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Table of Contents

The table of contents on the right has been linked to the individual sections in this compendium. Click on the text to jump to a specific section.

If you prefer to search for words or phrases used in any of the enclosed applications notes, click on the Search botton below to open Acrobat's Search window.

Search

Solution Segments

Consumer Products

Dietary Supplements, Natural Compounds & Additives

Food Authenticity

Food Processing & Packaging

Pesticides, Mycotoxins & Other Contaminants

Soil Quality & Fertilizer Analysis

Trace Metals

Veterinary Drugs

Index



Consumer Products

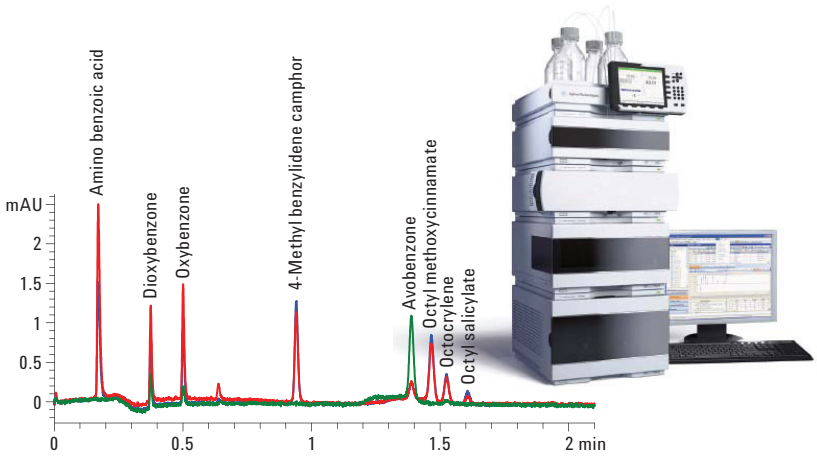
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> Return to Table of Contents
> Search entire document

Agilent 1290 Infinity LC with Agilent Poroshell columns for simultaneous determination of eight organic UV filters in under two minutes

Application Note

Consumer Products



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Abstract

Levels of UV filters in personal care products are regulated by the FDA and European Pharmacopeia (EP). Liquid chromatographic (LC) methods are widely accepted analytical techniques for the qualitative and quantitative analysis of these UV filters. Most of these traditional LC methods require about 25–50 minutes. In this Application Note, the Agilent 1290 Infinity LC, in combination with Agilent Poroshell columns, were used for development of a short, sensitive, robust and well resolved separation of eight FDA/EP approved active UV filter ingredients in 99 seconds. Standard deviation (SD) and relative standard deviation (RSD) values of retention time for replicate injections confirmed the excellent performance of the Agilent 1290 Infinity Binary Pump. Exceptional performance of the Agilent 1290 Infinity Diode Array Detector was established by minimum area RSD values and a wide linear range with standard organic UV filters in amounts from 0.25 ng to 200 ng on-column. In addition, the method was effectively used to identify active UV filters extracted from six international personal care products.

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Introduction

The wavelength of UV rays lies between X-rays and visible light (~ 10 – 400 nm)[1]. Production of melanin, a pigment that causes the darkening of skin, is a natural defense of the human body against UV radiation. Melanin absorbs UV radiation and dissipates the energy as harmless heat, though the response to UV radiation and production of melanin pigmentation depends on skin color and other genetic factors [2, 3]. The intensity of UV radiation and length of exposure are the main parameters involved in sunburn, irrespective of skin tone and ability of the skin to produce melanin. The major classifications of UV light are presented in Table 1[4].

Sl No:	Component	Wavelength (nm)	Effects of over exposure on skin
1	UV-A I	340-400	Can cause tanning but has minimal erythemal effect. Can cause long term damage. Penetrates deeply. it can contribute to skin cancer via indirect DNA damage.
2	UV-A II	320-340	Slightly erythemal contribution
3	UV-B	290-320	Causes sunburn and is a major contributor to skin cancer development.
4	UV-C	100-290	Very energetic radiation. Absorbed by the ozone layer. Direct DNA damage

Table 1
Major classifications of UV radiation.

Sunscreens protecting the skin against sunburn contain one or more of the following types of active ingredients:

- Organic chemicals that absorb UV light.
- Inorganic particulates that reflect, scatter, and absorb UV light.
- Organic particulates that have all the above features.

Organic UV filters are usually aromatic compounds conjugated with carbonyl groups. The FDA has approved seven UV-A filter compounds and nine UV-B filter compounds for sunscreen formulations in the United States, while the European Commission has approved the use of ten additional UV filters in European countries[5].

Though several approved UV filters are available in the market, extensive use of these UV filters may have several major concerns. Some sunscreen ingredients have been shown to have carcinogenic properties. Additionally, older and more widespread sunscreen chemicals cannot dissipate the energy of the excited state as efficiently as melanin, therefore the penetration of these sunscreen ingredients into the lower layers of the skin may increase the amount of free radicals and reactive oxygen species[6]. Therefore, extensive testing of sunscreens is advisable to reveal the efficacy of the ingredients. This Application Note discusses a short LC method to separate eight widely used UV filters within 99 seconds. Cosmetic manufacturers can adopt this method to simplify the analysis of sunscreen raw materials and personal care products in product development, regulatory compliance, and quality control to increase the efficiency of analysis.

Experimental

Instrument configuration

An Agilent 1290 Infinity LC, controlled by ChemStation (Version B.04.02) and equipped with a binary pump with integrated vacuum degasser, autosampler, thermostatted column compartment and a diode array detector, was used for data collection. The injection volume was set to 1 µL and the needle wash was enabled using acetonitrile for three seconds. The sample thermostat was set at 5 °C, while the columns were operated at 55 °C. The binary pump was operated at a flow rate of 1 mL/min. The detector was programmed for three different wavelengths (288, 304 and 358 nm) and operated at a sampling acquisition rate of 80 Hz (response time 0.062 seconds, >0.003 min). An Agilent Poroshell 120 EC-C18 column (75 mm × 2.1 mm, 2.7 µm) was used for the chromatographic separation.

Chemicals and standards

All eight UV filter standards and acetic acid (mobile phase modifier) were purchased from Aldrich (India). Super gradient grade acetonitrile (ACN) was purchased from Lab-Scan (Bangkok, Thailand). HPLC grade water was freshly taken from a Milli-Q water purification system. Six different brands of international sunscreen formulations were purchased locally. The details of organic UV filter standards used in this study are tabulated in Table 2.

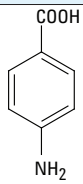
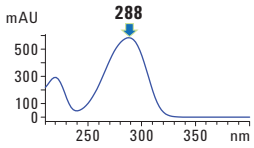
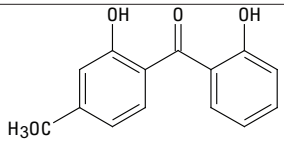
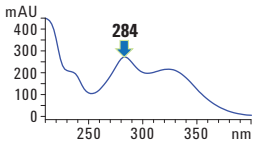
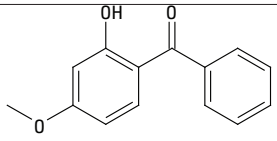
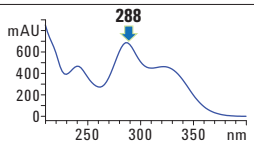
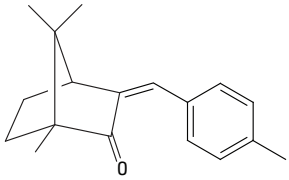
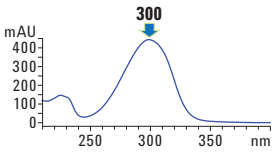
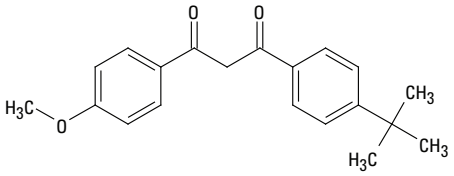
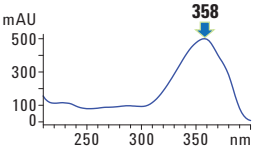
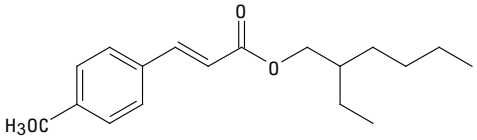
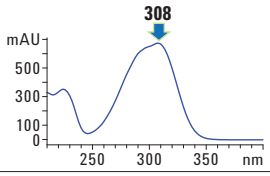
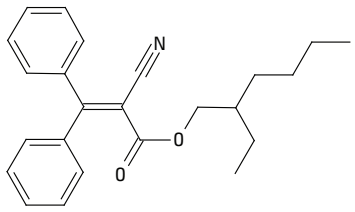
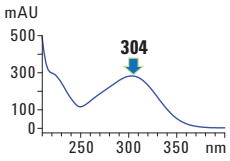
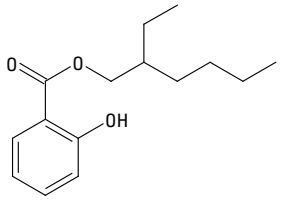
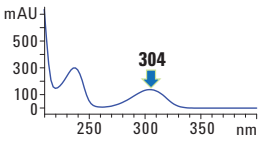
Compound number	Compound	Structure	UV Spectra
1	Name: Aminobenzoic acid CAS No: 150-13-0 Mol Formula: $C_7H_7NO_2$ Mol Wt: 137.14 Detection: 288 nm		
2	Name: Dioxybenzone CAS No: 131-53-3 Mol Formula: $C_{14}H_{12}O_4$ Mol Wt: 244.24 Detection: 288 nm		
3	Name: Oxybenzone CAS No: 131-57-7 Mol Formula: $C_{14}H_{12}O_3$ Mol Wt: 228.24 Detection: 288 nm		
4	Name: 4-Methyl benzylidene camphor CAS No: 36861-47-9 Mol Formula: $C_{18}H_{22}O$ Mol Wt: 254.37 Detection: 304 nm		
5	Name: Avobenzone CAS No: 70356-09-1 Mol Formula: $C_{20}H_{22}O_3$ Mol Wt: 310.39 Detection: 358 nm		
6	Name: Octylmethoxycinnamate CAS No: 5466-77-3 Mol Formula: $C_{18}H_{26}O_3$ Mol Wt: 290.397 Detection: 304 nm		
7	Name: Octocrylene CAS No: 6197-30-4 Mol Formula: $C_{24}H_{27}NO_2$ Mol Wt: 361.48 Detection: 304 nm		
8	Name: Octyl salicylate CAS No: 118-60-5 Mol Formula: $C_{15}H_{22}O_3$ Mol Wt: 250.33 Detection: 304 nm		

Table 2
Detailed list of organic UV filter standards used in this study.

LC parameters

Premixed solutions of 0.1% acetic acid in water and acetonitrile in the ratio 90:10 (A) and 10:90 (B) were used as mobile phase. The gradient used for the study is presented in Table 3. A post run time of 1 minute was set for column re-equilibration.

Time (min)	B (%)
0	50
0.1	70
2	85

Table 3
Gradient used for experiment.

Standard mix

A premixed solution of acetonitrile and 0.1% acetic acid in the ratio 50:50 was used as the diluent. A stock solution of each standard was prepared individually at a concentration of 1000 ppm (1000 ng/µL). A standard mixture of p-aminobenzoic acid, dioxybenzone, oxybenzone, 4-methyl benzylidene camphor, avobenzone, octyl methoxycinnamate, octocrylene and octyl salicylate, all 100 ppm (ng/µL) each, was prepared by diluting individual standard stock solutions using diluent. For detector linearity analysis, seven more mixed standard solutions with analyte concentrations of 50, 25, 10, 5, 1, 0.5 and 0.25 ng/µL were prepared by subsequent dilution of the higher concentrated standard mix.

Extraction of UV filters from formulation samples

UV filters from six different locally available international brands were extracted by a simple extraction procedure using acetonitrile. Two hundred fifty milligrams of each formulation were extracted with 5 mL of acetonitrile, sonicated and centrifuged. The supernatant liquid was filtered using 0.2 µ Agilent syringe filters (p/n 5061-3361). The resulting filtrate was diluted five times with diluent to get a stock solution of extracted sample. A diluted extracted sample for injection was prepared by further diluting the stock solution with equal volumes of diluent and injecting 1µL. Extracted samples spiked with standards were prepared by mixing equal volumes of 100 ppm standard mix solution and extracted sample stock solution. This spiked sample was used for the confirmation of peak identity in extracted samples by means of retention time and UV spectra.

Procedure

A blank injection was performed in all trials to check the chromatographic interference in the resolution. Standard mix, linearity levels, diluted extracted samples and spiked diluted extracted samples were also injected. The retention time of each standard was confirmed by individual standard injections.

Results and Discussion

LC chromatogram of standard mixture

The results showed excellent baseline separation of all eight active sunscreen ingredients, without chromatographic blank interference. The last peak of the standard mix (octyl salicylate) eluted at 1.62 minutes. A chromatographic representation of the standard mix is as shown in Figure 1. An unknown peak was observed at approximately 0.65 minute, which is an impurity present in the avobenzone standard. A peak purity check by spectral scanning in the range of 200 to 400 nm revealed that all eight compounds eluted without co-elution of any detectable impurities. Three different wavelengths were selected for detection as the maximum absorbance values vary for individual components. The peak width (half height), peak symmetry, USP tailing factor, and resolution values confirm the baseline separation of all the standard analytes in 99 seconds using the Agilent Poroshell 120 EC-C18 column (Table 4).

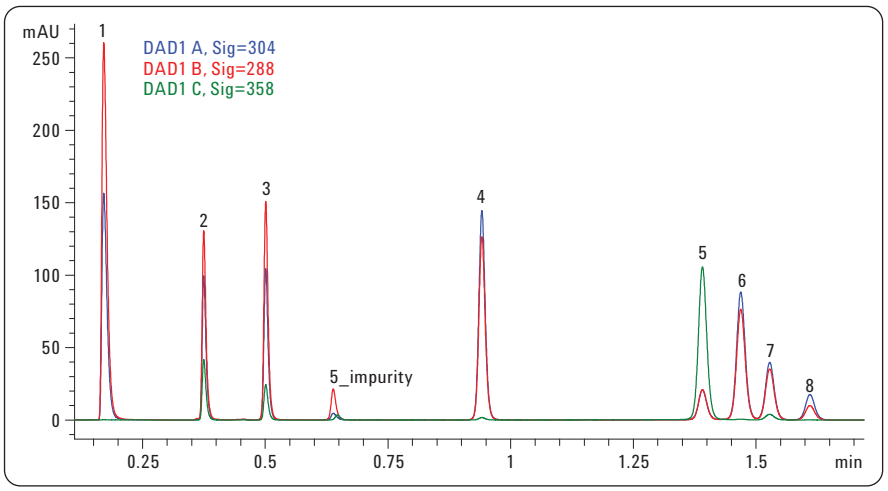


Figure 1
Chromatographic elution profile of eight organic UV filters in 99 seconds using an Agilent 1290 Infinity LC and an Agilent Poroshell 120 EC-C18 column (three different wavelengths are overlaid).

Comp No:	Name of the compound	Half peak width	Symmetry	USP tail	Resolution
1	Aminobenzoic acid	0.011	0.556	1.438	NA
2	Dioxybenzone	0.008	0.648	1.449	12.662
3	Oxybenzone	0.009	0.720	1.306	8.794
4	4-Methyl benzylidene camphor	0.014	0.862	1.112	22.988
5	Avobenzone	0.018	0.896	1.090	16.456
6	Octyl methoxycinnamate	0.019	0.920	1.070	2.453
7	Octocrylene	0.019	0.912	1.061	1.805
8	Octyl salicylate	0.020	0.910	1.061	2.447

Table 4
Peak width (half height), peak symmetry, USP tailing factor, and resolution values of the eight sunscreen ingredient standards in an injection of a standard mix (50 ng) with detection at 304 nm. Injection volume was 1 µL.

The chromatographic overlay of six replicates at 10 ng/µL confirms the excellent reproducibility of the data (Figure 2). Despite similarities in chemical structures, the components are well resolved within 99 seconds. At this level, the observed standard deviation (SD) value for retention time (RT) was < 0.0005, relative standard deviation (RSD) was < 0.32% and area RSD value was < 1.43%.

Signal-to-noise ratio

Figure 3 shows the chromatogram of the standard mix where all the analytes were at 0.25 ng on-column concentration. The observed signal-to-noise (S/N) values for each standard is calculated by taking the signal from a readily detectable peak height for each component and noise as absolute noise from the baseline in a compound-free area. The S/N values along with SD, and RSD values are tabulated in Table 5. At this concentration, S/N values for the first six compounds are > 20 and a least S/N value of 5 was observed for octyl salicylate (compound 8).

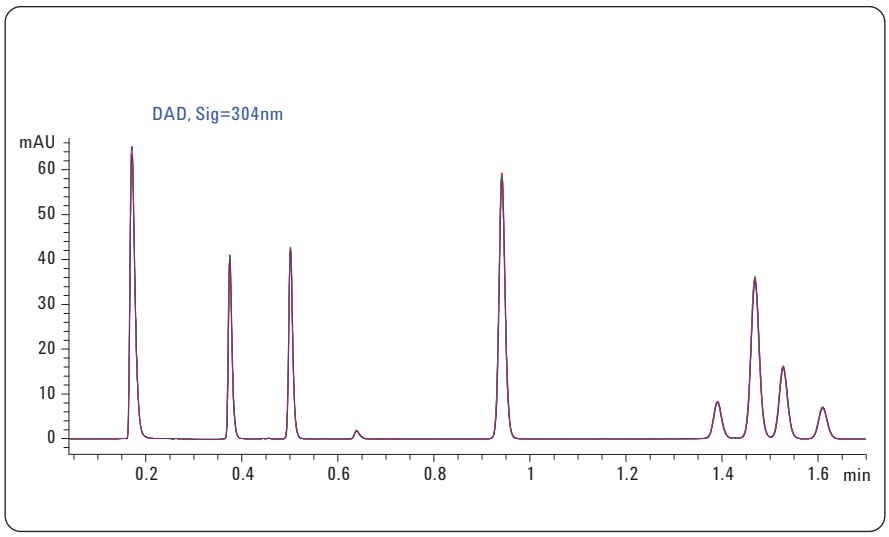


Figure 2
Chromatographic overlay of six replicates of standard mix injections at 10 ng/µL level.

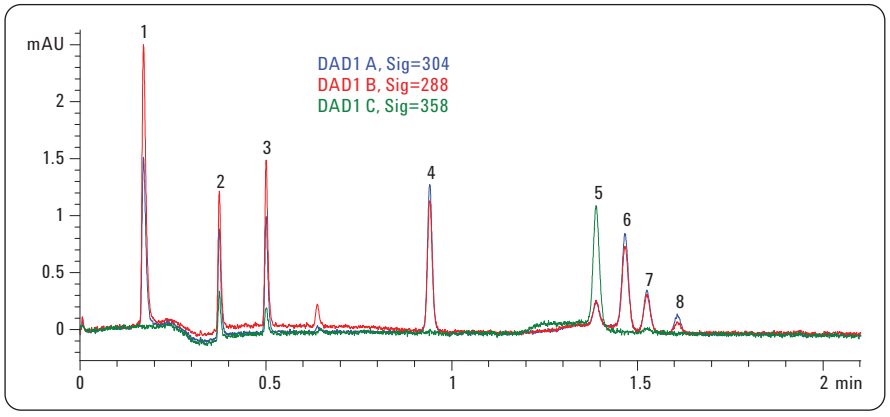


Figure 3
Chromatogram of standard mix where all the analytes were at 0.25 ng on-column concentration (three different wavelengths are overlaid).

	Comp 1	Comp 2	Comp 3	Comp 4	Comp 5	Comp 6	Comp 7	Comp 8
Injections	**288 nm**			**304 nm**	**358 nm**	**304 nm**		
3	51.5	26.5	31	36.1	21.1	22.9	10.4	4.6
5	50.3	25.5	28.8	34.7	20.2	22	9.4	4.4
6	56.5	27.3	31.8	34.1	20.8	22	10	4.6
7	50.2	25.6	30	35.1	19.8	21.9	9.5	4.5
8	52.8	26.1	31.4	35.7	19.3	22.2	9.7	4.4
9	50.2	25.3	29.8	35.3	18.6	21.8	9.8	4.5
Average	51.9	26.1	30.5	35.2	20.0	22.1	9.8	4.5
SD	2.47	0.75	1.13	0.71	0.94	0.40	0.36	0.09
RSD (%)	4.75	2.89	3.71	2.02	4.68	1.80	3.71	1.99

Table 5
Signal-to-noise values for each standard at 0.25 ng on-column concentration.

Linearity

A linearity study was performed in the concentration range of 0.25 ng to 200 ng (nine levels and five replicates) on-column concentration. The levels were 200 ng, 100 ng, 50 ng, 25 ng, 10 ng, 5 ng, 1 ng, 0.5 ng, 0.25 ng. The precision of area and retention time was demonstrated by calculating the SD and RSD values of five replicate injections for each level. The graphical representation of RSD for RT is shown in Figure 4. The observed RSD values are well within the acceptance limit of 1.0% confirming the excellent precision in retention time.

The results show an excellent assurance of area reproducibility above 0.5 ng on-column for all components except octyl salicylate (compound 8). For compound 8, from 1 ng and above, the area RSD values are well within the allowed limit of 2.0%. A smaller peak area showed poor UV response of octyl salicylate compared to other standards. This was the reason for a higher RSD value. The observed area RSD values throughout the linearity levels are tabulated in Table 6.

A calibration graph was constructed by plotting the peak area of each standard against nominal concentrations (0.25 ng, 0.5 ng, 1 ng, 5 ng, 10 ng, 25 ng, 50 ng, 100 ng, 200 ng). The linearity of the relationship between peak area and concentration is established by the correlation coefficients (R^2) > 0.9997. The overlaid linearity curves for all standards are shown in Figure 5. Observed R^2 values for individual components are tabulated in Table 7.

Extracted sample analysis

A spectral library was generated for all the standards to confirm peak identities and to provide data for spectral peak purity or the absence of coelution. UV filters from six different international brands were extracted and analyzed. Observed elution patterns for all the samples are overlaid and shown in Figure 6. An unknown peak was

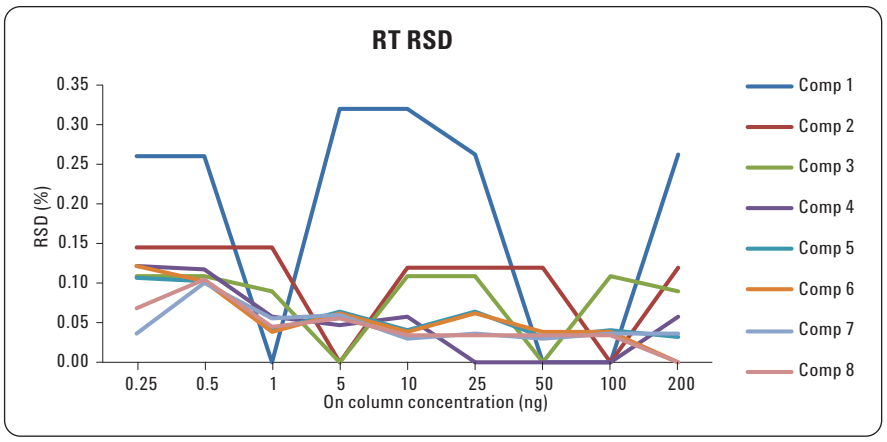


Figure 4
Retention time RSD.

On-column Concentration (ng)	Area RSD values (%)							
	Comp 1	Comp 2	Comp 3	Comp 4	Comp 5	Comp 6	Comp 7	Comp 8
0.25	1.70	4.40	3.57	1.53	1.95	2.80	5.93	9.67
0.5	1.48	1.46	1.48	1.45	0.98	1.46	1.80	4.24
1	0.47	0.55	0.73	0.87	1.00	1.34	1.20	1.94
5	0.22	1.37	0.06	0.09	0.31	0.21	0.20	0.50
10	0.95	1.02	0.96	1.10	1.16	1.29	1.43	1.21
25	0.27	0.45	0.50	0.46	0.55	0.54	0.61	0.53
50	1.11	1.16	1.02	0.98	0.80	0.80	0.70	0.79
100	0.11	0.16	0.20	0.26	0.25	0.29	0.27	0.33
200	0.16	0.24	0.19	0.16	0.19	0.18	0.22	0.11

Table 6
Area RSD values for all compounds at all linearity levels (n=5). Values > 2.0 are marked in red.

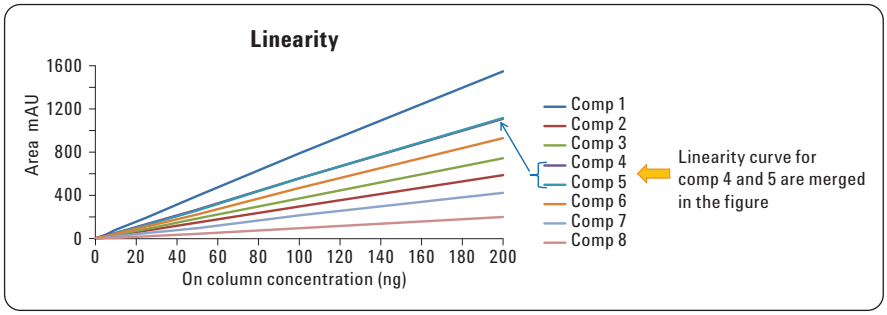


Figure 5
Overlaid linearity curves for all standards.

Compound	Name	R^2
1	Aminobenzoic acid	0.9999
2	Dioxybenzone	1.0000
3	Oxybenzone	0.9999
4	4-Methyl benzylidene camphor	0.9999
5	Avobenzone	0.9997
6	Octyl methoxycinnamate	0.9998
7	Octocrylene	0.9997
8	Octyl salicylate	0.9998

Table 7
Observed R^2 values for individual components.

observed in sample 5 at 1.71 min. Results show that avobenzone and octocrylene are the most widely used UV filter components in the sunscreen personal care products tested. From the extracted sample chromatogram, it is clear that samples b and d provide a broad range of protection against UVA, UVB and UVC rays.

Sunscreen products with higher SPF values may contain higher amounts of sunscreen components and consequently chances are high that these products contain significant amounts of impurities. The Agilent 1290 Infinity LC provides an overall picture of impurity profiles in personal care products in the shortest amount of time, as demonstrated for sample e in Figure 7.

Conclusions

This Application Note demonstrates the baseline separation of eight FDA/EP approved sunscreen compounds in 99 seconds using the Agilent 1290 Infinity LC and the Agilent Poroshell 120 EC-C18 column. The minimum observed resolution value in the standard mix chromatogram was > 1.8. S/N values for each component at 0.25 ng level (on-column concentration) were demonstrated. Linearity was demonstrated from 0.25 ng to 200 ng on-column for all compounds. The poorest R^2 value is 0.9997 (nine levels and five replicates). Across the linearity levels, the highest observed RT standard deviation value was 0.0018 and the highest observed RT RSD value was 0.32% (n=5). This method can be effectively used to chromatograph UV filters and impurities present in sunscreen and personal care cosmetic products.

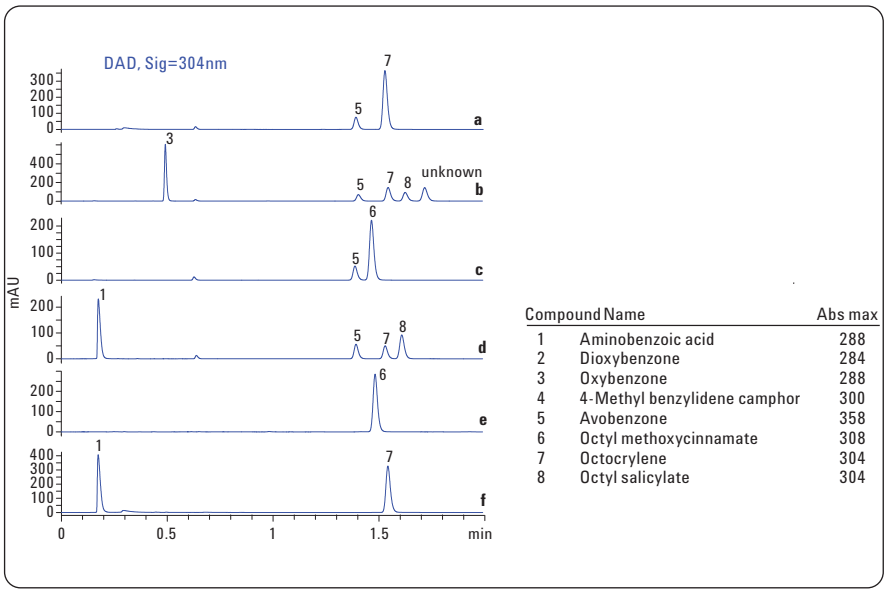


Figure 6
Overlay of chromatogram for all six extracted samples.

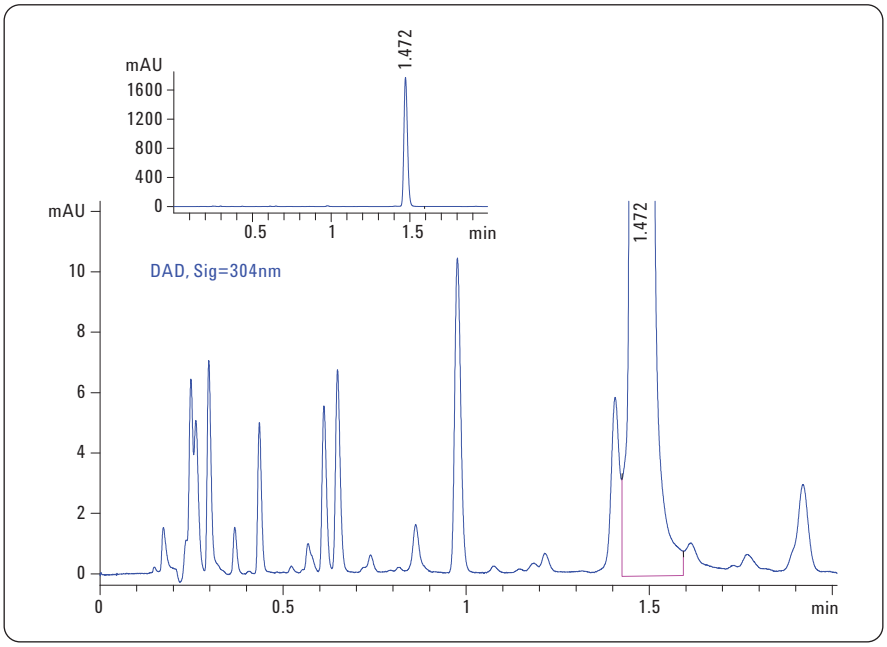


Figure 7
Full scale and zoomed chromatogram of sample "e", which demonstrates well resolved impurity peaks. These minor unidentified peaks may be parabens or other listed ingredients of the formulations and related impurities.

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Agilent Technologies

Profiling capsaicinoids in spicy foods and food products using the Agilent 1290 Infinity LC system and Agilent ZORBAX Poroshell 120 2.7 µm columns with UV/Vis diode array and fluorescence detection

Application Note

Food

Author

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Abstract

The Agilent 1290 Infinity LC system has significant capabilities for a wide range of HPLC and UHPLC applications. It exhibits a broader power range (for example, the combination of pressure and flow capabilities), and the flexibility to operate a wide range of column dimensions and particle sizes than any other commercially available system. Advanced optical design in the diode array detector allows a wide dynamic range and high sensitivity, both of which are critical in the monitoring of natural and synthetic food flavorings.

The combined benefits are demonstrated by a separation of primary capsaicinoid components found in chili pods, sauces and spices. Capsaicin is the primary component recognized as the "hot" component of chili peppers, members of the capsicum family. The capsaicinoid class is comprised of eight or more compounds that variably contribute a heat component to flavor. Capsaicin or synthetic capsaicin (nonivamide) has also been used in topical creams and applications to relieve arthritis, itching, neuropathy and other ailments. The core structure is phenolic and primary variation is found in the hydrophobic alkyl chain.

The high pressure capability of the system allows the use of methanol, and acetonitrile, to explore the selectivity of the two solvents. Various column configurations, including porous 1.8 µm and superficially porous 2.7 µm materials, were evaluated. The structure of capsaicin is shown in Figure 1.

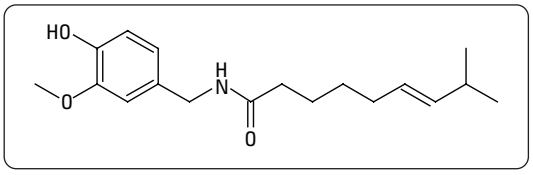


Figure 1
The structure of capsaicin ((E)-N- (4-hydroxy-3-methoxybenzyl) - 8-methylnon-6-enamide).

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The distinctive spectrum of capsaicin allows one to use the diode array detector to interrogate peaks in capsaicinoid regions to determine if the compound is likely to be an active component. The UV ratio for 229 nm/280 nm is nominally 2.4 (Figure 2).

After optimization of the standard mixture, a number of extracted samples were chromatographed (Figure 3).

Cayenne and other pure chili spices and fresh pods were easily analyzed with UV detection. Low level samples such as American paprika and complex blends such as chili and curry powders were more likely to show interferences. The solution to this problem was the Agilent 1200 Series fluorescence detector (G1321A) as shown in Figure 4. The remarkable selectivity and sensitivity of the FLD minimized or eliminated non-capsaicinoid peaks from the analyte region.

Configuration

- Agilent 1290 Infinity Binary Pump with Integrated Vacuum Degasser (G4220A)
- Agilent 1290 Infinity Autosampler (G4226A)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1200 Series Diode Array Detector (G1315C)

Conclusion

The flexible solvent and column selection features, and high pressure capability, of the system allows one to use highly efficient columns to rapidly develop separations with remarkable resolution while conserving solvent over the use of 4.6 mm id columns. The added selectivity of fluorescence detection provides solutions to unexpected interferences that would otherwise have required redevelopment of the separation method.

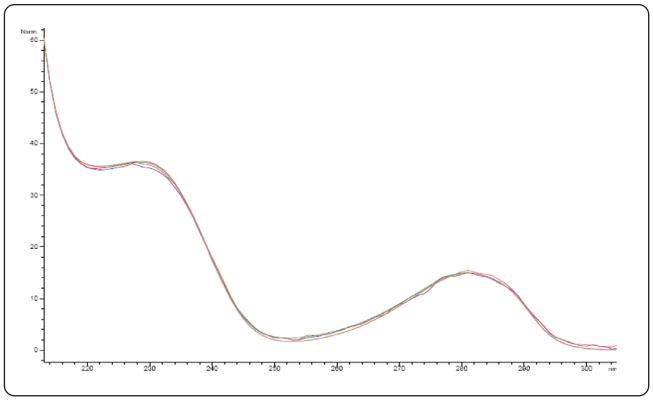


Figure 2
Overlaid extracted UV spectra of capsaicinoid compounds in chili.

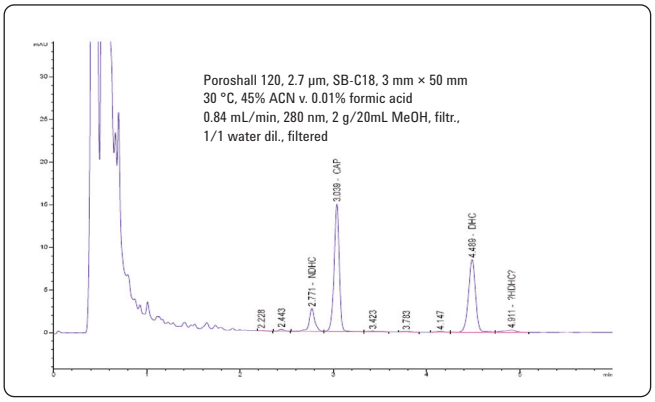
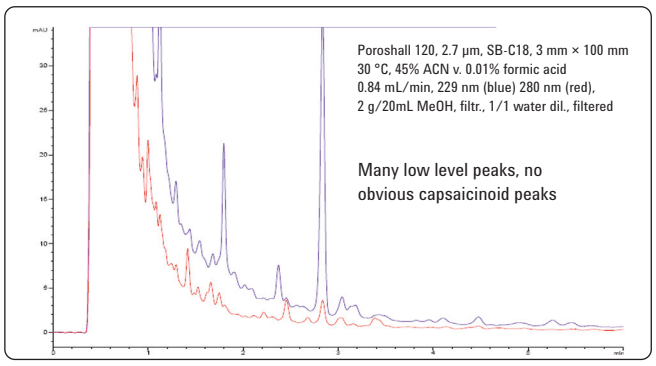


Figure 3
Separation of cayenne dried ground spice (CAP- capsaicin, NDHC – nordihydrocapsaicin, DHC – dihydrocapsaicin).



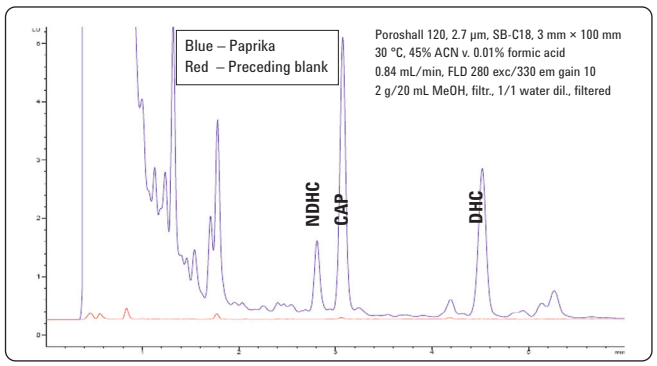


Figure 4
Comparison of UV and fluorescence signals for American Paprika extract (two frames).

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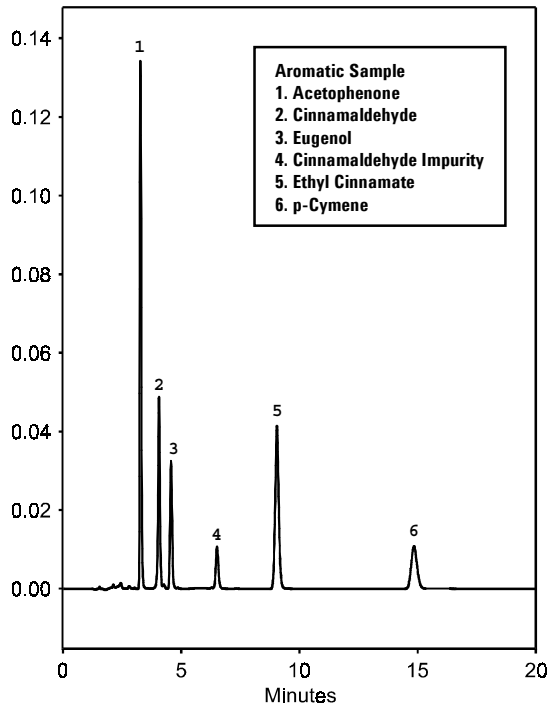
Aromatics

Application

Food Analysis

Robert Ricker

Aromatics are used in both the flavor and fragrance industries. Only a few of the many compounds that can be classified as aromatic were separated for this application note. The odors of the various aromatics in this study are as follows: acetophenone has an orange-blossom-like odor; cinnamaldehyde is found in some cinnamon oils and has a characteristic cinnamon odor; eugenol has an odor of cloves and is also used as a dental analgesic; ethyl cinnamate has a fruity and balsamic odor reminiscent of cinnamon. Cymene occurs in a number of essential oils, but no odor is described in the literature.



Conditions:
Column: ZORBAX Eclipse XDB-Phenyl, 4.6 x 150 mm (3.5µm) Agilent P/N: 963967-912
Mobile Phase: H_2O : MeOH, 40:60
UV: 254 nm; Flow: 1.0 mL / min.; 35°C

Highlights

- *Neutral compounds have excellent peak shape on Agilent ZORBAX Eclipse XDB-Phenyl columns.*
- *Efficiency can be increased with the use of smaller size (3.5 µm vs. 5.0 µm) particles. Plate counts of 13,000 - 18,000 were achieved for the five compounds in this application.*

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Robert Ricker is an application chemist based at Agilent Technologies, Wilmington, Delaware.

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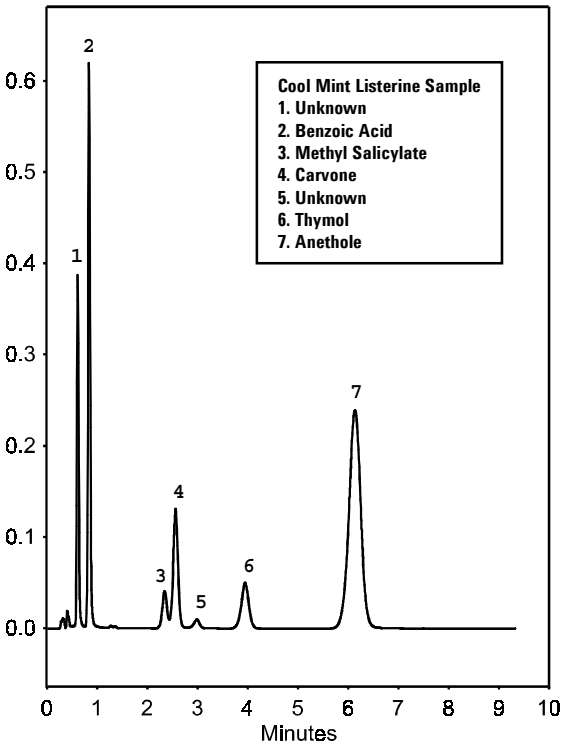
Flavoring Agents

Application

Food Analysis

Robert Ricker

Several flavoring agents can be found in Cool Mint Listerine, a popular mouthwash. Carvone is oil of caraway, anethole is a constituent of anise and fennel oils, which has a licorice flavor and methyl salicylate (wintergreen oil). This sample also contains the antiseptic, thymol and the preservative, benzoic acid.



Conditions:
Column: Agilent ZORBAX SB-Phenyl, Narrow-Bore LC/MS, 2.1 x 50 mm (5µm), Agilent PN 860975-912
Mobile Phase: 0.3% TFA : ACN, 65:35
UV: 254 nm; Flow: 0.3 mL / min.; Ambient

Highlights

- *Rapid analysis of complex mixtures while maintaining resolution can be achieved with shorter length (50 mm) columns.*
- *Sample preparation is minimal for liquid samples which can be diluted and injected directly onto the column.*
- *This method can be applied directly to LC/MS analysis.*
- *Sterically protected bonded phases provide superior lifetime at low pH.*

Agilent Technologies

Robert Ricker is an application chemist based at Agilent Technologies, Wilmington, Delaware.

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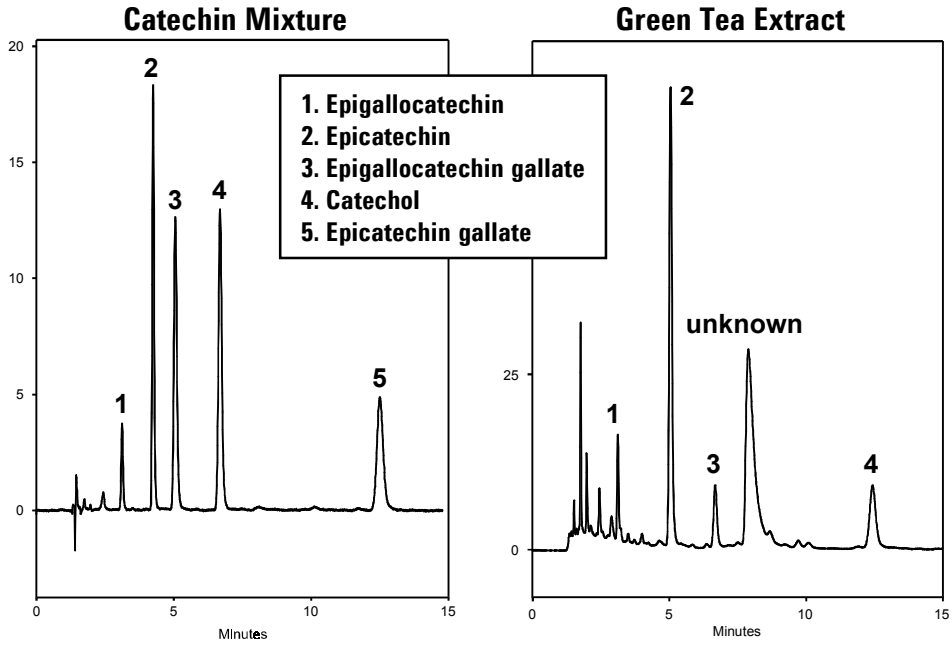
Epigallocatechin 3-O-Gallate Extract from Green Tea

Application

Food Analysis

Robert Ricker

Epigallocatechin 3-O-gallate (EGCG) belongs to a class of compounds called flavonoids and is further subclassified as a flavanol, due to the level of oxidation in its chemical structure. This compound has been recognized as a cancer-preventive compound due to its ability to inhibit urokinase, an enzyme which has been associated with excelerated tumor cell growth. Due to the interest in holistic-type, preventative medicine approaches in today's society, a method was developed for a series of catechins and an actual extract of green tea to serve as a general interest application.



ZORBAX SB-C8 (4.6 x 150 mm; 3.5 µm) (Agilent Part No. 863953-906)
Mobile Phase: 75% 0.1% Trifluoroacetic acid: 25% Methanol
Inj. Vol. 5 µL, 1 mL/min, 40°C
Det. UV (280 nm)

Highlights

- *Good peak shape and resolution are maintained for a group of catechins on Agilent ZORBAX SB-C8 at low pH.*
- *Sterically protected bonded phases, like SB-C8, offer extended column lifetime even with TFA-containing (low-pH) mobile phases.*
- *Good retention of the catechins allows adequate separation from other UV-absorbing compounds in the actual tea extract.*

Agilent Technologies

Robert Ricker is an application chemist based at Agilent Technologies, Wilmington, Delaware.

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Scale-Up of Anthocyanin Separations and Re-Analysis of Collected Fractions on an Agilent Prep-C18 Column

Application

Biochemical

Cliff Woodward



Highlights

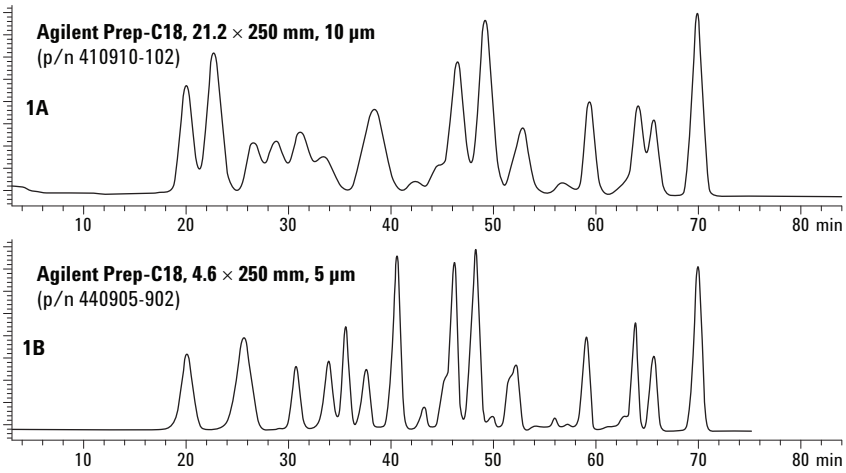
- The Agilent Prep-C18 columns demonstrate excellent scalability, allowing method development on analytical scale columns
- The Agilent Prep-C18 enables high resolution and high purity fractionation
- The Purification software of the Agilent ChemStation combined with the Agilent Prep-C18 makes complex purifications easy

Anthocyanins, potent anti-oxidants, are now recognized as health important components of many foods. Blueberries, of all natural foods, contain the highest concentrations of these interesting compounds. The purification and identification of various anthocyanins is an important step in understanding which components are most beneficial to human health. Anthocyanins are highly retained on C18 columns; in addition, they require high concentrations of organic solvents for extraction. In attempting to separate large quantities of some of the components for use as standards or for further structural workups, the analyst may encounter solubility issues, which can limit the loadability. The factors affecting loadability of anthocyanins were explored in previous work [1].

The first step in preparative purification is to prove that scale-up works. Once that is known, preparative chromatography is simple. Fractions can be collected and rechromatographed to demonstrate purity. Figure 1 shows the scalability of the Agilent Prep-C18 column. The resolution of the analytical column is, of course, better than the prep column.



An Agilent Prep-C18 column is shown above with an Agilent 1100 HPLC system.



1A

Agilent Prep-C18, 21.2 × 250 mm, 10 µ

Temperature:	Ambient
DAD wavelength:	525 nm
Injection:	2000 µL
Sample:	Blueberry extract, 46.1 mg/mL total dissolved solids (~5 mg/mL anthocyanins)
Flow:	21.2 mL/min

1B

Agilent Prep-C18, 4.6 × 250 mm, 5 µ

Temperature:	Ambient
DAD wavelength:	525 nm
Injection:	100 µL
Sample:	Blueberry extract, 46.1 mg/mL total dissolved solids (~5 mg/mL anthocyanins)
Flow:	1.0 mL/min

Mobile phase

A = 0.1% TFA in water

B = 0.1% TFA in methanol

Gradient timetable

Time (min)	% Solvent B
0.00	23.0
35.00	26.0
85.00	53.5

Figure 1. Scalability of Agilent Prep columns.

By using the ChemStation fraction collection software, fractions are obtained that are significantly purer than the separation would seem to allow. See Figure 2. Fraction 1 is >99% pure Delphinidin–3–galactoside and Fraction 2 is >97% pure Delphinidin–3–glucoside. This purity was obtained using the threshold and slope settings of the software to cut the fractions appropriately. Identities of the fractions were verified by liquid chromatography/mass spectrometry (LC/MS) (data not shown).

The conditions for the Agilent Prep-C18 columns below are the same as in Figure 1.

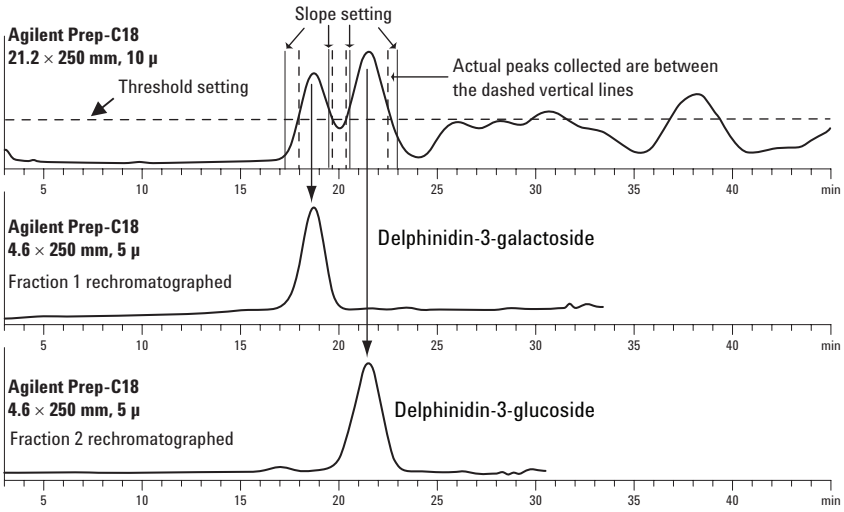


Figure 2. Fraction collection and rechromatography to demonstrate purity.

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Separation of Paraben Preservatives by Reversed-Phase HPLC

Application

Foods, Beverages, and Cosmetics

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Abstract

Paraben preservatives are shown to be readily and quickly analyzed using reversed-phase HPLC with a ZORBAX Eclipse XDB-C18 Rapid Resolution column.

Introduction

Preservatives are a class of chemical agents that are commonly used to prevent the growth of bacteria in foods, beverages, and cosmetics. The paraben preservatives (4-hydroxybenzoic acid esters) are among the most widely used. These preservatives were developed in the 1930's to stabilize creams.

Synthetic methyl, ethyl, and propyl parabens were developed from benzoic acid and were considered effective and economical since they were inexpensive to use as both a cosmetic and food grade preservative. It is estimated that 99% of all cosmetic and body care products contain some form of paraben preservatives. Methyl and propyl parabens are generally recognized as safe (GRAS) substances. Recently, however, this preservative system has come into question as these substances were found in cancerous tissues, especially breast tissue.

A study by the Journal of Pharmaceutical Science revealed that after receiving multiple doses of a gentamicin formula containing paraben preservatives, six infants found traces of up to 82.6% of the parabens in urine samples.

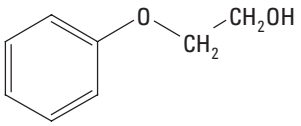
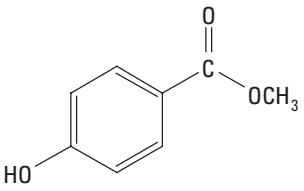
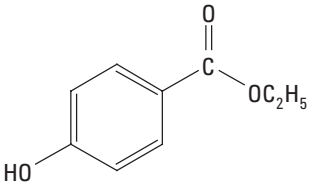
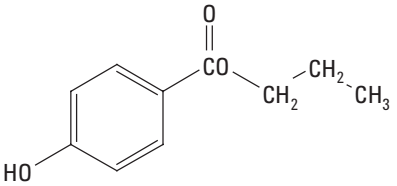
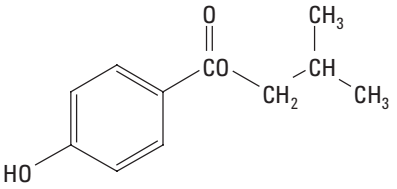
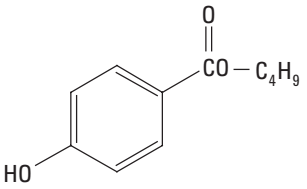
Researchers of the Department of Biology and Biochemistry of Brunel University in the United Kingdom found that the greatest concern regarding parabens focuses on their estrogen-mimicking ability in laboratory animals. In addition, 2-phenoxyethanol (2PX), a chemical substance also used as a preservative in several vaccines, is sometimes used in conjunction with parabens. Paraben mixtures have the advantages of being broad-spectrum, leading to reduced inventory levels and cost savings. It is easier to handle one liquid in reasonable quantities rather than several small quantities of powders or liquids. Phenonip, a product of Clariant Ltd, Horsforth, Leeds, United Kingdom, is a mixture of parabens in 2PX solution. This product is probably the best known of the paraben mixtures and is often copied.

Analysis of these substances at formulation and trace levels in foods and cosmetics is of great interest. HPLC is an ideal method for their separation and analysis.

Chemical Characteristics

The structures of 2PX and the parabens are depicted in Table 1. Due to their phenyl ring, these compounds are UV-detectable at extremely low concentrations. Since they have no ionic functional groups, they are considered lipophilic. Due to this lipophilicity, some accumulation in fatty tissues of the body would be expected. Parabens are slightly soluble in water, with the solubility decreasing as the ester chain length increases. For example, methyl paraben dissolves at the 0.25% (w/w) level at 20 °C while butyl paraben is soluble at the 0.02% (w/w) level. Most of the parabens are freely soluble in alcohol, acetone, ether and a number of other organic solvents. With such solubility properties, reversed-phase chromatography (RPC) is an ideal separation technique. Many reversed-phase separations of parabens are published in the chromatography literature [1–4].

Table 1. Structures and Concentrations of Preservative Compounds

2PX:	2-Phenoxyethanol (1.4 mg/mL)	
MEP:	Methylparaben (0.30 mg/mL)	
ETP:	Ethylparaben (0.07 mg/mL)	
PRP:	Propylparaben (0.04 mg/mL)	
IBP:	Isobutylparaben (0.04 mg/mL)	
BTP:	Butylparaben (0.08 mg/mL)	

Chromatographic Conditions

Column:	ZORBAX Eclipse XDB-C18 Rapid Resolution, 4.6 mm × 150 mm, 3.5 µm	
Mobile phase:	Solvent A: Water Solvent B: Methanol	
Gradient:	Time	% MeOH
	0	38
	5	38
	6	60
	16	60
	17	62
	20	38
Flow rate:	0.8 mL/min	
Temperature:	40 °C	
Detector:	UV 254 nm	
Injection volume:	5 µL	

Results and Discussion

The separation of the parabens and 2PX contained in a paraben product mix is depicted in Figure 1.

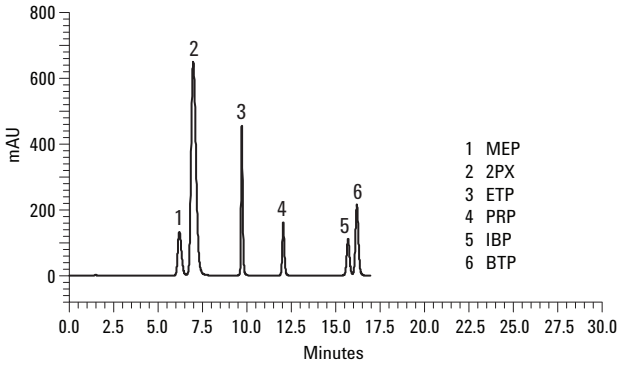


Figure 1. Separation of preservatives by reversed-phase HPLC.

Sample preparation merely involved dilution of the sample mix with methanol. All components were separated to baseline. On other columns, the separation of MEP and 2PX and IBP and BTP is usually quite difficult, especially in such a short analysis time (16 min). The method is reproducible with good separation efficiency.

Conclusion

Paraben preservatives are readily and quickly analyzed using reversed-phase HPLC with a ZORBAX Eclipse XDB-C18 Rapid Resolution column, 4.6 mm × 150 mm, 3.5 µm.

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Agilent Technologies

Column Selection for the Analysis of Fatty Acid Methyl Esters

Application

Food Analysis

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Abstract

The analysis of fatty acid methyl esters (FAMEs), derived from food, is a very important food characterization procedure. These esters are normally analyzed on columns coated with polar stationary phases, such as polyethylene glycols or cyanopropyl silicones, allowing separation of fatty acids according to their carbon number, the degree of unsaturation, the *cis-trans* configuration, and the location of the double bonds.

In this application note, three different stationary phases are compared for the separation of FAMEs. Polyethylene glycol columns gave good separation for the less complex samples, but they did not separate *cis-trans* isomers. A medium polar cyanopropyl column (DB23) provided excellent separation for complex FAME mixtures and also achieved some *cis-trans* separation. For more detailed *cis-trans* separation, the highly polar HP-88 cyanopropyl column is preferred.

Introduction

The analysis of FAMEs is used for the characterization of the lipid fraction in foods, and is one of the most important analyses for food. Lipids mainly consist of triglycerides, being esters of one glycerol molecule and three fatty acid molecules. Most edible fats and oils contain mainly fatty acids ranging from lauric acid (dodecanoic acid) to arachidic acid (eicosanoic acid). Besides the linear saturated fatty acids, branched fatty acids, mono-unsaturated, di-unsaturated, and higher unsaturated fatty acids can also occur. An overview of the most important fatty acids and their abbreviations appears in Table 1.

Table 1. Fatty Acids, Common Names, and Abbreviations

Fatty acid	Common Name	Abbreviation
Butanoic acid	Butyric acid	C4:0
Decanoic acid	Caproic acid	C10:0
Dodecanoic acid	Lauric acid	C12:0
Tetradecanoic acid	Myristic acid	C14:0
Hexadecanoic acid	Palmitic acid	C16:0
Hexadecenoic acid	Palmitoleic acid	C16:1
Octadecanoic acid	Stearic acid	C18:0
cis-9-Octadecenoic acid	Oleic acid	C18:1- *cis* (n9)
trans-9-Octadecenoic acid	Elaidic acid	C18:1- *trans* (n9)
all *cis*-9,12-Octadecadienoic acid	Linoleic acid	C18:2 - *cis* (n6)
all *trans*-9,12-Octadecadienoic acid	Linolelaidic acid	C18:2 - *trans* (n6)
all *cis*-9,12,15-Octadecatrienoic acid	α-Linolenic acid	C18:3 (n3)
all *cis*-6,9,12-Octadecatrienoic acid	γ-Linolenic acid	C18:3 (n6)
Eicosanoic acid	Arachidic acid	C20:0
cis-11-Eicosenoic acid		C20:1 (n9)
all *cis*-11,14-Eicosadienoic acid		C20:2 (n6)
all *cis*-11,14,17-Eicosatrienoic acid		C20:3 (n3)
all *cis*-8,11,14-Eicosatrienoic acid	Dihomogammalinolenic acid	C20:3 (n6)
all *cis*-5,8,11,14-Eicosatetraenic acid	Arachidonic acid	C20:4 (n6)
all *cis* 5,8,11,14,17-Eicosapentenoic acid	EPA	C20:5 (n3)
Docosanoic acid	Behenic acid	C22:0
cis-13-Docosenoic acid	Erucic acid	C22:1 (n9)
all *cis*-7,10,13,16-Docosatetraenoic acid		C22:4 (n6)
all *cis* 4,7,10,13,16,19-Docosahexenoic acid	DHA	C22:6 (n3)
Tetracosanoic acid	Lignoceric acid	C24:0
cis-15-tetracosenoic acid	Nervonic acid	C24:1 (n9)

For the characterization of the lipid fraction, the triglycerides are hydrolyzed (saponified) into glycerol and free fatty acids. Although the free fatty acids can be analyzed directly on polar stationary phases (such as an HP-FFAP column), more robust and reproducible chromatographic data are obtained if the fatty acids are derivatized to the methyl esters. For the derivatization, including hydrolysis and methylation, different methods are available [1]. These methods are easy to use and do not require expensive reagents or equipment. A typical sample preparation method is described in the sample preparation section.

After preparation of the FAMEs, they are separated according to the carbon number (number of carbon atoms in the fatty acid chain, excluding the methyl ester carbon) and the degree of unsaturation. Moreover, the position of the double bond(s) and the geometric configuration (*cis/trans*) are also important parameters and their determination adds additional information to the characterization of the lipid fraction in food.

In this application note, three stationary phases are compared for the separation of FAMEs. The first method uses DB-Wax, a polyethylene glycol column, where FAMEs from C4 (butyric acid) to C24 (lignoceric acid) can be separated according to carbon number and degree of unsaturation. On these columns, no separation of *cis*- and *trans*-isomers is obtained, and for complex mixtures, such as fish oils, some FAMEs are difficult to separate. However, the separation of FAMEs on polyethylene glycol columns is widely used and are applied to the characterization of “classical” samples, such as vegetable oils from corn, maize, olive, and soybean. Moreover, animal fats can also be

analyzed. One important application is the analysis of butyric acid in milk fat. The concentration of butyric acid in milk is an important indicator of milk quality, and its analysis is therefore very important in milk, dairy, and chocolate products.

For the analysis of complex samples, such as fish oils, additional resolution of the FAMEs is needed, and is obtained using a capillary column coated with a cyanopropyl-stationary phase, such as a DB-23. On this column, highly unsaturated fatty acids, such as all *cis* 5, 8, 11, 14, 17-eicosapentenoic acid methyl ester (EPA, C20:5 ω3) and all *cis* 4,7,10,13,16,19-docosahexenoic acid methyl ester (DHA, C22:6 ω3) are separated from other FAMEs. This analysis is very important in the framework of recent interest in omega-3 fatty acid determination. On the cyanopropyl column, separation of the *cis*- and *trans*-isomers is also possible. Due to stronger interaction of the *cis*-isomer with the cyano-dipole, the *trans*-isomers elute before the *cis*-isomers. In this way, the determination of *trans*-fatty acids is also performed, however, the polarity of the stationary phase is not sufficient to fully separate complex *cis-trans* mixtures.

For the separation of a complex FAME mixture containing a relatively large amount of *trans*-fatty acids, a highly polar HP-88 column is preferred. On this highly polar column excellent separation between different *cis*- and *trans*-isomers is obtained, however, some higher molecular weight fatty acids are more difficult to separate.

An overview of columns and their application area is summarized in Figure 1.

Experimental

Samples

Reference standards of FAMEs can be obtained from different sources as solutions or as neat compounds. For analysis, the standards are typically dissolved in hexane at a 0.01%–0.1% (w/v) concentration.

For column check-out, a 37-component mixture (Supelco #18919) was used. The mixture is available as a 100-mg neat mixture, containing C4–C24 FAMEs (2%–4% relative concentration). The whole sample was diluted in 10-mL hexane (final concentration = 0.2–0.4 mg/mL per FAME) before use. Oil and fat samples can be prepared using different methods [1–5].

Sample Preparation Method [5]

Weigh 100-mg sample in a 20-mL test tube (with screw cap) or reaction vial. Dissolve the sample in 10-mL hexane. Add 100-µL 2 N potassium hydroxide in methanol (11.2 g in 100 mL). Close the tube or vial and vortex for 30 s. Centrifuge. Transfer the clear supernatent into a 2-mL autosampler vial.

Analytical Conditions

The analyses were performed on an Agilent 6890 GC equipped with a flame ionization detector (FID). Automated split injection was performed using an Agilent 7683 autosampler. The instrumental configuration and analytical conditions are summarized in Table 2 (DB-Wax column), Table 3 (DB-23 column) and Table 4 (HP-88 column).

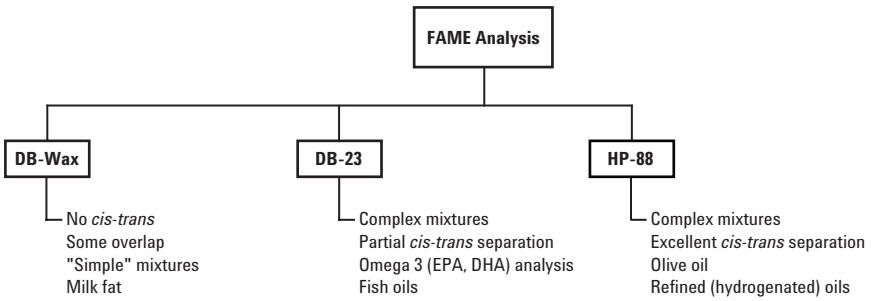


Figure 1. Overview of column selection for FAMEs analysis.

Table 2. DB-Wax Method 1

Instrumentation	
Chromatographic system:	Agilent 6890 GC
Inlet	Split/Splitless
Detector	FID or Agilent 5973 MSD
Automatic Sampler	Agilent 7683
Liner	Split liner (p/n 5183-4647)
Column	30 m × 0.25 mm ID, 0.25 µm DB-Wax (J&W 122-7032)
Experimental Conditions GC-FID	
Inlet temperature	250 °C
Injection volume	1 µL
Split ratio	1/50
Carrier gas	Hydrogen
Head pressure	53 kPa constant pressure (36 cm/s at 50 °C)
Oven temperature	50 °C, 1 min, 25 °C/min to 200 °C, 3 °C/min to 230 °C, 18 min.
Detector temperature	280 °C
Detector gases	Hydrogen: 40 mL/min; Air: 450 mL/min; Helium make-up gas: 30 mL/min.

Table 3. DB-23 Method 2

Instrumentation	
Chromatographic system:	Agilent 6890 GC
Inlet	Split/Splitless
Detector	FID or Agilent 5973 MSD
Automatic Sampler	Agilent 7683
Liner	Split liner (p/n 5183-4647)
Column	60 m × 0.25 mm ID, 0.15 µm DB-23 (J&W 122-2361)
Experimental Conditions GC-FID	
Inlet temperature	250 °C
Injection volume	1 µL
Split ratio	1/50
Carrier gas	Helium
Head pressure	230 kPa constant pressure (33 cm/s at 50 °C)
Oven temperature	50 °C, 1 min, 25 °C/min to 175 °C, 4 °C/min to 230 °C, 5 min.
Detector temperature	280 °C
Detector gases	Hydrogen: 40 mL/min; Air: 450 mL/min; Helium make-up gas: 30 mL/min.

Table 4. HP-88 Methods 3A and 3B

Instrumentation	
Chromatographic system:	Agilent 6890 GC
Inlet	Split/Splitless
Detector	FID or Agilent 5973 MSD
Automatic Sampler	Agilent 7683
Liner	Split liner (p/n 5183-4647)
Column A	100 m × 0.25 mm ID, 0.2 µm HP-88 (J&W 112-88A7)
Column B	60 m × 0.25 mm ID, 0.2 µm HP-88 (J&W 122-8867)
Experimental Conditions GC-FID	
Inlet temperature	250 °C
Injection volume	1 µL
Split ratio	1/50
Carrier gas A	Hydrogen
Carrier gas B	Helium
Head pressure	2 mL/min constant flow
Oven temperature A	120 °C, 1 min, 10 °C/min to 175 °C, 10 min, 5 °C/min to 210 °C, 5 min 5 °C/min to 230 °C, 5 min
Oven temperature B	175 °C, 10 min, 3 °C/min, 220 °C, 5 min
Detector temperature	280 °C
Detector gases	Hydrogen: 40 mL/min; Air: 450 mL/min; Helium make-up gas: 30 mL/min.

Results

A typical chromatogram for the analysis of the 37-compound FAMEs reference standard, obtained on the DB-Wax column is shown in Figure 2.

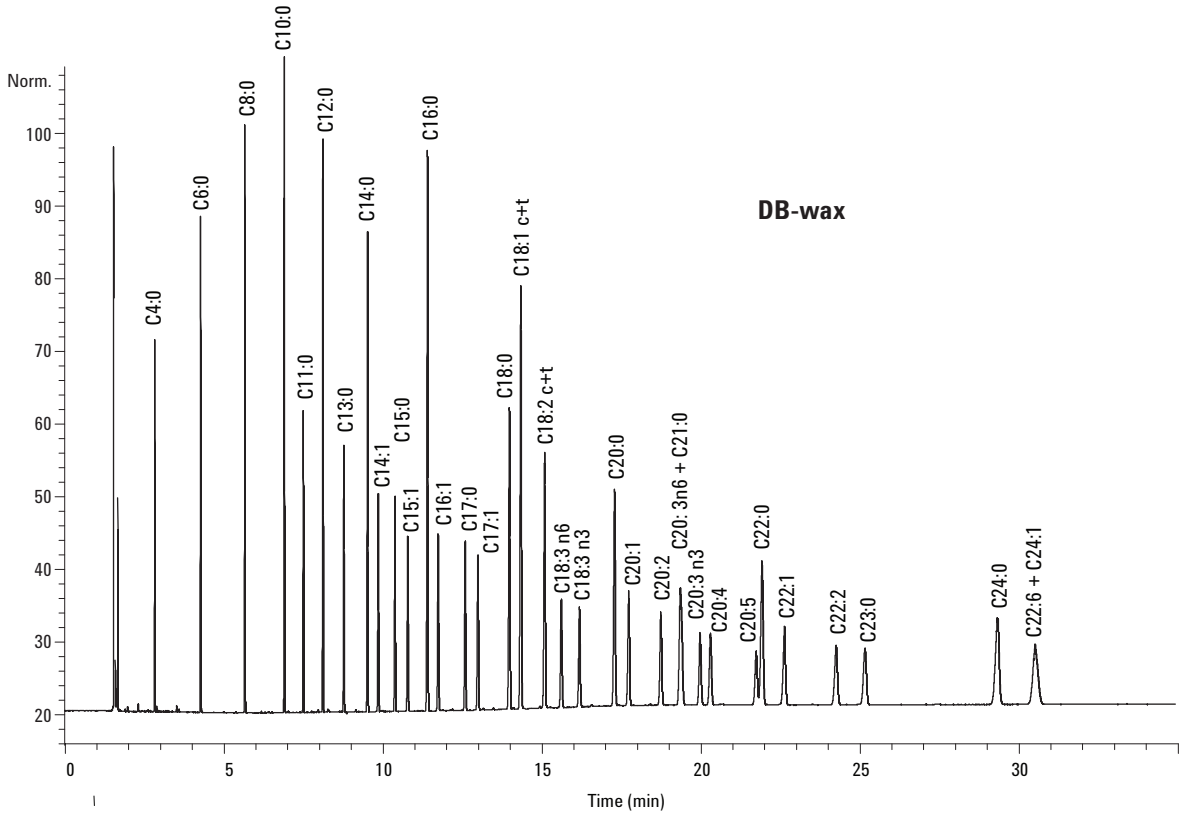


Figure 2. GC-FID analysis of 37-component FAMEs standard mixture on a 30 m × 0.25 mm ID, 0.25 µm DB-Wax column using Method 1. (See Table 2).

A good separation is obtained, except for the following compounds: *cis*- and *trans*-C18:1 coelute at 14.38 min, *cis*- and *trans*-C18:2 coelute at 15.13 min, C20:3 n6 and C21:0 coelute at 19.44 min, and C22:6 and C24:1 coelute at 30.73 min. However, this separation is sufficient for some classical oil and fat characterization methods. Butyric acid elutes at 4.28 min and can be determined in milk fat using this method. This is demonstrated in Figure 3, showing the analysis of a certified reference sample of milk fat (CRM 164, [6]).

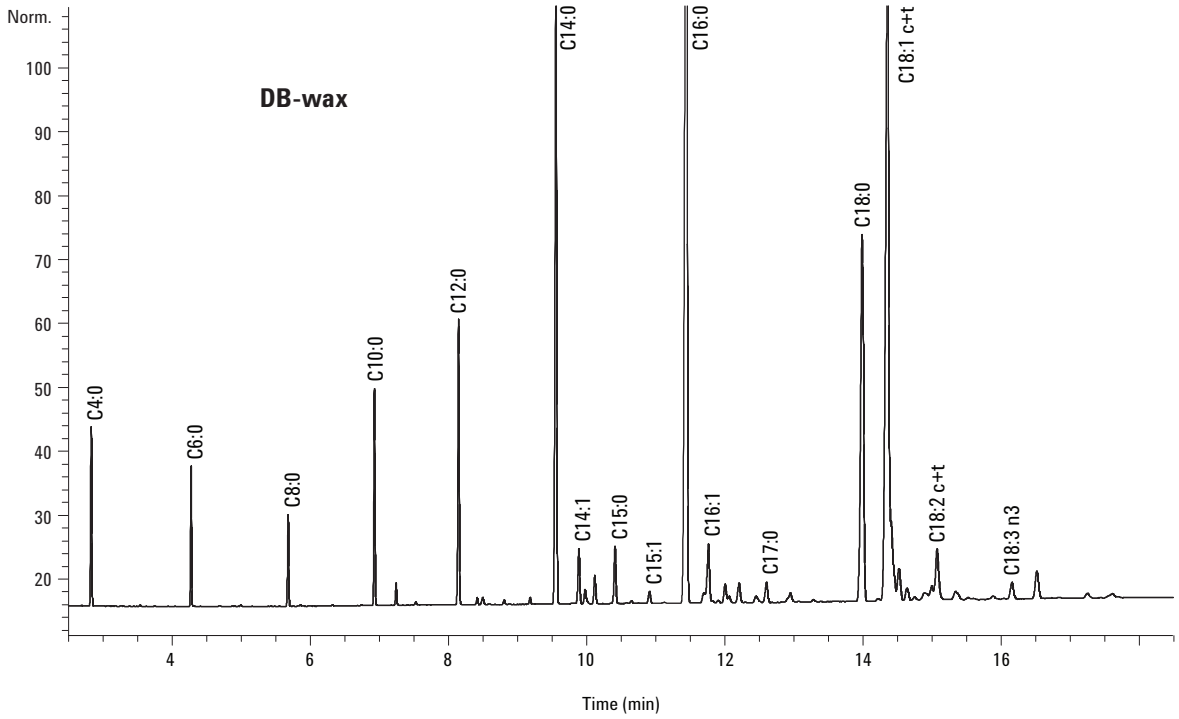


Figure 3. GC-FID analysis of milk fat (CRM 164) fatty acids on a 30 m × 0.25 mm ID, 0.25 µm DB-Wax column using Method 1, Table 2.

The separation of the 37-compound FAME standard mixture on the 60 m × 0.25 mm ID, 0.15 µm DB-23 column is shown in Figure 4.

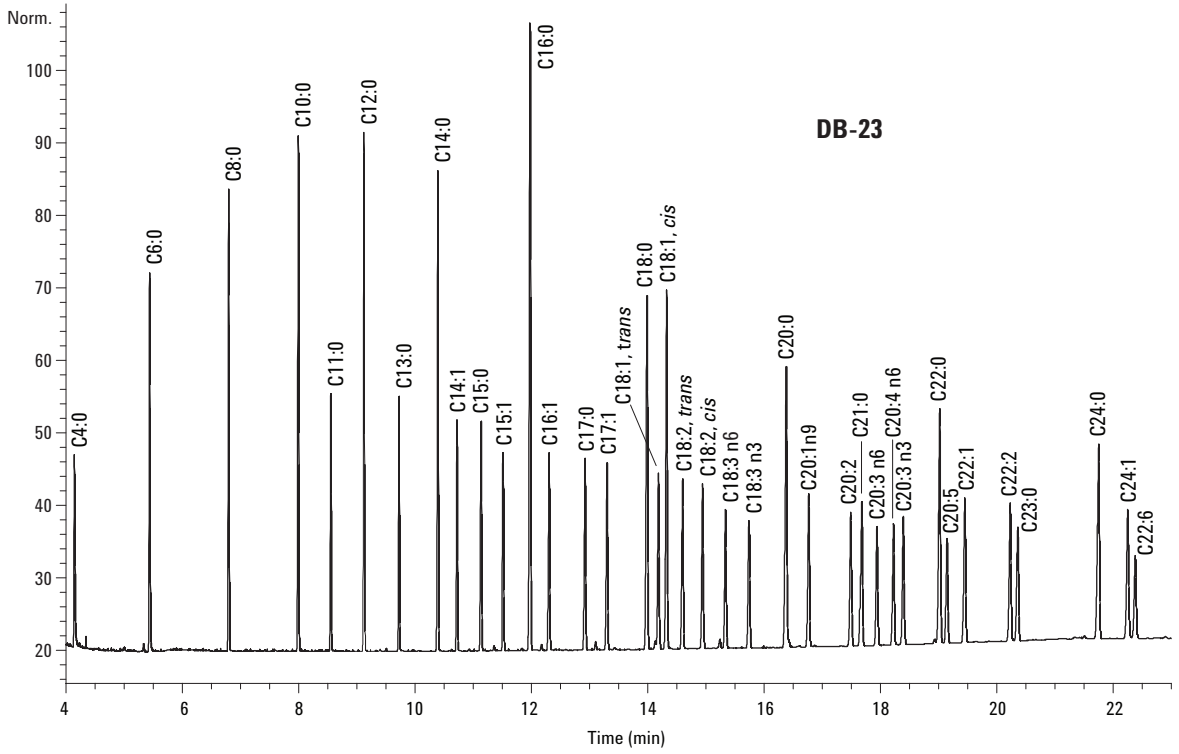


Figure 4. GC-FID analysis of FAMEs standard mixture on a 60 m × 0.25 mm ID, 0.15 µm DB-23 column using Method 2, Table 3.

Using these conditions, all compounds in the standard mixture are well resolved. Important is the separation of the *cis/trans* isomers and the separation of EPA (19.15 min) and DHA (22.38 min) components. This method is very useful for the analysis of fatty acid in complex mixtures, and especially for the determination of omega-3 fatty acids (such as EPA and DHA). An example of the separation obtained for a mixture of polyunsaturated fatty acids from a marine source is given in Figure 5. EPA and DHA can easily be detected and quantified.

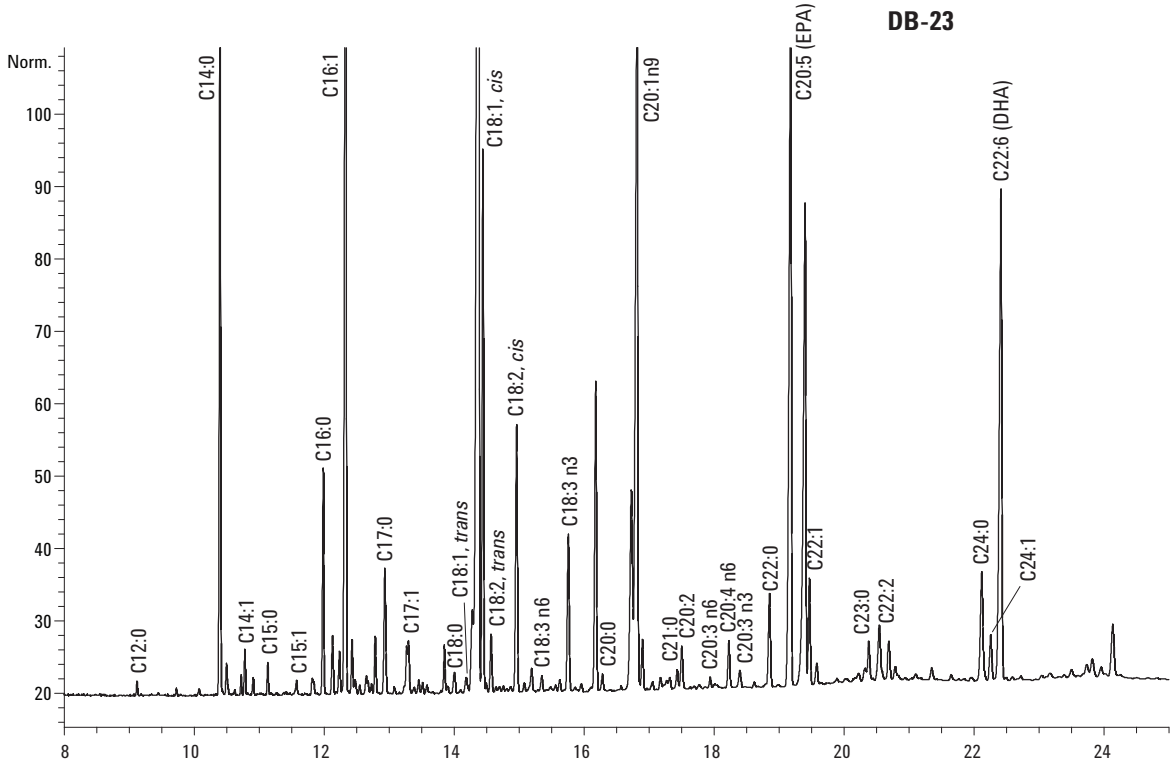


Figure 5. GC-FID analysis of unsaturated fatty acid mixture from marine origin on a 60 m × 0.25 mm ID, 0.15 µm DB-23 column using Method 2, Table 3.

The separation of the 37-compound mixture on the highly polar HP-88 column is shown in Figure 6.

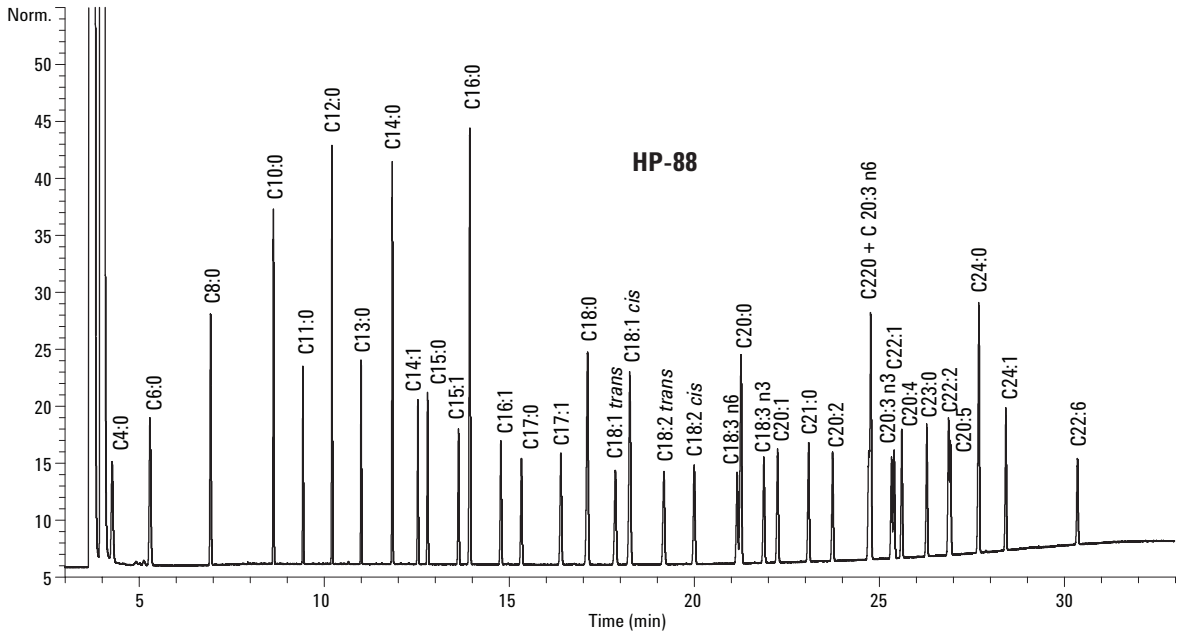


Figure 6. GC-FID analysis of 37-component FAMEs standard mixture on a 100 m × 0.25 mm ID, 0.2 µm HP-88 column using Method 3A, Table 4A.

Again a quite good separation is obtained, except for the separation of C22:0 and C20:3 (n-6) that coelute at 24.7 min. Using this column, however, the separation of *cis*- and *trans*-isomers is excellent. This is illustrated by the separation of a standard mixture containing five C18:1 isomers. The *cis*- and *trans*- positional isomers are well separated, as shown in Figure 7.

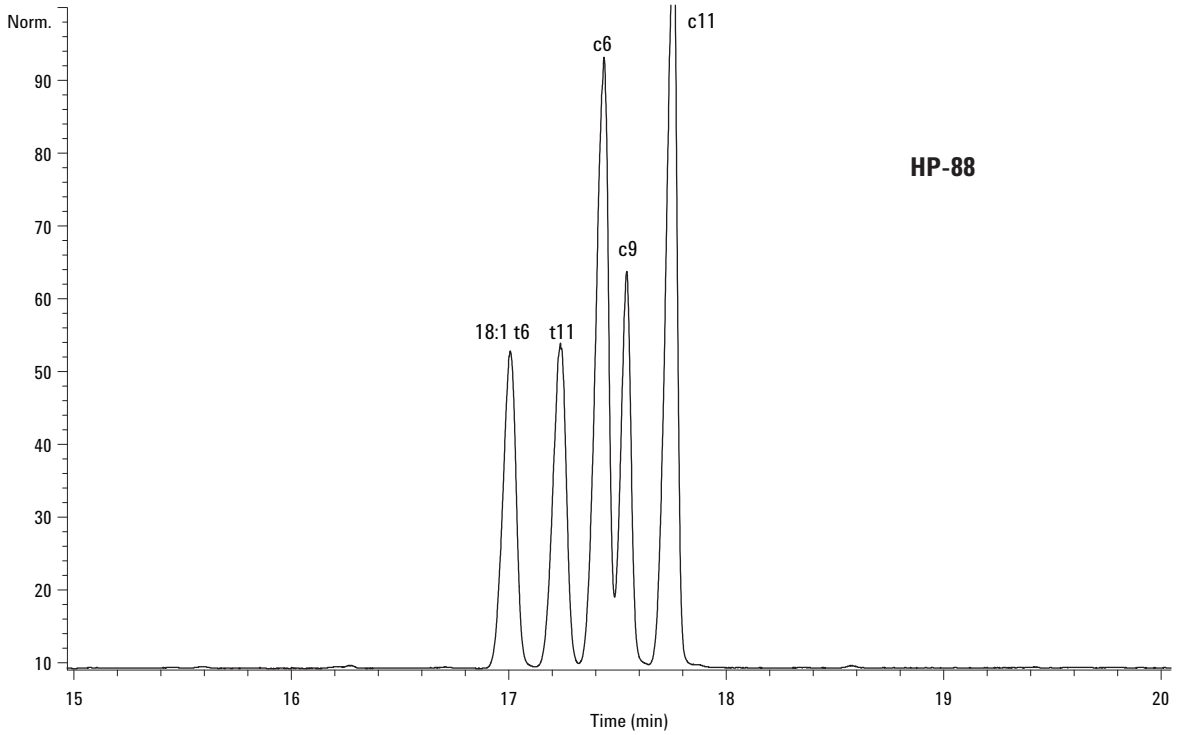


Figure 7. GC-FID analysis of C18:1 isomers on a 100 m × 0.25 mm ID, 0.2 µm HP-88 column using Method 3A, Table 4A.

Equally good separation is obtained for four C18:2 isomers (*trans-trans, cis-trans, trans-cis* and *cis-cis*), as shown in Figure 8.

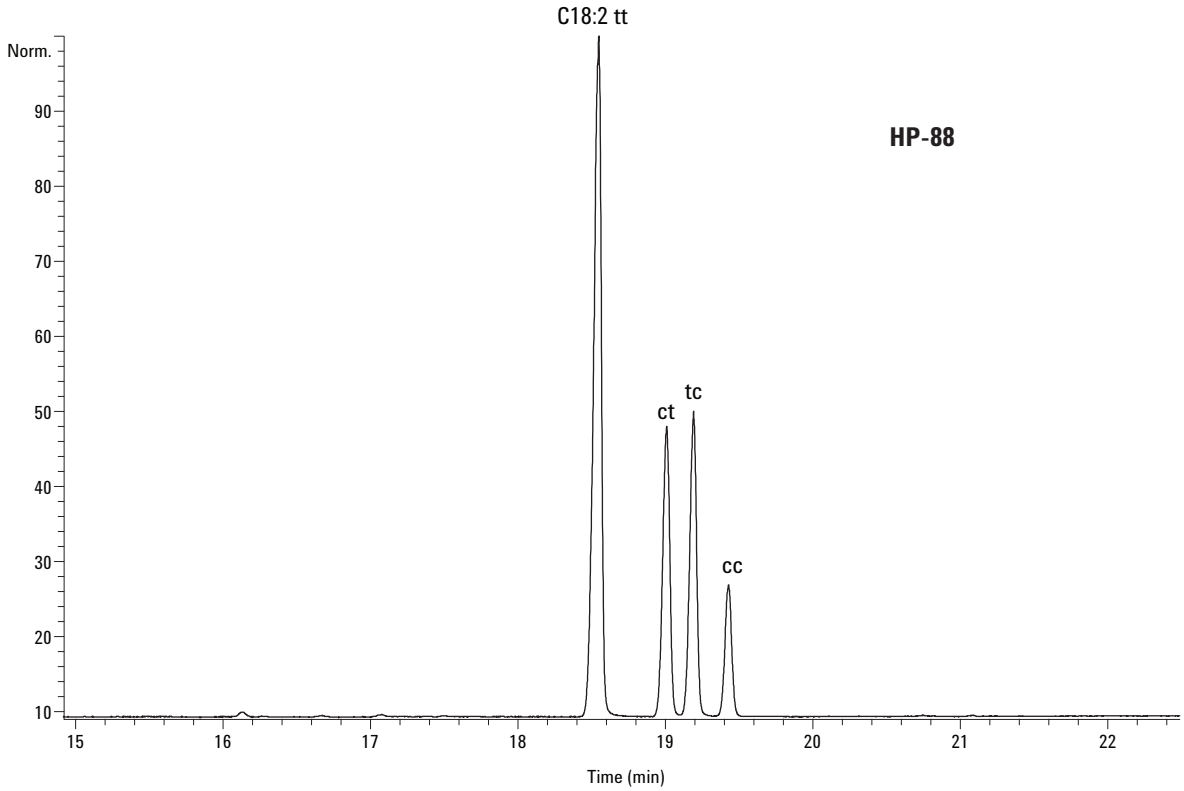


Figure 8. GC-FID analysis of C18:2 isomers on a 100 m × 0.25 mm ID, 0.2 µm HP-88 column using Method 3A, Table 4.

A comparison between a DB-23 and a HP-88 column was made for the separation of a highly hydrogenated oil. Due to the hydrogenation process, all possible positional and geometrical (*cis-trans*) isomers are formed. The sample was analyzed on a DB-23 column and an HP-88 column respectively, both isothermally at 180 °C. the chromatograms are compared in Figure 9 (details of C18:1 elution window). Although Figure 4 shows baseline separation of *trans*-C18:1(n9) and *cis*-C18:1 (n9) in the 37-component standard, Figure 9 demonstrates that for real samples containing several C18:1 isomers, the *cis-trans* separation with the HP-88 is a preferred column choice.

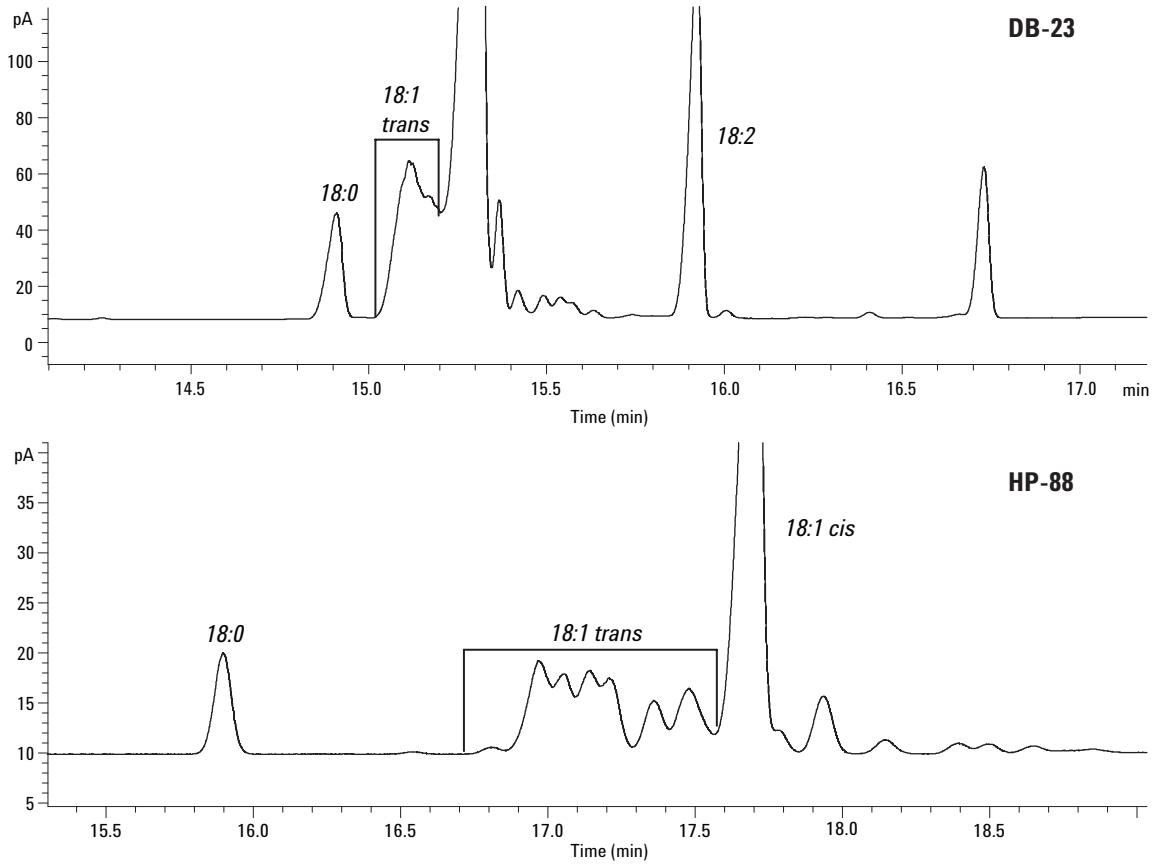


Figure 9. Comparison of the separation of C18:1 isomers from a hydrogenated oil obtained on a 60 m x 0.25 mm ID, 0.15 µm DB-23 column (upper window) and on a 100 m × 0.25 mm ID, 0.2 µm HP-88 column. Both analyses were performed at 180 °C isothermally.

The application of the HP-88 is demonstrated by the analysis of a partially hydrogenated rapeseed oil. The separation of the *trans* fatty acids is clearly illustrated in Figure 10. The valley between *trans*- and *cis*-isomers can easily be determined. Also the other *trans*-isomers (C18:2 and C18:3) can be detected.

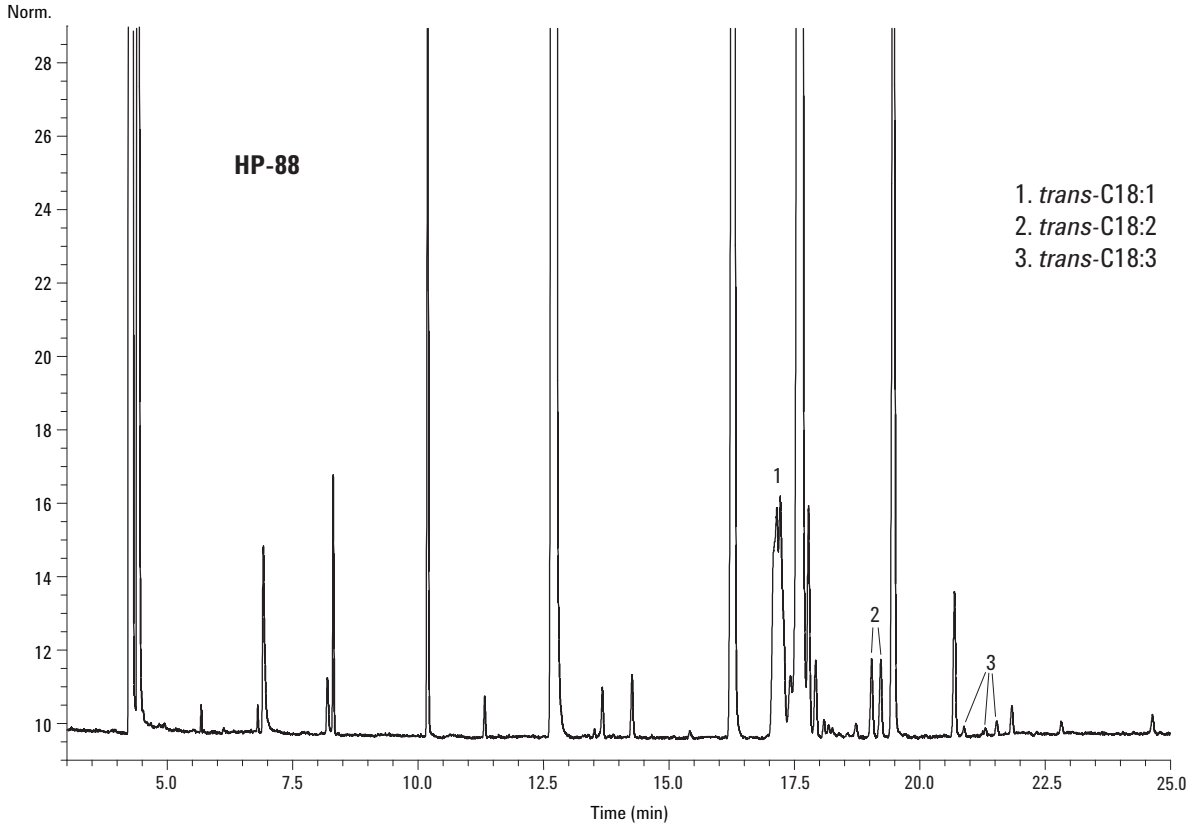


Figure 10. GC-FID analysis of FAMEs from a partially hydrogenated rapeseed oil on a 100 m × 0.25 mm ID, 0.2 µm HP-88 column using Method 3A. (See Table 4).

The same column can also be used for quality control of olive oil according to EC regulation 2568/91 [5]. Using the method described in Table 4 (Column A – Method A), the analysis time for the 100-m column is approximately 35 min using hydrogen as carrier gas. For the QC analysis of olive oil, a 60-m column can also be used (Table 4 – column B). Using helium as carrier gas and a different temperature program (that is, oven temperature B, Table 4), an excellent separation is obtained in less than 20 min, as shown in Figure 11. The obtained separation fully conforms to the EC regulation [5].

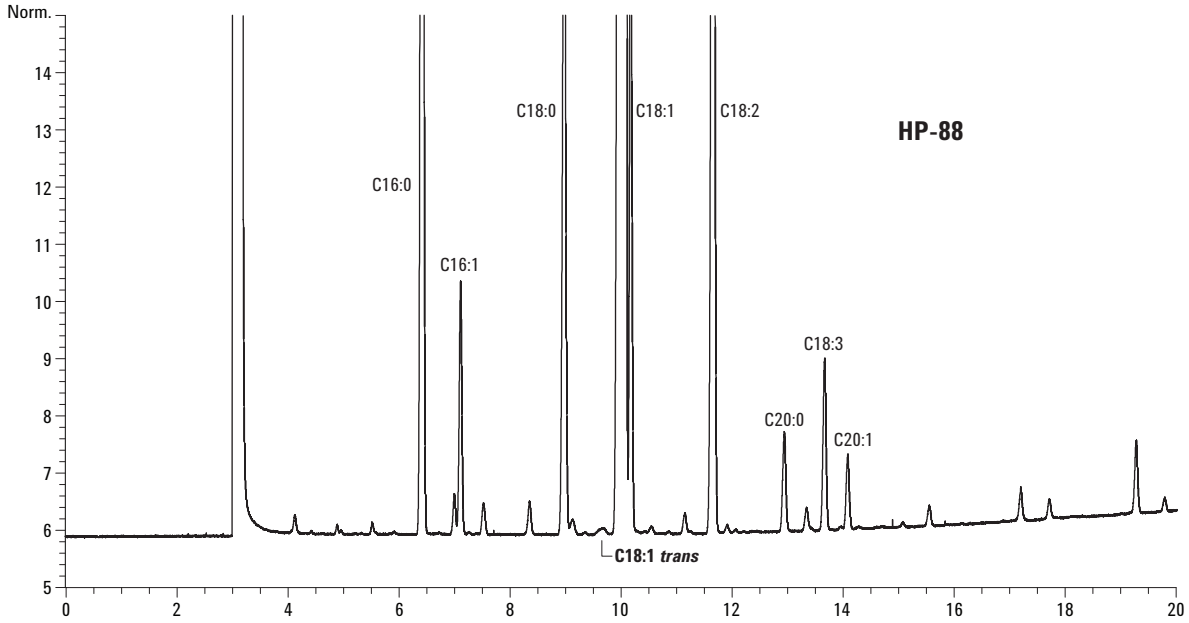


Figure 11. GC-FID analysis of olive oil FAMEs on a 60 m × 0.25 mm ID, 0.2 µm HP-88 column using Method 3B, Table 4.

Conclusions

Three types of stationary phases can be used for the analysis of FAMEs.

1. A DB-Wax column, is useful for the analysis of classical edible oils and fats, including the determination of butyric acid in milk fat. Using this column, however, no separation of *cis-trans* isomers is obtained.
2. A medium polar DB-23 cyanopropyl column is excellent for the analysis of complex FAME mixtures, including fish oils, allowing the determination of omega 3 fatty acids such as EPA and DHA. Partial *cis-trans* separation is obtained.
3. For the most demanding separation of *cis-trans* separation, an HP-88 column is recommended. This column is also the column of choice for olive oil QC analysis.

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High Throughput Separation of Xanthines by Rapid Resolution HPLC

Application Note

Biochemistry, Food and Beverage, Pharmaceutical

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Abstract

Xanthines were found to be optimally separated by reversed-phase HPLC on a C18 column. By a reduction in column length and particle size, the separation time for a mixture of the xanthines investigated was reduced from 8 minutes to 1.5 minutes without a major loss in resolution. A simple isocratic HPLC method was used and described to analyze theobromine, theophylline and caffeine in liquid refreshments (tea, chocolate syrup, and cocoa).

Introduction

Xanthines are a group of alkaloids that are commonly used for their effects as mild stimulants and as bronchodilators, notably in treating the symptoms of asthma. The most common xanthine is caffeine and it is found in foods such as coffee beans, tea, kola nuts, and in small amounts in cacao beans. Surprisingly, chocolate is a weak stimulant due to its content of theobromine, theophylline, and caffeine which are all methylxanthines. The chemical structures of these xanthines and some of their metabolites are depicted in Figure 1.

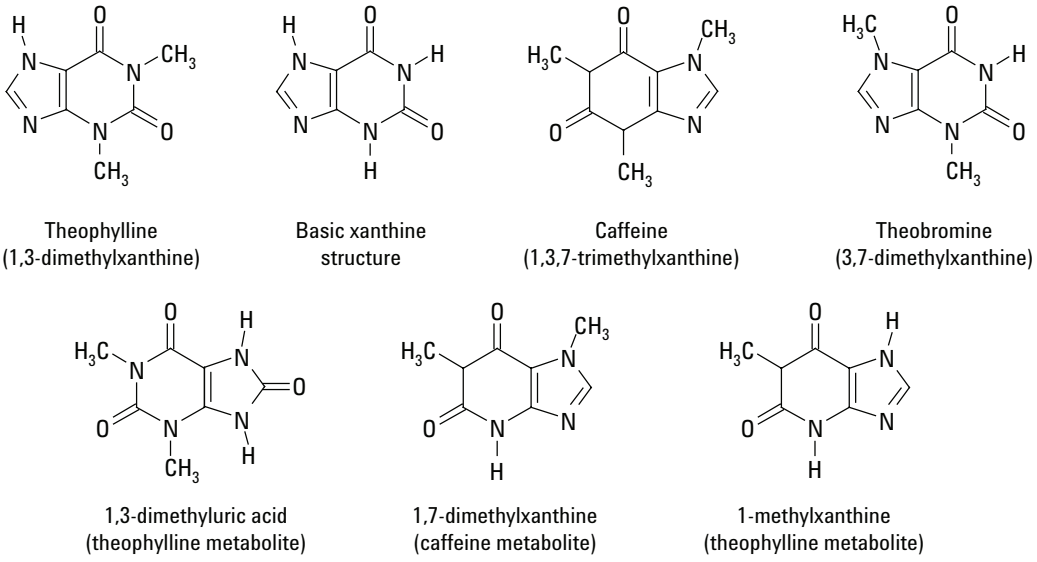


Figure 1. Structures of selected xanthines and metabolites used in this study.

The xanthines are absorbed in the body almost 100% and they appear in the blood in a few minutes after ingestion. Xanthines stimulate the central nervous system, can affect the circulatory system, and relax muscles in the bronchi. Caffeine is well known for its effect on reducing drowsiness and fatigue and improving alertness. These common xanthines are metabolized to a variety of compounds that may have physiological effects on the human body.

The xanthines are most often separated by reversed-phase HPLC (RP-HPLC) on a C18 column [1–3]. Although ion pair chromatography has been used for xanthine separation [2], RP-HPLC with buffered water and acetonitrile requires a much simpler mobile phase system. This application note will show how different stationary phases may impart different selectivities for xanthine separations and will also investigate the effect of particle size and column length on the separation speed. Finally, it will show an application of a method for the analysis of caffeine and theobromine in chocolate-based drinks.

Selection of the Stationary Phase for the Separation of Xanthines

HPLC allows for the resolution of peaks of interest in the shortest possible time. Selection of the appropriate stationary phase is an important step in method development. Initially, several different stationary phases were tried in order to choose an appropriate one for these investigations. Figure 2 shows the separation of the xanthine components in the test mixture using ZORBAX StableBond phases [cyano (CN), phenyl, C18] and a polar embedded stationary phase (ZORBAX Bonus RP).

Although the particle size was different for the Bonus column, our objective here was to choose the phase with the best selectivity and the shortest retention time. Under the conditions employed, the CN column gave least retention (by virtue of its shorter alkyl chain length), but the column failed to resolve two of the test xanthines. The SB-C18 column gave the best overall separation in the shortest time and thus became the stationary phase of choice.

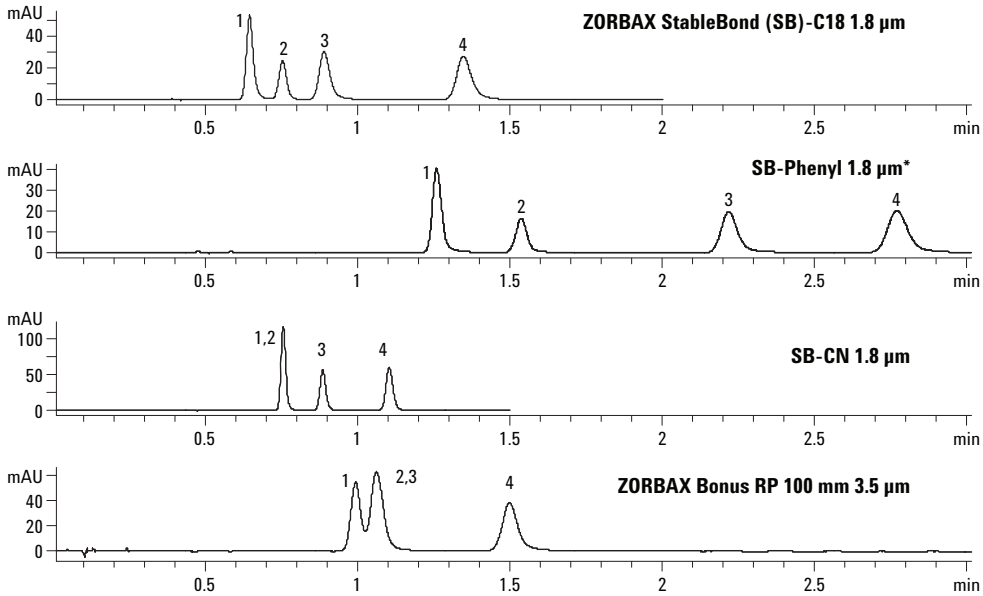


Figure 2. ZORBAX stationary phase selectivity comparisons for xanthines.

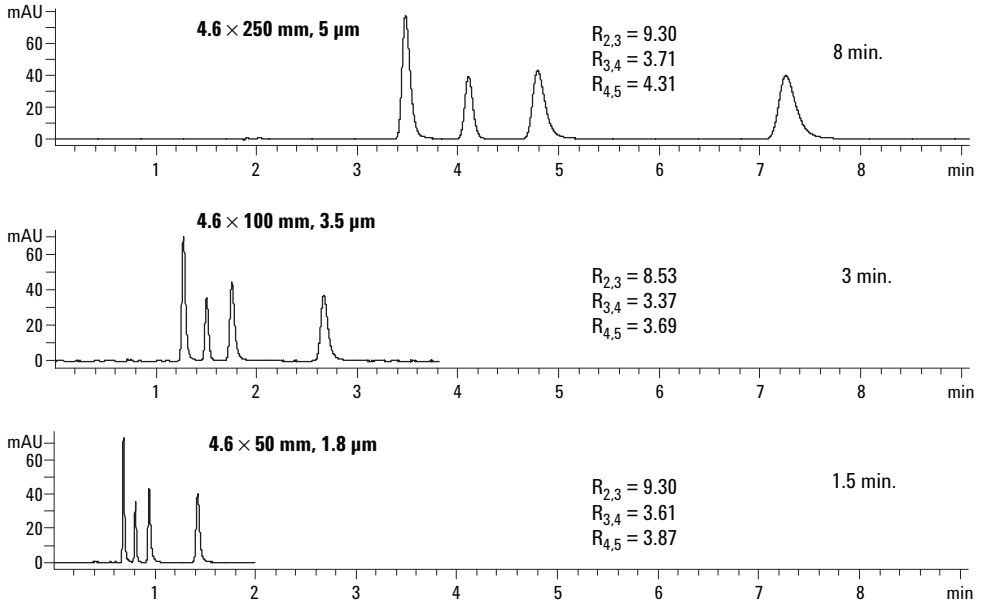
The chromatographic conditions chosen for subsequent experiments appear below:

LC Conditions

Column:	ZORBAX SB-C18 (various lengths and particle diameters shown on chromatograms),
Mobile phases:	A= 0.2% Formic acid (FA) B=Acetonitrile with 0.2% FA
Isocratic composition:	98% A 2% B (v/v)
Flow rate:	1.5 mL/min; Injection volumes are shown on chromatograms
Detection:	DAD, 254 nm
Flowcell:	3 µL, 2-mm flow path

The Effect of Particle Size and Column Length on the Separation of Xanthines

Recent trends in HPLC have pointed to the use of shorter columns with smaller particles. The end result is a faster separation with the same or similar resolution. Figure 3 depicts the isocratic separation of the xanthine test mixture on three different columns (250 mm, 100 mm, and 50 mm) packed with three different particle sizes of ZORBAX StableBond C18 (5 µm, 3.5 µm, 1.8 µm, respectively). As the column length decreases, one would expect to see shorter retention times, proportional to the decrease in length. Indeed Figure 3 clearly shows a decrease in overall separation time from 8 minutes to 1.5 minutes.



Solutes:
1 1-methylxanthine
2 1,3-dimethyluric acid
3 3,7-dimethylxanthine
4 1,7-dimethylxanthine

Column:	ZORBAX SB-C18 (see chromatogram for dimensions and particle size)
Mobile phase:	A = 0.2% FA B = AcCN w 0.2% FA
Isocratic composition:	98% A 2% B (v/v)
Flow rate:	1.5 mL/min
Injection volume:	2, 4, 6 µL, respectively
Detector:	DAD, 254 nm
Flowcell:	3 µL, 2-mm flow path

Figure 3. Column scalability: change in column configuration to increase speed while maintaining resolution.

On the other hand, one would also expect to see a reduction in column efficiency. However, by reducing the particle size, the overall efficiency and resolution is nearly the same. The calculated resolution for all pairs of xanthines is shown on Figure 3. This is the rapid resolution concept where a combination of shorter columns and smaller particles led to equivalent separations at greatly reduced separation time. Since the flow rate is the same, in this case 1.5-mL/min, the solvent use is decreased proportional to column length resulting in an overall cost reduction. Another advantage when converting to shorter columns is that the peaks are narrower. Thus, if the same sample mass is injected the resulting increase in peak height provides greater sensitivity. In Figure 3, the sample volume was reduced proportional to column length to keep peaks nearly the same peak height.

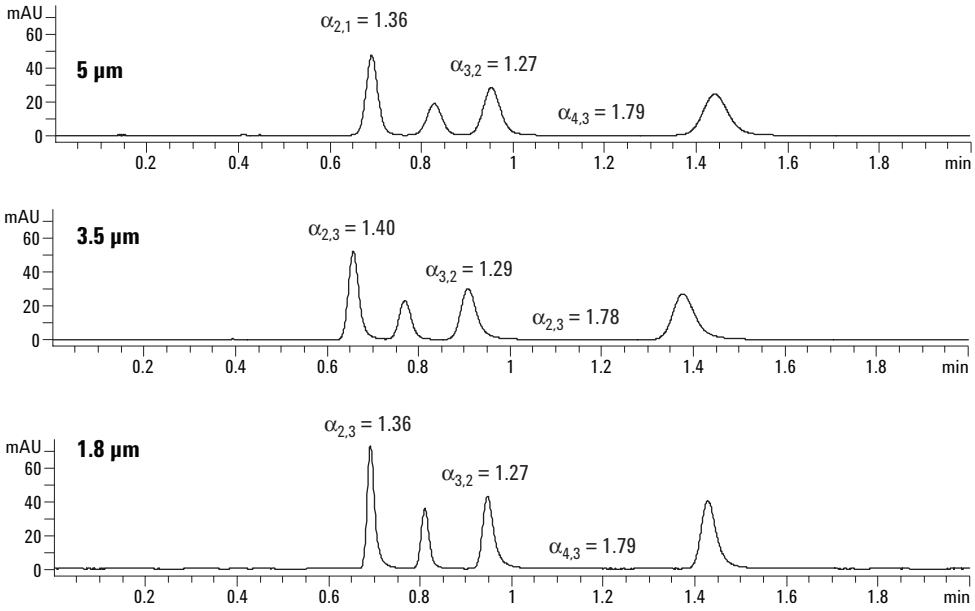
Of course, as one decreases the particle size of a column, the column backpressure increases with the inverse square of the particle diameter. Thus, if the same column length was used, the pressure at the same flow rate (or more correctly the linear velocity), the pressure would go up by a factor of 2 for a 3.5-µm particle versus a 5.0-µm particle and a factor of almost 8 for a 1.8-µm particle. However, with the increase in plate count for the smaller particles, columns can be shortened and the actual pressure increase is more nominal as can be seen in Table 1. Agilent's engineered particle size distribution helps to keep the pressure lower than what one would anticipate for a 1.8-µm column.

Table 1. Pressure as a Function of Particle Diameter and Column Length*

Particle diameter, um	Column length, mm	Pressure, bar	Pressure increase (relative to 5.0)
5	250	181	1.0
3.5	100	155	0.86
1.8	50	264	1.46

* Conditions of Figure 3

In order to demonstrate that a change in the particle size of the column packing has a minimal effect on selectivity, the isocratic separation of the xanthine test mix as a function of particle size at constant column length was investigated. Figure 4 shows a minimal variation in retention but a significant decrease in peak width in going from the 5-µm column to the 1.8-µm column. In other words, the column showed more efficiency and subsequent better resolution for the 1.8-um column but the selectivity was mostly unaffected. The HPLC conditions are shown on the chromatogram in Figure 4.



Solutes:
1 1-methylxanthine
2 1,3-dimethyluric acid
3 3,7-dimethylxanthine
4 1,7-dimethylxanthine

Column: ZORBAX SB-C18
Mobile phase: A = 0.2% FA
B = 0.2% FA in MeCN
Isocratic composition: 98% A 2% B (v/v)
Flow rate: 1.5 mL/min
Injection volume: 2 µL
Flowcell: 2 µL, 3-mm flow path

Figure 4. Column selectivity as a function of particle size.

Analysis of Xanthines in Liquid Refreshments

The three most common xanthines are caffeine, theophylline, and theobromine. These xanthines may be present in a variety of drinks, either as part of the flavoring or added to enhance taste or increase alertness. We developed a simple isocratic method to analyze for them in chocolate drink and tea. Using the same chromatographic conditions described earlier; an excellent separation of a standard xanthine mixture was achieved. See Figure 5.

Next, three different liquid refreshments-hot cocoa, chocolate syrup, and black tea (bag) were prepared using directions on the container, but using sonication for mixing. After preparation, all solutions were centrifuged and then the aqueous portion was filtered through a 0.45-micron filter to remove any particulates that may foul the HPLC column. In particular, the hot cocoa gave a distinctive fat layer, but only the aqueous layer was sampled for analysis. For each sample, a 3-µL injection of the aqueous extract was made. See results in Figure 6.

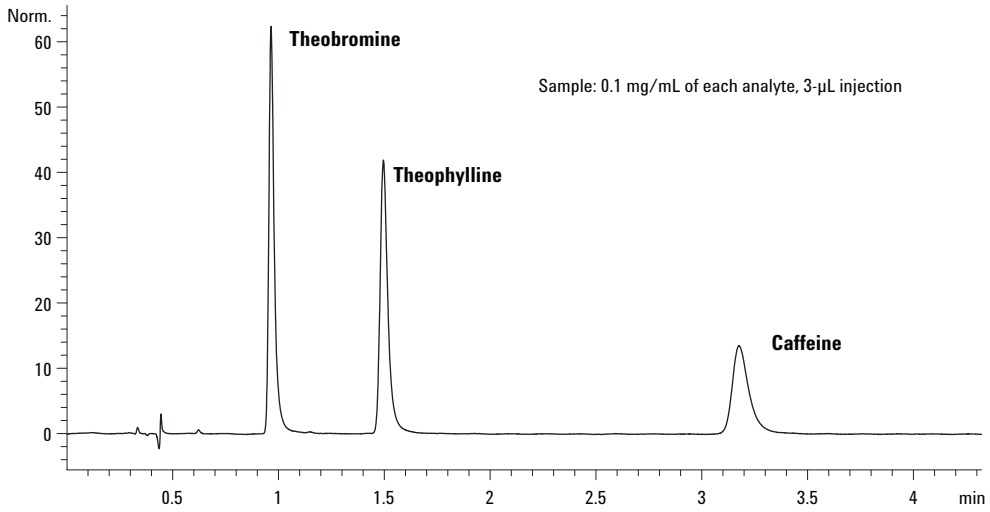
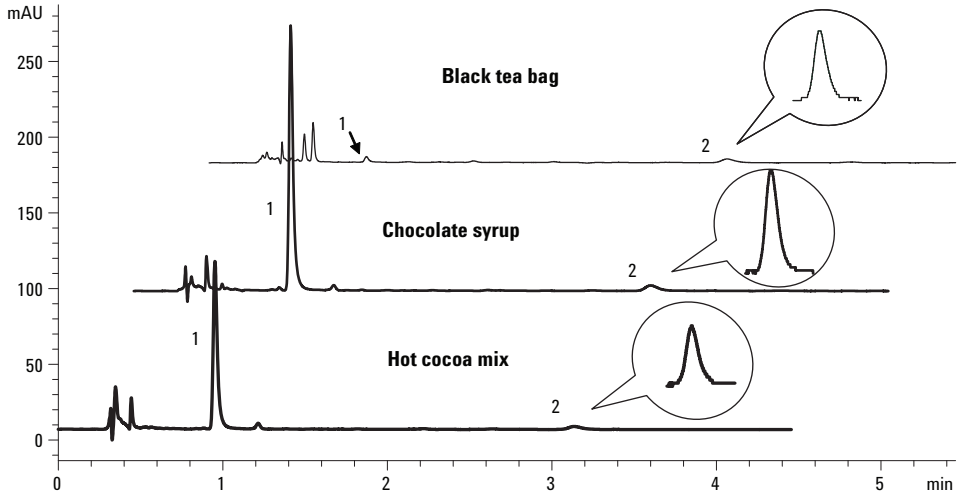


Figure 5. Separation of xanthine standards.



Peak 1: Theobromine
Peak 2: Caffeine (callouts show expanded absorbance range X10)

Figure 6. Analysis of liquid refreshments for xanthines.

From the raw areas we were able to do a semi-quantitative analysis (single-point calibration) of the three xanthines in the drinks. As can be seen from Table 2, in the chocolate drinks, relatively large levels of theobromine were observed but smaller amounts of caffeine while for the tea sample, caffeine was in an excess. The results of Table 2 were based on a weight/weight basis and not on total milligrams in the drink solution itself. These results are within the concentrations expected based on the manufacturer's approximations. No theophylline was observed in any of the drinks.

Table 2. Determination of Xanthines in Liquid Refreshments

Beverage	Theobromine (%, wt/wt)	Caffeine (%, wt/wt)
Hot chocolate	0.15	0.011
Chocolate syrup	0.13	0.011
Tea	0.056	0.17

Conclusions

Xanthines were found to be optimally separated by reversed-phase HPLC on a C18 column. By a reduction in column length and particle size, the separation time for a mixture of the xanthines investigated was reduced from 8 minutes to 1.5 minutes without a major loss in resolution. A simple isocratic HPLC method was used to analyze theobromine, theophylline and caffeine in liquid refreshments (tea, chocolate syrup, and cocoa).

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Analysis of Suspected Flavor and Fragrance Allergens in Cosmetics Using the 7890A GC and Capillary Column Backflush

Application

Food

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Abstract

Flavor and fragrance allergens are determined in cosmetics using GC-MS. After simple sample preparation by nonselective extraction/dilution, extracts were injected and analyzed under fast screening conditions and locked retention times. After elution of the target solutes, the low-volatility matrix constituents, such as detergents, were effectively removed using capillary column backflush. Column and detector contamination were thereby strongly reduced and sample throughput was significantly increased.

Introduction

According to EU directive 2003/15/EC [1], 27 fragrance compounds in cosmetic products should be labeled if their concentrations exceed 100 ppm (mg/kg) for wash-off products, such as shower gels or soaps, or 10 ppm for leave-on products, such as perfumes or creams. Therefore, qualitative and quantitative methods are needed to monitor these target solutes in various types of cosmetic products.

Depending on the sample matrix and solute concentrations, different sample preparation methods are developed and applied [2]. For the determination of allergens in cosmetic products, one of the major problems is related to the presence of less volatile or nonvolatile constituents, such as detergents (nonionic or ionic), waxes, lipids, etc. These constituents will contaminate the analytical system if the samples are introduced without selective sample preparation. Selective extraction or selective sample introduction is not easy, however, since the target compounds cover a broad volatility range (from limonene to benzyl benzoate) and polarity range (from relatively polar benzyl alcohol to apolar benzyl benzoate). The method of choice should therefore give ppm sensitivity on one hand, and avoid discrimination of the target solutes based on relative volatility or polarity, on the other hand. Moreover, for routine analysis in a quality control environment, sample preparation should be minimized and direct injection of a nonselective solution or extract is preferred. Recently, liquid sample introduction with selective retention of nonvolatiles in a packed PTV liner in combination with automated liner exchange was developed and validated [3]. This approach, however, requires a dedicated sampler.

In this application, an alternative method is proposed using a standard split/splitless inlet and Capillary Flow Technology. A QuickSwap device is used at the end of the column (coupled to the mass spectrometer transfer line), thereby allowing

column outlet pressure to be controlled with auxiliary electronic pneumatic control (EPC). By lowering the inlet pressure and raising the outlet pressure after the last peak of interest has eluted from the column, sample components remaining in the column are forced back out the head of the column into the split inlet and are subsequently trapped on the split vent trap.

The analysis is performed by GC-MS under retention-time locked conditions. The reference method, using a 30 m × 0.25 mm id × 0.25 µm HP-5MS column operated under helium [2], was translated to a fast screening method for maximum throughput, using a 15-m column and hydrogen as the carrier gas. The analysis time needed for the separation of the target solutes was thereby reduced from 24 to 8 minutes (3X speedup). The low-volatility sample matrix constituents are backflushed from the column, avoiding column and detector contamination, baseline shifts, and ghost peaks due to carryover into subsequent runs.

Sample Preparation

Samples are diluted to the 5% level (50 mg/mL) in an appropriate solvent (typically, acetone or dichloromethane is used). The sample is placed in an ultrasonic bath for 15 minutes to completely dissolve the target solutes in the solvent. After extraction and dissolution, the sample can be centrifuged and the supernatant transferred to an autosampler vial.

In this application, data were obtained on a shampoo sample containing fragrance compounds and nonionic detergents.

GC conditions

All analyses were performed on an Agilent 7890A GC-5975 MSD combination. Injection was done using a 7683 ALS. The GC-MS conditions can be summarized as follows:

GC-MS Conditions

Column	15 m x 0.25 mm id x 0.25 µm HP-5MS	Agilent P/N 19091-431
Carrier gas and pressure	Hydrogen	11.050 psi constant pressure
Column outlet and pressure	QuickSwap	4 psi via auxiliary EPC
Inlet	Split/splitless in split mode	250 °C, split ratio = 50:1
Oven temperature program	Fast analysis (3X speedup*)	50 °C (0.33 min) → 240 °C at 24°C/min
MSD setpoints	Transfer line temperature	250 °C
	Source temperature	300 °C
	Quad temperature	150 °C
Tune	Autotune	EMV +0V
QuickSwap restrictor	17 cm x 110 µm id (4 psi)	P/N G3185-60063
Detection	MS in scan mode	40–350 amu, samples = 2[1]
MSD events	Solvent delay	1.5 min
	Detector OFF (during backflush)	8.0 min

* Under these conditions, alpha isomethyl ionone elutes at 5.17 min, corresponding to a speed gain factor of 3 in comparison to a previously published retention time locking (RTL) method [2].

Backflush conditions (initiated at 8 min)

Inlet pressure	2 psi
Auxiliary pressure	70 psi
Backflush time	2.75 min
Backflush temperature	240 °C

Results

First, the shampoo extract was analyzed in a typical mode—without applying backflush and programming the oven to 320 °C to ensure that late eluters were eluted. In Figure 1, the overlay of the total ion chromatograms of 10 consecutive runs is shown. Excellent retention time and peak area repeatability is obtained in the first part of the chromatogram.

In this sample, some allergens could be detected, including limonene (peak 1), linalool (2), eugenol (3), lilial (4), hexyl cinnamaldehyde (5), benzyl benzoate (6), and benzyl salicylate (7). After 8 minutes, no target solutes elute, but peaks corresponding to nonionic detergents are detected. Even using a bakeout at 320 °C, these compounds are not completely removed from the column. This can be seen from the appearance of ghost peaks (for instance, one at 11.7 minutes indicated by an arrow). This peak and others due to carryover increase regularly with added sample injections, clearly indicating that not all low-volatility sample material elutes from the column. Also, an increasing baseline is clearly observed after 10 minutes. It should be noted that from this 14-minute run, only the first 8 minutes are in fact needed for the necessary separation and quantitation of the target allergens. The remaining time represents the common practice of trying to removing highly retained sample components from the column by "baking the column out." As demonstrated here, this is not so easily accomplished.

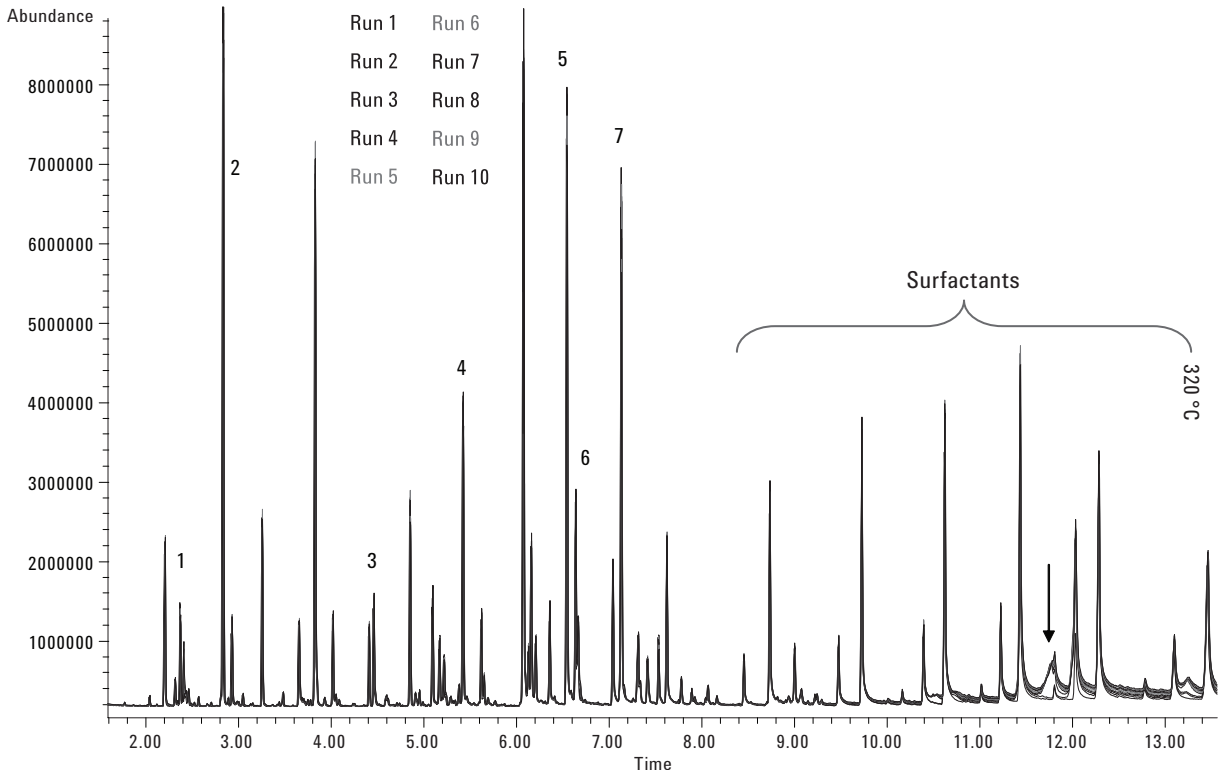


Figure 1. Overlay of 10 consecutive analyses of shampoo extract (oven temperature programmed to 320 °C, no backflush).

After this sequence of 10 sample runs, two blank runs were made. The chromatograms are given in Figure 2. Some contaminant peaks (probably extractables from repeated penetration of sample vial septum) elute around 6 to 8 minutes and are constant in both blank runs. The large peaks, eluting after 10 minutes, clearly show that high molecular weight materials were building up in the column and that these compounds were not removed, even by programming to 320 °C.

In a subsequent experiment, another six consecutive runs of the shampoo extract were made. For each analysis, the run was stopped at 8 minutes after the retention time of the most highly retained target allergen. After the sample runs, two blanks were run: one with the same temperature program as the samples, ending at 240 °C (8 minutes), and another in which the temperature program continued to 320 °C. The chromatograms of the sixth sample analysis, the first blank (stopped at 8 minutes) and the second blank (run to 320 °C) are overlaid in Figure 3.

Some ghost peaks appear within the 8-minute analysis time window, even in the first blank. From the second blank run to 320 °C, it is clear that low-volatility solutes were accumulating in the column from each injected sample. Accumulation of sample material such as that shown in this example quickly leads to column deterioration and greatly reduces the ability to detect and quantify minor sample components. By following the typical approach of attempting to remove late-eluting sample components (cleaning off the column) at high temperature, not only is the column prone to premature degradation due to oxidation and cleavage of stationary phase polymer, but the contamination is moved from the column into the mass spectrometer source, degrading its performance and requiring more frequent cleaning.

Next, a backflush method was set up and 10 new sample runs were made, followed by a blank run. The chromatograms of the sample analyses are shown in Figure 4.

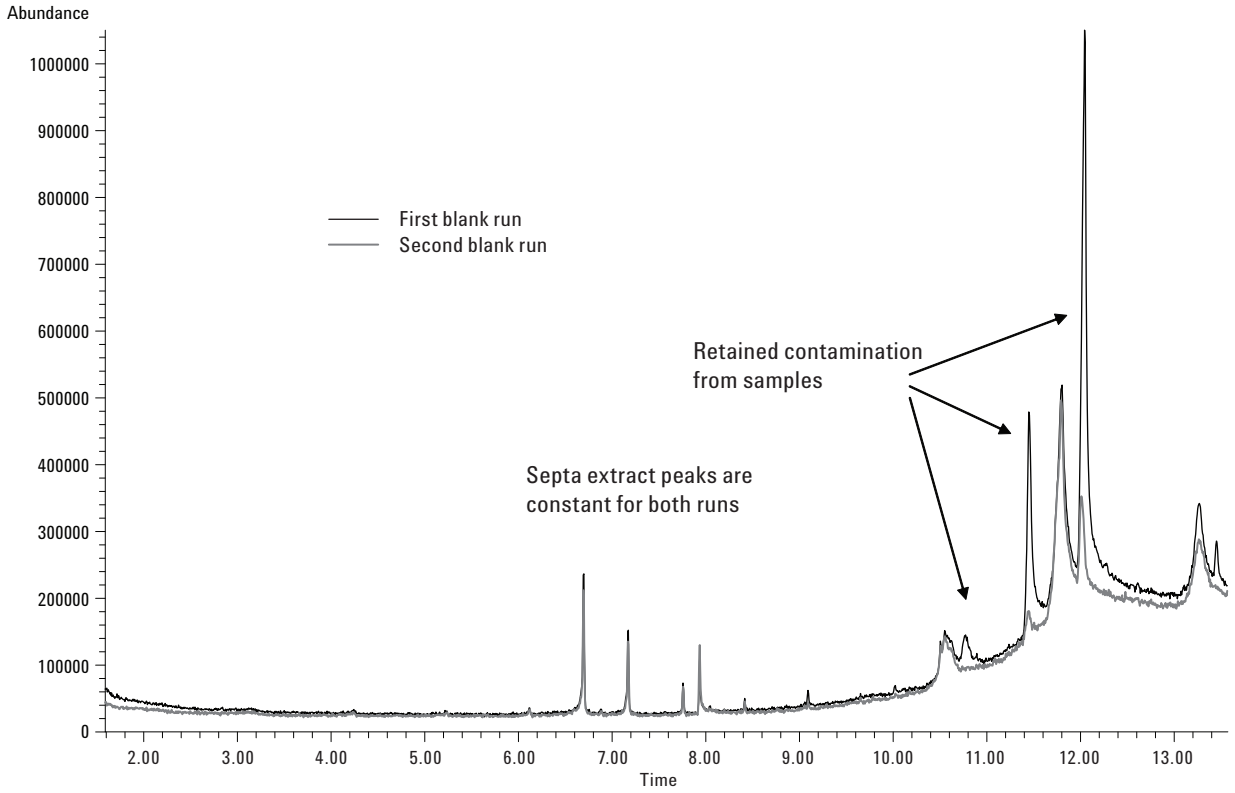


Figure 2. Two consecutive blank runs after analysis of shampoo extract.

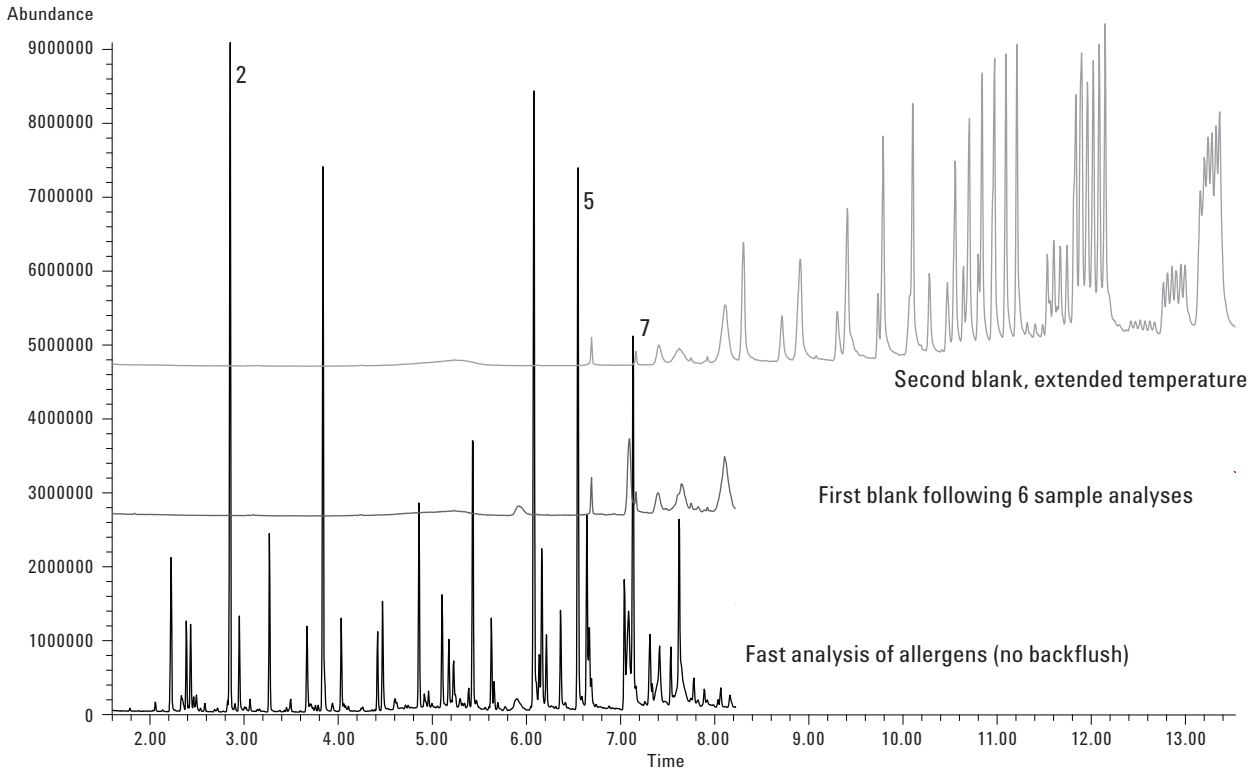


Figure 3. Overlay of sixth analysis of shampoo extract with run stopped at 240 °C (bottom), first blank run to 240 °C (middle), and second blank run to 320 °C (top).

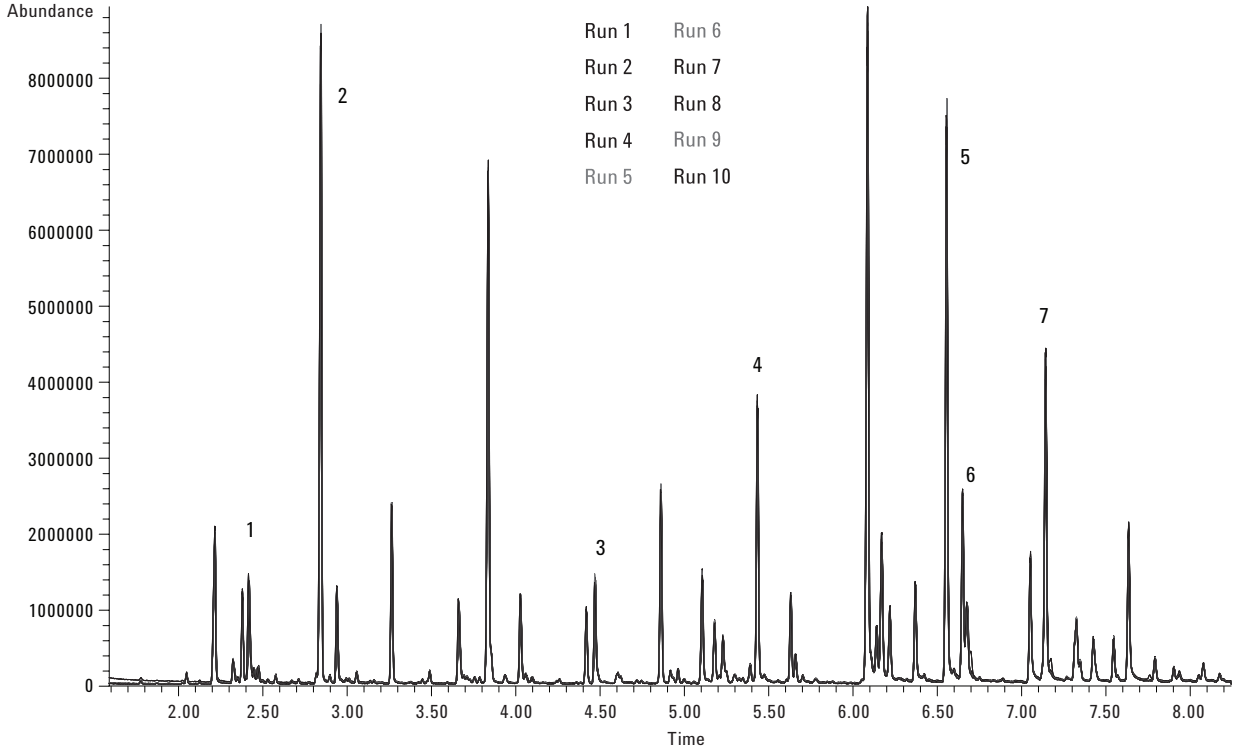


Figure 4. Overlay of 10 consecutive analyses of shampoo extract (oven temperature programmed to 240 °C, with backflush).

From Figure 4, it is clear again that excellent retention time and peak area repeatability were obtained with no evidence of carryover: no emerging ghost peaks; no increasing baseline.

In Figure 5, the tenth run is overlaid with a blank that was run immediately following it. In the blank run, only contaminant peaks coming from the solvent vial septum are observed. The detergent peaks were efficiently and effectively removed from the column.

The total analysis time was reduced from 13.6 min (programmed to 320 °C, with a 2-minute hold) to 11 minutes (programmed to 240 °C, with 2.75-minute backflush). Moreover, all low-volatile material was removed from the column, which was not the case with the longer run without backflush. An added bonus was that the oven cooldown and equilibration times were reduced because of the lower final oven temperature.

Retention time peak area repeatability was determined for each of the seven identified allergens and is listed in Table 1. The standard deviation on the retention times is better than 0.002 minute (RSD < 0.03%). Also, excellent values are obtained for peak area repeatability.

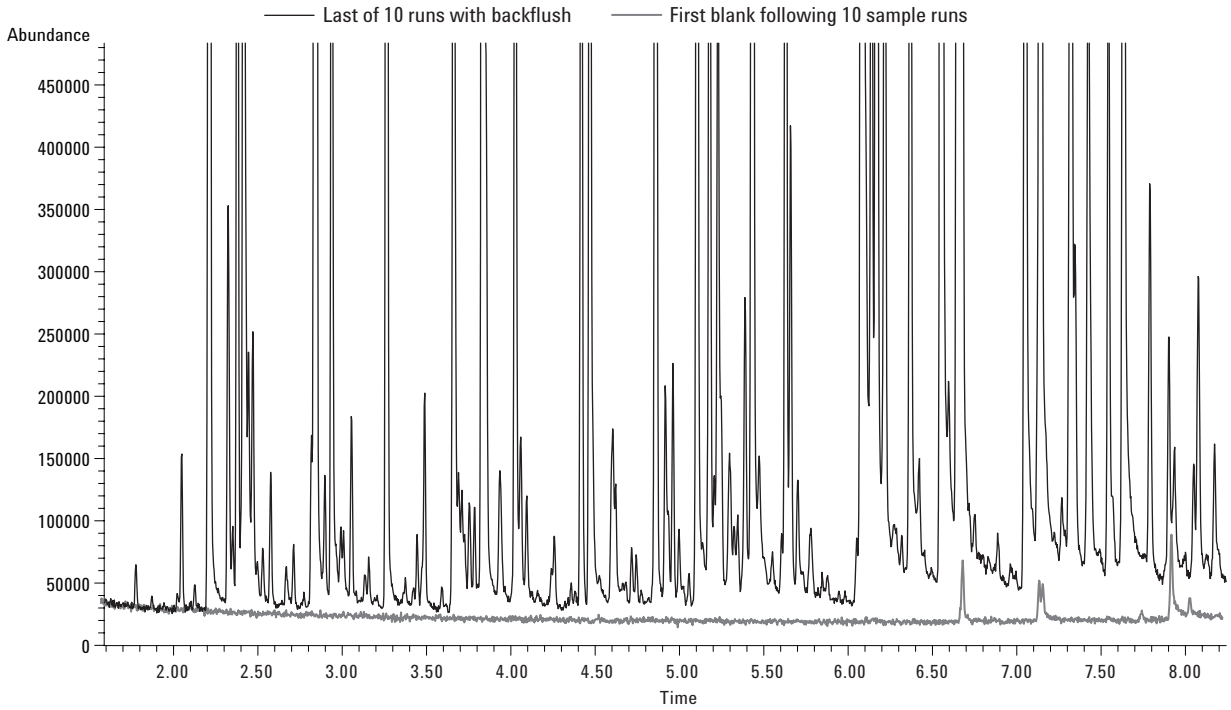


Figure 5. Overlay of 10 analyses of shampoo extract (oven programmed to 240 °C) with backflush (top) and subsequent blank run (bottom).

Table 1. Seven Identified Allergens

	RT min	RT SD min	RT RSD %	Area RSD %
Limonene	2.3771	0.0005	0.020	1.80
Linalool	2.8372	0.0004	0.015%	1.60
Eugenol	4.4671	0.0003	0.007	1.60
Lilial	5.4312	0.0015	0.028	1.53
Hexyl cinnamaldehyde	6.5514	0.0016	0.022	2.00
Benzyl benzoate	6.6467	0.0000	0.000	2.00
Benzyl salicylate	7.1405	0.0013	0.018	2.98
Average		**0.0008**	**0.015**	**1.95**

Conclusions

For the determination of flavor and fragrance allergens in cosmetics, direct sample injection in a split/splitless inlet can be used. In comparison to a previously presented retention time locked method, the analysis time was reduced by a factor of three using a shorter column and hydrogen as carrier gas in combination with 5975 MSD. Contamination of the column and detector was minimized using the backflush method with the 7890A GC. A 20% reduction of the run time is obtained, with faster oven recycle times. Ghost peaks from previous injections were eliminated. Excellent retention time repeatability and peak area repeatability were obtained.

Since the analysis of flavor and fragrance compounds is also performed on columns with a polar stationary phase and limited maximum operating temperature, for example, polyethylene glycol columns (MAOT 250 °C), the capillary column backflush technique using Capillary Flow Technology with the 7890A GC is a very interesting tool to remove highly retained sample components at moderate temperatures.

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Analysis of Suspected Flavor and Fragrance Allergens in Perfumes Using Two-Dimensional GC with Independent Column Temperature Control Using an LTM Oven Module

Application Note

Food and Flavors

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Abstract

Several different analytical methods based on GC/MS are used for the determination of flavor and fragrance allergens in raw materials and cosmetic products in accordance with EU Directive 2003/15/EC. For complex perfume samples with possible coelution of target compounds with other solutes, two-dimensional GC with heartcutting is preferred.

In this application note, a multidimensional capillary GC method is presented coupling Deans switch heartcutting with GC/MS and a low thermal mass (LTM) column module for optimal separation and quantitation of regulated allergens in complex samples. The method was applied to a perfume sample containing several regulated allergens. By using an LTM column module, the temperature of the second column could be controlled independently from the primary column in the main GC oven. Allergens were heartcut to the LTM at 50 °C, where they were focused and then later separated in an independent temperature program, resulting in optimum selectivity and better resolution of target compounds from sample matrix.

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Introduction

Recent European regulation requires allergen compounds to be monitored in fragranced products [1]. The target compounds include some common organic compounds such as limonene, citral, and cinnamic aldehyde. These compounds are often detected in natural products but can cause irritation to sensitive skin. According to the regulation, cosmetic products should therefore be labeled if the allergens are present above specified concentrations (10 ppm in "leave-on" and 100 ppm in "rinse-off" products). Consequently, effective methods are needed for qualitative and quantitative determination of the targeted compounds in these complex matrices.

The official target compound list includes 24 compounds. Some of the solutes consist of more than one chemical identity. Citral consists of two isomers: neral (Z citral) and geranial (E citral). Lyral also contains two isomers: (3- and 4-(4-hydroxy-4-methylpentyl)-3-cyclohexene-1-carboxaldehyde). Farnesol consists of at least four possible isomers, of which the Z,E (farnesol 1) and E,E isomer (farnesol 2) are the predominant compounds observed. In addition, some related compounds, such as phenylacetaldehyde, estragole, methyl 2-nonynoate, and methyleugenol are also monitored [2]. In total, 31 target compounds are analyzed. The list of solutes is given in Table 1 and the first dimension separation is shown in Figure 1.

Table 1. Target Allergen List in Order of Elution on the Agilent J&W HP-5MS Column

Peak number	Compound
1	Limonene
2	Benzyl alcohol
3	Phenyl acetaldehyde
4	Linalool
5	Estragol
6	Methyl 2-octynoate (= folione)
7	Citronellol
8	Neral
9	Geraniol
10	Geranial
11	Cinnamaldehyde
12	Anisyl alcohol
13	Hydroxy citronellal
14	Methyl 2-nonynoate (methyl octane carbonate)
15	Cinnamic alcohol
16	Eugenol
17	Methyleugenol
18	Coumarin
19	Isoeugenol
20	Alpha isomethyl ionone
21	Lilial (BMHCA)
22	Amyl cinnamaldehyde
23	Lyral 1
24	Lyral 2
25	Amyl cinnamyl alcohol
26	Farnesol 1
27	Farnesol 2
28	Hexyl cinnamaldehyde
29	Benzyl benzoate
30	Benzyl salicylate
31	Benzyl cinnamate

The range of matrices in which the target compounds have to be measured is very broad and includes natural essential oils, synthetic mixtures of flavor and fragrance compounds, natural product extracts, and finished products, such as soaps, gels, shower gels, lipsticks, and other cosmetic products. Moreover, the range of concentrations of the fragrance compounds in these matrices is very wide (from high ppb to percent). It is clear that to analyze all target compounds in all classes of matrices using one single method would be impossible. Therefore we have proposed classifying the different matrices into four classes [3]. For each class, dedicated analytical methods have been developed and validated. Direct injection of a diluted sample and analysis by one-dimensional GC/MS either in scan mode [4] or selected ion monitoring (SIM) mode is effective for samples that contain solutes that elute on an apolar column between decane (retention index 1000) and docosane (retention index 2200), providing that the sample complexity and analyte concentration range are not high, and that no nonvolatile matrix compounds are present [2]. One such method was developed using an Agilent J&W HP-5MS (apolar) column. The conditions and corresponding retention time locked information [5] and a complete allergens deconvolution reporting software (DRS) database with peak deconvolution are available from the Agilent Technologies Web site (www.agilent.com).

For highly complex samples (> 100 solutes) containing only volatile and semivolatile solutes, or for samples with a very broad concentration range of components (for example: very low concentrations of target compounds in a very high concentration of matrix compounds), a single-dimension GC separation is not effective. For these, the added power of two-dimensional capillary GC (GC/GC, 2D GC) has been shown to be helpful [3]. Using multiple heartcuts from a primary apolar column, target compounds can be isolated and resolved from interfering sample components on a polar secondary column, making accurate quantification possible even in cases where MS deconvolution of one-dimensional GC/MS data fails.

In this paper, the application of capillary flow technology Deans switching is demonstrated for the 2D GC analysis of a complex perfume sample. For even more method flexibility and separation power, the second-dimension column was housed in a low thermal mass (LTM) oven module for independent control of the column temperature. With this configuration, multiple heartcuts could be focused on the cooler secondary column and then released with an independent temperature program, which could be independently optimized for best separation of target compounds from complex sample matrix.

Experimental

The perfume sample was diluted to 5% (50 mg/mL) in acetone. Standard solutions were prepared from pure compounds at 100 ng/µL in acetone.

The analyses were performed on a 7890A GC/5975 MSD combination. The GC was equipped with an SSL inlet, FID detector, a capillary flow technologies based Deans switching system (p/n G2855B), a PCM flow module (option #309), and an LTM system controller bundle (p/n G6579A).

As illustrated in Figure 2, the primary column was installed in the GC oven and configured from the split/splitless inlet to the Deans switch. "Long leads" were requested when ordering the column for the LTM so that the inlet end could be connected directly to the Deans switch. The outlet of the column was cut close to the column module and connected to the MSD via uncoated but deactivated fused silica (FS) tubing using an Agilent Ultimate Union (p/n G3182-61580). This configuration results in better method translation of conditions than when the long lead is left on the outlet end of the column because this 1 m extends into the GC column oven and becomes an isothermal (third) separation zone that broadens peaks and can alter the relative retention and resolution achieved at the exit of the LTM module. A restrictor (uncoated but deactivated retention gap) was also connected between the second output of the Deans switch and a monitoring FID. The conditions are summarized in Table 2.

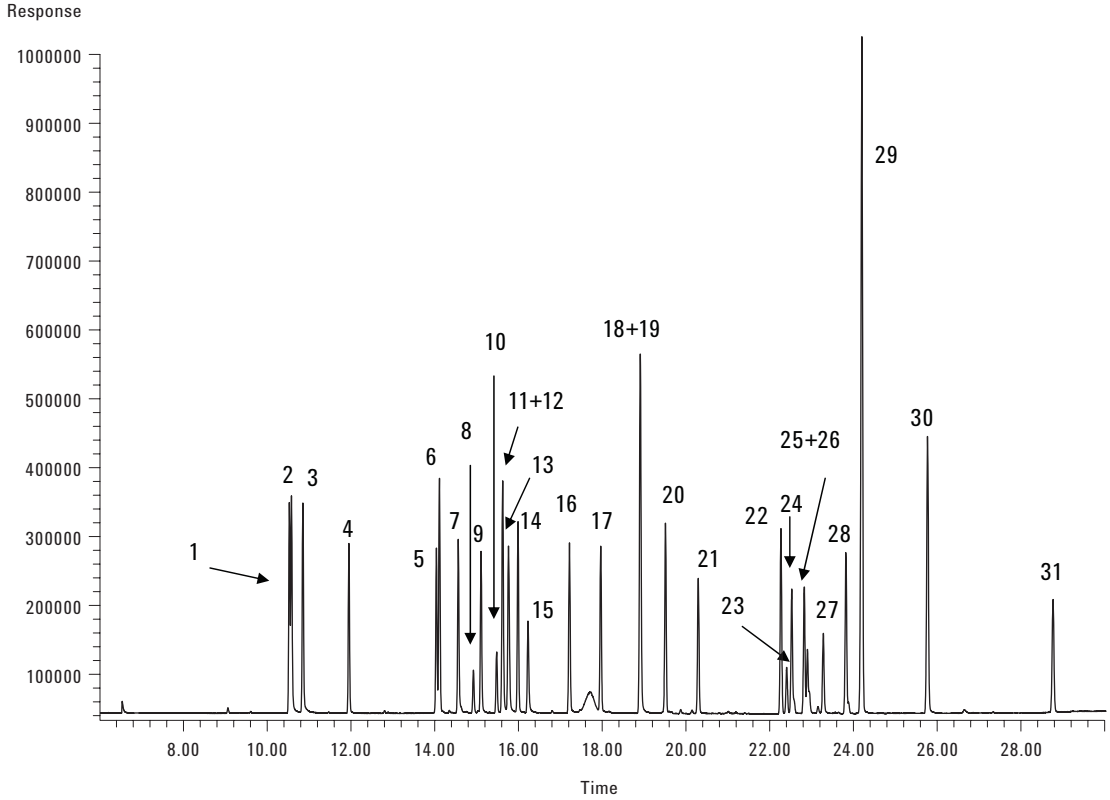


Figure 1. Separation of flavor and fragrance allergen test mixture (100 ppm) on the first dimension column (Agilent J&W HP-5MS) and FID detection. Peak identification is given in Table 1.

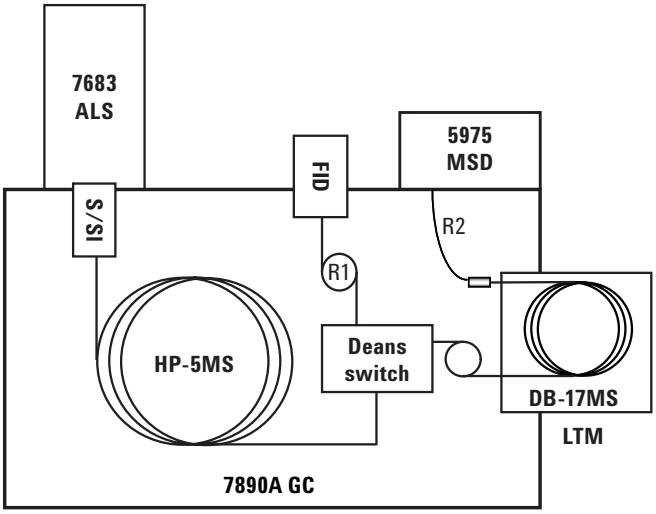


Figure 2. System configuration.

Results and Discussion

First, a standard mixture containing all target compounds at 100 ng/µL was analyzed. No heartcutting was used. The resulting chromatogram from the separation on the J&W HP-5MS column on the monitor FID is given in Figure 2. A good separation was obtained. Some coeluting pairs can effectively be resolved by mass spectral deconvolution (specific ions), as is done with DRS methods.

Next, the perfume sample was run under the same conditions. The chromatogram from the monitor FID detector shown in Figure 3A shows that the perfume is very complex, making determination of target compounds difficult. Some target solutes, such as linalool (peak 4) and alpha-isomethyl ionone (peak 20) are clearly resolved and can be determined. However, the elution window between 22 and 24.5 min, is quite complex. In this window, several target allergens elute,

Table 2. Analytical Conditions

Injection	1.0 µL
Inlet	S/Sl, 250 °C, split ratio = 1:25
Column 1 (Carrier gas = He)	30 m × 0.25 mm id × 0.25 µm Agilent J&W HP-5MS, p/n 19091S-433 Flow = 1.4 mL/min; constant flow mode (185 kPa at 50 °C) Inlet = SSL; outlet = PCM1
Column 2 (LTM) (Carrier gas = He) Flow (PCM1)	30 m × 0.25 mm id × 0.25 µm Agilent J&W DB-17ms, p/n 122-4732LTM with "long leads" (1 m at each end not wrapped) 2 mL/min constant flow mode (120 kPa at 50 °C) for first experiment, 120 kPa (1 min) → 256 kPa (28 min) at 4.35 kPa/min for second experiment
Restrictors	R1 = 63 cm × 100 µm id deactivated FS (cut from, for example, p/n 160-1010-5) R2 = 1 m × 250 µm id deactivated FS (p/n 160-2255-1)
GC oven temperature	50 °C (1 min) → 300 °C (27.75 min) at 8 °C/min Total run time = 60 min
LTM oven	50°C (25 min, after last heartcut) → 250 °C (1 min) at 6 °C/min (Total run time = 60 min)
FID monitor detector	300 °C, 30 mL/min H_2, 400 mL/min air
Deans switch heartcutting	Initially OFF Cut 1: ON at 10.2 min, OFF at 11.0 min Cut 2: ON at 15.3 min, OFF at 16.4 min Cut 3: ON at 22.0 min, OFF at 24.5 min
MS data acquisition	Autotune, scan mode, 41–300 u, samples = 2^2
MSD transfer line	300 °C
MS solvent delay	5 min
MS temperatures	Source = 300 °C, quad = 150 °C

including amyl cinnamaldehyde, lyral (two isomers), amyl cinnamyl alcohol (with a related impurity), farnesol (two isomers), hexyl cinnamaldehyde, and benzyl benzoate. Within the same window, interfering perfume constituents such as methyl dihydrojasmonate, ionones, and sesquiterpenes elute. Most of these have mass spectra with strong fragmentation, resulting in many nonspecific low mass ions, interfering significantly with target ion spectra and ion ratios. Traditional selective detection and quantification using SIM data or deconvolved scan data from DRS that are effective with simpler samples would therefore be problematic with this sample.

For example, confirming the presence of lyral in this sample was difficult with the simpler approach. With GC-SIM-MS, it was not possible to accurately quantify lyral, and its qualifier ions did not fall within the specified range. Review of the scan data clearly showed the presence of coeluting interferences.

Next, the sample was rerun with three heartcuts, including the problematic region between 22 and 24.5 minutes, which were heartcut to the second column. Propylene glycol, used as "keeper" in some perfumes, is a potential interferent in the first window that contains limonene, benzylalcohol, and phenylacetaldehyde. Quantification and identification of hydroxycitronellal in the second heartcut window is another component that, in the presence of interferences, is sometimes problematic to quantify using standard methods. The chromatogram obtained on the monitor detector is shown in Figure 3B, wherein the three heartcut windows show up as flat sections in the baseline.

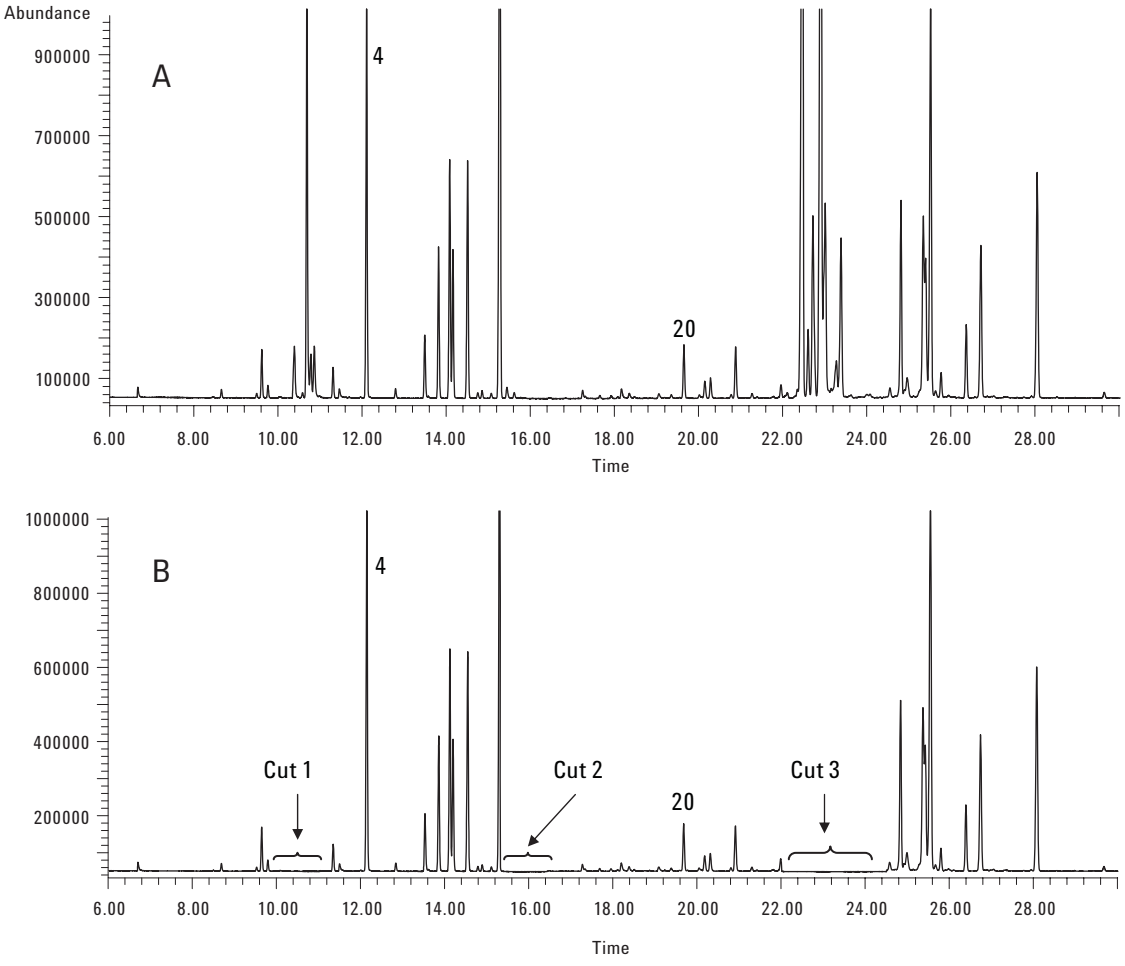


Figure 3. A) Separation of a perfume sample on the first-dimension column (Agilent J&W HP-5MS) using FID detection without heartcutting. Peaks: 4. Linalool; 20. Alpha-isomethyl ionone. B) Separation of a perfume sample on the first-dimension column (Agilent J&W HP-5MS) using FID detection with heartcutting (fractions: 10.2–11.0, 15.3–16.4, and 22.0–24.5 min).

The TIC chromatogram obtained after separation on the second-dimension column of the lyral fraction (heartcut 3) is shown in Figure 4A. First the analysis was performed using the same temperature program for the second column as for the first column (LTM program = 7890A oven program), emulating what would happen if the secondary column were housed in the GC oven (traditional configuration 2D GC). At least eight peaks were detected. The lyral isomers elute at 25.4 and 25.5 minutes. The second isomer, however, coelutes with another solute, and confirmation and quantification are not possible. The elution temperature of the lyral isomers in this case was around 240 °C. Both retention and selectivity at this temperature are low.

The experiment was repeated, this time with the J&W DB-17ms secondary column kept at 50 °C until the last heartcut was completed, and then the temperature was increased (at 6 °C/minute). Using this approach, the solutes are first focused at the head of the LTM column, and then elute at lower temperature (200 °C) during the temperature ramp, allowing both retention and selectivity to play more important roles. An added benefit is that the peak widths are narrowed due to the focusing, which improves peak resolution. Under these conditions, the isomers elute at 49.25 and 49.4 minutes and can be quantified without interference. The chromatogram of heartcut fraction 3 (22 to 24.5 minutes from column 1) is shown in Figure 4B. In contrast to Figure 4A, at least 20 peaks spanning a wide concentration range are clearly resolved. The presence of lyral isomers in the sample could thereby be confirmed and accurately quantified.

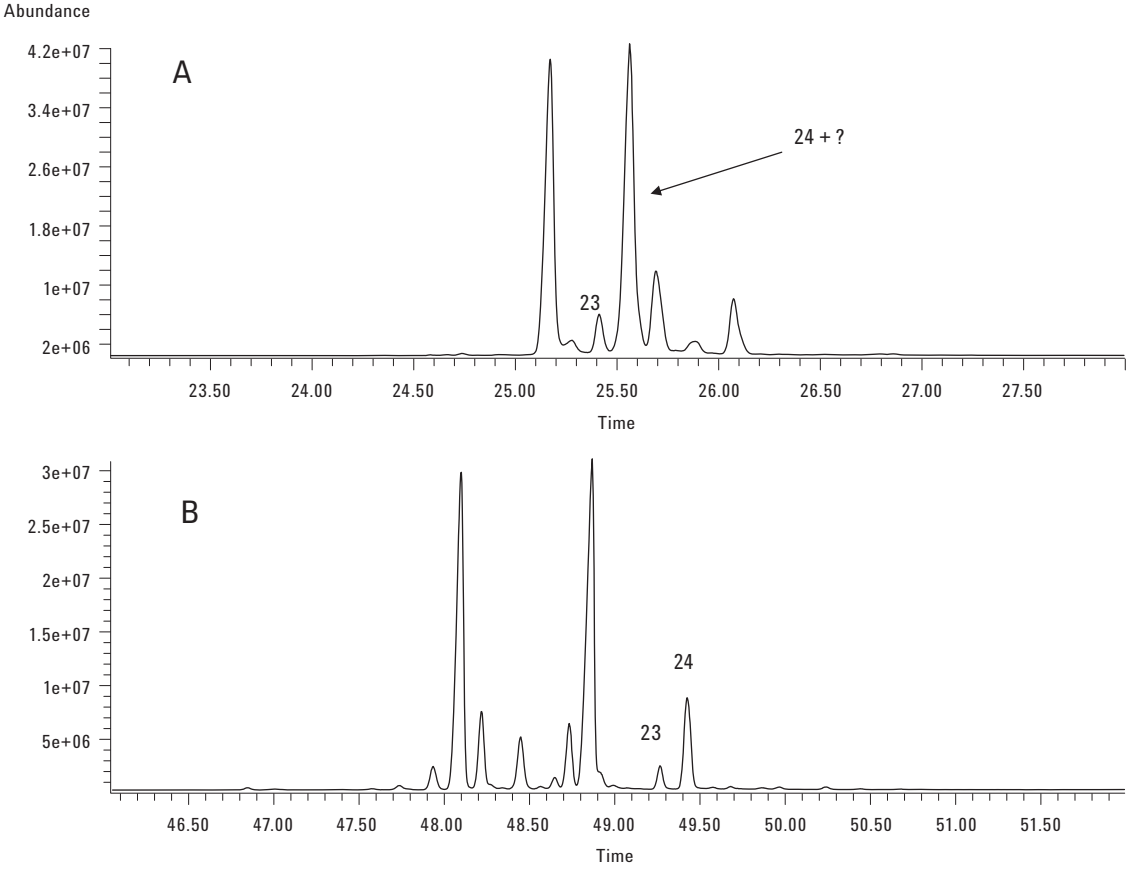


Figure 4. A) Separation of fraction 3 on the second-dimension column (Agilent J&W DB-17ms) using MS detection. Column 1 temperature = column 2 temperature: 50 °C (1 min) → 270 °C at 8 °C/min. Peaks: 23. Lyral 1; 24. Lyral 2. B) Separation of fraction 3 on the second-dimension column (Agilent J&W DB-17ms) using MS detection. Column 2 temperature: 50 °C (25 min) → 250 °C at 6 °C/min. Peaks: 23. Lyral 1; 24. Lyral 2.

By comparing the chromatograms in Figure 4, it is obvious that the independent temperature control of the second column in a 2D GC greatly increases the ability to optimize selectivity and resolution. This point was also demonstrated in the analysis of enantiomers using a chiral second-dimension column [6].

In addition to perfume samples, the approach presented herein can also be used for the determination of flavor and fragrance allergens in finished products. In these applications, any nonvolatile or late-eluting matrix compounds could be backflushed from the first-dimension column, as discussed in a manner similar to that described in an earlier application note [7].

Conclusions

Two-dimensional GC using Deans switch heartcutting in combination with MS can be used for the determination of flavor and fragrance allergens in complex perfume and cosmetic samples. Using LTM technology, the second dimension column temperature can be optimized independently from the primary column, resulting in better selectivity and resolution of target solutes from matrix interferences. Addition of an LTM module is more cost-effective, less cumbersome to configure, and takes up less space than if using a second GC as the independent zone.

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Potential Allergens in Aromatherapy Oils by GC/MS Using an Agilent J&W DB-XLB Capillary Column

Application Note

Consumer Products and Flavors and Fragrances

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Abstract

The European Union (EU) regulates 26 flavor and fragrance allergens. Twenty-four of these allergens are amenable to analysis by GC/MS. Some of these allergens are present at appreciable levels in essential oils used in aromatherapy. This note demonstrates a single quad GC/MS analysis of the GC/MS amenable listed allergens using the unique selectivity of an Agilent J&W DB-XLB capillary GC column. Representative total ion chromatograms of ylang ylang, lavender, and eucalyptus oils are shown. The DB-XLB column easily resolved the 24 analytes of interest, having both excellent peak shapes and high sensitivity.

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Introduction

The European Union (EU) regulates 26 flavor and fragrance allergens. GC/MS is an effective analytical technique for 24 of the 26 compounds listed as EU flavor and fragrance allergens. The GC/MS-amenable allergens (Table 1) are sufficiently volatile for this approach. Oak and tree moss extracts (the 2 remaining listed allergens) are less volatile and require LC/MS for effective analysis. The 24 GC/MS-amenable allergens are often components in fragrance, cosmetic and aromatherapy product formulations. Listed allergen limit targets are in place in the EU for rinse-off and leave-on fragrance and cosmetic products in the 10 – 100 ppm range [1]. Identification and detection of the listed allergens at these levels is readily obtainable using a single quad GC/MS approach. Acceptable levels and labeling requirements for aromatherapeutic products is less clear. These products can contain EU-listed allergens at levels more than ten times the acceptable level of 10 ppm for leave-on cosmetic products such as ointments and creams. Leave-on and rinse-off limits established for fragrance and cosmetic products serve as useful guides for analysis of aromatherapy products.

Professionals in the aromatherapy and massage industries strongly discourage the use of neat essential oils. These oils are strong concentrates that may contain irritants, sensitizers, and even potential carcinogens in addition to allergens [2]. Fortunately, these concentrated oils are typically mixed with carrier oils such as vegetable or mineral oil prior to use. Recipes for dilution often call for 1% to 5% dropwise preparation of the neat essential oils dispersed in the carrier oil. This practice only partially mitigates the potential harm from exposure to the chemicals present in the neat oils. It is imperative that both the practitioner providing the therapy and the patient having these products applied to their skin are aware of the potential risks present in order to use these products safely and effectively.

Continuous improvements in column manufacturing technology and GC/MS instrumentation have made the evaluation of aromatherapy oils a simpler and more reliable process. Detection and identification of the potential allergy-inducing components in these oils down to the single part per million (ppm) range is readily achievable. The quality of capillary GC/MS columns in terms of their bleed and surface activity profiles dramatically enhance the performance of this type of analysis with better analyte resolution and sharper peak shapes. Design improvement in GC/MS systems work with the improved columns to enhance GC/MS analyses in the low ppm range and become standard practice. Routine analysis of these oils for the 24 GC/MS-amenable allergens is now both a straightforward and robust process, if the peaks of interest can be resolved from matrix components. This application note highlights the separating power and chromatographic performance available today using Agilent J&W DB-XLB columns coupled with an Agilent 5975C series GC/MSD, capable of simultaneous SIM and Scan mode spectrometry.

Previous studies of this allergen set by GC/MS focused primarily on fragrance and cosmetic products. Several approaches have been described. The first one uses a deconvolution reporting software (DRS) approach on an Agilent J&W HP-5ms column. The second uses a two-dimensional column approach with a low thermal mass (LTM) device and Deans switch heart cutting to an Agilent J&W DB-17ms column. Finally, the third uses a single DB-17ms column single quad GC/MS approach to evaluate allergens in snack foods [3–5]. The focus of this application is a single column GC/MS separation of the allergens using the selectivity offered by the DB-XLB stationary phase. The selectivity of the midpolarity DB-XLB column is a useful tool in helping to resolve the allergens away from potential matrix interference. The sample matrices here are the essential oils used in the aromatherapy and massage industries.

Table 1. GC/MS-amenable EU Flavor and Fragrance Allergens

1	Limonene	7	Citral	13	Isoeugenol	19	Farnesol
2	Benzyl alcohol	8	Hydroxy cittronellal	14	a-isomeyhyl ionone	20	Amyl cinnamyl alcohol
3	Linalool	9	Anisyl alcohol	15	Coumarin	21	Hexyl cinnamaldehyde
4	Folione	10	Cinnamaldehyde	16	Lilial	22	Benzyl benzoate
5	Citronellol	11	Cinnaminic alcohol	17	Amyl-cinnamaldehyde	23	Benzyl salicilate
6	Geraniol	12	Eugenol	18	Lyral	24	Benzyl cinnamate

Experimental

This analysis was done with an Agilent J&W DB-XLB 30 m × 0.25 mm × 0.25 µm column (Agilent p/n 122-1232) on an Agilent 7890 GC System, together with an Agilent 5975C series GC/MSD. Details of the chromatographic conditions are shown in Table 2. Details of the flow path supplies used are listed in Table 3.

Table 2. Chromatographic Conditions for EU Allergen Analysis

GC/MS:	Agilent 7890A GC System, Agilent 5975C series GC/MSD, Triple Axis Detector
Sampler:	Agilent 7683B automatic liquid sampler, 5.0 µL syringe (Agilent p/n 5183-4729)
Injection:	1.0 µL
Carrier:	Helium, fixed pressure 11.06 psi
Inlet:	50:1 split ratio 250 °C, total flow 70.73 mL/min, 3 mL/min septum purge, gas saver on, 50 mL/min after 2 minutes
Inlet Liner:	MS certified liner (Agilent p/n 5188-6568)
Column :	Agilent J&W DB-XLB 30 m x 0.25 mm x 0.25 µm (Agilent p/n 122-1232)
Oven:	50 °C (1.0 min) to 100 °C (8 °C/min); 2 °C/min to 110 °C (2 min), 5 °C/min to 185, 30 °C/min to 280 °C (3 min)
MSD:	Transfer line 310 °C, source 350 °C, quadrupole 180 °C

Table 3. Flow Path Supplies

Vials:	Amber screw top glass vials (Agilent p/n 5183-2072)
Vial Caps:	Screw caps (Agilent p/n 5182-0723)
Vial inserts:	100 µL glass/polymer feet (Agilent p/n 5181-8872)
Syringe:	5 µL (Agilent p/n 5183-4729)
Septum:	Advanced Green (Agilent p/n 5183-4759)
Inlet Seal:	Gold plated inlet seal (Agilent p/n 5188-5367)
Inlet liners:	MS certified liner (Agilent p/n 5188-6568)
Ferrules:	0.4 mm id short; 85/15 Vespel/graphite (Agilent p/n 5181-3323)
20x magnifier:	20x Magnifier loop (Agilent p/n 430-1020)

Sample Preparation

Twenty-four individual 1000 ng/µL EU GC/MS flavor and fragrance standard solutions, obtained from AccuStandard, New Haven, CT were combined in equal portion with a laboratory prepared 1000 ng/µL internal standard solution to form a 40 ng/µL combined allergen standard solution. The internal standard 1,4 dibromobenzene was purchased from Sigma Aldrich, St. Louis, MO and prepared at a concentration of 1000 ng/µL in acetonitrile. The 40 ng/µL combined allergen solution was diluted 1:4 in acetonitrile to make a standard at a concentration of 10 ng/µL. Standards were prepared fresh, stored at 5 °C and used within seven days of preparation.

Ylang ylang, lavender and eucolyptus essential oil samples were obtained from a local retail store. All of the essential oils were labeled aromatherapeutic GC/IR verified grade and designated 100% pure and natural. The essential oil samples were diluted 1:20 in acetone and prepared fresh the day of the analysis.

Results and Discussion

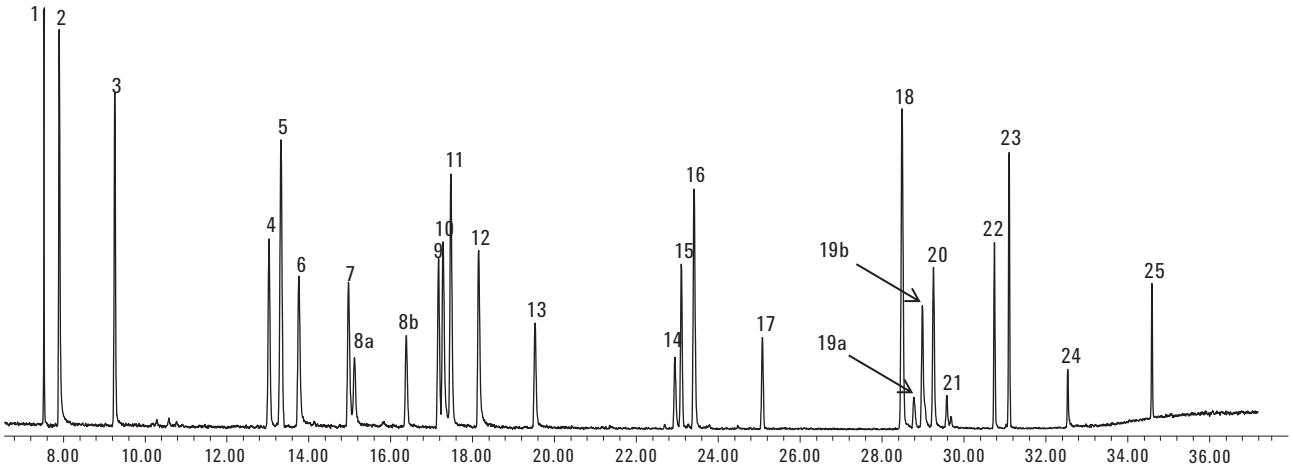
Most of the EU listed allergens fall into two chemical categories; aldehydes and alcohols. Both of these chemical classes of compounds are comprised of active analytes that can often lead to poor chromatographic performance. Using the DB-XLB column, excellent resolution and peak shape was achieved for these analytes.

Figure 1 shows the separation of 24 GC/MS-amenable allergens on a DB-XLB 30 m × 0.25 mm × 0.25 µm capillary GC column at a concentration of 40 ppm. Factoring in the 1-µL volume and a split ratio of 50:1 on-column loading of these analytes is 0.8 ng/component. This figure clearly shows that all 24 GC/MS-amenable allergens are easily detectable and identifiable at this level.

In this standard set citral, lyral, and farnesol have more than one isomer each and appear as more than one peak. Peak 19b is a combination of lyral 2 and farensol 1. Fortunately, the peaks for lyral 1 and farnesol 2 resolve nicely on the DB-XLB column enabling confirmation of either of these two analytes in the presence of each other over the 10–100 ppm range studied.

The dibromobenzene internal standard is included at the same concentration in both the standard and sample chromatograms to provide a fixed frame of reference in each of the figures. The Y scale in Figure 1 is magnified 25 times above the Y scale in the sample figures to highlight the sensitivity and peak shapes obtained with standard injection. The naturally occurring essential oil samples contain a wide variety of more concentrated components, plus the dibromobenzene internal standard at the same concentration as in the standard.

0.8 ng/component loading of 24 EU Allergens on an Agilent J&W DB-XLB Column

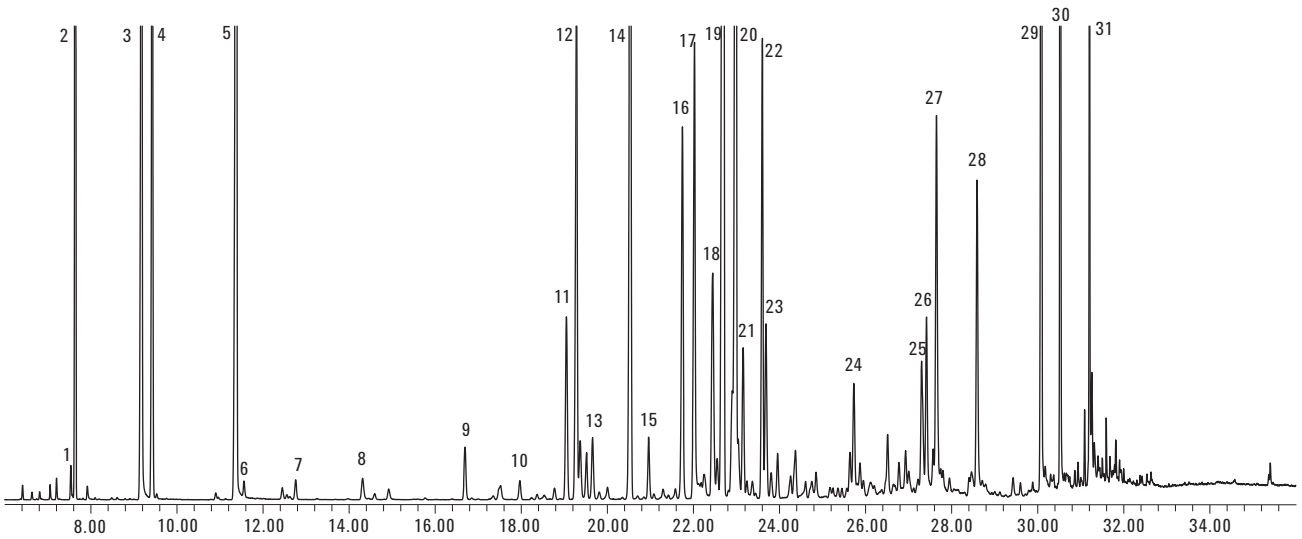


EU Flavor and Fragrance Allergens

1	Limonene	8a	Citral (neral)	14	Isoeugenol	19b	Farnesol 1
2	Benzyl alcohol	8b	Citral (geranial)	15	α-Isomethyl ionone	20	Amyl cinnamyl alcohol
3	Linalool	9	Hydroxy citronellal	16	Coumarin	21	Farnesol 2
4	Folione	10	Anisyl alcohol	17	Lilial	22	Hexyl cinnamaldehyde
5	Dibromobenzene (ISTD)	11	Cinnamaldehyde	18	Amyl cinnamaldehyde	23	Benzyl benzoate
6	Citronellol	12	Cinnaminic alcohol	19	Lyral 1	24	Benzyl salicilate
7	Geraniol	13	Eugenol	19b	Lyral 2	25	Benzyl cinnamate

Figure 1. Total ion chromatogram of a 1-µL injection of 40 ppm standard solution containing 24 EU allergens on an Agilent J&W DB-XLB 30 m × 0.25 mm × 0.25 µm column (p/n 122-4712). Y scale in this figure ranges from 0 to 2 × 10^3 counts, peak number 5 is the internal standard. GC conditions are in Table 2.

Figure 2 shows a total ion chromatogram of an ylang ylang essential oil sample diluted 1:20 in acetonitrile. The 1:20 dilution is representative of a typical 3% to 5% dropwise preparation for an essential oil in a mineral oil carrier. It serves as an excellent reference point for evaluating the allergen risk potential of an essential oil. The EU allergens detected and identified in this sample are in bold type below the figure. In this sample linalool, cinnamyl acetate, benzyl benzoate, and benzyl salicilate were all present. These potential allergens were present at levels more than 100 times the low level or 10 ppm investigated in this application.



Ylang Ylang Oil Major Components include 4 EU Allergens

1	Eucolyptol	9	p-Propyl anisol	17	***Cinnamyl acetate***	25	δ-Muurolene
2	p-Methyl anisol	10	α-Cubebene	18	t-Muurolene	26	t-Cardinol
3	***Linalool***	11	Copaene	19	Germacrene D	27	t-Muurolol
4	Methyl benzoate	12	β-Myrcene	20	α-Farnesene	28	β-Farnesene
5	Benzyl acetate	13	β-Elemene	21	α-Muurolene	29	***Benzyl benzoate***
6	Ethyl benzoate	14	Caryophyllene	22	δ-Cadinene	30	Caryophyllene
7	Dibromobenzene (ISTD)	15	β-Cubebene	23	γ-Cadinene	31	***Benzyl salicylate***
8	Cic-gernaniol	16	α-Caryophyllene	24	Carphyllene oxide		

Figure 2. Total ion chromatogram of ylang ylang oil diluted 1:20 in acetonitrile on an Agilent J&W DB-XLB ms 30 m x 0.25 mm × 0.25 µm column (p/n 122-4712). Y scale in this figure ranges from 0 to 5 x 10^6 counts, peak number 7 is the internal standard. GC conditions are in Table 2.

Figure 3 displays a total ion chromatogram of a lavender essential oil sample diluted 1:20 in acetonitrile. Linalool was present in this sample. Linalool is a main component in lavender oil, in this case, 1000 times the level of the 10 ppm standard.

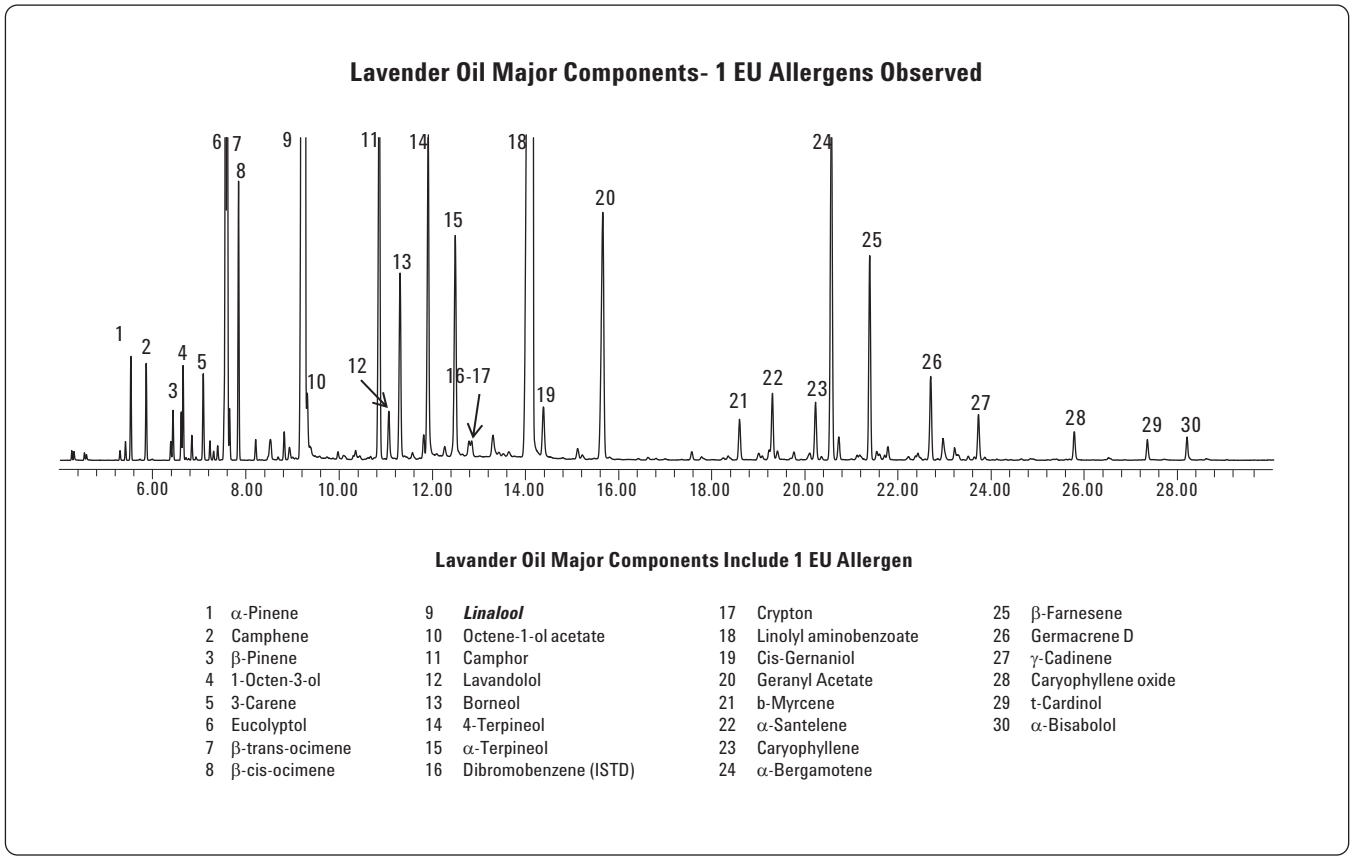


Figure 3. Total ion chromatogram of lavender oil diluted 1:20 in acetonitrile on an Agilent J&W DB-XLB ms 30 m × 0.25 mm × 0.25 µm column (p/n 122-4712). Y scale in this figure ranges from 0 to 5 x 10^6 counts, peak number 16 is the internal standard. GC conditions are in Table 2.

Figure 4 is a TIC of a eucalyptus essential oil sample diluted 1:20 in acetonitrile. In this sample, a single EU allergen was detected and identified. Linalool was present in this sample at a level approximately 5 times the 10-ppm standard. Eucalyptus oil was the simplest and most volatile of the essential oils investigated.

Conclusions

At least one EU-regulated allergen was identified in each of the essential oil samples investigated in excess of the 10-ppm limit set by the EU for leave-on cosmetic products. In ylang ylang and lavender oils, several of these allergens were present at more than 100 times the target limit for the compounds even for samples diluted 1 to 20. The 1 to 20 dilution chosen is representative of a typical 3% to 5% dropwise preparation done by massage and aromatherapists. It is imperative that professionals in the aromatherapy and massage industries are aware of the potential risks for these allergens to produce allergic responses, even in diluted form. Close monitoring by GC/MS analyses, appropriate labeling, and careful dilution of these products are required for safe use.

This application note successfully demonstrates the utility of a single quad GC/MS approach using an Agilent J&W DB-XLB column for analysis of the EU flavor and fragrance allergens in aromatherapy oils. Using the GC/MS conditions described, 24 of the EU-listed allergens amenable to GC/MS analysis are detectable and identifiable. Identification at the 10-ppm level, which is the leave-on limit set for fragrance and cosmetic products in the EU directive, was easily achievable.

Analyte peaks on the DB-XLB column were well resolved and sharp considering the active nature of the alcohol and aldehyde components in this sample set. The resolution observed on this column for the allergen peaks shows that reliable and robust analysis of potential allergen components in essential oils is both achievable and straightforward.

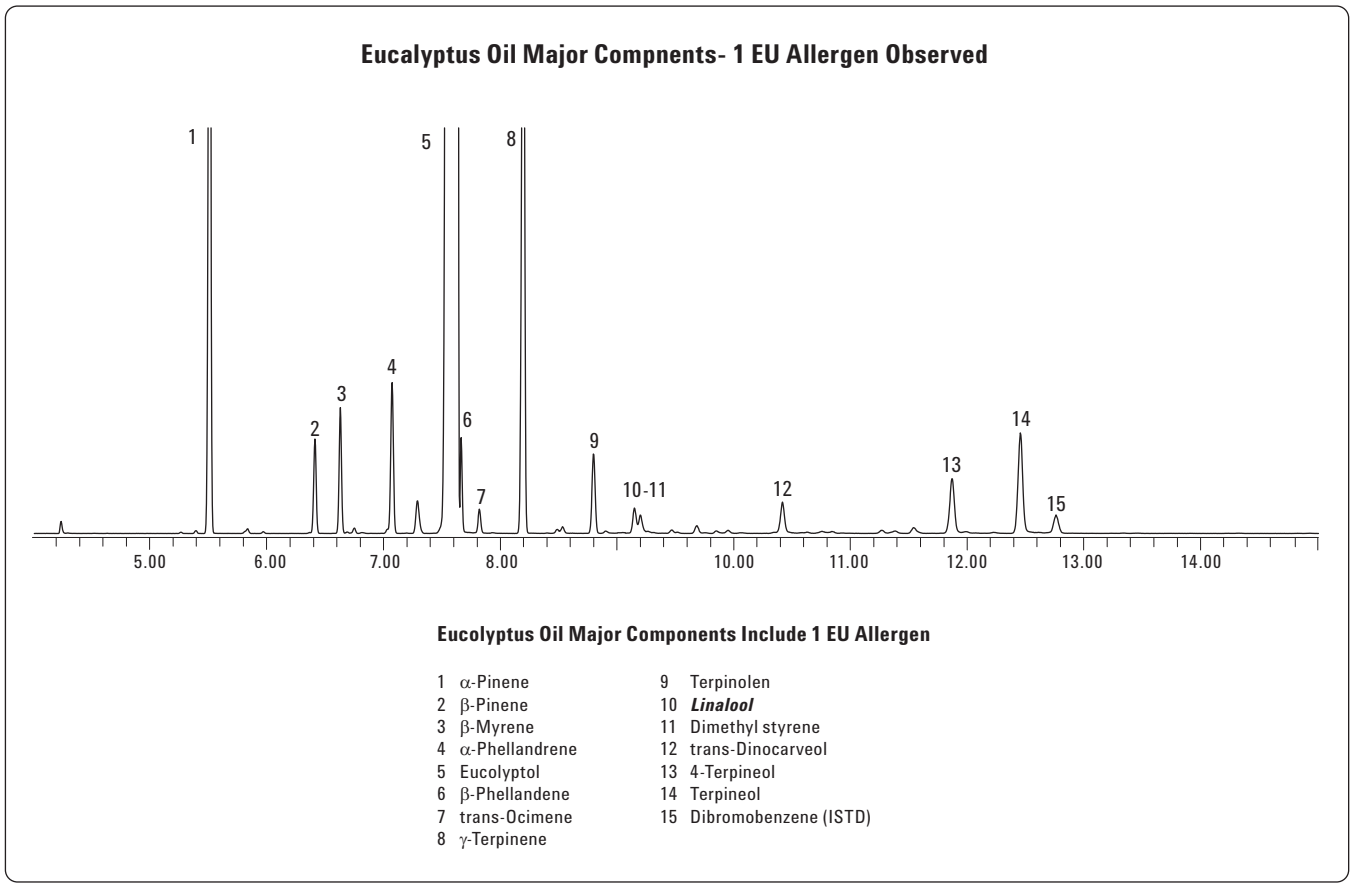


Figure 4. Total ion chromatogram of eucalyptus oil diluted 1:20 in acetonitrile on an Agilent J&W DB-XLB 30 m × 0.25 mm × 0.25 µm column (p/n 122-4712). Y scale in this figure ranges from 0 to 5 x 10^6 counts, peak number 15 is the internal standard. GC conditions are in Table 2.

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Determination of Phthalate Concentration in Toys and Children's Products

Gas Chromatography/Mass Spectrometry

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Abstract

Due to public health concerns, the Chinese government and the US Consumer Product Safety Commission (CPSC) both introduced test methods for the analysis of phthalate content in childcare items and toys using GC/MS in 2008. These regulatory methods require determination of the presence of six regulated phthalate esters including dibutyl phthalate (DBP), benzyl butyl phthalate (BBP), bis(2-ethylhexyl)-phthalate (DEHP), di-n-octyl phthalate (DNOP), di-isononyl phthalate (DINP), and di-isodecyl phthalate (DIDP). These six restricted phthalate esters along with other common phthalates such as dimethyl phthalate (DMP), diethyl phthalate (DEP), dihexyl phthalate (DHP), and bis(2-n-butoxyethyl)phthalate (DBEP) were analyzed with an Agilent 5975C Series GC/MSD and Agilent J&W DB-5ms Ultra Inert column, resulting in accurate calibration coefficients, and excellent reproducibility and recovery.

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Introduction

Phthalates are the most commonly used plasticizers, which are added to soft polyvinyl chloride (PVC) toys and baby products to increase polymer flexibility. This is due to their function as intermolecular "lubricants". Because they are additives and not reagents, they are not chemically bound in the polymer and are therefore able to leach from the matrix. Concerns have been raised over the effect of phthalate exposure from plastic materials because they may disrupt the hormonal development of children and can lead to early puberty, reproductive defects, and other health problems. This health concern has resulted in regulations regarding the type and levels of phthalates allowable in plastic toys and other articles.

In December 1999, the European Union (EU) implemented a restriction on six phthalate esters in toys and childcare products meant to be placed in the mouths of children under the age of three with a maximum concentration (total phthalate content) not exceeding 0.1% (w/w). The phthalate esters of interest include:

- dibutyl phthalate (DBP)
- benzyl butyl phthalate (BBP)
- bis(2-ethylhexyl)phthalate (DEHP)
- di-n-octyl phthalate (DNOP)
- di-isononyl phthalate (DINP)
- di-isodecyl phthalate (DIDP)

China is one of the major exporters of toys and children's products in the world. Chinese regulation GB/T 22048-2008 was issued in June 2008 and came into effect in May 2009 to strengthen the supervision of these harmful compounds. Similar to EU regulation, the identification and quantification of the six regulated phthalate esters were determined using GC/MS with an external standard method [2].

In August 2008, US Congress enacted the Consumer Product Safety Improvement Act (CPSIA 2008), which established a 0.1% (w/w) limit for these six phthalate esters. The US Consumer Product Safety Commission (CPSC) subsequently introduced a test method for the analysis of phthalate content in childcare items and toys using GC/MS with an internal standard method [3].

In addition to these six restricted phthalate esters, different regional regulations imposed provisions on other phthalates such as dimethyl phthalate (DMP), and diethyl phthalate (DEP) according to different regulations. The Statutory Order of Danish Ministry of Environment and Energy defines all diesters of o-phthalic acid as phthalates, indicating that most of the commonly used phthalates in children's products are prohibited by the Danish regulations.

This application note demonstrates the determination of common phthalates in plastic toys using the Agilent J&W DB-5ms Ultra Inert column based on the framework of the original Chinese GB/T 22048-2008 and US CPSC methods.

Experimental

The experiments were performed using an Agilent 7890 Gas Chromatograph System with split/splitless capillary inlet, an Agilent 5975C Mass Spectrometer with triple axis detector, and an Agilent 7683 automatic liquid sampler (ALS). The split/splitless inlets were fitted with long-lifetime septa (Agilent Part No. 5183-4761). Injections were made using a 10-µL syringe (Agilent Part No. 9301-0714).

Phthalate esters

The following phthalate esters were purchased from Sigma-Aldrich and AccuStandard:

- dibutyl phthalate (DBP)
- di-n-octyl phthalate (DNOP)
- di-isodecyl phthalate (DIDP)
- benzyl butyl phthalate (BBP)
- bis(2-ethylhexyl)phthalate (DEHP)
- di-isononyl phthalate (DINP)
- bis(2-butoxyethyl)phthalate (DBEP)
- diheptyl phthalate (DHP)
- dimethyl phthalate (DMP)
- diethyl phthalate (DEP)

Benzyl benzoate was obtained from Restek Corp.

As shown in Figure 1, these phthalates are based on the 1,2-benzenedicarboxylic acid structure. There are an infinite number of possible alkyl side chains (R) and an infinite number of combinations of the side groups (R and R'). For example, the di-isononyl phthalate (DINP) and di-isodecyl phthalate (DIDP) consists of an array of compounds due to the isomeric branched-chain alkyl groups on both side chains. For phthalate esters with saturated alkyl side chains (without oxygen), the most intense peak in the electron impact (EI) ionization mass spectrum at 70 eV is at *m/z* 149, with a signal-to-noise ratio approximately 10–100 times higher than that of other ions in the spectrum. The only exception is for dimethyl phthalate where both R and R' represent CH_3 and so the H on the oxygen is replaced by CH_3. Consequently, *m/z* 163 becomes the base peak. Several less intense ions were selected as application ions in order to eliminate matrix interferences from unwanted phthalates for quantitation, or due to isomeric congeners. For example, the quantitation ions for DINP and DIDP are low abundance ions at *m/z* 293 and *m/z* 307, respectively. Therefore, when preparing the calibration solutions the concentrations for DINP and DIDP normally are quite higher than other phthalates in most of the regulatory

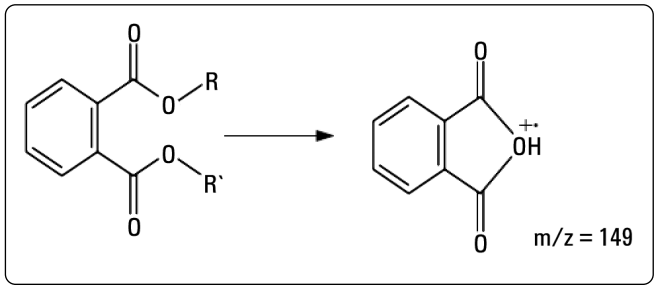


Figure1. Primary structure of phthalates and major fragmentation of phthalate esters in EI ionization (The dominant ion in the EI spectrum is typically m/z 149. The exception is for dimethyl phthalate where both R and R' are CH_3, so the H on the oxygen is replaced by CH_3 and m/z 163 becomes the base peak.)

methods.

Calibration solutions for Chinese GB/T 22048-2008 analytical methods

According to Chinese GB/T 22048-2008 analytical methods, five calibration solutions were prepared by dilution in dichloromethane (DCM). The concentration range of DBP, BBP, DEHP, and DNOP was from 0.5 µg/mL to 10 µg/mL. The concentration range of DINP and DIDP was from 5 µg/mL to 100 µg/mL in calibration solutions.

The experimental conditions are listed in Table 1. Table 2 presents the selected target ion and three qualifying ions.

Table 1. Gas Chromatography and Mass Spectrometers Conditions (GB/T 22048-2008)

GC Conditions	
Column	Agilent J&W DB-5ms Ultra Inert capillary column, 30 m × 0.25 mm, 0.25 µm (p/n 122-5532UI)
Inlet Temperature	Split @ 300 °C, spilt 20:1, split injection liner (Agilent p/n 5188-4647).
Carrier Gas	Helium, constant flow mode, 1.2 mL/min
Injection Volume	1 µL,
Oven Program	180 °C for 0.5 min; to 280 °C at 20 °C/min and hold for 7 min
MS Conditions	
Solvent Delay	4.2 min
MS Temp	230 °C (Source); 150 °C (Quad)
Transfer Line Temp	280 °C
MS	EI, SIM/Scan
Scan Mode	mass range (50-500 amu)
SIM Mode	ions (see Table 2)

Table 2. Typical quantitation ions for phthalate plasticisers

No	Compounds	CAS No.	Tgt ion	Q1	Q2	Q3
1	Dibutyl phthalate (DBP)	84-74-2	149	150	223	205
2	Benzyl butyl phthalate (BBP)	85-68-7	149	091	206	238
3	Bis(2-ethylhexyl)phthalate (DEHP)	117-81-7	149	167	279	150
4	Di-n-octyl phthalate (DNOP)	117-84-0	279	149	150	261
5	Di-isononyl phthalate (DINP)	28553-12-0	293	149	127	167
6	Di-isodecyl phthalate (DIDP)	26761-40-0	307	149	141	150

Calibration solutions for CPSC Analytical Method

The CPSC analytical method was used for determination of six restricted phthalate esters in childcare items and toys:

- DBP
- BBP
- DEHP
- DNOP
- DINP
- DIDP

Benzyl benzoate was adopted as an internal standard. In addition to these six restricted phthalate esters, dimethyl phthalate (DMP), diethyl phthalate (DEP), dihexyl phthalate (DHP) and bis(2-butoxyethyl)phthalate (DBEP) were included in this study because they are prohibited by regulations in other geographical locations.

Five calibration solutions including DBP, BBP, DEHP, DNOP, DINP, DIDP, DMP, DEP, DHP and DBEP at 0.25, 0.5, 1, 2, 5 and 10 µg/mL were prepared by dilution in cyclohexane, along with one calibration blank (cyclohexane). Each standard solution contained 5 µg/mL of benzyl benzoate as the internal standard. The experimental conditions are listed in Table 3. Suggested quantitative ions are in bold in Table 4.

Table 3. Gas Chromatography and Mass Spectrometery Conditions (CPSC Analytical Method)

GC Conditions	
Column	Agilent J&W DB-5ms Ultra Inert capillary column, 30 m × 0.25 mm, 0.25 µm (p/n 122-5532UI)
Inlet Temperature	290 °C
Carrier Gas	Helium at 1 mL/min
Injection Mode	Splitless, pulse injection at 35 psi for 0.5 min, splitless injection liner (Agilent p/n 5188-3316).
Injection Volume	1 µL
Oven Program	50 °C for 1 min to 280 °C at 30 °C/min to 310 °C at 15 °C/min hold for 4 min
MS Conditions	
Solvent Delay	5 min
MS Temp	230 °C (Source); 150 °C (Quad)
Transfer Line Temp	280 °C
MS	EI, SIM/Scan
Scan Mode	mass range (50-500 amu)
	For other parameters, see Table 4

Table 4 Phthalate Esters, CAS Number, Molecular Weights (mol wt) and Corresponding Ions

Compound Name	CAS No	Mol wt	Corresponding Ions (m/z)
Dimethyl phthalate (DMP)	131-11-3	194	194, **163***
Diethyl phthalate (DEP)	84-66-2	222	222, 177, **149**
Benzyl benzoate (BB)**	120-51-4	212	212, 91, 194,**105**
Dibutyl phthalate (DBP)	84-74-2	278	149, 167, 205, **223**
Dihexyl phthalate (DHP)	84-75-3	334	251, 233, **149**
Benzyl butyl phthalate (BBP)	85-68-7	312	91, 149, **206**
Bis(2-n-butoxyethyl)phthalate (DBEP)	117-83-9	366	176,193, **149**
Bis(2-ethylhexyl)phthalate (DEHP)	117-82-8	390	149, 167, **279**
Di-n-octyl phthalate (DNOP)	117-84-0	390	149, 167, 261, **279**
Di-isononyl phthalate (DINP)	28553-12-0	418	149, 167, **293**
Di-isodecyl phthalate (DIDP)	26761-40-0	446	149, 167, **307**

* Suggested quantitative ions are in bold.

** Benzyl benzoate is internal standard

Sample preparation for Real Samples

Sample #1 was a PVC toy and sample #2 was an infant pacifier. Both samples were ground or cut into pieces sized at less than 3 mm × 3 mm. One gram of cut pieces was Soxhlet extracted in 120 mL of dichloromethane for 6 h at 60-80 °C. The extract was concentrated to about 10 mL using a rotary evaporator and then diluted with DCM to 25 mL. For samples having very large amounts of phthalates (for example, 45%), further dilution may be needed to reduce concentration levels into the calibration range.

The spiked samples were treated according to the procedure described above.

Results and Discussion

Chinese GB/T 22048-2008 analytical methods

Calibration standards

The total ion chromatogram (TIC) of the six phthalate esters (DBP, BBP, DEHP, DNOP, DINP and DIDP) is shown in Figure 2. While DBP, BBP, DNOP and DEHP appeared as single peaks in the chromatogram, DINP and DIDP each appeared as typical finger peaks because of an array of possible isomers. The extracted ion chromatogram (EIC) of DINP at *m/z* 293 and DIDP at *m/z* 307 is shown in Figure 3 where area summation integration was used for the quantitation of these compounds.

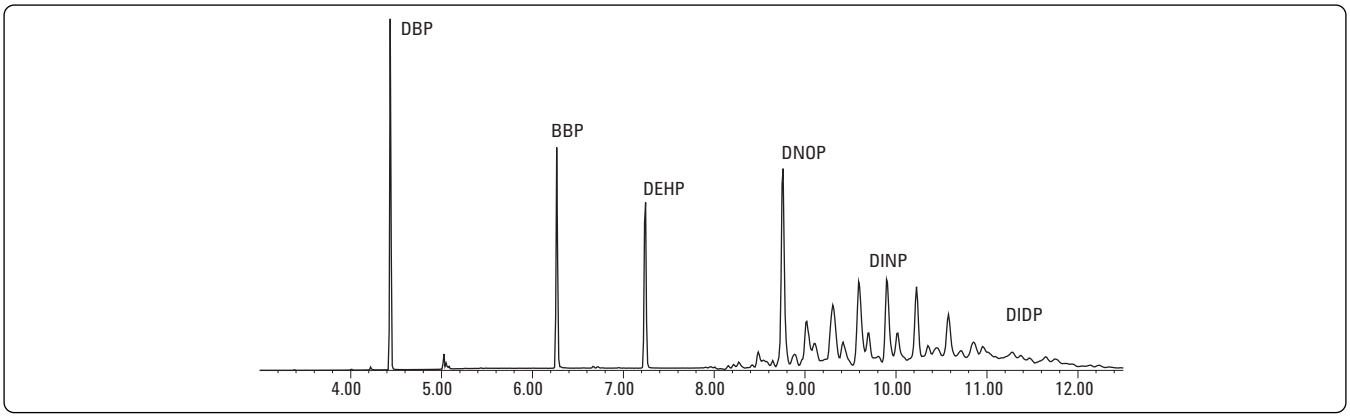


Figure 2. TIC of six regulated phthalates (DBP, BBP, DEHP and DNOP, 5 ppm each; DINP and DIDP 50 ppm each).

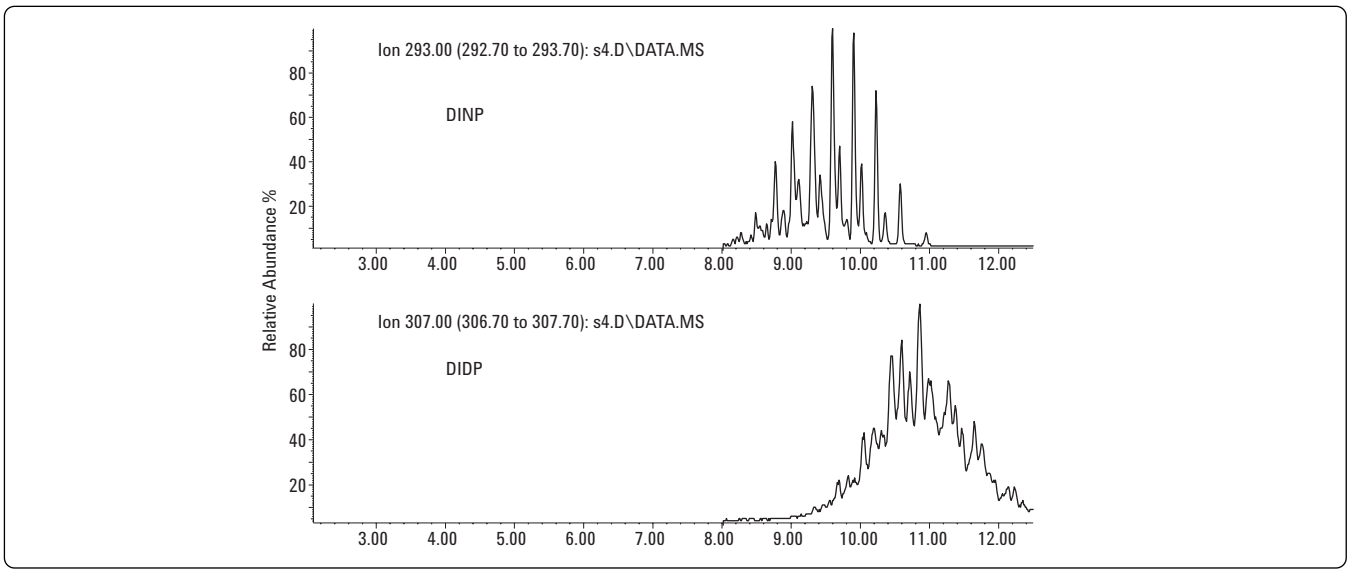


Figure 3. Extracted ion chromatogram of DINP at m/z 293 and DIDP at m/z 307.

Calibration curves were constructed from data obtained by 1-µL injections of standards. All the phthalate esters had excellent calibration coefficients as indicated in Table 5. Table 6 presents the recovery data for spiked samples, which were treated according to the procedure described in the sample preparation. The DINP and DIDP recoveries were determined at concentration levels of 50 mg/L and 100 mg/L, whereas the recoveries of other phthalates were determined at concentration levels of 5 mg/L and 10 mg/L. Excellent recoveries were obtained for all the compounds, ranging from 92% to 107% with relative standard deviations (RSD) being less than 7%.

Table 5. Calibration of Phthalate Esters

	Compound Name	Range of linearity (mg/L)	Correlation coefficient (R^2)
1	Dibutyl phthalate	0.5 ~10	0.9992
2	Benzyl butyl phthalate	0.5 ~ 10	0.9991
3	Bis(2-ethylhexyl)phthalate	0.5 ~ 10	0.9990
4	Di-n-octyl phthalate	0.5 ~ 10	0.9990
5	Di-isononyl phthalate	5 ~ 100	0.9986
6	Di-isodecyl phthalate	5 ~ 100	0.9979

Table 6. Recovery Data of Phthalate Ester Spikes in Sample

Compound Name	Spiked amount (mg/L)	No of replicates	Recovery mean,%	RSD%
Dibutyl phthalate	5	5	98.78	5.09
	10	5	102.76	4.05
Benzyl butyl phthalate	5	5	93.27	3.35
	10	5	99.38	4.03
Bis(2-ethylhexyl)phthalate	5	5	102.16	3.75
	10	5	106.21	5.29
Di-n-octyl phthalate	5	5	103.19	4.58
	10	5	98.43	4.42
Di-isononyl phthalate	50	5	104.81	5.01
	100	5	101.78	3.99
Di-isodecyl phthalate	50	5	104.48	6.11
	100	5	92.12	5.33

Real Sample

The chromatogram for sample #1 is provided in Figure 4 along with the chromatogram for the calibration standard. DEHP and DINP were detected in this particular PVC toy. However, the total % weight of phthalates was 0.037 %, which is well below the regulatory limits of 0.1% (w/w).

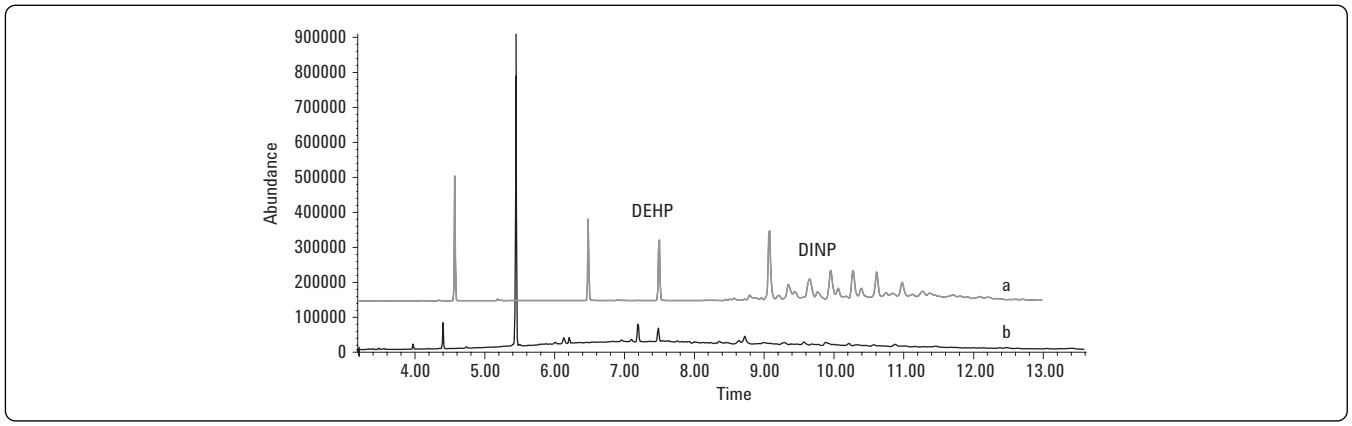


Figure 4. Chromatographic results for the GC/MS analysis of one PVC toy sample. (a) TIC of phthalates standards, (b) TIC of real sample.

CPSC Analytical Method

Calibration Standard

The total ion chromatogram of 10 phthalate esters (DMP, DEP, DBP, DHP, BBP, DBEP, DEHP, DNOP, DINP and DIDP) and internal standard benzyl benzoate at 5 ppm is shown in Figure 5. DNOP, DINP and DIDP were partially co-eluting because of isomeric congeners. As discussed earlier, in order to eliminate interferences, less intense ions at *m/z* 279, *m/z* 293 and *m/z* 307 were chosen as the quantitation ions for DNOP, DINP and DIDP, respectively.

According to the CPSC method, the calibration range is from 0.25 to 10 mg/L for each phthalate. The concentration range of DINP and DIDP in this method is lower compared to that for most other regulatory methods. As indicated in Table 7, all of the phthalates achieved excellent linearity with the linear coefficient R^2 being greater than 0.999. Figure 6 demonstrates an excellent injection-to-injection reproducibility of DIDP at 1 ppm.

Table 7. Calibration of Phthalate Esters

Compound Name	Range of linearity (mg/L)	RT (min)	Correlation coefficient (R^2)
Dimethyl phthalate (DMP)	0.25-10	6.56	0.9991
Diethyl phthalate (DEP)	0.25-10	7.13	0.9995
Benzyl benzoate (BB)*	5	7.89	IS
Dibutyl phthalate (DBP)	0.25-10	8.47	0.9993
Dihexyl phthalate (DHP)	0.25-10	9.71	0.9991
Benzyl butyl phthalate (BBP)	0.25-10	9.81	0.9990
Bis(2-n-butoxyethyl)phthalate (DBEP)	0.25-10	10.13	0.9995
Bis(2-ethylhexyl)phthalate (DEHP)	0.25-10	10.34	0.9992
Di-n-octyl phthalate (DNOP)	0.25-10	11.04	0.9994
Di-isononyl phthalate (DINP)	0.25-10	10.92-11.80	0.9987
Di-isodecyl phthalate (DIDP)	0.25-10	11.41-12.20	0.9990

* Benzyl benzoate is internal standard

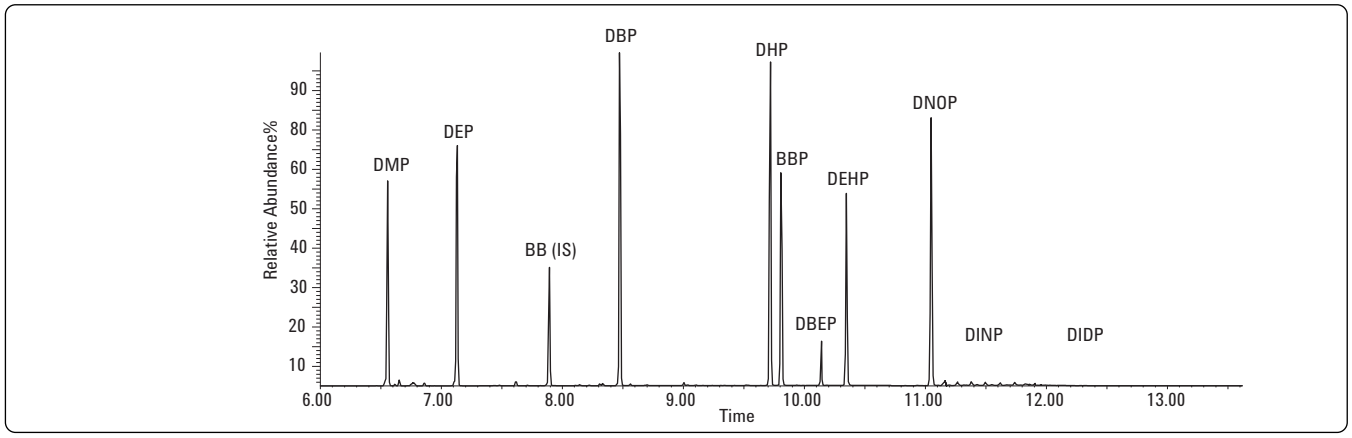


Figure 5. TIC of phthalates at 5 ppm.

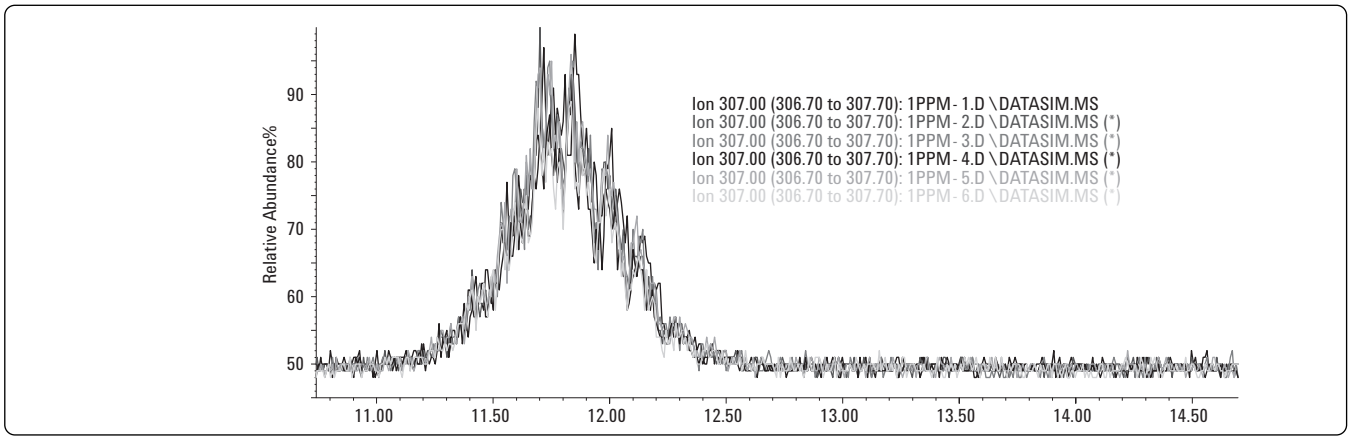


Figure 6. Reproducibility of DIDP from six injections at 1 ppm.

Real Sample

The extract and matrix spiked extract for sample #2 are shown in Figure 7. No regulated phthalates were detected in this sample #2 extract. A peak for butylated hydroxytoluene was evident, which is an antioxidant commonly added to polymers.

The spiked samples were treated according to the sample-preparation procedure described above. The recovery data and their statistics were based on five replicates of matrix spikes with 10 target phthalate esters at the 2-ppm level. Good recoveries were obtained for all investigated phthalates, ranging from 90% to 110% with relative standard deviations (RSD) less than 13.3%. Among these phthalate esters, excellent results for DMP, DEP, DBP, DHP, BBP, DEHP and DNOP were achieved, with the range of recovery at 97–105% and RSD repeatability of less than 5%.

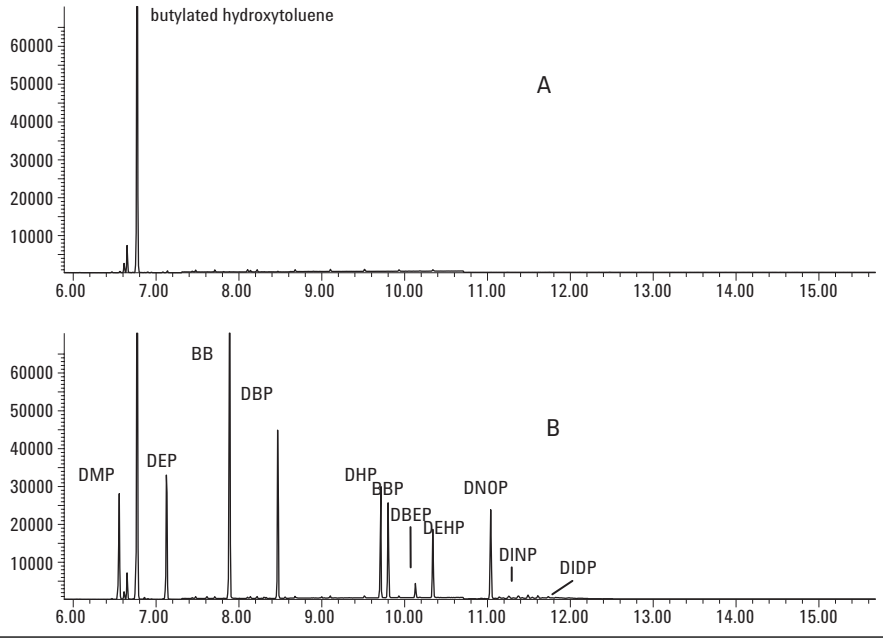


Figure 7. TIC of infant pacifier extract (A) and sample # 2 (pacifier) extract spiked with 2-ppm phthalate mixture (B).

Conclusion

According to the approved China standard and US CPSC method, two GC/MS methods were developed for the determination of phthalate esters in toy and children's products using the Agilent 7890A GC System and an Agilent 5975 Series GC/MSD with an Agilent J&W DB-5ms Ultra Inert column. These methods well meet the requirements of both regulatory methods and have good linearity, repeatability, and recoveries for all target phthalate esters.

References

1. Official Journal of the European Communities Decision 198/815/EC. 1999, European Commission; European Union Scientific Committee on Toxicology, Ecotoxicology, and the Environment.
2. U.S. Congress enacted the Consumer Product Safety Improvement Act (CPSIA 2008). http://www.cpsc.gov/cpsia.pdf
3. The US Consumer Product Safety Commission (CPSC) http://www.cpsc.gov/about/cpsia/cpsia.html

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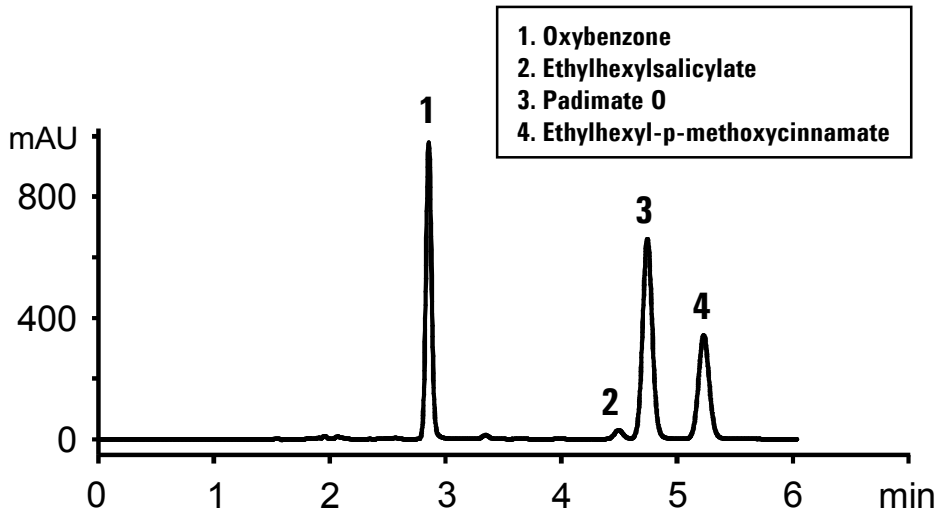
Analysis of Sunscreen Lotion Extract

Application

Pharmaceutical

Robert Ricker

Sunscreen lotions are becoming increasingly popular, as people are more concerned with health effects of exposure to UV radiation. This popularity has triggered improvements in sunscreen performance. Many products now use active ingredients that are lipid soluble. Lipid solubility increases their resistance to removal from skin, increasing SPF (sun protection factor) and durability (sweat-proof or waterproof). Little is known of skin penetration and systemic distribution of UV absorbers.



Conditions: LC: Agilent 1100
Column: ZORBAX SB-Phenyl (3.5µm), 4.6 x 150 mm, Agilent P/N: 863953-912
Mobile Phase: MeOH : H_2O (84:16), 0.1% TFA total
UV: 310 nm; Flow: 1.0 mL / min.; 30°C; Inj. Vol.: 5 µL

Highlights

- *ZORBAX Rapid-Resolution columns are highly efficient: N>100,000 / meter.*
- *ZORBAX StableBond columns are rugged at low pH, including 0.1% TFA (pH 2.1).*
- *ZORBAX StableBond columns provide symmetrical peak shape for improved peak quantitation.*

Agilent Technologies

Robert Ricker is an application chemist based at Agilent Technologies, Wilmington, Delaware.

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www.agilent.com/chem

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April 25, 2002
5988-6412EN



Agilent Technologies

Reversed Phase HPLC of Fatty Acids

Application Note

Author

Linda Lloyd
Agilent Technologies, Inc.

Introduction

The analysis of lipids is of particular importance to the food industry; a variety of lipid compounds are used commercially, particularly in processed foods. Meat and cereals contain naturally-occuring lipids ranging from polar phospholipids, fatty acids, diglycerides and cholesterol to non-polar fats and oils (triglycerides). Processed foods may also contain additional spray dried or encapsulated fats and emulsifiers. Health issues related to excessive dietary fat intake include obesity, increased risk of some forms of cancer and cholesterol deposition in cardiovascular diseases such as atherosclerosis (hardening of the arteries). Identification and quantification of the different types of lipid and their fatty acid composition is therefore required, but is complicated by the difficulty in detection due to the absence of a strong UV chromophore.

Fatty acids are long hydrocarbon chains with terminal carboxylate groups, and form a major component of triacylglycerides, phospholipids and sphingolipids. More than 1000 naturally-occuring fatty acids have been identified, but most common lipids contain only a few of this extensive group. Biological systems usually contain fatty acids with an even number of carbon atoms, between 14 and 24, the most common between 16 and 18 carbon atoms. In animals, these chains are invariably unbranched. The hydrocarbon chain can contain one or more *cis* configuration double bonds. These double bonds dramatically affect the physical properties of the fatty acids. Stearic and oleic acid are both 18 carbon atoms long, but oleic acid has one double bond and a melting point of 13.4 °C, in comparison to stearic acid which is saturated and has a melting point of 69.6 °C.

Fatty acids that contain no double bonds, when analyzed by reversed phase chromatography, are separated by chain length, the shortest eluting first.

Agilent Technologies

A PLRP-S column can be used to separate fatty acids in a variety of media. These columns are robust enough to be stable at pH 1-14 and cope with vigorous clean up procedures and aggressive eluents. The Agilent evaporative light scattering detector is an ideal detector for the analysis of fatty acids. Although these acids can be detected by UV at 210 nm, the tetrahydrofuran itself will absorb. However, the changing composition of the eluent does not present a problem for the Agilent ELSD, as it is evaporated before reaching the light scattering cell. This method of detection produces a flat, stable baseline, as illustrated in the following examples using the same experimental conditions.

Conditions

Column: PLRP-S 100Å 5 µm, 250 x 4.6 mm (p/n PL1512-5800)
Eluent A: 60 mM Acetic acid
Eluent B: ACN
Eluent C: THF
Gradient: 35:60:5 to 0:90:10 in 20 min
Flow Rate: 0.5 mL/min
Detection: Agilent ELSD (neb=80 °C, evap=70 °C, gas=1.0 SLM)

Results and Discussion

Figure 1 shows good separation of seven fatty acids and Figure 2 shows two of the fatty acids in evening primrose oil. Good baseline resolution was achieved through the use of PLRP-S reversed phase material.

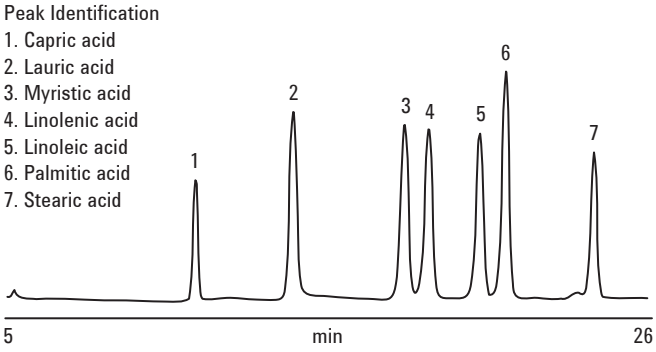


Figure 1. Separation of seven fatty acids using PLRP-S media.

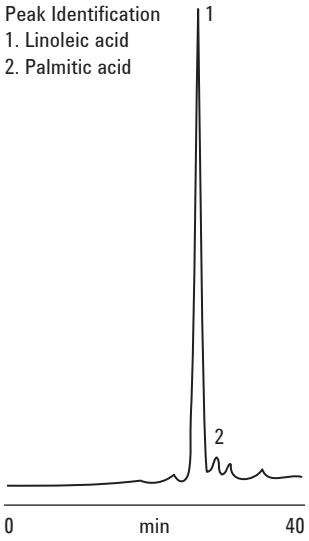


Figure 2. Fatty acid composition of evening primrose oil revealed by PLRP-S.

Conclusion

Coupling a PLRP-S column with the Agilent ELSD provides an ideal system for the quantitation of fatty acids, essential for their structural analysis and solute identification. As a single column, PLRP-S operates across the entire range of HPLC eluents. It is chemically stable and physically robust and so it is possible to switch between organic modifiers, such as ACN and tetrahydrofuran, and eluent pH 0 to 14.

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Agilent Technologies

L-Ascorbic Acid Stability in Orange Juice in TetraBrik Cartons

Application Note

Author

Linda Lloyd
Agilent Technologies, Inc.

Introduction

In recent years the value of L-ascorbic acid in human nutrition has become increasingly apparent to health conscious consumers. This has led to an upsurge in the manufacture of soft drinks based on fruit juices containing ascorbic acid. Orange juice in TetraBrik cartons is one such example. In this product the juice is processed and packed aseptically to reduce thermal loads during manufacture.

This note assesses the instability of L-ascorbic acid in reconstituted single strength orange juice in TetraBrik cartons stored at different temperatures. The effect of dissolved oxygen in the juice was also examined. Ion suppression reversed phase HPLC was used, with PLRP-S columns. PLRP-S is a rigid macroporous styrene/divinylbenzene HPLC phase which is chemically stable with the acidic eluent.

Agilent Technologies

Materials and Reagents

The samples were ready to drink commercial orange juice without artificial flavors, colors, preservatives or added sugar. Samples were stored at different temperatures for differing lengths of time. Before analysis all samples were centrifuged at 10000 g for 45 minutes then filtered through a 0.45 µm cellulose nitrate membrane.

Conditions

Columns: 2 x PLRP-S 100Å 5 µm, 150 x 4.6 mm (p/n PL1111-3500)
Eluent: 0.2 M NaH_2PO_4, pH 2.14
Flow rate: 0.5 mL/min
Detector: UV, 268 nm

Materials and Reagents

Figure 1 shows the degradation of L-ascorbic acid over time, with concentrations varying from 60.4% after 64 days at 4 °C to 3.6% after three days at 105 °C. The marked fall in L-ascorbic acid seems to coincide with an initial drop in dissolved oxygen concentrations (Figure 2). This dramatic loss of oxygen appeared to correlate with the higher decomposition rate of the acid at the beginning of storage (Figure 3). It seems the degradation mechanism is initially aerobic but continues anaerobically at a lesser rate once the level of dissolved oxygen has reached equilibrium.

The complete data set and analysis is available in Kennedy *et al.* (1992).

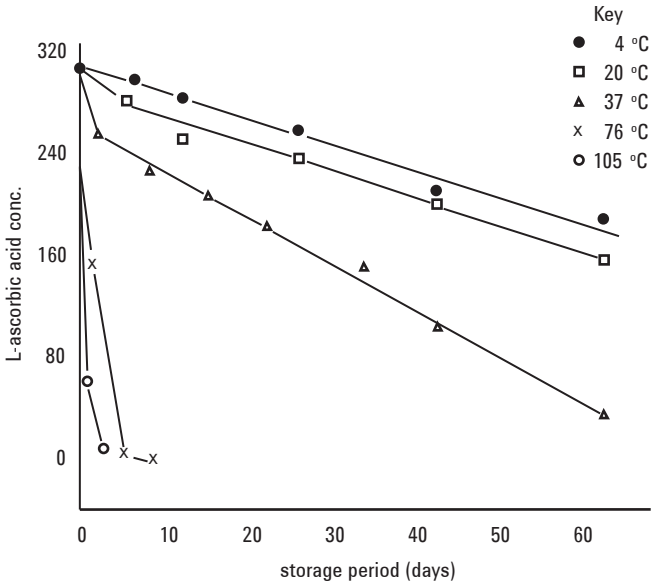


Figure 1. L-ascorbic acid concentration over time in orange juice in TetraBrik cartons at different storage temperatures.

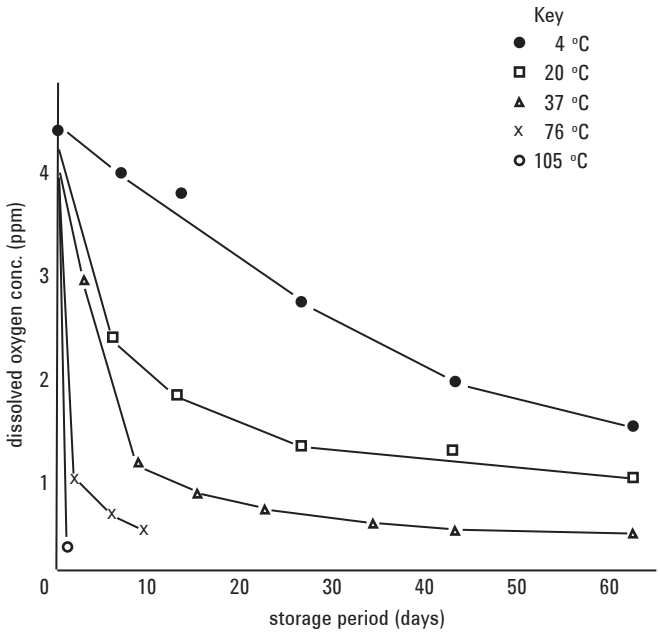


Figure 2. Dissolved oxygen levels vs storage time in TetraBrik cartons at different temperatures.

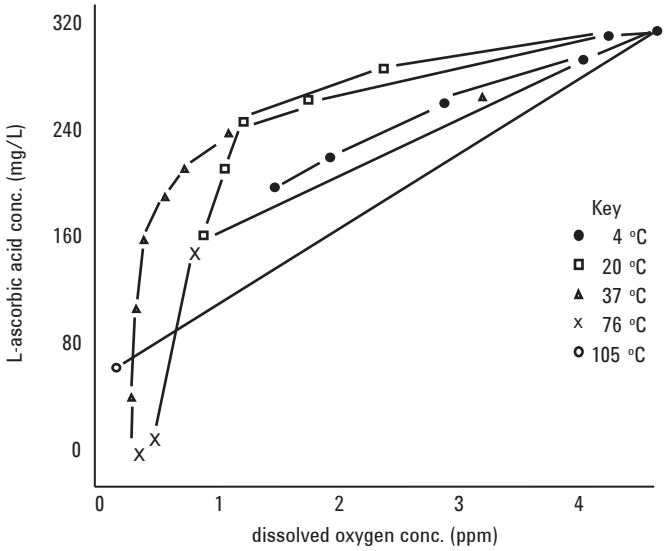


Figure 3. Loss of L-ascorbic acid vs dissolved oxygen level.

Conclusion

Ion suppression HPLC using PLRP-S columns successfully revealed the decline in L-ascorbic acid content of reconstituted orange juice. The amount of dissolved oxygen at carton filling had a significant effect on the acid's content., and the rate of oxygen consumption depended on the L-ascorbic acid content. Aerobic and anaerobic decomposition occurred in the same system.

Reference

Kennedy, JF, Rivera, ZS, Lloyd, LL, Warner, FP, Jumel, K (1992) L-ascorbic acid stability in aseptically processed orange juice in TetraBrik cartons and the effect of oxygen. *Food Chem.,* 45, 327-331.

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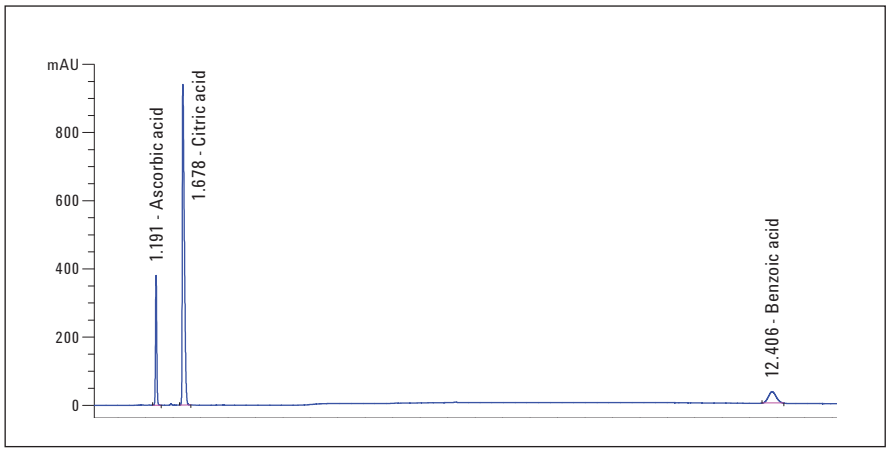
Analysis of ascorbic acid, citric acid and benzoic acid in orange juice

Application Note

Food

Author

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Abstract

Food additives, such as antioxidants and preservatives, are added to increase the shelf life of food items. In this Application Note, we describe a method to quantify an antioxidant (vitamin C) and preservatives (citric acid, benzoic acid) in orange juice. The method was developed on an Agilent 1260 Infinity LC system using an Agilent Poroshell EC-C18 column. Partial method validation was performed in aqueous samples to demonstrate linearity, robustness and precision in area and retention time. The limit of detection (LOD) for benzoic acid was found to be 0.2 µg/mL. During sample recovery studies, greater than 90% recovery was obtained for all three compounds. The method was effectively converted to a short ultra high performance liquid chromatography (UHPLC) method using an Agilent 1290 Infinity LC system. This new method was five times faster with the same LOD for benzoic acid. Both methods can be effectively applied by food manufacturers for quality control of food additives.

Agilent Technologies

Introduction

Antioxidants, such as ascorbic acid prevent oxidation by decreasing the available oxygen in the environment. Ascorbic acid is preferentially oxidized to the dehydroascorbic acid (DHA) form, thus preventing the oxidation of the matrix. Preservatives, such as citric acid or benzoic acid prevent or inhibit the growth of microorganisms in food. While some fruit naturally contain ascorbic acid, citric acid and benzoic acid[1], these components are added additionally added to fruit juices to increase the shelf life. Although the regulatory limit for benzoic acid in fruit juices is 400 to 600 µg/mL, concerns exist regarding the liberation of carcinogenic benzene by reaction of benzoic acid with ascorbic acid under certain conditions[2,3].

The amount of ascorbic acid is reported to diminish with time, temperature and other factors[4] to DHA. The AOAC Official Method 967.22 describes the analysis of vitamin C content by first oxidizing ascorbic acid to DHA followed by derivatization and fluorescence detection. Regarding UV based analysis, DHA has little absorbance above 220 nm, while ascorbic acid has absorbance from 244–265 depending on the pH of the buffer[5]. The AOAC Official Method 994.11 shows the UV based detection of benzoic acid in orange juice.

In this Application Note, a method is described to simultaneously quantify ascorbic acid, citric acid, and benzoic acid using UV based detection and a simple extraction procedure.

Reagent and materials

All the chemicals and solvents used were HPLC grade. Highly purified water used was from a Milli Q water purification system (Millipore Elix 10 model, USA). Acetonitrile 'super gradient' was purchased from Lab-Scan (Thailand) and potassium phosphate monobasic was obtained from Fluka (Germany). O-Phosphoric acid was purchased from Fluka (Switzerland). Standards of ascorbic acid, citric acid, and benzoic acid were from Sigma-Aldrich (India). International brand named orange juice manufactured in India were purchased.

Experimental

Instruments and Software

An Agilent 1260 Infinity Binary LC system consisting of the following modules was used:

- Agilent 1260 Infinity Binary Pump (G1312B)
- Agilent 1260 Infinity Autosampler and Thermostat (G1367E, G1330B)
- Agilent 1260 Infinity Thermostatted Column Compartment (G1316A)
- Agilent 1260 Infinity Diode Array Detector (G4212B) with 10-mm Max-Light flow cell

The UHPLC analysis was developed and performed using an Agilent 1290 Infinity LC system consisting of the following modules:

- Agilent 1290 Infinity Binary Pump (G4220A)
- Agilent 1290 Infinity Autosampler and Thermostat (G4226A, G1330B)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1290 Infinity Diode Array Detector (G4212A) with 10-mm Max-Light flow cell

Columns:

- Agilent Poroshell 120 EC-C18, 4.6 × 100 mm, 2.7µm (p/n 697975-302)

Software:

- Agilent ChemStation B.04.02

Chromatographic parameters

The chromatographic parameters used for reverse phase liquid chromatography and UHPLC are shown in Table 1.

Preparation of standards

Ascorbic acid, citric acid, and benzoic acid were accurately weighed out and dissolved in mobile phase A to obtain stock solutions of 5,000 µg/mL (ppm), 50,000 ppm and 100 ppm respectively. A 10 minute sonication was required to completely dissolve benzoic acid. Linearity levels were prepared by subsequent dilution from these stock solutions using mobile phase A as shown in Table 2. Mobile phase A has a pH of 2.5 which prevents the conversion of ascorbic acid to other ionization forms.

Sample preparation

An appropriate amount of o-phosphoric acid was added to 5 mL of orange juice, to adjust pH to 2.5 and vortexed. The solution was spun at 1879 x g for 5 minutes and filtered through Agilent Regenerated Cellulose Econofilter, 0.2 µm (p/n 5185-5830). The filtered solution was directly used for sample analysis.

Procedure

A 5 µL amount of mobile phase A was injected as blank, followed by each linearity level in six replicates. Area and retention time (RT) information for each level was used to calculate relative standard deviation (RSD) values. The limit of detection (LOD) and limit of quantitation (LOQ) were established from the lower linearity level injections for benzoic acid. Prior to determining the linearity level, the extracted orange juice was injected to measure the approximate concentration of ascorbic acid, citric acid, and benzoic acid. The average area of each linearity level was plotted against the concentration to obtain a linearity curve.

Chromatographic conditions

Parameter	Agilent 1260 Infinity LC system		Agilent 1290 Infinity LC system	
TCC temperature	20 °C		20 °C	
Acquisition rate	40 Hz		40 Hz	
DAD wavelength (nm)	210.0, 230.0, 243.5		210.0, 230.0, 243.5	
Flow cell	10 mm, 1 µL		10 mm, 1 µL	
Sample thermostat	4 °C		4 °C	
Mobile phase A	20 mM monobasic phosphate buffer (KH_2PO_4), pH 2.5 adjusted by o-phosphoric acid		20 mM monobasic phosphate buffer (KH_2PO_4), pH 2.5 adjusted by o-phosphoric acid	
Mobile phase B	60% methanol - 40% acetonitrile		60% methanol - 40% acetonitrile	
Gradient	Time (min)	%B	Time (min)	%B
	0	5	0	5
	2	5	0.5	5
	2.1	25	0.6	25
	13.0	25	3.0	25
	13.1	90	3.1	70
	18.0	90	3.9	70
	18.1	5	4.0	5
	25.0	5	5.0	5
Flow	1.0 mL/min		1.5 mL/min	
Injection volume	5 µL with 3.0 s flush port wash		4 µL with 5.0 s flush port wash	

Table 1
Chromatographic parameters used for the Agilent 1260 Infinity LC and Agilent 1290 Infinity LC systems.

Concentration levels	Ascorbic acid (µg/mL)	Citric acid (µg/mL)	Benzoic acid (µg/mL)
1	10	5500	0.2
2	45	6000	1
3	63	6500	2
4	90	7000	3
5	108	7500	5
6	144	8000	10
7	162	8500	20
8	180	9000	35
9	225		50

Table 2
Dilution table for the three analytes.

To perform the recovery studies, the pH of the orange juice was adjusted to 2.5. Low and high amount of vitamin C, citric acid, and benzoic acid were spiked to obtain low and high concentration spiked samples. The difference between the high and low concentration spiked samples was used for recovery calculations. To evaluate the robustness of the method, four critical method parameters were changed – flow rate ± 2%, TCC temperature ± 5%, injector ± 5%, and wavelenth ± 3%.

For each variation, a standard spike mix concentration of 108 ppm of vitamin C, 7,000 ppm of citric acid, and 5 ppm of benzoic acid were injected in seven replicates. Three different brands of orange juices were analyzed to determine the concentration of the three acids.

The method was then effectively transferred to an UHPLC method. LOD, LOQ and linearity of each standard was evaluated and precision of the method was established by Area and RT RSD.

Results and Discussion

Separation and detection

The separation of vitamin C, citric acid, and benzoic acid were tested on various columns. Samples used were standards spiked into orange juice and standards dissolved in mobile phase A to determine matrix interference. Agilent's phenyl-hexyl and Poroshell EC-C18 column showed good separation for aqueous standards.

Agilent Poroshell EC-C18 was used for further experiments. A low temperature of the TCC, 60% methanol –40% acetonitrile in mobile phase B gave better separation of standards from the matrix peaks. Ascorbic acid has the maximum peak absorbance at 243.5 nm in its acidic form at pH 2.5. Ascorbic acid is easily quantifiable since there are less absorbing matrix peaks at this wavelength. Citric acid was monitored at 210.0 nm while benzoic acid was monitored at 230.0 nm. Since ascorbic acid is stable at low temperatures, the autosampler was maintained at 4 °C during the analysis.

Margolis et al.[6], reported a significant drop in ascorbic acid concentration when stored in autosampler vials for 22 hrs. A loss of 89% in the concentration of ascorbic acid was shown in different lots of autosampler vials, however vials cleaned by a base-acid wash procedure described by Margolis showed a maximum loss of only 4%.

In this study, three different vials were tested:

- MS verified vials (p/n 5190-2280)
- ALS vials (p/n 5182-0716)
- ALS vials cleaned by base-acid wash procedure

Since the calibration standards were stored in buffer at 4 °C in thermostatted ALS, after 16 hours, ascorbic acid showed 3% loss in area, while citric acid and benzoic acid showed less than 1% loss in area in all three vials. This study suggests that any of the vials can be used in this analysis. For this application, MS verified vials were used. Figure 1 shows the chromatogram of ascorbic acid, citric acid, and benzoic acid separated using an Agilent 1260 Infinity LC system. A step gradient to 25% mobile phase B was necessary to elute out benzoic acid away from the matrix peak. A hold at higher percentage organic beyond 13 minutes was necessary to remove matrix peaks from orange juice.

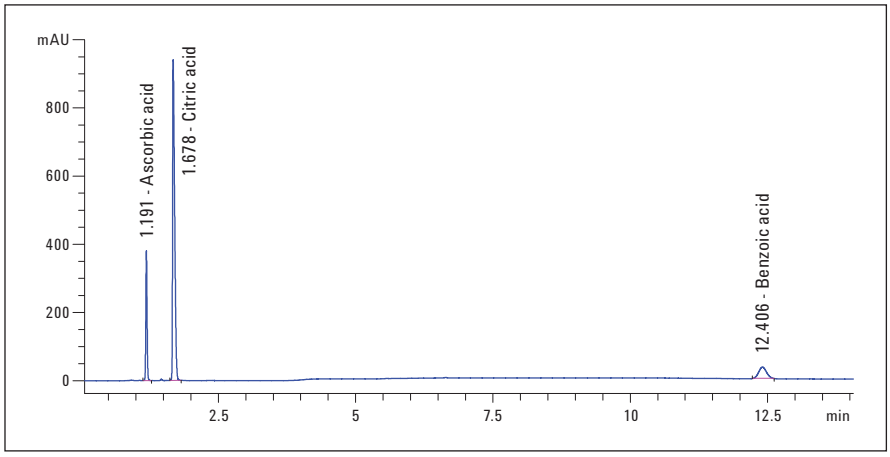


Figure 1
Separation of three standards ascorbic acid, citric acid and benzoic acid using an Agilent Poroshell 120 EC-C18 column. The chromatogram was collected at 230 nm.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The analyte concentration that provides a signal to noise ratio (S/N) of > 3 was considered as LOD and analyte concentration with S/N > 10 was considered as LOQ. Peak to peak method was used to calculate noise and compared with analyte peak height to obtain S/N values. In this application note, benzoic acids' LOD and LOQ were measured. LOD was 0.05 µg/mL with S/N = 3 and LOQ was 0.2 µg/mL with S/N = 16.

Linearity

Linearity curves with different concentration ranges were plotted for the three compounds. For benzoic acid, the linearity level was established starting from the LOQ level. Each linearity solution was injected six times and its average was used to construct the calibration curve. The linearity ranges cover the compounds' amount in orange juice. The linearity level for ascorbic acid is displayed in Figure 3. LOD and LOQ values, along with the linearity results are included in Table 3.

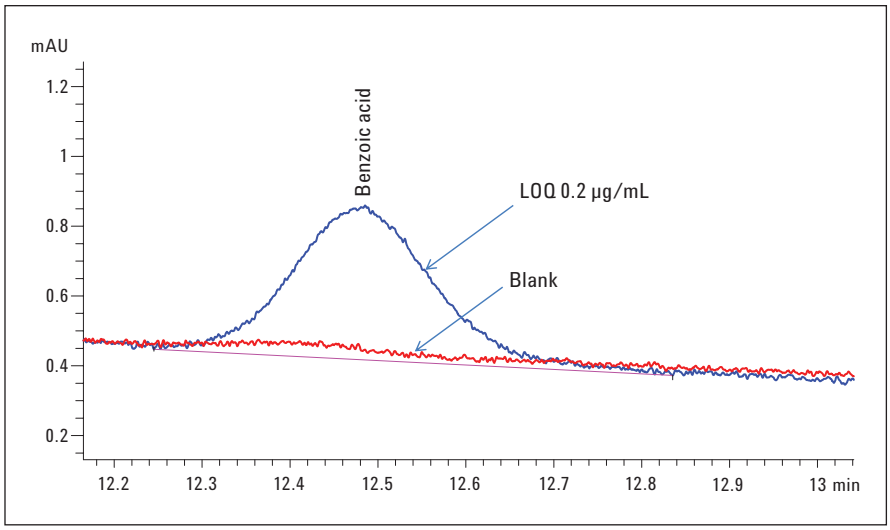


Figure 2
A 0.2 µg/mL (1 ng on column) solution of benzoic acid (at LOQ level) overlaid with blank injection. S/N ratio obtained at this concentration was 16.

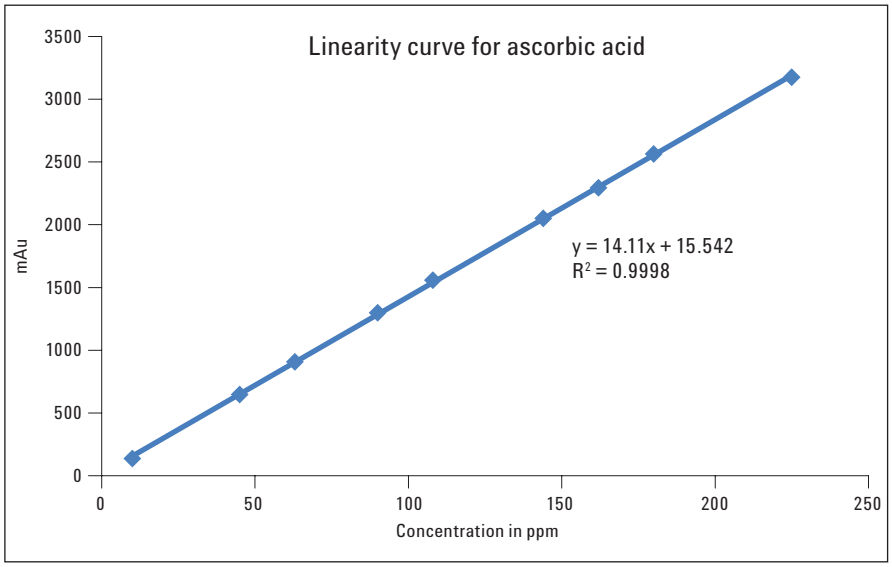


Figure 3
Linearity level of ascorbic acid from 10 µg/mL to 225 µg/mL showing the coefficient value.

Sl No:	Name	LOD µg/mL	LOD S/N	LOQ µg/mL	LOQ S/N	Linearity range (µg/mL)	R^2 value	Levels, replicate = 6	Accuracy
1	Ascorbic acid	–	–	–	–	10–225	0.9998	9	L1=87% (98%–100%)
2	Citric acid	–	–	–	–	5500–9000	0.9995	8	99%–101%
3	Benzoic acid	0.05	3	0.2	16	0.2–50	1	9	L1 = 106% 96–101%

Table 3
Linearity levels for ascorbic acid, citric acid and benzoic acid. The linearity range tested covers the compounds' content in orange juice.

Precision of retention time (RT) and area

The area precision was measured as RSD (%) across the linearity levels. The maximum RSD value of 1.9% for level 1 (L1) is obtained for benzoic acid as shown in Figure 4. Similarly, RT precision calculation showed a maximum RSD value of only 0.13%. The low RSD values for area and RT show acceptable reproducibility and precision of the method. Graphical representation of area RSD values is shown in Figure 4.

Robustness

To test the robustness of the method, a standard mix solution containing 108 µg/mL of ascorbic acid, 7,000 µg/mL of citric acid and 5 µg/mL of benzoic acid was used. Four critical method parameters (flow, TCC temperature, injector volume, and wavelength) were tested and data was collected in seven replicate injections. Analyte response areas from the last six replicates were used for the analysis. Allowed deviation for the area and retention time was set to ± 5% and ± 3% respectively.

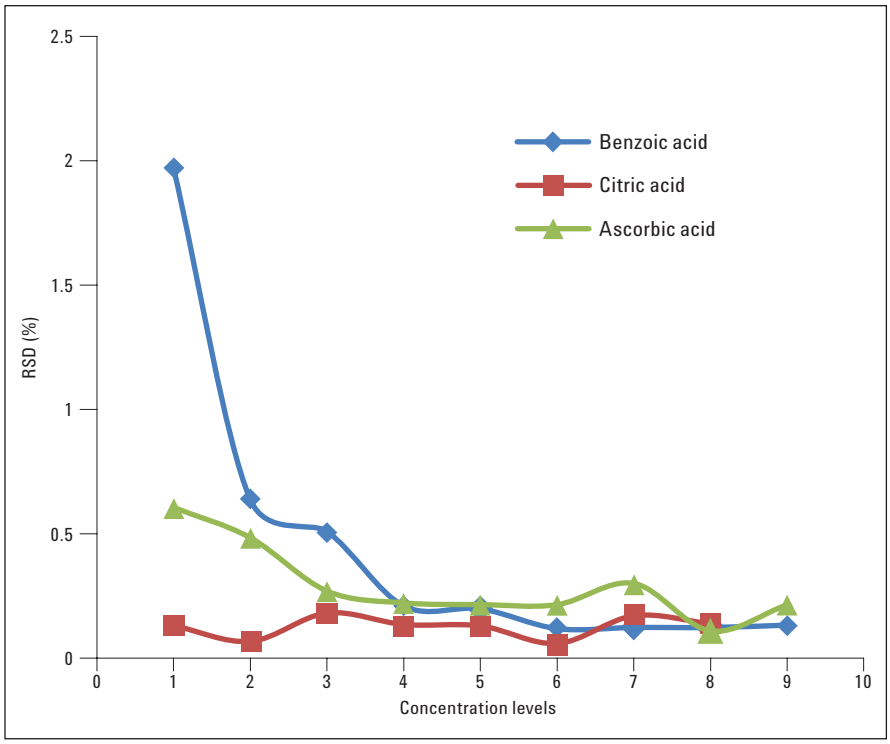


Figure 4
Area precision measured as RSD (%) for six replicates at each concentration level.

Parameters	Changes	Ascorbic acid % area	Ascorbic acid % RT	Resolution of citric acid at 230 nm % resolution	Citric acid % area	Citric acid % RT	Benzoic acid % area	Benzoic acid % RT
Flow: 1.0 mL/min ± 2%	High: 1.02 mL/min	6.2	2.1	0.0	2.2	2.2	3.4	1.8
	Low: 0.98 mL/min	3.3	1.8	0.6	1.2	1.3	0.5	1.7
TCC: 20 °C ± 5%	High: 21 °C	7.6	0.4	0.1	0.1	0.6	2.0	1.4
	Low: 19 °C	18.1	0.5	0.7	0.4	0.9	2.7	1.5
Injector: 5 µL ± 5%	High: 5.25 µL	2.4	0.2	1.6	5.6	0.3	4.3	0.9
	Low: 4.75 µL	8.2	0.0	0.5	5.0	0.2	6.1	0.2
Wavelength: 210.0, 230.0, 243.5 nm ± 3 nm	High: 213.0, 233.3, 246.5 nm	2.5	0.1	0.1	5.6	0.2	5.2	0.5
	Low: 207.0, 227.0, 240.5 nm	3.3	0.1	0.8	3.3	0.1	3.4	0.3

Table 4
Robustness test method results. The red numbers in Table 4 indicate that the allowed deviation was exceeded more than the allowed limit compared to the standard method.

The results of the robustness tests are summarized in Table 4. The red numbers indicate combinations where the allowed deviation was exceeded. A flow rate change of 2% results in a change in both area and RT for ascorbic acid. However, the peak area exceeds the allowed limit of 5% while the RT remained within the acceptable limits. Similarly, for ascorbic acid, the peak area is also found to have the greatest deviation caused by small variations in TCC temperature. These results show the importance of maintaining the column temperature during the analysis. Area reproducibility for citric acid and benzoic acid were found to be affected most by injector precision and wavelength accuracy. It is important that UV DAD is appropriately calibrated and passes accuracy tests. Robustness results indicate that the method is reliable for normal usage and to a great extent the performance remains unaffected by deliberate change in parameters. However, some parameters are critical and must be carefully controlled.

Recovery from sample matrix

As the blank matrix was not available, the recoveries of the three analytes were tested by spiking experiments. A low concentration standard spike solution contained ascorbic acid (300 µg), citric acid (4,000 µg) and benzoic acid (20 µg) spiked into orange juice (pH adjusted to 2.5 using o-phosphoric acid). Another high concentration spiking mix containing ascorbic acid (600 µg), citric acid (8,000 µg), and benzoic acid (40 µg) was also spiked into a separate orange juice sample. The analytes were extracted from the orange juice sample as described above. Using the aqueous linearity curve (see paragraph "Linearity" and Figure 3 on page 5), the area was converted to concentration values. The low concentration spiking mix concentration was subtracted from the high concentration value and the difference was compared with the difference in spiking amounts to obtain recovery values. This difference method would account for the degradation of any compound/matrix during analysis. The recovery experiment was performed in triplicate and the results are shown in Table 5. Ascorbic acid shows excellent recovery because of its unique absorbance maximum at 243.5 nm where background absorbance was minimum. Greater than 90% recovery for all three analytes were observed.

Compound name	Recovery (%)
Ascorbic acid	100 ± 3
Citric acid	91 ± 12
Benzoic acid	98 ± 6

Table 5
Recovery values results from spiking experiment performed in triplicates.

Sample analysis

In this study, the content of ascorbic acid, citric acid, and benzoic acid in orange juice was determined using the extraction procedure and the developed chromatographic method. Orange juices, labeled here as O-juice 1, O-juice 2, and O-juice 3, were analyzed using three different aliquots from each orange juice. Samples were prepared as described above. The results of the analysis were compared against the linearity equation to obtain the concentration values (Table 6) and show that the amount of the three analytes varies with the brand of orange juice. The O-juice 1 nutrition label claims 111 µg/mL of ascorbic acid. The actual amount found was 145 µg/mL. This is probably because ascorbic acid is also naturally present in orange juice thereby increasing the content level, or due to a batch variation in the product. O-juice 1 also claims to add no preservatives however, a trace amount of benzoic acid was found. O-juice 3 claims to add (without specifying the amount) antioxidant E300 and acid regulator E330 which corresponds to ascorbic acid and citric acid respectively. Both of these compounds were detected and quantified using this method. However, 2.25 µg/mL of benzoic acid was also detected in this sample as shown in Figure 5.

Orange juice samples	Ascorbic acid µg/mL	Citric acid µg/mL	Benzoic acid µg/mL
O-juice1	145 ± 2	8895 ± 21	0.62 ± 0.07
O-juice 2	93 ± 3	8188 ± 43	0 ± 0
O-juice 3	35.4 ± 0.3	3160 ± 8	2.25 ± 0.06

Table 6
Amount of ascorbic acid, citric acid and benzoic acid present in three different brands of orange juice. (Note that the added amount of most of these compounds are not specified on the nutrition label).

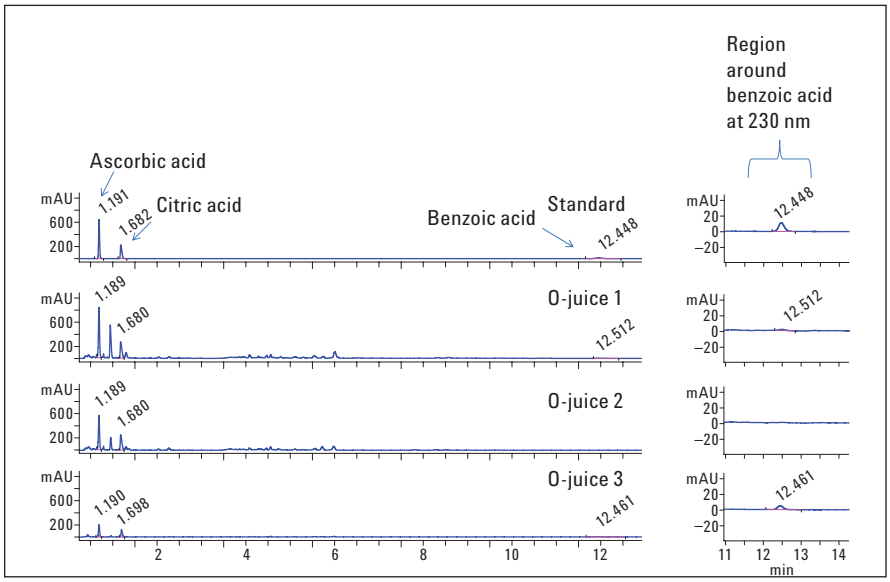


Figure 5
Three different orange juice brands analyzed at 230 nm for benzoic acid.

UHPLC Method

The HPLC method was transferred to an UHPLC method on an Agilent 1290 Infinity LC system. It was developed to provide a shorter and faster method using the same mobile phase but a shorter column. The resulting UHPLC method is only five minutes long as shown in Figure 6 compared to 25 minutes in the HPLC method. This 5× increase in speed results in 68% of solvent saving. A quick method also overcomes the possible loss of ascorbic acid during sample analysis. Note that the maximum percentage of solvent B in the UHPLC method goes up to 70% rather than 90%. Both 90% and 70% were tested and each can be used for the Agilent 1290 LC Infinity method. The LOD for benzoic acid is 0.05 µg/mL while the LOQ is 0.2 µg/mL (S/N =16), which is similar to that obtained by the Agilent 1260 Infinity LC method. The calibration curve for ascorbic acid was found to be linear as shown in Figure 7.

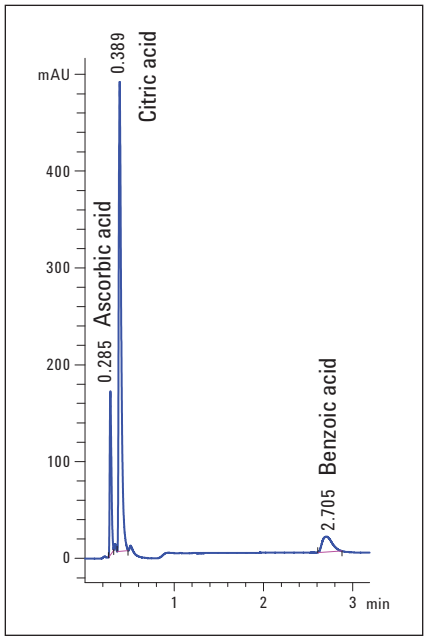


Figure 6
An UHPLC method separating the standards for ascorbic acid, citric acid, and benzoic acid on an Agilent Poroshell 120 EC-C18 column 3.0 × 75 mm, packed with 2.7-µm particles.

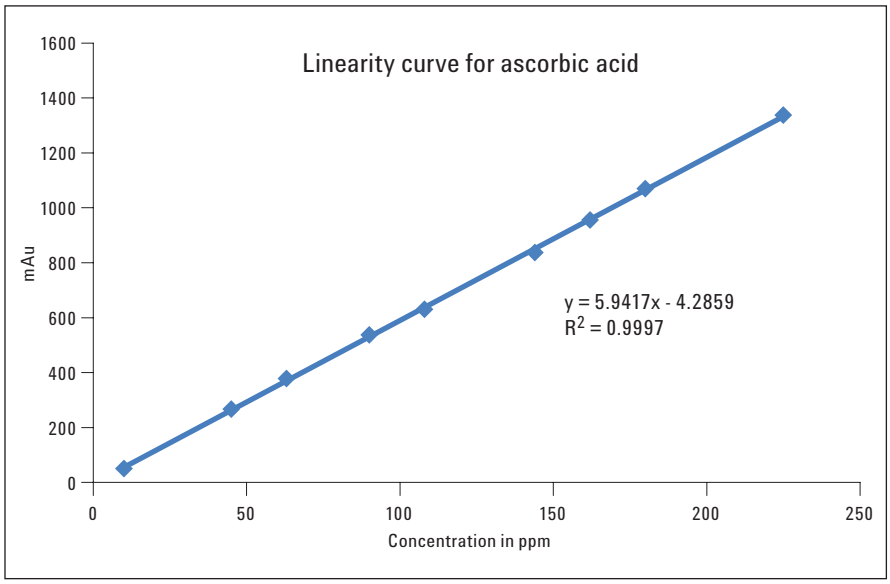


Figure 7
Linearity level of ascorbic acid from 10 µg/mL to 225 µg/mL (50 ng to 1125 ng on column) showing the coefficient value on an Agilent 1290 Infinity LC System.

RSD(%) deviation on area and RT was calculated for concentration levels same as in HPLC method. The results show that RSD(%) on area deviation was less than 2.5% for all concentration levels, as shown in Figure 8 while RSD on RT was less than 0.5%.

Conclusion

Ascorbic acid, citric acid and benzoic acid were separated and quantified using Agilent Poroshell 120 EC-C18 columns. A partially validated 25 minute method was developed. This method quantifies ascorbic acid, citric acid and benzoic acid in various orange juices with little or no matrix interference as seen by >90% recovery values. A method transfer to an Agilent 1290 Infinity LC system was effectively carried out by increasing the flow rate and using a smaller length column resulting in a 5 minute UHPLC method. Both the methods are linear and give precise results. These methods can therefore be applied to determine ascorbic acid, citric acid and benzoic acid in quality control of orange juices.

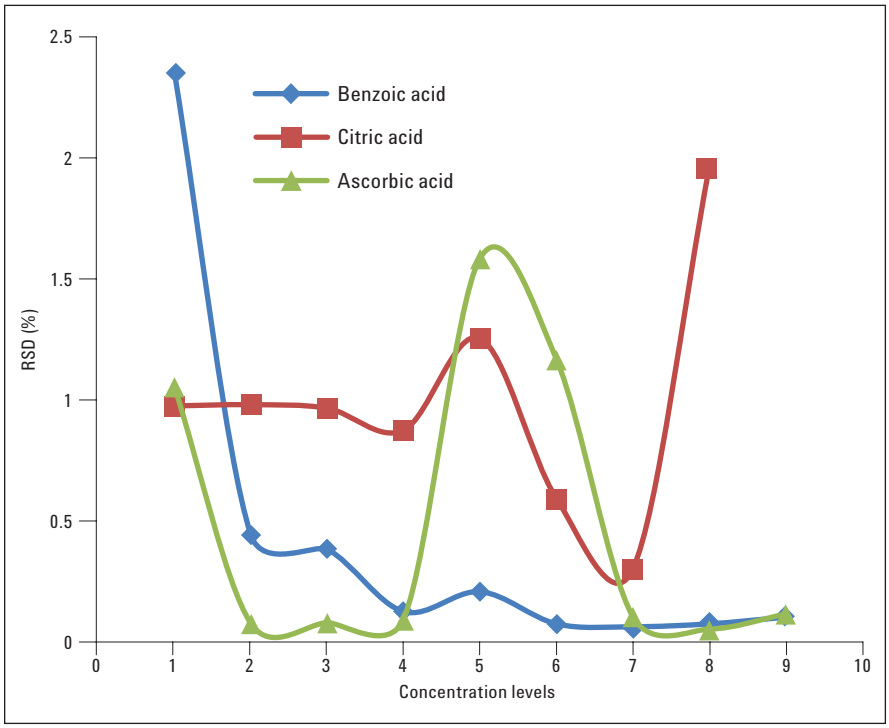


Figure 8
An UHPLC method showing area precision measured as RSD (%) for three different compounds, six replicates for each concentration level.

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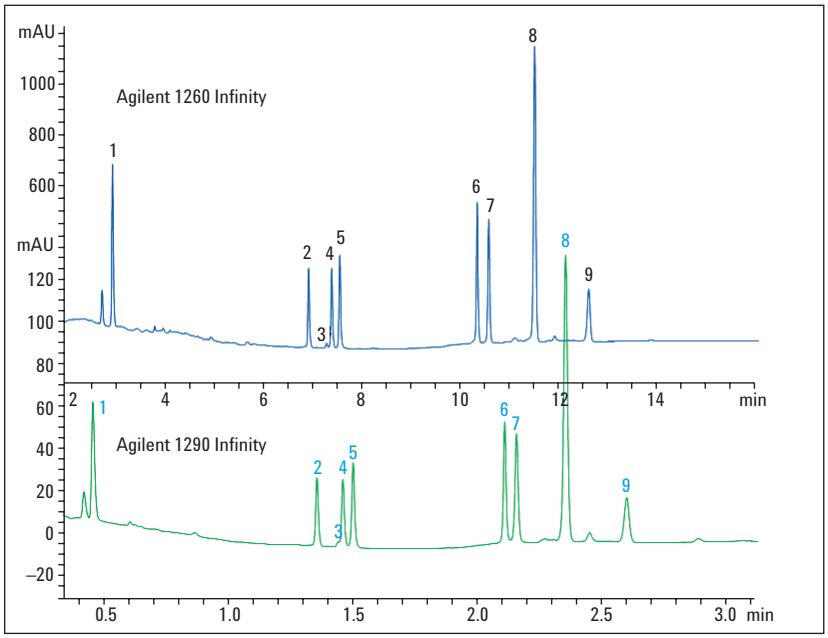
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Agilent Application Solution

Analysis of fat-soluble vitamins from food matrix for nutrition labeling

Application Note

Food



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Abstract

This Application Note shows how to carry out qualitative and quantitative analysis of fat soluble vitamins from two different food matrixes. A robust reverse phase high performance liquid chromatographic (RP-HPLC) method for simultaneous determination of nine fat soluble vitamins was developed. Separation and quantification was achieved by an Agilent 1260 Infinity LC System using an Agilent Poroshell EC-C18 column. The advanced feature of the Agilent 1260 Infinity Diode Array Detector (DAD) to select multiple wavelengths was utilized effectively to detect various vitamins at their maximum absorbance. Robustness of the method for routine nutrition labelling analysis was established by partial validation and the method was verified by analyzing vitamin D content from two different food matrices. Finally, using an Agilent 1290 Infinity LC System, this HPLC method was effectively transferred to a short Ultra High Pressure Liquid Chromatographic (UHPLC) method.

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Introduction

Vitamins are nutrients required in trace amounts by an organism for its healthy growth and must be obtained from the diet. In general, vitamins can be classified into two groups, water- or fat-soluble. As the level of vitamins present in a food may vary from nanograms to milligrams, labelling these vitamins in a matrix like infant formula is a mandatory requirement by the US Food and Drug Administration (FDA). The diversity in the chemical nature of vitamins made concurrent extraction and analysis of all vitamins challenging. Due to this, analysis of water soluble and fat soluble vitamins will be usually performed separately. Simultaneous analyses of ten water soluble vitamins are described in another Agilent Application Solution[1]. In this study, simultaneous analysis on nine fat soluble vitamins are discussed. The list of vitamins used in this study covers vitamins A, D, E, and K, which are the common fat soluble vitamins present in food medium. Even though extraction procedures for various fat soluble vitamins are different, developing a single analytical method simplifies the analytical challenge by quantifying the analytes in a single LC run.

In this Application Note, we described an approximately 15 minute long, sensitive, and robust method for simultaneous determination of nine fat soluble vitamins with UV detection. Also, this HPLC method transferred to a 3.5 minute UHPLC method for customers where short analytical times are critical requirement.

Experimental

Instruments and Software

An Agilent 1260 Infinity Quaternary LC System consisting of the following modules was used:

- Agilent 1260 Infinity Quaternary Pump with vacuum degasser (G1311B)
- Agilent 1260 Infinity High-Performance Autosampler (G1367E)
- Agilent 1260 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1260 Infinity Diode Array Detector (G4212B) with 60-mm Max-Light flow cell

The UHPLC analysis was developed and performed using an Agilent 1290 Infinity LC System consisting of the following modules:

- Agilent 1290 Infinity Binary Pump with integrated vacuum degasser (G4220 A) and 100-µL Jet Weaver mixer
- Agilent 1290 Infinity High Performance Autosampler (G4226A)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C):
- Agilent 1290 Infinity Diode Array Detector (G4212A) with 10-mm Max-Light flow cell

Columns:

- Agilent Poroshell 120 EC-C18 columns with internal diameters of 2.1 mm and lengths of 75 mm, packed with 2.7-µm particles (697775-902)
- Agilent Poroshell 120 EC-C18 column 3.0 × 150 mm, 2.7-µm (693975-302)

Software:

- Agilent ChemStation B.04.02

Reagents and materials

All the chemicals and solvents used were HPLC grade. Highly purified water was used from a Milli Q water purification system (Millipore Elix 10, Millipore, USA). Acetonitrile and methanol were of super gradient grade and were purchased from Lab-Scan (Bangkok, Thailand) and biotech grade tetra hydro furan (THF) was purchased from Sigma (Germany). Eluent additive grade acetic acid was purchased from Fluka (Germany) and all other chemicals used in this study were from Aldrich (India). Standards of menadione (K3), Linolenic acid (6-omega fatty acid), Retinol (alcohol form of vitamin A), retinoic acid (acid form of vitamin A), 9-cis retinal (aldehyde form of vitamin A), vitamin K2, Cholecalciferol (D3), tocopherol (a form of vitamin E), and vitamin K1 were purchased from Aldrich (India). The infant formula and multivitamin tablets used in this study for recovery and nutrition labelling analysis were purchased locally.

Chromatographic parameters

Chromatographic parameters used for reverse phase liquid chromatography and UHPLC are shown in Table 1.

Parameter	Agilent 1260 Infinity Quaternary LC system	Agilent 1290 Infinity LC system
Column oven:	45 °C	45 °C
Acquisition rate:	20 Hz	80 Hz
Data acquisition:	216, 246, 266, 326, 356, 376 nm	216, 246, 266, 326, 356, 376 nm
Flow cell:	60 mm path	10 mm path
Injection volume:	5 µL (needle with wash, flush port active for 5 seconds)	1 µL (needle with wash, flush port active for 3 seconds)
Sample thermostat:	5 °C	5 °C
Mobile phase A:	95:5; water:THF with 0.05% acetic acid	95:5; water:THF with 0.05% acetic acid
Mobile phase B:	75:25:5; Acetonitrile:Methanol:THF with 0.035% acetic acid	75:25:5;Acetonitrile:Methanol: THF with 0.035% acetic acid
Gradient:	At 0 min → 30%B At 3 min → 75%B At 8 min → 100%B At 15 min → 100%B At 15.1 min → 30%B	At 0 min → 50%B At 0.6 min → 75%B At 1.7 min → 100%B At 3.4 min → 100%B At 3.5 min → 50%B
Post run time:	5 minutes	1 minute
Flow rate:	0.9 mL/min	0.9 mL/min

Table 1
Chromatographic parameters used for the Agilent 1260 Infinity LC system and the Agilent 1290 Infinity LC system.

Fat soluble vitamin standard solution

Vitamin standards of menadione, linolenic acid, retinol, retinoic acid, retinal, vitamin K1, K2, D3, and tocopherol were prepared individually by weighing approx. 50 mg of the vitamin powder and transferring it to a 25-mL volumetric standards flask. Mobile phase B was added to form a stock solution of 2.0 mg/mL (2,000 ppm). Sonication was used when required. Fat-soluble vitamin stock solutions were wrapped with aluminum foil and stored at + 4.0 °C in the dark when not in use.

Mixed standard solution and linearity levels

About 100 µL of each standard were precisely mixed to get a 1,000-µL standard mix of fat soluble vitamins at concentration of 200 ppm each. Linearity levels were prepared by subsequent dilution of this 200-ppm standard spike mix, using mobile phase B as diluent. The linearity standard solutions were covering a range of 5 pg/µL to 100 ng/µL (10 levels and 6 replicates)

Sample preparation for nutrition labelling and recovery studies

Two different types of samples, infant formula and vitamin tablets were used for nutrition labelling and recovery studies. Vitamin D was the analyte used in this study to evaluate recovery efficiency and further perform nutrition labelling analysis. Vitamin D from 5-g infant formula was extracted by alkaline hydrolysis, using ethanolic potassium hydroxide and the refluxing time was about 45 minutes[2]. Pyrogallol was used as an antioxidant during the refluxion. A 10-mL mixture of hexane and diethyl ether in the ratio 1:1 was used to extract vitamin D from the saponified digest. A 1.0 mL amount of this organic layer was evaporated to dryness after successive washing with water and further reconstituted with 200 µL mobile phase B and injected in HPLC for nutrition labelling analysis. Recovery studies were performed using spiked and unspiked samples of infant formula. An on-column concentration of 25 ng vitamin D standard was used for sample spiking. Extraction procedure was same as before.

In the case of multi vitamin tablets, recovery and nutrition labelling studies were carried out from one tablet.

Precautions

Vitamins are known to be highly sensitive to light and heat. To extend the stability in solution form, all the prepared solutions were wrapped in aluminum foils and stored in a refrigerator at 4 °C in the dark, when not in use. The thermosttated autosampler tray was maintained at 4 °C during the analysis. Extractions of vitamin D from samples were performed under dark conditions.

Procedure

5 µL of mobile phase B was injected as blank and followed by each linearity level in six replicates. Area and retention time (RT) information of each level were utilized to calculate standard deviation (SD) and relative standard deviation (RSD) values. LOD and LOQ were established from the lower linearity level injections. Average area of vitamin peaks in each linearity levels was plotted against the concentration to construct linearity curves.

Six critical method parameters were changed to evaluate the robustness of the method. A standard spike mix was injected in six replicates and data was used for robustness study.

For nutrition labelling analysis, amount of vitamin-D present in infant formula and multivitamin tablet were quantified and compared with the label value.

Recovery studies using infant formula and multivitamin tablets were performed by injecting with or without spiking 25 ng vitamin D standard to 5 g or per tablet respectively.

The method was effectively transferred to UHPLC. LOD, LOQ, and linearity of each vitamin were evaluated and precision of the method was established by area and RT RSD. Linearity curves for all vitamins using UHPLC method were also plotted.

Results and Discussion

Separation and detection

Excellent separation of nine fat soluble vitamins in 13 minutes was achieved using an Agilent Poroshell 120 EC-C18 (3.0 mm × 150 mm, 2.7 µm) column. The absorbance maximum was found to be different for different vitamins due to the diversity in molecular structures. The chromatographic elution patterns of nine vitamins are shown in Figure 1 and the list of vitamins with individual absorbance maxima are tabulated in Table 2. The observed base line drift at 216 nm can be explained by the change in amount of modifier in the mobile phase during the gradient run. We used the peak purity feature in the ChemStation software to check the purity of each peak, and thus specificity of method was evaluated. Precision, linear range, accuracy, specificity, recovery, and robustness studies were used to validate the method.

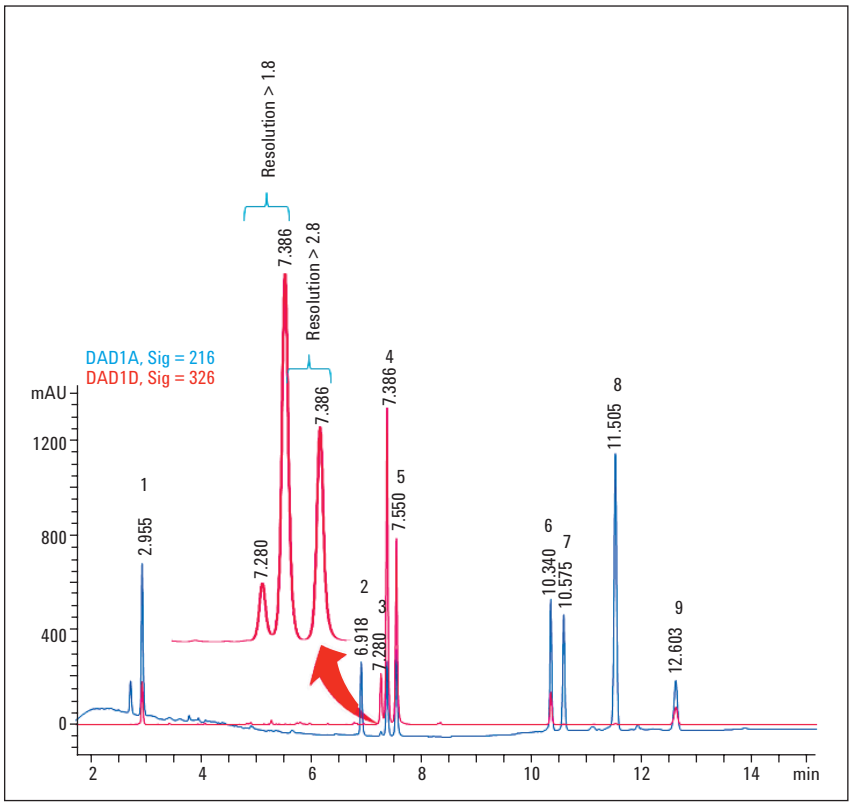


Figure 1
Separation of nine fat soluble vitamins using a 15-cm Agilent Poroshell 120 EC-C18 column. The excellent separation of three different forms of vitamin A is shown in the insert.

Sl no.	Name	Synonym	Absorbance max.	RT
1	Menadione	vitamin k3	250	2.965
2	Linolenic acid	6-omega fatty acid	< 216	6.918
3	Retinol	vitamin A alcohol	326	7.280
4	Retinoic acid	vitamin A acid	356	7.386
5	9-cis Retinal	vitamin A aldehyde	376	7.550
6	Vitamin K2	vitamin K2	246	10.340
7	Cholecalciferol	vitamin D3	266	10.575
8	Tocopherol	a form of vitamin E	< 220	11.505
9	Vitamin K1	vitamin k1	246	12.603

Table 2
List of vitamins used in this study and observed absorbance maxima for each vitamin.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The analyte concentration that provides a signal to noise ratio (S/N) of > 3 was considered as LOD and analyte concentration with S/N > 10 was considered as LOQ. Observed LOD and LOQ value of each vitamin are tabulated in Table 3. As an example, overlay of LOD, LOQ chromatograms of cholecalciferol (Vitamin D) with blank is shown in Figure 2.

Linearity

All the prepared linearity levels were injected in six replicates and linearity curves for each vitamin were constructed from the LOQ level to a highest concentration level in the study using area response and concentration values. This linearity range covers the usual vitamin content values in common food matrices. The observed regression coefficients for all vitamins were also tabulated in Table 3. The linearity curve for cholecalciferol is shown as an example in Figure 3.

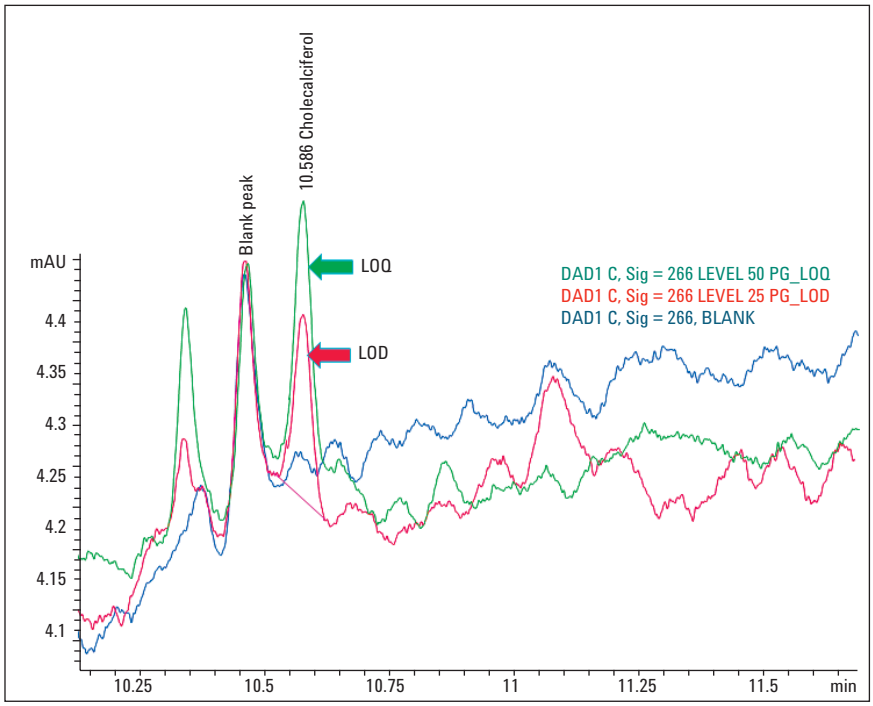


Figure 2
LOD (25 pg) and LOQ (50 pg) chromatograms of cholecalciferol (vitamin D) overlaid with blank.

Sl no.	Name	LOD (ng)	LOQ (ng)	Linearity range (ng) on-column	R^2 value	Total levels, replicates = 6	Linearity equation
1	Menadione	0.025	0.05	0.05–250	0.99997	10	y = 33.0129976 x + 6.4314273
2	Linolenic acid	1.5	2.5	2.5–500	0.99989	6	y =1.86128287 x + 0.1410729
3	Retinol	0.5	1.5	1.5–500	0.99990	7	y = 0.60131392 x + 1.2872412
4	Retinoic acid	0.025	0.05	0.05–250	0.99987	10	y = 40.3653646 x + 70.38627
5	Retinal	0.025	0.05	0.05–250	0.99956	10	y = 42.7776135 x + 50.084652
6	Vitamin K2	0.025	0.05	0.05–500	0.99996	11	y = 15.2586556 x – 16.251493
7	Cholecalciferol	0.025	0.05	0.05–500	1.00000	11	y = 17.9806249 x – 5.667246
8	Tocopherol	5	25	25–500	0.99896	4	y = 9.74071376 x – 229.06229
9	Vitamin K1	0.05	0.15	0.15–500	0.99989	10	y = 14.6792864 x – 19.932064

Table 3
LOD, LOQ, and linearity results of all nine vitamins.

Precision of retention time (RT) and area

To establish the method precision, relative standard deviation (RSD) values for retention time (RT) and area of all nine vitamins at 50 ng on-column concentration were calculated. The highest observed area RSD value was 0.035% and RT RSD was 0.04%. Graphical representation of area and RT RSD values of nine vitamins are shown in Figure 4.

Robustness

Robustness of the method was evaluated by deliberately varying six critical method parameters and the resulting deviation in area and retention time was calculated and compared to the original method. As a sample, a standard spike mix solution of vitamins was injected in six replicates. Allowed deviation for retention time and area was set to ± 3% and ± 5% respectively. Results from robustness study are summarized in Table 4.

The deviation of retention time is within the allowed limit for robustness studies with increasing flow rate, mobile phase composition and column temperature. However, the impact of gradient steepness variation was higher and observed RT deviations were above the limit. Area deviations for eight vitamins were found to be within the limit for majority of tests, except for vitamin K2. The area of vitamin K2 was found to be very sensitive to flow rate, gradient steepness, column temperature, mobile phase composition, and detection wavelength. Another parameter which needs to be carefully controlled is the detection wavelength. Robustness results indicate that, the method is reliable to use for normal usage and, to a great extent, the performance remains unaffected by deliberate change in parameters.

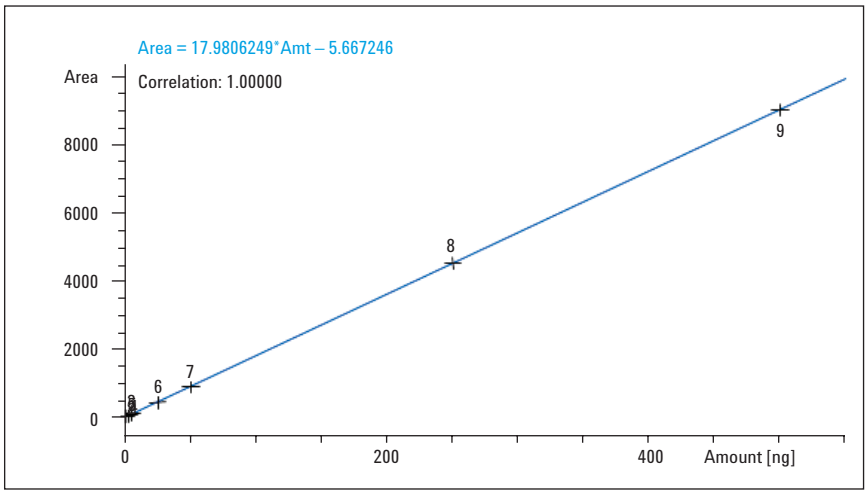


Figure 3
Linearity curve of cholecalciferol from 0.05 ng to 500 ng (on-column concentration) showing excellent coefficient value.

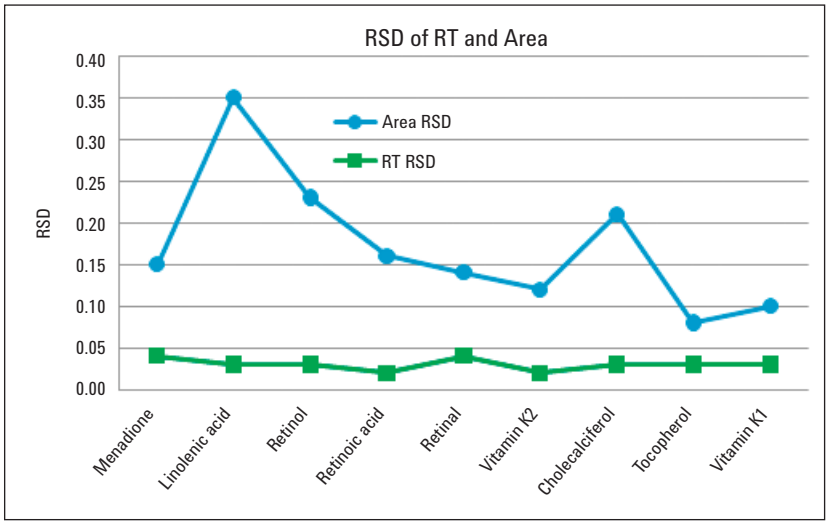


Figure 4
Excellent RT and area RSD values for all vitamins at 50 ng on-column concentration.

Sl no.	Parameter (actual value)	Measured deviation	Modified value	RT deviation (Allowed limit is ± 3.0%)	Area deviation (Allowed limit is ± 5.0%)
1	Flow rate (0.9)	2%	0.92 mL/min	Passed	Passed for 8 compounds
			0.88 mL/min	Passed for 8 compounds	Passed for 6 compounds
2	Injection volume (5 µL)	2%	5.1 µL	Passed	Passed for 8 compounds
			4.9 µL	Passed	Passed for 8 compounds
3	Gradient steepness (5, 75 to 100 in 5 minutes)	10%	4.5 (77.5 to 100 in 5 minutes)	Passed for 5 compounds (Failed for 4 compounds which are eluting in the initial gradient time frame)	Passed for 8 compounds
			5.5 (72.5 to 100 in 5 minutes)	Passed for 5 compounds (Failed for 4 compounds which are eluting in the initial gradient time frame)	Passed for 8 compounds
4	Mobile phase composition (ACN 75%)	5%	Acetonitrile 80% (80:15:5)	Passed	Passed for 7 compounds
			Acetonitrile 70% (70:25:5)	Passed	Passed for 8 compounds
5	Column temperature (45 °C)	5%	47.2 °C	Passed	Passed for 8 compounds
			42.8 °C	Passed	Passed for 8 compounds
6	Wavelength (216, 246, 266, 326, 356, 376 nm)	3 nm	(219, 249, 269, 329, 359, 379 nm)	Passed	Passed for 6 compounds
			(213, 243, 263, 323, 353, 373 nm)	Passed	Passed for 5 compounds

Table 4
Robustness test result summary.

Recovery of vitamin D from sample matrix

Recovery analyses for vitamin D from multi vitamin tablets or infant formula were carried out by a standard addition method[3]. A standard solution of vitamin D at 25 ng/µL was used for this analysis. In this study, the area of the vitamin D peak in the spiked sample, unspiked sample and standard chromatogram were measured separately. The difference in detector response between spiked and unspiked sample was compared against response observed in standard chromatogram and expressed in percentage as recovery. The recovery results from multivitamin tablets and infant formula were 94% and 62% respectively. The low recovery value of vitamin D from infant formula can be justified by the challenge involved in vitamin extraction from a matrix containing a high amount of proteins and fat. The chromatograms observed for spiked, unspiked, and standard vitamin solutions are shown in Figure 5.

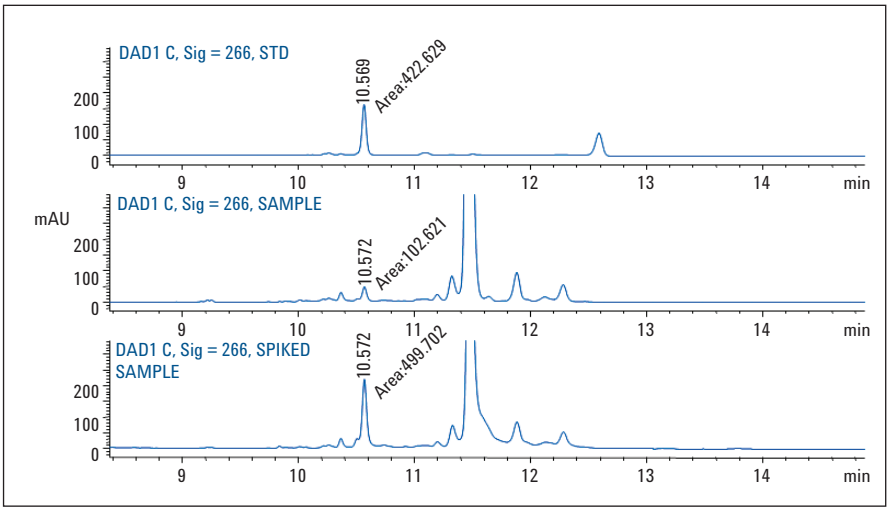


Figure 5
Overlay of spiked, unspiked, and standard chromatograms used for multivitamin tablets.

Nutrition labelling

In this study, the amount of vitamin D present in infant formula and multivitamin tablets was estimated using the area response and compared with the concentration claimed on the label. Linearity equations originated from linearity curves were used for the calculation. Extraction was carried out using ethanolic KOH refluxion. The label claim for vitamin D in infant formula and multivitamin tablets were 8.3 µg/100 g and 10 µg/tablet respectively. The calculated values with recovery corrections were 6.0 µg/100 g and 12.0 µg/tablet respectively. The results show excellent suitability of the method to quantify vitamins in food sample.

UHPLC Method

A UHPLC method with diode array detection was developed for the separation of nine fat soluble vitamins. The resulting UHPLC method shows excellent resolution and saves about 66% percent analysis time and solvent compared to the 15 minutes long HPLC gradient (Figure 6). The Retinol peak is slightly co-eluting with the retinoic acid peak. This will not be a problem for analyzing vitamins, because in a given sample matrix, the possibility of vitamin A being present in the alcohol (retinol) and acid (retinoic acid) form simultaneously is very rare. The LOD and LOQ levels and linearity of each vitamin, except for retinol, was established using the UHPLC method. The observed LOD, LOQ and linearity results are tabulated in Table 5. Excellent linearity observed for vitamin D is shown in Figure 7. RSD values for RT and area for an on-column concentration of 100 ppm with an injection volume of 1 µL was calculated. The highest observed RSD for area was 1.25% and that for RT was 0.12%. The results can be graphically represented as in Figure 8. Low RSD values for area and RT confirmed precision of the method.

These results prove the reliability and sensitivity of the developed UHPLC method. Quick nutrition labeling analysis of food samples is possible using this method.

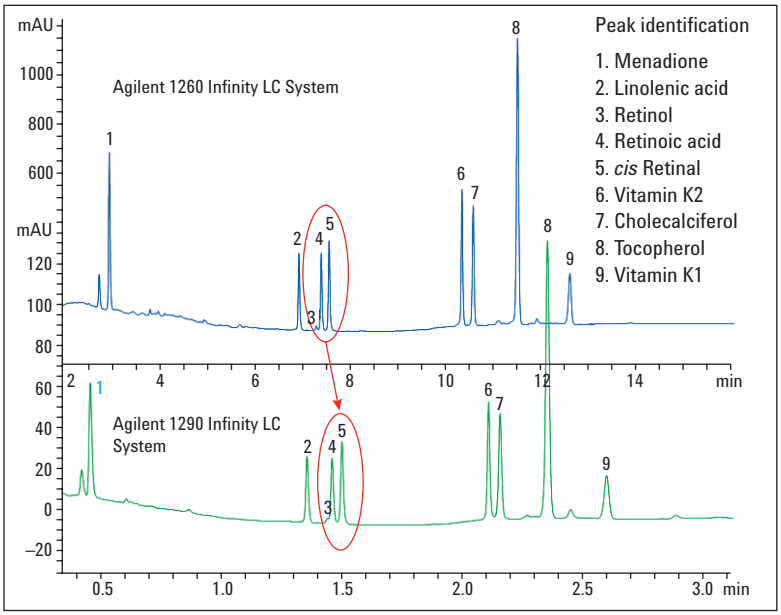


Figure 6
Overlay of separation of nine fat soluble vitamins using HPLC method on Agilent 1260 infinity and UHPLC method on 1290 Infinity LC system.

Sl no.	Name	LOD (ng)	LOQ (ng)	Linearity range (ng) on-column	R^2 value	Total levels, replicates = 6
1	Menadione	0.025	0.05	0.05–200	0.99995	10
2	Linolenic acid	0.5	1	1–500	0.99990	7
3	Retinoic acid	0.025	0.05	0.05–200	0.99999	10
4	Retinal	0.05	0.1	0.1–200	1.00000	9
5	vitamin K2	0.05	0.1	0.1–500	0.99999	10
6	Cholecalciferol	0.05	0.1	0.1–500	0.99998	10
7	Tocopherol	0.5	1	1–500	0.99966	7
8	vitamin K1	0.1	0.25	0.25–500	0.99990	9

Table 5
LOD and LOQ values derived from the UHPLC method using Agilent 1290 Infinity LC system.

Conclusion

Nine fat soluble vitamins were separated and quantified using an Agilent Poroshell 120 EC-C18 column. Using the Agilent 1260 Infinity LC System, a robust, 15 minute long, HPLC gradient method was developed. The method can be used successfully to quantify vitamins like menadione, Linolenic acid, various form of vitamin A, D, E, and K. The linearity range of the current study is broad to accommodate various possible concentrations of these vitamins in many food matrices. Gradient conditions ensured better chromatographic resolution improved sensitivity, and less matrix interference. The method is simple, specific, sensitive, rapid, and also provides good precision, linearity and recovery values. Efficient use of this method was demonstrated by quantifying vitamin D from infant formula and multivitamin tablet matrix. Later, this method was transferred to a short 3 minute UHPLC method using the Agilent 1290 Infinity LC System which in turn saves 66% of analysis time and solvent. These methods using the Agilent 1260 Infinity LC and Agilent 1290 Infinity LC systems can be applied for accurate routine nutrition labeling analysis of fat soluble vitamins.

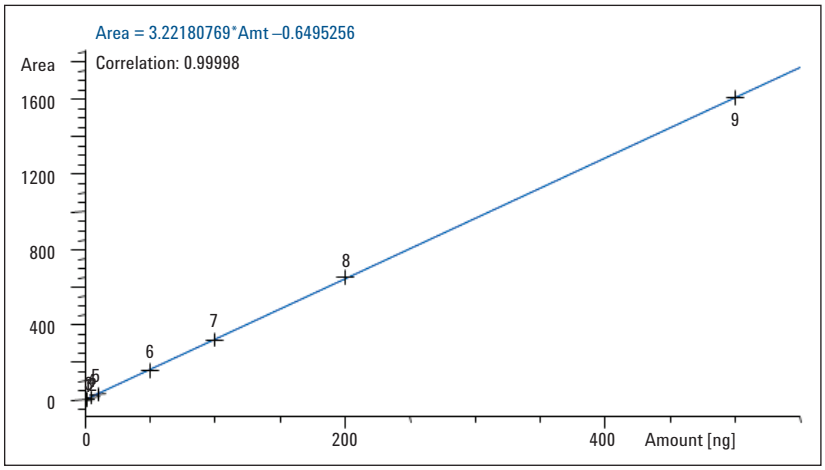


Figure 7
Linearity of vitamin-D from 0.05 ng to 500 ng showing a correlation of 0.99998 (9 levels and six replicates). Injection volume is 1 µL.

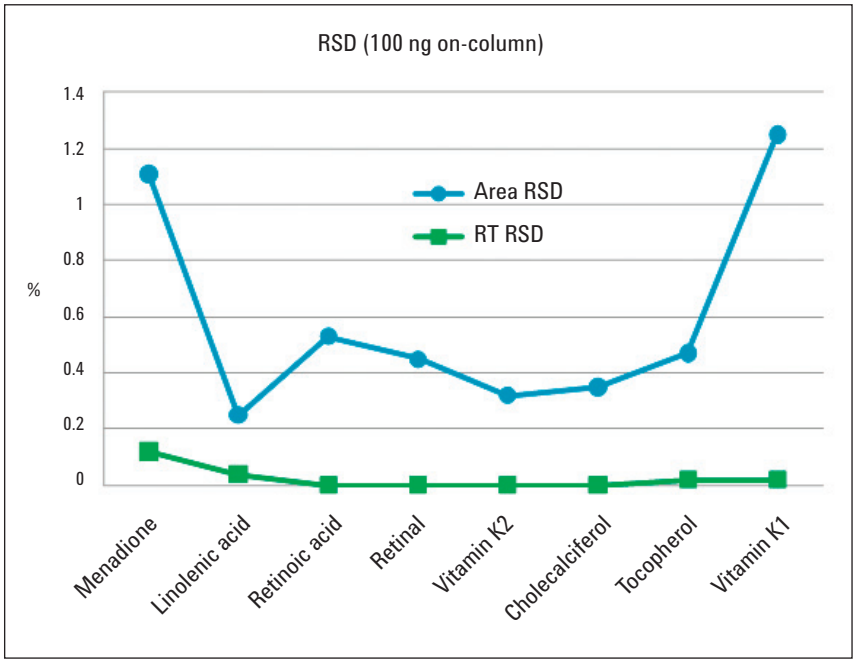


Figure 8
Area and RT RSD values from UHPLC results for all vitamins except retinol at an on-column concentration of 100 ppm. Injection volume is 1 µL and six replicates.

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Agilent Technologies

Fast analysis of resveratrol in red wine using the Agilent 1290 Infinity LC System

Application Note

Food

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Abstract

Red wine contains various polyphenols, which function as antioxidants. This Application Note shows the analysis of one of the antioxidant resveratrol in red wine. A recent study found that resveratrol improves health and extends maximum lifespans in various species[1]. The fast and highly sensitive analysis of resveratrol was done on an Agilent 1290 Infinity LC using an Agilent ZORBAX RRHD sub-2 µm column and the Max-Light high-sensitivity flow cell with 60 mm path length.

Configuration

Agilent 1290 Infinity LC System
Agilent 1290 Infinity Lc Binary Pump (G4220A)
Agilent 1290 Infinity LC Autosampler (G4226A)
Agilent 1290 Infinity LC Thermostatted Column Compartment (G1316C)
Agilent 1290 Infinity LC Diode Array Detector with 60 mm Max-Light high-sensitivity flow cell (G4212A)
System control by OpenLab CDS ChemStation Edition software

Analytical conditions

Column:	Agilent ZORBAX RRHD SB-C18 2.1 x 100 mm,1.8 µm
Mobile phase:	A = Water + 0.1% formic acid, B = Acetonitrile + 0.1 % formic acid
Isocratic:	25 % B
Flow rate:	0.5 mL/min
Column temp.:	40 °C
Injection vol.:	1 µL
Detection:	320 nm/4, reference off

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Experimental

A chromatogram of the standard solution is shown in Figure 1. The retention time of resveratrol is 1.91 min. Figure 2 shows the overlaid chromatograms of standard solutions at different concentration levels of 0.001, 0.0025 and 0.01 mg/L. With a S/N of 4.2 for the 0.001 mg/L solution even the lowest concentration was well over the limit of detection (LOD, S/N > 3). For the calibration, a set of external standards was used giving a good linearity with a R^2 value of 0.999 between concentrations from 0.001 to 0.1 mg/L (Figure 3). Precision of retention time and area was 0.12 % (RSD) and 3.32 % (RSD), respectively. For the analysis of a real-life sample, red wine was filtered through a 0.45 µm filter before injection. Figure 4 shows the chromatogram of a red wine sample containing 0.014 mg/L of resveratrol. For compound confirmation, the retention time of resveratrol was used and the UV spectrum of the peak was matched with a standard with a match factor 981. The total run time of the analysis was less than 3.5 minutes

Conclusion

This Application Notes shows the analysis of the antioxidant resveratrol in red wine in less than 4 minutes run time with excellent sensitivity, precision of area and retention time and linearity. The analysis of a real-life sample confirmed the good results.

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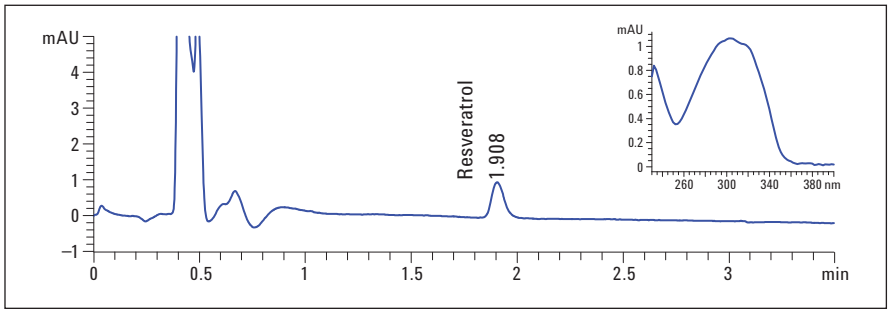


Figure 1
Chromatogram of reference solution (0.01 mg/L) and spectrum of resveratrol.

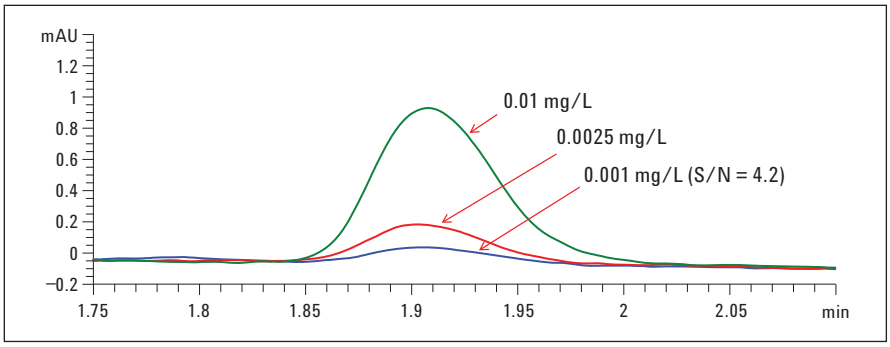


Figure 2
Chromatogram of standard solutions with different concentrations.

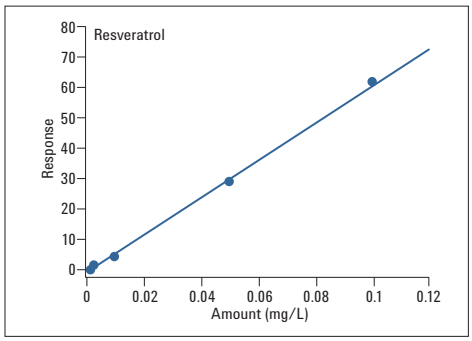


Figure 3
Calibration curve.

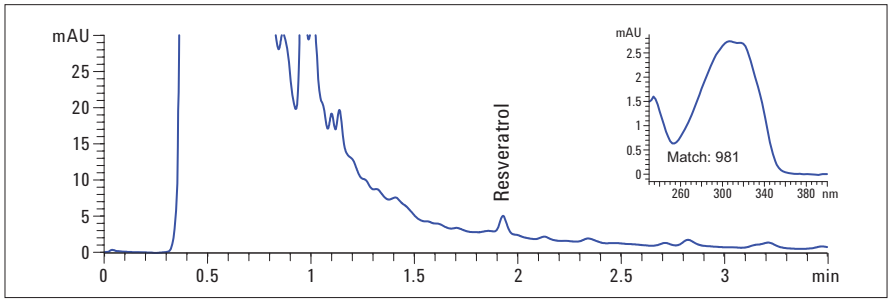


Figure 4
Chromatogram of red wine and spectrum of resveratrol.

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Metabolomic Profiling of Wines using LC/QTOF MS and MassHunter Data Mining and Statistical Tools

Application Note

Food Testing and Agriculture

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Abstract

A non-targeted metabolomic analysis approach to the classification of wine varieties was developed, employing LC/QTOF MS and MassHunter Workstation software. Molecular feature extraction, data filtering, and statistical analysis utilizing ANOVA and PCA identified 26 marker compounds that were then used to build a PLSDA prediction model. An overall accuracy of 95.6% in discriminating between Cabernet Sauvignon, Merlot and Pinot Noir red wine varieties was obtained using the model. This approach may be widely applicable to the analysis and characterization of foods.

Introduction

Wine is a beverage widely consumed throughout the world, with consumption estimated at 2.65 billion 9-liter cases in 2008, and wine consumption in the United States registered its 15th consecutive annual gain [1]. Thus wine is an important global food commodity of high commercial value. In order to protect this valuable market, wine authenticity control, mainly in terms of varieties, geographical origin and age, is continuously required to detect any adulteration and to maintain wine quality [2]. Wine is a complex matrix of water, alcohol, sugars and a wide range of minor organic and inorganic constituents. Many factors influence the concentration levels of these compounds, including grape variety, climate, wine-growing area, and, last but not least, the winemaking process. Establishing wine authenticity can thus be a very challenging proposition.

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In the past few years, the emerging field of metabolomics has become an important strategy in many research areas such as disease diagnostics, drug discovery and food science. It has been used for informative, discriminative, and predictive purposes associated with food quality and safety. Metabolomic studies aim at the comprehensive analysis of numerous targeted or non-targeted metabolites (compounds with molecular weight typically below 1000 daltons) in a biological sample. As the metabolites significantly differ in both physicochemical properties and abundance, these analyses require sophisticated analytical technologies.

One of the techniques most widely used in metabolomics is liquid chromatography/mass spectrometry (LC/MS). For processing of large chromatographic and/or spectral data sets typically generated by metabolomic analyses, effective software tools capable of rapid data mining procedures and aligning algorithms have to be used. Advanced chemometric tools for reduction of data dimensionality are also employed to maximize utilization of the information present in the multivariate records obtained from data mining, including principal component analysis (PCA) and Analysis of Variance (ANOVA).

This application note describes a non-targeted metabolomic analysis approach to the classification of wine varieties that employs liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (LC/QTOF MS). The Agilent Series 1200 SL Rapid Resolution LC system was coupled to an Agilent 6530 accurate-mass Q-TOF MS with electrospray ionization (ESI) enabled with Agilent Jet Stream technology. MassHunter Workstation software, including Qualitative Analysis and Mass Profiler Professional, was used for molecular feature extraction and data filtering, followed by multivariate analysis by PCA and one-way ANOVA, eventually leading to the construction of a classification model using Partial Least Square Discrimination Analysis (PLSDA). The end result was the use of 26 compounds that enabled a predictive model that was 95.6% accurate in discriminating between Cabernet Sauvignon, Merlot and Pinot Noir red wine varieties. The results of this study have been published [3].

Experimental

Reagents and standards

The reagents and standards used were as specified in the previous publication [3]. HPLC grade methanol was purchased from Honeywell Burdick and Jackson (Muskegon, MI, USA), and deionized water was produced by a Milli-Q purification system (Millipore, Bedford, MA, USA). Ammonium formate, ammonium acetate, acetic acid, and formic acid, used as mobile phase additives (each of purity ≥ 99%), were supplied GFS Chemicals (Powell, OH, USA) using doubly distilled formic acid, acidic acid and ammonium hydroxide at the appropriate concentrations.

Samples

In total, 45 red wine samples of Cabernet Sauvignon (CS, n = 15), Merlot (M, n = 16), and Pinot Noir (PN, n = 14) varieties were purchased from reliable sources at Czech Republic and U.S. retail markets. Samples represented wines of various geographic origin (Australia, Bulgaria, Czech Republic, France, Germany, Hungary, Chile, Italy, Macedonia, Slovakia, Spain, and USA), and vintage (2004–2008), comprising a highly variable sample set. Wines were uncorked and an aliquot of sample was transferred into a 2-mL autosampler amber vial (filled to its capacity). Samples were stored in the dark at 2 °C, until LC/MS measurements were taken. Samples were analyzed in random order.

Instruments

This study was performed on an Agilent Series 1200 SL Rapid Resolution LC system coupled to an Agilent 6530 accurate-mass Q-TOF MS with electrospray ionization (ESI) enabled with Agilent Jet Stream technology. The instrument conditions are listed in Table 1.

To assure the desired mass accuracy of recorded ions, continuous internal calibration was performed during analyses with the use of signals at *m/z* 121.0509 (protonated purine) and *m/z* 922.0098 (protonated hexakis,1H, 1H, 3H-tetrafluoropropoxy, phosphazine or HP-921) in positive ion mode; in negative ion mode, ions with *m/z* 119.0362 (proton abstracted purine) and *m/z* 980.0164 (acetate adduct of HP-921) were used.

Table 1. LC and Mass Spectrometer Conditions

LC Run Conditions	
Column	Agilent ZORBAX Eclipse Plus C-18, 2.1 × 100 mm, 1.8 µm (p/n 959931-902)
Column temperature	40 °C
Injection volume	2 µL
Autosampler temperature	4 °C
Needle wash	Flush port (50:25:25 H_2O, IPA:MeOH:H_2O, 5 sec)
Mobile phase	Positive ionization mode: A = 5 mM Ammonium formate B = Methanol Negative ionization mode: A = 5 mM Ammonium formate B = Methanol
Flow rate	0.3 mL/min during gradient run 0.5 mL/min during column equilibration
Gradient	B = 5% to 65%, 0 to 13 min B = 65% to 100%,13 to 16 min B = 100% from 16 to 20 min B = 5% from 20 to 24 min (column equilibration)
Analysis time	20 min
MS Conditions	
Ion mode	Positive and negative, ESI+APCI multimode ionization
Drying gas temperature	300 °C
Vaporizer temperature	170 °C
Drying gas flow	11 L/min
Nebulizer pressure	40 psi
Capillary voltage	4500 V positive ion mode 3000 V negative ion mode
Skimmer voltage	65 V
Octapole DC1	47 V
Octapole RF	750 V
Fragmentor voltage	125 V
Spectra acquisition rate	1.4 spectra/second
MS/MS Conditions	
Ion mode	Positive ion
Isolation window	4 amu
Spectra acquisition rate	1.4 spectra/second

Data Processing and Statistical Analysis

MassHunter Workstation software, including Qualitative Analysis (version 3.01) and Mass Profiler Professional (version B.02.00), was used for processing both raw MS and MS/MS data, including molecular feature extraction, background subtraction, data filtering, statistical analysis by ANOVA and PCA, followed by the construction of the predictive classification model, molecular formula estimation, and database searching.

To perform subtraction of molecular features (MFs) originating from the background, analysis of a blank sample (deionized water) was carried out under identical instrument settings and background MFs were removed. Using background subtracted data, files in compound exchange format (CEF files) were created for each sample and exported into the Mass Profiler Professional (MPP) software package for further processing.

Results and Discussion

Wine analysis

This metabolomic profiling study (Figure 1) began with the analysis of a total of 45 red wine samples from around the world comprising three different categories based on the grape varieties used to produce them: Cabernet Sauvignon (15), Merlot (16), and Pinot Noir (14). To avoid any possible discrimination of metabolites present, no sample pre-treatment procedures such as extraction or dilution were performed prior to LC/ MS analysis, and the wine samples were injected directly onto the separation column. As this was an untargeted study, generic settings were applied to both LC separation and MS analysis to obtain profiles containing as many compounds as possible. Both reversed-phase chromatography and hydrophilic interaction liquid chromatography (HILIC) were used to separate the wide variety of compounds present in wine. In addition, Agilent Jet Stream technology enabled electrospray and multimode ion sources were used to collect the MS data. The multimode ion source provided simultaneous APCI and electrospray ionization. The most useful data was collected with the reversed-phase Agilent ZORBAX Eclipse Plus C18, 2.1 × 100, 1.8 µm particle size column.

The wine sample analyses were quite complex, exhibiting a multitude of peaks, each containing multiple compounds (Figure 2). In addition, the solvent blank contained contaminant peaks that needed to be subtracted in order to generate valid data. Data mining was therefore required to extract meaningful data from the results.

EXPERIMENTAL SETUP

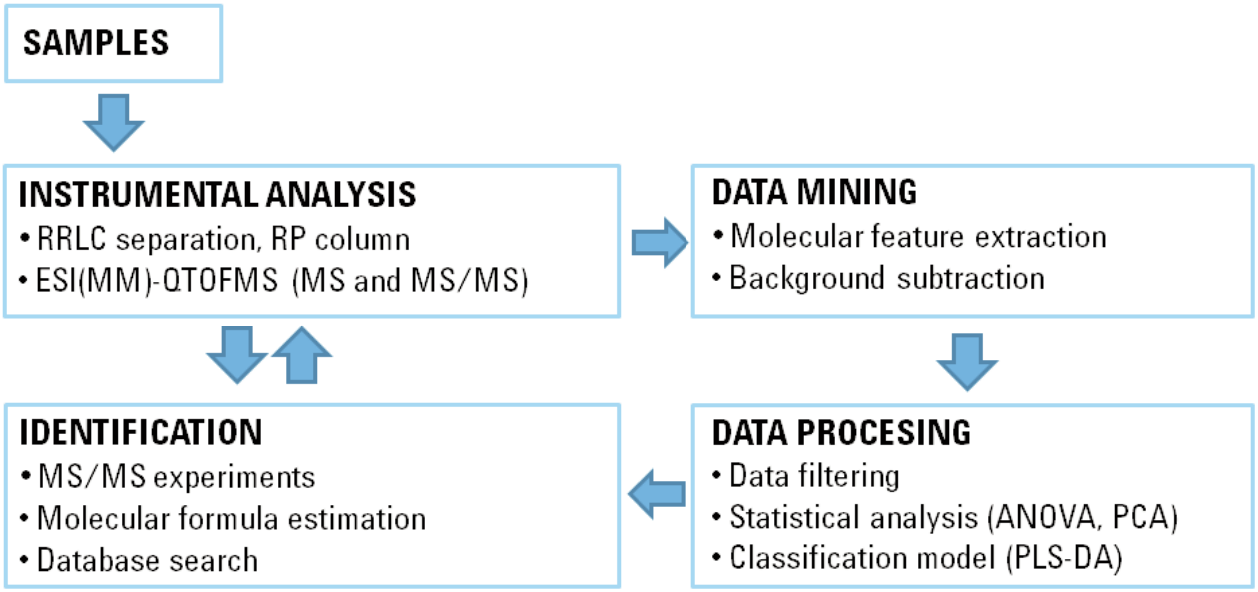


Figure 1. Workflow for a metabolomic study to generate a predictive model for wine classification and identify the marker compounds.

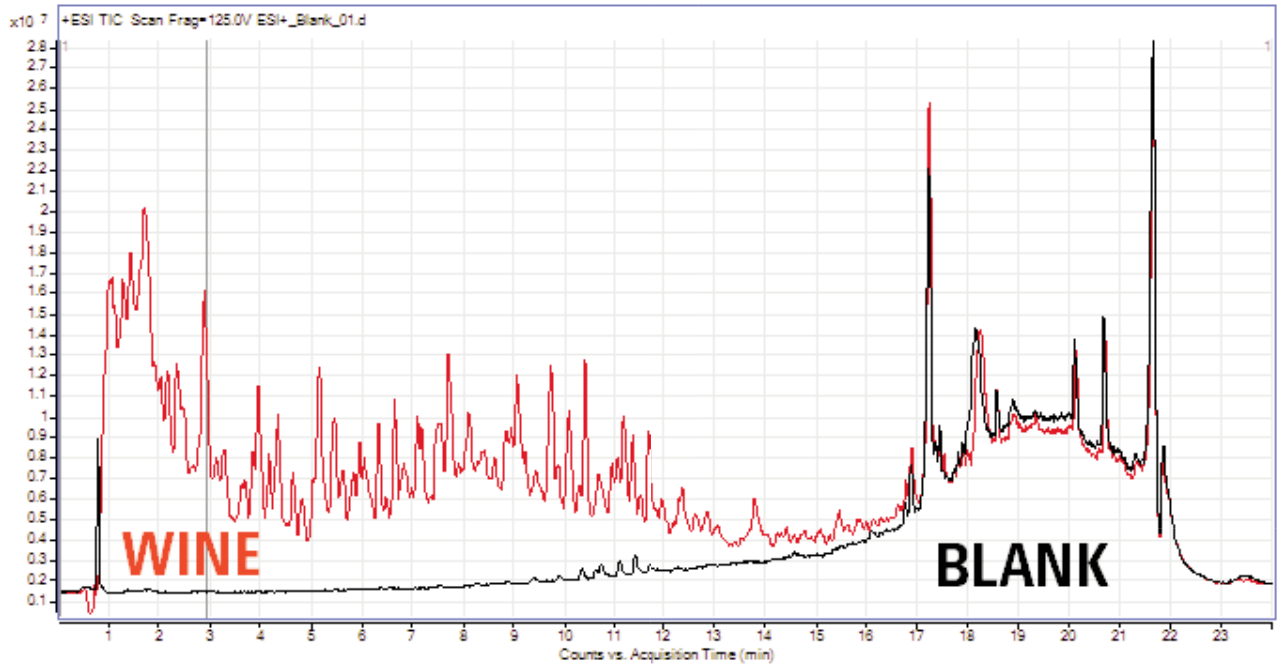


Figure2. Total Ion Current (TIC) chromatogram of a wine sample Ilustrating its complexity. Each peak contains several compounds. The blank must also be subtracted in order to properly interpret the data.

Data mining

The Molecular Feature Extractor (MFE) algorithm in the MassHunter workstation software was used to perform meaningful data mining. This algorithm removes data points that correspond to persistent or slowly-changing background, searches for features that have a common elution profile and groups ions into one or more *compounds* (features) containing *m/z* values that are related (correspond to peaks in the same isotope cluster, different adducts or charge states of the same entity). Molecular feature extraction also enables subtraction of chromatographic background caused by impurities in the mobile phase, extracting all compounds found in the solvent blank. The results are then used as a background subtraction dataset for all sample files, excluding all compounds that were in the blank from the evaluation of the samples.

The settings for the molecular feature extractor are important, because the algorithm has been *tuned* for different types of large and small molecules. Figure 3 shows the selection for the small molecules of interest in this study.

The masses found in the blank can be copied and pasted right into the MFE tab for automated background subtraction (Figure 4). Once all the settings are made for the data processing method, including the appropriate MFE algorithm and the threshold counts (Figure 3), it is saved and used to process all data files in a batch mode using the offline utility. The batch data processing includes finding compounds using MFE and then creating a compound exchange format (.CEF) file of the results. Those results are saved in a project folder that will then be used by Mass Profiler Professional for data filtering and statistical analysis.

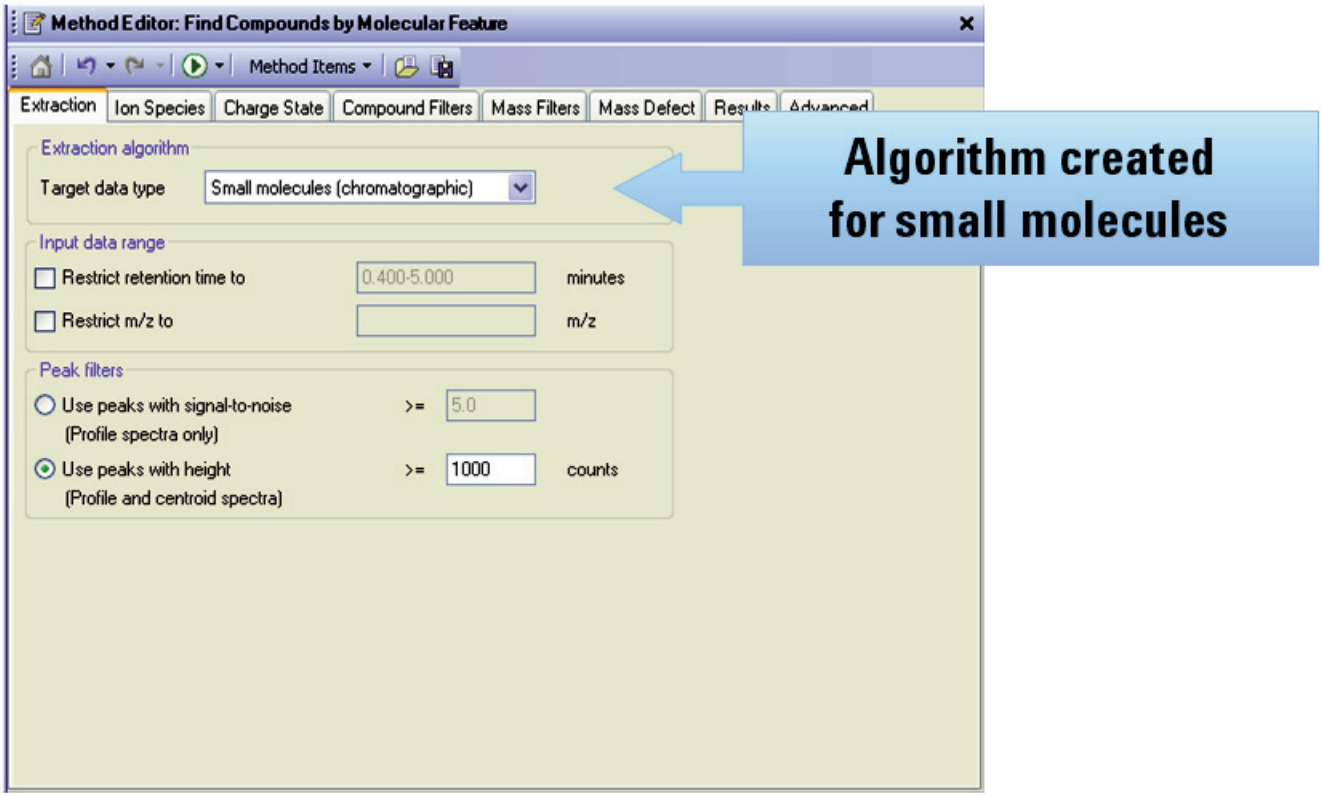


Figure 3. Settings used by the Molecular Feature Extractor (MFE) to extract entities (compounds) from the LC/QTOF MS data.

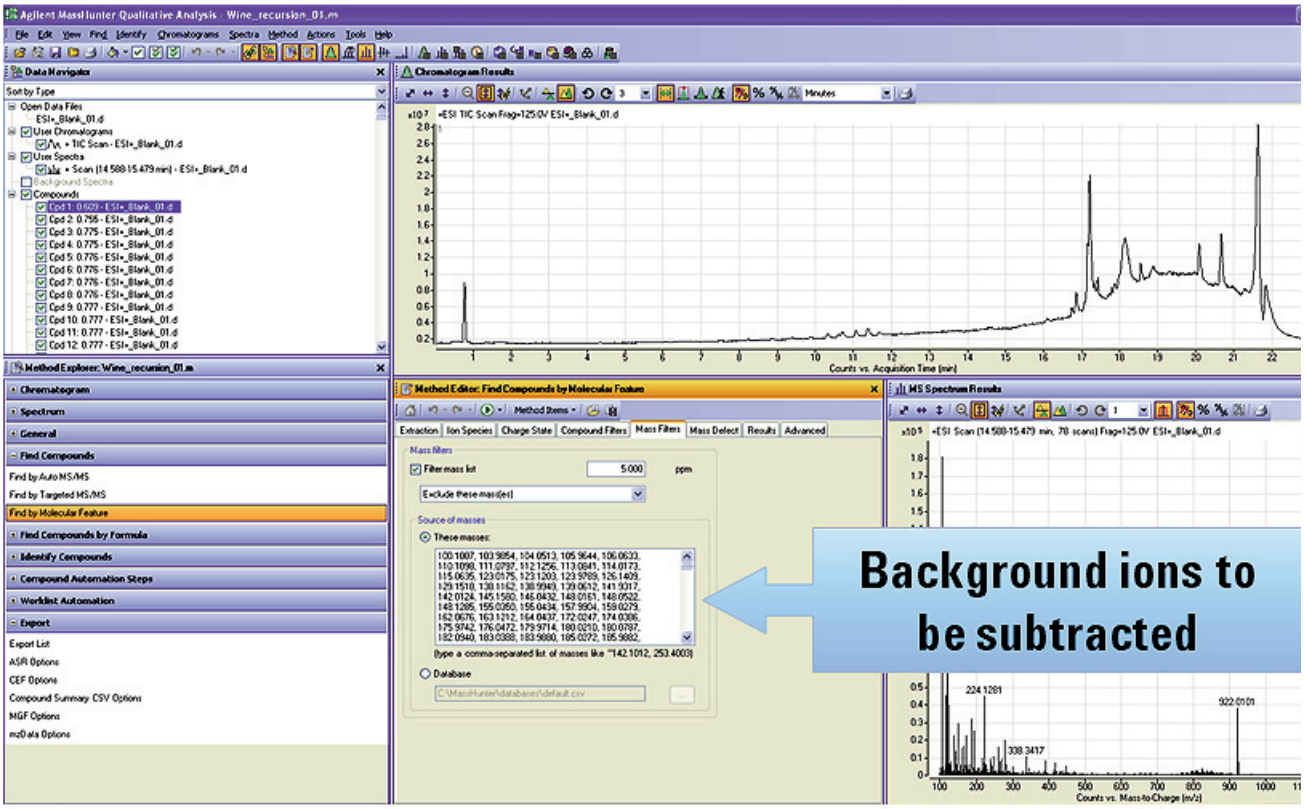


Figure 4. The resulting masses found in the blank can be copied and pasted right into the MFE tab for subtraction from all of the datasets.

Data processing

Data processing for metabolomic studies is often very tedious and time intensive when using complicated statistical software written to handle ASCI or text type results. Mass Profiler Professional (MPP) is ideal for the sophisticated data management, filtering, statistical analysis, interpretation, model creation, and prediction required to efficiently utilize metabolic data. It provides an easy-to-follow guided workflow that helps the user decide how best to evaluate the data. Expert users can go directly to the data processing they wish to use (see the Mass Profiler Professional brochure 5990-4164EN for further details).

MPP uses eight steps for data evaluation, starting with a summary report, revealing that the wine data set contains over 20,000 entities (or possible compounds) found throughout the samples. These are determined by the accurate mass of the cluster of ions and their chromatographic alignment. The next step is experiment grouping into classes, and in this case the three classes are Cabernet Sauvignon (CS), Merlot (M) and Pinot Noir (PN).

Data filtering

Entity filtering permits the creation of a higher quality data set so that subsequent multivariate analysis is more meaningful. The first filter determined which entities (compounds) were in at least one group 100% of the time (frequency analysis). That is, the compound must be in that group in all the samples. This frequency filter reduced the possible markers from 20506 entities to 663. However, by setting the frequency to 100%, some important markers could be filtered out. In the case of Pinot Noir, one compound was removed, even though it was present in 14 out of the 15 samples (Figure 5). Setting the frequency filter to 50% allows the inclusion of many more entities without excluding those like the compound in 14 of 15 Pinot Noir samples. Using the 50% filter reduced the number of entities from 20506 to 3600.

The next step was to filter the Analysis of Variance (ANOVA) results, which determine what level of variance is accepted as significant for a given entity. Using a probability p value of .05 (variance from one sample to another has a 95% probability that it is significant), the 3600 entities from the frequency filter were reduced to 40 significant compounds.

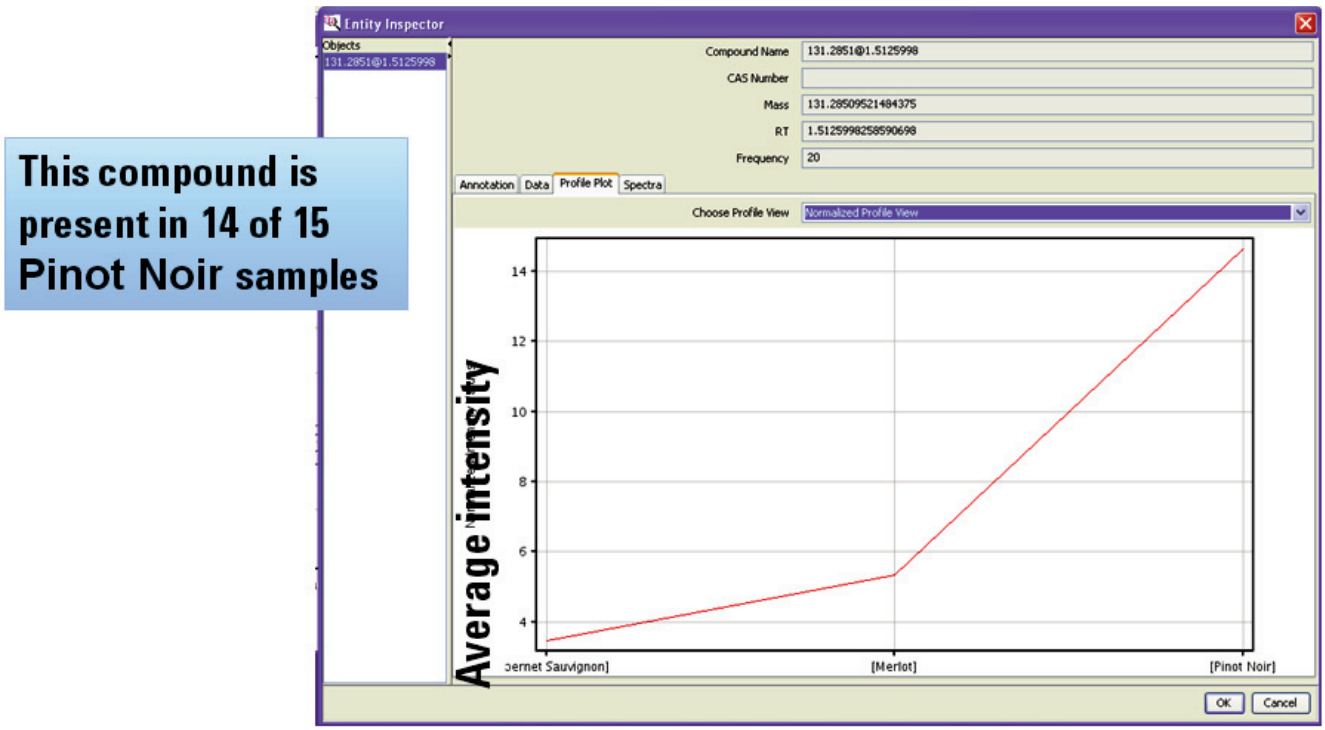


Figure 5. Using a 100% frequency filter can remove useful markers from the dataset, such as this compound that is present in Cabernet Souvignon and Merlot samples at very low frequency, but shows up in 14 of 15 Pinot Noir samples.

Fold Change was the final data processing filter applied, in order to identify entities with large abundance differences between the selected data classes, that is, those that differ in concentration by 2 fold, 3 fold, 4 fold, etc. between the three data classes (CS, M and PN). Examining the data at higher fold change than two, however, eliminated possible discriminating compounds of Cabernet and Merlot, and thus a 2-fold filter was applied (Figure 6). This reduced the data set from 40 to 26 possible compounds.

The next step in the processing of the data was recursion. Recursion allows the re-examination of the data to assure that each entity is a real peak and that those entities not found in a sample are not there. MassHunter Qualitative Analysis software automatically re-extracted the final group of 26 markers from the raw data to generate extracted ion chromatograms (EICs). A careful inspection of the resultant EICs was performed to eliminate false positives (not a real peak) and false negatives (a real peak is in a sample but was missed in the molecular feature extraction data mining step). Once the 26 entities were confirmed as real, statistical analysis was performed using Principle Component Analysis (PCA).

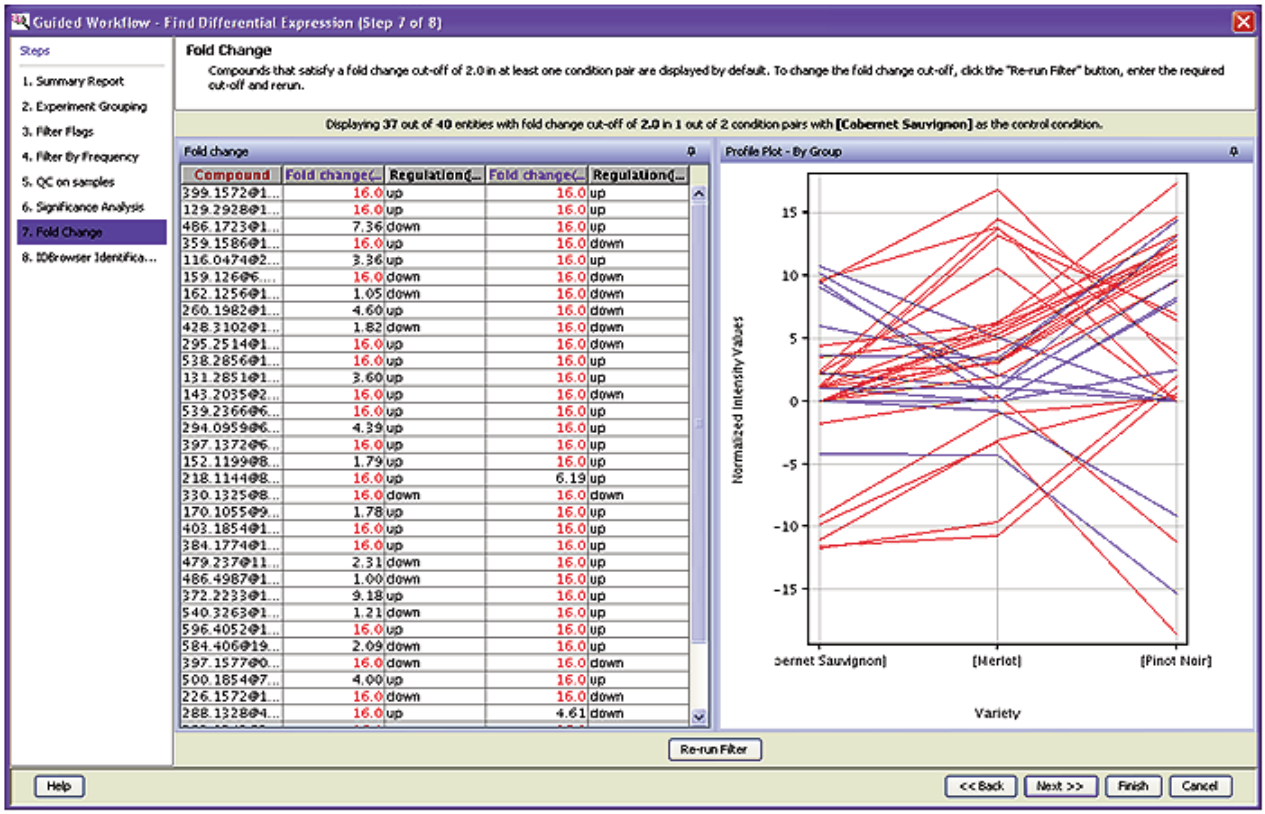


Figure 6. Fold Change Analysis is used to identify entities with significantly different abundance in selected classes. Examining the data at fold change higher than two eliminates possible Cabernet and Merlot markers, so a 2-fold filter was applied. This reduced the data set from 40 to 26 possible markers.

Statistical analysis

PCA is a frequently employed unsupervised multivariate analysis technique enabling data dimensionality reduction, while retaining the discriminating power in the data. It is performed using the transformation of measured variables into uncorrelated principal components, each being a linear combination of the original variables. The goal is to identify possible relationships within the classes of data. Performing PCA on the unfiltered data set and even the frequency-filtered data set did not reveal any relationships that would enable the data to be classified into varieties of wine. However, PCA of the 26 identified marker entities revealed distinctive grouping of the data into the three wine classes (Figure 7). Note that PCA does not make the statistical distinction between varieties; it only reveals that there are distinctions. The compounds that distinguish one variety of grapes from another were selected by the frequency filtering, ANOVA, and fold change filtering of the entities identified by the molecular feature extractor, and then qualified by the recursion analysis.

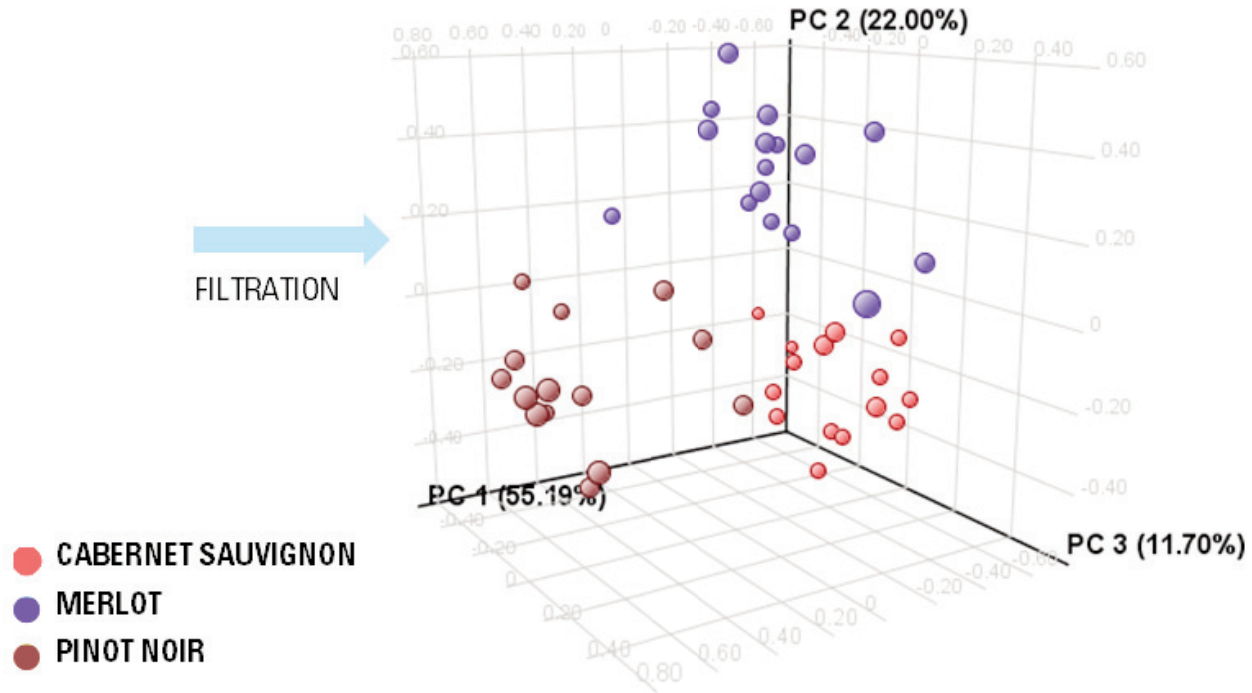


Figure 7. Principle Component Analysis (PCA) of the 26 markers remaining after the ANOVA and Fold Change filters were applied to the dataset. The markers for each of the three wine varieties group together well, indicating their utility for predicting the variety of an unknown wine.

Classification model

Having established three data classes with the filtered compounds that were selected through processing with Mass Profiler Professional, the next step was to create a model that can predict the variety of a wine. The Partial Least Square Discrimination Analysis (PLSDA) model in MPP best fit the mass spectral data. The first step in building the classification model was to train the model with the data.

The PLSDA algorithm produces a Confusion Matrix, which is a table with the true class in rows and the predicted class in columns. The diagonal elements represent correctly classified experiments, and cross diagonal elements represent misclassified experiments (Table 2). The table also shows the predictive accuracy of the model as the percentage of correctly classified experiments in a given class. The accuracy of this training set for each class, as well as overall, was 100%.

The next step was to test the model with the same data. Although redundant, this is a valid statistical procedure. The same class prediction model was used for the validation of the trained model. Note that Merlot was incorrectly identified in two cases, resulting in an accuracy of prediction for Merlot of 87.5%, and an overall predictive ability of 95.6% (Table 2). The use of more samples would likely improve the predictive ability of the model by improving the statistical power of the analysis.

Table 2. Confusion Matrix Illustrating the Classification Results Using the PLSD Model

	Cabernet Sauvignon (CS)	Merlot (M)	Pinot Noir (PN)	Accuracy (%)
Model training	**15**	**0**	**0**	
CS	0	16	0	**100.0**
M	0	0	14	**100.0**
PN				**100.0**
Recognition ability (%)				**100.0**
Model validation				
CS	15	0	0	**100.0**
M	1	14	1	**87.5**
PN	0	0	14	**100.0**
Predictive ability (%)				**95.6**

In order to demonstrate the predictive ability of the model, five wines were purchased that were not among the wines used to find the markers and develop the model. An additional wine was purchased whose identity was not revealed to the scientists conducting the experiment. Analysis of these wines and applying the classification model correctly predicted the variety of all five wines plus the unknown (Figure 8). These results demonstrate the feasibility of developing markers and using a model to accurately determine wine variety.

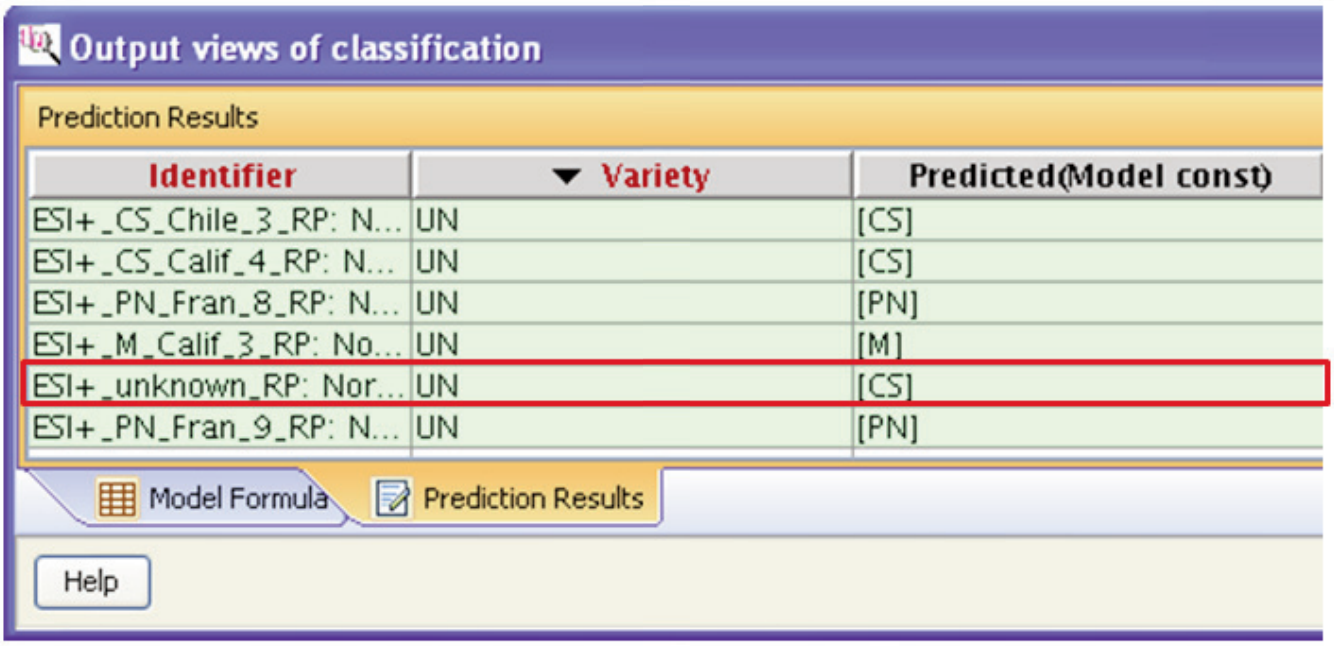


Figure 8. Table showing the results of the predictive model when applied to known wine samples, as well as one unknown. All samples were correctly classified.

Identification of wine markers

While it is not necessary to know the identity of the compounds used as wine markers, the availability of standards for the marker compounds could facilitate simpler tests for identification of wine variety. Using the single MS accurate mass data for the pseudo molecular ion and its isotopes, a molecular formula for each marker compound can be generated. Those formulae that provide a best fit of this data can be used to search private and public databases of possible compounds. Of the 26 markers, only one gave a result from a database. The molecular formula estimation and subsequent tentative identification of a selected marker compound for Pinot Noir (*m/z* 449.1078, RT 11.16 min), present in the final group of markers, were performed based on single MS. Using this data to search the PubChem database resulted in a suspect identification of this marker compound as cyanidin-3-O-glucoside, which is anthocyanidin pigment. Using the Q-TOF, accurate mass MS/MS of the *m/z* 449.1078 gave further indication that this is the identity of the marker compound. Without any idea of what a compound might be, even accurate MS and MS/MS data would be difficult to interpret to obtain a compound structure. Final confirmation would require a standard of the indicated compound.

Conclusions

Metabolomic studies are valuable tools for the profiling of complex food products such as wines. Using the highly accurate and reproducible data generated by the Agilent LC/Q-TOF MS system, a predictive model can be constructed to determine the variety of a wine. This may be broadly applicable to other foodstuffs. Sophisticated software tools such as MassHunter Qualitative Analysis and Molecular Profiler Professional can be used to conduct data mining, filtering and statistical analysis to identify markers specific to particular food varieties (such as wine varieties) and use those markers to build classification models that can determine the variety of a foodstuff. Such models can be highly accurate, with the overall accuracy of the wine variety classification model described here being 96.5%. While it is not necessary to know the identity of the marker compounds for the model to be accurate, the same instrument system can be used to generate MS/MS spectra that can enable identification of the marker compounds used in the model. If standards for the putative compounds are available, confirmation of the compound's identity can then be made.

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Chemical Markers to Classify Wine

A Prediction Model for Determining Wine Variety using the Agilent LC/MS Q-TOF and Agilent Mass Profiler Professional

FOOD AUTHENTICITY



Using the Agilent ToF or Q-TOF LC/MS system and Mass Profiler Professional unique markers can be discovered and applied without knowing the compounds identities.



Wine is a beverage produced and consumed throughout the world and is a highly valued commodity. Its classification and authenticity can be very important. The constituents of wine are complex and include compounds that impart taste, color, and other characteristics that determine the quality of the beverage. One component is the type of grape used and the question this study examines is whether there are specific compounds in wine that distinguish one grape from another. Using wines obtained from around the world, the power of accurate mass and high resolution is put to use by analyzing three varieties of wine, Pinot Noir, Merlot, and Cabernet Sauvignon. Using Agilent's Mass Profiler Professional software, the resulting single MS data containing over 26000 entities are statistically evaluated. Once filtered on differences, principle component analysis shows that the wine variety can be grouped by specific compounds found in the wine samples. With this knowledge, a model based on partial least squares differentiation is made and unknown wines can be classified. All this is done without knowing the identity of the marker compounds that can distinguish one grape from another.

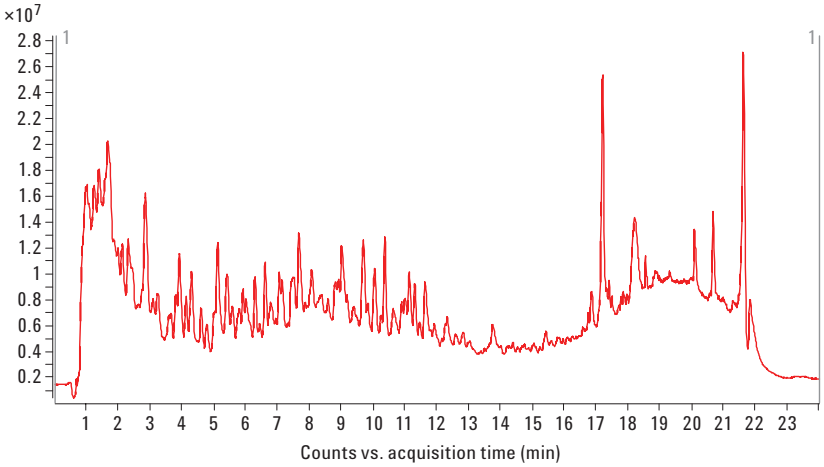
Using the Agilent 6530 Accutate-Mass Q-TOF LC/MS, MS/MS can be performed on the ions shown to correlate specific grapes and identification can be pursued with the excellent accurate mass measurements. It is noted that the identification of true unknowns, compounds not found in any database, is a difficult task. However, identification is not necessary for this type of determination and even the unidentified compounds can be used with their MS/MS signatures for routine classification. This study demonstrates the power of the Agilent LC/MS Q-TOF in combination with Mass Profiler Professional's multivariate statistical capabilities designed specifically for MS data processing.

Key Benefits

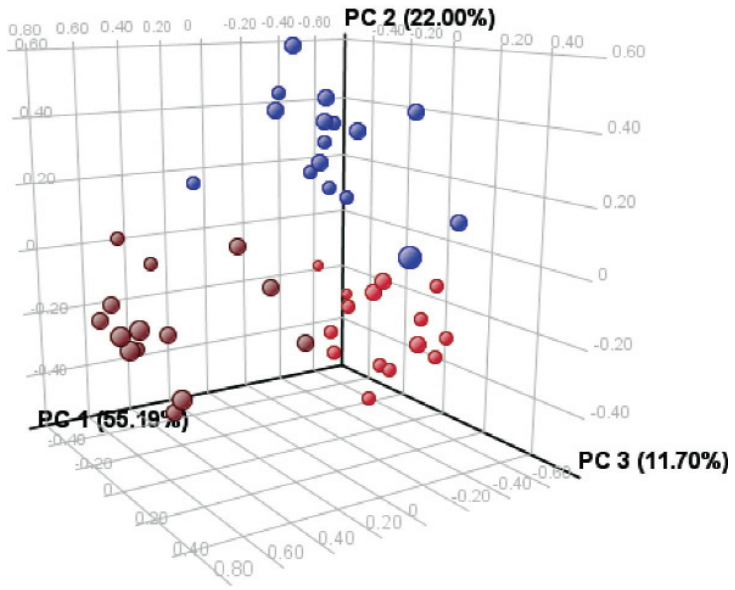
- Agilent 6500 Series Q-TOF LC/MS provides the sensitivity, mass accuracy and resolution needed to separate unique compounds recognizing a wines variety
- Mass Profiler Professional provides the needed multivariate statistics directly on accurate mass spectral data
- Mass Profiler Professional provides powerful models for prediction analysis
- Comprehensive workflow wizards guide the experimenter through statistical analysis of their data to obtain powerful correlations and visualization of the results
- With Q-TOF MS/MS the identity of marker compounds may be obtained

This work was reported in L. Vaclavik, O. Lacina, J. Hajslova, J. Zweigenbaum, Analytica Chimica Acta 2011, 685, 45 (http://www.sciencedirect.com/science/article/B6TF4-51GHWXP-1/2/e70f1f1928475f12c9341d8b67e05310).

A Prediction Model for Determining Wine Variety using the Agilent LC/MS Q-TOF and Mass Profiler Professional



Total Ion Chromatogram on wine sample injected directly shows the complexity of the data.



Principal component analysis of filtered results shows that Merlot, Cabernet Savagnoun, and Pinot Noir wines can be distinguished by these marker compounds.

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Direct Analysis of Milk Powder by Axially-Viewed Simultaneous ICP-OES

Application Note

Inductively Coupled Plasma-Optical Emission Spectrometers

Authors

Andrew Tame

Dennis Hoobin

Introduction

The elemental analysis of milk is important both as an indicator of environmental contamination and because milk is a significant pathway for toxic metal intake and a source of essential nutrients [1] for humans.

The accepted methods for elemental analysis of milk have traditionally included either wet digestion or dry ashing [3–6]. These are time consuming and involve procedures using potentially hazardous chemicals. A direct method of analysis by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-OES) would obviously be preferable, but this relies upon the instrument tolerating high levels of dissolved solids while providing sufficient sensitivity to measure toxic trace elements and the dynamic range to measure nutritional major elements.

The use of a sequential ICP-OES for direct analysis of milk has been previously described by Ryan [2]. This work describes the direct analysis of milk powder using standard quantitative calibration with aqueous standards using a simultaneous ICP-OES system. Viscosity effects of the milk powder solutions are corrected using scandium (361.383 nm—ionic line) as an internal standard. Major, minor and trace elements were determined in a single analysis. Less sensitive lines are used for the determination of major elements allowing both major and minor elements to be determined from a single solution.

Many of the major constituents in milk powder such as Na, K and Ca are Easily Ionized Elements (EIE) which can cause ionization interferences. Previous work [2] has shown that the addition of caesium as an ionization suppressant and internal standard to the standards and samples was beneficial. The ionization suppressant and internal standards are conveniently introduced into the plasma on-line, via the third channel of the peristaltic pump. The accuracy and validity of the method was assessed by the use of National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) 8435 Whole Milk Powder.

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Experimental

Instrumental

The Vista simultaneous ICP-OES axially viewed plasma was used for the analysis. The Vista features a free running, air cooled, 40 MHz RF generator and cooled cone interface. The Vista's optical system is based on an echelle polychromator with Charged Coupled Device (CCD) detector. The polychromator is thermostatted to 35 °C for stability, and the unique CCD detector features 70,000 pixels (detectors) arranged to exactly match the 2 dimensional echellogram. Sophisticated design of the detector has resulted in a rapid readout with excellent detection limits [4]. The instrument was controlled by an IBM computer with an Intel Pentium processor and Agilent's Vista worksheet software running under Microsoft's Windows NT operating system.

In this work a Vista with manual gas pressure regulator was used. Mass flow control of the nebulizer flow, which allows the nebulizer gas flow to be automatically adjusted, is available as an option. This work took advantage of the third channel pump to add internal standard and ionization suppressant on line. The Vista instrument is available with either the two or three channel pump option.

Table 1. Instrument Operating Conditions

Power	1.35 kW
Plasma gas flow	15.0 L/min
Auxiliary gas flow	1.5 L/min
Spray chamber type	Sturman-Masters
Torch	Standard axial torch with 2.3 mm id injector
Nebulizer	V-groove
Nebulizer pressure	240 kPa
Pump tube Inlet	PVC black-black
Outlet	PVC, blue-blue, 1.65 mm id
Polychromator purge	Boost (3.7 L/min)
Pump speed	15 rpm
Sample uptake rate	0.6 mL/min
Integration time	3 seconds
No. of replicates	3
Sample delay time	20 seconds
Fast pump	On
Stabilization time	20 seconds
Background correction	Fitted, 2 points/peak

To make sure that the milk powder sample was evenly mixed and in suspension while being aspirated, the solution was continuously stirred with a magnetic stirrer. Sodium and potassium were internally standardized with the caesium 372.328 nm line, using added caesium as both the ionization suppressant and internal standard. Scandium was used as an internal standard for all other lines.

Standard Preparation

Aqueous standards were prepared from 1000 mg/L single element standards (Spectrosol, BDH Chemicals). The standards were made up in 18 MΩ Milli-Q water with 0.5% v/v HNO_3 and 0.002% v/v Triton X100 prepared from a 1% w/v Triton X100 solution.

Table 2: Calibration Standards Prepared

Standard	Elements and concentration (mg/L)
Standard 1	Ba (0.5), Mn (0.5), Zn (0.5), Sr (0.5)
Standard 2	Ba (1.0), Fe (1.0), Mn (1.0), Zn (2.0), Sr (5.0)
Standard 3	Mg (5.0), Fe (5.0)
Standard 4	Mg (25.0), Na (25.0), Ca (25.0)
Standard 5	Na (100), Ca(100), K (100)
Standard 6	K (200)
Standard 7	S (10.0), P (50.0)
Standard 8	S (50.0), P (150.0)
Standard 9	S (100.0), P (200.0), Ca (250.0)
Standard 10	Ca (1000)
Standard 11	K (1000)
Standard 12	Na (1000)

Rinse and calibration blank solutions were prepared from 18 MΩ Milli-Q water with 0.5% HNO_3 and 0.002% Triton X100.

Sample Preparation

Solutions were prepared from samples supplied by the Ministry of Agriculture and Fisheries (MAF), New Zealand and SRM 8435 Whole Milk Powder.

Milk powder suspensions containing 2% w/v were prepared for all samples. The sample was accurately weighed and then transferred into a volumetric flask. The flask was filled approximately 3/4 full with 18 MΩ Milli-Q water and gently shaken until the milk powder was evenly mixed. Triton X100 was added to give a concentration of 0.002%. Acid was not added to the samples as it causes the precipitation of protein.

The samples were made up to the mark and placed in an ultrasonic bath for 5 minutes. The samples were then shaken vigorously for 1 minute.

A separate rinse containing 0.002% v/v Triton X100 and no acid was used for rinsing between samples.

Both caesium and scandium were used as internal standards in the analysis. Caesium also acted as an ionization suppressant. A bulk solution of 1% CsCl and scandium (0.5 mg/L) and was added to all solutions via the third channel of the peristaltic pump.

Results and Discussion

Milk Powder Analysis

The results presented in Tables 3–5 represent the concentrations of constituent elements in the milk powder on a dry weight basis. Previous work [2] had shown that the milk powder samples typically have low moisture contents and the samples were determined directly from the raw material without drying.

Table 3. Analysis of Standard Reference Material, S.R.M. 8435

Element	Wavelength (nm)	2.0% S.R.M. 8435 whole milk powder (ppm)	S.R.M. 8435 certified value (ppm)
Ba	493.408	0.72 ± 0.03	0.58 ± 0.23
Ca	373.690	8990 ± 340	9220 ± 490
K	404.721	12580 ± 210	13630 ± 470
Fe	238.204	1.07 ± 0.01	1.80 ± 1.1
Mg	285.213	838 ± 27	814 ± 76
Mn	257.610	0.17 ± 0.02	0.17 ± 0.05
Na	588.995	3810 ± 40	3560 ± 400
P	185.878	7400 ± 300	7800 ± 490
S	180.669	2320 ± 90	2650 ± 400
Sr	407.771	4.10 ± 0.20	4.35 ± 0.50
Zn	202.548	25.2 ± 0.9	28.0 ± 3.1

Table 4: Analysis of 2% Milk Powder

Element	Wavelength (nm)	2.0% Milk powder sample (wt%)
Ba	493.408	0.97
Ca	373.690	8909
K	404.721	11657
Fe	238.204	1.76
Mg	285.213	796
Mn	257.610	0.37
Na	588.995	3118
P	185.878	7729
S	180.669	2278
Sr	407.771	5.48
Zn	202.548	28.4

Table 5. Analysis of MAF#1

The "Known values" are values supplied by the New Zealand Dairy Research Institute.

Element	Wavelength (nm)	2.0% MAF#1 sample (ppm)	Known value (ppm)
Ba	493.408	3.24 ± 0.01	–
Ca	373.690	13750 ± 9	13274
K	404.721	15600 ± 120	17040
Fe	238.204	1.36 ± 0.04	2.0
Mn	257.610	0.310 ± 0.0001	–
Na	588.995	4000 ± 50	3490
P	185.878	10600 ± 140	9930
S	180.669	3280 ± 40	3280
Sr	407.771	3.99 ± 0.03	–
Zn	202.548	40.2 ± 0.8	37.33

Summary

The concentrations of various elements of both nutritional and environmental interest in milk powder samples were determined directly using the axially viewed Vista simultaneous ICP-OES. Aqueous calibration solutions were used and the scandium and caesium internal standards successfully corrected for the viscosity effects of the samples.

The addition of caesium as an ionization suppressant eliminated ionization interferences and the need for dilution, allowing both major and minor constituents to be measured in a single solution. Both trace and major element concentrations were able to be determined in the 2% w/v milk powder samples, with less sensitive analytical lines chosen for the elements present in the greatest concentrations, such as Ca. This wavelength selection flexibility, provided by the optical and detector design of the Vista, avoided the need to re-measure samples using the less sensitive radial optical configuration.

All measured values are in very good agreement with the certified values for the standard reference material, validating the accuracy of the method.

Acknowledgments

The authors wish to thank the New Zealand Dairy Research Institute for providing samples and test results, as well as Stewart Carter for help in preparing this manuscript.

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Rapid Gradient and Elevated Temperature UHPLC of Flavonoids in Citrus Fruit

Application Note

General Chromatography, Food Industry

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Abstract

Citrus fruits were analyzed by gradient UHPLC for naringin and other flavonoids. Gradient reversed phase HPLC with long (150 mm) sub-2-µm columns is a powerful technique for analyzing complex matrices with numerous analytes such as grapefruit juice. Sufficiently flushing the column with strong solvent and re-equilibration with weak solvent starting conditions is necessary but time consuming, possibly impinging on overall lab productivity. An Agilent ZORBAX Rapid Resolution High Definition (RRHD) Eclipse Plus C18, 2.1 mm × 150 mm, 1.8 µm column was able to resolve over 70 peaks in grapefruit juice in seven minutes. The column was then properly flushed with strong solvent and re-equilibrated in the next three minutes by operating at a high flow rate (0.8 mL/min). Orange and lime fruit were similarly analyzed.

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Introduction

Plant extracts such as lime juice and peel are ideal candidates for gradient analysis due to the wide range of polarity of the compounds found in the matrix; from hydrophilic small organic acids (citric acid) and sugars (fructose) to moderately polar polyphenols to the hydrophobic essential oil of lime. An isocratic method cannot accommodate the wide range of peak retention in a reasonable time whereas a gradient method can.

Gradient profiles of plant extracts, or fingerprints, are often used for identification, authentication and quality control. Agilent ZORBAX Rapid Resolution High Definition (RRHD) columns are designed for 1200 bar operation making them ideal for fast or high-resolution gradient separations, and a reliable tool for the food and herbal medicine industries.

High resolution chromatographic profiles or fingerprints of three types of citrus fruit were created using UHPLC conditions, highlighting several flavonoids. Citrus flavonoids are thought to have health benefits, based on antioxidant activity. These benefits include anticancer, antiviral, and anti-inflammatory properties. Some citrus flavonoids provide a bitter or sweet taste.

Gradient analyses often take longer than isocratic analyses due to flushing and re-equilibrating the column. One way to reduce analysis time is to increase the flow rate.The analyses in this application note take advantage of high flow rates.

Experimental

An Agilent 1290 Infinity LC System comprised of the following was used to obtain all data:

- Agilent 1290 Infinity Diode Array Detector (DAD) and 10 mm, 1 µL Max-Light flow cell
- Agilent 1290 Infinity Thermostatted Column Compartment (TCC)
- Agilent 1290 Infinity Automatic Liquid Sampler (ALS)
- Agilent 1290 Infinity Binary Pump with Jet Weaver V35 mixer

UHPLC columns and chromatographic conditions were:

Columns:	Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 mm × 150 mm, 1.8 µm p/n 959759-902 Agilent ZORBAX RRHD StableBond C18, 2.1 mm × 150 mm, 1.8 µm p/n 859700-902	
Mobile phase:	Solvent A: Water (0.1% formic acid) Solvent B: Acetonitrile (0.1% formic acid)	
Flow rate and gradient:	0.8 mL/min	
	Time	%B
	0	0
	0.5	0
	7	40
	7.1	100
	8.6	100
	8.7	0
	10	end
Column temperature:	40 °C or 90 °C	
Detection:	UV 276 nm, 4 nm, Ref. OFF	
Injection volume:	1.0 µL	

Samples

Rinds were peeled from the fruit flesh and promptly ground with a food processor until grounds were about 1–3 mm in diameter. Two hundred milligrams of ground rind were then extracted with 10 mL MeOH (0.1% KOH) in an ultrasonic bath for 10 min, then 0.45-µm syringe filtered. Fruit flesh was hand squeezed and the collected juice was 0.45 µm filtered with a 0.45 µm filter into amber autosampler vials.

Citrus flavonoid standards

The following standards included rutin, naringin, hesperidin, quercetin, and naringenin. The calibration samples were made by diluting standard stock solutions with water to the 10–1000 ppm range. Most of the standard stock solutions were aqueous, but some were made in methanol/H_2O due to solubility issues. A chromatogram of the standard mixture including the elution order of the flavonoids, and two calibration curves are shown in Figure 1.

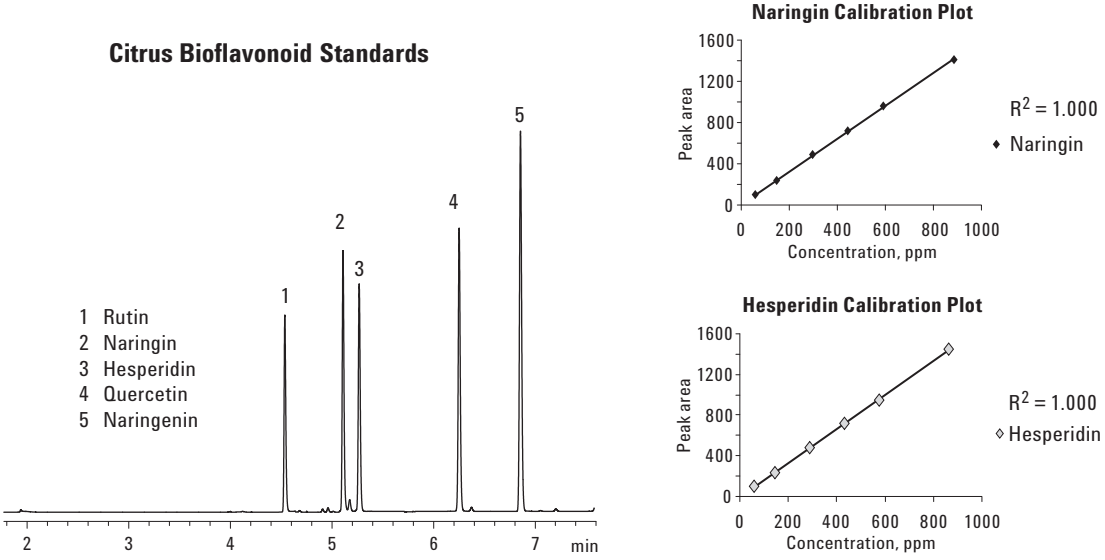


Figure 1. Chromatogram of the flavonoid standard mixture and calibration curves for naringin and hesperidin. Chromatographic conditions are listed in experimental section.

Results and Discussion

Higher mobile phase linear velocity (flow rate) using sub-2-µm columns has less of an adverse effect on the height of a theoretical plate (H) than larger particle columns allowing faster flow without significant resolution loss (Figure 2). This small loss in efficiency at high flow rate, an RRHD column characteristic, is welcome in gradient analysis because an increase in flow rate when holding k* constant, results in a decrease in gradient time:

Equation 1: $t_G = k^* V_m/F$

Where

- k^* is the gradient retention factor
- t_G is the gradient time
- F is the flow rate
- V_m is the column volume, assumed to be the same between the two columns

Increasing the number of column volumes per unit time (flow rate) in gradient analysis also reduces the other gradient segments including the isocratic hold, strong solvent flush and re-equilibration, and consequently reduces analysis time.

Narrow bore (2.1 mm id) columns are often used over larger bore columns for their compatibility with MS detectors, increased sensitivity, and reduced solvent usage.

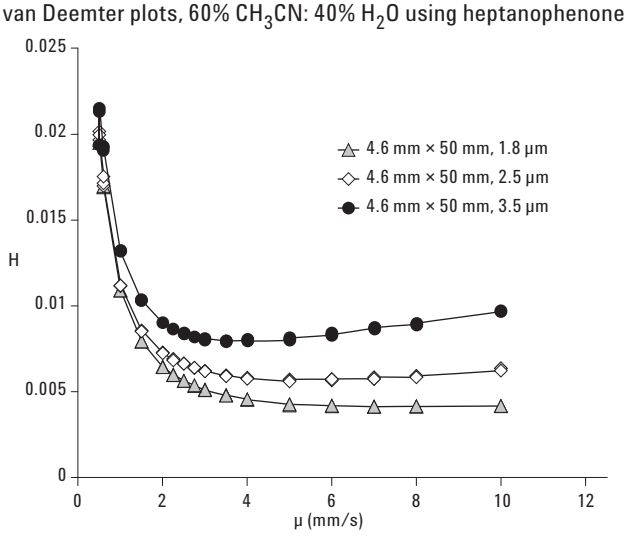


Figure 2 Plots of plate height versus velocity. Plate heights for sub-2-µm columns remain relatively constant compared to larger particle sized columns as mobile phase velocity (flow rate) increases.

Conventional 2.1 mm × 150 mm columns packed with 5 µm particles typically use flow rates of about 0.2 mL/min. The 0.2 mL/min flow rate combined with the relatively large column size and extra column volume contributes to long analysis times. For example, re-equilibrating the column to starting conditions with four column volumes of mobile phase at starting conditions takes at least six min. This does not include flushing out any delay volume (the volume of the flow path from the gradient formation point to the head of the column). Column re-equilibration time is calculated from a number of column volumes divided by the flow rate:

Equation 2: $n (\pi r^2 L (0.6)) / F = t$

or for the 2.1 × 150 mm column (F = 0.2 mL/min) example

$4 (\pi 0.105^2 15 (0.6)) / 0.2 = 6.3$ min

Where:

- n is the number of column volumes
- r is column internal radius (cm)
- L is column length (cm)
- 0.6 is the void fraction of the packed column
- F is the flow rate (mL/min)
- t is the re-equilibration time in minutes

Operating under UHPLC conditions, and using a faster flow rate (0.8 mL/min.) with 1.8 µm particles, column re-equilibration time is only 1.6 min, which is four times faster. The gradient and pressure profiles, and column volumes are shown graphically with a lime juice chromatogram in Figure 3. Only four column volumes were necessary for re-equilibration. This

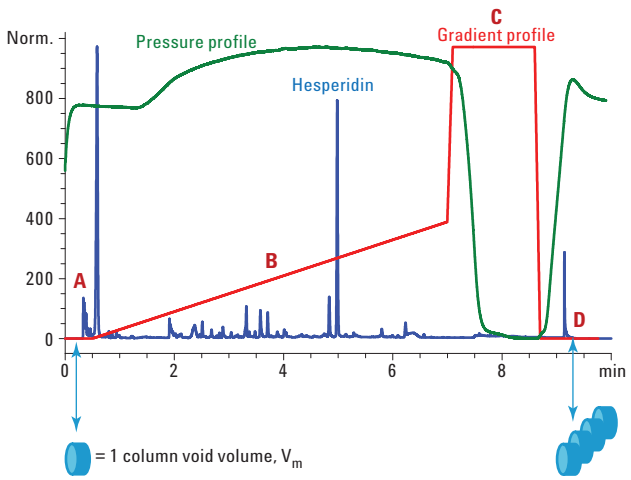


Figure 3. UHPLC of grapefruit juice with pressure and gradient profiles, and column volumes needed for re-equilibration. Segments of the gradient include A: initial isocratic hold, B: linear elution ramp, C: strong solvent column flush, D: re-equilibration.

was proven by injecting samples repeatedly without any change in k*, α, or resolution (Figure 4).

In addition to the grapefruit, lime and orange extracts were also analyzed with the method, each producing a distinct chromatographic profile. Several flavonoids were identified using retention time matching of standards as shown in Figures 5,6,7. Hesperidin or Narginin were quantified using an

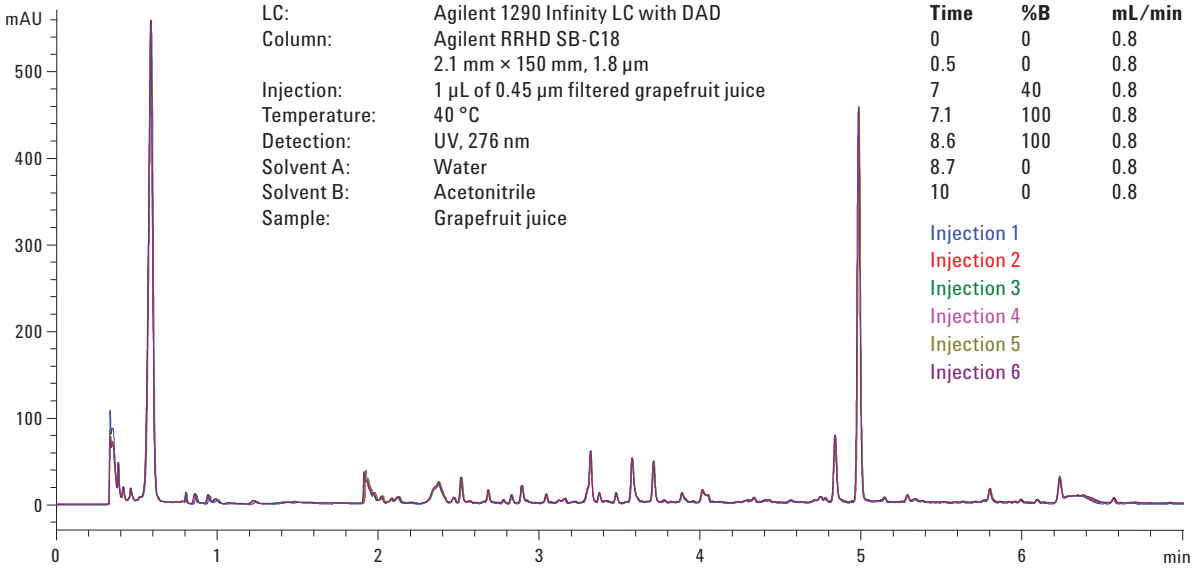


Figure 4. Overlay of six analyses with no variability in retention, selectivity or resolution, indicating sufficient column flushing and re-equilibration.

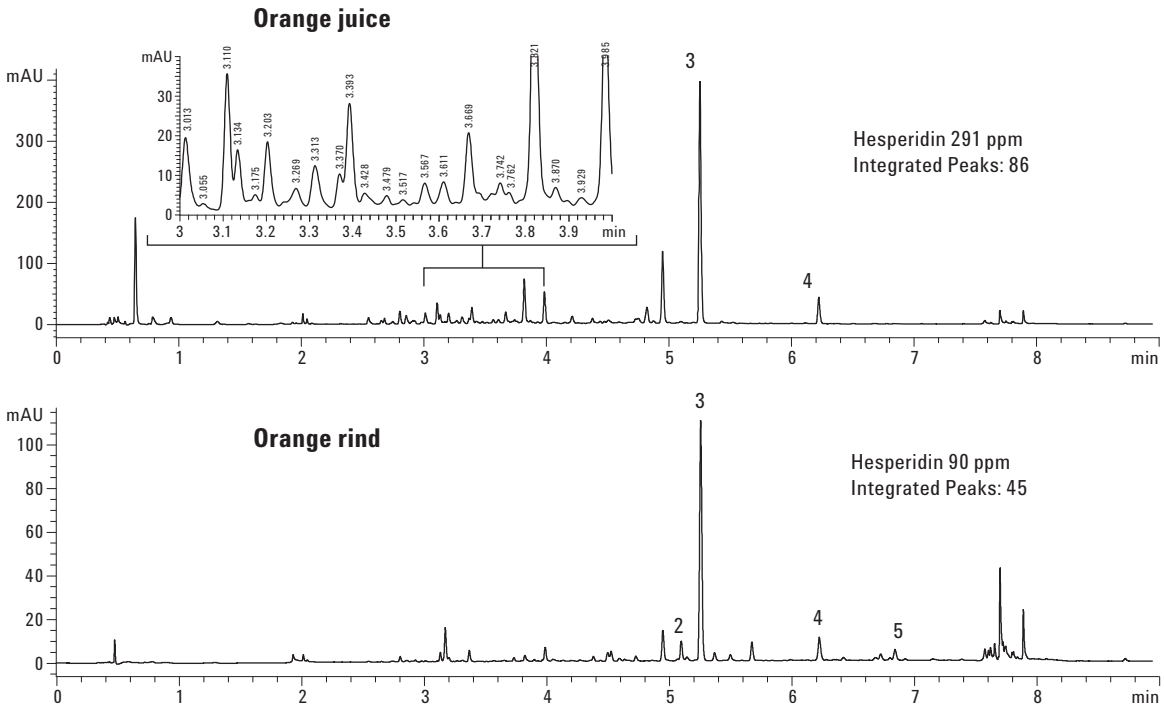


Figure 5. UHPLC fingerprint of orange juice and rind analyzed by an Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 mm × 150 mm (p/n 959759-902). Chromatographic conditions are listed in experimental section.

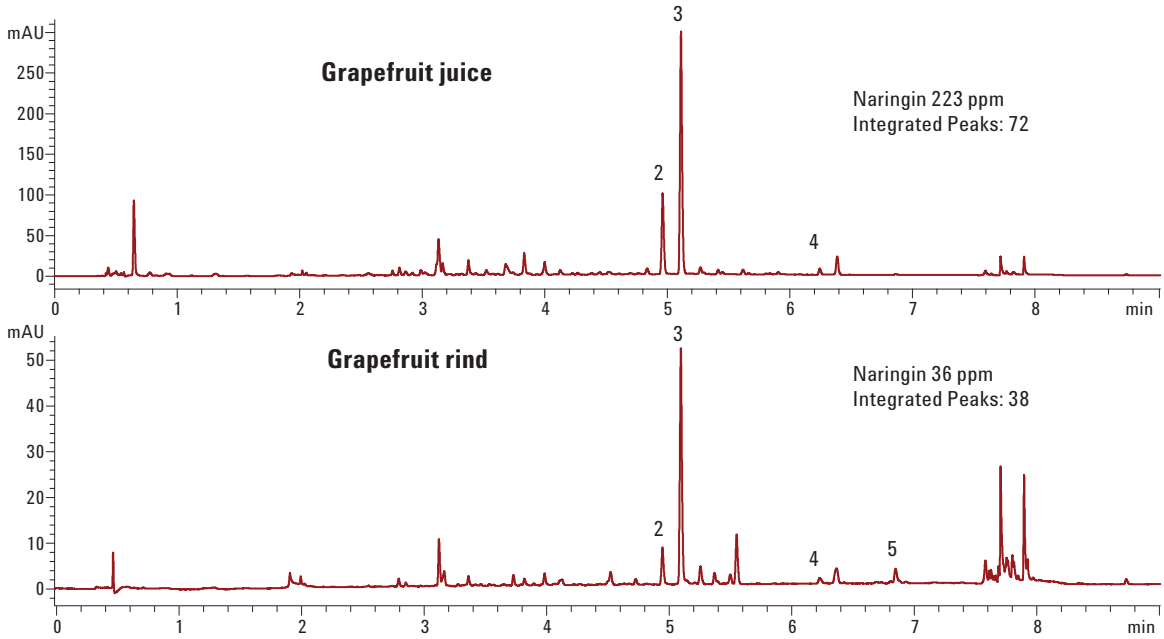


Figure 6. UHPLC fingerprint of grapefruit juice and rind analyzed by an Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 mm × 150 mm (p/n 959759-902). Chromatographic conditions are listed in experimental section.

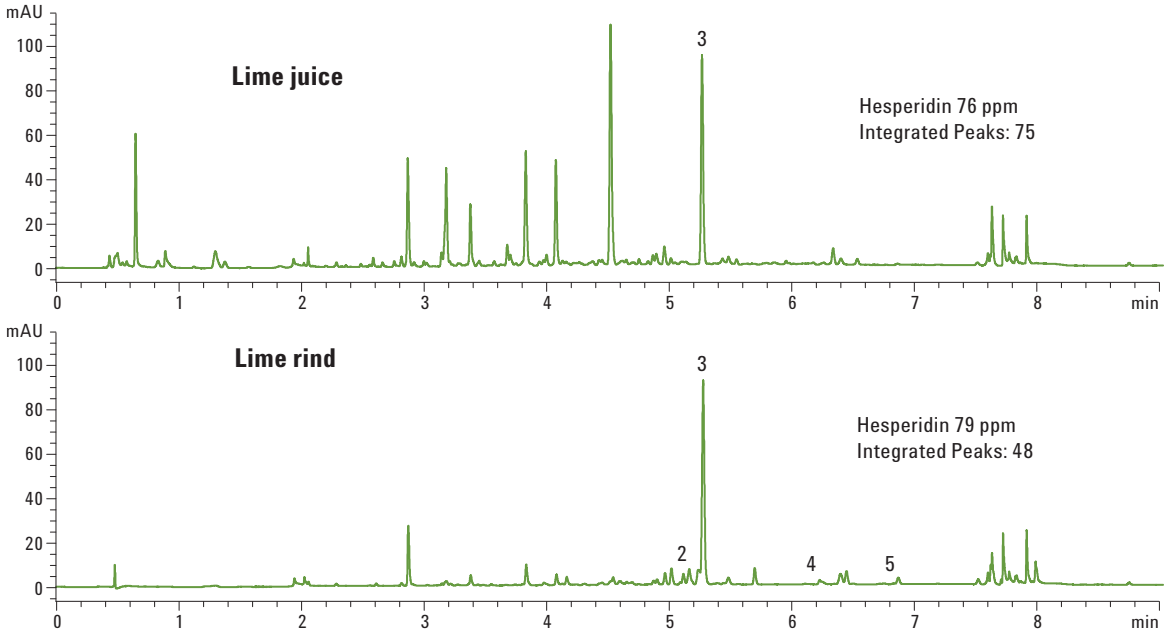


Figure 7. UHPLC fingerprint of lime juice and rind analyzed by an Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 mm × 150 mm (p/n 959759-902). Chromatographic conditions are listed in experimental section.

external standard six point calibration curve and concentrations listed in the figures. A subsection of the orange juice chromatogram is expanded to more clearly show the number of compounds present in the sample.

When using 2.1 mm × 150 mm RRHD columns, the high flow rate mitigates the additional analysis time inherent in gradient methods associated with isocratic holds, strong solvent flushing and re-equilibration. Sensitivity gains from narrower peaks however, are still preserved. Figure 8 indicates signal-to-noise

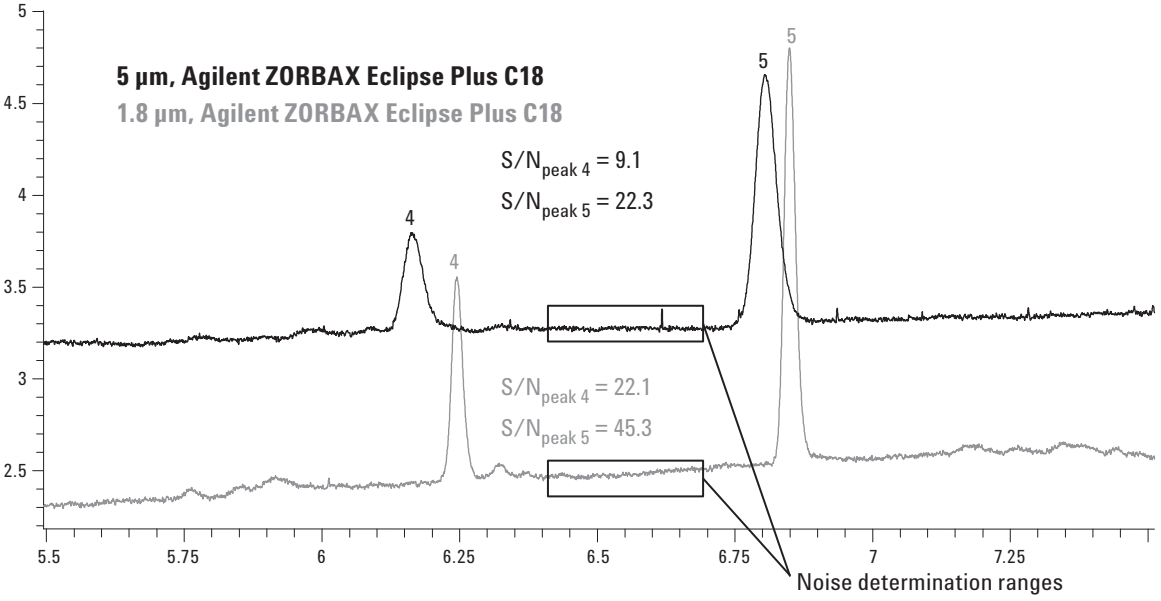


Figure 8. Signal-to-noise comparison of 5 and 1.8 µm Agilent ZORBAX Eclipse Plus C18 columns, 2.1 mm × 150 mm using a 1 ppm each quercetin and naringenin standard. Chromatographic conditions are listed in the experimental section.

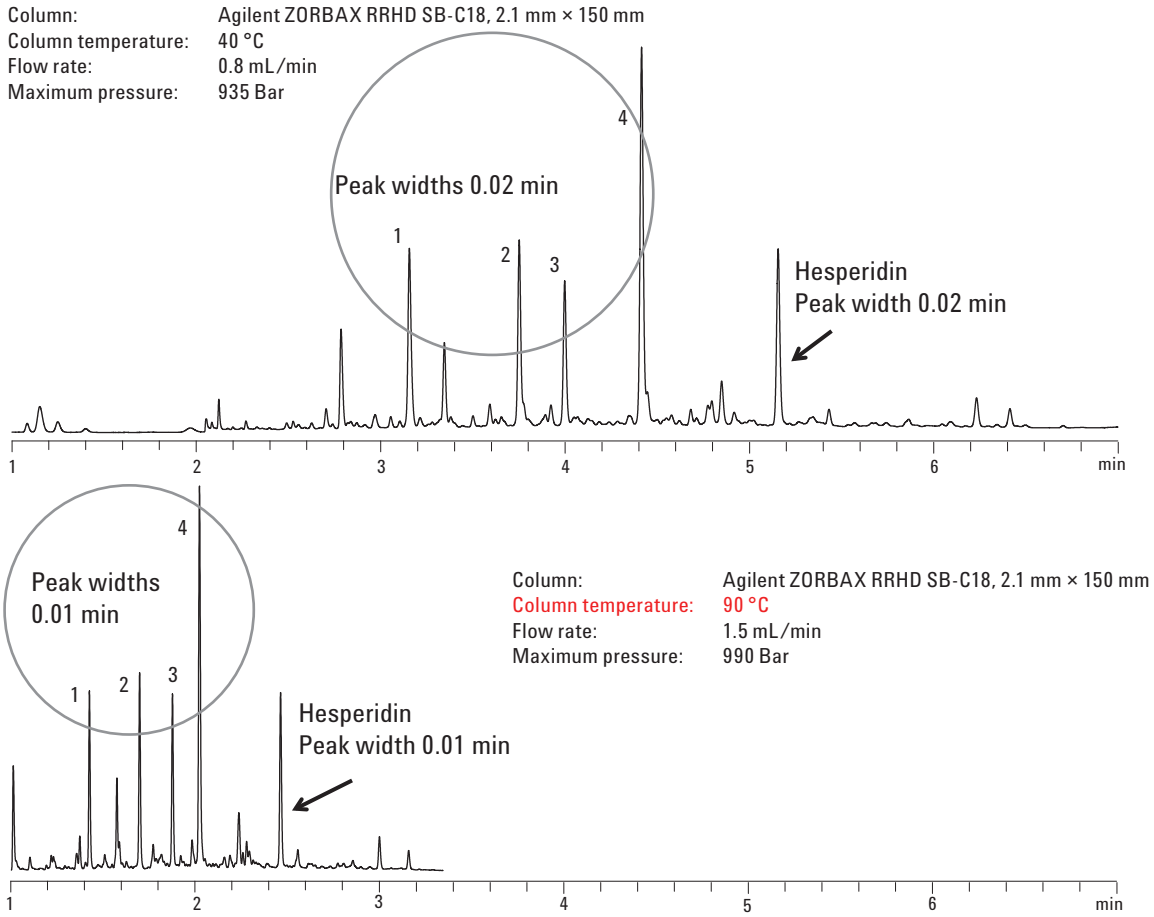


Figure 9. UHPLC of lime juice including high temperature and faster flow rates further reduce analysis time using an Agilent ZORBAX RRHD SB-C18 column. Chromatographic conditions listed in experimental section.

ratios of an Agilent ZORBAX RRHD Eclipse Plus C18 2.1 mm × 150 mm, 1.8 µm column is 50% to 60% greater compared to an Agilent ZORBAX Eclipse Plus C18, 2.1 mm × 150 mm, 5 µm column. The limit of quantification of quercetin is 1 ppm for the 5 µm column, and 0.5 ppm for the RRHD column.

Narrower peaks were achieved by using a faster flow rate. The temperature was raised to lower mobile phase viscosity, and the Eclipse Plus C18 phase was replaced with StableBond-C18 to withstand the extreme temperature. The temperature limit of the SB-C18 column is 90 °C, while the temperature limit of the Eclipse Plus C18 column is 60 °C. The flow rate could be increased to 1.5 mL/min at 90 °C. Gradient times were decreased by a factor of 1.875 to maintain k* for the flow rate change:

Equation 3: $k^* = (t_G \times F) \div V_m$

The 90 °C temperature, and 1.5 mL/min. rate produced 0.01 min wide peak widths at half height, which is half as wide as the 40 °C method. It also reduced analysis time proportionally to 5.3 minutes (Figure 9). RRHD column ruggedness allows these columns to operate at pressures up to 1200 bar, making them a perfect fit for the Agilent 1290 Infinity LC system. All RRHD columns can be used with other UHPLCs, however, the flow rate must be adjusted downward to allow pressure (or flow rate) to be in the operating range of the other vendor's UHPLC.

Conclusion

Complex natural products such as fruit juices and their rinds were separated into their many components in a relatively short time using gradient elution, high flow rate, and long (150 mm) highly efficient RRHD Eclipse Plus sub-2-µm columns on a UHPLC system – the Agilent 1290 Infinity LC system.

The benefit of the long column length allows challenging separations to be accomplished with high resolution and moderate analysis times. The benefit of 1.8 µm particles is that higher mobile phase flow rates can be used with little loss in efficiency. Detection sensitivity also is intrinsically gained when converting from larger particle columns to 1.8 µm columns. Higher temperature decreased mobile phase viscosity, permitting an even higher flow rate and consequently an even faster analysis.

Agilent's ZORBAX RRHD columns are ideal for fast or high-resolution gradient separations. They contain 1.8 µm particles for high resolution and are designed for stability up to 1200 bar for high flow rates on the Agilent 1290 Infinity LC system or other vendor UHPLC's.

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The Direct Analysis of Milk Powder on the Liberty Series II ICP-OES with the Axially-Viewed Plasma

Application Note

Inductively Coupled Plasma-Optical Emission Spectrometers

Author

Andrew Ryan

Introduction

The analysis of milk is important because milk is an indicator of environmental contamination, a significant pathway for toxic metal intake by humans and a source of essential nutrients. Previously, milk liquid and milk powder analysis was carried out using flame and graphite furnace atomic absorption spectrometry and even anodic stripping voltammetry [1]. Inductively coupled plasma atomic emission spectrometry (ICP-OES) and inductively coupled plasma mass spectrometry (ICP-MS) are now preferred for routine determinations because of the rapid multi-element analysis capabilities of these techniques.

Generally, milk samples are prepared by either wet digestion or dry ashing [1-5]. These are time consuming and involve procedures using potentially hazardous chemicals. Direct slurry nebulization combined with standard additions calibration has also been reported for ICP-MS with varying success [5,6]. The accuracy of standard additions is greatly influenced by calibration linearity and the presence of spectral interference. The ICP-MS technique has the advantage of sensitivity but is unable to analyzse high dissolved solid contents for long periods of time and the instrumentation is more expensive than ICP-OES.

This work describes the direct analysis of milk powder using standard quantitative calibration with aqueous standards. Viscosity effects of the milk powder solutions are corrected for using scandium (361.384 nm—ionic line) as an internal standard. Major, minor and trace elements were determined in a single analysis. Less sensitive lines are used for the determination of major elements allowing both major and minor elements to be determined from a single sample solution.

Many of the major constituents in milk powder such as Na, K and Ca are easily ionized elements (EIE) that have been reported to cause ionization interferences. Ionization interferences tend to cause a reduction in signal intensity with increasing concentration of EIE and the effect is prominent at interferent concentrations at or above 100 mg/L. The atomic lines of Na and, K, and to a lesser extent Ca (422.673 nm) and Li, exhibit signal enhancement with increasing concentrations of

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EIE. The effect can be easily minimized or eliminated on a radially-viewed ICP-OES by adjusting the viewing height. For the more sensitive axially-viewed ICP-OES, many reports of interferences due to EIE have been described [7,8]. In one report, scandium used as an internal standard was found to compensate for part of the signal depression [8]. Ionization interferences on the axially-viewed plasma have been found to be reduced by lowering the nebulizer pressure and increasing the power. Increasing the power increases the electron density in the plasma thus reducing the effect of electrons contributed by the EIE. Generally, when analyzsing samples that contain high levels of EIE, it is recommended that all standards have similar levels of EIE added (matrix matching).

An alternative is to saturate the plasma with a high concentration of another EIE such as caesium. Therefore, the effect of adding caesium as an ionization suppressant to the standards and samples was also investigated.

The accuracy and validity of the method was assessed by the use of National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) 8435 Whole Milk Powder.

Experimental

Instrumental

An Agilent Liberty Series II ICP-OES with the axially-viewed plasma was used for the analysis.

The Liberty Series II ICP features a 40 MHz free running RF generator, a 0.75 m Czerny-Turner monochromator with a 1800 grooves/mm holographic grating used in up to four orders. The resolution of the optical system ranges from 0.018 nm in the 1st order to 0.006 nm in the 4th order.

The instrument was controlled with a Digital Equipment Corporation (DEC) Venturis computer with an Intel Pentium processor and Agilent's Plasma 96 software running under Microsoft's Windows 95 operating system.

The instrument operating conditions are listed in Table 1.

Table 1: Instrument Operating Conditions

Power	1.30 kW
Plasma gas flow	15.0 L/min
Auxiliary gas flow	1.5 L/min
Spray chamber type	Sturman- Masters
Torch	Standard axial torch with 2.3 mm id injector
Nebulizer	V-groove
Nebulizer pressure	240 kPa
Pump tube	Inlet - PVC grey-grey, 1.30 mm id
	Outlet - PVC, blue-blue, 1.65 mm id
Pump speedrate	15 rpm
Sample uptake rate	1.5 mL/min
Integration time	1 second for Ca, K, Mg, Na, P, S and Sr
	3 seconds for Ba, Fe, Mn, Zn and Sc
No. of replicates	3
Sample delay time	25 seconds
Fast pump	On
Stabilization time	20 seconds
Background correction	Polynomial plotted background
PMT voltage	600 V

For the determination of sulfur, an Auxiliary Gas Module-2 (AGM-2) is required. The AGM provides a nitrogen purge for the monochromator to extend the working wavelength range from 189 nm down to 175 nm.

To make sure that the milk powder sample was evenly mixed and in suspension while being aspirated, the solution was continuously stirred with a magnetic stirrer.

Standard Preparation

Aqueous standards were prepared from Custom-Grade Multi-element Solutions Var Cal 1, Var Cal 2 and Var Majors 1 (Inorganic Ventures, Inc.) and from 1000 mg/L single element standards (Spectrosol, BDH Chemicals). The standards were made up in 18 MΩ Milli-Q water with 0.5% v/v HNO_3 and 0.002% v/v Triton X100 prepared from a 1% w/v Triton X100 solution. Scandium was added to each solution as an internal standard with a final concentration of 0.5 mg/L.

The following calibration standards were prepared.

Table 2. Calibration Standards

Standard	Concentration (mg/L)	Elements
Standard 1	0.2	Ba, Mn, Zn, Sr
Standard 2	1.0	Ba, Mn, Zn, Sr, Fe
Standard 3	5.0	Mg, Fe
Standard 4	25	Mg, Na, Ca
Standard 5	100	Na, Ca, K
Standard 6	200	K
Standard 7	13.350 and 32.614	S and P, respectively
Standard 8	66.752	S
Standard 9	163.069	P

Rinse and calibration blank solutions were prepared from 18 MΩ Milli-Q water with 0.5% HNO_3 and 0.002% Triton X100.

Sample Preparation

Solutions were prepared from an instant full cream milk powder sample purchased at a local supermarket and SRM 8435 Whole Milk Powder.

Milk powder solutions containing 0.5% and 4% w/v were prepared for both samples. The sample was accurately weighed and then transferred into a volumetric flask. The flask was filled approximately 3/4 full with 18MΩ Milli-Q water and gently shaken until the milk powder was evenly mixed. Triton X100 was added to give a concentration of 0.002%. Acid was not added to the sample solutions as it causes the precipitation of protein. Scandium (0.5 mg/L) was added as an internal standard.

The scandium bulk standard was stabilized with acid and it was necessary to dilute the scandium by preparing a secondary standard before adding it to the milk powder solutions because even a small concentration of acid will cause the precipitation of protein. Adding dilute ammonia solution to the samples to adjust the pH to 7.5 [6] can overcome this problem, but it was not required in this case.

The solutions were made up to the mark and placed in an ultrasonic bath for 5 minutes. The solutions were then shaken vigorously for 1 minute.

A separate rinse containing 0.002% v/v Triton X100 and no acid was used for rinsing between samples.

For the study of the effect of the addition of an ionization suppressant, 1% w/v Cs as CsCl was added to all sample, standard and rinse solutions. Caesium was chosen as an ionization suppressant as it has a low energy of ionization, is not very sensitive by ICP-OES and, therefore, spectral interference is generally not a problem. Caesium chloride is available in a very pure form and does not build up in the torch injector tube as readily as other alkali salts.

Results and Discussion

Milk Powder Analysis

The results presented in Tables 3–6 represent the concentrations of constituent elements in the milk powder on a dry weight basis. Moisture content in the two milk powder samples was determined by accurately weighing the undried samples and then reweighing the samples after drying in an air oven for 4 hours at 85 °C. Moisture content was small and represented only 1.3% and 1.9% of the total weight for the full cream milk powder sample and NIST SRM 8435, respectively.

The mean results of the triplicate analyses for the determination of major constituent elements in milk powder without the addition of 1% w/v Cs are listed in Table 3.

The mean results of the triplicate analyses for the determination of minor and trace constituent elements in milk powder without the addition of 1% w/v Cs are listed in Table 4.

The mean results of the triplicate analyses for the determination of major constituent elements in milk powder with the addition of 1% w/v Cs are listed in Table 5.

The mean results of the triplicate analyses for the determination of minor and trace constituent elements in milk powder with the addition of 1% w/v Cs are listed in Table 6.

The effect of ionization interference from the EIE can be seen for K and Sr and to a smaller extent Ca, in Tables 3 and 4. Ionic lines were used for Ca and Sr and signal suppression for these lines was evident as a value lower than the certified value was found. With the addition of Cs to all solutions, Tables 5 and 6, a value very close to the certified value was found for both lines.

For K, without the addition of Cs, a lower than expected result was found. This is unexpected because signal enhancement is usually observed for K. An explanation for this could be that standard 5 contained reasonably high levels of other EIE and therefore the signal enhancement for K in standard 5 was higher than that for the sample. The sample was remeasured for K using separate standards for K. The measured K concentrations for the full cream milk powder sample and SRM 8435 Whole Milk Powder were 1.14 and 1.26 wt%, respectively. These results are closer to the certified value for the SRM, but are still unexpectedly lower. With the addition of Cs, the result for K was very close to the certified value.

Na was also expected to be affected by the presence of other EIE but this was not evident in this analysis. This is probably due to the presence of other EIE's in both the standards and samples.

The remaining elements did not appear to be greatly affected by the EIE because much of the signal depression was corrected by the internal standard.

For the 4% milk powder solutions without added Cs, EIE concentration was approximately 1000 mg/L and ionization interference is reportedly [8] quite significant at these levels. Despite this, results very close to the certified values were found for Ba, Fe and Mn. This suggests the internal standard not only successfully corrected for the different viscosity of the samples but also corrected for ionization interference for these elements.

Table 3. Major Elements in Milk Powder Without the Addition of Caesium

Element	Wavelength (nm)	0.5% Milk powder sample (wt%)	0.5%S.R.M. 8435 Whole milk powder (wt%)	S.R.M. 8435 Certified value (wt%)
Ca	315.887	0.849 ± 0.033	0.876 ± 0.008	0.922 ± 0.049
K	769.896	0.844 ± 0.044	0.975 ± 0.020	1.363 ± 0.047
Na	588.995	0.277 ± 0.010	0.376 ± 0.004	0.356 ± 0.040
P	213.618	0.761 ± 0.031	0.784 ± 0.019	0.780 ± 0.049
S	180.731	0.263 ± 0.019	0.268 ± 0.006	0.265 ± 0.035

Table 4. Minor and Trace Elements in Milk Powder Without the Addition of Caesium

Element	Wavelength (nm)	0.5% Milk powder sample (mg/kg)	4% Milk powder sample (mg/kg)	0.5% S.R.M. (mg/kg) 8435 Whole milk powder (mg/kg)	4% S.R.M. 8435 Whole milk powder (mg/kg)	S.R.M. 8435 Certified value (mg/kg)
Mg	285.213	754 ± 23		808 ± 22		814 ± 76
Sr	407.771	4.65 ± 0.04		3.77 ± 0.03		4.35 ± 0.50
Zn	213.856	27.5 ± 1.5		25.5 ± 0.8		28.0 ± 3.1
Ba	455.403		0.70 ± 0.03		0.57 ± 0.02	0.58 ± 0.23
Fe	259.940		2.30 ± 0.05		1.70 ± 0.03	1.8 ± 1.1
Mn	257.610		0.242 ± 0.001		0.151 ± 0.004	0.17 ± 0.05

Table 5. Major Elements in Milk Powder With the Addition of 1% (w/v) Caesium

Element	Wavelength (nm)	0.5% Milk powder sample (wt%)	0.5%S.R.M. 8435 Whole milk powder (wt%)	S.R.M. 8435 Certified value (wt%)
Ca	315.887	0.931 ± 0.019	0.899 ± 0.021	0.922 ± 0.049
K	769.896	1.304 ± 0.032	1.397 ± 0.024	1.363 ± 0.047
Na	588.995	0.298 ± 0.004	0.378 ± 0.004	0.356 ± 0.040
Na	330.237	0.280 ± 0.004	0.360 ± 0.007	0.356 ± 0.040
P	213.618	0.775 ± 0.017	0.758 ± 0.004	0.780 ± 0.049
S	180.731	0.252 ± 0.007	0.254 ± 0.006	0.265 ± 0.035

Table 6. Minor and Trace Elements in Milk Powder With the Addition of 1% (w/v) Caesium

Element	Wavelength (nm)	0.5% Milk powder sample (mg/kg)	4% Milk powder sample (mg/kg)	0.5% S.R.M. (mg/kg) 8435 Whole milk powder (mg/kg)	4% S.R.M. 8435 Whole milk powder (mg/kg)	S.R.M. 8435 Certified value (mg/kg)
Mg	285.213	761 ± 14		775 ± 13		814 ± 76
Sr	407.771	5.53 ± 0.05		4.40 ± 0.07		4.35 ± 0.50
Zn	213.856	30.0 0.7		26.3 ± 0.5		28.0 ± 3.1
Ba	455.403		0.79 ± 0.02		0.62 ± 0.01	0.58 ± 0.23
Fe	259.940		2.23 ± 0.07		1.76 ± 0.06	1.8 ± 1.1
Mn	257.610		0.269 ± 0.004		0.167 ± 0.007	0.17 ± 0.05

Long Term Stability

The long term stability was determined for the most concentrated milk powder solution to show that good stability can be obtained over a reasonable period of time for a solution containing a high content of dissolved and undissolved solids. The effect of internal standardization on long term stability was also studied.

Long term stability was evaluated by continuously aspirating 4% instant full cream milk powder solution over a period of time. A one minute rinse was performed at the maximum pump rate of 50 rpm (fast pump) between measurements.

The precision of the measurements over one and a half hours with internal standardization correction ranged from 1.2 and 2.0 %RSD.

The long term stability plots for major constituents Ca and Mg and minor constituents Ba, Mn and Sr with and without internal standardization are displayed in Figures 1 and 2, respectively.

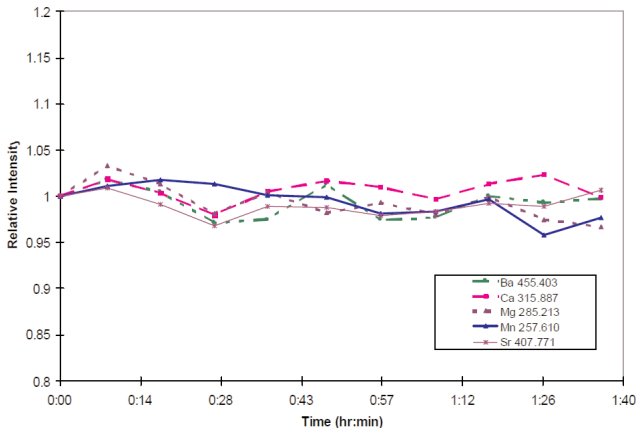


Figure 1. Signal stability over one and a half hours for a 4% full cream milk powder solution with internal standardization.

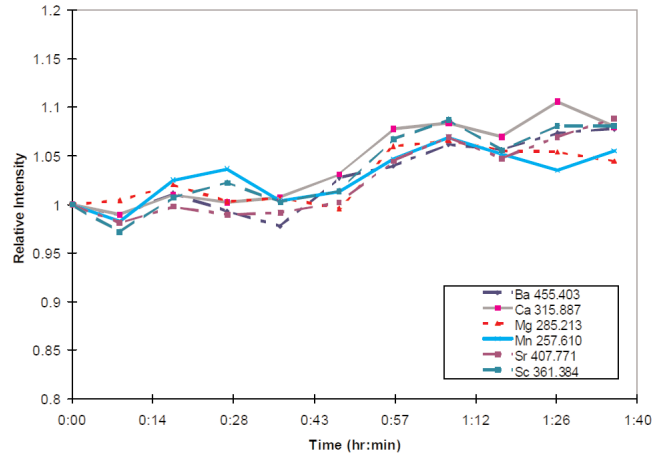


Figure 2. Signal stability over one and a half hours for a 4% full cream milk powder solution without internal standardization.

After the milk powder solution had been aspirated for over one and a half hours, the injector tube showed signs of blockage due to deposition of the milk powder. Figure 2 shows the effect of the build up of milk powder on the signal. Not only does the internal standard correct for viscosity effects and some ionization interferences, from Figure 1 it can be seen that it is also very effective in compensating for drift caused by the gradual build up of milk powder in the injector tube of the torch.

Five replicates were measured at an integration time of three seconds for each line. The precision for each measurement ranged from 0.1 to 1.6 %RSD for the major constituent elements and 0.2 to 3.0 %RSD for minor and trace constituent elements.

Sample Delay and Stabilization Times

When differing matrices are aspirated into a spray chamber, some time must be allowed so that the solution can reach the plasma and for the signal to stabilize. The time required to allow the system to stabilize when switching from aqueous solutions to the milk powder solutions was studied.

This was evaluated by firstly aspirating the rinse solution followed by a 4% full cream milk powder solution. The pump speed was set to 50 rpm (fast pump speed), which is the pump rate used during the sample delay stage, and the signal was monitored for Sc.

A plot of the signal over a short period of time is displayed in Figure 3.

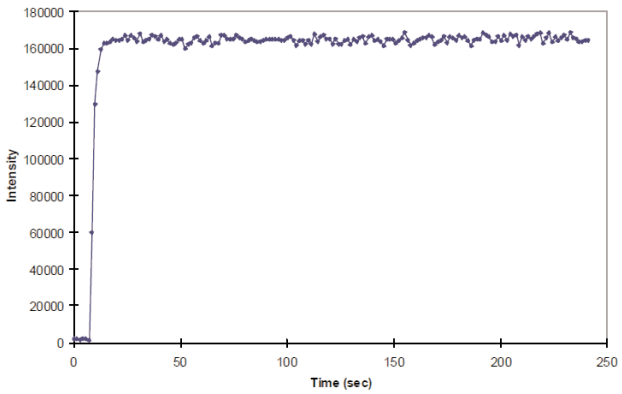


Figure 3. Stabilization profile for a 4% full cream milk powder solution.

When switching from the rinse solution to the 4% milk powder solution, the signal was found to stabilize after 20–25 seconds. Therefore, for the analysis of the milk powder solutions, a sample delay time of 25 seconds was used. The stabilization time was set to 20 seconds. The stabilization time is the time allowed for the pump to slow down from full speed to the analysis pump speed—in this case, 15 rpm.

It was observed that the presence of Triton X100 detergent, which is often used for slurry nebulization to help keep the sample in suspension, greatly reduced the stabilization time.

Summary

The concentrations of various elements of both nutritional and environmental interest in milk powder samples were determined on the Liberty Series II with the axially-viewed plasma.

Aqueous calibration solutions were used and the scandium internal standard successfully corrected for the viscosity effects of the samples, improved the long term stability of the analysis and corrected for some ionization interferences due to the high levels of EIE.

The time required for the system to stabilize after switching from aqueous solutions to the milk powder solutions was found to be very short.

The addition of caesium as an ionization suppressant eliminated ionization interferences and the need for dilution, allowing both major and minor constituents to be measured in a single solution. For the determination of trace elements such as strontium where a more concentrated milk powder solution is required, the addition of caesium becomes an important factor.

With the addition of 1% caesium, all measured values are in very good agreement with the certified values for the standard reference material, validating the accuracy of the method.

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HPLC Determination of Synthetic Pyrethroid Insecticides in Shampoos and Lotions

Application

Environmental, Consumer Products

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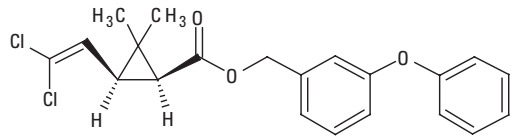
Abstract

The separation of synthetic pyrethroids, *cis*- and *trans*-permethrin, and the synergist piperonyl butoxide in shampoo and lotion was easily accomplished by dilution, filtration, and injection into a ZORBAX Eclipse XDB-C18 RPC HPLC column. All components could be separated to baseline with symmetrical peaks using a water-methanol isocratic mobile phase.

Introduction

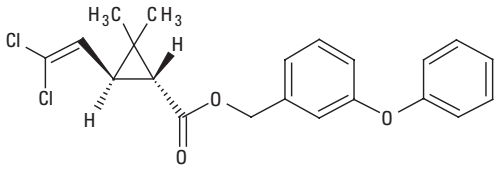
Permethrin (Figures 1A and 1B) is a pyrethroid insecticide that is a chlorinated, synthetic form of pyrethrum, a popular natural insecticide derived from chrysanthemum flowers. Permethrin has multiple uses as an insecticide in household insect foggers, tick and flea spray for yards and pets, as a spray for agricultural and livestock products, and for mosquito abatement. When combined with piperonyl butoxide (Figure 1C), a synergist that is used to enhance the affect of a wide variety of insecticides, permethrin formulations are widely used to combat head-lice and are available in the form of a liquid, gel, or shampoo. Recently, the increase of opportunistic infections in HIV-infected patients, like scabies, has lead to an increasing interest in formulations of these substances for therapy. Thus, the analysis of permethrin and it's synergist, piperonyl butoxide (PBO), is important at the formulation level as well as the trace level.

A



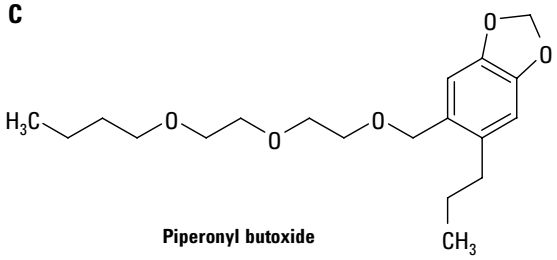
cis-**permethrin**

B



trans-**permethrin**

C



Piperonyl butoxide

Figure 1. Chemical structures for *cis*-permethrin (A), *trans*-permethrin (B), and piperonyl butoxide (C)

Chemical Properties

Permethrin has two diastereomers that possess different chemical, physical, and toxicological properties. Both permethrin and PBO are rather hydrophobic and have no polar functional groups. They are very insoluble in water but soluble with water-miscible organic solvents. They can be separated by reversed phase chromatography (RPC) without the use of buffers. Depending on the matrix, however, pH control could be useful to resolve matrix components from the insecticides.

Chromatographic Method and Application

In the present example, the analysis of these compounds in shampoo and lotion presented no unusual matrix effects, so sample preparation merely involved dilution of the sample with methanol, followed by filtration through a 0.45-µm membrane filter, and injection into the HPLC column. Figure 2 shows the RPC separation of the three standards; all three compounds are well separated isocratically on a 4.6 × 150-mm ZORBAX Eclipse XDB-C18 column with 3.5-µm particles, operated at 35 °C. Using a water-methanol mobile phase, peaks were quite symmetrical and the separation time was just over 6 minutes. A diode array detector (DAD) was used at 272 nm for detection. Small amounts of unidentified impurities are observed in Figure 2. Experimental conditions are listed in Table 1.

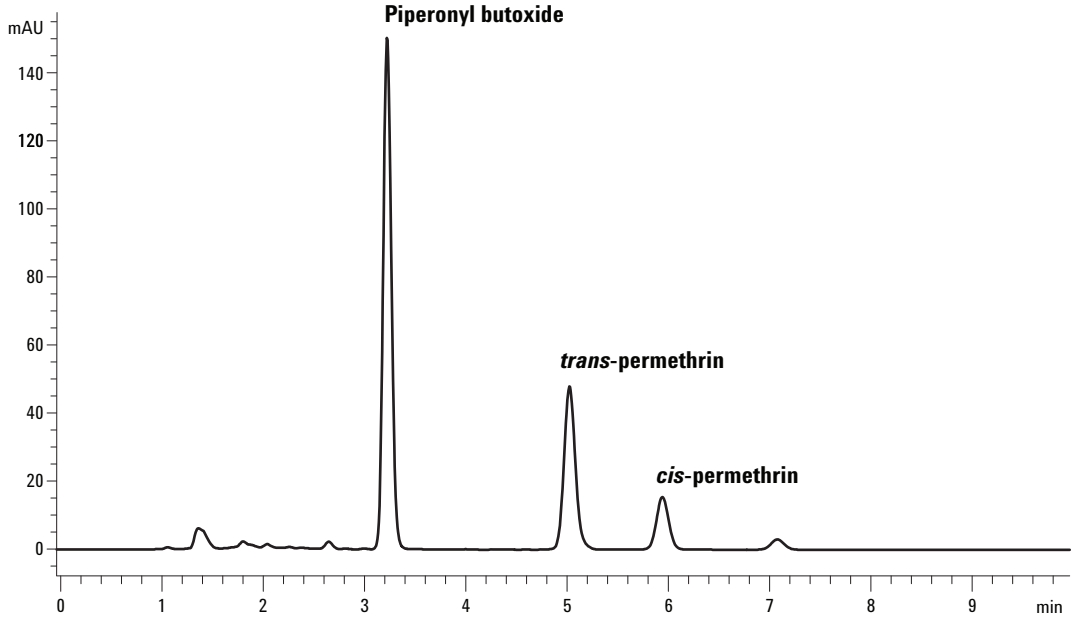


Figure 2. Chromatogram of permethrin and piperonyl butoxide standards.

Table 1. Experimental Conditions

Instrument:	Agilent Series 1100
Column:	ZORBAX Eclipse XDB-C18, 4.6- × 150-mm, 3.5-µm
Mobile phase:	Water-Methanol (10:90 V/V) (G1311A, Quaternary pump)
Flow rate:	1 mL/min
Temperature:	35 °C (G1316A Column Oven)
Detector:	Series 1100 Diode Array Detector (DAD) (G1315A), 272 nm (10): Ref. 360 nm (100)
Injection volume:	20 µL
Standards:	Permethrin = 0.064 mg/mL; PBO = 0.24 mg/mL
Preparation of standards:	Dissolve 40 mg of each permethrin and 120 mg piperonyl butoxide in 100-mL methanol. Dilute 4 mL to 25 mL with methanol.
Preparation of samples:	Dissolve 160 mg of either shampoo or lotion in 25-mL methanol followed by filtration through a 0.45-µm membrane filter.

The HPLC chromatogram of a diluted and filtered sample of lotion containing the permethrin *cis*- and *trans*-diastereomers and the PBO is shown in Figure 3. The chromatogram is rather clean, with only a small additional unknown peak eluting shortly after the void volume (1.4 min). A shampoo extract, shown in Figure 4, revealed the presence of another compound eluting after the PBO, and some enhanced level of lightly retained compounds just after the void volume.

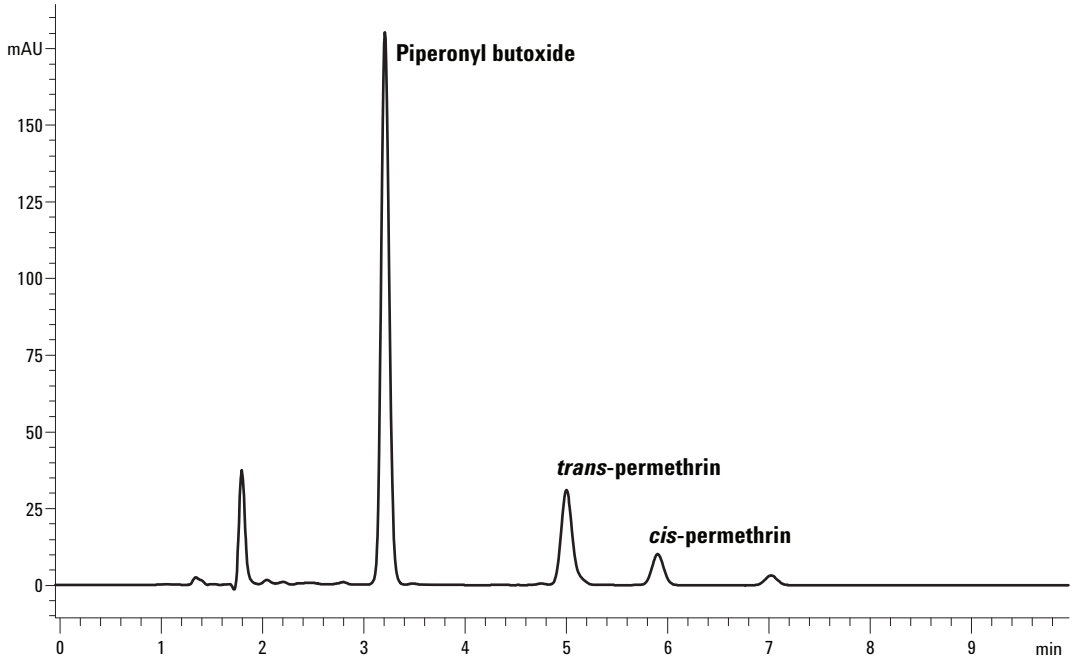


Figure 3. Determination of permethrin and piperonyl butoxide in lotion.

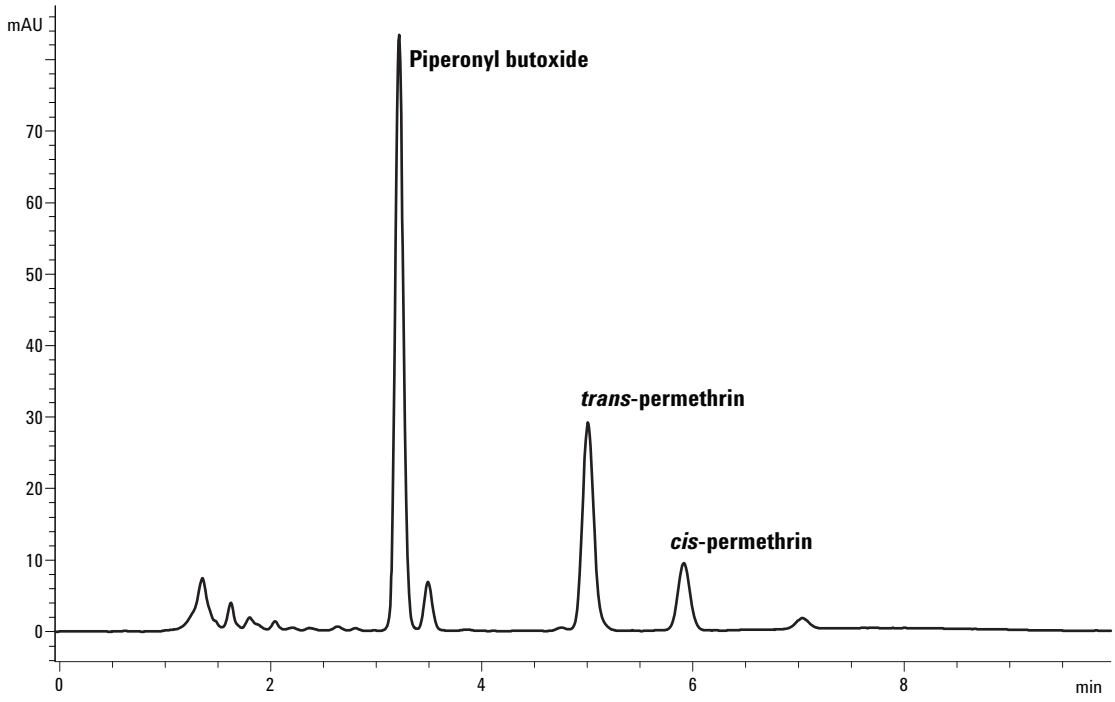


Figure 4. Determination of permethrin and piperonyl butoxide in shampoo.

Conclusions

The separation of synthetic pyrethroids, *cis*- and *trans*-permethrin, and the synergist PBO in shampoo and lotion was easily carried out by dilution, filtration, and injection into a C18 RPC HPLC column. All components could be separated to baseline with symmetrical peaks using a water-methanol isocratic mobile phase.

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Agilent Technologies

Application Note SI-01236

Accurate Analysis of Triterpene Glycosides in Black Cohosh by HPLC with ELSD

Stephen Bullock
Polymer Laboratories, now a part of Varian, Inc.

Introduction

The Varian evaporative light scattering detector is universal and its response is not dependent on the optical properties of the compound. Consequently, its good discriminating power and sensitivity is well suited to compounds such as triterpene glycosides that possess weak or no UV chromophores.

Triterpene glycosides (saponins) are molecules that have a four- or five-ring planar-base containing 30 carbon atoms (aglycone) with various attachments of sugar molecules such as glucose, galactose, glucuronic acid or xylose. The great complexity of triterpene glycoside structures arises from the variability of the aglycone structure, the nature of the side chains and the position of attachment of these sugar moieties. The isolation, analysis and structural determination of triterpene glycosides demands accurate and sophisticated techniques due to their unique chemical nature. The task of isolating these compounds from plant material is further complicated by the presence of many closely related substances in the plant tissue and by the fact that most of the saponins lack a chromophore.

HPLC is typically used as part of the purification process to isolate and identify triterpene glycosides in plant material, in order to achieve high purity extracts. Once the saponins have been purified, analytical techniques such as MS, NMR and infrared spectroscopy are used to elucidate their structure.

Triterpene glycosides occur in a number of plant species, such as black cohosh root (Cimicifuga racemosa (L.) Nutt.), which is taken as a dietary supplement in the belief that it relieves symptoms of the menopause and hot flushes. The primary active constituents of black cohosh root are the triterpene glycosides actein, 27 deoxyactein and cimifugoside, although biologically active substances including alkaloids, flavonoids and tannins are also thought to contribute to the herb's potency.

Instrumentation

Column: C18 5 µm, 150 x 4.6 mm
Detection: Varian ELSD (neb=30 °C, evap=50 °C, gas=1.4 SLM); UV-vis, 230 nm

Materials and Reagents

Eluent A: 0.1 % Formic acid in water
Eluent B: ACN

Sample Preparation

Sample: Black cohosh tablet

Conditions

Flow Rate: 1.0 mL/min
Injection Volume: 20 µL
Gradient: 30-40 % B in 30 min, 40-60 % B in 30 min, 60-30 % B in 10 min

Results and Discussion

Evaporative light scattering detection is a better alternative than UV detection for the analysis of triterpene glycosides because these compounds possess weak or no UV chromophores, thus limiting their sensitivity and the ability to run gradient elution on account of the need to analyze at short UV wavelengths (eg 230 nm).

This is highlighted in Figure 1, which shows the analysis of a black cohosh sample by UV and ELSD. To ensure the potency of black cohosh, commercially available extracts from manufacturer to manufacturer are standardized to contain 2.5 % triterpene glycosides.

However, the determination of two commercial, standardized tablets by ELSD, as shown in Figure 2, reveals differences in the composition and concentration of the active components.

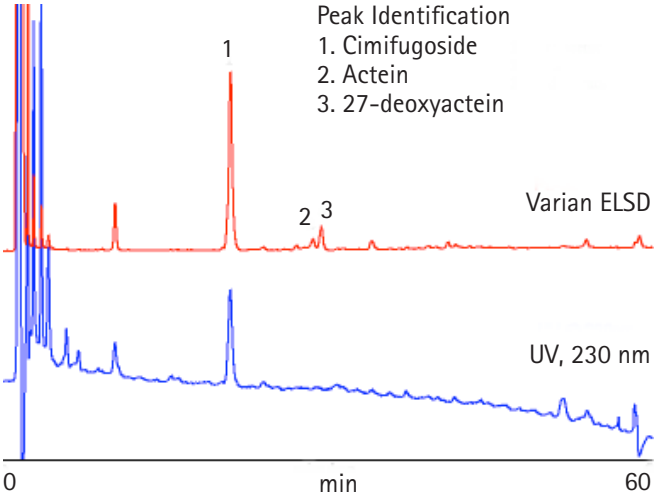


Figure 1. Separation of black cohosh tablets showing the superiority of the Varian evaporative light scattering detector over UV detection.

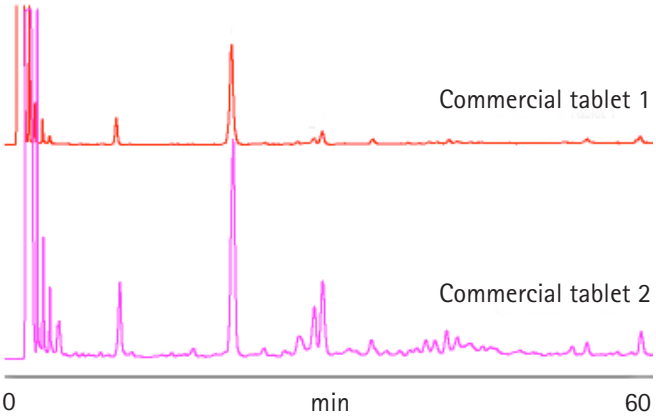


Figure 2. Highlighting the differences in active components in black cohosh tablets from different manufacturers using the Varian evaporative light scattering detector.

Conclusion

There is pressure to establish industry-wide standards to help ensure that dietary supplements are manufactured consistently with regard to their identity, purity, quality, strength and composition. Reliable and accurate analytical techniques are required to meet these objectives. HPLC with Varian evaporative light scattering detection are ideal for the elucidation of active ingredients in dietary supplements, providing important quality control data for industry, regulators and consumers.

These data represent typical results. For further information, contact your local Varian Sales Office.

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Application Note SI-01236 / TB1071

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Analysis of Complex Triglycerides in Starflower Oil from Borage by HPLC using ELSD

Stephen Bullock
Polymer Laboratories, now a part of Varian, Inc.

Introduction

The Varian evaporative light scattering detector is universal and independent on the optical properties of the compound under analysis. Consequently, its good discriminating power and sensitivity is well suited to compounds, such as triglycerides, that possess weak or no UV chromophores.

Starflower oil (borage oil) is extracted from the annual herb, borage (Borago officianalis). The oil is rich in the fatty acid gamma linolenic acid (GLA), which is an important intermediary in the metabolic conversion of linolenic acid into prostaglandin E1 (which modulates the action of many hormones). It is claimed to be beneficial for a wide range of conditions, such as premenstrual syndrome, eczema, rheumatoid arthritis as well as reducing cholesterol and blood pressure. An optimal dose and length of treatment for borage oil has not been established, although manufacturers recommend a dose of approximately 3 g of borage oil per day, providing approximately 700 mg of GLA.

The majority of dietary supplements, such as borage oil, are not subject to review or approval by the US Food and Drug Administration (FDA). As a result, the amounts of active ingredients or contaminants which they contain, may vary between brands or between different batches of the same brand. In addition, few reliable studies into the safety and efficacy of borage oil have been done. Consequently, the side effects of long term exposure to the oil are unknown, and so there is a need for accurate, analytical methods that characterize the composition of borage oil supplements, in order to provide a means of standardizing the quality of the herbal product from batch to batch and between manufacturers.

Borage oil contains a complex mixture of triglycerides that can be analyzed using several methods. Gas chromatography (GC) of methyl ester derivatives and HPLC coupled with refractive index (RI) detection have been reasonably successful for triglyceride analysis.

However, derivatization in GC is problematic for complex mixtures, whereas refractive index lacks sensitivity and is not compatible with gradient elution. HPLC with UV detection is a better choice than refractive index, but due to the low UV wavelengths required to analyze triglycerides the choice of mobile phase solvents is limited, and baseline drift is common with gradient elution. Evaporative light scattering detection with Varian's ELSD is superior to both RI and UV detection.

Instrumentation

Column: C18 5 µm, 250 x 4.6 mm
Detection: Varian ELSD (neb=25 °C, evap=50 °C, gas=1.4 SLM)

Materials and Reagents

Eluent A: ACN
Eluent B: DCM

Sample Preparation

Sample: 2 mg borage oil/mL

Conditions

Flow Rate: 1.0 mL/min
Injection Volume: 20 µL
Gradient: 30-50 % B in 40 min, 50-90 % in 2 min, hold three min

Results and Discussion

The excellent baseline stability of the Varian ELSD is shown in Figure 1.

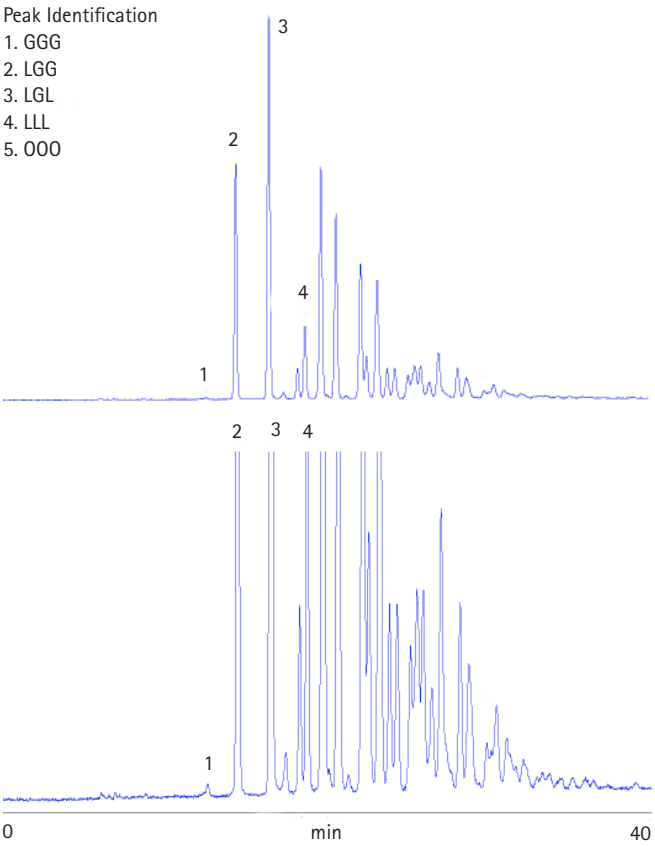


Figure 1. HPLC separation of borage oil using ELS detection (above) (with zoomed baseline (below)) showing the excellent baseline stability of the Varian ELSD.

Table 1. Abbreviations for fatty acids.

Symbol	Compound	No of Carbons
P	Palmitic acid	16
O	Oleic acid (cis-9)	18
L	Linoleic acid (cis, cis-9, 12)	18
Ln	α-Linolenic acid	18
G	γ-Linolenic acid (GLA)	18

Conclusion

The Varian ELSD revealed the true composition of triglycerides in borage oil, due to its sensitivity to compounds that possess weak or no UV chromophores. As the Varian ELSD is universal and independent of the optical properties of the compound, it detects any compound that is less volatile than the mobile phase, For samples, such as borage oil, that possess a complex triglycerides profile, gradient elution is required to provide optimum resolution and reduce run time. This is no problem for the Varian ELSD because it is compatible with a wide range of solvent gradients, displaying excellent baseline stability.

The Varian ELSD surpasses other ELSDs for low temperature HPLC applications with semi-volatile compounds. Its innovative design represents the next generation of ELSD technology, providing optimum performance across a diverse range of HPLC applications. The Varian ELSD's unique gas control permits evaporation of high boiling solvents at very low temperatures. For example, 100 % water at a flow rate of 5 mL/min can be removed at 30 °C. The novel design of the Varian ELSD provides superior performance compared to competitors' detectors for the analysis of semi-volatile compounds.

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Single Run Analysis of St John's Wort Tablets by HPLC with ELSD

Stephen Bullock
Polymer Laboratories, now a part of Varian, Inc.

Introduction

St John's Wort *(Hypericum perforatum L.)* has been used extensively in Europe for the relief of mild neuralgia and to help reduce the effects of depression, mild anxiety and nervous tension. Although St John's Wort has been the focus of a number of studies, doubt remains as to its mode of action. The herb contains a complex mixture of chemicals, including hypericin and hyperforin, which are believed to be the two main active ingredients in St John's Wort. Hyperforin, in particular, has been noted as a potent uptake inhibitor of the neurotransmitters serotonin (5-HT), norepinephrine, dopamine and GABA, which are believed to be responsible for feelings of anxiety, depression and related conditions.

The US FDA classifies St John's Wort as a dietary supplement and, as a result, the herbal product can be sold without requiring studies on dosage, safety or effectiveness. However, work has shown that St John's Wort can interact with certain drugs, and these interactions can be dangerous depending on the dose of the herb. Therefore, the need to monitor and characterize herbal formulations is paramount, because the strength and quality of St John's Wort products are often unpredictable. Products can differ in content not only from brand to brand, but also from batch to batch.

While HPLC has proved an ideal method of separating the complex components within St John's Wort, detection methods such as fluorescence[1] are too specific to reveal all of the components within the formulation. UV[2] detection is less specific but the optical differences between compounds within St John's Wort require multiple wavelength analysis, which requires multiple chromatographic runs.

A better alternative for the detection of St John's Wort by HPLC is to use the Varian evaporative light scattering (ELS) detector, which is universal and not dependent on the optical properties of the compound. Consequently, the instrument detects all of the components within St John's Wort in a single chromatographic run, if the separation has been optimized to separate the individual compounds within the formulation.

The advantanges of the Varian ELS detector are highlighted in the isolation and quantification of hypercin and hyperforin in different herbal formulations.

Instrumentation

Column: C18 5 µm, 150 x 4.6 mm
Detection: Varian ELSD (neb=30 °C, evap=50 °C, gas=1.6 SLM)

Materials and Reagents

Eluent A: 0.1 % Ammonium formate, adjusted to pH 2.5
Eluent B: Acetonitrile

Sample Preparation

Sample: St John's Wort tablets

Conditions

Flow Rate: 1.0 mL/min
Injection Volume: 20 µL
Gradient: 50-95 % B in 10 min, hold 5 min

Results and Discussion

The chromatographic profile varies at different evaporator temperatures due to the loss of semi-volatile components within the St John's Wort formulation. For the analysis of the active ingredients, hypericin and hyperforin, 50 °C is the optimum temperature. However, some of the minor peaks show a reduction in response (Figure 1).

The Varian ELS detector can be used to compare the composition of St John's Wort tablets from different manufacturers, as shown in Figure 2.

These chromatograms show the differences in composition between standardized St John's Wort tablets from different manufacturers. The ratio of hypericin to hyperforin varies between the tablets, as well as the number and concentration of minor peaks. This shows how the Varian ELS detector can provide a fast, cost effective method for characterizing St John's Wort extract.

Peak Identification
1. Hypericin
2. Hyperforin
3. Adhyperforin

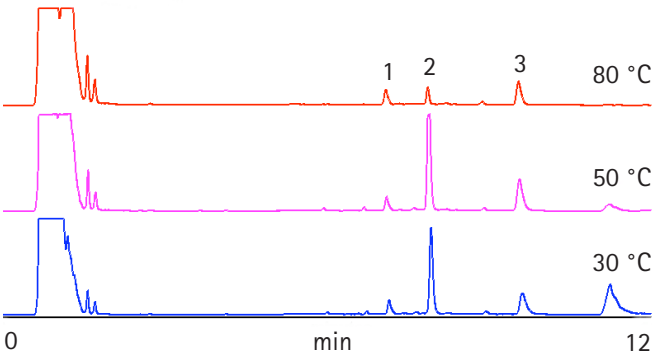


Figure 1. Chromatographic profile varies at different evaporator temperatures and some of the minor peaks show a reduction in response.

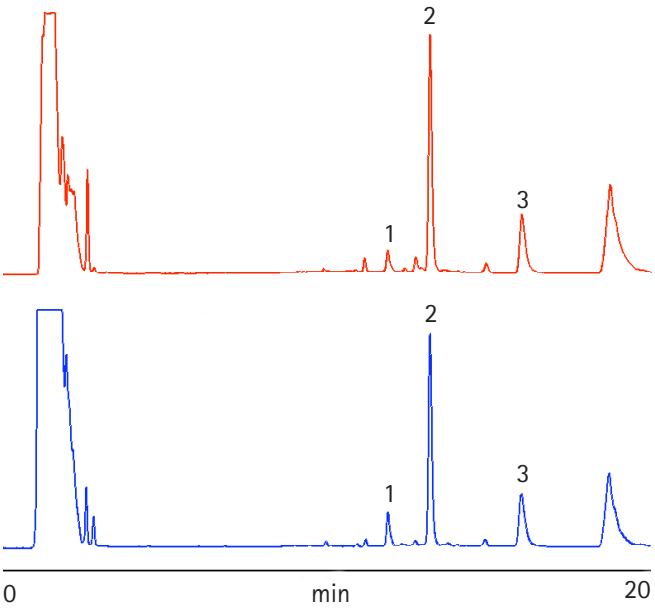


Figure 2. Differences in composition of St John's Wort tablets from two different manufacturers revealed by Varian ELSD.

Conclusion

ELSD is capable of detecting any compound that is less volatile than the mobile phase. The Varian ELS detector can evaporate highly aqueous eluents at 25 °C, facilitating the detection of semi-volatile compounds. For complex mixtures, such as St John's Wort, which contain compounds of differing volatilities, operating the Varian ELS detector at low temperature offers several advantages over standard detction methods.

References

[1]Draves, A. and Walker, S. 2000. Determination of hypericin and pseudohypericin in pharmaceutical preparations by HPLC with fluorescence detection. J. Chromatogr. B, 749: 57-66

[2]Ang, C.Y. et al. 2002. Determination of St John's Wort components in dietary supplements and functional foods by liquid chromatography. J. AOAC Int., 85: 1360-1369

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Application Note SI-01228 / TB1063

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Excellent Separation of Ginseng Root Constituents by HPLC with ELSD

Stephen Bullock
Polymer Laboratories, now a part of Varian, Inc.

Introduction

The root of the ginseng plant (Panax quinquefolium L.) has been used in Chinese medicine for thousands of years, due to its many reported health benefits. Ginseng is said to help people with low and high blood pressure, energizes those suffering from fatigue and helps calm people prone to stress or nervousness. It also has a reputation for improving mental clarity and memory, enhancing physical stamina and bolstering the immune system. The biologically active constituents of ginseng are complex mixtures of triterpene saponins, known as ginsenosides. At least 30 ginsenosides have been isolated and characterized, with R_{g1}, R_{b1}, R_{b2}, R_c and R_d being the most important in ginseng root. Many different techniques have been employed to identify and quantify ginsenosides, such as TLC, colorimetry, GC and HPLC. HPLC-UV methods provide excellent separation of ginsenosides. However, ginsenosides possess poor UV chromophores, which limit their sensitivity and the ability to run gradient elution owing to the need to analyze at short wavelengths. The Varian evaporative light scattering (ELS) detector can recognize any compound less volatile than the mobile phase, regardless of its optical properties. It is for this reason that the ELS detector is often referred to as 'universal'.

Instrumentation

Column: C8 5 µm, 300 x 7.7 mm
Detection: Varian ELSD (neb=50 °C, evap=70 °C, gas=1.4 SLM)

Materials and Reagents

Eluent A: Water
Eluent B: Acetonitrile

Conditions

Gradient: 20-60 % B in 30 min
Flow Rate: 0.8 mL/min
Injection Volume: 20 µL

Results and Discussion

The benefits of evaporative light scattering detection with the Varian ELSD is apparent in the analysis of ginseng root powder (Figure 1) and two of its major ginsenosides, R_{g1} and R_{b1} (Figure 2). Since the Varian ELSD evaporates all mobile phase solvents before detection, it shows no response to change in solvent composition during gradient elution.

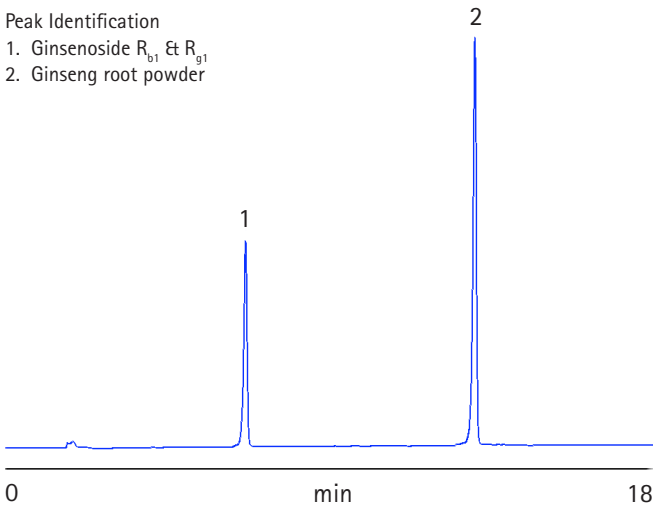


Figure 1. Ginseng root powder separation with the Varian ELSD.

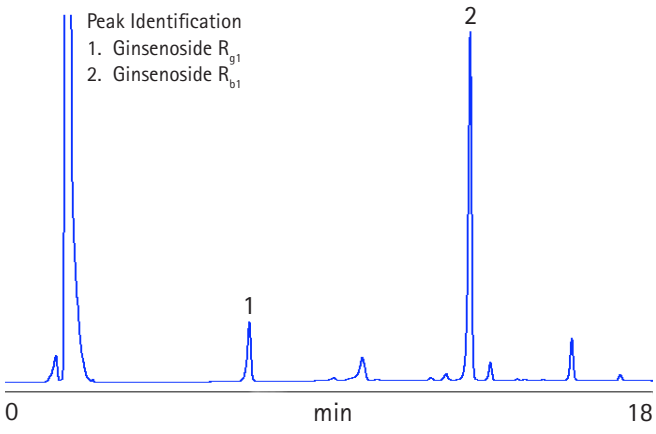


Figure 2. Good discrimination of R_{g1} and R_{b1} ginsenosides by the Varian ELSD.

Conclusion

Separation and identification of ginseng root constituents is straightforward using evaporative light scattering detection at the ambient temperatures available with the Varian ELSD. The Varian ELSD surpasses other ELSDs for low temperature HPLC applications with semi-volatile compounds. Its innovative design represents the next generation of ELSD technology, providing optimum performance across a diverse range of HPLC applications. The Varian ELSD's unique gas control permits evaporation of high boiling solvents at very low temperatures. For example, 100 % water at a flow rate of 5 mL/min can be removed at 30 °C. The novel design of the Varian ELSD provides superior performance compared to detectors from other vendors for the analysis of semi-volatile compounds.

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Application Note SI-01219 / TB1053

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Application Note SI-01215

Determination of the Limit of Detection for Caffeine by HPLC with ELSD

Stephen Bullock
Polymer Laboratories, now a part of Varian, Inc.

Introduction

Caffeine is widely used in the food and drink industry as well as being incorporated into some nutraceutical formulations. HPLC is often employed in assays for the quantification of caffeine. In this particular example, a gradient HPLC method was used to analyze caffeine at low concentrations to determine the on-column limit of detection (LOD). The Varian evaporative light scattering (ELS) detector was employed because of its superior performance, as the detection method is independent of the optical properties of the compound under consideration.

Instrumentation

Column: C8 3 µm, 50 x 4.6 mm
Detector: Varian ELSD (neb=50 °C, evap=50 °C, gas=1.4 SLM)

Materials and Reagents

Eluent A: 5 mM Ammonium acetate in Water
Eluent B: 5 mM Ammonium acetate in Acetonitrile

Conditions

Gradient: 10-100 % B in 5 min, 1 min hold at 100 % B
Flow Rate: 1.0 mL/min
Injection Volume: 10 µL

Results and Discussion

In this case, the LOD is defined as a peak height of greater than three times the baseline noise. The chromatograms in Figure 1 illustrate the signal to noise ratio (S/N) obtained for injections of 97 ng and 19 ng on column. The 19 ng loading gave a S/N = 15 and so, for this application, the LOD is well below 19 ng on-column.

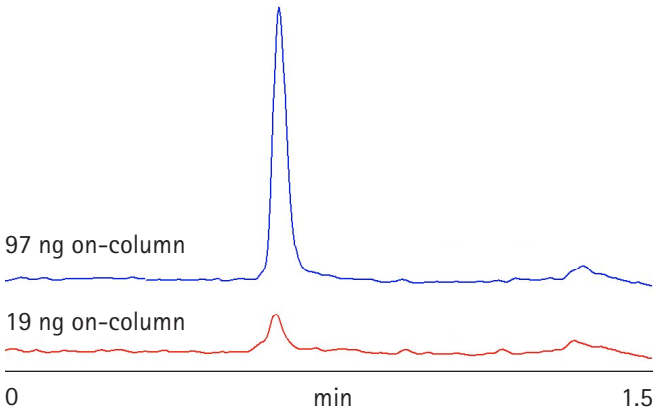


Figure 1. Very low signal to noise ratios achieved by Varian ELSD.

Conclusion

The Varian ELS detector successfully revealed an LOD for caffeine below 19 ng on-column. The Varian ELS detector surpasses other ELSDs for low temperature HPLC applications with semi-volatile compounds. Its innovative design represents the next generation of ELSD technology, providing optimum performance across a diverse range of HPLC applications. The Varian ELS detector's unique gas control permits evaporation of high boiling solvents at very low temperatures. For example, 100 % water at a flow rate of 5 mL/min can be removed at 30 °C. The novel design of the Varian ELS detector provides superior performance compared to detectors from other vendors for the analysis of semi-volatile compounds.

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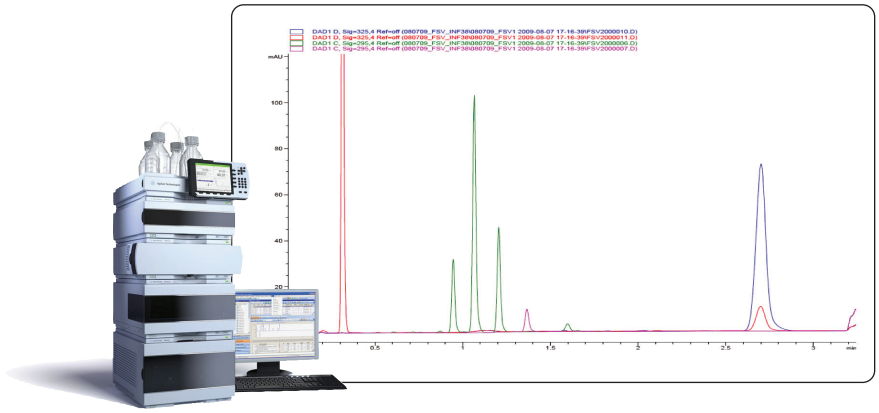
Fast analysis of fat soluble vitamins using the Agilent 1290 Infinity LC and ZORBAX RRHT and RRHD 1.8 µm columns

Application Note

Food Analysis

Author

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USA



Abstract

The Agilent 1290 Infinity LC has significant capabilities for a wide range of HPLC and UHPLC applications. With a broader power range (that is, the combination of pressure and flow capabilities) than any other commercially available system, and the flexibility to operate a wide range of column dimensions and particle sizes, it is extremely useful for method transfer from any HPLC or UHPLC to the 1290 Infinity LC system. It allows the user to access capabilities not otherwise available.

Introduction

The speed and high resolution are demonstrated by a separation of fat-soluble vitamin isomers and esters, at a high pressure and flow rate. At 2 mL/min, utilizing a simple 1-min gradient and a 3.0 x 50 mm, 1.8 µm column, the analysis time is only 3 min including the late eluting retinyl palmitate component. The separation of the main components is shown in Figure 1.

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The speed, resolution and flexibility of the system are further demonstrated by a separation of vitamins D2 and D3. At 2 mL/min, utilizing a simple isocratic condition and a 3.0 mm × 150 mm, 1.8 µm column, the analysis time is only 3 min. The separation of the main components, at three column temperatures including sub-ambient, is shown in Figure 2. Sub-ambient column temperature control, a standard feature of the Agilent Thermostatted Column Compartment, has significant advantages for many difficult isomer separations, including enantiomeric separations, and for shape-selective separations such as polycyclic aromatic hydrocarbons.

Configuration

- G4220A 1290 Infinity Binary Pump with Integrated Vacuum Degasser
- G4226A 1290 Infinity Autosampler
- G1316C 1290 Infinity Thermostatted Column Compartment
- G4212A 1290 Infinity Diode Array Detector

Conclusion

Taking advantage of the combined high flow and high pressure capability of the system allows one to use high efficiency 3 mm id columns (having up to 40% higher efficiency than comparable 2.1 mm id columns) to produce rapid separations with remarkable resolution while conserving solvent over the use of 4.6 mm id columns.

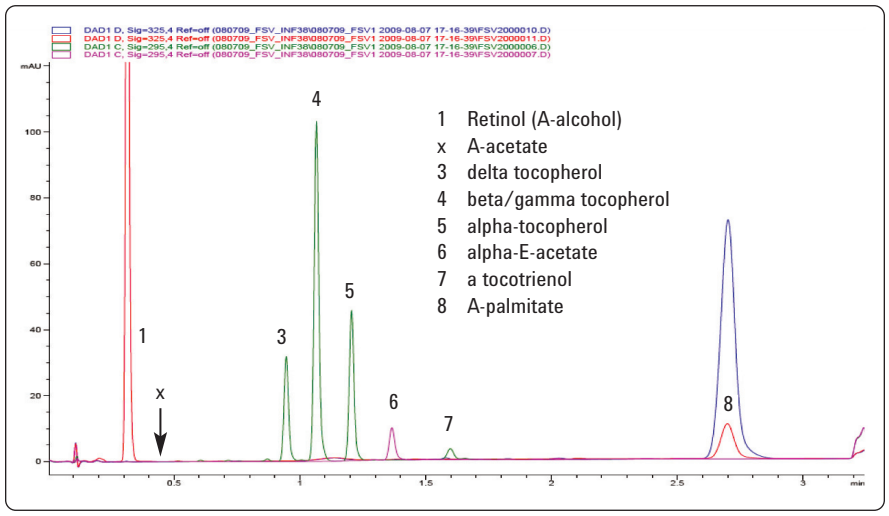


Figure 1
Analysis of important vitamins A and E components on the 1290 Infinity LC. Sample: solution of alcohols and esters of retinol and tocopherol. Conditions: 2.0 mL/min, 90% to 100% ACN at 1 min, hold to 3, run 4 min, ZORBAX RRHT StableBond C18, 3 mm × 50 mm, 1.8 µm, 45 °C.

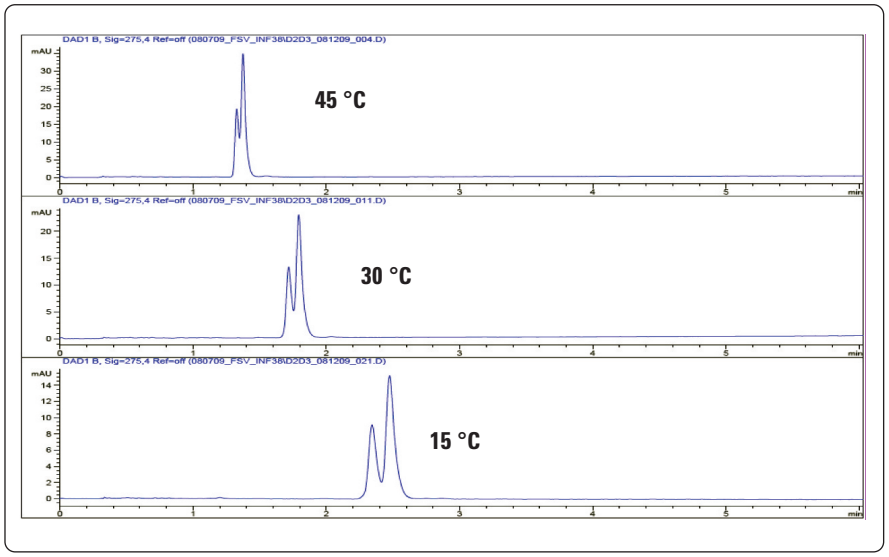


Figure 2
Analysis of vitamins D2 and D3 (order of elution) on the 1290 Infinity LC. Sample: standard mix (Sigma-Aldrich). Conditions: 2.0 mL/min, 75/25 ACN/MeOH isocratic, 280 nm UV ZORBAX RRHD StableBond C18, 3 mm × 150 mm, 1.8 µm, 45 °C, 30 °C and 15 °C.

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Agilent SampliQ OPT Solid Phase Extraction Sorbent in the Clean-up of Alkaloids in Goldenseal by HPLC-DAD

Application Note

Pharmaceutical

Authors

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Abstract

Sample clean-up of alkaloids of Goldenseal commercial products (hydrastine and berberine) was achieved by solid phase extraction employing Agilent's SampliQ Optimized Polymer Technology (OPT) sorbents. Separation of the products with a 0.1% phosphoric acid/methanol mobile phase was carried out on an Agilent 1200 series HPLC coupled with a diode array detector (DAD) on an Agilent ZORBAX Eclipse Plus C18 column (4.6 mm × 75 mm × 3.5 µm) using gradient elution with a 6 min total run time. The recovery for hydrastine ranged from 101% to 106% (n = 8) while that of berberine ranged from 71% to 82%, (n = 8), each with % relative standard deviation (RSD) of less than 1. The limits of detection and quantification for hydrastine were 0.50 and 1.65 µg/mL respectively while those of berberine were 0.47 and 1.55 µg/mL, respectively. Goldenseal sample from Willow contained 17 µg/mL hydrastine and 35 µg/mL berberine while Goldenseal sample from Solga contained 6 µg/mL hydrastine and 12 µg/mL berberine.

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Introduction

Goldenseal (*Hydrastis canadensis* L.) is a perennial herb in the Ranunculaceae family native to southeastern Canada and northeastern United States of America (USA) and is amongst the oldest herbal medicinal plants, most commonly employed in the Traditional Chinese Medicines (TCM) [1, 2]. The biological activities of Goldenseal are associated with the isoquinoline alkaloids hydrastine and berberine (see structures in Figure 1) even though the plant also contains other alkaloids including hydrastinine, tetrahydroberberine and canadine [2]. Goldenseal has been used for the treatment of infections, inflammation and as an immune system booster [3]. However, due to the imminence of new directives and legislation intended to regulate both the herbal and nutraceutical industries, there is a growing need for robust and highly sensitive analytical methods involving sample handling, which includes sampling, clean up and pre-concentration.

Sample handling is considered to be a fundamental step in the analytical procedure because it helps to achieve the low detection limits set by regulatory authorities [4]. Solid phase extraction (SPE) is one of the most popular sample clean up techniques used in sample handling prior to analysis of environmental, food, pharmaceutical, and biological samples by high-performance liquid chromatography (HPLC) or gas chromatography (GC). SPE has many advantages over traditional liquid-liquid extraction, such as the use of minimal amounts of organic solvent, ease of automation, lower cost, and reduced volumes of toxic residues [5]. In recent years, many reports have described the development of new SPE materials, for example mixed-mode sorbents as well as restricted access sorbents, immunoaffinity extraction sorbents, molecularly imprinted polymers, and conductive polymers [6, 7, 8].

This application note presents a method that has been optimized for SPE of hydrastine and berberine in Goldenseal employing Agilent SampliQ Optimized Polymer Technology (OPT) cartridges, which utilize polymeric sorbents, with significant reduction of matrix interferences, resulting in improved analysis.

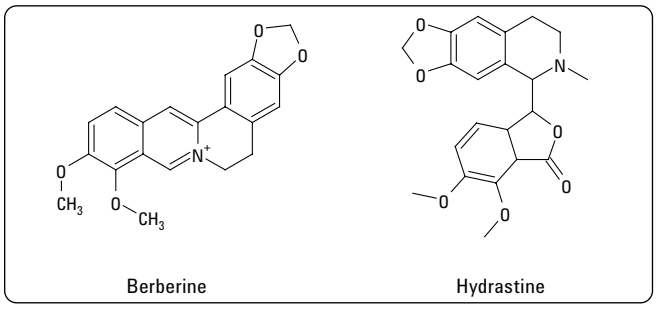


Figure 1. Structures of hydrastine and berberine.

Experimental

Materials and Chemicals

Berberine hydrochloride and hydrastine hydrochloride standards were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Phosphoric acid and potassium hydroxide were purchased from Merck Chemicals (Gauteng, South Africa) while HPLC grade methanol was purchased from Merck KGaA (Darmstadt, Germany). Potassium dihydrogen phosphate was from Saarchem Analytic (Krugersdorp, South Africa). Goldenseal capsules (Golden Seal Capsules, Willow Products, Port Elizabeth, South Africa and Solga Full Potency Herbs, Solgar Corporation, Leonia, NJ, USA) were purchased from a local herbal store in Grahamstown, South Africa. SPE cartridges were Agilent SampliQ OPT, 1 mL/30 mg tubes. Analysis was performed on an Agilent 1200 series gradient HPLC coupled with a diode array detector (DAD). The analytical column was an Agilent ZORBAX Eclipse Plus C18 column (4.6 mm × 75 mm × 3.5 µm).

Preparation of Stock and Working Solutions

The stock solution of hydrastine and berberine, 1000 µg/mL each, were prepared in methanol and stored at 4 °C when not used. All other standard solutions were prepared from the stock solution as required.

Sample Preparation

The contents of the capsules were first homogenized. Then, 200 mg of the homogenized sample were mixed with 50 mL of methanol and then stirred with a magnetic stirrer for 1 h, resulting in a suspension with undissolved particulates floating in it. The extracts were then filtered using a hydrophobic polyvinlyidene fluoride (PVDF) 0.45 µm Millipore Millex – HV membrane filter (Billerica, MA, USA). The methanolic extracts were diluted 1:3 with water and the pH adjusted to ~ 7 with 0.01 M potassium hydroxide.

Separation

A 5 µL aliquot of a hydrastine-berberine mixed standard (50 µg/mL of each) was injected into the HPLC column to optimize their separation. The separation of both alkaloids was monitored at 242 nm while wavelengths 294 and 350 nm were used for quantifying berberine and hydrastine, respectively. The HPLC conditions are as outlined in Table 1 below:

Table 1. HPLC Conditions

Column	Agilent ZORBAX Eclipse Rapid Resolution Plus C18, column, 4.6 mm × 75 mm, 3.5 µm (p/n 959933-902)			
Flow rate	1 mL/min			
Injection volume	5 µL			
Column temperature	35 °C			
Mobile phase	A: 0.1% Phosphoric acid B: Methanol			
Run time	6 min			
Post time	2 min			
Detection	UV 294 nm (berberine), 350 nm (hydrastine), 242 nm (for monitoring both)			
Gradient	Time	0	0.5	5
	%B	20	20	50

SPE Procedure

A systematic study of a series of conditioning, loading, washing and elution solvents was performed. The procedure was optimized by evaluating the isolation of hydrastine and berberine from a standard solution. Figure 2 shows the results of the optimization process.

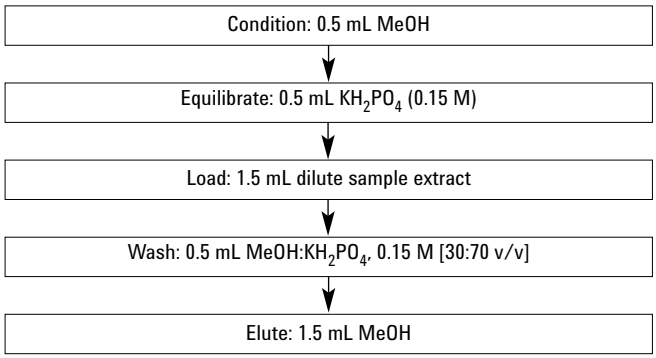


Figure 2. SPE procedure for cleaning alkaloids in Goldenseal using SampliQ OPT sorbent.

Results and Discussion

Separation and SPE Clean Up

Although the hydrastine and berberine standards could be separated isocratically, the initial analysis time was rather long (15 min) so gradient elution was essential to reduce the run time. Using the optimized HPLC conditions outlined in Table 1, Figure 3 shows the well-separated symmetrical peaks for the hydrastine and berberine standards in just over 4.5 minutes. A blank carried through the entire procedure showed no discernible peaks in the baseline. Next, a simple filtered extract from the Willow Goldenseal sample was injected prior to (Figure 4a) and after sample cleanup using the SPE procedure (Figure 4b). Note the decrease in the number of small peaks in Figure 4b indicating that the SPE treatment removed a number of potentially interfering species. A second sample of Goldenseal from Willow was spiked and treated in a similar manner to confirm the peak assignments. The results in Figure 5a and 5b showed that the peak intensity was increased and interfering peaks were significantly reduced.

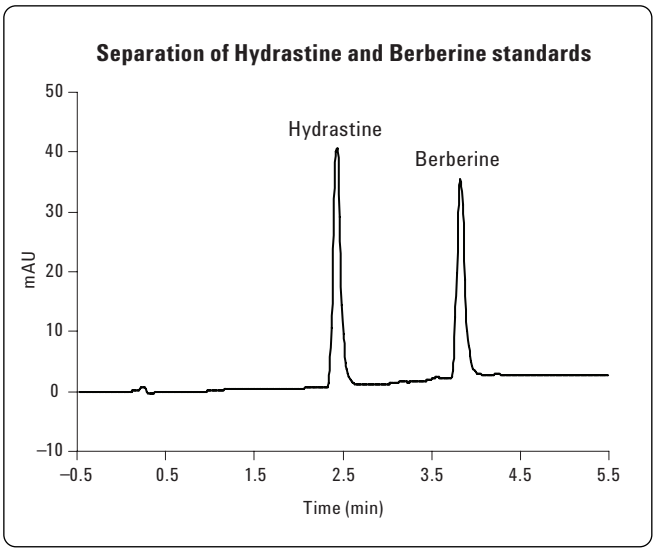


Figure 3. Chromatogram of hydrastine (120 µg/mL) and berberine (100 µg/mL) standard mix.

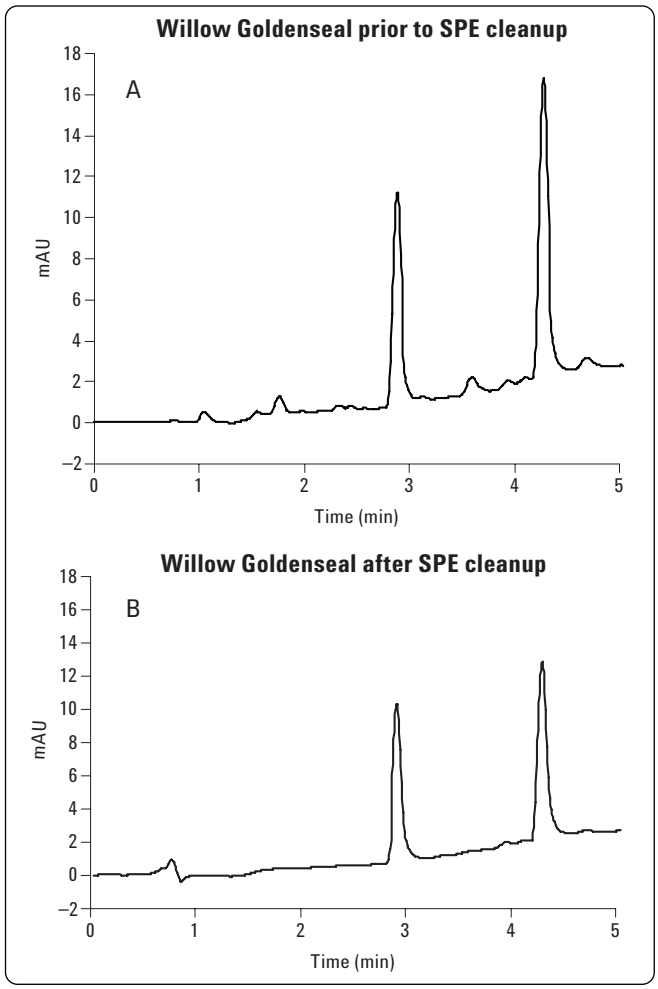


Figure 4. Chromatograms of Goldenseal samples before (a) and after (b) clean up.

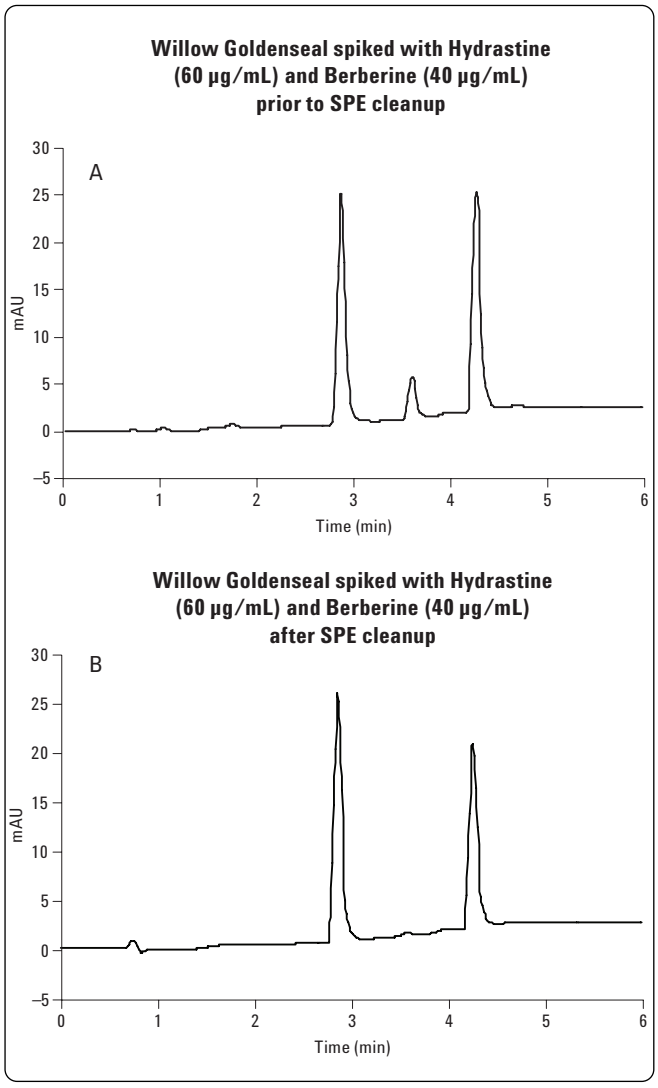


Figure 5. Chromatograms of Goldenseal Willow spiked samples (hydrastine and berberine, 60 and 40 µg/mL, respectively) before (a) and after (b) clean-up with SampliQ OPT sorbent.

Recovery and Reproducibility

The recoveries and reproducibility of berberine and hydrastine were evaluated by analyzing eight replicates of the commercial sample (Goldenseal Willow capsules) that were spiked at three different concentration levels of hydrastine and berberine within a day and then introduced to the SPE procedure. The background level of the spiked samples was determined for each concentration before clean up. Recovery was calculated by comparison of peak areas of unclean to those of the cleaned extracts. The recovery and reproducibility values for berberine and hydrastine are as outlined in the Table 2 below. The % RSDs were all less than 1, quite acceptable for an SPE cleanup and HPLC analysis procedure.

Table 2. Recovery and Reproducibility Data for the Two Alkaloids Standards

Compound	Level spiked (µg/mL) n=8	% Recovery	% RSD
Hydrastine	40	101	0.16
	60	102	0.56
	100	106	0.75
Berberine	10	71	0.17
	40	71	0.40
	80	82	0.32

Calibration Curves

The calibration curves were determined by preparing appropriate concentrations in methanol from berberine and hydrastine stock solutions and injecting directly into the HPLC column without SPE procedure. The method was found to be linear in the concentration ranges of 0–120 µg/mL for hydrastine and 0–100 µg/mL of berberine each with r^2 of 0.9994.

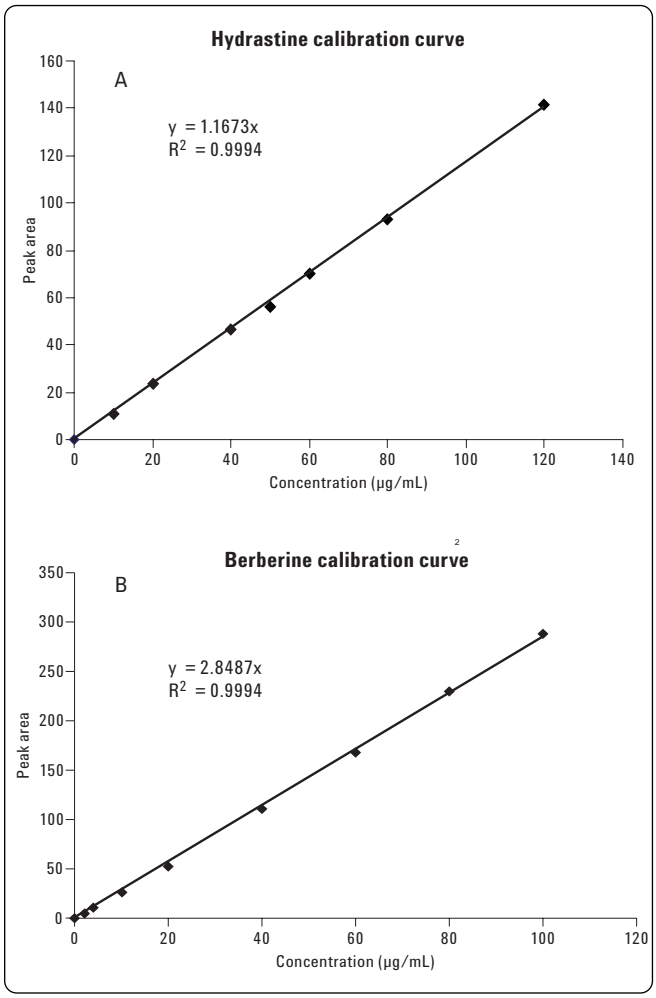


Figure 6. Calibration curves of (a) hydrastine and (b) berberine.

Linearity of the SPE Method

Linearity was studied on the SampliQ OPT sorbent by spiking sample extracts with increasing concentrations of hydrastine and berberine followed by SPE clean-up. At concentrations higher than 200 µg/mL for berberine, linearity was no longer observed (Figure 7). This is due to the fact that the SPE sorbent was overloaded and could no longer retain the alkaloid berberine. Most of the sample was lost at the washing step while some was lost even at the loading stage. For hydrastine, SampliQ OPT sorbent showed linearity for up to 500 µg/mL.

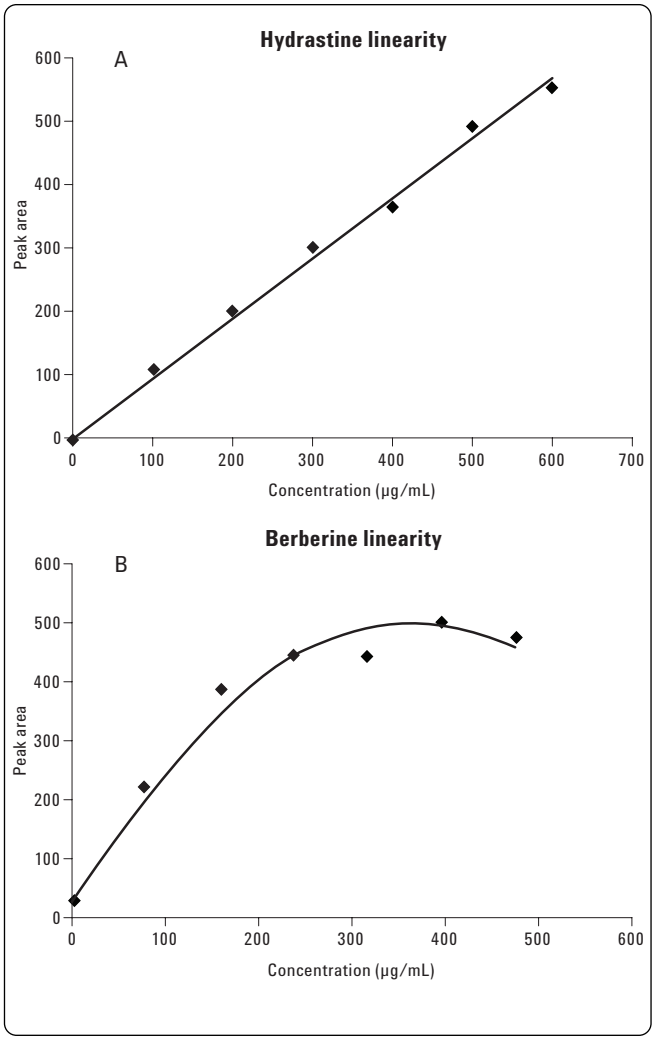


Figure 7. Linearity of (a) hydrastine and (b) berberine at higher concentrations.

Analysis of the Commercial Product

The method described was successfully applied to the analysis of the commercial products, Goldenseal capsules from Willow and Solga. Both products contained low but quantifiable amounts of the alkaloids as indicated in Table 3.

Table 3. Concentrations of hydrastine and berberine in Commercial Samples

	Concentration (µg/mL)	
	Hydrastine	Berberine
Willow	17	34
Solga	6	12

Limit of Detection and Limit of Quantification

The limits of detection were calculated using the intercept, y_B, and the standard error of the regression line, s_B at 3 times the standard error and LOD values were calculated using equations 1 and 2 [9,10].

$$y_{LOD} = y_B + 3S_B \quad \text{(Eqn 1)}$$

$$LOD = (y_{LOD} - y_B)/m \quad \text{where m = gradient} \quad \text{(Eqn 2)}$$

LOQ values were calculated using the same method as in equations 1 and 2, but using 10 times the standard error of regression line, (equations 3 and 4).

$$y_{LOQ} = y_B + 10S_B \quad \text{(Eqn 3)}$$

$$LOQ = (y_{LOQ} - y_B)/m \quad \text{(Eqn 4)}$$

The limit of detection and quantification for hydrastine were found to be 0.50 and 1.65 µg/mL, respectively, while that of berberine was 0.47 and 1.55 µg/mL, respectively.

Conclusions

Agilent SampliQ OPT cartridges achieved effective sample clean up of the TCM Goldenseal for the separation and analysis of hydrastine and berberine. The results demonstrated that the method was reproducible and reliable with good recoveries (101% to106% for hydrastine and 71% to 86% for berberine) at n = 8 and RSD less than 1%. The limits of detection and quantification for hydrastine were 0.50 and 1.65 µg/mL respectively while those of berberine were 0.47 and 1.55 µg/mL respectively. Goldenseal sample from Willow contained 17 µg/mL hydrastine and 35 µg/mL berberine while Goldenseal sample from Solga contained 6 µg/mL hydrastine and 12 µg/mL berberine.

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Extraction and HPLC Analysis of Alkaloids in Goldenseal

Application

Consumer Products and Drug Manufacturing/QA/QC

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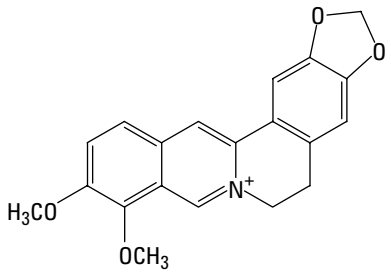
Abstract

An ambient extraction of goldenseal root powder followed by HPLC analysis of the alkaloids on a Zorbax Rapid Resolution Eclipse XDB-C18 column provides an accurate method for the determination of key alkaloids in goldenseal, including berberine and hydrastine. The extraction and HPLC analysis can be applied to several other alkaloids, including canadine, hydrastinine, and palmatine, and may be applicable to other berberine-containing plant roots. The Rapid Resolution Eclipse XDB-C18 column is used for an isocratic separation with high resolution of all components in under 15 minutes.

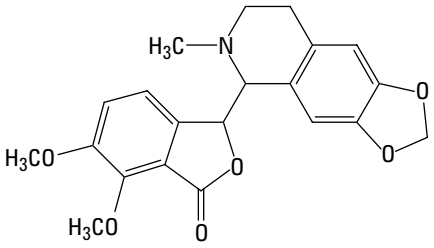
Introduction

Goldenseal, *Hydrastis canadensis* L., is one of the oldest herbal medicinal plants and is of current interest as a natural medicine. There are two alkaloids present in goldenseal that are the expected active components, berberine and hydrastine. Canadine, hydrastinine, berberastine, and canadaline are minor alkaoloids. Palmatine, which is closely related to berberine, is not found in *H. canadensis*, but is found in *Coptis*, another berberine-containing plant [1].

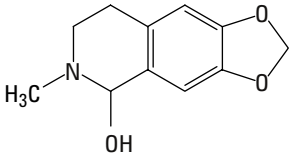
Goldenseal has been used as an anti-inflammatory and antibiotic. It has also been used to treat nasal congestion, cold, flu, and a variety of intestinal disorders. The whole root of the plant is used and is currently available in bulk (dried or powdered roots), tablets, capsules, and tinctures. Goldenseal plants have been overharvested and many are now grown on farms for use as herbal supplements. Inconsistent quantities of alkaloids are present in the products sold as herbal supplements. A simple process for extracting and analyzing the alkaloids is highly desirable to evaluate product quality. Figure 1 shows the structures of the alkaloids in goldenseal.



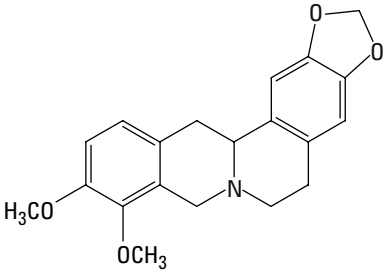
Berberine



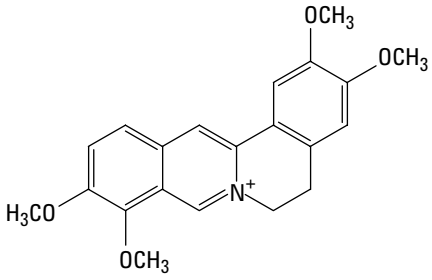
Hydrastine



Hydrastinine



Canadine



Palmatine

Figure 1. Structures of key alkaloids in goldenseal and related plants.

Experimental

Extraction Procedure

Literature reports by Betz and Anderson [1] and Burney [2] indicate that ambient extraction of alkaloids from *H. canadensis* is possible. This is followed by an HPLC determination for accurate quantitation of the alkaloid extracts. The optimized ambient extraction conditions used for the analysis of neat goldenseal root powder are summarized in the steps below.

1. Weigh ~ 0.5 g of root powder
2. Mix with 100 mL of acetonitrile:water:H_3PO_4 (70:30:0.1, v/v/v)
3. Sonicate 5 min, shake (wrist-action shaker) 10 min, centrifuge 5 min
4. Dilute extracts 1/5
5. Direct HPLC analysis of diluted extracts

HPLC Analysis

The HPLC analysis of alkaloids present in goldenseal needs to resolve the major alkaloids. In addition, it is desirable to resolve palmatine, because it is present in other berberine-containing plants. An HPLC method was developed to resolve all of these components. Optimum resolution and peak shape were obtained using the Zorbax Eclipse XDB-C18 column with an ammonium acetate buffer and acetonitrile. The separation is shown in Figure 2 with a complete list of the optimized conditions used. For consistent retention times, temperature control was required at 30°C [3].

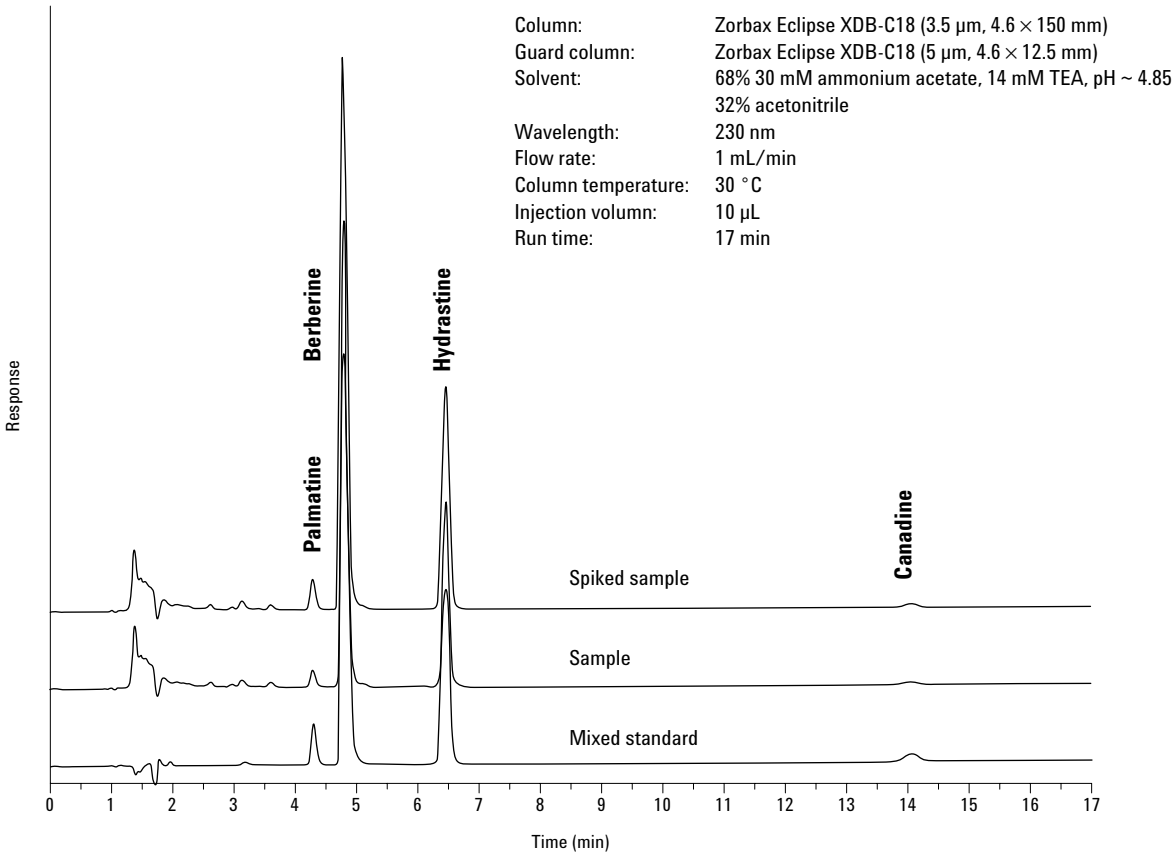


Figure 2. HPLC separation of goldenseal extract on Eclipse XDB-C18 column.

Method Validation

This HPLC method was applied in the validation of the ambient extraction method. Linearity, precision, and alkaloid recovery were investigated. Table 1 shows these results. The precision of the method is excellent, and recoveries of the different alkaloids ranged from 92%–102%, excellent for a quantitative method. The linearity was very good (Figure 3) and sensitivity was good. From the standard data, the calculated LOD (limit of detection) for berberine was 0.50 mg/mL and the LOQ (limit of quantitation) was 1.65 mg/mL, so that accurate quantitation down to low levels is possible, making it easy to test the quality of different goldenseal products.

Table 1. Validation Results of the Ambient Extraction Method

	Palmatine	Berberine	Hydrastine	Canadine
Precision (n = 10)	0.18 ±0.002(s)%	3.06 ±0.05(s)%	2.04 ±0.01(s)%	0.08 ±0.001(s)%
Alkaloid recovery (~ 0.3–2 g of GS) (~ 0.6–1 mg/mL) (n = 12)	0.18 ±0.003(s)	3.10 ±0.06(s)%	2.05 ±0.02(s)%	0.08 ±0.001(s)%
Spike and recovery (n = 3)	Spike level = ~ 0.15% 92.2 ±5.5(s)%	Spike level = ~ 2.0% 101.5 ±0.2(s)%	Spike level = ~ 2.0% 101.9 ±0.2(s)%	Spike level = ~ 0.10% 101.9 ±7.9(s)%

(s) = standard deviation

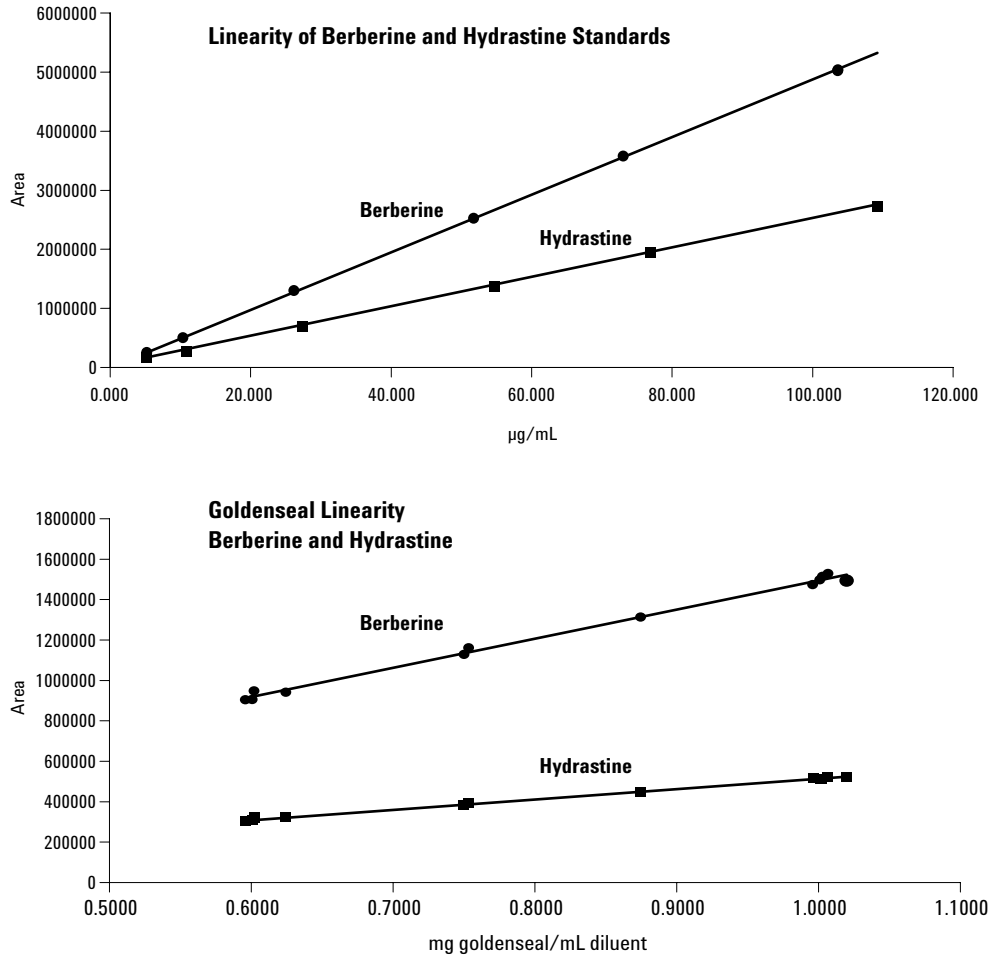


Figure 3. Linearity of berberine and hydrastine as standards and from goldenseal extracts.

Results

Goldenseal Testing

The ambient extraction and HPLC analysis method were applied to six lots of goldenseal root powder from three different vendors to determine the alkaloid content. The results are summarized in Table 2. These results demonstrate variability among vendors and the reason a good quantitative HPLC method is desirable.

Table 2. Results of Testing Goldenseal Root Powder from Multiple Lots and Multiple Vendors with the Ambient Extraction Method and HPLC

Vendor	Lot number	% Palmatine	% Berberine	% Hydrastine	% Canadine	Total weight % of known alkaloids
1	A	nd	3.27	2.36	0.09	5.94
	B	nd	3.29	2.40	0.07	5.98
2	C	0.19	3.01	1.99	0.09	5.53
	D	0.18	3.06	2.04	0.08	5.36
3	E	nd	4.60	4.06	0.12	8.99
	F	nd	3.93	2.67	0.20	6.93

Conclusions

This ambient extraction method of goldenseal is simple and reliable. This is followed by an isocratic HPLC analysis with a Zorbax Rapid Resolution Eclipse XDB-C18 column, which provides high resolution and excellent peak shape of six alkaloids in 15 minutes. This analysis provides reliable quantitative results of the alkaloids in goldenseal, including berberine and hydrastine. The method was applied to goldenseal from three different vendors and may be applicable to other berberine-containing plants.

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Separation of Hypericin in St. John's Wort

Application

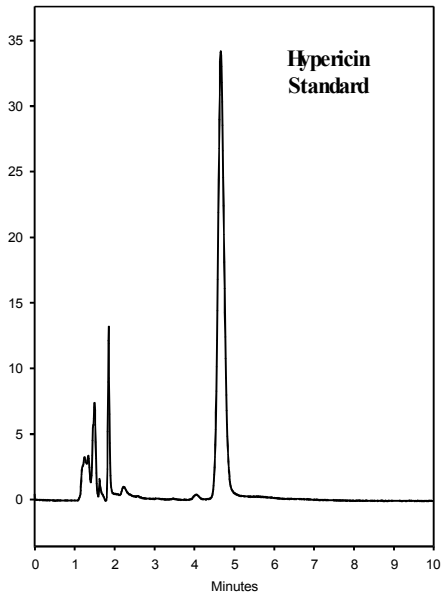
Pharmaceutical

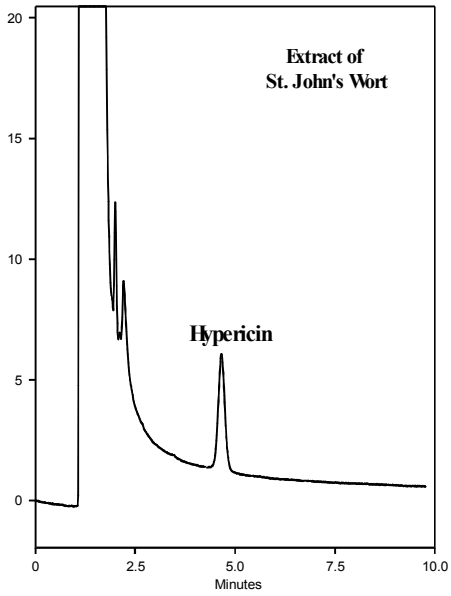
Robert Ricker

Hypericin is a major component in the herbal extract of Hypericum Perforatum, otherwise known as St. John's Wort. St. John's Wort has been used to treat depression but has also been recommended as an appetite suppressant. Due to the increasing interest in natural product therapies and the growing concern regarding government regulation of these manufacturers, a method was developed for hypericin and an herbal product of St. John's Wort.

Highlights

- *Good peak shape for hypericin at neutral pH on an Agilent ZORBAX Eclipse XDB-C8 column.*
- *Double endcapped Eclipse XDB-C8 yields longer column lifetime with neutral-pH mobile phases.*
- *Good retention of the hypericin allows adequate separation from other UV-absorbing compounds in the actual extract.*





Conditions:
Column: ZORBAX XDB-C8 (4.6 x 150 mm) (Agilent P/N: 993967-906)
Mobile Phase: 23% 25 mM Na_2HPO_4, Dibasic (pH 7.0 with H_3PO_4): 77% Methanol
Inj. Vol.: 2µL
Flow Rate: 1 mL/min
Temp.: 35°C
Detect.: UV (254 nm)

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Agilent Technologies

Multi-Element Determination of Heavy Metals in Dietary Supplements Using Collision/Reaction Cell ICP-MS

Application

Foods

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Abstract

Determination of 11 metals (including arsenic, chromium, mercury, iron, copper, nickel, zinc, selenium, lead, cadmium, and thallium) in UK-consumed dietary supplements was carried out using ICP-MS. The instrument used was the Agilent 7500ce, which is equipped with a collision/reaction cell (Octopole Reaction System), and was operated in no-gas, helium, and hydrogen modes, all acquired within a single method. Samples were microwave digested with nitric acid/hydrogen peroxide/hydrofluoric acid and quantified using external calibration. The method was assessed by the analysis of two certified reference materials (LGC7160 and SRM1577b), and recoveries were 100 ± 15% of the certified value for all elements.

Introduction

Heavy metals are natural components of the earth's crust and are widely used in agricultural, construction, manufacturing, and food/material processing industries. As trace elements, some heavy metals are metabolically essential to humans at low levels, but at higher concentrations they can become toxic. Heavy metal toxicity can result from high ambient air concentrations near emission sources, drinking-water contamination, or intake via the food chain.

Conversely, as our lifestyles have become increasingly busy, leaving less time to prepare and eat well-balanced meals, our diets are at an increased risk of becoming nutrient deficient. Dietary supplements, sold in capsule, tablet, or liquid form offer a simple and convenient way to supplement our diets and reduce the risk of nutrient deficiency.

In the UK, most products described as dietary supplements are regulated as foods and are subject to the general provisions of The Food Safety Act 1990 and secondary legislation on safety and labeling (Food Labeling Regulations 1996 [as amended] and the Food Supplements Directive 2002/46/EC). Past investigations have shown that some dietary supplements can contain elevated concentrations of metals and other elements. While some metals, such as selenium, iron, copper, chromium, and zinc [1], are essential at low concentrations, others, such as arsenic, cadmium, lead, and mercury, are toxic [2]. In the UK, arsenic is the only element for which there is legislation on permitted levels in foods. Arsenic is regulated under the "Arsenic in Food (as amended) Regulations 1959," which states a limit of 1 mg/kg in "general" food [3]. Cadmium, lead, mercury, and tin in specific foods are regulated under Commission Regulation 1881/2006 [4].

It is important that up-to-date information on the levels of metals and other elements in dietary supplements consumed in the UK is obtained, in order to assess whether there is any risk to consumers. The purpose of this study was to provide the Food Standards Agency (FSA) with up-to-date and accurate information on the concentration of a suite of metals contained within dietary supplements consumed in the UK.

To do this, microwave acid digestion was used for sample preparation, followed by multi-element determination by collision/reaction cell inductively coupled plasma mass spectrometer (CRC-ICP-MS) using He and H_2 collision gases to remove spectral interferences. The suite of metals included arsenic (As), chromium (Cr), iron (Fe), copper (Cu), nickel (Ni), zinc (Zn), selenium (Se), lead (Pb), mercury (Hg), cadmium (Cd), and thallium (Tl). Most elements were measured in standard mode (no gas) since multi-isotope ICP-MS data obtained for the food samples suggested the absence of significant polyatomic interferences. However, the ICP-MS detection of three of these elements, two essential (Fe and Se) and one toxic (As), was found to be strongly hampered by polyatomic ions. The purpose of this application is to demonstrate the ability of the Agilent 7500ce using the Octopole Reaction System (ORS) to eliminate these interferences (Table 1), providing accurate determination of these three elements in food supplements.

Experimental

Samples

Two hundred different dietary supplements (either tablet, capsule, liquid, or powder form) commercially available in the UK were sourced. The average weight of each tablet/capsule was determined using an electronic balance.

Sample Preparation

Tablets were crushed with a pestle and mortar. Crushed tablets, liquids, and powders were subsampled after thorough mixing. Oil capsules were digested whole. Approximately 0.7 g of sample was accurately weighed and microwave digested with 7 + 3 + 0.2 mL of nitric acid + hydrogen peroxide + hydrofluoric acid, respectively. The microwave program consisted of heating the samples to 180 °C over 20 min and holding for a further 10 min. Once cool, the digests were made up to 100 g using deionized water, and the resultant solutions were subjected to element determination by ICP-MS. Approximately 10% of the samples were digested in duplicate. A blank and QC material were included in each digestion run, containing a maximum of 12 samples.

QC Materials

Two certified reference materials were analyzed to assess the accuracy of the methodology. These were bovine liver SRM 1577b (NIST, Gaithersburg, USA) containing 0.73 ± 0.06 mg/kg Se and 184 ± 15 mg/kg Fe and crab paste LGC7160 (LGC, Teddington, UK) containing 11 ± 1 mg/kg As.

Instrumentation

Sample digestion was undertaken in a Mars 5 microwave (CEM, Buckingham, UK). Elemental measurements were performed using an Agilent 7500ce CRC-ICP-MS operating in hydrogen mode for Se and Fe, and helium mode for As to remove spectral interferences (Table 1). All other elements were measured in standard (no gas) mode within the same method. Typical operating conditions are illustrated in Table 1. The Integrated Sample Introduction System (ISIS) was used with a pump speed set at 0.1 rps during the analysis and washout in order to minimize the amount of matrix onto the interface and optimize sample throughput.

Table 1. Instrumental Conditions for 7500ce ORS Collision/Reaction Cell Mode

Parameter	ORS Cell Mode	
	He	H_2
Elements measured	As	Se, Fe
Spectral interferences removed by ORS gas	$^{40}Ar^{35}Cl^+$ on $^{75}As^+$ $^{40}Ca^{35}Cl^+$ on $^{75}As^+$	$^{38}Ar^{40}Ar^+$ on $^{78}Se^+$ $^{40}Ar^{37}Cl^+$ on $^{77}Se^+$ $^{40}Ca^{37}Cl^+$ on $^{77}Se^+$ $^{40}Ar^{16}O^+$ on $^{56}Fe^+$ $^{40}Ca^{16}O^+$ on $^{56}Fe^+$
RF power (W)	1520	
Carrier gas (L/min)	0.9	
Make-up gas (L/min)	0.26	
Nebulizer	Glass concentric, MicroMist	
Spray chamber	Quartz cooled to 2 °C	
Interface cones	Ni	
Cell gas	He	H_2
Cell gas flow rate (mL/min)	2.5	2.2
Points per peak	3	
Repetitions	10	
Integration time per mass (sec)	0.3	

Measurement

Five-point external calibrations with standards traceable to the National Institute of Standards and Technology (NIST, Gaithersburg, USA) were used to quantify the elements in the digests. Rhodium (Rh) was used as an internal standard and added on-line (1:1 with samples). The internal standard solution also contained 4% propanol to compensate for enhancement of As and Se signal from any residual carbon in the samples. Water

Research Council's (WRC) Aquacheck solutions (used in a proficiency testing scheme) with known concentrations of the analytes (660.3 ng/g Fe, 12.6 ng/g As, and 13.27 ng/g Se) were also analyzed as independent checks on the accuracy and precision of each ICP-MS run.

Results and Discussion

All sample results were checked against legislative limits and/or limits agreed with the FSA. The action limits used were 1 mg/kg arsenic, lead, and cadmium and 0.5 mg/kg for mercury. Samples that exceeded these limits were reanalyzed to confirm the results.

As illustrated in Table 2, five samples were confirmed to contain As concentrations greater than the 1 mg/kg limit recommended in The Arsenic in Food (as amended) Regulations 1959 [3]. A further two samples were found to contain As concentrations between 0.75 mg/kg and 1 mg/kg. It should be noted that several of these supplements were derived from marine animals, and the form of the As in such materials is likely to be nontoxic arsenobetaine. The majority of supplements (> 75%) were found to contain < 0.1 mg/kg As and Se, and > 20 mg/kg Fe.

Table 2. Samples with Arsenic Concentrations Above Recommended Limit

Product	Form	As in sample (mg/kg)
Product 1	Tablet	3.3 ± 0.7
Product 2	Capsule	2.5 ± 0.6
Product 3	Capsule	20.5 ± 4.8
Product 4	Capsule	1.5 ± 0.4
Product 5	Capsule	7.3 ± 1.7

Legislative limit = 1 mg/kg [3]
The uncertainty quoted is the expanded uncertainty calculated using a coverage factor of 2, which gives a level of confidence of approximately 95%. The uncertainty was calculated based upon the principles of the Eurachem Guide [5].

None of the samples was found to contain an elevated concentration of Cd, and only one sample was close to the limit for Hg. Ten samples were confirmed to contain concentrations of Pb above 1 mg/kg.

Results in mg/kg were calculated back to mg/tablet in order to allow comparison with label claims. For a number of supplements, differences were found between the measured concentrations of Se, Fe, Zn, Cu, Cr, or Ni and the values stated on the label. This makes it difficult for the FSA to accurately assess the dietary intake of these elements from such supplements based on label claim alone and demonstrates the necessity of this survey.

The limits of detection (LOD) and quantification (LOQ) of the described procedure, calculated according to International Union of Pure and Applied Chemistry (IUPAC) guidelines [6], are presented in Table 3. For 25% of the dietary supplements tested the Se concentration was found to be less than the LOD.

Table 3. Limits of Detection and Quantification of Se, Fe, and As

	Concentration (mg/kg)[1]		
	Se	Fe	As
LOD	0.009	0.072	0.006
LOQ	0.029	0.240	0.022

[1] Values shown are based on the average weight of dietary supplement tablet/liquid/capsule digested (0.67 g).

Recovery results for the QC materials and Aquacheck solutions were very good, with results for all elements falling within 100 ± 15% (n = > 9) of the certified/expected value. The recoveries obtained for the reference materials analyzed are illustrated in Figure 1. A number of sample and blank solutions were also spiked with Se, Fe, and As prior to microwave digestion. The resultant recoveries fell within 100 ± 10% (n = > 5) of the expected value. As a check on the repeatability of

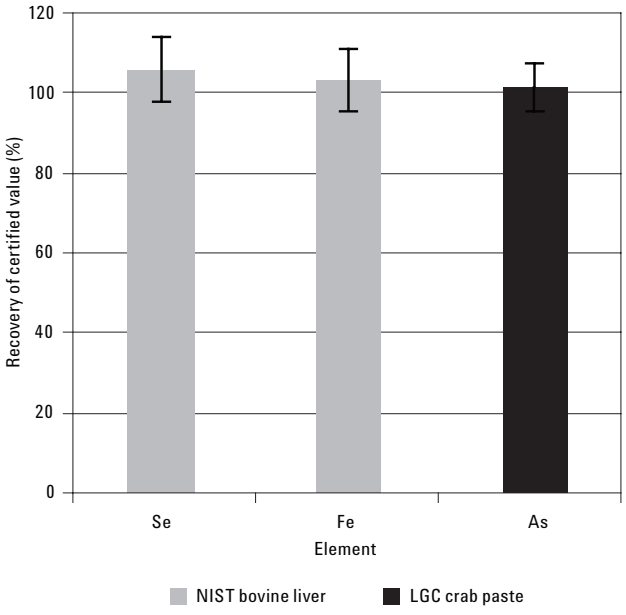


Figure 1. Recovery of Se, Fe, and As from the CRMs analyzed.

the method, approximately 10% of the samples were also independently digested and analyzed in duplicate. For these, coefficient of variation values of < 5% were found for all three elements measured in collision/reaction mode on the 7500ce ICP-MS.

Conclusions

Microwave digestion followed by analysis by ICP-ORS-MS has been shown to be a simple, reliable method for the multi-element determination of trace metals in nutritional supplements and foodstuffs. A number of supplements were found to contain Se, Fe, Zn, Cu, Cr, and Ni at concentrations that deviated from the label claim. Five samples for As and 10 samples for Pb were found to contain elevated concentrations above the recommended 1 mg/kg limit. The data generated in this survey has provided the FSA with up-to-date concentrations of these metals in a range of dietary supplements. The results have enabled the risk of metal toxicity from the consumption of dietary supplements to be assessed and published in a Food Standard Agency's Food Surveillance Information sheet [7].

References

1. World Health Organization, Trace Elements in Human Nutrition and Health, Geneva, 1996
2. The Ministry of Agriculture, Fisheries and Food (1982), Survey of Arsenic in Food. Food Surveillance Paper No. 8, Published by HMSO
3. The Arsenic in Food Regulations 1959 (S.I. [1959] No. 831), as amended by The Arsenic in Food (Amendment) Regulations 1960 (S.I. [1960] No. 2261) and The Arsenic in Food (Amendment) Regulations 1973 (S.I. [1973] No. 1052). The Stationery Office.
4. Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs.
5. Eurachem, Quantifying uncertainty in analytical measurement. Laboratory of the Government Chemist. London 1995 (ISBN 0 948926-08).
6. "A Statistical Overview of Standard (IUPAC and ACS) and New Procedures for Determining the Limits of Detection and Quantification: Application to Voltammetric and Stripping Techniques," *Pure & Appl. Chem.* Vol 69, No. 2, pp 297–328, 1998
7. Food Standard Agency's Food Surveillance Information sheet No 85/05 December 2005, Survey of Metals and Other Elements in Dietary Supplements.

Acknowledgements

The authors would like to thank Malcolm Burn and Kam Lee from the Food Chemistry team at LGC for performing the sample preparation/digestion, and Sheila Merson, Linda Evans, and Dave Curtis from the Specialised Techniques team for conducting the elemental determinations.

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Food Authenticity

Global sourcing and the diversity of products available to consumers has created a need to ensure the authenticity and the origin of those products. Often unscrupulous food manufacturers use additives for cost savings, like using apple juice in place of more expensive fruit juices, or, adding melamine to artificially increase the apparent protein level in animal food and baby formula. Whether screening for accidental contamination or checking for intentional fraud, Agilent Technologies has the intrumentation for the analysis of protein and DNA to determine authentictiy, genetic identity, and for GMO screening.

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Agilent Technologies

Identification of Oxidation Products of L-Ascorbic Acid by HPLC

Linda Lloyd
Polymer Laboratories, now a part of Varian, Inc.

Introduction

Ascorbic acid is a water soluble sugar acid with antioxidant properties. The L-isomer of ascorbic acid is commonly known as vitamin C and is found naturally in fruits and vegetables. It is also added to fruit juices and other processed products as an antioxidant. Vitamin C is an essential nutrient in the human diet in the manufacture of collagen. In humans, absence of the vitamin leads to scurvy, a deficiency disease.

The acid has strong reducing power but when oxidized is converted to several compounds that do not have the same antiscorbutic or reducing properties. Given the importance of the vitamin in human health and its widespread use as an antioxidant in processed foods, study of its degradation products is merited. This note describes aspects of the rate of degradation of L-ascorbic acid and the nature of some of its degradation products using PLRP-S columns from Polymer Laboratories, now a part of Varian, Inc. PLRP-S is a rigid macroporous styrene/divinylbenzene HPLC phase with outstanding chemical and physical stability. The high surface area of the 100Å pore size enables retention of water soluble solutes.

The aim of this study was to develop a rapid HPLC method for the quantitative and qualitative analysis of L-ascorbic acid.

Materials and Reagents

Reference samples: commercial L-ascorbic acid, oxalic acid, L-dehydroascorbic acid (DHAA) dimer

Conditions

Columns: 2 x PLRP-S 100Å 5 µm, 150 x 4.6 mm
Eluent: 0.2 M NaH_2PO_4, pH 2.14
Flow rate: 0.5 mL/min
Inj Vol: 20 µL
Detector UV, 268 and 220 nm

Results and Discussion

Reference materials

The retention of reference compounds is shown in Figure 1. L-ascorbic acid was well resolved from its possible degradation products.

Bromine oxidation of L-ascorbic acid

HPLC of freshly prepared solutions of L-ascorbic acid treated with bromine produced the curves shown in Figure 2. Peak

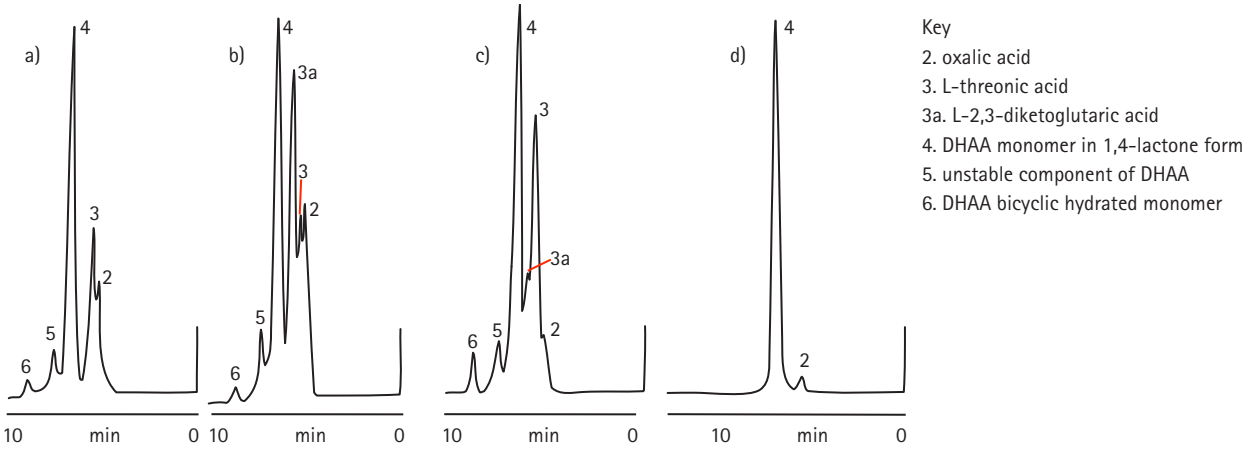


Figure 1. HPLC separation of a) L-dehydroascorbic acid solution spiked with b) potassium L-2,3-diketoglutaric acid and c) calcium threonate reference materials. d) is L-dehydroascorbic acid produced by Dietz's method, at 220 nm (0.2 AUFS).

1 is a high response at 220 nm, whereas peak 2 is a smaller response later identified as DHAA monomer with a free side chain. Peak height intensity was proportional to the amount of bromine added. Further addition of bromine was made to a cold solution of L-ascorbic acid (2 °C) to control degradation of DHAA. Homocysteine was then added and the reduction reaction went ahead at room temperature.

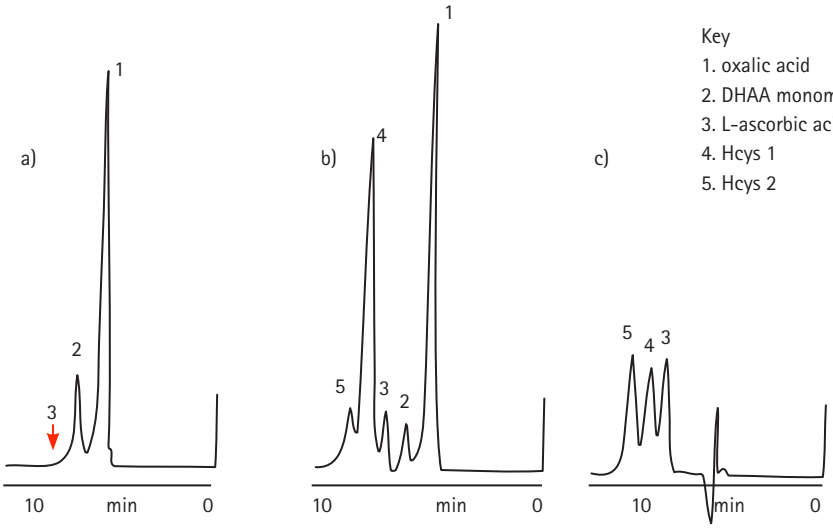


Figure 2. HPLC separation of a) bromine-oxidized L-ascorbic acid, at 220 nm (0.2 AUFS), and b) after addition of homocysteine (1:1), at 220 nm (0.1 AUFS) and c) at 268 nm (0.02 AUFS).

Effect of homocysteine on DHAA

The reaction of homocysteine with DHAA was examined to distinguish the DHAA peak from surrounding peaks in the commercial samples (Figure 3). *Inter alia*, L-ascorbic acid (peak 4a) increased very rapidly after reaction with homocysteine, and peaks 4 and 6 represent monomeric forms of DHAA.

The complete data set and analysis is available in Kennedy *et al.* (1989).

Conclusion

PLRP-S columns successfully revealed the identities of some degradation products of vitamin C in the development of a qualitative and quantitative HPLC method for the fast analysis of L-ascorbic acid.

Reference

Kennedy, JF, White, CA, Warner, FP, Lloyd, LL and Rivera, ZS (1989) The identification and analysis of the oxidation products of L-ascorbic acid by HPLC C. *J. Micronut. Anal.*, **5**, 91-109.

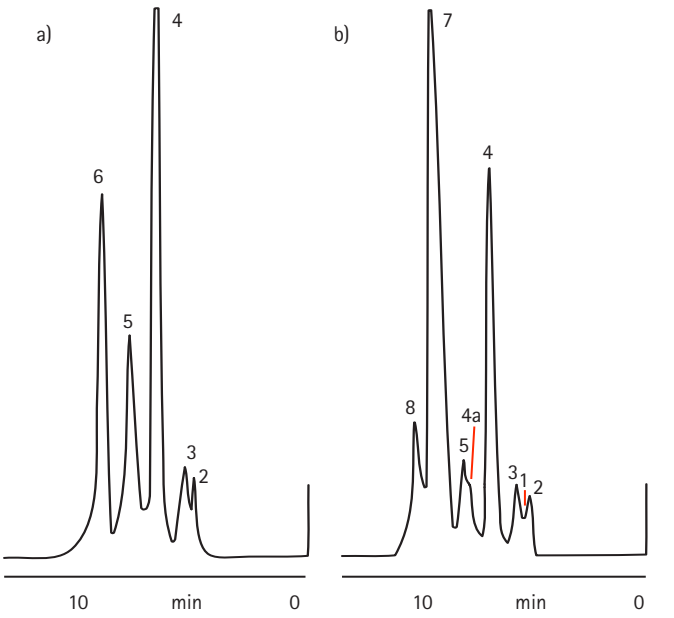


Figure 3. HPLC separation of L-dehydroascorbic acid solution a) before and b) after reduction reaction with homocysteine, at 220 nm (0.05 AUFS), using optimized conditions.

These data represent typical results. For further information, contact your local Varian Sales Office.

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Accurate analysis of organic acids and alcohols by HPLC

Linda Lloyd
Polymer Laboratories, now a part of Varian, Inc.

Introduction

Hi-Plex H columns from Polymer Laboratories, now a part of Varian, Inc., enable the separation of glucose, fructose and malic acid from wine while avoiding the common problem of the malic acid co-eluting with the glucose, which results in a false indication of sweetness. In addition, analysis of malic acid can show the age of a wine. Hi-Plex H is also the column of choice for the analysis of sugar alcohols and sugar molecules.

Instrumentation

Figure 1
Column: Hi-Plex H 8 µm, 300 x 7.7 mm
Hi-Plex H Fast Acid 8 µm, 100 x 7.7 mm
Hi-Plex H Guard, 50 x 7.7 mm
Detector: RI

Figure 2
Column: Hi-Plex H, 300 x 7.7 mm
Detector: UV, 210 nm

Materials and Reagents

Figure 1
Eluent: 0.005 M H_2SO_4

Figure 2
Eluent: 5 mM H_2SO_4

Conditions

Experimental conditions, specifically temperature, must be carefully optimized to ensure separation and prevent co-elution. The strength of the acid eluent also affects the resolution. The optimum temperature for resolution is greatly reduced by changing from 5 mM H_2SO_4 to 4 mM H_2SO_4.

Figure 1
Flow Rate: 0.6 mL/min
Temp: 76 °C

Figure 2
Flow Rate: 0.6 mL/min
Temp: 55 °C

Results and Discussion

Figure 1 shows good separation of three constituents of wine, and Figure 2 reveals the excellent separation of six organic acids, both separations using Hi-Plex H columns.

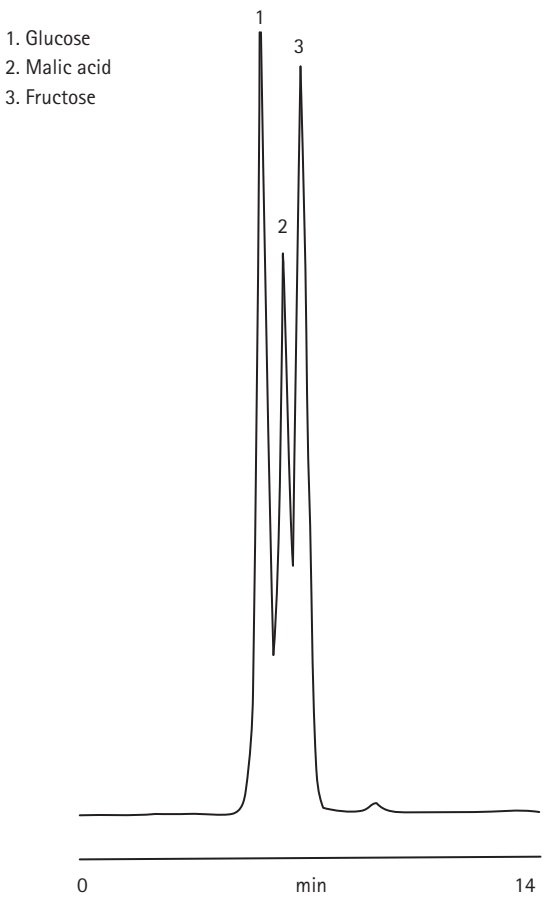


Figure 1. Good separation of wine components using PL Hi-Plex H columns, with no co-elution of malic acid and glucose.

Key

1. Oxalic Acid
2. Citric Acid
3. Malic Acid
4. Succinic Acid
5. Formic Acid
6. Acetic Acid

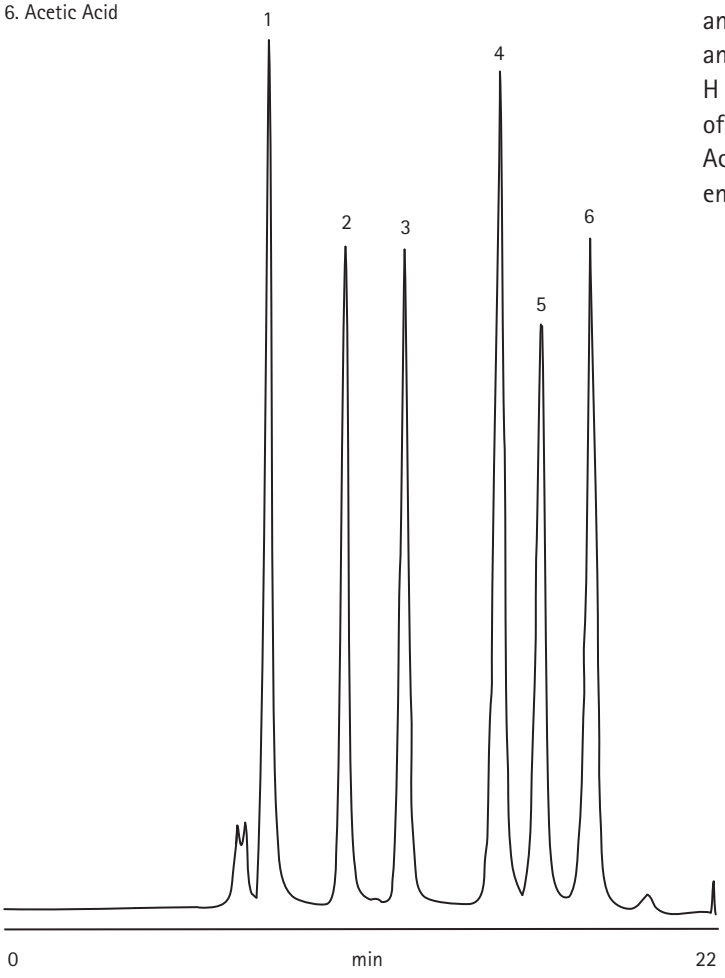


Figure 2. Hi-Plex H columns resolve organic acids with very good separation.

Conclusion

Hi-Plex columns are packed with sulfonated resin giving a fundamental improvement in performance. The monodisperse packing overcomes the problems of low efficiencies and high back pressures encountered with 'soft' gels. The columns are available in hydrogen form for the analysis of organic acids and alcohols. Hi-Plex H is also the column of choice for the analysis of sugar alcohols and sugar molecules. The Hi-Plex H Fast Acid column is also available for direct injecting of samples onto the column, with results within minutes. Accurate determination of composition and content is ensured.

These data represent typical results. For further information, contact your local Varian Sales Office.

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Application Note SI-01411 / TB2004/2005

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Profiling juice carbohydrates with evaporative light scattering detection

Stephen Bullock
Polymer Laboratories, now a part of Varian, Inc.

Introduction

The predominant sugars in fruit juice are the monosaccharides, glucose and fructose, and the disaccharide, sucrose. The ratio of these three sugars differs in each type of fruit juice. However, for each single fruit the ratio is relatively constant. In the production of "pure" fruit juices, the juice must be extracted by mechanical means and must not contain any added material. The mechanical extraction and concentration of the juice is normally carried out in the country where the fruit is grown. The concentrate is shipped to an intermediate country or the country where the juice is to be consumed, where it is reconstituted to produce the "pure" fruit juice product. The juice concentrate is an expensive commodity and one way of increasing profit is to extend the juice by the addition of sucrose and water, which are considerably cheaper. This extension of the juice is a form of adulteration, not detectable by the measurement of °Brix, as this assesses total dissolved sugar and not the ratio of the individual sugars.

Instrumentation

Hi-Plex resins from Polymer Laboratories, now a part of Varian, Inc., are ideal for the analysis of processed foods that contain a significant quantity of sugar, and provide a profile of both naturally occurring and added sugars. To determine the ratio of sucrose, glucose and fructose, a rapid, reliable and robust method is required. There are a number of HPLC methods to quantify these three sugars, with one of the most simple being the use of a calcium ligand exchange column, Hi-Plex Ca, with water as the eluent.

Sugars do not have a UV chromophore and therefore the RI detector has to be used if the solutes are to be analyzed without any derivatization. An improvement in sensitivity for carbohydrates can be achieved by using an evaporative light scattering detector, PL-ELS 1000, also from Polymer Laboratories. In addition to an increase in sensitivity, this detector gives a more stable, drift-free baseline which can improve the precision of the quantitation.

Column: Hi-Plex Ca, 300 x 7.7 mm
Detector: RI or PL-ELS 1000 (neb=80 °C, evap=80 °C, gas=1.0 SLM)

Materials and Reagents

Eluent: Water

Conditions

Flow Rate: 0.6mL/min
Temp: 85 °C

Results and Discussion

Figures 1 and 2 show the separation of orange juice and apple juice, respectively, and clearly indicate that the ratio of the three sugars differs in the two juices, sucrose being the main sugar in the orange juice and fructose in the apple juice. The superior stability provided by the PL-ELS 1000 ELSD is evident in the carbohydrate separations of two additional samples of orange and apple juice, as shown in Figure 3 and Figure 4.

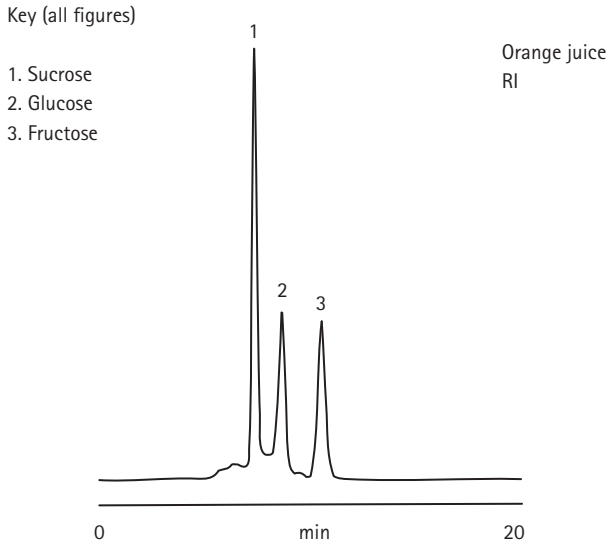


Figure 1. Sugar ratios in orange juice revealed by HPLC with a Hi-Plex Ca column and RI detection.

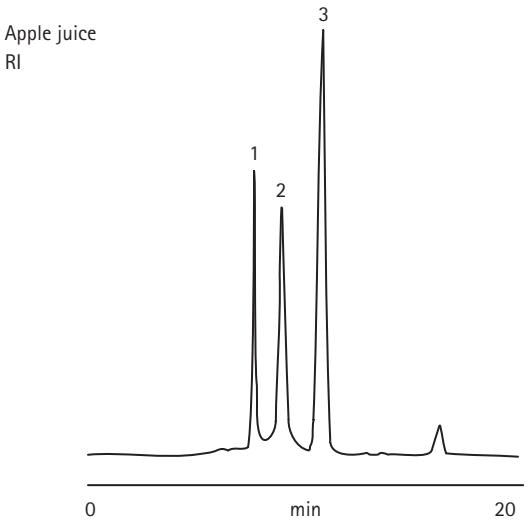


Figure 2. Sugars ratios in apple juice revealed by HPLC with a Hi-Plex Ca column and RI detection.

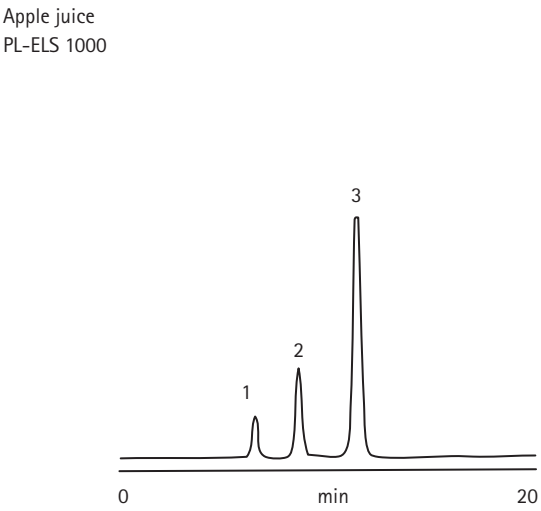


Figure 3. Sugar ratios in apple juice revealed by HPLC with a Hi-Plex Ca column and PL-ELS 1000 evaporative light scattering detector.

Conclusion

Hi-Plex columns are packed with sulfonated resin giving a fundamental improvement in performance to overcome the problems of low efficiencies and high back pressures encountered with 'soft' gels. The columns are available in calcium form for the analysis of carbohydrates in fruit juices, to meet the growing demand for more detailed product information for labelling and control purposes. The robust design of the PL-ELS 1000 evaporative light scattering detector allows the nebulizer and evaporator to operate at very high temperatures, efficiently handling the high boiling point solvents for this type of analysis that other ELSDs simply cannot manage. By using Hi-Plex columns and the PL-ELS 1000 ELSD, accurate determination of composition and content is assured.

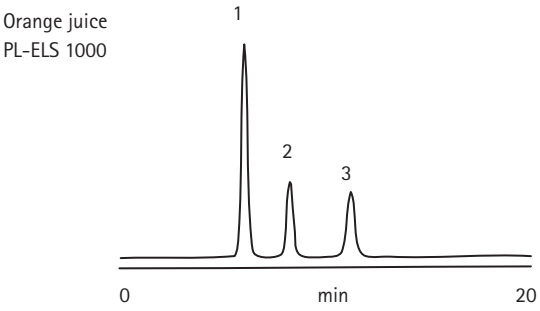


Figure 4. Sugar ratios in orange juice revealed by HPLC with a Hi-Plex Ca column and PL-ELS 1000 evaporative light scattering detector.

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Application Note SI-01151

Hi-Plex columns for fingerprinting organic acids in wine

Stephen Ball
Polymer Laboratories, now part of Varian, Inc.

Introduction

The Hi-Plex H is a high performance ligand exchange chromatography column from Polymer Laboratories, now a part of Varian, Inc. The column is based on polystyrene divinylbenzene with an 8% crosslinking and hydrogen counter ion. Typically used for the analysis of sugars, sugar alcohols and organic acids, its monodisperse sulfonated packing gives improved column efficiency, lower column pressure and assured batch to batch reproducibility.

The superior separation ability of Hi-Plex H is demonstrated in the quantitative analysis of organic acids in four different samples of wine: red, white, rosé and dessert wine. This type of analysis is important for the wine quality control, because the classes and content of organic acids give a characteristic taste to the finished product. Acetic acid, lactic acid, succinic acid, malic acid, citric acid and tartaric acid are the main organic acids in wine.

The use of a ligand exchange chromatography column such as Hi-Plex H significantly reduces the need for complicated sample preparation (typically involving elution through an ion exchange resin bed), as retention is brought about by not only ion exchange, but also by ion exclusion and partitioning on this type of column.

Materials and Reagents

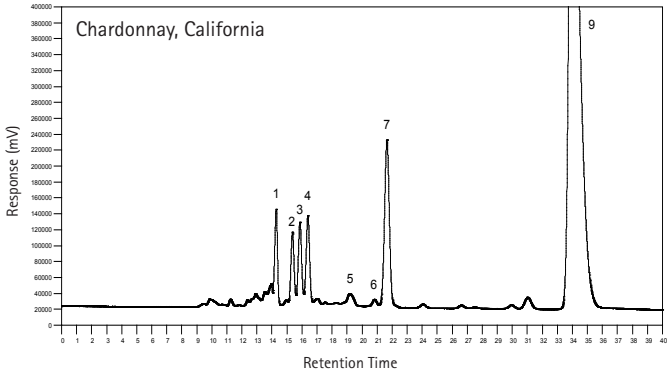
Columns: Hi-Plex H, 8 µm, 300 x 7.7 mm
(PL1170-6830)

Sample Preparation

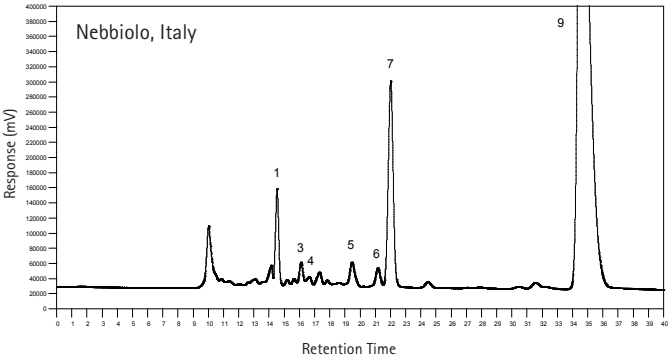
In order to obtain a complete RI profile, each wine was directly injected onto the column without any sample pre-treatment. The only exception to this being the Inniskillin Eiswein which was diluted by a factor of five with HPLC grade water, and the Marsala Wine which was diluted by a factor of three with water. Injection volume was 20 µL.

Conditions

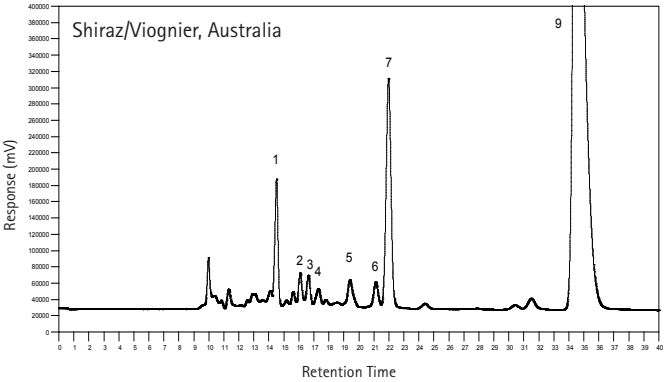
Mobile Phase: 0.004 M H_2SO_4
Flow Rate: 0.4 mL/min
Temp.: 75 °C
Detector: Varian 356-LC Refractive Index Dectector



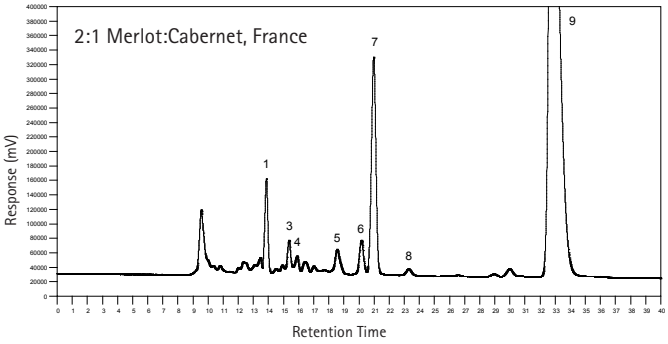
White wine contains a wide variety of organic acids and sugars.



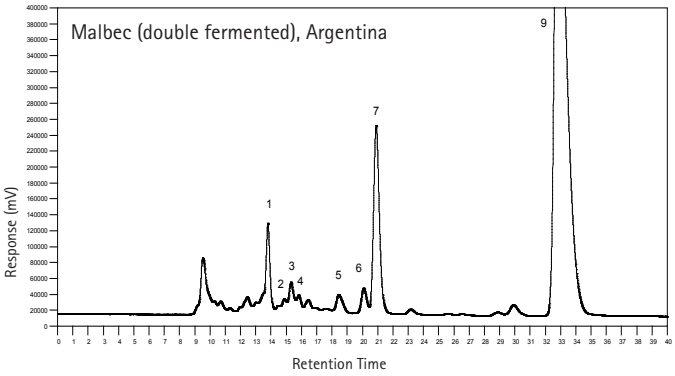
The main constituents of this red wine are tartaric acid, succinic acid and glycerol.



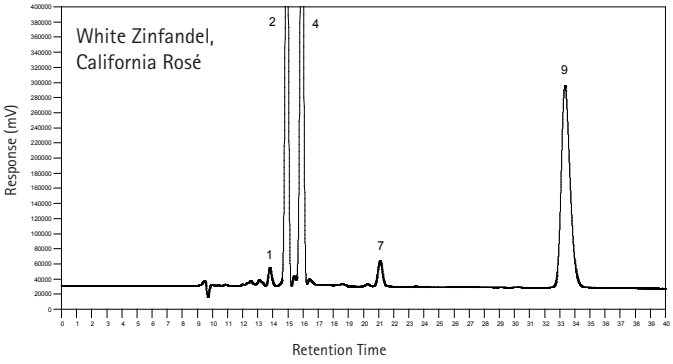
This wine contains slightly higher levels of malic acid and fructose than the Nebbiolo.



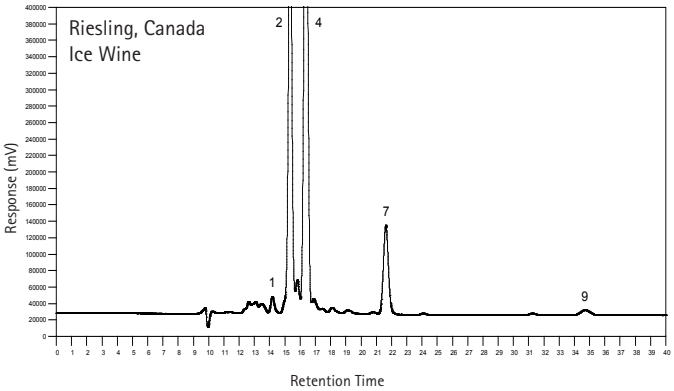
As shown, this red wine contains a slightly higher level of lactic acid than the other wines.



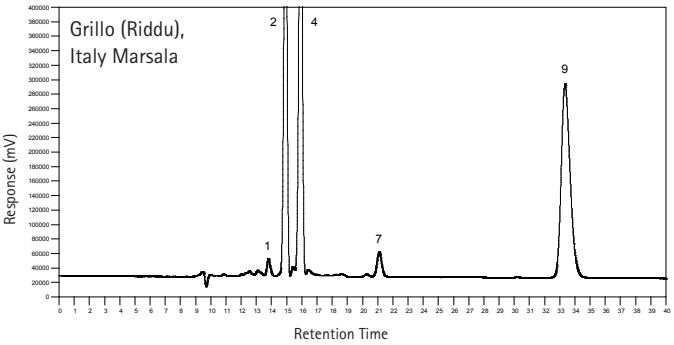
This wine has another unique profile, with differing levels of organic acids and sugar.



Rosé wine contains fewer organic acids and a significantly higher fructose content.



This dessert wine contains very high levels of malic acid, fructose and glycerol, but little else.



As expected, this dessert wine also contains high amounts of malic acid and fructose.

Key
1). Tartaric acid, 2). Malic acid, 3). Glucose, 4). Fructose, 5). Succinic acid, 6). Lactic acid, 7). Glycerol, 8). Acetic acid, 9). Ethanol

Results and Discussion

The results of this investigation prove that is possible to distinguish between different types of wine (i.e. red, white, rosé, dessert) by analyzing them via HPLC. By using an RI detector, levels of organic acids and sugars can be quantified simultaneously.

The rosé and the dessert wines all contain very high levels of malic acid and the fruit sugar fructose. In fact, Inniskillin Eiswein and Masala Wine, both dessert wines, contain up to five times as much sugar as ordinary rosé wine and 70 times as much sugar as the red and white wines. Eiswein (commonly called Ice Wine) is produced from grapes that have been frozen causing some of the water to freeze out, leaving the sugars and other solids dissolved in the remaining juice. The resulting wine is therefore very sweet but has a great deal of balancing acidity, which also explains the high level of malic acid in the wine samples.

The chromatograms of the red and white wines look very different to the others, in that they have much lower levels of sugar but much higher levels of lactic acid and glycerol. Red wine is made from the must (pulp) of red or black grapes that undergo fermentation together with the grape skins, while white wine is usually made by fermenting juice pressed from white grapes. During the fermentation process, yeast converts most of the sugars in the grape juice into ethanol and carbon dioxide, which explains the lows levels of glucose and fructose in the wine samples. Some wines also undergo malolactic fermentation, where bacteria convert malic acid into the milder lactic acid. All of these factors, and the levels of sugars and organic acids produced by the various fermentation processes contribute to the differing taste that each wine has and give each one a unique profile when analyzed by HPLC.

Some of the other peaks that appear in the chromatograms are likely to be as a result of tannins (bitter tasting plant polyphenols) present in the skins and seeds of the grapes used in the fermentation process.

Conclusion

The analysis of wines demonstrates how Hi-Plex H columns provide optimum resolution of closely eluting compounds, enabling quantitation of each. These columns are ideal for the analysis of sugar alcohols and sugar molecules using water as the mobile phase. The Hi-Plex H is also the column of choice for the analysis of organic acids, using dilute mineral acid as eluent. By using the columns at higher operating temperatures, closely eluting compounds can be resolved.

References

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Schneider, A., Gerbi, V. and Redoglia, M. (1987) A rapid HPLC method for separation and determination of major organic acids in grape musts and wines. American Journal of Enology and Viticulture, **38** (2), 151-155.

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Fast Analysis of Carbohydrates in Chocolate using Ligand Exchange Chromatography with ELSD

Stephen Bullock
Polymer Laboratories, now a part of Varian, Inc.

Introduction

Methods of detection for carbohydrates are severely limited as they do not normally possess chromophores or fluorophores. Detection can sometimes be accomplished in the low UV range, 190-200 nm, but unless high purity eluents are used and extensive sample preparation employed, excessive interference from other compounds may occur.

The refractive index detector is routinely used but RI is relatively insensitive, relying on a refractive index difference between solute and eluent. Where increased sensitivity is required, a pulsed amperometric detector is employed, but for uniform response, the carbohydrate must be in a high pH environment.

A better detector for the analysis of carbohydrates is the PL-ELS 1000 evaporative light scattering detector from Polymer Laboratories, now a part of Varian, Inc. When the PL-ELS 1000 is used in combination with Hi-Plex ligand exchange columns, alos from Polymer Laboratories, rapid isocratic separations of mono-, di- and oligosaccharides are achieved. The PL-ELS 1000 does not require the solutes of interest to have any particular optical properties. The principle of operation is a three stage process; the first stage involves the nebulization of the eluent, the second the evaporation of the solvent to leave solute particles and the third the detection of the light scattered by the solid solute particles as they pass through the light beam. Therefore, the only requirement for using PL-ELS 1000 is that the eluent is more volatile than the solutes.

When using Hi-Plex columns for the analysis of carbohydrates, water, with no buffer or added salt, is used as the eluent and therefore this is an ideal application for the PL-ELS 1000 detector as neutral carbohydrates have little UV activity. Sugars may be detected with the PL-ELS 1000 detector and a Hi-Plex column that has strong, cation exchange resins available in differing ionic forms.
The sulfonated column resin gives a fundamental improvement in performance and overcomes the problems of low efficiencies and high back pressures encountered with 'soft' gels. The separation mechanism is achieved initially by size exclusion, with larger oligosaccharides eluting before smaller monosaccharides, and then by ligand exchange - interaction of the numerous hydroxyl groups on the sugar molecules with the metal ion associated with the resin. Hi-Plex columns are used at elevated temperature with isocratic eluents.

Chocolate is produced in three distinct forms: dark chocolate, milk chocolate and white chocolate. The predominant sugar in the three varieties is the disaccharide sucrose. However, the milk sugar, lactose, will also be present in milk and white chocolate. The amount of lactose present will be indicative of the amount of milk solids used in the production process. As both sucrose and lactose are disaccharides, the Hi-Plex Pb column is the preferred choice for the analysis and quantification of these two components. Hi-Plex resins are available in 8% crosslinked calcium forms for the analysis of mono- and disaccharides, and hydrogen (acid) forms for the analysis of sugar alcohols and organic acids. Also available is a 4% crosslinked sodium form for the separation of high molecular weight oligosaccharides, such as corn syrups, to Dp 14.

Instrumentation

Column: Hi-Plex Pb, 300 x 7.7 mm
Detector: PL-ELS 1000 (neb=80 °C, evap=75 °C, gas=1.0 SLM)

Materials and Reagents

Eluent: Water

Sample Preparation

Aqueous solutions were prepared at a concentration of 100 mg chocolate/mL and 2 µL injection volumes were used for the quantitation.

Conditions

Flow Rate: 0.6 mL/min

Results and Discussion

Table 1 summarizes the quantitation of the two disaccharides, sucrose and lactose. Sucrose is present in all four samples with the plain chocolate having the highest level. Lactose, the milk sugar, can be seen in the other three samples. Differences in the sucrose and lactose content of the two milk chocolate samples from different manufacturers are evident.

Table 1. Disaccharide content of commercial chocolate samples expressed as a percentage by weight of chocolate.

Carbohydrate	Milk Sample 1	Milk Sample 2	Plain	White
Lactose	7	17	nd	9
Sucrose	30	41	69	42
Total	37	58	69	51

Nd - not detected

The disaccharide composition of four commercial chocolate samples is shown in Figure 1.

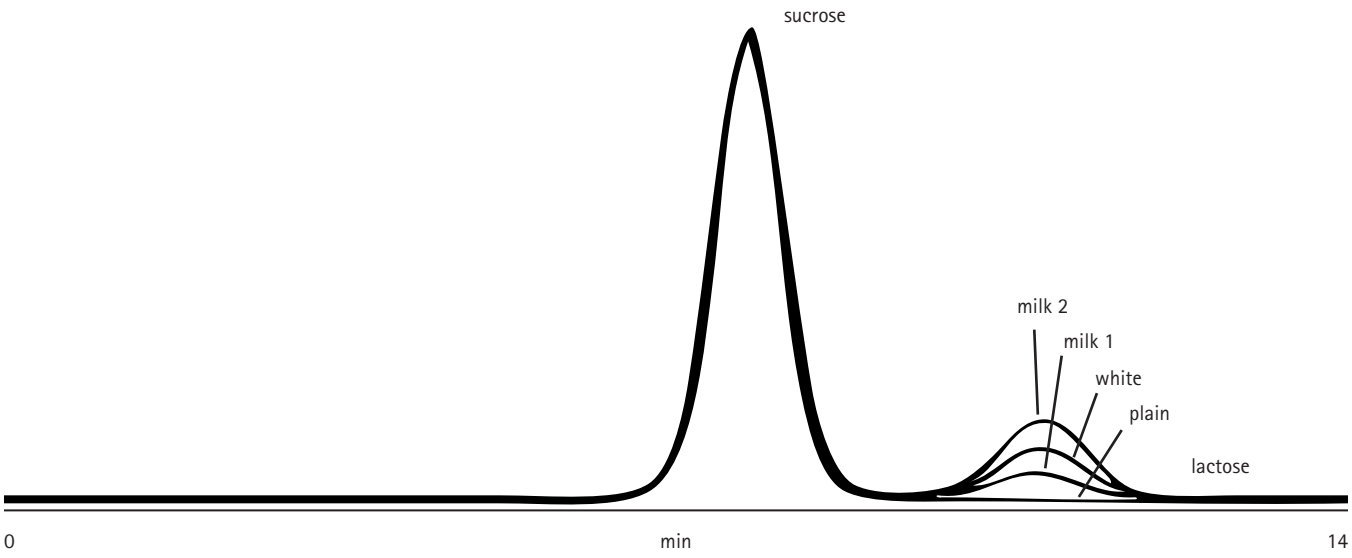


Figure 1. HPLC chromatograms of four commercial samples of chocolate, normalized to the height of the sucrose peak.

Conclusion

The composition of chocolate and levels of added milk solids in milk and white chocolate is readily achieved using water as the mobile phase with a Hi-Plex Pb column and the PL-ELS 1000 evaporative light scattering detector. The robust design of PL-ELS 1000 allows the nebulizer and evaporator to operate at very high temperatures, efficiently handling the high boiling point solvents that other ELSDs simply cannot manage.

This system avoids the use, high cost and disposal implications of toxic acetonitrile when separations are performed on amino silica columns. In addition, Hi-Plex stays active in the presence of sugar molecules. Together with fast dissolution, this benefit results in long lifetimes compared to amino silica columns.

These data represent typical results. For further information, contact your local Varian Sales Office.

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Determination of Melamine, Ammeline, Ammelide and Cyanuric Acid in Infant Milk-Based Formula and Other Food and Feed Products Using the Varian 220-MS Ion Trap GC/MS/MS and V:Results™ GC/MS Software

Haibo Wang and Anaïs Viven
Varian, Inc.

Introduction

In 2007, several pet food manufacturers recalled their products after finding melamine contamination, which caused serious illnesses in animals that consumed the food. In a follow-up investigation, the US Food and Drug Administration (FDA) and the Food Safety and Inspection Service (FSIS) found melamine and its analogs, cyanuric acid, ammeline and ammelide (Figure 1), in various food and feed ingredients, including bakery meal, pet food, swine, poultry and fish feed[1]. In September 2008, it was reported that milk products, especially infant formula, were contaminated with melamine in China. The melamine sickened at least 15,000 infants across the country and killed at least four. Allegedly, melamine was added to the milk formula and other vegetable protein products, such as wheat gluten and rice protein, to artificially increase the apparent protein levels due to its high nitrogen content. Although melamine itself may have low or no toxicity, it is believed that melamine and its analogs form insoluble crystals in urine, causing kidney stones and eventual acute renal failure[2].

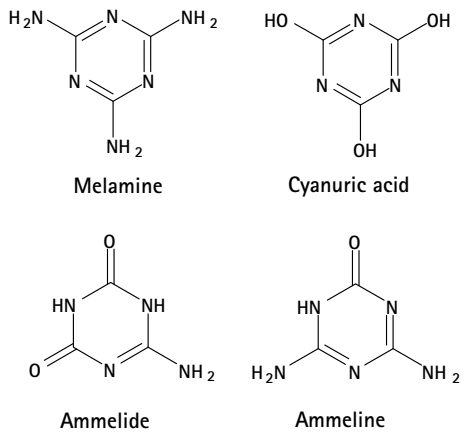


Figure 1. Melamine and related analogs.

Consequently, the US FDA developed a GC/MS method for screening and confirmation of melamine and its related analogs[1]. However, the method was not evaluated for quantitative analysis. In this note, we evaluated and developed a method using the Varian 220-MS ion trap mass spectrometer (Figure 2) to determine melamine and its analogs qualitatively and quantitatively based on the framework of the original FDA method.

Instrumentation

- Varian 220-MS Ion Trap Mass Spectrometer
- 431-GC gas chromatograph
- 8400 Trap AutoSampler
- Pierce Reacti-Therm/Reacti-Vap sample preparation system
- V:Results GC/MS software

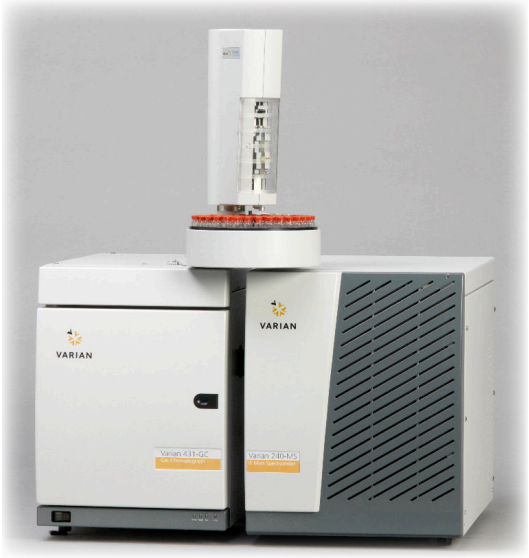


Figure 2. Varian 220-MS ion trap mass spectrometer with 431-GC gas chromatograph and 8400 AutoSampler.

Methods and Materials

Diethylamine (DEA) and pyridine (Sigma-Aldrich Co.)
Acetonitrile (Acros)
Extraction solvent: 10:40:50 DEA/water/acetonitrile

Sylating reagent: BSTFA with 1% TMCS:bis(trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane (Supelco)
Melamine and cyanuric acid (Sigma-Aldrich Co.)
Ammelide and ammeline (TCA America)
Dry dog food, cat food, and infant milk- based formula from a local supermarket.

Extraction Procedure

Approximately 0.5 g of a representative sample was weighed into a scintillation vial and extracted with 10 mL of extraction solvent (10:40:50 DEA/H_2O/ acetonitrile). The sample was mixed thoroughly, sonicated for 30 min, and centrifuged for 10-30 min at 15,000 rpm. The supernatant fluid was filtered using a 0.45 µm membrane.

Trimethylsiloxane (TMS) Derivatives

Transfer 200 µL filtrate from previous step to a 2 mL vial; evaporate to dryness at 70 °C with a low flow stream of dry nitrogen using the sample preparation system. Add 200 µL of pyridine and 200 µL BSTFA with 1% TMCS to the GC vial. Vortex to mix and incubate at 70 °C for 45 min.

Standard Curve

Prepare a stock solution containing melamine and its analogs (cyanuric acid, ammelide and ammeline) at 250 µg/mL 20:80 (v/v) in a mixture of DEA/H_2O. Dilute the stock solution to prepare calibration standards at 0.04, 0.1, 0.4, 1, 4, and 10 ppm in 20:80 (v/v) DEA/H_2O. Transfer 200 µL of each individual standard into in a 2.0 mL GC vial. Follow the same TMS-derivatization procedure as used for sample preparation in pyridine. Vortex to mix, and incubate at 70 °C for 45 min. The final concentration of the derivatized standards is 20, 50, 200, 500, 2000, and 5000 ppb.

GC Conditions

Column:	FactorFour™ VF-Xms, 30 m x 0.25 mm x 0.25 µm, with 5 m EZ-Guard™ (Part no. CP9018)
Inlet Temperature:	280 °C
Injection Volume:	1 µL
Carrier Gas Flow:	Helium at 1 mL/min
Injection Mode:	Splitless
Oven Program:	75 °C for 1 min to 300 °C at 15 °C/min, and hold 4 min for a total run time of 20 min

MS Conditions

Filament Delay:	6 min
Manifold Temp:	50 °C
Transfer Line Temp:	280 °C
Trap Temp:	220 °C
Emission Current:	15 µamp
Full Scan Mass Range:	100-400

MS/MS Parameters*

Time (min)	Parent ion (m/z)	Excitation storage (m/z)	Excitation amplitude (m/z)	Product ion mass range (m/z)
7.0 - 9.45	345.2	105	60	100 - 380
9.45 - 10.24	344.2	105	65	100 - 380
10.24 - 10.90	328.3	105	72	100 - 380
10.90 - 12.50	327.3	105	80	100 - 380

*Using non-resonant waveforms

Results and Discussion

Melamine and its analogs, cyanuric acid, ammelide and ammeline, were analyzed in both full scan and MS/MS operation modes. The multiple reaction monitoring (MRM) trace of the TMS derivatives of these four compounds is shown in Figure 3.

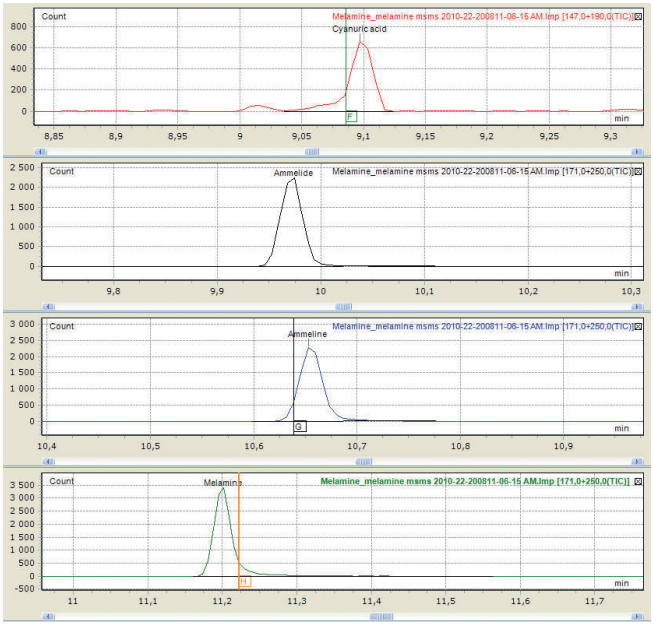


Figure 3. MRM of melamine and analogs in MS/MS operation mode at 10 ppb concentration.

This method provided excellent separation and identification of all melamine analogs. The quantitative determination of melamine was conducted from 20 to 2000 ppb in MS/MS mode. The calibration results are included in Table 1 and Figure 4. All compounds showed excellent linearity in MS/MS mode. The product ion spectra of melamine and its analogs are illustrated in Figure 5.

Table 1. Calibration of melamine and analogs.

Compound	Correlation coefficient (r^2)	Calibration range
Cyanuric acid	0.9997	20-2000
Ammelide	0.9975	20-2000
Ammeline	0.9994	20-2000
Melamine	0.9996	20-2000

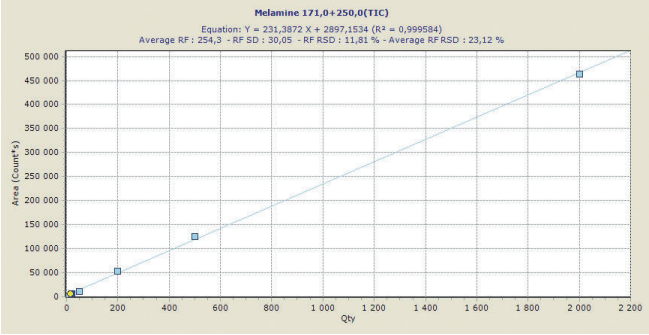


Figure 4. Calibration of melamine in MS/MS mode.

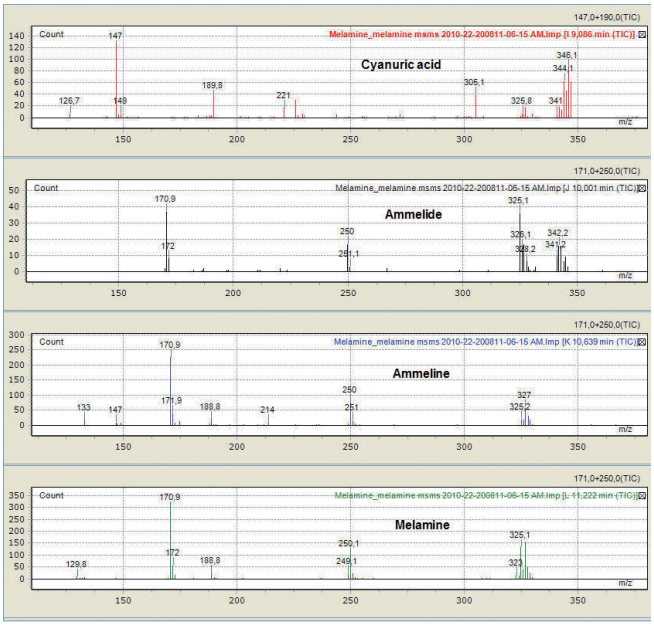


Figure 5. MS/MS product ion spectra of melamine and its analogs.

Three matrices were used to evaluate the robustness of this method. Melamine and its analogs (20 µg/g) were spiked in dry dog and cat foods and 5 µg/g was spiked in infant milk-based formula. The total ion chromatogram (TIC) of the spiked infant formula extract in full scan is shown in Figure 6. As indicated in the chromatogram, significant matrix interference was observed in full scan acquisition even when displaying the extracted ion chromatogram (EIC). In MS/MS mode, the interference from the matrix was eliminated, as shown in Figure 7 with the infant milk-based formula extract.

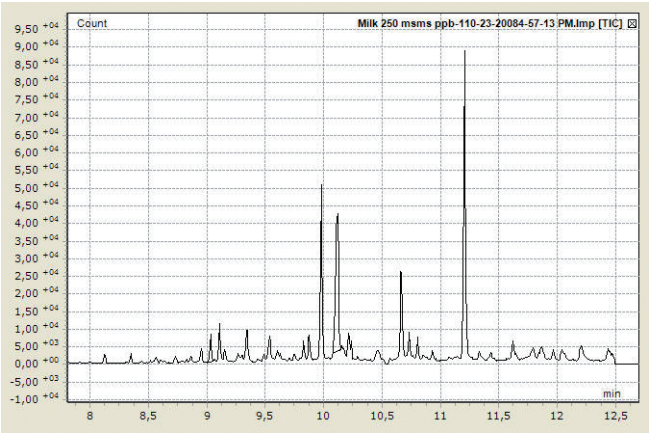


Figure 6. EIC of melamine and analogs spiked at 250 ppb in infant milk extract in full scan mode.

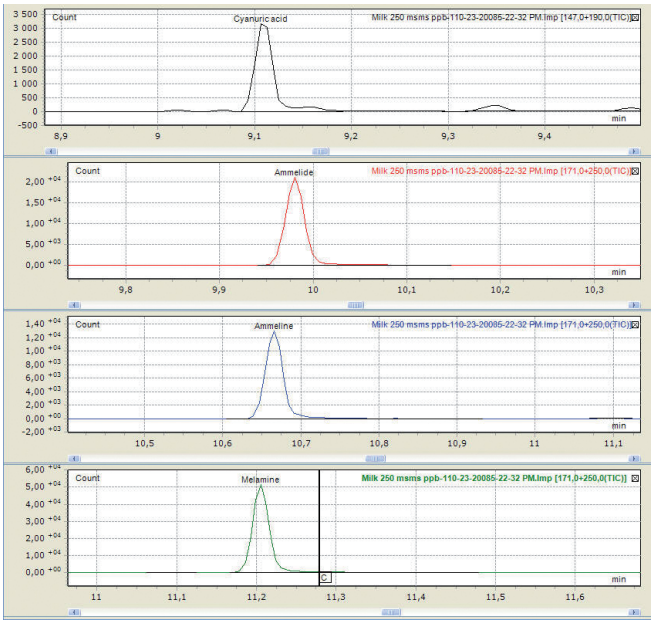


Figure 7. MRM of melamine and analogs spiked at 250 ppb in the infant formula extract in MS/MS mode.

Recovery studies of melamine, cyanuric acid, ammelide and ammeline were conducted in all three matrices at different concentrations (Table 2). Most compounds showed good recovery with excellent %RSD. Recovery of ammeline in infant milk was poorer overall with higher %RSD. This may have been due to variable extraction efficiency of ammeline from the infant milk matrix. Use of an internal standard, such as 2,6-diamino-4-chloropyrimidine, may alleviate the variation.

Table 2. Recovery and %RSD of melamine, cyanuric acid, ammelide and ammeline in MS/MS mode in dry dog food (n=3), dry cat food (n=3) and infant formula (n=6).

Average percentage recovery (%RSD)					
Matrix	Spiked (µg/g)	Cyanuric acid	Ammelide	Ammeline	Melamine
Dog food	20	132 (5)	104 (6)	102 (10)	119 (7)
Cat food	20	125 (8)	123 (9)	100 (9)	113 (8)
Infant milk	5	113 (12)	123 (8)	56 (22)	108 (6)

Conclusions

The method reported here, using the Varian 220-MS ion trap GC/MS/MS, screened and quantitated melamine and its analogs, cyanuric acid, ammelide and ammeline, in pet foods and infant formula at a concentration as low as 0.4

µg/g, which was 25 times lower than the FDA recommended level. MS/MS significantly eliminated matrix interference, providing an extra layer of confidence to positively identify and quantify target analytes. The linearity ranges studied were from 20 to 2000 ppb, and the detection limit was below 10 ppb for all four compounds.

References

1. FDA (2009) GC-MS Screen for the presence of Melamine, Ammeline, Ammelide and Cyanuric acid. US Food and Drug Administration, Silver Springs, MD, USA.
2. FDA (2009) Interim melamine and analogues safety/risk assessment. US Food and Drug Administration. US Food and Drug Administration, Silver Springs, MD, USA.

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Application Note SI-02407

Authentication of Scotch Whisky by GC Analysis Using an Agilent J&W CP-Wax 57 CB Basic Column

Application Note

Author

Johan Kuipers
Agilent Technologies, Inc.

Introduction

The production of genuine Scotch whisky is strictly regulated to ensure its quality and to protect whisky as a premium product. Various analytical methods are employed to confirm the authenticity of Scotch whisky brands. GC analysis is one of the techniques frequently used in the identification of counterfeit whisky products.

The characteristics of Scotch whisky are strongly influenced by the cereals used in fermentation and by distillation, maturation and blending. This leads to characteristic analytical profiles for the alcohol congeners, which can be used as reference points in authenticity analysis. The CP-Wax 57 CB Basic column is highly suited for this alcohol congener identification separating the most critical peak pairs:

- 2-methyl-1-butanol and 3-methyl-1-butanol
- Isobutanol and isoamyl acetate
- Ethylacetate and acetal

Due to the intensive crosslinking of the CP-Wax 57 CB liquid phase, the column shows an excellent stability and robustness for injections of water/alcohol related samples such as spirits. The base treatment of the Wax 57 liquid phase ensures the interference free elution of furfural from acetic acid making this particular GC column highly suited for the analysis of whisky and related spirits.

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Conditions

Technique:	GC-FID
Column:	CP-Wax 57 CB Basic, 50 m x 0.25 mm x 0.20 µm (part number CP97723B)
Sample Volume:	0.5 µL
Carrier Gas:	3.0 mL/min H2, constant flow
Injector:	200 °C, split 1:25
Temperature:	35 °C. 6 °C/min, 120 °C
Detector:	FID, 210 °C

Discussion

The suspect whisky (Figure 1) exhibits a different alcohol profile compared to genuine blended Scotch whisky (Figure2). Relative concentration levels for n-propanol and isobutanol are much lower compared to a Scotch whisky, this as a result of a higher distillation efficiency used in the manufacturing process of the counterfeit product. Also the 3-methyl-1-butanol / 2-methyl-1-butanol ratio is relatively high (> 5.0) indicating a spirit of non-cereal origin (Figure 3).

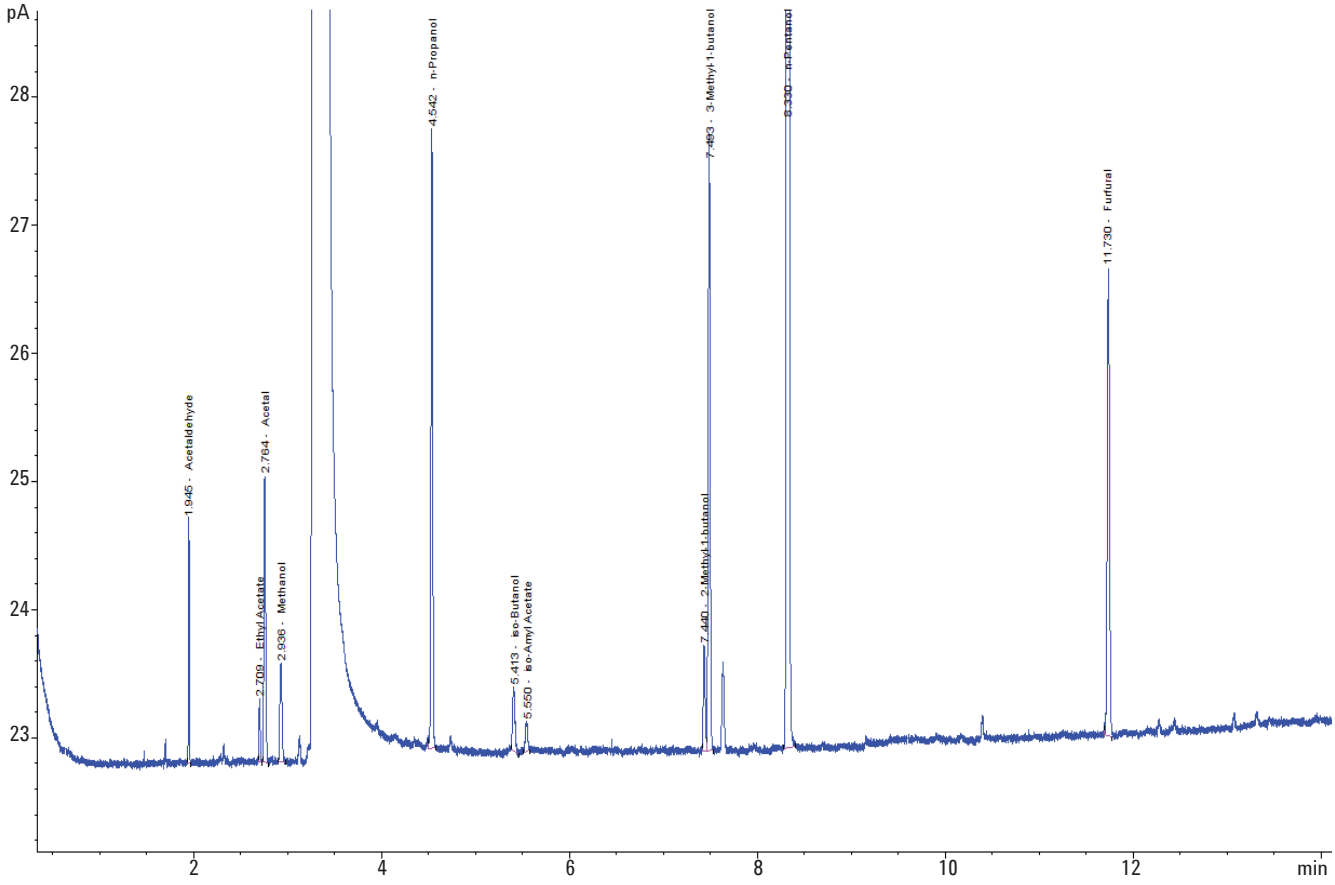


Figure 1. GC analysis of counterfeit Scotch whisky on CP-Wax 57 CB Basic

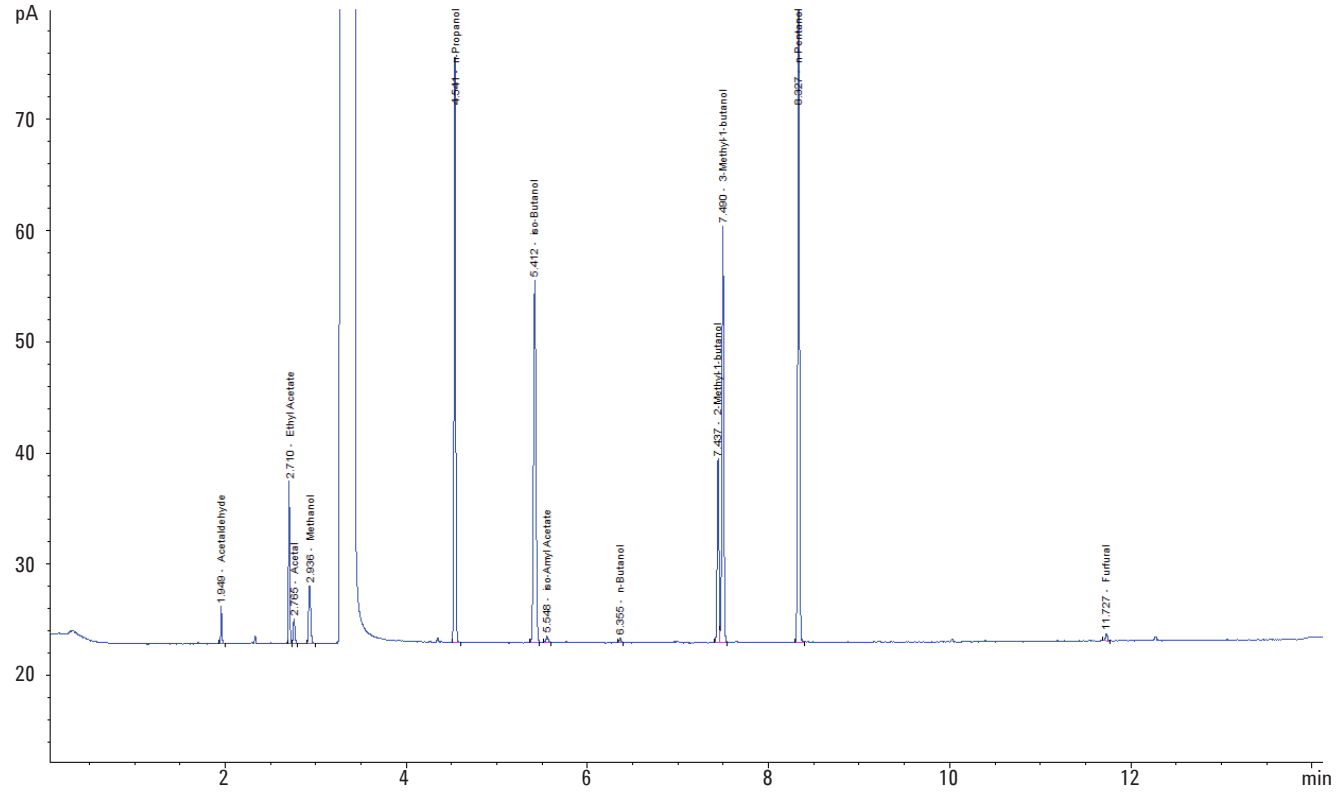


Figure 2. GC analysis of genuine blended Scotch whisky on CP-Wax 57 CB Basic

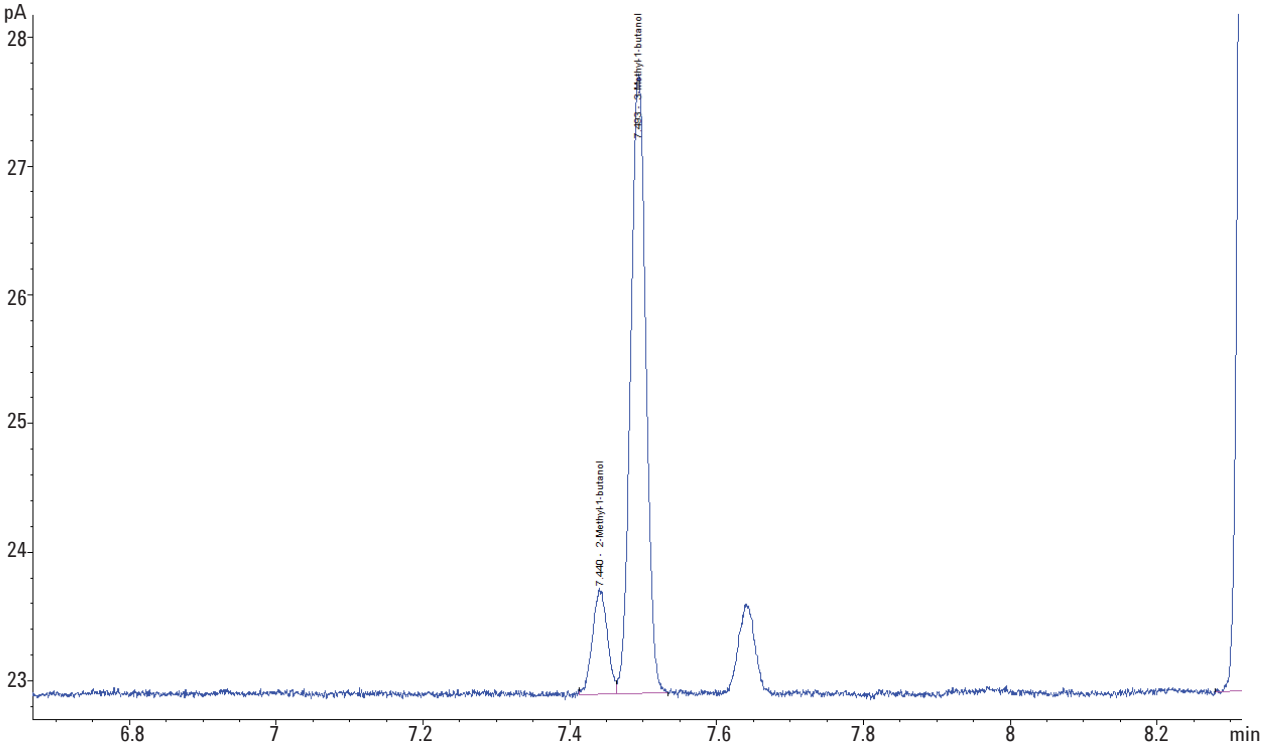


Figure 3. Expanded view of counterfeit Scotch whisky analysis on CP-Wax 57 CB Basic

Conclusions

The authentication of whisky products can be successfully carried out using the CP-Wax 57 CB Basic column because of its excellent separation of important chemical whisky descriptors such as the 2-methyl-1-butanol and 3-methyl-1-butanol. The highly durable CP-Wax 57 CB Basic column will provide a reliable GC analysis of alcoholic spirits for many hundreds of samples.

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Development of meat speciation assays using the Agilent 2100 bioanalyzer

Application

John Dooley
Steve Garrett

Abstract

The use of real-time PCR assays for quantitative PCR is becoming more frequent. During the development of such assays it is necessary to match both the PCR primers and the fluorescent probe (used for detection) in a single reaction. The probe production costs are high compared to the primer production costs. It is, therefore, useful to know that the newly designed PCR primers are functioning in an expected manner before the cost of probe production is incurred. This Application Note describes the use of the Agilent 2100 bioanalyzer with the DNA 500 LabChip® kit to confirm that primer sets are suitable before the probe is finally produced.

Introduction

Campden & Chorleywood Food Research Association (CCFRA) is interested in the development of PCR based methods for food authenticity, particularly in relation to the detection and quantification of meat species in meat products. The assays must be applicable to processed foods and, therefore, use small DNA targets as the extracted DNA is often degraded. One approach is to develop real-time methods based on the ABI Prism 7700 Sequence Detection System, known as TaqMan. The method is suitable for amplicon detection in the range 60–150 base pairs. It is common during the TaqMan assay development stage to find several suitable probes, each with several different primer sets. Although, theoretically, these assays should all work to consistent levels, practically there are variations between them and some assays are unlikely to work at all. Therefore, it is advisable to confirm that the primers designed are specific (produce only a single PCR product with no primer-dimerization) and will work under TaqMan conditions (high $MgCl_2$ concentration and strict cycling parameters). These conditions are fundamental to the accurate quantification of samples. Although confirmation of primers can be performed using traditional agarose gel methods, the Agilent 2100 bioanalyzer has several advantages over the agarose methods including speed of analysis, accurate quantification of PCR yield and sizing of products. In addition, safety is improved as there is a reduced risk from handling DNA staining dyes such as ethidium bromide. Using the DNA 500 LabChip kit allows accurate sizing of small PCR products. This is advantageous for real-time PCR where the amplicon size required is small (less than 150 bp). We describe the use of the Agilent 2100 bioanalyzer to assist in the development of assays suitable for sensitive detection of one meat species in another.

Materials and Methods

Design of PCR assay

TaqMan PCR assays (primers and probes) were designed using the Primer Express software (Applied Biosystems, Warrington, Cheshire, UK). Primers (forward and reverse) were designed to amplify single genomic DNA targets from pig, cow, sheep, turkey and chicken of less than 150 bp, in accordance with TaqMan design restraints. Primers were produced by MWG-Biotech, UK.

Performance of PCR reaction

PCR was performed in 25-µl volumes using 300 nM of each primer, 5 mM $MgCl_2$ and 100 ng of template DNA. A TaqMan-based amplification protocol (30 cycles of a two-step reaction consisting of 95 °C for 15 seconds and 60 °C for one minute) was applied to the reactions. PCR was finished with a final 10-minute step at 72 °C.

DNA 500 LabChip preparation

Chips were primed according to Agilent's instructions, provided with the chips. Samples (1µl) of each PCR reaction were loaded onto the DNA 500 LabChip following Agilent protocols and the chips were loaded into the Agilent 2100 bioanalyzer. The analysis of the DNA products was performed using the DNA 500 protocol of the accompanying software.

Results and Discussion

To perform absolute quantification it was necessary to design two assay types, a species-specific assay and a total meat assay that would be suitable for all meat species.

Species-specific assay development

Figure 1 shows examples of results. Figure 1A was obtained following amplification of different mammal or poultry species with a specific turkey assay. A single band was obtained with turkey (lane 5) only, i.e. there was no amplification with chicken, pork,

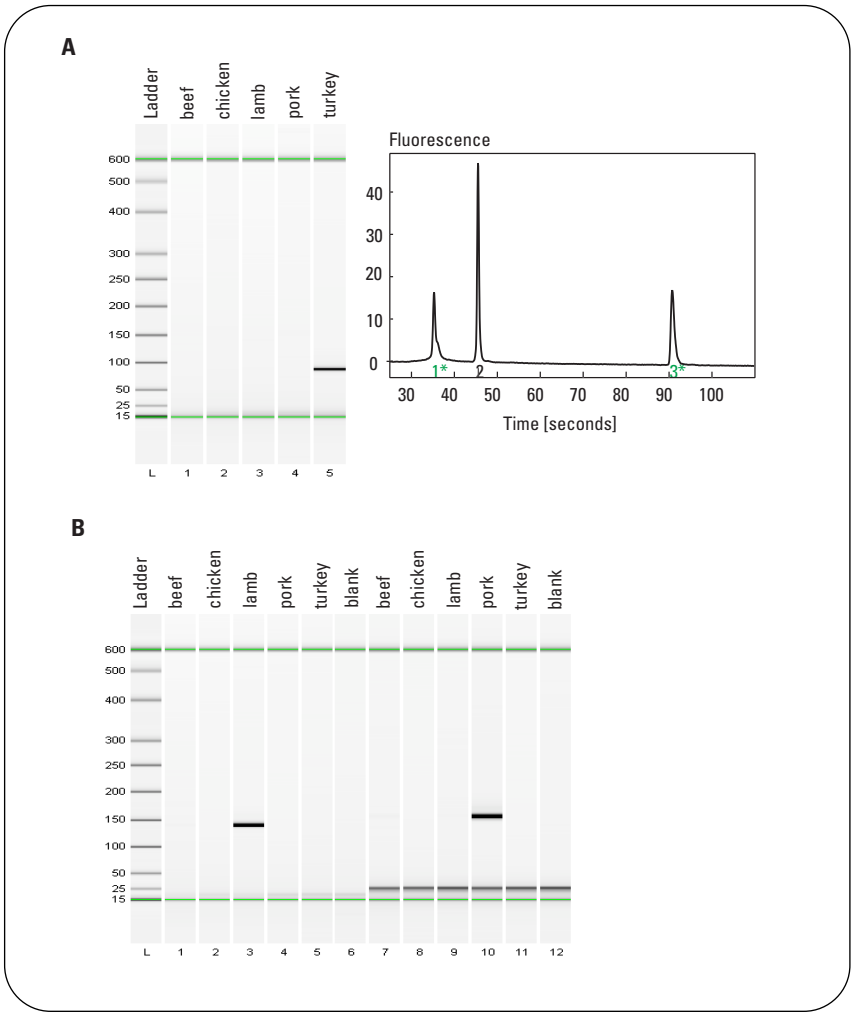


Figure 1
Species specific PCR amplification of meat samples.
A) With turkey specific primers only turkey samples are amplified. The electrophoretic trace confirms the high purity of the fragment.
B) Different sets of primers can be designed that allow the species specific PCR amplification of lamb (lanes 1-6) or pork (lanes 7-12).

beef and lamb. This band was not seen in any other poultry or mammal species. Figure 1B shows results of amplification with a set of specific mammal primers. A fragment can only be seen in the correct species, with no amplification detectable in any other species. Similar results were obtained for all five species under investigation. These results suggested that these primer sets would be suitable for individual species detection on the TaqMan. The appropriate probes were produced and the assays optimized for TaqMan usage. Species-specific amplification was observed on the TaqMan system.

Total meat assay development

Figure 2A shows the results of designing a total meat assay. The aim was to develop an assay that would amplify all meat species with the same degree of efficiency. The assay was also designed to show no amplification with non-meat species, including fish. Figure 2A shows that a single band of equal intensity was observed in all five species, whether of mammal or poultry origin. Figure 2B shows the overlay of the electrophoretic traces for these PCR products. The yields for all species were similar (mean 5.43± 0.64 ng/µl suggesting that this assay would be suitable for developing TaqMan-based, absolute quantification protocols. No amplification was observed in non-meat samples tested, including maize, soya, wheat and fish (figure 2A lanes 6-12). Figure 3 shows results from the complete TaqMan assays where the primers and probes were combined. Poor clarity (smudging) of the bands is possibly due to the use of dUTP in the TaqMan assay as opposed to the

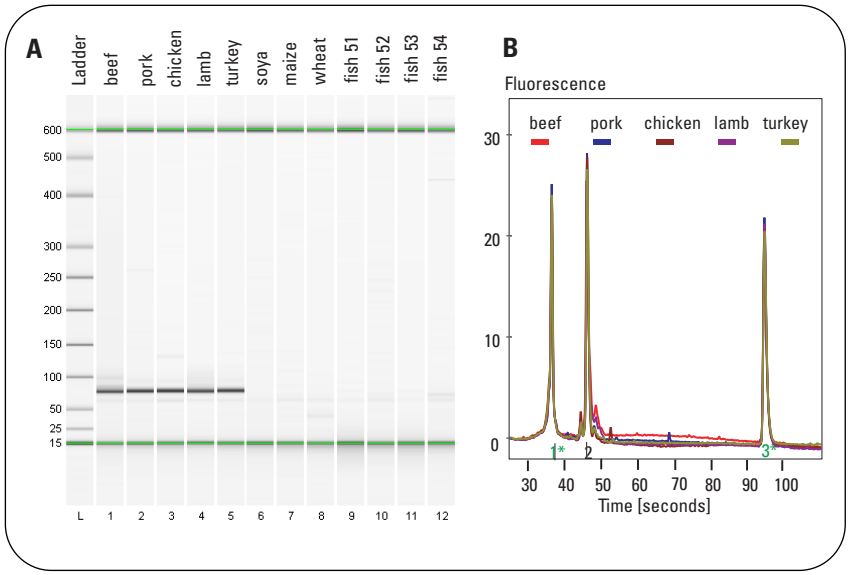


Figure 2
Development of a meat specific assay.
A) A set of primers can be designed that amplifies specifically all meat samples but does not amplify grain or fish.
B) The overlay of the electrophoretic traces reveals uniform amplification levels for different meat species.

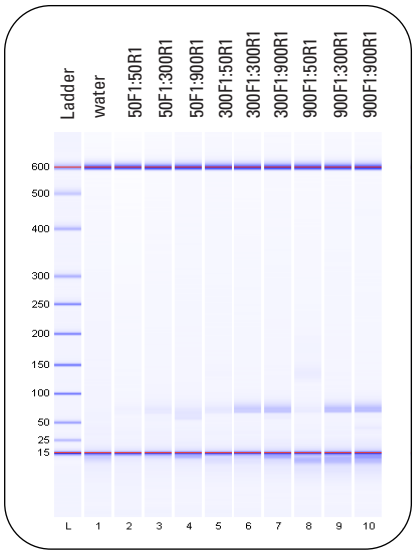


Figure 3
PCR products using TaqMan probes. Results from assy optimization test using pork DNA. Primers (forward [F] or reverse [R]) were used at 50, 300 or 900 nM concentration. Results show that at least 300 nM of F or R primer is required for amplification. 300 nM of each primer was found to be optimal for this amplification.

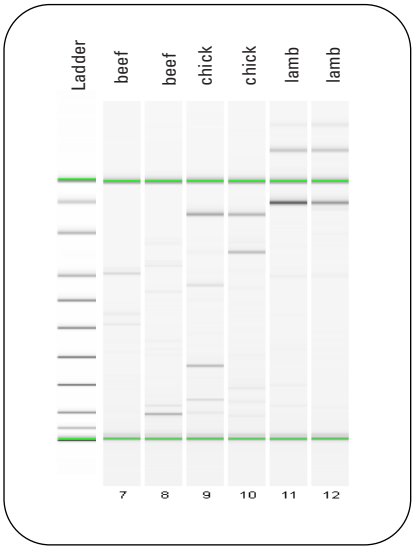


Figure 4
Non-specific amplification using non-optimal sets of primers.

use of dTTP, which was used in the conventional PCR reactions. An example of a primer set, although designed to be specific, is not specific in practice, is shown in figure 4. This assay was designed to amplify a single target in all meat species. As can be seen the number, size and yield of PCR fragments varies between the species. This primer pair was, therefore, inappropriate for use but having used the Agilent 2100 bioanalyzer to check the primers before purchasing the probe saved a considerable expense.

Conclusion

We believe the Agilent 2100 bioanalyzer provides a quick, visual method to confirm primer specificity and suitability for use in TaqMan assays. Although it would be possible to perform similar checks using SYBR Green DNA stains in the TaqMan machine itself, it is not possible to determine if the observed fluorescent change is due to primer-dimer formation or from the target of interest. The Agilent 2100 bioanalyzer allows confirmation of this and also confirmation that only a single target of expected size is being amplified. The ability of the Agilent 2100 bioanalyzer to quantify PCR yields is useful especially if assays being designed are required for quantitative or semi-quantitative determination, or as in our case to design a single assay suitable for detecting multiple species.

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Agilent Technologies

Analysis of genetically modified soya using the Agilent 2100 bioanalyzer

Application

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Abstract

In this Application Note we describe how the Agilent 2100 bioanalyzer was used to analyze multiple PCR products from Roundup Ready soya DNA. The multiplex assay assessed the effects of heat and low pH on subsequent amplification of genetically modified DNA and estimated levels of Roundup Ready soya within a sample.

Agilent Technologies

Introduction

Genetically modified organisms (GMOs) and derived food ingredients are regulated throughout the European Union (EU). Legislation requires appropriate labeling of products containing GM DNA. Whilst DNA methods based on the polymerase chain reaction (PCR) are suitable for monitoring known GMOs in raw materials and processed foods, there is concern that the analytical methods are less reliable for quantification purposes. Particular concern is for processed foods, where soya and maize ingredients, which are most likely to contain GMOs, are only a minor component of the finished product.

Food processing has a significant effect on the quality of DNA. Physical and chemical factors such as shear forces, heat treatment, nuclease activity and low pH will lead to degradation of the DNA. Soya is a common component of a wide range of foods, used as flour, protein isolate or concentrate. Soybeans are usually defatted by pressing and/or solvent extraction. In both processes, the soybeans are heated to 60–80 °C and the resulting protein meal can then be concentrated by extraction using weak acid (pH 4.5). These processes combined with further processing during product manufacture significantly reduce the quality of the soya DNA in the final product. This fragmentation of DNA reduces the probability of PCR detection particularly if the fragment sizes are smaller than the DNA sequence that is amplified by the primers.

Studies indicate that small sequences of DNA remain detectable following all but the most extreme processing conditions. In routine screening analysis for GMOs the use of small targets (<200 bp) is common. However, if there is differential degradation in these small targets (some sequences will be more susceptible to degradation than others) quantifying GMOs in processed foods using amplification of two similar but slightly differently sized targets may affect the accuracy of the results.

The aim of this project was to study the effect of food processing on PCR-based amplification and quantification of GM DNA. The approach was to develop a simple model assay system to observe differences in detection when using small targets in Roundup Ready (RR) soya heat treated at low pH. The Agilent 2100 bioanalyzer was used in post-PCR analysis to measure the concentration and number of differently sized PCR products.

Results

Assay development

The aim was to develop a model assay that could be used to assess the quality of DNA extracted from heat-processed soya flour samples, in particular, to investigate differences in PCR amplification between small DNA targets. A single multiplex PCR assay was developed that enabled four GM soya targets to be analyzed in a single reaction mix. Primer concentration was optimized in order to obtain four PCR products resolved by gel electrophoresis which corresponded in size to the soya lectin gene target of 80 bp, and the EPSPS (5-enolpyruvyl-shikamate-3-phosphate synthase) gene targets of 117 bp, 150 bp and 202 bp respectively. These latter targets are only found in Roundup Ready GM soya (Monsanto).

Although gel-based analysis enables sizing of PCR products, it cannot be used to provide accurate information on the quantity of a PCR product. Therefore, post-PCR analysis was performed using the Agilent 2100 bioanalyzer, which can accurately size and quantify PCR products. Initially, this was carried out using the DNA 7500 LabChip® kit. Four peaks were observed, corresponding to the PCR products within the lectin and EPSPS genes. However, the 80 bp peak from the lectin gene was not completely resolved from the alignment marker so quantification was not possible. Subsequently, post-PCR analysis was performed using the DNA 500 LabChip® when it was made available and resolution of all four peaks was observed (figure 1).

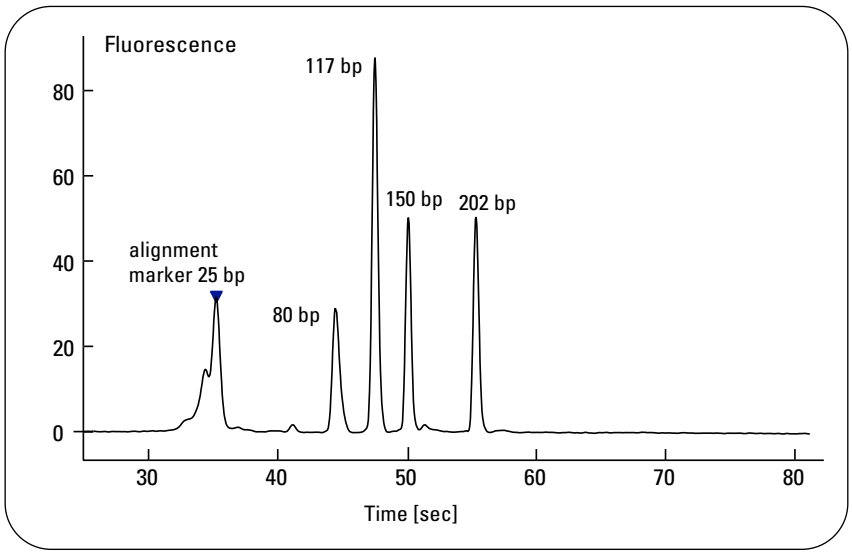


Figure 1
Multiplex assay for GM soya. Peaks produced by the four PCR products when analyzed with the Agilent 2100 bioanalyzer and DNA 500 LabChip kit.

The multiplex PCR assay was applied to DNA from RR soya flour reference materials (figure 2). The results show that there is an increase in PCR product concentration of the 117 bp, 150 bp and 202 bp products and little change in the concentration in the 80 bp product. This increase corresponds to the increase in RR content of the soya flour. No increase in the product from the lectin gene was expected, as it is common to both the GM- and non-GM soya. It should therefore be possible to estimate levels of GM soya in an unknown material by applying the multiplex assay and comparing the ratio of lectin product to the other products in the sample with ratios produced from certified reference materials (CRMs). The assays would have to

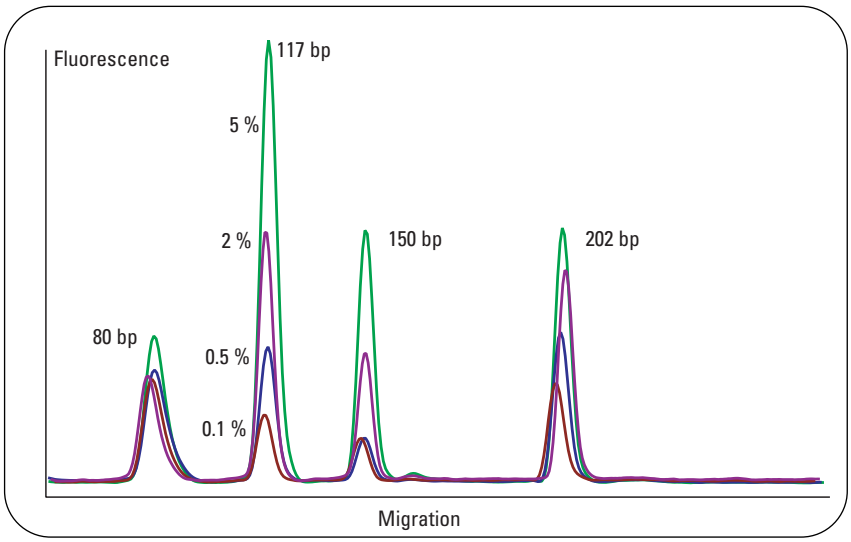


Figure 2
Peaks produced by the Agilent 2100 bioanalyzer using the multiplex assay on CRMs containing different levels of GM soya

Sample	Ratio 117/80	Ratio 150/80	Ratio 202/80
Extract 1a	1.50	0.68	1.04
Extract 1b	1.45	0.68	1.04
Extract 2a	1.40	0.46	0.71
Extract 2b	1.54	0.65	1.10
5% CRM	2.10	1.00	0.94
2% CRM	0.74	0.21	0.68
1% CRM	0.41	0.18	0.43

Table 1
Analysis of a soya flour

be performed using a limited number of PCR cycles in order to perform the end-point detection during linear stages of amplification. The reference materials and the unknown samples would also have to be similar in nature.

Analysis of a soya flour containing GM soya

The multiplex assay was applied in duplicate to two DNA extracts prepared from a soya flour sample which had given a positive result when screened for a common GM promoter sequence (CaMV 35S promoter). The assay was also applied to 1 %, 2 %, and a 5 % CRMs. Ratios of each EPSPS product compared to the lectin product were calculated (table 1). The same extracts were analyzed using a real-time PCR method for quantitative determination of GM soya. Results from the real-time analysis indicated that the sample contained approximately 5 % GM soya while the multiplex assay gave ratios indicating that the sample contained between 2 % and 5 % GM soya.

The effect of heating time and pH on detection and quantification of GM-DNA

The multiplex PCR assay was applied to soya flour samples containing approximately 1.3 % GM soya and boiled at either pH 3.3, 4.3 or 6.7 for up to 21 minutes. For accurate determination of the quantity of each PCR product, the samples were applied to the DNA 500 LabChip. The concentration of each PCR product was calculated using the Agilent 2100 bioanalyzer software. At pH 3.3 where an effect of heating time was observed, the amount of each PCR product at each time point was compared to the amount of each product at 0 minutes (table 2). At pH 3.3, the relative amount of the 80 bp product was reduced to 48 % after 15 minutes and no product was detected at 18 or 21 minutes. After 15 minutes, the relative amounts products of 118 bp and 150 bp were reduced to 27 % and 16 % respectively and the 202 bp product was not detected. None of the products were detected after 18 or 21 minutes.

Time at 100 °C and pH 3.3 (min)	Amount of PCR product*			
	80 bp	118 bp	150 bp	202 bp
0	100	100	100	100
3	74	77	73	67
6	57	58	21	6
9	36	23	24	15
12	67	33	47	21
15	48	27	16	0
18	0	0	0	0
21	0	0	0	0

* % product determined relative to the amount at 0 minutes

Table 2
The effect of heating time on RR flour held at pH 3.3, determined using the multiplex PCR method.

To eliminate any variation due to amount of DNA in each PCR reaction, the ratio of the lectin 80 bp product to each of the other three products was determined for all experiments (table 3), that is, normalized with respect to the 80 bp product. The ratios of each would be expected to remain constant if no degradation of the target DNA occurred or if the degree of degradation between the 80 bp target and the other targets was comparable. At pH 3.3 the ratios tended to increase with increasing heating time. This suggests that at low pH there were differences in the detectability of the three EPSPS targets compared to the smaller lectin target, with the

Time at 100 °C (min)		Ratio lectin 80bp/ RR-117bp	Ratio lectin 80bp/ RR-150bp	Ratio lectin 80bp/ RR-202bp
pH 3.3	0	1.8	3	1.9
	3	1.8	3	2.1
	6	1.7	7.8	17.5
	9	3	5	5
	12	3.6	4.4	6
	15	3.8	9	NP
	18	NP	NP	NP
pH 4.3	0	2	4.4	1.9
	3	2.2	2.9	1.8
	6	1.3	2	1.9
	9	1.3	2.2	2.3
	12	1.5	2.6	2.6
	15	1.8	3.7	2.7
	18	1.9	3.9	3
pH 6.7	0	1.8	4.2	1.7
	3	1.7	3.9	1.6
	6	1.2	2.3	1.5
	9	1.5	2.4	1.7
	12	1	1.9	1.3
	15	1.2	2.1	1.3
	18	1.4	2	1.4
	21	1.6	2.4	1.4

NP= no PCR products observed

Table 3
The effect of heating time on RR flour held at pH 3.3, 4.3 and 6.7, determined using the multiplex PCR method

80 bp target being degraded at a slower rate compared with the other targets. At pH 4.3, the 80/118 bp and 80/145 bp ratios decreased during the first 3–9 minutes of heating, then increased returning to the their original value, whereas the 80/202 bp ratio increased with heating time. Similar trends were observed at pH 6.7 except for the 80/202 bp ratio where little change occurred. However, further analyses are required to replicate these observations and focus around the pH where an effect is observed. These initial results indicate that the different targets used in PCR are not detected equally in these experiments.

Other studies show similar results. In 1998 Hüpfer et al.[1] demonstrated that PCR detection of GM maize in polenta could be influenced by pH during thermal treatment of the product. They showed that detection of a 1,914 bp segment of the cry1A(b) gene was not possible after boiling at neutral pH for 30 minutes, whereas a 211 bp fragment was detected after boiling for 105 minutes. At pH 2-3, the larger segment was not detected after boiling for 5 minutes and the smaller fragment was not detected after 15 minutes. As a result of such observations, it is common practice to use small target sequences in screening methods for GMOs. However, the work reported here suggests that at low pH, degradation of DNA results in differences in detection of very small target sequences. This may not be important for qualitative analysis, however, it is likely to have significance for the accuracy of quantitative analysis of processed foods with low levels of GM-DNA, when two target sequences are analyzed simultaneously as in real-time PCR.

Conclusion

The RR multiplex assay was used to quantify the amount of GM soya in a soya flour and assess the effects of pH and heat on the detection of GM soya DNA. A key component of the assay is the Agilent 2100 bioanalyzer which is used to accurately quantify the four PCR products simultaneously. This user-friendly instrument replaces gel based analysis and offers enormous potential for the routine screening of raw materials for levels of genetically modified organisms.

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Agilent Technologies

Detecting genetically modified organisms with the Agilent 2100 bioanalyzer

Application

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Hafida Oujati
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Genolife

Abstract

Labeling of food containing more than 1% of genetically modified organisms (GMOs) has been obligatory in Europe since January 2000. To guarantee transparency and labeling, methods to distinguish between transgenic food and their traditional counterparts must be available. Genolife developed a method to detect Ready RoundUp soy (RRS) and a multiplex PCR to detect five corn transgenes (Bt176, Bt11, MON810, T25 and GA21). The Agilent 2100 bioanalyzer and DNA 500 LabChip® kit provided a simple, high throughput and standardized way to analyze multiplex PCR products. The methods developed by Genolife allow detecting 0.01% of RRS in food ingredients on the one hand and 10 copies of transgene MON810, GA21 and Bt11, and 100 copies of transgene Bt176 and T25 on the other.

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Introduction

The rapid development of biotechnology has launched products and ingredients derived from genetically modified organisms (GMOs) into the food market. The general public, however, has shown anxiety about this new technology. Information and transparency regarding these products are essential in order to become accepted by the consumers. In Europe, labeling of GMOs is regulated by the Novel Food directives 258/97/EEC[1] and 1139/98/EEC[2]. More recently, the "threshold regulation" 49/2000/EEC[3] has been approved, specifying that foodstuffs are subject to labeling. When the proportion of an individual food component is higher than 1 % manufacturers must label their products. Moreover, the presence of GMOs must be adventitious and therefore, food manufacturers must be able to supply evidence that they have taken appropriate steps to avoid using GMOs. A key factor to guarantee transparency and labeling is the availability of methods to distinguish between transgenic food and their traditional counterparts, not only in raw materials but also in food products.

Several analytical methods using polymerase chain reaction (PCR) technology have been developed to qualitatively detect the presence of a modified sequence of nucleic acid in transgenic food.[4,5] But these analytical methods detect only one modified sequence of one genetically modified organism. It would be advantageous to detect more than one sequence per genetically modified organism (one endogenous gene and several transgenic markers) or to screen several GMOs in one analysis. We therefore developed a method to detect RoundUp Ready soy (RRS) from Monsanto in one part, and another method to detect one endogenous maize gene and five genetically modified maize genes – four are authorized in Europe (Bt176, Bt11, MON810 and T25) and one is non-authorized (GA21). The Agilent 2100 bioanalyzer and DNA 500 LabChip® kit provided a simple, rapid and standardized alternative to analyze multiplex PCR products.

Results and discussion

RRS detection in food ingredients

DNA was extracted from commercial transgenic soybean reference standards (Fluka) and different food samples (lecithin, soybean proteins, soybean flour) with specific protocol developed by Genolife. For each sample, four PCR reactions were done — one for amplification of an endogenous gene (ACC1, 115 bp) to check the quality of the extracted DNA, and three PCR reactions for specific RR soybean sequences (T1: 167 bp, T2: 141 bp and T3: 189 bp). After amplification, PCR products were mixed and 1 µl of each mixed PCR was analyzed on the Agilent 2100 bioanalyzer using the DNA 500 LabChip kit, which allows analysis of DNA fragments ranging in size from 25 to 500 bp. Twelve samples were analyzed simultaneously and the 2100 bioanalyzer produced raw data and analysis in multiple formats. It displayed a simulated gel view an electropherogram. A data table labels each of the peaks and furnishes information about the size and concentration for each fragment. Results are shown in figure 1. Non transgenic soy gives

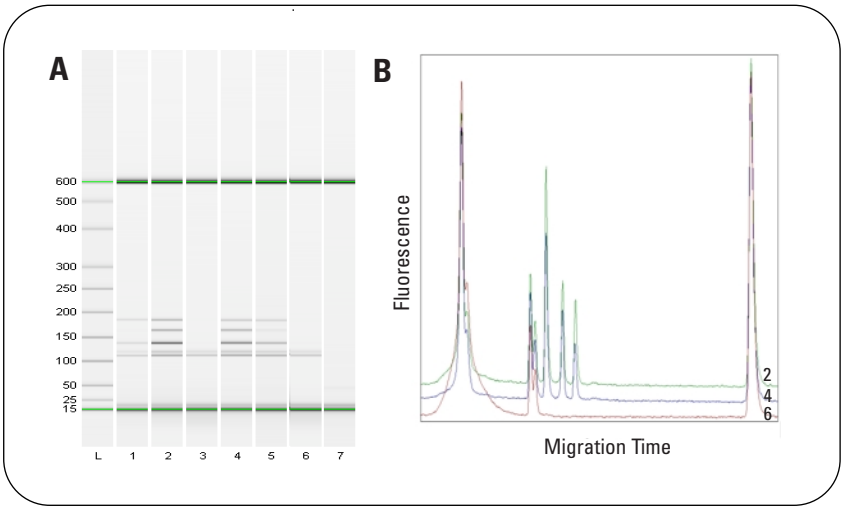


Figure 1
RR soybean detection
A) Gel view: 1-soya protein, 2- lecithin, 3-soybean flour, 4-RR soy 1 %, 5-RR soy 0.1 %, 6-non transgenic soy, 7-PCR blank.
B) Overlay of the electrophoretic traces of lanes 2, 4 and 6.

one band corresponding to the endogenous gene (115 bp) while RR soy 1 % and RR soy 0.1 % show four bands corresponding to the endogenous gene (115 bp) and the three specific RR soybean sequences (141, 167 and 189 bp). The Agilent 2100 bioanalyzer software compares unknown samples with commercial transgenic soybean reference standards. Soybean proteins and lecithin are transgenic (four bands at 115, 141, 167 and 189 bp) and soybean flour is not transgenic (only one band at 115 bp corresponding to endogenous gene). The 2100 bioanalyzer performs quantification using an internal standard (marker) added to each sample before loading, and the software calculates the DNA concentration in each band. Soybean protein contains less than 0.1 % and lecithin more than 1 % RR soy, compared to concentration DNA in transgenic band obtained with commercial transgenic soybean reference standards (table 1). The detection limit of this RR soy PCR is 0.01 %.

	RR1 %	RR 0.1 %	Soya protein	Lecithin
141 bp	1	0.24	0.12	1.3
167 bp	0.1	-	-	0.34
189 bp	1	0.56	0.48	1.3

Table 1
DNA concentration (ng/µl) of the band corresponding to the transgenic markers

GMO maize detection in food ingredients by multiplex PCR

In Europe, four GMOs maize were authorized — two insect-resistant corn species from Novartis (Bt11 and Bt176), one insect-resistant corn from Monsanto (MON810) and one glufosinate-tolerant corn developed by Agrevo (T25). We developed a PCR multiplex to detect these four corn lines and one endogenous gene to check the integrity of the extracted DNA. We also added a couple of primers to detect a glyphosate-tolerant corn GA21 produced by Monsanto. This glyphosate-tolerant corn is authorized in the USA and can be exported in Europe with authorized corn. The PCR multiplex amplified a 152-bp fragment for endogenous gene, a 343-bp fragment for Bt176 corn, a 149-bp fragment for T25, a 199-bp fragment for MON810, a 110-bp fragment for Bt11 and a 270-bp fragment for GA21. Results are presented in figure 2. The endogenous gene was amplified in all lanes except PCR blank. Only Bt176 fragment (343 bp) was obtained when only Bt176 corn was present in PCR tube (lane 4). Specificity was checked for each corn (lane 5: Bt11, lane 6: T25, lane 7: GA21 and lane 8: MON810). Lanes 1 to 3 presented PCR products when all corn lines were analyzed together. This multiplex PCR allows detection of five corn lines present at 0.2 % each (lane 3). The detection limit of this multiplex PCR is 10 copies for transgene MON810, GA21 and Bt11 and 100 copies of transgene Bt176 and T25.

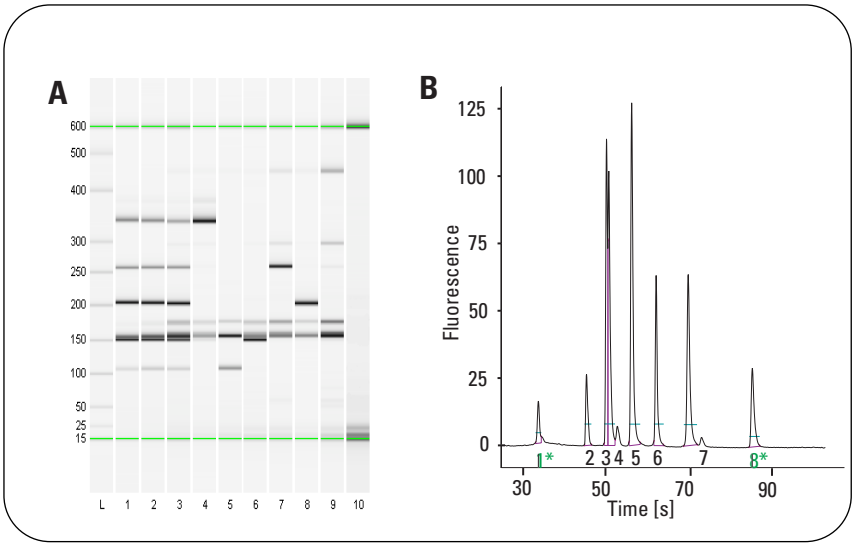


Figure 2
Multiplex PCR to detect GMO corn
A) 1-Bt176 5 %, Bt11 2 %, T25 5 %, GA21 5 %, MON810 5%, 2-Bt176 2 %, Bt11 2 %, T25 2 %, GA21 2 %, MON810 2 %, 3-Bt176 0.2 %, Bt11 0.2%, T25 0.2 %, GA21 0.3 %, MON810 0.3%, 4-Bt176 2 %, 5-Bt11 2 %, 6-T25 2 %, 7-GA21 2 %, 8-MON810 2 %, 9-non GMO corn, 10-PCR blanc
B) Electrophoregram of lane 2 (multiplex PCR with mix of 2 % corn). The peaks are: Bt11 corn (2), T25 corn (3), endogenous gene (4), MON810 corn (5), GA21 corn (6) and BT176 corn (7).

Conclusion

We developed detection methods for GMO soy and corn in food ingredients. The Agilent 2100 bioanalyzer performed the analysis of multiplex PCR product and allowed a semi quantification of GMO content. Two detection methods were presented— one for RR soy and one for GMO corn detection. The sensibility of the soya detection method is 0.01 % and 10 to 100 copies of transgene for corn mulitplex detection.

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Agilent Technologies

Characterization of Transgenic Soybean Seedlines by Protein Expression with the Agilent 2100 Bioanalyzer

Application

Food

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Abstract

This application note describes how the Agilent Technologies 2100 Bioanalyzer can be used to analyze protein extracts from transgenic seedlines. Accuracy and precision in the determination of protein size and concentration was sufficiently good to allow for the characterization of experimental seedlines based solely on expressed protein profiles.

Introduction

ß-conglycinin(7S) and glycininin(11S) are the primary seed storage proteins in soybean, comprising about 70% of the total storage proteins. Because these proteins make up such a large portion of soya protein, they are of critical economic importance. Characterizing the expression of these proteins in various soybean seed lines is also essential in expanding the range of soy protein applications in food. The relative levels of these two proteins have been shown to significantly impact the nutrition, taste, and texture of food products derived from soy protein extracts [1]. For this reason, soybean lines that preferentially express the 11S or 7S proteins continue to be an active target in the efforts to improve seed quality.

Both conglycinin and glycinin are complex aggregates made of smaller protein subunits. β-conglycinin is a 7S protein with a trimeric structure and is composed of 53, 70, and 76 kDa units. Glycinin is an 11S hexameric protein consisting of six monomer units, where each monomer is made up of 40 and 20 kDa subunits [2].

Given the sizes of protein subunits, it is relatively straightforward to characterize the levels of these two proteins by electrophoresis. The Agilent 2100 Bioanalyzer, an automated microfluidic electrophoresis platform, is well suited for the analysis of proteins in this size range. The Protein 200 LabChip has a size range of 14-200 kDa. Samples of soy protein isolate can be loaded, separated, and analyzed for relative protein composition in less than 45 minutes. In this application we describe the use of the Agilent 2100 Bioanalyzer in the analysis of soybeans, to determine the level of expression of 7S and 11S proteins.

Experimental

Extraction Protocol

Grind the seed into a fine powder. Place a 30 mg sample in an Eppendorf® tube and add 1000 µL of extraction buffer (50 mM Tris [pH 7.5], 10 mM 2-mercapto-ethanol, 0.1% SDS). Agitate the mixture on a rotary shaker for 30 minutes and then centrifuge at 15,000 g for 10 minutes. Remove the supernatant and introduce the extract directly into the Protein 200 LabChip to begin the assay protocol.

If the extracts contain an excessive amount of oil, the supernatant may be removed and further diluted prior to beginning the Protein 200 protocol.

Methodology

To determine if a transgenic line of soybeans preferentially expressed the β-conglycinin or glycinin protein groups, seed extracts were compared to extracts made from wild-type seed lines that strongly expressed either the 7S or the 11S group. Twenty extracts from the unknown seedline were prepared as described above. The protein profiles were determined by separating the proteins in the Agilent 2100 Bioanalyzer. Because of the size range of the proteins, the Protein 200 Plus chip (14-200 kDa) was used for the separation. The concentration of the individual proteins was determined by a comparison with an internal concentration marker (myosin). The ratio of 7S/11S proteins was then calculated from those values and the ratio was compared to the ratio of wild-type seedlines that preferentially expressed either 7S or 11S protein groups. Figure 1 shows the electropherogram for a control seedline, a 7S wild-type, an 11S wild type, and a representative sample from the unknown transgenic seedline. A simulated gel image of the same traces is shown in Figure 2.

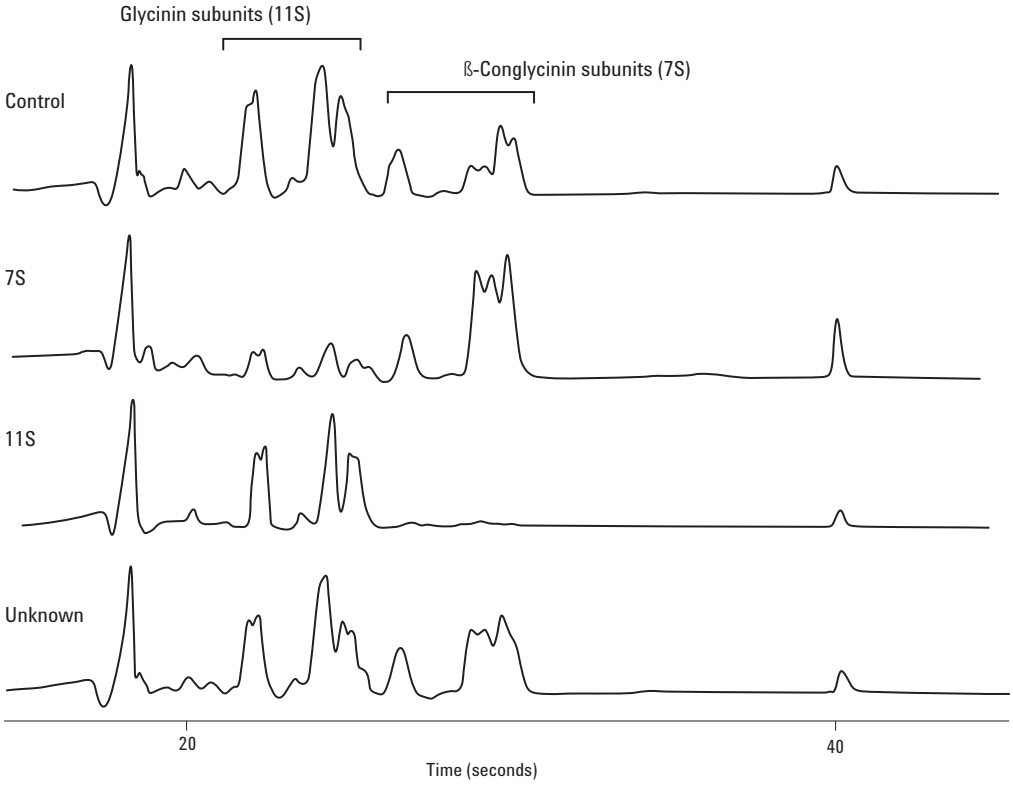


Figure 1. Electropherograms of soya protein extracts.

The ratio of 7S to 11S for the 20 sample extracts was calculated by integrating the individual components comprising the 7S and 11S groups and then determining the summed areas of the two groups. The levels of extracted protein and the 7S/11S ratios for the control 7S, 11S, and unknown groups are summarized in Table 1.

The ratios determined for the high 11S and high 7S seedlines indicate the range of expected 7S/11S ratios should fall between 0.04-3.4. The ratios determined for both the controls and unknown extracts both fall within this range. All 20 unknown samples showed a higher 7S/11S ratio than the control. Average ratio values for 20 unknown extracts and 5 control extracts was 0.72 and 0.39, respectively. Measurement precision was excellent for both sample sets.

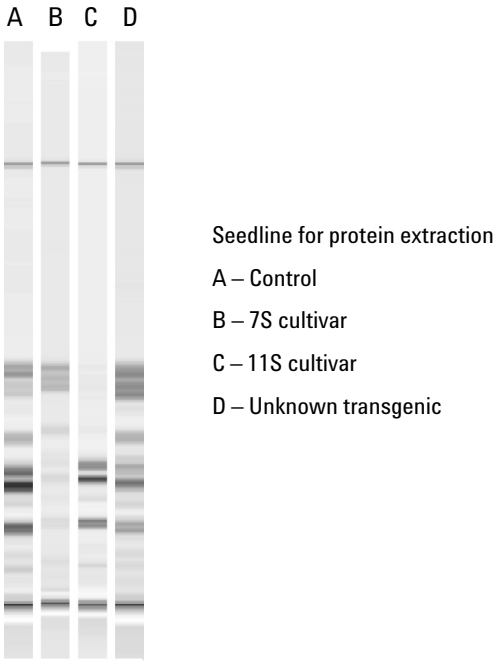


Figure 2. Gel simulation of electropherograms for soya protein extracts.

Table 1. Summary of Extracted Protein Levels and 7S/11S Protein Ratios

SeedLine	Extracted protein level µg/mL	7S/11S Ratio
Control	14,000	0.39 ±0.004 (n=5)
7S	5,200	3.4
11S	14,000	0.04
Unknowns	13,000	0.72 ±0.1 (n=20)

Conclusions

This application note describes the use of the Agilent 2100 Bioanalyzer and the Protein 200 LabChip Kit for evaluating the relative expression of β-conglycinin and glycininin in unknown seedlines. In the 20 protein extracts taken from the unknown seedline, the average ratio was 0.72 ±0.1. This ratio lays in range that is characteristic of high 7S expression seedlines. Given the precision of the ratio determination, it is clearly apparent that the assignment of this unknown seedline to the high 7S group is statistically significant. This conclusion is further supported by a comparison to a normal control seedline where the ratio of 7S/11S is 0.39 ±0.004.

The Agilent 2100 Bioanalyzer together with the Protein 200 LabChip Kit are quick and efficient tools for the determination of relative protein expression. The resulting protein expression profiles are in turn a highly effective means for the characterization of new transgenic seedlines.

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Nested Multiplex Polymerase Chain Reaction for the Determination of DNA From Genetically Modified Corn and Soy Beans Using the Agilent 2100 Bioanalyzer

Application

Agriculture

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Abstract

This application note describes how the Agilent Technologies 2100 bioanalyzer and the DNA 500 LabChip can be used to detect polymerase chain reaction products corresponding to genetically modified elements and endogenous sequences in corn and soy beans. The DNA extraction protocol used in the preparation of polymerase chain reaction samples was characterized using the Protein 200 Plus LabChip.

Introduction

Six years after the introduction of genetically modified organisms (GMO), consumer concerns about the presence of such modified organisms in food remains an ongoing issue. There has been a continuous debate surrounding issues of how food products that contain GMO ingredients should be regulated and labeled. This debate has been further complicated by disagreements over how GMOs should be detected and the significance of the detected levels. In spite of these issues, the number of available transgenic events has continued to grow. At the current time there are 14 transgenic varieties of corn and soy beans that have been deregulated by the Animal and Plant Health Inspection Service of the United States Department of Agriculture (USDA).

Although enzyme immunoassay is an efficient means for detecting transgenic proteins in raw products, only DNA analysis has proved to be effective for the entire range of sample matrices from raw materials to highly processed foods. The polymerase chain reaction (PCR) has been widely accepted as a method for the detection of DNA from genetically modified ingredients such as soya or corn [1, 2]. PCR detection of GMOs can be done either as a screening test using endpoint PCR or as a quantitative test using real-time fluorescence detection of the PCR product.

Quantitation by real-time PCR is an expensive analysis requiring assay calibration for each sample lot and multiple replicates of each unknown sample. Typical service charges for a single analysis are between $150–$300. Since each transgenic event must be evaluated individually, the cost of rigorously testing an unknown food sample for all the possible current transgenic events is cost prohibitive.

These cost constraints make it necessary to screen samples for DNA components that are present in

most GMOs prior to any quantitative analysis. This type of screening analysis can be carried out with a commercially available PCR test kit, such as the Biosmart Allin 1.0 GMO Screening System from Promega (Madison, Wisconsin). This kit provides a protocol and reagents for a nested multiplex PCR assay for the detection of DNA from modified organisms containing the 35S promoter. This genetic element is derived from the cauliflower mosaic virus and is found in most transgenic crops. In addition to the 35S promoter, the multiplex PCR reaction also detects sequences for soya (lectin), corn (zein) and an internal positive control. These multiplex PCR products are all easily resolved and detected using the DNA 500 LabChip and the Agilent 2100 bioanalyzer.

Experimental

DNA Extraction

Three DNA extraction protocols were evaluated prior to the PCR analysis. These protocols were the DNeasy Plant Mini Kit from Qiagen N. V. (Venlo, Netherlands), the Wizard Genomic DNA Purification Kit from Promega (Madison, Wisconsin) and a cetyltrimethylammonium bromide (CTAB) precipitation procedure that was developed in-house. Detailed descriptions of the DNeasy and Wizard Genomic kit protocols can be found in the technical manuals that accompany these products. The CTAB protocol is described below.

CTAB Extraction protocol:

1. A 50-mg sample was added to a 1.0-mL aliquot of CTAB extraction buffer (0.055 M CTAB (cetyltrimethylammonium bromide), 0.1 M Tris, 1.4 M NaCl, 0.2 M disodium EDTA pH 8.0), heated at 65 °C for 15 min and then placed on ice.
2. The extract mixture was centrifuged at 15,000 g for 10 min to remove particulates.
3. The supernatant was removed and stored in a new microfuge tube at 4 °C. (Samples with high starch levels, for example corn, were treated with 0.2 µL of α-amylase and incubated at 37 °C for 30 min.)
4. The recovered supernatant was combined with an equal volume of chloroform mixed for 30 s and then centrifuged for 10 min at 15,000 g. The upper (aqueous) phase was transferred to a new tube.
5. Two volumes of the CTAB precipitation solution (0.014 M CTAB, 0.04 M NaCl) were added to aqueous extract and mixed.
6. The mixture was incubated at 25 °C for 30 min and then centrifuged for 10 min at 15,000 g. The supernatant was discarded.
7. The precipitate was redissolved by adding 250 µL of the 1.2 M NaCl and incubating 37 °C for 10 min. The mixture was centrifuged for 5 min at 15,000 g and the supernatant was transferred to a new tube.
8. One volume of isopropanol was added to the mixture and then the mixture was stored at 4 °C for at least 30 min.
9. The solution was centrifuged for 10 min at 15,000 g and then the supernatant was discarded.
10. The pellet was washed with 500 µL of cold 70% ethanol, the mixture was centrifuged for 5 min at 15,000 g, and then the ethanol solution was discarded.
11. After drying the tube, the DNA pellet was redissolved in 25 µL of Tris buffer (0.01 M Tris, 0.001 M EDTA pH 8.0).

The soya reference standards were used in the evaluation of the DNA extraction because soya contains a high level of protein that can be accurately tracked through each step of the extraction procedure. Although the soya protein is not problematic for the PCR assay, it serves as a useful indicator of overall DNA purity. Protein levels in the final DNA extracts were determined by a protein measurement using the Protein 200 Plus LabChip and the Agilent 2100 bioanalyzer. Absorbance measurements were made with the ND-1000 spectrophotometer from NanoDrop Technologies, Inc. (Wilmington, Delaware).

PCR Protocol

Samples used in the GMO analysis consisted of Institute for Reference Materials and Measurements (IRMM) Certified Reference GMO Soy and GMO Corn, as well as commercial corn meal and soya powder. The PCR analysis was carried out using the Biosmart Allin 1.0 GMO Screening System from Promega (Madison, Wisconsin). A modified PCR cycling protocol was developed for use with the PTC 200 Peltier Thermal Cycler from MJ Research, Inc. (Waltham, Massachusetts). The PCR protocol is described below.

Multiplex PCR Protocol

1. Dilute the DNA sample to an initial concentration of 5–50 ng/µL. (This corresponds to an optical density, (OD), at 260 nm of 0.1–1.0 for a 1-cm cell.)
2. For the first stage of the multiplex PCR combine the following per reaction:

   17-µL 2X Qiagen Multiplex PCR Master Mix

   27-µL Allin Mix 1

   5-µL Internal control

   1-µL Extracted DNA
3. Vortex the solution.
4. Perform first stage of PCR amplification using the following cycling program.

Step	Time	Temperature (°C)
1	15 min	95
2	15 s	95
3	60 s	55
4	30 s	72
5	Repeat steps 2–4 an additional 39 times	
6	3 min	72

5. For the second stage of the Multiplex PCR combine the following per reaction

   24.5-µL 2X Qiagen Multiplex PCR Master Mix

   24.5-µL Allin Mix 2

   1.0-µL Product from first stage PCR
6. Perform second stage of PCR amplification using the following cycling program.

Step	Time	Temperature (°C)
1	15 min	95
2	15 s	95
3	60 s	57
4	30 s	72
5	Repeat steps 2–4 an additional 39 times	
6	3 min	72

Analysis of PCR Products

The PCR products were analyzed using the DNA 500 LabChip and the Agilent 2100 bioanalyzer. For comparison, the same PCR products were also characterized by gel electrophoresis in a 4% NuSieve 3:1 Plus Gel. The electrophoretic separation conditions were 150 V for 60 min in a 1X Tris-borate-EDTA (TBE) buffer.

Results

DNA Extraction

DNA extraction protocols were evaluated for both yield and purity of DNA. Protein levels in the DNA extracts were determined using the Protein 200 Plus LabChip [3]. The results of this comparison are summarized in Table 1.

Table 1. Summary of DNA Extraction

Method	Yield (%)	DNA/Protein w/w	A_{260}/A_{280}
CTAB	0.008	2.7	1.82
DNeasy	0.004	2.5	1.89
Wizard Genomic	0.008	0.3	1.62

The CTAB and Wizard Genomic protocols showed the highest levels of DNA recovery with final DNA yields of 0.008% of the initial sample weight. The DNA/protein ratio of 0.3 found in the Wizard DNA extract indicated that this extract still contained a high level of protein. The corresponding ratio for CTAB was 2.7, indicating that the protein content of the CTAB extract was only 1/9 of the Wizard protocol.

The A_{260}/A_{280} ratio is also a useful indicator of DNA purity, with high purity DNA having an absorbance ratio of 1.8–1.9. A comparison of the A_{260}/A_{280} ratios for the CTAB and Wizard extracts, 1.82 and 1.62 respectively, confirmed the higher DNA purity in the CTAB process. The DNeasy extraction process produced DNA of comparable quality to the CTAB process, but at a significantly lower yield. Since the CTAB extraction protocol gave the highest yield of high purity DNA, this protocol was used to prepare the DNA extracts used in the PCR analysis.

Analysis of PCR Products

The Biosmart Allin 1.0 GMO Screening System is a multiplex PCR kit that is capable of generating four PCR products. These PCR products include the following:

Base pairs (bp)	Product
118	Soy lectin gene
150	35S Promoter
217	Internal control (corn zein sequence added to the PCR mix)
278	Corn zein gene

All four of these products can be found in either the positive or the negative control reactions using the Biosmart GMO Screening System. The DNA 500 LabChip has ample separation to resolve all of these PCR products. This resolution is illustrated in Figure 1, which shows a composite of the bioanalyzer electropherograms for the positive and negative controls.

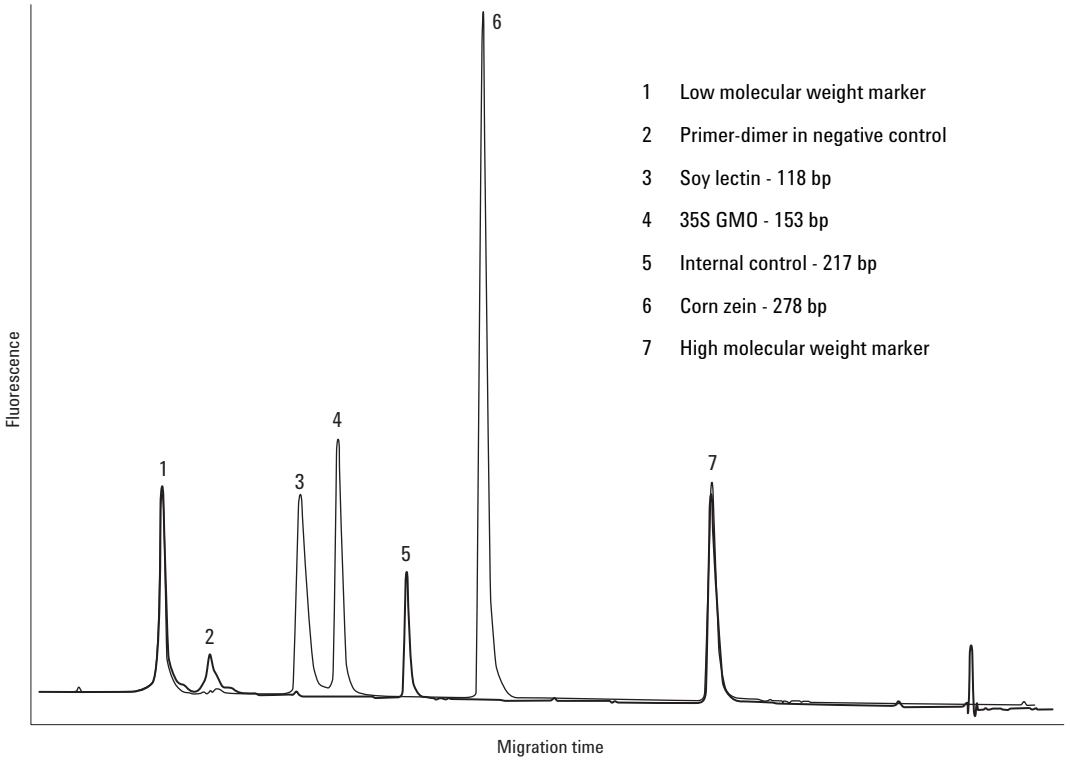


Figure 1. Composite of bioanalyzer electropherograms for positive and negative controls.

The PCR analysis was carried out on corn and soya sample sets. The corn sample set consisted of IRMM Certified Reference MON810™ corn standards at the following levels: 0% MON810, 0.1% MON810, 0.5% MON810, 1.0% MON810, 2.0% MON810, and 5.0% MON810, and commercial corn meal. The soya sample set was made of IRMM Certified Reference Roundup Ready® soya samples at the following levels: 0% Roundup Ready, 0.1% Roundup Ready, 0.5% Roundup Ready, 1.0% Roundup Ready, 2.0% Roundup Ready, and 5.0% Roundup Ready and commercial soya powder. Both sample sets contained a positive control with 0.5% each Bt176 corn and Roundup Ready soya, and deionized water as a negative control.

Figure 2 shows the electrophoretic gel separation and a gel-like bioanalyzer image for all of the samples listed above. The PCR products for the corn samples are shown in Figures 2A and 2C. Figures 2B and 2D show the soya results.

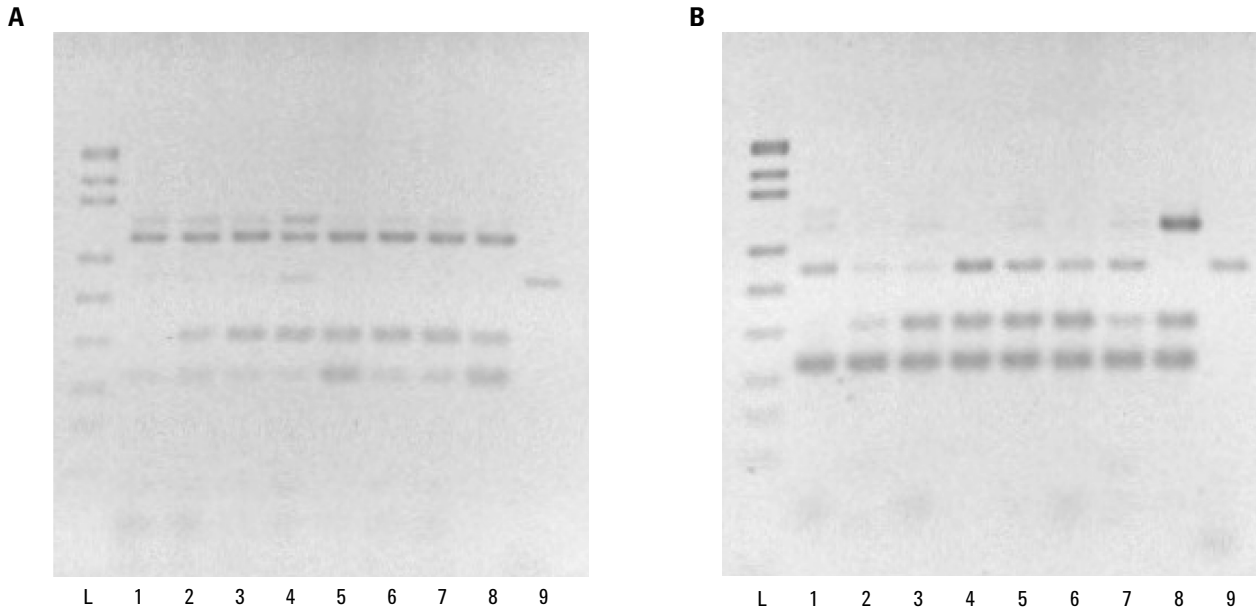


Figure 2A and 2B. 4% NuSieve 3:1 Plus Gel, 150 V for 60 min.

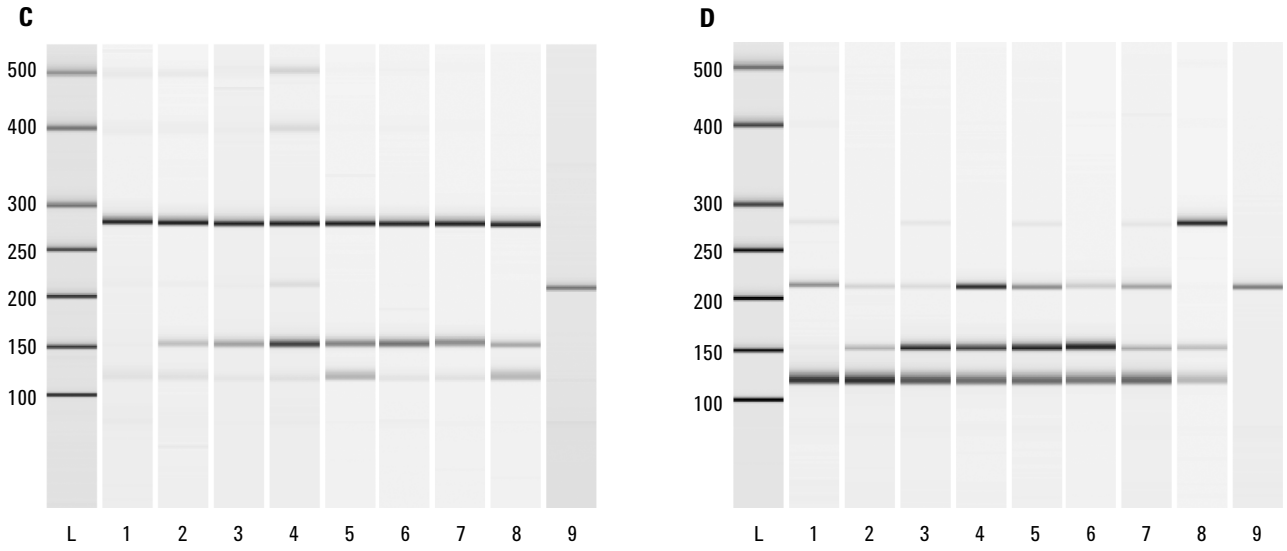


Figure 2C and 2D. Gel-like bioanalyzer image.

Figure 2. 2A (gel) and 2C (bioanalyzer)
L) Molecular weight ladder: 501, 489, 404, 353, 242, 190, 147, 110, 89, 67, 34, 34, and 26 bps **1**) 0% MON810 corn, **2**) 0.1% MON810 corn, **3**) 0.5% MON810 corn, **4**) 1.0% MON810 corn, **5**) 2.0% MON810 corn, **6**) 5.0% MON810 corn, **7**) Commercial corn meal, **8**) Allin positive control (0.5% Bt 176 maize and 0.5% Roundup Ready soybean), and **9**) Negative control (deionized water).

2B (gel) and 2D (bioanalyzer)
L) Molecular weight ladder, **1**) 0% Roundup Ready soya, **2**) 0.1% Roundup Ready soya, **3**) 0.5% Roundup Ready soya, **4**) 1.0% Roundup Ready soya, **5**) 2.0% Roundup Ready soya, **6**) 5.0% Roundup Ready soya, **7**) Commercial soya powder, **8**) Allin positive control (0.5% Bt 176 maize and 0.5% Roundup Ready soybean), and **9**) Negative control (deionized water).

Comparison of Gel and Bioanalyzer Response

The bioanalyzer DNA 500 LabChip clearly shows superior resolution and uniformity of band location compared to the 4% NuSieve gels. The enhanced reproducibility of band location in the gel-like bioanalyzer image is readily apparent in a visual comparison of Figures 2C and 2D to Figures 2A and 2B. Both the 4% gel and the bioanalyzer have sufficient sensitivity to visualize the 35S PCR product at the minimum corn and soy reference standard levels of 0.1% GMO.

A comparison of the initial 35S level in the sample to the amount of 35S PCR product shows that the 35S GMO PCR band increases as the GMO content increases. The PCR response in both corn and soy samples appears to saturate before the maximum GMO standard level of 5% is reached. This effect can be clearly demonstrated using the bioanalyzer's ability to measure PCR product concentrations. Tables 2 and 3 show the concentrations of the corn, soy, and 35S PCR products. For the corn samples, the PCR product response saturates at 1.0% GMO. In the soy samples, the saturation occurs at 0.5% GMO.

Table 2. GMO Corn Response - Corn and 35S PCR Products

Sample	278 bp-Corn amplicon (ng/µL)	153 bp-35S amplicon (ng/µL)	Ratio of 35S/ corn amplicons
0% MON810 Corn	2.5	0.03	0.01
0.1% MON810 Corn	3.4	1.2	0.4
0.5% MON810 Corn	6.2	3.8	0.6
1.0% MON810 Corn	3.5	4.3	1.2
2.0% MON810 Corn	5.2	4.4	0.9
5.0% MON810 Corn	4.7	4.7	1.0
Corn meal	6	5.2	0.9

Table 3. GMO Soya Response - Soya and 35S PCR Products

Sample	118 bp-Soya amplicon (ng/µL)	153 bp-35S amplicon (ng/µL)	Ratio of 35S/ soya amplicons
0% Roundup Ready soy	5.1	0.1	0.02
0.1% Roundup Ready soy	5.4	0.8	0.15
0.5% Roundup Ready soy	4.9	3.4	0.7
1.0% Roundup Ready soy	5.3	4.0	0.8
2.0% Roundup Ready soy	5.1	4.4	0.9
5.0% Roundup Ready soy	4.8	5.0	1.0
Soya powder	4	0.9	0.2
Positive control	4.8	2.2	0.5

Although concentrations of PCR products can be determined quite accurately, some care must be exercised in the interpretation of these quantitative results. Under conditions where a correlation can be seen between the GMO content and the concentration of GMO amplicon, for example, GMO <0.5%, a rough estimate of GMO concentration can be made. However, such an estimate is only reliable if the same PCR master mix and thermocycler are used for all the amplification reactions. In addition, sample and calibration standard matrices must also be highly similar.

For the Biosmart GMO Screening System, quantitative conclusions cannot be made at concentrations >0.5% GMO because the PCR response is saturated. Once the PCR product concentrations have reached this level, small differences in amplicon concentrations are not useful in making quantitative estimates. An example of this can be seen in Table 3. The 0.5% GMO soy reference has a 35S/soy amplicon ratio of 0.7. In the positive control, where the sample has 0.5% GMO soy and 0.5% GMO corn, the corresponding ratio is only 0.5. Since the positive control contains at least twice as much 35S GMO marker as the 0.5% soy reference, this ratio would be expected to be greater than 0.7. It is difficult to explain why the ratio is lower in the positive control. This behavior could be the result of the complex reaction kinetics in a multiplex nested PCR assay or may simply reflect the imprecision in endpoint PCR amplicon concentrations.

PCR Product Composition

The internal control in the Biosmart Allin 1.0 GMO Screening System is a 217 bp corn sequence that uses the same primer sequences as the corn PCR product. According to the manufacturer, when high levels of corn DNA are present, competition for primer may result in the loss of the internal control PCR product. An examination of the corn PCR products in Figure 2C shows this effect in that the 217 bp is either absent or visible only at trace levels. However, in the soy PCR assay in Figure 2D, the 217 bp fragment can easily be seen in all the samples except the positive control that contains corn DNA.

In the corn sample set, a weak band can be seen at around 120 bp. This suggests that during the DNA isolation step, trace amounts of soya DNA were introduced into the corn samples. Likewise, the presence of a band at 280 bp in some of the soy samples indicates low levels of corn DNA were present in several of the soy samples. It is not surprising that trace levels of cross contamination should be apparent in a nested PCR assay. Since all of the samples undergo a net 80 cycles of amplification, even a few copies of soy or corn DNA will be sufficient to produce a detectable PCR product.

In PCR assays using a large number of amplification cycles, it is not uncommon for amplicons of similar sequence to cross hybridize. In the case of the Biosmart Allin 1.0 GMO Screening System, both the corn PCR product and the internal control share a region of common DNA sequence. When these two amplicons cross-hybridize, the resulting product has both single-stranded and double-stranded regions. These structures, known as heteroduplexes, have substantially lower mobility than a corresponding double-stranded structure. The relative mobility shift depends on such factors as gel composition, ionic strength, and gel temperature [4]. In Figure 2, PCR products that are larger than the 278 bp corn amplicon are observed. These bands occur at about 320 bps in the 4% gel and at 400 and 500 bps in the bioanalyzer electropherogram. Cross hybridization of the corn and the internal control amplicons is probably responsible for these products.

Conclusion

This application note described the use of the Agilent 2100 bioanalyzer with the Protein 200 Plus and DNA 500 LabChip Kits in the evaluation of sample preparation and the analysis of multiplex PCR products. The Protein 200 Plus was used to determine which DNA extraction procedure was most effective in removing residual protein. The DNA 500 LabChip was used to characterize the Biosmart Allin 1.0 GMO Screening System, a nested multiplex PCR assay for the genetically modified corn and soy beans. Resolution and sensitivity in these assays was sufficient to identify all of the targeted multiplex PCR amplicons and to differentiate these targets from PCR artifacts. Sensitivity of the assay was sufficient to detect GMO content even at the minimum GMO standard level of 0.1% in both corn and soy reference standards.

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Determination of PCR-RFLP Profiles for Fish Species Using the Agilent 2100 Bioanalyzer

Application

Food Safety

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Abstract

This application note shows how the Agilent 2100 Bioanalyzer was used in polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) fragment analysis for fish species identification. A 464bp cytochrome-b target sequence, found in all vertebrate fish, was first amplified and then digested with restriction enzymes. The fragments were resolved on the DNA 500 LabChip®, allowing simple comparison with authentic species profiles due to the accuracy of fragment size determination. Use of the Bioanalyzer offered significant benefits over traditional gel electrophoresis and DNA staining techniques for PCR-RFLP fragment analysis.

Introduction

The diversity of fish products available to consumers has increased significantly in recent years. Products can range from premium grade fish steaks to low cost fish fingers. As fish are caught, processed, and distributed by a global network of operators, there is a need to ensure the authenticity and the origin of fish used in the products.

There is, therefore, a need to have reliable and simple species identification methods to support enforcement and compliance with labelling legislation (EC Council Regulation No. 104/2000 and EC Commission Regulation No. 2065/2001).

Methods of fish species identification based on morphological characteristics are suited to whole fish; however, fish species identification becomes more problematic once it is processed. Protein profiling is used for fish identification; however, this method requires the analysis of species reference materials alongside the samples and is less reliable when applied to processed food products as the proteins become denatured. DNA based methods offer an alternative approach to species identification as DNA remains detectable in all but the most heavily processed samples.

Direct sequencing is the most definitive method of identification; however, it cannot easily be applied to samples suspected or known to contain more than one species. Alternative techniques, using polymerase chain reaction (PCR), were developed to identify fish species based on DNA fingerprint patterns. Methods used include RAPDs (random amplified polymorphic DNA), SSCP (single strand conformation polymorphism) and PCR-RFLPs.

A PCR-RFLP technique, which involved digesting an amplified 464bp region of the cytochrome b gene with restriction enzymes to generate DNA profiles, was previously developed for the identification of salmon species [1, 2].

The aim of this work was to improve the method for identification of salmon and other species by replacing conventional gel electrophoresis and staining steps with the Agilent 2100 Bioanalyzer. The generation of species-specific PCR-RFLP profiles on the 2100 Bioanalyzer combined with accurate sizing and quantification of individual DNA fragments, offered significant advantage over gel-based approaches in terms of ease-of-use, speed, and accuracy of identification.

Materials and Methods

All chemicals used for this work, unless otherwise stated, were supplied by Sigma-Aldrich and were of molecular biology grade or equivalent. PCR primers were supplied by MWG-Biotech UK Ltd. PCR-RFLP profiles were generated using a DNA500 LabChip and the Agilent 2100 Bioanalyzer. AmpliTaq® Gold DNA polymerase from Applied Biosystems was used in all PCR reactions. Restriction enzymes were obtained from New England Biolabs and used per the manufacturer's instructions.

Fish Samples

Commercially important salmon and white fish species samples were obtained from appropriate fishery research laboratories in the UK, Canada, Alaska, New Zealand, and Japan. Five individuals were used to minimize the effects of polymorphic variation within the population. Additional samples of each fish species were obtained from local UK fishmongers and retailers.

DNA Extraction

DNA extraction was performed using a modification of the CTAB method. Samples (2 g wet weight) were suspended in 5 mL of CTAB buffer (2% CTAB [hexadecyltrimethylammonium bromide], 100-mM Tris-HCl, 20-mM EDTA, 1.4-M NaCl, pH 8.0) and 40 µL of Proteinase K solution (20 mg/mL) was added. Samples were mixed thoroughly and then incubated overnight at 60 °C. After incubation, 1 mL of supernatant was transferred to a 2.0-mL Eppendorf tube, cooled to room temperature (RT), and centrifuged at 13,000g for 10 minutes. The clear supernatant was recovered and an equal volume of chloroform was added. The solution was vortexed and then centrifuged at 16,000g for 15 minutes before the upper aqueous layer was transferred to a clean 1.5-mL Eppendorf tube. An equal volume of isopropanol was added and the DNA precipitated at RT for 30 minutes. DNA was pelleted by centrifugation at 16,000g for 15 minutes, washed in 70% ethanol and air dried for 30 minutes at RT. The DNA pellet was resuspended in 100 µL of sterile distilled water (SDW) and purified using Promega's Wizard® Purification Resin per the manufacturer's protocol. DNA extracts were recovered in 50 µL of 1×TE (10-mM Tris-HCl, pH 7.4, 1-mM EDTA, pH 8.0) buffer. Final DNA concentrations (ng/µL) were determined using a GeneQuant pro DNA calculator (Pharmacia).

DNA Amplification

PCR products (464bp target from the cytochrome b gene) were produced by amplification of DNA extracts (50 ng) in 20-µL reactions containing 1× AmpliTaq Gold PCR buffer (Applied Biosystems), 300 nM of each primer (L14735: 5'-AAA AAC CAC CGT TGT TAT TCA ACT A-3' and H15149: 5'-GCI CCT CAR AAT GAY ATT TGT CCT CA-3'), 200-nM dNTPs, 5-mM $MgCl_2$ and 0.05-U/µL of AmpliTaq Gold (Applied Biosystems). Amplification profiles (94 °C for 5 minutes [denaturation]; 40 cycles of: 94 °C for 40 s, 50 °C for 80 s, 72 °C for 80 s [amplification]; 72 °C for 7 minutes [final extension] were applied using a PE9600 PCR machine (Applied Biosystems). Unpurified PCR products (1 µL) were applied to the 2100 Bioanalyzer to confirm amplification.

PCR-RFLP Profiling

Unpurified PCR product (2.5 µL) was digested for 3 or more hours with two to five units of enzyme in a total volume of 5 µL. Reactions were terminated by incubation at 65 °C for 10 minutes. Digested PCR products (5 µL) were mixed with 5 µL of 20-mM EDTA, to achieve a final concentration of 10-mM EDTA, prior to loading on to DNA500 LabChips. Aliquots (1 µL) of the reaction mix were analyzed on the 2100 Bioanalyzer, per manufacturers' instructions.

Results

Evaluation of PCR-RFLP Profiles Generated on the 2100 Bioanalyzer for Species Identification

An initial evaluation of the PCR-RFLP method was performed using salmonid species.

Following cleavage of the amplified DNA fragment with restriction enzymes, species-specific PCR-RFLP patterns were resolved on the 2100 Bioanalyzer. An example of a PCR-RFLP pattern is given in Figure 1, which shows PCR-RFLP profiles for salmon and trout samples generated with enzymes DdeI and HaeIII.

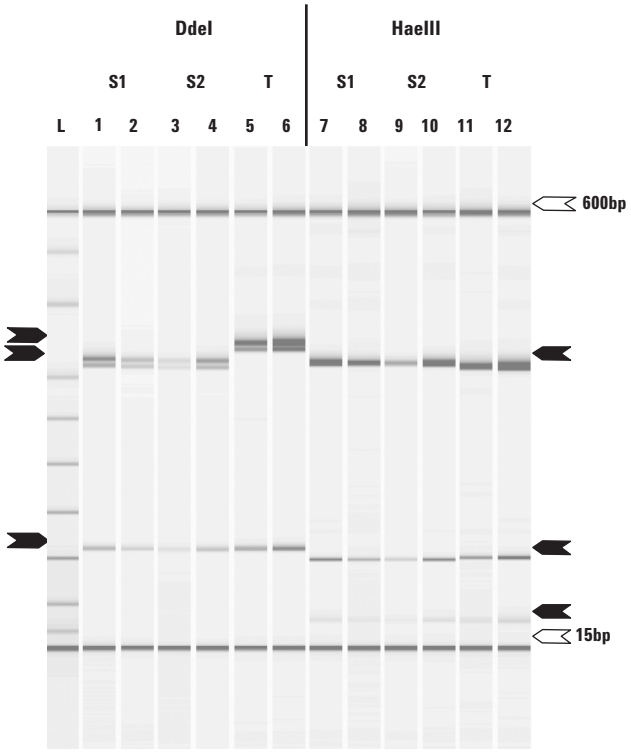


Figure 1. PCR-RFLP patterns obtained from salmon and trout with enzymes DdeI and HaeIII. PCR-RFLP patterns obtained when amplified DNA from two salmon (S1, S2) and one trout (T) samples were digested with DdeI (lanes 1–6) or HaeIII (lanes 7–12). A 15bp–600bp ladder (L) is shown. All wells contain 15bp and 600bp size markers. DNA fragments of note are indicated (arrows).

Observed and expected fragment sizes for a selection of five enzymes and four salmonids are shown in Table 1. As can be seen, patterns were similar to those reported previously [1, 2].

Expected DNA fragments of greater than about 25bp were readily detected; however, some smaller fragments were not detected because they could not be distinguished from the lower 15bp size marker or were outside the detection range (25bp–500bp) of the LabChip. Small DNA fragments (25bp–100bp), which were not reported previously, were observed in some digests [2]. This highlights the improved band resolution of this method in comparison to gel electrophoretic methods. This improved resolution is also highlighted by profiles generated with DdeI, where all four species have an extra fragment that is about 9bp smaller than the expected larger fragment. This is due to an extra DdeI site situated in primer H15149

Table 1 shows that *O. gorbuscha* and *O. mykiss* profiles generated with NlaIII have only two fragments when three are expected. This is believed to be due to the comigration of the two larger fragments. An analysis of the sequence of the *O. mykiss* 464bp amplicon indicated that cleavage of the amplicon with NlaIII should produce fragments of 192bp, 180bp and 91bp. These respectively equate to the 210bp, 190bp and 100bp fragments reported by Russell *et al.* (2000) and appear in Table 1. From the sequence data there is

Table 1. Expected and Observed PCR-RFLP Fragment Sizes Obtained with Five Restriction Enzymes and Four Salmonid Species

		Expected* (E) and observed (O) fragment sizes for each enzyme (bp)				
Species		**DdeI****	**Bsp1286I**	**HaeIII**	**NlaIII**	**Sau3AI**
O. nerka						
(Red salmon)	E	360, 130	300, 200	350, 130	310, 180	390, 120
	O	353, 346, 114	289, 172	320, 102, 35 or 421	272, 160	340, 115
O. gorbuscha						
(Pink salmon)	E	360, 130	U/C***	U/C	210, 190, 100	390, 120
	O	349, 343, 112	464	421	181, 92	338, 115
S. salar						
(Atlantic salmon)	E	350, 130	300, 200	350, 130	U/C	410, 110
	O	321, 312, 110	287, 172	318, 98, 35	438	370, 88
O. mykiss						
(Rainbow trout)	E	360, 130	300, 200	350, 130	210, 190, 100	U/C
	O	348, 339, 111	279, 174	313, 100, 33	185, 92	451

*Sizes as reported by Russell *et al.* (2000).

**Extra fragments in observed DdeI profiles are due to restriction site introduced by primer H15149.

***U/C Uncut with enzyme.

no evidence that the smaller 180bp fragment contains a higher proportion of heavier A or G bases or the larger 192bp fragment a higher proportion of lighter C or T bases, which could cause their respective molecular weights to converge. The calculated molecular weight difference (3277 Daltons) between the two fragments is equivalent to the difference in the number of bases. This makes it unlikely that comigration is due to molecular weight similarities between the two fragments. The comigration of these two fragments as a single fragment is consistently observed and does not detract from the identification of these species.

Overall, the profiles generated by the 2100 Bioanalyzer matched those expected or previously reported [1, 2], which supports the use of this approach for the identification of fish species. Further work was performed using Atlantic salmon and trout and a smaller number of enzymes to confirm the application of this approach.

Experimental Repeatability

In order to determine the experimental repeatability (LabChip-to-LabChip variability) of the complete assay, duplicate PCRs were produced from two salmon and one trout sample. Amplified fragments were cleaved with DdeI and digests stored at 4 °C until required. PCR-RFLP patterns were separated on four occasions using different DNA500 LabChips primed with two different batches of gel matrix, A and B. Two LabChips were run using a freshly prepared gel matrix (matrix A) while a third LabChip was run when gel matrix A was 1-week old. The fourth LabChip was run on the same date as the third LabChip but using a second, fresh batch of gel matrix (matrix B). Overall variation (encompassing variation due to LabChip-to-LabChip, PCR and gel matrix) appears in Table 2, which shows the results of analysis with the four LabChips following digestion with enzyme DdeI. Results are the mean fragment sizes observed on each LabChip from two PCR replicates of each species. Absolute fragment size variations within a single LabChip, that is, for PCR replicates of the same sample or between the two salmon samples, were less than 2bp and were only observed between the larger (>300bp) fragments. The overall absolute size variation for each fragment, which included variation due to different LabChips, PCRs and gel matrices, was slightly greater; the biggest variation was 6bp for the 320bp fragment in salmon (321bp to 327bp).

Fish Species Identification

Using sequence data generated from 10 different white fish species, theoretical PCR-RFLP profiles were generated from a range of restriction enzymes. Closer examination of these theoretical profiles indicated that only three enzymes would be needed to differentiate all the white fish species. Experimental profiles were generated to confirm that species identification was possible using just these three enzymes. Results of this analysis are shown in Figure 2, which shows that 9 of the 10 species could be identified based on profiles generated with only one enzyme. The remaining two species were differentiated using the other two enzymes.

Table 2. PCR-RFLP Fragment Sizes Obtained Following Separation of DNA Cleaved with DdeI on Four Different DNA500 LabChips

	Observed fragment sizes on each LabChip (bp)					
Expected band size (bp)	LabChip 1 (Fresh matrix A)	LabChip 2 (Fresh matrix A)	LabChip 3 (Week old matrix A)	LabChip 4 (Fresh matrix B)	Mean	%RSD
Salmon analysis*						
117	111	111	110	111	111	0.34
312	314	316	314	317	315	0.43
320	323	325	323	326	324	0.51
Trout analysis						
116	111	111	110	111	111	0.45
339	338	341	338	343	340	0.72
348	347	349	347	352	349	0.61

*An expected 27bp fragment from salmon is not detected by the 2100 Bioanalyzer.

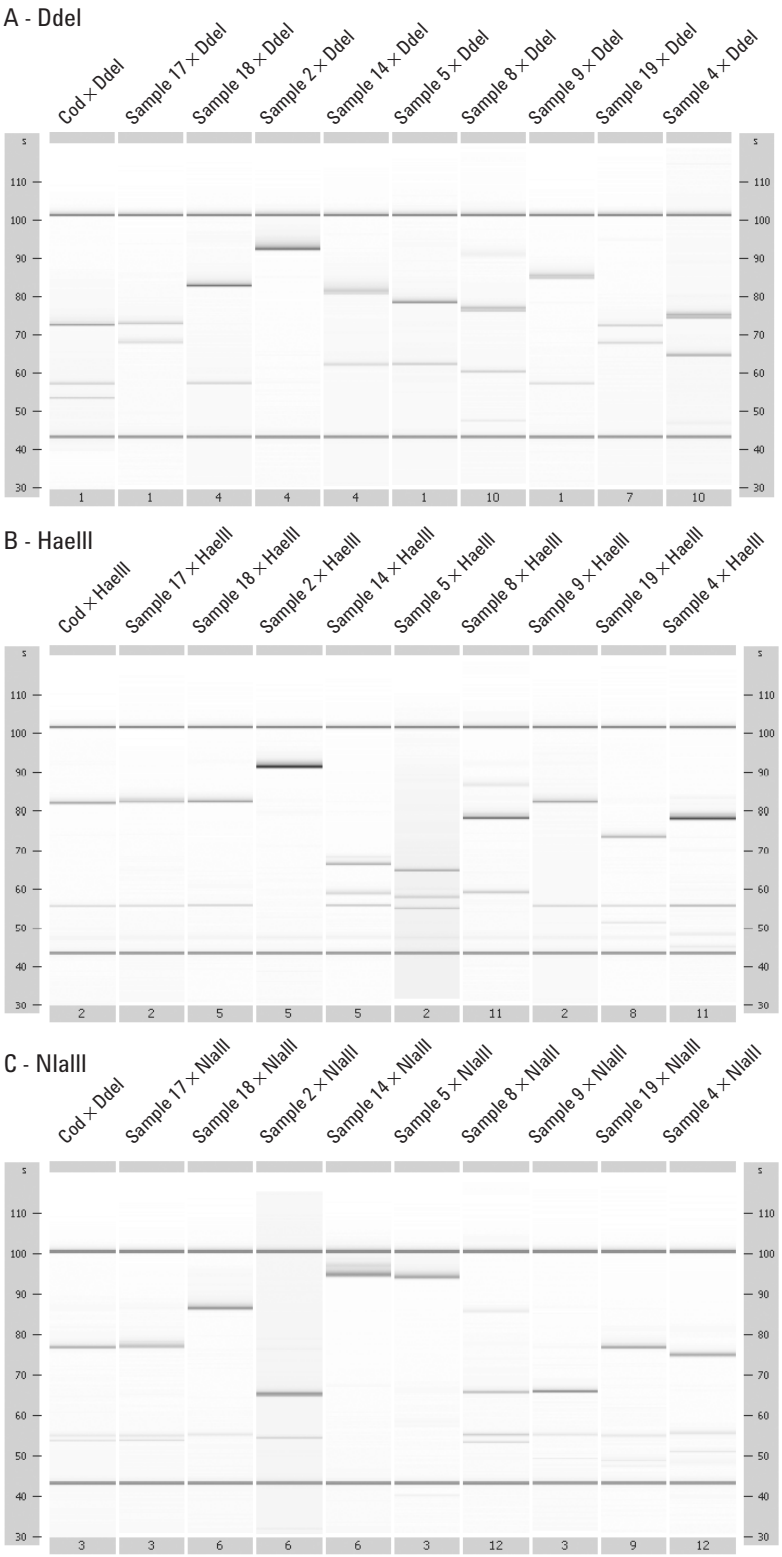


Figure 2. PCR-RFLP profiles from the 10 white fish species used in this study. Profiles were generated using enzymes DdeI (A), HaeIII (B) or NlaIII (C). Each sample number indicates a different fish species.

Analysis of a further nine salmon species was performed using these three enzymes only (data not shown). Again it was found that the salmonids could be differentiated using the three enzymes. A list of all the species studied is found in Table 3.

Table 3. Fish Species that Could be Differentiated Using PCR-RFLP with Enzymes Dde I, Hae III and NlaIII

Common name (UK)	Latin name
Atlantic cod	*Gadus morhua*
Pacific cod	*Gadus macrocephalus*
Coley (Saithe)	*Pollachius virens*
Haddock	*Melanogrammus aeglefinus*
European hake	*Merluccius merluccius*
South African hake	*Merluccius paradoxus*
European plaice	*Pleuronectes platessa*
Whiting	*Merlangus merlangus*
Alaskan(Walleye) Pollock	*Theragra chalcogramma*
Hoki	*Macruronus novaezelandiae*
Atlantic salmon	*Salmo salar*
Red / Sockeye salmon	*Oncorhynchus nerka*
Pink / Humpback salmon	*Oncorhynchus gorbuscha*
Chinook salmon	*Oncorhynchus tschawytscha*
Coho / Silver salmon	*Oncorhynchus kisutch*
Keta / Chum salmon	*Oncorhynchus keta*
Cut-throat trout	*Oncorhynchus clarki clarki*
Dolly Varden	*Salvelinus malma malma*
Cherry salmon	*Oncorhynchus masou masou*

Discussion

To identify species present in a sample when no prior knowledge of the sample is available requires a universally applicable method. PCR-RFLP profiling of a common region of the vertebrate cytochrome b gene, which is present in all fish species, enabling comparison with profiles in a database is one such universal approach.

The conventional PCR-RFLP fragment analysis involves gel electrophoresis, on large (over 30 cm^2), thin (<2 mm) acrylamide gels, to resolve the PCR-RFLP patterns. This makes handling and staining difficult and requires the use of large equipment and volumes of solution. All this makes these methods potentially hazardous and time consuming and can sometimes produce variable results. This type of detection is, therefore, not suited to use in enforcement and quality control laboratories where a rapid, robust detection method is required.

As an alternative, the 2100 Bioanalyzer incorporates conventional capillary electrophoresis (CE) technology into an easy-to-use chip-based format, which enables accurate sizing and quantification of individual DNA fragments. Coupled with the small (2 cm^2) size of the LabChip, this gives the system a significant advantage over conventional gel-based approaches in terms of ease-of-use, speed, and safety. This makes the 2100 Bioanalyzer ideally suited to the analysis of multiple small DNA fragments such as those found in PCR-RFLP profiles.

Using the 2100 Bioanalyzer it was possible to generate PCR-RFLP profiles that resembled those previously published for salmon species. However, generating PCR-RFLPs on the 2100 Bioanalyzer produced profiles with improved fragment resolution and detection, especially of smaller fragments that were not detected using conventional gel electrophoresis. PCR-RFLP profiles generated on the 2100 Bioanalyzer were also more consistently produced compared to gel electrophoresis.

Consequently, profiles based on three enzymes DdeI, NlaIII and HaeIII were developed for 19 commercially important species. Further studies should increase the range of species in the database, making it an important tool for the study of the authenticity of fish products.

Complete details of the development of the assays, application to fish species identification in products, and an interlaboratory study were recently published [3, 4, 5].

Acknowledgements

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Agilent 2100 bioanalyzer

Application compendium

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Agilent 2100 bioanalyzer

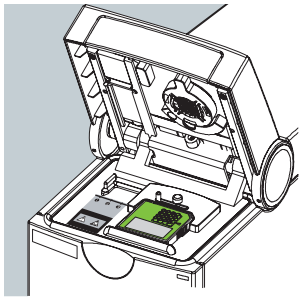
Application compendium

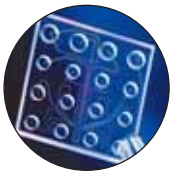


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- Eliminate potential mistakes that can occur while interpreting and storing data. The Agilent 2100 bioanalyzer automatically incorporates steps some researchers might otherwise ignore in the interest of time.

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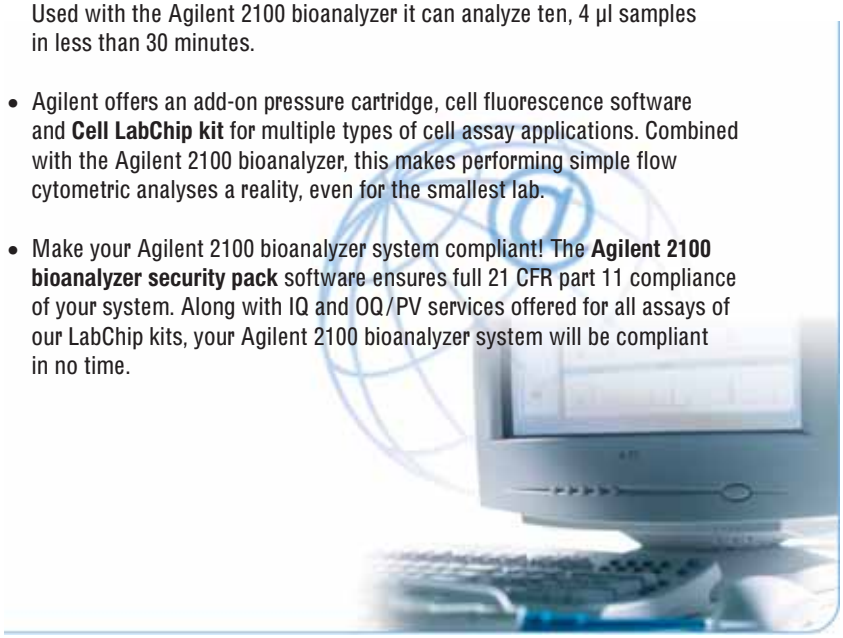
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Contents Page

I. Cell fluorescence analysis 5

Protein expression monitoring

Cell surface antibody staining - CD4 in CCRF CEM T-cells 6
Cell surface antibody staining - CD3 in T-cell leukemia 7
CD3 expression in T-cell leukemia via on-chip staining 8
Intracellular glucocorticoide receptor (GR) antibody staining in H4 hepatocytes 9
Analyzing a limited number of cells 10
Baculovirus titre determination 11
Upregulated gene expression in primary cells 12

Transfection efficiency monitoring

Green fluorescent protein in CHO cells 13
On-chip staining of GFP expression for optimizing transfection conditions with different DNA:lipid ratios 14
Verification of stable transfected cell clones by on-chip antibody staining 15
Transfection of primary cells 16

Apoptosis detection

Detection of phosphatidylserine on the cell surface via Annexin V binding 17
Intracellular Caspase-3 antibody staining assay 18
Fast Annexin protocol for time course of apoptosis induction via anti-FAS antibody 19-20
Apoptosis detection in primary cells 21

Gene silencing in cell culture

siRNA transfection optimization 22
Monitoring of gene silencing experiments 23

Contents

Page

II. DNA analysis 24

Restriction digest analysis
- Sizing range exemplified by the separation of Adenovirus 2/Dra I 25
- Detection of single base mutations 26-27

PCR product analysis
- Separation of 3 different mixtures of PCR products 28
- Determination of PCR product impurity 29-30
- Multiplex PCR analysis of bacteria in chicken 31
- Multiplex PCR with 19 products 32

Gene expression analysis
- mRNA expression study by comparative multiplex PCR 33
- Standardized end-point RT-PCR 34
- Co-amplification of GAPDH and hsp72 35
- Co-amplification of GAPDH and hsp72 - response curves 36
- Competitive PCR 37

Food analysis
- Development of meat specific assays 38-39
- Fish species identification by RFLP 40

GMO detection
- Development of a multiplex assay for soya 41
- DNA stability during food processing 42
- GMO detection by nested multiplex PCR 43

Oncology
- Tumor cell detection from carcinoma patient blood 44
- SNP analysis in cancer related P16 gene 45
- K-ras gene SNP detection 46
- METH-2 downregulation in lung carcinomas 47
- Label-free analysis of microsatellite instability in carcinoma 48

Clinical research
- Genotyping of H. pylori 49
- Duplications and deletions in genomic DNA 50

Forensic testing
- Optimization of PCR on mtDNA 51
- Pitfalls in mtDNA sequencing 52

Contents Page

III. RNA analysis 53

Analysis of total RNA
- RNA integrity 54
- Standardization of RNA Quality Control 55
- Reproducibility of quantitation 56
- Genomic DNA contamination 57

Low amounts of total RNA
- Detection of low levels of RNA 58
- RNA integrity with the RNA 6000 Pico kit 59
- RNA quality after staining and microdissection 60
- Analysis of minimum RNA amounts 61
- Genomic DNA in low concentrated RNA extracts 62
- Low RNA amounts from kidney sections 63

Analysis of mRNA
- RNA integrity 64
- Ribosomal RNA contamination in mRNA samples 65

Analysis of Cy5-labeled samples
- Analysis of cRNA with and without dye in gel matrix 66
- Optimization of labeling reactions 67
- cRNA fragmentation 68

Analysis of T7-RNA transcripts
- Size estimation 69

Contents

Page

IV. Protein analysis 70

Protein expression
- Analysis of cell lysates - protein induction 71

Protein purification:
- Comparison between lysate and flow through 72
- Analysis of protein purification 73
- GFP Streptag fusion protein purification 74
- Analysis of column capacity 75
- Analysis of column fractions to optimize conditions 76
- His-tag protein purification with Ni++ ZipTips® 77
- Enzymatic removal of His Tags from recombinant proteins 78
- Complementing RP-HPLC protein purification 79

Antibody Analysis
- Analysis of antibodies under reducing and non-reducing conditions 80
- Quantitation of the half-antibody content in IgG_4 preparations 81
- Comparison of SDS-PAGE, CGE and Agilent 2100 bioanalyzer for humanized monoclonal antibody analysis 82
- Absolute quantitation of IgG 83
- Quality control of stressed antibodies 84
- Separation of bispecific antibodies chains 85

Food analysis
- Bovine milk analysis 86
- Protein pattern of different transgenic seedlines 87

Protein - others
- Absolute protein quantitation 88
- Glycoprotein sizing 89
- Protein quality control prior to MS-analysis 90
- Depletion of high abundant proteins from blood samples 91
- Increased sensitivity by desalting protein samples 92

V. Literature 93

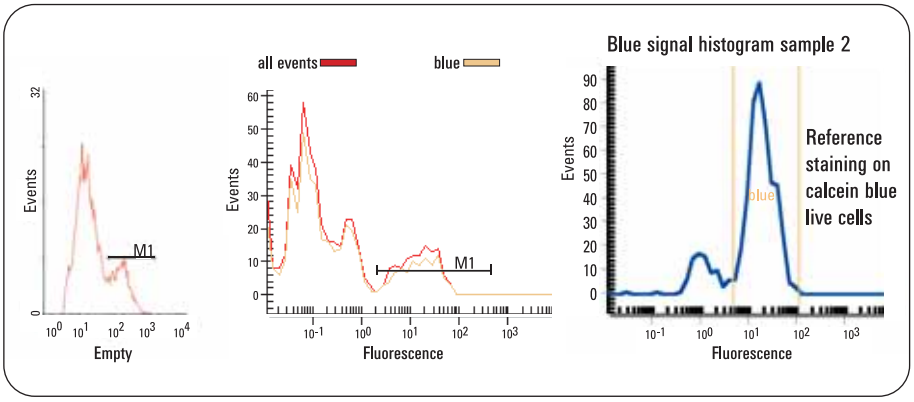
I. Cell fluorescence analysis

	Agriculture / Food	Drug Discovery / Drug Development	Drug Manufacturing QA / QC	Genomics	Pharmaceuticals	Proteomics	Forensics / Homeland Security
Protein expression monitoring							
Cell surface antibody staining - CD4 in CCRF CEM T-cells		●			●	●	
Cell surface antibody staining - CD3 in T-cell leukemia		●			●	●	
CD3 expression in T-cell leukemia via on-chip staining		●			●	●	
Intracellular glucocorticoide receptor (GR) antibody staining in H4 hepatocytes		●			●	●	
Analyzing a limited number of cells		●			●	●	
Baculovirus titre determination		●	●		●	●	
Upregulated gene expression in primary cells		●		●	●	●	
Transfection efficiency monitoring							
Green fluorescent protein in CHO cells		●	●			●	
On-chip staining of GFP expression for optimizing transfection conditions with different DNA:lipid ratios		●	●			●	
Verification of stable transfected cell clones by on-chip antibody staining		●	●			●	
Transfection of primary cells		●				●	
Apoptosis detection							
Detection of phosphatidylserine on the cell surface via Annexin V binding		●			●	●	
Intracellular Caspase-3 antibody staining assay					●		
Fast Annexin protocol for time course of apoptosis induction via anti-FAS antibody		●			●	●	
Apoptosis detection in primary cells		●			●		
Gene silencing in cell culture							
siRNA transfection optimization		●		●	●	●	
Monitoring of gene silencing experiments		●		●	●	●	

Protein expression monitoring

Cell surface antibody staining - CD4 in CCRF CEM T-cells

Flow cytometer (10,000 Events) Agilent 2100 bioanalyzer (500 Events)



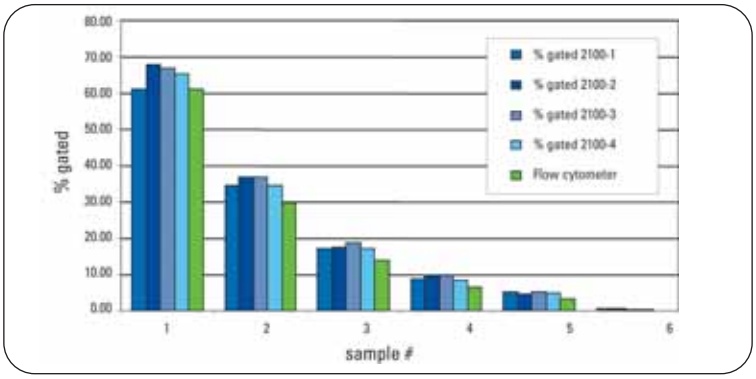
Kit: Cell fluorescence kit
Assay: Antibody staining assay
Application: CCRF-CEM cells were stained with hCD4-APC labeled antibodies and calcein live dye. 65% of all CCRF-CEM live cells (yellow curve) are expressing CD4 protein which is good in comparison to conventional flow cytometer results.

Corresponding application note: 5988-4322EN

Protein expression monitoring

Cell surface antibody staining - CD3 in T-cell leukemia

Averaged data per instrument



Mean % CD3+ cells				
2100-1	2100-2	2100-3	2100-4	Flow cyt.
60.9	67.8	66.6	65.0	60.9
34.4	36.7	36.7	34.3	29.8
17.3	17.6	18.7	17.2	13.8
8.9	9.4	9.9	8.3	6.5
5.1	4.4	5.3	4.9	3.2
0.8	0.6	0.3	0.3	0.0

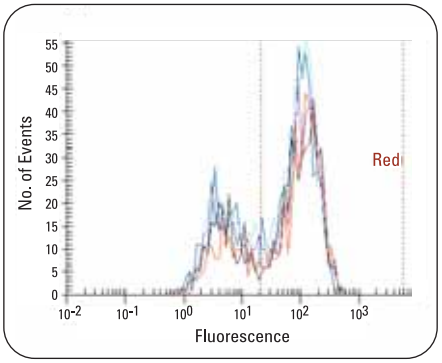
Kit: Cell fluorescence kit
Assay: Antibody staining assay
Application: Jurkat (T-cell leukemia) cells were stained with calcein alone or with calcein and APC-labeled anti-CD3 antibody. To mimic different subpopulation sizes, mixtures of both populations were prepared at various ratios.
Samples were analyzed with 4 Agilent 2100 bioanalyzer instruments on 5 chips and compared to a flow cytometer reference instrument. Interestingly, small subpopulations (like 10 - 20%) could be analyzed with good accuracy and reproducibility.

Corresponding application note: 5988-4322EN

Protein expression monitoring

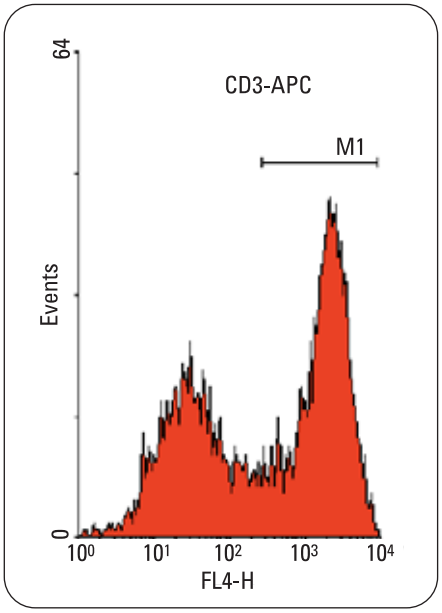
CD3 expression in T-cell leukemia via on-chip staining

A. On-chip Agilent 2100 bioanalyzer



Sample	% of Gated	Sample	% of Gated
1	-	4	64.6
2	66.9	5	66.7
3	67.2	6	72.0

B. Conventional flow cytometry



Kit: Cell fluorescence kit
Assay: Antibody staining assay
Application: Jurkat cells were stained on-chip with anti hCD3-APC prediluted 1:5.5 in cell buffer and Calcein (1:50 in cell buffer). After an incubation time of 25 minutes in the chip, samples were measured in the Agilent 2100 bioanalyzer. The faster and easier on-chip staining procedure has the advantage here of reducing cell consumption 17 fold and antibody reagent costs 80 fold.

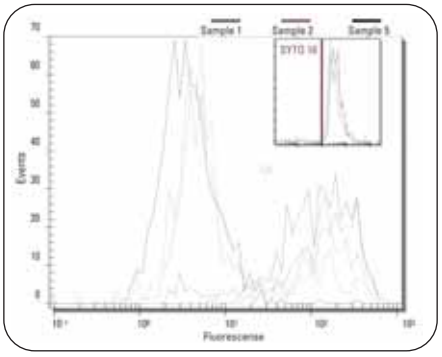
A) Overlay of representative histograms of calcein and antibody treated cells.
B) Comparison between on-chip staining data and data obtained by measuring cells stained by conventional staining on a flow cytometer.

Corresponding application note: 5988-7111EN

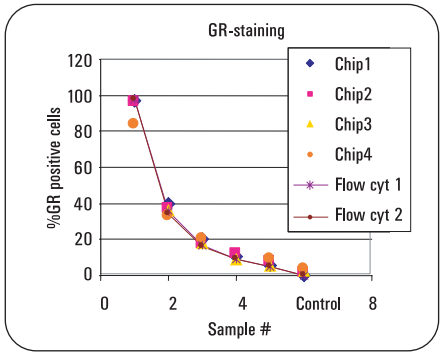
Protein expression monitoring

Intracellular glucocorticoide receptor (GR) antibody staining in H4 hepatocytes

Chip histogram overlay from 700 cells/sample



Correlation of chip vs. flow cytometer results

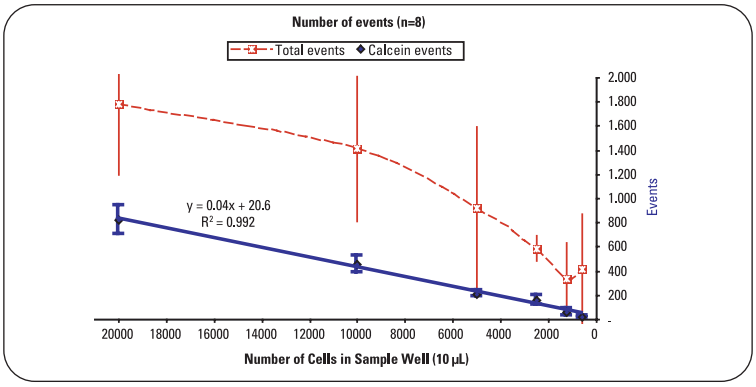


Kit: Cell fluorescence kit
Assay: Generic assay
Application: H4 hepatocytes cells were stained with SYTO16 DNA dye alone or with SYTO16 and GR primary antibody. After washing, both cell preparations were stained with APC-labeled secondary antibody. Mixtures of both populations were prepared at various ratios.
The insert in the left picture shows the overlay of all six cell samples in the blue reference color. The black histogram represents data from the control sample, no GR detected.
All other 5 samples have significant staining above marked fluorescence intensity in the red.
Good chip to chip reproducibility and comparison to flow cytometer is demonstrated.

Corresponding application note: 5988-4322EN

Protein expression monitoring

Analyzing a limited number of cells



Cells	Live-CD3+	STD(n=4)
20,000	83.7%	3.5%
10,000	85.6%	4.1%
5,000	87.7%	4.2%
2,500	84.0%	3.0%
1,250	89.8%	6.5%
625	90.0%	9.3%

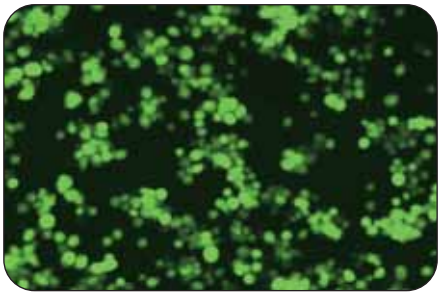
Kit: Cell fluorescence kit
Assay: On-chip antibody staining assay
Application: The direct comparison of different input numbers of cells (down to 625 cells in 10 µl) for the on-chip staining protocol reveals that even with a much lower number than the recommended 20000 cells/10 µl for the standard protocol reliable and meaningful results can be achieved with good reproducibility. The data shown were generated with CD3-positive Jurkat cells stained with an anti-CD3 antibody for the CD3 protein and counterstained with the live cell stain Calcein AM. Similar results were obtained with primary human dermal fibroblasts (PHDF) indicating the usefulness of this method for scarce specimen. The lack of sensitivity, automation and convenient quantitation found with other methods can be circumvented easily by using the Agilent 2100 bioanalyzer.

Corresponding application note: 5989-0746EN

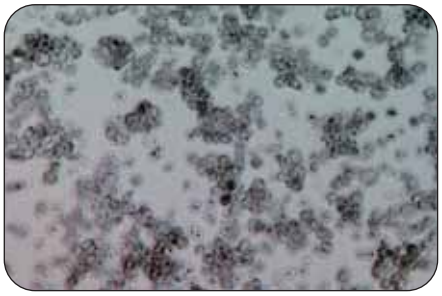
Protein expression monitoring

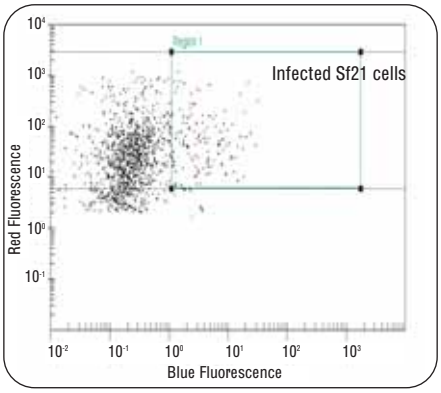
Baculovirus titre determination

Fluorescent Light



Transmitted Light





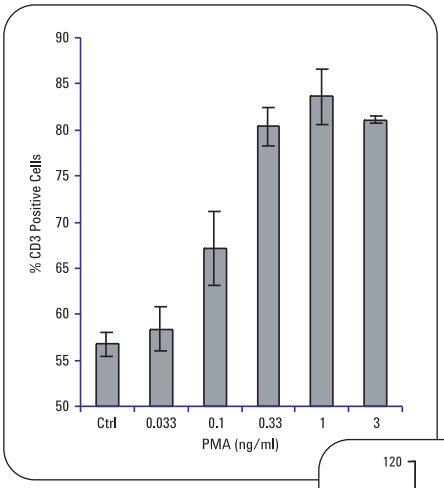
Kit: Cell fluorescence kit
Assay: GFP Assay
Application: A fast and convenient method exists for the calculation of baculovirus titre for expression systems facilitating insect cells. Using GFP-linked co-expression plasmids, the Agilent 2100 bioanalyzer and the flow cytometry set allows the calculation of the viral titre for six samples in approximately 90 minutes. It is superior to traditional plaque assays in terms of labor time, automation and user-to-user variability.

Corresponding application note: 5989-1644EN

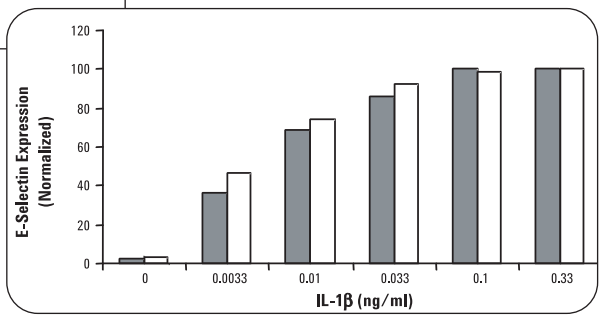
Protein expression monitoring

Upregulated gene expression in primary cells

A



B



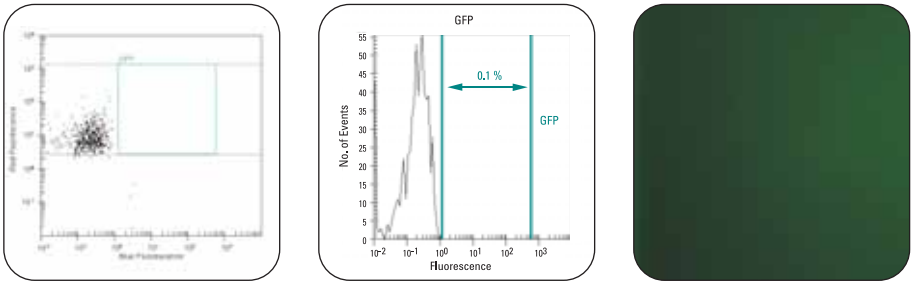
Kit: Cell fluorescence kit
Assay: On-chip antibody staining assay
Application: Flow cytometric analysis of primary cells can present a challenge for researchers due to limited availability and life span of primary cells. A dose-respondent upregulation of protein expression in primary cells using only a minimum number of cells in a fast on-chip-staining approach is shown here. Activation of peripheral blood lymphocytes by phorbol-12-myristate-13-acetate (PMA) leads to increased expression of the T cell receptor CD3 (Figure A, mean from 3 experiments).
For HUVECs (human umbilical vein endothelia cells) the induction of E-selectin (CD62E) expression upon IL-1β treatment is shown (Figure B, white bars) in comparison to results from a conventional flow cytometer (white bars).

Corresponding application note: 5989-2718EN

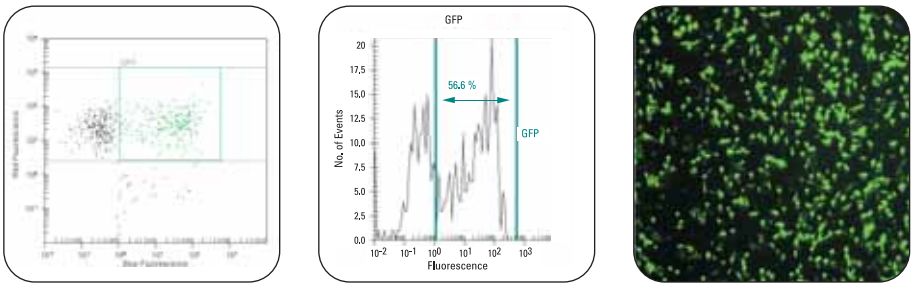
Transfection efficiency monitoring

Green fluorescent protein in CHO cells

Mock transfected cells



GFP transfected cells

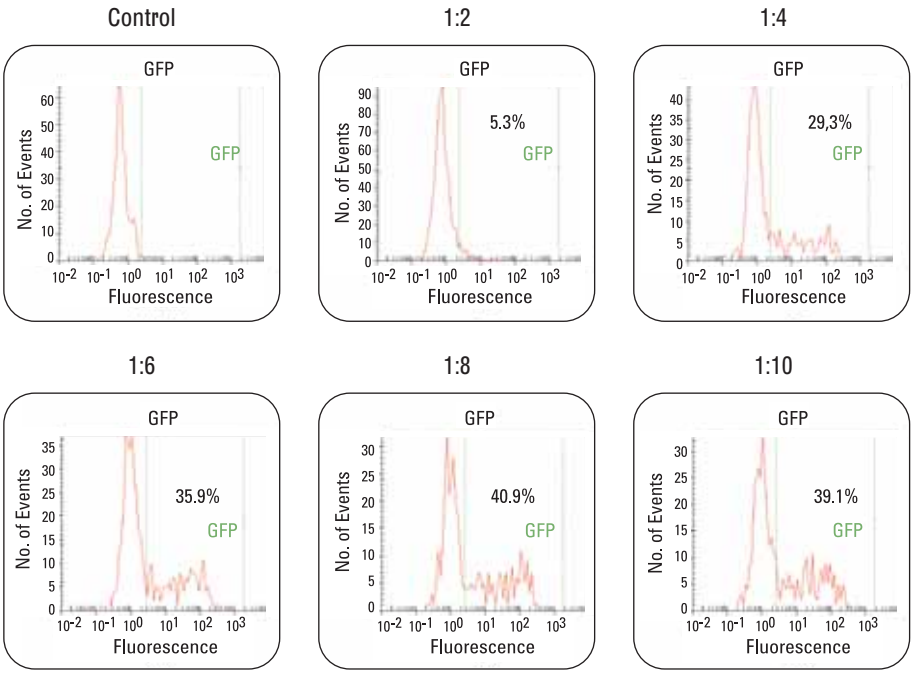


Kit: Cell fluorescence kit
Assay: GFP assay
Application: Chinese hamster ovary (CHO-K1) cells were transfected with EGFP DNA by a lipofection method. The upper panel shows the control mock transfection; here cells don't express GFP. Examples for data evaluation in dotplot view and histogram view are shown in comparison to the microscopy view. For analysis on the Agilent 2100 bioanalyzer, cells were stained with a red dye for live cells (reference stain). The transfection efficiency of 56% can be easily determined with the Agilent 2100 bioanalyzer.

Corresponding application note: 5988-4320EN

Transfection efficiency monitoring

On-chip staining of GFP expression for optimizing transfection conditions with different DNA:lipid ratios



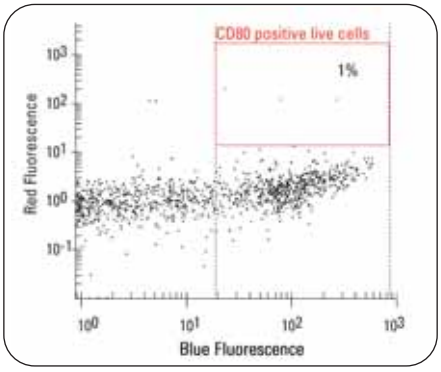
Kit: Cell fluorescence kit
Assay: On-chip GFP assay
Application: Chinese hamster ovary (CHO-K1) cells were transfected with EGFP DNA by alipofection method. Optimization of transfection conditions were done on one chip. Several DNA:lipofectamine ratios were tried. A ratio of 1:8 gave the best transfection efficiency. All cells were reference stained with a red live dye. On-chip staining was applied, minimizing the staining time, reagent usage and cell consumption.

Corresponding application note: 5988-7296EN

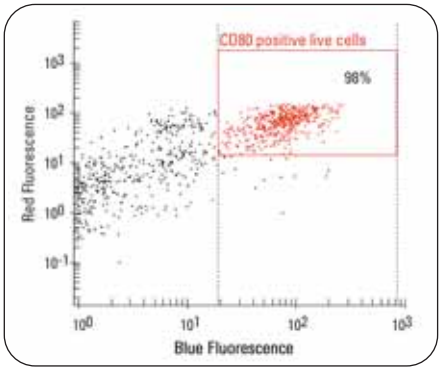
Transfection efficiency monitoring

Verification of stable transfected cell clones by on-chip antibody staining

Hek 293 control cells



CD 80 stable clone

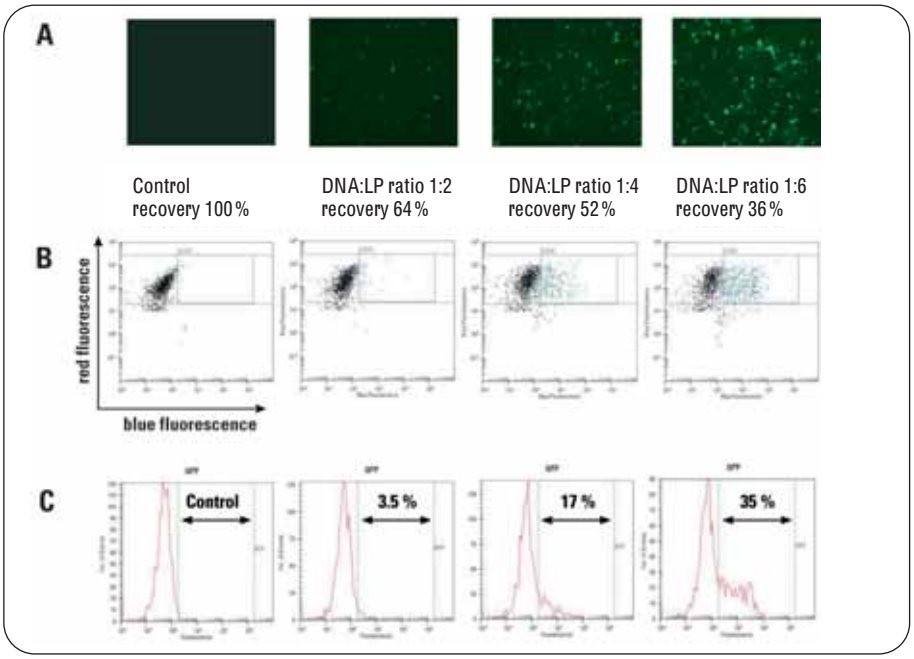


Kit: Cell fluorescence kit
Assay: On-chip antibody staining assay
Application: Verification of CD80 protein expression in stable transfected Hek 293 cells with the Agilent 2100 bioanalyzer. Control (left dot plot) and CD80 transfected cells (right) are stained on-chip with blue calcein live dye and anti-CD80-CyChrome antibody. Red region marks CD80 protein expressing 293 cells within live cell population - confirming expression in the CD80 stable clone Hek 293 cells.

Corresponding application note: 5988-7111EN

Transfection efficiency monitoring

Transfection of primary cells

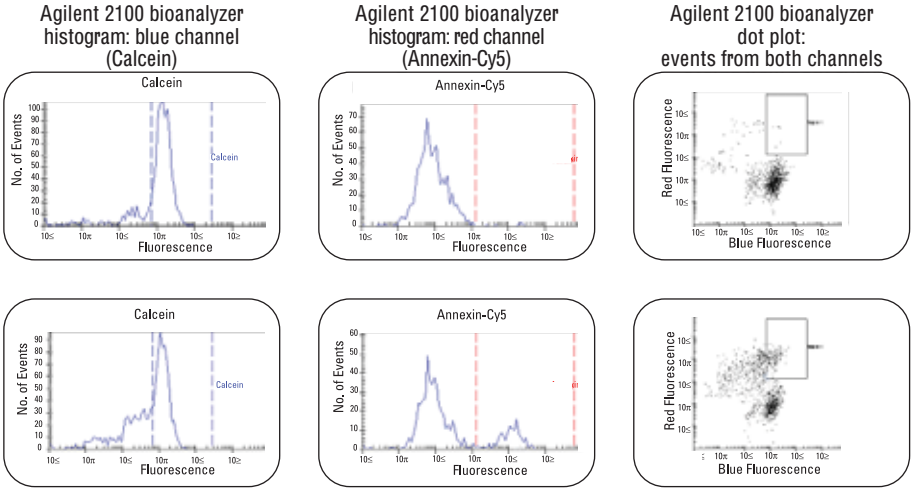


Kit: Cell fluorescence kit
Assay: GFP assay
Application: Monitoring the transfection efficiency in primary cells requires low cell consumption, high reproducibility of results, a fast on-chip staining procedure and ease-of-use all provided by the Agilent 2100 bioanalyzer. The transfection efficiency using a GFP-coding plasmid (pEGFP-C2) at varying plasmid:lipofectamine ratios (DNA:LP ratio) obtained with human umbilical vein endothelial cells (HUVEC) is measured in this optimization series. Images from a fluorescence microscope (A) and dot plots (B), as well as histograms (C) of control- and GFP-transfected cells are shown. Using increasing ratios, better transfection efficiency was achieved, whereas the toxicity of LP caused decreased recovery of living cells. Such data facilitates optimizing transfection conditions.

Corresponding application note: 5988-8154EN

Apoptosis detection

Detection of phosphatidylserine on the cell surface via Annexin V binding

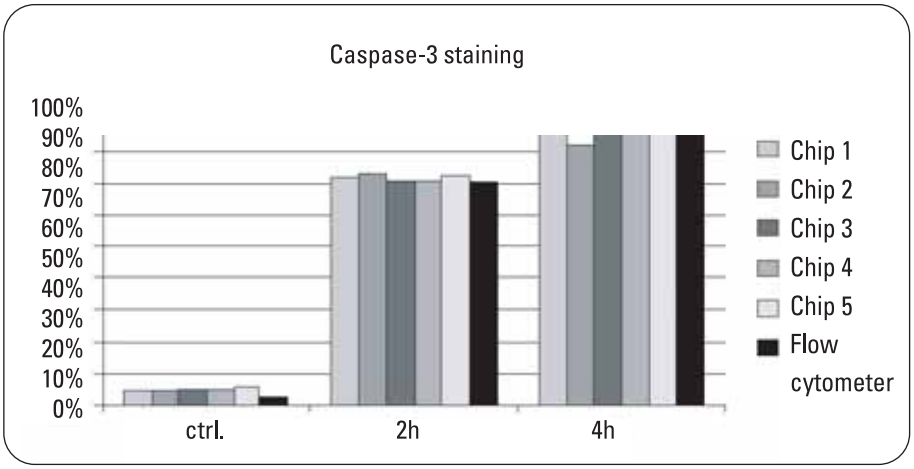


Kit: Cell Fluorescence kit
Assay: Apoptosis assay
Application: Apoptosis (programmed cell death) in Jurkat cells was induced with camptothecin. Cells treated for 16 hours and untreated cells were stained with calcein and Annexin-Cy5. Annexin-V binds to phosphatidylserine - a membrane lipid which is kept to the inner leaflet of the cell membrane of intact cells. Exposure of phopshatidylserine on the outer leaflet is an early indicator of apoptotic processes. Annexin-V binding is made detectable by Cy5 staining of the Annexin-V via a biotin-streptavidin interaction. Calcein staining of cells is used as a live control to distinguish living and apoptotic cells from dead cells. Calcein enters the cell via the membrane as a non-fluorescent ester. The ester is cleaved inside the cell which results in fluorescence.

The histograms on the left show the number and intensity value of all events which generated a signal in the blue channel, corresponding to calcein-stained cells. The histograms on the right shows all events which generated a signal in the red channel, corresponding to Annexin-V binding to apoptotic cells. While the control shows only low intensity values (background noise), the treated sample shows high intensity values (within the red markers) corresponding to apoptotic cells. The dot plot of the treated sample nicely shows the subpopulation of all live cells which are apoptotic.
Corresponding application note: 5988-4319EN

Apoptosis detection

Intracellular Caspase-3 antibody staining assay



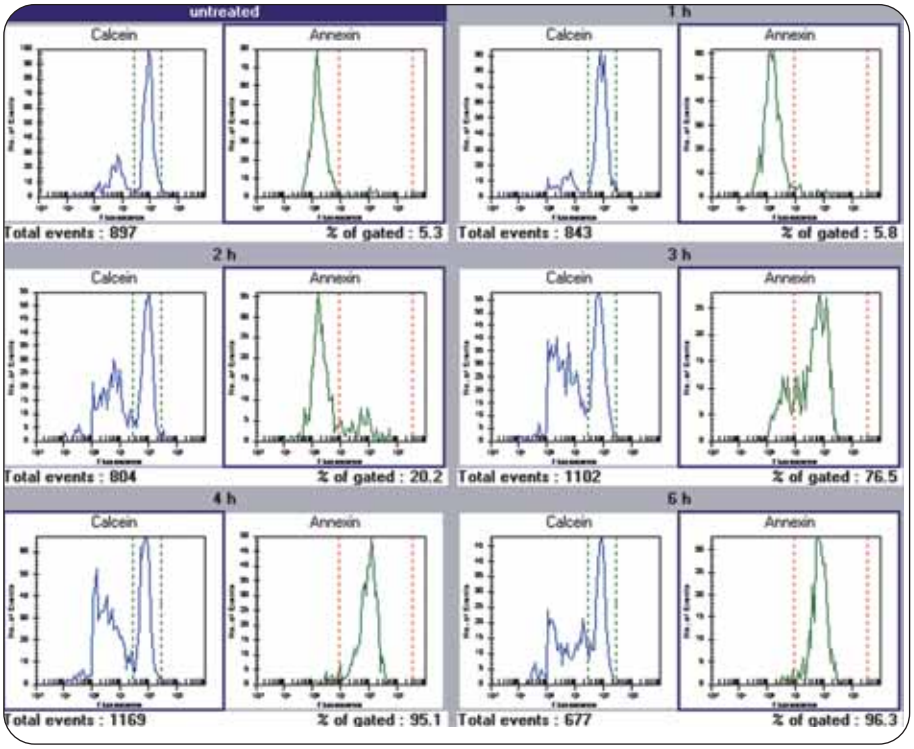
Kit: Cell fluorescence kit
Assay: Generic assay
Application: Induction of apoptosis in Jurkat cells was done with anti-FAS antibody treatment. Intracellular staining with specific antibodies against 'active' Caspase-3 were performed. Reference staining was done with SYTO16 DNA dye. Good chip to chip reproducibility and good comparison to conventional flow cytometer results were obtained.

Corresponding application note: 5988-4319EN

Apoptosis detection

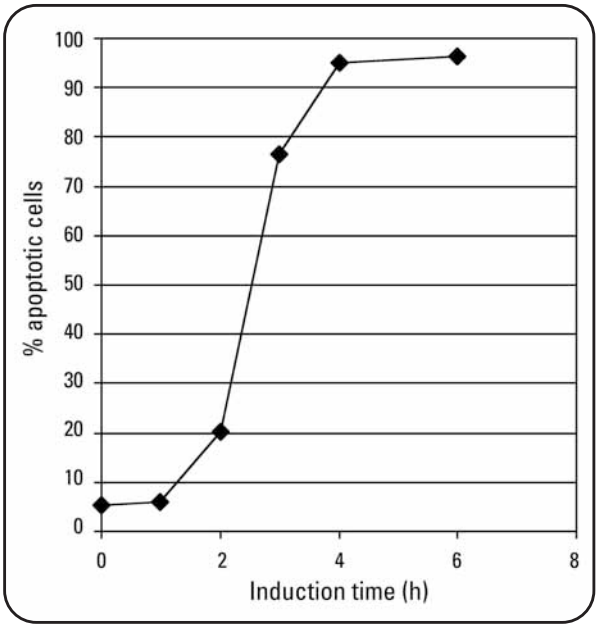
Fast Annexin protocol for time course of apoptosis induction via anti-FAS antibody

A



Kit: Cell fluorescence kit
Assay: Apoptosis assay
Application: Apoptosis (programmed cell death) in Jurkat cells was induced with anti-FAS antibody. Cells treated for 0,1,2,3,4 and 6 hours were stained with calcein and Annexin-Cy5. Annexin-V binds to phosphatidylserine - a membrane lipid which is kept to the inner leaflet of the cell membrane of intact cells. Exposure of phopshatidylserine on the outer leaflet is an early indicator of apoptotic processes. Annexin V binding is detectable by Cy5 staining of the Annexin-V via a biotin-streptavidin interaction. Calcein staining of cells is used as a live control to distinguish living and apoptotic cells from dead cells. Calcein enters the cell via the membrane as non-fluorescent ester. The ester is cleaved inside the cell which results in fluorescence and indicates apoptosis.

B

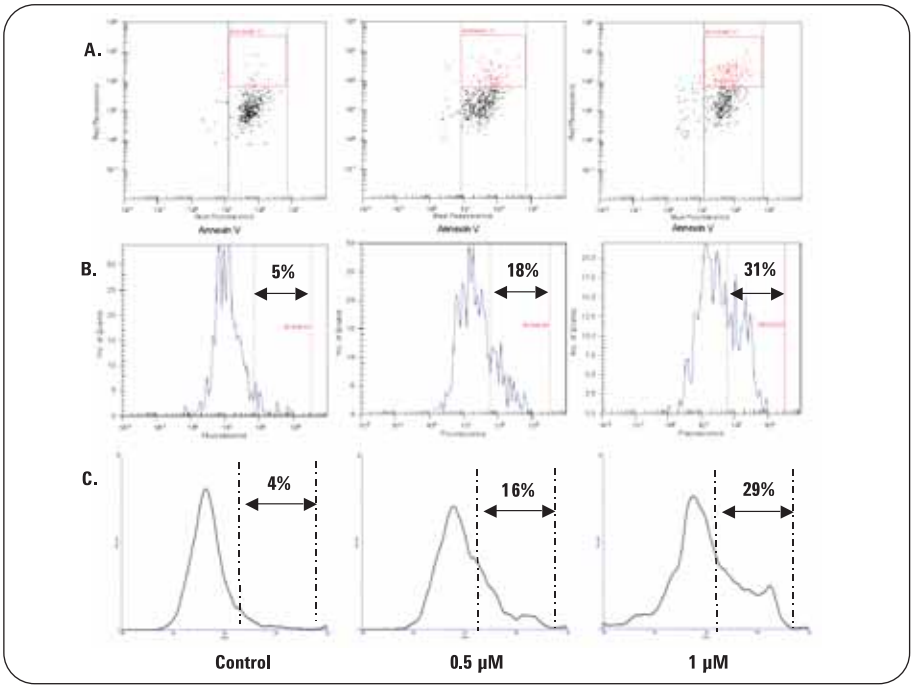


The histograms on page 18 (A) show the number and intensity value of all events which generated a signal in the blue channel, corresponding to calcein-stained cells.
The histograms on the right show all events which generated a signal in the red channel, corresponding to Annexin-V binding to apoptotic cells. While the control shows only low intensity values (background noise), the treated sample shows high intensity values (within the red markers) corresponding to apoptotic cells.
(B) Time course of the induction of apoptosis by anti-FAS antibody in Jurkat cells. Apoptosis is detectable in a significant amount of cells after 2 hours. Following a treatment of 4 hours, approximately 95% of the cells are apoptotic.

Corresponding application note: 5988-4319EN

Apoptosis detection

Apoptosis detection in primary cells



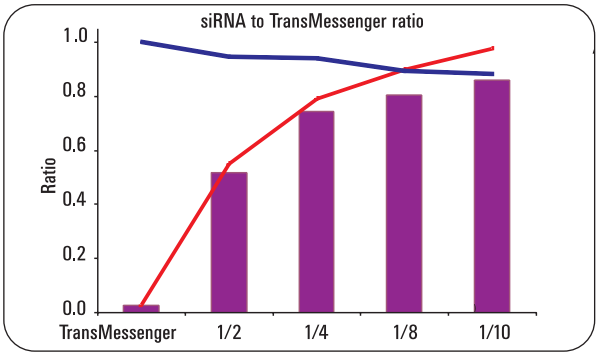
Kit: Cell fluorescence kit
Assay: On-chip antibody staining assay
Application: The Agilent 2100 bioanalyzer has been used to study induced apoptosis by monitoring annexin V-binding in primary human endothelial cells (HUVEC, not shown) and human dermal fibroblasts (NHDF, shown). A simple and fast assay protocol was used on cells left untreated or treated for 5 hours with different concentrations of staurosporine, which induces apoptosis. See row A for dot blots and B for histograms at different concentrations. Evaluation of the same samples on a conventional flow cytometer (row C) yielded similar results.

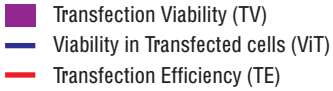
Corresponding application note: 5989-2934EN

Gene silencing in cell culture

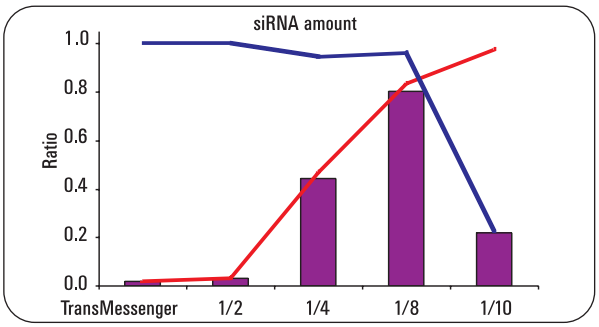
siRNA transfection optimization

A





B

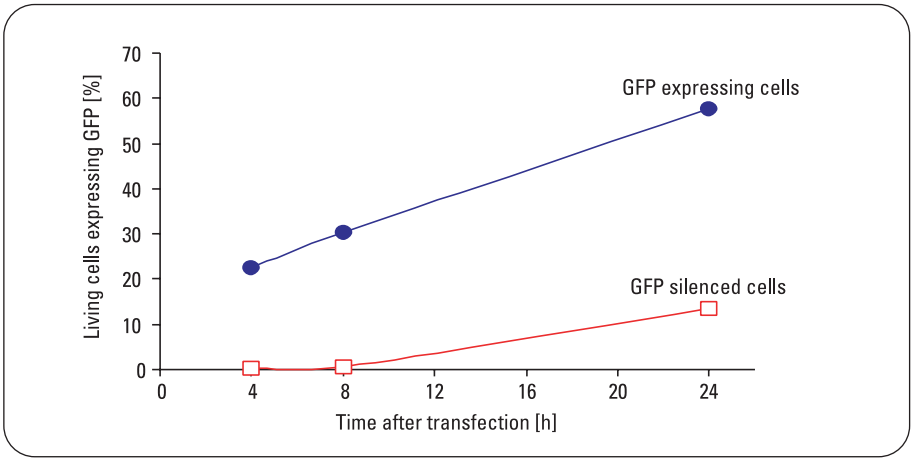


Kit: Cell fluorescence kit
Assay: On-chip antibody staining assay
Application: In gene silencing experiments (HeLa cells) we found that increasing amounts of transfection reagent (TransMessenger™)to a constant amount of siRNA leads to a plateau of transfection viability (panel A). Transfection viability reflects the product of the viability of the transfected cells and the transfection efficiency. With a constant siRNA/transfection reagent ratio of 1:4 and increasing total amounts of introduced siRNA (panel B) the viability of transfected cells decreases at a certain point although the transfection efficiency increases. Thus, there are experimental conditions where the number of living and transfected cells are at a maximum. The Agilent 2100 bioanalyzer features on-chip staining and leads to excellent results with a minimal consumption of cells and reagents.

Corresponding application note: 5988-9872EN

Gene silencing in cell culture

Monitoring of gene silencing experiments



Kit: Cell fluorescence kit
Assay: GFP Assay
Application: After co-transfection of a GFP plasmid and Cy5-labeled siRNA (GFP-specific), GFP expression and viability of cells were detected. The course of GFP expression in control (GFP only) and siRNA/GFP transfected cells was measured on the Agilent 2100 bioanalyzer. Accurate results were obtained fast and in an automated manner. They easily allow the efficiency and reliability of a given protocol and transfection reagents to be judged. Thus, such an experiment provides efficient monitoring and optimization of any gene silencing experiment.

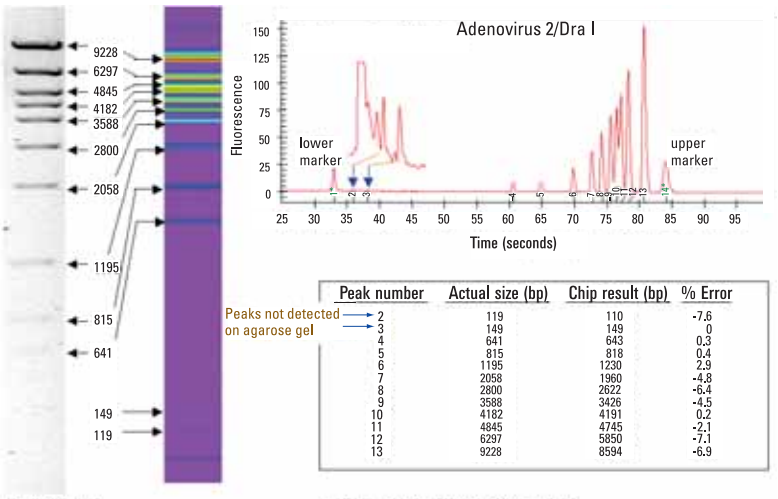
Corresponding application note: 5989-0103EN

II. DNA analysis

	Agriculture/Food	Drug Discovery/Drug Development	Drug Manufacturing QA/QC	Genomics	Pharmaceuticals	Proteomics	Forensics/Homeland Security
Restriction digest analysis							
Sizing range exemplified by the separation of Adenovirus 2/Dra I				●			
Detection of single base mutations (I)				●			●
Detection of single base mutations (II)				●			●
PCR product analysis							
Separation of 3 different mixtures of PCR products				●			●
Determination of PCR product impurity				●			●
Multiplex PCR analysis of bacteria in chicken	●			●			●
Multiplex PCR with 19 products	●			●			●
Gene expression analysis							
mRNA expression study by comparative multiplex PCR	●	●		●			
Standardized end-point RT-PCR	●	●		●			
Co-amplification of GAPDH and hsp72				●			
Co-amplification of GAPDH and hsp72 - response curves				●			
Competitive PCR	●			●			●
Food analysis							
Development of meat specific assays (I)	●			●			
Development of meat specific assays (II)	●			●			
Fish species identification by RFLP	●			●			
GMO detection							
Development of a multiplex assay for soya	●			●			
DNA stability during food processing	●			●			
GMO detection by nested multiplex PCR	●			●			
Oncology							
Tumor cell detection from carcinoma patient blood		●		●			
SNP analysis in cancer related P16 gene		●		●			
K-ras gene SNP detection		●		●			
METH-2 downregulation in lung carcinomas		●		●			
Label-free analysis of microsatellite instability in carcinoma		●		●			
Diagnostic research							
Genotyping of H. pylori	●	●		●			●
Duplications and deletions in genomic DNA		●		●			
Forensic testing							
Optimization of PCR on mtDNA				●			●
Pitfalls in mtDNA sequencing				●			●

Restriction digest analysis

Sizing range exemplified by the separation of Adenovirus 2/Dra I



Peak number	Actual size (bp)	Chip result (bp)	% Error
2	119	110	-7.6
3	149	149	0
4	641	643	0.3
5	815	818	0.4
6	1195	1230	2.9
7	2058	1960	-4.8
8	2800	2622	-6.4
9	3588	3426	-4.5
10	4182	4191	0.2
11	4845	4745	-2.1
12	6297	5850	-7.1
13	9228	8594	-6.9

Agarose gel

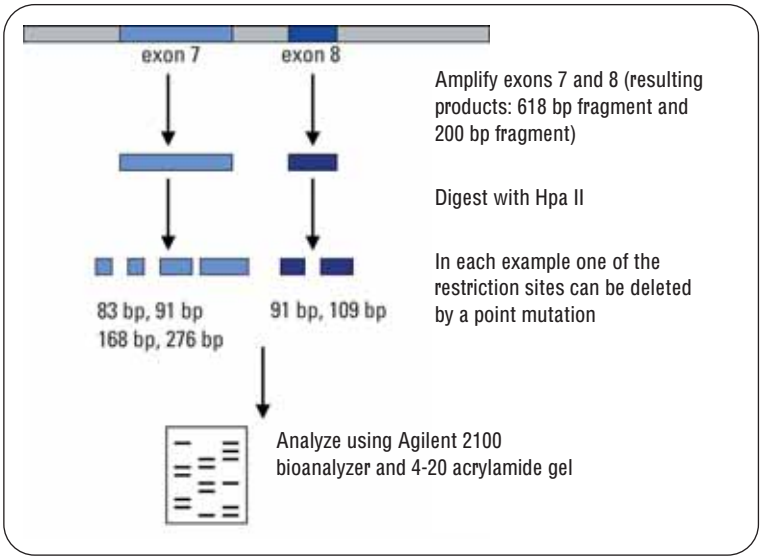
Agilent 2100 bioanalyzer data

Kit: DNA 12000 kit
Assay: DNA 12000 assay
Application: Restriction digest analysis of Adenovirus 2/Dra I. For restriction fragment analysis the large linear dynamic range of the lab-on-a-chip approach is very advantageous. Analyzing samples with large and short fragments on slab gels can be difficult because of bands running off the gel and insufficient staining (or over-staining) of bands.

Corresponding application note: 5968-7501EN

Restriction digest analysis

Detection of single base mutations (I)

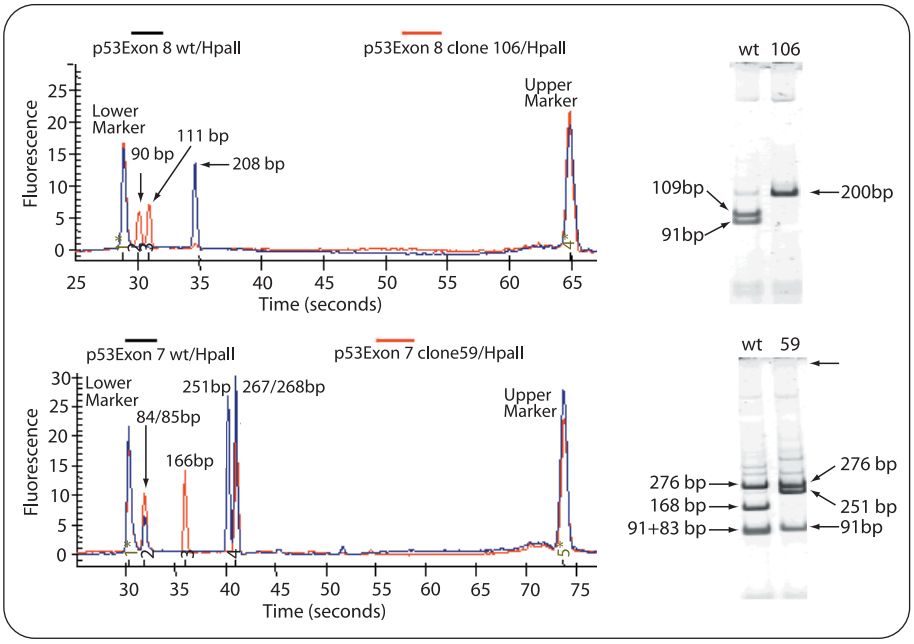


Kit: DNA 7500 kit
Assay: DNA 7500 assay
Application: Mutation detection by RFLP highlights the use of the Agilent 2100 bioanalyzer. Two different regions of the p53 gene were amplified with specific primers and digested with Hpa II, which cuts in a location that is prone to mutations. In the presence of a point mutation, the enzyme Hpa II does not cleave the DNA, leaving larger fragments that can be revealed by gel electrophoresis or by analysis with the DNA 7500 LabChip kit (see next page).

Corresponding application note: data not published

Restriction digest analysis

Detection of single base mutations (II)

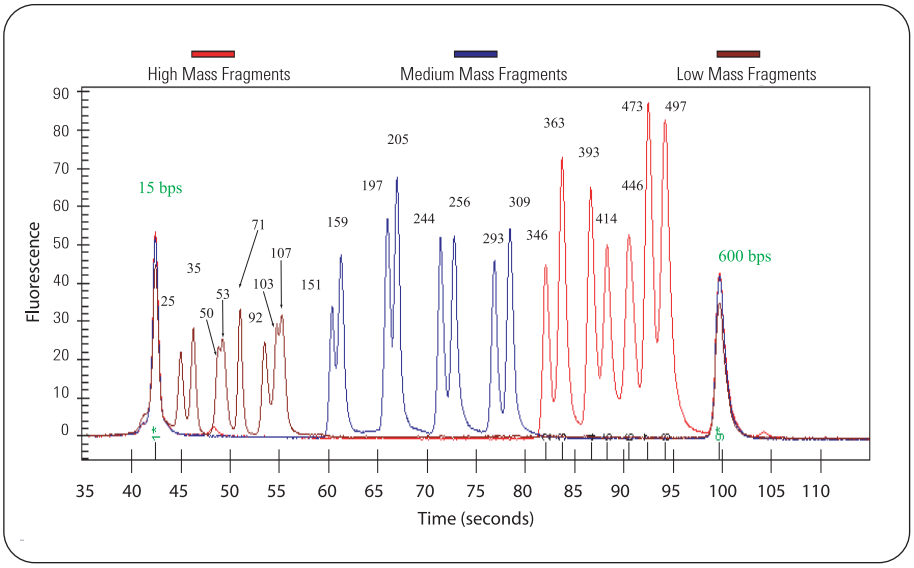


Kit: DNA 7500 kit
Assay: DNA 7500 assay
Application: Analysis on the chip showed an identical pattern of digest fragments as seen on the slab gel for the wildtype and Exon 7 & 8 PCR products. Comparison of the calculated sizes of the bands shows 1-2% variance with the LabChip assay, which allows fast and accurate detection of point mutations.

Corresponding application note: 5968-7496EN

PCR product analysis

Separation of 3 different mixtures of PCR products

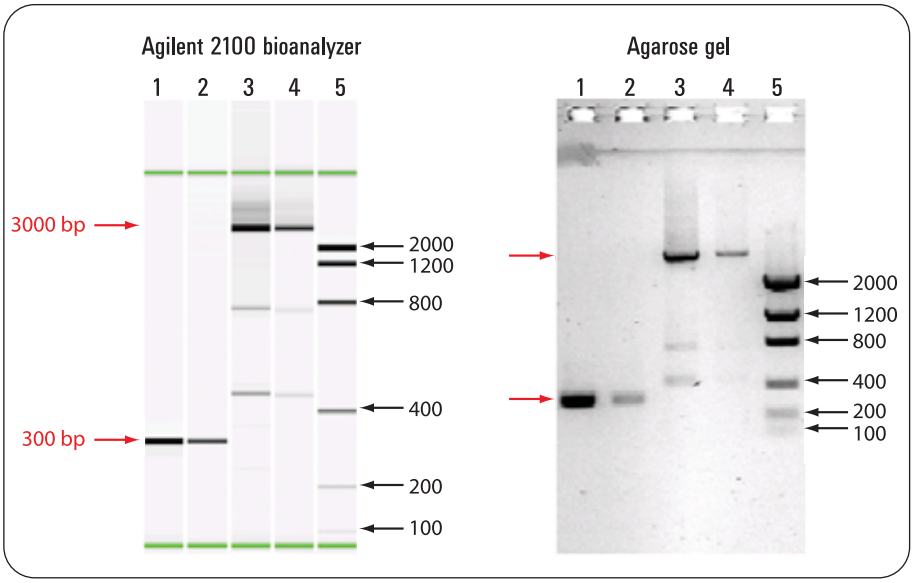


Kit: DNA 500 kit
Assay: DNA 500 assay
Application: Overlay of three different electropherograms, which are mixtures of PCR samples ranging from 25 to 500 base pairs in size. The two closest eluting bands (50 bp and 53 bp) are partially separated and identified by the software as two separate peaks. The DNA 500 assay achieves a resolution of five base pairs from 25 to 100 base pairs and a 5% resolution from 100 to 500 base pairs where the sizing error is less than 10% over the entire size range.

Corresponding application note: 5988-3041EN

PCR product analysis

Determination of PCR product impurity

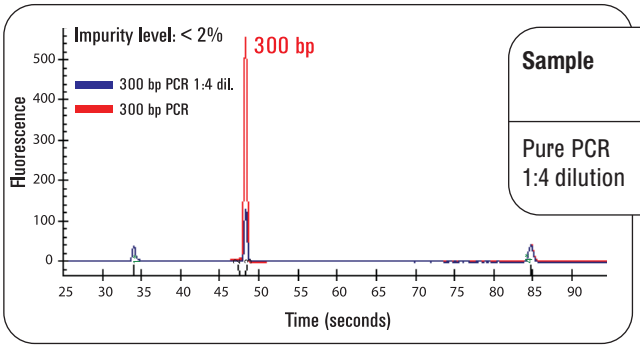


Kit: DNA 7500 kit
Assay: DNA 7500 assay
Application: Comparison between the analysis of two PCR reactions (300 and 3000 bp products) using the DNA 7500 LabChip kit vs. an agarose gel. Two different concentrations are shown side by side for each PCR reaction (undiluted and 1:4 dilution). The Agilent 2100 bioanalyzer shows superior performance in locating impurities over a broader concentration range than the gel. The 300 bp fragment appears to be uncontaminated in both the gel and on the Agilent 2100 bioanalyzer. The 3000 bp fragment shows few impurities on the gel, which become invisible at the 1:4 dilution. These impurities can easily be detected with the Agilent 2100 bioanalyzer.

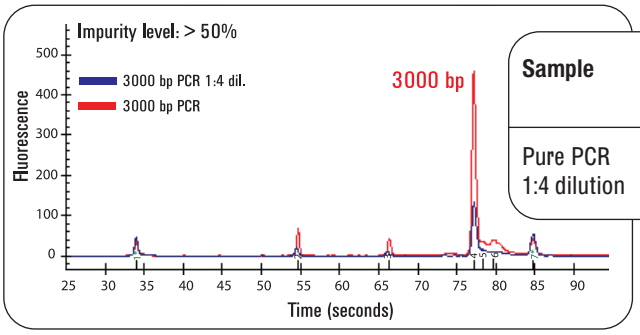
Corresponding application note: 5968-7496EN

PCR product analysis

Determination of PCR product impurity



Sample	c (DNA) All peaks	Main peak (300bp)
Pure PCR	41.4 ng/µl	40.7 ng/µl
1:4 dilution	9.6 ng/µl	9.6 ng/µl



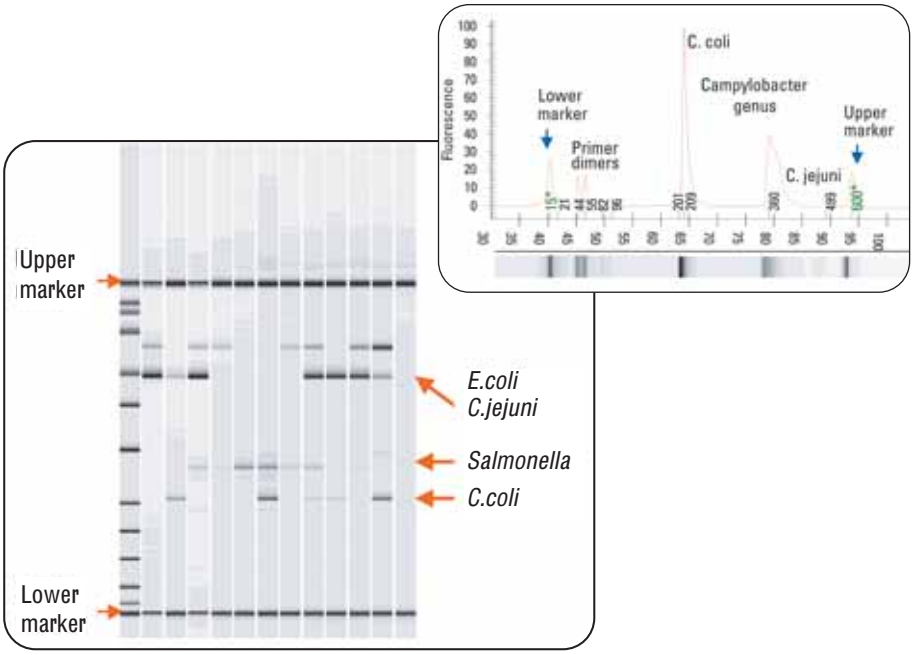
Sample	c (DNA) All peaks	Main peak (3000bp)
Pure PCR	61.9 ng/µl	40.7 ng/µl
1:4 dilution	14.8 ng/µl	9.8 ng/µl

Kit: DNA 7500 kit
Assay: DNA 7500 assay
Application: The quantitative data generated by the Agilent 2100 bioanalyzer indicate the amount of impurity or non-specific products in the PCR reactions from the previous page. Even in the 300 bp fragment a small impurity can be detected, while the 3000 bp fragment shows more than 50% impurities.

Corresponding application note: 5968-7496EN

PCR product analysis

Multiplex PCR analysis of bacteria in chicken



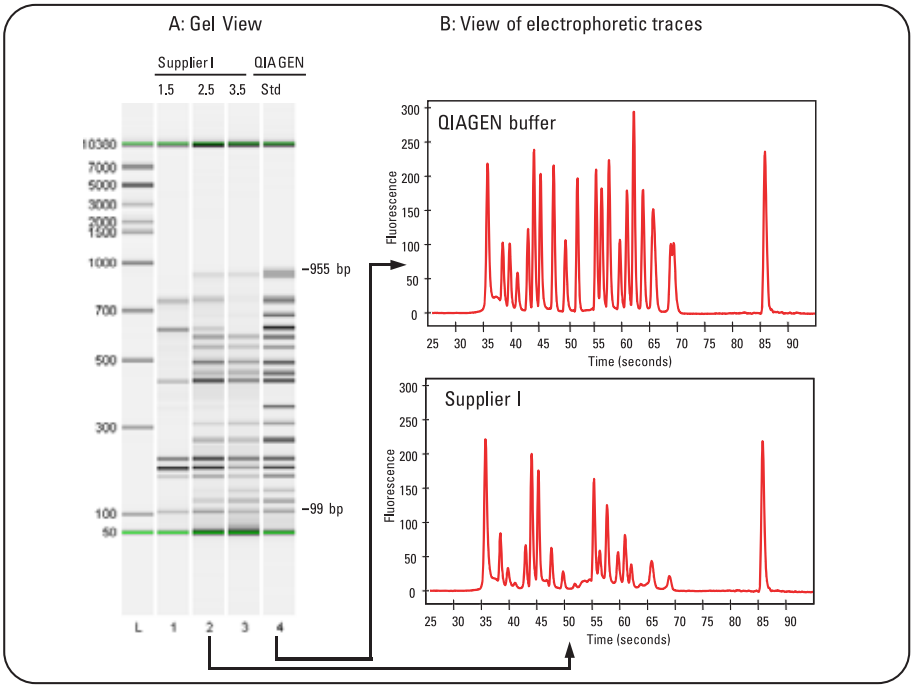
Data kindly provided by GenPoint, NL

Kit: DNA 500 kit
Assay: DNA 500 assay
Application: Multiplex PCR with four primer pairs, each one specific for a certain DNA sequence from one of the 4 bacteria to be tested for. Total DNA was extracted from chicken and subjected to PCR. The gel-like image shows traces from different chicken samples with bands showing up when an amplicon could be detected. The electropherogram is one example where bacterial DNA from two species of the *Campylobacter* genus could be detected.

Corresponding application note: data not published

PCR product analysis

Multiplex PCR with 19 products



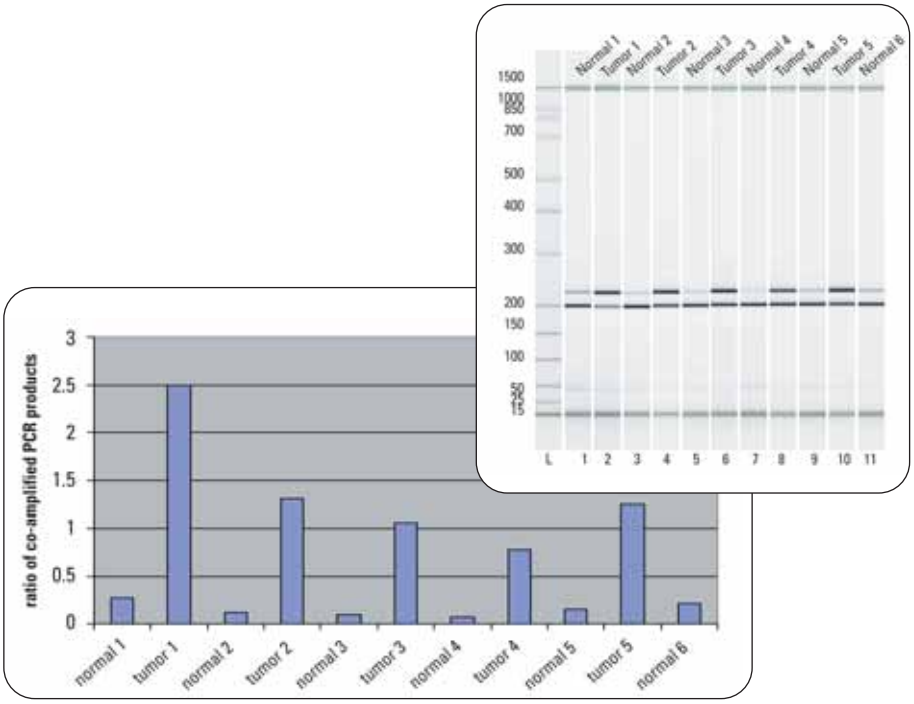
Data kindly provided by Qiagen, Germany

Kit: DNA 7500 kit
Assay: DNA 7500 assay
Application: Many molecular applications include PCR multiplexing as shown above with a PCR that yields 19 products. Applications are genotyping of transgenic organisms, detection of pathogens or GMs and microsatellite genotyping (e.g. short tandem repeat (STR) and variable number tandem repeat (VNTR) analyses). The sample shows optimization of PCR conditions (Mg^{2+} concentration) performed to ensure annealing of the multiple primers under identical conditions. Visualization and evaluation of the results can be performed efficiently with the Agilent 2100 bioanalyzer because of the high resolution, the accurate sizing, quantitation and extended linear range.

Corresponding application note: 5988-9342EN

Gene expression analysis

mRNA expression study by comparative multiplex PCR



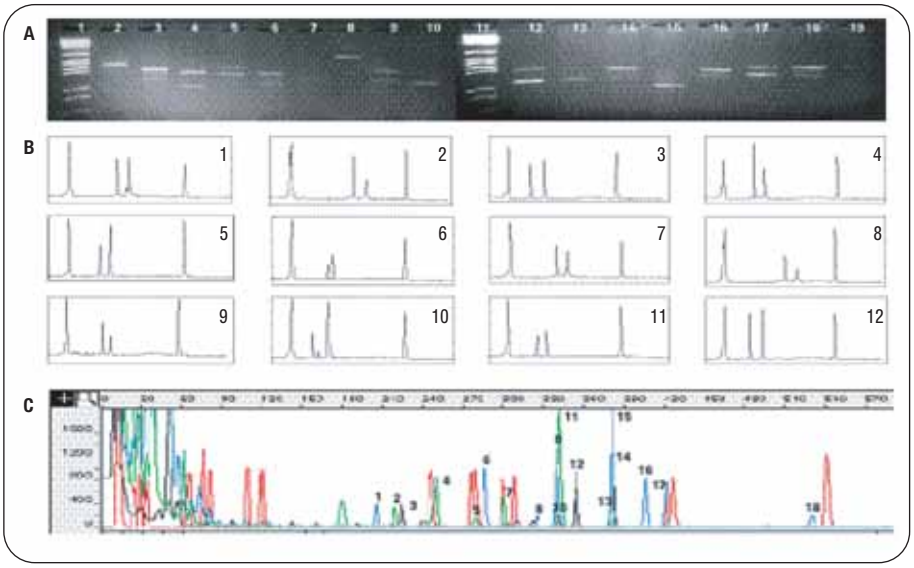
Data kindly provided by the Roy Castle Centre

Kit: DNA 1000 kit
Assay: DNA 1000 assay
Application: Two genes were co-amplified in this study. A tumor specific gene (upper band) along with a housekeeping gene (lower band). The upregulation of the tumor gene is visualized via analysis on the Agilent 2100 bioanalyzer. Building the ratio of the concentration values obtained from the Agilent 2100 bioanalyzer, numerical values are obtained that are normalized with regard to the RT-PCR amplification efficiency. This way tumor tissue can be distinguished from normal tissue more unambiguously.

Corresponding application note: data not published

Gene expression analysis

Standardized end-point RT-PCR



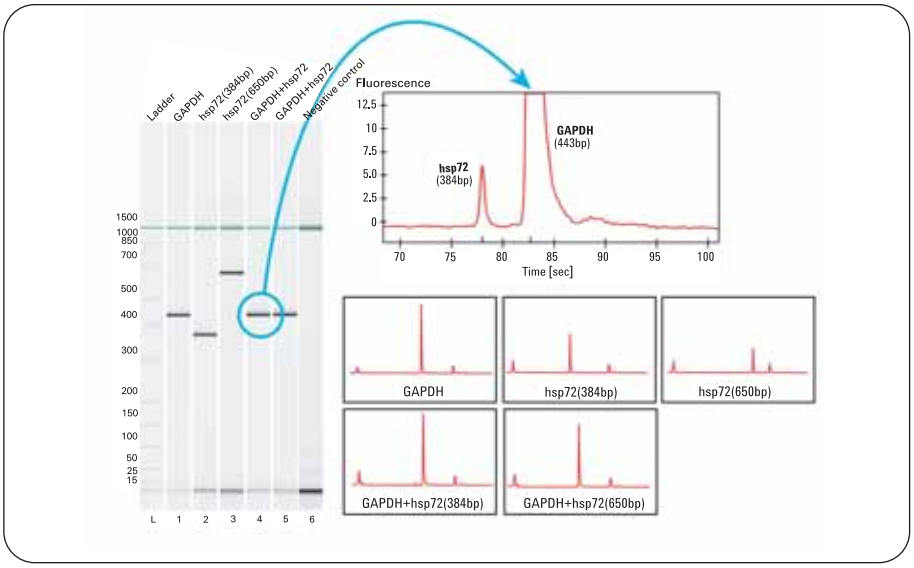
Data kindly provided by the Medical College of Ohio

Kit: DNA 7500 kit
Assay: DNA 7500 assay
Application: Complementary DNA from bronchial epithelial cells (BEC) was analyzed by a Standardized RT-PCR (StaRT) for the expression of 15 different genes. This analysis can be performed at the end-point of PCR without the need for real-time measurement at each cycle of PCR. Three methods for evaluation of representative results were compared (see above). The coefficient of variance (CV) from at least 3 measurements was calculated. The direct comparison of the reproducibility for agarose gel analysis (A, CV = 0.50) and the ABI Prism310 Genetic Analyzer (C, CV = 0.39) with the Agilent 2100 bioanalyzer (B, CV = 0.29) reveals that the Agilent 2100 bioanalyzer is superior. It is a reliable and valuable tool in quantitative gene expression analysis.

Corresponding application note: 5988-3674 EN

Gene expression analysis

Co-amplification of GAPDH and hsp72



Data kindly provided by Dr. Eric Gottwald, Forschungszentrum Karlsruhe, Germany

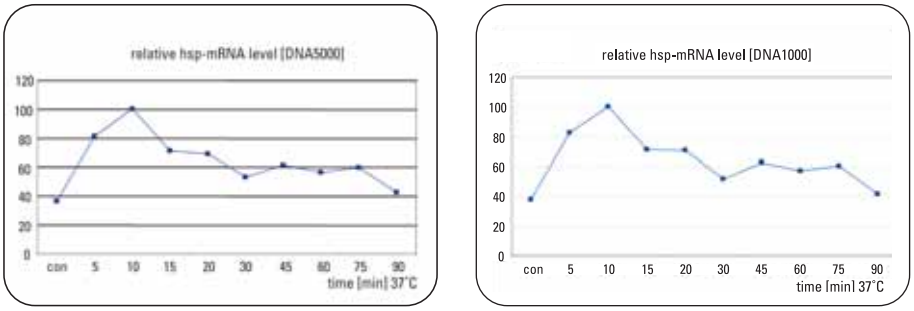
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Kit: DNA 1000 kit
Assay: DNA 1000 assay
Application: Gel-like image and electropherograms showing the results of separate amplifications and co-amplifications of GAPDH and hsp72 in unstimulated HepG2 cells. Primers for GAPDH yield a PCR product of 443 bp (lane 1), primers for hsp72 yield PCR products of 384 and 650 bp (lane 2 and 3). Lane 4 and 5 show the results of the co-amplification reactions. Due to the competitiveness of the reaction, very little hsp72 products could be detected in lane 4 (insert) and no product was detected in lane 5 (lane 6 = negative control). The broad linear dynamic range of the analysis allows detection of weak bands next to strong bands and helped in the determination of gene expression in this case.

Corresponding application note: 5988-4556EN

Gene expression analysis

Co-amplification of GAPDH and hsp72 - response curves



Data kindly provided by Dr. Eric Gottwald, Forschungszentrum Karlsruhe, Germany

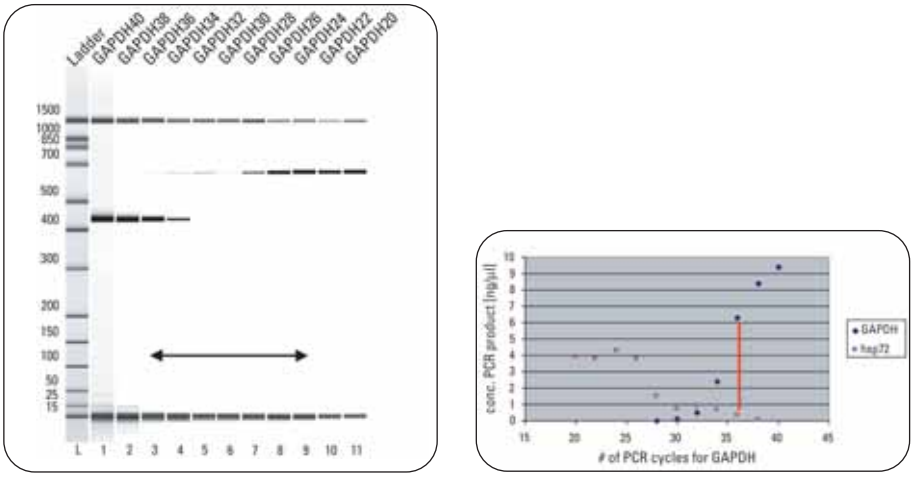
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Kit: DNA 1000 kit
Assay: DNA 1000 assay
Application: The optimized PCR conditions were used to monitor the response of a stimulus to hsp. Gene expression was monitored by comparing the RT-PCR amplification of a housekeeping gene with the co-amplification of hsp. In the current case, the highest gene expression was measured after about 10 minutes. As a comparison, the same set of samples was analyzed using the DNA 500 kit. Virtually identical results are obtained with both kits, demonstrating thatlab-on-a-chip technology can serve as a standardized approach to gel electrophoresis.

Corresponding application note: 5988-4556EN

Gene expression analysis

Competitive PCR



Data kindly provided by Dr. Eric Gottwald, Forschungszentrum Karlsruhe, Germany

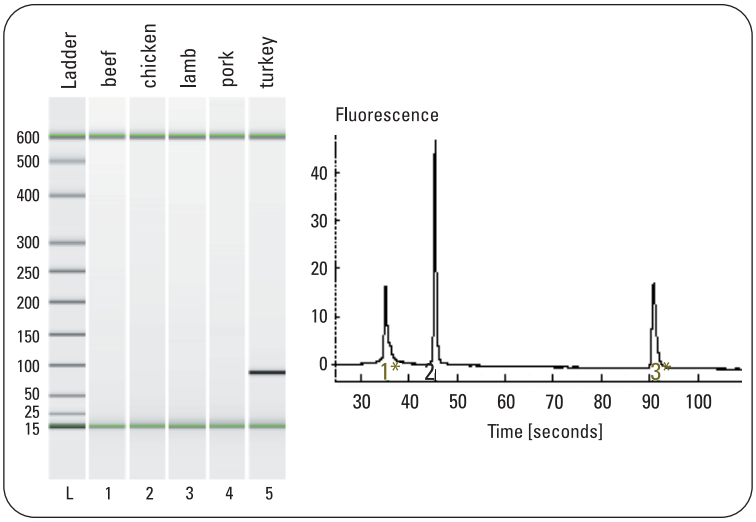
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Kit: DNA 1000 kit
Assay: DNA 1000 assay
Application: Two genes were reverse transcribed and co-amplified in one reaction tube. The PCR products were analyzed using the DNA 1000 LabChip kit. Primers for hsp72 were present from the beginning of the PCR reactions, while primers for GAPDH were added after various cycle numbers ranging from 20 to 40 cycles (primer dropping method). This allowed optimization of this competitive PCR reaction. The left graph displays the dynamic range (arrow) in the gel like view, whereas the right graph indicates conditions with greatest sensitivity (red line).

Corresponding application note: 5988-4556EN

Food analysis

Development of meat specific assays (I)



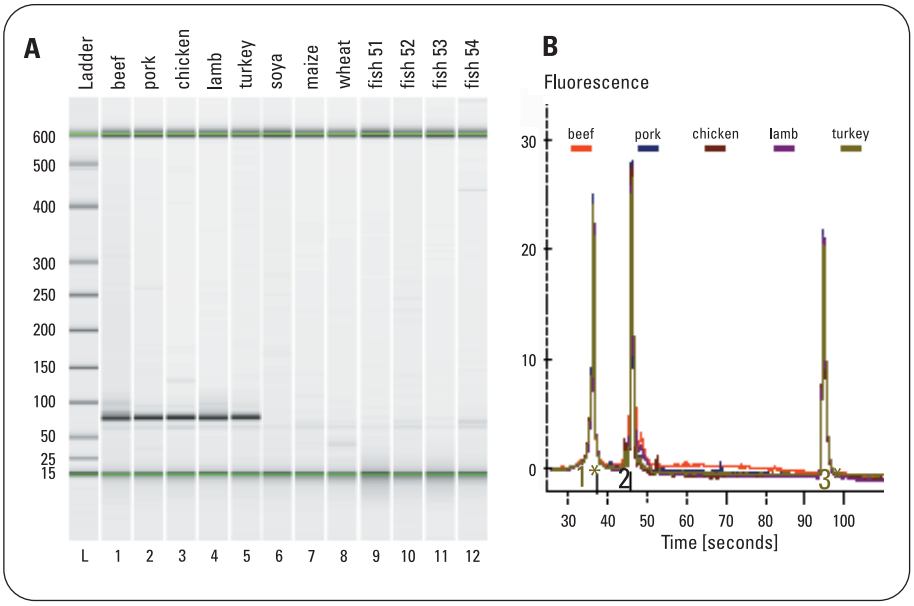
Data kindly provided by CCFRA, UK

Kit: DNA 500 kit
Assay: DNA 500 assay
Application: For detection of individual species in processed food, PCR assays with specific sets of primers can be developed. Example: turkey specific primers do not amplify any other meat species, including beef, chicken, lamb, or pork (see lane 5 and respective electropherogram).

Corresponding application note: 5988-4069EN

Food analysis

Development of meat specific assays (II)



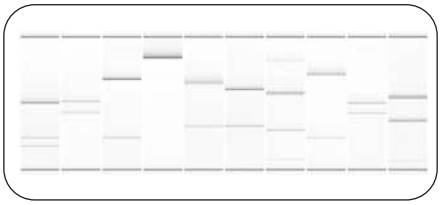
Data kindly provided by CCFRA, UK

Kit: DNA 500 kit
Assay: DNA 500 assay
Application: For detection of individual component types in processed food, PCR assays with specific sets of primers can be developed. Example: Primers that amplify any type of meat, but do not amplify other food constituents, including soya, maize, wheat or fish.

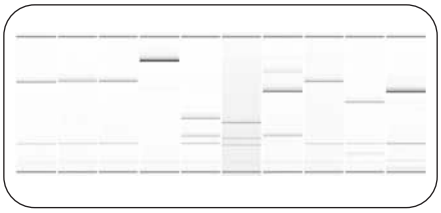
Corresponding application note: 5988-4069EN

Food analysis

Fish species identification by RFLP



Restriction enzyme: DdeI



Restriction enzyme: HaeIII



Restriction enzyme: NlaIII

Common name (UK)	Latin name
Atlantic Cod	Gadus morhua
Pacific Cod	Gadus macrocephalus
Coley (Saithe)	Pollachius virens
Haddock	Melanogrammus aeglefinus
European Hake	Merluccius merluccius
South African Hake	Merluccius paradoxus
European Plaice	Pleuronectes platessa
Whiting	Merlangus merlangus
Alaskan (Walleye) Pollock	Theragra chalcogramma
Hoki	Macruronus novaezelandiae
Atlantic Salmon	Salmo salar
Red / Sockeye Salmon	Oncorhynchus nerka
Pink / Humpback Salmon	Oncorhynchus gorbuscha
Chinook Salmon	Oncorhynchus tschawytscha
Coho / Silver Salmon	Oncorhynchus kisutch
Keta / Chum Salmon	Oncorhynchus keta
Cut-throat Trout	Oncorhynchus clarki clarki
Dolly Varden	Salvelinus malma malma
Cherry Salmon	Oncorhynchus masou masou

Data kindly provided by CCFRA, UK

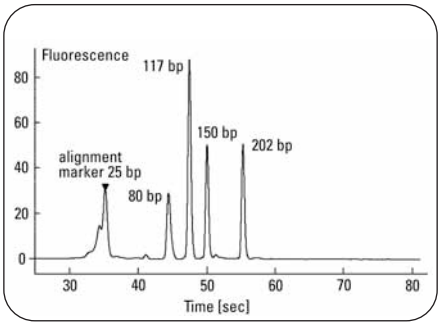
Kit: DNA 500 kit
Assay: DNA 500 assay
Application: Identification of white fish and salmon species in the processed state presents a challenge. However, evaluation of PCR-RFLP profiles (PCR-restriction fragment length polymorphism) of a 464 bp region from the cytochrom b gene cut separately with three restriction enzymes facilitated the differentiation of 19 commercially important species. Analysis of the restriction digests was performed with the Agilent 2100 bioanalyzer. This approach was successfully tested in an interlaboratory study.

Corresponding application note: 5989-2982EN

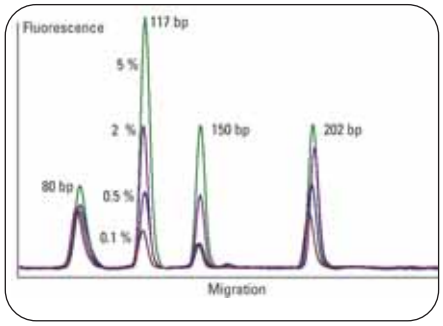
GMO detection

Development of a multiplex assay for soya

A



B



Data kindly provided by CCFRA, UK

Kit: DNA 500 kit
Assay: DNA 500 assay
Application: Multiplex assay for genetically modified (GM) soya. The aim was to develop a model assay that could be used to assess the quality of DNA extracted from heat-processed soya flour samples, in particular, to investigate differences in PCR amplification between small DNA targets. A single multiplex PCR assay was developed that enabled three GM soya targets and one control to be analyzed in a single reaction mix. Primer concentration was optimized in order to obtain four PCR products resolved by gel electrophoresis which corresponded in size to the soya lectin gene target of 80 bp, and the EPSPS (5-enolpyruvyl-shikamate- 3-phosphate synthase) gene targets of 117 bp, 150 bp and 202 bp respectively. These latter targets are only found in Roundup Ready GM soya. Figure A: Peaks produced by the four PCR products when analyzed with the Agilent 2100 bioanalyzer and DNA 500 LabChip kit. Figure B: Analysis of certified reference materials containing known amounts of GM soya.

Corresponding application note: 5988-4070EN

GMO detection

DNA stability during food processing

Time at 100°C and pH 3.3 (min)	Amount of PCR product*			
	80 bp	118 bp	150 bp	202 bp
0	100	100	100	100
3	74	77	73	67
6	57	58	21	6
9	36	23	24	15
12	67	33	47	21
15	48	27	16	0
18	0	0	0	0
21	0	0	0	0

* % product determined relative to the amount at 0 minutes

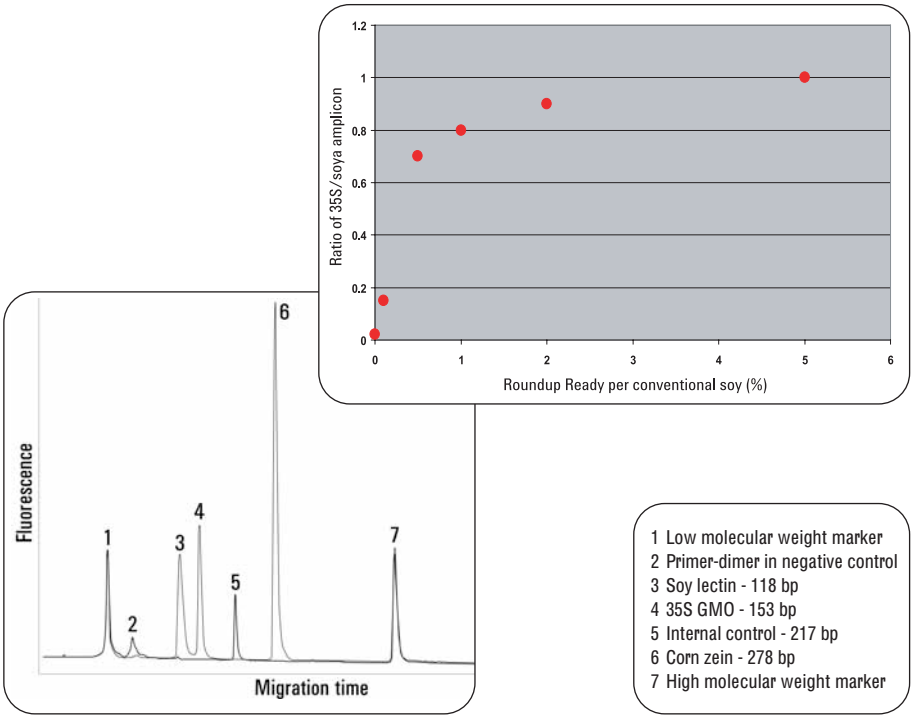
Data kindly provided by CCFRA, UK

Kit: DNA 500 kit
Assay: DNA 500 assay
Application: The multiplex PCR assay was applied to soya flour samples containing approx. 1.3 % GM soya and boiled at either pH 3.3, 4.3 or 6.7 for up to 21 minutes. For accurate determination of the quantity of each PCR product, the samples were applied to the DNA 500 LabChip. The concentration of each PCR product was calculated using the Agilent 2100 bioanalyzer software. At pH 3.3 where an effect of heating time was observed, the amount of each PCR product at each time point was compared to the amount of each product at 0 minutes (Table 2). At pH 3.3, the relative amount of the 80 bp product was reduced to 48 % after 15 minutes and no product was detected at 18 or 21 minutes. After 15 minutes, the relative amounts of products of 118 bp and 150 bp were reduced to 27 % and 16 % respectively and the 202 bp product was not detected. None of the products were detected after 18 or 21 minutes.

Corresponding application note: 5988-4070EN

GMO detection

GMO detection by nested multiplex PCR

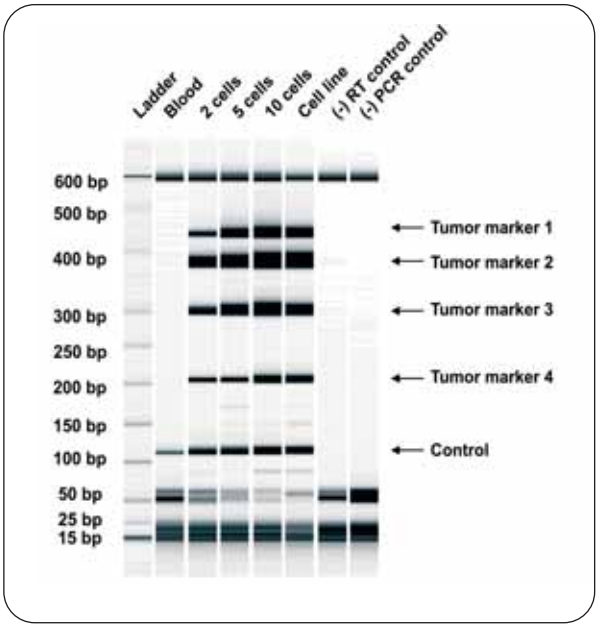


Kit: DNA 1000 kit
Assay: DNA 1000 assay
Application: GMO detection by multiplex PCR is widely used for soy and corn. Often sequences from the transgene and species specific controls or internal standard are co-amplified by endpoint PCR in a screening procedure. Multiple products can be analyzed with the Agilent 2100 bioanalyzer at high resolution and sensitivity. Quantification and comparison of product amounts may already lead to qualification of a positive screening result prior to analysis by expensive quantitative real time PCR.

Corresponding application note: 5989-0124EN

Oncology

Tumor cell detection from carcinoma patient blood



Data kindly provided by AdnaGen

Kit: DNA 500 kit
Assay: DNA 500 assay
Application: A combined method of specific tumor cell enrichment and a high sensitivity tumor cell detection by multiplex PCR allows analysis of several tumor marker genes. The method is so sensitive that it allows the detection of only a few tumor cells per 5 ml EDTA-blood. The Agilent 2100 bioanalyzer provides the performance to detect the PCR products with high sensitivity and automated result flagging. This method offers new possibilities for monitoring and prognosis in routine diagnosis, and may facilitate an appropriate selection of patients for adjuvant therapy.

Corresponding application note: 5988-9341EN

Oncology

SNP analysis in cancer related P16 gene

Sample	1	2	3	4	5	6	7	8	9	10	11
Genotype316	C	C	CG	CG	CG	CG	CG	C	C	C	G
Genotype356	C	C	CT	CT	CT	C	C	CT	CT	T	C
gel-like view Agilent 2100 bioanalyzer											
Main Band	198	198	197	198	198	198	198	198	199	198	195
Extra Band 1			204	204	204						
Extra Band 2			216	215	215	214	214				

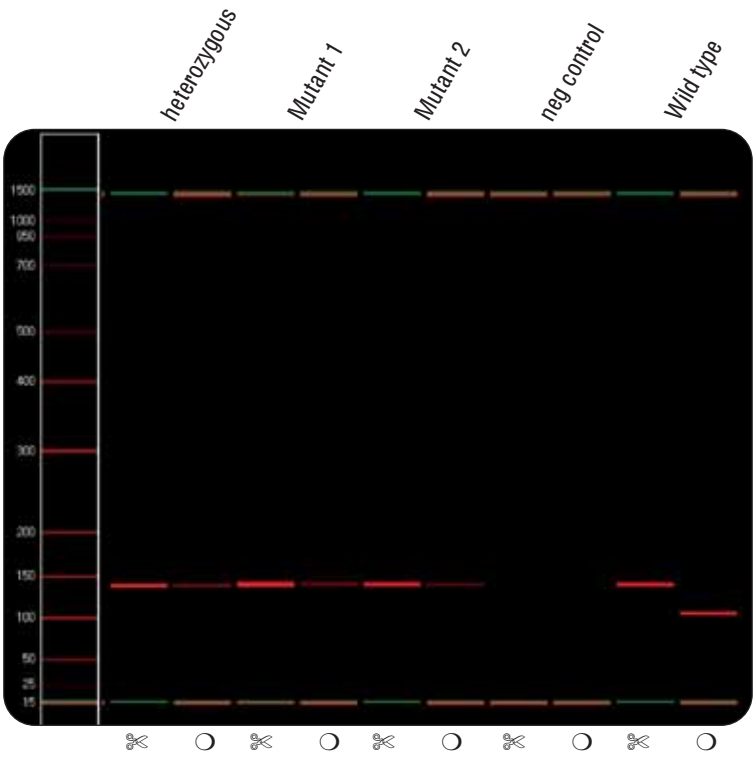
Data kindly provided by SAIC-Frederick

Kit: DNA 1000 kit
Assay: DNA 1000 assay
Application: Mutations in the exon 3 region of P16 gene are closely related to human cancer. A PCR yields 198 bp fragments with single, expected bands or additional, multiple bands in the Agilent 2100 bioanalyzer analysis. These observations correspond perfectly to genotyping sequencing data of normal and mutant tissues. The pattern of bands is visible due to slower mobility of the heteroduplex formed by heterozygote mutant of the samples. The method provides fast and reliable acquisition of genetic diagnostic data from cancer patients, also on single nucleotide polymorphisms (SNP).

Corresponding application note: 5989-0487EN

Oncology

K-ras gene SNP detection



Data kindly provided by SAIC-Frederick

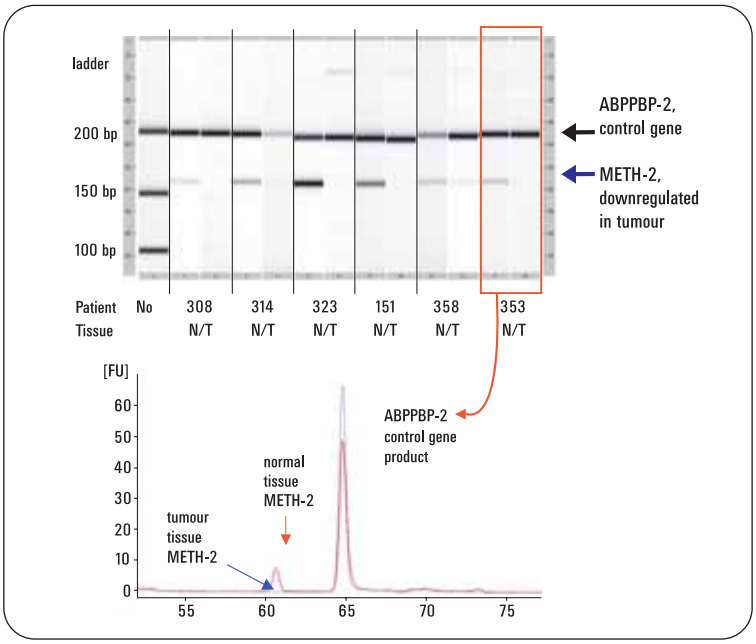
✂ - PCR product
❍ - Restriction

Kit: DNA 1000 kit
Assay: DNA 1000 assay
Application: Mutations in the K-ras gene coding 12 region can lead to cancer in different human tissues. A dedicated combination of PCR and specific restrictions (BstNI digest) reveals the underlying single nucletide polymorphisms (SNPs). The integral element within this test is the rapid and precise analysis of short amplicons (135 bp, see PCR-product lanes above) and fragments (106 bp, visible in lanes labeled with restriction) with the lab-on-a-chip technique. The test was used to ultimately determine a cancer patient's eligibility for a clinical trial for a peptide vaccine.

Corresponding application note: 5989-0487EN

Oncology

METH-2 downregulation in lung carcinomas



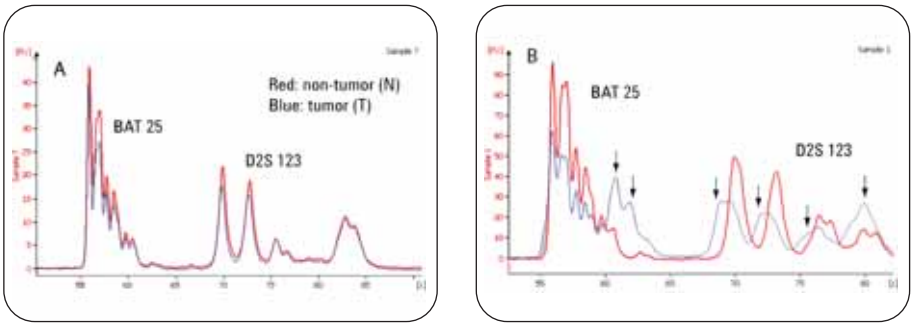
Data kindly provided by Roy Castle Lung Cancer Research Programme,
University of Liverpool, UK

Kit: DNA 1000 kit
Assay: DNA 1000 assay
Application: Microarray analysis reveals under- or over-representations of transcripts. Screening of several cell lines for independent validation of such observations can be done with different techniques such as comparative multiplex PCR. This application shows the downregulation of a characteristic antiangiogenetic factor (METH-2) for a series of patient samples. Expression in normal tissue and tissue from the non small lung carcinomas is compared. Results from the array experiments were confirmed on a broad basis. Fast and convenient analysis with the Agilent 2100 bioanalyzer with given quantitation capability fit perfectly in such analytical workflow.

Corresponding application note: 5989-3514EN

Oncology

Label-free analysis of microsatellite instability in carcinoma

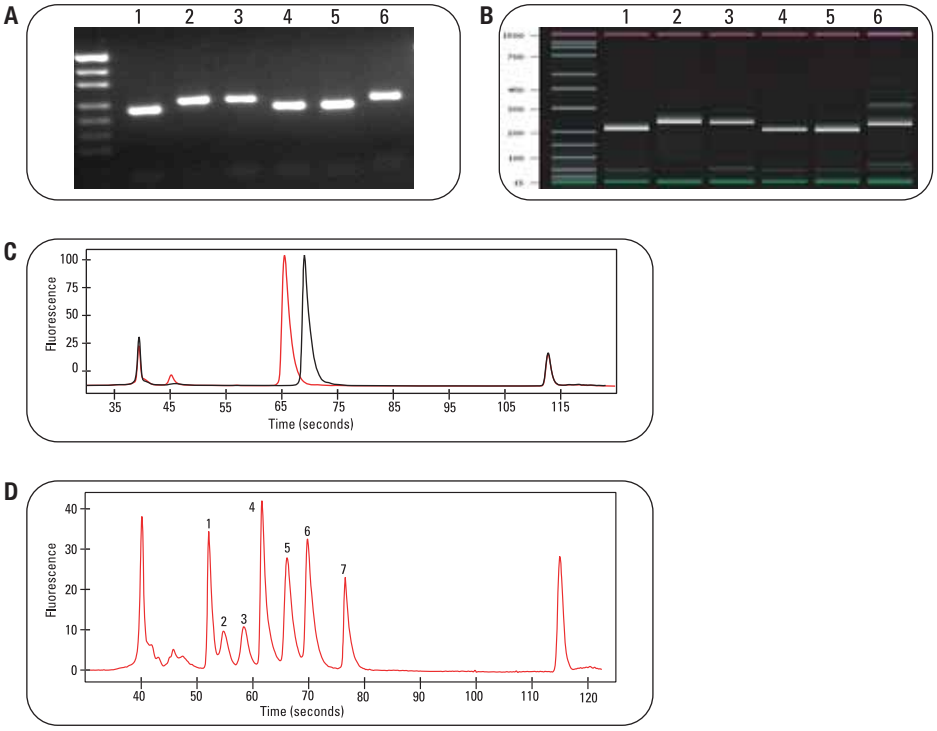


Kit: DNA 1000 kit
Assay: DNA 1000 assay
Application: Microsatellite instability (MSI) is caused by a failure of the DNA mismatch repair system and occurs frequently in various types of cancer. Given that conventional techniques used for MSI detection, for example, polyacrylamide gel electrophoresis (PAGE) or capillary electrophoresis, turned out to be laborious or expensive, this study aimed to develop a simple and efficient procedure of MSI detection. Detection of MSI could be demonstrated by microsatellite loci-associated, well defined deviations in the electropherogram profiles of tumor and non-tumor material and confirmed the classification of the MSI cases performed by conventional technology (95% concordance rate). Whereas the results of the MSI detection were comparable to conventional techniques, the on-chip electrophoresis on the Agilent 2100 bioanalyzer was superior in terms of speed, usability and data management.

Corresponding application note: 5989-2626EN

Clinical research

Genotyping of H. pylori



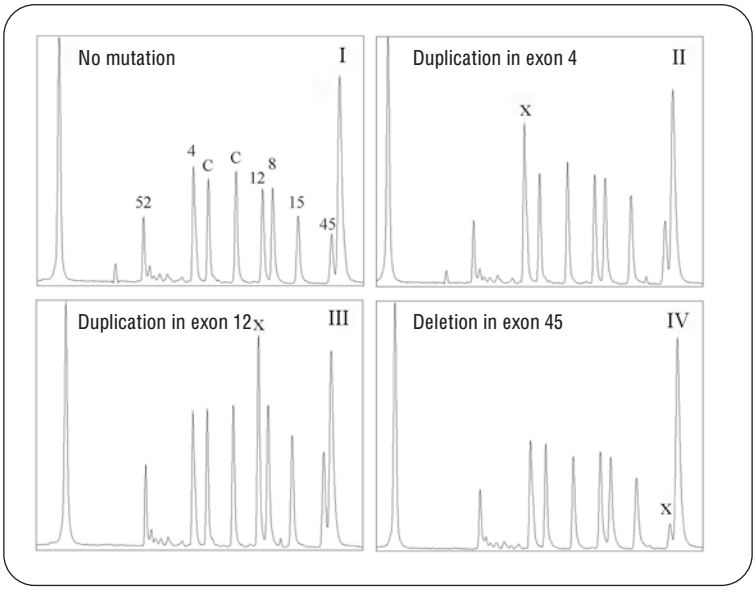
Data kindly provided by Institute for Pathology, Cologne

Kit: DNA 1000 kit
Assay: DNA 1000 assay
Application: Different allelic variants are associated with different stages of H. pylori virulence. Multiplex PCR on five alleles with products in the range of 102 to 301 bp were used to analyze DNA from paraffin embedded tissues. Agarose gel (A) yields only limited distinctiveness, whereas gel-like images (B) and electropherograms (C) show good resolution and superior reproducibility allowing convenient analysis of all desired products in parallel (D). An extended spectrum of prognostic or therapeutic relevant information is now routinely accessible for simultaneous analysis.

Corresponding application note: 5989-0078EN

Clinical research

Duplications and deletions in genomic DNA



Data kindly provided by Center for Human and Clinical Genetics, Leiden

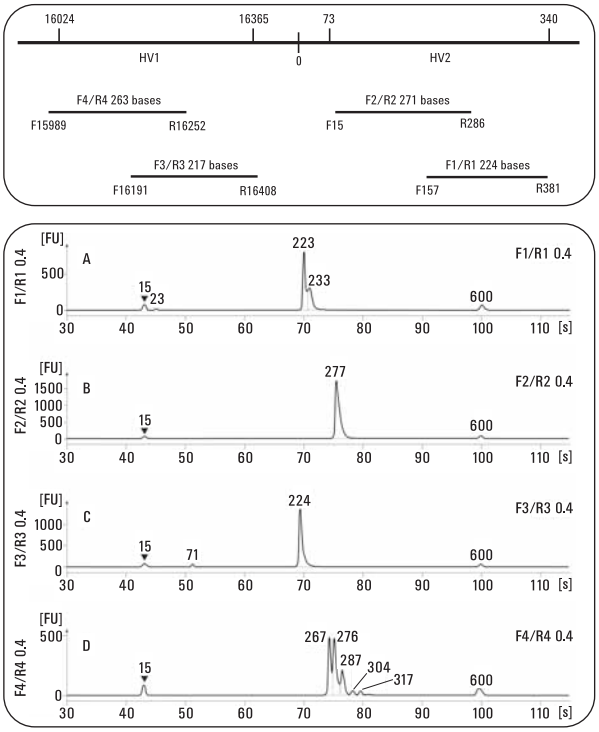
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Kit: DNA 500 kit
Assay: DNA 500 assay (in expert software)
Application: Multiplex amplifiable probe hybridization (MAPH) and multiplex ligation-dependent amplification (MLPA) are high throughput techniques for the detection of reordered genomic segments. These methods include hybridization of amplifiable probes with either stringent washing or ligation events prior to amplification. Exact and reproducible sizing and quantitation of multiple products are important prerequisites which are delivered by the Agilent 2100 bioanalyzer and lead to quick and simple analysis of genetically related diseases.

Corresponding application note: 5989-0192EN

Forensic testing

Optimization of PCR on mtDNA



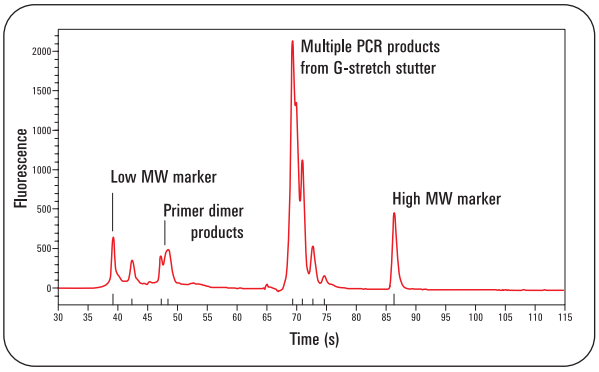
Amplified areas
in human mtDNA

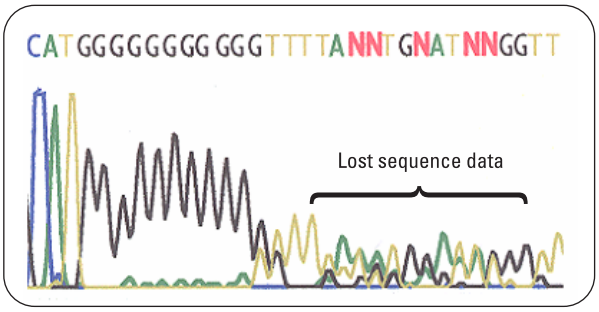
Kit: DNA 500 kit
Assay: DNA 500 assay
Application: Human mitochondrial DNA (mtDNA) is amplifiable even from small or badly degraded samples, even if genomic DNA is not available. Lanes B and C show homogenous PCR products which can subsequently be sequenced for identification. However, careful optimization of PCR parameters, like pH, Mg2+ concentration or polymerase amount is necessary and shown in detail in this application note. For example, a high Taq concentration increased the yield but also increased the level of byproducts. PCR for samples in lane D (impurities) and lane A (C-heteroplasmy) need to be improved. The Agilent 2100 bioanalyzer provides a rapid quantitative analysis over the broad size and concentration range needed for optimization and QC. It has proven to be an indispensable tool for forensic labs.

Corresponding application note: 5989-3107EN

Forensic testing

Pitfalls in mtDNA sequencing

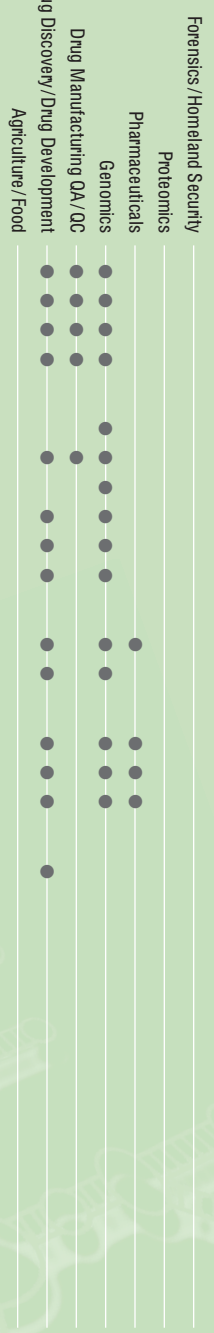




Kit: DNA 500 kit
Assay: DNA 500 assay
Application: Analysis of the non-coding sequence of human mitochondrial DNA (mtDNA) is performed for the purpose of identification in forensics. PCR amplification of limited or degraded mtDNA is done prior to sequencing. Quantitation and quality control of these PCR products (10-100 ng/ml, homogenous fragment in the range of 200-500 bp) was performed. Difficult PCR templates may cause G-stutters or other unintended byproducts of higher or lower mass (left). This may lead to indistinct sequence readings (right). Therefore, e.g. FBI guidelines enforce a 10% impurity level at the most. Fulfillment of this prerequisite can be satisfactorily verified with the Agilent 2100 bioanalyzer.

Corresponding application note: 5989-0985EN

III. RNA analysis



Analysis of total RNA
- RNA integrity
- Standardization of RNA Quality Control
- Reproducibility of quantitation
- Genomic DNA contamination

Low amounts of total RNA
- Detection of low levels of RNA
- RNA integrity with the RNA 6000 Pico kit
- RNA quality after staining and microdissection
- Analysis of minimum RNA amounts
- Genomic DNA in low concentrated RNA extracts
- Low RNA amounts from kidney sections

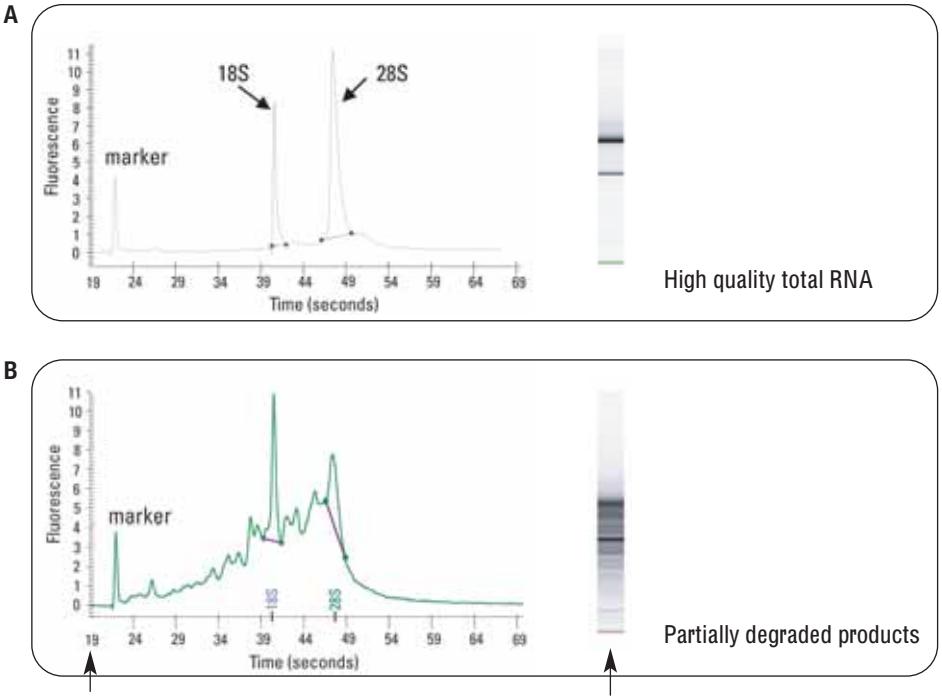
Analysis of mRNA
- RNA integrity
- Ribosomal RNA contamination in mRNA samples

Analysis of Cy5-labeled samples
- Analysis of cRNA with and without dye in gel matrix
- Optimization of labeling reactions
- cRNA fragmentation

Analysis of T7-RNA transcripts
- Size estimation

Analysis of total RNA

RNA integrity

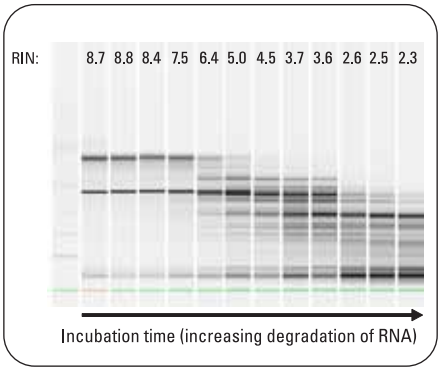


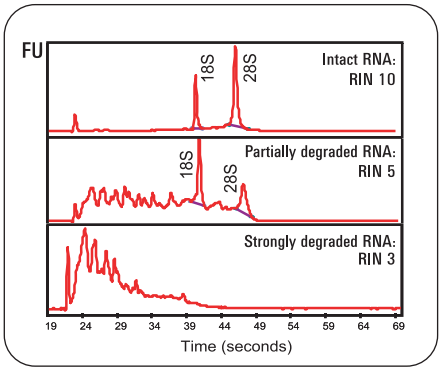
Kit: RNA 6000 Nano kit
Assay: Eukaryote total RNA Nano assay
Application: Analysis of total RNA integrity - a typical first QC step during cDNA or cRNA sample prep for microarrays. In Figure A the upper electropherogram and gel-like image show the analysis of high quality total RNA with the 18S and 28S subunit as two distinct bands. Figure B shows the analysis of a partially degraded total RNA sample. Many degradation products appear between the two ribosomal bands and below the 18S band. With the help of the Agilent 2100 bioanalyzer and the RNA 6000 Nano kit the important sample QC step prior to an expensive microarray experiment can be easily and quickly achieved.

Corresponding application note: 5968-7493EN

Analysis of total RNA

Standardization of RNA Quality Control





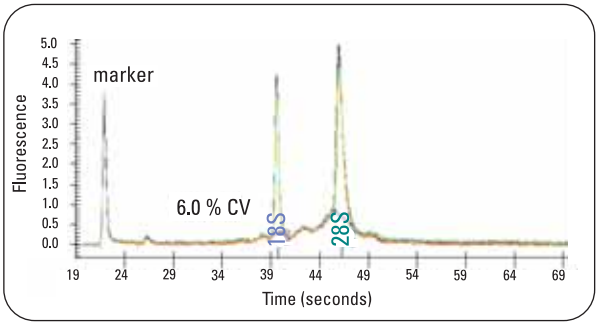
Kit: RNA 6000 Nano kit
Assay: Eukaryote total RNA Nano assay
Application: The RNA integrity number (RIN) is calculated by a dedicated software algorithm (expert 2100 software, starting with Rev 02.01) to assess the quality of RNA preparations. The RIN tool is a major step in the standardization of user-independent RNA evaluation and delivers more meaningful information than simple ratio calculations for ribosomal RNA peaks. It is not influenced by instrument, sample integration and most important, concentration variability, thereby facilitating the comparison of samples and avoiding cost-intensive experiments with low quality RNA preparations. The RIN algorithm is based on a large collection of RNA data of various tissues and qualities. Furthermore, anomalies like genomic DNA contaminations are indicated with weighted error messages (critical/non-critical) to achieve a maximum of reliability.

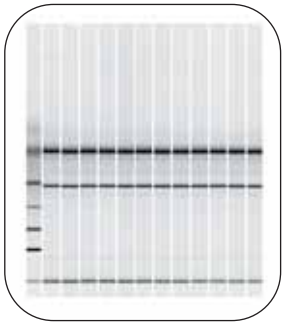
Corresponding application note: 5989-1165EN

Analysis of total RNA

Reproducibility of quantitation

Reproducibility for 12 consecutive runs



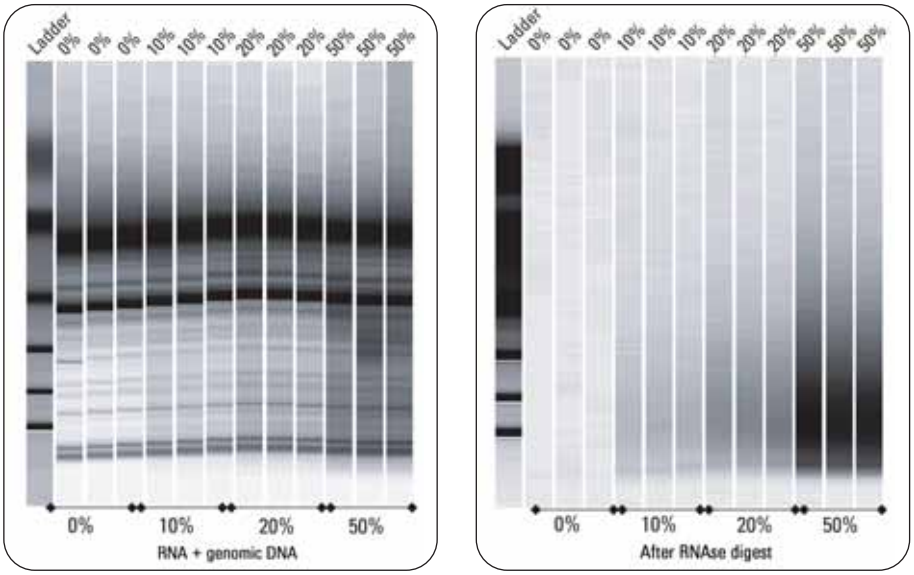


Kit: RNA 6000 Nano kit
Assay: Eukaryote total RNA Nano assay
Application: Alongside the quality control of RNA samples, measurement of RNA concentration is important for (bio-)chemical reactions, such as labeling reactions in the context of microarray experiments. With the RNA 6000 Nano kit good reproducibility can be achieved (here 6% CV), which is little affected by sample contaminants, such as phenol.

Corresponding application note: 5988-7650 EN

Analysis of total RNA

Genomic DNA contamination

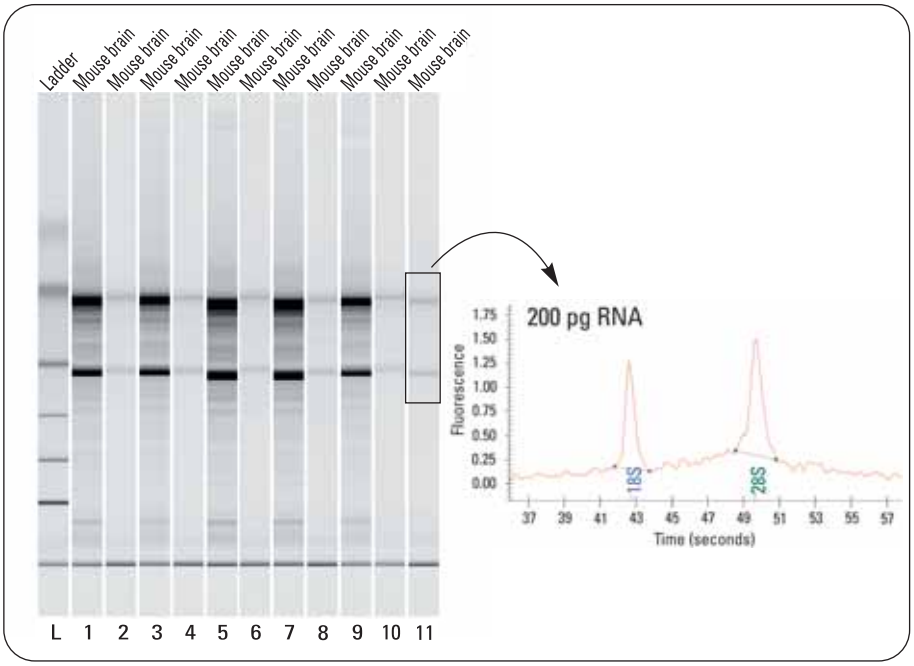


Kit: RNA 6000 Nano kit
Assay: Eukaryote total RNA Nano assay
Application: Gel representation of a chip run with total RNA samples (mouse brain) spiked with varying amounts of herring sperm genomic DNA before and after treatment with RNase. The left panel shows the intact RNA with broad bands in the low MW region stemming from the genomic DNA. After the RNase digest (right panel) only the DNA bands remain, ranging in intensity according to the amount of DNA spiked into the sample.

Corresponding application note: data not published

Low amounts of total RNA

Detection of low levels of RNA



Analysis of mouse brain RNA at two different concentrations

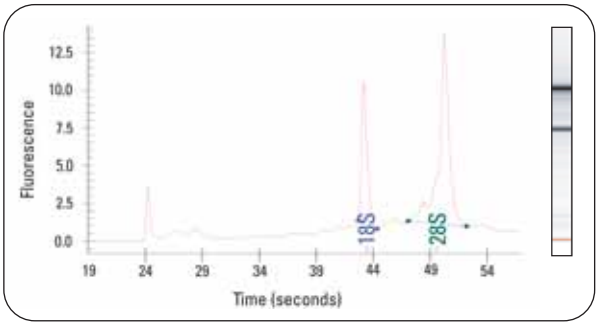
Kit: RNA 6000 Pico kit
Assay: Eukaryote total RNA Pico assay
Application: The RNA 6000 Pico kit is complementary to the RNA 6000 Nano kit and is suitable for all applications where the amount of RNA (or cDNA) is limited, e.g. for biopsy samples, samples from microdissection experiments, QC of cDNA made from total RNA, microarray samples, etc. Here Agilent 2100 bioanalyzer results obtained from mouse brain RNA (Ambion) at 200 and 1000 pg/ìl are shown. By analysis in repetitions the reproducibility of quality control is demonstrated. Detection of 200 pg total RNA could be achieved without problems.

Corresponding application note: data not published

Low amounts of total RNA

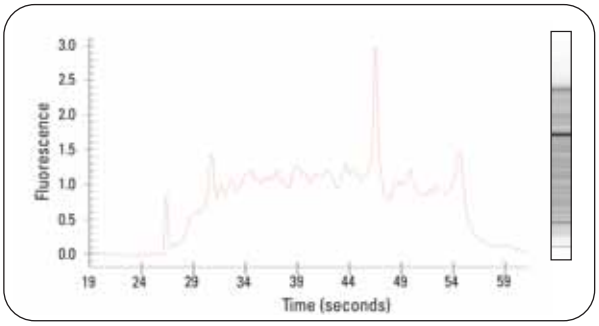
RNA integrity with the RNA 6000 Pico kit

A



Intact RNA

B



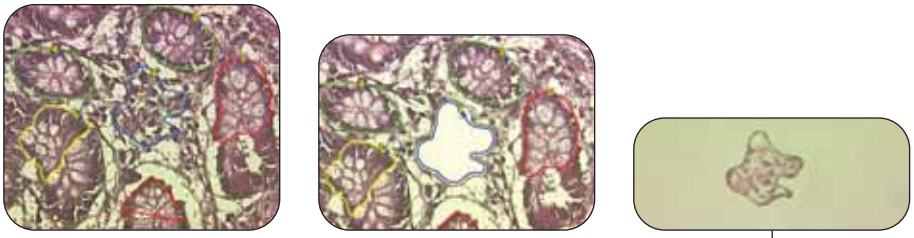
Degraded RNA

Kit: RNA 6000 Pico kit
Assay: Eukaryote total RNA Pico assay
Application: Detection of RNA degradation with the RNA 6000 Pico kit. Sample: mouse liver total RNA (Ambion) concentration: 1 ng. Degradation was accomplished by adding a low amount of RNase. In Figure A the upper electropherogram and gel-like image show the analysis of high quality total RNA with the 18S and 28S subunit as two distinct bands. Figure A shows the analysis of a partially degraded total RNA sample. Many degradation products appear between the two ribosomal bands and below the 18S band.

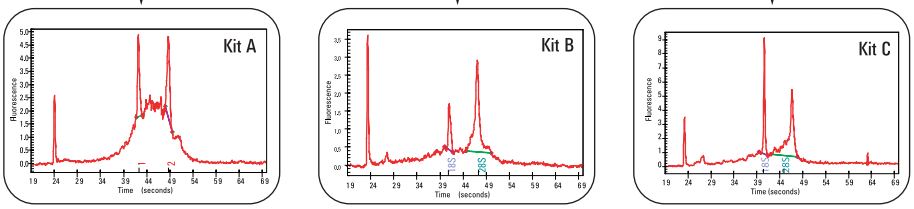
Corresponding application note: data not published

Low amounts of total RNA

RNA quality after staining and microdissection



Laser micro dissection



Check and optimize RNA quality and yield

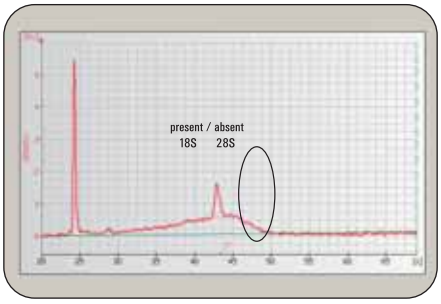
Kit: RNA 6000 Pico kit
Assay: Eukaryote Total RNA Pico assay
Application: RNA derived from laser-microdissected tissue isolated by the PALM®MicroBeam system was shown to be of high quality by convenient analysis with the RNA 6000 Pico assay. RNA-purification kits from different manufacturers and various common staining procedures have been tested and yielded 130-700pg/µl RNA from 1000 cells with different quality (see above). The RNA 6000 Pico kit was well suited to show differences in RNA quality and yield and, therefore, is an ideal tool to optimize and adapt experimental conditions to individual tissue. The experiments were accompanied by a more laborious real time PCR that revealed similar results. Due to its unprecedented sensitivity, the RNA 6000 Pico assay is an indispensable tool for quality control in the context of microdissection experiments, ensuring successful gene expression profiling experiments.

Corresponding application note: 5988-9128EN

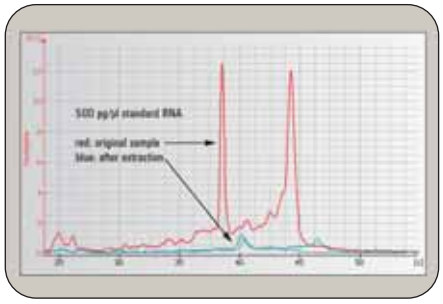
Low amounts of total RNA

Analysis of minimum RNA amounts

A



B



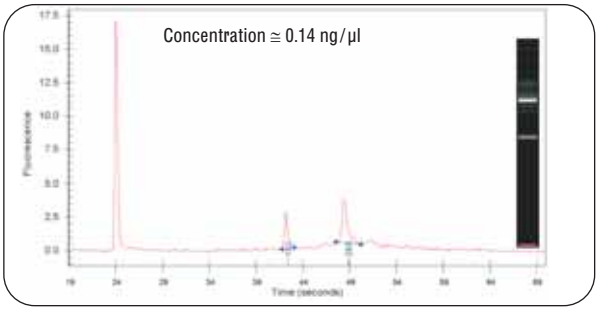
Kit: RNA 6000 Pico kit
Assay: Eukaryote Total RNA Pico assay
Application: The challenge of analysis of minimal amounts of RNA from e.g. laser micro dissections calls for detailed knowledge of extraction conditions. Some commonly used RNA isolation kits and buffer components were assessed in detail. The majority of the kits had no negative effect on the performance of the analysis, whereas, some kits include buffers which lead to shifted, missing and diminished RNA-peaks. In figure A, RNA isolated after microdissection shows lack of the 28S-peak due to high salt concentration introduced during the isolation process. In figure B, a standard RNA was diluted in water and subsequently extracted with a commercially available RNA extraction kit. The original samples (red) and the eluates after extraction are shown. These data show the importance of evaluating the individual method used for RNA extraction to exclude misleading conclusions.

Corresponding application note: 5989-0712EN

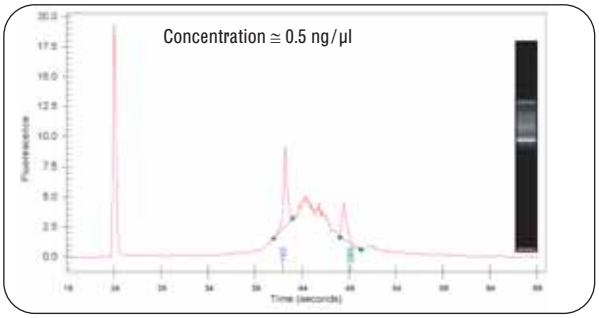
Low amounts of total RNA

Genomic DNA in low concentrated RNA extracts

A



B

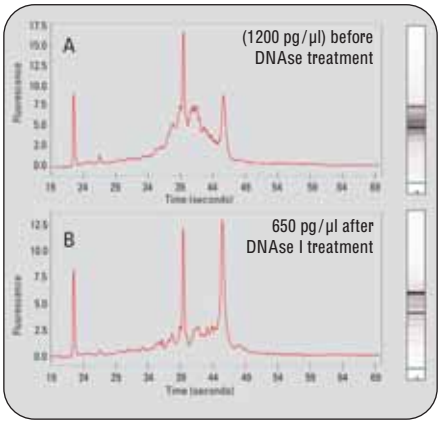


Kit: RNA 6000 Pico kit
Assay: Eukaryote Total RNA Pico assay
Application: Laser capture microdissection enables collection of cells from small tissue areas. A low RNA yield is in the nature of the extraction method from such a specimen that usually complicates quality assessment – a fact that can be circumvented by taking advantage of the Agilent 2100 bioanalyzer capabilities. A comparative study using mouse kidney cryosections showed that on-column DNase digestion is indispensable to obtain a reasonable result for integrity and yield (figure A). Experiments with omitted on-column DNA digestion confirmed that the peak visible in the inter-region consists of genomic DNA which caused overestimation of extracted RNA amounts (figure B).

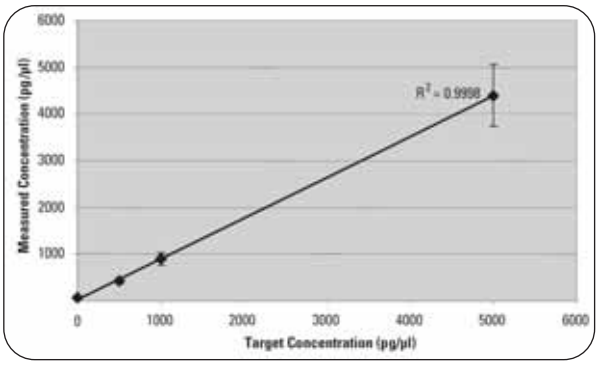
Corresponding application note: 5989-0991EN

Low amounts of total RNA

Low RNA amounts from kidney sections



Renal medulla RNA

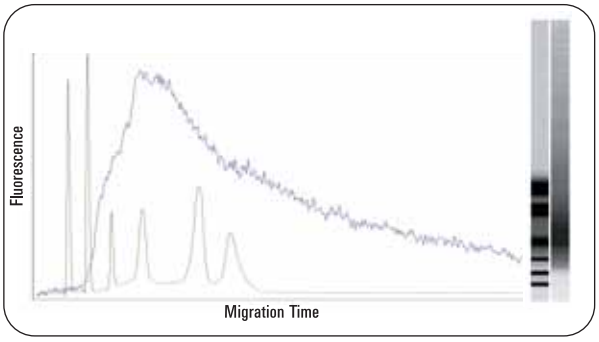


Kit: RNA 6000 Pico kit
Assay: Eukaryote Total RNA Pico assay
Application: High sensitivity quality control of RNA samples using the RNA 6000 Pico LabChip kit are demonstrated for microdissected samples (0.1 mm^3). DNAse I digestion revealed that DNA contamination was present in the sample. Removal of DNA revealed total RNA with a low degree of degradation. Under ideal conditions, the RNA Pico assay has a linear response curve and, therefore, allows estimation of RNA concentrations.

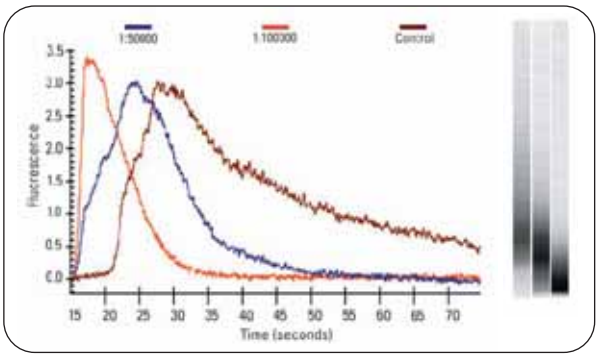
Corresponding application note: 5988-8554EN

Analysis of mRNA

RNA integrity



Highly enriched
Poly (A)+ RNA



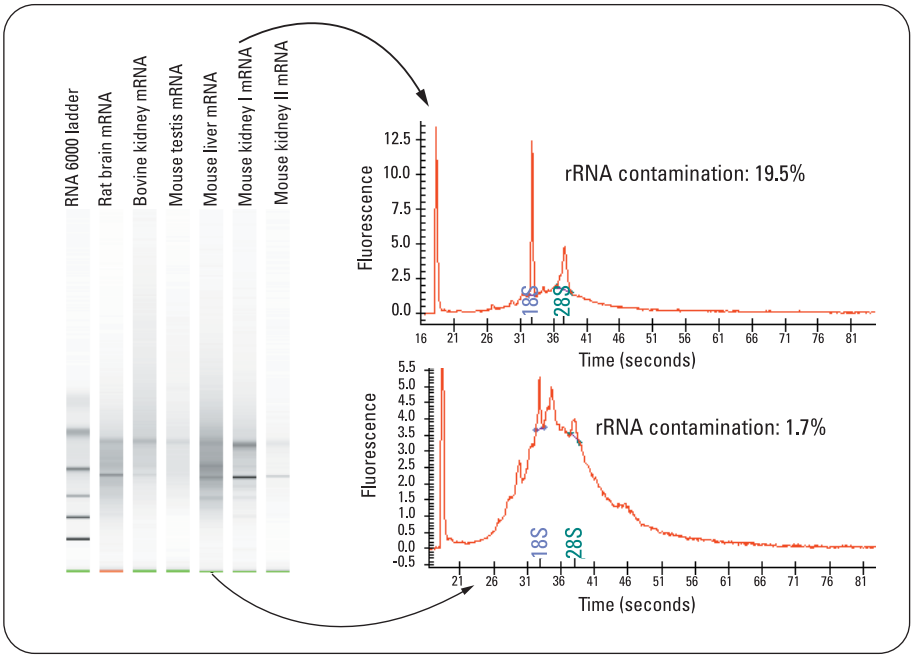
Progressive degradation
of Poly (A)+ RNA

Kit: RNA 6000 Nano kit
Assay: mRNA Nano assay
Application: Progressive degradation of Poly (A)+ RNA. Poly (A)+ RNA (60 ng/µL) from cultured Jurkat cells was incubated for 15 minutes at room temperature with very dilute RNase A (1 x 10-6 and 2 x 10-6 mg/mL, respectively). A progressive shift towards shorter fragment sizes can be observed. Even with a mild degradation, the absence of very long transcripts can be noticed.

Corresponding application note: 5968-7495EN

Analysis of mRNA

Ribosomal RNA contamination in mRNA samples

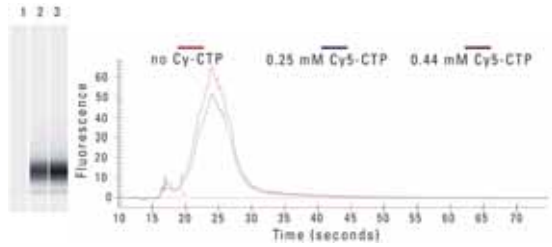


Kit: RNA 6000 Nano kit
Assay: mRNA Nano assay
Application: Ribosomal contamination in mRNA samples. During the isolation of mRNA, varying amounts of ribosomal RNA can remain in a sample. Since the purity of mRNA is of importance for a number of downstream applications, samples should be checked on the Agilent 2100 bioanalyzer. This slide shows the analysis of 6 commercially available RNA samples from different suppliers. Analysis on the Agilent 2100 bioanalyzer reveals large differences in the purity of the mRNA samples.

Corresponding application note: 5968-7495EN

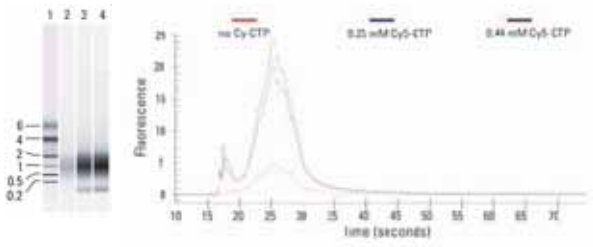
Analysis of Cy5 labeled samples

Analysis of cRNA with and without dye in gel matrix



Assay conditions:
- Cy5 labeled nucleic acids
- no intercalating dye used
- 5nM Cy5 dCTP added to gel matrix and sample buffer for focusing

Lanes:
1. Unlabeled cRNA
2. Cy5 labeled cRNA
3. Cy5 labeled cRNA



Assay conditions:
as above, but intercalating dye included in gel matrix

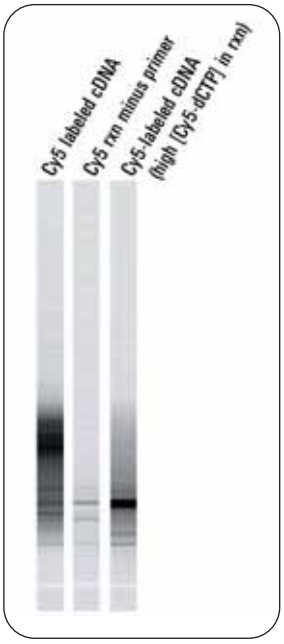
Lanes:
1. RNA transcript ladder
2. Unlabeled cRNA
3. Cy5 labeled cRNA
4. Cy5 labeled cRNA

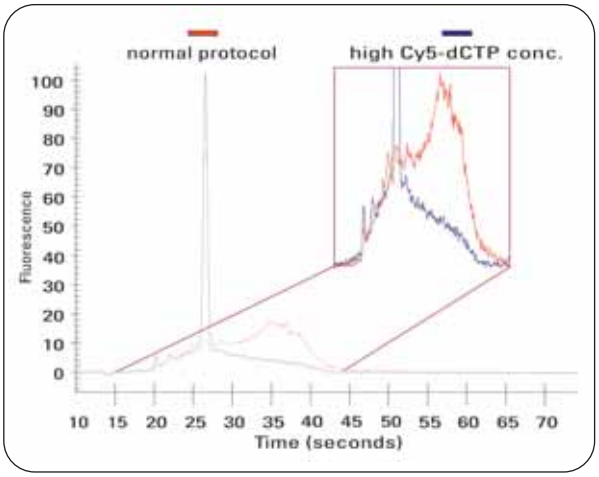
Kit: RNA 6000 Nano kit
Assay: mRNA Nano and Cy5 labeled nucleic acids Nano assay
Application: Analysis of Cy5 labeled and non-labeled cRNA samples. Cy5-labeled samples show the combined signals of the fluorescent label and the RNA signal created by the fluorescence of the RNA 6000 dye. If the RNA 6000 dye is omitted from the gel matrix, only the signal created by Cy5 is detected, allowing the determination of dye incorporation after a labeling reaction. Please note that for Cy3 labeled samples the intactness of the sample can be verified, but the dye incorporation can not be checked.

Corresponding application note: 5980-0321EN

Analysis of Cy5 labeled samples

Optimization of labeling reactions



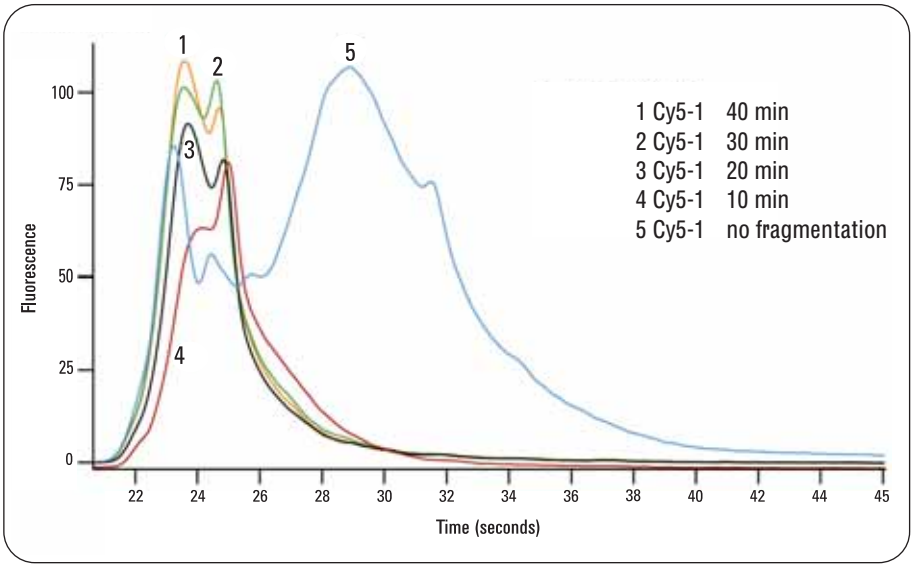


Kit: RNA 6000 Nano kit
Assay: Cy5 Labeled Nucleic Acids Nano assay
Application: An experiment was designed to check the influence of Cy5 dCTP concentration on labeling efficiency. Lane 2 represents the negative control (primer ommitted from the reaction mixture), while lane 3 shows the analysis of a reaction with a 6-fold increased Cy5 dCTP concentration. A look at the electropherograms reveals that the high Cy5 dCTP concentration not only gave a high peak of unincorporated Cy5, but also the labeling efficiency for longer fragments was very low. This approach allows the optimization of labeling reactions.

Corresponding application note: 5980-0321EN

Analysis of Cy5 labeled samples

cRNA fragmentation

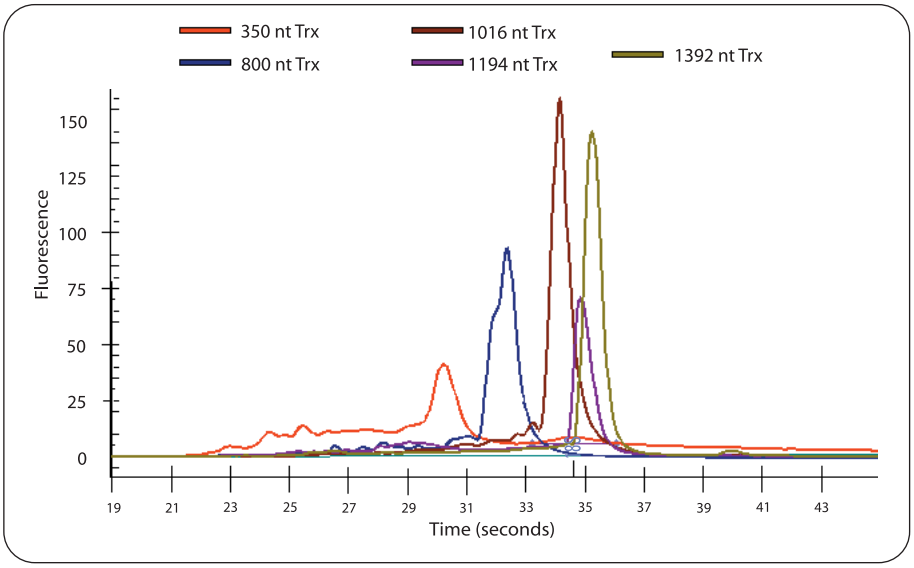


Kit: RNA 6000 Nano kit
Assay: Eukaryote total RNA Nano assay
Application: The RNA 6000 Nano LabChip kit can be used to monitor completion of a cRNA fragmentation reaction. In this example, the profile of a Cy5 labeled cRNA sample was monitored at different time points during a fragmentation reaction. It can be seen that after 10 minutes most of the fragments are in the desired size range. After 20 minutes, no further shift of fragmentation can be observed indicating completion of the fragmentation reaction.

Corresponding application note: 5988-3119EN

Analysis of T7 RNA transcripts

Size estimation



Kit: RNA 6000 Nano kit
Assay: Eukaryote total RNA Nano assay
Application: A number of RNA transcripts, ranging from 350 to 1400 nt in size, were analyzed on the RNA 6000 Nano LabChip kit. Although the assay runs under native conditions and the transcripts exhibit a certain degree of secondary structure, a good size estimation can be achieved.

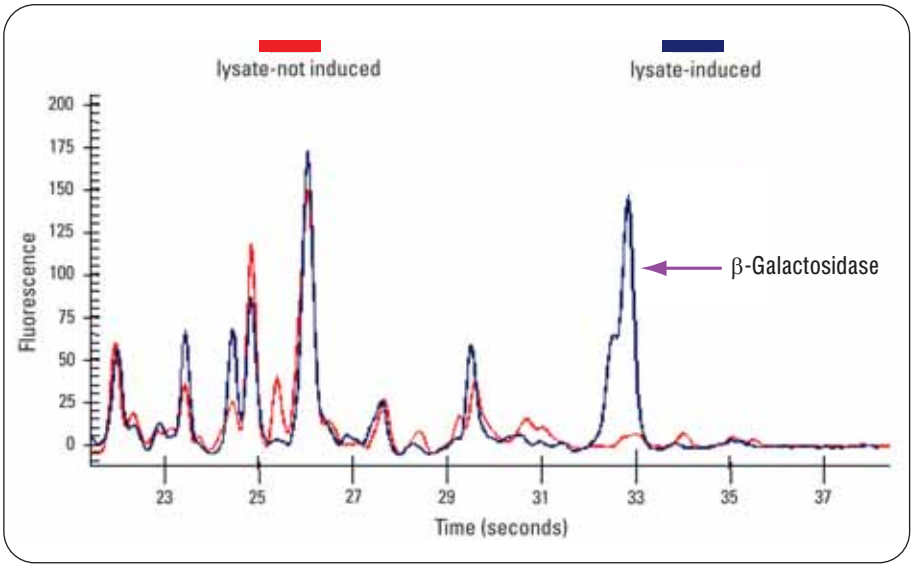
Corresponding application note: data not published

IV. Protein analysis

	Agriculture / Food	Drug Discovery / Drug Development	Drug Manufacturing QA / QC	Genomics	Pharmaceuticals	Proteomics	Forensics / Homeland Security
Protein expression							
Analysis of cell lysates - protein induction			●			●	
Protein purification:							
Comparison between lysate and flow through		●			●		
Analysis of protein purification		●		●		●	
GFP Streptag fusion protein purification		●				●	
Analysis of column capacity						●	
Analysis of column fractions to optimize conditions		●	●			●	
His-tag protein purification with Ni++ ZipTips®		●	●			●	
Enzymatic removal of His-tags from recombinant proteins			●			●	
Complementing RP-HPLC protein purification		●			●	●	
Antibody analysis							
Analysis of antibodies under reducing and non-reducing conditions		●	●				
Quantitation of the half-antibody content in IgG_4 preparations		●	●		●		
Comparison of SDS-PAGE, CGE and Agilent 2100 bioanalyzer for humanized monoclonal antibody analysis		●	●			●	
Absolute quantitation of IgG		●	●			●	
Quality control of stressed antibodies		●	●				
Separation of bispecific antibodies chains		●	●		●	●	
Food analysis							
Bovine milk analysis	●					●	
Protein pattern of different transgenic seedlines	●					●	
Protein - others							
Absolute protein quantitation	●		●		●	●	
Glycoprotein sizing	●		●			●	
Protein quality control prior to MS-analysis			●			●	
Depletion of high abundant proteins from blood samples		●			●		
Increased sensitivity by desalting protein samples	●	●			●	●	

Protein expression

Analysis of cell lysates - protein induction

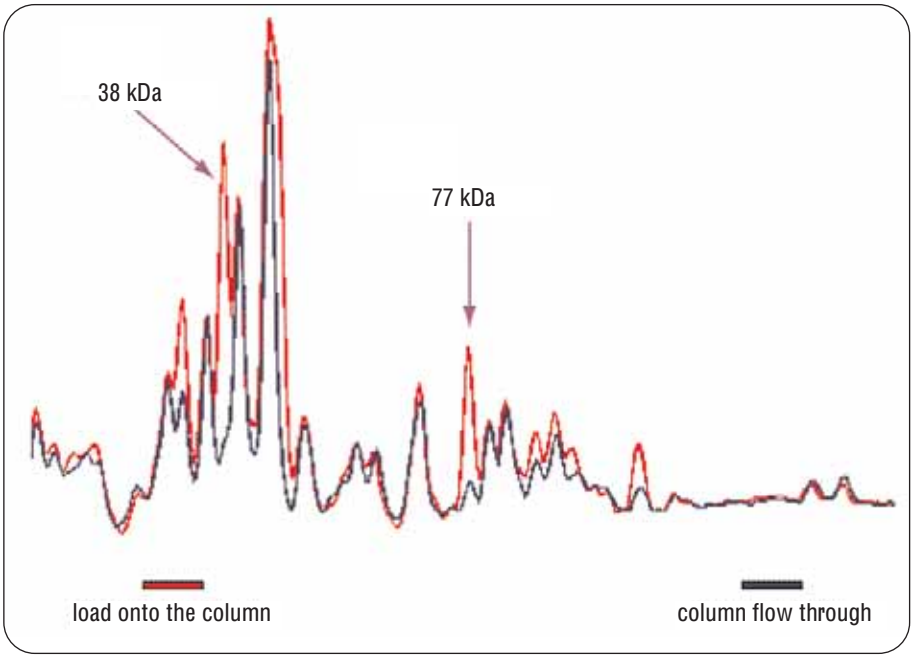


Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: Two cell lysates, induced and non-induced were compared to verify the induction of protein expression. The overlay feature of the bioanalyzer software allows quick sample comparison. The blue electropherogram trace shows the cell lysate highly expressing β-galactosidase (128 kDa).

Corresponding application note: data not published

Protein purification

Comparison between lysate and flow through

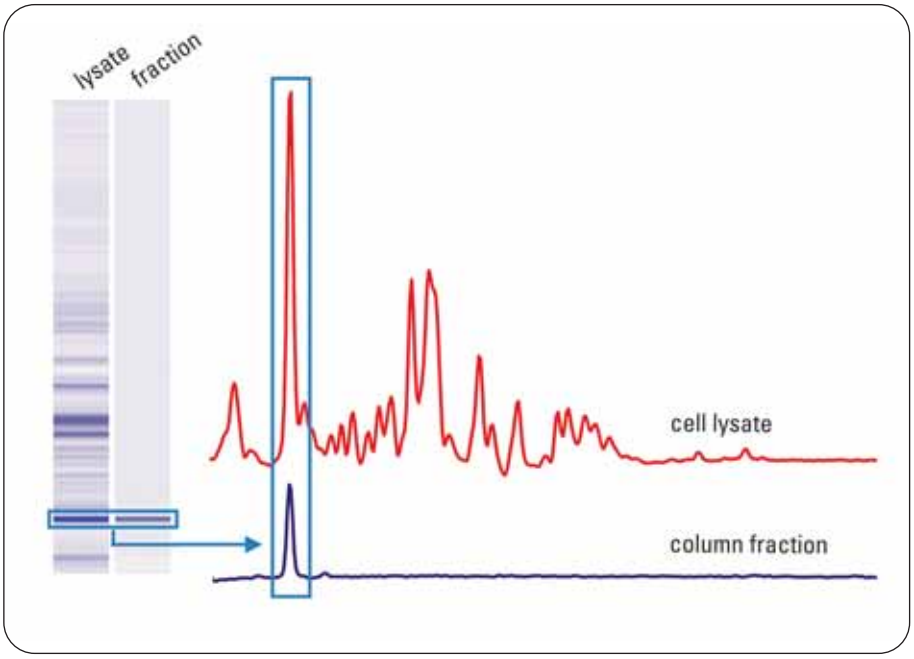


Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: Cells were lysed using the Pierce B-Per kit and then loaded onto an affinity column. The protein of interest, a 38 kDa protein, should bind to the column and not show up in the flow through. By overlaying the 2 electropherograms from both samples, the lysate and the flow through, it is visible that the protein of interest has bound to the column as expected. In addition, a 77 kDa protein has bound to the column, which could be attributed to unspecific binding or the binding of a dimer.

Corresponding application note: data not published

Protein purification

Analysis of protein purification



Courtesy of P. Sebastian and S.R. Schmidt GPC-Biotech AG, Martinsried, Germany

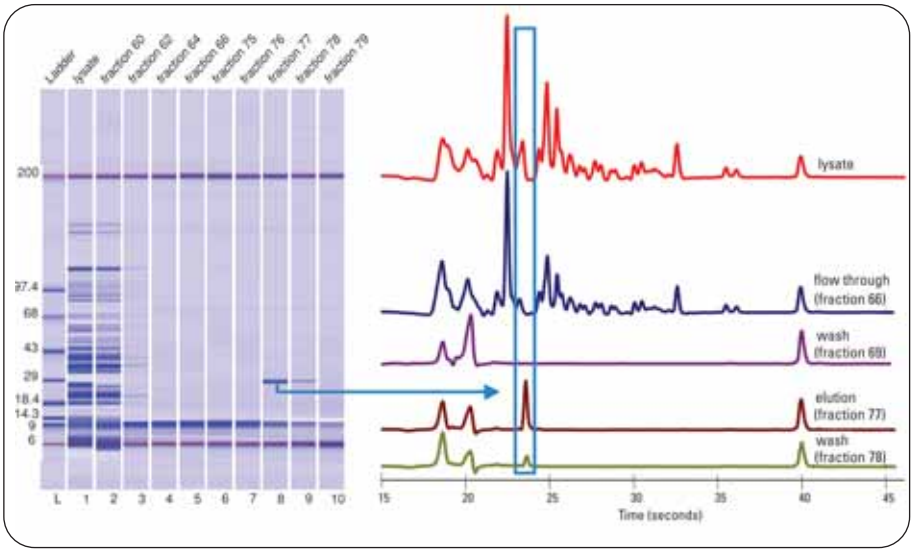
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Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: A 18 kDa protein was purified using affinitiy chromatography. The starting material and the column fraction were analyzed with the protein assay. The protein of interest was determined to be 99% pure and the concentration in the column fraction was 167 ng/µl.
The protein assay allows protein purity and concentration to be determined in one step, in addition it calculates protein size for reconfirmation.

Corresponding application note: data not published

Protein purification

GFP Streptag fusion protein purification



Courtesy of P. Sebastian and S.R. Schmidt GPC-Biotech AG, Martinsried, Germany

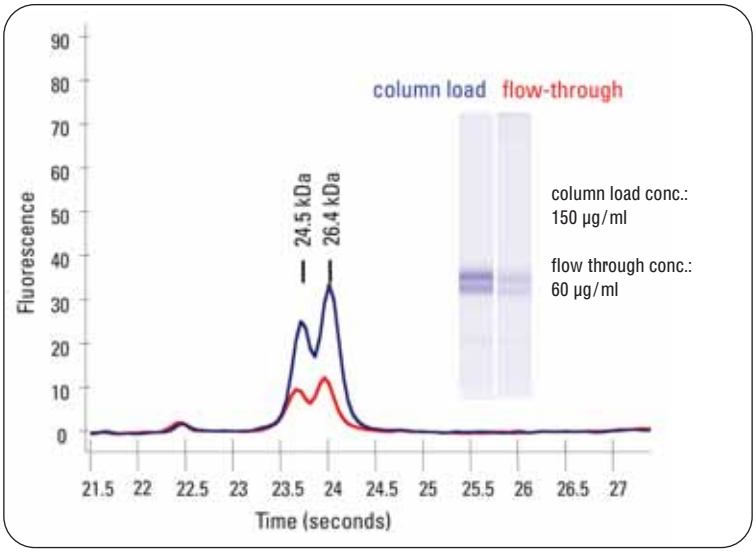
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Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: This example shows the analysis of various steps during the purification workflow of a GFP Streptag fusion protein (28 kDa). The protein was expressed in *E.coli* and purified via affinity chromatography with Strep Tactin Poros as the column matrix. The protein assay allows each purification step from the cell lysis to the elution of the purified protein to be monitored and optimized.

Corresponding application note: 5988-5025EN

Protein purification

Analysis of column capacity

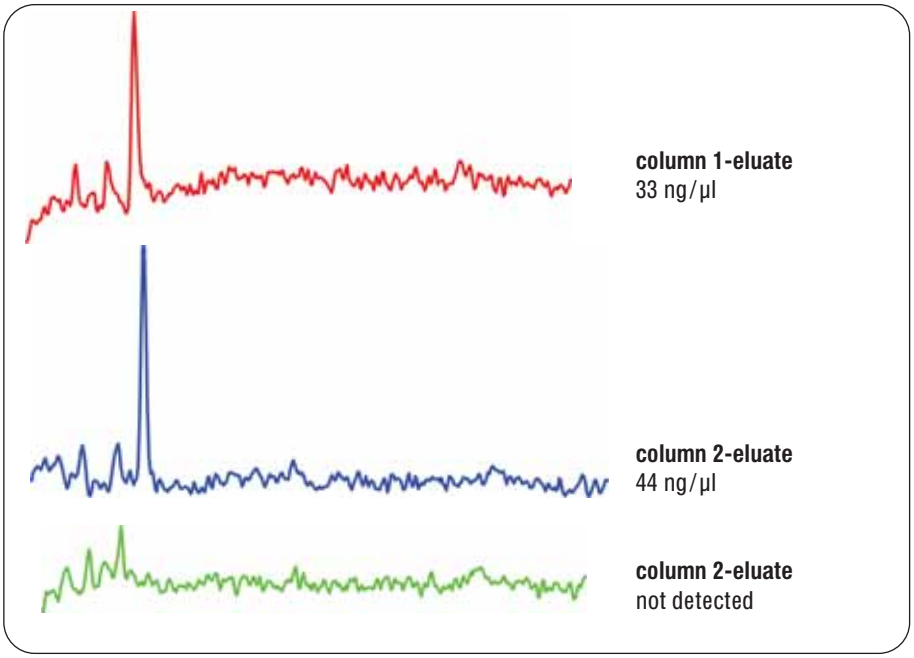


Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: The binding of a recombinant antibody Fab fragment to a Sepharose column with immobilized Protein G was analyzed to determine the column capacity and prevent column overloading. The protein assay allows this purification step to be monitored and quickly optimized.

Corresponding application note: 5988-4022EN

Protein purification

Analysis of column fractions to optimize conditions



Courtesy of P. Sebastian and S.R. Schmidt GPC-Biotech AG, Martinsried, Germany

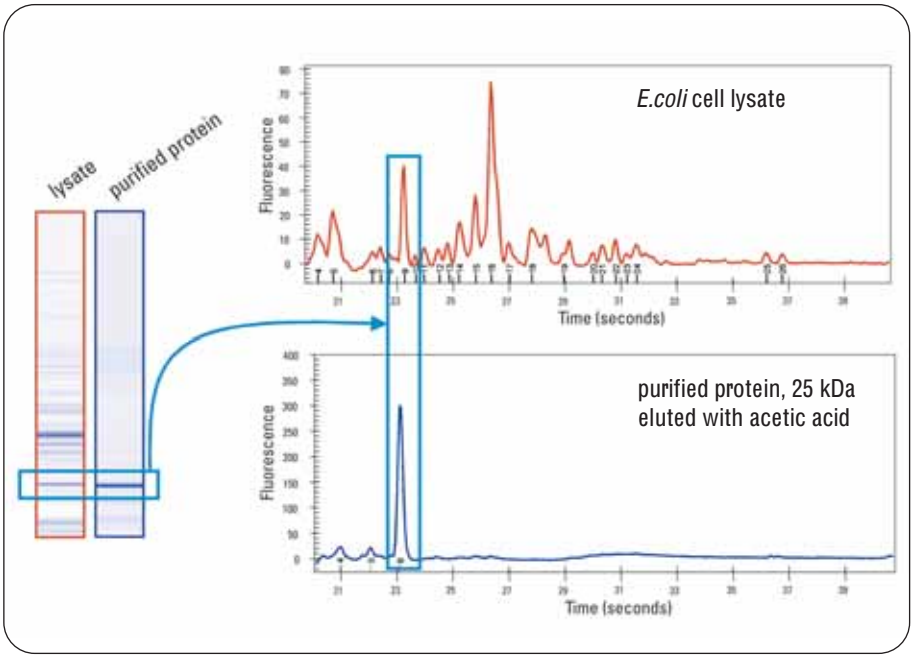
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Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: Different column conditions were tested to optimize the purification conditions for a 30 kDa protein. The column fractions were analyzed for protein purity and concentration to identify the optimal conditions providing a highly purified protein in a good yield. Using the protein assay it was possible to determine the optimum purification conditions in a short time frame.

Corresponding application note: data not published

Protein purification

His-tag protein purification using Ni++ZipTips®

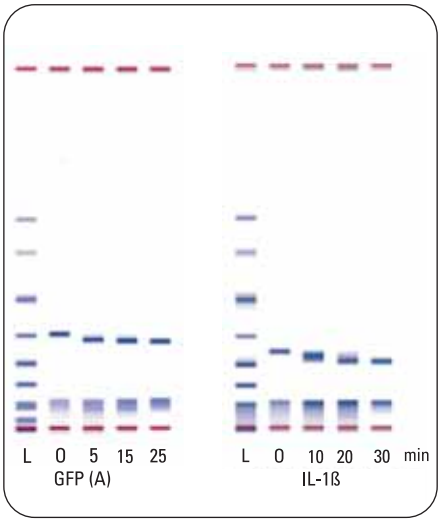


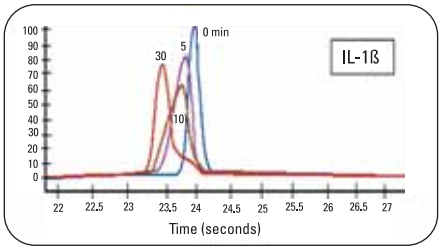
Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: ZipTips loaded with a Ni^{2+}-resin (in development by Millipore) were used to purify a His-tagged protein expressed in *E.coli*. Both the cell lysate and the purified protein were analyzed with the Agilent 2100 bioanalyzer to demonstrate the performance of the tips.
The purification with the tips takes approximately 5 minutes, usually followed by the analysis of the samples with SDS-PAGE analysis which takes a further 2 hours. The SDS-PAGE analysis was substituted by the much faster Protein 200 Plus assay run on the Agilent 2100 bioanalyzer.

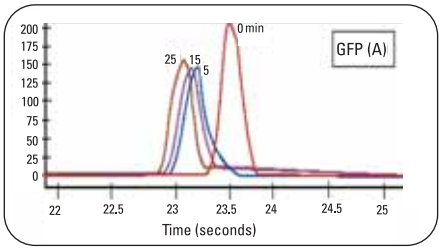
Corresponding application note: data not published

Protein purification

Enzymatic removal of His-tags from recombinant proteins







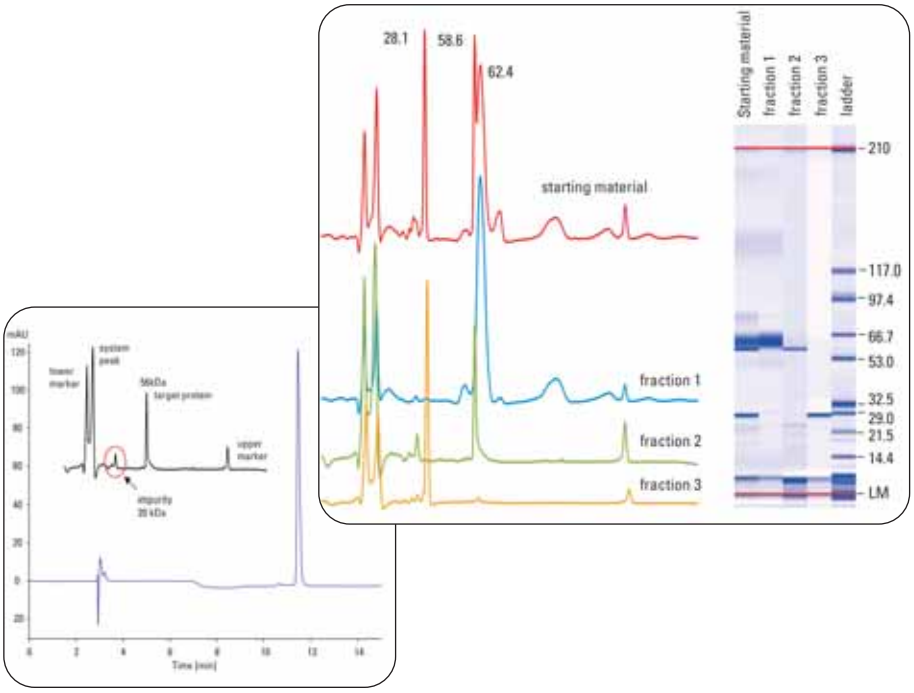
Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: For some applications, it might be necessary to remove the His-tag after the protein purification because of its effects on enzymatic activity or protein structure. Here the TAGZyme system (Qiagen) was used to remove the N-terminal His-tag from two different proteins, a GFP variant and a recombinant Interleukin 1β. Samples were taken at different time points to study the kinetics of the enzymatic cleavage. The dipeptide cleavage can be detected by a size shift on the gel-like images and the electropherograms. The fast analysis with the bioanalyzer allows multiple kinetic studies to done in one day instead of waiting until the next day for the results from SDS-PAGE analysis.

Poster presented at ABRF Conference, March 2002 by
F. Schäfer, K. Steinert, C. Feckler, J.Drees, and J.Ribbe, QIAGEN GmbH, Hilden, Germany

Corresponding application note: 5988-8144 EN

Protein purification

Complementing RP-HPLC protein purification

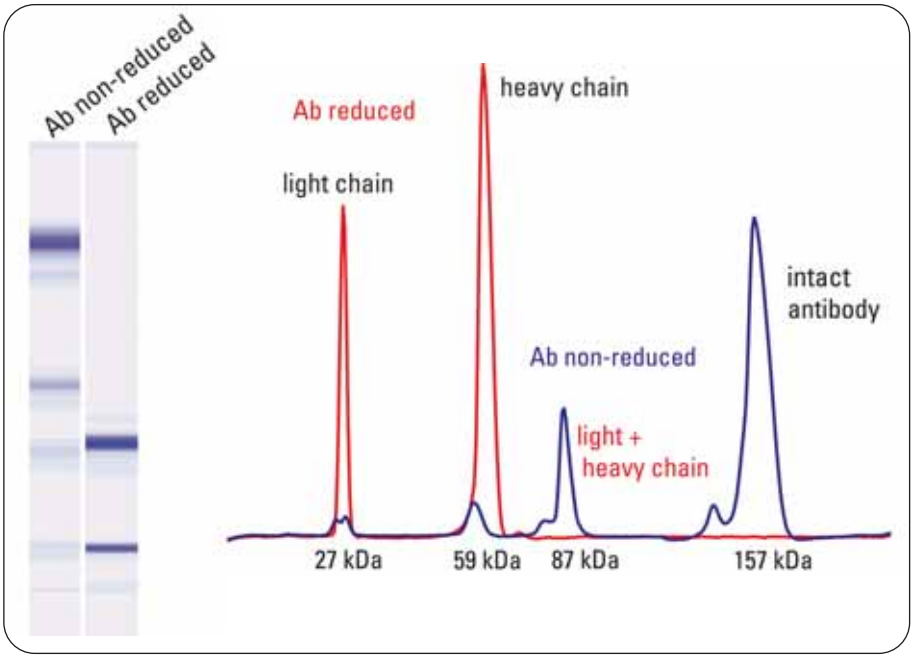


Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: Protein purification and characterization was carried out facilitating an Agilent 1100 Series purification system for reverse phase HPLC assisted by the Agilent 2100 bioanalyzer. The final polishing of a 56 kDa protein by RP HPLC from a pre-purified sample (starting material, right: red electropherogram and gel) and the analysis of three HPLC-fractions containing the major components are shown (fractions 1-3). No impurity is visible by RP HPLC reanalysis (left chromatogram, fraction 2) of the fraction containing the target protein. However, because the Agilent 2100 bioanalyzer is an orthogonal technique compared to reverse phase HPLC a 20 kDa protein could be found as an impurity (see insert). The reverse phase HPLC purification leads to a purity of only 76% for the protein of interest and the Agilent 2100 bioanalyzer reveals the necessity of further purification.

Corresponding application note: 5988-8630EN

Antibody analysis

Analysis of antibodies under reducing and non-reducing conditions

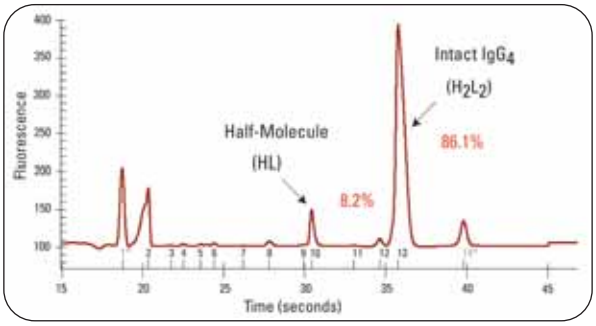


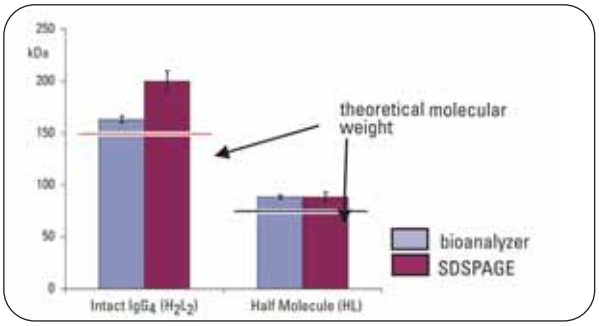
Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: The protein kit allows analysis of both reduced and non-reduced antibodies on the same chip. This is not possible using SDS-PAGE, as the reducing agent will diffuse within the gel and will also reduce other samples. Under non-reducing conditions, it is expected to detect the intact antibody around 160 kDa. Here the single light and heavy chains and half-antibodies are also visible. Under reducing conditions this is all completely reverted to single light and heavy chains, due to the reduction of the disulfide bonds.

Corresponding application note: data not published

Antibody analysis

Quantitation of the half-antibody content in IgG_4 preparations





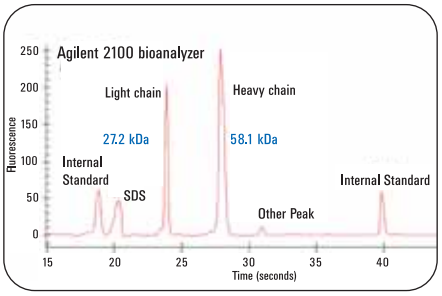
Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: In the given host cell line for antibody production usually up to 30% of IgG_4 is secreted as half molecule (half antibody). The half-molecule has only a single disulfide bond between the heavy and light chains, the inter-heavy chain disulfide bonds are absent. The protein assay allows the half-antibody content in IgG_4 preparations to be determined automatically.
In addition, the sizing provided by the Agilent 2100 bioanalyzer compares very well to the theoretical size and is superior to SDS-PAGE in terms of accuracy and reproducibility.

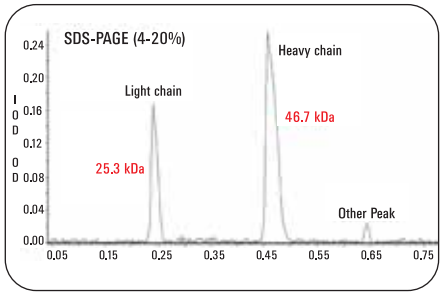
Poster presented at WCBP Conference, January 27-30, 2002 by E. Vasilyeva, H. Fajardo, P. Bove, F. Brown and M. Kretschmer. BIOGEN, Cambridge, MA , USA

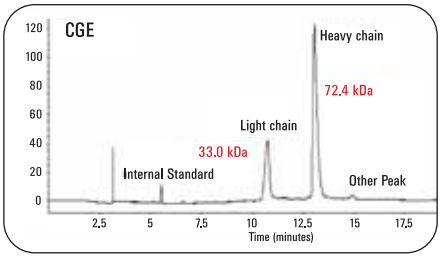
Corresponding application note: data not published

Antibody analysis

Comparison of SDS-PAGE, CGE and Agilent 2100 bioanalyzer for humanized monoclonal antibody analysis







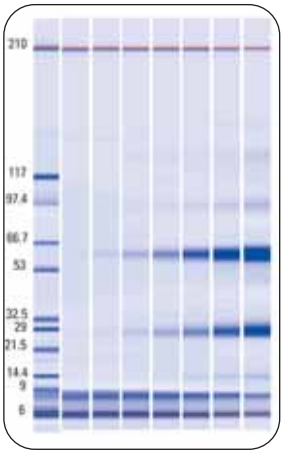
Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: The analysis of a humanized monoclonal antibody under reducing condition was compared using 3 different techniques, the Agilent 2100 bioanalyzer, 4-20% SDS-PAGE, stained with Coomassie, and capillary gel electrophoresis. All 3 techniques result in a similar separation pattern showing the light and the heavy chain of the antibody. In addition, the determined sizes of the light and heavy chain were comparable for all 3 techniques and compared well to the molecular weights determined by MALDI-TOF (light chain: 23762 Da, heavy chain: 51003 Da). However, the Agilent 2100 bioanalyzer provides significant time saving compared to the other techniques.

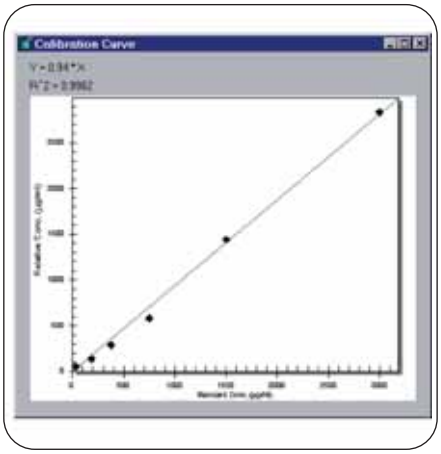
Poster presented at WCBP Conference, January 2002 by
S.H. Bowen, M. Chan, P. McGeehan, J. Smith, L. Inderdass, R. Strouse, M. Schenerman
MedImmune Inc., Gaithersburg, MD, USA

Corresponding application note: data not published

Antibody analysis

Absolute quantitation of IgG



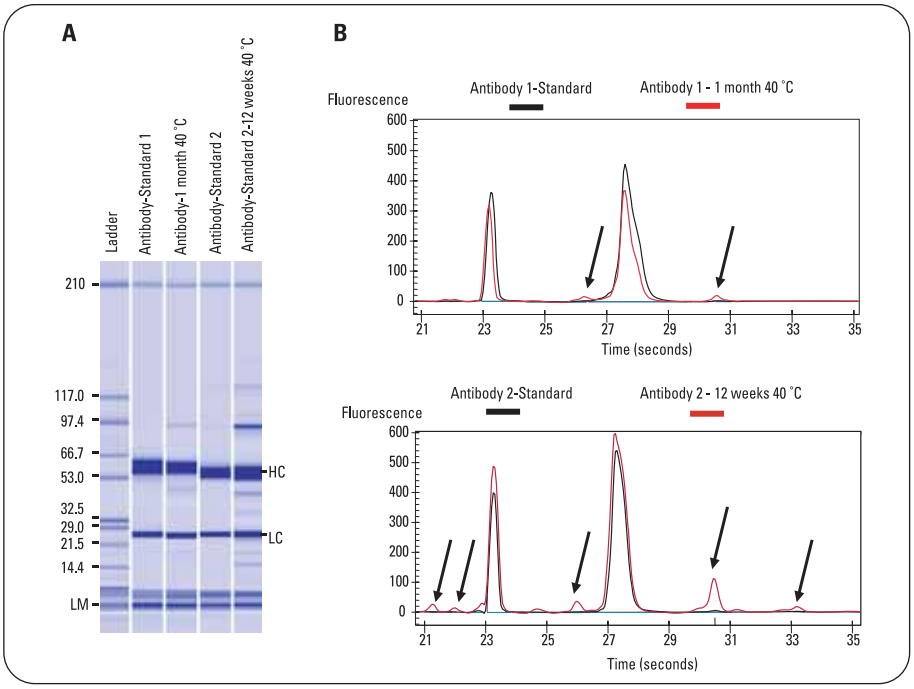


Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: The calibration feature of the software allows determination of the absolute antibody concentration in comparison to user defined standards with known concentration, accurate determination of IgG concentrations and carrying out batch comparison during antibody QA/QC.

Corresponding application notes: 5988-4021EN and 5988-6576EN

Antibody analysis

Quality control of stressed antibodies

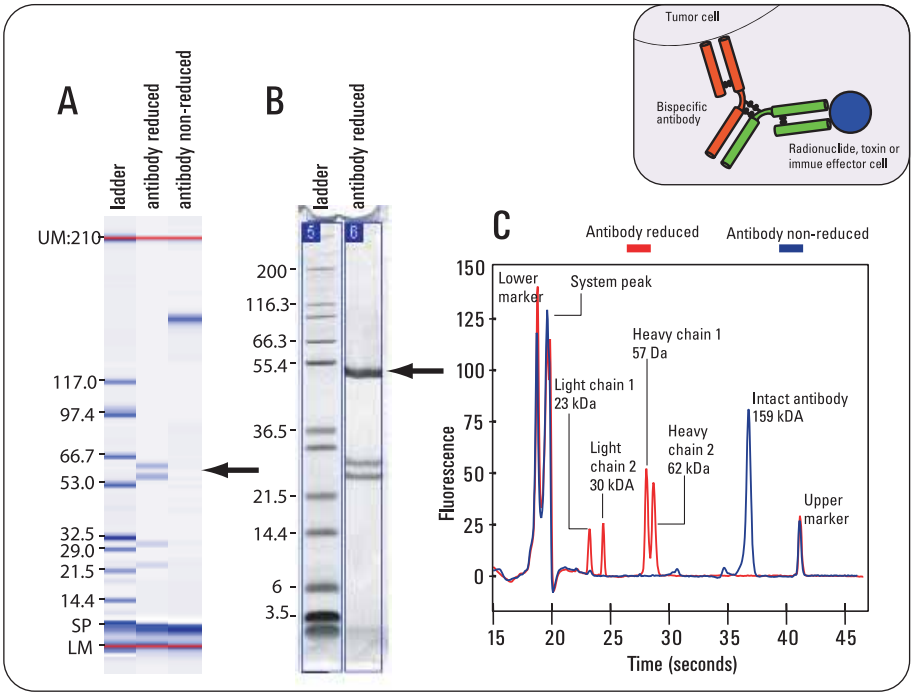


Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: A quality control step in pharmaceutical QA/QC departments is to trigger typical degradation and aggregation patterns for a specific antibody. The given samples from heat stress stability studies show expected protein byproducts after aging at elevated temperatures. The content of heavy and light chain, representing the intact antibody, is reduced by 5% or 13% within 1 month or respectively 12 weeks. Excellent reproducibility in the range from 0.6 to 1.7% CV for this quantification was achieved in a validation study with three different users and two bioanalyzer instruments over several days.

Corresponding application note: 5988-9648EN

Antibody analysis

Separation of bispecific antibodies chains

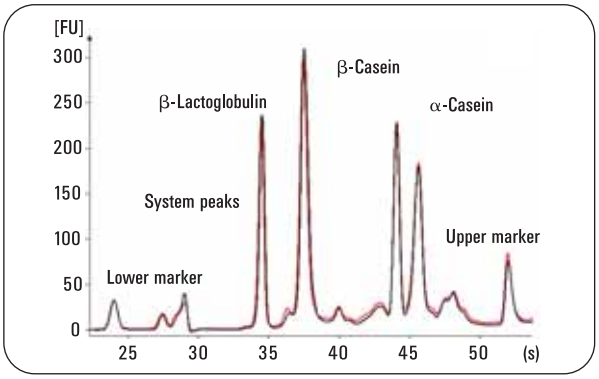


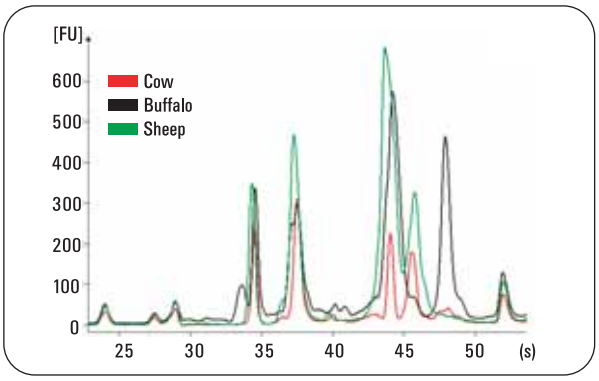
Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: In general, antibodies are biopharmaceuticals of great interest. Especially bispecific antibodies often require high resolution to allow analysis of both sets of chains (Agilent 2100 bioanalyzer: A, gel like view, resolved heavy chains; C electropherogram). A labor intensive SDS-PAGE could not resolve the heavy chains (B, marked by an arrow) in the given sample. In contrast, the Agilent 2100 bioanalyzer is a superior tool for antibody quality control since it is a convenient, fast and easy to standardize method which additionally enables quantitative analysis.

Corresponding application note: 5988-9651EN

Food analysis

Bovine milk analysis



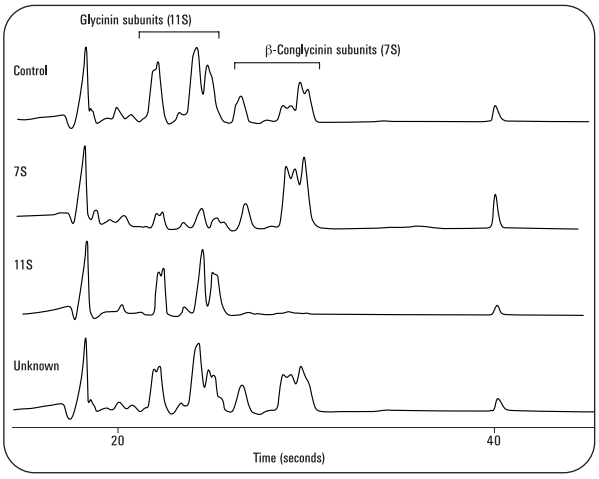


Kit: Protein 50 kit
Assay: Protein 50 assay
Application: The Protein 50 LabChip is suitable for analysis of diary products such as milk. The kit delivers an excellent reproducibility, as shown in this example (A, bovine milk diluted 1:10). Here the main protein fractions can be identified running the individual purified proteins for comparison (not shown). The overlay of the electropherograms from two separate runs under reducing conditions demonstrates the high reproducibility of the assay. Furthermore, milk from different animals could be distinguished based on their protein pattern (B) which facilitates a fast incoming inspection in routine labs.

Corresponding application note: data not published

Food analysis

Protein pattern of different transgenic seedlines



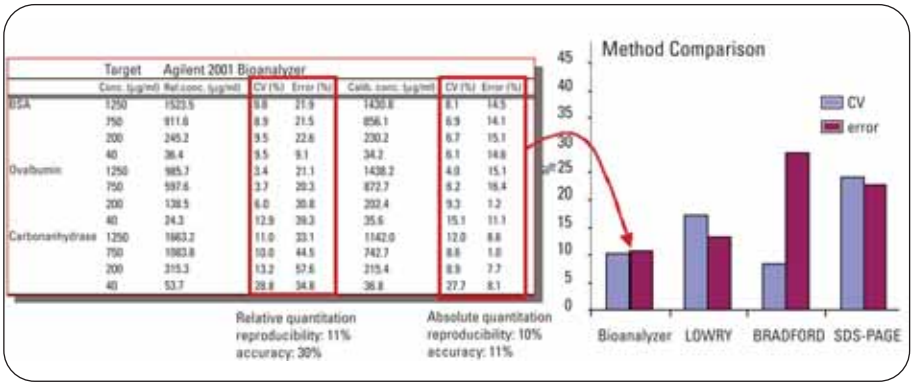
SeedLine	Extracted protein level µg/ml	7S/11S Ratio
Control	14,000	0.39±0.004(n=5)
7S	5,200	3.4
11S	14,000	0.04
Unknows	13,000	0.72±0.1(n=20)

Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: Determination of protein size and concentration with sufficient accuracy and precision allows the highly efficient characterization of transgenic seed lines. Expressed protein was available after grinding, extraction of seeds and dilution with buffer. Electropherograms were evaluated by integration of regions specific for 7S or 11S seed storage proteins. The elevated ratio of 7S/11S for the analyzed unknown line shows significant changes in the expression profile in comparison with the control.

Corresponding application note: 5988-9441EN

Protein - others

Absolute protein quantitation

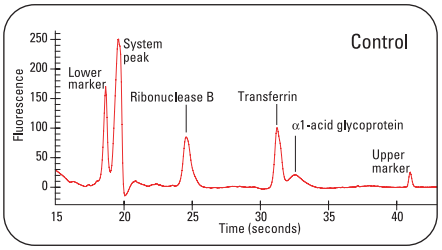


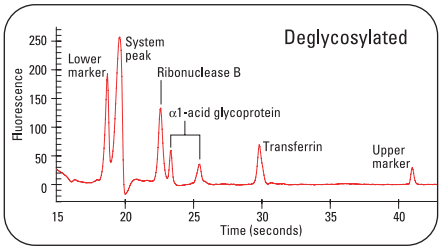
Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: A comparative analysis of different techniques used for absolute protein quantitation was performed analyzing 3 different proteins (CA, BSA, OV) in 4 different concentrations (40 - 1250 ug/ml). The same samples were quantitated using the Agilent 2100 bioanalyzer, two commonly used total protein quantitation assays, Lowry and Bradford, and SDS-PAGE, stained with Coomassie. The relative standard deviation (CV) and the error compared to the target concentration were determined. A comparison shows that the CV and error for the Agilent 2100 bioanalyzer are better than for the SDS-PAGE by a factor of 2. This data demonstrates that the Agilent 2100 bioanalyzer is a viable alternative for protein quantitation. It allows the quantitation of individual proteins and simultanous determination of protein purity and size.

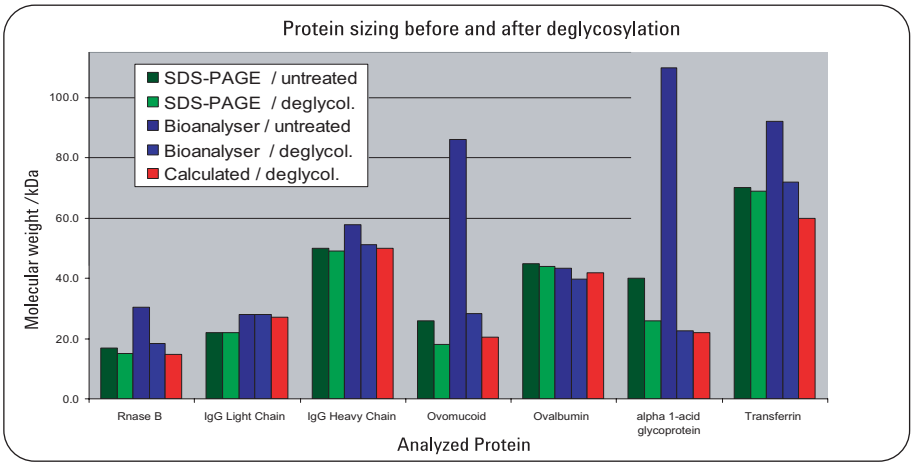
Corresponding application notes: 5988-4021EN and 5988-6576EN

Protein - others

Glycoprotein sizing





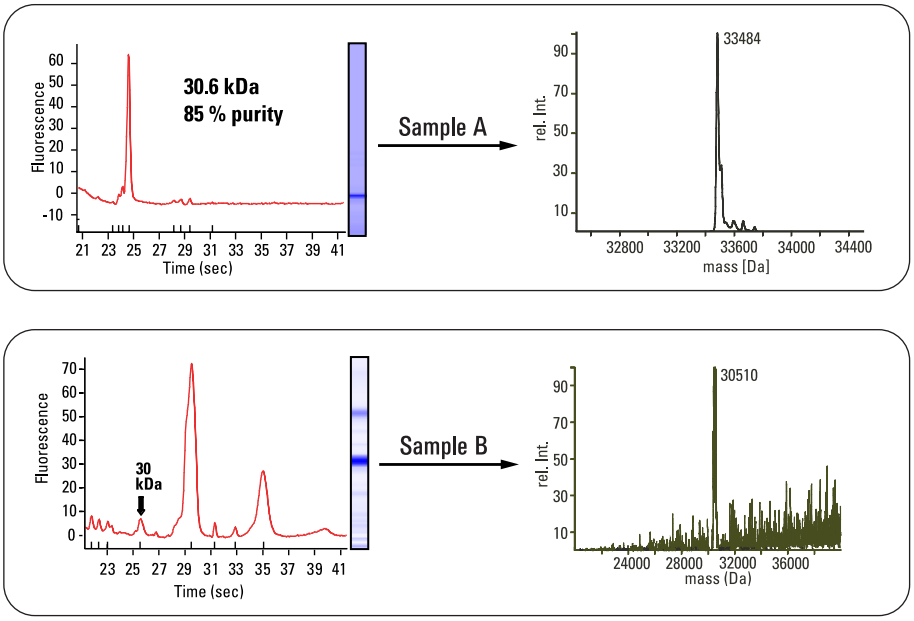


Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: Due to large carbohydrate moieties glycosylated proteins can differ in amount of incorporated SDS and shape of the protein/SDS-complex from non-glycosyslated proteins. This may lead to different migration times in SDS-PAGE, as well as in the Protein 200 Plus assay run on the Agilent 2100 bioanalyzer. The data compare deglycosylation of a mixture of three proteins (electropherogram on the left) with a commercial N-Glycosidase F Deglycosylation kit. Sizing experiments comparing glycosylated and non glycosylated states for additional proteins are compared and summarized on the right. Such an approach avoids misinterpretation of sizing due to glycosylation and allows detection of a posttranslational modification of unknown proteins.

Corresponding application note: 5989-0332EN

Protein - others

Protein quality control prior to MS-analysis

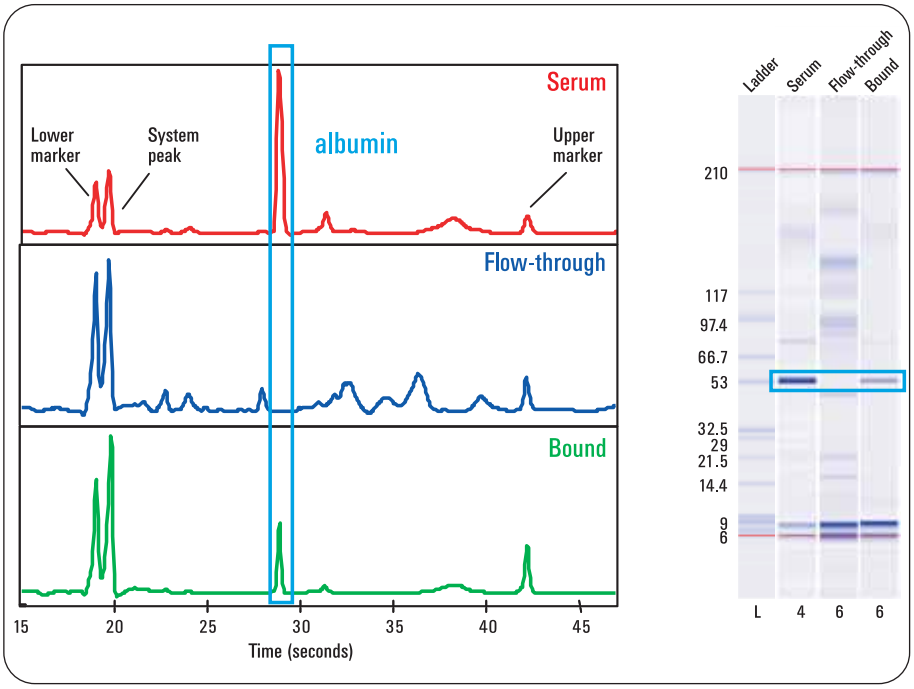


Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: By applying soft ionization methods like MALDI or ESI mass spectroscopy (MS), mass information from proteins up to 300kDa can be obtained. However, proper sample preparation is an important precondition. Concentration, purity and assumed size are valuable ex ante information usually given by biochemists to MS-analysis services. Two different examples for proteins analyzed by an LC/MS-method (right panel) with good results for sample A and discrepancies for sample B are shown. An analysis of the samples with the Protein 200 Plus assay (left panel) showed an impure protein preparation for sample B. Here, two major peaks at higher masses (66 and 132kDa) potentially caused by aggregates were encountered. The protein of interest (30kDa) yielded high noise background in the MS. A quality check of the sample with the Protein 200 Plus assay, therefore, may avoid an unproductive MS analysis or data evaluation.

Corresponding application note: 5989-0771EN

Protein - others

Depletion of high abundant proteins from blood samples

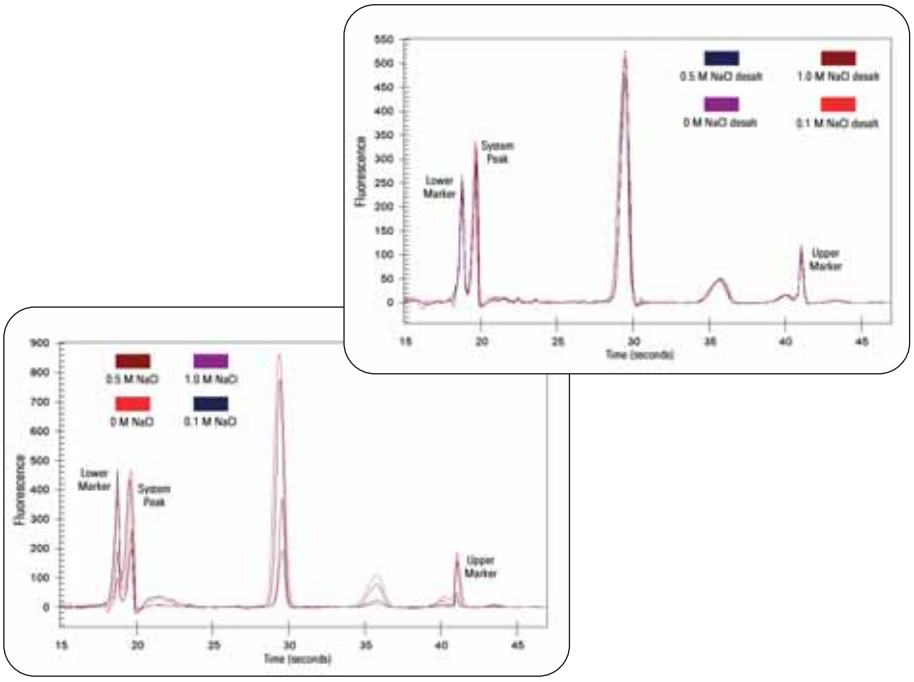


Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: Depletion of high abundant proteins in human blood plasma was facilitated by the Agilent Multiple Affinity Removal System. Unprocessed serum, the flow through (i.e. the immunodepleted serum) and the bound proteins after specific elution were analyzed on the Agilent 2100 bioanalyzer. Equivalent amounts were analyzed and resulting electropherograms (left) and gel like images (right) show in comparison the completeness of separation. The Agilent 2100 bioanalyzer, in combination with the Protein 200 Plus LabChip kit, proves to be an excellent method for evaluation of serum processed with albumin removal kits. The system offers a rapid and accurate method to detect proteins both quantitatively and qualitatively.

Corresponding application note: data not published

Protein - others

Increased sensitivity by desalting protein samples



Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: Protein purification steps, such as ion exchange chromatography, often implicate high salt concentrations. Nevertheless, quantitation of these samples is effective since the upper marker serves as internal protein standard and is subjected to the same conditions. However, under high salt conditions lower amounts of protein are injected into the microfluidic channels for analysis. Therefore, the sensitivity can be increased by usage of convenient desalting spin columns. Comparably higher and homogeneous peak intensities are obtained while the recovery after such treatment is good.

Corresponding application note: 5989-0228EN

Literature

Microfluidics application notes from Agilent Technologies

To download an application note visit the library section at:
www.agilent.com/chem/labonachip

Description	Publication number
DNA	
Quantitative analysis of PCR fragments with DNA 7500 LabChip kit	5968-7496EN
High precision restriction fragment sizing with DNA 12000 LabChip kit	5968-7501EN
Comparing the Agilent 2100 bioanalyzer performance to traditional DNA analysis	5980-0549EN
Agilent 2100 bioanalyzer replaces gel electrophoresis in prostate cancer research	5988-1086EN
High resolution DNA analysis with the DNA 500 and DNA 1000 LabChip kits	5988-3041EN
Quantitative end-point RT-PCR gene expression using DNA 7500 LabChip kit	5988-3674EN
Development of meat speciation assays using the Agilent 2100 bioanalyzer	5988-4069EN
Analysis of genetically modified soya using the Agilent 2100 bioanalyzer	5988-4070EN
Detecting genetically modified organisms with the Agilent 2100 bioanalyzer	5988-4847EN
Sensitive detection of tumor cells in peripheral blood of carcinoma patients by a reverse transcription PCR method	5988-9341EN
Highly efficient multiplex PCR using novel reaction chemistry	5988-9342EN
Microfluidic analysis of multiplex PCR products for the genotyping of Helicobacter pylori	5989-0078EN
Nested multiplex PCR for the determination of DNA from genetically modified corn and soy beans using the Agilent 2100 bioanalyzer	5989-0124EN
Rapid detection of genomic duplications and deletions using the Agilent 2100 bioanalyzer	5989-0192EN
Mutation detection for the K-ras and P16 genes	5989-0487EN
Use of the Agilent 2100 bioanalyzer and the DNA 500 LabChip in the analysis of PCR amplified mitochondrial DNA	5989-0985EN
Assessing genomic DNA contaminations of total RNA isolated from kidney cells obtained by Laser Capture Microdissection using the Agilent RNA 6000 Pico assay	5989-0991EN
Integrating high-throughput, on-chip electrophoresis analysis into PCR diagnostics projects	5989-1870EN
DNA QC for Oligonucleotide Array CGH (aCGH) with the Agilent 2100 bioanalyzer	5989-2487EN
Label-free analysis of microsatellite instability in colorectal carcinoma by on-chip electrophoresis	5989-2626EN
Determination of PCR-RFLP Profiles for Fish Species Using the Agilent 2100 bioanalyzer	5989-2982EN
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Use of the Agilent 2100 Bioanalyzer for Basmati Rice Authenticity Testing

Application

Food

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Abstract:

Ensuring integrity of raw food materials, ingredients, and products is both a product quality and regulatory compliance concern. Food suppliers and manufacturers may suffer economic and legal damages if proven to be supplying incorrectly labeled products. For example, EU Commission 1549/04 grants lower import tax on nine basmati rice varieties. A quick and cost-effective analytical method utilizing the Agilent 2100 bioanalyzer and DNA 1000 assay is shown as an alternative method to establish the authenticity of basmati rice products and to estimate the level of some varieties of non-basmati rice in ground rice products.

Introduction

The integrity of raw food materials, ingredients, and products must be maintained to ensure that they meet appropriate quality and legislative requirements. Food ingredient suppliers, manufacturers, and retailers can face legal action if proven to be supplying materials or products that are incorrectly labeled due to substitution or contamination.

One food area which has been under the spotlight in recent years is the supply of basmati rice to the UK from India and Pakistan. In Europe, Commission Regulation 1549/04 grants a lower import tax on nine basmati varieties: Basmati 370, Dehradun (Type 3), Basmati 217, Taraori, Ranbir Basmati, Kernel, Basmati 386, Pusa Basmati, and Super Basmati. Other basmati rice varieties approved by India, Pakistan, and the UK include Basmati 198, Basmati 385, Haryana Basmati, Kasturi, Mahi Suganda, and Punjab Basmati. In a Code of Practice developed by Indian, Pakistani, and UK industry and enforcement organizations that came into effect for products packed and labeled after January 2006, the level of non-basmati rice in a basmati rice product must not exceed 7% (see www.riceassociation.org.uk).

In order to check the supply of basmati rice, a DNA variety testing method using PCR amplification of eight rice microsatellite sequences has been developed for UK compliance (www.foodstandards.gov.uk/multimedia/pdfs/fsis4704basmati.pdf). During 2003, the UK Food Standards Agency carried out a surveillance exercise on basmati rice products using this method and revealed that 74% of them contained > 7% non-basmati varieties.

This study evaluates the use of the Agilent 2100 bioanalyzer to differentiate approved and non-approved varieties using three primer sets and to estimate the level of non-basmati using reference rice admixtures.

Experimental

Method

All reference basmati rice samples were obtained from the UK Food Standards Agency via the University of Wales (Bangor, UK). Other samples were from in-house basmati rice sample collections.

PCR amplification was performed using either a PE9600 or PE2400 PCR machine (Applied Biosystems).

Extraction

DNA was extracted from ground rice grains using Qiagen's DNeasy Plant Mini Kit or Promega's Maxwell 16 automated DNA extractor

PCR

DNA extracts were diluted 1 to 1 in sterile distilled water (SDW) to produce template DNA prior to use in PCRs. Amplification was performed in 25-µL PCRs containing 1x Amplitaq Gold PCR buffer (Applied Biosystems), 60 nM of each primer, 200 nM dNTPs, 3 mM $MgCl_2$, 0.05 U/µL of Ampli-Taq Gold (Applied Biosystems), and 2 µL of template DNA.

Marker	Forward Primer
RM201	CTC gTT TAT TAC CTA CAg TAC C
RM212	CCA CTT TCA gCT ACT ACC Ag
RM339	GTA ATC gAT gCT gTg ggA Ag
Marker	**Reverse Primer**
RM201	CTA CCT CCT TTC TAg ACC gAT A
RM212	CAC CCA TTT gTC TCT CAT TAT g
RM339	gAg TCA TgT gAT AgC CgA TAT g

Amplification profiles (95 °C for 15 minutes [denaturation]; 50 cycles of: 95 °C for 1 minute, 60 °C for 1 minute [amplification]; 72 °C for 10 minutes [final extension]) were used in all PCR reactions. DNA amplification was confirmed by separating PCR products using the Agilent 2100 bioanalyzer.

Capillary Gel Electrophoresis on 2100 Bioanalyzer

Reagents were prepared following manufacturers' instructions. Batches (~500 µL) of gel matrix (used to fill LabChip capillaries) were prepared as required or at 4 weekly intervals. All reagents were stored at 4 °C and allowed to reach room temperature for 1 hour before use. PCR products (1 µL)

Table 1. Analysis of Authenticated Basmati Rice Varieties Using Three Microsatellite Primer Sets

Rice variety	List of microsatellite amplification product sizes obtained with primer set*		
	RM201	RM212	RM339
Varieties listed in Commission Regulation 1549/04			
Basmati 370	162 (**162**)	134 (**139**)	200 (**193**)
Dehra Dun (Type 3)	162	134 (**139**)	200 (**195**)
Basmati 217	162	134	200
Ranbir	162	134	200
Taraori	162	134 (**139**)	200 (**195**)
Basmati 386	162	134 (**138**)	200 (**195**)
Kernel	162	134	200
Pusa	162	134 (**139**)	200 (**194**)
Super	162	134 (**140**)	204 (**196**)
Other varieties approved as basmati by UK Food Standards Agency			
Basmati 198	162	152	200
Basmati 385	162	152	200
Kasturi	162	132	166
Haryana Basmati	162	152	166
Mahi Sugandha	176	152	166
Punjab Basmati	162	152	200
Non-approved varieties			
Basmati 2000	162	152	204
Shaheen Basmati	162	152	200
Sherbati	178 (**176**)	130 (**135**)	166 (**167**)
Mugad Sugandha	178	132	166
Pak 386	178	130 (**135**)	166 (**167**)
Superfine	178	132	166
Pusa Sugandha	162	132	178
Yamini	162	134	200

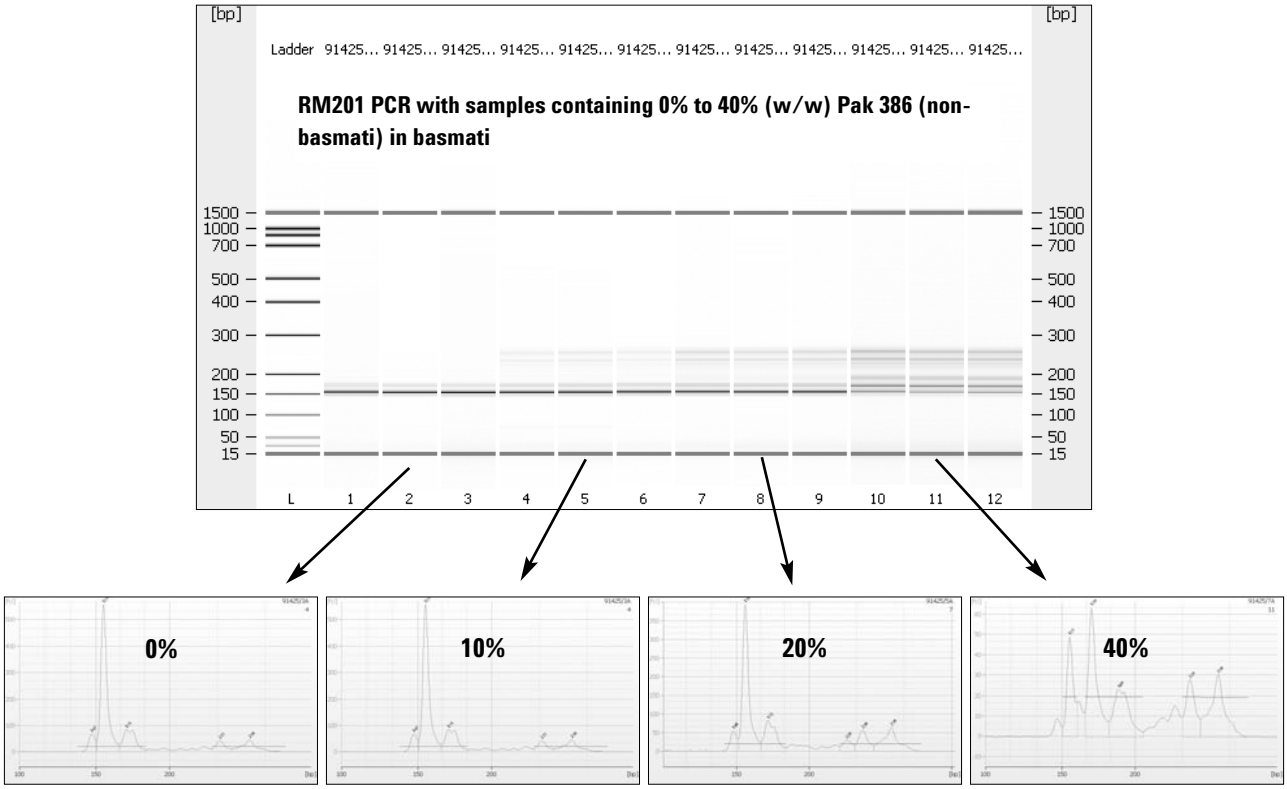
*These are from the FSA method developed by the University of Wales, Bangor. Actual size of fragments determined by the bioanalyzer using a DNA 500 chip kit are shown in brackets. The variation in bioanalyzer-determined fragment sizes can be about 5%. Shaded cells show how the varieties can be grouped using the three primer sets with analysis performed on the bioanalyzer.

were loaded directly onto prepared Series I DNA 500/DNA 1000 or Series II DNA 1000 labchips. All analysis was performed on the 2100 bioanalyzer, as per the manufacturers' instructions.

Results and Discussion

The different-sized PCR products generated when these three primer sets are applied to the different varieties are easily resolved on the bioanalyzer using the DNA 500 or DNA 1000 chip. RM 212 primers produce a 139 bp product with varieties listed in Commission Regulation 1549/04 and a 154 bp product with FSA-approved varieties apart from Kasturi and a few non-basmati varieties. The other primer sets enable separation of these varieties apart from the Yamini variety, which produces fragments similarly sized to the EC-approved varieties for all three primer sets. This variety is also difficult to distinguish from approved varieties using the standard method. The other FSA-approved basmati varieties could be distinguished from the EC-approved varieties but not from Basmati 2000 or Basmati 386 using these microsatellite primer sets. Use of further microsatellites that give PCR products that can be separated on the bioanalyzer will give improved differentiation of non-basmati rice varieties.

a) Primer set RM201



b) Primer set RM339

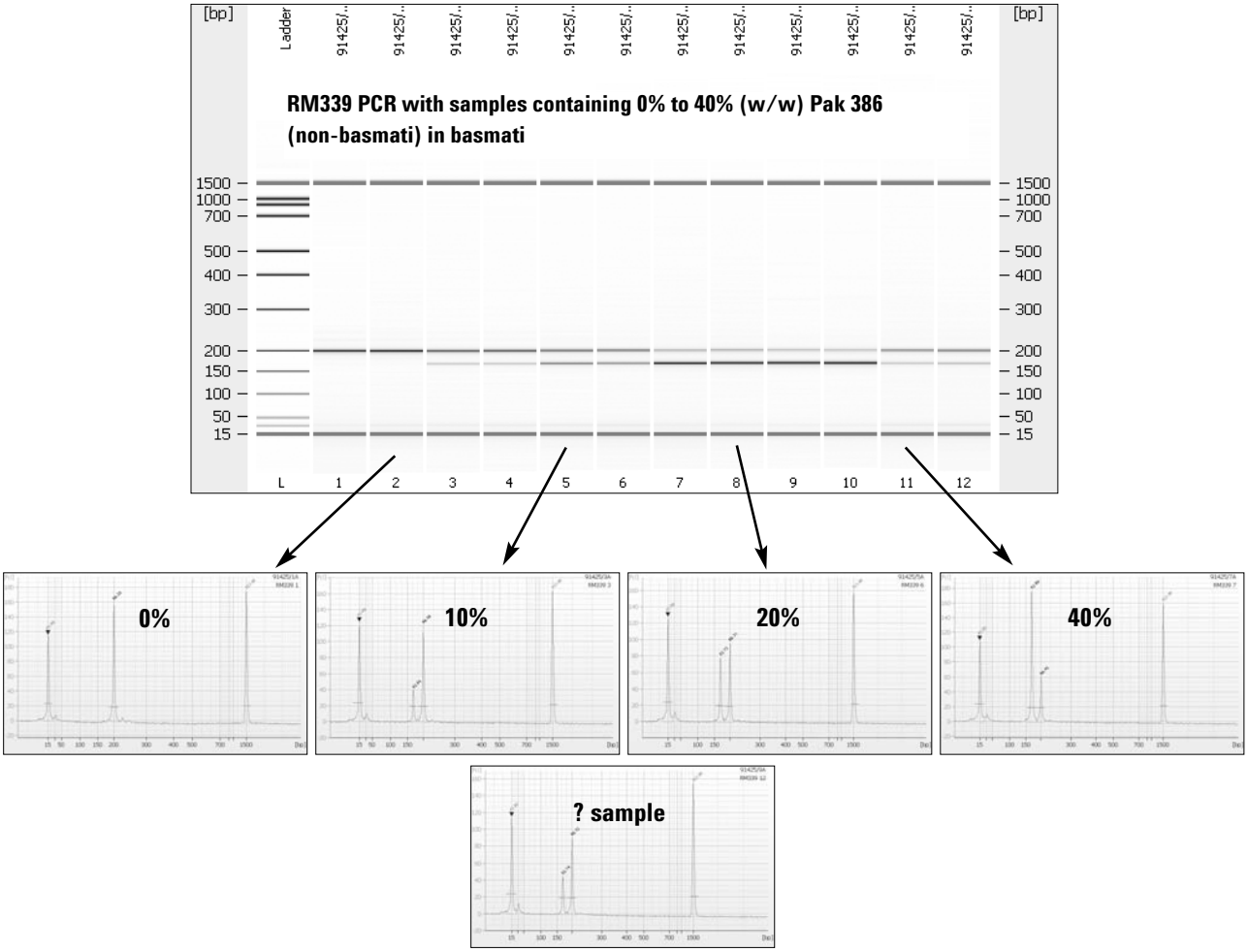


Figure 1. Analysis of non-basmati/basmati rice admixtures using two microsatellite primer sets. Estimation of percentage non-basmati variety (Pak 386) in an unknown sample.

Table 2. Experimental Summary

% Pak 386 in basmati rice	Primer RM201 mean ratio 156 bp/170 bp PCR fragments (n = 3)	Primer RM339 mean ratio 199 bp/169 bp PCR fragments (n = 2)
0	9.6	0
10	3.5	3.1
20	2.7	1.1
40	0.6	0.4
50	0.7	0.3
Unknown sample estimated to contain between 10–20% Pak 386	3.1	2.1

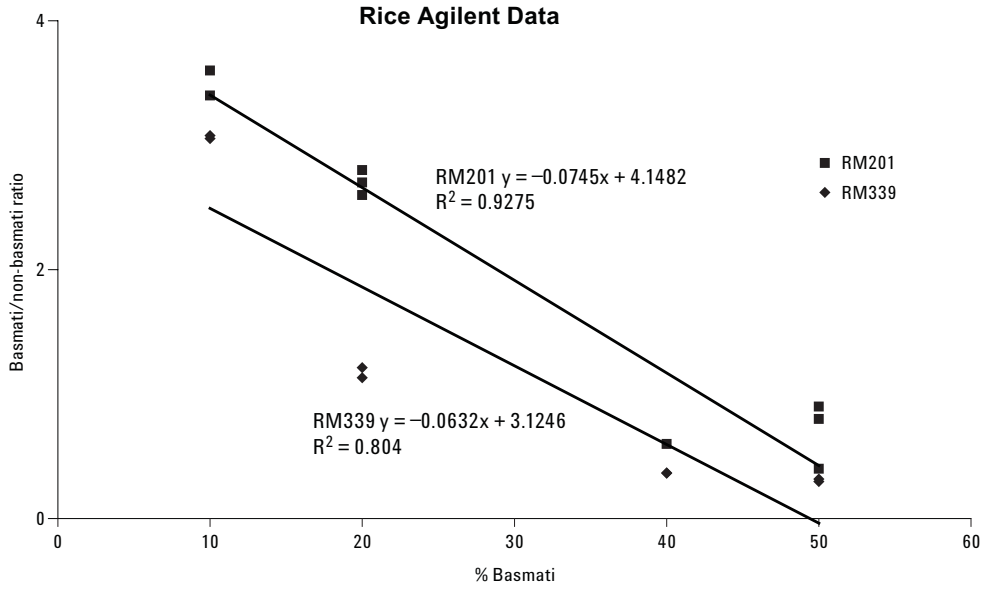


Figure 2. Standard curves based on the PCR product concentration ratios.

Both the RM201 and RM339 primer sets (Figure 1 and Table 2) could be used to produced separate and measurable PCR products with the PAK 386 basmati rice admixtures. The RM201 primers set gave a 170 bp product generated from the basmati rice and a 156 bp product from the Pak 386 variety, whereas RM339 primers gave 199 bp and 169 bp products. RM281 also gave other larger PCR products, which seemed to correlate with the presence of Pak 386 in the admixture. Standard curves (Figure 2) were produced using the PCR product concentration ratios. An unknown admixture sample was also analyzed and estimated to contain between 10 and 20% non-basmati rice in basmati.

Results show that the bioanalyzer can be used to estimate the level of non-basmati rice (Pak 386) in a basmati rice which produces different sized microsatellite PCR products from the Pak 386.

Conclusions

The Agilent 2100 bioanalyzer can be used as a quick and cost-effective alternative to establish the authenticity of basmati rice products and to estimate the level of some varieties of non-basmati rice. It should be feasible to develop further primer sets that would allow the bioanalyzer to be used to identify individual varieties. Simple manual and automated DNA extraction followed by fast PCR and post-PCR analysis on the bioanalyzer would allow rapid screening of rice materials prior to export from India and Pakistan and also allow enforcement bodies to efficiently test for microsatellite markers from non-approved basmati rice varieties in imported products.

Acknowledgements

Thanks to Mark Woolfe at UK Food Standards Agency and John Gorham and Katherine Steele at The University of Wales, Bangor, for the supply of primer set details and authentic basmati rice materials.

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Agilent Technologies

Rapid wheat varietal identification using the Agilent 2100 bioanalyzer and automated pattern-matching

Application Note

Dr Dhan Bhandari



Abstract

Accurate identification of wheat varieties is of paramount importance to the milling industry in many countries. This Application Note describes how the Agilent 2100 bioanalyzer and the Protein 230 assay can be used in conjunction with a third-party software, to analyze wheat proteins for varietal identification.

Agilent Equipment:

• 2100 Bioanalyzer Protein 230 Kit

Application Area:

• Food analysis

Agilent Technologies

Introduction

Authentication of varieties is important for the cereal industry for maintenance and testing of grain quality to meet market requirements. The charge-based separation of proteins by the acid-PAGE (polyacrylamide gel electrophoresis) technique is widely used for wheat varietal identification. However, this requires highly skilled operators to prepare, run and scan the gels and interpret band patterns. Also, there are safety concerns regarding the toxicity of unpolymerised acrylamide. While acid-PAGE is effective in analytical laboratories, the routine method can take up to two days. This is too slow for use at mill intake, which requires assessment of the wheat shipment during the period of delivery – typically under one hour. In this Application Note we demonstrate the use of the Agilent 2100 assay, with bioanalyzer and Protein 230 the Nonlinear Dynamics' Phoretix 1D Advanced (TotalLab TL120 DM) computerized pattern-recognition software to provide a better alternative to acid-PAGE. The aim of this study was to develop a robust, automated method for rapid identification of wheat varieties.

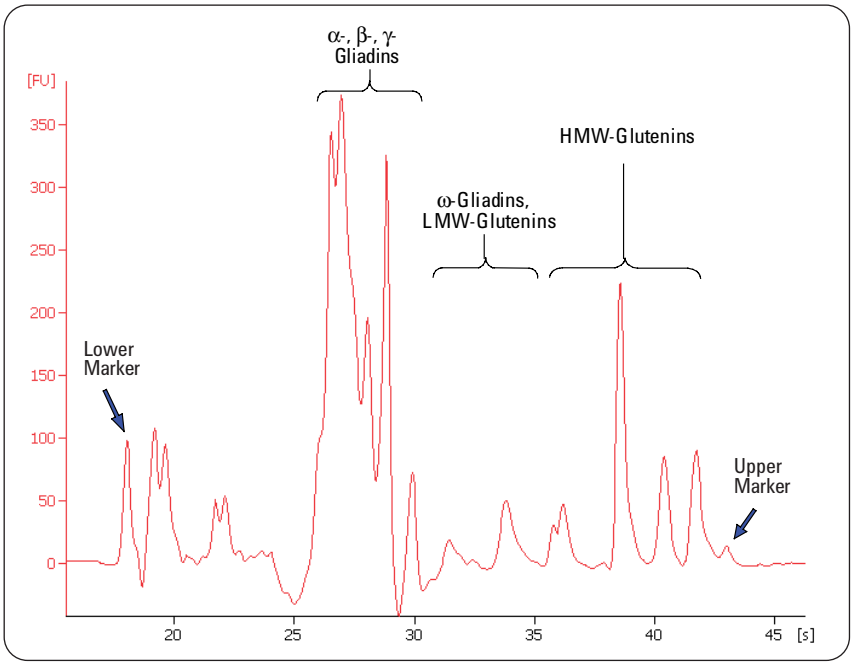


Figure 1
Typical wheat protein separation by the Protein 230 assay.

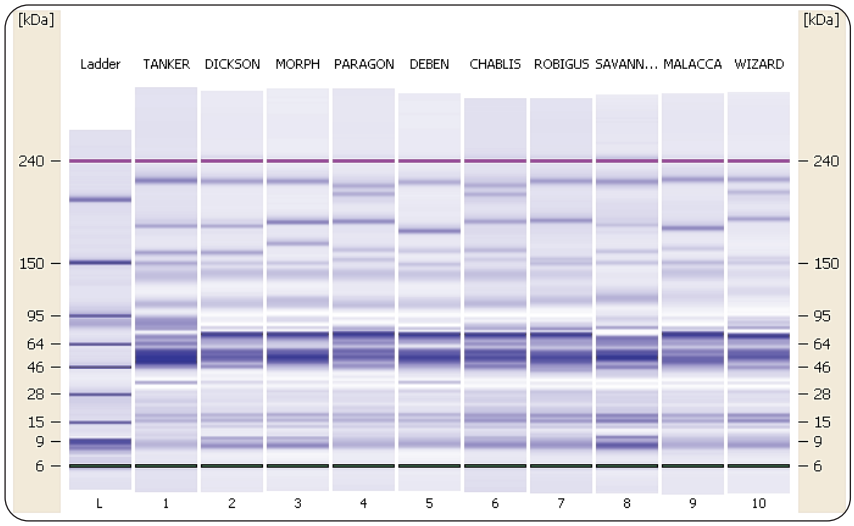


Figure 2
Bioanalyzer gel-like image of MW standards and 10 different wheat varieties.

Methods

Total wheat proteins (including glutenins) were extracted from individual grains in 0.4 mL of 2M urea, 15 % glycerol, 0.1 M DTT and 0.1 M Tris/HCl, pH 8.8, using an ultra-sonic water bath for 15 minutes. Extracts were centrifuged at 11,000 g for 5 minutes and treated with the Protein 230 assay reagents in according to the assay protocol. The samples were separated on the Agilent 2100 bioanalyzer, with each analysis of 10 samples plus ladder taking less than 25 minutes. Replicates of 34 wheat cultivars representing the 2004/5 UK Recommended List varieties were analyzed. The electropheromram profiles were processed using the Phoretix 1D Advanced and 1D Database (Nonlinear Dynamics) software for pattern-matching purposes.

Results

The Protein 230 assay produced well-resolved protein profiles, suitable for varietal discrimination (figures 1 & 2). The Phoretix software was able to compare the electropherogram profiles. Figure 3 shows an example of a dendrogram where all the replicates of three different varieties are correctly grouped. A prototype wheat library was developed by selecting the most representative varietal profile. Results showed that 90 % of test samples could be identified within the top three matches. Work is in progress to optimize the performance of this library. The practicality of the method and the robustness of the system is bourne out by the fact that the system is now in routine use in UK commercial mill intake laboratories.

Conclusions

Our study has demonstrated that using the Agilent 2100 bioanalyzer with the Phoretix system offers a standardized, objective method for rapid varietal discrimination. The ease of use and total analysis time of less than 50 minutes makes it most suitable for mill intake use. The optimized system will enable millers to make more confident decisions in accepting grain consignments, and could become widely adopted as an effective policing tool within the grain industry. A number of UK mills have purchased the combined systems for screening wheat deliveries at intake.

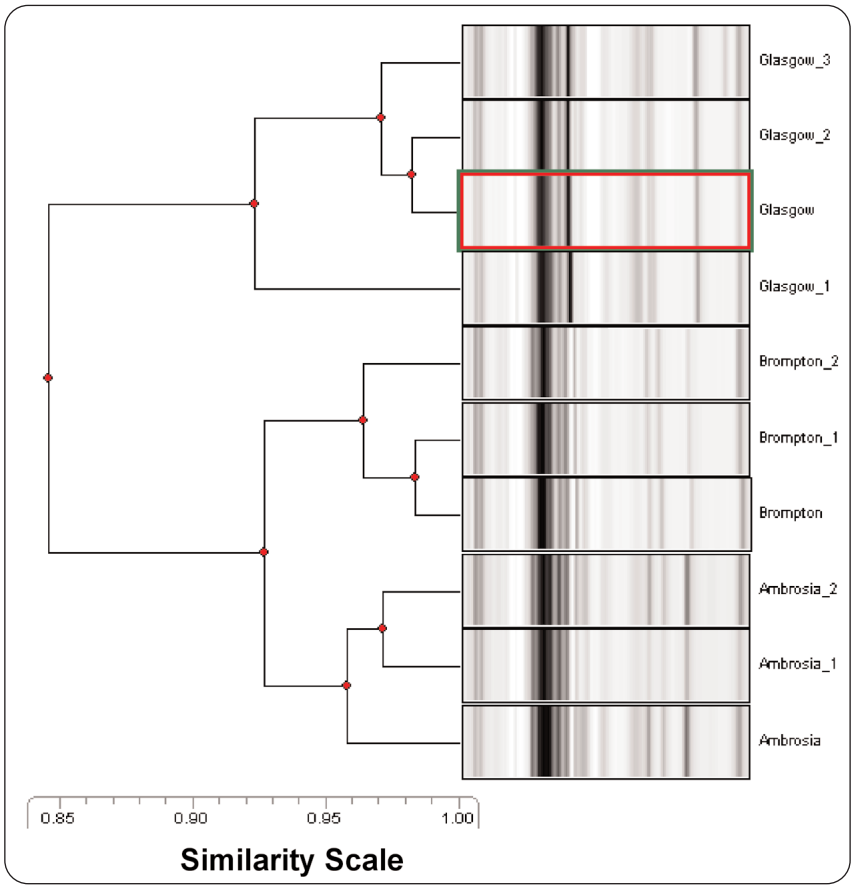


Figure 3
Dendrogram illustrating pattern-matching of replicate profiles of 3 wheat varieties.

Acknowledgements

CCFRA is grateful to the National Association of British and Irish Millers (nabim) for sponsoring this study.

Dr Dhan Bhandari is Senior Scientist at Campden & Chorleywood Food Research Association (CCFRA), Chipping Campden, Gloucestershire, GL55 6LD, UK.

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Strawberry and Raspberry Fruit Differentiation Using the Agilent CE 2100 Bioanalyzer

Application Note

Food

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Abstract

Food authenticity is an important and rapidly expanding area that requires development of molecular approaches to help avert fraudulent replacement of expensive food ingredients, and to ensure correct ingredient levels are present in prepacked foods. We report here on the development of a method that uses polymerase chain reaction (PCR) and the DNA 1000 chip to distinguish between DNA derived from the fruits of strawberry and raspberry. Using this approach, characteristic profiles from strawberry and raspberry DNA are generated on the Agilent 2100 capillary electrophoresis system, which may prove useful in food authentication studies.

Agilent Technologies

Introduction

Food authentication is an important and rapidly expanding analytical area that is needed to ensure that food conforms to current international legislation and that policies on food labeling and ingredients are enforced.

Raspberry (genus: *Rubus*) is used in many foods, including purées, jellies, jams, pies, cakes, pastries, dessert toppings, juices, wines, and dairy products such as ice cream and yogurt, as well as being eaten fresh or stored frozen for consumption later. The leaves of raspberry are often used in herbal teas, and the fruit is also used for potential health benefits. Strawberry (genus: *Fragaria*) is also a common type of fruit that is cultivated worldwide and is of global economic significance.

However, recent studies [1, 2] report that some food and drinks (fruit juices in particular) labeled as containing a particular fruit, contain little or no fruit of that particular species, or may have substituted or mixed that fruit with other edible fruits. This may occur through either deliberate adulteration or unintentional processing errors (via contamination through inefficient washing procedures or coprocessing of fruits). Such instances are in contravention of the law, and stakeholders (food retailers, enforcement agencies, etc.) all require access to methods that allow the accurate identification of food ingredients to ensure regulatory compliance and protect consumers. Additionally, correct identification of ingredients in food products is needed to support the authentic composition of food, especially in relation to the declared presence of allergens in a food product, and also in the fraudulent replacement of more expensive food ingredients.

A novel application of the Agilent 2100 capillary electrophoresis (CE) system to differentiate between DNA derived from strawberry and raspberry fruits, is reported here. This approach makes use of microsatellite markers that allow differentiation of strawberry and raspberry DNA based on presence/absence or size differentiation of PCR products, easily measured using the DNA 1000 chip.

Experimental

PCR Primers

Microsatellite markers that have been previously described [3] were used to differentiate between strawberry and raspberry DNA:

Fvi11 Forward: GCATCATCGTCATAATGAGTGC
Fvi11 Reverse: GGCTTCATCTCTGCAATTCAA

Fvi20 Forward: GAGTTTGTCATCCTCAGACACC
Fvi20 Reverse: AGTGACCCAGAACCCAGAA

Samples

Authenticated DNA for Samples A and B were kindly provided by the SCRI (Dundee, UK) and consisted of strawberry samples derived from a numbered selection from a commercial breeding program, and Glen Moy (raspberry), respectively.

Raspberries (Sample D) were bought from a UK supermarket chain as prepacked fruit (225 g) labeled as raspberries (produce of Spain). Strawberries (Sample C) were purchased from the same UK supermarket store at the same time, as prepacked fruit labeled as strawberries. Two individual DNA extractions were taken from each fruit batch and labeled as Samples C1 and C2, and Samples D1 and D2.

DNA Extraction

For Samples C and D, DNA was extracted from strawberry and raspberry fruits using a cetyl trimethylammonium bromide (CTAB) buffer (50 mM tris HCl; 4 M NaCl, 1.8% CTAB; 25 mM EDTA). Approximately 100 mg fruit samples were weighed and homogenized, DNA was extracted using the above CTAB buffer, resuspended in 100 µL of 1x TE buffer, and quantified using a spectrophotometer.

Thermal Cycle Conditions

25 µL PCR reaction mixes were made based on the following components: 12.5 µL of 2x Fast Start PCR Master mix (Product number 04710436001, Roche); 300 nM of appropriate forward primer; 300 nM of appropriate reverse primer; 15 ng of extracted genomic DNA; and sterile distilled water to make a final volume of 25 µL.

Thermal cycle conditions (MJ Research Tetrad #2 PCR machine) consisted of 95 °C for 6 min; 40 cycles of 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min; followed by 72 °C for 10 min and hold at 4 °C.

Use of the DNA 1000 Chips on the Agilent 2100 CE System

All chips were prepared according to the instructions provided with the Agilent DNA 1000 LabChip kit. The gel-dye mix was prepared by mixing 400 µL of the gel matrix with 20 µL of the dye concentrate, then filtering the mixture through a spin filter. The separation chip was filled with the gel matrix/dye mixture, and 5 µL of the markers was added to each sample well. After adding samples (1 µL each) to the sample wells and the DNA sizing ladder (1 µL) to the assigned ladder well, the chip was vortexed and run on the Agilent 2100 bioanalyzer.

Results and Discussion

Fvi11 Assay

According to published literature [3, 4], Fvi11 is based on a $(GA)_{16}$ repeat motif and should give an amplicon of around 137 bp in length with strawberry, but also exhibits polymorphism in amplicon size between *Fragaria* varieties. However, Fvi11 should not cross-react with raspberry, and so no amplicon should be present.

The results from this preliminary study shown in Figures 1A and 1B indicate that Fvi11 gives an amplicon of around 122 bp in the samples that contained strawberry (A and C). Additional amplicons were also sometimes observed at 282 and 290 bp, but within the confines of the limited experimental data presented here, not on a repeatable basis. In line with expectations, Figures 1A and 1C show that Fvi11 did not cross-react with samples that contained raspberry (B and D).

Negative controls showed no detectable amplification. Additionally, Fvi11 showed a positive result and amplified the same 122-bp fragment when tested on a commercially available strawberry sauce sample with a listed ingredient of 40 percent whole strawberries (Figure 1D), inferring the assay's applicability to processed food samples containing fruit.

Fvi20 Assay

According to published literature [3, 4], microsatellite marker

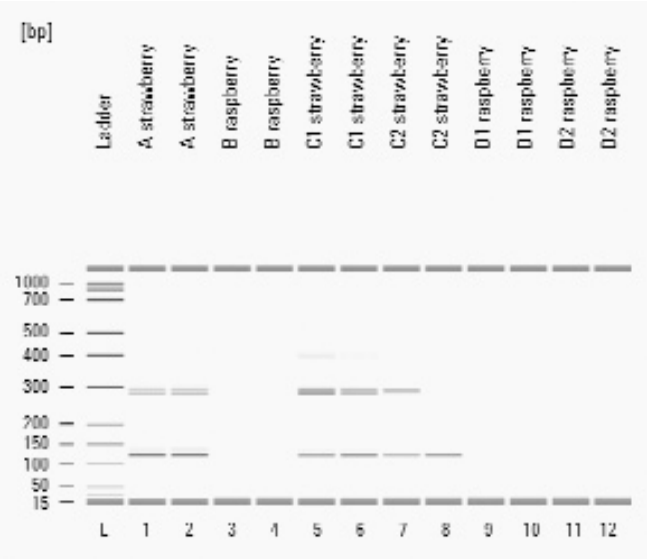


Figure 1A: Gel-like image based on Fvi11 assay on all samples.

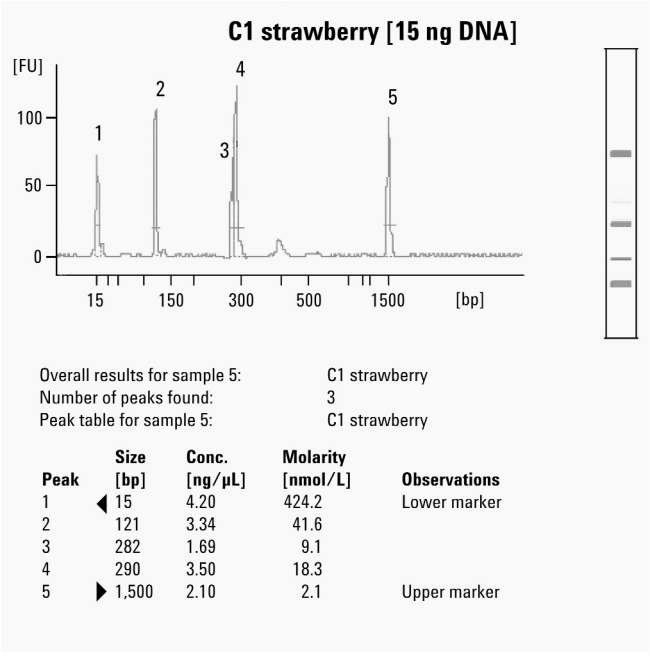


Figure 1B. Electropherogram to show profile generated using Fvi11 with strawberry DNA.

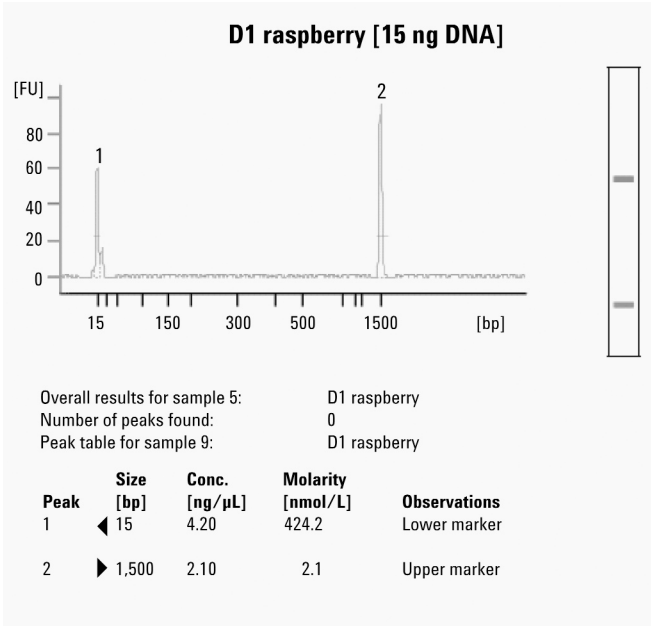
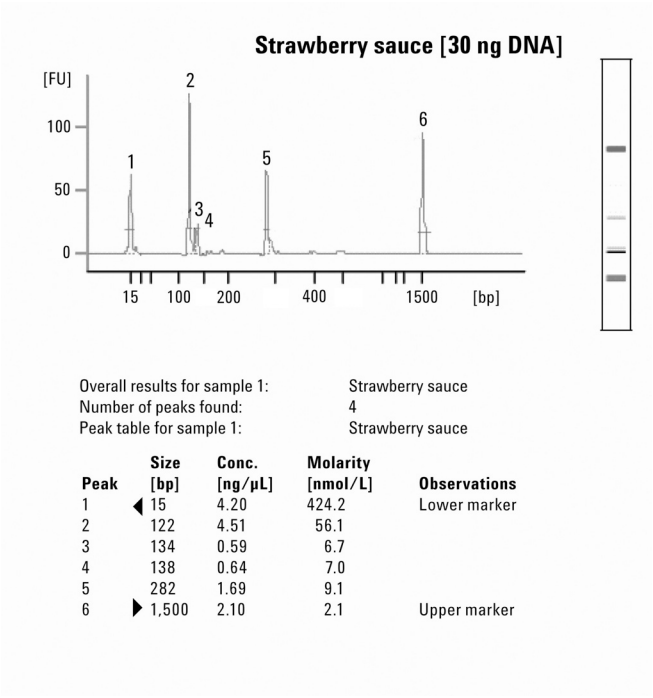


Figure 1C. Electropherogram to show absence of bands when using Fvi11 with raspberry DNA.



Overall results for sample 1: Strawberry sauce
Number of peaks found: 4
Peak table for sample 1: Strawberry sauce

Peak	Size [bp]	Conc. [ng/µL]	Molarity [nmol/L]	Observations
1	◀ 15	4.20	424.2	Lower marker
2	122	4.51	56.1	
3	134	0.59	6.7	
4	138	0.64	7.0	
5	282	1.69	9.1	
6	▶ 1,500	2.10	2.1	Upper marker

Figure 1D. Electropherogram to show characteristic strawberry DNA profile generated following application of Fvi11 to a strawberry sauce sample.

Fvi20 is based on a $(GA)_{20}$ simple sequence repeat motif sequence, and has been shown to give a single or multiple bands around 162 bp in length for *Fragaria* varieties, but only a single amplicon with raspberry (*Rubus*) varieties.

Based on the results from this preliminary study, shown in Figures 2A and 2B, the application of the Fvi20 assay to samples that contained strawberry (A and C) gave single or multiple amplicons at around 144, 162, and/or 175 bp. In the limited strawberry samples tested in this study, the 144-bp fragment was present and predominated, while the occurrence of the other bands was less repeatable.

The application of the Fvi20 marker locus to samples that contained raspberry (B and D), shown in Figures 2A and 2C, showed the presence of a single band at 136 bp, which was easily distinguished from the 144-bp amplicon characteristic of strawberry cultivars.

Negative controls and extraction blanks showed no detectable amplification. Furthermore, the application of Fvi20 to the commercially available strawberry sauce sample showed bands around 143 and 161 bp, characteristic of strawberry DNA being present (Figure 2D).

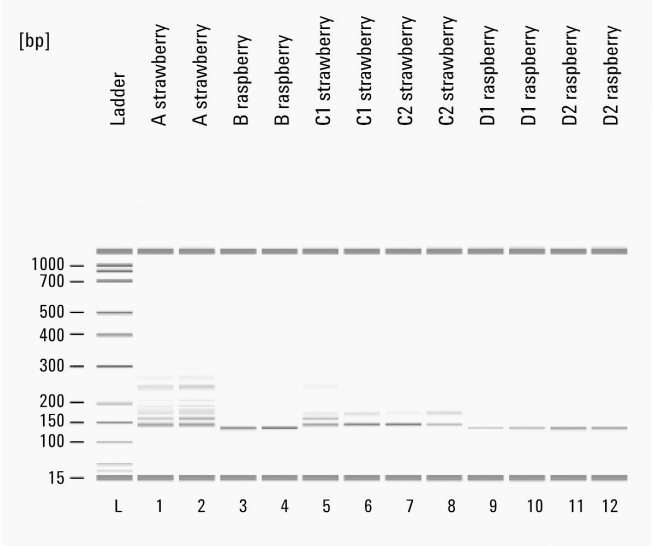
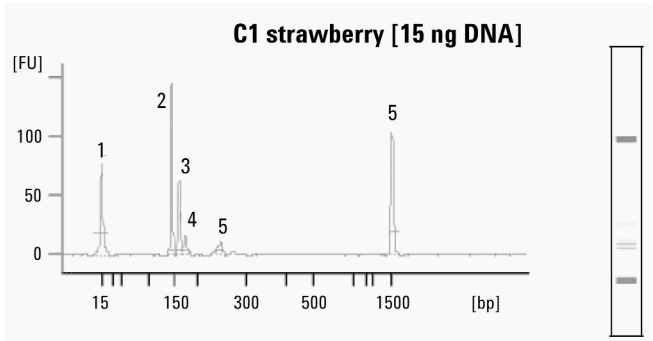


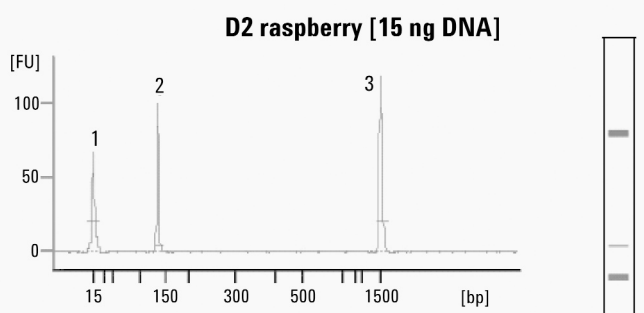
Figure 2A. Gel-like image based on Fvi20 assay on all samples.



Overall results for sample 5: C1 strawberry
Number of peaks found: 3
Peak table for sample 5: C1 strawberry

Peak	Size [bp]	Conc. [ng/µL]	Molarity [nmol/L]	Observations
1	◀ 15	4.20	424.2	Lower marker
2	144	4.13	43.6	
3	161	2.01	18.9	
4	174	0.76	6.6	
5	248	0.60	3.7	
6	▶ 1,500	2.10	2.1	Upper marker

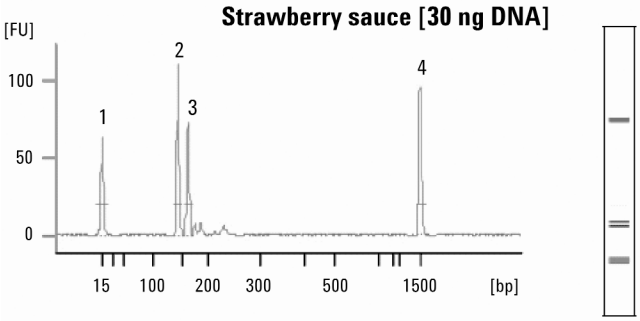
Figure 2B. Electropherogram to show profile generated using Fvi20 with strawberry DNA.



Overall results for sample 5: D2 raspberry
Number of peaks found: 1
Peak table for sample 12: D2 raspberry

Peak	Size [bp]	Conc. [ng/µL]	Molarity [nmol/L]	Observations
1	◀ 15	4.20	424.2	Lower marker
2	135	2.44	27.4	
3	▶ 1,500	2.10	2.1	Upper marker

Figure 2C. Electropherogram to show presence of one band when using Fvi20 with raspberry DNA.



Overall results for sample 1: Strawberry sauce
Number of peaks found: 2
Peak table for sample 1: Strawberry sauce

Peak	Size [bp]	Conc. [ng/µL]	Molarity [nmol/L]	Observations
1	◀ 15	4.20	424.2	Lower marker
2	143	3.71	39.4	
3	161	3.01	28.2	
4	▶ 1,500	2.10	2.1	Upper marker

Figure 2D. Electropherogram to show characteristic strawberry DNA profile generated following application of Fvi20 to the strawberry sauce sample.

Conclusions

Originally, the microsatellite markers of Fvi11 and Fvi20 were used to assess genetic variability in strawberry (*Fragaria*) varieties. We have shown the novel application of these primer pairs using PCR and the Agilent CE 2100 system, to differentiate between samples that contain strawberry and raspberry DNA. Based on initial studies and amplicon sizes, these may prove useful in food authentication studies. The results from this small study are only representative of specific varieties of *Fragaria* and *Rubus*, but demonstrated clear differentiation between strawberry and raspberry DNA using traditional PCR followed by resolving the PCR products on the Agilent 2100 CE system.

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2. A. Stój and Z. Targoński, "Use of amino acid analysis for estimation of berry juice authenticity," *Acta Sci. Pol., Technol. Aliment* (2006) 5(1):61–72
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4. K.S. Lewers, S.M.N. Styan, S.C. Hokanson, "Strawberry GenBank-derived and Genomic Simple Sequence Repeat (SSR) Markers and Their Utility with Strawberry, Blackberry, and Red and Black Raspberry," *J. Amer. Soc. Hort. Sci*. (2005) 130(1):102–115

Acknowledgements

We gratefully acknowledge provision of strawberry Sample A and raspberry Sample B as authenticated samples from Julie Graham, SCRI, for the purposes of this study.

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Agilent Technologies

Analysis of the Purity and Storage Stability of L-Ascorbic Acid Powders

Application Note

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Introduction

Most previous studies of the storage stability of L-ascorbic acid were done on solutions of the acid. However, L-ascorbic acid is usually kept in the dry powder form, batches of which may contain breakdown products or other impurities arising from the production process. The presence of these contaminants is suspected to adversely affect the stability of the acid and/or contribute to color changes in the stored product over time.

This note describes a trial of the purity and stability of stored L-ascorbic acid powder samples from four different manufacturers, and assesses degradation compounds and/or precursors from the production process. Ion suppression reversed phase HPLC was used, with PLRP-S columns. PLRP-S is a rigid macroporous styrene/divinylbenzene HPLC phase with outstanding chemical and physical stability with acidic eluents.

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Materials and Reagents

Samples: L-ascorbic acid powders from different manufacturers produced from 1971 to 1987
Impurity reference compounds: L-dehydroascorbic acid (DHAA), 2,3-diketo-L-gulonic acid, L-threonic acid, oxalic acid, furfural, 5,6-*O*-isopropylidene-L-ascorbic acid, D-gulonic acid-1,4-lactone

Conditions

Column: PLRP-S 100Å 5 µm, 150 x 4.6 mm (p/n PL1111-3500)
Eluent: 0.2 M NaH_2PO_4, pH 2.14
Flow rate: 0.5 mL/min
Detector: UV, 268 and 220 nm

Results and Discussion

A plot of manufacturing date against purity is given in Figure 1, showing a gradual decline in L-ascorbic acid with age even in dry powder formulations. Comparison of the retention times of sample peaks with those of the reference compounds (Table 1) did not reveal any sign of degradation products or precursors associated with the manufacture or storage of L-ascorbic acid. The absence of these compounds suggests that other degradation routes are present when L-ascorbic acid is stored under near anaerobic conditions of low water activity.

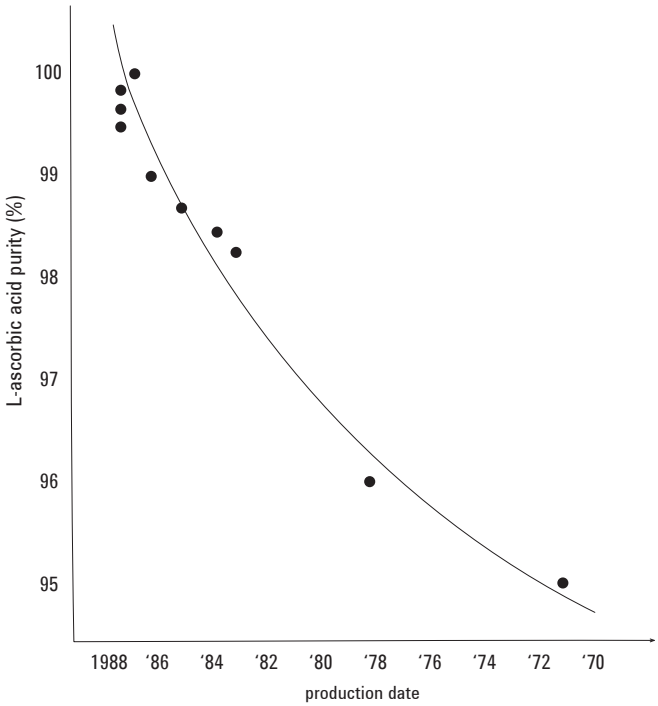


Figure 1. Graph of L-ascorbic acid purity and production date of powder samples of L-ascorbic acid.

Table 1. Retention times of reference compounds.

Sample	L-ascorbic acid association	Retention time (min)	Limit of detection (µmol)
oxalic acid	degradation product	6.00	0.039
L-threonine	degradation product	6.30	0.053
2,3-diketo-L-gulonic acid	precursor	6.60	-
D-gulonic acid-1,4-lactone	precursor	6.60	-
L-dehydroascorbic acid	degradation product	7.40	0.017
L-ascorbic acid	-	8.70	
5,6-*O*-isopropylidene-L-ascorbic acid	precursor	8.70	
fufural	degradation product	12.10	

The samples varied in their degree of whiteness regardless of the date of production, suggesting that differences in whiteness were not indicators of purity but may have been be due to differing production methods. However, the aged samples manufactured in 1971 and 1978 had a brown appearance, most evident in the 1978 sample. Measurement of the L-ascorbic acid content of these samples was lower compared to newer samples, but no impurities were detected.

The oxidative degradation of a solution of L-ascorbic acid was assessed, following the pathway shown in Figure 2.

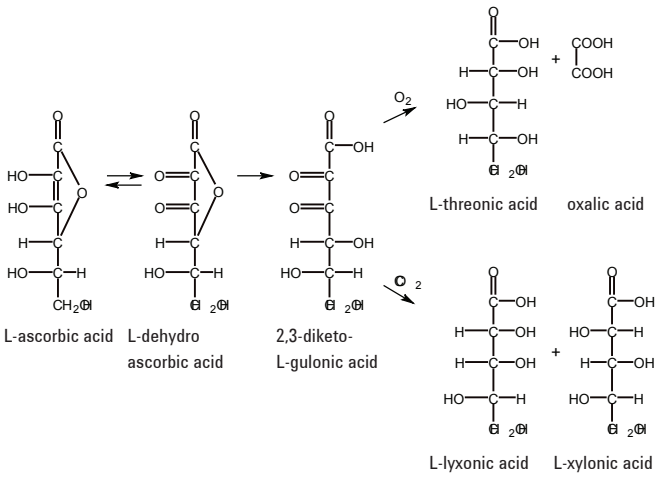


Figure 2. The proposed oxidative degradation path of L-ascorbic acid.

Table 1 shows the minimum limits of detection of the degradation products L-dehydroascorbic acid, oxalic acid and L-threonic acid from this assay. It was not possible to carry out a test for 2,3-diketo-L-gulonic acid because a pure reference compound was not available.

The complete data set and analysis is available in Kennedy *et al.* (1989).

Conclusion

Ion suppression HPLC using PLRP-S columns successfully elucidated the effects of age on the gradual deterioration of dry powder L-ascorbic acid, and suggested the presence of alternative degradation mechanisms when the acid is stored in dry powder form.

Reference

Kennedy, JF, Rivera, ZS, Lloyd, LL, Warner, FP, White, CA (1989) The use of reversed-phase HPLC for the determination of purity and stability on storage of L-ascorbic acid samples. *J. Micronutr. Anal.,* 5, 281-289.

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Agilent Technologies

Identification of different meat species by the Agilent Fish ID solution on the Agilent 2100 Bioanalyzer

Application Note

Food Authenticity, Food Testing

Authors

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Abstract

In food processing plants, a large number of various ingredients originating from different animal species are used as part of the production process. Sometimes this is done on the same production line. The Agilent Fish ID solution was developed to identify the species of fishes from food samples of different processing levels. This application note investigates its abilities to detect the presence of non-fish species, specifically mammalian or avian DNA originating from dairy products or meat.

Introduction

The Agilent Fish ID solution was introduced to allow fast and easy identification of fish species from raw, cooked, or otherwise processed fish samples. Food manufacturers are using a large number of ingredients in the preparation of their products. This includes dairy products and meat from various mammalian or avian species. These can be part of seafood preparations, either as wanted ingredient or as a contamination from the production process. The aim of this study was to determine the ability of the Fish ID solution to detect mammalian or avian DNA using meat samples.

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Materials and Methods

Meat samples

Beef, pork, wild boar, lamb, chicken, turkey, and duck samples were obtained from local supermarkets or butchers.

Isolation of DNA from meat tissue

DNA was isolated using the protocol described in the Agilent DNA Isolation kit which comes as part of the Fish ID Ensemble. DNA concentrations and 260/280, 260/230 ratios were checked using a Nanodrop ND-2000 spectrophotometer (Thermo Fisher Scientific).

Amplification of *Cyt*b target sequence

For the amplification of the *Cyt*b target sequence, 1 µl of the isolated DNA was used according to the protocol of the Agilent PCR-RFLP Fish ID kit. As a positive control, the kit supplied *Salmo salar* DNA was also amplified as described in the manual. Successful amplification of all samples as well as a clear negative control (NTC) was validated using a DNA 1000 assay on the Agilent 2100 Bioanalyzer.

Restriction digestion of the PCR products

Following the protocol of the Agilent PCR-RFLP Fish ID kit, a 2.5 µL aliquot of the PCR reaction was used in the restriction digestion with each of the kit supplied enzymes *Dde* I, *Hae* III, and *Nla* III.

Analysis of restriction patterns using the Bioanalyzer

The digested samples and the positive control salmon DNA were run on a DNA 1000 chip according to protocol. For each sample, the three independent digests were loaded in consecutive wells, allowing the analysis of four samples per chip. The resulting electropherograms were analyzed using the 2100 Bioanalyzer Expert software.

Results

DNA of sufficient yield and quality was prepared from all tissue samples using the Agilent DNA Isolation kit provided as part of the Fish ID solution. Two independent DNA samples for each of the meat tissues were used in the PCR together with the salmon positive control DNA supplied by the kit and a no template control. The results of the PCR reactions were validated on the 2100 Bioanalyzer using a DNA 1000 assay (Figure 1). For mammalian samples, a single clear PCR product was obtained similar to the salmon positive control. Turkey showed a reproducible double band, whereas chicken and duck samples resulted in a large number of unspecific amplicons present in the reaction.

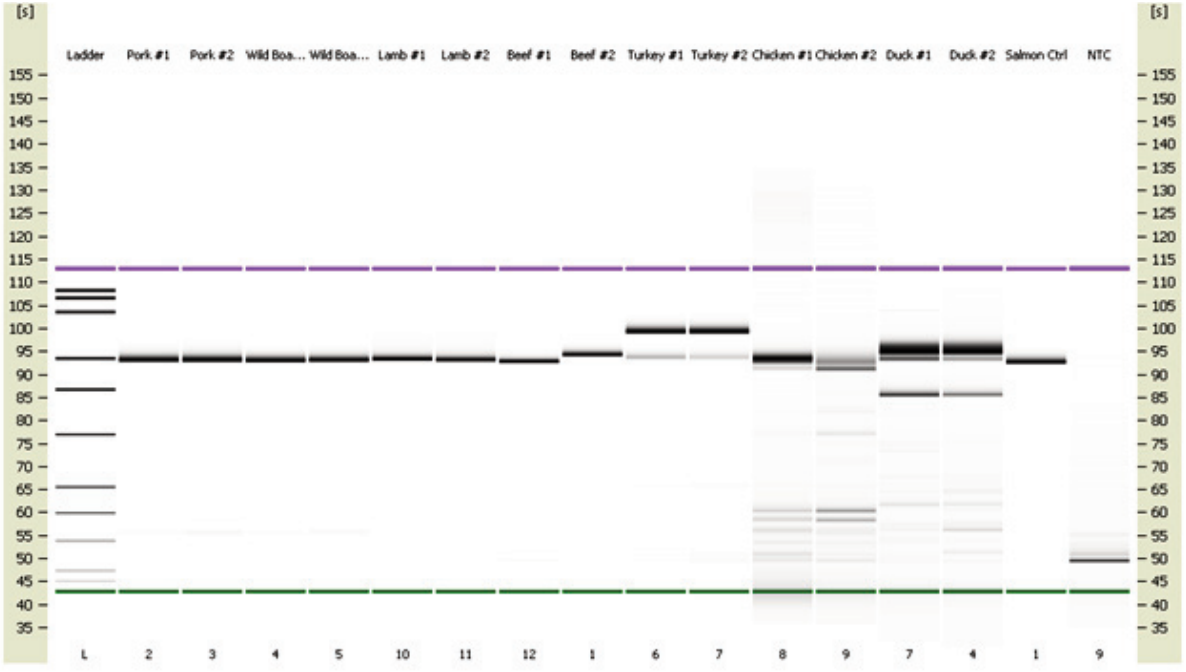


Figure 1: ***Analysis of Cytb PCR results.*** *Two independent meat DNA samples, the salmon (S. salar) positive control and a no-template control were analyzed on a Bioanalyzer using a DNA1000 assay. The virtual gel image shows a comparison file assembled of all samples using the Expert software. Lane 1: Ladder, lane 2 + 3: Pork, lane 4 + 5: Wild boar, lane 6 + 7: Lamb, lane 8 + 9: Beef, lane 10 + 11: Turkey, lane 12 + 13: Chicken, lane 14 + 15: Duck, lane 16: S. salar positive control, lane 17: NTC.*

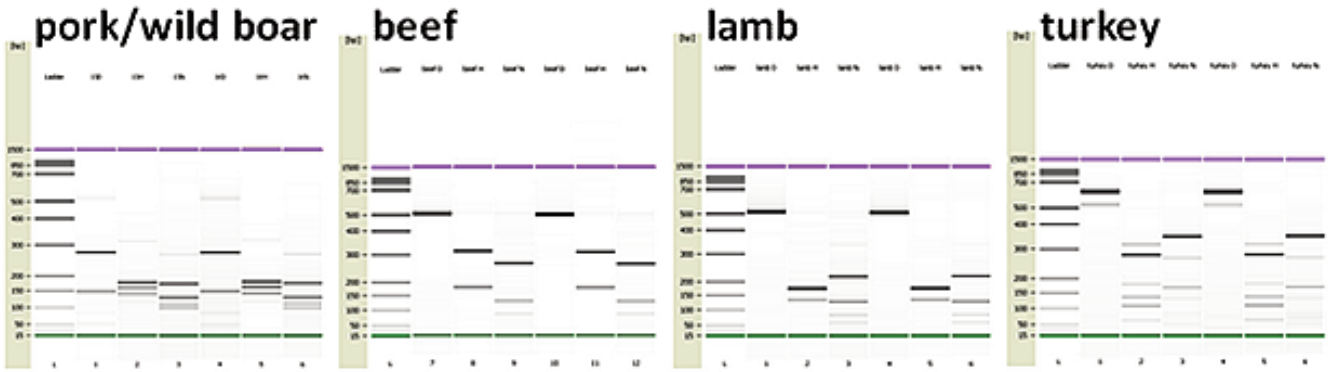


Figure 2. Restriction digestion analysis on the Agilent 2100 Bioanalyzer. The virtual gel images show the pattern combination for the restriction digestion of PCR products obtained from two independent meat tissue samples of pork, beef, lamb and turkey. Each panel shows a lane containing the ladder, Dde I, Hae III, and Nla III digestion pattern for sample 1, followed by sample 2 for the same tissue. Pork and wild boar resulted in the same pattern, therefore only the pork results are shown.

All PCR reactions, with the exception of the NTC, were subsequently used in a restriction digestion according to the manual of the PCR-RFLP Fish ID kit. As expected from the PCR results, both chicken and duck resulted in a multitude of fragments not producing a conclusive pattern (data not shown). In mammalian tissue and turkey, unique and easily identifiable patterns could be obtained (Fig. 2). Pork and wild boar gave rise to the same pattern combination. Fragment sizes are summarized in Table 1.

Table 1. Fragment Sizes and Standard Deviation in Bp for Pork, Wild Boar, Beef, Lamb and Turkey. The 132 Bp Fragment in Brackets was Consistently Present in Nla III Digests of Turkey but was not Detected with Default Settings in the Expert Software as it was Below the Peak Threshold

	***Dde* I**	***Hae* III**	***Nla* III**
Pork	276 ± 0.6, 149 ± 0.6	180 ± 1.3, 162 ± 1.3, 142 ± 1.7	175 ± 1.0, 130 ± 0.5, 109 ± 1.3, 98 ± 0.8
Wild boar	274 ± 1.0, 149 ± 0.8	181 ± 1.5, 162 ± 1.0, 143 ± 2.0	174 ± 0.8, 130 ± 0.8, 111 ± 1.0, 99 ± 0.6
Beef	505 ± 6.0	313 ± 3.1, 180 ± 0.6	267 ± 1.7, 131 ± 0.6, 87 ± 0.5
Lamb	517 ± 4.2	178 ± 1.0, 171 ± 1.0, 135 ± 1.0	220 ± 1.3, 129 ± 1.0, 84 ± 0.8, 58 ± 0.5
Turkey	624 ± 0.7, 525 ± 0.7	319 ± 0.0, 280 ± 1.4, 180 ± 1.4, 137 ± 0.7, 110 ± 0.7, 64 ± 0.7	352 ± 1.4, 271 ± 0.7, 169 ± 0.7, (132 ± 0.7)

Summary

Due to the design of the primers to cover a wide range of fish species, it was expected that non-fish species would be detected. A small scale validation with a few common mammalian and avian meat tissues showed the capability of the kit to successfully identify pork, beef and lamb. Avian *Cyt*b target DNA on the other hand seems to differ substantially from its mammalian or fish homolog. Only turkey gave rise to a simple, easily identifiable pattern, although producing two different PCR products. A full scale meat species identification solution will require a different primer design or even a different target sequence.

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Agilent DNA Fish ID solution

Discrimination of Different Meat Species by the Agilent Fish ID Solution on the Agilent 2100 Bioanalyzer System

FOOD AUTHENTICITY



A quick, robust and easy to use protocol to identify the species based on a well accepted PCR-RFLP method allows analysis from sample (includes most processed meats and fish) to result in one working day and yields good discrimination results

In food processing plants, a large number of various ingredients originating from different animal species are used as part of the production process. Sometimes this is done on the same production line. The Agilent Fish ID solution was developed to identify the species of fishes from food samples of different processing levels. Here, we look at its abilities to detect the presence of non-fish species, specifically mammalian or avian DNA originating from dairy products or meat.

DNA based testing methods allow sensitive detection and identification from almost all but the most heavily processed food samples.

The Agilent DNA Fish ID ensemble provides all reagents and enzymes to complete a PCR-RFLP method for a Cytb PCR target sequence and analysis of restriction fragment patterns on the Agilent 2100 Bioanalyzer to identify fish species based on this mitochondrial sequence. The availability of commercial screening solutions allows for more reliable and robust test results through well-matched components and facilitates testing for screening purposes by the use of mastermix formulations and streamlined protocols. The Agilent DNA Fish ID solution was evaluated for the purpose of identifying the species of other meat products that may be intentionally or inadvertently mixed into a finished product. Good results were obtained for beef, pork, wild boar, lamb and turkey.

Table 1: Fragment sizes and standard deviation in bp for pork, wild boar, beef, lamb and turkey. The 132 bp fragment in brackets was consistently present in Nla III digests of turkey but was not detected with default settings in the Expert software as it was below the peak threshold.

	***Dde* I**	***Hae* III**	***Nla* III**
Pork	276 ± 0.6, 149 ± 0.6	180 ± 1.3, 162 ± 1.3, 142 ± 1.7	175 ± 1.0, 130 ± 0.5, 109 ± 1.3, 98 ± 0.8
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Turkey	624 ± 0.7, 525 ± 0.7	319 ± 0.0, 280 ± 1.4, 180 ± 1.4 , 137 ± 0.7, 110 ± 0.7, 64 ± 0.7	352 ± 1.4, 271 ± 0.7, 169 ± 0.7, (132 ± 0.7)

Key Benefits

- Agilent 2100 Bioanalyzer has the widest range of assays available on the market covering applications for RNA, DNA or protein
- Fast, reproducible and robust method replacing gel electrophoresis
- Agilent method using convenient mastermix format reagents will have you identifying your fish within hours, no fish standard or guesswork required.
- Flexible modular system: In-house accredited methods can replace individual building blocks of the Fish ID solution
- Overlay to the Expert software allows rapid identification of restriction fragments and close matches.
- Agilent method is now useful for testing some other meats such as beef, pork, lamb, wild boar or turkey as well as most fresh or processed fish.

Discrimination of Different Meat Species by the Agilent Fish ID Solution on the Agilent 2100 Bioanalyzer System

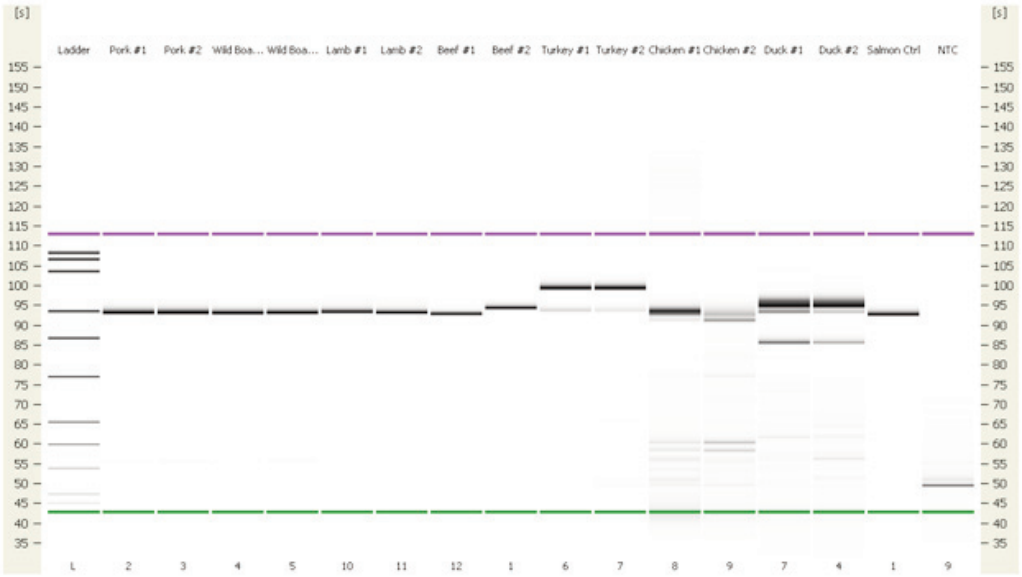


Figure 1: Analysis of Cytb PCR results. Two independent meat DNA samples, the salmon (S. salar) positive control and a no template control were analyzed on a Bioanalyzer using a DNA1000 assay. The virtual gel image shows a comparison file assembled of all samples using the Expert software. Lane 1: Ladder, lane 2 + 3: Pork, lane 4 + 5: Wild boar, lane 6 + 7: Lamb, lane 8 + 9: Beef, lane 10 + 11: Turkey, lane 12 + 13: Chicken, lane 14 + 15: Duck, lane 16: S. salar positive control, lane 17: NTC.

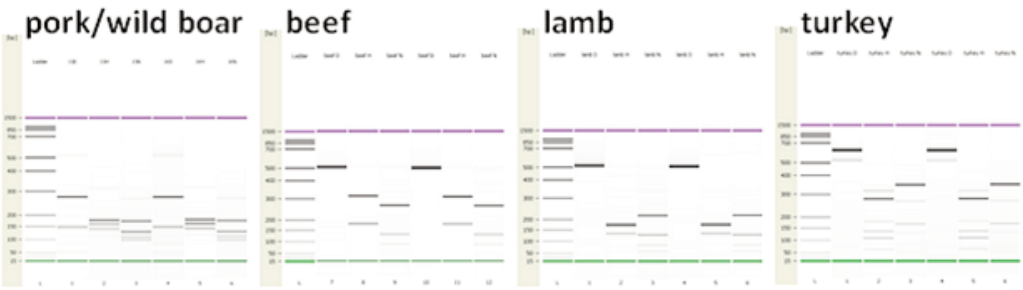


Figure 2. Restriction digestion analysis on the Agilent 2100 Bioanalyzer. The virtual gel images show the pattern combination for the restriction digestion of PCR products obtained from two independent meat tissue samples of pork, beef, lamb and turkey. Each panel shows a lane containing the ladder, Dde I, Hae III, and Nla III digestion pattern for sample 1, followed by sample 2 for the same tissue. Pork and wild boar resulted in the same pattern, therefore only the pork results are shown.

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Determination of Melamine Residue in Milk Powder and Egg Using Agilent SampliQ Polymer SCX Solid Phase Extraction and the Agilent 1200 Series HPLC/UV

Application Note

Food Safety

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Abstract

This method was developed for the determination of melamine in milk powder and egg. Solid phase extraction (SPE) and HPLC/UV are used consistent with the Chinese regulatory method. The sample preparation is performed using a polymeric mixed mode strong cation exchange resin. The separation and detection are performed by HPLC/UV. The limit of detection is 10 µg/kg. Linear calibration curves were obtained over the calibration range of 1 to 20 mg/kg. Overall recoveries range from 84 to 96 percent, with RSD values below 3.0 percent.

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Introduction

Melamine made headlines in September 2008 because it was found to be the contaminant responsible for the deaths of several infants and making many more sick. Melamine is used as an adulterant in milk and milk products because it causes a false positive value in a protein content measurement. The presence of melamine in foods is strictly regulated worldwide. Because milk is used in many other products for human and animal consumption, melamine is now detected in other food products in China and many other countries. The analysis for melamine is proceeding in many labs all over the world.

The Chinese government released a government regulation (GB/T 22388-2008) that established analytical methods for melamine in raw milk and dairy products and set a maximum residue limit (MRL) for melamine in food of 2 mg/kg. This application note describes the implementation and optimization of the solid phase extraction (SPE) method used for milk powder and egg described in GB/T 22388-2008.

Experimental

Reagents and Chemicals

Melamine was obtained from Sigma-Aldrich (Shanghai, China). A 1 mg/kg melamine stock solution was prepared in methanol and kept in the freezer (–4 °C). Appropriate dilutions using mobile phase generated working solutions that were prepared fresh daily.

Sample Extraction for Milk Powder or Egg

For egg, homogenize, place in a clean, sealed container, and store in the freezer at –4 °C.

For homogenized egg and milk powder, weigh 2.0 ± 0.01 g of sample and add to a 50-mL centrifuge tube. Add 15 mL of 1% trichloroacetic acid in water and 5 mL of acetonitrile, then cap. Sonicate for 10 minutes and then place samples on a vertical shaker for 10 minutes. Centrifuge the samples for 10 minutes at 4,000 rpm. Wet a filter paper with 1% trichloroacetic acid in water, then filter the supernatant into a 25.0-mL volumetric flask and bring to volume with 1% trichloroacetic acid in water. Transfer a 5.0 mL aliquot of the extract into a glass tube, and then add 5.0 mL purified water. Mix thoroughly on a vortex mixer.

SPE Purification

Agilent SampliQ SCX SPE cartridges (p/n 5982-3236, 3 mL, 60 mg) were used to clean up sample extracts. All SPE steps, including conditioning, sample load, wash, and the final elution, are performed without vacuum with a flow rate between 0.5 and 1 mL/min. For the drying step, vacuum is applied to quickly dry the cartridges. The procedure used for the SPE extraction is shown in Figure 1. Cartridges were conditioned with 3 mL of methanol, then 5 mL of water. A 10-mL prepared sample solution (equivalent to a 0.4-g sample) was passed through the cartridge. After the sample effused completely, the cartridge was washed with 3 mL of water and 3 mL of methanol. The entire effluent was discarded. The cartridge was dried under negative pressure below 2.0 kPa for 3 minutes. Finally, the cartridge was eluted with 6 mL of 5% ammonium hydroxide in methanol. The eluent was collected and dried under nitrogen at 45 °C. The resulting residue was resuspended and made to a constant volume of 1 mL using the mobile phase. Then the residue was filtered through a 0.45-µm filter membrane (p/n 5185-5836) and analyzed.

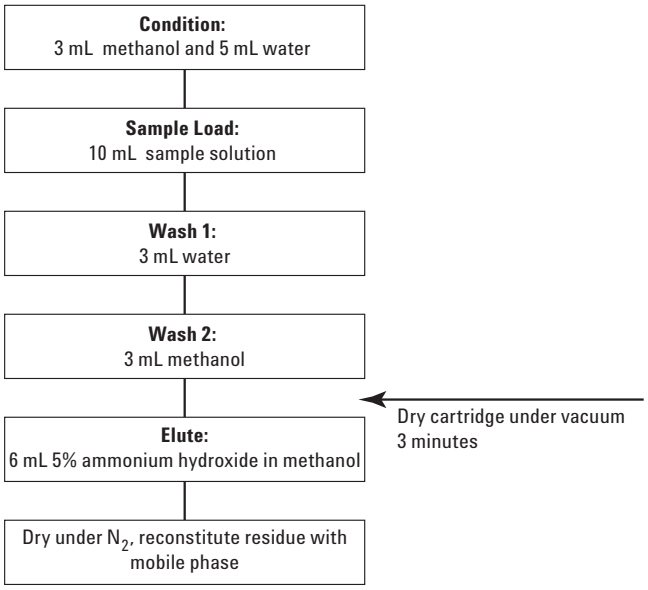


Figure 1. Melamine SPE procedure.

HPLC Conditions

Samples were analyzed on an Agilent 1200 Series HPLC with a diode array detector.

Column:	Agilent ZORBAX SB-C8 5 µm, 250 mm × 4.6 mm id (p/n 880975-906)
Flow rate:	1.0 mL/min
Column temperature:	40 °C
Detector wavelength:	240 nm
Injection volume:	20 µL
Mobile phase:	Acetonitrile-Buffer (15:85)
Buffer:	10 mmol/L citric acid and 10 mmol/L sodium octanesulfonate solution with a pH 3.0
Chromatography:	Isocratic

Results and Discussion

Linearity, Limit of Detection

Working standards were prepared at concentrations of 1, 5, 10, and 20 mg/kg by dilution of the stock solution with mobile phase. Linear regressions were calculated for melamine using the areas and the solution concentrations. The limit of detection (LOD) was the injection concentration whose signal-to-noise ratio was between 2 and 3. The linear range was between 1 and 20 mg/kg. The linearity and LOD results are shown in Table 1. The analysis of matrix blanks spiked with melamine shows no difference in area compared to solution based standards.

Table 1. Linearity and LOD of Melamine

Compound	Regression equation	Correlation coefficient	LOD (µg/kg)
Melamine	Y = 77.4698x + 0.2117	0.9999	10

Recovery and Reproducibility

The precision of the method is expressed as recovery of spiked melamine standard in milk or egg at concentrations of 2, 5, and 10 mg/kg levels. The analysis was performed in replicates of six at each level. Concentrations in the samples were calculated based on the external standard calibration curves. The chromatograms of the blank and spiked standard (2 mg/kg) are shown in Figures 2 to 5. The recovery and reproducibility data are shown in Table 2.

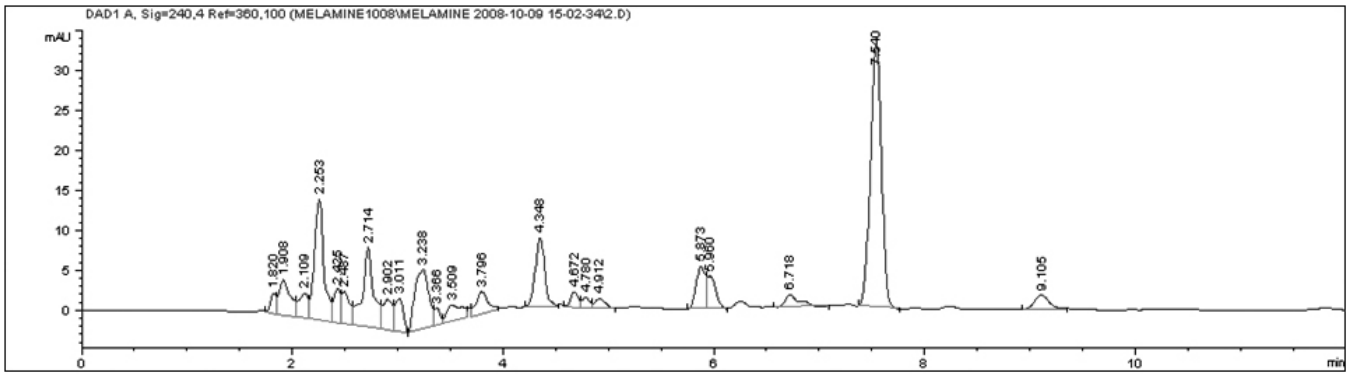


Figure 2. Chromatogram of a milk powder blank.

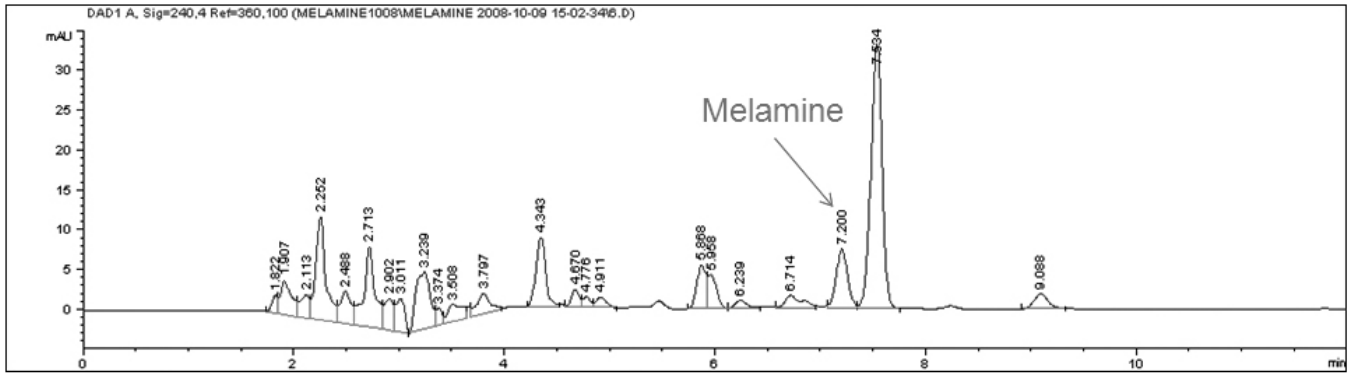


Figure 3. Chromatogram of a milk powder sample spiked at 2 mg/kg.

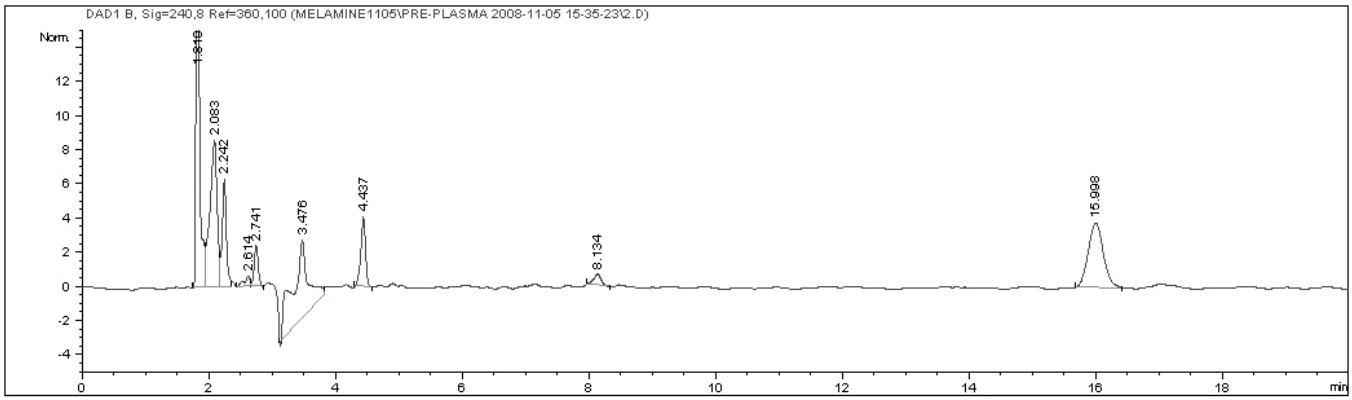


Figure 4. Chromatogram of an egg blank.

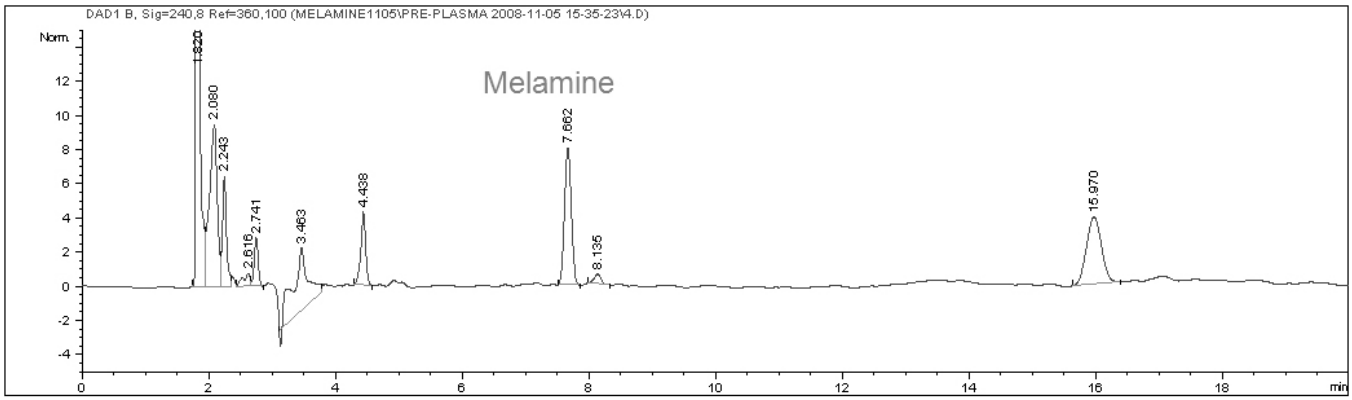


Figure 5. Chromatogram of an egg sample spiked at 2 mg/kg.

Table 2. Recoveries and Relative Standard Deviations of Melamine in Milk Powder and Egg Using Agilent SampliQ SCX SPE

Compound	Sample	Spiked level (mg/kg)	Recovery (%)	RSD (%)
Melamine	Milk powder	2	84.5	2.83
		5	85.3	2.56
		10	86.7	1.18
	Egg	2	95.2	3.00
		5	93.0	2.01
		10	95.7	2.89

Conclusions

Agilent SampliQ SCX solid phase extraction provides an effective single cartridge method for the purification and enrichment of melamine in milk powder and egg. The method is simple and complements any analytical procedure, such as LC/MS/MS or GC/MS. The recovery and reproducibility results, based on solution standards, are acceptable for melamine residue determination in milk powder and egg under the Chinese regulation. The impurities from milk and egg were minimal and did not interfere with the detection of melamine. The LOD (0.01 mg/kg) of melamine was significantly lower than the MRL (2 mg/kg).

Reference

GB/T 22388-2008, Determination of Melamine in Raw Milk and Dairy Products

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Quantitative Liquid Chromatography Analysis of Melamine in Dairy Products Using Agilent's 1120 Compact LC and 1200 Rapid Resolution LC

Application Note

Food Safety

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Abstract

In this application, three different LC methods are developed for the determination of melamine in dairy products. The first is a modification of the U.S. FDA method [1]. An Agilent LC system (1120 or 1200) is used with a ZORBAX SB-C8 LC column to run in reversed-phase ion-pair mode for routine quantitation of melamine. The second method is targeted for high throughput using an Agilent Rapid Resolution LC (RRLC) system (1200SL) to speed melamine analysis by more than three times with a Rapid Resolution High Throughput (RRHT) column. The third is an alternative ion-exchange LC method where a ZORBAX 300SCX column is employed to successfully retain melamine using a simple mobile phase of buffered water/acetonitrile without the presence of ion-pair reagent. Due to the complexity of dairy product matrices, a cleanup step using solid phase extraction (SPE) is required for the above methods. The Agilent SampliQ SCX, a mixed-mode polymer SPE cartridge with combined reversed-phase and strong cation exchange properties, is used to successfully remove matrix interferences.

Agilent Technologies

Introduction

In March 2007, imported pet food ingredients contaminated with melamine caused renal failure in dogs and cats across the United States. Once again, this compound is in the news as an illicit adulterant in milk and milk products. The same contaminant is now being detected in other food products that contain milk imported from China and as global concern rises, widespread testing for melamine is proceeding.

The published analytical approaches include LC, LC/MS, and GC/MS. The LC method is being used for quantitative analyses of melamine. Liquid chromatographic separation of this small polar compound can be achieved by reversed-phase ion-pair liquid chromatography. The U.S. FDA developed this methodology for melamine in pet food in 2007. With a slight modification of the proportion of mobile phase, the method can be successfully applied to separate melamine from a variety of dairy product matrices.

The disadvantage of conventional HPLC is time and solvent consumption. The Agilent 1200 Series Rapid Resolution LC system is designed for highest throughput without loss of resolution or with better resolution in combination with the Agilent ZORBAX RRHT columns. In this application note, the conventional HPLC method is transferred from a 4.6 mm × 250 mm, 5 µm ZORBAX SB-C8 column to a 4.6 mm × 50 mm, 1.8 µm RRHT ZORBAX SB-C8 column with equivalent results, and the LC run time is shortened from almost 20 minutes to 6 minutes.

An alternative approach for liquid chromatographic separation of this small polar compound is ion exchange chromatography. Agilent ZORBAX 300SCX is an ionic bonded-phase column packing used for cation exchange high-performance liquid chromatography. This packing consists of an aromatic sulfonic acid moiety covalently bonded to ZORBAX porous silica. This column is successfully applied to retain melamine using a simple mobile phase of buffered water/acetonitrile without the presence of ion-pair reagent.

For complex dairy product matrices, it is necessary to remove interferences such as protein, sugar, and fat before LC injection. Solid-phase extraction (SPE) is a simple way to clean up the complex matrix extract. SampliQ is a new family of SPE cartridges from Agilent with a wide range of sorbent chemistries. Among this family, the mixed-mode SampliQ Strong Cation Exchange (SCX) cartridge is a sulfonic acid-modified divinyl benzene polymer with both ion exchange and reversed-phase retention properties. This makes the SampliQ SCX very effective for cleanup after solvent extraction.

Experimental

Standard Preparation

A stock solution of melamine at 1,000 µg/mL is prepared in methanol by sonication. Dilutions in mobile phase are made up at 0.05, 0.1, 0.5, 1.0, 5.0, 10.0, 50.0, and 100.0 µg/mL concentrations.

Sample Preparation

The sample preparation process is a modification of the China national standard [2].

Sample Extraction Procedure

For liquid milk, milk powder, yogurt, ice cream, and creamy candy samples:

- Weigh 2 ± 0.01 g of sample and add to a 50-mL centrifuge tube, add 15 mL of 5% trichloroacetic acid in water and 5 mL of acetonitrile, then cap.
- Sonicate for 10 min and then place samples on vertical shaker for 10 min. Centrifuge for 10 min at 4000 rpm.
- Wet filter paper with 5% trichloroacetic acid in water, then filter the supernatant into a 25.0-mL volumetric flask, and bring to volume with 5% trichloroacetic acid in water.
- Transfer a 5.0-mL aliquot of the extract into a glass tube, and then add 5.0 mL purified water. Vortex to mix thoroughly.

For cheese, cream, and chocolate samples:

- Weigh 2 ± 0.01 g of sample, grind with 8~12 g of sea sand in a mortar, and then transfer into a 50-mL centrifuge tube.
- Wash the used mortar with 5 mL of 5% trichloroacetic acid in water three times, transfer washings into a 50-mL centrifuge tube, and then add 5 mL of acetonitrile.
- Proceed with the sonication and other steps as described in the previous preocedure..
- If the sample is very fatty, defat the extract using liquid-liquid extraction with hexane saturated with 5% trichloroacetic acid in water before cleanup by SPE.

Sample Cleanup Procedure

A SampliQ SCX SPE cartridge (p/n 5982-3236, 3 mL, 60 mg, or p/n 5982-3267, 6 mL, 150 mg) can be used to clean up sample extracts; the latter is used in this application note. All SPE elution steps, including conditioning, sample load, washing, and the final elution, are performed with a flow rate of less than 1 mL/min except for drying the cartridge by applying vacuum.

- Condition the SPE cartridge with 5 mL of methanol followed by 6 mL of water.
- Load the above sample extract to the conditioned cartridge. Wash the cartridge with 5 mL of water followed by 5 mL of methanol.
- Dry the cartridge by applying vacuum, and then elute with 5 mL of 5% ammonium hydroxide in methanol.
- Evaporate the eluate to dryness under a stream of nitrogen at approximately 50 °C.
- Reconstitute the dried extract in 1.0 mL of mobile phase, vortex for 1 min, and filter through a 0.2-µm regenerated cellulose membrane filter (p/n 5064-8222) into a glass LC vial.

Instrumentation and Conditions

Conventional HPLC method using 1120 Compact LC or 1200 LC:

- Agilent 1120 Compact LC system with gradient pump (degasser inside), autosampler, column compartment, and variable wavelength detector (VWD) or equivalent 1200 Series components
- EZChrom Elite Compact software or ChemStation software (Ver. B.04.01 or later)

Column	ZORBAX SB-C8 (also known as ZORBAX Rx-C8), 4.6 mm × 250 mm, 5 µm (p/n 880975-906)
Buffer	10 mM citric acid, 10 mM sodium octane sulfonate, adjusted to pH 3.0
Mobile phase	92:8 buffer:acetonitrile
Flow rate	1.5 mL/min
Injection volume	20 µL
Column temperature	30 °C
Detection wavelength	240 nm
Run time	20 min

High-Throughput Method Using 1200SL RRLC:

- Agilent 1200SL Series binary pump, degasser, wellplate sampler, thermostatted column compartment and diode array detector (DAD)
- ChemStation software (Ver. B.04.01 or later)

Column	ZORBAX SB-C8 RRHT, 4.6 mm × 50 mm, 1.8 µm (p/n 827975-906)
Buffer	10 mM citric acid, 10 mM sodium octane sulfonate, adjusted to pH 3.0
Mobile phase	92:8 buffer:acetonitrile
Flow rate	1.5 mL/min
Injection volume	8 µL
Column temperature	30 °C
Detection wavelength	240 nm
Run time	6 min

Ion Exchange Chromatography Method with 1120 Compact LC or 1200 LC:

- Agilent 1200 Series binary pump, degasser, wellplate sampler, thermostatted column compartment and variable wavelength detector (VWD) or equivalent 1120 Series components
- EZChrom Elite Compact software or ChemStation software (Ver. B.04.01 or later)

Column	ZORBAX 300SCX, 4.6 mm × 150 mm, 5 µm (p/n 883952-704)
Buffer	50 mM ammonium formate solution, adjust to pH 3.0 with formic acid
Mobile phase	15:85 buffer:acetonitrile
Flow rate	1.0 mL/min
Injection volume	10 µL
Column temperature	30 °C
Detection wavelength	240 nm
Run time	5.5 min

Results and Discussion

Separation of Melamine in Dairy Products by Reversed-Phase Ion-Pair LC

Melamine is not retained by reversed-phase LC and thus elutes with the solvent and unretained matrix interferences. However, using an ion-pairing reagent with reversed-phase chromatography, melamine can be well retained and separated from interferences. Figure 1 (a) is the chromatogram of melamine standard by reversed-phase ion-pair LC. Figure 1 (b) is the chromatogram of a positive yogurt sample after cleanup with the Agilent SampliQ SCX SPE cartridge.

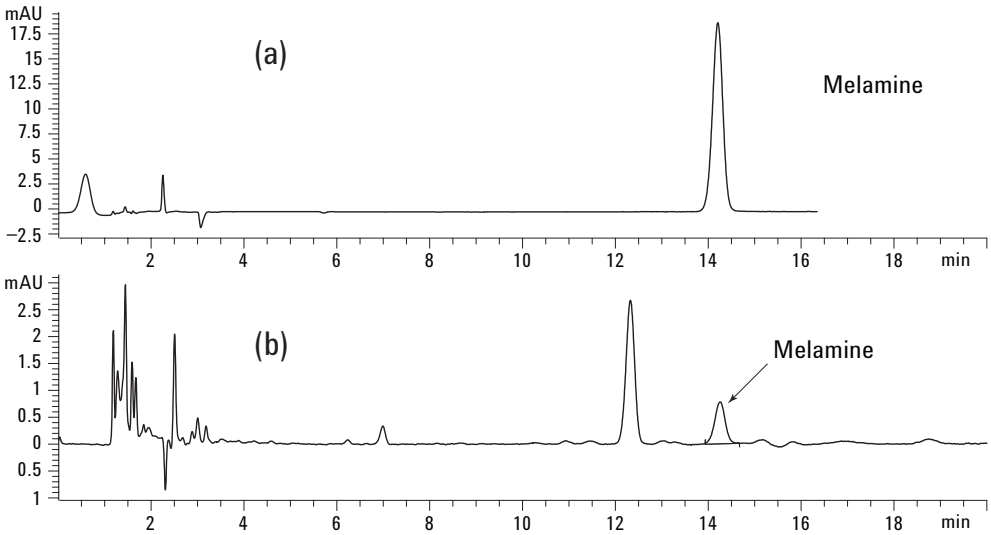


Figure 1. Separation of (a) 20 µg/mL melamine standard, and (b) positive yogurt sample after cleanup by SampliQ SCX SPE cartridge. Retention time of melamine is 14.2 minutes.

High-Throughput Analysis by Agilent 1200SL RRLC with RRHT Column

With the Agilent 1200 Series RRLC system, high throughput is possible. In combination with the Agilent ZORBAX RRHT columns, excellent chromatographic resolution can be achieved at much shorter run times than with a conventional LC system. A RRLC method is developed to dramatically increase the sample throughput for the determination of melamine in dairy products. Figure 2 (a) is the chromatogram of a melamine standard by the RRLC method with the retention time of melamine at 2.8 minutes.

Figure 2 (b) is the chromatogram of the same yogurt sample in Figure 1 (b). In order to make sure that the column is clear for the next injection, the total run time is extended to 6 minutes. The high throughput RRLC method is applied in the variety of dairy products matrices, including yogurt, liquid milk, and milk powder to demonstrate that the same resolution is achieved as with the conventional HPLC method. The calibration curve for the RRLC method is shown in Figure 3. The calibration includes 0.8, 2.0, 20.0, 40.0, and 80.0 µg/mL. The instrumental LOD (limit of detection) of the RRLC method is 0.03 µg/mL.

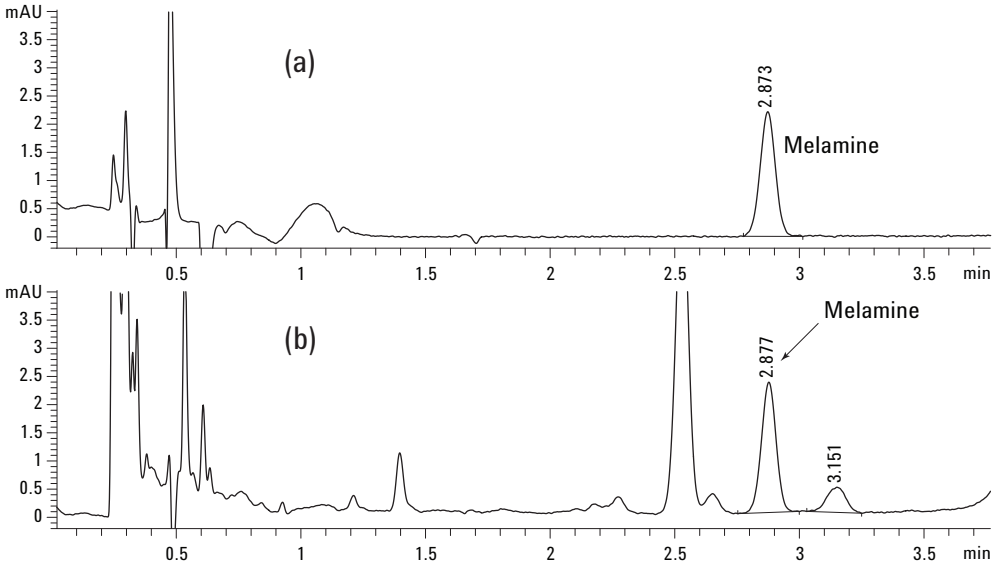


Figure 2. Separation of (a) 0.8 µg/mL melamine standard, and (b) positive yogurt sample after cleanup by SampliQ SCX SPE cartridge. Retention time of melamine is 2.8 minutes.

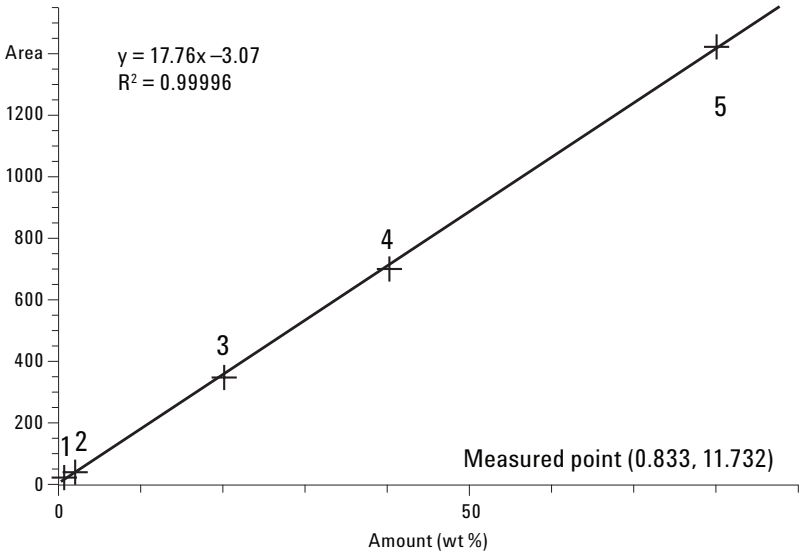


Figure 3. Calibration curve of RRLC method.

Ion Exchange Chromatographic Method

An alternative to ion-pair reversed-phase chromatography for melamine is ion exchange chromatography (IEC). The Agilent ZORBAX 300SCX is used for cation exchange high-performance liquid chromatography (HPLC). This column is employed to separate melamine in dairy product matrices with sufficient retention to separate matrix interferences. Figure 4 shows the separation of melamine from interferences without the SPE cleanup step. Generally, the total run time of the ion exchange chromatography is only 5.5 minutes with an LOD of 0.05 µg/mL, as shown in Figure 5. The calibration curve for the IEC method is shown in Figure 6. The calibration points include 0.5, 1.0, 5.0, 10, 50, and 100 µg/mL. Although the separation is shown to be interference free for raw milk and liquid milk without any additive, it is still recommended that the cleanup step be included to ensure robust methodology for running many samples and samples of different matrices.

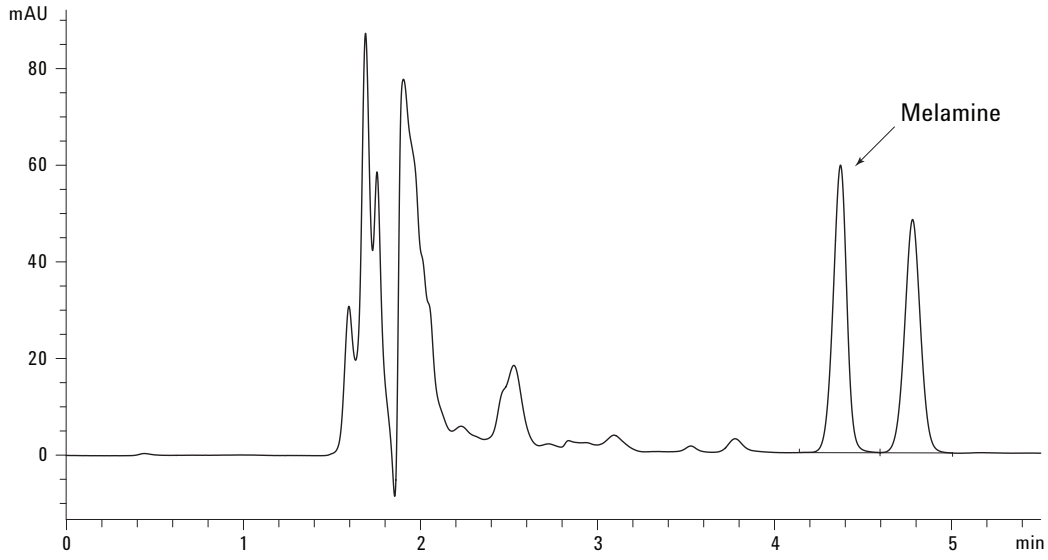


Figure 4. Separation of melamine in milk powder sample by IEC without cleanup by SPE.

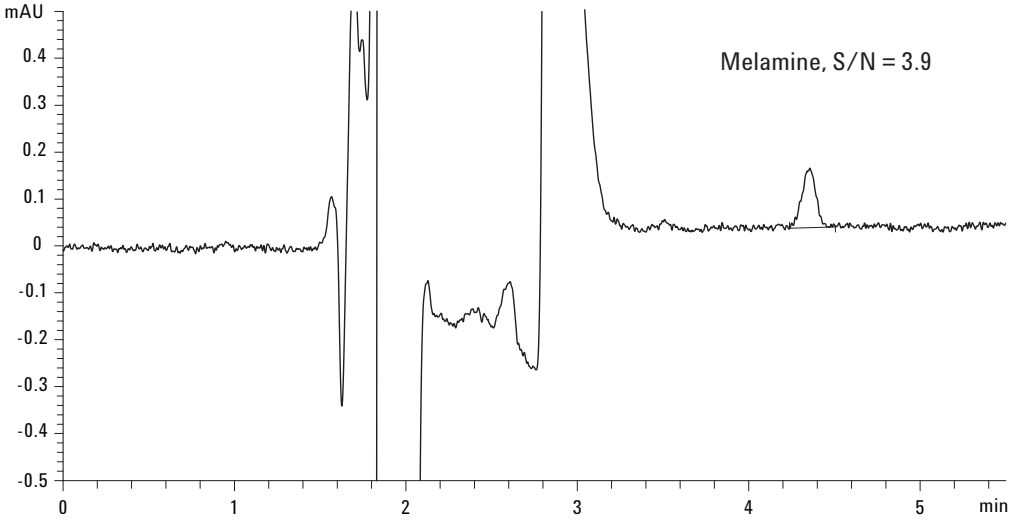


Figure 5. Limit of detection (LOD) for melamine at the concentration of 0.05 µg/mL.

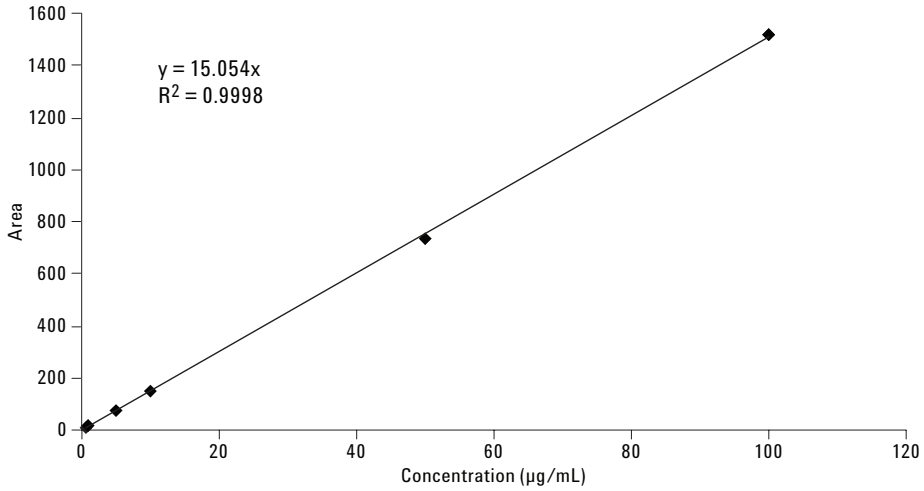


Figure 6. Calibration curve of IEC method.

Conclusions

Three approaches are described in this application note; the first is a reversed-phase ion-pair LC method employing Agilent 1120 compact LC or 1200 HPLC with an SB-C8 column. The second is a high-throughput method, which reduces the LC run time from 20 minutes to 6 minutes using the Agilent 1200 RRLC with the ZORBAX RRHT SB-C8 column. The last is an IEC method using the Agilent ZORBAX 300SCX column. Each successfully separates melamine from matrix interferences and provides identification by retention time and quantitative results. The results of this study, including sample cleanup with SampliQ SCX SPE cartridges and the three separation approaches, show that a complete solution from Agilent for the determination of melamine in dairy products is provided. The reversed-phase ion-pair method is based on the FDA and China national standards. However, the IEC method is simple, quick, sensitive, and robust. With this method, melamine can be successfully retained using a simple mobile phase without the presence of an ion-pair reagent.

References

1. Updated FCC Developmental Melamine Quantitation (HPLC-UV), April 2, 2007
2. GB/T 22388-2008 Determination of melamine in raw milk and dairy products, October 7, 2008

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A Total Solution for the Analysis of Melamine and Cyanuric Acid in Pet Food by GC/MS and Aqueous Normal-Phase LC/MS/MS

Application

Food

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Abstract

The Agilent 7890A-5975C GC/MS and 1200SL-6410 Triple Quadrupole LC/MS are used to analyze melamine and cyanuric acid in pet food and related raw material. The GC/MS method employs extraction and then trimethylsilyl derivatization and is used here for screening. The detection limit is 10 µg/g. The LC/MS/MS method requires only extraction and then a simple isocratic elution on a normal-phase silica column (Rx-Sil) with water and acetonitrile mobile phase containing 5 mM ammonium acetate. The separation of the two compounds takes 5 min. The linear range is from 50 pg/mL to 50 ng/mL for melamine and from 1 ng/mL to 100 ng/mL for cyanuric acid. The LC/MS/MS method is demonstrated by analysis of the target compounds in complex food matrix.

Introduction

The illicit additives melamine and cyanuric acid in pet food have caused the death of cats and dogs. This resulted in the U.S. FDA recalling millions of packages of pet food in the past year. Consequently, it became a hot issue to determine the content of melamine and cyanuric acid in pet food and related raw materials worldwide.

A screening method using GC/MS for both these compounds was published by the U.S. FDA [1]. According to this procedure, the detection limit for GC/MS screening method is 10 µg/g using a trimethylsilyl (TMS) derivatization process before injection. Although this method is used for screening, it could be used as well for confirmation and quantitation using the SIM/SCAN function of the Agilent 5975. In comparison with the GC/MS method, the LC/MS/MS method presented here simplifies the sample preparation process without derivatization and provides confirmation and quantitation in one step. Additionally, the high sensitivity and selectivity of LC/MS/MS could cover trace-level analysis in animal fluids and tissue to high levels in food and food ingredients.

Experimental

Sample Preparation

Sample preparation for GC/MS was done as per the FDA method [1]. To a 0.5-g sample, a mixed solvent (10:40:50 diethylamine:water:acetonitrile) is added and mixed well to thoroughly wet the entire sample. The mixture is then sonicated for 10 min, centrifuged at 5000 rpm for 10 min, and then filtered into a vial using a 0.45 µm nylon disc. Derivatization is done by transferring 200 µL of the extract to an autosampler vial and evaporating to dryness with a low flow of dry nitrogen at 70 °C. To the dried extract is added 200 µL pyri-

dine and 200 µL N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA). The mixture is vortexed and allowed to incubate at 70 °C for 45 min.

Sample preparation for LC/MS/MS was done by grinding about 5 g sample by a mill and mixing thoroughly. To a 0.5-g sample, 20 mL of acetonitrile/ water (50:50) is added, capped, and vortexed. The solution is sonicated for 30 minutes and then filtered with a 0.20-µm PTFE syringe filter (Agilent P/N 5185-5843). If the concentration of the target compounds is beyond the linear range of the method, further dilution of the extract with acetonitrile:water should be made. Note that if cyanuric acid and melamine are in the same solution at high concentrations, 10:40:50 diethylamine: water:acetonitrile is needed to disrupt the ion pair.

GC/MS Method Details

Instrument:	7890A GC with 5975C MSD
Software:	MSD ChemStation E.01.00 with NIST 05a MS Library 2.0d

GC Conditions

Column:	DB-5MS 30 m × 0.25 mm × 0.25 µm (P/N: 122-5532)
Inlet temperature:	290 °C
Injection mode:	Splitless or split (1:20)
Injection volume:	1 µL
Carrier gas flow:	He (constant flow) at 1.3 mL/min
Oven program:	75 °C (hold 1 minute) to 300 °C at 30 °C /minute (hold 2 min)
Total run time:	10.5 min.
Transfer line temperature:	280 °C

MS Conditions (SIM/Scan Mode)

Tune:	Autotune
Acquisition mode:	EI; SIM/scan mode
Solvent delay:	3.5 minutes
MS temperature:	230 °C (Source); 150 °C (Quad)

Scan Parameters

Scan range:	40–450 amu
Sampling rate:	2 (scan rate at ~3.6 scans/sec)
Threshold:	100

SIM Parameters

GROUP 1

Group identification:	Auto_1							
Resolution:	Low							
Plot 1 Ion:	345.10							
Ions/dwell in group:	Mass	Dwell	Mass	Dwell	Mass	Dwell		
	188.00	25	330.10	25	345.10	25		

GROUP 2

Group identification:	Auto_2							
Resolution:	Low							
Group start time:	6.76							
Plot 1 ion:	327.20							
Ions/dwell in group:	Mass	Dwell	Mass	Dwell	Mass	Dwell	Mass	Dwell
	197.00	25	285.10	25	327.20	25	342.20	25

LC/MS Method Details

Instrument:	1200 SL RRLC with 6410 Triple Quad MS
Software:	MassHunter B.01.03

LC Conditions

Column:	ZORBAX Rx-Sil, 2.1 mm × 150 mm, 5 µm (P/N 883700-901)
Column temperature:	40 °C
Mobile phase:	A = 5 mM ammonium acetate in water B = 5 mM ammonium acetate in acetonitrile
Flow rate:	0.4 mL/min
Injection volume:	10 µL
Isocratic:	95% B
Stop time:	5.5 minutes
Needle wash:	50:50 acetonitrile/water; flush port 10 seconds

MS Conditions

Ion source:	ESI
Polarity:	Positive and negative
Nebulizer gas:	Nitrogen
Ion spray voltage:	4000 V
Source temperature:	350 °C
Resolution:	Q1 (unit) Q3 (unit)
Scan mode:	Multiple reaction monitoring (MRM)
Segment:	Segment 1 = 0~2 min negative mode for cyanuric acid Segment 2 = 2~5.5 min positive mode for melamine
Delta EMV:	600 V

Parameters of multiple reaction monitoring (MRM) are shown in Table 1.

Table 1. MRM Parameters for Cyanuric Acid and Melamine

Time	Compound	Precursor	Product	Dwell (ms)	Fragmentor (V)	Collision Energy (V)
1.45	Cyanuric acid	128	42	200	100	30
		128	85	200	100	5
4.92	Melamine	127	85	200	100	20
		127	68	200	100	35

Results and Discussion

The Selection of Monitoring Ions for SIM Mode in GC/MS

Selective ion monitoring (SIM) is often used to improve the detection limit and quantitative reproducibility. With SIM mode, the MS monitors only a few ions for each target compound within the retention time (RT) range that the target elutes from the column. By monitoring only a few specific ions, the signal-to-noise ratio (S/N) improves significantly. Therefore, the selection of the ions monitored for SIM mode is very important for good analytical result. Usually, a few ions of highest abundance are chosen for SIM to maximize the response of the target compounds. However, if the interference from the matrix is significant, the most characteristic ions should be chosen to minimize signal disturbance even if their abundance is relatively lower. In this application, the influence of ion selection was demonstrated in Figure 1. With the use of more characteristic ions, the chromatogram has much less interference than the chromatogram with the highest abundance ions. The use of SIM/SCAN allows both the highest sensitivity and reproducibility with the additional benefit of full identification contained in the complete spectra of the analytes.

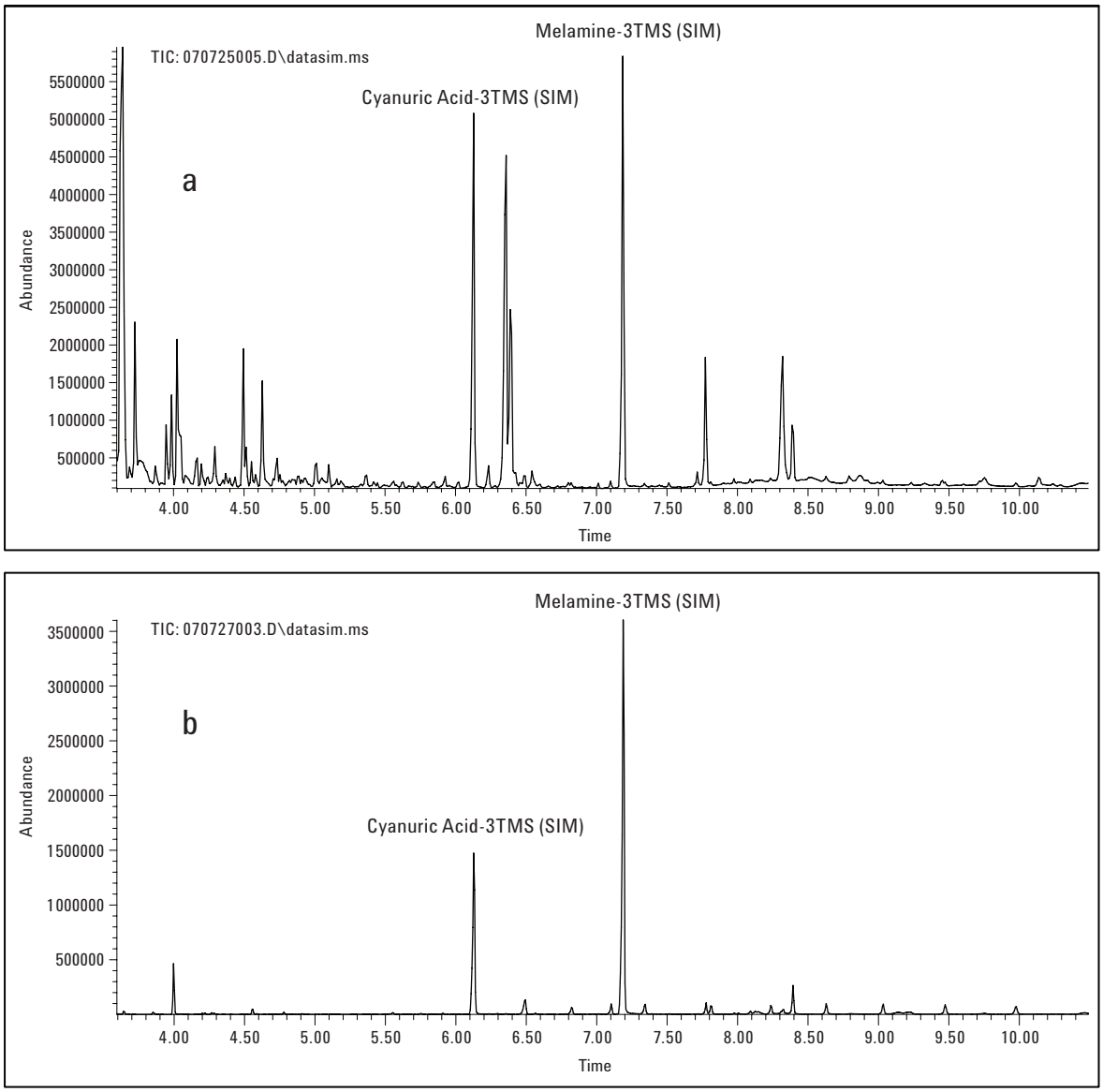


Figure 1. The influence of the selection of the ions monitored in SIM mode shown with spiked dry cat food; a) the ions of highest abundance were used (cyanuric acid: *m/z* 73, 147, 171; melamine: *m/z* 327, 330, 342, 345) and b) characteristic ions were used (cyanuric acid: *m/z* 188, 330, 345; melamine: *m/z* 197, 285, 327, 342).

The Retention of Melamine and Cyanuric Acid on a Normal-Phase Column

Liquid chromatographic separation of small highly polar compounds like melamine and cyanuric acid can be achieved by retention mechanisms such as ion-exchange and ion-pair reversed-phase liquid chromatography (IP-RPLC). In general, the ion-exchange approach is not very suited to the electrospray interface (ESI) due to the use of buffers with high ionic strength. Likewise, volatile ion-pair reagents can provide satisfactory results, but again are not well suited for routine use with LC/MS. In addition, ion pair separation of both an acid and a base in the same analysis is difficult.

A much more suitable approach for these compounds is known as aqueous normal-phase chromatography or hydrophilic interaction chromatography (HILIC) [2]. This mode of separation is defined as a partition of a polar analyte between a relatively nonpolar mobile phase and a polar semi-immobilized liquid phase associated with the stationary phase. Normal phase chromatography is usually associated with adsorption of the analyte on the stationary phase. It is believed that in this mode water is trapped on the surface of the stationary phase and evokes partitioning instead of adsorption. Both cyanuric acid and melamine can be successfully retained by the Rx-Sil column with 1.45 minutes and 4.92 minutes of retention time, respectively, under these conditions.

Quantitative Analysis of Melamine and Cyanuric Acid in Different Matrices

A time segment program was used to switch between the negative mode for cyanuric acid and the positive mode for melamine. Linearity of the developed method was studied by three replicate injections at each concentration level. The calibration curves of melamine and cyanuric acid are displayed in Figure 2. The linear range of the method is 50 pg/mL to 50 ng/mL for melamine and 1 ng/mL to 100 ng/mL for cyanuric acid. The detection limits for melamine and cyanuric acid are shown in Figure 3. The S/N ratio (peak-to-peak) is 12.7 for 50 pg/mL melamine and 12.8 for 1 ng/mL cyanuric acid.

Pet food, wheat gluten, corn meal, and rice protein samples were extracted and analyzed for both target compounds. Examples of a spiked wheat gluten sample are given in Figure 4. A mixture of acetonitrile and water (50:50) is most efficient for the extraction of melamine and cyanuric acid from the different matrices. When injecting 10 µL of sample, the extract should be diluted to 5% or less water or the peaks will be distorted. Otherwise, reducing the injection volume to 1 µL is the easiest way to eliminate the distortion.

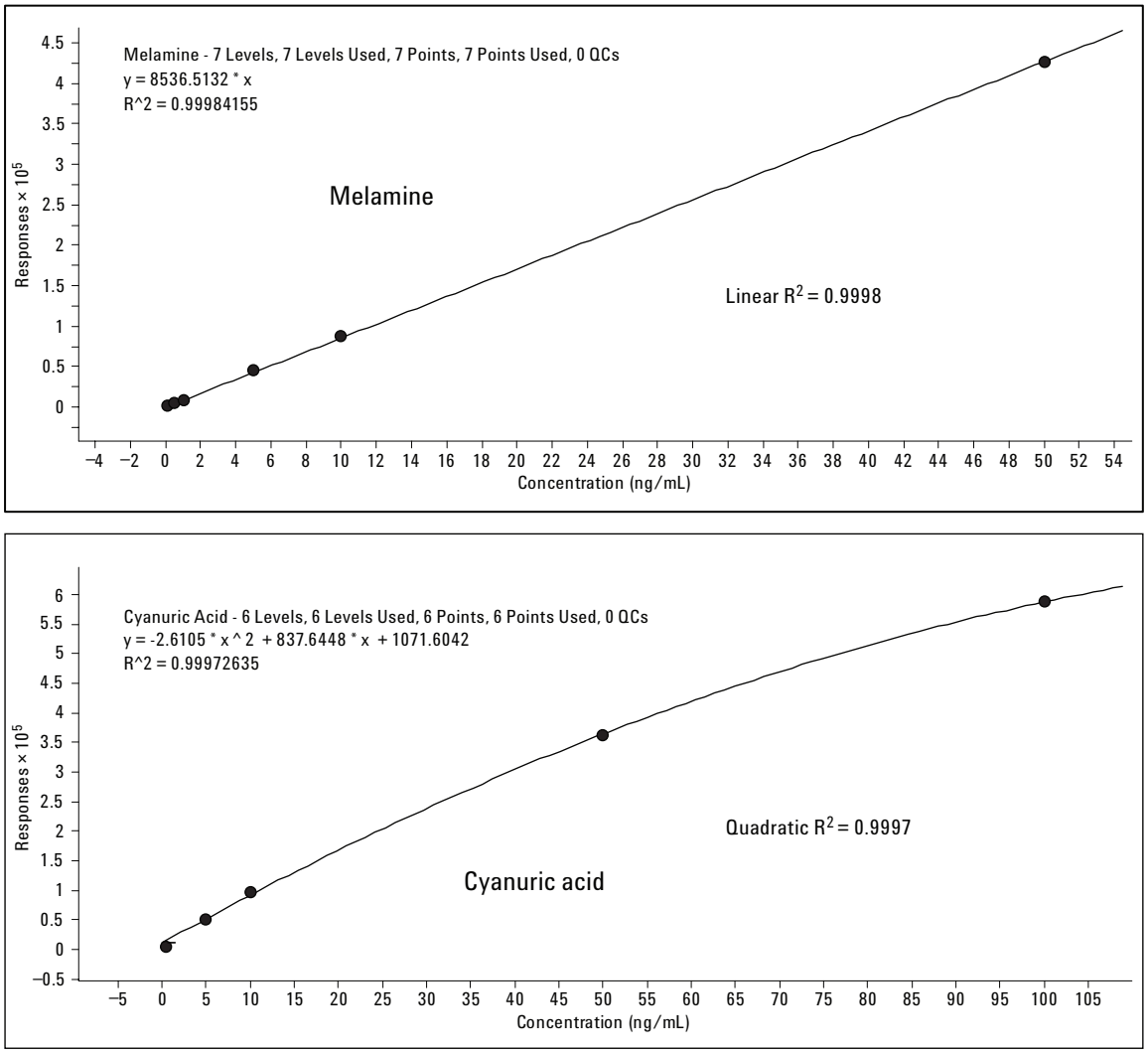


Figure 2. Linearity of melamine and cyanuric acid.

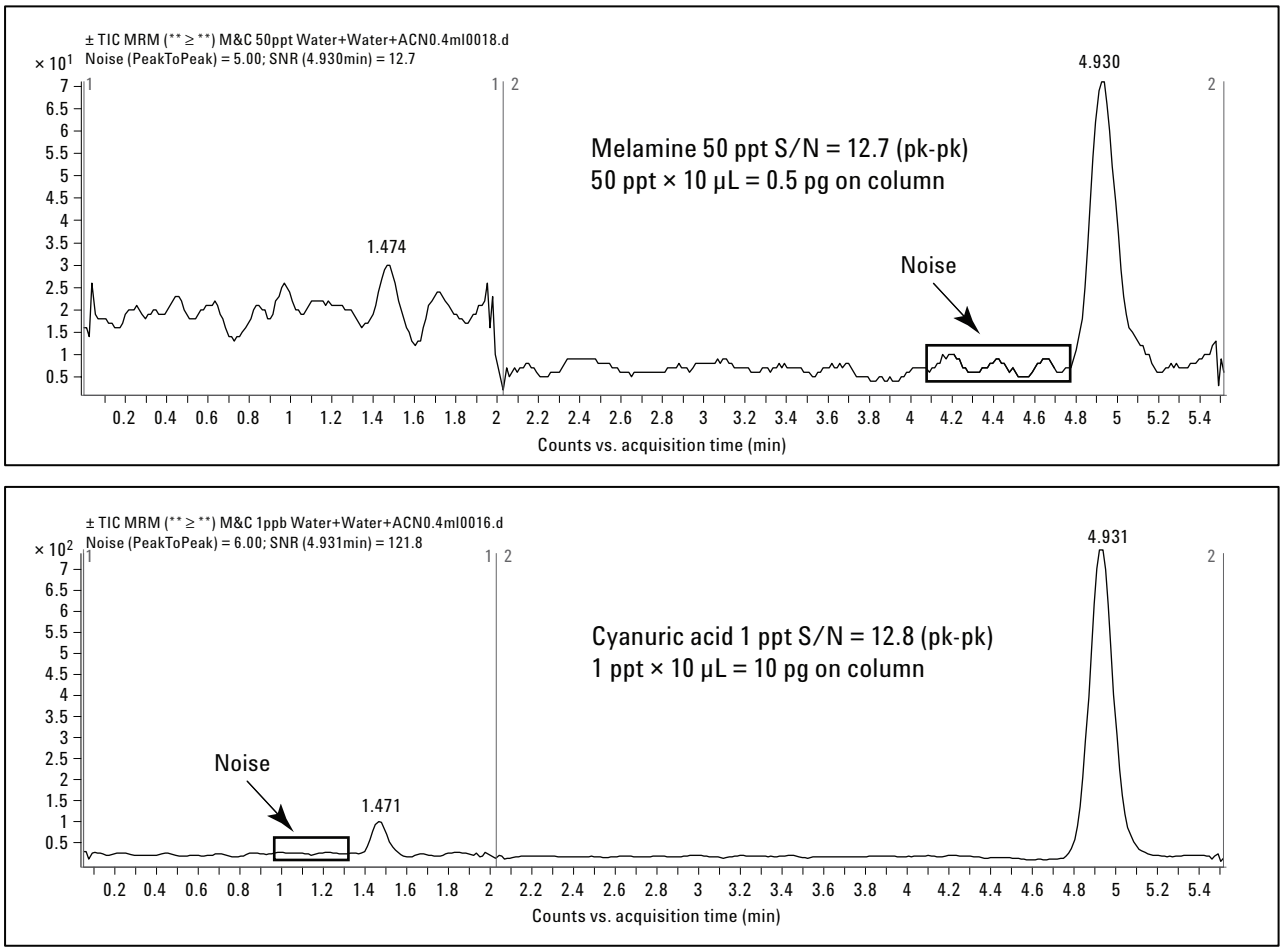


Figure 3. Detection limits for melamine and cyanuric acid.

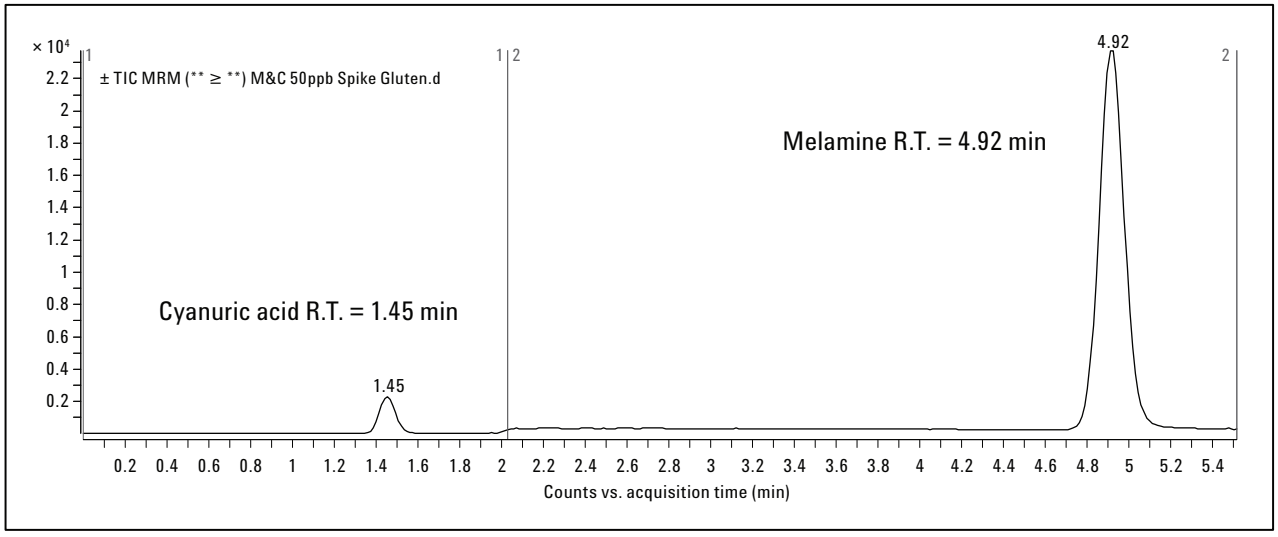


Figure 4. Total reaction monitoring chromatogram of spiked wheat gluten sample.

Conclusions

This application demonstrates that GC/MS or LC/MS/MS methods can readily be used for the analysis of melamine and cyanuric acid in pet food and its ingredients. In the example shown, the GC/MS method was used to screen the presence of melamine and cyanuric acid in pet food using SIM mode. However, the method can be used for quantitation and confirmation using SIM/SCAN so long as very low detection levels are not required. In addition, an LC/MS/MS method has been shown with both a simple sample preparation procedure and an aqueous normal-phase separation. The method is highly sensitive and selective and is used both for the confirmation and quantitation of melamine and cyanuric acid in pet food and related raw materials. The detection limits of 50 pg/mL for melamine and 1 ng/mL for cyanuric acid would readily allow for the analysis of these compounds at trace levels in animal fluids and tissues.

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Rapid Screening of Melamine and Cyanuric Acid in Milk Products Using Agilent J&W HP-5ms GC Column and Agilent 7890A/5975C GC/MSD with Column Backflushing

Application Note

Food Safety

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Abstract

A rapid screening method for the determination of melamine and cyanuric acid in milk products is developed using Agilent 7890A/5975C GC/MSD along with Agilent J&W HP-5ms GC columns. With the backflushing by capillary flow technologies, this method eliminates the time consumption for column bakeout after elution of target compounds, so as to significantly shorten the GC run time from more than 70 minutes to 14.5 minutes. Good linearity was obtained within the range of 10 to 200 µg/g, with correlation coefficients greater than 0.9996. The recoveries of both target compounds are greater than 95%.

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Introduction

The contamination of food with melamine and cyanuric acid has attracted much attention all over the world. For the analysis of milk products, one of the major problems is the presence of less-volatile and nonvolatile matrix components, such as protein and fat. These components might contaminate the analytical system if the sample is introduced without selective sample preparation. The usual way to remove the matrix is to bake the column at high temperature, which often takes much longer than the sample run time for the analysis of interest. For example, the application of a milk extract of FDA method [1] usually takes about 70 minutes. Selective extraction or selective sample introduction is not easy, since the target compounds cover a broad volatility and polarity range. Moreover, for a routine QC analysis, laboratories want to reduce the typical cycle time by 25 to 60 minutes to improve productivity. The Agilent 7890A/5975C GC/MSD system meets this demand by using backflushing and faster cooling.

In this application note, a modified U.S. FDA method is developed using a standard split/splitless inlet and Agilent Capillary Flow Technology. A three-way splitter device with a makeup gas is placed at the end of the column and connected to MSD with a restrictor, thereby allowing column outlet pressure to be controlled with auxiliary electronic pneumatic control (EPC). By decreasing the column inlet pressure and increasing the column outlet pressure after the last peak of interest eluted from the column, the column flow is reversed, and the matrix interference, especially high boiler, can be removed out of the inlet end of the column. [2]

Experimental

Standards and Reagents

The standards and reagents used in the experiment are listed in Table 1. Stock solutions of melamine and cyanuric acid, each at a concentration of 1,000 µg/mL, were separately prepared in methanol. Stock solutions of standards are stored in the refrigerator.

Table 1. Standards and Reagents

Standard	Melamine	Sigma-Aldrich	>99% purity
	Cyanuric acid	Sigma-Aldrich	>99% purity
Solvent	Methanol	Fisher Scientific	HPLC grade
	Pyridine	Fisher Scientific	Certified A.C.S. Reagent
Silylating reagent	BSTFA with 1% TMCS*	Supelco	/

* BSTFA: bis(trimethylsilyl)trifluoroacetamide, TMCS: Trimethylchlorosilane

Instrument

The experiment was performed on an Agilent 7890A gas chromatograph equipped with a split/splitless capillary inlet, an Agilent 5975C GC/MSD with Triple-Axis Detector, and an Agilent 7683B automatic liquid sampler (ALS). The split/splitless inlet is fitted with a long-lifetime septum (P/N 5183-4761) and split injection liner (P/N 5188-4647). Injections are made using a 10-µL syringe (P/N 9301-0714). The instrument conditions are listed in Table 2.

Table 2. Gas Chromatograph and Mass Spectrometer Conditions

GC Conditions	
Column:	HP-5ms, 30 m × 0.25 mm × 0.25 µm (P/N 19091S-433)
Inlet temperature	EPC, split/splitless @ 250 °C
Injection volume	1 µL, split ratio at 3:1
Carrier gas	Helium, constant flow mode, 1.3 mL/min
Oven program	75 °C (1 min), 30 °C/min to 300 °C (1 min)
Post-run	280 °C, hold for 5 min (backflushing duration)
Transfer line	280 °C
MS Conditions	
MS	EI, SIM/scan
Solvent delay	4.2 min
MS temperature	230 °C (source), 150 °C (quad)
Scan mode	Mass range (40 to 450 amu)
SIM mode	Ion (melamine: 342, 327*, 171, 99; cyanuric acid: 345*, 330, 188)
Backflush Conditions	
Device	3-way splitter (P/N G3183B)
Restrictor	0.706 m × 180 µm id
Outlet	PCM (P/N G1530-63309)
Outlet pressure	2 psi (60 psi for post-run)
Inlet pressure	2 psi (for post-run)

* Quantitative ion

Sample Preparation

Extraction

0.5 g of sample (powder or liquid) was weighed into a 20-mL polypropylene centrifuge tube; 5 mL of methanol was added. The sample was capped, vortex mixed, and then sonicated for 10 minutes. After the sample was centrifuged at 4,000 rpm for 6 minutes, the supernatant fluid was filtered through a 0.45-µm PTFE filter into a glass GC vial.

Derivatization

40 µL of the above extract was transferred into a glass GC vial. The extract was evaporated to dryness under a stream of nitrogen at approximately 70°C. 50 µL of pyridine and 50 µL of BSTFA were added. The sample was vortex mixed and incubated at 70 °C for 30 minutes.

Results and Discussion

7890A/5975C GC/MSD with a Backflush System for Milk Extracts

Milk extract usually contains many low-volatile or nonvolatile constituents. These compounds may stay near the front of the column until the oven temperature is high enough to move them through the column. In this application, a three-way splitter with makeup gas was employed to perform the backflush. The device has makeup gas supply tubing and four connectors, one connector for the analytical column and three connectors for the restrictor tube connecting to three available detectors. Since only an MSD is used as a detector in this application, the first two connectors were capped in the direction of makeup flow, the third connector was for the column in, and the last connector for the restrictor out to the MSD. This flow configuratin was used to avoid peak broadening due to improper flow sweeping. The length and internal diameter of the restrictor tubing is 0.706 m and 0.18 mm, respectively. The schematics of the GC/MS system configuration and tubing connection for the three-way splitter are shown in Figure 1.

First, a milk extract was analyzed in a typical mode – without backflush – by programming the oven to 300 °C to ensure that late eluters were eluted. It took more than 70 minutes to elute all the constituents in the extract (Figure 2A). Then, the extract was analyzed with a 5-minute backflush (Figure 2B). The backflush was accomplished by increasing the pressure in the outlet and decreasing the inlet pressure. The column flow was reversed to push the "heavy" constituents through the column inlet and out of the split vent. Figure 2C shows a 70-minute blank solvent run after the backflush. The blank run shows that the column was clean after backflushing, except for some peaks coming from the vial septa. Instead of baking the column at 300 °C for 55 minutes, the heavy matrix components were effectively removed from the column through backflushing. This reduced the run time from 70 minutes to 14.5 minutes, or a 4.8-fold increase in speed.

For a complex matrix, even baking for a long time cannot thoroughly remove the high boiler, which may result in peak retention time shift in the following injection. In Figure 3, two consecutive runs of the extract with 5-minute backflushing are shown. Excellent retention time and peak area repeatability were obtained, with no evidence of carryover, no emerging ghost peaks, and no increasing baseline. This demonstrated

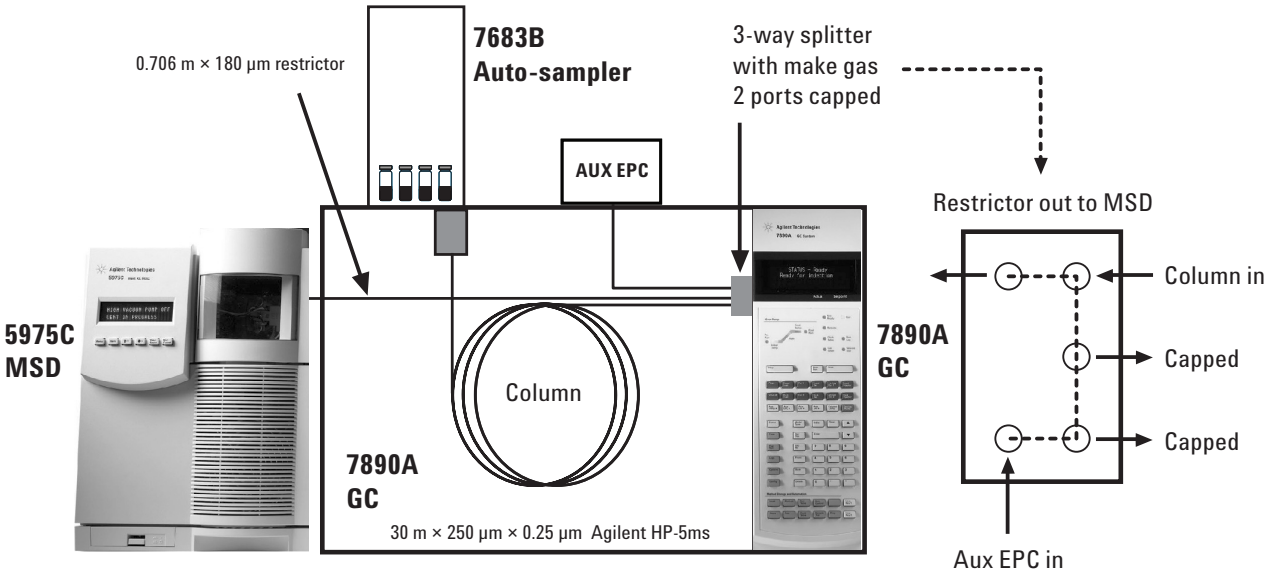


Figure 1. Schematics of GC/MS system configuration and tubing connection for three-way splitter.

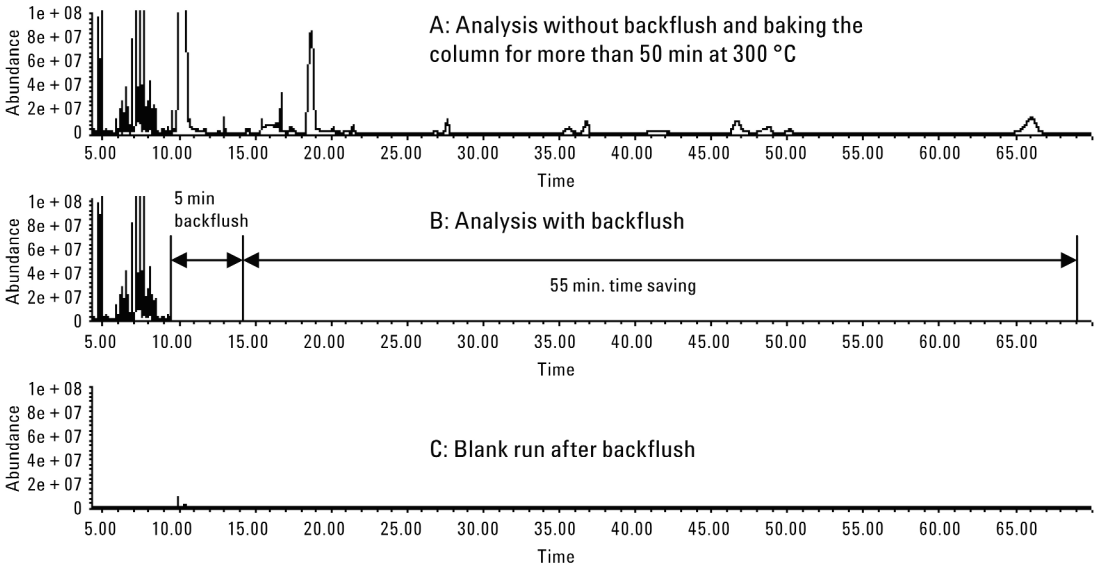


Figure 2. Time savings using backflush.

that backflushing is a perfect solution to avoid both high-temperature baking and retention time shift from run to run. Meanwhile, the faster oven cooling down capability of the Agilent 7890A allows for shorter cycle time. Additional time savings can be realized by using the three-way splitter used in this application, so that the liner and column can be changed without venting the MSD.

Figure 4 shows the total ion chromatograms (TIC) of a spiked milk powder sample. Synchronous SIM/scan was used to monitor ions of interest with high-sensitivity SIM mode and to simultaneously acquire library-searchable scan data in one run. This helped simplify the process of confirming positive or negative results. Figure 5 shows the mass spectra of cyanuric acid tri-TMS derivative (6.335 minutes) and melamine tri-TMS derivative (7.341 minutes), respectively.

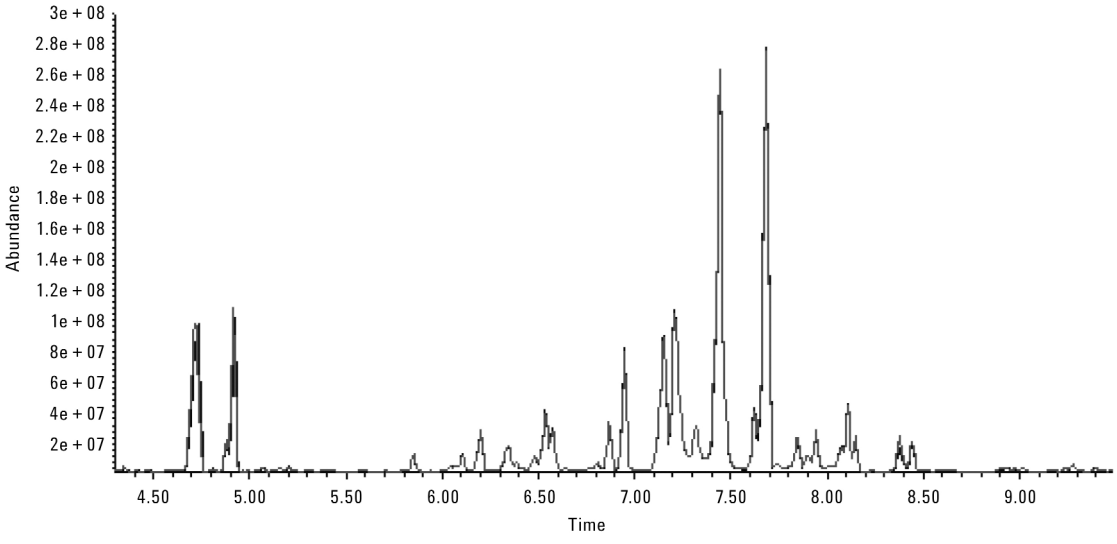


Figure 3. Overlay of total ion chromatogram spectra of two consecutive runs of a powdered infant formula extract.

Linearity and Recovery

A matrix blank milk sample is employed for a linearity experiment. 0.5 g of milk samples were spiked with four levels of cyanuric acid and melamine (10, 20, 80, and 200 µg/g). Excellent linearity was obtained for the two compounds within the range of 10 to 200 µg/g with a correlation coefficient higher than 0.9996.

To check the applicability of the method, a powdered infant formula (blank matrix) spiked with 40 µg/g of targeted analytes was analyzed. Excellent recoveries were obtained, with 96.1% for cyanuric acid and 95.6% for melamine (see Table 3).

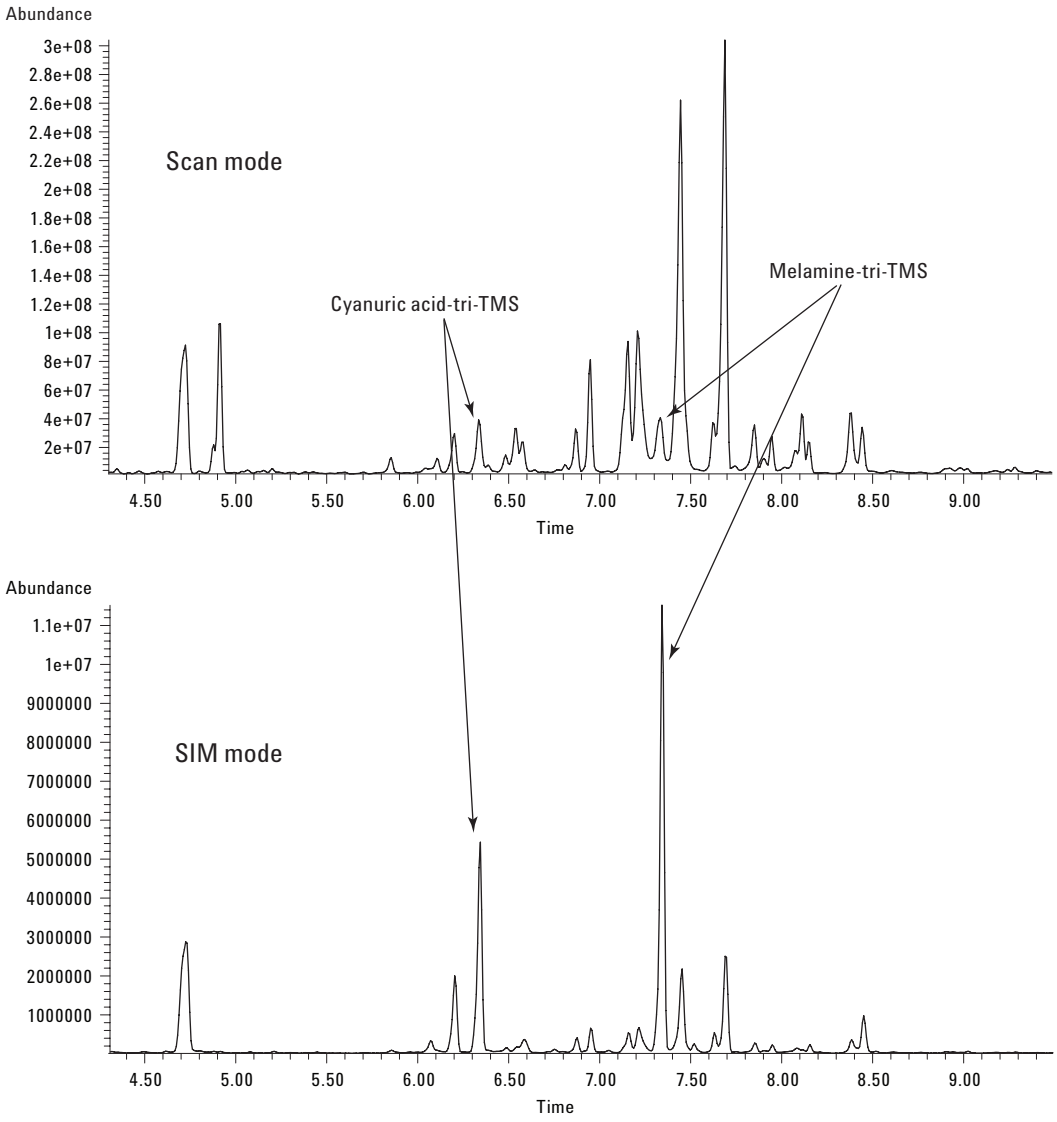


Figure 4. TICs of powdered infant formula sample.

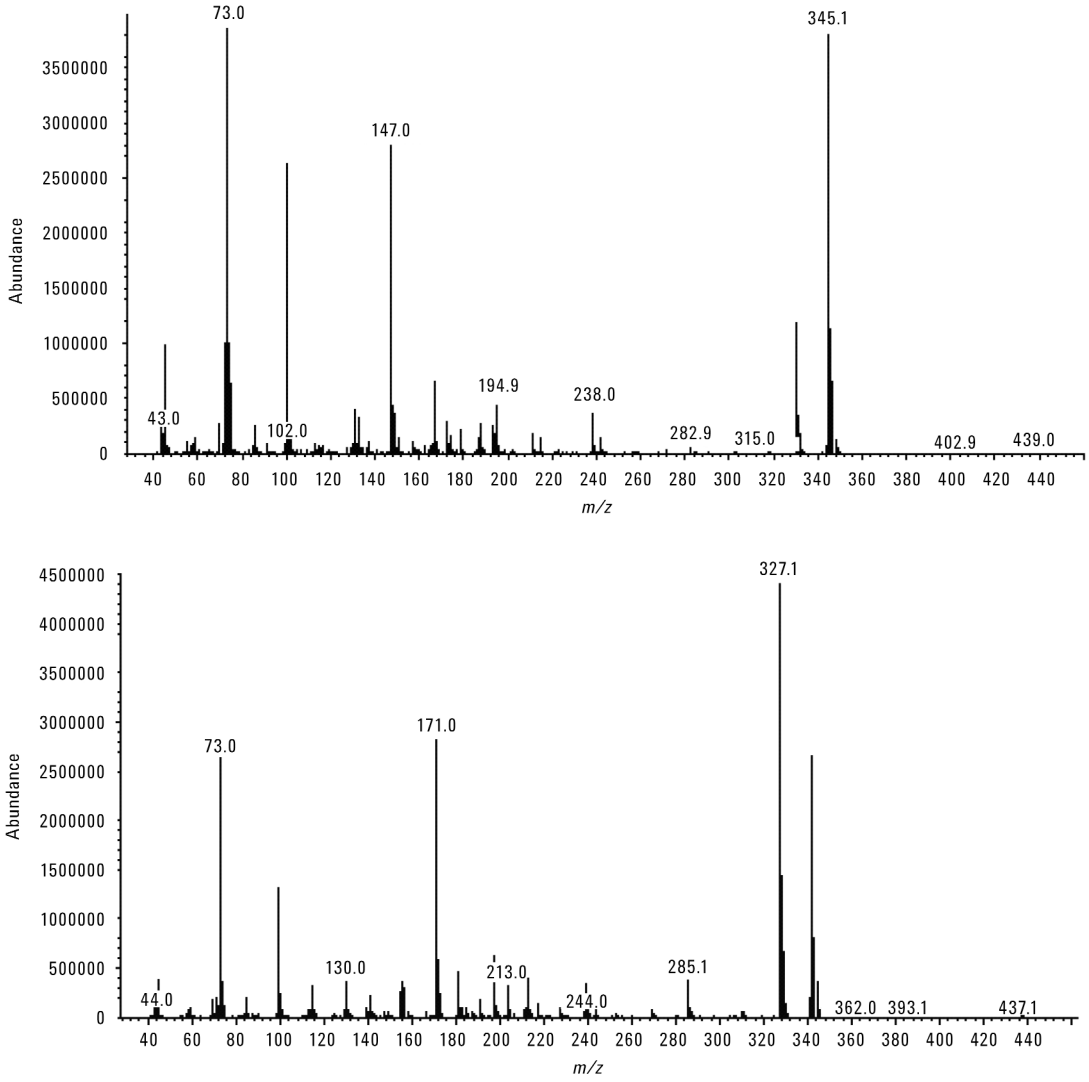


Figure 5. Mass spectra of cyanuric acid tri-TMS derivative (top) and melamine tri-TMS derivative (bottom).

Table 3. Recovery of Spiked Sample

Compound	RT (min)	Spiked level (µg/g)	Measured level (µg/g)	Recovery (%)
Cyanuric acid tri-TMS	6.319	40	38.42	96.1
Melamine tri-TMS	7.333	40	38.23	95.6

Real Sample Analysis

A brand of liquid milk was analyzed with backflush using the previously described method. The two targeted compounds were identified in less than 10 minutes, with cyanuric acid at 34.90 µg/g and melamine at 3.72 µg/g (see Figure 6).

Conclusions

The work described here is a rapid screening and quantitation method for the analysis of melamine and cyanuric acid in milk products that provides excellent linearity and recovery. Using Agilent 7890A/5975C GC/MSD combined with backflushing, the analysis time was cut down to five times shorter than conventional method. This method is fast and suitable for quality control of milk products for the determination of melamine and cyanuric acid.

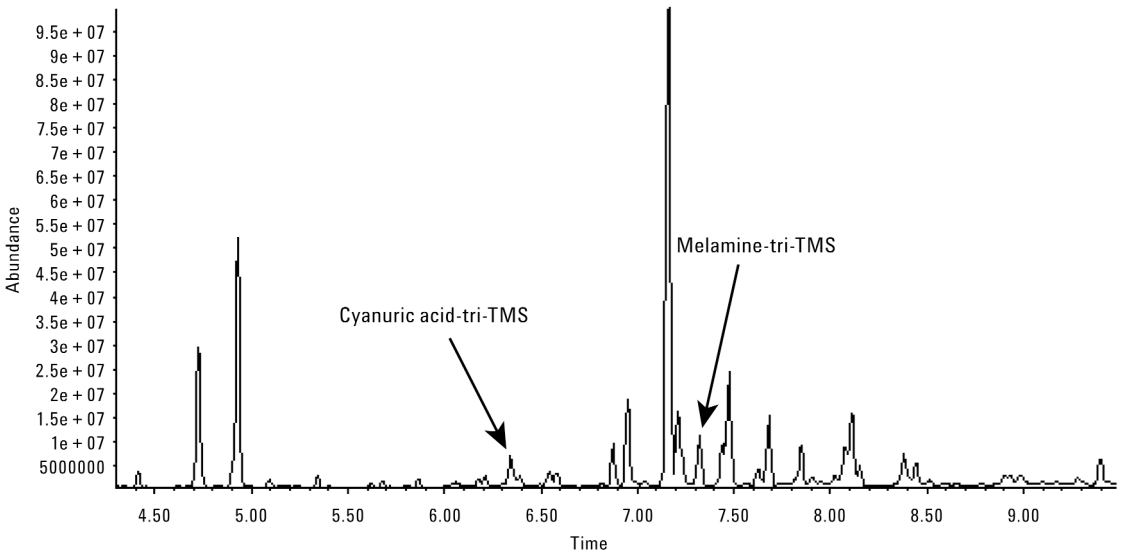


Figure 6. Total ion chromatogram of a contaminated brand of liquid milk.

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Rapid Screening and Confirmation of Melamine Residues in Milk and Its Products by Liquid Chromatography Tandem Mass Spectrometry

Application Note

Food

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Abstract

This rapid method uses the Agilent 6410 Triple Quadrupole (QQQ) with a cation ion exchange column for the liquid chromatography tandem mass spectrometry (LC/MS/MS) analysis of dairy products for melamine. Milk and milk products are prepared with a simple SPE cleanup method employing the new Agilent SampliQ SCX cartridge. The residue is quantified in the multiple reaction monitoring (MRM) mode. The selectivity of the QQQ can easily eliminate any matrix interferences that may occur in the separation and provide excellent response. The method provides good results with respect to precision, repeatability, and spiked recovery. The recovery of 80 ppb and 50 ppb melamine spikes in milk powder using the external standard calculation is 62.5 and 83.4 percent, respectively, and the RSD is less than 3 percent.

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Introduction

Melamine, a nitrogen-based compound used in industrial and commercial plastics, can cause kidney failure, and has been found in infant formula and other milk products.

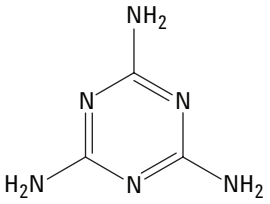


Figure.1 Structure of melamine $C_3H_6N_6$, MW = 126.1199.

In this work, a highly selective, sensitive LC/MS/MS method is developed and, compared to the GC/MS method, requires no derivatization. The method can both confirm and quantify in a single analysis and can achieve very low detection limits in complex matrices such as milk and milk products.

The ZORBAX 300SCX ion-exchange column is simple, fast, and equivalent to the column used in the China GB method and can easily meet the analysis requirements. Therefore, the method can improve lab efficiency/productivity and obtain more reliable and defendable results. It is suitable for the confirmation and quantitation for positive result screening by HPLC.

Experimental

Reagents and Chemicals

The acetonitrile is HPLC grade purchase from Dikma (Beijing, China). The HPLC water is prepared with a Milli-Q system.

Trichloroacetic acid solution: Weigh 50 g trichloroacetic acid and dissolve into 1 L of water.

Ammoniacal methanol solution: Weigh 5 mL ammonium hydroxide and 95 mL methanol

LC Parameters

Column	Agilent ZORBAX 300SCX, 2.1 mm × 150 mm, 5 µm (p/n 883700-704)
Injection volume	10 µL
Flow rate	0.2 mL/min
Temperature	40 °C
Mobile phase	A: 10 mM NH_4 acetate/acetic acid pH adjusted to 3.0 B: ACN A:B = 20:80
Stop time	10 min

MS Parameters

Agilent 6410A LC/MS Triple Quadrupole	
Ion source	Electrospray
Polarity	Positive
Nebulizer gas	Nitrogen
Ion spray voltage	4000 V
Dry gas temperature	350 °C
Dry gas flow rate	9 L/min
Nebulizer pressure	40 psi
Resolution	Q1 (unit) Q3 (unit)

MRM Setting

Rt	Compound	Precursor	Product	Dwell (ms)	Fragmentor (V)	Collision Energy (V)
7 min	Melamine	127	85	200	100	20
		127	68	200	100	35

Sample Preparation

1. Standards solution: dissolve melamine into mobile phase to concentration level at 1, 5, 10, 50, 100, and 500 ppb.
2. Liquid milk, milk powder, yogurt and ice-cream sample preparation:

   2.1 Extraction
   Weigh 2 g of the sample into a 50-mL plastic centrifuge tube with cup, add 15 mL 5% trichloroacetic acid in water solution and 5 mL acetonitrile, sonicate for 10 min, vortex for 10 min, and then centrifuge 10 min at 4000 rpm. Wet filter paper with 5% trichloroacetic acid and filter the supernatant and using a 25.0-mL volumetric flask, bring to
   volume with 5% trichloroacetic acid solution. Transfer 5.0 mL and then add 5 mL water for further cleanup.

   2.2 SPE Cleanup
   Load the above solution onto the SPE cartridge, 6 mL/150 mg SampliQ SCX (p/n 5982-3267). Condition the SPE cartridge before use by washing with 5 mL methanol and then 6 mL water to activate. After loading the sample wash with 5 mL water and then 5 mL methanol, vacuum to almost dry, and elute with 5 mL 5% ammoniacal methanol solution. Control the flow rate at less than 1 mL/min. Dry the eluate under 50 °C nitrogen. Then dissolve the residue (equivalent 0.4 g sample) with 1.0 mL mobile phase, vortex 1 min, and filter via 0.2 µ regenerated cellulose membrane filter (p/n 5064-8222) before injection.

Results and Discussion

Milk and relevant milk products contain hundreds of compounds and it is necessary to distinguish the illicitly added melamine among them. For confirmation and accurate quantification beyond the capability of LC, the LC/MS/MS method is an excellent tool. The high selectivity of the first and third quadrupoles, each working as a "mass filter" in MRM mode, allows selection of the precursor ion and two characteristic fragments, one used for quantitation and the other a qualifier ion (with both present at the corresponding ratio to the standard providing confirmation). Thus, mass spectrometry is a tool that can provide melamine screening laboratories and dairy product manufacturers accurate and precise quantitation and confirmation for samples screened positive.

The chromatograms in Figure 3 show that the Agilent 6410 QQQ determination removes chemical interferences using the high selectivity of the LC/MS/MS in MRM mode. Both the quantitation ion and the qualifier ion have little noise and no matrix interfering peaks, providing accurate and precise quantitation and confirmation. Confirmation is obtained by comparing both the ion ratios and the retention time to the results obtained for melamine standards.

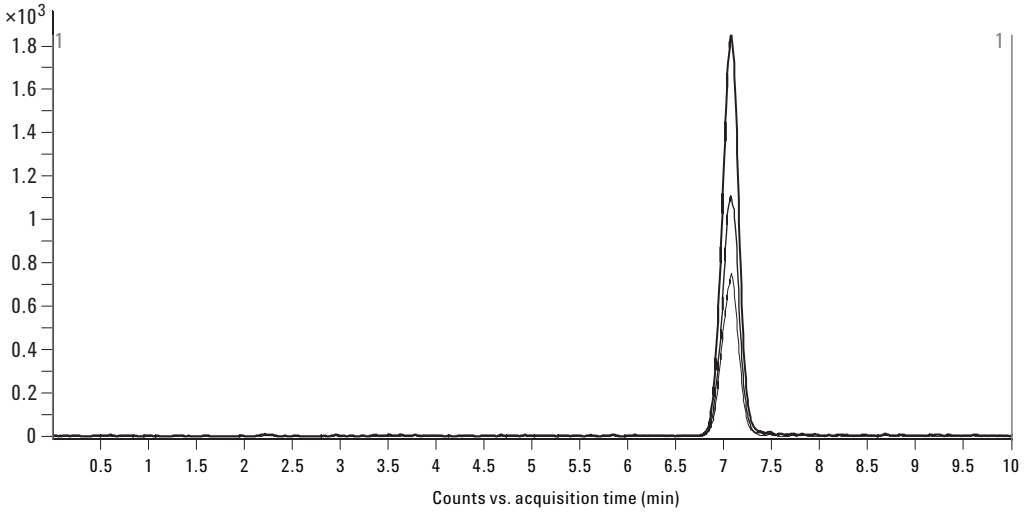


Figure 2. Result in solvent showing the precursor ion and two transition ions at the 10 ppb level.

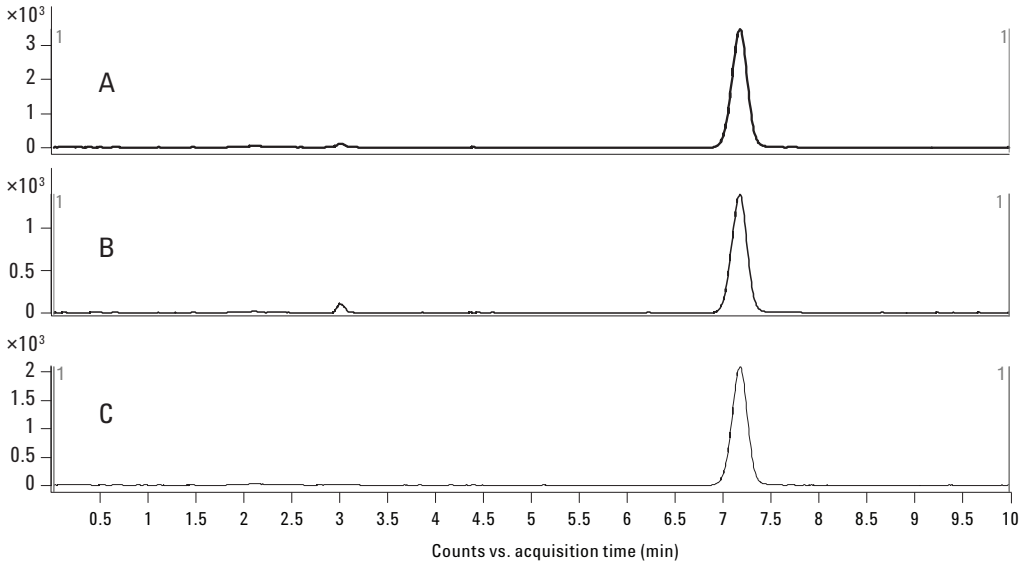


Figure 3. Results of 18.3 ppb level in milk powder with a 10 µL injection, A) total ion chromatogram (TIC) , B) qualifier ion, and C) quantitation ion.

Linearity

Another advantage of QQQ technology is the wide dynamic range for the different levels of sample concentrations as seen in Figure 4.

As shown in Figure 3, it is quite easy to obtain very low detection and, at the same time, analyze high-concentration samples. Samples screened positive by LC/UV detection with possible melamine concentrations above ppm level should be diluted for LC/MS/MS confirmation to avoid contamination of the highly sensitive MS system.

Sensitivity

Using LC/MS/MS excellent sensitivity can be obtained even in complex and dirty matrices. There is almost no background even at very low levels in milk samples.

Figure 5. shows a milk sample spiked at 1 ppb with melamine. Using these data, the calculated result for the limit of quantitation (LOQ) (S/N >10 peak to peak) is 0.5 ppb and limit of detection (LOD) (S/N > 3 peak to peak) is 0.2 ppb.

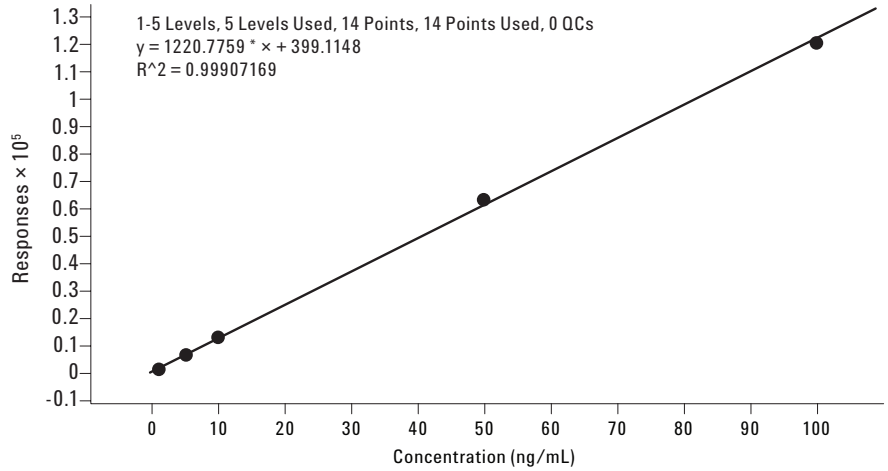


Figure 4. Linearity result for melamine using the MRM quantitation ion.

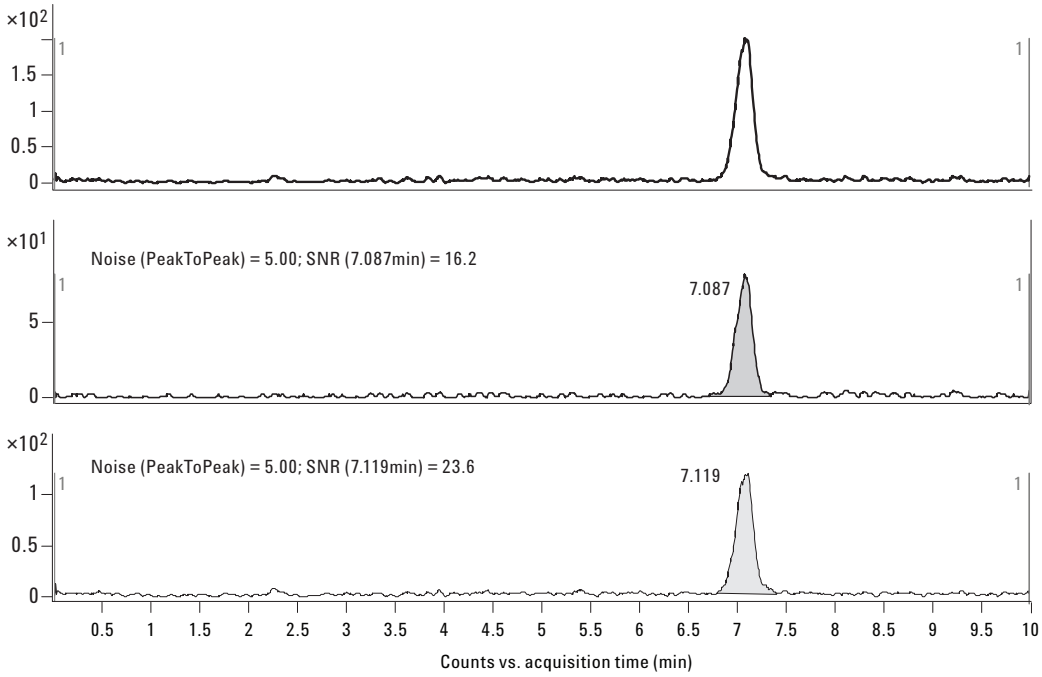


Figure 5. Response of melamine in a milk sample spiked at 1 ppb.

Repeatability

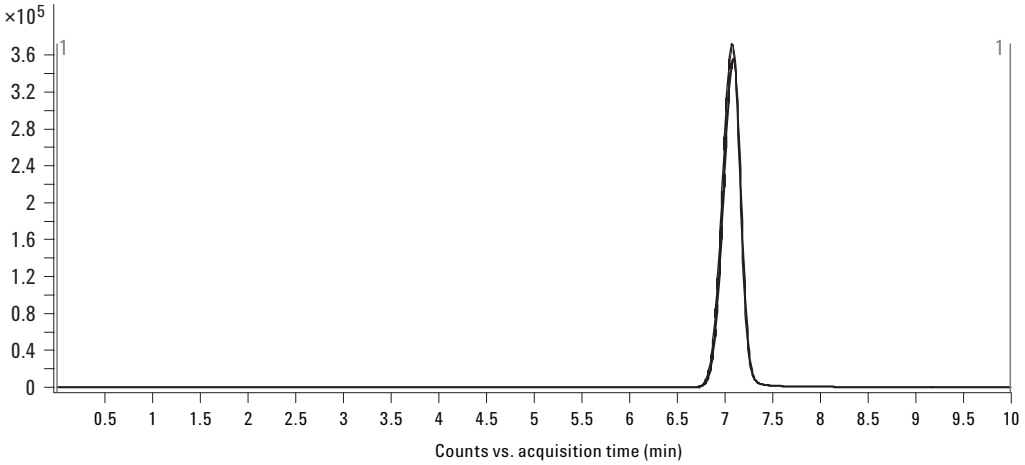


Figure 6. Replicate injections in liquid milk (n = 3) at 10 ppb level.

Table 1. Repeatability in Real Milk Samples with n = 3

Melamine concentration in real milk sample (ppm)	RSD of retention time (%)	RSD of MS response (%)
1	0.30	1.15
100	0.04	1.02

Excellent repeatability of this method is shown in Figure 6 and Table 1. This can ensure good results, even after day-to-day analysis of running samples.

Recovery

Using a calibration curve based on melamine standard in solvent, recovery data in milk powder is shown in Table 2.

Table 2. Recovery in Milk Powder

	Conc. = 80 ppb (n = 3)	Conc. = 50 ppb (n = 3)
Recovery (%)	62.5	83.4
RSD (%)	1.02	2.78

Saturation of the MS detector is observed at about 100 ppb. Using an internal standard method is recommended for future analysis with stable isotope labeled melamine.

Conclusions

A sensitive and specific method for the detection and quantitation of melamine in milk and milk products has been demonstrated. The method is robust and allows for the analysis of a large number of samples in complex matrices. Derivatization is not needed, and the method provides confirmation and quantitation in a single analysis at very low detection limits. This method can be readily used for confirmation of positive results obtained with less selective LC screening methods. The results of this study show that the Agilent 6410 LC/MS Triple Quadrupole, SampliQ SCX SPE cartridges, and a ZORBAX 300SCX HPLC column provide a robust, sensitive, and repeatable methodology.

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Trace-Level Analysis of Melamine in Milk Products on Agilent 7890A/5975C GC/MSD Using a New Agilent J&W DB-5ms Ultra Inert Column and SampliQ SCX Cartridges

Application Note

Gas Chromatography/Mass Spectrometry/Food Safety

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Abstract

A GC/MS method is presented for the quantitative determination and confirmation of melamine residues in milk products. The milk sample was cleaned up using Agilent's new SampliQ SCX SPE cartridges before derivatization. The derived extracts were analyzed by GC/MS with EI in synchronous SIM/scan mode on a new Agilent J&W DB-5ms Ultra Inert column. The method has good linearity in the range of 0.025 to 2.000 µg/mL with a correlation coefficient of 0.9986. The average recoveries of melamine, for milk spiked at 0.080, 0.800, and 1.600 mg/kg, were over 80 percent, with RSDs of less than 5 percent. The LOD is 0.0025 mg/kg in the milk matrix, which is suitable for trace-level analysis of melamine in milk products.

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Introduction

Melamine is an organic base chemical most commonly found in the form of white crystals rich in nitrogen. Recently, melamine in adulterated milk product has attracted much attention all over the world. The main analytical methods available for melamine quantification in milk involve GC/MS, HPLC, and LC/MS, such as China GB/T method 22388-2008 [1]. An HPLC method can be used to quantitatively analyze melamine at ppm level. But it is inadequate for qualitative analysis and trace-level analysis. LC/MS is a sensitive technique for quantitative and qualitative analysis. However, due to the high price of equipment, it is not widely used in practical applications. GC/MS is an economic and commonly used technique for most of analytical laboratories.

Milk products are complex matrixes that contain interfering compounds, such as protein and fat. These compounds may interfere with the analysis of the target analytes or contaminate the analytical system if the sample is introduced without selective sample preparation.

Solid-phase extraction (SPE) is commonly used as a sample preparation method for a variety of analytical procedures. Agilent's mixed-mode SampliQ SCX polymeric resin is a sulfonic acid-modified divinyl benzene polymer with both ion exchange and reverse-phase retention properties. The SampliQ SCX resin exhibits excellent retention for both basic and neutral compounds. It ensures fast, reliable extraction of basic compounds like melamine.

GB/T method 22388-2008 requires melamine analysis with an LOQ of 0.05 mg/kg in a GC/MS method. For a complex matrix like milk sample, an inert GC/MS column is crucial for trace-level analysis. The new Agilent J&W Ultra Inert column allows for the best and most consistent column inertness [3], providing better sensitivity and peak shape for active compounds and reliable results for trace-level analysis. Combined SampliQ SCX SPE with the Agilent J&W DB-5ms Ultra Inert column, a sensitive GC/MS method, is put forward on trace-level analysis of melamine in milk products.

Experimental

Standards and Reagents

The standards and reagents used in the experiment are listed in Table 1.

A stock solution 1,000 µg/mL of melamine was prepared in methanol. Working solutions were prepared by diluting the stock solution with methanol. After being transferred to an autosampler vial, the 1-mL working solution was allowed to evaporate to dryness with nitrogen. 600 µL pyridine and 200 µL BSTFA were then added, vortexed briefly to mix, and incubated at 70 °C for 30 minutes. With this procedure, a series of standard solutions of melamine trimethylsilyl (TMS) derivatives (0.025, 0.050, 0.200, 0.500, 1.000, and 2.000 µg/mL) were prepared for linearity analysis.

Table 1. Standards and Reagents

Melamine	> 99% purity
Methanol	HPLC grade
Pyridine	Certified A.C.S. reagent
1% trichloroacetic acid (TAA) solution	Dissolve 10.0 g of TAA in reagent water. Dilute to 1.0 L with reagent water.
22 g/L lead acetate solution	Dissolve 22.0 g of lead acetate in reagent water. Dilute to 1.0 L with reagent water.
5% ammonium hydroxide solution	Mix 5 mL of ammonium hydroxide and 95 mL of methanol together for use.
Silylating reagent	BSTFA with 1% TMCS: bis(trimethylsilyl) trifluoroacetamide with 1% Trimethylchlorosilane

Instruments

Table 2 lists the GC chromatography and mass spectrometry conditions of the method.

Table 2. Gas Chromatograph and Mass Spectrometer Conditions

GC Conditions	
Instruments	Agilent 7890A/5975C GC/MSD Agilent 7683 Automatic Liquid Sampler (ALS)
Column	Agilent J&W DB-5ms Ultra Inert, 30 m × 0.25 mm × 0.25 µm (P/N 122-5532UI)
Inlet temperature	EPC, split/splitless @ 250 °C
Injection volume	1 µL, split 3:1
Carrier gas	Helium, constant flow mode, 1.3 mL/min
Oven program	75 °C (1 min); 30 °C/min to 300 °C (2 min)
Transfer line	290 °C
MS Conditions	
MS	EI, SIM/scan
Solvent delay	4.2 min
MS temperature	230 °C (source); 150 °C (quad)
Scan mode	Mass range (40 to 450 amu)
SIM mode	Ion (342, 327*, 171, 99)

*Quantitative ion

Sample Preparation

Sample preparation was performed according to the scheme in Figure 1. A SampliQ SCX SPE cartridge (60 mg, 3 mL, P/N 5982-3236) was used to clean up sample extracts. All SPE elution steps, including conditioning, sample loading, washing, and elution were performed without the application of vaccum. Vacuum was only applied to dry the cartridges.

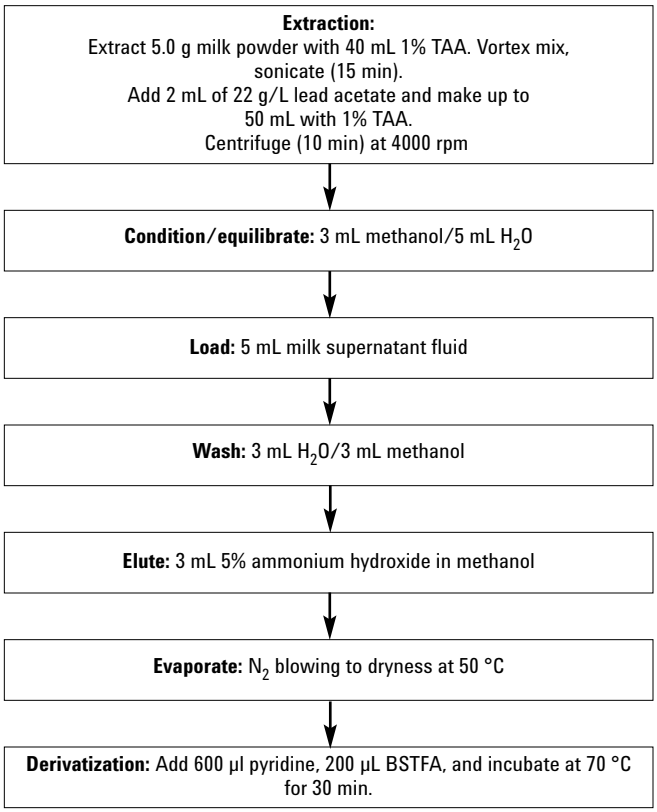


Figure 1. Scheme of sample preparation process.

The derivatization of melamine is shown is Figure 2.

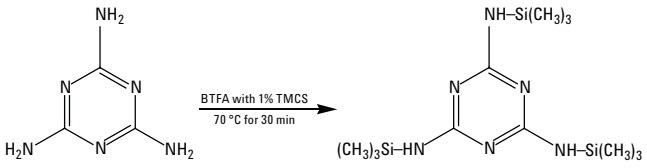


Figure 2. Melamine derivatization reaction.

Results and Discussion

GC/MS Analysis of Melamine

GC/MS was used for the identification (full-scan mode) and quantification (SIM mode) of melamine. It helped simplify the process of confirming positive or negative results.

Figure 3A shows the chromatogram of a sample that was only extracted with methanol without any SPE cleanup [4]. It highlights that there was much sample matrix interference in milk extract for the analytes of interest. Especially after 10 minutes, large amounts of high boilers eluted from the column. Undoubtedly, it increased the analysis time to ensure that the late eluters were eluted. Furthermore, it could contaminate the analytical system and shorten the lifetime of the ion source.

Figure 3B shows the chromatogram of a sample prepared according to the procedure in Figure 1. Figure 3C is the chromatogram of melamine tri-TMS for reference. The SCX SPE effectively removed interferences from the sample matrix. Downtime is reduced due to less detector contamination.

Figure 4 is the corresponding mass spectrum of melamine tri-TMS in Figure 3. After SPE cleanup, it eliminates some interference ions from the matrix and the mass spectra shows better "match" factors over 90. It further demonstrates that it makes the qualitative and quantitative analysis easier and more accurate.

As you can see in Figure 3, sharp, symmetrical peaks were observed for melamine tri-TMS on Agilent J&W DB-5ms Ultra Inert GC columns. Sharper peaks greatly enhance the height of the signal and therefore provide better signal-to-noise ratios and greater sensitivity. The ultra inert column ensures minimal compound adsorption, for more accurate quantification. It proved that Agilent J&W Ultra Inert GC columns allow you to perform trace-level analysis of melamine with the utmost confidence.

Linearity and Recovery

The linearity of the method was studied using the standard working solutions after derivatization. Figure 5 is the calibration curve for melamine tri-TMS. The method has good linearity in the range of 0.025 to 2.000 µg/mL, with a correlation coefficient of 0.9986.

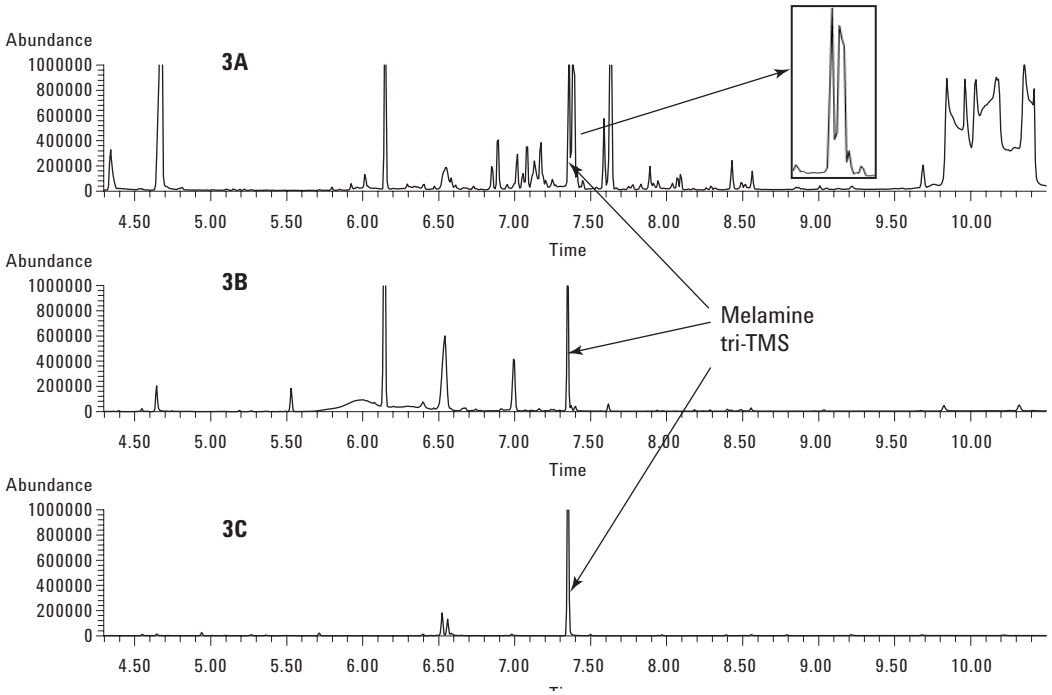


Figure 3. GC/MS SIM chromatogram of melamine tri-TMS. (3A: Sample without SPE cleanup; 3B: Sample with SPE cleanup; 3C: Standard)

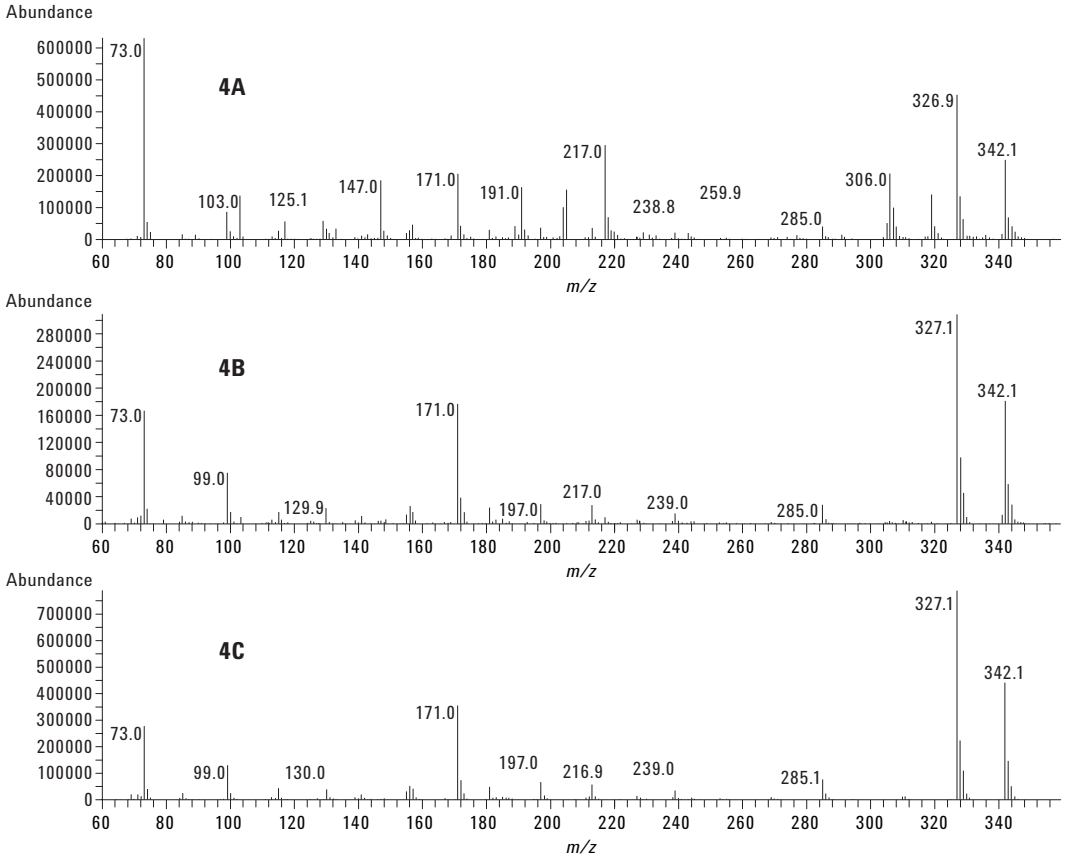


Figure 4. GC/MS mass spectrum of melamine tri-TMS. (4A: Sample without SPE cleanup; 4B: Sample with SPE cleanup; 4C: Standard)

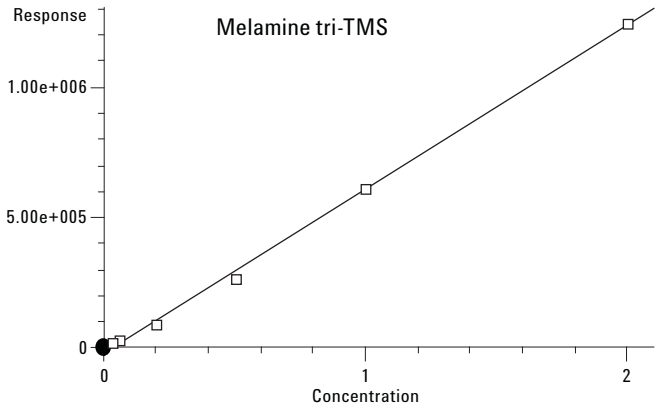


Figure 5. Calibration curve for melamine tri-TMS.

The recovery of the method was evaluated for melamine spiked at 0.080, 0.800, and 1.600 mg/kg levels. For each concentration level, the repeatability was evaluated on six duplicate samples. The spiked samples were treated according to the sample-preparation procedure described above. The recovery and repeatability data are listed in Table 3. The method resulted in good recoveries (over 80 percent) and repeatability with RSDs less than 5 percent at three levels. The LOD is calculated from the level of 0.08 mg/kg with a signal-to-noise ratio of 3. The LOD is 0.0025 mg/kg in the milk matrix, which is applicable for the trace-level analysis of melamine.

Table 3. Recovery and Repeatability of Spiked Samples

Compound	Spiked level (mg/g)	Recovery (%)	RSD (%) (n = 6)
Melamine tri-TMS	0.080	82.1	2.04
	0.800	82.8	4.88
	1.600	80.8	3.58

Conclusions

This application demonstrates a sensitive GC/MS method for melamine analysis in milk products using an Agilent J&W DB-5ms Ultra Inert GC column and SampliQ SCX SPE cartridges. It is suitable for trace-level analysis of melamine in raw milk and diary products.

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Rapid Screening and Confirmation of Melamine and its Analogs in Baby Formula and Soy Products Using Triple Quadrupole GC/MS and Backflushing

Application Note

Food

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Abstract

A rapid method for the screening and confirmation of melamine, ammelide, ammeline and cyanuric acid in baby formula and soy meal was developed using the Agilent 7890A/7000A Series Triple Quadrupole GC/MS and backflushing with a Purged Ultimate Union. The extraction and derivatization procedures are the same as those used in the FDA GC/MS method. Excellent linearity ($R^2 > 0.99$) was obtained in the range of 0.16 to 2.5 ppm, with run times less than 15 minutes.

Agilent Technologies

Introduction

The adulteration of food with melamine has quickly become an international problem as it has been detected in baby formula produced in the US, chocolates distributed in Canada, biscuits sold in the Netherlands, condensed milk in Thailand and eggs in Hong Kong. In response, many countries have established allowable limits for melamine, with the FDA maximum residue limit (MRL) as 1 part per million (ppm) for infant baby formula and 2.5 ppm for other products. The FDA GC-MS screening method [1] is capable of detecting melamine and its analogs (ammeline, ammelide and cyanuric acid) at 2.5 ppm. However, the FDA import alert of February 2009 requires that a testing method with a sensitivity of 0.25 ppm for melamine and its analogs be used to assure compliance to the MRLs. Therefore this method cannot be used to screen for melamine and its analogs under the new regulations, and confirmation would require an additional orthogonal method.

This application note describes a modification of the FDA GC-MS method for use on the new Agilent 7000A Series Triple Quadrupole GC/MS. The new method, which does not require a change in sample extraction and derivatization procedures, employs a purged union GC column configuration and backflushing to provide run times under 15 minutes. Melamine and its analogs can all be detected at 0.25 ppm, with highly reproducible and accurate quantification. Most importantly, this method provides screening, quantification and confirmation of melamine and its analogs, all in one short run.

Experimental

Standards and Reagents

The standards and reagents used are listed in Table 1. Stock solutions of melamine, ammelide, ammeline and cyanuric acid, each at a concentration of 1,000 µg/mL, were separately prepared in a mixture of DEA/H20 (20/80) and stored at 4 °C. Internal standard (2,6-Diamino-4-chloropyrimidine, or DACP) was prepared at a concentration of 57.7 ng/mL in pyridine. The above solutions were used to prepare matrix-matched standards as described in the FDA method [1]. Matrix samples were generously provided by the FDA.

Table 1. Standards and Reagents

Standard	Melamine	Sigma-Aldrich	>99% purity
	Cyanuric acid	TCI-America	>98.0%
	Ammelide	TCI-America	>98.0%
	Ammeline	TCI-America	>95.0%
	Internal standard†	Sigma-Aldrich	98%
Solvent	Diethylamine (DEA)	Sigma-Aldrich	SigmaUltra grade
	Pyridine	Fisher Scientific	Certified A.C.S. reagent
	Acetonitrile	Fisher	HPLC grade
Silylating reagent	BSTFA with 1% TMCS* (SYLON BFT)	Sigma-Aldrich	Derivatization grade

† DACP (2,6-Diamino-4-chloropyrimidine)
* BSTFA: bis(trimethylsilyl)trifluoroacetamide, TMCS: Trimethylchlorosilane

Instruments

The experiment was performed on an Agilent 7890A gas chromatograph equipped with a split/splitless capillary inlet, an Agilent 7000A Series Triple Quadrupole GC/MS with Triple-Axis Detector, and an Agilent 7683B automatic liquid sampler (ALS). The split/splitless inlet was fitted with a long-lifetime septum (p/n 5183-4761) and a deactivated, splitless single taper injection liner (p/n 5181-3316). Injections were made using a 10-µL syringe (p/n 9301-0714). The instrument conditions are listed in Table 2.

Table 2. Gas Chromatograph and Mass Spectrometer Conditions

GC Run Conditions	
Column	Two 15 m × 0.25 mm × 0.25 µm HP-5ms columns (p/n 19091S-431)
Inlet temperature	280 °C
Inlet pressure	12.9 psi
Carrier gas	Helium, constant flow mode, 1.2 mL/min
Pulsed splitless	25 psi at 0.5 min
Oven program	100 °C (1 min hold), 10 °C/min to 210 °C
Column velocity	41 cm/s
Injection volume	1 µL
Transfer line temperature	290 °C
GC Post-Run Conditions	
Backflush device	Purged Ultimate Union (p/n G3186-60580) controlled by a Pressure Control Module (p/n G3476-60501)
Backflush conditions	–3.6 mL/min at 300 °C for 1.3 min
MS Conditions	
Tune	Autotune
Delta EMV	400 V
Acquisition parameters	EI; selected reaction monitoring
Solvent delay	6 minutes
MS temperatures	Source 230 °C; Quadrupoles 150 °C

Sample Preparation

A 0.5-g amount of a representative portion of the sample was weighed into a 50-mL polypropylene centrifuge tube. An extraction solvent of DEA/Water/Acetonitrile (10/40/50) was prepared, and 20 mL added to the weighed sample. Diethylamine dissociates the melamine-cyanuric acid complex, thus reducing the risk of false negative measurements. DEA also improves the solubility of ammelide and ammeline, which have extremely low solubility in traditional extraction solvents. The sample was capped, vortex mixed, and then sonicated for 30 minutes. After the sample was centrifuged at 5,000 g or higher for 10 minutes, the supernatant fluid was filtered through a 0.45-µm nylon filter.

Derivatization

A 160-µL amount of the filtrate was transferred to a glass GC vial. The extract was evaporated to dryness under a stream of nitrogen at approximately 70 °C, and 600 µL of ISTD and 200 µL of BSTFA with 1% TMCS were added. The sample was vortex mixed and incubated at 70 °C for 45 minutes before injecting.

Analysis Parameters

The parameters used in the analysis of melamine and its analogs, as well as the internal standard, are shown in Table 3.

Table 3. Analysis Parameters

		Triple Quadrupole GC/MS		
Compound	**RT**	**SRM**	**Dwell time (ms)**	**Collision energy (EV)**
Melamine	12.467	327 → 171	20	17
		342 → 285	150	20
		342 → 213	150	22
Ammelide	10.801	344 → 171	50	22
		344 → 214	50	15
		329 → 171	50	20
Ammeline	11.748	328 → 171	50	25
		343 → 214	50	20
		343 → 171	50	30
Cyanuric acid	9.613	345 → 215	50	8
		345 → 188	50	12
		330 → 215	50	4
DACP (ISTD)	11.185	273 → 237	150	12
2,6-Diamino-4-chloropyrimidine		273 → 99	150	20

Results and Discussion

Backflushing with a Purged Ultimate Union System

A backflushing configuration was employed to remove higher boiling substances from the column prior to each subsequent run by flushing late eluting peaks out of the inlet split flow vent instead of driving them though the entire column and into the MSD. Backflushing reduces chemical noise and the cycle time of the analysis, thus increasing throughput. System uptime is also increased, due to reduced maintenance of the columns and MS detector. The suite of Agilent Capillary Flow Technology modules comprises a proprietary solution that enables easy and rapid backflushing with small dead volumes for improved resolution, and ferrules and fittings that eliminate leaks. All Capillary Flow Technology modules require the use of an Auxiliary Electronic Pneumatic Control (EPC) module or a Pneumatic Control Module (PCM) to provide a precisely-controlled second source of gas that directs the column flow to the appropriate column or detector. In normal operation, the PCM pressure is at or slightly above the pressure of the carrier gas through the column. During backflush, the inlet pressure is dropped to 1 psi and the PCM pressure is increased, forcing the flow to reverse through the column and out the purged inlet.

A unique, alternative approach to backflushing is the use of a Capillary Flow Technology device in the middle of the analytical column [2, 3]. Instead of using a 30-m column, two 15-m columns are used and connected by an ultra-low dead volume Purged Ultimate Union (Figure 1). The PCM adds just enough makeup gas to match that from the first column. Therefore, there is very little flow addition and subsequent decrease in sensitivity due to sub-optimal carrier gas flows into the mass spectrometer. Backflushing in this configuration is accomplished by reducing the flow and pressure in the first column and increasing them in the second column.

Figure 2 shows an example of backflushing with the purged union configuration. The top chromatogram shows six standards, where the third peak is considered the last analyte of interest and the fourth peak is the first of the late-eluting interferences. The middle chromatogram shows (a) the same

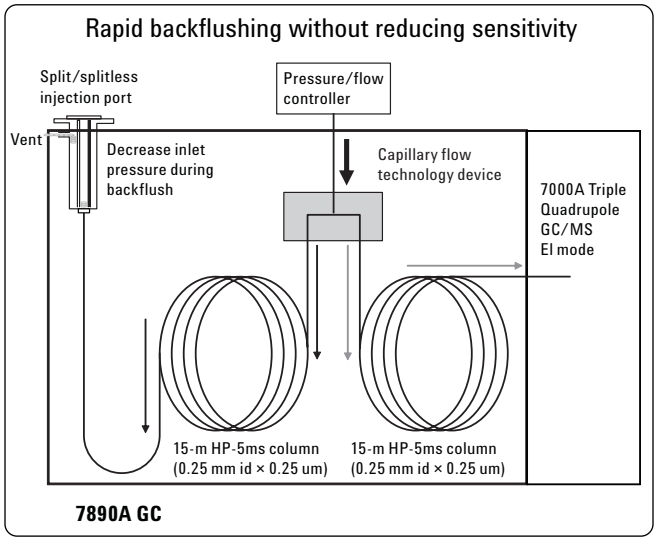


Figure 1. Schematic of the Purged Ultimate Union GC/MS configuration.

standard with backflushing beginning at 10.1 minutes, where flow is dropped in the first 15-m column and (b) where the flow in the second column is increased. The time between points a and b is the residence time of the last analyte compound in the second column. The last analyte is retained, but the late eluters never enter the MS detector. The bottom chromatogram demonstrates the lack of carryover in a subsequent blank run. Alternatively, backflushing can begin after the last peak of interest has eluted (point b). This eliminates the need to experimentally determine the residence time of the last target compound in the second column, while slightly increasing the cycle time.

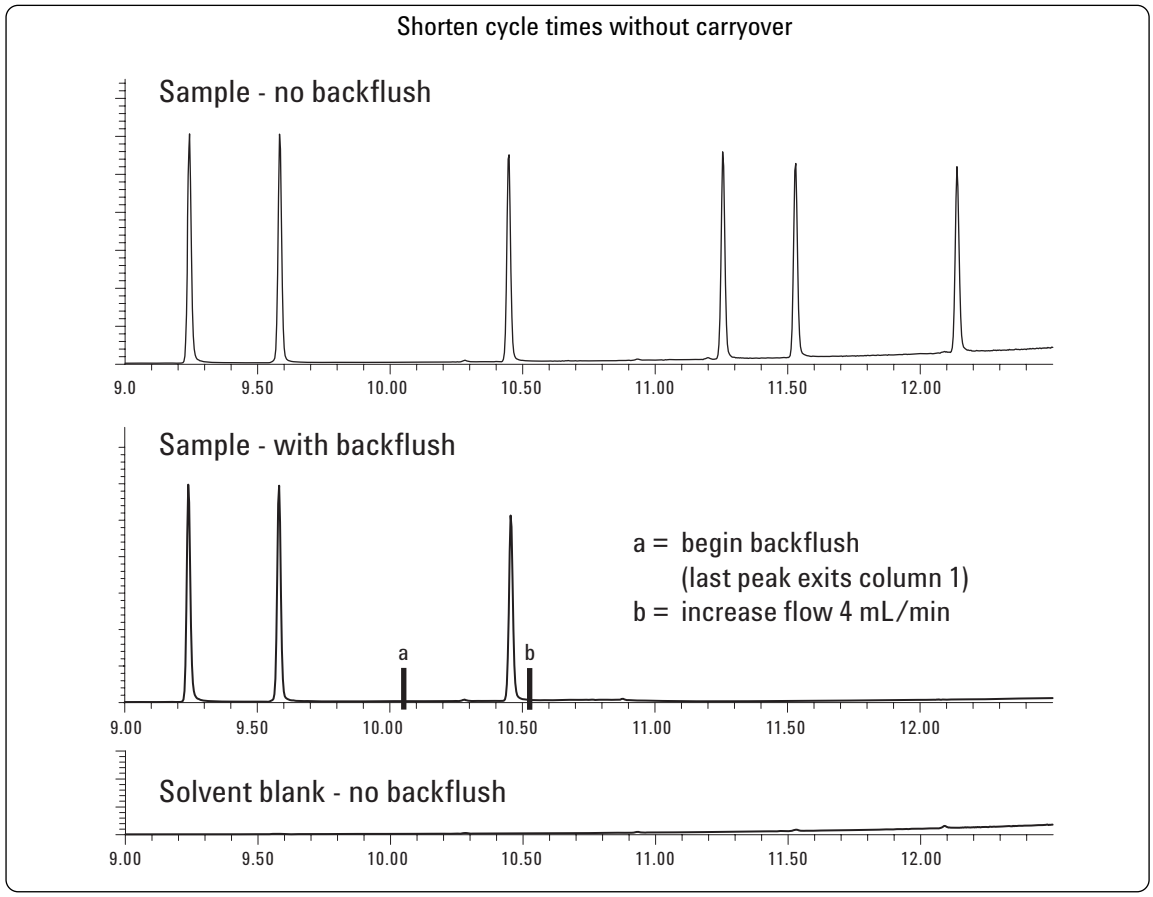


Figure 2. Backflushing with a purged ultimate union configuration. Top: no backflushing. Middle: Backflushing beginning at 10.1 minutes (a) until the third analyte elutes off the second column (b). Bottom: Subsequent blank injection showing no carryover.

Analysis of Melamine and its Analogs

The method developed on the Triple Quadrupole GC/MS system provides excellent separation and analysis of melamine, ammelide, ammeline and cyanuric acid in one run, and in less than 15 minutes (Figure 3). The significant improvement in the sensitivity and selectivity of the new Triple Quadrupole GC/MS method versus the GC/MS SIM method is vividly illustrated in Figure 4. While the new method provides a very clean analysis of the quantifying transition of melamine at 0.25 ppm, the GC/MS SIM method is less effective at reducing chemical noise at 2.5 ppm, using any of the SIM ions.

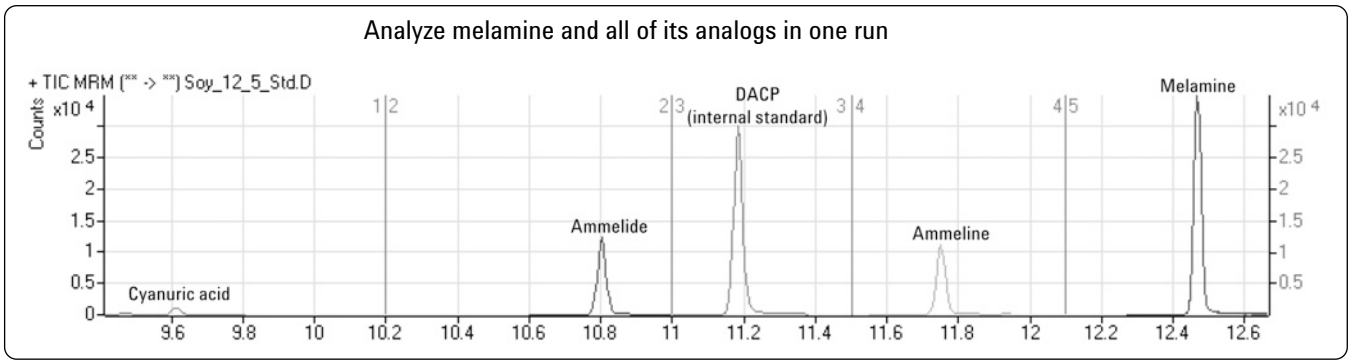


Figure 3. Reconstructed Total Ion Current Chromatogram (RTICC) resulting from SRM analysis, illustrating the resolution of melamine and its analogs.

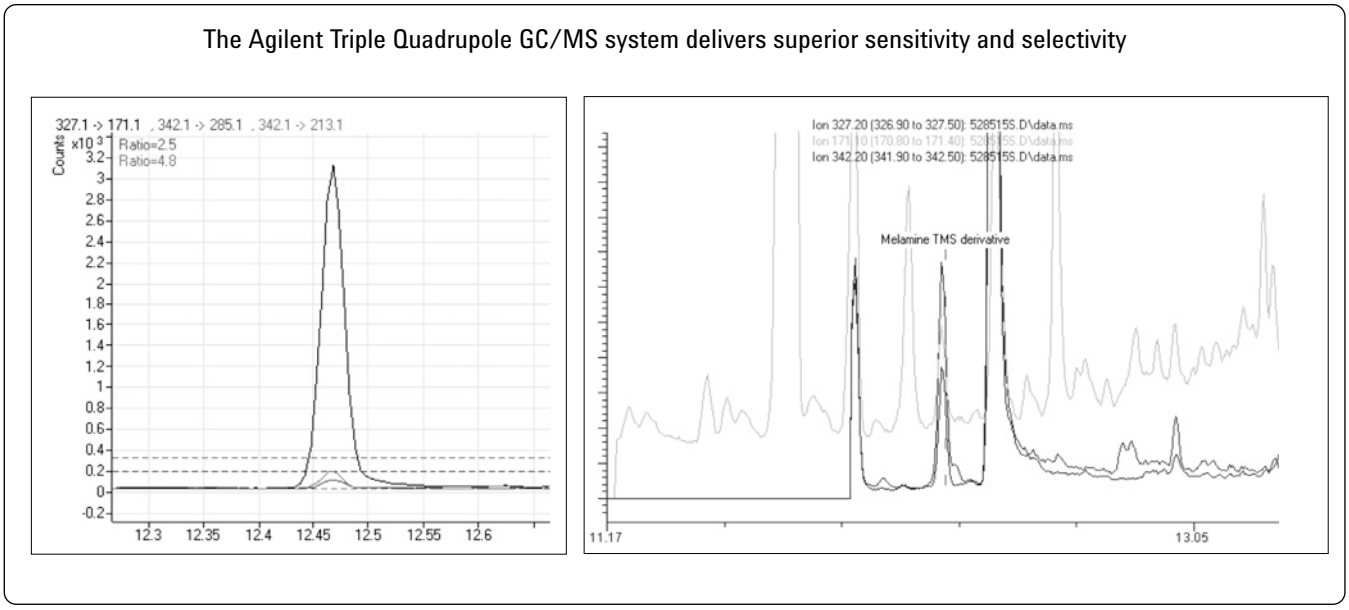


Figure 4. Comparison of detection of 0.25 ppm melamine in soy meal using the Triple Quadrupole GC/MS method (a), versus the GC/MS SIM method at 2.5 ppm (b). The quantifying transition used with the Triple Quadrupole GC/MS method was m/z 327.1→171.1, and the qualifying transitions were m/z 342.1→295.1 (2.5% of the peak area of the quantifying transition) and m/z 342.1→217.1 (4.8% peak area). The uncertainty bands are shown in (a) as well. The SIM ions used in the GC/MS method were m/z 342.2, 327.2, and 171.1 (b).

Sensitivity and Quantification

Each of the standards for melamine and its three analogs was added to matrix (both baby formula and soy meal) at concentrations of 0.78, 1.25, 3.9 and 12.5 ng/mL, corresponding to detection levels of 0.16 to 2.5 ppm. Calibration curves were constructed for each of the four compounds in each matrix.

Figures 5 and 6 illustrate the excellent linearity obtained for melamine and its three analogs, with R^2 values very close to 1.00. The accuracy of quantification was also very good for all four compounds in both matrices as illustrated in Tables 4 and 5.

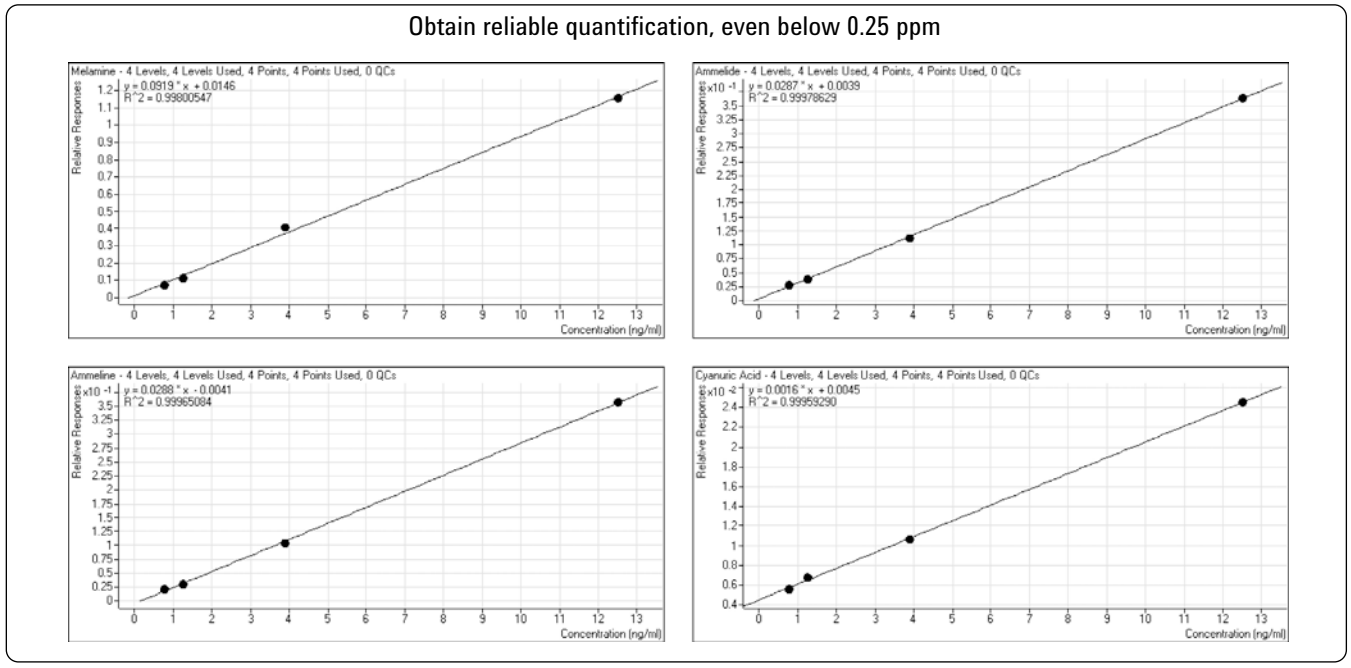


Figure 5. Calibration curves for quantification of melamine and its derivatives in baby formula based on a linear fit.

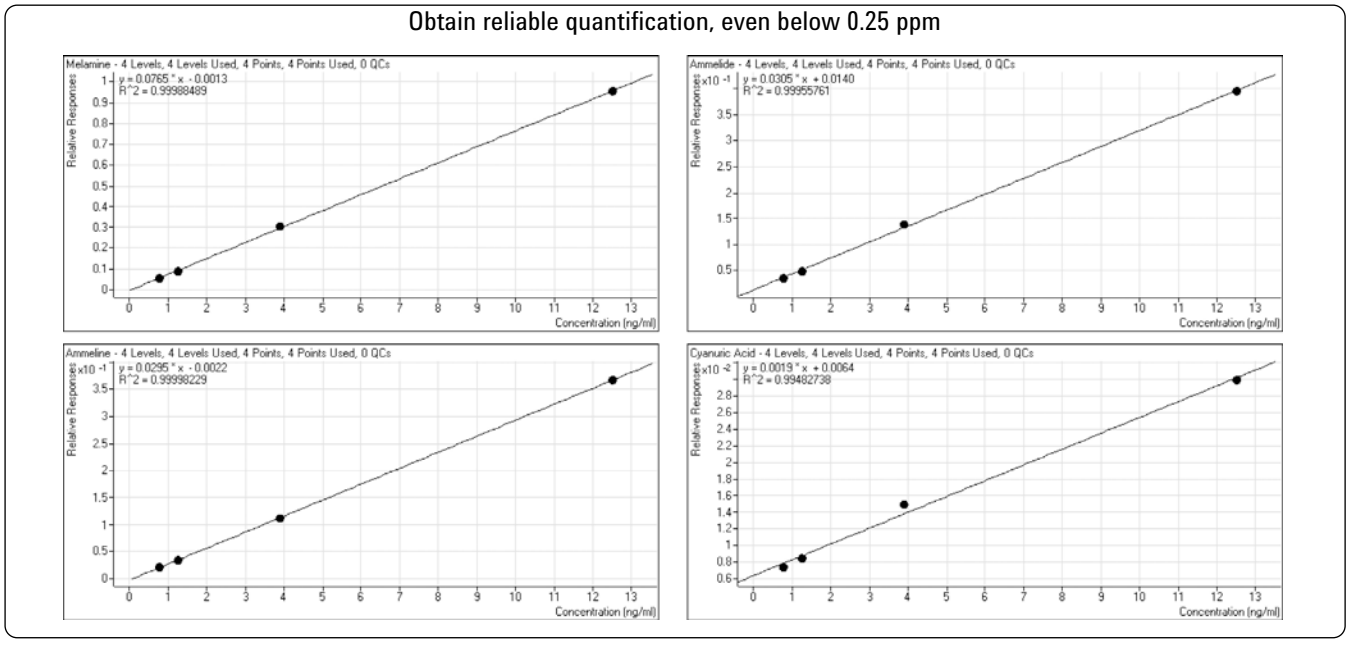


Figure 6. Calibration curves for quantification of melamine and its derivatives in soy meal based on a linear fit.

Table 4. Calibration Data for Quantification of Melamine and its Derivatives in Baby Formula Based on Matrix-Matched Standards

	Standard Concentration (ng/mL)	Measured Concentration (ng/mL)	Accuracy of Quantification (%)
Melamine	0.78	0.79	101.3
	1.25	1.23	99.1
	3.90	4.39	112.5
	12.5	12.50	100.0
Ammelide	0.78	0.86	110.3
	1.25	1.25	99.9
	3.90	3.79	97.2
	12.5	12.52	100.2
Ammeline	0.78	0.90	115.5
	1.25	1.22	97.2
	3.90	3.78	97.0
	12.5	12.52	100.3
Cyanuric acid	0.78	0.67	86.1
	1.25	1.40	111.9
	3.90	3.85	98.8
	12.5	12.51	100.1

Table 5. Calibration Data for Quantification of Melamine and its Derivatives in Soy Meal Based on Matrix-Matched Standards

	Standard Concentration (ng/mL)	Measured Concentration (ng/mL)	Accuracy of Quantification (%)
Melamine	0.78	0.76	97.7
	1.25	1.20	96.3
	3.90	3.98	102.2
	12.5	12.48	99.8
Ammelide	0.78	0.72	92.9
	1.25	1.18	94.2
	3.90	4.07	104.4
	12.5	12.46	99.7
Ammeline	0.78	0.81	103.7
	1.25	1.22	97.9
	3.90	3.90	99.9
	12.5	12.50	100.0
Cyanuric acid	0.78	0.71	91.3
	1.25	1.22	94.5
	3.90	4.49	115.1
	12.5	12.01	96.1

Confirmation

The identification point system was developed by EU scientists to define an acceptable procedure for scientifically confirming the presence of regulated substances. The more identification points provided by the analytical method, the more certain is the confirmation of the compound. Three points are required for compounds with an MRL. When no MRL can be defined because of the toxicity of the compound, it is banned at all levels. These compounds require four identification points. While four ions need to be monitored by GC/MS to provide four identification points, only two SRM transitions need to be monitored when using triple quadrupole GC/MS/MS. Analysis of melamine and its analogs was performed using at least two SRM transitions for each compound on the triple quadrupole GC/MS system to provide screening and positive confirmation in the same run.

Figures 7 and 8 illustrate the quantifying and qualifying transition profiles for the GC separation of each of the four compounds in both baby formula and soy meal. In each case the qualifying transitions have been normalized to the quantifying transition in order to better illustrate the identical peak shape obtained from both. These transitions therefore provide a positive confirmation of each of the four compounds in each of the sample matrices.

Conclusions

The FDA GC/MS method for screening for melamine, ammelide, ammeline, and cyanuric acid has been modified for use on the Agilent Triple Quadrupole GC/MS system in order to provide screening, quantification and confirmation in one short run. This method does not require any changes in extraction or derivatization procedures, and cycle time is about 15 minutes. In addition, this method meets the new FDA requirement for sensitivity of 0.25 ppm, and it demonstrates excellent linearity of quantification up to 2.5 ppm. Accuracy of quantification is greater than 97% and two SRM transitions for each of the four compounds have been demonstrated in order to provide sufficient identification points for a positive confirmation.

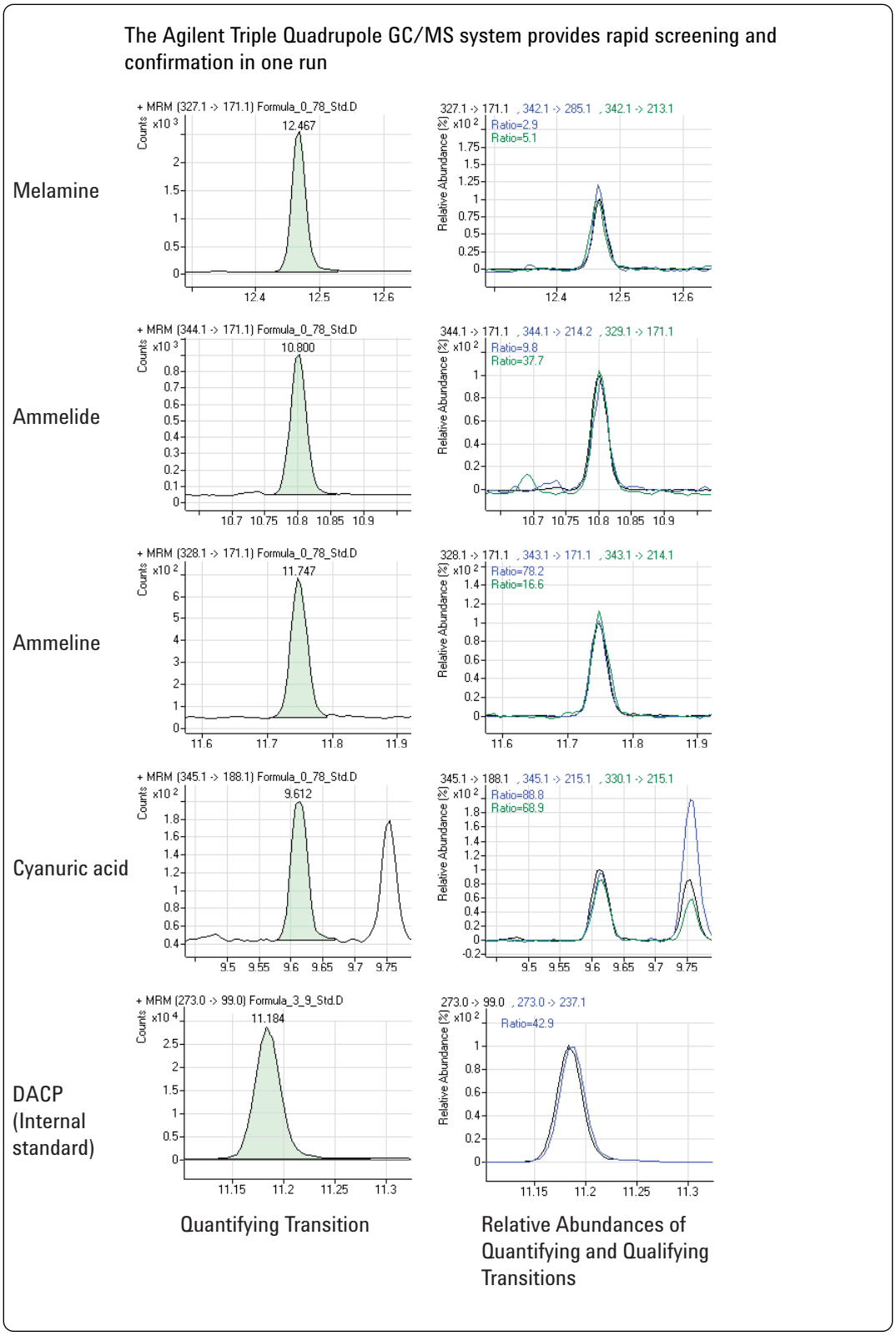


Figure 7. The Agilent Triple Quadrupole GC/MS system provides confirmation and screening in one run: quantifying and normalized qualifying transitions for melamine and its analogs at 0.78 ng/mL in baby formula.

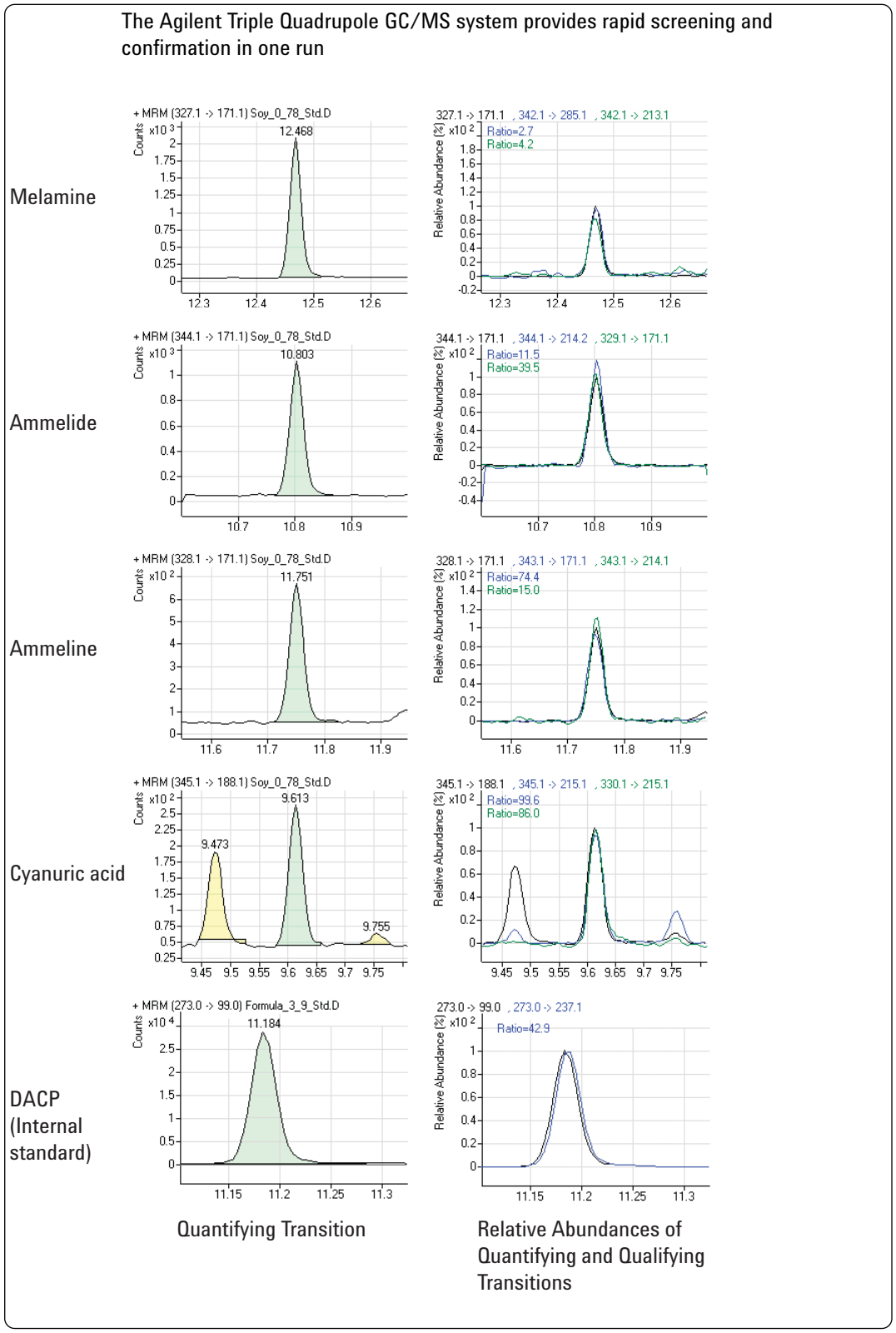


Figure 8. Quantifying and normalized qualifying transitions for melamine and its analogs at 0.78 ng/mL in soy meal.

Acknowledgement

The author gratefully acknowledges the guidance and material assistance of Greg Mercer, Pacific Regional Laboratory Northwest, Food and Drug Administration.

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2. H. Prest, C. Foucault and Y. Aubut, "Capillary Flow Technology for GC/MS: Efficacy of the Simple Tee Configuration for Robust Analysis Using Rapid Backflushing for Matrix Elimination," Agilent Technologies publication 5989-9359EN.
3. H. Prest, Capillary Flow Technology for GC/MS: "A Simple Tee Configuration for Analysis at Trace Concentrations with Rapid Backflushing for Matrix Elimination," Agilent Technologies publication 5989-8664EN.

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Food Processing & Packaging

Plastics and other polymers used during food processing and packaging applications often contain many additives that may significantly influence the final properties of these products. Analysis of the final product is essential to creating a high quality, environmentally safe product. Agilent helps you identify contaminates introduced during processing and offers solutions to determine the point in the manufacturing process that the contamination is occuring.

> Return to Table of Contents
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Analysis of elastomers by GPC/SEC

Application compendium

Authors

Greg Saunders and Ben MacCreath
Agilent Technologies, Inc.



The Measure of Confidence

Agilent Technologies

Contents Page

Introduction 3

Natural rubbers

High sensitivity analysis of natural rubbers with evaporative light scattering detection 5

Analysis of natural rubber with triple detection 6

Synthetic rubbers

Analysis of synthetic polybutadiene with triple detection 7

General synthetic elastomer analysis 8

Polydimethyl siloxane analysis using viscometry 10

Analysis of poly(styrene/butadiene) copolymers by conventional GPC 11

More Agilent solutions for the analysis of rubber 12

Ordering information 14

Suggestions for further reading 15

Polymer Laboratories was formed in 1976 to offer high quality columns, standards, instruments, and software for GPC/SEC. For over 30 years the company developed many market-leading products, including PLgel, PL aquagel-OH, PlusPore, PLgel Olexis, PolarGel columns, and EasiVial standards. Built on advanced in-house manufacturing technology, PL's products have the highest reputation for quality and performance, backed up by world-class technical and applications support.

With the aquisition of PL, Agilent offers an even wider range of GPC/SEC solutions for all types of polymer characterization of synthetic and bio-molecular polymers, with options for conventional GPC all the way up to complex determinations using multi-column and multi-detection methods.

Introduction

Elastomer is a general term used to describe rubbers – polymers that exhibit elasticity. Elasticity is the ability to deform under external stress but return to the original form after removal of the stress. Elastomers may be thermosets that require curing, or thermoplastics that contain both plastic and elastomeric species, and may be natural or synthetic in origin. Thermosetting elastomers are composed of polymeric chains joined by crosslinks, formed by curing reactions such as the vulcanization of natural rubber, creating a loose lattice structure. This allows chains to move relative to one another during deformation but return to their original positions when relaxed, allowing the material to reversibly extend. Without the crosslinkages the applied stress results in a permanent deformation. Thermoplastic elastomers contain plastic and elastomeric regions within the structure, with weaker non-covalent interactions between chains providing the anchor points, allowing the material to return to its original form after removal of the external force.

Gel permeation chromatography (GPC, also known as size exclusion chromatography, SEC) is a well-known technique for assessing the molecular weight distribution of polymers such as rubber, a property that influences many of their physical characteristics as shown in Table 1. In general, increasing molecular weight leads to higher performance, while an increase in the width of the distribution (the polydispersity) leads to a loss of performance but an increase in the ease of processing.

This application compendium describes the analysis of pre-cure thermosetting and thermoplastic elastomers by GPC.

Agilent Technologies, Inc. produces the most extensive range of GPC columns, standards and instruments that are ideally suited to the analysis of synthetic and natural rubber.

Agilent's columns are rugged and reliable, making them ideal for applications that rely on extremely reproducible analysis, such as in quality control environments. With extensive options in particle and pore size, you can select the column to match the molecular weight of the material under investigation, thereby ensuring you get the best quality data from your analysis. To complement our columns we also manufacture narrow polydispersity standards with very highly characterized molecular weights that are used as calibrants in the GPC analysis of rubber polymers.

The quality of our columns and standards is matched by an array of Agilent GPC instruments that cover the widest possible temperature range, from ambient to 220 °C.

These instruments perform all types of GPC/SEC experiments and can be used to analyze the complete range of polymer materials. Multiple detection options may be included in the instruments, such as light scattering and viscometry, or supplied in stand-alone formats, such as the Agilent evaporative light scattering range. Dedicated analysis software allows the biodegradation properties of the materials to be monitored.



The Agilent PL-GPC 50 Integrated GPC/SEC System

GPC/SEC analysis of natural rubbers

The first commercial elastomeric material was natural rubber, derived from the sap of the rubber tree **Hevea brasiliensis**. This substance is composed of a polymer of isoprene, most often cis-1,4-polyisoprene, although some natural rubber sources are composed of trans-1,4-polyisoprene. In the natural unrefined form, isoprene materials are accompanied by small amounts of proteins, fatty acids, resins and inorganic materials. Natural rubber is an elastomer and a thermoplastic. However, reacting the material with sulfur crosslinks the isoprene chains (a process known as vulcanizing), turns the polymer into a thermosetting material. Analysis of a natural rubber by GPC is always of the non-crosslinked, thermoplastic material.

Table 1. Effects of molecular weight distribution on the properties of elastomers

	Strength	Toughness	Brittleness	Melt Viscosity	Chemical Resistance	Solubility
Increasing Mw	+	+	+	+	+	-
Decreasing distribution	+	+	-	+	+	+

High sensitivity analysis of natural rubber with evaporative light scattering detection

Solutions of natural rubber are generally very difficult to prepare for GPC due to the fact that the polymer contains relatively high levels of 'gel' that are partially crosslinked. Normally, an aliquot of the eluent is added to the weighed sample. This is allowed to swell and dissolve overnight, and then the gel material is filtered out (0.5 µm) prior to GPC analysis.

In this case, the actual polymer concentration can be significantly lower than the original concentration prepared, depending on the gel content of the sample, and therefore detector response, usually RI, tends to be quite poor. The Agilent 380-ELSD evaporative light scattering detector exhibits significantly increased sensitivity compared to an RI and gives much greater response for this application. In addition, RI baseline drift, which commonly occurs, is very much emphasized when the actual peak response is so small. The 380-ELSD always gives a flat baseline which, together with the improved response, makes baseline and peak setting much more reliable for GPC calculations.

RI is also sensitive to system peaks around total permeation that usually occur even when samples are prepared in an aliquot of the eluent. These system peaks can interfere with low molecular weight components that are commonly found in natural rubber samples. With the 380-ELSD, system peaks are eliminated due to evaporation, leaving unadulterated sample peaks in the additives region.

The Agilent PLgel 10 µm MIXED-B columns, with their high efficiency (>35,000 plates/meter) and broad resolving molecular weight range (up to 10,000,000 daltons relative to polystyrene), are the columns of choice for high molecular weight polymers and demanding eluents. Separation of natural rubber reveals that the combination of PLgel MIXED-B columns with the 380-ELSD comprises a highly sensitive system for the discrimination of additives (Figure 1).

Columns: 3 x PLgel 10 µm MIXED-B, 300 x 7.5 mm
Eluent: Toluene
Flow Rate: 1.0 mL/min
Detection: 380-ELSD

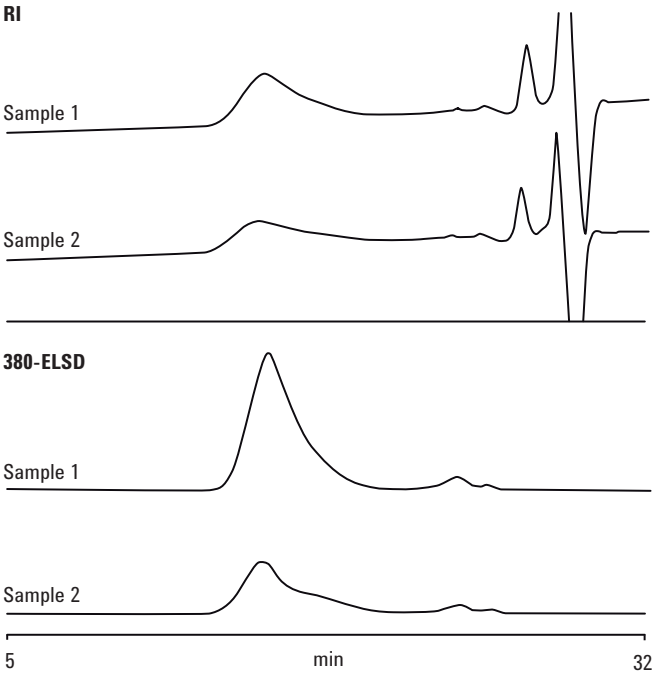


Figure 1. *Stable baseline and no interference from system peaks using the 380-ELSD evaporative light scattering detector (below) compared to RI detection (above) illustrating the advantages of ELSD in this application*

Figure 2 is a magnified view of the additive area revealing the unadulterated peaks in this region of interest.

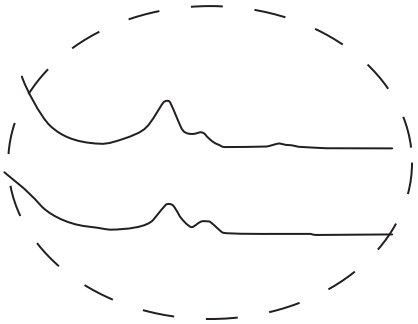


Figure 2. *Magnified view of the 380-ELSD plots showing the additive region - this region is masked with DRI detection, and so ELSD is a better detector for this application*

Analysis of natural rubber with triple detection

Triple detection GPC employs a concentration detector, a viscometer and a light scattering detector to assess the molecular weight distribution and molecular structure of polymers without having to rely on column calibrations. This can be important when analyzing complex materials for which no structurally similar standards are available.

Two samples of natural rubber were analyzed by GPC with triple detection. The objective was to determine why one of the materials had failed in end use. An integrated GPC system was used for the analysis.

The samples were analyzed using an Agilent PL-GPC 50 Integrated GPC/SEC System with differential refractive index detector, an Agilent PL-BV 400RT Online Integrated Viscometer, an Agilent PL-RTLS 15/90 Light Scattering Detector, and Agilent PLgel 10 µm MIXED-B columns. These columns provide high resolution of polymers that have high molecular weights, even in demanding eluents.

Figure 3 is a chromatogram of a natural rubber sample showing responses from the different detectors.

Samples:	2 x Natural rubber
Columns:	3 x PLgel 10 µm MIXED-B, 300 x 7.5 mm
Eluent:	Toluene
Injection Volume:	200 µL
Flow Rate:	1.0 mL/min
Temperature:	50 °C
Detection:	PL-GPC 50 with PL-BV 400RT Viscometer and PL-RTLS 15/90 light scattering detector

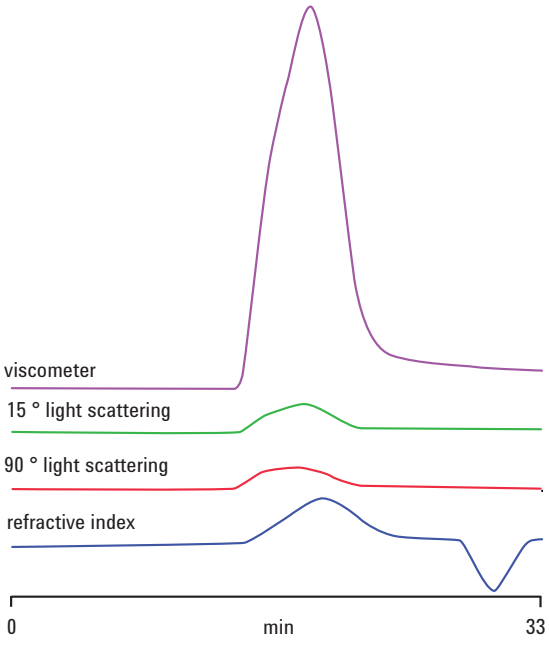


Figure 3. *Raw triple detection data for a natural rubber showing typical peak shapes observed for these materials*

Figure 4 indicates that one of the samples is considerably higher in molecular weight than the other, although the Mark-Houwink plots show that the two materials are structurally similar (Figure 5).

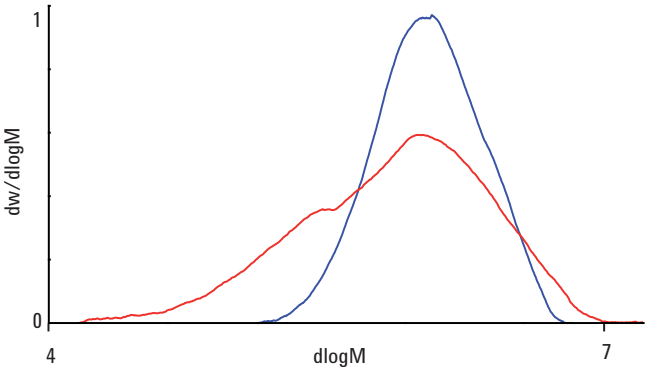


Figure 4. *Overlaid triple detection molecular weight distributions of two natural rubbers with very different distributions and therefore very different final properties*

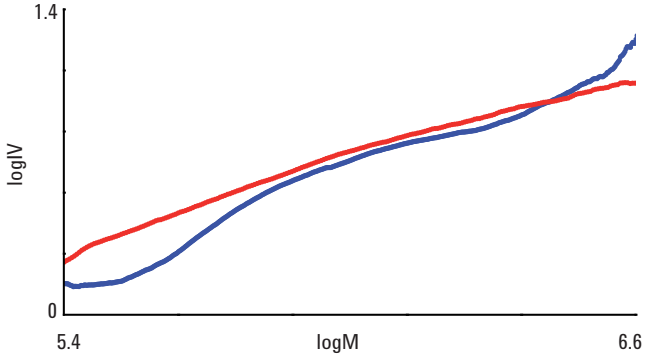


Figure 5. *Overlaid Mark-Houwink plots for two natural rubbers showing the similarity in structure of the materials, with deviation only at low molecular weight*

GPC/SEC analysis of synthetic rubbers

Analysis of synthetic polybutadiene with triple detection

Polybutadiene was one of the first types of synthetic elastomer to be invented and has largely replaced natural rubber in a wide variety of industrial applications.

Two Agilent PLgel 5 µm MIXED-C columns were used for this analysis with the results shown in Figures 6 and 7. The polybutadiene sample was prepared accurately at a nominal concentration of 2 mg/mL in tetrahydrofuran and injected into the system without further treatment. For the purpose of light scattering calculations, an average dn/dc was used for the sample.

Mark-Houwink (log intrinsic viscosity versus log M) plots (Figure 8) were generated from the viscometry and light scattering data. The curvature in the Mark-Houwink plot may be a result of structural changes in the polymer as a function of molecular weight.

Sample:	Polybutadiene
Columns:	2 x PLgel 5 µm MIXED-C, 300 x 7.5 mm
Eluent:	THF
Injection Volume:	100 µL
Flow Rate:	1.0 mL/min
Detection:	PL-GPC 50 with PL-BV 400RT and PL-RTLS 15/90

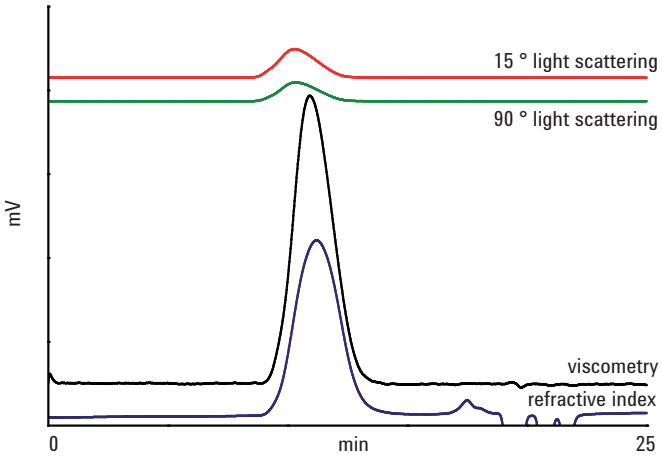


Figure 6. *Triple detection of a polybutadiene showing typical data for this type of sample*

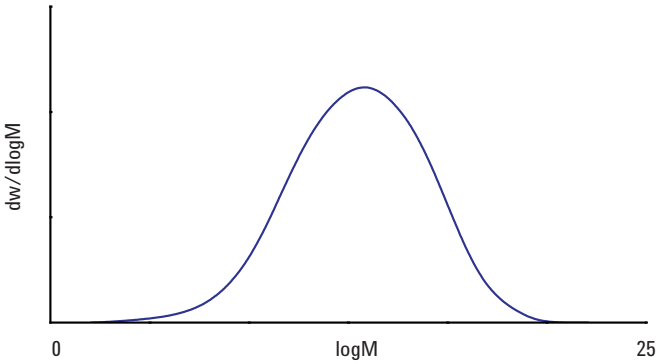


Figure 7. *Molecular weight distribution of a polybutadiene with a broad gaussian peak shape*

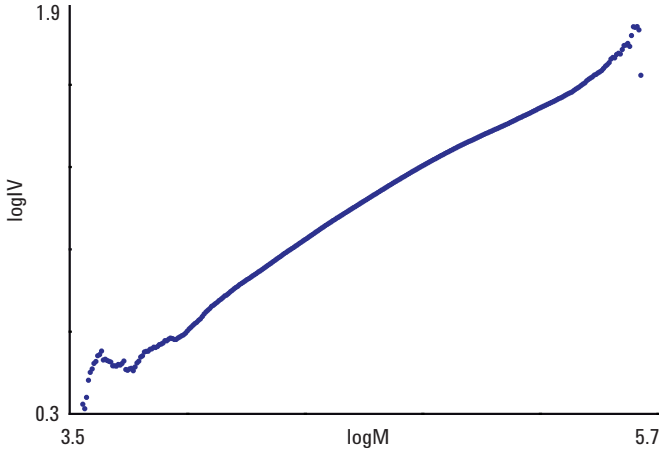


Figure 8. *Mark-Houwink plot of a polybutadiene with curvature that may be attributed to structural changes as a function of molecular weight*

General synthetic elastomer analysis

Polyisoprene can be produced synthetically. Together with polybutadiene and styrene butadiene, it is a common elastomeric material. Polybutadiene is a synthetic rubber manufactured from the monomer 1,3-butadiene. With high wear resistance, it is commonly used in tire manufacture, and to coat electronic assemblies due to its extremely high electrical resistivity. Polybutadiene exhibits 80% recovery after stress, one of the highest stress-recovery values of a synthetic material.

Styrene butadiene rubber (SBR) is a synthetic rubber copolymer of styrene and butadiene. With good abrasion resistance it is widely used in car tires, after blending with natural rubber.

The extended operating range of the PLgel 10 µm MIXED-B column (up to 10,000,000 MW) makes it ideally suited to the analysis of a wide range of high molecular weight elastomers (Figure 9). Sample solutions are routinely filtered prior to injection to remove insoluble "gel fractions", common to most elastomers.

Columns:	3 x PLgel 10 µm MIXED-B, 300 x 7.5 mm
Eluent:	THF
Flow Rate:	1.0 mL/min
Loading:	0.2% w/v, 100 µL
Temperature:	40 °C
Injection Volume:	200 µL
Detection:	Agilent PL-GPC 220

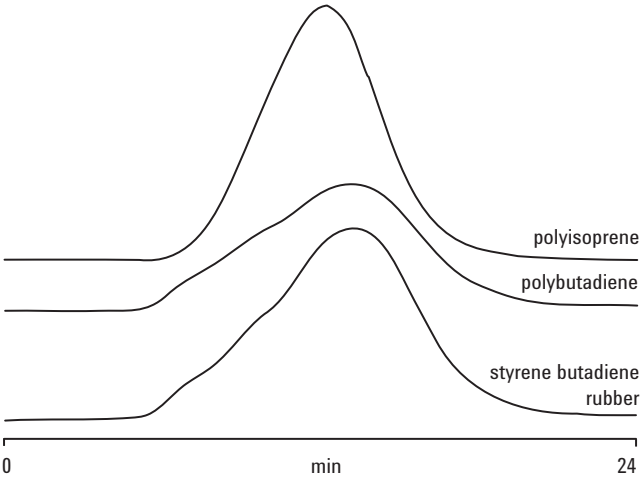


Figure 9. *Chromatograms of three types of synthetic rubber with multimodal peak shapes*

Hexane is a good solvent for butyl rubber although it can be chromatographed using other solvents, such as THF. The polarity of hexane is very low compared with more traditional solvents for GPC such as THF. However, it can be used successfully with PLgel columns (Figure 10).

Columns:	3 x PLgel 10 µm MIXED-B, 300 x 7.5 mm
Eluent:	Hexane
Flow Rate:	1.0 mL/min
Detection:	PL-GPC 50

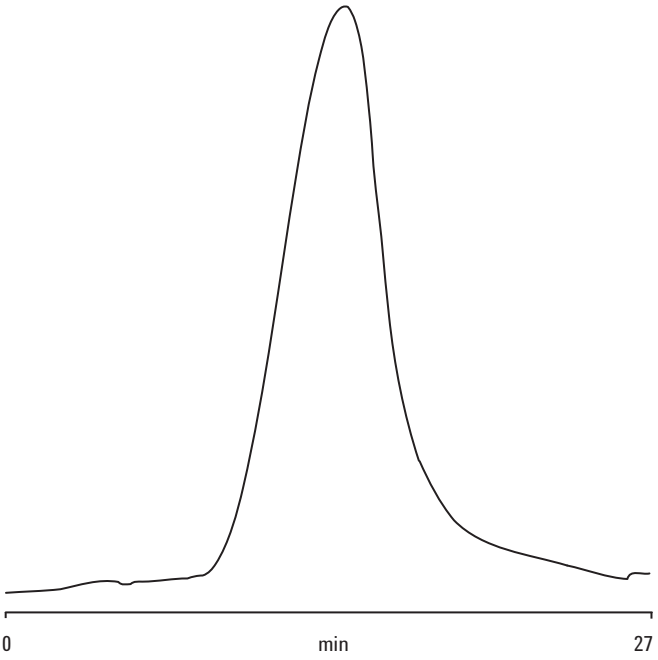


Figure 10. *Butyl rubber chromatogram in hexane showing that a good peak shape can be obtained*

Commercial grades of styrene butadiene rubber can contain very high molecular weight fractions and so, for successful GPC separations, the sample concentration must be minimized in order to avoid viscous streaming effects. Some grades of SBR can also contain low molecular weight mineral oil as a modifier (known as oil-extended grades) that can be resolved from the polymer peak, thus permitting quantification using Agilent ELSD (Figure 11).

Columns:	2 x PLgel 20 µm MiniMIX-A, 250 x 4.6 mm
Eluent:	THF
Flow Rate:	0.3 mL/min
Loading:	1 mg/mL, 100 µL
Detection:	380-ELSD (neb=45 °C, evap=90 °C, gas=0.7 SLM)

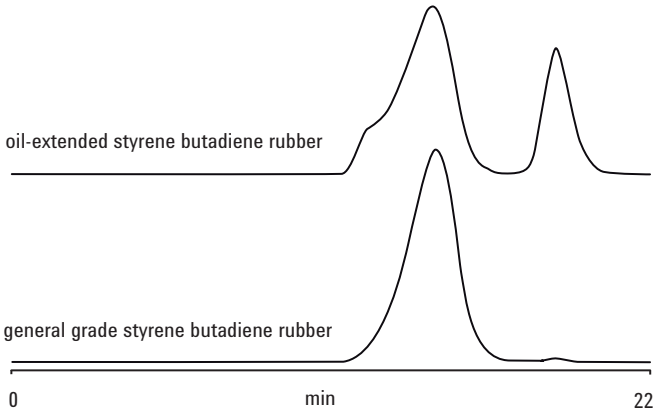


Figure 11. *Chromatograms of two styrene butadiene rubbers, one oil-extended, showing the presence of the low molecular weight additive*

The sample of oil-containing SBR, shown in Figure 12, was analyzed using refractive index detection. To ensure dissolution, the sample was warmed to 50 ºC and gently stirred for three hours. Filtering using 0.5 µm filters is recommended to remove any gel fractions. The PLgel MIXED-B packing permits resolution of both polymer and oil peaks.

Columns:	2 x PLgel 10 µm MIXED-B, 300 x 7.5 mm
Eluent:	THF
Flow Rate:	1.0 mL/min
Injection Volume:	100 µL
Detection:	PL-GPC 50

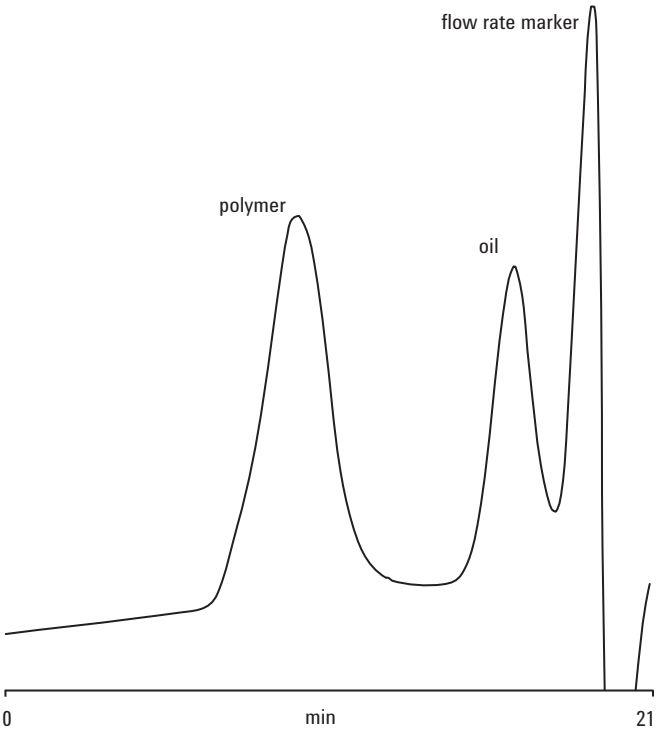


Figure 12. *Chromatogram of an oil-extended styrene butadiene rubber showing the presence of a low molecular weight oil additive*

Polydimethyl siloxane analysis using GPC/ viscometry

Polydimethyl siloxane (PDMS) is a non-toxic, non-flammable silicon-based polymeric material noted for its unusual rheological behavior. Composed of polymer chains of formula $(CH_3)_3SiO[SiO(CH_3)_2]_nSi(CH_3)_3$, PDMS is a viscoelastic material which, with long flow times or at high temperatures behaves like a liquid, and with short flow times or at low temperatures behaves like a rubber. PDMS is produced in a range of grades from liquids through to rubbery semi-solids depending on the molecular weight of the constituent chains. It is a widely used material and can be found in applications such as silicone caulks, lubricants, damping fluids and heat transfer fluids, as well as in breast and knuckle implants. It is also a food additive (E900), used as an anti-foaming and anti-caking agent.

PDMS was analyzed by GPC using the PL-GPC 50 Integrated GPC/SEC System. Due to the importance of the viscometric properties of the material in many final applications, a PL-BV 400RT viscometer was included in the PL-GPC 50 as well as the standard refractive index detector. Results are shown in Figures 13 and 14. This combination of detectors also allows analysis of the material by the Universal Calibration method, giving accurate molecular weights that are not reliant on the chemistry of the standards used for calibration (in this case, polystyrene standards). Although PDMS is soluble in tetrahydrofuran, it is also isorefractive with this solvent and, therefore, THF is not suitable for the analysis and toluene is a more suitable solvent.

Columns:	2 x Agilent PolyPore, 300 x 7.5 mm
Eluent:	Toluene
Flow Rate:	1.0 mL/min
Injection Volume:	100 µL
Detection:	PL-GPC 50, PL-BV 400RT

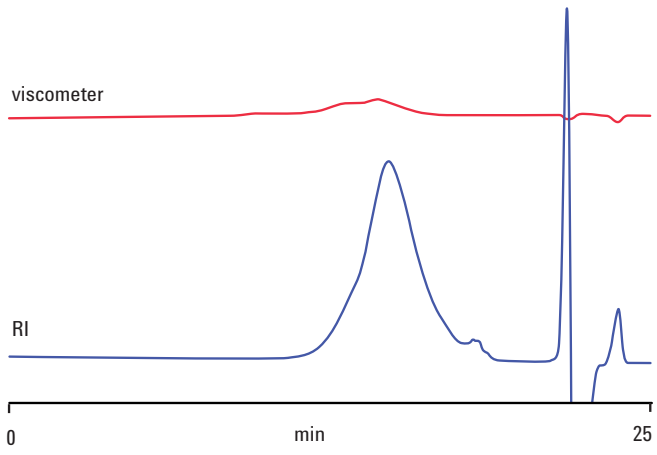


Figure 13. *Example overlaid refractive index and viscometer chromatograms for a sample of polydimethyl siloxane showing typical peak shapes*

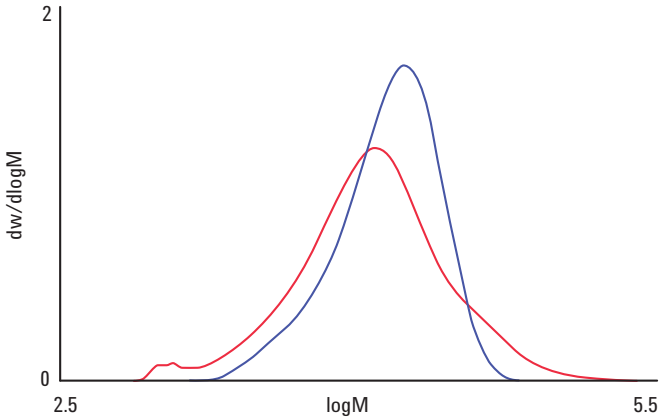


Figure 14. *Overlaid molecular weight distributions for two different grades of polydimethyl siloxane with different performance characteristics*

Although quite different in molecular weight, the Mark-Houwink plot (Figure 15) shows that the two materials are structurally very similar, indicating that their viscoelastic behavior as a function of molecular weight would be comparable.

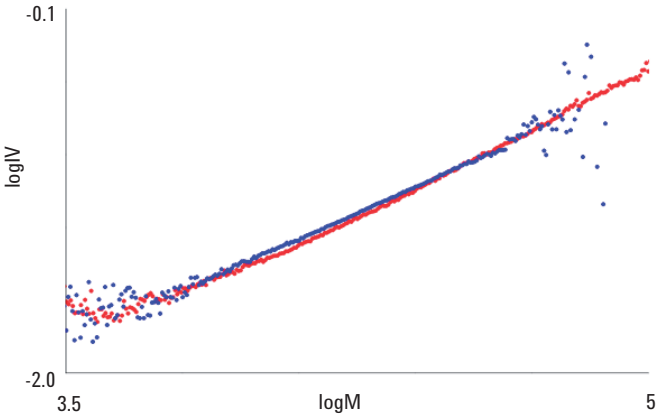


Figure 15. *Mark-Houwink plots calculated for two samples of polydimethyl siloxane show that the materials are structurally very similar*

Analysis of poly(styrene/butadiene) copolymers by conventional GPC

A poly(styrene/butadiene) block copolymer mimics many of the properties of natural rubber and has applications in a wide variety of industrial areas. Its characteristics are provided by the hard polystyrene chains being surrounded by a network of rubbery polybutadiene, which provides strength and flexibility over a large temperature range. The copolymer is a thermoplastic elastomer and therefore can be easily used in manufacturing by injection molding, or blended into an existing product to increase elasticity or impart toughness. The molecular weight distribution is critical, as any homopolymer will significantly affect the resultant end properties.

In the analysis described here distinct differences were observed arising from the presence of homopolymers along with the intended copolymer (Figures 16 and 17).

Columns:	2 x PLgel 5 µm MIXED-C, 300 x 7.5 mm
Calibration Standards:	Polystyrene EasiVial
Eluent:	THF (250 ppm BHT)
Temperature:	40 °C
Injection Volume:	100 µL
Detection:	PL-GPC 50

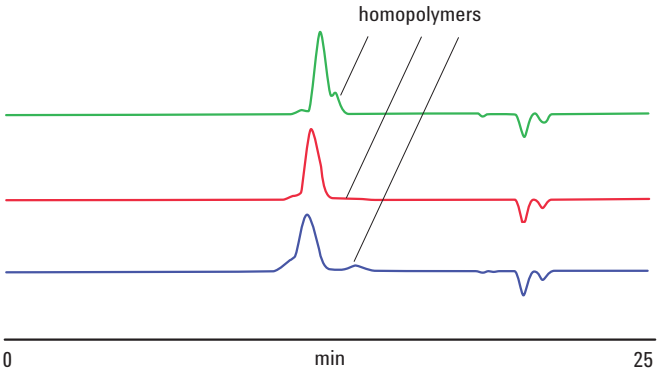


Figure 16. *Chromatograms for styrene butadiene rubbers showing the presence of homo- and copolymers*

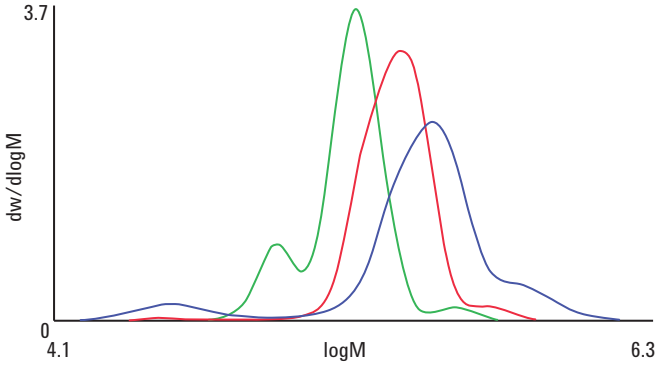


Figure 17. *Overlaid molecular weight distributions for the styrene butadiene rubber samples showing a clear change in molecular weight distribution between samples*

More Agilent solutions for the analysis of rubber

Although well-suited to the analysis of natural and synthetic rubber, GPC is not the only analytical technique available for the examination of these important materials. Agilent offers a range of instruments and consumables that can be used to test elastomers in environmental protection, recycling schemes, confirmation of equivalents, deformulation, quality control and failure analysis. Agilent instruments are particularly valuable for the detection of additives or modifiers, ubiquitous components found in most commercially useful rubbers.

Spectroscopy

Both the AA and ICP-OES techniques are suitable for determining the "filler" and trace levels of metals in compounded rubber products. For toys made from rubber materials, there are worldwide standards applicable that require the determination of a range of toxic elements.

For example, the US Consumer Product Safety Improvement Act of 2008 (CPSIA), EN 71 Part 3 and AS/NZS ISO 8124.3:2003 standards define requirements for testing the migration of inorganic elements from toys and children's products. Agilent AA and ICP-OES instruments meet and exceed these testing requirements. Analysts may also use AA and ICP-OES for monitoring and evaluating the environmental impact of waste rubber products (especially used tires) in land fill sites, artificial reefs, or assessing trace element levels in fly ash from tire-derived fuel.

NMR

Rubber NMR is a well-established subfield of Solids NMR. MAS (magic angle spinning) is used to improve shimming, and rubber spectra could be run in either a Solids MAS probe or a Nanoprobe. More advanced experiments that measure relaxation properties are employed to analyze dynamics and morphology, such as domain sizes, and the results have been used to explain the dielectric and mechanical properties of rubber. Agilent's 400, 500 or 600 MHz NMR systems are ideal for such tasks when equipped with accessories for solid state NMR.



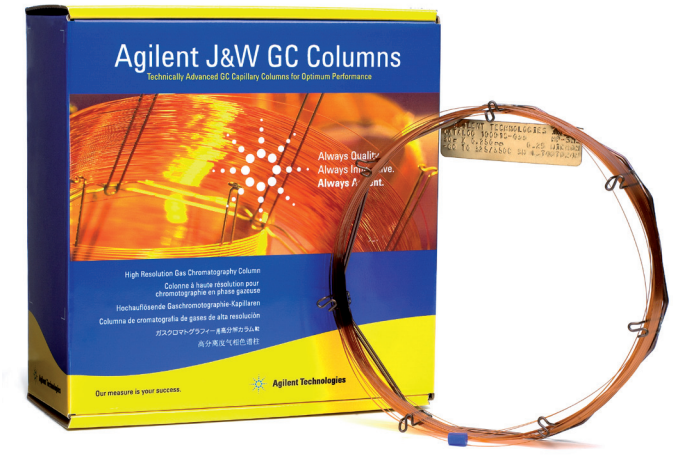
The Agilent 400-MR Magnetic Resonance Spectrometer is the system of choice for laboratories requiring a compact and easy-to-use instrument that delivers fast and reliable results day in and day out

GC

Toxic gases that are emitted during the processing and curing of rubber goods can be monitored using Agilent gas chromatography. The same technique helps ensure product safety when determining levels of plasticizers or nitrosamines in food rubbers, such as dummies for babies or teats for baby bottles. For the two ASTM rubber methods, we recommend Agilent J&W CP-PoraBOND Q GC columns to detect acrylonitrile in nitrile rubber.



The Agilent 7890A GC with 7693A Series Automatic Liquid Sampler



Agilent J&W offers the largest selection of the highest quality GC columns available today

FTIR

FTIR spectroscopy has a powerful ability to provide both chemical and structural information of a sample. Rapid, non-destructive, chemical identification is possible with easy-to-use Attenuated Total Reflectance (ATR) accessories. Agilent also provides thermally controlled accessories that enable users to investigate their elastomer sample under their desired environmental conditions. Or, if combined with thermogravimetric analysis (TGA), Agilent's hyphenated TGA/FTIR analyzer delivers real-time monitoring of gases evolved from rubber samples, and enables complex chemistries to be unravelled and understood. Our TGA/FTIR systems consist of an FTIR spectrometer and TGA/IR interface accessory with data interpretation performed using powerful Resolutions Pro software.



The Agilent 600 Series FTIR provides the highest level of sensitivity combined with detailed structural and compositional information for information-rich detection

Ordering information

Agilent offers robust instrumentation, application-based consumables, and customer-focused services, backed by our global team of product and applications experts, ready to help you solve your analytical challenges. Whether you're monitoring impurities in drinking water, designing new therapeutic drugs or developing cleaner fuels, our solutions deliver the sensitivity, flexibility and productivity your laboratory requires.

Columns

Description	Part No.
Agilent PLgel 5 µm MIXED-C, 300 x 7.5mm	PL1110-6500
Agilent PLgel 10 µm MIXED-B, 300 x 7.5 mm	PL1110-6100
Agilent PLgel 20 µm MiniMIX-A, 250 x 4.6 mm	PL1510-5300
Agilent PolyPore, 300 x 7.5 mm	PL1113-6500
Agilent EasiVial PS-H (2 mL)	PL2010-0201
Agilent EasiVial PS-H (4 mL)	PL2010-0200
Agilent EasiVial PS-L (2 mL)	PL2010-0401
Agilent EasiVial PS-L (4 mL)	PL2010-0400
Agilent EasiVial PS-M (2 mL)	PL2010-0301
Agilent EasiVial PS-M (4 mL	PL2010-0300
Agilent PS-H 2 mL Tri-Pack (90 Vials)	PL2010-0202
Agilent PS-H 4 mL Tri-Pack (90 Vials)	PL2010-0203
Agilent PS-L 2 mL Tri-Pack (90 Vials)	PL2010-0402
Agilent PS-L 4 mL Tri-Pack (90 Vials)	PL2010-0403
Agilent PS-M 2 mL Tri-Pack (90 Vials)	PL2010-0302
Agilent PS-M 2 mL Tri-Pack (90 Vials)	PL2010-0303

Standards

Description	Part No.
Agilent EasiVial PS-H (2 mL)	PL2010-0201
Agilent EasiVial PS-H (4 mL)	PL2010-0200
Agilent EasiVial PS-L (2 mL)	PL2010-0401
Agilent EasiVial PS-L (4 mL)	PL2010-0400
Agilent EasiVial PS-M (2 mL)	PL2010-0301
Agilent EasiVial PS-M (4 mL	PL2010-0300
Agilent PS-H 2 mL Tri-Pack (90 Vials)	PL2010-0202
Agilent PS-H 4 mL Tri-Pack (90 Vials)	PL2010-0203
Agilent PS-L 2 mL Tri-Pack (90 Vials)	PL2010-0402
Agilent PS-L 4 mL Tri-Pack (90 Vials)	PL2010-0403
Agilent PS-M 2 mL Tri-Pack (90 Vials)	PL2010-0302
Agilent PS-M 2 mL Tri-Pack (90 Vials)	PL2010-0303

Instruments

Description	Part No.
Agilent PL-GPC 220 Integrated GPC/SEC System	PL0820-0000
Agilent PL-GPC 50 Integrated GPC/SEC System	PL0870-8500
Agilent PL-BV 400HT Online Integrated Viscometer	PL0810-3050
Agilent PL-BV 400RT Online Integrated Viscometer	PL0810-3060
Agilent PL-HTLS 15/90 Light Scattering Detector for PL-GPC 220	PL0640-1200
Agilent PL-RTLS 15/90 Light Scattering Detector for PL-GPC 50	PL0640-1210
Agilent 380-LC Evaporative Light Scattering Detector (110 V)	PL0890-0110
Agilent 380-LC Evaporative Light Scattering Detector (240 V)	PL0890-0240



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Suggestions for further reading

Agilent has published application compendia on biodegradable polymers, engineering polymers, polyolefin analysis, and low molecular weight resins. In addition, we also offer a comprehensive and informative range of literature for all aspects of GPC/SEC, including application notes, datasheets and technical overviews.

Publication	Publication number
Introduction to GPC/SEC	5990-6969EN
GPC/SEC column selection guide	5990-6868EN
Biodegradable polymers	5990-6920EN
Engineering polymers	5990-6970EN
Polyolefin analysis	5990-6971EN
Low molecular weight resins	5990-6845EN

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Agilent Technologies

Analysis of polyolefins by GPC/SEC

Application Compendium

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The Measure of Confidence

Agilent Technologies

Contents Page

Polyolefin analysis by GPC/SEC

Introduction....3
The GPC separation mechanism....4
GPC system requirements for polyolefin analysis....5

Sample preparation

Agilent PL-SP 260VS Sample Preparation System....6

System, software and standards

The Agilent PL-GPC 220 Integrated GPC/SEC System....7
Agilent Cirrus GPC Software – the universal GPC solution....9
Standards for column calibration in polyolefin analysis....10
Recommendations for setting up a GPC/SEC system for polyolefin analysis....11

Columns for GPC analysis of polyolefins

Agilent PLgel Olexis....12

Polyolefin applications

Columns for high-molecular-weight polyolefins....14
Columns for lower-molecular-weight polyolefins....15
Repeatability study 1....16
Repeatability study 2....17

Specialist detectors

Multi-detector options for polyolefin analysis....19
GPC viscometry – analysis using a concentration detector and viscometer....19
GPC light scattering – analysis employing a concentration detector and a light scattering detector....19
GPC triple detection – analysis using concentration, viscometry and light scattering data....20
Comparisons between conventional GPC, GPC viscometry, GPC light scattering and GPC triple detection....20

Branching

Comparing long-chain branching in polyethylenes....21
Branching analysis of polyethylenes with Cirrus GPC Multi Detector Software....22
Analysis of branching in linear low-density polyethylene (LLDPE)....24
GPC/FTIR analysis of polyethylene....25

More Agilent solutions for polyolefin analysis....27

Polyolefin analysis by GPC/SEC

Introduction

Polyolefin is a general term describing polymers created from simple olefins or alkenes. Many different types of olefin exist, from the most simple, ethylene, to alpha-olefins of increasing complexity. Polyolefins are of great interest as two of them, polyethylene (polythene) and polypropylene, are among the highest tonnage polymers produced in the world. Interest in the analysis of polyolefins comes from the desire to create new materials with custom properties, from the development of new catalysts and from the need to perform quality control on polymer production.

Agilent has a long history of involvement in the analysis of polyolefins by gel permeation chromatography (GPC, also known as size exclusion chromatography, SEC). This application booklet describes Agilent's product portfolio for polyolefin analysis. Instrumentation, software, columns and standards are described, providing a complete package for the analysis of these important products. In addition, a wide range of applications are included that illustrate the performance of the complete solutions for polyolefin analysis offered by Agilent.

Gel permeation chromatography is a well-known technique for assessing the molecular weight distribution of polymers such as polyolefins. Molecular weight influences many of their physical characteristics, as shown in Table 1. In general, increasing molecular weight leads to higher performance, while an increase in the width of the distribution (the polydispersity) leads to a loss of performance but an increase in the ease of processing.

Many polyolefins, typically those containing over 10% ethylene and polypropylene monomers, are of limited solubility in a number of solvents. This is because the characteristic high strength and toughness of these materials results from their high crystallinity. Increased crystallinity requires break up of any inter-chain bonds in order to dissolve the material. Several solvents can be used, but in general the most effective is trichlorobenzene, a viscous solvent with a distinct odor. Ortho-dichlorobenzene is also used in some laboratories, but solubility in this solvent is less effective.

Table 1. *Effects of molecular weight (Mw) and the impact of decreasing the width of distributon of Mw on polyolefins*

	Strength	Toughness	Brittleness	Melt viscosity	Chemical resistance	Solubility
Increasing Mw	+	+	+	+	+	-
Decreasing distribution	+	+	-	+	+	+

Polymer Laboratories was formed in 1976 to offer high quality columns, standards, instruments, and software for GPC/SEC. For over 30 years the company developed many market-leading products, including PLgel, PL aquagel-OH, PlusPore, PLgel Olexis, PolarGel columns, and EasiVial standards. Built on advanced in-house manufacturing technology, PL's products have the highest reputation for quality and performance, backed up by world-class technical and applications support.

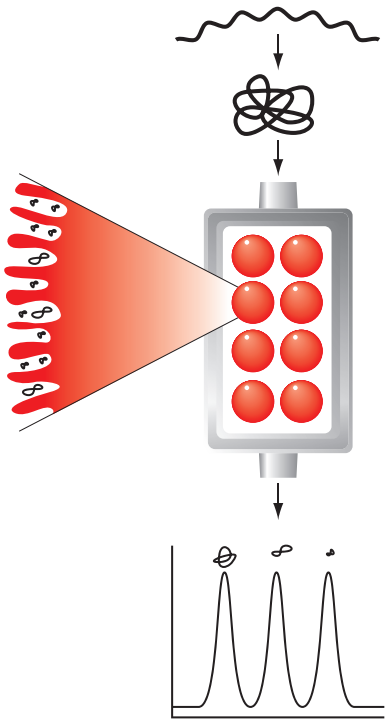
With the acquisition of PL, Agilent offers an even wider range of GPC and SEC solutions for all types of polymer characterization of synthetic and bio-molecular polymers, with options for conventional GPC all the way up to complex determinations using multi-column and multi-detection methods.

The GPC separation mechanism

- Polymer molecules dissolve in solution to form spherical coils with size dependent on molecular weight
- Polymer coils introduced to eluent flowing through a column
- Column packed with insoluble porous beads with well-defined pore structure
- Size of pores similar to that of polymer coils
- Polymer coils diffuse in and out of the pores
- Result is elution based upon size – large coils first, smaller coils last
- Size separation converted to molecular weight separation by use of a calibration curve constructed by the use of polymer standards

Highly crystalline polymers such as polyethylene are soluble only at high temperatures. This is because elevated temperatures are required to break down the ordered crystalline structure, and on cooling the material will re-crystallize and precipitate from solution. For these applications, high temperature is required throughout the entire analysis to ensure that the samples remain in solution. This places several requirements on the instrument for the successful analysis of polyolefins.

- Solvent choice is limited, mainly to 1,2,4-trichlorobenzene (TCB)
- Elevated temperature is required for dissolution, typically for 1 to 4 hours depending on molecular weight and crystallinity
- Column selection must be appropriate for the application in terms of molecular weight resolving range and efficiency of separation
- A high temperature GPC system is required to maintain all components at the analysis temperature, typically 135 to 170 °C, depending on molecular weight and crystallinity



Key

Smaller coils can access many pores

Larger coils can access few pores

Very large coils access very few pores

GPC system requirements for polyolefin analysis

Autosampler, detectors, columns, injection valve and transfer tubing must all be capable of handling elevated temperatures during polyolefin analysis. A typical system schematic is shown in Figure 1.

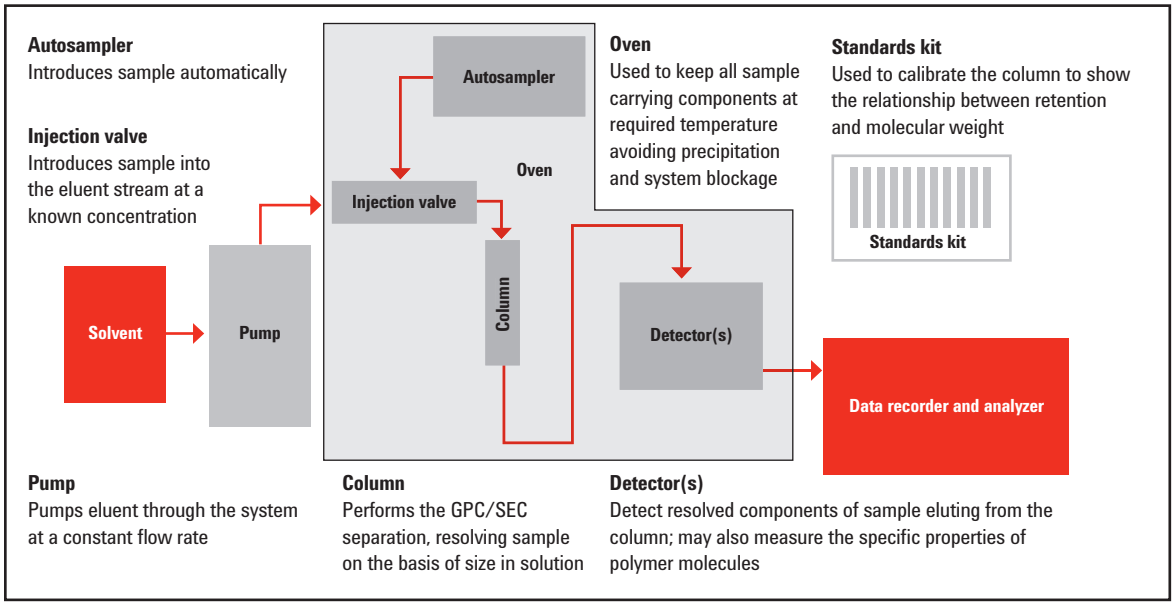


Figure 1. *Schematic of a GPC system for polyolefin analysis*

Sample preparation

Preparing polyolefin samples is time-consuming because high temperatures and long heating times are required to dissolve the sample (Table 2). Many polyolefins also display a lower density than common analytical solvents such as TCB, and so agitation of the sample is essential to ensure complete dissolution. Filtration may also be necessary to remove insoluble material such as fillers.

Table 2. *Preparing a polyolefin sample for analysis*

Material	Typical concentration (mg/mL)	Typical prep temp (°C)	Typical heating time (h)
Olefin wax	2 to 3	150	1
General PE or PP	2	150	4
Ultra-high-molecular-weight polyolefin	0.25 to 0.5	150	4 to 8

Agilent PL-SP 260VS Sample Preparation System

The PL-SP 260VS is designed for the manual dissolution and filtration of samples such as polyolefins prior to GPC analysis. The unit combines controlled heating across a temperature range of 30 to 260 °C (± 2 °C), with gentle agitation, user-selectable between 85 to 230 (± 10%) rpm. With its temperature range and speed capabilities, the PL-SP 260VS is ideal for a wide range of polymer types, including even the most difficult of samples such as ultra-high-molecular-weight polyethylene.

Choice of vial types

The removable aluminium blocks for the heated compartment are available in several formats to accommodate a variety of vial types. The Standard Accessory Kit is used with standard sample preparation 20 mL vials (supplied) and either PL-GPC 220 2 mL autosampler vials or 4 mL autosampler vials from other vendors. The Custom Accessory Kits let you choose alternative vials, if necessary.



Agilent PL-SP 260VS Sample Preparation System

Efficient dispensing

A unique pipettor device efficiently dispenses filtered sample solution from the sample preparation vial directly into destination (autosampler) vials with minimal handling.

Choice of filtration media

Filtration of polyolefin samples is often required to remove insoluble fillers or gel content (Figure 2). Two filter media are available:

- Glass-fiber (nominal porosity 1 µm) – the preferred system for general applications (Figure 2)
- Porous stainless steel (nominal porosity 0.5, 5, and 10 µm)

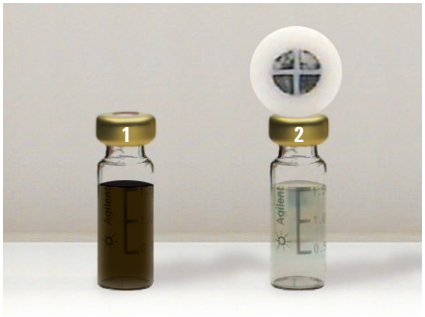


Figure 2. *Filtering a carbon black polyethylene solution – 1. without filtration, 2. after filtration using a 1 µm glass-fiber filter*

System, software and standards

The Agilent PL-GPC 220 Integrated GPC/SEC System for polyolefin analysis

The PL-GPC 220 is a leading system for the analysis of polyolefins at high temperature. Containing a number of features that have been specifically designed for polyolefin analysis, the PL-GPC 220 is the most versatile instrument for gel permeation chromatography.

Widest temperature range

The PL-GPC 220 features the widest operating range available: 30 to 220 °C, permitting analysis of virtually any polymer in any solvent. The multi-heater, forced-air oven is extremely stable, and accurately controls the temperature to within 0.05 °C. This minimizes detector baseline drift, ensuring the reproducible retention times so important in GPC.

High-precision isocratic pump – unrivalled reproducibility for precise results

The PL-GPC 220 incorporates a high-precision pump for the best pump performance available. Unbeatable flow reproducibility of 0.07% is achieved, not only in THF at near-ambient temperature, but also in TCB at temperatures above 140 °C.

Easy-access oven – changing columns and routine maintenance made simple

The column oven can comfortably hold six, 300 x 7.5 mm GPC columns. The oven operates at a convenient angle to allow for easy access for changing columns and the injector loop, providing comfortable and safe operation.



Agilent PL-GPC 220 Integrated GPC/SEC System

Enhanced RI sensitivity and stability

The improved refractive index (RI) detector includes a new photodiode and uses fiber optic technology to maximize sensitivity while minimizing baseline drift and noise, vital for good GPC/SEC. This RI detector delivers outstanding signal-to-noise ratios, even at 220 °C (Figure 3).

Conditions

Columns: 2 x Agilent PLgel 10 µm MIXED-B, 300 x 7.5 mm (Part No. PL1110-6100)
Flow Rate: 1 mL/min
Inj Vol: 200 µL
Detector: PL-GPC 220

Peak Identification

1. Mp = 1,460,000, conc. = 0.62 mg/mL
2. Mp = 9,860, conc. = 1.08 mg/mL

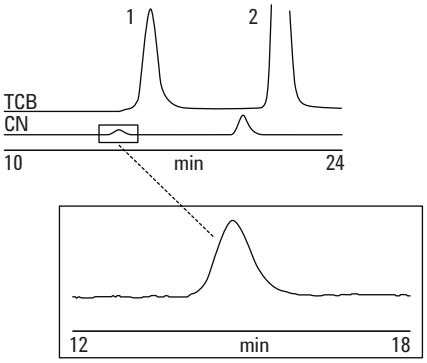


Figure 3. *Excellent signal-to-noise demonstrated in the separation of polystyrene standards*

Safety first – solvent leak detection and automated shutdown

Agilent's GPC/SEC systems incorporate integral sensors that constantly monitor the system. Vapor sensors are fitted in both the solvent module and column oven. The sensors can be programmed for sensitivity according to the solvent in use.

In the case of an unattended error, the system selects and activates the appropriate shutdown sequence depending on the nature of the error. Low solvent flow will be maintained, where possible, to avoid damage to valuable GPC columns.

An audit trail feature offers full status and error logging for system traceability.

Customized upgrade solutions

The oven easily handles multiple-detector upgrades such as light scattering and viscometry, and coupling to other techniques such as TREF (temperature rising elution fractionation), FTIR (fourier transform-infrared spectroscopy) and ELSD (evaporative light scattering detection). The oven holds up to four detectors in combination. For example, integrating RI, viscometry and light scattering would provide complete polymer characterization.

PC control – easy to program, easy to use

The PL-GPC 220 system for polymer characterization up to 220 °C features intuitive, comprehensive PC software control for full and flexible system management. With safety a pre-requisite, PC control uniquely permits remote use so that you do not need to be in the laboratory.

Interactive color-coded graphics provide ease-of-use. Simply click on the color-coded modules via the main screen to alter any run parameters. Flow rate, temperature and autosampler sequence are quickly and easily updated, and on-screen help is always available, if required (Figure 4).

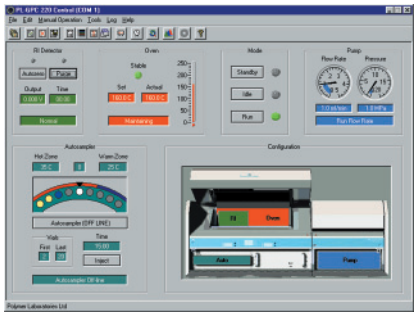


Figure 4. *Software control of the PL-GPC 220*

The time estimator calculates the amount of solvent you will need to run your samples. Input the day and time you want the system to start, then load your samples into the autosampler and let the PL-GPC 220 take care of the analysis for you.

The PL-GPC 220 is designed for true unattended operation. The system gradually heats to the analysis temperature, while the pump maintains a low flow of solvent through the column set. Once temperature is reached and stable, the pump ramps gradually to the flow rate required to run your sample. The PL-GPC 220 then automatically purges the RI detector and autozeros the baseline. Detector output is monitored and when stable, the autosampler loads and injects the first sample. Once the run sequence is complete, the flow rate automatically reduces to conserve solvent.

Integrated solvent delivery – safety by design

The solvent module in the PL-GPC 220 provides a safe, controlled environment in which solvent and waste are managed. Solvent handling is fully integrated and vented for operator safety, and the system does not need to be located in a fume hood.

The PL-GPC 220 includes an integral solvent degasser with a choice of solvent reservoir from 2 L bottles up to a 13 L stainless-steel tank. The solvent delivery module is thermostatically controlled to 30 °C, which ensures efficient, continuous and reproducible solvent delivery, even if the solvent is viscous or may be solid at near-ambient temperature (Figure 5).



Figure 5. *Agilent PL-GPC 220 integrated solvent delivery system*

Dual-zone-heated autosampler – no degradation of samples before injection

Agilent's innovative autosampler accommodates 39 samples in industry-standard 2 mL vials. Injection precision has been measured at better than 1% RSD with no cross contamination between samples, and without the need for rinse vials. The autosampler design features dual-zone heating to minimize thermal degradation. The warm and hot zones are independently programmable from ambient to 220 °C, and so the samples in the carousel waiting for injection are maintained at a lower holding temperature, then heated to analysis temperature prior to injection.

The vial is transferred to the column oven where the sample equilibrates before injection. This minimizes baseline disturbance and completely eliminates the risk of sample precipitation.

Agilent Cirrus GPC Software – the universal GPC solution

Cirrus is the powerful suite of GPC/multi-detector software from Agilent. Polymer Laboratories, now a part of Agilent, has been a supplier of industry-standard GPC software since the 1980s. Cirrus makes GPC calculations easy, whether in conventional GPC using a concentration detector or for multi-detector analysis with light scattering and viscosity.

Integration with existing LC software

Powerful, yet easy to use and learn, Cirrus is available for standalone GPC or for integrating GPC with LC. Cirrus utilizes the latest advances in software design to provide comprehensive calculation options, customized reporting, and high-resolution data capture with the Agilent PL DataStream.

Modular, flexible, and scalable

Cirrus is made to grow as your needs change. A suite of modules provides support for a variety of GPC techniques, such as multi-detector GPC, online FTIR detection and short-chain branching (SCB). Cirrus can be run on a standalone PC or provide a networked GPC solution.

Easy-to-use interface

Cirrus uses an intuitive graphical-user interface, so straightforward that new users can report results within an hour of installing the software. Cirrus is based on Agilent's Workbook concept to provide:

- A simple 'container' for data, parameters and results
- Automatic archiving of chromatograms, calibrations, and results
- Data traceability and data integrity
- Templates allowing predefinition of parameters and report content

Comprehensive calibration and calculation options

Cirrus offers a choice of calibration options.

- Conventional calibration using narrow standards
- Universal Calibration by viscometry or using Mark-Houwink coefficients
- Replicate entries of calibration points
- Three broad-standard calibration methods
- Averages and distributions can be calculated for any number of peaks in a chromatogram
- % of material can be reported for specific MW limits

A calibration overlay facility lets you view the effects of column performance over time.

Reviewing, collating, and condensing results

Cirrus meets the requirements of both QC/Routine and R&D environments, providing fully automated or interactive analysis. The software offers a number of powerful options to review, compare and extract information from archived data and results for inclusion into final reports.

Chromatograms and results can be reviewed both textually and graphically. This information can be exported in a variety of industry-standard formats. A powerful report designer provides total flexibility in report content and presentation. In Cirrus, all parameters relating to a chromatogram or results file are easily accessible via a comprehensive range of export options. Cirrus also ensures that data integrity and traceability are maintained throughout all operations.

Standards for column calibration in polyolefin analysis

Polymer standards from Agilent Technologies are the ideal reference materials for generating accurate, reliable GPC/SEC column calibrations, with the assurance of the ISO 9001:2000 quality standard. Additional applications for our highly characterized homopolymers exhibiting unique characteristics are as model polymers for research and analytical method development. These quality polymer standards are supplied with extensive characterization that utilize a variety of independent techniques (e.g. light scattering and viscometry) and high performance GPC to verify polydispersity and assign that all important peak molecular weight (Mp).

For polyolefin analysis, polyethylene and polystyrene standards are commonly employed. Agilent provides you with the widest choice of these materials to maximize your specific characterization needs. In addition, we supply other polymers as individual molecular weights, and broad distribution polymers for system validation or broad standard calibration procedures. A range of polymer standards available from Agilent are listed in Table 3.

Table 3. *Standards selection guide*

Polymer type	Individual Mw	Calibration kits	Agilent EasiCal	Agilent EasiVial	Type of GPC/SEC
Polystyrene	Yes	Yes	Yes	Yes	Organic
Polymethylmethacrylate	Yes	Yes		Yes	Organic
Polyethylene	Yes	Yes			Organic

Recommendations for setting up a GPC/SEC system for polyolefin analysis

The following questions will help you find the recommended columns and standards for any given application, as well as the system parameters such as injection volumes.

Choosing a column for GPC/SEC of polyolefins

Columns shown in bold are the best initial choice

Question	Answer	Recommendation	Comments
1. What is the expected molecular weight? *It may seem strange to ask this question, but in GPC/SEC the resolution of a column is related to the resolving range. Knowing something of the expected molecular weight of a sample helps to choose the best column that will give optimum results.*	High (up to several millions)	**PLgel Olexis**	PLgel Olexis is specifically designed for polyolefin analysis, offers optimal performance, also suitable for light scattering
		PLgel 10 µm MIXED-B or PLgel 20 µm MIXED-A	The PLgel MIXED-A column resolves higher than the PLgel MIXED-B but at lower efficiency due to larger particle size
		PLgel MIXED-B LS or PLgel MIXED-A LS	Suitable for light scattering
	Intermediate (up to hundreds of thousands)	PLgel 5 µm MIXED-C or PLgel 5 µm MIXED-D	These PLgel columns are the most widely applicable for the majority of applications
	Low (up to tens of thousands)	**PLgel 5 µm 500Å**	The PLgel column provides high resolution and is designed for low-molecular-weight applications
	Very low (a few thousand)	**PLgel 5 µm 100Å**	The PLgel column gives high resolution at low Mw
	Unknown	**PLgel Olexis**	This PLgel column is designed for polyolefin analysis
2. How many columns to use? *The greater the particle size of the media in the column (which is dependent on the expected molecular weight of the samples), the lower the resolution and the more columns are required to maintain the quality of the results. For higher molecular weight samples, larger particles are necessary to reduce the danger of shear degradation of samples during analysis.*	Depends on the particle size of the columns	Particle size 20 µm, use 4 columns	Increased number of columns required for large particle sizes to make up for low efficiences – PLgel Olexis is 13 µm
		Particle size 13 µm, use 3 columns	
		Particle size 10 µm, use 3 columns	
		Particle size 5 µm, use 2 columns	
3. What standard is best? *Depending on analysis there are two options.*		**Polystyrene (PS)** or polyethylene (PE)	Polystyrene is the most commonly used standard in convenient EasiVial format, polyethylene is useful for generating PE based molecular weights

Columns for GPC analysis of polyolefins

Agilent produces a broad array of columns for the analysis of synthetic polymers and many of them are suitable for the analysis of polyolefins. However, the PLgel Olexis column is specifically designed for polyolefins with a wide range of molecular weights.

Agilent PLgel Olexis

PLgel Olexis is the optimum column choice for the analysis of very high-molecular-weight polymers such as polyolefins. Designed and manufactured specifically for these compounds, the column resolves up to 100,000,000 g/mol (polystyrene in THF). Packed with 13 µm particles for maximum resolution with minimal polymer shear, the columns also operate up to 220 °C for the analysis of highly crystalline materials. The column packing exhibits the excellent mechanical stability and robustness expected from the PLgel product range.

No shear degradation

The columns have a particle size of 13 µm, selected to give good efficiency in excess of 30,000 plates/m. In addition, the excellent size consistency of the particles (Figure 6) results in a very narrow particle size distribution that ensures no shear degradation.

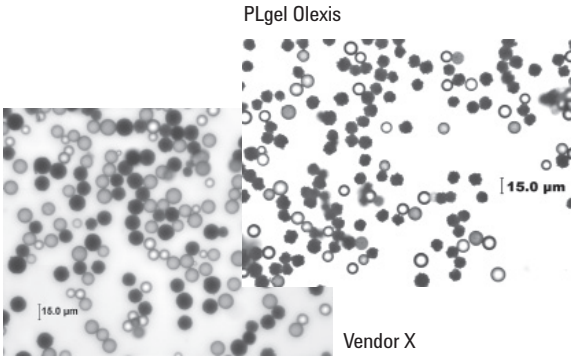


Figure 6. *The superior size consistency of PLgel Olexis particles is clearly evident*

High resolving range

Many new types of polyolefins have been developed recently with very high polydispersities. Determination of accurate polydispersities and modalities is critical in the research and development of these new polymers. PLgel Olexis completely satisfies this demand, for all polyolefin applications up to 100,000,000 g/mol.

Easy extrapolation

The large pore size of the particles makes them effective with many types of polyolefin. Linearity was introduced into the Agilent manufacturing process as a control criterion to ensure linear resolution across the operating range (Figure 7). The result is simplified extrapolation for calibrations.

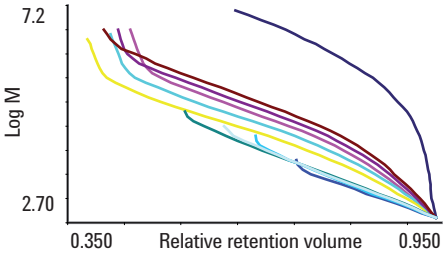


Figure 7. *Some of the components of PLgel Olexis that contribute to its lack of artifacts*

One column for all polyolefin applications

As the packing material in PLgel Olexis is an accurate blend of many components, smooth distributions are produced that truly reflect the sample composition (Figure 8). Dislocations are absent, so you can be sure that any unusual peak shapes represent the true nature of the sample and are not artifacts.

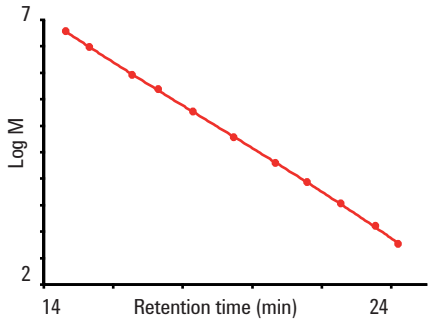


Figure 8. *Careful blending delivers highly linear polystyrene calibrations with PLgel Olexis in TCB*

The quality of the blending in PLgel Olexis columns means that polyolefins of very different polydispersity can be confidently analyzed on the same column set. Once again, PLgel Olexis provides trustworthy, clean and mono-modal peaks.



Polyolefin applications

The applications in this booklet illustrate the diversity of polyolefin samples, and reveal the flexibility of PLgel columns and the necessity for the PL-GPC 220 in addressing the analysis of such compounds.

Columns for high-molecular-weight polyolefins

Polyolefins range from low-molecular-weight hydrocarbon waxes to ultra-high-molecular-weight rigid plastics. The molecular weight distributions of polyolefins is directly related to physical properties such as toughness, melt viscosity and crystallinity. High-molecular-weight polyolefins tend to exhibit very broad molecular weight distribution (MWD). For such samples, small particles with small pore sizes are not desirable since shear degradation may occur, and so the high-pore-size particles of PLgel Olexis are recommended.

Conditions

Samples:	Polyethylenes
Columns:	3 x PLgel Olexis, 300 x 7.5 mm (Part No. PL1110-6400)
Eluent:	TCB + 0.015% BHT
Flow Rate:	1 mL/min
Inj Vol:	200 µL
Temp:	160 °C
Detector:	PL-GPC 220 (RI) + viscometer

Artifacts known as dislocations can arise in blended columns, resulting from a mismatch of the pore volume of components in the blend. Dislocations lead to false modalities and polydispersities. Avoiding dislocations was an integral part of the design brief for PLgel Olexis columns. Accurate blending of these components produces a column that gives a smooth molecular weight distribution, providing a true reflection of the shape of the MWD (Figure 9). PLgel Olexis is perfect for studies that require accurate polydispersity index and modality information.

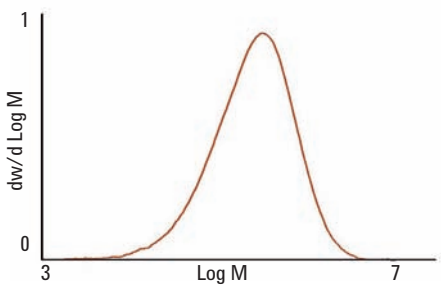


Figure 9. *True representation of polyolefin molecular weight distribution with PLgel Olexis*

Figure 10 shows a range of polyolefin samples analyzed on a PLgel Olexis column, covering the spread of molecular weights. There are no dislocations and the peak shape of the very broad samples shows true sample modality.

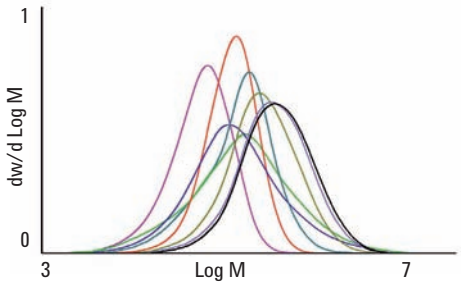


Figure 10. *PLgel Olexis reveals true modalities across the range of polyolefins*

Given the accurate resolving power of PLgel Olexis you can be sure that unusual peak shapes are real and not artifacts; unusual peak shapes of some samples will be true reflections of their modality. This is important for studies into reaction mechanisms and catalyst behavior (Figure 11).

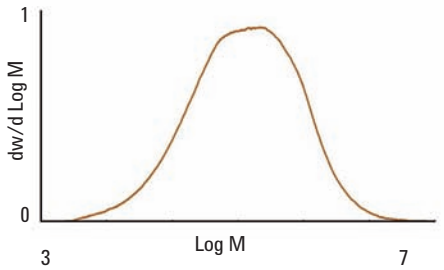


Figure 11. *A true change in peak shape revealed by PLgel Olexis of a multi-modal material manufactured from a multi-site catalyst*

Columns for lower-molecular-weight polyolefins

Crude oil, or petroleum, is the main source of organic chemicals for industry. The major chemicals are derived from two constituents of oil, xylene and naphtha. These raw materials are then broken down into more basic products, e.g. polyethylene, polypropylene, elastomers, asphalts and liquid hydrocarbons. Characterization of such products is commonly achieved using GPC. This involves a liquid chromatographic separation from which a molecular weight distribution calculation can be made following calibration of the system with suitable polymer standards. The diversity of petroleum products demands a variety of GPC column types for optimized analysis. Low-molecular-weight liquid hydrocarbons require high resolution of individual components. This is illustrated in Figure 12, where three linear hydrocarbons are resolved easily to base-line in a reasonably short analysis time.

Conditions

Samples:	Linear hydrocarbons
Columns:	2 x Agilent PLgel 5 µm 100Å, 300 x 7.5 mm (Part No. PL1110-6520)
Eluent:	TCB
Flow Rate:	1 mL/min
Temp:	145 °C
Detector:	PL-GPC 220

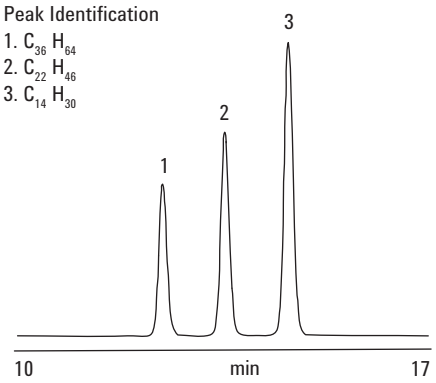


Figure 12. *Linear hydrocarbons separated to base-line on a PLgel column set*

Figure 13 shows the separation of a selection of low-molecular-weight linear hydrocarbons.

Conditions

Samples:	Linear hydrocarbons
Columns:	2 x Agilent PLgel 3 µm 100Å, 300 x 7.5 mm (Part No. PL1110-6320)
Eluent:	TCB
Flow Rate:	0.8 mL/min
Inj Vol:	20 µL
Temp:	145 °C
Detector:	PL-GPC 220

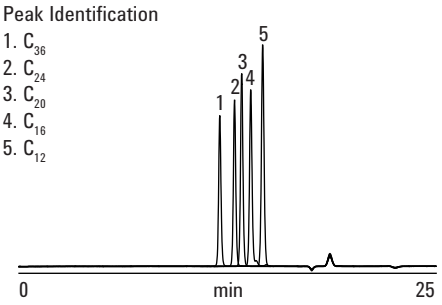


Figure 13. *Separation of low-molecular-weight hydrocarbons*

The PLgel 100Å columns have a GPC exclusion limit of 4,000 molecular weight (polystyrene equivalent). Intermediate products can be analyzed using the PLgel MIXED-D column that has a linear molecular weight resolving range up to an exclusion limit of around 400,000 molecular weight. The 5 µm particle size maintains high column efficiency and thus fewer columns are required and analysis time is relatively short.

Figure 14 shows a chromatogram of a relatively low-molecular-weight hydrocarbon wax obtained on PLgel 5 µm MIXED-D columns.

Conditions

Samples:	Linear hydrocarbons
Columns:	2 x Agilent PLgel 5 µm MIXED-D, 300 x 7.5 mm (Part No. PL1110-6504)
Eluent:	TCB
Flow Rate:	1 mL/min
Inj Vol:	200 µL
Temp:	160 °C
Detector:	PL-GPC 220

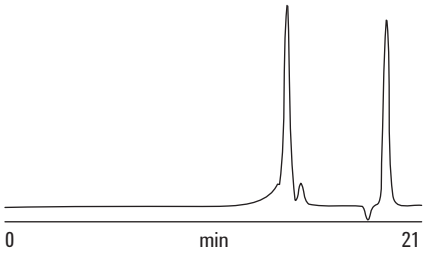


Figure 14. *A low-molecular-weight wax*

Figure 15 shows the analysis of an asphalt used in road surfacing. Subsequently derived information regarding the molecular weight distribution of such materials is invaluable in determining their processibility and final properties.

Conditions

Columns:	2 x PLgel 5 µm MIXED-D, 300 x 7.5 mm (Part No. PL1110-6504)
Eluent:	THF
Flow Rate:	1 mL/min
Temp:	40 °C
Detector:	RI

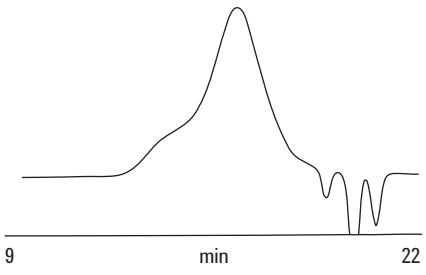


Figure 15. *Fast analysis of asphalt on PLgel 5 µm MIXED-D columns*

Repeatability study 1

A commercial sample of high-density polyethylene (HDPE) was prepared at 2 mg/mL using the PL-SP 260VS Sample Preparation System, with a dissolution temperature of 160 °C and a dissolution time of two hours. Eight aliquots of the master batch solution were dispensed into PL-GPC 220 autosampler vials and placed in the autosampler carousel of the PL-GPC 220 where the hot zone temperature was 160 °C and the warm zone 80 °C (Figure 16).

Conditions

Columns:	3 x PLgel 10 µm MIXED-B, 300 x 7.5 mm (Part No. PL1110-6100)
Eluent:	TCB + 0.0125% BHT
Flow Rate:	1 mL/min
Inj Vol:	200 µL
Temp:	160 °C
Detector:	PL-GPC 220

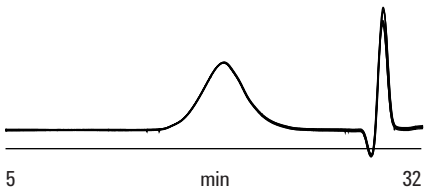


Figure 16. *Overlay of the raw data chromatograms obtained for eight consecutive injections of HDPE*

The data were analyzed against a polystyrene standards calibration using the following Mark-Houwink parameters to obtain the polypropylene equivalent molecular weight averages that are shown in Table 4.

Polystyrene in TCB[1] $K = 12.1 \times 10^{-5}$ $\alpha = 0.707$

Polyethylene in TCB[2] $K = 40.6 \times 10^{-5}$ $\alpha = 0.725$

Table 4. *Summary of results from eight injections of HDPE*

Injection number	Mn	Mp	Mw
1	17,289	76,818	333,851
2	16,988	77,434	335,496
3	17,428	77,514	332,616
4	17,521	77,052	335,635
5	17,348	76,520	334,212
6	17,487	77,728	333,511
7	16,898	77,578	335,642
8	17,457	77,288	334,923
Mean	17,302	77,241	334,485
Std Dev	220	387	1,048
% Variation	1.3	0.5	0.3

Figure 17 shows an overlay of the molecular weight distribution calculated for the eight consecutive injections of the HDPE sample, and illustrates the excellent repeatability obtained with the PL-GPC 220 using PLgel 10 µm MIXED-B columns.

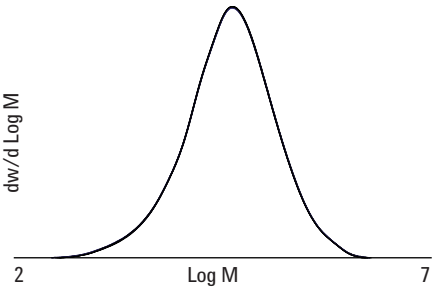


Figure 17. *Molecular weight overlay of eight consecutive injections of HDPE*

Repeatability study 2

A commercial sample of high-density polypropylene (HDPP) was prepared at 1.5 mg/mL using the PL-SP 260VS Sample Preparation System with a dissolution temperature of 160 °C and a dissolution time of two hours. Six aliquots of the master batch solution were dispensed into PL-GPC 220 autosampler vials and placed in the carousel where the hot zone temperature was 160 °C and the warm zone 80 °C.

Figure 18 shows an overlay of the raw data chromatograms obtained for six consecutive injections of the sample.

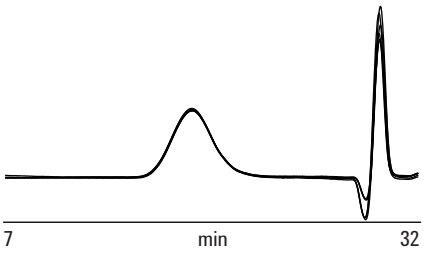


Figure 18. *Overlay of the raw data chromatograms obtained for six consecutive injections of HDPP*

The data were analyzed against a polystyrene standards calibration using the following Mark-Houwink parameters to obtain the polypropylene-equivalent molecular weight averages that are shown in Table 5.

Polystyrene in TCB[1] $K = 12.1 \times 10^{-5}$ $\alpha = 0.707$

Polypropylene in TCB[2] $K = 19.0 \times 10^{-5}$ $\alpha = 0.725$

Table 5. *Overlay of the raw data chromatograms obtained for six consecutive injections of HDPP*

Injection number	Mp	Mn	Mw
1	127,132	65,086	185,795
2	131,893	65,089	185,236
3	128,673	66,802	186,202
4	132,062	67,417	188,048
5	131,625	69,320	188,679
6	130,227	69,677	186,188
Mean	130,202	67,232	186,691
Std Dev	1,693	1,815	1,239
% Variation	0.13	2.70	0.66

Conditions

Columns:	3 x PLgel 10 µm MIXED-B, 300 x 7.5 mm (Part No. PL1110-6100)
Eluent:	TCB + 0.0125 BHT
Flow Rate:	1 mL/min
Inj Vol:	200 µL
Temp:	160 °C
Detector:	PL-GPC 220

Figure 19 shows an overlay of the molecular weight distribution calculated for the six consecutive injections of the HDPP sample that illustrates the excellent repeatability obtained with the PL-GPC 220 using PLgel 10 µm MIXED-B columns.

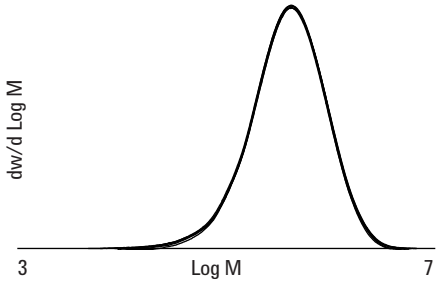


Figure 19. *Molecular weight overlay of six consecutive injections of HDPP*

References

[1] H. Coll and D. K. Gilding (1970) Universal calibration in GPC: a study of polystyrene, poly-α-methylstyrene, and polypropylene. *Journal of Polymer Science Part A-2: Polymer Physics*, 8, 89-103.

[2] T. G. Scholte , N. L. J. Meijerink, H. M. Schoffeleers and A.M.G. Brands (1984) Mark-Houwink equation and GPC calibration for linear short chain branched polyolefins, including polypropylene and ethylene-propylene copolymers. *Journal of Applied Polymer Science*, 29, 3763.

Specialist detectors

Multi-detector options for polyolefin analysis

Conventional GPC employs a refractive index or other concentration detector. However, polyolefins can be analyzed by multi-detector GPC that combines a concentration detector with a viscometer, a static light scattering detector, or both.

GPC viscometry – analysis using a concentration detector and viscometer

A viscometer may be housed inside the oven of the PL-GPC 220 to allow analysis of polyolefins by GPC viscometry. Using GPC viscometry, molecular weights are determined using the Universal Calibration method. A plot of molecular size as log (molecular weight x intrinsic viscosity) versus retention time is constructed for a series of narrow standards, based on the relationships in Equations 1 and 2.

Equation 1:

Hydrodynamic volume α molecular weight x intrinsic viscosity

Equation 2:

Log (MW x intrinsic viscosity) versus retention time ≃ log (hydrodynamic volume) versus retention time

PLgel Olexis columns are separated and calibrated in terms of size and so a Universal Calibration is obtained (Figure 20).

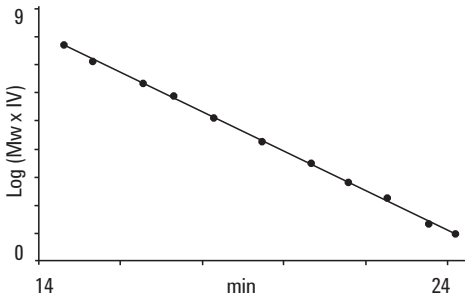


Figure 20. *Multi-detector GPC Universal Calibration of a PLgel Olexis column*

The Universal Calibration technique gives polyolefin molecular weights regardless of the calibrants used in the analysis. This allows cheaper calibrants such as polystyrene to be used while still providing accurate polyolefin results.

- Intrinsic viscosities are measured from the viscometer and concentration detector
- Accurate molecular weights are calculated assuming that the sample obeys the Universal Calibration (pure size exclusion is obtained)
- Radius of gyration is calculated using a model for the polymer behavior in solution

GPC light scattering – analysis employing a concentration detector and a light scattering detector

A dual-angle light scattering detector can be sited inside the oven of the PL-GPC 220 to allow analysis of polyolefins by GPC light scattering, employing the dissymmetry method. In GPC light scattering, accurate molecular weights are determined directly by using the response of the light scattering detector and the intensity of scattered light, as described in Equation 3.

Equation 3:

$R_\theta = CM\,(dn/dc)^2\,P_\theta K_\theta$

R_θ is the detector response, CM is concentration x mass, dn/dc is the specific refractive index increment, P_θ is the particle scattering function and K_θ is the light scattering constant.

- Molecular weights are calculated directly from the light scattering response, calculating the particle scattering function from the ratio of intensities at 15° and 90°
- Radius of gyrations are determined from the particle scattering function by comparison of the two angles, but only if the molecule is over about 10 nm in size and the scattering intensity shows angular dependence
- Intrinsic viscosity is calculated using a model for the polymer behavior in solution

GPC triple detection – analysis using concentration, viscometry and light scattering data

In this technique, both a viscometer and a dual-angle light scattering detector are housed inside the PL-GPC 220. With GPC triple detection, molecular weights are determined directly using the response of the light scattering detector as described above.

- Molecular weights are calculated directly from the light scattering response, calculating the particle scattering function from the ratio of intensities at 15 ° and 90 °
- Radius of gyrations are determined from the particle scattering function by comparison of the two angles but only if the molecule is over about 10 nm in size and the scattering intensity shows angular dependence
- Intrinsic viscosity is calculated from the viscometer trace

Comparisons between conventional GPC, GPC viscometry, GPC light scattering and GPC triple detection

Conventional GPC using only a concentration detector generates molecular weights on the basis of comparison to a series of calibration standards. However, unless the standards and samples are of the same chemistry and therefore same size in solution at any given molecular weight, the results are only relative as the GPC column separates on the basis of size not molecular weight. Conventional GPC only gives accurate results if standards of the same chemistry as the samples under investigation are used.

GPC viscometry and GPC light scattering, or GPC triple detection, can be used to determine 'absolute' molecular weights of samples, independent of the chemistry of standards used in the column calibration (GPC viscometry) or independent of column calibration entirely (GPC light scattering and GPC triple detection).

The values of molecular weight can vary between these techniques because the viscometer and light scattering detectors respond to different properties of the polymer, the viscometer to molecular density, and the light scattering detector to size in solution. Therefore, molecular weights calculated by these approaches will not necessarily have the same values.

Branching

Comparing long-chain branching in polyethylenes

Multi-detector GPC combined with branching calculations is an excellent way of comparing and identifying different kinds of polyethylene. These different materials, although of the same basic chemical structure, differ in their mode of manufacture and have very different physical properties.

LDPE – low-density polyethylene

Low-density polyethylene was the first grade of polyethylene manufactured in the 1930s. It exhibits relatively low crystallinity compared to other forms of polyethylene due to the presence of long branches on the polymer backbone (on about 2% of the carbon atoms). As a result, the tensile strength of the material is lower while resilience is higher. These long-chain branches are a result of 'backbiting' reactions in the synthetic processes used to manufacture the material. Multi-detector GPC can measure the level of branching in LDPE.

HDPE – high-density polyethylene

High-density polyethylene is manufactured using different catalysts than those used for LDPE, selected to give very low levels of branching from the backbone. HDPE therefore has higher density and crystallinity than LDPE, resulting in a tougher, more temperature-stable product. HDPE does not display long-chain branching.

LLDPE – linear low-density polyethylene

Linear low-density polyethylene is a newer material manufactured by incorporation of small quantities of alpha-olefins such as butane, hexane or octene into the polymer. LLDPE materials are more crystalline than LDPE, but are elastomeric and have a higher tensile strength and puncture resistance. Multi-detector GPC employing a viscometer and/or light scattering detector cannot be used to investigate the branching in LLDPE as changes in the density and size of the molecules compared to linear materials are very small and cannot be detected. GPC-FTIR is employed for short-chain branching analysis, as discussed on page 24.

Investigating branching in polyolefins

In multi-detector GPC, branching is assessed by investigating changes in molecular size or intrinsic viscosity as a function of increasing molecular weight. In all cases for polymers of the same chemistry, branched molecules always have lower Rg and IV values than linear analogs due to the presence of branch points.

In all methods, branching calculations can be performed on either the intrinsic viscosity (measured or calculated) or radius of gyration (measured or calculated) data. The quality of the branching results will depend on the quality of the source data (intrinsic viscosity or radius of gyration). Contraction factors are determined from the Mark-Houwink (log intrinsic viscosity versus log MW) or conformation (log radius of gyration versus log MW) plots using the relationships in Equation 4.

Equation 4:

Radius of gyration contraction factor

$$g = \left(\frac{\text{Rg branched}}{\text{Rg linear}} \right)_{MW}$$

Intrinsic viscosity contraction factor

$$g' = \left(\frac{\text{IV branched}}{\text{IV linear}} \right)_{MW}$$

where $g = g'^{(1/\epsilon)}$

ϵ (structure factor) = 0.5 to 1.5, typically 0.75

The value of g (directly or taken from the value of g' and an estimation of the structure factor, typically 0.75) is used along with the branching repeat unit (the molecular weight of the monomer multiplied by 1,000) to obtain branching numbers using a branching model. In the absence of structural data for the sample, a number-average ternary-branching model is used as shown in Equation 5.

Equation 5:

$$g = [(1 + B_n/7)^{1/2} + 4B_n/9\pi]^{-1/2}$$

where B_n = branches per 1,000 carbons

Branching numbers are expressed as number of branches per 1,000 carbons (from polyethylene investigations). If the polymer in question is not polyethylene then the actual branching number may not be directly meaningful. However, comparison between samples is still possible.

Analysis of branching in polyethylenes

Samples of LDPE, HDPE and LLDPE were analyzed with the PL-GPC 220 by triple detection.

Conditions

Columns:	3 x PLgel Olexis, 300 x 7.5 mm (Part No. PL1110-6400)
Eluent:	TCB + 0.015% BHT
Flow Rate:	1.0 mL/min
Inj Vol:	200 µL
Temp:	160 °C
Detector:	PL-GPC 220 (RI) + viscometer + dual-angle light scattering

Refractive index, dual-angle light scattering and viscometry detectors were employed and the data was analyzed with Cirrus GPC Multi Detector Software. A polystyrene standard was used to generate the detector constants for the triple detection analysis.

Figure 21 shows the molecular weight distributions for the three samples. Although there was some overlap, the samples clearly had significantly different molecular weights.

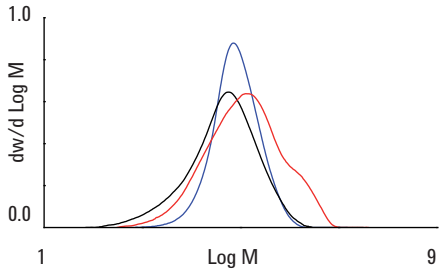


Figure 21. *Overlaid molecular weight distributions for three samples of polyethylene, HDPE – black, LLDPE – blue, LDPE – red*

Figure 22 shows the Mark-Houwink plots for the three samples using intrinsic viscosities generated from the viscometer and molecular weights from the light scattering detector.

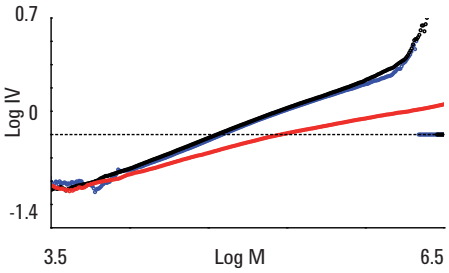


Figure 22. *Overlaid Mark-Houwink plots for three samples of polyethylene, HDPE – black, LLDPE – blue, LDPE – red*

The Mark-Houwink plot describes the change in the viscosity of the polymers as a function of increasing molecular weight. The HDPE and LLDPE samples overlay on the Mark-Houwink plot, indicating that the polymers have very similar structures. The Mark-Houwink parameters K (the intercept) and alpha (the slope) indicate that the materials contain no branching that can be detected by multi-detector GPC. However, the LDPE shows a clear deviation from the HDPE and LLDPE lines, with a decreasing slope as molecular weight increases. This is due to increased branching of the LDPE compared to the other materials as molecular weight increases lead to a reduction in viscosity.

Branching analysis of polyethylenes with Cirrus GPC Multi Detector Software

The presence of long-chain branching (over six carbons in length) in polyolefins strongly influences physical properties such as melt viscosity and mechanical strength. The distribution chain branches in polyolefins are determined by the polymerization mechanism and there is significant interest in the production of materials with well-defined and characterized molecular weight and branching distributions for specific applications.

Three samples of polyethylene, one HDPE and two LDPE, were analyzed using the PL-GPC 220 by GPC/viscometry. Two of the samples had been synthesized by a mechanism to promote branching, while the third was a standard linear reference material, NBS 1475.

Refractive index viscometry detectors were employed and the data was analyzed with Cirrus GPC Multi Detector Software using the Universal Calibration approach. Polystyrene standards were used to generate the Universal Calibration and the unbranched sample was used as a linear model in the determination of branching.

Figure 23 shows the molecular weight distributions for the three samples. The black plot is for the unbranched sample. Although there was some overlap, the samples clearly had significantly different molecular weights.

Figure 24 shows the Mark-Houwink plots for the three samples. The upper-most sample is the unbranched material. The other two samples have lower intrinsic viscosities at any given molecular weight, with the unbranched polymer indicating the presence of branching. This can be expressed in terms of g, the branching ratio, defined in Equation 6, where ϵ is a constant.

Equation 6:

$$g = \left(\frac{\text{IV branched}}{\text{IV linear}} \right)^{1/\epsilon}$$

Conditions

Samples:	Polyethylenes
Columns:	3 x PLgel Olexis, 300 x 7.5 mm (Part No. PL1110-6400)
Eluent:	TCB + 0.015% BHT
Flow Rate:	1.0 mL/min
Inj Vol:	200 µL
Temp:	160 °C
Detector:	PL-GPC 220 (RI) + viscometer

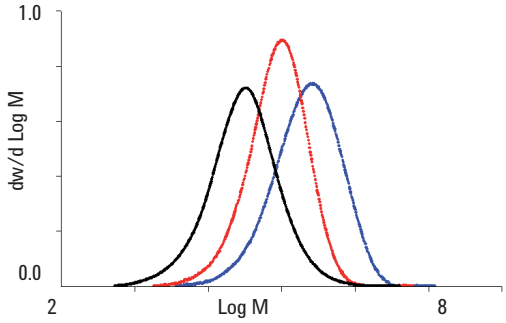


Figure 23. *Molecular weight distribution plots for three polyethylene samples – the black plot is the unbranched sample*

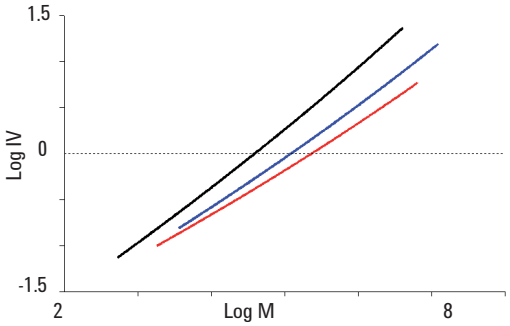


Figure 24. *Mark-Houwink plots for three samples of polyethylene*

The unbranched sample was used as the linear model and so gives a g value of unity (except at high molecular weight due to scatter in the data). The other two samples both exhibit a decrease in g as a function of molecular weight, indicating that as molecular weight increases the number of branches also increases. Based on these calculated g values, a branching number or number of branches per 1,000 carbon atoms can be generated. This is achieved by fitting the data into a model. The Cirrus GPC Multi Detector Software offers a selection of branching models that can be employed in this approach. In this case a model was used that calculates a number-average branching number assuming a random distribution of branches on the polymer. Figures 25 and 26 show the g plots and branching number plots obtained for the samples.

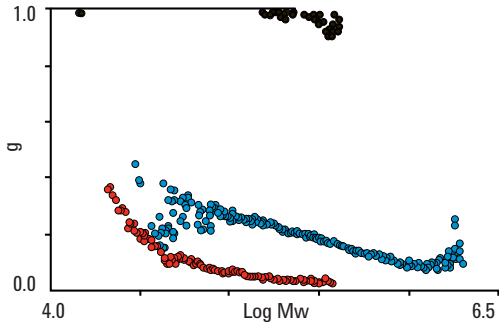


Figure 25. *Branching ratio g plots for three polyethylene samples – the black plot is the unbranched sample*

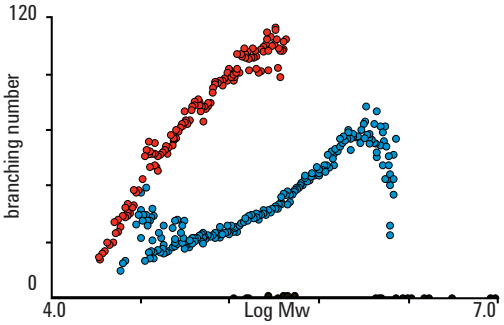


Figure 26. *Calculated branching numbers as a function of molecular weight for three samples of polyethylene – the black plot is the unbranched sample*

The results show that of the two, branched samples, the trend in molecular weight distribution does not follow the trend in branching distribution. The sample showing the most branching at any given molecular weight has a lower molecular weight than the second sample. Clearly, understanding both the molecular weight and branching distributions will give an insight into the processibility of the two materials.

Analysis of branching in linear low-density polyethylene (LLDPE)

Fourier transform-infrared (FTIR) spectroscopy is a well-established technique used in compositional analysis of materials through the measurement of vibrational absorption bands. Polymers typically exhibit relatively simple absorption spectra, allowing them to be readily identified by comparison to library data and are therefore well suited to analysis by FTIR. Coupling FTIR detection with gel permeation chromatography is particularly advantageous as FTIR detection can be utilized as both concentration detector for molecular weight calculations and as a spectroscopic tool for compositional analysis, significantly enhancing the information available from a single GPC experiment.

Coupling a PL-GPC 220 system to one of the range of Agilent's FTIR spectrometers can be achieved using the PL-HTGPC-FTIR interface, which consists of a heated flow cell, a heated transfer line, and a temperature control box. The flow cell and transfer line can be heated up to 175 °C with an accuracy of ± 0.5 °C for polyolefin applications. To obtain good quality spectra, the FTIR spectrometer is fitted with a fast MCT (mercury-cadmium-telluride) detector. Data acquisition is performed through the spectrometer's time-resolved data-acquisition software.

GPC/FTIR analysis of polyethylene

Highly crystalline polyethylene is difficult to analyze by GPC due to its limited solubility in most organic solvents, and the high temperatures required for dissolution (typically over 135 °C). Trichlorobenzene (TCB) is the most commonly used solvent for these materials. TCB is also a suitable solvent for GPC analysis with FTIR detection as the solvent has a good absorption window between about 3,500 and 2,700 cm^{-1}, which corresponds to the >C-H stretching region. CH vibrations dominate the solid-state spectra of polyethylene and so this absorption region is of key importance.

Focusing on the >C-H stretching region, differences in the proportions of $>CH_2$ and $–CH_3$ groups in a sample can be seen in the relative intensities of the absorption bands. This dependence of the infrared spectra on the presence of $–CH_3$ and $>CH_2$ groups can be used to measure the level of short-chain branching (SCB) in polyethylene[1]. These are branches less than six carbons long introduced by co-polymerization of ethylene with other alpha-olefins that cannot be detected by traditional multi-detector GPC experiments, as they do not affect the viscosity of the polymer. The level of SCB does, however, strongly influence crystallinity, density, and stress-crack resistance of polyethylene. By measuring the spectra of polyethylene containing SCB, the relative intensities of the stretching vibrations due to $–CH_3$ and $>CH_2$ groups can be measured and, providing that the monomers used to introduce SCB are known, the level of SCB can be estimated using chemometrics. Coupling the detector to a GPC system allows the SCB to be assessed (as a function of molecular weight).

Analysis of an ethylene-hexene copolymer by GPC/FTIR

A sample of ethylene co-polymerized with hexane was analyzed using the PL-GPC 220 coupled to an Agilent FTIR to assess the levels of short-chain branching.

Conditions

Column:	2 x PLgel Olexis, 300 x 7.5 mm (Part No. PL1110-6400)
Eluent:	Trichlorobenzene (with BHT)
Inj Vol:	200 µL
Flow Rate:	1.0 mL/min
Temp:	160 °C
Data Collection:	Time-resolved Agilent Resolutions Pro software collecting at 8.0 cm^{-1} resolution with 16 scan accumulations for 11 minutes, range 3,500 – 2,700 cm^{-1} with automatic solvent background subtraction
Detection:	Agilent PL-HTGPC-FTIR interfaced to an Agilent FTIR spectrometer fitted with an MCT detector

Cirrus GPC-FTIR SCB software was used to perform the experiments, calculating SCB based on a rigorous chemometrics approach. To determine molecular weight, the FTIR data was used as a concentration source for the generation of Figure 27, showing an overlay of the polymer weight and short-chain branching distribution obtained for a copolymer of ethylene and another alpha-olefin by FTIR. Clearly, in this case the level of co-monomer incorporation was uniform across the distribution.

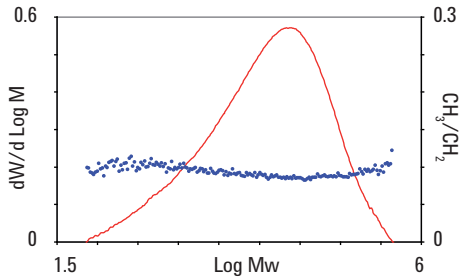


Figure 27. *Overlaid chromatogram of polymer weight and short-chain branching distribution for a sample of ethylene-hexene copolymer*

Reference

[1] P.J. DesLauriers, D.C. Rohlfing and E.T. Shieh (2002) Quantifying short chain branching microstructures in ethylene-1-olephin copolymers using size exclusion chromatography and Fourier transform infrared spectroscopy (SEC-FTIR). *Polymer*, 43, 159-170.

Ordering Information

Columns

Description	Part No.
Agilent PLgel 3 µm 100Å, 300 x 7.5 mm	PL1110-6320
Agilent PLgel 5 µm 100Å, 300 x 7.5 mm	PL1110-6520
Agilent PLgel 5 µm MIXED-D, 300 x 7.5 mm	PL1110-6504
Agilent PLgel 10 µm MIXED-B, 300 x 7.5 mm	PL1110-6100
PLgel 10 µm MIXED-B LS, 300 x 7.5 mm	PL1110-6100LS*
PLgel 20 µm MIXED-A, 300 x 7.5 mm	PL1110-6200
PLgel 20 µm MIXED-A LS, 300 x 7.5 mm	PL1110-6200LS*
Agilent PLgel Olexis, 300 x 7.5 mm	PL1110-6400

Standards

Description	Part No.
Agilent PS-H EasiVial 2 mL pre-weighed polystyrene calibration kit	PL2010-0201
Agilent PS-M EasiVial 2 mL pre-weighed polystyrene calibration kit	PL2010-0301
Agilent E-M-10 polyethylene calibration kit, 10 x 0.2 g	PL2650-0101
Agilent E-MW-10 polyethylene calibration kit, 10 x 0.1 g	PL2650-0102
Agilent E-SCB polyethylene short-chain branching calibration kit, 10 x 0.1 g	PL2650-0103

Instruments

Description	Part No.
Agilent PL-SP 260VS Sample Preparation System**	
Agilent PL-GPC 220 Integrated GPC/SEC System	PL0820-0000
Agilent PL-HTGPC-FTIR**	
Agilent PL-BV 400HT Online Integrated Viscometer	PL0810-3050
Agilent PL-HTLS 15/90 Light Scattering Detector	PL0640-1200
Agilent custom accessory kit**	

Software

Description	Part No.
Agilent Cirrus GPC Multi Detector Software	PL0570-2020
Agilent Cirrus GPC Software	PL0570-2000
Agilent GPC-FTIR SCB Software	PL0570-2300

** Low shedding for light scattering applications*
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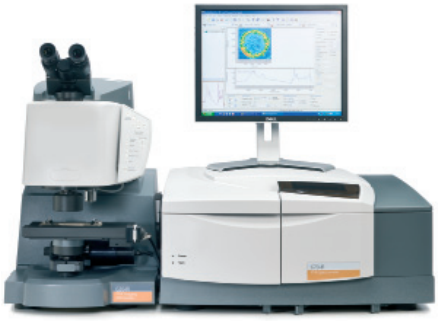
More Agilent solutions for polyolefin analysis

As well as high-temperature GPC, Agilent offers other solutions for the analysis of polyolefins.

FTIR

Fourier transform-infrared spectroscopy is an essential tool in the analysis of polymer films and other materials. Applications range from quality testing of raw materials to failure analysis of large objects. Our solutions feature Agilent's high performing 600-IR Series spectrometers and microscopes, software and accessories.

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The Agilent 600-IR series provides the highest level of sensitivity combined with detailed structural and compositional information for information-rich detection

NMR

Agilent NMR has long been an effective tool for the characterization of polymers. 1D and 2D NMR methods have been routinely used for many years. A more advanced method developed at Agilent uses pulsed-field gradient-heteronuclear multiple-bond correlation with 2D NMR to detect weak signals in the presence of much larger resonances. This technique permits assignment of signals from minor structures such as chain ends and defects, essential information for a full understanding of these complex synthetic compounds.

The Agilent 400-MR provides unmatched productivity for diverse chemical applications by combining easy-to-use software with the outstanding performance of DirectDrive and DirectDigital spectrometer architecture. Push-button experiments, along with straightforward processing and data export capabilities, make the 400-MR the best choice for compound detection, quantification and structure confirmation.

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Agilent Technologies

Analysis of engineering polymers by GPC/SEC

Application Compendium

Authors

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Agilent Technologies, Inc.



The Measure of Confidence

Agilent Technologies

Contents Page

Polymer analysis by GPC/SEC

Introduction 3

Agilent's GPC/SEC technology

GPC/SEC columns 4

GPC/SEC standards 4

GPC/SEC instruments 4

GPC/SEC of polymers in aggressive solvents

Aggressive solvents 5

GPC analysis of polyether ether ketone (PEEK) 5

GPC analysis of polybutylene terephthalate (PBT) resins in HFIP 6

Polyethylene terephthalate analysis in o-chlorophenol as an alternative solvent 7

GPC/SEC of polymers at high temperature

Column selection for polyolefin analysis 8

High temperature GPC analysis of polypropylene on the Agilent PL-GPC 220 Integrated GPC/SEC System – repeatability study 9

Branching analysis of polyethylenes with Agilent Cirrus GPC Multi Detector Software 10

Polyphenylene sulfide analysis 11

Companion products 13

Other GPC/SEC resources from Agilent 14

Polymer analysis by GPC/SEC

Introduction

Increasingly, plastics are being utilized to perform structural and mechanical roles in the construction and engineering industries. The favorable properties of polymers, such as mechanical strength, durability, and resistance to chemical and physical degradation, coupled with their relative cheapness, means that polymers outperform many traditional materials, including wood and metals, in key applications. With the creation of new polymeric materials, this shift towards plastics is becoming even more pronounced as materials with new properties are designed and developed. An understanding of the behavior of polymers is key to designing new materials with appropriate performance characteristics for specific applications. Analysis of these materials is therefore a critical component of the development and manufacture of engineering polymers.

Gel permeation chromatography (GPC, also known as size exclusion chromatography, SEC) is a well-known technique for assessing the molecular weight distribution of polymers, a property that influences many of their physical properties. Generally, increasing molecular weight leads to higher performance characteristics, while an increase in the width of the distribution (the polydispersity) leads to a loss of performance but an increase in the ease of processing.

Engineering polymers are particularly difficult to analyze – they are generally tough and difficult to dissolve, often requiring aggressive solvents and elevated temperatures. For these applications at high temperature, a high performance integrated GPC system, such as the Agilent PL-GPC 220 Integrated GPC/SEC System, is a necessity. The PL-GPC 220 has the highest temperature range of any system on the market. The following applications show the analysis of various types of engineering polymers and illustrate the conditions and equipment required.



Table 1. *Effects of molecular weight distribution on the properties of engineering polymers*

	Strength	Toughness	Brittleness	Melt viscosity	Chemical resistance	Solubility
Increasing Mw	+	+	+	+	+	–
Decreasing distribution	+	+	–	+	+	+

Agilent's GPC/SEC technology

Agilent produces the most extensive range of GPC columns, standards, instruments and software, ideally suited to the analysis of engineering polymers.

GPC/SEC columns

Agilent's GPC columns are the most rugged and reliable on the market, making them ideal for applications performed in viscous solvents and at elevated temperatures where column lifetime can be an issue. The extensive column range includes products tailored to the analysis of engineering polymers that generally have high molecular weights and high viscosities, and includes specialist columns such as Agilent PLgel Olexis that are designed for the analysis of a specific material. With extensive options in particle and pore size, Agilent's columns can be selected to match the molecular weight of the material under investigation, thereby ensuring that the best quality of data is obtained from the GPC/SEC experiment.



Agilent offers a selection of GPC/SEC column dimensions

GPC/SEC standards

Narrow polydispersity polymer standards with very highly characterized molecular weights are used as calibration standards in the GPC analysis of engineering polymers. Polystyrene standards are the first choice for many organic solvents, either for conventional GPC column calibration or for calibrating light scattering and viscosity detectors.



Agilent's EasiVial calibration kit

GPC/SEC instruments

Complementing Agilent's column technology is the most extensive collection of integrated GPC/SEC instrumentation on the market, covering the widest temperature range available, from ambient to 220 °C.

Agilent's PL-GPC 220 Integrated GPC/SEC System features unbeatable reproducibility for any GPC/SEC application, across the entire operating range. The PL-GPC 220 is an extremely flexible system, designed to run almost all polymer, solvent and temperature combinations, from 30 to 220 °C.

The PL-GPC 220 allows all forms of the GPC/SEC experiment to be performed and can be used to analyze the complete range of engineering materials, including those that require analysis at extremely high temperatures. Multiple detection options can be included in the instruments, such as light scattering and viscometry, and dedicated analysis software is available that allows the properties of engineering polymers to be analyzed in detail. Agilent's complete range of columns and instrumentation offer a clear advantage in the analysis of engineering polymers.



PL-GPC 220 Integrated GPC/SEC System

For more information on Agilent's GPC/SEC products, visit **www.agilent.com/chem/gpcsec**

GPC/SEC of polymers in aggressive solvents

Aggressive solvents

Many polymers, especially those used in engineering applications, show only limited solubility in a small number of solvents. This is because high strength and toughness are usually a result of high molecular weight and/or high crystallinity. Increasing molecular weight requires untangling the molecular chains to dissolve the material, whereas increased crystallinity requires break-up of any inter-chain bonds that may be present.

The PL-GPC 220 Integrated GPC/SEC System is designed to allow the use of even the most aggressive solvents. The following applications illustrate the analysis of a range of engineering polymers that require aggressive solvents for solubility or as eluents during the analysis.

GPC analysis of polyether ether ketone (PEEK)

Application areas: High performance components, tubing in liquid chromatography

Polyether ether ketone (PEEK) was developed in 1977 by ICI and was one of the first of the new generation of engineering thermoplastics developed for chemical resistance, high mechanical strength and high thermal stability – the useful properties of the material are retained up to temperatures as high as 315 °C. A crystalline material with repeat units of two ethers and a ketone group in the polymer backbone, PEEK is a high cost material. For many applications, such as the manufacture of piston components in engines, the insulation of cables and the production of high performance aircraft parts, this cost is justified as there are no other plastics that can offer the same performance properties. The industrial performance of PEEK makes analysis of this material by GPC difficult. PEEK has excellent chemical resistance and is unaffected by many organic and inorganic chemicals, dissolving only in strong or concentrated anhydrous oxidizing agents. Previous methods for analyzing PEEK have involved mixtures of trichlorobenzene and phenol running at high temperatures.

For this analysis, the PEEK sample was dissolved in a small volume of dichloroacetic acid at 120 °C for two hours. After dissolution, the sample was diluted to the required concentration of 0.2% (w/v) with chloroform and injected into a system running at temperature after filtration to remove undissolved material.

The PEEK sample eluted as a broad polymer peak with an MW of 70,000 g/mol and a polydispersity of 2.2. The large system peak observed at the end of the run was due to the excess dichloroacetic acid used in the preparation of the sample.

Conditions

Sample:	Polyether ether ketone (PEEK)
Columns:	2 x Agilent PLgel 10 µm MIXED-B, 300 x 7.5 mm (Part No. PL1110-6100)
Eluent:	80% chloroform 20% dichloroacetic acid
Flow Rate:	1.0 mL/min
Inj Vol:	200 µL
Detector:	PL-GPC 220

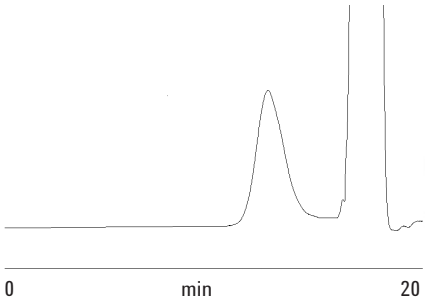


Figure 1. *Chromatogram of a PEEK sample*

GPC analysis of polybutylene terephthalate (PBT) resins in HFIP

Application areas: Machined parts

Polybutylene terephthalate (PBT) resins are used in a wide variety of applications in which toughness and resistance to damage are highly advantageous. However, mechanical and thermal stress during the production of molded parts can cause degradation, giving a reduction in desirable physical properties. The molecular weight distribution of the resin is a key measure of the onset of degradation and therefore of estimating the mechanical strength of the final product. PBT is soluble in 1,1,1,3,3,3-hexafluoroisopropanol (HFIP), a polar organic solvent, which is excellent for dissolving polar polymers such as polyamides and polyesters. The analysis was carried out in HFIP modified by the addition of 20 mM sodium trifluoroacetate to prevent aggregation. Two Agilent PL HFIPgel columns, designed specifically for HFIP applications, were employed for the analysis at a temperature of 40 °C. The PL-GPC 220 Integrated GPC/SEC System was used with differential refractive index and viscometry detection. GPC coupled with a molecular weight-sensitive viscometer allowed calculation of molecular weights based on hydrodynamic volume using the Universal Calibration approach, leading to molecular weights independent of the standards used to generate the column calibration. Agilent polymethylmethacrylate (PMMA) standards were employed to generate the Universal Calibration.

Table 2 shows the molecular weight averages and intrinsic viscosity for the sample before and after molding, as determined by GPC/viscometry. Clearly, the molecular weight distribution indicates that after molding, the material has suffered from degradation and is less robust than the virgin material.

Table 2. *Molecular weight averages and intrinsic viscosity for the PBT resin sample*

	Mn/g mol^{-1}	Mw/g mol^{-1}	Intrinsic viscosity/g^{-1}
Virgin resin	24,400	48,600	0.535
Molded part	11,200	24,000	0.306

Conditions

Samples: PBT resin
Columns: 2 x PL HFIPgel, 300 x 7.5 mm (Part No. PL1114-6900HFIP)
Eluent: HFIP + 20 mM NaTFA
Flow Rate: 1.0 mL/min
Inj Vol: 200 µL
Temp: 40 °C
Detectors: PL-GPC 220, viscometer

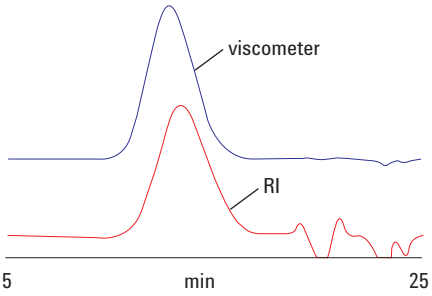


Figure 2. *Example overlay of a dual-detector chromatogram of the virgin PBT resin before molding*

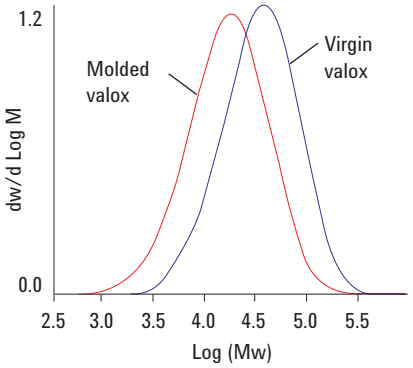


Figure 3. *Molecular weight distributions of the two samples*

Polyethylene terephthalate analysis in o-chlorophenol as an alternative solvent

As an alternative to the use of HFIP, PET can be analyzed in o-chlorophenol. This viscous solvent requires elevated temperatures and is a hazardous substance.

The samples were dissolved by heating to 110 °C for 30 minutes. The polymer remains in solution at room temperature but the high viscosity of the eluent means that high temperature GPC is necessary. Three grades of PET, with different intrinsic viscosities, were analyzed and compared, showing minor differences between the materials.

Conditions

Samples:	PET resin
Columns:	2 x PLgel 10 µm MIXED-B, 300 x 7.5 mm (Part No. PL1110-6100)
Eluent:	o-chlorophenol
Flow Rate:	1.0 mL/min
Temp:	100 °C
Detection:	PL-GPC 220

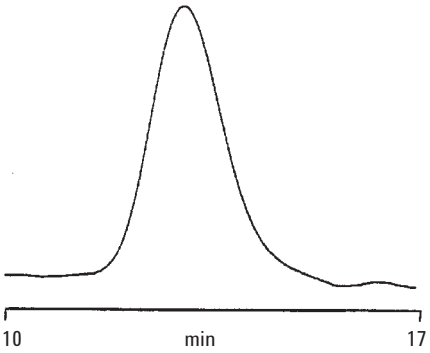


Figure 4. *Chromatogram of a PET sample*

Peak Identification
1. IV=0.72
2. IV=0.75
3. IV=0.84

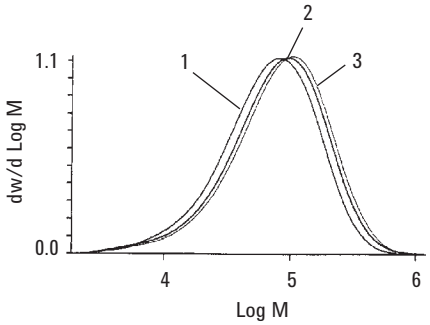


Figure 5. *Molecular weight distributions of the PET samples*

GPC/SEC of polymers at high temperature

High temperature analysis

Some highly crystalline polymers such as polyethylene show only limited solubility and only then at high temperatures. This is due to the fact that temperature is required to break down the ordered crystalline structure, and on cooling, the material will re-crystallize and precipitate from solution. For these applications, high temperature is required throughout the entire analysis to ensure that the samples remain in solution during the experiment. The PL-GPC 220 Integrated GPC/SEC System is capable of maintaining a constant temperature up to 200 °C between the point of injection, the columns and the detector cells, until the point of elution. The following applications illustrate the analysis of crystalline polymers at high temperatures using the PL-GPC 220.

Column selection for polyolefin analysis

Polyolefins range from low molecular weight hydrocarbon waxes to ultra high molecular weight rigid plastics. The molecular weight distribution of polyolefins is directly related to physical properties such as toughness, melt viscosity, and crystallinity. GPC/SEC is widely accepted as the preferred technique to fully characterize the molecular weight distribution of polyolefins.

The selection of a column set for the analysis of a polyolefin is dependent on the molecular weight range of the sample. Low molecular weight samples can be analyzed using high efficiency, relatively low pore size columns. Higher molecular weight materials require large particle size media to minimize shear effects, with a wide pore size distribution.

Figures 6 to 8 show typical data for four very different polyolefin samples, all obtained with the PL-GPC 220.

Conditions

Sample:	Linear hydrocarbons
Columns:	2 x Agilent PLgel 3 µm 100Å, 300 x 7.5 mm (Part No. PL1110-6320)
Eluent:	TCB
Flow Rate:	0.8 mL/min
Inj Vol:	20 µL
Temp:	145 °C
Detector:	PL-GPC 220

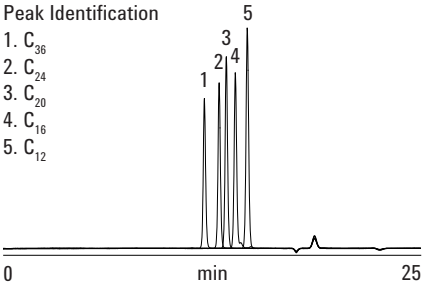


Figure 6. *Separation of a selection of low molecular weight linear hydrocarbons analyzed using two PLgel 3 µm 100Å columns*

Conditions

Sample:	Hydrocarbon wax
Columns:	2 x Agilent PLgel 5 µm MIXED-D, 300 x 7.5 mm (Part No. PL1110-6504)
Eluent:	TCB
Flow Rate:	1.0 mL/min
Inj Vol:	100 µL
Temp:	160 °C
Detector:	PL-GPC 220

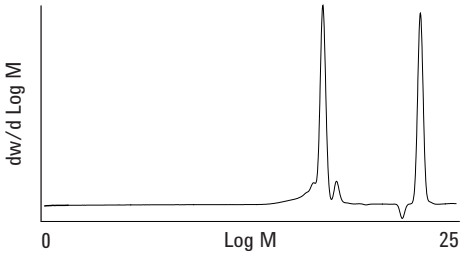


Figure 7. *Chromatogram of a relatively low molecular weight hydrocarbon wax obtained on two PLgel 5 µm MIXED-D columns*

Conditions

Sample:	Polyethylene
Columns:	3 x PLgel Olexis, 300 x 7.5 mm (Part No. PL1110-6400)
Eluent:	TCB
Flow Rate:	1.0 mL/min
Inj Vol:	200 µL
Temp:	160 °C
Detector:	PL-GPC 220

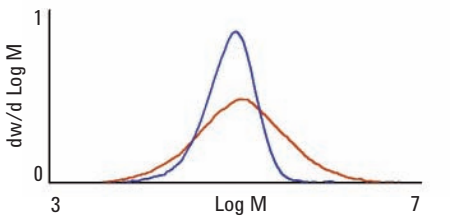


Figure 8. *Overlaid molecular weight distributions of medium and high polydispersity polyethylene samples*

High molecular weight materials require analysis on high pore size PLgel Olexis columns that minimize shear degradation, and are able to resolve up to 100,000,000 g/mol with a 13 µm particle size. These applications illustrate the diversity of polyolefin samples and indicate the flexibility of the PLgel series of columns in addressing the analysis of such samples.

High temperature GPC analysis of polypropylene on the PL-GPC 220 – repeatability study

Application areas: Plastic pipes, bottles and containers

The PL-GPC 220 Integrated GPC/SEC System is ideally suited to the analysis of polypropylene. A commercial sample of polypropylene (PP) was prepared at 1.5 mg/mL using the Agilent PL-SP 260VS Sample Preparation System with a dissolution temperature of 160 °C and a dissolution time of two hours. Six aliquots of the master batch solution were dispensed into the PL-GPC 220 autosampler vials and placed in the carousel where the hot zone temperature was 160 °C and the warm zone 80 °C.

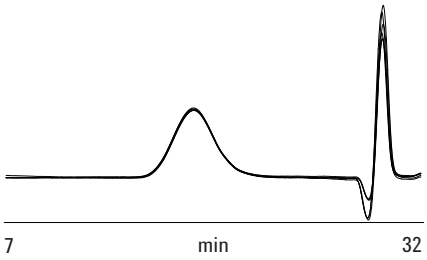


Figure 9. *Overlay of the raw data chromatograms obtained for six consecutive injections of polypropylene*

The data was analyzed against a polystyrene standards calibration using the following Mark-Houwink parameters to obtain the polypropylene equivalent molecular weight averages that are shown in Table 3.

Polystyrene in TCB[1] $K=12.1x10^{-5}$ $\alpha=0.707$

Polypropylene in TCB[2] $K=19.0x10^{-5}$ $\alpha=0.725$

Table 3. *Calculated molecular weights for six injections of polypropylene and calculated % variation*

Injection Number	Mp	Mn	Mw
1	127,132	65,086	185,795
2	131,893	65,089	185,236
3	128,673	66,802	186,202
4	132,062	67,417	188,048
5	131,625	69,320	188,679
6	130,227	69,677	186,188
Mean	130,202	67,232	186,691
Std Dev	1,693	1,815	1,239
% Variation	0.13	2.70	0.66

The results illustrate the excellent repeatability obtained with the PL-GPC 220 using PLgel 10 µm MIXED-B columns.

Conditions

Sample:	Polypropylene
Columns:	3 x PLgel Olexis, 300 x 7.5 mm (Part No.PL1110-6400)
Eluent:	TCB + 0.0125% BHT
Flow Rate:	1.0 mL/min
Inj Vol:	200 µL
Temp:	160 °C
Detector:	PL-GPC 220

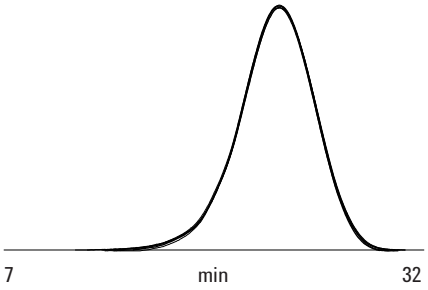


Figure 10. *Overlay of the molecular weight distribution calculated for six consecutive injections of the polypropylene sample*

References

[1] H. Col and D. K. Giddings, *J. Polym. Sci.*, (A2) **8** (1970) 89

[2] T.G. Scholte et al, *J. Appl. Polym. Sci.*, **29** (1984) 3763

Branching analysis of polyethylenes with Cirrus GPC Multi Detector Software

Application areas: Plastic bags and containers

The presence of long chain branching (over 6 carbons in length) in polyolefins strongly influences physical properties such as melt viscosity and mechanical strength. The distribution chain branches in polyolefins are determined by the polymerization mechanism and there is significant interest in the production of materials with well-defined and characterized molecular weight and branching distributions for specific applications.

Here we describe the analysis of three samples of polyethylene with the PL-GPC 220 by GPC/viscometry. Two of the samples had been synthesized by a mechanism to promote branching while the third was a standard linear reference material NBS 1475. The analysis was carried out at 160 °C with three PLgel Olexis columns in trichlorobenzene (TCB) with 0.015% butylated hydroxytoluene (BHT) as a stabilizer.

Refractive index and viscometry detectors were employed and the data was analyzed with Cirrus GPC Multi Detector Software using the Universal Calibration approach. Polystyrene standards were used to generate the Universal Calibration and the unbranched sample was used as a linear model in the determination of branching.

Figure 11 shows the molecular weight distributions for the three samples. The black plot is for the unbranched sample. Although there was some overlap, the samples clearly had significantly different molecular weights.

Figure 12 shows the Mark-Houwink plots for the three samples. The uppermost sample is the unbranched material. The other two samples have lower intrinsic viscosities at any given molecular weight, with the unbranched polymer indicating the presence of branching. This can be expressed in terms of g, the branching ratio, defined as follows, where ϵ is a constant:

$$g = \left[\frac{\text{Intrinsic viscosity (branched)}}{\text{Intrinsic viscosity (linear)}} \right]^{1/\epsilon}$$

Conditions

Samples:	Polyethylenes
Columns:	3 x PLgel Olexis, 300 x 7.5 mm (Part No. PL1110-6400)
Eluent:	TCB + 0.015% BHT
Flow Rate:	1.0 mL/min
Inj Vol:	200 µL
Temp:	160 °C
Detector:	PL-GPC 220 + viscometer

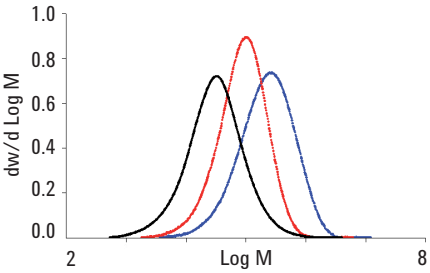


Figure 11. *Molecular weight distribution plots for the three polyethylene samples – the black plot is for the unbranched sample*

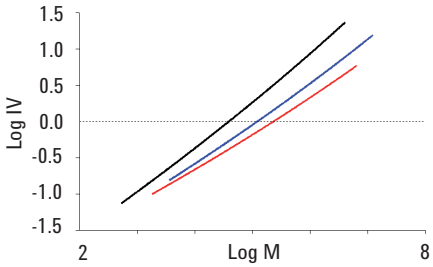


Figure 12. *Mark-Houwink plots for three samples of polyethylene*

The unbranched sample was used as the linear model and so gives a g value of unity (except at high molecular weight due to scatter in the data). The other two samples both exhibit a decrease in g as a function of molecular weight, indicating that as molecular weight increases, the number of branches increases. Based on these calculated g values, a branching number or number of branches per 1,000 carbon atoms can be generated. This is achieved by fitting the data into a model. The Cirrus GPC Multi Detector Software offers a selection of branching models that can be employed in this approach. In this case a model was used that calculates a number-average branching number, assuming a random distribution of branches on the polymer. Figures 13 and 14 show the g plots and branching-number plots obtained for the samples.

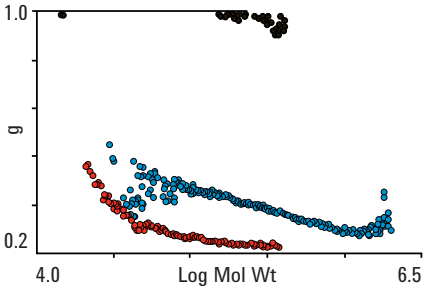


Figure 13. *Branching ratio g plots for the three polyethylene samples – the black plot is the unbranched sample*

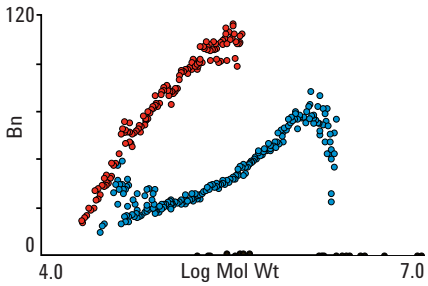


Figure 14. *Calculated branching numbers as a function of molecular weight for three samples of polyethylene – the black plot is the unbranched sample*

The results show that of the two branched samples, the trend in molecular weight distribution does not follow the trend in branching distribution. The sample showing the most branching at any given molecular weight has a lower molecular weight than the second sample. Clearly, understanding both the molecular weight and branching distributions will give an insight into the processibility of the two materials.

Polyphenylene sulfide analysis

Application areas: High performance membranes, felts and insulators

Polyphenylene sulfide (PPS) is an engineering polymer with a rigid backbone of alternating aromatic rings linked by sulfur atoms. It is useful as a structural material due to its high resistance to both chemical and thermal attack, and the material is very stiff, even at high temperatures. PPS is used in a number of applications, including as a filter fabric for coal boilers, in felts used in paper making, in electrical insulation applications and in the manufacture of specialty membranes. PPS is naturally insulating, although the addition of a dopant can be used to make the material semi-conducting.

PPS is particularly difficult to analyze by GPC. The high chemical and thermal resistance of the material means that it is only soluble in specialist solvents such as ortho-chloronaphthalene at elevated temperatures of around 200 °C. The PL-GPC 220 is capable of operation at these temperatures, and the PLgel column material can perform the analysis of PPS.

Conditions

Columns: 3 x PLgel 10 µm MIXED-B, 300 x 7.5 mm (Part No. PL1110-6100)
Eluent: o-chloronaphthalene
Flow Rate: 1.0 mL/min
Temp: 210 °C
Detector: PL-GPC 220

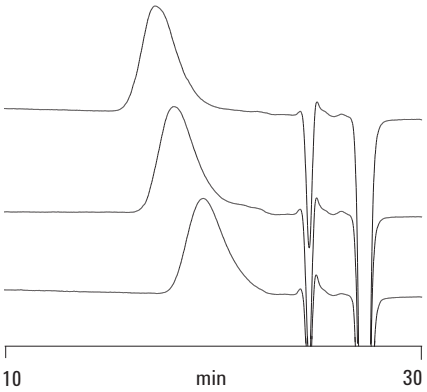


Figure 15. *Overlaid chromatograms of three samples of polyphenylene sulfide*

Ordering information

The following products are featured in this application compendium. For a full list of GPC/SEC part numbers, visit **www.agilent.com/chem/store**

Columns

Description	Part No.
Agilent PLgel 3 µm 100Å, 300 x 7.5 mm	PL1110-6320
Agilent PLgel 5 µm MIXED-D, 300 x 7.5 mm	PL1110-6504
Agilent PLgel 5 µm MIXED-C, 300 x 7.5 mm	PL1110-6500
Agilent PLgel 10 µm MIXED-B, 300 x 7.5 mm	PL1110-6100
Agilent PLgel 20 µm MIXED-A, 300 x 7.5 mm	PL1110-6200
Agilent PLgel 20 µm MIXED-A LS, 300 x 7.5 mm	PL1110-6200LS*
Agilent PLgel Olexis, 300 x 7.5 mm	PL1110-6400
Agilent PL HFIPgel, 300 x 7.5 mm	PL1114-6900HFIP

Standards

Description	Part No.
Agilent PS-H EasiVial 2 mL pre-weighed polystyrene calibration kit	PL2010-0201
Agilent PS-M EasiVial 2 mL pre-weighed polystyrene calibration kit	PL2010-0301
Agilent PS-L EasiVial 2 mL pre-weighed polystyrene calibration kit	PL2010-0401
Agilent EasiCal PS-1 pre-prepared polystyrene kit	PL2010-0501
Agilent EasiCal PS-2 pre-prepared polystyrene kit	PL2010-0601
Agilent PM EasiVial 2 mL pre-weighed polymethylmethacrylate calibration kit	PL2020-0201
Agilent PM EasiVial 4 mL pre-weighed polymethylmethacrylate calibration kit	PL2020-0200

Instruments

Description	Part No.
Agilent PL-GPC 220 Integrated GPC/SEC System	PL0820-0000
Agilent PL-BV 400HT Online Integrated Viscometer	PL0810-3050
Agilent PL-HTLS 15/90 Light Scattering Detector	PL0640-1200
Agilent PL-SP 260VS Sample Preparation System**	

Software

Description	Part No.
Agilent Cirrus GPC Software	PL0570-2000
Agilent Cirrus GPC Multi Detector Software	PL0570-2020

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Companion products

The term engineering polymers includes a wide range of materials, and although gel permeation chromatography is the paramount technique in their analysis, there are other analytical techniques that may be employed. Agilent makes a range of instruments in molecular spectroscopy and X-ray crystallography that can be used for the investigation of these types of material. Agilent's advanced instruments elucidate their characteristics and composition, leading to a better understanding of the behavior and fitness for purpose of these increasingly valuable products.

UV-Vis-NIR spectroscopy

Agilent's Cary range of UV-Vis-NIR spectrophotometers has been synonymous with excellence and high performance for over 60 years. The Cary spectrophotometer series is the standard for researchers wanting to extend the boundaries of spectrophotometric measurement. The range is equally at home in routine laboratories where reliability and ease of use are vital for the quality control of polymers.



The Agilent 600-IR series provides the highest level of sensitivity combined with detailed structural and compositional information for information-rich detection

Fluorescence spectroscopy

Agilent's Cary Eclipse Fluorescence Spectrophotometer offers the high performance you've come to expect from a Cary, at a surprisingly low price. With xenon flash lamp technology, plug-and-identify electronics and feature-packed, intuitive software, the instrument embodies the Agilent and Cary names.

FTIR spectroscopy

The compositional analysis of polymers is made easy with Agilent's FTIR spectrometers and microscopes that provide the ability to extract specific chemical information from extremely small sample areas. Exclusive to Agilent and taking full advantage of our FTIR attenuated total reflection (ATR) imaging technology, the Specac Imaging Golden Gate Diamond ATR accessory also provides the highest quality chemical images that are distortion- and aberration-free with a preserved aspect ratio, while maintaining the Golden Gate's robustness and ease of use.

Raman spectroscopy

Raman spectroscopy delivers qualitative and quantitative information on chemical species that make up engineering polymers. Raman complements IR spectroscopy, particularly for the study of crystalline polymers. Agilent's Synergy FT-Raman module is the most compact FT-Raman accessory on the market, maintaining the versatility required by research spectroscopists.

X-ray crystallography

X-ray crystallography was famously used to decipher the structure of the DNA polymer in the early 1950s. These days, Rosalind Franklin would probably use Agilent's Xcalibur E, the expert diffractometer for the modern chemical crystallography laboratory and the most popular choice for single wavelength, small molecule crystallography.

Other GPC/SEC resources from Agilent

Agilent has published application compendia on biodegradable polymers, polyolefin analysis, elastomers, and low molecular weight resins. In addition, we also offer a comprehensive and informative range of literature for all aspects of GPC/SEC, including application notes, datasheets and technical overviews.

Publication	Publication number
Biodegradable polymers	5990-6920EN
Polyolefin analysis	5990-6971EN
Elastomers	5990-6866EN
Low molecular weight resins	5990-6845EN
Introduction to GPC/SEC	5990-6969EN
GPC/SEC reference poster	5990-6882EN
Column selection guide	5990-6868EN

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Agilent Technologies

Biodegradable polymers - analysis of biodegradable polymers by GPC/SEC

Application compendium

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The Measure of Confidence

Agilent Technologies

Contents Page

Studying biodegradable polymers 3

Synthetic polymers

Poly(lactide-co-glycolide) 4

Polycaprolactam 4

Polyvinyl alcohol 5

Polyethylene glycol 5

Naturally-occuring polymers

Natural rubber 8

Polyacrylic acid 9

Chitosan 12

Cellulosic polymer 13

Carboxymethyl cellulose 13

Hydroxyethyl cellulose 14

Methyl cellulose 15

Cellulose acetate 16

Pectins 17

More Agilent solutions for biodegradable polymers 19

Studying biodegradable polymers

Biodegradation is the degradation of a material by environmental factors such as sunlight, temperature changes or the action of microbes. In polymer science and engineering, the design of polymers susceptible to biodegradation is of increasing importance for two reasons – polymers that degrade naturally in the body to harmless products may be used in biological devices and in drug delivery, and polymers that break down in the environment are significantly 'greener' than traditional plastics.

Biodegradation is key to the suitability of materials for use in drug delivery devices or in temporary structures within the body, such as sutures. For these applications, the ability of the body to naturally break down the material used either as part of the application or post-event is very important, making the removal of the polymer simply a case of allowing the natural process of degradation to occur. Many materials are being investigated for these applications as medical science progresses.

The landfill crisis has made the production of non-polluting polymers for packaging and engineering uses a high priority. These materials need to be able to perform their function, but also break down in the environment with time, a difficult proposition.

For these materials, the rate of degradation and therefore the lifetime and performance of the polymer in the natural environment is related to the length of the polymer chains in the material, with degradation leading to scission of the polymer chains and a shortening of their length.

Gel permeation chromatography (GPC, also known as size exclusion chromatography, SEC), a well-known technique for determining the molecular weight distribution of polymers, is therefore key to studying biodegradable materials by giving an insight into the rate at which a material might degrade, and revealing the presence of degraded polymer chains in a sample.

This application compendium shows examples of GPC applications involving different biodegradable polymers, derived from synthetic and natural sources.

Agilent Technologies produces the most extensive range of GPC/SEC columns, standards and instruments that are ideally suited to the analysis of biodegradable polymers.

Agilent's columns are the most stable available, and include ranges suited for use in organic and aqueous eluents, solvent mixtures and high polarity organic eluents, covering the requirements of the diversity of biodegradable materials. With extensive options in particle and pore size, Agilent's columns can be specifically selected to match the molecular weight of the material under investigation, thereby ensuring that the best quality data is obtained from the GPC/SEC experiment.

Agilent's GPC/SEC columns are the most rugged and reliable on the market, making them ideal for applications that rely on extremely reproducible analysis such as in quality control environments.

Given that many biodegrabale materials are destined for use in vivo, ensuring the quality of materials is of the upmost importance.

Agilent also manufactures narrow polydispersity standards with very highly characterized molecular weights that are used as calibration standards in the GPC/SEC analysis of biodegradable polymers.

Complementing Agilent's column technology is the most extensive collection of integrated GPC/SEC instrumentation on the market covering the temperature range from ambient to 220 °C.

These instruments allow all forms of the GPC/SEC experiment to be performed and can be used to analyze the complete range of biodegradable materials. Multiple detection options can be included in the instruments, such as light scattering and viscometry, and dedicated analysis software is available that allows the biodegradation properties of the materials to be monitored.

Agilent's complete range of columns and instrumentation offer a clear advantage in the analysis of biodegradable polymers.



Narrow dispersity polymer calibrants

Synthetic polymers

Poly(lactide-*co*-glycolide)

Application area: Drug delivery

Poly(lactide-*co*-glycolide) copolymers have found extensive applications in the pharmaceutical industry. The molecular weight distribution of the polymer can affect the properties of the end product, and is therefore of interest in both the areas of development and quality control.

The copolymer is quite polar in nature, but can be dissolved in several solvents suitable for gel permeation chromatography (GPC), notably tetrahydrofuran (THF) and chloroform.

Low boiling solvents like chloroform can suffer from outgassing effects. When employing refractive index detection, this can lead to chromatograms with noisy or drifting baselines. The Agilent 380-ELSD and Agilent 385-ELSD (cooled) evaporative light scattering detectors, on the other hand, always deliver baselines which are stable and drift-free. Furthermore, due to its evaporative nature, it provides chromatograms which are free from system peaks around total permeation which are commonly associated with refractive index detectors. Agilent's 380-ELSD and 385-ELSD also offer superior sensitivity compared to refractive index.

Poly(lactide-*co*-glycolide) copolymers are relatively low in molecular weight.

The Agilent PLgel 5 µm MIXED-D columns with their high efficiency (>50,000 plates/meter) and broad resolving molecular weight range (up to 400,000 daltons relative to polystyrene), are the columns of choice for this application.

Figure 1 shows a typical raw data chromatogram for a poly(lactide-co-glycolide) sample.

Columns: 2 x PLgel 5 µm MIXED-D, 300 x 7.5 mm (Part No. PL1110-6504)
Eluent: Chloroform
Flow Rate: 1.0 mL/min
Detector: Agilent ELSD

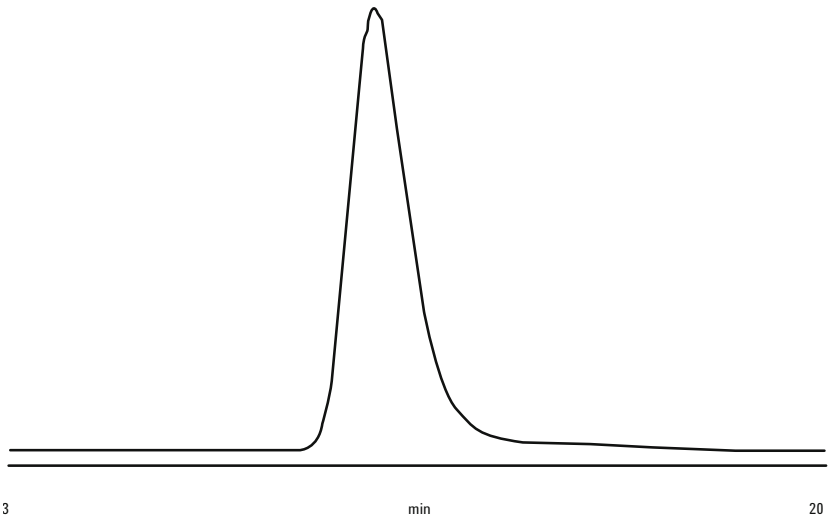


Figure 1. *Typical raw data chromatogram for a poly(lactide-co-glycolide) sample showing a typical gaussian peak shape*

Polycaprolactam

Application area: Drug delivery

Traditional drug delivery systems, such as the oral contraceptive pill, have a major disadvantage - the release of the active species is very non-linear, with typically a high dosage at the time of introduction followed by a steady decline as the drug is metabolized. A release profile of this kind is inefficient. Ideally, the dosage of the active compound into the body should remain at a constant level during treatment. The controlled delivery of drugs in vitro to produce linear dosing regimes is a major goal of therapeutic research. Polycaprolactam is a well-known polymer that biodegrades by enzymatic cleavage of ester bonds under conditions found within the human body. Introducing an active drug contained in a matrix of polycaprolactam into the body leads to the steady release of drug as the polymer matrix degrades. Appropriate inclusion of the drug into the matrix controls the rate of release

A critical parameter controlling the rate of degradation of biodegradable polymers is the molecular weight of the starting material. The higher the average molecular weight, the slower the rate of biological degradation. Measuring the molecular weight distributions of biodegradable polymers by gel permeation chromatography (GPC) is a critical part of research into controlled drug release with polymers. The chromatogram below shows polycaprolactam obtained in THF using two Agilent PLgel 5 µm MIXED-C columns. The polymer eluted as a broad peak with an average molecular weight of 80,000 g/mol and a polydispersity of 2.5.

Sample:	Polycaprolactam
Columns:	2 x PLgel 5 µm MIXED-C, 300 x 7.5 mm (Part No. PL1110-6500)
Eluent:	THF (stabilized)
Flow Rate:	1.0 mL/min
Inj Vol:	200 µL
Detector:	RI

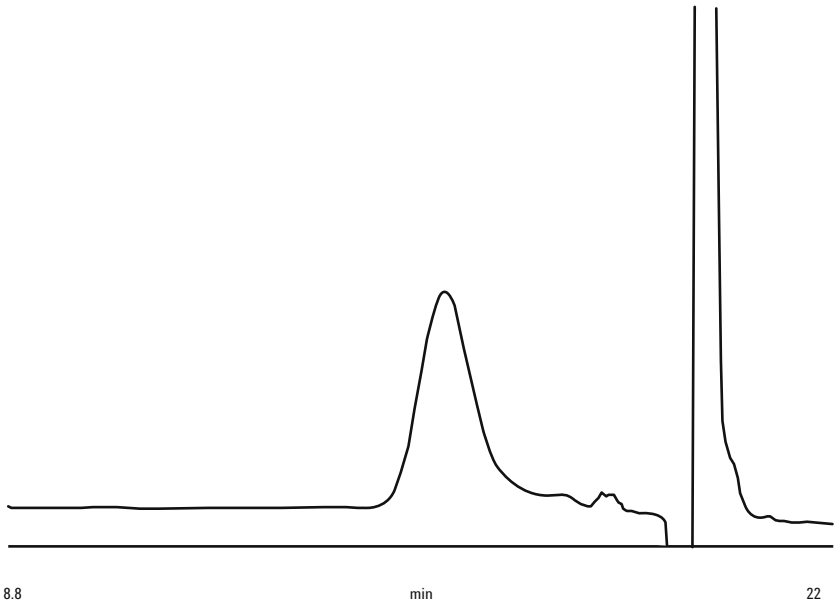


Figure 2. *Typical raw data chromatogram for a sample of polycaprolactam containing low molecular weight components and showing a large system peak*

Polyvinyl alcohol

Application areas: Adhesive, surfactant, surface properties

Fully or partially hydrolyzed grades of polyvinyl alcohol are normally specified according to their viscosity in solution. Aqueous SEC can be used to characterize these polymers in terms of molecular weight distribution. Three samples with the same degree of hydrolysis were compared by overlaying their molecular weight distributions. This is a convenient method of fingerprinting materials for quality control, and is more informative in production control and end-use performance evaluation than single point viscosity measurements.

Calibrants:	Pullulan polysaccharides
Columns:	2 x Agilent PL aquagel-OH 40 8 µm, 300 x 7.5 mm (Part No. PL1149-6840)
Eluent:	0.25M $NaNO_3$, 0.01M NaH_2PO_4, pH 7
Flow Rate:	1.0 mL/min
Detector:	RI

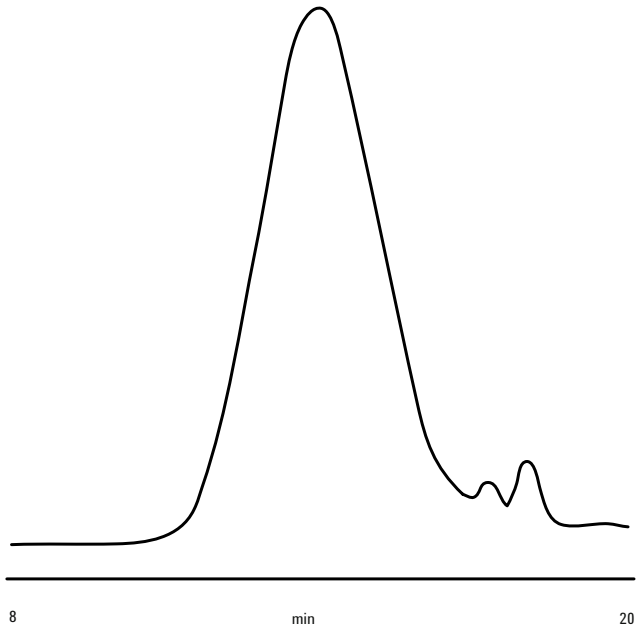


Figure 3. *Raw data chromatogram (sample A) showing the presence of low molecular weight components along with the polymer peak*

Table 1. Correlation of GPC results with polymer specification for PVA

Sample	Viscosity (mPa.s)	Mn	Mw
A	4.0	9771	29470
B	10.0	23339	80174
C	20.0	31210	102309

Polyethylene glycol (PEG)

Application areas: Excipient, dispersant, antifreeze

Polyethylene glycols are inert, water-soluble and biodegradable polymers used in a range of applications from medical formulations, protein conjugates to cosmetic products and antifreeze solutions. Manufactured by living polymerization processes, PEGs have narrow molecular weight distributions with physical properties controlled by their molecular weight.

Agilent PL aquagel-OH 30 8 µm high performance columns are ideal for relatively low molecular weight separations, combining low exclusion limit, high pore volume and high column efficiency (>35,000 plates/meter) for maximum resolution.

The separation below shows a range of PEG samples.

Columns: 2 x PL aquagel-OH 30 8 µm, 300 x 7.5 mm (Part No. PL1120-6830)
Eluent: Water
Flow Rate: 1.0 mL/min
Detector: RI

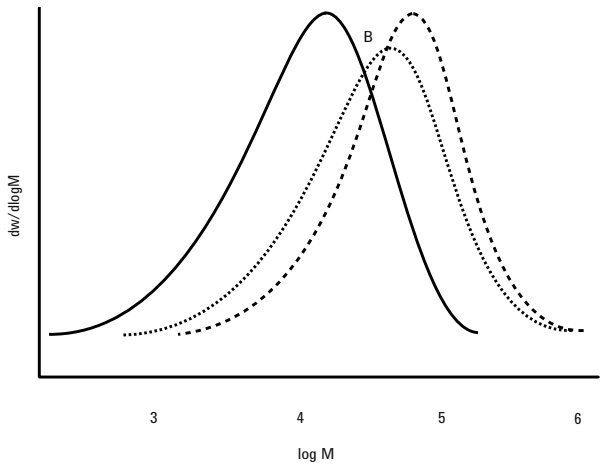


Figure 4. *Example molecular weight distributions of the three polyvinyl alcohols with very different physical properties*

Columns: 2 x PL aquagel-OH 30 8 µm, 300 x 7.5 mm (Part No. PL1120-6830)
Eluent: Water
Flow Rate: 1.0 mL/min
Detector: RI

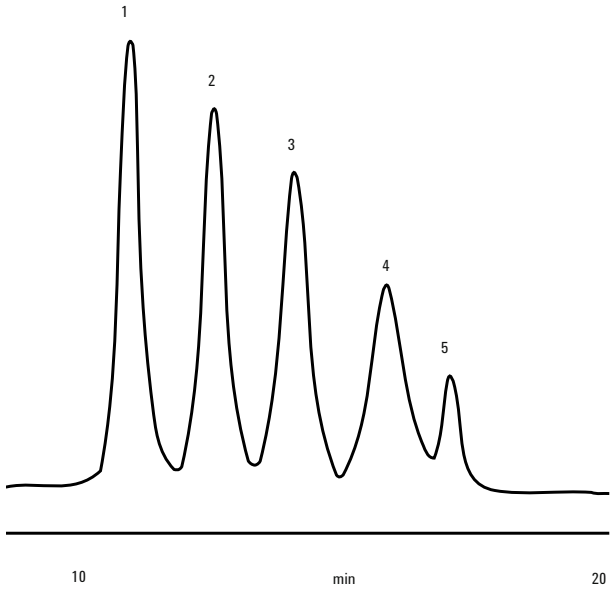


Figure 5. *Resolution achieved in the separation of five PEG samples used for calibration*

Naturally-occurring polymers

Natural rubber

Application area: Engineering material
A biodegradable polymer produced from tree sap, natural rubber is an elastomer that has many uses in a wide range of industrial and household products. It is degraded slowly by bacterial action.

Solutions of natural rubber samples are generally very difficult to prepare for GPC due to the fact that the polymer contains relatively high levels of 'gel', which is partially crosslinked. An aliquot of the eluent is added to the weighed sample. It is allowed to swell and dissolve overnight, and then the gel material is filtered out (0.5 µm) prior to GPC analysis.

In this case, the actual polymer concentration can be significantly lower than the original concentration prepared depending on the gel content of the sample, and therefore detector response, usually RI, tends to be quite poor. ELSD exhibits significantly increased sensitivity compared to an RI and subsequently gives much greater response for this application. In addition, RI baseline drift, which commonly occurs, is very much emphasized when the actual peak response is so small.

ELSD always gives a flat baseline which, together with the improved response, makes baseline and peak setting much more reliable for GPC calculations.

A further problem with RI is sensitivity to system peaks around total permeation, which usually occurs even when samples are prepared in an aliquot of the eluent. These system peaks can interfere with low molecular weight components which are commonly found in natural rubber samples. This situation is very much improved when ELSD is employed, as system peaks are eliminated due to evaporation, leaving unadulterated sample peaks in the additives region.

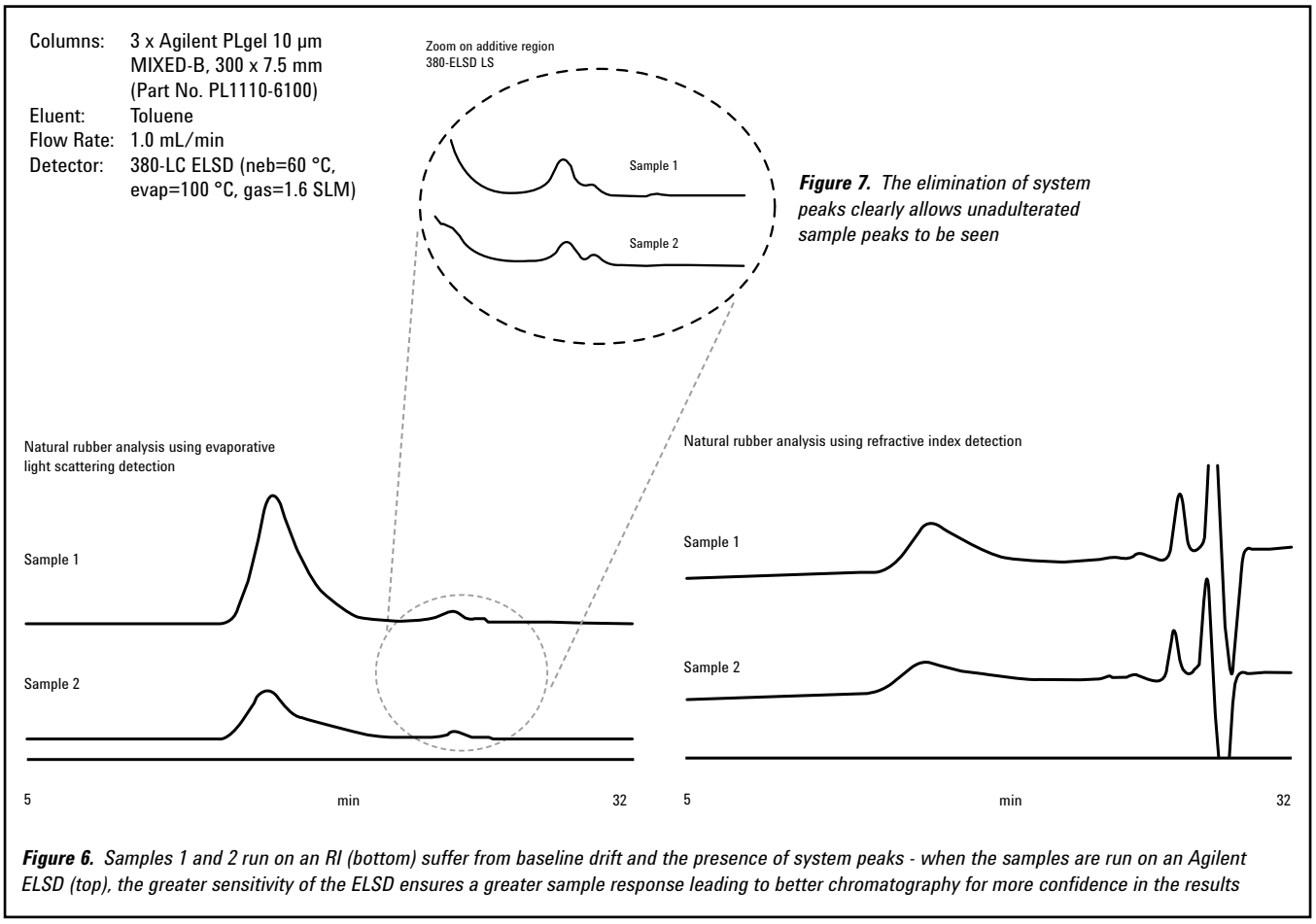


Figure 7. *The elimination of system peaks clearly allows unadulterated sample peaks to be seen*

Figure 6. *Samples 1 and 2 run on an RI (bottom) suffer from baseline drift and the presence of system peaks - when the samples are run on an Agilent ELSD (top), the greater sensitivity of the ELSD ensures a greater sample response leading to better chromatography for more confidence in the results*

Polyacrylic acid

Application areas: Adhesive, water treatment

Polyacrylic acid is a biodegradable water soluble polymer with numerous industrial applications, including as a super adsorbent (e.g. in disposable nappies), in water treatment as a metal ion scavenger and in the treatment of metal surfaces prior to coating.

The molecular weight distribution (MWD) of this material is an important parameter, as it strongly affects the end use properties of the polymer. Aqueous SEC is an ideal analytical tool for the measurement of the MWD of polyacrylic acid. Since polyacrylic acid is a polyelectrolyte, care must be taken in selecting the appropriate SEC conditions. In the SEC described below, a buffered mobile phase with a high electrolyte content was used to minimize non-size exclusion effects.

Agilent PL aquagel-OH MIXED-H columns were selected to provide good resolution over a wide molecular weight range. Column calibration was achieved using Agilent polyethylene oxide (PEO) EasiVial standards, see Figure 8.

Columns:	2 x PL aquagel-OH MIXED-H 8 µm, 300 x 7.5 mm (Part No. PL1149-6800)
Eluent:	0.2M $NaNO_3$ + 0.01M NaH_2PO_4, adj to pH 7
Flow Rate:	1.0 mL/min
Inj Vol:	200 µL
Sample Conc:	PEO standards: 0.1-0.5 mg/mL Polyacrylic acid: approx 0.2% w/v
Detector:	RI

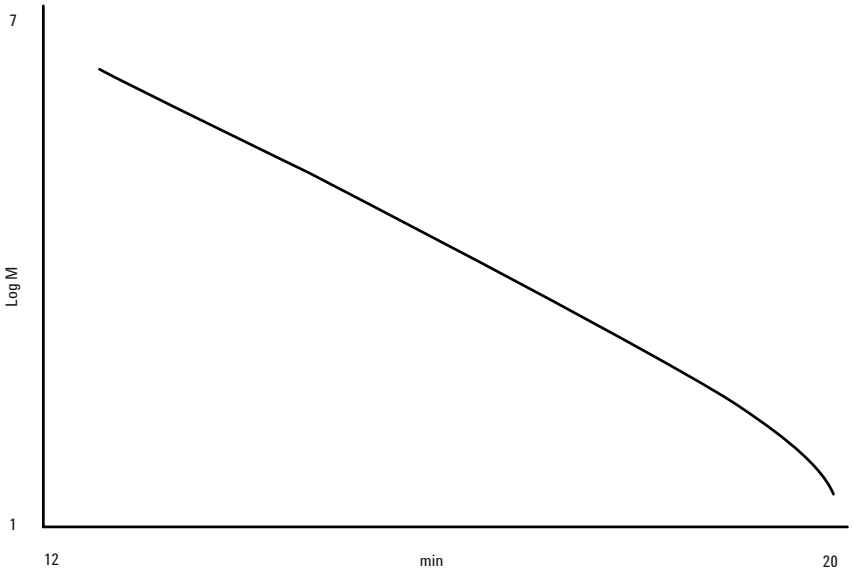


Figure 8. *PL aquagel-OH MIXED-H 8 µm column calibration using PEO EasiVial standards showing the relationship between retention time and the log of molecular weight*

EasiVial standards

EasiVial standards provide a rapid and convenient means of constructing an aqueous SEC column calibration curve over a wide molecular weight range (typically 100 to 1,200,000 g/mol). Each vial contains a mixture of four individual, highly characterized, narrow dispersity standards. The amount of each individual standard is carefully controlled during manufacture, allowing their use in SEC-viscometry which requires accurate concentrations.

Refractive index chromatograms obtained from each PEO EasiVial are presented in Figure 9.

Three polyacrylic acid samples (A, B and C) were chromatographed and their corresponding molecular weight distribution compared, see Figure 10.

Table 2. Comparison of molecular weight averages for the three samples of polyacrylic acid

Sample	Mn (g/mol)	Mw (g/mol)	PD
A	33,450	89,430	2.67
B	7,990	14,930	1.87
C	7,880	13,490	1.71

Sample A was found to possess a significantly higher molecular weight distribution compared to Samples B and C, which were found to be similar. Consequently, Sample A was expected to possess significantly different rheological properties compared to the remaining two samples. Closer examination of the samples showed Sample A to be significantly more viscous than B and C, which were similar. In addition, the MWD of Sample A was found to be bi-modal, which suggests that the sample may be a blend of more than one component. In conclusion, differences in the molecular weight distributions of the polyacrylic acids were identified. These differences were corroborated through visual examination of the samples' bulk viscosity.

Columns: 2 x PL aquagel-OH MIXED-H 8 µm, 300 x 7.5 mm (Part No. PL1149-6800)
Eluent: 0.2M $NaNO_3$ + 0.01M NaH_2PO_4, adj to pH 7
Flow Rate: 1.0 mL/min
Inj Vol: 200 µL
Sample Conc: PEO standards: 0.1-0.5 mg/mL
Polyacrylic acid: approx 0.2% w/v
Detector: RI

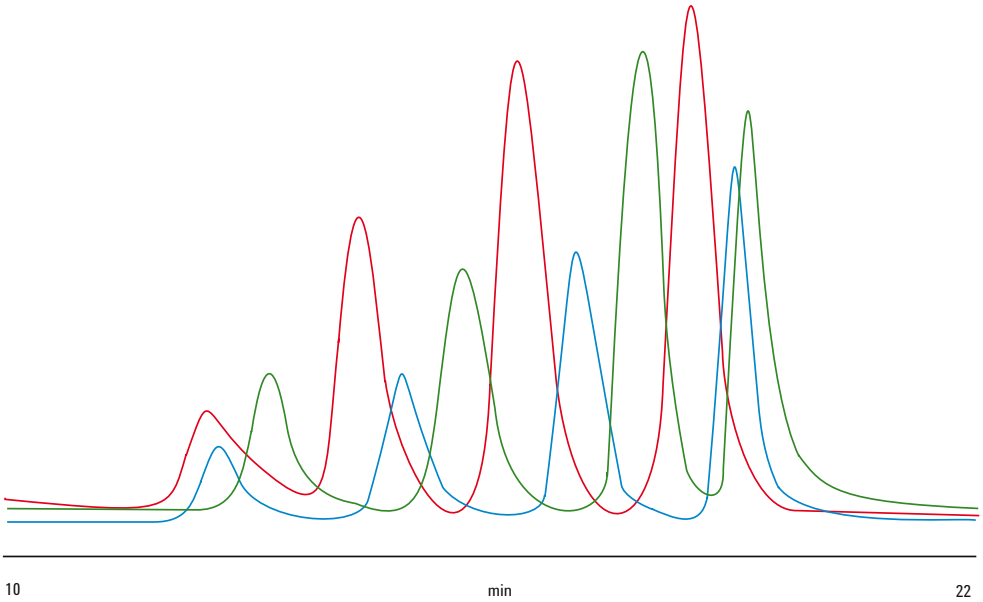


Figure 9. *Example chromatograms of PEO EasiVial standards used to create the calibration*

Columns:	2 x PL aquagel-OH MIXED-H 8 µm, 300 x 7.5 mm (Part No. PL1149-6800)
Eluent:	0.2M $NaNO_3$ + 0.01M NaH_2PO_4, adj to pH 7
Flow Rate:	1.0 mL/min
Inj Vol:	200 µL
Sample Conc:	PEO standards: 0.1-0.5 mg/mL
	Polyacrylic acid: approx 0.2% w/v
Detector:	RI

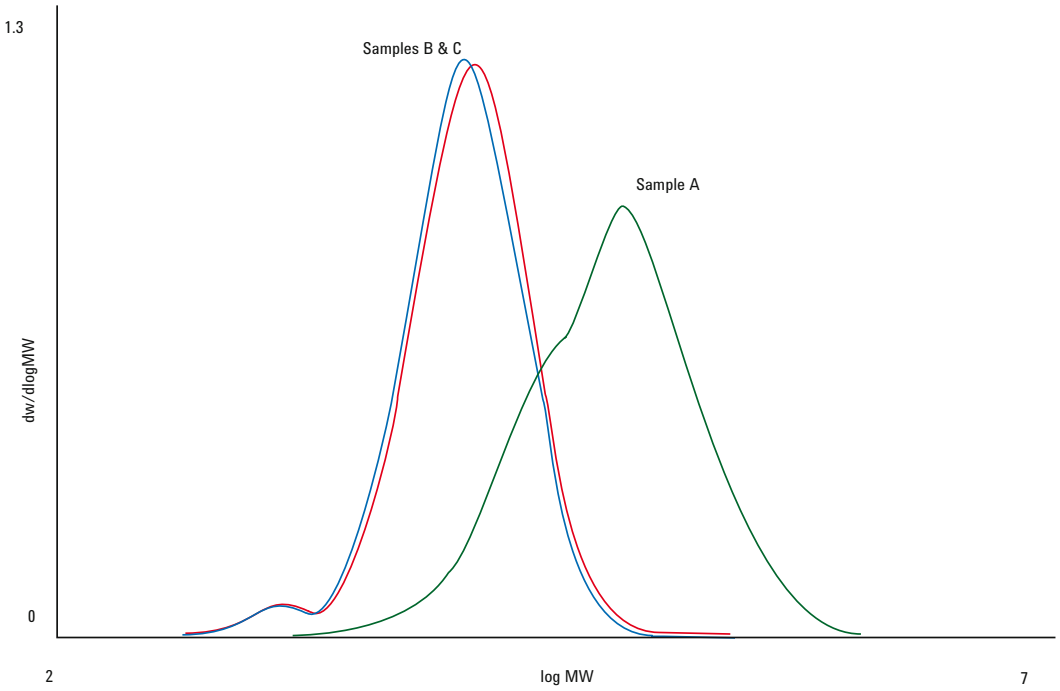


Figure 10. *Molecular weight distribution of three polyacrylic acid samples showing a clear difference between one of the samples (A) and the other two (B and C)*

Chitosan

Application areas: Drug delivery, paper production

Chitosan is a naturally-occurring polysaccharide made by alkaline N-deacetylation of chitin which is believed to be the second most abundant biomaterial after cellulose. The term chitosan does not refer to a uniquely-defined compound, but merely refers to a family of copolymers with various fractions of acetylated units containing both chitin and chitosan monomers.

The main interest in chitosan derives from its cationic nature in acidic solutions which provides unique properties relative to other polysaccharides, which are usually neutral or negatively charged. Application areas of chitosan include biomedical (e.g. wound healing, burn treatment and use as a hemostatic agent), paper production, textile finishes, photographic products, cements, heavy metal chelating agents and waste removal.

GPC/SEC can be used as a quality control tool for the determination of MW and MWD. Different molecular weights would be appropriate to particular applications.

Three grades of chitosan were analyzed using a column set comprising 2 x PL aquagel-OH MIXED 8 µm columns. These columns offer resolution over a wide molecular weight range (up to 10,000,000 relative to PEO/PEG).

Due to the cationic nature of the samples, they were prepared in strong acid and were allowed to stand overnight to aid dissolution. They were analyzed in 0.5M sodium nitrate buffer and at low pH.

Raw data chromatograms and weight average molecular weight values (Mw) for the three chitosan samples are shown below.

The system was calibrated with narrow pullulan polysaccharide standards and the resulting calibration curve is illustrated below.

Columns:	2 x PL aquagel-OH MIXED-H 8 µm, 300 x 7.5 mm (Part No. PL1149-6800)
Eluent:	0.5M $NaNO_3$, 0.01M NaH_2PO_4, pH 2
Flow Rate:	1.0 mL/min
Detector:	RI

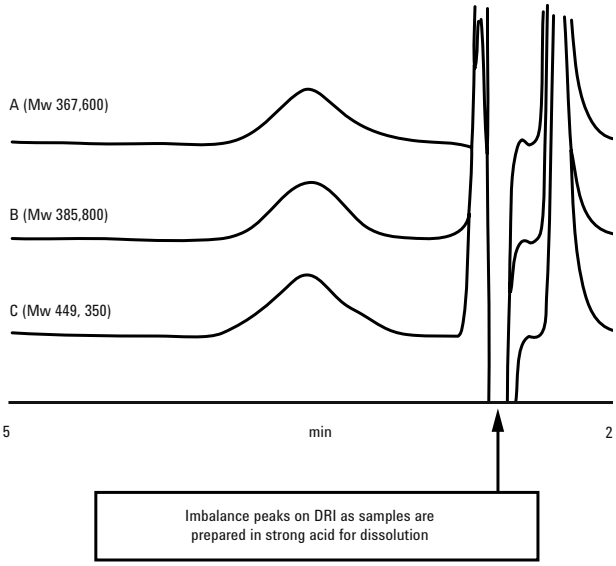


Figure 11. *Raw data chromatograms of three chitosan samples showing typical peak shapes with strong imbalance peaks due to dissolution conditions*

Columns:	2 x PL aquagel-OH MIXED-H 8 µm, 300 x 7.5 mm (Part No. PL1149-6800)
Eluent:	0.5M $NaNO_3$, 0.01M NaH_2PO_4, pH 2
Flow Rate:	1.0 mL/min
Detector:	RI

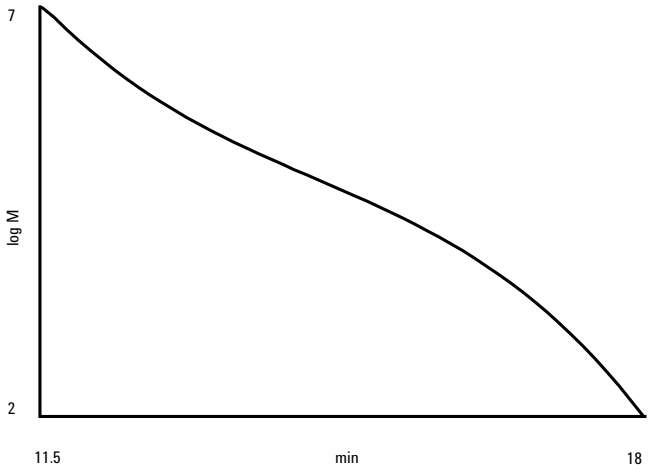


Figure 12. *SEC calibration showing the resolving range of the PL aquagel-OH MIXED-H 8 µm column set*

Cellulosic polymer

Application areas: Thickening agent and viscosity modifier
The most abundant component of plant matter, cellulose, can be modified to produce a number of biodegradable materials with useful properties.

Carboxymethyl cellulose (CMC) is a cellulose derivative with some of the hydroxyl groups of the glucopyranose monomers of cellulose modified to contain carboxymethl groups. CMC is a thickener used in the food industry where it has E number E466, and is also used to stabilize emulsions in ice cream. It is also a constituent of many non-food products, including toothpaste and water-based paints. Hydroxyethyl cellulose has some of the hydroxyl groups modified with ethyl chains, and is used as a gelling and thickening agent in cosmetics, cleaning solutions, and other household products.

Carboxymethyl cellulose

Calibrants:	Pullulan polysaccharides
Columns:	2 x Agilent PL aquagel-OH 60 8 µm, 300 x 7.5 mm (Part No. PL1149-6860)
	1 x PL aquagel-OH 40 8 µm, 300 x 7.5 mm (Part No. PL1149-6840)
Eluent:	0.5M Na_2SO_4
Flow Rate:	1.0 mL/min
Detector:	RI

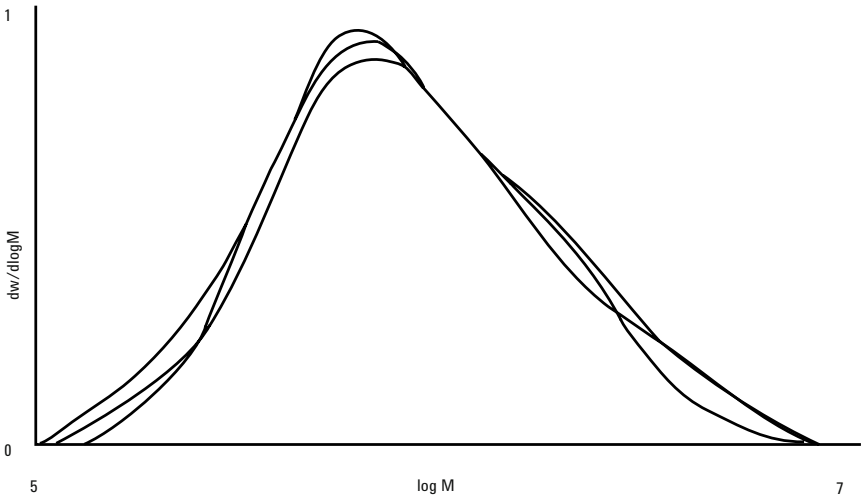


Figure 13. *Example molecular weight distributions of three samples of carboxymethyl cellulose with subtle differences between samples*

Hydroxyethyl cellulose

The correlation between the two measurements is good, showing that GPC is a viable measurement technique to viscosity when ensuring the quality of these samples.

Table 3. Correlation of viscosity data with GPC results for hydroxyethyl cellulose

	A	B	C
Viscosity Range (cps)	75-112	250-324	1500-2500
Mn	60,300	413,000	914,000
Mw	179,000	849,000	2,016,000
Mz	39,000	1,552,000	3,422,000

Calibrants:	Pullulan polysaccharides
Columns:	2 x PL aquagel-OH 60 8 µm, 300 x 7.5 mm (Part No. PL1149-6860)
	1 x PL aquagel-OH 40 8 µm, 300 x 7.5 mm (Part No. PL1149-6840)
Eluent:	0.05M NaH_2PO_4, 0.25M NaCl, pH 7
Flow Rate:	1.0 mL/min
Detector:	RI

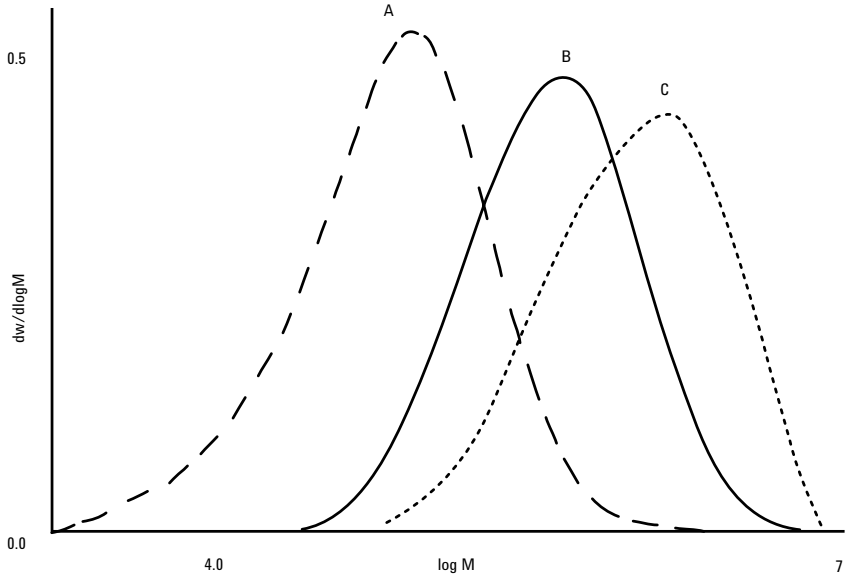


Figure 14. *Example molecular weight distributions of three samples, A, B and C with very different properties*

Methyl cellulose

Application areas: Emulsifier, treatment of constipation

Table 4. Correlation of viscosity data with GPC results for methyl cellulose

	A	B
Viscosity Range (cps)	85-115	4000-6000
Mn	131,000	484,000
Mw	369,000	1,023,000
Mz	691,000	1,884,000

There is good correlation between the viscosity data and molecular weight averages.

Calibrants:	Pullulan polysaccharides
Columns:	2 x PL aquagel-OH 60 8 µm, 300 x 7.5 mm (Part No. PL1149-6860)
	1 x PL aquagel-OH 40 8 µm, 300 x 7.5 mm (Part No. PL1149-6840)
Eluent:	0.05M NaH_2PO_4, 0.25M NaCl, pH 7
Flow Rate:	1.0 mL/min
Detector:	RI

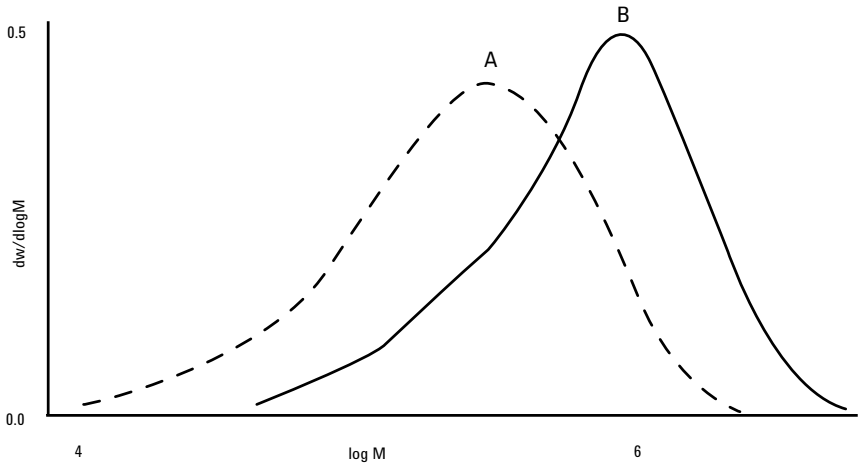


Figure 15. *Example molecular weight distributions for two samples of methyl cellulose which behave very differently*

Cellulose acetate analysis

Application areas: Photographic film base, adhesives, synthetic fibers
Used extensively in the photographic and packaging industries, cellulose acetate is soluble in a limited number of solvents. Here, dissolution was achieved in dimethylacetamide after gentle heating and stirring of the sample solution. Lithium chloride was added to the eluent to counter any polyelectrolyte effects.

Columns:	3 x PLgel 10 µm MIXED-B, 300 x 7.5 mm (Part No. PL1110-6100)
Eluent:	DMAc+0.5% LiCl
Flow Rate:	1.0 mL/min
Loading:	0.2% w/v, 100 µL
Temp:	60 °C
Detector:	GPC (RI)

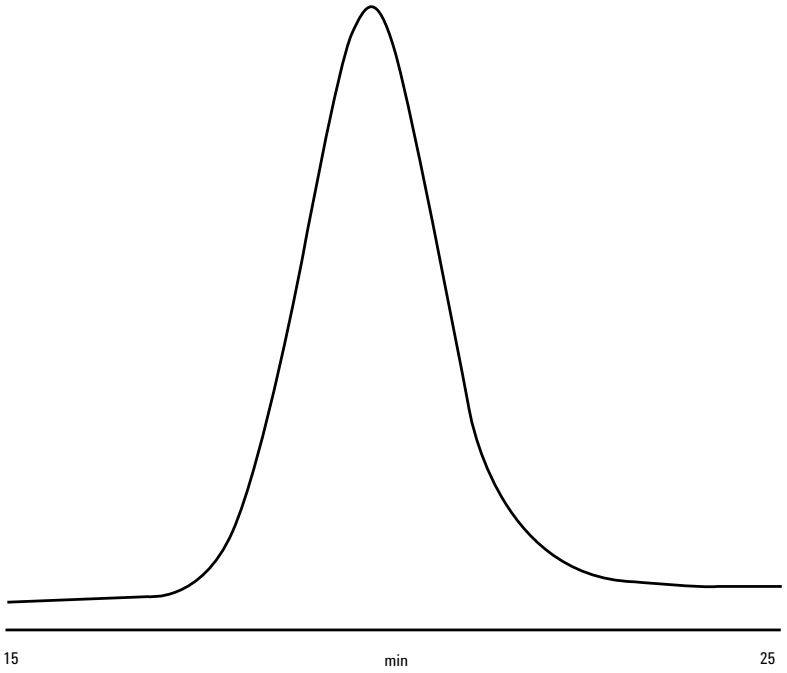


Figure 16. *Example chromatogram for a sample of cellulose acetate analysis showing a typical polymer peak shape*

Pectins

Application area: Gelling agent in food

Pectins are a class of polysaccharide gum found naturally in fruits such as apples, plums, grapes and cranberries. Structurally complex, pectins consist of 'smooth' and 'hairy' regions. The smooth regions are linear partially methylated poly(D-galacturonic) acid, the hairy regions alternating L-rhamnosyl and D-galacturonosyl residues containing L-arabinose and D-galactose branch points up to 20 residues long. As a result of this heterogenous nature, pectins adopt complex structures in solution.

Applications of pectin are related to the formation of crosslinks through hydrogen bonding of the carboxylic acid groups, and include use as gelling agents, thickeners and water binders. Triple detection size exclusion chromatography employs a concentration detector, a viscometer and a light scattering detector to assess the molecular weight distribution and molecular structure of polymers without having to rely on column calibrations. This can be important when analyzing complex materials for which no structurally similar standards are available.

In this application, a sample of pectin was analyzed on the Agilent PL-GPC 50 integrated GPC system running at 30 °C fitted with a refractive index detector, an Agilent PL-BV 400 four capillary bridge viscometer and an Agilent PL-LS 15/90 dual angle light scattering detector (collecting scattered light at 15° and 90°). Two PL aquagel-OH MIXED 8 µm columns were used for the analysis with a 200 µL injection loop and a buffer solution of 0.2M $NaNO_3$, 0.01M NaH_2PO_4, adjusted to pH 7, as the eluent. The sample was prepared accurately at nominally 2 mg/mL in the eluent and filtered before injection through a 0.45 µm disposable filter. For the purpose of light scattering calculations, an average dn/dc value was used for the sample.

Figure 17 shows an overlay of the triple detector chromatograms for the pectin sample. The chromatograms obtained on the refractive index and light scattering detectors were clearly multimodal, as expected for a structurally heterogeneous material.

Columns: 2 x PL aquagel-OH MIXED 8 µm, 300 x 7.5 mm (Part No. PL1149-6800)
Eluent: 0.2M $NaNO_3$ + 0.01M NaH_2PO_4, adj pH 7
Flow Rate: 1.0 mL/min
Inj Vol: 200 µL
Detectors: PL-GPC 50, RI, PL-BV 400, PL-LS 15/90

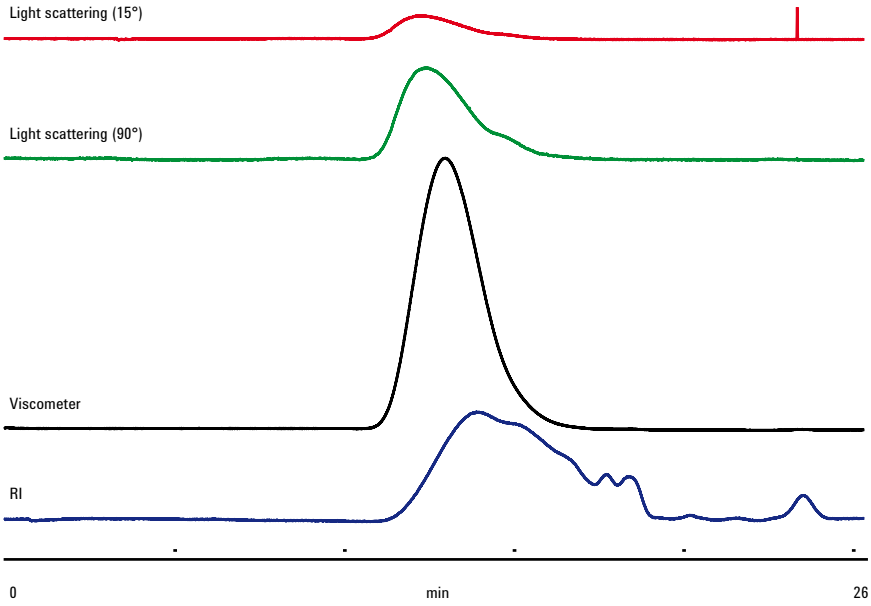
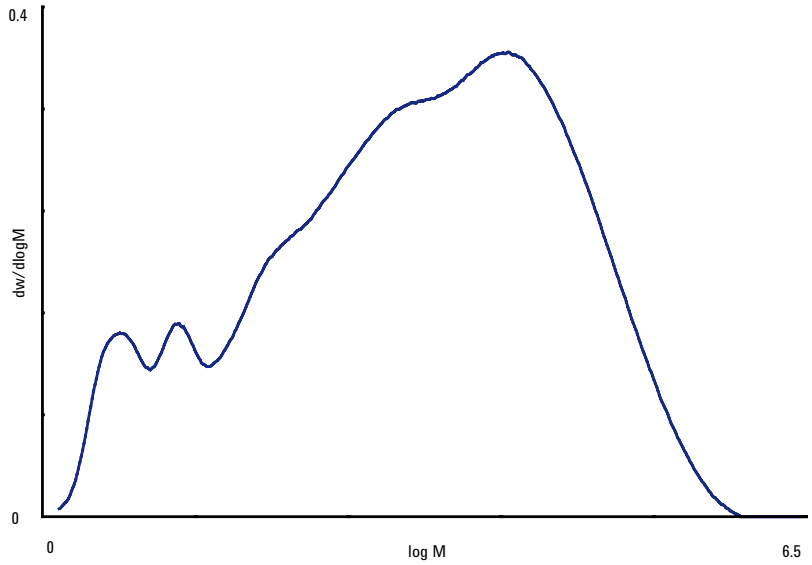


Figure 17. *A typical multi detector overlay of chromatograms for a sample of pectin, showing the different responses of the detector*

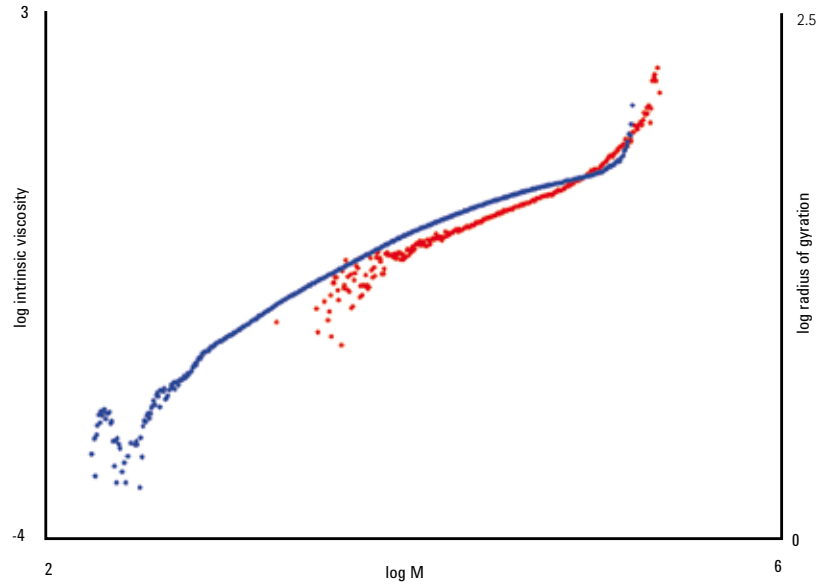
From the viscometry and light scattering data, Mark-Houwink (log intrinsic viscosity versus log M) and conformation (log radius of gyration versus log M) plots were generated for the pectin, shown overlaid in Figure 19.

The Mark-Houwink, and to some extent, the conformation plots show curvature over the entire molecular weight distribution, indicating a change in molecular density as a function of molecular weight, resulting from a variation in the relative amounts of 'smooth' and 'hairy' regions. This application demonstrates how the new PL-GPC 50 can be used for the analysis of structurally complex but commercially important materials by multi detector GPC.



Columns:	2 x PL aquagel-OH MIXED 8 µm, 300 x 7.5 mm (Part No. PL1149-6800)
Eluent:	0.2M $NaNO_3$ + 0.01M NaH_2PO_4, adj pH 7
Flow Rate:	1.0 mL/min
Inj Vol:	200 µL
Detectors:	PL-GPC 50, RI, PL-BV 400, PL-LS 15/90

Figure 18. *Molecular weight distribution calculated for the pectin showing a complex shape*



Columns:	2 x PL aquagel-OH MIXED 8 µm, 300 x 7.5 mm (Part No. PL1149-6800)
Eluent:	0.2M $NaNO_3$ + 0.01M NaH_2PO_4, adj pH 7
Flow Rate:	1.0 mL/min
Inj Vol:	200 µL
Detectors:	PL-GPC 50, RI, PL-BV 400, PL-LS 15/90

Figure 19. *Mark-Houwink and conformation data showing differences in structure between the two materials*

More Agilent solutions for biodegradable polymers

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Agilent Technologies

SEC Analysis of Pectin

Application Note

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Introduction

Pectin is a natural product used extensively as a jellifying, thickening and stabilizing agent in the food industry. It is produced from plant raw materials such as apple, citrus and beet. The extracts are processed to derive pectins with specific properties. Although pectin chemical composition is key to its application, rheological behavior is critical to performance, and determination of the molecular weight distribution can help to predict rheological behavior. SEC and Agilent PL aquagel-OH MIXED-H 8 µm columns are ideal for resolving pectins. With their wide molecular weight resolving range (up to 10 million g/mol relative to PEO/PEG) and high efficiency (>35,000 plates/meter), PL aquagel-OH are the columns of choice for this application.

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Conditions

Pectin samples were prepared at 2 mg/mL, left to fully dissolve overnight and filtered through a 0.45 µm membrane. The column set was calibrated with narrow pullulan standards and, therefore, all molecular weight values quoted are relative to these. The calibration curve is shown is Figure 1.

Samples: Pectin
Columns: 2 x PL aquagel-OH MIXED-H 8 µm, 300 x 7.5 mm (part number PL1149-6800)
Eluent: 0.2 M $NaNO_3$ + 0.01 M NaH_2PO_4 at pH 7
Flow Rate: 1.0 mL/min
Detection: RI

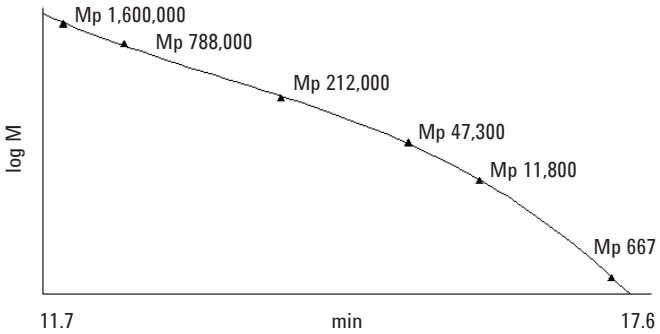


Figure 1. Pullulan standard calibration curve for PL aquagel-OH MIXED-H 8 µm

Results and Discussion

Raw data chromatograms for the pectin samples are illustrated in Figure 2.

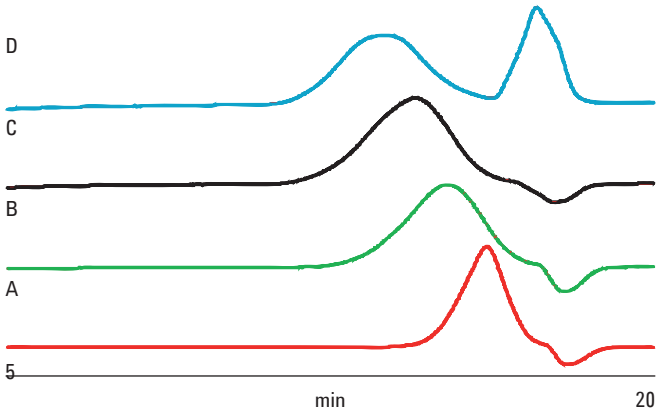


Figure 2. Chromatograms of four pectin samples

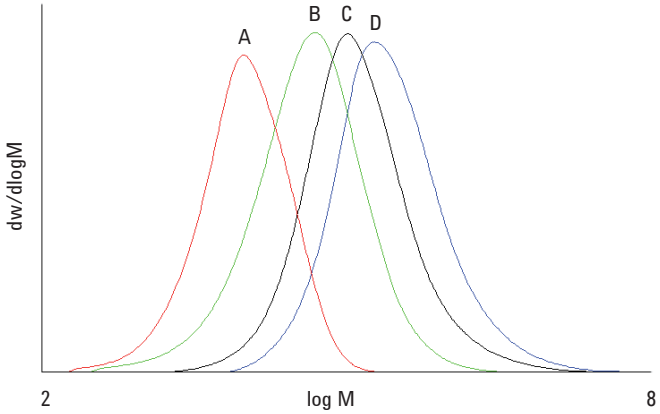


Figure 3. Molecular weight distributions of four pectin samples

Unlike the other samples, sample D exhibits a strong, positive peak around total permeation. This sample is a slow setting grade and contains buffer salts added to modify its properties. Molecular weight averages for the samples are given in the table below.

Table 1. Molecular weight averages for the four pectin samples

Sample	Mn	Mw	Mw/Mn
A	6,520	17,560	2.7
B	21,720	88,480	4.1
C	67,980	243,120	3.6
D	128,360	459,990	3.6

Conclusion

The wide molecular weight operating range of PL aquagel-OH MIXED-H 8 µm columns makes them particularly suited to the analysis of water soluble polymers with intermediate to high molecular weight. The use of a simple buffer solution as the eluent for the analysis of pectins reduces interaction between the sample and the columns ensuring that good chromatography is obtained.

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Agilent Technologies

SEC Analysis of Dextrans

Application Note

Authors

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Introduction

Dextran polysaccharides comprise linked a-D-glucose units with short side chains. These compounds are synthesized in aqueous solutions from fermentation disaccharides produced by lactobacillae such as *Leuconostoc mesenteroides* and *Streptococcus mutans*. In medicine, dextran is used as an antithrombotic to reduce blood viscosity and as an intravenous fluid to solubilize other factors, such as iron, or to replace lost blood in emergencies. In addition, it has a role as a lubricant in some eye drops, and in SEC matrices such as Sephadex. Dextrans are also used as starting or intermediate reagents by food, biotech, photographic and chemical manufacturers.

Size exclusion chomatography will reveal differences in the molecular size profiles of dextrans. Agilent PL aquagel-OH 40 and 60 8 µm columns are ideal for this purpose because they combine low exclusion limit, high pore volume and high column efficiency (>35,000 plates/meter) for maximum resolution. In this case, two different PL aquagel-OH columns were connected in series to cover a molecular weight range from 10^4 to 10^7.

Agilent Technologies

Conditions

Samples: Four dextrans
Columns: 2 x PL aquagel-OH 60 8 µm, 300 x 7.5 mm (p/n PL1149-6860 + 1 x PL aquagel-OH 40 8 µm, 300 x 7.5 mm (p/n PL1149-6840)
Eluent: 0.2 M NaH_2PO_3 + 0.2 M NaCL at pH 7
Flow Rate: 1.0 mL/min
Detection: RI

Results and Discussion

Figure 1 shows the differences in molecular weights of four commercial dextrans.

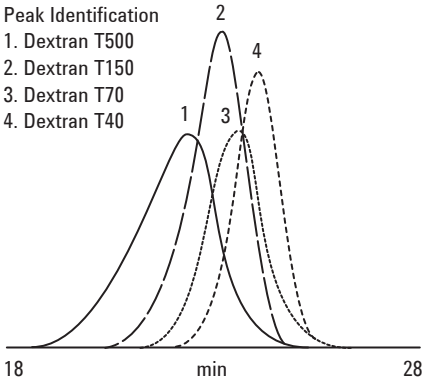


Figure 1. Overlay chromatograms showing the differences in molecular weights of four commercial dextrans

Conclusion

SEC and PL aquagel-OH columns successfully resolved four samples of dextran. The 'neutral' surface and ability to operate across a wide range of eluent conditions equip PL aquagel-OH for the high performance analysis of analytes with neutral, ionic and hydrophobic moieties, singly or combined.

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Identification of Unknown Reaction By-Products and Contaminants in Epoxyphenolic-Based Food Can Coatings by LC/TOF-MS

Application

Food Safety

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Abstract

This application illustrates how time-of-flight mass spectrometry can be used in the safety evaluation of new and existing can coatings used in the food industry. The accurate mass provides information for the parent compound and fragment ions greatly increase the confidence in the identification process.

Introduction

The internal surface of metal cans used to pack foodstuffs is often coated to form a barrier between the food and the metal of the can. The coating formulation may contain various components such as resins, crosslinking agents, catalysts, lubricants, wetting agents, and solvents. The potential exists for these ingredients, or by-products of reactions between them, to migrate from the can coating into foods. Thus existing and especially new coatings need to be evaluated for their safety for contact with food and beverages.

We will illustrate this evaluation using the example of epoxyphenolic can coatings based on bisphenol A epoxy resins. These are cured by stoving with phenolic resins to produce a three-dimensional crosslinked network to provide the chemical and pack resistance required for food and beverage cans. The epoxy monomer bisphenol A diglycidyl ether (BADGE, see Figure 1) participates in these polymerization reactions via its reactive epoxide groups. However, it can also undergo addition from attacking nucleophiles such as water or solvents to give lower molecular weight products that might migrate into the packed food [1–3]. These potential migrants need to be identified.

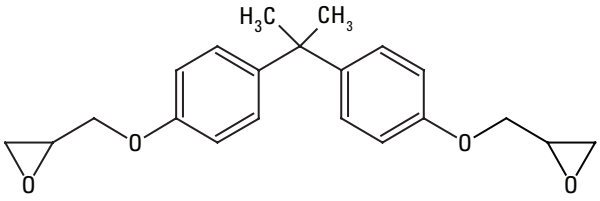


Figure 1. Chemical structure of BADGE, $C_{21}H_{24}O_4$.

The accurate mass measurements provided by time-of-flight (TOF) mass spectrometry (MS) for unknown compounds makes this identification process possible without the need for authentic standards of every possible minor impurity and reaction by-product.

Experimental

Sample Extraction

A metal panel (250 cm^2) coated with an epoxyphenolic lacquer and stoved under industrial conditions was cut into pieces (approximately 1 cm^2) and extracted by immersion in acetonitrile (100 mL). After 18 hours the extract was evaporated to a small volume (1 mL).

LC Conditions

Instrument:	Agilent LC 1200 SL
Mobile phases:	A: water B: acetonitrile
Gradient:	20% B to 50% B over 25 min, hold 20 min, 100% B at 60 min, hold 10 min, return to 20% B over 10 min
Flow rate:	0.2 mL/min
Column:	Agilent ZORBAX Eclipse XDB, 100 mm × 2.1 mm, 3.5-µm particles Part number 961753.902
Injection:	5 µL

MS Conditions

Instrument:	Agilent 6210 LC/MS TOF in positive ion ESI mode
Nebulizer press.:	30 psi
Capillary:	4000 V
Gas temp.:	300 °C
Drying gas:	7 L/min

Results and Discussion

TOF-MS parameters were optimized using solvent standards of BADGE, as mainly BADGE derivatives were expected to be extracted from the coating [1]. A fragmentor value of 150 V was used first, to cause no fragmentation, and so molecular ion adducts were seen. Figure 2 shows the TIC for the acetonitrile extract of the epoxyphenolic coating. There are many unknown peaks, and the one at 27.2 min was chosen for this example.

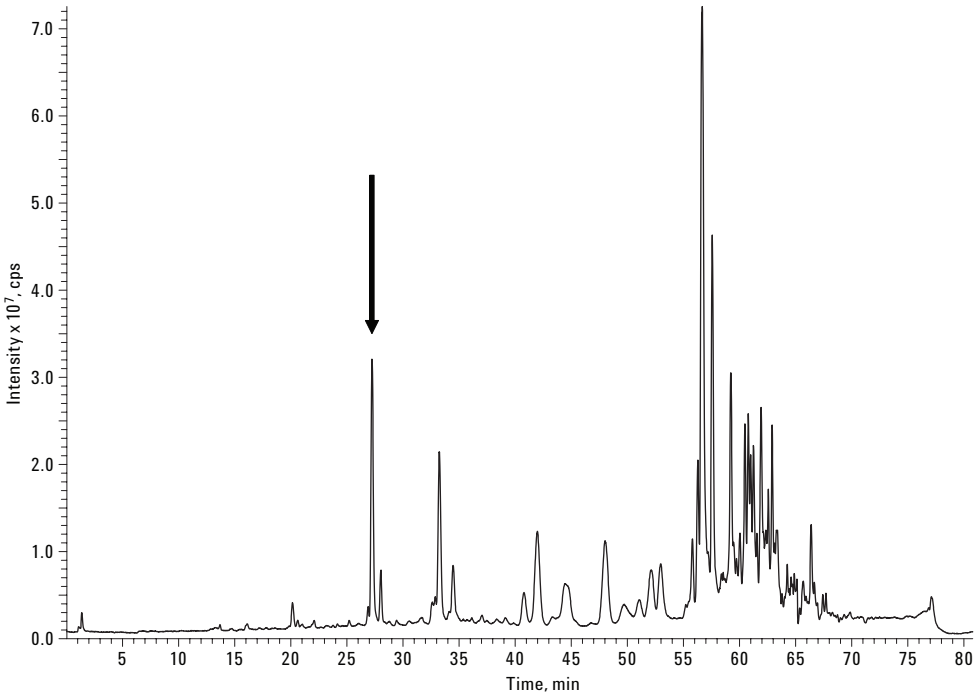


Figure 2. Total ion chromatogram of the acetonitrile extract of the epoxyphenolic coating.

Figure 3 shows the mass spectrum of the peak at 27.2 min. The differences in masses between the ions suggest that these are due to the protonated, ammoniated, sodiated, and potassiated molecule. No ammonia, sodium, or potassium was added to the mobile phase, and it is likely that these adducts arose due to contamination from other work carried out on the instrument, or were present in the solvents used in the mobile phase.

The formula calculator was used to propose identities for the peak, using the accurate mass determined for [M+NH4]+, as it was the most intense. Only one possible empirical formula was provided limiting the elements to C, H, O, and only one N within the 5 ppm mass error limit used.

For the experimentally derived mass 494.3118, the formula $C_{27}H_{44}O_7N$ was proposed (theoretical mass 494.3112, 1.15 ppm error). As it is proposed that this is the ammoniated adduct (subtract NH_4), this gives a formula of $C_{27}H_{40}O_7$ for the unknown peak. Furthermore, it is suspected that this peak is a BADGE derivative (subtract $C_{21}H_{24}O_4$ from the formula) and this suggests that the unknown peak is BADGE + $C_6H_{16}O_3$.

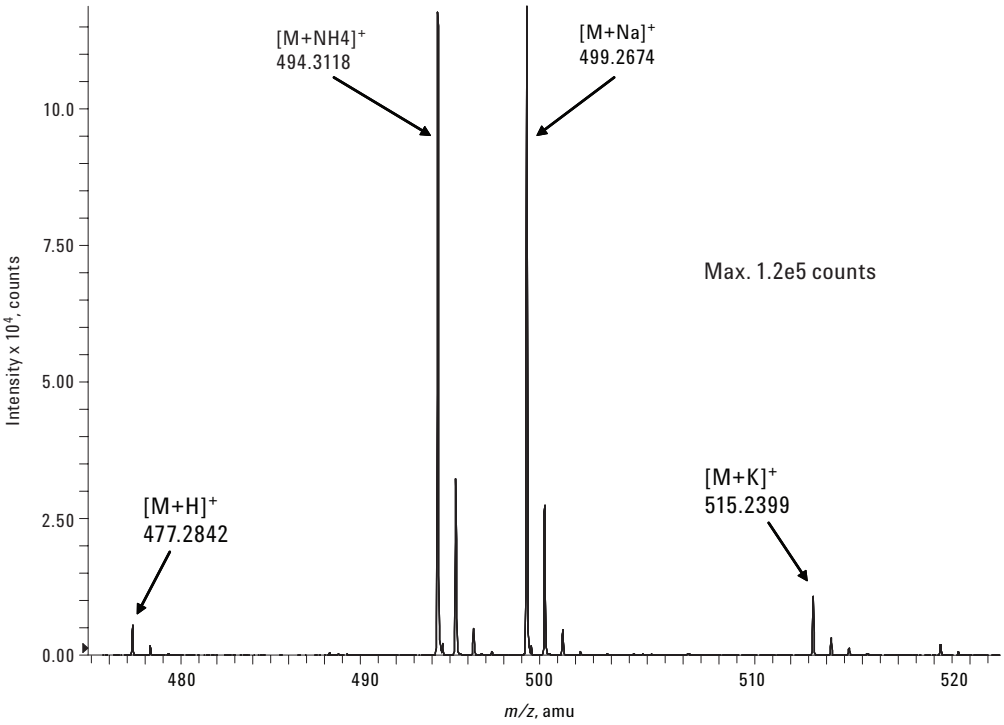


Figure 3. Mass spectrum of the unknown peak at 27.2 min (fragmentor = 150 V).

Fragmentation experiments were carried out to aid the identification process. A fragmentor value of 275 V dissociated the ammoniated molecular adduct into fragment ions, see Figure 4.

The accurate masses of the fragment ions were put into the formula calculator and the structures of the ions were theorized from the proposed empirical formulae. The fragment ions confirmed the presence of the BADGE unit (*m/z* 341.1727), that one of the epoxide rings had reacted with water (fragment ion at *m/z* 209.1149), and the other had reacted with butoxyethanol (BuOEtOH, $C_6H_{16}O_3$) (fragment ion at *m/z* 309.2036), a solvent used in the manufacturing process of the coating formulation. Figure 5 shows the structure of BADGE.H_2O.BuOEtOH.

Using the same approach, the identity of virtually all of the peaks in Figure 2 was established and different can coating chemistries have been studied.

Conclusions

Solvent extracts of epoxyphenolic can coatings have been analyzed by LC/TOF-MS to identify potential migrants into food and beverages. Accurate mass data of the parent compound and the fragment ions allows confident assignment of previously unknown peaks. Using the LC/TOF-MS has helped the testing of existing can coatings and guided the development of new coating chemistries.

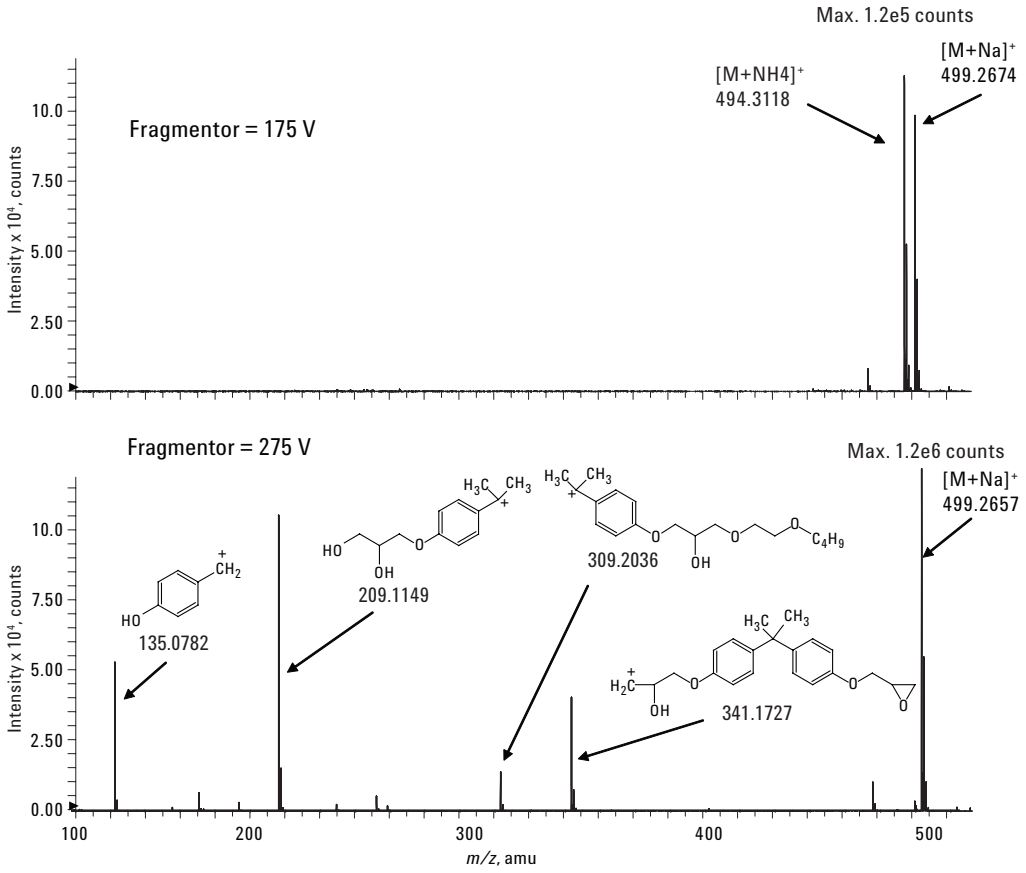


Figure 4. TOF-MS of the unknown peak at 27.2 min.

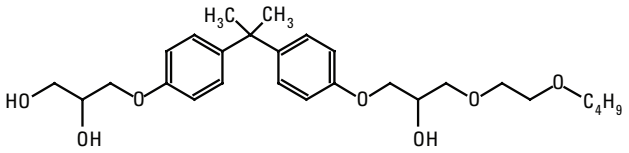


Figure 5. Structure of the identified compound: BADGE.H_2O.BuOEtOH.

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Identification of Unknown Polyester Oligomers in New Polyester Food Can Coatings by LC/TOF-MS Using Molecular Feature Extraction and Database Searching

Application

Food Safety

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Abstract

This application illustrates how time-of-flight mass spectrometry (TOF-MS) can help in the safety evaluation of new and existing polyester-based coatings used on the inside of food cans. A database of over 1,000 possible migrants based on small polyester oligomers that may not be incorporated into the polymer network of the coating was prepared from likely starting materials and the exact mass of each one was calculated. Automated database searching using the Agilent Data Analysis software and molecular feature extraction (MFE) compared the accurate mass information provided by the TOF-MS analysis to the database to identify previously unknown chromatographic peaks.

Introduction

The development of new and improved internal coatings for food and beverage cans must be cognizant of the legislative requirements on food contact materials [1]. If not, otherwise promising developments with good technical performance could come to a wasteful dead end. Coating formulations usually contain various components such as resins, cross-linking agents, catalysts, lubricants, wetting agents, and solvents. The potential exists for these ingredients, or by-products of reactions between them, to migrate from the can coating into foods. Thus, existing and especially new coatings must be evaluated for their safety for contact with food and beverages.

An earlier application described the analysis of a can coating based on epoxy resins [2]. Polyester-based coatings provide an alternative to epoxy resins and in these systems the three-dimensional polymer network is built up from a number of possible poly-functional alcohol and carboxylic acid monomers [3]. Oligomers are by-products of the polymerization process and can migrate from the coating into the food [4]. These oligomers can be formed from all possible combinations of the monomers used to construct the polymer. Some of the most common monomers used to prepare polyester resins are given in Table 1.

Combination of the different monomers provides a large number of possible polyester oligomers with the potential to migrate from the can coating into food. The accurate mass measurements provided by TOF-MS makes the identification of potential migrants possible without the need for authentic standards of every possible polyester oligomer. The Agilent MFE data analysis tool allows very fast searching of the unknown chromatographic peaks against a large user-prepared database of possible polyester oligomers.

Experimental

Sample Extraction

A metal panel (250 cm^2) coated with a polyester lacquer and stoved under industrial conditions was cut into pieces (approximately 1 cm^2) and extracted by immersion in acetonitrile (100 mL). After 18 h the extract was concentrated to a small volume (1 mL) for LC/TOF-MS analysis.

LC Conditions

Instrument	Agilent 1200 SL
Column	Agilent ZORBAX XDB-C18 100 mm × 2.1 mm, 3.5 µm Agilent p/n: 961753-902
Mobile phases	A: Water B: Acetonitrile
Gradient	20% B to 50% B over 25 min, hold 20 min, 100% B at 60 min, hold 10 min, return to 20% B over 10 min
Flow rate	0.2 mL/min
Injection volume	5 µL

MS Conditions

Instrument	Agilent 6210 TOF-MS in positive ion ESI mode
Nebulizer pressure	30 psi
Capillary:	4000 V
Gas temperature	325 °C
Drying gas	10 L/min

Data Analysis (DA) Parameters

DA operation	Molecular Feature Extraction	
Report type	Include confirmation screening by database search	
Processing option/peak detection		
	S/N threshold	50
	Minimum relative volume	2.5%
Adduct	H, NH_4, Na, K	
Confirmation screen		
	Mass tolerance	5 ppm

Results and Discussion

Figure 1 shows the total ion chromatogram (TIC) of the polyester coating extract. Although the amounts detected are low, these peaks need to be identified for a complete safety evaluation of the coating intended for food cans.

Because an unknown number of different polyols and polyacids can be used to make polyester resins (see Table 1 for some common examples), many polyester oligomers are theoretically possible. An Excel spreadsheet was prepared to calculate the exact mass of all possible oligomers and this contained over 1,000 possibilities [2,3]. An excerpt is given in Table 2. Although this is seemingly a very cumbersome and time-consuming process, once the database is constructed it allows very rapid and efficient data analysis and identification of unknown peaks in all further polyester-based coatings analyzed. The Excel spreadsheet was converted to .CSV format and used with the Agilent Data Analysis software to automatically search the chromatogram of unknown peaks (Figure 1) and compare it to the oligomer masses. Use of MFE, even with over 1,000 entries, makes this process very fast.

Table 3 gives the identities of the 13 polyester oligomers detected and identified using the MFE software. The confidence in the identification is good as all mass error values (difference between the measured and theoretical masses) are less than 5 ppm. Analysis revealed the polyester was based on phthalic acids esterified with five of the polyols listed in Table 1. Twelve of the 13 oligomers are cyclic and only one is linear. This is reasonable because cyclic oligomers cannot be incorporated into the polymeric network of the coating, making them more readily available to migrate.

The TIC (Figure 1) shows more peaks than the 13 polyester oligomers identified in Table 2. This is due to isomeric forms of the oligomers that chromatograph differently. These can arise from (a) different isomers of the starting substances (for example, ortho-, meta-, or para-phthalic acid, or isomeric polyols); (b) oligomers having the same composition but different structures (for example, linear PA+EG+PA+EG+PA+NPG versus linear PA+EG+PA+NPG+PA+EG); (c) two or more oligomers having the same empirical formula but a different identity (for example, 3PA+2EG+NPG and 3PA+3PG both have the formula $C_{33}H_{30}O_{12}$); (d) diastereoisomers formed when incorporating the chiral 1,3-propylene glycol monomer.

The identity of those peaks not assigned as polyester oligomers were proposed based on chemical knowledge and analysis of the starting materials for the lacquer. One of these was proposed to be a plasticizer found in a starting material, three were from lubricants (two found in starting materials) and two from surfactant-type molecules (not found in starting materials).

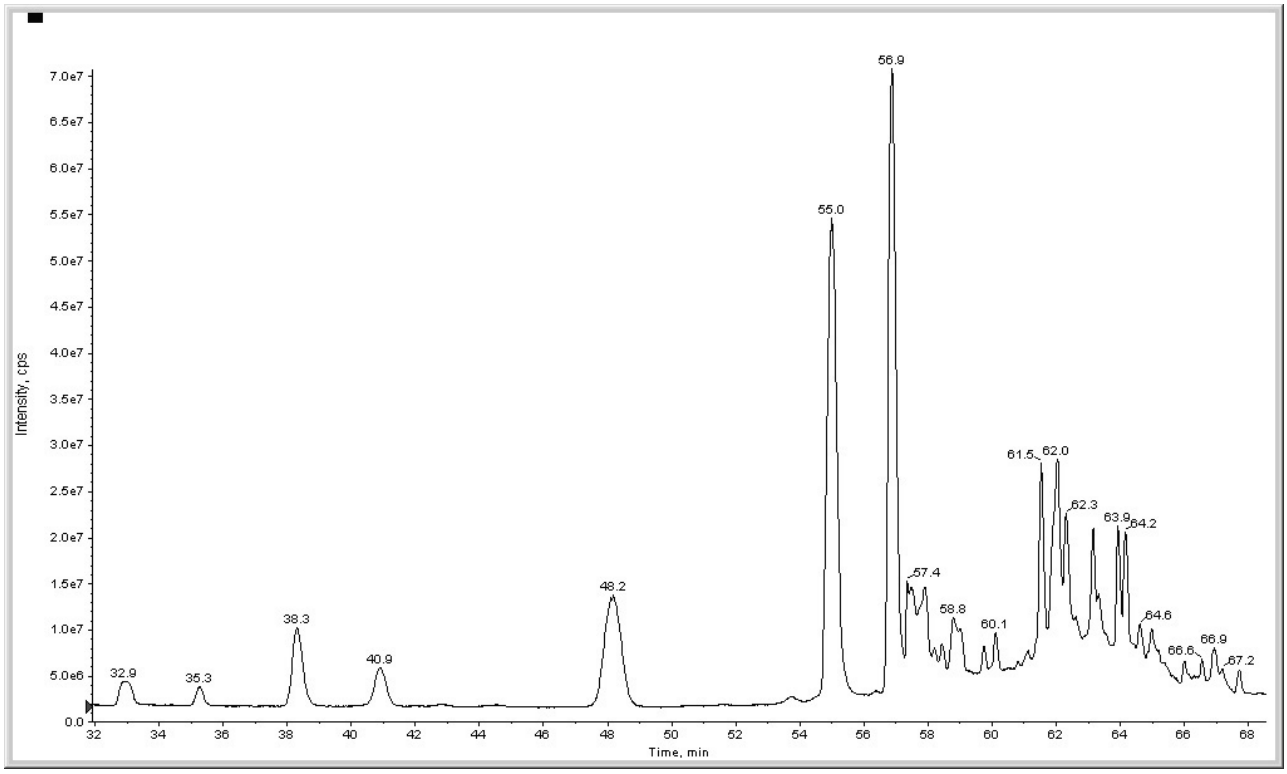


Figure 1. Total ion chromatogram (TIC) of the polyester coating extract.

Table 1. Commonly Used Monomers for Polyester Resins [2,3]

Common name	Abbreviation	Formula
Polyols		
Ethylene glycol	EG	$C_2H_6O_2$
Propylene glycol (1,2- and 1,3-)	PG	$C_3H_8O_2$
Butanediol (1,3- + isomers)	BD	$C_4H_{10}O_2$
Diethylene glycol	DEG	$C_4H_{10}O_4$
Neopentyl glycol	NPG	$C_5H_{12}O_2$
1,6-Hexanediol	HD	$C_6H_{14}O_2$
Tris(hydroxymethyl)propane	HMP	$C_6H_{14}O_3$
Cyclohexyldimethanol	CHDM	$C_8H_{16}O_2$
2,2,4-Trimethylpentane-1,3-diol	TMP	$C_8H_{18}O_2$
Polyacids		
Adipic acid	AA	$C_6H_{10}O_4$
Phthalic acid (o, m, and p-isomers)	PA	$C_8H_6O_4$
Trimellitic acid	TMA	$C_9H_6O_6$

Table 2. Excerpt from the Polyester User-Prepared Database (See Table 1 for Abbreviations)

		AA	TMA	PA	CHDM	BD	EG	DEG	PG	HD	HMP	TMP	NPG	H_2O	MW
PA+EG	Linear			1			1							1	210.0528
EG+PA+EG	Linear			1			2							2	254.0790
PA+EG+PA+EG	Linear			2			2							3	402.0951
PA+EG+PA+EG	Cyclic			2			2							4	384.0845
PA+EG+PA+EG+PA	Linear			3			2							4	550.1111
PA+EG+PA+EG+PA+NPG	Linear			3			2						1	5	636.1843
PA+EG+PA+NPG+PA+EG	Linear			3			2						1	5	636.1843
PA+PG+PA+PG+PA+PG	Linear			3					3					5	636.1843
PA+PG+PA+PG+PA+PG	Cyclic			3					3					6	618.1737

Table 3. Polyester Oligomers Identified Using Molecular Feature Extraction and Database Searching

Mass of compound	Formula predicted	Mass error (ppm)	Proposed identity	Notes
384.0845	$C_{20}H_{16}O_8$	1.4	2PA+2EG	Cyclic
426.1315	$C_{23}H_{22}O_8$	1.4	2PA+EG+NPG	Cyclic
428.1107	$C_{22}H_{20}O_9$	1.1	2PA+EG+DEG	Cyclic
466.1630	$C_{26}H_{26}O_8$	0.36	2PA+CHDM+EG	Cyclic
468.1784	$C_{26}H_{28}O_8$	0.50	2PA+2NPG	Cyclic
508.2114	$C_{29}H_{32}O_8$	3.3	2PA+CHDM+NPG	Cyclic
618.1737	$C_{33}H_{30}O_{12}$	0.84	3PA+2EG+NPG or 3PA+3PG	Cyclic
660.2238	$C_{36}H_{36}O_{12}$	4.6	3PA+EG+2NPG	Cyclic
700.2520	$C_{39}H_{40}O_{12}$	0.82	3PA+CHDM+EG+NPG	Cyclic
702.2703	$C_{39}H_{42}O_{12}$	4.8	3PA+3NPG	Cyclic
704.2469	$C_{38}H_{40}O_{13}$	0.79	3PA+CHDM+2PG	Linear
742.3003	$C_{35}H_{50}O_{17}$	0.74	3PA+CHDM+2NPG	Cyclic
782.3330	$C_{45}H_{50}O_{12}$	4.2	3PA+2CHDM+NPG	Cyclic

Conclusions

Solvent extracts of polyester can coatings have been analyzed by LC/TOF-MS to identify potential migrants into food and beverages. Accurate mass data of the parent compounds and automated data analysis software with MFE allowed confident assignment of previously unknown peaks by searching a user-prepared database of possible polyester oligomers. The database allows rapid identification of these oligomers in many complex samples. In this work LC/TOF-MS has helped to ensure the safety of food can coatings and has guided the development of new coating chemistries.

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LC-TOF-MS As a Tool to Support Can Coating/Food Interaction Studies

Application

Food Safety

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Abstract

This application illustrates how time-of-flight mass spectrometry has been used in the studies of interactions at the can coating/food interface of internally coated metal cans intended for use in the food industry. Previously unconfirmed migrants were confidently identified using accurate mass information provided.

Introduction

The internal surface of metal cans used to pack foodstuffs is often coated to form a barrier between the food and the metal of the can. The coating formulation may contain various components, such as resins, cross-linking agents, catalysts, lubricants, wetting agents, and solvents. The potential exists for these ingredients, or by-products of reactions between them, to migrate from the can coating into foods.

Food ingredients such as fat or water can cause some coatings to swell, which may enhance any migration, particularly if the food is heat processed in its packaging. Migration can also depend on other factors: contact time and temperature, the type and thickness of the coating, and the molecular mass and size of the migrating species. Studies, in particular migration modeling, of interactions between the can coating and the foodstuff are important in understanding, and eventually reducing, migration of compounds from the can coating into the foodstuffs.

In previous applications we have described the analysis of can coatings based on epoxy resins [1] and polyester resins [2] and how the accurate mass information for the parent compounds and fragment ions greatly increased the confidence in the identification of unknown compounds. In this application we describe how liquid chromatography/time-of-flight mass spectrometry (LC/TOF-MS) was used as a tool to support studies on can coating/food interactions

Experimental

Coated Panels

The epoxyphenolic (EPH) lacquer was applied to metal panels and cured in an oven at 200 °C for 10 min. Small test specimens with an area of 9 cm^2 were cut from the panel and folded to a concertina shape ready for migration studies.

Exposure to Sunflower Oil

The folded test specimens of coated panel were submerged in sunflower oil in a pressurised vial at 121 °C in a silicon oil bath for 10, 20, 30, 40, 50, 60, 70, and 120 min. After exposure, the coated

metal test specimens were wiped to remove the oil and submerged in acetonitrile (25 mL) overnight. A portion of the acetonitrile (1 mL) was passed through a 0.2-mm PTFE filter.

Hydrochlorination

Concentrated hydrochloric acid (100 µL) was added to a portion (500 µL) of the concentrated (10 times) EPH acetonitrile extract. This was allowed to react at 60 °C for 18 h in a sealed vial and made up to 1 mL with acetonitrile.

Liquid Chromatography-Fluorescence Detection (LC-FLD)

Instrument:	Agilent 1200 Series LC and G1321A FLD
Mobile phases:	A = 0.1% acetic acid in water B = acetonitrile
Gradient:	At t = 0, B = 35%; t = 5 min, B = 50%; t = 10 min, B = 50%; t = 20 min, B = 100%; t = 25 min, B = 100%
Flow rate:	1.0 mL/min
Column:	Agilent ZORBAX Eclipse XDB, 100 mm × 2.1 mm, 3.5-µm particle size (part number 961753.902)
Injection:	20 µL
Excitation wavelength:	275 nm
Emission wavelength:	305 nm
Gain:	2^10

LC-FLD-TOF-MS

Instrument:	Agilent 1200 Series LC, G1321A FLD and TOF positive electrospray
Mobile phases:	A = 0.1% acetic acid in water B = acetonitrile
Gradient:	At t = 0 min, B = 35%; t = 5 min, B = 50%; t = 20 min, B = 50%, t = 30 min, B = 100%; t = 40 min, B = 100%
Flow rate:	0.3 mL/min
Column:	Agilent ZORBAX Eclipse XDB, 100 mm × 2.1 mm, 3.5-µm particle size (part number 961753.902)
Injection:	5 µL
Excitation wavelength:	275 nm
Emission wavelength:	305 nm
Gain:	2^10
Nebulizer pressure:	30 psi
Capillary:	4000 V
Gas temperature:	325 °C
Drying gas:	10 L/min
Fragmentor:	150 V

Acylation

A method reported by Biedermann and Grob was adapted for use [3]. A portion (5 mL) of the EPH acetonitrile extract was evaporated to dryness under nitrogen. Acetic anhydride (25 µL) and pyridine (25 µL) were added and allowed to react for 15 min. The excess reagents were removed by evaporation under nitrogen and the residue was redissolved in acetonitrile (500 µL).

Another portion (5 mL) of EPH acetonitrile extract was evaporated under nitrogen and treated with acetic anhydride as described above. After removal of the excess reagents by evaporation, trifluoroacetic acid (TFAA, 100 µL) was added. This was allowed to react for 15 min and the excess reagent removed by evaporation under nitrogen. The dry residue was redissolved in acetonitrile (500 µL).

Results and Discussion

During migration tests using panels coated in a generic EPH coating, two co-eluting peaks were studied by LC-FLD. The identity of a pair of peaks at a similar retention time has been previously reported in the literature as two isomers of cyclo-di-BADGE [4]. The structure of cyclo-di-BADGE is shown in Figure 1.

The EPH coated panels were exposed to sunflower oil at 121 °C for increasing periods of time and the co-eluting peaks were seen to behave differently, as shown in Figure 2. The profile of the two peaks, 2-1 and 2-2, was seen to change: as the length of exposure increased, the relative peak height of peak 2-1 decreased compared to that of peak 2-2, suggesting that this compound was migrating at a greater

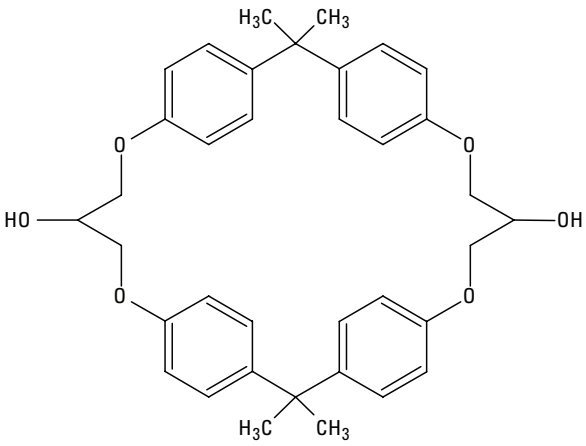


Figure 1. Structure of cyclo-di-BADGE, $C_{36}H_{40}O_6$.

rate. After 120 min, the two peaks had approximately equal peak heights.

The identification power of TOF-MS shown in previous applications [1-2] was used to investigate the EPH extracts with the aim of confidently identifying the co-eluting peaks and explaining the migration behavior seen. The flow rate of 1.0 mL/min used in the LC-FLD was deemed too high to be used on the LC-TOF-MS (although this ESI source can accept a flow rate of 1.0 mL/min), so the LC conditions were adapted. The LC-TOF-MS apparatus had a FLD in series. Figure 3 shows the FLD chromatogram of a concentrated acetonitrile EPH extract. The slower gradient means that the peaks are now eluting later (22.4 min and 23.15 min) and there are now three peaks, because the extra time on the column has allowed greater interactions with the stationary phase, which in turn has allowed greater resolution between the peaks.

This becomes clearer when looking at the TOF-MS data for this chromatogram (Figure 4). There are three peaks: 4-1 at 22.13 min, 4-2 at 22.61 min, and 4-3 at 23.45 min. Peaks 4-2 and 4-3 have the same mass spectra and molecular formula ($C_{36}H_{40}O_5$), as shown in Figure 5 and Table 1. Peak 4-1 has a different molecular formula ($C_{25}H_{34}O_5$).

From this data the molecular formula of peak 4-1 was proposed as $C_{25}H_{34}O_5$ with an identity of BADGE.BuOH. This identity was deduced based on methods reported in an earlier application [1]. The identity was confirmed by the addition of HCl to the extract. As expected, the BADGE.BuOH peak disappeared and was replaced by a peak with a

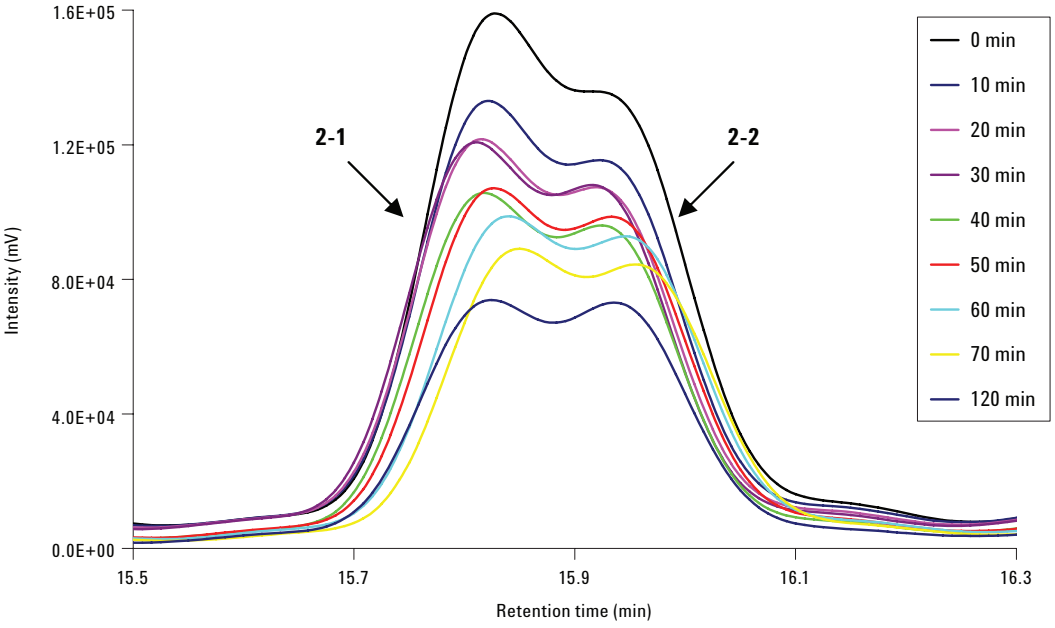


Figure 2. LC-FLD chromatogram obtained after exposing the EPH can coating to sunflower oil for different lengths of time.

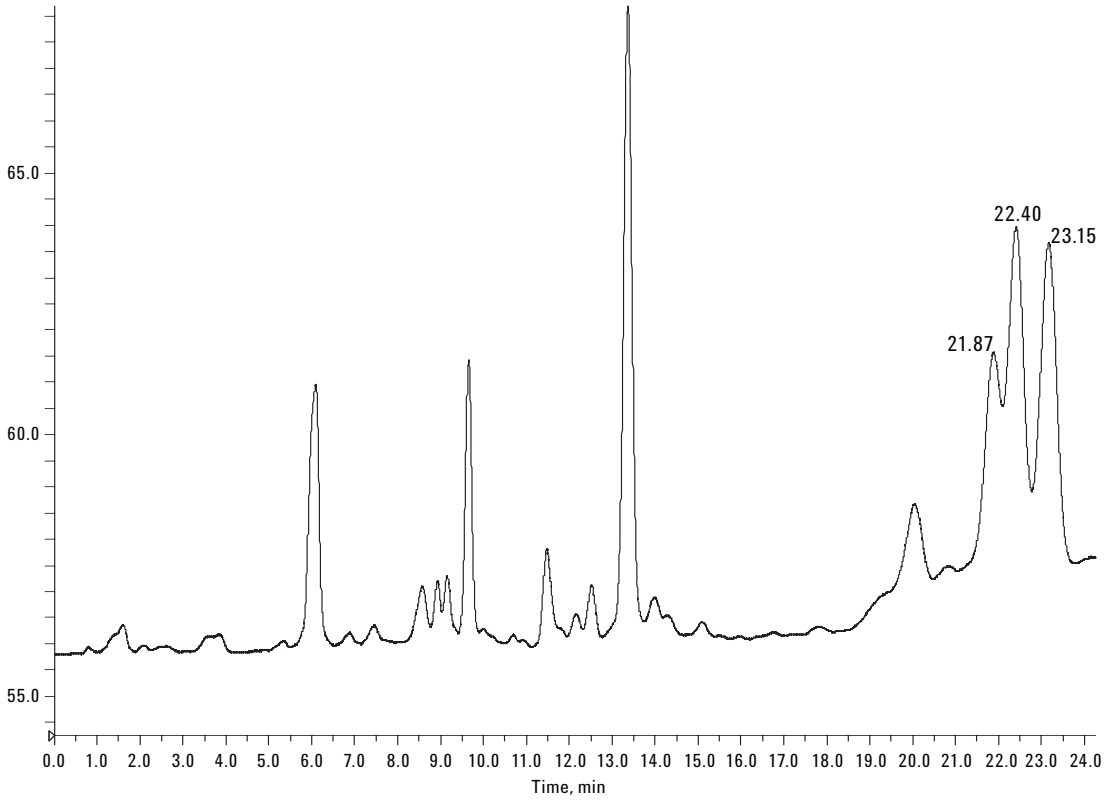


Figure 3. LC-FLD chromatogram of the concentrated EPH acetonitrile extract.

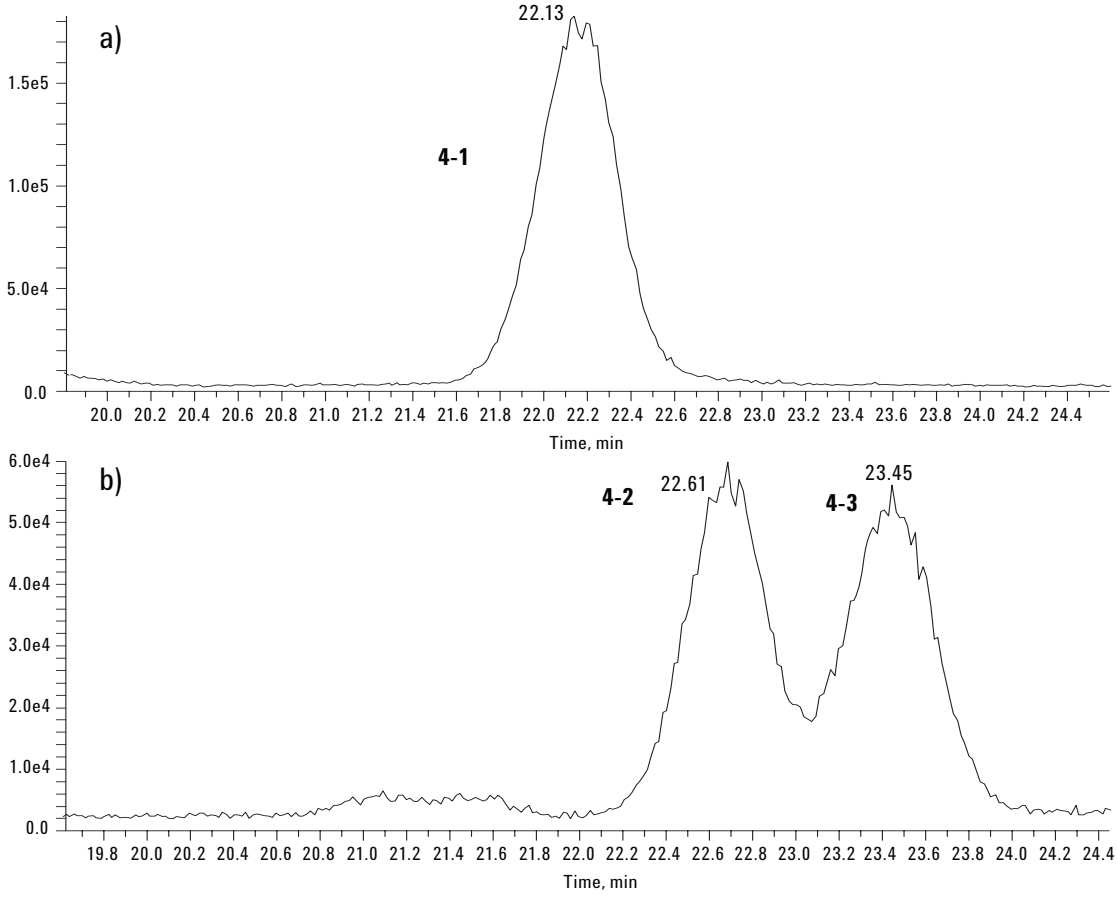


Figure 4. Extracted ion chromatograms for the three peaks of interest: a) *m/z* 436.98 - 437.48 and b) *m/z* 591.03 - 591.53.

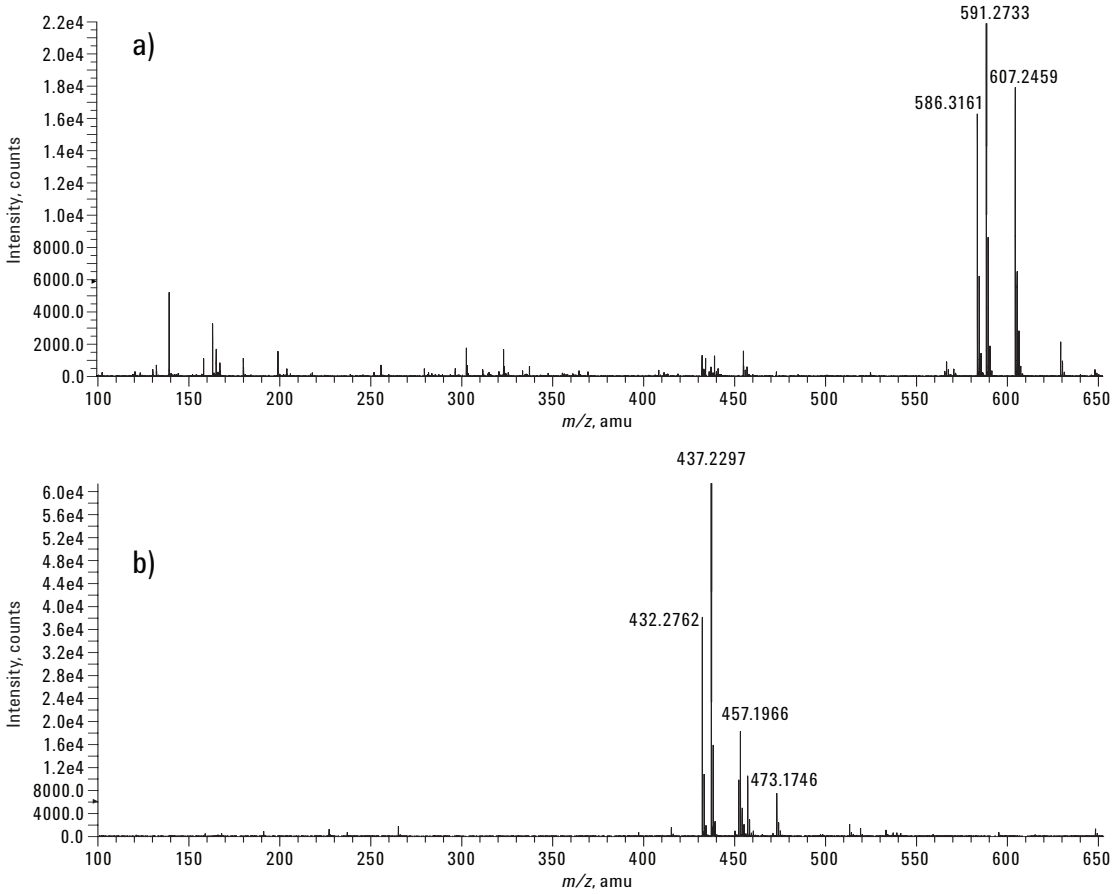


Figure 5. Mass spectrum of peak a) 4-1, b) 4-2, and 4-3.

Table 1. Molecular Formula Database Information for Peaks 4-1, 4-2 and 4-3

Peak	Mass	Molecular formula predicted	Theoretical mass	Mass error (PPM)	Molecular adduct
4-1	432.2746	$C_{25}H_{38}NO_5$	432.2744	0.12	$M+NH_4$
	437.2300	$C_{25}H_{34}O_5Na$	437.2298	0.35	M+Na
4-2	586.3163	$C_{36}H_{44}NO_6$	586.3163	–0.026	$M+NH_4$
	591.2722	$C_{36}H_{40}O_6Na$	591.2717	0.83	M+Na
4-3	586.3161	$C_{36}H_{44}NO_6$	586.3163	–0.37	$M+NH_4$
	591.2725	$C_{36}H_{40}O_6Na$	591.2717	1.3	M+Na

molecular formula consistent with that of BADGE.BuOH.HCl, as the HCl adds across the remaining epoxide ring. Figure 6 shows the relevant extracted ion chromatograms. Figure 7 shows the mass spectrum of BADGE.BuOH.HCl, with excellent correlation between experimental and theoretical chlorine isotope patterns.

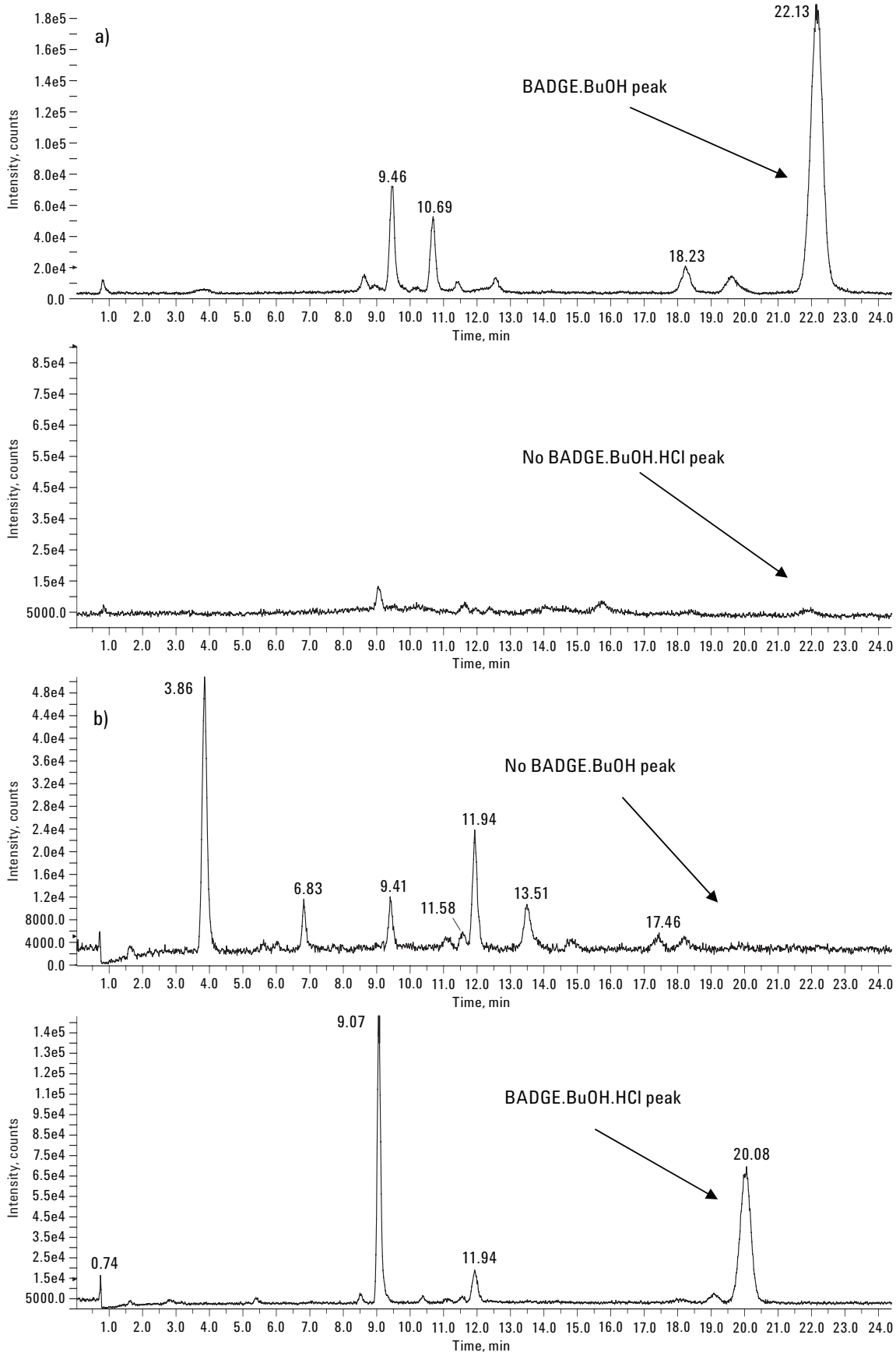


Figure 6. Extracted ion chromatograms for BADGE.BuOH (*m/z* 437 – 438) and BADGE.BuOH.HCl (*m/z* 451 – 452) for a) untreated EPH extract and b) EPH extract treated with HCl.

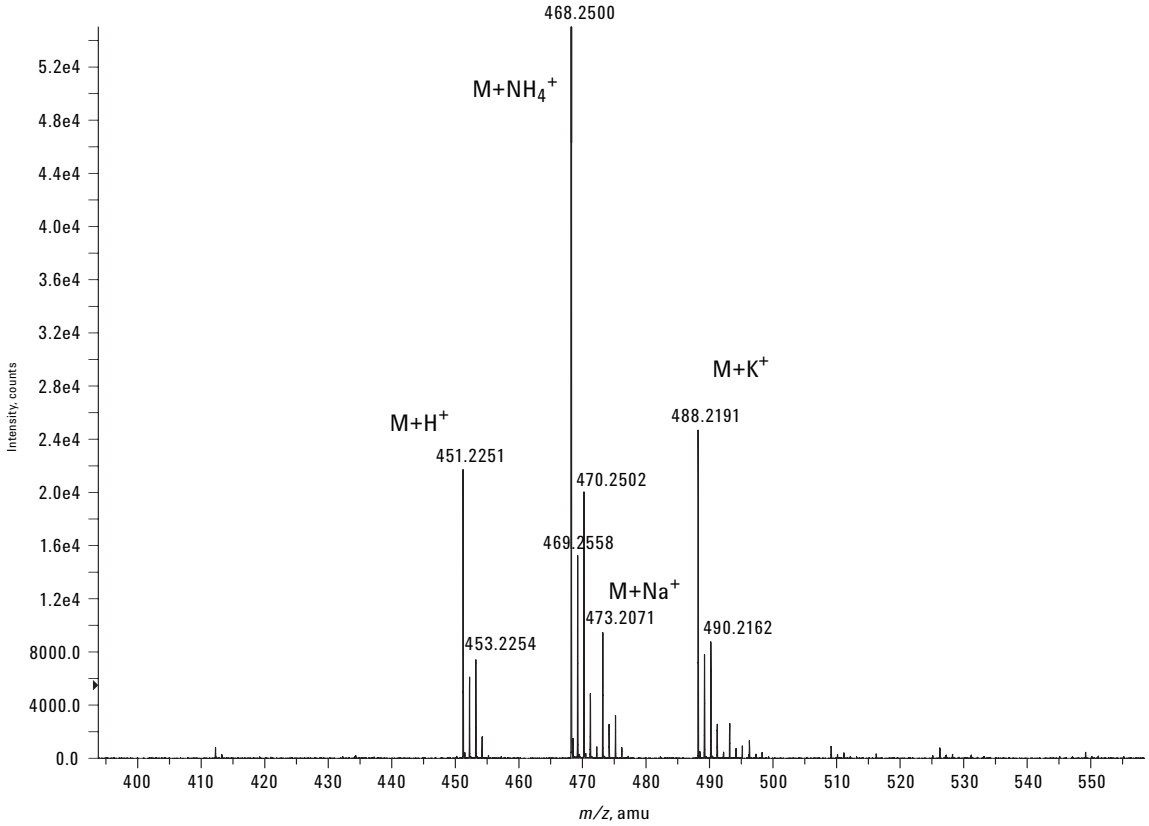


Figure 7. Mass spectrum of the peak at 20.1 min, corresponding to BADGE.BuOH.HCl.

Peaks 4-2 and 4-3 had the same mass spectra and were proposed to be $C_{36}H_{40}O_6$. As well as corresponding to cyclo-di-BADGE as suggested above, this could also correspond to BADGE.BPA, a linear BADGE derivative with the same molecular formula (see Figure 8).

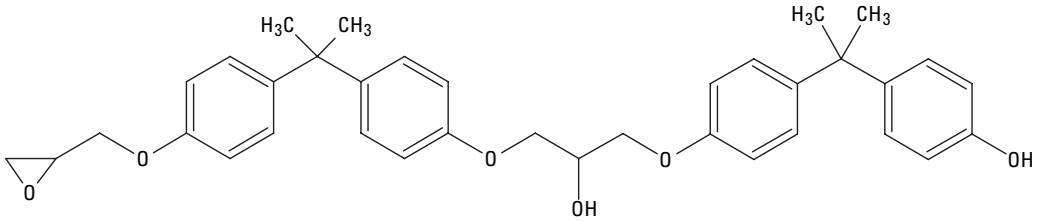


Figure 8. Structure of BADGE.BPA, $C_{36}H_{40}O_6$.

The addition of HCl did not change the two co-eluting peaks, which suggests that they are in fact due to cyclo-di-BADGE and not BADGE.BPA, as any peaks due to BADGE.BPA would be expected to disappear as the HCl will add across the epoxide ring. This conclusion was tested further; one of the differences between the proposed structures is that cyclo-di-BADGE has two hydroxide groups but BADGE.BPA has additional epoxide functionality as well as two hydroxide groups. It has been reported that acetic anhydride and TFAA react differentially with hydroxide and epoxide groups [3]. Acetic anhydride causes acylation of free hydroxide groups while further addition of TFAA causes acylation across the epoxide ring (see Figure 9). LC-TOF-MS analysis of the EPH extract after treatment with acetic anhydride showed the presence of a doubly acylated compound, either compound 9-1 or 9-2 ($C_{40}H_{44}O_8$). After further addition of TFAA the absence of a peak corresponding to compound 9-3 suggests that the two co-eluting peaks are in fact both due to cyclo-di-BADGE. The reason for two peaks is proposed to be because of the presence of stereoisomers, with the two hydroxide groups being cis- or trans- to each other, depending upon the side from which the phenol group attacks the epoxide ring during formation [3].

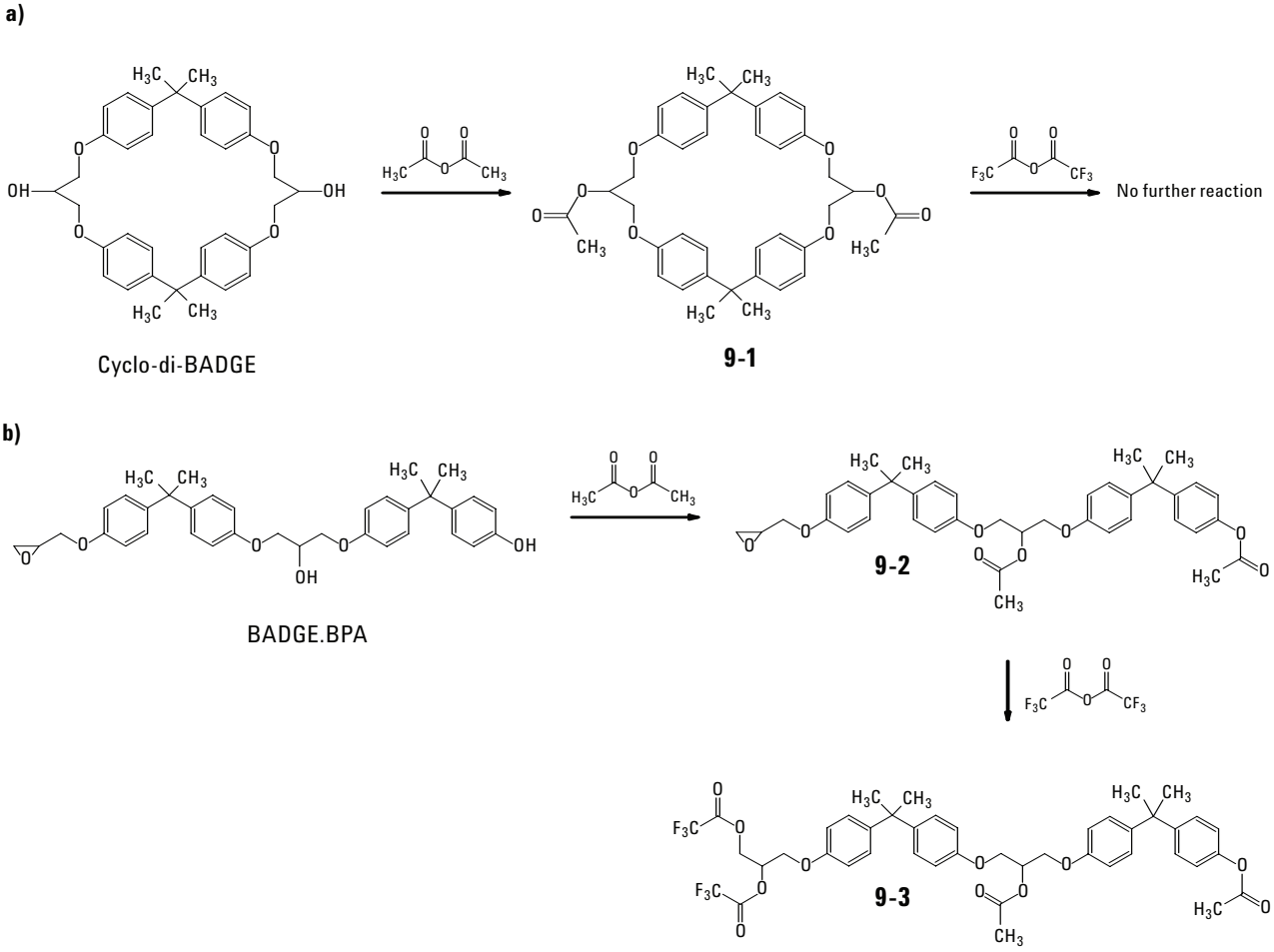


Figure 9. Acylation reactions for a) cyclo-di-BADGE and b) BADGE.BPA.

Conclusions

During migration studies at Leeds University, two co-eluting peaks were seen to behave differently during exposure to sunflower oil at different temperatures. There were in fact three co-eluting peaks and these were identified using LC-TOF-MS. The first peak was identified as BADGE.BuOH, and the second and third peaks were confirmed as *cis*- and *trans*- isomers of cyclo-di-BADGE. It is suggested that the differences seen in the original migration studies were due to the differences in structure between BADGE.BuOH and cyclo-di-BADGE, with the BADGE.BuOH migrating faster than cyclo-di-BADGE into the simulants, and the two stereoisomers of cyclo-di-BADGE migrating at the same rate as each other, which would account for the changes in peak height.

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Pesticides, Mycotoxins & Other Contaminants

Pesticides are widely used in many areas of agriculture as they are considered economically important for high yield production. Mycotoxins enter our food chain as a result of fungal infection of crops that are either eaten directly by people or are fed to animals. In today's world of extensive importing and exporting of food goods, the analysis and monitoring of pesticides and other contaminants is essential. Agilent can help you screen for, confirm, and quantitate mycotoxins and more than 900 pesticides in use worldwide.

> Return to Table of Contents
> Search entire document

Organophosphorus Pesticides in Apple Matrix by GC/MS/FPD using an Agilent J&W DB-35ms Ultra Inert GC Column

Application Note

Environmental and Food Safety

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Abstract

The Agilent J&W DB-35ms Ultra Inert (UI) 20 m × 0.18 mm × 0.18 µm column effectively resolved the analytes of interest producing excellent peak shape for even the more problematic organophosphorus (OP) pesticides. The detection limits for most of the pesticides were 15-25 ng/mL. Recovery studies were performed by spiking with a standard solution to achieve the desired concentrations in an apple matrix; 150, 300 and 750 ng/mL GC/MS/SIM and 50, 100, 250 ng/mL FPD in phosphorus mode. Recoveries were >77% for most of the pesticides by GC/MS/SIM and >75% by GC/FPD.

This application note details a quick and effective analytical method for the determination of low ppm and trace level organophosphorus pesticides residues in apple extract. A capillary flow technology (CFT) device was installed post-column to split the effluent between the MSD and FPD, implementing an automated backflush that diminished residual sample carryover and reduced instrument cycle times. This multi-signal configuration allowed for full scan, selective ion monitoring (SIM), and flame photometric detection from a single injection.

A simplified QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method provided sufficient sample matrix cleanup while preserving low level analyte detection.

Agilent Technologies

Introduction

Organophosphorous (OP) pesticides are widely used in the agricultural industry for crop protection. Human toxicities for this class of molecules have shown acute as well as chronic effects from pesticide poisoning. OP pesticides affect the nervous system of insects and mammals by inhibiting an enzyme, acetylcholinesterase, important in helping regulate nerve impulses [1].

Children are considered more susceptible to organophosphate toxicity because their pesticide dose per body weight is larger compared to that of adults [2]. Children also have lower levels of detoxifying enzymes that deactivate OP pesticides, contributing to their vulnerability to pesticide exposure [3,4]. Recent studies have shown a correlation between OP pesticides exposure and an increased risk for attention deficit hyperactivity disorder (ADHD) and other neurodevelopmental deficits in children [3,5,6,7]. Because the main source of exposure for children is through consumption of food containing OP pesticide residues [2,8], analytical testing capable of determining residual pesticides in food samples is critical.

The multiresidue determination of pesticides in fruits and vegetables usually involves an organic extraction of the pesticides from the plant matrix, followed by a cleanup procedure to remove co-extractives and other interferences. Anastassiades et al [9] developed a QuEChERS method for the analysis of pesticide residues in produce. This approach simplifies the traditional, labor intensive extraction and cleanup procedure, while providing a fast, robust, and cost effective method suitable for extracting pesticide residues.

Chromatographically active compounds such as organophosphorus pesticides can adsorb onto active sites in the sample flow path, particularly at trace levels, compromising an analyte's response. These pesticides tend to show peak tailing through interaction with active sites in a chromatographic system. This makes analysis challenging, particularly in difficult sample matrices. Minimizing activity in the GC column is essential to ensure accurate quantitation. Agilent's J&W DB-35ms Ultra Inert (UI) column minimizes column activity so difficult and active analytes can be consistently analyzed at trace levels. The use of the midpolarity DB-35ms UI phase also offers additional selectivity over a nonpolar phase, which can assist in resolving potentially coeluting peaks, or shift a peak of interest away from matrix interferences.

A gas chromatographic system capable of multisignal detection can provide complementary data for identification, confirmation, and quantitation of target analytes from a single injection. This method provides simultaneous detection of organophosphorus pesticides by GC/MS/SIM and FPD in phosphorus mode by splitting the column effluent between the MSD and FPD. The approach chosen here uses a GC/MSD/FPD system to identify and confirm the order of elution for peaks of interest. Once the elution order is established, the chromatographic parameters can easily be transferred to a GC/FPD system. The use of FPD detection without flow splitting is expected to increase sensitivity threefold, further improving lower level detection.

The GC/MS system was also equipped with backflush capability, which shortens instrument cycle time by backflushing late-eluting matrix components through the inlet purge valve. Long bakeout times between injections are avoided by using this technique. Backflushing has the additional benefit of increasing the time intervals for source cleaning by effectively clearing deleterious matrix components from the system [10].

Experimental

An Agilent 7890 GC with an Agilent 5975C MSD equipped with a flame photometric detector and Agilent 7683B automatic liquid sampler were used for this series of experiments. A purged two-way capillary flow technology (CFT) device was used to split the effluent 3:1 to the MSD:FPD. The CFT device also allowed for post-column backflush. Table 1 lists the chromatographic conditions used for these analyses. Table 2 lists the flow path consumable supplies used in these experiments.

Table 1. Chromatographic Conditions

GC/MSD:	Agilent 7890 GC/Agilent 5975C Series GC/MSD
Sampler:	Agilent 7683B automatic liquid sampler, 5.0 µL syringe (Agilent p/n 5181-1273)
CFT Device:	Purged 2-way splitter (Agilent p/n G3180B) Split Ratio 3:1 MSD:FPD
MSD Restrictor:	1.2 m × 0.15 mm id deactivated fused silica tubing
FPD Restrictor:	1.4 m × 0.15 mm id deactivated fused silica tubing
PCM 1:	3.8 psi constant pressure
Inlet:	1 µL splitless; 250 °C, purge flow 60 mL/min at 0.25 min, gas saver on at 2 min 20 mL/min
Column:	Agilent J&W DB-35ms UI 20 m × 0.18 mm × 0.18 µm (Agilent p/n121-3822UI)
Carrier:	Helium, constant pressure 43.5 psi at 95 °C
Oven:	95 °C (1.3 min), 15 °C/min to 125 °C, 5 °C/min to 165 °C, 2.5 °C/min to 195 °C, 20 °C/min to 280 °C (3.75 min)
Postrun Backflush:	5 min at 280 °C, PCM 1 pressure 70 psi during backflush, 2 psi inlet pressure during backflush
MSD:	310 °C transfer line, 310 °C source, 150 °C quad

Table 2. Flow Path Supplies

Vials:	Amber crimp top glass vials (Agilent p/n 5183-4496)
Vial Caps:	Crimp caps (Agilent p/n 5181-1210)
Vial inserts:	250 µL glass/polymer feet (Agilent p/n 5181-8872)
Syringe:	5 µL (Agilent p/n 5181-1273)
Septum:	Advanced Green (Agilent p/n 5183-4759)
Inlet liner:	Deactivated dual taper Helix liner (Agilent p/n 5188-5398)
Ferrules:	0.4 mm id short; 85/15 vespel/graphite (Agilent p/n 5181-3323)
PCT fittings:	Internal nut (Agilent p/n G2855-20530)
PCT ferrules:	SilTite ferrules, 0.25 mm id (Agilent p/n 5188-5361)
20x magnifier:	20x Magnifier loop (Agilent p/n 430-1020)

Reagents and Chemicals

All reagents and solvents were HPLC or Ultra Resi grade. Acetonitrile (ACN) from Honeywell (Muskegon, MI, USA), toluene from Burdick & Jackson, and acetone from JT Baker was purchased through VWR International (West Chester, PA, USA). The 12-component custom pesticide standard was prepared by Ultra Scientific (N. Kingstown, RI, USA).

Solutions and Standards

The OP pesticide stock standard solution (100 µg/mL of 12 organophoshorus pesticides) was diluted in acetone to yield spiking solutions 1 and 10 µg/mL. A surrogate standard, triphenyl phosphate (TPP), was prepared at concentrations of 1, 15 and 100 µg/mL in toluene. The spiking solutions were used to prepare the calibration curves in the matrix blank extract by appropriate dilution.

Sample Preparation

An organic apple sample was purchased from a local grocery store. The apple was chopped into small cubes and frozen at –80 °C overnight. The samples were then comminuted thoroughly to achieve sample homogeneity. The sample extraction method used the QuEChERS method. Figure 1 illustrates the sample preparation procedure graphically in a flow chart.

Samples containing 15 (± 0.1) grams of apple were weighed into centrifuge tubes. QC samples were spiked with appropriate amount of spiking solutions to yield QC samples with quantitative concentrations relative to the 3:1 split ratio of 150, 300, and 750 ng/mL levels for GC/MS-SIM determination, and 50, 100, and 250 ng/mL by flame photometric detection. Each sample received a 15-mL aliquot of ACN. Two ceramic bars (Agilent p/n 5982-9313) were added to each sample to aid in sample extraction. The samples were vortexed for 1 minute. An Agilent original QuEChERS extraction salt packet (Agilent p/n 5982-5555) containing 6 grams of $MgSO_4$ and 1.5 grams sodium chloride was added to each centrifuge tube. The capped tubes were shaken on a Geno/Grinder @1500 rpm for 1 minute. The samples were centrifuged at 4000 rpm for 5 min.

An 8 mL aliquot of the upper layer was transferred to an Agilent QuEChERS General Fruits and Vegetables dispersive SPE 15 mL tube (Agilent p/n 5982-5058). The dSPE tube was vortexed for 1 minute and then centrifuged at 4000 rpm for 3 minutes to complete the sample extraction. The extract from the dSPE tube was transferred to a GC vial and analyzed by SIM GC/MS and GC/FPD using the chromatographic conditions listed in Table 1.

Extractions of water and acetonitrile aliquots were prepared in the same manner as the samples and served as reagent blanks.

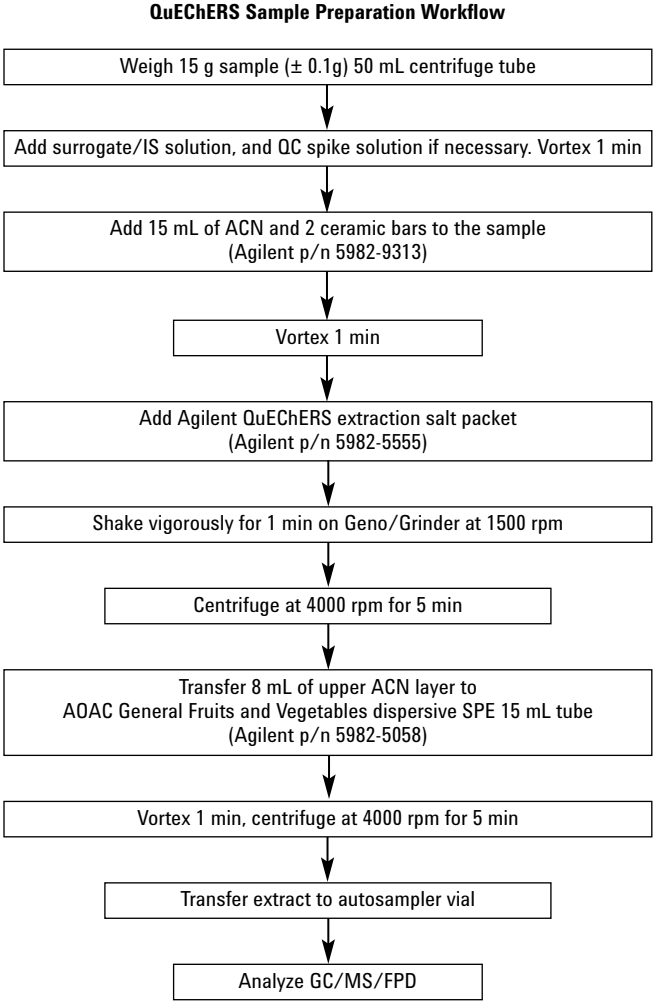


Figure 1. Flow chart of the Agilent QuEChERS extraction procedure for apple samples.

Discussion of Results

The organophosphorus pesticides were resolved on an Agilent J&W DB-35ms UI 20 m × 0.18 mm × 0.18 µm analysis column in about 30 minutes. The 12-component pesticide matrix-matched standard shown in Figure 2 shows good peak shapes for the pesticides in both the GC/MS/SIM and FPD chromatograms. Organophosphorus pesticides, particularly the more polar pesticides can be problematic, often yielding broad peak shapes or excessive tailing making reliable quantitation at low levels difficult. The high level of inertness of the DB-35ms UI column results in better peak shape and decreased sample adsorption allowing lower detection limits. Figure 3 depicts the excellent peak shape seen for four of the more polar OP pesticides (oxydemeton-methyl, methamidophos, mevinphos, acephate) with the DB-35ms UI column.

The performance of the DB-35ms UI high efficiency column yielded excellent linearity and recovery over the calibration range of this study. The linearity of the column as defined by the r^2 values of the pesticide standard curve was ≥0.992 for all the pesticides using both detectors. The individual OP pesticide analyte values are shown in Table 3.

The GC/MS/SIM analysis was able to detect down to the 15–20 ng/mL range for most of the pesticides. A higher SIM signal is necessary to quantify the more volatile pesticides below the 30 ng/mL range due mainly to matrix interferences. Because flame photometric detection in phosphorus mode is selective only for analytes containing phosphorus, it is able to detect low levels of OP pesticides in complex matrices without the matrix interferences. The FPD was able to detect the OP pesticides down to 15 ng/mL with the exception of naled, which could only be detected at higher levels (>25 ng/mL). Naled can undergo debromination, which can have an impact on detection, especially at trace levels. The detection levels for the targeted OP pesticides were well below the US maximum residue levels (MRLs) in an apple matrix, except in the case of chlorpyrifos, which has an MRL of 10 ppb for apples and grapes [11]. Analysis by GC/FPD without flow splitting offers increased sensitivity to monitor the lower levels of detection needed for chlorpyrifos.

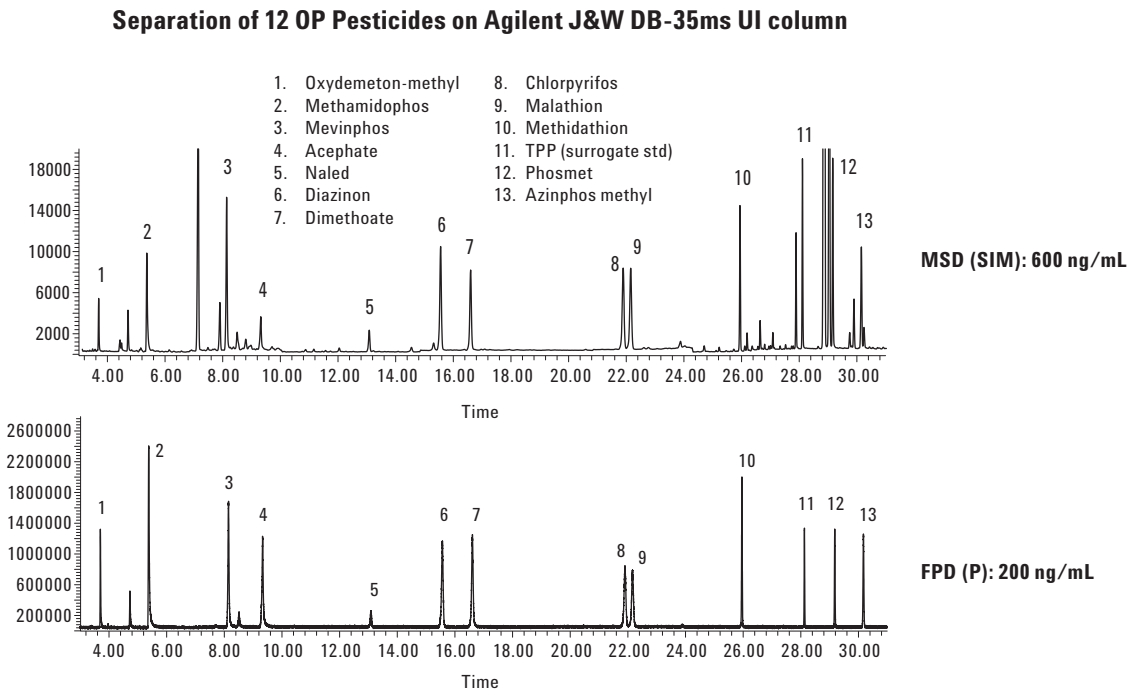


Figure 2. GC/MS-SIM and FPD chromatograms of a matrix matched organophosphorus pesticides standard analyzed on an Agilent J&W DB-35ms UI 20 m × 0.18 mm × 0.18 µm capillary GC column (Agilent p/n 121-3822UI). Chromatographic conditions are listed in Table 1. The effluent split ratio is MSD:FPD = 3:1.

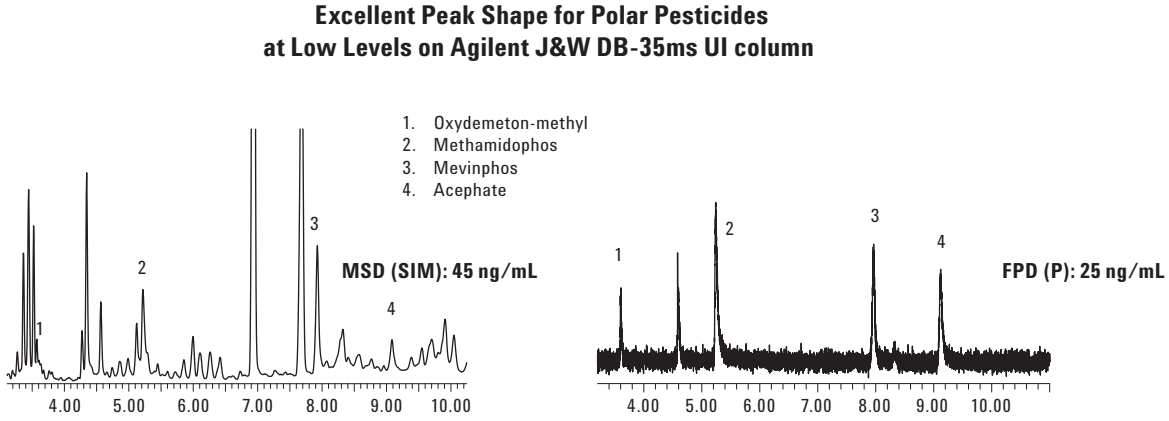


Figure 3. Enlarged section of GC/MS/SIM and FPD chromatograms of the more problematic polar pesticides analyzed on an Agilent J&W DB-35ms UI capillary column (Agilent p/n 121-3822UI). Chromatographic conditions are listed in Table 1. The effluent split ratio is MSD:FPD = 3:1.

Table 3. Correlation Coefficients for the OP Pesticides Calibration Standards Analyzed by GC/MS-SIM and FPD in Phosphorus Mode with a Split Ratio for MSD:FPD = 3:1

Excellent Linearity of OP Pesticides on Agilent J&W DB-35ms UI Column

	MSD	***FPD***
Oxydemeton-methyl	0.994	0.997
Methamidophos	0.997	0.997
Mevinphos	0.997	0.999
Acephate	0.997	0.999
Naled	0.992	0.996
Diazinon	0.996	0.997
Dimethoate	0.997	0.999
Chlorpyrifos	0.997	0.998
Malathion	0.995	0.999
Methidathion	0.996	0.999
TPP	0.999	0.997
Phosmet	0.997	0.999
Azinphos methyl	0.995	0.999

r^2 values for 30, 75, 150, 300, 525, 750, 1500 ppb MSD Calibration Levels
25, 50, 100, 175, 250, 500 ppb FPD Calibration Levels

The extraction process using the QuEChERS method was effective in retaining the OP pesticides in the spiked apple sample and providing sufficient cleanup of the sample matrix for GC/MS analysis. Figure 4 shows the organophosphorus pesticide mix spiked into an apple matrix sample. The matrix was prepared using a QuEChERS sample preparation approach that included extraction/partitioning and dispersive-SPE. A GC/MS/SIM blank matrix trace is shown below the analyte trace to indicate the level of potential matrix interference with the analytes of interest. Peak shapes for the organophosphorus pesticides are still quite sharp and well resolved indicating excellent performance on the DB-35ms UI column in fruit matrix.

Recoveries were determined by GC/MS/SIM at the 150, 300, and 750 ng/mL levels, and 50, 100, and 250 ng/mL using the FPD in phosphorus mode. The recoveries for most of the pesticides were greater than 75% with average RSDs below 10%. Recoveries for the individual OP pesticides are listed in Table 4. Lower recoveries were noted for the more polar pesticides: oxydemeton-methyl, methamidophos, and acephate. One possible explanation is that these polar, highly water soluble pesticides may have been partially lost through incomplete partitioning into the aqueous layer during the extraction step [12].

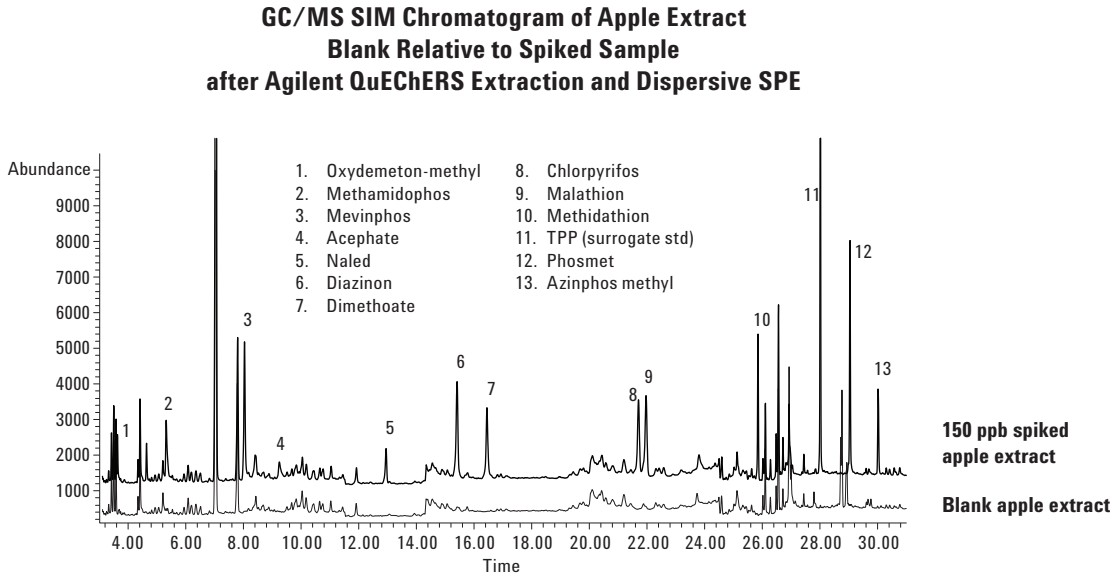


Figure 4. GC/MS/SIM chromatogram of the apple extract blank and a 150 ng/mL spiked apple extract analyzed on an Agilent J&W DB-35ms UI capillary column (Agilent p/n 121-3822UI). Chromatographic conditions are listed in Table 1.

Table 4. Recovery and Repeatability of OP Pesticides in Spiked Apple Matrix with an Agilent J&W DB-35ms UI Column (Agilent p/n 121-3822UI) (continued)

Recovery and Repeatability of OP Pesticides in Spiked Apple Matrix by SIM GC/MS with Agilent J&W DB-35ms UI Column

	150 ng/mL fortified QC		300 ng/mL fortified QC		750 ng/mL fortified QC	
Analysis	**% Recovery**	**RSD (n=6)**	**% Recovery**	**RSD (n=6)**	**% Recovery**	**RSD (n=6)**
Oxydemeton methyl	64.6	5.5	64.0	5.0	61.9	8.2
Methamidophos	68.9	8.5	78.6	3.9	83.8	3.9
Mevinphos	88.7	4.3	93.4	2.6	97.0	3.2
Acephate	77.5	6.4	80.3	5.2	84.3	2.6
Naled	92.9	6.2	87.6	2.4	80.3	5.7
Diazinon	84.5	3.0	89.0	2.7	90.9	3.0
Dimethoate	90.6	2.9	92.9	3.3	96.6	3.6
Chlorpyrifos	87.0	3.7	91.7	3.1	95.6	3.6
Malathion	92.5	3.8	91.9	3.4	97.3	3.6
Methidathion	89.6	4.4	92.1	3.4	99.2	3.7
TPP (surrogate std)	100.6	3.8	101.5	3.1	100.1	3.0
Phosmet	85.9	5.1	86.8	3.4	95.1	3.8
Azinphos methyl	88.6	4.2	84.3	3.5	95.4	3.9

Table 4. Recovery and Repeatability of OP Pesticides in Spiked Apple Matrix with an Agilent J&W DB-35ms UI Column (Agilent p/n121-3822UI)

Recovery and Repeatability of OP Pesticides in Spiked Apple Matrix by GC/FPD with Agilent J&W DB-35ms UI Column

	150 ng/mL fortified QC		300 ng/mL fortified QC		750 ng/mL fortified QC	
Analysis	**% Recovery**	**RSD (n=6)**	**% Recovery**	**RSD (n=6)**	**% Recovery**	**RSD (n=6)**
Oxydemeton methyl	45.8	8.5	60.6	9.4	62.4	7.7
Methamidophos	63.4	9.2	75.5	5.9	83.7	4.3
Mevinphos	80.5	5.0	90.1	3.5	93.5	4.1
Acephate	64.1	11.1	78.5	7.5	81.3	7.9
Naled	97.2	12.0	87.6	8.0	78.6	8.7
Diazinon	80.1	2.3	86.7	2.9	90.3	3.5
Dimethoate	80.6	7.2	91.0	3.6	93.6	4.6
Chlorpyrifos	80.8	4.7	91.5	5.5	96.6	4.4
Malathion	84.7	4.4	92.9	4.5	96.9	4.8
Methidathion	84.7	7.9	93.9	2.6	96.1	4.1
TPP (surrogate std)	99.6	2.3	99.7	6.0	95.9	3.2
Phosmet	76.6	5.5	89.3	2.6	92.4	4.5
Azinphos methyl	79.4	7.6	88.5	4.5	93.8	3.2

Conclusions

This application note successfully shows a quick and efficient analytical method to monitor low and trace level organophosphorus pesticides residue in apple samples. Splitting the column effluent to both an MSD and FPD facilitated selectivity, identification, and confirmation of OP pesticides from a single injection, thereby increasing laboratory productivity. Using GC/MS in Full Scan mode enabled identification of specific pesticides, while SIM mode offered selectivity and sensitivity for quantitation of the pesticides at trace levels. Confirmation and further specificity was achieved by FPD in phosphorus mode. FPD detection was effective at minimizing matrix interferences enabling lower detection.

The Agilent QuEChERS method for general fruits and vegetables was successful at providing enough sample cleanup to minimize matrix interferences while still maintaining low level analyte detection. The simple QuEChERS extraction method allows for faster sample prep facilitating higher sample throughput. Residual sample matrix carryover is removed through use of backflush, which eliminates the need for a bakeout cycle, significantly reducing analytical run times.

The Agilent J&W DB-35ms UI capillary column resolves the targeted OP pesticides and provides excellent peak shapes for the polar pesticides allowing for more reliable quantitation at low levels. Detection levels for the OP pesticides were at or below the US maximum residue levels (MRLs) for various fruits. Matrix-matched calibration standards yielded regression coefficients $r^2 \geq 0.992$ and recoveries from fortification studies were greater than 75% with an average RSD <10% for both GC/MS/SIM and FPD, further demonstrating the effectiveness of using an Agilent J&W DB-35ms UI column for residual pesticide determination.

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Sensitive Femtogram Determination of Aflatoxins B_1, B_2, G_1 and G_2 in Food Matrices using Triple Quadrupole LC/MS

Application Note

Food Safety

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Abstract

A simple and inexpensive sample cleanup procedure based on a dispersive solid phase adsorption approach (C18) is effective in removing background matrix contaminants for reliable determination of aflatoxins in food at the femtogram level by triple quadrupole LC/MS. This application demonstrates fast analysis time (< 6 min) with good chromatographic resolution and separation for all four aflatoxins. Standard curves for each aflatoxin analyte show good linearity (> 0.998) across a wide concentration range (0.1–100 µg/L). Recoveries using the dispersive solid phase adsorption approach were between 85–110% for each aflatoxin for all four spiked food matrices and were comparable to other widely used SPE routines. The limit of detection was determined to be < 0.15 µg/kg and the limit of quantitation < 0.5 µg/kg for all four sample matrices. Precision data was typically below 5% RSD for all analytes.

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Introduction

Aflatoxins are a group of mycotoxins produced as metabolites by the fungi aspergillus flavus and aspergillus parasiticus [1]. They can be found in various foods including grains, nuts, and spices [2]. There are four major naturally occurring aflatoxins: B_1, B_2, G_1 and G_2 (Figure 1). Exposure to them can cause cancer in humans and live stock, therefore reliable and sensitive analytical methods for the determination of aflatoxins are required to safeguard our food supply.

Experimental

These analyses were performed using an Agilent G6460A Triple Quadrupole LC/MS/MS System equipped with Agilent Jet Stream Technology [3] using an Agilent 1200 Series SL LC. The LC system consisted of a binary pump (G1312B), vacuum degasser (G1379B), a low carryover automatic liquid sampler (G1367D), thermostatted column compartment (G1316B) and MassHunter data system.

Aflatoxin standards and foods

Purified aflatoxin standards (B_1, B_2, G_1 and G_2) were obtained from Sigma-Aldrich. Aflatoxin-free corn flour, wheat, peanut and walnut samples obtained from a local grocery store were used for recovery studies.

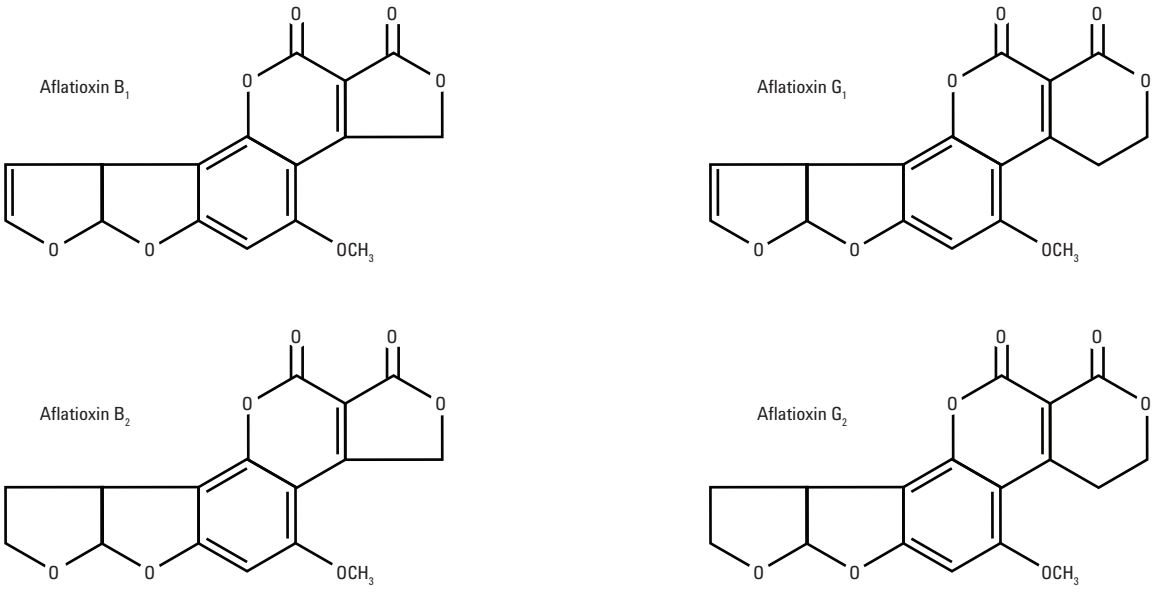


Figure 1. Structures of aflatoxins B_1, B_2, G_1 and G_2.

Instrumentation

Rapid Resolution HPLC Conditions and Configuration:

- Agilent 1200 Series Binary Pump SL (G1312B)
- High Performance WP Sampler SL Plus (G1367D)

  Sampler Thermostat (G1330B)
- Thermostatted Column Compartment SL, including 10P/Two-Position switching valve (G1316B with option #057)

Method Conditions:

Column:	Agilent ZORBAX Eclipse Plus C18, 2.1 x 50 mm, 1.8 µm	
Column temperature:	40 °C	
Injection volume:	5 µL	
Autosampler temp:	4 °C	
Needle wash:	Flushport (100% methanol), 5 seconds	
Mobile phase:	A = 10 mM NH_4 acetate in water B = 100% methanol	
Gradient flow rate:	0.6 mL/min (no split)	
Gradient:	Time (min)	%B
	0	5
	5	100
	6	100
Analysis time:	6 min	
Equilibration time:	1.5 min	
Total run time:	7.5 min	

Mass Spectrometer Source Conditions and Configuration:

Agilent 6460 Triple Quadrupole LC/MS equipped with Agilent Jet Stream Technology.

Ion Source Conditions:

Ion Mode:	ESI/Agilent Jet Stream, Positive ionization
Capillary Voltage:	4000 V
Drying gas (nitrogen):	10 L/min
Drying gas temperature:	325 °C
Nebulizer gas (nitrogen):	50 psi
Sheath Gas temperature:	350 °C
Sheath Gas flow:	11 L/min
Nozzle Voltage:	0 V
Q1 and Q2 Resolution:	0.7 amu [autotune]
Delta EMV:	400V

The Triple Quadrupole MS MRM parameters are listed in Table 1. All fragmentor voltage (frag) settings and respective collision energies (CE) and the most abundant MS/MS product ions per analyte were determined automatically using the Agilent MassHunter Optimizer Software.

Table 1. MRM Transitions for Aflotoxins and Respective Internal Standards

Name	Retention time (min)	Fragmentor voltage (V)	Precursor ion (m/z)	Product ion (m/z)	Collision energy (eV)
Aflatoxin B_1	4.68	130	313.1	241.1	35
				285.1	20
				269.1	25
Aflatoxin B_2	4.57	130	315.1	287.1	25
				259.1	25
				243.1	40
Aflatoxin G_1	4.40	130	329.1	243.1	25
				311.1	20
				283.1	20
Aflatoxin G_2	4.26	130	331.1	245.1	30
				285.1	25
				313.1	25
Isotope B_1	4.68	130	330.1	301.1	20
				255.1	40
Isotope B_2	4.57	130	332.1	303	25
				273.0	30
Isotope G_1	4.40	130	346.1	257.1	25
				299.1	25
Isotope G_2	4.26	130	348.1	330.1	25
				259.1	30

Sample Preparation and Recovery Studies

Corn flour, ground wheat, peanut and walnut samples (10 g each) were spiked with a mixture of four aflatoxin standards, each at 5 and 25 ng/g. This was then extracted using 40 mL of acetonitrile-water (84:16, v/v) for 30 min with shaking at room temperature. The extract was cleaned up using both C18 powdered adsorbent material (ODS SPE bulk sorbent, Agilent p/n 5982-1182) and MycoSep 226 multifunctional SPE (Romer). Aliquots (0.4 mL) of the cleaned up extracts were diluted with 0.6 mL 10 mM ammonium acetate in water.

The sample was then centrifuged at 14,000 rpm for 3 min prior to LC/MS/MS analysis.

Each food matrix and spike level was conducted in seven replicates to represent and maintain statistical integrity. A schematic of this sample preparation is illustrated in Figure 2.

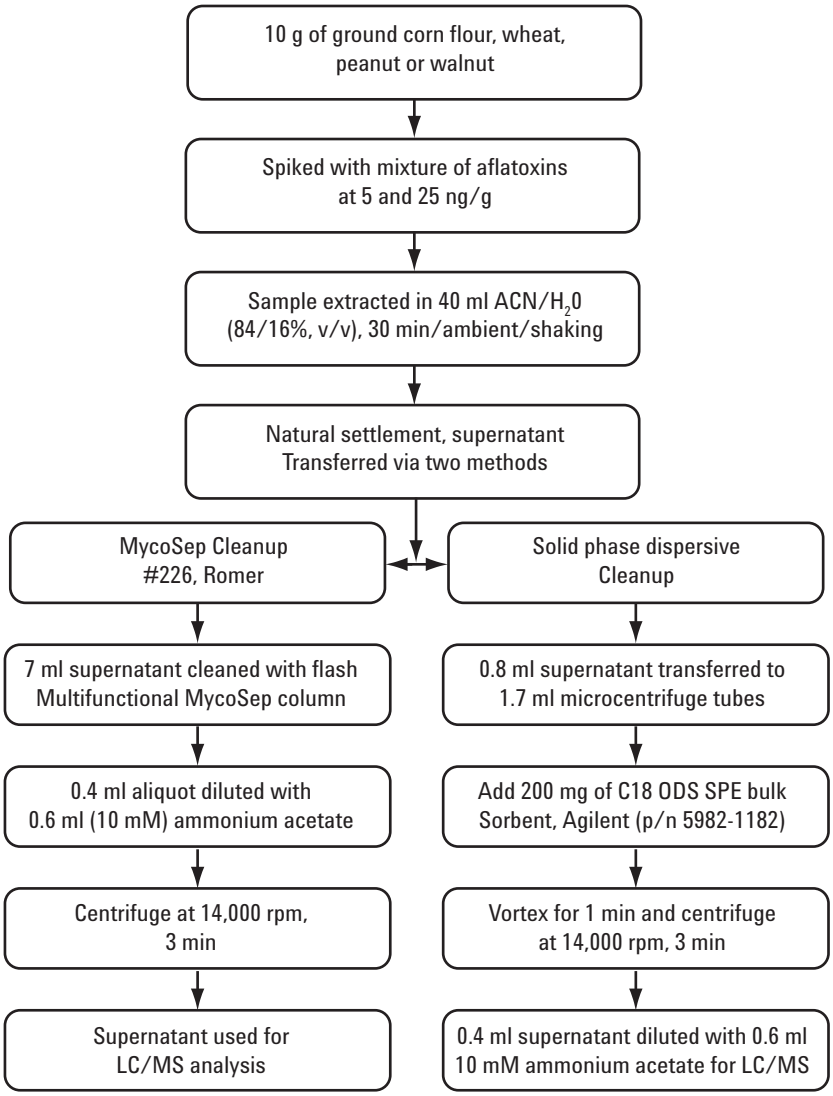


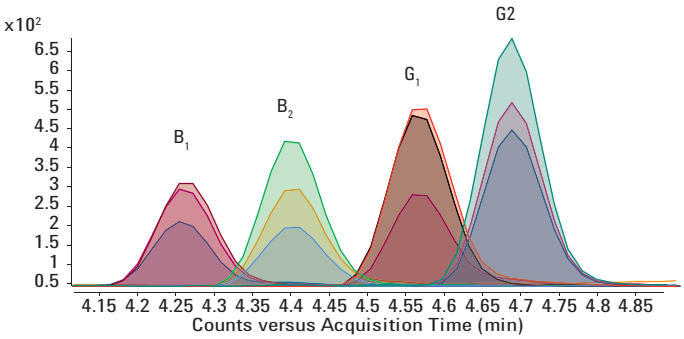
Figure 2. Schematic matrix sample preparation workflow showing the dispersive solid phase adsorption approach versus a widely used SPE approach.

Results and Discussion

The rapid chromatography conditions as outlined in the experimental section yielded good chromatographic resolution for each aflatoxin analyte and each analysis was completed in six minutes. A typical chromatogram is shown in Figure 3(a), which illustrates 1 ppb concentration level of each aflatoxin together with the corresponding isotopically labeled internal standards at a concentration level of 2.5 ppb (Figure 3(b)). These chromatograms show overlaid extracted ion chromatograms (EICs.)

Standard curves for aflatoxins B1, B2, G1 and G2 all showed a good linearity through the concentration range 0.1 to 100 ppb each with a linear correlation (R^2) of greater than 0.999. Figure 4 illustrates an overlay of each standard curve on the same scale, but without internal standard correction. The use of internal standards effectively adjusted for matrix differences, as shown in Figure 5.

(a) Overlaid aflatoxin EICs Quant ion and two Qualifiers (1 ppb)



(b) Overlaid Isotopically labelled aflatoxin EICs Quant ion and Qualifier ion (2.5 ppb)

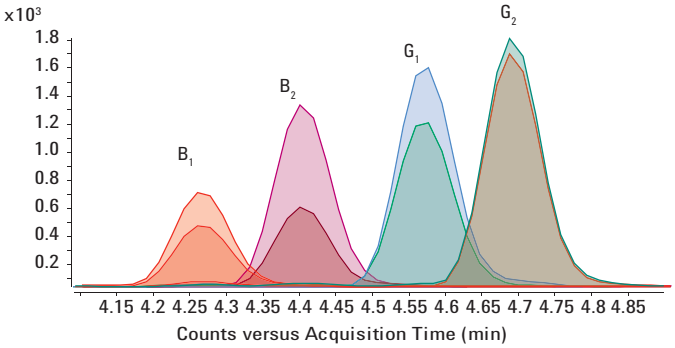


Figure 3. LC/MS/MS chromatogram of aflatoxin B_1, B_2, G_1 and G_2 standards at 1 ppb with corresponding isotopically labelled internal standards at 2.5 ppb.

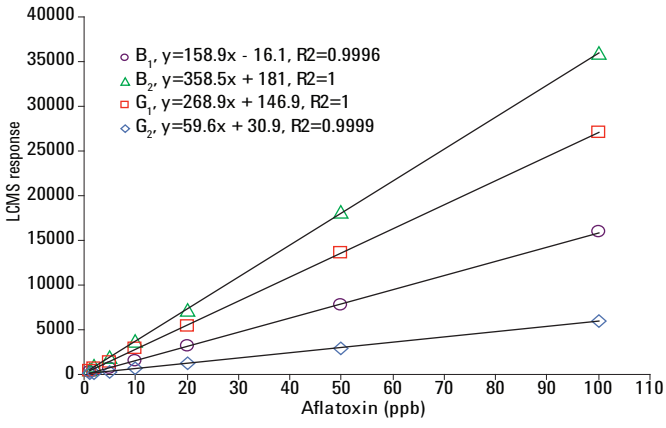


Figure 4 Overlaid standard curves for aflatoxins B_1, B_2, G_1 and G_2.

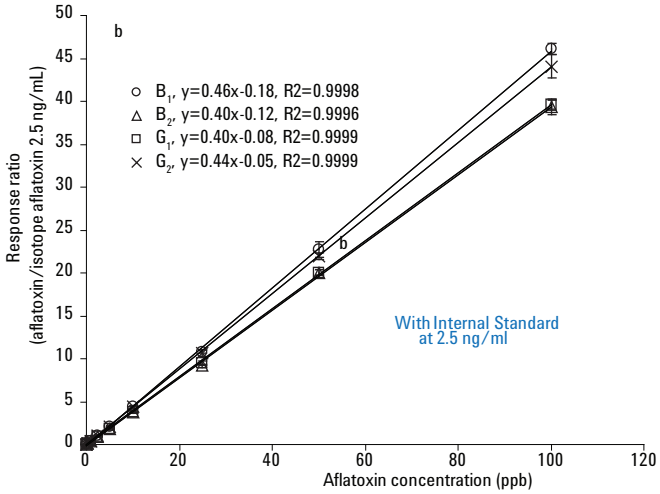


Figure 5. Overlaid standard curves for aflatoxins B_1, B_2, G_1 and G_2 with internal standard correction.

In order to determine the limits of detection (LOD) and reporting (LOR), seven separate and mutually exclusive batches of each food matrix were tested via the two sample preparation protocols and analytical methodology outlined previously. The results outlined in this document are derived from the average values across the seven batches of each matrix (N=7.)

Limits of detection were determined using the protocol of chromatographic signal-to-noise ratio of above 3/1 (peak to peak.) Limits of reporting were determined using the protocol of chromatographic signal-to-noise ratio above 10/1 (peak to peak.)

Table 2 details the observed LODs and LORs for each aflatoxin across the series of four food matrices. The limit of detection overall was determined to be < 0.15 µg/kg and the limit of quantitation < 0.5 µg/kg for all four sample matrices and both sample preparation routines.

Tables 3 and 4 summarize the LOD data obtained across the seven batches. Table 3 data is presented with respect to dispersive solid phase adsorption only, and Table 4 data using the Mycosep SPE sample preparation only.

Table 2. Limits of Detection and Reporting Observed for Aflatoxins B_1, B_2, G_1 and G_2 Across Four Food Matrices via Dispersive SPA and Mycosep SPE Sample Preparation Approaches

	Aflatoxin	Mycosep (#226, Romer)		Dispersive C18 ODS SPE Bulk Sorbent, Agilent (p/n 5982-1182)	
Food Matrix		**LOD ng/g (S/N>3)**	**LOR ng/g (S/N>10)**	**LOD ng/g (S/N>3)**	**LOR ng/g (S/N>10)**
Corn	B_1	0.047	0.16	0.060	0.20
(Ave 7-batches)	B_2	0.036	0.12	0.085	0.28
	G_1	0.08	0.28	0.10	0.35
	G_2	0.046	0.15	0.033	0.11
Wheat	B_1	0.068	0.23	0.012	0.042
(Ave 7-batches)	B_2	0.11	0.36	0.037	0.12
	G_1	0.14	0.47	0.15	0.50
	G_2	0.038	0.13	0.11	0.36
Peanut	B_1	0.051	0.17	0.056	0.19
(Ave 7-batches)	B_2	0.045	0.15	0.069	0.23
	G_1	0.07	0.23	0.05	0.15
	G_2	0.052	0.17	0.14	0.45
Walnut	B_1	0.12	0.41	0.093	0.31
(Ave 7-batches)	B_2	0.035	0.12	0.098	0.33
	G_1	0.03	0.10	0.12	0.40
	G_2	0.047	0.16	0.04	0.13

Table 3. LOD Results Observed for Aflatoxins B_1, B_2, G_1 and G_2 via the Dispersive SPA

Food Matrix	B_1 LOD (ng/g)	B_2 LOD (ng/g)	G_1 LOD (ng/g)	G_2 LOD (ng/g)
Corn	0.060	0.085	0.100	0.033
Wheat	0.012	0.037	0.150	0.110
Peanut	0.056	0.069	0.050	0.140
Walnut	0.093	0.098	0.120	0.040
Average	0.055	0.072	0.105	0.080
Mass On-Column (fg)	275	360	525	400

Table 4. LOD Results Observed for Aflatoxins B_1, B_2, G_1 and G_2 via the Mycosep, #226 SPE Sample Preparation Approach

Food Matrix	B_1 LOD (ng/g)	B_2 LOD (ng/g)	G_1 LOD (ng/g)	G_2 LOD (ng/g)
Corn	0.047	0.036	0.080	0.046
Wheat	0.068	0.110	0.140	0.038
Peanut	0.051	0.045	0.070	0.052
Walnut	0.120	0.035	0.030	0.047
Average	0.072	0.057	0.080	0.046
Mass On-Column (fg)	360	283	400	229

Sample Preparation Approach

Recovery studies were extensively undertaken for both sample cleanup techniques in parallel across the four matrices and across the seven batches for each aflatoxin analyte. Figures 6 (a) and (b) graphically depict the recovery trends across the four food matrices for the dispersive C18 cleanup approach and the Mycosep SPE cleanup, respectively. As illustrated, the recoveries for both sample cleanup procedures were between 85-110 % for each of the aflatoxins for all four spiked food matrices, with the MycoSep cleanup method only marginally better than the C18 one for walnut samples .

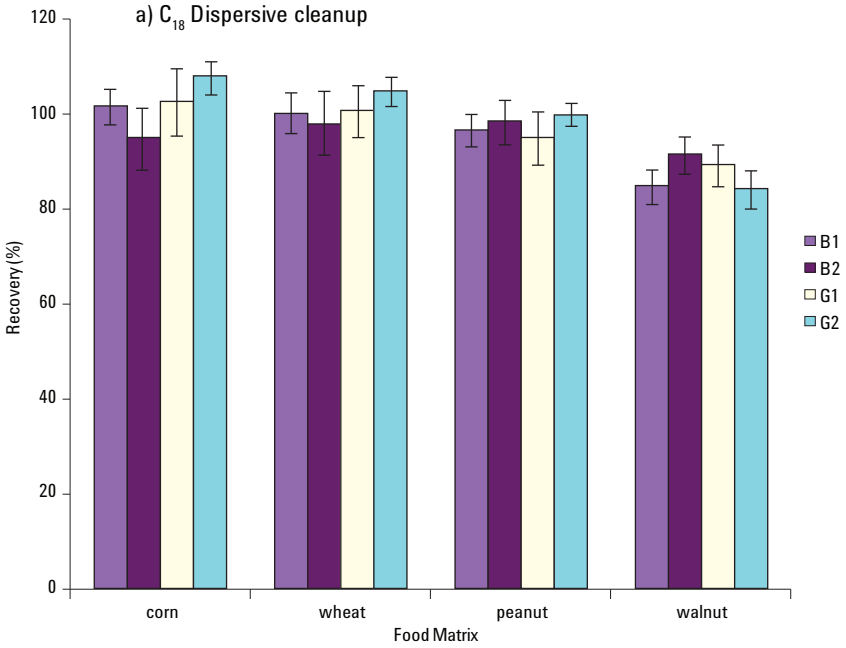


Figure 6(a). Recovery of aflatoxin B_1, B_2, G_1 and G_2 from food matrices using C_{18} dispersive cleanup.

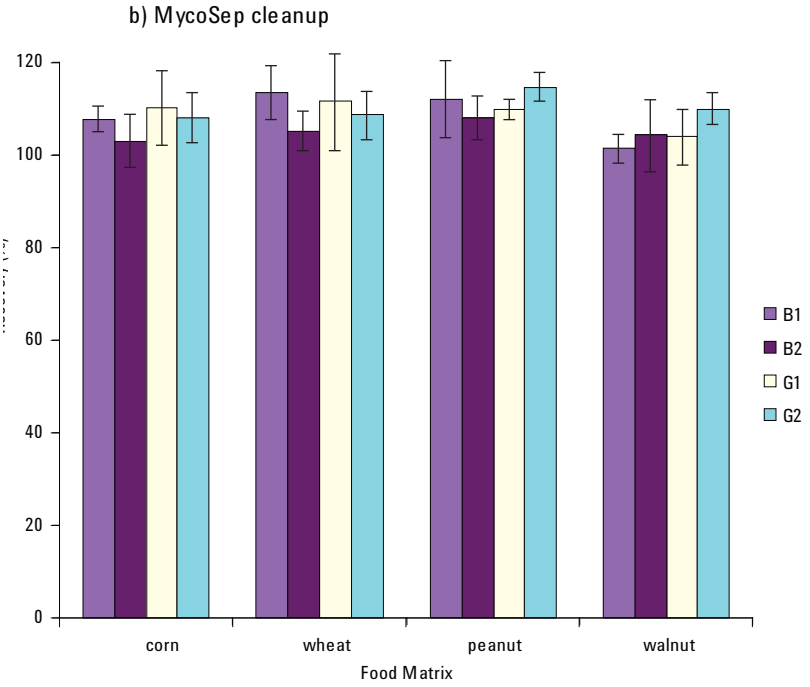


Figure 6(b). Recovery of aflatoxin B_1, B_2, G_1 and G_2 from food matrices using Mycosep, #226 SPE cleanup.

Aflatoxin analyte recovery data for each separate food matrix is detailed in Tables 5 through 8 and was undertaken at two concentration spiked levels of 5 ng/g and 25 ng/g. Each sample batch tested was split and divided between the two cleanup procedures outlined in this document following the natural settlement and supernatant transfer step outlined in Figure 2(a).

Table 5. Spiked Corn Samples – Recovery Studies (% Recovery, ± RSD, N=7)

	Aflatoxin	Corn spiked at 5 ng/g C18 cleanup	Corn spiked at 25 ng/g C18 cleanup	Corn spiked at 5 ng/g Mycosep#226	Corn spiked at 25 ng/g Mycosep#226
No Internal Standard	B_1	101.7 ± 3.7	95.7 ± 3.0	107.8 ± 2.8	105.4 ± 2.8
	B_2	95 ± 6.3	95.4 ± 1.4	103.0 ± 5.7	105.3 ± 2.0
	G_1	102.7 ± 7.1	96.8 ± 1.75	110.2 ± 7.9	103.7 ± 3.0
	G_2	107.9 ± 3.5	97.8 ± 0.88	108.1 ± 5.4	104.3 ± 2.4
Internal Standard	B_1	102.3 ± 2.9	100.1 ± 2.4	108.2 ± 5.1	97.8 ± 3.3
	B_2	100.0 ± 7.9	94.0 ± 3.1	101.7 ± 4.7	92.8 ± 3.5
	G_1	107.3 ± 3.5	97.0 ± 6.0	110.3 ± 3.6	102.5 ± 1.8
	G_2	101.3 ± 5.6	100.4 ± 3.8	104.9 ± 5.4	97.2 ± 6.3

Table 6. Spiked Wheat Samples – Recovery Studies (% Recovery, ± RSD, N=7)

	Aflatoxin	Wheat spiked at 5 ng/g C18 cleanup	Wheat spiked at 25 ng/g C18 cleanup	Wheat spiked at 5 ng/g Mycosep#226	Wheat spiked at 25 ng/g Mycosep#226
No Internal Standard	B_1	100.1 ± 4.4	96.6 ± 2.9	113.5 ± 5.9	100.6 ± 1.8
	B_2	98.2 ± 6.9	96.4 ± 2.6	105.1 ± 4.5	102.1 ± 4.4
	G_1	100.5 ± 5.5	105.4 ± 3.8	111.5 ± 10.4	106.1 ± 4.0
	G_2	104.9 ± 3.2	106.7 ± 1.3	108.6 ± 5.2	103.7 ± 2.9
Internal Standard	B_1	100.9 ± 3.6	109.3 ± 4.7	107.5 ± 4.8	111.7 ± 4.9
	B_2	85.2 ± 7.7	99.8 ± 2.8	92.4 ± 6.3	101.0 ± 4.0
	G_1	110.6 ± 7.8	112.8 ± 1.8	117.6 ± 7.7	109.3 ± 5.7
	G_2	108.4 ± 6.2	108.3 ± 3.9	115.6 ± 7.1	109.8 ± 3.6

Table 7. Spiked Peanut Samples – Recovery Studies (% Recovery, ± RSD, N=7)

	Aflatoxin	Peanut spiked at 5 ng/g C18 cleanup	Peanut spiked at 25 ng/g C18 cleanup	Peanut spiked at 5 ng/g Mycosep#226	Peanut spiked at 25 ng/g Mycosep#226
No Internal Standard	B_1	96.7 ± 3.4	97.0 ± 4.6	112.0 ± 8.4	104.9 ± 1.7
	B_2	98.3 ± 4.7	97.4 ± 2.9	108.0 ± 4.6	104.5 ± 2.0
	G_1	95.0 ± 5.6	95.0 ± 4.9	109.9 ± 2.1	105.7 ± 3.4
	G_2	100.0 ± 2.3	100.0 ± 2.0	114.7 ± 3.2	106.3 ± 1.1
Internal Standard	B_1	101.8 ± 3.6	96.1 ± 2.0	100.0 ± 6.8	103.0 ± 3.5
	B_2	102.5 ± 5.5	100.2 ± 5.0	99.4 ± 4.1	102.9 ± 2.7
	G_1	105.7 ± 7.3	99.2 ± 2.2	105.2 ± 4.3	101.7 ± 5.2
	G_2	107.5 ± 10.9	104.9 ± 6.7	109.3 ± 8.7	102.4 ± 3.1

Table 8. Spiked Walnut Samples – Recovery Studies (% Recovery, ± RSD, N=7)

	Aflatoxin	Walnut spiked at 5 ng/g C18 cleanup	Walnut spiked at 25 ng/g C18 cleanup	Walnut spiked at 5 ng/g Mycosep#226	Walnut spiked at 25 ng/g Mycosep#226
No Internal Standard	B_1	84.9 ± 3.7	85.2 ± 2.2	101.4 ± 3.2	101.0 ± 2.3
	B_2	91.5 ± 3.9	89.8 ± 2.8	104.2 ± 7.9	106.3 ± 2.9
	G_1	89.4 ± 4.4	86.7 ± 1.5	103.9 ± 5.9	101.7 ± 4.2
	G_2	84.0 ± 4.0	83.1 ± 1.3	109.9 ± 3.4	106.3 ± 1.5
Internal Standard	B_1	106.5 ± 4.9	98.9 ± 4.1	93.8± 1.4	100.2 ± 2.9
	B_2	99 ± 5.4	96.5 ± 3.5	92.4 ± 2.7	98.7 ± 4.4
	G_1	103.2 ± 5.9	94.9 ± 2.5	102.8 ± 9.0	102.1 ± 3.9
	G_2	100.2 ± 6.2	97.5 ± 4.6	99.5 ± 6.8	101.2 ± 3.8

Conclusions

An inexpensive and rapid LC/MS/MS method has been developed for the analysis and confirmation of aflatoxins B_1, B_2, G_1 and G_2 in cereals and nuts, with a detection limit of less than 1 ppb. This method is inclusive of sample preparation using a dispersive C18 solid phase adsorption approach (Agilent bulk sorbent p/n 5982-1182.) The performance of this simple sample cleanup procedure was comparable to that of a widely used and generally accepted SPE approach in terms of matrix cleanup and aflatoxin recoveries.

Aflatoxin limits of detection were determined to be less than 0.15 µg/kg and aflatoxin limits of reporting were all less than 0.5 µg/kg for all four sample matrices (corn, wheat, peanut and walnut.)

Standard curves for aflatoxins B_1, B_2, G_1 and G_2 showed a good linearity through the concentration range of 0.1 to 100 ppb with a linear correlation (R^2) of greater than 0.999 for all analytes.

Aflatoxin recoveries were between 85-110% for each of the aflatoxins for all four spiked food matrices using the dispersive C18 solid phase adsorption approach.

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Improved Isolation and Analysis of Mycotoxins from Cereals, Beer and Wine

Application Note

Authors

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Introduction

Trichothecenes are important mycotoxins produced primarily by the genus fusarium. The toxic effects of fusarium toxins on human and animals are well documented and reliable and sensitive analysis methods are therefore required to protect the population and its food sources[1]. This application shows the optimized extraction and clean-up of 12 type A- and B-trichothecenes (Neosolaniol [NEO], HT-2 toxin [HT-2], T-2 toxin [T-2], T-2 triol, T-2 tetraol, Monoacetoxyscirpenol [MAS], Diacetoxyscirpenol [DAS], Deoxynivalenol [DON], 3-Acetyl-deoxynivalenol [3ADON], 15-Acetyl-deoxynivalenol [15ADON], Nivalenol [NIV], Fusarenon [FUS]) and Zearalenone (ZEA) in cereals and cereal-based food, as well as the four polar mycotoxins DON, T-2, HT-2 and ZEA in wheat beer and sake wine matrices. The clean-up was optimized on Bond Elut Mycotoxin, a newly developed solid phase extraction (SPE) sorbent. The LC-MS-MS method presented here gives quantitative and qualitative information and has the accuracy and resolution required for reporting results to European and US governmental agencies[2].

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Sample Preparation

Cereals and cereal-based food samples

Typical clean-up methods of trichothecenes and ZEA from cereals and cereal-based foods use commercially available polar clean-up columns[3]. Substances that interfere with the detection of the mycotoxins are retained while trichothecenes are not. This purification method, however, gives low recoveries for the polar toxins: NIV, T-2 tetraol, and DON.

To address these problems, the extraction step was optimized by marginally increasing the polarity of the extraction solvent to ACN/H_2O (80/20; v/v) as previously described in Varian Application Note 295[4]. Trials with the polar DON reference material from Food Analysis Performance Assessment Scheme (FAPAS) confirm that the best recovery data is achieved with the Bond Elut Mycotoxin method (Table 1).

Table 1. Recovery comparison of Food Analysis Performance Assessment Scheme (FAPAS) certified reference material for DON applying LC-MS-MS

Method 1: Clean up on Bond Elut Mycotoxin
Method 2: Clean up on polar charcoal-alumina sorbent (PS)

Reference Material	Certified Value (µg/kg)	Method 1 (µg/kg)	Method 2 (µg/kg)
FAPAS T2210	463 ± 167	495 ± 5	395 ± 15

Wheat beer and sake wine samples

Prior to clean-up by the Bond Elut cartridge, the wheat beer and sake wine samples were degassed by sonication for 30 min at room temperature. The degassed samples were filtered through Whatman no.1 filter paper (Florham Park, NJ), and the solution of multiple mycotoxins was added to a final concentration of either 35 or 350 ng/g with an internal standard (ZAN) concentration of 50 ng/g. This mixture was applied to a Bond Elut Mycotoxin column. Fully automated clean-up was performed on a Zymark SPE workstation (Hopkinton, MA) according to the Bond Elut Mycotoxin method (Table 2).

Table 2. Bond Elut Mycotoxin Method

1. Pass 4 mL of the filtrated sample extract through a Bond Elut Mycotoxin column (part number 12165001B).
2. Evaporate 2 mL of the eluate to dryness at 50 °C under a gentle stream of nitrogen.
3. Reconstitute in 0.5 mL acetonitrile/water (20/80; v/v).
4. Inject into LC-MS-MS.

LC-MS-MS

The LC-MS-MS system utilized in these experiments is the 320-MS triple quadrupole mass spectrometer fitted with an electrospray ionization (ESI) source, two ProStar 210 liquid chromatography pumps, and an Agilent ProStar 430 autosampler. Separation was according to the gradient program shown in Table 3.

Table 3. Gradient program and LC/API conditions

Time (min)	A (%)	B (%)	Flow (mL/min)
0:00	100	0	0.3
16:00	40	60	0.3
16:01	40	60	0.3
40	30	70	0.3
40:01	85	15	0.4
50:00	85	15	0.4

LC Conditions

Column:	Polaris C18-A 5 µm, 150 mm × 3.0 mm id (p/n A2000150X030)
Buffer A:	5 mM ammonium acetate, 1% acetic acid in 10% methanol
Buffer B:	5 mM ammonium acetate, 1% acetic acid in 100% methanol
Injection Solvent:	Buffer A
Injection Volume:	30 µL

API Conditions

Ionization Mode:	ESI (positive and negative)
Collision Gas:	1.8 mTorr argon
API Drying Gas:	30 psi at 250 °C
API Nebulizing Gas:	50 psi
Needle:	4500 V
Capillary:	Scanning
Detection:	1900 V

Results and Discussion

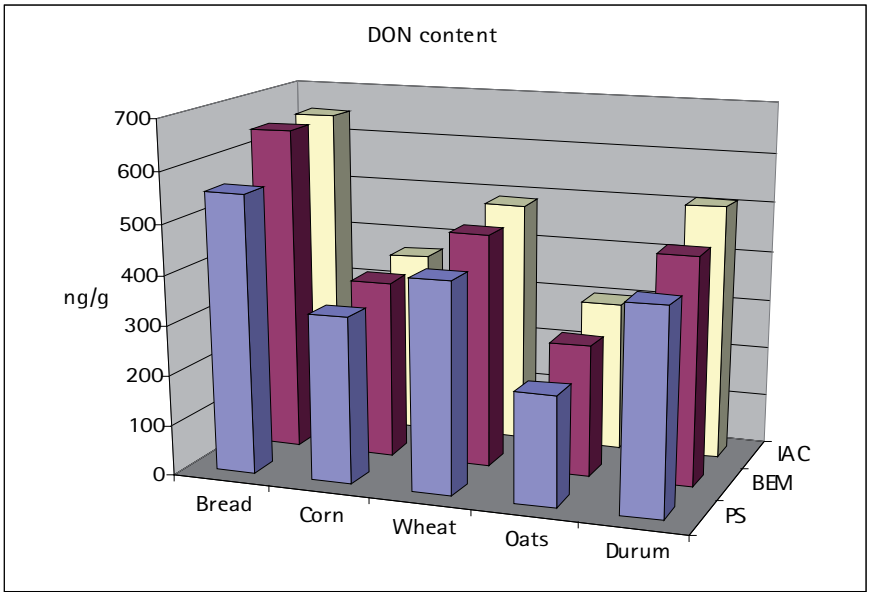
Cereals and cereal-based food samples

With the low increase of 6% water in the extraction solvent and with the clean-up step on Bond Elut Mycotoxin, recoveries (especially for the polar toxins DON, NIV, 3ADON and T-2 tetraol) were increased up to 31%.

If the determination of DON alone is of interest, then the highest content can be achieved with an extraction of 100% water and clean up with the more expensive IAC; however, for the determination of 12 trichothecenes with different polarities, the Bond Elut Mycotoxin cartridge provides good results.

Figure 1 shows the trichothecene content of 5 naturally contaminated samples after 3 different clean-up methods.

Up to 43% higher values were achieved using Bond Elut Mycotoxin for the clean-up of naturally contaminated samples containing the polar toxins DON, NIV, 3ADON, 15ADON and T-2 tetraol in comparison to the charcoal-alumina based polar method.



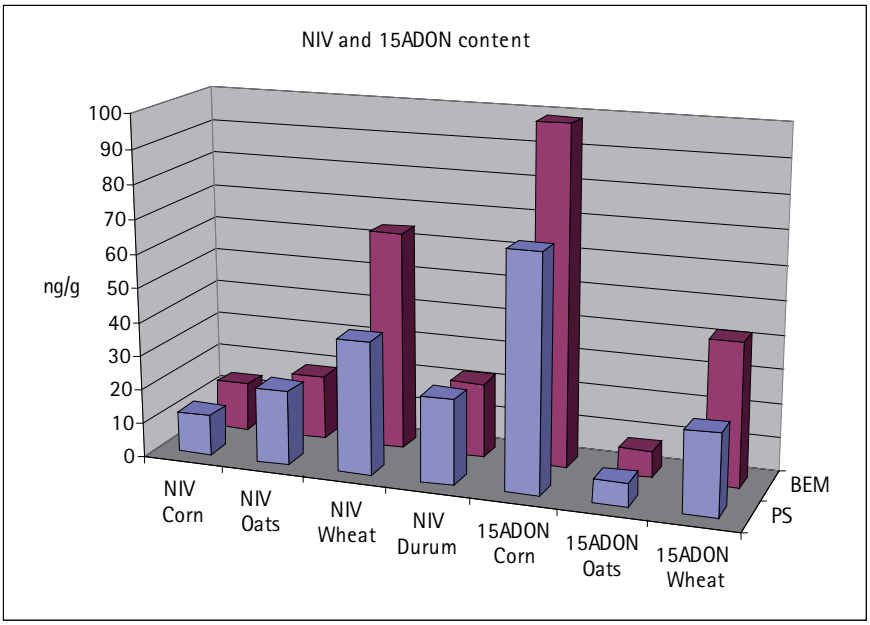


Figure 1. Trichothecene contents of 5 naturally contaminated samples analyzed with DONPrep (IAC), Polar charcoal-alumina sorbent (PS) and Bond Elut Mycotoxin cartridges (BEM) (n=3)

Wheat beer and sake wine samples

A mixture of HT-2, T-2, ZEA and ZAN (internal standard) at a low-level concentration of 35 ng/g was spiked into a complex matrix of wheat beer and/or sake wine.

Figure 2 shows the chromatograms with excellent peak shape and high signal response of the mycotoxins in a wheat beer matrix.

Table 4 shows the recovery rates calculated for high concentration of 350 ng/g and low concentration of DON, ZEA, T-2 and HT-2 from wheat beer and sake after clean-up on Bond Elut Mycotoxin. Very good recoveries (> 90%) and excellent reproducibility (n=3) are displayed for DON and ZEA. The recoveries of T-2 and HT-2 are >60% and they satisfy the European requirements. The EU guideline 2005/38/EG sets recovery limits for DON and ZEA in the range of 60–120% and 60–130% for T-2 and HT-2.

Summary

This application note describes a new, reliable, and cost-efficient clean-up method for the determination by triple quadrupole LC-MS-MS, of 12 type A- and B-trichothecenes in cereals and cereal-based foods. In addition, it shows the determination of four polar mycotoxins DON, T-2, HT-2 and ZEAN in wheat beer and sake wine matrices. The extracted mycotoxins are purified by means of the newly developed Bond Elut Mycotoxin SPE cartridge. The method exceeds the required limits of detection as well as ion ratio requirements for European Directives (ion ratios of <30%), such as SANCO D.1(06)D/412820.

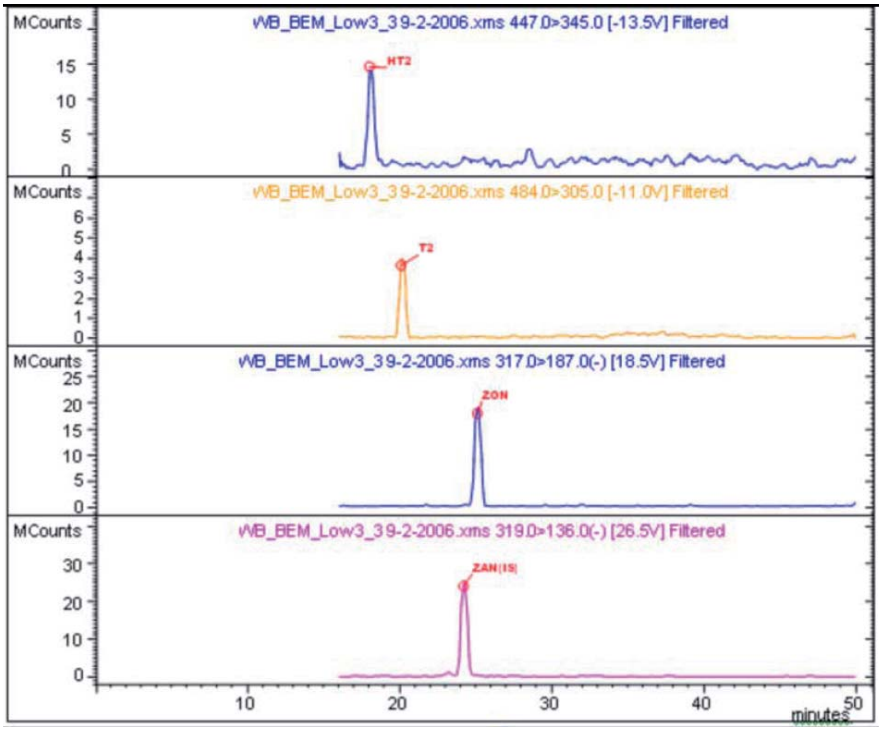


Figure 2. Example chromatograms of low-level spikes for various mycotoxins in a wheat beer matrix. Shown (top to bottom) are: HT-2, T-2, ZEA and ZAN (internal standard)

Table 4. Rates of recovery for selected mycotoxins after a clean-up step on Bond Elut Mycotoxin from wheat beer and sake at two concentrations; relative standard deviation is calculated from three replicates

	Wheat beer matrix			
	% Recovery	**% RSD**	**% Recovery**	**% RSD**
Mycotoxin	35 ng/g		350 ng/g	
DON	92	2.6	95.5	1.5
ZEA	116	6.1	101.9	1.3
T-2	61.3	12.6	60.1	1.1
HT-2	81.8	5.6	76.1	1.4

	Sake wine matrix			
	% Recovery	**% RSD**	**% Recovery**	**% RSD**
Mycotoxin	35 ng/g		350 ng/g	
DON	94.3	7.4	96.8	0.5
ZEA	99.3	1.3	99.8	0.8
T-2	101.3	1.3	66.0	0.9
HT-2	113.9	8.3	111	1

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[1] European Commission, Scientific Commitee on Food; Opinion on Fusarium Toxins Part 6: Group evaluation of T-2 toxin, HT-2 toxin, nivalenol and deoxynivalenol; http://www.europa.eu.int/comm/food/food/chemicalsafety/contaminants/fusarium_en.htm (adopted: 02/27/2002)

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Agilent Technologies

Fast separation of EU and EPA Regulated PAHs on Agilent J&W FactorFour VF-17ms for PAH

Application Note

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Introduction

When analyzing polycyclic aromatic hydrocarbons (PAHs) the bottleneck is the number of PAHs with the same mass. With MS detection different compounds with the same mass cannot be resolved. To resolve these PAHs the column selectivity and the oven program are very important. The Agilent FactorFour VF-17ms for PAH has perfect selectivity and optimized film thickness to analyze the PAHs, addressing the increasing need of many laboratories for improved productivity.

PAHs enclose a large group of organic compounds which contain two or more aromatic rings. Hundreds of individual PAHs can be formed during incomplete combustion or pyrolysis of organic matter, industrial processes and cooking and food processing. PAHs are therefore analyzed in food as well as environmental samples. Because of the difference in European (EU) and American (EPA) legislation, different sets of PAHs are prescribed (Table 1). The EU PAH list is used for food samples while the EPA list concerns environmental samples. This application note describes the analysis of EU and EPA PAHs in a single run, which can therefore be used for environmental as well as for food samples.

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Conditions

Technique:	GC/MS
Column:	VF-17ms for PAHs, 20 m x 0.15 mm, df = 0.05 µm (part number CP9009)
Temperature:	70 °C (0.5 min), 70 °C/min, 180 °C (1 min), 5 °C/min, 245 °C (1 min), 4 °C/min, 270 °C (1.5 min), 15 °C/min, 350 °C
Carrier Gas:	Helium, constant flow 1 mL/min
Injector:	300 °C, Splitless mode
Detector:	Quadrupole MS, EI in SIM, ion source 275 °C, transfer line 300 °C
Sample:	Concentration approx. 1 µg/mL
Injection Volume:	1 µL

Table 1. Peak Identification for Figure 1

Peak	Compound	MW	EU PAH	EPA PAH
1	Naphthalene	128		X
2	Acenaphthylene	152		X
3	Acenaphthene	154		X
4	Fluorene	166		X
5	Phenanthrene	178		X
6	Anthracene	178		X
7	Fluoranthene	202		X
8	Pyrene	202		X
9	Benzo(c)fluorene	216	X	
10	Benz(a)anthracene	228	X	X
11	Cyclopenta(c,d)pyrene	226	X	
12	Triphenylene	228		
13	Chrysene	228	X	X
14	6-Methylchrysene	242		
15	5-Methylchrysene	242	X	
16	Benzo(b)fluoranthene	252	X	X
17	Benzo(k)fluoranthene	252	X	X
18	Benzo(j)fluoranthene	252	X	
19	Benzo(a)pyrene	252	X	X
20	Indeno(1,2,3-cd)pyrene	276	X	X
21	Benzo(b)triphenylene	278		
22	Dibenz(a,h)anthracene	278	X	X
23	Benzo(g,h,i)perylene	276	X	X
24	Dibenzo(a,l)pyrene	302	X	
25	Dibenzo(a,e)pyrene	302	X	
26	Dibenzo(a,i)pyrene	302	X	
27	Dibenzo(a,h)pyrene	302	X	

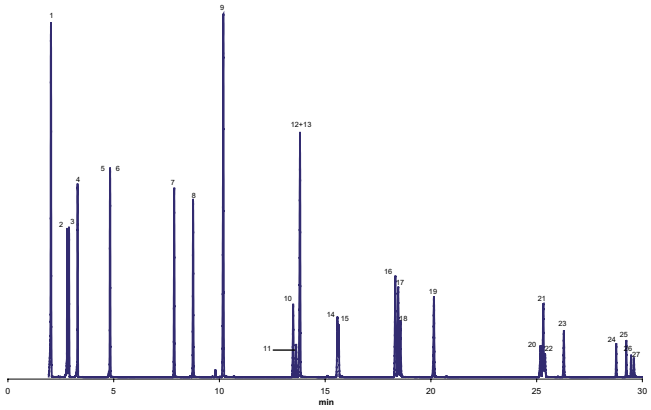


Figure 1. GC/MS analysis of EU and EPA PAHs on VF-17ms 20 m x 0.15 mm x 0.05 µm

Results and Discussion

When analyzing PAHs some peak groups are difficult to resolve on the MS because of their identical mass. One of the groups, Benz(a)anthracene, Cyclopenta(c,d)pyrene, Chrysene and Triphenylene, has compounds with different masses, but the difference between these masses, m/z 226 and 228, is difficult to distinguish with MS. Therefore these compounds should be resolved chromatographically (Figure 2). The same problem occurs when resolving Indendo(1,2,3-cd)pyrene, Benzo(b)triphenylene and Dibenz(a,h)anthracene with m/z 276 and 278 (Figure 4).

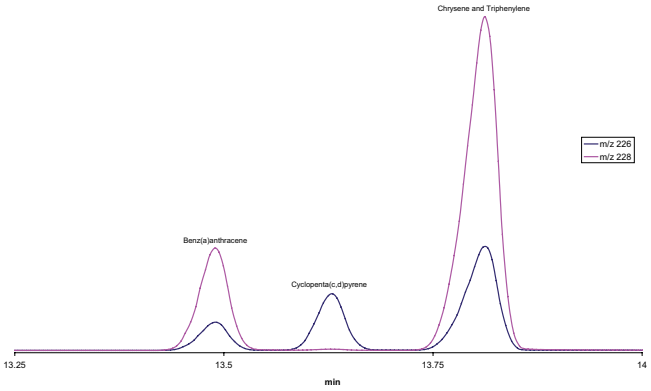


Figure 2. Separation of m/z 226 and 228

In the other difficult group, the Benzofluoranthene isomers, all three compounds have the same mass (m/z 252). With most analyses, Benzo(b)fluoranthene and Benzo(k)fluoranthene are not resolved very well. However, in this analysis, not only are Benzo(b)fluoranthene and Benzo(k)fluoranthene fully resolved, but also Benzo(j)fluoranthene (Figure 3).

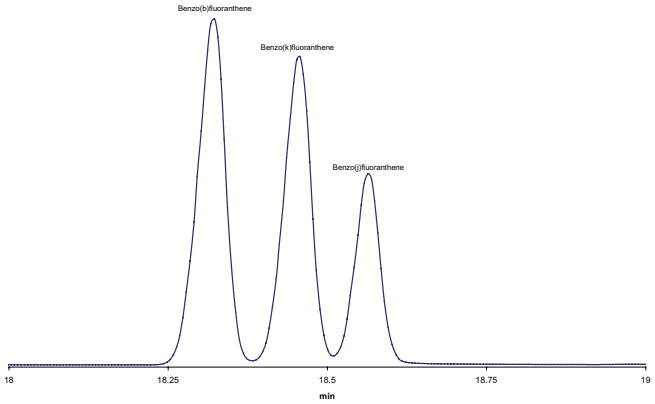


Figure 3. Separation of Benzofluoranthene isomers (m/z 252)

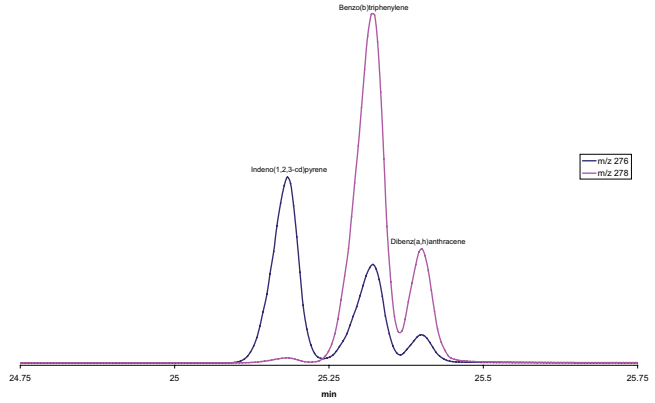


Figure 4. Separation of m/z 276 and 278

Conclusion

In this application note the EU and EPA regulated PAHs are perfectly separated using the VF-17ms for PAH in less than 30 minutes. With the optimized oven program the difficult groups are nearly baseline resolved. However, it should be noted that Triphenylene, which is not listed in either EPA or EU lists, can cause a false positive because of its co-elution with Chrysene.

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Agilent Technologies

Multi-residue Analysis of PAHs, PCBs and OCPs using an Agilent J&W FactorFour VF-35ms Column

Application Note

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Introduction

Multi-residue analysis involves the separation of different groups of compounds in a single operation. One of the most important advantages of multi-residue analysis is the opportunity to screen and quantify a multitude of components in a short time span, reducing analysis costs. A multi-residue method for the separation of polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs) is described here.

PAHs, PCBs and OCPs have different sources and belong to different chemical classes. PAHs contain two or more aromatic rings and are formed during incomplete combustion or pyrolysis of organic matter. OCPs are pesticides containing mainly carbon, hydrogen and chlorine atoms. They break down slowly and can remain in the environment long after application, and bioaccumulate in organisms during prolonged exposure. PCBs are characterized by two phenyl groups, with varying numbers of chlorine atoms. PCBs are used in many industrial and commercial applications, for example, as plasticizers in paints, plastics and rubber products, and in pigments and dyes. All of these compounds are typically very persistent in sediments, plants and animals.

The 16 EPA PAHs,17 PCBs (including the 6 EU marker PCBs) and 24 common OCPs were analyzed in a single run at different concentration levels. The concentration of the PAHs was ten times higher than the concentration of PCBs and OCPs, a concentration difference that is common also in real environmental samples.

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Conditions

Technique:	GC/MS
Column:	VF-35ms, 30 m x 0.25 mm, df=0.25 µm (part number CP8877)
Sample Conc:	OCPs and PCBs 0.1 µg/mL, PAHs 1 µg/mL
Temperature:	75 °C (1 min), 20 °C/min, 220 °C, 5 °C/min, 260 °C (2 min), 7 °C/min, 305 °C, 2 °C/min, 330 °C
Injection Volume:	1.0 µL
Carrier Gas:	Helium, constant flow, 1.0 mL/min
Injection:	100 °C (0.4 min), 600 °C/min, 300 °C (15 min), 50 °C/min, 120 °C, splitless
Detection:	Quadrupole MS, EI in SIM, Source 230 °C, Transfer line 280 °C

Results and Discussion

The VF-35ms column delivered a multi-residue analysis of 57 PAHs, PCBs and OCPs in 32.5 minutes (Figure 1). For PAHs, two pairs are difficult to resolve, namely benzo[b] fluoranthene/benzo[k]fluoranthene and indeno[1,2,3-c,d] pyrene/dibenz[a,h]anthracene. The first pair has the same mass and therefore cannot be separated by MS alone. The second pair have different masses (276 and 278 respectively) and again are difficult to resolve using only MS.

For PCB and OCP groups, PCB 138/PCB 163 and p,p'-DDD/o,p'-DDT have the same mass spectra and cannot be separated by MS. The OCP group also has another pair difficult to resolve, namely cis-heptachlor epoxide and trans-heptachlor epoxide. These compounds both have the main m/z 353 in their mass spectra. Confirmation of both compounds therefore requires an additional m/z.

Figures 2 to 6 show the peak pairs that are difficult to resolve.

Table 1. Peak Identification and SIM ions

Peak	Compound	Ions
1	Naphthalene	128
2	Acenaphthylene	152
3	Acenaphthene	154
4	Fluorene	166
5	Hexachlorobenzene	284, 249
6	α-HCH	181, 219
7	PCB 18	256, 186
8	γ-HCH	181, 219
9	β-HCH	181, 219
10	Phenanthrene	178
11	Anthracene	178
12	PCB 28	256, 186
13	PCB 31	256, 186
14	Heptachlor	272, 100
15	δ-HCH	181, 219
16	PCB 20	256, 186
17	PCB 52	292, 220
18	Aldrin	66, 263
19	PCB 44	292, 220
20	trans-Heptachlor epoxide	353, 81
21	cis-Heptachlor epoxide	81, 183
22	PCB 155	360, 290
23	trans-Chlordane	373, 326
24	PCB 101	326, 254
25	o,p'-DDE	246, 318
26	cis-Chlordane	373, 237
27	Fluoranthene	202
28	Endosulfan I	195, 241
29	p,p'-DDE	246, 318
30	Dieldrin	79, 263
31	Pyrene	202
32	o,p'-DDD	235, 165
33	PCB 118	326, 254
34	PCB 149	360, 290
35	Endrin	263, 81
36	PCB 153	360, 290
37	o,p'-DDT	235, 165
38	p,p'-DDD	235, 165
39	Endosulfan II	241, 195
40	PCB 105	326, 254
41	PCB 163	360, 290
42	PCB 138	360, 290
43	p,p'-DDT	235, 165
44	Endrin-aldehyde	345, 67
45	Endosulfan sulfate	272, 387
46	PCB 180	396, 324
47	Methoxychlor	227
48	Benz[a]anthracene	228
49	Chrysene	228
50	PCB 170	396, 324
51	PCB 194	430, 358
52	Benzo[b]fluoranthene	252
53	Benzo[k]fluoranthene	252
54	Benzo[a]pyrene	252
55	Indeno[1,2,3-c,d]pyrene	276
56	Dibenz[a,h]anthracene	278
57	Benzo[g,h,i]perylene	276

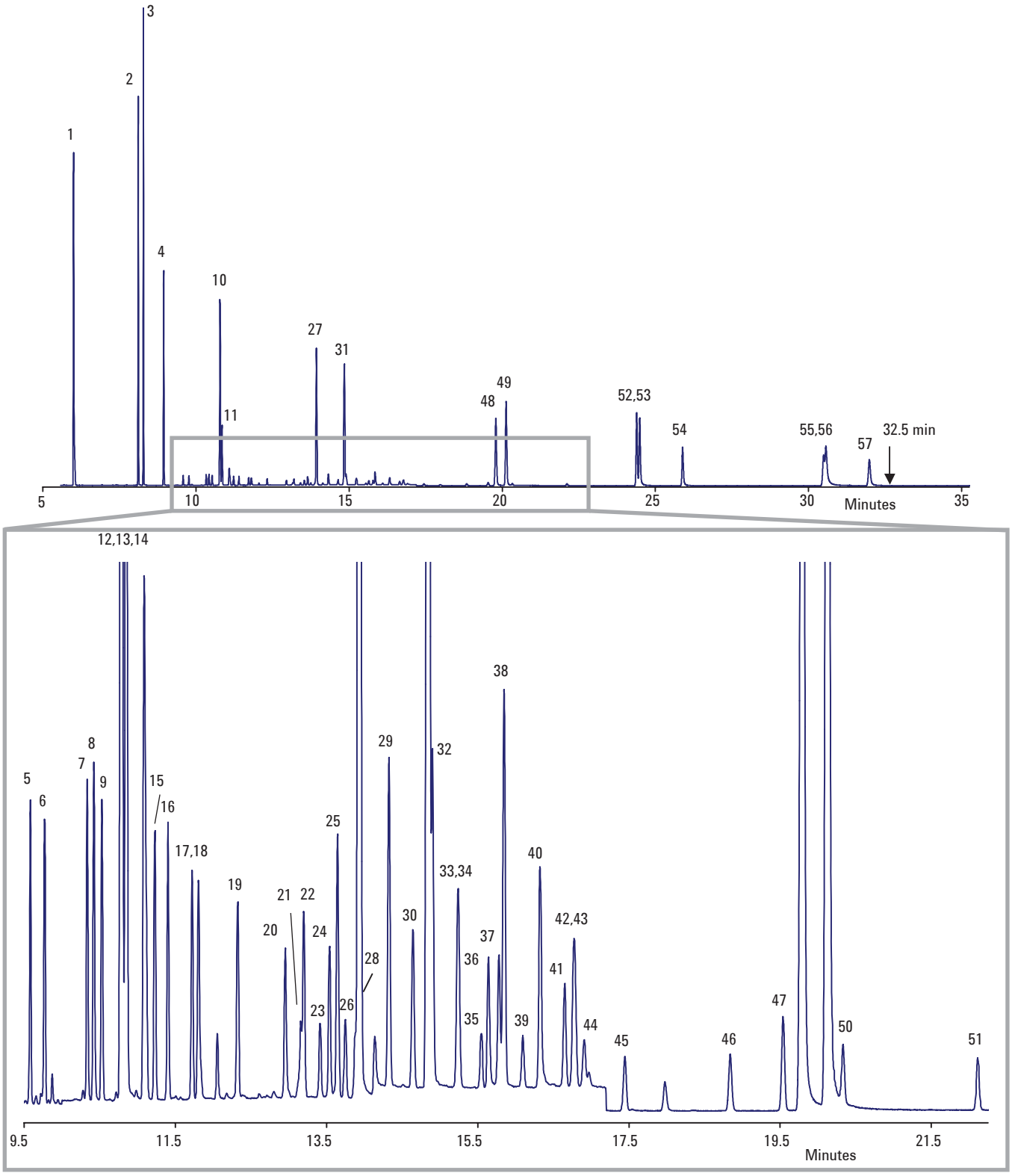


Figure 1. Total ion chromatogram multi-residue analysis on a VF-35ms column.

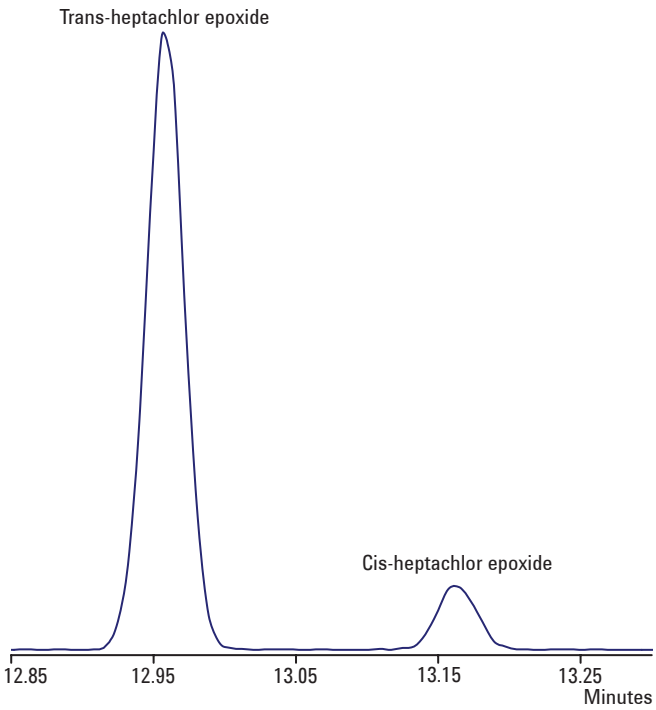


Figure 2. Zoomed total ion chromatogram of cis-heptachlor epoxide / trans-heptachlor epoxide

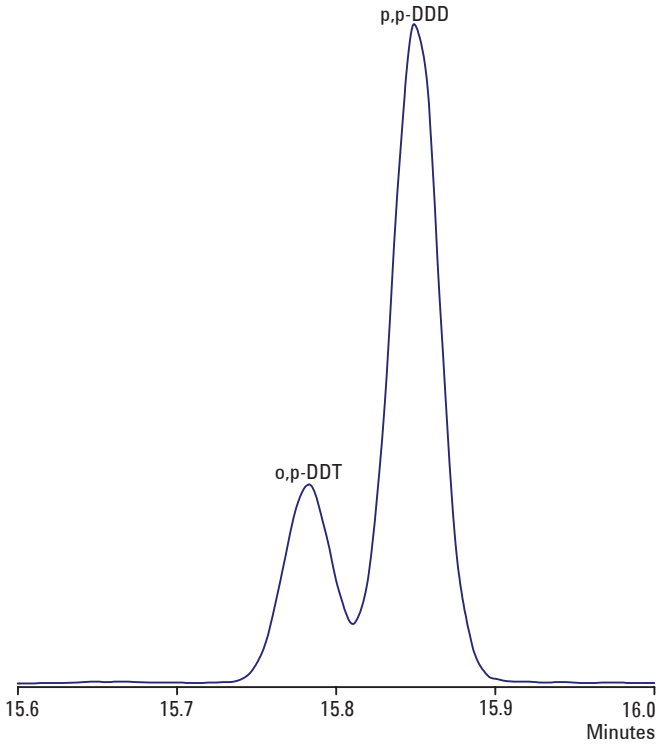


Figure 3. Selected ion chromatogram (m/z 235) of p,p'-DDD / o,p'-DDT

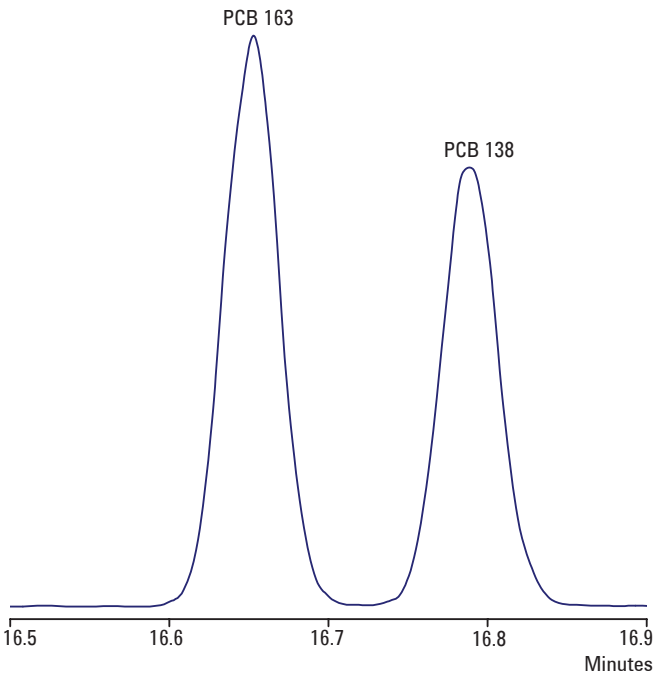


Figure 3. Selected ion chromatogram (m/z 360) of PCB 138/PCB 163

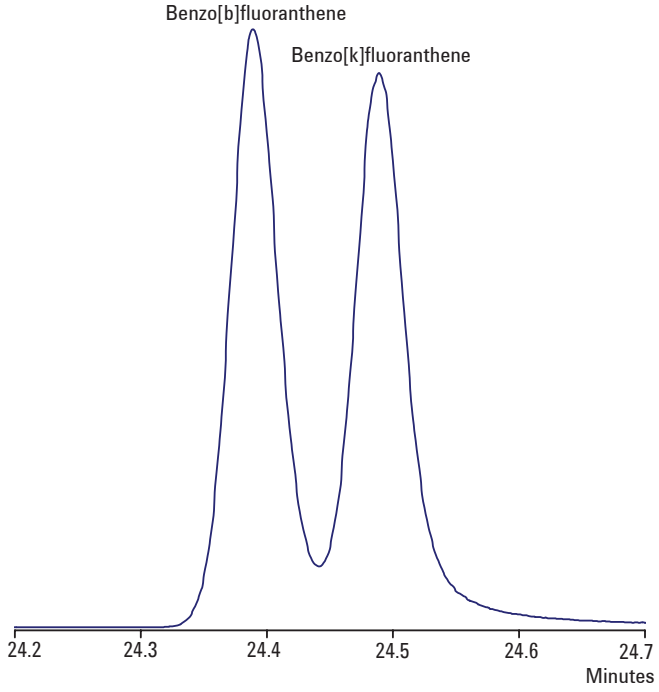


Figure 4. Zoomed total ion chromatogram of benzo[b]fluoranthene / benzo[k]fluoranthene

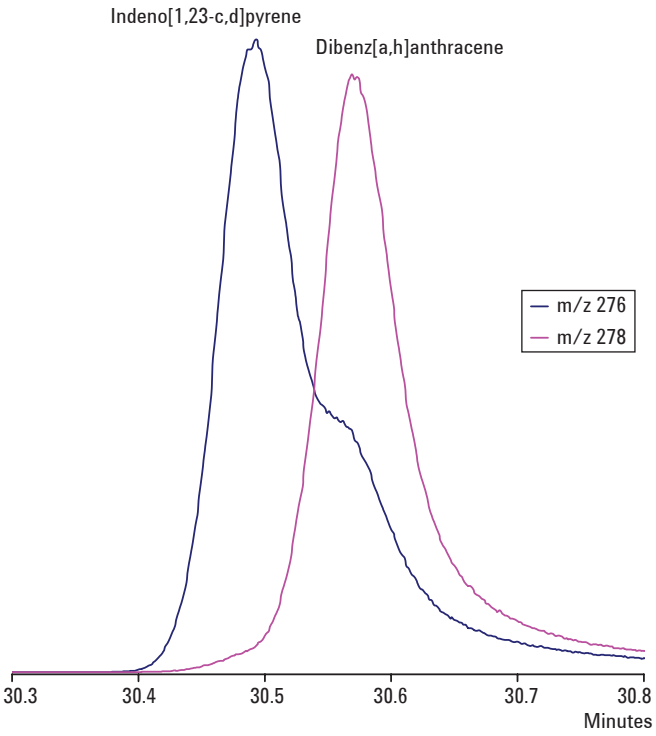


Figure 6. Selected ion chromatogram (m/z 276 and 278) of indeno[1,2,3-c,d]pyrene and dibenz[a,h]anthracene

Conclusion

Analyzing all of these compounds in a single run is normally problematic because every group presents its own difficulties in separation. However, with the VF-35ms column and optimized oven program, multi-residue separation is achieved in about 32 minutes. The VF-35ms column is based on a medium polarity, highly robust and inert phase, making it the ideal choice for demanding trace environmental and chemical analysis.

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Extra Fast Separation of 16 US EPA 610 Regulated PAHs on Agilent J&W Select PAH

Application Note

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Agilent Technologies, Inc.

Introduction

The difficulty in analyzing polycyclic aromatic hydrocarbons (PAHs) is that a number of them have the same mass. This makes their separation with GC/MS problematic, so that column selectivity and an optimized oven program are necessary for their resolution.

PAHs contain two or more aromatic rings and are formed during incomplete combustion or pyrolysis of organic matter, industrial processes, and cooking and food processing. Due to their carcinogenic activities, it is necessary to analyze PAHs in environmental and food samples. However, in PAH analysis, there is a difference between the European (EU) and American (EPA 610) legislation, with the EPA list used for environmental samples and the EU PAHs analyzed in food samples (Table 1). We describe here an optimized oven program for the Select PAH column that separates PAH in less than 7 minutes (Figure 1).

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Conditions

Technique:	GC/MS, Triple Quad
Column:	Select PAH, 15 m x 015 mm, df=0.10 µm (part number CP7461)
Sample:	SRM 1647c, concentration approximately 0.8-21 µg/mL (www.nist.com)
Injection Volume:	1 µL
Temperature:	70 °C (0.40 min), 120 °C/min, 180 °C, 40 °C/min, 350 °C (5 min)
Carrier Gas:	Helium, constant flow 1.2 mL/min
Injector:	300 °C, Splitless mode, 0.25 min @ 100 mL/min
Detector:	Triple Quad, EI in FS or SIM mode, ion source 275 °C, transfer line 300 °C

Results and Discussion

Conditions were optimized to give a fast separation of the 16 EPA PAHs. The most important isomers to resolve were phenanthrene and anthracene (m/z 178), benzo[b and k] fluoranthene (m/z 252), and indeno[1,2,3-c,d]pyrene and dibenzo[a,h]anthracene with m/z 276 and 278.

Table 1. Peak identification for Figure 1

Peak	MW	Compound	EPA 610	SFC1 and EFSA2 PAHs (15+1)	CAS
1	128	Naphthalene	x		91-20-3
2	152	Acenaphthylene	x		208-96-8
3	154	Acenaphthene	x		83-32-9
4	166	Fluorene	x		86-73-7
5	178	Phenanthrene	x		85-01-8
6	178	Anthracene	x		120-12-7
7	202	Fluoranthene	x		206-44-0
8	202	Pyrene	x		129-00-0
9	228	Benzo[a]anthracene	x	x	56-55-3
10	228	Chrysene	x	x	218-01-9
11	252	Benzo[b]fluoranthene	x	x	205-99-2
12	252	Benzo[k]fluoranthene	x	x	207-08-9
13	252	Benzo[a]pyrene	x	x	50-32-8
14	278	Dibenzo[a,h]anthracene	x	x	53-70-3
15	276	Indeno[1,2,3-c,d]pyrene	x	x	193-39-5
16	276	Benzo[g,h,i]perylene	x	x	191-24-2

[1] Scientific Committee on Food, one of the committees providing the European Commission with scientific advice on food safety. [2] European Food Safety Authority.

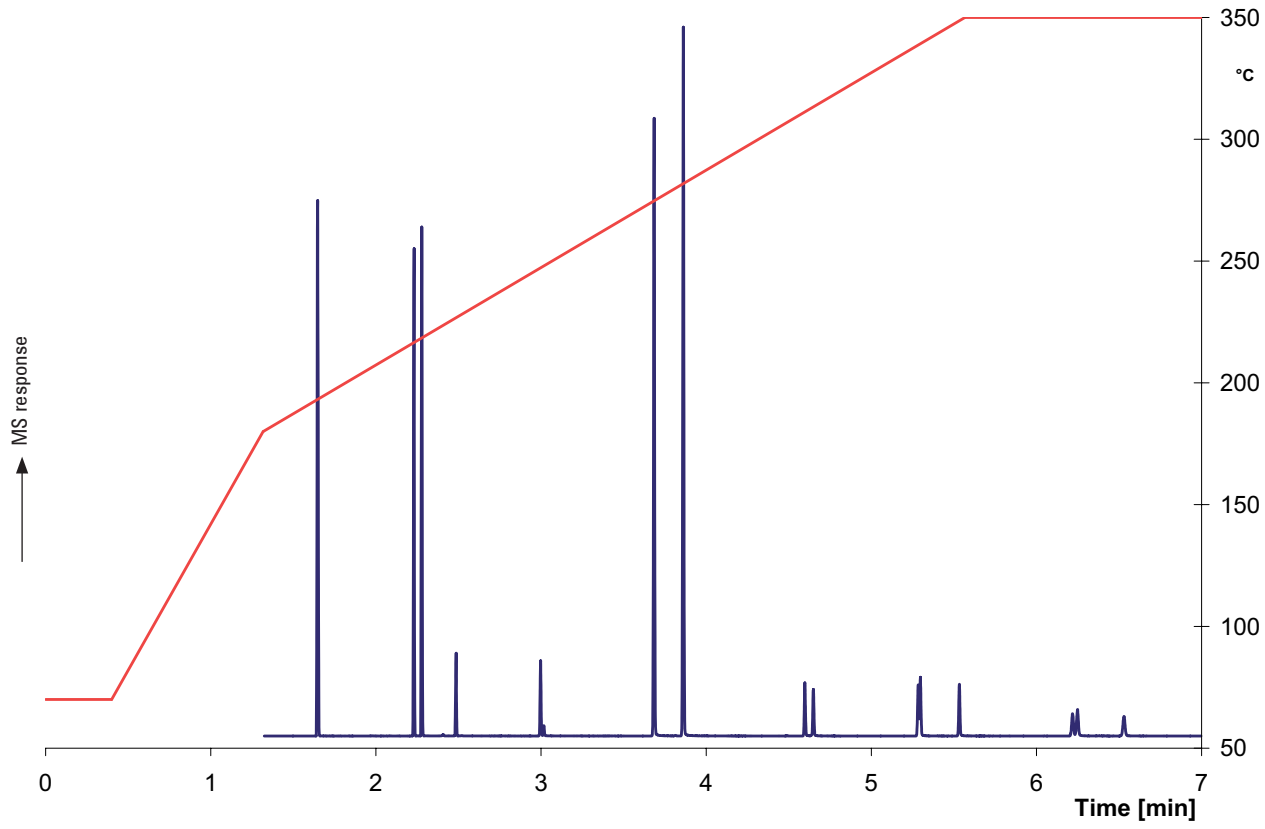


Figure 1. Fast GC/MS analysis of 16 US EPA PAHs on Select PAH

Table 2. Peak Identification for Figure 2

Peak	MW	Compound	EPA 610	CAS
1	128	Naphthalene	x	91-20-3
2	152	Acenaphthylene	x	208-96-8
3	154	Acenaphthene	x	83-32-9
4	166	Fluorene	x	86-73-7
5	178	Phenanthrene	x	85-01-8
6	178	Anthracene	x	120-12-7
7	202	Fluoranthene	x	206-44-0
8	202	Pyrene	x	129-00-0
9	228	Benzo[a]anthracene	x	56-55-3
10	228	Chrysene	x	218-01-9

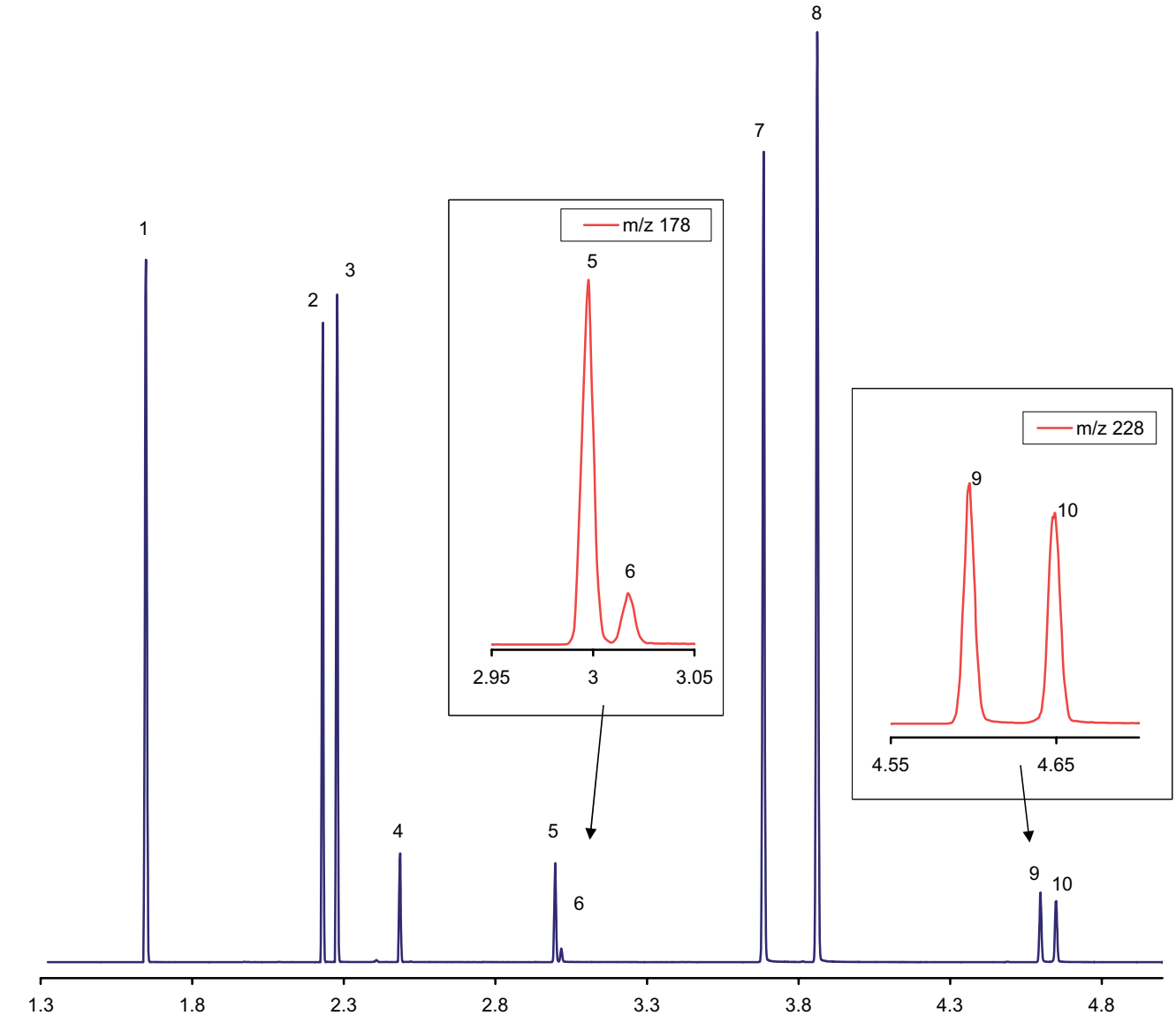


Figure 2. Details and peak identification, peak numbers 1 to 10

Table 3. Peak Identification for Figure 3

Peak	MW	Compound	EPA 610	CAS
11	252	Benzo[b]fluoranthene	x	205-99-2
12	252	Benzo[k]fluoranthene	x	207-08-9
13	252	Benzo[a]pyrene	x	50-32-8
14	278	Dibenz[a,h]anthracene	x	53-70-3
15	276	Indeno[1,2,3-c,d]pyrene	x	193-39-5
16	276	Benzo[g,h,i]perylene	x	191-24-2

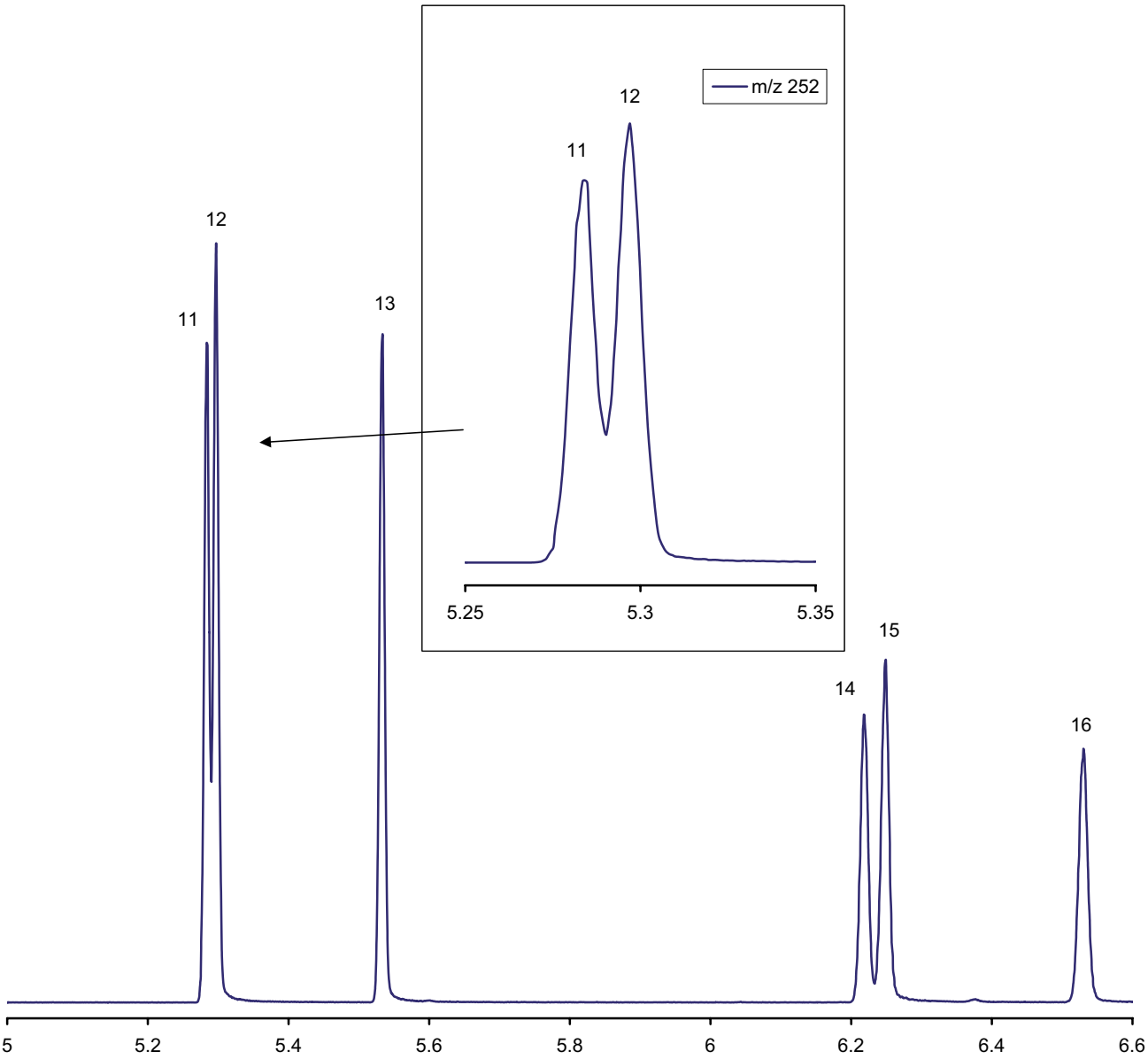


Figure 3. Details and peak identification, peak numbers 11 to 16

Conclusion

With the Select PAH GC column and the optimized oven program described here, all 16 EPA PAHs were resolved in a single run with a runtime of less than 7 minutes.

The Select PAH column also offers enhanced resolution of PAHs, preventing co-elution of interfering PAHs that can cause false positives and inaccurate results. Typical interferences are triphenylene on chrysene, and benzo[j] fluoranthene on benzo[k]fluoranthene. Application note SI-02259 describes the separation of 54 PAHs, including the 16 EPA PAHs and their interferences.

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SI-02282

Agilent Technologies

Separation of EU and EPA Regulated PAHs on Agilent J&W FactorFour VF-17ms Column

Application Note

Author

Laura Provoost
Agilent Technologies, Inc.

Introduction

The difficulty in analyzing polycyclic aromatic hydrocarbons (PAHs) is the number of PAHs with the same mass. This makes the separation of these PAHs with GC/MS rather difficult, and column selectivity and an optimized oven program are necessary to resolve these PAHs. In this application note an optimized oven program for the FactorFour VF-17ms column is described.

PAHs are compounds which contain two or more aromatic rings. They are formed during incomplete combustion or pyrolysis of organic matter, industrial processes and cooking and food processing. PAHs are therefore analyzed in both environmental and food related matrices.

In PAH analysis there is a difference between the European (EU) and American (EPA) legislation. These regulatory bodies both describe a different set of PAHs (Table 1). The EPA list is especially used for environmental samples while the EU PAHs are analyzed in food samples. This application note shows a GC/MS method which resolves all EU and EPA PAHs with the VF-17ms column (Figure 1).

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Conditions

Technique:	GC/MS
Column:	VF-17ms, 30 m x 0.25 mm, df = 0.15 µm (part number CP8981)
Temperature:	70 °C (1 min), 40 °C/min, 180 °C, 2.5 °C/ min, 245 °C, 2.0 °C/min, 270 °C (10 min), 8.0 °C/min, 350 °C (5 min)
Carrier Gas:	Helium, constant flow 1 mL/ min
Injector:	300 °C, Splitless mode
Detector:	Quadrupole MS, EI in SIM, ion source 275 °C, transfer line 300 °C
Sample:	Concentration approx. 1 µg/ mL
Injection Volume:	1 µL

Table 1. Peak Identification for Figure 1

Peak	Compound	MW	EU PAH	EPA PAH
1	Naphthalene	128		X
2	Acenaphthylene	152		X
3	Acenaphthene	154		X
4	Fluorene	166		X
5	Phenanthrene	178		X
6	Anthracene	178		X
7	Fluoranthene	202		X
8	Pyrene	202		X
9	Benzo(c)fluorene	216	X	
10	Benz(a)anthracene	228	X	X
11	Cyclopenta(c,d) pyrene	226	X	
12	Triphenylene	228		
13	Chrysene	228	X	X
14	6-Methylchrysene	242		
15	5-Methylchrysene	242	X	
16	Benzo(b) fluoranthene	252	X	X
17	Benzo(k) fluoranthene	252	X	X
18	Benzo(j)fluoranthene	252	X	
19	Benzo(a)pyrene	252	X	X
20	Indeno(1,2,3-cd) pyrene	276	X	X
21	Benzo(b) triphenylene	278		
22	Dibenz(a,h) anthracene	278	X	X
23	Benzo(g,h,i)perylene	276	X	X
24	Dibenzo(a,l)pyrene	302	X	
25	Dibenzo(a,e)pyrene	302	X	
26	Dibenzo(a,i)pyrene	302	X	
27	Dibenzo(a,h)pyrene	302	X	

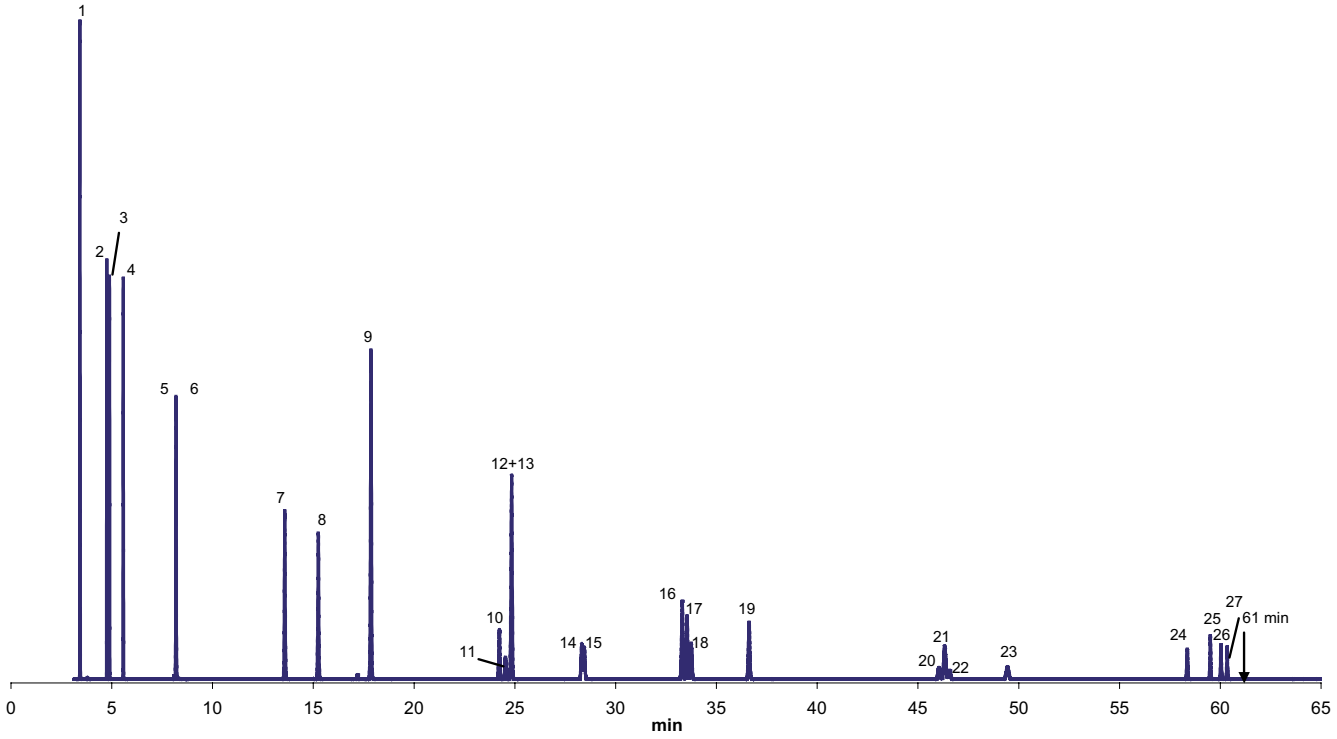


Figure 1. GC/MS analysis of EU and EPA PAHs on VF-17ms 30 m x 0.25 mm x 0.15 µm

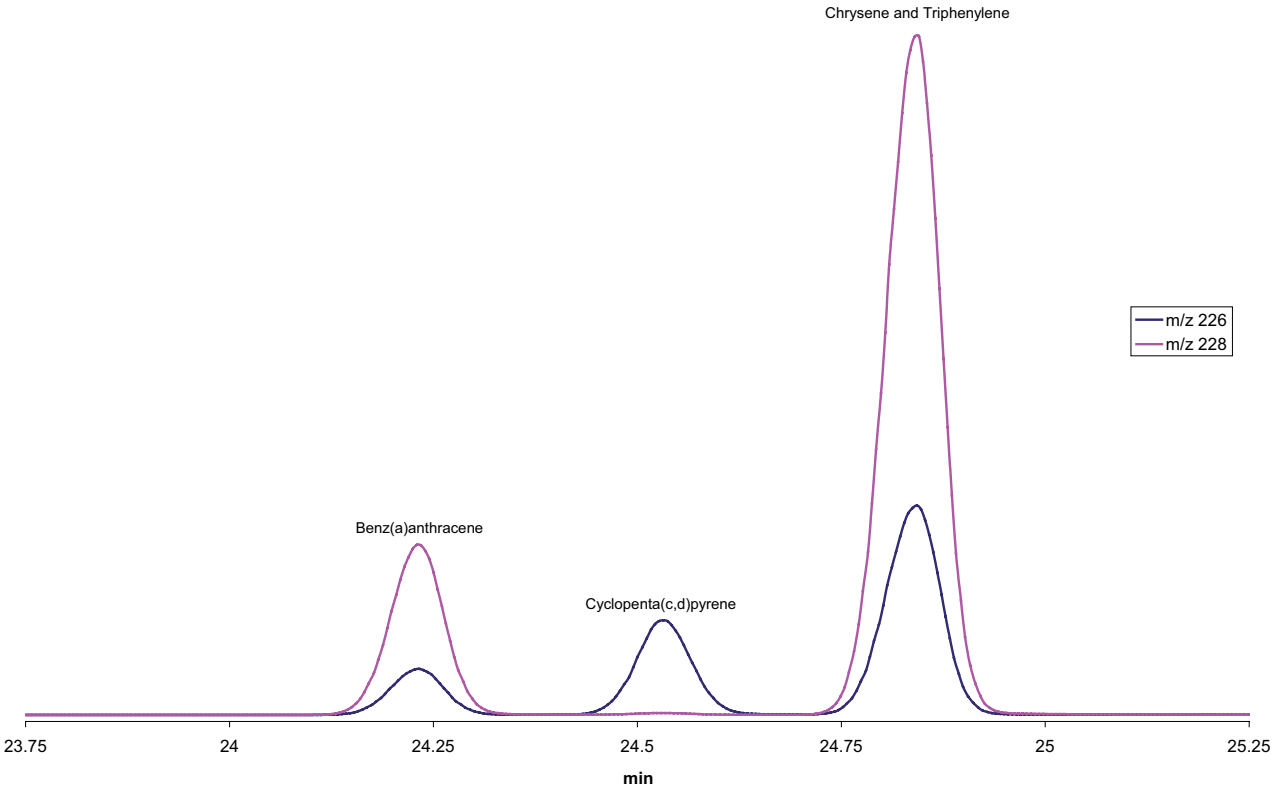


Figure 2. Separation of m/z 226 and 228

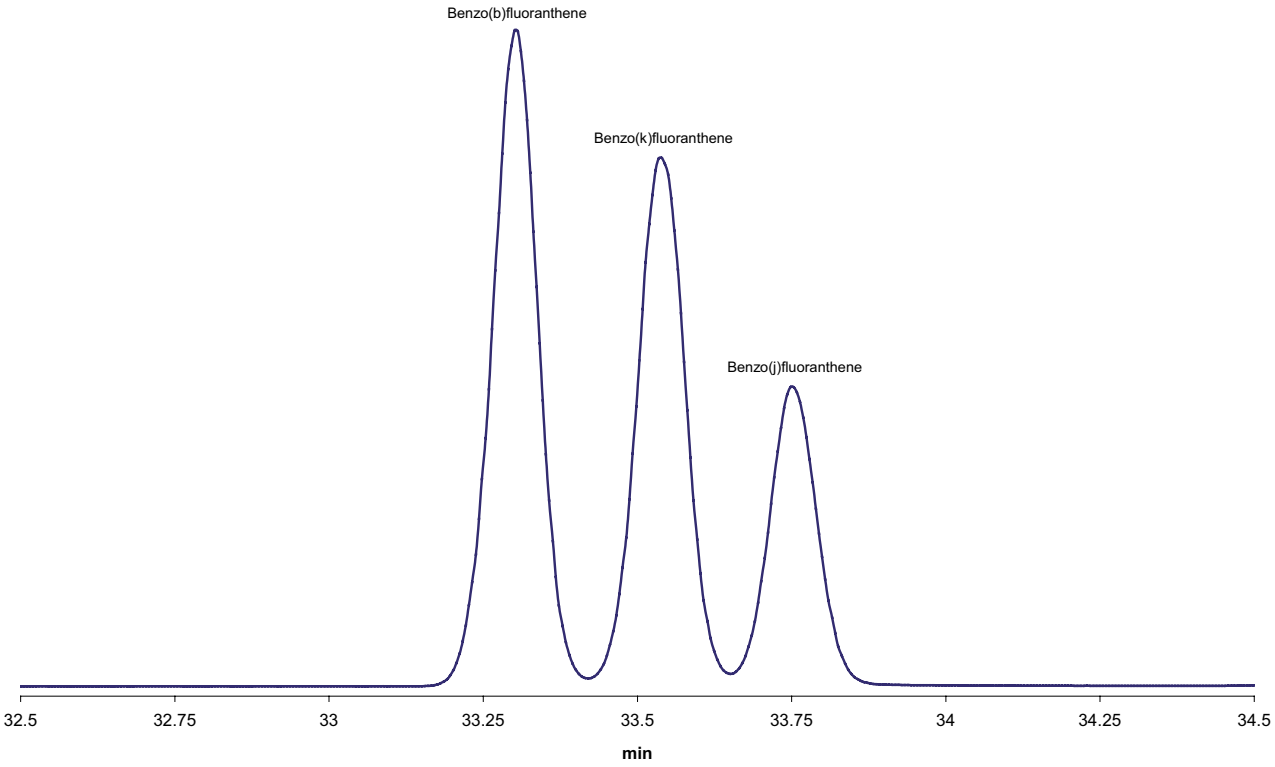


Figure 3. Separation of Benzofluoranthenes (m/z 252)

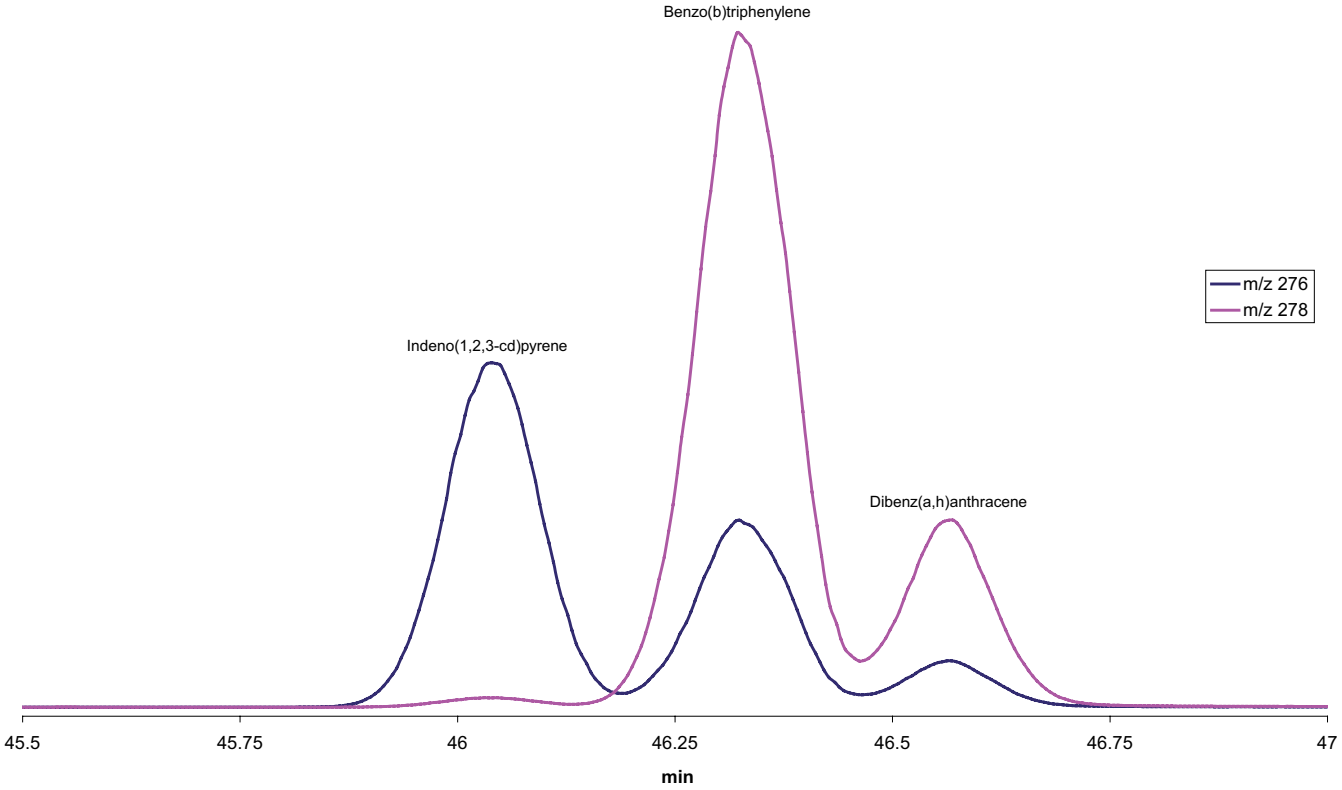


Figure 4. Separation of m/z 276 and 278

Results and Discussion

When performing this analysis there are 3 sets of peaks which are difficult to resolve. The first set, Benz(a) anthracene, Cyclopenta(c,d)pyrene, Chrysene and Triphenylene, has different masses, m/z 226 and 228. The compounds with m/z 228 also contain some m/z 226 and this makes it difficult to resolve this set only with MS (Figure 2). The same problem occurs when separating Indendo(1,2,3-cd)pyrene, Benzo(b)triphenylene and Dibenz(a,h)anthracene with m/z 276 and 278 (Figure 4).

The third set of PAHs which are difficult to resolve are the Benzofluoranthene isomers. These three isomers, Benzo(b) fluoranthene, Benzo(j)fluoranthene and Benzo(k)fluoranthene, have the same mass and can't be resolved with MS only (Figure 3).

For the above reasons the column has to resolve these components chromatographically which is showed in the figures.

Conclusion

The VF-17ms column phase as well as the GC oven program affect the separation of the difficult to resolve PAH sets. With the optimized oven program described in this application note all EU and EPA PAHs are resolved. It should be taken in mind that Triphenylene can cause a false positive because of the co-elution with Chrysene.

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Benefit of Low Temperature ELSD for the Analysis of Herbicides by HPLC

Stephen Bullock
Polymer Laboratories, now a part of Varian, Inc.

Introduction

Phenylurea compounds are used extensively in agriculture as selective herbicides, for pre- and post-emergence, by inhibiting photosynthesis. They are easily taken up from soil solution by the root systems of plants and rapidly translocated into stems and leaves by the transpiration system. Common phenylurea herbicides, such as diuron, have a relatively low tendency to adsorb to soils and sediments, while its degradation products have a relatively long lifetime. Consequently, the mobility and relative persistence of these herbicides increases the chance of migration to ground water via leaching and surface run-off. Developing analytical methods for the analysis of phenylurea herbicides and their metabolites in surface and ground water is vital to the understanding of herbicide fate and degradation pathways in the environment.

Unlike the majority of pesticides, the application of gas chromatography (GC) to the analysis of phenylurea herbicides is difficult because they are thermally unstable and degrade rapidly to isocyanates and amines[1]. In addition, thermal reactions in the GC detector and on column result in a lack of reproducibility preventing the quantitative screening of the degradation products. A better approach for thermally sensitive compounds is to use HPLC, coupled with evaporative light scattering detection.

Evaporative light scattering detectors respond to all compounds that are less volatile than the mobile phase. In the past, high ELSD operating temperatures were required to fully evaporate aqueous eluents, making the detection of semi-volatile analytes difficult or even impossible.

The Varian ELS detector is designed to evaporate highly aqueous mobile phases at ambient temperature. This is achieved by introducing a stream of dry nitrogen during the evaporation stage, which increases the vapor loading capacity of the surrounding gas and enables complete evaporation of the eluent to take place without raising the temperature. The advantage of characterizing samples at ambient temperature is demonstrated by the analysis of a mixture of ten phenylurea herbicides.

Instrumentation

Column: C18 5 µm, 250 x 4.6 mm
Detection: Varian ELSD (neb=25 °C, evap as shown, gas=1.6 SLM)

Materials and Reagents

Eluent A: Water
Eluent B: ACN

Sample Preparation

Sample: Phenylurea herbicide mixture

Conditions

Flow Rate: 0.7 mL/min
Injection Volume: 20 µL
Gradient: 10-80 % B in 40 min

Results and Discussion

The herbicide mixture contains compounds of different volatilities and the highly volatile herbicides are not detected as the ELSD temperature is increased. Only seven of the ten herbicides are detected at 70 °C, compared to all ten at 25 °C. Therefore, by operating the Varian ELS detector at ambient temperature the loss of semi-volatile components is minimized, the sample integrity is preserved and maximum sensitivity is achieved, as shown in Figure 1.

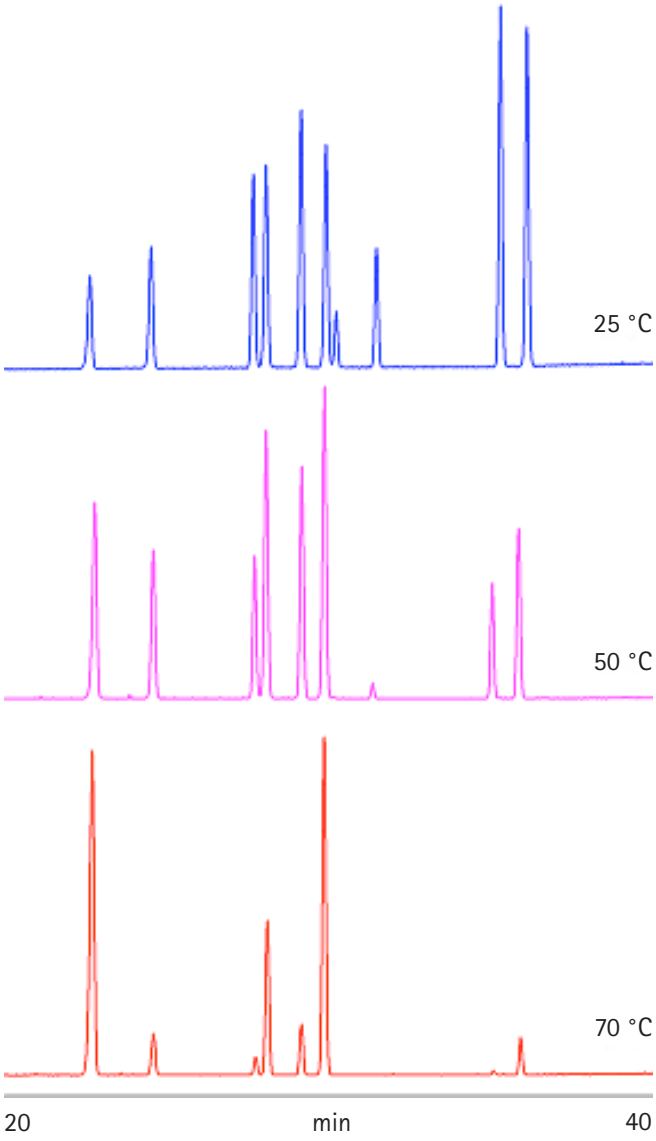


Figure 1. Varian ELSD reveals the presence of semi-volatile herbicides when operated at ambient temperature.

Conclusion

Separation and identification of semi-volatile compounds is straightforward using evaporative light scattering detection at the ambient temperatures available with the Varian ELS detector. The Varian ELS detector surpasses other ELSDs for low temperature HPLC applications with semi-volatile compounds. Its innovative design represents the next generation of ELSD technology, providing optimum performance across a diverse range of HPLC applications. The Varian ELS detector's unique gas control permits evaporation of high boiling solvents at very low temperatures. For example, 100 % water at a flow rate of 5 mL/min can be removed at 30 °C. The novel design of the Varian ELS detector provides superior performance compared to detectors from other vendors for the analysis of semi-volatile compounds.

References

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Application Note SI-01229 / TB1064

Fast analysis of coal tar polycyclic aromatic hydrocarbons on Agilent J&W Select PAH

Application Note

Author

John Oostdijk
Agilent Technologies, Inc.

Introduction

The difficulty in analyzing polycyclic aromatic hydrocarbons (PAHs) is the number of PAHs with the same mass. This makes their separation by GC/MS rather difficult, and so column selectivity and an optimized oven program are necessary for the resolution of PAHs. We describe here the fast analysis of a coal tar sample using an optimized oven program and a 15 m x 0.15 mm x 0.10 µm Select PAH column.

Coal tar is a brown or black liquid of high viscosity that smells of naphthalene and aromatic hydrocarbons. It is obtained from the destructive distillation of coal. In the past, coal tar was sourced as a by-product from the manufacture of coal gas but is now produced during the production of coke for steel making. The crude tar contains many organic compounds, such as benzene, naphthalene, methylbenzene and phenols, which can be obtained by distillation, leaving a residue of pitch. At one time coal tar was the major source of organic chemicals, most of which are now derived from petroleum and natural gas. Coal tar pitch is mainly used as binding agent in the production of carbon electrodes, anodes and Søderberg electrodes, for instance, by the aluminium industry. It is also used as a binding agent for refractories, clay pigeons, active carbon, coal briquetting, road construction and roofing. In addition, small quantities are used for heavy-duty corrosion protection.

The standard reference material for coal tar analysis (SRM 1597a, NIST) is a natural, combustion-related mixture of PAHs from a medium crude coke-oven tar that is dissolved in toluene. It is intended for use in the evaluation and validation of analytical methods for the determination of PAHs. A sample of SRM 1597a was therefore analyzed using an optimized oven program for the Select PAH column, demonstrating the excellent performance of the column with this reference material.

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Conditions

Technique:	GC/MS, Triple Quad
Column:	Select PAH, 15 m x 0.15 mm, df = 0.10 µm (part number CP7461)
Sample:	SRM 1597a, concentration approximately 0.2-1030 mg/kg (www.nist.gov)
Injection Volume:	1.0 µL SIM mode, 0.1 µL FS mode
Temperature:	70 °C (0.40 min), 70 °C/min, 180 °C
Program:	7 °C/min, 230 °C (7 min), 50 °C/min, 280 °C (7 min), 30 °C/min, 350 °C (4 min)
Carrier Gas:	Helium, constant flow 1.2 mL/min
Injection:	300 °C, Splitless mode, 0.5 min @ 100 mL/min
Detection:	Triple Quad, EI in FS or SIM mode, ion source 275 °C, transfer line 300 °C

Results and Discussion

The sample was analyzed directly in full scan and SIM mode using the same conditions as for a standard with regulated and interfering PAHs. Chromatograms with peak identifications are shown in Figures 1 to 3. Figure 1 shows chrysene (66 mg/kg) separated from triphenylene (12 mg/kg). Benzo[b,k,j]fluoranthene (66, 37 and 41 mg/kg) was separated from benzo[a]pyrene (94 mg/kg). The MS spectrum from chrysene is also shown. As well as m/z 228, other ions, such as m/z 226 and m/z 229, were formed. PAHs were mostly very stable and only low fragment ions were observed, for example m/z 113 and 114 in the mass spectrum.

Table 1. Peak identification for Figure 1

Peak	Name
BaA	Benzo[a]anthracene
BaP	Benzo[a]pyrene
BbFA	Benzo[b]fluoranthene
BkFa	Benzo[k]fluoranthene
BjFA	Benzo[j]fluoranthene
CHR	Chrysene
CPP	Cyclopenta[c,d]pyrene
TP	Triphenylene

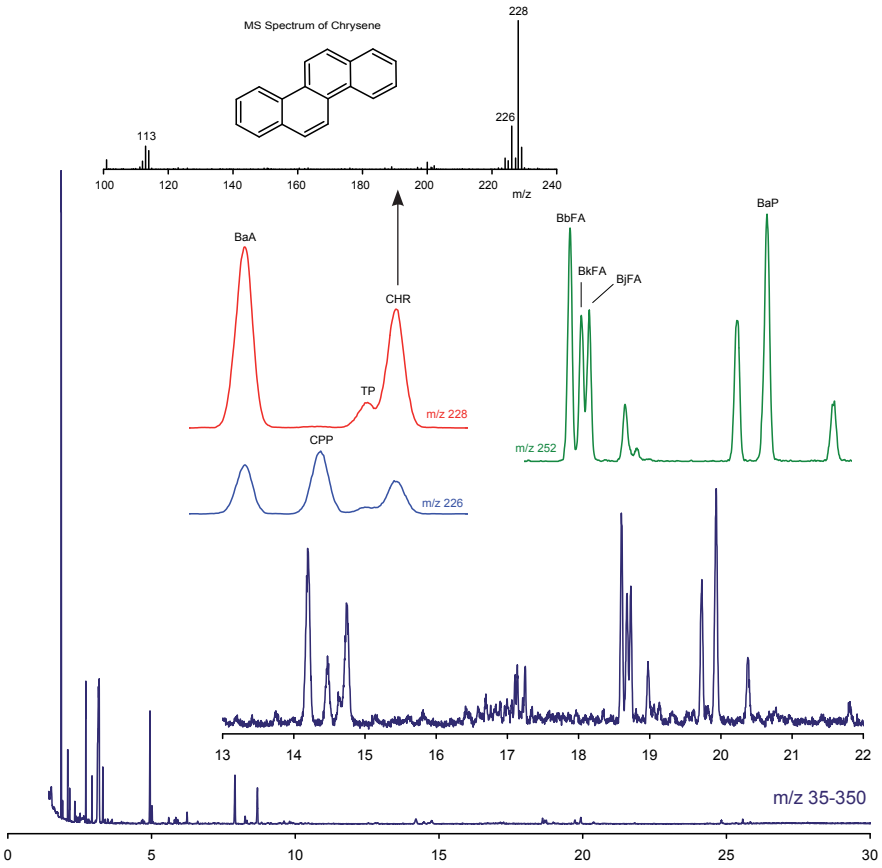


Figure 1. GC/MS analysis of 0.1 µL of SRM 1597a in full scan mode (m/z 35-350) on Select PAH

Table 2. Peak identification for Figure 2

Peak	Name
ACL	Acenaphthylene (263 mg/kg)
BaA	Benzo[a]anthracene (98 mg/kg)
BaP	Benzo[a]pyrene (94 mg/kg)
BghiP	Benzo[g,h,i]perylene (51 mg/kg)
DBaeP	Dibenzo[a,e]pyrene (9 mg/kg)
FA	Fluoranthene (327 mg/kg)
PHE	Phenanthrene (454 mg/kg)
PY	Pyrene (240 mg/kg)
NA	Naphthalene (1030 mg/kg)

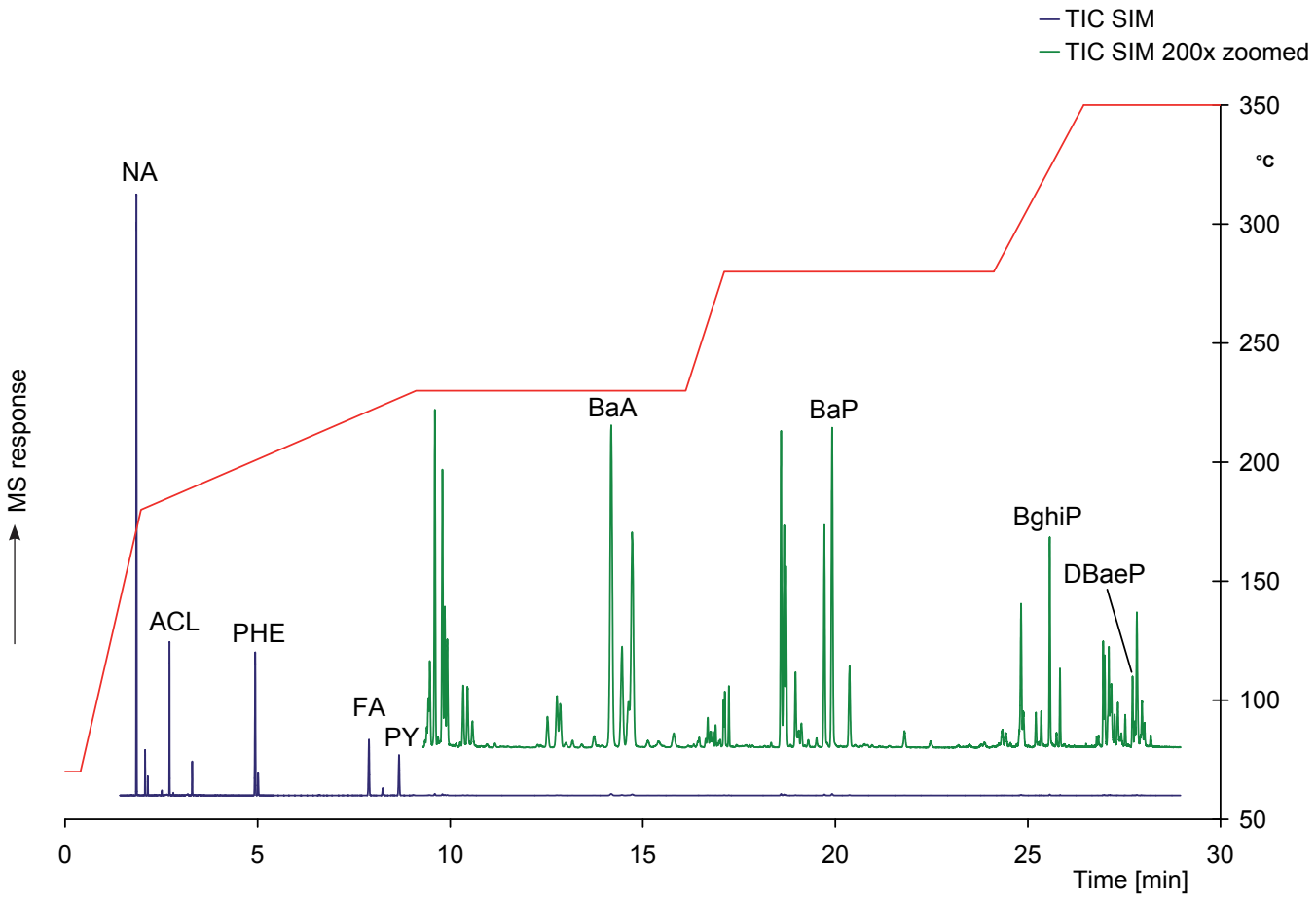


Figure 2. GC/MS analysis of 1 µL of SRM 1597a in SIM mode on Select PAH. The final part of the chromatogram was enlarged because of the large concentration difference in the sample

Table 3. Peak identification for Figure 3

Peak	Name
ATR	Anthanthrene (Dibenzo[d,e,f,m,n,o] chrysene)
BaFA	Benzo[a]fluoranthene
BaFL	Benzo[a]fluorene
BaP	Benzo[a]pyrene
BbCHR	Benzo[b]chrysene
BbFA	Benzo[b]fluoranthene
BbFL	Benzo[b]fluorene
BbNTP	Benzo[b]naphto[2,1-d]thiophene

Peak	Name
BbPer	Benzo[b]peryelene
BbTP	Benzo[b]triphenylene
BcFL	7H-benzo[c]fluorene
BeP	Benzo[e]pyrene
BghiP	Benzo[g,h,i]perylene
BjFA	Benzo[j]fluoranthene
BkFA	Benzo[k]fluoranthene
Cor	Coronene
DBaeP	Dibenzo[a,e]pyrene

Peak	Name
DBahA	Dibenzo[a,h]anthracene
DBahP	Dibenzo[a,h]pyrene
DBaiP	Dibenzo[a,i]pyrene
DBalP	Dibenzo[a,l]pyrene
IP	Indeno[1,2,3-cd]pyrene
6MC	6-Methylchrysene
5MC	5-Methylchrysene
Per	Perylene
Pic	Picene

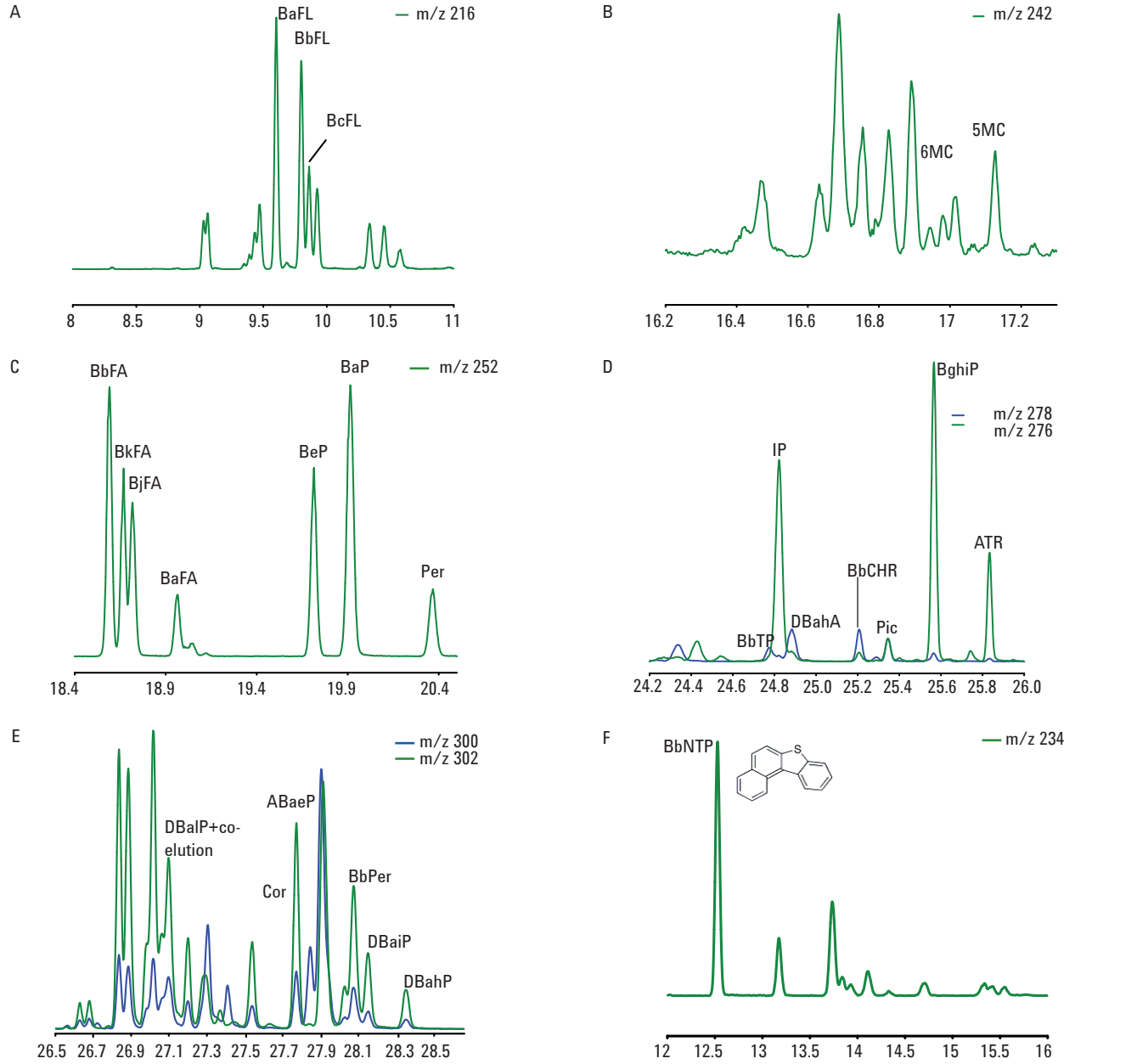


Figure 3. GC/MS analysis of 1 µL of SRM 1597a in SIM mode on Select PAH . Some examples of critical separations are shown: A. Benzofluorenes, B. Methylchrysenes, C. Benzofluoranthenes and isomers, D. Dibenzoanthracenes and isomers, E. Dibenzopyrenes and isomers, F. Benzo[b]naphtho[ ] thiophene isomers

Conclusion

The Select PAH column separated target PAHs in a complex mixture of coal tar in a single run with a run time of 29 min. Together with an optimized oven program, this unique column is the only easy-to-use, single-shot solution that separates all regulated PAH isomers, with no false positives or inaccurate results. With high speed, high resolution and high temperature stability it is the perfect column for accurate PAH analysis.

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Agilent Technologies

Fast Separation of 16 US EPA 610 Regulated PAHs on Agilent J&W Select PAH GC Columns

Application Note

Author

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Agilent Technologies, Inc.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are compounds that contain two or more aromatic rings. They are formed during incomplete combustion or pyrolysis of organic matter, industrial processes, and cooking and food processing. PAHs are therefore analyzed in environmental and food samples. The difficulty in determining PAHs is that several of their isomers have the same mass. This makes the separation of PAHs with GC/MS rather difficult, and so column selectivity and an optimized oven program are necessary for their resolution. This application note describes the interference-free analysis of 16 PAHs listed in EPA 610 using the J&W Select PAH GC column.

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Conditions

Technique:	GC/MS, Triple Quad
Column:	Select PAH, 30 m x 0.25 mm, df = 0.15 µm (part number CP7462)
Sample:	SRM 1647c, concentration appr. 0.8-21 µg/mL (www.nist.com)
Injection Volume:	1 µL
Temperature:	70 °C (0.80 min), 60 °C/min, 180 °C, 20 °C/min 350 °C (5 min)
Carrier Gas:	Helium, constant flow 2.0 mL/min
Injector:	300 °C, Splitless mode, 0.75 min @ 50 mL/min
Detector:	Triple Quad, EI in SIM mode, ion source 275 °C, transfer line 300 °C

Results and Discussion

When performing this analysis, the conditions are optimized to give a fast separation of the 16 EPA PAHs. The most important isomers to resolve are phenanthrene and anthracene (m/z 178), benzo[b and k]fluoranthene (m/z 252), and indeno[1,2,3-c,d]pyrene and dibenz[a,h]anthracene with m/z 276 and 278. These compounds are all resolved by the Select PAH column, as shown in Figure 1. Figures 2 and 3 provide detailed information on the 16 EPA PAHs.

Table 1. Peak identification for Figure 1

Peak	MW	Compound	CAS
1	128	Naphthalene	91-20-3
2	152	Acenaphthylene	208-96-8
3	154	Acenaphthene	83-32-9
4	166	Fluorene	86-73-7
5	178	Phenanthrene	85-01-8
6	178	Anthracene	120-12-7
7	202	Fluoranthene	206-44-0
8	202	Pyrene	129-00-0
9	228	Benz[a]anthracene	56-55-3
10	228	Chrysene	218-01-9
11	252	Benzo[b]fluoranthene	205-99-2
12	252	Benzo[k]fluoranthene	207-08-9
13	252	Benzo[a]pyrene	50-32-8
14	278	Dibenz[a,h]anthracene	53-70-3
15	276	Indeno[1,2,3-c,d]pyrene	193-39-5
16	276	Benzo[g,h,i]perylene	191-24-2

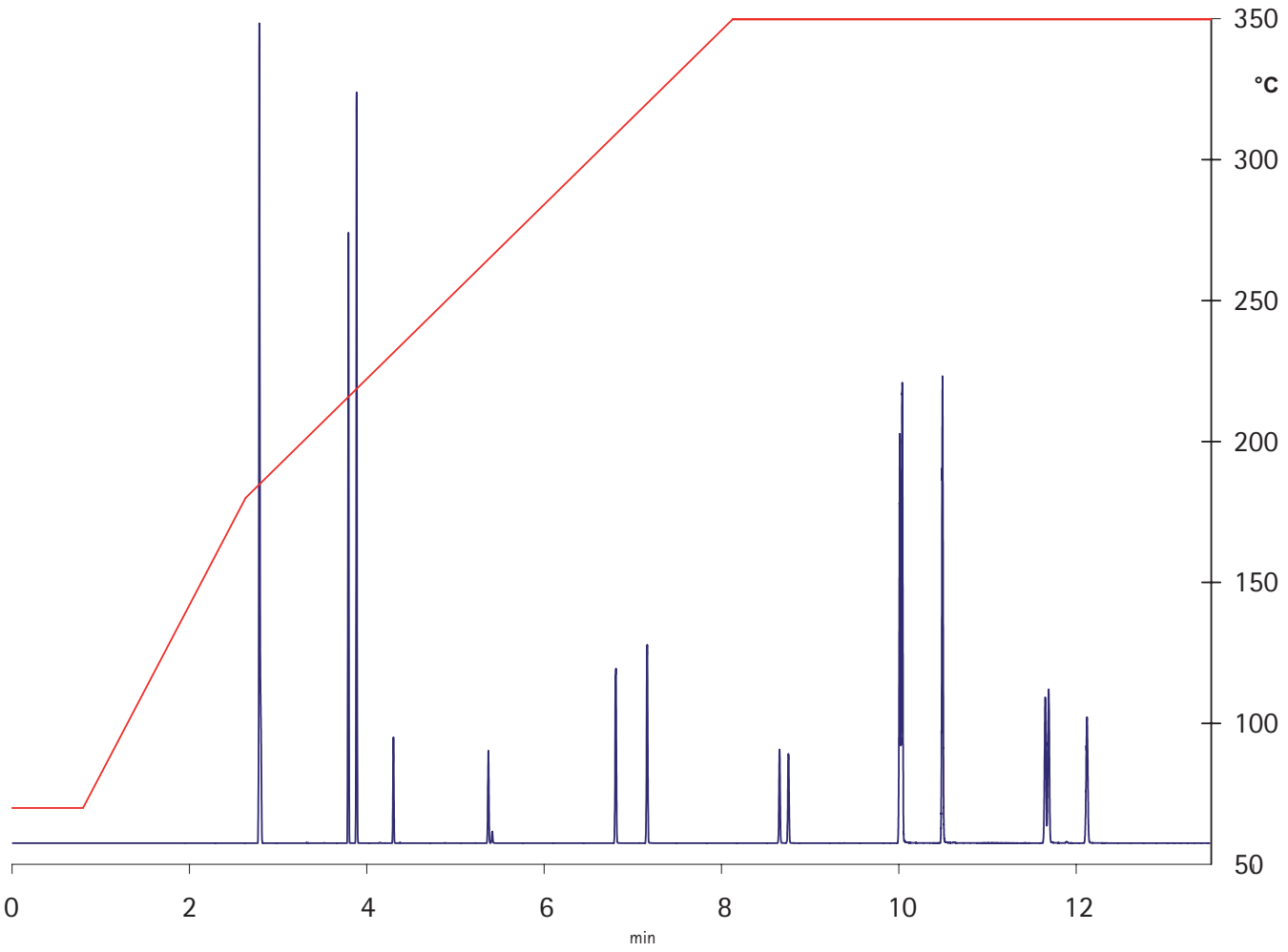


Figure 1. Fast GC/MS analysis of 16 US EPA PAHs on Select PAH, 30 m x 0.25 mm x 0.15 µm

Table 2. Peak Identification for Figure 2

Peak	MW	Compound	CAS
1	128	Naphthalene	91-20-3
2	152	Acenaphthylene	208-96-8
3	154	Acenaphthene	83-32-9
4	166	Fluorene	86-73-7
5	178	Phenanthrene	85-01-8
6	178	Anthracene	120-12-7
7	202	Fluoranthene	206-44-0
8	202	Pyrene	129-00-0
9	228	Benz[a]anthracene	56-55-3
10	228	Chrysene	218-01-9

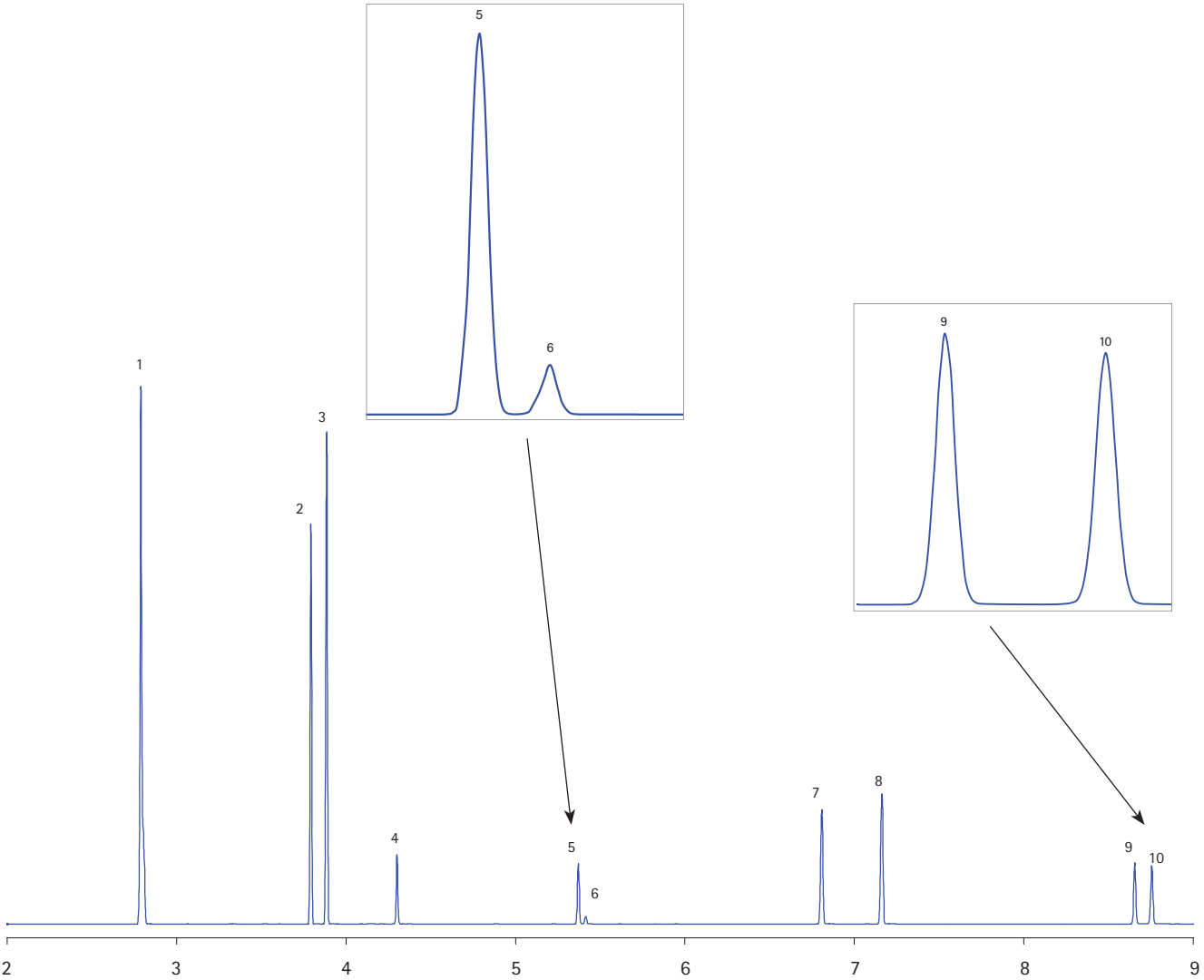


Figure 2. Details and identification of PAH peaks 1 –10

Table 3. Peak Identification for Figure 3

Peak	MW	Compound	CAS
11	252	Benzo[b]fluoranthene	205-99-2
12	252	Benzo[k]fluoranthene	207-08-9
13	252	Benzo[a]pyrene	50-32-8
14	278	Dibenz[a,h]anthracene	53-70-3
15	276	Indeno[1,2,3-c,d]pyrene	193-39-5
16	276	Benzo[g,h,i]perylene	191-24-2

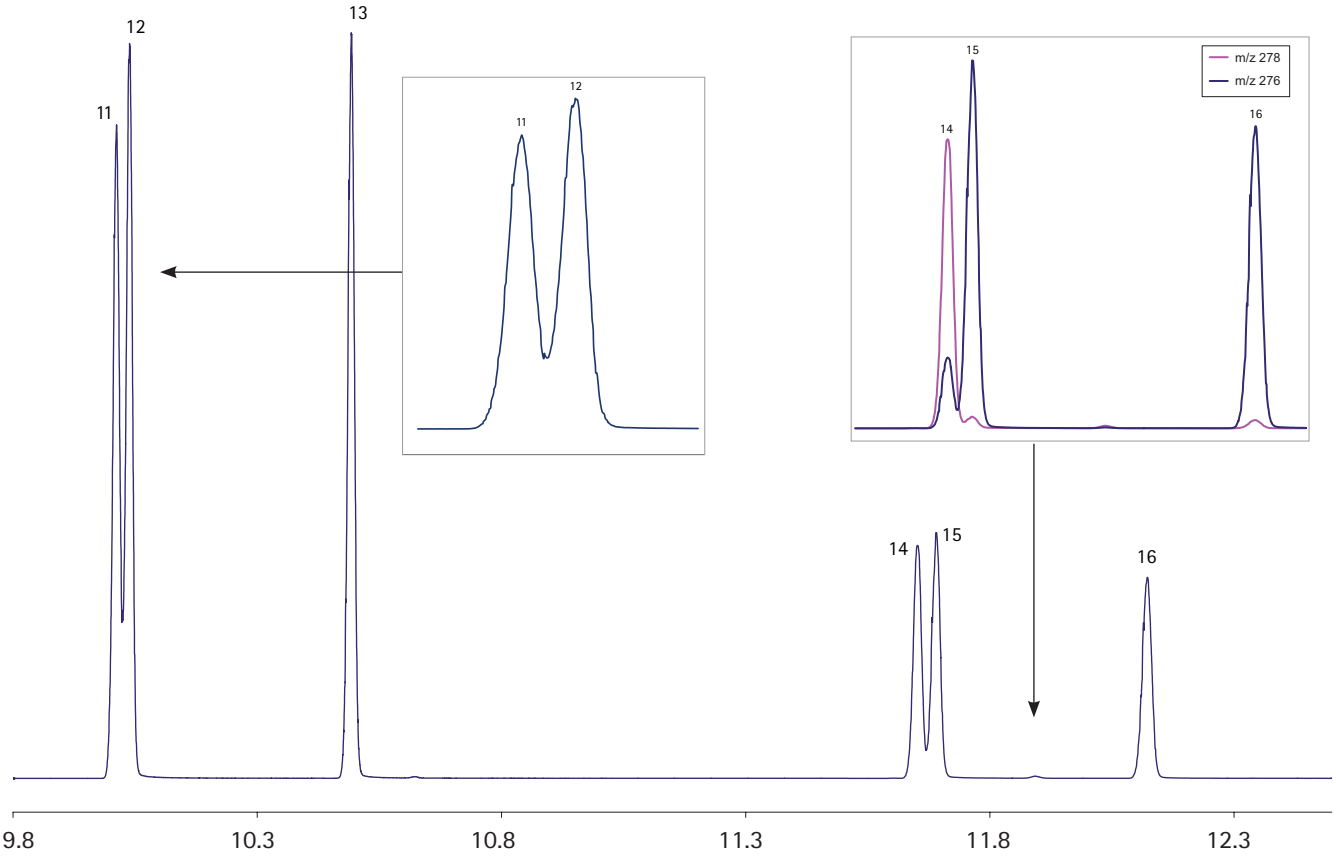


Figure 3. Details and identification of PAH peaks 11–16

Conclusion

Both GC column phase and oven program affect the separation of difficult to resolve PAH sets. With the optimized oven program described here, all 16 EPA PAHs are resolved in a single run with a runtime of less than 13 min. The Select PAH column also offers enhanced resolution of PAHs, preventing co-elution of interfering PAHs that can cause false positives and inaccurate results. Typical interferences are triphenylene on chrysene, and benzo[j]fluoranthene on benzo[k]fluoranthene. Application note SI-02232 describes the separation of 54 PAHs, including the 16 EPA PAHs and their interferences.

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SI-02263

Agilent Technologies

Fast Separation of EU and US EPA Regulated PAHs on Agilent J&W Select PAH GC Columns

Application Note

Author

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Agilent Technologies, Inc.

Introduction

Several polycyclic aromatic hydrocarbons (PAHs) have the same mass, making them difficult to separate using GC/MS. To resolve these PAHs, the use of an appropriate column and an optimized oven program are necessary. In addition, speed of analysis is important for high productivity, but faster speeds often lead to lower resolution. With the 15 m x 0.15 mm ID version of the J&W Select PAH GC column, it is possible to perform high resolution PAH analysis at high speed with no loss of resolution. In this application note, we demonstrate high resolution analysis of 54 PAHs within 30 minutes.

PAHs are formed during incomplete combustion or pyrolysis of organic matter, during industrial processes and in cooking and food processing. Due to their carcinogenic properties, they are pollutants of concern and are subject to monitoring in food and environmental samples. However, different legislations describe different PAHs. The US Environmental Protection Agency (EPA) focuses on environmental PAH contamination whereas the EU legislation is more concerned with PAHs in foodstuffs.

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Conditions

Technique:	GC/MS, Triple Quad
Column:	Select PAH, 15 m x 0.15 mm, df = 0.10 µm (part number CP7461)
Sample Conc:	Approx. 0.1-0.3 µg/mL
Injection Volume:	1 µL
Temperature:	70 °C (0.4 min), 70 °C/min, 180 °C, 7 °C/min, 230 °C (7 min), 50 °C/min, 280 °C (7 min), 30 °C/min, 350 °C (4 min)
Carrier Gas:	Helium, constant flow 1.2 mL/min
Injector:	300 °C, Splitless mode, 0.5 min @ 100 mL/min
Detector:	Triple Quad, EI in SIM mode, ion source 275 °C, transfer line 300 °C

Results and Discussion

There are three groups of peaks that are difficult to resolve when analyzing PAHs. Benz[a]anthracene, cyclopenta[c,d] pyrene, chrysene and triphenylene have different masses, m/z 226 and 228. However, the compounds in this group with m/z 228 also contain some m/z 226, making resolution problematic using only MS. The same complication occurs when separating indeno[1,2,3-c,d]pyrene, benzo[b] triphenylene and dibenz[a,h]anthracene with m/z 276 and 278.

The third set of difficult to resolve PAHs are the benzofluoranthene isomers, benzo[b]fluoranthene, benzo[j] fluoranthene and benzo[k]fluoranthene, which have the same mass and, as before, cannot be resolved with MS only.

Therefore, resolution of these isomers must be done chromatographically. This is straightforward and fast using the Select PAH column and the optimized oven program, as shown in Figure 1 (all PAHs) and Figures 2-8 (details and information).

Table 1. Peak identification of Figure 1

Peak	MW	Compound	EPA 610	SFC & EFSA PAHs (15+1)	CAS
1	136	Naphthalene-d8			1146-65-2
2	128	Naphthalene	x		91-20-3
3	142	2-Methylnaphthalene			91-57-6
4	142	1-Methylnaphthalene			90-12-0
5	152	Acenaphthylene	x		208-96-8
6	164	Acenaphthene-d10			15067-26-2
7	154	Acenaphthene	x		83-32-9
8	166	Fluorene	x		86-73-7
9	188	Phenanthrene-d10			1517-22-2
10	178	Phenanthrene	x		85-01-8
11	178	Anthracene	x		120-12-7
12	202	Fluoranthene	x		206-44-0
13	202	Pyrene	x		129-00-0
14	216	Benzo[a]fluorene			238-84-6
15	216	Benzo[b]fluorene			243-17-4
16	216	7H-Benzo[c]fluorene		x	205-12-9
17	234	Benzo[b]naphtho[2,1-d] thiophene			239-35-0
18	226	Benzo[g,h,i]fluoranthene			203-12-3
19	228	Benzo[c]phenanthrene			195-19-7
20	228	Benz[a]anthracene	x	x	56-55-3
21	226	Cyclopenta[c,d]pyrene		x	27208-37-3
22	240	Chrysene-d10			1719-03-5
23	228	Triphenylene			217-59-4
24	228	Chrysene	x	x	218-01-9
25	242	6-Methylchrysene			1705-85-7
26	242	5-Methylchrysene		x	3697-24-3
27	252	Benzo[b]fluoranthene	x	x	205-99-2
28	252	Benzo[k]fluoranthene	x	x	207-08-9
29	252	Benzo[j]fluoranthene		x	205-82-3
30	252	Benzo[a]fluoranthene			203-33-8
31	252	Benzo[e]pyrene			192-97-2
32	252	Benzo[a]pyrene	x	x	50-32-8
33	264	Perylene-d12			1520-96-3
34	252	Perylene			198-55-0
35	268	3-Methylcholanthrene			56-49-5
36	330	9,10-diphenylanthracene			216-105-1
37	279	Dibenzo[a,h]acridine			226-36-8
38	279	Dibenzo[a,j]acridine			224-42-0
39	278	Dibenz[a,j]anthracene			224-41-9
40	292	Dibenzo[a,h]anthracene D14			13250-98-1
41	278	Benzo[b]triphenylene			215-58-7
42	276	Indeno[1,2,3-cd]pyrene	x	x	193-39-5
43	278	Dibenz[a,h]anthracene	x	x	53-70-3
44	278	Benzo[b]chrysene			214-17-5
45	278	Picene			213-46-7
46	27	Benzo[g,h,i]perylene	x	x	191-24-2
47	276	Dibenzo[def,mno]chrysene			191-26-4
48	267	7H-Dibenzo[c,g]carbazole			194-59-2
49	302	Dibenzo[a,l]pyrene		x	191-30-0
50	302	Dibenzo[a,e]pyrene		x	192-65-4
51	300	Coronene			191-07-1
52	302	Benzo[b]perylene			197-70-6
53	302	Dibenzo[a,i]pyrene		x	189-55-9
54	302	Dibenzo[a,h]pyrene		x	189-64-0

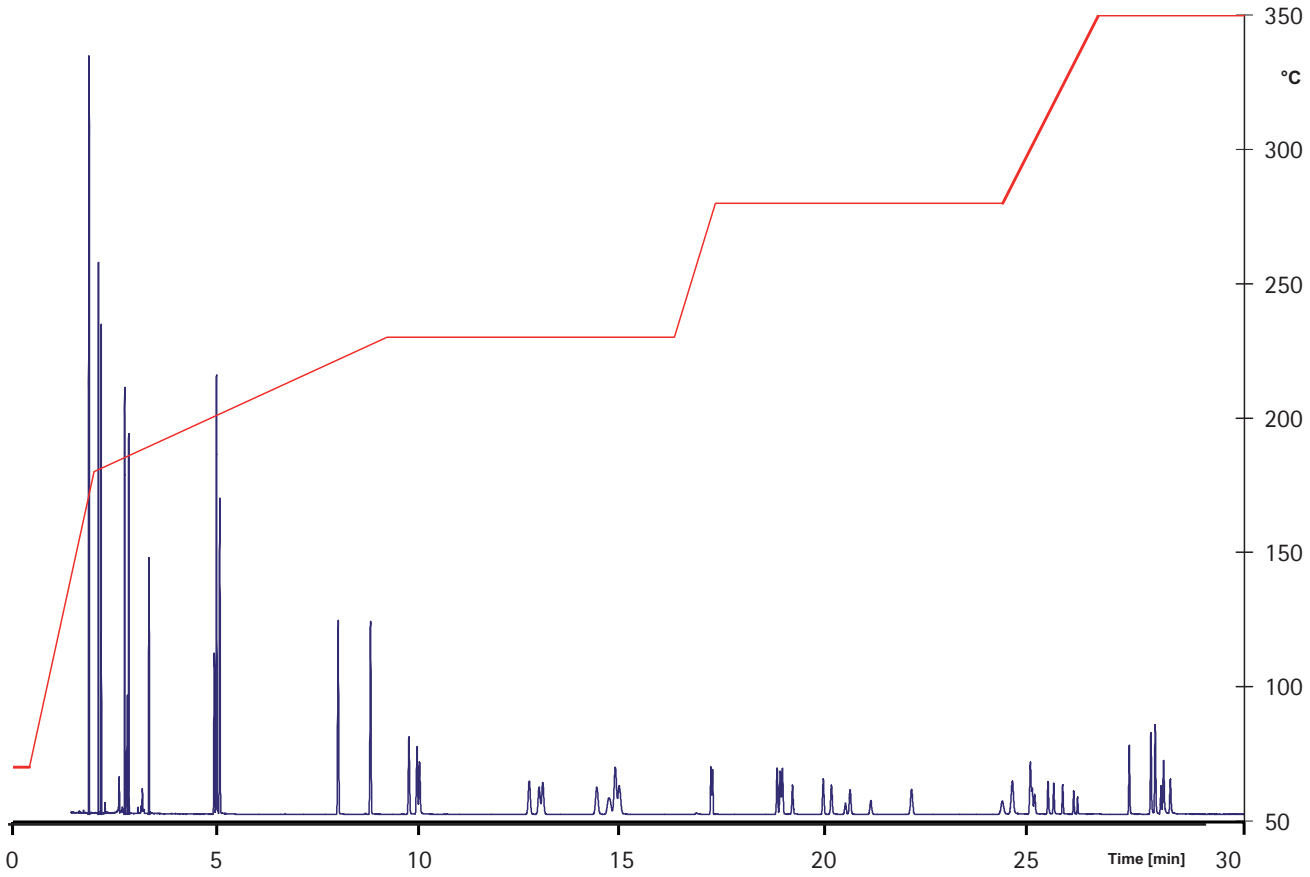


Figure 1. GC/MS analysis of EU and EPA PAHs on Select PAH 15 m x 0.15 mm x 0.10 µm

Table 2. Peak Identification for Figure 2

Peak	MW	Compound	EPA 610	CAS
1	136	Naphthalene-d8		1146-65-2
2	128	Naphthalene	x	91-20-3
3	142	2-Methylnaphthalene		91-57-6
4	142	1-Methylnaphthalene		90-12-0
5	152	Acenaphthylene	x	208-96-8
6	164	Acenaphthene-d10		15067-26-2
7	154	Acenaphthene	x	83-32-9
8	166	Fluorene	x	86-73-7

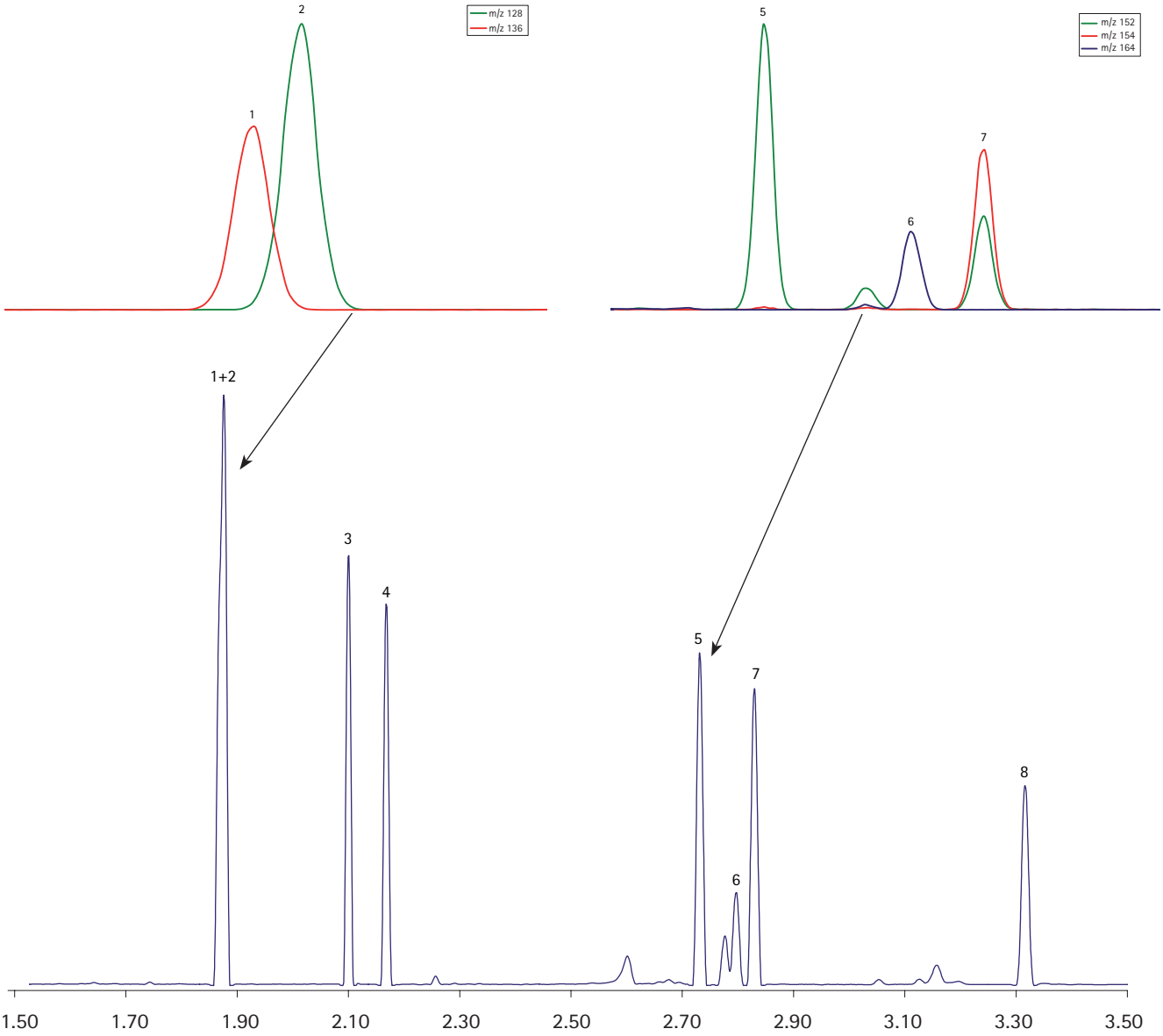


Figure 2. Details and identification of peaks 1 to 8

Table 3. Peak Identification for Figure 3

Peak	MW	Compound	EPA 610	CAS
9	188	Phenanthrene-d10		1517-22-2
10	178	Phenanthrene	x	85-01-8
11	178	Anthracene	x	120-12-7
12	202	Fluoranthene	x	206-44-0
13	202	Pyrene	x	129-00-0

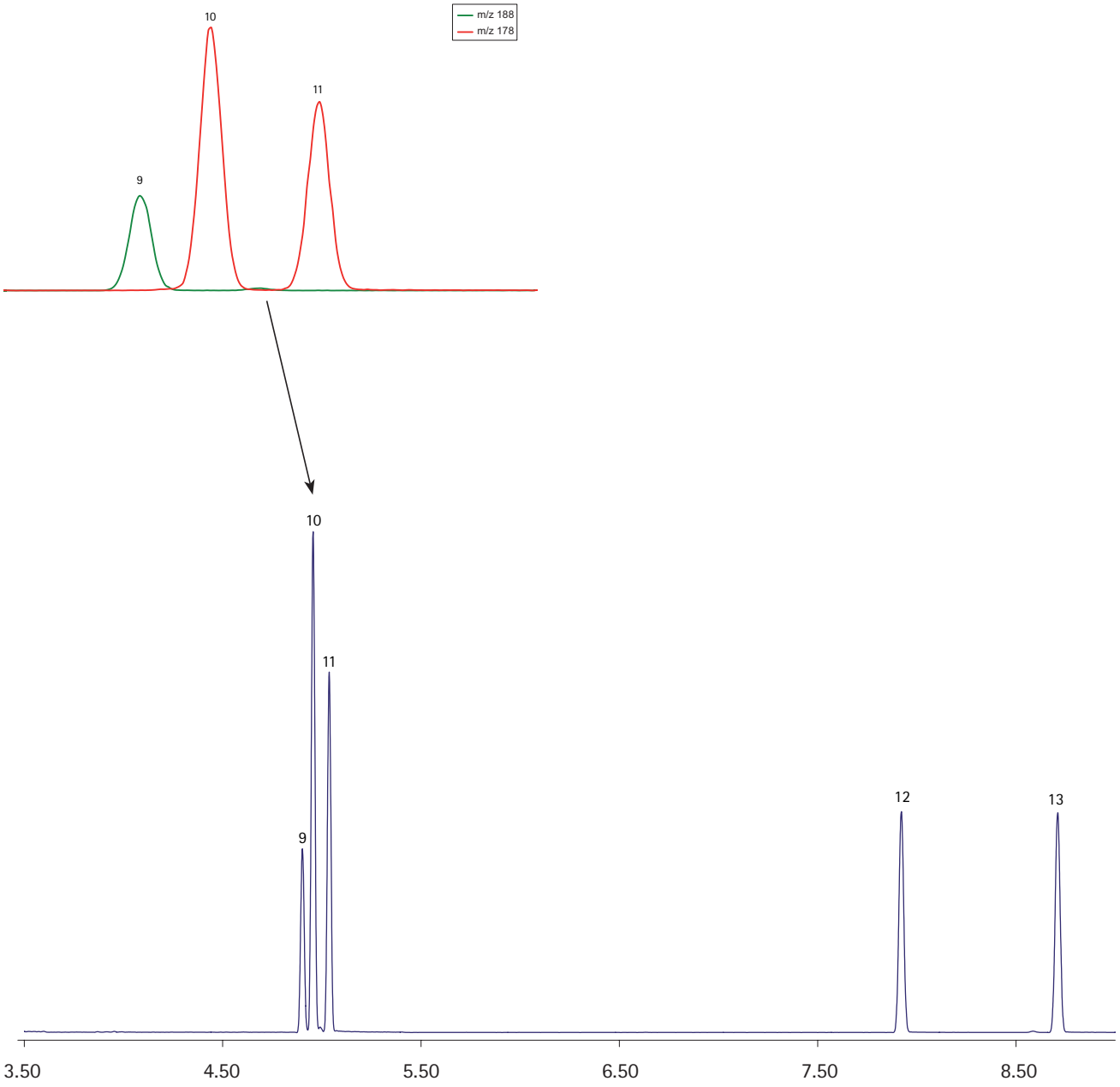


Figure 3. Details and identification of peaks 9 to 13

Table 4. Peak Identification for Figure 4

Peak	MW	Compound	EPA 610	SFC & EFSA PAHs (15+1)	CAS
14	216	Benzo[a]fluorene			238-84-6
15	216	Benzo[b]fluorene			243-17-4
16	216	7H-Benzo[c]fluorene		x	205-12-9
17	234	Benzo[b]naphtho[2,1-d] thiophene			239-35-0
18	226	Benzo[g,h,i]fluoranthene			203-12-3
19	228	Benzo[c]phenanthrene			195-19-7
20	228	Benz[a]anthracene	x	x	56-55-3
21	226	Cyclopenta[c,d]pyrene		x	27208-37-3
22	240	Chrysene-d10			1719-03-5
23	228	Triphenylene			217-59-4
24	228	Chrysene	x	x	218-01-9

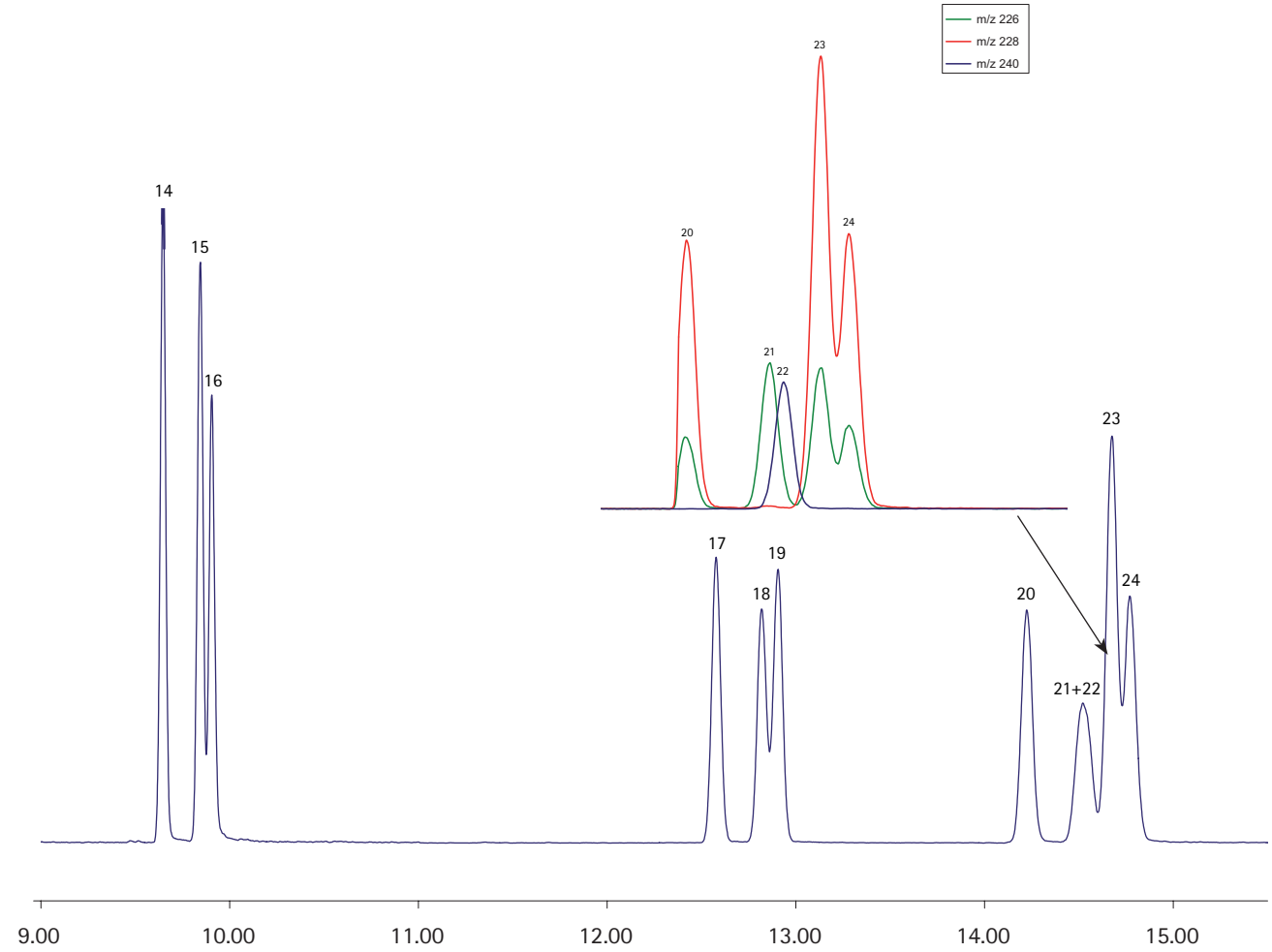


Figure 4. Details and identification of peaks 14 to 24

Table 5. Peak Identification for Figure 5

Peak	MW	Compound	EPA 610	SFC & EFSA PAHs (15+1)	CAS
25	242	6-Methylchrysene			1705-85-7
26	242	5-Methylchrysene		x	3697-24-3
27	252	Benzo[b]fluoranthene	x	x	205-99-2
28	252	Benzo[k]fluoranthene	x	x	207-08-9
29	252	Benzo[j]fluoranthene		x	205-82-3
30	252	Benzo[a]fluoranthene			203-33-8
31	252	Benzo[e]pyrene			192-97-2
32	252	Benzo[a]pyrene	x	x	50-32-8
33	264	Perylene-d12			1520-96-3
34	252	Perylene			198-55-0
35	268	3-Methylcholanthrene			56-49-5
36	330	9,10-diphenylanthracene			216-105-1

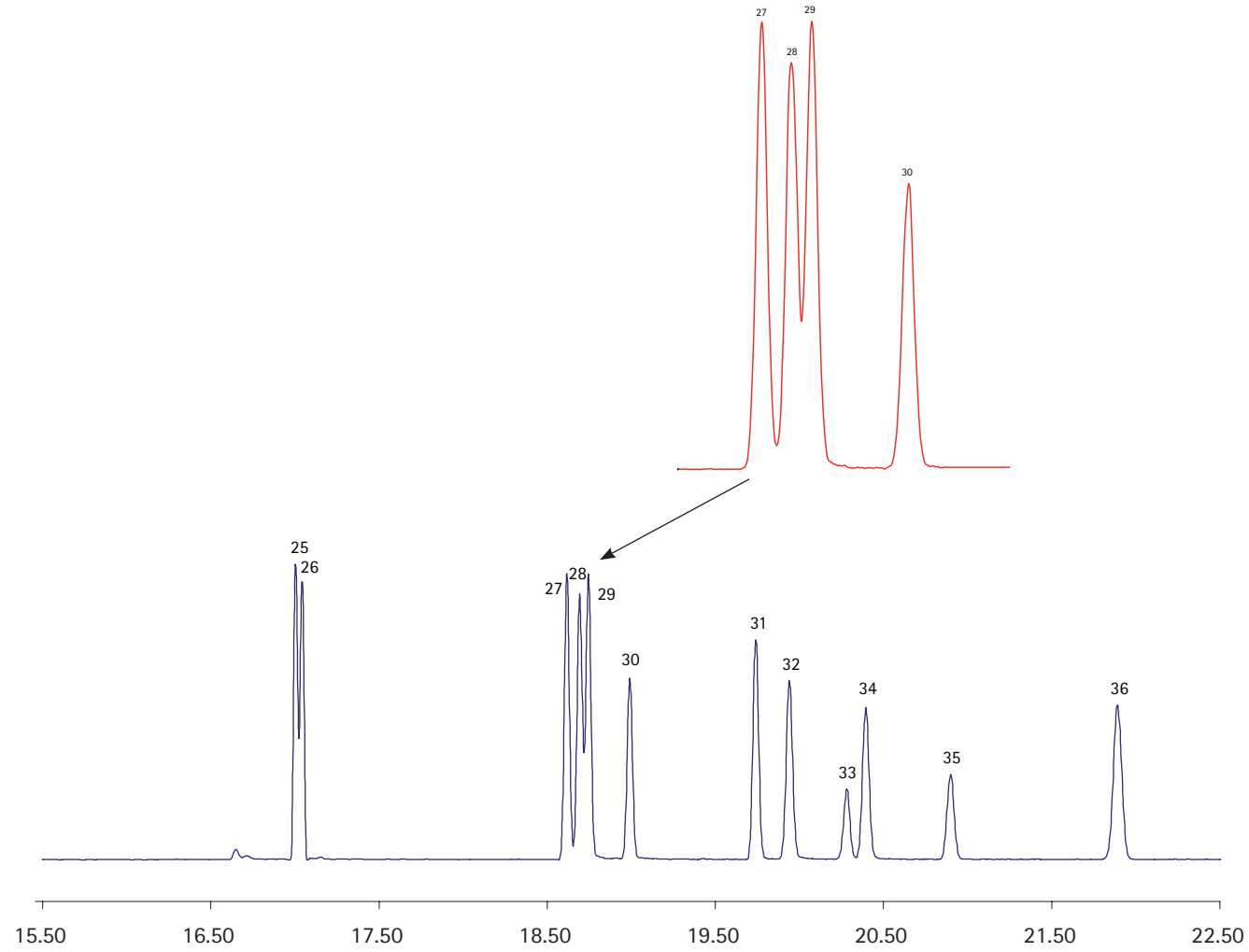


Figure 5. Details and identification of peaks 25 to 36

Table 6. Peak Identification for Figure 6

Peak	MW	Compound	EPA 610	SFC & EFSA PAHs (15+1)	CAS
37	279	Dibenzo[a,h]acridine			226-36-8
38	279	Dibenzo[a,j]acridine			224-42-0
39	278	Dibenz[a,j]anthracene			224-41-9
40	292	Dibenzo[a,h]anthracene D14			13250-98-1
41	278	Benzo[b]triphenylene			215-58-7
42	276	Indeno[1,2,3-c,d]pyrene	x	x	193-39-5
43	278	Dibenz[a,h]anthracene	x	x	53-70-3
44	278	Benzo[b]chrysene			214-17-5
45	278	Picene			213-46-7
46	27	Benzo[g,h,i]perylene	x	x	191-24-2
47	276	Dibenzo[def,mno]chrysene			191-26-4
48	267	7H-Dibenzo[c,g]carbazole			194-59-2
49	302	Dibenzo[a,l]pyrene		x	191-30-0
50	302	Dibenzo[a,e]pyrene		x	192-65-4
51	300	Coronene			191-07-1
52	302	Benzo[b]perylene			197-70-6
53	302	Dibenzo[a,i]pyrene		x	189-55-9
54	302	Dibenzo[a,h]pyrene		x	189-64-0

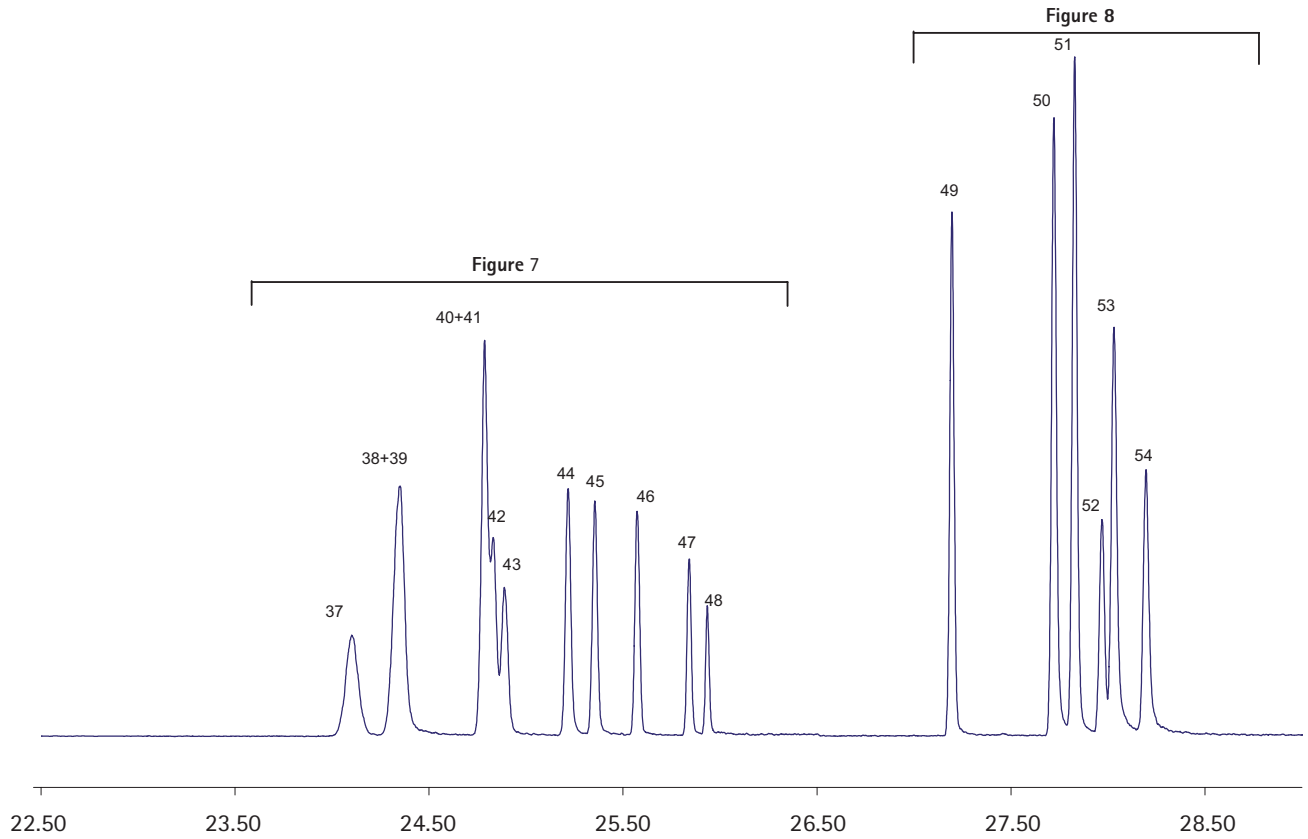


Figure 6. Details and identification of peaks 37 to 54

Table 7. Peak Identification for Figure 7

Peak	MW	Compound	EPA 610	SFC & EFSA PAHs (15+1)	CAS
37	279	Dibenzo[a,h]acridine			226-36-8
38	279	Dibenzo[a,j]acridine			224-42-0
39	278	Dibenz[a,j]anthracene			224-41-9
40	292	Dibenzo[a,h]anthracene D14			13250-98-1
41	278	Benzo[b]triphenylene			215-58-7
42	276	Indeno[1,2,3-c,d]pyrene	x	x	193-39-5
43	278	Dibenz[a,h]anthracene	x	x	53-70-3
44	278	Benzo[b]chrysene			214-17-5
45	278	Picene			213-46-7
46	27	Benzo[g,h,i]perylene	x	x	191-24-2
47	276	Dibenzo[def,mno]chrysene			191-26-4
48	267	7H-Dibenzo[c,g]carbazole			194-59-2

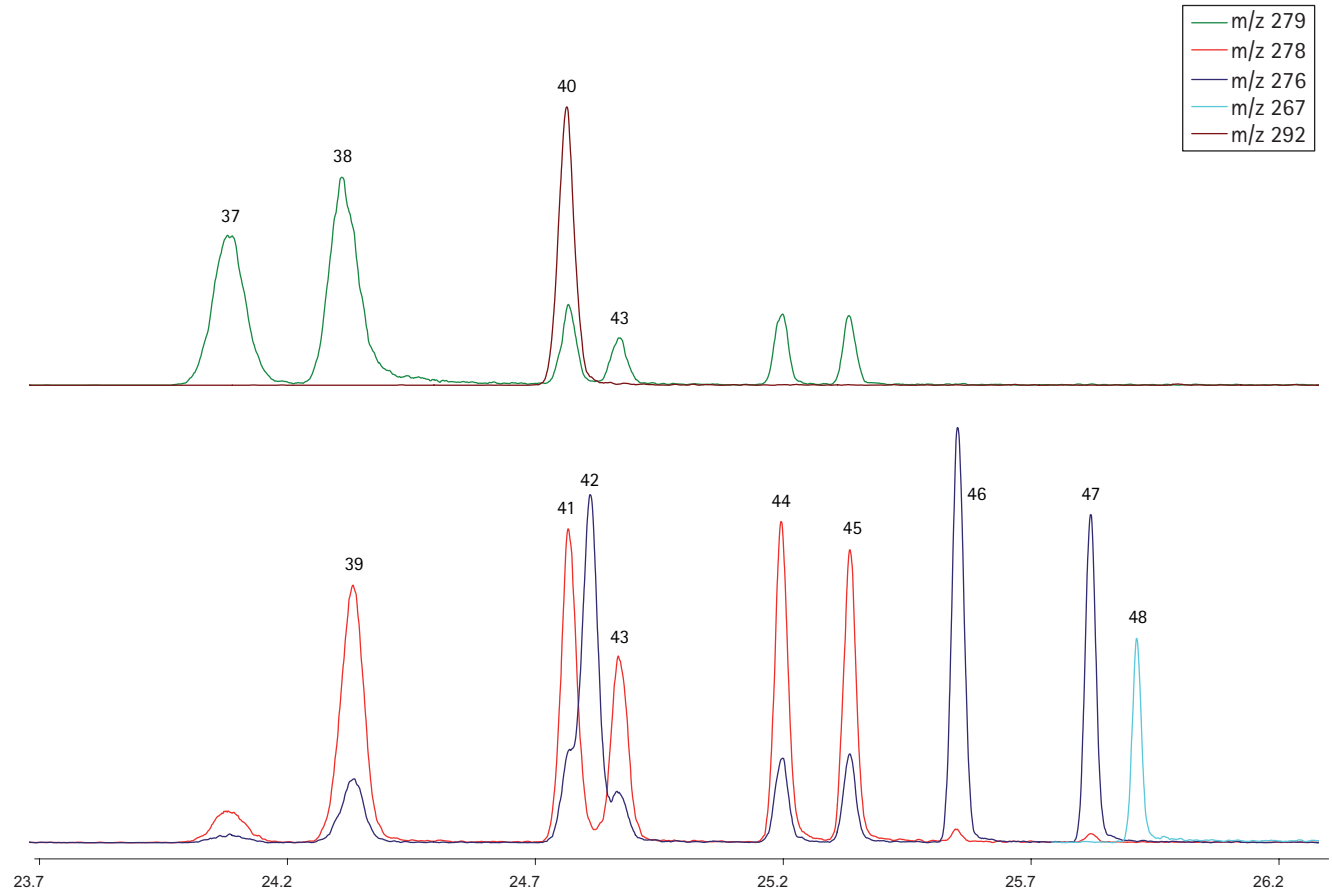


Figure 7. Details and identification of peaks 37 to 48

Table 8. Peak Identification for Figure 8

Peak	MW	Compound	EPA 610	SFC & EFSA PAHs (15+1)	CAS
49	302	Dibenzo[a,l]pyrene		x	191-30-0
50	302	Dibenzo[a,e]pyrene		x	192-65-4
51	300	Coronene			191-07-1
52	302	Benzo[b]perylene			197-70-6
53	302	Dibenzo[a,i]pyrene		x	189-55-9
54	302	Dibenzo[a,h]pyrene		x	189-64-0

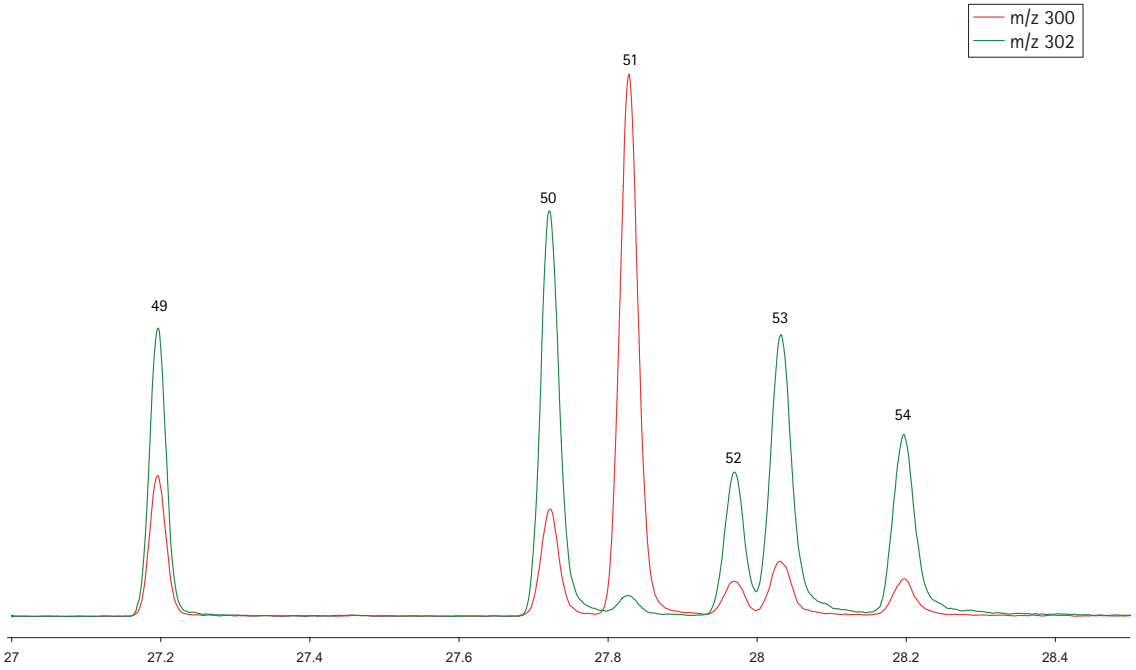


Figure 8. Details and identification of peaks 49 to 54

Conclusion

The J&W Select PAH column phase, as well as the GC oven program, affects the separation of difficult to resolve PAH sets. With the optimized oven program described in this application note, all EU and EPA PAHs, and known interferences, are resolved in a single run with a runtime of 29 min.

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Bordajandi LR et al., (2008) Optimisation of the GC-MS conditions for the determination of the 15 EU foodstuff priority polycyclic aromatic hydrocarbons, J. Sep. Sci., 31, 1769-1778.

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SI-02259

Agilent Technologies

GC Analysis of Halogenated Pesticides using the Agilent J&W FactorFour VF-1701ms with EZ-Guard

Application Note

Author

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Agilent Technologies, Inc.

Introduction

Pesticides analyses can be challenging due to complex sample matrices. Guard columns are often used to protect the analytical column from non-volatile compounds in the sample matrix. While prolonging the lifetime of the analytical column, the guard can introduce the risk of leakage through the coupling, or adsorption of analytes due to the influence of the connector. Leakage decreases column lifetime and adsorption causes asymmetrical peaks, lower detection response, and decreases the sensitivity of the analysis.

EZ-Guard is an integrated guard column that eliminates the risk of leakage and adsorption. When the resolution or the analyte response diminishes, a coil is removed from the EZ-Guard column so that column performance will improve.

The VF-1701ms is often used as a conformation column for pesticide analysis. Now with EZ-Guard, the VF-1701ms can be used for such samples in complex matrices. This application note demonstrates the performance of the VF-1701ms with EZ-Guard in the separation of pesticides.

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Materials and Methods

Column:	VF-1701ms, 30 m x 0.25 mm ID, df=0.25 µm, + 10 m EZ-Guard (part number CP9177)
Sample Size:	2 µL, Split ratio 1:50
Sample Conc:	0.1% in hexane
Temp Program:	220 °C
Carrier Gas:	H2
Pressure:	90 kPa
Injector Temp:	275 °C, split
Detector Temp:	300 °C, ECD

Results and Discussion

As shown in the chromatogram (Figure 1) all compounds have excellent peak shape and high response, including unstable endrin and p, p'-DDT, demonstrating the inertness of the whole GC column.

Conclusion

The J&W FactorFour VF-1701ms with EZ-Guard offers improved column lifetimes and high performance in the analysis of pesticides in complex matrices. Peak shapes are excellent, maximizing the sensitivity of the analysis. In addition, EZ-Guard columns are fast and easy to install and operate, boosting productivity and delivering improved efficiency. Also, the last meter is uncoated, functioning as an integrated transfer line, providing faster detector stabilization.

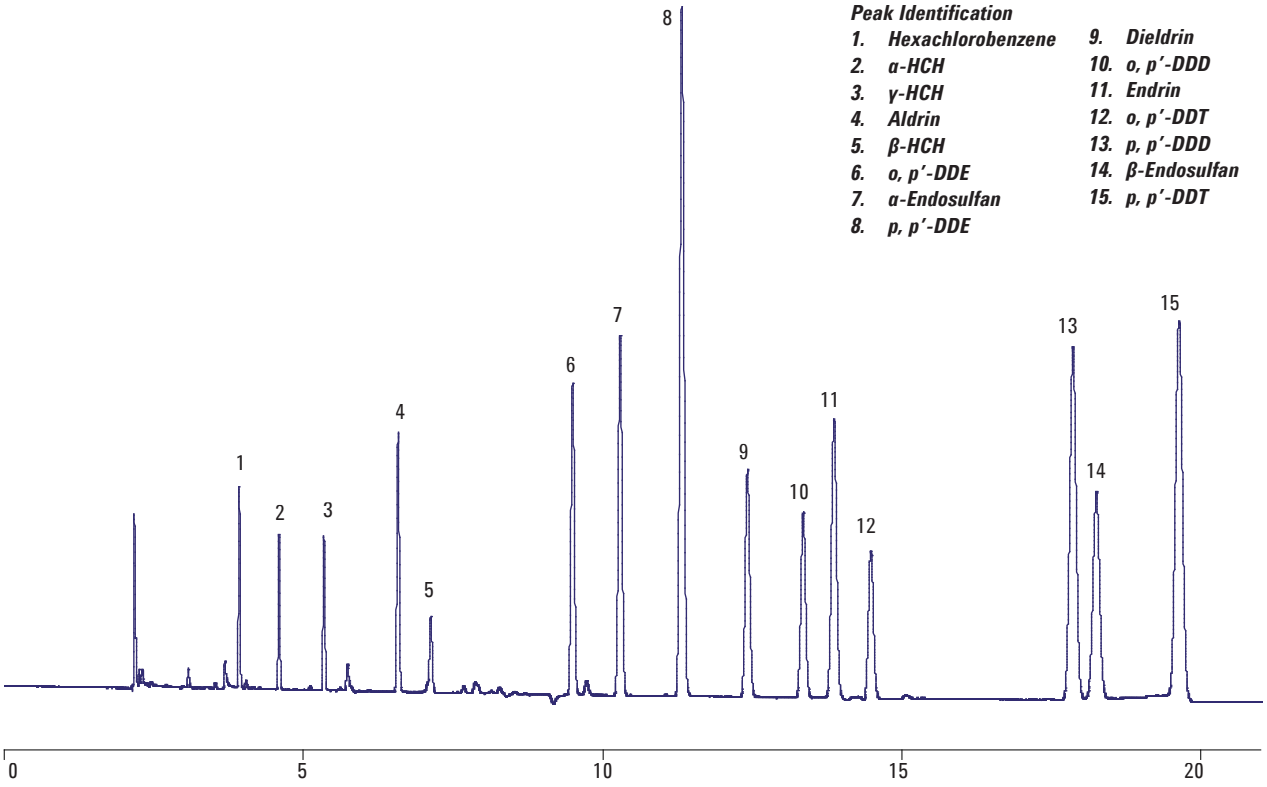


Figure 1. Excellent resolution of 12 halogenated pesticides on a VF-1701ms GC column with EZ-Guard

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Agilent Technologies

Separation of 54 PAHs on an Agilent J&W Select PAH GC Column

Application Note

Author

John Oostdijk
Agilent Technologies, Inc.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are compounds that contain two or more aromatic rings. They are formed during incomplete combustion or pyrolysis of organic matter, industrial processes and cooking and food processing. PAHs are therefore analyzed in environmental and food samples.

The difficulty in analyzing PAHs lies in separating PAH isomers. These isomers have the same chemical structure and same ion fragment and therefore cannot be separated by mass spectrometers. The Select PAH capillary column has enhanced selectivity towards PAHs, separating the isomers and enabling accurate PAH analysis. This application note describes an optimized oven program for the Select PAH column. In PAH analysis, there is a difference between the European (EU) and American (EPA) legislation. The legislations both describe a different set of PAHs and address different matrix origins. Table 1 lists the PAH regulated by EU and EPA legislation, including the potential interferences. For this application note, we chose a sample containing 54 PAHs to demonstrate the unique selectivity of the Select PAH.

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Conditions

Technique:	GC/MS, Triple Quad
Column:	Select PAH, 30 m x 0.25 mm, df=0.15 µm (part number CP7462)
Sample Conc:	approx 0.1-0.3 µg/mL
Injection Volume:	1 µL
Temperature:	70 °C (0.7 min), 85 °C/min, 180 °C, 3 °C/min, 230 °C (7 min), 28 °C/min, 280 °C (10 min), 14 °C/min, 350 °C (3 min)
Carrier Gas:	Helium, constant flow 2 mL/min
Injector:	300 °C, Splitless mode, 1 min @ 50 mL/min
Detector:	Triple Quad, EI in SIM mode, ion source 275°C, transfer line 300°C

Results and Discussion

Figure 1 shows the separation of all 54 PAH isomers and Figures 2-6 reveal the finer details.

When performing this analysis, there are three sets of peaks that are difficult to resolve. The first set, comprising benzo[a]anthracene, cyclopenta[c,d]pyrene, chrysene and triphenylene, has different masses, m/z 226 and 228. The compounds with m/z 228 also contain some m/z 226, making it difficult to resolve this set by MS alone. The same problem occurs when separating indeno[1,2,3-c,d]pyrene, benzo[b]triphenylene and dibenzo[a,h]anthracene with m/z 276 and 278. The third set of PAH isomers difficult to resolve are the benzofluoranthenes. These three isomers, benzo[b] fluoranthene, benzo[j]fluoranthene and benzo[k]fluoranthene, have the same mass and cannot be resolved by MS.

However, the unique selectivity of Select PAH resolves all the PAH isomers and enables accurate quantification of all PAHs.

Peaks 1 and 2 show some tailing, which can be resolved by further optimization of the splitless injection conditions.

Conclusion

The Select PAH column resolves 54 PAHs in a single GC/MS run, including the hard to separate PAH isomers. The optimized GC oven program performs the run time in less than 45 minutes.

Table 1. Peak Identification for Figure 1

Peak	MW	Compound	EPA 610	SFC & EFSA PAHs (15+1)	CAS
1	136	Naphthalene-d8			1146-65-2
2	128	Naphthalene	X		91-20-3
3	142	2-Methylnaphthalene			91-57-6
4	142	1-Methylnaphthalene			90-12-0
5	152	Acenaphthylene	X		208-96-8
6	164	Acenaphthene-d10			15067-26-2
7	154	Acenaphthene	X		83-32-9
8	166	Fluorene	X		86-73-7
9	188	Phenanthrene-d10			1517-22-2
10	178	Phenanthrene	X		85-01-8
11	178	Anthracene	X		120-12-7
12	202	Fluoranthene	X		206-44-0
13	202	Pyrene	X		129-00-0
14	216	Benzo[a]fluorene			238-84-6
15	216	Benzo[b]fluorene			243-17-4
16	216	7H-Benzo[c]fluorene		X	205-12-9
17	234	Benzo[b]naphto[2,1-d]-thiophene			239-35-0
18	226	Benzo[g,h,i]-fluoranthene			203-12-3
19	228	Benzo[c]phenanthrene			195-19-7
20	228	Benz[a]anthracene	X	X	56-55-3
21	226	Cyclopenta[c,d]pyrene		X	27208-37-3
22	240	Chrysene-d10			1719-03-5
23	228	Triphenylene			217-59-4
24	228	Chrysene	X	X	218-01-9
25	242	6-Methylchrysene			175-85-7
26	242	5-Methylchrysene		X	3697-24-3
27	252	Benzo[b]fluoranthene	X	X	205-99-2
28	252	Benzo[k]fluoranthene	X	X	207-08-9
29	252	Benzo[j]fluoranthene		X	205-82-3
30	252	Benzo[a]fluoranthene			203-33-8
31	252	Benzo[e]pyrene			192-97-2
32	252	Benzo[a]pyrene	X	X	50-32-8
33	264	Perylene-d12			1520-96-3
34	252	Perylene			198-55-0
35	268	3-Methylcholanthrene			56-49-5
36	330	9,10-Diphenylanthracene			216-105-1
37	279	Dibenzo[a,h]acridine			226-36-8
38	279	Dibenzo[a,j]acridine			224-42-0
39	278	Dibenzo[a,j]anthracene			224-41-9
40	292	Dibenzo[a,h] anthracene-d14			13250-98-1
41	278	Benzo[b]triphenylene			215-58-7
42	276	Indeno[1,2,3-c,d]pyrene	X	X	193-39-5
43	278	Dibenzo[a,h]anthracene	X	X	53-70-3
44	278	Benzo[b]chrysene			214-17-5
45	278	Picene			213-46-7
46	276	Benzo[g,h,i]perylene	X	X	191-24-2
47	276	Dibenzo[def,mno]-chrysene			191-26-4
48	267	7H-Dibenzo[c,g]-carbazole			194-59-2
49	302	Dibenzo[a,l]pyrene		X	191-30-0
50	302	Dibenzo[a,e]pyrene		X	192-65-4
51	300	Coronene			191-07-1
52	302	Benzo[b]perylene			197-70-6
53	302	Dibenzo[a,i]pyrene		X	189-55-9
54	302	Dibenzo[a,h]pyrene		X	189-64-0

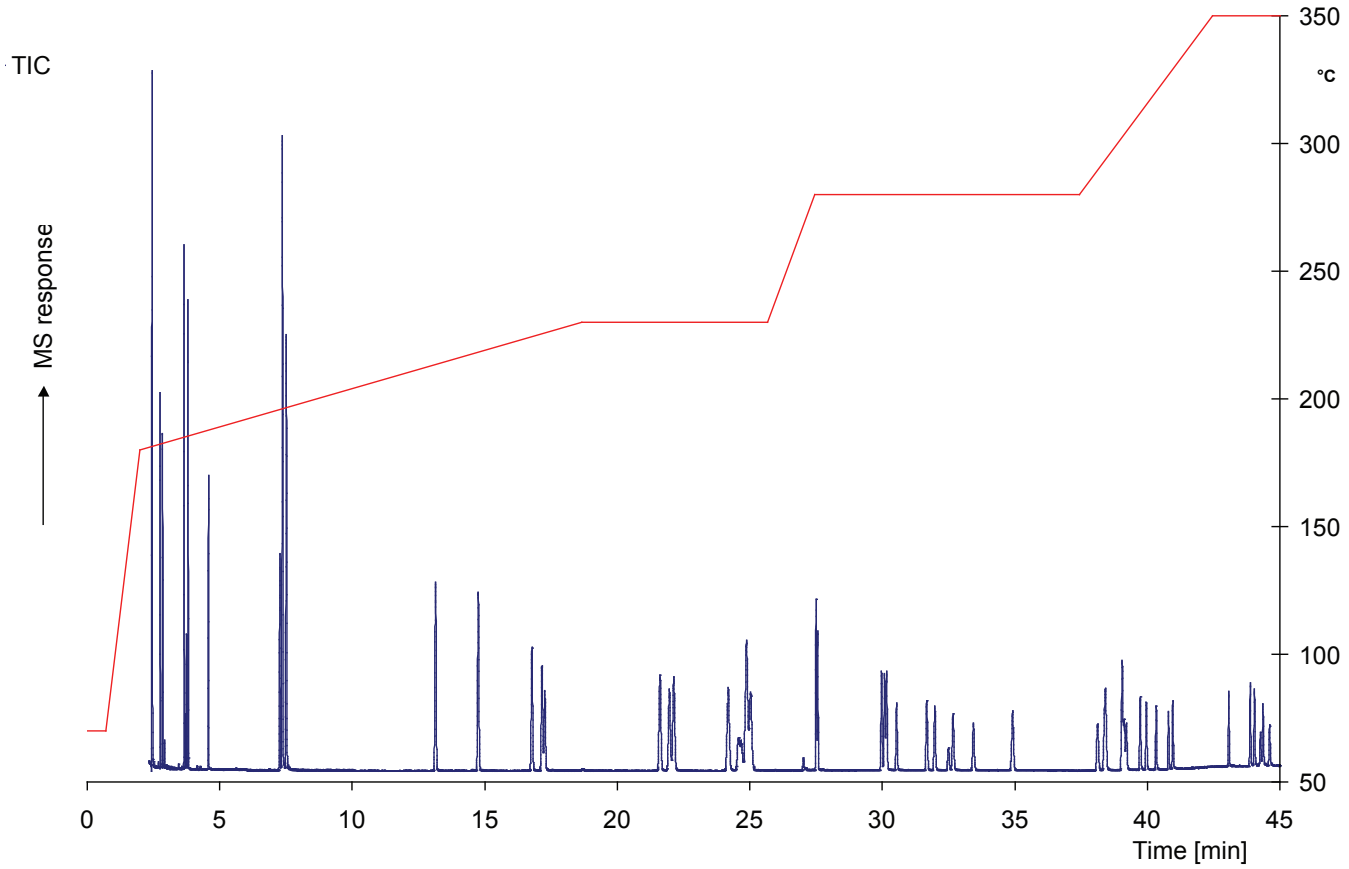


Figure 1. GC/MS analysis of EU and EPA PAHs on Select PAH column, 30 m x 0.25 mm x 0.15 µm

Table 2. Peak Identification for Figure 2

Peak	MW	Compound	EPA 610	CAS
1	136	Naphthalene-d8		1146-65-2
2	128	Naphthalene	X	91-20-3
3	142	2-Methylnaphthalene		91-57-6
4	142	1-Methylnaphthalene		90-12-0

Peak	MW	Compound	EPA 610	CAS
5	152	Acenaphthylene	X	208-96-8
6	164	Acenaphthene-d10		15067-26-2
7	154	Acenaphthene	X	83-32-9
8	166	Fluorene	X	86-73-7

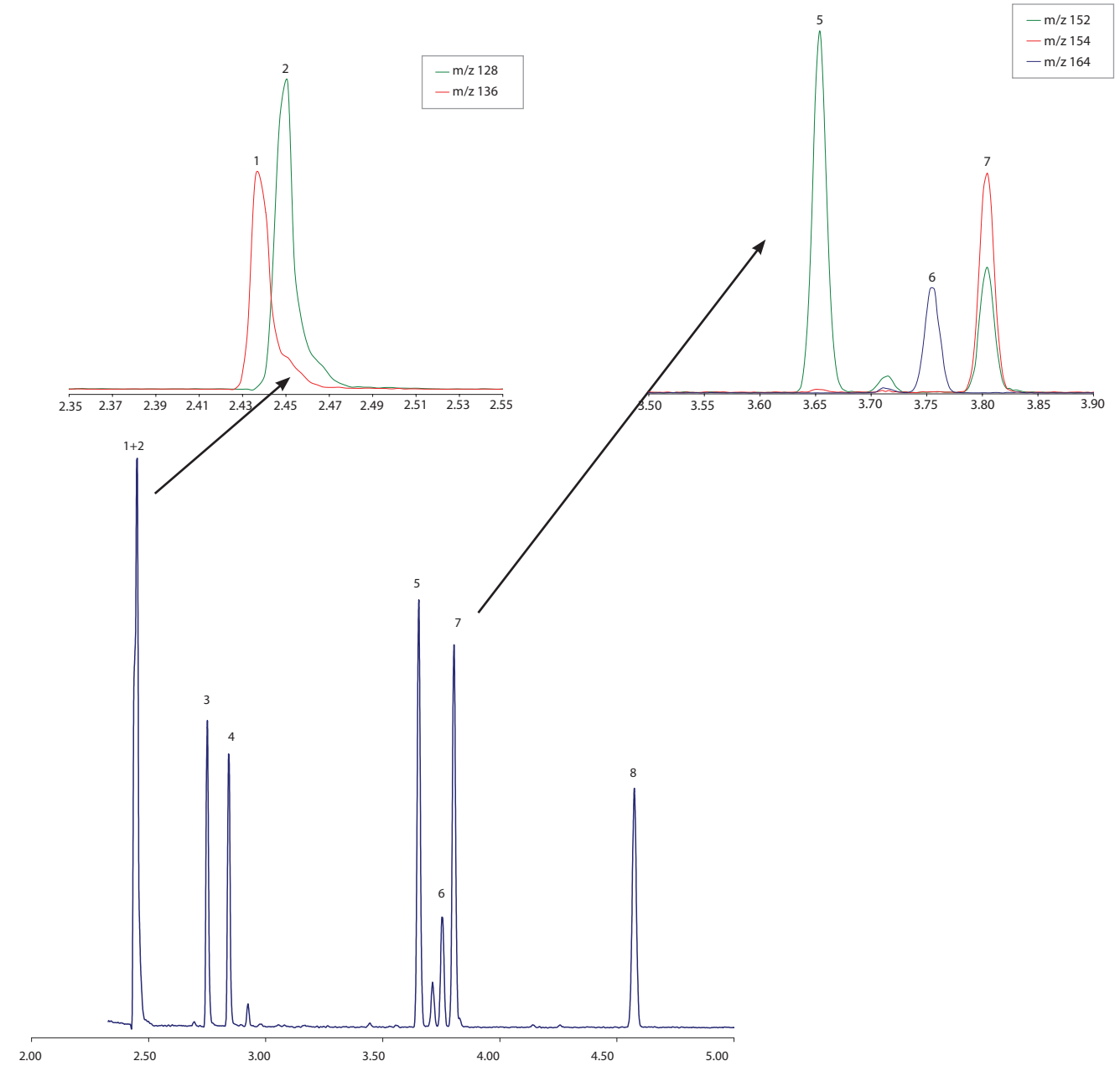


Figure 2. Details and identification of peaks 1 to 8

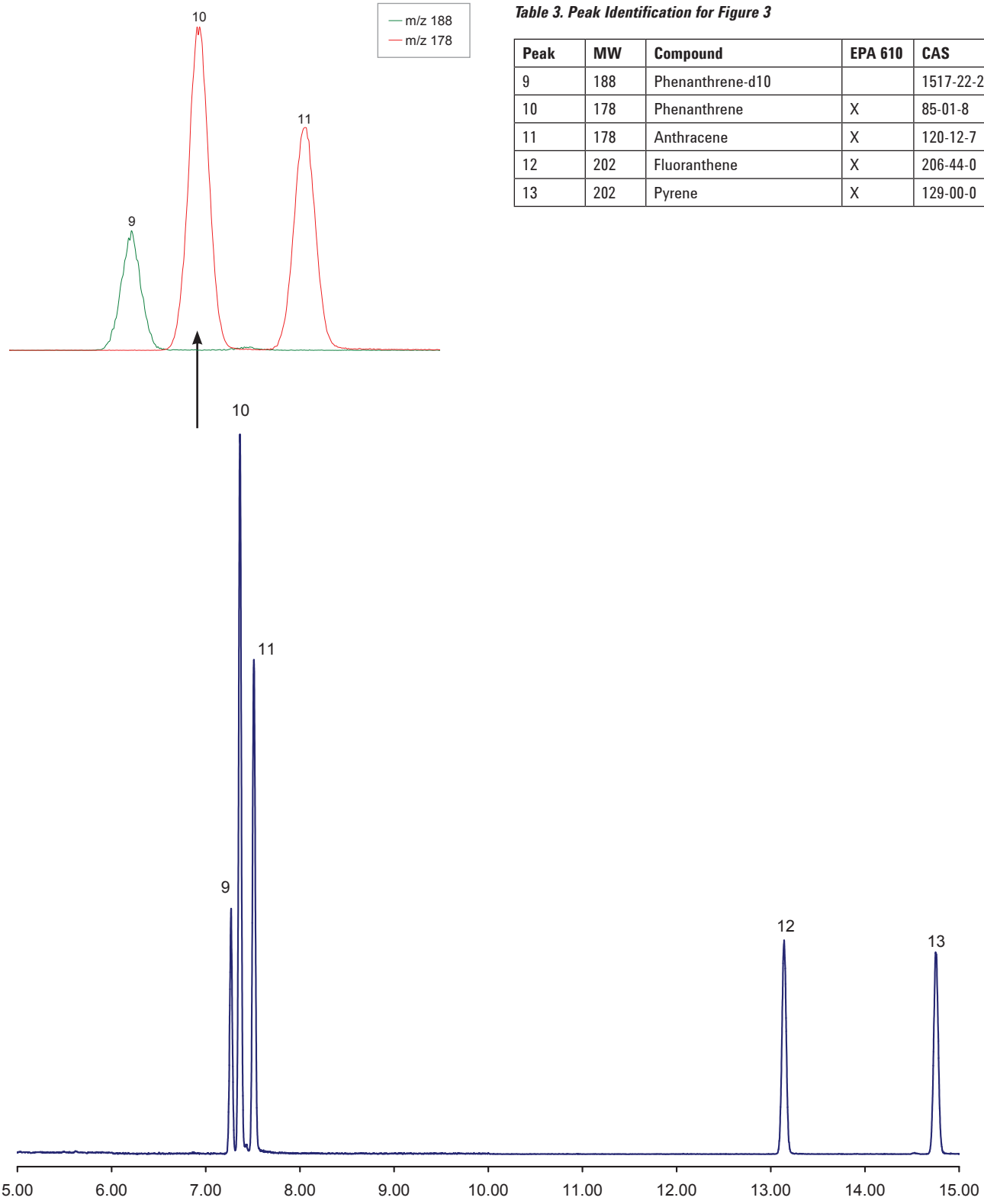


Figure 3. Details and identification of peaks 9 to 13

Table 3. Peak Identification for Figure 3

Peak	MW	Compound	EPA 610	CAS
9	188	Phenanthrene-d10		1517-22-2
10	178	Phenanthrene	X	85-01-8
11	178	Anthracene	X	120-12-7
12	202	Fluoranthene	X	206-44-0
13	202	Pyrene	X	129-00-0

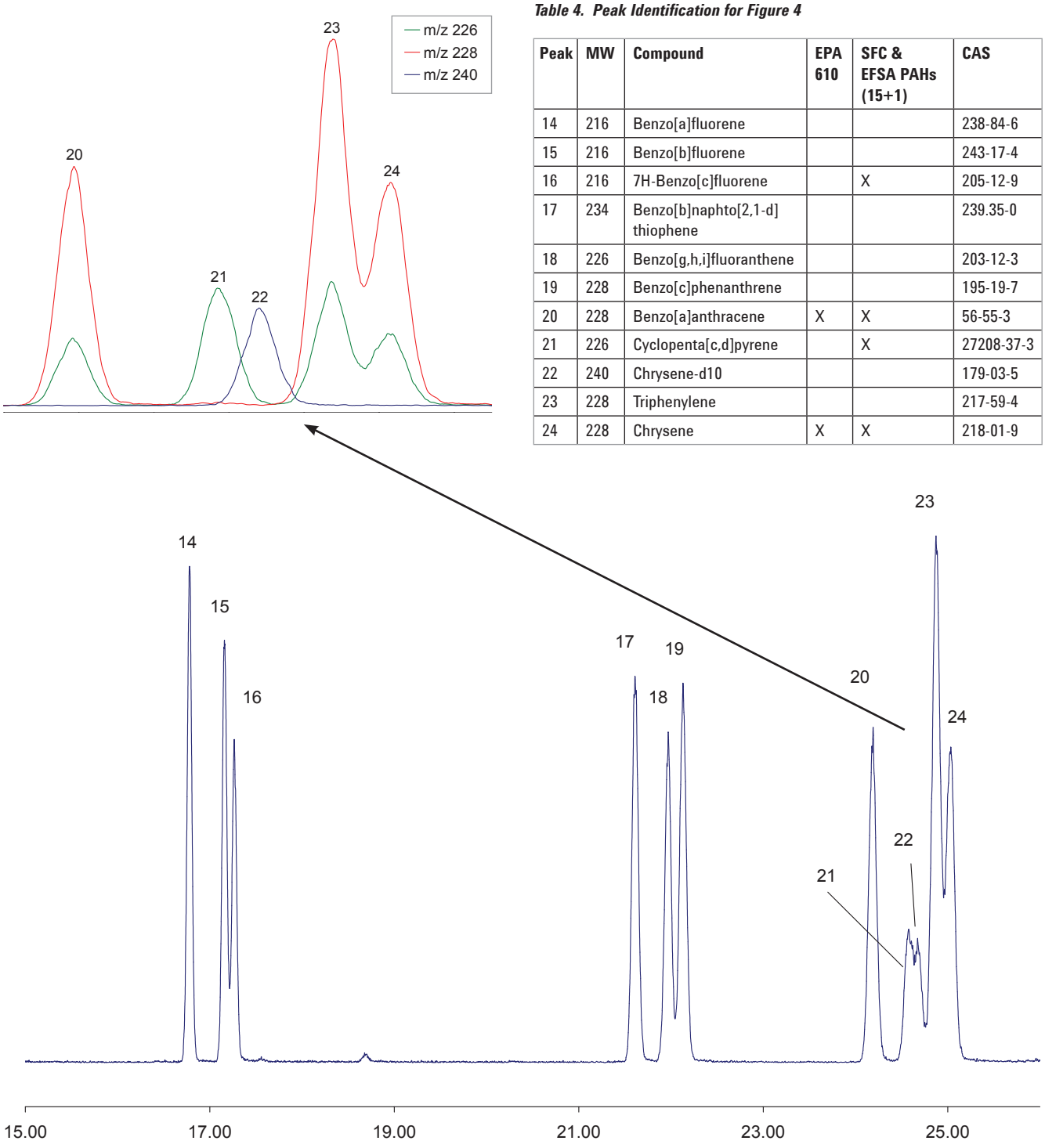


Figure 4. Details and identification of peaks 14 to 24

Table 4. Peak Identification for Figure 4

Peak	MW	Compound	EPA 610	SFC & EFSA PAHs (15+1)	CAS
14	216	Benzo[a]fluorene			238-84-6
15	216	Benzo[b]fluorene			243-17-4
16	216	7H-Benzo[c]fluorene		X	205-12-9
17	234	Benzo[b]naphto[2,1-d] thiophene			239.35-0
18	226	Benzo[g,h,i]fluoranthene			203-12-3
19	228	Benzo[c]phenanthrene			195-19-7
20	228	Benzo[a]anthracene	X	X	56-55-3
21	226	Cyclopenta[c,d]pyrene		X	27208-37-3
22	240	Chrysene-d10			179-03-5
23	228	Triphenylene			217-59-4
24	228	Chrysene	X	X	218-01-9

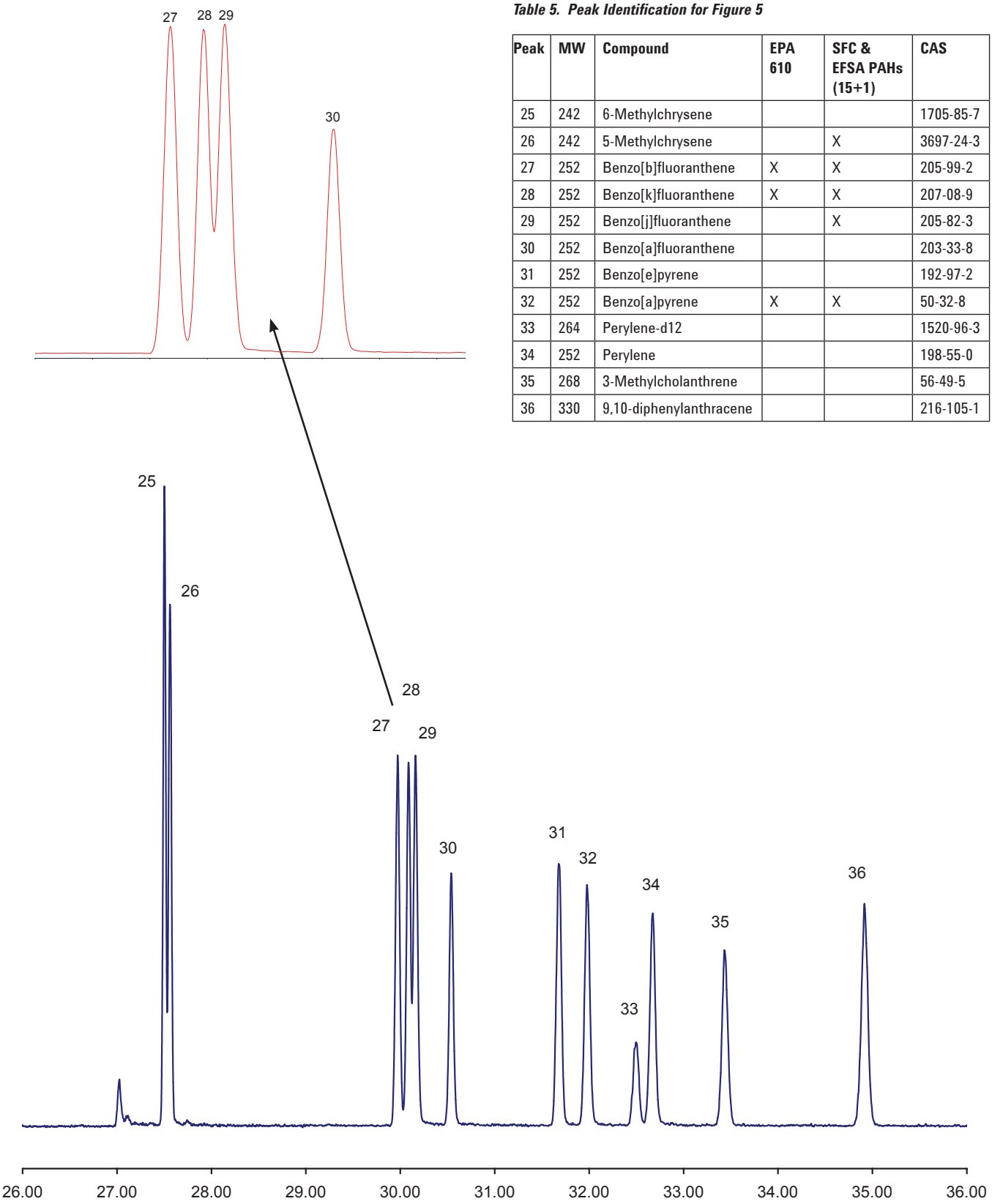


Figure 5. Details and identification of peaks 25 to 36

Table 5. Peak Identification for Figure 5

Peak	MW	Compound	EPA 610	SFC & EFSA PAHs (15+1)	CAS
25	242	6-Methylchrysene			1705-85-7
26	242	5-Methylchrysene		X	3697-24-3
27	252	Benzo[b]fluoranthene	X	X	205-99-2
28	252	Benzo[k]fluoranthene	X	X	207-08-9
29	252	Benzo[j]fluoranthene		X	205-82-3
30	252	Benzo[a]fluoranthene			203-33-8
31	252	Benzo[e]pyrene			192-97-2
32	252	Benzo[a]pyrene	X	X	50-32-8
33	264	Perylene-d12			1520-96-3
34	252	Perylene			198-55-0
35	268	3-Methylcholanthrene			56-49-5
36	330	9,10-diphenylanthracene			216-105-1

Table 6. Peak Identification for Figure 6

Peak	MW	Compound	EPA 610	SFC & EFSA PAHs (15+1)	CAS
37	279	Dibenzo[a,h]acridine			226-36-8
38	279	Dibenzo[a,j]acridine			224-42-0
39	278	Dibenzo[a,j]anthracene			224-41-9
40	292	Dibenzo[a,h] anthracene-d14			13250-98-1
41	278	Benzo[b]triphenylene			215-58-7
42	276	Ideno[1,2,3-c,d]pyrene	X	X	193-39-5
43	278	Dibenzo[a,h]anthracene	X	X	53-70-3
44	278	Benzo[b]chrysene			214-17-5
45	278	Picene			213-46-7

Peak	MW	Compound	EPA 610	SFC & EFSA PAHs (15+1)	CAS
46	276	Benzo[g,h,i]perylene	X	X	191-24-2
47	276	Dibenzo[def,mno]chrysene			191-26-4
48	267	7H-Dibenzo[c,g]carbazole			194-59-2
49	302	Dibenzo[a,l]pyrene			191-30-0
50	302	Dibenzo[a,e]pyrene		X	192-65-4
51	300	Coronene			191-07-1
52	302	Benzo[b]perylene			197-70-6
53	302	Dibenzo[a,i]pyrene		X	189-55-9
54	302	Dibenzo[a,h]pyrene		X	189-64-0

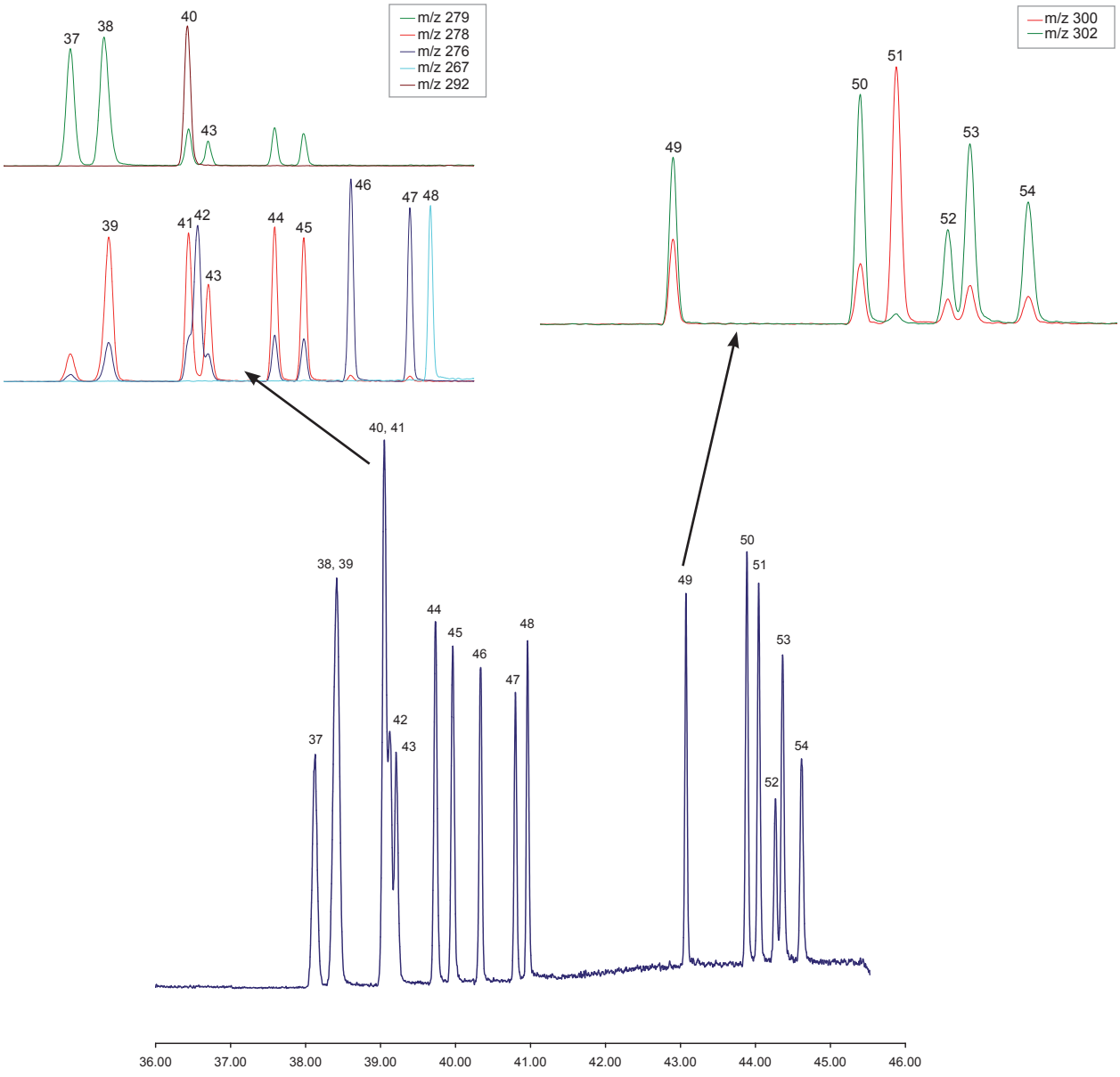


Figure 6. Details and identification of peaks 37 to 54

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Agilent Technologies

GC Analysis of PAHs using an Agilent J&W FactorFour VF-17ms Column with EZ-Guard

Application Note

Author

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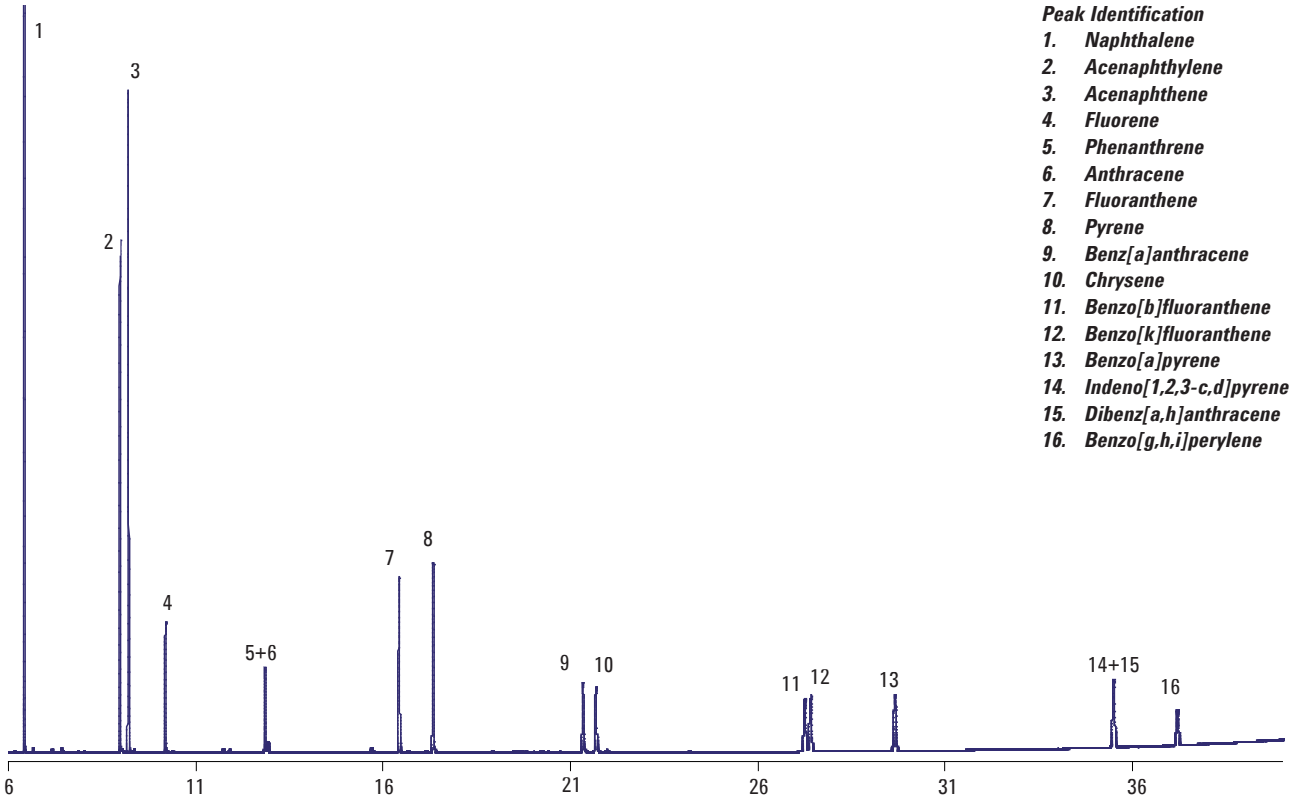
Introduction

Polycyclic aromatic hydrocarbons (PAHs) can be formed during incomplete combustion or pyrolysis of organic matter, industrial processes and cooking and food processing. US and European Union legislation require the monitoring of pollutant PAHs in air, waste, sludge, drinking water and food because of their carcinogenic properties. The matrix of the sample can be complex, depending on the nature of the sample. In particular, food, waste water and sludge can introduce non-volatiles into the column, despite sample preparation. These non-volatile components originating from the sample matrix will adhere to the analytical column and diminish separation and detector response.

By using an EZ-Guard in front of the analytical column, the non-volatile components are trapped on the integrated guard. When column performance diminishes, a meter of the EZ-Guard can be cut off to restore column performance with no loss of resolution in the analytical column.

The VF-17ms GC column is widely used for analyzing PAHs, being chosen for its high selectivity towards certain PAH isomers. This application note demonstrates the selectivity of the VF-17ms with EZ-Guard in the analysis of these important pollutants.

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Analysis of polycyclic aromatic hydrocarbons using a VF-17ms column with EZ-Guard

Materials and Methods

Technique:	GC-FID
Column:	FactorFour VF-17ms, 30 m x 0.25 mm ID, df=0.25 µm, + 10 m EZ-Guard (part number CP9025)
Temp Program:	70 °C (1.7 min) to 180 °C (20 °C/min) to 280 °C (8 °C/min) 5 min isothermal, to 350 °C (4 °C/min), 10 min isothermal
Carrier Gas:	He
Pressure:	2.0 bar
Injector Temp:	275 °C
Detector Temp:	325 °C, FID
Splitless Time:	1 min (split 100 ml/min)
Sample Size:	2 µL
Sample Info:	4-27 ug/mL, solvent acetonitrile

Results and Discussion

The figure above shows the performance of the VF-17ms with EZ-Guard for PAH analysis. EZ-Guard does not interfere with the column's inertness, as shown by the excellent peak shapes.

Conclusion

With the VF-17ms with EZ-Guard, Agilent introduces a GC column ideal for PAH analysis in complex matrices.

The EZ-Guard extends the lifetime of the GC column by trapping the non-volatile components arising from the sample matrix. In addition, EZ-Guard columns are quick, and easy to install and operate, boosting productivity and delivering improved efficiency as the integrated transfer line provides faster detector stabilization.

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Analysis of all 136 tetra- through octa- polychlorinated dibenzo-p-dioxins and dibenzofurans using an Agilent J&W FactorFour VF-Xms GC column

Application Note

Authors

Johan Kuipers
Agilent Technologies, Inc.

Vyacheslav N. Fishman
The Dow Chemical Company

Introduction

Out of the 136 polychlorinated dibenzo-p-dioxins (PCDD) and polychlorinated dibenzofurans (PCDF) congeners (tetra- through octa-), there are 17 congeners with chlorines in the 2378-positions that are considered to be of toxicological significance and are typically used for Toxic Equivalence (TEQ) calculations.

In order to establish TEQ values, an accurate determination of isomer-specific concentrations of all 17 2378-substituted dioxins and furans is required. Most of the regulatory methods for the PCDD/PCDF analysis have been developed based upon a dual column approach using a combination of non-polar and polar GC columns.

In this note, data is presented for the elution order of all 136 tetra- through octa- polychlorinated dioxins and furans, including the separation of 16 out of 17 2378-substituted dioxins and furans from other close-eluted isomers. We used a high Si-arylene modified liquid phase column, VF-Xms, with no need for a second column.

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Materials and Methods

Column:	VF-Xms Fused Silica, 60 m x 0.25 mm, df = 0.25 µm (part number CP8809)
Carrier Gas:	He, constant pressure at 290 kPa or 42 psi
Oven Temp:	160 ºC for 1 min 160-230 ºC at 35 ºC/min, hold for 18 min 230-250 ºC at 10 ºC/min, hold for 25 min 250-310 ºC at 10 ºC/min, hold for 6 min
Interface Temp:	290 ºC
Injection:	Splitless, 270 ºC, 1 µL
Detector:	MAT-95XP double focusing high raesolution magnetic sector mass spectrometer, EI 42eV, Ion source 270 °C, Acceleration voltage 4700V, SIM mode at resolution > 10,000 (10% valley)
Sample:	128 qualitative standards (tetra- through hexa-) PCDD/PCDF with an approximate concentration of 25 ng/mL in nonane. (Cambridge Isotope Laboratories Andover, MA, USA). In addition, native HpCDD, HpCDF, OCDD and OCDF were added to the above mixture

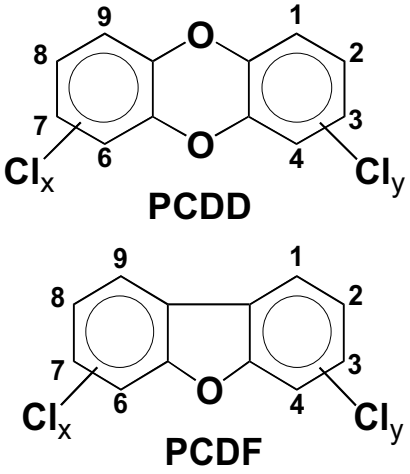


Figure 1. Chemical structures of PCDD and PCDF congeners

Table 1. Number of isomers of the PCDD and PCDF

Chlorines (x+y)	Acronyms	PCDD number of isomers	PCDF number of isomers
4	TCDD/TCDF	22	38
5	PnCDD/ PnCDF	14	28
6	HxCDD/ HxCDF	10	16
7	HpCDD/ HpCDF	2	4
8	OCDD/OCDF	1	1
4-8	Total	49	87

Results

Figures 2 and 3 show the separation of PCCD/PCDF where quantitative results can be easily achieved on 16 out of 17 2378-substituted congeners. Note that critical 2378-TCDD and 2378-TCDF separations can be easily achieved with better than 25% valley as typically required by regulatory methods.

Conclusion

The VF-Xms liquid phase provides superior performance towards dioxins and furans separations compared to other GC columns, delivering a high degree of quantification accuracy for 2378-substituted congeners. The ability to separate 2378-TCDF at the baseline from other closely eluting isomers means there is no need for confirmatory analysis by a complementary GC column when strictly following US and Canadian regulatory methods.

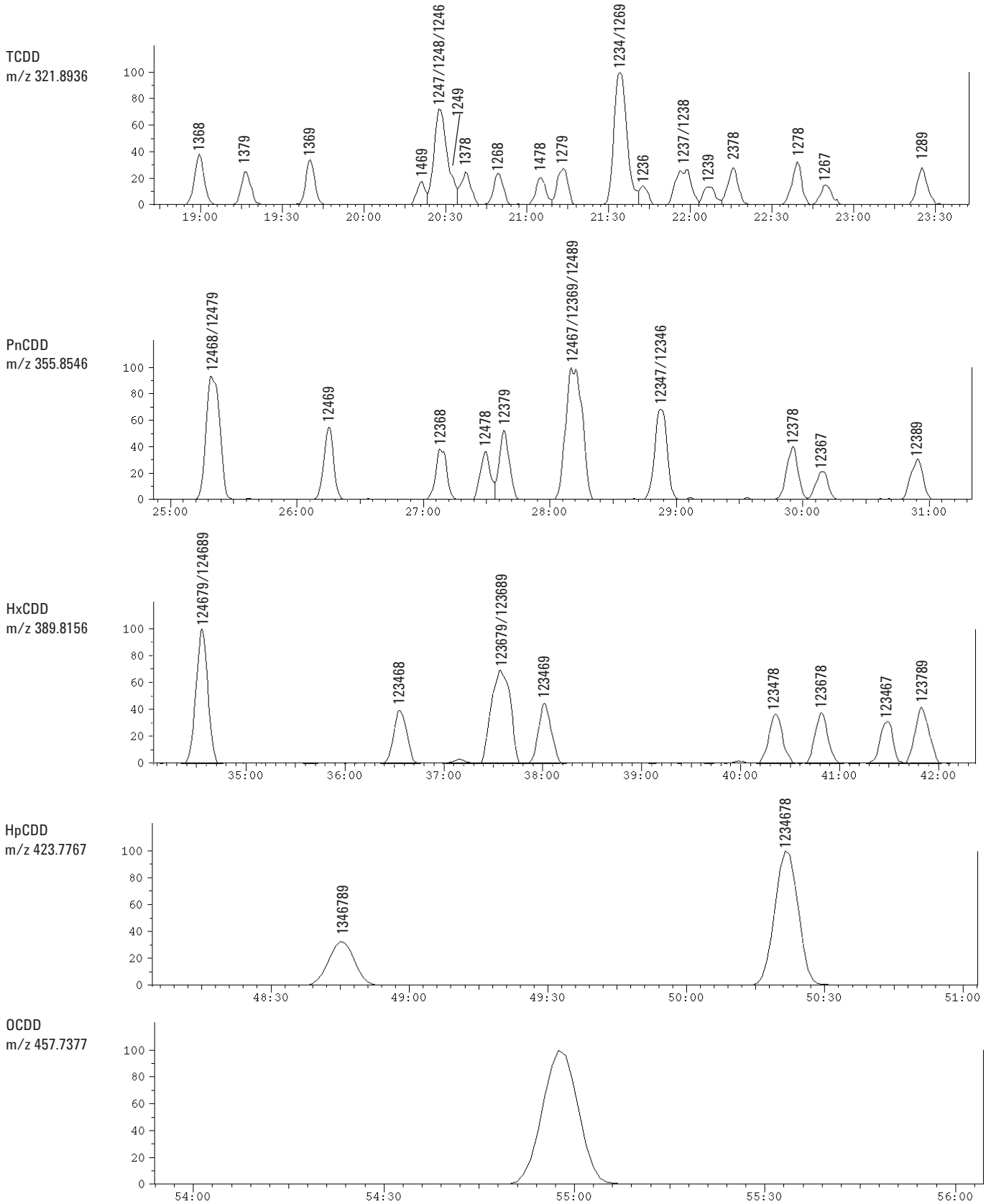


Figure 2. Separations of polychlorinated dibenzo-p-dioxins on a VF-Xms GC column

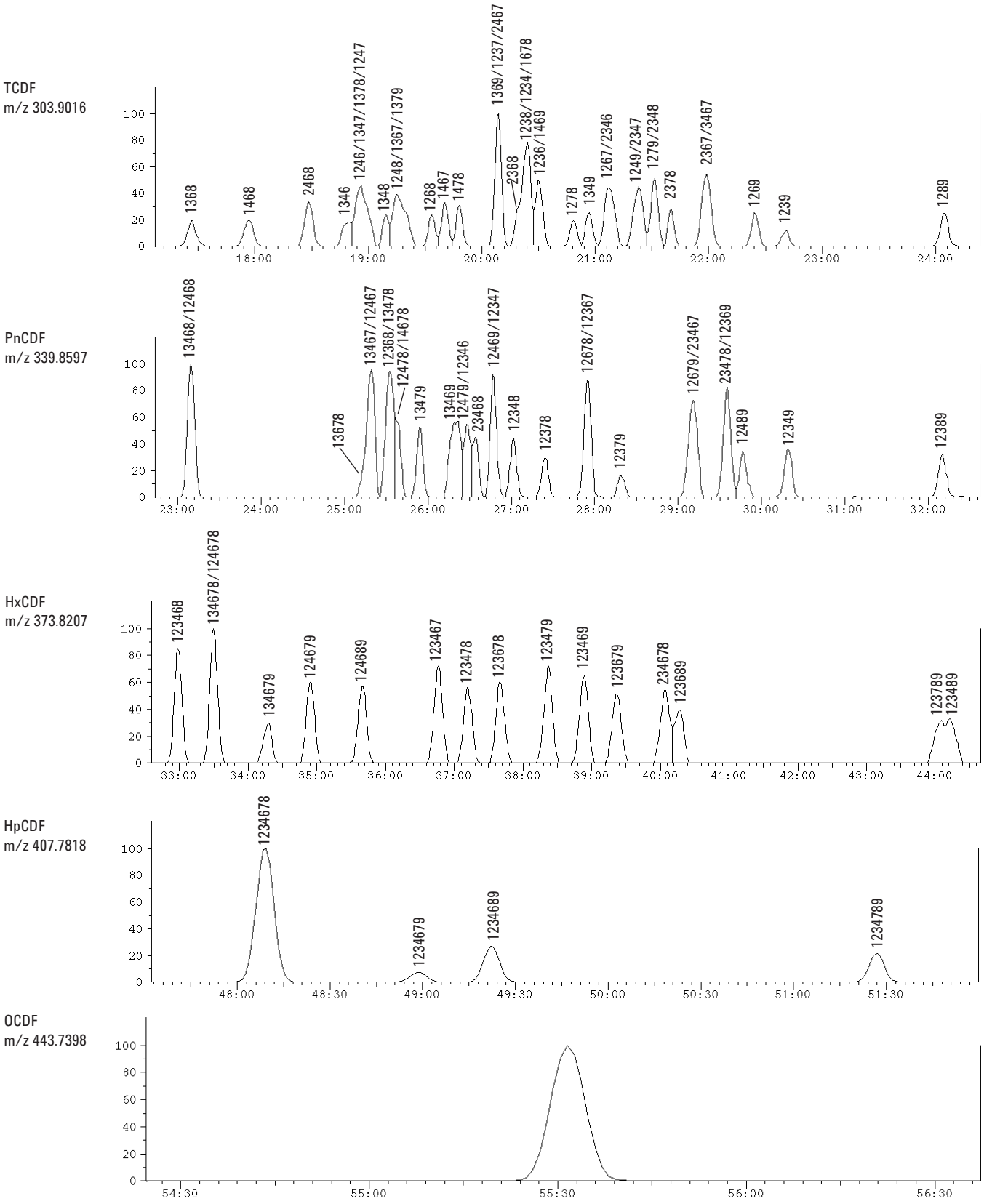


Figure 3. Separations of polychlorinated dibenzofurans on a VF-Xms GC column

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Agilent Technologies

Fast GC Analysis of EPA and EU Regulated PAHs

Application Note

Authors

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Agenzia Regionale per la Protezione Ambientale della Toscana

Introduction

After assessing 33 PAHs, the European Commission Scientific Committee on Food (SCF) in 2002 found that due to their toxicity, 15 of them were of major concern to human health and should therefore be monitored. The FAO/WHO Expert committee on Food Additives identified benzo(c)fluorine in 2005 as an additional compound that should be analyzed. These 15+1 EU priority PAHs differ from the 16 PAHs covered under the US EPA regulations.

With the increase of the number of target PAH analytes and the fact that many PAHs and their isomers exhibit identical MS mass fragmentation, the chromatographic separation and selectivity of the stationary phase for the analysis of PAHs has become more important.

This application note illustrates the successful use of an Agilent J&W FactorFour VF-17ms column from Agilent Technologies, Inc. for the fast GC/MS analysis of most PAHs listed in the EU and EPA regulations (Figure 1).

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Conditions

Technique:	GC/MS
Column:	VF-17ms 20 m x 0.15 mm df = 0.15 mm (p/n CP5884), retention gap 2.5 m x 0.53 mm (p/n CP8009)
Temperature:	90 °C (1 min), 78 °C/min, 130 °C (0.4 min), 21 °C/min, 190 °C (1.90 min), 15.7 °C/min, 320 °C (12 min)
Carrier Gas:	Helium, constant flow 1 mL/min
Injector:	Splitless mode, pulsed pressure 50 psi for 0.2 min, 280 °C
Detector:	Quadrapole MS, EI in SIM, ion source 230 °C, transfer line 280 °C
Sample:	Concentration 5 ppm, isotope ISTDs 0.5 ppm, solvent toluene
Injection Vol:	1 µL

Results

Critical peak pairs such as the benzofluoranthene isomers (m/z 252) are almost baseline resolved (Figure 3). Although the VF-17ms stationary phase exhibits an excellent selectivity towards difficult to separate PAHs, the coelution of some peak pairs cannot be avoided. Indeno[1,2,3-cd]pyrene (m/z 276) and dibenzo[a,h]anthracene (m/z 278) coelute but can be distinguished by their difference in molecular masses (Figure 4). Crysene cannot be unambiguously quantified due to the possible coelution with triphenylene and identical quantifying ions (m/z 228), but is well resolved from benzo(a) anthracene (Figure 5). The four EU priority dibenzopyrenes (m/z 302) are well separated by VF-17ms (Figure 6). However, due to the large number of isomers with the same molecular mass, possible interferences may influence results.

The temperature program was carefully optimized to obtain optimal results in terms of separation and speed of analysis.

Discussion

The VF-17ms column efficiently separates all 16 US regulated PAHs as well as the 15+1 EU priority PAHs. The 50% phenyl groups incorporated in the liquid phase provide additional selectivity based on π/π stacking interactions and electrostatic supported separation mechanisms. The smaller 0.15 mm ID column delivers fast analysis within 25 minutes without compromizing the resolution efficiency needed for the separation of critical PAH peak pairs. Quantification of the 23 PAHs of this particular analysis is carried out by means of isotope dilution using deuterium labelled PAH internal standards. Perylene D12 is used as an additional standard to correct for response variations between standard and sample analysis due to matrix influences during injection.

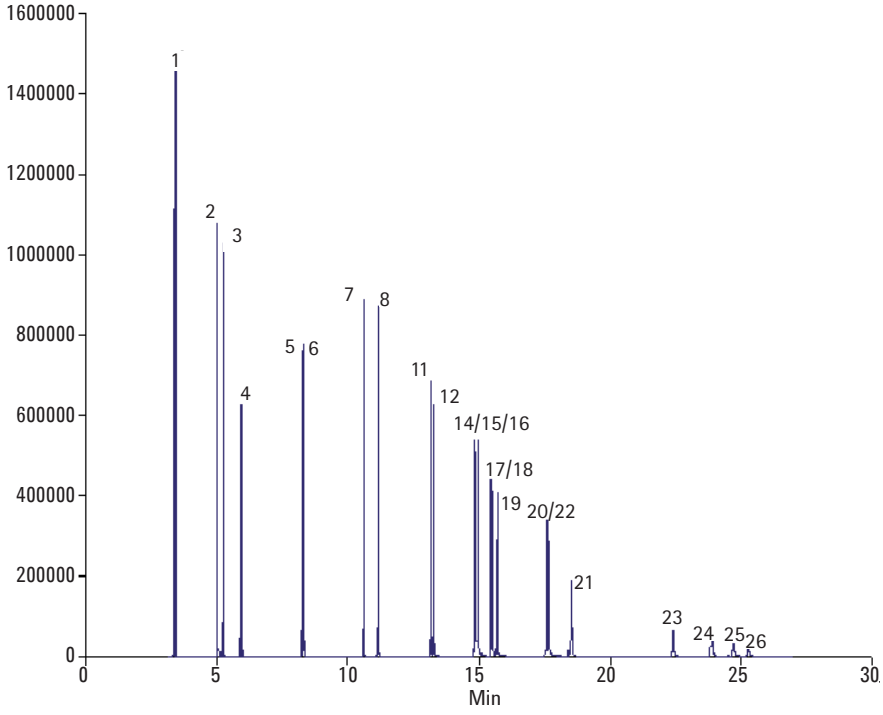


Figure 1. Fast GC/MS analysis of EU and EPA PAHs on VF-17ms 20 m x 0.15 mm x 0.15 µm

Peak Identification

	PAH	Abbre-viation	MW	EU PAHs	EPA PAHs
1	Napthalene	Nap	128		x
2	Acenaphthylene	Acy	152		x
3	Acenaphtene	Acp	154		x
4	Fluorene	Flr	166		x
5	Phenanthrene	Phen	178		x
6	Anthracene	Ant	178		x
7	Fluoranthene	Flt	202		x
8	Pyrene	Pyr	202		x
9	Benzo[c]fluorene	B[c]F	216	x	
10	Cyclopenta[cd]pyrene	CCP	226	x	
11	Benzo[a]anthracene	BaA	228	x	x
12	Chrysene	Chr	228	x	x
13	5-Methylchrysene	5-MC	242	x	
14	Benzo[b]fluoranthene	BbF	252	x	x
15	Benzo[k]fluoranthene	BkF	252	x	x
16	Benzo[j]fluoranthene	BjF	252	x	
17	Benzo[e]pyrene	BeP	252		
18	Benzo[a]pyrene	BaP	252	x	x
19	Perylene	Per	252		
20	Indeno[1,2,3-cd]pyrene	IcdP	276	x	x
21	Benzo[ghi]perylene	BghiP	276	x	x
22	Dibenzo[a,h]anthracene	DahA	278	x	x
23	Dibenzo[a,l]pyrene	DalP	302	x	
24	Dibenzo[a,i]pyrene	DaiP	302	x	
25	Dibenzo[a,e]pyrene	DaeP	302	x	
26	Dibenzo[a,h]pyrene	DahP	302	x	

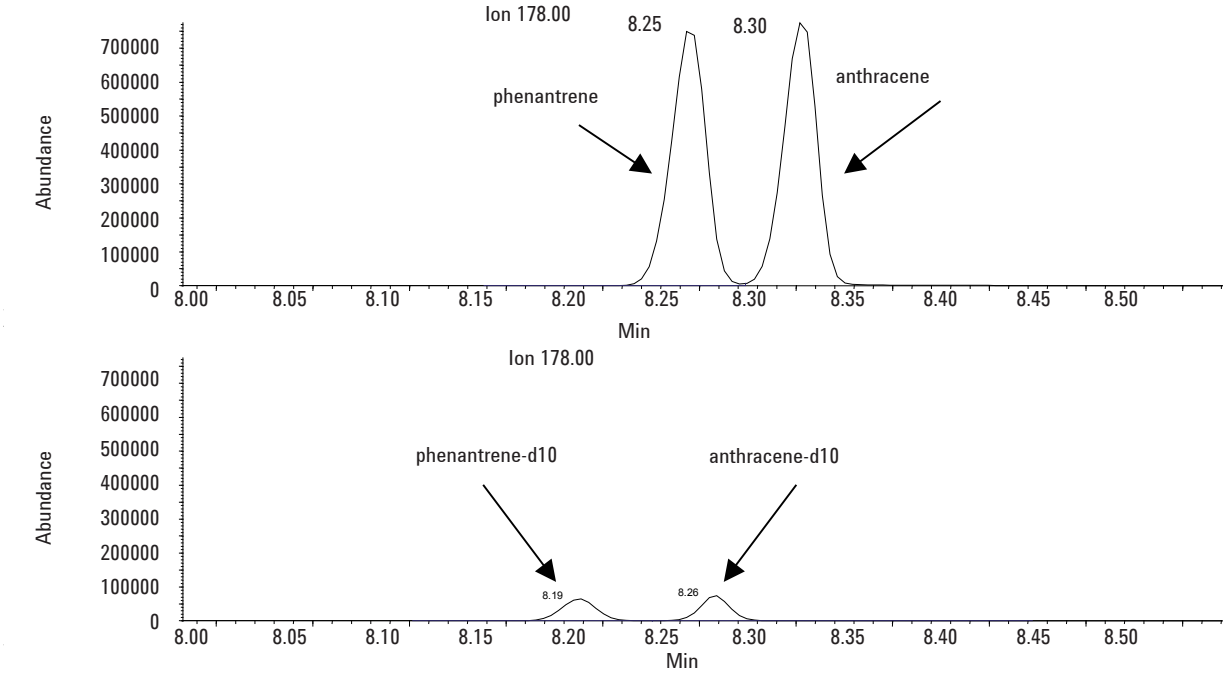


Figure 2. Phenanthrene and Fluoranthene both with m/z 178 are efficiently separated

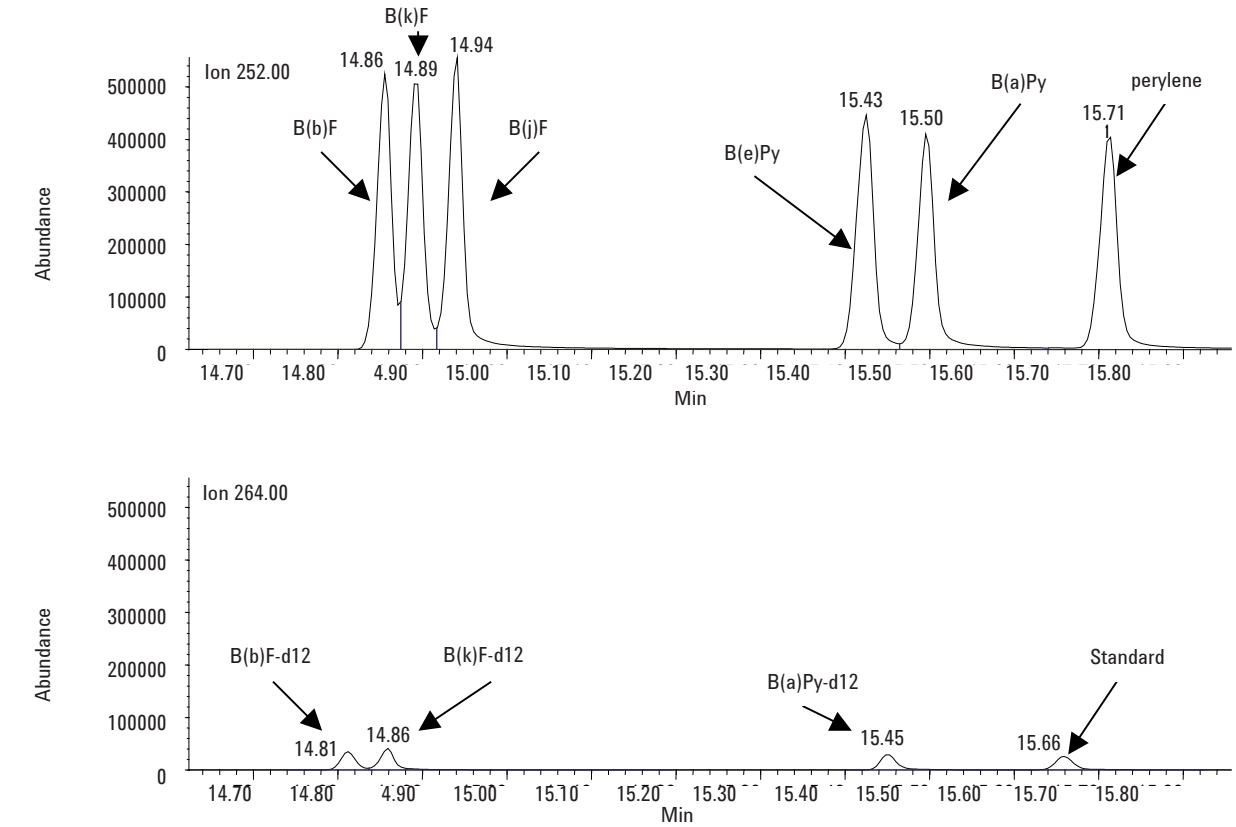


Figure 3. The most critical separations of the PAHs with m/z 252. The benzofluoranthenes isomers are almost baseline resolved

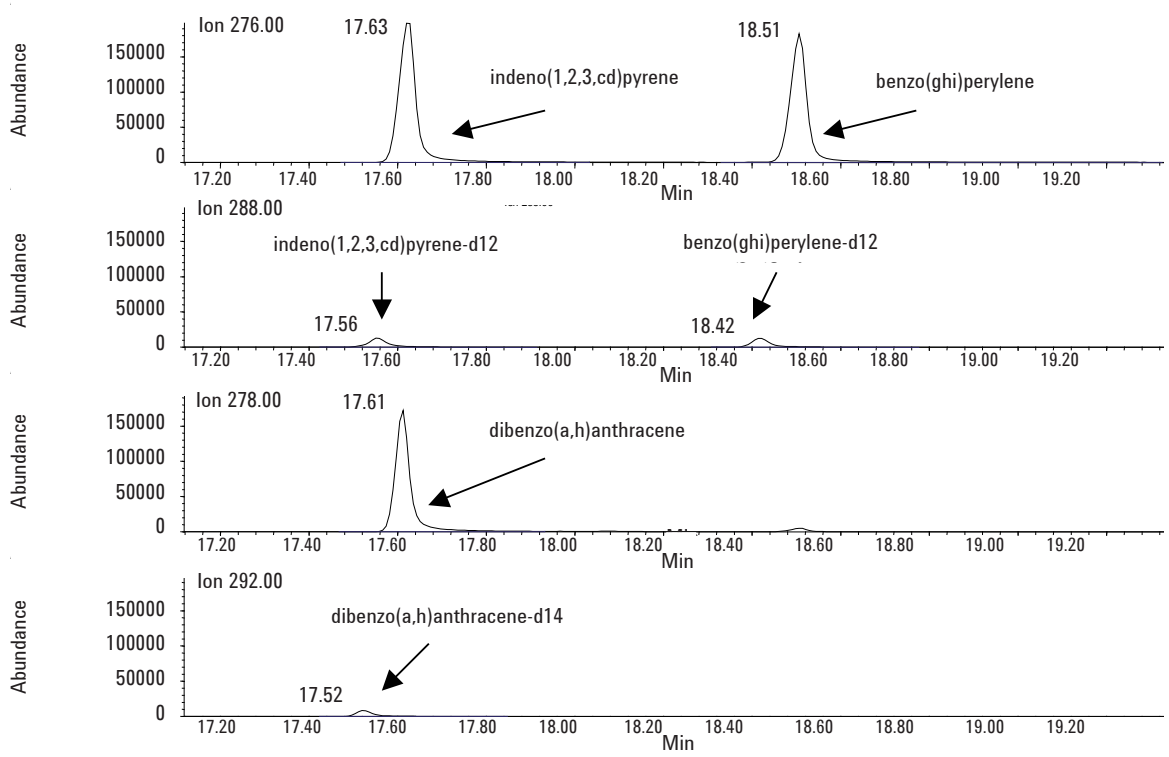


Figure 4. The VF-17ms is able to separate indeno(1,2,3,cd) pyrene from dibenzo(a,h)anthracene using different temperature programs, in this analysis they are discriminated by their difference in molecular masses

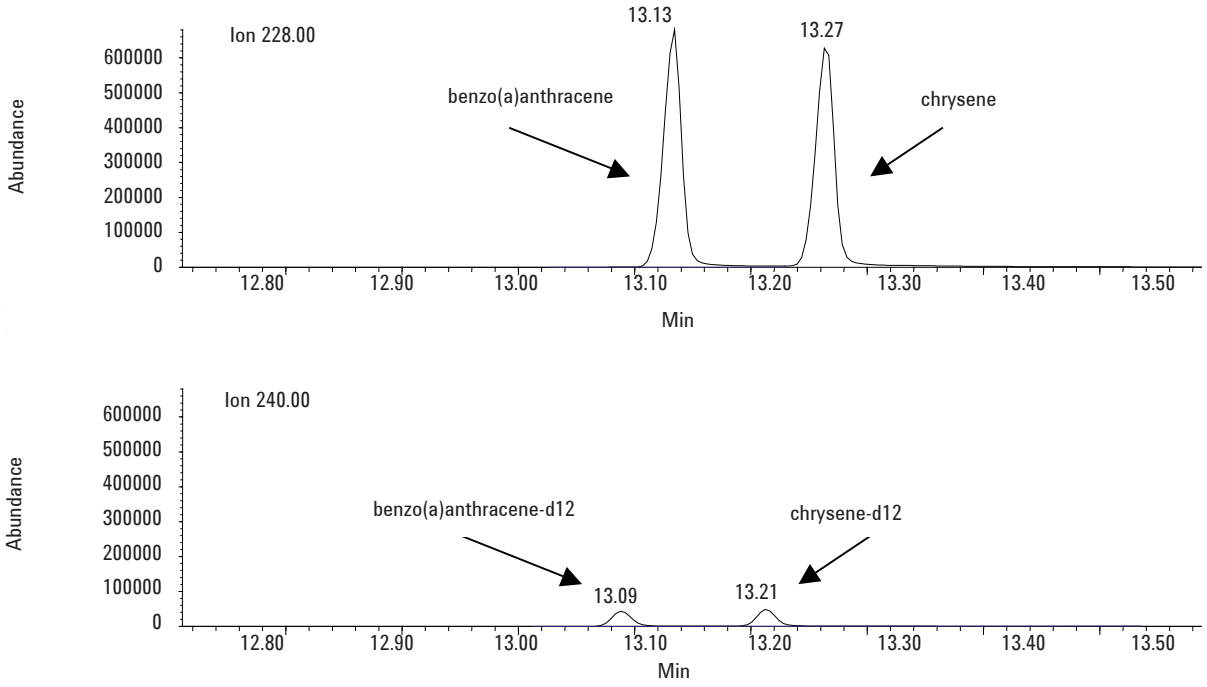


Figure 5. Benzo(a)anthracene and chrysene are well resolved

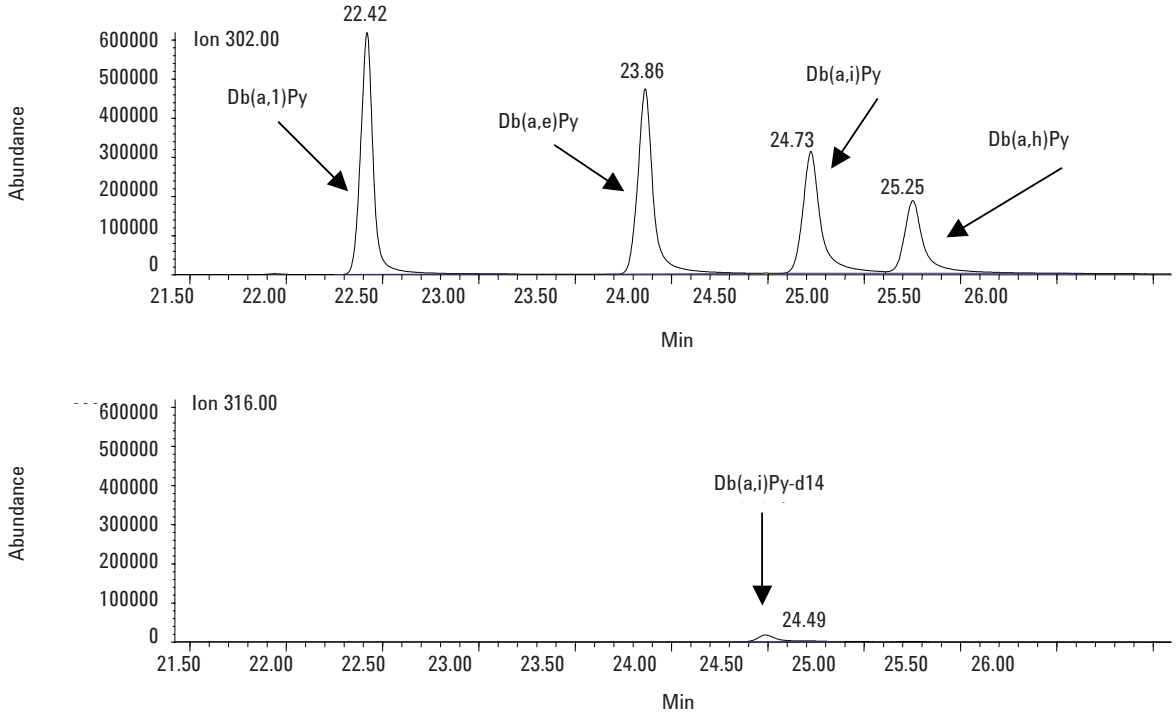


Figure 6. The last target compound dibenzo(a,h)pyrene elutes in about 25 minutes

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New SPE Sorbent for Clean-up of Fusarium Toxin-contaminated Cereals & Cereal-based Foods, Bond Elut Mycotoxin

Application Note

Fusarium Fungi, Cereals

Authors

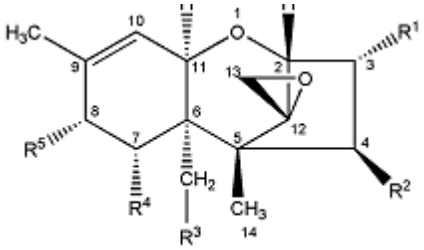
Marianna Klötzel, Uwe Lauber
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Introduction

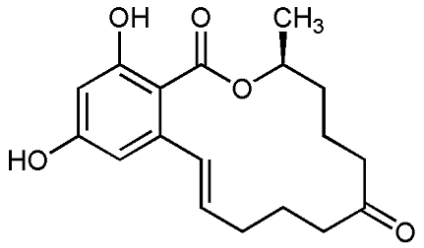
Fusarium fungi are probably the most prevalent toxin-producing fungi of the northern temperate regions and are commonly found on cereals grown in the temperate regions of America, Europe and Asia. A variety of fusarium fungi produce different toxins of the class of trichothecenes. More than 140 of these compounds have been isolated from fungi cultures and this number is still increasing[1]. The toxic effects of fusarium toxins on humans and animals are well-documented[2,3] and reliable and sensitive analysis methods, which comply with the European regulations for mycotoxin determination in food and feed, are required[4, 5]. Traditional sample preparation for trichothecene analysis typically involves extraction with acetonitrile/water and clean-up via charcoal-alumina columns[6]. As the trichothecenes differ considerably in polarity and solubility, recoveries of the more polar analytes are often compromised with this approach. Another approach is the use of immunoaffinity columns (IAC)[7]. These provide highly selective extractions with high recoveries, however, separate IAC columns are needed for each toxin. To overcome the limitations of these methods, there was a need to develop an extraction and clean-up method for the simultaneous determination of several trichothecenes with high recoveries for polar toxins by minimizing the matrix effects.

This application note shows the optimized extraction and clean-up step of 12 type A- and B-trichothecenes and zearalenone (ZEA) in cereals and cereal-based food on Bond Elut Mycotoxin, a newly developed extraction sorbent. Structures and names of the 12 toxins investigated in this application are shown in Figure 1.

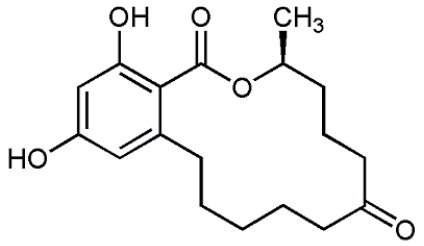
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Trichothecenes



Zearalenone



Zearalanone



A healthy wheat head (left) next to one showing severe symptoms of Fusarium head blight disease (right) (Photo by Keith Weller, ARS US Department of Agriculture)

	Trichothecene	R1	R2	R3	R4	R5
Type A	Neosolaniol (NEO)	OH	OAc	OAc	H	OH
	HT-2 toxin (HT-2)	OH	OH	OAc	H	$OCOCH_2CH(CH_3)_2$
	T-2 toxin (T-2)	OH	OAc	OAc	H	$OCOCH_2CH(CH_3)_2$
	T-2 triol	OH	OH	OH	H	$OCOCH_2CH(CH_3)_2$
	T-2 tetraol	OH	OH	OH	H	OH
	Monoacetoxyscirpenol (MAS)	OH	OH	OAc	H	H
	Diacetoxyscirpenol (DAS)	OH	OAc	OAc	H	H
Type B	Deoxynivalenol (DON)	OH	H	OH	OH	=O
	3-Acetyl-DON (3ADON)	OAc	H	OH	OH	=O
	15-Acetyl-DON (15DON)	OH	H	OAc	OH	=O
	Nivalenol (NIV)	OH	OH	OH	OH	=O
	Fusarenon-X (FUS)	OH	OAc	OH	OH	=O

Figure 1. Chemical structure of type A- and B-trichothecenes, zearalenone (ZEA) and zearalanone (ZAN). Type A trichothecenes have various groups at ring position 8, type B-trichothecenes have a carbonyl function at position 8

Extraction and Clean-up

Clean-up methods of trichothecenes and ZEA from cereals and cereal-based foods widely use commercially available polar clean-up columns. Analytical interfering substances are retained while trichothecenes are not adsorbed on the packing material. This purification method, however, gives low recoveries for the polar toxins NIV, T-2 tetraol and DON. New studies[9] point out that one possible reason for the low recoveries for the polar toxins might be the low water content in the acetonitrile/water mixture (ACN/H_20; 84/16; v/v), which is used for the extraction of the mycotoxins from the matrix. Furthermore, to elute these polar compounds from the column containing polar adsorbents like alumina, a hydrophilic solvent is needed. When using more polar extraction mixtures like ACN/H_20 (75/25; v/v), the recoveries of the polar toxins NIV, T-2 tetraol and DON could be raised. However, the higher the content of water in the extraction solvent resulted in co-extraction of more matrix compounds and led therefore to strong ion suppression in the LC-MS analysis.

To address these problems, we optimized the extraction step by marginally increasing the polarity of the extraction solvent to ACN/H_20 (80/20; v/v) and used the Bond Elut Mycotoxin cartridge to clean up the extracts. Trials with the polar DON reference material from Food Analysis Performance Assessment Scheme (FAPAS) confirm that the best recovery data were achieved with the Bond Elut Mycotoxin method (Table 1).

Table 1. Recovery comparison of Food Analysis Performance Assessment Scheme (FAPAS) certified reference material for DON applying LC-MS/MS.

Reference Material	Certified Value (µg/kg)	Method 1 (µg/kg)	Method 2 (µg/kg)
FAPAS T2210	463 ± 167	495 ± 5	395 ± 15

Method 1: Clean-up on Bond Elut Mycotoxin
Method 2: Clean-up on competitor column (charcoal-alumina)

To calculate the amount of ZEA, the extracted matrices were spiked with a defined amount of zearalanone (ZAN) standard solution before the clean-up step on Bond Elut Mycotoxin. The measured value of ZEA was corrected by the value of ZAN, as previously described by Berthiller et al[8]. Using ZAN as internal standard, the recovery of ZEA was about 100%.

Bond Elut Mycotoxin Method

1. Extract 25 g of finely ground sample with a solution of 100 mL acetonitrile/water (80/20; v/v) by blending at high speed for 3 minutes. For simultaneous determination of zearalenone (ZEA), spike extract at a level of 50 ng/g sample with zearalanone (ZAN) solution in acetonitrile as internal standard.

2. Filter.

3. Pass 4 mL of the filtrate through a Bond Elut Mycotoxin column (part number 12165001B).

4. Evaporate 2 mL of the eluate to dryness at 50 °C under a gentle stream of nitrogen.

5. Reconstitute in 0.5 mL acetonitrile/water (20/80; v/v). Inject 10 µL into LC-MS/MS for analysis.

Results and Discussion

The Bond Elut Mycotoxin product provides a single column method for the clean-up of 12 type A- and B-trichothecenes plus ZEA (corrected via internal standard ZAN).

Table 2 shows the average recoveries and RSDs obtained for 12 trichothecenes and ZEA from spiked wheat, corn, durum, oats, bread, muesli and cereal infant food samples after clean-up with Bond Elut Mycotoxin columns. By combining an increased polarity of the extraction solvent with the clean-up step on Bond Elut Mycotoxin, recoveries, especially for the polar toxins DON, NIV, 3ADON and T-2 tetraol, were increased up to 31% when compared to the extraction method on charcoal-alumnia cartridges[9].

Table 3 shows the trichothecene content of 6 naturally contaminated samples after 3 different clean-up methods. Up to 43% higher values were achieved in the analysis of naturally contaminated samples for the polar toxins DON, NIV, 3ADON, 15ADON and T-2 tetraol in comparison to the charcoal-alumina based method. If the determination of DON alone is of interest, then the highest content can be achieved with an extraction of 100% water and clean-up with IAC; however, for the determination of 12 trichothecenes with different polarities, the Bond Elut Mycotoxin provides comparable results.

Conclusion

As the performance of the Bond Elut Mycotoxin cartridges is similar or better, and the columns are more cost effective, the new clean-up procedure is a very good alternative to other standardized methods commonly used.

Table 2. Average recovery and RSD in percentage obtained for 12 trichothecenes and ZEA from spiked wheat, corn, durum, oats, bread, muesli and cereal infant food samples (spiking levels of 50/100, 200/400 and 500/1000 ng/g for trichothecenes/DON and 50 ng/g for ZEA and ZAN), after clean-up with Bond Elut Mycotoxin columns, (n=3). Data reported by Klötzel et al[10].

Toxin	Recovery [%] ± RSD [%], 3 levels, n = 3						
	Wheat	Corn	Durum	Oats	Bread	Muesli	Infant Food
DON	90 ± 5.2	93 ± 2.8	98 ± 3.8	96 ± 5.1	87 ± 1.7	87 ± 3.7	88 ± 12
NIV	67 ± 5.9	74 ± 2.5	67 ± 6.3	73 ± 10	65 ± 5.7	71 ± 13	66 ± 10
3ADON	89 ± 9.3	88 ± 7.6	97 ± 6.6	93 ± 11	100 ± 5.5	101 ± 7.1	91 ± 9.4
15ADON	92 ± 13	87 ± 15	89 ± 11	89 ± 11	96 ± 9.5	98 ± 8.3	96 ± 6.6
FUS	91 ± 10	94 ± 4.2	91 ± 7.8	91 ± 7.8	98 ± 8.5	97 ± 6.4	96 ± 4.3
T-2	87 ± 7.6	88 ± 8.8	84 ± 2.2	84 ± 2.2	83 ± 8.2	75 ± 11	70 ± 7.3
HT-2	82 ± 7.3	91 ± 3.3	85 ± 5.0	85 ± 5.0	79 ± 3.3	70 ± 7.7	74 ± 0
NEO	91 ± 2.6	78 ± 11	68 ± 18	68 ± 18	80 ± 2.0	104 ± 10	71 ± 6.3
DAS	82 ± 8.3	89 ± 3.6	85 ± 5.2	85 ± 5.2	75 ± 3.7	82 ± 6.8	68 ± 4.6
MAS	86 ± 13	85 ± 12	93 ± 4.2	93 ± 4.2	86 ± 11	88 ± 16	91 ± 14
T-2 triol	69 ± 9.1	66 ± 1.2	83 ± 2.8	83 ± 2.8	76 ± 9.3	82 ± 3.3	71 ± 7.9
T-2 tetraol	69 ± 12	75 ± 6.8	73 ± 10	73 ± 10	65 ± 11	67 ± 17	70 ± 16
ZEA	110 ± 5.9	113 ± 5.0	108 ± 4.8	108 ± 4.8	111 ± 6.0	102 ± 2.7	116 ± 6.7

Table 3. Trichothecene contents of six naturally contaminated samples analyzed with DONPrep IAC, MycoSep 227 and Bond Elut Mycotoxin cartridges, (n=3). Data reported by Klötzel et al[10].

Sample	Clean-up	DON [ng/g]	NIV [ng/g]	15ADON [ng/g]	HT-2 [ng/g]	T-2 [ng/g]	T2 tetraol [ng/g]
Bread	IAC	690 ± 18					
Bread	Mycosep	557 ± 19					
Bread	Bond Elut Mycotoxin	648 ± 21					
Corn	IAC	368 ± 8.4					
Corn	Mycosep	333 ± 14	12 ± 0	69 ± 2.0			
Corn	Bond Elut Mycotoxin	356 ± 3.8	14 ± 0	99 ± 2.5			
Wheat	IAC	488 ± 5.5					
Wheat	Mycosep	421 ± 16					
Wheat	Bond Elut Mycotoxin	468 ± 19					
Oats	IAC	299 ± 11					
Oats	Mycosep	220 ± 5.3	22 ± 3.3	7.0 ± 0.4	93 ± 12	15 ± 4.1	91 ± 6.2
Oats	Bond Elut Mycotoxin	264 ± 13	19 ± 1.2	7.7 ± 0.1	78 ± 4.9	12 ± 4.3	106 ± 3.2
Wheat	IAC	1680 ± 32					
Wheat	Mycosep	1590 ± 40	39 ± 3.7	24 ± 2.0			
Wheat	Bond Elut Mycotoxin	1750 ± 120	64 ± 1.4	42 ± 2.2			
Durum	IAC	512 ± 15					
Durum	Mycosep	407 ± 25	25 ± 4.1				
Durum	Bond Elut Mycotoxin	456 ± 42	22 ± 1.3				

Ordering Information

Part number	Description
12165001B	Bond Elut Mycotoxin 1 gm in JR cartridge, 100 cartridges/pk
12131009	Reservoir 6 mL 100 tubes/pk
12131015	Reservoir 6 mL with 2 x 20 µm polypropylene filter pre-installed 100/pk
12131021	20 µm polypropylene filter for 6 mL cartridges
12234105	Vac Elut 20 Manifold SPE Cartridge Processing Station with collection rack for 10x75 mm test tubes

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Multi-Residue Confirmation of Pesticides in Honey using Solid Supported Liquid Extraction

Application Note

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Introduction

In recent years, several Belgian beekeepers were asked to shut down their hives due to withering of the hive. The cause of the withering was unknown. A multi-factoral study was initiated, which included a multi-class pesticide residue study from honey, to determine if pesticides were the cause of the decline. The extensive distribution of pesticides causes bees that have been fed on contaminated blossoms to transfer pesticide residues into honey. Multi-residue confirmation methods to identify and quantify widely used pesticides, which could have been the source of the bees decline, needed to be developed. Previously published papers already report determination methods for pesticides in honey, however, most of them analyze only one or two pesticide groups, such as organochlorine or organophosphorous residues. This application note shows the development and validation of 17 pesticides and metabolites of different chemical classes:

Insecticides: Carbofuran (Ca), Methiocarb (Mh), Pirimicarb (Pi), Dimethoate (Dm), Fipronil (Fi), Imidacloprid (Im)

Herbicides: Amidosulfuron (Am), Rimsulfuron (Ri), Atrazine (At), Simazine (Si), Chlorotoluron (Ch), Linuron (Li), Isoxaflutole (Is), Metosulam (Mo)

Fungicides: Diethofencarb (De)

Metabolites: Methiocarb sulfoxide (Mhs), 2-Hydroxytertbutylazine (TOH)

The application described here is based on solid supported liquid-liquid extraction method (SLE) followed by LC/MS/MS. The results are compared to data from standard liquid-liquid extraction (LLE) to check extraction efficiency and appropriateness of the SLE method. More detailed information on the method development and validation has been published previously[1].

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Chem Elut - the solid support

The solid support consists of specially processed, wide-pore, diatomaceous earth packed into clean polypropylene cartridges. The aqueous sample is applied to the dry Chem Elut sorbent. The sample is distributed as a thin film over the chemically inert support, which acts as a stationary phase. Subsequently, elution takes place using immiscible organic solvents. The lipophilic substances are extracted from the aqueous into the organic phase, while the aqueous phase remains on the Chem Elut sorbent. A phase-separation filter is incorporated into the cartridge as a safeguard to ensure that organic eluents remain uncontaminated by aqueous matrix. The extraction on Chem Elut is carried out with gravity only - no vacuum is required (Figure 1).

Sample Preparation and Clean Up

SLE procedure on 5 mL Chem Elut cartridge (part number 12198006)

Method:

1. Spike 1 g honey sample with 20 µL of surrogate standard solution (concentrations listed in Figure 2).

2. Mix with 1.25 mL water and 2.5 mL acetone.

3. Add 1.25 mL of NaCl solution (20 g/100 mL).

4. Apply sample to Chem Elut cartridge by gravity flow.

5. Allow 15 mins for complete adsorption to take place.

6. Elute twice with 10 mL ethyl acetate.

7. Evaporate at 30 °C.

8. Reconstitute with 200 µL acetonitrile/water (10/90).

9. Inject 20 µL into LC/MS/MS.

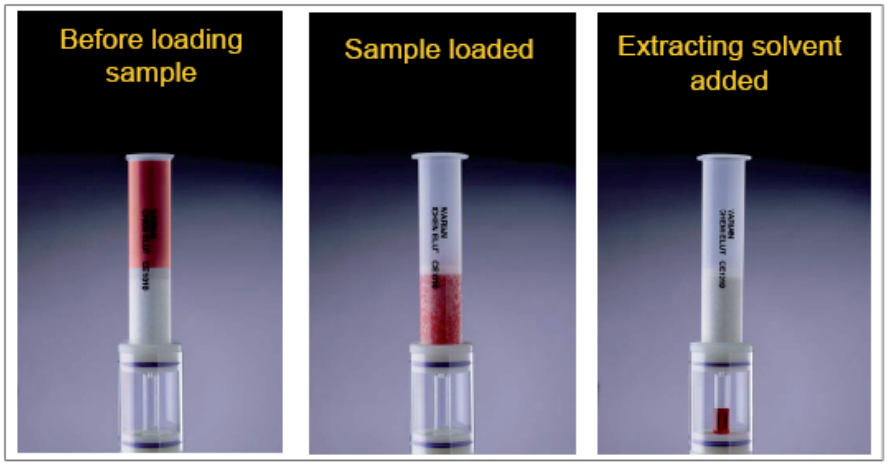


Figure 1. Solid Supported Extraction on Chem Elut cartridges

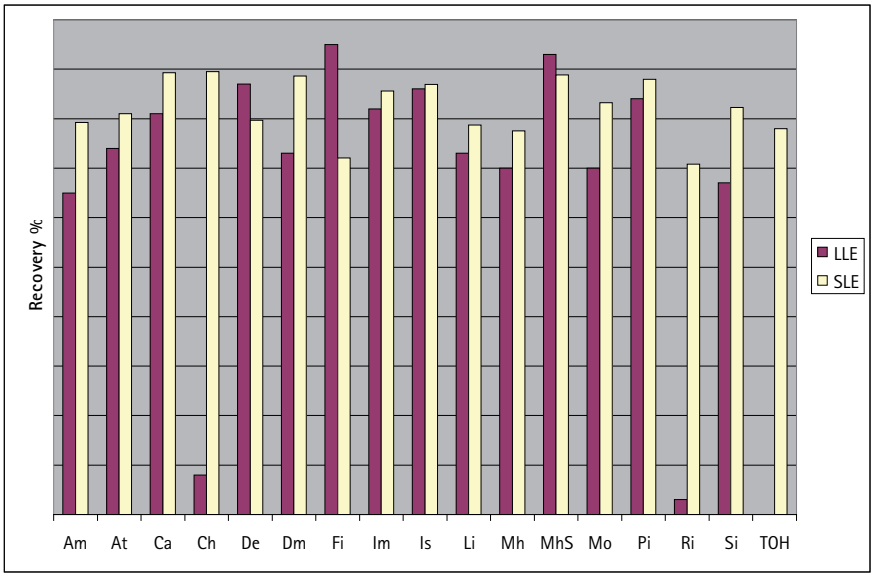
Analysis Conditions

The method is based on HPLC coupled to mass spectrometry (MS) operating in tandem mode (MS/MS) according to EU advice 2002/657/EC [2].

Column:	Polaris C18-A 3 µm, 2.0 x 150 mm (part number A2001150X020)
Temperature:	40 °C
Mobile Phase A:	Water + 0.1% acetic acid
Mobile Phase B:	Acetonitrile + 0.1% acetic acid
Linear Gradient Conditions:	held 10% B for 1 min, to 80% B in 14 mins, to 100% B in 2 mins, held 100% B for 2 mins
Flow Rate:	0.4 mL/min

Results and Discussion

Figure 2 shows the comparison between recoveries obtained after SLE on Chem Elut and classical LLE. The LLE was performed by mixing 1 g honey with 2 mL water and 6.5 mL acetonitrile for 30 mins. After centrifugation the organic layer is evaporated to 100 µL and 100 µL of water is added prior to LC/MS. The comparison shows that the Chem Elut extraction provides similar or even higher extraction efficiency than LLE for most compounds. The greatest advantage of SLE is that the SLE technique avoids emulsion formation, which is standard in LLE, significantly easing the extraction procedure. Key advantages of Chem Elut cartridges are their ease of use and the wide range of compounds that can be extracted efficiently.



Pesticide (ng/mL):
Am 0.4, At 0.4, Ca 0.4, Ch 20.0, De 2.0, Dm 2.0, Fi 10.0, Im 2.0, Is 2.0, Li 2.0, Mh 10.0, MhS 20.0, Mo 2.0, Pi 0.4, Ri 0.4, Si 2.0, TOH 1.0

Figure 2. Recovery comparison of pesticides between solid supported liquid-liquid extraction (SLE) on Chem Elut and classical liquid-liquid extraction (LLE)

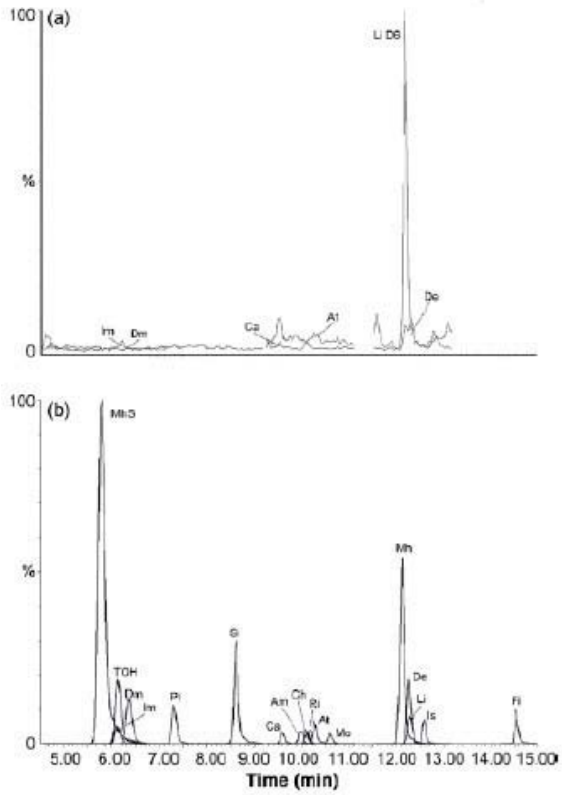


Figure 3. Chromatogram obtained for a honey blank matrix (a) and for a methanolic standard solution (b) using the Polaris C18-A column. Concentrations between 0.4 ng/mL and 20 ng/mL

Figure 3 shows the chromatogram of a methanolic standard solution with pesticide concentration between 0.4 and 20 ng/mL and a blank honey matrix. The total analysis time was 23 mins. The Polaris C18-A column is based on ultra-pure silica with a polar group placed between the primary C18 chain and the silica surface. The resultant packing material contains a surface, which is easily "wetted" with polar eluents and shows unique selectivity for a broad range of chemically different compounds. Furthermore, the polar group shields reactive silanols from polar silanophilic compounds, which improves peak symmetry and minimizes the "collapse" of the C18 chains in high-aqueous eluents. Seventeen pesticides were separated by optimizing the LC gradient, and the co-eluted pesticides with different masses were identified using MRM mode.

Conclusion

A rapid, reliable, time- and resource-saving analytical method is reported for the measurement of 17 pesticides of different chemical classes used in apiculture or in the surrounding agriculture in the context of a bee mortality study. The multi-residue analytical procedure developed in this study was based on a solid supported liquid-liquid extraction step using diatomaceous earth as inert solid support. Extracts were analyzed without further purification by LC/MS/MS in ESI mode. The SLE with Chem Elut cartridges has proven to be efficient for a wide range of pesticides, nearly independent of their polarity.

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GC/μECD Analysis and Confirmation of PCBs in Fish Tissue with Agilent J&W DB-35ms and DB-XLB GC Columns

Application Note

Food Analysis

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Abstract

A fish sample obtained from a local grocery store was analyzed for 19 PCB (Polychlorinated Biphenyl) compounds using a GC with μECD. A QuEChERS procedure (Quick, Easy, Cheap, Effective, Rugged, and Safe) with dispersive solid phase extraction (dSPE) cleaned the sample prior to analysis. A dual μECD and dual capillary GC column approach performed simultaneous primary and confirmatory analysis. The primary column, Agilent J&W DB-35ms 30 m × 0.25 mm, 0.25 μm and confirmatory column Agilent J&W DB-XLB 30 m × 0.25 mm, 0.50 μm effectively resolved all 19 PCBs. The method was calibrated at the 10, 20, 50, 100, 250, and 400 ng/mL PCB levels, yielding excellent linearity and reproducibility. Spiked recoveries ranged between 72 and 116% at 50 and 200 ng/mL levels in the fish matrix.

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Introduction

Omega-3 fatty acids lower cholesterol, cancer risks, and blood pressure levels. The human body does not produce this fatty acid that is mainly obtained through diet or supplements. Fish is a good source of omega-3 fatty acids, which is especially high in fatty fish. However, even though fish has many beneficial aspects, fish may also contain contaminants such as heavy metals, PCBs or other pollutants.

Even though polychlorinated biphenyls (PCBs) were banned in the late 1970s in the United States and other countries, PCB contamination remains a concern. PCBs are slow to break down and are persistent in the environment, collecting in the sediment of rivers and lakes [1,2]. PCBs are highly lipophilic and bioaccumulate in the fatty tissue of fish throughout the food chain. Consumption of contaminated fish is a significant source of human exposure [3]. Many agencies such as the EPA and local state governments have issued fish advisories recommending monthly or annual fish consumption limits [4].

A dual column, dual µECD system with an Agilent J&W DB-35ms 30 m × 0.25 mm, 0.25 µm primary analysis column and an Agilent J&W DB-XLB 30 m × 0.25 mm, 0.50 µm confirmatory column is shown to separate and analyze the PCBs in the fish sample. Continuous improvements and stringent process control with respect to column activity make this column pair a particularly good choice for analysis of PCBs in a challenging fish tissue matrix.

The QuEChERS (quick, easy, cheap, effective, rugged, and safe) AOAC sample preparation approach is used for extraction and cleanup of 19 PCB congeners in fish tissue. This approach involves an initial extraction in a buffered aqueous and acetonitrile system, an extraction and partitioning step following a salt addition, and a cleanup step using dispersive solid phase extraction [5].

Experimental

An Agilent 7890A GC system equipped with dual µECD detection was used for this series of experiments enabling simultaneous identification and confirmation from a single injection. The GC was also fitted with an unpurged two-way splitter capillary flow technology (CFT) device, simplifying maintenance and reducing system downtime. Table 1 lists the chromatographic conditions used for these analyses. Table 2 lists flow path consumable supplies used in these experiments.

Table 1. Chromatographic Conditions

GC:	Agilent 7890A equipped with dual µECD detection
Sampler:	Agilent 7873B 5.0 µL syringe (Agilent p/n 5181-1273)
CFT device:	2-way unpurged splitter capillary flow technology (Agilent p/n G3181B)
Carrier:	Hydrogen 85 cm/s, constant flow 3.5 mL/min
Injection:	1.0 µL splitless; 250 °C, Purge flow 50 mL/min at 0.3 min, Gas saver 50 mL/min at 2 min
Column 1:	Agilent J&W DB-35 ms 30 m × 0.25 mm, 0.25 µm (Agilent p/n 122-3832)
Column 2:	Agilent J&W DB-XLB 30 m × 0.25 mm, 0.50 µm (Agilent p/n 122-1236)
Oven:	110 °C (0.1 min), 25 °C/min to 200 °C (0.5 min), 10 °C/min to 240 °C (0.5 min), 30 °C/min to 325 °C (1.5 min)
Injection:	1µL, 250 °C splitless, purge 50 mL/min at 0.3 min, gas saver 50 mL/min on at 2 min
Dual µECD:	350 °C, N_2 makeup; constant column + makeup = 30 mL/min

Table 2. Flow Path Supplies

Vials:	Amber screw top glass vials (Agilent p/n 5183-2072)
Vial caps:	Blue screw caps (Agilent p/n 5182-0717)
Vial inserts:	100 µL glass/polymer feet (Agilent p/n 5181-8872)
Syringe:	5 µL (Agilent p/n 5181-1273)
Septum:	Advanced green (Agilent p/n 5183-4759)
Inlet seal:	Gold plated inlet seal (Agilent p/n 5188-5367)
Inlet liners:	Deactivated dual taper direct connect (Agilent p/n G1544-80700)
Ferrules:	0.4 mm id short; 85/15 vespel/graphite (Agilent p/n 5181-3323)
CFT fittings:	Internal nut (Agilent p/n G2855-20530)
CFT ferrules:	SilTite ferrules, 0.25 mm id (Agilent p/n 5188-5361)
20x magnifier :	20x Magnifier loop (Agilent p/n 430-1020)

Reagents and Chemicals

All reagents and solvents were HPLC or Ultra Resi grade. Acetonitrile (ACN) was from Honeywell (Muskegon, MI, USA), acetic acid (HAc) was from Sigma-Aldrich (St. Louis, MO, USA), and acetone was from VWR International (West Chester, PA, USA). The PCB congeners standard (RPCM-8082) and surrogate standard (ISM-320) were purchased from Ultra Scientific (N. Kingstown, RI, USA).

Solutions and Standards

A 1% acetic acid solution in ACN was prepared by adding 10 mL of acetic acid to 1 L of ACN.

The PCB stock standard solution (100 µg/mL of 19 congeners) was diluted in acetone to yield spiking solutions of 1 and 5 µg/mL. A 10 µg/mL surrogate spiking solution was prepared by diluting the stock surrogate (200 µg/mL) solution in acetone. The spiking solutions were used to prepare the calibration curves in the matrix blank extract by appropriate dilution.

Sample Preparation

A Swai fish sample was purchased from a local grocery store. The fish was chopped into small cubes and frozen at –80 °C overnight. The samples were then comminuted thoroughly to achieve sample homogeneity. The sample extraction method used the QuEChERS method followed by dSPE [5]. Figure 1 illustrates the sample preparation procedure graphically in a flow chart.

A 3.0 g sample of fish was weighed into a centrifuge tube. QC samples were fortified with appropriate amount of PCB spiking solution to yield QC samples with concentrations of 10, 50, and 200 ng/mL. A 150-µL amount of surrogate spiking standard (10 µg/mL) was added to each QC sample to yield a 100 ng/mL concentration. Each sample received a 12.0-mL aliquot of deionized water and 15-mL aliquot of 1% HAc in ACN. The samples were vortexed at 1500 rpm for 1 minute. Two ceramic bars (Agilent p/n 5982-9313) were added to each sample to aid in sample extraction. An Agilent SampliQ QuEChERS AOAC extraction salt packet (Agilent p/n 5982-5755) containing 6 g of $MgSO_4$ and 1.5 g sodium acetate was added to each centrifuge tube. The capped tubes were shaken on a Geno/Grinder at 1500 rpm for 1 minute. The samples were centrifuged at 4000 rpm for 5 minutes.

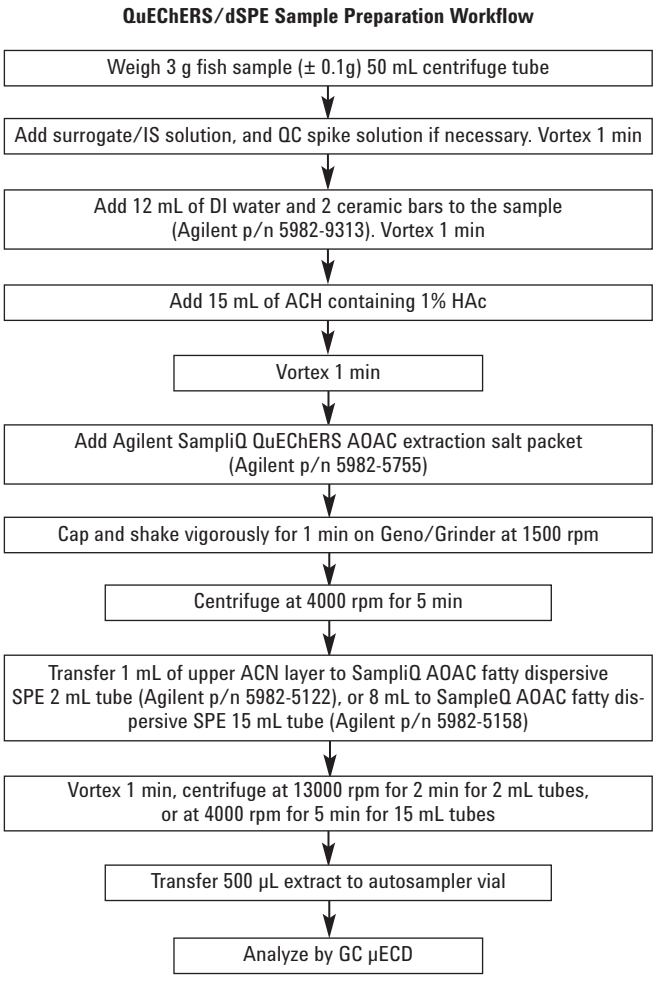


Figure 1. Flow chart of the Agilent SampliQ QuEChERS modified AOAC extraction procedure for fish sample [5].

An 8-mL aliquot of the upper layer was transferred to an Agilent SampliQ QuEChERS fatty sample dispersive SPE 15 mL tube (Agilent p/n 5982-5158). The dSPE tube was vortexed for 1 minute and then centrifuged at 4000 rpm for 5 minutes to complete the sample extraction. The liquid from the dSPE tube was transferred to a GC vial and analyzed by GC-µECD using the chromatographic conditions listed in Table 1.

Extractions of water and acetonitrile aliquots were prepared in the same manner as the samples and served as reagent blanks.

Table 3. r^2 Values for the PCB Congeners Calibration Standards over the 10 ng/mL to 400 ng/mL Range of this Study

Analytes	DB-35ms r^2	DB-XLB r^2
IUPAC 1	0.9994	0.9994
Tetrachloro-m-xylene (surrogate)	0.9913	0.9923
IUPAC 5	0.9993	0.9998
IUPAC 18	0.9998	0.9995
IUPAC 31	0.9980	0.9984
IUPAC 52	0.9986	0.9992
IUPAC 44	0.9988	0.9993
IUPAC 66	0.9990	0.9993
IUPAC 101	0.9992	0.9994
IUPAC 87	0.9984	0.9991
IUPAC 110	0.9939	0.9991
IUPAC 151	0.9998	0.9996
IUPAC 153	0.9981	0.9993
IUPAC 141	0.9993	0.9998
IUPAC 138	0.9984	0.9994
IUPAC 187	0.9989	0.9996
IUPAC 183	0.9993	0.9998
IUPAC 180	0.9994	0.9998
IUPAC 170	0.9993	0.9997
IUPAC206	0.9995	0.9996
Decachlorobiphenyl (surrogate)	0.9910	0.9895

Results and Discussion

The PCB and surrogate standards were resolved on the DB-35ms 30 m × 0.25 mm, 0.25 µm primary analysis column in less than 12 min. Figure 2 shows the separation of a 50 ng/mL PCB standard solution (100 ng/mL surrogate standard). Figure 3 shows a chromatogram of the same 50 ng/mL PCB standard (100 ng/mL surrogate standard) injection on the DB-XLB 30 m x 0.25 mm, 0.50 µm confirmatory analysis column.

The performance of the dual column set yielded acceptable linearity and recovery over the calibration range of this study. The method limit of quantitation (MLQ) of 10 ppb is substantially lower than the current regulatory guideline set by the FDA of 2,000 ppb for PCBs in food grade fish, and below the Agency for Toxic Substance and Disease Registry (ATSDR) maximum residue limit (MRL) of 0.02 mg/kg/day for these analytes [6]. The linearity of the column set as defined by the r^2 values of the PCB congeners standard curve ranged from 0.9939-0.9998. The individual PCB congener values are shown in Table 3. The lowest calibration standard on the column set also achieved excellent signal-to-noise ratios as shown in Figure 4.

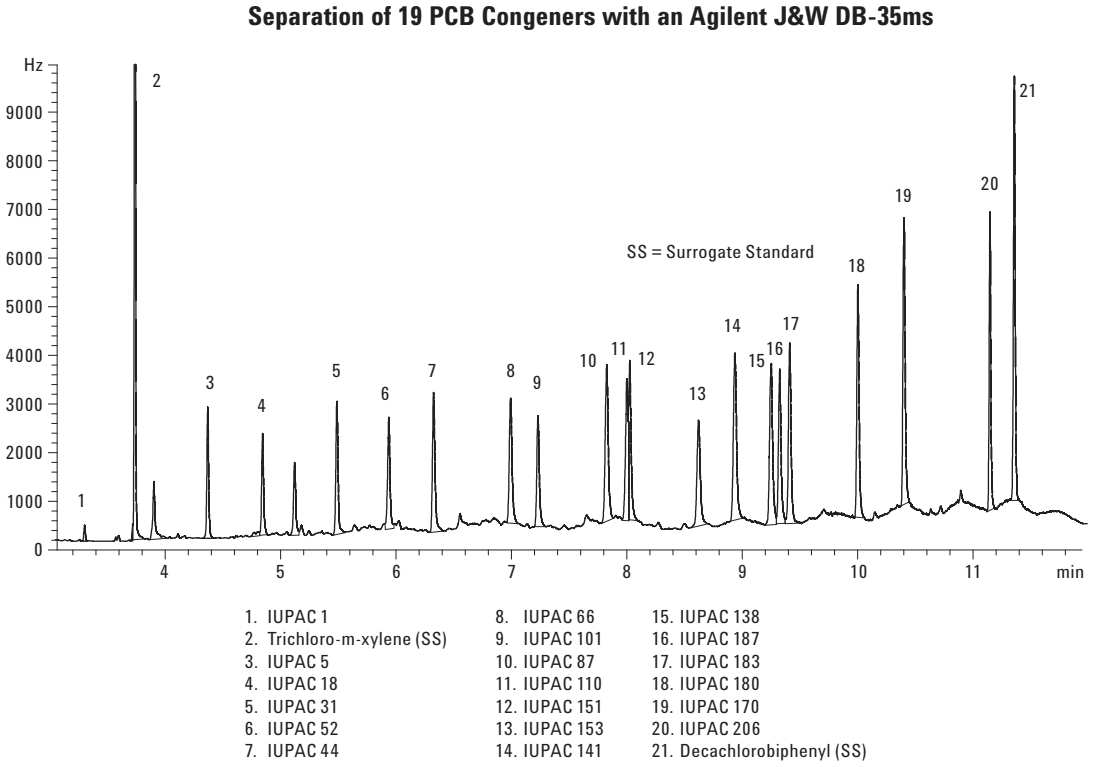


Figure 2. GC/µECD chromatogram of the 50 ng/mL PCB congeners standard analyzed on an Agilent J&W DB-35ms 30 m × 0.25 mm, 0.25 µm capillary GC column (Agilent p/n 122-3832). Chromatographic conditions are listed in Table 1.

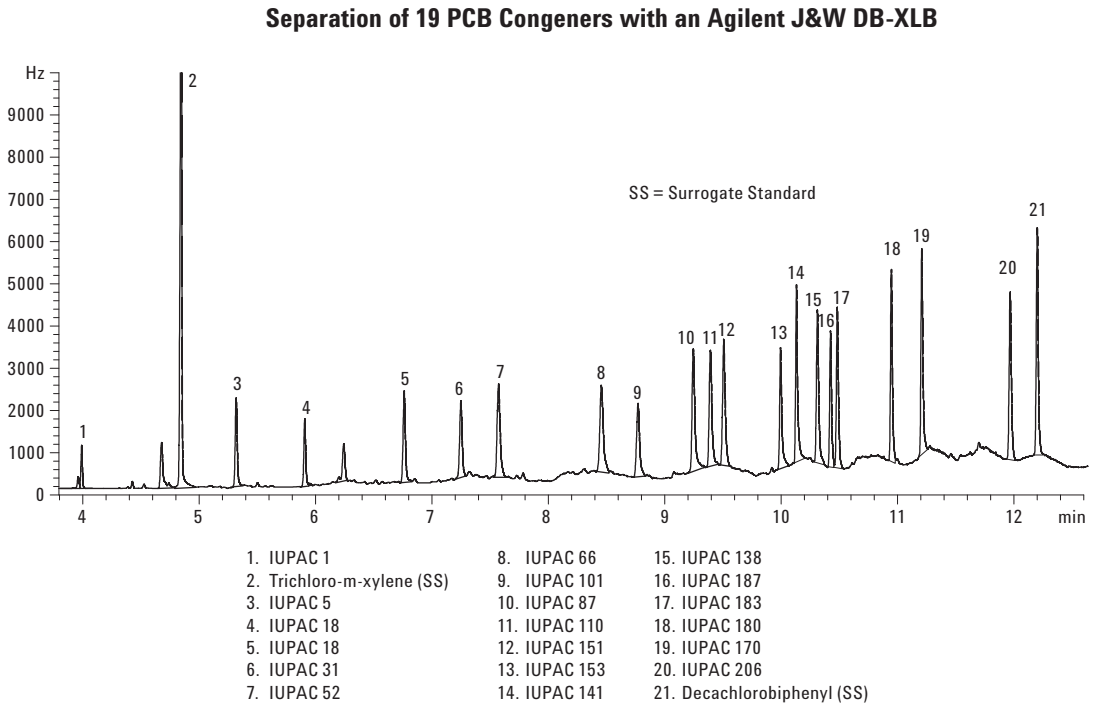


Figure 3. GC/µECD chromatogram of the 50 ng/mL PCB congeners standard analyzed on an Agilent J&W DB-XLB 30 m × 0.25 mm, 0.50 µm capillary GC column (Agilent p/n122-1236). Chromatographic conditions are listed in Table 1.

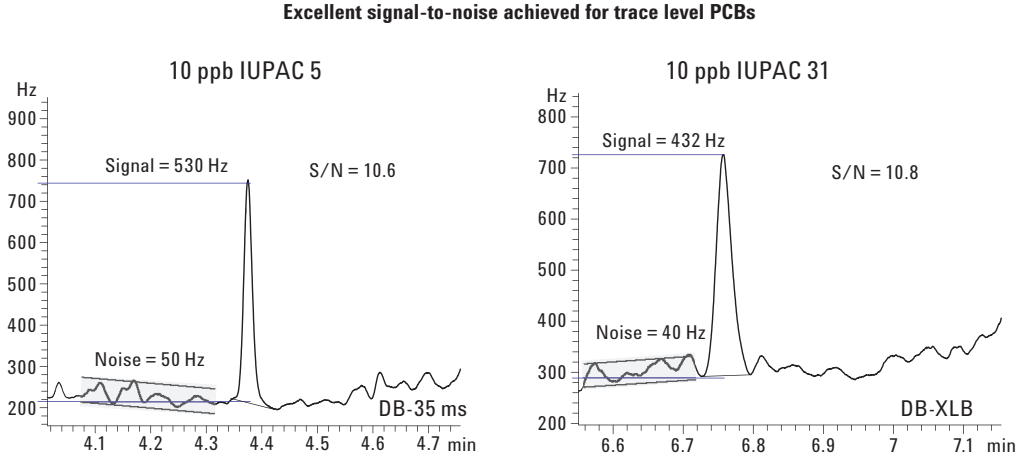
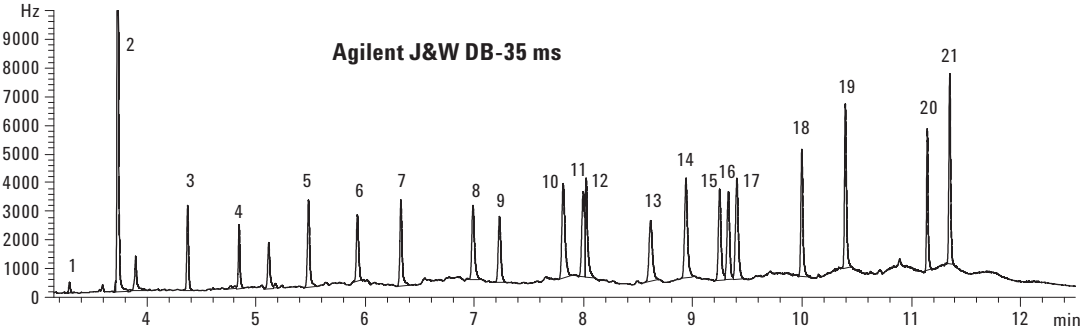


Figure 4 Enlarged view chromatogram of two individual congeners in the 10 ng/mL PCB calibration standard analyzed on the Agilent J&W DB-35ms and DB-XLB capillary columns. Chromatographic conditions are listed in Table 1.

The extraction process using the QuEChERS followed by dispersive SPE was effective in retaining the PCBs in the spiked fish sample and providing sufficient cleanup of the sample matrix for GC-µECD analysis. Figure 5 shows the separation of the extracted PCBs in a spiked fish sample on the primary and confirmation column set.

50 ppb PCBs in fish after Agilent's SampliQ QuEChERS extraction and dispersive SPE for fatty samples



1. IUPAC 1
2. Trichloro-m-xylene (SS)
3. IUPAC 5
4. IUPAC 18
5. IUPAC 31
6. IUPAC 52
7. IUPAC 44
8. IUPAC 66
9. IUPAC 101
10. IUPAC 87
11. IUPAC 110
12. IUPAC 151
13. IUPAC 153
14. IUPAC 141
15. IUPAC 138
16. IUPAC 187
17. IUPAC 183
18. IUPAC 180
19. IUPAC 170
20. IUPAC 206
21. Decachlorobiphenyl (SS)

Figure 5. GC/µECD chromatogram of the 50 ng/mL fortified fish extract analyzed on Agilent's J&W DB-35ms and DB-XLB GC columns. Chromatographic conditions are listed in Table 1.

The recoveries were determined at the 10, 50, and 200 ng/mL PCB levels. Recoveries for the individual PCBs on each column are shown in Tables 4 and 5. The mid and high level recovery ranges were excellent with the DB-35ms column (72 to 112%), and with the DB-XLB column (72 to 116%) for all PCBs investigated.

Lower recoveries were noted for three of the 19 congeners in the low level 10 ppb QC sample. The co-elution of IUPAC 110 and IUPAC 151 on the DB-35ms column contributed to the lower recovery seen for the IUPAC 110, but both congeners were resolved on the XLB column yielding recoveries over 72%.

Because the sensitivity of the ECD is relative to the amount of chloro substituents present, the response for the PCBs generally increases with increased chlorine content. Since IUPAC 1 is a monochlorinated biphenyl (2-chlorobiphenyl) it exhibits poor ECD sensitivity. This was found to contribute to the low recovery for IUPAC 1 at the 10 ng/mL level, however recovery and reproducibility were excellent at 50 ng/mL (average recovery 111%, average reproducibility 2.4%).

Table 4. Recovery and Repeatability of PCBs in Fortified Swai Fish with Agilent J&W DB-35ms column (Agilent p/n122-3832)

	10 ng/mL fortified QC		50 ng/mL fortified QC		200 ng/mL fortified QC	
Analytes	**%Recovery**	**RSD (n=6)**	**%Recovery**	**RSD (n=6)**	**%Recovery**	**RSD (n=6)**
IUPAC 1	43.2	4.0	111.9	2.4	99.0	1.6
Tetrachloro-m-xylene (surrogate)	107.5	1.2	110.4	1.4	103.9	1.8
IUPAC 5	68.5	1.3	107.0	1.8	99.6	2.2
IUPAC 18	99.3	2.9	108.5	1.9	95.1	2.5
IUPAC 31	56.7	3.3	110.5	2.0	101.8	2.3
IUPAC 52	75.7	3.4	91.8	2.1	100.5	1.9
IUPAC 44	61.4	1.7	107.8	2.2	100.6	2.1
IUPAC 66	66.9	2.0	97.2	2.8	96.2	2.4
IUPAC 101	65.8	2.5	99.5	2.8	94.7	2.2
IUPAC 87	75.7	2.8	99.1	1.5	101.4	2.4
IUPAC 110	29.7	2.6	100.0	2.6	102.8	2.8
IUPAC 151	100.1	2.1	99.8	2.6	94.5	1.9
IUPAC 153	49.9	1.9	89.9	1.6	91.5	2.9
IUPAC 141	67.7	2.1	93.1	1.5	92.2	2.3
IUPAC 138	52.2	2.9	95.9	2.4	93.4	2.2
IUPAC 187	57.0	3.4	92.1	2.3	89.5	2.2
IUPAC 183	62.1	3.2	87.3	2.2	85.7	2.2
IUPAC 180	63.1	3.2	88.6	1.1	84.0	2.6
IUPAC 170	65.4	4.2	91.0	1.6	86.7	2.3
IUPAC 206	58.8	3.2	77.7	1.5	72.5	2.4
Decachlorobiphenyl (surrogate)	75.0	2.9	81.1	2.4	75.9	2.7

Table 5. Recovery and Repeatability of PCBs in Fortified Swai Fish with Agilent J&W DB-XLB column (Agilent p/n 122-1236)

	10 ng/mL fortified QC		50 ng/mL fortified QC		200 ng/mL fortified QC	
Analytes	**%Recovery**	**RSD (n=6)**	**%Recovery**	**RSD (n=6)**	**%Recovery**	**RSD (n=6)**
IUPAC 1	56.5	3.2	116.2	2.2	99.4	2.0
Tetrachloro-m-xylene (surrogate)	108.0	1.6	110.9	1.3	105.1	1.9
IUPAC 5	87.4	2.5	112.1	1.5	100.7	1.9
IUPAC 18	69.9	1.6	112.3	2.1	100.8	2.5
IUPAC 31	63.1	3.5	108.3	1.8	103.2	2.3
IUPAC 52	59.6	3.5	104.7	2.6	100.5	1.8
IUPAC 44	75.7	2.9	105.0	1.9	101.7	2.0
IUPAC 66	83.1	2.9	101.5	2.7	98.2	2.0
IUPAC 101	73.0	2.4	98.4	1.9	96.6	2.0
IUPAC 87	60.7	2.7	109.2	1.8	100.7	1.6
IUPAC 110	72.9	2.6	103.0	2.3	100.1	1.6
IUPAC 151	75.1	3.6	93.9	2.9	93.5	2.6
IUPAC 153	36.0	6.9	104.5	3.0	91.1	1.9
IUPAC 141	74.4	2.6	98.0	1.6	91.9	2.3
IUPAC 138	65.4	3.3	98.9	2.5	93.1	2.1
IUPAC 187	68.4	2.2	94.6	1.0	88.7	2.0
IUPAC 183	72.6	3.7	92.2	2.0	86.1	2.4
IUPAC 180	76.0	3.2	92.2	2.0	84.5	2.7
IUPAC 170	74.9	8.7	94.6	2.2	87.5	2.3
IUPAC 206	60.1	3.2	78.3	2.4	72.6	2.7
Decachlorobiphenyl (surrogate)	74.6	2.4	81.4	1.6	76.7	2.3

Conclusions

This application note shows a robust, inexpensive, analytical method that sufficiently monitors PCBs in fish samples to address food safety concerns. This method demonstrates the feasibility of using a dual column µECD approach for routine fish screening as an alternative to GC/MS.

The Agilent SampliQ QuEChERS AOAC method for fatty samples followed by dSPE is effective at providing enough sample cleanup to avoid matrix interferences, while maintaining low level analyte detection.

The dual column set of an Agilent J&W DB-35ms primary analytical column and an Agilent J&W DB-XLB confirmatory column on one instrument allows simultaneous identification and confirmation of the presence of the PCBs. The DB-35ms primary analysis column and the DB-XLB confirmatory column with dual µECD detection were effective at analyzing 19 PCBs in a fish matrix following sample matrix cleanup. The single injection, dual column approach improves productivity by saving instrument and analyst time. Continuous improvements and stringent process control with respect to column activity make the DB-35ms and DB-XLB column pair an excellent choice for analysis of analytes such as PCBs.

The performance of the dual column set DB-35ms and DB-XLB with GC µECD had excellent linearity over the range of concentrations studied with r^2 values between 0.9939 and 0.9998 for the PCB compounds. Recovery and reproducibility was shown to be greater than 77% with an RSD below 3.0 at 50 ng/mL. The method limit of quantitation for the PCB congeners using this approach was significantly lower than currently regulated MRLs in fish. The results achieved with this method shows determination of PCBs by µECD as a reliable alternative to GC/MS.

Acknowledgements

The authors wish to thank Joan Stevens for her help and suggestions with the Agilent SampliQ QuEChERS AOAC extraction procedure.

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A Low Femtogram Target Screen Method for Perfluorinated Compounds in Food Matrices and Potable Water Using the Agilent 6460 Triple Quadrupole LC/MS System Equipped with Agilent Jet Stream Technology

Application Note

Environmental, Food Safety

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Abstract

In this application note, we outline a viable method for reliably detecting low-femtogram levels of perfluorinated carboxylates (PFCA) and sulphonates (PFSA) without background component interference, with an inline contaminant trap LC modification. Good chromatographic resolution of all perfluorinated compounds was observed. A representative suite of PFCAs and PFSAs were analyzed herein and were all detected at on-column levels lower than 75 fg in drinking water matrices (S/N >3.) The most sensitive analytes PFHxS, PFDS and PFBS were detected at 2.6, 3.2 and 5 fg levels, respectively. Method detection limits for spiked pork liver matrix extract samples were below 600 fg on-column for the entire analyte suite. No detectable background contamination was observed in blank injections for any analyte in this study. Linearity for up to five orders of magnitude with R^2 values above 0.996 for the entire suite were recorded.

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Introduction

Exposure, bioaccumulation and potential toxicity continue to be issues in environmental biota and their food webs from emerging contaminants such as perfluoronated byproducts (PFC) from industrial processing. Since background levels of such analytes are significant and stable in the atmosphere already, it is difficult to obtain reliable and accurate low-level on-column measurements.

In this application note, we present a case in which a suite of perfluorinated carboxylates (PFCA) and sulfonates (PFSA) were screened at low fg on-column levels in a potable water matrix and in spiked (pork) liver samples with zero background interference using dynamic multiple reaction monitoring (dynamic MRM) [1]. This approach allowed us to gain reliable positive identifications and extremely low limits of detection. By utilizing an inline contaminant trap configuration we assured cleanliness of the HPLC system and allowed use of inline membrane degassing without compromising system dead-volume or analysis speed.

A comprehensive evaluation of a suite of PFCAs & PFSAs including isotopically labeled ISTDs was undertaken which examined sensitivity and linearity of each component. Appropriate dynamic MRM transitions were identified using automatic instrument optimizations of fragmentor (frag) and collision energy (CE) voltages and applied to the chromatographic method dynamically to maximize analyte signal quality at lower concentrations. Optimal settings for the Agilent Jet Stream Technology [2] were determined for the complete PFCA & PFSA suite effectively increasing the sensitivity to around 14x that of normal electrospray ionization (ESI) conditions.

Table 1. Compounds Analyzed for this Study

Target compounds	
perfluoro-1-butanesulfonate	(PFBS)
perfluoro-n-hexanoic acid	(PFHxA)
perfluoro-n-heptanoic acid	(PFHpA)
perfluoro-1-hexanesulfonate	(PFHxS)
perfluoro-n-octanoic acid	(PFOA)
perfluoro-n-nonanoic acid	(PFNA)
perfluoro-1-octanesulfonate	(PFOS)
perfluoro-n-undecanoic acid	(PFUA)
perfluoro-1-decanesulfonate	(PFDS)
perfluoro-n-dodecanoic acid	(PFDoA)
perfluoro-n-tridecanoic acid	(PFTriA)
perfluoro-n-tetradecanoic acid	(PFTA)

Experimental

This analysis was performed using an Agilent 6460A triple quadrupole LC/MS with an Agilent 1200SL Series LC system. The LC system consisted of a binary pump (G1312B), vacuum degasser (G1379B), a low carryover automatic liquid sampler (G1367D), thermostatted column compartment (G1316B) and MassHunter data system.

Sample Handling

Sample handling is a critical element in the measurement of trace amounts of perfluorinated carboxylates and sulphonates, since background levels can be prevalent and derived from laboratory consumables and protective lab-wear. The series of analyses outlined in this application note considered this and precautions were taken to eliminate any such cross-contamination. Silanized glass vials were used with aqueous diluents that had been passed through a solid-phase extraction. Nitrile rubber vial caps and non PTFE-containing pipette tips were used. Only nitrile rubber derived protective laboratory gloves were worn.

Instrumentation

Rapid Resolution HPLC Conditions and Configuration

Configuration:

Agilent 1200 Series Binary Pump SL:	(G1312B)
High Performance WP Sampler SL Plus:	(G1367D)
Sampler Thermostat:	(G1330B)
Thermostatted Column Compartment SL	(G1316B)

Method Conditions:

Column:	Agilent ZORBAX Eclipse Plus C18, 2.1 mm × 50 mm, 1.8 µm	
Column temperature:	55 °C	
Injection volume:	1 µL	
Autosampler temp:	4 °C	
Needle wash:	Flushport (100% methanol), 5 seconds	
Mobile phase:	A = 2 mM NH_4 acetate in water	
	B = 2 mM NH_4 acetate in methanol	
Gradient flow rate:	0.5 mL/min	
Gradient:	Time (min)	%B
	0	6
	0.5	6
	6	95
	8	95
Total run time:	9.0 min (including 1 min equilibration time)	

Mass Spectrometer Dynamic MRM Conditions and Configuration

Configuration:

Agilent 6460 Triple Quadrupole Mass Spectrometer equipped with Agilent Jet Stream Technology

Ion Source Conditions:

Ion mode:	ESI/Agilent Jet Stream, Negative
Capillary voltage:	3750 V
Nozzle voltage:	0 V
Drying gas (nitrogen):	4 L/min
Drying gas temperature:	320 °C
Nebulizer gas (nitrogen):	60 psi
Sheath gas temperature:	350 °C
Sheath gas flow:	12 L/min

Dynamic MRM acquisition:

Cycle time:	250 ms
Total dynamic MRMs:	29
Maximum concurrent MRMs:	12
Retention time window:	30 sec
Minimum/maximum dwell:	17.33/246.50 ms
Q1 and Q2 resolution:	0.7 amu [unit]
Delta EMV:	0 V

Dynamic MRM triple quad MS parameters are listed in Table 2. All fragmentor voltage (frag) settings, respective collision energies (CE), and most abundant MS/MS product ions per analyte were determined automatically using the Agilent MassHunter Optimizer software.

Ion Source Optimization

In order to achieve the optimal and most sensitive Agilent 6460 ESI MS source conditions for the complete suite of analytes, each dynamic MRM method transition was measured using a single mixed standard repetitively. In addition, each subsequent sample injection was also measured using a systematic and single source parameter change. This was to obtain the best and most sensitive method conditions for an optimized method, but only had to be undertaken once.

In reality, a single set of source parameter conditions are not necessarily the optimum settings for all analytes in a suite (or assay) so a compromise set of conditions were determined for the suite of perfluorinated analytes. A subsequent

Table 2. Dynamic MRM PFSA/PFCA Settings

Compound name	Precursor ion mass	Q1-resolution	Product ion mass	Q2-resolution	Fragmentor voltage	Collision energy (eV)	Retention time (min)	RT delta (min)	Ion polarity
PFBS	298.9	unit	80	unit	133	45	3.623	1	Negative
PFBS (Q)	298.9	unit	98.9	unit	133	29	3.623	1	Negative
PFDA	512.9	unit	469	unit	102	5	5.543	1	Negative
PFDA (C13)2	514.9	unit	469.9	unit	102	5	5.542	1	Negative
PFDoA	612.9	unit	569	unit	97	5	5.961	1	Negative
PFDoA (C13)2	614.9	unit	570	unit	97	5	5.961	1	Negative
PFDoA (Q)	612.9	unit	169	unit	97	25	5.961	1	Negative
PFDS	598.9	unit	80	unit	205	94	5.752	1	Negative
PFHpA	362.9	unit	319	unit	66	5	4.626	1	Negative
PFHpA (Q)	362.9	unit	169	unit	66	13	4.626	1	Negative
PFHxA	312.9	unit	268.9	unit	66	5	4.143	1	Negative
PFHxA (C13)2	314.9	unit	269.9	unit	66	5	4.141	1	Negative
PFHxS	398.9	unit	80	unit	174	49	4.671	1	Negative
PFHxS (O18)2	402.9	unit	83.9	unit	174	49	4.671	1	Negative
PFHxS (Q)	398.9	unit	99	unit	174	45	4.671	1	Negative
PFNA	462.9	unit	418.9	unit	66	5	5.296	1	Negative
PFNA (C13)5	467.9	unit	423	unit	66	5	5.296	1	Negative
PFNA (Q)	462.9	unit	169	unit	66	17	5.296	1	Negative
PFOA	412.9	unit	368.9	unit	86	5	5.003	1	Negative
PFOA (C13)4	416.9	unit	371.9	unit	86	5	5.001	1	Negative
PFOA (Q)	412.9	unit	169	unit	86	13	5.003	1	Negative
PFOS	498.9	unit	80	unit	210	50	5.302	1	Negative
PFOS (C13)4	502.9	unit	80	unit	210	50	5.301	1	Negative
PFOS (Q)	498.9	unit	99	unit	210	50	5.302	1	Negative
PFTA	712.9	unit	669	unit	112	9	6.255	1	Negative
PFTriA	662.9	unit	619	unit	102	9	6.117	1	Negative
PFUA (C13)2	564.9	unit	519.9	unit	92	5	5.764	1	Negative
PFUA	562.9	unit	519	unit	92	5	5.762	1	Negative
PFUA (Q)	562.9	unit	169	unit	92	21	5.762	1	Negative

technical note that details the complete source optimization of this suite of compounds will soon be published.

Results and Discussion

Inline Contaminant Trapping

For highly sensitive measurements of PFCAs and PFSAs at low femtogram on-column levels, it was necessary to ensure the removal of background PFC contamination derived from sample work-up, mobile phase impurities or instrument components. PFCAs and PFSAs are typically hard to break down naturally. Their precursors are widely released into the atmosphere which are degraded to terminal PFCAs and PFSAs.

One approach is to stop PFCAs and PFSAs from entering the high-pressure HPLC flow system by effectively trapping them using a small inline reverse phase column or cartridge immediately after the respective pump head, prior to the point at which the gradient mix is achieved. Figure 1 schematically shows this configuration with a low dead-volume binary pump setup.

The positioning of the inline contaminant trap [Agilent ZORBAX Eclipse Plus C18 (4.6 mm × 30 mm, 3.5 µm, p/n-959936-902)] was prior to the mixing point of the gradient pump and on the aqueous pump channel (in this case Pump A.) It was exposed to a 100% isocratic aqueous mobile phase and effectively trapped all PFCA and PFSA contaminants from entering the HPLC flow path. Further, since the inline trap was before the gradient mix point, it had zero dead-volume implications to the HPLC separations. *It must be noted that this is a nonstandard configuration and as such may not be supported by Agilent Technologies.*

Moreover, extreme care regarding sample handling techniques was observed so that PFCAs and PFSAs were not introduced artificially during the preparation process. For example, careful choice of silanized glass vials with rubber septa were a necessity as were the use of non PTFE-containing pipette tips and protective clothing (nitrile rubber gloves). Sample diluent was also isocratically pumped through a C18 flash column to remove background PFCAs and PFSAs prior to use.

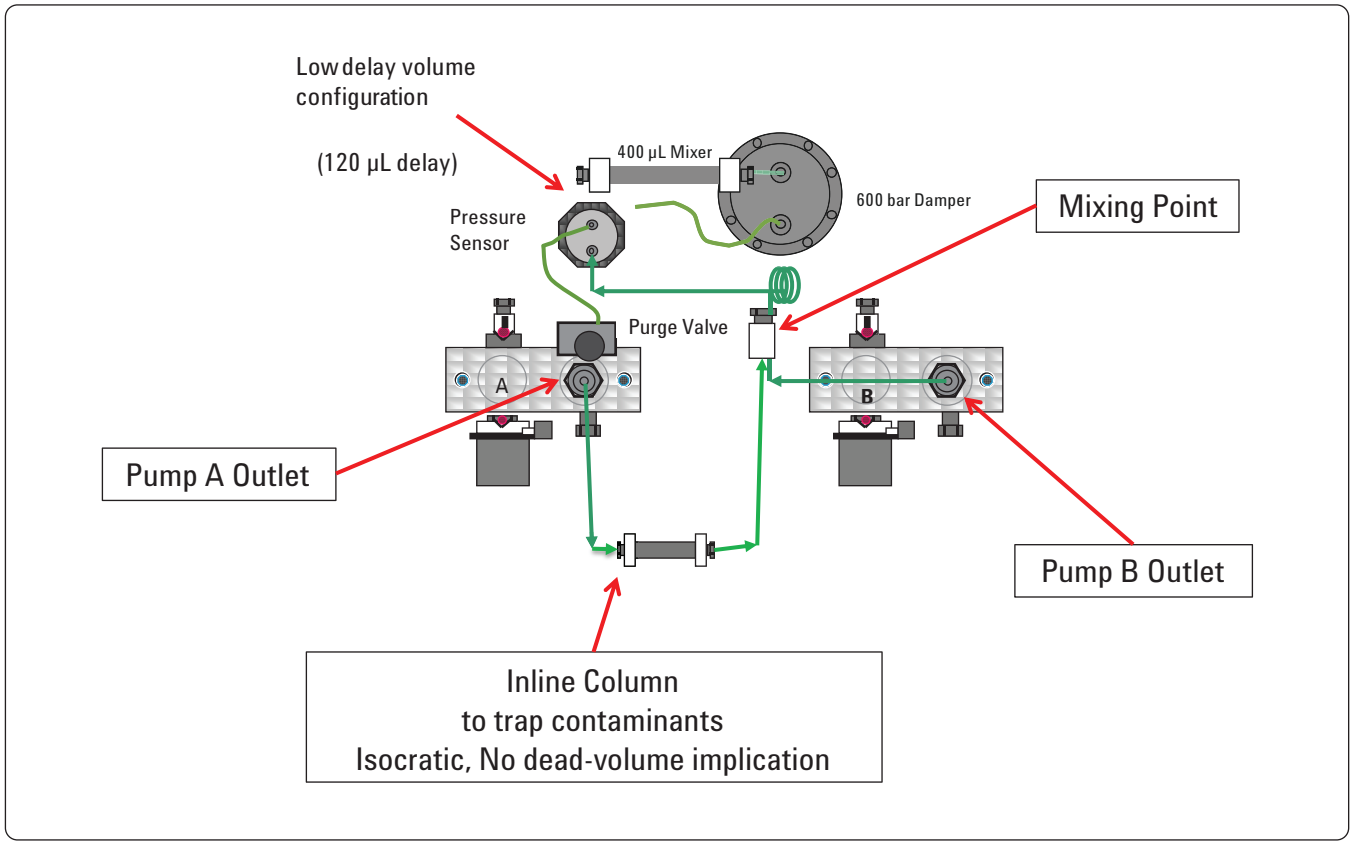


Figure 1. Inline contaminant trapping schematic.

A typical low-level injection (10 fg on-column) featuring a PFHxS (transition 398.9→80 *m/z*) is illustrated in Figure 2 in triplicate, together with a blank injection prior to these analyses. The blank sample baseline was completely clear of residual PFHxS, as a result of the cleanliness of the HPLC system from the inline contaminant trap. This was also true for all other analytes in this study; due to space restrictions, only PFHxS is shown here. The complete set of data will be published in a future application note.

Figure 2 also indicates the outstanding high level of sensitivity of the Agilent 6460 triple quad MS for the negative polarity analysis of such perfluorinated analytes spiked into potable water matrix. In this study, LODs for PFHxS were the lowest for the suite, giving an average of 2.6 fg on-column over triplicate injections and defined as having a signal-to-noise (S/N) ratio of greater than 3. All other analytes in the PFC suite exhibited LODs of less than 75 fg on-column. Figure 3 illustrates an overlaid chromatogram for all PFC analytes at a level of 100 fg on-column.

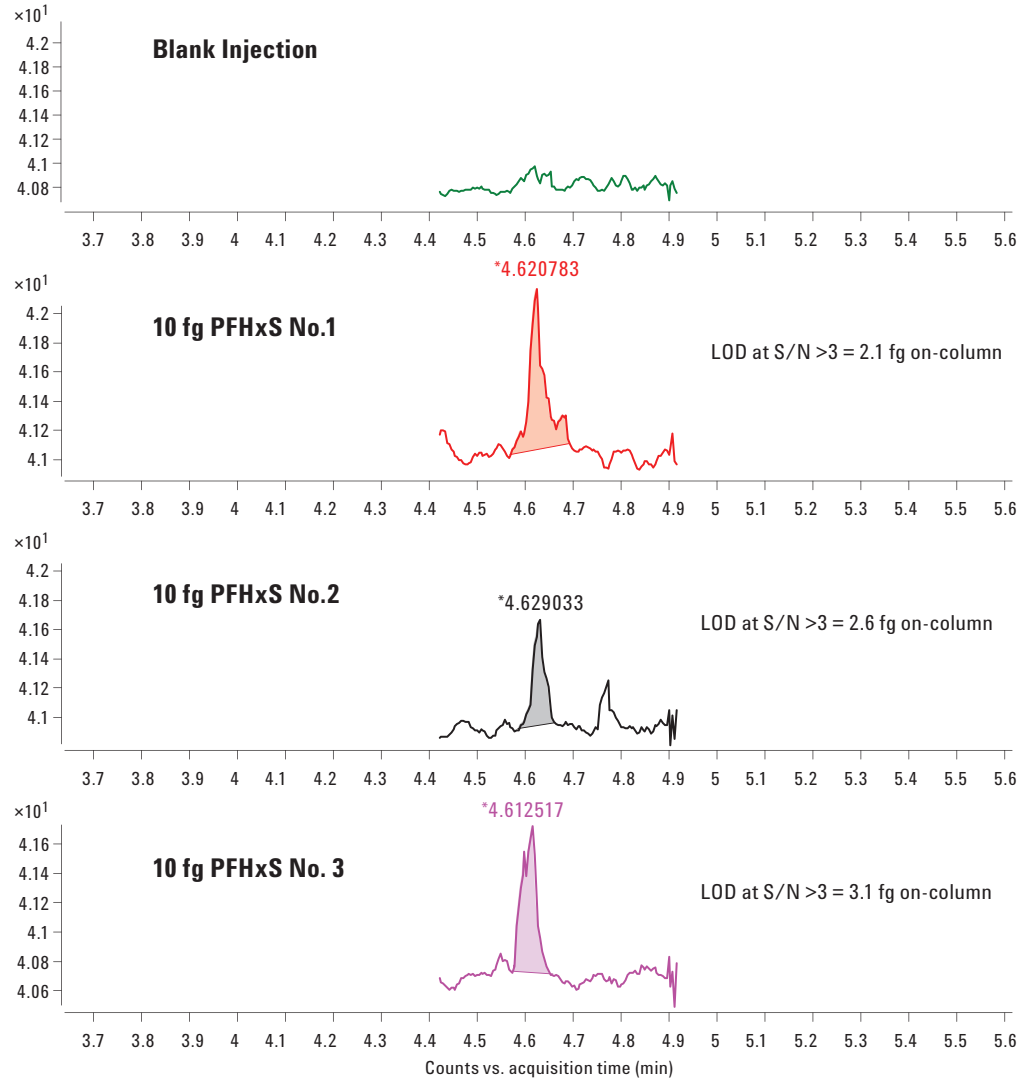


Figure 2. Blank injection with 3x replicates of PFHxS Standard, 10 fg on-column, spiked potable water.

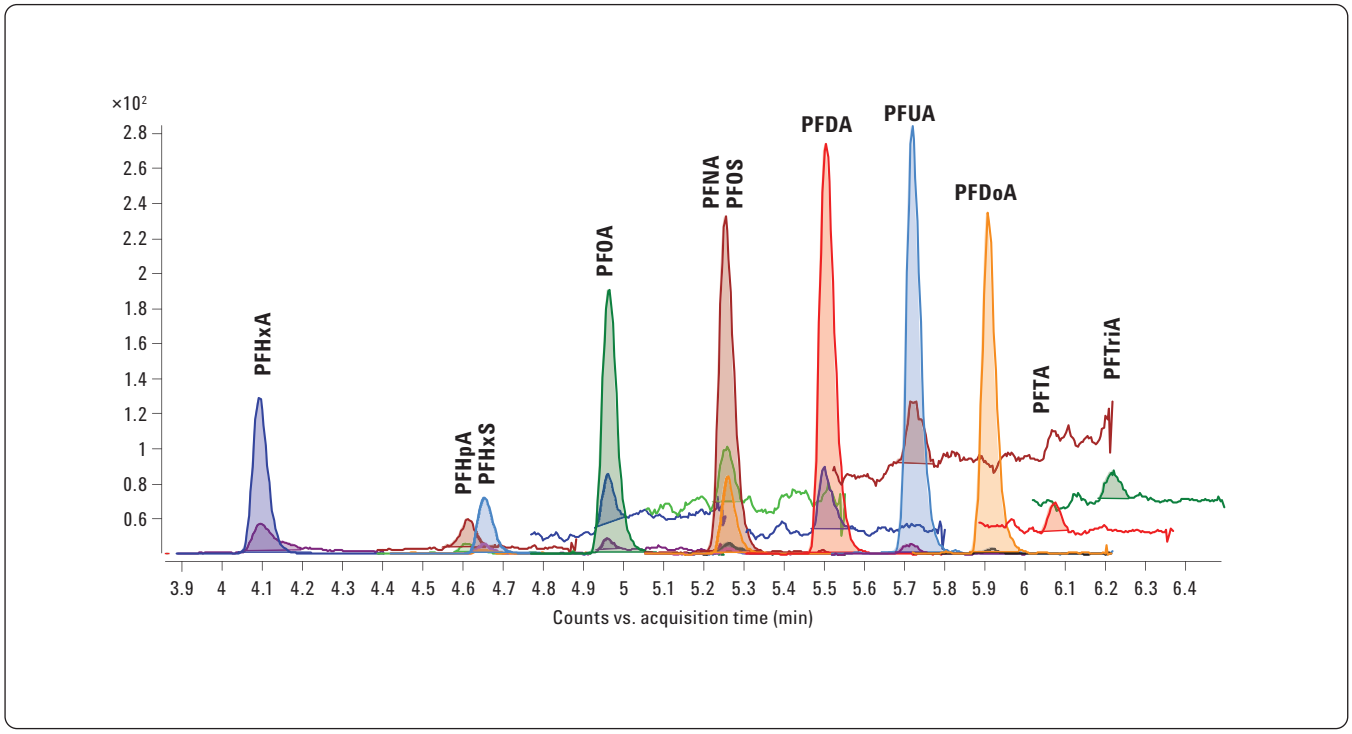


Figure 3. PFCA/PFSA Suite dynamic MRM chromatogram (overlaid) at 100 fg with quantifier and qualifier ions (spiked potable water).

Limits Of Detection (Potable Water Spiked Samples)

Table 3 outlines the limit of detection (LOD) values observed for the suite of PFCs undertaken in this study and spiked into untreated potable water matrix. All PFCA/PFSA LODs in this evaluation were below a value of 75 fg on-column. They were achieved with no background carryover at extremely high sensitivity by careful optimization of fragmentation and collision energy parameters and careful fine-tuning of Agilent Jet Stream and ion source parameters.

A typical ISTD-corrected calibration curve for one of the analytes in the suite (PFOS) is outlined in Figure 4. The linearity R^2 value was found to be 0.99957820 for triplicate injections for more than five orders of magnitude.

Table 3. LOD Results for Spiked Potable Water Samples

Compounds	LOD (fg on column, S/N >3)
PFBS	5
PFHxA	8.4
PFHpA	12.2
PFHxS	2.6
PFOA	43.7
PFNA	75
PFOS	5.7
PFDA	36.3
PFUA	44
PFDS	3.2
PFDoA	55.9
PFTriA	74.2
PFTA	21.7

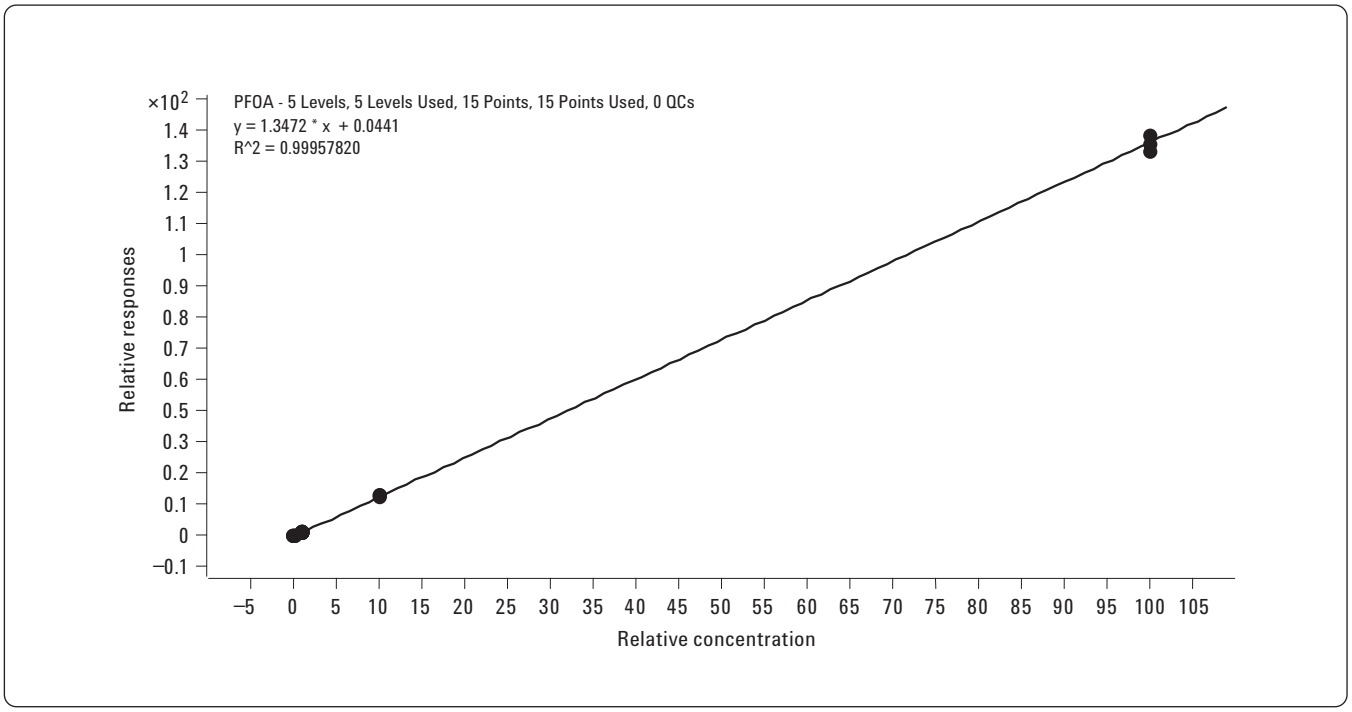


Figure 4. PFOA linearity over five orders of magnitude in potable water (10 fg – 100 pg on-column. [N = 3]).

Method Detection Limits (Spiked Pork Liver Samples)

Table 4 summarizes the method detection limits (MDL) which were observed for each individual PFCA or PFSA analyte when applied to spiked liver extracts.

More than half of the compounds showed a precision value significantly less than 10% RSD (based on peak area) at this challenging MDL concentration.

MDL values in spiked pork liver extracts ranged between 600 fg and 45 fg on-column for this reported methodology.

Table 4. PFCA/PFSA Method Detection Limits

Compounds (spiked pork liver extract)	Method detection limit (fg on column, S/N >10)
PFBS	97.7
PFHxA	110.5
PFHpA	249
PFHxS	44.62
PFOA	291.5
PFNA	421.3
PFOS	58.3
PFDA	275.3
PFUA	303.9
PFDS	54.9
PFDoA	594.5
PFTriA	494.5
PFTA	503.2

Conclusions

A highly sensitive low-femtogram dynamic MRM Agilent 6460 triple quad LC/MS method has been presented for the analysis of a suite of PFCAs and PFSAs analytes that illustrates excellent precision at low-femtogram on-column levels in a complex food matrix and potable water.

Background PFCA and PFCS interferences normally associated with low-level analyses of such perfluorinated suites were eliminated by careful preparation of samples, sample handling and an inline flow contaminant trapping cartridge set-up within the HPLC flow path.

Complete ion source optimization was undertaken for each of the analytes in the suite. This effectively increased the analytical sensitivity by at least a factor of 14x compared with standard ESI source settings.

References

1. "New Dynamic MRM Mode Improves Data Quality and Triple Quad Quantification in Complex Analyses," Agilent Technologies publication 5990-3595EN.
2. "Agilent Jet Stream Thermal Gradient Focussing Technology," Agilent Technologies publication 5990-3494EN.

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Analysis of Polycyclic Aromatic Hydrocarbons in Fish with Agilent SampliQ QuEChERS AOAC Kit and HPLC-FLD

Application Note

Food

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Abstract

An HPLC-Florescence Detection (FLD) method was developed and validated for the determination of sixteen polycyclic aromatic hydrocarbons (PAHs) in fish fillets. The analyzed compounds included naphthalene (Nap), acenaphthylene (Acy), acenaphthene (Ace), fluorene (Flu), phenanthrene (Phe), anthracene (Ant), fluoranthene (Fln), pyrene (Pyr), 1,2-benza[a]anthracene (BaA), chrysene (Chr), benzo[e]pyrene (BeP), benzo[e]acenaphthylene (BeA), benzo[k]fluoranthene (BkF), dibenzo[a,h]anthracene (DahA), benzo[g,h,i]perylene (Bghi)P and indeno[1,2,3-cd]pyrene (InP). The method employs a quick, easy, cheap, effective, rugged and safe (QuEChERS) multiresidue sample preparation procedure adopted from the Association of Analytical Communities (AOAC) Official method 2007.01 for extraction and cleanup. The analytes were separated on an Agilent ZORBAX Eclipse PAH HPLC column (4.6 mm × 50 mm, 1.8 µm) by gradient elution with a binary system of acetonitrile - water and subsequent fluorescence detection set at appropriate excitation and emission wavelengths. The analyte recoveries ranged from 83.4% to 101% with relative standard deviations ranging from 0.6 to 1.9% at three different fortification levels. The limits of detection and quantification ranged from 0.04 to 0.84 and 0.1 to 2.80 ng/g, respectively.

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Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a large group of organic compounds included in the European Union and US Environmental Protection Agency (US EPA) priority pollutant list because of their mutagenic and carcinogenic properties [1]. Excluding smokers and occupationally vulnerable populations, most individuals are exposed to PAHs predominantly from dietary sources [2]. In the marine environment, PAHs are bioavailable to marine species via the food chain, as waterborne compounds, and contaminated sediments. As lipophilic compounds they can easily cross lipid membranes and have the potential to bioaccumulate in aquatic organisms. Although for most people, fish and seafood represents only a small part of the total diet, the contribution of this food group to the daily intake of PAHs in some individuals may be comparatively important [3].

The AOAC QuEChERS method has been widely applied in the analysis of pesticides in food since it was introduced by USDA scientists [4-5]. In general, there are two major steps: extraction and dispersive SPE cleanup. The method uses a single step buffered acetonitrile extraction while simultaneously salting out water from the aqueous sample using anhydrous magnesium sulfate ($MgSO_4$) to induce liquid-liquid partitioning. After removing an aliquot from an organic layer, for further cleanup, a dispersive solid phase extraction (dSPE) step is conducted using a combination of primary secondary amine (PSA) sorbent to remove organic acids from other components and anhydrous $MgSO_4$ to reduce the remaining water in the extract. Other sorbents, such as graphitized carbon black (GCB), may be added to remove pigments and sterol, or C18 to remove lipids and waxes.

This application note presents a method for the analysis of PAHs at trace levels in fish tissue with HPLC-FLD. The HPLC methods are useful for PAH analysis since UV and fluorescence detection offer enhanced selectivity over other techniques such as GC with flame ionization detection [6]. The method includes sample preparation with SampliQ AOAC Buffered Extraction kit (p/n 5982-5755) and SampliQ AOAC Fatty Dispersive SPE 15 mL kit (p/n 5982-5158). Chemical structures of the PAHs in this study are shown in Figure 1.

Experimental

Reagents and Chemicals

All reagents were analytical or HPLC grade. Acetonitrile (CH_3CN) and PAHs were purchased from Sigma-Aldrich (St. Louis, MO, USA). The water used was from a MilliQ system (Milford, Mass, USA). The mobile phase was filtered through a Whatman membrane filter (47 mm diameter and 2 µm pore size).

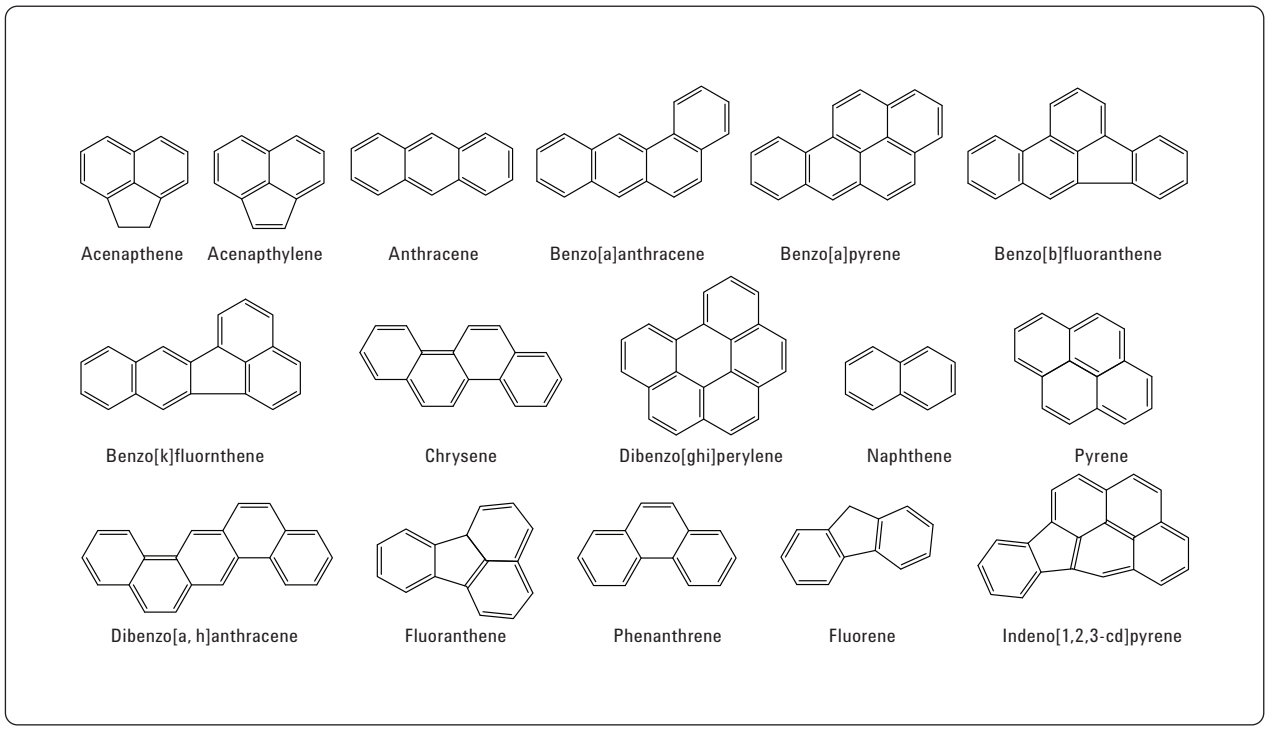


Figure 1. Chemical structures for the polycyclic aromatic hydrocarbons used in the study.

Standard Solutions

Standard stock solutions (1 mg/mL) were prepared by dissolving 10 mg of the desired PAH in 10 ml CH_3CN and stored at –20 °C. All working solutions were prepared fresh daily by serial dilution with CH_3CN.

Equipment and Material

The analysis was performed on an Agilent 1200 Series HPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with a binary pump and a fluorescence detector (FLD) set at varying excitation and emission wavelengths (Table 1). The selection of the excitation and emission wavelengths for detection was based on the optimum responses for the various PAHs. Separation of the compounds was achieved on an Agilent ZORBAX Eclipse PAH column (4.6 mm × 50 mm, 1.8 µm), p/n 959941-918. The data was processed by HPLC 2D Chemstation software.

Extraction and cleanup were achieved with Agilent SampliQ Buffered QuEChERS AOAC Extraction kit, p/n 5982-5755 and SampliQ QuEChERS AOAC Dispersive SPE kit, p/n 5982-5158, (Agilent Technologies).

A Kenwood Grinder (obtained from a local appliance store) was employed for homogenizing the fish sample.

Instrument conditions

HPLC conditions

Table 1. HPLC Conditions used for Separation of PAHs

Column	Agilent ZORBAX Eclipse PAH C18 4.6 × 50 mm, 1.8 µm	
Flow rate	0.8 mL/min	
Column temperature	18 °C	
Injection volume	5 µL	
Mobile phase	A = Deionized H_2O	B = CH_3CN
Gradient	T (min)	% B
	0	60
	1.5	60
	7	90
	13	100
Detection	UV at 230 nm (Acy) and varying fluorescence excitation (Ex) and emission (Em) wavelengths	
Wavelengths:		
Time (min)	**Ex/Em wavelengths (nm)**	**PAH detected**
0 – 5 (dark blue)	260/352	Nap, Ace, Flu, Phe, Chr
0 – 14 (red)	260/420	Ant, Pyr, BeP, DahA, BghiP
0 – 14 (light blue)	260/460	Fln, 1,2-BaA,BeA, BkF, InP

Sample preparation

The fish fillets were purchased from a local food store, minced, and deep frozen until analysis.

Extraction

A 5.0 g sample of fish homogenate was placed into a 50 mL centrifuge tube from the SampliQ QuEChERS AOAC Extraction kit and the tube was centrifuged for 20 s. Samples were then spiked with appropriate spiking solutions to yield appropriate working solutions for recoveries and reproducibility studies. A 2000 µL volume of spiking solution was added to all samples except the blank, and the tubes were shaken vigorously for 1 min. Next, 8 mL of CH_3CN, then an Agilent SampliQ QuEChERS AOAC extraction salt packet (p/n 5082-5755) containing 6 g of anhydrous $MgSO_4$ and 1.5 g of anhydrous NaOAc were added to the tubes. The sample tubes were hand shaken vigorously for 1 min, then further centrifuged at 4000 rpm for 5 min.

Dispersive SPE Cleanup

A 6.0 mL aliquot of the upper CH_3CN layer was transferred into a SampliQ QuEChERS AOAC Dispersive SPE 15 mL tube. This SPE tube contained 400 mg of PSA, 400 mg of C18EC, and 1200 mg of anhydrous $MgSO_4$. After one minute of shaking, the tubes were centrifuged at 4000 rpm for 5 min. A 4 mL aliquot of the extract was filtered through a 0.45 µm PVDF syringe filter, then 1000 µL of the extract was placed in an autosampler vial for HPLC-FLD analysis.

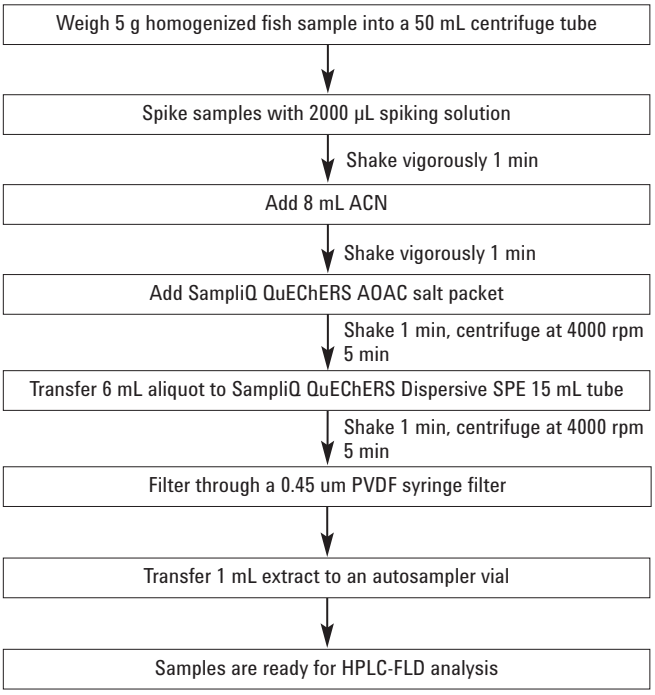


Figure 2. Flow chart of QuEChERS AOAC sample preparation procedure.

Results and Discussion

Chromatographic results

Figure 3 shows an overlay of color-coded chromatograms at various fluorescence conditions (Table 1) of the standard mixture of the 16 PAHs. A chromatogram of the blank fish extract is presented in Figure 4. Overlay chromatograms of the spiked fish sample at spiking level 1 are shown in Figure 5.

QuEChERS extraction

The use of CH_3CN as an extracting solvent in a salting-out condition, without the need to add co-solvents, attained high extraction yields as shown by the recoveries in Table 4. The CH_3CN solvent is compatible with the HPLC – FLD procedure in this application note. Therefore no evaporation or reconstitution solvent was required. This is particularly important for the PAHs since some of these compounds (naphthalene, acenaphthene and fluorene) are extremely volatile and may be lost during an evaporation step [1].

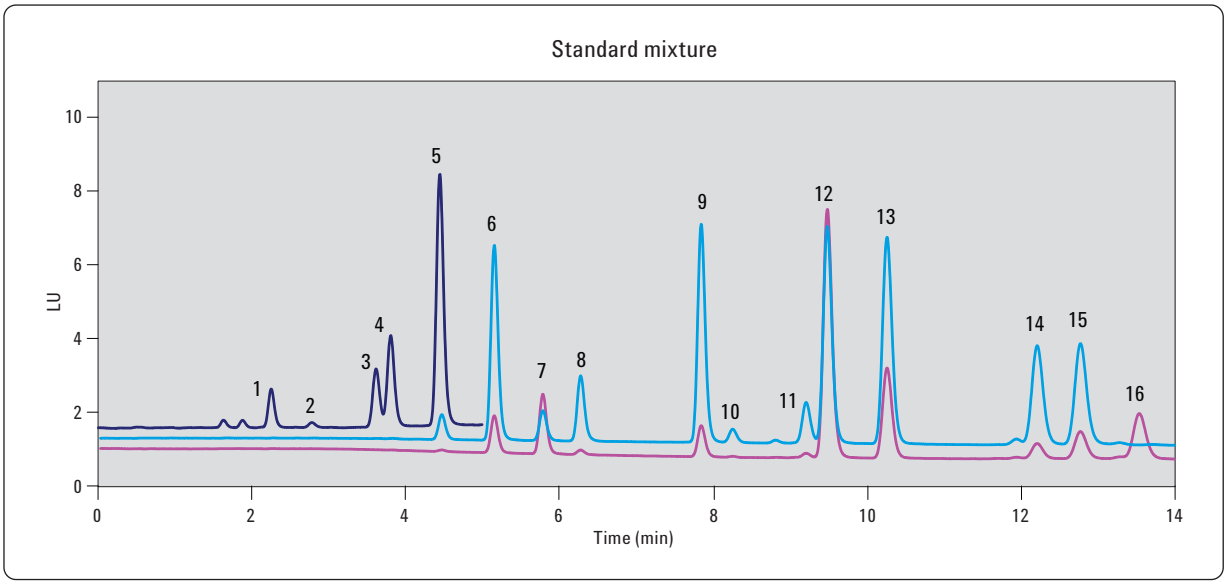


Figure 3. Overlay HPLC – FLD chromatograms of the standard mixture containing: 1. Nap 2. Acy 3. Ace 4. Flu 5. Phe 6. Ant 7. Fln 8. Pyr 9. BaA 10. Chr 11. BeP 12. BeA 13. BkF 14. DahA 15. BghiP 16. InP . The concentration of the PAHs was 1 mg/mL. The blue portion of the chromatogram used the following excitation/emission wavelengths: 260-nm/352-nm; the red portion 260-nm/420-nm; the light blue-portion: 260-nm/440-nm. For acenaphthylene, UV detection at 230-nm was used. Chromatographic conditions are shown in Table 1.

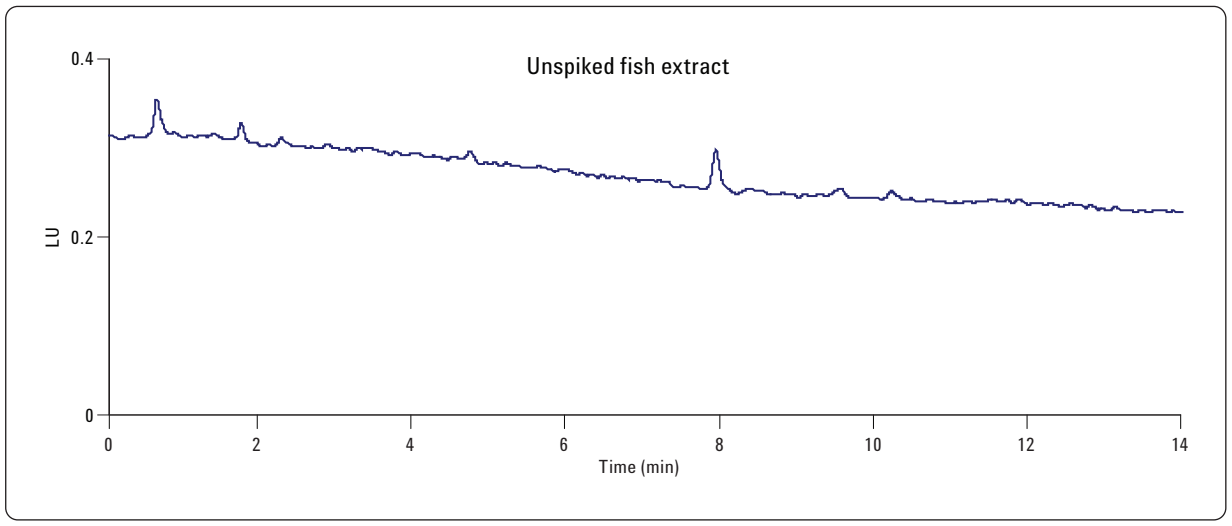


Figure 4. Chromatogram of the blank fish extract. Chromatographic conditions are shown in Table 1. The baseline chromatogram used the following excitation/emission wavelengths: 260-nm/352-nm. The other excitation/emission conditions showed no other interferences.

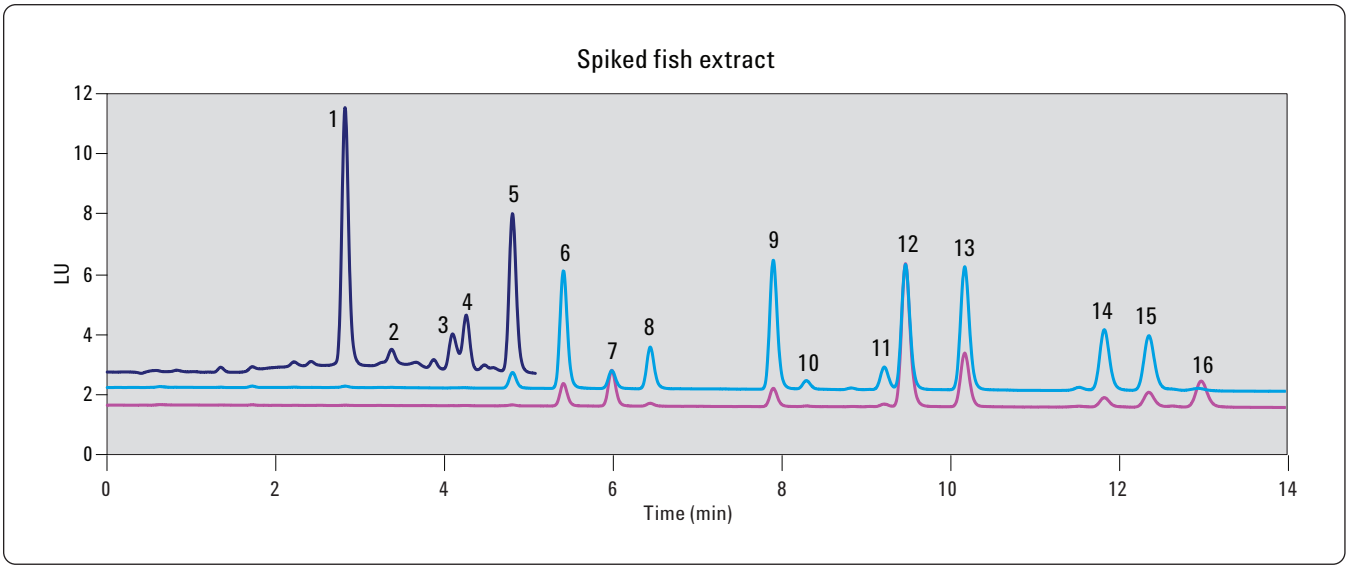


Figure 5. Overlay HPLC – FLD chromatograms of the spiked fish sample containing: 1. Nap 2. Acy 3. Ace 4. Flu 5. Phe 6. Ant 7. Fln 8. Pyr 9. BaA 10. Chr 11. BeP 12. BeA 13. BkF 14. DahA 15. BghiP 16. InP. The spiking level for this sample was level 1 (see Table 3). The blue portion of the chromatogram used the following excitation/emission wavelengths: 260-nm/352-nm; the red portion 260-nm/420-nm; the light blue portion: 260-nm/440-nm. For acenaphthylene, UV detection at 230-nm was used. Chromatographic conditions are shown in Table 1.

Linearity, Limit of Detection (LOD) and Limit of Quantification (LOQ)

Linearity

The linear calibration curves were obtained by plotting the peak area for each analyte versus its concentration. Curves were generated by spiking the sample blanks at a concentration range of 0 – 300 ng/g.

Limits of Detection and Quantification

The limits of detection and quantification were estimated from the concentration of sulfonamides required to give a signal-to-noise ratio of 3 and 10 respectively. Table 2 shows the regression equation, correlation coefficients, and very acceptable limits of detection and quantification.

Table 2. Linearity, LOD and LOQ for the Sixteen Polycyclic Aromatic Hydrocarbons

PAH	Regression equation	R^2	LOD	LOQ
Naphthalene	Y = 0.0222x + 0.1366	0.9991	0.62	2.07
*Acenaphthylene	Y = 0.0544x – 0.0130	0.9993	0.25	0.83
Acenaphthene	Y = 0.0184 x – 0.0204	0.9998	0.56	1.87
Fluorene	Y = 0.0323x – 0.1717	0.9990	0.12	0.40
Phenanthrene	Y = 0.0950x + 0.0086	0.9995	0.18	0.60
Anthracene	Y = 0.0838x – 0.1265	0.9991	0.24	0.80
Fluoranthene	Y = 0.0247x – 0.0237	0.9994	0.04	0.16
Pyrene	Y = 0.0218x – 0.0432	0.9998	0.09	0.30
1,2-Benzanthracene	Y = 0.0120x – 0.0103	0.9994	0.03	0.10
Chrysene	Y = 0.0052x + 0.0086	0.9990	0.28	0.93
Benzo[e]pyrene	Y = 0.0144x – 0.0037	0.9997	0.04	0.16
Benz[e]acenaphthylene	Y = 0.1186x – 0.032	0.9995	0.07	0.23
Benzo[k]fluoranthene	Y = 0.0464x + 0.0969	0.9997	0.05	0.16
Dibenzo[a,h]anthracene	Y = 0.0531x + 0.0001	0.9990	0.84	2.80
Benzo[g,h,i]perylene	Y = 0.0440x + 0.0722	0.9993	0.11	0.36
Indeno[1,2,3-cd]pyrene	Y = 0.0324x – 0.0912	0.9993	0.05	0.18

* UV detection at 230 nm

Recovery and Reproducibility

The recovery and reproducibility (RSD) were evaluated on spiked samples at three different levels (Table 3). The analysis was performed in replicates of six (n = 6) at each level. Table 4 shows the very good to excellent recoveries, and excellent RSD values for the sixteen polycyclic aromatic hydrocarbons.

Conclusions

A simple and fast mulitiresidue method based on SampliQ QuEChERS AOAC and HPLC-FLD has been developed for the simultaneous determination of sixteen polycyclic aromatic hydrocarbons at parts-per-billion (ppb) levels in fish tissue. High recoveries with excellent RSD were attained, therefore the method should be applied for quality control of PAHs in real samples.

Table 3. PAHs Spiking Levels

PAH	Spiking level (ng/g)		
	1	2	3
Naphthalene	20	100	200
*Acenaphthylene	20	100	200
Acenaphthene	10	50	100
Fluorene	10	50	100
Phenanthrene	10	50	100
Anthracene	10	50	100
Fluoranthene	10	50	100
Pyrene	10	50	100
1,2-Benzanthracene	5	20	50
Chrysene	10	50	100
Benzo[e]pyrene	5	20	50
Benz[e]acenaphthylene	5	20	50
Benzo[k]fluoranthene	5	20	50
Dibenzo[a,h]anthracene	5	20	50
Benzo[g,h,i]perylene	5	20	50
Indeno[1,2,3-cd]pyrene	5	20	50

* UV detection at 230 nm

Table 4. Recoveries and RSDs for the Sixteen Polycyclic Aromatic Hydrocarbons in Fish Sample (n = 6)

PAH	Level of spiking (ng/g) (n = 6)					
	1		2		3	
	%Recovery	%RSD	%Recovery	%RSD	%Recovery	%RSD
Naphthalene	94.7	1.4	97.9	1.1	93.8	1.4
*Acenaphthylene	87.8	1.7	96.3	1.2	85.6	0.8
Acenaphthene	92.1	1.5	93.0	1.8	96.7	0.8
Fluorene	98.1	1.5	89.9	1.0	97.2	0.9
Phenanthrene	90.6	0.9	93.8	0.8	83.1	1.7
Anthracene	96.7	1.0	87.6	0.8	92.1	0.6
Fluoranthene	83.4	1.3	93.9	1.5	95.9	1.2
Pyrene	93.5	1.8	86.1	1.3	95.0	1.4
1,2-Benzanthracene	94.5	1.3	89.6	1.6	94.9	1.0
Chrysene	101.0	1.4	97.8	1.7	87.2	1.6
Benzo[e]pyrene	88.8	1.5	85.2	1.9	95.0	1.4
Benz[e]acenaphthylene	95.5	0.7	92.7	0.7	89.2	0.9
Benzo[k]fluoranthene	93.5	0.8	94.6	0.9	98.9	0.8
Dibenzo[a,h]anthracene	88.2	0.9	97.3	1.1	97.1	0.6
Benzo[g,h,i]perylene	98.4	0.8	95.5	1.6	98.2	0.7
Indeno[1,2,3-cd]pyrene	91.5	1.5	97.9	0.9	94.3	0.7

* UV detection at 230 nm

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Determination of Acrylamide in Cooking Oil by Agilent SampliQ QuEChERS Acrylamide Kit and HPLC-DAD

Application Note

Food Safety

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Abstract

This application note describes a method based on a quick, easy, cheap, effective, rugged and safe (QuEChERS) multiresidue sample preparation procedure. The QuEChERS method presents an extraction and cleanup protocol for the determination of acrylamide employing methacrylamide as the internal standard. The analyte and internal standard were separated on an Agilent ZORBAX HILIC Plus column (4.6 mm × 50 mm, 3.5 µm) by isocratic elution employing 3% 5 mM CH_3COOH and 97% CH_3CN with subsequent diode array detection at 210 nm. The acrylamide recoveries ranged from 84 to 93.8% with relative standard deviation of less than 4%. The limits of detection and quantification were 32.4 and 108 ng/mL respectively.

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Introduction

Acrylamide (Figure 1) is an organic compound used to manufacture plastic materials, paper, dyes, cosmetics and polyacrylamide, which is a water stabilizer for sewage water treatment and gel electrophoresis [1]. Acrylamide also occurs naturally as a byproduct of the cooking process and its presence in food was first confirmed by Swedish researchers in 2002 [2]. The Swedish findings about high levels of acrylamide in heat treated foods were quickly confirmed by the UK Food Standards Agency through its official website notification on 17 May 2002 and US Environmental Protection Agency (USEPA) found the limit for acrylamide in drinking water to be extremely low (0.5 µg/kg) [3]. Carbohydrate-rich foods such as french fries processed at high temperatures and under low moist conditions are of concern because high concentrations of acrylamides are produced in this process [4]. Acrylamide, at high concentrations, has adverse effects as a human neurotoxin and has also been classified as a probable carcinogen and genotoxicant [5]. Acrylamide vapors irritate the eyes and the skin and can cause paralysis of the cerebrospinal system [6].

The QuEChERS method, which was initially designed for the analysis of pesticides in food has since been adapted to include the extraction of a variety of analytes such as acrylamide [5]. In general, there are two major steps: extraction and dispersive SPE cleanup. The method uses a single step buffered acetonitrile extraction while simultaneously salting out water from the sample using anhydrous magnesium sulfate ($MgSO_4$) to induce liquid-liquid partitioning. After removing an aliquot from an organic layer for further cleanup, dispersive solid phase extraction (dSPE) is conducted using a combination of primary secondary amine (PSA) to remove fatty acids, and anhydrous $MgSO_4$ to reduce the remaining water in the extract.

This application note presents a method for the analysis of acrylamide in cooking oil with HPLC-DAD. The method includes sample preparation with SampliQ QuEChERS Extraction kit for acrylamides (p/n 5982-5850) and SampliQ EN Fruits and Vegetables with Fats and Waxes Dispersive SPE kit (p/n 5982-5156).

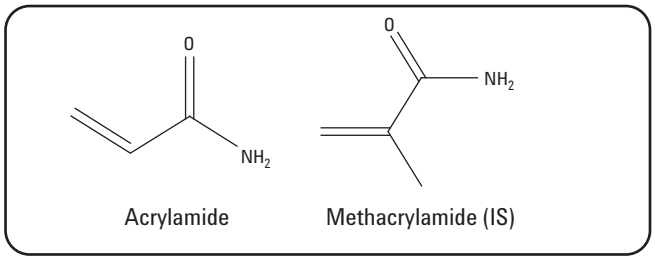


Figure 1. Chemical structures for acrylamide and methacrylamide (IS).

Experimental

Reagents and Chemicals

All reagents were analytical or HPLC grade. Acetonitrile (CH_3CN), n-hexane, acrylamide and methacrylamide were purchased from Sigma-Aldrich (St. Louis, MO, USA). The water used was from a MilliQ system from Millipore (Milford, Mass, USA). The mobile phase was filtered through a Whatman membrane filter (47 mm diameter and 2 µm pore size).

Standard Solutions

Standard stock solutions (1 mg/mL) were prepared by dissolving 10 mg of the acrylamide/methacrylamide in 10 mL MilliQ water and stored at 4 °C. All working solutions were prepared daily by serial dilution also in MilliQ water.

Equipment and Material

The analysis was performed on an Agilent 1200 Series HPLC, Agilent Technologies Inc. (Santa Rosa, CA, USA) equipped with a binary pump and a diode array detector (DAD) set at 210 nm. Separation of the compounds was achieved on an Agilent ZORBAX HILIC Plus column (4.6 mm × 50 mm, 3.5 µm, p/n 959943-901). The data was processed by Agilent ChemStation for LC/MS 2D system software.

Extraction and cleanup were achieved with Agilent SampliQ QuEChERS Extraction kit for acrylamides, p/n 5982-5850 and SampliQ QuEChERS EN Dispersive SPE kit, p/n 5982-5156.

Instrument conditions

HPLC conditions

Table 1. HPLC Conditions used for Separation of Acrylamide and Methacrylamide

Column	Agilent ZORBAX HILIC Plus 4.6 × 50 mm, 3.5 µm
Flow rate	0.2 mL/min
Column temperature	30 °C
Injection volume	5 µL
Mobile phase	Isocratic elution: A = 3% 5 mM acetic acid B = 97% acetonitrile
Run time	10 min
Post time	3 min
Detection	DAD @ 210 nm

Sample preparation

The cooking oil (sunflower-based) was purchased from a local store.

Extraction

Figure 2 outlines the extraction procedure. A 1-g sample of cooking oil was placed into a 50-mL centrifuge tube from the SampliQ QuEChERS Extraction kit. Samples were spiked appropriately to yield working solutions for recoveries and reproducibility studies. Samples, with exception of the blank, were fortified with 1000 µL spiking solution and mixed with 9 mL of water. After shaking vigorously for 1 min, 10 mL of CH_3CN were added, followed by an addition of Agilent SampliQ QuEChERS extraction salt mixture for acrylamides (p/n 5082-5850). The QuEChERS extraction packet contained 4 g of anhydrous $MgSO_4$ and 0.5 g NaCl. Due to the high concentration of long chain fatty acids in the cooking oil, 5 mL of hexane were added to the extraction mixture. The sample tubes were hand-shaken vigorously for 1 min and then centrifuged at 4000 rpm for 5 min. Note that in the centrifuge tube, the hexane forms a third layer [hexane (top layer): acetonitrile (middle layer): water + salts (bottom layer). The hexane layer was discarded prior to the dSPE cleanup.

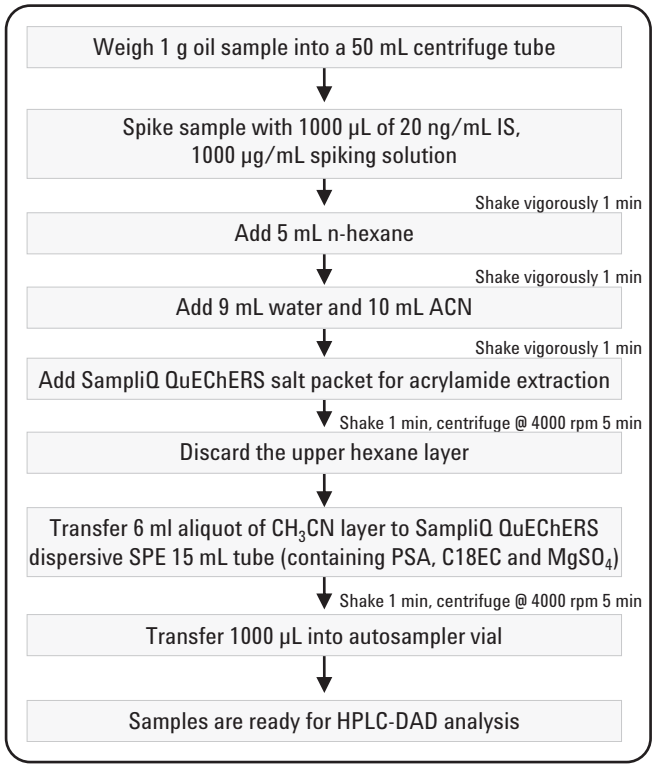


Figure 2. Flow chart for the QuEChERS sample preparation procedure.

Dispersive SPE cleanup

A 6-mL aliquot of the CH_3CN layer (now the top layer after hexane removal) was transferred into a SampliQ QuEChERS EN Dispersive SPE 15 mL tube. The SPE tube contained 150 mg PSA, 150 mg C18EC and 900 mg $MgSO_4$. The tubes were then further centrifuged at 4000 rpm for 5 min. A 1000-µL amount of extract was placed in an autosampler vial for an HPLC-DAD analysis.

Results and Discussion

Chromatographic analysis

The separation of acrylamide and methacrylamide (internal standard) was achieved on an Agilent ZORBAX HILIC Plus column (4.6 mm × 50 mm, 3.5 µm, p/n 959943-901) using isocratic elution, with 3% 5 mM acetic acid and 97% acetonitrile as the mobile phase. The column temperature was set at 30 °C while the flow rate was set at 0.2 mL/min. Figure 3 shows a typical chromatogram for the injection of the standard mixture. Different mobile phase polarity compositions, from 100% water to 100% acetonitrile, were evaluated. The best retention with a short run time was obtained with 97% acetonitrile and 3% acetic acid.

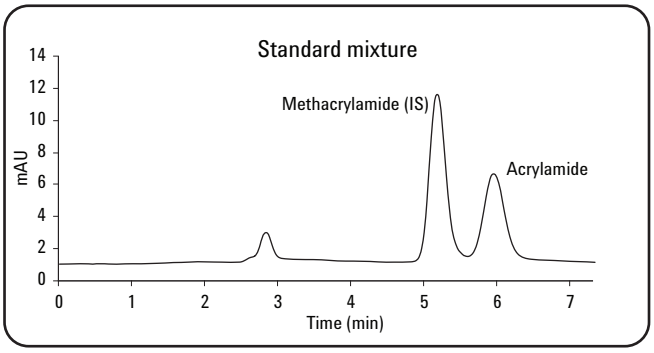


Figure 3. Chromatogram of the standard mixture of acrylamide and methacrylamide (IS).

QuEChERS method

QuEChERS salt packet (p/n 5982–5850) containing 4 g $MgSO_4$ and 0.5 g NaCl was used to extract acrylamide from 1 g of cooking oil sample. The addition of the salt induced the acetonitrile–water phase separation [5]. A 5-mL volume of n-hexane was added to the samples for defatting, which removed long chain fatty acids that could create challenges in chromatographic analysis by giving peaks overlapping with the analyte or clogging the column [6]. Dispersive SPE was employed for sample cleanup.

The QuEChERS protocol in this application note is simple and does not require evaporating the extracting solvent. This is beneficial because acrylamides are usually lost during this step, leading to low recoveries. [7].

Linearity, limit of detection (LOD) and limit of quantification (LOQ)

Linearity

A linear calibration curve (Figure 4) was obtained by plotting the relative responses of analyte (peak area of analyte / peak area of IS) to the relative concentration of analyte (concentration of analyte / concentration of IS). The curve was generated by spiking the sample blanks at a concentration range of 0 - 1500 ng/mL. Good linearity was demonstrated with $r^2 = 0.9992$.

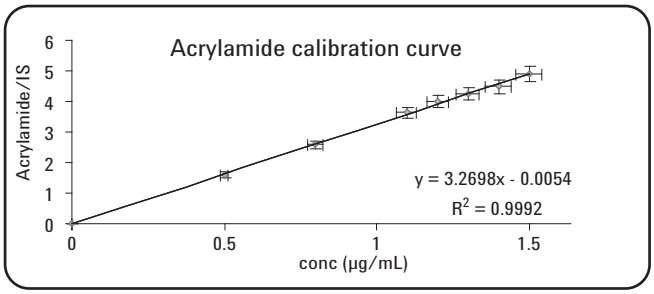


Figure 4. Acrylamide calibration curve.

Limits of Detection and Quantification

The limits of detection and quantification were evaluated from the concentration of acrylamide required to give a signal-to-noise ratio of 3 and 10 respectively. The limit of detection (LOD) was found to be 32.4 ng/mL while the limit of quantification (LOQ) was 108 ng/mL.

Recovery and Reproducibility

The recovery and reproducibility (RSD) were evaluated on spiked samples at three different fortification levels: 500, 1000, and 2000 ng/mL. The analysis was performed in replicates of six (n = 6) at each level. Table 2 shows the recoveries and RSD values for acrylamide.

Table 2. Recoveries and RSDs for the Acrylamide in Oil Sample (n = 6)

Concentration (ng/mL)	Recovery % (n = 6)	RSD % (n = 6)
500	84.0	3.2
1000	93.8	2.2
2000	92.2	1.5

The chromatograms of the oil blank and the spiked oil sample, after the QuEChERS extraction and cleanup, are shown in Figures 5 and 6 respectively. The blank oil extract did not show any detectable amounts of acrylamide. Similar results were reported on a study of acrylamide content in commercial frying oils [7]. The frying oils in that study did not contain any detectable amounts of acrylamide (detection limit of 0.02 µg/mL) prior to processing food samples.

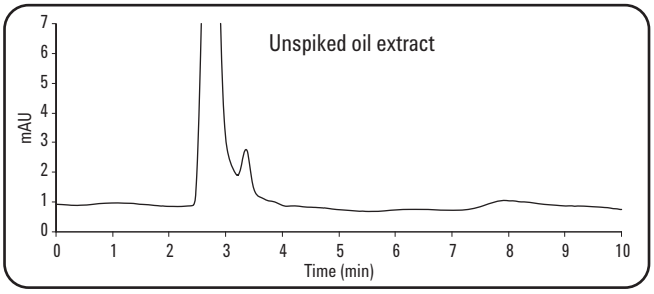


Figure 5. Chromatogram of the blank oil extract.

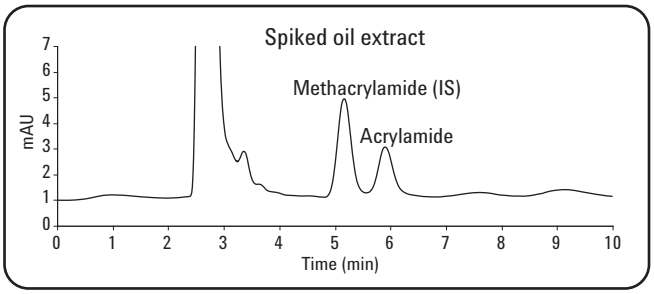


Figure 6. Chromatogram of the spiked oil extract.

Conclusions

A simple and fast multiresidue method using SampliQ QuEChERS for acrylamide extraction and cleanup with an HPLC-DAD analysis on a HILIC column has been developed. High extraction yields with excellent RSD, LOD (32.4 ng/mL), and LOQ (108 ng/mL) were obtained. Therefore, the method may be applied for quality control of acrylamide in real samples.

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Analysis of Pesticide Residues in Spinach Using Agilent SampliQ QuEChERS AOAC Kits by GC/MS

Application Note

Food Safety

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Abstract

This application note describes the use of a quick, easy, cheap, effective, rugged, and safe (QuEChERS) AOAC sample preparation approach for extraction and cleanup of 18 GC-amenable multiple pesticide class residues in spinach. The method employed involves initial extraction in a buffered aqueous/acetonitrile system, an extraction/partitioning step after the addition of salt, and a cleanup step using dispersive solid phase extraction (dispersive SPE). In order to address the significant loss of planar pesticides caused by graphitized carbon black (GCB) in dispersive SPE, a modified method with addition of toluene was employed for the planar pesticides. The target pesticides in the spinach extracts were then analyzed by gas chromatography/mass spectrometry (GC/MS) operating in selective ion monitoring (SIM) mode. The method was validated in terms of recovery and reproducibility. The limit of quantitation (LOQ) for most pesticides is 10 ng/g; however folpet has an LOQ of 50 ng/g in spinach. This application, employing SampliQ QuEChERS kits, produced results well below the maximum residue limits (MRLs) for all pesticides screened. The spiked levels for the recovery experiments were 10, 50, and 200 ng/g.

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Introduction

The AOAC QuEChERS method has been widely applied for the analysis of pesticides in food since it was introduced by USDA scientists. [1-3] In summary, the method uses a single-step buffered acetonitrile (1% HAc) extraction while simultaneously salting out water from the sample using anhydrous magnesium sulfate ($MgSO_4$) to induce liquid-liquid partitioning. For cleanup, a dispersive SPE step is employed using a combination of primary secondary amine (PSA) to remove fatty acids as well as other components, and anhydrous $MgSO_4$ to reduce the remaining water in the extract. After mixing and centrifugation, the upper layer is ready for analysis. According to different food matrices, other ingredients may be added in this step, such as graphitized carbon black (GCB) to remove pigments and sterol, or C18 to remove more lipids and waxes.

Spinach is considered to be a highly pigmented matrix since it contains large amounts of chloryophyll. Therefore, the dispersive SPE kits with GCB were selected for further clean-up. GCB adsorbs planar molecules such as pigments and sterols; therefore it is very helpful in cleaning-up pigmented matrix like spinach. However, GCB also adsorbs pesticides with planar structure, such as carbendazim, chlorothalonil, and coumaphos. As a result, this kind of dispersive SPE kit is not recommended for the analysis of planar pesticides. Previously, we discussed the impact of toluene addition to the dispersive SPE tube on the analysis of pesticides in pigmented matrices [4]. It turned out that this modification can greatly increase the extraction efficiency of those problematic pesticides. With the combination of the original (w/o toluene) and modified (w/toluene) dispersive SPE, the performance of SampliQ AOAC Buffered Extraction Kits and SampliQ AOAC Dispersive SPE kits for pigmented produce was demonstrated to be excellent for the analysis of LC amenable pesticides in spinach. [5]

In this study, the performance of the SampliQ AOAC Buffered Extraction kit (p/n 5982-5755) and SampliQ AOAC Dispersive-SPE kits for Pigmented Fruits and Vegetables (p/n 5982-5222 and 5982-5258) was evaluated for the extraction of volatile and semi-volatile pesticides. Analysis was performed by GC/MS. Seventeen GC-amenable pesticides were selected which represent multiple classes, including non-polar organochlorine pesticides (OCs), certain organophosphorus pesticides (OPs) and organonitrogen pesticides (ONs). Table 1 shows the chemical and regulatory information for these pesticides in spinach.

Experimental

Reagents and Chemicals

All reagents and solvents were high-performance liquid chromatography (HPLC) or analytical grade. Methanol (MeOH) and toluene were from Honeywell (Muskegon, MI, USA), acetonitrile (ACN) and glacial acetic acid (HAc) were from Sigma-Aldrich (St Louis, MO, USA). Formic acid (FA) was from Fluka (Sleinheim, Germany). The pesticide standards and internal standard (triphenyl phosphate, TPP) were purchased from Sigma-Aldrich (St Louis, MO, USA), Chem Service (West Chester, PA, USA), or Ultra Scientific (North Kingstown, RI, USA).

Solutions and Standards

A 1% acetic acid in ACN solution was prepared by adding 10 mL of HAc to 1 L of ACN.

Standard and internal standard (IS) stock solutions (2 mg/mL) were made in MeOH, respectively, and stored at –20 °C. Three QC spiking solutions of 1.5, 7.5 and 30 µg/mL were made fresh daily in 1:1 ACN/H2O containing 0.1% FA. A 2.5 µg/mL standard solution in ACN containing 0.1% FA was used to prepare the calibration curves in the matrix blank extract by appropriate dilution. A 15 µg/mL of TPP spiking solution in 1:1 ACN/H2O containing 0.1% FA was used as the internal spiking standard (IS).

Equipment and Material

Agilent Gas Chromatograph (Agilent Technologies Inc., Santa Clara, CA, USA).

Agilent 5975C Mass Spectrometer (Agilent Technologies Inc., Santa Clara, CA, USA).

SampliQ QuEChERS AOAC Extraction kits, p/n 5982-5755 (Agilent Technologies Inc., Wilmington, DE, USA).

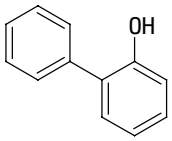
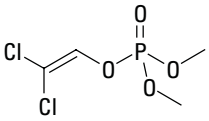
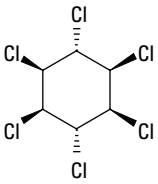
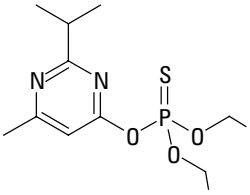
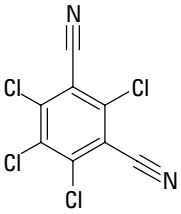
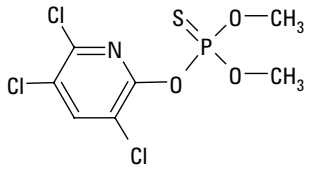
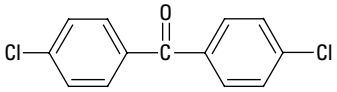
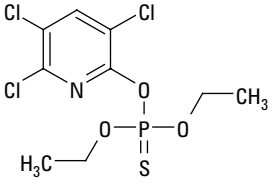
SampliQ QuEChERS AOAC dispersive SPE kits for Pigmented Fruits and Vegetables, p/n 5982-5222 and 5982-5258 (Agilent Technologies Inc., Wilmington, DE, USA).

CentraCL3R Centrifuge (Thermo IEC, MA, USA)

Bottle top dispenser (VWR, So Painfield, NJ, USA)

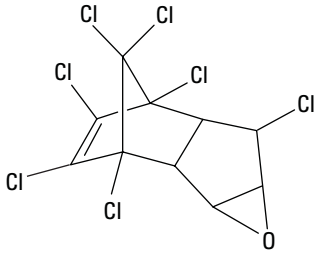
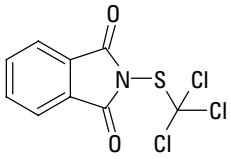
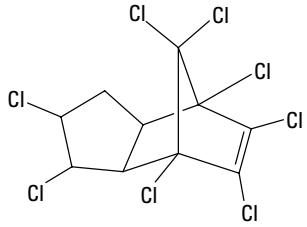
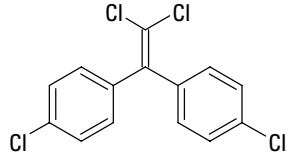
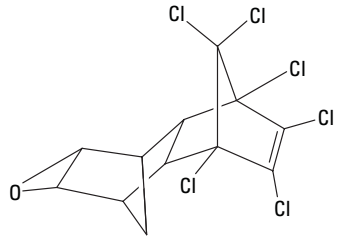
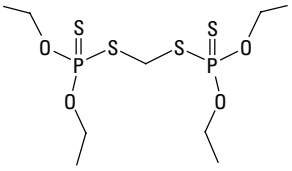
Eppendorf microcentrifuge (Brinkmann Instruments, Westbury, NY, USA)

Table 1. Pesticides Chemical and Regulatory Information [6–9]

Name	Category	Log P	pKa	Structure	MRLs in spinach (ng/g)*
σ-Phenylphenol	Phenol	3.18	9.4		2000
Dichlorvos	Organophosphate	1.9	NA		10
Lindane	Organochlorine	3.69	NA		10
Diazinon	Organophosphate	3.69	2.6		100
Chlorothalonil	Chloronitrile	2.94	NA		4000
Chlorpyrifosmethyl	Organophosphate	4.00	NA		30
Dichlorobenzophenone	Organochlorine	4.44	NA		500
Chlorpyrifos	Organophosphate	4.7	NA		10

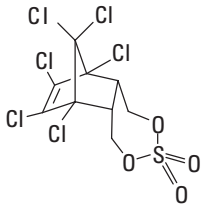
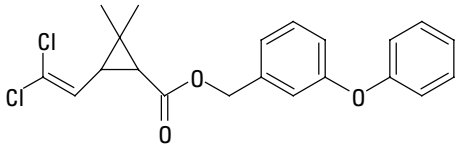
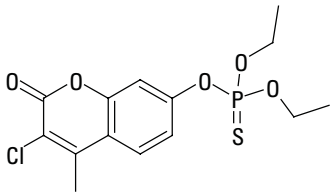
(Continued)

Table 1. Pesticides Chemical and Regulatory Information [6–9]

Name	Category	Log P	pKa	Structure	MRLs in spinach (ng/g)*
Heptachlor epoxide	Organochlorine	5.83	NA		30
Folpet	Phthalimide	3.02	NA		2000
Chlordane	Cyclodiene organochlorine	2.78	NA		20
DDE	Organochlorine	6.55	NA		50
Dieldrin	Chlorinated hydrocarbon	3.7	NA		10
Ethion	Organophosphate	5.07	NA		300

(Continued)

Table 1. Pesticides Chemical and Regulatory Information [6–9]

Name	Category	Log P	pKa	Structure	MRLs in spinach (ng/g)*
Endosulfan sulfate	Organochlorine	3.13	NA		50
Permethrins	Pyrethroid	6.1	NA		50
Coumaphos	Organothio phosphate	3.86	NA		100

*The MRLs numbers list in the table are for apple or lowest level in other fruit and vegetables. They could be higher in different commodities.

Instrument Condition

An Agilent GC/MS method for pesticides analysis was used for this study. [10]

GC conditions

Inlet:	Splitless
Inlet liner:	Helix double taper, deactivated (p/n 5188-5398)
Carrier gas:	Helium
Inlet pressure:	19.6 psi (constant pressure mode) during run 1.0 psi during backflush
Inlet temperature:	250 °C
Injection volume:	1.0 µL
Purge flow to split vent:	30 mL/min at 0.75 min
Oven temperature program:	70 °C (1 min), 50 °C/min to 150 °C (0 min), 6 °C /min to 200 °C (0 min), 16 °C/min to 280 °C (6 min)
Post run:	3 min
Capillary flow technology:	Purged Ultimate Union (p/n G3186B) - used for backflushing the analytical column and inlet.
	Aux EPC gas: Helium plumbed to Purged Ultimate Union
Aux EPC pressure:	4.0 psi during run, 80.0 psi during backflush
Column:	Agilent J&W HP-5MS Ultra Inert 15 m × 0.25 mm, 0.25 µm (p/n 19091S-431UI)
Connections:	Between inlet and Purged Ultimate Union (p/n: G3186B)
Restrictor:	65 cm x 0.15 mm, 0.15 µm DB-5MS Ultra Inert
Connections:	Between the Purged Ultimate Union and the MSD

MS conditions

Tune file	Atune.u
Mode	SIM (refer to Table 2 for settings in detail)
Source, quad, transfer line temperature	230 °C, 150 °C and 280 °C respectively
Solvent delay	2.30 min
Multiplier voltage	Autotune voltage

Sample Preparation

The sample preparation procedure includes sample comminution, extraction and partitioning and dispersive SPE clean-up. This process has been described in detail in previous application notes. [8] The procedure used for spinach was similar with the exception of the dispersive SPE clean-up step which includes toluene addition.

The frozen chopped organic spinach was homogenized thoroughly. A 15 g (±0.1 g) amount of homogenized sample was placed into a 50 mL centrifuge tube. Samples were fortified with appropriate QC spiking solutions (100 µL) when necessary, then fortified with 100 µL of IS spiking solution (15 µg/mL of TPP). After vortexing the sample for 30s, 15 mL of 1% HAc in ACN was added to each tube using the dispenser. To each tube, an Agilent SampliQ QuEChERS AOAC extraction salt packet (p/n 5982-5755) was added directly. Sample tubes were capped tightly, and hand-shaken vigorously for 1 min. Tubes were centrifuged at 4000 rpm for 5 min.

Next, the ACN extracts were separated into two parts for both original and modified dispersive SPE methods. The modified dispersive SPE method has a different procedure; therefore, it is described below in detail. The volume of ACN extracts (about 14 mL) will be enough for simultaneously processing samples with original and modified dispersive SPE when using the 2 mL size dispersive SPE tube. If you are using the 15 mL size tube, then 14 mL of ACN extracts from the one sample will not be enough for processing dispersive SPE by the two methods (since 8 mL are required for each dispersive SPE method). Therefore, another sample must be extracted from the beginning.

Table 2. Instrument Acquisition Data Used for the Analysis of 18 Pesticides by GC/MS

Analyte	SIM	Collection window (min)	RT (min)
(1) Dichlorvos	184.9	2.3 – 4.0	2.88
(2) σ-Phenylphenol	170.1, 169.1	4.0 – 5.0	4.35
(3) Lindane	180.9, 182.9	5.0 – 6.9	6.67
(4) Diazinon	137.1, 179.1	6.9 – 7.7	7.19
(5) Chlorothalonil	265.8, 263.8	6.9 – 7.7	7.34
(6) Chlorpyrifos-methyl	285.9, 287.9	7.7 – 8.6	8.25
(7) Dichlorobenzophenone	250.0,139.0	8.6 – 10.0	9.55
(8) Chlorpyrifos	196.8, 198.8	8.6 – 10.0	9.57
(9) Heptachlor epoxide	352.8, 354.8	10.0 – 10.4	10.31
(10) Folpet	259.9, 261.9	10.4 – 10.85	10.75
(11) γ-Chlordane	372.8, 374.8	10.85 – 11.6	10.97
(12) DDE	245.9, 317.9	10.85 – 11.6	11.21
(13) α-Chlordane	372.8, 374.8	10.85 – 11.6	11.50
(14) Dieldrin	262.9, 264.9	11.0 – 12.3	11.89
(15) Ethion	230.9	12.3 – 13.6	12.97
(16) Endosulfan sulfate	273.8	12.3 – 13.6	13.35
TPP (IS)	325.1, 326.1	13.6 – 15.0	13.84
(17) Permethrin	183.1	15.0 – 23.0	15.69, 15.79
(18) Coumaphos	362.0, 225.9	15.0 – 23.0	15.83

A 1 mL aliquot of the upper ACN layer was transferred into an Agilent SampliQ QuEChERS dispersive SPE 2 mL tube (p/n 5982-5222); or an 8 mL aliquot was transferred into an Agilent SampliQ QuEChERS dispersive SPE 15 mL tube (p/n 5982-5258). The 2 mL tube contained 50 mg of PSA, 50 mg of GCB and 150 mg of anhydrous $MgSO_4$; while the 15 mL tube contained 400 mg of PSA, 400 mg of GCB and 1200 mg of anhydrous $MgSO_4$.

Next, 375 µL of toluene were added to the 2 mL tube, and 3 mL of toluene were added to the 15 mL tube. The tubes were tightly capped and vortexed for 1 min. We suggest vortexing the tubes for a few seconds before adding the sample, to prevent possible agglomerates. The 2 mL tubes were centrifuged with a micro-centrifuge at 13,000 rpm for 2 min, and the 15 mL tubes were centrifuged in a standard centrifuge at 4000 rpm for 5 min. An 825 µL amount of extract was then transferred into another tube, and dried by N_2 flow. Samples were reconstituted into 600 µL of ACN containing 0.1% FA. After vortexing and sonicating, the reconstituted samples were transferred directly into autosampler vials for GC/MS injection. The reconstituted blank samples were directly used to prepare the calibration curve.

Another aliquot of ACN extracts was processed following the original dispersive SPE clean-up procedure. Figure 1 shows the flow chart of the whole extraction procedure (original and modified dispersive SPE, 2 mL size) for spinach samples.

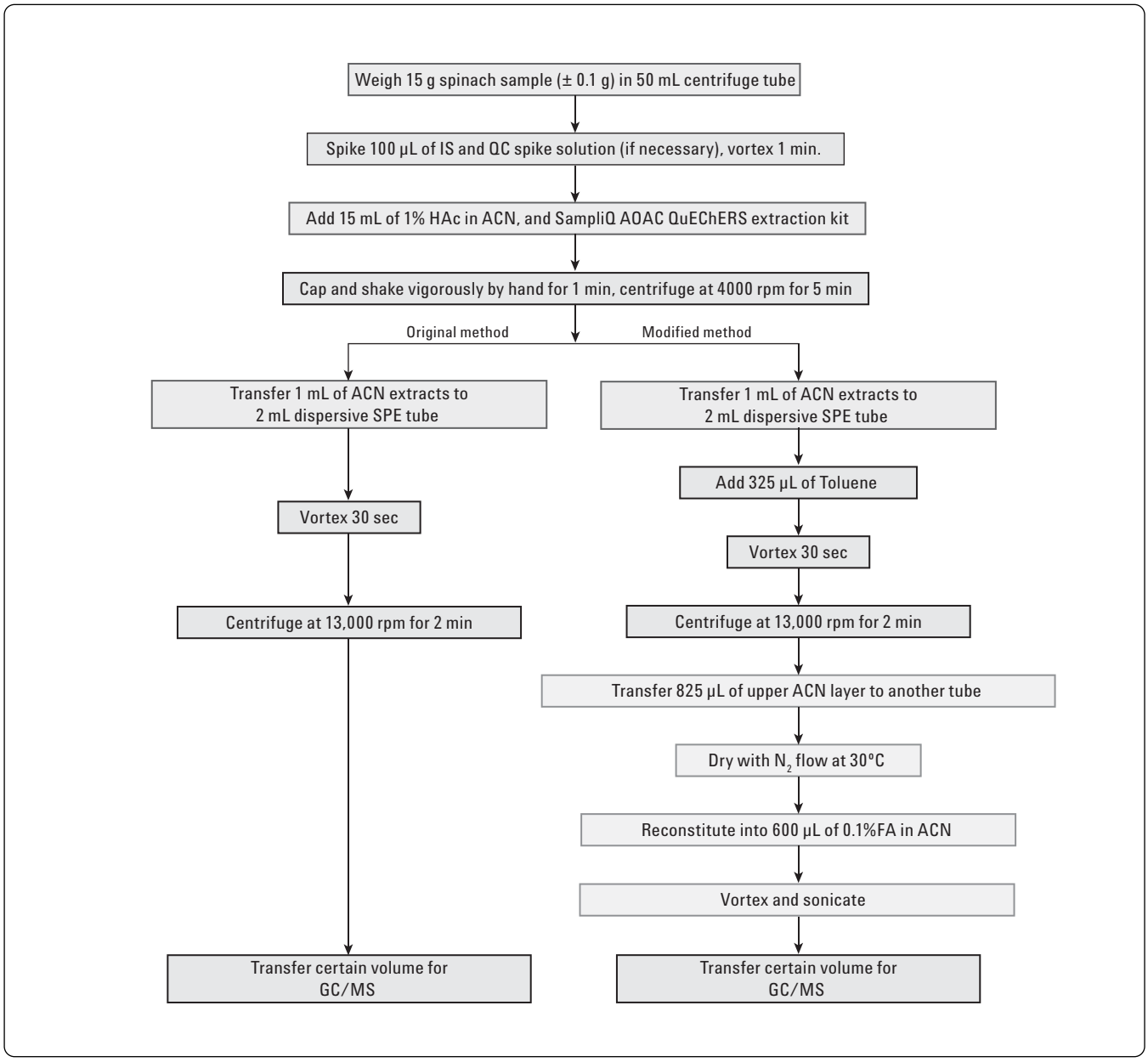


Figure 1. Flow chart of the QuEChERS AOAC extraction procedure (original and modified dispersive SPE, 2 mL size) for spinach sample.

Results and Discussion

The QuEChERS method for pesticide residues analysis provides high-quality results in a fast, easy, inexpensive approach. For the pigmented fruits and vegetables, the addition of GCB in the dispersive SPE tube can greatly remove pigments and sterols. In order to address the significant loss of planar pesticides, toluene was added to increase the extraction efficiency of those pesticides. Previously we discussed that the addition of toluene retained more matrix impurities in the final sample. [4] In the application using LC/MS/MS, there's no chromatographic differences between the samples processed by the original and modified methods due to the powerful selectivity of LC/MS/MS. The selectivity of GC/MS (SIM mode) is not as powerful as that of LC/MS/MS (MRM mode). In GC/MS there are interference peaks apparent in the blank chromatogram. Fortunately most of the pesticides tested are free of co-eluting interferences. There was also an interference eluting at a retention time very close to that of σ-phenylphenol, and this cannot be differentiated for quantitation. The response of this interferent within the blank was integrated to be less than 20% of the response of the σ-phenylphenol peak at the LOQ (10 ng/g) sample. Therefore, it was considered selectivity-acceptable for this compound. Finally, the GC/MS blank chromatograms showed minor differences from samples processed by the original and modified methods, but these differences did not affect the analysis of the target analytes. Figure 2 and Figure 3 show the GC/MS chromatograms of matrix blank (IS spiked) and 50 ng/g fortified spinach extract processed by the original and modified dispersive SPE methods.

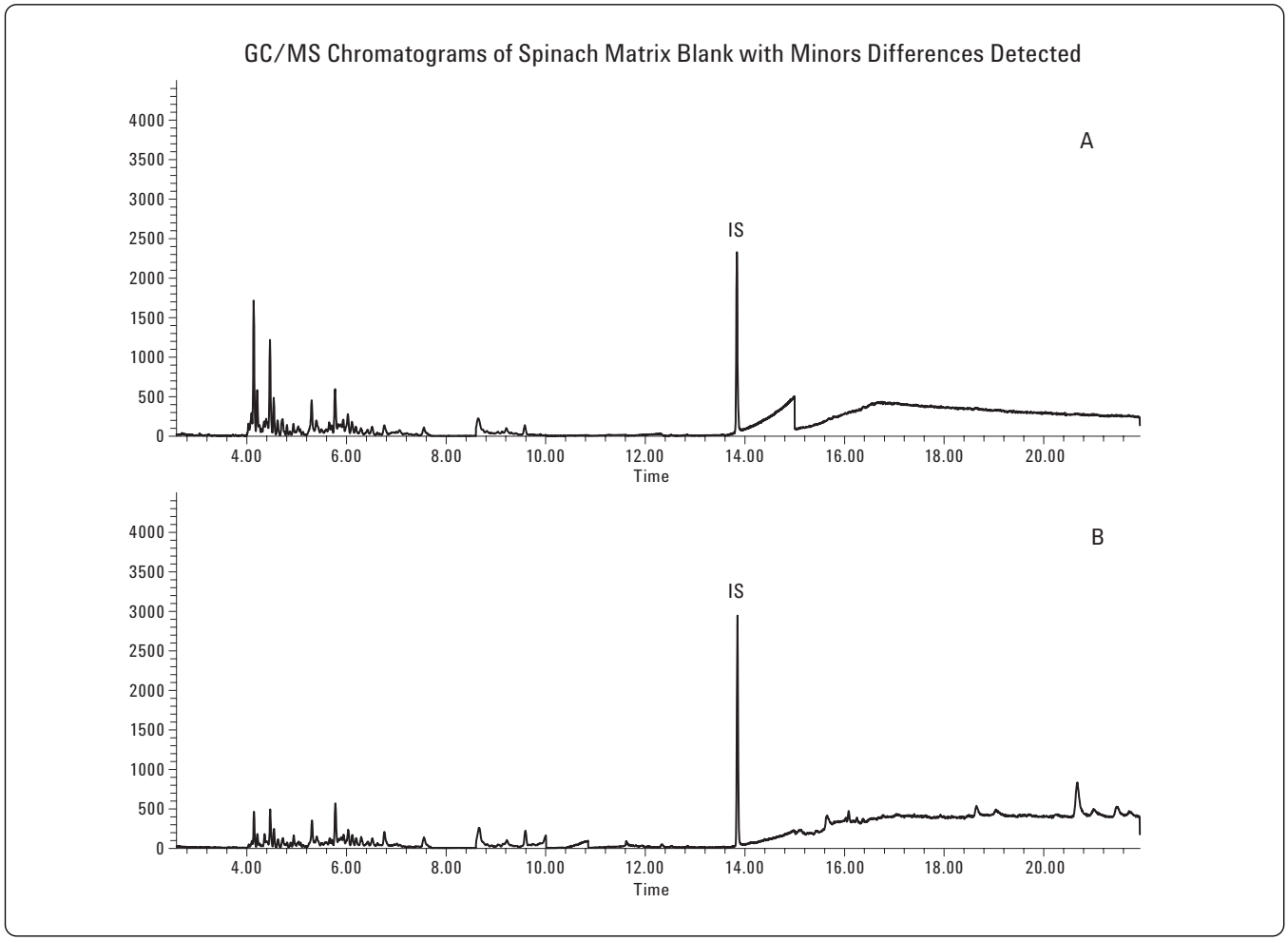


Figure 2. GC/MS chromatograms of spinach matrix blank processed by original dispersive SPE (A) and modified dispersive SPE (B). IS: Internal Standard TPP.

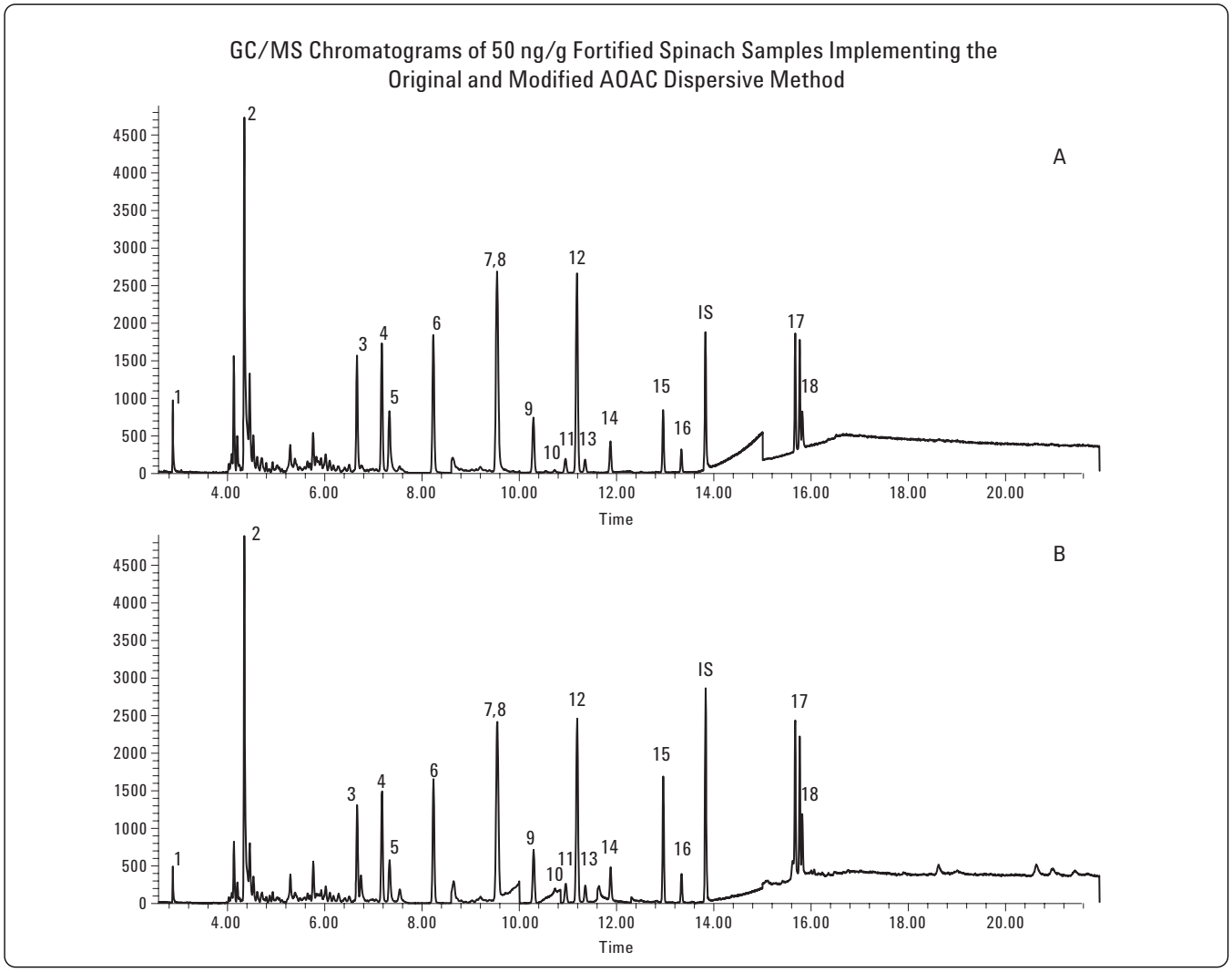


Figure 3. GC/MS chromatograms of 50 ng/g fortified spinach sample extracts processed by original dispersive SPE (A) and modified dispersive SPE (B). Peak identification: 1. Diachlorvos, 2. σ-Phenylphenol, 3. Lindane, 4. Diazinon, 5. Chlorothalonil 6. Chloropyrifos methyl 7. Dichlorobenzophenone, 8. Chlorpyrifos, 9. Heptachlor epoxide, 10. Folpet, 11. γ-Chlordane, 12. DDE, 13. α-Chlordane, 14. Dieldrin, 15. Ethion, 16. Endosulfan sulfate, 17. Permethrin, 18. Coumaphos. IS: Internal Standard, TPP.

Linearity and Limit of Quantification (LOQ)

The linear calibration range for all of the pesticides was 10–400 ng/g, except folpet, which was 50–400 ng/g. For the sample processed by original and modified methods, the corresponding matrix blank was used to prepare the calibration curves respectively. Calibration curves, spiked in matrix blanks, were made at levels of 10, 20, 50, 100, 250, and 400 ng/g. The TPP was used as an internal standard at 100 ng/g. The calibration curves were generated by plotting the relative responses of analytes (peak area of analyte/ peak area of IS) to the relative concentration of analytes (concentration of analyte / concentration of IS). The 10 ng/g quantification limits LOQ (10 ppb) and 50 ng/g LOQ for folpet (50 ppb) established for the pesticides are substantially lower than many MRLs of those pesticides in fruit and vegetables. The regression fit used for the calibration curves was the average response factor. Table 3 shows the linear regression equation and correlation coefficient (R2) for both 1 mL and 8 mL dispersive SPE.

Table 3. Linearity of 17 Pesticides in Spinach Extract

	1 mL dispersive SPE		8 mL dispersive SPE	
Pesticide	**Linear Term**	**RF Rel Std Dev (%)**	**Linear Term**	**RF Rel Std Dev (%)**
Dichlorvos	5.55e-001	9.1	4.71e-001	6.8
σ-Phenylphenol	2.93e+000	7.9	2.30e+000	9.3
Lindane	8.34e-001	9.5	6.98e-001	8.1
Diazinon	1.03e+000	8.7	9.25e-001	11.2
Chlorothalonil *	7.67e-001	14.2	7.83e-001	13.7
Chlorpyrifos methyl	1.26e+000	12.5	1.20e+000	10.3
Dichlorobenzophenone *	3.03e+000	9.8	2.61e+000	12.2
Chlorpyrifos	6.46e-001	6.9	5.99e-001	11.5
Folpet *,**	4.36e-002	10.2	3.33e-002	11.4
γ-Chlordane	1.73e-001	6.2	1.38e-001	6.4
DDE	2.98e+000	7.4	2.49e+000	6.2
α-Chlordane	1.37e-001	8.1	1.07e-001	8.1
Dieldrin	3.41e-001	5.1	2.92e-001	10.6
Ethion	1.07e+000	15.6	1.42e+000	12.2
Endosulfan sulfate	3.06e-001	4.6	2.42e-001	4.2
Permethrin	1.14e+000	6.4	1.22e+000	9.7
Coumaphos *	3.45e-001	6.4	2.64e-001	11.3

* Results from modified dispersive SPE

** Calibration curve range: 50 – 400 ng/g.

Recovery and Reproducibility

The recovery and reproducibility were evaluated by spiking pesticides standards in comminuted spinach sample at levels of 10, 50 and 200 ng/g. These QC samples were quantitated against the matrix spiked calibration curve. The analysis was performed in replicates of six (n = 6) at each level. The recovery and reproducibility (shown as % RSD) data for 1 mL and 8 mL dispersive SPE are shown in Table 4 and Table 5, respectively. Since it was demonstrated that the dispersive SPE size (1 mL and 8 mL) didn't affect the results, the 8 mL size modified dispersive SPE test was not performed due to sample volume limitation. In the 18 GC-amenable pesticides we screened, four pesticides, chlorothalonil, dichlorobenzophenone, folpet, and coumaphos, were found to be adversely affected by the GCB in the dispersive SPE step. With the addition of toluene, the recoveries of those pesticides were increased from 50% to 200% with better precision. However, the modified method also reduced the recovery of certain pesticides that had generated good results originally.

Therefore, the quantitation results shown here are the combination of 14 pesticides from original dispersive SPE and four pesticides from the modified method. It can be seen from the results that the 14 pesticides processed by the original method give out good recoveries (average of 88.8% for 1 mL and 86.3% for 8 mL) and precision (average of 5.4% RSD for 1 mL and 4.8% RSD for 8 mL). Although the four pesticides processed by the modified method give lower recovery (average of 75.3% for 1 mL) but great precision (average of 6.1% RSD for 1 mL), the results were much better than the results obtained by original methods (average recovery of 41.7% with 14.9% average RSD). Please refer to the previous application note [4] for discussions in more detail. Folpet was quantified, but the LOQ was found to be 50 ng/g due to poor sensitivity.

Table 4. Spinach AOAC Dispersive, 1 mL Sample Volume, 2 mL Tube, LC/MS/MS Results

Pesticide	Low QC (10 ng/g) Recovery	RSD	Mid QC (50 ng/g) Recovery	RSD	High QC (200 ng/g) Recovery	RSD
Dichlorvos	94.0	3.0	91.7	10.5	80.9	4.6
σ-Phenylphenol	95.0	2.2	92.0	7.9	78.7	3.8
Lindane	83.7	3.1	93.9	12.2	91.8	3.3
Diazinon	97.3	4.3	95.6	9.9	91.8	3.3
Chlorothalonil *	47.5	6.8	44.9	6.6	49.4	4.3
Chlorpyrifos methyl	74.1	4.6	71.7	4.5	72.2	5.8
Dichlorobenzo Phenone *	97.5	7.6	66.8	3.9	68.8	6.8
Chlorpyrifos	88.3	3.0	79.6	3.5	77.0	3.5
Heptachlor epoxide	74.9	1.9	81.6	11.7	78.2	3.9
Folpet *	NA	NA	98.8	6.0	77.7	6.7
γ-Chlordane	106.0	4.9	112.2	3.3	93.6	5.3
DDE	80.3	2.2	86.8	9.6	75.4	3.5
α-Chlordane	107.6	4.2	108.4	3.5	91.6	3.7
Dieldrin	99.7	2.6	93.7	9.6	78.9	3.4
Ethion	91.4	3.4	100.0	5.0	107.4	7.6
Endosulfan sulfate	93.7	4.8	97.3	8.8	89.8	4.3
Permethrin	84.7	5.7	74.8	9.9	84.6	6.0
Coumaphos *	98.4	5.5	84.2	9.5	81.2	3.2

* Results from modified dispersive SPE method.

Table 5. Spinach AOAC Dispersive 8 mL Volume, 15 mL Tube, Results by GC/MS

Pesticide	Low QC (10 ng/g) Recovery	RSD	Mid QC (50 ng/g) Recovery	RSD	High QC (200 ng/g) Recovery	RSD
Dichlorvos	93.7	2.6	92.5	4.2	86.2	5.9
σ-Phenylphenol	87.9	5.1	92.5	6.6	95.2	6.3
Lindane	83.1	5.1	85.4	2.9	84.5	5.2
Diazinon	85.8	6.9	85.2	2.9	87.3	5.5
Chlorothalonil*	21.1	49.7	23.6	14.3	23.2	14.0
Chlorpyrifos methyl	76.4	2.4	73.9	2.7	73.8	3.6
Dichlorobenzophenone*	93.3	4.1	56.6	2.0	61.4	4.5
Chlorpyrifos	77.8	3.6	70.2	4.6	69.0	3.1
Heptachlor epoxide	78.6	4.6	79.6	2.6	85.3	5.1
Folpet*	NA	NA	60.3	17.4	53.7	10.7
γ-Chlordane	106.8	5.7	110.7	3.5	100.4	4.9
DDE	80.8	4.2	81.8	2.6	81.3	4.9
α-Chlordane	104.2	6.2	103.6	3.3	95.8	5.3
Dieldrin	96.4	6.2	93.0	1.2	79.3	5.3
Ethion	83.8	4.0	82.8	2.3	85.3	4.9
Endosulfan sulfate	90.5	8.8	87.5	7.3	84.5	6.4
Permethrin	84.0	4.9	78.4	6.8	79.5	10.7
Coumaphos*	61.2	22.2	42.6	28.8	35.3	20.6

*Poor results caused by GCB added in dispersive SPE, can be improved by addition of toluene in the dispersive SPE.

Conclusions

Agilent SampliQ QuEChERS AOAC buffered extraction kits and dispersive SPE kits for pigmented fruits and vegetables provide a simple, fast and effective method for the purification of representative volatile to semi-volatile pesticides in spinach. The modified dispersive SPE method with the addition of toluene provides a very useful option to improve the loss of planar pesticides caused by GCB in dispersive SPE tubes. The recovery and reproducibility, based on matrix spiked standards, were acceptable for multiclass, multi-residue pesticide determination in spinach. The impurities and matrix effects from spinach did not interfere with the quantitation of target compounds. As the selected pesticides represented a broad variety of different classes and properties, the Agilent SampliQ QuEChERS AOAC Buffered Extraction and Dispersive kits for Pigmented Fruits and Vegetables can be used for other pesticides in similar pigmented matricies.

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Agilent Technologies

PAH Analyses with High Efficiency GC Columns: Column Selection and Best Practices

Food Quality and Environmental

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Abstract

The European Union (EU) regulates a series of PAHs found primarily in food matrices. This list is referred to as the EU 15 +1 list. The US Environmental Protection Agency (EPA) regulates a series of 16 PAHs historically addressed as environmental pollutants. Both lists contain unique analytes that present different separation challenges. However, there are eight analytes that are common to both lists. In this study, resolution of all 24 combined regulated PAHs is achieved, in under 28 minutes using an Agilent J&W DB-EUPAH 20 m x 0.18 mm, 0.14 µm High Efficiency GC column. Resolution of 23 of the 24 combined regulated PAHs is shown using an Agilent J&W DB-5ms 20 m x 0.18 mm, 0.18 µm High Efficiency GC column in under 22 minutes. Both the Agilent J&W DB-EUPAH and DB-5ms columns are excellent column choices for analysis of the regulated PAHs. The Agilent J&W DB-EUPAH is recommended when separation of benzo[b,j,k]fluoranthene isomers is required.

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Instruction

Polycyclic aromatic hydrocarbons (PAHs) are a large class of organic compounds containing two or more fused aromatic rings. PAHs often result from the incomplete combustion of organic substances such as wood, coal, and oil. The European Union PAH regulation has focused on these substances as potential contaminants in the food supply. A main source of potential human exposure to PAHs is through heat processing of meat and dairy products, such as grilling and smoking [1]. There are serious health concerns regarding PAHs since many are classified as carcinogenic or mutagenic [2].

In 2005, the European Commission recommended the monitoring of fifteen EU priority PAHs along with an additional PAH highlighted by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) [3]. The EU 15+1 priority PAHs along with the US-EPA-regulated PAHs are provided in Table 1 [4]. There are eight PAHs that are common to both the EU-15 +1 list and the US-EPA list.

A 5% phenyl methylpolysiloxane stationary phase column is the most commonly used GC column for PAH analysis. This nonpolar column yields good resolution for the 16 US-EPA PAHs [5,6], however, three critical pairs of the 15+1 EU PAHs co-elute and are difficult to resolve by mass spectrometry [7,8]. These challenging pairs are benz[a]anthracene-cyclopenta[c,d]pyrene- chrysene,benzo[b]fluoranthene-benzo[k]fluoranthene-benzo[j]fluoranthene, and indeno[1,2,3-cd] pyrene-dibenz[a,h]anthracene. Agilent J&W DB-EUPAH, a midpolar GC column, improves the resolution of these critical pairs allowing for more accurate detection and quantitation of the 15 +1 EU priority PAHs.

Another set of challenging analytes is the four dibenzopyrene isomers. Due to their high molecular weight (MW 302), these isomers are prone to discrimination and poor peak shape. Broad, tailing peaks make reliable quantitation difficult and decrease the signal-to-noise ratio, resulting in an increase in the limits of detection. Limiting analyte dwell time on the column and in the GC/MS interface can offset these deleterious chromatographic effects. Shorter columns with thinner film thickness and high operating temperatures are all factors that collectively can improve peak shapes for these analytes.

Table 1. EU and US-EPA Regulated PAH Compounds

Peak #	Component	CAS #	MW	EU 15+1	EPA
1	Naphthalene	91-20-3	128		x
2	Acenaphthylene	208-96-8	152		x
3	Acenaphthene	83-32-9	154		x
4	Fluorene	86-73-7	166		x
5	Phenanthrene	85-01-8	178		x
6	Anthracene	120-12-7	178		x
7	Fluoranthene	206-44-0	202		x
8	Pyrene	129-00-0	202		x
9	*Benzo[c]fluorene*	205-12-9	216	x	
10	**Benz[a]anthracene**	56-55-3	228	x	x
11	*Cyclopenta[c,d]pyrene*	27208-37-3	226	x	
12	**Chrysene**	218-01-9	228	x	x
13	*5-Methylchrysene*	3697-24-3	242	x	
14	**Benzo[b]fluoranthene**	205-99-2	252	x	x
15	**Benzo[k]fluoranthene**	207-08-9	252	x	x
16	*Benzo[j]fluoranthene*	205-82-3	252	x	
17	**Benzo[a]pyrene**	50-32-8	252	x	x
18	**Indeno[1,2,3-cd]pyrene**	193-39-5	276	x	x
19	**Dibenz[a,h]anthracene**	53-70-3	278	x	x
20	**Benzo[g,h,i]perylene**	191-24-2	276	x	x
21	*Dibenzo[a,l]pyrene*	191-30-0	302	x	
22	*Dibenzo[a,e]pyrene*	192-65-4	302	x	
23	*Dibenzo[a,i]pyrene*	189-55-9	302	x	
24	*Dibenzo[a,h]pyrene*	189-64-0	302	x	

Regulated PAH compounds shown in plain text are included only in the US-EPA set, compounds in Italic are included only in the EU 15+1 list, and the compounds in bold are included in both the US-EPA and EU 15+1 lists.

Experimental

GC EU PAH standard (Agilent p/n 5190-0487) and US-EPA mixture (Agilent p/n 8500-6035) were diluted separately to a concentration of 2 µg/ml using class A glassware and pipets. These solutions were then mixed 1:1 to for a final concentration of 1-2 µg/ml of all 24 regulated PAHs.

Table 2. Chromatographic Conditions DB-EUPAH Column

Sample:	0.5 µL 1-2 µg/mL EU + EPA PAH combined standards (EU PAH standard Agilent p/n 5190-0487 and EPA PAH standard Agilent p/n 8500-6035)
GC/MS:	Agilent 7890A GC System with an Agilent 5975C Series GC/MSD, TAD, and an Agilent 7873B automatic liquid sampler
Column:	Agilent J&W DB-EUPAH 20 m × 0.18 mm, 0.14 µm (Agilent p/n 121-9627)
Carrier:	Helium 60 cm/sec 1.8 ml/min constant flow
Oven:	70 °C (0.8 min), 70 °C/min to 180 °C, 7 °C/min to 230 °C (6 min), 40 °C/min to 280 °C (5 min) 25 °C/min to 335 °C (5 min)
Inlet:	300° C splitless, purge 100 mL/min at 0.25 min
Inlet liner:	Helix double taper deactivated (Agilent p/n 5188-5398)
MSD:	Sim/Scan mode 50-400 AMU, transfer line 340 °C, source 340 °C, quad 150 °C

Table 3. Chromatographic Conditions DB-5ms Column

Sample:	0.5 µL 1-2 µg/mL EU + EPA PAH combined standards (EU PAH standard Agilent part # 5190-0487 and EPA PAH standard Agilent part # 8500-6035)
GC/MS:	Agilent 7890A GC System with an Agilent 5975C Series GC/MSD, TAD, and an Agilent 7873B automatic liquid sampler
Column:	Agilent J&W DB-5 ms 20 m × 0.18 mm, 0.18 µm (Agilent p/n 121-5522)
Carrier:	Helium 60 cm/sec 1.8 ml/min constant flow
Oven:	55 °C (0.4 min), 25 °C/min to 200 °C, 8 °C/min to 280 °C, 10 °C/min to 320 °C (2 min) 25 °C/min to 335 °C (5 min)
Inlet:	300 °C splitless, purge 100 mL/min at 0.25 min
Inlet liner:	Helix double taper deactivated (Agilent part # 5188-5398)
MSD:	Sim/Scan mode 50-400 AMU, transfer line 340 °C, source 340 °C, quad 150 °C

Table 4. Flow Path Supplies

Vials:	Amber screw top glass vials (Agilent p/n 5183-2072)
Vial Caps:	Screw caps (Agilent p/n 5182-0723)
Vial inserts:	100 µL glass/polymer feet (Agilent p/n 5181-8872)
Syringe:	5 µL (Agilent p/n 5183-4729)
Septum:	Advanced green (Agilent p/n 5183-4759)
Inlet Seal:	Gold plated inlet seal (Agilent p/n 5188-5367)
Inlet liners:	Helix double taper deactivated (Agilent p/n 5188-5398)
Ferrules:	0.4 mm ID short; 85/15 vespel/graphite (Agilent p/n 5181-3323)
20 × magnifier :	20 × Magnifier loop (Agilent p/n 430-1020)

Discussion of Results

Figure 1 shows the separation of all 24 analytes included in the EU 15 + 1 and US-EPA PAH lists on a DB-EUPAH column. This separation was accomplished in under 28 minutes on an Agilent J&W DB-EUPAH 20 m x 0.18 mm, 0.14 µm column (Agilent p/n 121-9627). The selectivity of this midpolar column is necessary to resolve the benzo(b,j,k) fluoranthene isomers. High temperature stability is also required for elution of the high boiling point dibenzopyrenes.

The injection volume was reduced to 0.5 µL in order to scale the separation to the 0.18 or high efficiency GC (HEGC) format. This is often a necessary step when working with high efficiency columns because the higher efficiency of narrow bore columns is at the expense of sample loading capacity. Here the separation focused on achieving faster analysis. An example of a separation maximizing resolution with longer retention on this column was described previously [9].

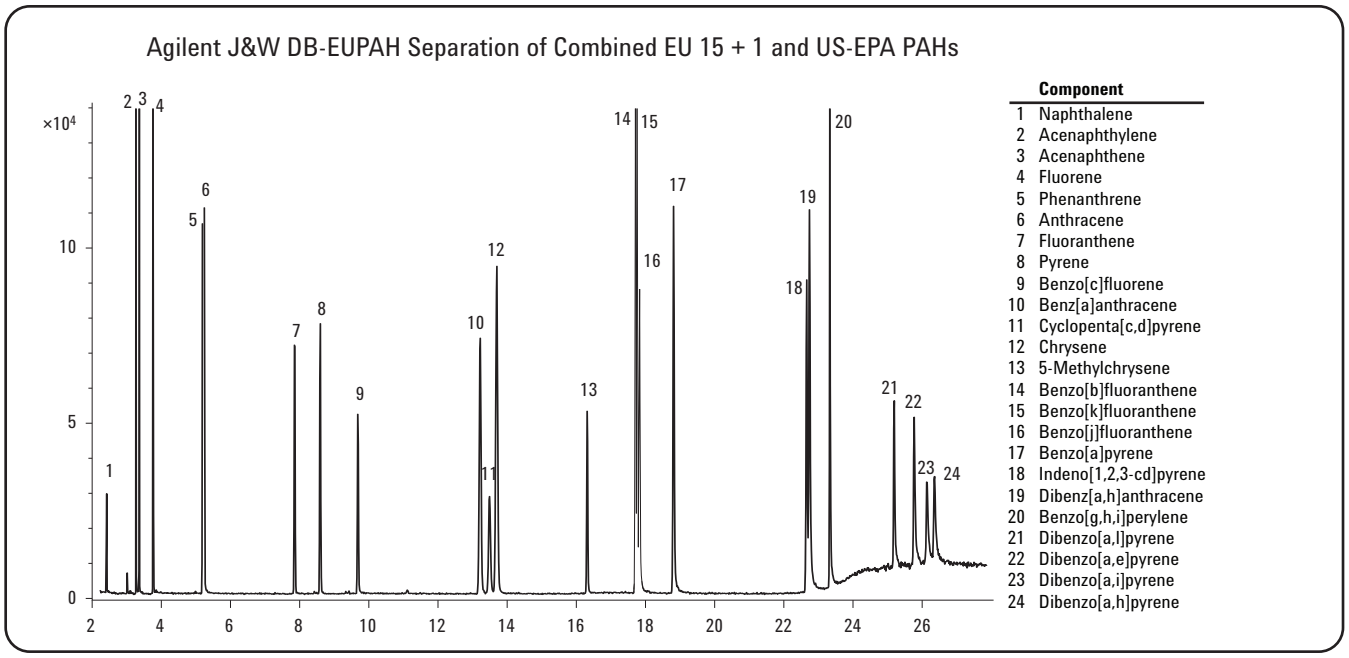


Figure 1. EU and US-EPA regulated PAH separation on an Agilent J&W DB-EUPAH 20 m × 0.18 mm, 0.14 µm column (Agilent p/n 121-9627). Chromatographic conditions as in Table 2 and flow path supplies as in Table 4.

Figure 2 shows the resolution of 23 of the 24 analytes included in the EU 15 + 1 and US-EPA PAH lists on an Agilent J&W DB-5ms column. Benzo[j] fluoranthene is not resolved from benzo[k]fluoranthene using this column. However, when it is sufficient to report the sum of the benzoflouranthene isomers, the Agilent J&W DB-5ms column is an excellent choice for the 24 regulated PAHs. This separation was accomplished in under 22 minutes on an Agilent J&W DB-5ms 20 m × 0.18 mm, 0.18 µm column (Agilent p/n 121-5522). The Agilent J&W DB-5ms separation offers a 27 % faster analysis time when compared to the DB-EUPAH separation shown in Figure 1.

There are a number of best practices to consider when optimizing a GC/MS system for PAH analysis. The use of retention gaps and/or inlet backflushing can reduce maintenance and cycle times. Close examination of injection parameters such as injection volume, inlet temperature, purge time activation, solvent focusing and holding the oven temperature stable during injection can all contribute to better results. Minimizing inlet and system dwell time by operating at high linear velocities can also improve results. Another best practice for PAHs is to keep heated zones well insulated and hot to reduce the potential for system cold spots and the resultant signal loss.

Conclusions

All 24 (EU 15 + 1 and US-EPA) regulated PAHs are resolved using an Agilent J&W DB-EUPAH 20 m × 0.18 mm, 0.14 µm column. The benzo[b,j,k]fluoranthene isomers were adequately separated for individual quantitation. This is the column of choice when resolution of benzo[j]fluoranthene is required. This separation was accomplished in under 28 minutes.

23 of 24 (EU 15 + 1 and US-EPA) regulated PAHs resolve using an Agilent J&W DB-5ms 20 m × 0.18 mm, 0.18 µm column. This column is an excellent choice when benzoflouranthene isomers are reported as a sum of the isomers and speed of analysis is critical. This separation was accomplished in under 22 minutes.

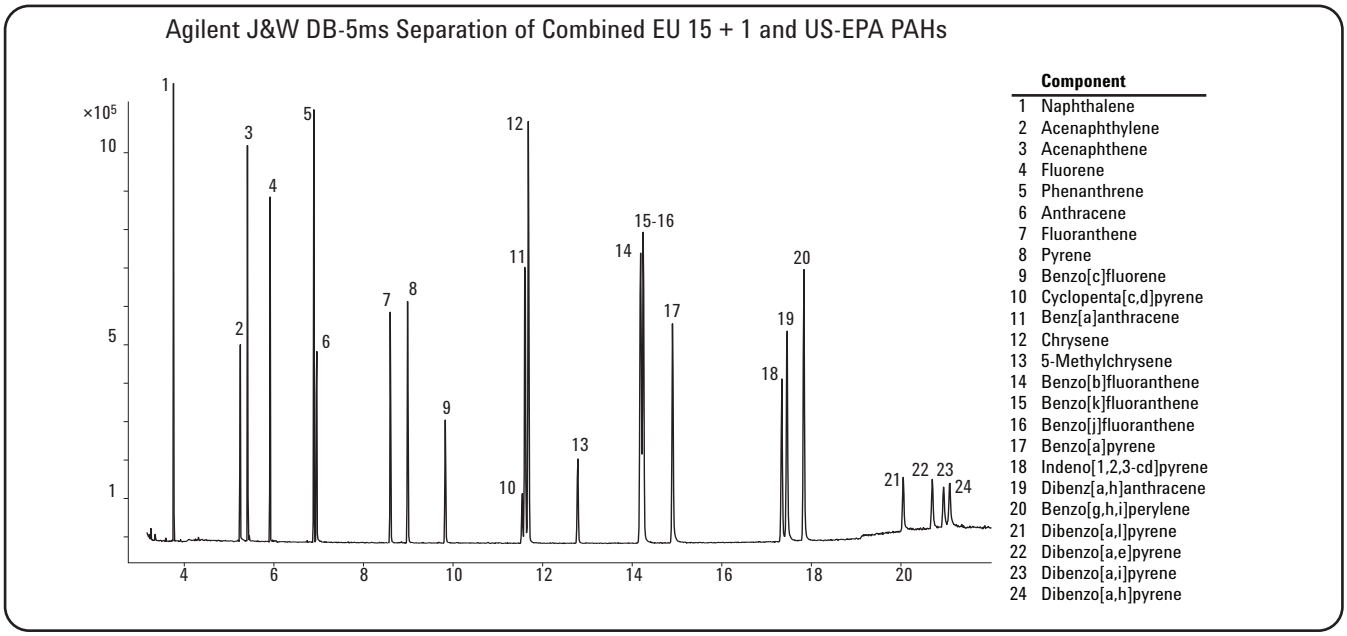


Figure 2. EU and US-EPA regulated PAH separation on an Agilent J&W DB-5 ms 20 × 0.18 mm, 0.18 µm (Agilent p/n 121-5522) column. Chromatographic conditions as in Table 3 and flow path supplies as in Table 4.

Factors to consider in optimizing EU 15 +1 and US-EPA PAH analyses

- Choose an Agilent J&W DB-EUPAH when the resolution of benzo[b,j,k]fluoranthene isomers (24 of 24 peaks in combined set) is required.
- Choose an Agilent J&W DB-5ms when benzo[b,j,k]fluoranthene isomers can be reported as a sum of the isomers. The Agilent J&W DB-5ms resolves 23 of 24 regulated PAHs in 27 % faster cycle time than the Agilent J&W DB-EUPAH column.
- Consider the use of retention gaps and inlet backflushing to reduce cycle time and maintenance.
- Achieve faster analysis times with no loss of resolution using 0.18 mm id high efficiency GC columns.
- Optimize injection volume, temperature, purge time, and solvent focusing for best results on your instrument.
- Minimize inlet and system dwell time with high linear velocities.
- Keep heated zones hot to avoid cold spots and signal loss.

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Analysis of Pesticide Residues in Spinach Using Agilent SampliQ QuEChERS EN Kit by LC/MS/MS Detection

Application Note

Food Safety

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Abstract

This application note describes the use of a quick, easy, cheap, effective, rugged, and safe (QuEChERS) EN sample preparation approach for extraction and cleanup of 13-pesticide residues representing various classes in spinach. Because spinach is considered a highly pigmented matrix, the EN dispersive SPE kit for highly pigmented fruits and vegetables is selected. Graphitized carbon black (GCB) in the amount of 7.5 mg/mL of ACN extract is added to the kit. The target pesticides in the spinach extracts are then determined by liquid chromatography coupled to an electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) operating in positive ion multiple reaction monitoring (MRM) mode. GCB is reported to have a significantly negative impact on the extraction of pesticides with planar structure. However, with the small amount of GCB addition in the EN dispersive SPE kit, our results show that the impact of GCB on planar pesticides is negligible and acceptable quantitation results are obtained. The 5 ng/g limit of quantitation (LOQ) for pesticides in spinach shown in this application is well below the maximum residue limits (MRLs). The spiking levels for the recovery experiments are 10, 50, and 200 ng/g. Mean recoveries range between 60 and 99% (85.4% on average), with an RSD below 11% (5.5% on average).

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Introduction

The EN QuEChERS method has been widely employed in the analysis of pesticides in food, especially in Europe. [1-2] The method uses acetonitrile extraction, followed by salting out water from the sample using anhydrous magnesium sulfate ($MgSO_4$), NaCl and buffering citrate salts to induce liquid-liquid partitioning. For cleanup, a dispersive solid phase extraction (dispersive SPE) is conducted using a combination of primary secondary amine (PSA) to remove fatty acids from among other components, and anhydrous $MgSO_4$ to reduce the remaining water in the extract. According to different food matrices, other ingredients may be added in this step, such as graphitized carbon black (GCB) to remove pigments and sterol, or C18 to remove more lipids and waxes.

Spinach is considered to be a highly pigmented vegetable since it contains high levels of chlorophyll. Therefore, the EN dispersive SPE kits for highly pigmented commodities were selected for this application. In these kits, besides 25 mg of PSA and 150 mg of $MgSO_4$, 7.5 mg of GCB is added per mL of ACN extracts. GCB adsorbs planar molecules like pigments and sterols; hence it is very helpful in cleaning up pigmented matrices like spinach. The efficiency of cleanup is dependent upon the amount of GCB used. The more GCB used, the more planar molecules are absorbed, and therefore, a cleaner sample matrix is obtained. The main difference between the EN method and AOAC method for cleaning up the highly pigmented matrix is the amount of GCB used in the dispersive SPE step. Instead of the relatively high amount of GCB used in AOAC method (50 mg of GCB per mL of ACN extracts), a much lower amount of GCB was used in the EN methods (2.5 mg of GCB per mL of ACN extracts for "pigmented" produce, or 7.5 mg of GCB per mL of ACN extracts for "highly pigmented" produce). The GCB impacted the extraction of planar pesticides differently, depending upon the method used. The AOAC method generated much cleaner final sample matrix but caused significant loss of planar pesticides; the EN method, on the contrary, caused little to no loss of planar pesticides but generated a more complicated sample matrix.

Previously, we described that a modified AOAC method with toluene addition in the dispersive SPE step greatly increased the extraction efficiency of planar pesticides in a pigmented matrix such as spinach. [3] Subsequently, we demonstrated the performance of SampliQ QuEChERS AOAC kit for the analysis of pesticides in spinach using combination of the modified (with toluene addition) and the original AOAC method (without toluene addition). [4, 5] In this study, 13 pesticides were used for evaluating the performance of the Agilent EN Buffered Extraction kit (p/n 5982-5650) and SampliQ QuEChERS EN Dispersive SPE kits for Highly Pigmented Fruits and Vegetables (p/n 5982-5321 and 5982-5356). The method was validated in terms of recovery and reproducibility. Table 1 shows the chemical and regulatory information for these pesticides in spinach.

Experimental

Reagents and Chemicals

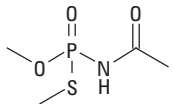
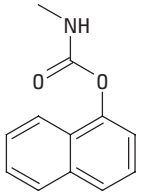
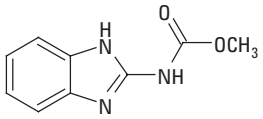
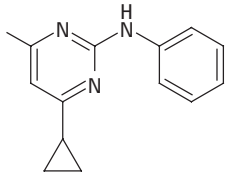
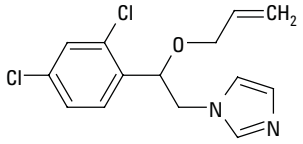
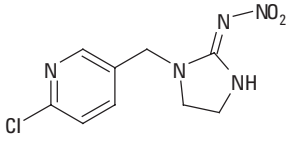
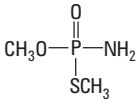
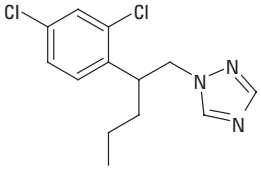
All reagents and solvents were HPLC or analytical grade. Methanol (MeOH), and toluene were from Honeywell (Muskegon, MI, USA). Acetonitrile (ACN), dimethyl sulfoxide (DMSO) and glacial acetic acid (HAc) were from Sigma-Aldrich (St Louis, MO, USA). Ammonium acetate (NH_4OAc) was from Fisher Chemicals (Fair Lawn, NJ, USA). Formic acid (FA) was from Fluka (Sleinheim, Germany). The pesticide standards and internal standard (triphenyl phosphate, TPP) were purchased from Sigma-Aldrich (St Louis, MO, USA), ChemService (West Chester, PA, USA), Ultra (Kingstown, RI, USA), or AlfaAesar (Ward Hill, MA, USA).

Solutions and Standards

A 1 M ammonium acetate pH 5 stock solution was made by dissolving 19.27 g NH_4OAc powder in 250 mL Milli-Q water, pH adjusted to 5 with acetic acid monitored with a pH meter. The solution was stored at 4 °C. A 5 mM ammonium acetate in 20:80 MeOH/H_2O solution, pH 5, was made by combining 200 mL MeOH and 800 mL Milli-Q water, adding 5 mL of 1 M ammonium acetate pH 5 stock solution and mixing well. A 5 mM ammonium acetate in ACN was prepared by adding 5 mL of 1 M ammonium acetate pH 5 stock solution to 1 L ACN, mixing well and sonicating 5 min. A 1% formic acid in ACN solution was prepared by adding 1 mL of formic acid to 100 mL of ACN, and mixing well.

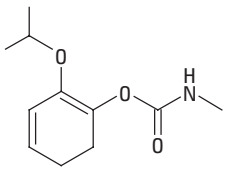
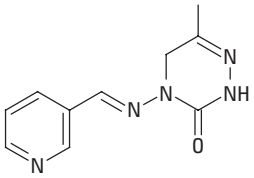
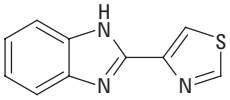
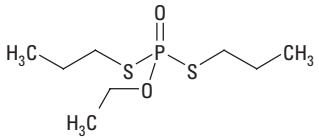
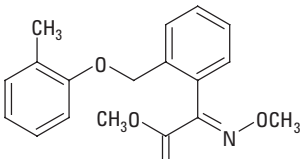
Standard and internal standard (IS) stock solutions (2.0 mg/mL for all except 0.5 mg/mL for carbendazim) were made in MeOH, 0.1% FA in ACN, or DMSO, respectively, and stored at -20 °C. Three QC spiking solutions of 1, 5, and 20 µg/mL, were made fresh daily in 1:1 ACN/H_2O with 0.1% FA. A 10 µg/mL standard spiking solution in 1:1 ACN/H_2O with 0.1% FA was made also for the preparation of a calibration curve in the matrix blank extract by appropriate dilution. A 15 µg/mL of TPP in 1:1 ACN/H_2O with 0.1% FA was made as an IS spiking solution.

Table 1. Pesticides Chemical and Regulatory Information [6–8]

Name	Class	Log P	pKa	Structure	MRLs in spinach (ng/g)*
Acephate	Organophosphate	–0.89	8.35		20
Carbaryl	Carbamate	2.36	10.4		50
Carbendazim	Benzimidazole	1.48	4.2		100
Cyprodinil	Anilinopyrimidine	4	4.44		500
Imazalil	Imidazole	3.82	6.53		20
Imidacloprid	Neonicotinoid	0.57	NA		1000
Methamidophos	Organophosphate	-0.79	NA		10
Penconazole	Triazole	3.72	1.51		50

(Continued)

Table 1. Pesticides Chemical and Regulatory Information [6–8]

Name	Class	Log P	pKa	Structure	MRLs in spinach (ng/g)*
Propoxur	Carbamate	0.14	NA		2000
Pymetrozine	Pyridine	–0.19	4.06		600
Thiabendazole	Benzimidazole	2.39	4.73 12.00 0		50
Ethoprophos	Organophosphate	2.99	NA		5
Kresoxim-methyl	Strobilurin	3.4	NA		50

*The MRLs numbers list in the table are for spinach or other vegetables. They could be higher in different commodities.

Equipment and Material

Agilent 1200 Series HPLC with Diode Array Detector (Agilent Technologies Inc., CA, USA).

Agilent 6410 triple quadrupole LC/MS system with Electrospray Ionization (Agilent Technologies Inc., CA, USA).

Agilent SampliQ QuEChERS EN Extraction kits, p/n 5982-5650, and SampliQ QuEChERS EN dispersive SPE kits for Highly Pigmented Fruits and Vegetables, p/n 5982-5321 and 5982-5356 (Agilent Technologies Inc., DE, USA).

CentraCL3R Centrifuge (Thermo IEC, MA, USA)

Bottle top dispenser (VWR, So. Painfield, NJ, USA)

Eppendorf microcentrifuge (Brinkmann Instruments, Westbury, NY, USA)

Instrument Conditions

The previous LC/MS/MS method was used. [9]

HPLC conditions

Column:	Agilent ZORBAX Solvent Saver Plus Eclipse Plus Phenyl-Hexyl, 3.0 x 150 mm, 3.5 µm (p/n 959963-312)
Flow rate:	0.3 mL/min
Column Temperature:	30 °C
Injection volume:	10 µL
Mobile Phase:	A, 5 mM ammonium acetate, pH 5.0 in 20:80 MeOH/H_2O B, 5 mM ammonium acetate, pH 5.0 in ACN
Needle wash:	1:1:1:1 ACN/MeOH/IPA/H_2O w/0.2% FA.

Gradient:

Time	% Acetonitrile	Flow rate (mL/min)
0	20	0.3
0.5	20	0.3
8.0	100	0.3
10.0	100	0.3
13.0	STOP	

Post run:	4 min
Total cycle time:	17 min

MS conditions

Positive mode	
Gas temp.:	350 °C
Gas flow:	10 L/min
Nebulizer:	40 Psi
Capillary:	4000 V

Other conditions relating to the analytes are listed in Table 2.

Sample Preparation

The sample preparation procedure includes sample comminution, extraction/partitioning and dispersive SPE cleanup. It was described in detail in the previous application notes. [9] The procedure used in spinach was similar to the one used in apple, except that the dispersive SPE kit was for highly pigmented produce rather than general fruits and vegetables.

Briefly, the frozen chopped organic spinach was homogenized thoroughly. A 10 g (±0.1g) of homogenized sample was placed into a 50 mL centrifuge tube. Samples were fortified with appropriate QC spiking solutions (100 µL) when necessary, and then 66.7 µL of IS spiking solution (15 µg/mL of TPP). After vortexing sample for 30 s, 10 mL of ACN was added to each tube using the dispenser. Tubes were then capped and shaken by hand for 1 min. To each tube, an Agilent SampliQ QuEChERS EN extraction salt packet (p/n 5982-5650), containing 4 g anhydrous $MgSO_4$, 1 g NaCl, 1 g Na_3Citrate, and 0.5 g Na_2HCitrate sesquihydrate, was added directly. Sample tubes were capped tightly, and hand-shaken vigorously for 1 min. Tubes were centrifuged at 4000 rpm for 5 min.

A 1 mL aliquot of upper ACN layer was transferred into Agilent SampliQ QuEChERS EN dispersive SPE 2 mL tube (p/n 5982-5321); or 6 mL aliquot into Agilent SampliQ QuEChERS EN dispersive SPE 15 mL tube (p/n 5982-5356). The 2 mL tube contains 25 mg of PSA, 150 mg of anhydrous $MgSO_4$ and 7.5 mg of GCB; while the 15 mL tube contains 150 mg of PSA, 900 mg of anhydrous $MgSO_4$ and 45 mg of GCB. The tubes were capped tightly and vortexed for 1 min. The 2 mL tubes were centrifuged with a micro-centrifuge at 13,000 rpm for 2 min, and the 15 mL tubes in a standard centrifuge at 4000 rpm for 5 min. A 200 µL aliquot of extract was transferred into an autosampler vial. An aliquot of 10 µL 1% FA in ACN was added immediately. Then 800 µL of water or appropriate standard solutions (prepared in water) were added. The samples were capped and vortexed thoroughly for LC/MS/MS analysis.

Table 2. Instrument Acquisition Data Used for the Analysis of 13 Pesticides by LC/MS/MS

Analyte	MRM channels (*m/z*)	Fragmentor (V)	CE (V)	RT (min)
Acephate	1) 184.0 > 94.9 2) 184.0 > 111.0	60	3 15	2.55
Methamidophos	1) 142.0 > 94.0 2) 142.0 > 124.9	60	8 8	2.54
Pymetrozine	1) 218.1 > 105.0 2) 218.1 > 78.0	115	20 50	2.97
Carbendazim	1) 192.1 > 160.0 2) 192.1 > 105.0	95	18 40	5.07
Imidacloprid	1) 256.1 > 209.1 2) 256.1 > 175.0	60	12 18	5.53
Thiabendazole	1) 202.1 > 175.0 2) 202.1 > 131.0	110	27 38	5.65
Propoxur	1) 210.1 > 111.0 2) 210.1 > 92.9	50	12 15	6.89
Carbaryl	1) 202.0 > 145.0 2) 202.0 > 115.0	50	3 40	7.30
Ethoprophos	1) 243.1 > 130.9 2) 243.1 > 172.9	80	15 15	8.50
Imazalil	1) 297.1 > 158.9 2) 297.1 > 200.9	80	22 15	8.52
Penconazole	1) 284.1 > 158.9 2) 284.1 > 172.9	80	32 32	8.95
Cyprodinil	1) 226.1 > 93.0 2) 226.1 > 108.0	120	35 35	9.23
Kresoxim methyl	1) 314.0 > 222.1 2) 314.0 > 235.0	70	10 10	9.44
TPP (IS)	1) 327.1 > 77.0 2) 327.1 > 151.9	70	45 45	9.49

1) Quantifier transition channel
2) Qualifier transition channel

Results and Discussion

The QuEChERS method for pesticide residue analysis provides high-quality results in a fast, easy, inexpensive approach. For the pigmented fruits and vegetables, the addition of GCB in the dispersive SPE tube can improve the removal of pigments and sterols. The cleaning efficiency of the method with GCB is related to the amount of GCB added. The more GCB used, the cleaner the matrix after treatment and less matrix interferences remaining in the final sample. Since GCB can also cause the removal of planar pesticides during the extraction procedure, smaller amounts of GCB used in the EN dispersive SPE step has less of an effect on the planar pesticides. Compared to the AOAC method, the EN method for pigmented produce uses much less GCB in the dispersive SPE step. For normal pigmented commodities like carrots and romaine lettuce, 2.5 mg of GCB can be used per mL of ACN extract; and for highly pigmented commodities like spinach or red sweet pepper, 7.5 mg of GCB can be used per mL of ACN extract. [1]

According to the recommendation, the EN dispersive SPE kit for highly pigmented products was used for spinach in our study. Given the highly pigmented kit, the amount of GCB used in the EN method is still much lower than that used in AOAC method, which is 50 mg of GCB per mL of ACN extract. Therefore, visually, the efficiency of matrix cleanup provided by the EN method was much weaker than that provided by AOAC method. The final sample processed by EN method still appeared dark green in color; while the previous final sample processed by AOAC method showed almost colorless transparency. The matrix blank differences are also shown in the UV chromatogram at λ = 254 nm shown in Figure 1. More interference peaks appear in the matrix blank processed by the EN method. Also more impurities may have accumulated

in the column or ionization source, which can have negative effects on the column and MS instrument. However, with the powerful selectivity provided by LC/MS/MS, the MRM chromatogram of matrix blank did not show any interference peaks to the target analytes. Figure 2 shows the LC/MS/MS chromatograms of matrix blank (IS spiked) and 50 ng/g fortified spinach extract processed by EN dispersive SPE method.

Four pesticides including Carbendazim, Thiabendazole, Cyprodinil, and Pymetrozine, with planar structure showed significant loss by the original AOAC dispersive SPE method. In addition, the modified method with toluene in the dispersive SPE step increased the extraction efficiency. [3,4] In order to investigate the impact of GCB on the planar pesticides, a comparison experiment with and without toluene addition in the dispersive SPE step for spinach samples fortified with the same level of pesticide standard (50 ng/g) was performed. The results showed little to no loss of planar pesticides caused by the small amount of GCB used in the EN method, and no significant improvement obtained by the addition of toluene. Therefore, the original EN method was employed for subsequent experiments. The method was validated in terms of recovery and reproducibility, and the quantitation results are discussed subsequently.

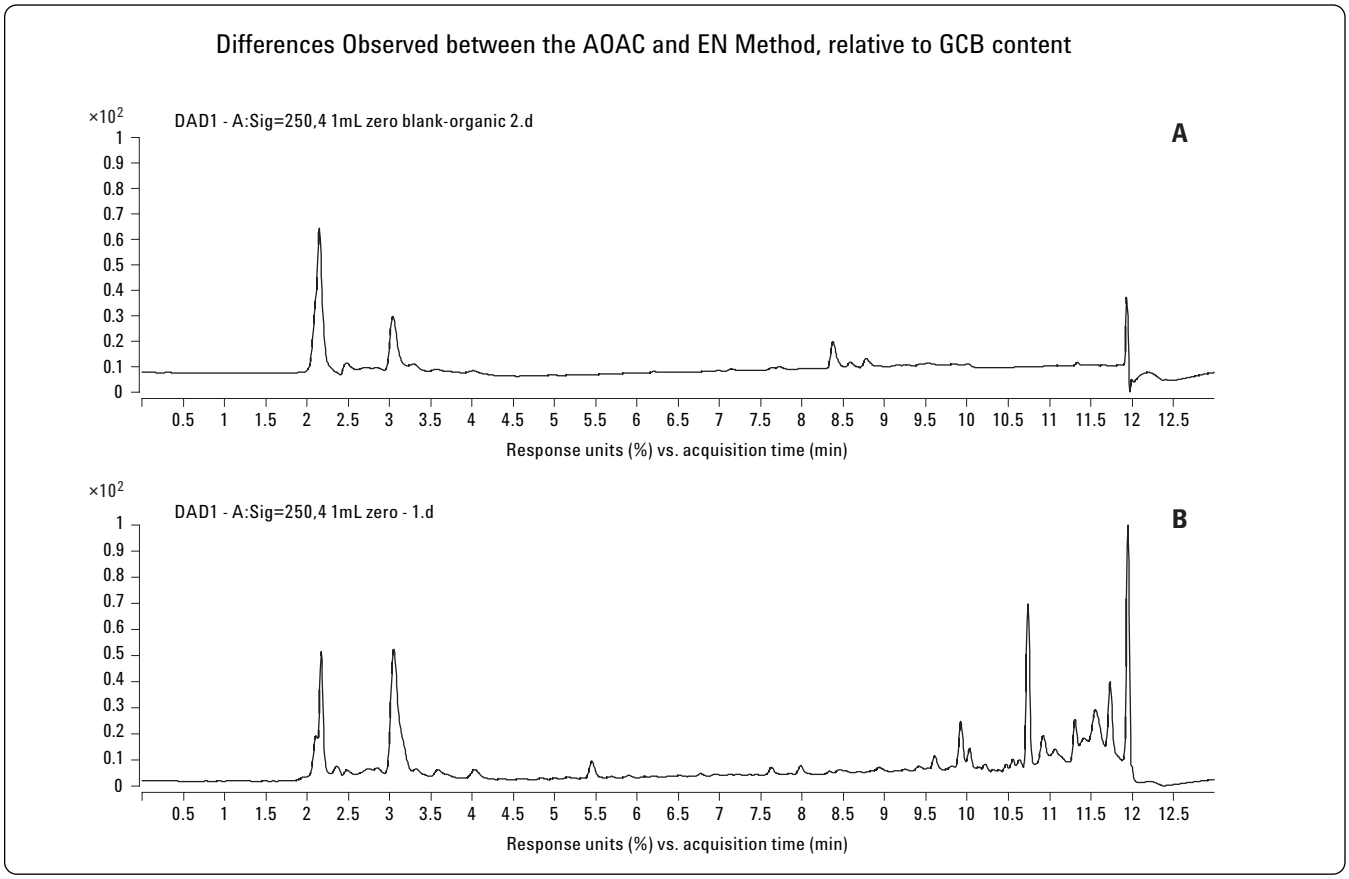


Figure 1. UV chromatogram (λ = 254nm) of spinach matrix blank processed by AOAC method (A) and EN method (B).

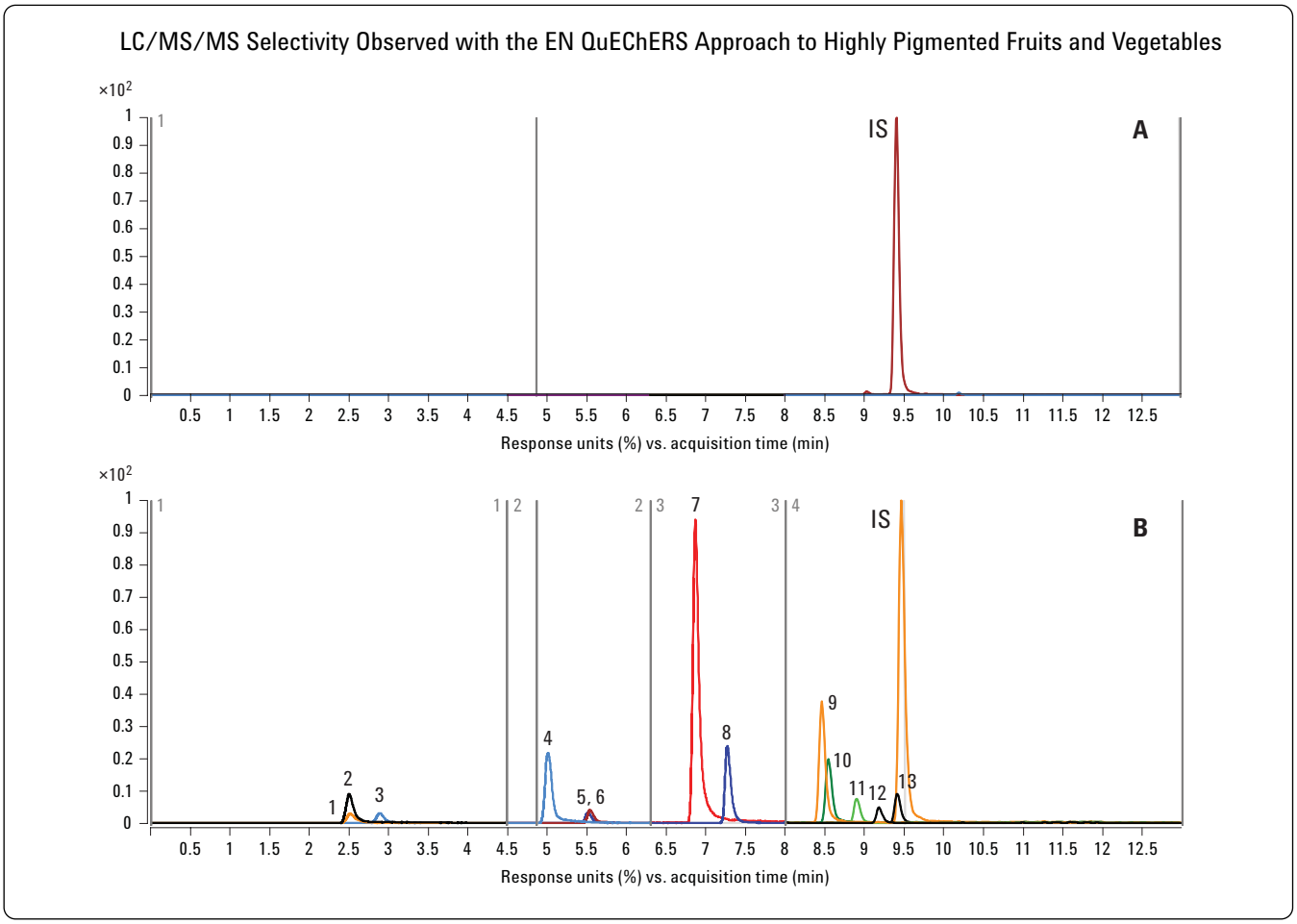


Figure 2. MRM chromatograms of spinach matrix blank (A) and 50 ng/g fortified sample (B) processed by EN method. Peak identification: 1. Methamidophos, 2. Acephate, 3. Pymetrozine, 4. Carbendazim, 5. Imidacloprid 6. Thiabendazole, 7. Propoxur, 8. Carbaryl, 9. Ethoprophos, 10. Imazalil, 11. Penconazole, 12. Cyprodinil, 13. Kresoxim methyl IS: Internal Standard, TPP.

Linearity and limit of quantification (LOQ)

The linear calibration range for all of the pesticides tested was 5–250 ng/g. Calibration curves, spiked in matrix blanks, were made at levels of 5, 10, 50, 100, 200, and 250 ng/g. The TPP was used as an internal standard at 100 ng/g. The calibration curves were generated by plotting the relative responses of analytes (peak area of analyte/peak area of IS) to the relative concentration of analytes (concentration of analyte/concentration of IS). The 5 ng/g quantification limits LOQ (5 ppb) established for all of the pesticides is lower than the MRLs of these pesticides in fruits and vegetables. Table 3 shows the linear regression equation and correlation coefficient (R^2) for both 1 mL and 6 mL dispersive SPE.

Table 3. Linearity of Pesticides in Spinach Extract

Analytes	1 mL dispersive SPE Regression equation	R^2	6 mL dispersive SPE Regression equation	R^2
Methamidophos	Y = 0.2220X + 0.0005	0.9950	Y = 0.2244X + 0.0003	0.9893
Acephate	Y = 0.0814X + 0.0008	0.9972	Y = 0.0797X + 0.0005	0.9974
Pymetrozine	Y = 0.2063X + 0.0009	0.9559	Y = 0.1544X - 0.0006	0.9946
Carbendazim	Y = 0.9015X + 0.0164	0.9945	Y = 0.8526X + 0.0008	0.9917
Imidacloprid	Y = 0.0630X + 0.0001	0.9814	Y = 0.0682X - 0.0002	0.9952
Thiabendazole	Y = 0.3028X + 0.0059	0.9539	Y = 0.2315X + 0.0007	0.9968
Propoxur	Y = 1.3721X + 0.0018	0.9983	Y = 1.3304X + 0.0003	0.9981
Carbaryl	Y = 0.3459X + 0.0009	0.9968	Y = 0.3224X – 0.0003	0.9963
Ethoprophos	Y = 0.7588X - 0.0011	0.9979	Y = 0.7211X – 0.0023	0.9984
Imazalil	Y = 0.4644X + 0.0007	0.9889	Y = 0.4203X + 0.0002	0.9990
Penconazole	Y = 0.1647X – 0.0010	0.9937	Y = 0.1595X – 0.0008	0.9979
Cyprodinil	Y = 0.2575X + 0.0010	0.9884	Y = 0.2272X + 0.0007	0.9987
Kresoxim methyl	Y = 0.1175X – 0.0003	0.9976	Y = 0.1779X – 0.0008	0.9962

Recovery and Reproducibility

The recovery and reproducibility were evaluated by spiking pesticides standards in comminuted spinach sample at levels of 10, 50 and 200 ng/g. These QC samples were quantitated against the matrix spiked calibration curve. The analysis was performed in replicates of six at each level. The recovery and reproducibility (shown as RSD) data of 1 mL and 6 mL dispersive SPE are shown in Tables 4 and Table 5, respectively. It can be seen from the results that the nine pesticides with non-planar structure give excellent recoveries (average of 90.4% for 1 mL and 94.3% for 6 mL) and precision (average of 4.7% RSD for 1 mL and 5.3% RSD for 6 mL). The four pesticides with planar structure give lower but still acceptable recovery (average of 71.8% for 1 mL and 79.8% for 6 mL) but good precision (average of 5.8% RSD for 1 mL and 4.8% RSD for 6 mL).

The impact of GCB on planar pesticides is visible and varies with different compounds. Cyprodinil gave excellent recovery and precision. Carbendazim gave excellent recovery and precision for low and mid level QCs, but poorer recovery for high level QC. Pymetrozine and thiabendazole gave lower recovery but still acceptable precision. The data in Table 6 show that the results of planar pesticides generated by EN method and AOAC modified method (with toluene addition) are comparable.

Table 4. Recovery and Reproducibility of Pesticides in Fortified Spinach with 1 mL Dispersive SPE Tube (p/n 5982-5321)

Analytes	10 ng/g fortified QC		50 ng/g fortified QC		200 ng/g fortified QC	
	Recovery	RSD (n=6)	Recovery	RSD (n=6)	Recovery	RSD (n=6)
Methamidophos	85.5	4.1	84.4	3.8	87.5	6.2
Acephate	83.7	8.3	84.6	5.9	91.6	5.8
Pymetrozine *	60.0	6.4	57.8	4.7	61.4	9.1
Carbendazim *	78.0	7.1	87.7	3.9	49.8	6.8
Imidacloprid	96.5	6.2	91.1	4.6	94.6	4.6
Thiabendazole *	64.3	7.0	71.5	6.5	71.5	5.8
Propoxur	93.7	4.7	92.0	4.1	86.7	4.3
Carbaryl	93.8	5.6	89.4	3.6	91.4	4.1
Ethoprophos	97.1	4.6	89.8	2.6	83.7	4.1
Imazalil	86.6	5.7	80.6	4.9	84.2	4.8
Penconazole	107.8	4.9	94.4	3.2	81.2	3.7
Cyprodinil *	89.6	4.4	88.6	4.5	80.8	3.9
Kresoxim methyl	101.5	3.8	94.6	1.4	92.8	3.8

* Pesticides with planar structure.

Table 5. Recovery and Reproducibility of Pesticides in Fortified Spinach with 6 mL Dispersive SPE Tube (p/n 5982-5356)

Analytes	10 ng/g fortified QC		50 ng/g fortified QC		200 ng/g fortified QC	
	Recovery	RSD (n=6)	Recovery	RSD (n=6)	Recovery	RSD (n=6)
Methamidophos	85.0	8.3	87.7	2.7	95.0	9.4
Acephate	88.6	5.1	84.6	3.1	94.6	9.3
Pymetrozine *	68.7	3.7	65.7	1.5	71.9	10.8
Carbendazim *	94.0	5.4	91.4	2.7	53.5	9.3
Imidacloprid	102.0	8.9	85.4	6.1	100.1	7.7
Thiabendazole *	77.2	4.4	77.6	2.4	79.2	9.7
Propoxur	98.2	5.7	96.3	1.8	93.9	7.2
Carbaryl	98.5	3.6	94.0	1.7	97.4	7.2
Ethoprophos	102.3	6.0	95.3	1.7	91.0	6.8
Imazalil	88.8	6.4	86.8	2.8	93.5	7.7
Penconazole	104.5	2.5	96.4	2.0	84.6	5.5
Cyprodinil *	101.5	4.2	92.2	2.4	86.8	7.6
Kresoxim methyl	99.7	6.1	97.4	1.6	95.3	6.9

* Pesticides with planar structure.

*Table 6. Results Comparison of Planar Pesticides Generated by EN Method and Modified AOAC Method (With Toluene Addition)**

Analytes	EN method for highly pigmented matrix		Modified AOAC method by toluene addition	
	Mean recovery (%)	Mean RSD (%)	Mean recovery (%)	Mean RSD (%)
Carbendazim	75.7	5.9	98.5	2.5
Cyprodinil	89.9	4.5	63.1	3.2
Pymetrozine	64.3	6.0	65.2	3.7
Thiabendazole	73.2	4.9	69.7	2.7

*The data can be found in reference [4].

Conclusions

Agilent SampliQ QuEChERS EN buffered extraction kits and dispersive SPE kits for highly pigmented fruits and vegetables provide a simple, fast and effective method for the purification of representative pesticides in spinach. The small amount of GCB used in dispersive SPE does not impact the extraction of planar pesticides significantly, which makes the extraction procedure in this highly pigmented matrix as simple as the one used in general fruit and vegetables. The recovery and reproducibility, based on matrix spiked standards, are acceptable for multiclass, multi-residue pesticide determination in spinach. However, the final extract matrix contains more impurities, which may result in more negative impacts on the column and MS instrument. The selected pesticides represent a broad variety of different classes and properties; therefore, the Agilent SampliQ QuEChERS EN Buffered Extraction and Dispersive kits for Highly Pigmented Fruits and Vegetables can be used for other pesticides in similar highly pigmented matrices.

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Analysis of Pesticide Residues in Spinach Using Agilent SampliQ QuEChERS AOAC Kit by LC/MS/MS Detection

Application Note

Food Safety

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Abstract

This application note describes the use of a quick, easy, cheap, effective, rugged, and safe (QuEChERS) AOAC sample preparation approach for the extraction and cleanup of 13 pesticide residues representing various pesticide classes in spinach. The original AOAC method employed involves initial extraction in a buffered aqueous/acetonitrile system, an extraction/partitioning step after the addition of salt, and a cleanup step using dispersive solid-phase extraction (dispersive SPE). In order to address the significant loss of planar pesticides caused by graphitized carbon black (GCB) in dispersive SPE, a modified method with the addition of toluene was employed. The presence of the target pesticides in the spinach extracts were then determined by liquid chromatography coupled to an electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) operating in positive ion multiple reaction monitoring (MRM) mode. With the combination of original and modified dispersive SPE, the method was validated in terms of recovery and reproducibility for all of the analytes of interest. The 5 ng/g limit of quantitation (LOQ) for pesticides in spinach shown in this application was well below the maximum residue limits (MRLs). The spiking levels for the recovery experiments were 10, 50, and 200 ng/g. Mean recoveries ranged between 64% and 108% (average of 91.9%), with RSD below 10% (average of 3.3%).

Agilent Technologies

Introduction

The AOAC QuEChERS method has been widely applied in the analysis of pesticides in food since it was introduced by USDA scientists. [1-3] In general, it contains two major steps: extraction and dispersive SPE cleanup. In the extraction step, the method uses a single step buffered 1% acetonitrile (ACN) extraction while simultaneously salting out water from the sample using anhydrous magnesium sulfate ($MgSO_4$) to induce liquid-liquid partitioning. For cleanup, a dispersive solid phase extraction (dispersive SPE) step is employed using a combination of primary secondary amine (PSA) to remove fatty acids as well as other components, and anhydrous $MgSO_4$ to reduce the remaining water in the extract. According to different food matrices, other ingredients may be added in this step, such as graphitized carbon black (GCB) to remove pigments and sterol, or C18 to remove more lipids and waxes.

Spinach is considered to be a highly pigmented vegetable since it contains high levels of chlorophyll. Therefore, the dispersive SPE kits with GCB were selected for further cleanup. In these kits, 50 mg of GCB per mL of ACN extracts are added to 50 mg of PSA and 150 mg of $MgSO_4$. GCB adsorbs planar molecules such as pigments and sterols. Therefore, it is helpful in the cleanup of pigmented matrices such as spinach. However, GCB also adsorbs pesticides with planar structure such as carbendazim, and thiabendazole. As a result, this type of dispersive SPE kit is not recommended for use with planar pesticides. This limitation will have a negative impact on the analysis of planar pesticides from pigmented matrices.

In the previous Application Note, [4] we discussed the impact of toluene addition to the dispersive SPE tube on the analysis of pesticides in pigmented matrix. This Application Note illustrated that this modification can greatly increase the extraction efficiency for problematic pesticides. GCB was employed for the analysis of planar pesticides in pigmented matrices such as spinach with the addition of toluene. In this study, 13 pesticides were used for evaluating the performance of the Agilent AOAC Buffered Extraction kit (p/n 5982-5755) and SampliQ QuEChERS AOAC Dispersive SPE kits for Pigmented Fruits and Vegetables (p/n 5982-5222 and 5982-5258). With the combination of original and modified dispersive SPE, the method was validated in terms of recovery and reproducibility. Table 1 shows the chemical and regulatory information for these pesticides in spinach.

Experimental

Reagents and Chemicals

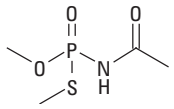
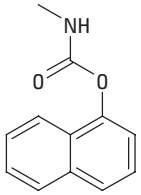
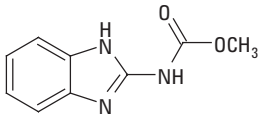
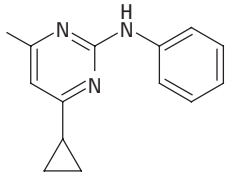
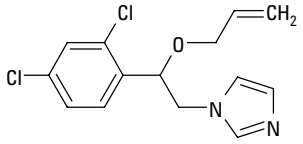
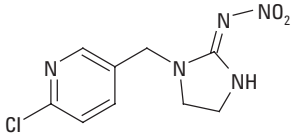
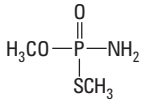
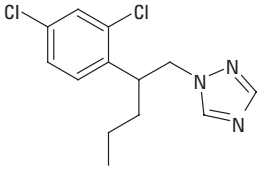
All reagents and solvents were HPLC or analytical grade. Methanol (MeOH), and toluene were from Honeywell (Muskegon, MI, USA). Acetonitrile (ACN), dimethyl sulfoxide (DMSO) and glacial acetic acid (HAc) were from Sigma-Aldrich (St Louis, MO, USA). Ammonium acetate (NH_4OAc) was from Fisher Chemicals (Fair Lawn, NJ, USA). Formic acid (FA) was from Fluka (Sleinheim, Germany). The pesticide standards and internal standard, triphenyl phosphate, (TPP) were purchased from Sigma-Aldrich (St Louis, MO, USA), ChemService (West Chester, PA, USA), Ultra (Kingstown, RI, USA), or AlfaAesar (Ward Hill, MA, USA).

Solutions and Standards

The 1M NH_4OAc pH 5 stock solution was made by dissolving 19.27 g NH_4OAc powder in 250 mL Milli-Q water. The pH was adjusted to 5 with HAc monitored with a pH meter. The solution was stored at 4 °C. A 5 mM NH_4OAc solution in 20:80 MeOH/H_2O, pH 5, was made by combining 200 mL MeOH and 800 mL Milli-Q water, adding 5 mL of 1 M NH_4OAc pH 5 stock solution and mixing well. A 5 mM NH_4OAc in ACN solution was prepared by adding 5 mL of 1 M NH_4OAc pH 5 stock solution to 1 L ACN, mixing well and sonicating 5 min. A 1% HAc in ACN solution was prepared by adding 10 mL of HAc to 1 L of ACN, and mixing well.

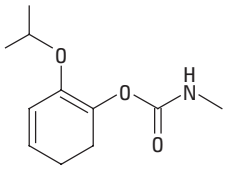
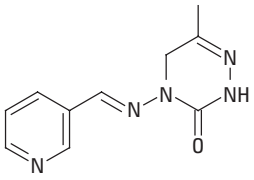
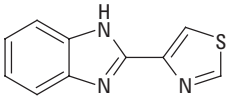
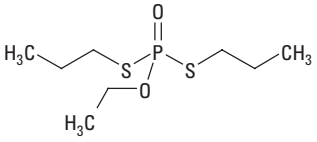
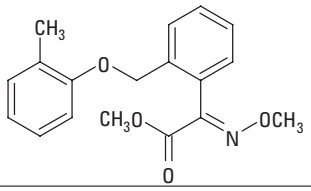
Standard and internal standard (IS) stock solutions (2.0 mg/mL for all, except 0.5 mg/mL for carbendazim) were made in MeOH, 0.1% FA in ACN, or DMSO, respectively, and stored at –20 °C. Three QC spiking solutions of 1.5, 7.5 and 30 µg/mL were made fresh daily in 1:1 ACN/H_2O containing 0.1% FA. A 10 µg/mL standard spiking solution in 1:1 ACN/H_2O containing 0.1% FA was made for the preparation of calibration curves in the matrix blank extract by appropriate dilution. A 15 µg/mL IS spiking standard of TPP was made in 1:1 ACN/H2O containing 0.1% FA.

Table 1. Pesticides Chemical and Regulatory Information [5–7]

Name	Class	Log P	pKa	Structure	MRLs in apple (ng/g)*
Acephate	Organophosphate	–0.89	8.35		20
Carbaryl	Carbamate	2.36	10.4		50
Carbendazim	Benzimidazole	1.48	4.2		100
Cyprodinil	Anilinopyrimidine	4	4.44		500
Imazalil	Imidazole	3.82	6.53		20
Imidacloprid	Neonicotinoid	0.57	NA		1000
Methamidophos	Organophosphate	–0.79	NA		10
Penconazole	Triazole	3.72	1.51		50

(Continued)

Table 1. Pesticides Chemical and Regulatory Information [5–7]

Name	Class	Log P	pKa	Structure	MRLs in apple (ng/g)*
Propoxur	Carbamate	0.14	NA		2000
Pymetrozine	Pyridine	–0.19	4.06		600
Thiabendazole	Benzimidazole	2.39	4.73 12.00 0		50
Ethoprophos	Organophosphate	2.99	NA		5
Kresoxim-methyl	Strobilurin	3.4	NA		50

*The MRLs numbers listed in the table are for spinach or other vegetables. They could be different in different commodities.

Equipment and Material

Agilent 1200 Series HPLC with Diode Array Detector (Agilent Technologies Inc., CA, USA).

Agilent 6410 triple quadrupole LC/MS/MS system with Electrospray Ionization (Agilent Technologies Inc., CA, USA).

Agilent SampliQ QuEChERS AOAC Extraction kits, p/n 5982-5755, and SampliQ QuEChERS AOAC dispersive SPE kits for Pigmented Fruits and Vegetables, p/n 5982-5222 and 5982-5258 (Agilent Technologies Inc., DE, USA).

CentraCL3R Centrifuge (Thermo IEC, MA, USA)

Bottle top dispenser (VWR, So. Painfield, NJ, USA)

Eppendorf microcentrifuge (Brinkmann Instruments, Westbury, NY, USA)

Instrument Condition

The previous LC/MS/MS method was directly used. [8]

HPLC conditions

Column:	Agilent ZORBAX Solvent Saver Plus Eclipse Plus Phenyl-Hexyl, 3.0 x 150 mm, 3.5 µm (p/n 959963-312)
Flow rate:	0.3 mL/min
Column Temperature:	30 °C
Injection volume:	10 µL
Mobile Phase:	A: 5 mM NH_4OAc, pH 5.0 in 20:80 $MeOH/H_2O$ B: 5 mM NH_4OAc, pH 5.0 in ACN
Needle wash:	1:1:1:1 ACN/MeOH/isopropyl alcohol (IPA)/H_2O w/0.2% FA.

Gradient:

Time	% B	Flow rate (mL/min)
0	20	0.3
0.5	20	0.3
8.0	100	0.3
10.0	100	0.3
10.01	20	0.5
13.0	STOP	

Post run:	4 min
Total cycle time:	17 min

MS conditions

Positive mode	
Gas temperature:	350 °C
Gas flow:	10 L/min
Nebulizer:	40 psi
Capillary:	4000 V

Other conditions relating to the analytes are listed in Table 2.

Sample Preparation

The sample preparation procedure includes sample comminution, extraction/partitioning and dispersive SPE cleanup. It has been described in detail in previous Application Notes. [8] The procedure used in spinach was similar except for the addition of toluene to the dispersive SPE cleanup step.

Frozen chopped organic spinach was homogenized thoroughly. A 15 g (± 0.1g) amount of homogenized sample was placed into a 50 mL centrifuge tube. Samples were fortified with appropriate QC spiking solutions (100 µL) when necessary, and then 100 µL of IS spiking solution (15 µg/mL of TPP) were added. After vortexing sample for 30 s, 15 mL of 1% HOAc in ACN was added to each tube using the dispenser. To each tube, an Agilent SampliQ QuEChERS AOAC extraction salt packet (p/n 5982-5755) was added directly. Sample tubes were capped tightly, and hand-shaken vigorously for 1 min. Tubes were centrifuged at 4000 rpm for 5 min.

The ACN extracts were separated into two samples for both original and modified dispersive SPE methods. The modified dispersive SPE method follows a different procedure, therefore it is described below in detail. The volume of ACN extracts (~14 mL) was enough for simultaneously processing samples with original and modified dispersive SPE when using 2 mL size dispersive SPE tubes. When 15 mL size tubes were used, 14 mL of ACN extracts from one sample was not enough for processing dispersive SPE by two methods simultaneously. Therefore, another sample was extracted from the beginning.

A 1 mL aliquot of the upper ACN layer was transferred into an Agilent SampliQ QuEChERS dispersive SPE 2 mL tube (p/n 5982-5222); or 8 mL aliquot into an Agilent SampliQ QuEChERS dispersive SPE 15 mL tube (p/n 5982-5258). The 2 mL tube contained 50 mg of PSA, 50 mg of GCB and 150 mg of anhydrous $MgSO_4$; while the 15 mL tube contained 400 mg of PSA, 400 mg of GCB and 1200 mg of anhydrous $MgSO_4$. Next, 375 µL of toluene were added to the 2 mL tube, or 3 mL of toluene was added to 15 mL tube. The tubes were tightly capped and vortexed for 1 minute. The tubes were vortexed for a few seconds before sample addition, to prevent agglomerates. The 2 mL tubes were centrifuged with a microcentrifuge at 13,000 rpm for 2 min, and the 15 mL tubes in a standard centrifuge at 4000 rpm for 5 min. An 825 µL amount

Table 2. Instrument Acquisition Data for the Analysis of 13 Pesticides by LC/MS/MS

Analyte	MRM channels (*m/z*)	Fragmentor (V)	CE (V)	RT (min)
Acephate	1) 184.0 > 94.9 2) 184.0 > 111.0	60	3 15	2.55
Methamidophos	1) 142.0 > 94.0 2) 142.0 > 124.9	60	8 8	2.54
Pymetrozine	1) 218.1 > 105.0 2) 218.1 > 78.0	115	20 50	2.97
Carbendazim	1) 192.1 > 160.0 2) 192.1 > 105.0	95	18 40	5.07
Imidacloprid	1) 256.1 > 209.1 2) 256.1 > 175.0	60	12 18	5.53
Thiabendazole	1) 202.1 > 175.0 2) 202.1 > 131.0	110	27 38	5.65
Propoxur	1) 210.1 > 111.0 2) 210.1 > 92.9	50	12 15	6.89
Carbaryl	1) 202.0 > 145.0 2) 202.0 > 115.0	50	3 40	7.30
Ethoprophos	1) 243.1 > 130.9 2) 243.1 > 172.9	80	15 15	8.50
Imazalil	1) 297.1 > 158.9 2) 297.1 > 200.9	80	22 15	8.52
Penconazole	1) 284.1 > 158.9 2) 284.1 > 172.9	80	32 32	8.95
Cyprodinil	1) 226.1 > 93.0 2) 226.1 > 108.0	120	35 35	9.23
Kresoxim methyl	1) 314.0 > 222.1 2) 314.0 > 235.0	70	10 10	9.44
TPP (IS)	1) 327.1 > 77.0 2) 327.1 > 151.9	70	45 45	9.49

1) Quantifier transition channel
2) Qualifier transition channel

of extract was then transferred into another tube, and dried by N_2 flow. Samples were reconstituted into 600 µL of ACN containing 0.1%FA. After vortexing and sonicating, 200 µL of extract were transferred into an autosampler vial, and then 800 µL of water or other appropriate standard solution (prepared in water) were added. The samples were capped and vortexed thoroughly for LC/MS/MS analysis.

Another aliquot of ACN extracts was processed following the original dispersive SPE clean-up procedure. Figure 1 shows the flow chart of the whole extraction procedure (original and modified dispersive SPE) for a spinach sample.

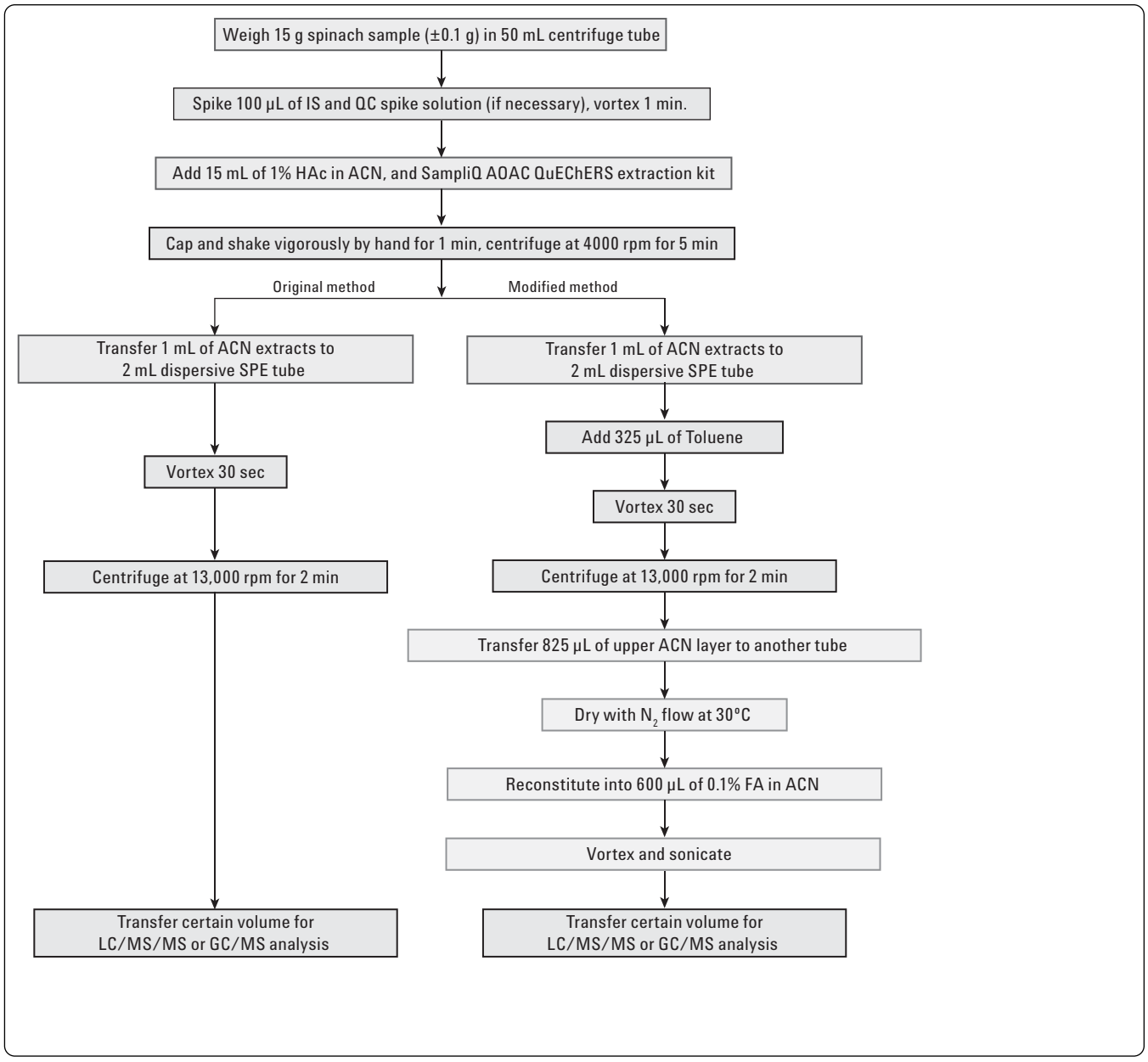


Figure 1. Flow chart of the QuEChERS AOAC extraction procedure (original and modified dispersive SPE, 2 mL size) for a spinach sample.

Results and Discussion

QuEChERS method for pesticide residues analysis provides high-quality results in a fast, easy, inexpensive approach. For the pigmented fruits and vegetables, the addition of GCB in the dispersive SPE tube can improve the removal of pigments and sterols. Toluene was added to increase the extraction efficiency of planar pesticides. Previously it was established that the addition of toluene produces a yellow final sample, indicating that matrix impurities are retained. [4] However, with the powerful selectivity provided by LC/MS/MS, there have not been any chromatographic differences found between the samples processed with the original and modified methods. Figures 2 and 3 show the LC/MS/MS chromatograms of matrix blank (IS spiked) and 50 ng/g fortified spinach extract processed by original and modified dispersive SPE method.

Four pesticides with planar structure showed significant loss by the original dispersive SPE method. The modified method with toluene addition increased the recovery of those four pesticides by two to three times, from 20% to 40% and 60% to 100%. In addition, the repeatability improved from >15% to <5% RSD. The addition of toluene had no affect on the quantitation results of other pesticides. Therefore, the results from the original method for high recovered pesticides were combined with the results from modified method for planar pesticides. The method was validated in terms of recovery and reproducibility, and the quantitation results are discussed.

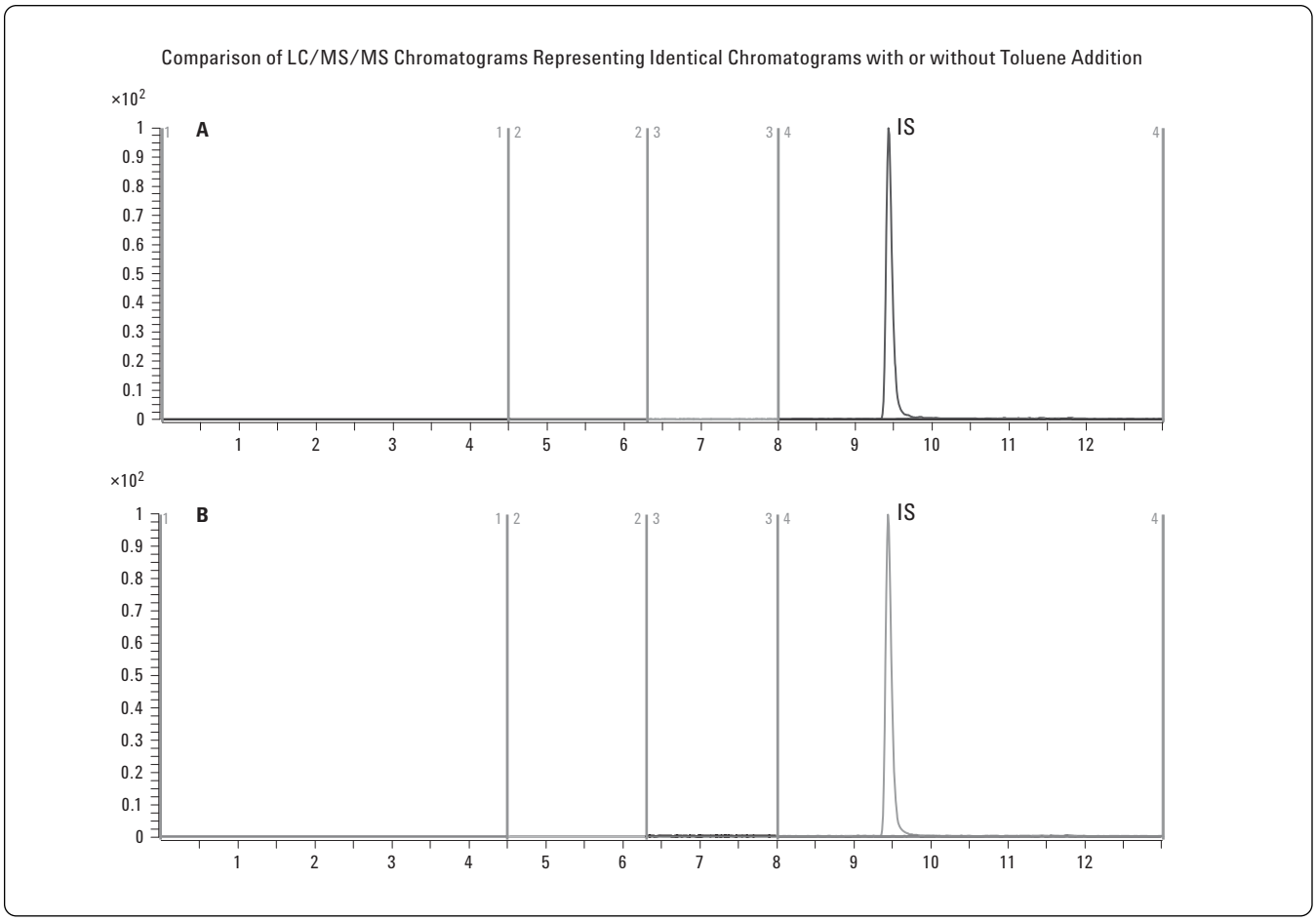


Figure 2. LC/MS/MS chromatograms of spinach matrix blank processed by original dispersive SPE (A) and modified dispersive SPE (B). IS: Internal Standard TPP.

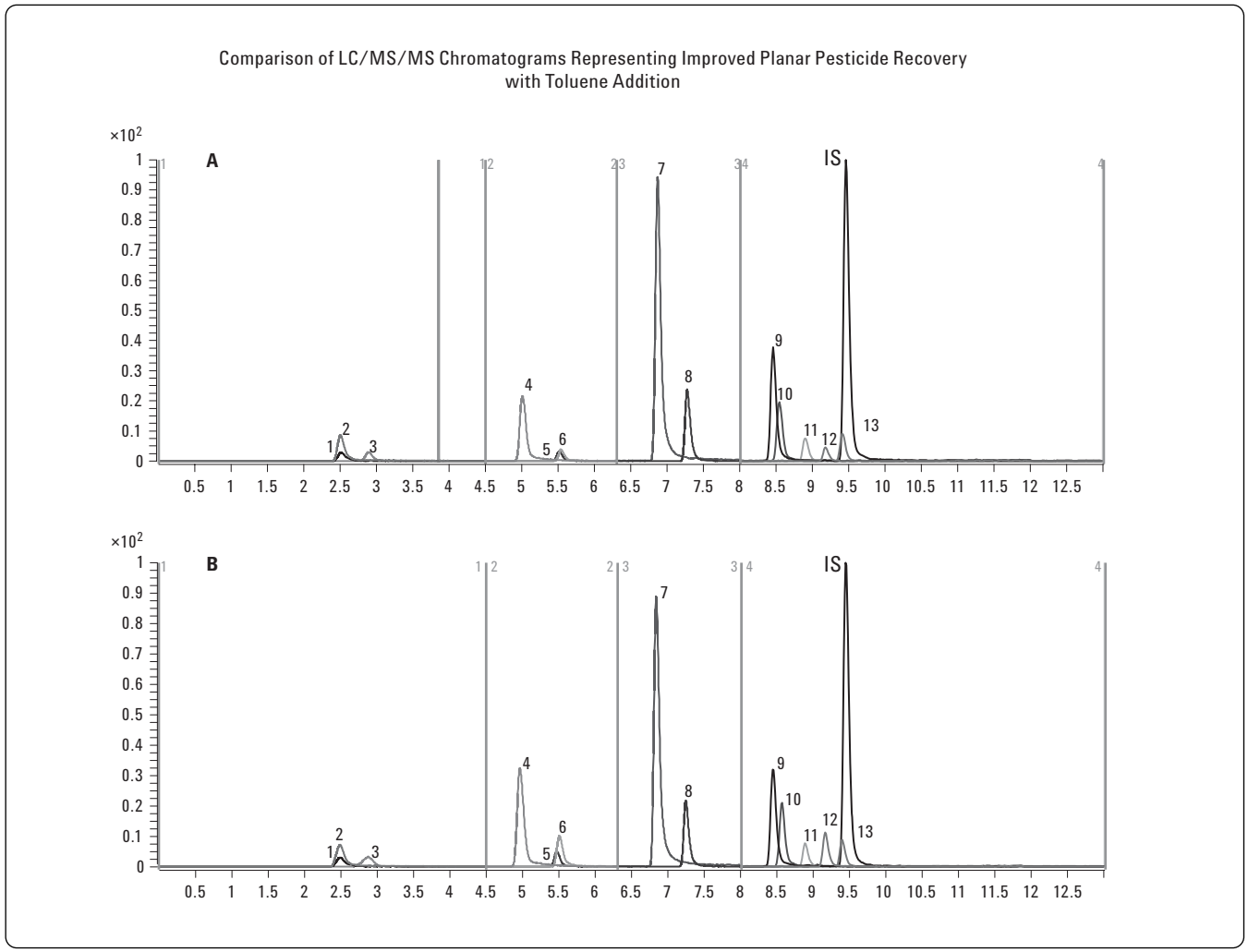


Figure 3. LC/MS/MS chromatograms of 50 ng/g fortified spinach sample extracts processed by original dispersive SPE (A) and modified dispersive SPE (B). Peak identification: 1. Methamidophos, 2. Acephate, 3. Pymetrozine, 4. Carbendazim, 5. Imidacloprid 6. Thiabendazole, 7. Propoxur, 8. Carbaryl, 9. Ethoprophos, 10. Imazalil, 11. Penconazole, 12. Cyprodinil, 13. Kresoxim methyl IS: Internal Standard, TPP.

Linearity and Limit of Quantification (LOQ)

The linear calibration range for all of the pesticides was 5–250 ng/g. For samples processed by original and modified methods, the corresponding matrix blanks were used to prepare the calibration curves respectively. Calibration curves, spiked in matrix blanks, were made at levels of 5, 10, 50, 100, 200, and 250 ng/g. The TPP was used as an internal standard at 100 ng/g. The calibration curves were generated by plotting the relative responses of analytes (peak area of analyte / peak area of IS) to the relative concentration of analytes (concentration of analyte / concentration of IS). The 5 ng/g quantification limits LOQ (5 ppb) established for all of the pesticides was lower than or equal to the MRLs of these pesticides in fruits and vegetables. Table 3 shows the linear regression equation and correlation coefficient (R^2) for both 1 mL and 8 mL dispersive SPE.

Table 3. Linearity of Pesticides in Spinach Extract

Analytes	1 mL dispersive-SPE Regression equation	R^2	8 mL dispersive-SPE Regression equation	R^2
Methamidophos	Y = 0.2358X – 0.0008	0.9976	Y = 0.2164X – 0.0014	0.9983
Acephate	Y = 0.0862X – 0.0003	0.9975	Y = 0.0804X – 0.0006	0.9942
Pymetrozine *	Y = 0.2073X – 0.0002	0.9995	Y = 0.2034X – 0.0013	0.9978
Carbendazim *	Y = 0.8375X + 0.0032	0.9915	Y = 0.8383X + 0.0002	0.9982
Imidacloprid	Y = 0.0652X – 0.0007	0.9905	Y = 0.0620X – 0.0011	0.9742
Thiabendazole *	Y = 0.4081X - 0.0008	0.9995	Y = 0.4102X – 0.0011	0.9975
Propoxur	Y = 1.9253X – 0.0042	0.9995	Y = 1.8253X – 0.0037	0.9996
Carbaryl	Y = 0.4243X – 0.0013	0.9979	Y = 0.3993X – 0.0019	0.9946
Ethoprophos	Y = 0.7859X – 0.0012	0.9983	Y = 0.7420X – 0.0012	0.9985
Imazalil	Y = 0.4586X + 0.0002	0.9954	Y = 0.4229X + 0.0005	0.9903
Penconazole	Y = 0.1643X – 0.0014	0.9923	Y = 0.1468X – 0.0003	0.9944
Cyprodinil *	Y = 0.3274X – 0.0024	0.9904	Y = 0.3067X – 0.0013	0.9978
Kresoxim methyl	Y = 0.1809X – 0.0015	0.9975	Y = 0.1659X – 0.0008	0.9928

* Results from modified dispersive SPE method.

Recovery and Reproducibility

The recovery and reproducibility were evaluated by spiking pesticides standards in communited spinach sample at levels of 10, 50 and 200 ng/g. These QC samples were quantitated against the matrix spiked calibration curve. The analysis was performed six times at each level. The recovery and reproducibility (shown as RSD) data of 1 mL and 8 mL volume dispersive SPE are shown in Tables 4 and 5, respectively. The results show that the nine pesticides processed with the original method resulted in excellent recoveries (average of 97.8% for 1 mL and 103.4% for 8 mL) and precision (average of 3.6% RSD for 1 mL and 4.3% RSD for 8 mL). Although the four pesticides processed with the modified method resulted in lower recoveries (average of 78.5% for 1 mL and 69.7% for 8 mL) but high precision (average of 2.7% RSD for 1 mL and 3.3% RSD for 8 mL). The results from the modified method were much better than the results obtained by original methods. Please refer to previous Application Note [4] for a detailed discussion.

Table 4. Excellent Recovery and Reproducibility of Pesticides in Fortified Spinach with a 1 mL volume, 2 mL Dispersive SPE Tube (p/n 5982-5222)

Analytes	10 ng/g fortified QC		50 ng/g fortified QC		200 ng/g fortified QC	
	Recovery	RSD (n=6)	Recovery	RSD (n=6)	Recovery	RSD (n=6)
Methamidophos	91.8	4.2	93.3	3.7	93.8	5.7
Acephate	93.4	3.3	91.3	5.6	101.9	7.8
Pymetrozine *	74.0	2.9	71.1	3.2	70.3	2.9
Carbendazim *	105.3	4.0	109.1	2.5	88.9	1.7
Imidacloprid	98.2	4.5	100.4	3.7	100.0	2.7
Thiabendazole *	79.0	2.7	76.6	2.3	75.5	1.8
Propoxur	100.0	1.7	98.1	3.5	93.0	4.0
Carbaryl	110.8	3.2	108.1	1.0	105.1	3.2
Ethoprophos	98.8	1.6	98.2	3.3	95.1	3.1
Imazalil	84.0	3.8	89.6	2.5	89.8	1.7
Penconazole	103.1	5.4	98.4	3.5	97.2	1.9
Cyprodinil *	69.1	4.7	62.0	2.9	61.3	1.1
Kresoxim methyl	104.4	4.8	101.2	5.0	102.6	3.0

* Results from modified dispersive SPE method.

Table 5. Excellent Recovery and Reproducibility of Pesticides in Fortified Spinach with a 8 mL volume, 15 mL Dispersive SPE Tube (p/n 5982-5258)

Analytes	10 ng/g fortified QC		50 ng/g fortified QC		200 ng/g fortified QC	
	Recovery	RSD (n=6)	Recovery	RSD (n=6)	Recovery	RSD (n=6)
Methamidophos	98.6	3.8	94.2	7.1	97.8	2.9
Acephate	95.5	8.9	91.5	6.3	105.6	5.7
Pymetrozine *	62.4	4.3	53.9	3.4	59.3	5.4
Carbendazim *	95.7	1.6	98.6	1.9	93.3	2.9
Imidacloprid	112.7	4.2	107.6	7.7	110.4	3.7
Thiabendazole *	58.0	3.5	62.1	3.3	66.8	2.8
Propoxur	104.9	1.4	103.3	3.7	99.0	3.3
Carbaryl	116.9	2.2	114.6	2.4	110.8	2.1
Ethoprophos	105.3	2.5	105.7	2.8	103.0	2.3
Imazalil	86.3	3.9	94.9	4.3	93.9	3.4
Penconazole	103.5	10.4	106.9	3.6	99.2	6.4
Cyprodinil *	63.1	2.8	60.6	4.8	62.7	2.9
Kresoxim methyl	111.2	4.5	106.6	3.2	112.0	3.0

* Results from modified dispersive SPE method.

Figure 4 shows the recovery and precision results obtained by 1 mL and 8 mL volume dispersive SPE. To simplify the comparison, the average recovery and precision of three fortification concentrations were used for all pesticides. The results of two dispersive SPE cleanup approaches appeared to be independent of volume used. Apparently, both approaches provided efficient and similar sample cleanup, and thus generated relatively equivalent results. However, if 8 mL size dispersive SPE volume is used, two duplicated extractions must be performed initially to complete both original and modified dispersive SPE. If 1 mL size dispersive SPE is used, only one extraction is needed to provide enough volume to perform both original and modified dispersive SPE simultaneously. This is more cost effective saving time, sample amount, and labor. The extractions can be performed according to the user's requirements and regulations.

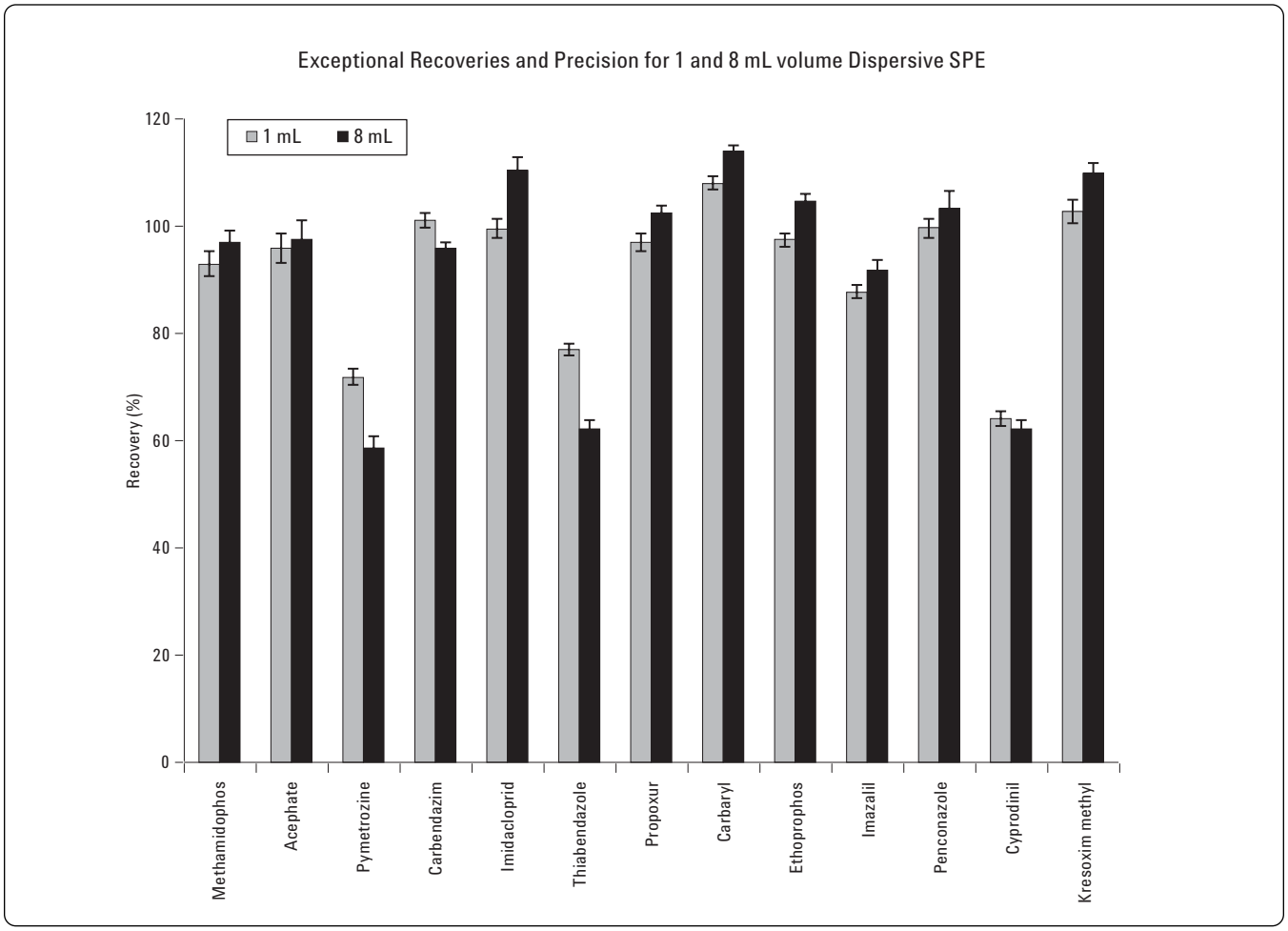


Figure 4. The recovery and precision results for 1 mL dispersive SPE and 8 mL dispersive SPE.

Conclusions

Agilent SampliQ QuEChERS AOAC buffered extraction kits and dispersive SPE kits for pigmented fruits and vegetables provide a simple, fast and effective method for the purification of representative pesticides in spinach. The modified dispersive SPE method with the addition of toluene provides a very useful way to limit the loss of planar pesticides caused by GCB in dispersive SPE. The recovery and reproducibility of this method, based on matrix spiked standards, were acceptable for multiclass, multi-residue pesticide determination in spinach. The impurities and matrix effects from spinach were minimal and did not interfere with the quantitation of any target compound. As the selected pesticides represented a broad variety of different classes and properties, the Agilent SampliQ QuEChERS AOAC Buffered Extraction and Dispersive kits for Pigmented Fruits and Vegetables can be used for other pesticides in other similar pigmented matrices.

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Optimizing Recoveries of Planar Pesticides in Spinach Using Toluene and Agilent SampliQ AOAC QuEChERS Kits with Graphitized Carbon

Application Note

Food Safety

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Abstract

This application note describes the impact of toluene addition in the dispersive solid phase extraction (SPE) step on the analysis of pesticides in spinach using Agilent SampliQ QuEChERS AOAC kits for highly pigmented fruits and vegetables. Graphitized carbon black (GCB) is required in the dispersive SPE kits in order to remove high levels of pigments from the matrix. However, it also retains pesticides with planar structures resulting in poor recovery and precision. The eight problematic pesticides found in the original AOAC method, by either LC/MS/MS or GC/MS, generated poor results with about 20% to 60% recovery with >15% relative standard deviation (RSD). In the modified AOAC method, an aliquot of toluene was added to the dispersive SPE clean-up tube, in a ratio of 8:3 (acetonitrile (ACN) extracts/toluene). It significantly improved the extraction efficiency of the problematic planar pesticides. With the modified AOAC method, the eight problematic pesticides generated substantially improved recoveries, 50% to 100%, and < 10% RSD. However, the addition of toluene also introduced more matrix impurities into the final sample, and caused problems for some pesticides which gave good results originally. Therefore, the modified AOAC method cannot be considered a "drop in" replacement for the original AOAC method; but it can be a very useful alternative for the problematic pesticides affected by GCB in the pesticides analysis of highly pigmented matrix.

Agilent Technologies

Introduction

The AOAC quick, easy, cheap, effective, rugged, safe (QuEChERS) method has been widely applied in the analysis of pesticides in food since it was introduced by USDA scientists. [1-3] In general, it contains two major steps: extraction and dispersive SPE clean-up. In the extraction step, the method uses a single-step buffered acetonitrile (1% HAc) extraction while simultaneously salting out water from the sample using anhydrous magnesium sulfate ($MgSO_4$) to induce liquid-liquid partitioning. For cleanup, a dispersive solid phase extraction (dispersive SPE) step is employed using a combination of primary secondary amine (PSA) to remove polar organic acids as well as other components, and anhydrous $MgSO_4$ to reduce the remaining water in the extract. After mixing and centrifugation, the upper layer is ready for analysis.

Various food matrices require modifications to the dispersive SPE clean-up step. For general fruits and vegetables, 50 mg PSA and 150 mg $MgSO_4$, per mL of ACN extracts are used for clean-up to remove polar organic acids, some sugars and lipids, and excess water. Pigmented fruits and vegetables kits, besides PSA and $MgSO_4$, include 50 mg GCB per mL of ACN extracts to remove pigments like chlorophyll and carotinoides. For fruits and vegetables with fats and waxes, 50 mg C18 per mL of ACN extracts is added with PSA and $MgSO_4$ removing lipids and sterols. Therefore, according to the food matrix, analysts need to select a suitable dispersive SPE kit in order to analyze pesticides of interest.

Previously, we demonstrated the excellent performance of SampliQ QuEChERS AOAC buffered AOAC extraction kits and dispersive SPE kits for general fruits and vegetables on a representative group of pesticides in apple by LC/MS/MS and GC/MS. [4, 5] For the SampliQ QuEChERS AOAC kits for pigmented fruits and vegetables, spinach was selected as the matrix in order to evaluate the extraction and performance of the dispersive kit. GCB was added to the dispersive SPE kit to remove the high level of pigments, such as chlorophyl and carotinoides, which can cause more matrix effect and introduce more interferences. Conversely, GCB can cause a significant loss of planar pesticides, for example, thiobendazole, chlorothalonil, coumaphos, cyprodinil. [3, 6] Therefore, the use of GCB is recommended when planar pesticides are not being analyzed; greatly limiting the usefulness of GCB to the clean-up of pigmented matrix. In previous GCB SPE column extractions [7], solvent mixtures containing toluene were commonly used to elute pesticides through GCB columns. ACN/toluene (3:1) mixtures have been used for the multiclass multiresidue method (MRM) elution of pesticides through tandem GCB-NH_2 [8], GCB-PSA [9], and GCB SAX-PSA. [10] In this study, toluene was added into the ACN extracts in the second step of QuEChERS, the dispersive SPE clean-up. We determined that the ratio of 8:3 (ACN extract toluene) generated higher recoveries (50% to 300% higher), and substantially better precision (< 10% RSD) for the eight GCB retained pesticides. However, it was noted that the addition of toluene caused adverse affects, such as additional matrix impurities in the final extracted samples, lower recovery and higher imprecision for certain pesticides which originally produced good results without the addition of toluene.

Experimental

Reagents and Chemicals

All reagents and solvents were HPLC or analytical grade. Methanol (MeOH), and toluene were from Honeywell (Muskegon, MI, USA). Acetonitrile (ACN), dimethyl sulfoxide (DMSO) and acetic acid, glacial (HAc) were from Sigma-Aldrich (St Louis, MO, USA). Ammonium acetate (NH_4OAc) was from Fisher Chemicals (Fair Lawn, NJ, USA). Formic acid (FA) was from Fluka (Sleinheim, Germany). The pesticide standards and internal standard triphenyl phosphate, (TPP) were purchased from Sigma-Aldrich (St Louis, MO, USA), ChemService (West Chester, PA, USA), Ultra (North Kingstown, RI, USA), or AlfaAesar (Ward Hill, MA, USA).

Solutions and Standards

The 1 M ammonium acetate pH 5 stock solution was made by dissolving 19.27 g NH_4OAc powder in 250 mL Milli-Q water, and the pH adjusted to 5 with glacial acetic acid. The solution was stored at 4 °C. Methanol/H_2O (20:80) containing 5 mM ammonium acetate pH 5 was made by combining 200 mL MeOH and 800 mL Milli-Q water, adding 5 mL of 1M ammonium acetate pH 5 stock solution and mixing well. A 5 mM ammonium acetate in ACN solution was prepared by adding 5 mL of 1 M ammonium acetate pH 5 stock solution to 1 L ACN, mixing well and sonicating 5 min. 1% HAc in ACN was prepared by adding 10 mL of glacial acetic acid to 1 L of ACN, and mixing well.

Standard and internal standard (IS) stock solutions (2.0 mg/mL for all, except 0.5 mg/mL for carbendazim) were made in MeOH, 0.1% FA in ACN, or DMSO, respectively, and stored at –20°C. Three QC spiking solutions of 1.5, 7.5, and 30 µg/mL were made fresh daily in 1:1 ACN/H_2O containing 0.1% FA. A 10 µg/mL standard spiking solution in 1:1 ACN/H_2O containing 0.1% FA was made for preparation of LC/MS/MS calibration curves in the matrix blank extract by appropriate dilution.

A 2.5 µg/mL standard solution in ACN containing 0.1% FA was used to prepare the GC/MS calibration curves in the matrix blank extract by appropriate dilution. A 15 µg/mL IS spiking standard of TPP in 1:1 ACN/H_2O containing 0.1% FA was made.

Equipment and material

Agilent 1200 Series HPLC with Diode Array Detector (Agilent Technologies Inc., CA, USA).

Agilent 6410 triple quadrupole MS/MS system with Electrospray Ionization (Agilent Technologies Inc., CA, USA).

Agilent Gas Chromatograph (Agilent Technologies Inc., Santa Clara, CA, USA).

Agilent 5975C Mass Spectrometer (Agilent Technologies Inc., Santa Clara, CA, USA).

Agilent SampliQ QuEChERS AOAC Extraction kits, p/n 5982-5755, and SampliQ QuEChERS AOAC dispersive SPE kits for Pigmented Fruits and Vegetables, p/n 5982-5222 and 5982-5258 (Agilent Technologies Inc., DE, USA).

CentraCL3R Centrifuge (Thermo IEC, MA, USA)

Bottle top dispenser (VWR, South Painfield, NJ, USA)

Eppendorf microcentrifuge (Brinkmann Instruments, Westbury, NY, USA)

Instrument Conditions

HPLC conditions

Column:	Agilent ZORBAX Solvent Saver Plus Eclipse Plus Phenyl-Hexyl, 3.0 × 150 mm, 3.5 µm (p/n: 959963-312)
Flow rate:	0.3 mL/min
Column temperature:	30°C
Injection volume:	10 µL
Mobile phase:	A: 5 mM ammonium acetate, pH 5.0 in 20:80 MeOH/H2O; B: 5 mM ammonium acetate, pH 5.0 in ACN
Needle wash:	1:1:1:1 ACN/MeOH/IPA/H2O w/0.2% FA.

Gradient:

Time	% B	Flow rate (mL/min)
0	20	0.3
0.5	20	0.3
8.0	100	0.3
10.0	100	0.3
10.01	20	0.5
13.0	STOP	

Post run:	4 min
Total cycle time:	17 min.

GC conditions

Inlet:	Splitless
Inlet liner:	Helix double taper, deactivated (p/n: 5188-5398)
Carrier gas:	Helium
Inlet pressure:	19.6 psi (constant pressure mode) during run 1.0 psi during back flush
Inlet temperature:	250 °C
Injection volume:	1.0 µL
Purge flow to split vent:	30 mL/min at 0.75 min
Oven temperature program:	70 °C (1 min), 50 °C/min to 150 °C (0 min), 6 °C /min to 200 °C (0 min), 16 °C/min to 280 °C (6 min)
Post run:	3 min
Capillary flow technology:	Purged Ultimate Union (p/n: G3186B) – used for backflushing the analytical column and inlet.
Aux EPC gas:	Helium plumbed to Purged Ultimate Union
Aux EPC pressure:	4.0 psi during run, 80.0 psi during backflush
Column:	Agilent J&W HP-5ms Ultra Inert 15 m × 0.25 mm, 0.25 µm (p/n: 19091S-431UI)
Connections:	Between inlet and Purged Ultimate Union (p/n: G3186B)
Restrictor:	65 cm x 0.15 mm, 0.15 µm DB-5 ms Ultra Inert.
Connections:	Between the Purged Ultimate Union and the MSD.

For the instrument acquisition data of MS/MS in LC/MS/MS and MS in GC/MS relating to the analytes, please refer to the acquisition data table in the previous Agilent publications. [4, 11]

Sample Preparation

The sample preparation procedure includes sample comminution, extraction/partitioning and dispersive SPE clean-up. The QuEChERS method employing spinach as the vegetable matrix is similar to the method described in detail in previous application notes [4,5], with the exception of the dispersive SPE step which includes a toluene addition.

The frozen chopped organic spinach was homogenized thoroughly. Fifteen grams (± 0.1g) of homogenized sample was placed into 50 mL centrifuge tubes. Samples were fortified with appropriate QC spiking solutions (100 µL) if necessary, and then 100 µL of IS spiking solution (15 µg/mL of TPP). After vortexing the samples for 30 s, 15 mL of 1% HAc in ACN was added to each tube. An Agilent SampliQ QuEChERS AOAC extraction salt packet (p/n 5982-5755) was added directly to each tube. Sample tubes were sealed tightly, and hand-shaken vigorously for 1 min. Tubes were centrifuged at 4,000 rpm for 5 min.

A 1 mL aliquot of the upper ACN layer was transferred into an Agilent SampliQ QuEChERS dispersive SPE 2 mL tube (p/n 5982-5222); or an 8 mL aliquot was transferred into an Agilent SampliQ QuEChERS dispersive SPE 15 mL tube (p/n 5982-5258). The 2 mL tube contained 50 mg of PSA, 50 mg of GCB and 150 mg of anhydrous $MgSO_4$, while the 15 mL tube contained 400 mg of PSA, 400 mg of GCB and 1200 mg of anhydrous $MgSO_4$. Subsequently, 375 µL of toluene were added to the 2 mL tubes, and 3 mL of toluene were added to the 15 mL tubes. The tubes were tightly capped and vortexed for 1 min. The 2 mL tubes were centrifuged with a micro-centrifuge at 13,000 rpm for 2 min, and the 15 mL tubes centrifuged in a standard centrifuge at 4,000 rpm for 5 min. An 825 µL amount of extract was transferred into a 15 mL centrifuge tube and dried by N_2 flow. Samples were reconstituted with 600 µL of ACN containing 0.1% FA, vortexed and sonicated. A 200 µL aliquot of the extract was transferred into an autosampler vial, and 800 µL of water or appropriate standard solutions (prepared in water) were added. The samples were capped and vortexed thoroughly prior to LC/MS/MS analysis. For samples analyzed by GC/MS, a 600 µL reconstituted sample was either transferred directly to an autosampler vial or used to prepare the calibration curves.

In order to determine toluene's affect on the dispersive SPE procedure, another aliquot of ACN extracts was processed following the original dispersive SPE clean-up procedure.

Figure 1 shows the dispersive SPE procedure scheme according to the original method (w/o toluene) and the modified method (w/ toluene).

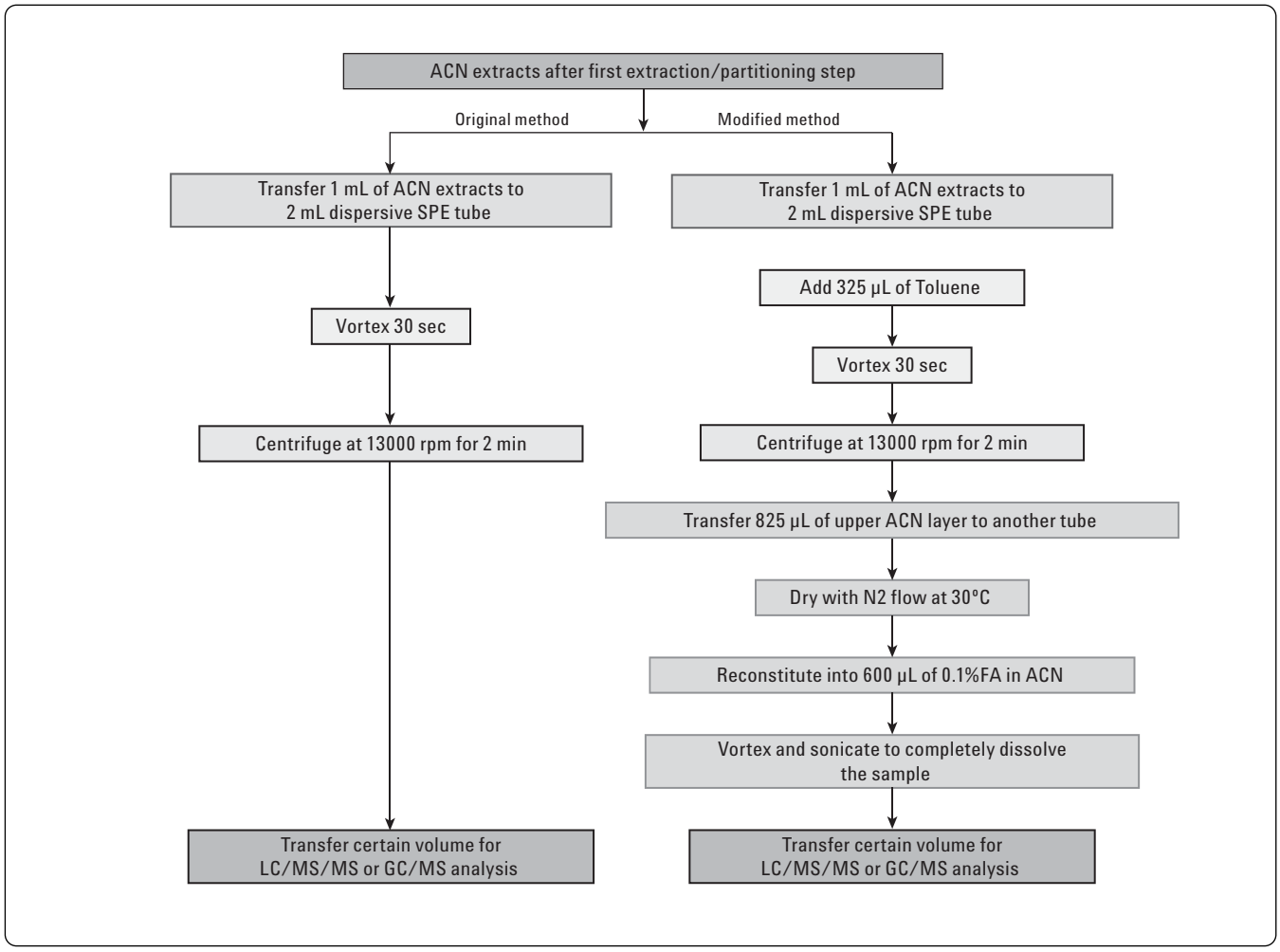


Figure 1. Dispersive SPE procedures of original method (w/o toluene) and modified method (w/toluene).

Results and Discussion

Impact on the Clean-up of Matrix

The QuEChERS methodology for pesticide residue analysis provided high-quality results with a fast, easy, inexpensive approach. For pigmented fruits and vegetables, the addition of GCB in the dispersive SPE tube can greatly remove pigments and sterols. This was clearly shown by the color of the extracts. The spinach ACN extract after the first salt extraction step was very dark green in color. When a dispersive SPE kit for pigmented produce (with GCB) was employed for dispersive SPE clean-up, the upper ACN extract layer became clear with an almost colorless to very light yellow color. On the contrary, when a dispersive SPE kit for general fruits and vegetables was used without GCB, the upper layer was still a dark green to black color. The dispersive SPE extracts modified by the addition of toluene gave a bright yellow color after vortexing and centrifuging. The increase of color for the extracts suggested that the addition of toluene either reduced the affinity of GCB for those pigment molecules, or back-extracted those molecules from the GCB. The addition of toluene resulted in more impurities in the final extracted sample which is demonstrated by the comparison of the UV chromatograms (λ = 254 nm) for the two matrix blanks as shown in Figure 2.

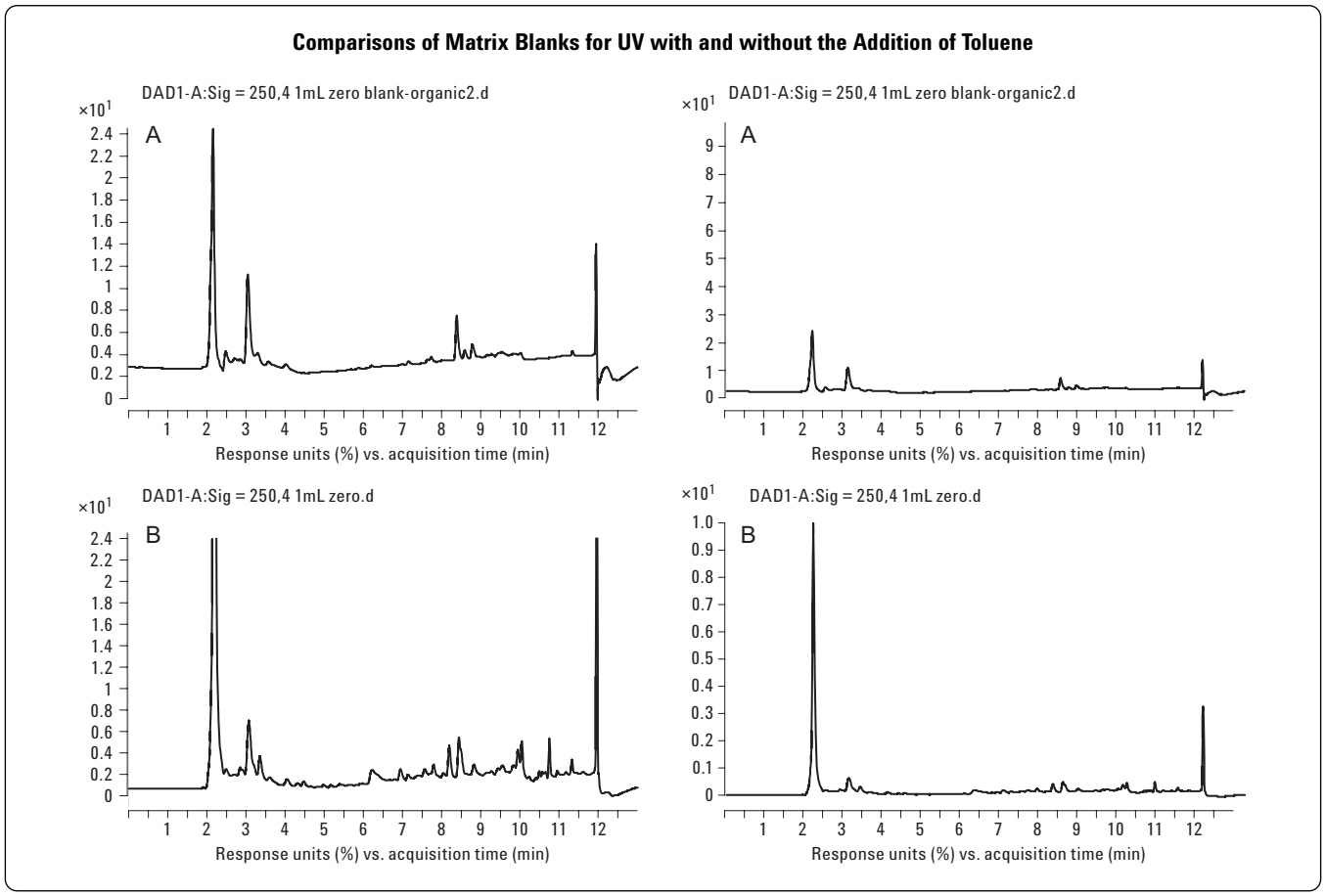


Figure 2. UV chromatogram (λ = 254 nm) comparison of matrix blank obtained with original method without toluene (A) and modified method with addition of toluene (B). Left chromatograms shown in small scale for detail comparison, and right chromatograms shown full scale for big interference peaks comparison. In both cases, the same scale was used for blank A and B chromatograms.

However, the increase in matrix impurities didn't affect the LC/MS/MS or GC/MS pesticide analysis. Figure 3 shows the spinach matrix blank LC/MS/MS chromatograms processed by the modified method with addition of toluene (A) and the original method without addition of toluene (B). With the enhanced selectivity of LC/MS/MS, the two blank samples (A and B) showed similarly clean chromatograms. Figure 4 shows the spinach matrix blank GC/MS chromatograms processed by the modified method with the addition of toluene (A) and the original method without the addition of toluene (B). The two blank chromatograms show some minor differences, but similarities are confirmed.

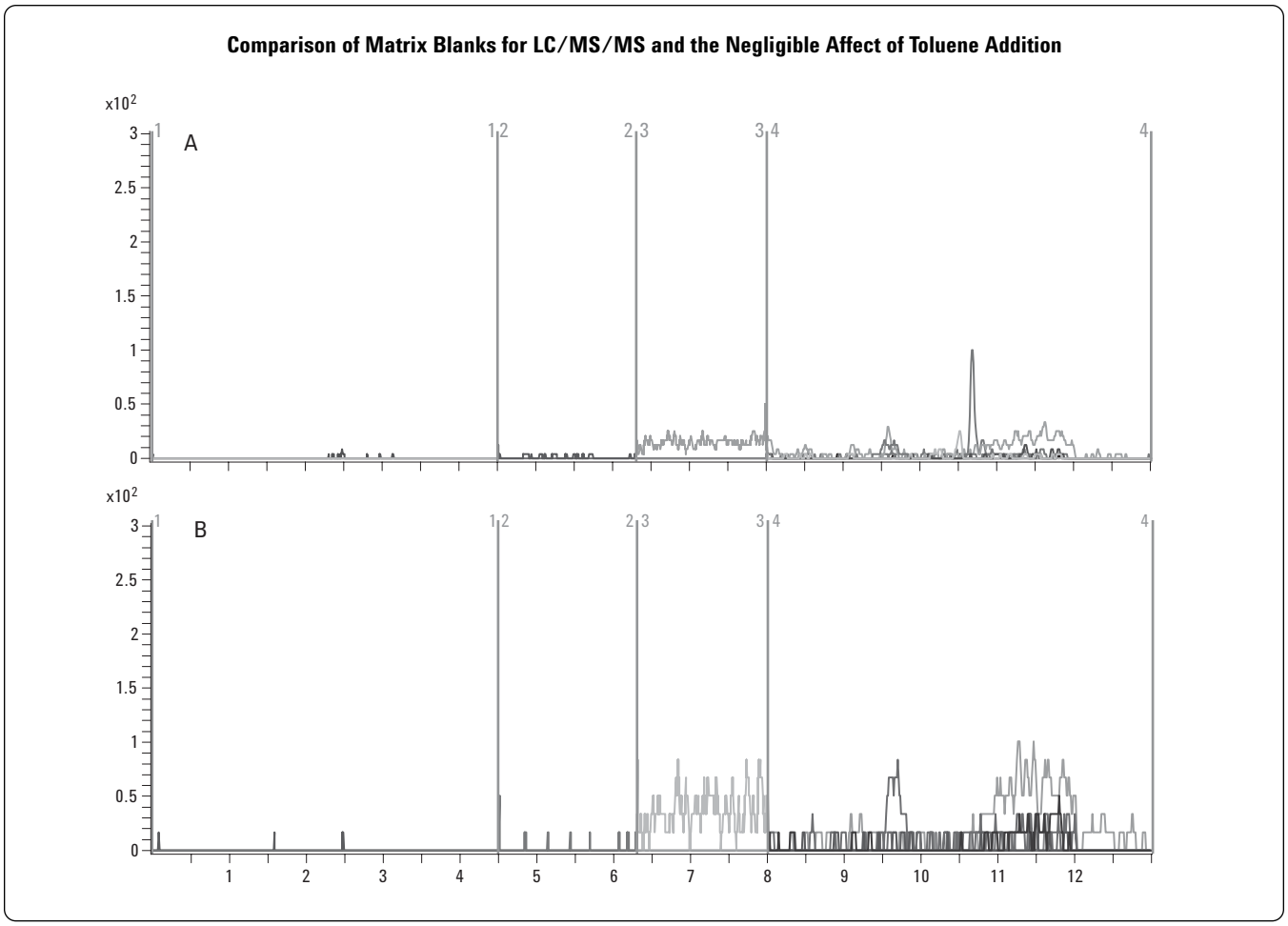


Figure 3. Spinach matrix blank LC/MS/MS chromatogram. A. Spinach matrix blank processed by modified method (w/toluene); B. Spinach matrix blank processed by original method (w/o toluene).

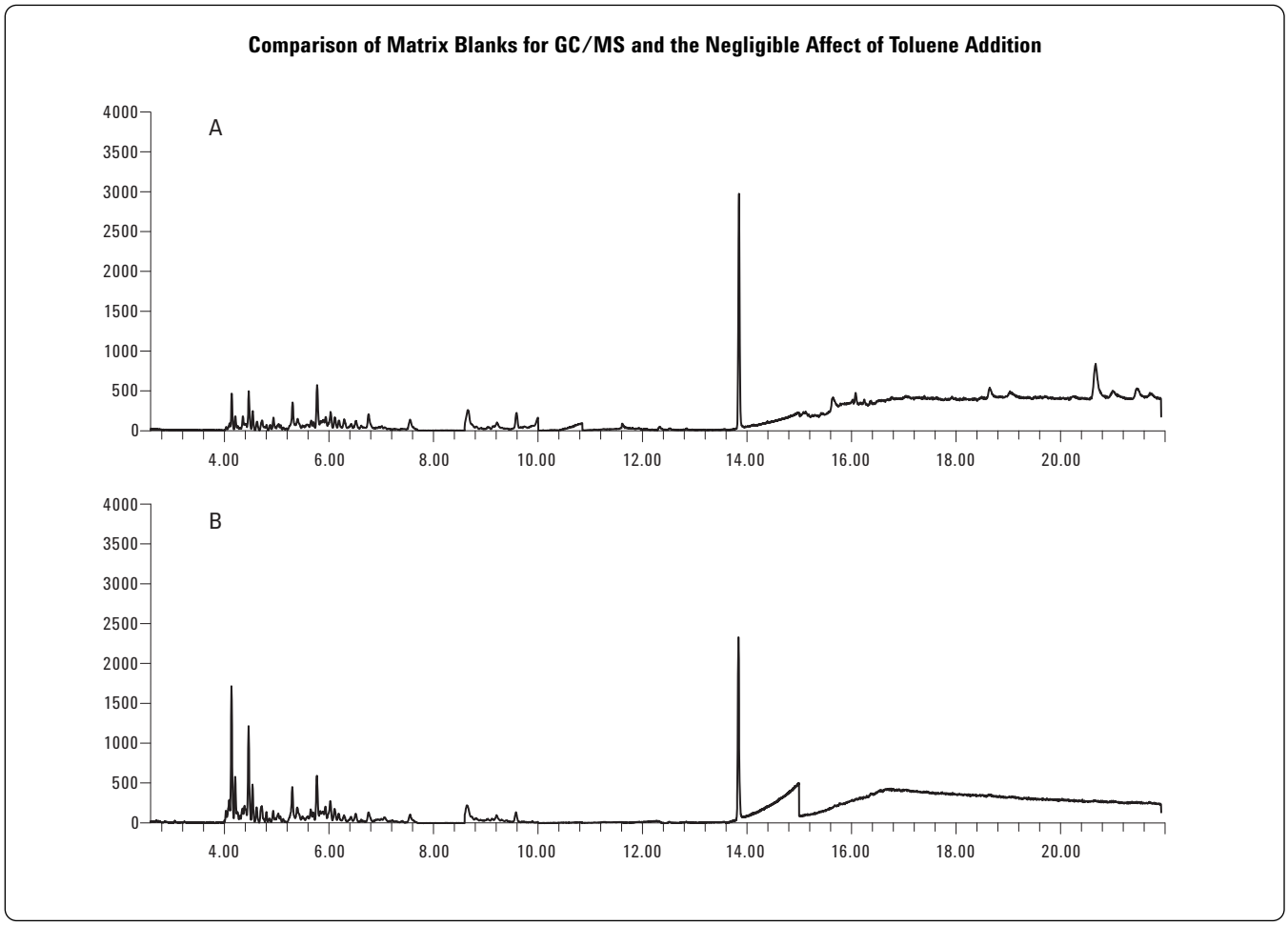


Figure 4. Spinach matrix blank GC/MS chromatograms. A. Spinach matrix blank processed by modified method (w/toluene); B. Spinach matrix blank processed by original method (w/o toluene).

Significant Improvement Made on Some Pesticides

The improvements made by the addition of toluene on certain pesticides was very significant (50% to 300% increase in recovery). Because GCB adsorbs planar compounds, the method produced very low recoveries (20% to 60%) of pesticides with planar compounds and poor precision (>14% RSD). These problematic pesticides determined by the original method included carbendazim, thiabendazole, pymetrozin, cyprodinil, chlorthalonil, coumaphous, dichlorobenzophenone, and folpet. The first four pesticides were analyzed by LC/MS/MS, and the second four pesticides by GC/MS.

The optimum volume of toluene addition was determined by parallel spinach samples spiked at the same concentration level and subjected to buffered salt extraction. An 8 mL aliquot of ACN extract was transferred into a 15 mL dispersive tube. Different volumes of toluene were added according to the following ratios: 8:1, 8:2 and 8:3 (ACN extracts/toluene, n = 3). Samples without the addition of toluene were also processed for comparison. The final samples were analyzed by LC/MS/MS, and an average of analyte responses (peak area of analyte/peak area of IS) were used for response comparison. As shown in Figure 5, the addition of toluene increased the extraction efficiency, as indicated by a 200% to 300% higher analyte response. In general, the more toluene added, the higher the responses obtained. Therefore, the addition of toluene at a ratio of 8:3 was selected for both the LC/MS/MS and GC/MS experiments. This ratio is comparable to the ratio of 3:1 ACN/toluene that Schenck recommended. [7]

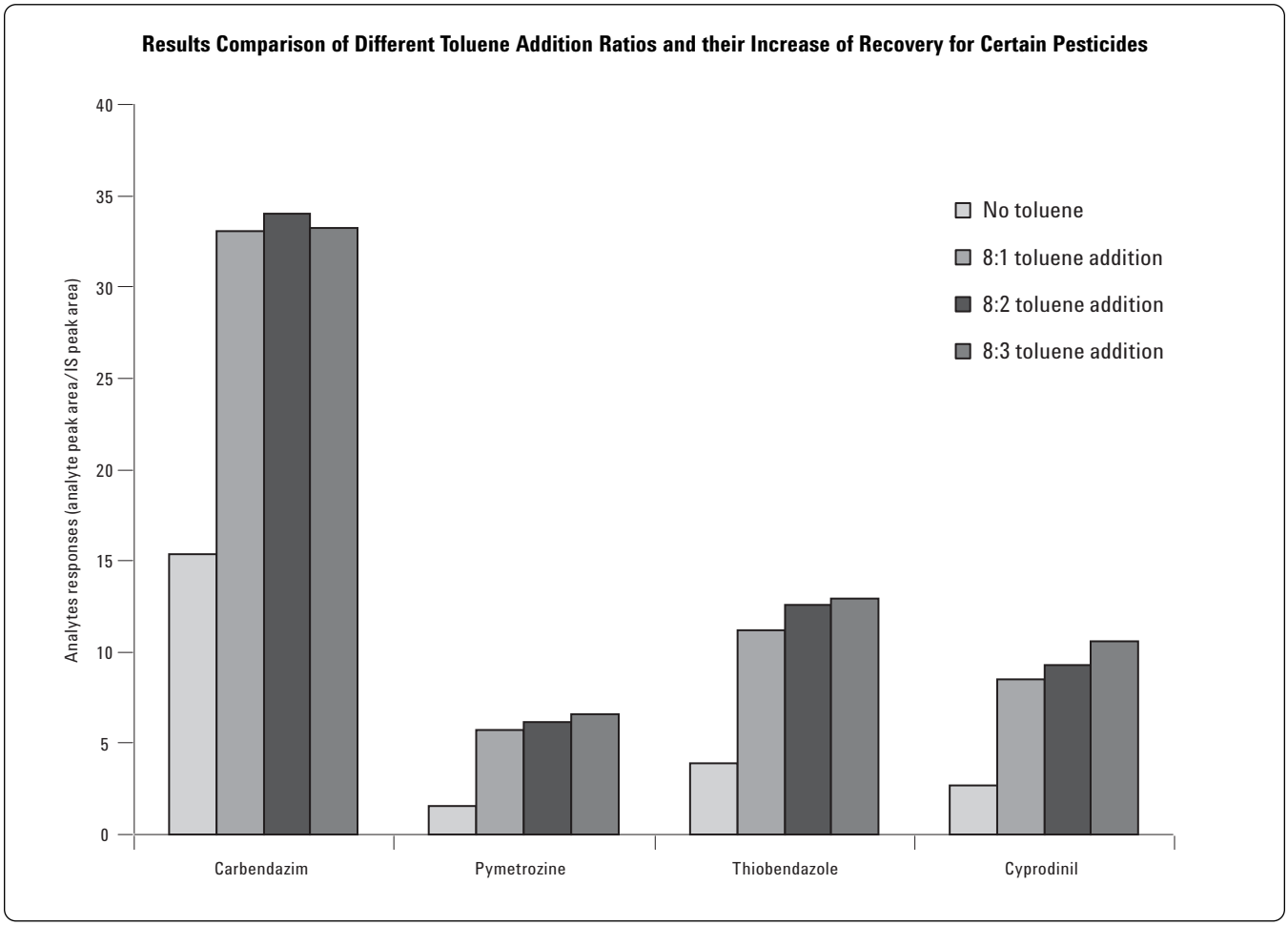


Figure 5. Results comparison of different toluene addition volumes. First column: results generated with no toluene addition; second column: results generated with toluene addition at ratio of 8:1 (ACN extracts/toluene); third column: results generated with toluene addition at ratio of 8:2; fourth column: results generated with toluene addition at ratio of 8:3.

The two different sizes of dispersive SPE (1 mL and 8 mL) were also compared for toluene addition. According to the ratio of 8:3, 3 mL of toluene were added to the 8 mL tubes; while 375 µL of toluene were added to the 1 mL tubes. The results obtained by the modified method were also compared to those from the original method. As shown in Figure 6, both dispersive SPE volumes incorporating the modified method significantly increased the recovery of the difficult pesticides by 200-300%, and gave a substantial improvement in precision. The 1 mL volume dispersive SPE provided slightly higher recovery compared to the 8 mL volume dispersive SPE, especially for pymetrozine and thiabendazole. Processing a single sample with the buffered salt extraction and partitioning step produced about 14 mL of ACN extract, which is enough to process dispersive SPE by both the original and modified methods at a 1 mL volume simultaneously. Additionally, a smaller amount of toluene was required. Therefore, the use of the1 mL volume dispersive SPE kits with the modified method is recommended for problematic pesticides. This eliminates the need for another buffered salt extraction, saving analyst time, labor and additional sample and solvents.

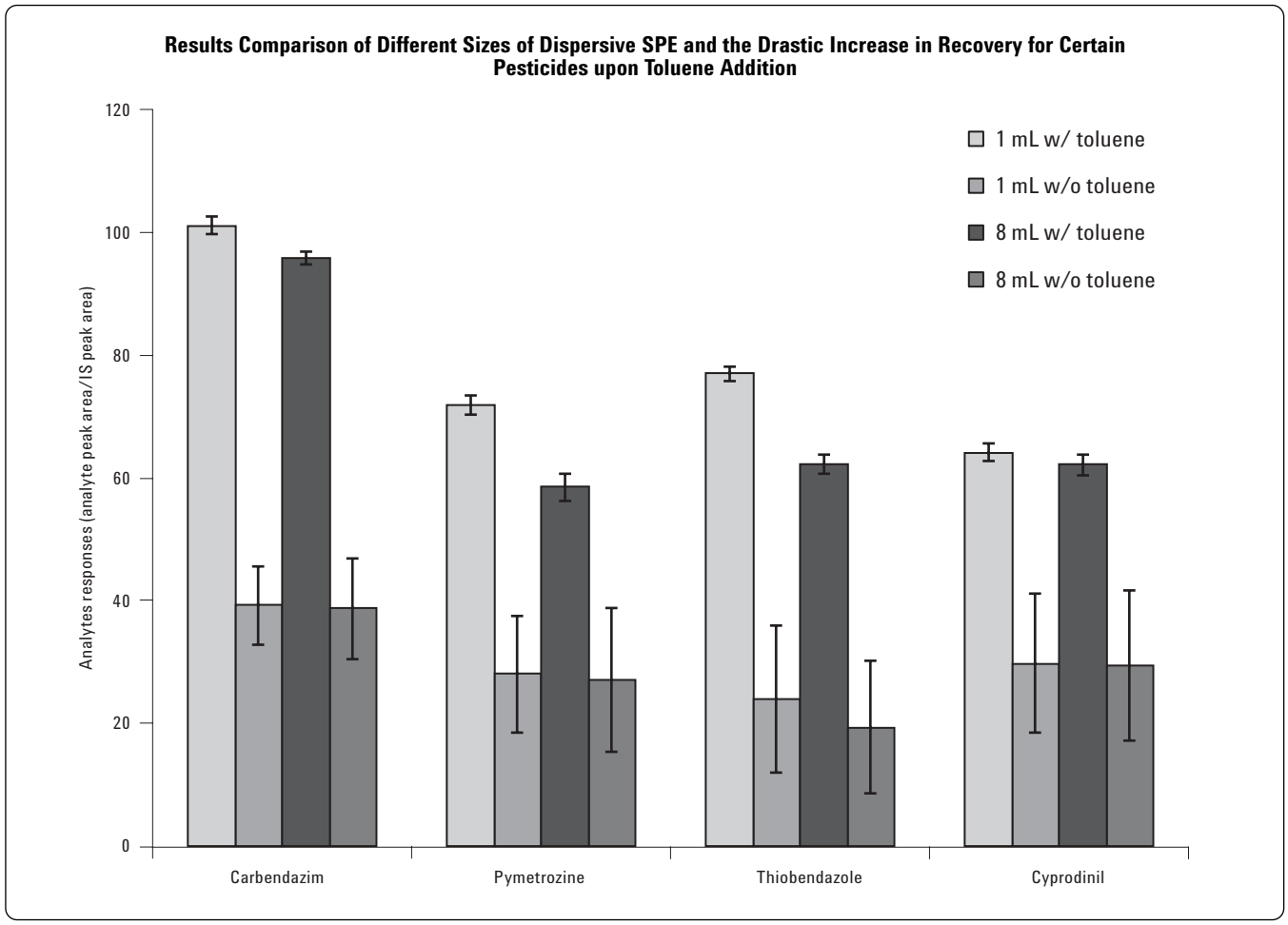


Figure 6. Results comparison of 1 mL and 8 mL dispersive SPE with the modified method (w/ toluene) and the original method (w/o toluene).

Impact on Other Pesticides

The impact of toluene addition on other pesticides was monitored and the results used to classify these pesticides into three groups. The first group of pesticides showed the same recovery and precision from both the original method and modified method. The second group of pesticides were those in which the addition of toluene generated about 10% to15% less recovery, but still showed acceptable precision. The third group included only one pesticide, dichlorvos, from the 34 pesticides screened by LC/MS/MS or GC/MS. For this pesticide, the addition of toluene adversely affected the analysis of dichlorvos producing much lower recovery and unacceptable precision. In general, these negative impacts were observed more on GC amenable pesticides than LC amenable pesticides, and may be linked to the additional drying step in the modified method.

Table 1 shows the impact the addition of toluene made on the modified dispersive SPE analysis of representative pesticides.

Table 1. The Impact on Certain Pesticides by the Modified Dispersive-SPE with Addition of Toluene

Analytes	Original method (w/o toluene)		Modified method (w/ toluene)		Impact with modified method	Detection method
	Recovery	RSD (n=6)	Recovery	RSD (n=6)		
Carbendazim	38.9	14.6	98.5	2.5	Positive	LC/MS/MS
Thiabendazole	21.8	19.7	69.7	2.7	Positive	LC/MS/MS
Pymetrozine	27.6	21.2	65.2	3.7	Positive	LC/MS/MS
Cyprodinil	29.6	23.4	63.1	3.2	Positive	LC/MS/MS
Chlorthalonil	21.1	16.4	47.3	5.9	Positive	GC/MS
Coumaphos	30.1	24.0	87.9	6.1	Positive	GC/MS
Dichlorobenzophenone	53.7	4.5	77.7	6.1	Positive	GC/MS
Folpet	62.0	14.6	88.2	6.3	Positive	GC/MS
Dichlorvos	88.8	6.0	20.4	89.8	Greatly negative	GC/MS
σ-Phenylphenol	88.6	4.6	73.7	7.4	Slightly negative	GC/MS
Diazinon	94.9	5.9	81.3	4.0	Slightly negative	GC/MS
Chlordane	103.9	4.5	101.3	4.5	None	GC/MS
Permethrin	81.4	7.2	83.3	5.1	None	GC/MS
Acephate	95.5	5.6	99.8	4.7	None	LC/MS/MS
Carbaryl	108.0	2.5	109.1	1.9	None	LC/MS/MS
Propoxur	97.0	3.1	96.7	2.5	None	LC/MS/MS

Conclusion

This application note discusses the impact of the addition of toluene on the AOAC QuEChERS method for the analysis of multiclass pesticide residues using Agilent SampliQ AOAC buffered extraction kits and SampliQ AOAC dispersive SPE kits for pigmented fruits and vegetables. The addition of toluene at a ratio of 8:3 (ACN extracts/toluene) to the dispersive SPE step can significantly increase the recovery of problematic pesticides with planar structure by 50% to 300% and improve precision. The addition of toluene can also generate some negative effects, by introducing more matrix impurities, and reducing the recovery of certain pesticides. Therefore, the modified method should not be considered a direct replacement for the original method. It does provide an option for problematic pesticides affected by GCB in the analysis of a highly pigmented matrix. The extraction will not have to be repeated from the beginning. The ACN extracts after the first buffered salt extraction step can be processed by both the original and modified AOAC methods simultaneously with Agilent SampliQ 2 mL dispersive SPE kits for pigmented matrix, saving the analyst additional sample preparation and solvent usage. By combining the results from the original and modified methods, analysts can obtain extremely impressive results and analyze a greater variety of multiclass pesticides in pigmented fruits and vegetables relative to the original method.

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Analysis of Pesticide Residues in Apple Using Agilent SampliQ QuEChERS EN Kits by GC/MS

Application Note

Food Safety

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USA

Abstract

This application note describes the use of a quick, easy, cheap, effective, rugged, and safe (QuEChERS) sample preparation approach described in the European Committee (EN) for extraction and cleanup of 17 GC-amenable multiple pesticide class residues in apple. The method involves initial extraction in an aqueous/acetonitrile system, an extraction/partitioning step after the addition of salt, and a cleanup step using dispersive solid phase extraction (dispersive SPE). The two different dispersive SPE cleanup approaches (1 mL and 6 mL aliquot volumes) were evaluated simultaneously after sample extraction. The target pesticides in the apple extracts were then analyzed by gas chromatography/mass spectrometry (GC/MS) operating in selective ion monitoring (SIM) mode. The method was validated in terms of recovery and reproducibility. The limit of quantitation (LOQ) for pesticides in apple is 10 ng/g. This application employed Agilent's SampliQ QuEChERS kit and produced results well below the maximum residue limits (MRLs) for all the pesticides screened. The spiked levels for the recovery experiments were 10, 50, and 200 ng/g. Recoveries ranged between 68 and 112% (86.0% on average), with RSD below 15% (4.7% on average).

Agilent Technologies

Introduction

The QuEChERS method for pesticide analysis was first introduced by USDA scientists in 2003. [1] The method was modified to address some problematic pesticides by including a buffered extraction system. [2] The EN method 15662:2007 is a European variation to the QuEChERS method. [3, 4] The method uses acetonitrile extraction, followed by the salting out of water from the sample using anhydrous magnesium sulfate ($MgSO_4$), NaCl and buffering citrate salts to induce liquid-liquid partitioning. A dispersive solid phase extraction (dispersive SPE) is conducted for cleanup using a combination of primary secondary amine (PSA) to remove fatty acids among other components and anhydrous $MgSO_4$ to reduce the remaining water in the extract. After mixing and centrifugation, the upper layer is ready for analysis.

Although the EN and AOAC are similar methods, they do have several differences. First, the extraction buffered system in the EN method uses sodium chloride, sodium citrate and disodium citrate sesquihidrate instead of sodium acetate in the AOAC extraction step. Second, in the dispersive SPE step, the EN method uses 25 mg PSA per mL of extract rather than 50 mg PSA per mL of extract as stated in the AOAC method.

Gas Chromatography/Mass Spectrometry (GC/MS) has been widely used in pesticide analysis for many years. Many pesticides are volatile or semi-volatile, which makes them GC-amenable compounds. Previously, we evaluated the performance of Agilent's SampliQ EN buffered extraction kit and SampliQ EN dispersive SPE kits for the analysis of polar pesticides in apple using LC/MS/MS for detection and quantification. [5] In this study, the performance of the SampliQ EN Buffered Extraction kit (p/n 5982-5650) and SampliQ EN dispersive SPE kit for General Fruits and Vegetables (p/n 5982-5021 and 5982-5056) was evaluated for the extraction of volatile and semi-volatile pesticides. Analysis was performed by GC/MS. Seventeen GC-amenable pesticides were selected which represent multiple classes, including non-polar organochlorine pesticides (OCs), certain organophosphorus pesticides (OPs) and organonitrogen pesticides (ONs). The MRLs of these pesticides are a function of both the pesticide class and food matrix and have been set at 10 ng/g or higher. Table 1 shows the chemical and regulatory information for these pesticides in apple.

Experimental

Reagents and Chemicals

All reagents and solvents were HPLC or analytical grade. Acetonitrile (ACN), methanol (MeOH) were from Honeywell (Muskegon, MI, USA). Formic acid (FA) was from Fluka (Sleinheim, Germany). The pesticide standards and internal standard (triphenyl phosphate, TPP) were purchased from Sigma-Aldrich (St Louis, MO, USA), Chem Service (West Chester, PA, USA), or Ultra Scientific (North Kingstown, RI, USA).

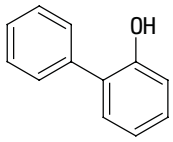
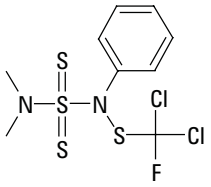
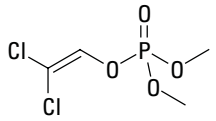
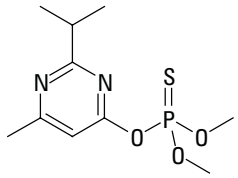
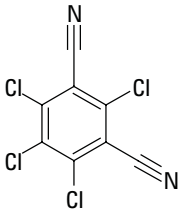
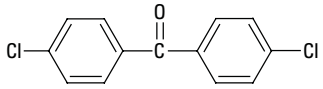
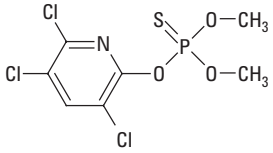
Solutions and Standards

Standards and internal standard (IS) stock solutions (2 mg/mL) were made in MeOH, respectively, and stored at –20 °C. Three QC spiking solutions of 1, 5 and 20 µg/mL were made fresh daily in 1:1 ACN/H2O (0.1% FA). A 2.5 µg/mL standard solution (17 pesticides) in ACN (0.1% FA) was used to prepare the calibration curves in the matrix blank extract by appropriate dilution. A 10 µg/mL amount of TPP spiking solution in 1:1 ACN/H_2O (0.1% FA) was used as the internal spiking standard (IS).

Equipment and Materials

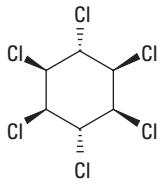
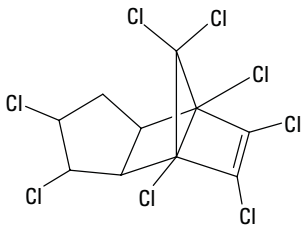
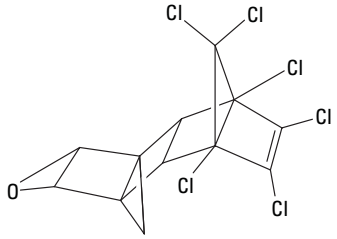
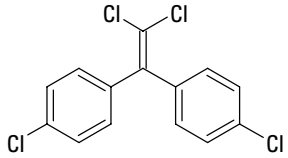
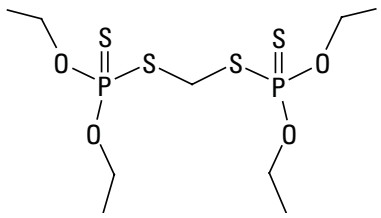
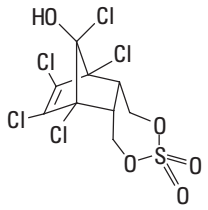
- Agilent Gas Chromatograph (Agilent Technologies Inc., Santa Clara, CA, USA).
- Agilent 5975C Mass Spectrometer (Agilent Technologies Inc., Santa Clara, CA, USA).
- SampliQ QuEChERS EN Extraction kit, p/n 5982-5650 (Agilent Technologies Inc., Wilmington, DE, USA).
- SampliQ QuEChERS EN dispersive SPE kits for General Fruits and Vegetables, p/n 5982-5021 and 5982-5056 (Agilent Technologies Inc., Wilmington, DE, USA).
- CentraCL3R Centrifuge (Thermo IEC, MA, USA)
- Bottle top dispenser (VWR, So Painfield, NJ, USA)
- Eppendorf microcentrifuge (Brinkmann Instruments, Westbury, NY, USA)
- Grinder (St Joseph, MI USA)

Table 1. Pesticides Chemical and Regulatory Information [6–9]

Name	Category	Log P	pKa	Structure	MRLs in apple (ng/g)*
σ-Phenylphenol	Phenol	3.18	9.4		20
Dichlofluanid	Sulphamide	3.7	NA		5000
Dichlorvos	Organophosphate	1.9	NA		10
Diazinon	Organophosphate	3.69	2.6		100
Chlorothalonil	Chloronitrile	2.94	NA		10
Dichlorobenzophenone	Organochlorine	4.44	NA		500
Chlorpyrifosmethyl	Organophosphate	4.00	NA		500

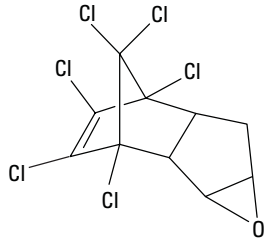
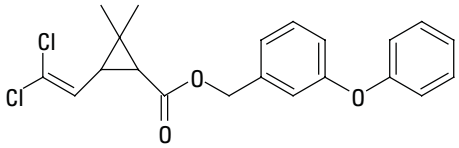
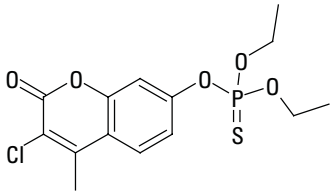
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Table 1. Pesticides Chemical and Regulatory Information [6–9]

Name	Class	Log P	pKa	Structure	MRLs in apple (ng/g)*
Lindane	Organochlorine	3.69	NA		10
Chlordane	Cyclodiene organochlorine	2.78	NA		20
Dieldrin	Chlorinated hydrocarbon	3.7	NA		10
DDE	Organochlorine	6.55	NA		50
Ethion	Organophosphate	5.07	NA		300
Endosulfan sulfate	Organochlorine	3.13	NA		50

(Continued)

Table 1. Pesticides Chemical and Regulatory Information [6–9]

Name	Class	Log P	pKa	Structure	MRLs in apple (ng/g)*
Heptachlor epoxide	Organochlorine	5.83	NA		10
Permethrins	Pyrethroid	6.1	NA		50
Coumaphos	Organothiophosphate	3.86	NA		100

*The MRLs numbers list in the table are for apple or lowest level in other fruit and vegetables. They could be higher in different commodities.

Instrument Condition

An Agilent GC/MS method for pesticides analysis was used for this study. [10]

GC conditions

Inlet	Splitless
Inlet liner	Helix double taper, deactivated (p/n 5188-5398)
Carrier gas	Helium
Inlet pressure	20.18 psi (constant pressure mode) during run 1.0 psi during backflush
Inlet temperature	250 °C
Injection volume	1.0 µL
Purge flow to split vent	30 mL/min at 0.75 min
Oven temperature program	70 °C (1 min), 50 °C/min to 150 °C (0 min), 6 °C /min to 200 °C (0 min), 16 °C/min to 280 °C (6 min)
Post run	3 min
Capillary flow technology	Purged Ultimate Union (p/n G3186B) – used for backflushing the analytical column and inlet.
Aux EPC gas	Helium plumbed to Purged Ultimate Union
Aux EPC pressure	4.0 psi during run, 80.0 psi during backflush
Column	Agilent J&W HP-5ms Ultra Inert 15 m × 0.25 mm × 0.25 µm (p/n 19091S-431UI)
Connections	Between inlet and Purged Ultimate Union (p/n G3186B)
Restrictor	65 cm × 0.15 mm × 0.15 µm DB-5MS Ultra Inert.
Connections	Between the Purged Ultimate Union and the MSD

MS conditions

Tune file	Atune.u
Mode	SIM (refer to Table 2 for settings in detail)
Source, quad, transfer line temperatures	230 °C, 150 °C and 280 °C respectively,
Solvent delay	2.30 min
Multiplier voltage	Autotune voltage

Table 2. Instrument Acquisition Data Used for the Analysis of 17 Pesticides by GC/MS.

Analyte	SIM	Collection window (min)	RT (min)
(1) Dichlorvos	184.9	2.3 – 4.0	2.88
(2) σ-Phenylphenol	170.1, 169.1	4.0 – 5.0	4.35
(3) Lindane	180.9, 182.9	5.0 – 6.9	6.67
(4) Diazinon	137.1, 179.1	6.9 – 7.7	7.19
(5) Chlorothalonil	265.9, 263.9	14.65 – 16.0	14.8
(6) Chlorpyrifos-methyl	285.9, 287.9	7.7 – 8.6	8.25
(7) Dichlofluanid	123, 167.0	8.6 – 9.35	9.16
(8) Dichlorobenzophenone	139, 249.9	18.8 – 20.5	19.2
(9) Heptachlor epoxide	352.8, 354.8	10.0 – 10.4	10.31
(10) γ-Chlordane	372.8, 374.8	10.85 – 11.6	10.97
(11) DDE	245.9, 317.9	10.85 – 11.6	11.21
(12) α-Chlordane	372.374.8	10.85 – 11.6	11.50
(13) Dieldrin	262.9, 264.9	11.0 – 12.3	11.89
(14) Ethion	230.9	12.3 – 13.6	12.97
(15) Endosulfan sulfate	273.8	12.3 – 13.6	13.35
TPP (IS)	325.1, 326.1	13.6 – 15.0	13.84
(16) Permethrin	183.1	15.0 – 23.0	15.69, 15.79
(17) Coumaphos	362.0, 225.9	15.0 – 23.0	15.83

Sample preparation

Sample comminution

Organically grown, pesticide free apples were purchased from a local grocery store. Approximately three pounds of apples were chopped into small, bean sized cubes. Skin was included, but the core was discarded. The chopped apple cubes were then placed into a clean plastic bag and frozen at –20 °C overnight. The bag was massaged occasionally to make sure the cubes remained separate. The following day, only the required amount of frozen apple cubes was removed and thoroughly blended. Dry ice was added while comminuting, when possible. Samples were comminuted thoroughly to get the best sample homogeneity. It was verified that no pieces of apple were visible in the final sample.

Extraction/Partitioning

A 10 g (± 0.1 g) amount of previously homogenized sample was placed into a 50 mL centrifuge tube. QC samples were fortified with 100 µL of appropriate QC spiking solution. 100 µL of IS spiking solution (10 µg/mL of TPP) was added to all the samples except the control blank to yield a 100 ng/g concentration in the samples. Tubes were capped and vortexed for 1 min. A 10 mL aliquot of ACN was added to each tube using the dispenser. Tubes were capped and shaken by hand for 1 min. An Agilent SampliQ QuEChERS EN extraction salt packet (p/n 5982-5650), containing 4 g anhydrous $MgSO_4$, 1 g NaCl, 1 g Na_3Citrate, and 0.5 g Na_2H Citrate sesquihydrate, was added directly to each tube. The salt bag was massaged carefully to loosen any clumped salts before pouring. No powders were left in the threads or rims of the tubes. Tubes were sealed tightly and shaken vigorously for 1 min by hand to ensure that the solvent interacted well with the entire sample and crystalline agglomerates were broken up sufficiently. Sample pH was checked and 5M NaOH solution was used to adjust the pH to 5–5.5, if necessary. Sample tubes were centrifuged at 4000 rpm for 5 min.

Dispersive SPE Cleanup

A 1 mL aliquot of upper ACN layer was transferred into Agilent SampliQ QuEChERS EN dispersive SPE 2 mL tube (p/n 5982-5021); or a 6 mL of aliquot was transferred into Agilent SampliQ QuEChERS EN dispersive SPE 15 mL tube (p/n 5982-5056). The 2 mL tube contained 25 mg of PSA and 150 mg of anhydrous $MgSO_4$; while the 15 mL tube contained 150 mg of PSA and 900 mg of anhydrous $MgSO_4$. The tubes were capped tightly and vortexed for 1 min. The 2 mL tubes were centrifuged with a micro-centrifuge at 13,000 rpm for 2 min, and the 15 mL tubes in a standard centrifuge at 4000 rpm for 5 min. A 500 µL portion of the extract was transferred into an autosampler vial and 25 µL of 1% FA in ACN was added immediately.

Figure 1 shows the flow chart for the QuEChERS EN sample extraction procedure.

Results and Discussion

Using the SampliQ QuEChERS kits, the entire procedure is fast, easy, and offers time and labor savings, while ensuring consistency. An analyst can process 40-50 samples in just a few hours. Agilent's SampliQ extraction salts are uniquely prepared in an anhydrous package. The addition of a food sample with a high content of water directly to the salts creates an exothermic reaction, which can affect analyte recoveries, especially for volatile pesticides. The unique SampliQ anhydrous salts packet allows salt addition **AFTER** the addition of organic solvent to the sample, as specified in the original QuEChERS methodology.

Our previous study demonstrated good performance of Agilent's SampliQ QuEChERS EN kits on the extraction of a broad variety of semi-polar to polar pesticides analyzed by LC/MS/MS. [5] It is also advantageous to evaluate the performance of the EN kit for the analysis of volatile and semi-volatile pesticides using GC/MS, since these classes of pesticides have been widely used for many years. The selectivity of GC/MS (SIM mode) is not as powerful as that of LC/MS/MS (MRM mode). Furthermore, the final QuEChERS prepared samples still contain some food matrix impurities, which can be observed in the GC/MS chromatogram of blank apple extract. Therefore, it is critical to carefully choose the selected ions of each compound for monitoring when setting up the SIM method. In general, the most abundant ions were selected in order to achieve the best sensitivity; however in a few instances the sensitivity was compromised to obtain better selectivity by using more unique but less abundant ions.

Another potential issue with the use of GC/MS for the analysis of QuEChERS samples is the contamination of the ionization source and deterioration of the GC column. QuEChERS food samples usually still contain high-boiling indigenous impurities, which can accumulate on the head of the column, causing peak tailing and retention time shift. Over time, these impurities can migrate to the mass spectrometer (MS) source, causing contamination of the source. Decreased sensitivity and peak shape distortion, especially for the semi-polar compounds, were observed when additional QuEChERS samples were injected into the GC/MS system. Therefore, column backflushing was employed to increase column life as well as preserve the MS source. Agilent's capillary flow technology makes column backflushing routine [10–12]. Several different capillary flow devices can be used for this purpose.

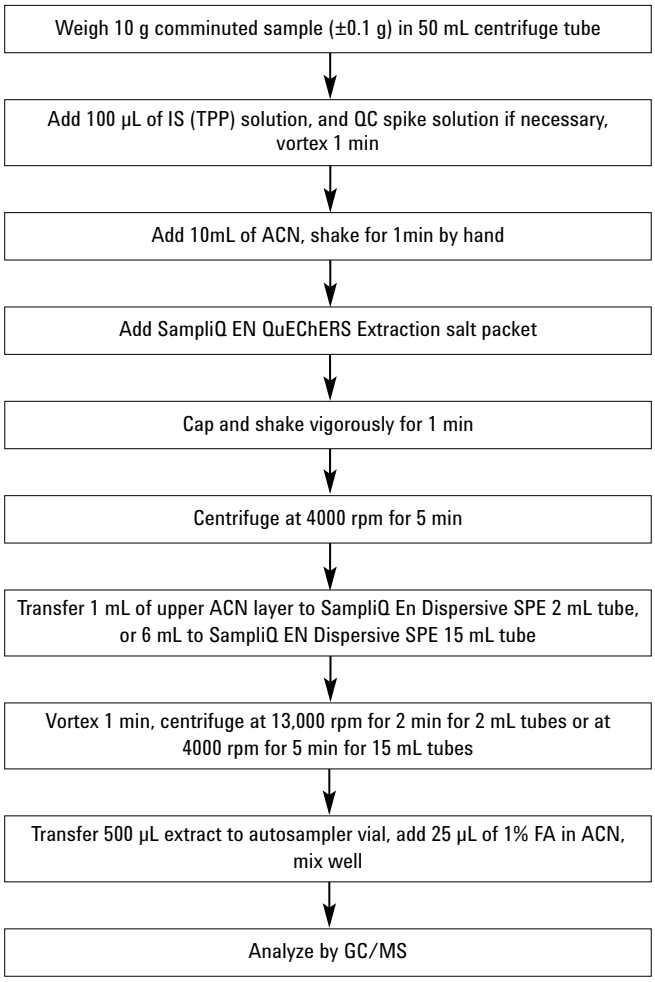


Figure 1. Flow chart of the Agilent SampliQ QuEChERS EN extraction procedure.

In this study, the GC/MS system used a Purged Ultimate Union. The analytical column was connected to the capillary flow device. A short restrictor (65 cm × 0.15 mm × 0.15 µm of DB-5ms Ultra Inert column) was used to couple the capillary flow device to the mass spectrometer. In a previous application note [10], there are figures showing the backflush system, that was used in this study.

Figure 2(a, b) shows the chromatograms of a blank apple extract and a 50 ng/g fortified apple extract. As shown in Figure 2a, interference peaks are found in the blank chromatogram; fortunately most pesticides are free of co-eluting interferences. There was an interference eluting at a retention time very close to that of σ-phenylphenol (peak 2), and cannot be differentiated for integration. The average response of this interference in the blank extract was 215 (n=4), while the average response of α-phenylphenol in the LOQ (10 ng/g)

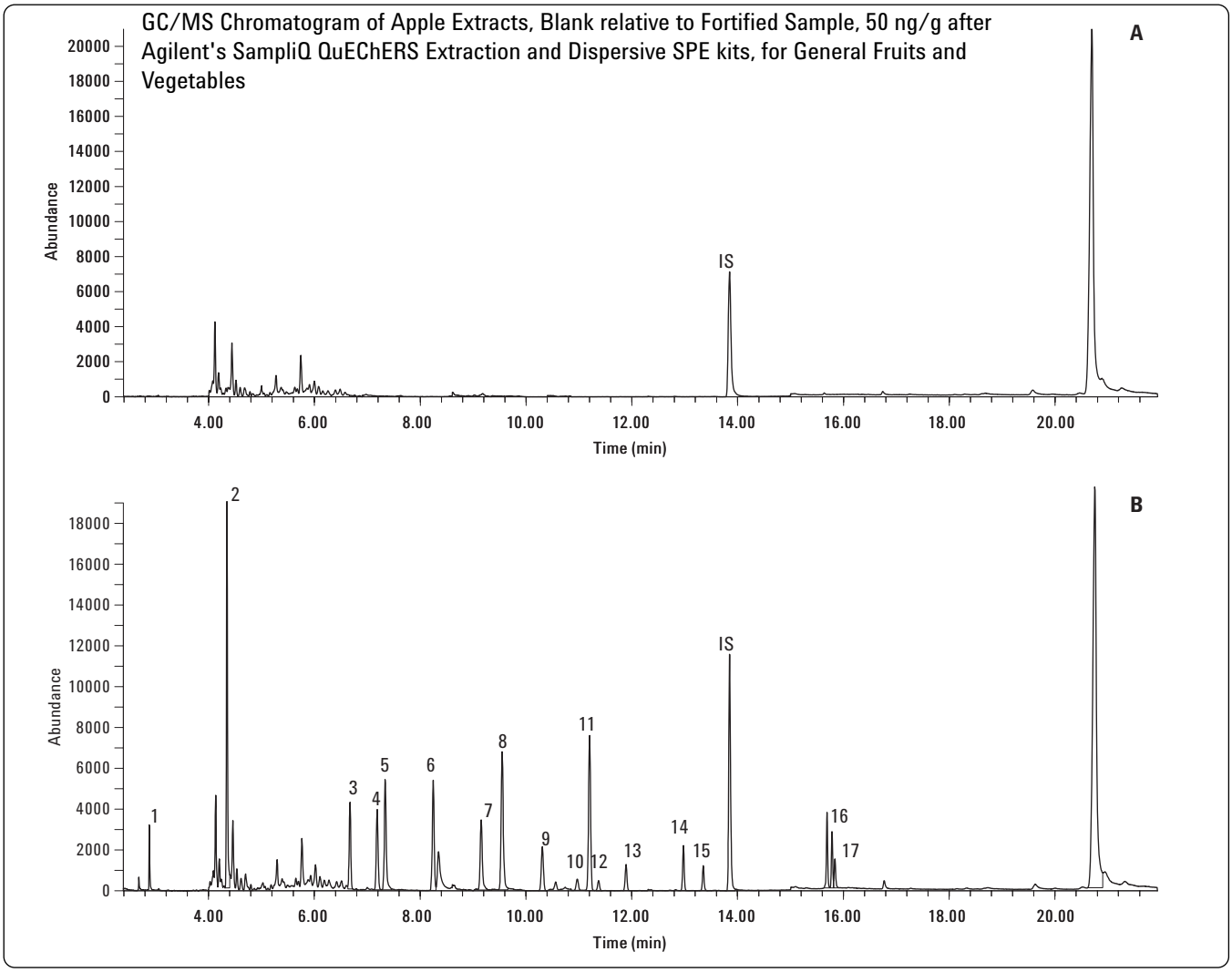


Figure 2. GC/MS chromatogram of apple extract. (A) apple extract blank; (B) 50ng/g fortified apple extract. Peak Identification: 1. Dichlorvos, 2. σ-Phenylphenol, 3. Lindane, 4. Diazinon, 5. Chlorothalonil, 6. Chlorpyrifos-methyl, 7. Dichlofluanid, 8. Dichlorobenzophenone, 9. Heptachlor epoxide, 10. γ-Chlordane, 11. DDE, 12. α-Chlordane, 13. Dieldrin, 14. Ethion, 15. Endosulfan sulfate, 16, Permethrin, 17. Coumaphos. IS. Triphenyl phosphate (TPP)

was 3196 (n=12). The interference response was less than 20% of the response of the σ-phenylphenol peak at the LOQ (10 ng/g) sample. Therefore the selectivity was considered acceptable for this compound.

Linearity and Limit of Quantification (LOQ)

The linear calibration range for all of the pesticides was 0–400 ng/g. Two different dispersive SPE volumes (1 mL and 6 mL) were used for evaluation and comparison; therefore, two calibration curves were generated from matrix blanks prepared from each size. Calibration curves were made at levels of 10, 20, 50, 100, 250, and 400 ng/g. The TPP was the internal standard (IS) at 100 ng/g in all cases. The calibration curves were generated by plotting the relative responses of analytes (peak area of analyte/peak area of IS) to the relative concentration of analytes (concentration of analyte/concentration of IS). Table 1 shows that the 10 ng/g quantification limits LOQ (10 ng/g or 10 ppb) established for the pesticides are substantially lower than many MRLs for the pesticides in fruit and vegetables. The regression fit used for the calibration curves was the average response factor. Table 3 shows the linear term and RF relative standard deviation (%) for both 1 mL and 6 mL dispersive SPE samples. The RF relative SD is an important parameter for the evaluation of the linearity of calibration. In general, the smaller the value the better linearity of the curve, and it is usually acceptable for less than

Table 3. Linearity of 17 Pesticides in Apple Extract

	1 mL dispersive SPE		6 mL dispersive SPE	
Analytes	**Linear Term**	**RF Rel Std Dev (%)**	**Linear Term**	**RF Rel Std Dev (%)**
Dichlorvos	4.53e–001	7.9	5.52e–001	8.6
σ-Phenylphenol	2.41e+000	7.5	2.82e+000	9.3
Lindane	6.79e–001	11.5	8.09e–001	9.2
Diazinon	8.35e–001	15.0	9.32e–001	13.6
Chlorothalonil	1.39e+000	14.1	1.69e+000	14.1
Chlorpyrifos-mehyl	1.32e+000	14.7	1.31e+000	16.5
Dichlofluanid	1.03e+000	11.7	1.29e+000	12.9
Dichlorobenzophenone	6.08e–001	10.0	7.13e–001	10.4
Heptachlor epoxide	5.41e–001	12.4	5.58e–001	12.3
γ-Chlordane	1.77e–001	9.3	1.83e–001	9.1
DDE	2.44e+000	10.7	2.67e+000	9.5
α-Chlordane	1.34e–001	10.0	1.38e–001	9.4
Dieldrin	2.85e–001	9.8	3.09e–001	6.9
Ethion	7.06e–001	27.8	7.30e–001	27.9
Endosulfan sulfate	2.95e–001	11.2	3.29e–001	11.5
Permethrin	8.73e–001	11.8	8.20e–001	17.6
Coumaphos	2.36e–001	19.0	2.16e–001	28.7

20% RF relative SD. The data in Table 3 shows that the linearity of the calibration curve for most analytes is perfect with less than a 15% of RF relative SD value. However, the two standard curves of Ethion (1 mL and 6 mL) and one standard curve of Coumaphos generated RF relative SD values higher than 20%, possibly due to the matrix effect.

Recovery and Reproducibility

The recovery and reproducibility were evaluated by spiking pesticides standards in comminuted apple sample at levels of 10, 50 and 200 ng/g. These QC samples were quantitated against the matrix spiked calibration curve. The analysis was performed in replicates of six (n=6) at each level. The recovery and reproducibility (shown as % RSD) data for 1 mL and 6 mL dispersive SPE sample are shown in Table 4 and 5, respectively. It can be seen from the results that all of the pesticides give good recoveries (average of 84.7% for 1 mL and 87.2% for 6 mL) and precision (average of 4.3% RSD for 1 mL and 5.1% RSD for 6 mL). Compared to the results of these pesticides extracted with AOAC QuEChERS method [13], the EN QuEChERS method gives slightly lower recovery (recovery 5–6% lower on average) but similar precision (RSD 4–5% on average for both methods). Variance may be possible due to a different buffering system and solvent volume used in the first extraction step.

Table 4. Recovery and Repeatability of Pesticides in Fortified Apple With Agilent's SampliQ Dispersive SPE Tube, 2 mL (p/n 5982-5021); Recovery 84.7%, RSD 4.3% (avg)

	10 ng/g fortified QC		50 ng/g fortified QC		200 ng/g fortified QC	
Analytes	**Recovery**	**RSD (n=6)**	**Recovery**	**RSD (n=6)**	**Recovery**	**RSD (n=6)**
Dichlorvos	97.6	5.1	90.8	6.2	81.0	6.9
σ-Phenylphenol	94.4	5.5	83.1	6.6	76.3	5.2
Lindane	87.4	4.9	80.0	6.1	73.3	3.8
Diazinon	83.6	5.6	79.6	5.5	69.6	5.0
Chlorothalonil	68.3	4.9	71.8	5.8	69.6	5.0
Chlorpyrifos-mehyl	79.3	4.5	80.7	4.8	83.1	3.0
Dichlofluanid	91.8	5.6	85.8	6.9	65.2	4.7
Dichlorobenzo phenone	83.9	6.4	83.0	4.8	80.0	3.1
Heptachlor epoxide	80.0	4.7	82.9	5.0	81.4	1.7
γ-Chlordane	79.6	4.3	80.5	5.4	78.3	1.6
DDE	80.5	3.2	80.3	5.1	76.8	1.2
α-Chlordane	84.8	3.3	83.1	4.7	78.6	1.3
Dieldrin	83.4	3.1	80.5	4.3	76.2	1.1
Ethion	97.7	4.4	104.9	4.8	91.7	1.4
Endosulfan sulfate	93.2	5.4	88.5	4.5	87.9	1.1
Permethrin	88.8	6.2	93.9	4.6	104.3	0.7
Coumaphos	101.4	4.7	111.9	3.9	111.2	1.6

Table 5. Recovery and Repeatability of Pesticides in Fortified Apple With Agilent's SampliQ Dispersive SPE Tube, 15 ML (p/n 5982-5056), Recovery 87.2%, RSD 5.1% (avg)

	10 ng/g fortified QC		50 ng/g fortified QC		200 ng/g fortified QC	
Analytes	**Recovery**	**RSD (n=6)**	**Recovery**	**RSD (n=6)**	**Recovery**	**RSD (n=6)**
Dichlorvos	99.4	8.2	90.9	2.6	85.7	4.4
σ-Phenylphenol	76.9	8.9	81.6	1.6	82.0	3.6
Lindane	87.8	7.0	88.9	2.7	86.3	2.7
Diazinon	87.0	8.3	86.6	1.8	89.3	2.7
Chlorothalonil	71.7	11.1	77.9	1.8	75.9	3.8
Chlorpyrifos-mehyl	77.7	9.8	82.7	2.3	86.7	2.2
Dichlofluanid	80.0	7.8	86.5	6.1	76.6	5.1
Dichlorobenzo phenone	86.2	6.1	87.6	2.4	85.7	1.5
Heptachlor epoxide	82.6	5.7	86.7	2.8	85.9	1.9
γ-Chlordane	89.6	7.1	85.1	2.9	83.6	2.1
DDE	91.9	5.5	88.7	3.5	83.8	1.8
α-Chlordane	90.3	4.4	88.0	3.1	84.0	1.3
Dieldrin	93.6	7.3	88.1	4.3	83.2	1.6
Ethion	81.0	6.7	94.2	4.0	91.1	1.7
Endosulfan sulfate	96.4	5.3	91.2	3.9	89.8	1.2
Permethrin	89.3	5.6	95.5	3.9	108.9	1.3
Coumaphos	89.0	10.8	90.7	6.6	97.1	2.0

Figure 3 shows the recovery and precision results for 1 mL dispersive SPE and 6 mL dispersive SPE. The two different dispersive SPE clean-ups were performed by transferring 1 mL or 6 mL of ACN extract from the same sample following the extraction step. In order to simplify the comparison, the average recovery and precision of three fortification concentrations were used for all of the pesticides. The results of each dispersive SPE clean-up appeared to be independent of volume used. Both approaches provided similar efficient sample clean-up and generated relatively equivalent results.

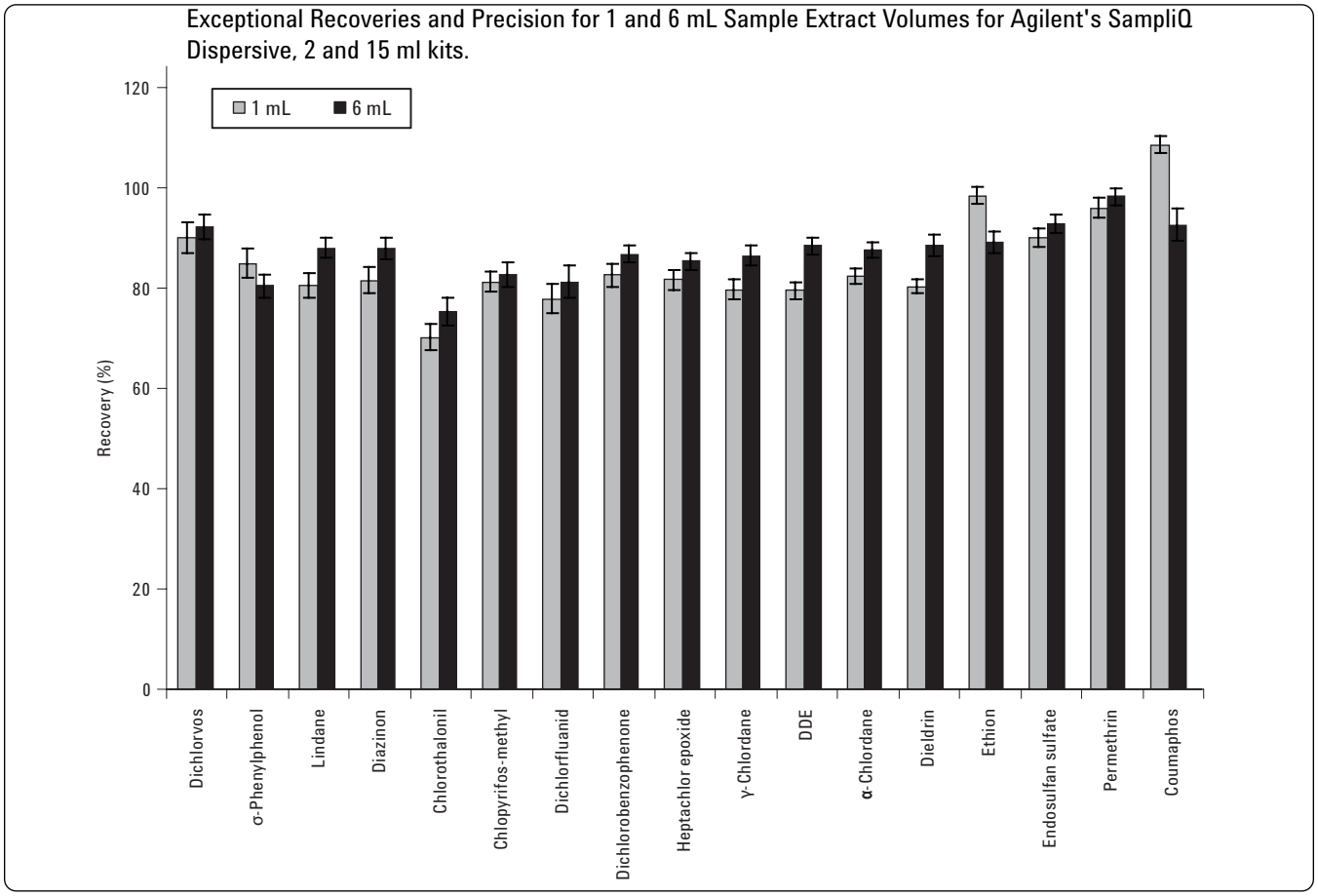


Figure 3. The recovery and precision results of 1 and 6 mL sample volumes employing Agilent's SampliQ Dispersive SPE, 2 and 15 mL kits, respectively.

Conclusions

Agilent's SampliQ QuEChERS EN Extraction and Dispersive SPE kits for General Fruits and Vegetables provide a simple, fast and effective method for the purification and enrichment of representative volatile to semi-volatile pesticides in apple. The recovery and reproducibility, based on matrix spiked standards, were acceptable for multi-class, multi-residue pesticide determination in apple. The impurities and matrix effects from apple did not interfere with the quantitation of target compounds. The LOQs of the pesticides were lower than regulated MRLs in apple. Since the selected pesticides represented a broad variety of different classes and properties, the Agilent SampliQ QuEChERS EN Extraction and Dispersive SPE kits for General Fruits and Vegetables is an excellent choice for other pesticides in similar food matricies

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Agilent Technologies

Analysis of Pesticide Residues in Apple Using Agilent SampliQ QuEChERS AOAC Kits by GC/MS

Application Note

Food Safety

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Abstract

This application note describes the use of a quick, easy, cheap, effective, rugged, and safe (QuEChERS) AOAC sample preparation approach for extraction and cleanup of 17 GC-amenable pesticide residues from multiple classes, in apple. The method employed involves initial extraction in a buffered aqueous/acetonitrile system, an extraction/ partitioning step after the addition of salt, and a cleanup step utilizing dispersive solid phase extraction (dispersive SPE). The two different dispersive SPE clean-up approaches used either a 1 mL or 8 mL sample volume and were evaluated in parallel after sample extraction. The target pesticides in the apple extracts were then analyzed by gas chromatography/mass spectrometry (GC/MS) operating in selective ion monitoring (SIM) mode. The method was validated in terms of recovery and reproducibility. The limit of quantitation (LOQ) for most pesticides is 10 ng/g; however, the pesticide Folpet has an LOQ of 50 ng/g in apple. This application employing SampliQ QuEChERS kits produced results well below the maximum residue limits (MRLs) for all the pesticides screened. The spiked levels for the recovery experiments were 10, 50, and 200 ng/g. Recoveries ranged between 70 and 136% (92.5% on average), with RSD below 15% (5.0% on average).

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Introduction

The QuEChERS method for pesticide analysis was first introduced by USDA scientists in 2003. [1] The method was modified to address problematic pesticides by including a buffered extraction system [2]. After a full validation for more than 200 pesticides, this improved method was formalized and adopted as AOAC Official Method 2007.01. [3] In summary, the method uses a single-step buffered acetonitrile (1% HAc) extraction while simultaneously salting out water from the sample using anhydrous magnesium sulfate ($MgSO_4$) to induce liquid-liquid partitioning. For cleanup, a dispersive solid phase extraction (dispersive SPE) step is employed using a combination of primary secondary amine (PSA) to remove fatty acids as well as other components, and anhydrous $MgSO_4$ to reduce the remaining water in the extract. After mixing and centrifugation, the upper layer is ready for analysis.

Gas Chromatography/Mass Spectrometry (GC/MS) has been widely used in pesticide analysis for many years, because many pesticides are volatile or semi-volatile they are GC-amenable. Previously, we evaluated the performance of a SampliQ AOAC buffered extraction kit and SampliQ AOAC dispersive SPE kits for the analysis of polar pesticides in apple using LC/MS/MS for detection and quantification. [4] In this study, the performance of the SampliQ AOAC Buffered Extraction kit (PN 5982-5755) and SampliQ AOAC dispersive-SPE kits for General Fruits and Vegetables (p/n 5982-5022 and 5982-5058) were evaluated for the extraction of volatile and semi-volatile pesticides. Analysis was performed by GC/MS. Seventeen GC-amenable pesticides were selected which represent multiple classes, including non-polar organochlorine pesticides (OCs), certain organophosphorus pesticides (OPs) and organonitrogen pesticides (ONs). The MRLs of these pesticides are a function of both the pesticide class and food matrix and have been set at 10 ng/g or higher. Table 1 shows the chemical and regulatory information for these pesticides in apple.

Experimental

Reagents and Chemicals

All reagents and solvents were HPLC or analytical grade. Acetonitrile (ACN), and methanol (MeOH) were from Honeywell (Muskegon, MI, USA), and acetic acid (HAc) was from Sigma-Aldrich (St Louis, MO, USA). Formic acid (FA) was from Fluka (Sleinheim, Germany). The pesticide standards and internal standard (triphenyl phosphate, TPP) were purchased from Sigma-Aldrich (St Louis, MO, USA), Chem Service (West Chester, PA, USA), or Ultra Scientific (North Kingstown, RI, USA).

Solutions and Standards

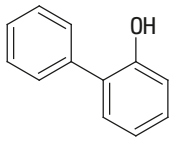
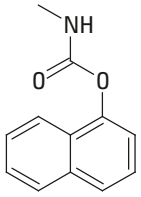
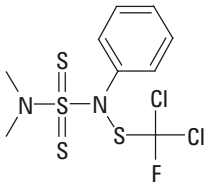
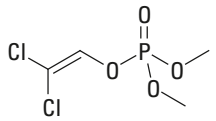
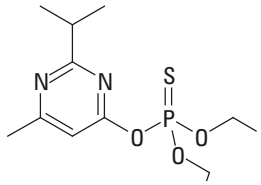
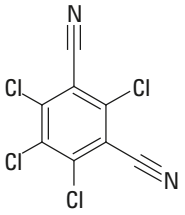
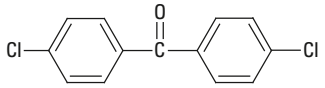
A 1% acetic acid solution in ACN was prepared by adding 10 mL of acetic acid to 1 L of ACN.

Standard and internal standard (IS) stock solutions (2 mg/mL of 11 pesticides) were made in MeOH, respectively, and stored at –20 °C. A commercially available mix of 6 pesticides, at 20 µg/mL in hexane was used directly. Three QC spiking solutions of 11 pesticides at 1.5, 7.5 and 30 µg/mL were made fresh daily in 1:1 ACN/H_2O containing 0.1% FA, while the 20 µg/mL of 6 pesticides mix was directly used for QC spike. A 2.5 µg/mL standard solution of 17 pesticides in ACN containing 0.1% FA was used to prepare the calibration curves in the matrix blank extract by appropriate dilution. A 15 µg/mL of TPP spiking solution in 1:1 ACN/H_2O containing 0.1% FA was used as the internal spiking standard (IS).

Equipment and Material

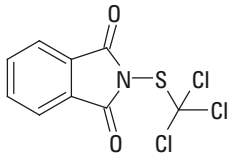
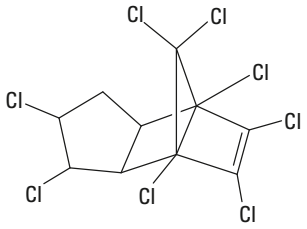
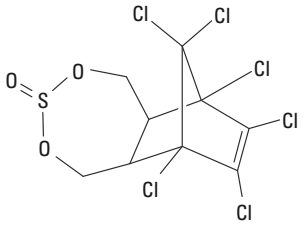
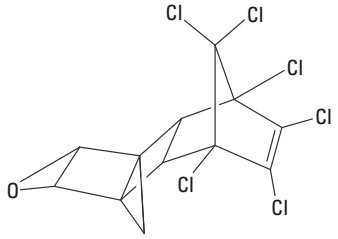
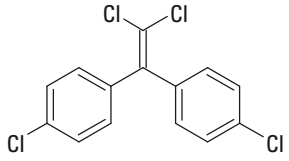
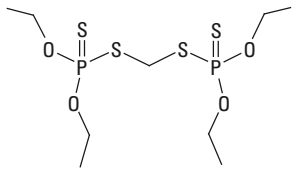
- Agilent Gas Chromatograph (Agilent Technologies Inc., Santa Clara, CA, USA).
- Agilent 5975C Series GC/MSD (Agilent Technologies Inc., Santa Clara, CA, USA).
- Agilent SampliQ QuEChERS AOAC Extraction kits, p/n 5982-5755 (Agilent Technologies Inc., Wilmington, DE, USA).
- Agilent SampliQ QuEChERS AOAC dispersive SPE kits for General Fruits and Vegetables, p/n 5982-5022 and 5982-5058 (Agilent Technologies Inc., Wilmington, DE, USA).
- CentraCL3R Centrifuge (Thermo IEC, MA, USA)
- Bottle top dispenser (VWR, So Painfield, NJ, USA)
- Eppendorf microcentrifuge (Brinkmann Instruments, Westbury, NY, USA)

Table 1. Pesticides Chemical and Regulatory Information [5–8]

Name	Category	Log P	pKa	Structure	MRLs in apple (ng/g)*
σ-Phenylphenol	Phenol	3.18	9.4		20
Carbaryl	Carbamate	2.36	10.4		50
Dichlofluanid	Sulphamide	3.7	NA		5000
Dichlorvos	Organophosphate	1.9	NA		10
Diazinon	Organophosphate	3.69	2.6		100
Chlorothalonil	Chloronitrile	2.94	NA		10
Dichlorobenzo phenone	Organochlorine	4.44	NA		500

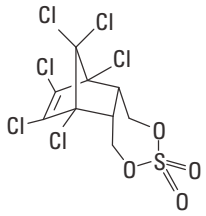
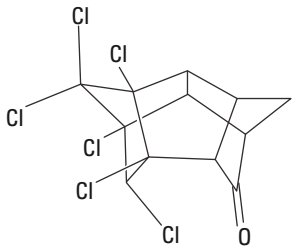
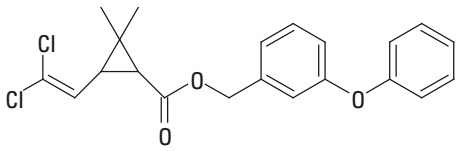
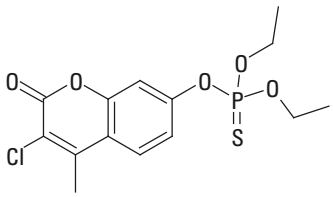
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Table 1. Pesticides Chemical and Regulatory Information [5–8]

Name	Class	Log P	pKa	Structure	MRLs in apple (ng/g)*
Folpet	Phthalimide	3.02	NA		3000
Chlordane	Cyclodiene organochlorine	2.78	NA		20
Endosulfan	Organochlorine	3.13	NA		50
Dieldrin	Chlorinated hydrocarbon	3.7	NA		10
DDE	Organochlorine	6.55	NA		50
Ethion	Organophosphate	5.07	NA		300

(Continued)

Table 1. Pesticides Chemical and Regulatory Information [5–8]

Name	Class	Log P	pKa	Structure	MRLs in apple (ng/g)*
Endosulfan sulfate	Organochlorine	3.13	NA		50
Endrin ketone	Organchlorine	4.99	NA		10
Permethrins	Pyrethroid	6.1	NA		50
Coumaphos	Organothio phosphate	3.86	NA		100

*The MRLs numbers list in the table are for apple or lowest level in other fruit and vegetables. They could be higher in different commodities.

Instrument Condition

An Agilent GC/MS method for pesticides analysis was used for this study. [9]

GC conditions

Auto-sampler:	Agilent 7683 automatic liquid sampler
Inlet:	Splitless
Column:	Agilent 30 m × 0.25 mm × 0.25 µm HP-5MS Ultra Inert (p/n 19091S-433UI)
Carrier gas:	Helium in the constant pressure mode
Retention time locking:	Chlorpyrifos-methyl locked to 16.596 min (nominal column head pressure = 22.0 psi)
Oven temperature program:	70 °C (2 min), 25 °C/min to 150 °C (0 min), 3 °C /min to 200 °C (0 min), 8 °C/min to 280 °C (11.5 min)
Injection volume:	1.0 µL

MS conditions

Tune file:	Atune.u
Mode:	SIM (refer to Table 2 for settings in detail)
Source, quad, transfer line temperature:	230 °C, 150 °C and 280 °C respectively,
Solvent delay:	3.00 min
Multiplier voltage:	Autotune voltage

Sample preparation

Sample comminution

Organically grown, pesticide-free apples were purchased from a local grocery store. Approximately three pounds of apples were chopped into small, bean sized cubes. Skin was included, but the seeds were discarded. The chopped apple cubes were then placed into a clean plastic bag and frozen at –20 °C overnight. The bag was massaged occasionally to make sure the cubes remained separate. The following day, only the required amount of frozen apple cubes was removed and thoroughly blended. Dry ice was added while comminuting, when possible. Samples were comminuted thoroughly to get the best sample homogeneity, ensuring there were no pieces of apple visible in the final sample.

Extraction/Partitioning

A 15 g (± 0.1g) amount of previously homogenized sample was placed into a 50 mL centrifuge tube (from the SampliQ QuEChERS extraction kit). QC samples were fortified with 100 µL of appropriate QC spiking solution (11 pesticides) and 7.5, 37.5, and 150 µL of 20 µg/mL stock solution (6 pesticides mixture), respectively, yielding QC samples with concentra-

Table 2. Instrument Acquisition Data Used for the Analysis of 17 Pesticides by GC/MS.

Analyte	SIM	Collection window (min)	RT (min)
(1) Dichlorvos	184.9	3.0 – 6.5	5.8
(2) σ-Phenylphenol	170.1, 169.1	6.5 – 9.5	8.8
(3) Diazinon	137.1, 179.1	13.5 – 14.65	14.5
(4) Chlorothalonil	265.9, 263.9	14.65 – 16.0	14.8
(5) Carbaryl	144	16.0 – 17.5	16.8
(6) Dichlofluanid	123, 167.1	17.5 – 18.8	18.4
(7) Dichlorobenzophenone	139, 249.9	18.8 – 20.5	19.2
(8) Folpet	259.9, 261.9	21.35 – 21.8	21.6
(9) γ-Chlordane	372.9, 374.9	21.8 – 22.3	22.0
(10) Endosulfan	240.8, 238.8	22.3 – 23.2	22.6
(11) Dieldrin	262.8	23.2 – 25.0	23.9
(12) DDE	245.9, 317.9	23.2 – 25.0	24.0
(13) Ethion	230.9	25.0 – 26.4	26.0
(14) Endosulfan sulfate	273.8	26.4 – 27.2	26.8
TPP (IS)	325.1, 326.1	27.2 – 28.0	27.7
(15) Endrin ketone	316.9	28.0 – 28.5	28.2
(16) Permethrin	183.1	30.0 – 32.5	31.4, 31.6
(17) Coumaphos	362.0	30.0 – 32.5	31.7

tions of 10, 50 and 200 ng/g. A 100 µL amount of internal standard spiking solution (15 µg/mL of TPP) was added to all samples except the control blank to yield a 100 ng/g concentration in each sample. Tubes were capped and vortexed for 1 min. A 15 mL amount of 1% HAc in ACN was added to each tube using the dispenser. An Agilent SampliQ QuEChERS extraction salt packet from the kit (PN 5982-5755) containing 6 g of anhydrous $MgSO_4$, and 1.5 g of anhydrous NaOAc was added directly to the tubes. The salt bag was massaged carefully to break up any salt clumps before pouring. The tubes were examined to ensure that no powder was left in the threads or rims of the tubes. Sample tubes were sealed tightly and shaken vigorously for 1 min by hand to ensure that the solvent interacted with the entire sample and crystalline agglomerates were dispersed. Sample tubes were centrifuged at 4000 rpm for 5 min.

Dispersive SPE Cleanup

A 1 mL aliquot of the upper ACN layer was transferred to an Agilent SampliQ QuEChERS dispersive SPE 2 mL tube (p/n 5982-5022). An 8 mL aliquot was transferred to an Agilent SampliQ QuEChERS dispersive SPE 15 mL tube (p/n 5982-5058). The 2 mL tube contained 50 mg of PSA and 150 mg of anhydrous $MgSO_4$; while the 15 mL tube contained 400 mg of PSA and 1200 mg of anhydrous $MgSO_4$. The tubes were tightly capped and vortexed for 1 min. The 2 mL tubes were centrifuged with a micro-centrifuge at 13,000 rpm for 2 min, and 15 mL tubes in a standard centrifuge at 4000 rpm for 5 min. An aliquot from the extract, 500 µL was transferred into an autosampler vial, and analyzed by GC/MS.

Figure 1 shows the flow chart for the QuEChERS AOAC sample extraction procedure.

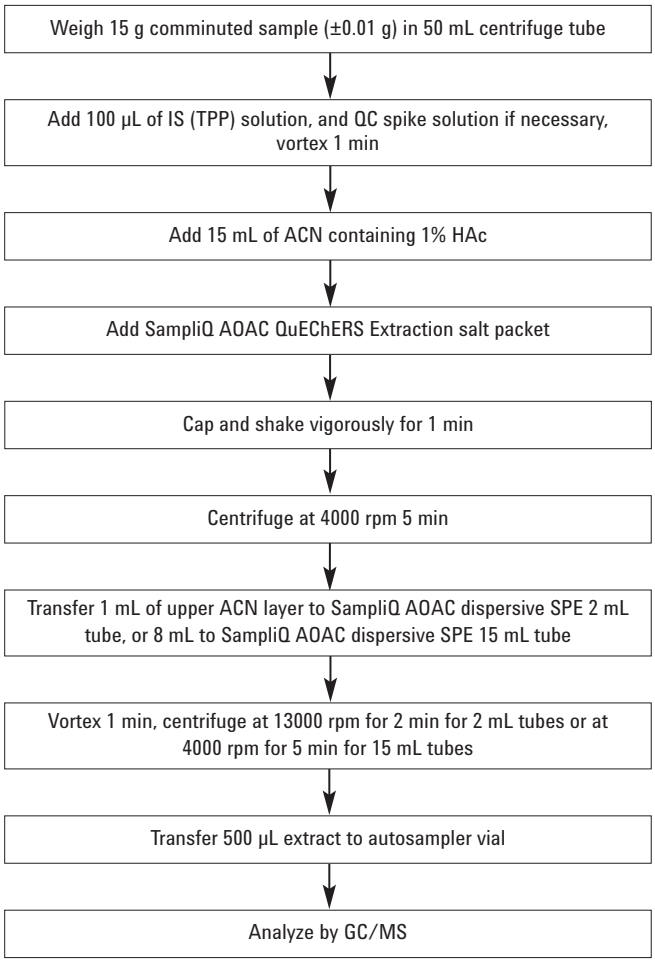


Figure 1. Flow chart of the Agilent SampliQ QuEChERS AOAC extraction procedure.

Results and Discussion

Using the SampliQ QuEChERS kits, the entire procedure is faster, easier, offers time and labor savings, while ensuring consistency. An analyst can process 40–50 samples in just a few hours. The addition of a food sample with a high content of water directly to the salts creates an exothermic reaction, which can affect analyte recoveries, especially for volatile pesticides. Agilent's SampliQ extraction salts are uniquely prepared in an anhydrous package. The unique SampliQ anhydrous salts packet allows addition after adding organic solvent to the sample, as specified in the original QuEChERs method.

In our previous study, the new design of SampliQ QuEChERS AOAC kits demonstrated excellent recovery and precision for a broad variety of semi-polar to polar pesticides using LC/MS/MS. [4] There are many semi-volatile and volatile pesticides, so the use of GC/MS is applicable for the performance evaluation of the AOAC kits for the analysis of these groups of pesticides. The selectivity of GC/MS (SIM mode) is not as effective as that of LC/MS/MS (MRM mode). Furthermore, the final QuEChERS samples still contained food matrix impurities, which can be observed in the GC/MS chromatogram of blank apple extract. Therefore, it is important to carefully choose the monitored ions of each compound when setting up the SIM method. In general, the most abundant ions were selected in order to achieve the best sensitivity; however in a few instances the sensitivity was compromised to obtain better selectivity by using more unique but less abundant ions. As shown in Figure 2a, there are interference peaks apparent in the blank chromatogram; fortunately most pesticides are free of co-eluting interferences. There was an interference eluting at a retention time very close to that of σ-phenylphenol, and can not be differentiated for quantitation.

The response of this interferent within the blank was integrated to be less than 20% response of σ-phenylphenol peak at the LOQ (10 ng/g) sample. Therefore, the selectivity was considered acceptable for this compound. Figure 2 (a, b) shows the chromatograms of a blank apple extract and 50 ng/g fortified apple extract.

Linearity and Limit of Quantification (LOQ)

The linear calibration range for all of the pesticides was 0–400 ng/g; excluding Folpet at 50–400 ng/g due to poor sensitivity. Two different dispersive SPE volumes (1 mL and 8 mL) were used for evaluation and comparison; therefore, two calibration curves were generated from matrix blanks prepared from each size. Each calibration curve was made at levels of 10, 20, 50, 100, 250, and 400 ng/g. The TPP was the internal standard (IS) at 100 ng/g in all cases. The calibration curves were generated by plotting the relative responses of analytes (peak area of analyte/peak area of IS) to the relative concentration of analytes (concentration of analyte/concentration of IS). Table 1 shows that the 10 ng/g quantification limits LOQ (10 ppb) and 50 ng/g LOQ for Folpet (50 ppb) established for pesticides are substantially lower than many MRLs for the pesticides in fruit and vegetables. The regression fit used for the calibration curves was the average response factor. Table 3 shows the linear term and RF relative standard deviation (%) for both 1 mL and 8 mL dispersive SPE.

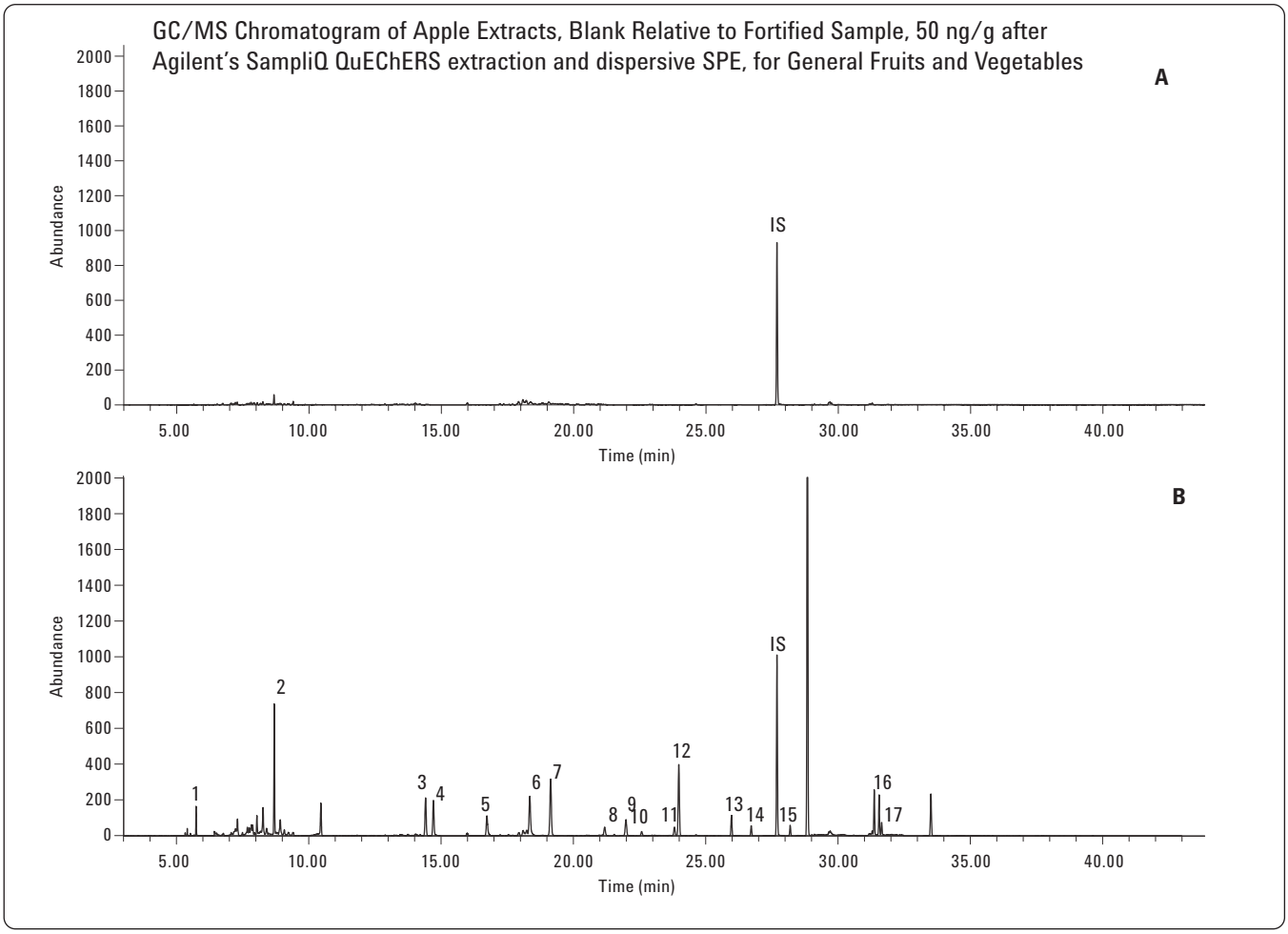


Figure 2. GC/MS chromatogram of apple extract. (A) apple extract blank; (B) 50-ng/g fortified apple extract. Peak Identification: 1. Dichlorvos, 2. σ-Phenylphenol, 3. Diazinon, 4. Chlorothalonil, 5. Carbaryl, 6. Dichlofluanid, 7. Dichlorobenzophenone, 8. Folpet, 9. γ-Chlordane, 10. Endosulfan, 11. Dieldrin, 12. DDE, 13. Ethion, 14. Endosulfan sulfate, 15. Endrin ketone, 16, Permethrin, 17. Coumaphos. IS. Triphenyl phosphate (TPP).

Table 3. Linearity of 17 Pesticides in Apple Extract

	1 mL dispersive SPE		8 mL dispersive SPE	
Analytes	**Linear Term**	**RF Rel Std Dev (%)**	**Linear Term**	**RF Rel Std Dev (%)**
Dichlorvos	3.47e-001	11.4	3.87e-001	4.6
σ-Phenylphenol	1.37e-000	10.7	1.50e-000	11.4
Diazinon	7.04e-001	10.9	7.39e-001	6.5
Chlorothalonil	6.84e-001	13.7	8.02e-001	8.9
Carbaryl	8.07e-001	14.1	1.01e-000	10.8
Dichlofluanid	1.04e-000	12.8	1.08e-000	8.6
Dichlorobenzophenone	4.55e-001	11.4	4.60e-001	8.2
Folpet	3.88e-002	19.5	4.52e-002	20.1
γ-Chlordane	3.23e-001	10.4	3.31e-001	9.2
Endosulfan	8.56e-002	15.2	8.26e-002	8.8
Dieldrin	2.71e-001	6.2	2.59e-001	5.9
DDE	1.43e-000	8.4	1.39e-000	7.5
Ethion	5.87e-001	19.7	5.63e-001	17.0
Endosulfan sulfate	2.72e-001	9.6	2.74e-001	9.5
Endrin ketone	2.75e-001	10.1	2.75e-001	7.8
Permethrin	9.71e-001	9.4	9.29e-001	8.0
Coumaphos	2.70e-001	15.6	2.72e-001	15.7

Recovery and Reproducibility

The recovery and reproducibility were evaluated by spiking pesticides standards in comminuted apple sample at levels of 10, 50 and 200 ng/g. These QC samples were quantitated against the matrix-spiked calibration curve. The analysis was performed in replicates of six (n=6) at each level. The recovery and reproducibility (shown as % RSD) data for 1 mL and 8 mL volume dispersive SPE are shown in Table 4 and Table 5, respectively. It can be seen from the results that all of the pesticides give excellent recoveries (average of 90.8% for 1 mL and 94.2% for 8 mL) and precision (average of 5.7% RSD for 1 mL and 4.3% RSD for 8 mL). As mentioned above, an interferent was eluted very closely with σ-phenylphenol. The selectivity was still acceptable because the interferent contributed less than 20% of LOQ; however, the contribution of the interference peak resulted in the higher recovery of this compound at low levels. Folpet is a notoriously unstable pesticide, and the main problems dealing with degradation and instability come from the N-trihalomethylthio functional group. [3, 10] Folpet was quantified, but the LOQ was found to be 50 ng/g due to poor sensitivity, however, recovery and reproducibility at 50 ng/g and above were acceptable (average recovery 85.5%, average reproducibility 10%).

Table 4. Recovery and Repeatability of Pesticides in Fortified Apple With Agilent SampliQ 2 mL Dispersive SPE Tube (p/n 5982-5022); Recovery 90.8%, RSD 5.7% (avg)

Analytes	10 ng/g fortified QC		50 ng/g fortified QC		200 ng/g fortified QC	
	Recovery	RSD (n=6)	Recovery	RSD (n=6)	Recovery	RSD (n=6)
Dichlorvos	86.8	7.0	83.9	11.6	81.5	5.5
σ-Phenylphenol	113.4	6.3	96.3	6.5	100.5	3.6
Diazinon	98.6	2.3	87.3	2.8	90.4	4.9
Chlorothalonil	86.1	10.0	84.4	5.3	93.2	7.6
Carbaryl	96.1	9.0	93.8	8.3	99.1	8.2
Dichlofluanid	90.0	7.0	84.6	2.9	94.6	5.0
Dichlorobenzo phenone	97.8	7.6	95.0	6.2	102.2	4.3
Folpet	–	–	74.4	9.1	95.7	11.0
γ-Chlordane	79.6	4.4	88.9	4.3	95.3	4.4
Endosulfan	69.8	9.2	91.2	5.3	96.2	5.2
Dieldrin	90.6	10.9	86.6	3.2	92.8	4.8
DDE	84.0	4.8	89.4	3.8	95.4	4.5
Ethion	90.9	1.8	103.5	1.4	116.5	5.0
Endosulfan sulfate	79.8	1.9	80.4	4.6	86.8	5.6
Endrin ketone	85.2	12.0	80.7	3.6	91.8	4.5
Permethrin	87.9	2.8	93.8	2.0	94.0	4.4
Coumaphos	87.8	5.1	89.7	3.0	90.0	6.4

Table 5. Recovery and Repeatability of Pesticides in Fortified Apple With Agilent SampliQ 15 mL Dispersive SPE Tube (p/n 5982-5058); Recovery 94.2%, RSD 4.3% (avg)

Analytes	10 ng/g fortified QC		50 ng/g fortified QC		200 ng/g fortified QC	
	Recovery	RSD (n=6)	Recovery	RSD (n=6)	Recovery	RSD (n=6)
Dichlorvos	103.4	4.2	85.6	8.1	97.2	7.2
σ-Phenylphenol	125.8	8.7	99.2	4.4	105.4	5.0
Diazinon	96.0	4.5	82.3	2.1	88.4	6.3
Chlorothalonil	96.5	3.0	82.8	5.2	97.7	4.5
Carbaryl	97.7	3.9	91.4	4.4	101.9	5.0
Dichlofluanid	91.7	5.1	83.7	1.0	93.7	5.1
Dichlorobenzo phenone	98.8	9.3	96.2	4.7	105.3	4.3
Folpet	–	–	88.4	4.0	72.5	6.0
γ-Chlordane	80.9	3.5	87.5	3.3	94.8	5.0
Endosulfan	80.3	7.3	84.1	3.6	98.6	3.0
Dieldrin	81.2	3.4	93.1	2.0	98.7	3.9
DDE	86.1	1.8	92.4	3.4	98.9	3.9
Ethion	106.5	3.6	122.2	2.0	136.3	4.2
Endosulfan sulfate	91.6	4.6	87.7	4.0	93.0	4.1
Endrin ketone	76.2	3.3	82.4	3.9	91.8	4.1
Permethrin	97.9	1.6	104.7	1.1	106.6	4.2
Coumaphos	82.3	6.7	86.5	2.5	89.3	5.1

Figure 3 shows the recovery and precision results for 1 mL dispersive SPE and 8 mL dispersive SPE. The two different dispersive SPE clean-ups were performed by transferring 1 mL or 8 mL of ACN extract from the same sample following the extraction step. In order to simplify the comparison, the average recovery and precision of three fortification concentrations were used for all pesticides. The results of each dispersive SPE clean-up appeared to be independent of volume used. Both approaches provided efficient and similar sample clean-up, and thus generated relatively equivalent results.

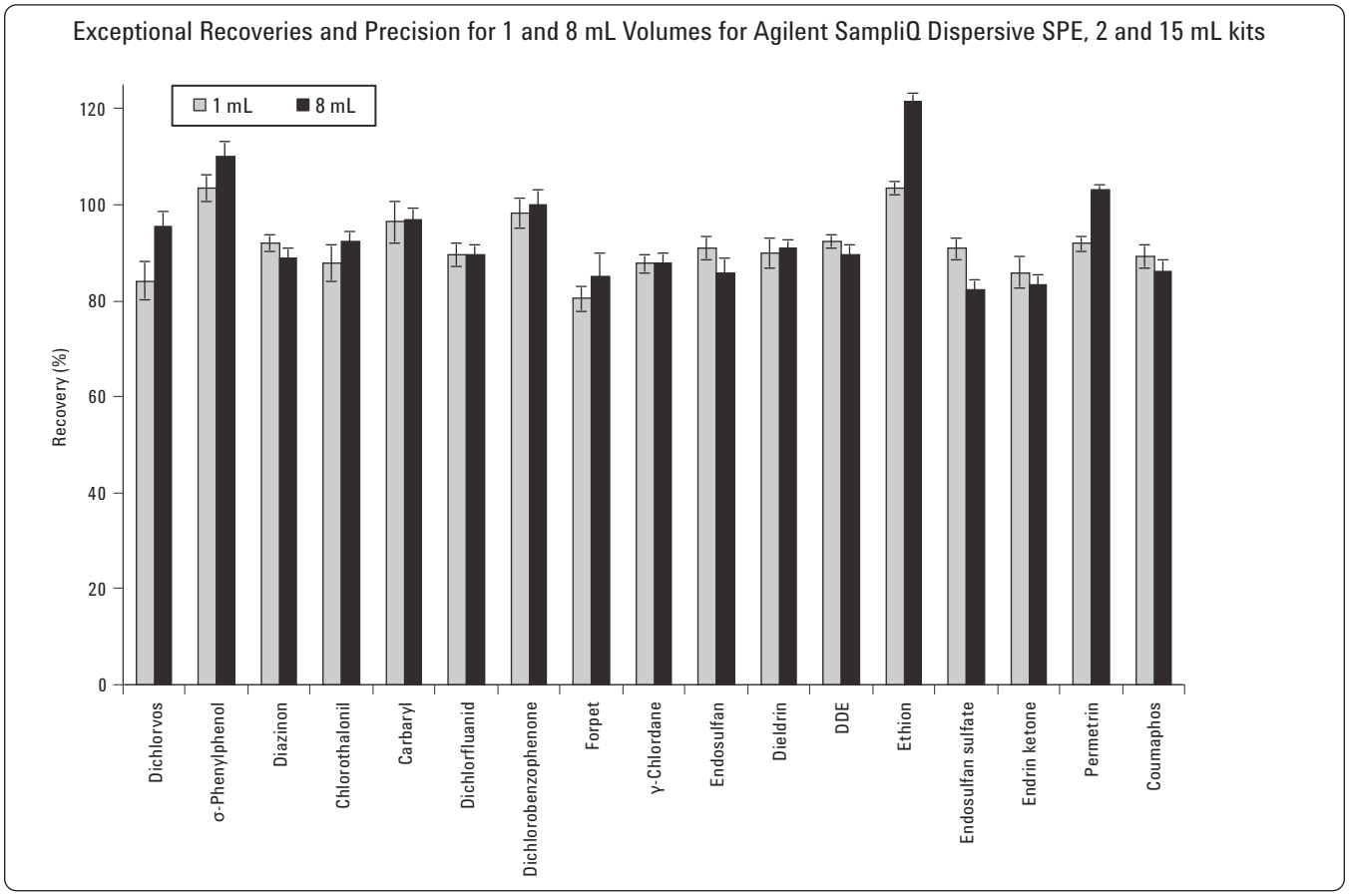


Figure 3. Recoveries and precision for 1 and 8 mL sample volumes employing Agilent SampliQ Dispersive SPE, 2 and 15 mL kits, respectively.

Conclusions

Agilent SampliQ QuEChERS AOAC method for General Fruits and Vegetables: Extraction and Dispersive SPE kits provided a simple, fast and effective method for the purification and enrichment of representative volatile to semi-volatile pesticides in apple. The recovery and reproducibility, based on matrix spiked standards, were acceptable for multiclass, multi-residue pesticide determination in apple. The impurities and matrix effects from apple did not interfere with the quantitation of target compounds. The LOQs of the pesticides were lower than regulated MRLs in apple. As the selected pesticides represented a broad variety of different classes and properties, the Agilent SampliQ QuEChERS AOAC Extraction and Dispersive SPE kits for General Fruits and Vegetables is an excellent choice for other pesticides in similar food matricies.

Acknowledgements

The authors wish to thank Dr. Meng, Chin K. for his help with the GC/MS instrument, and Dr. Ball, Carol H. for many valuable suggestions.

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Analysis of Pesticide Residues in Apples using Agilent SampliQ QuEChERS AOAC Kit by LC/MS/MS Detection

Application Note

Food Safety

Authors

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Abstract

This application note describes the use of a quick, easy, cheap, effective, rugged, and safe (QuEChERS), Association of Analytical Communities (AOAC) Official Method 2007.01; sample preparation approach for extraction and cleanup of 16 pesticide residues in apple. The 16 pesticides chosen represent various classes of interest. The method employed involves initial extraction in a buffered aqueous/acetonitrile system, an extraction/partitioning step after the addition of salt, and then a cleanup step utilizing dispersive solid phase extraction (dispersive SPE). The two different dispersive SPE clean-up approaches (1 mL and 8 mL) were evaluated simultaneously after sample extraction. The target pesticides in the apple extracts were then determined by liquid chromatography coupled to an electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) operating in positive ion multiple reaction monitoring (MRM) mode. The method was validated in terms of recovery and reproducibility. The 5 ng/g limit of quantitation (LOQ) for pesticides in apple shown in this application was well below the maximum residue limits (MRLs). The spiking levels for the recovery experiments were 10, 50, and 200 ng/g. Mean recoveries ranged between 76 and 117% (95.4% on average), with RSD below 15% (4.3% on average).

Agilent Technologies

Introduction

Multi-residue analysis of pesticides in fruits, vegetables, and other foods is the primary function of many regulatory, industrial, and contract laboratories throughout the world. Because of the wide variety of pesticides and complexity of food matrices, the sample must be initially cleaned up using a sample preparation technique prior to analysis. Without question, the most efficient approach to pesticide analysis involves the use of multiclass, multi-residue methods. Once the preliminary analytical quality requirements, including accuracy, precision, sensitivity, selectivity and dynamic range, have been met to suit the needs of a particular analysis, other considerations should be evaluated. These additional considerations include sample throughput, ruggedness, ease of use, cost of materials and labor, toxic solvent usage, and waste generation.

The QuEChERS method was introduced first by USDA scientists in 2003. [1] The method was then modified to address some problematic pesticides by using a buffered extraction system. [2] After a full validation for more than 200 pesticides, this improved method was formalized and adopted as AOAC Official Method 2007.01. [3] In summary, the method uses a single-step buffered acetonitrile (1% HAc) extraction while salting out water from the sample using anhydrous magnesium sulfate ($MgSO_4$) to induce liquid-liquid partitioning. After removing an aliquot from the organic layer, for further cleanup a dispersive solid phase extraction (dispersive SPE) is conducted using a combination of primary secondary amine (PSA) to remove fatty acids from other components and anhydrous $MgSO_4$ to reduce the remaining water in the extract. After mixing and centrifugation, the upper layer is ready for analysis.

In this study, 16 pesticides were used for evaluating the performance of the Agilent AOAC Buffered Extraction kit (p/n 5982-5755) and SampliQ QuEChERS AOAC dispersive SPE kit for General Fruits and Vegetables (p/n 5982-5022 and 5982-5058), suitable for common fruit and vegetable applications. Apple was selected as the fruit matrix for the evaluation. Most of the pesticides are from the original "representative pesticides" list [2]. According to their experience, a method working well for these representative pesticides should work equally well for nearly all of the other pesticides that are routinely monitored in multiclass, multi-residue methods. These pesticides are from 9 different pesticide classes, including acidic, basic, neutral, base-sensitive and acid-labile pesticides. Furthermore, the selected pesticides are suitable for LC/MS/MS analysis. The MRLs of these pesticides are a function of both the pesticide class and food matrix and have been set at 10 ng/g or higher. Table 1 shows the chemical and regulatory information for these multiple class pesticides in apple.

Experimental

Reagents and Chemicals

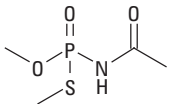
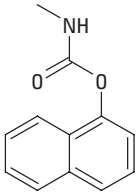
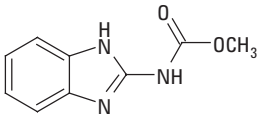
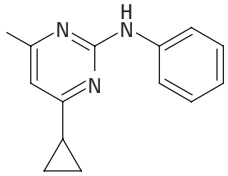
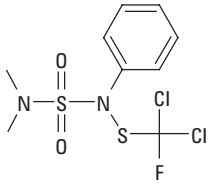
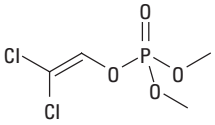
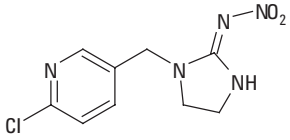
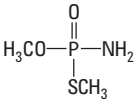
All reagents and solvents were HPLC or analytical grade. Acetonitrile (ACN), methanol (MeOH) were from Honeywell (Muskegon, MI, USA). Dimethyl sulfoxide (DMSO) and acetic acid (HAc) were from Sigma-Aldrich (St Louis, MO, USA). Ammonium acetate (NH_4OAc) was from Fisher Chemicals (Fair Lawn, NJ, USA). Formic acid (FA) was from Fluka (Sleinheim, Germany). The pesticide standards and internal standard, triphenyl phosphate (TPP), were purchased from Sigma-Aldrich (St Louis, MO, USA), ChemService (West Chester, PA, USA), Ultra Scientific (North Kingstown, RI, USA), or AlfaAesar (Ward Hill, MA, USA).

Solutions and Standards

A stock solution of 1M ammonium acetate pH 5 was made by dissolving 19.27 g NH_4OAc powder in 250 mL Milli-Q water. The pH was adjusted to 5 with HAc monitored with a pH meter. The solution was stored at 4 °C. MeOH/H_2O (20:80) containing 5 mM NH_4OAc pH 5 was made by combining 200 mL MeOH and 800 mL Milli-Q water, adding 5 mL of 1M NH_4OAc pH 5 stock solution. 5 mM NH_4OAc in ACN was prepared by adding 5 mL of 1M NH_4OAc pH 5 stock solution to 1 L ACN, mixing well and sonicating 5 min. 1% HAc in ACN was prepared by adding 10 mL of acetic acid to 1 L of ACN.

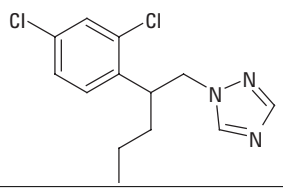
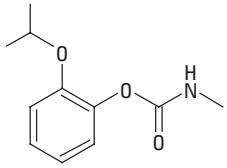
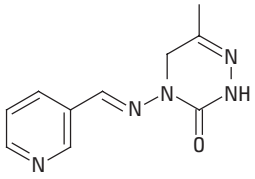
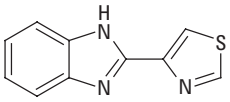
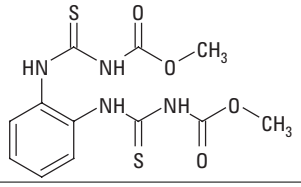
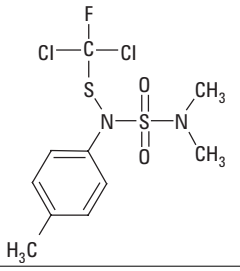
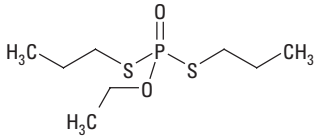
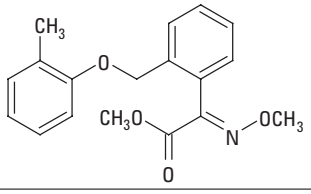
Standard and internal standard (IS) stock solutions (2.0 mg/mL for all, except 0.5 mg/mL for carbendazim) were made in MeOH, 0.1% FA in ACN, or DMSO, respectively, and stored at –20 °C. Three QC spiking solutions of 1.5, 7.5, and 30 µg/mL were made fresh daily in 1:1 ACN/H_2O (0.1% FA).

Table 1. Pesticides Chemical and Regulatory Information [4–6]

Name	Class	Log P	pKa	Structure	MRLs in apple (ng/g)*
Acephate	Organophosphate	–0.89	8.35		20
Carbaryl	Carbamate	2.36	10.4		50
Carbendazim	Benzimidazole	1.48	4.2		100
Cyprodinil	Anilinopyrimidine	4	4.44		50
Dichlofluanid	Sulphamide	3.7	NA		5000
Dichlorvos	Organophosphate	1.9	NA		10
Imidacloprid	Neonicotinoid	0.57	NA		500
Methamidophos	Organophosphate	-0.79	NA		10

(Continued)

Table 1. Hormones Used in this Study

Name	Class	Log P	pKa	Structure	MRLs in apple (ng/g)*
Penconazole	Triazole	3.72	1.51		50
Propoxur	Carbamate	0.14	NA		1000
Pymetrozine	Pyridine	-0.19	4.06		20
Thiabendazole	Benzimidazole	2.39	4.73 12.00		50
Thiophanate-methyl	Benzimidazole	1.45	7.28		100
Tolylfluanid	Sulphamide	3.9	NA		3000
Ethoprophos	Organophosphate	2.99	NA		5
Kresoxim-methyl	Strobilurin	3.4	NA		50

*The MRLs numbers list in the table are for apple or lowest level in other fruit and vegetables. They could be higher in different commodities.

A 10 µg/mL standard solution in 1:1 ACN/H_2O (0.1% FA) was made for preparation of calibration curves in the matrix blank extract by appropriate dilution. A 15 µg/mL of TPP in 1:1 ACN/H_2O (0.1% FA) was used as an internal standard (IS).

Equipment and Material

- Agilent 1200 HPLC with Diode Array Detector (Agilent Technologies Inc., Santa Clara, CA, USA).
- Agilent 6410 Triple Quadrupole LC/MS/MS system with Electrospray Ionization (Agilent Technologies Inc., Santa Clara, CA, USA).
- Agilent SampliQ Buffered QuEChERS AOAC Extraction kit, p/n 5982-5755, and SampliQ QuEChERS AOAC Dispersive SPE kit for General Fruits and Vegetables, p/n 5982-5022 and 5982-5058 (Agilent Technologies Inc., Wilmington, DE, USA).
- CentraCL3R Centrifuge (Thermo IEC, MA, USA)
- Bottle top dispenser (VWR, So. Plainfield, NJ, USA)
- Eppendorf microcentrifuge (Brinkmann Instruments, Westbury, NY, USA)
- Grinder (St. Joseph, MI, USA)

Instrument Condition

HPLC conditions

Column:	Agilent ZORBAX Solvent Saver Plus Eclipse Plus Phenyl-Hexyl, 3.0 x 150 mm, 3.5 µm (p/n 959963-312)
Flow rate:	0.3 mL/min
Column Temperature:	30 °C
Injection volume:	10 µL
Mobile Phase:	A: 5mM NH_4OAc, pH 5.0 in 20:80 MeOH/H_2O B: 5 mM NH_4OAc, pH 5.0 in ACN
Needle wash:	1:1:1:1 ACN/MeOH/IPA/H_2O (0.2% FA)

Gradient:

Time	% B	Flow rate (mL/min)
0	20	0.3
0.5	20	0.3
8.0	100	0.3
10.0	100	0.3
10.01	20	0.5
12.0	100	0.5
13.0	STOP	

Post run:	4 min
Total cycle time:	17 min

MS conditions

Positive mode	
Gas Temperature:	350 °C
Gas Flow:	10 L/min
Nebulizer:	40 psi
Capillary:	4000 V

Other MS conditions relating to the analytes are listed in Table 2.

Table 2. Instrument Acquisition Data Used for the Analysis of 16 Pesticides by LC/MS/MS

Analyte	MRM channels (*m/z*)	Fragmentor (V)	CE (V)	RT (min)
Acephate	1) 184.0 > 94.9 2) 184.0 > 111.0	60	3 15	2.55
Methamidophos	1) 142.0 > 94.0 2) 142.0 > 124.9	60	8 8	2.54
Pymetrozine	1) 218.1 > 105.0 2) 218.1 > 78.0	115	20 50	2.97
Carbendazim	1) 192.1 > 160.0 2) 192.1 > 105.0	95	18 40	5.07
Dichlorvos	1) 221.0 > 109.0 2) 221.0 > 95.0	110	13 40	6.57
Thiophanate methyl	1) 343.1 > 151.0 2) 343.1 > 117.9	105	17 65	7.08
Propoxur	1) 210.1 > 111.0 2) 210.1 > 92.9	50	12 15	6.89
Carbaryl	1) 202.0 > 145.0 2) 202.0 > 115.0	50	3 40	7.30
Cyprodinil	1) 226.1 > 93.0 2) 226.1 > 108.0	120	35 35	9.23
Dichlorfluanid	1) 333.0 > 123.0 2) 333.0 > 223.9	85	28 5	9.40
Ethoprophos	1) 243.1 > 130.9 2) 243.1 > 172.9	80	15 15	8.50
Penconazole	1) 284.1 > 158.9 2) 284.1 > 172.9	80	32 32	8.95
Tolyfluanid	1) 347.0 > 136.9 2) 347.0 > 238.0	60	25 3	9.73
Thiabendazole	1) 202.1 > 175.0 2) 202.1 > 131.0	110	27 38	5.65
Imidacloprid	1) 256.1 > 209.1 2) 256.1 > 175.0	60	12 18	5.53
TPP	1) 327.1 > 77.0 2) 327.1 > 151.9	70	45 45	9.49
Kresoxim methyl	1) 314.0 > 222.1 2) 314.0 > 235.0	70	10 10	9.44

1) Quantifier transition channel
2) Qualifier transition channel

Sample preparation

Sample comminution

In order to get the most reliable statistical results, it is important to spend the necessary effort and time on conducting proper sampling and homogenization procedures. Organically grown, pesticide-free apples were purchased from a local grocery store. Approximately three pounds of apples were chopped into small, bean-sized cubes. Skin was included, but pit was discarded. The chopped apple cubes were put into a clean plastic bag and frozen at –20 °C overnight. The bag was massaged occasionally to make sure the cubes were frozen loosely, to avoid clumping. The following day, a portion of frozen apple cubes were removed and thoroughly blended. Certain precautions were exercised while blending the sample. First, the chopped apple cubes remained in the freezer until the point of blending. Only the portion of apple cubes necessary for homogenizing were removed; the rest were kept in the freezer until the next comminution. Dry ice was added, when possible, while comminuting to keep the temperature low. Second, the blender container was kept dry to prevent clumping. In between blending, the container was rinsed and dried. Third, samples were comminuted thoroughly to obtain the best sample homogeneity. No pieces of apple were visible in the final sample.

Extraction/Partitioning

A 15 g (±0.05 g) previously homogenized sample was placed into a 50 mL centrifuge tube from the SampliQ QuEChERS Extraction kit. QC samples were fortified with 100 µL of appropriate QC spiking solution yielding QC samples with concentrations of 10, 50, and 200 ng/g. One hundred microliters of IS spiking solution (15 µg/mL of TPP) were added to all samples except the control blank to yield a 100 ng/g concentration in each sample. Tubes were capped and vortexed for 1 min. Fifteen milliliters of 1% HAc in ACN were added to each tube using the dispenser. To each tube, an Agilent AOAC Buffered Extraction packet from the kit (p/n 5982-5755) containing 6 g of anhydrous $MgSO_4$ and 1.5 g of anhydrous NaOAc, was added directly to the tubes. No powders were left in the threads or rims of the tubes. Tubes were sealed tightly and shaken vigorously for 1 min by hand to ensure that the solvent interacted well with the entire sample and crystalline agglomerates were broken up. Sample tubes were centrifuged at 4000 rpm for 5 min.

Dispersive SPE Cleanup

A 1 mL aliquot of the upper ACN layer was transferred into a SampliQ QuEChERS AOAC 2 mL dispersive SPE tube (p/n 5982-5022) or 8 mL aliquot were transferred into an SampliQ QuEChERS AOAC 15 mL dispersive SPE tube (p/n 5982-5058). The 2 mL tube contained 50 mg of PSA and 150 mg of anhydrous $MgSO_4$; while the 15 mL tube contained 400 mg of PSA and 1200 mg of anhydrous $MgSO_4$. The tubes were tightly capped and vortexed for 1 min. The 2 mL tubes were centrifuged with a micro-centrifuge at 13000 rpm for 2 min, and the 15 mL tubes were centrifuged in a standard centrifuge at 4000 rpm for 5 min. Two hundred microliters of extract were transferred into an autosampler vial. Then 800 µL of water or another appropriate standard solution (prepared in water) were added. The samples were capped and vortexed thoroughly. The samples were then ready for LC/MS/MS analysis.

The flow chart in Figure 1 illustrates the sample preparation procedure.

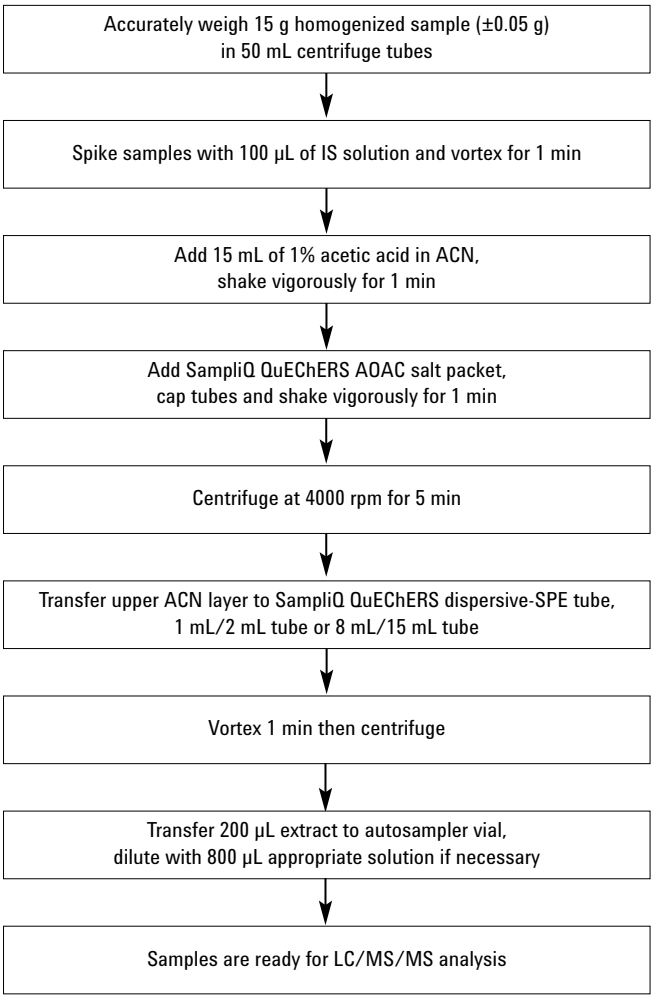


Figure 1. QuEChERS AOAC sample preparation procedures flow chart.

Results and Discussion

In addition to being fast, easy, cheap, effective, rugged and safe, an additional key feature of the QuEChERS method is the potential for the simultaneous analysis of multi-pesticide residues. With the new design of SampliQ QuEChERS kits, the whole procedure is even faster, easier, and offers more time and labor savings, while ensuring consistency. An analyst can process 40–50 samples in just a few hours. Adding a food sample with a high percentage of water directly to the salts may create an exothermic reaction that can affect analyte recovery. Agilent's SampliQ salts and buffers are uniquely prepared in anhydrous packages. This allows addition **AFTER** adding solvent to the sample, as specified in the QuEChERS methodology. The final QuEChERS sample may contain food matrix impurities because it is a very simple sample extraction and cleanup procedure. The final apple extract appeared light green. But with the powerful selectivity of LC/MS/MS multiple reaction monitoring (MRM) mode, the extracted apple blank appeared to be clean and free of impurities, indicating the blank apple extract did not contribute any interferences with the target compounds. Figure 2 shows the chromatograms of a blank apple extract and a 10 ng/g fortified apple extract.

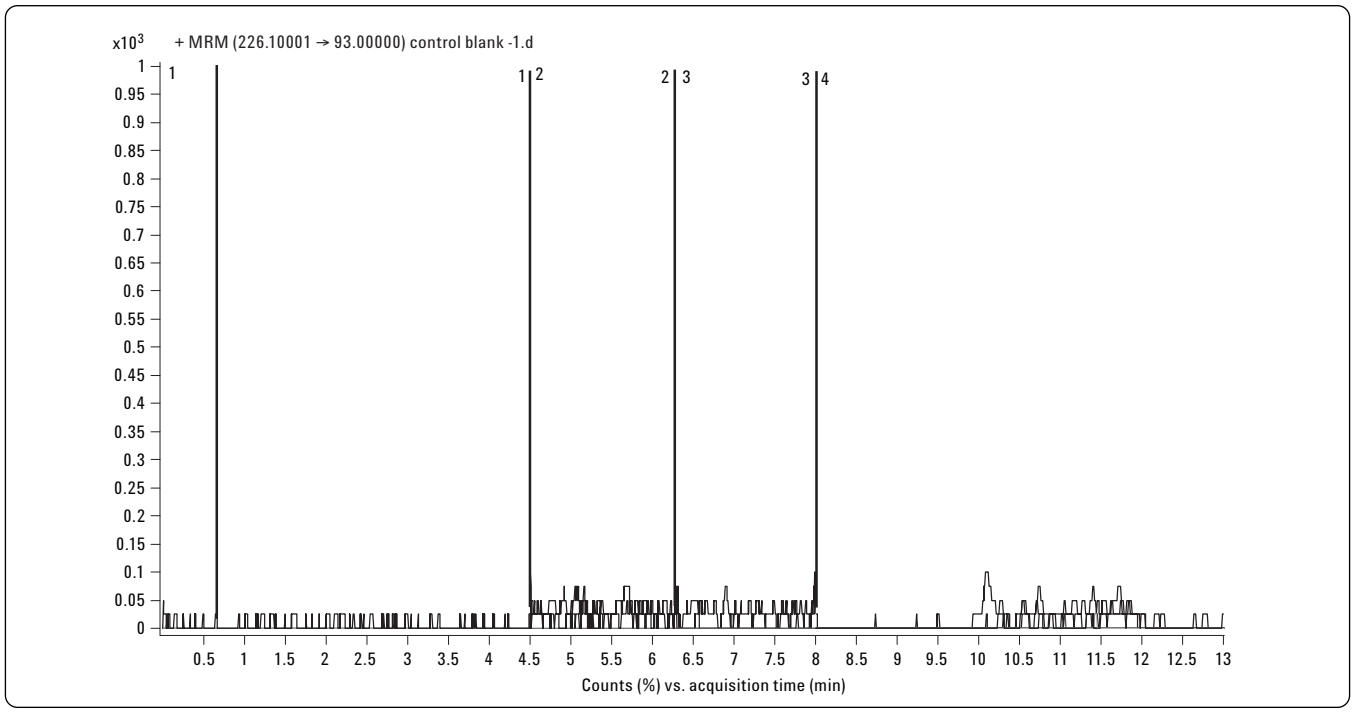


Figure 2a Chromatograms of apple extract blank. No interference was found in the blank.

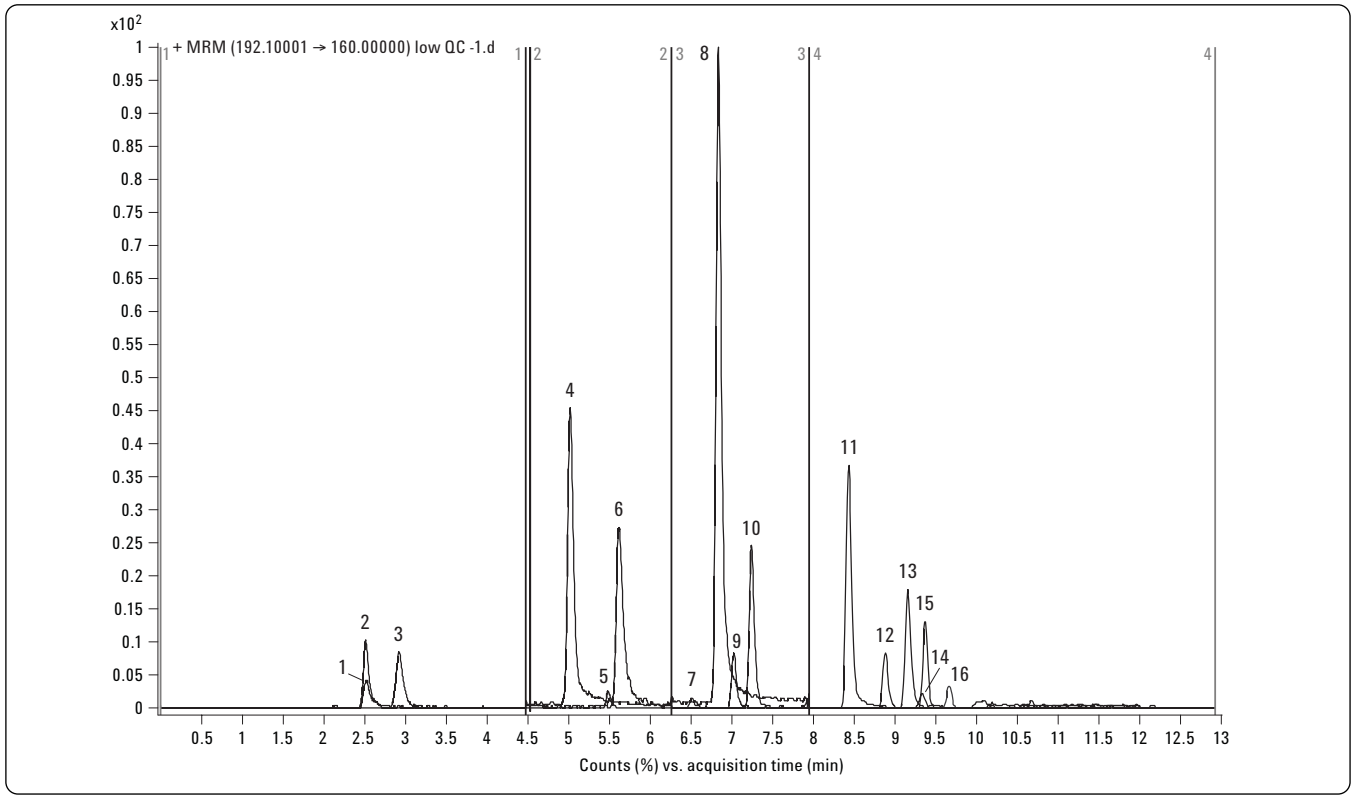


Figure 2b. Chromatogram of 10 ng/g fortified apple extract. Peak identification: 1. Methamidophos, 2. Acephate, 3. Pymetrozine, 4. Carbendazim, 5. Imidacloprid, 6. Thiabendazole, 7. Dichlorvos, 8. Propoxur, 9. Thiophanate methyl, 10. Carbaryl, 11. Ethoprophos, 12. Penconazole, 13. Cyprodinil, 14. Dichlofluanid, 15. Kresoxim methyl, 16, Tolyfluanid.

Linearity and Limit of Quantification (LOQ)

The linear calibration range for all the pesticides was 5 – 250 ng/g. Since two different dispersive SPE volumes (1 mL and 8 mL) were used for evaluation and comparison, two sets of calibration curves were generated respectively. Matrix blanks were prepared for each size. Calibration curves, spiked in matrix blanks, were made at levels of 5, 10, 50, 100, 200, and 250 ng/g. The TPP (IS) was used at 100 ng/g level.

The calibration curves were generated by plotting the relative responses of analytes (peak area of analyte/peak area of IS) to the relative concentration of analytes (concentration of analyte/concentration of IS). Table 1 shows that the 5 ng/g quantification limits LOQ (5 ppb) established for all of the pesticides is lower than the MRLs of these pesticides in fruit and vegetables. Table 3 shows the regression equation and correlation coefficient (R^2) for both 1 mL and 8 mL dispersive SPE volumes.

Table 3. Linearity of Pesticides in Apple Extract

Analytes	1 mL dispersive SPE Regression equation	R^2	8 mL dispersive SPE Regression equation	R^2
Methamidophos	Y = 0.2349X - 0.0013	0.9949	Y = 0.2300X - 0.0007	0.9981
Acephate	Y = 0.1118X - 0.0012	0.9881	Y = 0.1094X - 0.0014	0.9980
Pymetrozine	Y = 0.2671X - 0.0016	0.9950	Y = 0.2290X - 0.0014	0.9975
Carbendazim	Y = 0.9441X + 0.0063	0.9895	Y = 0.8583X + 0.0006	0.9968
Imidacloprid	Y = 0.0513X - 0.0009	0.9905	Y = 0.0500X - 0.0007	0.9933
Thiabendazole	Y = 0.7049X + 0.0044	0.9868	Y = 0.6198X + 0.0043	0.9961
Dichlorvos	Y = 0.0265X + 0.0001	0.9884	Y = 0.0247X + 0.0006	0.9439
Propoxur	Y = 2.0348X - 0.0091	0.9951	Y = 2.0264X - 0.0090	0.9965
Thiophanate methyl	Y = 0.2024X - 0.0054	0.9307	Y = 0.5090X - 0.0041	0.9682
Carbaryl	Y = 0.4984X - 0.0002	0.9965	Y = 0.4889X - 0.0029	0.9976
Ethoprophos	Y = 0.8203X - 0.0064	0.9952	Y = 0.8536X - 0.0076	0.9971
Penconazole	Y = 0.1775X - 0.0006	0.9903	Y = 0.1783X - 0.0019	0.9848
Cyprodinil	Y = 0.3529X - 0.0023	0.9960	Y = 0.3528X - 0.0022	0.9958
Dichlorfluanid	Y = 0.0453X - 0.0004	0.9869	Y = 0.0460X - 0.0006	0.9954
Kresoxim methyl	Y = 0.2498X - 0.0024	0.9932	Y = 0.2490X - 0.0013	0.9927
Tolyfluanid	Y = 0.0718X - 0.0016	0.9823	Y = 0.0755X - 0.0006	0.9788

Recovery and Reproducibility

The recovery and reproducibility were evaluated by spiking pesticide standards in homogeneous apple samples at levels of 10, 50, and 200 ng/g. These QC samples were quantitated against the matrix spiked calibration curve. The analysis was performed in replicates of six (n = 6) at each level. The recovery and reproducibility (RSD) data of 1 mL and 8 mL dispersive SPE sample volumes are shown in Tables 4 and 5, respectively. It can be seen from the results that all of the pesticides give acceptable recoveries (average of 97.5% for 1 mL and 93.3% for 8 mL) and precision (average of 4.5% RSD for 1 mL and 4.1% RSD for 8 mL). The notoriously base-sensitive pesticides such as dichlorfluanid and tolyfluanid showed excellent recovery and precision. Acid labile pesticide, pymetrozine, also showed acceptable recovery and precision.

Table 4. Recovery and Repeatability of Pesticides in Fortified Apple With 2 mL Dispersive SPE Tube (p/n 5982-5022)

	10 ng/g fortified QC		50 ng/g fortified QC		200 ng/g fortified QC	
Analytes	**Recovery**	**RSD (n=6)**	**Recovery**	**RSD (n=6)**	**Recovery**	**RSD (n=6)**
Methamidophos	83.6	5.6	81.3	2.6	83.4	1.4
Acephate	106.8	5.8	95.6	2.3	97.3	2.0
Pymetrozine	78.3	11.4	76.6	11.6	108.1	5.3
Carbendazim	101.0	6.5	98.5	4.3	91.0	2.6
Imidacloprid	107.0	6.5	97.6	3.4	107.4	3.0
Thiabendazole	106.2	6.6	103.7	2.6	95.5	2.0
Dichlorvos	78.2	11.4	94.2	7.2	95.8	1.8
Propoxur	106.3	0.8	105.7	1.2	101.2	1.6
Thiophanate methyl	79.0	15.4	76.7	15.4	102.2	8.1
Carbaryl	93.4	1.9	98.4	2.2	97.5	1.1
Ethoprophos	95.8	4.5	96.1	1.8	94.7	1.3
Penconazole	117.0	4.8	111.9	2.3	111.0	1.6
Cyprodinil	106.9	4.0	102.0	2.8	102.4	1.8
Dichlorfluanid	92.5	6.5	96.3	2.2	99.4	2.6
Kresoxim methyl	98.2	9.3	101.9	2.7	104.1	1.8
Tolyfluanid	96.6	9.5	105.1	1.8	102.2	1.7

Table 5. Recovery and Repeatability of Pesticides in Fortified Apple With 15 mL Dispersive SPE Tube (p/n 5982-5058)

Analytes	10 ng/g fortified QC		50 ng/g fortified QC		200 ng/g fortified QC	
	Recovery	RSD (n=6)	Recovery	RSD (n=6)	Recovery	RSD (n=6)
Methamidophos	80.6	9.3	79.4	2.9	83.1	2.5
Acephate	94.6	7.0	93.7	3.4	95.1	2.5
Pymetrozine	88.8	12.1	87.7	10.1	118.4	5.5
Carbendazim	85.9	3.9	90.4	2.7	85.5	2.2
Imidacloprid	101.8	3.5	99.3	3.7	106.0	0.9
Thiabendazole	92.5	6.4	92.2	2.6	89.5	1.5
Dichlorvos	73.7	14.8	91.8	7.3	95.5	2.0
Propoxur	96.2	1.6	98.2	0.6	97.2	1.2
Thiophanate methyl	81.4	4.9	78.2	13.4	102.3	5.8
Carbaryl	86.5	2.6	90.3	1.4	91.1	1.2
Ethoprophos	89.6	2.9	92.1	1.0	94.1	1.1
Penconazole	102.1	2.5	106.0	3.0	111.0	1.6
Cyprodinil	93.9	3.7	97.4	0.9	99.7	2.0
Dichlorfluanid	81.7	8.7	96.9	5.6	98.1	2.6
Kresoxim methyl	91.8	5.8	93.9	2.0	98.3	1.2
Tolyfluanid	94.1	7.9	95.2	4.0	97.5	2.6

Figure 3 shows the recovery and precision results for 1 mL and 8 mL dispersive SPE. The two different dispersive SPE clean-ups were performed by using 1 mL or 8 mL of ACN extract from the same sample tube after the extraction step. In order to simplify the comparison, the average recovery and precision of three fortification concentrations were used for all pesticides. The results of two dispersive SPE clean-up approaches appeared to be independent of volume used. Both approaches provided efficient sample clean-up, and generated relatively equivalent results.

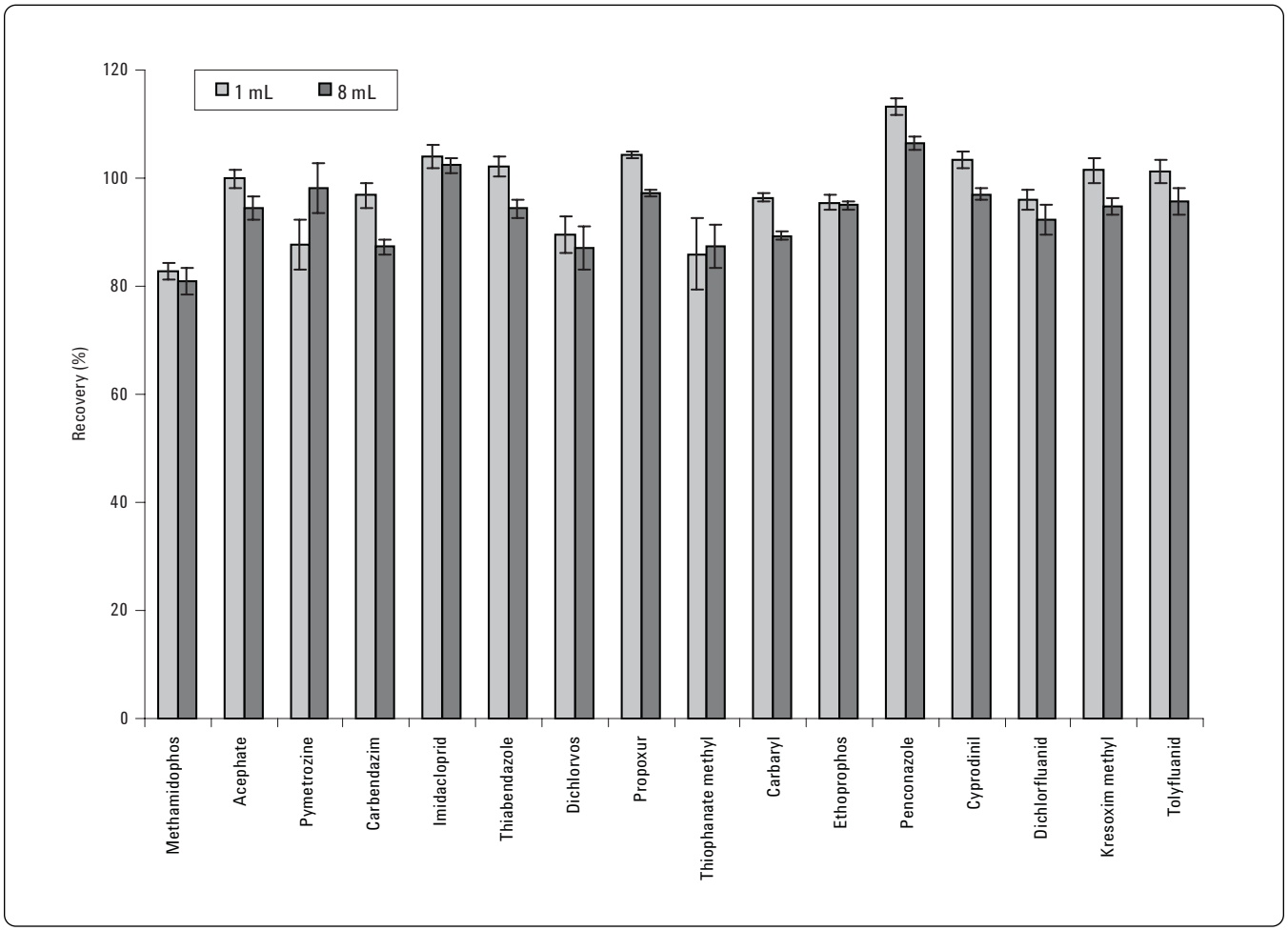


Figure 3. Results comparison of 1 mL and 8 mL dispersive SPE sample volume.

Conclusions

Agilent SampliQ AOAC Buffered Extraction kit and SampliQ AOAC dispersive SPE kit for General Fruits and Vegetables provided a simple, fast, and effective method for the purification of representative pesticides in apple. The recovery and reproducibility, based on matrix spiked standards, were acceptable for multiclass, multi-residue pesticide determination in apple. The impurities and matrix effects from apple were minimal and did not interfere with the quantitation of any target compound. The LOQs of the pesticides were significantly lower than their regulated MRLs in apple. As the selected pesticides represented a broad variety of different classes and properties, the Agilent SampliQ QuEChERS AOAC Extraction and Dispersive kit for General Fruits and Vegetables can be used for other pesticides in similar fruit matrices.

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Introduction

Multi-residue methods are efficient and cost-effective for analysis of pesticide residues. For methods with a very wide scope, generic sample preparation procedures are usually employed. Inherent to this approach is that clean up of extracts is only possible to a limited extent[1]. When applying such methods to complex matrices like baby food, herbs, spices and tobacco, enhanced selectivity in detection is required to make up for the low selectivity in sample preparation.

Your Challenges

The analytical challenge is to maximize the number of pesticides, minimize the variety of methods, keep run times short and achieve limits of detection (LOD's) at or below the maximum residue limits (MRLs) which are specified for pesticides under EU legislation.

As regulations in the European Union require very low MRLs for pesticide residues, the latest challenge has been to reach part-per-billion level concentrations for hundreds of pesticides in complex matrices, which in turn has required greater sensitivity and efficiency in pesticide screening. Quantitation and confirmation of identity of trace level compounds can be complicated by the matrix, resulting in qualifier ion ratios out of range, or target ions buried in the high chemical background noise. With single quadrupole mass spectrometry, selected ion monitoring (SIM) is often used to improve the detection limit and quantitative reproducibility. In SIM mode, only a few ions are monitored for each target within the retention time (RT) range that the target elutes from the column. SIM may not work well for trace levels in matrix as the interferences in SIM are the same as in full scan mode.

Our Integrated Approach

Triple quadrupole mass spectrometry allows for drastic reduction or elimination of matrix interferences that limit the accuracy and detection limits of SIM methods. This process, referred to as Multiple Reaction Monitoring (MRM), has two fundamental advantages over SIM. First, detection is based secondary "product ion" produced by the collisional dissociation of an analyte "precursor ion". The analyte precursor ion (isolated in Q1 by a SIM mechanism) has the same selectivity as SIM, but there is a high probability that at least one of the resultant product ions will be unique to the precursor and not the interference. The increase selectivity of MRM is often apparent by the reduced offset of the baseline as compared to SIM. Secondly, during the mass filtering process in Q1, all lower m/z ions from the sample are eliminated. The unique product ions from the collisional dissociation are measured in this "zero" noise region of the spectrum. The combination of a unique product ions (more selectivity) and the elimination of background noise results in consistently low limits of detection even for complex matrices.



This application brief describes the analysis of pesticides in fruit and vegetable extracts using the Agilent 7000 Series Triple Quadrupole GC/MS system in MRM mode and in combination with Retention Time Locking[2] and Agilent Capillary Flow Technology to provide backflushing of high-boiling materials.[3]

Column backflushing is essential for the analysis of complex samples such as food extracts[4] because they usually contain high-boiling indigenous compounds. In just a few runs, these materials can collect on the head of the column, causing peak tailing, retention time shifts and increased chemical noise. Over time, they can migrate from the column to the ionization source, which would eventually have to be cleaned. Agilent's proprietary capillary flow technology makes column backflushing routine and easy to setup for non experts.

The method robustness is drastically increased and the analysis cycles are shortened[5]. In conclusion the system up-time is maximized allowing significant productivity gains. The need for maintenance is reduced by keeping the chromatographic system and MS ion source cleaner between each injection.



Figure 1
Agilent 7890A/7000A Triple Quadrupole GC/MS system with the new high capacity 7693 ALS.

Experimental

Samples were prepared using the QuEChERS[6] method. QuEChERS stands for Quick Easy, Cheap, Effective, Rugged and Safe and is a food sample preparation for multi-class, multi-residue pesticide analysis. See more at www.agilent.com/chem/Quechers

Instrumentation

The Triple quadrupole GC/MS system used for these experiments are described in Table 1 and shown in Figure 1.

Instrument conditions

Instrumentation	
GC/MS Triple Quadrupole:	Agilent 7000A
GC:	Agilent 7890A
Inlet:	PTV, in splitless mode, 1µL Injection, Multi-baffle liner 80 °C for 0.5 min, then 500 °C/min to 280 °C for 2 min
Capillary flow technology device:	3-way splitter with analytical column in and restrictor out to the triple quadrupole helium pressure provided by Aux EPC at 1 psi
Column:	Agilent J&W HP-5ms Ultra Inert 30 m x 0.25 mm ID, 0.25 µm HP-5MSUI
Restrictor:	80 cm x 0.180 mm deactivated fused silica
Carrier gas:	Helium 30.883 psi (constant pressure mode)
Oven temperature:	70 °C (1 min), 25 °C/min to 150 °C (0 min), 3 °C/min to 200 °C (0 min), 8 °C/min to 280 °C (10 min)
Backflush:	Time 5 min, inlet press. 1 psi, Aux EPC 80 psi, oven temp. 280 °C
Retention time locking:	Chlorpyrifos-methyl locked to 16.53 min
Collision cell gases:	N_2 2.60 psi and He 6.25 psi
Inert source temperature:	260 °C
Quadrupole temperature:	150°C

Table 1
Instrument conditions.

Method Development

In order to maximize the response of the instrument for each residue the choice of precursor ion, product ion and collision energy were optimized. The spectra of a typical pesticide in full scan mode (50-500 m/z) e.g. Dicloran (MW = 206) is displayed in Figure 2. The product ion scan spectra of 206 m/z at different collision energy are displayed in Figure 3.

Results

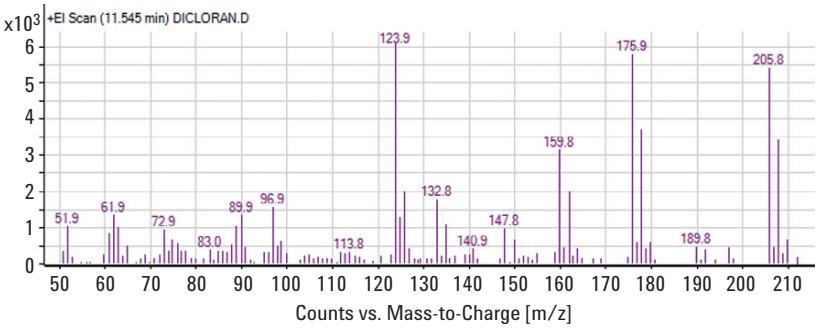


Figure 2
Full scan spectrum of Dicloran under EI ionization mode.

Results

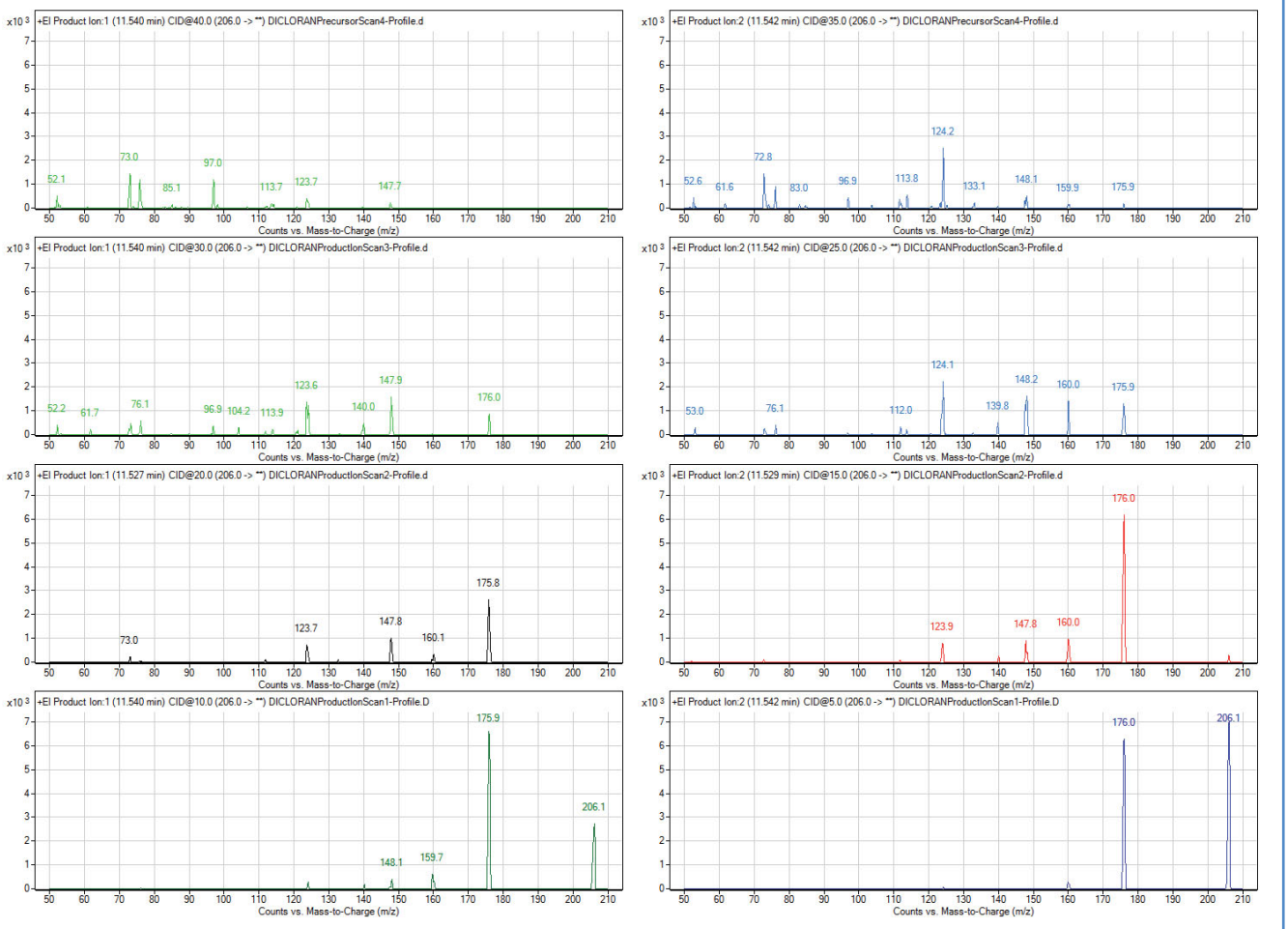


Figure 3
Product ion scan of Dicloran for the Precursor (206 m/z) at different collision energies (5-40 V).

The optimum collision energy for the 206>176 transition product ion was found to be at 10V and the resulting MRM chromatogram is shown in Figure 4.

Results and discussion

Figure 5 shows the TIC chromatogram acquired in MRM mode for 360 pesticides. Each MRM segment is indicated by a grey marker line. An enhanced view on a selected part of the analysis with an overlay of all MRM for the compounds in this part is shown Figure 6. The MRM mode allows for accurate quantification of many coeluting analytes as shown between 13.6 and 14.2 minutes. Figure 7 demonstrates the identity confirmation of Diclobenil in a peppermint extract at 10 pg on column using two MRM transitions. The dashed lines indicate the allowed range of the ion ratio as specified in the method.

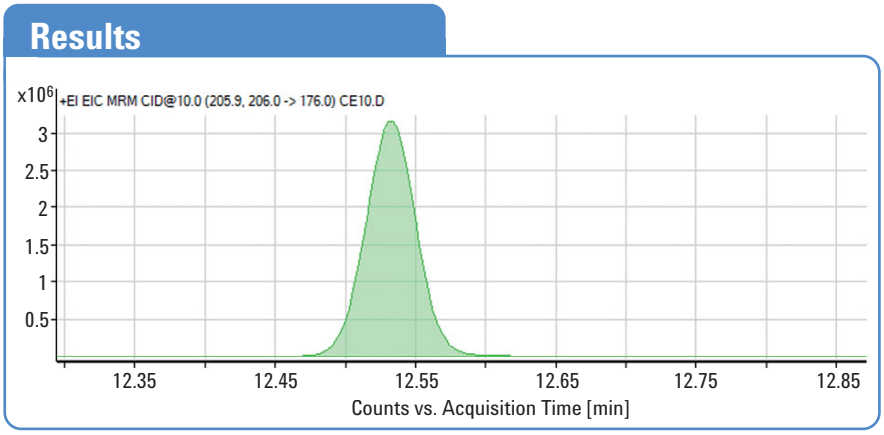


Figure 4
MRM chromatogram of Dicloran at 10ppb.

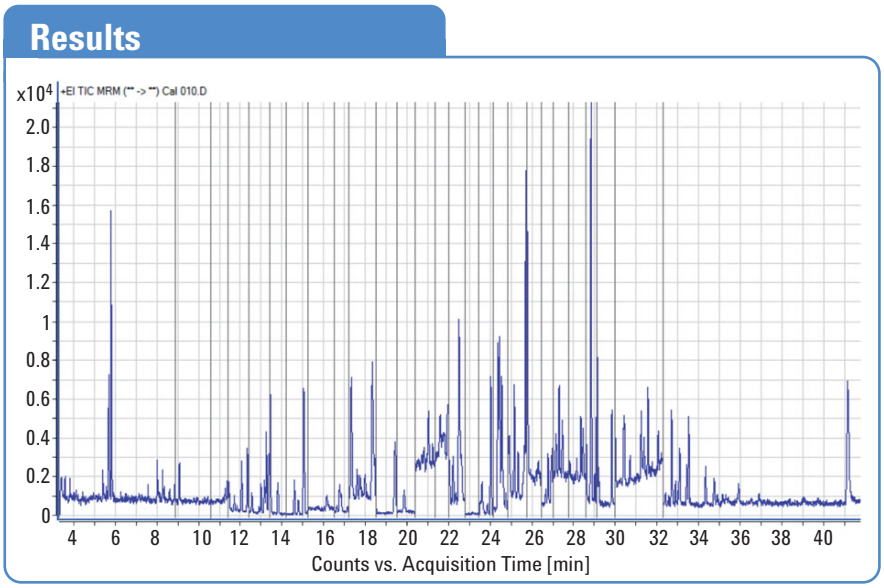


Figure 5
Total ion chromatogram of the vegetable extract by GC/MS/MS.

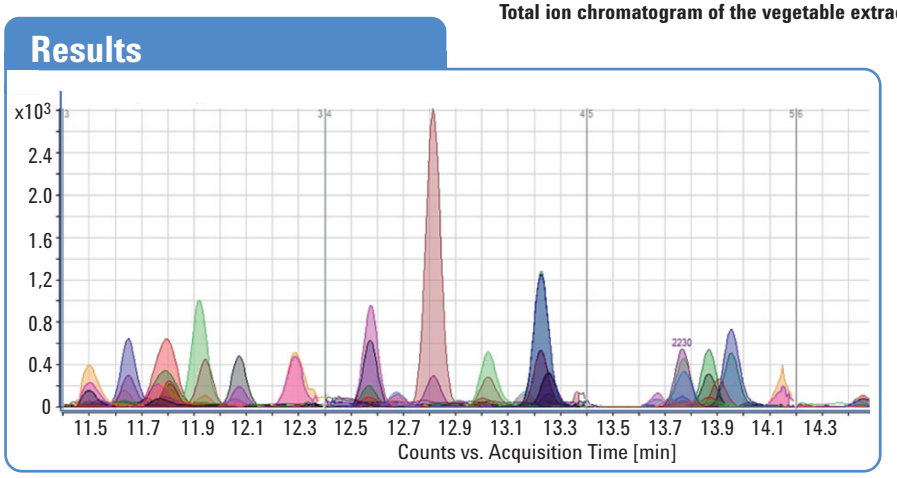


Figure 6
Overlay of extracted MRM transitions.

Results

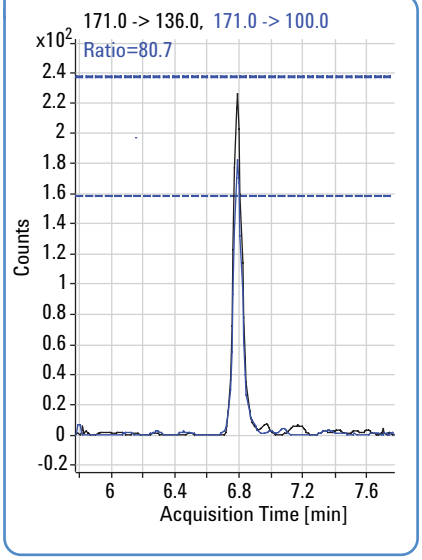


Figure 7
Two transitions identifying Diclobenil in a peppermint extract at 10 pg on column. The dashed lines indicate the allowed range of the ion ratio.

Linearity was also tested with five levels between 1 and 200 ppb for 360 pesticides and Figure 8 shows the calibration curves for Diclobenil and Chlormefos. The correlation coefficients of the external standard calibration curve were 0.99 on average. The LOD was estimated based on the calculated S/N of the 10 pg standard. For the majority of pesticides, LODs were below 2 pg on column (based on S/N >3:1 Peak to Peak).

Retention time reproducibility was also tested to demonstrate the robustness of the analytical method. Figure 9 shows the outstanding retention time stability of one representative compound: Trifluralin. Calculated %RSD is 0.0306 at 6.073 min for one hundred consecutive injections of lettuce extract into the GC/MS/MS system. Only 3 minute Backflush was necessary to remove all high boiling matrix compounds, the total cycle time for this stability test was 21 hours.

Results

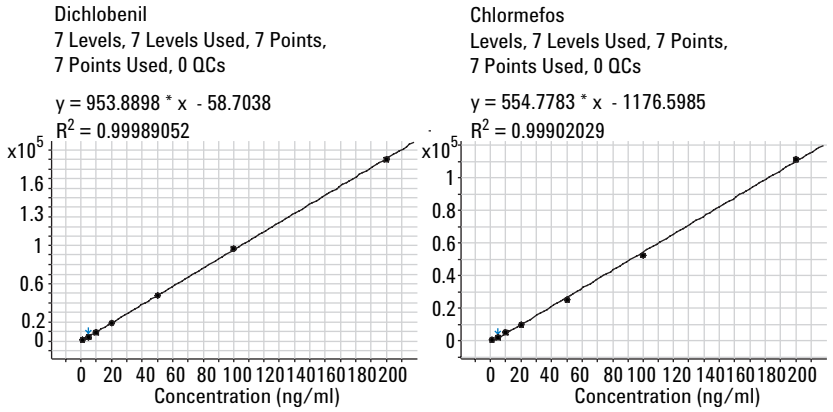


Figure 8
Calibration curves showing excellent linearity over the concentration from 1 ppb to 200 ppb range R^2= 0.999 respectively for Dichlobenil and Chlormefos.

Results

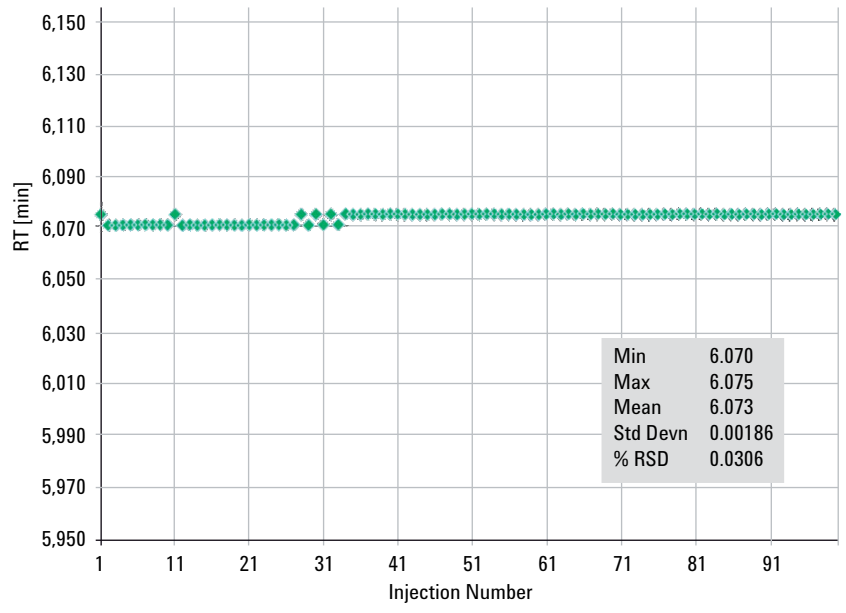


Figure 9
Exceptional retention time stability of Trifluralin with 100 injections of lettuce extract thanks to column backflushing.



Conclusion

- Agilent's 7000 Series Triple Quadrupole GC/MS in combination with the 7890 GC is a sensitive and rugged tool for target pesticide analysis in complex matrices. The single multi-residue method we developed also meets the performance and identity confirmation criteria defined by the stringent EU regulations.

- Excellent selectivity has been achieved to allow unambiguous confirmation of identity for these 360 pesticides even in very complex food matrices and generic sample clean up. Agilent SampliQ QuEChERS kits enable you to prepare food samples for multiresidue, multi-class pesticide analysis with just a few simple steps.

- For this new GC/MS/MS method, the Agilent Retention Time Locking (RTL) database was used to calibrate the retention times of all pesticides. Therefore, the presented GC/MS/MS method can be easily transferred to other Agilent 7000 Series Triple Quadrupole systems with minimum effort and time.

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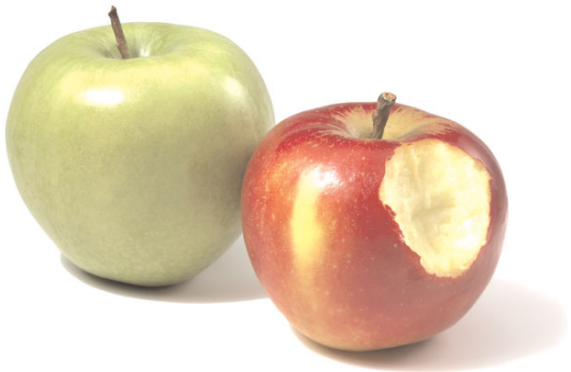
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Agilent Technologies

A Method for the Trace Analysis of 175 Pesticides Using the Agilent Triple Quadrupole GC/MS/MS

Application Note

Food Safety

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Abstract

A GC/MS/MS multiple reaction monitoring (MRM) method has been developed on the Agilent 7890A/7000A GC triple quadrupole mass spectrometer system (GC/QQQ) for 175 commonly analyzed pesticides. Numerous fruit and vegetable extracts were analyzed by this method and by a GC single quadrupole mass spectrometer (GC/Q) for comparison. The GC/Q was operated in the selected ion monitoring (SIM) mode and in the scan mode. Scan results were evaluated using Agilent's Deconvolution Reporting Software (DRS) with the RTL Pesticide and Endocrine Disruptor Database. The GC/Q instrument was equipped with a Multimode inlet and injections of 5 µL were made in the cold splitless mode. These were compared to 1 µL injections of the same extracts on the GC/QQQ. The GC/QQQ was found to be far more sensitive and selective than either GC/Q approach, primarily because there is much less interference from co-extracted matrix. There is, however, still a need for GC/Q methods that use DRS to screen for more than 900 pesticides and other contaminants since the GC/QQQ in the MRM mode is only for target compound analysis.

Agilent Technologies

Introduction

Pesticide residue analysis is a complex task requiring the analyst to search for dozens, or even hundreds, of compounds in a wide variety of crop matrices. Extraction techniques, such as the QuEChERS method [1–3] leave large amounts of indigenous materials in the extract. The use of more extensive cleanup steps risks removing pesticide residues in addition to the matrix. As required detection limits for many pesticides fall to 10 µg/Kg (10 ppb) or lower, more sophisticated analytical tools are needed.

For GC-amenable pesticides, many laboratories are using two complementary techniques for screening and confirmation purposes. For broad screening at the 5 to 100 ppb level, GC/single quadrupole (GC/Q) is employed with Deconvolution Reporting Software (DRS) and the RTL Pesticide and Endocrine Disruptor library from Agilent Technologies [4–6]. This is a scan method to screen for 927 GC-amenable pesticides and endocrine disruptors in a single GC/MS run. Detection limits for most pesticides vary from approximately 5 to 100 ppb, depending upon the matrix and the injection volume [4]. For target pesticide analysis in the most complex matrices, the Agilent 7890A/7000A GC/triple quadrupole (GC/QQQ) is unmatched.

This paper compares three mass spectral techniques for the analysis of pesticide residues in a variety of crop matrices. Spiked and unspiked samples were analyzed by GC/Q in the selected ion monitoring (SIM) mode and in the scan mode with DRS analysis. The same samples were also analyzed by GC/QQQ using a multiple reaction monitoring (MRM) method for 175 pesticides. The objective was to compare the ability of these GC/Q and GC/QQQ methods to detect low levels of pesticides in several different crop matrices.

Experimental

Samples

Spiked and unspiked extracts of fresh produce were provided by the U.S. Food and Drug Administration (U.S. FDA, CFSAN, College Park, MD) and the U.S. Department of Agriculture (USDA ARS, ERRC, Wyndmoor, PA). Samples from the FDA were prepared using the QuEChERS [1–3] method modified to include the use of activated carbon as an additional sorbent. The resultant toluene solution contained 4.5 grams of produce per milliliter of extract. Samples from the USDA were extracted using the published QuEChERS method and contained 1 gram of produce per milliliter of acetonitrile solvent.

Instrumentation

The GC/Q and GC/QQQ systems used for these experiments are described in Tables 1 and 2.

Table 1. Instrumentation and Analytical Conditions for the GC/Q System

GC	Agilent 7890A Series
Autosampler	Agilent 7693A Injector and sample tray
Inlet	Multimode inlet
Carrier gas	Helium
Inlet pressure	18.420 psi (constant pressure mode) during run 2.0 psi (during backflush)
Splitless Mode Inlet Parameters	
Temperature	250 °C
Inlet liner	Helix double taper, deactivated (P/N 5188-5398)
Injection volume	1 µL
Purge flow to split vent	30 mL/min at 0.75 min
Cold Splitless Mode Inlet Parameters	
Temperature program	60 °C (0.01 min), 700 °C/min to 280 °C (hold)
Inlet liner	Helix double taper, deactivated (P/N 5188-5398)
Injection volume	5 µL
Purge flow to split vent	30 mL/min at 1.25 min
Oven temperature program	70 °C (1 min), 50 °C/min to 150 °C (0 min), 6 °C/min to 200 °C (0 min), 16 °C/min to 280 °C (5 min)
Capillary flow technology	2-way splitter with one port capped – used for backflushing the analytical column and retention gap
Pneumatic Control Module (PCM)	Helium plumbed to 2-way splitter
PCM pressure	4.0 psi during run, 60.0 psi during backflush
Analytical column	Agilent J&W HP-5ms UI 15 m × 0.25 mm × 0.25 µm (P/N 19091S-431UI)
Connections	Between retention gap and 2-way splitter
Retention gap	2.0 m × 0.25 mm Siltek deactivated fused silica tubing (Restek, Bellefonte, PA)
Connections	Between inlet and analytical column using an Ultimate Union (P/N G3182-61580) to couple the retention gap to the column
Restrictor	80 cm × 0.15 mm deactivated fused silica tubing (Agilent)
Connections	Between the 2-way splitter and the MSD
Initial column flow rate	2.705 mL/min (nominal)
Retention time locking	Chlorpyrifos-methyl locked to 8.298 min
Mass selective detector	Agilent 5795C Series with performance turbo pump
Mode	Electron impact
Transfer line temperature	280 °C
Source temperature	230 °C
Quadrupole temperature	150 °C
Threshold	100
Sampling rate	A/D = 4
Gain factor	1
SIM dwell times	Variable from 4 to 25 ms
Trace ion detection	On
Solvent delay	2.5 min
Backflushing Conditions	
Timing	5 min duration during post-run
Oven temperature	280 °C

(Continued)

Table 1. Instrumentation and Analytical Conditions for the GC/Q System (continued)

Aux EPC pressure	60 psi
Inlet pressure	2 psi
Software	
GC/MSD	Agilent GC/MS ChemStation control and data analysis software (P/N G1701EA E.02.00 SP1)
Deconvolution Reporting Software	Agilent P/N G1716AA (Ver. A.04.00)
Library Searching Software	NIST MS Search (Ver 2.0d) (comes with NIST mass spectral library – Agilent P/N G1033A)
Deconvolution software	Automated Mass Spectral Deconvolution and Identification Software (AMDIS_32 version 2.62 or greater; comes with NIST mass spectral library – Agilent P/N G1033A)
MS Libraries	NIST 08 mass spectral library (Agilent P/N G1033A) Pesticide and Endocrine Disruptor Database (Agilent P/N G1672AA)

Table 2. Instrumentation and Analytical Conditions for the GC/QQQ system

GC	Agilent 7890A Series
Autosampler	Agilent 7683A Injector and sample tray
Inlet	Split/splitless
Inlet liner	Helix double taper, deactivated (P/N 5188-5398)
Carrier gas	Helium
Inlet pressure	18.350 psi (constant pressure mode) during run 1.0 psi (during backflush)
Inlet temperature	250 °C
Injection volume	1 µL
Purge flow to split vent	30 mL/min at 0.75 min
Gas saver	On (20 mL/min at 2.0 min)
Oven temperature program	70 °C (1 min), 50 °C/min to 150 °C (0 min), 6 °C/min to 200 °C (0 min), 16 °C/min to 280 °C (5.5 min)
Capillary flow technology	Purged Ultimate Union (P/N G3186B) – used for backflushing the analytical column and retention gap
Aux EPC gas	Helium plumbed to Purged Ultimate Union
Aux EPC pressure	4.0 psi during run, 80.0 psi during backflush
Analytical column	Agilent J&W HP-5ms UI 15 m × 0.25 mm × 0.25 µm (P/N 19091S-431UI)
Connections	Between retention gap and Purged Ultimate Union (P/N G3186B)
Retention gap	2.0 m × 0.25 mm Siltek deactivated fused silica tubing (Restek, Bellefonte, PA)
Connections	Between inlet and analytical column using ultimate union (P/N G3182-61580) to couple the retention gap to the column
Restrictor	65 cm × 0.15 mm deactivated fused silica tubing (Agilent)
Connections	Between the Purged Ultimate Union (P/N G3186B) and the MSD
Initial column flow rate	2.688 mL/min (nominal)
Retention time locking	Chlorpyrifos-methyl locked to 8.298 min
Triple Quadrupole Mass Spectrometer	Agilent 7000A Series
Mode	Electron impact
Transfer line temperature	280 °C
Solvent delay	2.3 min
Source temperature	300 °C
Quadrupole temperature	Q1 and Q2 = 150 °C
MRM Mode Conditions	
MS1 resolution	1.2 u
MS2 resolution	1.2 u
Collision gas flows	Nitrogen at 1.5 mL/min, Helium at 2.35 mL/min
Backflushing Conditions	
Timing	3 min duration during post-run
Oven temperature	280 °C
Aux EPC pressure	80 psi
Inlet pressure	1 psi
Software	
Data acquisition	Agilent MassHunter Data Acquisition Software (Ver. B.04.00)
Qualitative analysis	MassHunter Workstation Software for Qualitative Analysis (Ver. B.03.01)
Quantitative analysis	MassHunter Workstation Software for Quantitative Analysis (Ver. B.03.01)

Results and Discussion

GC Configuration

Both GC systems used a 15-m × 0.25 mm × 0.25 µm Agilent J&W HP-5ms UI column and were running the standard Agilent pesticide method [7] at 2X speed. This method uses an initial oven temperature of 70 °C, which works for most GC solvents without using a retention gap. However, 1-µL injections of samples in toluene lead to poor peak shape, so a 2-m deactivated retention gap was coupled to the front of the column. This improved the peak shapes.

Column backflushing is essential for the analysis of food extracts [4, 8–9] because they usually contain high-boiling indigenous compounds. In just a few runs, these materials can collect on the head of the column (or retention gap), causing peak tailing and retention time shifts. Over time, they can migrate from the column to the mass spec source, which would eventually have to be cleaned.

Agilent's capillary flow technology makes column backflushing routine (4, 8–9) and several different capillary flow devices can be used for the purpose. The GC/QQQ system used a Purged Ultimate Union, while the GC/Q system used a two-way splitter (with one port capped). In both cases, the analytical column was connected to the capillary flow device. A short restrictor was used to couple the capillary flow device to the mass spectrometer. Figure 1 shows the configuration of each instrument.

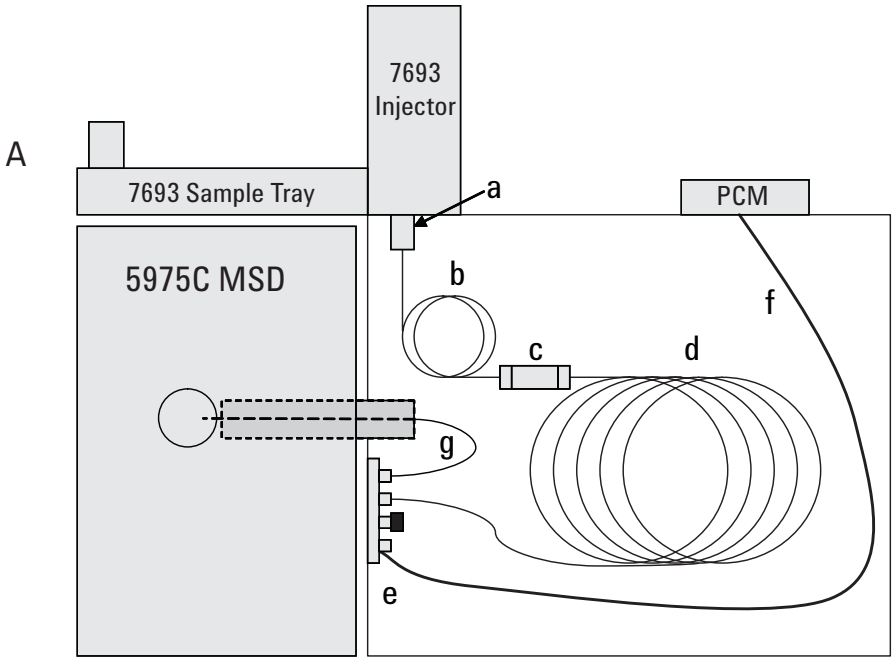


Figure 1A. The GC/MSD used for scan and SIM analyses was configured with a) Multimode inlet, b) 2 m × 0.25 mm deactivated retention gap, c) Ultimate Union, d) 15 m × 0.25 X 0.25 µm Agilent J&W HP-5ms UI column, e) two-way purged splitter with one port capped, f) helium purge flow controlled by a pneumatic control module (PCM), and g) 80 cm × 0.15 mm deactivated restrictor.

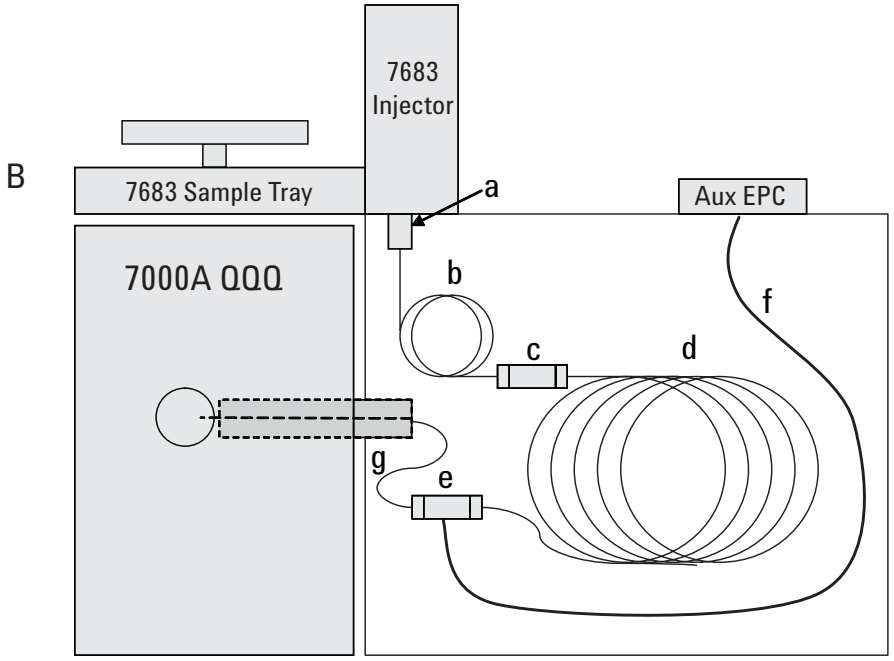


Figure 1B. The GC/QQQ used for MRM analyses was configured with a) split/splitless inlet, b) 2 m × 0.25 mm deactivated retention gap, c) Ultimate Union d) 15 m × 0.25 × 0.25 µm Agilent J&W HP-5ms UI column, e) Purged Ultimate Union, f) helium purge flow, and g) 65 cm × 0.15 mm deactivated restrictor.

MRM Method

A method was developed for the analysis of 175 commonly analyzed pesticides. Two transitions were determined for each compound and the collision energy was optimized for each. Since the method was locked to the Agilent Pesticide method (running at twice the original speed), the retention times correspond to those recorded in Agilent's RTL Pesticide and Endocrine Disruptor Database (P/N G1672AA) divided by two. There are small differences in RT between the database and values shown here because this method used a retention gap, capillary flow device, and a restrictor. Table 3 lists the pesticides in alphabetical order with their retention times, quant and qual transitions, and the collision energies for each.

Table 3. Target and Qualifier Transitions for 175 Pesticides

		Quant transition			Qual transition		
Compound name	**RT (min)**	**Precursor ion**	**Product ion**	**CE**	**Precursor ion**	**Product ion**	**CE**
Acrinathrin	15.371	181.1	152.1	25	181.1	127.1	30
Akton	11.403	282.9	219.0	10	282.9	184.0	25
Alachlor	8.507	188.1	130.1	40	188.1	160.1	10
Aldrin	9.247	262.9	192.9	40	262.9	190.9	40
Allethrin	10.908	123.1	81.1	10	123.1	79.1	20
Atrazine	6.581	200.1	122.1	10	200.1	104.0	20
Azamethidaphos (Azamethiphos)	13.248	215.0	171.0	15	215.0	128.0	30
Azinphos-methyl	14.835	160.1	77.1	20	160.1	132.1	0
Benfluralin	5.842	292.1	264.0	10	292.1	160.1	20
BHC, α-	6.025	181.0	145.0	15	181.0	109.0	30
BHC, ß-	6.595	181.0	145.0	15	181.0	109.0	30
BHC, δ-	7.266	181.0	145.0	15	181.0	109.0	30
Bifenthrin	14.428	181.1	165.1	30	181.1	166.1	15
Bromacil	9.186	205.0	132.0	30	205.0	187.9	20
Bromophos	10.020	330.9	315.9	20	330.9	285.9	35
Bromophos-ethyl	11.261	358.9	302.9	15	358.9	284.8	35
Bromopropylate	14.320	183.0	155.0	15	183.0	76.0	35
Captan	10.617	79.1	77.1	10	79.1	51.1	25
Carbophenothion	13.316	157.0	121.0	25	157.0	75.1	40
Chlordane, *cis*-	11.410	372.9	265.9	40	372.9	263.9	30
Chlordane, *trans*-	11.010	372.9	265.9	20	372.9	263.9	25
Chlordene, α-	8.562	230.0	160.0	40	230.0	195.0	25
Chlordene, ß-	9.376	230.0	160.0	35	230.0	195.0	25
Chlordene, γ-	9.314	230.0	160.0	40	230.0	195.0	25
Chlorfenvinphos, ß-	10.779	267.0	159.0	20	267.0	81.0	40
Chlorobenzilate	12.706	139.0	111.0	15	139.0	75.0	30
Chloroneb	4.323	191.0	113.0	15	191.0	141.0	10
Chlorothalonil	7.395	265.9	133.0	40	265.9	230.9	20
Chlorpyrifos	9.606	196.9	168.9	15	196.9	107.0	40
Chlorpyrifos-methyl	8.284	286.0	93.0	25	286.0	270.9	20
Chlorthiophos	13.051	268.9	205.0	15	268.9	177.0	25
Coumaphos	15.859	362.0	109.0	15	362.0	81.0	40
Cyanazine	9.694	212.1	123.1	20	212.1	151.1	10
Cyanophos	6.887	243.0	109.0	10	243.0	79.0	30
Cyfluthrin 1	16.144	163.0	127.1	5	163.0	91.1	15
Cyfluthrin 2	16.212	163.0	91.1	15	163.0	127.1	5

(Continued)

Compound name	RT (min)	Quant transition			Qual transition		
		Precursor ion	Product ion	CE	Precursor ion	Product ion	CE
Cyfluthrin 3	16.273	163.0	127.1	5	163.0	91.1	15
Cyfluthrin 4	16.307	163.0	127.1	5	163.0	91.1	15
Cyhalothrin, λ-	15.208	181.1	152.1	30	181.1	127.1	35
Cypermethrin 1	16.381	181.1	152.1	25	181.1	127.1	35
Cypermethrin 2	16.463	181.1	152.1	30	181.1	127.1	35
Cypermethrin 3	16.531	181.1	152.1	25	181.1	127.1	30
Cypermethrin 4	16.558	181.1	152.1	25	181.1	127.1	30
Dacthal (DCPA) (Chlorthal-Dimethyl)	9.721	300.9	222.9	25	300.9	166.9	40
DDD, o,p'-	12.170	235.0	165.1	30	235.0	199.1	15
DDD, p,p'-	12.841	235.0	165.1	25	235.0	199.1	20
DDE, o,p'-	11.241	246.0	176.1	40	246.0	211.0	20
DDE, p,p'-	12.007	246.0	176.1	40	246.0	175.1	40
DDT, o,p'-	12.882	235.0	165.1	30	235.0	199.1	20
DDT, p,p'-	13.492	235.0	165.1	30	235.0	199.1	20
DEF (Tribufos)	12.054	169.0	57.1	5	169.0	112.9	5
Deltamethrin	18.016	181.1	152.1	25	181.1	127.1	25
Demeton-S	6.303	88.1	60.0	5	88.1	59.0	20
Demeton-S-methyl	5.230	88.1	60.0	5	88.1	59.0	15
Dialifos	15.432	208.0	102.1	40	208.0	89.0	40
Diallate 1	5.957	234.1	150.0	20	234.1	192.0	10
Diallate 2	6.127	234.1	150.0	20	234.1	192.0	10
Diazinon	7.226	179.1	121.1	40	179.1	137.2	20
Dicapthon	9.694	262.0	216.0	15	262.0	123.0	40
Dichlofenthion	8.067	279.0	223.0	15	279.0	205.0	30
Dichlofluanid	9.199	123.0	77.1	20	123.0	51.1	40
Dichlorobenzophenone, 4,4'-	9.593	139.0	111.0	15	139.0	75.1	30
Dichlorvos	2.905	109.0	79.0	5	109.0	47.0	15
Diclobenil	3.367	171.0	100.0	25	171.0	136.0	15
Dicloran	6.269	206.0	176.0	10	206.0	124.0	30
Dieldrin	11.926	262.9	192.9	40	262.9	190.9	35
Dimethachlor	8.080	134.1	105.1	15	134.1	77.1	30
Dioxathion	15.934	125.0	97.0	5	125.0	65.0	25
Disulfoton	7.260	88.1	60.0	5	88.1	59.0	25
Ditalimfos	11.586	130.0	102.1	15	130.0	75.0	30
Edifenphos	13.377	173.0	109.0	15	173.0	65.1	40
Endosulfan ether	7.660	240.9	205.9	20	240.9	203.9	20
Endosulfan I	11.308	240.9	205.9	15	240.9	136.0	40
Endosulfan II	12.570	195.0	125.0	25	195.0	159.0	10
Endosulfan sulfate	13.377	271.9	236.9	20	271.9	116.9	40
Endrin	12.366	262.9	193.0	35	262.9	190.9	35
Endrin aldehyde	12.956	249.9	214.9	35	249.9	141.9	40
Endrin ketone	14.116	316.9	101.0	20	316.9	245.0	20
EPN	14.333	157.0	77.1	25	157.0	110.0	15
Ethalfluralin	5.632	276.1	105.1	35	276.1	202.0	20
Ethion	12.997	231.0	128.9	25	231.0	174.9	10
Ethoprop (Ethoprophos)	5.357	158.0	97.0	15	158.0	114.0	5
Etridazole	3.963	183.0	139.9	20	183.0	108.0	40

(Continued)

Compound name	RT (min)	Quant transition			Qual transition		
		Precursor ion	Product ion	CE	Precursor ion	Product ion	CE
Famphur	13.329	218.0	109.0	15	218.0	79.0	30
Fenamiphos (Phenamiphos)	11.803	303.1	80.0	40	303.1	154.0	20
Fenarimol	15.222	139.0	111.0	15	139.0	75.0	35
Fenchlorphos (Ronnel)	8.650	284.9	269.9	15	284.9	239.9	35
Fenitrothion	9.030	277.0	109.0	20	277.0	260.0	5
Fenpropathrin	14.503	181.1	152.1	30	181.1	127.1	35
Fensulfothion	12.780	292.0	156.0	25	292.0	109.0	20
Fenthion	9.552	278.0	109.0	20	278.0	125.0	20
Fenvalerate 1	17.202	167.1	125.0	15	167.1	89.1	40
Fenvalerate 2	17.412	167.1	125.0	10	167.1	89.1	35
Fluchloralin	7.321	306.1	264.1	5	306.1	206.0	15
Flucythrinate 1	16.571	199.1	107.1	30	199.1	157.1	10
Flucythrinate 2	16.741	199.1	107.1	25	199.1	157.1	5
Fluridone	16.944	328.1	259.0	30	328.1	189.1	40
Fluvalinate τ- 1	17.412	250.1	55.1	15	250.1	200.1	20
Fluvalinate τ- 2	17.480	250.1	55.1	15	250.1	200.1	25
Folpet	10.807	147.1	103.1	5	147.1	76.0	30
Fonophos	6.934	246.1	109.0	15	246.1	137.0	5
Heptachlor	8.379	271.9	236.8	25	271.9	116.9	40
Heptachlor exo-epoxide isomer A	10.474	183.0	118.9	30	183.0	154.9	15
Heptachlor exo-epoxide isomer B	10.352	352.9	262.8	25	352.9	281.9	20
Hexachlorobenzene	6.168	283.9	213.9	35	283.9	248.8	25
Hexazinone	13.702	171.1	71.1	15	171.1	85.1	15
Iprobenfos (IBP)	7.660	204.0	91.1	10	204.0	121.0	40
Iprodione	14.211	187.0	124.0	25	187.0	159.0	15
Isazophos	7.517	161.1	119.0	10	161.1	146.0	5
Isofenfos	10.813	213.1	121.0	20	213.1	185.0	5
Jodfenphos (Iodofenphos)	11.776	376.9	361.9	20	376.9	93.0	35
Leptophos	14.876	171.0	77.1	25	171.0	124.1	10
Lindane (γ-BHC)	6.710	181.0	145.0	15	181.0	109.0	30
Malathion	9.396	173.1	99.0	15	173.1	117.0	10
Methidathion	11.146	145.0	85.1	5	145.0	58.1	15
Methoxychlor, o,p'-	13.730	227.1	121.1	15	227.1	91.1	35
Methoxychlor, p,p'-	14.442	227.1	141.1	40	227.1	169.1	30
Metolachlor	9.450	162.1	133.1	15	162.1	132.1	25
Mevinphos	3.782	127.0	109.0	10	127.0	95.0	15
Mirex	14.923	271.9	236.9	15	271.9	116.9	40
Nonachlor, *cis*-	12.848	408.8	109.0	20	408.8	299.9	25
Nonachlor, *trans*-	11.539	408.8	299.8	25	408.8	301.8	30
Oxadiazon	12.210	175.0	112.0	15	175.0	76.1	40
Parathion	9.633	291.1	109.0	10	291.1	81.0	40
Parathion methyl	8.284	263.0	109.0	10	263.0	79.0	35
Pentachloroaniline	7.761	264.9	193.9	30	264.9	155.9	30
Pentachlorobenzene	4.459	249.9	214.9	25	249.9	142.0	40
Pentachlorobenzonitrile	6.866	274.9	239.9	20	274.9	204.9	35
Pentachlorophenyl methyl ester	6.283	264.9	236.9	10	264.9	142.9	40
Pentachlorothioanisole	9.016	295.9	245.8	40	295.9	262.9	15

(Continued)

Compound name	RT (min)	Quant transition			Qual transition		
		Precursor ion	Product ion	CE	Precursor ion	Product ion	CE
Permethrin, *cis-*	15.703	183.1	153.1	15	183.1	168.1	15
Permethrin, *trans-*	15.798	183.1	155.1	10	183.1	165.1	10
Phenanthrene-d10	6.863	188	160	10	188	186	10
Phenothrin	14.713	183.1	153.1	15	183.1	168.1	15
Phenthoate	10.861	274.0	121.0	10	274.0	125.0	20
Phorate	5.961	231.0	128.9	25	231.0	174.9	10
Phosalone	14.855	182.0	111.0	15	182.0	75.1	40
Phosmet	14.259	160.0	77.1	30	160.0	133.0	15
Pirimiphos ethyl	10.332	318.1	166.1	15	318.1	182.1	15
Pirimiphos methyl	9.138	290.1	125.0	25	290.1	233.0	10
Procymidone	10.983	283.0	96.1	10	283.0	67.1	40
Profenofos	11.953	207.9	63.1	40	207.9	99.0	25
Propachlor	5.164	120.1	77.1	20	120.1	92.1	5
Propargite	13.858	135.1	107.1	15	135.1	77.1	30
Propazine	6.676	214.1	172.0	10	214.1	104.0	20
Propetamphos	6.948	138.0	110.0	5	138.0	64.0	15
Propyzamide	6.975	173.0	145.0	15	173.0	109.0	35
Prothiophos	11.878	162.0	63.1	40	162.0	98.0	20
Pyraclofos	15.439	360.0	96.9	35	360.0	194.0	15
Pyrazophos	15.351	221.1	193.1	10	221.1	149.1	15
Pyridaphenthion	14.272	340.1	199.1	5	340.1	97.0	40
Quinalphos	10.827	146.1	118.1	10	146.1	91.1	30
Quintozene	6.832	236.9	118.9	25	236.9	142.9	30
Resmethrin	13.994	123.1	81.1	5	123.1	95.1	5
Simazine	6.473	201.1	173.1	5	201.1	138.1	10
Sulfotep-ethyl	5.902	322.0	146.0	25	322.0	65.0	40
Sulprofos	13.180	322.0	97.0	30	322.0	156.0	5
Tebupirimfos	7.687	261.1	137.1	15	261.1	153.1	20
Tecnazene (TCNB)	5.110	202.9	83.0	25	202.9	142.9	20
Tefluthrin	7.524	177.1	127.1	20	177.1	137.0	20
Temephos	20.525	125.0	47.0	20	125.0	79.0	10
Terbufos	6.890	231.0	128.9	25	231.0	174.9	10
Terbuthylazine	6.907	214.1	104.0	20	214.1	132.0	10
Tetrachloroaniline, 2,3,5,6-	5.293	230.9	158.0	25	230.9	122.0	40
Tetrachlorvinphos	11.478	329.0	109.0	25	329.0	79.0	35
Tetramethrin I	14.299	164.1	107.1	15	164.1	135.1	10
Tetramethrin II	14.421	164.1	107.1	10	164.1	135.1	5
Thiometon	6.161	125.0	47.0	20	125.0	79.0	10
Tolclofos methyl	8.392	265.0	250.0	15	265.0	93.0	25
Tolyfluanid	10.623	137.0	91.1	20	137.0	65.1	35
Triallate	7.470	268.0	183.9	25	268.0	226.0	15
Triazophos	13.241	161.0	134.1	10	161.0	91.1	20
Trifluralin	5.808	306.1	264.0	5	306.1	160.0	30
Triphenyl phosphate	13.865	326.1	169.1	35	326.1	233.0	10
Vinclozolin	8.311	212.0	145.0	25	212.0	109.0	40

Carrot Extract

A carrot extract with incurred pesticide residues was analyzed in the scan and SIM modes with the GC/Q. In each case, 5-µL injections were made using Agilent's new Multimode inlet operated in the cold splitless mode. Three SIM methods were used to monitor > 170 compuonds with about 60 pesticides in each method. Four ions were monitored for each compound. The scan data were analyzed automatically using Agilent's Deconvolution Reporting Software, together with the 927-compound RTL Pesticide and Endocrine Disruptor Database.

The same carrot sample was also analyzed on the 7890A/7000A GC/QQQ system using the MRM transitions listed in Table 3. An 11-point calibration curve was prepared in carrot matrix for 170 pesticides from 3.33 µg/kg (ppb) to 6670 µg/kg. Table 4 shows the results of these analyses.

Table 4. Results from the Analysis of a Carrot Extract with Incurred Pesticides by GC/MS in the Scan Mode with DRS Analysis, by GC/MS in the SIM Mode, and by GC/MS/MS in the MRM Mode (An X implies that the compound was found by that method.)

	GC/Q 5 µL (Multimode inlet)		GC/QQQ[a] 1 µL
Pesticide	Cold SL scan + DRS	Cold SL SIM	Hot SL (ppb)
Diclobenil			0.38[b]
Pentachlorobenzene			0.75[b]
Trifluralin			2.3[b]
Tefluthrin			0.53[b]
4,4'-Dichlorobenzophenone			1.2[b]
Chlorpyrifos			24.7
o,p'-DDE			3.7
p,p'-DDE	X	X	240
o,p'-DDD			9
p,p'-DDD o,p'-DDT	X X		Sum = 45
p,p'-DDT	X	X	130
Fenazaquin	X	Not in method	Not in method

a. The actual concentration of these compounds was lower in the original carrot sample by a factor of 4.5 since the extraction method results in 4.5 g of produce per mL of extract.

b. The reported values fall below the lowest point on the calibration curve.

The single quad methods were not quantitative, so Table 4 only indicates (with an X) if a pesticide was found, either by DRS or by manual examination of the SIM data. Since the triple quad method was calibrated, the amount of each pesticide could be determined. The amounts reported are those found in the extract. Because the extraction method concentrated this sample by a factor of 4.5:1 (4.5 g of carrot to 1.0 mL of final extract), the pesticide concentrations in the original carrot samples were actually lower by this factor.

The scan method with DRS analysis has the capability to find any of the 927 compounds in the database, while the SIM and MRM methods are limited to the 175 target compounds listed in Table 3. DRS found fenazaquin, a pesticide that was not in the SIM or MRM methods. This demonstrates the advantage of using GC/MS with DRS for screening purposes in combination with GC/MS/MS for target compound analysis.

In spite of the concentrated carrot matrix, the GC/QQQ was able to detect three pesticides below 1 ppb (1 µg/kg) and three more below 5 ppb. The lowest level calibration standard was prepared at 3.33 ppb, so numbers reported below that level are extrapolated values. The optimal MRM transitions for p,p'-DDD and o,p'-DDT are the same and, since these two compounds were only partially resolved chromatographically, they are reported together.

Figure 2A shows the extracted quant ion (*m/z* 246) for p,p'-DDE from the scan analysis of the carrot sample. Interferences in these chromatograms make it harder to do an accurate quantitative analysis without first deconvoluting the spectrum. After deconvolution (Figure 2B), ChemStation integration is trivial. Figure 2C shows the EIC (*m/z* 246) from the GC/MS SIM analysis of the same sample. Although the signal/noise ratio (S/N) is 10-fold better, there appear to be more interferences.

It is easy to see the advantage of the GC/QQQ for target compound analysis. A 1-µL injection of the carrot extract on this instrument gave a clean MRM chromatographic peak (Figure 2D) with better S/N (434) than was obtained for the 5-µL GC/Q SIM analysis (S/N = 375)(Figure 2C).

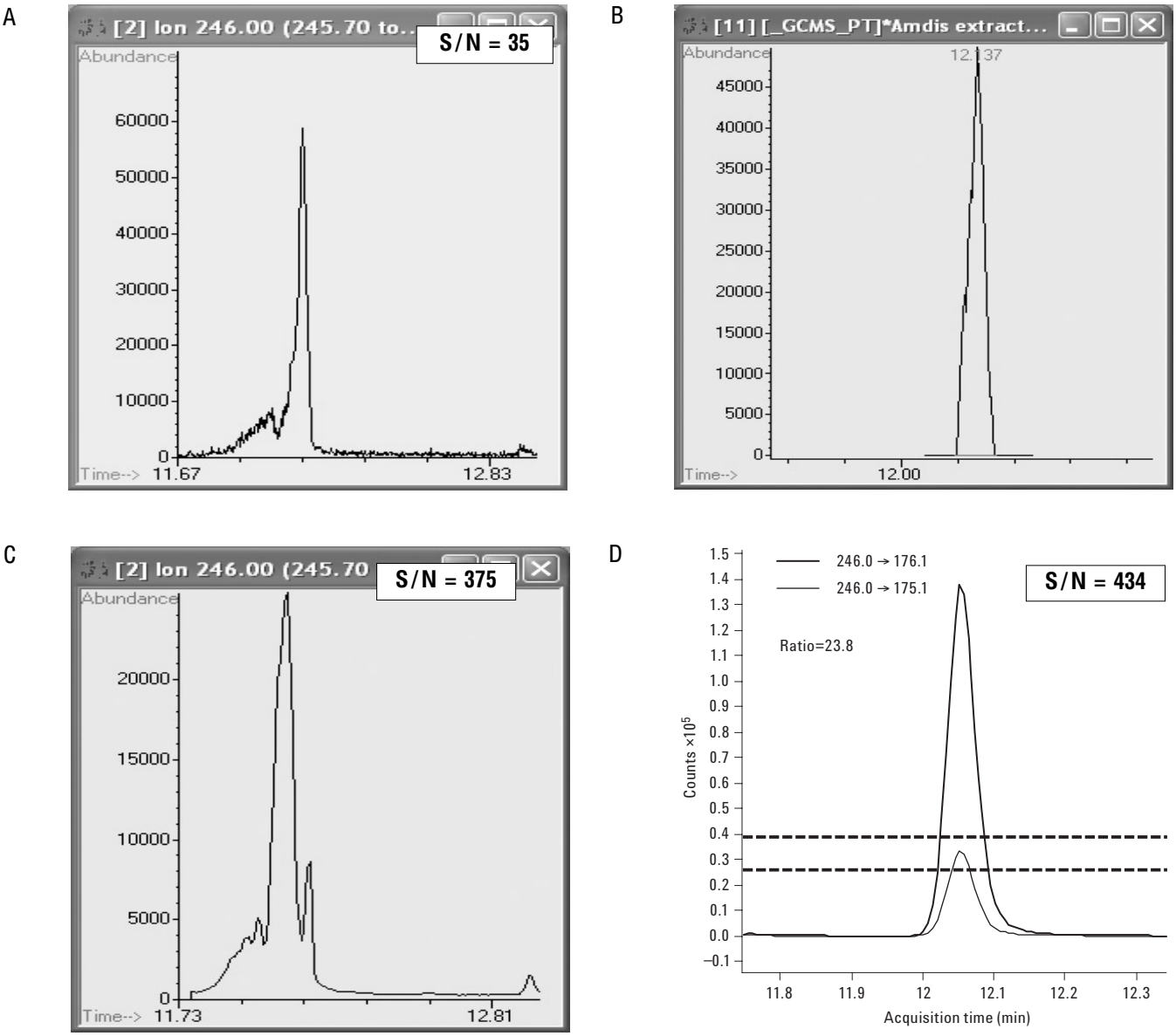


*Figure 2. A) p,p'-DDE quant ion (*m/z *246) extracted from the scan chromatogram obtained from a 5-µL cold splitless injection of a carrot extract with incurred pesticides. B) Same as in (A) but after deconvolution. C) p,p'-DDE quant ion (*m/z *246) extracted from a SIM chromatogram obtained from a 5-µL cold splitless injection of the same sample. D) Quant and qualifier transitions (246.0 → 176.1 and 246.0 → 175.1, respectively) for the GC/MS/MS analysis of a 1-µL hot splitless injection of the same carrot extract. Peak-to-peak signal/noise ratios for the extracted ions and the quant transition are shown. The ratio of the two transition ions (D) is 23.8, confirming the presence of p,p'-DDE.*

Comparing GC/MS SIM to GC/MS/MS MRM – Various Matrices

Figure 3 compares GC/MS SIM results to GC/MS/MS MRM results for p,p'-DDE spiked into various commodities at 10 ppb. On the left, the SIM EICs for the quant ion (*m/z* 246) show increasing amounts of matrix interference from the apple, cabbage, ginseng, orange, and spinach samples. In contrast, the p,p'-DDE GC/MS/MS transitions shown on the right have no interferences from any of the extracts. The large S/N values shown for the quant transition (246.0 → 176.1) suggest that one should be able to detect p,p'-DDE at the sub-ppb level.

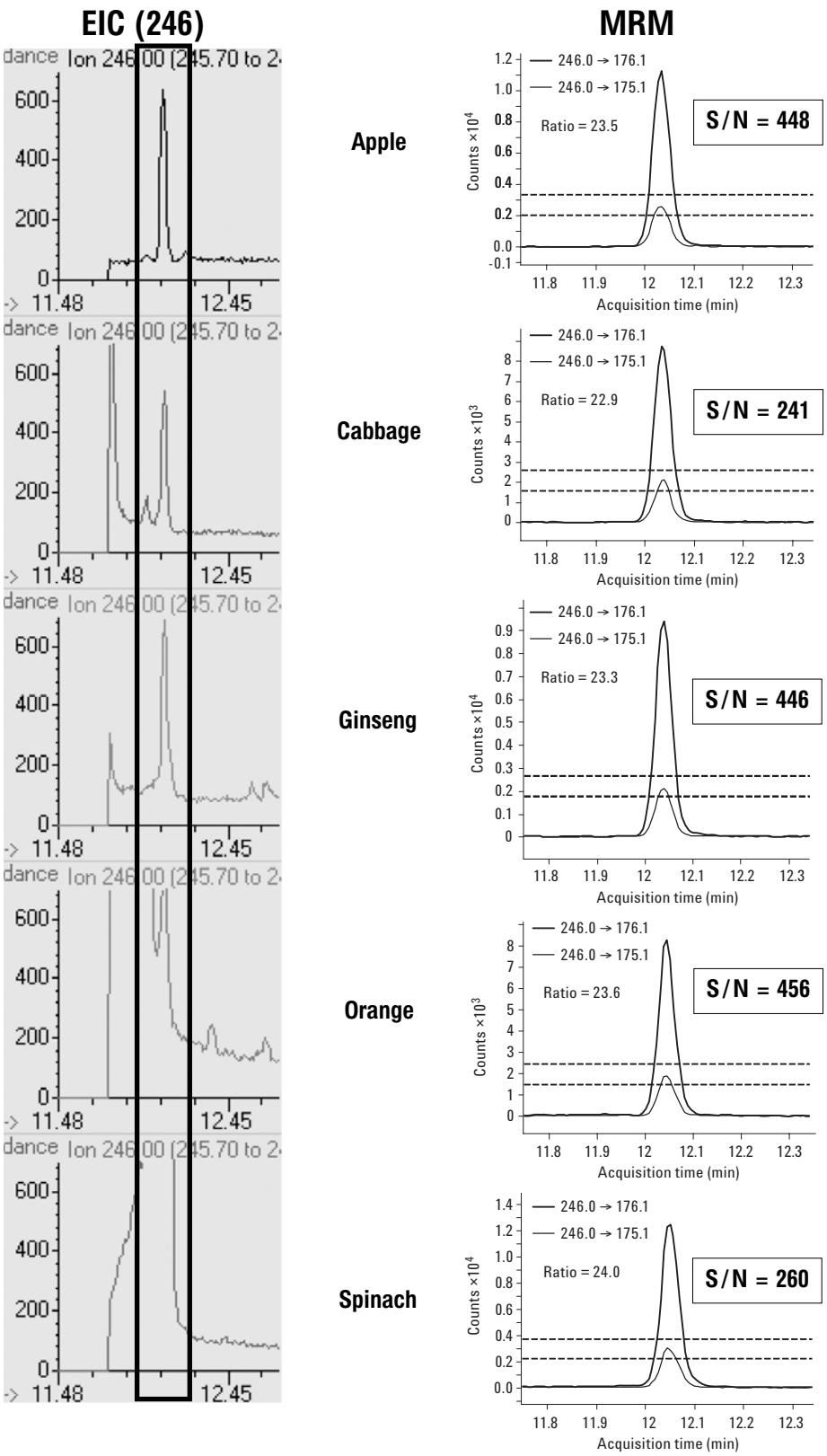


*Figure 3. A comparison of GC/MS SIM and GC/MS/MS MRM analysis for p,p'-DDE spiked at 10 ppb (10 µg/kg) into five different produce extracts. On the left, the EICs for the p,p'-DDE quant ion (*m/z *246) show increasing amounts of interference from the matrix. The transitions on the right (246.0 → 176.1 and 246.0 → 175.1) for p,p'-DDE are clean, with peak-to-peak S/N values ranging from 241 to 448. All injections were 1 µL.*

Tomato Extract

All three techniques being discussed were able to identify incurred chlorothalonil in a tomato extract, which was present at 1 ppm. However, only the GC/QQQ was able to identify pentachlorobenzonitrile, a chlorothalonil metabolite, which it measured at 9.3 ppb. Figure 4 shows the MRM transitions for pentachlorobenzonitrile and a calibration curve for the compound ranging from 3.33 ppb to 6670 ppb.

Backflushing the Column

The norm when analyzing dirty samples by GC/MS is to replace the inlet liner and clip the column frequently. Many labs do this daily. Otherwise, matrix accumulates in the liner and column, degrading the chromatography. Over time, these materials migrate through the GC column and contaminate the source, which then needs to be cleaned. This problem may be compounded with a GC/QQQ instrument because one does not see much evidence of the matrix and the temptation is to ignore maintenance until the source (and sometimes the first quadrupole) needs to be cleaned.

The Agilent 7000A Series triple quad MS uses the same inert source and gold-plated quartz quadrupole that are found in the 5975C MSD. These can be heated up to 350 °C and 200 °C, respectively, which greatly minimizes the need for cleaning, even when high-boiling matrix compounds do reach the detector.

The best way to prevent chromatographic degradation and reduce the need for source cleaning is to backflush the GC column during or after each run. With the configurations shown in Figure 1, backflushing is done for 3 to 5 minutes after the run by raising the pressure at the capillary flow device (two-way splitter or the Purged Ultimare Union) and lowering the inlet pressure. This reverses the flow through the column and purges high-boiling matrix components from the head of the column and out through the inlet's split vent.

During the course of this work, approximately 100 1-µL injections of concentrated food extracts were made into the GC/QQQ system with no evidence of column or MS performance problems. Nearly 300 µL of these same extracts were injected into the GC/Q system before column and inlet maintenance was required. With the capillary flow device installed, you can do this maintenance without venting the mass spectrometer.

Conclusions

Agilent's 7890A/7000A triple quadrupole MS system is a sensitive and rugged tool for target pesticide analysis. There is far less interference from matrix than one sees in single quadrupole methods, making it much easier to quantify pesticides at the low ppb levels required by today's legislation. In many cases a 1-µL injection into the GC/QQQ produced far better results than a 5-µL injection into the GC/Q. Nevertheless, there is still a need for screening methods that look for hundreds of pesticides. For this, we recommend using large-volume injection with Agilent's new Multimode inlet, GC/Q analysis in the scan mode, and data analysis using Deconvolution Reporting Software with Agilent's Pesticide and Endocrine Disruptor Database. The combination of these two approaches is the best way to screen for more than 900 contaminants (by GC/Q with DRS) while performing ultra-trace analysis for a smaller list of target compounds (using GC/QQQ). Both approaches benefit from column backflushing, which is highly recommended when analyzing dirty samples, such as food extracts.

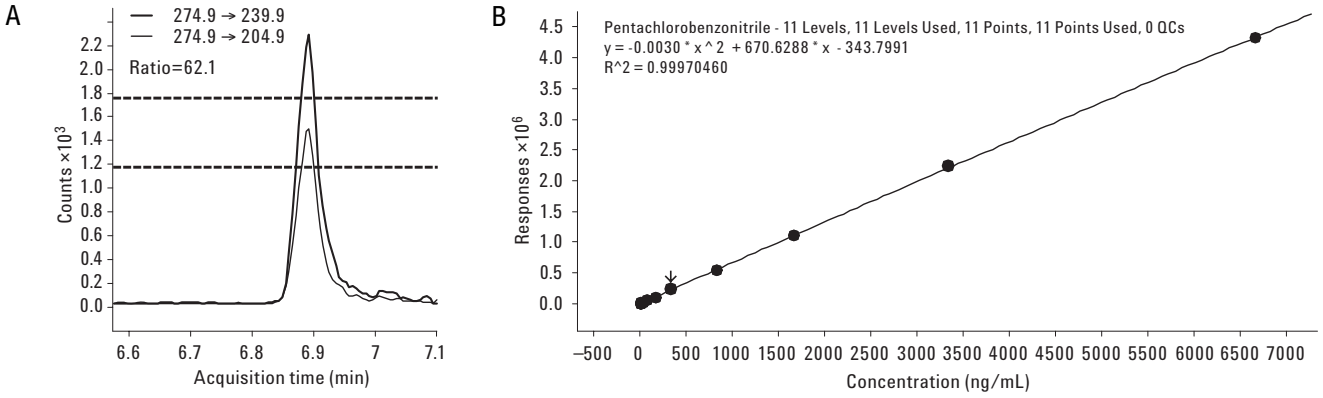


Figure 4. A) MRM transitions identifying pentachlorobenzonitrile at 9.3 ppb in a tomato extract. B) A calibration curve for pentachlorobenzonitrile from 3.33 to 6,670 ppb with a quadratic curve fit > 0.999.

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Agilent Technologies

Organophosphorus Pesticides Analysis Using an Agilent J&W DB-5ms Ultra Inert Capillary GC Column

Application Note

Environmental

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Abstract

Agilent Technologies Inc. has implemented new testing procedures to more effectively evaluate GC column inertness performance. This new testing procedure employs deliberately aggressive probes to thoroughly investigate and verify column inertness and quality. In challenging separations, knowing that the GC column has been thoroughly investigated for column inertness gives analysts higher confidence in the accuracy of their results.

Trace- and ultra trace-level pesticide analyses are important tools for accessing food supply and environmental quality worldwide. In this application note, trace-level organophosphorus pesticide analysis is demonstrated using electron impact single quadrupole scanning mass spectrometry. Agilent's J&W DB-5ms Ultra Inert capillary GC column provides excellent peak shape for even the most problematic pesticides.

Agilent Technologies

Introduction

Pesticides are commonly used in agricultural and residential applications throughout the world. Organophosphorus pesticides make up approximately 70 percent of the insecticides currently in use. Unfortunately, these highly toxic materials have three main routes of human exposure: inhalation, ingestion, and skin penetration. Sources of these exposures include consumption of foodstuff containing pesticide residues, aerosol inhalation, and dermal contact during pesticide application. [1]

Organophosphorus pesticides use the same mechanism of action as deadly nerve agents such as sarin, soman, and VX. These pesticides affect the nervous system of insects, mammals, and wildlife by inhibiting the enzyme cholinesterase, important in helping regulate nerve impulses. Inactivation of cholinesterase leads to the accumulation of the neurotransmitter acetylcholine in the central and peripheral nervous system, which leads to depressed motor function and respiratory depression. Human toxicities for this class of molecules have shown acute as well as chronic effects from pesticide poisoning. [2,3]

Organophosphorus pesticides tend to be difficult to quantify due to poor peak shape, as evidenced by broad, asymmetrical peaks. An EPA Method 525.2 standard containing organophosphorus pesticides along with a custom pesticide mix acquired from Ultra Scientific (North Kingstown, RI) were analyzed to highlight the value of using a 30-m Agilent J&W DB-5ms Ultra Inert capillary GC column for difficult pesticide analysis. Many pesticides are sensitive to chromatographic system activity and will readily breakdown. The Ultra Scientific custom mix contains several types of these pesticides, which are useful in quickly evaluating system performance with particularly challenging pesticide analytes. Capillary GC column activity as a potential source of result uncertainty has been virtually eliminated with the Ultra Inert series of columns. [4]

Experimental

An Agilent 6890N GC/5975B MSD equipped with a 7683B autosampler was used for this series of experiments. Table 1 lists the chromatographic conditions used for these analyses. Table 2 lists flow path consumable supplies used in these experiments.

Table 1A. Chromatographic Conditions for EPA Method 525.2 Calibration Standards

GC	Agilent 6890N/5975B MSD
Sampler	Agilent 7683B, 5.0-µL syringe (Agilent p/n 5181-1273) 1.0-µL splitless injection
Carrier	Helium 44 cm/sec, 1.5 mL/min constant flow
Inlet	Pulsed splitless; 250 °C, 40 psi until 0.75 min, purge flow 50 mL/min at 1.0 min
Inlet liner	Deactivated dual taper direct connect (Agilent p/n G1544-80700)
Column	Agilent J&W DB-5ms Ultra Inert 30 m × 0.25 mm × 0.25 µm (Agilent p/n 122-5532UI)
Oven	40 °C (1 min) to 110 °C (50 °C/min), 7 °C/min to 190 °C, 12 °C/min to 285 °C, hold 2 min.
Detection	MSD source at 250 °C, quadrupole at 150 °C, transfer line at 280 °C, EI mode, scan range 45–450 amu

Table 1B. Chromatographic Conditions for Ultra Scientific Calibration Standards

GC	Agilent 6890N/5975B MSD
Sampler	Agilent 7683B, 5.0-µL syringe (Agilent p/n 5181-1273) 1.0-µL splitless injection
Carrier	Helium 52 cm/s, constant flow
Inlet	Pulsed splitless; 250 °C, 40 psi until 0.75 min, purge flow 50 mL/min at 1.0 min
Inlet liner	Deactivated dual taper direct connect (Agilent p/n G1544-80700)
Column	Agilent J&W DB-5ms Ultra Inert 30 m × 0.25 mm × 0.25 µm (Agilent p/n 122-5532UI)
Oven	75 °C to 175 °C (15 °C/min), 10 °C/min to 275 °C (1 min)
Detection	MSD source at 250 °C, quadrupole at 150 °C, transfer line at 280 °C, EI mode, scan range 45–450 amu

Table 2. Flow Path Supplies

Vials	Amber crimp-top glass vials (Agilent p/n 5183-4496)
Vial caps	Crimp caps with 11-mm septa (Agilent p/n 5181-1210)
Vial inserts	100-µL glass/polymer feet (Agilent p/n 5181-8872)
Syringe	5 µL (Agilent p/n 5181-1273)
Septum	Advanced Green (Agilent p/n 5183-4759)
Inlet liners	Deactivated dual taper direct connect (Agilent p/n G1544-80700)
Ferrules	0.4 mm id short; 85/15 Vespel/graphite (Agilent p/n 5181-3323)
20x magnifier	20x magnifier loupe (Agilent p/n 430-1020)

Sample Preparation

A six-component EPA Method 525.2 pesticide standard mix and internal/surrogate standard mix were purchased from Accu-Standard (New Haven, CT) and used to prepare a six-level calibration standard set. The stock pesticide solution as delivered had a nominal concentration of 1,000 µg/mL. The internal/surrogate solution as delivered had a nominal concentration of 500 µg/mL. The calibration standards were prepared with component concentrations of 10, 5, 2, 1, 0.5, and 0.1 µg/mL and a constant level of 5 µg/mL of internal/surrogate standard as per EPA Method 525.2. All solutions were prepared in acetone using class A volumetric pipettes and flasks. Acetone used was JT Baker Ultra Resi Grade purchased thorough VWR International (West Chester, PA). Acetone was used as a reagent blank and syringe wash solvent.

An 11-component pesticide standard mix was purchased from Ultra Scientific and used to prepare a seven-level calibration standard set. The stock pesticide solution as delivered had a nominal concentration of 1,000 µg/mL. The calibration standards were prepared with component concentrations of 10, 5, 2.5, 1, 0.5, 0.25, and 0.1 µg/mL. All solutions were prepared in 2,2,4-trimethylpentane using class A volumetric pipettes and flasks. The 2,2,4-trimethylpentane used was JT Baker Ultra Resi Grade purchased thorough VWR International (West Chester, PA). 2,2,4-Trimethylpentane was used as a reagent blank and syringe wash solvent.

Results and Discussion

Baseline Inertness Profile for Ultra Inert Columns

The basic approach for inertness verification for the Agilent J&W Ultra Inert series of capillary GC columns is testing with aggressive active probes at low concentration and low temperature. [5] This is a rigorous approach that establishes consistent baseline inertness profiles for each column in the Agilent J&W Ultra Inert GC column series. The baseline inertness profile then serves as a predictor for successful analysis of chemically active species that tend to adsorb onto active sites, particularly at trace level, like the organophosphorus pesticides in this application example. A more detailed description of the test mix and additional application examples can be found in references 6 through 8.

Organophosphorus Pesticide Analysis

In this application note, a multilevel pesticide calibration curve set was evaluated over the concentration range of 0.1 to 10 µg/mL on an Agilent J&W Ultra Inert DB-5 ms 30 m × 0.25 mm × 0.25 µm (Agilent p/n 122-5532UI). Separate calibration curves were developed for both the EPA 525.2 organophosphorus and Ultra Scientific standards. The standard levels used for the 525.2 calibration were 0.1, 0.5, 1, 2, 5, and 10 µg/mL, while the Ultra Scientific calibration levels were 0.1, 0.25, 0.5, 1, 2.5, 5, and 10 µg/mL. The custom pesticide standard from Ultra Scientific was used to determine system performance by analyzing difficult pesticides, such as endrin and p,p'-DDT, which are prone to analyte breakdown.

No tailing was observed for any of the organophosphorus pesticide peaks across the range studied in either standard set. Sharp, symmetrical peak shapes were noted for all the organophosphorus pesticides analyzed. Good resolution was obtained for each of the pesticides investigated.

Linearity for the 525.2 standard components was excellent across the range studied, giving R^2 values of 0.997 or greater in all cases but fenamiphos, which had an R^2 value of 0.978. This value increases to 0.991 at the midlevel concentrations as suggested by EPA Method 525.2 Sec. 13.2.3.3. Figure 5 indicates the correlation coefficients for each of the individual pesticides and shows an example linear regression plot for disulfoton.

Linearity for the Ultra Scientific standard components was also quite good across the range studied. R^2 values of 0.990 or greater were obtained for the organophosphorus pesticides. Figure 6 indicates the correlation coefficients for each of the individual pesticides and shows an example linear regression plot for mevinphos.

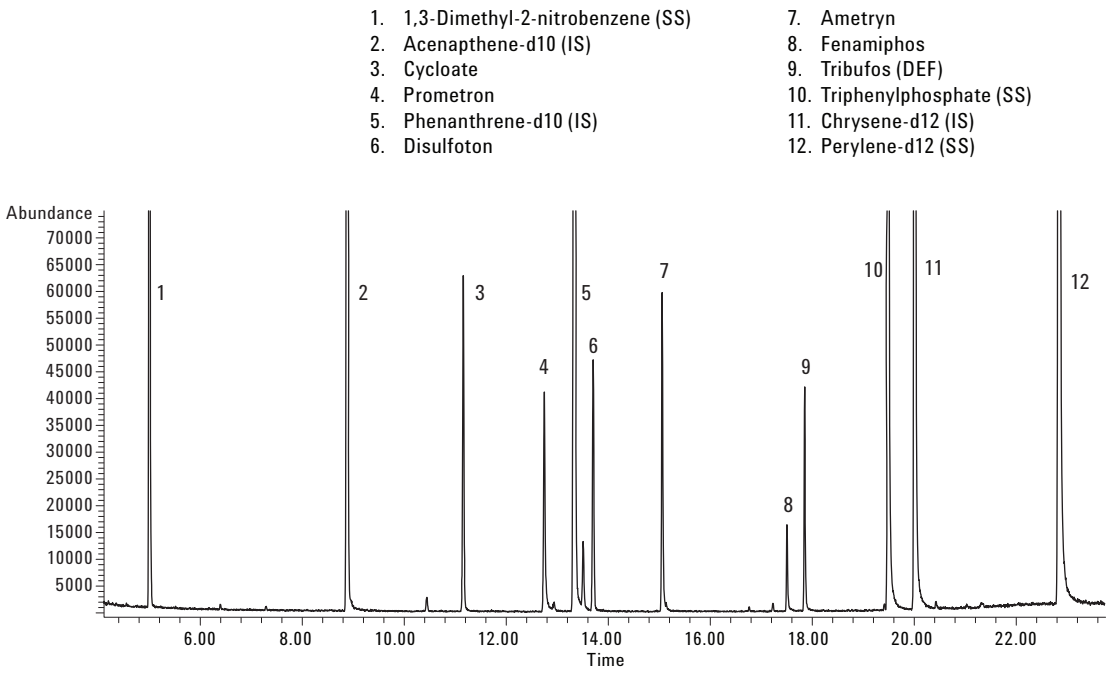


Figure 1. Total ion chromatogram (scan mode) of the 1-ng on-column EPA Method 525.2 standard solution loading on an Agilent J&W DB-5ms Ultra Inert 30 m × 0.25 mm × 0.25 µm capillary GC column (p/n 122-5532UI). Chromatographic conditions are listed in Table 1A.

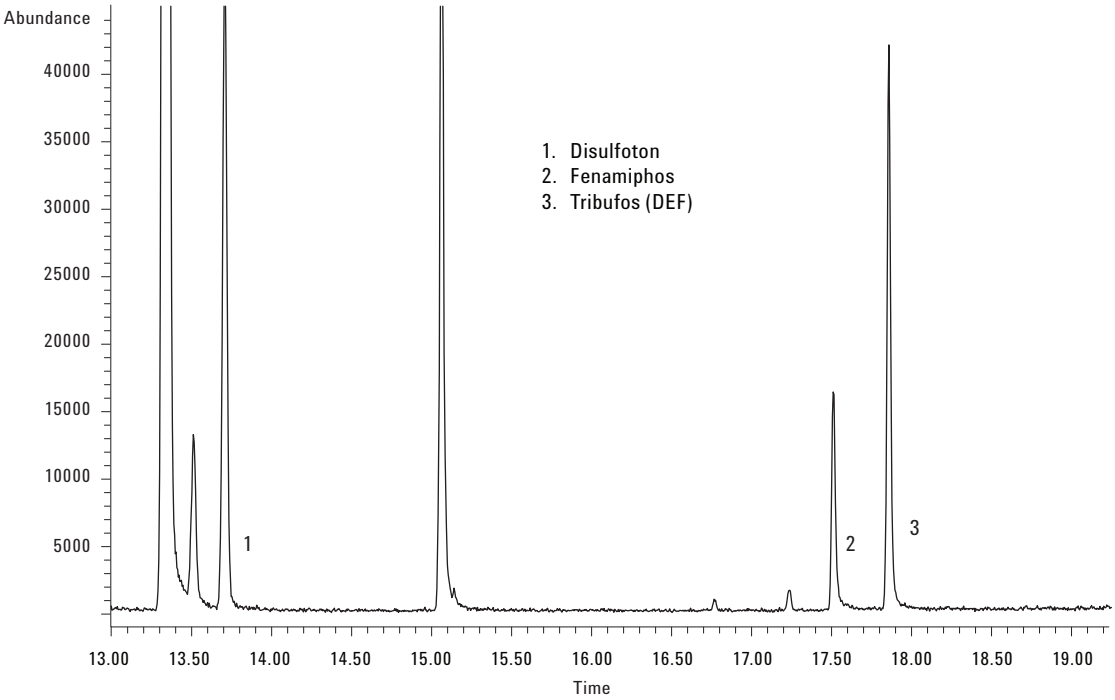


Figure 2. Enlarged section of the total ion chromatogram (scan mode) for a 1-µL injection of 1.0 µg/mL EPA Method 525.2 standard pesticide mix. The peaks noted in the figure are the three organophosphorus pesticides of interest. Chromatographic conditions are listed in Table 1A.

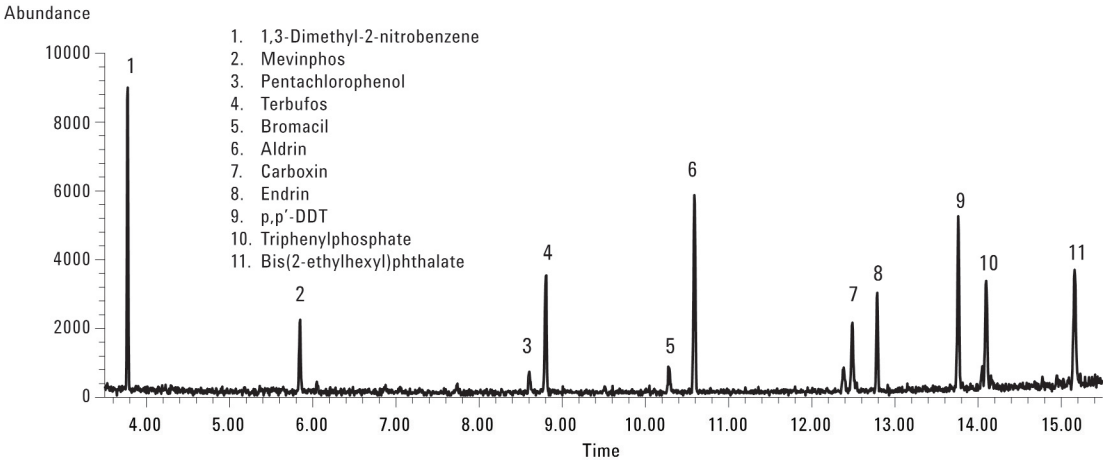


Figure 3. Total ion chromatogram (scan mode) of the 0.1-ng on-column Ultra Scientific standard solution loading on an Agilent J&W DB-5ms Ultra Inert 30 m × 0.25 mm × 0.25 µm capillary GC column (p/n 122-5532UI). Chromatographic conditions are listed in Table 1B.

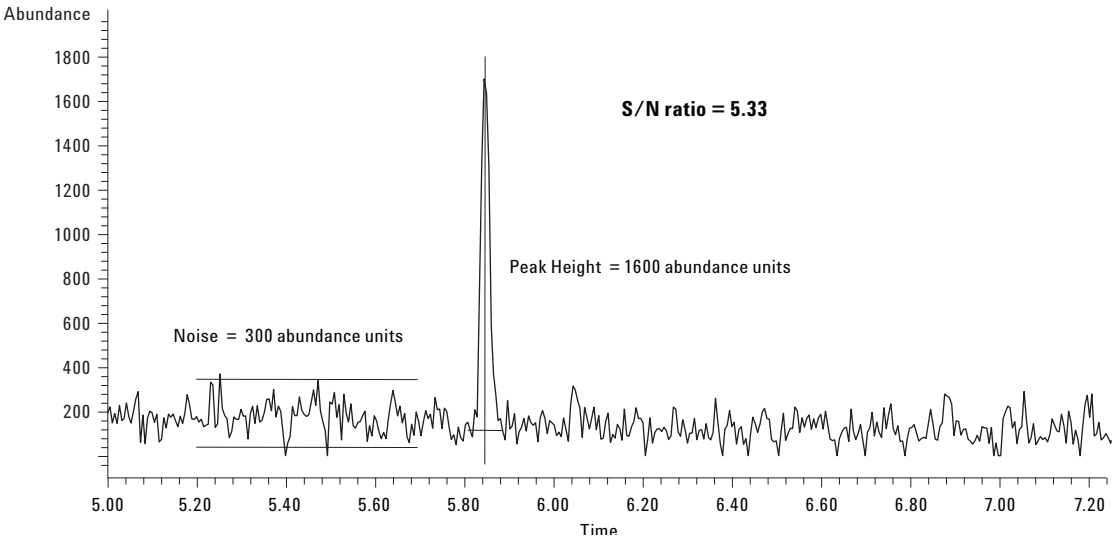


Figure 4. Enlarged section of the total ion chromatogram (scan mode) for a 1-µL injection of 0.1 µg/mL Ultra Scientific standard pesticide mix on an Agilent J&W DB-5ms Ultra Inert 30 m × 0.25 mm × 0.25 µm capillary GC column (p/n 122-5532UI). The peak in the figure is mevinphos, an organophosphorus pesticide of interest. This injection represents an on-column loading of 0.1 ng per component. Chromatographic conditions are listed in Table 1B.

Component	R^2
Cycloate	1.000
Prometon	0.999
Disulfoton	0.999
Ametryn	0.999
Fenamiphos	0.978
Tribufos (DEF)	0.997

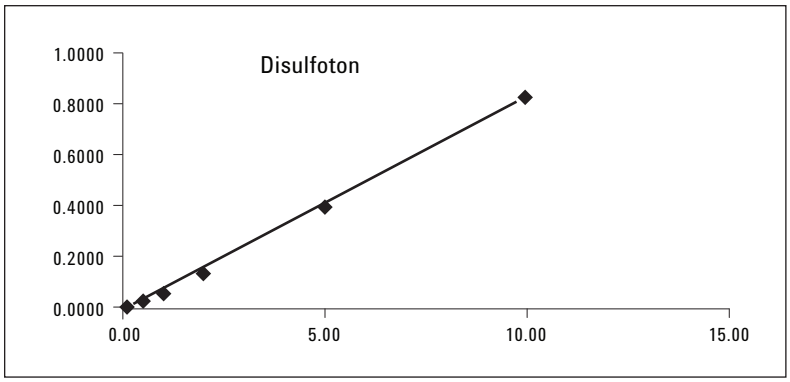


Figure 5. Correlation coefficients for the EPA Method 525.2 pesticide components over the 0.1 to 10 µg/mL range of this study and an example linear regression plot for disulfoton.

Component	R^2
1,3-Dimethyl-2-nitrobenzene	0.999
Mevinphos	0.990
Pentachlorophenol	0.989
Terbufos	0.996
Bromacil	0.988
Aldrin	0.999
Carboxin	0.996
Endrin	0.998
p,p'-DDT	0.996
Triphenylphosphate	0.997
DEHP	0.996

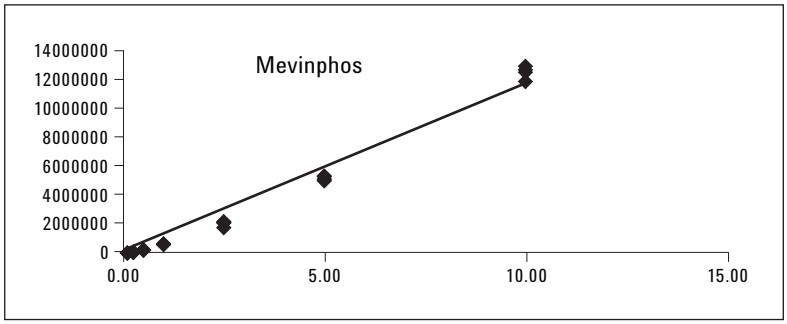


Figure 6. Correlation coefficients for the Ultra Scientific pesticide components over the 0.1 to 10 µg/mL range of this study and an example linear regression plot for mevinphos.

Conclusions

This application successfully demonstrates the use of an Agilent J&W DB-5ms Ultra Inert capillary GC column for trace-level organophosphorus pesticides. Linearity was excellent for all organophosphorus pesticides studied, yielding 0.99 or greater R^2 values down to a 0.1-ng on-column loading of each component. One of the reasons for excellent linearity and high R^2 values is the highly inert surface of the column. The lack of chemically active sites makes these columns an excellent choice for trace-level applications.

This study was done using scan mode on an Agilent 6890/5975B GC/MSD equipped with an inert electron impact source. The signal-to-noise ratio for a 0.1-ng on-column loading of mevinphos was greater than 5 to 1 with this system. This result shows clearly the power of using an Agilent J&W DB-5ms Ultra Inert column for trace-level organophosphorus pesticides analysis. Lower limits of quantification are expected when using one of Agilent's latest GC/MS offerings, such as the 7890/5975C GC/MSD Triple-Axis Detector coupled with an Agilent J&W DB-5ms Ultra Inert GC capillary column.

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The Analysis of Chlorinated Pesticides and PCBs Using the HP-608 Capillary Column

Application Note
228-236

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Abstract

Chlorinated pesticides and PCBs targeted in EPA Methods 608, 8080, 8081, and CLP pesticides for wastewater and solid wastes are analyzed under optimum conditions at a constant flow of 2.4 ml/min. The merits of splitless and on-column injection techniques using the Agilent 5890 Series II GC with electronic pressure control (EPC) are compared.

Key Words: chlorinated pesticides, PCBs, on-column injection, splitless injection, HP-608 capillary column, EPA 608, EPA 8080/8081, CLP pesticides, electronic pressure control.

Introduction

Chlorinated pesticides and PCBs have been banned in the U.S. for several years. However, because of their persistence in the environment, EPA methods 8080/8081 and CLP pesticides target 16 to 20 chlorinated organic pesticides in the evaluation of solid waste. This includes pesticides, their degradation products, technical grades of chlordane, toxaphene, and PCBs in solid waste.[1,2] EPA Method 608 targets similar pesticides in industrial and wastewater discharges.[3] EPA Methods 608 and 8080 prescribe packed-column analysis, whereas Methods 8081 and CLP pesticides prescribe capillary column analysis.

These EPA Methods allow laboratories to substitute columns of their choice provided that performance data such as chromatographic resolution, analyte breakdown, and MDLs (minimum detectable levels) are equal to or better than those provided with the EPA methods.

The HP-608 is a wide bore (530 µm-id) capillary column specially designed for the analysis of organic pesticides. GC/ECD separations of chlorinated pesticides and PCBs were done using the HP-608 column with both on-column and splitless inlet sample introductions. In both cases, the HP-608 provided superior chromatographic resolution, excellent reproducibility, and minimal analyte breakdown for the analysis of pesticides and PCBs.

Experimental

A 30 m x 530 µm x 0.5 µm HP-608 column (part no. 19095S-023) was used under constant carrier gas flow using the 5890 Series II GC with EPC equipped with a split/splitless inlet and a cool on-column inlet. Equipment included the 7673 automatic sampler with tray and the electron capture detector (ECD).

Samples were introduced in both the on-column and splitless modes. The Merlin™ Microseal septum (part no. 5181-8816) was used in the split/splitless inlet to replace the conventional inlet septum. A deactivated tapered glass liner (part no. 5181-3316) was used for all splitless injection runs. GC conditions were controlled using the HP 3365

Table 1. Experimental Conditions

Instrument Requirements	
Gas chromatograph:	Agilent 5890 Series II with EPC
Injection ports:	Split/splitless inlet with temperature and pressure programmable features On-column inlet with temperature and pressure programmable features
Column:	HP-608, 30 m x 530 µm x 0.5 µm (Part number 19095S-023)
Detector:	ECD
Sample introduction:	7673 splitless fast injection On-column injection
Data collection:	3365 ChemStation and HP Vectra 486/133T
Experimental Conditions	
Column:	HP-608, 30 m x 530 µm x 0.5 µm (Part number 19095S-023)
Carrier gas:	He, 20 cm/sec, 2.2 psi at 80°C with EPC under constant flow of 2.4 ml/min
Oven:	First ramp: 80°C (hold 1 min) to 190°C at 30°C/min Second ramp: 190°C to 280°C (hold 1 min) at 6°C/min Third ramp: 280°C to 300°C (hold 2 min) at 20°C/min
Injection:	Splitless: 1 µl, inlet temperature of 250°C On-column: 1 µl oven track for inlet temperature program
Detector:	ECD (330°C), 65 ml/min N_2 makeup, 6 ml/min anode purge
Sample:	Pesticides and PCB standard solutions in isooctane

ChemStation. Data was managed with a HP Vectra PC (486/33T). Instrument parameters and experimental conditions are listed in **Table 1**.

Pesticide solutions containing 16 to 22 components were prepared from the dilution of certified standards (part no. 8500-5873 and 8500-5876, mixes A and B: level 2) with isooctane (pesticide residue grade from Burdick & Jackson). Pesticide standards (part no. 5062-3589), including four vials of 16 EPA-608 pesticides and two vials of two component inlet check solutions (endrin/DDT concentrations are 50 ppb/100 ppb), were used without further dilution. These pesticide compounds are listed in **Table 2**.

Figure 1. Chromatograms of the 16 chlorinated pesticides under optimum GC conditions, 100 pg of each pesticide injected. Peak identification in Table 2.

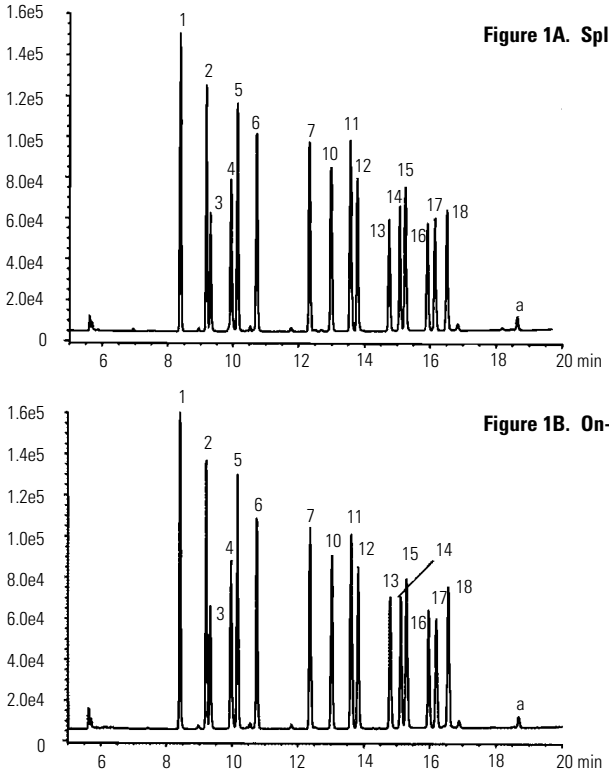


Table 2. Chlorinated Pesticides

Peak No.	EPA-608	Compound Name EPA-8080/8081	EPA-CLP Pesticides
1	alpha-BHC	alpha-BHC	alpha-BHC
2	Lindane	Lindane	Lindane
3	beta-BHC	beta-BHC	beta-BHC
4	Heptachlor	Heptachlor	Heptachlor
5	delta-BHC	delta-BHC	delta-BHC
6	Aldrin	Aldrin	Aldrin
7	Heptachlor epoxide	Heptachlor epoxide	Heptachlor epoxide
8		Chlordane-gamma	Chlordane-gamma
9			Chlordane-alpha
10	Endosulfan I	Endosulfan I	Endosulfan I
11	4,4′-DDE	4,4′-DDE	4,4′-DDE
12	Dieldrin	Dieldrin	Dieldrin
13	Endrin	Endrin	Endrin
14	4,4′-DDD	4,4′-DDD	4,4′-DDD
15	Endosulfan II	Endosulfan II	Endosulfan II
16	4,4′-DDT	4,4′-DDT	4,4′-DDT
17	Endrin aldehyde	Endrin aldehyde	Endrin aldehyde
18	Endosulfan sulfate	Endosulfan sulfate	Endosulfan sulfate
19		Methoxychlor	Methoxychlor
20	a-Degradation product		Endrin ketone
SS1			Tetrachloro-m-xylene
SS2			Decachlorobiphenyl

Results and Discussion

Splitless Analysis

Figure 1A shows the analysis of a standard solution containing the 16 EPA-608 targeted pesticides at a constant column flow of 2.4 ml/minute. One microliter of sample (100 pg of each component) was introduced in splitless mode at 250°C under the conditions[4] listed in **Table 1**. All 16 components were well resolved in sharp symmetric peaks, and the analysis was completed in less than 17 minutes. The 30-m HP-608 (530 µm id) column possesses sufficient efficiency to completely resolve the complex pesticides mix, including chlorinated compounds with similar or isomeric structures. The absence of coeluting peaks on the HP-608 column permitted fast and accurate identification and quantitation.

Low-Temperature On-Column Analysis

Figure 1B shows the same pesticides standard mix using the cool on-column injection technique. On-column injection of 1 µl of sample at 80°C resulted in little sample degradation, minimal byproducts, and good sensitivity (see **Table 3**). Common to both **Figures 1A** and **1B** is the absence of tailing peaks, including the endrin aldehyde peak (peak 17), indicating the HP-608 column surface is very inert.

Reproducibility

Reproducibility for the analysis of chlorinated pesticides using HP-608 columns with the HP GC/ECD system was excellent (see **Table 3**). The RSD (relative standard deviation) in absolute area counts for all 16 EPA targeted pesticides was less than 2% for on-column runs (two sets of six replicate injections). Similarly, the peak area counts reproducibility for all splitless injection runs (three sets of six replicate injections) was in the 1% to 2% RSD range using the same standard sample.

The standard deviation of retention times was within 0.003–0.005 minutes and 0.002 minutes for on-column and splitless runs, respectively. In comparison, the standard deviation of retention times for EPA Method 8081 analysis (**Table 10**, reference 1) using wide-bore capillary columns ranged from 0.007 minutes to 0.013 minutes for the same set of pesticides. This clearly demonstrates that chromatographic reproducibility obtained using the HP-608 capillary column is better than that obtained using the capillary columns stipulated in EPA Method 8081.

Table 3. Reproducibility of Pesticide Analysis

	Retention Times, min			Area Counts		
Pesticides	**Mean**	**Std Dev**	**% RSD**	**Mean**	**Std Dev**	**% RSD**
A. On-column injection (100 pg each component)						
alpha-BHC	8.423	0.004	0.047	431643	7497	1.74
Lindane	9.225	0.004	0.046	393514	6496	1.65
beta-BHC	9.352	0.004	0.046	208287	3428	1.65
Heptachlor	9.984	0.004	0.042	310294	5430	1.75
delta-BHC	10.181	0.005	0.044	390027	7428	1.90
Aldrin	10.760	0.004	0.039	359246	6996	1.95
Heptachlor epoxide	12.385	0.003	0.028	359586	5740	1.60
Endosulfan I	13.036	0.004	0.031	321622	5478	1.70
4,4′-DDE	13.623	0.004	0.026	341930	7070	2.07
Dieldrin	13.838	0.004	0.027	336042	4832	1.44
Endrin	14.814	0.004	0.025	268560	5298	1.97
4,4′-DDD	15.135	0.004	0.024	254389	3017	1.19
Endosulfan II	15.311	0.004	0.025	297580	4326	1.45
4,4′-DDT	15.975	0.003	0.021	259369	3881	1.50
Endrin aldehyde	16.208	0.004	0.022	205588	1876	0.91
Endosulfan sulfate	16.570	0.003	0.021	281397	4143	1.47
a, Degradation product	18.690	0.003	0.017	3416	97	2.83
B. Splitless injection (100 pg each component)						
alpha-BHC	8.351	0.002	0.020	376446	7222	1.92
Lindane	9.146	0.002	0.020	317405	6592	2.08
beta-BHC	9.273	0.002	0.018	165105	3129	1.90
Heptachlor	9.898	0.002	0.018	207924	4637	2.23
delta-BHC	10.097	0.001	0.013	301779	6113	2.03
Aldrin	10.671	0.002	0.015	308689	6422	2.08
Heptachlor epoxide	12.289	0.001	0.011	289985	6216	2.14
Endosulfan I	12.938	0.002	0.014	253489	5496	2.17
4,4′-DDE	13.527	0.001	0.011	313249	6102	1.95
Dieldrin	13.735	0.002	0.014	209054	3925	1.88
Endrin	14.710	0.002	0.013	160235	3104	1.94
4,4′-DDD	15.034	0.002	0.013	168113	3094	1.84
Endosulfan II	15.207	0.002	0.015	228810	4868	2.13
4,4′-DDT	15.874	0.002	0.012	168810	2129	1.26
Endrin aldehyde	16.103	0.002	0.010	148655	3687	2.48
Endosulfan sulfate	16.467	0.002	0.013	190284	3003	1.58
a, Degradation product	18.584	0.002	0.012	21513	1747	8.12

Comparison of Sample Introduction Techniques

For all on-column injection runs, degradation was negligible due to the low initial column temperature (80°C) and the direct introduction of a liquid sample plug into an inert column. As a result, inlet-related sample discrimination, alteration, and degradation were eliminated, while the advantages of solvent focusing and stationary phase focusing were maximized. Routine analysis of the inlet check solution (specified by the EPA methods) showed that the average degradation was less than 3% for endrin and 1% for DDT.

As demonstrated by the clean baseline in **Figure 1A**, little sample degradation occurred at an inlet temperature of 250°C. However, a small endrin ketone peak (RT of 18.69 minutes) appeared on the chromatograms from the GC runs with both on-column and splitless injection shown in **Figures 1A** and **1B**. A closer look (**Table 3**), shows that the area counts for endrin ketone (peak a, a byproduct of endrin degradation) measured 5 times larger in the splitless runs than for the on-column runs (average absolute area counts of 3,400 versus 21,000). The GC runs of the inlet check standard (after 200 repeated splitless injections), showed a 7% endrin degradation and 10% DDT degradation. These values were well below the EPA requirement of 15% degradation for both endrin and DDT.

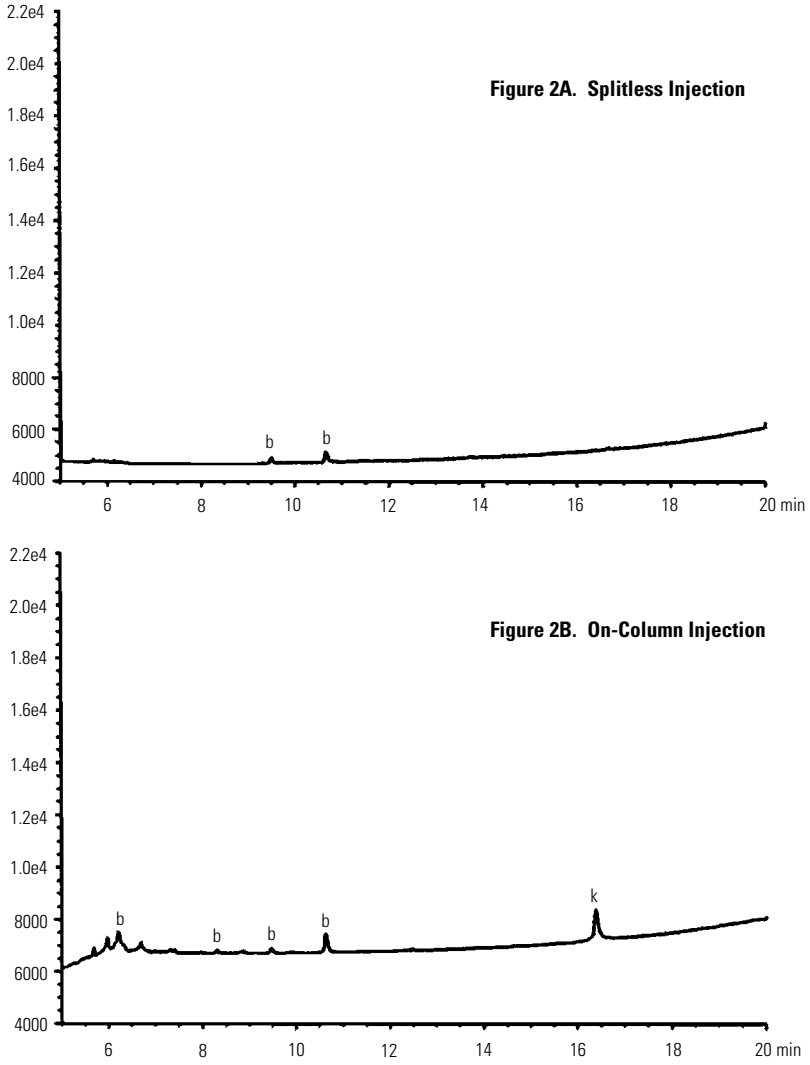
Use of the Merlin™ Microseal[5] and the deactivated glass liner also contributed directly to the low degradation rate in the splitless mode. The Microseal is designed to provide a good inlet seal without using a conventional septum. By eliminating the introduction of particulates into the inlet liner from conventional septum, useful life for the inlet liner is extended, down time (to change a liner and a conventional septum) is reduced, and laboratory throughput is increased.

The use of splitless injection technique may also prevent interference from extraneous and high boiling materials in dirty samples. This is demonstrated in **Figures 2A** and **2B**. **Figure 2** shows the analysis of isooctane solvent (pesticide-residue grade) using both splitless (**Figure 2A**) and on-column injection (**Figure 2B**). The late-eluting peak (peak k) , at 16.69 minutes retention time in the on-column run, does not appear in the chromato-gram of the splitless run (**Figure 2A**).

This peak, possibly a high boiling contaminant in isooctane, appears again in **Figure 3B**. **Figures 3A** and **3B** show analyses of a 10-ppb pesticide standard using splitless injection and on-column injection, respectively. The peak (peak k) eluting just before endosulfan sulfate (peak 18) may cause a higher value for the determination of trace endosulfan sulfate in the sample.

Both area counts and peak heights for the splitless runs were smaller than those for the on-column injection runs (see **Table 3**). For example, the average counts of lindane from the splitless runs were approximately 80% of those from the on-column injections (**Table 3**). Therefore, on-column injection is a good choice for clean samples and trace analyses demanding high sensitivity and low detection limits (large area counts).

Figure 2. Chromatograms of isooctane under optimum GC conditions, 1 µl injected. (b,k=solvent contaminants)



Analysis of PCBs and EPA Methods 8080, 8081, and CLP Pesticides

For wastewater and solid waste samples, the EPA recommends splitless injection for the determination of pesticides and PCBs. Using splitless injection under optimum 5890 Series II GC conditions, all 17 pesticides targeted by EPA Method 8080B are resolved as shown in **Figure 4**.

Among the 20 components targeted by EPA Methods 8081 and CLP pesticides, all but alpha-chlordane and endosulfan I (they are partially separated) are well resolved by the HP-608 column (**Figure 5**). Since the HP-608 column can effectively separate the complex mix of these pesticides, it is a good column choice for the determination of PCBs and multiple-peak response pesticides such as chlordane and toxaphene. **Figure 6** shows a comparison of chromatograms for technical grade chlordane and toxaphene, while **Figure 7** is a comparison of chromatograms for seven PCBs, all analyzed under the same GC conditions using the HP-608 capillary column.

Figure 3. Chromatograms of dilute pesticides mix under optimum GC conditions; 10 pg of each pesticide injected. (Peak ID, see Table 2)

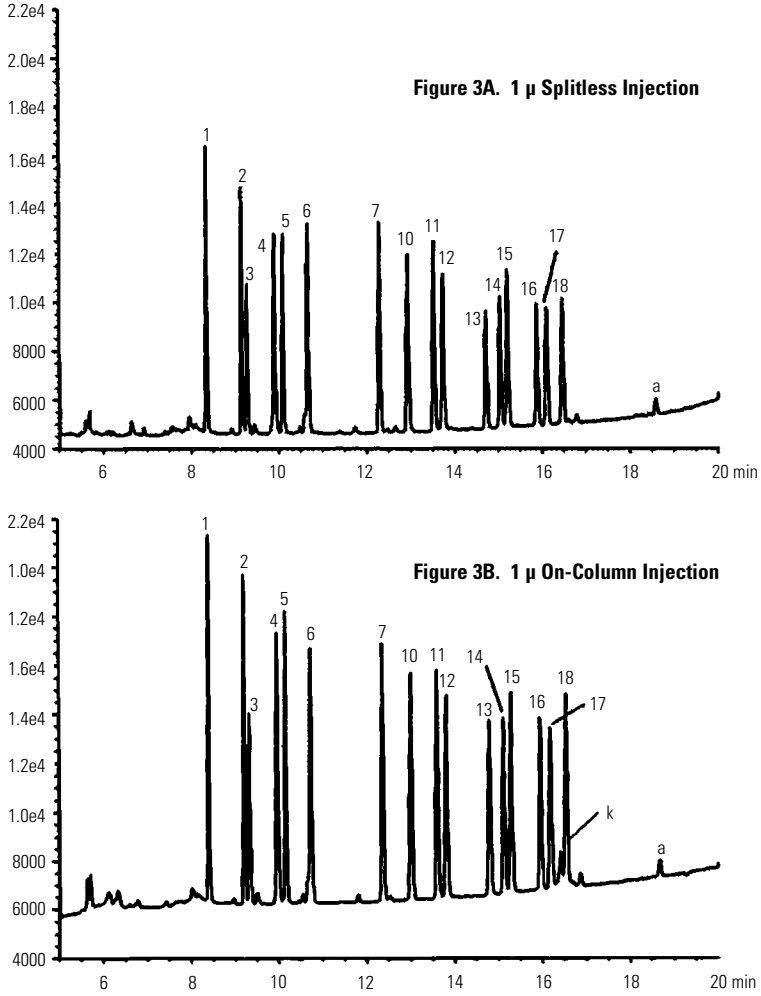
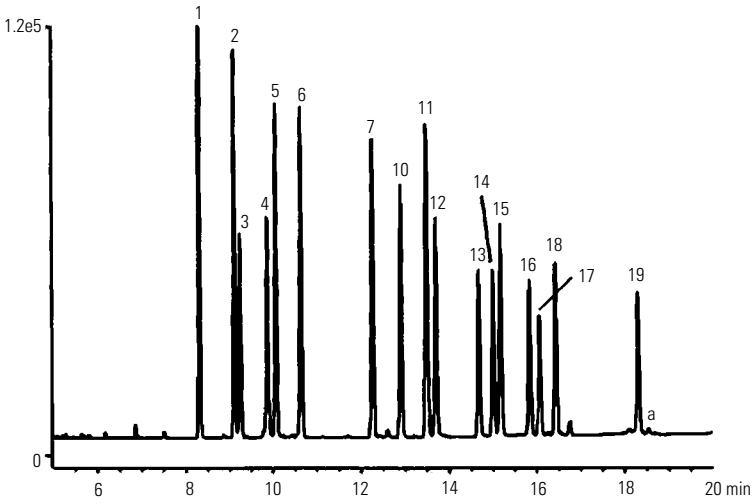


Figure 4. Chromatograms of the EPA-Method 8080 pesticides under optimum GC conditions. Splitless injection of 100–200 pg per component. (Peak ID, see Table 2)



Conclusion

Under optimal conditions, the HP-608 column separates 16 EPA-608 pesticides in 17 minutes and 20 EPA-CLP pesticides (and EPA-8081 pesticides) in 19 minutes (22 minutes including the surrogate, decachlorobiphenyl). Both splitless and on-column injections yield little sample degradation and provide excellent reproducibility of retention times and area responses. On-column injection is more suitable for clean samples and trace analysis, while splitless injection is better used for wastewater and waste samples.

Figure 5. Chromatogram of pesticides targeted in EPA-method 8081 and CLP pesticides under optimum GC conditions. Splitless injection of 50–100 pg per component. (Peak ID, see Table 2)

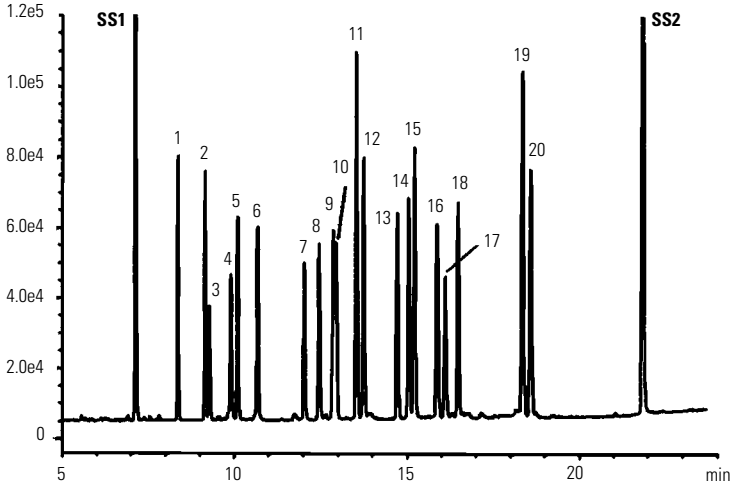


Figure 6. Chromatogram of technical grade toxaphene and chlordane under optimum GC conditions. Splitless injection of 1 µl 2.5 ppm mix

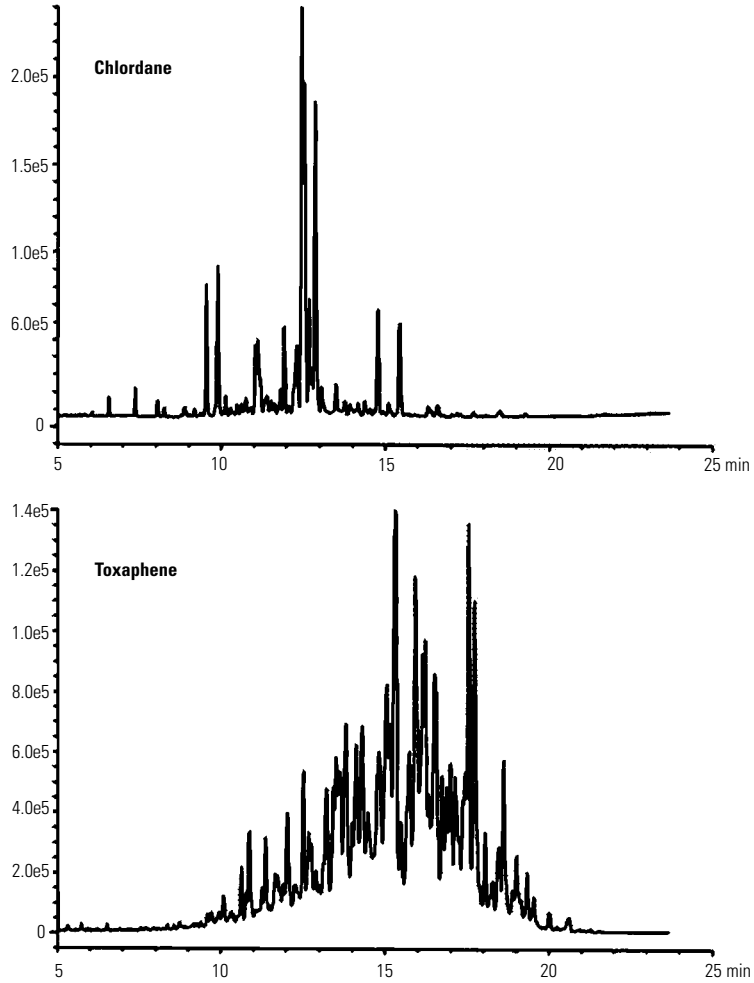
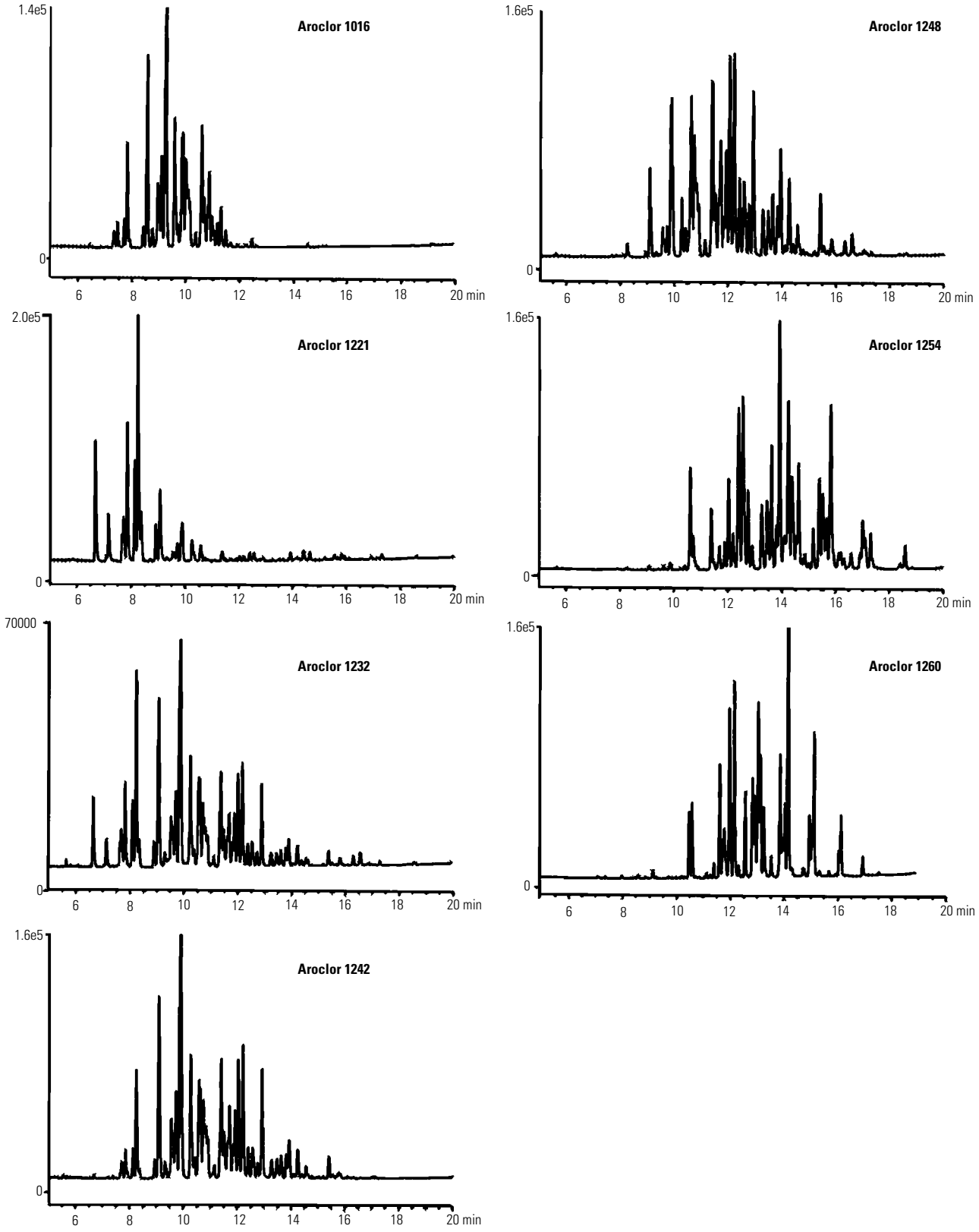


Figure 7. A comparison of seven PCBs under optimum GC conditions. Splitless injection of 1 µl 2.5 ppm each



Acknowledgment

The authors wish to thank Dr. D. Pautler for his many helpful discussions.

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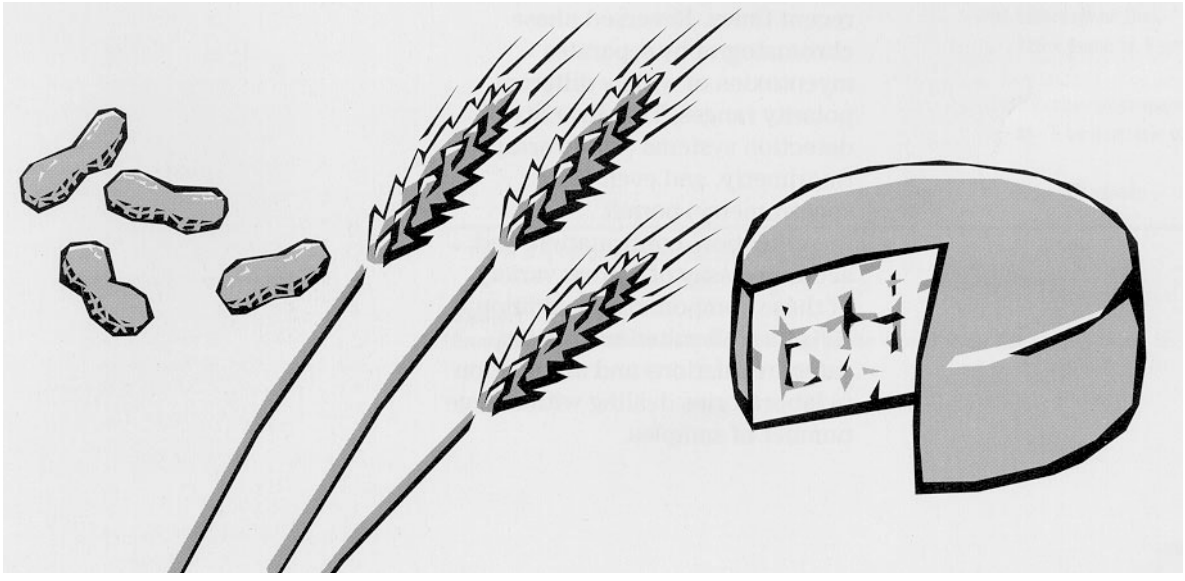
Analysis of Mycotoxins by HPLC with Automated Confirmation by Spectral Library

Application Note

Food Analysis

Rainer Schuster
Gerhard Marx
Michael Rothaupt

This note describes the sample preparation, chromatographic separation and detection of four different types of mycotoxins in food samples. Deciding which approach to adopt for analyzing these depends on the sample matrix and the type of fungus it has been contaminated with. Various professional organizations have proposed a variety of sample preparation methods–those for aflatoxins, ochratoxin A, patulin and zearaleneone are described here. All HPLC separations have been performed on reversed phase material (normal phase chromatography; a diol column can be used for patulin) and monitored with UV-visible absorbance diode-array detection (DAD) and fluorescence detection (FLD) for aflatoxins, ochratoxin and zearalenones or mass spectrometry (MS) for aflatoxins. Most compounds have been identified and confirmed by UV-visible absorbance spectral library search, purity control and by retention time tagging.



Introduction

Mycotoxins are highly toxic compounds produced by fungi. These toxins can contaminate foodstuffs when storage conditions are favorable to fungal growth. Mycotoxin nomenclature very often results from the fungi where the substance was first detected, for example aflatoxins in Aspergillus flavus strains, ochratoxin in Aspergillus ochraceus, patulin in Penicillium and Aspergillus, zearalenone in Fusarium. Most of these mycotoxins have been identified after cases of poisoning in livestock or the population at large. In 1969 more than 100,000 turkeys died of an unknown condition (so-called Turkey X) that was finally traced to peanuts–a component of their feed contaminated with Aspergillus flavus. During the wartime winter of 1940 in the USSR many people died after eating grain poorly stored and highly contaminated with Fusarium toxins such as zearalenone and fusarin C. A similar case occurred in 1965 in South Africa with ochratoxins found in cereals accumulated unmetabolized in animal kidneys.

Aflatoxins are known to be mutagens, teratogens (causing fetal abnormalities) and carcinogens (particularly in cancer of the liver or kidneys). Ochratoxins cause nephropathies in pigs, are teratogenic, and carcinogenic particularly in the liver and kidneys. Zearalenone shows estrogenic effects in sows and poultry, and affects the liver and kidneys. Patuline is a powerful mutagenic and cytotoxic compound. The intake of these mycotoxins over a long period at very low concentrations may be highly dangerous, yet difficult to combat since the small quantities are difficult to trace.

Currently most mycotoxins are still assayed using thin-layer chromatography (TLC), which permits effective compound separation and characterization. Such assay may be performed with satisfactory sensitivity when the compounds to be detected are fluorescent–a fluorodensitometer reads the plates quantitatively and objectively and has become indispensable to the control laboratory. However due to its higher separation power and shorter analysis times, use of HPLC has expanded rapidly in recent times. Reversed phase chromatography separates mycotoxins of widely different polarity ranges. The diversity of detection systems (diode array, fluorimetry, and even mass spectrometry) permit identification, confirmation and accurate assay of a great variety of these compounds. In addition HPLC is well suited to existing safety regulations and automation in laboratories dealing with a large number of samples.

The complexity in composition of processed foodstuffs makes a fixed routine necessary for analysis:

1. sampling protocol that ensures representative data from any one batch
2. extraction of mycotoxins, using mostly chloroform, acetone, or methanol
3. purification of the extract with clean-up methods
4. concentration of the extract
5. qualitative detection and assay of the mycotoxins.

In this paper we describe the analysis of 4 different types of mycotoxins. First we describe the chemical nature and occurrence of these toxins.

Experimental

Table 1 gives a short overview of analysis conditions for the four different mycotoxins aflatoxins, ochratoxin A, zearalenone and patuline.

Compound class	Matrix	Sample preparation	Chromatographic conditions
Aflatoxins G_2, G_2, B_2, B_1, M_2, M_1	Nuts, spices, animal food, milk, dairy products	Extraction §35LMBG	Hypersil ODS 100 x 2.1-mm id, 3 µm particles *HP 799160D-352* Water-methanol-acetonitrile 63:26:11 as isocratic mixture* Flow 0.3 ml/min at 25°C
Ochratoxin A	Cereals, flour, figs	Extraction §35LMBG Acidify with HCI. Extract with toluene. Clean up SiO_2. Elute toluene-CH_3COOH 9: 1	Lichrospher 100 RP18 125 x 4-mm id, 5 µm particles *HP 799250D-564-3* Water with 2 % acetic acid/acetonitrile, 1 : 1* Flow 1 ml/min at 40°C
Zearalenone	Cereals	Extract with toluene. Sep-pak clean up. Elute toluene-acetone 95: 5.	Hypersil ODS 100 x 2.1-mm id, 3 µm particles *HP 799160D-352* Water-methanol-acetonitrile 5:4:1 as isocratic mixture* Flow 0 : 45 ml/min. at 45°C
Patuline	Apple products	Clean-up on Extrelut Silica gel clean up Elute toluene-ethyl acetate 3: 1.	a)Superspher RP 18 125 x 4-mm id, 4 µm particles *HP 799250D-464,* Water-acetonitrile, 95 % to 5 % gradient Flow 0.6 ml/min at 40° C b) Lichrospher Diol 125 x 4-mm id, 5 µm particles Hexane-isopropanl 95:5 as isocratic mixture Flow 0.6 ml/min

* 100 % B is recommended for cleaning the column.

Table 1. Sample preparation and chromatographic conditions for mycotoxins in foodstuffs

Compound types:

Aflatoxins–are chemical derivatives of difurancoumarin (figure 1). Although a number of different aflatoxin metabolites are known, interest is usually focused on the four main aflatoxins B_1, B_2, G_1, G_2 and the so-called milk toxin M_1.

Aflatoxin B_1 is in the majority of cases the most abundant toxin, the most toxic and the most potent carcinogen. Maximum levels for B1 are usually given for the individual compound, 2 ppb in Germany, 5 ppb in France and 1 ppb in Switzerland, for example. United States legislation regulates the aflatoxin content of a contaminated product as the sum of B_1-plus-B_2-plus-G_1-plus-G_2, which may not exceed 20 ppb. Aflatoxins are most often analyzed in nuts, for example peanuts and pistachios, cereals, figs, bread, meat, eggs, butter, milk, margarine, juices, cottonseed products, and cocoa beans. Considering the complexity of these matrices, sample preparation is the most important step for reliable results.

Since fungal growth–and therefore contamination by aflatoxin–is not homogeneous, normal sampling gives mediocre results. The US Department of Agriculture (USDA) tackled the problem by defining a sampling protocol for peanuts which involves as many as eight assays on four samples of more than
1 kg. Sampling is not specified in European countries. At best, a few hundred grams are taken to determine the mean level of aflatoxins in a batch as large as of a couple of thousand tons of grain.

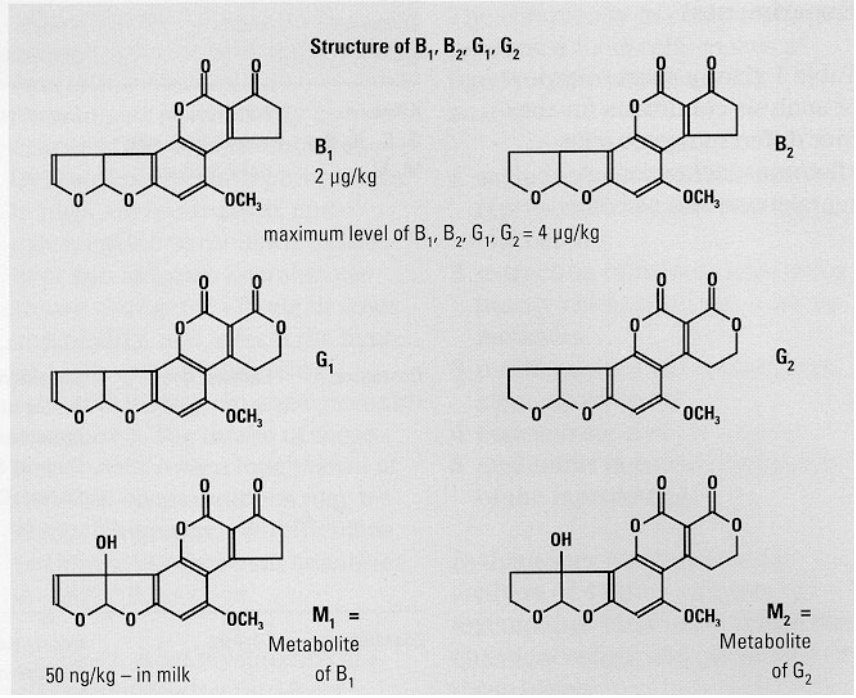


Figure 1. Structure of aflatoxins and their maximum permitted concentrations (given for Germany[1])

Ochratoxins–The mycotoxin ochratoxin A can be produced by different fungi including Aspergillus and Penicillium. Of the ochratoxins A, B, and C, the latter two so far have not been found in naturally contaminated products. Beside nephrotoxicity, ochratoxin A has hepatoxic, teratogenic and carcinogenic properties in the kidneys. Ochratoxin A was found in various foodstuffs. Analysis of more that 900 plant samples show a contamination rate of about 13 %, mostly in barley, oats and wheat. The concentrations of ochratoxin A found varied from 0.1 to 200 µg/kg.[2] A review of results from various countries, covering around 7000 samples, reported that contamination was about 14 %.[3] Ochratoxin A is the primary agent in so-called mycotoxic porcine nephropathy (MPN) a disease prevalent in pigs. The toxicity is a third that of the toxicity of aflatoxin B_1 in rats. The main human intake is assumed to be through the consumption of pork and wholemeal products.

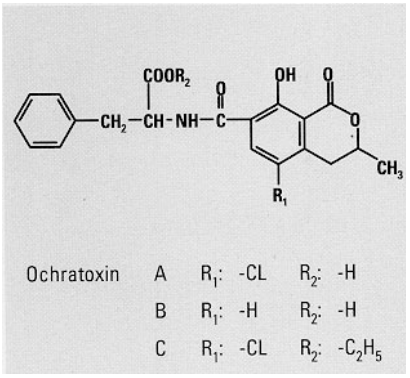


Figure 2. Structure of three common ochratoxins

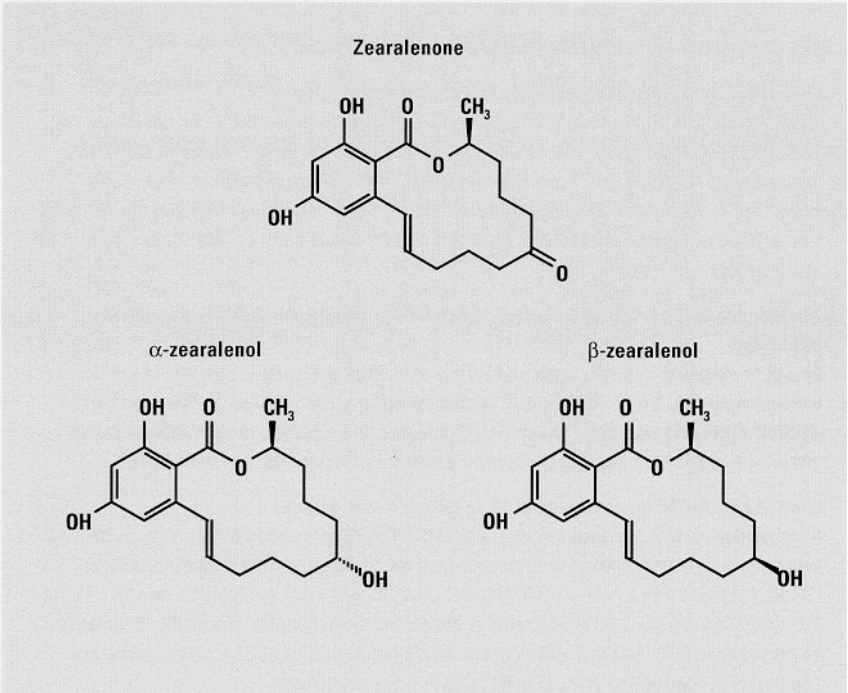


Figure 3. Chemical structures of zearalenone, a–and b-zearalenol and a -zearalanol (Zeranol)

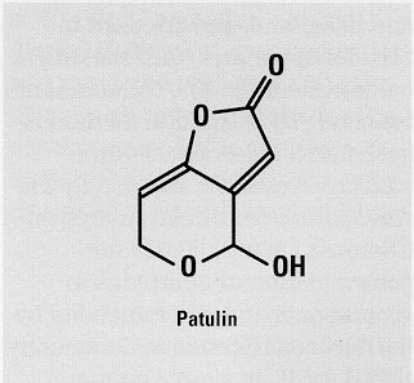


Figure 4. Chemical structure of patulin

Zearalenone–an estrogenic-efficient mycotoxin produced by Fusarium, occurs mainly in a variety of natural products such as corn and other grains. Whereas the acute toxicity of zearalenone is low, its intake is linked to various possible estrogenic disease effects in children.[4] After carcinogenity was determined in rodents, a recent risk assessment resulted in an estimated safe intake of not more than 0.05 µg for each kilogram body weight per day for humans.[12] Use of contaminated animal feed means that these compounds are present in cow's milk as zearalenone and the diastereomer metabolites and b-zearalenol. Another zearalenone derivative is the synthetic a -zearalanol (also known as Zeranol) which is used in some countries for fattening cattle. The recommended limit for zearalanol is 10 µg kg for liver and 2 µg/kg for other meats.[7] Their use as anabolic agents is prohibited in the European Community. investigations of food and animal feedstuffs have shown zearalenone concentrations between 0.001 and 2.0 mg/kg.[5] The highest levels, 1700 mg/kg, were found in silo-corn.[6]

Patulin–unstable in cereals, mainly occurs in fruit, especially apples, is a metabolite of several fungi of Penicillium and Aspergillus. Most of the survey work has been done on apple juices and apple-based products.

Aflatoxin sample preparation

For sample preparation different methods are described in the literature.[8,9,18] Lipid should be eliminated after analyte extraction if lipid content exceeds 5 %. Solvents in which the aflatoxins are insoluble are hexane, petroleum ether, pentane and isooctane, and are used in Soxhlet apparatus (6h), shaking or column clean up. The contaminants branch (CB) extraction method is based on a chloroform-water mixture, a method adopted by the Association of Official Analytical Chemists (AOAC) 20.029 for determination of aflatoxins in ground nuts and recommended by the European Economic Community (EEC) for B_1 in simple animal nutrition foodstuffs.

Whatever extraction method is used the resulting extract still contains, besides the aflatoxins, various impurities (lipids, pigments and so on) requiring an extra clean-up step. Apart from purification by precipitation or by liquid-liquid partition, the most commonly used technique is column adsorption chromatography.

Extraction of aflatoxins with chloroform

CB method (AOAC and EEC) for extraction of aflatoxins

50 g of finely ground sample are mixed with 25 g of diatomaceous earth and moistened with 25 ml of water. This mixture is carefully shaken, diluted in 250 ml of chloroform and shaken vigorously for 30 minutes on a vibration shaker. A 50 ml portion of chloroform extract is collected for purification and assay. The addition of water facilitates chloroform penetration into substrates derived from plants, while the diatomaceous earth retains various substances like pigments.

Method §35 *Lebensmittel und Bedaffspeyenstands Gesetz* (LMBG)[8] for extraction of aflatoxins

20 g of finely ground sample are mixed with 20 g of silica gel, particle size 20–45 μm (for example celit 545, Serva, Germany). This mixture is diluted with 200 ml of chloroform and 20 ml of water and vigorously shaken for 30 minutes on a vibration shaker. After filtration, 100 ml are evaporated close to dryness on a rotary evaporator (temperature 40 °C).

Method §35 LMBG[9] for extraction of M_1 in milk and milk powder

50 ml acetone and 5 g sodium chloride and 1 ml 1 N H_3PO_4 are added to 50 g milk, or 10 $_9$ milk powder homogenized in 40 g water, and shaken for 10 minutes. After addition of 100 ml dichloromethane and a further 10 minutes shaking, 25 g of silica gel, particle size 20–45 μm (for example celit 545, Serva, Germany) is added and shaken again. The dichloromethane/ acetone phase is filtered and 100 ml of the filtrate (equivalent to 33.33 g milk or 6.66 g milk powder) is evaporated to dryness at 40 °C in a rotary evaporator.

Purification of aflatoxins

Method according to AOAC/ EEC and §35 LMBG regulations[8]

A glass column (400 x 30mm) is filled in succession with 5 g sodium sulfate, 10 g of silica gel (63–200 μm, dried at 105 °C for lie), and 15 g anhydrous sodium sulfate topped up with some cotton-wool. The extract to be cleaned is added on top and eluted with 15–20 ml chloroform. Then the column is washed with 150 ml hexane and 150 ml diethylether to remove lipids and other interfering compounds from the aflatoxins. The aflatoxins are eluted with 150 ml of a chloroform/methanol (97: 3) mixture. The eluate is dried down and redissolved in a suitable solvent for assay by HPLC (methanol).

Extraction of aflatoxins from milk, AOAC 26.139

To 25 ml of milk, 10 drops of NH_4OH are added, swirled and diluted by 70 ml acetonitrile. The mixture is shaken for 1 minute and centrifuged for 5 minutes at 1000 rpm. The aqueous alkaline acetonitrile supernatant is transferred and evaporated on a rotary evaporator at 45 °C. The residue is acidified with 15 drops (about 500 μl) of HCl to pH 1.3 and partitioned into methylene chloride on the liquid-liquid extraction column, ChemElut™ (Analytichem, United Kingdom). The dichloromethane is evaporated off by rotary evaporation. After cooling, the residue is redissolved in 1.5 ml of dichloromethane, evaporates and redissolves in 500 μl hexane-dichloromethane (1: 9). Clean-up is performed on a BondElut NH_2 cartridge (Analytichem, United Kingdom) conditioned with hexane-dichloromethane (1: 9).100 μl of the milk extract is transferred to the column; fats are removed with 230 μl hexane-dichloromethane (1: 9) while zearalenone is eluted with 1 ml of methanol. After evaporation of methanol the residue is dissolved in 500 μl of mobile phase.

Extraction of ochratoxins with toluene

According to §35 LMBG Method 15-00-1, AOAC 26.100–26.125

30 ml of 2 M HCl in 50 ml of 0.4 M magnesium chloride solution is added to 20 g of ground and mixed sample. After homogenization, 100 ml toluene is added and shaken vigorously for 60 minutes. The suspension is separated by centrifuge and 50 ml of the toluene supernatant is passed through a preconditioned Sep Pak silica gel column. The column is washed with two 10-ml aliquots of hexane, 10 ml of toluene/acetone (95: 5) and 5 ml of toluene. Ochratoxin A is eluted with two aliquots of 15 ml toluene/acetic acid (9: 1) and dried down at 40 °C. The residue is redissolved in 1 ml of mobile phase and filtered.

Ochratoxin sample preparation

Methylester derivatives of the ochratoxins can also be analyzed. From the extracted sample, 500 µl is evaporated to dryness and redissolved with 1 ml of dichloromethane and 2 ml of 14 % boron trifluoride in methanol. The solution is heated for 15 minutes at 50–60 °C and after cooling diluted in 30 ml of distilled water and extracted with three 10-ml aliquots of dichloromethane. The organic phase is filtered through sodium sulfate, dried down and dissolved in 500 µl of mobile phase.

Zearalenone sample preparation

Thanks to Eppley s technique,[13] zearalenone, aflatoxins and ochratoxin can be simultaneously extracted on Sep-Pak silica cartridges. The sample is added as a toluene extract, washed with toluene and zearalenone is eluted with 10 ml of toluene-acetone (95: 5) mixture.

Patulin sample preparation

Two approaches are documented.[1] Fruit juices can be cleaned on an Extrelut cartridge followed by analyte extraction on a silica gel column with toluene-ethyl acetate (3: 1) before HPLC assay.[14] The analyte can be extracted into ethyl acetate, followed by partition extraction into 1.4 % Na_2CO_3 solution and back into ethyl acetate. After evaporation of the ethyl acetate at 40 °C, the residue is dissolved in methanol-ethyl acetate (9: 1) if it is to be analyzed on a reversed-phase column packing material or in hexane-isopropanol if a diol-phase column packing material is used (details of suitable columns are given in table 1).[18]

Chromatographic separations, peak confirmation and quantification

We used a Hewlett-Packard HP 1090 Series M liquid chromatograph with DR 5 binary solvent-delivery system, variable-volume auto-injector, temperature-controlled column compartment and solvent-preheating device. Mobile phase methanol and acetonitrile were of HPLC reagent quality (Baker, Gross-Gerau, Germany). A diode-array UV-Visible absorbance detector was used together with HPLC^{3D} ChemStation software to automatically quantify the mycotoxins and identify them using spectral libraries. Fluorescent species were detected using an HP 1046A programmable flourescence detector (FLD) under the control of the HPLC^{3D} ChemStation, using lex 265 nm, lem 455 nm for aflatoxins, lex 247 nm, lem 480 nm for ochratoxin A and lex 236 nm, lem 464 nm for zearalenone. Aflatoxins were also determined using mass spectrometry on an HP 5989 MS Engine equipped with negative ion detection and Thermospray options. The electron filament capability was used to provide higher sensitivity. LC eluant passed through a capillary tube and was simultaneously heated to approaching the boiling point. The resulting liquid-vapor was injected into the mass spectrometer where it was ionized and analyzed. The mass spectrometer was controlled and the data were analyzed by the HP 59940A MS ChemStation (HP-UX series).

Results and discussion

Aflatoxin assay by HPLC-DAD and HPLC-FLD

Thin layer chromatography can be replaced by reversed phase HPLC, improving accuracy, and dramatically speeding up the time required to assay, for example B_1 takes three hours by TLC, M_1 four hours. Figure 6 shows a separation of the common aflatoxins M_2 (5 ng), M_1 (10 ng), G_2 (1.5 ng), G_1 (5 ng), B_2 (1.5 ng), B_1 (5 ng) on a reversed phase column (refer to table 1 for conditions).

Flow rate	0.30 ml/min
Mobile phase	Isocratic water–methanol–acetonitrile (63: 26: 11) mixture
Detection:	
Fluorescence	lex 365 m, lem 455 nm
Diode-array	365 nm

Due to the extreme differences in fluorescence yields for B_2 and B_1 respectively G_2 and G_1 (B_2 FLD yield is about 60 times higher than B_1) it can be useful to run both detectors in series. Diode-array detection in addition gives us the UV-visible absorbance spectra dimension for further identification of the aflatoxins. As an alternative to the isocratic run with subsequent 100 % B wash, a gradient analysis from 35 % B (methanol-acetonitrile, 26: 11) to 55 % B in 10 min and 100 % B in 14 min (at 35 °C) might be used. Peaks become much sharper than under isocratic conditions, with higher signal-to-noise, and less polar compounds in the food extract are eluted in this run.

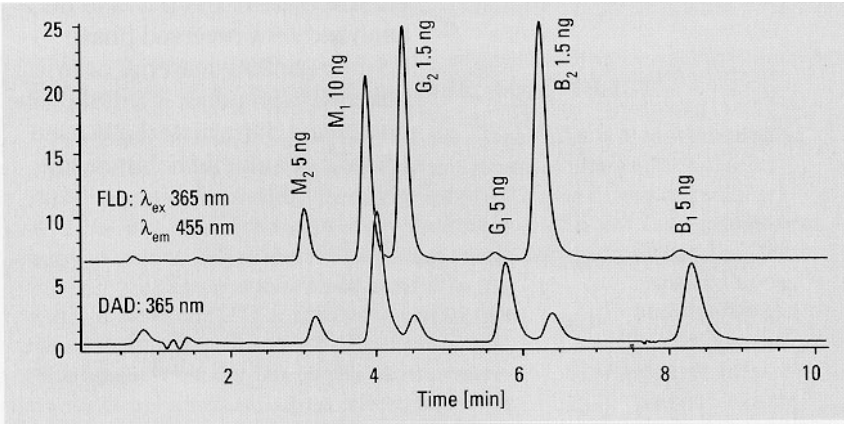


Figure 5. Analysis of the common aflatoxins by fluorescence and diode-array detection

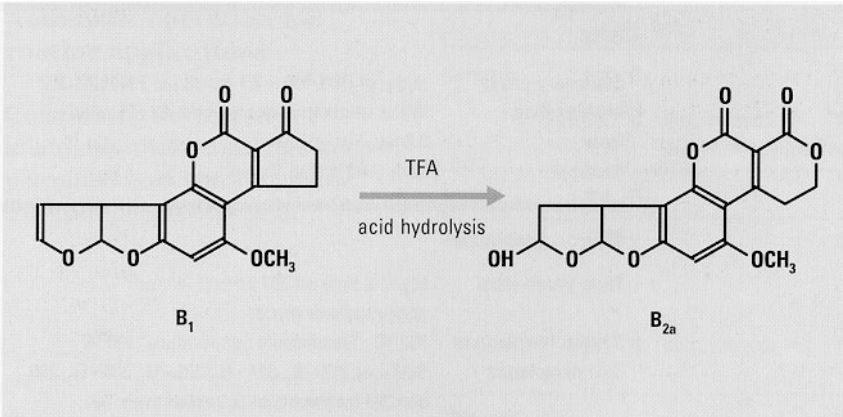


Figure 6. Structure of aflatoxins after hydrolysis with trifluoracetic acid (TFA), from B_1 to B_{2a}

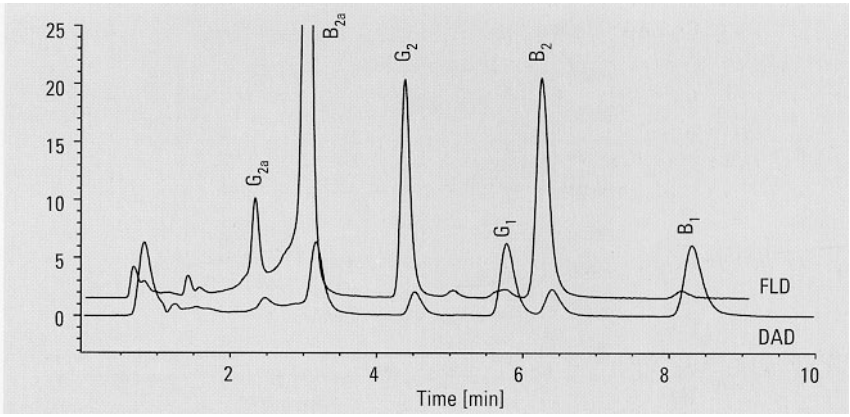


Figure 7. Analysis of the aflatoxins G_2, G_1, B_2, B_1 and G_{2a} and B_{2a} (hemiacetals), with conditions as for figure 1

If fluorescence is used alone it might be desirable to improve the B_1 and G_1 fluorescence yield by hydrating the double bond of the furanic ring (figure 6) with trifluoroacetic acid (TFA) to form the corresponding hemiacetals B_{2a} and G_{2a}. This approach can also be used as a confirmation tool for B_1 and G_1. A separation of the four aflatoxins and the hemiacetals B_{2a} and G_{2a} is shown in figure 7.[17]

Sensitivity of the B_1 can also be improved by formation of an iodine derivative[10] or by modification of the flow cell to a cell filled with fine silica particles.[11]

Aflatoxin assay by LC-MS

For highest sensitivity and selectivity we have investigated the use of mass spectrometry. The aflatoxin standard (not including M_2 and M_1) was diluted fivefold and 1 µl was injected resulting in concentrations of 1 ppm for G_1 and B_1, and 300 ppb for G_2 and B_2 (figure 8). A further dilution of 1: 10 and the 1-µl injection is shown in figure 9. Detection limits for G_1, B_2 and B_1 are less than 50 ppb for this separation. An example of thermospray application is shown in figure 11.

HPLC	
Stationary phase	Hypersil ODS 100 x 2.1 mm, 3 µl 799160D-352
Mobile phase	Water-methanol-acetonitrile 163: 26: 11)
Flow	0.3 ml
Gradient	32 %–60 % B in 10 min
MS	
Tune parameters	Manual tune on 367 adduct ion for polypropylene glycol
Source temperature	250°C Quadropole temperature 120 °C
SIM parameters	SIM ions 312–B_1, 314–B_2, 328–G_1, 330–G_2, 286 and 284 fragments of G_1 respectively G_2
Dwell time	600 msec
Electron multiplier	2500 V On Mode negative
Thermospray stem temperature	95 °C Filament ON

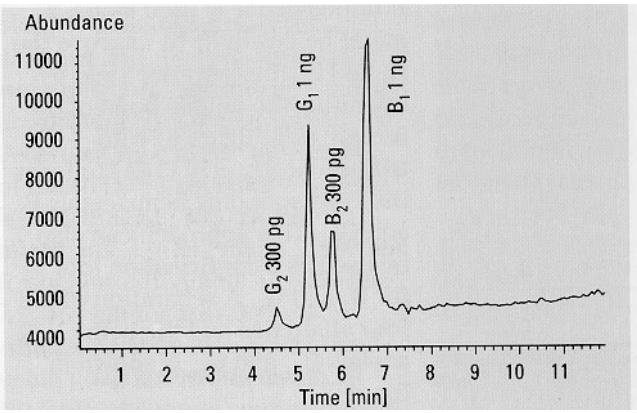


Figure 8. Analysis of G_2, G_1, B_2 and B_1 using HPLC and thermospray mass spectrometry

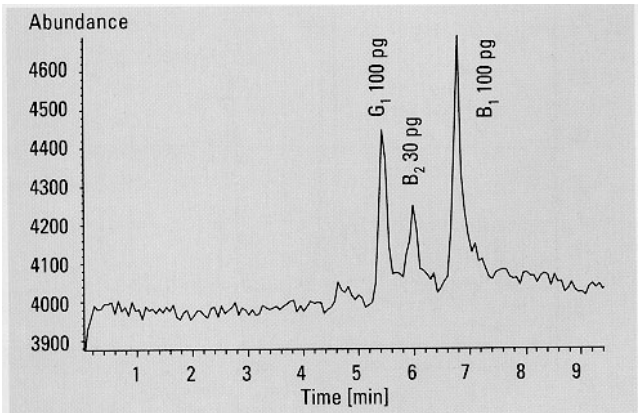


Figure 9. G_1, B_2, and B_1 in the low picogram range at 1-µl injection volume

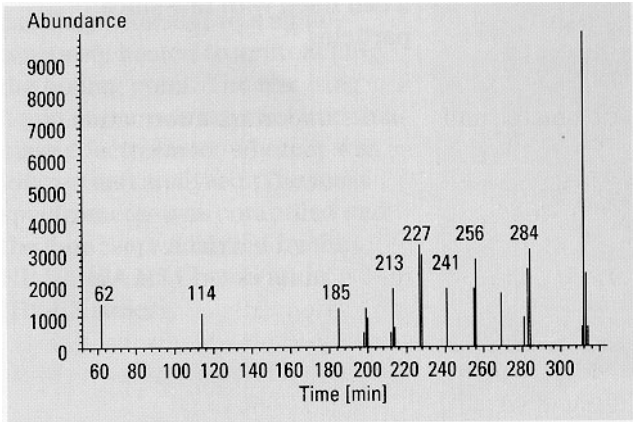


Figure 10. Total ion monitoring spectrum of aflatoxin B_1

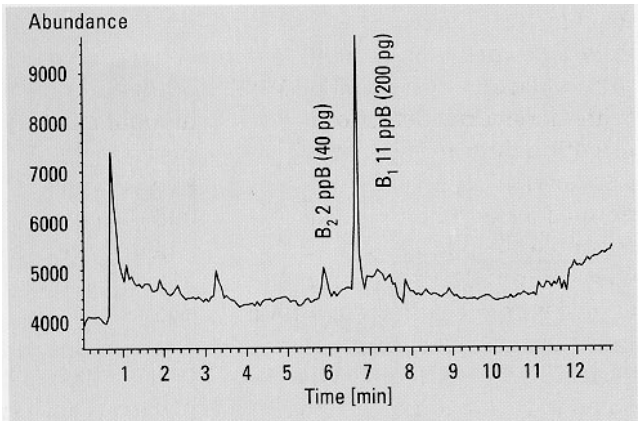


Figure 11. Extract of pistachio nut according to § 35 LMBG with 1-µl injection (see also figure 12 with 2-µl injection volume)

Automatic operation for routine applications

Considering the toxicity of aflatoxins, most countries keep permitted concentrations low, for examples see table 2.

	Aflatoxin B_1	Total aflatoxin	Milk toxin
Germany	2 ppb B_1	4 ppb $\sum\{B_1, B_2, G_1, G_2\}$	50 ppt M_1 milk
	20 ppb B_1 animal feed	50 ppt $\sum\{B_1, B_2, G_1, G_2\}$	
	diet and baby food		
France	5 ppb B_1		
Switzerland	1 ppb B_1	5 ppb $\sum\{B_1, B_2, G_1, G_2\}$	50 ppt M_1 in milk
			20 ppt M_1 baby food
			250 ppt M_1 cheese
	2 ppb B_1 corn, cereals	10 ppt $\sum\{B_1, B_2, G_1, G_2\}$	
		diet and baby food	
USA, FDA		20 ppb $\sum\{B_1, B_2, G_1, G_2\}$	500 ppt M_1 milk
WHO, FAO	5ppb B_1	10 ppb $\sum\{B_1, B_2, G_1, G_2\}$	

Table 2. Limits for Aflatoxins in different countries

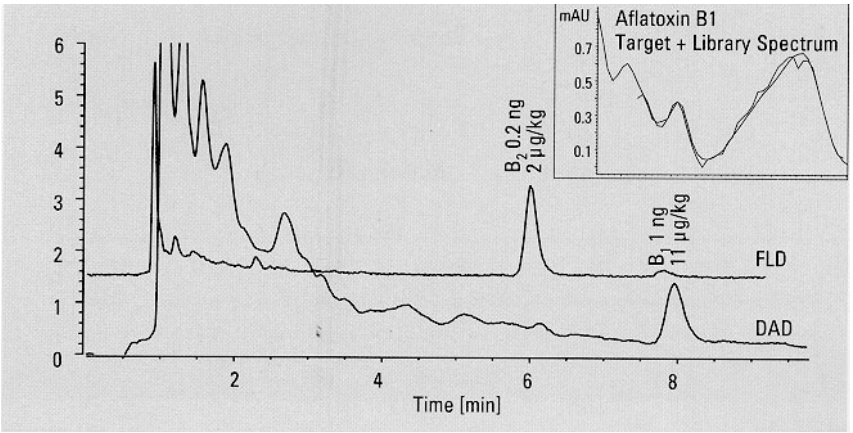


Figure 12. The analysis of a 2-µl injection of pistachio-nut extract (§35 LMBG) detected by FLD and DAD and UV-Spectrum

Sample preparation in the following applications was performed according to §35 LMBG. With diode-array detection, retention time and spectral information can both be incorporated automatically in the report. We created a library of standard mycotoxin spectra tagged with their HPLC retention times. After each run, peak spectra were automatically compared with library spectra, and their purity checked by overlaying several spectra taken in each peak. The customized report prints all this information retention times, chromatogram, library and calibration table, amounts, library search match and purity match factor. The method is fully automatic. Data acquisition and data evaluation including quantification and qualitative identification are performed in one run.

Figure 12 shows a 2-µl injection of pistachio-nut extract detected using FLD and DAD. B_1 is present in less than 1.0 ng (absolute) corresponding to 11 ppb (20 g of material extracted in 500 µl methanol of which 2 µl was injected corresponds to a multiplication factor of 12500 for the ppb value). This is close to the detection limit for fluorescence, while for UV-visible absorbance much lower values are detectable. To determine lower concentrations by FLD, larger injection volumes are needed. Fluorescence has the advantage of high selectivity- and therefore no matrix effects–whereas the diode-array detector shows much higher sensitivity for B_1 and G_1 and can be used for additional confirmation using the automatic library search program. Figure 13 shows the printout of such a search. A report header contains all the important information, such as data file name, the library used, search threshold values, peak purity, while the quantitative report contains the corresponding retention times (from library, calibration table and chromatogram), purity and library match factors and names of the identified compounds.

Report from automatic Library search

***** REPORT *****

Operator Name:	RS	Vial/Inj.No.: 100/1	
Date & Time:	10 Apr 92 4:06 pm		
Data File Name:	FOOD:AFLA-PIST		
Integration File Name:	DATA:AFLA1.I		
Calibration File Name:	FOOD:AFLA-DAD.Q		
Quantitation method:	ESTD	calibrated by Area response	
Sample Info:	PISTATIO NUTS according to $35 LMBG (reduced to 500 µl MeOH)		
Misc. Info:	20g extr. CH2CL2–clean Silicag–HEXANE, ETHER, CHC13		
Method File Name:	AFLA-DAD.M	Wavelength from: 250 to: 400 nm	
Library File Name:	FOOD:AFLATOX.L	Library Threshold: 959	
Reference Spectrum:	8.32 min	Peak Purity Threshold: 950	
Time window from:	5.0 % to: 5.0 %	Smooth Factor: 5	
Dilution Factor: 12.5	Sample Amount: 0.0	Resp.Fact.uncal.peaks: None	

Name	Amount [µg/kg]	Peak-Ret. [min]	Call-Ret. [min]	Lib.-Ret [min]	Purity Match factor	Library	Res.
B1	11.48	A 7.956	7.18	8.18	967	987	6.4
	11.48						

Figure 13. Printout of the report of the analysis in figure 12

A milk sample spiked with 600 ng/l of aflatoxin M_1 was prepared according to § 35 LMBG. 2 µl were injected and detected with FLD and DAD (figure 14). The sub-nanogram amounts of M_1 (0.6 ng) could be detected by DAD and a spectral search for confirmation was performed.

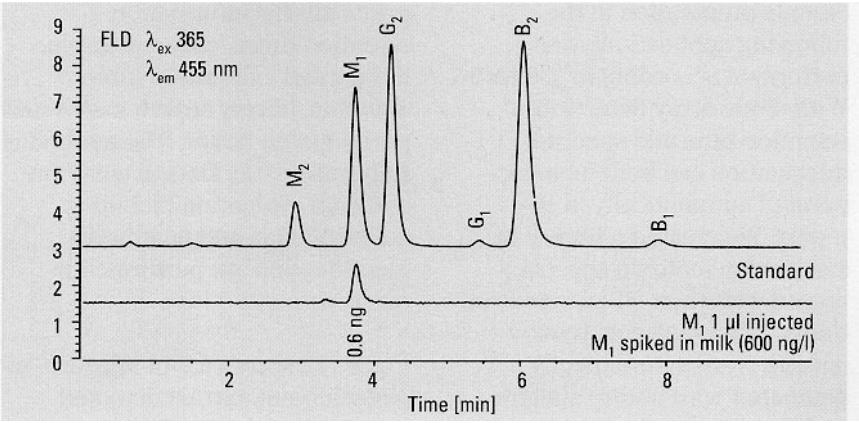


Figure 14. Analysis of spiked milk sample (600 ng/l) using sample preparation § 36 LMBG

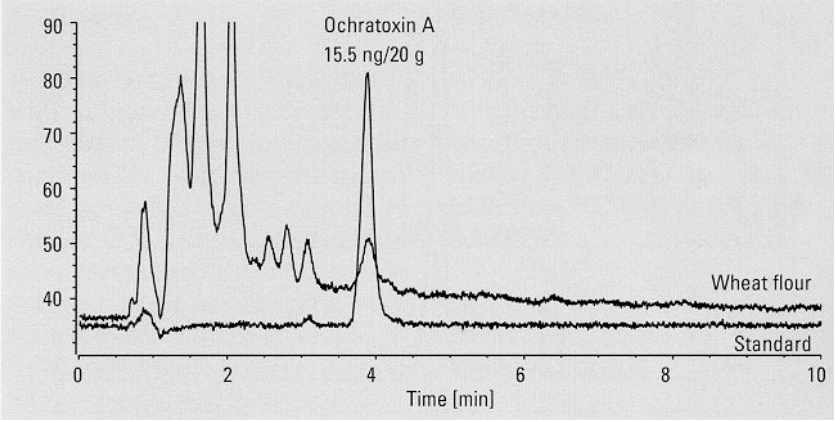


Figure 15. Analysis of ochratoxin A in wheat flour with the corresponding standard

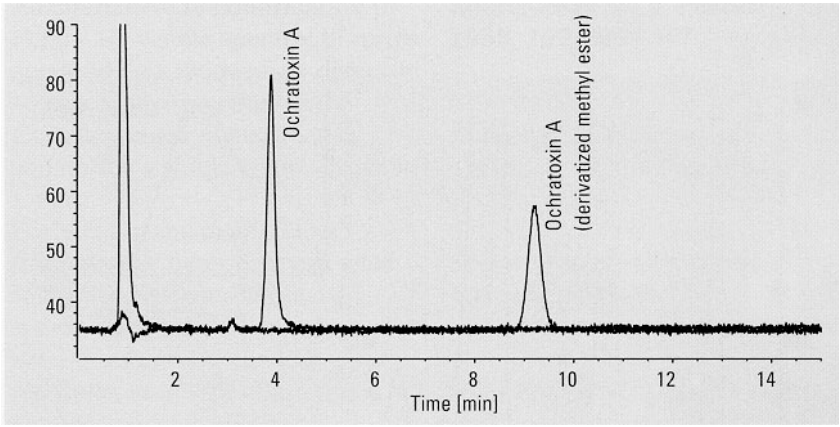


Figure 16. Separation of ochratoxin A and the ochratoxin A methylester derivative (1 ng absolute)

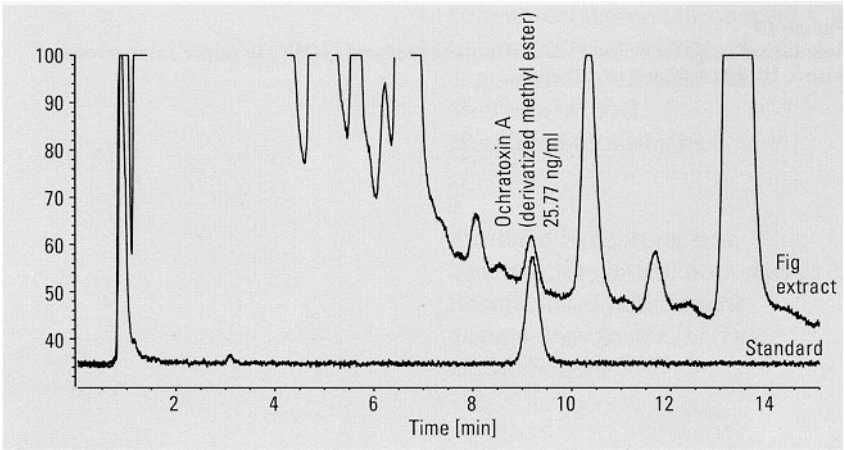


Figure 17. Analysis of a fig extract where ochratoxin A has been derivatized and overlaid with the corresponding methyl ester standard

Ochratoxin A assay by HPLC

Separation was achieved on a reversed phase column (LiChrospher 100 RP 18 125 x 4-mm id, 5 µm particles) with water /2 % acetic acid / acetonitrile (1: 1) and detected at lex 247 nm, lem 480 nm with a fluorescence detector, 20-µl injection volume and 40°C column temperature.

This analysis was confirmed with a derivatization of the mycotoxin to the methyl ester (figure 16).

The analysis also works well in more complicated matrices, for example, figs (figure 17).

Zearalenone assay by HPLC-DAD and HPLC-FLD

Separation was achieved on an Hypersil ODS narrow-bore column (100 x 2.1-mm id, 5-µm particles) using a 50 parts water, 40 parts acetonitrile, 10 parts methanol isocratic mobile phase mixture. DAD detection wavelength was 236 nm with 20 nm bandwidth, fluoresecence detection was at lex 236 nm, lem 464 nm. Figure 18 shows a standard composed of 5 ng a-zearalenol, 2 ng b-zearalenol and 8 ng zearalenone. We recommend the DAD for sensitivity and spectral confirmation, while higher selectivity is given by fluorescence.

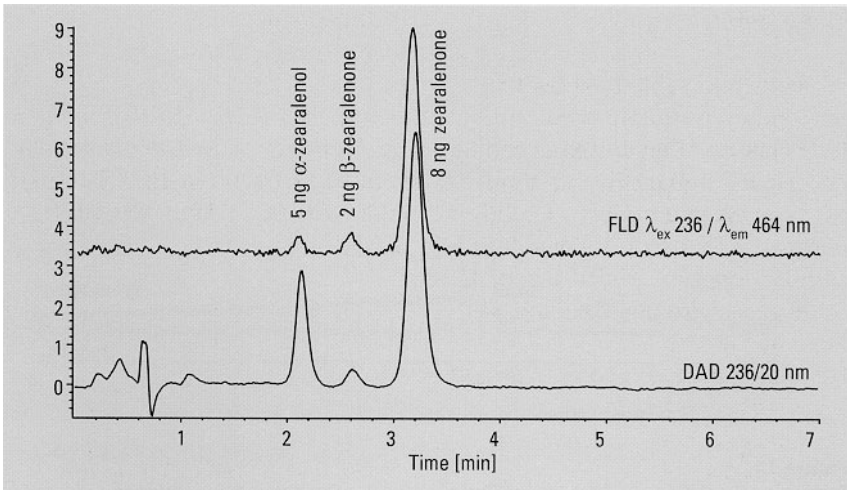


Figure 18. Analysis of zearalenone and its metabolites with FLD and DAD detection

Patulin assay by HPLC-DAD

A major problem for the analysis of patulin in apple products (juice, pies and so on) is the high content of 5-hydroxy methyl furfural (HMF) a compound that elutes close to patulin and absorbs light in the ultraviolet region also. Separation of both HMF and patulin was achieved on a silicagel column and also on a diol column (a reversed phase column based on silica gel with two hydroxyl endings). Figure 18 shows a separation of the compounds in an apple juice sample on a diol column (conditions given in table 1). Patulin was detected at 270 nm with subsequent identification using spectral library search.

With improved reverse phase column materials, separation can be done on Spherisorb RP 18, 5-µm particles using an acetonitrile gradient from 5 % to 100 % at 40 °C.[16]

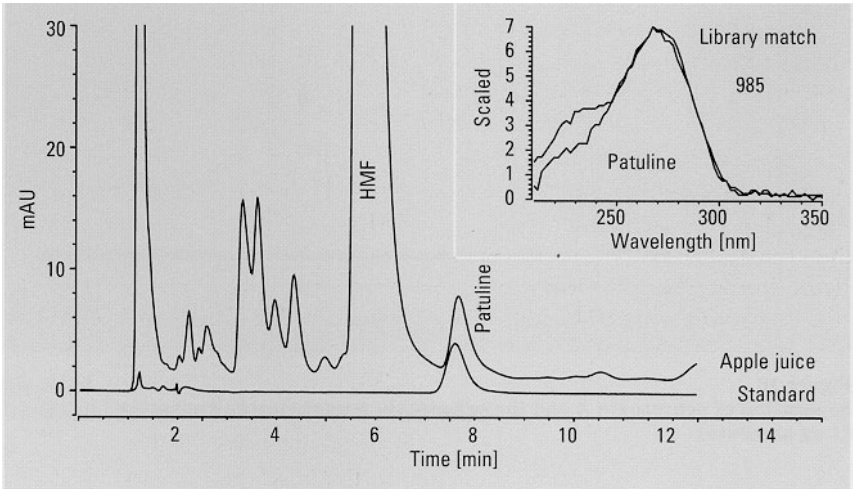


Figure 19. Resolution of patulin and 5-hydro methyl furfural (HMF) in apple juice overlaid with a 10-ng standard of patulin

Conclusion

We have been able to show that with suitable sample preparation four classes of mycotoxins can be successfully quantified at nanogram levels in a variety of solid and liquid foodstuffs. Considering sample complexities, a variety of approaches are possible and we have discussed these at length. HPLC separations were performed on reversed phase materials. Derivatization and subsequent fluorescence detection can improve selectivity for aflatoxins and ochratoxin A, and serve as an additional confirmatory analysis. An alternative to confirmation by FLD—UV-visible spectral libraries acquired on a diode-array detector—can be incorporated in the analytical run and automated, generating a single comprehensive report.

For most of the mycotoxins, fluorescence detection was used for high sensitivity. Mass spectrometry was able to lower detection limits to the low picogram range for aflatoxins, including confirmation via molecular mass.

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Fast Dual-Column GC/ECD Analysis of Chlorinated Pesticides—EPA Methods 608 and 8080

Application Note
228-305

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Abstract

Dual-column analysis with HP-35 and HP PAS-1701 columns was used to analyze chlorinated pesticides targeted in EPA Methods 608 and 8080 for wastewater and solid wastes. GC parameters were optimized using the Agilent 5890 Series II gas chromatograph (GC) with electronic pressure control (EPC), a dual injector, and a dual electron capture detector (ECD) system. The analysis of 18 pesticides was completed in 12 minutes.

Introduction

Currently, many testing laboratories use dual-column/dual-ECD GC systems to analyze the chlorinated pesticides specified in EPA Methods 608 and 8080[1,2]. For this application, EPC was used with an HP-35 column (35% phenyl, 65% methyl polysiloxane phase) as the primary column and the HP PAS-1701 column for confirmation.

The unique selectivity of the HP-35 column for this set of chlorinated pesticides permitted focus on the optimization of oven temperature for the HP PAS-1701 column. Individual EPC ports for each injector permitted individual regulation of column flow for both the HP-35 and the HP PAS-1701.

Experimental

EPA Method 608 and 8080 targeted pesticides were separated using 30 m x 0.53 mm x 1.0 µm HP-35 and HP PAS-1701 columns (part no. 19095G-123 and 19094U-023, respectively). Analyses were performed on an HP 5890 Series II GC with EPC, dual split/splitless inlets, and dual ECDs. An Agilent 7673 automatic liquid sampler was used to process the simultaneous splitless injections. A deactivated single-tapered glass liner with a small plug of glass wool (part no. 5181-3316) and a Merlin Microseal septum (part no. 5181-8816) were used with each split/splitless inlet. Instrumentation and GC conditions are listed in **Table 1**.

A test mix containing 18 pesticides (50 ppb per component) and two surrogates was prepared from the dilution of certified standard mixes with pesticide-grade hexane (Burdick & Jackson). Pesticides in the test mix are listed in **Table 2**.

Table 1. Experimental Conditions

Instrument Requirement	
Gas Chromatograph	Agilent Technologies 5890 Series II with EPC
Injection Ports	Dual split/splitless inlets
Column	HP-35, 30 m x 0.53 mm x 1.0 µm (Part no. 19095G-123)
	HP PAS-1701, 30 m x 0.53 mm x 1.0 µm (Part no. 19095S-123)
Detector	Dual ECD
Sample Introduction	7673 automatic sampler with dual injectors
Data Collection	3365 ChemStation and HP Vectra 486/33T PC
Experimental Conditions	
Injection	Splitless 1 µl, purge delay, 0.75 min, inlet temperature of 250°C
Carrier gas	(A) HP-35, pressure program: 8.6 psi (1 min) at 0.5 psi/min to 12 psi and at 3.0 psi/min to 25 psi (0 min)
	(B) HP-1701, helium, 10 ml/min constant flow
Oven	160°C (1 min) to 280°C at 10°C/min and to 300°C (2 min) at 25°C/min
Detector	ECD (300°C), 120 ml/min N_2 makeup, 6 ml/min anode purge

Results and Discussion

In a dual-column/dual-ECD system, samples introduced in a single injection can be split between two columns using a Y-connector and detected by different ECDs. However, when using a Y-connector without EPC, the split sample flow to each column cannot be optimized, and equal and consistent sample splits cannot be presumed. The only variable that can be optimized, in dual-column ECD analysis using a Y-connector is the oven temperature program, which can be optimally balanced for the two dissimilar columns. Using dual-column GC/ECD without EPC, it would typically require 45 to 60 minutes to obtain baseline separations for EPA Method 608 and 8080 targeted pesticides (see **Figure 1**).

A typical run from an environmental testing laboratory for a test mix containing 18 targeted pesticides and two surrogates is shown in **Figure 1**. A Yconnector was used to split samples for both columns, DB-608 and DB-1701, and good baseline separations were obtained for most analytes. This dual-column run was completed in 45 to 53 minutes using the following oven temperature program: 150°C (1 minute) to 260°C (18.34 minute) at 3°C/minute, then to 275°C (5 minutes) at 25°C/minute. Clearly this oven temperature program was optimized to separate critical pairs, such as DDE/dieldrin, DDD/endosulfan II, endosulfan sulfate/mehtoxychlor, and methosychlor/endrin ketone for both columns.

Table 2. Chlorinated Pesticides.

Peak No.	Pesticides
1	Tatrachloro-m-xylene (SS1)
2	alpha-BHC
3	Lindane
4	beta-BHC
5	Heptachlor
6	delta-BHC
7	Aldrin
8	Heptachlor epoxide
9	Endosulfan I
10	4,4′-DDE
11	Dieldrin
12	Endrin
13	4,4′-DDD
14	Endosulfan II
15	4,4′-DDT
16	Endrin aldehyde
17	Endosulfan sulfate
18	Methoxychlor
19	Endrin ketone
20	Decachlorobiphenyl (SS2)

Figure 2 shows chromatograms of the same pesticide test mix using the HP-35 and HP PAS-1701 columns and EPC. The oven program, 160°C (1 minute) to 280°C at 10°C/minute and to 300°C (2 minutes) at 25°C/minute, was optimized to separate the critical pairs, endosulfan

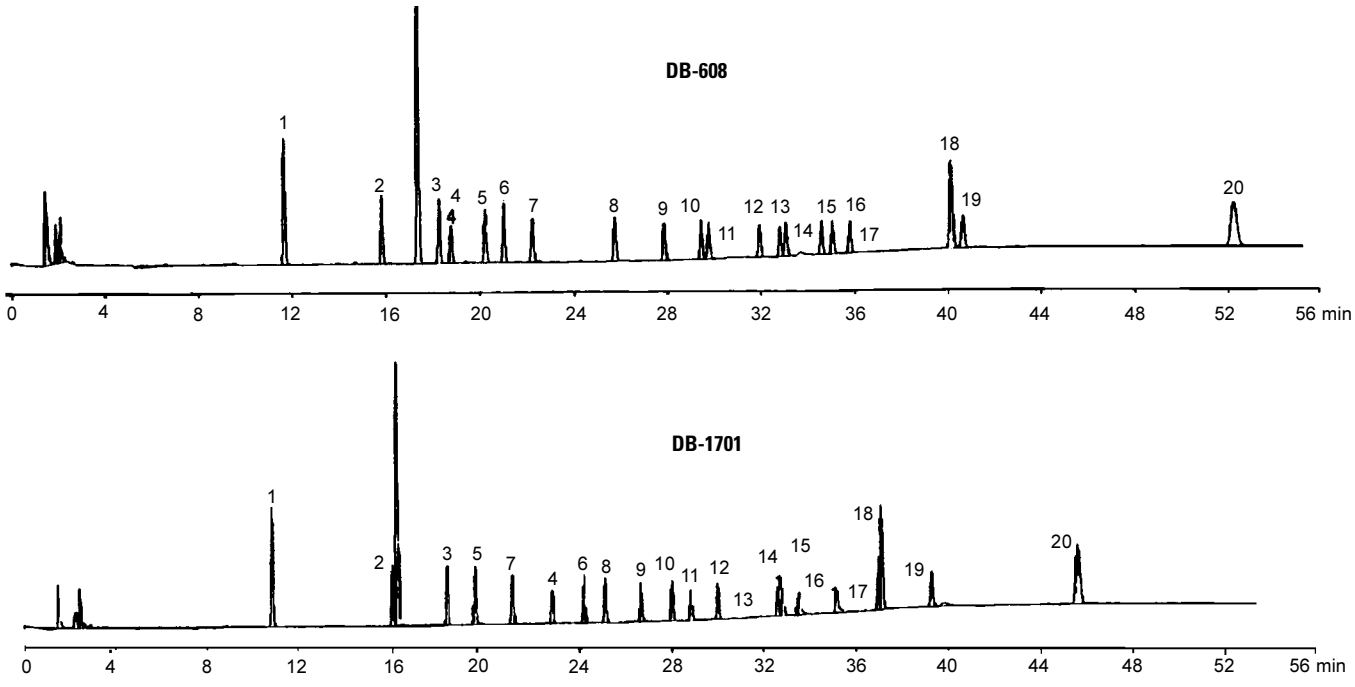


Figure 1. Typical chromatograms of a pesticides standard mix using DB-608 and DB-1701 columns under GC conditions used in environmental testing laboratories. (See Table 2 for peak identification.)

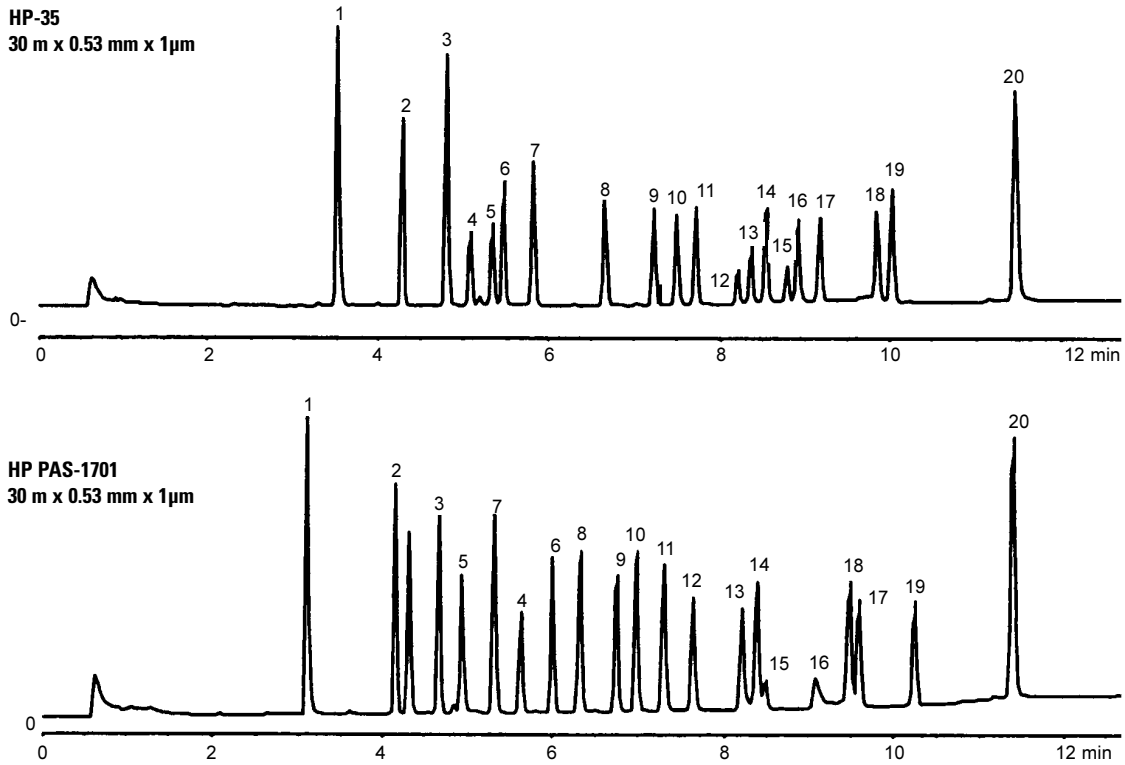


Figure 2. Chromatograms of a pesticides standard mix using HP-35 and HP PAS-1701 columns under the GC conditions listed in Table 1. (See Table 2 for peak identification.)

II/DDT and methoxychlor/endosulfan sulfate, for the HP PAS-1701 column. In this run, EPC provided a constant 10 ml/minute helium flow to the HP PAS-1701 column throughout the entire run.

For the HP-35 column, the following pressure program was used: 8.6 psi (hold 1 minute) at 0.5 psi/minute to 12 psi and at 3.0 psi/minute to 25 psi (hold for constant flow for the remaineder of the run). This pressure program actually provided a 10 ml/minute constant flow to elute most of the pesticides and an increased flow (up to 20 ml/minute) near the end of the run to elute the last analyte, surrogate decachlorobiphenyl and other high-boiling materials from the column.

GC parameters optimized for dual-column/dual-injector/dual-ECD analysis of chlorinated pesticides reduced analysis time to less than 12 minutes. In addition to speed, all EPA Methods 608 and 8080 targeted pesticides and surrogates were well resolved with good sharp peaks for accurate quantitation.

Conclusion

The use of EPC permitted individual column flow control to each ECD. The unique selectivity of the HP-35 column for chlorinated pesticides permitted focus on the optimization of oven temperature for the HP PAS-1701 column. Run time was 11.5 minutes with good baseline separations for all 20 target pesticides and surrogates. The result was a reduction in sample turnaround time from 54 to 11.5 minutes for a 400% increase in productivity. This is more than a twofold improvement in productivity when compared with conventional methods currently used at many environmental testing laboratories with DB-608 and DB-1701 columns.

Acknowledgement

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Agilent Technologies
Innovating the HP Way

Analysis of Organochlorine Pesticides and PCB Congeners with the Agilent 6890 Micro-ECD

Application

Gas Chromatography

June 1997

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Abstract

A new electron capture detector (ECD) for the Agilent 6890 Series gas chromatograph (GC) was used to analyze polychlorinated biphenyl congeners and organochlorine pesticides. The linearity of the 6890 Micro-ECD in the calibration range of 2 to 400 ppb was evaluated. The micro-ECD easily meets the linearity requirements of U.S. EPA contract laboratory programs for pesticides. Its limit of detection for these compounds goes down to less than 50 ppt. The micro-ECD also exhibits good reproducibility.

Key Words

Organochlorine pesticides, PCB congeners, 6890 GC, micro-ECD; pesticide analysis, ECD.

Introduction

The electron capture detector (ECD) is the detector of choice in many Contract Laboratory Programs (CLP)[1] and EPA methods for pesticide analysis because of its sensitivity and selectivity for halogenated compounds. However, there are drawbacks to the ECD design. The ECD is inherently nonlinear[2], with a limited linear range. The limited linear range means that dilution and reanalysis are frequently required for samples that are outside the calibration range.

Also, the typical ECD is designed to be compatible with both packed and capillary columns. This results in a flow cell that is larger than that required for capillary columns alone, which reduces detector sensitivity.

To address these problems, a new ECD was developed for the 6890 Series gas chromatograph (GC). The 6890 Micro-ECD has a smaller flow cell optimized for capillary columns and was redesigned to improve the linear operating range.

This application note examines the linearity, reproducibility, and limit of detection of the new ECD with mixtures of polychlorobiphenyl (PCB) congeners and organochlorine pesticides (OCPs).

Experimental

All experiments were performed on an 6890 Series GC with electronic pneumatics control (EPC) and the 6890 Micro-ECD. Table 1 shows the experimental conditions for PCB congeners and OCPs.

Table 1. Experimental Conditions for PCB Congener and OCP Analysis.

System Conditions	PCB Congener Analysis	OCP Analysis
Oven	80 °C (2 min); 30 °C/min to 200 °C; 10 °C/min to 320 °C (5 min).	80 °C (2 min); 25 °C/min to 190 °C; 5 °C/min to 280 °C; 25 °C/min to 300 °C (2 min).
Inlet	Split/splitless; 300 °C	Split/splitless; 250 °C
Carrier	Helium, 16.8 psi (80 °C); 1.3-mL/min constant flow	Helium, 23.9 psi (80 °C); 2.2-mL/min constant flow
Sampler	Agilent 7673, 10-µL syringe, 1-µL splitless injection	7673, 10-µL syringe, 1-µL splitless injection
Column	30-m, 250-µm id, 0.25-µm film HP-5MS (part no. 19091S-433)	30-m, 250-µm id, 0.25-µm film HP-5MS (part no. 19091S-433)
Detector	330 °C; makeup gas: nitrogen, constant column and makeup flow	330 °C; makeup gas: nitrogen, constant column and makeup flow

The solutions were prepared by making appropriate dilutions of a stock solution with isooctane. For PCB congeners, the stock solution was an EPA PCB congener calibration check solution (from Ultra Scientific Company, part number RPC-EPA-1). For OCPs, the solution was an OCP calibration check solution (part number 8500-5876).

Results and Discussion

Linearity and Response Factors

A series of dilutions of the PCB mixture from 2 ppb to 200 ppb and of the OCP mixture from 2 ppb to 400 ppb was injected into the 6890 Micro-ECD system. The linearity was determined by calculating the correlation coefficient from the resulting calibration curve.

Figures 1 and 2 present typical chromatograms of OCPs and PCBs at 20 or 40 ppb and 50 ppb, respectively. Figure 3 is a calibration curve of decachlorobiphenyl, typical of other PCB congeners. Figure 4 shows the calibration curve of 4, 4' DDE, typical of OCPs. The correlation coefficient,

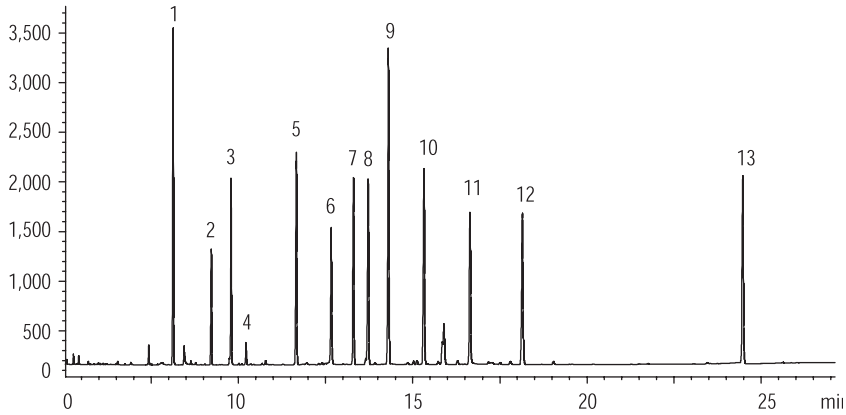


Figure 1. Typical chromatogram of OCPs at 20 or 40 ppb.
See table 1 for conditions. See table 5 for peak identification.

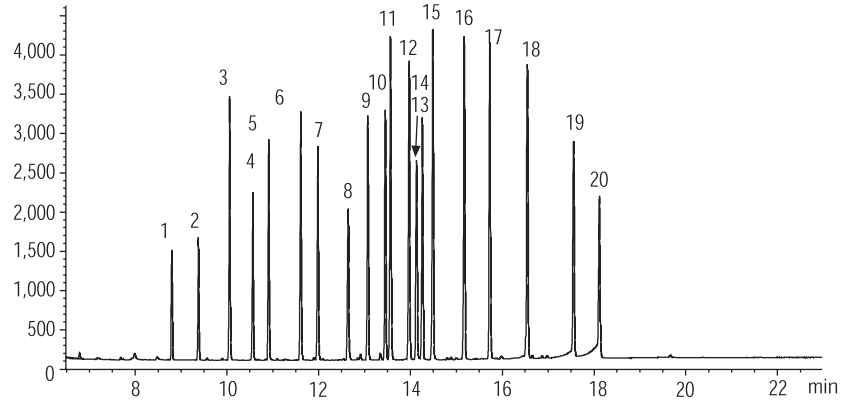


Figure 2. Typical chromatogram of PCB congeners at 50 ppb.
See table 1 for conditions. See table 4 for peak identification.

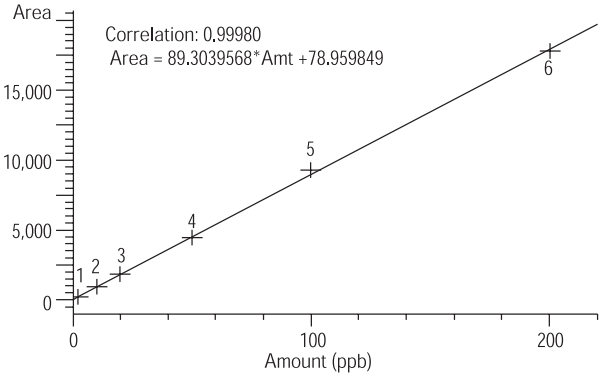


Figure 3. Typical linearity of PCB congener analysis: decachlorobiphenyl from 2-200 ppb.

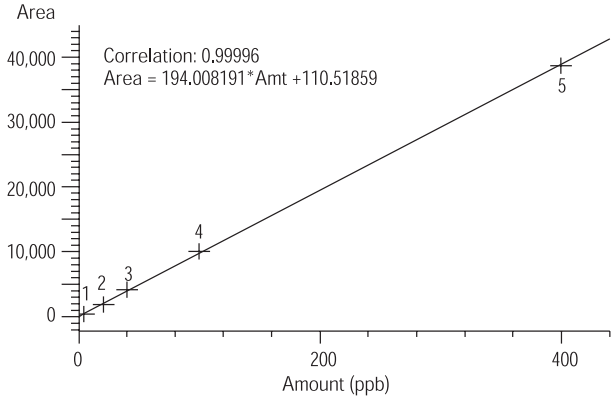


Figure 4. Typical linearity of OCP analysis: 4,4' DDE from 4 to 400 ppb.

average response factors, and percent relative standard deviation (%RSD) for the response factors for each analyte are shown in tables 2 and 3.

All correlation coefficients were at least 0.9996. In these experiments, the 6890 Micro-ECD is linear over this range. The typical range required by CLP methods is 5-80 ppb[1], so the 6890 Micro-ECD exceeds the range by almost twofold.

In addition, the CLP method requires the percent RSD of the response factors for most components to be less than 20 percent for a three-point calibration curve (5 to 80 ppb). As shown in tables 2 and 3, the percent RSD of the response factors ranged from 0.55 percent to 12. 5 percent for the PCB congeners and from 2.8 percent to 10 percent for the OCPs over a concentration range of two orders of magnitude (2 to 400 ppb). Furthermore, the average response factor of each analyte was so consistent and reproducible that the internal standard technique can be used to quantitate all OCPs and PCB congeners.

Table 2. PCB Congener Analysis: Linearity of the 6890 Micro-ECD 2 ppb to 200 ppb.
See table 1 for conditions.

Peak	Name	Average Response Factor	%RSD of Response Factor	Correlation (%)
1	2,4-Dichlorobiphenyl	2e-2	12.5	99.97
2	2,2′,5-Trichlorobiphenyl	2e-2	11.1	99.97
3	2,4,4′-Trichlorobiphenyl	8.5e-3	7.5	99.99
4	2,2′,5,5′-Tetrachlorobiphenyl	1.3e-2	10.2	99.97
5	2,2′,3,5-Tetrachlorobiphenyl	1e-2	9.4	99.98
6	2,3,4,4′-Tetrachlorobiphenyl	8e-3	6.7	99.99
7	2,2′,4,5,5′-Pentachlorobiphenyl	9e-3	8.8	99.98
8	3,3′,4,4′-Tetrachlorobiphenyl	1.2e-2	12.6	99.97
9	2,3,4,4′,5-Pentachlorobiphenyl	8e-3	5.5	99.99
10	2,2′,4,4′,5,5′-Hexachlorobiphenyl	8e-3	8.1	99.98
11	2,3,3′,4,4′-Pentachlorobiphenyl	6e-3	1.9	99.99
12	2,2′,3,4,4′,5-Hexachlorobiphenyl	6.5e-3	3.8	99.99
13	3,3′,4,4′,5-Pentachlorobiphenyl	9e-3	6.5	99.99
14	2,2′,3,4,5,5′,6-Heptachlorobiphenyl	8e-3	5.7	99.99
15	2,2′,3,3′,4,4′-Hexachlorobiphenyl	5.6e-3	1.8	99.99
16	2,2′,3,4,4′,5,5′-Heptachlorobiphenyl	5.8e-3	1.0	99.99
17	2,2′,3,3′,4,4′,5-Heptachlorobiphenyl	5.8e-3	0.57	99.99
18	2,2′,3,3′,4,4′,5,6-Octachlorobiphenyl	6e-3	0.78	99.99
19	2,2′,3,3′,4,4′,5,5′,6-Nonachlorobiphenyl	8e-3	3.1	99.96
20	Decachlorobiphenyl	1e-2	9.5	99.98

Table 3. OCP Analysis: Linearity of the 6890 Micro-ECD *2 or 4* ppb to *200 or 400* ppb.
See table 1 for conditions.

Peak	Name	Average Response Factor	% RSD of Response Factor	Correlation (%)
1	2,4,5,6-Tetra-m-xylene	4.2e-3	5.3	99.97
2	beta-BHC	1.1e-2	7.1	99.99
3	delta-BHC	6.4e-3	4.7	99.99
4	Aldrin	4.7e-3	9.5	99.97
5	Heptachlor epoxide	4.7e-3	5.4	99.99
6	gamma-Chlordane	6.6e-3	6.6	99.99
7	alpha-Chlordane	5e-3	4.3	99.98
8	4,4' DDE	5e-3	2.8	99.99
9	Endosulfan II	2.9e-3	4.4	99.98
10	Endrin aldehyde	4.5e-3	5.9	99.94
11	Endosulfan sulfate	5.1e-3	5.3	99.97
12	Endrin ketone	4.7e-3	9.0	99.89
13	Decachlorobiphenyl	3.7e-3	9.9	99.96

Reproducibility

The reproducibility of the 6890 Micro-ECD was established by analyzing each mixture using identical conditions five times. Each analyte in the PCB congener mixture was injected at a concentration of 50 ppb, and the analytes in the OCP mixture were 20 or 40 ppb. The results are shown in tables 4 and 5. The highest %RSD for any analyte is 3.69 percent for aldrin, which is well below the CLP maximum allowable RSD of 15 percent.[1]

Table 4. PCB Congener Analysis: Reproducibility of the 6890 Micro-ECD 50 ppb; N=5.
See table 1 for conditions.

Peak	Name	Average Area	RSD (%)
1	2,4-Dichlorobiphenyl	2229	1.26
2	2,2′,5-Trichlorobiphenyl	2547	1.29
3	2,4,4′-Trichlorobiphenyl	5687	1.41
4	2,2′,5,5′-Tetrachlorobiphenyl	3721	1.43
5	2,2′,3,5-Tetrachlorobiphenyl	4941	1.46
6	2,3,4,4′-Tetrachlorobiphenyl	5943	1.40
7	2,2′,4,5,5′-Pentachlorobiphenyl	5089	1.47
8	3,3′,4,4′-Tetrachlorobiphenyl	3822	1.72
9	2,3,4,4′,5-Pentachlorobiphenyl	6203	1.62
10	2,2′,4,4′,5,5′-Hexachlorobiphenyl	6189	1.44
11	2,3,3′,4,4′-Pentachlorobiphenyl	8375	1.68
12	2,2′,3,4,4′,5-Hexachlorobiphenyl	7538	1.56
13	3,3′,4,4′,5-Pentachlorobiphenyl	5092	2.02
14	2,2′,3,4,5,5′,6-Heptachlorobiphenyl	6224	1.69
15	2,2′,3,3′,4,4′-Hexachlorobiphenyl	8921	1.67
16	2,2′,3,4,4′,5,5′-Heptachlorobiphenyl	8527	1.82
17	2,2′,3,3′,4,4′,5-Heptachlorobiphenyl	8625	1.91
18	2,2′,3,3′,4,4′,5,6-Octachlorobiphenyl	8338	2.13
19	2,2′,3,3′,4,4′,5,5′,6-Nonachlorobiphenyl	6097	2.55
20	Decachlorobiphenyl	4622	2.85

Table 5. OCP Analysis: Reproducibility of the 6890 Micro-ECD; N=5.
See table 1 for conditions.

Peak	Name	Concentration (ppb)	Average Area	RSD (%)
1	2,4,5,6-Tetra-m-xylene	20	4785	0.7
2	beta-BHC	20	1802	0.81
3	delta-BHC	20	3251	1.50
4	Aldrin	20	402	3.69
5	Heptachlor epoxide	20	4316	1.58
6	gamma-Chlordane	20	2958	1.23
7	alpha-Chlordane	20	4219	1.06
8	4,4' DDE	40	4103	1.76
9	Endosulfan II	40	7176	1.27
10	Endrin aldehyde	40	4719	0.85
11	Endosulfan sulfate	40	4040	3.04
12	Endrin ketone	40	4386	2.52
13	Decachlorobiphenyl	40	5369	0.85

Detection Limit

To establish the lower limit of detection for the 6890 Micro-ECD with PCBs and OCPs, 1-µL injections were made at gradually decreasing concentrations. Figures 5 and 6 show chromatograms with analyte concentrations of 50 to 100 ppt.

All the analyte peaks for both the PCB congener and OCP mixtures are still easy to quantitate, and in fact smaller concentrations can be reliably analyzed. Aldrin, which has the lowest response of the OCPs, still exhibits an adequate signal-to-noise ratio at the 50 ppt level under these analysis conditions.

Conclusion

The Agilent 6890 Micro-ECD response was linear over the concentration range of 2 to 200 ppb, produced reproducible results, and exhibited excellent sensitivity for mixtures of PCB congeners and OCPs.

References

1. U.S. EPA Contract Laboratory Program, "Statement of Work for Organic Analysis," OLM03.1, August 1994.

2. R. Buffington and M.K. Wilson, eds., *Detectors for Gas Chromatography*, Agilent Technologies, Inc., Part Number 5958-9433, 1987.

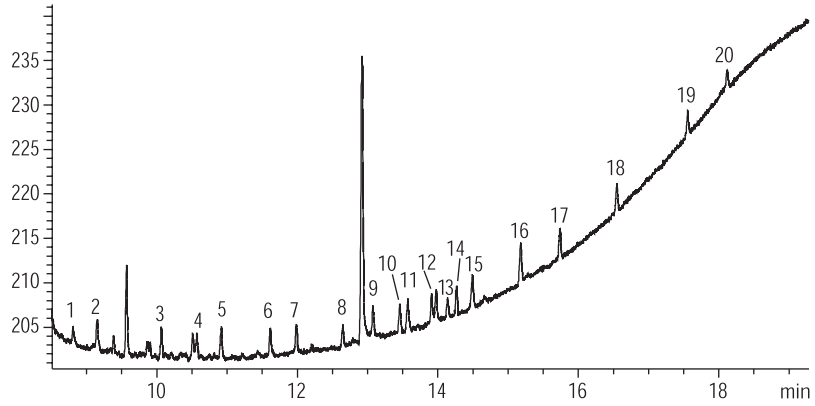


Figure 5. PCB congener mixture at 50 ppt each.
See table 1 for conditions. See table 4 for peak identification.

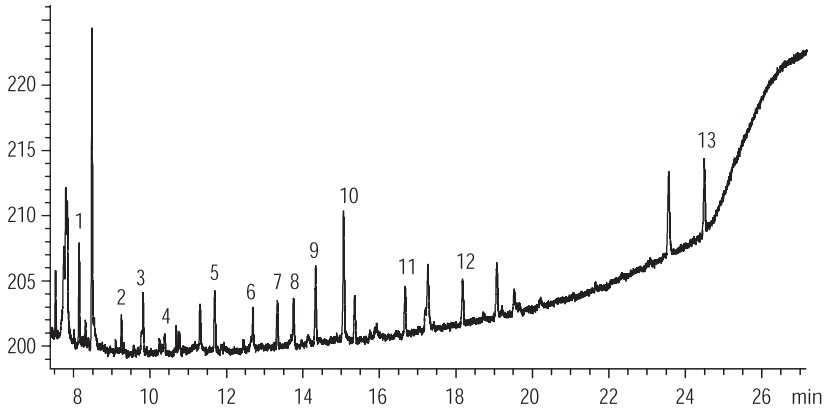


Figure 6. OCP Mixture at 50 to 100 ppt.
See table 1 for conditions. See table 5 for peak identification.

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HPLC Analysis of Aflatoxines in Pistachio Nuts using HPLC

Angelika Gratzfeld-Heusgen

Food

Abstract

The following mycotoxins have been analyzed: aflatoxins G_2, G_1, B_2, B_1, M_2, and M1, ochratoxin A, zearalenone, and patuline.

Mycotoxins are highly toxic compounds produced by fungi. They can contaminate food products when storage conditions are favorable to fungal growth. These toxins are of relatively high molecular weight and contain one or more oxygenated alicyclic rings. The analysis of individual mycotoxins and their metabolites is difficult because more than 100 such compounds are known, and any individual toxin is likely to be present in minute concentration in a highly complex organic matrix. Most mycotoxins are assayed with thin-layer chromatography (TLC). However, the higher separation power and shorter analysis time of HPLC has resulted in the increased use of this method. The required detection in the low parts per billion (ppb) range 4,[1, 2, 3] can be performed using suitable sample enrichment and sensitive detection.

Sample preparation

Samples were prepared according to official methods.[2] Different sample preparation and HPLC separation conditions must be used for the different classes of compounds. The table on the next page gives an overview of the conditions for the analysis of mycotoxins in foodstuffs.

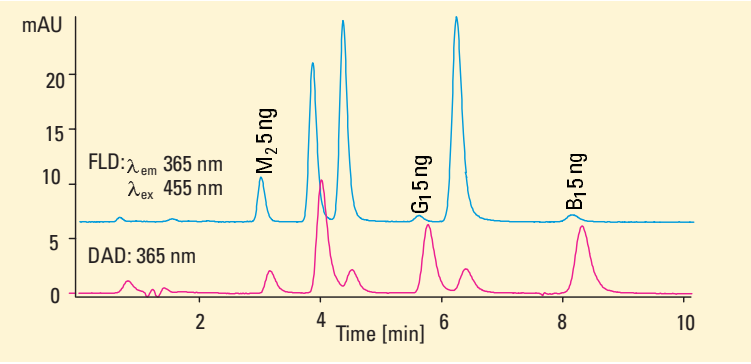


Figure 1
Analysis of aflatoxins with UV and fluorescence detection

Chromatographic conditions

The HPLC method presented here for the analysis of mycotoxins in nuts, spices, animal feed, milk, cereals, flour, figs, and apples is based on reversed-phase chromatography, multisignal UV-visible diode-array detection, and fluorescence detection. UV spectra were evaluated as an additional identification tool.

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HPLC method performance

Limit of detection 1–5 µg/kg

Repeatability
of RT over 10 runs <0.12 %
of areas over 10 runs <1.5 %

Linearity
of UV-visible DAD 1–500 ng
of fluorescence 30 pg to 2 ng

Column class	Matrix	Sample preparation	Chromatographic conditions
Aflatoxins G_2, G_1, B_2, B_1, M_2, M_1	nuts, spices, animal feed, milk, dairy products	• extraction according to Para. 35, LMBG* [4, 5]	Hypersil ODS, 100 × 2.1 mm id, 3-µm particles water/methanol/ACN (63:26:11) as isocratic mixture (100% B is recommended for cleaning the column flow rate: 0.3 ml/min at 25 °C DAD: 365/20 nm Fluorescence detector (FLD): excitation wavelength 365 nm, emission wavelength 455 nm
Ochratoxin A	cereals, flour, figs	• extraction according to Para. 35, LMBG • acidify with HCl • extract with toluene • SiO_2 cleanup elute toluene/acetic acid (9:1)	Lichrospher 100 RP18, 125 × 4 mm id, 5-µm particles water with 2 % acetic acid/ACN (1:1)* flow rate: 1ml/min at 40 °C FLD: excitation wavelength 347 nm, emission wavelength 480 nm
Zearalenone	cereals	• extract with toluene • Sep-pak cleanup • elute toluene/acetone (95:5) • AOAC 985.18:[3] α-zearalenol and zearalenone in corn	Hypersil ODS, 100 × 2.1 mm id, 3-µm particles water/methanol/ACN (5:4:1) as isocratic mixture* flow rate: 0.45 ml/min at 45 °C DAD: 236/20 nm FLD: excitation wavelength 236 nm, emission wavelength 464 nm
Patuline	apple products	• cleanup on Extrelut • silica gel cleanup • elute toluene/ethylacetate (3:1)	Superspher RP18, 125 × 4 mm id, 4-µm particles water 5 %–95 % ACN flow rate: 0.6 ml/min at 40 °C DAD: 270/20 nm or Lichrospher diol, 125 × 4 mm id, 5-µm particles hexane/isopropanol (95:5) as isocratic mixture flow rate: 0.6 ml/min at 30 °C DAD: 270/20 nm

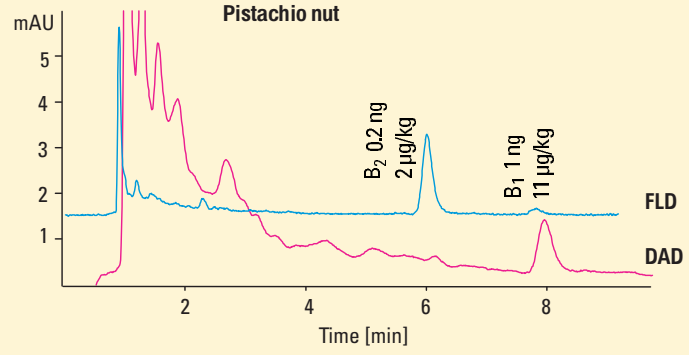


Figure 2
Analysis of aflatoxins in pistachio nuts with UV and fluorescence detection

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2. Lebensmittel- und Bedarfsgegenständegesetz, Paragraph 35, Germany.

3. *Official Methods of Analysis, Food Compositions; Additives, Natural Contaminants*, 15th ed; AOAC: Arlington, VA, 1990, Vol. 2.; AOAC Official Method 980.20: aflatoxins in cotton seed products; AOAC Official Method 986.16: Aflatoxins M_1, M_2 in fluid milk; AOAC Official Method 985.18: α-zearalenol.

4. Farrington et. al., "Food Additives and Contaminants, **1991**, Vol. 8, No. 1, 55-64".

5. *Official Methods of Analysis*; Horwitz, W., Ed.; 14th ed.; AOAC: Arlington, VA, **1984**; secs 12.018–12.021.

Equipment

Agilent 1100 Series
- degasser
- isocratic pump
- autosampler
- thermostatted column compartment
- diode array detector,
- fluorescence detector

Agilent ChemStation
+ software

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Analysis of Pesticides in Salad Samples and Spices using HPLC

Rainer Schuster

Food

Abstract

The following compound classes of pesticides have been analyzed: triazines, phenylurea-herbicides, methabenzthiazuron, diquat, paraquat, and mercaptobenzothiazol.

Carbamates and glyphosate also have been analyzed but with different equipment. In most countries, growing concern about the residues of pesticides in food products is evident. Therefore, regulations limiting the concentration of pesticides in foodstuffs have been introduced to protect consumers from contaminated food products. Several methods are used to control these limits. HPLC is recommended for the analysis of low volatile compounds and for compounds that are unstable when heated.

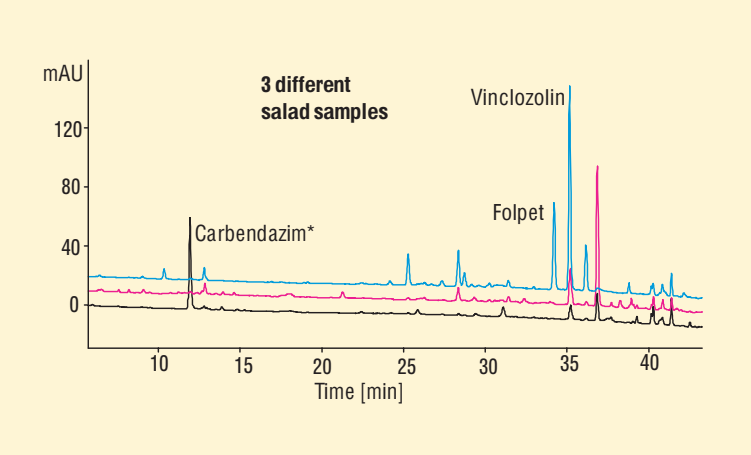


Figure 1
Analysis of pesticide residues in three different salad samples
* Carbendazim has a low recovery rate of only approximately 40 %

Conditions

Column 100 ˘ 3 mm Hypersil BDS, 3 µm
Mobile phase water/ACN (95:5)
Gradient at 10 min 25% ACN
at 26 min 42% ACN; at 34 min 60% ACN
Flushing time 10 min at 100% ACN
Post time 6 min
Flow rate 0.5 ml/min
Oven temp 42 °C
Injection vol 3–10 µl
Detector UV-DAD detection wavelengths 214/15 nm, 230/20 nm, and 245/20 nm reference wavelength 400/80 nm
Sample preparation
Salad was homogenized and then extracted with liquid/liquid extraction. The extract was cleaned with gel permeation chromatography using cyclohexane/ethyl acetate. Spices were prepared according to Specht 22 with gel permeation chromatography.

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Sample preparation

Sample preparation and enrichment depend strongly on the matrix. Drinking water samples, for example, must be extracted using solid-phase extraction, whereas vegetables are extracted with liquid/liquid extraction after homogenization, followed by additional cleaning and sample enrichment.

Chromatographic conditions

The HPLC method presented here was used for the analysis of pesticides in salad samples and spices.[1, 2]

HPLC method performance

Limit of detection
0.01 µg/l

Repeatability
of RT over 10 runs <0.2 %
of areas over 10 runs <1 %

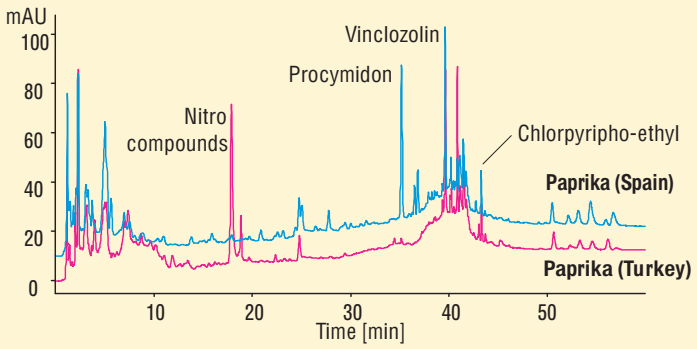
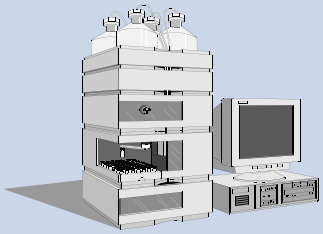


Figure 2
Analysis of pesticide residues in two paprika samples

Equipment

Agilent 1100 Series
- degasser
- quaternary pump
- autosampler
- thermostatted column compartment
- diode array detector, Agilent ChemStation + software



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Trace Level Pesticide Analysis by GC/MS Using Large-Volume Injection

Application

Gas Chromatography

September 1997

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Abstract

Large-volume injection (LVI) using the Agilent programmable temperature vaporizing (PTV) inlet can improve gas chromatography system detection limits by one to two orders of magnitude over standard methods that call for 1- or 2-µL injections. An Agilent 6890 Series gas chromatograph (GC), configured with a PTV inlet, a 6890 Series automatic liquid sampler (ALS), and an Agilent 5973 mass selective detector (MSD), was used for the analysis of pesticides in standards and several food extracts. By making 100-µL injections, several pesticides could be identified by scanning gas chromatography/mass spectrometry (GC/MS) at the 100 ppt (100 ng/L) level. The PTV inlet tolerated dirty food extracts very well; more than 1,500 µL of such samples were injected into a single PTV liner. This application note includes recommendations for doing LVI using the PTV/6890/5973 GC/MSD system.

Introduction

More than 700 pesticides are registered for use in the world[1] , and many more continue to persist in the environment, even though they are no longer being applied. For the protection of human health and the environment, pesticide residues are routinely monitored in food, water, soil, and tissue samples. "Acceptable" residue limits have been set for various foods and environmental samples by agencies such as the United States Environmental Protection Agency (U.S. EPA), the Codex Alimentarius Commission[2] , and many other governmental organizations around the world. A great many methods have been developed to screen for pesticides in food[3-7] and the environment[8-10] to ensure that risks associated with pesticide use are minimized.

Recently, concern has increased that certain pesticides and other synthetic chemicals may be acting as pseudo hormones which disrupt the normal function of the endocrine system in wildlife and humans. Birth defects, behavioral changes, breast cancer, lowered sperm counts, and reduced intelligence are among the many disorders that have been blamed on these "endocrine disrupting" compounds, though much research must be done to verify these assertions. In 1996, Colborn, Domanoski, and Myers[11] brought these issues into the public spotlight with the publication of their book *Our Stolen Future*. Recently, the United States Congress passed legislation calling for increased testing of suspected endocrine disrupters and monitoring their levels in food[12] and water[13] supplies. Because the endocrine system can be exquisitely sensitive to extremely low hormone concentrations, there is a need to measure concentrations of suspected endocrine disrupters (many of which are pesticides) at very low levels. Initiatives such as the Pesticide Data Program, developed by the United States Department of Agriculture[14] , seek to

determine the lowest measurable pesticide levels in various foods to develop a total exposure model. Clearly, there is pressure to push pesticide detection limits to even lower levels than are routinely achieved today. Most residue measurements are made by gas chromatography using a variety of element-selective or mass spectral detectors (GC/MS). Therefore, to achieve lower detection limits, it is necessary to improve the detection limits of these GC methods.

In GC, there are primarily four ways to improve method detection limits: 1) increase the concentration of analytes in a sample, usually by reducing the volume of an extract; 2) increase the sensitivity of the detector; 3) increase the selectivity of the detector to reduce chemical background "noise" or 4) increase the volume of sample injected. Because GC/ MS can be highly selective and extremely sensitive, it is often the method of choice for pesticide analysis and/or confirmation. However, for the reasons discussed above, there are occasions when even greater sensitivity is required. This application note describes a method for increasing GC/MS system detection limits by making large-volume injections (LVI) using Agilent's new programmable temperature vaporizing (PTV) inlet. Because this LVI technique is detector-independent, it is applicable to other GC configurations that may be used for pesticide residue analysis.

Experimental

Pesticide Standard Solution

Stock solutions of 14 pesticides were prepared at 1 mg/mL by adding 10 mg each of trifluralin, hexachlorobenzene, pentachloronitrobenzene, dichloran, chlorothalonil, chlorpyrifosmethyl, chlorpyrifos, endosulfan I, p,p'-DDE, propargite, iprodione, methoxychlor, and fenvalerate (mix of isomers I and II) to individual 20mL vials and diluting with 10.0 mL of acetone. Permethrin was obtained as a mixture of permethrin I and permethrin II comprising 32 percent and 27 percent of the sample, respectively, so 16.95 mg of this mixture was diluted with 10 mL of acetone giving a solution in which the combined permethrins represented 1 mg/mL. A stock mixture was prepared by adding 4 mL of the permethrin and fenvalerate solutions and 1 mL of each of the other stock solutions to a 100-mL volumetric flask and diluting to volume with acetone. The resultant solution contained 40 ng/µL each of the combined permethrin and fenvalerate isomers and 10 ng/µL each of the other 12. This sample was diluted further with acetone to prepare standards that were analyzed by LVI. All these pesticides were obtained in neat form from Chem Service (West Chester, PA USA).

Extracts

Fruit and vegetable extracts were obtained from the Florida Department of Agriculture and Consumer Services (Tallahassee, FL USA). Commodities were extracted using a version of the Luke procedure[15-17] that gave a final sample representing 1.75 g of the commodity per mL of extract.

Table 1. Instrumentation and Conditions Used for Pesticide Samples

GC/MS System	
Gas chromatograph	6890 Series GC
Automatic liquid sampler	6890 Series ALS
Mass spectral detector	5973 Series MSD
Programmable temperature vaporizing inlet	PTV with CO_2 cooling
Computer for data acquisition and analysis	HP Vectra XU 6/200
Software	G1701AA Version A.03.00 running Microsoft® Windows™ 95
Column	30 m x 0.25 mm x 0.25 µm Agilent HP-5MS
Instrumental Conditions	
GC Parameters	
Carrier gas	Helium
Inlet liner	Prototype deactivated borosilicate with fritted glass on interior walls (part no. 5183-2041)
Syringe size	50 µL
Injection volume	100 µL (Inject 10 µL 10 times)
Injection delay	12 sec
Inlet temperature program	40 °C (4.2 min), 200 °C/min to 320 °C (2 min)
Vent flow	400 mL/min Vent pressure 0.0 psi for 4.00 min
Purge flow to split vent	50.0 mL/min at 6.50 min
Column head pressure	0 psi (4 min) then 17.3 psi (constant pressure)
Oven temperature program	50 °C (6.13 min), 30 °C/min to 150 °C (2 min), 3 °C/min to 205 °C (0 min), 10 °C/min to 250 °C (20 min)
MSD Parameters	
Acquisition mode	Scan (35-550 amu)
Temperatures	Transfer line = 280 °C, MS quad = 150 °C, MS source = 230 °C

Instrumentation

Table 1 lists the instrumentation and chromatographic conditions used for LVI and GC/MS analysis of pesticide samples.

Brief PTV Tutorial

Before focusing on the PTV/GC/ MS analysis of pesticides, it is important to understand how the PTV inlet operates in the solvent vent mode for large-volume injections.

The PTV Inlet

The PTV inlet has the same basic functions as the split/ splitless inlet except that it is temperature programmable from -60 °C (using CO_2 cooling) or -160 °C (using liquid N_2 cooling) to 450 °C at rates up to 720 °C/min. However, the PTV's design has been optimized for its main uses-LVI and cold split/splitless injection. Although hot split and splitless injections may be made with or without a pressure pulse, care must be taken not to exceed the small internal volume of the PTV inlet. In practice, it is best to choose the Agilent split/splitless inlet for hot injections and the PTV inlet for LVI and cold split/ splitless techniques.

Most GC pesticide methods call for injecting 1-2 µL; splitless injection is used because it is compatible with dirty extracts of food, soil, or water. Pulsed splitless injection allows one to make injections of up to 5 µL using standard equipment[18]. Enormous gains in system sensitivity can be realized by using the PTV inlet in the "solvent vent" mode, which is compatible with injections of 5-1,000 µL. These large injections may be made manually or automatically using either a standard 6890 Series ALS in the multiple injection mode or by using a controlled speed injector available from Gerstel[19]. Because the injection process may take several minutes, manual injections are usually impractical and good precision may be hard to achieve.

The 6890 Series ALS is designed to make one or more injections of up to 25 µL into the PTV inlet. After the desired number of injections has been made, the inlet is heated and the chromatography begins. Though the system controls allow up to 99 injections, a reasonable upper limit is about 10, making 250 µL the typical injection volume limit for this system. For even larger injections, the controlled speed injector[19] should be used. For all of the analyses described below, 100 µL were injected by making 10 sequential injections of 10 µL each.

How the PTV Works in the Solvent Vent Mode

Figure 1 shows a diagram of the PTV inlet. For large-volume injections, three steps are required. These are: 1) injection and solvent elimination; 2) splitless sample transfer to the GC column; and 3) chromatographic separation and, if desired, a simultaneous inlet bake-out step. The steps are described more completely below.

Injection and Solvent Elimination (Step 1)

During injection, the column head pressure is set to 0 psi to eliminate or, in the case of GC/MS, reduce the flow through the column. When mass spectral detection is used, there is still

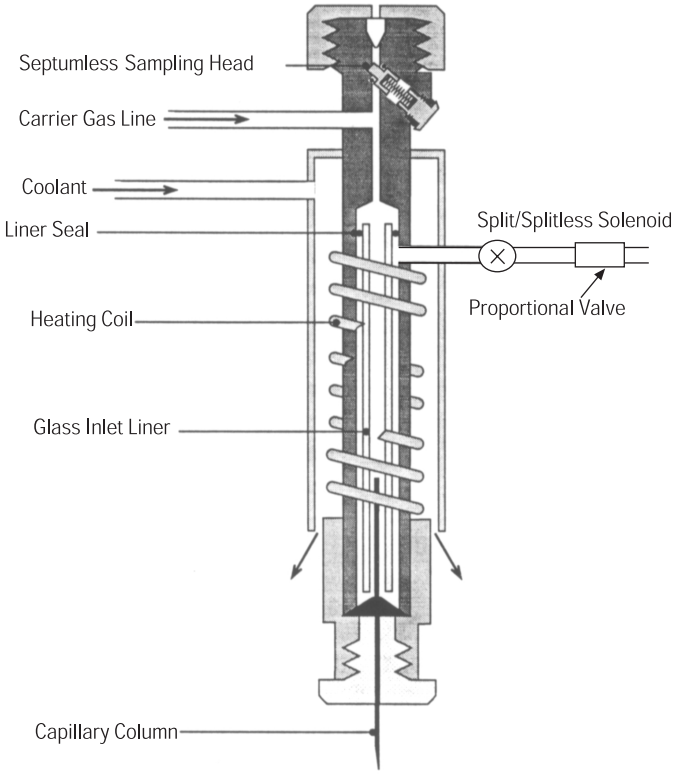
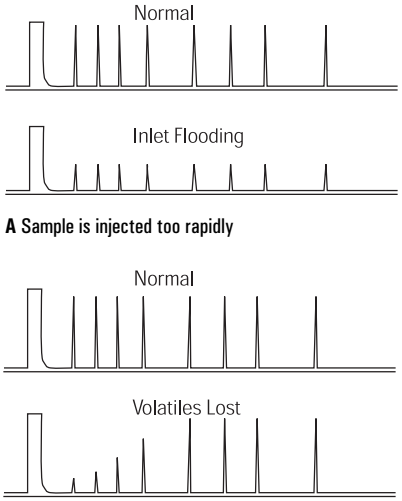


Figure 1. The PTV inlet shown with the septumless head. The inlet is also available with a septum head that may be equipped with a standard septum or a Merlin Microseal. (Figure reproduced with permission of Gerstel GMBH.)

some flow because the column outlet is under vacuum. At the same time, a steady stream of carrier gas passes through the inlet and out through the split vent. This flow is typically between 100 and 500 mL/min. The sample is injected into the cool liner where it remains as a liquid, dispersed over the liner walls or any packing material that may be in the liner. The steady flow of carrier gas through the liner causes the solvent (and any volatile fraction of the sample) to evaporate and be swept with the carrier gas out through the split vent. This is analogous to "blowing down" a sample with a stream of inert gas, except that this takes place inside the PTV inlet. When most of the solvent has evaporated, the next injection is made and the evaporation process repeats, accumulating more sample in the inlet. To recover an analyte completely, its boiling point should be at least 100 °C greater than that of the solvent; most pesticides fall into this category.



A Sample is injected too rapidly

B Solvent evaporates completely between injections

Figure 2. Chromatograms A and B illustrate the result of poor timing of multiple injections.

The timing of these multiple injections can be important. If the sample is introduced too rapidly, the liner may become flooded and liquid will be forced out through the split vent. Chromatographically, this shows up as reduced area counts for all analytes (see figure 2A). If there is too much time between injections, all of the solvent may evaporate and more of the volatile analyte fraction may be lost too. This results in poor recovery of volatiles but 100 percent recovery of the less volatile compounds (see figure 2B). Set-points such as inlet temperature, vent flow, and injection delay times can affect recovery of volatiles. Note that for 100 percent recovery, an analyte should have a boiling point at least 100 °C greater than the solvent. One can adjust the delay between injections by entering the desired value in the ChemStation software. Some experimentation is usually necessary when setting this delay for a new method. It will be dependent upon such factors as the solvent type, injection volume, vent flow, and inlet temperature.

Splitless Sample Transfer to the GC Column (Step 2)

Once the desired number of injections has been made, the column head pressure is restored and the vent flow is tur ned off. At this point, the inlet temperature is programmed up to a value that is sufficient to transfer all of the desired analytes to the GC column. This step is similar to a splitless injection, except that instead of flash vaporization, the sample is transferred as the inlet temperature is programmed up. For the most gentle treatment of labile analytes, slow ramp rates may be used. This allows analytes to be flushed into the column at the minimum temperature needed for volatilization. When sample decomposition is not a problem, the inlet may be heated as fast as 720 °C/min.

Chromatographic Separation (Step 3)

During sample transfer, the oven temperature is usually held between 30 °C below and 20 °C above the solvent's atmospheric boiling point, depending on whether the solvent effect is needed to focus the more volatile fraction of the analytes. Again, some experimentation is necessary to optimize peak shapes. After the sample has been transferred in step 2, the oven temperature is programmed up and chromatography begins.

After the inlet has reached its maximum temperature and sufficient time has elapsed to transfer the sample to the column, a purge flow of 30-50 mL/min is restored to the split vent. If desired, one can set a very large split flow for a few minutes and bake out the inlet at a higher temperature to remove nonvolatile impurities. To conserve carrier gas, gas saver should be turned on at the end of this bake-out step.

Entering PTV Inlet Parameters into the Agilent ChemStation

When preparing the PTV portion of a GC method, one should first decide on the sample size and how many injections are required. In this work, ten 10-µL injections were made for a total of 100 µL. When entering parameters into the ChemStation screen, the Injector icon is first selected (figure 3) under the "GC edit parameters" menu. Next, the Configure button is pressed to enter the syringe size and enable multiple injections. From the main injector screen, the injection volume (10 µL) and number of injections are entered[10] . For this work, a 12-second delay was chosen between injections to allow for solvent evaporation.

The estimated total injection time is listed on the Inlets screen (figure 4). This is helpful when setting the inlet and oven parameters. First, the vent flow rate (400 mL/min for these analyses) is chosen, which sets the vent pressure to 0 psi until the injection sequence is done and solvent from the last injection has largely evaporated (4.00 min in figure 4). This is done by entering these values in the following fields:

Vent Flow 400 mL/min
Vent pressure 0.0 psi until
4.00 min

Next, the purge flow and elapsed time are set by entering values in the following field:

Purge Flow to Split Vent
50.0 mL/min @ 6.50 min

Note that as an aid in setting up the method, the "estimated total injection time" is shown just above the previous data entry fields.

In this example, the normal column head pressure was restored and the vent flow was turned off at 4.00 min. This prepares the inlet for the splitless transfer of the sample to the column. The vent flow remained off until it was set to 50 mL/min at 6.5 min. Thus, there is a 2.5-min period for inlet temperature programming and splitless sample transfer to the column. In this example, the inlet was held at 40 ° C for 4.2 min, enough time to make 10 injections, turn off the purge flow, and restore the column head pressure; the PTV was then programmed to 320 ° C at 200 ° C/min (figure 4).

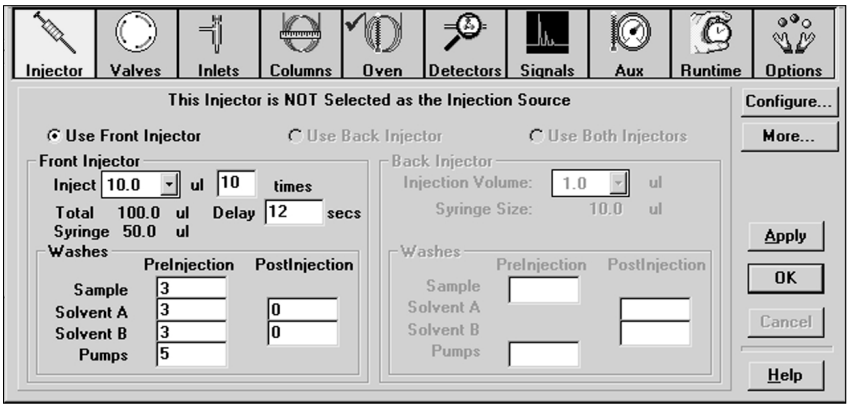


Figure 3. The injector screen from Agilent GC and GC/MS ChemStation software showing the setpoints available for multiple injections. To configure the sampler for multiple injections, set the syringe size, and choose slow injection, click on the Configure button.

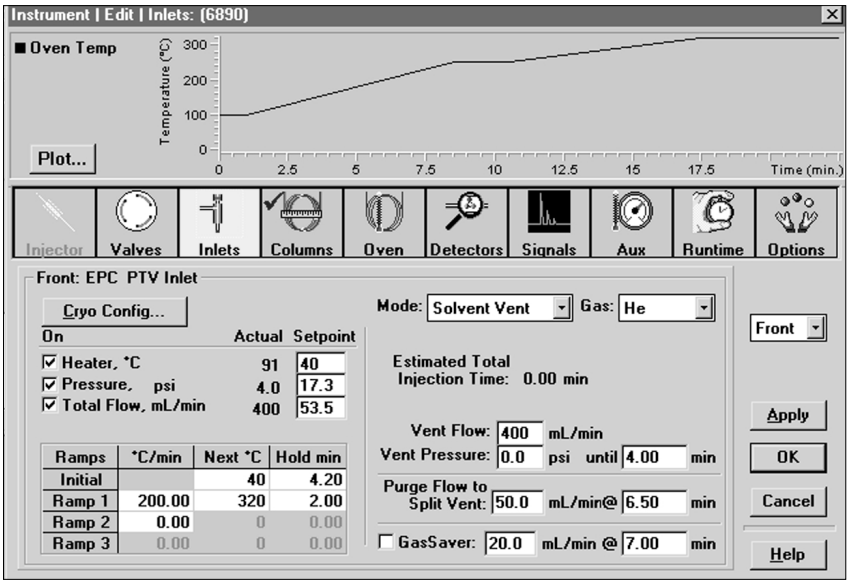


Figure 4. The inlets screen from Agilent GC and GC/MS ChemStation software showing the setpoints available for operation of the PTV inlet in the solvent vent mode.

Although not done for these analyses, the inlet could be baked out by setting the "purge flow to split vent" to a large value (perhaps 500 mL/min) at the end of the splitless time (6.50 min) and at the same time, program the inlet to a higher temperature. After the bake-out period, the inlet temperature is programmed downward and gas saver is turned on.

Normally, the GC oven is held at its starting temperature until the splitless injection is complete (6.50 min in this case) at which time oven temperature programming is begun. For this work, the oven temperature program was begun at 6.13 min so that the pesticide retention times would match a retention time data base that was in use. Figure 5 diagrams the PTV and GC oven setpoints used for this work.

PTV Inlet Liner Considerations

The correct liner choice is critical to the success of any pesticide analysis by PTV injection. The liner must be thoroughly deactivated or many labile pesticides may decompose or adsorb in the inlet. In general, any liner containing glass wool will be unsatisfactory for the analysis of labile pesticides, whether or not the glass wool is deactivated. At this time, two PTV liners are suggested for pesticide analysis:

- Part no. 5183-2037 is a deactivated, open multibaffled liner with no internal packing that may be used for single or multiple injections of 5 µL or less. This liner gives very good recovery for pesticides, even extremely difficult ones such as acephate and methamidophos.
- Part no. 5183-2041 is a deactivated liner with an internal coating of sintered glass to give it more surface area and is, therefore, suitable for single or multiple 25-µL injections. This liner gives better than 70 percent recovery for most pesticides, although tests have shown that acephate and methamidophos cannot be analyzed using this liner, and that recoveries of guthion are often less than 50 percent. A prototype version of this liner was used for all of the work described in this application note.

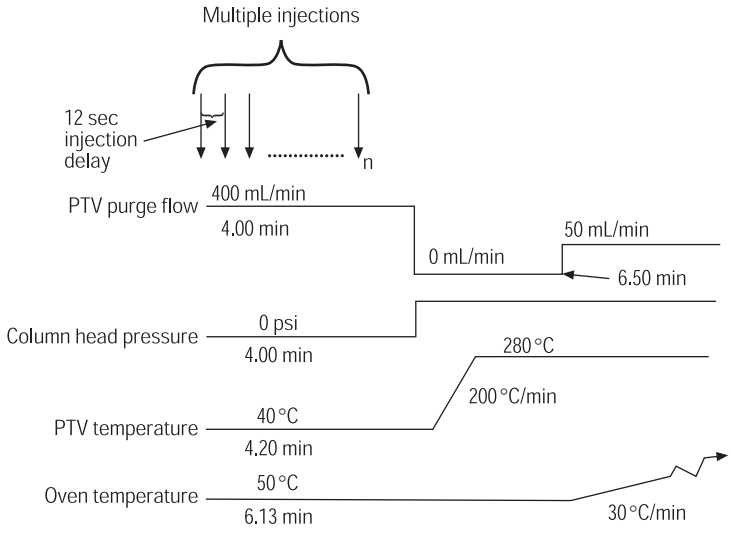
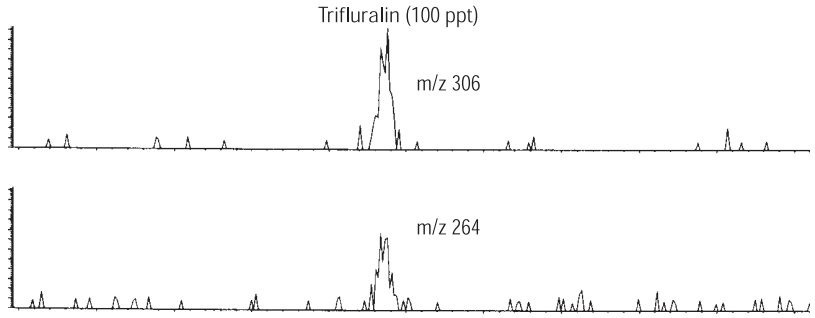


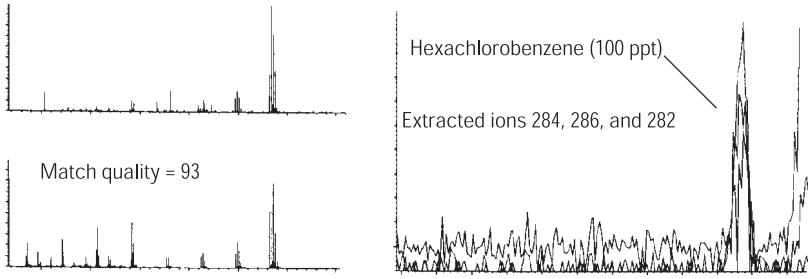
Figure 5. Illustration of the GC and sampler setpoints used for 100-µL injections of pesticide samples. Note that normally, the GC oven hold period would have been at least 6.5 min for this method. A value of 6.13 min pesticide retention times to a data base.

Results and Discussion

When compared to a typical 2-µL splitless injection, 100-µL PTV injections can often result in a 50-fold improvement in system detection limits. Selective detectors such as the MSD can help the analyst to realize the full measure of this sensitivity improvement by excluding background that may be introduced from solvent impurities, vial cap extract, and indigenous compounds coextracted with the analytes. In this application, it was possible to see most of the pesticides in the 14-component mixture at 100 ppt in the scan mode (400 ppt for the isomer mixes of permethrin and fenvalerate). Figure 6 shows extracted ion chromatograms for trifluralin and hexachlorobenzene (HCB) at 100 ppt. Library searching gave a match quality of 93 for the HCB peak. Fenvalerate isomers I and II were found in the solution in a ratio of about 78:22. Figure 7 shows extracted ion chromatograms for fenvalerate I at a concentration of 311 ppt.



A Extracted ion current chromatograms of trifluralin



B Extracted ion current chromatogram of HCB with its mass spectrum and library match

Figure 6. Scanning GC/MS results for a pesticide standard containing Trifluralin and Hexachlorobenzene at 100 ppt. (Ten 10-µL injections were made using the PTV inlet.)

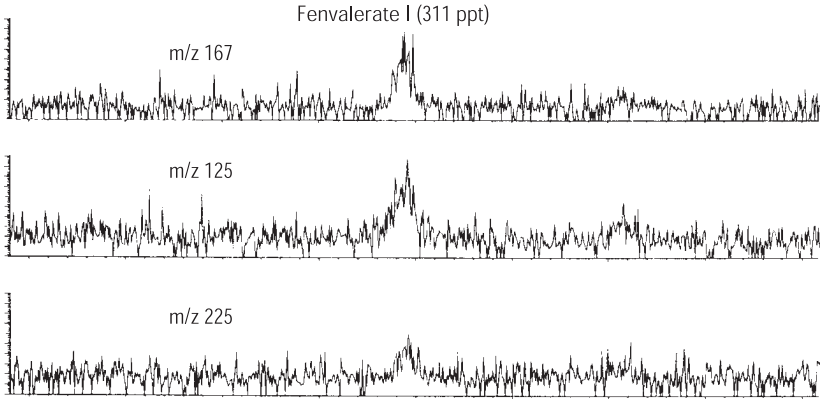


Figure 7. Extracted ion current chromatograms of Fenvalerate I at a concentration of 311 ppt in a pesticide standard. (Ten 10-µL injections were made using the PTV inlet.)

Analysis of a bell pepper extract revealed several pesticide residues. As seen in figure 8, chlorpyrifos and the endosulfans were easily detected. The Florida Department of Agriculture determined the concentration of chlorpyrifos, alpha-endosulfan, betaendosulfan, and endosulfansulfate to be 0.210, 0.011, 0.018, and 0.013 ppm, respectively. It is important to note that these compounds could be detected with very high selectivity by extracting high mass ions that are characteristic of these pesticides but not of the matrix. Using LVI, there is ample signal from these less abundant ions for good quantitation. With normal injection volumes, selectivity may have to be compromised and the most abundant ions extracted in a pesticide spectrum to gain sensitivity.

Phosmet, captan, and propoxur were all easily detected in a pear sample. The total ion current chromatogram (TIC) is shown in figure 9 along with spectrum obtained for captan juxtaposed with the library spectrum. Figure 10 shows the propoxur peak along with 2,4,6-tribromoanisole and 2,4,6-tribromophenol, two other compounds that were surprising to find in a pear sample. Though the origin of these brominated compounds is not known, a recent paper by Hoffmann and Sponholz 20 suggests that tribromophenol is used to treat storage palettes for the prevention of fire and mold growth, and that the anisole is formed from the phenol microbiologically. Perhaps these pears were shipped in containers that had been similarly treated.

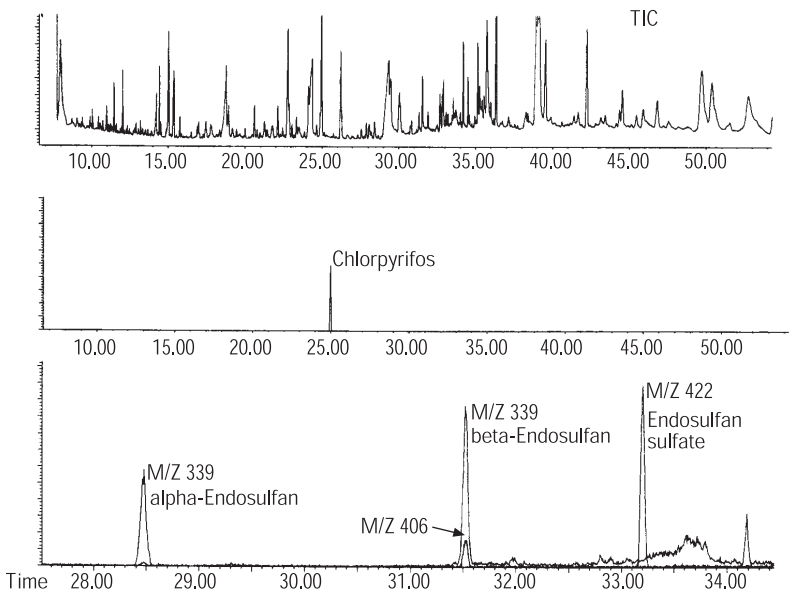


Figure 8. GC/MS Analysis of a bell pepper extract. (Ten 10-µL injections were made using the PTV inlet.) Using LVI, there was sufficient signal to use high mass ions with smaller abundances to achieve greater selectivity.

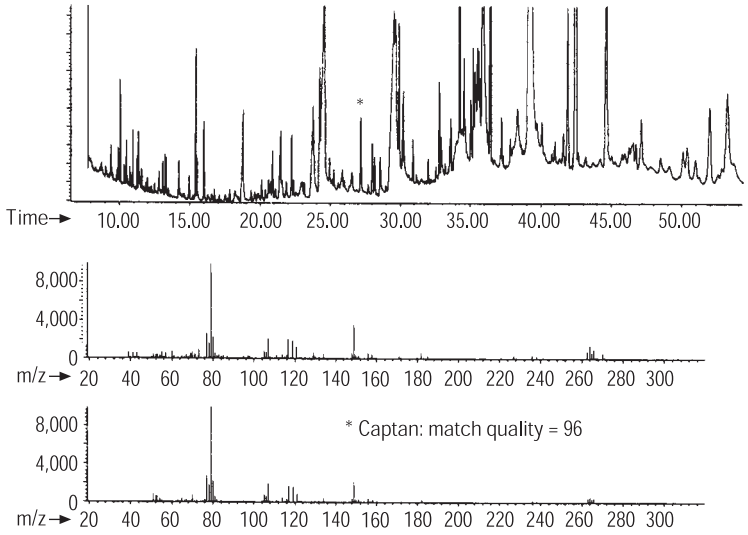


Figure 9. TIC of a pear extract resulting from a 100-µL Injection (10 x 10 µL). Captan was easily detected, and its spectrum gave a library match quality of 96.

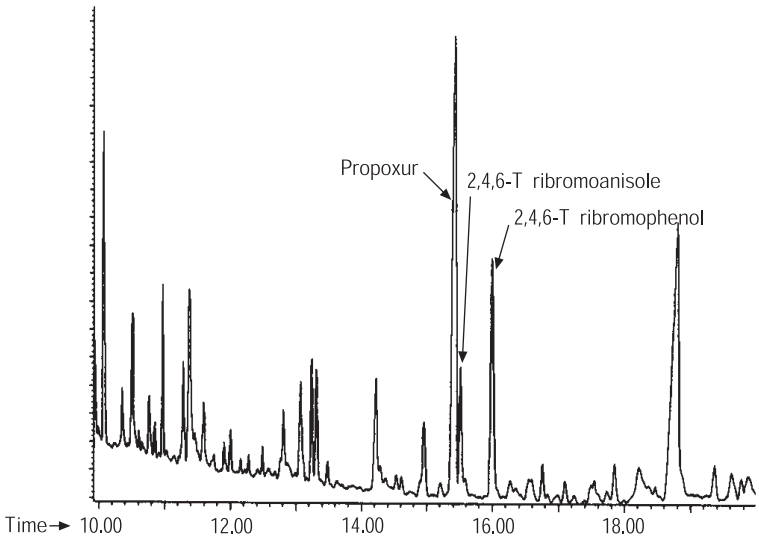


Figure 10. TIC of a pear extract resulting from a 100-µL Injection (10 x 10 µL). Propoxur and two brominated phenolics were easily identified.

A single sintered glass coated liner of the type described above (part no. 5183-2041) was used for about ten 50- and ten 100-µL injections (ca. 1,500 µL total) of vegetable extracts before it was replaced. All of the extracts were rather dirty, and an inlet bake-out step was not used. Although the liner looked somewhat discolored for about 2 cm where injections were made, it still performed well at the time it was replaced.

Conclusion

Using the PTV inlet in the solvent vent mode, it is relatively simple to increase system detection limits by one or two orders of magnitude. When combined with the Agilent 6890 Series automatic liquid sampler, multiple injections of up to 25 µL each into the inlet can be made, allowing the solvent to vent while pesticides and other less volatile analytes accumulate. After the desired sample volume has been introduced (typically 5-250 µL), the solvent vent is closed and the sample is transferred to the column in a temperature-programmed splitless injection. By making 100-µL injections into a PTV-equipped Agilent 6890 Series GC coupled to the Agilent 5973 MSD, it was possible to see several pesticides at the 100 ng/L level (100 ppt) in the scan mode. With such low detection limits, less abundant ions can be used to identify and quantitate pesticides at low ppb levels, thereby gaining in selectivity as well.

When performing LVI, there are several parameters to adjust and some method development time is usually required. However, the method described herein worked well and can be duplicated for the PTV/GC/MS analysis of pesticides in food.

Acknowledgment

The author wishes to thank Ms. Joanne Cook of the Florida Department of Agriculture and Consumer Services for supplying the food extracts used in these experiments and Dr. Bill Wilson (Agilent Technologies) for supplying liner deactivation test results.

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Analysis of Fumonisin Mycotoxins by LC/MS

Application Brief

Agilent 1100 Series LC/MSD
Foods, Environmental

Friedrich Mandel

Introduction

The fungus *Fusarium*, which is known to infest corn and corn products, produces a group of mycotoxins called fumonisins. The toxicities of the most abundant fumonisins, $B_{1\text{-}3}$, have been extensively studied, and a variety of species-specific toxicities have been published. These compounds may be carcinogenic to humans. Fumonisins are characterized by a 19-carbon aminopolyhydroxyalkyl chain that is diesterified with propane-1-2, 3- tricarboxylic acid. Analogues $B_{1\text{-}3}$ show a difference in the number and position of the hydroxyl groups (Figure 1). Fumonisins B_2 and B_3 have the same molecular weight.

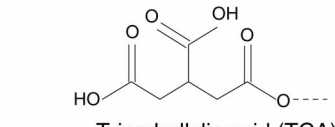
Most analytical methods exclude the detection of one or more of the known fumonisins. Traditional HPLC analysis requires the derivatization of the amino group. In this paper, we show that the Agilent 1100 Series LC/MSD can detect fumonisins without derivatization.

Experimental

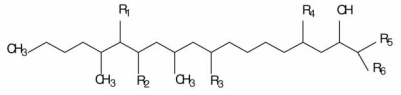
The system comprised of an Agilent 1100 Series binary pump, vacuum degasser, autosampler, thermostated column compartment, diode-array detector (DAD), and LC/MSD. The LC/MSD used electrospray ionization (ESI). Complete system control and data evaluation were done on the Agilent ChemStation for the LC/MSD.

Results and Discussion

The fumonisin analogues were analyzed in scan mode at a high concentration (25 ng) to determine the molecular ion and confirming fragments. The initial conditions showed the molecular ion $[M+H]^+$, but no significant fragment ions. Collision induced dissociation (CID) was used to generate more fragments for structural confirmation. Fumonisin B_2 and B_3, indistinguishable by their spectra, were easily separated chromatographically (Figure 2).



Tricarballylic acid (TCA)



	R1	R2	R3	R4	R5	R6	MW
B1	TCA	TCA	OH	OH	NH2	CH3	721.4
B2	TCA	TCA	H	OH	NH2	CH3	705.4
B3	TCA	TCA	OH	H	NH2	CH3	705.4

Figure 1. Structure of fumonisins.

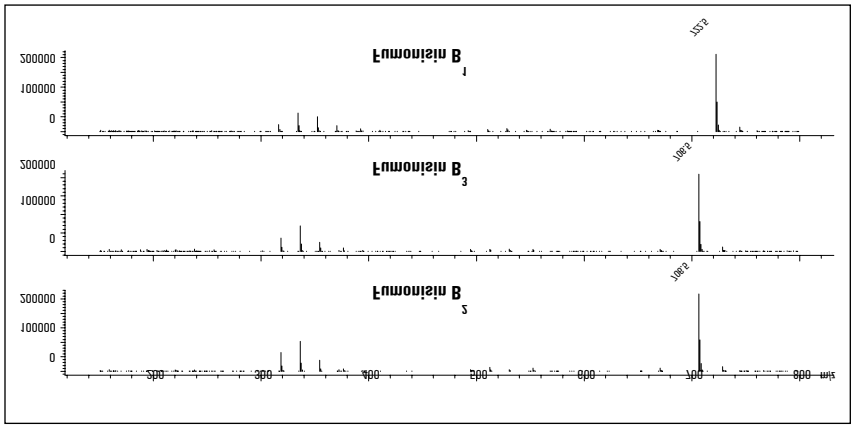


Figure 2. Mass spectra for fumonisin analogues.

The total ion chromatogram (TIC) shows very good sensitivity at 25 ng (Figure 3). To further improve sensitivity, the standards were run in the selected ion monitoring (SIM) mode.

Chromatographic Conditions

Column:	150 x 2.1 mm Zorbax Eclipse XDB, C18, 5 μm
Mobile phase:	A = 5 mM ammonium acetate in water, pH 3 B = acetonitrile
Gradient:	Start with 33% B at 8 min 60% B at 9 min 33% B
Flow rate:	250 μl/min
Injection vol:	5 μl
Column temp:	40°C
Diode-array detector:	Signal 220, 4 nm; reference 550,100 nm

MS Conditions

Source:	ESI
Ion mode:	Positive
Vcap:	4000 V
Nebulizer:	30 psig
Drying gas flow:	10 l/min
Drying gas temp:	350°C
Scan range:	120-820 amu
Step size:	0.1
Peak width:	0.15 min
Time filter:	On
Fragmentor:	Variable 230 V (100-680) 100 V (680-800)

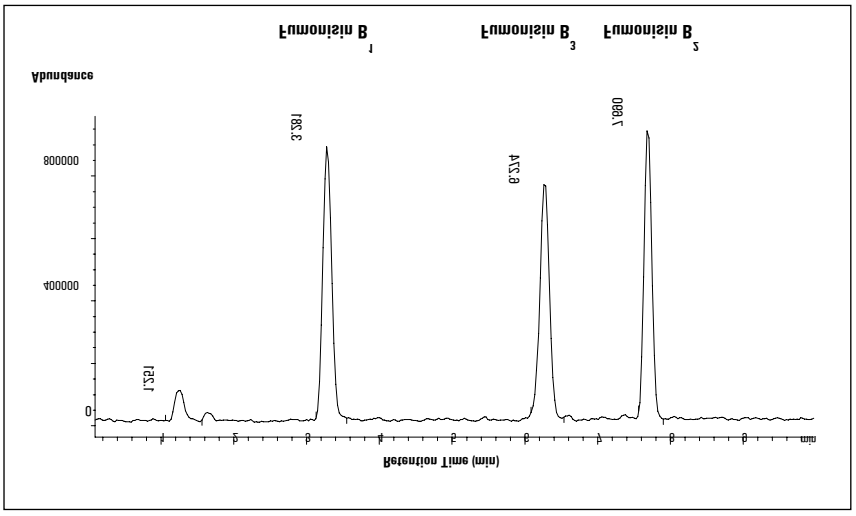


Figure 3. Chromatographic separation of fumonisin analogues at 25 ng.

Figure 4 shows the extracted ion chromatograms for 250 pg of fumonisins in a corn extract. The mass spectra showing the molecular and fragment ions provide high-confidence identification and quantification.

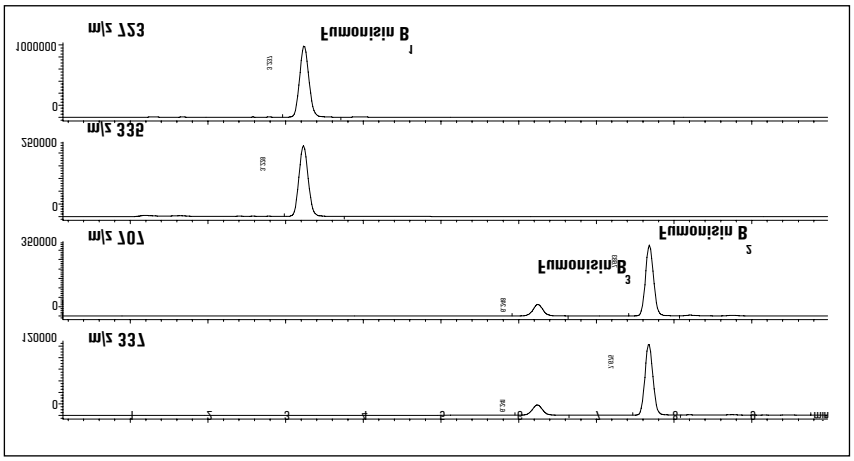


Figure 4. SIM of molecular and fragment ions for fumonisins in spiked corn extract.

Chromatographic Conditions

Column:	150 x 2.1 mm Zorbax Eclipse XDB, C18, 5 μm
Mobile phase:	A = 5 mM ammonium acetate in water, pH 3 B = acetonitrile
Gradient:	Start with 33% B at 8 min 60% B at 9 min 33% B
Flow rate:	250 μl/min
Injection vol:	5 μl
Column temp:	40°C
Diode-array detector:	Signal 220, 4 nm; Reference 550,100 nm

MS Conditions

Source:	ESI
Ion mode:	Positive
Vcap:	4000 V
Nebulizer:	30 psig
Drying gas flow:	10 l/min
Drying gas temp:	350°C
SIM ions:	at 0 min, 334.4, 352.4, 370.4, 722.5 at 5 min, 336.4, 354.4, 706.5
Step size:	0.1
Peak width:	0.15 min
Time filter:	On
Fragmentor:	Variable 230 V (334.5, 352.4, 370.4) 100 V (706.5, 722.5)



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Conclusion

The Agilent 1100 Series LC/MSD is capable of detecting fumonisins at low levels without derivatization. Mass spectrometry allows specific and sensitive detection in complex matrices such as corn extract.

Friedrich Mandel *is an application chemist at Agilent Technologies, Inc.*

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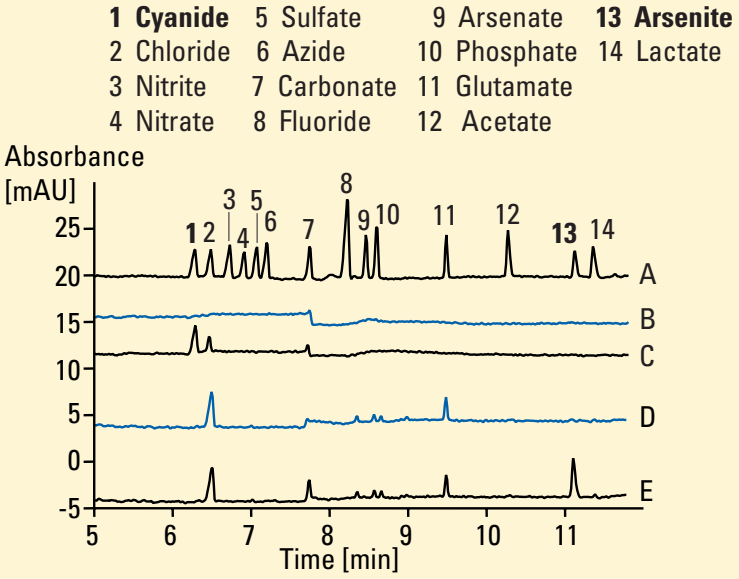
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Analysis of Poisoned Food by Capillary Electrophoresis

Tomoyoshi Soga
and Maria Serwe

Food

Abstract
In cases of poisoning, analytical tools are needed to determine the identity of the toxins quickly and accurately. This enables healthcare professionals to administer appropriate treatment as quickly as possible and helps police to find those responsible. A rapid determination of anionic toxins in adulterated foods and beverages is possible using capillary electrophoresis (CE) with indirect UV detection. Cyanide, arsenite, arsenate, selenate, azide and other anions can be detected within 15 minutes, requiring only minimal sample preparation.



Conditions

Injection
6 s @ 50 mbar
Capillary
fused silica capillary
total length 112.5 cm
effective length 104 cm
internal diameter 50 µm
Buffer
Agilent Basic Anion Buffer
Voltage
-30 kV
Temperature
30 °C
Detection
signal 350/20 nm
reference 275/10 nm

Figure 1
Analysis of cyanide and arsenite in food.
A = anion standard (50 ppm each), B= Oolong tea (1:100 diluted with H_2O), C= Oolong tea as in B, spiked with 100 ppm NaCN, D=curry (1:100 diluted with H_2O, filtered through 0.22 µm filter), E=curry as in D, spiked with 100 ppm $NaAsO_2$

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Experimental

Anion analysis was performed using the Agilent Capillary Electrophoresis system equipped with diode-array detection and computer control via Agilent ChemStation. The analysis is based on the Agilent Forensic Anion Analysis Kit (part number 5064-8208).

Prior to first use, a new capillary was flushed with run buffer for 15 minutes (at 1 bar). Between the analyses the capillary was flushed 2 minutes from the OutHome vial into waste, then 2 minutes from the InHome vial into waste. This procedure avoids baseline fluctuations as a result of buffer depletion. Buffer vials were replaced after 10 runs when using 2 ml vials, after 5 runs, when using 1 ml vials.
Sample preparation consisted simply of dilution with water, or dilution and additional filtration through a 0.22 µm filter, as indicated in figure 1.

Results

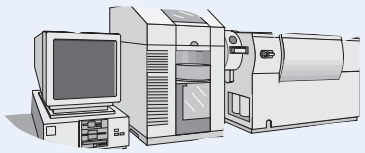
Figure 1 shows the analysis of food spiked with cyanide and arsenite. Depending on the results of this quick analysis, the sample can then undergo a more detailed analysis.

The assay was linear over the range 10–100 ppm with $r^2 > 0.999$. The method detection limit was 5–10 ppm. For the analysis of curry, the repeatability for arsenite (n = 6) was 0.06 % RSD for migration time and 2.7 % RSD for peak area. For cyanide in Oolong tea the respective values were 0.13 % RSD for migration time (n = 10) and 4 % for peak area (sample diluted in 0.01 N NaOH).

Other toxic anions that can be determined are arsenate, azide and selenate (which migrates between azide and carbonate). Compared to ion chromatography (IC), the advantages of CE for this type of analysis are the shorter analysis time and the minimal sample preparation needed for samples with a complex matrix (e.g. curry). Additionally, the analysis of azide and arsenate together with cyanide and arsenite is not possible in one run with IC.

Equipment

- Agilent Capillary Electrophoresis system
- Agilent ChemStation
- Agilent Forensic Anion Analysis Kit



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Fast Screening of Pesticide and Endocrine Disrupters Using the Agilent 6890/5973N GC/MSD System, Part I

Application

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January 2000

Abstract

Agilent Technologies' new, fast GC/MSD method can significantly speed up the screening of pesticides. Agilent's GC method translation software (available free from the Agilent Technologies Web site, http://www. chem. agilent.com/cag/servsup/usersoft/main.html#mxlator) was used in developing the new method based on the standard 42-min method. A 10 m x 0.1 mm x 0.1 µm HP-5 column was used to increase analysis speed up to fourfold. The time savings were implemented in increments (down to 10.5 minutes) to verify the predictability of scaling and the effect of scaling on the signal-to-noise ratio.

Key Words

RTL, pesticide, environmental, screening, fast GC, method translation, 5973, 6890, MTL

Introduction

Analysts want faster analyses to improve laboratory productivity. Often, when speeding up GC methods, an analyst will trade resolution for increased analysis speed. This loss of resolution can complicate peak identification, even with a mass selective detector (MSD).

Agilent Technologies has developed new techniques to solve the peak identification problem based on Agilent's retention time locking (RTL) software and a new mass spectral library that contains the locked retention times and characteristic ions for 567 of the most common pesticides and endocrine disrupters of concern worldwide. A GC/MSD method was developed based on the standard 42-min method[1] to screen for all 567 of the most common analytes. A specific combination of column stationary phase, carrier gas flow rate, and oven temperature programming is required to lock all the compounds to an expected retention timetable[2]. Compound identification based only on spectral searching alone is difficult when analyzing extracts containing significant sample matrix content because of overlapping peaks and noisy baselines.

The new screening tool, integrated within Agilent's ChemStation for MSD, searches for all 567 compounds by first checking and integrating four characteristic ions within the expected time window, and second by printing out a report showing "hits" and "possible hits" (ratios of characteristic ions that do not match the expected values in the library within specified limits).

In one application, the analysis time of the standard pesticide method was reduced by one half, two-thirds, and three-fourths. The faster methods were scaled exactly as predicted by using a combination of Agilent's method translation (MTL) and RTL software. Because scaling was exact, these faster methods can be used with precisely-scaled pesticide libraries, making the screening process even more powerful and adaptable to individual needs.

Experimental

The GC method translation software tool was used to find operating conditions for the faster methods. Figure 1 is a screen capture of MTL software data entry showing the original conditions and the new chromatographic conditions for a twofold speed gain. The column flow rate, which is helpful to avoid exceeding MSD pumping capacity[3], is also found in the table. A 16:1 split ratio was suggested in the table as a proportional scaling from the original column to the smaller i.d. column with corresponding lower capacity. The program also determined the required column head pressure and corresponding oven ramp. The Agilent 6890 GC fast oven option (220/240V in the U.S.) was required for the faster oven ramp used in this study.

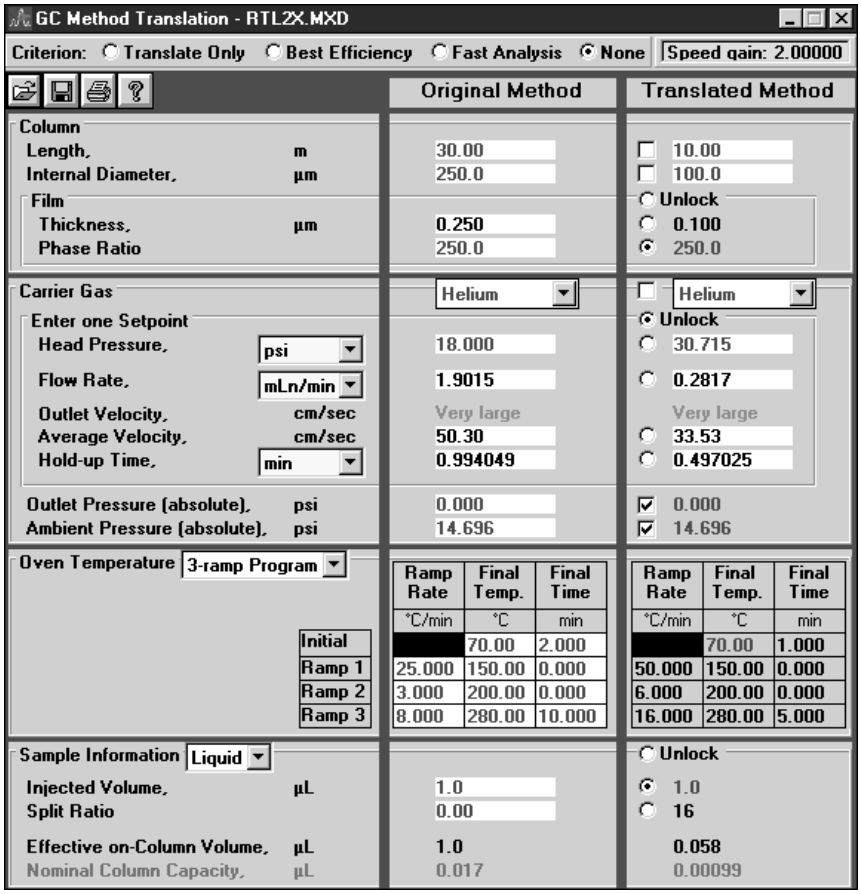


Figure 1. Screen capture showing the method translation (MTL) software data entry used in a twofold speed gain translation.

General chromatographic conditions are listed in table 1. The standard used was a mixture of 26 pesticides at 10 ppm. A 10 m x 0.1 mm x 0.1 µm HP-5 column (part number 19091J-141) was used. The head pressure determined by the method translation software (30.72 psi) was used as the starting point for retention time locking. The column head pressure required to lock retention times of the compounds to the library (the original retention time divided by 2) was determined using the automated RTL process integrated within the Agilent ChemStation for MSD. This process (first translate the method then lock the retention times) was repeated for the threefold and fourfold time reductions.

Table 1. Chromatographic Conditions

Speed	Onefold (1X)	Twofold (2X)	Threefold (3X)	Fourfold (4X)
GC	110 V	220/240 V		
Column	30 m x 0.25 mm x 0.25 µm HP5-MS (P/N 19091S-433)	10 m x 0.1 mm x 0.1 µm HP-5 (P/N 19091J-141)		
Injection mode	Splitless	16:1 split		
Column head pressure	18.0 psi	36.55 psi	63.17 psi	90.0 psi
Column flow (mL/min)	1.5	0.4	0.8	1.5
Inlet control mode	Constant pressure	Constant pressure		
Carrier gas	Helium	Helium		
Injector temperature	250 °C	250 °C		
Oven temperature	70 (2 min)	70 (1 min)	70 (0.67 min)	70 (0.5 min)
Ramp 1	25 °C/min	50	75	100
	150 (0 min)	150 (0 min)	150 (0 min)	150 (0 min)
Ramp 2	3 °C/min	6	9	12
	200 (0 min)	200 (0 min)	200 (0 min)	200 (0 min)
Ramp 3	8 °C/min	16	24	32
	280 (10 min)	280 (5 min)	280 (3.33 min)	280 (2.5 min)
Oven equilibration	2 min	2 min		
Injection volume	1 µL	1 µL		
Liner	5183-4647	5183-4647		
MS Conditions				
Solvent delay	3 min	1.8 min	1.2 min	0.9 min
Tune file	Atune.u	Atune.u		
Low mass	35 amu	35 amu		
High mass	500 amu	450 amu		
Threshold	150	250		
Sampling	2	2	1	1
Scans/sec	3.15	3.50	6.54	6.54
Quad temperature	150 °C	150 °C		
Source temperature	230 °C	230 °C		
Transfer line temperature	280 °C	280 °C		
Acquisition mode	Scan (EI)	Scan (EI)		

Figure 2 shows the results of the shortened analysis times. The three chromatograms look extremely similar, except that the time axis is scaled proportionally. Because MTL followed by RTL scales methods very precisely, scaled screening libraries for corresponding time reductions can be obtained by dividing the retention times in the library by the speed gain (which does not have to be an integer). The peak heights from all the methods are very similar. Although the sample was split 16:1 for the smaller column, the small column i.d. and faster oven ramp combination made the peaks narrower and higher, so there was minimal loss in the signal to noise ratio.

Conclusion

The highly accurate and reproducible pressure and temperature control of the Agilent 6890 GC allows precise scaling of the standard 42-min GC/MSD pesticide method. Run time was shortened to 10.5 minutes using a fast oven ramp rate and a 10-meter 100-micron column. The combination of MTL and RTL facilitated scaling and yielded exact scaling. RTL libraries can accurately be scaled to correspond to the faster analyses.

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2. H. Prest, P. L. Wylie, K. Weiner, and D. Agnew, "Efficient Screening for Pesticides and Endocrine Disrupters Using the HP 6890/ 5973 GC/MSD System," Agilent publication number 5968-4884E, April 1999.
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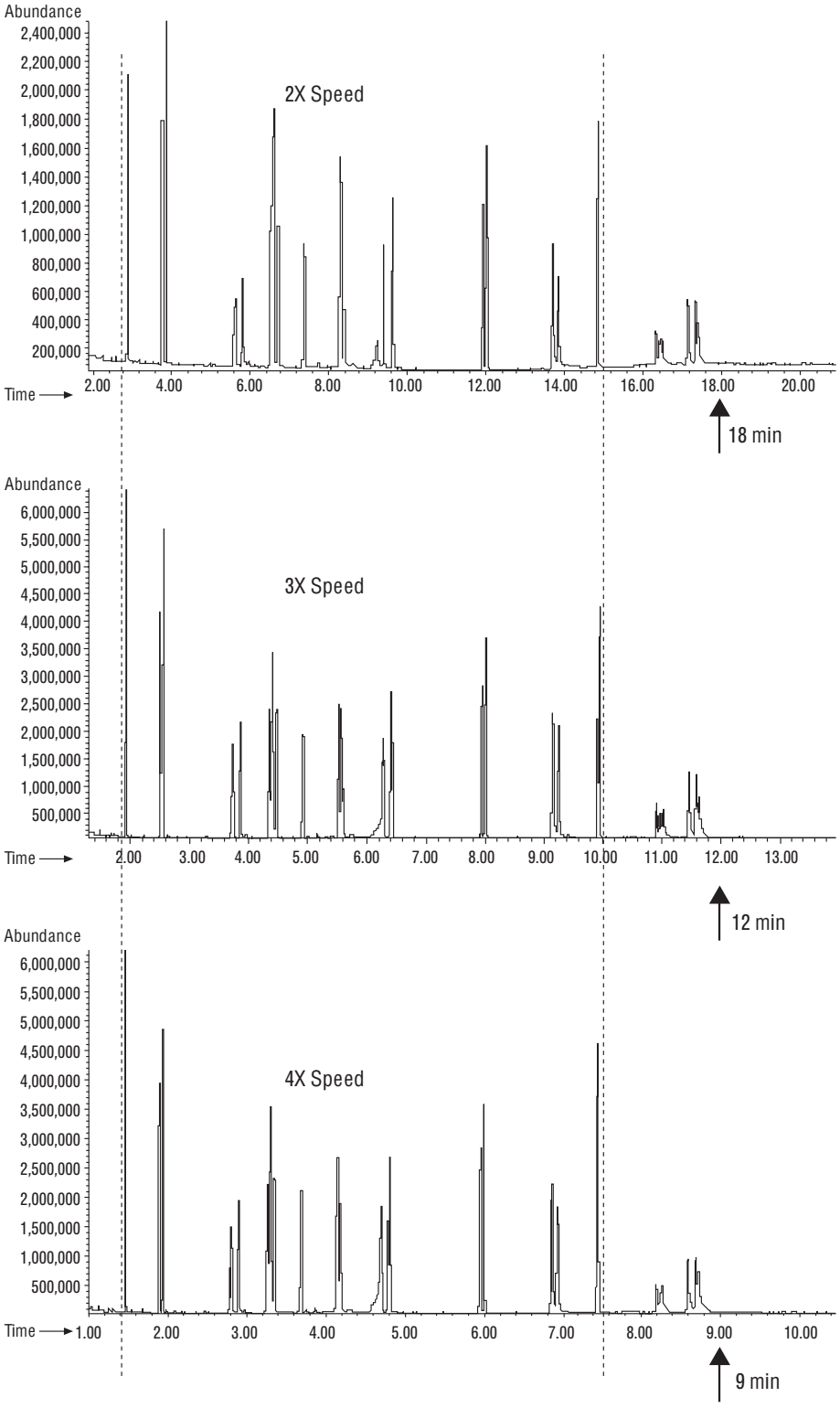


Figure 2. Three TICs of the 2X, 3X, and 4X speedups. The standard analysis (1X) was 42 minutes long. The two vertical lines on the figure are used as references to show the similarity of the TICs.

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Agilent Technologies
Innovating the HP Way

Gas Chromatography/Mass Spectrometry Approaches to the Analysis of Acrylamide in Foods

Application

Food Safety

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Abstract

Discovery of acrylamide in cooked foods has required an examination of foods for potential exposure. A classic approach employs extracting acrylamide from the food with water and converting the acrylamide to brominated derivatives. These derivatives are described here in terms of their spectra and response in electron impact and positive chemical ionization. Additionally, a more direct and simple approach involving extraction and direct injection and analysis of acrylamide by positive chemical ionization is described. This screening approach is rapid, robust, and provides low detection limits.

Introduction

The discovery announced in April 2002 by scientists at Sweden's National Food Administration of acrylamide (2-propenamide) in fried and baked foods at levels many times that allowed in water suggested a much higher exposure than previously estimated [1-3]. Acrylamide (Figure 1), a known neurotoxin, is considered a probable human carcinogen. The World Health Organization considers 0.5 µg/L the maximum level for acrylamide in water. However, foods such as french fries, baked potato chips, crisp breads, and other common cooked foods, were found to contain acrylamide between 100 and 1000 µg/kg. Acrylamide was not found in the raw foodstuffs and cooking by boiling produced no detectable levels. Recent work has suggested that acrylamide forms via the Maillard reaction, which occurs when amino acids and sugars (for example, asparagine and sucrose) are heated together [4]. The concern over these relatively high concentrations has led to studies of the occurrence of acrylamide in a wide variety of foods.

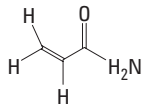


Figure 1 Acrylamide (2-propenamide), CH_2=CHCONH$_2$, 71.08 g/mole, CAS number 79-06-1.

Acrylamide Analytical Methodologies

A wide variety of instrumental approaches have been applied to acrylamide. Recent methods using

liquid chromatography with tandem mass spectrometry (MS-MS) detection have proved useful to approximately 50 µg/kg (ppb) or better using the 72 to 55 *m/z* transition (for example, [5]). This approach has appeared attractive in providing a simple sample preparation strategy. Gas chromatographic methods using MS detection with electron impact (EI) ionization typically suffer from the relatively small size of the molecule and therefore use derivatization. This application note presents alternative gas chromatography/ mass spectrometry (GC/MS) approaches aimed at more rapid screening, as well as the conventional, definitive quantitation via derivatization. These methods are rapid and relatively simple approaches to acrylamide analysis.

Rapid Screening via GC/MS-SIM with Positive Chemical Ionization

EI ionization mass spectrum for acrylamide (Figure 2) reveals very low mass ions; 71, 55, 44 *m/z*. Although there is good intensity at sub-ng levels, the ions are subject to interferences in food samples. The positive chemical ionization (PCI) spectrum achieved with ammonia provides more selective ionization and is of greater utility than EI in food matrices, Figure 3. Ammonia PCI results in two ions; 72 *m/z*, the protonated molecule, $[M+H]^+$, and 89 *m/z* due to the adduct, $[M+NH_4]^+$. PCI provides good selectivity and sensitivity for acrylamide–picogram amounts can be detected.

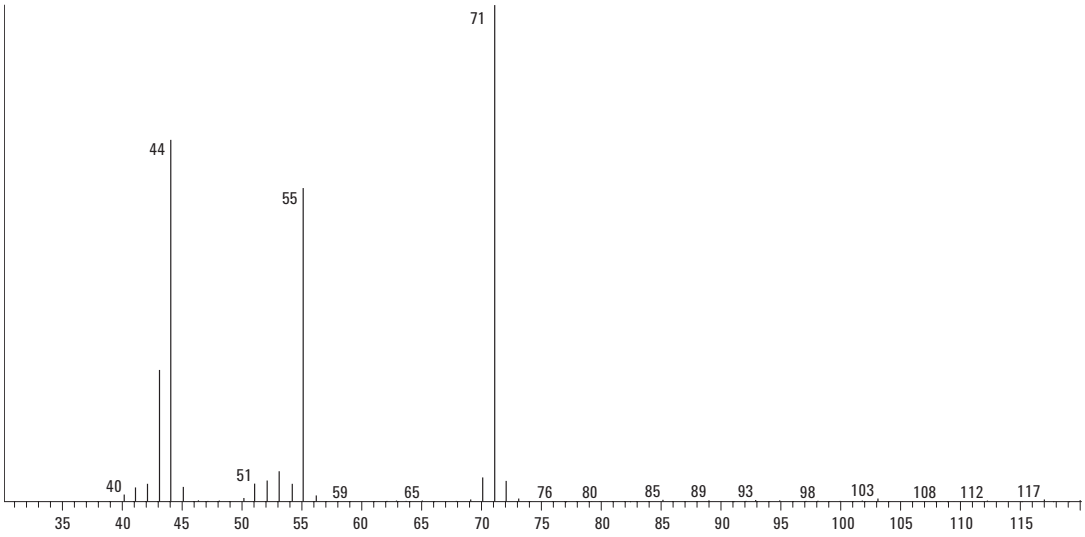


Figure 2 The EI ionization spectrum of acrylamide (40–120 amu).

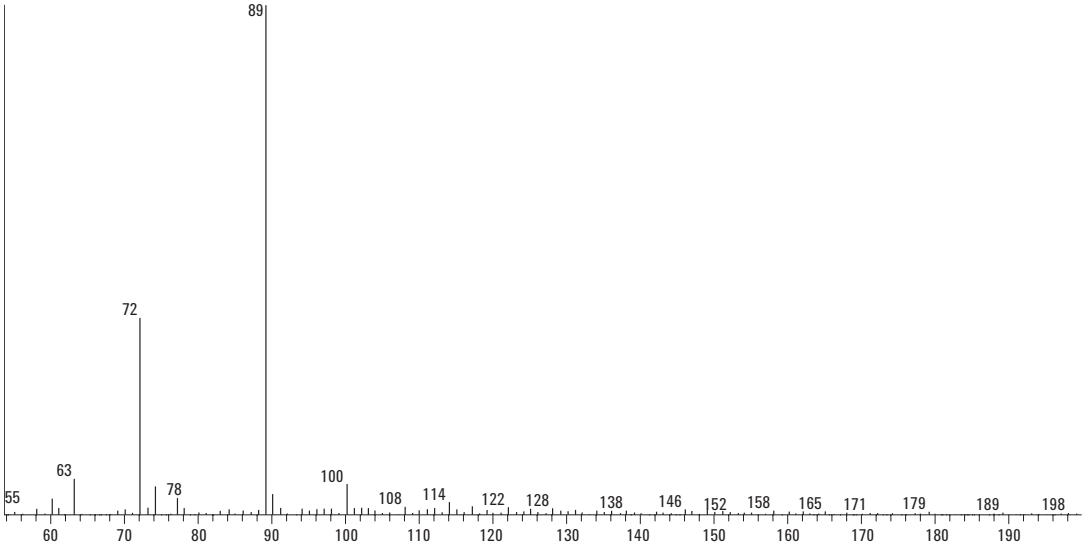


Figure 3 The PCI spectrum of acrylamide with ammonia reagent gas (60–200 amu).

Figure 4 shows a calibration curve from 100 pg to 10 ng collected under the method cited below in the section on Instrumental Parameters.

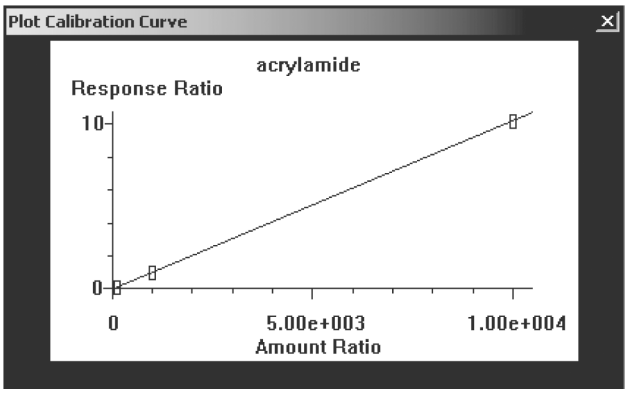


Figure 4 PCI-ammonia SIM calibration curve from 100-picograms to 10 ng ($R^2 = 1.00$).

Screening Sample Preparation

The enhanced specificity obtained through PCI can be used for rapid screening using a very simple and rapid sample cleanup. A food sample is homogenized and pulverized, and 0.4-g subsample is transferred to a centrifuge tube. The sample is extracted with 1 mL of methanol:water (9:1 v/v) solution for 10 minutes using an ultrasonic cleaner. Prior to sonication, 1 µg of labeled $^{13}C_3$-acrylamide is added to the 1-mL solution. After sonication, the sample is centrifuged for about 5 minutes at 8000 rpm. The upper layer is decanted and transferred to a vial for injection and analysis by GC/MS-PCI conditions with selected ion monitoring (SIM). See Table 1 for method parameters for PCI screening of native acrylamide.

Table 1. GC/MS Instrumental Method Parameters for PCI Screening of Native Acrylamide

Inlet parameters		
Liner:		Agilent p/n 5062-3587 Single-taper with glass wool
Temperature:		220 °C
Mode:		Pulsed splitless
Pulse pressure:		30.0 psi
Pulse time:		1.20 min
Purge flow:		50.0 mL/min
Purge time:		1.20 min
Total flow:		54.7 mL/min
Gas saver:		Off
Oven parameters		
Oven maximum:		260 °C
Oven equilibrium time:		0.20 min
Initial temperature:		60 °C
Initial time:		1.00 min
Ramp	Temperature	Time
12 °C/min	230 °C	10.00 min
Run time:		25.17 min
Column parameters		
Capillary column		Agilent 19091X-136 HP-INNOWax
Maximum temperature:		260 °C
Nominal length:		60.0 m
Nominal diameter:		250.00 µm
Nominal film thickness:		0.25 µm
Carrier:		Helium
Mode:		Constant flow 2.0 mL/min
Outlet and pressure:		MSD Vacuum
MSD Parameters		
Solvent delay		7.00 min
Tuning:		PCI Ammonia at 24% (1.2 mL/min)
EM Setting:		PCI Autotune + 400 V
Source temperature:		250 °C
Quad temperature:		150 °C
SIM Parameters		
Resolution:		High
Group ions		Dwell (ms)
72.0		60
75.0		60
89.1		60
92.1		60

Screening Method Results and Discussion

Figure 5 shows the extracted ion chromatograms for a sample of white bread. The baseline shows very little disturbance near the acrylamide analyte due to the selective nature of the PCI with ammonia. The extracted concentration is approximately 34 ng/mL or 85 ng acrylamide per gram white bread. Since acrylamide is formed when amino acids and sugars are heated together, it is logical to suspect the possibility of acrylamide formation in the inlet during injection. To test this possibility, the white bread extract was spiked with 100 ng of acrylamide and reanalyzed. The results calculated 135 ng/mL and suggest that either the relatively low temperature and short duration in the liner due to pressure pulsing mitigate acrylamide formation for this sample or acrylamide formed in the inlet is highly reproducible. This may not be the case in all extracts or under all similar conditions.

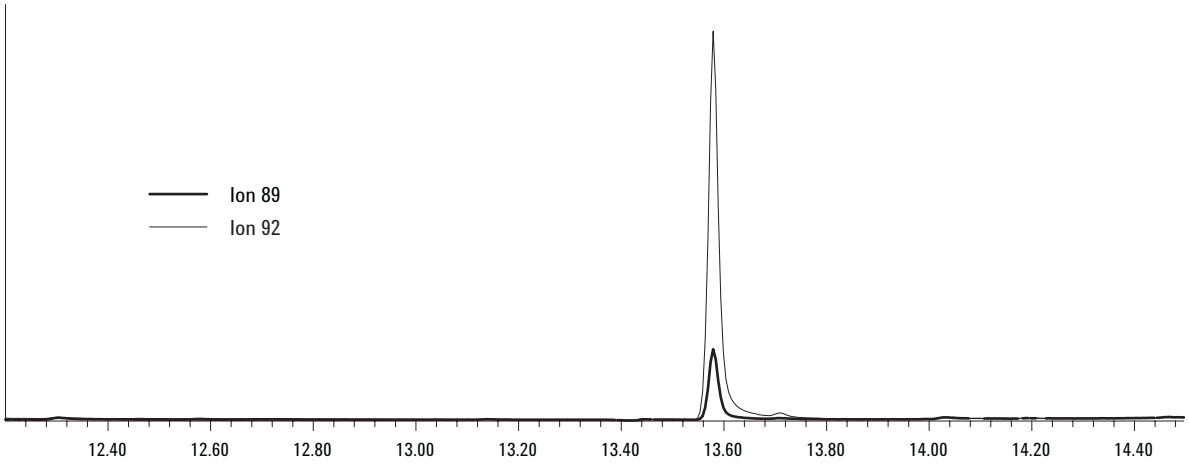
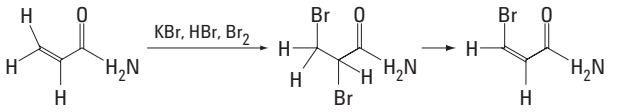


Figure 5. Extracted ion chromatograms for acrylamide (84 ng/g) in sample of white bread.

GC/MS Approaches to Acrylamide Involving Derivatives

Another approach to extraction from foods uses water, in situ derivatization, and liquid-liquid extraction [6, 7]. In this approach acrylamide in a homogenized sample is extracted with (hot) water, 1 g : 10 mL. A strong brominated agent is added and allowed to react. This reaction converts acrylamide to the 2,3-dibromopropionamide. Excess brominating reagent is removed by addition of sodium thiosulfate and the solution centrifuged and/or filtered. The 2,3-dibromopropionamide is extracted by partitioning into ethyl acetate. An option is to further treat this derivative to form a more stable analyte, the 2-bromopropenamide. The overall chemistry is given in Equation 1. Methacrylamide, CH_2=CH(CH_3)$CONH_2$, is frequently used as a recovery surrogate so its behavior is also reported here.



2,3-dibromopropionamide
$C_3H_5Br_2NO$
mol. wt. 230.9 g/mole

2-bromopropenamide
C_3H_4BrNO
mol. wt. 150.0 g/mole

Equation 1

Experimental

Acrylamide and methacrylamide were obtained as neat standards (Sigma-Aldrich Corp) and dissolved in HPLC grade methanol. Labeled acrylamide, 1,2,3-$^{13}C_3$-acrylamide, was obtained at 1 mg/mL methanol (Cambridge Isotope Laboratories, Andover, MA). The brominating reagent solution was made according to the literature [6] with reagent grade KBr, HBr, and bromine water (VWR, San Francisco,CA). Sodium thiosulfate was obtained as a 1-Normal solution (VWR, San Francisco,CA).

Derivatization also followed the literature [6] with addition of 1 mL of brominating reagent to solutions containing acrylamide; over-night derivatization, neutralization by 1-drop 1N sodium thiosulfate and extraction by 1-mL ethyl acetate (pesticide grade, VWR). The dibromo-derivatives were directly injected. The mono-bromo-derivatives were generated by addition of triethylamine.

Instrumental conditions for the dibromopropionamide and bromopropenamide are cited in Tables 2 and 3. All data was collected using 2-µL injections.

Table 2. GC/MS Instrumental Method Parameters for Dibromopropionamide (Dibromo-Derivative of Acrylamide) in EI and PCI with Methane and Ammonia

Inlet parameters		
Liner:		Agilent p/n5181-3315 double-taper
Temperature:		250 °C
Mode:		Pulsed splitless
Pulse pressure:		30.0 psi
Pulse time:		1.20 min
Purge flow:		50.0 mL/min
Purge time:		1.20 min
Total flow:		54.7 mL/min
Gas saver:		On
Oven parameters		
Oven maximum:		325 °C
Oven equilibrium time:		0.50 min
Initial temperature:		50 °C
Initial time:		1.00 min
Ramp	Temperature	Time
45 °C/min	300 °C	2.00 min
Run time:		8.56 min
Column parameters		
Capillary column		Agilent 122-3832 DB-35 ms
Maximum temperature:		340 °C
Nominal length:		30 m
Nominal diameter:		250 µm
Nominal film thickness:		0.25 µm
Carrier:		Helium
Mode:		Constant flow 1.2 mL/min
Outlet and pressure:		MSD Vacuum
MSD Parameters for EI and PCI		
Solvent delay		5.00 min
EI Parameters		
EI Tuning:		Autotune
EM Setting:		Autotune + 400 V
Source temperature:		230 °C
Quad temperature:		150 °C
EI SIM parameters		
Resolution:		Low

(Continued)

Table 2. GC/MS Instrumental Method Parameters for Dibromopropionamide (Dibromo-Derivative of Acrylamide) in EI and PCI with Methane and Ammonia (Continued)

Group ions	Dwell (ms)
2,3-dibromopropionamide	Acrylamide analyte
149.9	10 ms
151.9	10 ms
106.0	10 ms
$^{13}C_3$-2,3-dibromopropionamide	Internal standard
152.9	10 ms
154.9	10 ms
109.9	10 ms
2,3-dibromo-2-methylpropionamide	Ancillary surrogate
120.0	10 ms
122.0	10 ms
164.0	10 ms
166.0	10 ms
PCI Parameters	
PCI Tuning:	PCI Autotune
EM Setting:	PCI Autotune + 400 V
Source temperature:	250 °C
Quad temperature:	150 °C
PCI SIM Parameters	
Methane reagent gas:	MFC 20% (1.0 mL/min)
Resolution:	Low
Group ions	Dwell (ms)
2,3-dibromopropionamide	Acrylamide analyte
231.9	10 ms
233.9	10 ms
149.9	10 ms
151.9	10 ms
$^{13}C_3$-2,3-dibromopropionamide	Internal standard
234.9	10 ms
236.9	10 ms
2,3-dibromo-2-methylpropionamide	Ancillary surrogate
245.9	10 ms
247.9	10 ms
Ammonia reagent gas:	MFC 20% (1.0 mL/min)
Resolution:	Low
Group ions	Dwell (ms)
2,3-dibromopropionamide	Acrylamide analyte
248.9	10 ms
246.9	10 ms
250.9	10 ms
$^{13}C_3$-2,3-dibromopropionamide	Internal standard
251.9	10 ms
249.9	10 ms
253.9	10 ms
2,3-dibromo-2-methylpropionamide	Ancillary surrogate
262.9	10 ms
260.9	10 ms
264.9	10 ms

Table 3. GC/MS Instrumental Method Parameters for 2-bromopropenamide (Monobromo-Derivative of Acrylamide) in EI

Inlet parameters	
Liner:	Agilent p/n 5062-3587 Single-taper with glass wool
Temperature:	250 °C
Mode:	Pulsed splitless
Pulse pressure:	30.0 psi
Pulse time:	1.20 min
Purge flow:	50.0 mL/min
Purge time:	1.20 min
Total flow:	54.7 mL/min
Gas saver:	Off
Oven parameters	
Oven maximum:	325 °C
Oven equilibrium time:	0.50 min
Initial temperature:	50 °C
Initial time:	1.00 min
Column parameters	
Capillary column	Agilent 122-5533 DB-5MS

Ramp	Temperature	Time
25 °C/min	140 °C	0.00 min
45 °C/min	300 °C	1.50 min
Run time:		9.66 min

Maximum temperature:	350 °C
Nominal length:	30.0 m
Nominal diameter:	250 µm
Nominal film thickness:	1.00 µm
Carrier:	Helium
Mode:	Constant flow
1.2 mL/min	
Outlet and pressure:	MSD Vacuum
MSD Parameters for EI and PCI	
Solvent delay	5.00 min
EI Parameters	
EI Tuning:	Autotune
EM setting:	Autotune + 400V
Source temperature:	230 °C
Quad temperature:	150 °C
EI SIM Parameters	
Resolution:	Low
Group ions	Dwell (ms)
2-bromopropenamide	Native acrylamide
148.9	20 ms
150.9	20 ms
105.9	20 ms
$^{13}C_3$-2-bromopropenamide	Internal standard
151.95	20 ms
153.95	20 ms
2,3-dibromo-2-methylpropionamide	Ancillary surrogate
120.0	10 ms
122.0	10 ms
164.0	10 ms
166.0	10 ms

Results and Discussion

EI Ionization

Figures 6 and 7 show the EI mass spectrum of the 2,3-dibromopropionamide and the 2-bromopropenamide, respectively. Note the similar spectra for the two brominated acrylamide derivatives. In EI, the 2,3-dibromopropionamide loses bromide to generate the C_3H_5ONBr ion that shows the isotopic abundance expected from a monobrominated species. The addition of the triethylamine (base) leads to loss of HBr in solution, generating the monobrominated species C_3H_4ONBr which contains one less hydrogen than the dibromo-derivative and appears as the molecular ion in EI. The spectra share a common C_2H_3Br ion that accounts for the fragments at 105.9 and 107.9 *m/z*. Note that use of the $^{13}C_3$-acrylamide as an internal standard prohibits use of the 107.9 ion in acrylamide quantitation due this C_2H_3Br fragment. The dibromo-derivative shows greater response than the monobrominated compound and lacks the 149 fragment which is subject to interferences from phthalates which are ubiquitous in solvents and food packaging. Both compounds demonstrate good linearity over the range of 10 to 500 pg/µL in EI-SIM as shown in Figures 8 and 9, but better EI detection and elution at a higher oven temperature makes the dibromo-derivative more attractive than the monobromo-derivative. However, it has long been known that the 2,3-dibromopropionamide breaks down in the injection port to form the 2-bromopropenamide. The fraction converted is a function of the injection port activity hence the use of the double-tapered liner for the dibromopropionamide analysis as opposed to the single-tapered liner with wool for the bromopropenamide. Use of the ^{13}C-labeled surrogate is necessary to correct for the degradation of the dibromo-derivative but the methacrylamide surrogate may correct fairly well for recoveries of the mono-brominated acrylamide. Because of this and citations of its use in the literature, the EI spectrum for the brominated methacrylamide is shown in Figure 10 and ions are presented in the acquisition method tables. As the 2,3-dibromo-2-methylpropionamide, this surrogate elutes just prior to the 2,3-dibromopropioamide and much later than the 2-brompropenamide on the GC programs cited.

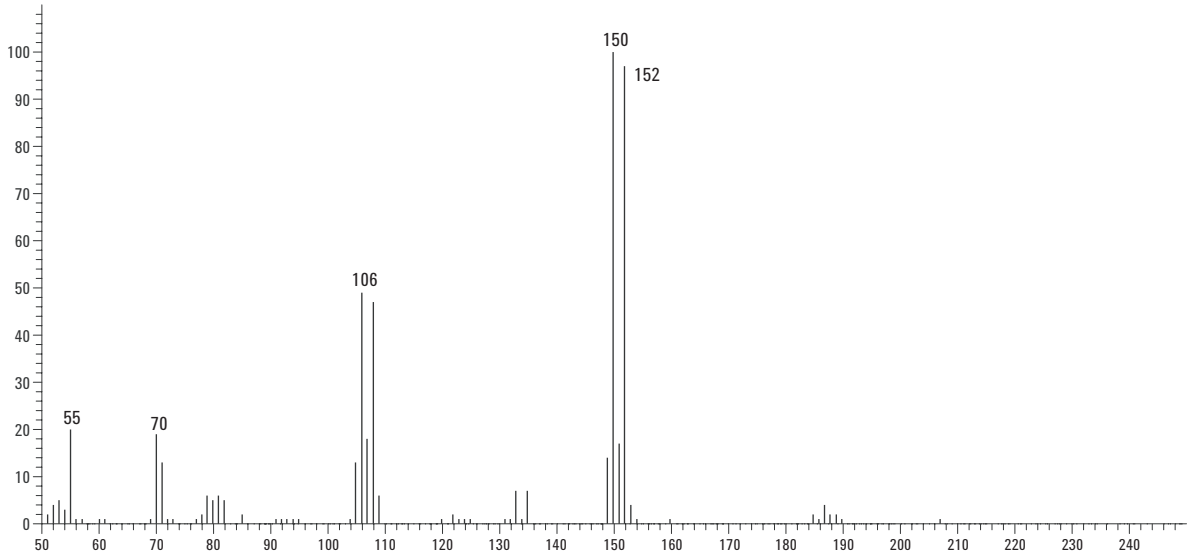


Figure 6. EI ionization spectrum of 2,3-dibromopropionamide.

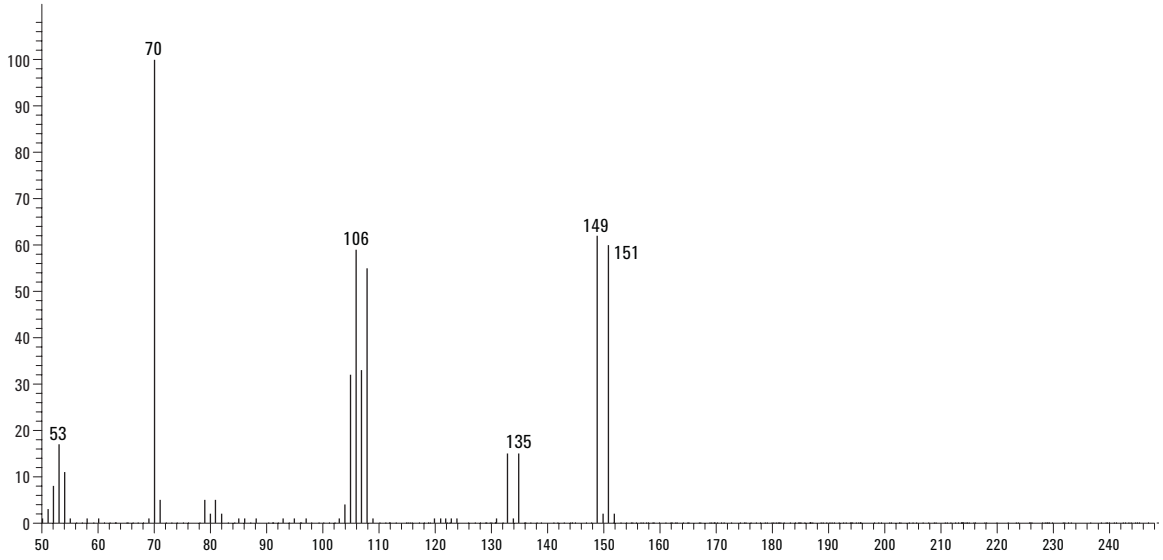


Figure 7. EI ionization spectrum of 2-bromopropenamide.

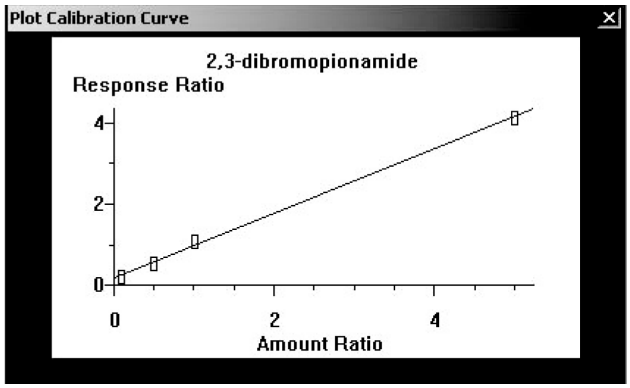


Figure 8. Calibration Curve plot for 2,3-dibromopropionamide from 10 to 500 pg/µL ($R^2 = 0.998$).

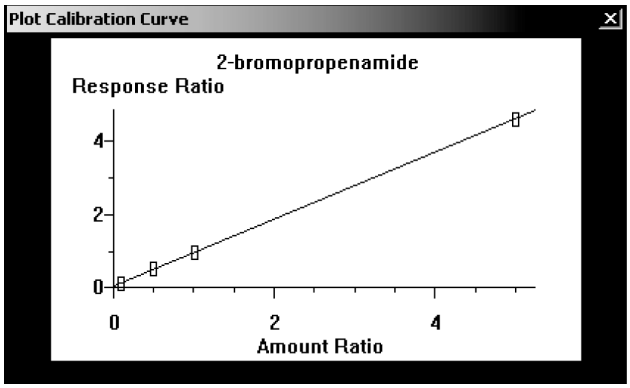


Figure 9. Calibration Curve plot for 2-bromopropenamide from 10 to 500 pg/µL ($R^2 = 0.999$).

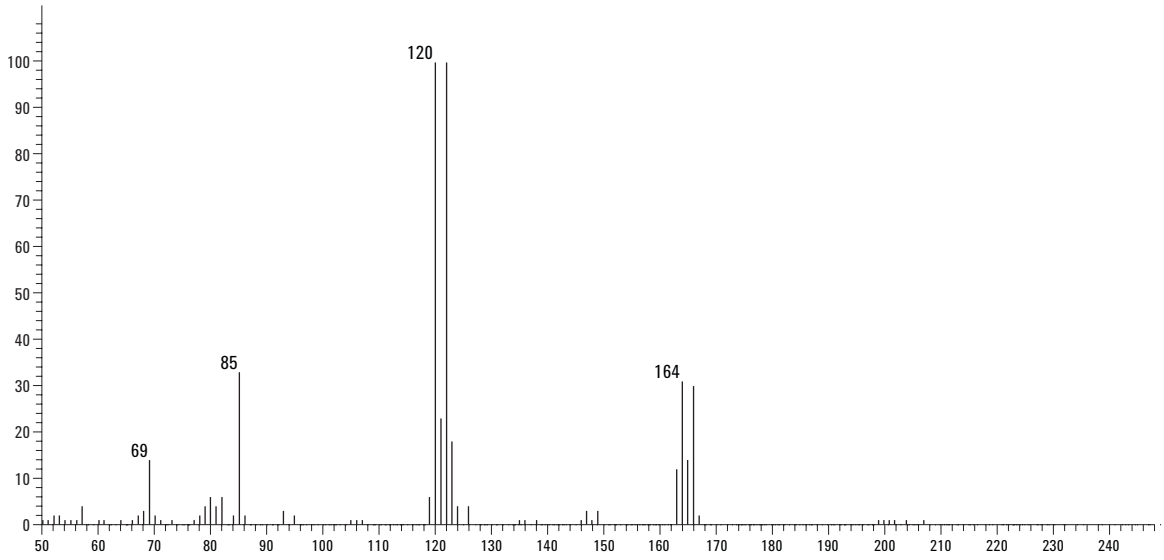


Figure 10. EI ionization spectrum of the alternative, methacrylamide surrogate, 2,3-dibromo-2-methylpropionamide.

PCI

The 2,3-dibromopropionamide spectra in PCI with methane and ammonia reagent gas are shown in Figures 11 and 12. In methane, the highest mass fragment is due to $[M+H]^+$ and in ammonia, $[M+NH_4]^+$. Response with methane is higher than with ammonia and would make a good choice in acrylamide quantitation in samples, if background for that particular food are not an issue. Calibration is similar to that in EI between 10 and 500 pg/µL for both methane and ammonia (R^2 >0.998). It is important that the lower mass fragments that occur in methane and ammonia PCI, *m/z* 72 and 89, respectively, are not used in SIM quantitation. These intense fragments apparently originate through elimination of Br_2 and do not coincide with the cited ions.

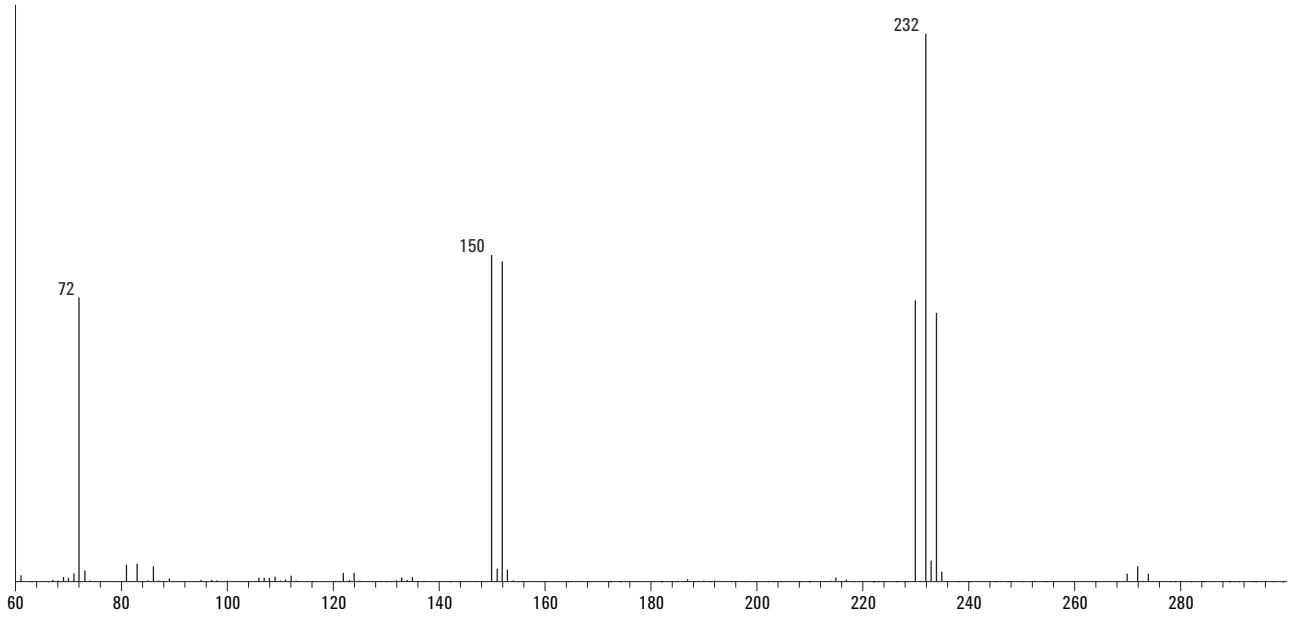


Figure 11. The PCI spectrum of 2,3-dibromopropionamide with methane reagent gas (60–300 amu).

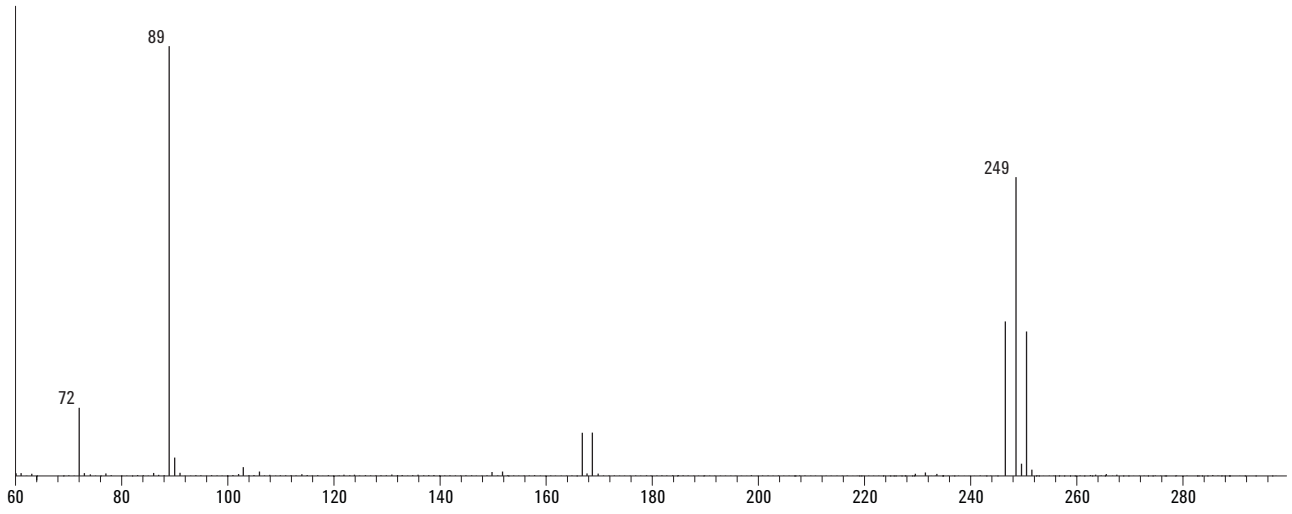


Figure 12. The PCI spectrum of 2,3-dibromopropionamide with ammonia reagent gas (60–300 amu).

Similar to the situation in EI, PCI response of the 2,3-dibromopropionamide exceeds that of the 2-bromopropenamide under either reagent gas. Spectra for this analyte using methane and ammonia are presented in Figures 13 and 14. Highest mass fragments for 2-bromopropenamide also are due to $[M+H]^+$ in methane and in ammonia, $[M+NH_4]^+$. For completeness, the spectra are also included for the brominated methacrylamide surrogate, Figure 15 and 16.

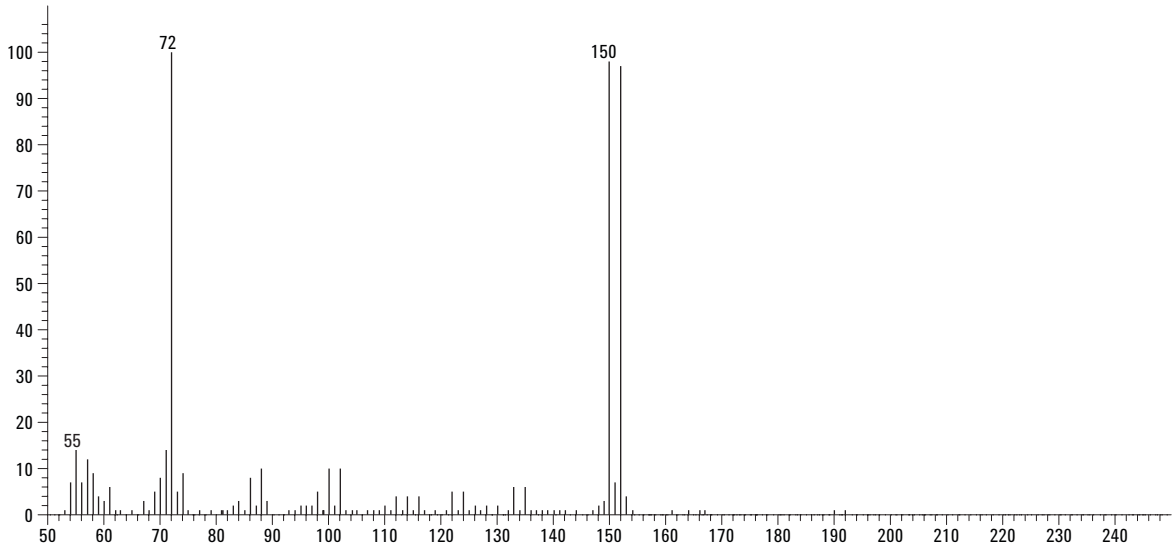


Figure. 13 The PCI spectrum of 2-bromopropenamide with methane reagent gas (50–250 amu).

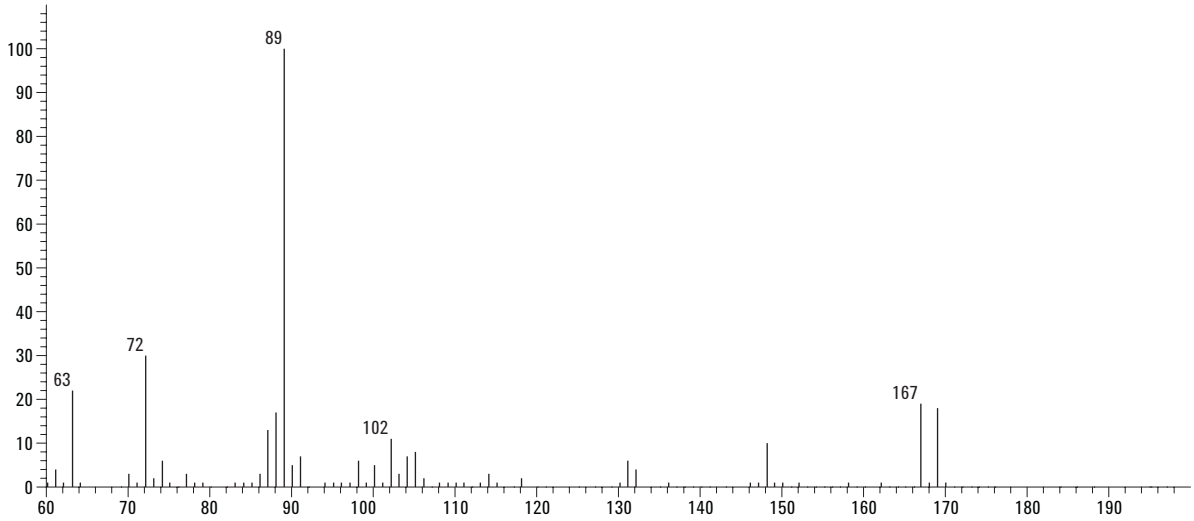


Figure 14. The PCI spectrum of 2-bromopropenamide with ammonia reagent gas (60–200 amu).

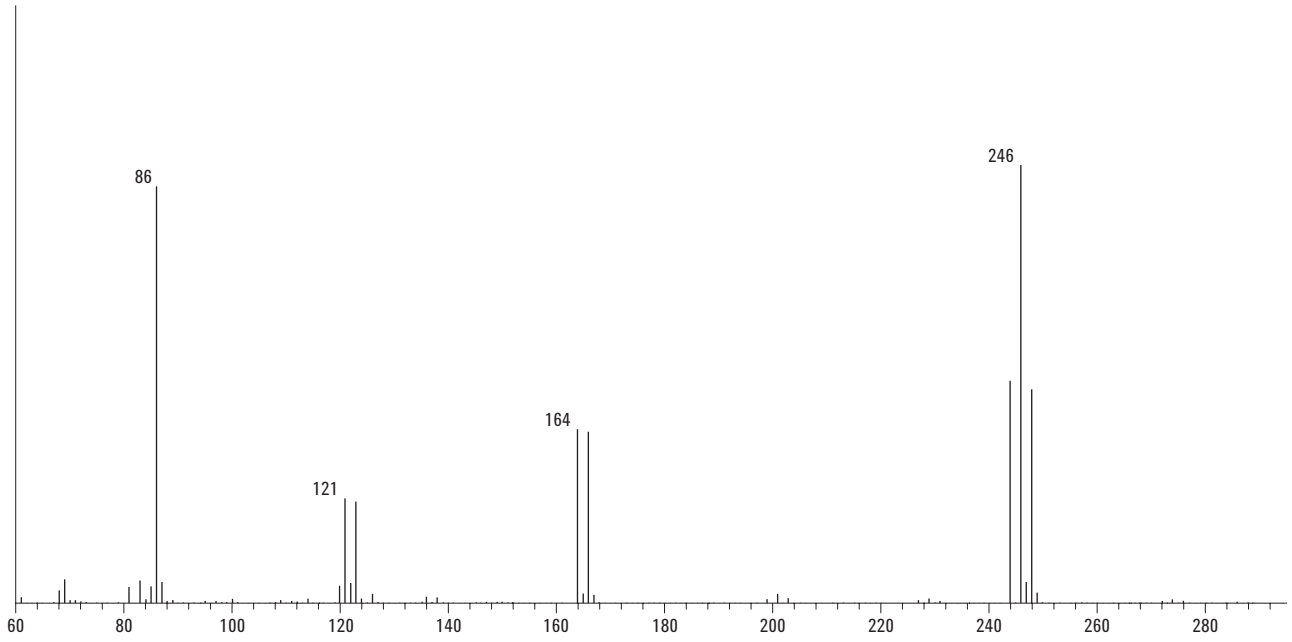


Figure 15. The PCI spectrum of 2,3-dibromo-2-methylpropionamide (the methacrylamide derivative) with methane reagent gas (60–300 amu).

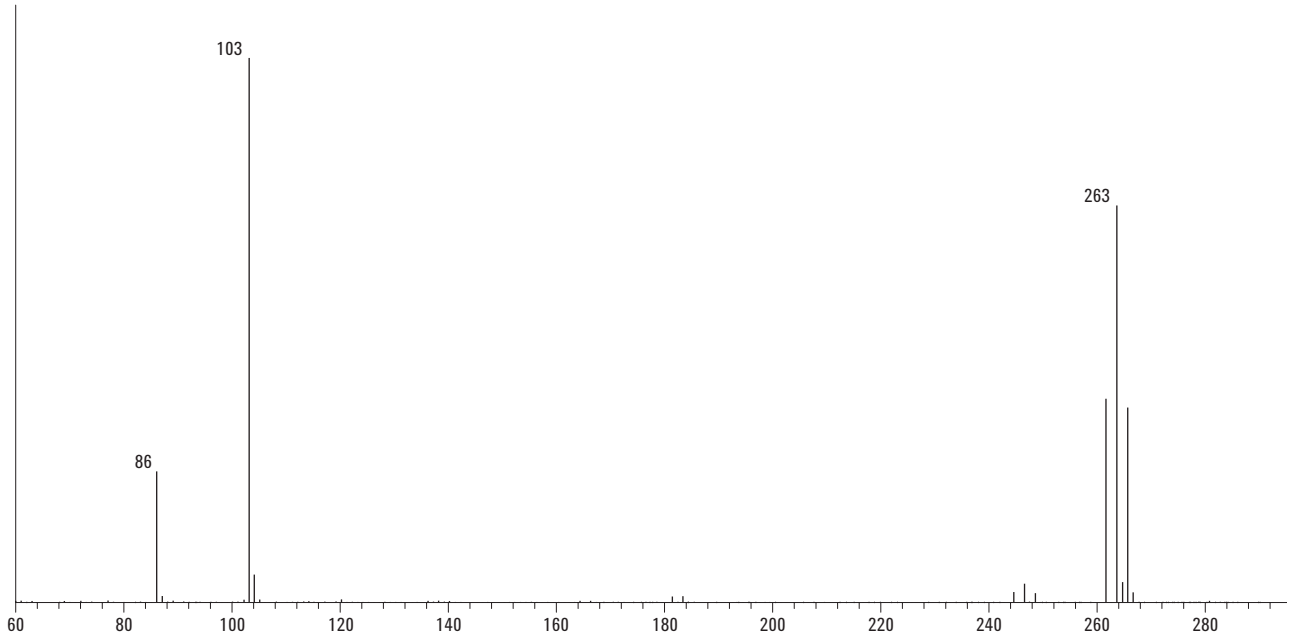


Figure 16. The PCI spectrum of 2,3-dibromo-2-methylpropionamide with ammonia reagent gas.

Conclusions

Since acrylamide was found in a wide range of foodstuffs, a variety of approaches were presented here. The rapid screening approach for native acrylamide using PCI provides a direct and simple method for sensitive detection and quantitation. For approaches using the brominated derivatives, the dibromopropionamide shows superior opportunities for detection and quantitation relative to the 2-bromopropenamide. If, for a particular food product, there are problems in EI, PCI will provide a worthwhile approach for exploration. Methane reagent gas provides about twice the response of ammonia. The degradation of the dibromopropionamide can and must be accounted for by an appropriate labeled internal standard. The methacrylamide surrogate also may be useful for recovery calculations. Data collected on potato chips, and not presented here, suggests this is the case.

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Agilent Technologies

A Blind Study of Pesticide Residues in Spiked and Unspiked Fruit Extracts Using Deconvolution Reporting Software

Application

Food

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Abstract

The Agilent Technologies mass selective detector (MSD) coupled with deconvolution reporting software (DRS) provides additional powerful data processing capabilities to the MSD ChemStation software. Reviewing full scan gas chromatography/mass spectrometry data for the confirmation of pesticide residues can be a labor-intensive and time-consuming process requiring great skill and concentration by an experienced analyst. The DRS is able to process a complex food extract total ion chromatogram in about 1 minute, whereas an experienced analyst may take more than 30 minutes to achieve the same quality result. Extensive data shown in this report supports the high confidence level that an analyst can have in results rapidly produced by the DRS.

Introduction

Typical mass spectral pesticide residue analysis requires finding target ions and meeting qualifier ion ratios. It is sometimes very difficult to confirm target compounds from high matrix background because the matrix affects the ion ratios of the target compounds or complicates the spectrum with additional ions. To be certain of the results, background subtraction and manual integration are often practiced. It is, therefore, a time-consuming process to confirm target compounds in a dirty matrix. It can take an experienced analyst 15 to 30 minutes to review/confirm one data file.

Two powerful gas chromatography/mass spectrometry (GC/MS) techniques - Retention Time Locking (RTL) and deconvolution were combined to create a quantitation and screening tool that can identify 567 pesticides and endocrine disrupters from a single run in 1–2 minutes. The Agilent Technologies GC/MSD-DRS provides the additional functionality to the MSD ChemStation.

Experimental

DRS Overview

A detailed overview of the DRS is given in an application note 5989-1157EN [1], available for download at www.agilent.com/chem. The operating principles of the DRS appear in Figure 1.

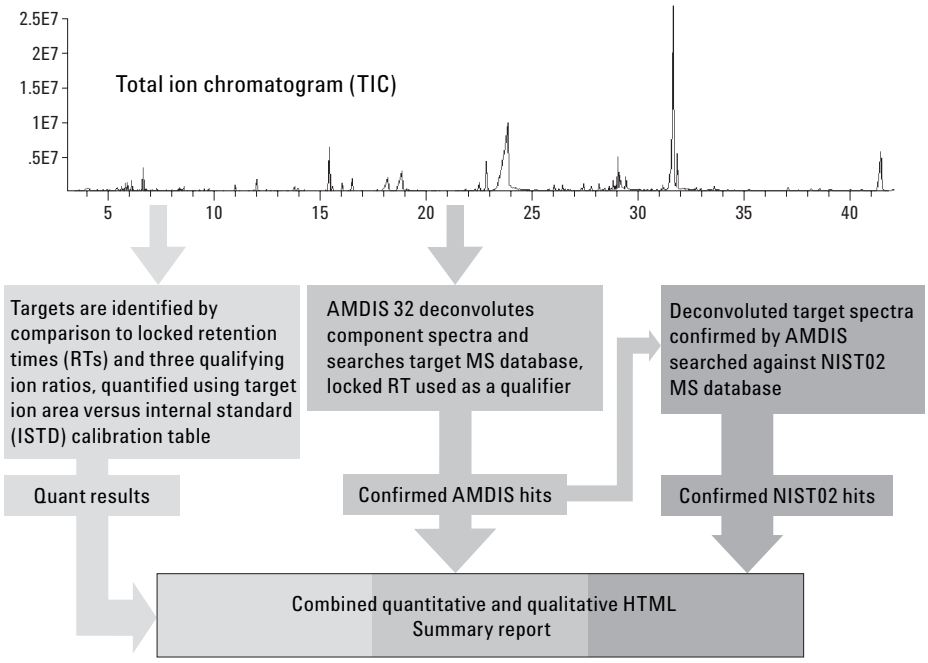


Figure 1. Schematic diagram summarizing the GC/MS DRS.

The quantitation capabilities of the MSD ChemStation are combined with the deconvolution power of the industry standard AMDIS program from NIST. AMDIS is able to separate spectra of interest from dirty matrix spectra present in samples analyzed for pesticides. A third level of confidence is obtained by sending the deconvoluted spectra for library searches of the NIST02 145,000 compound library. A comprehensive report is produced in about 1 minute.

Samples

Six samples of fruit extracts, supplied in 90/10 iso-octane/toluene solvent were received for analysis by GC/MS. The samples were prepared by an accredited food pesticide laboratory based in Scandinavia. Three of the samples were spiked with a number of pesticides at varying concentration levels. Although the range of concentrations of the pesticides in each sample was given, **neither the actual number of pesticides** spiked into each control sample **nor the identities were** supplied. Details of the samples appear in Table 1. The other three samples were 'real', unspiked extracts.

Table 1. Sample Details for Blind Study

Sample number	Matrix extracted	Number of pesticides	Concn range (mg/Kg)	Comments
1	Orange	20–40	0.02–0.20	Control sample - spiked
2	Lettuce	20–40	0.02–0.20	Control sample - spiked
3	Apple	20–40	0.01–0.20	Control sample - spiked
4	Grapes	2–4	0.1–1.0	Real sample
5	Orange	2–4	0.2–5.0	Real sample
6	Apple	2–4	0.05–2.0	Real sample

Instrumentation

The samples were analyzed by full-scan GC/MS using the analytical conditions given in Table 2. Data processing and reporting were performed using the default settings provided with the DRS.

Table 2. RTL GC/MS Analysis Conditions for Fruit Extract Samples

Gas chromatograph	**Agilent 6890N**
Column	30 m x 0.25 mm id x 0.25 µm HP-5MS (p/n 19091S-433)
Carrier gas	Helium
Flow rate	1.9 mL/min at 70 °C
Head pressure	18 psig, constant pressure mode Method RTLocked to methyl chlorpyrifos at 16.593 min
Injector type	PTV, septumless head
Injector temperature (°C), hold time (min), and ramp rate (°C/min)	90 °C (0.3 min) - 1720 °C/min - 250 °C
Vent time	0.2 min
Vent flow	30 mL/min
Vent pressure	0 psig
Purge flow	60 mL/min
Purge time	1.0 min
Syringe volume	50 µL
Injection volume	15 µL
Liner	Empty multibaffle
Oven program: temperature (°C), hold time (min), and ramp rate (°C/min)	70(2)-25-150(0)-3-200(0)-8-280(10)
MSD	**Agilent 5973 inert**
MS interface	280 °C
MS source	230 °C
MS quad	150 °C
Detection mode	EI, Scan 40–550 amu
EM voltage	ATUNE value

Results

The results for the three spiked extracts appear in Table 3 - note that the details of which pesticides were added to the spiked samples were not supplied until after the results were shown to the customer. Those pesticides confirmed by the DRS, are shown lightly shaded. The analytes, shown darkly shaded, are not present in the Agilent RTL Pesticides database. Analyte entries left unshaded were not confirmed.

Table 3. MSD-DRS Results for Three Spiked Fruit Extract Samples

	Sample 1: Control-orange, spiked		Sample 2: Control-lettuce, spiked		Sample 3: Control- apple, spiked	
	Pesticide	Added mg/kg	Pesticide	Added mg/kg	Pesticide	Added mg/kg
1	Methamidofos*	0.10	Diphenylamine	0.10	Mevinphos	0.05
2	Dichlorvos*	0.10	HCB	0.02	Trichlorfon	0.05
3	Acephate*	0.10	Lindane (HCH-gamma)	0.04	Heptenophos	0.02
4	Omethoate	0.10	Diazinon	0.04	Tecnazene	0.01
5	Propachlor	0.20	Chlortalonil	0.04	HCH alpha	0.01
6	Chlorprofam	0.10	Vinclozolin	0.04	HCH beta	0.02
7	Monocrotophos	0.10	Carbaryl	0.20	Dichloran	0.05
8	Dimethoate	0.04	Metalaxyl	0.10	Pyrimethanil	0.02
9	Quintozene	0.02	Pirimiphos-methyl	0.10	Etrimphos	0.02
10	Parathion-methyl	0.10	Malathion	0.10	Ethiofencarb	0.10
11	Dichlofluanid	0.10	Chlorpyrifos	0.10	Metribuzin	0.05
12	Fenpropimorph	0.10	Cyprodinil	0.04	Toclophos methyl	0.01
13	Triadimefon	0.04	Penconazole	0.04	Linuron	0.05
14	Thiabendazole	0.10	Captan	0.10	Aldrin	0.02
15	Tolylfluanid	0.04	Folpet**	0.10	Diethofencarb	0.02
16	Mecarbam	0.10	Procymidone	0.04	Trichloronate	0.02
17	Methidation	0.10	Endosulfan-a	0.04	Triadimenol	0.05
18	Vamidothion	0.10	pp-DDE	0.04	Disulfoton sulfoxide	0.20
19	Imazalil	0.10	Bupirimate	0.04	Disulfoton sulfone	0.02
20	Myclobutanil	0.10	Endosulfan-b	0.04	Fluazinam	0.05
21	Kresoxim methyl	0.10	Aclonifen	0.04	Chlorbenzilate	0.05
22	Tebuconazole	0.10	Ethion	0.04	Oxadixyl	0.05
23	Phosmet	0.10	Triazophos	0.04	Benalaxyl	0.05
24	Fenpropathrin	0.04	Endosulfan-sulfate	0.04	Dicofol	0.05
25	Tetradifon	0.04	Iprodione	0.04	Fenazaquin	0.02
26	Azinphos-methyl	0.10	Bromopropylate	0.10	Pyrazophos	0.05
27	Fenarimol	0.10	Methoxychlor	0.10	Acrinathrin	0.02
28	Azinpfos-ethyl	0.10	Phosalone	0.10	Bitertanol	0.05
29	Prochloraz	0.10	Lambda-Cyhalothrin	0.04	Cyfluthrin beta	0.05
30	Flucythrinate	0.10	Permethrin	0.10	Alpha cypermethrin	0.05
31	Esfenvalerate	0.04	Cypermethrin	0.10		
32	Azoxystrobin	0.04	Fenvalerate	0.04		
33			Deltamethrin	0.10		

* See Discussion item 1.

** See Discussion item 2.

The results for the three 'real' extracts appear in Table 4. Those pesticides confirmed by the DRS are shown lightly shaded. The darkly-shaded analytes are not present in the Agilent RTL Pesticides database. Analyte entries left unshaded were not confirmed. Analytes with an associated concentration were confirmed as present by the customer using NPD/ECD. Lightly-shaded analytes without a concentration label were detected and confirmed by the DRS, but not by the customer.

Table 4. MSD-DRS Results for Three 'Real' Fruit Extract Samples

Sample 4: Grapes
0.68 mg/Kg Captan
0.21 mg/Kg Cyprodinil
0.27 mg/Kg Fludioxinil
Diphenylamine
Sample 5: Orange
2.5 mg/Kg Imazalil
0.25 mg/Kg Medidathion
3.0 mg/Kg Thiabendazole
Sample 6: Apple
0.86 mg/Kg Diphenylamine
0.05 mg/Kg Chlorpyrifos
0.79 mg/Kg Thiabendazole
Dimethoate
Ethoxyquin
Methyl parathion
Endosulfan sulfate
Propargite

Discussion

1. Control - Orange spiked extract

This control sample was spiked with 32 pesticides at levels ranging between 0.02 and 0.10 mg/kg. Twenty-six pesticides were detected and confirmed by the DRS software, two were not reported since they are not present in the Agilent RTL Pesticide database and four were not detected. The spiking was done to the raw matrix, not to a matrix extract. For the polar pesticides (methamidofos and acephate), the recovery was in the 20%–30% range as confirmed by NPD/ECD. Therefore, that explains why these pesticides were not detected by DRS.

2. Control - Lettuce spiked extract

This control sample was spiked with 33 pesticides at levels ranging between 0.02 and 0.20 mg/kg. Twenty-nine pesticides were detected and confirmed by the DRS software, three were not reported since they are not present in the Agilent RTL Pesticide database and one was not detected. The one undetected analyte, (Folpet, marked with two asterisks in Table 3), was detected and confirmed if a higher sensitivity setting was used in the AMDIS deconvolution program.

3. Control - Apple spiked extract

This control sample was spiked with 30 pesticides at levels ranging between 0.01 and 0.20 mg/kg. Twenty-two pesticides were detected and confirmed by the DRS software, six were not reported since they are not present in the Agilent RTL Pesticide database and two were not detected.

Overall, of the 95 spiked analytes in the three control samples, 93% of the pesticides present in the Agilent RTL Pesticide database were detected and confirmed by full-scan library searching of the deconvoluted mass spectra.

4. 'Real' Grape extract

The customer had detected and confirmed three pesticide residues in the Grape extract sample - Captan, Cyprodinil, and Fludioxinil. Of these three analytes, Captan was confirmed by the DRS and Cyprodinil and Fludioxinil are not entries in the Agilent RTL Pesticide database. However, DRS also confirmed an additional pesticide residue - Diphenylamine, which was not reported by the customer.

5. 'Real' Orange extract

The customer had detected and confirmed three pesticide residues in the Orange extract sample - Imazilil, Methidathion, and Thiabendazole. All three of these pesticides were confirmed by the DRS software and no other analytes were confirmed.

6. 'Real' Apple extract

The customer had detected and confirmed three pesticide residues in the Apple extract sample - Diphenylamine, Chlorpyriphos, and Thiabendazole. All three of these pesticides were confirmed by the DRS. In addition, the DRS also confirmed the presence of five additional pesticide residues - Dimethoate, Ethoxyquin, Methyl Parathion, Endosulfan Sulfate, and Progargite. These five pesticides had not been reported by the customer.

Conclusions

The Agilent Technologies MSD-DRS provides additional powerful data processing capabilities to the MSD ChemStation software. Reviewing full scan GC/MS data for the confirmation of pesticide residues can be a labor-intensive and time consuming process requiring great skill and concentration by an experienced analyst.

The DRS is able to process a complex food extract TIC in the order of 1 minute, whereas an experienced analyst may take more than 30 minutes to achieve the same quality result. The DRS software was proven to report the lowest number of false positives and false negatives in the shortest time period.

In scan mode, the detection limit is not as low as in selected ion monitoring (SIM) mode; however, any prior knowledge of the target analytes (retention times or characteristic ions) is not required for the DRS.

The extensive data shown in this report, run under totally blind conditions, shows the high degree of confidence that an analyst can have in the results produced by the DRS in minutes.

Reference

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New Tools for Rapid Pesticide Analysis in High Matrix Samples

Application

Food Analysis

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Abstract

Recent developments in GC/MS hardware and software make it possible to analyze samples with high levels of matrix contamination much faster than ever before. New tools such as mass spectral deconvolution, reliable and inert effluent splitters, and column backflushing capabilities can be combined to produce large time savings. By accelerating the chromatographic run, post-run bakeout, and data interpretation steps, analysis times can be shortened by at least three-fold versus conventional methods. These tools are especially useful in analyses with high levels of matrix background, such as the inspection of the food supply for contaminants. In addition to monitoring for pesticide residues, the threat of terrorism has recently raised concerns over deliberate contamination of food with other toxic materials. This article describes a GC/MS system for the rapid screening of foodstuffs for chemical contaminants with a special emphasis on pesticides, organophosphorus, and organosulfur compounds.

Introduction

Techniques for decreasing the analysis time of gas chromatography (GC) methods have been developed in recent years.

Tools like Method Translation [1] have made it straightforward to reduce analysis time by a known factor and maintain the exact relative elution order of the analytes. The use of appropriate shorter and smaller diameter columns can maintain the same resolution while achieving a much shorter analysis time.

One application area where this approach has met difficulty, however, is the gas chromatography/mass spectrometry (GC/MS) analysis of pesticides in complex matrices like food. This application requires that speed-up schemes maintain column capacity in order to handle the large matrix peaks and achieve low detection limits for analytes. Since chromatography is governed by the triangle of speed, resolution, and capacity, resolution must be sacrificed to increase speed at the same capacity. The problem is that chromatographic resolution is also needed to confirm the identity of any target analytes detected in the presence of interferences from the sample. In this note, the reduction in chromatographic resolution in faster analysis is more than adequately compensated for by use of spectral deconvolution [2] and simultaneous element-selective detection for the confirmation step.

The system consists of a GC/MS with a dual-wavelength flame photometric detector (DFPD) for the simultaneous collection of phosphorus, sulfur, and mass spectral data.

The GC column effluent is split between the two detectors in the ratio of 2:1 in favor of the mass selective detector (MSD). The system is retention-time locked to the Agilent pesticide library [1] scaled to threefold faster times, which contains the

retention times and spectra for 567 pesticides used worldwide. Samples are analyzed with MS in full-scan electron-impact ionization (EI) mode. The combination of precise retention times, elemental, and mass spectral data is used to screen for specific target compounds. The flame photometric detector (FPD) data also highlights any non-target, P- or S-containing compounds for identification by MS.

The MS data is screened using the standard quantitation software based on retention time (RT), ion ratios, and spectral cross correlation. The MS data is also processed using spectral deconvolution software, which greatly reduces spectral interferences from the matrix. The deconvoluted spectra are then searched against a table of targets. Any hits are confirmed by searching against the main NIST library. This process is automated by the Agilent Deconvolution Reporting Software (DRS), also providing significant time savings in data interpretation.

The system described here uses column backflushing, a technique used to save large amounts of time with complex samples. Backflushing is done with the splitter hardware. This technique removes heavy residues from the column much faster and at lower temperatures than the conventional bakeout step at the end of the run. This reduces MS source contamination by preventing the higher levels of column bleed and heavy matrix components from entering the MSD. It also increases the column lifetime.

The approach used thus reduces analysis time in three major ways: shortening the chromatographic run time; automating data interpretation; and reducing bakeout time. Other notable advantages are the ability to change columns and/or inlet liners without venting the MSD, and a reduced need for MS source cleaning.

System Configuration

The system configuration used is shown in Figure 1. Key components are:

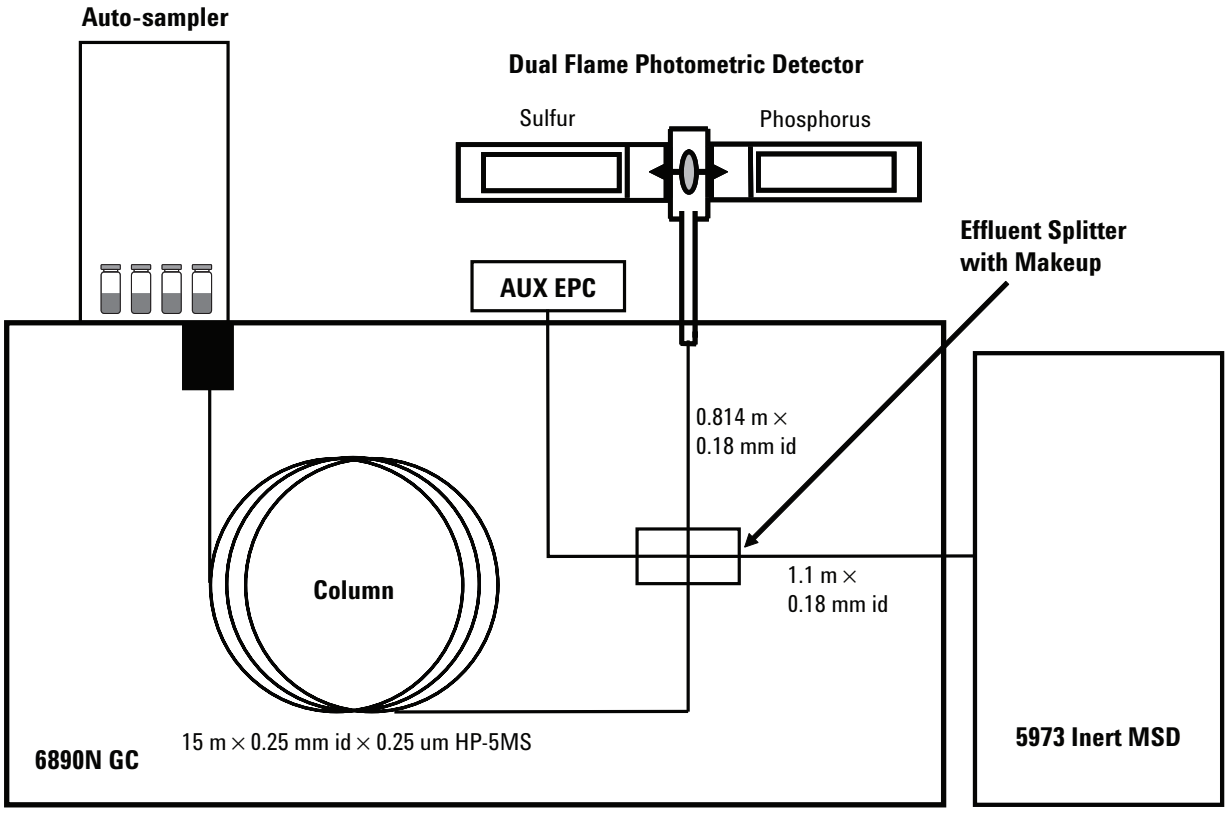


Figure 1. System configuration.

Key Components

Fast Oven With the 6890N 220V oven (option 002), the pesticide analysis method can be run precisely 3 times faster (14 min) using a 15 m HP-MS column. If the 220V GC is further equipped with SP1 2310-0236 (puts MSD interface in back of oven under rear injection port) and the G2646-60500 oven insert accessory (reduces oven volume twofold), the speed can be increased to 4.8 times faster (9 min). The cool-down time of the oven is also reduced.

Dual FPD 6890N Option 241 is a single flame photometric detector with two optical detection channels, one for sulfur and one for phosphorus. The signals from the DFPD are collected, stored, and processed by the MS ChemStation simultaneously with the MS data. The FPD data can be used in several ways. Nontarget organophosphorus compounds like new pesticides or designer nerve agents are highlighted. The presence of an element at the retention time of an identified compound can be used to support confirmation of identity. The response on the FPD can be used for quantitative or semi-quantitative analysis, especially for situations where no calibration standard is available for an identified analyte.

Microfluidic Splitter 6890N Option 889 uses diffusion bonded plate technology combined with metal column ferrules to make an inert, easy-to-use, leak-free, high-temperature column effluent splitter. The splitter uses Auxillary (Aux) electronic pneumatics control (EPC) for constant pressure makeup (6890N Option 301). The Aux EPC makeup can be pressure programmed at the end of the run to higher pressure, while at the same time the inlet pressure is lowered to near ambient. This causes the flow in the column to reverse direction, backflushing heavy materials out the split vent of the inlet. The Aux EPC also allows column changing and maintainance without venting the MSD. When the column fitting is removed from the splitter, helium from the makeup supply purges the fitting, preventing air from entering the MSD. If the column is attached to the splitter but removed from the inlet, helium flows backwards through the column and out the inlet end. Inlet maintainance or column headtrimming can be done without cooling and venting the MSD and air is not introduced into a hot source.

MSD System The 5973N Inert with Performance Electronics and performance turbo (G2579A) EI MSD is used. This configuration provides faster full scan rates while maintaining sensitivity. The scan rates are compatible with the narrower peaks generated by fast chromatography. The performance turbo pump is required to handle the higher flows associated with fast chromatography and backflushing.

DRS Software (G1716AA) Spectral deconvolution of the MS data allows identification of analytes in the presence of overlapped matrix peaks. This significantly reduces chromatographic resolution requirements, allowing much shorter analysis times. DRS utilizes the AMDIS deconvolution program from NIST, originally developed for trace chemical weapons detection in complex samples. DRS presents the analyst with three distinct levels of compound identification: (1) ChemStation, based on retention time and four ion agreement; (2) AMDIS, based on “cleaned spectra” full ion matching and locked retention time; (3) NIST02 search using a >147,000 compound library.

Instrument Operating Parameters

The recommended instrument operating parameters are listed in Table 1. These are starting conditions and may have to be optimized.

Split injection was used to match the amount of matrix to the column capacity. Citrus oils cause retention shifts if excess sample is injected. Splitless injection could be used for samples with significantly less matrix. The inlet liner was found to be of low activity, as it does not contain glass wool. Proper mixing for split injections is done by the internal liner geometry.

The 6890 220V oven was needed for the ramps described in Tables 1 and 2. This oven program is necessary for the precise 3× speed increase of the RTLocked pesticide database.

The 15-m HP-5ms column has the same phase ratio as the 30 m column traditionally used for the 1× method. This shorter column allows a flow rate for a 3× precisely scaled faster method. The outlet is listed as “unspecified” because the column connects to the splitter. The splitter pressure is operated at a constant 3.8 psig using an auxillary EPC module.

The 5973 inert Performance Electronics data acquisition sampling rate was set to 1, which is faster than the typical setting of 2. Signal-to-noise is improved over previous systems at faster sampling rates. More data points allows for easier integration and better deconvolution to compensate for the loss in resolution using a shorter column.

The microfluidic splitter parameters are chosen to provide the desired split ratio between detectors

while meeting the flow requirements of the detectors used. A primary consideration with the current system is to make sure that the flow to the MSD does not exceed ~4 mL/min while collecting analyte data. It was also desired to split the effluent 2:1 in favor of the MSD. These parameters were entered into the spreadsheet calculator (included with the splitter), which calculated the lengths and diameters of the detector restrictors

Table 1. Gas Chromatograph and Mass Spectrometer Operating Parameters

GC	Agilent Technologies 6890		
Inlet	EPC Split/Splitless		
Mode	Split, 1.0 µL injected		
Inlet temp	250 °C		
Pressure	23.84 psi		
Split ratio	10:1		
Split flow	44.1 mL/min		
Total flow	48.1 mL/min		
Gas saver	Off		
Gas type	Helium		
Inlet Liner	Siltek Cyclosplitter, 4 mm id, Restek part number 20706-214.1		
Oven	220V		
Oven ramp	°C/min	Next °C	Hold min
Initial		70	0.67
Ramp 1	75	150	0.00
Ramp 2	9	200	0.00
Ramp 3	24	280	3.33 (end of pesticide ramp)
Ramp 4	50	320	50.0 (end of oil elution)
Total run time	13.96 min to elute pesticides		
Total run time	64.76 min to elute heavy components from citrus oils		
Equilibration time	0.5 min		
Oven max temp	325 °C		
Column	Agilent Technologies HP-5MS, p/n 19091S-431		
Length	15.0 m		
Diameter	0.25 mm		
Film thickness	0.25 µm		
Mode	Constant Pressure = 23.84 psi		
Inlet	Front		
Outlet	Unspecified		
Outlet pressure	3.8 psi (aux pressure to splitter)		
Back Detector (FPD)			
Temperature	250 °C		
Hydrogen flow	75.0 mL/min		
Oxidizer flow	100.0 mL/min		
Oxidizer gas type	Air		
Mode	Constant makeup flow		
Makeup flow	60.0 mL/min		
Makeup gas type	Nitrogen		
Flame	On		
Lit offset	5.00		
Photo multiplier	On		

Table 1. Gas Chromatograph and Mass Spectrometer Operating Parameters (Continued)

Signal 1		Signal 2	
Data rate	5 Hz	Data rate:	5 Hz
Type	Back detector	Type:	Front detector
Save data	On	Save data:	On
Zero	0.0 (Off)	Zero:	0.0 (Off)
Range	0	Range:	0
Fast Peaks	Off	Fast Peaks:	Off
Attenuation	0	Attenuation:	0

AUX Pressure 5	
Description	
Gas type	Helium
Initial pressure	3.80 psi
Initial time	0.00 min (this value will follow oven ramp)
MSD	Agilent Technologies 5973 Inert Performance Electronics
Tune file	Atune.U
Mode	Scan
Solvent delay	1.00 min
EM voltage	Atune voltage
Low mass	45 amu
High mass	450 amu
Threshold	0
Sampling	1
Scans/sec	6.68
Quad temp	150 °C
Source temp	230 °C
Transfer line temp	280 °C
Splitter	Agilent 6890N Option 889
Split ratio	2:1 MSD:DFPD
MSD restrictor	1.1 m × 0.18 mm id deactivated fused silica tubing
DFPD restrictor	0.81 m × 0.18 mm id deactivated fused silica tubing

Backflush Instrument Operating Parameters

Instrument operating conditions for backflushing are shown in Table 2. These are starting parameters and will have to be optimized depending on sample matrix. Conditions listed here are only those that are different from the Table 1 conditions.

Instead of baking the column at 320 °C for 50 min, the heavy matrix material is backflushed through the column inlet, out the split vent. This is accomplished in 5 min at 300 °C, saving 45 min of run time, preserving column life, and shortening cool down time.

The column inlet pressure is reduced to 1.1 psi by using the ramped pressure feature of the EPC. At the end of the backflush time, it is ramped back to initial conditions.

Simultaneous with ramping the inlet pressure down to 1.1 psig, the Aux EPC splitter pressure is ramped up to 23 psig. This increase in pressure at the column outlet, along with the decrease in inlet pressure, backflushes the column. The backflush pressure for the Aux EPC is set to limit the flow to the MSD to < 8 mL/min. This is calculated using the Agilent Flow Calculation software program, also supplied with the splitter kit. The calculator is used to find the pressure which gives the desired flow through the MSD splitter restrictor (1.1 m × 0.18 mm id) at the backflushing temperature 300 °C. The result was 23.6 psig which would produce a flow of 8 mL/min, so 23 psig was used.

The backflush time is determined empirically and depends on the sample matrix. The process is to try a backflush run followed by a blank run with the conventional long-hold to see if the heavy materials

are completely removed. If not, the process is repeated with a longer backflush time. As a very rough guide, start with a backflush time of five void times for the backwards flow. Using the Agilent Flow Calculation software with the “inlet pressure” at 23 psig, the “outlet pressure” at 1.1 psig, and the temperature at 300 °C, the hold-up time (void time) is 0.423 min. The rough guide says that the column should be backflushed for 2.12 min. This works for removing most heavies, but 5 min is required in this case to remove them all.

At the end of the backflush time, the Aux EPC is ramped back to initial conditions.

The MSD is time-programmed to collect data over the time range of 1 to 13.96 min. All of the pesticides elute during this time range.

Table 2. Backflush Gas Chromatograph and Mass Spectrometer Operating Parameters. Use Table 1 Conditions Except for These Differences

Backflush Oven Conditions			
Oven ramp	°C/min	Next °C	Hold min
Initial		70	0.67
Ramp 1	75	150	0.00
Ramp 2	9	200	0.00
Ramp 3	24	280	3.33 (end of pesticide ramp)
Ramp 4	50	300	5.40 (end of oil backflush)
Total run time	13.96 min to elute pesticides		
Total run time	19.76 min to elute heavy components from citrus oils		
Backflush Column Conditions			
Mode	Ramped Pressure		
Press ramp	psi/min	Next psi	Hold min
Initial		23.84	13.96 (end of pesticide ramp)
Ramp 1	99	1.1	5.57 (backflush time)
Ramp 2	99	23.84	0.00
Backflush AUX 5 Conditions			
Press ramp	psi/min	Next psi	Hold min
Initial		3.8	13.96 (end of pesticide ramp)
Ramp 1	99	23.0	5.61 (backflush time)
Ramp 2	99	3.8	0.00
Backflush MSD Conditions			
Timed MS detector entries			
Time (min)	State (MS on/off)		
13.96	Off		

Results

A mixture of 25 pesticides was run at 1×, 3×, and 4.8× speeds. The Total Ion Chromatograms (TICs) are shown in Figure 2.

There is some loss in resolution, but the loss is limited because the shorter columns are run at flows closer to the optimum. The resolution losses are mitigated by using the faster scanning capabilities of the performance electronics together with DRS.

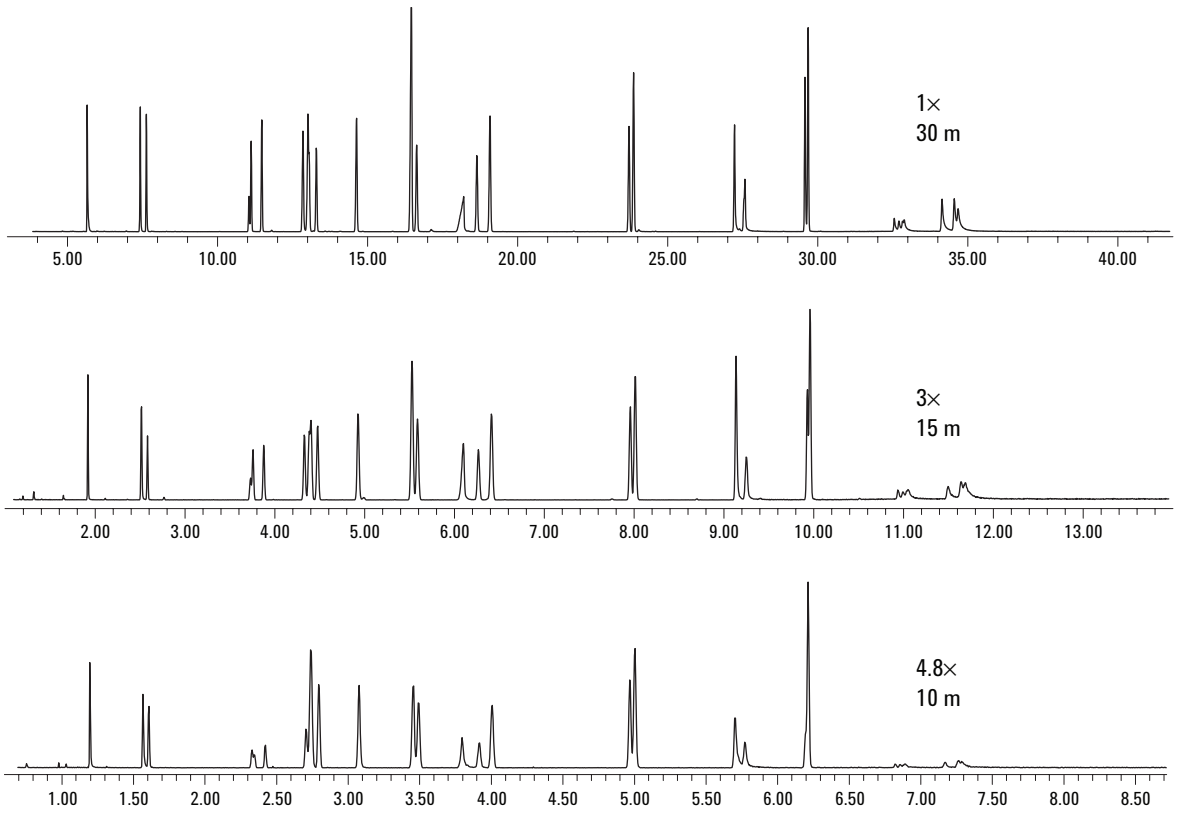


Figure 2. TICs of pesticide test mix at three different scaled speeds. All columns have the same phase ratio.

A neat lemon oil was analyzed using the 3× speed conditions. The TIC is shown in Figure 3 with the DFPD phosphorus (P) and sulfur (S) data channels. The ChemStation software can simultaneously acquire two signals of GC data with the MSD data. The pesticides elute within the 14 min window shown, but the matrix continues to elute for an additional 50 min at 320 °C.

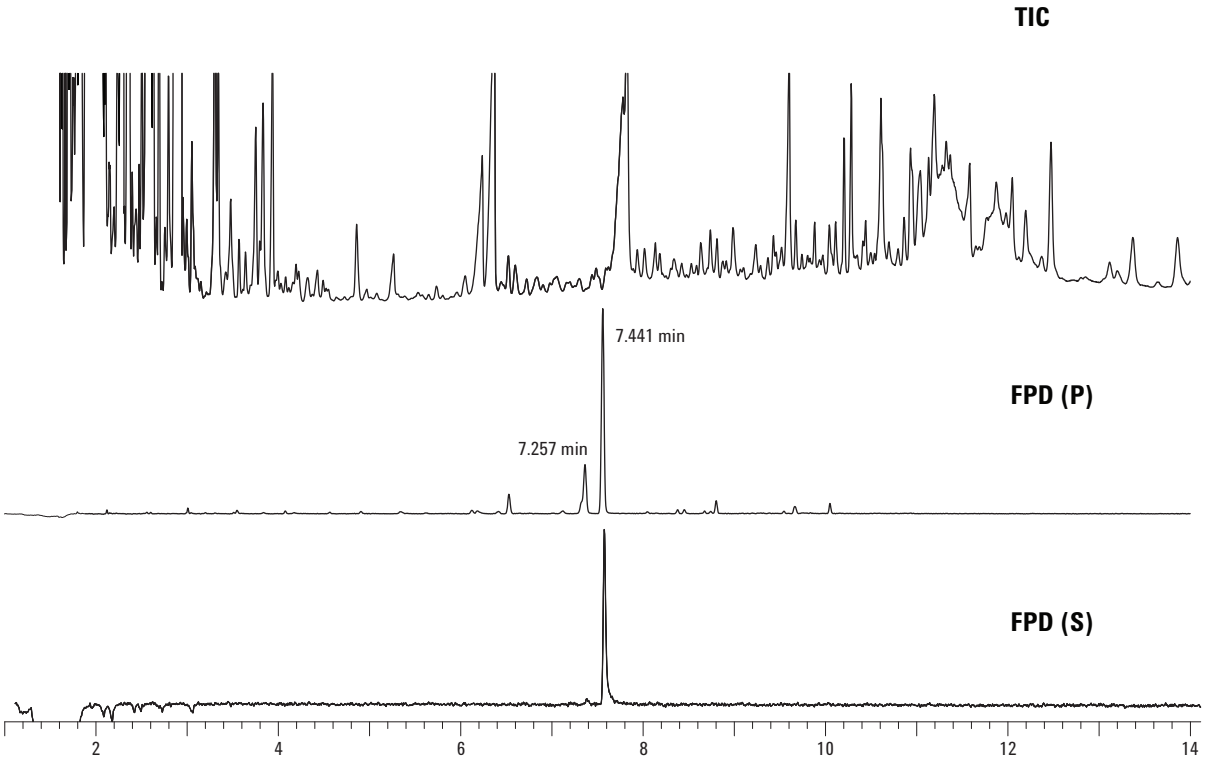


Figure 3. MS and DFPD data from lemon oil analyzed with 3x method.

The P and S chromatograms indicate the possible presence of numerous pesticides. The largest P peak, 7.441 min, also contains S. A PBM reverse Library search identified the peak as Methidathion ($C_6H_{11}N_2O_4PS_3$) with a match quality of 45. It was also identified using a target ion and three qualifier ions.

The raw apex spectrum of the P peak at 7.257 min is shown in the top of Figure 4. No match was found for a P-containing compound in the top 20 library search results. It was also not identified by the ChemStation target and qualifier ion criteria due to out-of-range ratios.

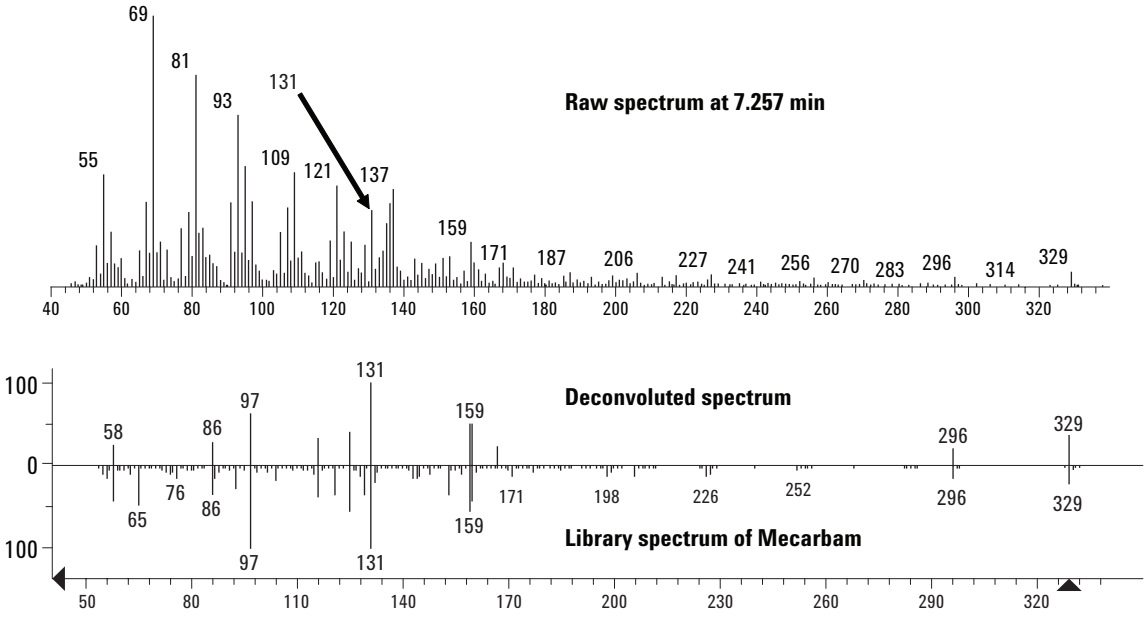
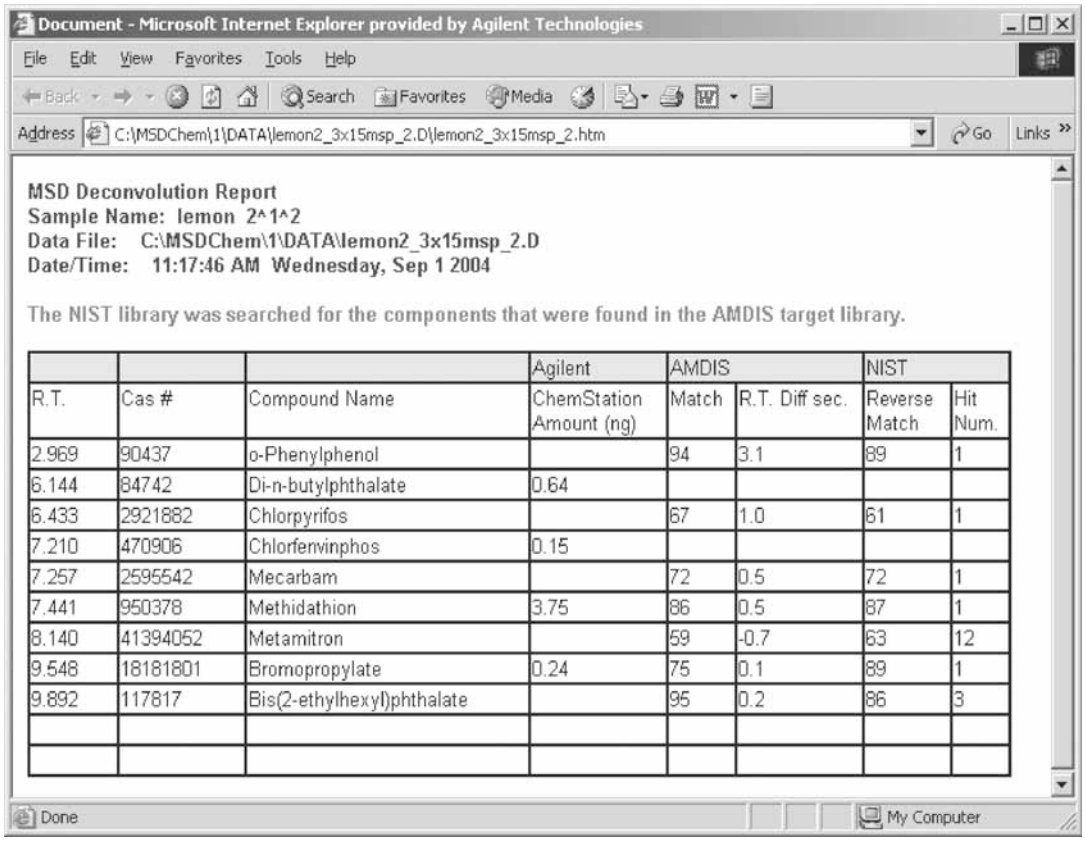


Figure 4. Top - Raw mass spectrum of peak at 7.257 min in lemon oil. Bottom - Deconvoluted spectrum of 7.257 min peak compared to NIST02 library spectrum of Mecarbam.

The DRS report is shown in Figure 5. The peak at 7.257 min is clearly identified as Mecarbam by the DRS software. The deconvoluted spectrum has a match factor of 72, compared to both the Agilent Pesticide AMDIS database and the NIST02 library. Additionally, the DRS report shows the elution time to be only 0.5 s from expected. Further confirmation is the presence of P with S barely visible. The deconvoluted spectrum for the peak at 7.257 min is shown at the bottom of Figure 4, together with the NIST library spectrum of Mecarbam.

The DRS report displays the amount for compounds found by the normal ChemStation quant process. The compounds must be properly calibrated to have a meaningful value. In this lemon oil, the ChemStation found four compounds. The AMDIS portion of DRS found two of the same compounds and an additional five compounds missed by the ChemStation. The NIST02 library search confirmed all of the compounds found by AMDIS using the NIST02 >147,000 compound library. The DRS results show good match quality at the locked retention times for seven target compounds.

No single software package can produce this same confidence level in compound identification. An experienced analyst would take 1–4 hours to process this complex sample manually with each of the three software packages used by DRS. DRS produced this report is less than two minutes.



			Agilent	AMDIS		NIST	
R.T.	Cas #	Compound Name	ChemStation Amount (ng)	Match	R.T. Diff sec.	Reverse Match	Hit Num.
2.969	90437	o-Phenylphenol		94	3.1	89	1
6.144	84742	Di-n-butylphthalate	0.64				
6.433	2921882	Chlorpyrifos		67	1.0	61	1
7.210	470906	Chlorfenvinphos	0.15				
7.257	2595542	Mecarbam		72	0.5	72	1
7.441	950378	Methidathion	3.75	86	0.5	87	1
8.140	41394052	Metamitron		59	-0.7	63	12
9.548	18181801	Bromopropylate	0.24	75	0.1	89	1
9.892	117817	Bis(2-ethylhexyl)phthalate		95	0.2	86	3

Figure 5. DRS Report for lemon oil.

Backflushing

Citrus oils contain significant amounts of material that elute after the last pesticide. This requires a 150-min hold at 320 °C to elute all of the heavy material with a 1× method. The total run time for the 1× method is therefore 195 min, as shown in Table 3.

Table 3. Method Run Time Comparison

Column	30 m	15 m	10 m
Speed	1×	3×	4.8×
Run time	min	min	min
Pesticides	42	14	8.75
No backflush matrix	195	65	40.6
With backflush matrix	n/a	20	12.5

The 3× method requires a 50-min hold at 320 °C, as shown at the top in Figure 6, resulting in a 65-min run time. With backflushing, all of this heavy material is removed in 5 min at 300 °C, as shown in the bottom of Figure 6. This is a 9-fold reduction in analysis time compared to the 1× method.

4.8x Method

Using the 220V oven, SP1 2310-0236, and oven insert accessory, the method can be scaled to 4.8× faster, as shown in Table 3. There is a practical limit to the amount of matrix that can be tolerated with the reduced resolution using a 10-m column. However, for matrices less complex than a citrus oil, the 4.8× method can be successfully used to save even more time. The Performance Electronics allows running at faster scan speeds while maintaining signal/noise ratio. Sufficient points across a peak are maintained even with faster chromatography.

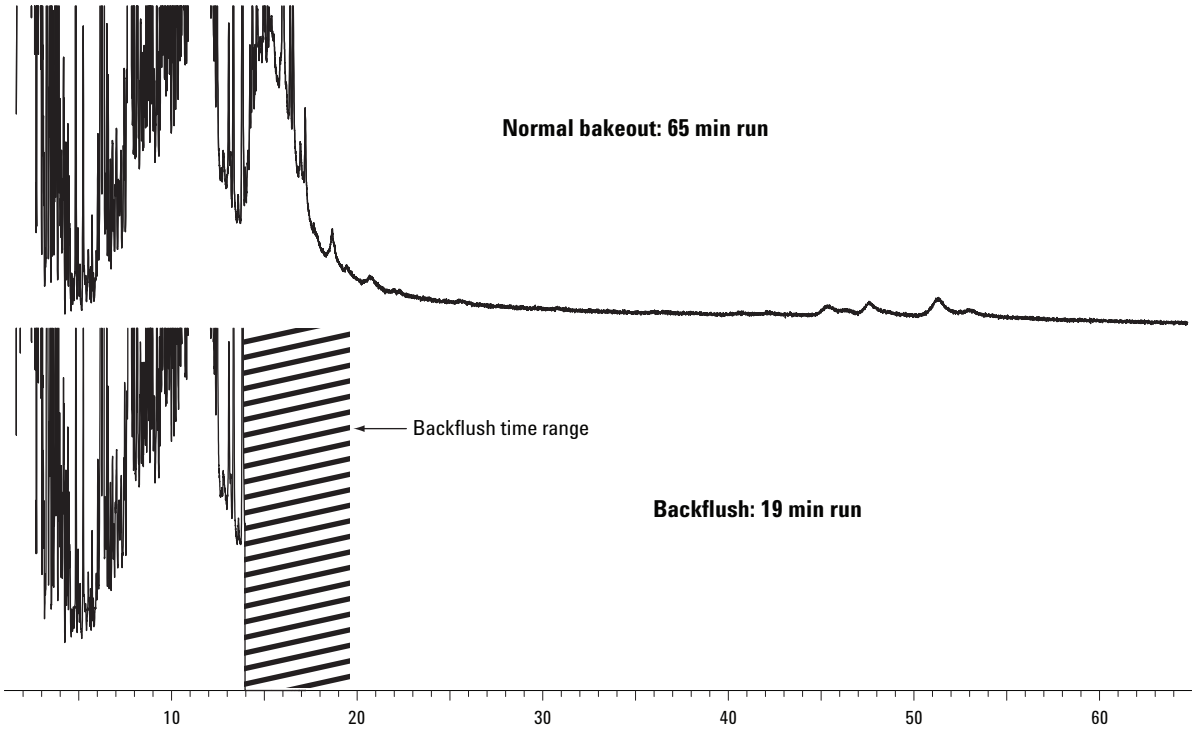


Figure 6. Top - 3× lemon oil analysis with 50 min bakeout at 320 °C. Bottom - 3× lemon oil analysis with 5 min backflush at 300 °C.

Conclusions

New tools are available for the analysis of pesticides in complex matrices. These tools can be combined to significantly reduce analysis and data processing time.

- Fast oven - programming rates needed for faster runs
- Shorter column - 3× faster runs with sufficient resolution
- Microfluidic splitter - confidence in results using selective detection simultaneous with MS data
- Backflush - additional 3× reduction in run time with lower column temperatures for extended lifetime
- Performance Electronics - maintain signal/noise at faster sampling rates
- DRS - three levels of target analyte identification in less than two minutes

Using the above tools, the run time for analysis of lemon oil was reduced from 195 minutes to 20 minutes (nine-fold). DRS reduced the data analysis from hours to minutes.

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Identification and Isolation of DSP-Toxins Using a Combined LC/MS-System for Analytical and Semipreparative Work

Application

Food Safety

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Abstract

The configuration and operation of a combined liquid chromatography/mass spectrometry (LC/MS) system to identify and isolate DSP-toxins is described. In the analytical mode, okadaic acid (OA) and dinophysistoxin-1 (DTX-1) are more selectively and sensitively monitored when compared to LC with fluorescence detection. With less sample preparation, the detection limits are decreased by a factor of 3–5, depending on the matrix. In semipreparative mode, OA and DTX-1 could be isolated from crude extracts of *Prorocentrum lima* algae using mass-based fraction collection with a purity >98%. Due to this method, reference standards of DSP toxins are now commercially available.

Introduction

Diarrheic shellfish poisoning (DSP) is a gastrointestinal syndrome that occurs in humans after the consumption of bivalve mollusks such as scallops, mussels, clams and oysters. The symptoms include abdominal pain, vomiting, nausea, headache, diarrhea, chills, and fever. DSP toxins can be classified in three groups: the okadaic acid (OA) group involving OA and the dinophysistoxins (DTXs), the pectenotoxin group (PTXs) and the yessotoxin group (YTXs).

Inside the OA group, OA and DTX-1 are the main toxins responsible for DSP outbreaks. The outbreaks led to the establishment of control programs for marine biotoxins in many countries. In Germany residues of DSP toxins in mussels are controlled at present under the regulation of the Fischhygiene-Verordnung of 8th June 2000. This Order requires the testing of shellfish for the presence of toxins by means of animal tests (mouse bioassays) or by chemical analytical procedures [1, 2]. Liquid chromatography with fluorescence detection is an established technique, but it requires the derivatization of the not naturally fluorescent DSP toxins. Using LC/MS coupled with electrospray ionization (ESI) more sensitive and selective results are attainable with less sample preparation.

The greatest problem regarding the analytical methods for monitoring DSP toxins is the availability of pure reference material. The DSP toxins OA and DTX-1 can be isolated from crude extracts of *Prorocentrum lima* algae (see Figure 1) using mass-based fraction collection in semipreparative mode. The present work describes the configuration, setup, and operation of a combined LC/MS system for analytical and semipreparative work.

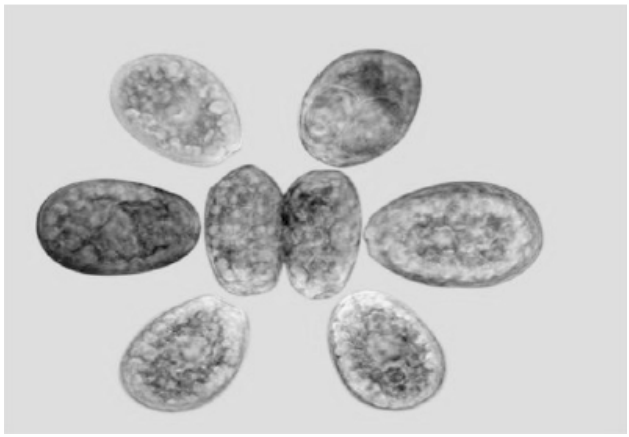
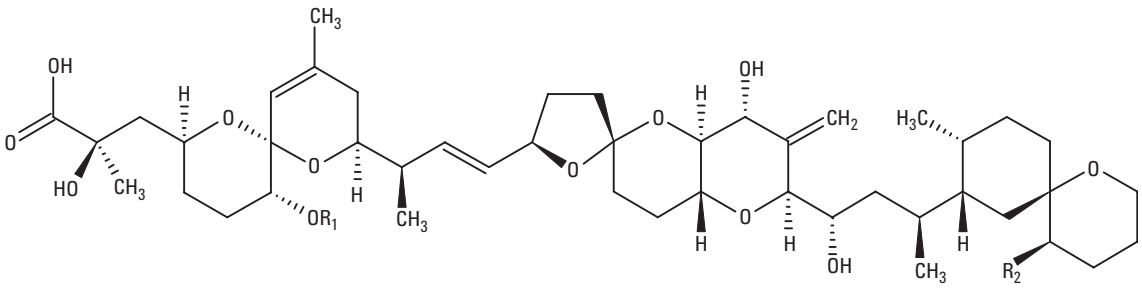


Figure 1. ***Prorocentrum lima*** **algae under the microscope.**

Experimental

The DSP toxins shown in Figure 2 were analyzed in this work. The analyses were conducted in two modes: Analytical and Semipreparative.



OA: $R_1 = H, R_2 = H$ $C_{44}H_{68}O_{13}$
DTX-1: $R_1 = H, R_2 = CH_3$ $C_{45}H_{70}O_{13}$

Chemical and physical properties: Polyether structure, Carboxylic acid
Lipophilic, and no chromophore

Figure 2. DSP toxins.

LC/MS Method Details - Analytical

LC Conditions

Instrument:	Agilent 1100 HPLC (Quaternary pump)
Column:	150 × 3.0 mm ZORBAX SB-C18, 5 µm
Mobile phase:	A Water (0.1% Formic acid) B Methanol
Gradient:	20% B at 0 min 20% B at 5 min 80% B at 20 min
Stop time:	28 min;
Post time:	4 min
Flow rate:	0.6 mL/min
Injection vol:	10 µL

MS Conditions

Instrument:	Agilent LC/MSD
Source:	Positive/Negative switching ESI
Drying gas flow rate	12 L/min
Nebulizer:	60 psig
Drying gas temp:	350 °C
V_{cap}:	3000 V (positive and negative)

LC/MS Method Details - Semipreparative

LC Conditions

Instrument 1:	Agilent 1100 HPLC (Quaternary pump)
Column:	50 × 9.4 mm ZORBAX SB-C18, 5 µm
Mobile phase:	A Water (0.1% Formic acid) B Methanol
Gradient:	20% B at 0 min 20% B at 5 min 80% B at 20 min
Stop time:	28 min
Post time:	4 min
Flow rate:	7.0 mL/min
Injection vol:	100 µL (250 µL using Multiple Draw Mode)
Instrument 2:	Agilent 1100 HPLC (Isocratic pump) for makeup flow
Flow rate:	0.8 mL/min (50% H_2O + 50% MeOH + 0.1% Formic acid)
Active splitter:	Split ratio 271:1

MS Conditions

Instrument:	Agilent LC/MSD
Source:	Negative ESI
Drying gas flow:	12 L/min
Nebulizer:	60 psig
Drying gas temp:	350 °C
V_{cap}:	3000 V (positive)
MSD Fraction Collection Setup	
FC Mode:	Use method target mass; Adducts: $(M–H)^-$

Results and Discussion

Analytical Work

In the analytical mode of the LC/MS system (Figure 3) the DSP toxins were monitored using ESI with positive/negative mode switching. The positive ion mode is four times more sensitive than the negative ion mode (Figure 4). Mass spectra for OA and DTX-1 show a sodiated molecular ion instead of a protonated molecular ion, and characteristic fragment ions $[M+H – nH_2O]^+$, where n = 1–4, formed by a sequential loss of water. In negative ion mode only the $[M–H]^-$ ion is detected. LC/MS provided a more selective and sensitive method for monitoring DSP toxins in comparison to LC with fluorescence detection (Figure 5), by a factor of 3–5.

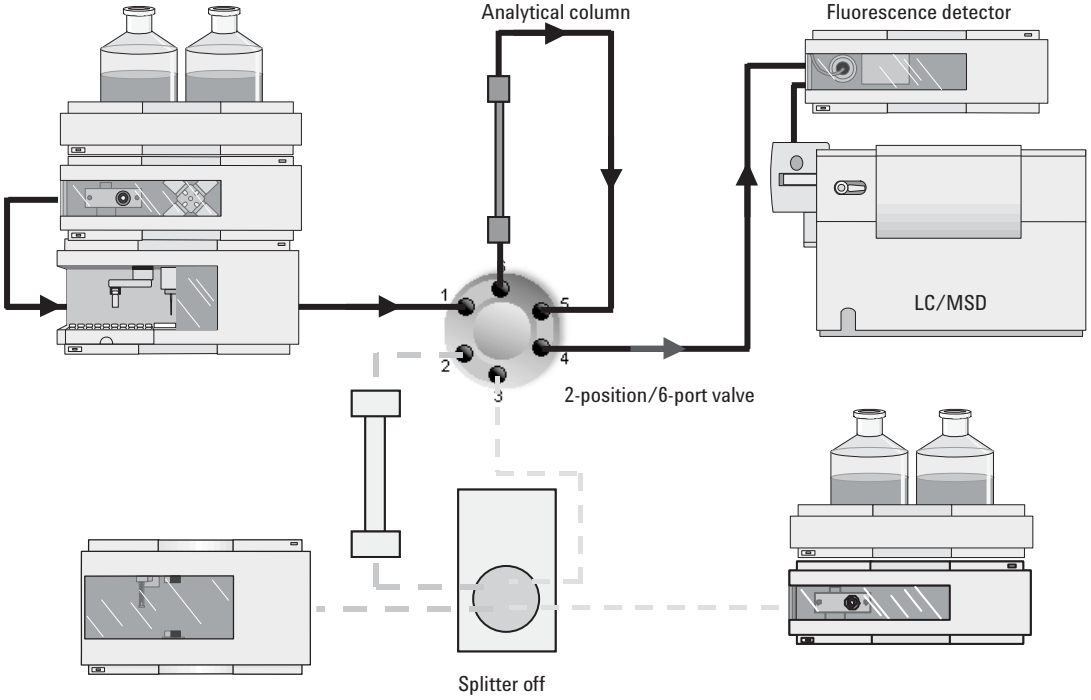


Figure 3. System diagram (analytical work).

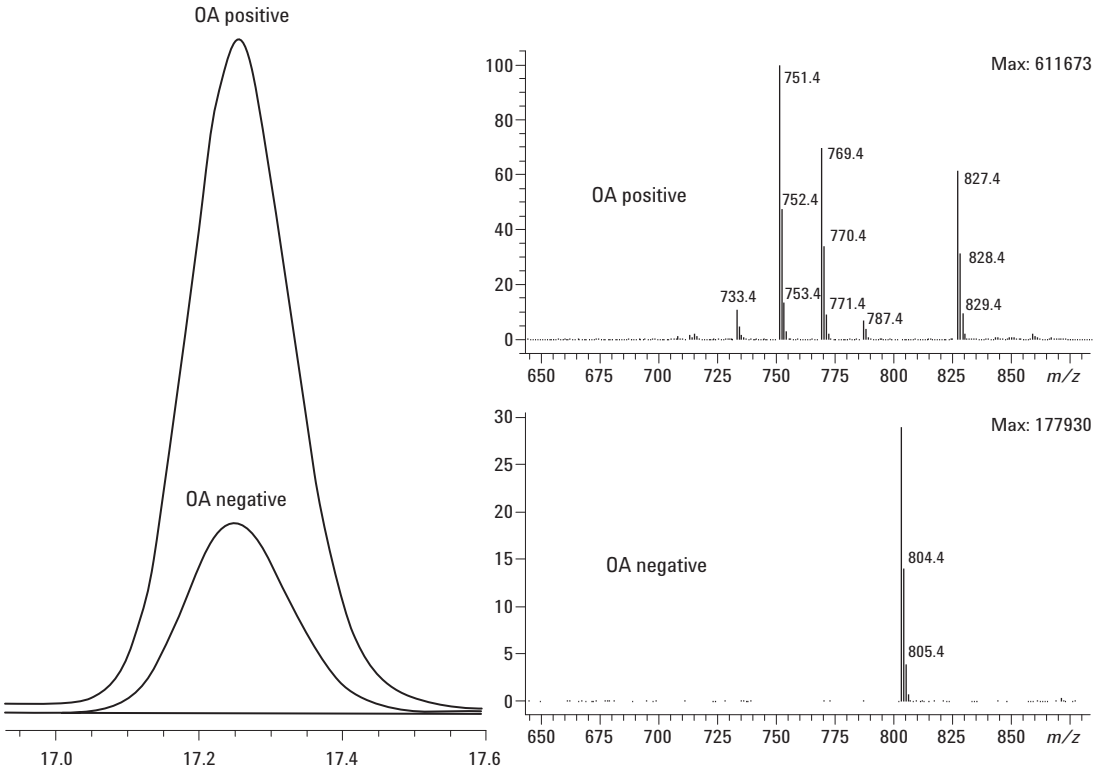


Figure 4. LC/MS analysis of OA.

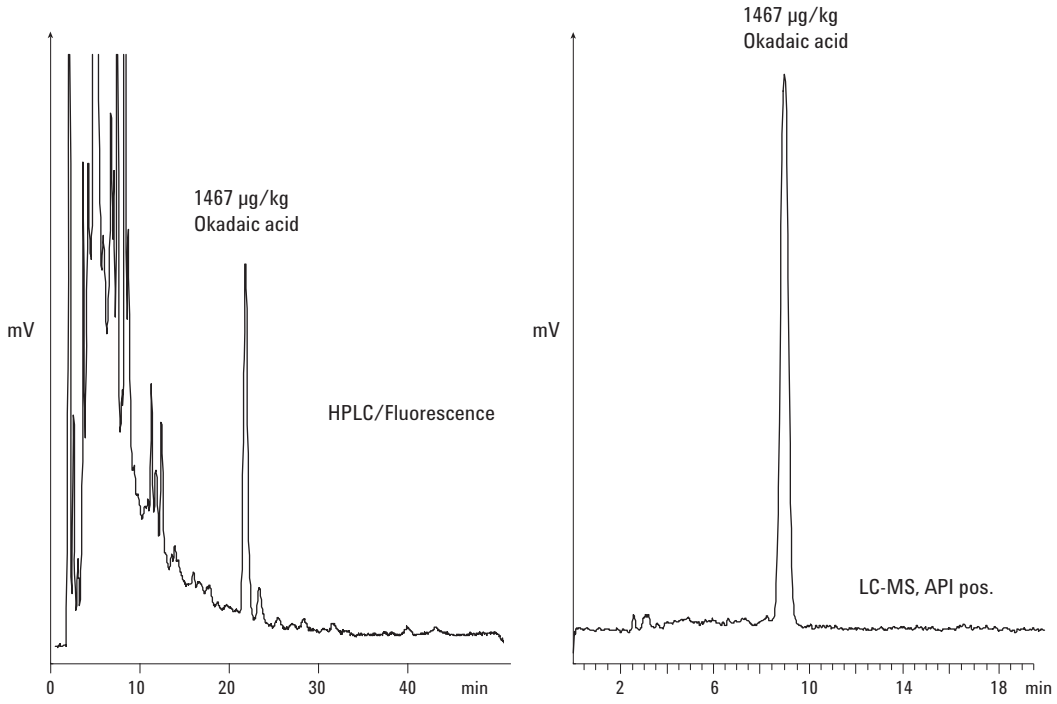


Figure 5. Comparative analysis of DSP toxins in shellfish.

Semipreparative Work

The reference standards could be obtained by switching the system to semipreparative mode (Figure 6). The valve is switched to position 2.

The main flow now goes to the semipreparative column and then through the splitter to fraction collector (AS). The make-up flow goes through the splitter where it picks up some of the compound from the main flow and goes to the MS-detector (MSD).

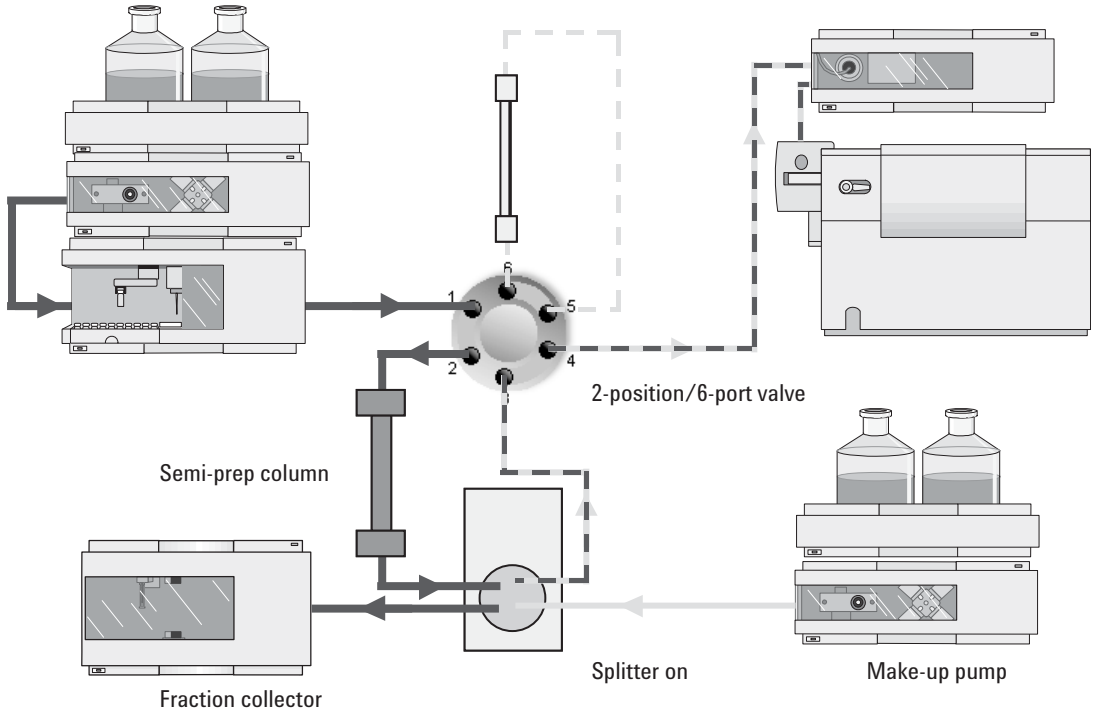


Figure 6. System diagram (semipreparative work).

Besides OA and DTX-1, a new OA-toxin with similar mass spectral properties could be isolated from crude extracts of *Prorocentrum lima* algae using mass-based fraction collection (Figure 7). The mass-based fraction collection of a methanolic extract of *Prorocentrum lima* algae results in three fractions: OA, DTX-1 and an unknown toxin. From MS^n experiments it can be determined that the molecular structure of the unknown toxin must be very similar to those of OA and DTX-1.

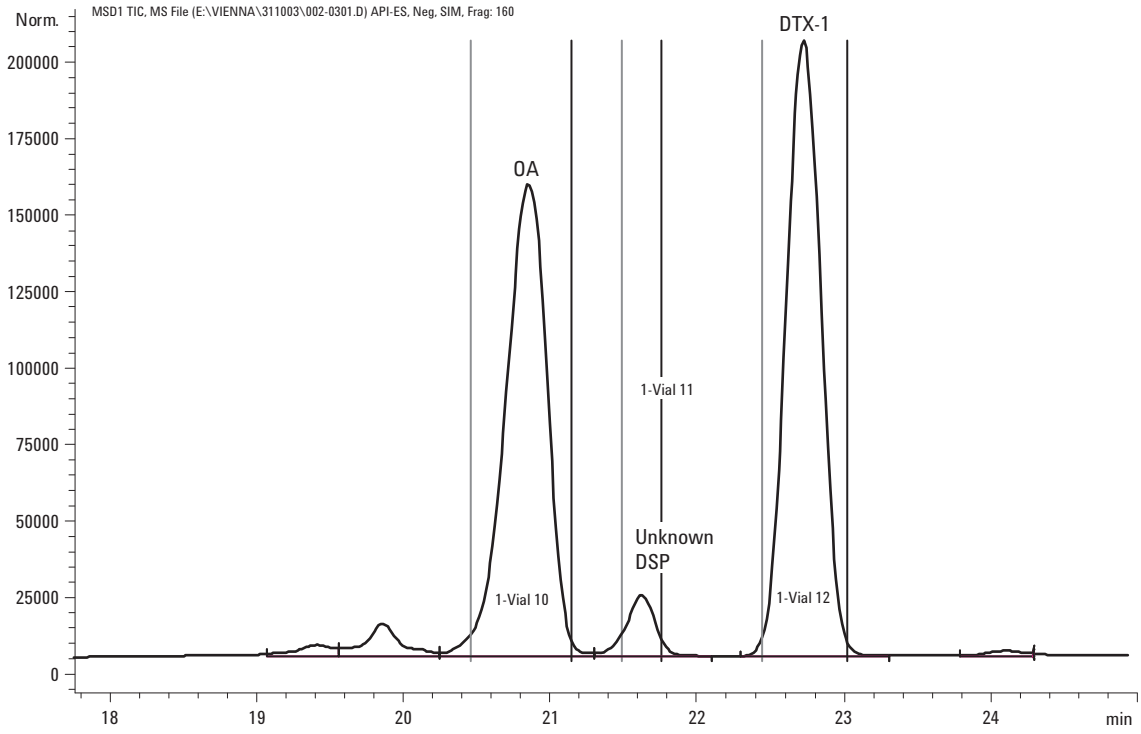


Figure 7. Mass-based fraction collection of DSP toxins.

Because of the low concentration, the target compounds had to be collected from multiple injections of the same sample, a process usually referred to as pooling (Figure 8). Reanalysis of the collected fractions gave results for purity >98%. This method is robust (Figure 9) and has now resulted in making reference standards of DSP toxins commercially available.

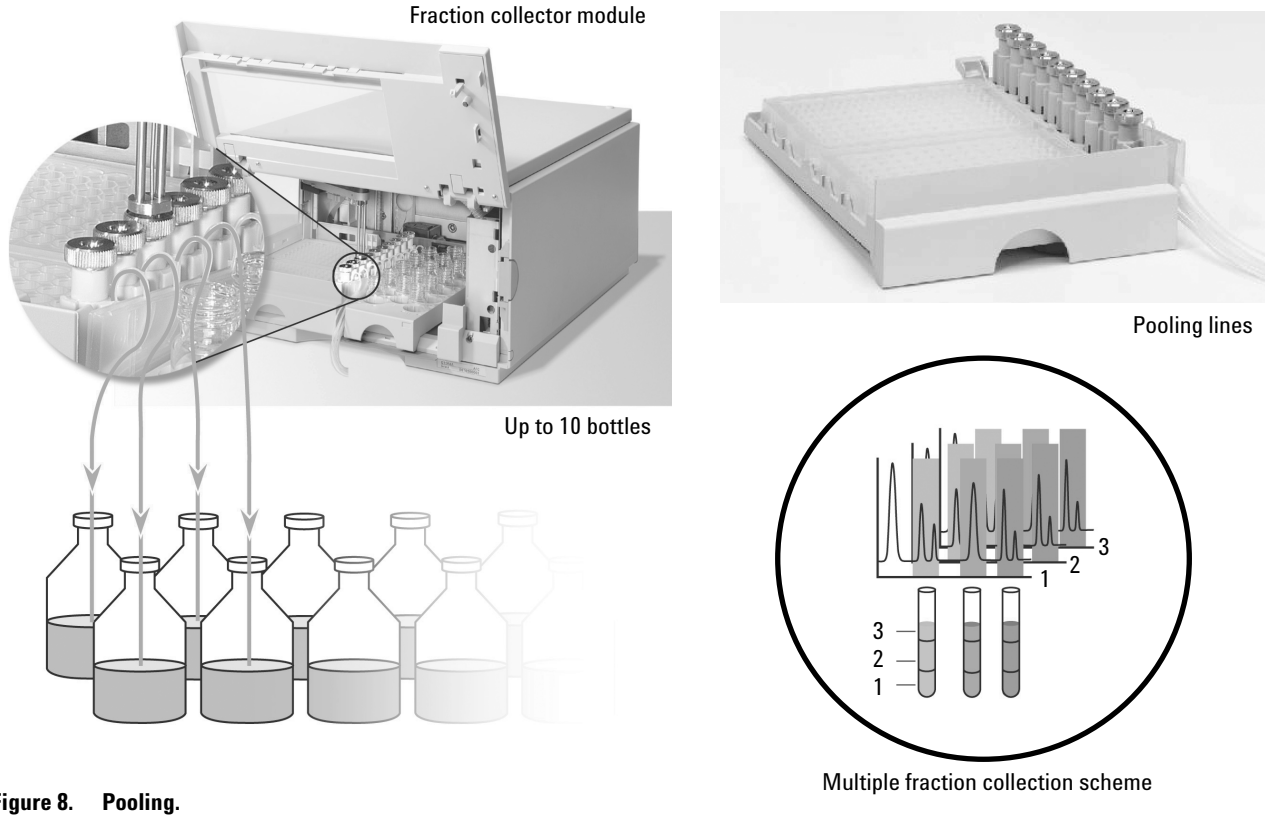


Figure 8. Pooling.

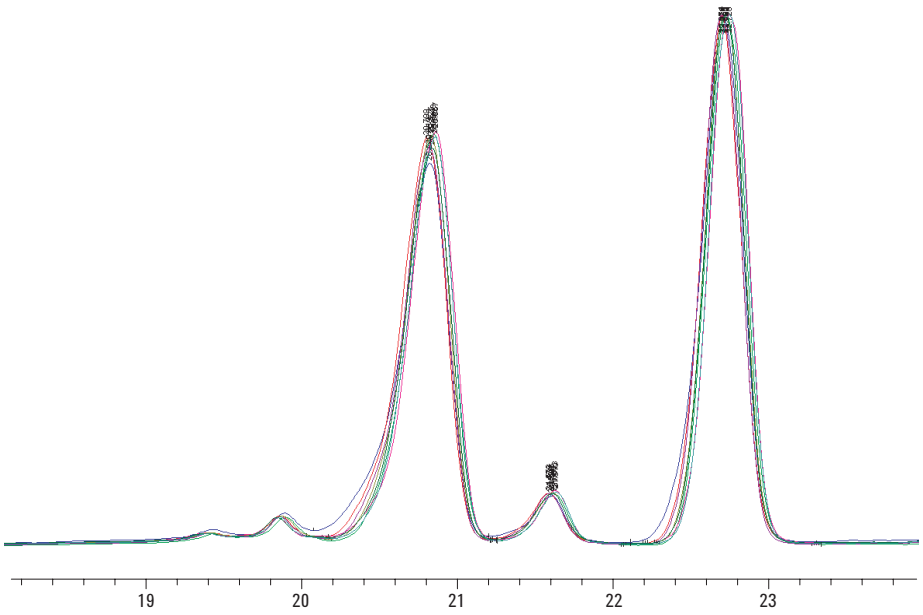


Figure 9. Robustness of the method - overlay of 10 mass-based fraction collection runs.

Conclusions

Configuration and operation of a combined LC/MS system to identify and isolate DSP toxins is described. In the analytical mode, OA and DTX-1 were monitored more selectively and sensitively than by using LC with fluorescence detection. With less sample preparation, the detection limits could be decreased by a factor of 3–5, depending on the matrix. In the semipreparative mode OA and DTX-1 could be isolated from crude extracts of *Prorocentrum lima* algae using mass-based fraction collection with a purity >98%. Due to this method reference standards of DSP toxins are now commercially available.

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Analysis of Terbuthylazine in Olive Oil by LC/Ion Trap MS and LC/Time-of-Flight MS

Application

Food safety

Authors

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Abstract

This application note describes the use of liquid chromatography/ion trap mass spectrometry (LC/ITMS) and liquid chromatography/time-of-flight mass spectrometry (LC/TOFMS) for the identification and quantitation of terbuthylazine in olive oil samples. The method includes a sample treatment step based on a preliminary liquid-liquid extraction, followed by matrix solid-phase dispersion (MSPD) using an aminopropyl-bonded silica as a sorbent material. A final clean-up step is performed with Florisil using acetonitrile as an eluting solvent. The analysis by ion trap was achieved in MS/MS mode, monitoring the characteristic fragment ion at *m/z* 174. The identification by LC/TOFMS was accomplished with the accurate mass (and the subsequently generated empirical formula) of the protonated molecule ($[M+H]^+$ *m/z* 230), along with the accurate mass of the fragment ion and the characteristic chlorine isotope cluster present in terbuthylazine. The accuracy typically obtained was routinely better than 2 ppm. The method sensitivity, linearity, precision, accuracy, matrix effects, and limit of detection were also studied; they supported the potential of LC/TOFMS and LC/ITMS for the routine quantitative analyses of terbuthylazine in olive oil.

Introduction

Olive oil is one of the most-used food products in Mediterranean countries. The positive effects of olive oil on health have prompted a demand for this product world-wide. “Virgin” olive oil is obtained from the fruit of the olive tree (*Olea Europaea*) exclusively by mechanical and physical processes without any further treatment, which may alter the olive oil quality. The most extensively applied agrochemicals in olive plantations of Mediterranean countries (Greece, Spain, and Italy) are herbicides and insecticides. Although herbicides are mainly applied to soils, some residues can persist to the harvest stage, thus contaminating the olives picked up from soil. This can result in the presence of trace amounts of these pesticides in olive oil. Consequently, both the European Union and the Codex Alimentarius Commission of the Food and Agriculture Organization (FAO) of the United Nations have established maximum pesticide residue levels in olives and olive oil. Currently, various olive oil pesticide residue regulatory programs are being carried out to update and to establish new and more stringent regulations concerning the maximum residue levels in these commodities [1]. This fact has fostered the development of more powerful analytical tools in order to provide enough sensitivity and selectivity to meet these requirements in food samples such as edible oils, which have a complex matrix due to the high fat content of the extracts obtained after the sample treatment step.

Many multiresidue procedures employing different clean-up techniques and a variety of detection methods are reported on the determination of pesticide residues in olive oil. The most commonly used methodology is based on GC after a comprehensive clean-up step, in most cases based on liquid-liquid partitioning or gel permeation chromatography to separate the low molecular mass pesticides from the higher molecular mass fat constituents of the oil, such as triglycerides [2, 3]. The preparation of oil samples for the determination of pesticides by GC requires the complete removal of the high-molecular-mass fat from the sample to maintain the chromatographic system in working order. Recently, a multiresidue method for the determination of triazines and organophosphorous pesticides using MSPD followed by GC/MS and ion trap MS techniques was reported [4]. In this work, we further explore the capabilities of LC/ITMS and LC/TOFMS techniques for the identification of terbuthylazine, one of the most common pesticides found in olive oil.

Experimental Methods

Olive Oil Extraction

MSPD was used for the extraction of terbuthylazine from olive oil after a preliminary liquid-liquid extraction with organic solvents.

1. The liquid-liquid extraction:
   - An aliquot of approximately 5 g (ca. 5.5 mL) of olive oil sample was mixed with 15 mL of petroleum ether, saturated with acetonitrile, in a 100-mL separatory funnel, in which a two-step liquid-liquid extraction was performed.
   - A solution of 25 mL of acetonitrile, saturated with petroleum ether, was added to the olive oil mix obtained previously. The funnel was shaken vigorously for 3 minutes, and the acetonitrile phase was separated from the petroleum ether phase.
   - Another 10 mL of acetonitrile saturated with petroleum ether was added to the petroleum ether extract. The mixture was shaken again for 3 minutes and the acetonitrile phase was collected and added to the previous one.
   - Finally, a 7-mL aliquot of the acetonitrile extract was transferred to a 10-mL glass test tube. The extract was then carefully evaporated down to a final volume of about 2 mL. This remaining extract was transferred to a glass mortar to be subjected to matrix solid-phase dispersion.
2. The MSPD:
   - The extract obtained in the liquid-liquid extraction step was homogenized with 2 g of aminopropyl-bonded sorbent (Bondesil-NH_2, 40-m particle size, Varian Inc.) until a fine powder was obtained.
   - The mixture was transferred to a commercially available minicolumn containing 2 g of Florisil (12-mL Bond-Elut Varian minicolumn, Varian Inc.). This minicolumn was connected to a vacuum system for solid phase extraction adjusting the flow to 3 mL/min.
   - An elution step was carried out with 2×5 mL of acetonitrile. The final extract was evaporated until near dryness, then dissolved with 1:1 acetonitrile:water.
   - Prior to LC/ITMS and LC/TOFMS analysis, the extract was filtered through a 0.45-m PTFE filter (Millex FG, Millipore, Milford, MA, USA).

Using the MSPD method, recoveries for terbuthylazine from olive oil samples were higher than 96% with a 6% relative standard deviation (RSD) (n = 5).

Agilent 1100 Series LC/MSD TOF Methods

LC conditions

LC Pumps were Agilent 1100 binary pumps

Injection volume:	50 µL with standard Agilent 1100 ALS
Column:	ZORBAX Eclipse XDB-C8, 4.6 mm × 150 mm, 5 µm, p/n 993967-906
Mobile phases:	A = acetonitrile and B = 0.1% formic acid in water
Gradient:	5 minutes isocratic at 10% A, followed by a linear gradient to 100% A in 25 minutes at a flow rate of 0.6 mL/min

MS conditions

LC/MSD TOF

Source:	Positive ESI (electrospray ionization)
Capillary:	4000 V
Nebulizer:	40 psig
Drying gas:	9 L/min
Gas temp:	300 °C
Fragmentor:	190 V
Skimmer:	60 V
Oct DC1:	37.5 V
Oct RF V:	250 V
Reference masses:	*m/z* 121.0509 and 922.0098
Resolution:	9500 ± 500 @ *m/z* 922.0098
Mass range:	50–1000 *m/z*
Reference A sprayer 2:	Constant flow during the run

Agilent 1100 Series LC/MSD Trap Methods

LC/MSD Trap

Methods identical to LC/MSD TOF for direct comparison of peaks.

LC Pumps:	HP 1100
Inject vol:	50 µL
Column:	ZORBAX Eclipse XDB-C-8, 4.6 mm × 150 mm, 5 µm, p/n 993967-906
Mobile phases:	A = acetonitrile (ACN) B = 0.1% formic acid in water
Gradient:	10% A, isocratic, for 5 minutes followed by linear gradient to 100% A in 25 min at a flow rate of 0.6 mL/min

LC/MSD Trap

Positive ESI:	Capillary 3200 V
Nebulizer:	40 psig
Drying gas:	9 L/min, gas temp 300 °C
Capillary exit:	70 V
Skimmer:	60 V
Trap accumulation:	50,000 counts with maximum accumulation time of 200 ms

Results and Discussion

Identification of Terbuthylazine by LC/ITMS and LC/TOFMS

Olive oil is one of the most difficult food matrices due to the presence of numerous interferences that show up in full-scan mode. For this reason, the ion trap method was optimized in MS/MS mode by isolating the precursor ion at *m/z* 230, which corresponds to $[M+H]^+$. An isolation mass window of *m/z* 2 and optimized fragmentation amplitude was used in order to enhance the signal-to-noise (S/N) ratio. An amplitude voltage of 0.8 V was used to obtain good fragmentation for terbuthylazine, which fragments by losing the terbutyl group (–56) forming the *m/z* 174 fragment ion.

Figure 1 shows the analysis of an olive oil sample (spiking level 0.025 mg/kg) by ion trap MS/MS. The extracted ion chromatogram (EIC) for *m/z* 174, the main fragment of terbuthylazine, as well as its mass spectrum is shown. The fragmentation occurs at the C–N bond via the cleavage of the terbutyl group. This represents a characteristic fragmentation for this analyte, allowing for the structural confirmation of terbuthylazine in a relative complex matrix such as olive oil.

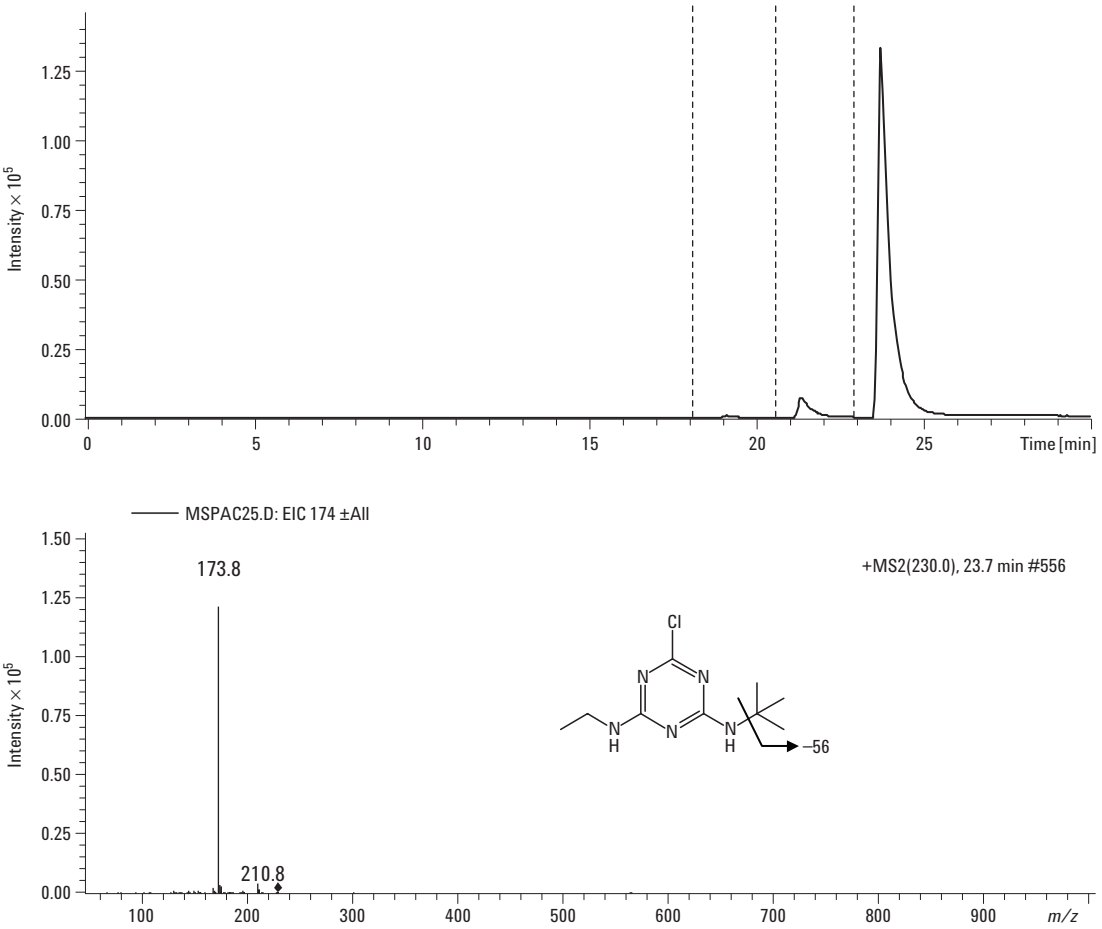


Figure 1. Ion Trap MS/MS chromatogram corresponding to the analysis of a spiked olive oil sample with terbuthylazine (0.025 mg/kg). The EIC for *m/z* 174 and its corresponding spectrum are shown.

LC/TOFMS analyses were optimized in terms of fragmentation. The in-source collisionally induced dissociation (CID) fragmentation is greatly enhanced at high fragmentor voltages. This provides highly valuable structural information since the accurate mass of the characteristic fragment ion can be used along with that of the molecular ion for confirmation purposes. The relative abundances for both the main fragment and the protonated molecule of terbuthylazine are summarized in Table 1 at three different voltages: 160 V (low), 190 V (medium), and 230 V (high). In order to obtain sufficient sensitivity for quantitative purposes (using the protonated molecule) and additional qualitative spectral information provided by the fragment ions generated by in-source fragmentation, a value of 190 V was chosen for further analyses. The accurate masses for both the main fragment and the protonated molecule of terbuthylazine in a spiked olive oil matrix are shown in Table 2.

Table 1. Effect of the Fragmentor Voltage on CID Fragmentation for LC/TOFMS

***m/z* ion**	**Relative abundance**		
	160 V	**190 V**	**230 V**
230 $[M+H]^+$	100	100	20
174 $[M+H–C_4H_8]^+$	5	30	100

Table 2. LC/TOFMS Accurate Mass Measurements for Terbuthylazine and its Main Fragment Ion in Olive Oil Matrix-Matched Standard (Fragmentor Voltage 190 V). Spiking Level: 0.025 mg/Kg

Elemental composition	Ion	Theoretical mass	Measured mass	Error mDa	Error ppm
$C_9H_{16}N_5{}^{35}Cl$	$[M+H]^+$	230.1167	230.1168	0.1	0.4
$C_9H_{16}N_5{}^{37}Cl$		232.1137	232.1134	–0.3	–1.5
$C_5H_9N_5{}^{35}Cl$	$[M+H–C_4H_8]^+$	174.0541	174.0542	0.1	0.6
$C_5H_9N_5{}^{37}Cl$		176.0511	176.0511	–0.05	–0.3

Along with the accurate mass of the protonated molecule and the information provided by the fragments obtained with an optimized in-source fragmentation, terbuthylazine presents another feature which enables its identification; the presence of a chlorine atom. The accurate mass pattern of the ^{37}Cl isotope signal confirms that the peak unequivocally contains only one chlorine atom (Figure 2). Therefore, the accurate mass of each ion as well as the presence of the chlorine signal, together with the characteristic retention time represent sufficient information to unequivocally identify and confirm this specie in such complex matrices.

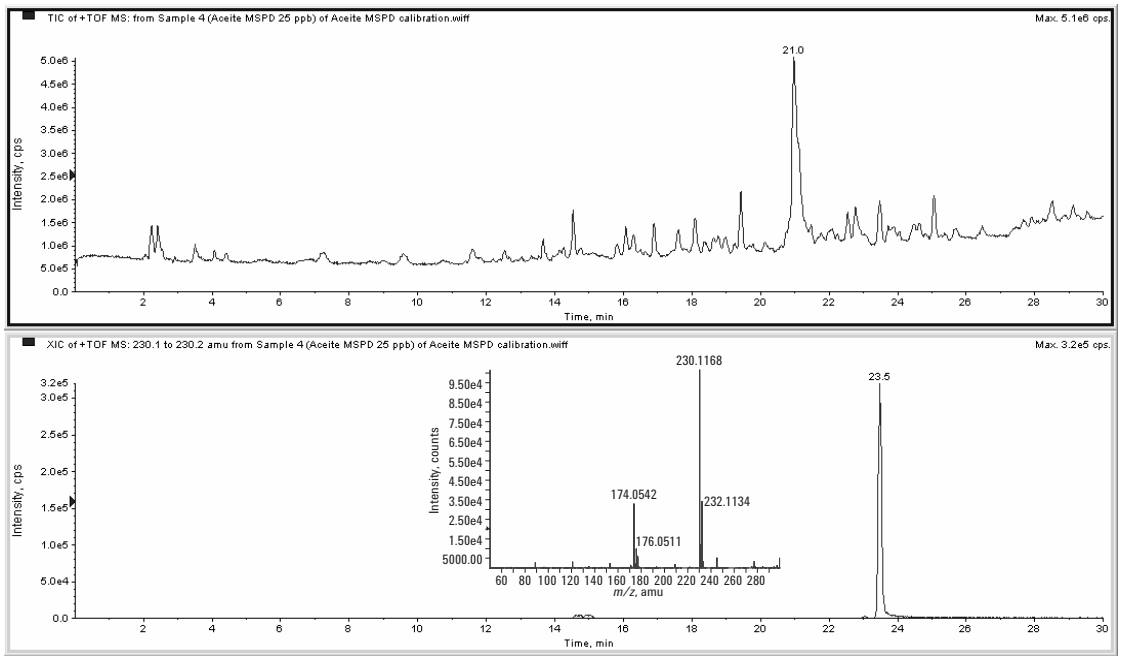


Figure 2. LC/TOFMS total ion chromatogram (TIC) corresponding to the analysis of a spiked olive oil sample with terbuthylazine (0.025 mg/kg). The EIC for *m/z* 230 and its corresponding spectrum are also shown.

Analytical Performance

The analytical performance of the proposed methods was studied in order to explore the ability to carry out quantitative analyses of terbuthylazine in these complex matrices with a high content of fat. The calibration was carried out using matrix-matched standards prepared by the extraction method based on MSPD described in the experimental section. Linearity was evaluated by analyzing solutions of matrix-matched standards at seven different concentration levels in the range 0.005–0.5 mg/kg. Using ion trap the quantitation was performed in MS/MS mode of the *m/z* 230 ion. Using LC/TOFMS the quantitation was carried out using the peak area from the EIC of the protonated molecule with a mass window of 0.1 Da. As an example, Figure 3 shows the linear calibration curve obtained by LC/TOFMS for terbuthylazine in an olive oil matrix compared to the curve obtained in pure solvent.

The limits of detection (LOD) were estimated from the injection of matrix-matched standard solutions with concentration levels giving a S/N ratio of about 3. The results obtained are shown in Table 3. The LOD obtained are remarkable since they are far below the maximum residue level regulations established for these pesticides. In this sense, LC/TOFMS analyses benefits of the use of narrow mass windows for quantitation purposes, which results in enhanced S/N ratio, thus providing lower detection limits. This fact illustrates the analytical potential of the proposed method based on MSPD and LC/TOFMS for the analyses of pesticides in complex matrices with high content of fat.

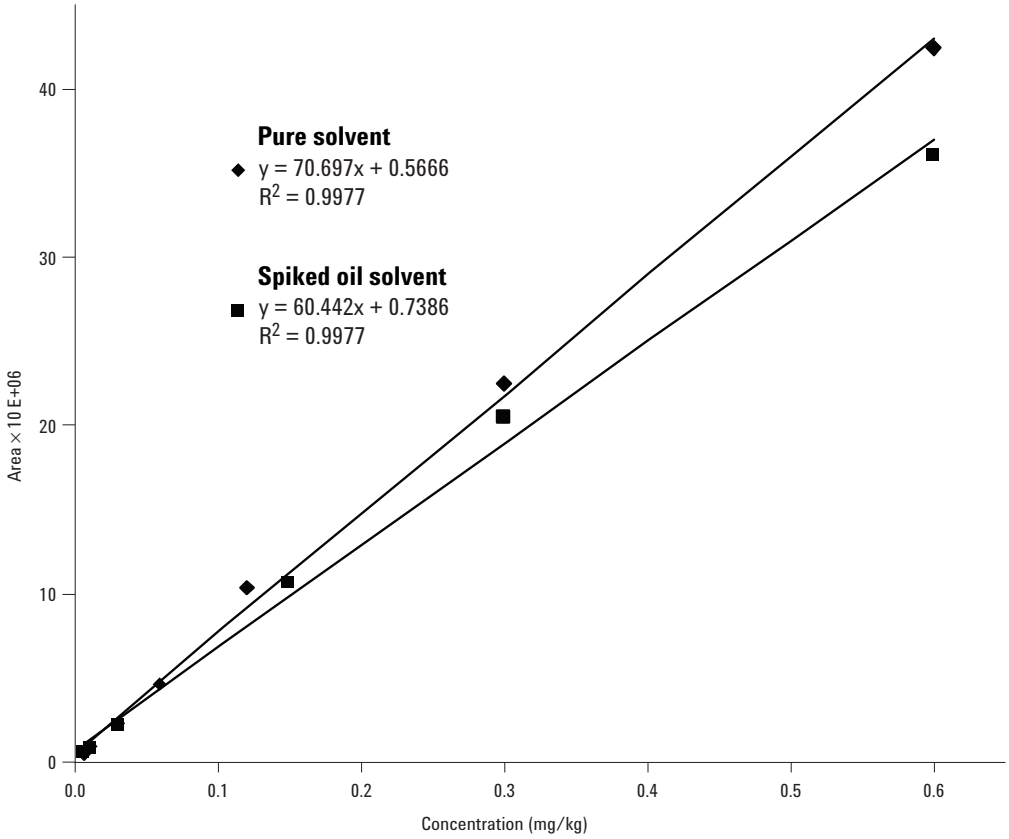


Figure 3. Calibration plot obtained from spiked olive oil samples versus solvent based samples by LC/TOFMS.

Table 3. Analytical Parameters for the Analysis of Terbuthylazine in Olive Oil Samples by Ion trap MS/MS and LC/TOFMS

Method	Concentration range (mg/kg)	Linearity (R^2)	LOD (µg/kg)	RSD (%) n = 5
LC/ITMS	0.005–0.5	0.991	0.2	5.5
LC/TOFMS	0.005–0.5	0.995	1	2

Analysis of Olive Oil Samples

To evaluate the effectiveness of the proposed method, it was applied to the analysis of olive oil samples. An example is shown in Figure 4 where traces of terbuthylazine were found in an olive oil extract; the EIC for *m/z* 230 is also shown. This is a real example illustrating the usefulness of the routine accurate mass measurement capabilities of LC/TOFMS, especially when analyzing traces of pesticides in complex samples such as olive oil. In this sense, the selectivity of LC/TOFMS relies on the resolving power of the instrument on the *m/z* axis, enabling discrimination between the target species and an "isobaric" interference within 0.05 Da of the mass difference (using 230 *m/z*, as example). In the case of terbuthylazine, it is easily overcome using the isotopic chlorine signal or a characteristic fragment not affected by other interfering species.

Conclusion

LC/ITMS and LC/TOFMS can be used for the identification and quantitation of terbuthylazine in olive oil samples after performing several preliminary sample treatments. The analysis by ion trap was achieved in MS/MS mode, monitoring the characteristic fragment ion at *m/z* 174. The identification by LC/TOFMS was accomplished with the accurate mass (and the subsequently generated empirical formula) of the protonated molecule ($[M+H]^+$ *m/z* 230), along with the accurate mass of the fragment ion and the characteristic chlorine isotope cluster present in terbuthylazine. The accuracy typically obtained was routinely better than 2 ppm. The method sensitivity, linearity, precision, accuracy, matrix effects, and LOD were also studied and they supported the potential of LC/TOFMS and LC/ITMS for the routine quantitative analyses of terbuthylazine in olive oil.

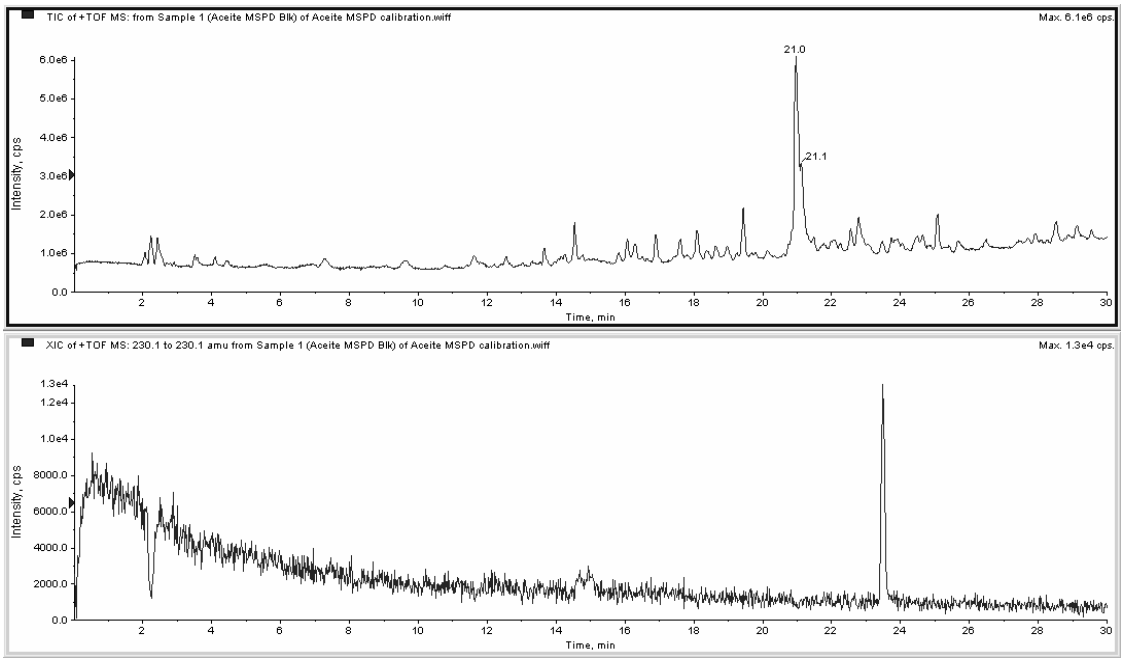


Figure 4. Upper: LC/TOFMS TIC of a "positive" for terbuthylazine in an olive oil sample. Lower: EIC of terbuthylazine using a 20 mDa mass window.

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Separation of Aflatoxins by HPLC

Application

Environmental, Food Safety

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Abstract

Four target aflatoxins (B_1, B_2, G_1, and G_2) were separated by HPLC using an isocratic ternary mixture of water, methanol and acetonitrile, and detected using UV 365 nm. A baseline separation was achieved in less than 5.5 min.

Introduction

Aflatoxins are mycotoxins that are produced by various *Aspergillus flavus* molds. Not only are these compounds extremely toxic, but they are also mutagenic, teratogenic (causing fetal abnormalities), and carcinogenic. Unfortunately, *A. flavus* is a common mold found in tropical and subtropical countries and has been found to cause aflatoxin contamination. This contamination is a result of inadequate storage conditions for certain agricultural commodities such as peanuts, cereal seeds, dried fruit, and a wide range of tree nuts such as pistachio, pecans, walnuts, almonds, and herbal seeds such as red and black pepper, cloves, and cinnamon. Because of their toxic and carcinogenic nature, there is a very low minimum detectable quantity (MDQ) for aflatoxin contamination in food.

Chemical Nature

Although 18 different aflatoxins have been identified, the four most prevalent aflatoxins are B_1, B_2, G_1, and G_2, whose chemical structures are shown in Figure 1. Aflatoxin B_1 is one of the most potent and abundant environmental mutagens and carcinogens known. Aflatoxins are quite stable in many foods and are fairly resistant to degradation. Collectively, the aflatoxins are chemical derivatives of difurancoumarin. Pure aflatoxin B_1 is a pale-white to yellow crystalline, odorless solid. Aflatoxins are freely soluble in moderately polar solvents such as chloroform, methanol, and dimethyl sulfoxide, and dissolve in water to the extent of 10–20 mg/L. In methanol, they have fairly strong extinction coefficients (around 10,000) at 265 nm and 360–362 nm. They fluoresce under UV radiation, and fluorescence detection is often used for trace analysis in HPLC. Since there are differences in fluorescence yields between B_1 and B_2, and between G_1 and G_2, it can be useful to run both UV and fluorescence detectors in series [1]. Aflatoxins have no polar functional groups, and can be separated by virtue of their hydrophobicity.

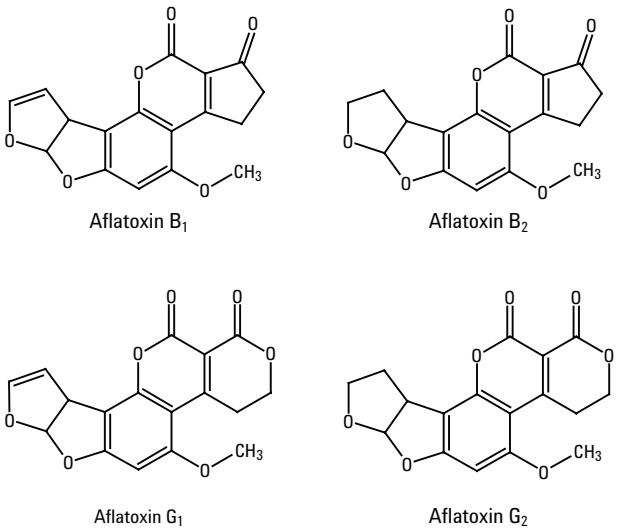


Figure 1. Chemical structures of target aflatoxins.

HPLC Methodology

While thin-layer chromatography was frequently used in the past, HPLC has been used in recent years because of its ease of operation and better quantitation. Most HPLC methods published to date have used reversed-phase HPLC on C18 bonded phases [1–4], where the aflatoxins are separated by their hydrophobicity. Most published separations have been performed on 5-µm columns of 25-cm in length. The use of smaller particle size packings in shorter columns with faster separation times are now in vogue. These columns show that the same separations can be achieved in less time than on the longer columns with similar resolution. In the present study, we desired to show that using such a column can provide improved results compared to the older methods. See Figure 2.

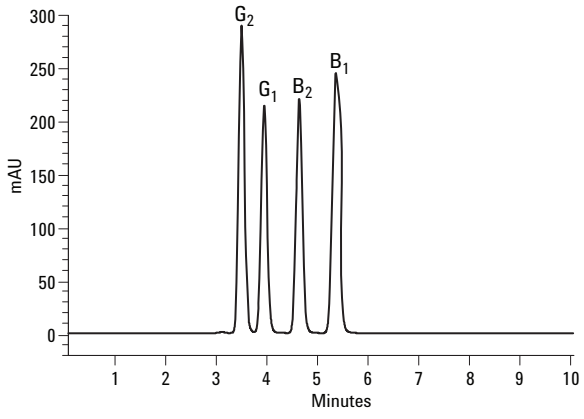


Figure 2. Reversed-phase separation of target aflatoxins using ZORBAX XDB-C18 Rapid Resolution column.

Experimental Conditions

Chemicals:	The aflatoxins were purchased from Sigma Aldrich (Madrid, Spain).

HPLC Conditions

Column:	ZORBAX Eclipse XDB-C18, 4.6 mm × 150 mm, 3.5 µm
Mobile phase:	Water/MeOH/ACN; 50/40/10 (V/V/V)
Flow rate:	0.8 mL/min
Temperature:	Ambient
Detector:	UV 365 nm
Injection volume:	10 µL (0.044 mg/mL)

Results and Discussion

All four aflatoxins were separated using an isocratic ternary mixture of water, methanol, and acetonitrile. A baseline separation was achieved in less than 5.5 min. Although UV detection was shown here, in some cases, lower levels of detection may be obtained for B_2 and G_2 using fluorescence (λ_{ex} = 365 nm, λ_{em} = 455 nm) detection. Mass spectroscopic detection has also been used [1].

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Screening for 926 Pesticides and Endocrine Disruptors by GC/MS with Deconvolution Reporting Software and a New Pesticide Library

Application Note

Food and Environmental

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Abstract

An updated and greatly expanded collection of mass spectral libraries has been introduced, replacing Agilent's RTL Pesticide Library and DRS pesticide solution. The new library contains 926 pesticides, endocrine disruptors, and related compounds – 359 more than the original library. Included are all compounds specified for GC/MS analysis in the new Japanese "Positive List" regulations. All compounds have locked retention times that can be accurately reproduced using an Agilent GC/MS system with the ChemStation's Retention Time Locking software. The new Database can be used as a standard GC/MS library for compound identification or with Agilent's Screener software for identifications based upon retention time and mass spectral matching. The greatest benefit accrues when these libraries are used with Agilent's new version of Deconvolution Reporting Software (part number G1716AA version A.03.00). This solution allows one to screen GC/MS files for all 926 pesticides and endocrine disrupters in about two minutes per sample. Deconvolution helps identify pesticides that are buried in the chromatogram by co-extracted materials. The new database was compared to the smaller one for the DRS analysis of 17 surface water samples. With the new database, DRS found 99 pesticides, metabolites, fire retardants, and related contaminants that were not contained in the original RTL Pesticide and Endocrine Disruptor Library.

Introduction

Several years ago Agilent Technologies introduced Retention Time Locking (RTL) for gas chromatography (GC) and GC with mass spectral detection (GC/MS). RTL software makes it possible to reproduce retention times from run-to-run on any Agilent GC or GC/MS, in any laboratory in the world, so long as the same nominal method and GC column are used (1). Since any laboratory can reproduce retention times generated in another, it is possible to create mass spectral libraries that contain locked retention times. By locking their method to the published database, users can screen GC/MS files for all of the library's compounds. "Hits" are required to have the correct retention time as well as the correct spectrum, which eliminates many false positives and gives more confidence in compound identifications (2).

More recently, Agilent introduced Deconvolution Reporting Software (DRS) that incorporates mass spectral deconvolution with conventional library searching and quantification. DRS results from a marriage of three different GC/MS software packages:

1) The Agilent GC/MS ChemStation,
2) The National Institute of Standards and Technology (NIST) Mass Spectral Search Program with the NIST '05 MS Library, and
3) The Automated Mass Spectral Deconvolution and Identification System (AMDIS) software, also from NIST.

The original DRS software was intended to be a comprehensive solution for pesticide analysis and, therefore, included the mass spectra (in AMDIS format) and locked retention times for 567 pesticides and suspected endocrine disrupters (3).

Recently, Agilent introduced an updated and greatly expanded Pesticide and Endocrine Disruptor Database (part number G1672AA) that now contains 926 entries. This represents the addition of 359 new compounds to the original library. At the same time, Agilent introduced a new version of the DRS software (part number G1716AA version A.03.00) that can be used with any Agilent-provided or user-developed DRS library.

Pesticide and Endocrine Disruptor Database Contents

The G1672AA Pesticide and Endocrine Disruptor Database contains virtually all GC-able pesticides, including those introduced very recently. In addition, the database includes numerous metabolites, more endocrine disruptors, important PCBs and PAHs, certain dyes (for example, Sudan Red), synthetic musk compounds, and several organophosphorus fire retardants.

This new database includes:

- A conventional mass spectral library for use with Agilent GC/MS ChemStations
- A screener database for use with Agilent's powerful screener software that is integrated into the GC/MS ChemStation
- Locked Retention Times for all 926 compounds that any Agilent 5975 or 5973 GC/MS user can reproduce in their laboratory
- Files for use with Agilent's G1716AA (A.03.00) Deconvolution Reporting Software
- An e-method that can be loaded into Agilent's G1701DA (version D.02.00 SP1 or higher) with instrument parameters for acquiring GC/MS files and analyzing the data with DRS. These parameters are listed in Table 1.
- Example files
- Application notes

On November 29, 2005, the Japanese Government published a "Positive List" system for the regulation of pesticides, feed additives, and veterinary drugs. Maximum Residue Limits (MRL) have been set for 758 chemicals while 65 others have been exempted from regulation. Fifteen substances must have no detectable residues. Other agricultural chemicals not mentioned have a uniform MRL of 0.01 ppm (4). This new regulation is scheduled to take effect on May 29, 2006.

Of the pesticides in the Japanese Positive List, 265 are to be analyzed by GC/MS. The new G1672AA Pesticide library contains mass spectra and locked retention times for all of these compounds. Thus, a laboratory could screen for all 265 "positive list" compounds and several hundred more pesticides in just 1–3 minutes after the GC/MS run.

Experimental

Table 1 lists the instrumentation, software, and analytical parameters used by Agilent for pesticide analysis. Depending upon the desired injection volume, a PTV inlet or split/splitless inlet can be used.

Table 1. Instrumentation and Conditions of Analysis

Gas Chromatograph	**Agilent 6890N**
Automatic Sampler	Agilent 7683 Injector and AutoSampler
Inlet	Agilent PTV operated in the solvent vent mode or Split/Splitless
Column	Agilent 30 m × 0.25 mm × 0.25 µm HP-5MSi (part number 19091S-433i)
Carrier gas	Helium in the constant pressure mode
Retention time locking	Chlorpyrifos-methyl locked to 16.596 min (nominal column head pressure = 17.1 psi)
Oven temperature program	70 °C (2 min), 25 °C/min to 150 °C (0 min), 3 °C /min to 200 °C (0 min), 8 °C /min to 280 °C (10–15 min)
PTV inlet parameters	Temp program: 40 °C (0.25 min), 1600 °C/min to 250 °C (2 min); Vent time: 0.2 min; Vent flow: 200 mL/min; Vent pressure: 0.0 psi; Purge flow: 60.0 mL/min; Purge time: 2.00 min
Injection volume	15 µL (using a 50-µL syringe)
Mass Selective Detector	**Agilent 5975 inert**
Tune file	Atune.u
Mode	Scan (or SIM with SIM DRS library)
Scan range	50–550 u
Source, quad, transfer line temperatures	230, 150, and 280 °C, respectively
Solvent delay	4.00 min
Multiplier voltage	Autotune voltage
Software	
GC/MSD ChemStation	Agilent part number G1701DA (version D02.00 sp1 or higher)
Deconvolution Reporting Software	Agilent part number G1716AA (version A.03.00) Deconvolution Reporting Software
Library searching software	NIST MS Search (version 2.0d or greater) (comes with NIST '05 mass spectral library – Agilent part number G1033A)
Deconvolution software	Automated Mass Spectral Deconvolution and Identification Software (AMDIS_32 version 2.62 or greater; comes with NIST '05 mass spectral library – Agilent part number G1033A)
MS Libraries	NIST '05 mass spectral library (Agilent part number G1033A) Agilent RTL Pesticide and Endocrine Disruptor Libraries in Agilent and NIST formats (part number G1672AA)

Results and Discussion

DRS, which has been described in preceding papers (3,5,6), can be summarized as follows:

Three separate, but complimentary, data analysis steps are combined into the DRS. First, the GC/MS ChemStation software performs a normal quantitative analysis for target pesticides using a target ion and up to three qualifiers. An amount is reported for all calibrated compounds that are detected. For other compounds in the database, an estimate of their concentration can be reported based upon an average pesticide response factor that is supplied with the DRS software. The DRS then sends the data file to AMDIS, which deconvolutes the spectra and searches the Agilent RTL Pesticide Library using the deconvoluted full spectra. A filter can be set in AMDIS, which requires the analyte's retention time to fall within a user-specified time window. Because RTL is used to reproduce the RTL database retention times with high precision, this window can be quite small – typically 10–20 seconds. Finally, the deconvoluted spectra for all of the targets found by AMDIS are searched against the 147,000-compound NIST mass spectral library for confirmation; for this step, there is no retention time requirement.

This approach was rapidly adopted by many laboratories because of its ability to identify pesticides in complex chromatograms containing high levels of co-extracted interferences. Indeed, the solution proved to be so useful that users began to create their own DRS libraries (7). Therefore, the DRS was unbundled from the pesticide database so that it could be used with any agilent-provided or user-created database.

The original 567-compound RTL Pesticide Library (G1049A) included pesticides, a few metabolites, and most of the GC-amenable endocrine disruptors that were known at the time. The new version of the library includes many more pesticides, endocrine disruptors, and metabolites. This update also contains important compounds from other classes of contaminants that have been found in food and water supplies. Included are eighteen polychlorinated biphenyls (PCBs), four polybrominated biphenyls (PBBs), several polynuclear aromatic hydrocarbons (PAHs), several organophosphorus fire retardants, three important toxaphene congeners, and three Sudan dyes.

Advantages of Deconvolution

Figure 1 shows a screen from AMDIS that illustrates the power of this deconvolution software. The white trace in Figure 1A is the total ion chromatogram while the other three are extracted ions of a deconvoluted peak (a "component" in AMDIS terminology). Note that the TIC and extracted ions are not scaled to each other and this component is actually obscured by co-eluting compounds. Figure 1B juxtaposes the deconvoluted component spectrum (white) with the complete "undeconvoluted" spectrum (black). Clearly, this component is buried under co-eluting peaks that would ordinarily obscure the analyte. Figure 1C shows that the deconvoluted peak (white spectrum) is a good library match for norflurazon (black spectrum). The locked retention time for norflurazon in the RTL Pesticide Database is 26.933 min, which is just 2.3 seconds away from its observed RT in this chromatogram. Confidence in peak identifications is greatly enhanced by the combination of spectral deconvolution and locked retention time filtering.

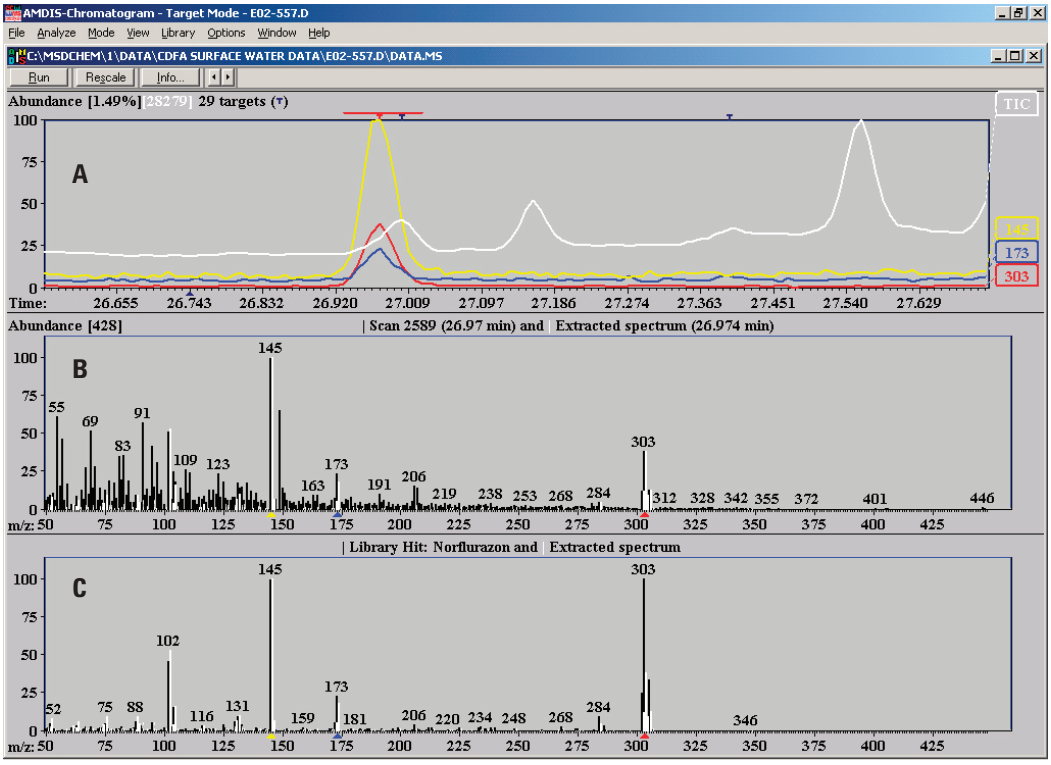


Figure 1. AMDIS screen showing the identification of norflurazon.
A) The total ion and extracted ion chromatograms where norflurazon elutes.
B) The deconvoluted component spectrum (white) juxtaposed with the spectrum at 26.972 min (black).
C) The deconvoluted component matched to the library spectrum of norflurazon.

Surface Water Analysis - Revisiting an Earlier Study

In an earlier study, a comparison was made between Agilent's DRS and conventional pesticide analysis (3). The California Department of Food and Agriculture (CDFA) provided data files for 17 surface water extracts that had been analyzed in their laboratory. Since the GC/MS chromatograms were locked to the Agilent pesticide method, it was possible to analyze these data files using DRS without having to re-run the samples. The original DRS analysis was made using the 567-compound RTL Pesticide Database. For comparison, these same data files were re-analyzed using the new 926-compound RTL Pesticide Database. The chromatogram (Figure 2) and the DRS report (Figure 3) from one of these samples are shown below.

Excluding phthalates, seven new compounds (shown with bold type in Figure 3) were identified using the 926-compound database: 4-chlorophenyl isocyanate (a phenylurea herbicide metabolite); 3,4-dichlorophenyl isocyanate (diuron metabolite); tris(2-chloroethyl) phosphate (a fire retardant); caffeine (a stimulant); Cyprodinil (a fungicide); desmethyl-norflurazon (a metabolite of norflurazon, an herbicide); and tris(2-butoxyethyl) phosphate (a fire retardant). Although caffeine is not generally considered to be dangerous, it is included in the database because it has been found frequently in sewage effluent and in numerous waterways together with a various pharmaceuticals and pesticides (8).

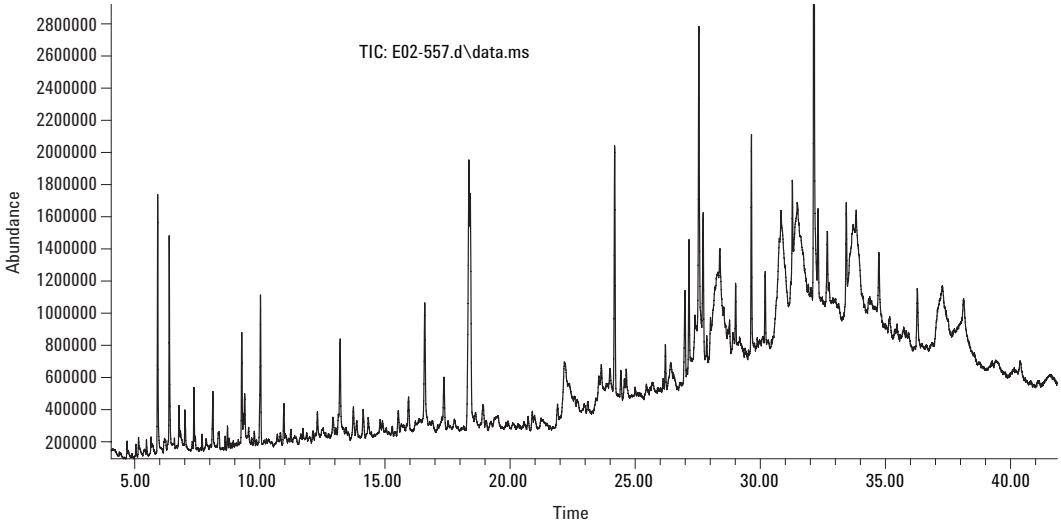


Figure 2. Chromatogram of a surface water extract that was analyzed by DRS using the new RTL Pesticide and Endocrine Disrupter Database. The results of this analysis are shown in Figure 3.

MSD Deconvolution Report
Sample Name: **E02-557**
Data File: **C:\MSDChem\1\DATA\CDFA surface water data\E02-557.d**
Date/Time: **11:24 AM Tuesday, Apr 4 2006**

The NIST library was searched for the components that were found in the AMDIS target library.

			Agilent			NIST	
RT	**Cas #**	**Compound name**	**ChemStation amount (ng)**	**AMDIS match**	**RT Diff (sec.)**	**reverse match**	**Hit number**
4.4689	106445	4-Methylphenol		62	3.2		
4.4689	0000	3-Carbobenzyloxy-4-ketoproline				48	1
4.8840	104121	**4-Chlorophenyl isocyanate**		84	–1.8	86	2
6.3879	102363	**Diuron Metabolite [3,4-Dichlorophenyl isocyanate]**		99	3.1	95	1
6.8357	759944	EPTC		84	2.0	85	1
7.6988	95761	3,4-Dichloroaniline		93	2.1	89	2
7.9342	131113	Dimethylphthalate		67	1.7	84	2
8.1112	25013165	Butylated hydroxyanisole		63	–7.7		
8.1112	0000	7-Methoxy-2,2,4,8-tetramethyltricyclo [5.3.1.0(4,11)]undecane				62	1
8.941	29878317	Tolyltriazole [1H-Benzotriazole, 4-meth-]	1.29				
9.7903	134623	N,N-Diethyl-m-toluamide		85	2.2	84	2
10.0019	84662	Diethyl phthalate		98	2.6	92	1
10.7109	119619	Benzophenone		86	2.6	88	2
10.9684	126738	Tributyl phosphate		96	3.0	90	1
11.6491	1582098	Trifluralin		83	0.7	74	1
12.9326	122349	Simazine		88	1.4	86	2
13.4309	115968	**Tris(2-chloroethyl) phosphate**		79	1.0	78	1
13.7478	1517222	Phenanthrene-d10		95	1.3	83	1
15.4048	58082	**Caffeine**		80	1.6	74	1
15.9474	84695	Diisobutyl phthalate		90	3.2	88	4
16.5988	5598130	Chlorpyrifos Methyl		97	0.4	90	1
17.3653	7287196	Prometryn		90	1.5	84	1
18.4213	84742	Di-n-butylphthalate		99	0.4	94	1
18.9214	51218452	Metolachlor		90	0.7	87	1
20.5633	121552612	**Cyprodinil**		69	–0.1		
20.5633	76470252	9,9-Dimethoxy-9-sila-9, 10-dihydroanthracene				70	1
26.4247	23576241	**Norflurazon, Desmethyl-**		87	–4.5	69	2
26.9700	27314132	Norflurazon		87	1.5	79	1
26.9992	85687	Butyl benzyl phthalate		94	–0.5	94	1
27.3984	51235042	Hexazinone		89	0.8	83	1
28.0127	78513	**Tris(2-butoxyethyl) phosphate**		75	3.3	83	1
29.6537	117817	Bis(2-ethylhexyl)phthalate		98	0.3	90	3
33.9298	84764	Di-n-nonyl phthalate		65	–1.9		
33.9298	0000	Phthalic acid, 3,4-dichlorophenyl propyl ester				71	1
13.739		Phenanthrene-d10	10				

Figure 3. **DRS report from the analysis of a surface water sample.** The compounds shown in bold type were found by the new RTL Pesticide Database but not the original one because these compounds were not included.

For this sample, the ChemStation identified only tolyltriazole at 8.941 min, but AMDIS did not confirm this assignment, nor could it be confirmed manually. Butylated hydroxyanisole was tentatively identified by AMDIS with a low match value, but the retention time is off by –7.7 seconds which is considerably more than most other hits. This compound is not in the NIST library so it could not be confirmed. The ChemStation method used for this analysis required that all three qualifier ions fall within ±20% (relative) which is a rigorous requirement for such a complex sample. This explains why so few compounds were found by the ChemStation.

Cyprodinil (20.563 min) was identified by AMDIS but the NIST library search failed to confirm its presence. The next line shows that the best NIST library match is an anthracene derivative that is nothing like cyprodinil. This result was obtained when AMDIS was configured to "use uncertain peaks" as shown in Figure 4. When this feature is turned off in DRS Compound Identification Configuration, the best NIST library hit for this spectrum is, indeed, cyprodinil. When a compound's identity is ambiguous, as with cyprodinil, it may be useful to perform the DRS search both ways and compare the results.

In the comparison described earlier (3), DRS was able to identify all 37 pesticides found by the CDFA chemist. However, DRS completed the task for all 17 samples in about 20 minutes compared to ~8 hours for the manual procedure (Table 2). Moreover, DRS identified one false positive in the CDFA report and found 34 additional pesticides and related compounds.

Using the new 926-compound Database, it took 32 minutes to analyze all of the samples and DRS was able to find an additional 99 pesticides, metabolites, fire retardants, and related compounds (Table 2).

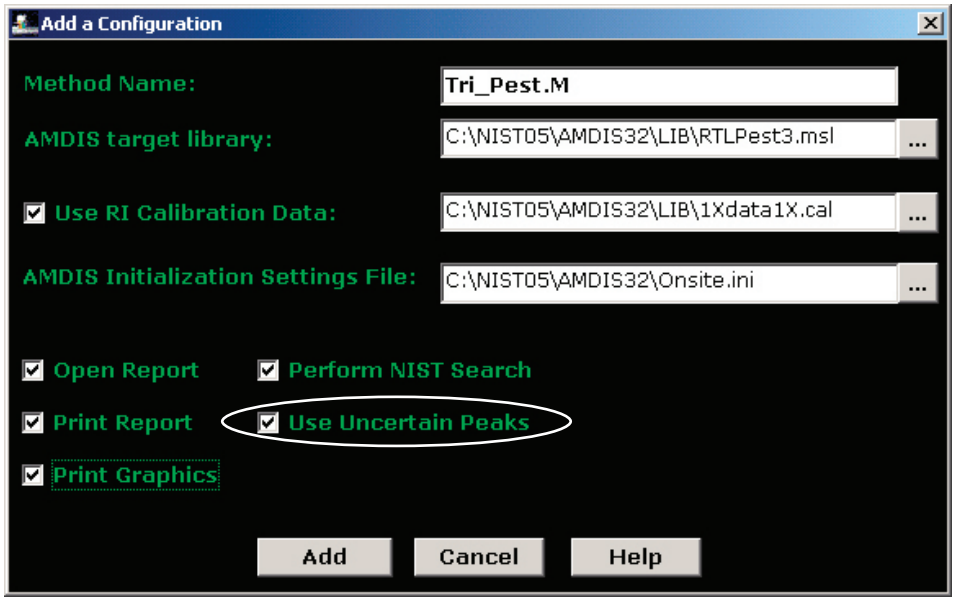


Figure 4. DRS configuration screen for the method called Tri_Pest. When the box labeled "Use Uncertain Peaks" is checked, AMDIS will use uncertain peaks for library searches. When unchecked, AMDIS ignores uncertain mass spectral peaks. Sometimes, this can affect the quality of a library match.

Table 2. Comparison of the Results Obtained by Screening 17 Surface Water Extracts Using Traditional Methods (CDFA) and Using DRS With Two Different Databases – the G1049A With 567 Compounds and the G1672AA With 926 Entries

	CDFA	Agilent DRS (Original G1049A database)	Agilent DRS (G1672 AA database)
Targets found (not counting ISTD)	37	Same 37 +34 more	Same 37 +99 more
False positives	1	0	0
Processing time	~8 hrs (ChemStation only)	20 minutes	32 min

Handling Stereoisomers

Many pesticides have multiple stereoisomers with virtually identical mass spectra. For example, cyfluthrin has four diastereomers arising from its three chiral centers. It is very difficult and sometimes impossible to determine the elution order of these isomers and most analysts report them as a sum of the isomer amounts. Agilent's G1049A RTL Pesticide database arbitrarily assigned each isomer a Roman numeral with I for the earliest eluting isomer, II for the next, and so on. The same Chemical Abstracts Service number (CAS #) was assigned to all of the isomers. Generally, it was a CAS # for the compound with "unstated stereochemistry." This caused some incompatibility with AMDIS as explained below.

AMDIS software differentiates among compounds using a "chemical identification number." The easiest and most consistent approach is to use each compound's CAS #. The default setting for AMDIS is to allow each CAS # to be used only once when analyzing a GC/MS data file. While this seems logical, it requires that each database entry have a different CAS #. It is possible to allow multiple hits per compound by checking the box in AMDIS found in the drop down menu under Analyze/Settings/Identif. However, this allows multiple peaks to be assigned the same compound name.

In the new RTL Pesticide Database (G1672AA), the Roman numeral designations remain and the first isomer in the series is given its genuine CAS #. Subsequent isomers in the series are given unique, but fictitious "CAS #s" generated by Agilent. The compound's real CAS # appears in braces after the compound name. For example, the cyfluthrin isomers are entered into the database as shown in Table 3.

Table 3. Method for Listing Compounds with Multiple Stereoisomers in the New G1672AA RTL Pesticide Database

RT	Compound name*		CAS #**
32.218	Cyfluthrin I		68359-37-5
32.359	Cyfluthrin II	{CAS # 68359-37-5}	999028-03-4
32.477	Cyfluthrin III	{CAS # 68359-37-5}	999029-03-7
32.536	Cyfluthrin IV	{CAS # 68359-37-5}	999030-03-4

* In a series, the earliest eluting isomer is identified with "I" and is assigned its legitimate CAS #. Subsequent isomers are assigned unique, but fictitious CAS #s (see footnote **). Their actual CAS # is put in braces behind the compound name.

** Cyfluthrin I has been given it's genuine CAS #. Cyfluthrin II-IV have been given unique numbers that can be distinguished from actual CAS numbers because they all have six digits before the first hyphen (9 total) and all begin with the series 999.

Figure 5 shows how permethrin was identified in a spinach sample using both databases with AMDIS configured to allow one hit per compound. Using the older 567-compound database (G1049A) only one permethrin isomer was identified because its CAS # could be used only once. With the new format used in the 926-compound RTL Pesticide Database (G1672AA), both isomers of permethrin were identified. Not surprisingly, the NIST library search found no hits with the same fictitious CAS # assigned to permethrin II. So, the software printed the best match on the following line. This compound, a cyclopropanecarboxylic acid derivative, is a permethrin isomer.

So long as the NIST library search is turned on in DRS, it will always print another line after reporting a compound with a fictitious CAS #. Note that these fictitious CAS #s always contain 9 digits and begin with 999.

A)

			Agilent			NIST	
RT	**Cas #**	**Compound name**	**ChemStation amount (ng)**	**AMDIS match**	**RT Diff (sec.)**	**reverse match**	**Hit number**
31.6158	52645531	Permethrin II		88	3.9	91	3

B)

			Agilent			NIST	
RT	**Cas #**	**Compound name**	**ChemStation amount (ng)**	**AMDIS match**	**RT Diff (sec.)**	**reverse match**	**Hit number**
31.4127	52645531	Permethrin I		78	2.6	81	3
31.6088	999046036	Permethrin II {CAS # 52645-53-1}		65	3.5		
31.6088	51877748	Cyclopropanecarboxylic acid, 3-(2,2-dichlorovinyl)-2,2-dimethyl-, (3-phenoxyphenyl)methyl ester, (1R-trans)-				95	1

Figure 5. A) A single isomer of permethrin was identified by DRS using the G1049A 567-compound database when AMDIS was not allowed to use multiple hits per compound.
B) Two permethrin isomers are identified by DRS with the G1672AA 926-compound database under the same circumstances.

Conclusions

The new G1672AA RTL Pesticide and Endocrine Disruptor library contains substantially more target analytes than its predecessor. With the addition of 359 new compounds, it is the most comprehensive library of its type available today. Many new pesticides, metabolites, and endocrine disruptors were added along with important PCBs, PBBs, PAHs, synthetic musk compounds, Sudan dyes, and organophosphorus fire retardants. The database contains all of the analytes specified for GC/MS analysis in the new Japanese "Positive List" regulations.

When combined with the complete DRS solution, one can screen GC/MS data files for all 926 compounds in about two minutes per sample. This is the fastest, most comprehensive, most accurate, and least tedious method for screening food and environmental samples for these compounds.

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Lists of Compounds in Databases

1,2,4-Trichlorobenzene
1,2-Dibromo-3-chloropropane
1,3,5-Tribromobenzene
1,3-Dichlorbenzene
17a-Ethynylestradiol
1-naphthalenol
2-(1-naphthyl)acetamide
2-(2-Butoxyethoxy)ethyl thiocyanate
2-(Octylthio)ethanol
2,3,4,5-Tertrachloronitrobenzene
2,3,4,5-Tetrachlorophenol
2,3,4,6-Tetrachlorophenol
2,3,5,6-Tetrachlorophenol
2,3,5,6-Tetrachloro-p-terphenyl
2,3,5-Trichlorophenol
2,3,5-Trimethacarb
2,3,6-Trichloroanisole
2,3,7,8-Tetrachlorodibenzofuran
2,3,7,8-Tetrachlorodibenzo-p-dioxin
2,4,5,6-Tetrachloro-m-xylene
2,4,5-T methyl ester
2,4,5-Trichloroaniline
2,4,5-Trichlorophenol
2,4,5-Trichloro-p-terphenyl
2,4,5-Trimethylaniline
2,4,6-Tribromoanisole
2,4,6-Tribromophenol
2,4,6-Trichloroanisole
2,4,6-Trichlorophenol
2,4-D methyl ester
2,4-D sec-butyl ester
2,4-DB methyl ester
2,4'-Dichlorobenzophenone (2,4'-Dicofol decomposition product)
2,4-Dichlorophenol
2,4-Dichlorophenyl benzenesulfonate
2,4-Dimethylaniline
2,4-Dimethylphenol
2,6-Dichlorobenzamide
2,6-Dichlorobenzonitrile
2,6-Dimethylaniline
2-[3-Chlorophenoxy]propionamide
2-Chlorophenol
2-Ethyl-1,3-hexanediol
2-ethyl-6-methylaniline
2-Hydroxyestradiol
2-Methyl-4,6-dinitrophenol
2-Methylphenol
2-Nitrophenol
2-Phenoxypropionic acid
3,4,5-Trimethacarb
3,4-Dichloroaniline
3,5-Dichloroaniline
3-Aminophenol
3-Chloro-4-fluoroaniline
3-Chloro-4-methoxyaniline
3-Chloroaniline
3-Hydroxycarbofuran
3-Indolylacetonitrile
3-Trifluormethylaniline
4,4'-Dichlorobenzophenone
4,4'-Oxydianiline
4,6-Dinitro-o-cresol (DNOC)
4-Aminodiphenyl
4-Bromoaniline
4-Chloro-2-methylaniline
4-Chloro-3-methylphenol
4-Chloroaniline
4-Chlorophenyl isocyanate
4-Isopropylaniline
4-Methylphenol
4-Nitrophenol
4-Nonylphenol
5,7-Dihydroxy-4'-methoxyisoflavone
9,10-Anthraquinone
Acenaphthene
Acenaphthylene
Acephate
Acequinocyl
acetamiprid
Acetochlor
Acifluorfen methyl ester
Aclonifen
Acrinathrin
Alachlor
Aldrin
Allidochlor
Ametryn
Amidithion
Aminocarb
Amitraz
Amitraz metabolite [Methanimidamide, N-(2,4-dimethylphenyl)-N'-methyl-]
Ancymidol
Anilazine
Aniline
Anilofos
Anthracene
Aramite I
Aramite II {CAS # 140-57-8}
Atraton
Atrazine
Atrazine-desethyl
Azaconazole
Azamethiphos
Azibenzolar-S-methyl
Azinphos-ethyl
Azinphos-methyl
Aziprotryn metabolite [2-Amino-4-isopropylamino-6-methylthio-1,3,5-triazine]
Aziprotryne
Azobenzene
Azoxybenzene
Azoxystrobin
Barban
Beflubutamid
Benalaxyl
Benazolin-ethyl
Bendiocarb
Benfluralin

Benfuracarb
Benfuresate
Benodanil
Benoxacor
Bentazone
Bentazone methyl derivative
Benthiocarb
Benzene, 1,3-*bis*(bromomethyl)-
Benzenesulfonamide
Benzidine
Benzo(a)anthracene
Benzo(a)pyrene
Benzo[b]fluoranthene
Benzo[g,h,i]perylene
Benzo[k]fluoranthene
Benzophenone
Benzoximate metabolite
Benzoylprop ethyl
Benzyl benzoate
b-Estradiol
BHC alpha isomer
BHC beta isomer
BHC delta isomer
BHC epsilon isomer
Bifenazate metabolite
(5-Phenyl-o-anisidine)
Bifenox
Bifenthrin
Binapacryl
Bioallethrin
Bioallethrin S-cyclopentenyl isomer
Bioresmethrin
Biphenyl
Bis(2,3,3,3-tetrachloropropyl) ether
Bis(2-butoxyethyl) phthalate
Bis(2-ethylhexyl)phthalate
Bisphenol A
Bitertanol I
Bitertanol II {CAS # 55179-31-2}
Boscalid (Nicobifen)
Bromacil
Bromfenvinphos-(E)
Bromfenvinphos-(Z)
Bromobutide
Bromocyclen
Bromophos
Bromophos-ethyl
Bromopropylate
Bromoxynil
Bromoxynil octanoic acid ester
Bromuconazole I
Bromuconazole II {CAS # 116255-48-2}
Bufencarb
Bupirimate
Buprofezin
Butachlor
Butafenacil
Butamifos
Butoxycarboxim
Butralin
Butyl benzyl phthalate
Butylate
Butylated hydroxyanisole
Cadusafos
Cafenstrole
Caffeine
Captafol
Captan
Carbaryl
Carbetamide
Carbofuran
Carbofuran-3-keto
Carbofuran-7-phenol
Carbophenothion
Carbosulfan
Carboxin
Carfentrazone-ethyl
Carpropamid
Carvone
Cashmeran
Cekafix
Celestolide
Chinomethionat
Chloramben methyl ester
Chloranocryl
Chlorbenside
Chlorbenside sulfone
Chlorbicyclen
Chlorbromuron
Chlorbufam
Chlordecone
Chlordene, *trans*-
Chlordimeform
Chlorethoxyfos
Chlorfenapyr
Chlorfenethol
Chlorfenprop-methyl
Chlorfenson
Chlorfenvinphos
Chlorfenvinphos, *cis*-
Chlorfenvinphos, *trans*-
Chlorflurecol-methyl ester
Chlormefos
Chlornitrofen
Chlorobenzilate
Chloroneb
Chloropropylate
Chlorothalonil
Chlorotoluron
Chlorpropham
Chlorpyrifos
Chlorpyrifos Methyl
Chlorthal-dimethyl
Chlorthiamid
Chlorthion
Chlorthiophos
Chlorthiophos sulfone
Chlorthiophos sulfoxide
Chlozolinate
Chrysene
Cinerin I
Cinerin II
Cinidon-ethyl
cis-Chlordane
Clodinafop-propargyl
Clomazone
Cloquintocet-mexyl
Coumaphos
Crimidine
Crotoxyphos
Crufomate
Cyanazine
Cyanofenphos
Cyanophos
Cyclafuramid
Cycloate
Cyclopentadecanone
Cycluron

Cyflufenamid
Cyfluthrin I
Cyfluthrin II {CAS # 68359-37-5}
Cyfluthrin III {CAS # 68359-37-5}
Cyfluthrin IV {CAS # 68359-37-5}
Cyhalofop-butyl
Cyhalothrin I (lambda)
Cyhalothrin (Gamma)
Cymiazole
Cymoxanil
Cypermethrin I
Cypermethrin II {CAS # 52315-07-8}
Cypermethrin III {CAS # 52315-07-8}
Cypermethrin IV {CAS # 52315-07-8}
Cyphenothrin *cis*-
Cyphenothrin *trans*- {CAS # 39515-40-7}
Cyprazine
Cyproconazole
Cyprodinil
Cyprofuram
Cyromazine
d-(*cis-trans*)-Phenothrin-I
d-(*cis-trans*)-Phenothrin-II {CAS # 260002-80-2}
Dazomet
DDMU [1-Chloro-2,2-*bis*(4'-chlorophenyl)]
Decachlorobiphenyl
Deltamethrin
Demephion
Demeton-S
Demeton-S-methylsulfon
Desbromo-bromobutide
Desmedipham
Desmetryn
Dialifos
Di-allate I
Di-allate II {CAS # 2303-16-4}
Diamyl phthalate
Diazinon
Diazinon-oxon
Dibenz[a,h]anthracene
Dicamba
Dicamba methyl ester
Dicapthon
Dichlofenthion
Dichlofluanid
Dichlofluanid metabolite (DMSA)
Dichlone
Dichlormid
Dichlorophen
Dichlorprop
Dichlorprop methyl ester
Dichlorvos
Diclobutrazol
Diclocymet I
Diclocymet II {CAS # 139920-32-4}
Diclofop methyl
Dicloran
Dicrotophos
Dicyclohexyl phthalate
Dicyclopentadiene
Dieldrin
Diethatyl ethyl
Diethofencarb
Diethyl dithiobis(thionoformate) (EXD)
Diethyl phthalate
Diethylene glycol
Diethylstilbestrol
Difenoconazol I
Difenoconazol II {CAS # 119446-68-3}
Difenoxuron
Diflufenican
Diisobutyl phthalate
Dimefox
Dimepiperate
Dimethachlor
Dimethametryn
Dimethenamid
Dimethipin
Dimethoate
Dimethomorph-(E)
Dimethomorph-(Z) {CAS # 110488-70-5}
Dimethylphthalate
Dimethylvinphos(z)
Dimetilan
Dimoxystrobin
Di-n-butylphthalate
Di-n-hexyl phthalate
Diniconazole
Dinitramine
Di-n-nonyl phthalate
Dinobuton
Dinocap I
Dinocap II {CAS # 39300-45-3}
Dinocap III {CAS # 39300-45-3}
Dinocap IV {CAS # 39300-45-3}
Di-n-octyl phthalate
Dinoseb
Dinoseb acetate
Dinoseb methyl ether
Dinoterb
Dinoterb acetate
Di-n-propyl phthalate
Diofenolan I
Diofenolan II {CAS # 63837-33-2}
Dioxabenzofos
Dioxacarb
Dioxathion
Diphacinone
Diphenamid
Diphenyl phthalate
Diphenylamine
Dipropetryn
Dipropyl isocinchomeronate
Disulfoton
Disulfoton sulfone
Ditalimfos
Dithiopyr
Diuron
Diuron Metabolite [3,4-Dichlorophenyl isocyanate]
Dodemorph I
Dodemorph II {CAS # 1593-77-7}
Drazoxolon
Edifenphos
Empenthrin I
Empenthrin II {CAS # 54406-48-3}
Empenthrin III {CAS # 54406-48-3}
Empenthrin IV {CAS # 54406-48-3}
Empenthrin V {CAS # 54406-48-3}
Endosulfan (alpha isomer)
Endosulfan (beta isomer)
Endosulfan ether
Endosulfan lactone
Endosulfan sulfate
Endrin
Endrin aldehyde
Endrin ketone

EPN
Epoxiconazole
EPTC
Erbon
Esfenvalerate
Esprocarb
Etaconazole
Ethalfluralin
Ethidimuron
Ethiofencarb
Ethiolate
Ethion
Ethofenprox
Ethofumesate
Ethofumesate, 2-Keto
Ethoprophos
Ethoxyfen-ethyl
Ethoxyquin
Ethylenethiourea
Etoxazole
Etridiazole
Etridiazole, deschloro- (5-ethoxy-3-dichloromethyl-1,2,4-thiadiazole)
Etrimfos
Eugenol
Exaltolide [15-Pentadecanolide]
Famoxadon
Famphur
Fenamidone
Fenamiphos sulfoxide
Fenamiphos-sulfone
Fenarimol
Fenazaflor
Fenazaflor metabolite
Fenazaquin
Fenbuconazole
Fenchlorazole-ethyl
Fenchlorphos
Fenchlorphos-oxon
Fenclorim
Fenfuram
Fenhexamid
Fenitrothion
Fenitrothion-oxon
Fenobucarb
Fenoprop
Fenoprop methyl ester
Fenothiocarb
Fenoxanil
Fenoxaprop-ethyl
Fenoxycarb
Fenpiclonil
Fenpropathrin
Fenpropidin
Fenson
Fensulfothion
Fensulfothion-oxon
Fensulfothion-oxon -sulfone
fensulfothion-sulfone
Fenthion
Fenthion sulfoxide
Fenthion-sulfone
Fenuron
Fenvalerate I
Fenvalerate II {CAS # 51630-58-1}
Fepropimorph
Fipronil
Fipronil, desulfinyl-
Fipronil-sulfide
Fipronil-sulfone
Flamprop-isopropyl
Flamprop-methyl
Fluacrypyrim
Fluazifop-p-butyl
Fluazinam
Fluazolate
Flubenzimine
Fluchloralin
Flucythrinate I
Flucythrinate II {CAS # 70124-77-5}
Fludioxonil
Flufenacet
Flumetralin
Flumiclorac-pentyl
Flumioxazin
Fluometuron
Fluoranthene
Fluorene
Fluorodifen
Fluoroglycofen-ethyl
Fluoroimide
Fluotrimazole
Fluoxastrobin *cis*-
Fluquinconazole
Flurenol-butyl ester
Flurenol-methylester
Fluridone
Flurochloridone I
Flurochloridone II {CAS # 61213-25-0}
Flurochloridone, deschloro-
Fluroxypyr-1-methylheptyl ester
Flurprimidol
Flurtamone
Flusilazole
Fluthiacet-methyl
Flutolanil
Flutriafol
Fluvalinate-tau-I
Fluvalinate-tau-II {CAS # 102851-06-9}
Folpet
Fonofos
Formothion
Fosthiazate I
Fosthiazate II {CAS # 98886-44-3}
Fuberidazole
Furalaxyl
Furathiocarb
Furilazole
Furmecyclox
Halfenprox
Haloxyfop-methyl
Heptachlor
Heptachlor epoxide isomer A
Heptachlor exo-epoxide isomer B
Heptenophos
Hexabromobenzene
Hexachlorobenzene
Hexachlorophene
Hexaconazole
Hexazinone
Hexestrol
Hydroprene
Imazalil
Imazamethabenz-methyl I
Imazamethabenz-methyl II {CAS # 81405-85-8}
Imibenconazole
Imibenconazole-desbenzyl

Indeno[1,2,3-cd]pyrene

Indoxacarb and Dioxacarb decomposition product [Phenol, 2-(1,3-dioxolan-2-yl)-]

Ioxynil

Ioxynil octanoate

Ipconazole

Iprobenfos

Iprodione

Iprovalicarb I

Iprovalicarb II {CAS # 140923-25-7}

Irgarol

Isazophos

Isobenzan

Isobornyl thiocyanoacetate

Isocarbamide

Isocarbophos

Isodrin

Isofenphos

Isofenphos-oxon

Isomethiozin

Isoprocarb

Isopropalin

Isoprothiolane

Isoproturon

Isoxaben

Isoxadifen-ethyl

Isoxaflutole

Isoxathion

Jasmolin I

Jasmolin II

Jodfenphos

Kinoprene

Kresoxim-methyl

Lactofen

Lenacil

Leptophos

Leptophos oxon

Lindane

Linuron

Malathion

Malathion-o-analog

MCPA methyl ester

MCPA-butoxyethyl ester

MCPB methyl ester

m-Cresol

Mecarbam

Mecoprop methyl ester

Mefenacet

Mefenpyr-diethyl

Mefluidide

Menazon

Mepanipyrim

Mephosfolan

Mepronil

Metalaxyl

Metamitron

Metasystox thiol

Metazachlor

Metconazole I

Metconazole II {CAS # 125116-23-6}

Methabenzthiazuron [decomposition product]

Methacrifos

Methamidophos

Methfuroxam

Methidathion

Methiocarb

Methiocarb sulfone

Methiocarb sulfoxide

Methomyl

Methoprene I

Methoprene II {CAS # 40596-69-8}

Methoprotryne

Methoxychlor

Methoxychlor olefin

Methyl (2-naphthoxy)acetate

Methyl paraoxon

Methyl parathion

Methyl-1-naphthalene acetate

Methyldymron

Metobromuron

Metolachlor

Metolcarb

Metominostrobin (E)

Metominostrobin (Z) {CAS # 133408-50-1}

Metrafenone

Metribuzin

Mevinphos

Mirex

Molinate

Monalide

Monocrotophos

Monolinuron

Musk amberette

Musk Ketone

Musk Moskene

Musk Tibetene (Moschustibeten)

Musk xylene

Myclobutanil

N,N-Diethyl-m-toluamide

N-1-Naphthylacetamide

Naled

Naphthalene

Naphthalic anhydride

Naproanilide

Napropamide

Nicotine

Nitralin

Nitrapyrin

Nitrofen

Nitrothal-isopropyl

N-Methyl-N-1-naphthyl acetamide

Nonachlor, *cis*-

Nonachlor, *trans*-

Norflurazon

Norflurazon, desmethyl-

Nuarimol

o,p'-DDD

o,p'-DDE

o,p'-DDT

Octachlorostyrene

o-Dianisidine

o-Dichlorobenzene

Ofurace

Omethoate

o-Phenylphenol

Orbencarb

ortho-Aminoazotoluene

Oryzalin

Oxabetrinil

Oxadiazon

Oxadixyl

Oxamyl

Oxycarboxin

Oxychlordane

Oxydemeton-methyl

Oxyfluorfen

p,p'-DDD
p,p'-DDE
p,p'-DDM [*bis*(4-chlorophenyl)methane]
p,p'-DDT
p,p'-Dibromobenzophenone
p,p'-Dicofol
Paclobutrazol
Paraoxon
Parathion
PBB 52 Tetrabrombiphenyl
PBB 101
PBB 15
PBB 169 Hexabrombiphenyl
PCB 101
PCB 105
PCB 110
PCB 118
PCB 126
PCB 127
PCB 131
PCB 136
PCB 138
PCB 153
PCB 169
PCB 170
PCB 180
PCB 30
PCB 31
PCB 49
PCB 77
PCB 81
p-Dichlorobenzene
Pebulate
Penconazole
Pendimethalin
Pentachloroaniline
Pentachloroanisole
Pentachlorobenzene
Pentachloronitrobenzene
Pentachlorophenol
Pentanochlor
Permethrin I
Permethrin II {CAS # 52645-53-1}
Perthane
Phantolide
Phenamiphos
Phenanthrene
Phenanthrene-d10
Phenkapton
Phenol
Phenothiazine
Phenothrin I
Phenothrin II
Phenoxyacetic acid
Phenthoate
Phorate
Phorate sulfone
Phorate sulfoxide
Phorate-oxon
Phosalone
Phosfolan
Phosmet
Phosphamidon I
Phosphamidon II {CAS # 13171-21-6}
Phthalide
Phthalimide
Picloram methyl ester
Picolinafen
Picoxystrobin
Pindone
Piperalin
Piperonyl butoxide
Piperophos
Pirimicarb
Pirimiphos-ethyl
Pirimiphos-methyl
Plifenat
p-Nitrotoluene
Potasan
Prallethrin, *cis*-
Prallethrin, *trans*- {CAS # 23031-36-9}
Pretilachlor
Probenazole
Prochloraz
Procymidone
Prodiamine
Profenofos
Profenofos metabolite (4-Bromo-2-chlorophenol)
Profluralin
Prohydrojasmon I
Prohydrojasmon II {CAS # 158474-72-7}
Promecarb
Promecarb artifact [5-isopropyl-3-methylphenol]
Prometon
Prometryn
Propachlor
Propamocarb
Propanil
Propaphos
Propargite
Propargite metabolite [Cyclohexanol, 2-(4-tert-butylphenoxy)]
Propazine
Propetamphos
Propham
Propiconazole-I
Propiconazole-II {CAS # 60207-90-1}
Propisochlor
Propoxur
Propyzamide
Prosulfocarb
Prothioconazole-desthio
Prothiofos
Prothoate
Pyracarbolid
Pyraclofos
Pyraflufen-ethyl
Pyrazon
Pyrazophos
Pyrazoxyfen
Pyrene
Pyrethrin I
Pyrethrin II
Pyributicarb
Pyridaben
Pyridaphenthion
Pyridate
Pyridinitril
Pyrifenox I
Pyrifenox II {CAS # 88283-41-4}
Pyriftalid
Pyrimethanil
Pyrimidifen
Pyriminobac-methyl (E)
Pyriminobac-methyl (Z) {CAS # 136191-64-5}

Pyriproxyfen
Pyroquilon
Quinalphos
Quinoclamine
Quinoxyfen
Quintozene metabolite (pentachlorophenyl methyl sulfide)
Quizalofop-ethyl
Rabenzazole
Resmethrin
Resmethrine I
Resmethrine II {CAS # 10453-86-8}
Rotenone
S,S,S-Tributylphosphorotrithioate
Schradan
Sebuthylazine
Sebuthylazine-desethyl
Secbumeton
Silafluofen
Silthiopham
Simazine
Simeconazole
Simetryn
Spirodiclofen
Spiromesifen
Spiroxamine I
Spiroxamine II {CAS # 118134-30-8}
Spiroxamine metabolite (4-tert-butylcyclo-hexanone)
Sudan I
Sudan II
Sudan Red
Sulfallate
Sulfanilamide
Sulfentrazone
Sulfotep
Sulfur (S8)
Sulprofos
Swep
Tamoxifen
TCMTB
Tebuconazole
Tebufenpyrad
Tebupirimifos
Tebutam
Tebuthiuron
Tecnazene
Tefluthrin, *cis*-
Temephos
Terbacil
Terbucarb
Terbufos
Terbufos-oxon-sulfone
Terbufos-sulfone
Terbumeton
Terbuthylazine
Terbuthylazine-desethyl
Terbutryne
Tetrachlorvinphos
Tetraconazole
Tetradifon
Tetraethylpyrophosphate (TEPP)
Tetrahydrophthalimide, *cis*-1,2,3,6-
Tetramethrin I
Tetramethrin II {CAS # 7696-12-0}
Tetrapropyl thiodiphosphate
Tetrasul
Thenylchlor
Theobromine
Thiabendazole
Thiazopyr
Thifluzamide
Thiofanox
Thiometon
Thionazin
Thymol
Tiocarbazil I
Tiocarbazil II {CAS # 36756-79-3}
Tolclofos-methyl
Tolfenpyrad
Tolylfluanid
Tolylfluanid metabolite (DMST)
Tolyltriazole [1H-Benzotriazole, 4-methyl-]
Tolyltriazole [1H-Benzotriazole, 5-methyl-]
Tonalide
Toxaphene Parlar 26
Toxaphene Parlar 50
Toxaphene Parlar 62
trans-Chlordane
Transfluthrin
Traseolide
Triadimefon
Triadimenol
Tri-allate
Triamiphos
Triapenthenol
Triazamate
Triazophos
Tributyl phosphate
Tributyl phosphorotrithioite
Trichlamide
Trichlorfon
Trichloronate
Triclopyr methyl ester
Triclosan
Triclosan-methyl
Tricresylphosphate, *meta*-
Tricresylphosphate, *ortho*-
Tricresylphosphate, *para*
Tricyclazole
Tridemorph, 4-tridecyl-
Tridiphane
Trietazine
Triethylphosphate
Trifenmorph
Trifloxystrobin
Triflumizole
Trifluralin
Triphenyl phosphate
Tris(2-butoxyethyl) phosphate
Tris(2-chloroethyl) phosphate
Tris(2-ethylhexyl) posphate
Triticonazole
Tryclopyrbutoxyethyl
Tycor (SMY 1500)
Uniconizole-P
Vamidothion
Vernolate
Vinclozolin
XMC (3,4-Dimethylphenyl N-methylcarbama
XMC (3,5-Dimethylphenyl N-methylcarbama
Zoxamide
Zoxamide decomposition product

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Rapid Determination of Hydroxymethylfurfural in Foods Using Liquid Chromatography-Mass Spectrometry

Application

Food Industry

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Abstract

Hydroxymethylfurfural is a product of food deterioration and is still under investigation for possible toxic effects. It can also be used to monitor food quality. A sensitive and selective LC/MS method for monitoring this compound is presented. The method can quantitatively determine hydroxymethylfurfural in food with a detection limit of 0.005 µg/g. Sample preparation and analytical conditions are given.

Introduction

Hydroxymethylfurfural (HMF) is recognized as an indicator of quality deterioration in a wide range of foods. It is formed as an intermediate in the Maillard reaction and is also formed during acid-catalyzed dehydration of hexoses. Formation of HMF in foods is especially dependent on temperature and pH [1].

In recent years, the presence of HMF in foods has raised toxicological concerns: the compound and its similar derivatives were shown to have cytotoxic, genotoxic, and tumoral effects. However, further studies suggest that HMF does not pose a serious health risk, but the subject is still a matter of debate.

Several HPLC techniques were reported for the determination of HMF in various foods. These techniques use UV detection because of the strong absorption of furfurals at approximately 280 to 285 nm. However, many compounds naturally present or formed in foods during processing may also absorb at this wavelength. Poor chromatographic resolution of these compounds may adversely affect the quantification of HMF during UV detection.

A rapid and reliable liquid chromatography/mass spectrometry (LC/MS) method was developed for the determination of HMF in foods. The method entailed aqueous extraction of HMF, solid-phase extraction (SPE) cleanup and analysis by LC/MS. The separation was performed on a narrow-bore column to shorten the chromatographic run.

Experimental

LC/MS experiments were performed using an Agilent 1100 series HPLC system consisting of a binary pump, an autosampler, and a temperature-controlled column oven, coupled to an Agilent 1100 MS detector equipped with atmospheric pressure chemical ionization (APCI) interface.

Data acquisition was performed in selected ion monitoring (SIM) mode using the interface parameters: drying gas (N_2, 100 psig) flow of 4 L/min, nebulizer pressure of 60 psig, drying gas temperatures of 325 °C, vaporizer temperature of 425 °C, capillary voltage of 4 kV, corona current of 4 µA, fragmentor voltage of 55 eV, and dwell time of 439 ms. Ions monitored for HMF were *m/z* 109 and *m/z* 127. The quantification was performed based on the signal response of the ion having *m/z* of 109.

LC/MS

Flow rate:	0.2 mL/min
Gradient:	ZORBAX Bonus RP, 100 mm × 2.1 mm, 3,5 µm
Mobile phase:	0.01 mM acetic acid in 0.2% aqueous solution of formic acid
Injection:	20 µL out of 1000 µL

MS conditions

Ionization mode:	Positive APCI
Nebulizer pressure:	60 psi
Drying gas flow:	4 L/min
Drying gas temperature:	325 °C
Vaporizer temperature:	425 °C
Skimmer:	20 V
Capillary voltage:	4kV
Fragmentor voltage:	55 eV
Dwell time:	439 ms

The chromatographic separations were performed on a ZORBAX Bonus RP Narrow Bore column (2.1 mm × 100 mm, 3.5 µm) using the isocratic mixture of 0.01 mM acetic acid in 0.2% aqueous solution of formic acid at a flow rate of 0.2 mL/min at 40 °C.

Method

Sample Preparation

Finely ground sample (1 g) was weighed into a 10-mL glass centrifuge tube with cap. Carrez I and II solutions were prepared by dissolving 15 g of potassium hexacyanoferrate and 30 g of zinc sulfate in 100 mL of water, respectively. A total of 100 µL Carrez I and 100 µL Carrez II solutions were added to the sample and the volume completed to 10 mL with 0.2 mM acetic acid. HMF was extracted by mixing the tube for 3 min using a vortex mixer. It was then centrifuged for 10 min at 5,000 rpm at 0 °C. The clear supernatant was further cleaned up by using Oasis HLB SPE cartridge. Prior to use, the SPE cartridge was conditioned by passing 1 mL of methanol and equilibrated by passing 1 mL of water at a flow rate of approximately two drops per second using a plastic 2-mL syringe. The excess water was removed from the cartridge by passing 2 mL of air. One milliliter of aqueous extract was eluted through the preconditioned cartridge at a flow rate of approximately one drop per second using a plastic syringe and the eluate was discarded. The cartridge was washed by passing 0.5 mL of water. Then the cartridge was dried under a gentle stream of nitrogen. HMF was eluted from the cartridge by passing 0.5 mL of diethyl ether at a flow rate of approximately one drop per second using a plastic 2-mL syringe. The eluate was collected in a conical bottom glass test tube placed in a water bath at 40 °C (Zymark Turbo Vap® LV Evaporator) and evaporated to dryness under nitrogen at 3 psig. The remaining residue was immediately redissolved in 1 mL of water by mixing in a vortex mixer for 1 min. Twenty microliters of this test solution was injected onto the HPLC system.

Results and Discussion

Positive APCI-MS analysis of HMF showed both the precursor [M+1] ion and the compound-specific ion [$C_6H_5O_2$] due to loss of water from the protonated molecule. See Figure 1. These characteristic

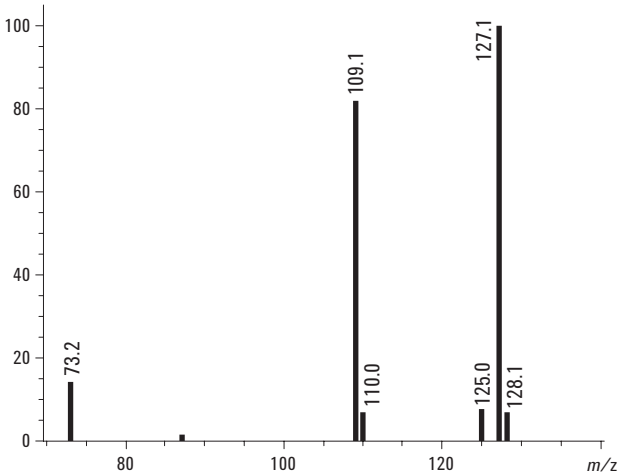


Figure 1. Mass spectrum for HMF obtained with positive APCI.

ions having *m/z* of 127 and 109 were used to monitor HMF in SIM mode. The ratio of these ions (response of ion 127/response of ion 109 = 1.12) was used to confirm the purity of HMF peak. The signal response was linear over a concentration range of 0.05 to 2.0 µg/mL for both ions with correlation coefficients of higher than 0.99. On the basis of a signal-to-noise ratio of 3, the limit of detection (LOD) was determined to be 0.005 µg/mL and 0.006 µg/mL for ions having *m/z* 127 and *m/z* 109, respectively. LC/MS with APCI was found to be a powerful tool that allowed us to determine HMF sensitively and precisely.

The chromatographic separation of HMF was performed on a ZORBAX Bonus RP narrow-bore column. The solution of 0.01 mM acetic acid in 0.2% aqueous solution of formic acid was used as the mobile phase at a flow rate of 0.2 mL/min to increase the ionization yield during MS detection with an adequate separation of HMF in the column from interfering matrix co-extractives. Under these conditions, HMF eluted at 5.087 min with good retention time reproducibility (5.09 ±0.04 min, n = 10). See Figure 2. The capacity factor (k') was determined to be 2.33 for HMF based on the hold-up time of 1.55 min.

Usual approach for the extraction of free furfurals from solid food matrices entails extraction with water followed by clarification using Carrez I and II reagents. Direct LC/MS analysis of aqueous extract showed the presence of interfering compounds. Oasis HLB cartridge packed with a macroporous copolymer of the lipophilic divinylbenzene and the hydrophilic N-vinylpyrrolidone was, there-

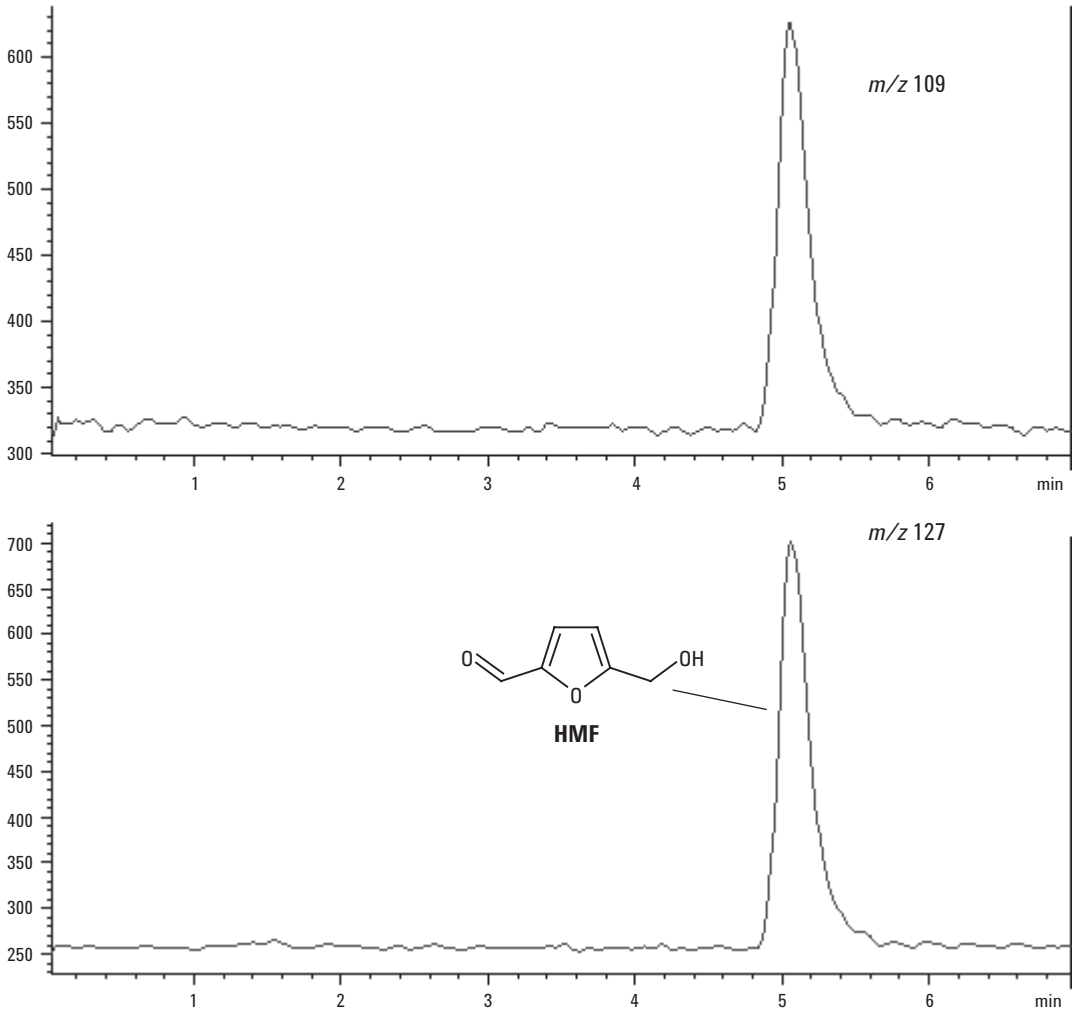


Figure 2. Extracted ion chromatogram (EIC) of an HMF standard (HMF concentration is 100 ng/mL).

fore, used to clean the extract prior to LC analysis. The clear aqueous extract was passed through a preconditioned cartridge. HMF present in the extract strongly interacted with the sorbent material while much of the co-extractives did not. HMF retained in the cartridge was then eluted with diethyl ether. It was determined that 0.5 mL of diethyl ether was sufficient to recover HMF from the cartridge completely.

SPE cleanup brought significant improvement for the detection of HMF using MS in SIM mode. Total ion chromatogram indicated the presence of three major peaks in the sample. HMF peak was identified by comparing both retention time and mass spectral data. The ratio of characteristic ions having *m/z* 127 and *m/z* 109 also confirmed the purity of HMF peak. The compound-specific ion [$C_6H_5O_2$] having *m/z* of 109 was found to be more selective than the parent compound ion. So, the quantification of HMF was performed using the signal response recorded for this ion.

The accuracy of the method was verified by analyzing spiked cereal-based baby foods. The recovery of HMF was determined by analyzing each of the

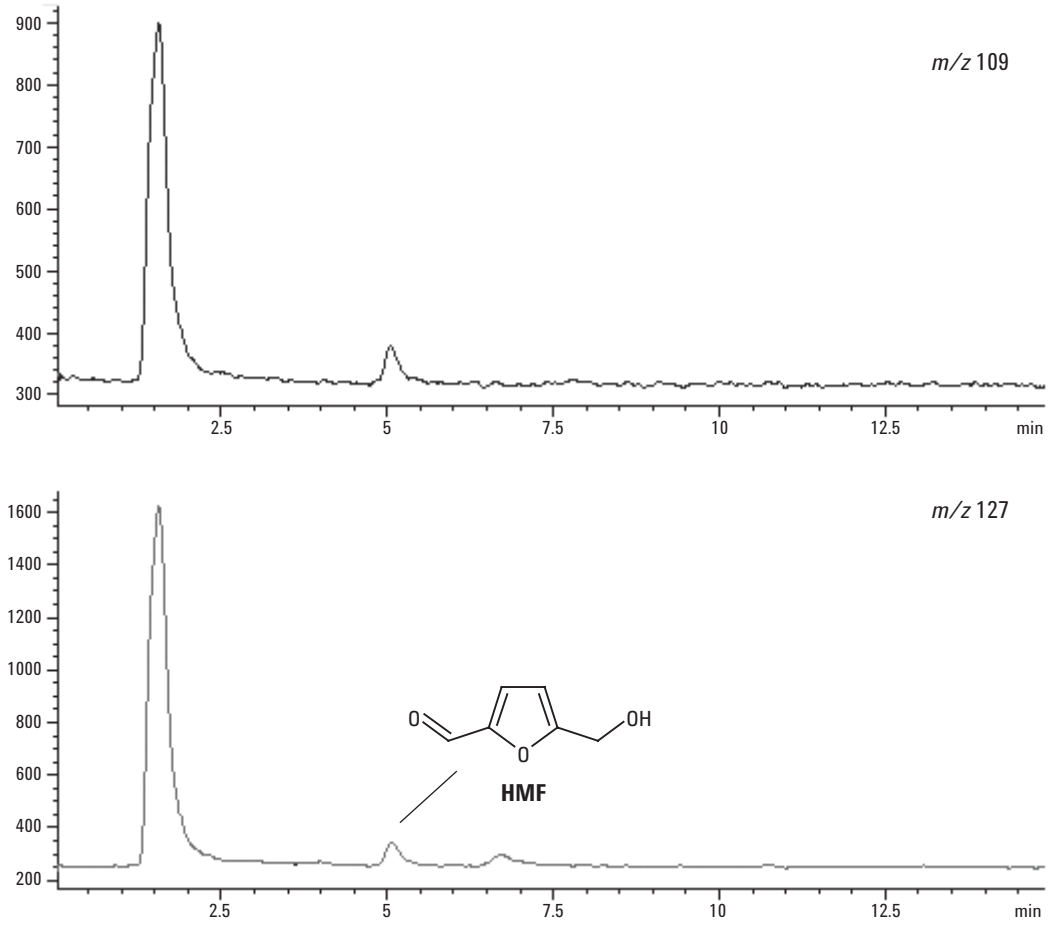


Figure 3. EIC of a fruited yogurt sample (HMF concentration is 0.2 µg/g).

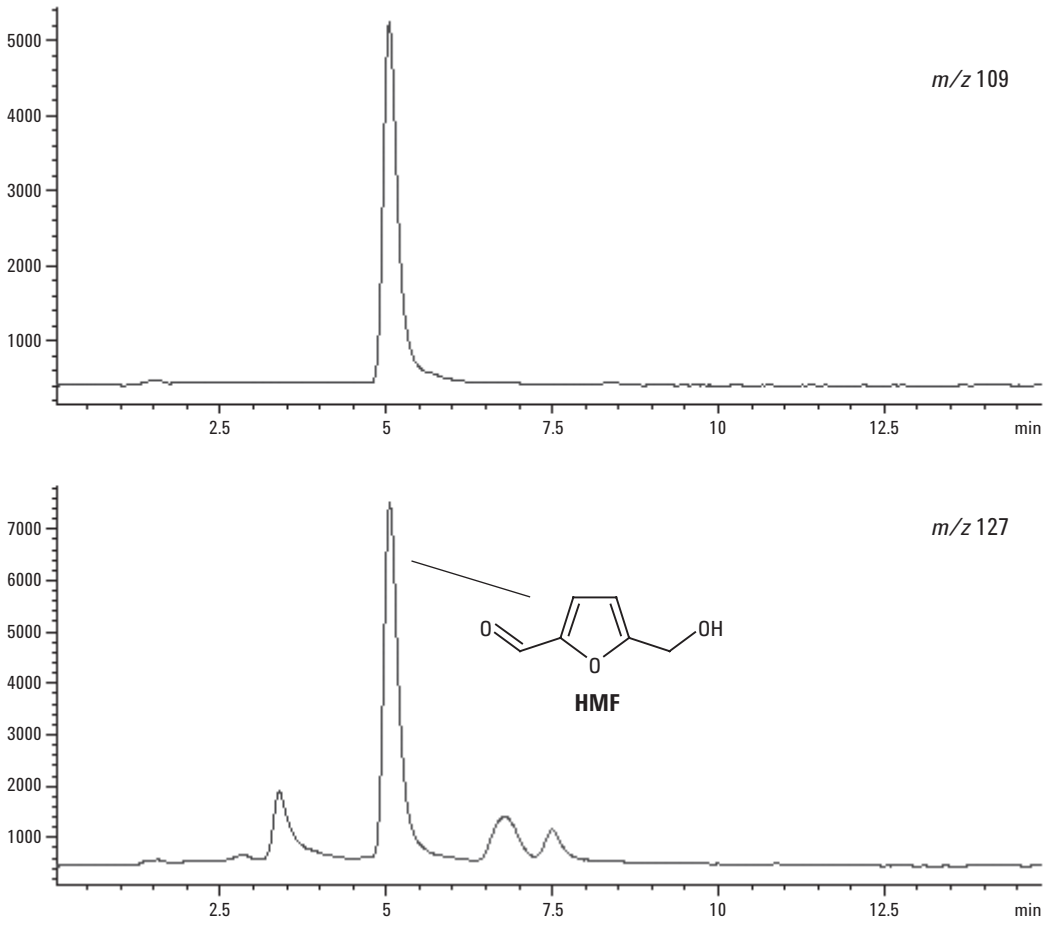


Figure 4. EIC of a crisp bread sample (HMF concentration is 17.5 µg/g).

spiked samples four times for spiking levels ranging from 0.25 to 5.0 µg/g. The mean percentage recoveries exceeded 90% for all levels.

The method is capable of low concentrations, but also high concentrations of HMF in foods precisely and accurately. Figure 3 illustrates the EICs of a fruited yogurt sample having 0.2 µg/g of HMF. It is difficult to measure such a low concentration of HMF using LC coupled to UV detection. Figure 4 illustrates the EICs of a crisp bread sample having 17.5 µg/g of HMF.

Conclusion

The growing attention of the scientific community with regard to the potentially toxic effects of HMF requires new efforts to be made to establish new rapid, reliable, and sensitive methods to determine HMF in real matrices. Previous methods usually dealt with the food items where HMF concentrations are comparatively higher and use extraction procedures that usually do not avoid potential interfering compounds prior to LC analysis. Presence of interferences may be problematic, particularly during the UV detection after LC separation when low concentrations of HMF are being measured in baby foods. The method described in this application combines 1) a rapid separation of HMF from the matrix co-extractives in a narrow-bore column, 2) an efficient cleanup of the extract using SPE, and 3) a selective detection of HMF using MS in a single analytical method.

Reference

1. V. Gökmen, H. Z. Senyuva, Improved method for the determination of hydroxymethylfurfural in baby foods using liquid chromatography-mass spectrometry, *Journal of Agricultural and Food Chemistry*, 2006, 54, 2845–2849.

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Determination of 44 Pesticides in Foodstuffs by LC/MS/MS

Application

Food Safety

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Abstract

A sensitive and selective analytical method for the determination of 44 pesticide residues in several foodstuffs using the Agilent G6410AA Triple Quadrupole Mass Spectrometer (QQQ) was developed. This method use two different sample preparation methods followed by LC/MS/MS (liquid chromatography/tandem mass spectrometry). The limits of detection for all pesticides were less than 10 ng/mL in foodstuff. The sensitivity of QQQ easily met the maximum residue limits (MRLs) of all investigated pesticides in Japan Food Hygiene Law.

Introduction

Pesticides are widely used in agricultural practices. The main application can be classified in production and post-harvest treatment of agricultural commodities for transport purposes. In this sense, production agriculture comprises the main category of use of pesticides subject to control requirements and, therefore, maximum residue levels (MRLs) have been fixed to assess food safety. In recent years, the established regulations regarding MRLs in commodities have been more and more stringent. In Japan, the positive list system was introduced this year, and MRLs have been set for over 500 pesticides in all foodstuffs. This new system sets different MRLs for each pesticide within each food group. Typically, the MRLs range from 0.01 to 3 µg/g depending on the commodities and pesticides. The low MRLs fostered the development of more sensitive analytical methods to meet the requirements of complex samples. In this sense, liquid chromatography/tandem mass spectrometry (LC/MS/MS) with QQQ in multiple reaction monitoring (MRM) mode has become so far the most widely used techniques for the quantitation of polar pesticides in food. MRM mode provides for more specific detection in a complex matrix such as food. In this work, 44 pesticides (Tables 1 and 2) are analyzed in two separate runs with sample analytical conditions. The sensitivity requirements set by the positive lost system for these pesticides are easily met.

Experimental

Chemicals

The acetonitrile was of LC/MS grade from Wako Pure Chemical Ind (Japan). Toluene, acetone, n-hexane, formic acid, sodium chloride, and anhydrous sodium sulfate were of analytical grade from Wako Pure Chemical. All SPE cartridges were purchased from Spelco Japan (Japan). Pesticide standards were obtained from Hayashi Pure Chemical (Japan).

Sample Preparation

Extraction

Vegetable and fruit samples were obtained from the local markets. A sample of 10 to 500 g was chopped in a food processor to obtain thoroughly mixed homogenates. A 20-g portion of sample homogenate was weighed in a 200-mL PTFE centrifuge tube. Then 50 mL of acetonitrile was added and blended in a Polytoron. The extract was then filtered by applying vacuum. The filtrate was collected and the residue was re-extracted with 20 mL of acetonitrile. The filtrates were combined in a 100-mL volumetric flask and made up to volume with acetonitrile. A 20-mL portion of the extract was transferred into a PTFE centrifuge tube, and 10 g of NaCl and 20 mL of 0.5 M phosphate buffer (pH 7.0) were added to the extract followed by shaking for 5 min. Five grams of anhydrous Na_2SO_4 were added to the acetonitrile layer obtained after salting out. After removing anhydrous Na_2SO_4, the extract was evaporated to dryness by rotary evaporator (water bath temperature did not exceed 40 °C). The residue was dissolved in 2 mL of acetonitrile-toluene (3:1).

Cleanup

Group 1 - The extract was loaded into a GCB/amino propyl SPE cartridge (500 ng/500 mg) preconditioned with 10 mL of acetonitrile-toluene (3:1). The 20 mL of acetonitrile-toluene (3:1) was further added to the SPE cartridge. All eluate was collected and evaporated by rotary evaporator. The residue was dissolved in 4 mL of methanol.

Group 2 - The extract was loaded into a silica gel SPE cartridge (500 mg) preconditioned with 10 mL each of methanol, acetone, and n-hexane (10 mL of methanol, 10 mL of acetone, and 10 mL of n-hexane, total volume is 30 mL). The 10 mL of acetone-triethylamine-n-hexane (20:0.5:80) was further added to the SPE cartridge. All eluate was discarded. The 20 mL of acetone-methanol (1:1) was applied and the eluate was collected and evaporated by rotary evaporator. The residue was dissolved in 4 mL of methanol.

Standard Preparation

Stock solutions of individual pesticides were prepared in methanol at 1 µg/mL. Serial dilutions using methanol produced a range of standard mixture solutions at 0.001 µg/mL to 1 µg/mL.

The blank matrix residues were fortified with a mixture of pesticides studied at 10 ng/g.

LC/MS/MS Instrument

The LC/MS/MS system used in this work consists of an Agilent 1100-series vacuum degasser, binary pump, well-plate autosampler, thermostatted column compartment, and the Agilent G6410 Triple Quadrupole Mass Spectrometer with an electrospray ionization source (ESI). The objective of the method development was to obtain a fast and sensitive analysis for quantifying pesticides in fruits and vegetables. For chromatographic resolution and sensitivity, different solvents and columns were optimized. It was found that a simple solvent system using water, acetonitrile, formic acid, formic acetate, and a 1.8-µm particle size C18 column would work very well.

LC Conditions

Instrument:	Agilent 1100 HPLC
Column:	ZORBAX Extend C18, 100 mm × 2.1 mm, 1.8 µm (p/n 728700-902)
Column temp:	40 °C
Mobile phase:	A = 0.1% formic acid +5 mM ammonium formate in water B= Acetonitrile
Gradient:	10% B at 0 min, 80% B at 30 min
Flow rate:	0.2 mL/min
Injection vol:	5 µL

MS Conditions

Instrument:	Agilent 6410 QQQ
Source:	Positive ESI
Drying gas flow:	10 L/min
Nebulizer:	50 psig
Drying gas temp:	350 °C
V_{cap}:	4000 V
Scan:	*m/z* 100 to 550
Fragmentor:	Variable 100 V
MRM ions:	Shown in Tables 1 and 2
Collision energy:	Shown in Tables 1 and 2

LC/MS/MS Method

Quantitative analysis was carried out using MRM mode with time program. The parameters of MRM transition are shown in Tables 1 and 2.

Table 1. Data Acquisition Parameters of MRM Transitions of Each Pesticide in Group 1

No	Pesticides	RT (min)	Molecular weight	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy(V)
1	Thiabendazole	5.018	201	202	175	20
2	Thiamethoxam	6.16	291	292	211	5
3	Clothianidin	7.83	249	250	169	10
4	Chloridazon	8.19	221	222	104	10
5	Imidacloprid	8.39	255	256	209	20
6	Dimethirimol	8.8	209	210	171	20
7	Oxycarboxine	11.02	267	268	175	10
8	Thiacloprid	11.03	252	253	126	20
9	Azamethiophos	12.87	324	325	183	10
10	Ferimzone(E)	13.21	254	255	124	20
11	Ferimzone(Z)	13.7	254	255	132	20
12	Phenmedipham	17.77	317	318	136	20
13	Azinphos-methyl	17.9	318	132	77	15
14	Simeconazole	18.5	293	294	70	15
15	Isoxaflutol	18.7	359	360	251	15
16	Pyriftalid	18.7	318	319	139	20
17	Tridemorph	19.21	297	298	130	15
18	Methoxyfenozide	20.06	312	313	149	20
19	Chromafenozide	20.57	394	175	141	20
20	Fenoxycarb	20.63	301	302	88	15
21	Naproanilide	21.27	291	292	171	10
22	Butafenacil	21.55	491	492	331	20
23	Cyazofamide	21.7	324	325	108	10
24	Anilofos	22.5	367	368	199	10
25	Pyrazolate	23.5	438	439	173	15
26	Benzofenap	24	430	431	105	20
27	Cyflufenamid	24.3	412	413	241	20
28	Indoxacarb	24.37	527	528	150	15
29	Clomeprop	24.78	372	373	299	5
30	Cloquincet-mexyl	24.8	335	336	238	15
31	Furathiocarb	25.7	365	383	195	15
32	Lactofen	26.3	478	479	344	15
33	Tralkoxydim	26.7	329	330	284	10

Table 2. Data Acquisition Parameter of MRM Transitions of Each Pesticide in Group 2

No	Pesticides	RT (min)	Molecular weight	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (V)
1	Flumetsulam	9.96	325	326	129	20
2	Thidiazuron	11.95	220	221	102	10
3	Imazaquin	12.25	311	312	267	20
4	Thifensulfuron-methyl	12.89	387	388	167	10
5	Florasulam	13.75	359	360	129	20
6	Forchlorfenuron-methyl	14.63	247	248	129	10
7	Clorasulam-methyl	16.41	429	430	398	10
8	Diclosulam	16.83	405	406	161	20
9	Fomesafen	18.27	438	456	344	10
10	Triflusulfuron-methyl	19.29	492	493	264	15
11	Haloxyfop	19.67	361	362	316	15

Results and Discussion

Optimization of MRM Transitions

Determination of the optimal MRM transitions for each pesticide was carried out using full scan mode followed by product ion scan mode using two pesticide standard mixtures at 1 µg/mL. TICs of these standard mixtures in full scan mode and product ion scan mode are shown in Figures 1 and 2. The mass spectrum of each pesticide by full scan mode exhibited protonated molecular ions; $[M+H]^+$ as the base peak ion except azinphos-methyl, furathiocarb, and fomesafen, which exhibited fragment ion and ammonium adduct ion $[M+NH_4]^+$. These ions were selected as precursor ions for MRM mode. It was possible to generate individual product ion MS/MS spectrum of each pesticide by using multiple acquisition and time programming mode. As shown in Tables 1 and 2, 10 time segments for 33 pesticides in group 1 and 7 time segments for 11 pesticides in group 2 were used for MRM mode.

Total ion chromatograms of pesticide standard mixture corresponding to the minimum MRL value for pesticides (10 ng/mL) are shown in Figure 3. These show excellent signal-to-noise (S/N) ratios for all pesticides. The limit of detection (LOD) for each pesticide was determined using an S/N ratio of 3 with an MRM chromatogram of each pesticide at 1 ng/mL (see Table 3). To evaluate the linearity of the calibration curves, various concentrations of pesticide standard solutions ranging from 0.001 ng/mL to 1 ng/mL were analyzed. As shown in Table 3, the linearity was very good for all pesticides with correlation coefficients (r^2) greater than 0.998

The matrix effect of this method was investigated by using orange, apple, potato, and cabbage extracts spiked with pesticide standards at 10 ng/mL. Typical MRM chromatograms of orange extract are shown in Figures 4 and 5. The other chromatograms of apple, potato, and cabbage extract are shown in Figure 6. There was not additional peak from sample matrix in all food when compared with the pesticide standard mixture. These results indicate that MRM mode has very high selectivity.

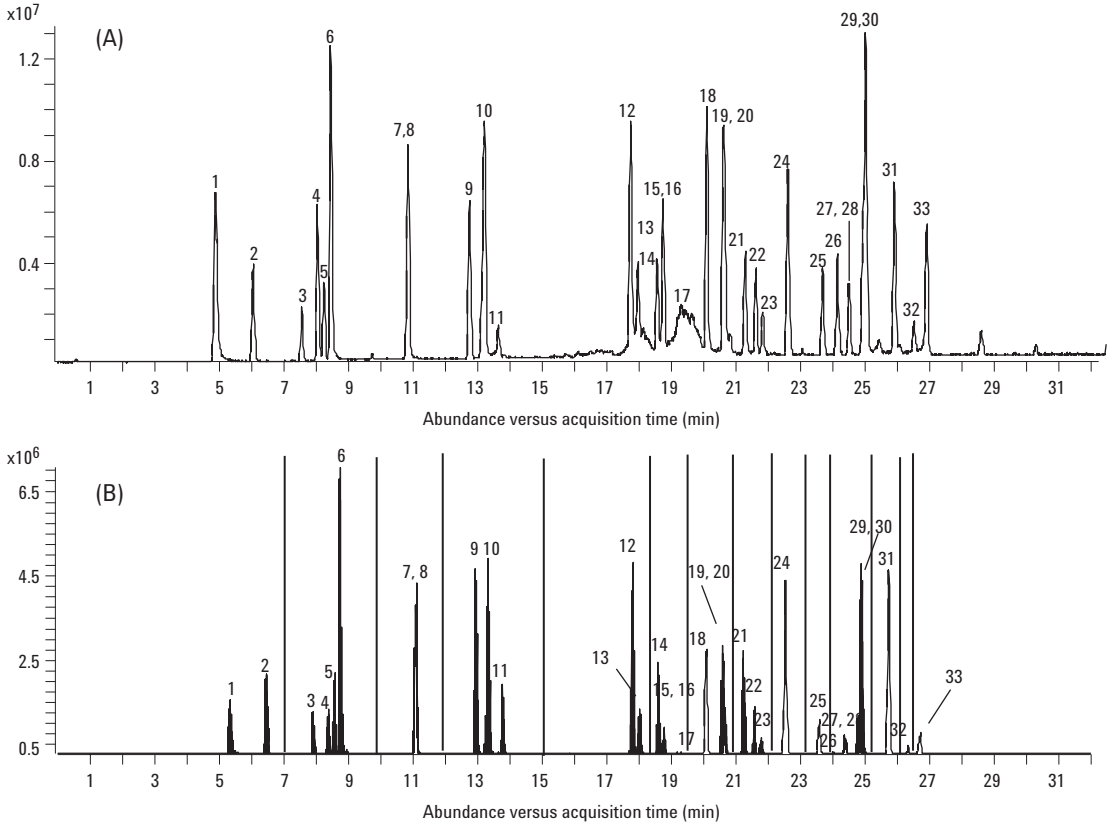


Figure 1. TIC of 33 pesticides standard in full scan mode (A) and product ion scan mode (B) at 1 µg/mL.

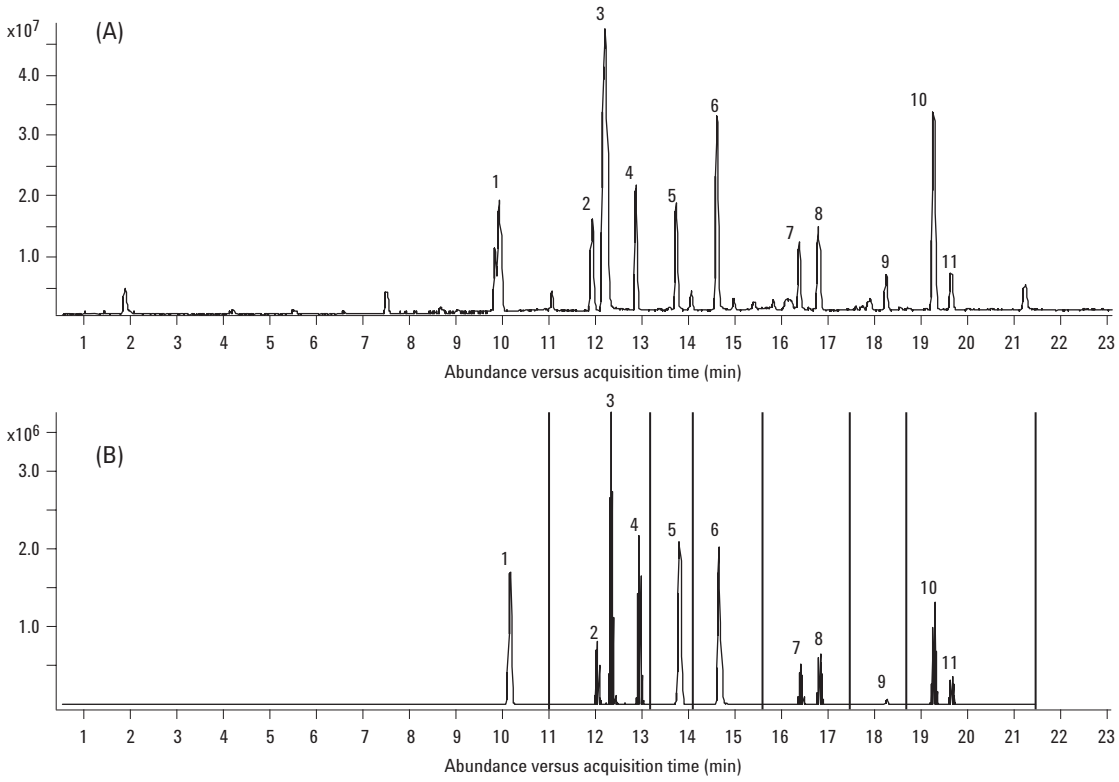


Figure 2. TIC of 11 pesticides standard in full scan mode (A) and product ion scan mode (B) at 1 µg/mL.

Furthermore, the change on the peak intensity of each pesticide by sample matrix was calculated by comparing with the peak intensity of pesticide standards. As these results show in Table 4, the relative intensity of each pesticide ranged from 91 to 116%. Thus, matrix effect such as ion suppression may be insignificant and it was possible to use external standards instead of matrix matched standards. The repeatability of each pesticide in orange extract is also shown in Table 4, and the RSD of each pesticide was in the range from 1.7 to 5.9%.

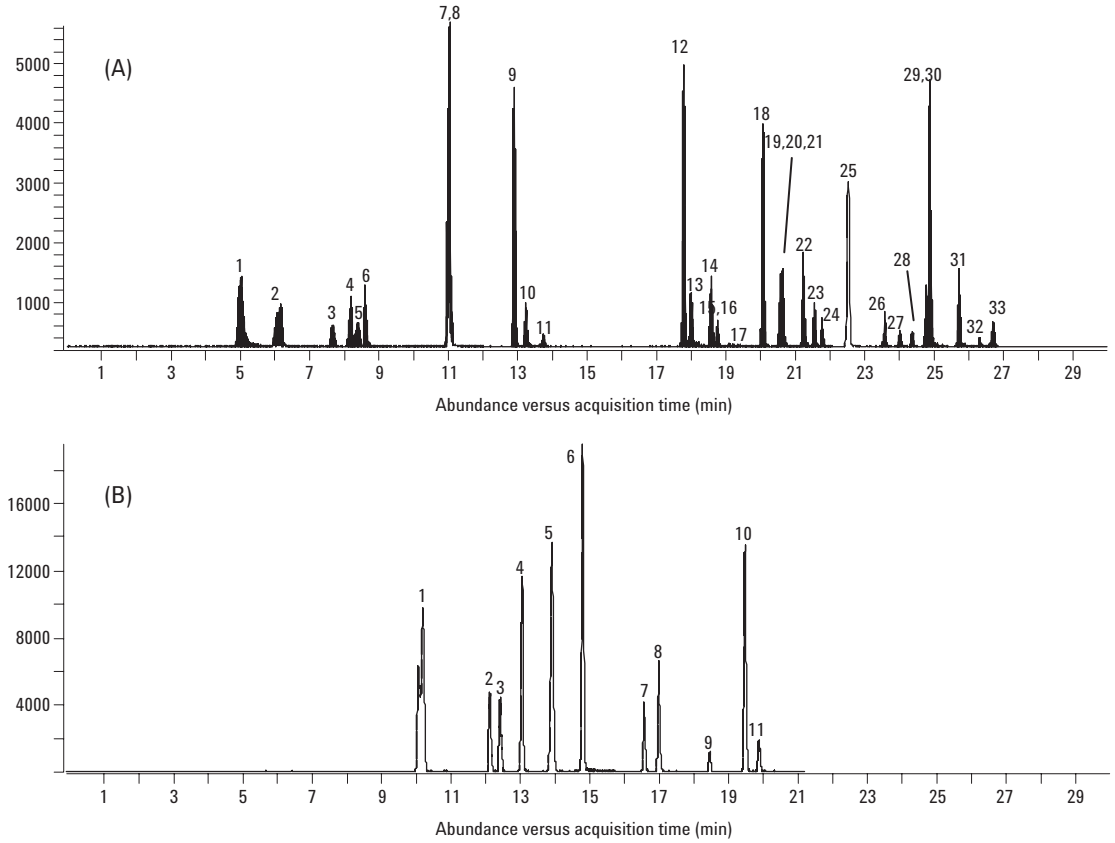


Figure 3. TIC of 33 pesticide standards (A) and 11 pesticides standard (B) at 10 ng/mL in MRM mode.

Table 3. Linearity and LOD of 44 Pesticide Standard Solutions

No	Pesticides	r^2	LOD (ng/mL)	No	Pesticides	r^2	LOD (ng/mL)
Group 1							
1	Thiabendazole	0.9999	<0.1	18	Methoxyfenozide	0.9993	0.55
2	Thiamethoxam	0.9992	<0.1	19	Chromafenozide	0.9992	0.49
3	Clothianidin	0.9999	<0.1	20	Fenoxycarb	0.9988	<0.1
4	Chloridazon	0.9993	<0.1	21	Naproanilide	0.9993	<0.1
5	Imidacloprid	0.9995	<0.1	22	Butafenacil	0.9994	<0.1
6	Dimethirimol	0.9989	<0.1	23	Cyazofamide	0.9987	0.43
7	Oxycarboxine	0.9993	<0.1	24	Anilofos	0.9991	<0.1
8	Thiacloprid	0.9991	<0.1	25	Pyrazolate	0.9990	0.51
9	Azamethiophos	0.9988	<0.1	26	Benzofenap	0.9982	0.49
10	Ferimzone(E)	0.9993	0.34	27	Cyflufenamid	0.9993	0.43
11	Ferimzone(Z)	0.9995	0.53	28	Clomeprop	0.9993	0.61
12	Phenmedipham	0.9993	<0.1	29	Indoxacarb	0.9991	1.04
13	Azinphos-methyl	0.9997	<0.1	30	Quinclorac-methyl	0.9988	0.63
14	Simeconazole	0.9992	<0.1	31	Furathiocarb	0.9987	<0.1
15	Isoxaflutol	0.9991	<0.1	32	Lactofen	0.9987	1.10
16	Pyriftalid	0.9988	<0.1	33	Tralkoxydim	0.9992	0.52
17	Tridemorph	0.9991	1.21				
Group 2							
1	Flumetsulam	0.9996	<0.1	7	Clorasulam-methyl	0.9987	<0.1
2	Thidiazuron	0.9994	<0.1	8	Diclosulam	0.9989	<0.1
3	Imazaquin	0.9992	<0.1	9	Fomesafen	0.9989	0.32
4	Thifensulfuron-methyl	0.9989	<0.1	10	Triflusulfuron-methyl	0.9992	<0.1
5	Florasulam	0.9969	<0.1	11	Haloxyfop	0.9995	0.19
6	Forchlorfenuron-methtyl	0.9977	<0.1				

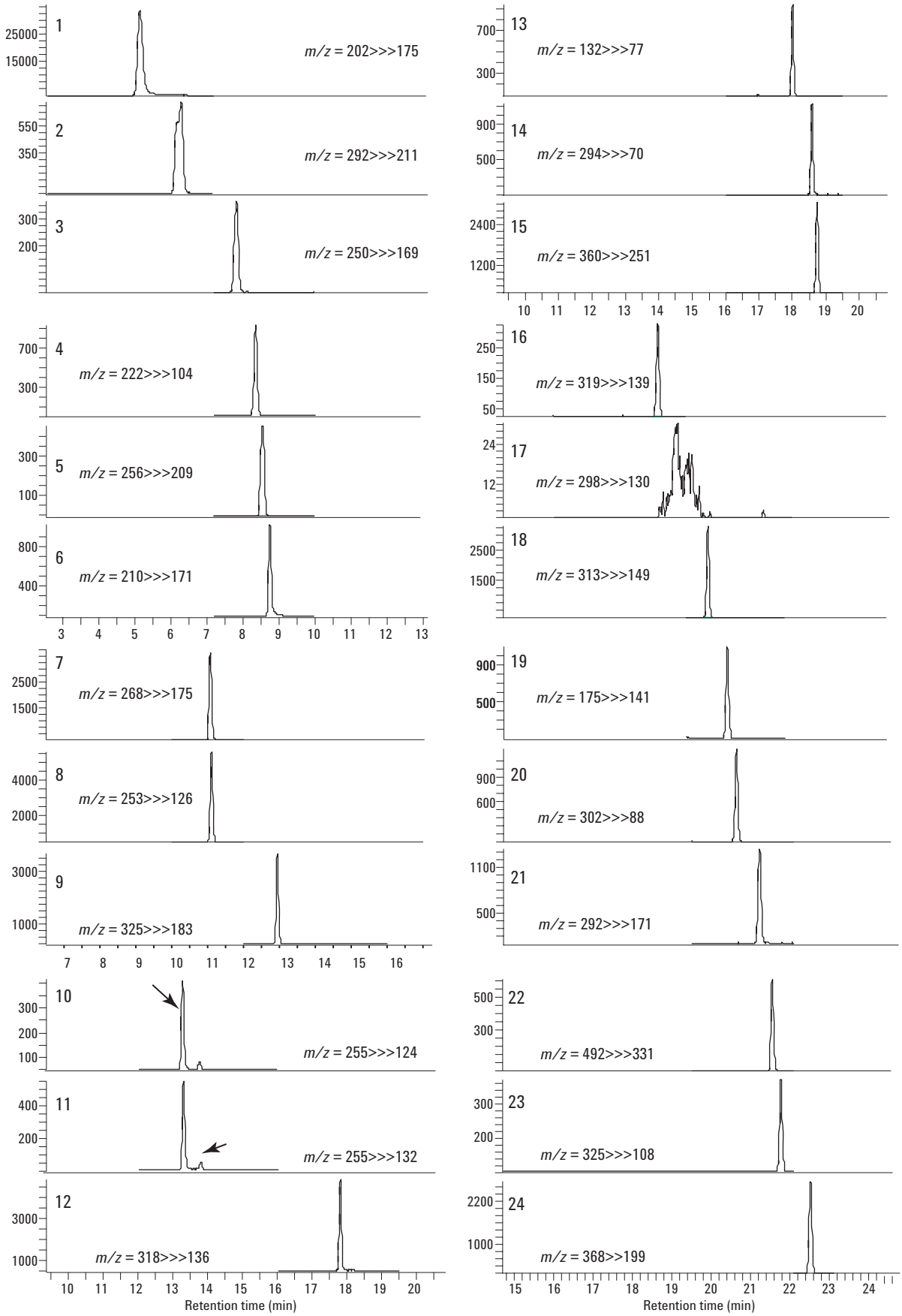


Figure 4. MRM of 33 pesticides in orange extract spiked at 10 ng / mL. (Continued)

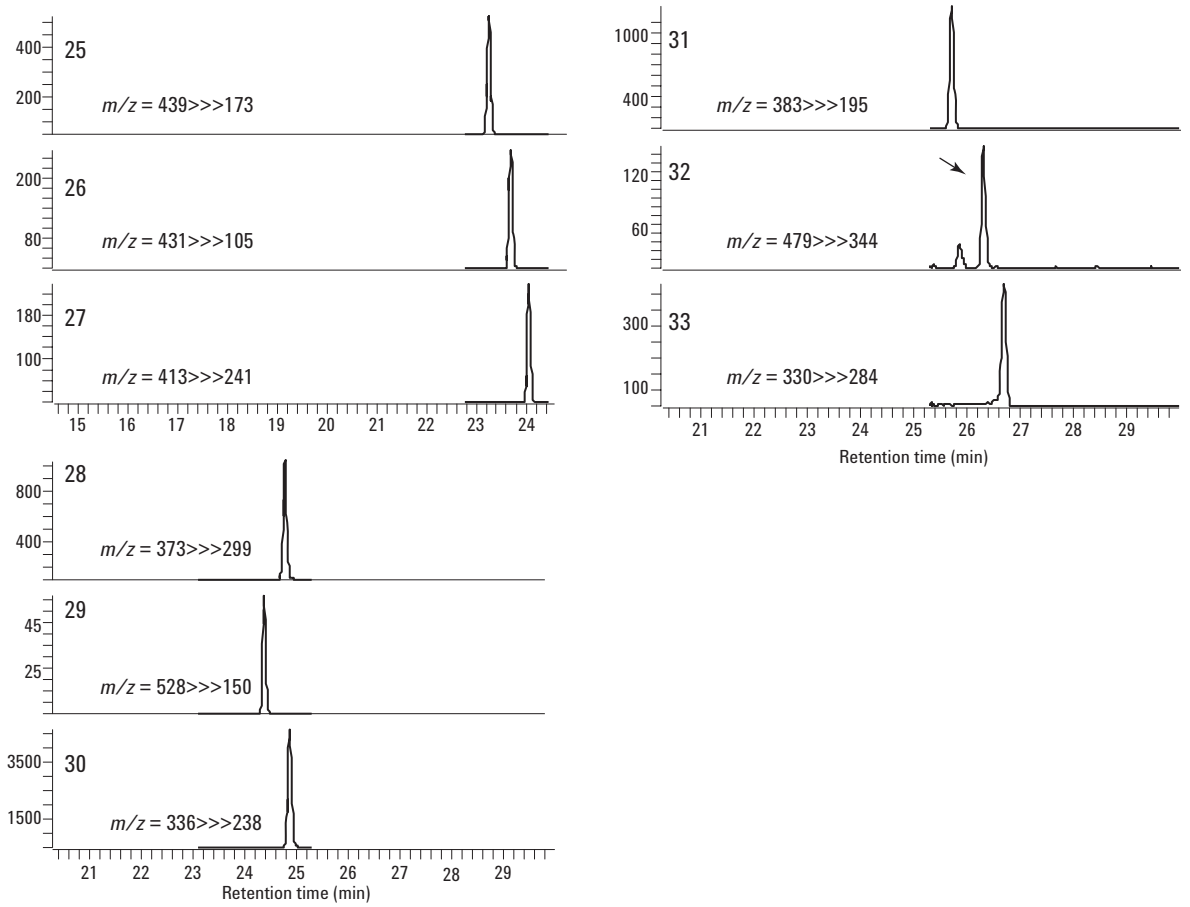


Figure 4. MRM of 33 pesticides in orange extract spiked at 10 ng / mL.

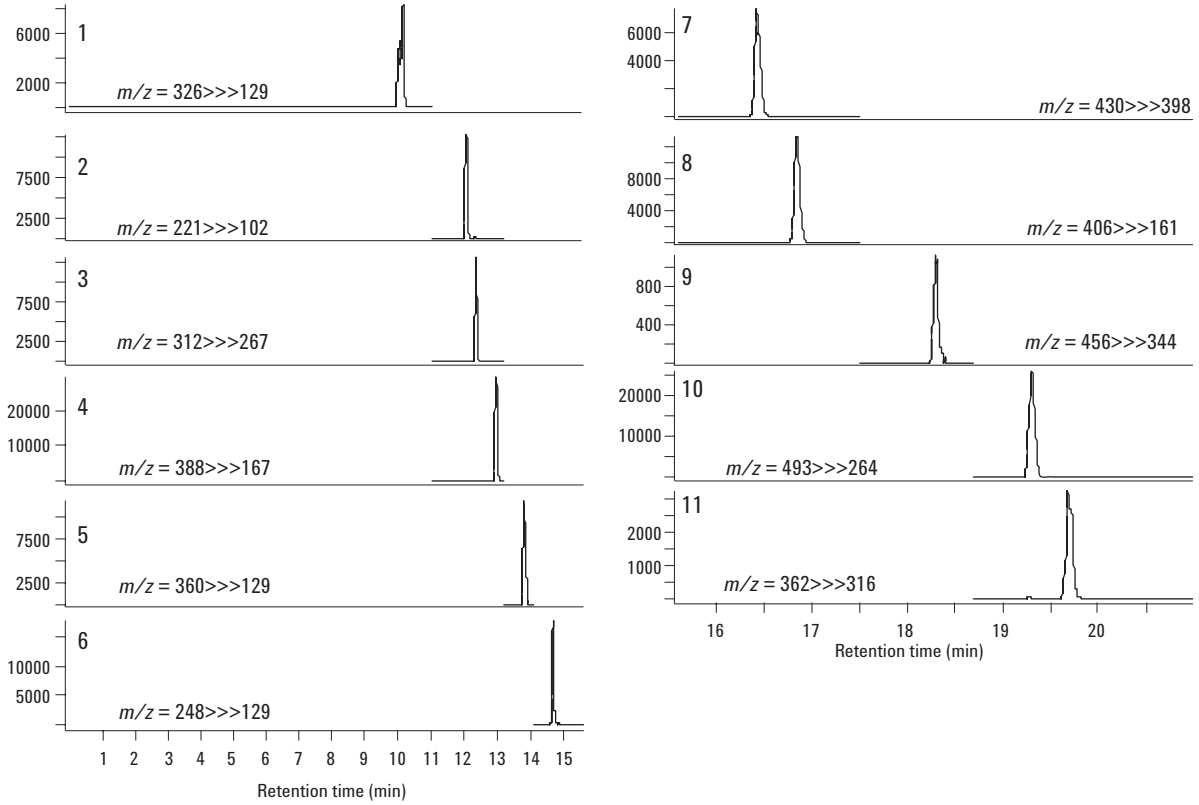


Figure 5. MRM of 11 pesticides in orange extract spiked at 10 ng / mL.

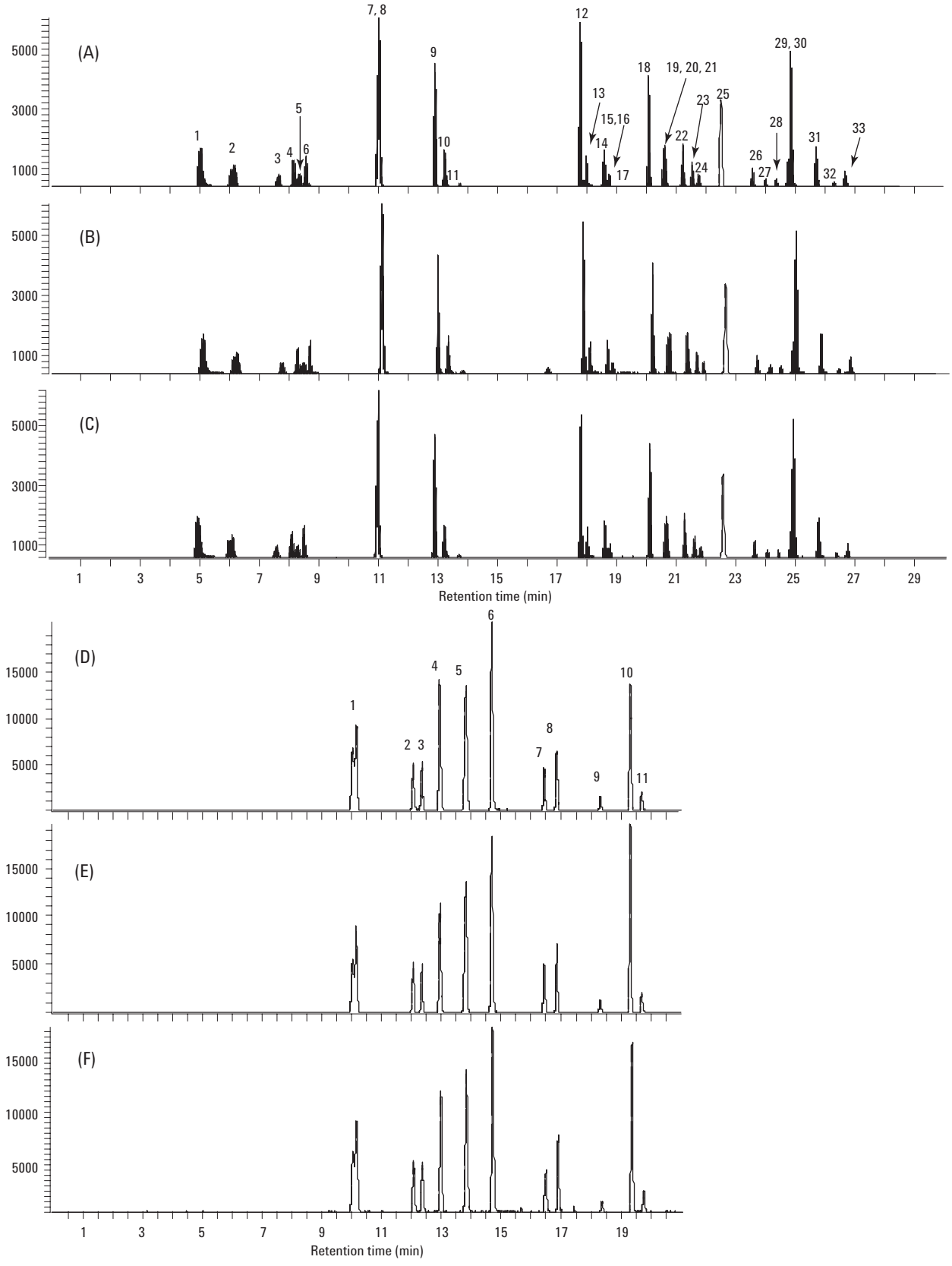


Figure 6. TIC of spiked at 10 ng/mL.

Table 4. Relative Intensity of Each Pesticide in Sample Extracts

No	Pesticides	Relative intensity(%)			
Group 1		**Orange***	**Cabbage**	**Apple**	**Potato**
1	Thiabendazole	105 (3.2)	101	116	107
2	Thiamethoxam	103 (2.1)	98	104	105
3	Clothianidin	106 (2.9)	101	109	112
4	Chloridazon	105 (3.3)	106	101	109
5	Imidacloprid	102 (1.7)	97	102	104
6	Dimethirimol	103 (4.6)	107	103	108
7	Oxycarboxine	106 (3.7)	102	104	106
8	Thiacloprid	104 (3.1)	104	106	108
9	Azamethiophos	93 (4.6)	90	94	84
10, 11	Ferimzone(E,Z)	116 (4.1)	109	102	112
12	Phenmedipham	96 (5.3)	99	100	104
13	Azinphos-methyl	90 (2.1)	103	104	110
14	Simeconazole	104 (4.4)	102	106	110
15	Isoxaflutol	102 (2.7)	104	108	103
16	Pyriftalid	97 (4.1)	103	104	93
17	Methoxyfenozide	92 (3.1)	99	104	97
18	Chromafenozide	96 (2.8)	102	103	101
19	Tridemorph	97 (3.4)	96	100	111
20	Fenoxycarb	99 (2.1)	105	102	101
21	Naproanilide	91 (4.3)	97	98	103
22	Butafenacil	102 (2.6)	114	104	114
23	Cyazofamide	93 (3.5)	92	87	95
24	Anilofos	102 (2.7)	105	103	107
25	Pyrazolate	103 (4.7)	101	103	97
26	Benzofenap	108 (5.2)	111	98	108
27	Cyflufenamid	108 (3.4)	110	105	101
29	Indoxacarb	109 (2.6)	105	100	111
28	Clomeprop	105 (4.2)	107	106	104
30	Cuinclorac-methyl	105 (4.1)	104	104	105
31	Furathiocarb	102 (1.8)	104	105	101
32	Lactofen	100 (3.7)	109	105	112
33	Tralkoxydim	101 (3.3)	111	102	117
Group 2					
1	Flumetsulam	97 (2.6)	110	156	104
2	Thidiazuron	104 (4.8)	101	102	113
3	Imazaquin	105 (3.1)	100	100	101
4	Thifensulfuron-methyl	106 (2.9)	112	116	113
5	Florasulam	99 (3.1)	106	103	109
6	Forchlorfenuron-methyl	101 (4.4)	103	100	108
7	Clorasulam-methyl	94 (3.9)	104	97	142
8	Diclosulam	95 (3.3)	102	96	107
9	Fomesafen	99 (5.9)	101	95	109
10	Triflusulfuron-methyl	97 (4.1)	111	104	108
11	Haloxyfop	108 (4.8)	114	110	124

*( ): RSD,% calculated based on five replicates within one day

Conclusions

The multiresidue method by LC/MS/MS described here was suitable for the determination of 44 pesticides in a variety of food samples due to its high sensitivity and high selectivity. Another advantage of this method is that ion suppression was not observed for all food samples studied. Thus, it may eliminate the need for matrix-matched standards, which make analysis more tedious for samples from different origins.

For more details concerning this application, please contact masahiko_takino@agilent.com

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Multiresidue Analysis of 100 Pesticides in Food Samples by LC / Triple Quadrupole Mass Spectrometry

Application

Food Safety

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Abstract

An analytical methodology for confirming the presence of a group of 100 pesticides in vegetable and fruit samples was developed using the Agilent G6410AA Triple Quadrupole Mass Spectrometer (QQQ). One transition per parent compound was monitored in a single chromatographic run containing two time segments. The sensitivity obtained meets the maximum residue levels (MRLs) established by the European Union regulation for food monitoring programs. The analytical performance of the method was evaluated for different types of fruit and vegetables — orange, tomato, and green pepper — showing little or no matrix effects. Linearity of response over two orders of magnitude was demonstrated (r > 0.99). This study is a valuable indicator of the potential of the QQQ for routine quantitative multiresidue analysis of pesticides in vegetables and fruits.

Introduction

In recent years, the established regulations regarding the maximum residue limits (MRLs) in commodities have become more and more stringent. The European Union (EU) has set new directives for pesticides at low levels in vegetables in order to meet health concerns. For fruits and vegetables intended for production of baby food, an MRL of 10 µg/kg is applicable for all pesticides, and compounds without a stated regulation also have the lowest MRLs at 10 µg/kg. The low MRLs have encouraged the development of more sensitive analytical methods to meet the requirements in complex samples. In this sense, liquid-chromatography tandem-mass spectrometry (LC-MS-MS) with triple quadrupole in multiple reaction monitoring (MRM) mode has become, so far, the most widely used technique for the monitoring and quantitation of pesticides in food, as reported extensively in the literature. On the other hand, high-resolving power mass spectrometric techniques, such as time-of-flight mass spectrometry (TOF-MS), have been applied recently for screening purposes as well. Nevertheless, the simplicity of methodologies using triple quadrupole as a detection technique, together with the low limits of detection achieved and the MS/MS capability make this technique a valuable tool for routine

monitoring programs established in regulatory official laboratories. The easiness of use is sometimes an essential for these types of regulatory agencies, which lack the high-skilled personnel required for more sophisticated techniques such as TOF-MS. Triple quadrupole technology is not new in the sense that it needs to be validated for monitoring purposes and its basis is already well-established for routine analysis.

Our study in this report is one of the first of its kind to examine the new Agilent Triple Quad for the analysis of pesticides in fruit and vegetables. This topic was chosen because of the relevance of these compounds and their significant use on food commodities. The sensitivity of the QQQ easily meets the levels required by the regulations on pesticides in food.

Experimental

Sample preparation

Pesticide analytical standards were purchased from Dr. Ehrenstorfer (Ausburg, Germany). Individual pesticide stock solutions (around 1,000 µg/mL) were prepared in pure acetonitrile or methanol, depending on the solubility of each individual compound, and stored at –18 °C. From these mother solutions, working standard solutions were prepared by dilution with acetonitrile and water.

Vegetable samples were obtained from the local markets. "Blank" vegetable and fruit extracts were used to prepare the matrix-matched standards for validation purposes. In this way, two types of vegetables and one fruit (green peppers, tomatoes, and oranges) were extracted using the QuEChERS method already described in a previous application [1]. The vegetable extracts were spiked with the mix of standards at different concentrations (ranging from 2 to 100 µg/kg) and subsequently analyzed by LC/MS/MS.

LC / MS / MS Instrumentation

LC Conditions

Column:	Agilent ZORBAX Eclipse® XDB C-8, 4.6 mm × 150 mm, 5 µm, (p/n 993967-906).
Column temperature:	25 °C
Mobile phase:	A = 0.1% formic acid in water B= Acetonitrile
Flow-rate:	0.6 mL/min
Gradient:	10% B at 0 min 10% B at 5 min 100% B at 30 min
Injection volumes:	1-5 µL

MS Conditions

Mode:	Positive ESI using the Agilent G6410AA Triple Quadrupole Mass Spectrometer
Nebulizer:	40 psig
Drying gas flow:	9 L/min
V capillary:	4000 V
Drying gas temperature:	350 °C
Q1 resolution:	Unit
Q2 resolution:	Unit
Fragmentor voltage:	70 V
Collision energy:	5–25 V
MRM:	1 transition for every compound as shown in Table 1
Dwell time:	15 msec

Results and Discussion

Optimization of LC / MS / MS conditions

A preliminary study of the optimal MRM transitions for every compound was carried out by injecting groups of analytes (around 10 analytes in one chromatographic run) at a concentration level of 10 µg/mL. Various collision energies (5, 10, 15, 20, and 25 V) were applied to the compounds under study. The optimum energies were those that gave the best sensitivity for the main fragment ion and, as a general rule, left about 10% of parent compound in the spectra, and they were selected as optimum ones. Only one fragment ion was chosen as the most abundant product ion for every target compound. Results are shown in Table 1.

Table 1. Analytical Conditions and Limits of Detection (LOD) for Each of the Compounds Tested

Compound name	Retention time (min)	Protonated molecule $[M+H]^+$	Product ion (*m/z*)	Collision energy	LOD (pg)
Segment 1					
Cyromazine	2.7	167	125	20	10
Thiosultap	2.7	312	232	10	90
Cartap	3	150	105	15	10
Thiocyclam	4.5	182	137	10	8
Aldicarb sulfoxide	6.4	207	89	5	9
Carbendazim	6.6	192	160	15	5
Thiabendazole	7.9	202	175	25	10
Aldicarb sulfone	10.8	223	148	5	50
Nitenpyram	11	271	225	10	7
Hydroxyatrazine	11.2	198	156	15	3
Methomyl	11.5	163	88	5	4
Deisopropylatrazine	11.9	174	132	15	18
Imazapyr	12.5	262	234	15	8
Metamitron	13.9	203	175	15	8
Fenuron	14.5	165	72	15	2
Deethylatrazine	14.8	188	146	15	4
Imidacloprid	14.8	256	209	10	7
Dimethoate	15.4	230	199	5	7
Acetamiprid	15.5	223	126	15	6
Prometon	15.7	226	184	20	4
Irgarol metabolite	16	214	158	15	0.8
Methiocarb sulfone	16.4	258	122	5	6
Nicosulfuron	16.9	411	182	15	6
Thiacloprid	17	253	126	15	3
Imazalil	17.2	297	159	15	7
Mebendazole	17.2	296	264	20	2
Aldicarb	17.5	213	89	10	10
Imazaquin	17.8	312	284	20	15
Oxadixyl	17.9	279	219	10	10
Fluroxypyr	17.9	255	209	10	120
Simazine	18	202	132	15	5
Monuron	18	199	72	10	2
Lenacil	18.4	235	153	10	20
Cyanazine	18.5	241	214	10	70
Metolcarb	18.5	166	109	5	2
Spiroxamine	18.6	298	144	15	10
Dichlorvos	18.7	221	109	15	10
Metribuzin	18.9	215	187	15	5
Chlorotoluron	19.4	213	72	15	3
Prometryn	19.5	242	200	20	2
Terbutryn	19.5	242	186	15	1
Carbofuran	19.6	222	165	10	2
Bendiocarb	19.7	224	167	5	2
Segment 2					
Spinosad A	20	732	142	5	12
Carbaryl	20.1	202	145	5	2
Irgarol 1051	20.3	254	198	15	0.1
Atrazine	20.3	216	174	15	0.3
Metalaxyl	20.4	280	248	10	5
Difenoxuron	20.4	287	123	15	5
Isoproturon	20.4	207	72	15	1
Bensultap	20.5	432	290	15	6

Table 1. Analytical Conditions and Limits of Detection (LOD) for Each of the Compounds Tested (Continued)

Compound name	Retention time (min)	Protonated molecule $[M+H]^+$	Product ion (*m/z*)	Collision energy	LOD (pg)
Diuron	20.5	233	72	15	5
Spinosad D	20.7	746	558	5	100
Ethiofencarb	20.7	226	107	5	5
Dimethomorph isomer 1	21.3	388	301	20	11
Propachlor	21.6	212	170	10	1
Dimethomorph isomer 2	21.7	388	301	20	8
Prochloraz	21.9	376	308	10	6
Propanil	22.2	218	162	15	10
Cyproconazole	22.5	292	70	10	6
Methiocarb	22.6	226	169	5	15
Terbutylazine	22.7	230	174	15	0.3
Bromuconazole isomer 1	22.8	376	159	20	6
Fenamiphos	23	304	217	15	0.7
Methidathion	23	303	145	5	5
Azoxystrobin	23.2	404	372	10	0.4
Phosmet	23.2	318	160	5	2
Captan	23.2	300	264	10	50
Dimethenamide	23.3	276	244	10	1
Promecarb	23.3	208	151	10	5
Bromuconazole isomer 2	23.7	376	159	20	6
Molinate	23.7	188	126	10	5
Diflubenzuron	24.1	311	158	10	9
Iprodione	24.6	330	245	10	8
Propiconazole isomer 1	24.7	342	159	20	5
Malathion	24.8	331	127	5	5
Propiconazole isomer 2	24.9	342	159	20	5
Metolachlor	24.9	284	252	10	2
Triflumizole	24.9	346	278	10	7
Alachlor	25	270	238	10	8
Acetochlor	25.1	270	224	10	8
Flufenacet	25.2	364	194	5	5
Difenoconazole isomer 1	25.3	406	251	20	4
Difenoconazole isomer 2	25.4	406	251	20	4
Chlorfenvinphos	25.5	359	155	10	8
Benalaxyl	25.8	326	294	5	5
Parathion ethyl	26.2	292	236	10	9
Triclocarban	26.4	315	162	15	8
Hexaflumuron	26.5	461	158	10	7
Buprofezin	26.7	306	201	10	1
Diazinon	26.8	305	169	15	1
Teflubenzuron	26.9	381	158	15	22
Chlorpyrifos methyl	27.1	322	212	15	15
Profenofos	27.6	373	303	10	7
Lufenuron	27.9	511	158	10	10
Prosulfocarb	28	252	91	15	2
Flufenoxuron	28.5	489	158	10	6
Butylate	28.7	218	57	10	2
Pendimethalin	29.2	282	212	5	5
Trifluralin	29.7	336	236	15	30

The MRM transitions were included in the method with a dwell time of 15 msec, and two different time segments were recorded in the chromatographic run (each one of them containing about half of the pesticides studied). Figure 1 shows the chromatogram corresponding to 100 pg on column for all the compounds studied. Extracted ion chromatograms are overlaid for each one of the target analytes according to their respective protonated molecule and product ion MRM transition.

Linearity and Limits of Detection

Linearity was evaluated by analyzing the standards solutions at five different concentration levels in the range 2 to 100 pg on column. As an example, the calibration curve generated for atrazine is shown in Figure 2. As it can be observed in this figure, the linearity of the analytical response across the studied range is excellent, with a correlation coefficient of 0.998. Similar results were obtained for the rest of the compounds analyzed.

The limits of detection (LOD) were estimated from the injection of standard solutions at concentration levels corresponding to a signal-to-noise ratio of about 3. The results obtained are included in Table 1 as well. The best limits of detection were obtained for the triazines (from 100 fg to 2 pg on column) and the highest limits of detection were for fluoroxypyr and spinosad D (above 100 pg).

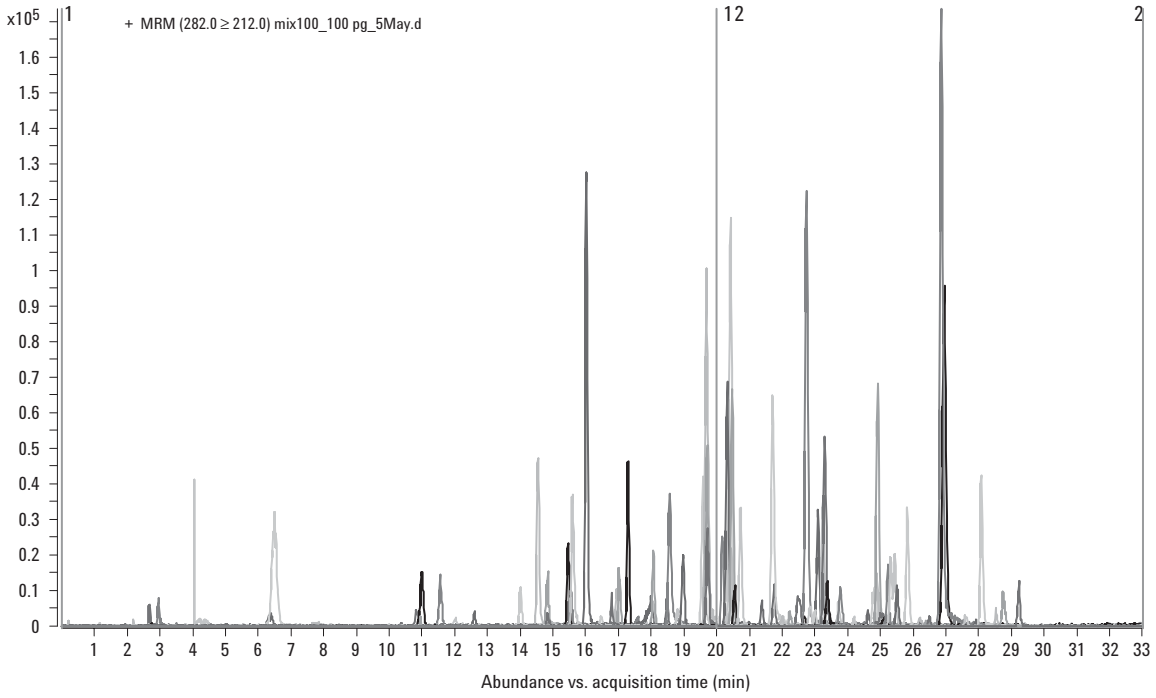


Figure 1. Product ion chromatograms of a mix of 100 pesticides (concentration: 100 pg on column).

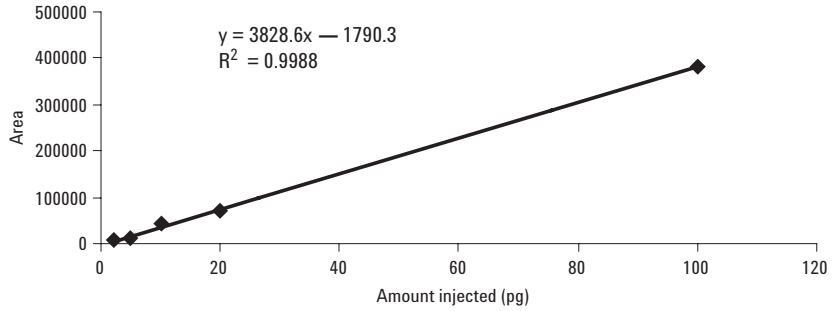


Figure 2. Calibration curve for atrazine using a linear fit with no weighting and no origin treatment.

Application to Vegetable Matrices

To confirm the suitability of the method for analysis of real samples, matrix-matched standards were analyzed in three different matrices — green pepper, tomato, and orange — at two different concentration levels (10 and 100 µg/kg). Figure 3 shows the analysis of a green pepper spiked with the pesticide mix at 10 µg/kg (10 pg on column). As it can be observed in two of the MS/MS extracted product ion chromatograms, for dimethoate and azoxystrobin, compounds can be easily identified in these complex matrices due to the selectivity of the MRM transitions, thus fulfilling the regulation limits imposed by the EU directives. In general, the LOD obtained meet the requirements regarding the MRLs imposed by the existing European regulations.

Reference

1. Imma Ferrer and E. Michael Thurman, "Determination of Fungicides in Fruits and Vegetables by Time-of-Flight and Ion Trap LC/MS" (2005) Agilent Technologies, publication 5989-2209EN www.agilent.com/chem.

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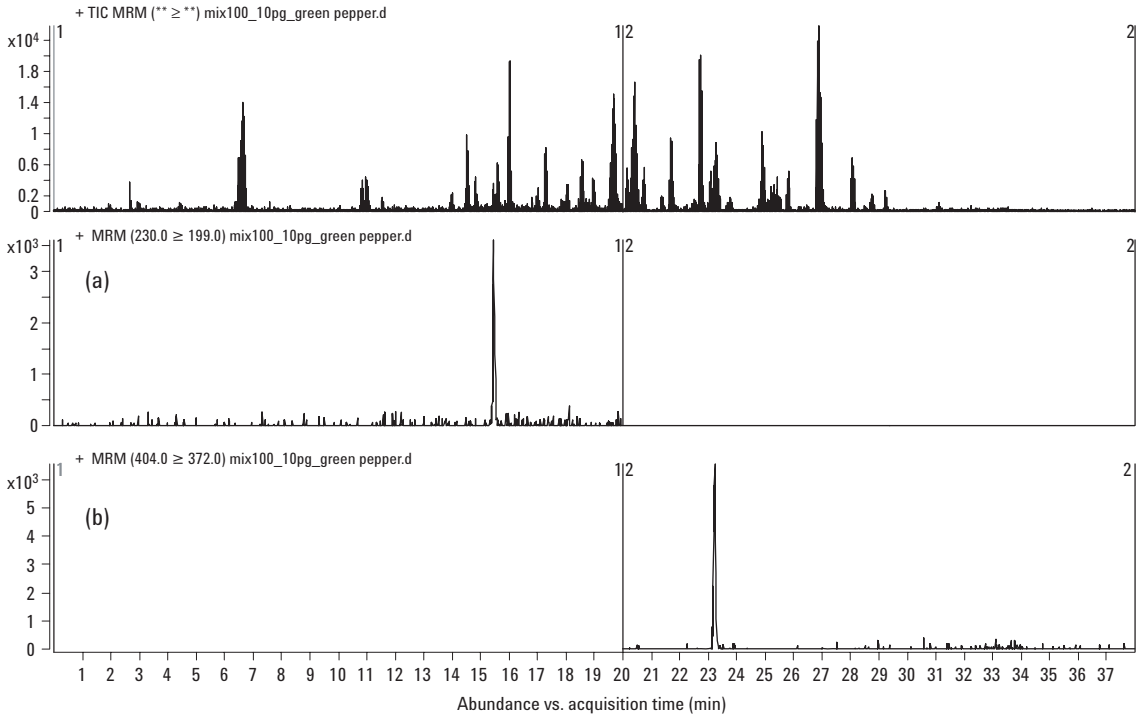


Figure 3. MRM chromatogram of a spiked green pepper sample at 10 µg/kg. Product ion chromatograms for (a) dimethoate and (b) azoxystrobin.

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Using RTL and 3-Way Splitter to Identify Unknown in Strawberry Extract

Application Brief

Chin-Kai Meng

Food Safety and Environmental

Fruit and vegetable extracts are usually very complex to analyze. It is common to use the very selective GC detectors, for example NPD, μECD, and FPD, to look for trace pesticide residues in the extracts. Mass spectrometry is most often used to confirm the hits from GC detectors. A previous application note [1] describes a GC/MS system with a three-way splitter added to the end of the column. The column effluent could be split three ways to two GC detectors and the MSD. The splitter system is therefore capable of providing up to four signals (two GC signals, SIM, and full-scan chromatograms) from a single injection. The combination of element selective detectors, SIM/scan, and Deconvolution Reporting Software (DRS) makes a very powerful pesticide analysis system [2]. The trade-off is the decrease of analyte concentration in any detector due to the flow splitting at the end of the column.

The system used for this study consists of an Agilent 7890A GC with split/splitless inlet, a three-way splitter, μECD, dual flame photometric detector (DFPD), and 5975C MSD. Figure 1 shows chromatograms from 2 separate injections (each injection provides two GC signals) of the same strawberry extract without any hardware or filter changes. All of the target compounds were found and confirmed by DRS, GC, and MS signals except the unknown peak at about 41 minutes. The peak shows responses from μECD, DFPD(S) and DFPD(P). However, no peak was observed in the MS full-scan signal. This makes it difficult to confirm the unknown peak using the full-scan TIC.

Since the analysis was retention time locked, it is therefore possible to find potential matches by examining the RTL pesticide database (part number G1672AA). The unknown compound, containing electron-capturing atoms (for example, Cl or O), P, and S atoms, would have a target retention time inside the

Highlights

Splitter—an inert, easy-to-use capillary flow technology that splits column effluent to multiple detectors (for example, MSD, DFPD, and μECD). The splitter configuration provides a comprehensive screening and quantitative system.

By combing RTL, element-selective detector chromatograms, and the RTL pesticide database, a trace level pesticide residue was identified without the full-scan mass spectrum.

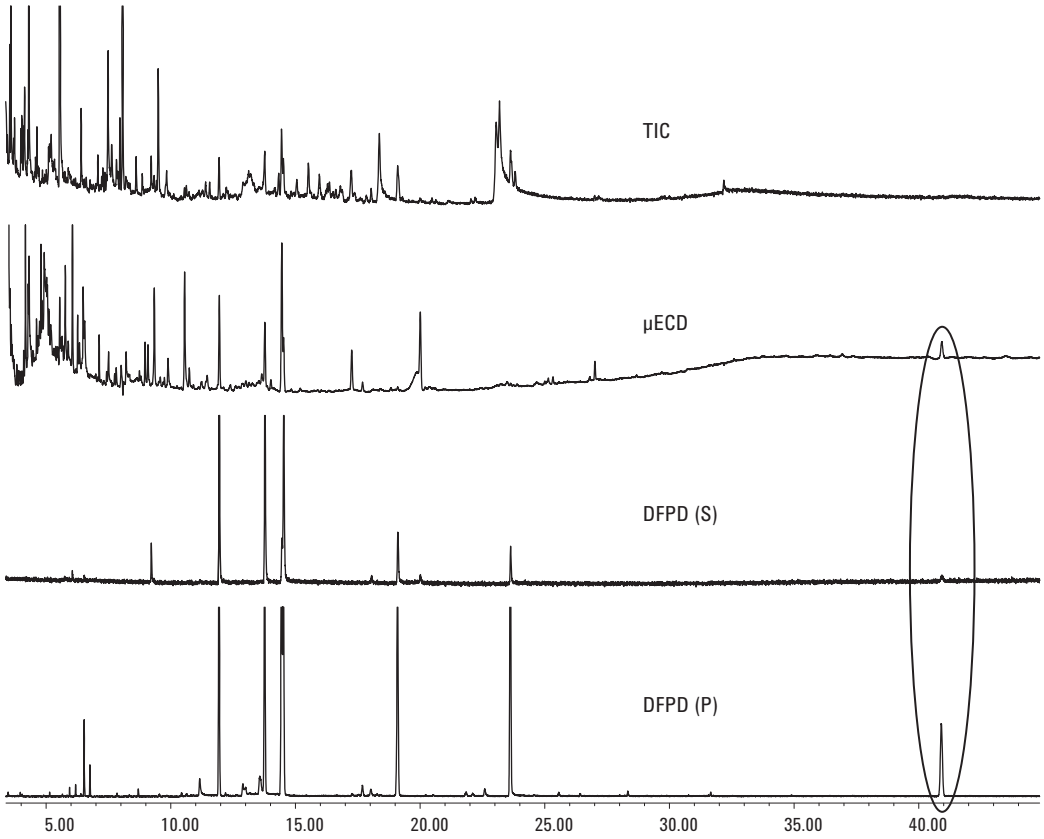


Figure 1. Unknown compound detected by GC signals not found in strawberry extract TIC.

Table 1. Compound List Extracted from the RTLPest3.tab File

Name	CAS	Mol form	Mol wt	R.T.	Target Ion	Q1	Q2	Q3
Fluthiacet-methyl	117337196	$C_{15}H_{15}ClFN_3O_3S_2$	403.9	39.10	403	56	405	232
Benzo[g,h,i]perylene	191242	$C_{22}H_{12}$	276.3	39.13	276	277	138	275
Temephos	3383968	$C_{16}H_{20}O_6P_2S_3$	466.5	40.74	466	125	93	109
PBB 169 hexabrombiphenyl	60044260	$C_{12}H_4Br_6$	627.6	40.93	308	468	148	154
Rotenone	83794	$C_{23}H_{22}O_6$	394.4	41.70	192	191	394	177

41 ± 0.5-minute window (that is, 40.5 to 41.5 min) in the database, if it is included in the database. Table 1 is a portion of the RTLPest3.tab file[1] opened in Microsoft Excel. The compound temephos at locked retention time 40.74 min meets all the criteria for the unknown peak. To further confirm peak identity, extracted ion chromatograms (EICs) of the four major ions of temephos were plotted. Figure 2 shows EICs of target ion and three qualifiers (ions 466, 125, 93, and 109 from Table 1) of temephos. Although the ion intensities were weak, the noticeable presence of all four ions at 40.9 min helped to confirm that the unknown peak was temephos.

1. The RTLPest3.tab file is created in the C:\Database directory while executing the Tools\List Screen Database... command (in MSD Enhanced Data Analysis software) and selecting the RTLPest3.scd from the C:\Database directory.

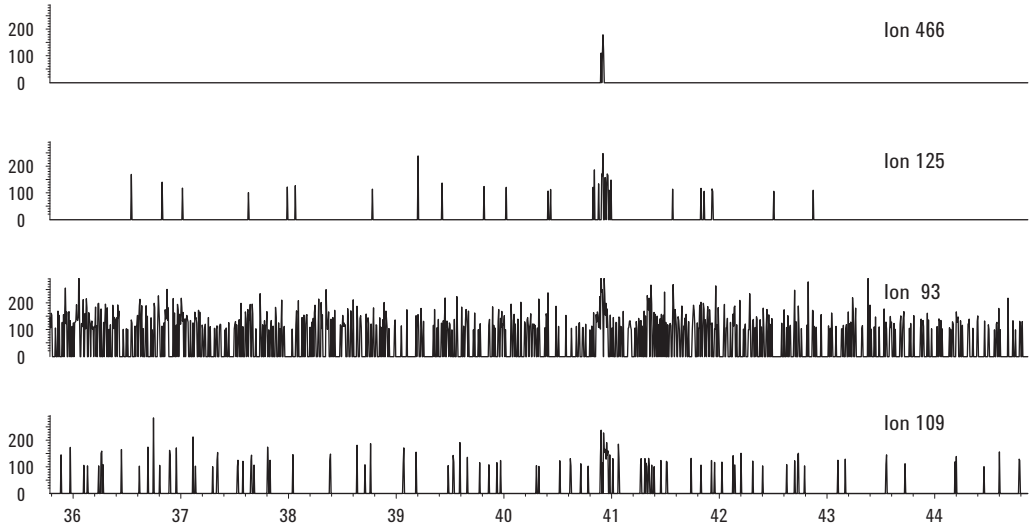


Figure 2. EICs of target ion 466 (temephos) and three qualifier ions.

References

1. Chin-Kai Meng and Bruce Quimby, "Identifying Pesticides with Full Scan, SIM, μECD, and FPD from a Single Injection," Application Note, 5989-3299, July 2005.

2. Mike Szelewski and Bruce Quimby, "New Tools for Rapid Pesticide Analysis in High Matrix Samples," Application Note, 5989-1716, October 2004.

Acknowledgement

Strawberry extract is courtesy of Dr. Steven Lehotay from USDA Agricultural Research Service in Wyndmoor, Pennsylvania, USA.

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Direct Injection of Fish Oil for the GC-ECD Analysis of PCBs: Results Using a Deans Switch With Backflushing

Application

Environmental and Pharmaceutical

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Abstract

A Deans switch, employing Agilent's Capillary Flow Technology, was configured on an Agilent 7890A gas chromatograph (GC) equipped with dual electron capture detectors (ECDs). A method was developed for the analysis of fish oil for polychlorinated biphenyl (PCB) contamination. The Deans switch was used to heart cut 7 indicator PCBs (IUPAC congeners 28, 52, 101, 118, 138, 153, and 180) from the primary DB-XLB column onto a DB-200 column for further separation. Fish oil from a supplement capsule was simply diluted 1:10 in isooctane and injected directly. In a separate experiment, the fish oil was analyzed by GC with a flame ionization detector (GC/FID) without backflushing. From these analyses, it was estimated that about two-thirds of the fish oil components would remain on the column after the 17.4-minute GC/ECD run. To prevent carryover, contamination, and retention time shifts, the Deans switch was used to backflush the primary column at the end of each run. Evidence shows that backflushing removed the fish oil residue, which otherwise would quickly degrade the chromatography.

Introduction

Fish oils contain high levels of eicosapentanoic acid (EPA) and docosahexanoic acid (DHA), omega-3 fatty acids that are thought to have beneficial health affects. In addition to eating fish, many people take fish oil as a supplement to their daily diet. However, fish, especially those high on the aquatic food chain, can bioaccumulate fat-soluble pollutants. Among these are polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and polychlorinated biphenyls (PCBs). Therefore, fish oil used in supplements undergoes a variety of analyses, including tests for halogenated pollutants.

One of the quality assurance tests is to analyze fish oil for PCB contamination. This is complicated by the fact that fish oil is a very complex mixture containing high-boiling fatty acids and triglycerides of fatty acids; chain lengths are mostly between 14 and 22 carbons. They also contain varying amounts of phospholipids, glycerol ethers, wax esters, and fatty alcohols. PCB analysis is complex by itself, with 209 possible congeners. Of these, 140 to 150 have been observed in commercial PCB mixtures called Aroclors. PCB analysis usually focuses on the 12 planar, dioxin-like PCBs and/or on seven indicator PCBs (IUPAC Numbers 28, 52, 101, 118, 138, 153, and 180).

To obtain sufficient sensitivity and selectivity for these compounds, analysts have traditionally employed very expensive techniques such as high-resolution mass spectrometry (HR/MS) or HR/MS/MS. Analysis of the fish oil generally follows a series of extraction and cleanup steps. This paper focuses on the analysis of the seven indicator PCBs in fish oil using an Agilent 7890A GC configured with a Deans switch, two columns of differing selectivity, and dual electron capture

detectors (ECDs). Fish oil from a commercially available supplement was simply diluted 10:1 in isooctane and injected into the GC. No cleanup steps were employed.

Experimental

The fish oil supplement was obtained from a local grocery store. According to the bottle's label, each gelatin capsule contains 1.0 g of fish oil of which 180 mg is EPA and 120 mg is DHA. Oil was removed from a capsule and diluted with isooctane (pesticide grade from Sigma-Aldrich, Milwaukee, WI, USA) to make a 10% solution. This solution was spiked with various Aroclors (Supelco, Bellefonte, PA, USA) or with individual PCB congeners (AccuStandard, New Haven, CT, USA).

Table 1 lists the instrumentation and experimental conditions for the analysis.

Table 1. Instrumentation and Experimental Conditions

Instrumentation and Software	
Gas chromatograph	Agilent 7890A
Automatic sampler	Agilent 7683B Series injector and tray
Primary column	J&W 30-m × 0.18-mm × 0.18-µm DB-XLB (P/N 121-1232)
Primary column connections	Split/splitless inlet to Deans switch
Secondary column	J&W 30-m × 0.25-mm × 0.50-µm DB-200 (P/N 122-2033)
Secondary column connections	Deans switch to back ECD
Restrictor	76.8-cm × 0.100-mm deactivated fused silica tubing
Restrictor connections	Deans switch to front ECD
Inlet liner	Agilent deactivated single taper with glass wool (P/N 5062-3587)
Auxiliary pressure control device	Agilent 7890A Pneumatic Control Module (PCM) Option # 309
Deans switch calculator software	Agilent Technologies Deans Switch Calculator (Rev. A.01.01)
Software for data acquisition and analysis	Agilent GC ChemStation (Rev. B.03.01)
Instrumental Conditions for Analysis	
Inlet	Split/splitless at 330 °C
Oven temperature program	80 °C (1 min), 50 °C/min to 200 °C (0 min), 10 °C/min to 290 °C (5 min)
Detectors	Dual ECD at 340 °C
ECD make-up gas	N_2 at 60 mL/min
Inlet pressure	H_2 at 41.040 psig (constant pressure mode)
PCM pressure to Deans switch	H_2 at 20.610 psig (constant pressure mode)
Post-Run Backflush Conditions	
Post-run duration	2.4 min
Inlet pressure	H_2 at 0 psig
PCM pressure	H_2 at 80 psig
Oven temperature during backflush	290 °C for 2.4 min

Results and Discussion

Without backflushing, the high-boiling components of fish oil can be retained by the GC column, causing severe carryover problems from one run to the next. After a few injections, so much of the fish oil residue builds up on the column that it causes PCB retention times to shift by a minute or more. Such dramatic retention time shifts would prevent the use of the Deans switch, where heart cuts are just a few seconds wide.

Deans Switch–Heart Cutting

The Deans switch is one of Agilent's new devices that employ Capillary Flow Technology. These devices have extremely low dead volumes, are inert, and do not leak, even with large cycles in oven temperature. Columns are easy to install into the Deans switch, which is mounted on the side of the oven wall (Figure 1).

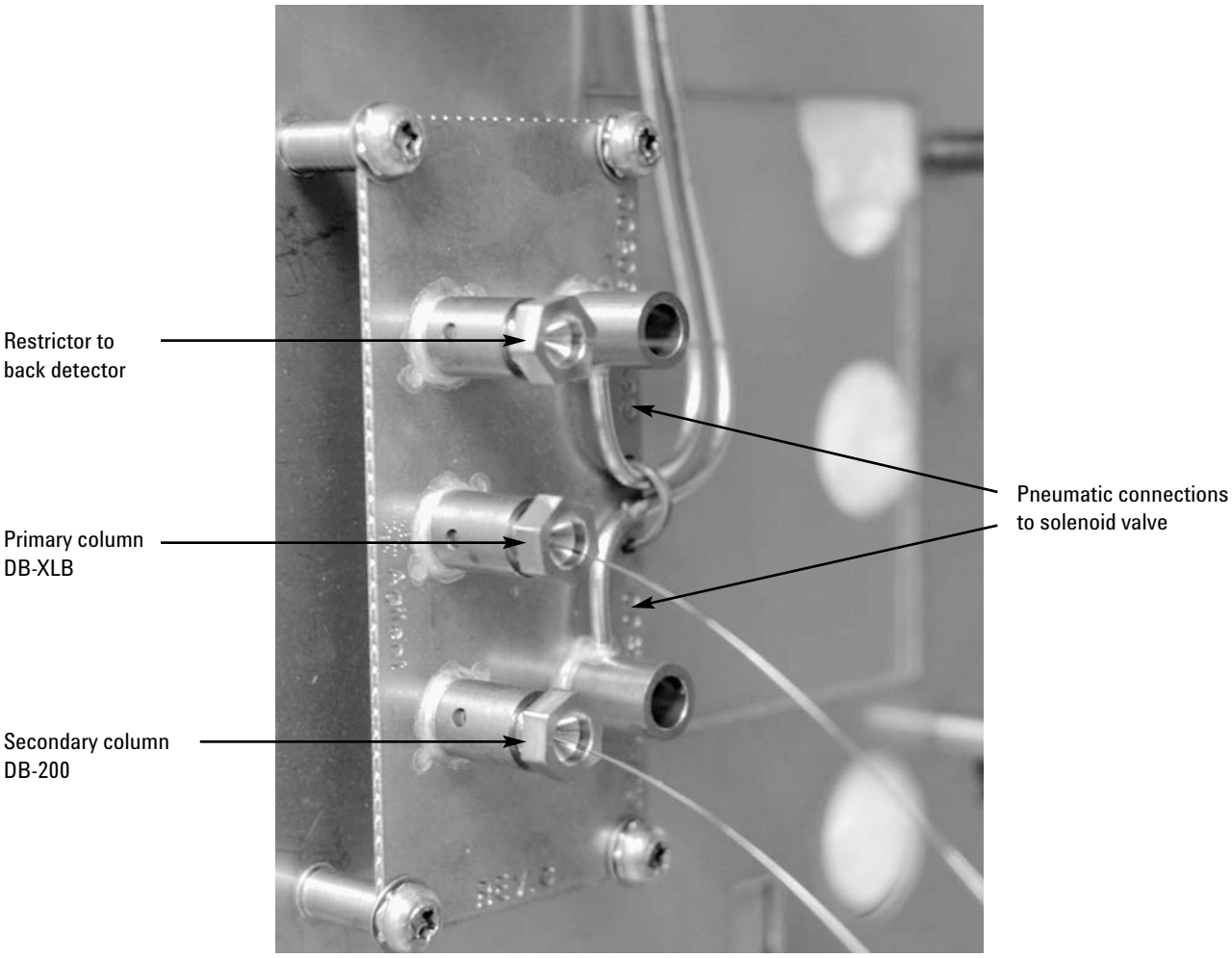


Figure 1. Photograph of the Deans switch installed on the side of the 7890A GC oven. The column and restrictor connections are indicated by an * in Figure 2a.

As shown in Figure 2a, the 30-m × 0.18-mm × 0.18-µm DB-XLB column is connected between the split/splitless inlet and the Deans switch. A short length of deactivated fused silica tubing (76.8 cm × 0.100 mm) connects the Deans switch to the front ECD. The secondary column (30-m × 0.25-mm × 0.5-µm DB-200) was chosen because it is more polar than the DB-XLB column and has a different selectivity for PCBs. It has an upper temperature limit of 300 °C, which is high enough to elute the PCBs of interest.

Figure 2a shows the Deans switch in the "normal" mode with the solenoid valve in the off position. In this mode, the effluent from the primary DB-XLB column is directed through the restrictor to the front ECD. When the solenoid valve is switched, the effluent is directed through the secondary DB-200 column to the back ECD (Figure 2b). The retention times for the seven indicator PCBs were initially determined with the valve in the *off* position. Using the timed events table in the ChemStation, the valve was switched to *on* just before each PCB peak and *off* immediately after. This produced seven heart cuts that were directed through the DB-200 column to the back ECD.

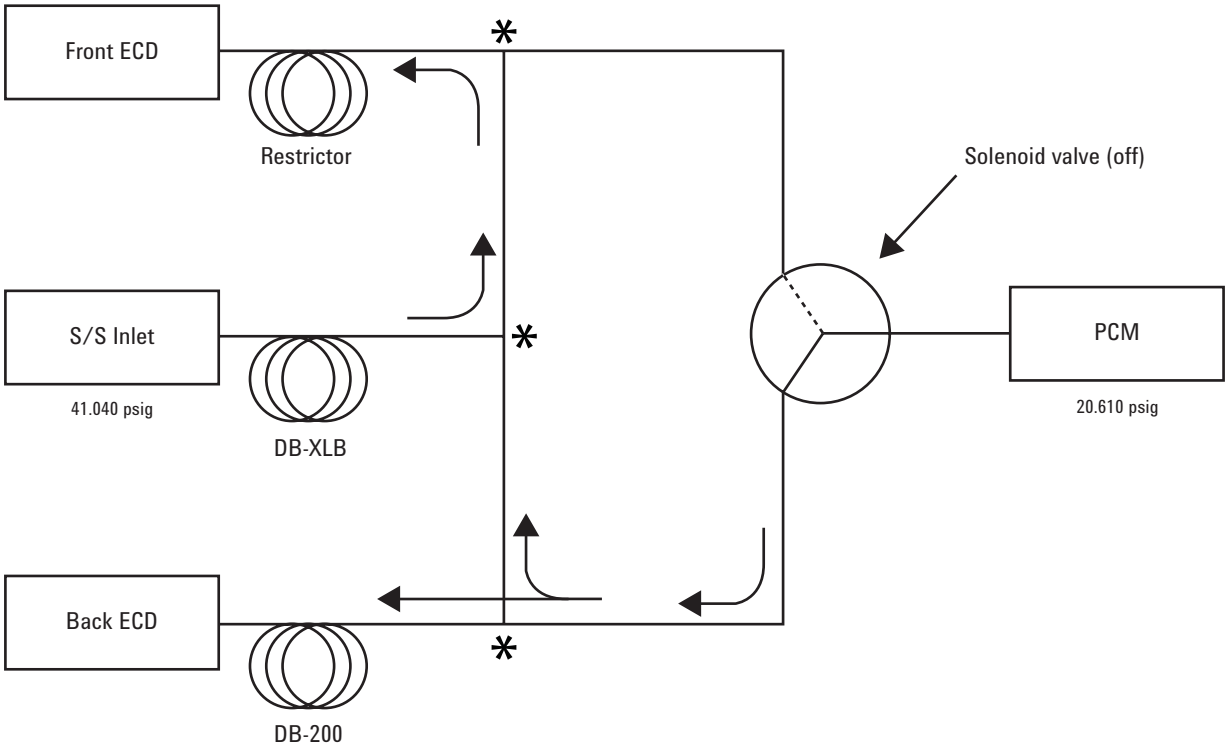


Figure 2a. Deans switch in the "no cut" position. The effluent from the DB-XLB column goes directly to the front ECD through the short restrictor. The intersections marked with an * are column and restrictor connections to the Deans switch plate (Figure 1).

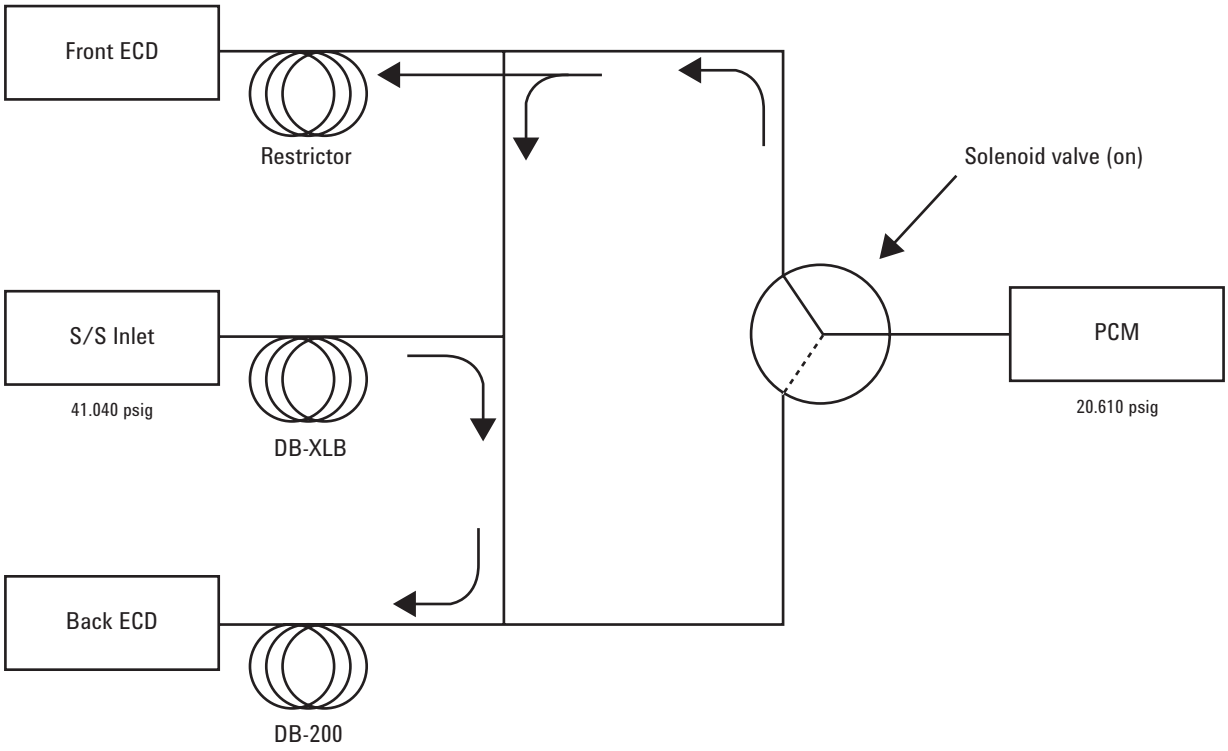


Figure 2b. Deans switch in the "cut" position. The effluent from the DB-XLB column goes to the DB-200 column and then to the back ECD.

In some Deans switch systems, the second column is placed in a separate GC oven or cryogenic cooling is used to trap the heart cut components at the head of the second column. In this case, both columns were mounted inside of the 7890A oven and cooling was not used to focus compounds at the head of the DB-200 column.

Figure 3a shows the chromatogram for a fish oil sample spiked with Aroclor 1260. PCBs 28, 52, 101, 118, 138, 153, and 180 were cut out of the primary chromatogram (Figure 3b) and sent to the second column (Figure 3c). The purpose of the DB-200 column is to resolve the target PCBs from other PCBs and matrix components that co-elute with them on the DB-XLB column. Six of the 7 PCBs appear to be well resolved on the DB-200 column. PCB 118 is only partially resolved by this method.

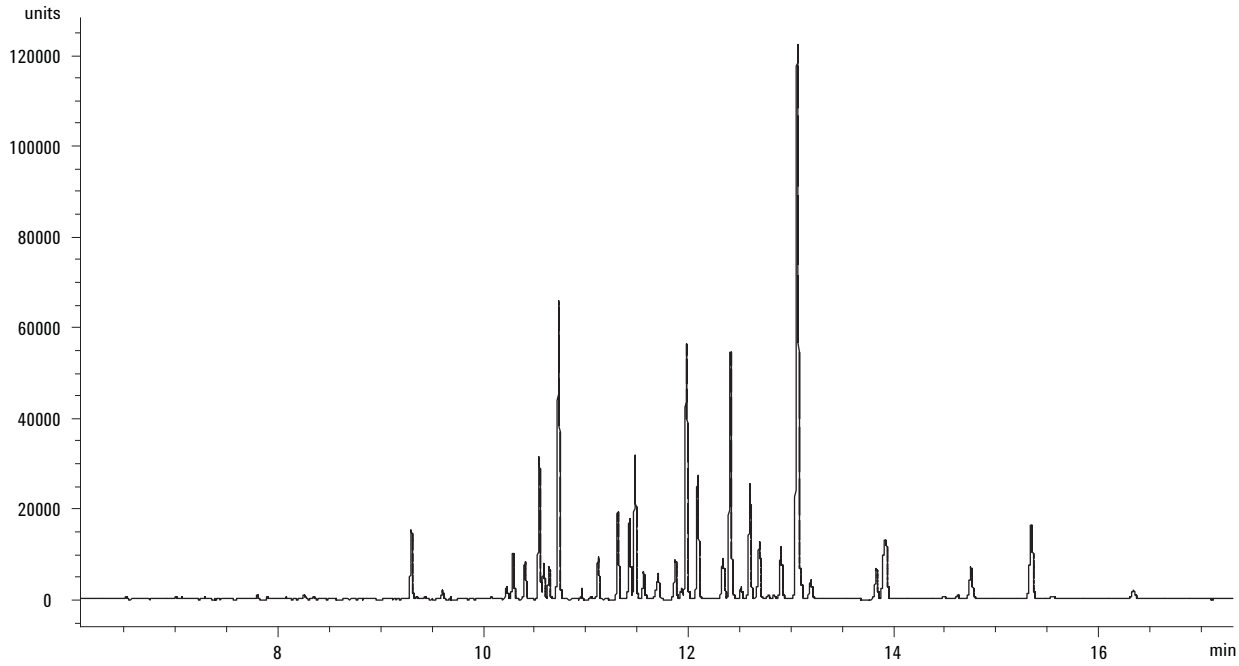


Figure 3a. GC/ECD chromatogram of Aroclor 1260 spiked into fish oil. This is the effluent from the primary DB-XLB column with seven heart cuts.

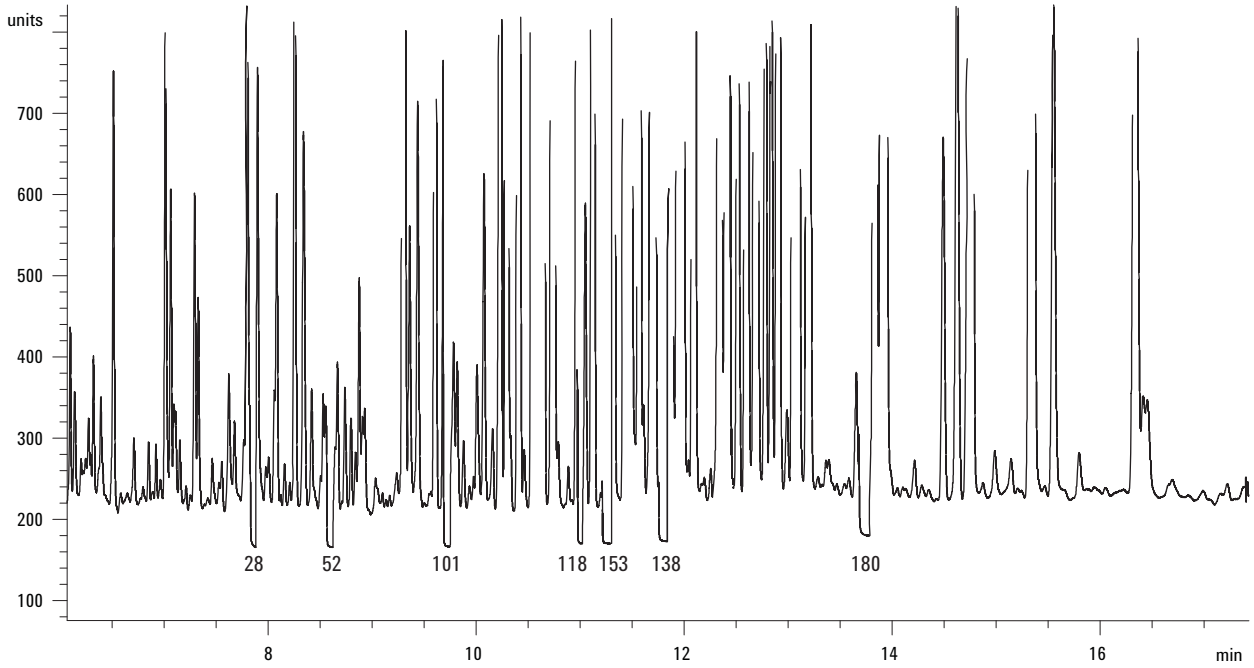


Figure 3b. Enlargement of the chromatogram in Figure 3a showing where heart cuts were made for the seven target PCBs.

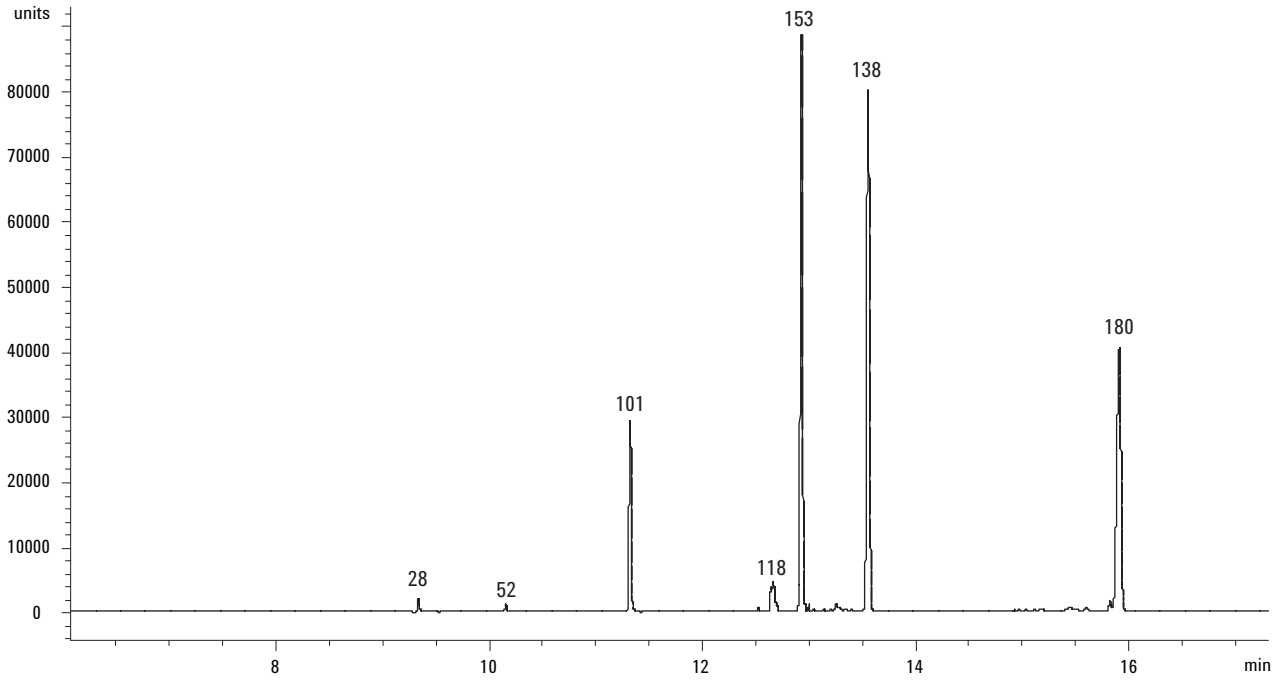


Figure 3c. GC/ECD chromatogram from the DB-200 column. The peaks in this chromatogram were heart cut from the DB-XLB column. Except for congener 118, the target PCBs were separated from co-eluting interferences by the DB-200 column.

Deans Switch–Backflushing

Data collection with the Deans switch system ended at 17.4 min with the oven at 290 °C. While it was assumed that a lot of the fish oil components remained on the column at this point, it was impossible to tell because the ECD responds poorly to these compounds. The fish oil does contribute some small peaks (both positive and negative) to the chromatogram, but it is impossible to see the full contribution of the fish oil. So a sample of the fish oil was analyzed on an identical DB-XLB column using a flame ionization detector (FID) with no Deans switch installed. The temperature was held at 290 °C for an extra 25 minutes to determine if high boiling compounds were still eluting.

Figure 4 shows that a great deal of the fish oil continued to elute after 17.4 minutes (arrow in figure). When a blank run was made with a final oven temperature of 310 °C, much more of the fish oil eluted from the column (Figure 4, middle chromatogram). A second blank run (Figure 4, top chromatogram) showed that fish oil components were still eluting from the column. In actuality, only about a third of the fish oil comes off the column under the Deans switch conditions. This is why other fish oil methods begin with a solvent extraction followed by solid phase extraction for sample cleanup.

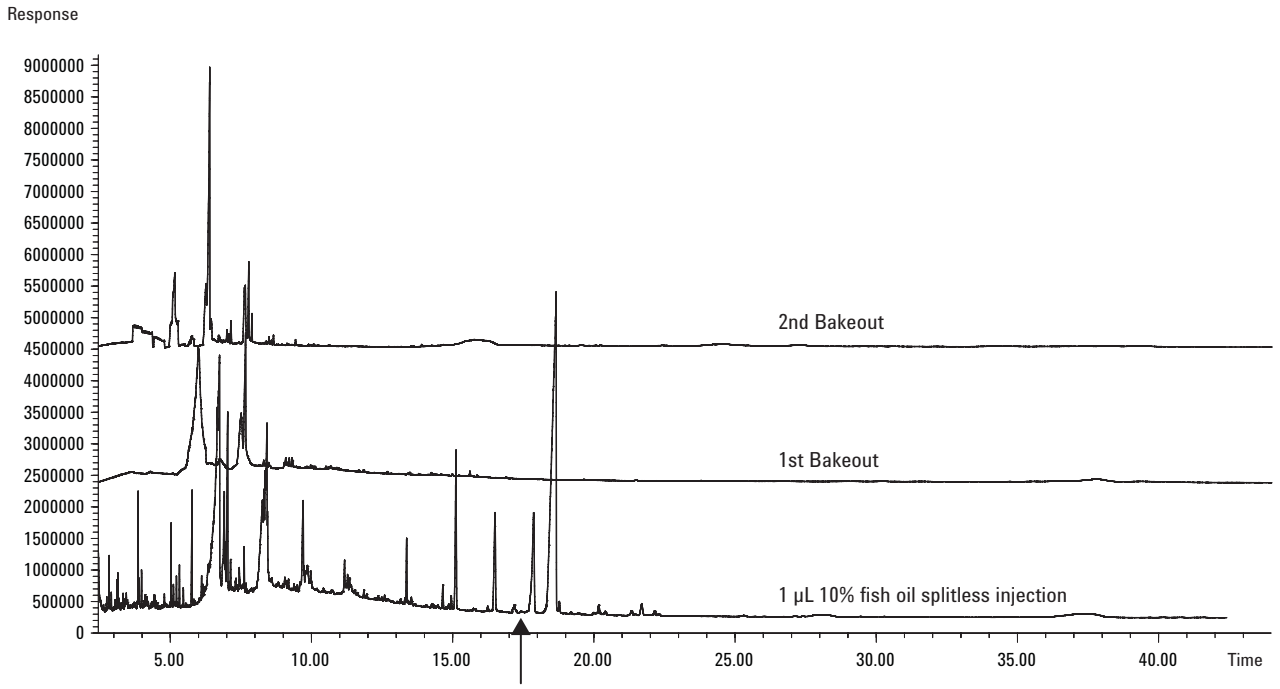


Figure 4. GC/FID chromatogram from a 1 µL splitless injection of 10% fish oil using a 30-m × 0.18-mm × 0.18 µm DB-XLB column. The arrow indicates where the GC/ECD method ends and the post-run backflush begins. In this case, there was no backflushing so the oven was held at 290 °C for an extra 25 min. The run was repeated two more times without injection but with the oven held at 310 °C for 30 minutes at the end of the run. Residue from the fish oil injection continued to elute, even during a second bakeout step.

The 7890A has been designed to make column backflushing a routine process. It has been shown empirically that backflushing should continue for about five times the holdup time. In this case the column was held at 290 °C during the post run backflush. At the same time, the inlet pressure was dropped to 0 psig while the PCM pressure was increased to 80 psig. Using Agilent's GC Pressure/Flow Calculator software, the H_2 flow rate backwards through the column was 3.81 mL/min and the holdup time was 0.466 min. Backflushing was, therefore, continued for 2.4 minutes, which is slightly more than five times the calculated holdup value. Figure 5 shows the Deans switch in the backflush mode.

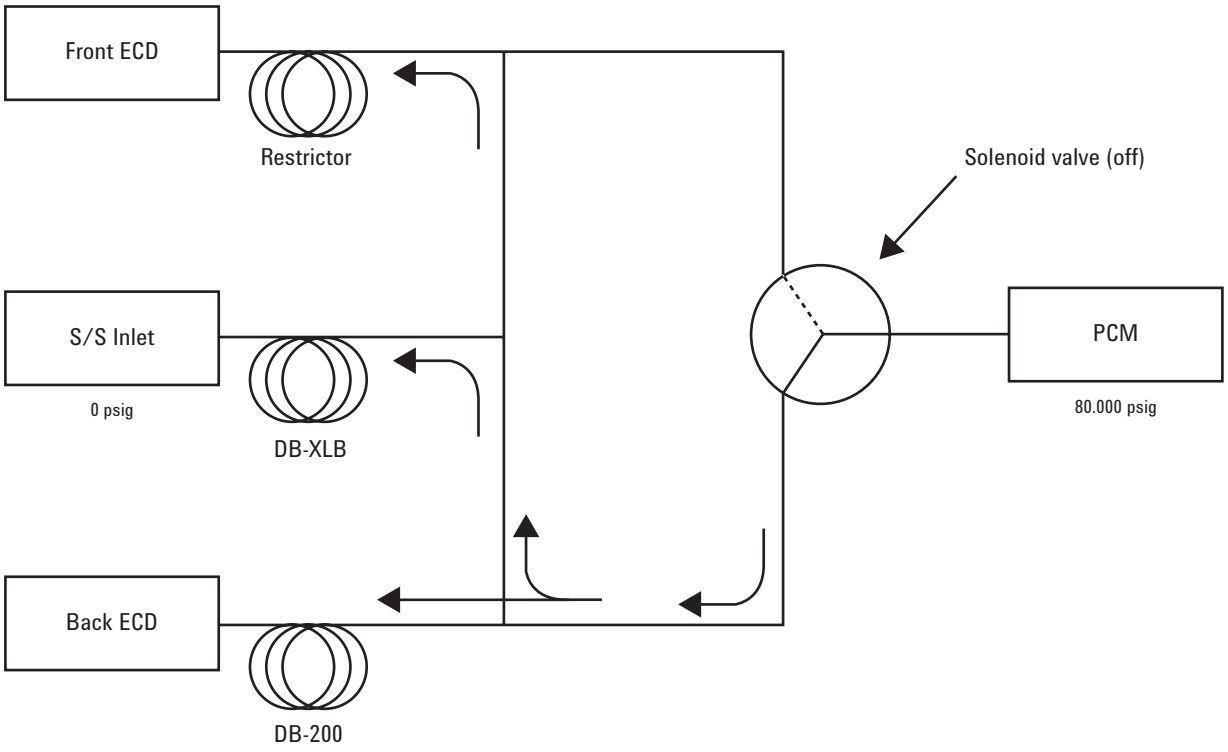


Figure 5. Deans switch in the "backflush" mode. The inlet pressure is dropped to 0 (or 1) psig while the PCM pressure is raised to 80 psig. This causes the carrier gas to flow backwards through the DB-XLB column. The reverse flow sweeps high-boiling fish oil components off the head of the column and out the split vent.

As mentioned earlier, just a few injections of fish oil can cause dramatic shifts in PCB retention times. Backflushing forces the remaining fish oil components backwards through the primary column and out through the split vent. This prevents fish oil buildup on the column, thus eliminating carryover and retention time shifts. Figure 6a compares the first and last chromatograms in a six-run sequence. One-µL splitless injections were made of 10% fish oil spiked with Aroclor 1260. This sequence was run after many previous injections of fish oil using this method, and it is clear that the retention times did not shift.

Figure 6b shows the seven PCBs that were heart cut from the two analyses shown in Figure 6a. Figure 6b shows no differences in the first and last heart cut chromatograms, providing further proof that there were not even subtle shifts in the PCB retention times. Each heart cut was just 4 to 5 seconds wide, so very small RT shifts in the first column would dramatically alter the results in the second.

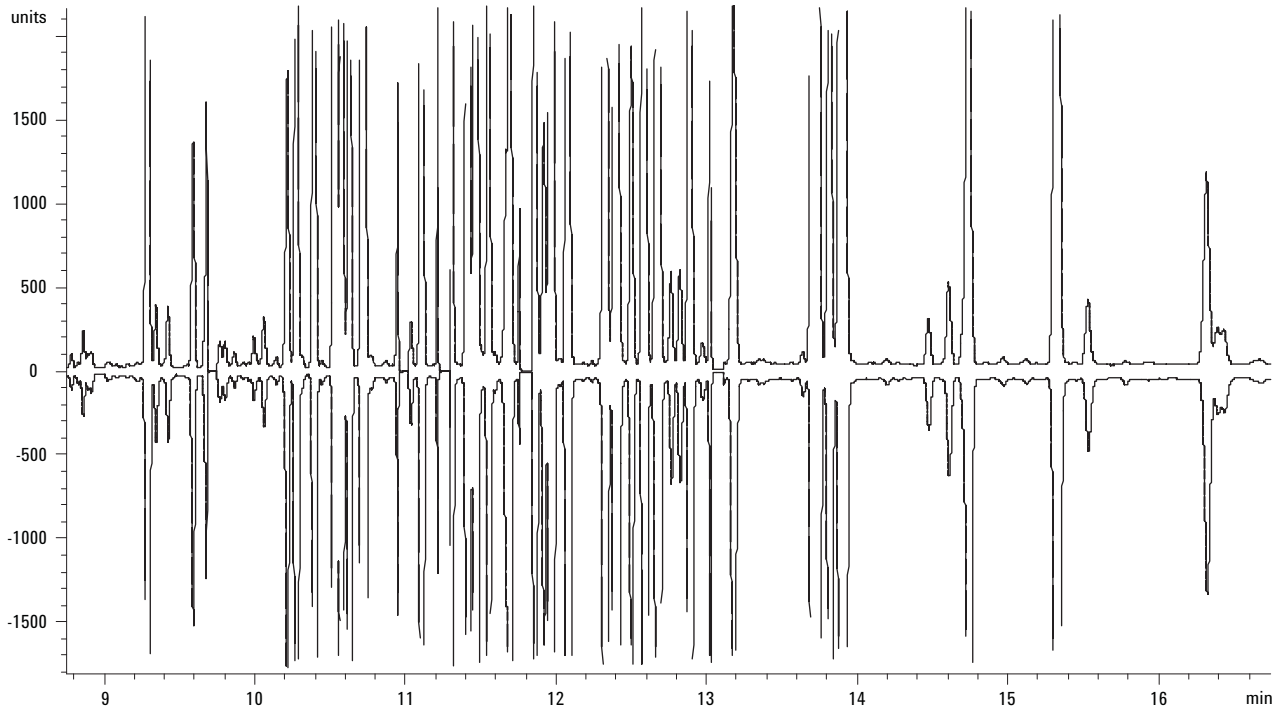


Figure 6a. First (top) and sixth (inverted) injections of 10% fish oil spiked with Aroclor 1260. Seven Deans switch cuts were made from this DB-XLB column in order to isolate PCBs 28, 52, 101, 118, 138, 153, and 180. The DB-XLB column was back-flushed after each run, preventing build-up of fish oil residue. The comparison shows that there was no shift in retention times caused by fish oil accumulation.

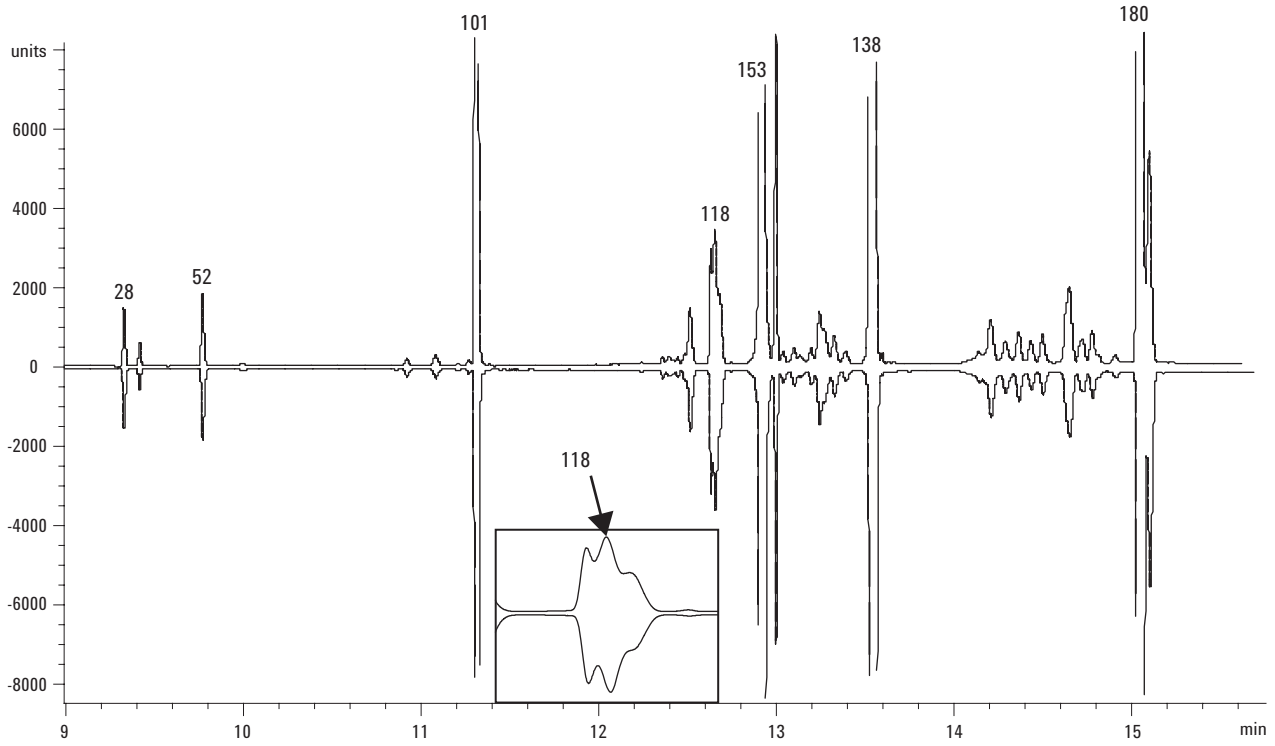


Figure 6b. Chromatogram of the seven PCB congeners and interferences that were cut from the DB-XLB column to the DB-200. The first chromatogram (top) and sixth (inverted) are identical, providing further proof of retention time stability. Any retention time shift on the primary column would severely alter the appearance of the secondary chromatogram.

Conclusions

This paper demonstrates that it is possible to analyze PCBs in fish oil without performing laborious sample cleanup prior to GC injection. A Deans switch was used to cut seven target PCBs (28, 52, 101, 118, 138, 153, and 180) from a DB-XLB column for further separation on a DB-200 column. This produced nearly baseline separation of the target PCBs. Only congener 118 was not well separated from co-eluting PCBs. Further refinement of the oven temperature program would be needed to isolate this congener.

It has been estimated that about two-thirds of the fish oil remained on the primary GC column at the end of the run. By setting the Deans switch to the backflush mode for just 2.4 minutes at the end of each run, this material was swept backwards through the column and out the split vent. There was no evidence for retention time shifts or carryover from run to run.

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Rapid Analysis of Crude Fungal Extracts for Secondary Metabolites by LC/TOF-MS – A New Approach to Fungal Characterization

Application

Food

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Abstract

A novel approach to studying the production of secondary metabolites by fungi using LC/TOF-MS has been developed. Fungi grown on culture media are solvent-extracted and directly analyzed by LC/TOF-MS. Searching against a database of 465 secondary metabolites, mycotoxins and other compounds of interest can be readily identified. The methodology was validated by spiking culture media with 20 mycotoxin standards and identifying these toxins in the crude solvent extracts. Subsequently, using seven different fungi from culture collections, after culturing for 7 to 14 days in three different media, anticipated metabolites were readily identified.

Introduction

From a food safety perspective there is a need to characterize molds (fungi) isolated from agricultural products, as these may represent a potential source of mycotoxin contamination in food.

Traditionally, this fungal characterization has been based on classical mycology, involving culturing the fungi on different media and then classifying depending on morphological and growth behavior characteristics. However, such classification can be time-consuming and is somewhat subjective, being dependant on the skill and experience of the mycologist. Additionally, such typing of fungi only provides anecdotal evidence about actual profiles of secondary metabolites, as it is based on previously observed secondary metabolism of particular fungal species. This empirical approach is further confounded by the fact that fungi of the same species can be both toxigenic and nontoxigenic; that is, some readily produce mycotoxins, but some otherwise indistinguishable fungi of the same species are genetically incapable of toxin production. Classification of fungal species alone therefore provides no real insight into mycotoxin production.

In the past, direct analysis of fungal culture media for the presence of mycotoxins has of necessity involved “target” analysis with the inevitable assumption as to which toxins should be sought. However, LC/TOF-MS offers new possibilities for studying the behavior of fungi with regard to toxin production. Providing that efficient extraction from the medium of toxins with widely differing polarity can be demonstrated, the specificity of TOF-MS means that any further sample clean-up is not necessary. Furthermore, targeted analysis is also unnecessary as the instrument can provide accurate mass measurement of molecular ions of any components detected in an LC run, and these can be identified by searching a database of exact masses of relevant secondary metabolites.

In this note we describe suitable conditions for extraction of secondary metabolites from cultured fungi and LC/TOF-MS conditions for subsequent analysis. The methodology has been validated by spiking aflatoxins, ochratoxin A, trichothecenes, zearalenone, and fumonisins into various growth media, and demonstrating good recovery from the media at low levels and subsequent identification by searching against a database of 465 secondary metabolites. The methodology has been applied to one *Penicillium* species and six *Aspergillus* species, which were obtained from a culture collection, and their secondary metabolites have been compared with the anticipated toxin profiles.

Experimental

All analytical work was performed using an Agilent 6210 TOF-MS coupled to an Agilent 1200 Series HPLC. The separation of mycotoxins and other fungal metabolites was also carried out using an HPLC system (consisting of vacuum degasser, autosampler with thermostat, binary pump, and DAD system) equipped with a reversed-phase C18 column (ZORBAX Eclipse XDB 100 × 2.1 mm, 1.8 µm). The TOF-MS was equipped with a dual-nebulizer electrospray source, allowing continuous introduction of reference mass compounds. The instrument was scanned from *m/z* 100 to 1,000 for all samples at a scan rate of 1 cycle/sec in 9,429 transient/scan. This mass range enabled the inclusion of two reference mass compounds, which produced ions at *m/z* 121.0508 and 922.0097. The injected sample volume was 5 µL.

The HPLC analysis used a mobile phase of acetonitrile and 2 mM ammonium acetate in an aqueous solution of 1% formic acid at a flow rate of 0.3 mL/min. The gradient elution started with 15% acetonitrile and reached 100% acetonitrile in 20 min. The column was washed with 100% acetonitrile for 5 min. and equilibrated for 5 min between chromatographic runs. UV spectra were obtained using diode array detection scanning every 0.4 sec from 200 to 700 nm with a resolution of 4 nm. The optimum TOF-MS conditions are given in Table 1. The data recorded were processed with Analyst-QS software with accurate mass application. The database of 465 mycotoxins and other fungal metabolites was created in Excel from reference sources [1,2], which were easily adapted to use in a search capacity using Agilent software.

Table 1. LC/MS-TOF Operational Conditions in ESI+ Ion Mode

Parameter	
Capillary voltage	3000 V
Nebulizer pressure	40 psig
Drying gas	10 L/min
Gas temperature	300 °C
Fragmentor voltage	150 V
Skimmer voltage	60 V
OCT* RF	250 V
OCT* DC	37.5 V
Mass range (*m/z*)	100–1000
Reference masses	121.050873; 922.009798

*Octapole

Fungal Extraction

Well-characterized isolates of *A. paraciticus (NRRL 2999)*, were obtained from the USDA culture collection and isolates of *A. flavus, (200198), A. ochraceus (200700), A. oryzae (200828), A. niger (200807), A. fumigatus (200418),* and *P. citrinum (501862)* were obtained from the TÜBITAK Mamara Research Center culture collection. Fungi were inoculated onto malt extract agar (MEA), potato dextrose agar (PDA), and yeast extract sucrose agar (YES) in petri dishes. After allowing the fungi to grow for 7 to 14 days at 25 °C, typical prolific growth of fungal colonies was observed on the surface of the media. Samples of fungal hyphae, together with underlying culture media, were taken by vertically cutting two 6-mm diameter plugs using a cork borer. The plugs were transferred to 5-mL disposable screw-cap bottles. Extraction conditions were modified from previous published methods [3,4]. One of the plugs was extracted twice with 2 mL ethyl acetate with 1% formic acid and then 2 mL isopropanol. The second plug was extracted twice with 2 mL ethyl acetate with 1% formic acid and then 2 mL acetonitrile, followed by 1 min vortexing and 30 min total ultrasonication. The extracts were filtered and evaporated gently under a nitrogen stream. The residues in both cases were dissolved in 1 mL methanol, ultrasonicated for 10 min and passed through a 0.2-µL disposable filter prior to HPLC analysis.

Results and Discussion

Optimization of LC/TOF-MS Conditions

The most important instrumental parameters, which were capillary voltage, nebulizer pressure, drying gas, gas temperature, and skimmer voltage, were initially optimized by autotune to achieve

maximum sensitivity. However, the fragmentor voltage also needed to be optimized to provide maximum structural information, which sometimes required a compromise. Optimization was carried out by varying the fragmentor voltage in the range of 55 to 250 V without changing any other conditions. The fragmentor voltage that provided minimum fragmentation was found at 150 V.

To validate the whole procedure, 20 commercially available standards (aflatoxins B_1, B_2, G_1, and G_2; aflatoxin M_1; ochratoxin A; zearalenone; 4-deoxynivalenol; 3-acetyldeoxynivalenol; 15-acetyldeoxynivalenol; diacetoxyscirpenol; fusarenone X; neosolaniol; fumonisins B_1, B_2, and B_3; nivalenol; HT-2 toxin; T2 toxin; and kojic acid) and internal standard (benzophenone) were mixed together. Using positive electrospray, the accurate masses of protonated molecule ions, retention times, and UV spectra were obtained in each case.

Construction of Database of Accurate Masses of Fungal Metabolites

An Excel spreadsheet was constructed containing the exact mass data for each of the 465 mycotoxins and fungal metabolites, together with their empirical formulas [1,2]. Theoretical monoisotopic exact masses of the compounds were calculated based on their molecular formula using an Excel spreadsheet (called "Formula DB Generator" and provided with the Agilent TOF) and put into csv (comma-separated values) format for use by the Agilent TOF automated data analysis software. The csv file is searched automatically by the LC/TOF-MS instrument at the completion of the sample run and a report is generated on compounds that were found in the database. The creation of the data analysis method is done using a data analysis editor. The editor allows selection of adducts (for example, in positive ion H^+, NH_4^+, Na^+, etc.) and neutral losses to be searched automatically, as well as mass accuracy and retention time tolerances, report options, and other search and detection criteria. Retention times are not required but if they are known add a degree of confidence to the identification.

We use samples of various growth media that had been spiked with the standard mixture of 20 mycotoxin standards to determine retention times. The standards were injected 10 times to establish the repeatability of those retention times. The criteria used for identification were a fit for the accurate mass of the M+1 ion to a mass tolerance of ± 5 ppm, a retention time match to ± 0.2 min (if standards available), a minimum peak height count, which is called the compound threshold of 1,000 counts (or a signal-to-noise ratio of ~10:1 or 0.06% relative volume), and, if present, good correspondence (to ± 5 ppm) with predicted adducts and neutral fragment losses.

Method Validation by Spiking and Analysis

Based on the above detection criteria, all 20 standards were correctly identified when spiked at 25 to 100 µg/kg into growth media, and analyzed as described above.

Utilization of the Method to Determine Metabolite Production from Well-Characterized Fungi

Rather than simply looking at theoretical situations with spiked growth media, the above technique was applied to the real situation of well-characterized fungi being cultured on various media. One *Penicillim* species and six *Aspergillus* species were grown on three different media. Using the simple solvent extraction described above, the crude extracts were directly analyzed by LC/TOF-MS. By way of illustration, Figure 1 shows the total ion chromatogram for an *A. flavus* extract indicating about 20 components detected. The peak eluting at 8.9 min on database searching was found to have an accurate mass of *m/z* 313.0712. Based on the $M+H^+$ ion this corresponded to aflatoxin B_1 with a 0.2 ppm mass match as compared to the database exact mass for aflatoxin B_1.

The software uses a molecular feature (MFE) algorithm that removes all ions that are not real peaks and groups the real ions into "molecular features." Those molecular features can be characterized by their relationship with each other and adducts, dimers, trimers, etc., and their isotopes (depicted as +1, +2, etc.) are deduced. The molecular features and accurate mass measurement of these species for the peak at a retention time of 8.9 min identified as aflatoxin B_1 are summarized in Table 2. Selecting a molecular feature, the software will calculate possible empirical formulas and score the isotopes for the "fit" to the proposed formula; this is also shown in Table 2. The formula with the score of 100 is that of aflatoxin B_1. This formula then can be automatically translated to a Web connection search with NIST, ChemIndex, and Medline. The search results in NIST indicated the formula and structure of aflatoxin B_1 as illustrated in Figure 2.

In addition to the identification of aflatoxin B_1 as a secondary metabolite from *A. flavus*, this fungi was also found to produce aflatoxin B_2, aflatoxin B_3, and aflatoxin G_1, which are consistent with

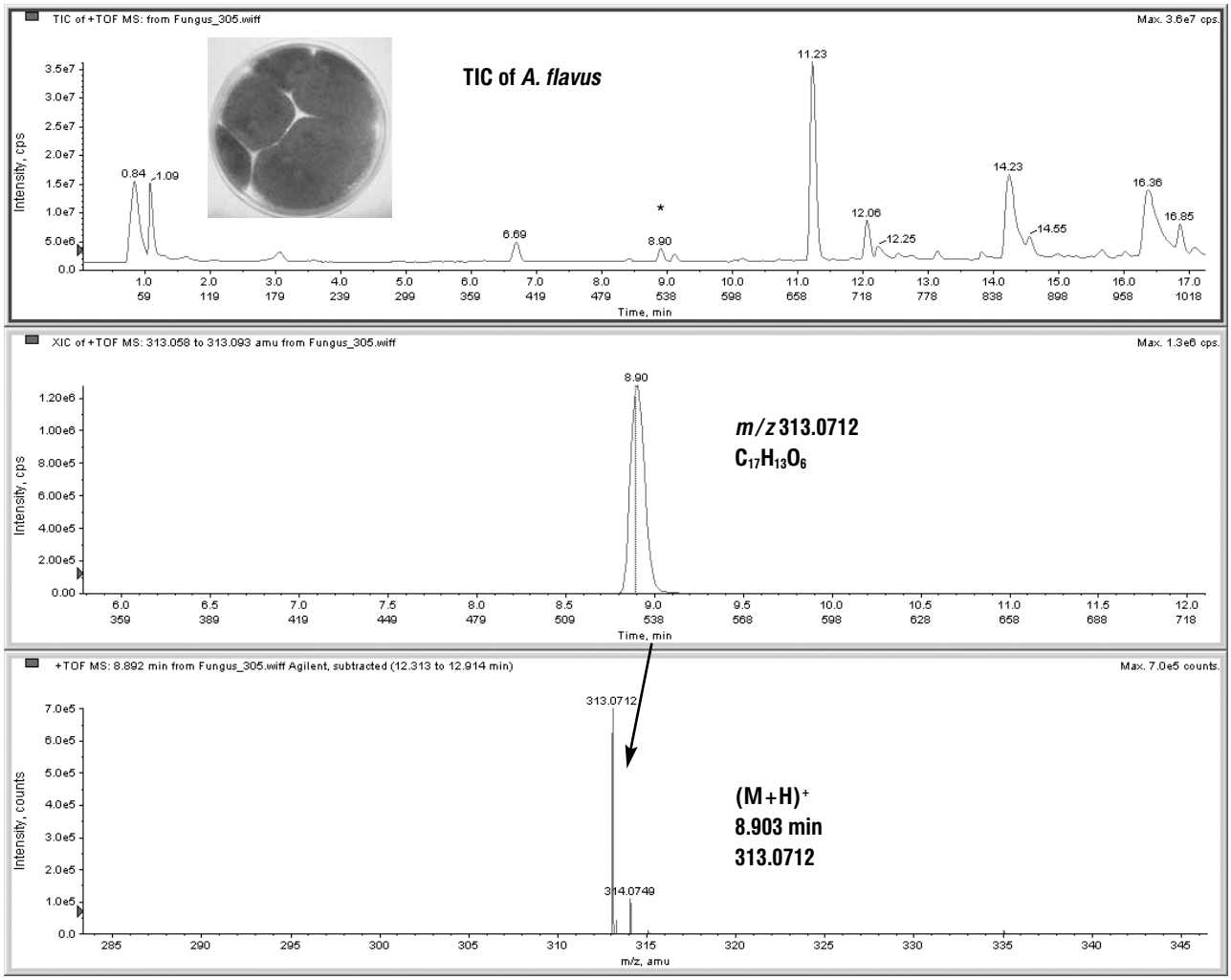


Figure 1. Analysis of an extract from *A. flavus* by LC/TOF-MS illustrating:
(a) Total ion chromatogram (TIC) with * peak corresponding to aflatoxin B_1,
(b) Extracted ion chromatogram from *m/z* 313.058 to 313.093 for aflatoxin B_1,
(c) Full-scan spectrum showing accurate mass with 0.2 ppm error for M+1 ion for aflatoxin B_1.

Table 2. Typical Clusters Seen in ESI+ LC/MS-TOF on the Peak Retention Time of 8.90 min, *m/z* 313.0706

MFE

Feature #27 (RT = 8.903)

Species	RT	*m/z*	Mass	Abundance	Width
M	8.903		312.0633	5541933	0.088
M+H	8.903	313.0706	312.0633	4035186	0.09
M+H+1	8.903	314.0744		622147	0.088
M+H+2	8.902	315.0766		74349	0.09
M+H+3	8.906	316.0795		7943	0.085
M+Na	8.904	335.0529	312.0637	86580	0.094
M+Na+1	8.904	336.0563		15898	0.097
M+Na+2	8.916	337.0593		1848	0.091
2M+H	8.906	625.1357	312.0642	741	0.049
2M+Na	8.902	647.1164	312.0636	226965	0.061
2M+Na+1	8.902	648.1202		65639	0.063
2M+Na+2	8.900	649.1228		14941	0.065
2M+Na+3	8.899	650.1256		2677	0.058
2M+Na+4	8.897	651.1290		257	0.048

MFE

Composition's chemical formula	dm(Da)	dm(ppm)	dm(ppm)	DBE	Score
$C_{17}H_{12}O_6$	0.0001	0.2	0.2	12	100
$C_{18}H_8N_4O_2$	0.0014	4.5	4.5	17	77
$C_{14}H_4N_{10}$	–0.0013	–4.2	4.2	18	68
$C_9H_{16}N_2O_8S$	–0.0006	–1.9	1.9	3	58
$C_{13}H_8N_6O_4$	–0.0026	–8.4	8.4	13	55

NIST
National Institute of Standards and Technology

Standard Reference Data Program | Data Gateway | Online Databasis | Chemistry WebBook

Aflatoxin B_1

- **Formula:** $C_{17}H_{12}O_6$
- **Molecular weight:** 312.27
- **IUPAC International Chemical Identifer:**
  - o InChI=1/C17H12O6/c1-20-10-6-11-14(8-4-5-21-17(8)22-11)15-13(10)7-2-3-9(18)12(7)16(19)23-15/h4-6,8,17H,2-3H2,1H3
- **CAS Registry Number:** 1162-65-8
- **Chemical structure:**

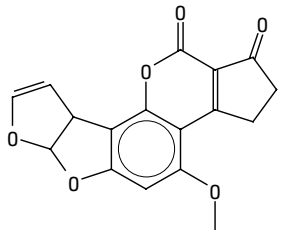


Figure 2. Database search result for emprical formula using NIST (Medline and ChemIndex results were the same but are not given here). Note molecular weights should not be searched in these databases as they are often the "average" molecular weight and not the monoisotopic weight.

known metabolic behavior. In Table 3 the screening results from the database search with a 5 ppm tolerance are shown with the accurate masses of some other peaks, which corresponded to known compounds. Kojic acid and methoxysterigmatocystin, which are a good match, are both well-known fungal metabolites that might be expected to be found from *A. flavus*. A good match was also found for cinnamic acid, which is not known as a metabolite.

When this new approach was applied in a preliminary study of a total of seven different fungi obtained from culture collections and grown on three different media, the results shown in Table 4 were obtained. In most cases the predicted metabolites were found, which gives good confidence in the methodology. Some of these initial results showed that predicted mycotoxins were not detected and unexpected metabolites were found. The possibility of a misidentified culture exists or that metabolites not previously reported were detected. While this demonstrates the power of the approach, these results do need to be followed up with more in-depth study.

Future Prospects

The use of accurate mass LC/TOF-MS combined

Table 3. Results of Automated Mycotoxin Database Search for *A. flavus* Extract (Extraction compound list is sorted in ascending order of retention time within 5 ppm error. Benzophenone was used as an internal standard.)

Mass Value = 142.03					
Formula	**Compound**	**Mass**	**Error (mDa)**	***Error (ppm)**	**Ret. Time Error**
$C_6H_6O_4$	Kojic acid	142.03	–0.10	–0.7	–
Mass Value = 148.05					
Formula	**Compound**	**Mass**	**Error (mDa)**	**Error (ppm)**	**Ret. Time Error**
$C_9H_8O_2$	Cinnamic acid	148.05	–0.08	–0.5	–
Mass Value = 328.06					
Formula	**Compound**	**Mass**	**Error (mDa)**	**Error (ppm)**	**Ret. Time Error**
$C_{17}H_{12}O_7$	Aflatoxin G_1	328.06	1.01	1.4	–0.05
Mass Value = 354.07					
Formula	**Compound**	**Mass**	**Error (mDa)**	***Error (ppm)**	**Ret. Time Error**
$C_{19}H_{14}O_7$	5-Methoxysterigmatocystin	354.07	0.99	2.8	–
Mass Value =312.06					
Formula	**Compound**	**Mass**	**Error (mDa)**	***Error (ppm)**	**Ret. Time Error**
$C_{17}H_{12}O_6$	Aflatoxin B_1	312.06	–0.05	–0.2	–0.11
Mass Value =312.06					
Formula	**Compound**	**Mass**	**Error (mDa)**	***Error (ppm)**	**Ret. Time Error**
$C_{17}H_{14}O_6$	Aflatoxin B_2	314.08	0.06	0.2	0.06
Mass Value = 338.08					
Formula	**Compound**	**Mass**	**Error (mDa)**	***Error (ppm)**	**Ret. Time Error**
$C_{19}H_{14}O_6$	Methylsterigmatocystin	338.08	–0.16	–0.5	–
Mass Value = 182.07					
Formula	**Compound**	**Mass**	**Error (mDa)**	***Error (ppm)**	**Ret. Time Error**
$C_{13}H_{10}O$	Benzophenone	182.07	0.73	4.0	–0.15

Table 4. A Comparison of Detected and Predicted Metabolites from Culture Collection Fungi Grown in MEA, YES, and PDA Medium

	Fungi						
Metabolites	*P. citrinum*	*A. flavus*	*A. paraciticus*	*A. niger*	*A. fumigatus*	*A. oryzae*	*A. ochraseus*
AFB1		✱ √	✱ √				
AFB2		✱ √	✱ √				
AFB3		✱ √	✱				
AFG1		✱ √	✱ √				
AFG2		✱	✱ √				
KA	✱ √	✱ √	✱ √		✱	✱	
MST		✱ √	√				
5-MST		√					√
OTA				✱ √			✱
RO-A					√		
FU-B					✱ √		
MA				✱ √			
AA		✱					√
Nig				✱ √			
Ter							√
Cit	✱						

√ - metabolites detected by LC/TOF-MS; ✱ - metabolites predicted to be present

Key:

AFB1	Aflatoxin B_1 etc.	FU-B	Fumigaclavine B
KA	Kojic acid	MA	Malformin (peptides)
MST	Methylsterigmatocystin	AA	Aspergillic acid
5-MST	5-methoxysterigmatocystin	Nig	Nigragillin
OTA	Ochratoxin A	Ter	Terrein
RO-A	Roquefortine A (isofumigaclavine A)	Cit	Citrinin

with database searching is a powerful example of a new, versatile identification technique that can be used in targeted analysis. In the area of fungal metabolites, the potential to screen fungi for a range of metabolites for which dedicated methods are not available has been demonstrated. This approach offers new possibilities for fungal typing based on metabolite production and rapid screening of agricultural products for mycotoxins of food safety interest. Where previously unknown metabolites are detected, although LC/TOF-MS can provide some insight, further work with a hybrid quadrupole time-of-flight LC/MS system (LC/QTOF-MS) will be required for structural elucidation.

Conclusions

A simple and rapid method has been developed using LC/TOF-MS to determine the profile of secondary metabolites produced by fungi under various culture conditions. The approach has been validated by spiking representative metabolites into solid cultures and demonstrating good recovery and identification by searching accurate masses against a metabolite database. Results for a range of well-characterized fungi from a culture collection showed that the anticipated toxins could be readily detected.

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Determination of Aflatoxins in Food by LC/MS/MS

Application

Food Safety

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Abstract

A sensitive and selective analytical method for the determination of aflatoxin G1, G2, B1, and B2 residues in cereals using the Agilent G6410AA LC/MS Triple Quadrupole Mass Spectrometer was developed. This method uses simple sample preparation methods followed by LC/MS/MS. The limits of detection for all aflatoxins were less than 1 ng/mL in cereals.

Introduction

Aflatoxins (AFs) belong to a closely related group of secondary fungal metabolites. These mycotoxins are severely toxic metabolites produced mainly by *Aspergillus flavus* and *A. parasiticus*, and exposure to them can cause cancer in humans and livestock [1]. Based on epidemiological evidence, AFs have been classified as human liver carcinogens by the World Health Organization and by the U.S. Environmental Protection Agency. Thus, accurate determination of AFs is required to avoid human disease from AF exposure and to advance worldwide surveillance of food. Analysis of AFs in food products is routinely performed by thin-layer chromatography (TLC) and liquid chromatography (LC) with fluorescence detection (FD) in combination with both precolumn derivatization and postcolumn derivatization. The LC/FD technology is often used due to the high selectivity and sensitivity of these methods. Furthermore, hyphenated techniques such as LC coupled to mass spectrometric (MS) detection have been developed and applied in residual analysis of foods. The high selectivity and sensitivity of MS detection methods associated with the resolution of LC provide decisive advantages to perform qualitative as well as quantitative analysis of a wide range of molecules at trace levels. Several papers describing different kinds of MS methods for the analysis of AFs have been published [2-4.]

Experimental

Sample Preparation

The samples analyzed (peanuts, corn, nutmeg, and red pepper) were obtained from a local market and did not include any AFs. The extraction and cleanup steps for AFs were carried out according to validated methods reported by Tanaka [5]. Briefly, 20 g fine ground sample was poured into a 200-mL Erlenmeyer flask, followed by adding 40 mL acetonitrile-water (9:1, v/v) for corn and cereals. After shaking for 30 min, the mixed solution was centrifuged for 5 min at 1,650 g. The supernatant obtained was filtered through a glass microfiber GF/B grade filter (Whatman Interna-

tional Ltd, Maidstone, UK). A 5-mL portion of the filtrate was applied to a MultiSep number 228 cartridge column for the cleanup. After passing through at a flow rate of 1 mL/min, 2 mL of the first eluate was collected. The eluate was evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue was reconstituted in 1 mL methanol-water (4:6 v/v) containing 10 mM ammonium acetate.

Standard Preparation

Each of the standard reagents, aflatoxin G_2 (AFG_2), aflatoxin G_1 (AFG_1), aflatoxin B_2 (AFB_2) and aflatoxin B_1 (AFB_1), was dissolved in acetonitrile at 1 mg/mL and was stored at 4 °C in the dark until use. To prepare the working standard for LC/MS analysis, each AF stock solution was equally pipetted and transferred to a vial, and it was then diluted with the mobile phase. The final concentration of each AF was 1 ng/mL.

Chemicals

The standards AFG_2, AFG_1, AFB_2, and AFB_1 were obtained from Sigma Aldrich Japan (Tokyo, Japan). The purity of these compounds was greater than 99%. Ammonium acetate, toluene, HPLC-grade acetonitrile, and HPLC-grade methanol were obtained from Wako Chemical (Osaka, Japan). Water was purified in-house with a Milli-Q system (Millipore, Tokyo, Japan). The cartridge column of MultiSep number 228 was purchased from Showa Denko (Kanagawa, Japan).

LC/MS Instrument

The LC/MS/MS system used in this work consists of an Agilent 1200 Series vacuum degasser, binary pump, well-plate autosampler, thermostatted column compartment, the Agilent G6410 Triple Quadrupole Mass Spectrometer with an electrospray ionization (ESI) source. The objective of the method development was to obtain a fast and sensitive analysis for quantifying AFs in foods. For chromatographic resolution and sensitivity, different solvents and columns were optimized. It was found that a simple solvent system using water, methanol, ammonium acetate, and a 1.8-µm particle size C18 column worked very well.

LC Conditions

Instrument:	Agilent 1200 HPLC
Column:	ZORBAX Extend C18, 100 mm × 2.1 mm, 1.8 µm (p/n 728700-902)
Column temp:	40 °C
Mobile phase:	A = 10 mM ammonium acetate in water B= Methanol 40% A/60% B
Flow rate:	0.2 mL/min
Injection volume:	5 µL

MS Conditions

Instrument:	Agilent 6410 LC /MS Triple Quadrupole
Source:	Positive ESI
Drying gas flow:	10 L/min
Nebulizer:	50 psig
Drying gas temp:	350 °C
V_{cap}:	4000 V
Scan:	*m/z* 100 – 550
Fragmentor:	Variable 100 V
MRM ions:	Shown in Table 1
Collision energy:	Shown in Table 1

LC/MS/MS Method

Quantitative analysis was carried out using MRM mode. The parameters for MRM transitions are shown in Table 1.

Table 1. Data Acquisition Parameters of MRM Transitions for Each Aflatoxin

No	Mycotoxins	RT (min)	Molecular weight	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (V)
1	Aflatoxin G_2	5.21	330	331	245	30
2	Aflatoxin G_1	6.61	328	329	243	30
3	Aflatoxin B_2	8.44	314	315	259	30
4	Aflatoxin B_1	10.89	312	313	241	30

Results and Discussion

Optimization of MRM Transitions

Determination of the optimal MRM transitions for each aflatoxin was carried out using single-MS full-scan mode followed by product ion scan mode using taflatoxin standard mixtures at 1 ug/mL. Mass spectra of these standard mixtures in full scan mode and product ion scan mode are shown in Figures 1 and 2. The mass spectrum of each aflatoxin by full-scan mode exhibited the protonated molecule $[M+H]^+$ as the base peak ion. These ions were selected as precursor ions for MRM mode. The optimum collision voltage is compound dependent. To establish the optimum collision voltage, this parameter was varied from 5 to 40 V using a step size of 5V. As shown in Figure 2, a distinct optimum in the intensity of the product ion of each AF was observed at 30 V. The product ions that indicated the highest intensity were *m/z* 245 (AFG_2), 243 (AFG_1), 259 (AFB_2), and 241 (AFB_1), respectively. On the basis of the above results, the collision voltage was set to 30 V.

Table 1 shows the parameters of MRM mode of each aflatoxin.

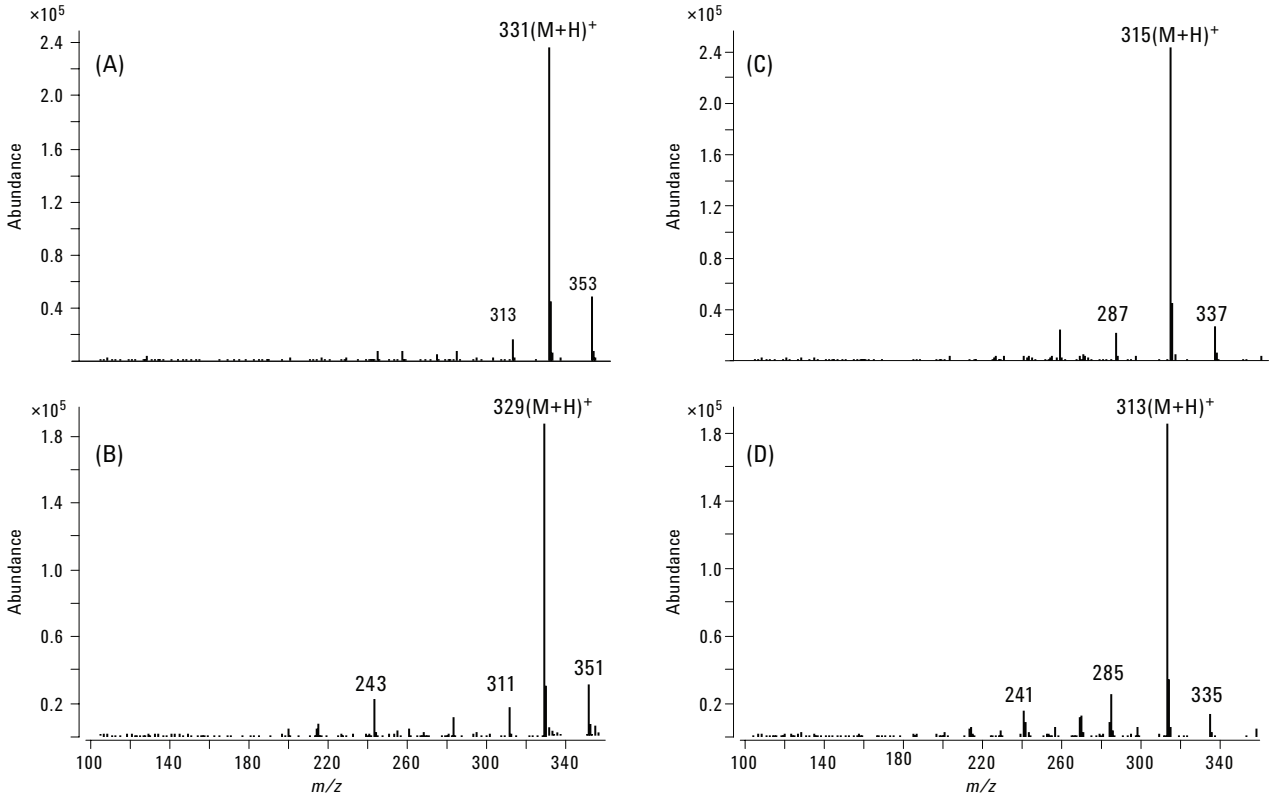


Figure 1. Mass spectra of four aflatoxins standard in single-MS full-scan mode at 1 µg/mL (A): aflatoxin G_2, (B): aflatoxin G_1, (C): aflatoxin B_2, and (D): aflatoxin B_1.

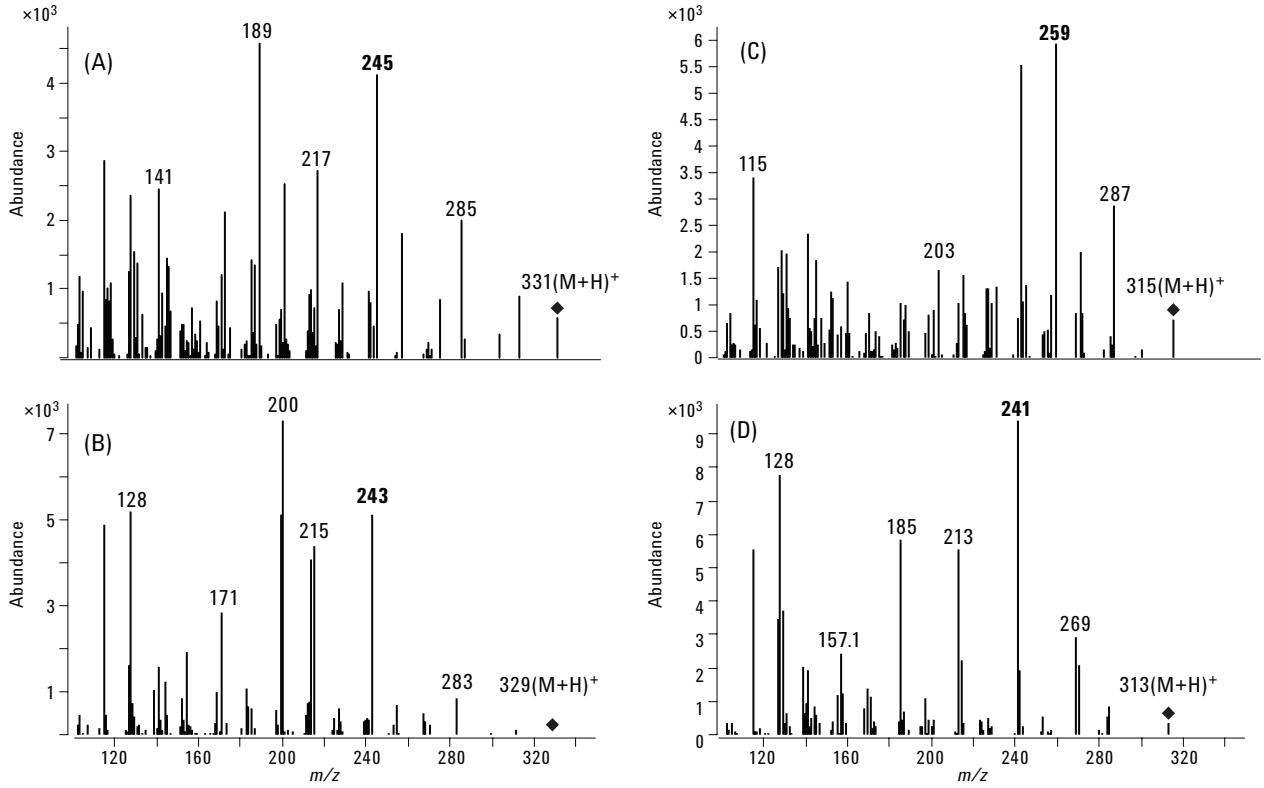


Figure 2. Mass spectra of four aflatoxins standard in product ion scan mode at 1µg/mL (A): aflatoxin G_2, (B): aflatoxin G_1, (C): aflatoxin B_2, and (D): aflatoxin B_1.

The MRM chromatogram of each aflatoxin at 0.1 ng/mL is shown in Figure 3. These show excellent signal-to-noise (S/N) ratios for all aflatoxins. The limit of detection (LOD) for each aflatoxin was determined using an S/N ratio of 3 with this MRM chromatogram and is shown in Table 2. To evaluate the linearity of the calibration curves, various concentrations of aflatoxin standard solutions ranging from 0.1 ng/mL to 100 ng/mL were analyzed. As shown in Figure 4 and Table 2, the linearity was very good for all aflatoxins with correlation coefficients (r^2) greater than 0.999.

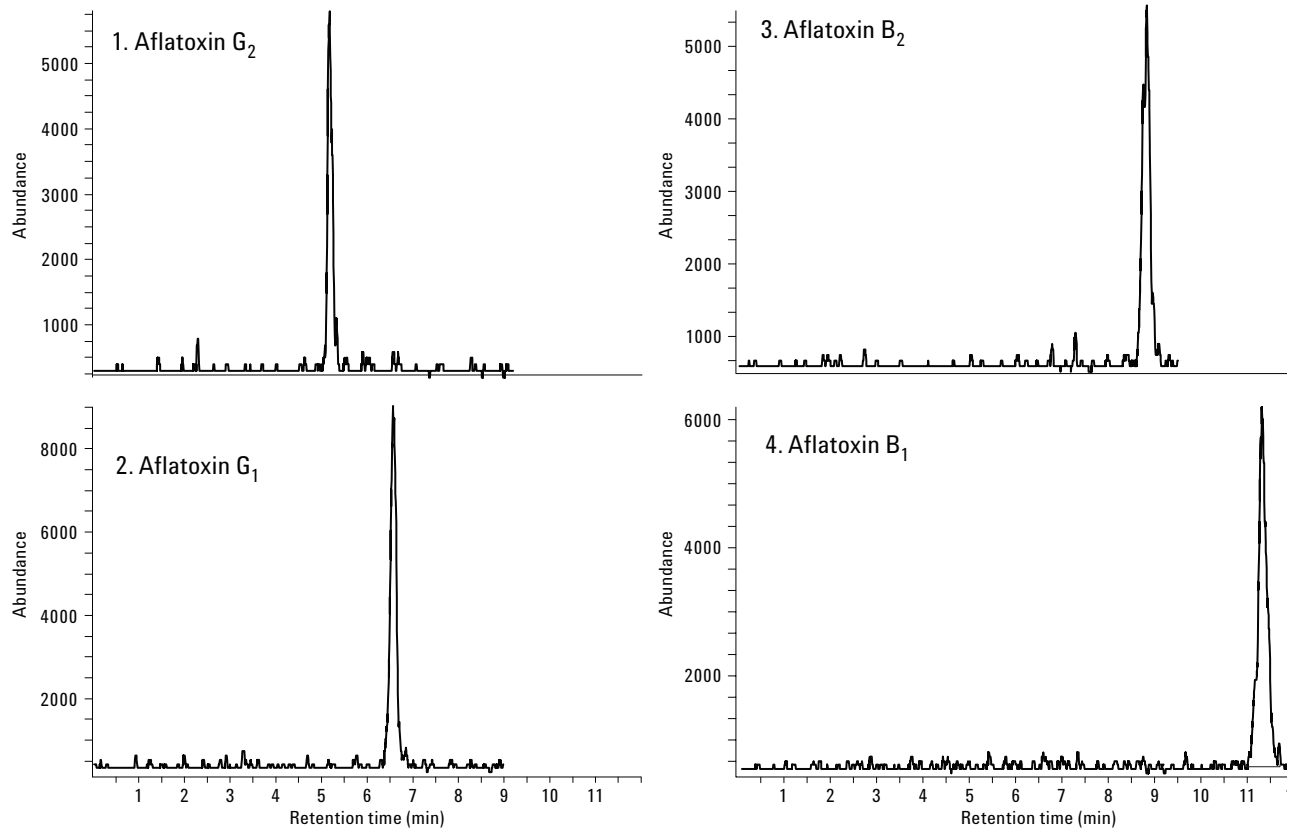


Figure 3. MRM chromatograms of four aflatoxin standards at 0.1 ng/mL in MRM mode.

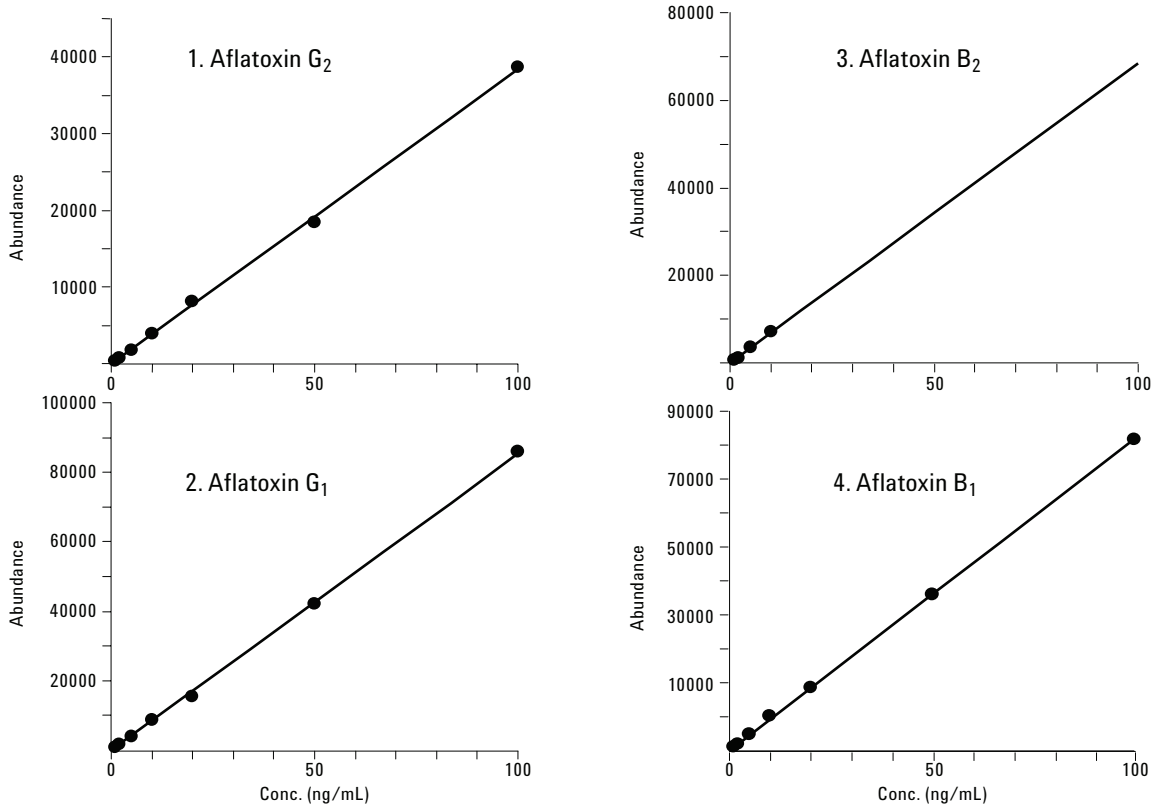


Figure 4. Calibration curves of four aflatoxins ranged from 0.1 ng/mL to 100 ng/mL.

Table 2. Linearity and LODs of Four Aflatoxins

No	Mycotoxins	r^2	LOD (ng/mL)
1	Aflatoxin G_2	0.9999	0.025
2	Aflatoxin G_1	0.9992	0.020
3	Aflatoxin B_2	0.9999	0.025
4	Aflatoxin B_1	0.9993	0.020

The matrix effect of this method was investigated by using cereal and corn extracts spiked with mycotoxin standards at 0.2 ng/mL. Typical MRM chromatograms of cereal and corn extract are shown in Figures 5 and 6, respectively. There were no additional peaks from sample matrix in either food when compared with the mycotoxin standard mixture. These results indicate that MRM mode has very high selectivity.

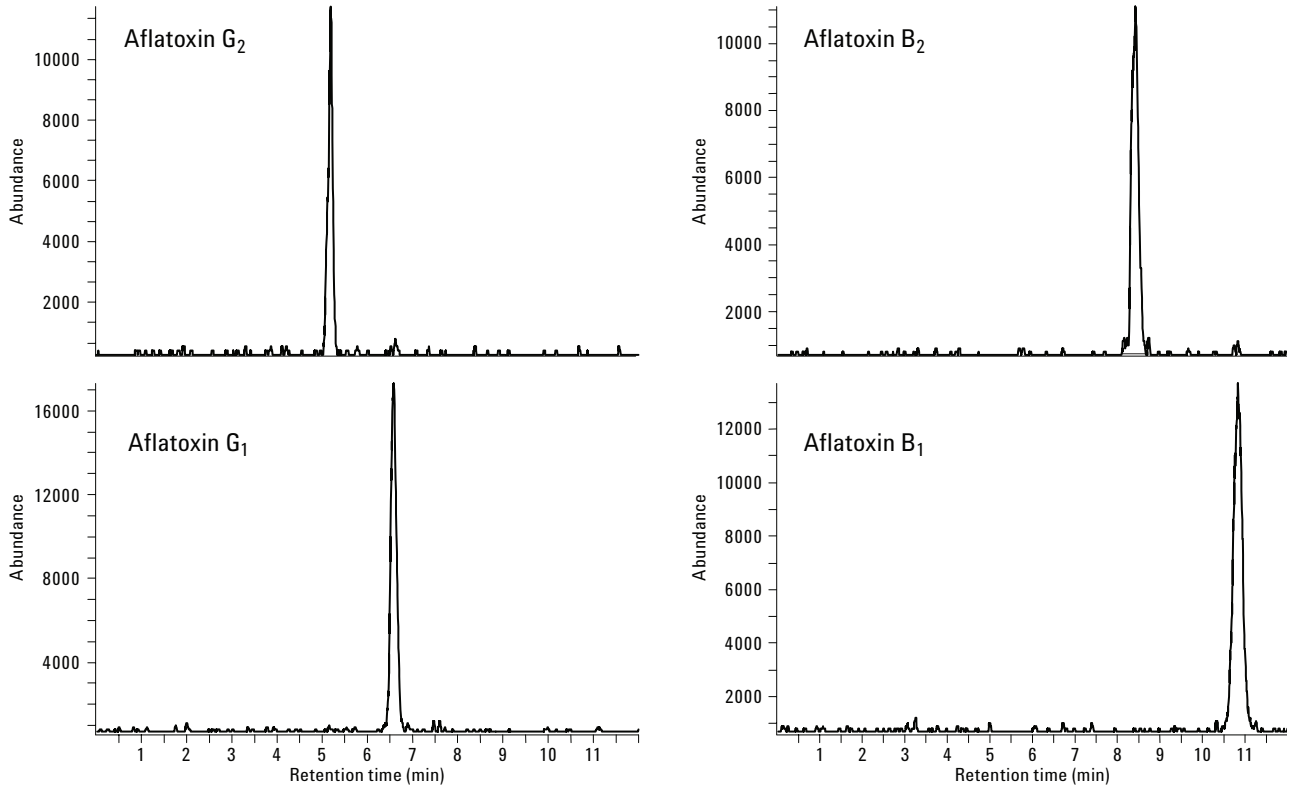


Figure 5. MRM of four aflatoxins in cereal extract spiked at 0.2 ng/g.

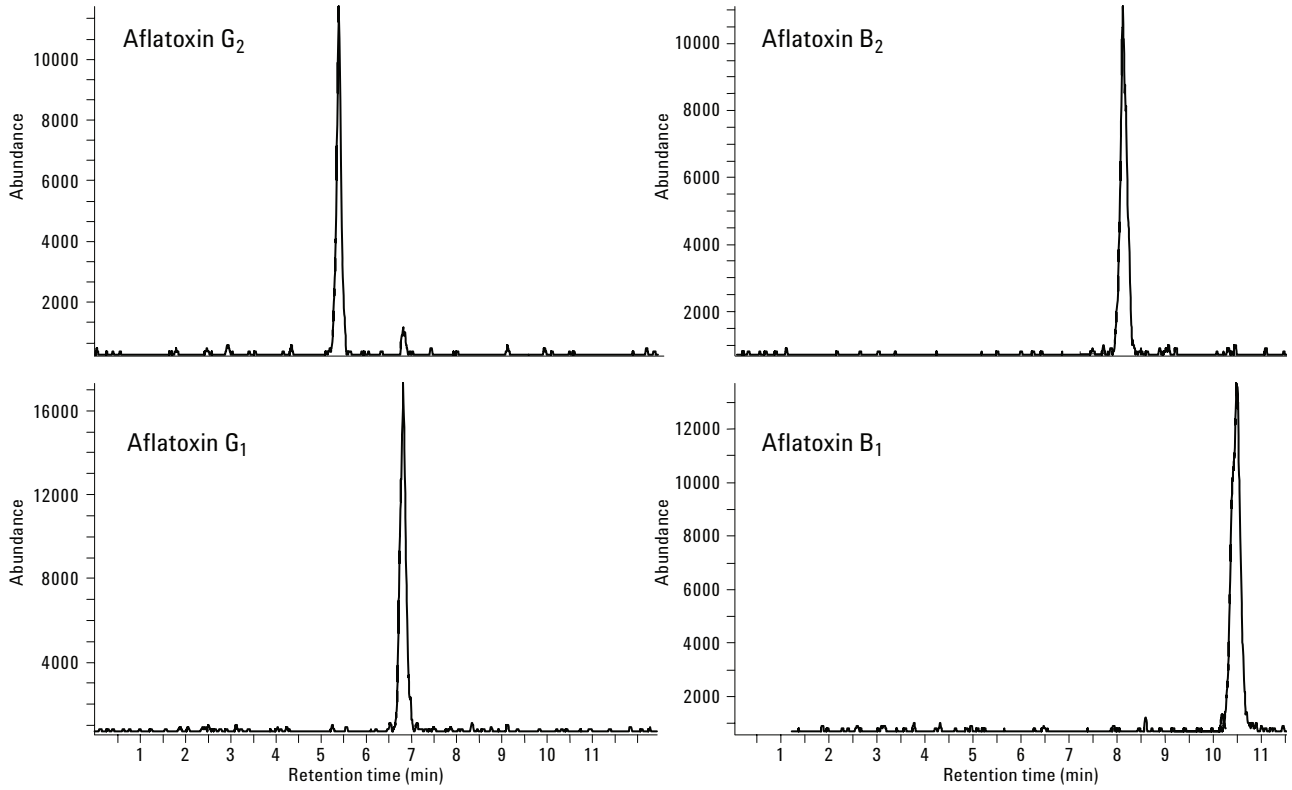


Figure 6. MRM of four aflatoxins in corn extract spiked at 0.2 ng/g.

Furthermore, the change on the peak intensity of each aflatoxin by the sample matrix was investigated by comparison with the peak intensity of aflatoxin standards. As these results show in Table 3, the relative intensity of each pesticide ranged from 88 to 96%. Thus, matrix effects such as ion suppression may be insignificant and it is possible to use external standards instead of matrix-matched standards.

Table 3. Relative Intensity of Each Aflatoxin in Sample Extracts

No	Mycotoxins	Relative intensity (%)	
		Cereal	Corn
1	Aflatoxin G_2	88	91
2	Aflatoxin G_1	92	94
3	Aflatoxin B_2	93	96
4	Aflatoxin B_1	97	95

Conclusions

The multi-aflatoxin method by LC/MS/MS described here was suitable for the determination of four aflatoxins in cereal and corn extract due to its high sensitivity and high selectivity. Another advantage of this method is that ion suppression was not observed for all food samples studied. Thus, it may eliminate the need for matrix-matched standards, which makes analysis more tedious for samples from different origins.

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Addressing the Challenges of Analyzing Trace Perfluorooctanoic Acid (PFOA) and Perfluorooctane Sulfonate (PFOS) Using LC/QQQ

Application

Food, Environmental

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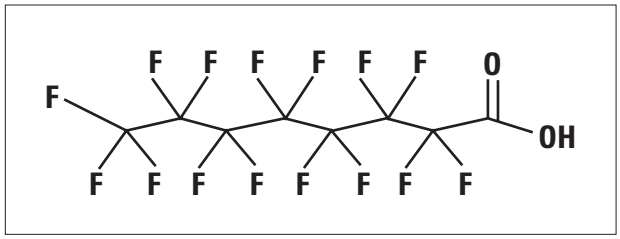
Abstract

An approach to the difficult task of quantifying trace quantities of perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) in complex matrix was developed using liquid chromatography and tandem mass spectrometry (LC/MS/MS). The technique uses isotopically labeled analytes for accurate quantitation (0.4 to 400 pg on column). It is important to recognize that if using the linear chain sample as standard for calibration, the quantitation results of real-world samples (branched and linear isomers mixed) will be off by as much as 40%.

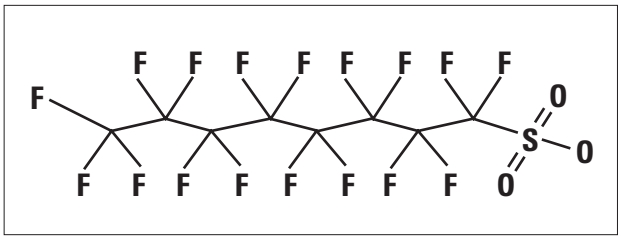
Introduction

Perfluorooctanoic acid (PFOA) is an industrial surfactant and a necessary processing aid in the manufacture of fluoropolymers [1]. Fluoropolymers have many valuable properties, including fire resistance and the ability to repel oil, stains, grease and water. One of the most common uses of PFOA is for processing polytetrafluoroethylene (PTFE), most widely known as Teflon®. PFOA is also a by-product from direct and indirect contact with food packaging (for example, microwave-popcorn bags, bags for muffins or french fries, pizza box liners, boxes for hamburgers, and sandwich wrappers), and in the fabrication of water- and stain-resistant clothes.

Perfluorooctanesulfonic acid (PFOS) is usually used as the sodium or potassium salt and is referred to as perfluorooctane sulfonate. See Figure 1.



PFOA



PFOS

Figure 1. Chemical structures for PFOA and PFOS. Note that both have C8 chains.

Analytical Methodology for PFOA/PFOS

- LC/MS/MS is the preferred detection methodology due to its high sensitivity and specificity in complex matrices.
- Multiple reaction monitoring (MRM) is used to quantitate, using two or more product ions for confirmation.
- The detection limit is typically in the range 1 to 100 pg/mL (ppt), requiring high-sensitivity detection.
- On-column or off-line solid-phase extraction (SPE) and concentration are needed to achieve low-level detection (1 pg/mL).

Measuring PFOS and PFOA

Issue 1: ***What transitions should be used to give the best accuracy when quantifying with a linear standard?***

Quantification of PFOS and PFOA is usually based on a linear standard, but actual samples show a series of branched isomers together with the linear isomer. The ratio of these isomers varies based upon biodegradation and industrial processes in their formation; therefore, it is unlikely that a standard can be formulated to mimic the actual sample. The relative intensities of the MRM transitions will vary based upon branching, making some transitions better than others. Branching impacts ionization efficiency and CID energy; therefore, it affects the accuracy of analytical measurement [2].

Issue 2: ***Can isotopically labeled standards in matrix be used to measure nonlabeled PFOS and PFOA?***

Most biological and environmental matrices have background levels of PFOS and PFOA; although matrix-matched calibrations are providing good results, the accuracy can be enhanced. The method of standard additions is a protocol to address this issue, but it adds several additional injections to the analysis. Matrix may have varying amount of background. Standard addition is not practical in analyzing many different matrices. Solvent calibrations do not correct for matrix effects.

Experimental

Sample Prep

- All solvent standards were prepared in methanol.
- Plasma extracts were prepared by acetonitrile precipitation and centrifuging, with the upper layer taken and spiked with known concentrations of PFOA or PFOS.

LC

- Agilent 1200 Rapid Resolution LC system
- ZORBAX Eclipse Plus C18 Rapid Resolution HT column 2.1 cm × 50 mm, 1.8-µm particles (P/N 959741-902)
- 20-µL injection, 0.4 mL/min column flow
- 0 to 100% B in 10 min, A = water with 2 mM ammonium acetate; B = MeOH

MS/MS

- Agilent QQQ
- Negative-ion detection
- 3500 V_{cap}, drying gas 9.5 L/min at 350 °C, nebulizer 45 psi
- Fragmentor voltages, collision energy (CE), and ion transitions are experimentally determined

Multiple Reaction Monitoring (MRM)

Figure 2 displays a cross-section of the Agilent 6410 QQQ above a hypothetical sequence of spectra characteristic of ion transitions within the instrument.

The ions are generated in the source shown at the far left of the figure. The precursor ion of interest is then selected from this mixture and isolated through the Q1 quadrupole, which acts as a mass filter. This is similar to selected ion monitoring (SIM). After Q1, characteristic fragments that are specific to the structure of the precursor ion are generated in the collision cell (Q2, although not a quadrupole). By using the Q3 quadrupole, these fragments are then selected for measurement at the detector. This is a selective form of collision-induced dissociation (CID), known as tandem MS/MS. By setting Q3 to a specific fragment ion existing in the collision cell, the chemical or background noise is almost totally eliminated from the analyte signal, therefore, significantly increasing the signal-to-noise ratio. Ion 210 is called the precursor ion and ions 158 and 191 are product ions. Each transition (210→191 or 210→158) is a reaction for a particular target. Typically, the QQQ is used to monitor multiple analytes or mass transitions, therefore, the term MRM. The 158 could be considered the quantitation ion, because it is the

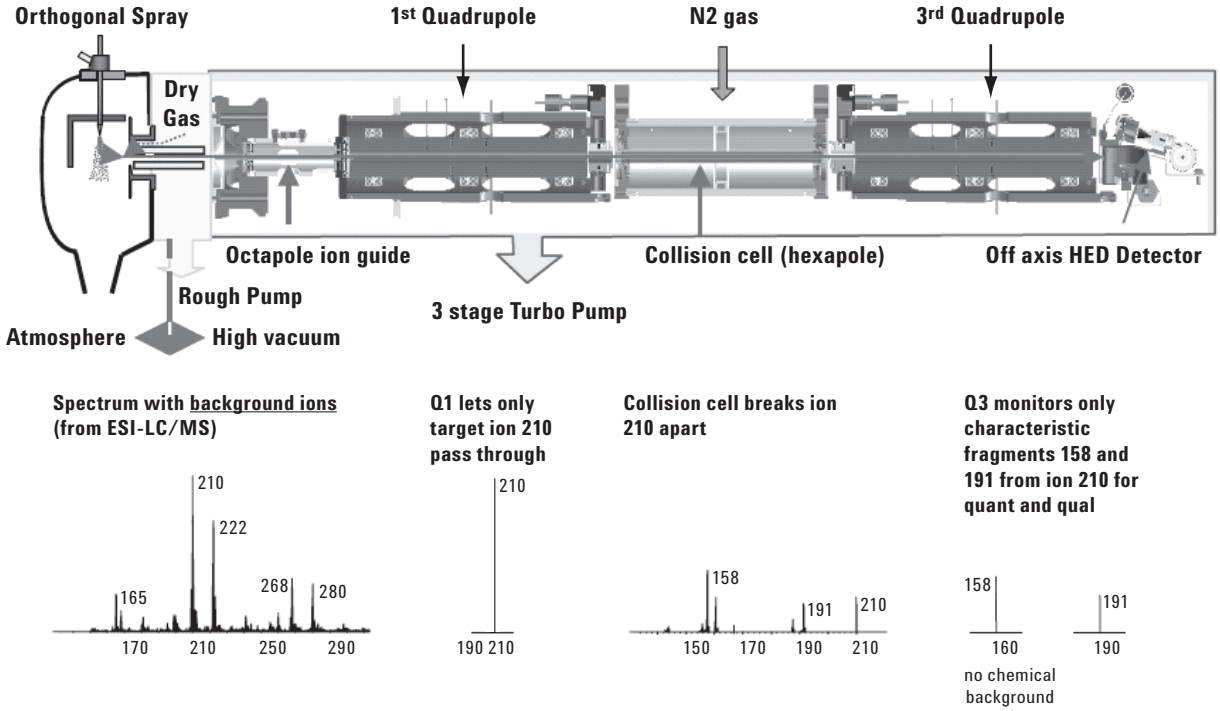


Figure 2. A cross-section of the Agilent 6410 QQQ above a sequence of spectra characteristic of ion transitions within the instrument for a hypothetical sample (*not* PFOA or PFOS). Note that the final spectrum is very clean, containing only the desired target ions. (HED = high-energy dynode electron multiplier)

most intense, and 191 could be used for confirmation by using the area ratio of the 191 qualifier to the 158 quantifier ion as a criterion for confirmation. With MRM, most chemical noise is eliminated in Q1, and again in Q3, allowing us to get ppt detection.

The fragmentor is the voltage at the exit end of the glass capillary where the pressure is about 1 mTorr. Fragmentor and collision energies need to be optimized. A fragmentor that is too small won't have enough force to push ions through the gas. A fragmentor that is too high can cause CID of precursor ions in the vacuum prior to mass analysis, thereby reducing sensitivity. The actual voltage used is compound-, mass-, and charge-dependent, and therefore needs to be optimized to get the best sensitivity. The CE in the collision cell needs to be optimized in order to generate the most intense product ions representative of each target compound. Collision cell voltage will depend on the bond strength, the molecular weight of the compound, and the path by which the ion is formed (directly from the precursor ion or through a series of sequential intermediates). Typically each product ion will exhibit a preferential collision energy that results in the best signal abundance.

The experimental operations required to arrive at optimal conditions are exemplified by the series of experiments shown in Figures 3 to 5.

Optimization of the fragmentor voltages for the $[M-H]^-$ ions of PFOA (*m/z* 413) and PFOS (*m/z* 499) are shown in Figure 3.

Note that there is little signal detected for PFOA at the optimal fragmentor voltage for PFOS (200 V). Ions 413 and 499 are called precursor ions. PFOA is relatively fragile; its precursor signal drops off at 160 V. PFOS shows that it is harder than PFOA to break apart; the best fragmentor voltage for PFOS is 200 V.

The appropriate collision energies for product ions *m/z* 369 $[M-CO_2H]^-$ and *m/z* 169 $[C_3F_7]^+$ are experimentally determined and used to quantify PFOA. See Figure 4.

In each case the collision energy producing the most intense peak for each ion is chosen for the analysis. PFOA takes little collision energy to break into ion *m/z* 369 (6 V for highest intensity).

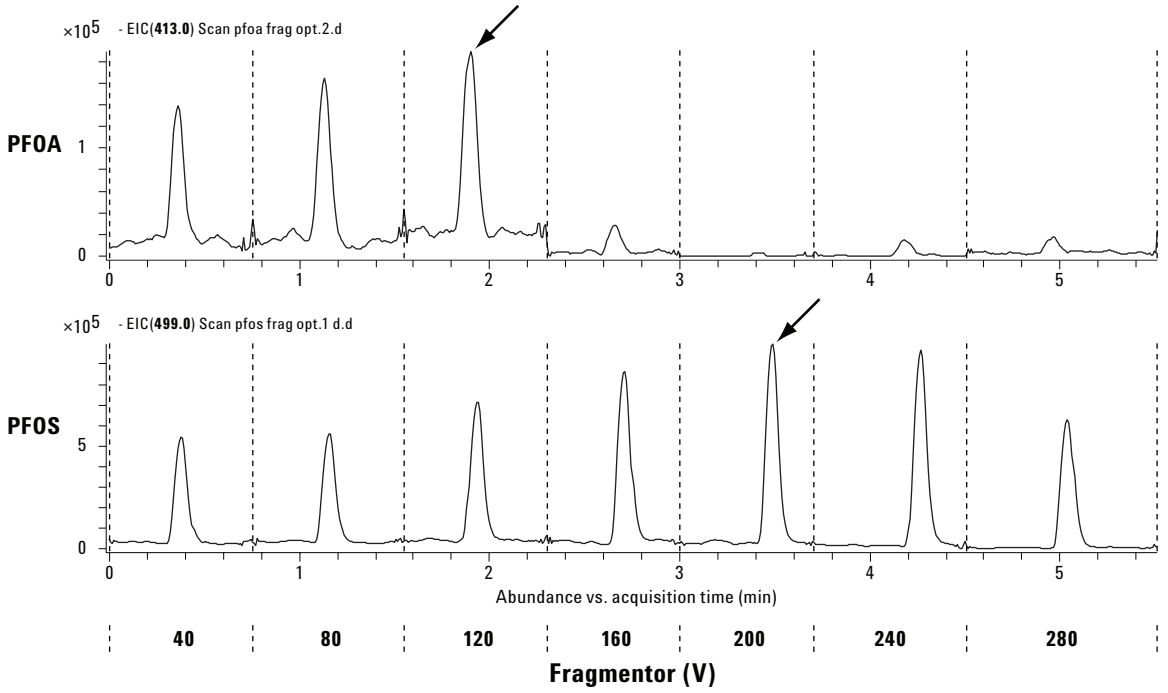


Figure 3. Determination of optimal fragmentor voltage using sequential plots of signal intensity versus applied voltage.

To maximize the intensity of the ion at *m/z* 169, the collision energy needs to go to 16 V.

The QQQ software can switch collision energies very rapidly. So in a method, the optimal collision voltage can be selected for each ion transition.

In the same manner, the appropriate collision energies for PFOS product ions at *m/z* 169, 99, and 80 are experimentally determined and used for its quantitation. The optimal collision energies for the three ion transitions are 45, 50, and 70 V. See Figure 5.

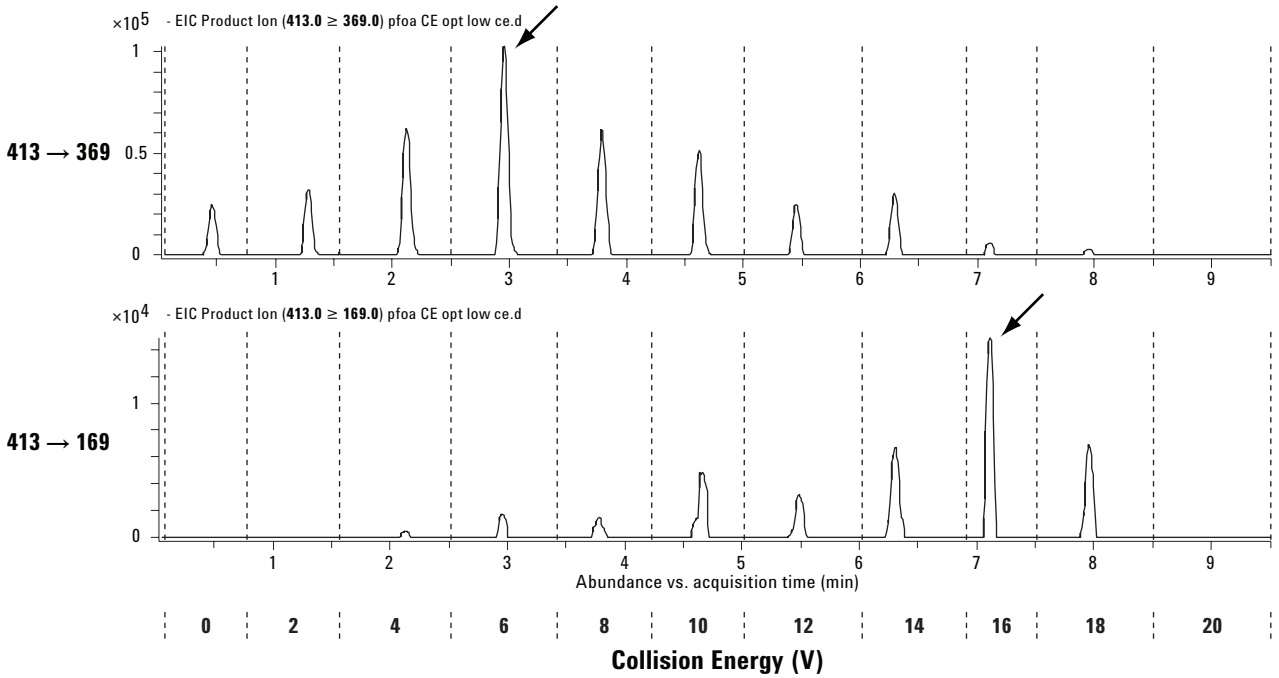


Figure 4. Signal intensity as a function of collision energy for PFOA product ions *m/z* 369 $[M\text{-}CO_2H]^-$ and *m/z* 169 $[C_3F_7]^+$.

Notice the big difference in collision energy between PFOA (6 to 16 V) and PFOS (45 to 70 V). We have seen from fragmentor optimization that PFOA is relatively fragile compared to PFOS, in which the optimum fragmentor voltages are 120 and 200 V for PFOA and PFOS, respectively. The CE reinforces that aspect.

Example calibration curves for the specified product ions used to quantitate PFOA and PFOS are shown in Figure 6. The analyst can also sum the intensities of these MRM transitions to get a calibration curve.

These five ion transitions exhibit linear correlation coefficients > 0.998, and are good for quantitation over three orders of magnitude. Notice that the lowest amount on column is 0.4 pg.

Regarding issue 1: ***What transitions should be used to give the best accuracy when quantifying with a linear standard?***

This is addressed using Figures 7 to 9.

Figure 7 exhibits chromatograms from these representative transitions for PFOA and PFOS for the linear standard and samples containing branches (10-min gradient).

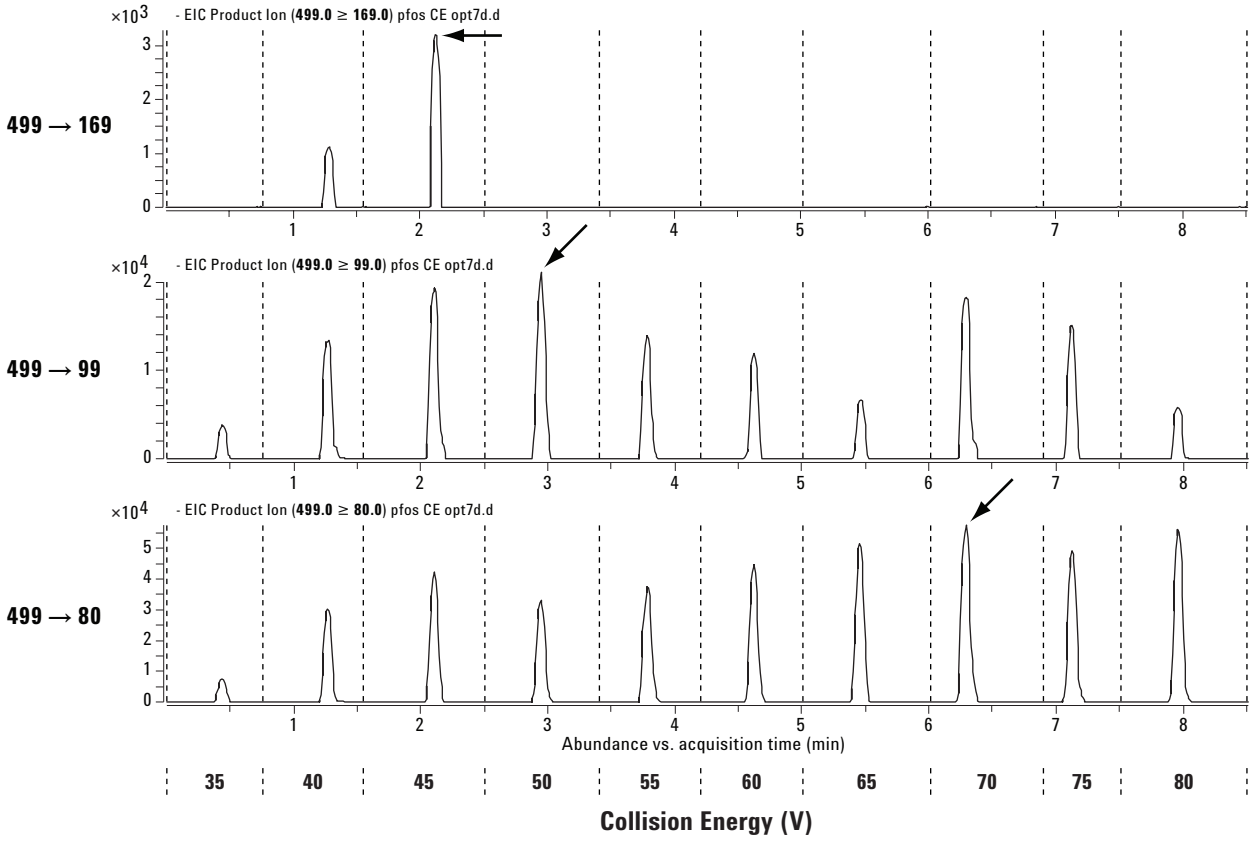
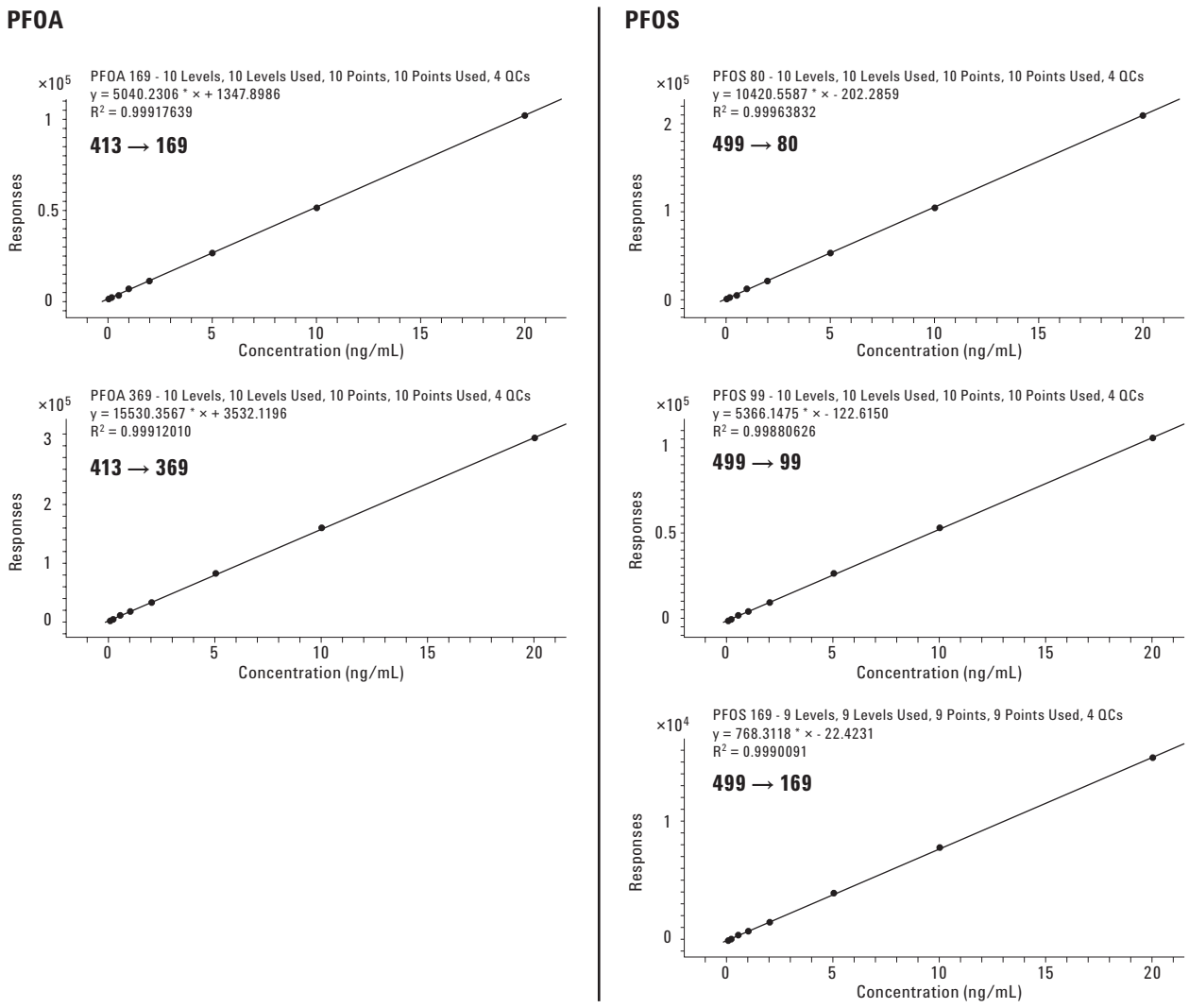


Figure 5. Signal intensity as a function of collision energy for PFOS product ions at *m/z* 169, 99, and 80.



Concentration range 0.02 to 20 ng/mL (0.4 to 400 pg injected on column)

Figure 6. Calibration curves for the product ions used to measure PFOA and PFOS.

Real-world samples have been detected with branched isomers due to manufacturing processes, metabolism, and degradation processes. The top chromatogram of Figure 7 shows only linear chain compounds from a standard. The bottom chromatogram is an actual sample from the environment. It shows additional peaks (shoulders) in the chromatogram resulting from branched isomers.

We examine those peaks in greater detail in Figure 8.

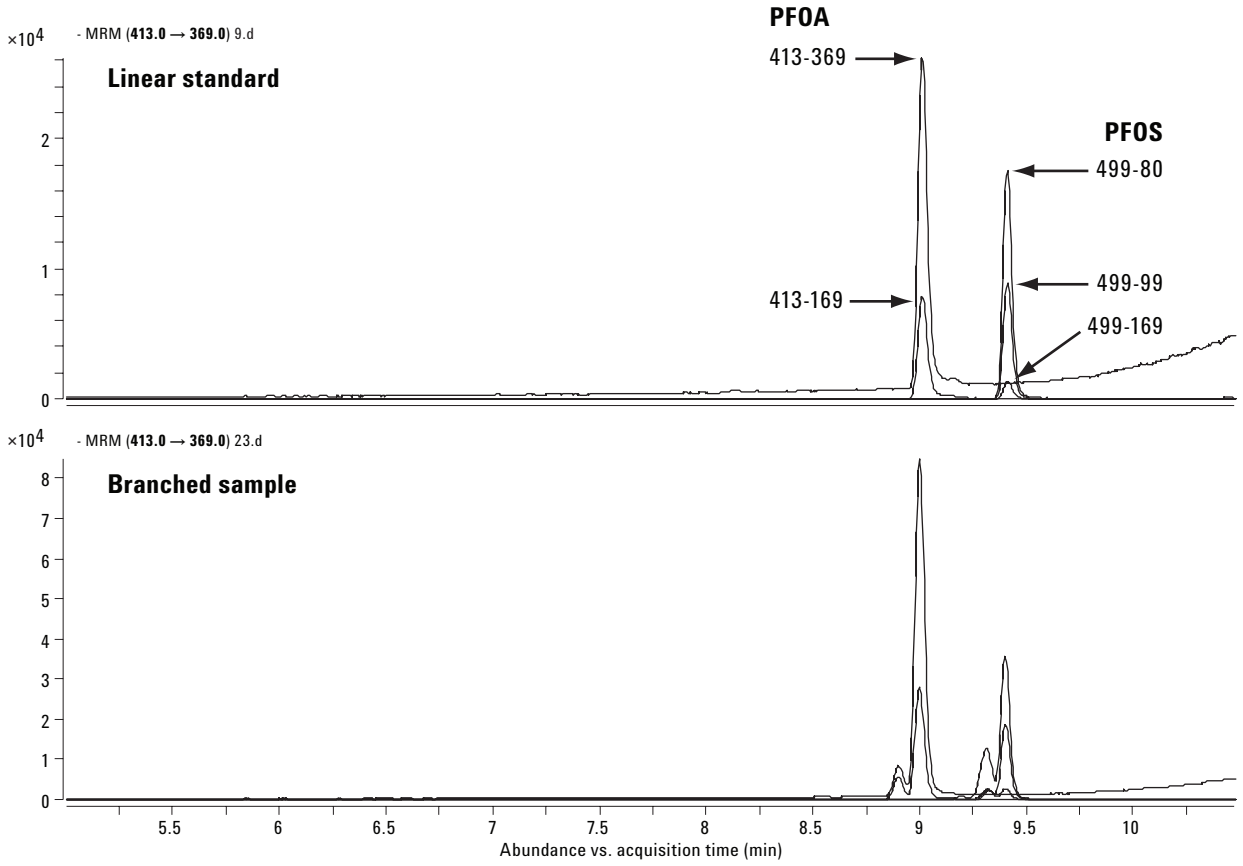


Figure 7. MRM chromatograms for PFOA and PFOS for both linear and branched samples.

The relative abundances for each MRM transition are dependent on the branching locations and the specific mass transitions. Figure 8 shows a 10-minute run. The chromatography can separate the linear from the branched isomers. The branched sample is typically a C7 chain with a methyl side group (isooctyl isomer). The most interesting part of the analysis is that the ion ratios for the branched compounds are very different from the linear chain compounds [3, 4, 5]. For linear PFOA, the ion at *m/z* 169 is about 30 to 40% of ion 369. The branched isomer shows that the ratio changed to 90 to 100%. For linear PFOS, the ion at *m/z* 99 is about 50% of ion 80 and is 500% of ion 169. The branched isomer shows that ion 99 is only 20 to 30% of ion 80, and 100% of ion 169. This is a cause of concern in terms of quantitation accuracy. This shows that CID stability is very different when the analyte is branched.

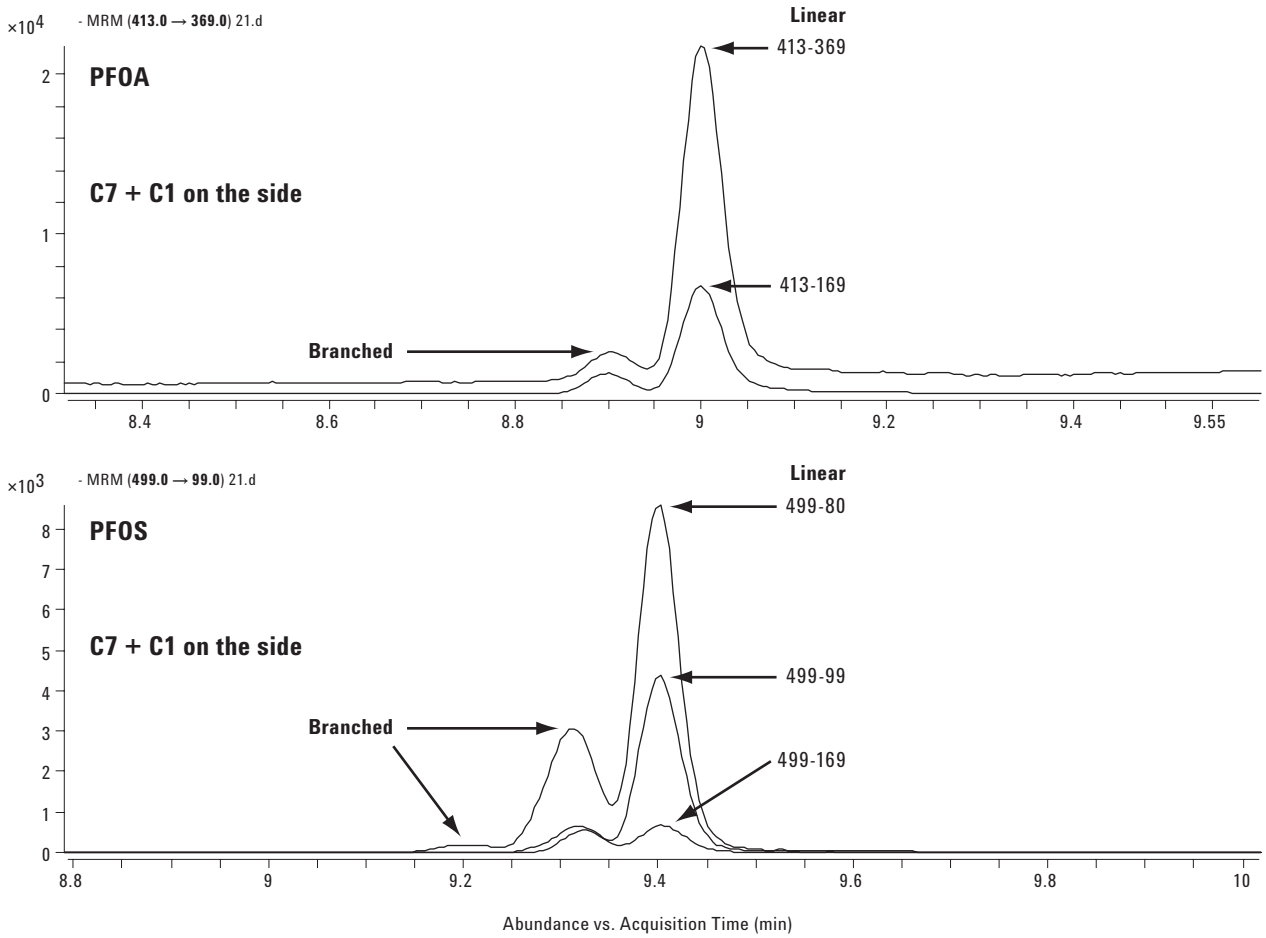


Figure 8. MRM chromatograms for PFOA and PFOS for both linear and branched samples.

Another variable in the analysis is the gradient time. Figure 9 compares the effect of a 3-min versus 10-min gradient.

In the fast gradient case (on the right), the branched isomers (dashed lines) are not resolved from the linear isomers (solid lines), resulting in a significant error in the measured value (most noticeable for PFOS).

The two chromatograms on the left are the same two that are shown in Figure 8. They are used here for comparison against the unresolved analytes shown on the right (3-min run). Although we would like to cut down on the analysis time, the branched and linear isomers need to be resolved in order to get accurate quantitation results.

Two samples of the same concentration. One sample is the pure linear isomer; the other sample has a mixture of branched isomers. If their MRM responses (ion ratios) are the same, they would show the same results as when the isomers are not resolved. This example shows that the responses are not the same when the isomers are not resolved. If you add the responses of the side chain analyte and the linear chain analyte of the same sample, the area of each ion transition is different from the pure linear chain analyte ion transition, as seen in the two chromatograms on the right, most apparent is for PFOS. If using the linear chain sample as standard for calibration, the results of real-world samples (branched and linear isomers mixed) will be off by as much as 40% (see Table 1). The quantitation falls apart.

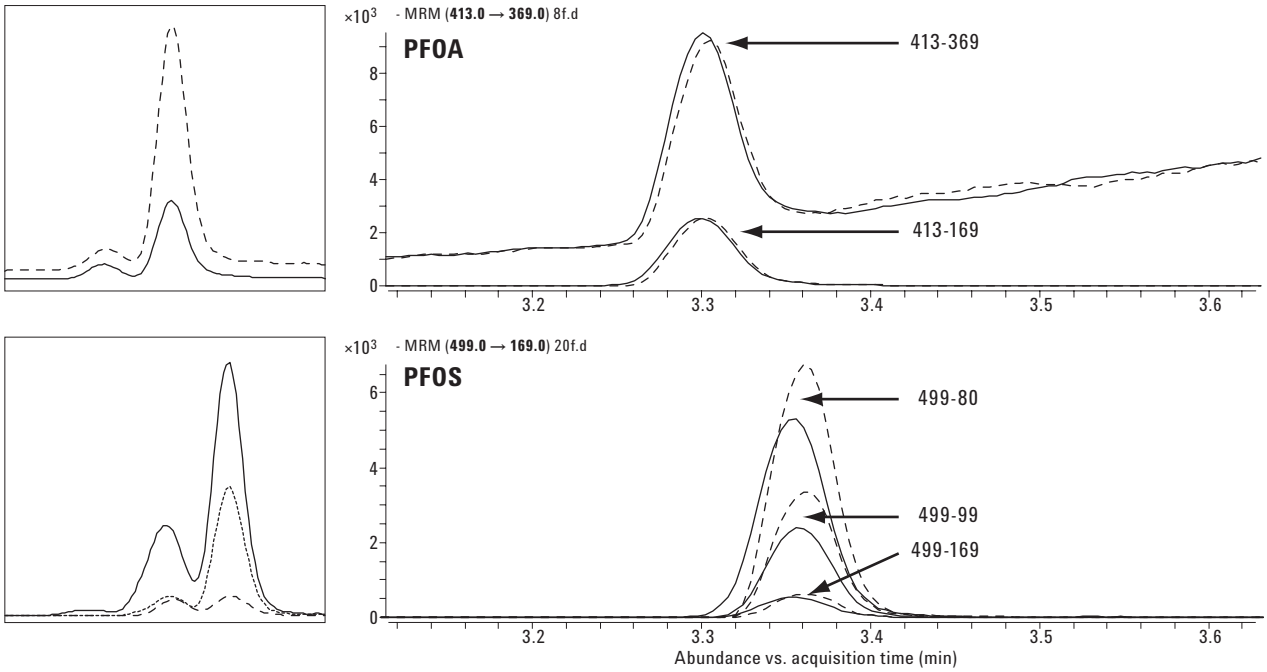


Figure 9. Comparison of PFOA and PFOS MRM chromatograms produced using both 10- and 3-minute gradients. The 3-minute gradient chromatograms are on the right.

The effect of measurement accuracy (*not* ion ratios) of total PFOA and PFOS in branched samples against *a linear standard for each MRM transition* is shown in Table 1.

Table 1. Measurement Accuracy (Target Is 100%) as Function of Compound, Transition, and Run Time

Compound	MRM transition	Percent response (n = 8)	
		10-min run	3-min run
PFOA	**413→369**	**105.9**	**108.2**
	413→169	**96.4**	**89.4**
PFOS	**499→169**	**102.5**	**112.2**
	499→99	75.0	73.3
	499→80	59.3	61.1

The best MRM ions are in bold type. The best results for PFOA can be obtained by averaging the results for the two MRM ions together.

Ion ratios can cause quantitation failure. For PFOA, it does not matter if it's a 3-min run or a 10-min run: the ion 369 transition response is always higher and the ion 169 transition response is always lower. The errors are larger for the 3-min run. The variations are greater for PFOS. In literature, PFOS analysis monitors the ion 80 transition, but it exhibits a large variation. It can be as low as 60%, as seen in Table 1. 499 → 169 is a good transition for quantitation. It is much more accurate, but it is less sensitive compared to 499 → 80 transition.

Regarding issue 2: *Can isotopically labeled standards in matrix be used to measure non-labeled PFOS and PFOA?*

This is addressed using Figures 10 to 12.

Observations regarding the effect of different matrices on signal responses are shown in Figure 10. The taller trace represents the response of PFOA in methanol. The response is lower as the same amount of PFOA is added into a plasma extract.

The matrix effect (common using electrospray ionization) can lead to signal suppression or enhancement; therefore, matrix-matched calibrations are required for accurate quantitation. Due to varying background levels of PFOS and PFOA in matrix, it may not be feasible to use matrix-matched calibrations for quantitating PFOS or PFOA concentrations in study samples. Also, the method of standard additions is not a practical alternative for many matrices with varying levels of target analytes.

As a practical alternative, measuring PFOA using isotopically labeled matrix-matched standards was examined. Results are shown in Figures 11 and 12.

Figure 11 shows that isotopically labeled standards can provide a good linear calibration curve over the quantitation range of 0.02 to 20 ng/mL (0.4 to 400 pg on column). Excellent linear correlation coefficients (≥ 0.9994) were obtained.

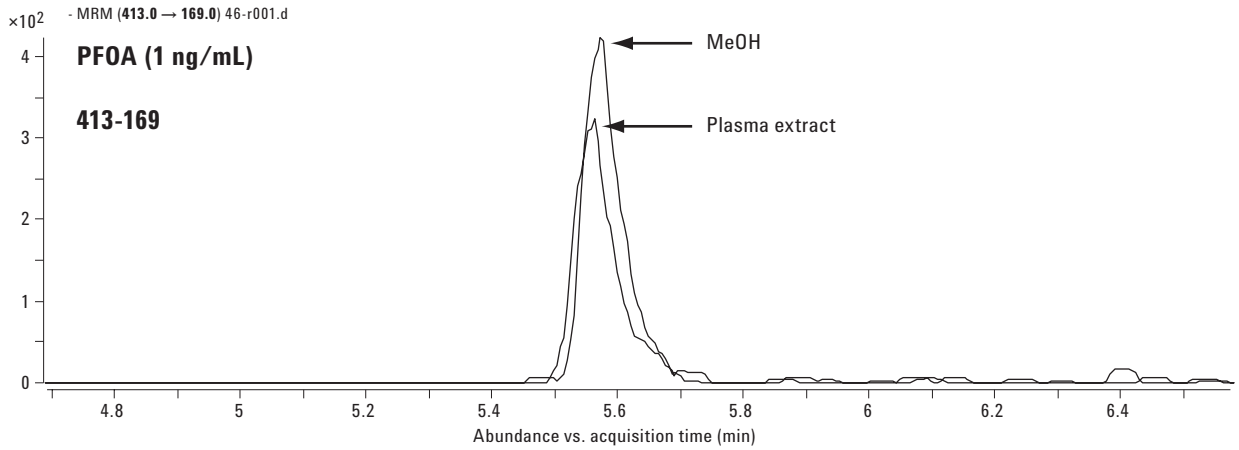


Figure 10. PFOA responses in MeOH and plasma extract at the same concentrations.

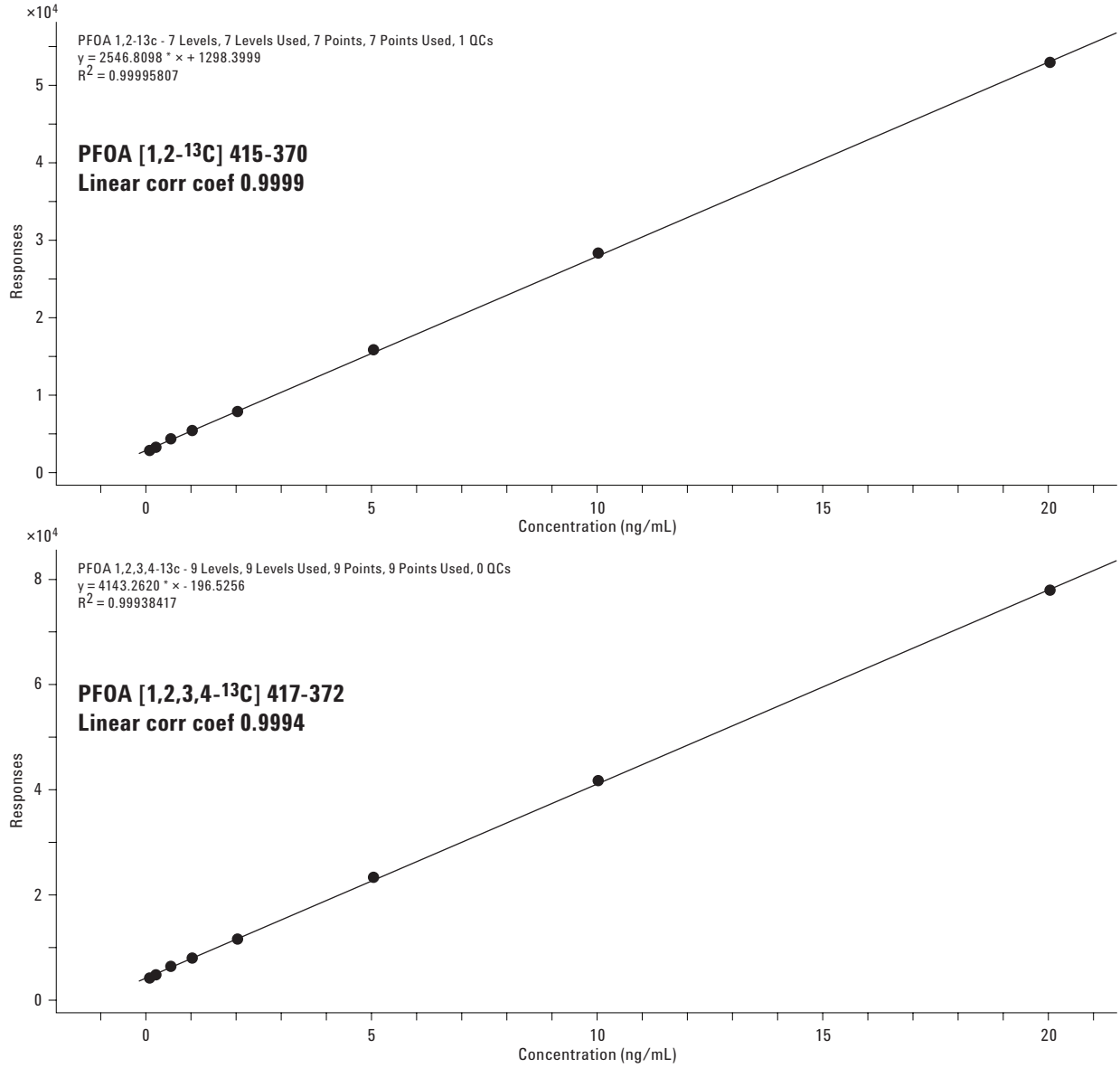


Figure 11. Linear correlations for PFOA using two different isotopically labeled calibration standards.

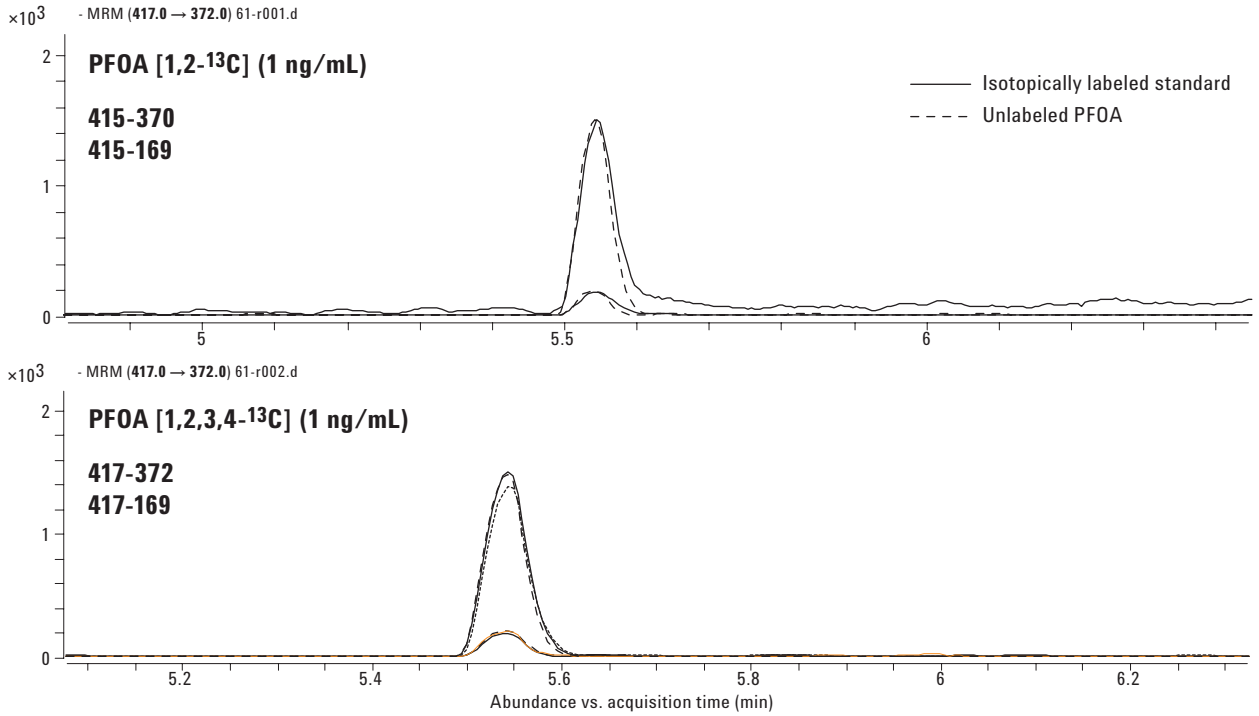


Figure 12. Both isotopically labeled PFOA compounds show good correlation to the unlabeled PFOA. The same transitions for the labeled and native forms of the PFOA were used.

Table 2. Comparison of Different Matrix-Matched Calibrations for Measuring PFOA in Plasma

	Calibration standard	Matrix for calibration	Plasma sample response (Std Dev)
1	PFOA	MeOH	71 (± 33 %)
2	PFOA [1,2-^{13}C]	Plasma	100.4 (± 3.1 %)
3	PFOA [1,2,3,4-^{13}C]	Plasma	97.3 (± 5.1 %)

Matrix-matched calibrations using isotopically labeled PFOA work well.

For row 1, the calibration standard used MeOH as the solvent, and the plasma sample exhibited a 71% response due to matrix suppression. Therefore, we cannot use a calibration standard in MeOH to quantitate samples in matrix; the variation can be as large as 30%. Rows 2 and 3 show that if the calibration is done using an isotopically labeled compound in matrix, the actual plasma sample yields accurate results: 100 and 97%.

Conclusions

- The Agilent LC/QQQ is an excellent instrument for quantifying trace target compounds in complex mixtures.
- The best ion transitions for analysis need to be determined experimentally.
- Fragmentor voltages and collision energies require experimental determination and optimization.
- Using MRM in the QQQ helps achieve the lowest detection limits in complex matrices.
- Branched PFOA/PFOS can affect quantitation accuracy as much as 40% unless it is corrected.
- Matrix suppression can cause the quantitation to be off by as much as 30%. Isotopically labeled analytes work well for accurate quantitation in spite of varying background levels of PFOA/PFOS in matrices.

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Acknowledgement

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Analysis of poisoned food by capillary electrophoresis

Application Note

Food

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Abstract

In cases of poisoning, analytical tools are needed to determine the identity of the toxins quickly and accurately. This enables healthcare professionals to administer appropriate treatment as quickly as possible and helps police to find those responsible. A rapid determination of anionic toxins in adulterated foods and beverages is possible using capillary electrophoresis (CE) with indirect UV detection. Cyanide, arsenite, arsenate, selenate, azide and other anions can be detected within 15 minutes, requiring only minimal sample preparation.

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Experimental

Anion analysis was performed using the Agilent Capillary Electrophoresis system equipped with diode-array detection and Agilent ChemStation software. The analysis is based on the Agilent Forensic Anion Solutions Kit (Agilent part number 5064-8208).

Prior to first use, a new capillary was flushed with run buffer for 15 minutes (at 1 bar). Between the analyses the capillary was flushed 2 minutes from the OutHome vial into waste, then 2 minutes from the InHome vial into waste. This procedure avoids baseline fluctuations as a result of buffer depletion. Buffer vials were replaced after 10 runs when using 2 mL vials, after 5 runs, when using 1 mL vials. Sample preparation consisted simply of dilution with water, or dilution and additional filtration through a 0.22 µm filter, as indicated in figure 1.

Equipment

- Agilent Capillary Electrophoresis system
- Agilent ChemStation
- Agilent Forensic Anion Solutions Kit

Results and discussion

Figure 1 shows the analysis of food spiked with cyanide and arsenite. Depending on the results of this quick analysis, the sample can then undergo a more detailed analysis.

The assay was linear over the range 10–100 ppm with $r^2 > 0.999$. The method detection limit was 5–10 ppm.

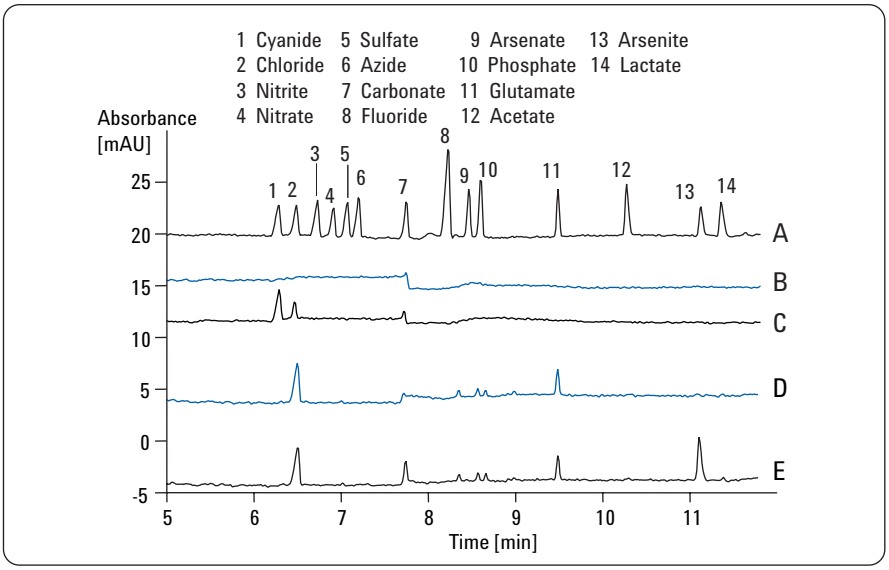


Figure 1
Analysis of cyanide and arsenite in food. A = anion standard (50 ppm each), B= Oolong tea (1:100 diluted with H_2O), C= Oolong tea as in B, spiked with 100 ppm NaCN, D=curry (1:100 diluted with H_2O, filtered through 0.22 µm filter), E=curry as in D, spiked with 100 ppm $NaAsO_2$.

Chromatographic conditions

Injection:	6 seconds at 50 mbar
Capillary:	Fused silica capillary, total length 112.5 cm, effective length 104 cm, internal diameter 50 µm (Agilent part number G1600-64211)
Buffer:	Agilent Basic Anion Buffer (Agilent part number 5064-8209)
Voltage:	-30 kV
Temperature:	30 °C
Detection:	Signal 350/20 nm, reference 275/10 nm

For the analysis of curry, the repeatability for arsenite (n = 6) was 0.06 % RSD for migration time and 2.7 % RSD for peak area. For cyanide in Oolong tea the respective values were 0.13 % RSD for migration time (n = 10) and 4 % for peak area (sample diluted in 0.01 N NaOH).

Other toxic anions that can be determined are arsenate, azide and selenate (which migrates between azide and carbonate). Compared to ion chromatography (IC), the advantages of CE for this type of analysis are the shorter analysis time and the minimal sample preparation needed for samples with a complex matrix (e.g. curry). Additionally, the analysis of azide and arsenate together with cyanide and arsenite is not possible in one run with IC.

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Determination of Benzimidazole Fungicides in Apple Juice by SampliQ Polymer SCX Solid-Phase Extraction with High-Performance Liquid Chromatography

Application Note

Food Safety

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Abstract

Solid-phase extraction (SPE) coupled with high-performance liquid chromatography (HPLC) was optimized for extraction and quantification of two fungicides (carbendazim and thiabendazole) in apple juice. Results indicate that SPE using Agilent SampliQ SCX (60 mg, 3 mL) and HPLC using an Agilent ZORBAX Eclipse Plus C18 column (4.6 mm × 100 mm, 3.5 µm) is an excellent combination for extraction and analysis of these compounds. Recoveries ranged from 92.1 to 99.4 percent with RSDs below 5 percent and limits of detection of 4 µg/kg.

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Introduction

Because of the indiscriminate use of pesticides for different applications, important environmental problems are emerging that are a risk to plant, animal, and human health. Fungicides are one group of these pesticides that are used primarily to control spoilage of crops as a result of fungal attack. Fungicides in general represent approximately 20 to 25 percent of all pesticides used. Benzimidazole fungicides are systemic pesticides, widely used in agriculture for pre- and post-harvest treatment for control of a wide range of pathogens. These substances are applied directly to the soil or sprayed over crop fields and hence are released to the environment. They readily penetrate plants through the roots and leaves and can directly enter natural waters by drainage from agricultural land. Most of these compounds persist in the environment after application; some even remain for years. Two of the main compounds in the benzimidazole family are carbendazim and thiabendazole (Table 1). Carbendazim has both protective and curative activity against a wide range of fungal diseases. It is toxic to humans, animals, and plants and also is very persistent in water, wastewater, soil, crops, and food. Thiabendazole is used to control fruit and vegetable diseases such as mold, rot, and blight, and is used as a veterinary drug to treat worms.

Agilent SampliQ SCX SPE cartridge was used to extract fungicides from apple juice. This application note describes the implementation and optimization of the method described in SN/T 1753-2006 and the results of validation.

Experimental

Materials and Chemicals

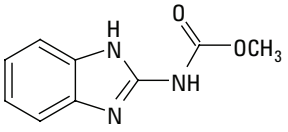
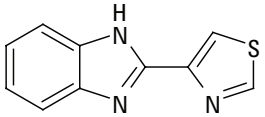
All reagents and solvents were HPLC or analytical grade. Fungicide standards were purchased from Sigma-Aldrich Trading Co. (Shanghai, China). Apple juice (food grade) was purchased from a local market.

Phosphate buffer: 1.38 g sodium dihydrogen phosphate and 1.41 g disodium hydrogen phosphate in 1,000 mL water, adjust pH to 3.0

Stock solution (0.1 mg/mL) was prepared in methanol and kept in the freezer (–20 °C). Working solutions were prepared using the stock solution diluted with methanol. The working solutions should be prepared every week and need to be stored at 4 °C.

The SPE cartridges were Agilent SampliQ SCX 3 mL, 60 mg (p/n 5982-3236). The analysis was performed on an Agilent 1200 HPLC with variable wavelength detector (VWD). The analytical column was an Agilent ZORBAX Eclipse Plus C18 3.5 µm 100 mm × 2.1 mm id (p/n 959793-902). Agilent 0.45-µm filter membranes (p/n 5185-5836) were used to filter sample solutions prior to HPLC analysis.

Table 1. Fungicides Used in this Study

No.	Name	pKa	Log P	Structure
1	Carbendazim CAS # 10605-21-7	4.48	1.45	
2	Thiabendazole CAS # 148-78-8	4.7	2.39	

HPLC Conditions

Column:	ZORBAX Eclipse Plus C18 100 mm × 4.6 mm 3.5 µm (p/n 959793-902)
Flow rate:	1.0 mL/min
Injection volume:	20 µL
Detection wavelength:	288 nm
Moblie phase:	Phosphate buffer-Acetonitrile (73:27)

Sample Preparation

Weigh 10 g apple juice, dilute to 100 mL with water, and mix with a glass rod for 1 minute. Transfer the diluted sample to a 250-mL Erlenmeyer flask and adjust pH to 10 with 2 mol/L NaOH solution. Divide the sample between two or three 50-mL polypropylene centrifuge tubes and centrifuge for 10 minutes at 4,000 rpm. Recombine the supernatants into a glass beaker.

SPE Purification

The procedure used for the SPE extraction is shown in Figure 1. The Agilent SampliQ SCX cartridges were conditioned with 3 mL of methanol, followed by 3 mL 0.15 mol/L NH_4OH solution with gravity flow (about 1 mL/min).

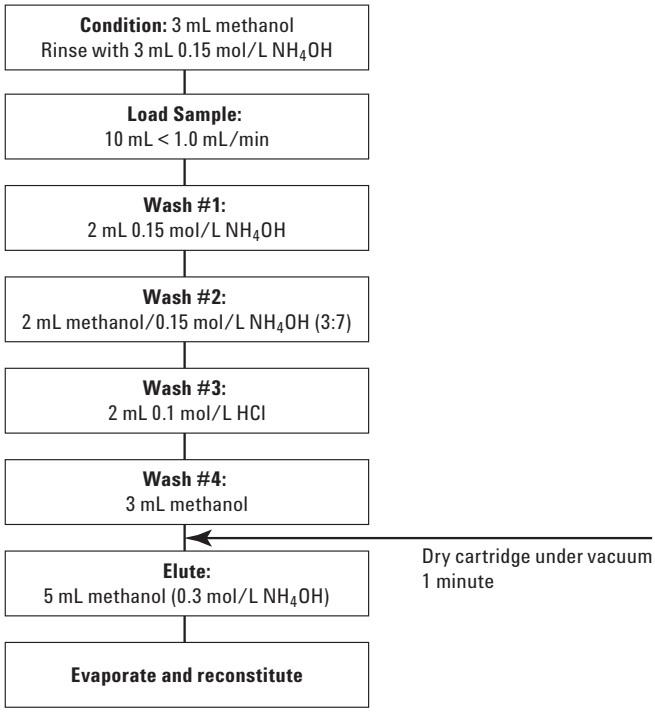


Figure 1. Fungicides in apple juice SPE procedure.

Load 10 mL supernatant liquid to SampliQ SCX cartridges at a speed about 1 mL/min. After the sample effuses completely, wash the cartridge with 2 mL of 0.15 mol/L NH_4OH, 2 mL of a solution of methanol and 0.15 mol/L NH_4OH (3:7), 2 mL of 0.1 mol/L HCl and 3 mL methanol. All three wash steps were under gravity flow. Discard all of the effluents. Dry the cartridge under negative pressure below 2.0 kPa for 1 minute. Finally, elute the cartridge with 5 mL of 0.5 mol/L NH_4OH in methanol, under gravity flow. Collect the eluent and dry it under nitrogen. Dissolve the resulting residue and bring it to a constant volume of 1 mL using the mobile phase. Then filter the residue through a 0.45-µm filter membrane and analyze.

Results and Discussion

Linearity, Limits of Detection

Stock solutions were diluted to different concentrations and analyzed by HPLC. Linear regressions were calculated for the tetracyclines using the areas and the solution concentrations. The limit of detection (LOD) was the injection concentration at which the signal-to-noise ratio was between 2 and 3. The linear range was between 25 and 500 µg/kg. The linearity and LOD are shown in Table 2.

Table 2. Linearity and LODs of Fungicides

Compound	Regression equation	Correlation coefficient	LOD (µg/kg)
Carbendazim	Y = 75.781× – 0.4018	0.9999	4
Thiabenzole	Y = 108.07× – 0.6984	0.9999	4

Recovery and Reproducibility

Recoveries were calculated for spiked fungicide standards in apple juice at 25, 50, and 100 µg/kg levels. The analysis was performed in replicates of six at each level. The chromatograms of the blank and spiked standard (100 µg/kg) are shown in Figure 2 and Figure 3. The recovery and reproducibility data are shown in Table 3.

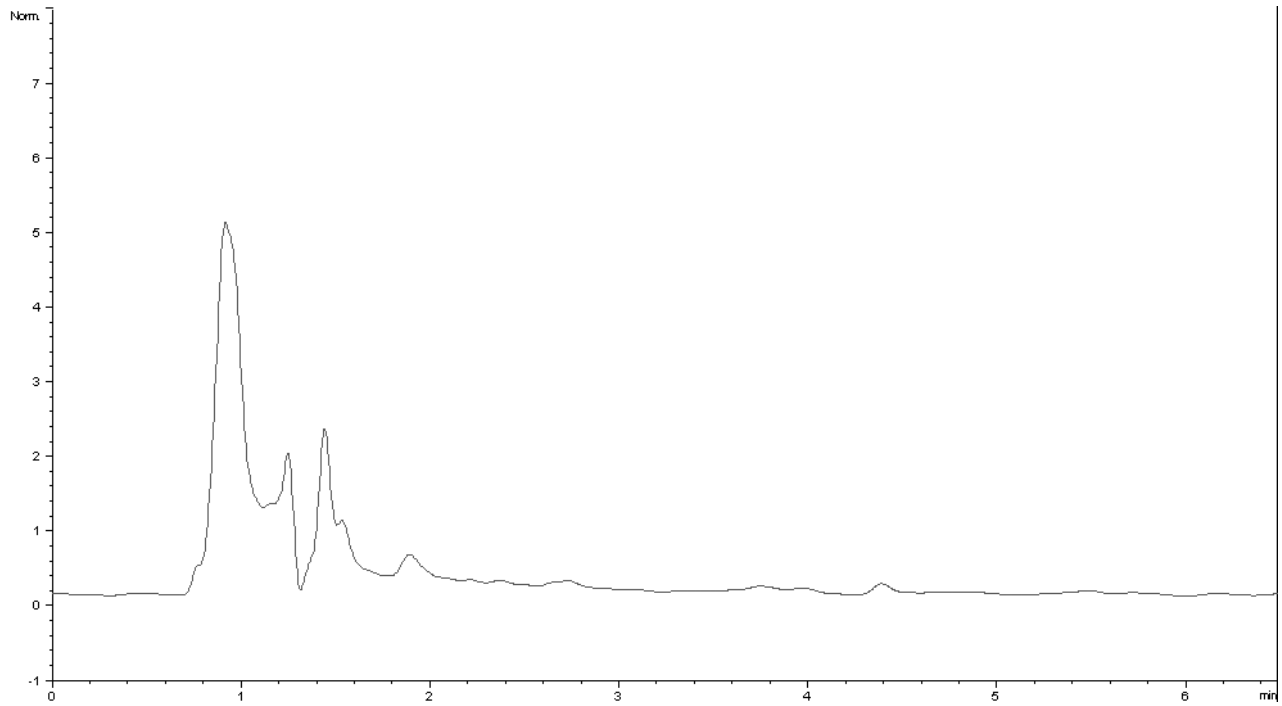


Figure 2. Chromatogram of apple juice blank.

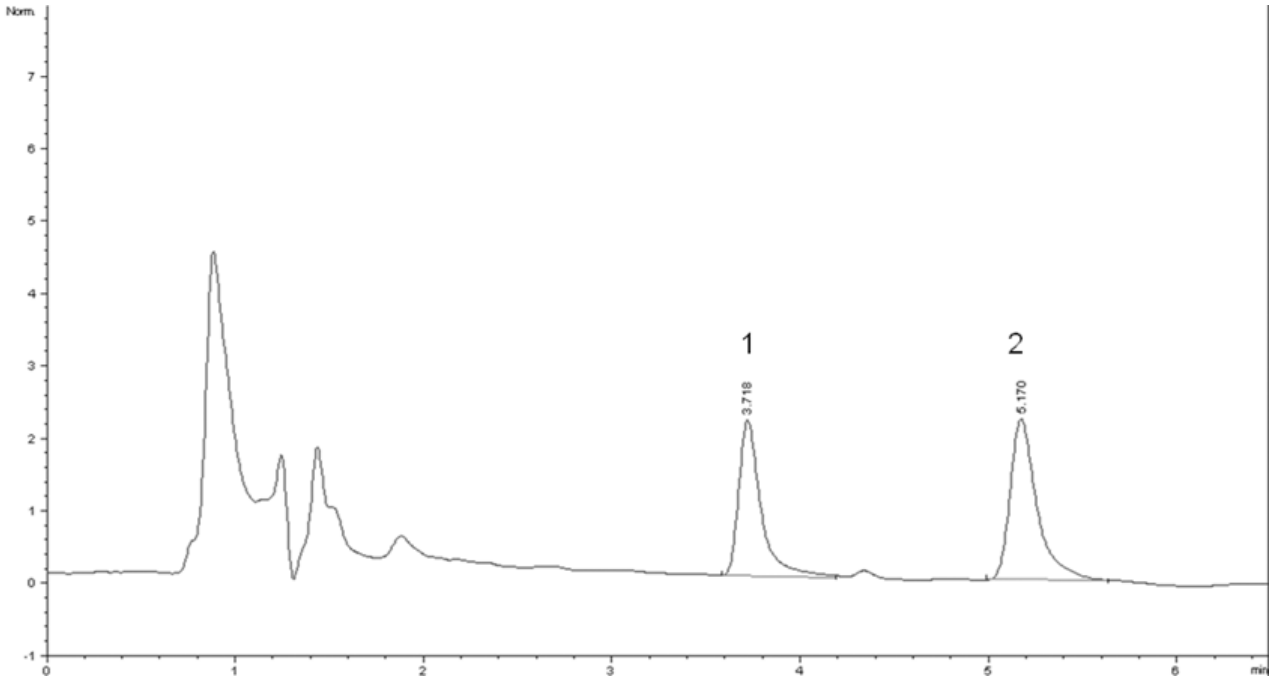


Figure 3. Chromatogram of apple juice sample spiked at 100 µg/kg (1 – Carbendazim, 2 – Thiabenzole).

Table 3. Recoveries and RSDs of Fungicides in Apple Juice by SPE

Compound	Spiked level (µg/kg)	Recovery (%)	% RSD (n = 6)
Carbendazim	25	98.6	3.99
	50	99.4	3.24
	100	95.9	3.27
Thiabenzole	25	99.0	2.38
	50	92.1	4.90
	100	93.0	3.79

Conclusions

Agilent SampliQ SCX provides a simple and effective single-cartridge SPE method for the purification and enrichment of fungicides in apple juice. The recovery and reproducibility results based on solution standards are acceptable for fungicide residue determination in apple juice under the Chinese regulation. The impurities from apple juice were minimal and did not interfere with any of the fungicides analyzed.

References

SN/T 1753-2006, Determination of thiabenzole and carbendazim residues in concentrated fruit juice for import and export – High Performance Liquid Chromatographic method.

For More Information

For additional information on Agilent SampliQ SPE products visit: www.agilent.com/chem/sampliq

For additional information on Agilent HPLC columns visit: www.agilent.com/chem/LCcolumns

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An Application Kit for Multi-Residue Screening of Pesticides using LC/TOF or Q-TOF with a Pesticide Personal Compound Database

Application Note

Food Safety and Environmental

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Abstract

An application kit for pesticide screening has been developed for the Agilent time-of-flight (TOF) and quadrupole time-of-flight (Q-TOF) mass spectrometers using a database with almost 1600 entries. It can be quickly and easily used for both food and environmental samples where the ability to detect and identify a large number of pesticides is necessary. The system allows the user to create custom databases containing retention times of compounds of interest for targeted analysis. Screening with this database thus provides both targeted and non-targeted pesticide detection. A test mix for both positive ion and negative ion modes is provided to demonstrate the functionality of the kit. An example of a general method for pesticide screening is given along with an example of a spinach extraction using the Agilent SampliQ extraction and dispersive SPE kits for complete food analysis.

Agilent Technologies

Introduction

Because over 1000 pesticides have been in use over the last century and new pesticides are being developed, there is a great need to perform both targeted and non-targeted screening in food and the environment. The Agilent time-of-flight (TOF) mass spectrometers provide both high mass resolution and mass accuracy that allow comparison of the measured mass to the exact mass of an ionized compound. In addition, the tandem hybrid quadrupole time-of-flight (Q-TOF) mass spectrometer provides the capability of both screening and confirming compounds in one instrument.[1] Both liquid chromatography (LC) combined with TOF MS and Q-TOF MS provide a robust and sensitive means to perform this type of screening at levels required by the international community. Because TOF is a pulsing instrument the resulting data is always full spectra, which allows the screening of compounds that are sought (targeted) and those that may not be expected (non-targeted).[2] In contrast LC/MS/MS with a triple quadrupole in its most sensitive mode, multi-reaction monitoring (MRM), provides targeted screening and confirmation only.[3]

Recently Agilent has introduced the Pesticide Personal Compound Database (PCD) consisting of 1600 compounds and pesticides. With PCD the analyst can use the pesticide database as is for non-targeted screening or create custom databases from the read-only supplied database. The custom database can be edited by changing entries, adding, and deleting entries. In addition, a powerful feature of updating retention times allows the users' custom database to be modified with retention times from the users' chromatographic conditions.[4] The analyst can create as many custom databases with LC-dependent retention times as needed. This allows easy targeted (compounds verified with standards run with specific conditions) and non-target analysis (compounds in the database that have not been verified). The ability to detect and identify compounds not being sought in food and environmental samples can be very important. However, this ability must not be confused with affirmation that compounds not detected are not present. This can only be done by validation studies showing that the specific LC/MS method employed on specific matrices can detect the compounds reported as not present at the levels of concern. As an example, the pesticide database contains compounds not amenable to LC/MS such as hexachlorobenzene. These are included for the added information of the user. In addition, confirmation of positives would always require standards run with chromatographic conditions that would provide indicative retention times and additional structural information that can be obtained from fragments generated by MS/MS. Even with these analytical considerations, screening for a large list of pesticides as enabled by the LC/TOF or Q-TOF with the Agilent Pesticide PCD can be very valuable in detecting and identifying compounds that should not be present.

Experimental

Reagents and Chemicals

Pesticide standards were from a variety of sources: Sigma, Ultra-Scientific, ChemService, and Dr. Ehrenstorfer. For trace analysis the highest purity mobile phases are recommended. B&J LC/MS grade acetonitrile and methanol are used here. Buffers should be prepared from the highest quality chemicals such as GFS doubly distilled acetic acid, formic acid and ammonium hydroxide. If solid ammonium acetate and ammonium formate is used it should be prepared in a concentrated solution and then any particulates removed with 0.2-µm filters. Agilent Pesticide Test Mix, p/n 5190-0469 acid and base diluted separately as instructed to 10 ppb in 10% acetonitrile/90% water. An Agilent SampliQ QuEChERS AOAC Extraction kit, p/n 5982-5755. Agilent SampliQ QuEChERS AOAC Dispersive SPE kits for Highly Pigment Fruits and Vegetables, p/n 5982-5321 (2 mL) and p/n 5982-5356 (15 mL).

LC/MS methods are given in the Appendices:

Appendix I, LC/MS/MS Conditions for Test Mix Positive and Negative Ion Samples.

Appendix II. Agilent 1200 Series SL LC Parameters.

Appendix III, Agilent 1290 Infinity LC Parameters.

Spinach sample preparation

- Weigh 15 g (±0.1 g) of homogenized spinach sample.
- Spike standards or IS solution if necessary.
- Vortex 30 s.
- Add 15 mL of 1% acetic acid in acetonitrile.
- Add 1 bag of extraction kit (p/n 5982-5982-5755) buffered QuEChERS extraction tubes, AOAC Method 2007.01 with 6 g $MgSO_4$, 1.5 g NaAcetate.
- Cap and hand shake vigorously for 1 min.
- Centrifuge at 4000 rpm for 5 min.
- Transfer 1 mL or 8 mL of the upper layer to the dispersive SPE kit (p/n 5982-5321 or p/n 5982-5356) for highly pigmented fruits and vegetables.
- Vortex 1 min.
- Centrifuge 2-mL tubes at 13000 rpm for 2 min, or 15-mL tubes at 4000 rpm for 5 min.

- Transfer 200 µL of the upper layer to the autosampler vial.
- Add 800 µL of water or appropriate standard spiking solution.
- Vortex 1 min, and prepare for LC/MS/MS analysis.

Results and Discussion

Fast and easy startup with Agilent test mix

To facilitate fast startup for pesticide screening, a positive and negative ion compound test mix is included with the Agilent Application Kit. This type of screening depends on obtaining accurate mass results and the TOF or Q-TOF should be operated with appropriate reference ions so that the best results will be obtained. Each of these test mixtures are prepared with a final injection concentration at 10 ppb, the accepted limit for pesticides worldwide. The extracted ion chromatogram (EIC) for each of the pesticides in the positive ion mix is shown in Figure 1. A method is provided with the kit that will allow the user to repeat this analysis. This method is an acquisition only method. Similar results demonstrate that the system is working properly. There are also two methods provided for work list automation data analysis that will generate the summary report of a database search of the Pesticide PCD. One method is the MFE_pesticide and this uses the “find compounds by molecular feature extraction (MFE)” algorithm in MassHunter Qualitative Analysis, a powerful data mining tool. This unique data mining program searches the data for all ions that can be associated with a real chromatographic peak and that may represent a "feature" of a molecule. This excludes reference ions and constant background ions and "spikes" that do not represent real compounds in the data file. MFE will create a compound list of all peaks in the data file that it has determined to represent real molecules. This algorithm is fast and generates good results with appropriate settings. The resulting report is shown in Table 1 for the positive test mix. This mixture contains only the compounds highlighted in the report. Please note that the database search screen does not confirm the presence of compounds and that compounds listed in the results does not indicate that they are conclusively present. Compounds listed could be from the blank, carry over or other sources.

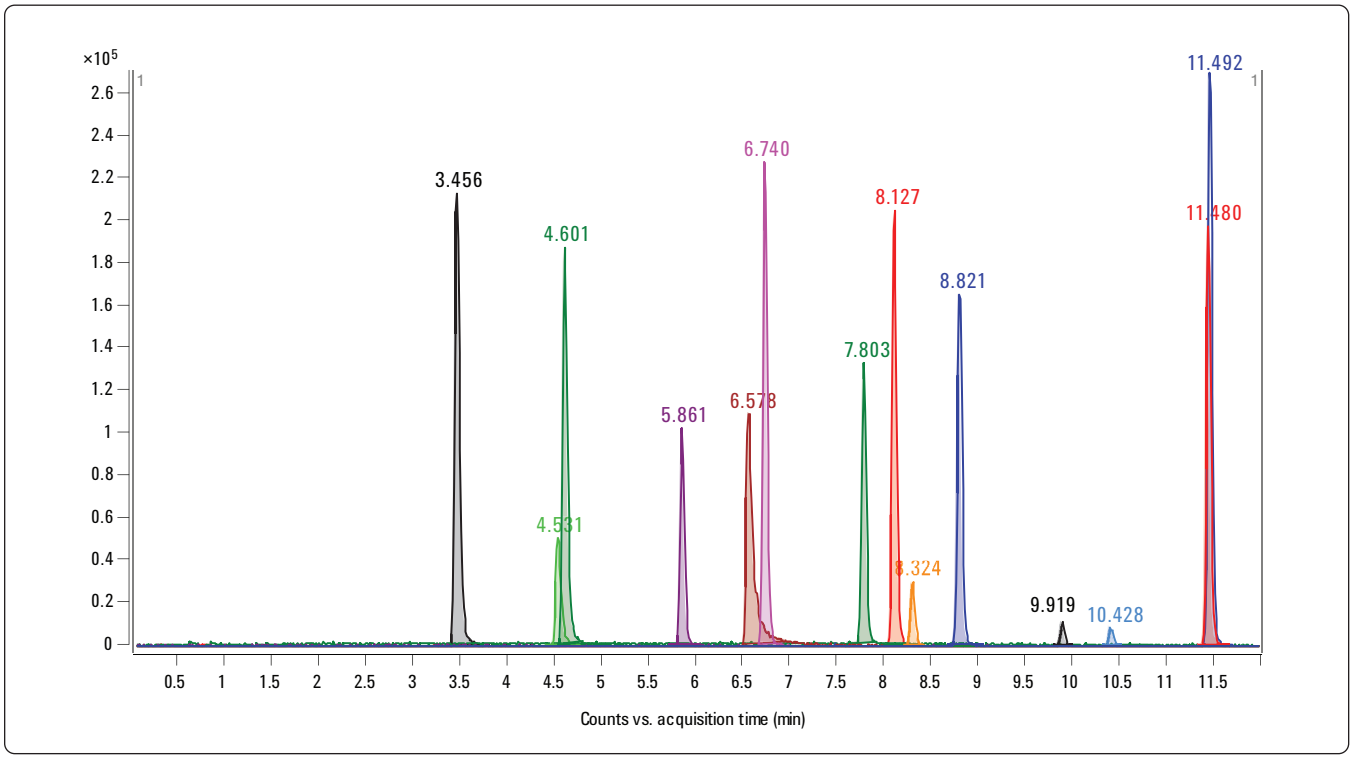


Figure 1. Extracted ion chromatogram of the positive ion test mix .

Table 1. Find Compounds by Molecular Feature Extractor with Pesticide Database Search Report for Positive Ion Test Mix

Data File	TestMix_pos_1.d	**Sample Name**	Test_Mix_pos_1
Sample Type	Sample	**Position**	P1-F2
Instrument Name	CAS6530_1	**User Name**	
Acq Method	Test_Mix_Pos.m	**Acquired Time**	6/1/2009 3:28:51 PM
IRM Calibration Status	Success	**DA Method**	MFE_Pesticide.m
Comment			

Compound Table

Compound Label		RT	Mass	Name	DB Formula	DB Diff (ppm)
Cpd 19:	Aminocarb	3.472	208.1213	Aminocarb	$C_{11}H_{16}N_2O_2$	–0.44
Cpd 40:	Imazapyr	4.543	261.1113	Imazapyr	$C_{13}H_{15}N_3O_3$	–0.03
Cpd 41:	Thiabendazole	4.612	201.036	Thiabendazole	$C_{10}H_7N_3S$	0.2
Cpd 52:	Ethiofencarb sulfoxide	5.176	241.0777	Ethiofencarb sulfoxide	$C_{11}H_{15}NO_3S$	–1.91
Cpd 62:	Dimethoate	5.866	228.9998	Dimethoate	$C_5H_{12}NO_3PS_2$	–0.75
Cpd 65:	Imazalil	6.549	296.0488	Imazalil	$C_{14}H_{14}Cl_2N_2O$	–1.58
Cpd 66:	Imazalil	6.579	296.0485	Imazalil	$C_{14}H_{14}Cl_2N_2O$	–0.65
Cpd 68:	Metoxuron	6.746	228.0666	Metoxuron	$C_{10}H_{13}ClN_2O_2$	–0.09
Cpd 85:	Carbofuran	7.805	221.1054	Carbofuran	$C_{12}H_{15}NO_3$	–1.05
Cpd 88:	Atrazine	8.138	215.094	Atrazine	$C_8H_{14}ClN_5$	–0.92
Cpd 89:	DEET	8.2	191.1309	DEET	$C_{12}H_{17}NO$	0.53
Cpd 90:	Tibenzate	8.323	228.0607	Tibenzate	$C_{14}H_{12}OS$	1
Cpd 91:	Metosulam	8.33	417.0069	Metosulam	$C_{14}H_{13}Cl_2N_5O_4S$	–0.98
Cpd 92:	Fluoroglycofen	8.33	419.0033	Fluoroglycofen	$C_{16}H_9ClF_3NO_7$	–3.28
Cpd 93:	Tibenzate	8.433	228.0608	Tibenzate	$C_{14}H_{12}OS$	0.39
Cpd 97:	Tibenzate	8.527	228.0609	Tibenzate	$C_{14}H_{12}OS$	–0.12
Cpd 99:	Metazachlor	8.837	277.0983	Metazachlor	$C_{14}H_{16}ClN_{30}$	–0.53
Cpd 107:	Molinate	9.927	187.1027	Molinate	$C_9H_{17}NOS$	2.02
Cpd 111:	Malathion	10.448	330.036	Malathion	$C_{10}H_{19}O_6PS_2$	0.2
Cpd 113:	Phenylacrylicacid	10.558	148.0522	Phenylacrylicacid	$C_9H_8O_2$	1.59
Cpd 121:	Tri-n-butyl phosphate	11.177	266.1645	Tri-n-butyl phosphate	$C_{12}H_{27}O_4P$	0.58
Cpd 123:	Tri-n-butyl phosphate	11.272	266.1646	Tri-n-butyl phosphate	$C_{12}H_{27}O_4P$	0.32
Cpd 125:	Pyraclostrobin	11.477	387.0989	Pyraclostrobin	$C_{19}H_{18}ClN_3O_4$	–0.9
Cpd 127:	Diazinon	11.497	304.1012	Diazinon	$C_{12}H_{21}N_2O_3PS$	–0.56

Database Search Results

Compound	Hits
Aminocarb	1

Compound	Best	Formula	Mass	Tgt Mass	Diff (ppm)	RT
Aminocarb	TRUE	$C_{11}H_{16}N_2O_2$	208.1213	208.1212	–0.44	3.472

Database Search Results

Compound	Hits
Imazapyr	1

Compound	Best	Formula	Mass	Tgt Mass	Diff (ppm)	RT
Imazapyr	TRUE	$C_{13}H_{15}N_3O_3$	261.1113	261.1113	–0.03	4.543

Database Search Results

Compound	Hits
Thiabendazole	1

Compound	Best	Formula	Mass	Tgt Mass	Diff (ppm)	RT
Thiabendazole	TRUE	$C_{10}H_7N_3S$	201.036	201.0361	0.2	4.612

Database Search Results

Compound	Hits
Ethiofencarb sulfoxide	1

Compound	Best	Formula	Mass	Tgt Mass	Diff (ppm)	RT
Thiabendazole	TRUE	$C_{10}H_7N_3S$	201.036	201.0361	0.2	4.612

Database Search Results

Compound	Hits
Ethiofencarb sulfoxide	2

Compound	Best	Formula	Mass	Tgt Mass	Diff (ppm)	RT
Ethiofencarb sulfoxide	TRUE	$C_{11}H_{15}NO_3S$	241.0777	241.0773	–1.91	5.176
Methiocarb sulfoxide	FALSE	$C_{11}H_{15}NO_3S$	241.0777	241.0773	–1.91	5.176

Database Search Results

Compound	Hits
Dimethoate	1

Compound	Best	Formula	Mass	Tgt Mass	Diff (ppm)	RT
Dimethoate	TRUE	$C_5H_{12}N0_3PS_2$	228.9996	228.9996	–0.75	5.866

Database Search Results

Compound	Hits
Imazalil	1

Compound	Best	Formula	Mass	Tgt Mass	Diff (ppm)	RT
Imazalil	TRUE	$C_{14}H_{14}Cl_2N_2O$	296.0488	296.0483	–1.58	6.549

Database Search Results

Compound	Hits
Imazalil	1

Compound	Best	Formula	Mass	Tgt Mass	Diff (ppm)	RT
Imazalil	TRUE	$C_{14}H_{14}Cl_2N_2O$	296.0485	296.0483	–0.65	6.579

Database Search Results

Compound	Hits
Metoxuron	1

Compound	Best	Formula	Mass	Tgt Mass	Diff (ppm)	RT
Metoxuron	TRUE	$C_{10}H_{13}ClN_2O_2$	228.0666	228.0666	–0.09	6.746

Database Search Results

Compound	Hits
Carbofuran	1

Compound	Best	Formula	Mass	Tgt Mass	Diff (ppm)	RT
Carbofuran	TRUE	$C_{12}H_{15}NO_3$	221.1054	221.1052	–1.05	7.805

Database Search Results

Compound	Hits
Atrazine	1

Compound	Best	Formula	Mass	Tgt Mass	Diff (ppm)	RT
Atrazine	TRUE	$C_8H_{14}ClN_5$	215.094	215.0938	–0.92	8.138

Database Search Results

Compound	Hits
DEET	1

Compound	Best	Formula	Mass	Tgt Mass	Diff (ppm)	RT
DEET	TRUE	$C_{12}H_{17}NO$	191.1309	191.131	0.53	8.2

Database Search Results

Compound	Hits
Tibenzate	1

Compound	Best	Formula	Mass	Tgt Mass	Diff (ppm)	RT
Tibenzate	TRUE	$C_{14}H_{12}OS$	228.0607	228.0609	1	8.323

Database Search Results

Compound	Hits
Metosulam	1

Compound	Best	Formula	Mass	Tgt Mass	Diff (ppm)	RT
Metosulam	TRUE	$C_{14}H_{13}Cl_2N_5O_4S$	417.0069	417.0065	–0.98	8.33

Database Search Results

Compound	Hits
Fluoroglycofen	1

Compound	Best	Formula	Mass	Tgt Mass	Diff (ppm)	RT
Fluoroglycofen	TRUE	$C_{16}H_9ClF_3NO_7$	419.0033	419.002	–3.28	8.33

Database Search Results

Compound	Hits
Tibenzate	1

Compound	Best	Formula	Mass	Tgt Mass	Diff (ppm)	RT
Tibenzate	TRUE	$C_{14}H_{12}OS$	228.0608	228.0609	0.39	8.433

Database Search Results

Compound	Hits
Tibenzate	1

Compound	Best	Formula	Mass	Tgt Mass	Diff (ppm)	RT
Tibenzate	TRUE	$C_{14}H_{12}OS$	228.0609	228.0609	–0.12	8.527

Database Search Results

Compound	Hits
Metazachlor	1

Compound	Best	Formula	Mass	Tgt Mass	Diff (ppm)	RT
Metazachlor	TRUE	$C_{14}H_{16}ClN_3O$	277.0983	277.0982	-0.53	8.837

Database Search Results

Compound	Hits
Molinate	1

Compound	Best	Formula	Mass	Tgt Mass	Diff (ppm)	RT
Molinate	TRUE	$C_9H_{17}NOS$	187.1027	187.1031	2.02	9.927

Database Search Results

Compound	Hits
Malathion	1

Compound	Best	Formula	Mass	Tgt Mass	Diff (ppm)	RT
Malathion	TRUE	$C_{10}H_{19}O_6PS_2$	330.036	330.0361	0.2	10.448

Database Search Results

Compound	Hits
Phenylacrylicacid	1

Compound	Best	Formula	Mass	Tgt Mass	Diff (ppm)	RT
Phenylacrylicacid	TRUE	$C_9H_8O_2$	148.0522	148.0524	1.59	10.558

Database Search Results

Compound	Hits
Tri-n-butyl phosphate	2

Compound	Best	Formula	Mass	Tgt Mass	Diff (ppm)	RT
Tri-n-butyl phosphate	TRUE	$C_{12}H_{27}O_4P$	266.1645	266.1647	0.58	11.177
Tri-iso-butyl phosphate		$C_{12}H_{27}O_4P$	266.1645	266.1647	0.58	11.177

Database Search Results

Compound	Hits
Tri-n-butyl phosphate	2

Compound	Best	Formula	Mass	Tgt Mass	Diff (ppm)	RT
Tri-n-butyl phosphate	TRUE	$C_{12}H_{27}O_4P$	266.1646	266.1647	0.32	11.272
Tri-iso-butyl phosphate		$C_{12}H_{27}O_4P$	266.1646	266.1647	0.32	11.272

Database Search Results

Compound	Hits
Pyraclostrobin	1

Compound	Best	Formula	Mass	Tgt Mass	Diff (ppm)	RT
Pyraclostrobin	TRUE	$C_{19}H_{18}ClN_3O_4$	387.0989	387.0986	–0.9	11.477

Database Search Results

Compound	Hits
Diazinon	1

Compound	Best	Formula	Mass	Tgt Mass	Diff (ppm)	RT
Diazinon	TRUE	$C_{12}H_{21}N_2O_3PS$	304.1012	304.1011	–0.56	11.497

The second method is Find_formula_pesticide. This method uses the “find by formula” algorithm of MassHunter Qualitative Analysis. This algorithm searches the data for the ions specified for each molecule in the database. For the supplied database this would entail generating extracted ion chromato-grams for each entry times each adduct (1600 for H^+, 1600 for Na^+, etc.). This is thorough but slower. However, if these searches are done automatically in a worklist, the processing time is reasonable. The analyst must determine what is the best fit-for-purpose procedure. Note that automatic database searching can be done during the worklist acquisition or after. Using the “worklist run” parameter of MassHunter acquisition, acquisition and data analysis can be selected, or data analysis only after the data has been collected. The data analysis methods can be added to the worklist by adding the column "Override DAMethod" to the MassHunter worklist and inserting the method to be used. (The qualitative analysis methods can be saved to the name of the acquisition method eliminating the need to add the "Override" column. (However, keeping acquisition methods and data analysis methods separate provides more flexibility.) All methods can be customized to meet the needs of a particular analysis.

Figure 2 shows the chromatogram of the negative ion compound test mix. Table 2 shows the results automatically generated for the negative ion mixture using the MFE_pesticide method. The report is generated using "Find and Identify" selection of compound automation and the 1600-compound pesticide database, pesticides.mtl, is searched. The worklist automation uses the "Compound automation and report" selection. To obtain the report shown, the "CompoundReportwithIdentificationHits.xltx" template of the "Common Reporting Options" in the General Navigation bar of MassHunter Qualitative analysis must be selected. This is important because as shown in the compound list of Table 2, the wrong isomer, dinoprop, is listed. This is the first isomer found in the database. The selected report template then lists the results of each database hit and the three isomers in the database are shown under this heading in the report. If the data were analyzed with the "Find by Formula" algorithm, the report would include all the isomers in the database in the main body of the report. If a retention time that matched the compound were in a custom database, only that isomer would be reported (targeted analysis). (Note that for the find by formula method to work within a worklist the Worklist Actions of the method should separately list "Compound Automation without report" and then "Generate Compound Report.")

The compound actually present is dinoseb and if this were a non-targeted analysis the analyst would need to confirm which one was present.

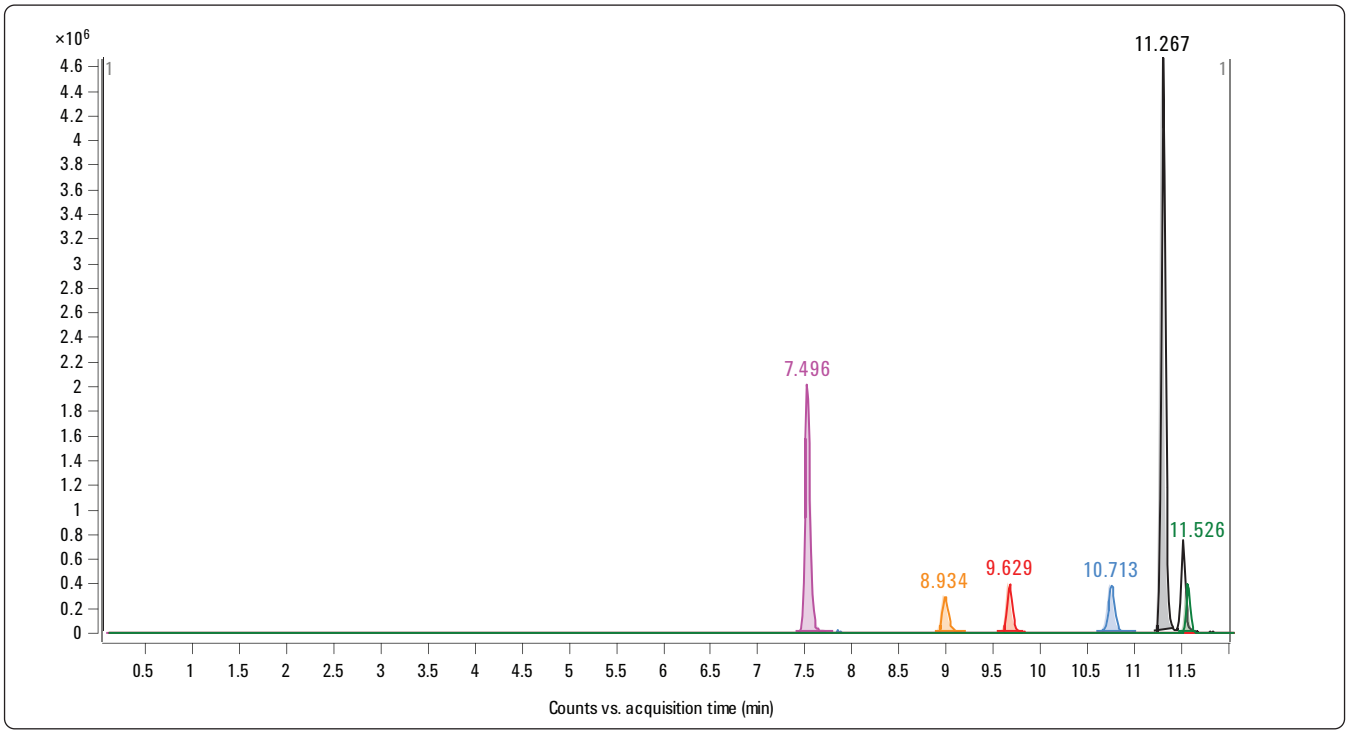


Figure 2. Extracted ion chromatogram of the negative ion test mix.

Table 2. Find compounds by Molecular Feature Extractor with Pesticide Database Search Report for Negative Ion Test Mix

Data File	Test_mix_neg_01.d	**Sample Name**	Test Mix Neg 1
Sample Type	Sample	**Position**	P1-F1
Instrument Name	CAS6530_1	**User Name**	
Acq Method	Test_mix_neg.m	**Acquired Time**	6/1/2009 1:33:54 PM
IRM Calibration Status	Success	**DA Method**	MFE_Pesticide.m
Comment			

Compound Table

Compound Label		RT	Mass	Name	DB Formula	DB Diff (ppm)
Cpd 12:	Bentazone	7.491	240.0573	Bentazone	$C_{10}H_{12}N_2O_3S$	–1.69
Cpd 15:	Dibutyl succinate	7.904	230.1517	Dibutyl succinate	$C_{12}H_{22}O_4$	0.5
Cpd 24:	2,4-D Methyl ester	8.768	233.9847	2,4-D Methyl ester	$C_9H_8Cl_2O_3$	1.7
Cpd 26:	2,4,5-T	8.934	253.9306	2,4,5-T	$C_8H_5Cl_3O_3$	–0.72
Cpd 32:	Silvex	9.623	267.9465	Silvex	$C_9H_7Cl_3O_3$	–1.6
Cpd 37:	Citronellal hydrate	10.219	172.1465	Citronellal hydrate	$C_{10}H_{20}O_2$	–1.25
Cpd 39:	Citronellal hydrate	10.37	172.1464	Citronellal hydrate	$C_{10}H_{20}O_2$	–0.47
Cpd 41:	Acifluorfen	10.716	360.9967	Acifluorfen	$C_{14}H_7ClF_3NO_5$	–0.55
Cpd 42:	Citronellal hydrate	10.736	172.1466	Citronellal hydrate	$C_{10}H_{20}O_2$	–1.37
Cpd 51:	Alantolactone	11.249	232.1462	Alantolactone	$C_{15}H_{20}O_2$	0.35
Cpd 52:	Dinoprop (see below)	11.267	240.075	Dinoprop	$C_{10}H_{12}N_2O_5$	–1.72
Cpd 56:	Hexaflumuron	11.53	459.982	Hexaflumuron	$C_{16}H_8Cl_2F_6N_2O_3$	–0.76

Database Search Results

Compound	Hits
Bentazone	1

Compound	Best	Formula	Mass	Tgt Mass	Diff (ppm)	RT
Bentazone	TRUE	$C_{10}H_{12}N_2O_3S$	240.0573	240.0569	–1.69	7.491

Database Search Results

Compound	Hits
Dibutyl succinate	1

Compound	Best	Formula	Mass	Tgt Mass	Diff (ppm)	RT
Dibutyl succinate	TRUE	$C_{12}H_{22}O_4$	230.1517	230.1518	0.5	7.904

Database Search Results

Compound	Hits
2,4,5-T	2

Compound	Best	Formula	Mass	Tgt Mass	Diff (ppm)	RT
2,4,5-T	TRUE	$C_8H_5Cl_3O_3$	253.9306	253.9304	–0.72	8.934
Tricamba		$C_8H_5Cl_3O_3$	253.9306	253.9304	–0.72	8.934

Database Search Results

Compound	Hits
Silvex	1

Compound	Best	Formula	Mass	Tgt Mass	Diff (ppm)	RT
Silvex	TRUE	$C_9H_7Cl_3O_3$	267.9465	267.9461	–1.6	9.623

Database Search Results

Compound	Hits
Citronellal hydrate	1

Compound	Best	Formula	Mass	Tgt Mass	Diff (ppm)	RT
Citronellal hydrate	TRUE	$C_{10}H_{20}O_2$	172.1465	172.1463	–1.25	10.219

Database Search Results

Compound	Hits
Citronellal hydrate	1

Compound	Best	Formula	Mass	Tgt Mass	Diff (ppm)	RT
Citronellal hydrate	TRUE	$C_{10}H_{20}O_2$	172.1464	172.1463	–0.47	10.37

Database Search Results

Compound	Hits
Acifluorfen	1

Compound	Best	Formula	Mass	Tgt Mass	Diff (ppm)	RT
Acifluorfen	TRUE	$C_{14}H_7ClF_3NO_5$	360.9967	360.9965	–0.55	10.716

Database Search Results

Compound	Hits
Citronellal hydrate	1

Compound	Best	Formula	Mass	Tgt Mass	Diff (ppm)	RT
Citronellal hydrate	TRUE	$C_{10}H_{20}O_2$	172.1466	172.1463	–1.37	10.736

Database Search Results

Compound	Hits
Alantolactone	1

Compound	Best	Formula	Mass	Tgt Mass	Diff (ppm)	RT
Alantolactone	TRUE	$C_{15}H_{20}O_2$	232.1462	232.1463	0.35	11.249

Database Search Results (Note that the following are isomers with the same formula even though the compound present is listed as "FALSE.")

Compound	Hits					
Dinoprop	3					
Compound	**Best**	**Formula**	**Mass**	**Tgt Mass**	**Diff (ppm)**	**RT**
Dinoprop	TRUE	$C_{10}H_{12}N_2O_5$	240.075	240.0746	–1.72	11.267
Dinoseb	FALSE	$C_{10}H_{12}N_2O_5$	240.075	240.0746	–1.72	11.267
Dinoterb		$C_{10}H_{12}N_2O_5$	240.075	240.0746	–1.72	11.267

Database Search Results

Compound	Hits					
Hexaflumuron	1					
Compound	**Best**	**Formula**	**Mass**	**Tgt Mass**	**Diff (ppm)**	**RT**
Hexaflumuron	TRUE	$C_{16}H_8Cl_2F_6N_2O_3$	459.982	459.9816	–0.76	11.53

Customized databases with user added retention times

One of the powerful benefits of the supplied database is that it can be saved to a user customized database. To create a read-write customizable database, the user selects the "File" menu item and the "New Database." The software then allows selection of an existing database and then the naming of a new database. A description can also be given. When "Create" is selected the database with the new name contains all the entries of the selected database. In this way multiple custom databases can be created. The technical note on the Pesticide PCD [4] shows how the user can run standards with unique chromatographic conditions and easily update retention times in their custom database. This is shown in Figures 3 and 4 for the positive and negative ion test mix respectively.

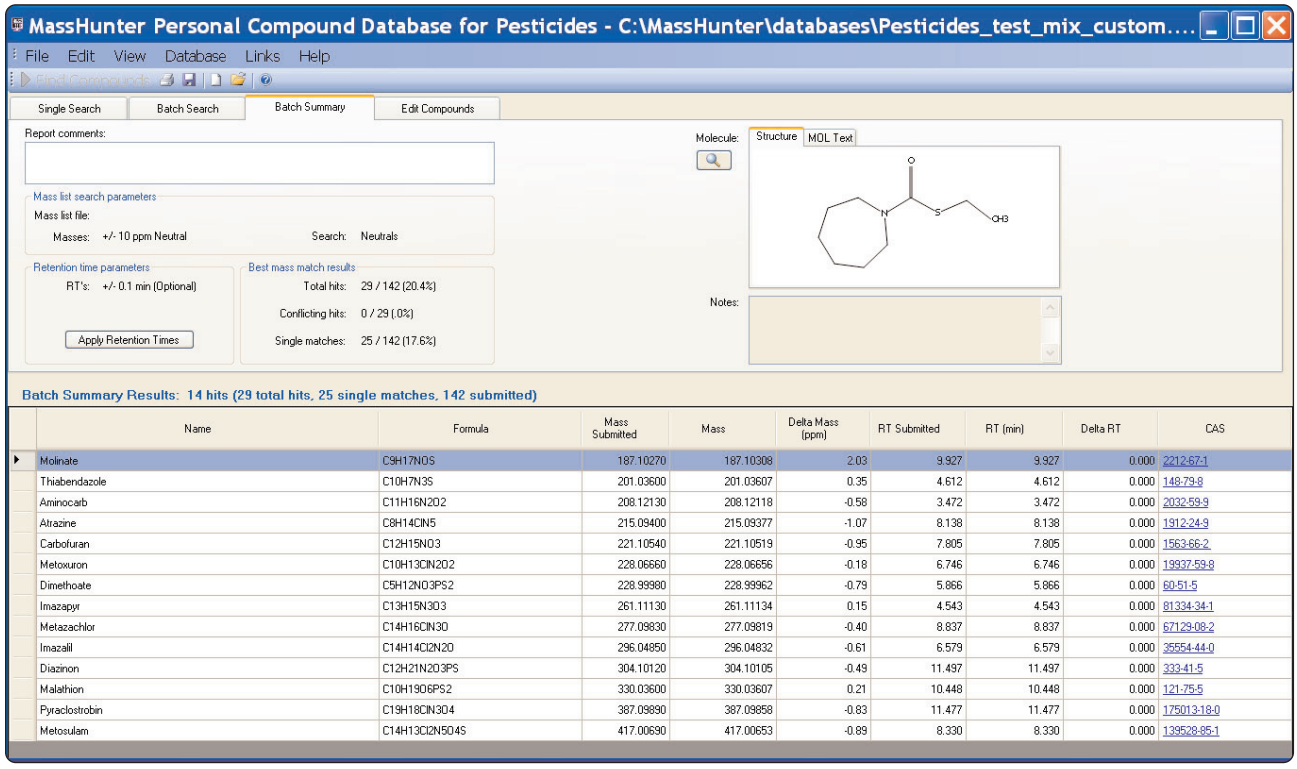


Figure 3. Pesticide Personal Compound Database (now with Library –PCDL, not shown) customized with retention times from positive ion test mix.

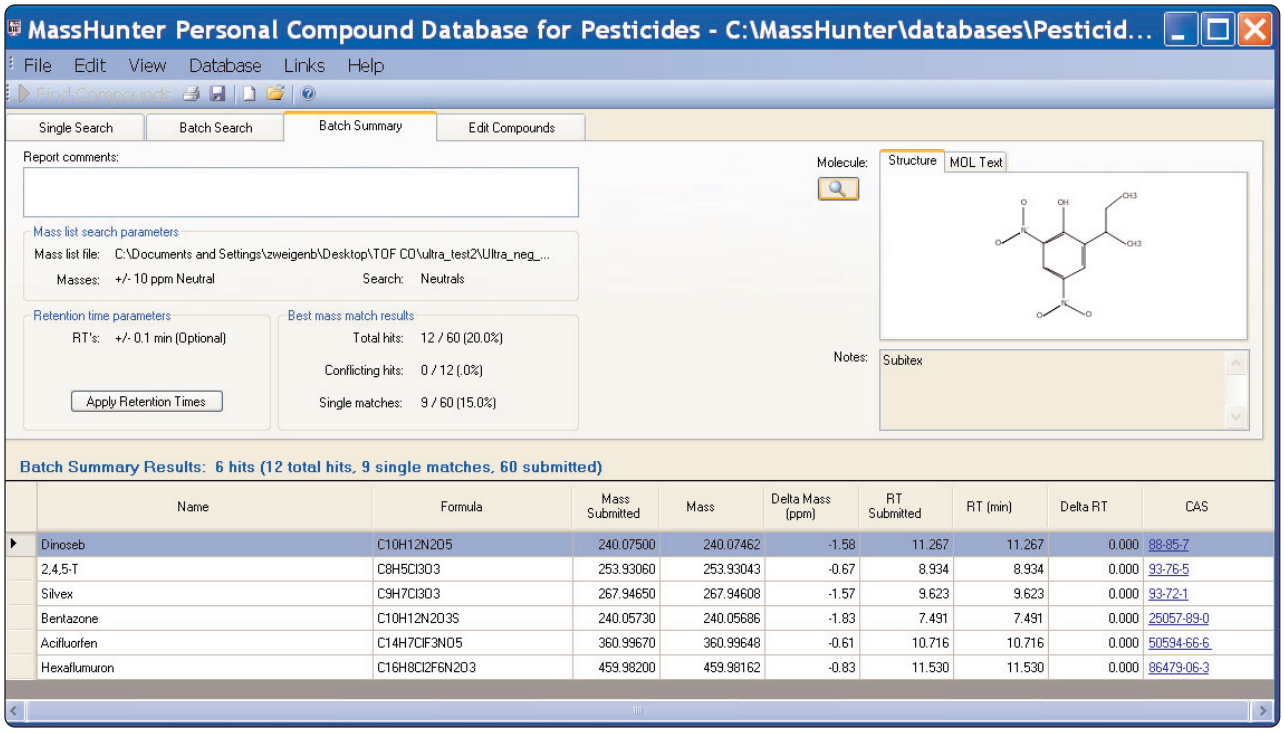


Figure 4. Pesticide Personal Compound Database (PCDL) customized with retention times for negative ion test mix.

If the analysis were for targeted compounds where retention times are known, dinoseb would be chromatographically separated from the other isomers. It is a simple exercise to take the results of the test mix, create a custom database from the provided pesticide database and update retention times. This would now create a targeted analysis. Either data analysis method can be modified for a targeted and non-targeted analysis by selecting "mass and retention time (optional)" for the search criteria. Targeted only analysis would be performed if "mass and retention time (required)" was checked. A report for a targeted and non-targeted analysis of the negative test mix with the method Find_by Formula and a custom database with the retention time for dinoseb would only list that compound. In this result, only dinoseb is reported because it is the compound in the custom database that matches the retention time. Even with retention times, identified compounds in the database must be confirmed. Both screening and confirmation can be done with the LC/Q-TOF.

Table 3. Find compounds by Formula with Pesticide Database Search Report for Negative ion Test Mix

Data File	Test_mix_neg_01.d	**Sample Name**	Test Mix Neg 1
Sample Type	Sample	**Position**	P1-F1
Instrument Name	CAS6530_1	**User Name**	
Acq Method	Test_mix_neg.m	**Acquired Time**	6/1/2009 1:33:54 PM
IRM Calibration Status	Success	**DA Method**	find_by_formula_pesticids.m
Comment			

Compound Table

Compound Label	RT	Mass	Abund	Name	Formula	Tgt Mass	DB Diff (ppm)
Cpd 1: Dichloromethoxybenzene	5.583	175.9796	9712	Dichloromethoxybenzene	$C_7H_6Cl_2O$	175.9796	0.11
Cpd 2: Bentazone	7.492	240.0573	108523	Bentazone	$C_{10}H_{12}N_2O_3S$	240.0569	1.69
Cpd 3: Dibutyl succinate	7.904	230.1517	7790	Dibutyl succinate	$C_{12}H_{22}O_4$	230.1518	–0.5
Cpd 6: Dichloroprop	8.764	233.9845	33463	Dichloroprop	$C_9H_8Cl_2O_3$	233.985	–2.39
Cpd 7: Disugran	8.764	233.9845	33463	Disugran	$C_9H_8Cl_2O_3$	233.985	–2.39
Cpd 4: Dichlorophenol 2,4-	8.764	161.9633	6051	Dichlorophenol 2,4-	$C_6H_4Cl_2O$	161.9639	–3.59
Cpd 5: 2,4-D Methyl ester	8.764	233.9845	33463	2,4-D Methyl ester	$C_9H_8Cl_2O_3$	233.985	–2.39
Cpd 9: Tricamba	8.941	253.9306	15646	Tricamba	$C_8H_5Cl_3O_3$	253.9304	0.75
Cpd 8: 2,4,5-T	8.941	253.9306	15646	2,4,5-T	$C_8H_5Cl_3O_3$	253.9304	0.75
Cpd 10: Trichlorophenol, 2,4,6-	9.613	195.9245	5877	Trichlorophenol, 2,4,6-	$C_6H_3Cl_3O$	195.9249	–2.12
Cpd 11: Silvex	9.624	267.9468	18804	Silvex	$C_9H_7Cl_3O_3$	267.9461	2.64
Cpd 14: Acifluorfen	10.708	360.9966	18261	Acifluorfen	$C_{14}H_7ClF_3NO_5$	360.9965	0.43
Cpd 13: Nitrofluorfen	10.708	317.0062	10928	Nitrofluorfen	$C_{13}H_7ClF_3NO_3$	317.0067	–1.41
Cpd 12: Azinphos-methyl	10.708	317.0061	9536	Azinphos-methyl	$C_{10}H_{12}N_3O_3PS_2$	317.0058	1
Cpd 15: Citronellal hydrate	10.732	172.1466	132453	Citronellal hydrate	$C_{10}H_{20}O_2$	172.1463	1.39
Cpd 16: Alantolactone	11.251	232.1462	10414	Alantolactone	$C_{15}H_{20}O_2$	232.1463	–0.54
Cpd 20: Ethiofencarb sulfoxide	11.262	241.078	23056	Ethiofencarb sulfoxide	$C_{11}H_{15}NO_3S$	241.0773	2.86
Cpd 21: Methiocarb sulfoxide	11.262	241.078	23056	Methiocarb sulfoxide	$C_{11}H_{15}NO_3S$	241.0773	2.86
Cpd 19: Dinoprop	11.262	240.0752	249379	Dinoprop	$C_{10}H_{12}N_2O_5$	240.0746	2.22
Cpd 17: Dinoseb	11.262	240.0752	249379	Dinoseb	$C_{10}H_{12}N_2O_5$	240.0746	2.22
Cpd 18: Dinoterb	11.262	240.0752	249379	Dinoterb	$C_{10}H_{12}N_2O_5$	240.0746	2.22
Cpd 22: Hexaflumuron	11.533	459.9823	19824	Hexaflumuron	$C_{16}H_8Cl_2F_6N_2O_3$	459.9816	1.44

The power of Q-TOF for screening and confirmation

As an example of the power of this technique, a strawberry extract was spiked and analyzed using an Agilent 1200 Series SL LC with an Agilent 6520 Q-TOF. The extracted ion chromatogram of the over 100 pesticides spiked into this sample is shown in Figure 5. A pesticide screen with a Q-TOF is the same as with a TOF. However, LC/Q-TOF MS offers the highly selective MS/MS with accurate mass measurement that provides a workflow for both screening and confirmation. [1]

Screening hundreds of target and non-target pesticides using the Agilent 1200 Series SL with 6230 TOF

A standard of over 200 pesticides is run in a similar fashion and the EIC generated from the pesticides detected in a "find compounds by molecular feature" extractor with database search is shown in Figure 4. This method employs the Agilent 1200 Series SL and the Agilent 6230 TOF with Jet Stream Technology. This is the preferred configuration as it provides additional sensitivity to meet the demanding needs of multi-residue analysis. The method for this analysis is also provided with the Agilent Pesticide Screen Application Kit for TOF and Q-TOF.

The highest quality results are obtained with good chromatographic and mass spectral resolution. The ability to detect and identify thousands of compounds lies in both these parameters and accurate mass measurement. However, for any given real food sample only a few pesticides will be found. This may not be the case for environmental samples but the possibility of no more than 10 to 20 per site would be realistic. Given this reality, the need to be able to validate that hundreds of compounds can be detected in a fast analysis would provide this capability. Figure 6 shows a 3-minute run of over 100 pesticides using the new Agilent 1290 Infinity LC connected to the new Agilent 6540 Q-TOF. Given the chromatographic resolution achieved and the mass spectral resolution obtained, this analysis is reasonable for screening pesticides in food and environmental samples. The quality of the mass spectral data is shown in Figure 7 and this was collected at rate of 10 spectra per second.

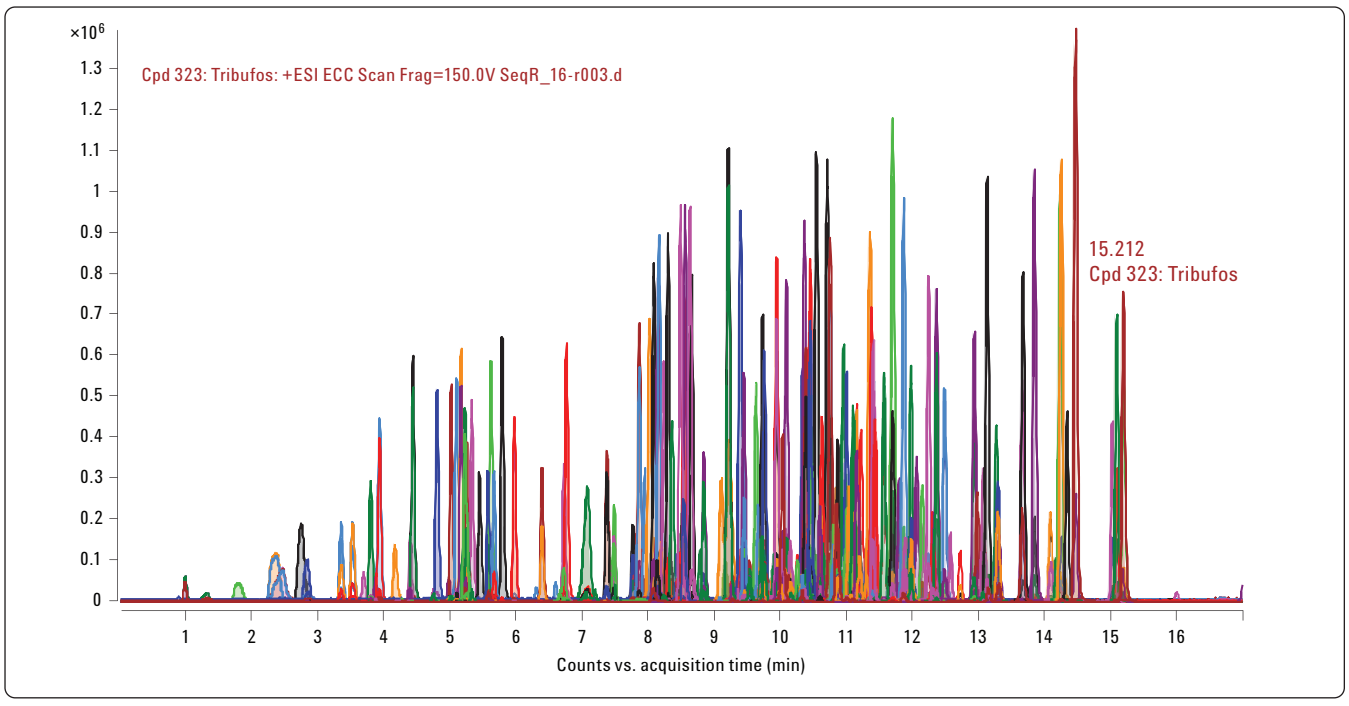


Figure 5. Extracted compound chromatogram (from compounds found by MFE) of 200 pesticides using the Agilent 1200 Series SL LC with the Agilent 6230 TOF.

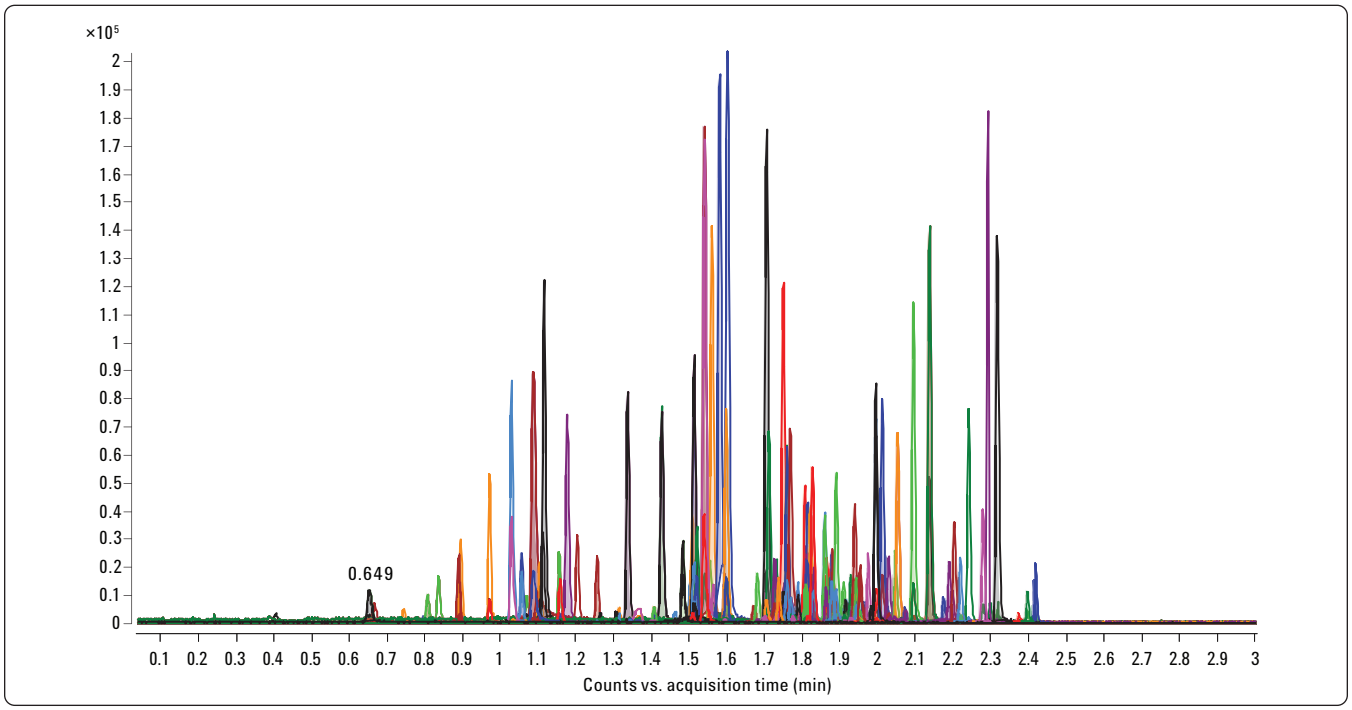
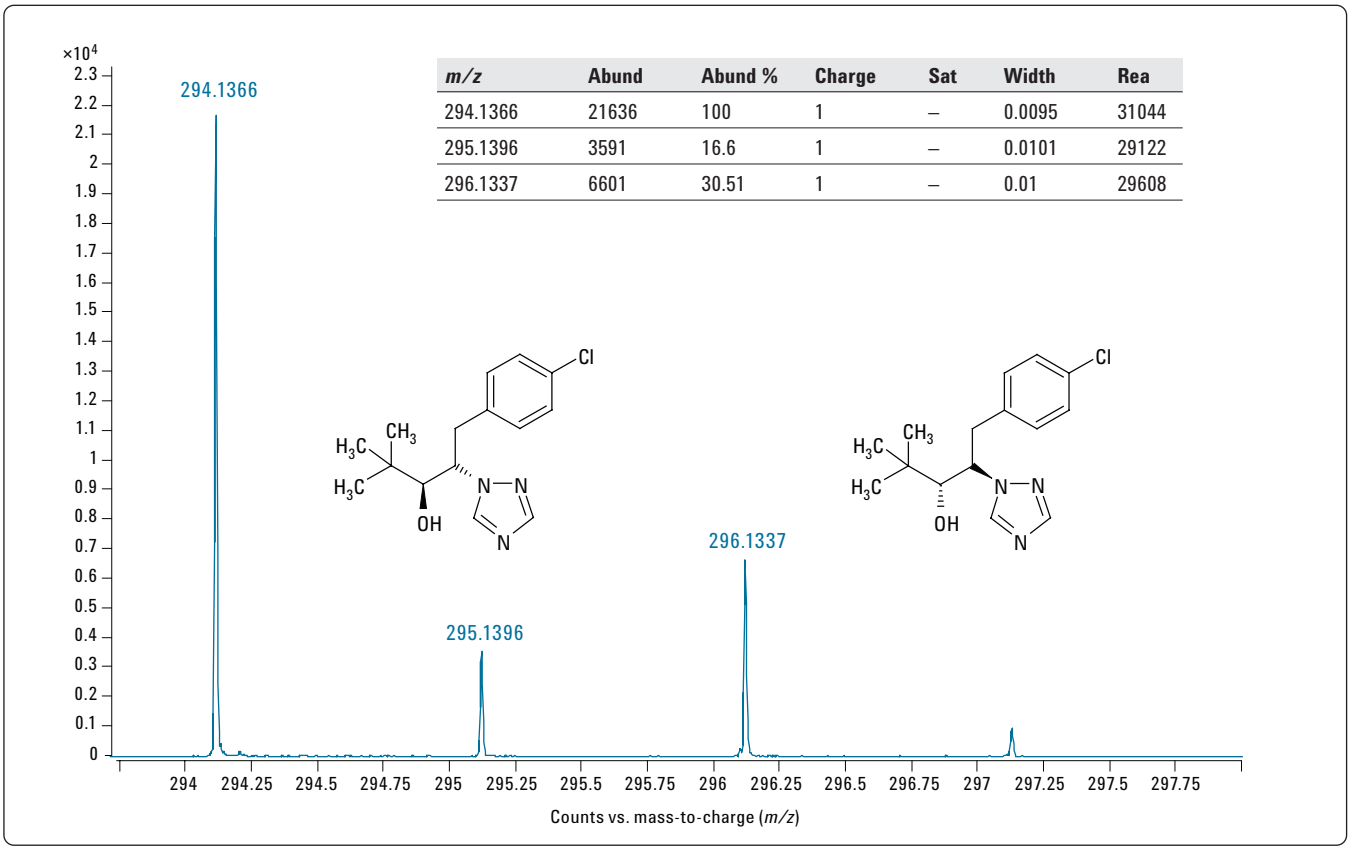


Figure 6. Extracted compound chromatogram of 100 pesticides in 3 min using the new Agilent 1290 Infinity LC with the new Agilent 6540 Q-TOF.



m/z	Abund	Abund %	Charge	Sat	Width	Rea
294.1366	21636	100	1	–	0.0095	31044
295.1396	3591	16.6	1	–	0.0101	29122
296.1337	6601	30.51	1	–	0.01	29608

Figure 7. Example mass spectrum from data on 3 min run with Agilent 1290 Infinity LC and Agilent 6540 Q-TOF. Note the mass resolution at 10 spectra per second.

Extraction to analysis with SampliQ extraction and SPE Kits

Finally, as an example of a complete analysis a spinach sample was spiked with pesticides at the 10-ppb level and extracted using the SampliQ QuEChERS Kit p/n 5982-5755. Then the Agilent SampliQ QuEChERS AOAC Dispersive SPE kit for highly pigmented fruits and vegetables, p/n 5982-5356 (15 mL), was used for clean-up. In addition, a reagent blank was prepared and run using an Agilent 1200 Series SL/6530 LC/Q-TOF and the standard screened with "find by molecular feature extractor" and the Pesticide database (not customized). The resulting mass list from the reagent blank was placed in the MFE settings by exporting the mass list to a .csv file, selecting "exclude these masses" under "Filter Mass", and using the exported .csv file as the database. In this way all the ions in the reagent blank will be removed from standards and samples processed with this method. The spiking solution (neat standard) was analyzed using the same acquisition method and the Worklist Automation. The results are given in Table 4 and represent the pesticides in the standard. It should be noted that if background removal is performed, the mass list should be searched by the database to make sure that compounds of concern will not be excluded. The .csv file is editable in Excel and masses can be removed from the exclusion list if necessary (for example, if pesticides are found in the blank).

Table 4. Neat Pesticide Standard for Spinach Extract

Data File	2ppb neat std .d	**Sample Name**	2ppb neat in 20:80 ACN/H2O
Sample Type	Sample	**Position**	P1-F1
Instrument Name	CAS6530_1	**User Name**	Jaz
Acq Method	MFE_Compound_report.m	**Acquired Time**	7/10/2009 12:43:56 PM
IRM Calibration Status	Success	**DA Method**	MFE_Pesticide_report.m
Comment			

Compound Table

Compound Label	RT	Mass	Name	DB Formula	DB Diff (ppm)	Hits (DB)
Cpd 12: Methamidophos	2.053	141.0012	Methamidophos	$C_2H_8NO_2PS$	0.96	1
Cpd 15: Acephate	2.467	183.0115	Acephate	$C_4H_{10}NO_3PS$	2.24	1
Cpd 18: Acephate	2.632	183.0119	Acephate	$C_4H_{10}NO_3PS$	–0.02	1
Cpd 24: Pymetrozine	3.242	217.0967	Pymetrozine	$C_{10}H_{11}N_5O$	–1.54	1
Cpd 25: Pymetrozine	3.361	217.0965	Pymetrozine	$C_{10}H_{11}N_5O$	–0.5	
Cpd 29: Carbendazim	4.259	191.0695	Carbendazim	$C_9H_9N_3O_2$	0.06	1
Cpd 35: Thiabendazole	4.633	201.0359	Thiabendazole	$C_{10}H_7N_3S$	0.81	1
Cpd 44: Imidacloprid	5.564	255.0527	Imidacloprid	$C_9H_{10}ClN_5O_2$	–1.43	1
Cpd 51: Imazalil	6.587	296.0489	Imazalil	$C_{14}H_{14}Cl_2N_2O$	–1.92	1
Cpd 54: Dicyclanil	7.172	190.0968	Dicyclanil	$C_8H_{10}N_6$	–0.77	1
Cpd 55: Thiophanate-methyl	7.426	342.0463	Thiophanate-methyl	$C_{12}H_{14}N_4O_4S_2$	–1.97	1
Cpd 86: Propoxur	7.621	209.1053	Propoxur	$C_{11}H_{15}NO_3$	–0.35	1
Cpd 87: Pyrocatechol	7.621	110.0368	Pyrocatechol	$C_6H_6O_2$	–0.59	1
Cpd 89: Norethynodrel	7.631	298.192	Norethynodrel	$C_{20}H_{26}O_2$	4.2	1
Cpd 91: Carbaryl	7.994	201.079	Carbaryl	$C_{12}H_{11}NO_2$	–0.07	7
Cpd 92: Naphthol, 1-	7.995	144.0575	Naphthol, 1-	$C_{10}H_8O$	0.01	1
Cpd 97: Ethoprop	9.908	242.0569	Ethoprop	$C_8H_{19}O_2PS_2$	–2	1
Cpd 99: Penconazole	10.219	283.0649	Penconazole	$C_{13}H_{15}Cl_2N_3$	–2.26	1
Cpd 101: Cyprodinil	10.482	225.1268	Cyprodinil	$C_{14}H_{15}N_3$	–0.68	1
Cpd 105: Kresoxim methyl	10.924	313.132	Kresoxim methyl	$C_{18}H_{19}NO_4$	–1.93	1

Figure 8 shows the extracted compound chromatogram from the "Molecular Feature Extractor" of the spinach extract. Even with the clean-up procedure and background ions removed, this is a complex sample. Table 5 shows the database search result for the spinach extract and all compounds detected in the standards were detected in the extract. If this were an analysis done for targeted and non-targeted analysis, all non-target positives (those without matching retention times) should be examined in MassHunter Qualitative Analysis before further analysis.

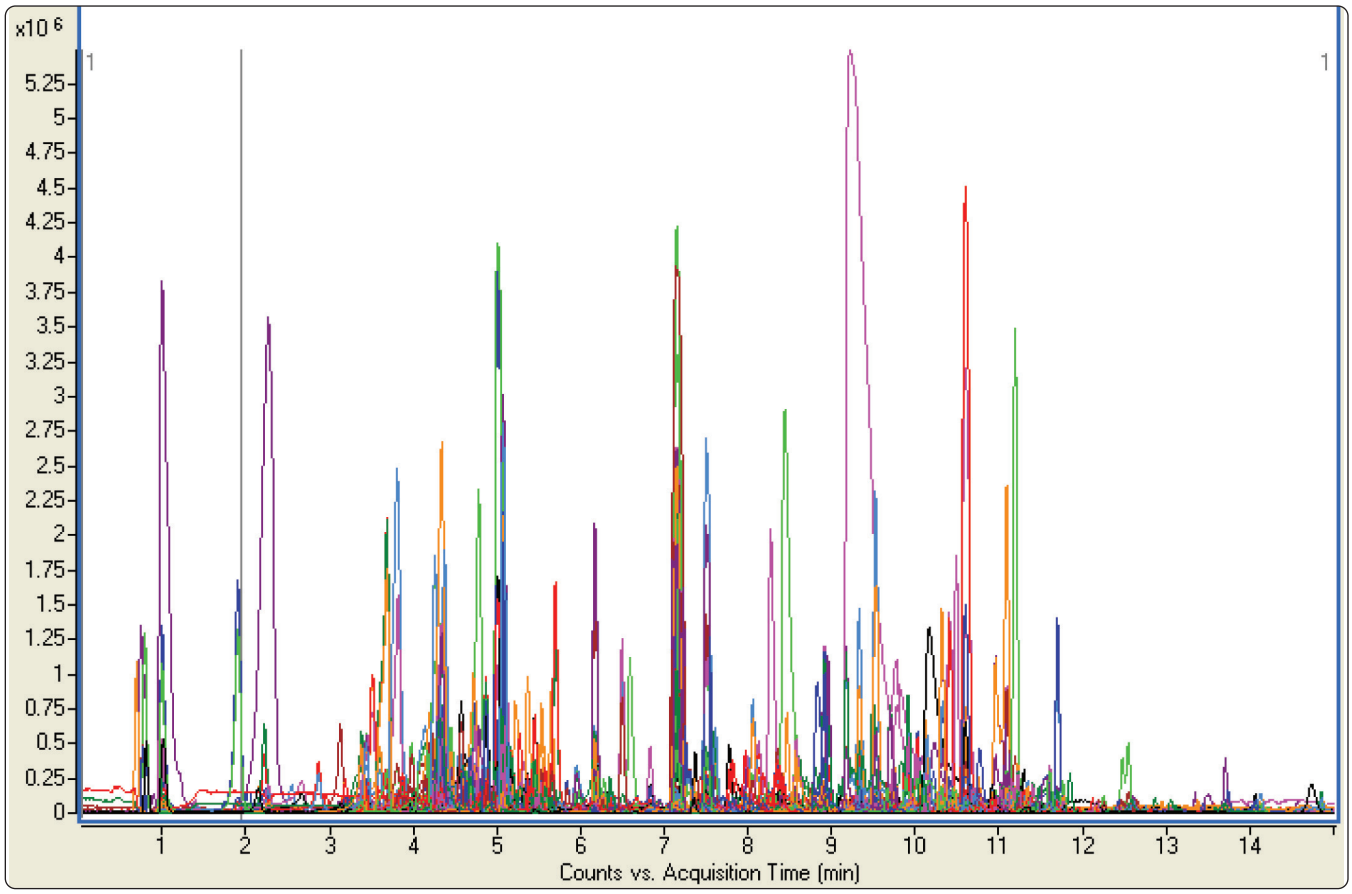


Figure 8. Extracted compound chromatogram of spinach sample with over 1200 compound features found .

Table 5. Results of Spinach Screen using Molecular Feature Extractor

Data File	Spinach AOAC 10ppb.d	**Sample Name**	Spinach AOAC 10 ppb (2 ppb in sample)
Sample Type	Sample	**Position**	P1-A4
Instrument Name	CAS6530_1	**User Name**	Jaz
Acq Method	MFE_Compound_report.m	**Acquired Time**	7/10/2009 12:40:59 PM
IRM Calibration Status	Some Ions Missed	**DA Method**	MFE_Pesticide_report.m
Comment			

Compound Table

Compound Label		RT	Mass	Name	DB Formula	DB Diff (ppm)
Cpd 36:	Methamidophos	2.042	141.001	Methamidophos	$C_2H_8NO_2PS$	2.37
Cpd 42:	Carbofuran-3-OH-7-phenol	2.226	180.0786	Carbofuran-3-OH-7-phenol	$C_{10}H_{12}O_3$	0.12
Cpd 44:	Metolcarb	2.233	165.0789	Metolcarb	$C_9H_{11}NO_2$	0.43
Cpd 74:	Acephate	2.614	183.0116	Acephate	$C_4H_{10}NO_3PS$	1.72
Cpd 94:	Decarbofuran	3.127	207.0896	Decarbofuran	$C_{11}H_{13}NO_3$	–0.45
Cpd 97:	Quinacetol sulfate	3.14	187.0631	Quinacetol sulfate	$C_{11}H_9NO_2$	1.39
Cpd 103:	3,5-Xylyl methylcarbamate	3.197	179.0945	3,5-Xylyl methylcarbamate	$C_{10}H_{13}NO_2$	0.78
Cpd 119:	Carbofuran, - 3 hydroxy	3.323	237.1003	Carbofuran, - 3 hydroxy	$C_{12}H_{15}NO_4$	–0.88
Cpd 121:	Pymetrozine	3.36	217.0963	Pymetrozine	$C_{10}H_{11}N_5O$	0.44
Cpd 136:	Propoxur	3.445	209.1053	Propoxur	$C_{11}H_{15}NO_3$	–0.42
Cpd 140:	3,5-Xylyl methylcarbamate	3.471	179.0953	3,5-Xylyl methylcarbamate	$C_{10}H_{13}NO_2$	–3.52
Cpd 161:	8-Hydroxyquinoline	3.59	145.0526	8-Hydroxyquinoline	C_9H_7NO	0.82
Cpd 198:	Metalaxyl	3.805	279.1478	Metalaxyl	$C_{15}H_{21}NO_4$	–2.52
Cpd 207:	Phenyl isocyanate	3.817	119.0373	Phenyl isocyanate	C_7H_5NO	–1.32
Cpd 221:	Aspidinol	3.946	224.1049	Aspidinol	$C_{12}H_{16}O_4$	–0.24
Cpd 284:	Phenoxyacetic acid	4.155	152.0472	Phenoxyacetic acid	$C_8H_8O_3$	1.13
Cpd 303:	Dimethyl phthalate	4.188	194.0579	Dimethyl phthalate	$C_{10}H_{10}O_4$	0.28
Cpd 310:	Trinexapac	4.21	224.0685	Trinexapac	$C_{11}H_{12}O_5$	0.08
Cpd 316:	Carbendazim	4.254	191.0695	Carbendazim	$C_9H_9N_3O_2$	–0.21
Cpd 323:	Geraniol	4.282	154.1357	Geraniol	$C_{10}H_{18}O$	0.55
Cpd 338:	Dimethyl phthalate	4.316	194.0579	Dimethyl phthalate	$C_{10}H_{10}O_4$	–0.1
Cpd 368:	Propoxur	4.372	209.1054	Propoxur	$C_{11}H_{15}NO_3$	–1.02
Cpd 386:	Aldicarb	4.443	190.0776	Aldicarb	$C_7H_{14}N_2O_2S$	–0.09
Cpd 455:	Phenoxyacetic acid	4.617	152.0474	Phenoxyacetic acid	$C_8H_8O_3$	–0.33
Cpd 461:	Thiabendazole	4.628	201.0362	Thiabendazole	$C_{10}H_7N_3S$	–0.66
Cpd 492:	Butopyronoxyl	4.723	226.1207	Butopyronoxyl	$C_{12}H_{18}O_4$	–0.65
Cpd 584:	Tiocarbazil	4.904	279.167	Tiocarbazil	$C_{16}H_{25}NOS$	–4.82

Compound Label		RT	Mass	Name	DB Formula	DB Diff (ppm)
Cpd 587:	Kresoxim methyl	4.905	313.132	Kresoxim methyl	$C_{18}H_{19}NO_4$	–1.89
Cpd 641:	Pyrethrin I	5.022	328.2041	Pyrethrin I	$C_{21}H_{28}O_3$	–0.8
Cpd 642:	Allethrin	5.022	302.1884	Allethrin	$C_{19}H_{26}O_3$	–0.79
Cpd 644:	Spiromesifen	5.022	370.2148	Spiromesifen	$C_{23}H_{30}O_4$	–1.01
Cpd 720:	Phosfon	5.19	396.1312	Phosfon	$C_{19}H_{32}Cl_3P$	–1.33
Cpd 721:	Santonin	5.197	246.1259	Santonin	$C_{15}H_{18}O_3$	–1.43
Cpd 740:	Dimethyl phthalate	5.255	194.0576	Dimethyl phthalate	$C_{10}H_{10}O_4$	1.68
Cpd 743:	Metaldehyde	5.265	176.1044	Metaldehyde	$C_8H_{16}O_4$	2.75
Cpd 804:	Phosfon	5.439	396.1301	Phosfon	$C_{19}H_{32}Cl_3P$	1.47
Cpd 816:	Allethrin	5.454	302.1882	Allethrin	$C_{19}H_{26}O_3$	–0.08
Cpd 830:	Buthiobate	5.498	372.1696	Buthiobate	$C_{21}H_{28}N_2S_2$	–0.67
Cpd 858:	Imidacloprid	5.57	255.0527	Imidacloprid	$C_9H_{10}ClN_5O_2$	–1.47
Cpd 976:	Kresoxim methyl	6.15	313.1321	Kresoxim methyl	$C_{18}H_{19}NO_4$	–2.14
Cpd 1047:	Kresoxim methyl	6.366	313.132	Kresoxim methyl	$C_{18}H_{19}NO_4$	–1.78
Cpd 1063:	Alantolactone	6.463	232.1463	Alantolactone	$C_{15}H_{20}O_2$	0.23
Cpd 1075:	Santonin	6.521	246.1258	Santonin	$C_{15}H_{18}O_3$	–0.98
Cpd 1089:	Imazalil	6.595	296.049	Imazalil	$C_{14}H_{14}Cl_2N_2O$	–2.29
Cpd 1124:	Salbuterol	6.838	239.1522	Salbuterol	$C_{13}H_{21}NO_3$	–0.34
Cpd 1144:	Butopyronoxyl	6.93	226.1206	Butopyronoxyl	$C_{12}H_{18}O_4$	–0.52
Cpd 1212:	Cinmethylin	7.152	274.1937	Cinmethylin	$C_{18}H_{26}O_2$	–1.56
Cpd 1242:	Dicyclanil	7.22	190.0968	Dicyclanil	$C_8H_{10}N_6$	–0.54
Cpd 1274:	Thiophanate-methyl	7.419	342.0457	Thiophanate-methyl	$C_{12}H_{14}N_4O_4S_2$	–0.24
Cpd 1331:	Bisphenol A	7.596	228.1141	Bisphenol A	$C_{15}H_{16}O_2$	4.06
Cpd 1335:	Propoxur	7.614	209.1052	Propoxur	$C_{11}H_{15}NO_3$	–0.1
Cpd 1337:	Pyrocatechol	7.615	110.0369	Pyrocatechol	$C_6H_6O_2$	–1.11
Cpd 1348:	Cinmethylin	7.7	274.1933	Cinmethylin	$C_{18}H_{26}O_2$	–0.16
Cpd 1357:	Bisphenol A	7.778	228.1147	Bisphenol A	$C_{15}H_{16}O_2$	1.28
Cpd 1394:	Naphthol, 1-	7.996	144.0574	Naphthol, 1-	$C_{10}H_8O$	0.69
Cpd 1395:	Carbaryl	7.996	201.0791	Carbaryl	$C_{12}H_{11}NO_2$	–0.39
Cpd 1410:	Spinosyn B	8.128	717.4462	Spinosyn B	$C_{40}H_{63}NO_{10}$	–1.38
Cpd 1429:	Spinosyn A	8.278	731.4619	Spinosyn A	$C_{41}H_{65}NO_{10}$	–1.43
Cpd 1438:	Spiroxamine	8.364	297.2675	Spiroxamine	$C_{18}H_{35}NO_2$	–2.27
Cpd 1477:	Spinosyn D	8.621	745.4775	Spinosyn D	$C_{42}H_{67}NO_{10}$	–1.29
Cpd 1494:	Embelin	8.752	294.1838	Embelin	$C_{17}H_{26}O_4$	–2.25
Cpd 1501:	Cinmethylin	8.797	274.1935	Cinmethylin	$C_{18}H_{26}O_2$	–0.9
Cpd 1506:	Santonin	8.81	246.1261	Santonin	$C_{15}H_{18}O_3$	–2.01
Cpd 1526:	Cinmethylin	8.908	274.1935	Cinmethylin	$C_{18}H_{26}O_2$	–0.82
Cpd 1574:	Cinmethylin	9.184	274.1936	Cinmethylin	$C_{18}H_{26}O_2$	–1.21
Cpd 1578:	Bromophos	9.22	363.8501	Bromophos	$C_8H_8BrCl_2O_3PS$	–2.55
Cpd 1604:	Bromophos	9.279	363.8502	Bromophos	$C_8H_8BrCl_2O_3PS$	–2.57
Cpd 1715:	Imazethapyr	9.883	289.1417	Imazethapyr	$C_{15}H_{19}N_3O_3$	3.08

Compound Label	RT	Mass	Name	DB Formula	DB Diff (ppm)
Cpd 1728: Ethoprop	9.911	242.0566	Ethoprop	$C_8H_{19}O_2PS_2$	–0.93
Cpd 1746: Imazethapyr	10.027	289.1419	Imazethapyr	$C_{15}H_{19}N_3O_3$	2.59
Cpd 1782: Penconazole	10.224	283.0649	Penconazole	$C_{13}H_{15}Cl_2N_3$	–1.99
Cpd 1797: Cinmethylin	10.325	274.1931	Cinmethylin	$C_{18}H_{26}O_2$	0.68
Cpd 1829: Cyprodinil	10.49	225.1271	Cyprodinil	$C_{14}H_{15}N_3$	–2.07
Cpd 1833: Chenodeoxycholic acid	10.526	392.291	Chenodeoxycholic acid	$C_{24}H_{40}O_4$	4.36
Cpd 1838: Embelin	10.541	294.1837	Embelin	$C_{17}H_{26}O_4$	–2.12
Cpd 1885: Chenodeoxycholic acid	10.749	392.2909	Chenodeoxycholic acid	$C_{24}H_{40}O_4$	4.45
Cpd 1908: Cinmethylin	10.852	274.1936	Cinmethylin	$C_{18}H_{26}O_2$	–1.24
Cpd 1927: Kresoxim methyl	10.933	313.1321	Kresoxim methyl	$C_{18}H_{19}NO_4$	–2.18
Cpd 1933: Cinmethylin	10.967	274.1934	Cinmethylin	$C_{18}H_{26}O_2$	–0.53
Cpd 1951: Cinmethylin	11.089	274.1939	Cinmethylin	$C_{18}H_{26}O_2$	–2.13
Cpd 1978: Carbofuranphenol	11.235	164.0841	Carbofuranphenol	$C_{10}H_{12}O_2$	–2.38
Cpd 2017: Spiroxamine	11.536	297.2666	Spiroxamine	$C_{18}H_{35}NO_2$	0.5
Cpd 2044: Spiroxamine	11.731	297.2663	Spiroxamine	$C_{18}H_{35}NO_2$	1.49
Cpd 2054: Spiroxamine	11.846	297.2673	Spiroxamine	$C_{18}H_{35}NO_2$	–1.65
Cpd 2062: Spiroxamine	11.955	297.2672	Spiroxamine	$C_{18}H_{35}NO_2$	–1.26
Cpd 2096: Etacelasil	12.24	316.1098	Etacelasil	$C_{11}H_{25}ClO_6Si$	3.42
Cpd 2205: Spiroxamine	12.797	297.267	Spiroxamine	$C_{18}H_{35}NO_2$	–0.58
Cpd 2206: Ivermectin B1b	12.806	860.488	Ivermectin B1b	$C_{47}H_{72}O_{14}$	4.9
Cpd 2253: Ivermectin B1b	13.128	860.4891	Ivermectin B1b	$C_{47}H_{72}O_{14}$	3.61
Cpd 2314: Ivermectin B1b	13.372	860.4896	Ivermectin B1b	$C_{47}H_{72}O_{14}$	3.04
Cpd 2474: Ivermectin B1b	14.122	860.4906	Ivermectin B1b	$C_{47}H_{72}O_{14}$	1.85

Conclusions

The Agilent TOF and Q-TOF Pesticide Application Kit has been developed to provide comprehensive screening of pesticides for both targeted and non-targeted compounds. The database includes almost 1600 compounds and gives the user great flexibility in its use.

The kit offers:

- Fast and easy startup of complex analyses
- A comprehensive pesticide database of almost 1600 compounds including:
    - Chemical structures, formulas and exact masses
    - Direct Chemical Internet links to PUBCHEM and Chemspider
    - IUPAC Names
    - The ability to create spectral libraries
    - Completely customizable additions/deletions and retention time additions for chromatographic conditions developed by the user
- Results can be searched directly from the PCDL software
- Results can be data-mined with powerful searching tools such as, the Molecular Feature Extractor and Find by Formula
- Searches of the database can be partially or completely automated using MassHunter Qualitative Analysis and the MassHunter Acquisition Worklist

References

1. Agilent Technologies publication 5990-3935EN, "Q-TOF LC/MS Screening and Confirming of Non-Targeted Pesticides in a Strawberry Extract."
2. Agilent Technologies publication 5989-5496EN, "Automated Screening of 600 Pesticides in Food by LC/TOF MS Using a Molecular-Feature Database Search."
3. Agilent Technologies publication 5990-4253EN, "Multi-Residue Pesticide Analysis with Dynamic Multiple Reaction Monitoring and Triple Quadrupole LC/MS/MS."
4. Agilent Technologies publication 5990-3976EN, "Pesticide Personal Compound Database for Screening and Identification."

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Appendix I

LC/MS/MS Conditions for Test mix Positive and Negative Ion Samples

Agilent 1200 Series SL LC Parameters

Column:	Agilent ZORBAX Eclipse Plus C18, 2.1 mm × 100 mm, 1.8 µm Agilent p/n 959764-902
Column temperature:	35 °C
Injection volume:	5
Autosampler temperature:	Ambient
Needle wash:	5 s with methanol
Mobile phase:	A = 5 mM acetic acid in water B = 100% acetonitrile
Flow rate:	0.3 mL/min
Gradient:	5% B at t = 0 to 95% B at t = 12 min
Stop time:	12 min
Post time:	3 min

Agilent 6530 Q-TOF Parameters

Jet Stream Conditions

Gas temperature:	250 °C
Gas flow:	7 L/min
Nebulizer:	40 psi
Sheath gas temperature:	325 °C
Sheath gas flow:	11 L/min
Capillary + ion:	3500 V
Nozzle voltage:	0 V
Capillary – ion:	2500 V
Nozzle voltage:	1500 V
Acquisition Mode:	MS1
Min Range	100 *m/z*
Max Range	1100 *m/z*
Scan Rate	1.4 per s
Reference Masses:	Positive ion 121.050873 ($M+H^+$ for purine) 922.009798 ($M+H^+$ for HP-921)
Reference Masses:	Negative ion 119.0362 ($M-H^-$ for purine) 980.016375 ($M+C_2H_3O_2^-$ for HP-921 acetate adduct)

Appendix II

Agilent 1200 Series SL LC Parameters

Agilent 1200 Series LC Parameters

Column:	Agilent ZORBAX Eclipse Plus C18, 2.1 mm × 100 mm, 1.8 µm Agilent p/n 959764-902
Column temperature:	55 °C
Injection volume:	5.0 µL
Autosampler temperature:	6 °C
Needle wash:	Flushport (MeOH:H_2O 75:25), 5 s
Mobile phase:	A = H_2O w/5 mM ammonium formate + 0.01% formic acid B = 5 mM ammonium formate + 0.01% formic acid in 95:5 acetonitrile:water
Flow rate:	0.3 mL/min

Gradient pump time table

Time	Flow	Pressure	Solv ratio B
0.5	No change	600	6
14	No change	600	95
17	No change	600	95

Stop time	17 min
Post time	3 min

Agilent 6230 TOF Parameters

Jet Stream Conditions

Drying gas temperature:	225 °C
Drying gas flow (nitrogen):	9 L/min
Nebulizer gas pressure (nitrogen):	25 psig
Capillary voltage:	4500 V
Sheath gas temperature:	350 °C
Sheath gas flow:	11 L/min
Nozzle voltage:	500 V
Acquisition Mode MS1	
Min Range	25 m/z
Max Range	3200 m/z
Scan Rate	3
Reference Masses:	Positive ion 121.050873 ($M+H^+$ for purine) 922.009798 ($M+H^+$ for HP-921)

Appendix III

Agilent 1290 Infinity LC Parameters

Column:	Agilent ZORBAX Eclipse Plus C18 HD, 2.1 mm × 100 mm, 1.8 µm Agilent p/n
Column temperature:	60 °C
Injection volume:	5.0 µL
Autosampler temperature:	6 °C
Needle wash:	Flushport (MeOH:H_2O 75:25) 5 s
Mobile phase:	A = H_2O w/5 mM ammonium formate + 0.01% formic acid B = 5 mM ammonium formate + 0.01% formic acid in 95:5 acetonitrile:water
LC flow rate:	1.0 mL/min

Gradient pump time table

Time	Flow	Pressure	Solv ratio B
0.15	No change	600	6
2.1	No change	600	95
3	No change	600	95

Stop time	3 min
Post time	1 min

6540 Q-TOF Parameters

Jet stream conditions

Drying gas temperature:	325 °C
Drying gas flow (nitrogen):	8 L/min
Nebulizer gas pressure (nitrogen):	60 psig
Capillary voltage:	4000 V
Sheath gas temperature:	350 °C
Sheath gas flow:	12 L/min
Nozzle voltage:	500
Acquisition Mode:	MS1
Min Range	100 *m/z*
Max Range	1000 *m/z*
Scan Rate	10 per s
Reference Masses:	Positive ion 121.050873 ($M+H^+$ for purine) 922.009798 ($M+H^+$ for HP-921)

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Agilent Technologies

Multi-Residue Pesticide Analysis with Dynamic Multiple Reaction Monitoring and Triple Quadrupole LC/MS/MS

Fast and Effective Method Development Using an Application Kit and a Pesticides Compound Parameter Database

Application Note

Food Safety and Environmental

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Abstract

The analysis of pesticide residues in food and environmental samples is challenging due to the low concentrations and large number of analytes that need to be monitored and quantified. In addition, method development for Liquid Chromatography/Mass Spectrometry/Mass Spectrometry (LC/MS/MS) with a triple quadrupole instrument is laborious and time consuming because of the compound dependent parameters that need to be optimized. This application note describes how pesticide residue LC/MS/MS methods can be set up quickly and efficiently using the Agilent Pesticides Application Kit. This Application Kit contains a pesticide test mix, a 600-compound pesticide MRM database, a quick start guide and several dynamic Multiple Reaction Monitoring (MRM) methods, which can easily be incorporated into a specific method for pesticide residue analysis. The Pesticides Dynamic MRM database contains compounds commonly monitored around the world and provides fast, customized method development of the analysts' list of pesticides. Results from a 100 and 300-compound mixture are demonstrated with an Agilent 1200 SL Series Rapid Resolution LC and the Agilent 6460 Series Triple Quadrupole LC/MS System with Agilent Jet Stream Technology. The 300-compound mixture was also analyzed using an Agilent 1290 Infinity Ultra High Pressure Liquid Chromatograph (UHPLC) and a 6460 LC/MS. With the higher pressure capabilities of the Agilent 1290 Infinity UHPLC, rapid separations with higher peak capacity and less peak overlap than the Agilent 1200 Series RRLC were produced. Using a spinach matrix spiked with 16 pesticides, the performance of a complete method with the SampliQ extraction and dispersive SPE kits and the Agilent LC/MS/MS triple quadrupole on a typical food matrix was

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Introduction

The analysis of target pesticide residues has traditionally been performed using Gas Chromatography/Mass Spectrometry (GC/MS) or Liquid Chromatography/Mass Spectrometry (LC/MS) methods. Because of the number of pesticides used and the sensitivity needed for monitoring hundreds of pesticides in a single analysis, both techniques are a requirement. GC/MS is needed for the less polar, more volatile pesticides and LC/MS for pesticides that are more polar or thermally labile and there is much overlap between them. However, many of the pesticides developed over the last 20 years are most amenable to LC/MS. The method of choice for trace analysis in complex matrices uses a triple quadrupole (QQQ) mass spectrometer incorporating multiple reaction monitoring (MRM). During an MRM analysis the QQQ monitors the product ions produced by collisions of precursor ions in the central quadrupole (the collision cell) of the mass spectrometer, as seen in Figure 1. An MRM analysis can generate a very sensitive and specific analysis of target compounds.

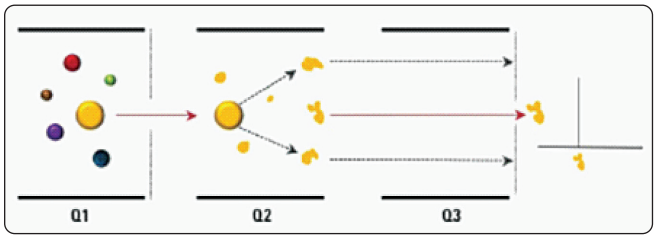


Figure 1. A schematic diagram of MRM mode on a triple quadrupole instrument. The precursor ion is selected in Q1, fragmentation occurs in Q2, and the product is selected by Q3. Since two stages of mass selectivity are used, there is very little interference from background matrix resulting in excellent sensitivity.

Over time regulating agencies have continually increased the number of pesticides and residues that must be monitored. It is now common that hundreds of residues need to be analyzed in a single LC/MS analysis. To address this challenge the MRM transitions that need to be monitored are switched using programmed time segments. This is called time segmented MRM. It is accomplished by programming the QQQ to monitor specific product ions in time segments during the LC/MS analysis. However, the method requires well defined elution time boundaries and must avoid time segment switches when compounds elute from the LC. If a time segmented MRM analysis is generated for a sample that contains hundreds of residues, the time segmented MRM analysis becomes subject to cycle and dwell time limitations that affect the sensitivity and specificity of the analysis. A new technique, Dynamic Multiple Reaction Monitoring (MRM) alleviates these limitations and also allows easier method development and future modifications of the method, such as the addition of new pesticides to be analyzed. Using Dynamic MRM, analyte ions are only monitored while they are eluting from the LC. This significantly improves the MS duty cycle time for very complex samples when compared with the time segment method and improves the sensitivity and specificity of an analysis.[1]

One of the challenges in developing an MRM method, whether it is a time segment or Dynamic MRM, is creating the time sequence of MS/MS events and mass spectrometer conditions necessary to maximize sensitivity and specificity. It is essential to generate a list of two or more MRM transitions and compound specific parameters, fragmentor voltage and collision energy for each compound being analyzed. The availability of a database containing over 600 pesticides with the MS/MS instrumental information that can be used with all Agilent triple quadrupoles eliminates the need to create this information via tedious manual procedures. The database allows easy import of selected compounds into the user's analytical method. A portion of this database is shown in Figure 2. In addition to creating custom methods, the read-only database allows the user to copy their customized database to meet his or her specific needs. A technical note describes this database in detail. [2] The Agilent Pesticides Application Kit also includes a pesticide test mixture that is used to demonstrate the performance of the system and pre-tested methods, allowing faster method development. Neither the kit nor the test mixture diminishes the need for each laboratory to define suitable QC/QA procedures and perform validation. Each laboratory must have QC tests fit-for-purpose and run analytical standards to validate analytical results.

This application note will demonstrate the use of the Agilent Pesticide Application Kit with a 600-compound parameter database and Dynamic MRM for the analysis of complex pesticide mixtures. The liquid chromatographic separations are performed using an Agilent 1200 SL Series RRLC or an Agilent 1290 Infinity UHPLC with an Agilent 6460 QQQ incorporating Jet Stream technology.[3] The methods described in the note are straightforward to generate using the Agilent MassHunter data analysis software and the Pesticide Dynamic MRM Database. Some limits of detection (LOD) of 100 fg or less were achieved using these methods with the Agilent 6460 Series QQQ LC/MS system. These methods are also compatible with all Agilent 6400 series LC/MS systems.

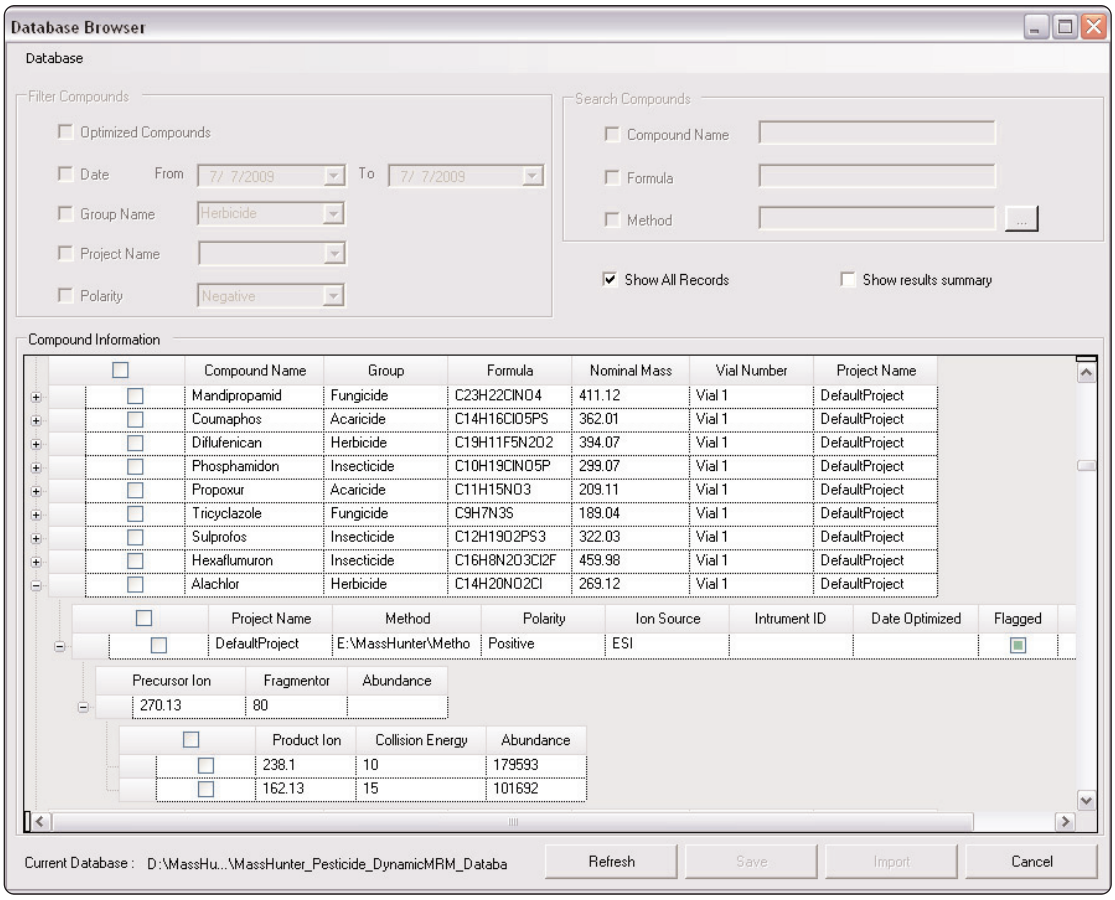


Figure 2. Compound Parameter Database with over 600 pesticides entries.

Experimental

Reagents and Chemicals

- Agilent Pesticide Test Mix, p/n 5190-0469 acid and base diluted separately as instructed to 10 ppb in 10% acetonitrile/90% water
- An Agilent SampliQ QuEChERS AOAC Extraction kit, p/n 5982-5755. Agilent SampliQ QuEChERS AOAC Dispersive SPE kits for Highly Pigmented Fruits and Vegetables, p/n 5982-5321 (2 mL) and p/n 5982-5356 (15 mL)
- Multiple pesticide standards were obtained from Sigma, Chemservice, and Dr. Erhenstofer

Instrument Settings

- *Appendix I: LC/MS/MS Conditions for Test mix Positive and Negative Ion Samples
- Appendix II: LC/MS/MS Conditions for a 100 Pesticide Methods
- *Appendix III: LC/MS/MS Conditions for 300-Pesticide Methods using the Agilent 1200 Series SL
- Appendix IV: LC/MS/MS Conditions for the 300-Pesticide Methods using the Agilent 1290 Infinity LC
- Appendix V: LC/MS/MS Conditions for Pesticides in Spinach using QuEChERS Extraction.
- *Appendix VI: LC/MS/MS Conditions for the 165-Pesticide Methods using the Agilent 1200 Series SL
- *Appendix VII: LC/MS/MS Conditions for the 224-Pesticide Methods using the Agilent 1200 Series SL
- Appendix VIII: LC/MS/MS Conditions for the 224-Pesticide Methods using Agilent 1290 Infinity LC

*Each of these methods are included with the Application Kit

Spinach Sample Preparation

- Weigh 15 g (±0.1 g) of homogenized spinach sample.
- Spike standards or IS solution if necessary.
- Vortex 30 s.
- Add 15 mL of 1% acetic acid in acetonitrile.
- Add 1 bag of extraction kit (p/n 5982-5982-5755) buffered QuEChERS extraction tubes, AOAC Method 2007.01 to 6 g $MgSO_4$ and 1.5 g NaAc.
- Cap and hand-shake vigorously for 1 min.
- Centrifuge at 4000 rpm for 5 min.
- Transfer 1 mL or 8 mL upper layer to the dispersive SPE kit (p/n 5982-5321 or p/n 5982-5356) for highly pigmented fruits and vegetables.
- Vortex 1 min.
- Centrifuge 2-mL tubes at 13000 rpm for 2 min, or 15 mL tubes at 4000 rpm for 5 min.
- Transfer 200 µL of the upper layer to the autosampler vial.
- Add 800 µL of water or appropriate standard spiking solution.
- Vortex 1 min, to prepare for LC/MS/MS analysis.

Results and Discussion

Positive and Negative Ion Test Mix

In addition to the 600-compound database, the Agilent Application Kit for pesticide residue analysis also includes a positive and negative ion test mix, with their analysis methods shown in Appendix I. The methods contain compound names, MRM transitions, fragmentor voltages, collision energies, and retention times for the Dynamic MRM. The test mix and the supplied method allow the analyst to demonstrate that the system is operating properly for pesticide analysis immediately after installation. The LC/MS/MS extracted ion chromatograms (EIC) from the test mix analyzed in the positive and negative ion mode using Dynamic MRM is shown in Figures 3 and 4.

The Application Kit Quick Start Guide [4] shows the analyst how to run the test mixes and create a Dynamic MRM method. To create new methods, standards are analyzed at higher concentrations with a one segment MRM method. The data is processed using the Agilent MassHunter Quantitative Data Analysis software to generate a custom report that now includes analyte retention times. A Dynamic MRM method is generated by importing the results from the custom report and specifying a delta retention time window. This process will be automated in the near future. Table 1 shows a partial listing of

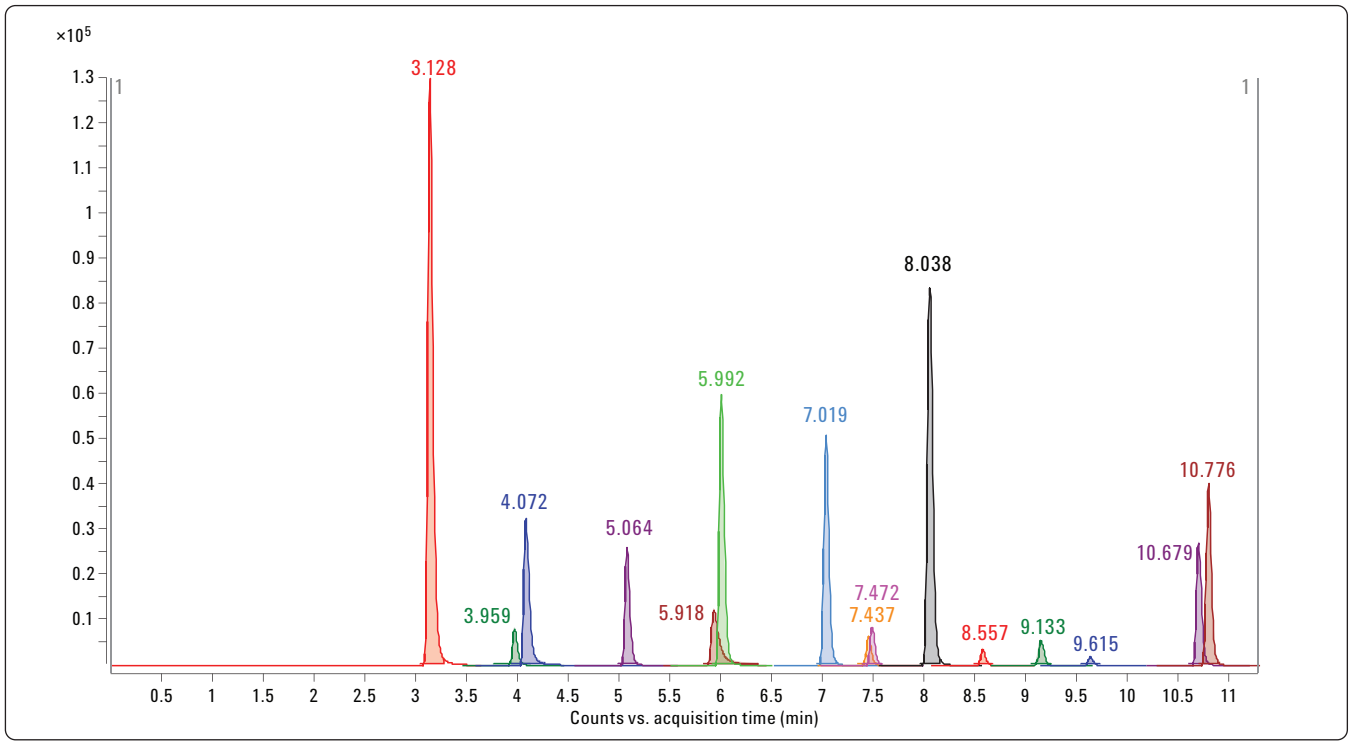


Figure 3. Positive ion test mix extracted ion chromatogram (see Appendix 1 for list of compounds matching retention times given in chromatogram).

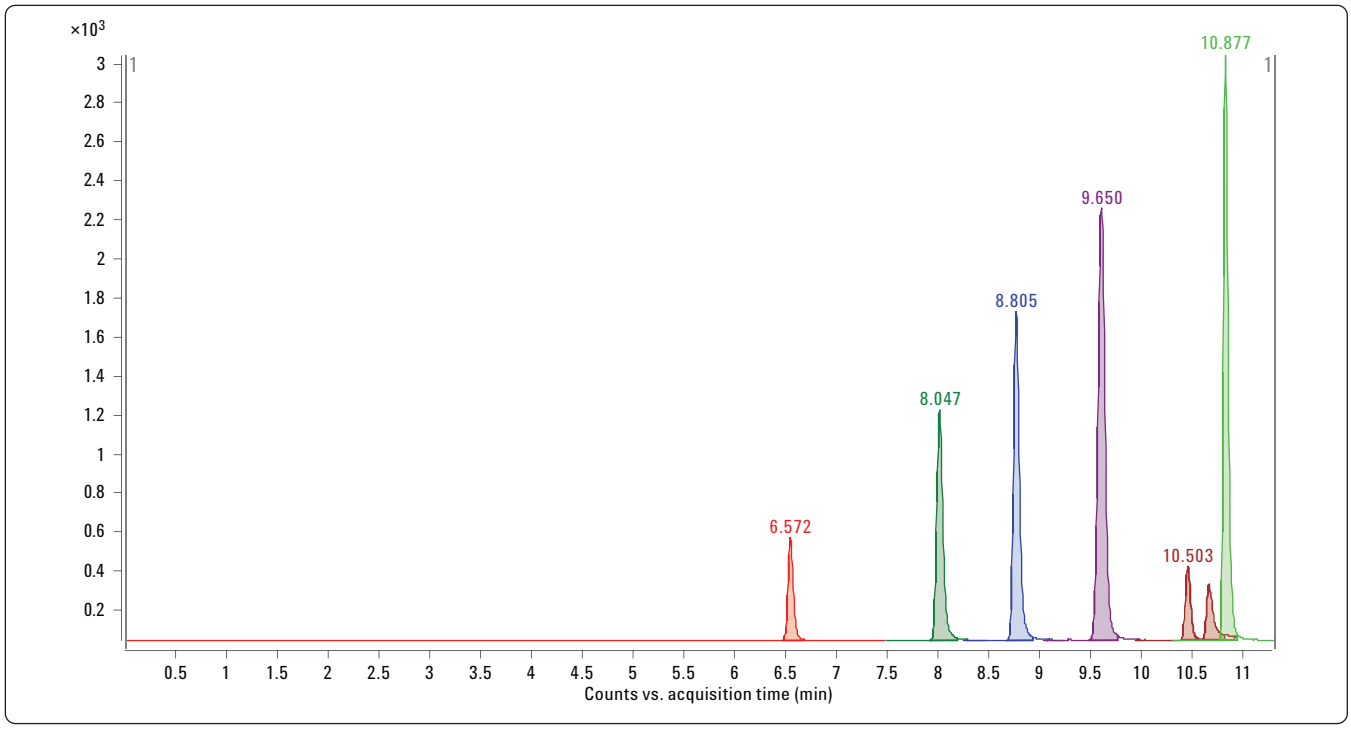


Figure 4. Negative Ion Test Mix extracted ion chromatogram (see Appendix 1 for list of compounds matching retention times given in chromatogram).

the acquisition parameters from a Dynamic MRM method. Note in this example the retention time window (Delta RT) is 2 min which is large for narrow peaks. A window this wide can be used to run standards where retention times have shifted and need to be updated in the users' customized method.

Table 1. Dynamic MRM Screen Capture of Acquisition Parameters

Acquisition | Source | Chromatogram | Instrument | Diagnostics

Scan segments

Compound Name	ISTD?	Precursor Ion	MS1 Res	Product Ion	MS2 Res	Fragmentor	Collision Energy	Ret Time (min)	Delta Ret Time
Acephate	☐	184	Unit	125	Unit	80	10	1.212	2
Aminocarb	☐	209	Unit	137	Unit	120	20	1.251	2
Atrazine	☐	216	Unit	132	Unit	120	20	7.602	2
Azinphos-methyl	☐	318	Unit	132	Unit	80	10	9.346	2
Carbofuran	☐	222	Unit	123	Unit	120	15	7.13	2
Chlorpyrifos methyl	☐	322	Unit	125	Unit	80	15	12.168	2
Diazinon	☐	305	Unit	153	Unit	160	20	11.822	2
Dimethoate	☐	230	Unit	171	Unit	80	10	4.645	2
Imazalil	☐	297	Unit	159	Unit	160	20	6.498	2

Dynamic MRM Parameters

Cycle Time 500 ms

Fast and effective screening of a 100-compound pesticide mix using Dynamic MRM

A 100-compound mix of pesticides was used to demonstrate the effectiveness of the Dynamic MRM. Appendix II contains the LC/MS/MS conditions and a partial listing of the Dynamic MRM method used to analyze a 100-pesticide mixture at the 100 pg/compound level. Note that the column used was 50 mm in length so faster analysis and less efficiency is obtained. The LC/MS/MS extracted ion chromatogram shown in Figure 5 illustrates the performance of the system. The complete LC analysis took less than 15 minutes. Figure 6 shows a 1-min time window where 11 compounds (22 MRM's) are eluting. Figure 7 shows the 1-min delta retention time window for each Dynamic MRM transition. Note the many peak overlaps in the chromatograms. This necessitates the use of dynamic transitions instead of time segmented transitions in order to achieve the needed cycle time so that each peak can have enough data points to adequately describe the peak for quantitation. Furthermore time segmented MRM has an inherent "dead time" data loss when monitoring analyte peaks eluting near or between time segment boundaries. Time segmented MRM methods may require duplicate monitoring of specific analytes which elute over adjoining time segments. In addition, Dynamic MRM maximizes the dwell times for overlapping peaks enhancing the signal-to-noise while maintaining constant cycle time. Note that the cycle time selected should ideally provide about 20 data points across a peak with a minimum of 64 data points in the retention time window (Delta Ret Window).

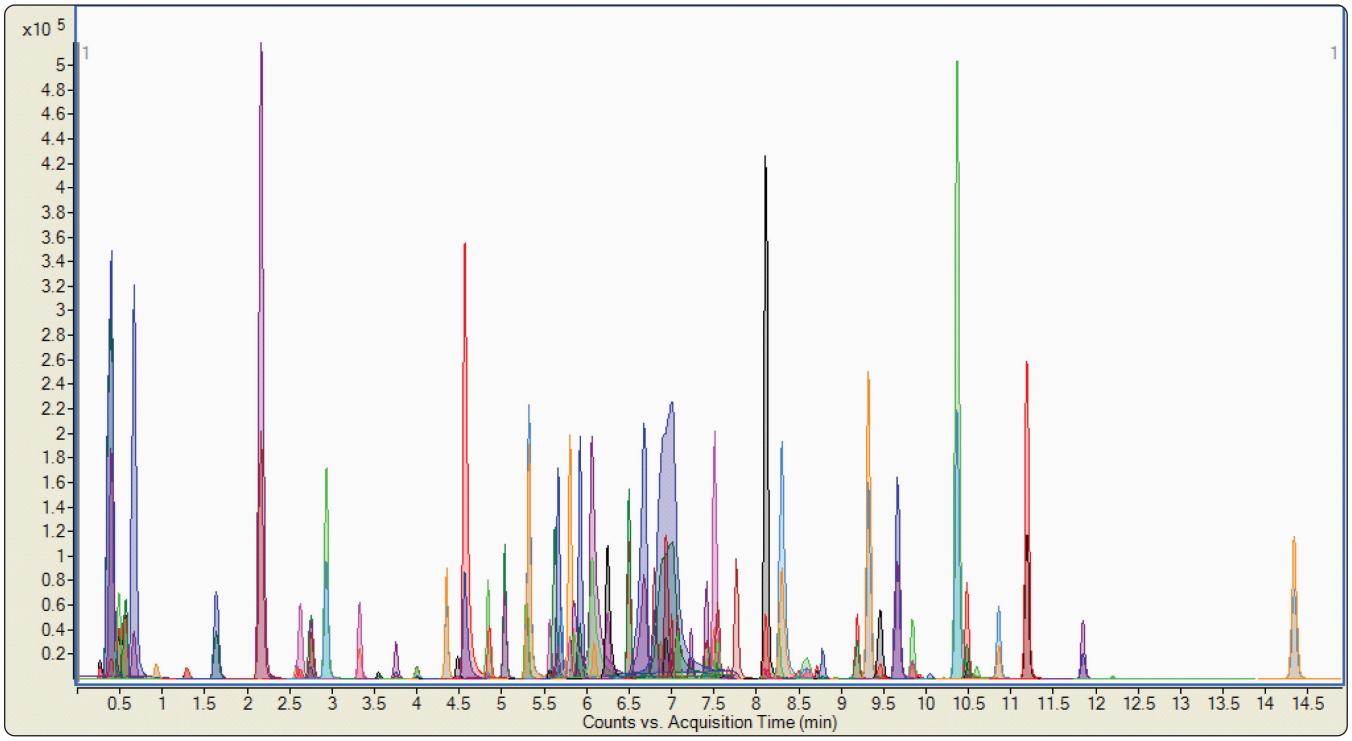


Figure 5. Extracted Ion Chromatograms of 100 compound pesticide mixture (100 pg level).

Compound name	Precursor ion	Product ion	Retention time
Cinosulfuron	414.1	183	5.579
Cinosulfuron (Q)	414.1	157	5.579
Chlorotoluron	213.1	72	5.642
Chlorotoluron (Q)	213.1	140	5.642
Atrazine	216.1	174	5.682
Atrazine (Q)	216.1	132	5.682
Carbaryl	202.1	145	5.736
Carbaryl (Q)	202.1	117	5.734
Carboxin	236.1	143	5.836
Carboxin (Q)	236.1	87	5.836
Chlorsulfuron	358.0	167	5.896
Chlorsulfuron (Q)	358.0	141	5.896
Ethiofencarb	226.1	107	5.937
Ethiofencarb (Q)	226.1	164	5.936
Dodemorph	283.3	116	6.073
Dodemorph (Q)	282.3	98	6.074
Diuron (Q)	233.0	160	6.101
Cyprodinil	226.1	108	6.245
Cyprodinil (Q)	226.1	93	6.246
Difenoxurone	287.1	123	6.509
Difenoxurone (Q)	287.1	72	6.509

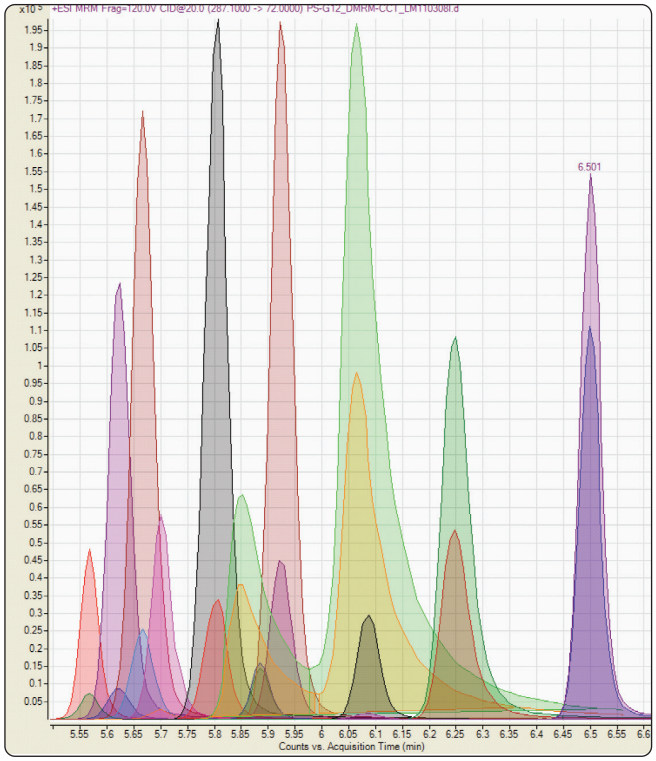


Figure 6. Left: Table of 11 compounds monitored during a 1 minute time window. Right: Dynamic MRM of compounds being monitored.

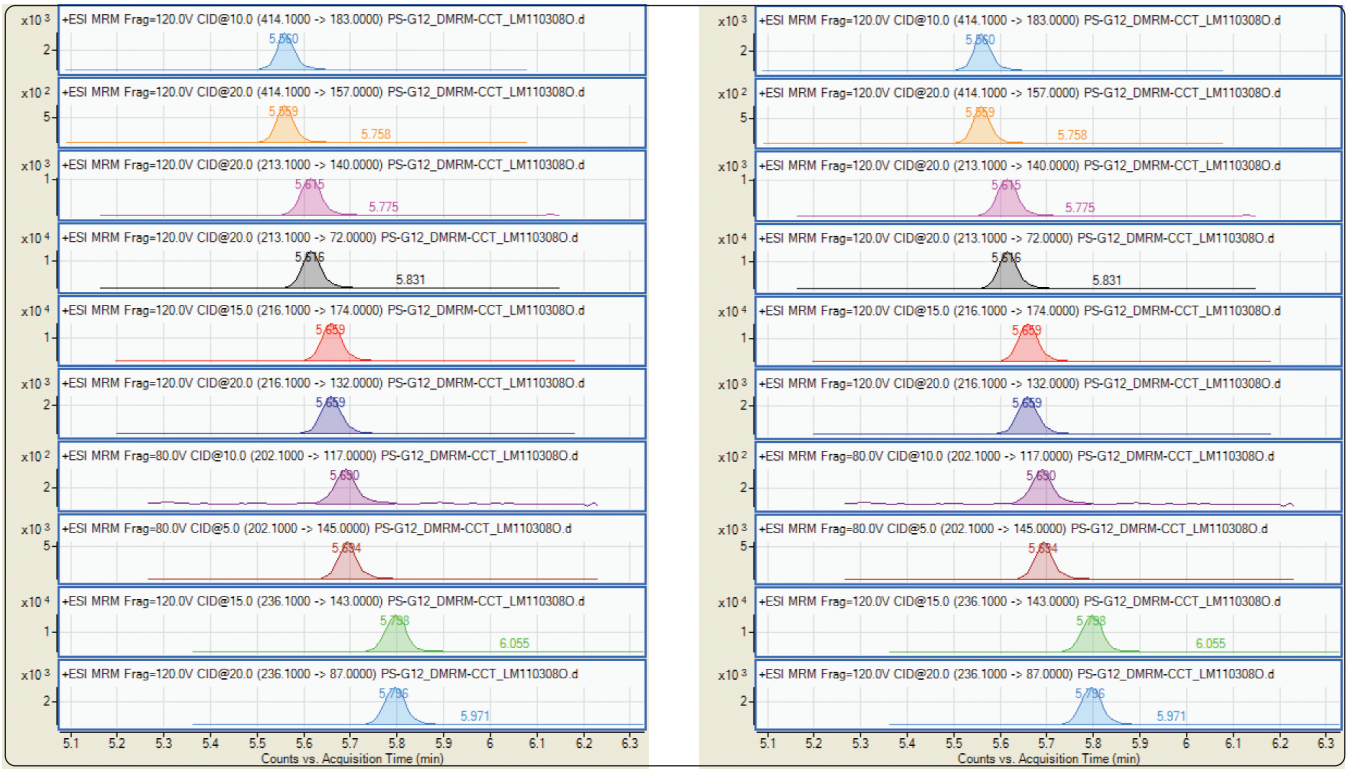


Figure 7. Dynamic MRM windows for each MRM transition.

Typical results achieved with the method are shown in Figure 8. It illustrates the results from one of the compounds, atrazine, in the 100-compound mixture. Note the 20 data points that were collected during the elution of atrazine. This provides a sufficient number of data points to assure quantitative accuracy and shows the effectiveness of Dynamic MRM.

Average signal height:	15,650
Average signal area:	50,966
RSD:	3.2%
Estimated LOQ:	100 fg or less

6–7 data points above FWHM

3 sec FWHM, 6 sec at 10% valley

20 data points baseline-to-baseline

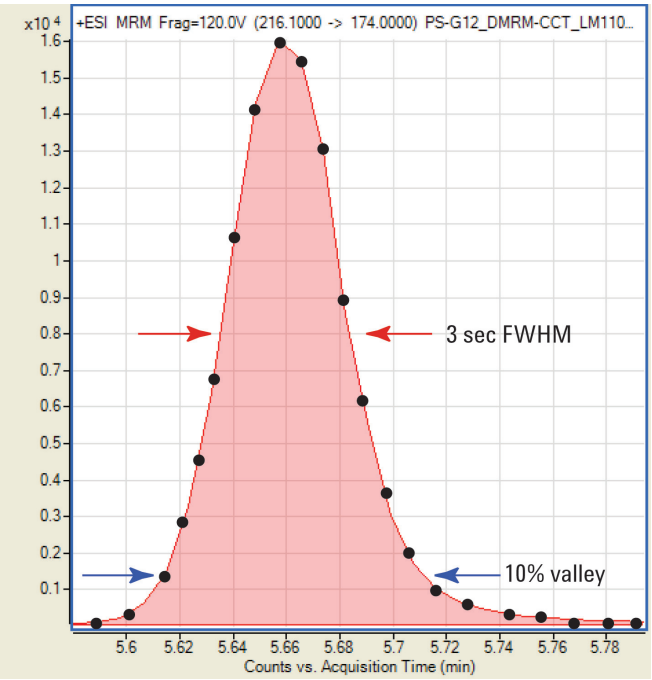


Figure 8. Typical analytical results shown with 10 pg of atrazine visualizing the effectiveness of Dynamic MRM.

The calibration data from four compounds in the mixture are illustrated in Figure 9. R^2's = 0.0998 are achieved for each pesticide. With constant cycle time maintained, the quantitative results with Dynamic MRM are excellent.

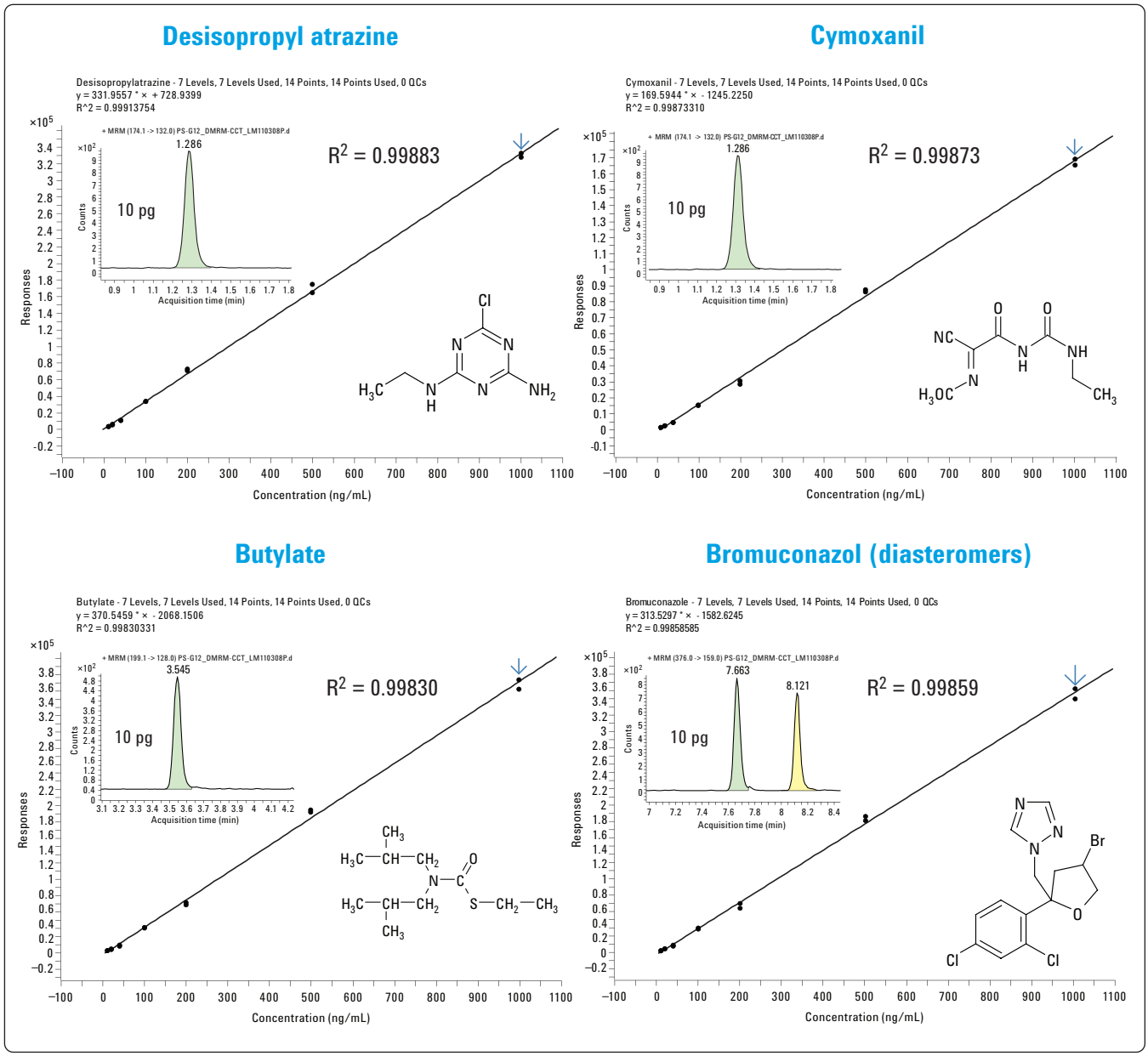


Figure 9. Dynamic MRM Calibration Plots, 10 pg–1 ng (7 levels).

Sharper peaks are produced with a 300-pesticide mix using the new Agilent 1290 Infinity LC

Appendix III contains a partial listing of the Dynamic MRM method used to analyze a 300-pesticide mixture at the 100 pg/compound level. The LC/MS/MS extracted ion chromatogram is shown in Figure 10. The analysis took less than 20 minutes using the Agilent 1200 Series SL RRLC and an Eclipse Plus C18 2.1 mm × 100 mm, 1.8 µm column at a flow rate of 0.5 mL/min. The same mixture was separated using an Agilent 1290 Infinity UHPLC with an Eclipse-Plus C18, 2.1 mm × 150 mm, 1.8 µm column. Figure 11, an extracted ion chromatogram and Figure 12, an expanded portion of the chromatogram, demonstrate that this complex mixture has been analyzed in about 15 minutes which is approximately 25% faster than with the Agilent 1200 Series SL RRLC. The Agilent 1290 Infinity UHPLC also produced a separation with higher peak capacity and less peak overlap than the Agilent 1200 Series SL RRLC. Typical peak ½ heights using atrazine as an example with the Agilent 1290 Infinity UHPLC are 1.8 s. This is because the longer column provides higher efficiency and the Agilent 1290 Infinity LC can operate at the pressure these conditions incurred (~900 bar).

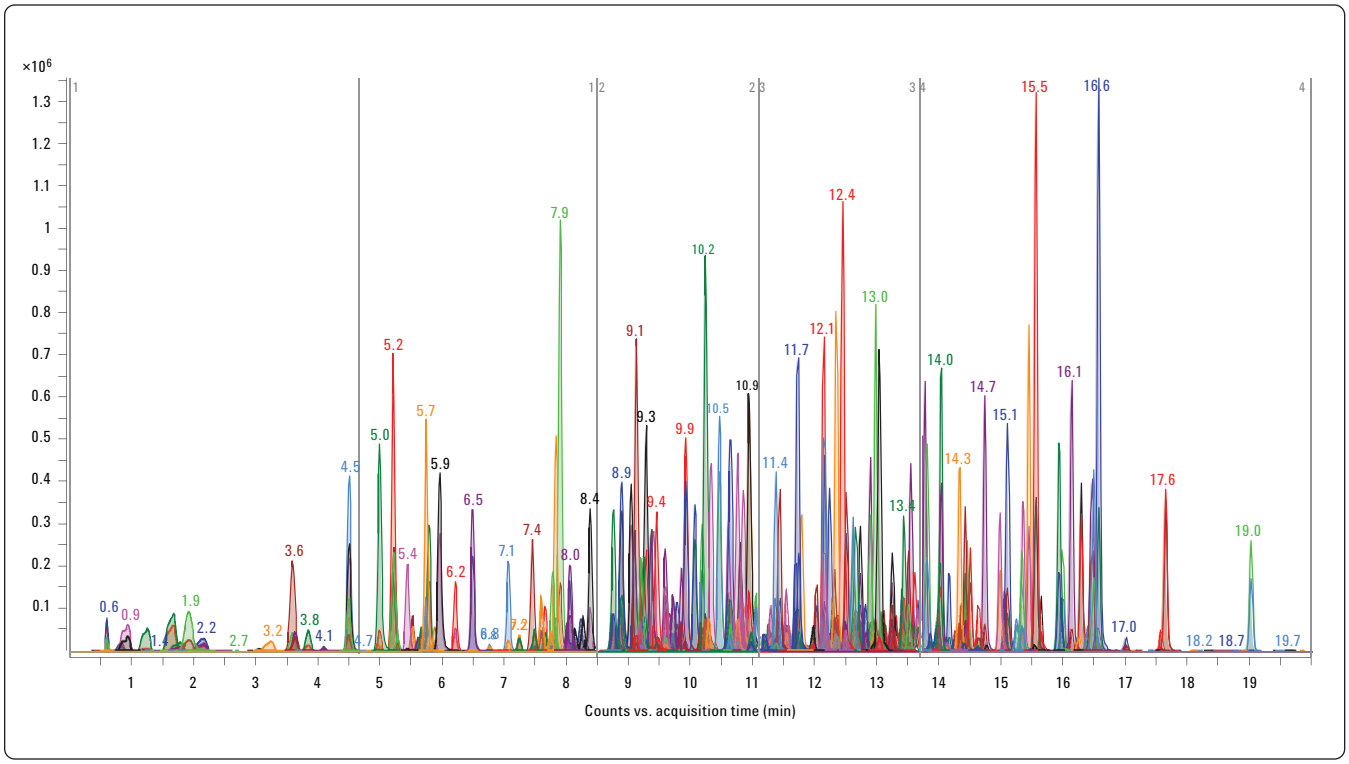


Figure 10. EIC of 300 compound pesticide mixture using an Agilent 1200 Series SL RRLC.

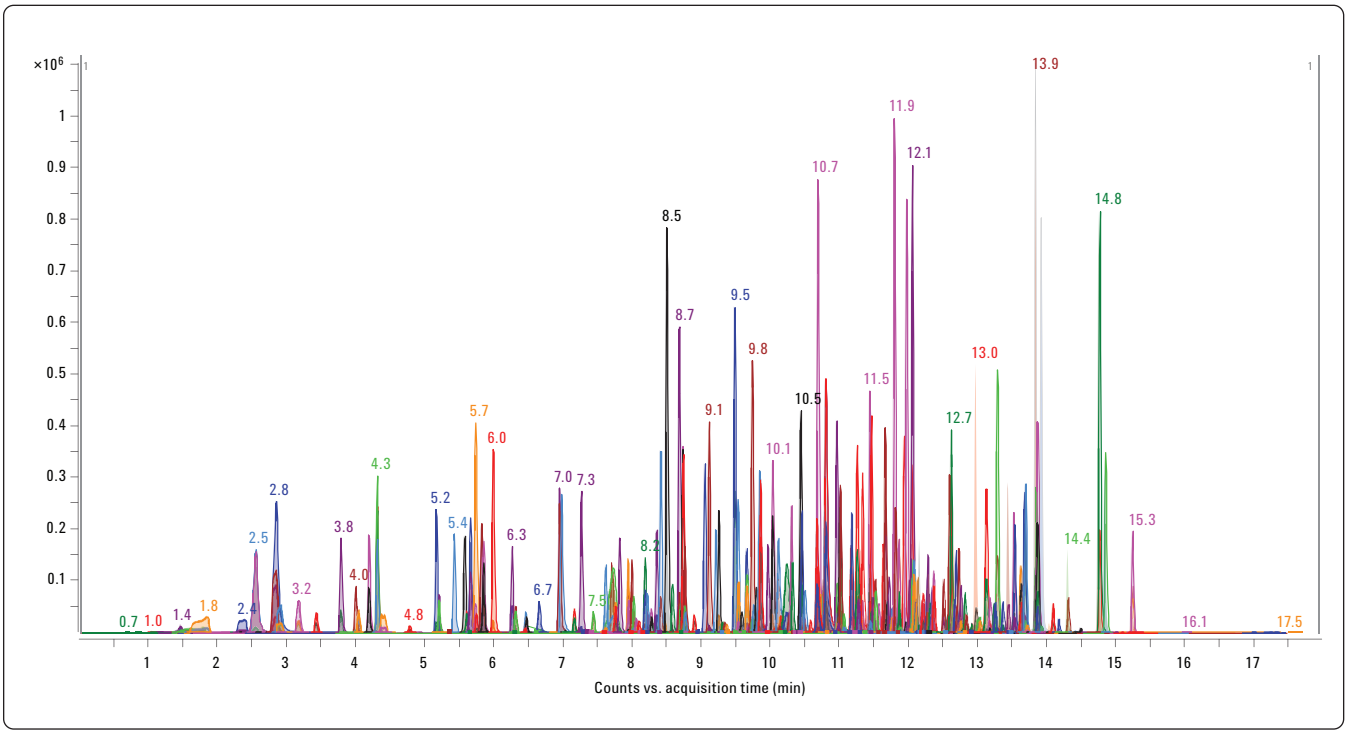


Figure 11. EIC of 300-compound pesticide mixture using the Agilent 1290 Infinity UHPLC.

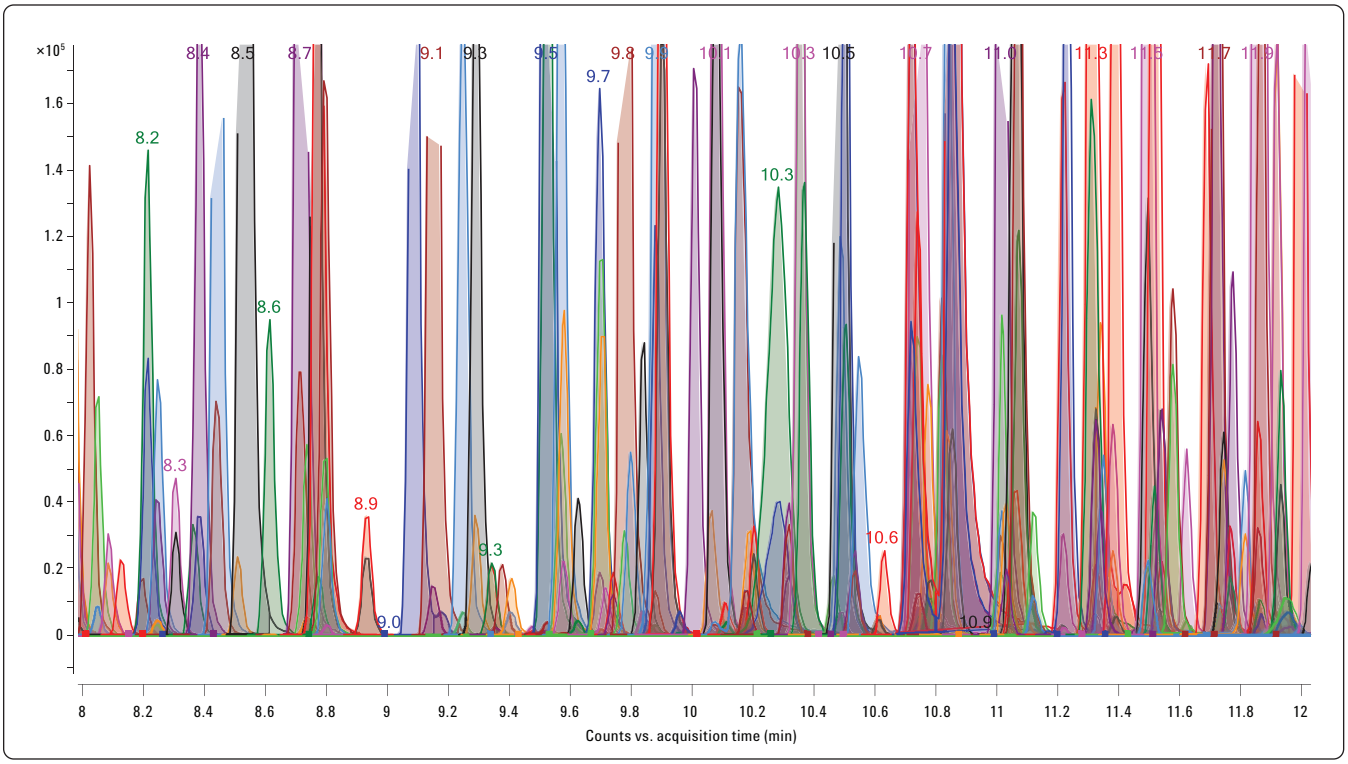


Figure 12. Expanded EIC of 300-compound pesticide mixture using an Agilent 1290 Infinity UHPLC illustrating the high peak capacity of the Agilent 1290 Infinity.

Faster analysis with a 224-pesticide mix using the new Agilent 1290 Infinity LC

Another advantage of the Agilent 1290 Infinity LC with the Agilent 6460 Series QQQ LC/MS is the ability to increase flow and decrease analysis time. Using the 1200 Series SL the analysis of 225 pesticides is performed in 15 min and shown in Figure 13. The method for this analysis is given in Appendix IV. With the Agilent 1290 Infinity LC the flow can be doubled and the gradient completed in half the time. This provides the same separation in less than 7 min as shown in Figure 14. The method for this analysis is given in Appendix V. Analyzing hundreds of pesticides in one run, it is best to obtain the highest peak capacity as shown in the 300-pesticide example. However, if speed of analysis is absolutely necessary, it is shown that the higher pressure capability of the Agilent 1290 Infinity LC and the higher pressure capability of the HD columns provide the performance needed.

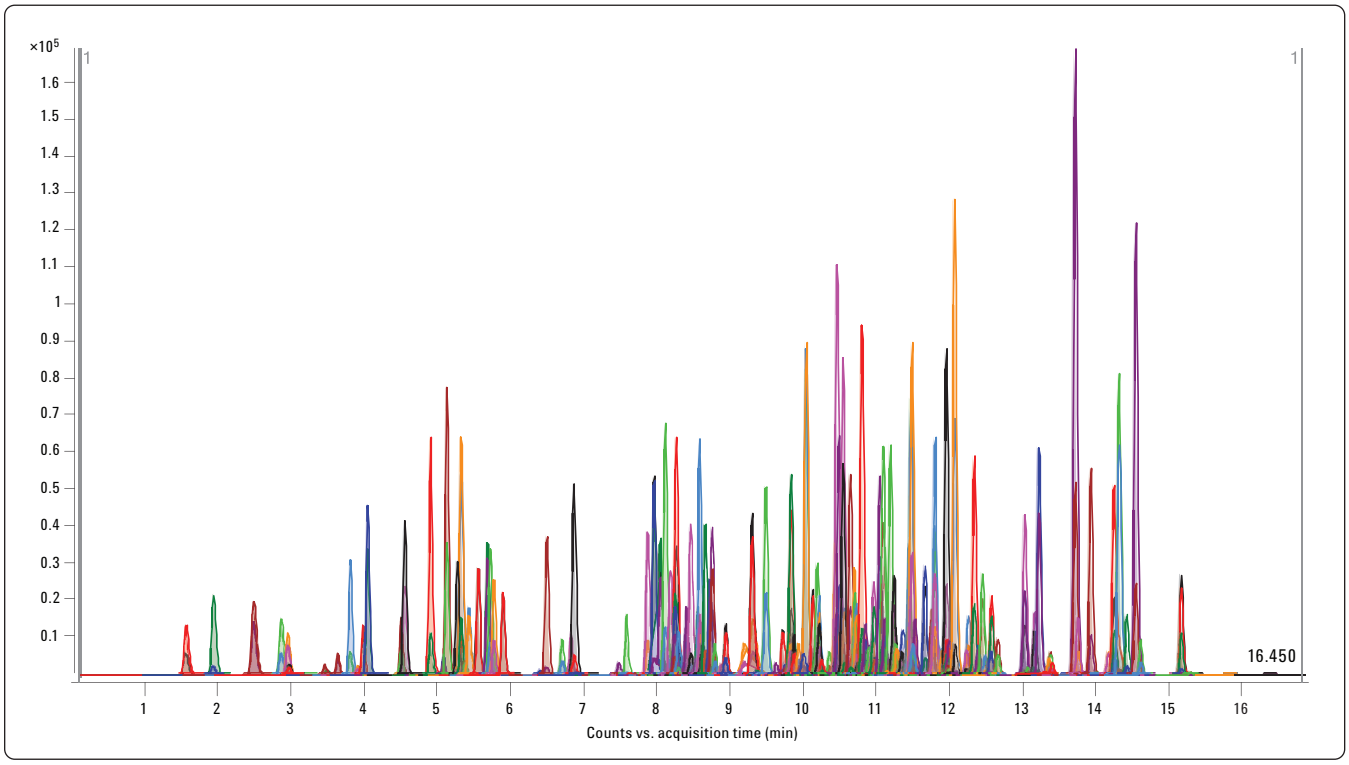


Figure 13. EIC of 224 pesticides using the Agilent 1200 Series SL LC and the Agilent 6460 QQQ LC/MS.

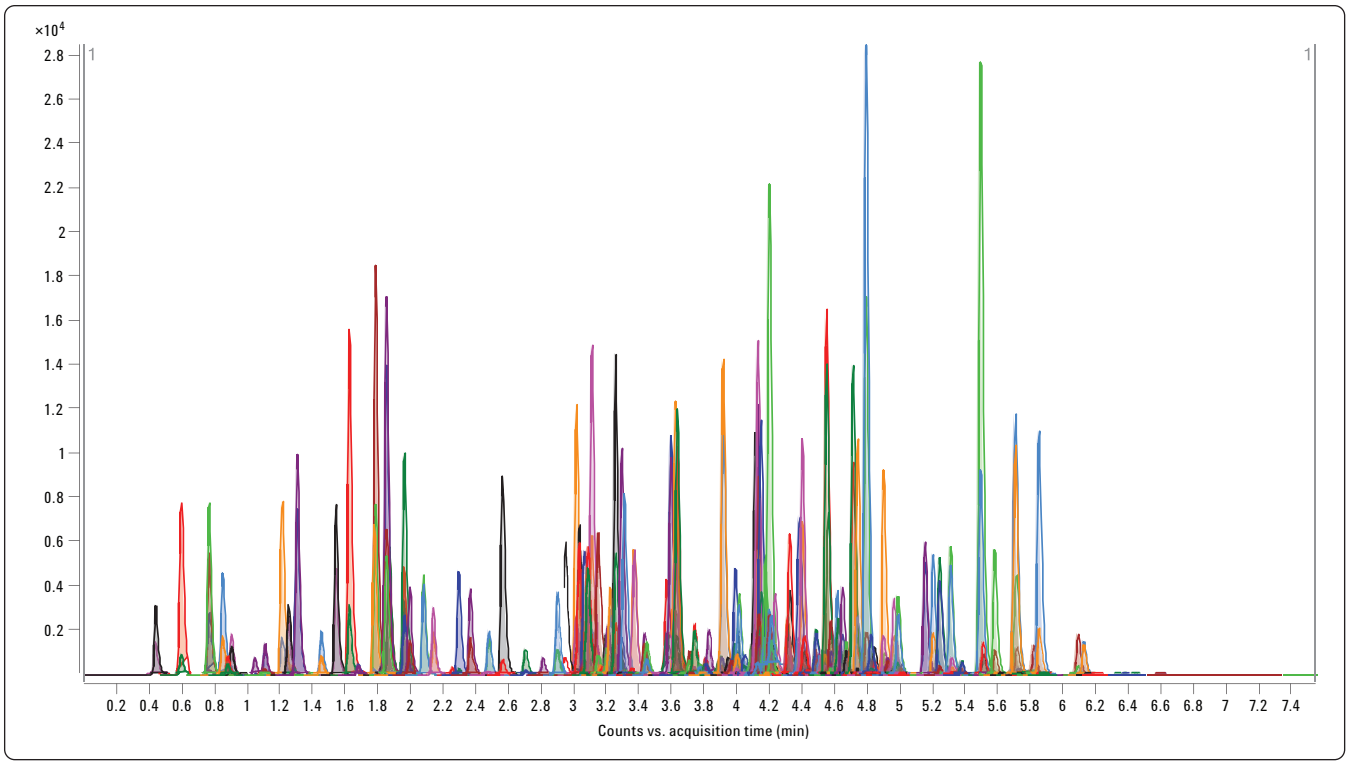


Figure 14. EIC of 224-pesticide mix analyzed with Agilent 1290 Infinity LC and the Agilent 6460 QQQ LC/MS.

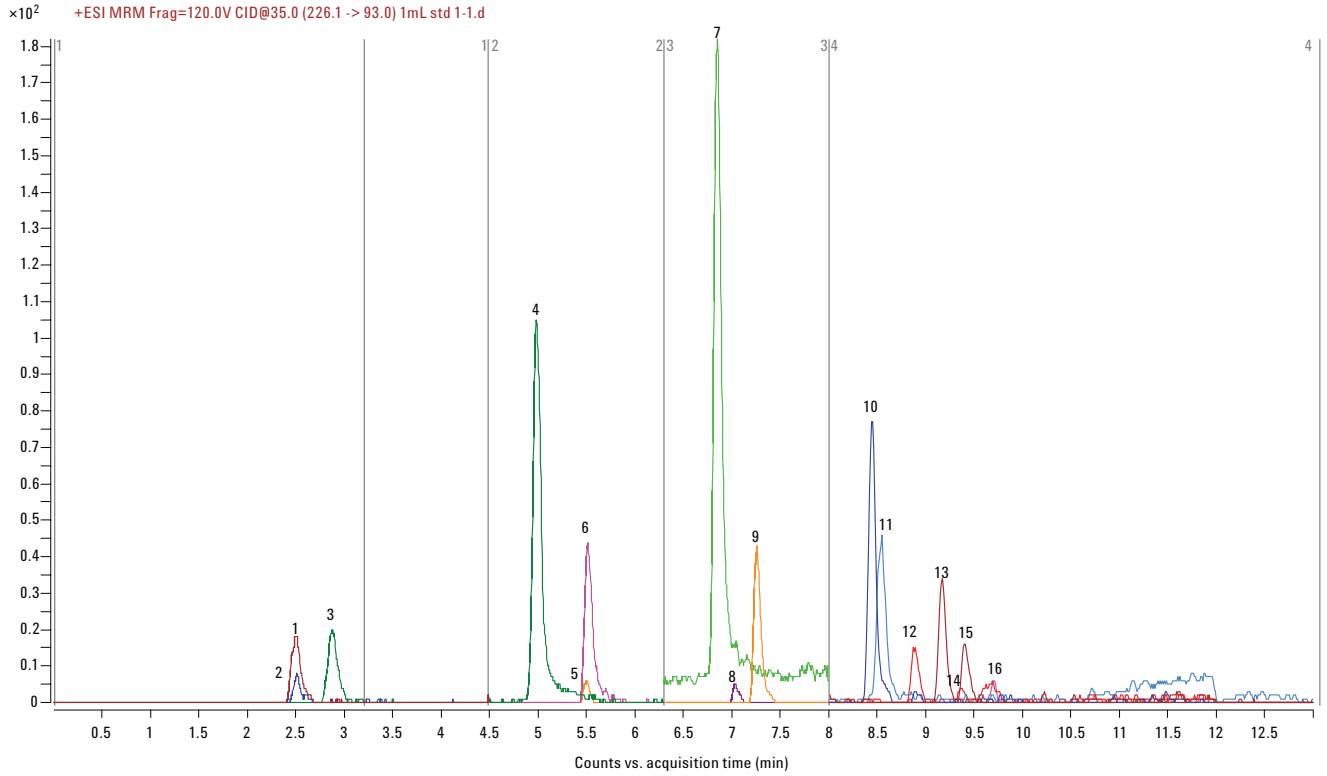
Pesticides Application Kit in a food matrix-Spinach using SampliQ Extraction and Dispersive SPE Kits

To demonstrate the use of the Agilent application kit for the analysis of a typical food product with Agilent's easy to use SampliQ extraction and dispersive SPE kits, a spinach matrix was spiked with 10 ppb of the 16 pesticides listed in Table 2. Triphenylphosphate (TPP) is the internal standard.

Table 2. List of 16 Pesticides and Instrument Parameters Spiked into Spinach Matrix at 10 ppb

Analyte	MRM channel (*m/z*) Quantifier	Qualifier	Fragmentor (V)	Collision energy (V) Quantifier	Qualifier	Retention Time (min)
Acephate	184.0 > 94.9	184.0 > 110.0	60	3	15	2.55
Methamidophos	142.0 > 94.0	142.0 > 124.9	60	8	8	2.54
Pymetrozine	218.1 > 105.0	218.1 > 78.0	115	20	50	2.97
Carbendazim	192.1 > 160.0	192.1 > 105.0	95	18	40	5.07
Imidacloprid	256.1 > 209.1	256.1 > 175.0	60	12	18	5.53
Thiabendazole	202.1 > 175.0	202.1 > 131.0	110	27	38	5.65
Propoxur	210.1 > 111.0	210.1 > 92.9	50	12	15	6.89
Thiophanate methyl	343.1 > 151.0	343.1 > 117.9	105	17	65	7.08
Carbaryl	202.0 > 145.0	202.0 > 115.0	50	3	40	7.30
Ethoprophos	243.1 > 130.9	243.1 > 172.9	80	15	15	8.50
Imazalil	297.1 > 158.9	297.1 > 200.9	80	22	15	8.52
Penconazole	284.1 > 158.9	284.1 > 172.9	80	32	32	8.95
Cyprodinil	226.1 > 93.0	226.1 > 108.0	120	35	35	9.23
Dichlorfluanid	333.0 > 123.0	333.0 > 223.9	85	28	5	9.40
Kresoxim methyl	314.0 > 222.1	314.0 > 235.0	70	10	10	9.44
Tolyfluanid	347.0 > 136.9	347.0 > 238.0	60	25	3	9.73
TPP (IS)	327.1 > 77.0	327.1 > 151.9	70	45	45	9.49

Figure 15 shows the EIC of the spinach sample spiked at the 10-ppb pesticide level. All the pesticides are easily detected at this level with a total analysis time less than ten minutes.



10 ppb of pesticides spiked into the spinach matrix.

Peak identification:

1. Acephate
2. Methamidophos
3. Pymetrozine
4. Carbendazim
5. Imidacloprid
6. Thiabendazole
7. Propoxur
8. Thiophanate methyl
9. Carbaryl
10. Ethoprophos
11. Imazalil
12. Penconazole
13. Cyprodinil
14. Dichlorfluanid
15. Kresoxim methyl,
16. Tolyfluanid

Internal standard peak (TPP) is not shown to get clear peak profile of other small peaks.

Figure 15. EIC of 10 ppb pesticides into spinach matrix..

An example of the linearity achieved for the spiked spinach matrix is shown in Figure 16. The calibration range was 5 – 250 ng/g and seven levels were used to generate the curve, 5, 10, 25, 100, and 250 ng/g. The curve was generated by plotting the ratio of the analyte peak area, carbaryl, to the internal standard (IS) peak area with the ratio of the analytes concentration to IS concentration. The $R^2 = 0.998$.

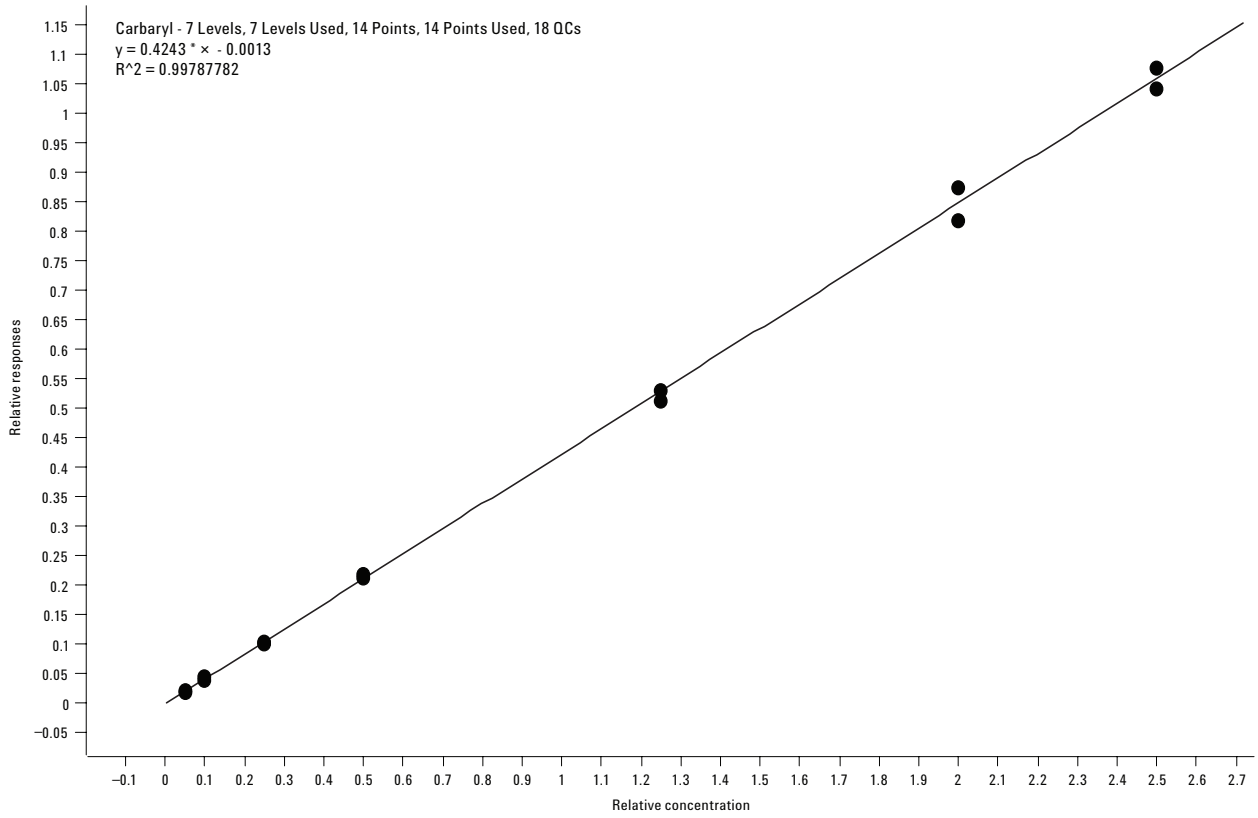


Figure 16. Carbaryl calibration curve.

Conclusions

The **Agilent Pesticide Application Kit** for LC/QQQ provides the user with fast method development for hundreds of pesticides with multiple transitions and the ability to develop those methods customized to his or her specific analytical needs.

This application note demonstrates the use of the Agilent Application Kit for Pesticides using several Agilent technologies for screening large numbers of compounds. The following technologies are used:

- ***600 compound pesticide MRM database*** and the **Agilent MassHunter Data Acquisition and Analysis software.** The combination gives users the ability to generate acquisition and analysis methods quickly. The methods can be easily customized and rapidly modified to meet the needs of future analyses.
- **Dynamic MRM** which maximizes the detection capability of the QQQ when hundreds of residues are being analyzed.
- **Agilent 1200 Series SL RRLC** interfaced to the **Agilent 6400 series triple quadrupoles** for fast and high resolution LC/MS/MS analysis. Use of the Agilent 6460 QQQ with Agilent's Jet Stream Electrospray Ion Source ensures lowest levels of detection of the pesticides. However, any of the Agilent 6400 series LC/QQQ will provide excellent results.
- Easy to use **SampliQ QuEChERS sample preparation kits** included in the Application Kit provide a fast and reproducible method to extract pesticide residues from complex food matrixes in a few simple steps.
- **Ready to use methods** with retention times for Dynamic MRM using the Agilent 1200 Series SL LC system. See all * Appendix methods.[4]

Use of these technologies allows methods to be quickly developed and enables screening of complex matrices containing hundreds of potential residues at femtomole concentrations.

This kit is compatible with all Agilent 1200 Series LC and 6400 series QQQ MS systems and will enable the user to quickly get started running multi-residue pesticides. For the most demanding analyses, the Agilent 1290 Infinity LC with the 6460 QQQ should be considered. Additional methods for this system should be available in the near future.

References

1. Application Note 5990-3595EN, New Dynamic MRM Mode Improves Data Quality and Triple Quad Quantification in Complex Samples.
2. Technical Note 5990-4255EN Pesticide Dynamic Multi-reaction monitoring Database.
3. Technical Note 5990-3494EN Agilent Jet Stream Thermal GradienFocusing Technology.
4. Agilent Publication 5990-4262EN Pesticide analysis with DRMRM database quick start guide.

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Appendix I

LC/MS/MS Conditions for Test mix Positive and Negative Ion Samples

Agilent 1200 Series SL LC Parameters

Column:	Agilent ZORBAX Eclipse Plus C18, 2.1 mm × 100 mm 1.8 µm Agilent p/n 959764-902
Column temperature:	35
Injection volume:	5
Autosampler temperature:	Ambient
Needle wash:	5 s with methanol
Mobile phase:	A = 5 mM acetic acid in water B = 100% acetonitrile
Flow Rate:	0.3 mL/min
Gradient:	5% B at t = 0 to 95% B at t = 12 min
Stop Time:	12 min
Post:	Time 3 min

Jet Stream Conditions

Gas temperature:	250 °C
Gas flow:	7 L/min
Nebulizer:	40 psi
Sheath gas temperature:	325 °C
Sheath gas flow:	1 L/min
Capillary + ion:	3500 V
Nozzle voltage:	0 V
Capillary – ion:	2500 V
Nozzle voltage:	1500 V

MS/MS Scans for positive ions

Compound Name	ISTD?	Prec Ion	MS1 Res	Prod Ion	MS2 Res	Frag (V)	CE (V)	Ret Time	Ret Window	Polarity
Aminocarb	❑	209	Unit	137	Unit	120	20	3.128	1	Positive
Imazapyr	❑	262	Unit	217	Unit	160	15	3.959	1	Positive
Thiabendazole	❑	202	Unit	131	Unit	120	30	4.072	1	Positive
Dimethoate	❑	230	Unit	171	Unit	80	10	5.064	1	Positive
Imazalil	❑	297	Unit	159	Unit	160	20	5.918	1	Positive
Metoxuron	❑	229.1	Unit	72.1	Unit	93	14	5.992	1	Positive
Carbofuran	❑	222	Unit	123	Unit	120	15	7.019	1	Positive
Atrazine	❑	216	Unit	132	Unit	120	20	7.437	1	Positive
Metosulam	❑	418	Unit	175	Unit	144	26	7.472	1	Positive
Metazachlor	❑	278.1	Unit	134.1	Unit	75	18	8.038	1	Positive
Molinate	❑	188.1	Unit	55.1	Unit	78	22	9.113	1	Positive
Malathion	❑	331	Unit	99	Unit	80	10	9.615	1	Positive
Pyraclostrobin	❑	388	Unit	163	Unit	120	20	10.679	1	Positive
Diazinon	❑	305	Unit	153	Unit	160	20	10.776	1	Positive

MS/MS Scans for negative ions

Compound Name	ISTD?	Prec Ion	MS1 Res	Prod Ion	MS2 Res	Frag (V)	CE (V)	Ret Time	Ret Window	Polarity
Bentazon	❑	239.1	Unit	132	Unit	80	32	6.572	1	Negative
2,4,5-T	❑	252.9	Unit	194.8	Unit	76	9	8.047	1	Negative
Silvex	❑	266.9	Unit	194.9	Unit	90	5	8.805	1	Negative
Acifluorfen	❑	360	Unit	315.9	Unit	78	5	9.650	1	Negative
Dinoseb	❑	239.1	Unit	207	Unit	154	21	10.503	1	Negative
Hexaflumuron	❑	459	Unit	438.9	Unit	102	5	10.877	1	Negative

Appendix II

LC/MS/MS Conditions for 100-Pesticide Methods

Agilent 1200 Series LC Parameters

Column:		Agilent ZORBAX Eclipse Plus-C18, 2.1 mm × 50 mm, 1.8 µm Agilent p/n 959741-902
Column temperature:		35 °C
Injection volume:		1.0 µL
Autosampler temperature:		6 °C
Needle wash:		Flushport (MeOH:H_2O 75:25), 5 s
Mobile phase:		A = 0.1% formic acid in water B = 0.1% formic acid in 95:5 acetonitrile:water
Flow rate:		0.6 mL/min
Gradient	Time	%B
	0	10
	10	70B
	15	90B
Stop time	20	10B
Post time	5	

Note that example transitions, fragmentor voltages, and collision energies for this method are shown in Figure 7.

Jet Stream Conditions

Drying gas temperature:	325 °C
Drying gas flow (nitrogen):	6 L/min
Nebulizer gas pressure (nitrogen):	35 psig
Capillary voltage:	4000 V
Sheath gas temperature:	400 °C
Sheath gas flow:	12 L/min
Nozzle voltage:	Off

Agilent 6460A QQQ settings

MS1 and MS2 resolution:	Unit
Time Filtering:	Peak width = 0.03 min
Dynamic MRM transitions:	200
Constant cycle time:	373 ms
Delta EMV:	400 V

Appendix III

LC/MS/MS Conditions for 300-Pesticide Methods using the Agilent 1200 Series SL

Agilent 1200 Series LC Parameters

Column:	Agilent ZORBAX Eclipse Plus-C18, 2.1 mm × 100 mm, 1.8 µm Agilent p/n 959764-902
Column temperature:	35 °C
Injection volume:	1.0 µL
Autosampler temperature:	6 °C
Needle wash:	Flushport (MeOH:H_2O 75:25), 5 s
Mobile phase:	A = H_2O w/5 mM ammonium formate + 0.01% formic acid B = 5 mM ammonium formate + 0.01% formic acid in 95:5 acetonitrile:water
Flow rate:	0.5 mL/min

Gradient pump time table

Time	Flow	Pressure	Solv ratio B
0.5	No change	600	6
18	No change	600	95
20	No change	600	95
20.01	No change	600	6
Stop time	20 min	10%B	
Post time	5 min		

Jet Stream Conditions

Drying gas temperature:	325 °C
Drying gas flow (nitrogen):	6 L/min
Nebulizer gas pressure (nitrogen):	35 psig
Capillary voltage:	4000 V
Sheath gas temperature:	400 °C
Sheath gas flow:	12 L/min
Nozzle voltage:	Off

Ten representative MS/MS Transitions from 300-Compound Methods

Compound Name	ISTD?	Prec Ion	MS1 Res	Prod Ion	MS2 Res	Frag (V)	CE (V)	Ret Time
Promecarb	❑	208.1	Unit	151	Unit	80	5	11.635
Promecarb	❑	208.1	Unit	109	Unit	80	10	11.635
Flurtamone	❑	334.1	Unit	303	Unit	120	20	11.644
Flurtamone	❑	334.1	Unit	247	Unit	120	30	11.644
Isoxaflutole	❑	377.1	Unit	360.1	Unit	100	5	11.669
Isoxaflutole	❑	360.1	Unit	251	Unit	120	10	11.669
Dimethenamide	❑	276.1	Unit	244	Unit	120	10	11.683
Dimethenamide	❑	276.1	Unit	168	Unit	120	15	11.683
Diethofencarb	❑	268.2	Unit	226	Unit	80	5	11.706
Diethofencarb	❑	268.2	Unit	152	Unit	80	20	11.706

Appendix IV

LC/MS/MS Conditions for 300-Pesticide Methods using the Agilent 1290 Infinity LC

Agilent 1290 LC Parameters

Column:	Agilent ZORBAX Eclipse Plus-C18, 2.1 mm × 150 mm, 1.8 µm RRHD 1200 Series bar columns Agilent p/n 959759-902
Column temperature:	60 °C
Injection volume:	35 µL (stacked injection, 5 µL sample + 30 µL H_2O
Autosampler temperature:	6 °C
Needle wash:	Flushport (MeOH:H_2O 75:25 + 0.01% formic acid), 10 s
Mobile phase:	A = H_2O w/5 mM ammonium formate + 0.01% formic acid B = MeOH w/5 mM ammonium formate + 0.01% formic acid
LC flow rate:	0.5 mL/min
LC gradient:	6% B (T = 0) to 98% B (T = 15 min), hold 3 min

MS Parameters

Sheath gas flow:	11 L/min
Sheath gas heater:	375 °C
Charging Electrode:	300 V (pos ion mode)
Capillary voltage:	–4 kV (pos ion mode)
Nebulizer pressure:	35 psig
Drying gas temperature:	325 °C
Drying gas flow:	8 L/min

Ten representative MS/MS Scan Segments from 300-Compound Methods

Compound Name	ISTD?	Prec Ion	MS1 Res	Prod Ion	MS2 Res	Frag (V)	CE (V)	Ret Time
Chloridazon	❑	222	Unit	104	Unit	120	25	5.841
Chloridazon	❑	222	Unit	92	Unit	120	30	5.841
Aminocarb	❑	209.1	Unit	152.1	Unit	120	10	5.841
Aminocarb	❑	209.1	Unit	137	Unit	120	20	5.841
Fluroxypyr	❑	255	Unit	209	Unit	80	10	5.845
Fluroxypyr	❑	255	Unit	181	Unit	80	15	5.845
Acetamiprid	❑	223.1	Unit	126	Unit	80	15	5.858
Acetamiprid	❑	223.1	Unit	56	Unit	80	15	5.858
Vamidothion	❑	288	Unit	146	Unit	80	10	5.996
Vamidothion	❑	288	Unit	118	Unit	80	20	5.996

Appendix V

LC/MS/MS Conditions for Pesticides in Spinach using QuECHERS Extraction

Agilent 1200 Series HPLC conditions

Column:		Agilent ZORBAX Eclipse Plus Phenyl-hexyl, 150 mm × 3 mm, 3.5 µm Agilent p/n 959963-312	
Column temperature:		30 °C	
Injection volume:		10 µL	
Mobile phase:		A = 5 mM ammonium acetate, pH 5.0 in 20:80 MeOH/H_2O B = 5 mM ammonium acetate, pH 5.0 in ACN	
Needle wash:		1:1:1:1 ACN/MeOH/IPA/H_2O w/0.2% FA	
Gradient:	Time (min)		Flow rate
	(min)	% B	(mL/min)
	0	20	0.3
	0.5	20	0.3
	8.0	100	0.3
	10.0	100	0.3
	10.1	20	0.5
	12.0	100	0.5
Stop time:	13.0 min		
Post run:	4 min		
Total cycle time:	17 min		

Agilent 6410 MS conditions

Positive mode	
Gas temperature:	350 °C
Gas flow:	10 L/min
Nebulizer:	40 psi
Capillary:	4000 V

Appendix VI

LC/MS/MS Conditions for 165-Pesticide Methods using the Agilent 1200 Series Infinity SL

Agilent 1200 Series Infinity SL LC Parameters

Column:	Agilent ZORBAX Eclipse Plus C18, 2.1 mm × 100 mm 1.8 µm Agilent p/n 959764-902
Column temperature:	35 °C
Injection volume:	5.0 µL
Autosampler temperature:	6 °C
Needle wash:	Flushport (MeOH:H2O 75:25) 5 s
Mobile phase:	A = H2O w/5mM ammonium formate + 0.01% formic acid B = 5 mM ammonium formate + 0.01% formic acid in methanol

Gradient — Pump Time Table

Time (min)	Solv ratio B (%)
0.00	10
1.00	10
18.00	100
20.00	100
20.10	10
25.00	10

Jet Stream Conditions

Spray Chamber Conditions

Gas temperature:	200 °C
Dry gas :	6 L/min
Nebulizer:	35 psi
Sheath gas temperature:	250 °C
Sheath gas flow:	12 L/min
Positive cap voltage:	4000 V
Nozzle voltage:	300 V

Ten Representative MS/MS Transitions from 167-Compound Methods

Compound Name	ISTD?	Prec Ion	MS1 Res	Prod Ion	MS2 Res	Frag (V)	CE (V)	Ret Time	Ret Window
Ethiofencarb-sulfon	❑	275	Unit	201	Unit	80	0	6.89	1
Ethiofencarb-sulfon	❑	275	Unit	107	Unit	80	10	6.89	1
Clothianidin	❑	250	Unit	169	Unit	90	5	7.064	1
Clothianidin	❑	250	Unit	132	Unit	90	15	7.064	1
Imidacloprid	❑	256.1	Unit	209	Unit	80	15	7.071	1
Imidacloprid	❑	256.1	Unit	175.1	Unit	80	20	7.071	1
Ethiofencarb-sulfoxid	❑	242	Unit	185	Unit	80	15	7.153	1
Ethiofencarb-sulfoxid	❑	242	Unit	107	Unit	80	5	7.153	1
Monalide	❑	257.1	Unit	200.1	Unit	105	4	7.165	1
Monalide	❑	257.1	Unit	137.1	Unit	105	8	7.165	1

Appendix VII

LC/MS/MS Conditions for 224-Pesticide Methods using the Agilent 1200 Series SL

Agilent 1200 Series LC Parameters

Column:	Agilent ZORBAX Eclipse Plus-C18, 2.1 mm × 100 mm, 1.8 µm Agilent p/n 959764-902
Column temperature:	55 °C
Injection volume:	5.0 µL
Autosampler temperature:	6 °C
Needle wash:	Flushport (MeOH:H_2O 75:25), 5 s
Mobile phase:	A = H_2O w/5 mM ammonium formate + 0.01% formic acid B = 5 mM ammonium formate + 0.01% formic acid in 95:5 acetonitrile:water
Flow rate:	0.3 mL/min

Gradient pump time table

Time	Flow	Pressure	Solv ratio B
0.5	No change	600	6
14	No change	600	95
17	No change	600	95

Stop time 17 min
Post time 3 min

Jet Stream Conditions

Drying gas temperature:	225 °C
Drying gas flow (nitrogen):	10 L/min
Nebulizer gas pressure (nitrogen):	25 psig
Capillary voltage:	4500 V
Sheath gas temperature:	350 °C
Sheath gas flow:	11 L/min
Nozzle voltage:	500 V

Ten representative MS/MS Transitions from 224-Compound Methods

Compound Name	ISTD?	Prec Ion	MS1 Res	Prod Ion	MS2 Res	Frag (V)	CE (V)	Ret Time	Ret Window
Buprofezin	❑	306.2	Unit	201.1	Unit	115	4	14.321	1
Buprofezin	❑	306.2	Unit	57.2	Unit	115	16	14.321	1
Sulprofos	❑	323	Unit	247.1	Unit	130	5	14.327	1
Sulprofos	❑	323	Unit	219	Unit	130	12	14.327	1
Eprinomectin B1a	❑	914.6	Unit	468.3	Unit	150	5	14.372	1
Eprinomectin B1a	❑	914.6	Unit	330.3	Unit	150	10	14.372	1
Chlorfluazuron	❑	540	Unit	383	Unit	115	16	14.402	1
Chlorfluazuron	❑	540	Unit	158	Unit	115	16	14.402	1
Fenpyroximat	❑	422.2	Unit	366.2	Unit	130	15	14.428	1
Fenpyroximat	❑	422.2	Unit	135	Unit	130	40	14.428	1

Appendix VIII

LC/MS/MS Conditions for 224-Pesticide Methods using the Agilent 1290 Infinity LC

Agilent 1200 Series LC Parameters

Column:	Agilent ZORBAX Eclipse Plus-C18, 2.1 mm × 100 mm, 1.8 µm Agilent p/n 959764-902
Column temperature:	55 °C
Injection volume:	5.0 µL
Autosampler temperature:	6 °C
Needle wash:	Flushport (MeOH:H_2O 75:25), 5 s
Mobile phase:	A = H_2O w/5mM ammonium formate + 0.01% formic acid B = 5 mM ammonium formate + 0.01% formic acid in 95:5 acetonitrile:water
Flow rate:	0.6 mL/min

Gradient pump time table

Time	Flow	Pressure	Solv ratio B
0.5	No change	600	6
7	No change	600	95
10	No change	600	95

Stop time 10 min
Post time 3 min

Jet Stream Conditions

Drying gas temperature:	225 °C
Drying gas flow (nitrogen):	10 L/min
Nebulizer gas pressure (nitrogen):	25 psig
Capillary voltage:	4500 V
Sheath gas temperature:	350 °C
Sheath gas flow:	11 L/min
Nozzle voltage:	500 V

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Analysis of Pesticide Residues in Apple by GC/MS using Agilent SampliQ QuEChERS Kits for Pre-injection Cleanup

Application Note

Food Safety

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Abstract

The QuEChERS method, which stands for Quick, Easy, Cheap, Effective, Rugged and Safe was developed in 2003 by scientists at the USDA. This method was created to easily clean up and prepare food samples for multi-class, multi-residue pesticide analysis. This application note describes the use of the original, non-buffered QuEChERS method to prepare apple samples for residue analysis by gas chromatography/mass spectrometry (GC/MS). Fifteen pesticides of different classes were studied. The experiments were done using Agilent QuEChERS extraction kits for 10-g samples and dispersive kits for 1-mL sample volumes. The analysis was done by GC/MS using selective ion monitoring (SIM) mode. The limit of quantitation for all the pesticides studied was 10 ng/g in apple using this method. At 200 ng/g, the recoveries ranged from 89% to 102%, and at 10 ng/g, the recoveries ranged from 72% to103%. The relative standard deviations associated with these recoveries were less than 11% in all cases.

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Introduction

Pesticides, which include herbicides, fungicides and other pest-control chemicals, have long been a part of agriculture. While their use can be widespread and beneficial, pesticides can also be harmful to both humans and animals. Because of this, pesticide handling is monitored by several agencies, including the United States Environmental Protection Agency (EPA) [1]. Of concern to the general population is the maximum residue levels (MRL) for pesticide in food items.

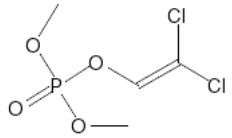
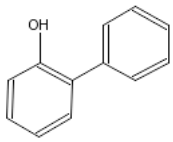
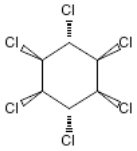
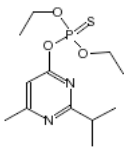
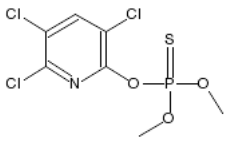
In 2003, scientists at the USDA developed a method for the quick and easy cleanup of food samples for pesticide analysis.[2] This method was given the acronym QuEChERS, which stands for Quick, Easy, Cheap, Effective, Rugged and Safe. The method has since been modified for other analyses. The method for this analysis incorporates a simple acetonitrile/water extraction facilitated by the addition of $MgSO_4$, which salts out water from the sample and includes a liquid/liquid extraction with these two solvents. The extraction step is followed by a dispersive solid phase extraction that combines both a primary secondary amine (PSA) and anhydrous $MgSO_4$ to remove fatty acids and reduce the remaining water in the extract respectively. See the Agilent SampliQ QuEChERs Kit brochure (publication 5990-3562EN) or www.agilent.com/chem/quechers for more information about QuEChERS and suggestions for analyses of different fruits and vegetables.

Experimental

Reagents and Chemicals

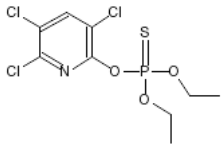
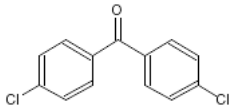
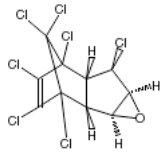
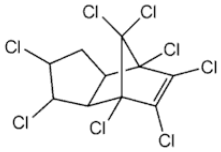
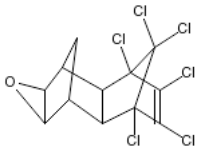
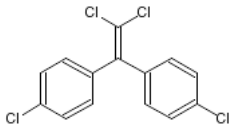
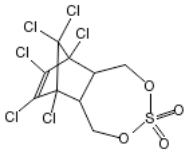
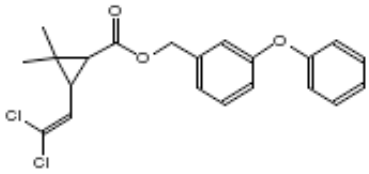
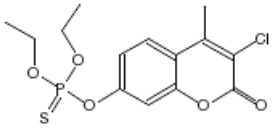
Water (EMD Chemicals, Gibbstown, NJ) and acetonitrile (Burdick and Jackson, Muskegan, MI) were HPLC grade. The pesticides were all analytical grade. Dichlorvos (98.9%), and diazinone (99.5%) were purchased from Ultra Scientific (Kingstown, RI). Coumaphos was purchased from Honeywell Riedel De Haen (Seelze, Germany). All other pesticides were purchased from Chem Service (West Chester, PA). See Table 1 for the chemical and regulatory information for the pesticides used in this study. [3-5] The internal standard, triphenyl phosphate (TPP) was purchased from Sigma-Aldrich (St. Louis, MO).

Table 1. Pesticide Chemical and Regulatory Information.

Analyte	Structure	Category	Log P	pKa	MRLs in apple
Dichlorvos		Organophosphate	1.9	NA	10
σ-phenylphenol		Phenol	3.18	9.4	20
Lindane		Organochlorine	3.69	NA	10
Diazinone		Organophosphate	3.69	2.6	100
Chlorpyrifosmethyl		Organophosphate	4.00	NA	500

(Continued)

Table 1. Pesticide Chemical and Regulatory Information.

Analyte	Structure	Category	Log P	pKa	MRLs in apple
Chlorpyrifos		Organophosphate	4.55	NA	100
dichlorobenzophenone		Organochlorine	4.44	NA	500
Heptachlor-epoxide		Organochlorine	5.83	NA	10
Chlordane		Cyclodiene organochlorine	2.78	NA	20
Dieldrin		Chlorinated hydrocarbon	3.7	NA	10
DDE		organochlorine	6.55	NA	50
Endosulfan sulfate		Organochlorine	3.13	NA	50
Permethrins		Pyrethroid	6.1	NA	50
Coumaphos		Organophosphate	3.86	NA	100

Solutions

Individual stock solutions of the pesticides (2 mg/mL) were prepared in methanol and stored at -20 °C. From these, the High-QC solution which was 20 µg/mL for all the pesticides was prepared in 50:50 acetonitrile/water. The internal standard was 20 µg/mL triphenyl phosphate in acetonitrile.

The High-QC solution was used to prepare all other spiking solutions. A mid range spiking solution (Mid-QC) with a concentration of 5 µg/mL was prepared in 50:50 acetonitrile/water. A low range spiking solution (Low-QC) with a concentration of 1 µg/mL was prepared in 50:50 acetonitrile/water.

Calibration Curve

A 2.5 µg/mL standard working solution was prepared using the High-QC solution. A six-point calibration curve (10, 20, 50, 100, 250 and 400 ng/mL) was created by adding the appropriate volume of this 2.5 µg/mL solution to the matrix blank extract. Internal standard solution was added to have a final concentration of 100 ng/mL.

Sample Preparation

Certified organic, pesticide-free red delicious apples were purchased at a local grocery. Approximately 3 pounds of apples were diced into approximately 1-cm cubes. The seeds were discarded, but the skin was included. The cubes were placed in a plastic bag and frozen at -20 °C overnight. For the first 5 hours in the freezer, the samples were massaged to prevent them from freezing together. When ready to perform the extraction, the amount of sample required was removed from the freezer. A coffee grinder (Mr. Coffee 2.3-oz coffee grinder, Shelton, CT) was used to comminute the sample. If necessary, dry ice may be added to aid this. The sample was checked to ensure that there were no large pieces or lumps remaining prior to extraction. [6].

QuEChERS Cleanup

Step 1, Extraction

The SampliQ Original QuEChERS Method (non-buffered) Extraction Kit, for use with 10g samples (p/n 5982-5550) was used for the extraction step. A 10-g (±0.05g) amount of the homogenized apple sample was placed in a 50-mL centrifuge tube. 100 µL of the appropriate spiking solution was added. The sample was vortexed (VWR vortex mixer model K-550-G, West Chester, PA) for 1 min, then 10 mL of acetonitrile were added. The sample was shaken vigorously for 1 min, then 4 g of $MgSO_4$ and 1 g of NaCl from the extraction salt packet (p/n 5982-5550) were added. The sample was vortexed for 1 min. A 100-µL amount of internal standard solution was added, then the sample was centrifuged (Eppendorf 5810R 15 amp, Westbury, NY) for 5 min at 5000 rpm. See Figure 1.

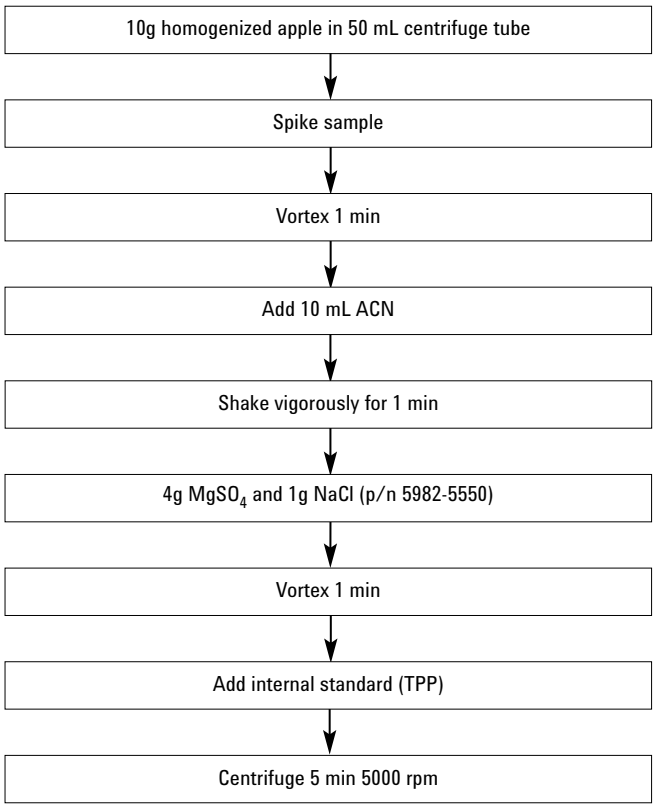


Figure 1. Extraction using SampliQ QuEChERS kit.

Step 2 Dispersive SPE cleanup

The SampliQ QuEChERS Dispersive Kit for General Fruits and Vegetables was used for dispersive SPE cleanup (p/n 5982-5022). This kit removes polar organic acids, some sugars and lipids. One milliliter of the resultant solution was transferred to a 2-mL centrifuge tube containing 50 mg of PSA and 1 50 mg of $MgSO_4$. This was vortexed for 30 sec, then centrifuged for 5 min (VWR micro-centrifuge model 235 B, West Chester, PA). A 0.5-mL amount of the resulting extract was transferred to a sample vial to be analyzed by GC/MS. See Figure 2.

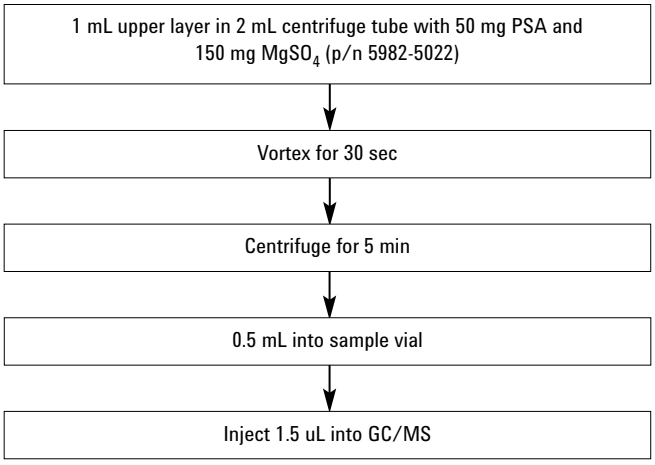


Figure 2. Dispersive SPE using SampliQ QuEChERS kit.

Instrument Conditions

Samples were analyzed using an Agilent 7890A GC system with an Agilent 5975C Series GC/MSD (Agilent Technologies Inc., Santa Clara, CA). An Agilent GC/MS method for pesticide analysis was used with some minor modifications. (7) See Tables 2 and 3 for instrument conditions.

Table 2. GCMS instrument conditions

GC conditions	
Injection source	Manual
Inlet	Splitless
Column	Agilent J&W HP-5ms Ultra Inert, 30 m × 0.250 mm, 0.25 µm film (PN: 190915-433UI)
Carrier Gas	Helium in constant flow mode
Oven Temperature Program	70 °C (2 min) 25 °C/min to 150 °C (0 min) 3 °C/min to 200 °C (0 min) 8 °C/min to 280 °C (7 min)
Injection volume	1 µL
MS Conditions	
Tune File	Atune.u
Mode:	SIM
Source temperature	230 °C
Quad temperature	150 °C
Transfer line temperature	280 °C
Solvent Delay	4.00 minutes
Multiplier Voltage	Autotune voltage

Table 3. Selective Ion Monitoring (SIM) mode conditions.

Peak	Analyte	RT (min)	SIM target	SIM qualifier	Collection window (min)
1	Dichlorvos	6.9	185.00	109.50	4.0–9.0
2	σ-phenylphenol	10.4	170.10	169.10	9.0–14.0
3	Lindane	15.7	180.90	182.90	14.0-16.0
4	Diazinone	16.6	137.10	179.10	16.0–18.0
5	Methyl-chlorpyrifos	18.9	285.90	287.90	18.0–21.0
6	Chlorpyrifos	21.5	196.90	–	21.0–22.0
7	Dichlorobenzophenone	21.5	139.00	–	21.0–22.0
8	Heptachlor-epoxide	23.0	352.90	354.90	22.0–23.6
9	γ-chlordane	24.0	372.90	374.90	23.6–24.3
10	α-chlordane	24.6	372.90	374.90	24.3–25.0
11	Dieldrin	25.4	79.10	–	25.0–27.0
12	DDE	25.5	246.00	317.90	25.0–27.0
13	Endosulfan Sulfate	27.9	271.80	273.80	27.0–28.0
I.S.	TPP	28.6	325.1	326.1	28.0–29.5
14	Permethrin	32.1	183.10	–	29.5–38.0
15	Coumaphos	32.2	96.90	109.00	29.5–38.0

Results and Discussion

As shown in Figure 3b, the apple matrix blank sample had only a few peaks spread across the experimental collection times for the pesticides using the chosen GC/MS method. In the spiked sample (3a), all compounds except coumaphos were free of interferences and gave good linearity as shown in Table 4.

The peak corresponding to coumaphos was difficult to integrate in some samples due to an irregular baseline, which is a possible reason for poor linearity. The QuEChERS method of sample preparation was proven to be quick, easy and effective for this type of analysis. When using the QuEChERS method, samples may still have some impurities that can show up in the chromatograms. In order to achieve the best sensitivity for the analytes of interest, SIM mode was used. Sensitivity for the pesticides was greatly increased by selecting ions corresponding to the analytes of interest to be monitored during different segments of the experiment. In most cases, the highest abundance ion for each analyte was chosen to give the best sensitivity. However, in some cases where selectivity was compromised by this choice, another less abundant ion was used for quantitation. For most of the analytes, a second qualifier ion was also used. The selected ions for each compound and the time segments during which they were monitored are given in Table 3.

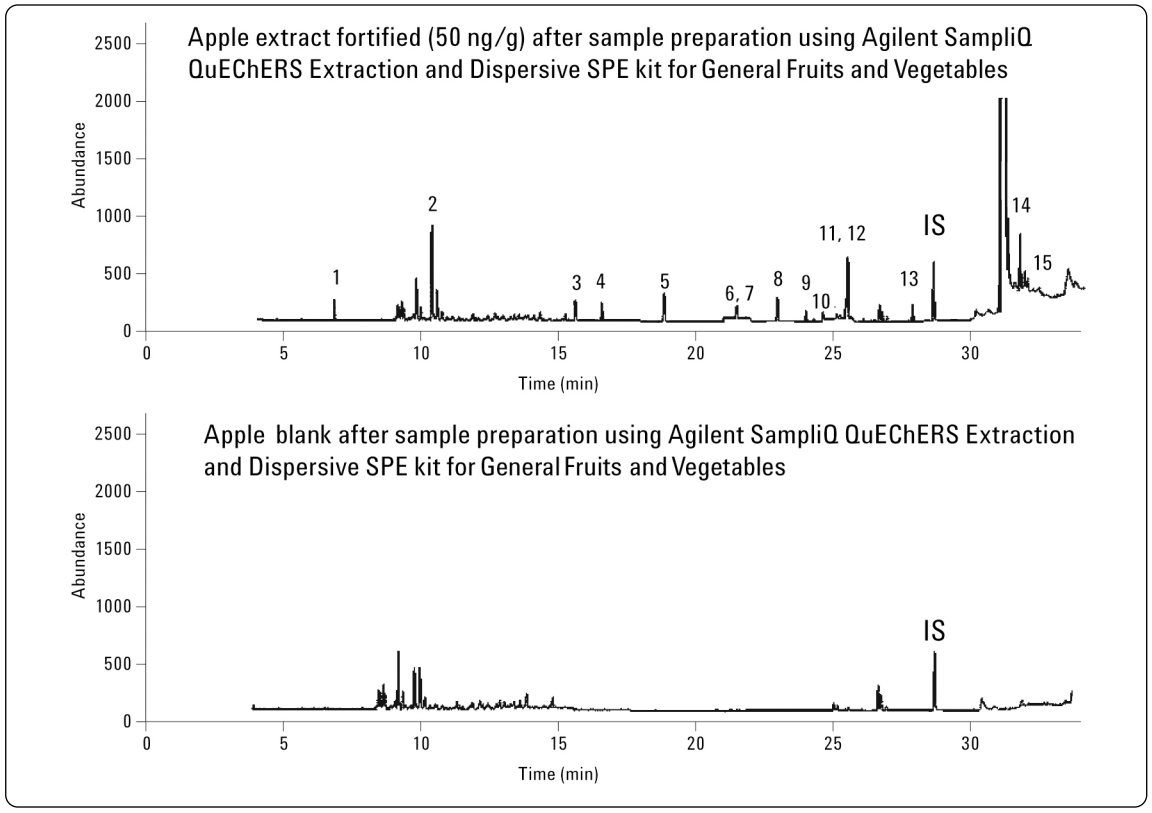


Figure 3. GC/MS chromatograms of apple extract. Peak identifications in Table 3.

Table 4. Regression data for pesticides

Pesticide	Regression Equation	R^2
Dichlorvos	y = 0.1243x - 0.01141	0.9965
σ-phenylphenol	y = 0.6885x - 0.03763	0.9965
Lindane	y = 0.1719x - 0.02280	0.9967
Diazinone	y = 0.1811x - 0.02608	0.9945
Methyl-chlorpyrifos	y = 0.3242x - 0.05026	0.9943
Chlorpyrifos	y = 0.1459x - 0.02455	0.9916
Dichlorobenzophenone	y = 0.1573x - 0.01840	0.9937
Heptachlor-epoxide	y = 0.1995x - 0.02828	0.9906
γ-chlordane	y = 0.07058x - 0.005587	0.9917
α-chlordane	y = 0.05601x - 0.001840	0.9927
Dieldrin	y = 0.2091x - 0.02544	0.9923
DDE	y = 0.4609x - 0.05950	0.9901
Endosulfan Sulfate	y = 0.1262x - 0.01675	0.9897
Permethrin	y = 0.1327x + 0.03232	0.9889
Coumaphos	y = 0.06985x + 0.01864	0.9543

Table 5 shows the recovery and reproducibility for each pesticide in the apple matrix spiked at three different concentrations (200 ng/g, 50 ng/g and 10 ng/g).

Table 5. Recovery and reproducibility of pesticides in apple using the original QuEChERS method (n=4).

Pesticide	High-QC 200ng/g		Mid-QC 50ng/g		Low-QC 10ng/g	
	Recovery	RSD	Recovery	RSD	Recovery	RSD
Dichlorvos	99.4	2.8	96.7	10.8	102.8	5.0
σ-phenylphenol	89.5	6.3	79.6	6.8	92.0	6.1
Lindane	92.6	4.2	88.5	9.7	97.9	2.0
Diazinone	102.1	4.4	98.8	5.5	90.5	9.1
Methyl-chlorpyrifos	98.5	3.1	90.0	4.3	88.7	7.1
Chlorpyrifos	100.2	1.2	95.6	4.0	93.5	6.5
Dichlorobenzophenone	99.4	0.6	89.1	6.4	90.3	5.0
Heptachlor-epoxide	95.4	3.9	85.6	5.4	87.0	3.2
γ-chlordane	95.9	2.0	90.0	6.8	92.3	3.5
α-chlordane	93.5	2.6	85.8	6.9	95.5	4.7
Dieldrin	99.9	1.8	93.6	5.3	99.4	4.2
DDE	92.7	1.9	87.1	5.7	94.5	4.2
Endosulfan Sulfate	99.5	2.3	90.8	2.8	97.8	2.3
Permethrin	97.6	2.1	93.0	3.4	100.7	4.8
Coumaphos	96.6	3.0	79.6	3.5	72.5	4.5

Conclusions

The results show that Agilent SampliQ QuEChERS kits offer an effective method of purification for pesticides in an apple matrix. The impurities remaining after the extraction and dispersive steps were minimal. When used in conjunction with the power of GC/MS in the SIM mode, this method of sample preparation offers a quick, easy and complete solution to quantitate pesticides in fruit matrices.

Acknowledgements

The authors would like to acknowledge Limian Zhao and David Schultz of Agilent Technologies for help with the GC/MS software and in acquiring the pesticide solutions. Also, thank you to Bill Davis and Abby Folk of Agilent Technologies for help with the GC/MS hardware configuration.

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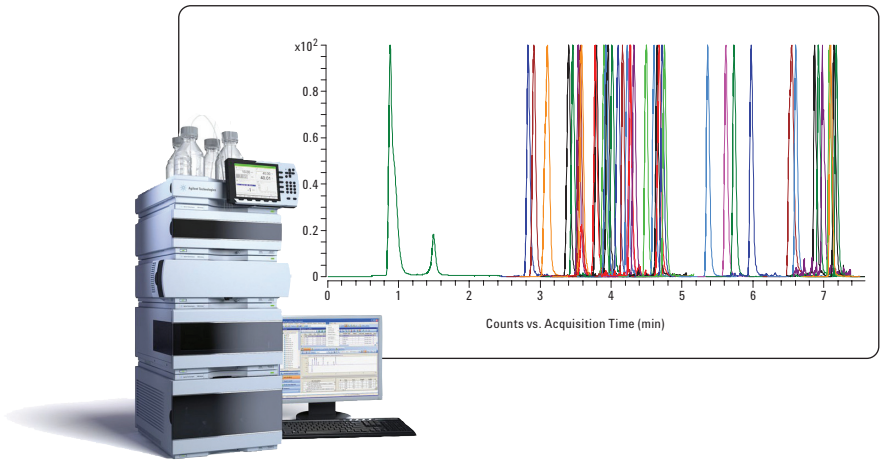
Determination of pesticides in baby food by UHPLC/MS/MS using the Agilent 1290 Infinity LC system and the Agilent 6460 triple quadrupole LC/MS

Application Note

Food

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Abstract

The qualitative and quantitative analysis of pesticides at trace levels in baby food matrices using UHPLC and triple quadrupole MS is demonstrated. Sample preparation is performed using an Agilent SampliQ QuEChERS kit for extraction and dispersive SPE. The extracts are analyzed by LC/MS/MS on an Agilent 1290 Infinity LC system coupled to an Agilent 6460 triple quadrupole LC/MS using Dynamic MRM. The method and extraction performance were evaluated in terms of repeatability, linearity and sensitivity. Moreover the influence of the additional dispersive SPE cleanup was investigated. Detection limits were between 500 ng/kg and 10 ng/kg (ppt), which is much lower than the maximum residue level (MRL) of 10 µg/kg (ppb) imposed by the European Union.

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Introduction

Due to diversity of pesticides used in food protection and the globalization of the food industry, the monitoring of programs that cover a large number of pesticides is important. The application of UHPLC systems combined with the new generation triple quadrupole mass spectrometers facilitate the analysis of pesticides in challenging matrices such as food samples. As a result of the high sensitivity and the high scan rate capabilities of the Agilent 6460A triple quadrupole mass spectrometer, the simultaneous qualitative and quantitative multiresidue analysis of a large set of pesticides at trace levels can be performed.

The high sensitivity is essential for the analysis of these compounds in derived products, where the concentrations will be a fraction of the concentration in the raw material. In this respect, baby food is a challenging matrix. This application notes describes the quantitative analysis of 40 pesticides in baby food at levels below the maximum residue level (MRL) (10 µg/kg fruit or vegetable) specified in EC Regulation 396/2005 which was implemented in September 2008.[1] A QuEChERS extraction and dispersive SPE method was applied to isolate the pesticides from the baby food matrix. An Agilent 1290 Infinity LC was used to perform the separation on a Rapid Resolution High Definition (RRHD) ZORBAX Eclipse Plus column. The total analysis time was 10 min (including 1.5 min re-equilibration) and detection limits ranged from 10 to 500 ng/kg using Dynamic MRM and two transitions (quantifier and qualifier) per compound. Three different baby food compositions were analyzed. Extraction performance criteria such as repeatability, recovery (accuracy) and sensitivity were investigated.

Experimental

Instrumentation

An Agilent 1290 Infinity LC system and an Agilent 6460A triple quadrupole LC/MS with Agilent jet stream technology were used. The 1290 Infinity LC system was configured as follows:

Part number	Description
G4220A	Agilent 1290 Infinity Binary Pump with integrated vacuum degasser
G4226A	Agilent 1290 Infinity Autosampler
G1316C	Agilent 1290 Infinity Thermostatted Column Compartment
G4212A	Agilent 1290 Infinity Diode Array Detector

Method parameters:		
Column	Agilent ZORBAX Eclipse Plus RRHD C18, 150 mm L × 2.1 mm id, 1.8 µm d_p	
Mobile phase	A = 0.05% (w/v) ammonium formate + 0.01% (v/v) formic acid in water B = Methanol	
Flow rate	0.5 mL/min	
Gradient	Min	% B
	0 to 5	10 to 65
	5 to 6.5	65 to 95
	6.5 to 8.5	95
	8.5 to 10	10
Temperature	45 °C	
Injection	2 µL, with needle wash (flushport, 5 s, water/methanol 1/1)	
Detection	MS/MS	
Ionization	Electrospray, positive ionization	
Jet Stream parameters		
Drying gas temperature	250 °C	
Drying gas flow	10 L/min	
Nebulizer pressure	30 psig	
Sheath gas temperature	340 °C	
Sheath gas flow	11 L/min	
Capillary voltage	4500 V	
Nozzle voltage	500 V	
Acquisition		
Dynamic MRM	See Table 1	
Delta EMV	50	
Cycle time	200 ms	

Compound		Precursor ion (*m/z*)	Product ion (*m/z*)	Fragmentor (V)	Collision energy (V)	Retention time (min)	Retention time window (min)
Cyromazine	Q	167.1	85.1	100	25	1.20	1.5
Cyromazine	q	167.1	125.1	100	25	1.20	1.5
Flonicamid	Q	230.1	203.1	80	15	2.85	0.8
Flonicamid	q	230.1	174.1	80	15	2.85	0.8
Thiamethoxam	Q	292.2	211.0	85	4	2.92	0.8
Thiamethoxam	q	292.2	181.0	85	16	2.92	0.8
Monocrotofos	Q	224.1	127.0	85	10	3.11	0.8
Monocrotofos	q	224.1	193.0	85	5	3.11	0.8
Dicrotofos	Q	238.1	112.1	90	5	3.41	0.8
Dicrotofos	q	238.1	127.0	90	15	3.41	0.8
Ethiofencarb-sulfone	Q	258.1	107.1	80	10	3.47	0.8
Ethiofencarb-sulfone	q	258.1	201.1	80	10	3.47	0.8
Imidacloprid	Q	256.1	175.1	90	20	3.55	0.8
Imidacloprid	q	256.1	209.0	90	15	3.55	0.8
Clothianidin	Q	250.0	169.1	90	7	3.58	0.8
Clothianidin	q	250.0	132.1	90	15	3.58	0.8
Ethiofencarb-sulfoxide	Q	242.1	107.1	80	15	3.60	0.8
Ethiofencarb-sulfoxide	q	242.1	185.1	80	15	3.60	0.8
Methiocarb-sulfoxide	Q	242.0	185.1	80	10	3.79	0.8
Methiocarb-sulfoxide	q	242.0	170.0	90	15	3.79	0.8
Thiofanox-sulfone	Q	251.1	57.1	100	15	3.80	0.8
Thiofanox-sulfone	q	251.1	76.1	100	15	3.80	0.8
Trichlorfon	Q	256.9	109.0	100	15	3.92	0.8
Trichlorfon	q	256.9	221.0	100	15	3.92	0.8
Vamidothion	Q	288.1	146.1	80	10	3.94	0.8
Vamidothion	q	288.1	118.1	80	20	3.94	0.8
Acetamiprid	Q	223.1	126.0	100	15	3.94	0.8
Acetamiprid	q	223.1	56.0	100	15	3.94	0.8
Carbofuran-3-OH	Q	238.1	163.1	85	5	3.96	0.8
Carbofuran-3-OH	q	238.1	181.1	85	5	3.96	0.8
Fenthion-oxon-sulfoxide	Q	279.0	104.1	125	30	4.03	0.8
Fenthion-oxon-sulfoxide	q	279.0	121.1	125	30	4.03	0.8
Carbendazim	Q	192.1	160.1	100	15	4.11	0.8
Carbendazim	q	192.1	132.1	100	25	4.11	0.8
Fenthion-oxon-sulfone	Q	295.0	217.1	125	25	4.18	0.8
Fenthion-oxon-sulfone	q	295.0	104.1	125	25	4.18	0.8
Cymoxanil	Q	199.2	128.0	65	5	4.24	0.8
Cymoxanil	q	199.2	111.0	100	20	4.24	0.8
Oxycarboxin	Q	268.1	175.0	100	10	4.27	0.8
Oxycarboxin	q	268.1	146.9	100	25	4.27	0.8
Chlothiamid	Q	205.9	189.0	85	20	4.29	0.8
Chlothiamid	q	205.9	172.0	85	20	4.29	0.8

Table 1
Dynamic MRM data acquisition parameters for the compounds under investigation. Q = quantifier, q = qualifier.

Compound		Precursor ion (*m/z*)	Product ion (*m/z*)	Fragmentor (V)	Collision energy (V)	Retention time (min)	Retention time window (min)
Thiacloprid	Q	253.1	126.0	100	20	4.34	0.8
Thiacloprid	q	253.1	186.0	100	10	4.34	0.8
Florasulam	Q	360.0	129.1	100	20	4.51	0.8
Florasulam	q	360.0	191.9	100	10	4.51	0.8
Tricyclazole	Q	190.1	163.2	100	20	4.62	0.8
Tricyclazole	q	190.1	136.2	100	25	4.62	0.8
Butocarboxim	Q	213.1	75.1	110	15	4.66	0.8
Butocarboxim	q	213.1	156.1	110	5	4.66	0.8
Thiabendazole	Q	202.0	175.0	120	25	4.69	0.8
Thiabendazole	q	202.0	131.0	120	35	4.69	0.8
Aldicarb	Q	208.0	116.0	70	0	4.73	0.8
Aldicarb	q	208.0	89.1	70	5	4.73	0.8
DMSA	Q	201.0	92.1	85	15	4.76	0.8
DMSA	q	201.0	137.1	85	10	4.76	0.8
Propoxur	Q	210.1	111.1	50	10	5.36	0.8
Propoxur	q	210.1	93.0	50	20	5.36	0.8
Carbaryl	Q	202.1	145.1	50	2	5.62	0.8
Carbaryl	q	202.1	127.0	50	20	5.62	0.8
Monolinuron	Q	215.2	126.0	100	20	5.75	0.8
Monolinuron	q	215.2	148.1	100	20	5.75	0.8
Fluazifop	Q	328.1	282.1	120	20	5.99	0.8
Fluazifop	q	328.1	254.1	120	20	5.99	0.8
Spiroxamine	Q	298.4	144.2	100	10	6.54	0.8
Spiroxamine	q	298.4	100.2	100	10	6.54	0.8
Pyrimethanil	Q	200.1	107.1	100	25	6.61	0.8
Pyrimethanil	q	200.1	82.0	100	30	6.61	0.8
Fenhexamid	Q	302.1	97.0	120	10	6.88	0.8
Fenhexamid	q	302.1	142.1	100	5	6.88	0.8
Fenbuconazole	Q	337.2	125.0	120	15	6.94	0.8
Fenbuconazole	q	337.2	194.1	120	15	6.94	0.8
Iprodion	Q	330.0	244.9	110	10	6.98	0.8
Iprodion	q	330.0	287.9	110	5	6.98	0.8
Kresoxim-methyl	Q	314.2	116.0	70	10	7.08	0.8
Kresoxim-methyl	q	314.2	222.0	70	10	7.08	0.8
Penconazole	Q	284.1	69.9	85	15	7.11	0.8
Penconazole	q	284.1	158.8	85	30	7.11	0.8
TPP	Q	327.1	77.0	180	40	7.14	0.8
TPP	q	327.1	151.9	180	40	7.14	0.8
Pyraclostrobin	Q	388.2	194.1	100	10	7.18	0.8
Pyraclostrobin	q	388.2	296.2	100	10	7.18	0.8

Table 1
Dynamic MRM data acquisition parameters for the compounds under investigation. Q = quantifier, q = qualifier. (continued)

Solutions and Samples

Stock solutions of the pesticides were prepared in acetonitrile. These solutions were diluted to the appropriate concentration (range 0.05 ppb to 1 ppm) in 1% v/v acetic acid in acetonitrile. An internal standard solution of triphenylphosphate (TPP, 20 µg/mL) was prepared in the same solvent.

Sample Preparation

Three baby food products were obtained from a local supermarket. According to the labels, the samples were composed of the following ingredients:

- Sample 1: carrots (40%), potatoes (18%), tomatoes (18%), beans (13%), beef (10%)
- Sample 2: water (37%), potatoes (30%), spinach (17%), chicken (10%)
- Sample 3: carrots (54%), potato (23%), water (16%), rice (7%)

The sample preparation was performed using Agilent SampliQ QuEChERS AOAC kits for extraction and dispersive SPE cleanup. The procedure is described below.

Extraction

1. Weigh 15 g of sample into a 50-mL centrifuge tube.
2. Add 100 µL TPP solution.
3. Add spiking solution, if necessary.
4. Vortex for 30 s.
5. Add 15 mL of 1% v/v acetic acid in acetonitrile and the SampliQ AOAC extraction salt (p/n 5982-5755).
6. Cap tubes and shake vigorously by hand for 1 min.
7. Centrifuge at 4,000 rpm for 5 min.
8. Filter 1 mL of sample through a syringe filter (0.2 µm pore size, regenerated cellulose, p/n 5061-3366) and analyze directly (no SPE) or (additional clean-up).

Dispersive SPE

1. Transfer 8 mL from the centrifuged extract into a 15-mL SampliQ AOAC dispersive SPE tube for fatty samples (p/n 5982-5158).
2. Vortex for 30 s.
3. Centrifuge at 13,000 rpm for 2 min.
4. Filter 1 mL through a syringe filter (0.2 µm pore size, regenerated cellulose, p/n 5061-3366) and analyze.

Results and discussion

State-of-the-art LC/MS/MS equipment enables fast multiresidue analysis of pesticides at low levels in complex matrices. The Agilent 1290 Infinity LC provides the necessary power to perform analysis of the 40 selected pesticides within the 10-min total analysis time (run time and equilibration time). A 15 cm column was preferred above a 10 or 5 cm column because of the higher resolving power. This is useful to minimize ion suppression or response enhancement due to matrix effects. Methanol was chosen as an organic modifier because of the significantly improved sensitivity compared to acetonitrile for this analysis.

During the analysis, a total of 82 transitions (2 per solute + 2 for IS) had to be performed. The dynamic MRM function allows MRM transition lists to be built based on a retention time window specified for each analyte. Consequently, the pesticides are only monitored during that elution window in the analytical run. This approach leads to equivalent or better results in terms of sensitivity and quantification (data points) compared to the traditional time segment based methods [2]. With the Dynamic MRM enabled, the maximum number of concurrent MRMs was 32. Using an MRM cycle time of 200 ms, the minimal and maximal transition dwell times were 2.75 and 96.50 ms (values given by MassHunter acquisition software), respectively. The resulting number of data points across the peaks was above 20 for all compounds which is largely sufficient for quantitation purposes.

The performance of the LC/MS/MS method was tested by the analysis of standard solutions. The chromatogram (overlaid MRMs of quantification ions) for a 10 ppb solution is shown in Figure 1. Figures of merit are summarized in Table 2. The injection precision was tested at two concentration levels (1 and 10 ppb). The standard solutions were each injected five consecutive times. The linearity of the method was evaluated between 0.05 and 20 ppb at eight levels (0.05, 0.10, 0.20, 0.50, 1,2,10, and 20 ppb). Each solution was injected once. The lowest level is below the detection limit for some compounds. For these analytes, the calibration curve was started at the limit of detection.

The sensitivity was excellent and all compounds could be analyzed at the sub-ppb level. An example of the ion traces (quantification ion transition and qualifier ion transition) and the corresponding calibration curves for fluazifop

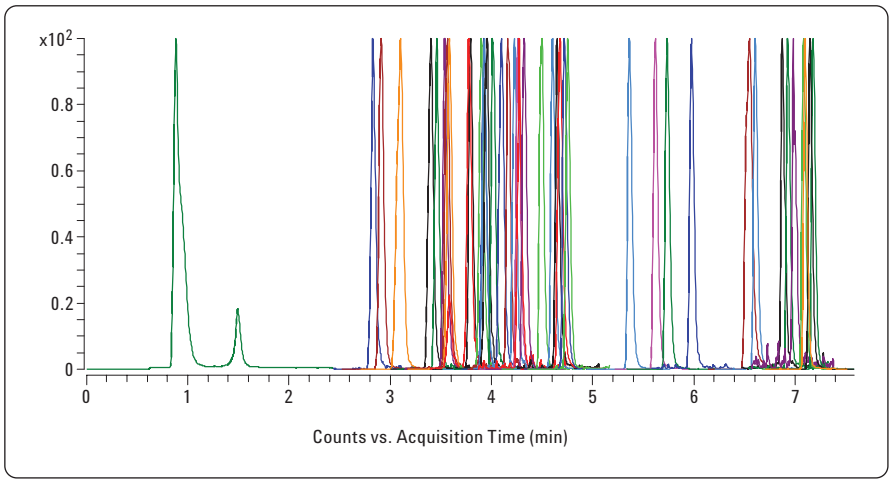


Figure 1
MRM of a 10 ppb standard solution (only quantifier transitions are shown).

Compound	Repeatability of injection (% RSD) 1 ppb	10 ppb	Linearity (R^2)	Detection limit (ppb) Q	q
Acetamiprid	2.20	1.62	0.9999	0.02	0.02
Aldicarb	4.82	2.03	0.9999	0.01	0.02
Butocarboxim	19.93	2.36	0.9996**	0.20	0.50
Carbaryl	1.70	1.73	0.9996	0.01	0.01
Carbendazim	2.93	1.28	0.9997	0.01	0.05
Carbofuran-3-OH	14.68	2.50	0.9996*	0.10	0.10
Chlothiamid	20.64	7.28	0.9979*	0.20	1.00
Clothianidin	7.69	2.14	0.9999*	0.10	0.20
Cymoxanil	7.30	3.88	0.9998*	0.10	0.50
Cyromazine	2.02	1.08	0.9993	<0.50[1]	0.50
Dicrotofos	3.69	1.01	0.9994	0.01	0.02
DMSA	5.13	2.36	0.9996	0.05	0.20
Ethiofencarb-sulfone	2.69	2.25	0.9998	0.05	0.20
Ethiofencarb-sulfoxide	6.24	2.02	0.9991*	0.10	0.10
Fenbuconazole	11.29	1.24	0.9994**	0.20	1.00
Fenhexamid	5.19	4.93	0.9988**	0.20	1.00
Fenthion-oxon-sulfone	13.96	7.10	0.9988	0.05	0.05
Fenthion-oxon-sulfoxide	13.13	2.90	0.9986	0.05	0.10
Flonicamid	10.87	2.55	0.9980	0.05	0.20
Florasulam	9.51	3.25	0.9999	0.05	0.20
Fluazifop	5.77	3.28	0.9998**	0.20	0.50
Imidacloprid	3.31	1.15	0.9998	0.05	0.05
Iprodione	24.53	4.28	0.9984***	0.50	5.00
Kresoxim-methyl	4.46	1.16	0.9999	0.01	0.05
Methiocarb-sulfoxide	4.02	2.85	0.9991*	0.10	0.20
Monocrotofos	1.71	1.45	0.9996	0.01	0.02
Monolinuron	0.67	0.27	0.9999	0.05	0.05
Oxycarboxin	5.92	1.93	0.9991	0.05	0.05
Penconazole	2.02	1.60	0.9997	0.01	0.02
Propoxur	0.70	0.94	0.9998	0.01	0.01
Pyraclostrobin	1.23	0.93	0.9996	0.01	0.02
Pyrimethanil	5.55	0.60	0.9997	0.02	0.05
Spiroxamine	0.91	0.87	0.9997	<0.01	<0.01
Thiabendazole	2.99	0.96	0.9999	0.02	0.02
Thiacloprid	1.57	1.10	0.9995	0.02	0.05
Thiamethoxam	1.38	1.89	0.9998	0.01	0.05
Thiofanox-sulfone	5.13	2.14	0.9998	0.05	0.10
Trichlorfon	6.34	4.31	0.9988	0.05	0.20
Tricyclazole	1.66	0.85	0.9999	0.02	0.02
Vamidothion	4.56	1.16	0.9997	0.01	0.01

* Detection limit is 0.10 ppb, calibration starts at 0.10 ppb.

** Detection limit is 0.20 ppb, calibration starts at 0.20 ppb.

*** Detection limit is 0.50 ppb, calibration starts at 0.50 ppb.

1 High due to interference of a system peak.

Table 2
Method performance results.

(a compound with relatively low sensitivity) and for propoxur (a compound with relatively good sensitivity) are shown in Figures 2 and 3, respectively. Most of the compounds have detection limits below 0.05 ppb. The sensitivity for spiroxamine is below the lowest level injected (0.01 ppb) which is significantly better compared to the other pesticides. No accurate detection limit could be determined for cyromazine due to a system peak that interfered at low levels.

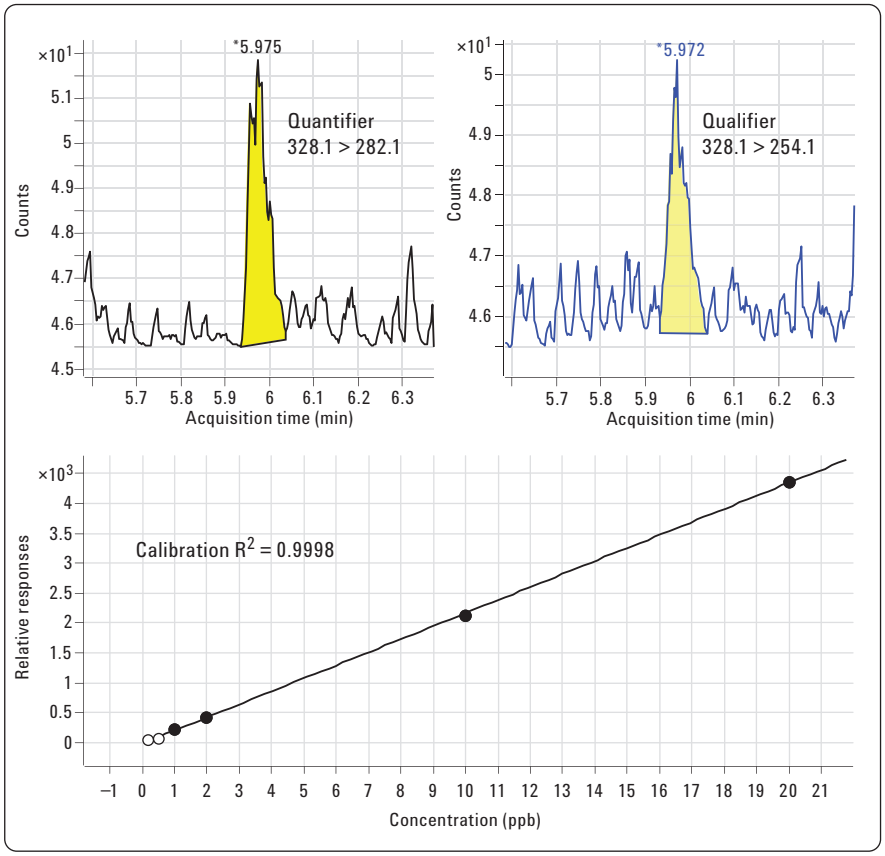


Figure 2
Ion traces for two transitions at the LOD (0.5 ppb standard solution) and calibration curve for fluazifop.

The QuEChERS sample preparation procedure was applied to three baby food samples. Extracts were analyzed with and without additional dispersive SPE cleanup. There were no target compounds detected above the LOD in non-spiked samples. The resulting chromatogram, shown as an overlay of quantification transitions for a sample spiked at 1-ppb level with all 40 pesticides, is depicted in Figure 4. The signals for the quantifier and qualifier transitions for fluazifop and propoxur in the spiked sample at 1-ppb level are shown in Figure 5. From these traces it is clear that excellent selectivity and sensitivity are obtained. The relative response of the quantification transition and qualifier transition are clearly within the limits for positive identification.

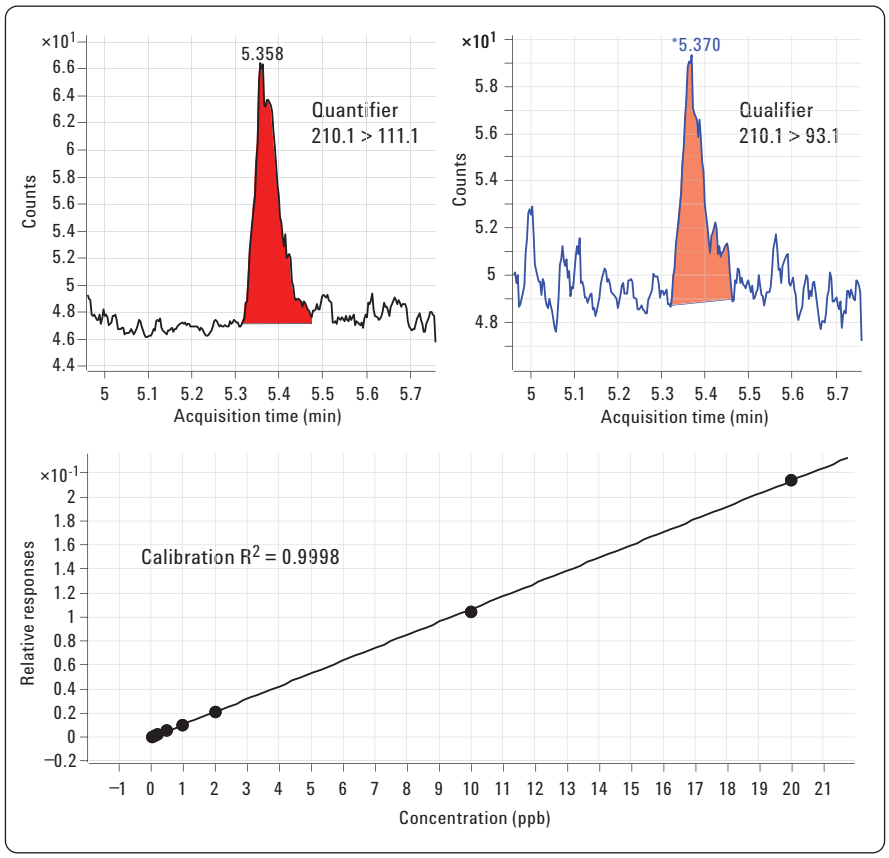


Figure 3
Ion traces for two transitions at the LOD (0.01 ppb standard solution) and calibration curve for propoxur.

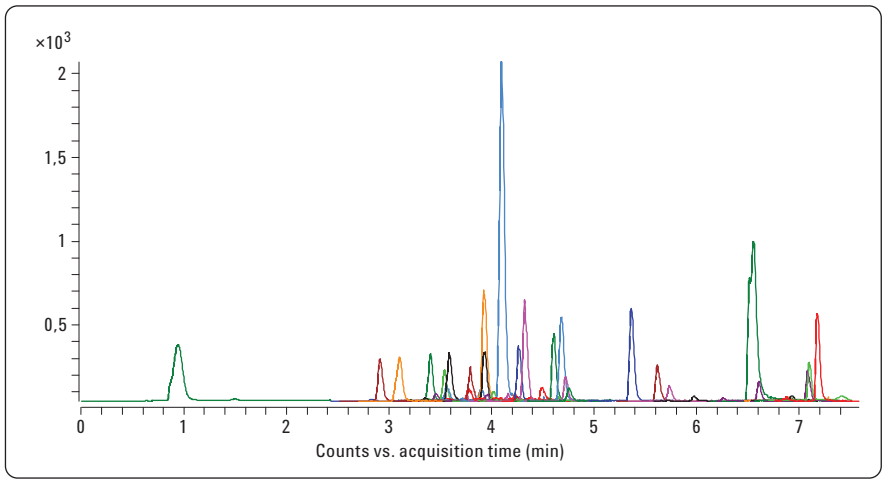


Figure 4
MRM of an extract of sample 2 spiked with 1 ppb (only quantifier transitions are shown). No dispersive SPE performed on the sample. The transition for the internal standard is not shown.

The performance criteria of the sample preparation and analysis method are summarized in Table 3. The extraction repeatability is calculated on sample 1, spiked at the 10-ppb level and repeated (extraction + analysis) five times. Most RSDs are below 10%, with the exception of iprodione and fluazifop, where higher values are obtained after SPE. The average recovery (response spike sample / response calibration sample) for the three different samples was between 70% and 110% at 1 and 10 ppb spike level in most cases. No significant differences were observed between the different matrices. The recovery is satisfactory even at the 1-ppb level and in most cases there is no significant difference between extracts that have been subjected to the additional SPE procedure and those that have not. For cyromazine, better values are obtained after SPE. For fluazifop, on the other hand, very low recoveries (and high RSD) are obtained when additional dispersive SPE is used. In this case, analysis without additional SPE is recommended.

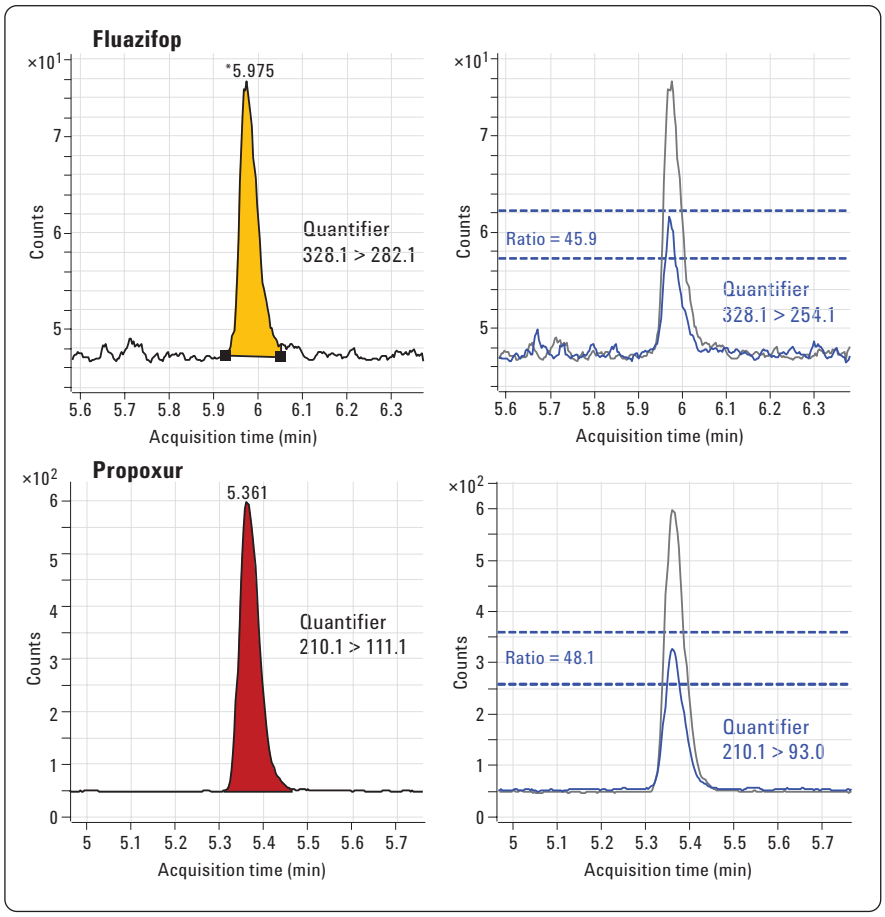


Figure 5
Ion traces for 2 transitions for fluazifop and propoxur in an extract of sample 2 spiked with 1 ppb. No dispersive SPE performed on the sample. The uncertainty was set at 20% (dotted lines).

	Repeatability of extraction (% RSD)1		Average recovery (%)2				Lowest level detected in extract (ppb)3
			1 ppb		10 ppb		
Compound	No IS SPE	No IS No SPE	SPE	No SPE	SPE	No SPE	
Acetamiprid	1.50	1.55	107.0	83.4	99.3	92.2	0.10
Aldicarb	1.74	1.69	91.9	82.8	92.4	81.8	0.10
Butocarboxim	4.41	4.17	95.4	79.6	91.8	86.9	1.00
Carbaryl	1.84	1.30	92.3	75.4	93.2	80.1	0.10
Carbendazim	1.87	1.23	88.6	79.4	90.5	78.1	0.10
Carbofuran-3-OH	6.13	4.62	85.8	114.4	100.5	98.2	1.00
Chlothiamid	4.59	9.54	87.5	105.1	91.8	70.1	1.00
Clothianidin	3.01	1.70	87.0	117.7	103.7	103.2	1.00
Cymoxanil	5.27	5.11	101.5	72.1	99.0	98.1	1.00
Cyromazine	1.70	0.54	108.7	87.1	73.0	57.8	<10.00[4]
Dicrotofos	2.73	1.98	102.9	83.0	93.5	83.5	0.10
DMSA	2.13	1.73	96.3	109.9	103.0	108.1	0.10
Ethiofencarb-sulfone	1.52	2.96	92.1	85.6	91.8	84.2	1.00
Ethiofencarb-sulfoxide	2.33	0.74	90.8	88.9	92.1	83.2	0.10
Fenbuconazole	3.77	6.28	107.5	80.6	90.8	99.8	1.00
Fenhexamid	5.22	7.29	74.0	100.8	91.5	83.5	1.00
Fenthion-oxon-sulfone	6.08	4.49	91.4	78.4	89.8	89.8	1.00
Fenthion-oxon-sulfoxide	2.63	0.90	122.8	96.5	100.8	90.0	0.10
Flonicamid	2.77	3.02	94.4	86.1	94.3	91.8	0.10
Florasulam	5.98	3.76	72.2	103.3	73.8	115.9	0.10
Fluazifop	20.71	1.45	14.4	117.7	18.8	92.0	1.00
Imidacloprid	2.98	2.35	115.2	114.9	111.5	117.6	0.10
Iprodion	14.30	4.37	87.0	90.8	89.8	91.0	1.00
Kresoxim-methyl	4.16	3.82	74.0	80.6	71.8	80.7	0.10
Methiocarb-sulfoxide	3.06	1.63	94.5	98.5	93.1	87.1	0.10
Monocrotofos	1.54	0.72	90.2	81.6	90.9	83.1	0.10
Monolinuron	1.66	0.71	90.0	80.9	92.9	84.3	0.10
Oxycarboxin	2.04	1.74	89.8	107.4	101.0	105.6	0.10
Penconazole	4.25	2.77	73.4	78.2	76.9	83.6	0.10
Propoxur	1.61	0.25	94.7	83.0	95.6	84.9	0.10
Pyraclostrobin	3.62	4.59	86.1	89.3	84.8	90.9	0.10
Pyrimethanil	1.99	2.22	85.5	84.4	86.8	78.3	0.10
Spiroxamine	3.91	1.50	79.6	91.7	78.4	85.8	0.10
Thiabendazole	1.29	1.52	92.7	74.5	91.1	78.7	0.10
Thiacloprid	2.51	1.74	96.7	90.7	94.3	86.4	0.10
Thiamethoxam	2.09	1.12	104.8	108.5	112.4	108.7	0.10
Thiofanox-sulfone	2.70	1.48	99.0	93.3	91.5	84.1	0.10
Trichlorfon	6.94	1.93	86.9	86.5	99.7	92.4	1.00
Tricyclazole	0.90	1.58	91.3	72.0	90.9	75.6	0.10
Vamidothion	1.75	2.63	92.4	79.2	90.0	81.8	0.10

1 Sample 1, spiked with 10 ppb, extracted 5 times. 1 injection per extract.

2 Average of samples 1 to 3, spiked at 1 ppb and at 10 ppb and extracted once. 1 injection per extract.

3 Samples were spiked at 0.1, 1, and 10 ppb level. Lowest detected level is reported.

4 High due to interference of a system peak.

Table 3
Extraction performance.

Conclusion

The multiresidue LC/MS/MS method enabled the analysis of 40 pesticides at low levels in baby food. Sample preparation was performed using an Agilent SampliQ QuEChERS AOAC kit. The total analysis time using the Agilent 1290 Infinity LC system and the Agilent 6460A triple quadrupole LC/MS was 10 min. All compounds could be detected at 0.5 µg/kg or lower in the samples, which is 20 times lower than the MRL for these compounds in baby food according to EU regulation. The extraction repeatability and recovery were good. No difference on extraction and analytical performance due to differences in sample matrix were observed. The optional dispersive SPE cleanup procedure can be applied but for some solutes larger standard deviation and lower recoveries were observed after SPE.

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PBDE Analysis Using an Agilent J&W DB-5ms Ultra Inert GC Column

Application Brief

Environmental

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USA

Introduction

Polybrominated diphenyl ether (PBDE) analyses are important tools for understanding food supply and environmental quality worldwide. In this application, trace-level PBDE analysis is demonstrated using electron impact GC–MS. For these challenging separations, knowing that each GC column has been thoroughly tested for column inertness gives the analyst higher confidence in the accuracy of the results.

Agilent has implemented new testing procedures to more effectively evaluate GC column inertness performance. These new testing procedures employ deliberately aggressive probes to thoroughly investigate column inertness and quality. These extremely active probes, including 1-propionic acid, 4-picoline and trimethyl phosphate, are used to verify each column's inertness performance. Capillary GC column activity as a potential source of result uncertainty has been virtually eliminated with the Ultra Inert series of columns.

PBDE Analyses

PBDE-209 is a particularly challenging analyte because of its long retention and tendency to degrade with high-temperature exposure. High-temperature thermal stability is an issue for this class of compounds, but is more pronounced for BDE-209, as it is highly brominated and well retained. One key to successful BDE analysis is to limit the time that these compounds are exposed to high temperatures. A 15-m long column, as opposed to a typical 30-m long column was used in this instance to limit residence time for BDE-209. Fortunately, the BDEs resolve well, with symmetrical peak shapes, when using Agilent J&W DB-5ms ultra inert phase, enabling successful separation on the shorter column. Figure 1 shows a total ion chromatogram of the eight BDEs investigated in this study.

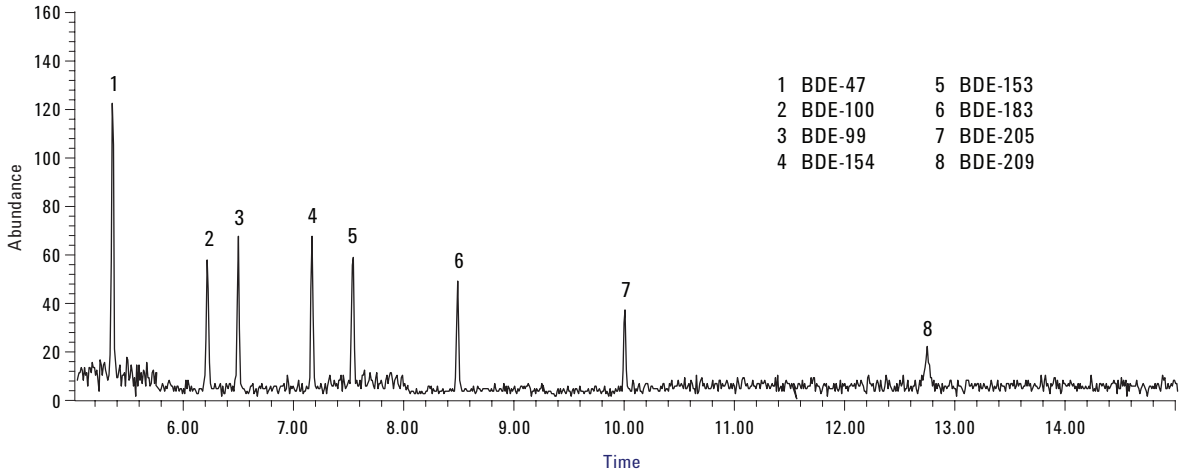


Figure 1. Total ion chromatogram (SIM mode) of a 0.005 ng (BDEs -47, -100, -99, -154, -153, -183 and -205) and 0.025 ng (BDE-209) on-column loading on an Agilent J&W DB-5ms Ultra Inert 15 m x 0.25 mm, 0.25 µm capillary GC column (p/n 122-5512UI).

Table 1 Chromatographic Conditions

GC	Agilent 6890N/5973B MSD
Sampler	Agilent 7683B, 5.0 µL syringe (Agilent p/n 5188-5246), 1.0 µL splitless injection, 5 ng each component on column
Carrier	Helium 72 cm/s, constant flow
Inlet	Pulsed splitless; 325 °C, 20 psi until 1.5 min, purge flow 50 mL/min at 2.0 min
Inlet liner	Deactivated dual taper direct connect (Agilent p/n G1544-80700)
Column	Agilent J&W DB-5ms Ultra Inert 15 m × 0.25 mm, 0.25 µm (Agilent p/n 122-5512UI)
Oven	150 to 325 °C (17 °C/min), hold 5 min
Detection	MSD source at 300 °C, quadrupole at 150 °C, transfer line at 300 °C, scan range 200–1000 amu

This application successfully demonstrates the use of a 15 m Agilent J&W DB-5ms Ultra Inert capillary GC column for trace-level BDEs in a 15-min analysis. Linearity was excellent for all eight BDEs studied, yielding 0.997 or greater R^2 values down to a 0.005 ng (0.025 ng for BDE-209) on-column loading of each component. One of the reasons for the excellent linearity and high R^2 values is the highly inert surface of the column. The lack of chemically active sites makes these columns an excellent choice for trace-level applications.

The Agilent 6890/5975B GC/MSD (SIM mode) equipped with an inert electron impact source had excellent sensitivity with even the most challenging BDE in this set, BDE-209. The signal-to-noise ratio for a 0.025 ng on-column loading of BDE-209 was greater than three to one with this system. This result shows clearly the power of using an Agilent J&W DB-5ms Ultra Inert capillary GC column for trace-level BDE analysis. Lower limits of quantification are expected when using one of Agilent's latest GC/MS offerings, such as the 6890N/5975C GC/MSD Triple-Axis Detector coupled with an Agilent J&W DB-5ms Ultra Inert GC capillary column.

Access the full application note at:
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PAH Analysis Using an Agilent J&W DB-5ms Ultra Inert Capillary GC Column

Application Brief

Environmental

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Introduction

Trace- and ultra trace-level polycyclic aromatic hydrocarbon (PAH) analyses are important tools for accessing environmental quality and foodstuff purity worldwide. In this application, trace-level PAH analyses are demonstrated using electron impact single quadrupole scanning mass spectrometry. In these challenging separations, knowing that the GC column has been thoroughly investigated for column inertness gives the analyst higher confidence in the accuracy of the results.

Agilent has implemented a new testing procedure to more effectively evaluate GC column inertness performance. This new testing procedure employs deliberately aggressive probes to thoroughly investigate column inertness and quality. These aggressive probes, including 1-propionic acid, 4-picoline and trimethyl phosphate, are used to verify each column's inertness performance. This is a rigorous approach that establishes consistent baseline inertness profiles for each column in the Agilent J&W Ultra Inert GC column series.

PAH Analysis

In this application, a 16-component PAH standard mixture was evaluated over a concentration range of 0.05 µg/mL to 5 µg/mL on an Agilent J&W DB-5ms Ultra Inert 30 m × 0.25 mm, 0.25 µm capillary GC column (p/n 122-5532UI). Excellent sensitivity was observed for each of these components across the range studied. Good resolution was obtained in a 15-min analysis for each of the PAHs, with the exception of indeno [1,2,3-c,d]pyrene and dibenz[a,h]anthrancene, which were only partially resolved. Figure 1 shows the total ion chromatogram for a standard injection at the 0.5 µg/mL level; GC conditions are listed in Figure 1.

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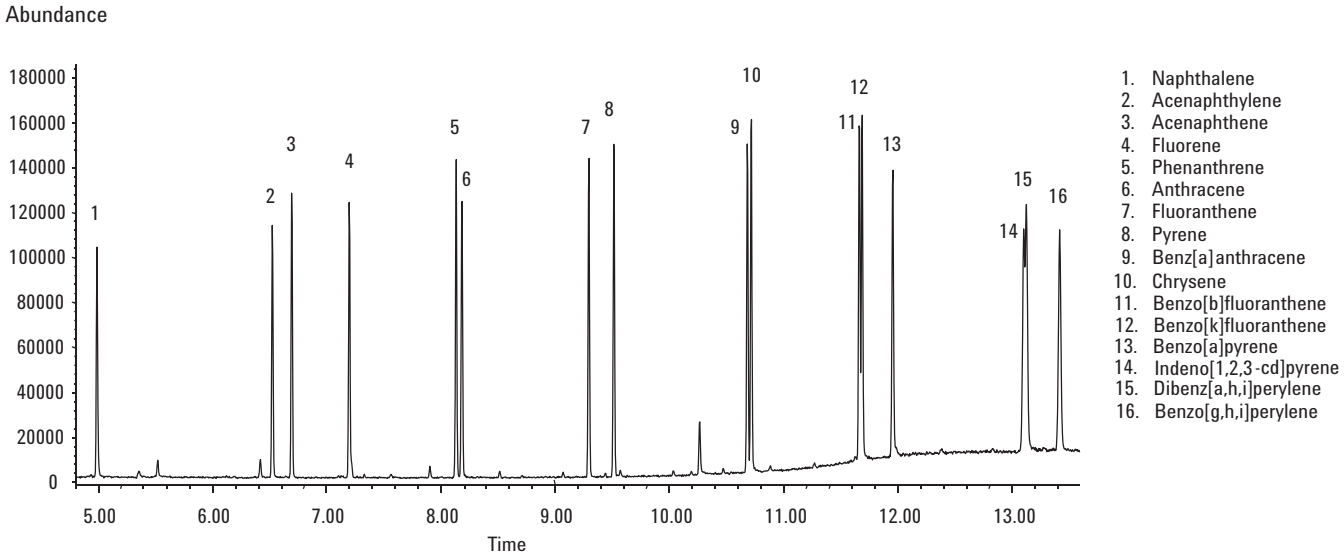


Figure 1. Total ion chromatogram (scanning mode) of a 1 µL injection of the 0.5 µg/mL standard solution on an Agilent J&W DB-5ms Ultra Inert 30 m × 0.25 mm, 0.25 µm capillary GC column (p/n 122-5532UI). This injection represents an on-column loading of 0.5 ng per component.

Table1. Chromatographic Conditions

GC	Agilent 6890N/5973B MSD
Sampler	Agilent 7683B, 5.0 µL syringe (Agilent p/n 5188-5246), 1.0 µL splitless injection, 5 ng each component on column
Carrier	Helium 45 cm/s, constant flow
Inlet	Pulsed splitless; 300 °C, 40 psi until 0.2 min, purge flow 30 mL/min at 0.75 min
Inlet liner	Deactivated dual taper direct connect (Agilent p/n G1544-80700)
Column	Agilent J&W DB-5ms Ultra Inert 30 m × 0.25 mm, 0.25 µm (Agilent p/n 122-5532UI)
Oven	55 °C (1 min) to 320 °C (25 °C/min), hold 3 min
Detection	MSD source at 300 °C, quadrupole at 180 °C, transfer line at 280 °C, scan range 45 to 450 AMU

This application successfully demonstrates the use of an Agilent J&W DB-5ms Ultra Inert capillary GC column for trace-level PAHs in a 15 minute analysis. Linearity was excellent for all 16 PAHs studied, yielding 0.995 or greater R^2 values down to a 0.05 ng column loading of each component. One of the reasons for excellent linearity and high R^2 values is the highly inert surface of the column. The lack of chemically active sites makes these columns excellent choices for trace-level applications.

This study was done exclusively using scanning mode on an Agilent 6890N/5975B GC/MSD equipped with an inert electron impact source. The signal-to-noise ratio for a 0.05 ng on-column loading of benzo[a]pyrene was greater than 9 to 1 with this system. This result clearly shows the power of using an Agilent J&W DB-5ms Ultra Inert column for trace-level PAH analysis.

Access the full application note at:
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Analysis of Low-level Polycyclic Aromatic Hydrocarbons (PAHs) in Rubber and Plastic Articles Using Agilent J&W DB-EUPAH GC column

Application Note

Gas Chromatography/Mass Spectrometry

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Abstract

Agilent J&W DB-EUPAH GC columns are designed for the analysis of EU-regulated priority PAHs. This application demonstrates a GC/MS method for the determination of 20 PAHs including 16 EPA-regulated priority PAHs and four commonly monitored PAHs including benzo(j)fluoranthene, benzo[e]pyrene, 2-methylnaphthalene and 1-methylnaphthalene in rubber and plastics using this type of column. To ensure the accuracy of results, the quantitation was performed with internal standardization using five isotopically-labeled PAHs including naphthalene-d8, acenaphthene-d10, phenanthrene-d10, chrysene-d12 and perylene-d12 along with p-terphenyl-d14 as the surrogate standard. All 26 compounds were separated well with the DB-EUPAH column. The resulting good linearity and sample recovery demonstrate the high selectivity of the described method in this application note for trace-level detection and confirmation of the targeted PAHs in complex sample matrices.

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Introduction

Polycyclic aromatic hydrocarbons (PAHs), containing two to eight aromatic rings [1], are identified as some of the most persistent organic pollutants (POPs). Due to their well known carcinogenic and mutagenic properties, some PAHs are classified as priority pollutants by both the U.S. EPA and the European Commission. The U.S. EPA designated 16 PAH compounds as priority pollutants, including naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, dibenz[a,h]anthracene, benzo[g,h,i]perylene, and indeno[1,2,3-cd]pyrene. The 16 EPA priority PAHs are often targeted for measurement in environmental samples.

PAHs may be present in oil, coal, rubber, and plastics. Some rubber products such as tires are produced using extender oils that may unintentionally contain various levels of PAHs. These extender oils along with the PAHs are incorporated into the rubber matrix and remain locked in the final products. In 2005, the European Commission adopted a Directive [2] restricting the marketing and use of certain PAHs in extender oils for tire production. All tires produced after 1 January 2010 are required to comply with the new Directive.

In the Directive, extender oils may not be used for the production of tires if they contain more than 1 mg/kg benzo(a)pyrene (BaP), or more than 10 mg/kg of the sum of the eight PAHs of concern including benzo(a)pyrene (BaP), benzo(e)pyren (BeP), benzo(a)anthracene (BaA), chrysene (CHR), benzo(b)fluoranthene (BbFA), benzo(j)fluoranthene (BjFA), benzo(k)fluoranthene (BkFA) and dibenzo(a,h)anthracene (DBAhA). BeP and BjFA were not included in 16 EPA-regulated priority PAHs.

Five percent phenyl methylpolysiloxane stationary phase was the most commonly-cited GC column for the analysis of the 16 EPA-regulated PAHs, [3,4]. The resolution of the Benzo(b,k,j)fluoranthenes isomers were not easily obtained using this GC column.

In this application note, a DB-EUPAH column was chosen to provide the necessary separation for all 20 PAHs of interest in rubber and plastic articles. This includes the resolution of all the critical isomers such as the benzo(b,k.j)fluoranthenes. With its exceptional thermal stability, low column bleed at elevated temperatures, and consistent column inertness, the Agilent J&W DB-EUPAH column delivers fast, reliable results while meeting demanding regulatory requirements.

Experimental

The experiments were performed on one Agilent 7890 gas chromatograph equipped with an Agilent 5975C series GC/MSD, and an Agilent 7683 Automatic Liquid Sampler (ALS). The instrument conditions are listed in Table 1.

Chemicals and Standards

All standards in the experiment were purchased from Sigma-Aldrich (St. Louis, MO, USA). The CAS numbers and nominal molecular mass are provided in Table 2.

Surrogate Spiking Solution

p-Terphenyl-d14 was used as a surrogate standard in this experiment. The surrogate spiking solution was prepared from aliquots of pure compound diluted with toluene to a concentration of 10 µg/mL, and served as a stock solution. Surrogate solution was added to all samples and all quality control samples prior to extraction.

Table 1. Gas Chromatograph and Mass Spectrometer Conditions

GC Conditions	
Column:	Agilent J&W DB-EUPAH, 20 m × 0.18 mm × 0.14 µm (Agilent p/n 121-9627）
Inlet temperature:	290 °C
Carrier gas:	Helium, constant flow mode, 52 cm/s
Injection mode:	Splitless, purge flow 50 mL/min at 0.75 min
Injection volume:	1 µL
Oven:	120 °C (1 min), 8 °C/min to 200 °C (0.5 min), 11 °C/min to 270 °C, 2 °C/min to 300 °C,
Post run:	320 °C (4 min)
MS Conditions	
Solvent delay:	2.8 min
MS temp:	250 °C (Source); 180 °C (Quad)
Transfer line:	290 °C
MS:	EI, SIM/Scan
Scan mode:	Mass range (50-450 amu)
For other parameters, see Table 2	
Miscellaneous Parts	
Septa:	Long-lifetime septa (Agilent p/n 5183-4761)
Liner:	Splitless deactivated dual taper direct connect liner (Agilent p/n G1544-80700).
Syringe	5 µL syringe (Agilent p/n 5181-1273)

Table 2. Polycyclic Aromatic Hydrocarbons, CAS Number, Nominal Molecular Mass and Corresponding Ions

Compound	CAS No.	Nominal molecular mass	Corresponding Ions
Naphthalene-d8*	1146-65-2	136	**136**, 108, 68
Acenaphthene-d10*	15067-26-2	164	**164**, 160
Phenanthrene-d10*	1719-06-8	188	**188**
Chrysene-d12*	1719-03-5	240	**240**, 236, 120
p-Terphenyl-d14**	1718-51-0	244	**244**, 122 ,212
Perylene-d12*	1520-96-3	264	**264**, 265, 260
Naphthalene	91-20-3	128	**128**, 127, 129
2-methylnaphthalene	91-57-6	142	**142**, 141, 115
1-methylnaphthalene	90-12-0	142	**142**, 141, 115
Acenaphthylene	208-96-8	152	**152**, 153, 151
Acenaphthene	83-32-9	154	**153**, 154, 152
Fluorene	86-73-7	166	**166**, 165, 167
Phenanthrene	85-01-8	178	**178**, 176, 179
Anthracene	120-12-7	178	**178**, 176, 179
Fluoranthene	206-44-0	202	**202**, 200, 101
Pyrene	129-00-0	202	**202**, 200, 101
Benzo(a)anthracene	56-55-3	228	**228**, 226, 229
Chrysene	218-01-9	228	**228**, 226, 229
Benzo(b)fluoranthene	505-99-2	252	**252**, 253, 126
Benzo(j)fluoranthene	205-82-3	252	**252**, 253, 126
Benzo(k)fluoranthene	207-08-9	252	**252**, 253, 126
Benzo(e)pyrene	192-97-2	252	**252**, 253, 126
Benzo(a)pyrene	50-32-8	252	**252**, 253, 126
Indeno(1,2,3-cd)pyrene	193-39-5	276	**276**, 138, 277
Dibenzo(a,h)anthracen	53-70-3	278	**278**, 139, 279
Benzo(g,h,i)perylene	191-24-2	276	**276**, 138, 277

1. * Internal standard
2. ** surrogate standard
3. Suggested quantitative ions are in bold.

Internal Standard Solution

The internal standard solution included naphthalene-d8, acenaphthlene-d10, phenanthrene-d10, chrysene-d12 and perylene-d12. The internal standard solution was made from aliquots of pure compounds, diluted with toluene to a concentration of 10 µg/mL, and served as a stock solution.

Calibration Solution

Calibrations solutions were prepared in five different concentrations ranging from 5 to 500 ppb by diluting commercially available certified solutions containing analytes of interest. Each standard solution contained 500 µg/L of internal standards (ISTDs).

Sample Preparation

According to ZLS standard ZEK 01.2-08 to prepare samples, rubber and plastic articles were cut into pieces no larger than 2–3 mm in size. Five hundred milligrams of cut pieces were extracted by 20 mL of toluene and mixed with internal standards for 1 hour in the ultrasonic bath at a temperature of 60 °C. After cooling to room temperature, an aliquot was taken from the extract for analysis.

One rubber sample and one plastic sample spiked with PAHs of interest at the 100 ppb level respectively were treated according to the procedure described above.

Results and Discussion

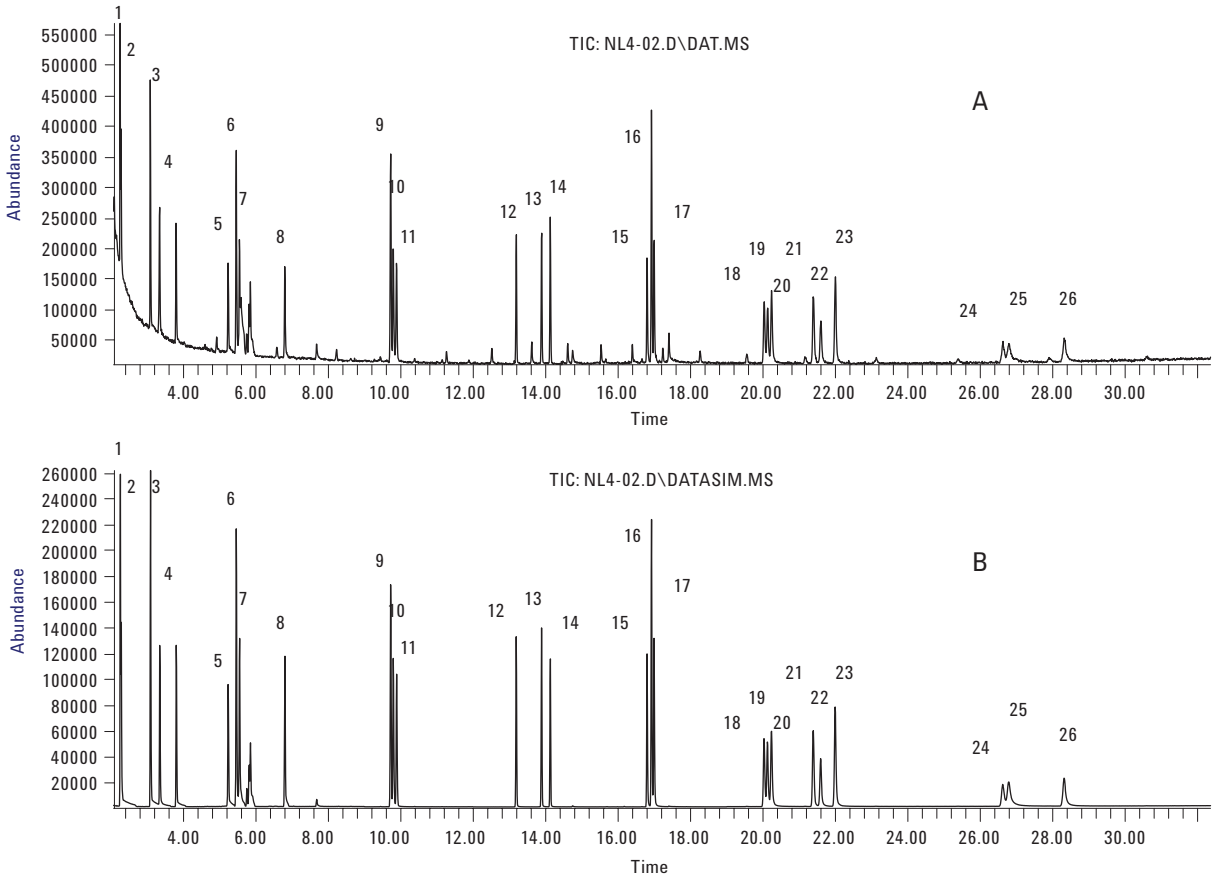
Figure 1 shows the GC/MS total ion chromatograms for the compounds of interest. Due to the presence of isomers, some compounds listed in Table 2 with the same quantitation ions could be eluting quite closely. Therefore good resolution is very important for these isomers to achieve accurate quantitative results. As shown in Figure 1, all 20 targeted PAH compounds were well-resolved with the DB-EUPAH column.

Figure 2 exhibits the baseline resolution of some critical PAH pairs of interest, including phenanthrene and anthracene (*m/z* 178), fluoranthene and pyrene (*m/z* 202), benzo[a]anthracene and chrysene (*m/z* 228), with excellent peak shapes using the DB-EUPAH column.

Benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(j)fluoranthene, benzo(e)pyrene and benzo(a)pyrene are isomers with the same quantitation ion (*m/z* 252). According to 2005/ 69/ EC Directive, the content of benzo(a)pyrene was restricted to be less than 1 mg/kg. Figure 2 shows that benzo(a)pyrene can be separated well, and the resolution of benzo(a)pyrene and benzo(e)pyrene is 2.82 with the DB-EUPAH column. Benzo(b, k,j)fluoranthenes are difficult-to-chromatograph isomers. Benzo(b)fluoranthene (BbFA) and benzo(j)fluoranthene (BjFA) often show coelution on the commonly used 5% phenyl methylpolysiloxane stationary phase GC column. The resolution of benzo(b,j,k)fluoranthene is 1.41

and 1.63 respectively, meeting the requirement of analysis. The three benzo(b,k,j)fluoranthene isomers could be baseline separated, however, with the compromise of a extra 10-min run time. Therefore, it is a trade-off between speed and resolution. The described method in this application note was chosen to provide a reasonable sample run time when meeting the regulatory requirements.

Calibration curves were constructed from the data obtained by the 1-µL injections of standards at 5, 50, 100, 250, 500 ppb. Each standard solution contained 500 ppb of internal standards (ISTDs). All the PAHs have excellent linearity with calibration coefficients greater than 0.998 as shown in Table 3.



1. Naphthalene-d8
2. Naphthalene
3. 2-methylnaphthalene
4. 1-methylnaphthalene
5. Acenaphthylene
6. Acenaphthene-d10
7. Acenaphthene
8. Fluorene
9. Phenanthrene-d10
10. Phenanthrene
11. Anthracene
12. Fluoranthene
13. Pyrene
14. p-Terphenyl-d14
15. Benzo(a)anthracene
16. Chrysene-d12
17. Chrysene
18. Benzo(b)fluoranthene
19. Benzo(k)fluoranthene
20. Benzo(j)fluoranthene
21. Benzo(e)pyrene
22. Benzo(a)pyrene
23. Perylene-d12
24. Indeno(1,2,3-cd)pyrene
25. Dibenzo(a,h)anthracen
26. Benzo(g,h,i)perylene

Figure 1. Twenty PAHs at 250 ppb each with ISTDs at 500 ppb each and surrogate at 250 ppb, using synchronous SIM/SCAN mode, A: SCAN mode B: SIM mode.

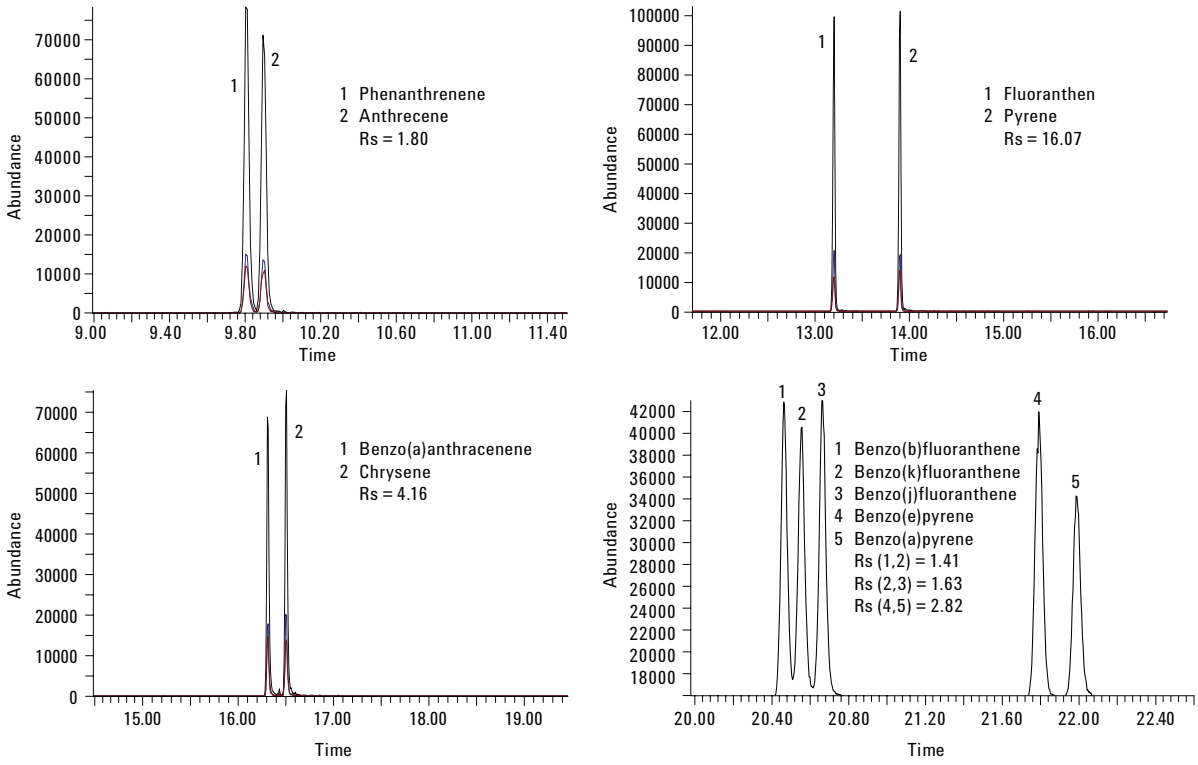


Figure 2. Resolution of Critical isomer Pairs with the DB-EUPAH column, 20 m × 0.18 mm × 0.14 µm (Agilent p/n 121-9627).

The GC/MS TIC for matrix spiked extract is illustrated in Figure 3. The spiked samples were treated according to the procedure described in the sample preparation. The recovery data for the spiked samples are listed in Table 4. All data were based on three replicates of matrix spikes with the 20 targeted PAHs at the 100 ppb level. Good recoveries were achieved for all the compounds, ranging from 73.5% to 119.4%, satisfying both the US-EPA and EU regulatory requirements.

Conclusion

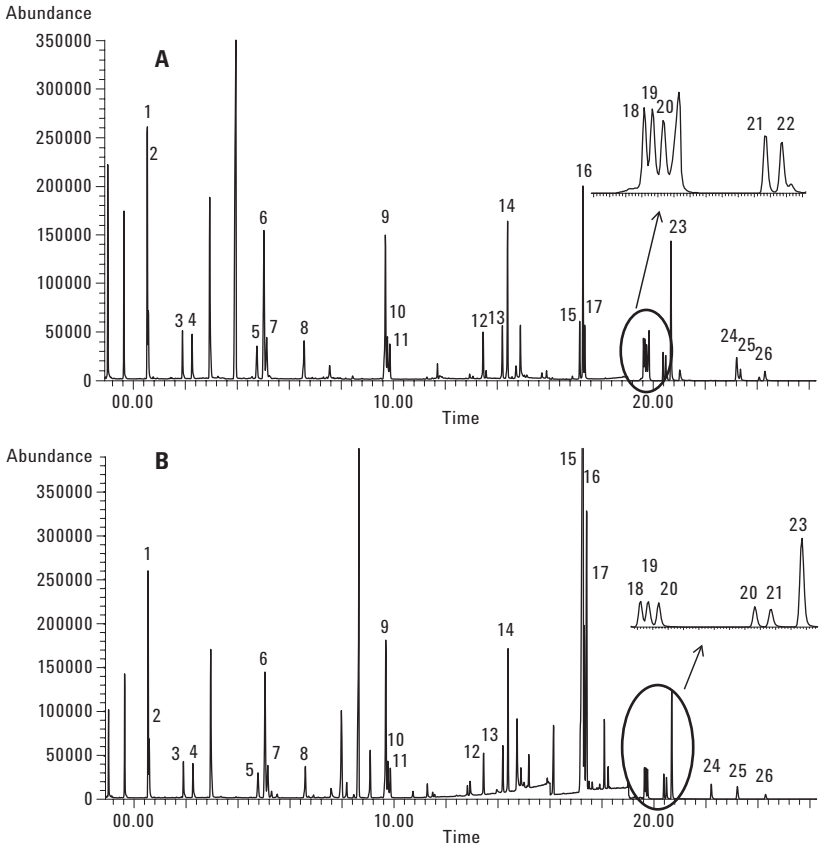
This application demonstrates a highly sensitive and selective GC/MS method for PAH analysis in rubber and plastic products using an Agilent J&W DB-EUPAH GC column. The DB-EUPAH column can effectively separate the 20 PAHs of interest, resolving all the critical, difficult-to-separate pairs. The system allows for trace-level detection of the PAHs in rubber and plastic articles. Good linearity and recoveries were achieved for all targeted compounds. The Agilent J&W DB-EUPAH column delivers fast, reliable results while meeting the requirements of both EPA and EU regulatory methods.

Table 3. The Regression Equations and Correlation Coefficient of PAHs

Compound	Range of linearity (ng)	Correlation coefficient (R^2)
Naphthalene	0.005-0.5	0.9997
2-Methylnaphthalene	0.005-0.5	0.9999
1-Methylnaphthalene	0.005-0.5	0.9998
Acenaphthylene	0.005-0.5	0.9996
Acenaphthene	0.005-0.5	0.9999
Fluorene	0.005-0.5	0.9994
Phenanthrene	0.005-0.5	0.9999
Anthracene	0.005-0.5	0.9992
Fluoranthene	0.005-0.5	0.9990
Pyrene	0.005-0.5	0.9996
Benzo[a]anthracene	0.005-0.5	0.9985
Chrysene	0.005-0.5	0.9998
Benzo[b]fluoranthene	0.005-0.5	0.9998
Benzo[j]fluoranthene	0.005-0.5	0.9983
Benzo[k]fluoranthene	0.005-0.5	0.9990
Benzo[e]pyrene	0.005-0.5	0.9992
Benzo[a]pyrene	0.005-0.5	0.9997
Indeno[1,2,3-c,d]pyrene	0.005-0.5	0.9989
Dibenzo[a,h]anthracene	0.005-0.5	0.9989
Benzo[g,h,i]perylene	0.005-0.5	0.9993

Table 4. Recoveries of PAHs in Substantial Plastic Sample and Rubble Sample

Compounds	Spiked (ppb)	Recovery (%) plastic sample	Recovery (%) rubber sample
Naphthalene	100	101.99	106.7
2-Methylnaphthalene	100	93.3	95.7
1-Methylnaphthalene	100	91.22	92.6
Acenaphthylene	100	111.32	120.8
Acenaphthene	100	98.45	109.0
Fluorene	100	107.66	114.6
Phenanthrene	100	92.54	109.1
Anthracene	100	106	110.8
Fluoranthene	100	110.52	119.4
Pyrene	100	111.62	104.0
Benzo[a]anthracene	100	102.61	118.5
Chrysene	100	107.67	95.9
Benzo[b]fluoranthene	100	103.18	114.6
Benzo[j]fluoranthene	100	103.02	118.9
Benzo[k]fluoranthene	100	91.13	88.8
Benzo[e]pyrene	100	90.8	92.3
Benzo[a]pyrene	100	106.58	119.2
Indenol(1,2,3-cd)pyrene	100	75.36	79.9
Dibenzo(a,h)anthracen	100	80.47	89.9
Benzo(g,h,i)perylene	100	76.45	73.5



1. Naphthalene-d8
2. Naphthalene
3. 2-methylnaphthalene
4. 1-methylnaphthalene
5. Acenaphthylene
6. Acenaphthene-d10
7. Acenaphthene
8. Fluorene
9. Phenanthrene-d10
10. Phenanthrene
11. Anthracene
12. Fluoranthene
13. Pyrene
14. p-Terphenyl-d14
15. Benzo(a)anthracene
16. Chrysene-d12
17. Chrysene
18. Benzo(b)fluoranthene
19. Benzo(k)fluoranthene
20. Benzo(j)fluoranthene
21. Benzo(e)pyrene
22. Benzo(a)pyrene
23. Perylene-d12
24. Indeno(1,2,3-cd)pyrene
25. Dibenzo(a,h)anthracen
26. Benzo(g,h,i)perylene

Figure 3. TIC of matrix spiked extract using Agilent GC/MS system and Agilent J&W DB-EUPAH 20 m × 0.18 mm, 0.14 µm column (Agilent p/n 121-9627) A: plastic, B: rubber.

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EU's Scientific Committee on Food PAH 151 Analysis Using a ZORBAX Eclipse PAH Column

Application Brief

Food

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Introduction

The 16 Environmental Protection Agency (EPA) priority pollutant polycyclic aromatic hydrocarbons (PAHs) are the dominant analytes in PAH methods. In fact, Agilent ZORBAX Eclipse PAH columns' ruggedness, longevity, batch-to-batch reproducibility and unvarying selectivity among three particle sizes were demonstrated using the EPA priority pollutants PAH mixture.[1] But for food and other environmental analyses, both subsets and additional PAHs must be separated. Different PAH separations including a fast food screening method and a complex environmental standard (24 PAHs) were previously shown.[2] A method for The European Union's (EU's) Scientific Committee on Food (SCF) 15 priority PAHs with benzo[c]fluorene, deemed relevant by the Joint FAO/WHO Experts Committee on Food Additives (JECFA), is presented here.

Experimental

This complex PAH separation was developed on an Agilent ZORBAX Eclipse PAH 2.1mm x 50 mm, 1.8 µm column, p/n 959741-918 and an Agilent Rapid Resolution 1200 Series LC (RRLC) system. It could be scaled up to a more traditional 4.6 mm x 150 mm, 5 or 3.5 µm column if desired, but the shorter, highly efficient, high throughput column was chosen to speed up method development. The "15+1" mixture was made by dissolving individual neat compounds in toluene or methylene chloride then combining them. Dilutions were made with ethanol.

Figure 1 shows the rapid analysis of 18 PAHs associated with food safety including the 15 classified as priority from the SCF (peaks #4–18), benzo[c]fluorene and two additional PAHs, benzo[c]phenanthrene and triphenylene that elute near benzo[c]fluorene. The short 50 mm column length and highly efficient 1.8 µm Eclipse PAH particles resolve the 18 components in 10 minutes, including re-equilibration.

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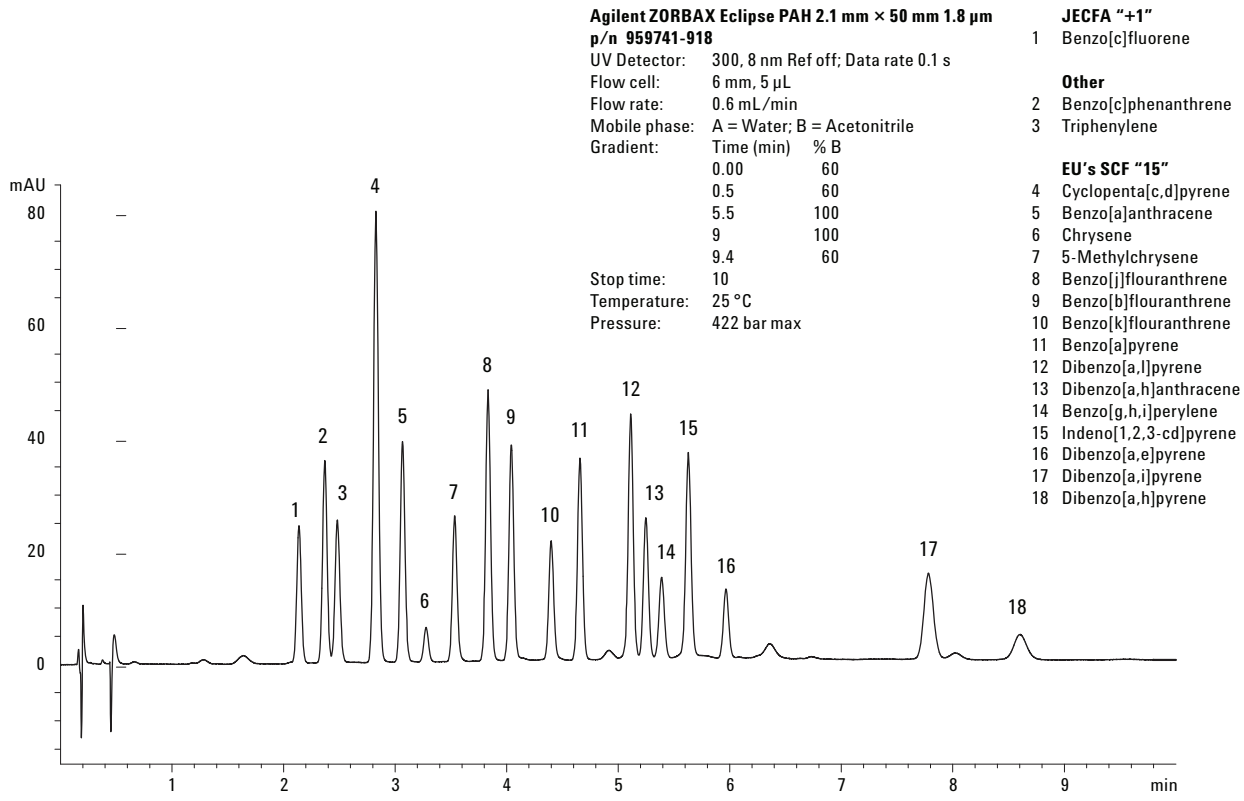


Figure 1. EU's SCF and JECFA "15+1" with two additional PAHs separated by the Agilent ZORBAX Eclipse PAH 2.1 mm x 50 mm, 1.8 µm column.

Conclusion

Many PAH methods are established for the EPA priority PAHs, but fewer have been established for the EU's SCF and JECFA "15+1" mix designed for the food industry. Agilent ZORBAX Eclipse PAH columns' proven robustness and selectivity make them ideal for the broad variety of PAH samples in matrices such as air, water, soil and food, including the "15+1" PAH mix. The 1.8 µm Eclipse PAH high throughput column produced a rapid separation of 18 PAHs associated with the food safety industry in 10 minutes, including re-equilibration. With choices in column lengths, diameters and particle sizes, the Eclipse PAH column gives the analyst valuable analysis options for difficult PAH sample matrices.

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GC-µECD Analysis and Confirmation of Contract Laboratory Protocol Pesticides in Olive Oil

Application Note

Food Safety and Environmental Markets

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Abstract

An olive oil sample obtained from a local grocery store is analyzed for 20 contract laboratory protocol (CLP) pesticides. A QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) with dispersive solid phase extraction (dSPE) procedure cleaned the sample prior to analysis. A dual µECD and dual capillary GC column approach accomplished simultaneous primary and confirmatory analysis. The primary column, an Agilent J&W DB-35ms 30 m × 0.25 mm × 0.25 µm and a confirmatory column, an Agilent J&W DB-XLB 30 m × 0.25 mm × 0.50 µm effectively resolved all 20 CLP pesticides. An unpurged two-way capillary flow technology splitter divided the flow from a single injection port to the two analytical GC columns. Endosulfan sulfate and endosulfan 1 were present in the olive oil sample.

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Introduction

Lower cholesterol and reduced cancer risk are among the reported beneficial health effects obtained from the antioxidants and monounsaturated fats found in olive oil. The potential health benefits have bolstered the popularity of using olive oil as a cooking oil, salad dressing, or direct replacement in baking for other traditional food oils. This rise in olive oil's popularity has led to higher demand and production levels.

Higher olive oil production levels increase the potential for olive crop destruction by pests. Therefore the industry is more reliant on the use of pesticides, leading to increased regulation of pesticide use [1]. For example, food safety concerns have led to tighter government regulation of pesticide residues found in foods such as olive oil. Production of olive oil has a tendency to concentrate pesticide residues in the oil because it takes four kilograms of olives to make one kilogram of olive oil [2]. Olive oil producers need inexpensive, robust analytical methods for necessary monitoring and testing.

In previous studies of pesticides residue analysis in olive oil, the extraction procedures varied from liquid-liquid followed by solid phase extraction (SPE), gel permeation chromatography (GPC), and more recently to the use of QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) with dispersive solid phase extraction (dSPE) [3-4]. QuEChERS and dSPE are convenient ways to clean up sample matrixes sufficiently to remove chromatographic interferences, while retaining sensitivity for analytes of interest. In this olive oil example, the high boiling lipid portion of the sample is removed along with its potential for carryover and interference with peaks of interest.

A dual column, dual µECD system with an Agilent J&W DB-35ms 30 m × 0.25 mm × 0.25 µm primary analysis column and an Agilent J&W DB-XLB 30 m × 0.25 mm × 0.50 µm confirmatory column was used to separate the CLP pesticides in olive oil [5-6]. Continuous improvements and stringent process control with respect to column activity make this column pair a particularly good choice for analysis of active analytes such as pesticides.

The GC was also fitted with an unpurged two-way splitter capillary flow technology (CFT) device. This device allows the operator to disassemble and service the inlet, the inlet transfer line or either of the analytical columns individually. System maintenance and troubleshooting are faster with reusable connections to the CFT.

Experimental

Standard Preparation

CLP pesticide (PPM-808C-1) and surrogate standards (ISM-320-1) were purchased from Ultra Scientific, 250 Smith St, N. Kingstown, RI. The CLP pesticide standard solution was prepared by diluting the standard to 8000 ng/mL with 2,2,4-Trimethylpentane (Ultra-Resi grade from VWR International, West Chester, PA). The 2000 ng/ml stock surrogate solution was made by diluting the surrogate standard with 2,2,4-Trimethylpentane. Working solutions of 4, 8, 80 and 800 ng/mL for the CLP pesticides and 1, 2, 20, and 200 ng/mL of the surrogates were used for spiking and calibration samples. 2,2,4-Trimethylpentane was used as a solvent blank and syringe wash solvent.

Sample Preparation

A sample of extra virgin olive oil was purchased at a local grocery store. The sample extraction method used the QuEChERS method followed by dSPE. Figure 1 illustrates the sample preparation procedure graphically in a flow chart.

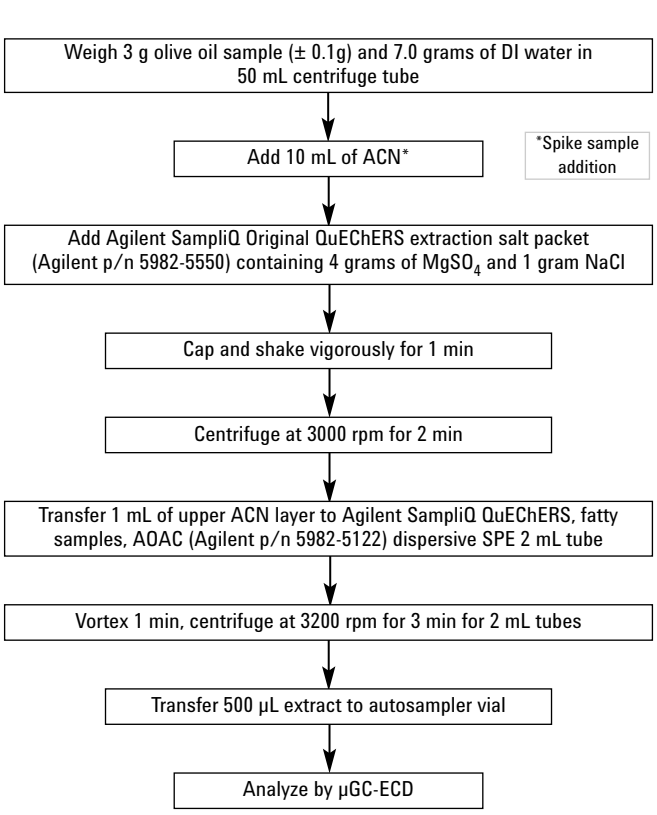


Figure 1: Flow chart of the Agilent SampliQ QuEChERS original extraction procedure for olive oil sample.

A 3.0-g olive oil aliquot and 7.0-g aliquot of deionized water were weighed into a centrifuge tube. The sample, spiked sample and blank each received 10-mL aliquots of acetonitrile (HPLC grade from VWR International). An Agilent SampliQ QuEChERS extraction salt packet (Agilent p/n 5982-5550) containing 4 g of $MgSO_4$ and 1 g NaCl was added to each centrifuge tube. The capped tubes were shaken for 1 min by hand and then 1 min on a mechanical shaker. The samples were centrifuged at 3000 rpm for 2 min.

A 1-mL aliquot of the upper layer was transferred into an Agilent SampliQ QuEChERS, fatty samples, AOAC (Agilent p/n 5982-5122) dispersive SPE 2 mL tube. The dSPE tube was vortexed for 1 min and then centrifuged at 3200 rpm for 3 min to complete the sample extraction. The liquid from the dSPE tube was transferred to a GC vial and run on the GC-µECD using the chromatographic conditions listed in Table 1 and Table 2.

Table 1. Chromatographic Conditions

GC/Dual µECD:	7890A equipped with dual µECD detection and a 7873B auto sampler
CFT Device:	2-way unpurged splitter capillary flow technology (Agilent p/n G3181B)
Column 1:	DB-35 ms 30 m × 0.25 mm × 0.25 µm (Agilent p/n 122-3832)
Column 2:	DB-XLB 30 m × 0.25 mm × 0.50 µm (Agilent p/n 122-1236)
Carrier Gas:	Hydrogen 56 cm/sec
Oven:	110 °C (1.4 min), 21 °C/min to 285 °C (1 min), 30 °C/min to 300 °C (2 min)
Injection:	1µL, 250 °C splitless, purge 50 mL/min at 0.3 min, gas saver 50 mL/min on at 2 min
Dual µ-ECD:	350 °C, N_2 makeup; constant column + makeup = 30 mL/min

Table 2 Flow Path Supplies

Vials:	Amber screw top glass vials (Agilent p/n 5183-2072)
Vial Caps:	Screw caps (Agilent p/n 5182-0723)
Vial inserts:	100 µL glass/polymer feet (Agilent p/n 5181-8872)
Syringe:	5 µL (Agilent p/n 5183-4729)
Septum:	Advanced green (Agilent p/n 5183-4759)
Inlet Seal:	Gold plated inlet seal (Agilent p/n 5188-5367)
Inlet liners:	Dual taper direct connect linear (Agilent p/n G1544-80700)
Ferrules:	0.4 mm id short; 85/15 vespel/graphite (Agilent p/n 5181-3323)
CFT fittings:	Internal nut (Agilent p/n G2855-20530)
CFT ferrules:	SilTite ferrules, 0.25 mm id (Agilent p/n 5188-5361)
20x magnifier :	20x Magnifier loop (Agilent p/n 430-1020)

To produce the spiked sample, a 3-mL aliquot of the 80 ng/mL CLP standard solution was added to the olive oil and water mixture, before the SampliQ QuEChERS original extraction salt packet addition. Extractions of water and acetonitrile aliquots in the same manner as the samples and the spiked sample served as reagent blanks.

Discussion of Results

The CLP pesticides and surrogates standards were resolved on the Agilent J&W DB-35ms 30 m × 0.25 mm × 0.25 µm primary analysis column in less than 12 min. Figure 2 show the separation of a 4.0 ng/mL CLP standard solution with the surrogate standards added at a concentration of 1.0 ng/mL. Peak numbers in the chromatogram label the peaks of interest and a compound key is included with the figure. Figure 3 shows a chromatogram of the same 4 ng/mL CLP standard (0.5 ng/mL surrogate standard) injection on the Agilent J&W DB-XLB 30 m × 0.25 mm × 0.50 µm confirmatory analysis column. Although peaks 10 and 11 (α-chlordane and endosulfan 1) are not completely resolved, the separation is suitable for confirming the presence of these analytes when observed on the primary analysis (DB-35ms) column.

The performance of the dual column set yielded acceptable linearity, limits of detection (LOD), and quantitative (LOQ) in accordance with current EU guidelines for these analytes. The linearity of the column set as defined by the R^2 values of the CLP pesticide standard curve ranged from 0.994-0.999. Individual pesticide values are shown in Table 3. The LOD (S/N = 3) and LOQ (S/N = 10) were determined through close examination of an expanded section of chromatograms on each column relative to a known concentration peak in the lowest standard. Figure 4A shows this comparison for the tetrachloro-m-xylene peak on the primary analysis DB-35ms column and Figure 4B shows the same comparison on the DB-XLB confirmation column. The average LOD (S/N = 3) and LOQ (S/N =10) across both columns using the lowest concentration (0.5 ng/mL) of the standard CLP pesticides was 0.3 ng/mL and 1.0 ng/mL respectively. The dual column set of a primary analytical column and a confirmatory column on one instrument allows simultaneous confirmation of the presence of the CLP pesticides. The single injection, dual column approach saves instrument and analyst time and offers the analyst an alternative to GC/MS screening for olive oil samples.

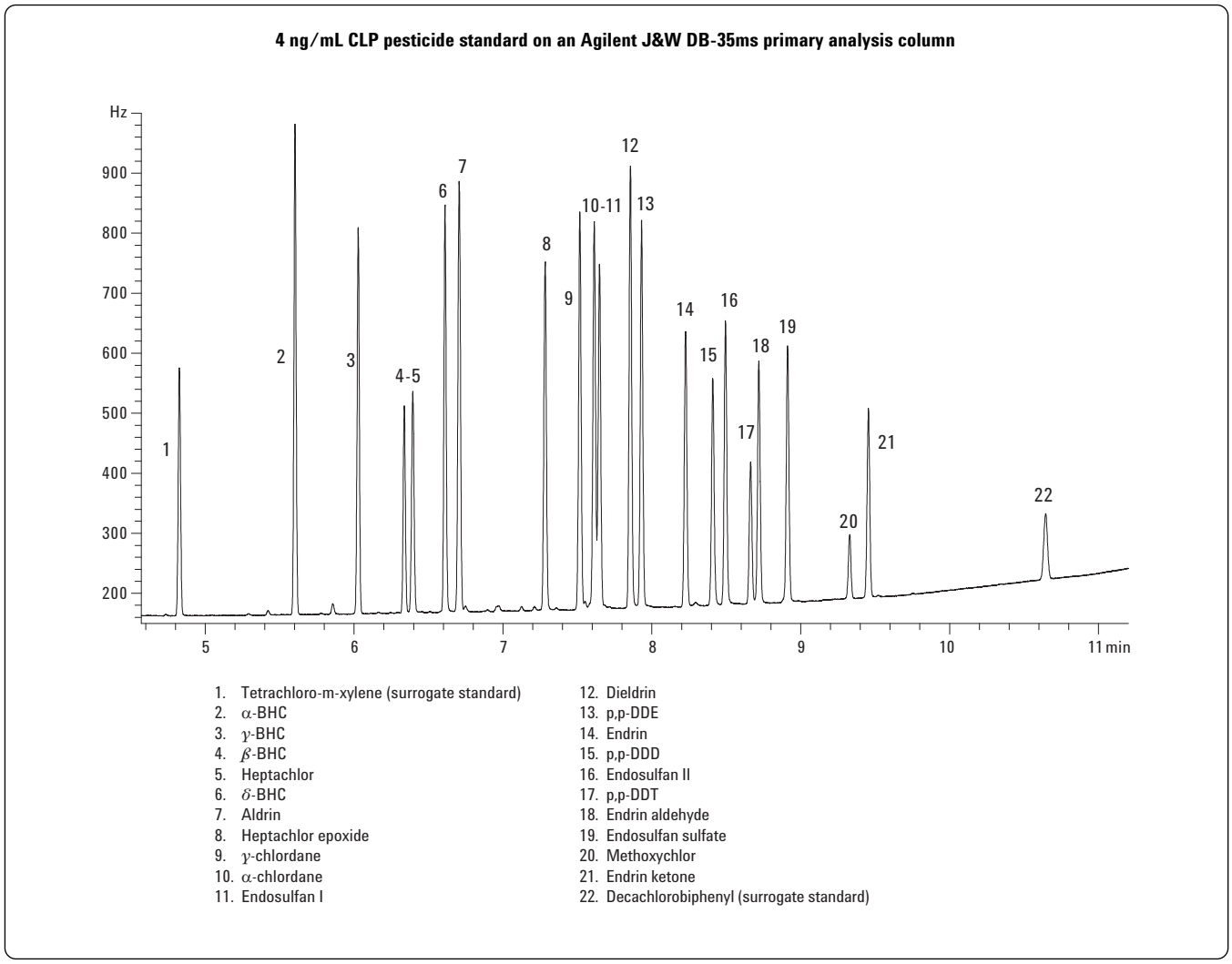


Figure 2. GC-μECD chromatogram of 4 ng/mL standard of CLP pesticides and surrogates standard analyzed on an Agilent J&W DB-35ms, 30 m × 0.25 mm × 0.25 μm column.

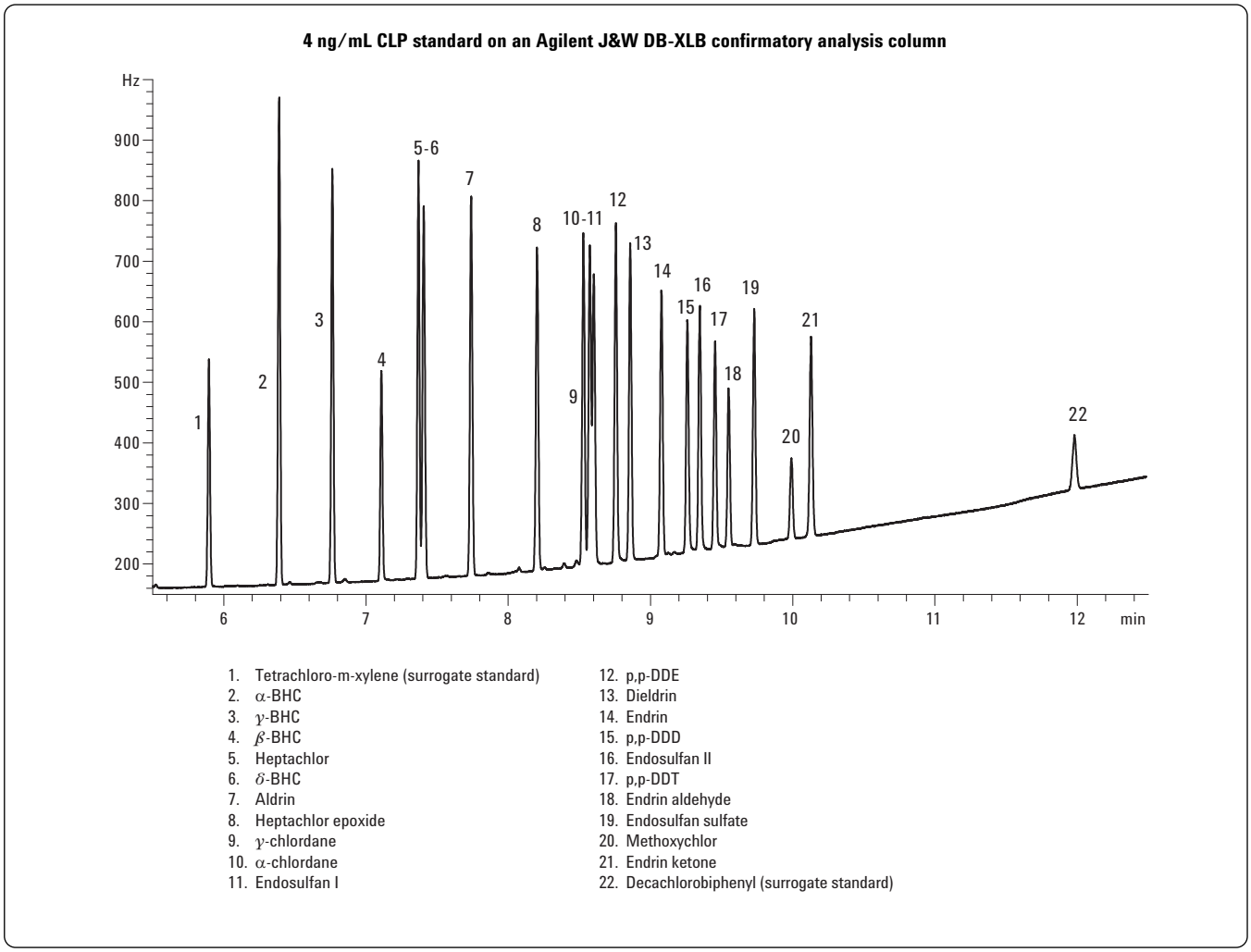


Figure 3. GC-μECD chromatogram of 4 ng/mL standard of CLP pesticides and surrogates standard analyzed on an Agilent J&W DB-XLB, 30 m × 0.25 mm × 0.50 μm column.

Table 3. Calibration Curve - Calibration Standards of 4, 8, 10, 20, and 40 ng/mL Were Prepared in 2,4-Trimethylpentane.

	DB-35 ms	DB-XLB
Compounds	r^2	r^2
α-BHC	0.998	0.998
γ-BHC	0.998	0.998
β-BHC	0.997	0.998
Heptachlor	0.998	0.998
δ-BHC	0.997	0.995
Aldrin	0.997	0.998
Heptachlor epoxide	0.998	0.998
γ-chlordane	0.998	0.997
α-chlordane	0.998	0.996
Endosulfan I	0.997	0.996
p,p-DDE	0.998	0.998
Dieldrin	0.997	0.998
Endrin	0.995	0.997
Endosulfan II	0.999	0.998
Endrin Aldhyde	0.998	0.995
p,p-DDD	0.998	0.998
p,p-DDT	0.999	0.998
Endosulfan sulfate	0.995	0.998
Methoxychlor	0.998	0.996
Endrin Ketone	0.998	0.994

A dual column µECD system resolved the 20 CLP pesticides in the spiked olive oil sample. The primary analytical column DB-35ms separated the 20 CLP pesticides with minor matrix interference in a sample of olive oil spiked with an 80 ng/mL CLP pesticide and surrogate standard. There are several olive oil matrix peaks observed in the spiked samples, including one large peak, that fortunately do not co-elute with the CLP pesticides. Figure 5 clearly shows the separation of all 20 CLP pesticides in an olive oil matrix. In Figure 6, the DB-XLB confirms the presence and separation of the CLP pesticides in the spiked olive oil sample.

Figures 7 and 9 show the overlaid chromatograms on the DB-35ms primary analysis column of the spiked and native olive oil sample with peaks for endosulfan sulfate and endosulfan 1 labeled. Figures 8 and 10 show the overlaid chromatograms on the DB-XLB confirmatory column with peaks for endosulfan sulfate and endosulfan 1 labeled. Chromatogram overlays of the native and spiked olive oil samples identify and confirm endosulfan sulfate and endosulfan 1 as pesticide residues in the olive oil sample. Concentrations from the CLP calibration standard curves were 23.1 ng/mL for endosulfan sulfate and 7.1 ng/mL for endosulfan 1.

The extraction process using the QuEChERS followed by dispersive SPE was effective in retaining the pesticides in the spiked olive oil sample as well as cleaning up the sample matrix for GC-µECD analysis. The fact that endosulfan sulfate and endosulfan 1 were present and detected at low levels in the olive oil sample investigated underscores this point.

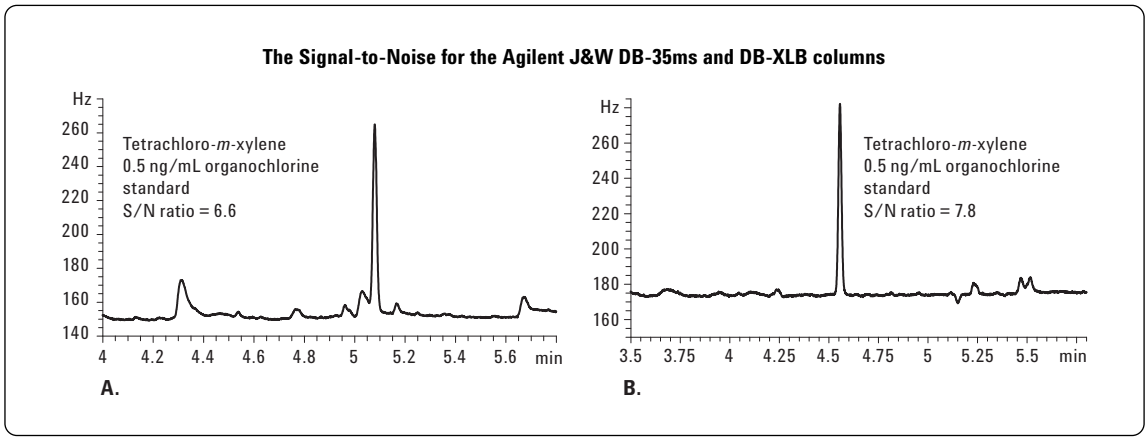


Figure 4. GC-µECD chromatogram of 0.5 ng/mL standard of CLP pesticides and surrogates standard. A.) The S/N on the Agilent J&W DB-35ms, 30 m × 0.25 mm × 0.25 µm column is 6.6. B.) The S/N on an Agilent J&W DB-XLB, 30 m × 0.25 mm × 0.50 µm column is 7.8.

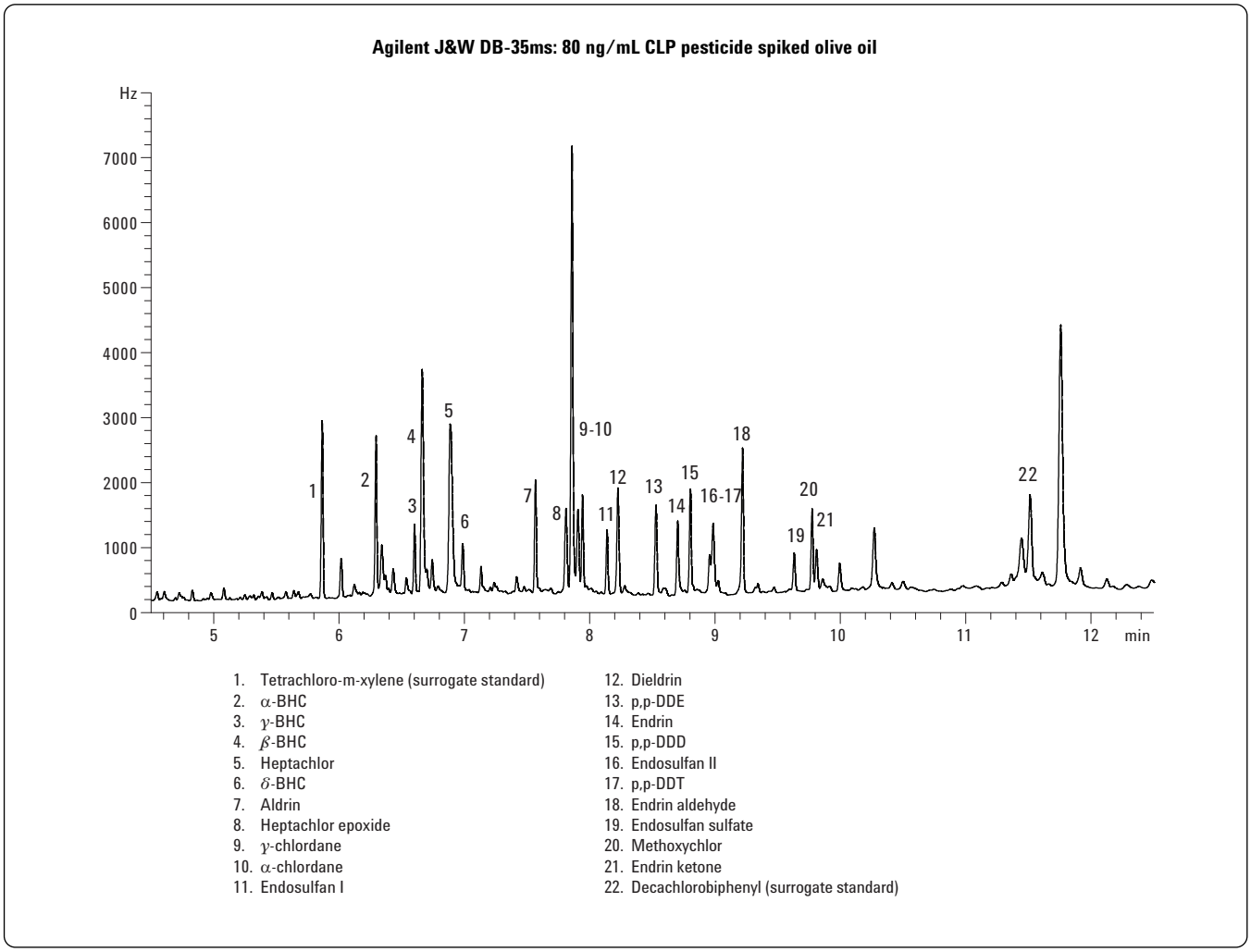


Figure 5. GC-µECD chromatogram of olive oil sample spiked with 80 ng/mL of CLP pesticides and 20 ng/mL surrogates standard analyzed on an Agilent J&W DB-35ms 30 m × 0.25 mm × 0.25 µm column.

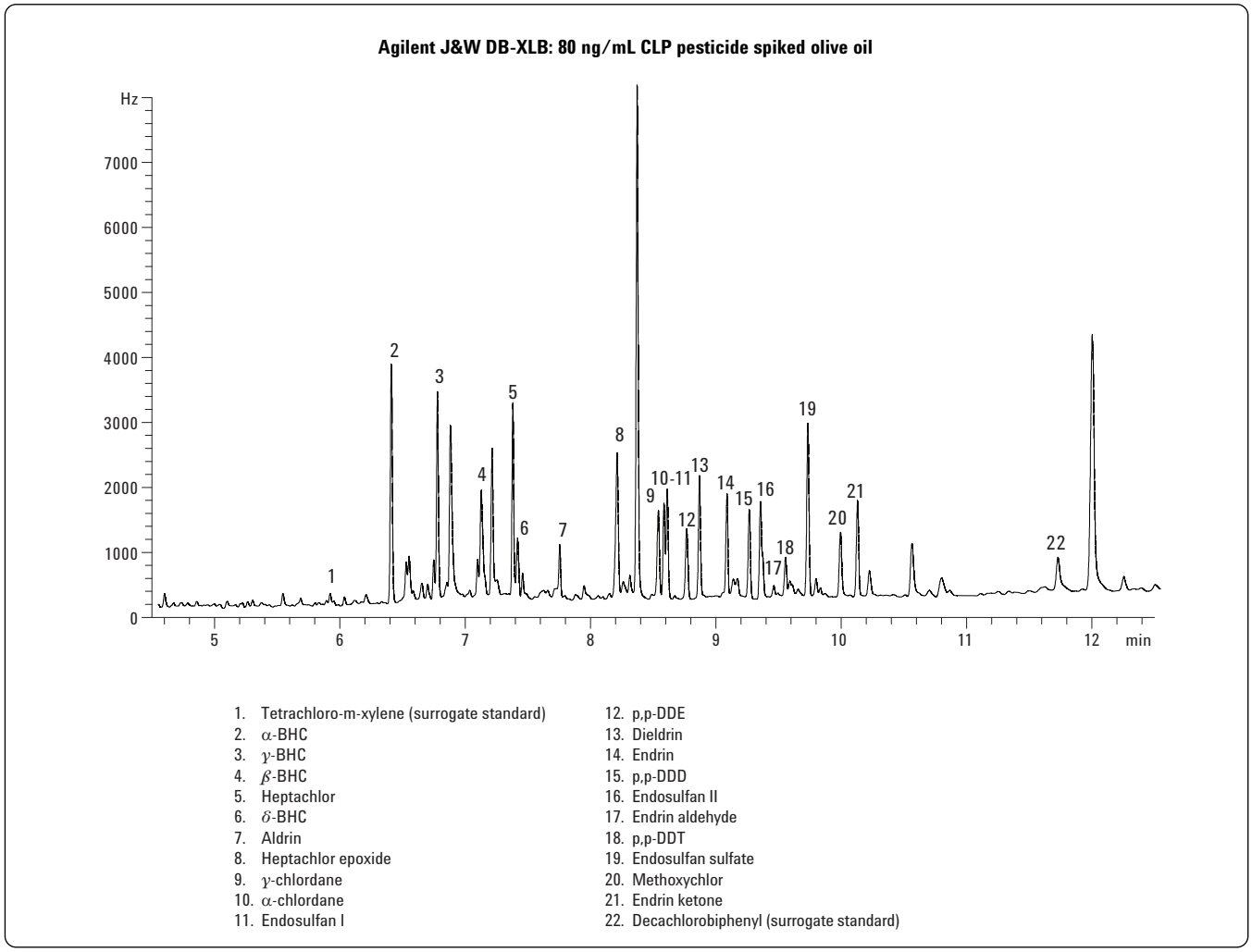


Figure 6. GC-µECD chromatogram of olive oil sample spiked with 80 ng/mL of CLP pesticides and 20 ng/mL surrogates standard analyzed on an Agilent J&W DB-XLB 30 m × 0.25 mm × 0.25 µm column.

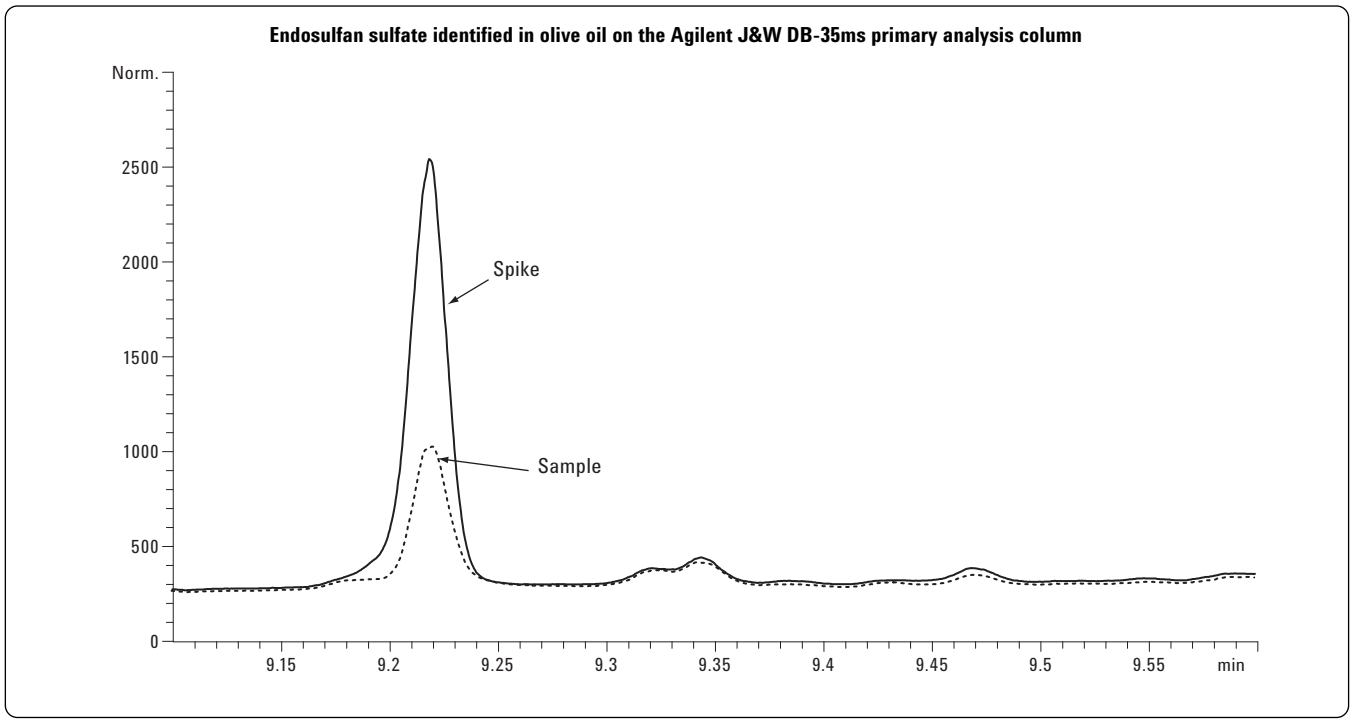


Figure 7. GC-µECD chromatogram of olive oil sample overlaid with olive oil sample spike with 80 ng/mL of CLP pesticides and 20 ng/mL surrogates standard analyzed on an Agilent J&W DB-35ms, 30 m × 0.25 mm × 0.25 µm column. This overlay offers more evidence of the presence of endosulfan sulfates on the Agilent J&W DB-35ms column.

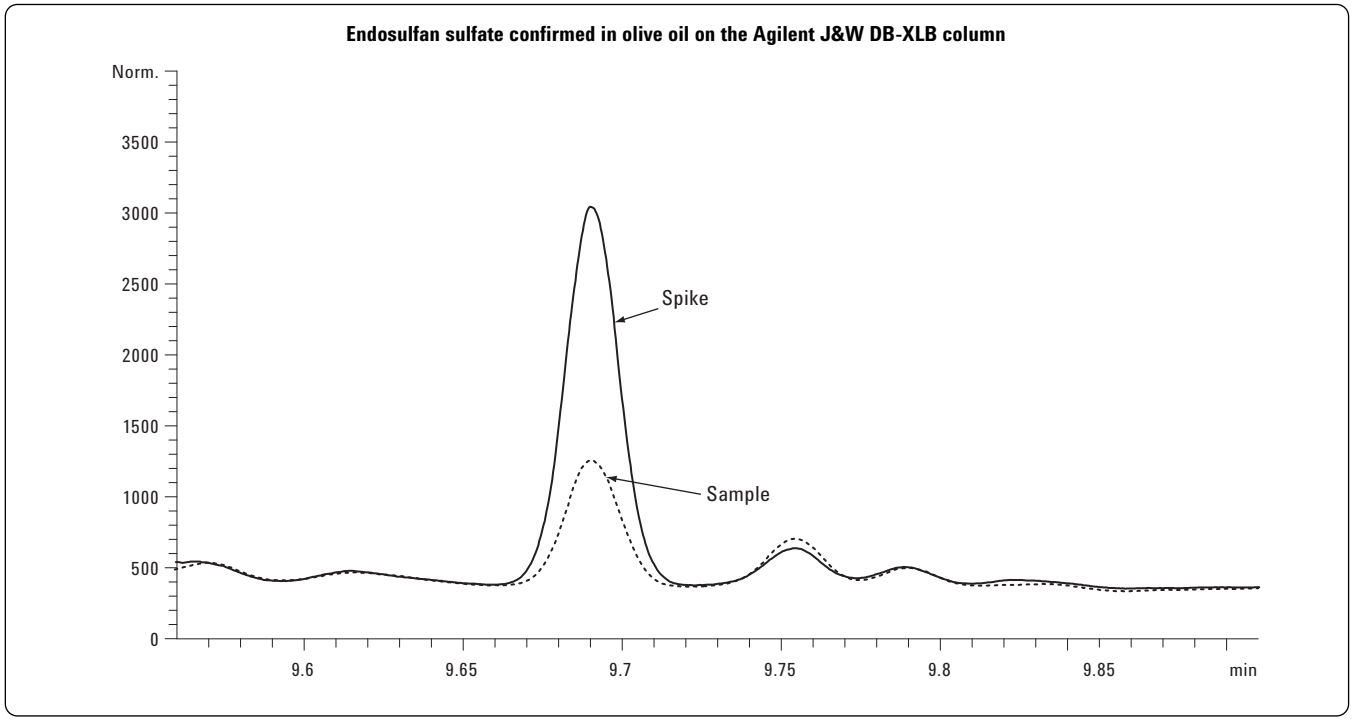


Figure 8. GC-µECD chromatogram of olive oil sample overlaid with olive oil sample spike with 80 ng/mL of CLP pesticides and 20 ng/mL surrogates standard analyzed on an Agilent J&W DB-XLB, 30 m × 0.25 mm × 0.50 µm column. This overlay offers more evidence of endosulfan sulfates presence on the Agilent J&W DB-XLB column.

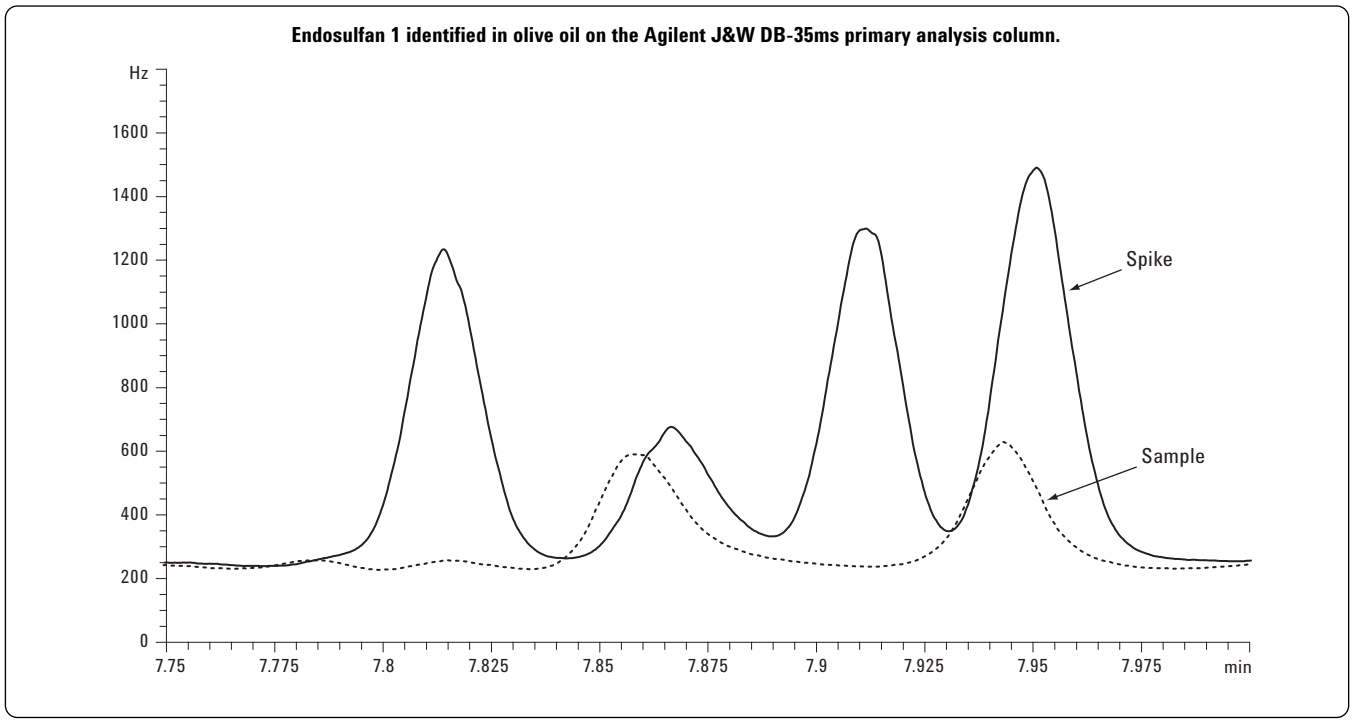


Figure 9. GC-µECD chromatogram of olive oil sample overlaid with olive oil sample spike with 80 ng/mL of CLP pesticides and 20 ng/mL surrogates standard analyzed on an Agilent J&W DB-35ms, 30 m × 0.25 mm × 0.25 µm column. This overlay offers more evidence of endosulfan 1 presence on the Agilent J&W DB-35ms column.

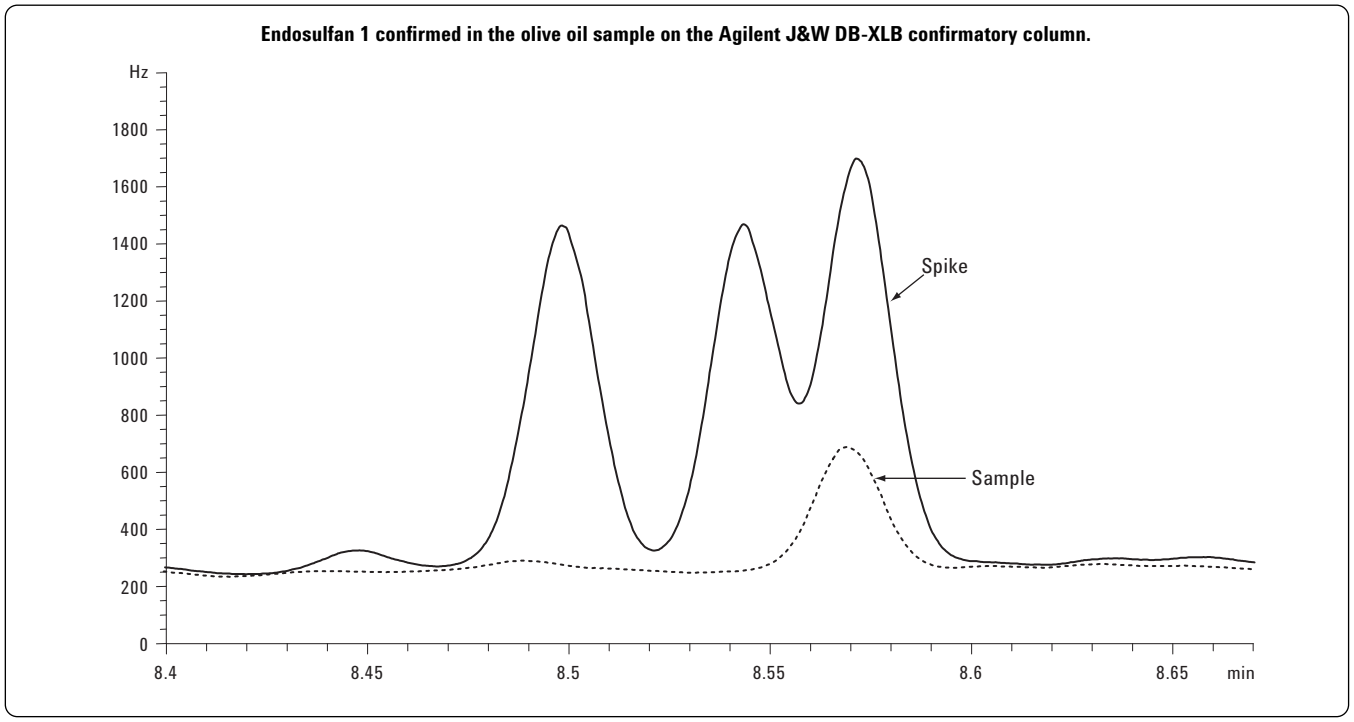


Figure 10. GC-µECD chromatogram of olive oil sample overlaid with olive oil sample spike with 80 ng/mL of CLP pesticides and 20 ng/mL surrogates standard analyzed on an Agilent J&W DB-XLB, 30 m × 0.25 mm × 0.50 µm column. This overlay offers more evidence of endosulfan 1 presence on the Agilent J&W DB-XLB column.

Conclusions

The dual column set of an Agilent J&W DB-35ms primary analytical column and an Agilent J&W DB-XLB confirmatory column on one instrument allows simultaneous identification and confirmation of the presence of the CLP pesticides. The DB-35ms primary analysis and a DB-XLB confirmatory column with dual µ-ECD detection were effective at analyzing 20 CLP pesticides in an olive oil matrix following sample matrix cleanup. The single injection, dual column approach improves productivity by saving instrument and analyst time. Continuous improvements and stringent process control with respect to column activity make the DB-35ms and DB-XLB column pair an excellent choice for analysis of active analytes such as pesticides.

This note successfully shows a robust, inexpensive analytical method to monitor CLP pesticides in olive oil suitable to address food safety concerns. This method demonstrates the feasibility of using a dual column µECD approach for routine olive oil screening as an alternative to GC/MS.

QuEChERS followed by dSPE were effective at providing just enough sample cleanup to avoid matrix interferences while still maintaining low-level analyte detection. The performance of the dual column set DB-35ms and DB-XLB on the GC-µECD had excellent linearity over the range of concentrations studied with R^2 values between 0.994 and 0.999 for the individual pesticides. The limits of detection (LOD) quantitative (LOQ) for this analysis were 0.3 ng/mL and 1.0 ng/mL, demonstrating low level detection capability. QuEChERS followed by dSPE also provided a fast and effective way to prepare the sample.

The primary analysis DB-35ms column identified endosulfan sulfate and endosulfan 1 in the olive oil sample. The DB-XLB confirmatory column confirmed that endosulfan sulfate and endosulfan 1 were present in the olive oil sample. Overlaid chromatograms of pesticide spiked and native olive oil samples on both columns provided additional evidence confirming the presence of these two pesticides through a process of pattern recognition. The evidence proves that these two pesticides were present in this grocery store olive oil.

The fact that a single randomly selected commercial olive oil sample came back positive for two pesticide residues indicates residues are present in these oils. Clearly, there is a need to monitor olive oil for pesticide residues through tests such as the dual column dual GC-µECD analysis described in this note.

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Determination of Dioxin-Like and Non-Dioxin-Like Polychlorinated Biphenyl Congeners in Foodstuffs and Animal Feed Using the Agilent 7000 Triple Quadrupole GC/MS System

Application Note

Food Safety

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Abstract

Two methods have been developed on the Agilent 7000 Triple Quadrupole GC/MS system for the analysis of polychlorinated biphenyl (PCB) congeners in foodstuffs and animal feed. The methods were shown to give linear response over the required concentration ranges. In addition, quantitative results for dioxin-like PCB (dl-PCB) congeners down to low pg TEQ/g levels and non-dioxin-like PCB (ndl-PCB) congeners at levels below 1 ng/g product were in good agreement with values obtained using a GC-High Resolution mass spectrometer. This application note demonstrates the determination of the 12 dl-PCB comprising eight mono-*ortho* PCB congeners (# 105, 114, 118, 123, 156, 157, 167 and 189) and four non-*ortho* PCB congeners (# 77, 81, 126 and 169) as well as the six ndl-PCB congeners (# 28, 52, 101, 138, 153 and 180) that are also known as “Indicator PCB” congeners.



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Introduction

Polychlorinated biphenyls (PCB) are highly toxic Persistent Organic Pollutants (POP) with properties that are detrimental to human health. They have been linked to cancer, endocrine disruption and reproductive disorders. Until their ban in the late 20th Century, PCBs were widely manufactured for use in hundreds of industrial and commercial applications including electrical products and hydraulic equipment and as plasticizers in paints, plastics and, rubber products. PCB congeners that have been released into the environment can bio-accumulate in animal tissues and thereby enter the human food chain.

Current legislation in the United States [1] and the European Union (EU) [2], [5] require the confirmation and quantitation of polychlorinated dibenzo-p-dioxins (PCDD) and polychlorinated dibenzofurans (PCDF) and dioxin-like polychlorinated biphenyl congeners (dl-PCB) in foodstuffs and animal feed by isotope dilution capillary gas chromatography–high resolution mass spectrometry (GC-HRMS). The analysis of dioxins and furans in foodstuffs and animal feed by gas chromatography-triple quadrupole mass spectrometry is shown in a previously published Agilent application note [3].

Maximum levels for PCDD, PCDF and dl-PCB congeners in foodstuffs and animal feed are given in additional EU regulations [4], [6]. dl-PCB congeners have each been assigned a Toxic Equivalency Factor (TEF) that relates the toxicity of each individual dl-PCB congener to 2,3,7,8 Tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD), which itself is assigned a TEF of 1. The individual concentration of each dl-PCB found in foodstuffs and animal feed samples is multiplied by it's respective TEF and after summation the total concentration is expressed as the Toxic Equivalent (TEQ) in terms of pg TEQ/g fat, pg TEQ/g fresh weight (fish) or ng TEQ/kg in 88% dry feed.

The World Health Organization (WHO) through the International Program on Chemical Safety (IPCS) originally established and then re-evaluated TEF for PCDD, PCDF and dl-PCB. Original TEF values were established by WHO/IPCS expert consultation in 1997 and re-evaluated in 2005. As a result, some TEF values have been changed and it is important to clearly state the set of TEF values used by indicating the year in which they were first expressed (TEF_{WHO98} or TEF_{WHO05}) and the resultant TEQ_{WHO98} and TEQ_{WHO05} values. TEF values assigned to PCDD, PCDF and dl-PCB are shown in Table 1.

The maximum levels (based on TEF_{WHO98} values) for PCDD, PCDF and dl-PCB in certain foodstuffs as prescribed by EU legislation are given in Table 2.

Table 1. WHO Toxic Equivalency Factors (TEF_{WHO98} and TEF_{WHO05}) for PCDD, PCDF and dl-PCB Congeners from Assessments Made in 1998 and 2005 (Changed Values in Italics)

Compound	TEF WHO_{98}	TEF WHO_{05}	Compound	TEF WHO_{98}	TEF WHO_{05}
Chlorinated dibenzo-p-dioxins			**Non-*ortho* substituted PCB**		
2378-TCDD	1	1	PCB-77	0.0001	0.0001
12378-PeCDD	1	1	PCB-81	0.0001	*0.0003*
123478-HxCDD	0.1	0.1	PCB-126	0.1	0.1
123678-HxCDD	0.1	0.1	PCB-169	0.01	*0.03*
123789-HxCDD	0.1	0.1			
1234678-HpCDD	0.01	0.01			
OCDD	0.0001	*0.0003*			
Chlorinated dibenzofurans			**Mono-*ortho* substituted PCB**		
2378-TCDF	0.1	0.1	PCB-105	0.0001	*0.00003*
12378-PeCDF	0.05	*0.03*	PCB-114	0.0005	*0.00003*
23478-PeCDF	0.5	*0.3*	PCB-118	0.0001	*0.00003*
123478-HxCDF	0.1	0.1	PCB-123	0.0001	*0.00003*
123678-HxCDF	0.1	0.1	PCB-156	0.0005	*0.00003*
234678-HxCDF	0.1	0.1	PCB-157	0.0005	*0.00003*
123789-HxCDF	0.1	0.1	PCB-167	0.00001	*0.00003*
1234678-HpCDF	0.01	0.01	PCB-189	0.0001	*0.00003*
1234789-HpCDF	0.01	0.01			
OCDF	0.0001	*0.0003*			

Table 2. Maximum Levels (Upperbound concentrations) for PCDD, PCDF and dl-PCB Congeners in Certain Foodstuffs, as Specified in EU Regulation (EC) No 1881/2006

Foodstuff	Maximum levels Sum of dioxins (WHO-PCDD/F-TEQ)	Maximum levels Sum of dioxins and dl-PCB (WHO-PCDD/F-PCB-TEQ)
Meat and meat products (excluding edible offal) of the following animals :		
- Bovine animals and sheep	3.0 pg/g fat	4.5 pg/g fat
- Poultry	2.0 pg/g fat	4.0 pg/g fat
- Pigs	1.0 pg/g fat	1.5 pg/g fat
- Raw milk and dairy products, including butter fat	3.0 pg/g fat	6.0 pg/g fat
- Hens eggs and egg products	3.0 pg/g fat	6.0 pg/g fat

The action levels for the sum of the 12 dl-PCB congeners in certain foodstuffs and certain feedstuffs are shown in Tables 3 and 4, respectively.

The six ndl-PCB congeners do not have TEF values and their results are expressed simply as the sum of the six individual congeners.

The chemical structures of the 12 dl-PCB congeners and 6 ndl-PCB congeners are shown in Figures 1 and 2, respectively.

This application note describes sensitive and reproducible methods for the screening of dl-PCB congeners and ndl-PCB congeners in foodstuffs and animal feed using the Agilent 7000 Triple Quadrupole GC/MS system that meets the requirements of EU Legislation for a screening method.

Table 3. Action Levels (Upperbound Concentrations) for the Sum of dl-PCB Congeners in Certain Foodstuffs, as Specified in EU Commission recommendation 2006/88

Food	Action level for dioxin-like PCBs (TEQ_{WHO98})
Meat and meat products of	
Runinants (bovine animals, sheep)	1.0 pg/g fat
Poultry and farmed game	1.5 pg/g fat
Pigs	0.5 pg/g fat
Liver and derived products of terrestrial animals	4.0 pg/g fat
Muscle meat of fish and fishery products and products thereof with the exception of eel	3.0 pg/g fresh weight
Muscle meat of eel (Anguilla anguilla) and products thereof	6.0 pg/g fresh weight
Milk and milk products, including butter fat	2.0 pg/g fat
Hens eggs and egg products	2.0 pg/g fat

Table 4. Action Levels (Upperbound Concentrations) for the Sum of dl-PCB Congeners in Certain Feedstuffs, as Specified in EU Regulation 2002/32/EC

Product intended for animal feed	Action threshold relative to a feeding stuff with a moisture content of 12%
Feed materials of plant origin with the exception of vegetable oils and their byproducts	0.35 ng TEQ/kg
Vegetable oils and their byproducts	0.5 ng TEQ/kg
Feed materials of mineral origin	0.35 ng TEQ/kg
Animal fat, including milk fat and egg fat	0.75 ng TEQ/kg
Other land animal products including milk and milk products and eggs and egg products	0.35 ng TEQ/kg
Fish oil	14.0 ng TEQ/kg

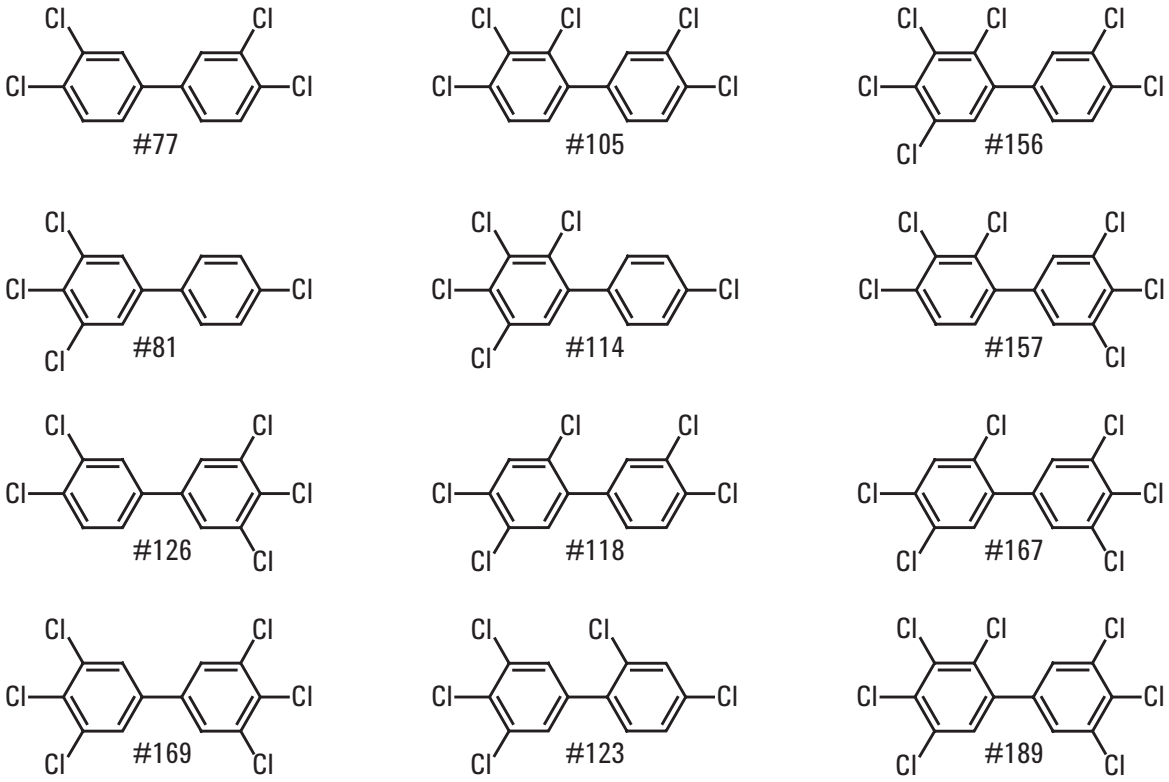


Figure 1. Chemical structures of dl-PCB congeners.

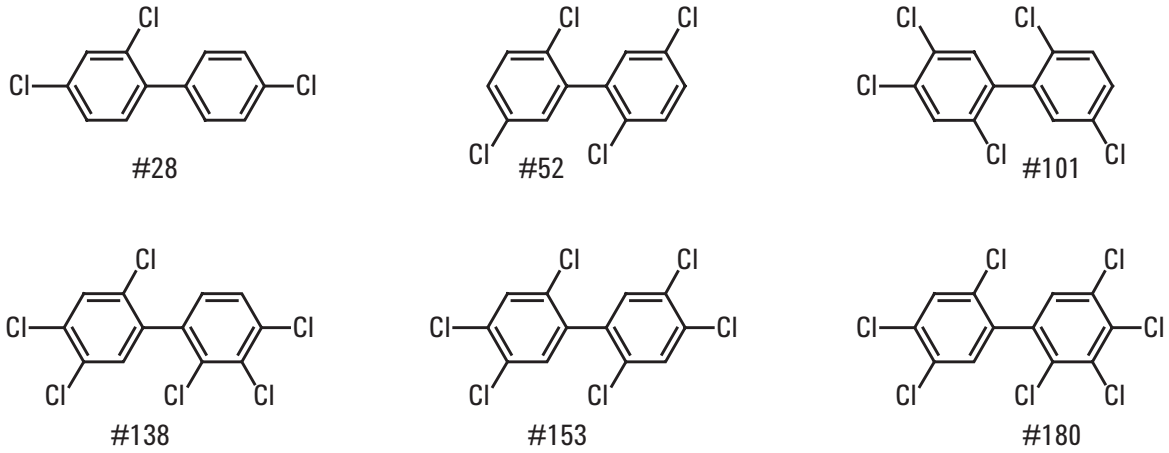


Figure 2. Chemical structures of ndl-PCB congeners.

Experimental

Calibration Standards

Calibration mixtures of native PCB congeners and their ^{13}C-isotope labelled internal standards were obtained from Cambridge Isotope Laboratories and Wellington Laboratories Inc.

Sample Preparation and Analysis

The most frequently used methods for the determination of PCDD, PCDF, dl-PCB congeners and ndl-PCB congeners in foodstuffs and animal feed combine fat extraction (for example, Soxhlet or extraction with organic solvents) with cleanup steps using different column chromatographies such as silica gel coated with sulfuric acid, florisil, alumina, and active carbon. The final extracts are collected as three fractions containing the mono-*ortho* PCB congeners and indicator PCB congeners (1a, Figure 3), non-*ortho* PCB congeners (1b, Figure 3) and PCDD/F (2, Figure 3), by eluting with various solvents. After addition of a syringe spike of ^{13}C- labelled PCB internal standards, the extracts were evaporated under a gentle stream of nitrogen and subsequently reconstituted with toluene and analyzed with GC/MS/MS. The PCDD/F fraction was reconstituted with 20 µL of toluene, the non-*ortho* PCB fraction with 40 µL of toluene and the mono-*ortho*/indicator PCB fraction with 250 µL of toluene.

A flow diagram summarizing the sample preparation steps is shown in Figure 3.

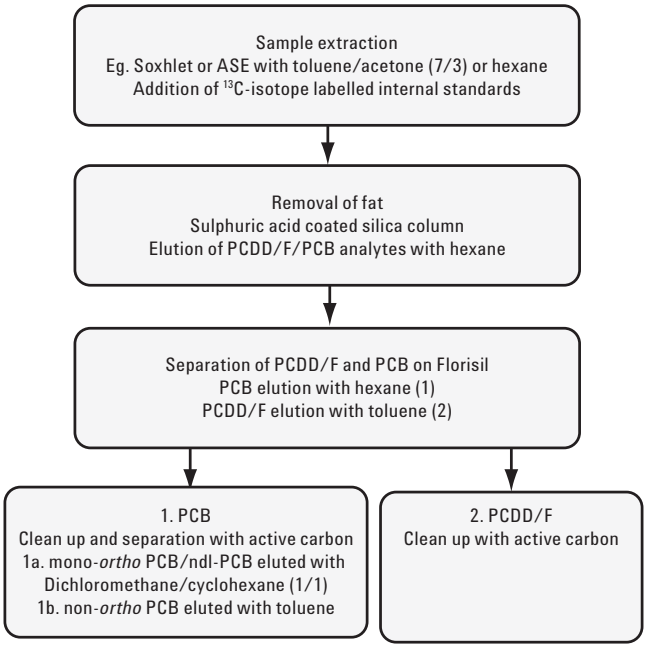


Figure 3. Flow diagram of the sample extraction and cleanup procedures.

The analyses were performed on an Agilent 7890 GC and an Agilent 7000 Triple Quadrupole GC/MS system. The 7890 Series GC was configured with a carbon dioxide cooled Multimode Inlet (MMI) and an HT-8 50 m × 0.22 mm, 0.25 µm capillary column.

The GC instrument conditions for the mono-*ortho* PCB congeners are listed in Table 5 and the GC instrument conditions for the non-*ortho* PCB congeners are given in Table 6. MS parameters, common to both sets of PCB congeners, are shown in Table 7. The 7000 Triple Quadrupole GC/MS was operated in MS/MS-EI (electron ionization) Multiple Reaction Monitoring (MRM) mode. Each analyte and its associated ^{13}C-Internal standard (ISTD) were measured using two different precursor ions and two different product ions.

Table 5. GC Conditions for Mono-ortho and dl-PCB Congeners

Column	HT-8 50 m × 0.22 mm id, 0.25 µm
Injection	2 µL cold splitless using CO_2 cooled Multimode Inlet (MMI)
Injection port liner	4 mm id, unpacked
Inlet temperature program	100 °C (0.02 min), 500 °C/min to 300 °C
Purge flow to split vent	50 mL/min at 1.0 min
Carrier gas	Helium, constant flow 1.2 mL/min
Oven program	80 °C (3.0 min hold), 20 °C/min to 160 °C (0 min), 4 deg °C/min to 300 °C (8 min), (Total run time = 50.0 minutes)
MS transfer line temp	280 °C

Table 6. GC Conditions for Non-ortho-PCB Congeners

Column	HT-8 50 m × 0.22 mm id, 0.25 µm
Injection	2 µL cold splitless using CO_2 cooled Multimode Inlet (MMI)
Injection port liner	4 mm id with glass wool
Inlet temperature program	100 °C (0.02 min), 500 °C/min to 300 °C
Purge flow to split vent	50 mL/min at 1.0 min
Carrier gas	Helium, constant flow 1.2 mL/min
Oven program	120 °C (2.0 min hold), 40 °C/min to 160 °C (0 min), 7 deg °C/min to 300 °C (10 min), (Total run time = 33.0 minutes)
MS transfer line temp	280 °C

Table 7. MS Setpoints for all PCB Congeners

Electron energy	–78 EV
Tune	EI Autotune
EM gain	100
MS1 resolution	Unit
MS2 resolution	Wide
Quant/Qual transitions	Table 8/Table 9
Dwell times	Table 8/Table 9
Collision energies	Table 8/Table 9
Collision cell gas flows	Nitrogen at 1.5 mL/min, Helium at 2.25 mL/min
MS temperatures	Ion source 280 °C, MS1 150 °C, MS2 150 °C

A full list of the analyte retention times, MRM settings and dwell times for the mono-*ortho* and ndl-PCB congeners and the non-*ortho* PCB congeners are given in Tables 8 and 9, respectively.

An Agilent 7693A Automatic Liquid Sampler with the sampler tray cooled to 5 °C was used and 2 µL cold splitless injections were made using a 10-µL syringe.

Table 8. MS/MS Settings for Native Mono-Ortho and ndl-PCB Congeners (ndl-PCB Congeners Shown in Bold Italics) and ^{13}C-Internal Standards

TS	Segment start time (min)	Analyte	RT (min)	Quant pre-cursor	Product	Dwell (ms)	CE (V)	Qual pre-cursor	Product	Dwell (ms)	CE (V)
1	22.0	***^{13}C-PCB 28***	***24.34***	***268.0***	***198.1***	***25***	***26***	***270.0***	***198.1***	***25***	***26***
		PCB 28	***24.35***	***256.0***	***186.0***	***75***	***26***	***258.0***	***186.0***	***75***	***26***
		^{13}C-PCB 52	***25.66***	***302.0***	***232.0***	***25***	***28***	***304.0***	***234.0***	***25***	***28***
		PCB 52	***25.67***	***289.9***	***220.0***	***75***	***28***	***291.9***	***222.0***	***75***	***28***
2	29.0	***^{13}C-PCB 101***	***30.15***	***335.9***	***266.0***	***25***	***28***	***337.9***	***268.0***	***25***	***28***
		PCB 101	***30.16***	***323.9***	***253.9***	***75***	***28***	***325.9***	***255.9***	***75***	***28***
		^{13}C-PCB 123	33.55	335.9	266.0	25	28	337.9	268.0	25	28
		PCB 123	33.56	323.9	253.9	75	28	325.9	255.9	75	28
		^{13}C-PCB 118	33.76	335.9	266.0	25	28	337.9	268.0	25	28
		PCB 118	33.77	323.9	253.9	75	28	325.9	255.9	75	28
		^{13}C-PCB 141	34.00	371.9	301.9	25	28	369.9	299.9	25	28
		^{13}C-PCB 114	34.19	335.9	266.0	25	28	337.9	268.0	25	28
		PCB 114	34.20	323.9	253.9	75	28	325.9	255.9	75	28
		^{13}C-PCB 153	***34.50***	***371.9***	***301.9***	***25***	***28***	***369.9***	***299.9***	***25***	***28***
		PCB 153	***34.51***	***359.8***	***289.9***	***75***	***28***	***357.8***	***287.9***	***75***	***28***
		^{13}C-PCB 105	35.15	335.9	266.0	25	28	337.9	268.0	25	28
		PCB 105	35.16	323.9	253.9	75	28	325.9	255.9	75	28
		^{13}C-PCB 138	***35.88***	***371.9***	***301.9***	***25***	***28***	***369.9***	***299.9***	***25***	***28***
		PCB 138	***35.89***	***359.8***	***289.9***	***75***	***28***	***357.8***	***287.9***	***75***	***28***
		^{13}C-PCB 167	37.64	371.9	301.9	25	28	369.9	299.9	25	28
		PCB 167	37.65	359.8	289.9	75	28	357.8	287.9	75	28
3	38.5	^{13}C-PCB 156	38.78	371.9	301.9	25	28	369.9	299.9	25	28
		PCB 156	38.79	359.8	289.9	75	28	357.8	287.9	75	28
		^{13}C-PCB 157	39.06	371.9	301.9	25	28	369.9	299.9	25	28
		PCB 157	39.07	359.8	289.9	75	28	357.8	287.9	75	28
		^{13}C-PCB 180	***39.17***	***407.8***	***337.9***	***25***	***30***	***405.8***	***335.9***	***25***	***30***
		PCB 180	***39.18***	***393.8***	***323.9***	***75***	***30***	***395.8***	***325.9***	***75***	***30***
		^{13}C-PCB 189	42.43	407.8	337.9	25	30	405.8	335.9	25	30
		PCB 189	42.44	393.8	323.9	75	30	395.8	325.9	75	30

Table 9. MS/MS Settings for Native Non-Ortho PCB Congeners and ^{13}C-Internal Standards

TS	Segment start time (min)	Analyte	RT (min)	Quant pre-cursor	Product	Dwell (ms)	CE (V)	Qual pre-cursor	Product	Dwell (ms)	CE (V)
1	19.0	^{13}C-PCB 81	20.74	301.9	232.0	25	28	303.9	234.0	25	28
		PCB 81	20.75	289.9	220.0	125	28	291.9	222.0	125	28
		^{13}C-PCB 77	21.12	301.9	232.0	25	28	303.9	234.0	25	28
		PCB 77	21.13	289.9	220.0	125	28	291.9	222.0	125	28
2	22.0	^{13}C-PCB 126	23.55	335.9	265.9	25	28	337.9	267.9	25	28
		PCB 126	23.56	323.9	253.9	125	28	325.9	255.9	125	28
3	25.0	^{13}C-PCB 169	26.26	371.9	301.9	25	28	369.9	299.9	25	28
		PCB 169	26.27	359.9	289.9	125	28	357.8	287.9	125	28

Results and Discussion

Chromatography

The multiple reaction monitoring (MRM) chromatograms for the native mono-*ortho* and ndl-PCB congeners, with an analysis time of 50 minutes, are shown in Figure 4. The multiple reaction monitoring (MRM) chromatograms for the native non-*ortho* PCB congeners, with an analysis time of 33 minutes, are shown in Figure 5.

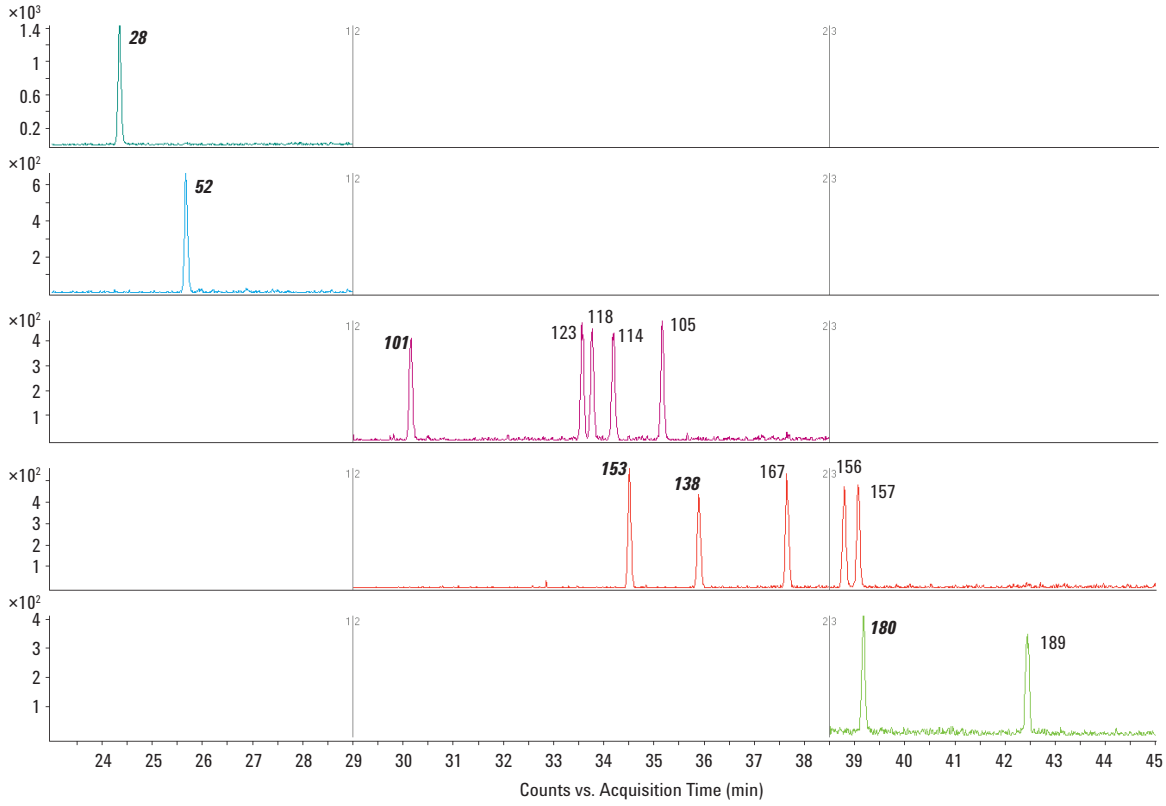


Figure 4. MRM chromatograms of native Mono-ortho and ndl-PCB congeners (ndl-PCB congeners labelled in bold italics).

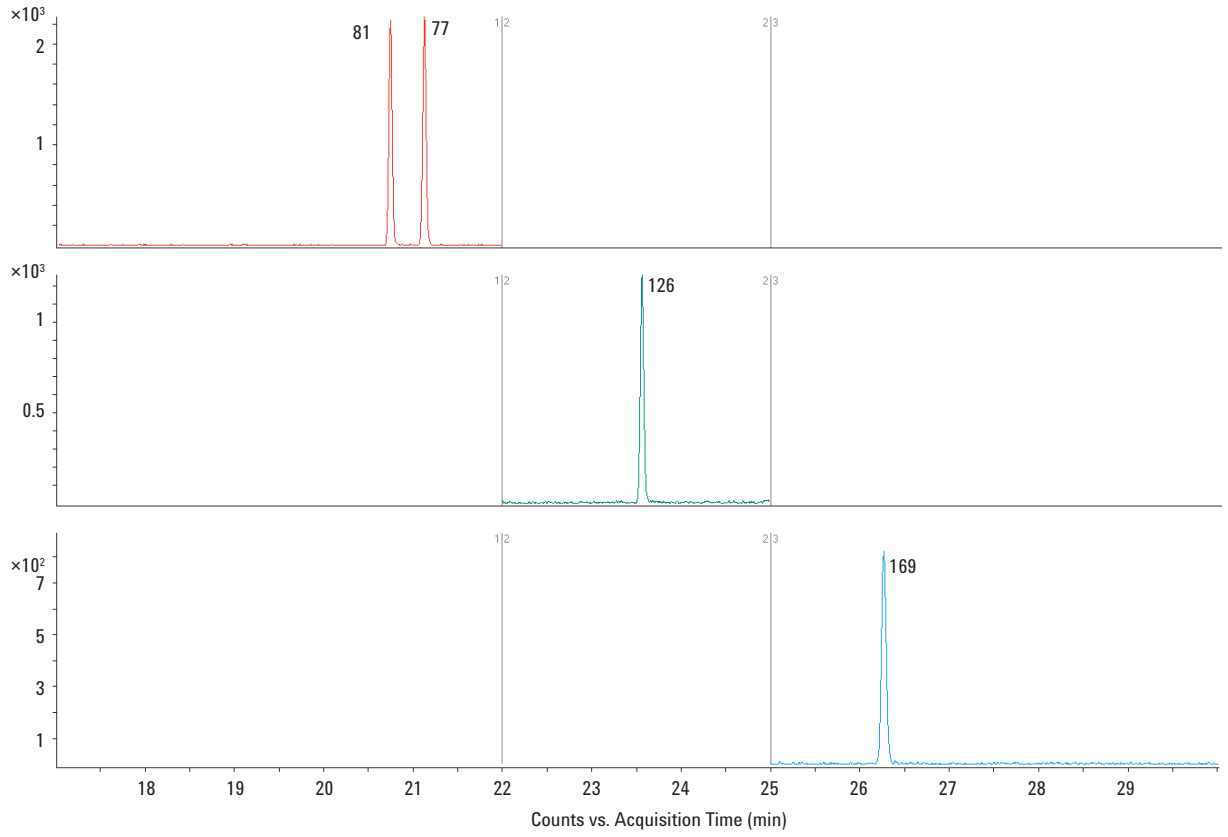


Figure 5. MRM chromatograms of native Non-ortho PCB congeners.

Linearity of Response

All PCB congeners were measured using ^{13}C-labelled internal standard (ISTD) calibration. Seven-point ISTD calibration curves were created using calibration standard solutions at the concentrations given in Tables 10 and 11, respectively.

Table 10. Concentration of Native Mono-Ortho and ndl-PCB Congeners and their ^{13}C-ISTD Calibration Standards

Mono-ortho PCB	Natives pg/µL	^{13}C pg/µL	^{13}C (PCB 180, 153, 138 141 = recovery) pg/µL
M1	0.05	5.00	50.0
M2	0.15	5.00	50.0
M3	0.50	5.00	50.0
M4	1.50	5.00	50.0
M5	5.00	5.00	50.0
M6	15.00	5.00	50.0
M7	50.00	5.00	50.0

Table 11. Concentration of Native Non-Ortho PCB Congeners and their ^{13}C- ISTD Calibration Standards

Non-Ortho PCB	Natives pg/µL	^{13}C pg/µL
C1	0.10	2.50
C2	0.25	2.50
C3	0.50	2.50
C4	1.00	2.50
C5	2.50	2.50
C6	5.00	2.50
C7	10.00	2.50

Excellent linearity was obtained over the required concentration range for all the PCB congeners and example calibration curves for PCB 126 and PCB 169 are shown in Figures 6 and 7, respectively.

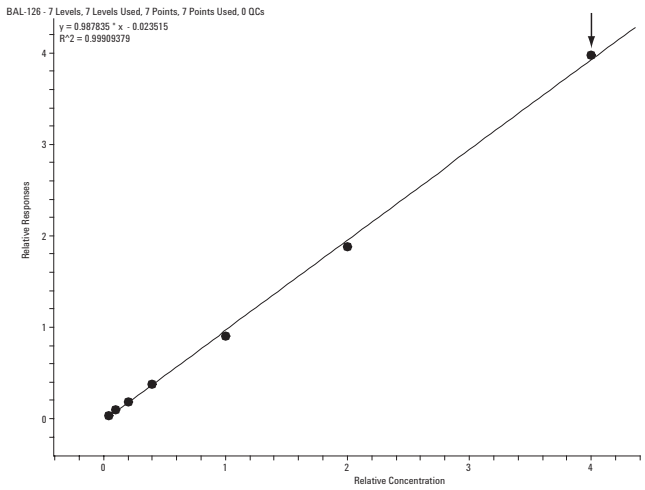


Figure 6. 7-point ISTD calibration curve for PCB 126 with linear fit.

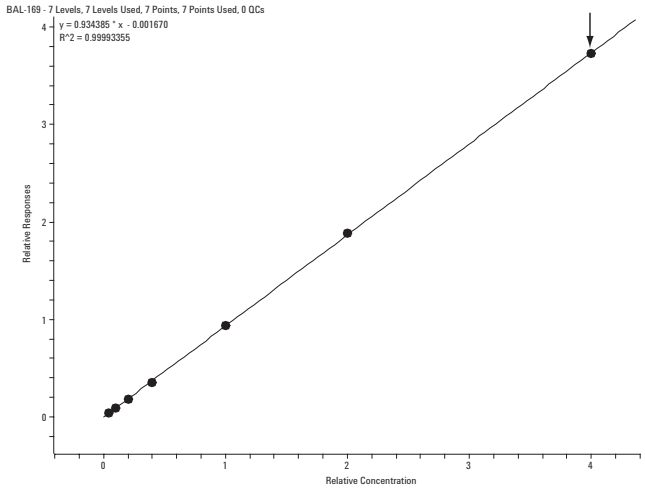


Figure 7. Seven-point ISTD calibration curve for PCB 169 with linear fit.

The linear calibration curve fits for all the PCB congeners are shown in Table 12. All analytes gave linear curve fit coefficients (R^2) greater than 0.998.

Sample Analysis

Eighty samples of four different foodstuffs and animal feed: animal feed (n = 45), cows' milk (n = 11), meat (n = 19) and liver (n = 5) were extracted and analyzed using a GC-High Resolution Mass Spectrometer (GC-HRMS) at a resolution of

Table 12. Linear Correlation Coefficients for Seven-Point ISTD Calibration Curves Over the Range 0.05 pg/µL – 50 pg/µL for Mono-Ortho and ndl-PCB Congeners and 0.1 pg/µL – 10 pg/µL for Non-Ortho PCB Congeners, Injection Volume = 2 µL

Mono-*ortho* PCB	R^2	Non-*ortho* PCB	R^2
PCB 28	0.9999	PCB 81	0.9992
PCB 52	0.9993	PCB 77	0.9991
PCB 101	0.9991	PCB 126	0.9991
PCB 123	0.9997	PCB 169	0.9999
PCB 118	0.9994		
PCB 114	0.9998		
PCB 153	0.9997		
PCB 105	0.9999		
PCB 138	0.9993		
PCB 167	0.9988		
PCB 156	0.9985		
PCB 157	0.9987		
PCB 180	0.9995		
PCB 189	0.9990		

R = 10,000. The same sample vials were then transferred to the Agilent 7000 Triple Quadrupole GC /MS system and reanalyzed.

Figure 8 shows the comparative sample results (total TEQ-dl-PCB, upperbound values) of the two sets of measurements expressed as the percentage difference between the results obtained by the GC-HRMS and GC/MS/MS analyses.

The agreement between the results obtained for the total of the 12 dl-PCB congeners on the GC-HRMS and the GC/MS/MS system for foodstuffs and animal feed samples at levels above 1 pg TEQ/g were within the range of ± 10%.

The comparative results for the 68 foodstuffs and animal feed samples that gave total dl-PCB results less than 1.2 TEQ pg/g are shown in Figure 9.

The agreement between the results obtained for the sum of the 12 dl-PCB congeners on the GC-HRMS and the GC/MS/MS system for foodstuffs and animal feed samples at levels between 0.1 and 1 pg TEQ/g was within the range of ± 15%. Only those animal feed samples with total dl-PCB congener concentrations below 0.1 TEQ pg/g gave some results with percentage differences greater than 15%.

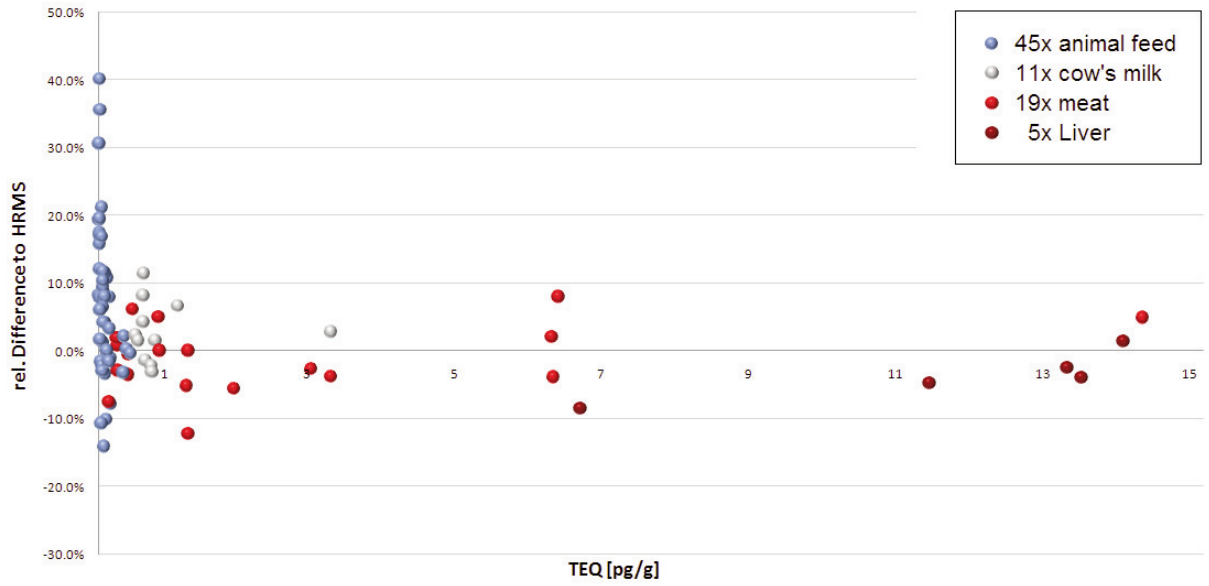


Figure 8. Comparative results for the sum of the 12 dl-PCB congeners (TEQ_{WHO98} upperbound values) for 80 foodstuffs and animal feed samples analyzed by GC-HRMS and GC/MS/MS.

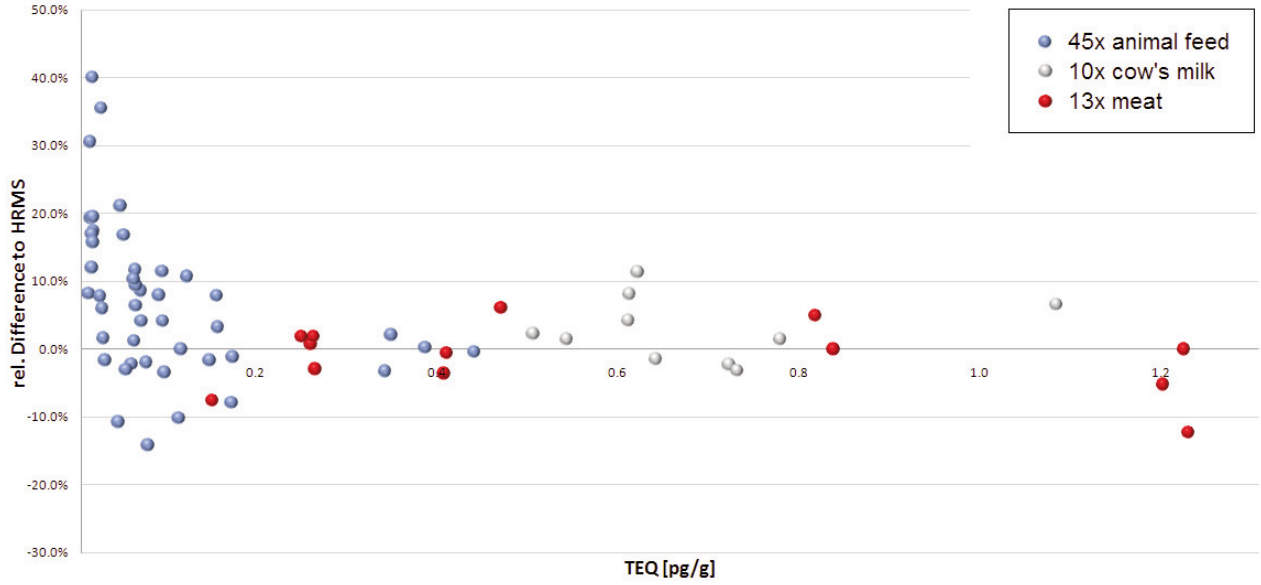


Figure 9. Comparative results for the sum of the 12 dl-PCB congeners (TEQ_{WHO98} upperbound values) for 68 foodstuffs and animal feed samples analyzed by GC-HRMS and GC/MS/MS that gave values less than ~1.2 pg TEQ/g product.

Figure 10 shows the comparative sample results (total ndl-PCB congeners, upperbound values) of the two sets of measurements expressed as the percentage difference between the results obtained by the GC-HRMS and GC/MS/MS analyses.

The agreement between the sum of the results obtained for the six ndl-PCB congeners on the GC-HRMS and the GC/MS/MS for foodstuffs and animal feed samples at levels between 0.5 and 10 ng/g was within the range of ± 10%. Some animal feed samples with total ndl-PCB congener concentrations below 0.5 ng/g gave results with percentage differences greater than + 10%.

Conclusion

The Agilent 7000 Triple Quadrupole GC/MS system provides linear, reproducible and sensitive detection of dl-PCB congeners in foodstuffs and animal feed samples down to low pg TEQ/g values. Comparison of analytical results for foodstuffs and animal feed samples by GC-HRMS and GC/MS/MS indicates the suitability of the Agilent 7000 Triple Quadrupole GC/MS system for the routine screening of dl-PCB congeners in foodstuffs and animal feed that meets the requirements of European Union legislation.

Additionally, the Agilent 7000 Triple Quadrupole GC/MS system has been shown to determine total ndl-PCB congeners in foodstuffs and animal feed samples at concentration levels of 1 ng/g product and below, which is also in good agreement with results obtained by GC-HRMS.

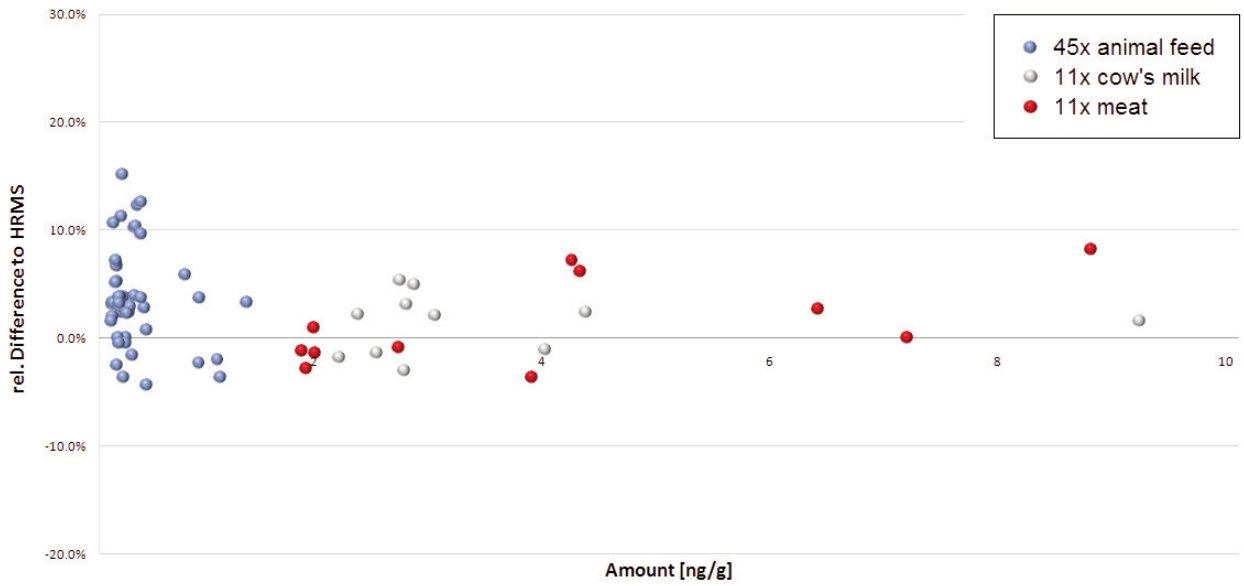


Figure 10. Comparative results for the sum of ndl-PCB congeners (upperbound values) for 67 foodstuffs and animal feed samples analyzed by GC-HRMS and GC/MS/MS that gave values less than 10 ng/g product.

References

1. EPA Method 1668, Revision A: Chlorinated Biphenyl Congeners in Water, Soil, Sediment and Tissue by HRGC/HRMS.
2. Commission Regulation (EC) No 1883/2006 of December 19, 2006, Laying down methods of sampling and analysis for the official control of levels of dioxins and dioxin-like PCBs in certain foodstuffs."
3. C Sandy, "Determination of Polychlorinated Dibenzo-p-dioxins (PCDD) and Polychlorinated Dibenzofurans (PCDF) in Foodstuffs and Animal feed using the Agilent 7000 Triple Quadrupole GC/MS System," Agilent Technologies publication 5990-6594EN (2010).
4. Commission Regulation (EC) No 1881/2006 of December 19, 2006 Setting maximum levels for certain contaminants in foodstuffs.
5. Commission Regulation (EC) No 152/2009 Annex V letter B of January 27, 2009 Laying down the methods of sampling and analysis for the official control of feed.
6. Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002, on undesirable substances in animal feed.

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Determination of Polychlorinated Dibenzo-p-dioxins (PCDD) and Polychlorinated Dibenzofurans (PCDF) in Foodstuffs and Animal Feed using the Agilent 7000 Triple Quadrupole GC/MS System

Application Note

Food Safety

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Abstract

A method has been developed on the Agilent 7000 GC Triple Quadrupole GC/MS system for the analysis of polychlorinated dibenzo-p-dioxins (PCDD) and polychlorinated dibenzofurans (PCDF) in foodstuffs and animal feed. The method was shown to give linear response over the required concentration range, good repeatability of response and quantitation down to low pg TEQ/g levels.



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Introduction

Polychlorinated dibenzo-p-dioxins (PCDD) and polychlorinated dibenzofurans (PCDF) are highly toxic persistent organic pollutants (POP) with properties that are detrimental to human health and have been linked to causing cancer, endocrine disruption, and reproductive disorders. PCDD and PCDF are not manufactured deliberately but are the byproducts of the combustion of contaminated chemical waste, chemical and pesticide manufacturing, pulp and paper bleaching processes and other sources. PCDD and PCDF are lipophilic chemicals that accumulate in the fatty tissues of animals that form part of the human food chain. It is estimated that more than 80% of human exposure to dioxins derives from food of animal origin.

There have been several incidents of dioxin contamination in the human food chain over the past 20 years. One of the most recent was in December 2008 when contaminated pork and beef products were discovered in the Republic of Ireland [1] during routine testing.

Current legislation in the United States [2] and the European Union, [3,4] requires the confirmation and quantitation of dioxins, furans, and dioxin-like polychlorinated biphenyls (dl-PCBs) in foodstuffs and animal feed by isotope dilution capillary gas chromatography/ high resolution mass spectrometry (GC/HRMS). Additionally, EU Legislation does make provisions for the screening of dioxins in foodstuffs and animal feed by other mass spectrometric techniques or by bioassays. The specific compounds covered by the EU Legislation are shown in Table 1, along with the Toxic Equivalency Factors (TEF) relating the toxicity of each individual analyte to 2,3,7,8 tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD), which is assigned a TEF value of 1. The individual concentration of each dioxin, furan, and dl-PCB found in foodstuff and animal feed samples is multiplied with the respective TEF and after summation the total concentration is expressed as the Toxic Equivalent (TEQ) in terms of pg TEQ/g fat .

Table 1. PCDD, PCDF and dl-PCB congeners specified in EU Legislation along with the TEF values stipulated in 1998 and 2005. (WHO_{05} changes indicated in italics)

Compound	TEF WHO_{98}	TEF WHO_{05}	Compound	TEF WHO_{98}	TEF WHO_{05}
Chlorinated dibenzo-p-dioxins			Non-ortho substituted PCBs		
2378-TCDD	1	1	PCB-77	0.0001	0.0001
12378-PeCDD	1	1	PCB-81	0.0001	*0.0003*
123478-HxCDD	0.1	0.1	PCB-126	0.1	0.1
123678-HxCDD	0.1	0.1	PCB-169	0.01	*0.03*
123789-HxCDD	0.1	0.1			
1234678-HpCDD	0.01	0.01			
OCDD	0.0001	*0.0003*			
Chlorinated dibenzofurans			Mono-ortho substituted PCBs		
2378-TCDF	0.1	0.1	PCB-105	0.0001	*0.00003*
12378-PeCDF	0.05	*0.03*	PCB-114	0.0005	*0.00003*
23478-PeCDF	0.5	*0.3*	PCB-118	0.0001	*0.00003*
123478-HxCDF	0.1	0.1	PCB-123	0.0001	*0.00003*
123678-HxCDF	0.1	0.1	PCB-156	0.0005	*0.00003*
234678-HxCDF	0.1	0.1	PCB-157	0.0005	*0.00003*
123789-HxCDF	0.1	0.1	PCB-167	0.00001	*0.00003*
1234678-HpCDF	0.01	0.01	PCB-189	0.0001	*0.00003*
1234789-HpCDF	0.01	0.01			
OCDF	0.0001	*0.0003*			

The maximum levels for PCDD, PCDF, and dl-PCB in certain foodstuffs as prescribed by EU legislation are given in Table 2.

Table 2. Maximum Levels for PCDD, PCDF and Dioxin-like PCB in Certain Foodstuffs, as Specified in EU Regulation (EC) No 1881/2006

	Maximum levels	
Foodstuff	Sum of Dioxins (WHO-PCDD/F-TEQ)	Sum of Dioxins and dl-PCB (WHO-PCDD/F-PCB-TEQ)
Meat and meat products (excluding edible offal) of the following animals :		
Bovine animals and sheep	3.0 pg/g fat	4.5 pg/g fat
Poultry	2.0 pg/g fat	4.0 pg/g fat
Pigs	1.0 pg/g fat	1.5 pg/g fat
Raw milk and dairy products, including butter fat	3.0 pg/g fat	6.0 pg/g fat
Hens' eggs and egg products	3.0 pg/g fat	6.0 pg/g fat

This application note describes a sensitive and reproducible method that meets the requirements of EU Legislation for the screening of PCDD and PCDF in foodstuffs using the Agilent 7000 Triple Quadrupole GC/MS/MS system.

Experimental

Calibration Standards

Native PCDD and PCDF calibration mixtures and their ^{13}C-isotope labeled internal standards were obtained from Cambridge Isotope Laboratories and Wellington Laboratories Inc.

Sample Preparation and Analysis

The most frequently used methods for the determination of PCDD/PCDF and dl-PCB in foodstuffs and animal feed combine fat extraction (for example, Soxhlet or extraction with organic solvents) with cleanup steps using different column chromatographies, such as silica gel coated with sulphuric acid, florisil, alumina, and active carbon. The isotope labeled analogues of all PCDD/PCDF with 2,3,7,8-chlorine substitution were added at the beginning of the extraction. The extract was collected as three fractions containing dioxins (2), mono-ortho-PCB and indicator PCB (1a), and non-ortho PCBs (1b), by eluting with various solvents. After addition of a syringe spike ($^{13}C_{12}$ -1,2,3,4-TCDD), the extracts were evaporated under a gentle stream of nitrogen, reconstituted with toluene, and analyzed with GC/MS/MS. The dioxin fraction was reconstituted with 20 µL of toluene, the non-ortho PCB fraction in 40 µL of toluene and the mono-ortho and indicator PCB fraction in 250 µL of toluene.

A flow diagram summarizing the sample preparation steps is shown in Figure 1.

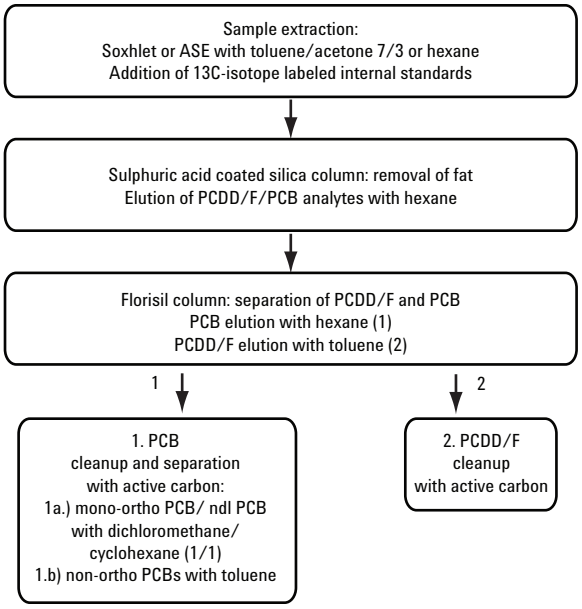


Figure 1. Flow diagram of the sample extraction and cleanup procedures.

The analysis was performed on an Agilent 7000 Triple Quadrupole GC/MS system with an Agilent 7890 GC. The 7890 GC was configured with a carbon dioxide cooled Multimode Inlet (MMI), a 2 m × 0.25 mm id uncoated deactivated capillary column linked to a pressure controlled tee (PCT) and an Agilent J&W DB-5ms UI 60 m × 0.25 mm, 0.25 µm capillary column. The chromatographic method was retention time locked (in direct connect mode) using PCB 105 to a retention time of 34.0 minutes.

The instrument conditions are listed in Table 3. A schematic diagram of the GC/MS/MS system is shown in Figure 2. The 7000 Triple Quadrupole GC/MS was operated in MS/MS-EI (electron ionization) Multiple Reaction Monitoring (MRM) mode. Each analyte and its associated ^{13}C-Internal standard was measured using two precursor ions and two different product ions. A full list of the analyte retention times and MRM settings are given in Table 4. The MRM settings consist of five time segments, each segment monitoring the tetra, penta, hexa, hepta, and octa dioxin and furan isomers, respectively. Dwell times were set to 75 ms for the native analytes and to 25 ms for all internal standards.

An Agilent 7693 Automatic Liquid Sampler with the sampler tray cooled to 5 °C was used to make 2-µL pulsed cold splitless injections using a 10-µL syringe.

Table 3. Gas Chromatograph and Mass Spectrometer Conditions

GC Conditions	
Column (1)	2.0 m x 0.25 mm uncoated siltek deactivated fused silica
Pressure controlled tee	Agilent p/n G3186B
Column (2)	Agilent J&W DB-5ms UI 60 m × 0.25 mm, 0.25 µm (122-5562UI)
Back Flush time	15.0 minutes after injection
Back flush flow rate	Column (1) - 5.0 mL/min, concurrent back flush
Injection	2 µL cold pulsed splitless using CO_2 cooled Multi Mode Inlet (MMI)
Inlet temperature program	100 °C (0.05 min), 600 °C/min to 300 °C
Injection Pulse Pressure	30 psi until 1.0 min
Purge Flow to Split Vent	40 mL/min at 1.5 min
Carrier Gas	Helium, Column (1) constant flow 0.9 mL/min Helium, Column (2) constant flow 1.0 mL/min
RTL Compound	PCB 105, Locked RT = 34.0 minutes
Oven program	130 °C (2.0 min hold), 10 °C/min to 200 °C (16 min), 5 deg °C/min to 235 °C (7 min), 5 °C/min to 350 °C
MS Transfer line temp	300 °C
MS Conditions	
Tune	EI Autotune
Gain	100
MS1 Resolution	Wide
MS2 Resolution	Wide
Dwell Times	Natives 75 ms, Labeled compounds 25 ms
Collision Energies	Table 4
Collision cell gas flows	Nitrogen at 1.5 mL/min, helium at 2.25 mL/min
MS Temperatures	Ion source 280 °C, quadrupoles 150 °C
Solvent delay	25.0 minutes

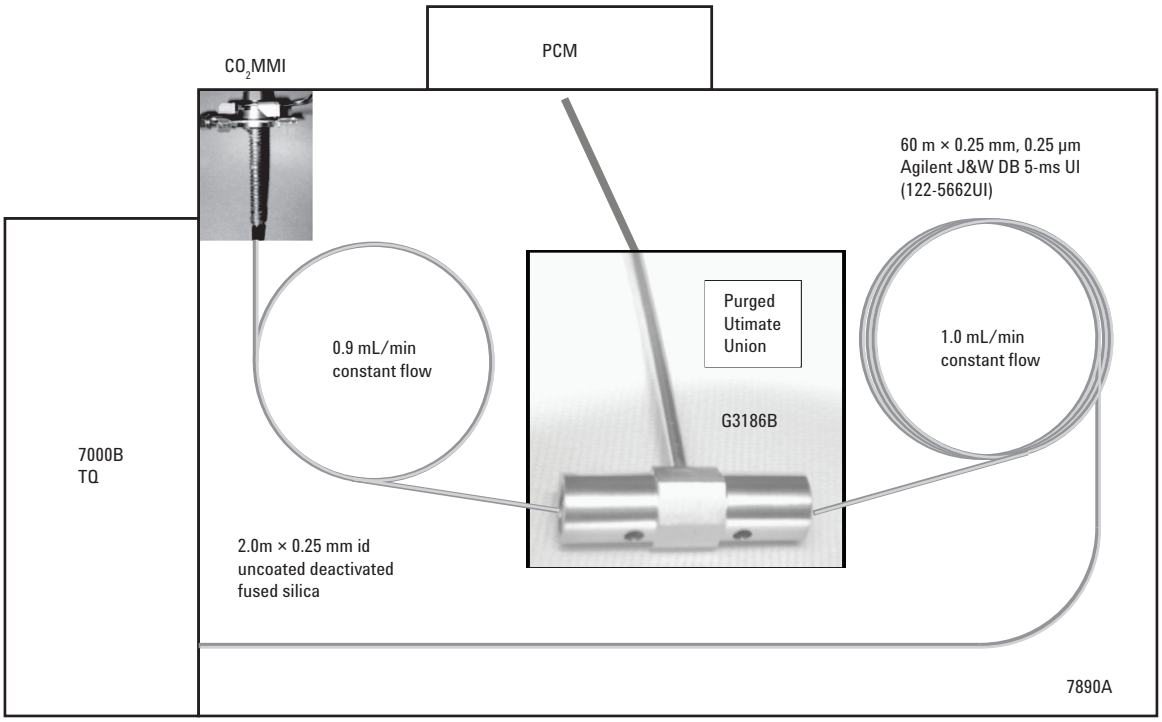


Figure 2. Schematic diagram of the GC/MS/MS hardware.

Table 4. MS/MS Settings for PCDD, PCDF and ^{13}C-Internal Standards

TS	Segment start time (min)	Peak number	Analyte	RT (min)	Quant precursor	Product	CE (V)	Qual precursor	Product	CE (V)
1	25.0									
		1	13C-2378-TCDF	35.43	315.9	251.9	33	317.9	253.9	33
		2	2378-TCDF	35.47	303.9	240.9	33	305.9	242.9	33
		3	13C-1234-TCDD	35.77	331.9	267.9	24	333.9	269.9	24
		4	13C-2378-TCDD	36.79	331.9	267.9	24	333.9	269.9	24
		5	2378-TCDD	36.80	319.9	256.9	24	321.9	258.9	24
2	40.0									
		6	13C-12378-PeCDF	42.55	351.9	287.9	35	349.9	285.9	35
		7	12378-PeCDF	42.56	339.9	276.9	35	337.9	274.9	35
		8	13C-23478-PeCDF	44.00	351.9	287.9	35	349.9	285.9	35
		9	23478-PeCDF	44.02	339.9	276.9	35	337.9	274.9	35
		10	13C-12378-PeCDD	44.45	365.9	301.9	25	367.9	303.9	25
		11	12378-PeCDD	44.48	355.9	292.9	25	353.9	290.9	25
3	46.0									
		12	13C-123478-HxCDF	48.04	385.8	321.9	35	387.8	323.9	35
		13	123478-HxCDF	48.06	373.8	310.9	35	375.8	312.9	35
		14	13C-123678-HxCDF	48.21	385.8	321.9	35	387.8	323.9	35
		15	123678-HxCDF	48.22	373.8	310.9	35	375.8	312.9	35
		16	13C-234678HxCDF	48.96	385.8	321.9	35	387.8	323.9	35
		17	234678-HxCDF	48.97	373.8	310.9	35	375.8	312.9	35
		18	13C-123478-HxCDD	49.17	403.8	339.8	25	401.8	337.9	25
		19	123478-HxCDD	49.19	389.8	326.9	25	391.8	328.8	25
		20	13C-123678-HxCDD	49.30	403.8	339.8	25	401.8	337.9	25
		21	123678-HxCDD	49.32	389.8	326.9	25	391.8	328.8	25
		22	13C-123789HxCDD	49.63	403.8	339.8	25	401.8	337.9	25
		23	123789-HxCDD	49.65	389.8	326.9	25	391.8	328.8	25
		24	13C-123789-HxCDF	50.04	385.8	321.9	35	387.8	323.9	35
		25	123789-HxCDF	50.06	373.8	310.9	35	375.8	312.9	35
4	51.0									
		26	13C-1234678-HpCDF	51.84	419.8	355.8	36	421.8	357.8	36
		27	1234678-HpCDF	51.86	409.8	346.8	36	407.8	344.8	36
		28	13C-1234678-HpCDD	53.11	437.8	373.8	25	435.8	371.8	25
		29	1234678-HpCDD	53.13	423.8	360.8	25	425.8	362.8	25
		30	13C-1234789-HpCDF	53.69	419.8	355.8	36	421.8	357.8	36
		31	1234789-HpCDF	53.70	407.8	344.8	36	409.8	346.8	36
5	55.0									
		32	13C-OCDD	56.23	469.7	405.8	26	471.7	407.8	26
		33	OCDD	56.24	457.7	394.8	26	459.7	396.8	26
		34	13C-OCDF	56.41	453.7	389.8	35	455.7	391.8	35
		35	OCDF	56.42	441.7	378.8	35	443.7	380.8	35

Capillary flow technology and backflushing have proven to be invaluable tools in improving method robustness and chromatographic integrity for GC/MS analysis of samples with high matrix content [5]. Backflushing removes high-boiling matrix components from the system that would otherwise remain behind from injection to injection, causing retention time shifts, loss of chromatographic peak shapes, and eventual contamination of the mass spectrometer ion source.

The 2-m precolumn and pressure controlled tee (PCT) were used to provide concurrent backflushing of the precolumn during the chromatographic run. Concurrent backflushing is a technique that works well in methods employing long (60 m) capillary columns that cannot be efficiently backflushed in postrun mode using a post-column connection to the PCT. The flow rate in the precolumn is reversed once all the analytes of interest have moved in to the 60-m analytical column. This is implemented by automatically reducing the pressure at the MMI 15 minutes after the sample injection takes place, which was determined experimentally by a sequence of standard injections with varying backflush times.

Results and Discussion

Chromatography

The chromatographic separation of the native PCDD and PCDF congeners is shown in Figure 3. The peak numbers refer to the entries in Table 4. The chromatographic run time for each sample was 60 minutes.

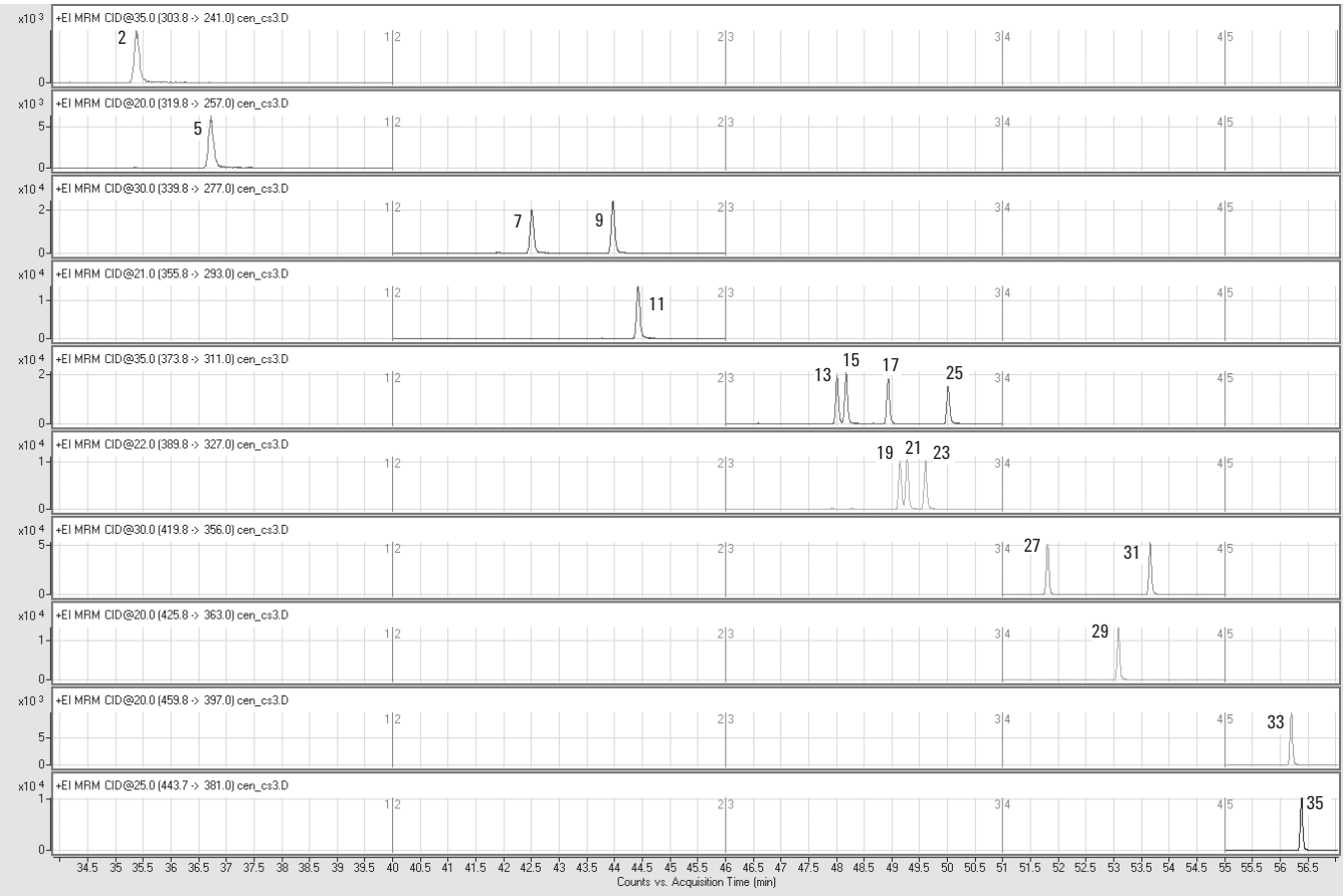


Figure 3. MRM chromatograms of native PCDD and PCDF congeners. (Peak numbers refer to analytes listed in Table 4).

Linearity of Response and Sensitivity

The PCDD and PCDF were measured using ^{13}C-labeled internal standard (ISTD) calibration. The seven-point ISTD calibration curves for 2,3,7,8-TCDD and 1,2,3,7,8-PeCDD are shown in Figures 4 and 5, respectively. Excellent linearity is shown for 2 µL injections of the calibration standards over the concentration range of 0.05 pg/µL to 5 pg/µL with R^2 values > 0.999. The insets in Figures 4 and 5 show the R^2 values for the average of response factors for these two dioxin congeners.

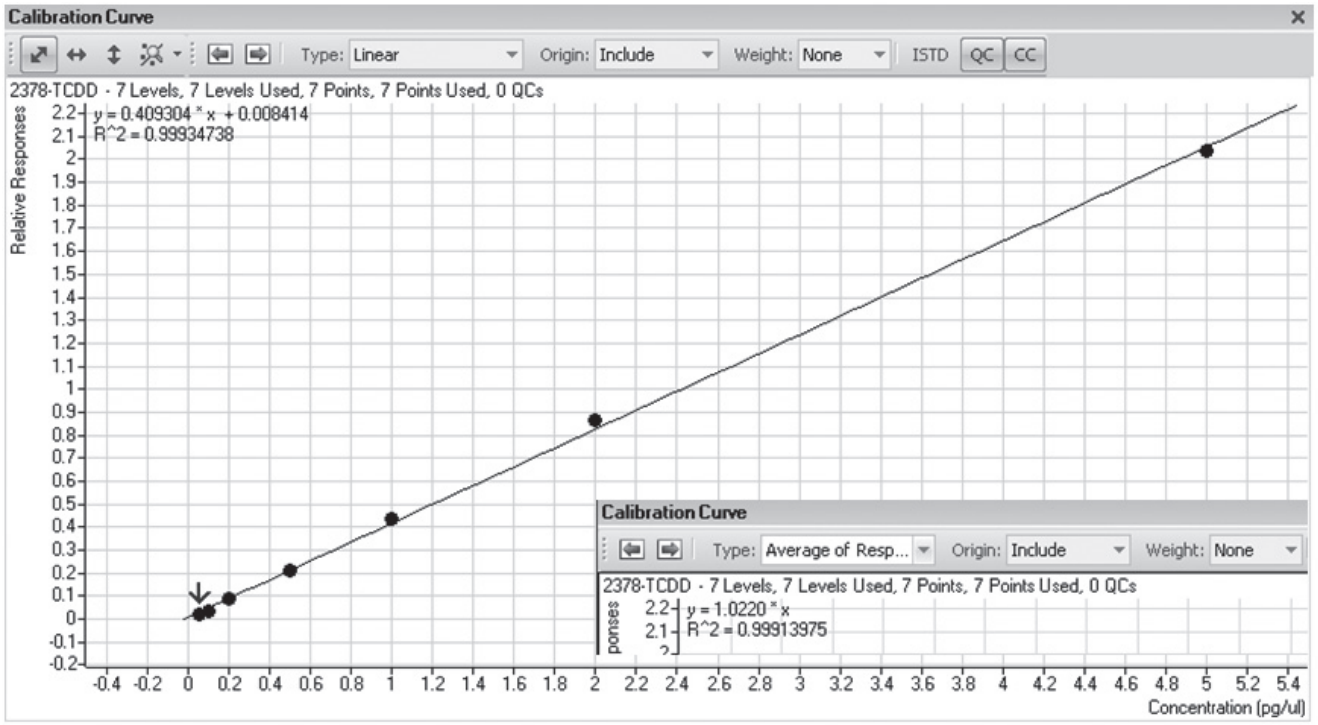


Figure 4. Calibration curve for 2,3,7,8-TCDD with both linear fit and average of response factors (inset).

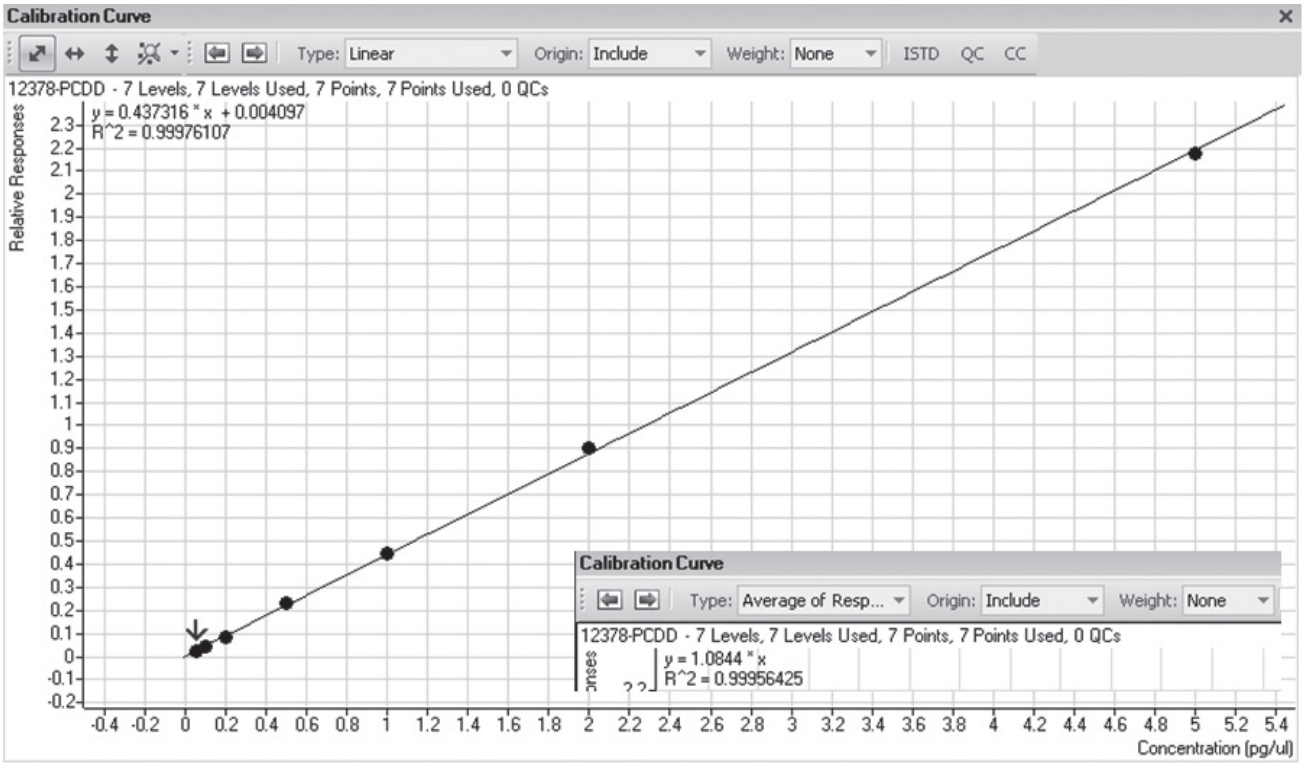


Figure 5. Calibration curve for 1,2,3,7,8-PeCDD with both linear fit and average of response factors (inset).

The linear calibration curve fits for all 17 PCDD and PCDF congeners are shown in Table 5.

*Table 5. Linear Correlation Coefficients for Seven-Point ISTD Calibration Curves over the Range 100 fg – 10 pg Injected. * (OCDD 500 fg – 50 pg injected)*

Analyte	R^2	Analyte	R^2
2378-TCDD	0.99934	2378-TCDF	0.99984
12378-PeCDD	0.99976	12378-PeCDF	0.99909
123478-HxCDD	0.99994	23478-PeCDF	0.99995
123678-HxCDD	0.99905	123478-HxCDF	0.99971
123789-HxCDD	0.99977	123678-HxCDF	0.99983
1234678-HpCDD	0.99945	234678-HxCDF	0.99953
OCDD*	0.99780	123789-HxCDF	0.99972
		1234678-HpCDF	0.99971
		1234789-HpCDF	0.99991
		OCDF	0.99907

The selected reaction monitoring (MRM) chromatograms for the native PCDD and PCDF congeners for the lowest calibration standard (0.1 pg on-column) are shown in Figure 6.

Peak Area Precision and Peak Area Ratio Precision

The peak area precision (raw peak area) for the native PCDD and PCDF congeners was determined by spiking a pork fat extract with native PCDD and PCDF at a concentration of 100 fg/µL and ^{13}C-ISTD at 1pg/µL, respectively. A sequence of replicate 2-µL cold pulsed splitless injections (n = 15) was made. The %RSD values for the peak areas of native PCDD/PCDF and ^{13}C-ISTD are shown in Figure 7. All native congeners gave precision values less than 10% except for 1,2,3,4,6,7,8-HpCDD, which gave a value of 11.9 %. This slightly higher result may be attributed to the somewhat lower absolute response of this particular analyte. The ^{13}C-ISTD gave %RSD values of 5% or lower.

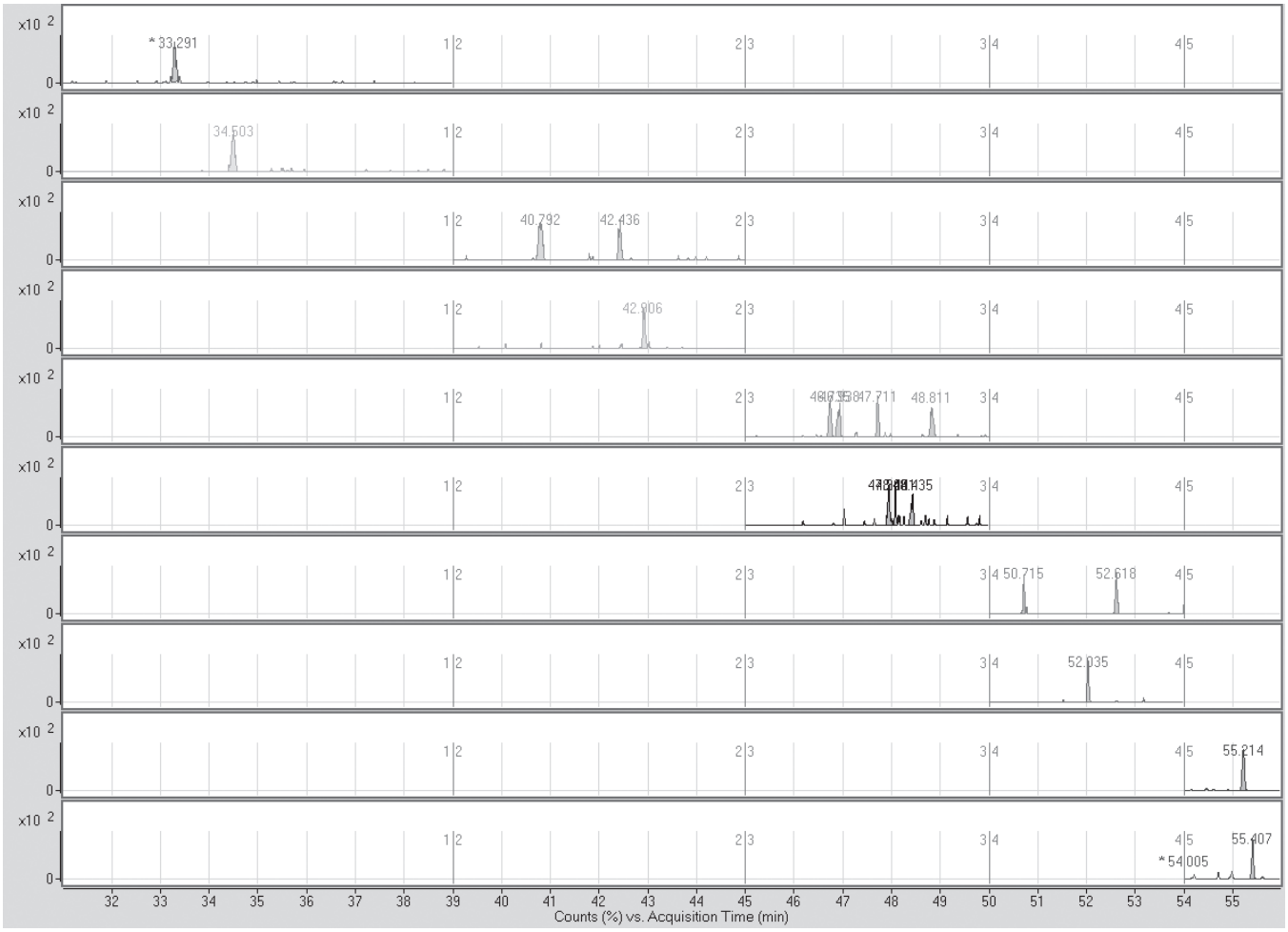


Figure 6. MRM chromatograms of native PCDD and PCDF congeners. Lowest calibration standard, 100 fg injected on-column (OCDD 500 fg injected on-column).

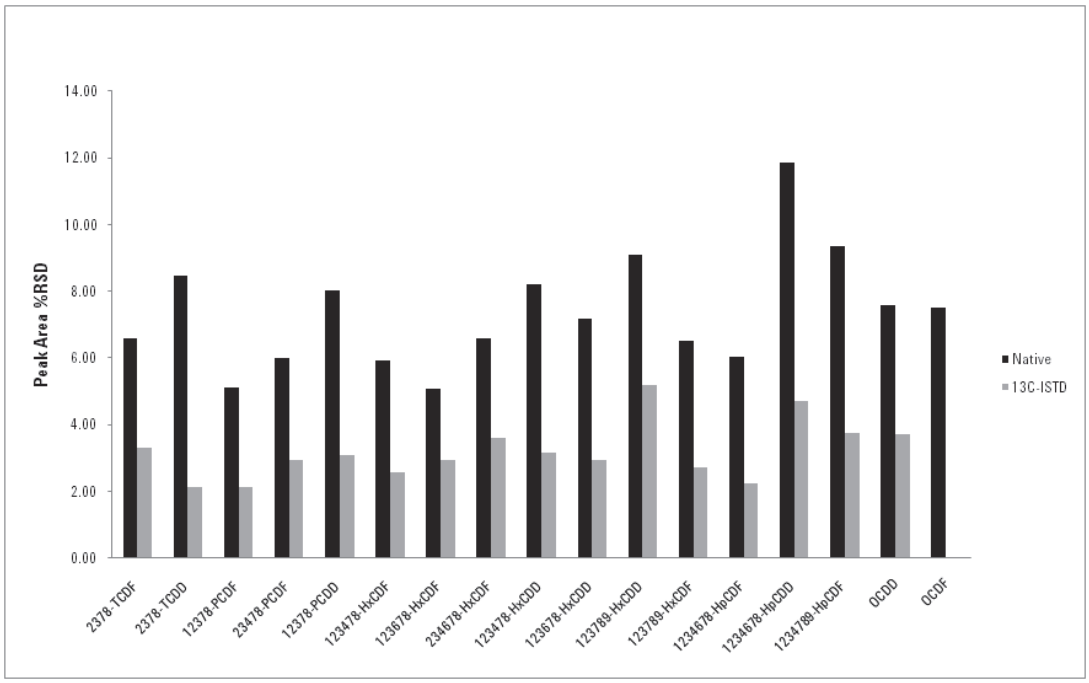


Figure 7. Repeatability of peak areas for native PCDD and PCDF congeners and ^{13}C-ISTD (n=15).

The peak area ratio precision (analyte peak area divided by its ^{13}C-ISTD peak area) was also determined for the 15 replicate injections. The %RSD values for the ratio of peak areas are shown in Figure 8. All analytes gave precision values less than 10% except for 1,2,3,4,6,7,8-HpCDD, which gave a %RSD value of 13.6 %.

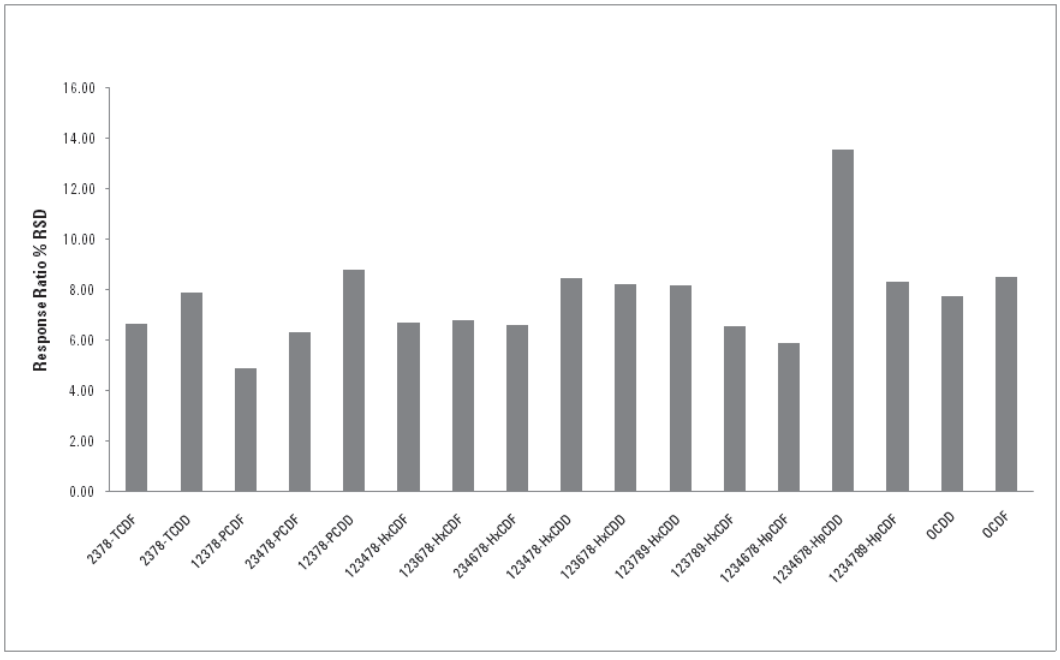


Figure 8. Repeatability of response ratios for native PCDD and PCDF congeners (n=15).

Sample Analysis

The MRM chromatograms for the tetra- and penta-CDF isomers present in a hen's egg extract are shown in Figure 9. The concentrations of the 2,3,7,8-TCDF, 1,2,3,7,8-PeCDF, and 2,3,4,7,8-PeCDF were determined as 15.5, 3.4, and 3.1 pg/g fat, respectively.

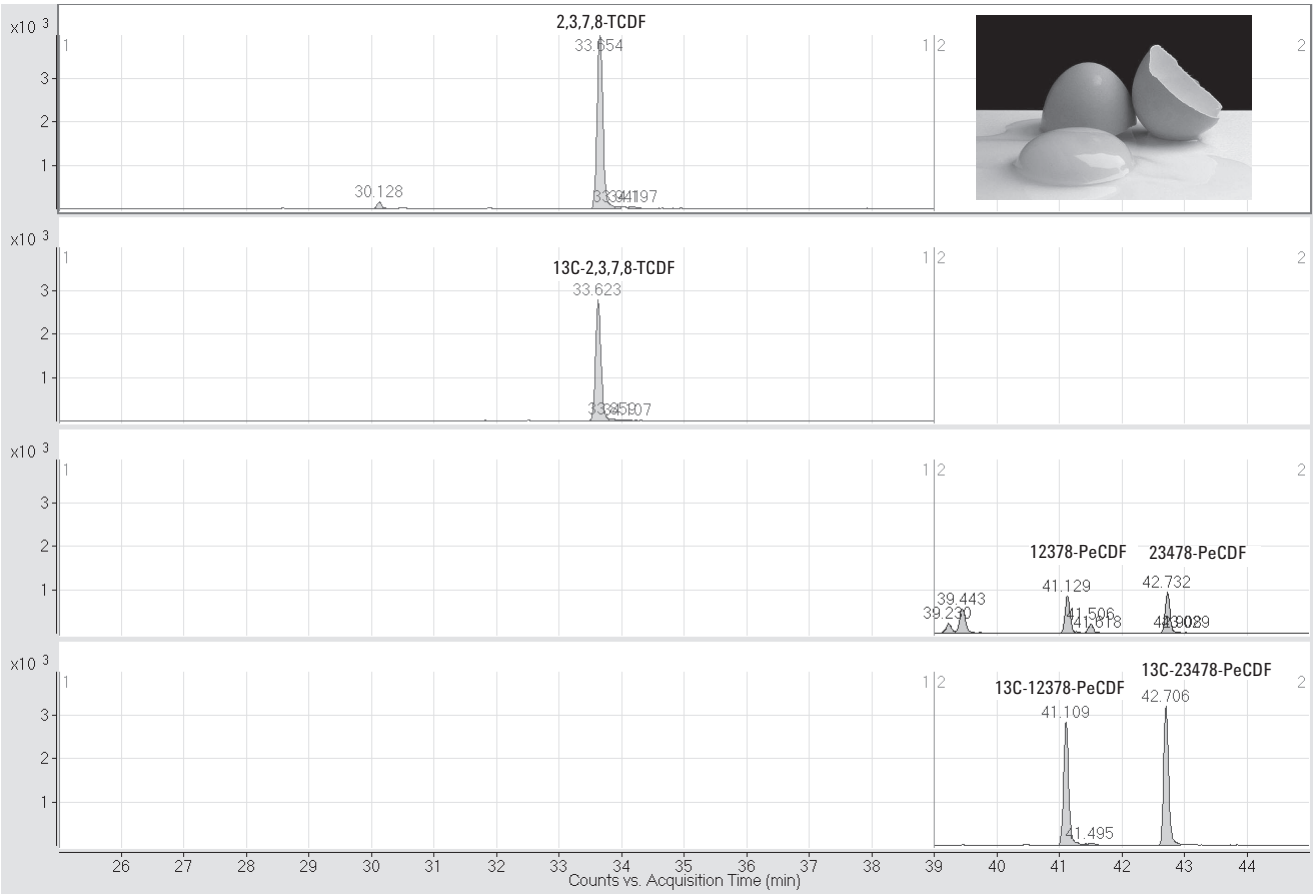


Figure 9. MRM chromatograms of tetra- and penta-CDF congeners and ^{13}C-ISTDs from a hen's egg extract.

An advantage of screening for dioxins and furans in foodstuffs and animal feed by GC/MS/MS, as opposed to using bio-assay, is that each congener is individually quantified. This allows the quantitative contribution of each PCDD and PCDF congener within the sample to be plotted. This, in turn, may provide a valuable clue as to the likely source of the contamination. The quantitative distribution of PCDD and PCDF congeners in a hen's egg extract is shown in Figure 10.

Samples of five different foodstuffs: liver (n=5), beef (n=4), poultry meat (n=6), hens' eggs (n=5), and animal feed (n=31) were extracted and analyzed using a GC High Resolution Mass Spectrometer (GC/HRMS) at a resolution of R=10,000. The same sample vials were then transferred to the Agilent 7000 GC/MS/MS system and reanalyzed.

Figure 11 shows the comparative sample results (upperbound values) of the two sets of measurements expressed as the percentage difference between the results obtained by the GC/HRMS and GC/MS/MS analyses.

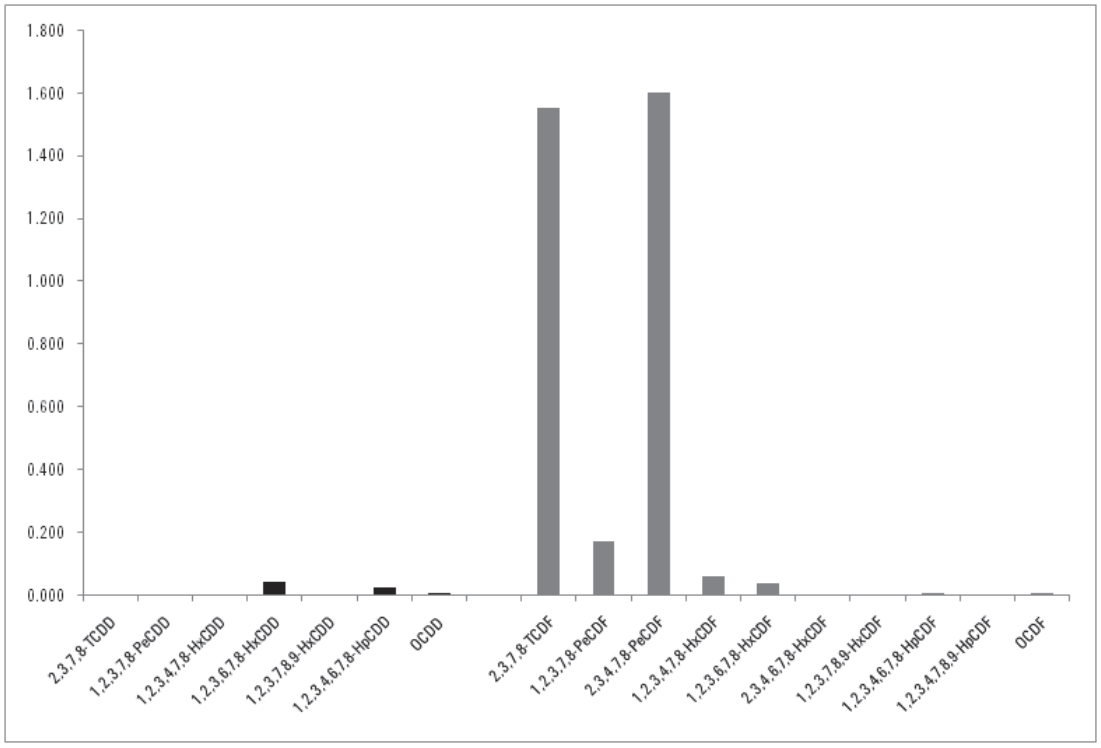


Figure 10. Quantitative distribution of PCDD and PCDF congeners in a hen's egg extract, units are pg TEQ/g fat.

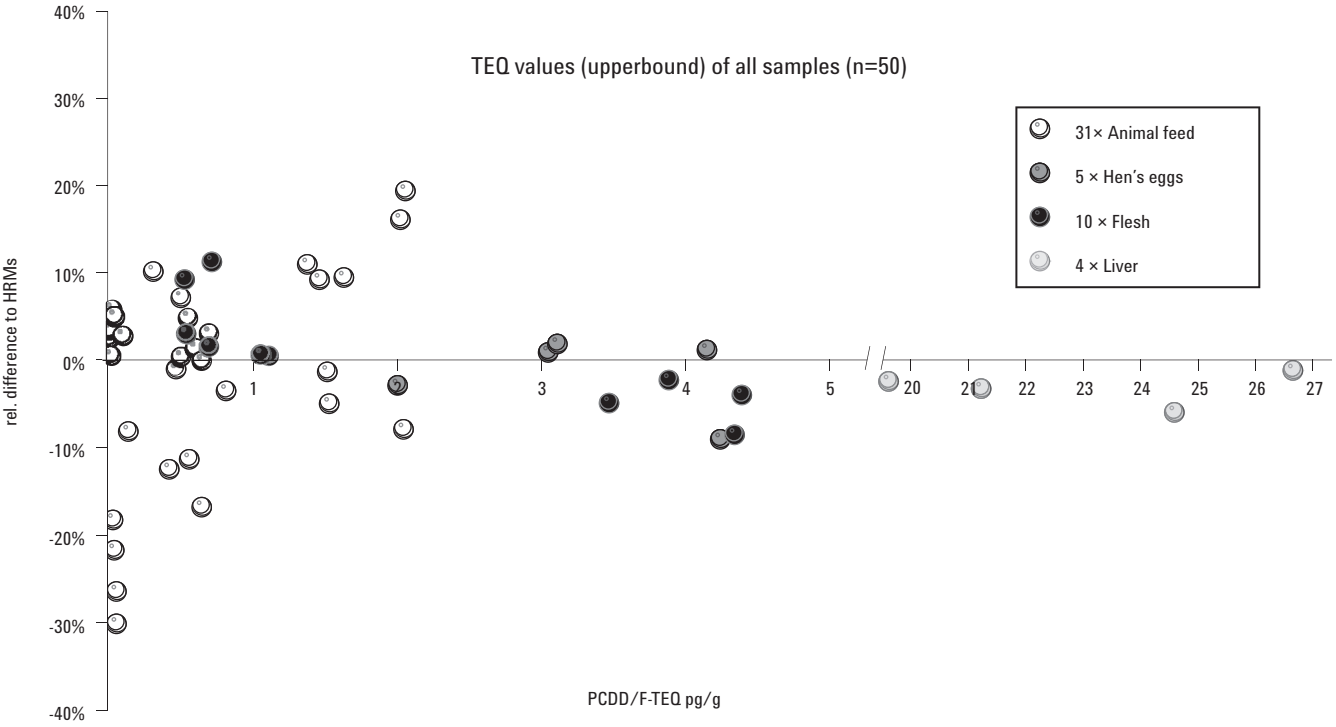


Figure 11. Comparative results (upperbound concentration values) for 50 food and feed samples analyzed by GC/HRMS and GC/MS/MS.

Figure 12 shows the comparative sample results (upperbound concentration values) of the two sets of measurements for those samples that gave values less than 3 pg TEQ/g. Additionally, Figure 12 is annotated with the Maximum Levels (ML) and Action Levels (AL) for poultry meat, hens' eggs, and animal feedstuff as prescribed by European Union Legislation.

Foodstuff samples that exhibited levels of total PCDD and PCDF congeners at upperbound values greater than 3 pg TEQ/g gave quantitative results by GC/MS/MS that were within ± 10% of the value obtained by GC/HRMS.

The agreement between the results obtained on the GC-HRMS and the GC/MS/MS for foodstuff and feedstuff samples at levels between 0.5 and 3 pg/g TEQ were within the range of ± 10 to ± 20%.

Only those animal feedstuff samples with results of 0.1–0.2 pg TEQ/g (well below the EU action level of 0.5 pg TEQ/g) gave result differences > 20% between the GC/HRMS and GC/MS/MS. This greater differential may be attributed to the results being expressed as the upperbound values and the lower limit of detection (LOD) achievable by the GC/HRMS system. In Animal Feedstuff samples, the GC/HRMS gave a range of LODs for the PCDD and PCDF congeners between 0.01–0.06 pg/g, whereas the GC/MS/MS gave 0.02–0.08 pg/g.

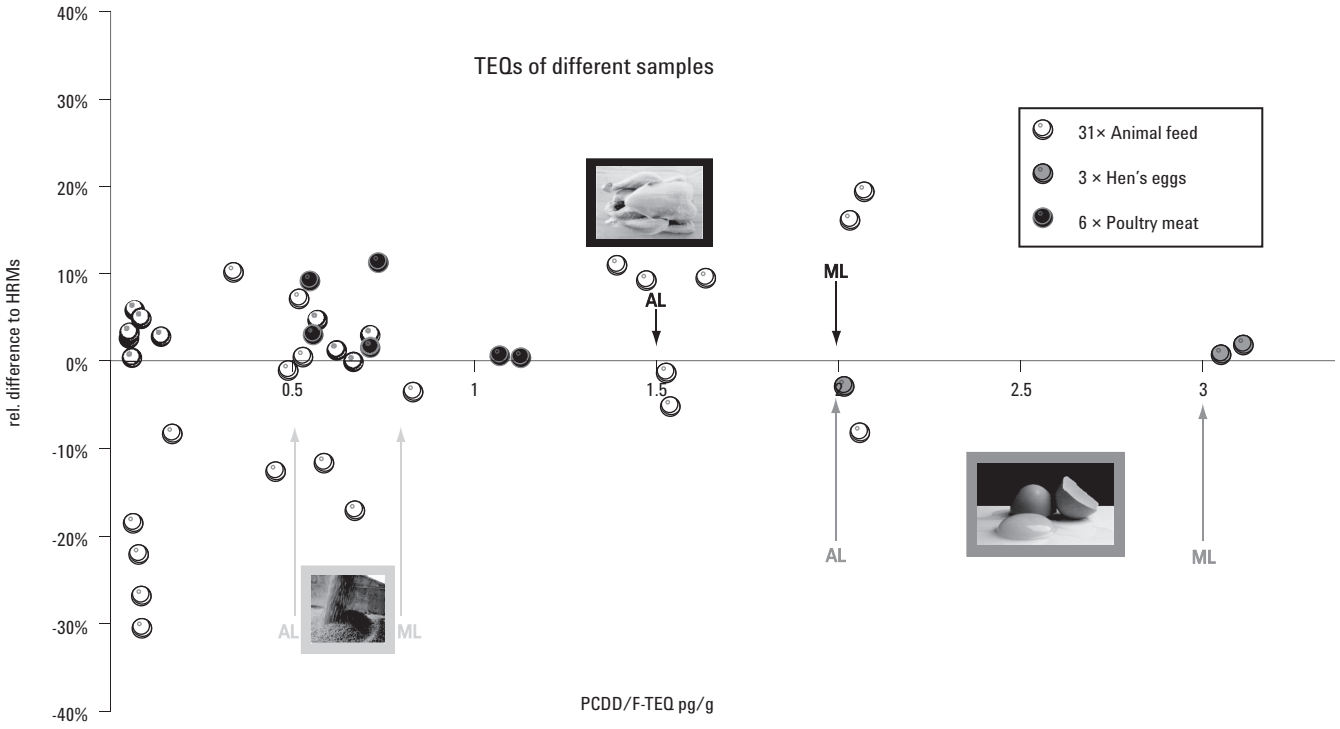


Figure 12. Comparative results (upperbound concentration values) for 40 food and feed samples analyzed by GC/HRMS and GC/MS/MS that gave values less than ~3 pg TEQ/g. ML= EU Maximum Level, AL = EU Action Level.

Conclusion

The Agilent 7000 Triple Quadrupole GC/MS system provides linear, reproducible and sensitive detection of PCDD and PCDF congeners in foodstuffs and animal feed samples down to low pg TEQ/g values. Comparison of analytical results of foodstuff and animal feed extracts by GC/HRMS and GC/MS/MS indicates the suitability of the Agilent 7000 Triple Quadrupole GC/MS system for routine screening of PCDD and PCDF congeners in foodstuffs and feedstuffs that meets the requirements of European Union legislation.

References

1. UK Food Standards Agency Ref 74/2008 - Emerging incident involving presence of dioxins in Irish pork meat.
2. US EPA Method 1613B - Tetra- through Octa-Chlorinated Dioxins and Furans by Isotope dilution HRGC/HRMS.
3. Commission Regulation (EC) No 1883/2006 of 19 December 2006 Laying down methods of sampling and analysis for the official control of levels of dioxins and dioxin-like PCBs in certain foodstuffs.
4. Commission Regulation (EC) No. 152/2009 Annexe V letter B, 27 January 2009, "Laying Down the Methods of Sampling and Analysis for the Official Control of Feed".
5. C. Sandy, "Improving GC-MS Method Robustness and Cycle Times using Capillary Flow Technology and Back Flushing," Agilent Technologies publication 5990-3367EN (2009).

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A reliable and routine GC/MS/MS

Method for the Determination of Chemical Contaminants in Marine Shellfish

FOOD SAFETY



Need a sensitive, reliable and robust method for the routine determination of organo-chlorine pesticides, polyaromatic hydrocarbons and polychlorinated biphenyls in marine shellfish?



Chemical contaminants that are released into the marine environment maybe ingested (absorbed) by fish and shellfish and thus become introduced in to the human food chain. Lipophillic chemicals such as Organo-chlorine pesticides (OCPs) and Polychlorinated biphenyls (PCBs) can bio-accumulate in the fatty tissues of marine fish and shellfish, the longer an organism is exposed to a contaminated environment, the higher the likely levels of contaminants.

The Clean Seas Environmental Monitoring Program (CSEMP) is an initiative designed to monitor the levels of chemical contamination in the United Kingdom's coastal and estuarine areas. The major drivers for this program are:

- To meet the mandatory monitoring requirements under Oslo and Paris Convention (OSPAR) Joint Assessment and Monitoring Program (JAMP)
- Compliance with EC Directives

Agilent Technologies has partnered with a leading European Analytical Laboratory to develop a sample preparation method based on a modified QuEChERS extraction along with a GC/MS/MS method for the determination of selected OCPs, PAHs and PCBs in marine shellfish (Mussel) tissue. The GC/MS/MS method provides reproducible and sensitive determination of OCPs, PCBs and PAHs that employs large volume (solvent vent) injection using a Multimode inlet (MMI) and post-column, post-run backflush in order to remove high boiling matrix components that would otherwise remain in the column between analyses and subsequently cause degradation of chromatographic performance and contamination of the mass spectrometer ion source.

The analytical method meets the detection limit requirements of 0.1 µg/Kg for OCPs and PCBs, and 0.5–1.0 µg/Kg for PAHs.

Compounds

- 16 Organo-chlorine pesticides
- 19 Polyaromatic hydrocarbons
- 7 Polychlorinated biphenyls

Method for the Determination of Chemical Contaminants in Marine Shellfish

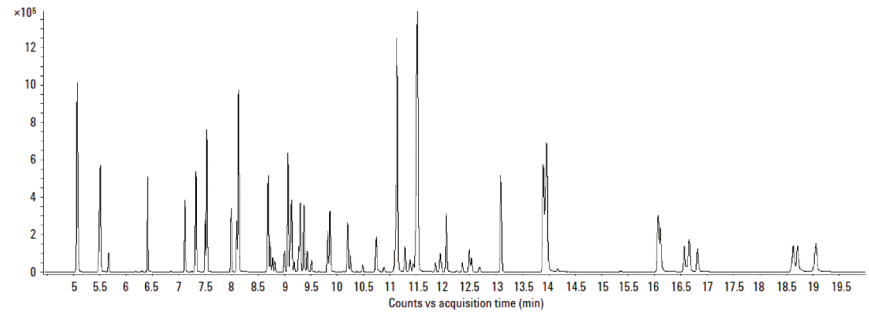


Figure 1. *TIC MRM chromatogram of a calibration standard mixture of OCPs, PAHs and PCBs*.*

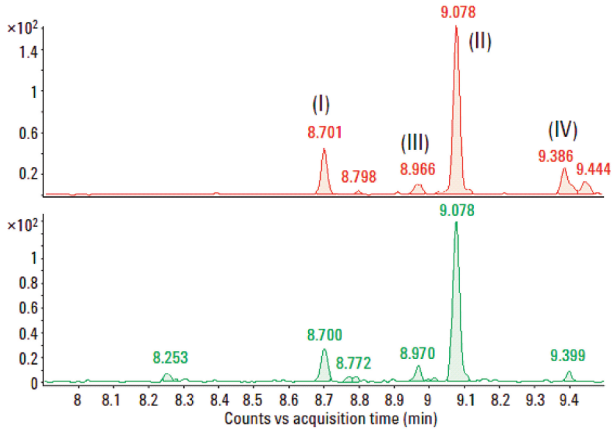


Figure 2. *MRM Chromatograms for (I) incurred a-HCH and (II) incurred g-HCH in mussel sample, Concentrations 0.06 and 0.30 µg/Kg, respectively. Peaks (III) and (IV) are traces of incurred b-HCH and d-HCH, respectively.*

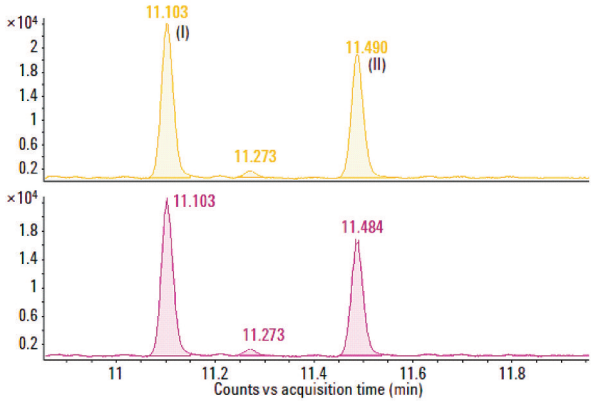


Figure 3. *MRM Chromatograms for (I) incurred Fluoranthene and (II) incurred Pyrene in mussel sample, Concentrations 8.64 and 5.83 µg/Kg, respectively.*

* Full analytical details are available in Agilent Technologies publication 5990-7714EN.

Key Benefits

- Sample extraction based on a modified QuEChERS method.
- Recoveries for all analytes in the range of 85.4% to 123.9%.
- Large volume (solvent vent mode) injection using a multimode inlet ensuring required detection limits are met.
- Retention time locked chromatographic method for ease of set up and on-going maintenance.
- Capillary flow technology provides post-column, post-run backflush to ensure chromatographic method robustness and prevent contamination of the MS ion source with high-boiling matrix.
- Mass Hunter software that is very powerful yet easy to master, providing excellent data review capabilities and easy, flexible reporting.

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Agilent 6460 LC-QQQ – Highly Sensitive and Robust Analysis for Lipophilic Marine Toxins in Shellfish

Application Note

Environmental, Food Safety

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Abstract

Marine biotoxins are increasingly threatening the human health in many parts of the world. While the toxins are formed by microscopic planktonic algae of several genera usually at very low concentrations, they can be accumulated in bivalve molluscs to reach toxic doses. The consumption of contaminated shellfish or fish can lead to human poisoning or even death. In animals and humans there are four recognized symptom types of shellfish poisoning: Diarrhetic shellfish poisoning (DSP), paralytic shellfish poisoning (PSP), neurotoxic shellfish poisoning (NSP) and amnesic shellfish poisoning (ASP).

The official standard reference method in the EU (Commission Regulation EC No. 2074/2005) for monitoring of lipophilic biotoxins is the mouse bioassay (MBA). Recently the MBA has been considered to be inadequate (The EFSA Journal, 2009, 1306, 1-1) by the European Food Safety Authority (EFSA) because of high variability, insufficient detection capability and limited specificity. A specific, alternative method for the determination of marine biotoxins with low limits of detection (LOD) has been requested.

This document describes a highly sensitive LC-MS/MS method for the determination of lipophilic marine toxins, including ocadaic acid (OA), dinophysistoxins (DTX toxins) and polyether toxins like azaspiracids, pectenotoxins and yessotoxins. Preliminary tests have shown that this method is also applicable to further lipophilic toxins like domoic acid, gymnodimine or spirolides, if reference compounds are available.



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Introduction

Marine biotoxins are formed as secondary metabolites by marine planctonic algae typically at very low concentrations. During an algal bloom the concentrations of the toxins can reach toxic levels in particular due to the accumulation of the toxins in bivalve mollusks. In the last two decades the number and intensity of harmful algae blooms has increased and a bigger number of toxic compounds have been found in the marine food chain (Marine biotoxins. FAO Food and Nutrition Papers (80) 2004). A more sensitive and more reliable method for the determination of lipophilic marine toxins has been requested by the European Food Safety Authority (EFSA) since the current official standard reference method in the EU, the mouse bioassay has been considered to be inadequate because of high variability, insufficient detection capability and limited specificity.

Your Challenges

The challenge is to have a sensitive and robust analytical method available for the determination of lipophilic marine toxins, including ocadaic acid, dinophysistoxins (DSP toxins) and polyether toxins like azaspiracids, pectenotoxins and yessotoxins in seafood. The method should have the potential to be extended to further lipophilic toxins like domoic acid, gymnodimine or spirolides. The limits of detection (LODs) need to be below the maximum residue limits (MRLs) which are specified for azaspiracids, the sum of okadaic acid, dinophysistoxins and pectenotoxins, and yessotoxins under EU legislation. Quantitation and confirmation of compounds at a trace level can be complicated by the matrix. As reference material for many compounds is not available tools are required to quantify several compounds based on the response of others.

Our integrated approach

Modern methods for the analysis of marine biotoxins are based on physicochemical techniques like LC-MS/MS. Triple quadrupole mass spectrometry allows for drastic reduction or elimination of matrix interferences. The Multiple Reaction Monitoring (MRM) is based on the detection of a secondary "product ion" produced by the collisional dissociation of an analyte "precursor ion". Whereas the analyte precursor ion (isolated in MS1 by a SIM mechanism) has the same selectivity as SIM, the resultant product ions (isolated in MS2 by a SIM mechanism) are more likely to be unique to the target compounds leading to an increased selectivity of the MRM. The combination of unique product ions (more selectivity) and the elimination of background noise results in consistently low limits of detection even for complex matrices. The method described here is a highly sensitive and specific method for the analysis of shellfish samples for lipophilic marine toxins using the Agilent 6460 Triple Quadrupole LC-MS system in MRM mode in combination with the Agilent 1200 SL Rapid Resolution HPLC and the Agilent MassHunter Workstation software. The described sample clean-up procedure is extremely simple, and thus the method is highly applicable to routine analysis. It allows for the analysis of OA, DTX-1, DTX-2 including their esters after hydrolysis, YTX, OH-YTX, PTX-1, PTX-2, AZA-1, AZA-2, and AZA-3. Due to the lack of commercially available standards some of the toxins have to be quantified using the calibration response of other compounds using the *CopyCalibrationLevel.quant.script.*

Methods and Operation

Modern methods for analysis of marine biotoxins are based on LC-MS/MS with Triple Quadrupole systems. The sample preparation is simply done by liquid extraction and subsequent filtration. Separation is based on HPLC and quantification is done by LC-ESI-MS/MS (MRM –positive and negative mode).

Analysis steps
Liquid Phase Extraction
HPLC Analysis with linear gradient
Detection by LC-ESI-MS/MS (MRM).

Sample preparation steps
An amount of 2 g of cooked, grinded shellfish tissue is weighed. Addition of 9 mL of methanol (80 %). This extraction procedure is done twice. The two extracts are combined and filled up to 50 mL in a volumetric flask. An aliquot is filtered to remove all remaining particles (filter RC 0.45 µm) and 10 µL of this sample extract is injected for the LC-MS/MS analysis.

Structures of analytes (example)

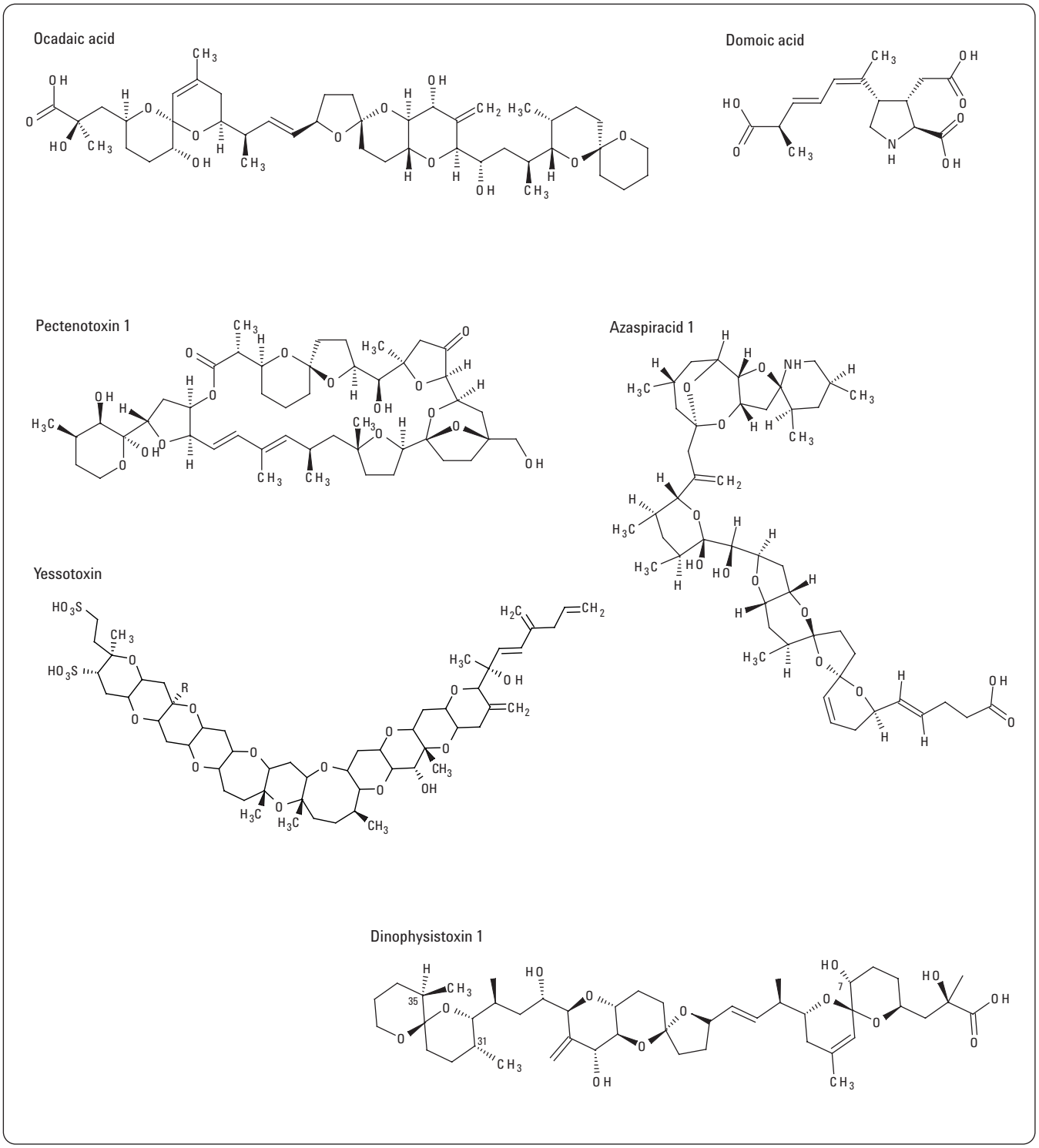


Figure 1
Chemical formulas of some marine toxins.

LC-MS/MS method

The total run time required to determine lipophilic marine toxins is less than 30 min. – The method runs in positive ionization mode for OA, DTX-1, DTX-2, PTX-1, PTX-2, AZA-1, AZA-2 and AZA-3. YTX is done in a separate run which requires a different column and mobile phase. The extract (10 µL) is injected directly to the LC-MS/MS system. For the LC method a solvent mixture of 0.1 % formic acid in Water (A) and MeOH (B) for positive ionization and 2 mM ammonium acetate in water (A) and MeOH (B) for negative ionization is used as mobile phase on a linear gradient. The column (Phenomenex Luna 5 µm C18(2) 100 Å 150 x 2.0 mm [pos. mode], ZORBAX Eclipse Plus C 8 4.6 x 75 mm 3.5 µm [neg. mode]) is held in an oven at 30 °C with a flow-rate of 0.2 mL/min.

Gradient* Time [min]	Solvent ratio B [%]
0	5
10	85
22	85
23	5
30	5

Table 1
Gradient settings.
*Applicable for positive and negative ionization mode.

Sample Preparation Steps

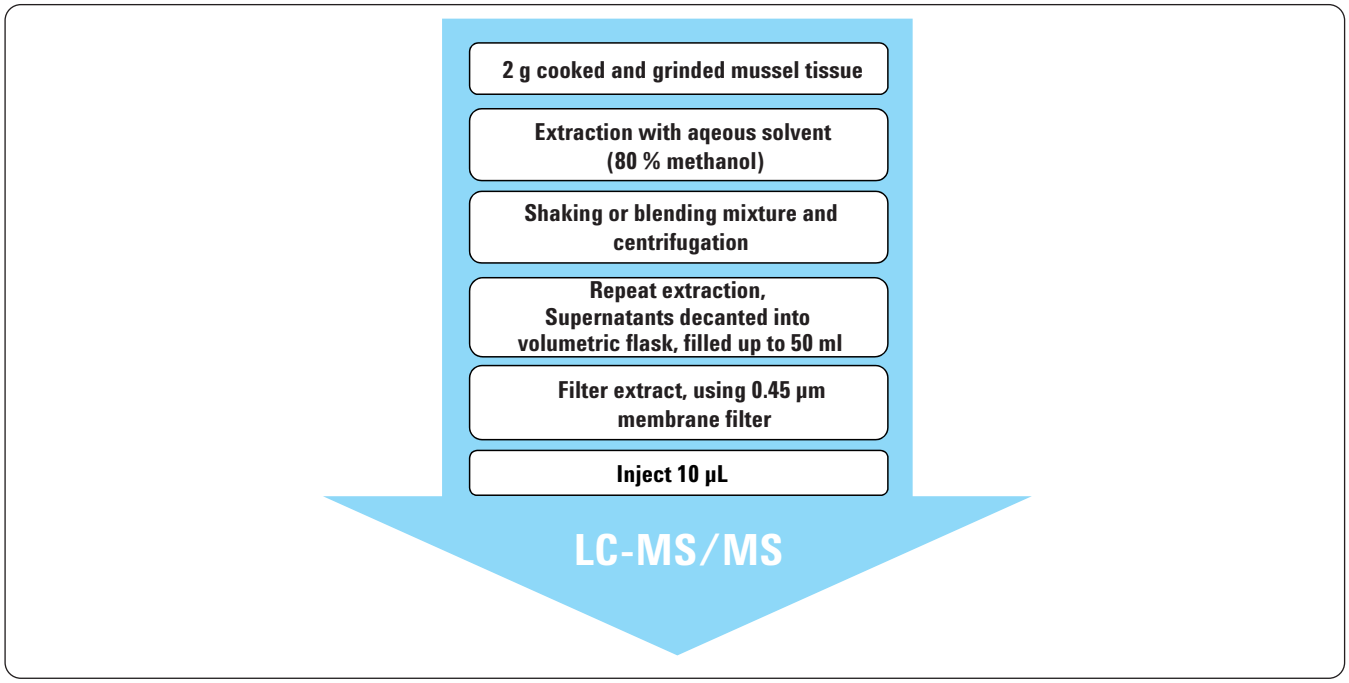


Figure 2
Sample preparation procedure for the determination of marine toxins in shellfish.
According to McNabb, P., A.I. Selwood, and P.T. Holland, Multiresidue method for determination of algal toxins in shellfish. J AOAC Int, 2005. 88: p. 761-772
Chapela, M.J., et al., Lipophilic toxins analyzed by liquid chromatography-mass spectrometry and comparison with mouse bioassay in fresh, frozen, and processed molluscs. J Agric Food Chem, 2008. 56(19): p. 8979-86.
Moutfort, D.O., T. Suzuki, and P. Trueman, Protein phosphatase inhibition assay adapted for determination of total DSP in contaminated mussels. Toxicon, 2001. 39: p. 383-390

Mass Spectrometer Settings and Jet Stream Parameter

Agilent 6460 QQQ ESI JetStream Source parameter

Gas Temperature:	300 °C
Gas Flow:	5 L/min
Nebulizer:	45 psi
Sheath Gas Temp:	250 °C
Sheath Gas Flow:	11 L/min
Capillary:	+ 3500 V
	- 3500 V
Nozzle Voltage:	+/- 500 V
Delta EMV	400

Agilent 6410 QQQ ESI-Source parameter

Ionization:	ESI
Gas Temperature:	300 °C
Gas Flow:	10 L/min
Nebulizer:	43 psi
Capillary:	+ 4500 V
	- 5200 V
Delta EMV:	400

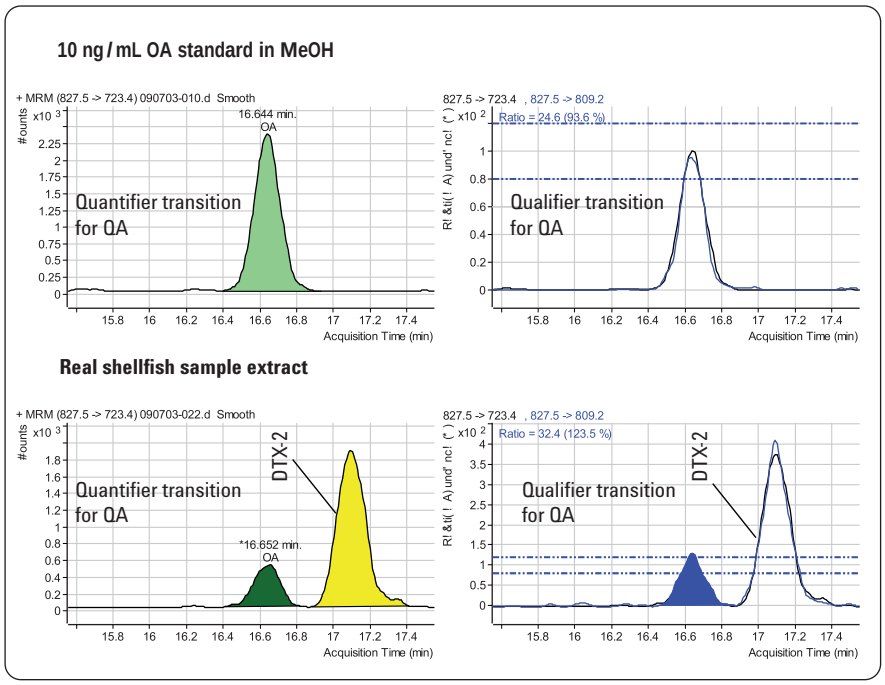


Figure 3
OA standard and real shellfish sample, containing OA and DTX-2 (same sample as shown in figure 4).
OA 23 µg/kg and DTX-2 130 µg/kg.

Analyte	Polarity	Prec Ion m/z	Prod Ion m/z	Frag [V]	CE [eV]	Quantifier
OA and DTX-2	pos	827.5	723.4	220	55	X
	pos	827.5	809.2	220	45	
DTX-1	pos	841.5	737.2	220	55	X
	pos	841.5	823.2	220	45	
PTX-1	pos	897.5	555.3	230	70	X
	pos	897.5	853.5	230	60	
PTX-2	pos	881.5	539.3	230	70	X
	pos	881.5	837.5	230	60	
PTX-2sa*	pos	899.5	855.5	230	60	X
	pos	899.5	557.3	230	70	
YTX	neg	1141.5	1061.3	135	35	X
	neg	1141.5	925.5	135	60	
Homo-YTX*	neg	1155.4	1075.5	135	35	X
OH-YTX	neg	1157.4	1077.5	135	35	X
OH-Homo-YTX*	neg	1171.4	1091.5	135	35	X
AZA-1	pos	842.5	824.5	200	40	X
	pos	842.5	806.5	200	55	
AZA-2	pos	856.5	838.5	200	40	X
	pos	856.5	820.5	200	55	
AZA-3	pos	828.5	810.5	200	40	X
	pos	828.5	792.5	200	55	

*Transitions based on literature information

Table 2
MRM transitions and MS settings.

Results

The method has been validated within an international collaborative study. The collaborative study was conducted in the framework of the working group §64 LFGB "Phycotoxins", which is hosted by the federal Office of Consumer Protection and Food Safety (BVL).

Compound	LOD	LOQ
[1]OA	6 µg/kg	20 µg/kg
[1]DTX-1 & 2	6 µg/kg	20 µg/kg
[2]AZA-1 to 3	6 µg/kg	20 µg/kg
[1]PTX-1 & 2	6 µg/kg	20 µg/kg
[3]YTX	10 µg/kg	35 µg/kg

[1] MRL in raw mussel material for sum of OA, DTX-1 & 2, PTX-1 & 2: 160 µg/kg OA equivalents

[2] MRL in raw mussel material for sum of azaspiracids: 160 µg/kg AZA-1 equivalents

[3] MRL in raw mussel material for sum of yessotoxins: 1000 µg/kg YTX equivalents

Table 3
LODs and LOQs of method for lipophilic marine toxins in cooked, grinded mussels.

Reproducibility S.D. is in the range from approx. 10 % to 35 % (depends on matrix, concentration, analyte). Extraction recovery is in the range from 75 % to 102 % (depends on analyte and matrix).

Benefits

- Highly sensitive and selective determination of marine toxins in shellfish with Agilent 6460 Triple Quadrupole LC-MS system and Agilent 1200 SL Rapid Resolution HPLC
- Simple and cost-effective sample preparation and easy workflow for routine sample analysis with high reliability
- Compliance with recent EFSA guidelines
- Flexibility to add other lipophilic toxins

Chromatogram of real shellfish samples

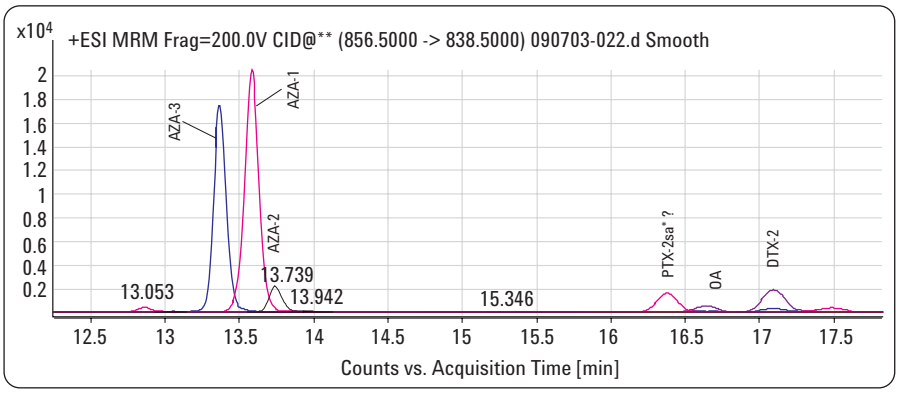


Figure 4
Real blue mussel sample extract.
Concentration: 96 µg/kg AZA-1, 22 µg/kg AZA-2, 50 µg/kg AZA-3, 23 µg/kg OA and 130 µg/kg DTX-2.
*PTX-2sa, tentatively assigned on the basis of transitions from literature, no standard available for PTX-2sa.

Chromatogram of real shellfish samples

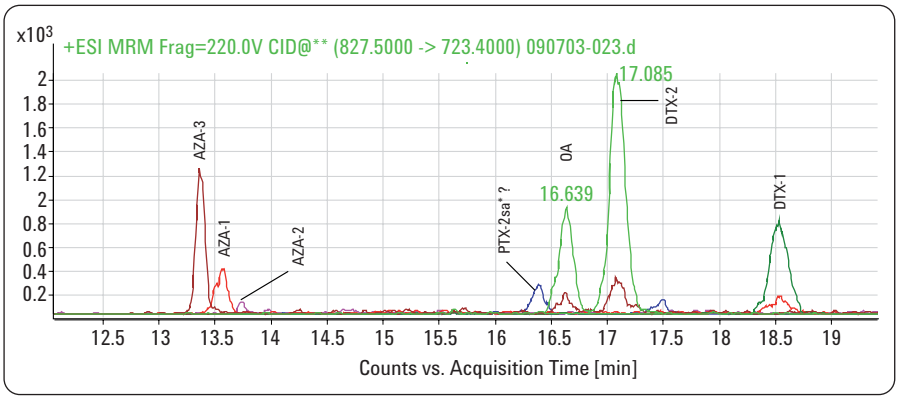


Figure 5
Real blue mussel extract, AZA-1 to -3 < LOQ 20 µg/kg.
Concentration: 37 µg/kg OA, 120 µg/kg DTX-2 and 69 µg/kg DTX-1.
*PTX-2sa, tentatively assigned on the basis of transitions from literature, no standard available for PTX-2sa.

Chromatogram of a QC sample

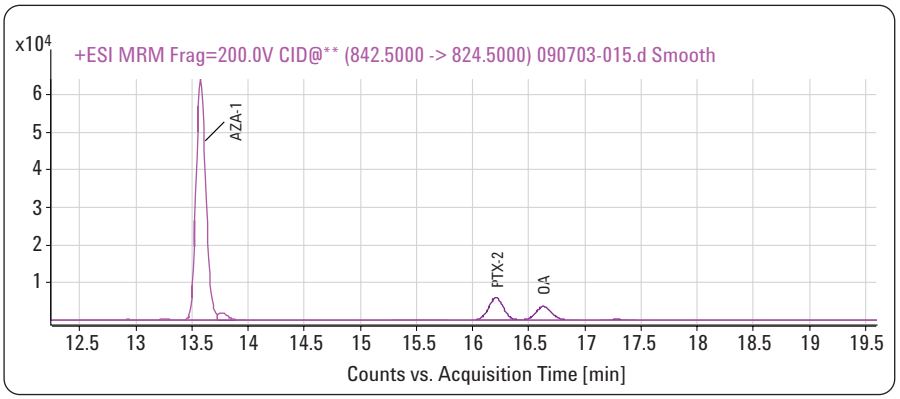


Figure 6
QC-Sample, 15 µg/kg for AZA-1, PTX-2 and OA, spiked in blue mussel extract.

Calibration curve (matrix matched)

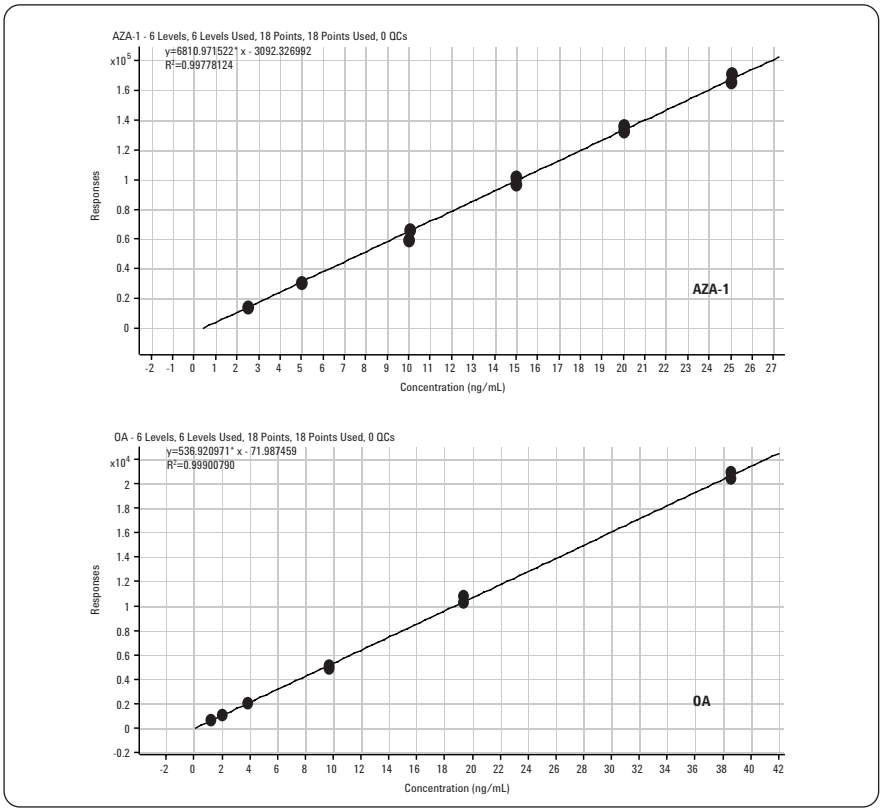


Figure 7
Calibration curve for AZA-1 and OA spiked in mussel extract, linear curve fit, origin included weighting none. Calibration range AZA-1 2.5 to 25 ng / mL and OA 1.1 to 38 ng / mL.

Calibration curve (in methanol)

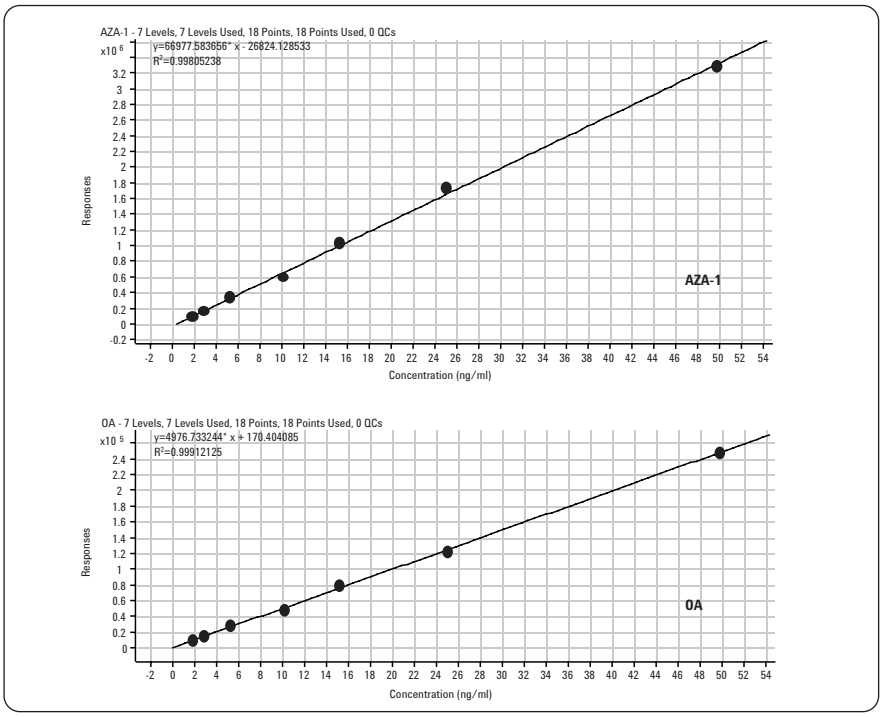


Figure 8
Calibration curve for AZA-1 and OA in MeOH, linear curve fit, origin included weighting none. Calibration Range 1.5 to 50 ng / mL for both.

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Determination of Chemical Contaminants in Marine Shellfish using the Agilent 7000 Triple Quadrupole GC/MS System

Application note

Food Safety

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Abstract

A sample preparation method based on a modified QuEChERS extraction has been developed along with a GC/MS/MS method for the determination of selected Organo-chlorine pesticides, Polyaromatic hydrocarbons and Polychlorinated biphenyl congeners. The analytical method meets the detection limit requirements for the organic chemical contaminants in marine shellfish tissue (mussel) stipulated in the United Kingdom's Clean Seas Environmental Monitoring Program.



Agilent Technologies

Introduction

The Clean Seas Environmental Monitoring Program (CSEMP) is an initiative designed to monitor the levels of chemical contamination in the UK's coastal and estuarine areas. The major drivers for this program are

- To meet the mandatory monitoring requirements under Oslo and Paris Convention (OSPAR) Joint Assessment and Monitoring Program (JAMP).
- Compliance with EC Directives.

The EC dangerous substance directive (76/464/EEC) requires the analysis of sediment or biota to determine the trend in the substances discharged. Organic compounds in Shellfish are also monitored to meet some requirements of Shellfish Water Directive (79/923/EEC), the Shellfish Hygiene Directive (91/492/EEC) and as amended by 97/61/EC, and Fisheries Products Directive (91/493/EEC) [1].

The program specifies 16 organo-chlorine compounds (OCPs), 28 polyaromatic hydrocarbons (PAHs) and 7 polychlorinated biphenyl congeners (PCBs). The Limit of Detection (LoD) requirements are 0.1 µg/Kg for OCPs and PCBs, and 0.5 – 1.0 µg/Kg for PAHs.

An extraction method for these organic contaminants in marine shellfish tissue (mussel), based on a modified QuEChERS [2], [3] extraction method, has been developed and the extracts from which were analysed by gas chromatography coupled to a triple quadrupole mass spectrometer (GC-QQQ). The chromatographic method includes a post-column pressure controlled tee which facilitates post-column, post-run backflush in order to remove high boiling matrix components that would otherwise remain in the column between analyses and subsequently cause degradation of chromatographic performance and contamination of the mass spectrometer ion source. The effectiveness of post-column back flush has been demonstrated in a previously published Agilent application note [4].

Experimental

Calibration Standards

Calibration mixtures of native PAHs and isotope labelled PAH internal standards were obtained from SPEX Certiprep and Cambridge Isotopes, respectively. Custom made mixture for OCPs and PCB congeners were procured from LGC Promochem. PCB 155 and isotope labelled OCP internal standards were obtained from QMX and CDN Isotopes.

Sample Preparation

2 g amounts of homogenized mussel tissue samples were extracted using a modified QuEChERS extraction method. The extraction and clean up workflow is shown in Figure 1.

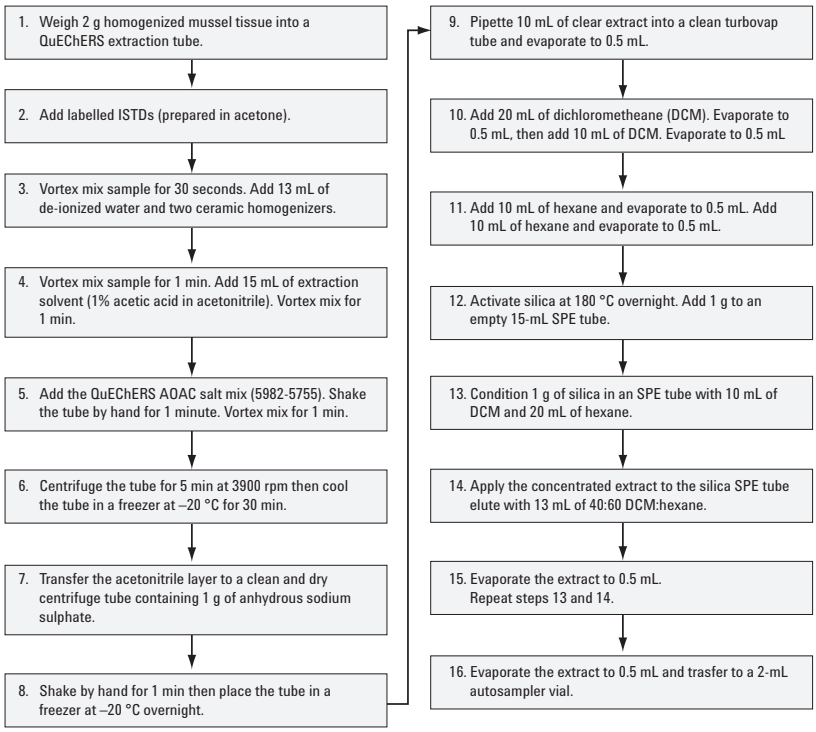


Figure 1. Flow diagram of the sample extraction and clean-up procedure.

GC/MS/MS Analysis

The analyses were performed on an Agilent 7890 GC / 7000 Triple Quadrupole GC/MS system. The 7890 Series GC was configured with a carbon dioxide cooled Multimode Inlet (MMI) and a 15 m × 0.25 mm id, 0.25 µm DB-5MSUI capillary column coupled to a 0.65 mm id × 0.15 mm id, 0.15 µm DB-5MSUI restrictor to the mass spectrometer via a capillary flow pressure controlled tee. A schematic diagram of the GC/MS/MS system configuration is shown in Figure 2.

The analytical column was operated in constant flow mode and the chromatography was retention time locked using PCB 118 as the locking compound at a retention time of 12.370 minutes. The pressure controlled tee was operated in constant pressure mode with helium controlled by a pneumatics control module (PCM).

An Agilent 7693A auto-liquid sampler with was employed and either 1 µL cold splitless injections using a 10 µL syringe (during GC/MS/MS method optimization) or 10 µL solvent vent injections made using a 25 µL syringe (for instrument calibration and sample analyses).

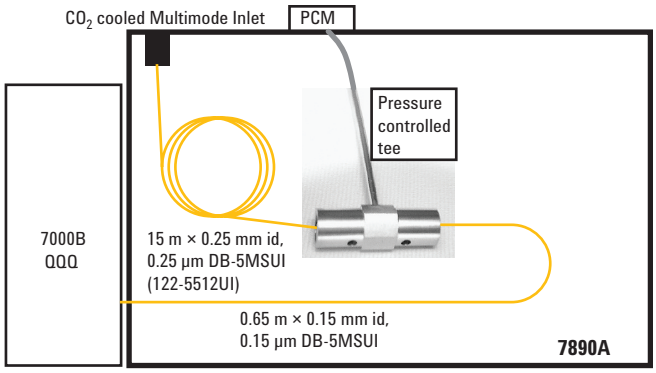


Figure 2. Schematic diagram of GC/MS/MS system configuration.

The GC instrument conditions are listed in Table 1.

The mass spectrometer was operated in electron impact ionization (EI) MS/MS mode using multiple reaction monitoring (MRM) for all the analytes and their associated internal standards. Mass spectrometer operating conditions are given in Table 2 and the full list of analytes with their respective retention times, monitoring ion transitions, collision energies and, dwell times are shown in Table 3.

Table 1. GC Analysis Conditions

Column (1)	15 m × 0.25 mm id, 0.25 µm DB-5MSUI (122-5512UI)
Column (2)	0.65 m × 0.15 mm id, 0.15 µm DB-5MSUI (cut from 165-6626)
Injection mode (1)	1 µL cold pulsed splitless using CO_2 cooled Multimode Inlet (MMI) and a 10 µL syringe
Inlet temperature program	50 °C (0.05 min), 600 °C/min to 325 °C
Inlet pressure pulse	13.0 psig for 0.75 min
Purge Flow to Split Vent	50 mL/min at 1.0 min
Injection port liner	2 mm id, multi-baffled (5190-2296)
Injection mode (2)	10 µL solvent vent using CO_2 cooled Multimode Inlet (MMI) and a 25 µL syringe
Inlet temperature program	40 °C (0.31 min), 600 °C/min to 325 °C
Inlet Vent pressure	5.0 psig
Inlet vent flow	100 mL/min
Inlet vent time	0.31 min
Outlet pressure	0 psig
Injection speed	100 µL/min
Purge Flow to Split Vent	50 mL/min at 1.0 min
Injection port liner	2 mm id, multi-baffled (5190-2296)
Carrier Gas	Helium, constant flow 1.2 mL/min
Oven temp program	50 °C (1) – 20 – 200 °C/min (0) – 10 °C/min – 300 °C (1.5)
RTL Compound	PCB 118, locked at 12.370 min
Pressure controlled tee	G3186B, operated at 2.0 psig constant pressure
Back flush conditions	Inlet pressure 1.0 psig, PCM pressure 60 psig, time 2.0 min

Table 2. Mass Spectrometer Operating Conditions

MS Transfer line temp	325 °C
MS Source	300 °C
MS Quad 1 , 2 temp	150 °C , 150 °C
Collision cell gases	Nitrogen 1.5 mL/min, Helium 2.25 mL/min
MS1 / MS2 Resolution	Wide/wide
MRM settings	See Table 3
Electron energy	-70 eV
Ionization mode	Electron impact (EI)
EI Autotune	Gain normalized
Gain factor	5

Table 3. MS/MS Settings for OCPs, PAHs, PCB Congeners and Labelled Internal Standards

TS	Time	Analyte	RT (min)	Precursor	Product	Dwell (ms)	CE(V)	Precursor	Product	Dwell (ms)	CE(V)
1	4.0	d3-135-TCB	5.050	182.9	147.9	25	35	182.9	110.9	25	35
		135-TCB	5.068	179.9	144.9	25	35	179.9	108.9	25	35
		d8-Napthalene	5.479	136.0	108.0	25	25				
		Naphthalene	5.504	128.0	102.0	25	22	128.0	127.0	25	20
		HCBD	5.658	224.9	189.9	25	22	224.9	187.9	25	22
2	6.8	d8-Acenapthylene	7.308	160.0	132.0	25	30	160.0	108.0	25	30
		Acenaphthylene	7.321	152.0	151.0	25	40	152.0	150.0	25	40
		d10-Acenapthene	7.494	164.0	162.0	25	30	164.0	160.0	25	30
		Acenaphthene	7.525	154.0	152.0	25	40	153.0	152.0	25	40
3	7.8	d10-Fluorene	8.099	176.0	174.0	15	30				
		Fluorene	8.131	166.0	165.0	15	30				
		d6-HCH - alpha	8.699	224.0	187.0	15	15	224.0	150.0	15	15
		HCH - alpha	8.730	181.0	145.0	15	15	181.0	109.0	15	30
		HCB	8.770	283.9	248.8	15	25	283.9	213.9	15	35
		HCH- beta	8.990	181.0	145.0	15	15	181.0	109.0	15	30
		d6-HCH- gamma	9.077	224.0	187.0	15	15	224.0	150.0	15	15
		HCH - gamma	9.107	218.8	183.0	15	5	181.0	109.0	15	30
		Dibenzothiophene	9.110	184.0	152.0	15	40	184.0	139.0	15	40
		d10-Phenanthrene	9.274	188.0	184.0	15	40	188.0	160.0	15	40
		Phenanthrene	9.299	178.0	176.0	15	34				
		Anthracene	9.367	178.0	176.0	15	34				
		HCH - delta	9.428	181.0	145.0	15	15	181.0	109.0	15	30
4	9.6	PCB 28	9.820	256.0	186.0	20	26	258.0	186.0	20	26
		PCB 52	10.250	289.9	220.0	20	28	291.9	222.0	20	28
		Aldrin	10.480	298.0	263.0	20	8	263.0	191.0	20	30
		Isodrin	10.880	262.9	193.0	20	35	262.9	191.0	20	35
5	11.0	d10-Fluoranthene	11.103	212.0	210.0	15	45	212.0	208.0	15	45
		Fluoranthene	11.128	202.0	201.0	15	30	202.0	200.0	15	50
		PCB 155	11.280	357.8	287.9	15	28	359.8	289.9	15	28
		op-DDE	11.375	248.0	176.0	15	30	246.0	211.0	15	20
		PCB 101	11.437	323.9	253.9	15	28	325.9	255.9	15	28
		d10-Pyrene	11.486	212.0	210.0	15	45	212.0	208.0	15	45
		Pyrene	11.512	202.0	201.0	15	30	202.0	200.0	15	45

Table 3. MS/MS Settings for OCPs, PAHs, PCB Congeners and Labelled Internal Standards (Continued)

TS	Time	Analyte	RT (min)	Precursor	Product	Dwell (ms)	CE(V)	Precursor	Product	Dwell (ms)	CE(V)
5		pp-DDE	11.857	248.0	176.0	15	30	246.0	211.0	15	20
		C13-Dieldrin	11.933	269.8	200.0	15	40	269.8	198.0	15	40
		Dieldrin	11.940	262.8	193.0	15	30	262.8	191.0	15	30
		op-DDD	11.956	237.0	165.0	15	20	235.0	200.0	15	8
6	12.15	Endrin	12.265	281.0	245.0	25	20	263.0	193.0	25	35
		PCB 118 (RTL compound)	12.370	323.9	253.9	25	28	325.9	255.9	25	28
		pp-DDD	12.500	237.0	165.0	25	20	235.0	199.1	25	8
		op-DDT	12.543	237.0	165.0	25	20	235.0	199.1	25	20
		PCB 153	12.698	357.8	287.9	25	28	359.8	289.9	25	28
		C13-pp-DDT	13.091	247.0	177.0	25	20	247.0	211.0	25	20
		pp-DDT	13.099	237.0	165.0	25	20	235.0	199.1	25	20
		PCB 138	13.112	357.8	287.9	25	28	359.8	289.9	25	28
7	13.5	Benzo[a]anthracene	13.897	228.0	226.0	40	38				
		d12-Chrysene	13.915	240.0	236.0	40	35				
		Chrysene / Triphenylene	13.965	228.0	226.0	40	38				
		PCB 180	14.175	393.8	323.9	40	30	395.8	325.9	40	30
8	15.0	Benzo[b+j]fluoranthene	16.06	252.0	250.0	75	42	250.0	248.0	75	40
		d12-Benzo[k]fluoranthene	16.084	264.0	260.0	75	40				
		Benzo[k]fluoranthene	16.116	252.0	250.0	75	42	250.0	248.0	75	40
		Benzo[e]pyrene	16.561	252.0	250.0	75	42	250.0	248.0	75	40
		d12-Benzo[a]pyrene	16.616	264.0	260.0	75	40				
		Benzo[a]pyrene	16.654	252.0	250.0	75	42	250.0	248.0	75	40
		Perylene	16.814	252.0	250.0	75	42	250.0	248.0	75	40
9	18.0	d12-Indeno[123-cd]pyrene	18.600	288.0	284.0	75	50				
		Indeno(123-cd)pyrene	18.631	276.0	274.0	75	42				
		d14-Dibenz[a,h]anthracene	18.662	292.0	288.0	75	50				
		Dibenz[a,h]anthracene	18.712	278.0	276.0	75	38				
		d12-Benzo[g,h,i]perylene	19.020	288.0	284.0	75	45				
		Benzo[ghi]perylene	19.064	276.0	274.0	75	38				

Results and Discussion

Chromatography

The total ion chromatogram (TIC) for all MRM transitions of all analytes is shown in Figure 3. For additional clarity, labelled TIC MRM chromatograms of the OCPs, PAHs and PCB congeners are shown in Figures 4, 5, and 6, respectively.

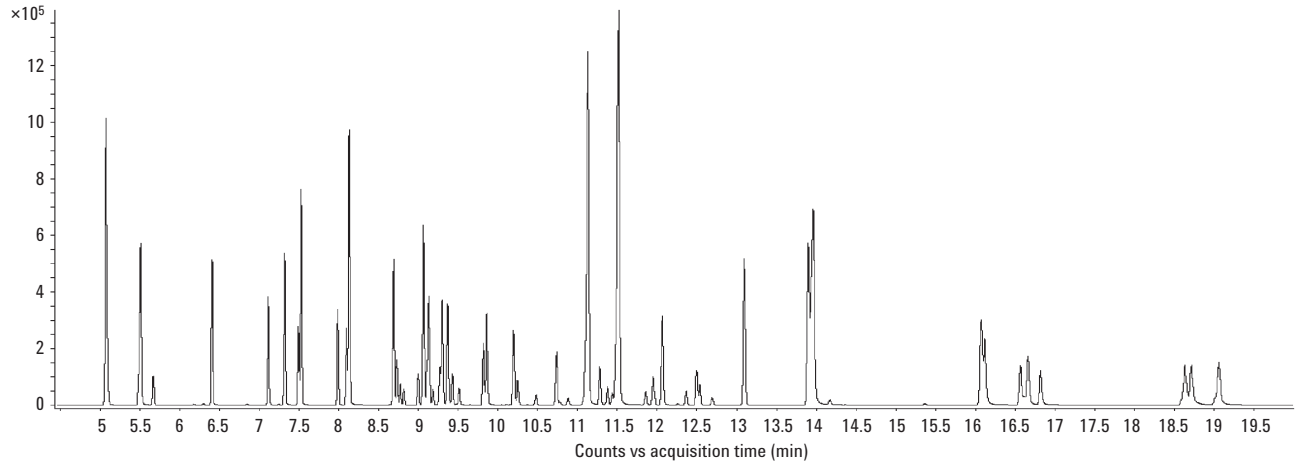


Figure 3. TIC MRM Chromatogram for a calibration standard.

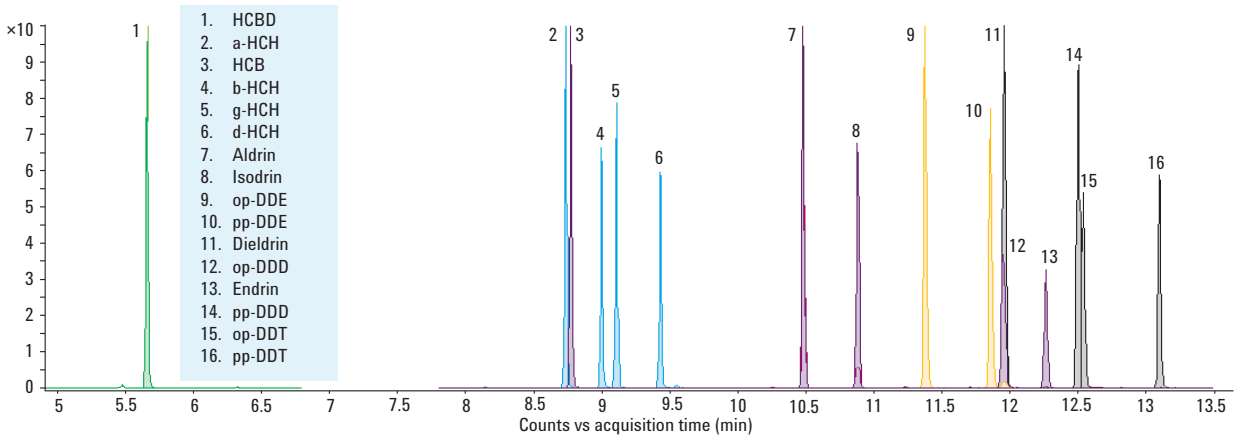


Figure 4. TIC MRM Chromatogram for OCP analytes.

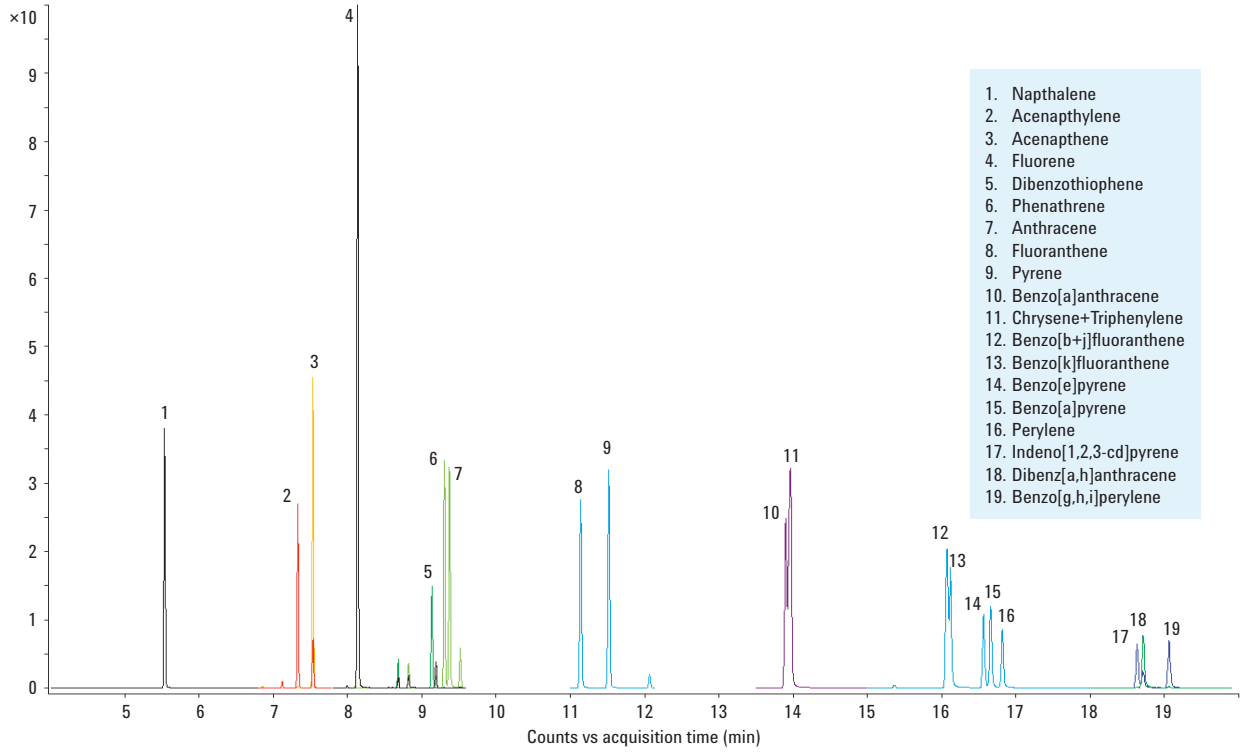


Figure 5. TIC MRM Chromatogram for PAH analytes.

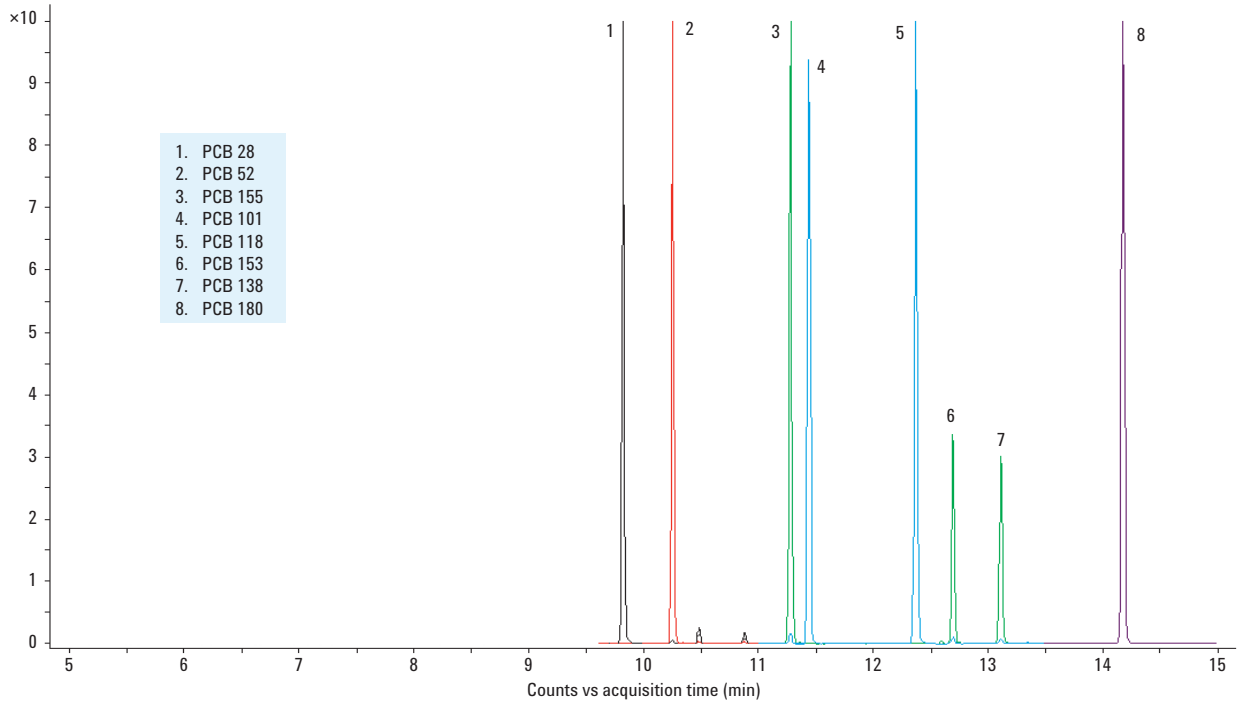


Figure 6. TIC MRM Chromatogram for PCB congeners.

Analyte calibration curves

The GC-MS/MS system was calibrated using a five-point internal standard (ISTD) calibration. The calibration standards for target analytes were prepared in hexane at concentrations of 0.4, 2.0, 8.0, 80.0, and 200.0 pg/µL. All ISTDs were added at 80.0 pg/µL. Calibration curves were created using 10 µL solvent vent mode injections. The calibration curves for all analytes gave correlation coefficients greater than 0.999. Table 4 shows curve fit types and correlation coefficient values.

Table 4. Curve Fits and Correlation Coefficients for ISTD Calibration Curves

Analyte	Curve fit	R^2
HCBD	Quadratic	0.9994
a-HCH	Linear	0.9996
HCB	Linear	0.9998
b-HCH	Linear	0.9995
g-HCH	Linear	0.9999
d-HCH	Linear	0.9991
Aldrin	Quadratic	0.9999
Isodrin	Quadratic	0.9999
op-DDE	Linear	0.9998
p,p-DDE	Linear	0.9993
Dieldrin	Quadratic	0.9992
op-DDD	Quadratic	0.9999
Endrin	Linear	0.9997
pp-DDD	Linear	0.9997
o,p-DDT	Linear	0.9992
p,p-DDT	Linear	0.9995
Napthalene	Linear	0.9997
Acenapthylene	Linear	0.9997
Acenapthene	Linear	0.9999
Fluorene	Linear	0.9997
Dibenzothiophene	Quadratic	0.9999
Phenanthrene	Linear	0.9999
Anthracene	Linear	0.9997
Fluoranthene	Linear	0.9992
Pyrene	Linear	0.9996
Benzo[a]anthracene	Linear	0.9998
Chrysene+Triphenylene	Quadratic	0.9999
Benzo[b+j]fluoranthene	Linear	0.9998
Benzo[k]fluoranthene	Quadratic	0.9997
Benzo[e]pyrene	Linear	0.9996
Benzo[a]pyrene	Linear	0.9998
Perylene	Linear	0.9999
Indeno[123-cd]pyrene	Quadratic	0.9996
Dibenz[a,h]anthracene	Quadratic	0.9999
Benzo[g,h,i]perylene	Quadratic	0.9997
PCB 28	Linear	0.9998
PCB 52	Linear	0.9998
PCB 101	Linear	0.9999
PCB 118	Linear	0.9996
PCB 153	Linear	0.9998
PCB 138	Linear	0.9998
PCB 180	Linear	0.9994

Example calibration graphs over the range of interest for g-HCH, PCB 118 and Benzo[a]pyrene are shown in Figure 7.

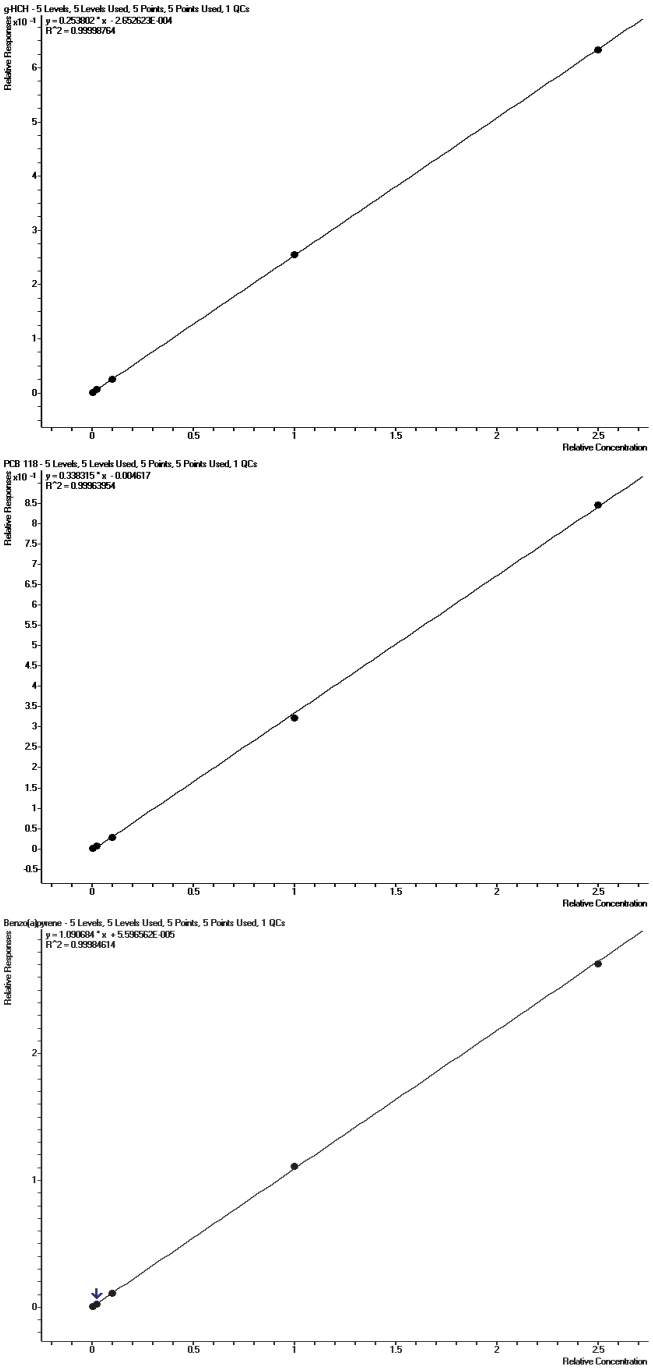


Figure 7. Five point ISTD calibration curves for g-HCH (Top), PCB 118 (middle) and Benzo[a]pyrene (bottom).

Recovery of target analytes and Quantitative Reproducibility

Five sample aliquots (2g) from homogenized mussel tissue were weighed into QuEChERS extraction tubes. The samples were spiked with an acetone solution of target analytes at a level equivalent to 4 µg/Kg (8 ng/2g sample) and internal standards. The extraction tubes were then vortex mixed for 1 minute and the samples were extracted using the procedure given in Figure 1.

Relative percent standard deviations (RSD%) and Spike Recoveries (Recovery %) were calculated for each of the target analytes as given below;

$$RSD\% = \frac{SD}{Mean} \times 100$$

$$\text{Recovery } \% = \frac{Mean(spiked)}{[Mean(unspiked) + Spike]} \times 100$$

where SD is the Standard Deviation.

The list of target analytes, grouped by chemical class (OCPs, PAHs and PCBs) plus their associated internal standards, quantitative reproducibility values and percentage recovery values are shown in Table 5. Percent recovery values for the OCPs, PAHs and PCB congeners from spiked mussel tissue are also shown graphically in Figure 8, (a), (b) and (c), respectively.

Table 5. Target Analytes, Their Associated ISTDs, RSD% Values for Quantitative Reproducibility and Recovery% Values

Analyte	ISTD	RSD% [n=5]	Recovery%
HCBD	d3-135-TCB	11.1	85.4
a-HCH	d6-g-HCH	3.9	115.1
HCB	d6-a-HCH	13.3	92.0
b-HCH	PCB-155	7.0	116.8
g-HCH	d6-g-HCH	2.3	114.1
d-HCH	PCB-155	6.7	123.9
Aldrin	PCB-155	15.8	108.9
Isodrin	PCB-155	13.9	108.7
op-DDE	PCB-155	3.5	120.4
p,p-DDE	PCB-155	4.8	121.5
Dieldrin	^{13}C-Dieldrin	4.0	93.4
op-DDD	PCB-155	4.0	119.9
Endrin	^{13}C-Dieldrin	7.7	112.7
pp-DDD	^{13}C-pp-DDT	6.1	101.6
o,p-DDT	^{13}C-pp-DDT	3.5	104.1
p,p-DDT	^{13}C-pp-DDT	1.1	100.0
Napthalene	d8-Napthalene	3.7	107.7
Acenapthylene	d8-Acenapthylene	7.4	98.5
Acenapthene	d10-Acenapthene	5.0	102.5
Fluorene	d10-Fluorene	8.4	100.9
Dibenzothiophene	d10-Fluorene	8.5	105.6
Phenanthrene	d10-Phenanthrene	7.8	102.6
Anthracene	d10-Phenanthrene	5.6	100.2
Fluoranthene	d10-Fluoranthene	0.9	101.0
Pyrene	d10-Pyrene	7.0	92.3
Benzo[a]anthracene	d12-Chrysene	4.1	103.5
Chrysene+Triphenylene	d12-Chrysene	1.1	104.5
Benzo[b+j]fluoranthene	d12-Benzo[k]fluoranthene	24.0	107.7
Benzo[k]fluoranthene	d12-Benzo[k]fluoranthene	5.4	104.1
Benzo[e]pyrene	d12-Benzo[a]pyrene	1.6	105.0
Benzo[a]pyrene	d12-Benzo[a]pyrene	3.3	102.9
Perylene	d12-Benzo[a]pyrene	1.1	106.4
Indeno[123-cd]pyrene	d14-Dibenz[a,h]anthracene	2.8	94.9
Dibenz[a,h]anthracene	d14-Dibenz[a,h]anthracene	2.4	103.0
Benzo[g,h,i]perylene	d14-Dibenz[a,h]anthracene	4.8	103.1
PCB 28	PCB-155	3.9	105.5
PCB 52	PCB-155	3.0	105.8
PCB 101	PCB-155	3.5	112.3
PCB 118	PCB-155	6.1	107.0
PCB 153	PCB-155	3.6	107.6
PCB 138	PCB-155	4.5	109.9
PCB 180	PCB-155	4.8	110.1

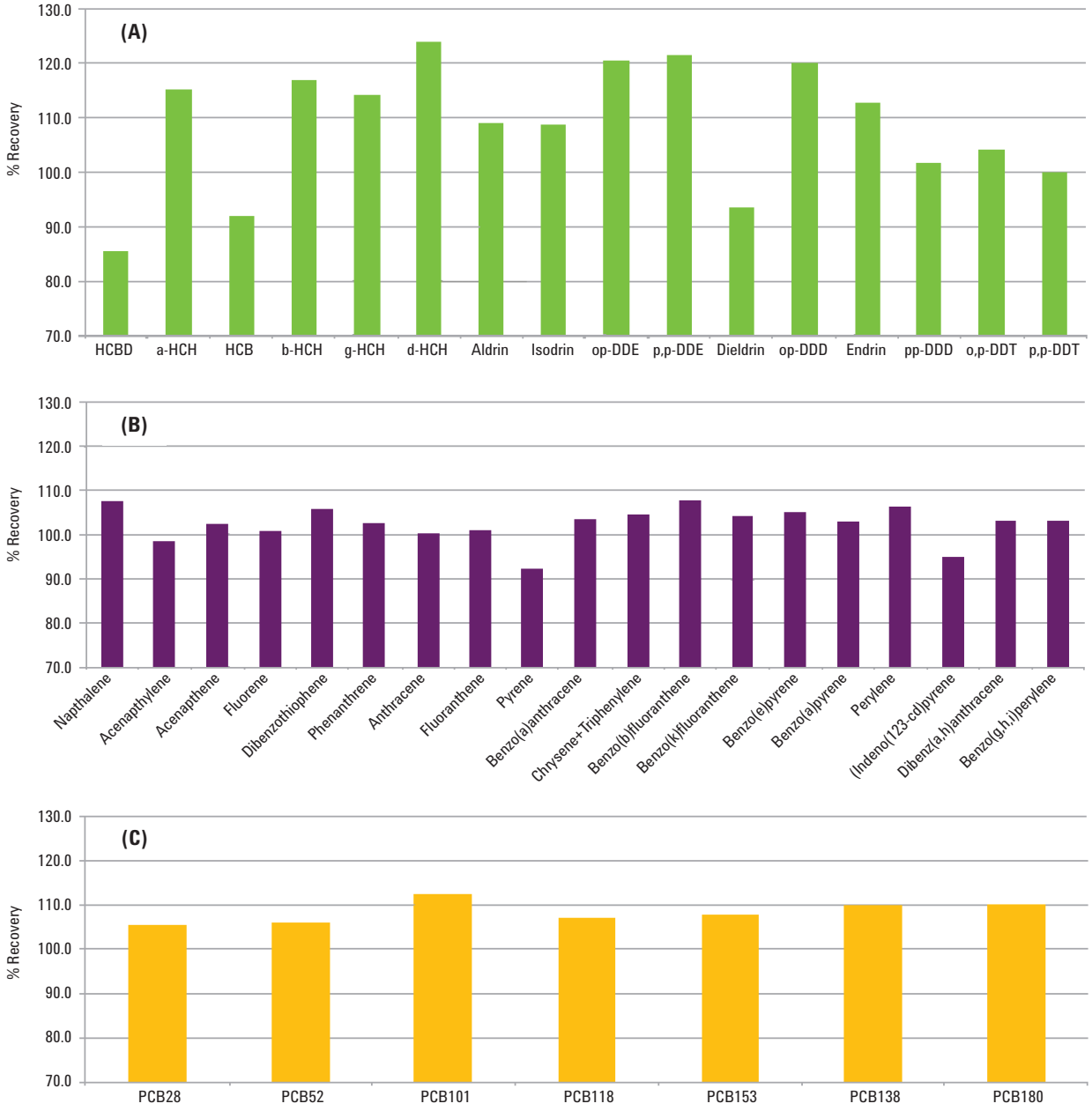


Figure 8. Graphical representation of analyte percent recovery values for (a) OCPs, (b) PAHs and (c) PCB congeners in spiked mussel tissue.

Sample Analysis

Marine mussel samples were sourced from local commercial shell fish suppliers, homogenized, extracted and analysed using the sample preparation and GC/MS/MS conditions as described. MRM chromatograms for the incurred HCH isomers quantified in a mussel sample are shown in Figure 9, the incurred Fluoranthene and Pyrene PAHs in Figure 10 and, the incurred PCB 180 congener in Figure 11, respectively.

Conclusion

A sample preparation method based on a modified QuEChERS extraction and clean up regime has been developed and applied to the extraction of OCPs, PAHs and PCB congeners from marine mussel tissue. The quantitative GC/MS/MS method demonstrated good reproducibility and recoveries for all analytes were in the range of 85.4% – 123.9% in spiked mussel tissue.

The Agilent 7000 Triple Quadrupole GC/MS system provided reproducible and sensitive detection of OCPs, PAHs and PCB congeners in mussel tissue down to concentration levels of 0.1 µg/Kg. The performance of the extraction/clean-up and analysis by GC/MS/MS meets the requirements of the CSEMP legislation.

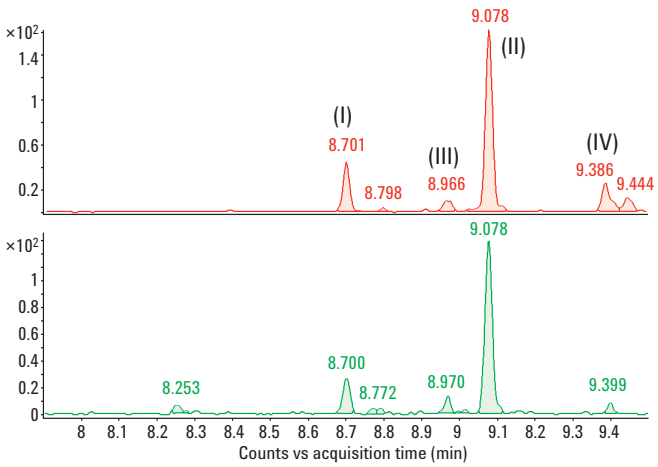


Figure 9. MRM Chromatograms for (i) incurred a-HCH and (ii) incurred g-HCH in mussel sample, Concentrations 0.06 and 0.30 µg/Kg, respectively. Peaks (iii) and (iv) are traces of incurred b-HCH and d-HCH, respectively.

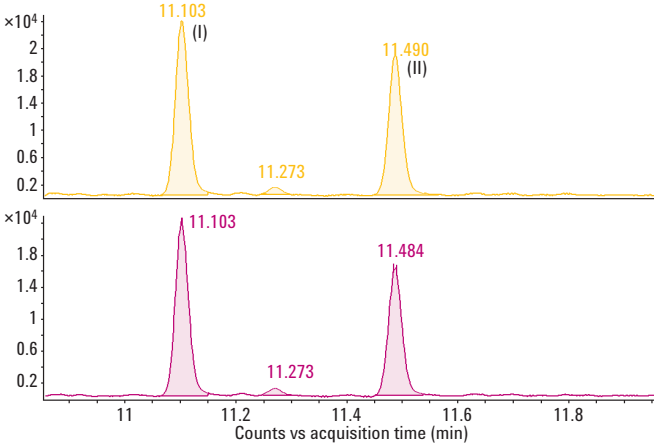


Figure 10. MRM Chromatograms for (i) incurred Fluoranthene and (ii) incurred Pyrene in mussel sample, Concentrations 8.64 and 5.83 µg/Kg, respectively.

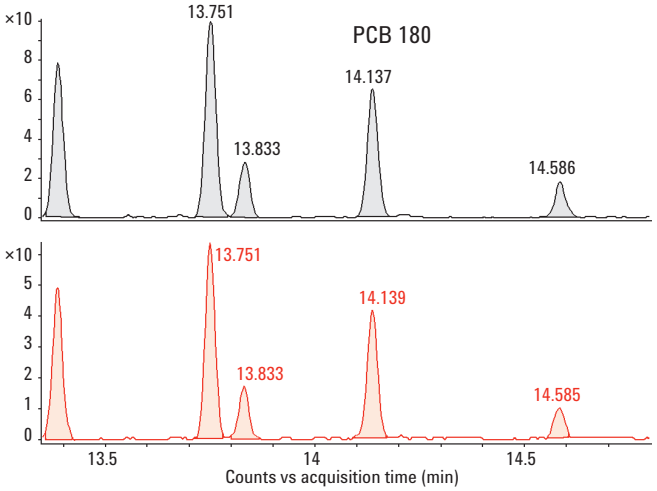


Figure 11. MRM Chromatograms for incurred PCB 180 in mussel sample, Concentration 0.14 µg/Kg.

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Analysis of Pesticide Residues in Apple using Agilent SampliQ QuEChERS European Standard EN Kits by LC/MS/MS Detection

Application Note

Food Safety

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Abstract

This application note describes the use of a quick, easy, cheap, effective, rugged, and safe (QuEChERS) sample preparation approach described in the European Committee Standard (EN) for extraction and cleanup of 16 multiple class pesticide residues of interest in apple. The method employed involves initial extraction in an aqueous/acetonitrile system, an extraction/partitioning step after the addition of salt, and then a cleanup step utilizing dispersive solid phase extraction (dispersive SPE). The two different dispersive SPE clean-up approaches (1 mL and 6 mL sample volume) are evaluated simultaneously after sample extraction. The target pesticides in the apple extracts are then determined by liquid chromatography coupled to an electrospray ionization tandem mass spectrometer (LC-ESI-MS/MS) operating in positive ion multiple reaction monitoring (MRM) mode. The method is validated in terms of recovery and reproducibility. The 5 ng/g of limits of quantitation (LOQ) for pesticides in apple established in this application is well below their regulatory maximum residue limits (MRLs). The spiking levels for the recovery experiments are 10, 50, and 200 ng/g. Excluding pymetrozine, recoveries of the pesticides ranged between 73 and 111% (87% on average), and RSDs below 20% (5.8% on average).

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Introduction

Multi-residue analysis of pesticides in fruits, vegetables, and other foods is a primary function of many regulatory, industrial, and contract laboratories throughout the world. Because of the wide variety of pesticides and complexity of food matrices, the sample must be initially cleaned up using a compatible sample preparation technique before injection into the detection system. It is unquestionable that the most efficient approach to pesticide analysis involves the use of multiclass, multi-residue methods. Once the preliminary analytical quality requirements of accuracy, precision, sensitivity, selectivity, and dynamic scope have been met to suit the need for a particular analysis, other practice considerations should be evaluated. These additional considerations include high sample throughput, ruggedness, ease of use, low cost, labor, minimal toxic solvent usage, and waste generation.

The QuEChERS method was introduced first by USDA scientists in 2003 [1]. The method was then modified to address some problematic pesticides by using a buffered extraction system. [2] There is also a European variation, the prEn method 15662: 2007 [3], [4]. In summary, the method uses acetonitrile extraction, followed by the salting out of water from the sample using anhydrous magnesium sulfate ($MgSO_4$), NaCl and buffering citrate salts to induce liquid-liquid partitioning. For cleanup, a dispersive solid-phase extraction (dispersive SPE) is conducted using a combination of primary secondary amine (PSA) to remove fatty acids from other components and anhydrous $MgSO_4$ to reduce the remaining water in the extract. After mixing and centrifugation, the upper layer is ready for analysis.

The EN methodology is similar in principal to the AOAC method, but has several differences. First, the extraction buffered system in the EN method uses sodium chloride, sodium citrate dehydrate, and disodium citrate hydrogenate sesquihidrate instead of sodium acetate in the extraction step. Second, in the dispersive SPE step, the EN method uses 25 mg PSA per mL of extract rather than 50 mg PSA per mL of extract used by the AOAC method.

In this study, 16 pesticides are used to demonstrate the performance of Agilent SampliQ QuEChERS EN Buffered Extraction kit (p/n 5982-5650) and EN dispersive SPE kit (p/n 5982-5021 and 5982-5056) for General Fruits and Vegetables, suitable for common fruit and vegetable applications. Most of the pesticides are from the original 'representative pesticides' list [1]. According to their experience, a method working well for these representative pesticides should work equally well for nearly all of the other pesticides that are routinely monitored in multiclass, multi-residue methods. These pesticides are from nine different pesticide classes, including acidic, basic, neutral, base-sensitive, and acid-labile pesticides. Furthermore, the selected pesticides are suitable for LC/MS/MS analysis. The MRLs of these pesticides have been set for 10 ng/g or higher. Table 1 shows the chemical and regulatory information of these pesticides.

Experimental

Reagents and Chemicals

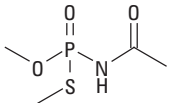
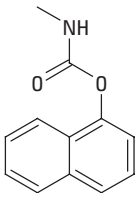
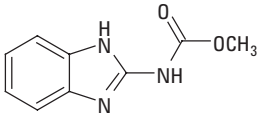
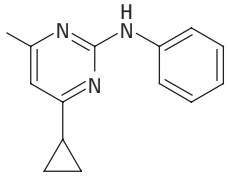
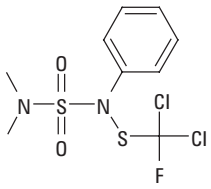
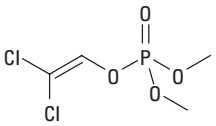
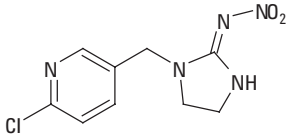
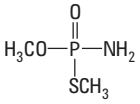
All reagents and solvents were HPLC or analytical grade. Acetonitrile (ACN), methanol (MeOH) were from Honeywell (Muskegon, MI, USA). Dimethyl sulfoxide (DMSO) was from Sigma-Aldrich (St Louis, MO, USA). Ammonium acetate (NH_4OAc) was from Fisher Chemicals (Fair Lawn, NJ, USA). Formic acid (FA) was from Fluka (Sleinheim, Germany). The pesticide standards and internal standard (triphenyl phosphate, TPP) were purchased from Sigma-Aldrich (St Louis, MO, USA), ChemService (West Chester, PA, USA), Ultra Scientific (North Kingstown, RI, USA), or AlfaAesar (Ward Hill, MA, USA).

Solutions and Standards

A 1M NH_4OAc pH 5 stock solution was made by dissolving 19.27 g NH_4OAc powder in 250 mL Milli-Q water. The pH was adjusted to 5 with HAc and monitored with a pH meter. The solution was stored at 4 °C. 20:80 MeOH/H_2O containing 5 mM NH_4OAc pH 5 was made by combining 200 mL MeOH and 800 mL Milli-Q water, adding 5 mL of 1M NH_4OAc pH 5 stock solution.

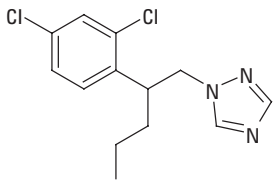
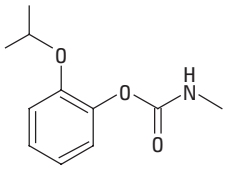
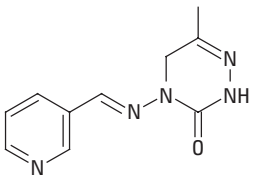
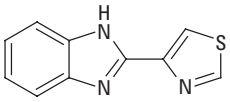
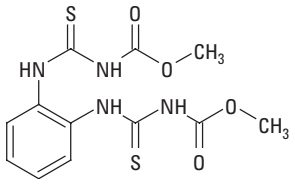
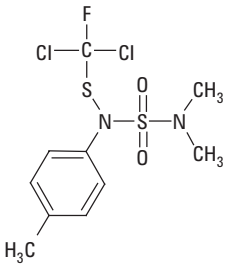
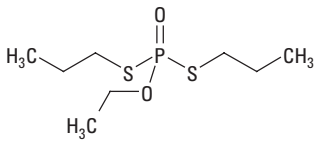
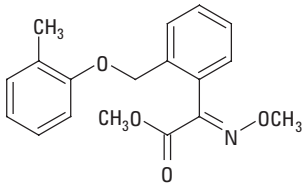
A 5 mM NH_4OAc in ACN solution was prepared by adding 5 mL of 1M NH_4OAc pH 5 stock solution to 1 L ACN, mixing well and sonicating 5 min. A 1% FA in ACN solution was prepared by adding 1 mL of FA to 100 mL of ACN.

Table 1. Pesticides Chemical and Regulatory Information [5–7]

Name	Class	Log P	pKa	Structure	MRLs in apple (ng/g)*
Acephate	Organophosphate	–0.89	8.35		20
Carbaryl	Carbamate	2.36	10.4		50
Carbendazim	Benzimidazole	1.48	4.2		100
Cyprodinil	Anilinopyrimidine	4	4.44		50
Dichlofluanid	Sulphamide	3.7	NA		5000
Dichlorvos	Organophosphate	1.9	NA		10
Imidacloprid	Neonicotinoid	0.57	NA		500
Methamidophos	Organophosphate	–0.79	NA		10

(Continued)

Table 1. Pesticides Chemical and Regulatory Information [5–7]

Name	Class	Log P	pKa	Structure	MRLs in apple (ng/g)*
Penconazole	Triazole	3.72	1.51		50
Propoxur	Carbamate	0.14	NA		1000
Pymetrozine	Pyridine	-0.19	4.06		20
Thiabendazole	Benzimidazole	2.39	4.73 12.00		50
Thiophanate-methyl	Benzimidazole	1.45	7.28		100
Tolylfluanid	Sulphamide	3.9	NA		3000
Ethoprophos	Organophosphate	2.99	NA		5
Kresoxim-methyl	Strobilurin	3.4	NA		50

*The MRLs numbers list in the table are for apple or lowest level in other fruit and vegetables. They could be higher in different commodities.

Standard and internal standard (IS) stock solutions (2.0 mg/mL for all, except 0.5 mg/mL for carbendazim) were made in MeOH, 0.1% FA in ACN, or DMSO, respectively, and stored at –20 °C. Three QC spiking solutions of 1,5, and 20 µg/mL were made fresh daily in 1:1 ACN/H_2O with 0.1% FA. A 10 µg/mL standard spiking solution in 1:1 ACN/H_2O with 0.1% FA was made for preparation of calibration curves in the matrix blank extract by appropriate dilution. A 10 µg/mL IS spiking standard of TPP was made in 1:1 ACN/H_2O (0.1% FA).

Equipment and Material

- Agilent 1200 HPLC with Diode Array Detector (Agilent Technologies Inc., Santa Clara, CA, USA).
- Agilent 6410 Triple Quadrupole LC/MS/MS system with Electrospray Ionization (Agilent Technologies Inc., Santa Clara, CA, USA).
- Agilent SampliQ QuEChERS extraction kit, p/n 5982-5650, and dispersive SPE tubes, p/n 5982-5021 and 5982-5056 (Agilent Technologies Inc., Wilmington, DE, USA).
- CentraCL3R Centrifuge (Thermo IEC, MA, USA)
- Bottle top dispenser (VWR, So. Plainfield, NJ, USA)
- Eppendorf microcentrifuge (Brinkmann Instruments, Westbury, NY, USA)
- Grinder (St. Joseph, MI, USA)

Instrument Condition

HPLC conditions

Column:	Agilent ZORBAX Solvent Saver Plus Eclipse Plus Phenyl-Hexyl, 3.0 x 150 mm, 3.5 µm (p/n 959963-312)
Flow rate:	0.3 mL/min
Column temperature:	30 °C
Injection volume:	10 µL
Mobile phase:	A: 5 mM ammonium acetate, pH 5.0 in 20:80 MeOH/H2O B: 5 mM ammonium acetate, pH 5.0 in ACN
Needle wash:	1:1:1:1 ACN/MeOH/IPA/H_2O (0.2% FA.)

Gradient:

Time	% B	Flow rate (mL/min)
0	20	0.3
0.5	20	0.3
8.0	100	0.3
10.0	100	0.3
10.01	20	0.5
12.0	100	0.5
13.0	STOP	

Post run:	4 min
Total cycle time:	17 min

MS conditions

Positive mode	
Gas temperature:	350 °C
Gas flow:	10 L/min
Nebulizer:	40 psi
Capillary:	4000 V

Other conditions relating to the analytes are listed in Table 2.

Table 2. Instrument Acquisition Data Used for the Analysis of 16 Pesticides by LC/MS/MS

Analyte	MRM channels (m/z)	Fragmentor (V)	CE (V)	RT (min)
Acephate	1) 184.0 > 94.9 2) 184.0 > 111.0	60	3 15	2.55
Methamidophos	1) 142.0 > 94.0 2) 142.0 > 124.9	60	8 8	2.54
Pymetrozine	1) 218.1 > 105.0 2) 218.1 > 78.0	115	20 50	2.97
Carbendazim	1) 192.1 > 160.0 2) 192.1 > 105.0	95	18 40	5.07
Dichlorvos	1) 221.0 > 109.0 2) 221.0 > 95.0	110	13 40	6.57
Thiophanate methyl	1) 343.1 > 151.0 2) 343.1 > 117.9	105	17 65	7.08
Propoxur	1) 210.1 > 111.0 2) 210.1 > 92.9	50	12 15	6.89
Carbaryl	1) 202.0 > 145.0 2) 202.0 > 115.0	50	3 40	7.30
Cyprodinil	1) 226.1 > 93.0 2) 226.1 > 108.0	120	35 35	9.23
Dichlorfluanid	1) 333.0 > 123.0 2) 333.0 > 223.9	85	28 5	9.40
Ethoprophos	1) 243.1 > 130.9 2) 243.1 > 172.9	80	15 15	8.50
Penconazole	1) 284.1 > 158.9 2) 284.1 > 172.9	80	32 32	8.95
Tolyfluanid	1) 347.0 > 136.9 2) 347.0 > 238.0	60	25 3	9.73
Thiabendazole	1) 202.1 > 175.0 2) 202.1 > 131.0	110	27 38	5.65
Imidacloprid	1) 256.1 > 209.1 2) 256.1 > 175.0	60	12 18	5.53
TPP	1) 327.1 > 77.0 2) 327.1 > 151.9	70	45 45	9.49
Kresoxim methyl	1) 314.0 > 222.1 2) 314.0 > 235.0	70	10 10	9.44

1) Quantifier transition channel
2) Qualifier transition channel

Sample preparation

Sample comminution

In order to get the most reliable statistical results, it is important to spend the necessary effort and time on conducting proper sampling and homogenization procedures. Organically grown, pesticide free apples were purchased from a local grocery store. Approximately three pounds of apples were chopped into small, bean sized cubes. Skin was included, but the pit was discarded. Then, the chopped apple cubes were put into a clean plastic bag and frozen at –20 °C overnight. The bag was massaged occasionally to make sure the cubes were frozen loosely to avoid clumping. The following day, a portion of frozen apple cubes were removed and thoroughly blended. Certain precautions were exercised while blending the sample. First, the chopped apple cubes remained in the freezer until the point of blending. Only the portion of apple cubes necessary for homogenizing were removed; the rest was kept in the freezer until the next comminution. Dry ice was added while comminuting to keep the temperature low. Second, the blender container was kept dry to prevent clumping. In between blending, the container was rinsed and dried. Third, samples were comminuted thoroughly to get the best sample homogeneity. There were not any pieces of apple visible in the final sample.

Extraction/Partitioning

A 10 g (±0.05g) previously homogenized sample was placed into a 50 mL centrifuge tube. QC samples were fortified with 100 µL of appropriate QC spiking solution. A 100 µL of IS spiking solution (10 µg/mL of TPP) were added to all of samples except the control blank to yield a 100 ng/g concentration in sample. Tubes were capped and vortexed for 1 min. Ten milliliters of ACN was added to each tube using the dispenser. Tubes were then capped and shaken by hand for 1 min. To each tube, an Agilent SampliQ QuEChERS EN extraction salt packet (p/n 5982-5650), containing 4 g anhydrous $MgSO_4$, 1g NaCl, 1g sodium citrate, and 0.5 g disodium citrate sesquihydrate, was added directly. No powders were left in the threads or rims of the tubes. Tubes were sealed tightly and shaken vigorously for 1 min by hand to ensure that the solvent interacted well with the entire sample and crystalline agglomerates were broken up sufficiently. Sample pH was checked with pH paper, and 5N NaOH was added to adjust the pH to 5-5.5. Sample tubes were centrifuged at 4000 rpm for 5 min.

Dispersive SPE Cleanup

A 1 mL aliquot of the upper ACN layer was transferred into an Agilent SampliQ QuEChERS EN dispersive SPE 2 mL tube (p/n 5982-5021); or 6 mL of aliquot was transferred into an Agilent SampliQ QuEChERS EN dispersive SPE 15 mL tube (p/n 5982-5056). The 2 mL tube contained 25 mg of PSA and 150 mg of anhydrous $MgSO_4$; while the 15 mL tube contained 150 mg of PSA and 900 mg of anhydrous $MgSO_4$. The tubes were capped tightly and vortexed for 1 min. The 2 mL tubes were centrifuged with a micro-centrifuge at 13,000 rpm for 2 min, and the 15 mL tubes with a standard centrifuge at 4000 rpm for 5 min. Ten microlitres of extract were transferred into an autosampler vial. A 10 µL of a 1% FA in ACN solution was added immediately, in addition to 800 µL of water or appropriate standard solution (prepared in water). The samples were capped and vortexed thoroughly, to prepare for LC/MS/MS analysis.

The flow chart in Figure 1 illustrates the sample preparation procedure.

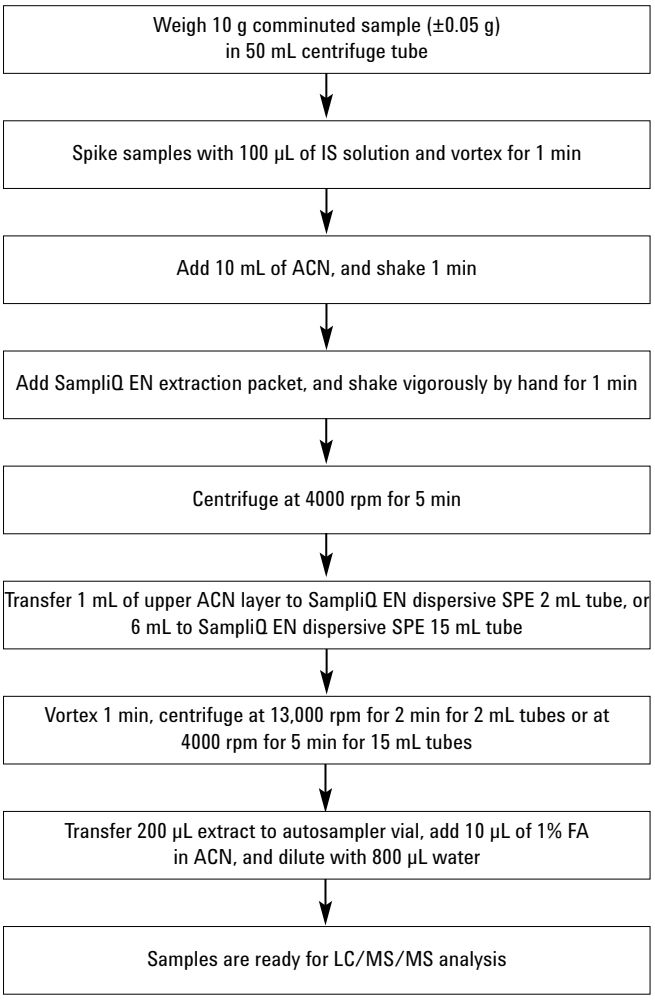


Figure 1. QuEChERS EN sample preparation procedures flow chart.

Results and Discussion

In addition to being fast, easy, cheap, effective, rugged and safe, an additional key feature of the QuEChERS method is the potential for the simultaneous analysis of multi-pesticide residues. With the new design of Agilent's SampliQ QuEChERS kits, the whole procedure is even faster, easier, and offers more time and labor savings, while ensuring consistency. An analyst can process 40–50 samples in just a few hours. Adding a food sample with a high percentage of water directly to the salts may create an exothermic reaction that can affect analyte recovery. Agilent's SampliQ salts and buffers are uniquely prepared in anhydrous packages. This allows addition **AFTER** adding solvent to the sample, as specified in the QuEChERS methology. The final QuEChERS sample still contains food matrix impurities because it is a very simple sample extraction and cleanup procedure. The final apple extract appeared light green. But with the powerful selectivity of LC/MS/MS multiple reaction monitoring mode, the extracted apple blank appeared to be clean and free of coeluting impurities, indicating that the cleaned-up apple extract did not contribute any interferences with the target compounds. Figure 2 shows the chromatograms of a blank apple extract and a 10 ng/g fortified apple extract.

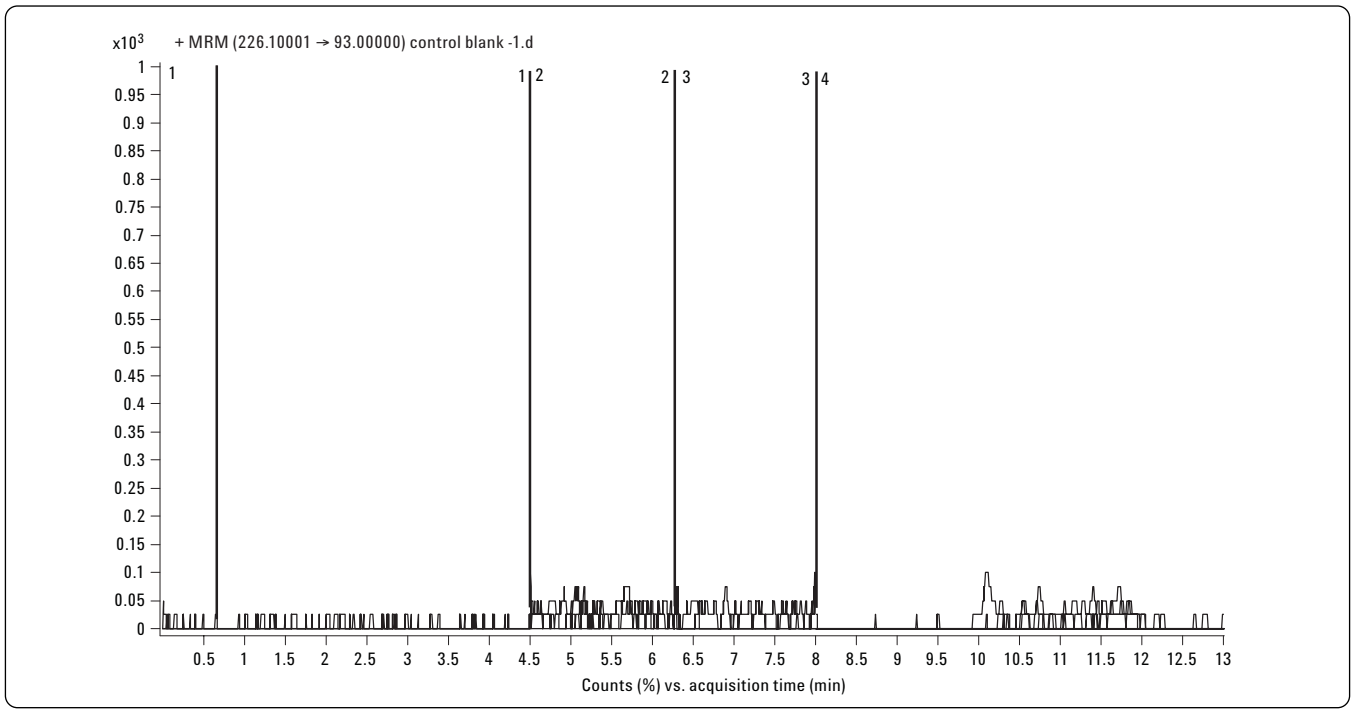


Figure 2a Chromatograms of apple extract blank. No interference was found in the blank.

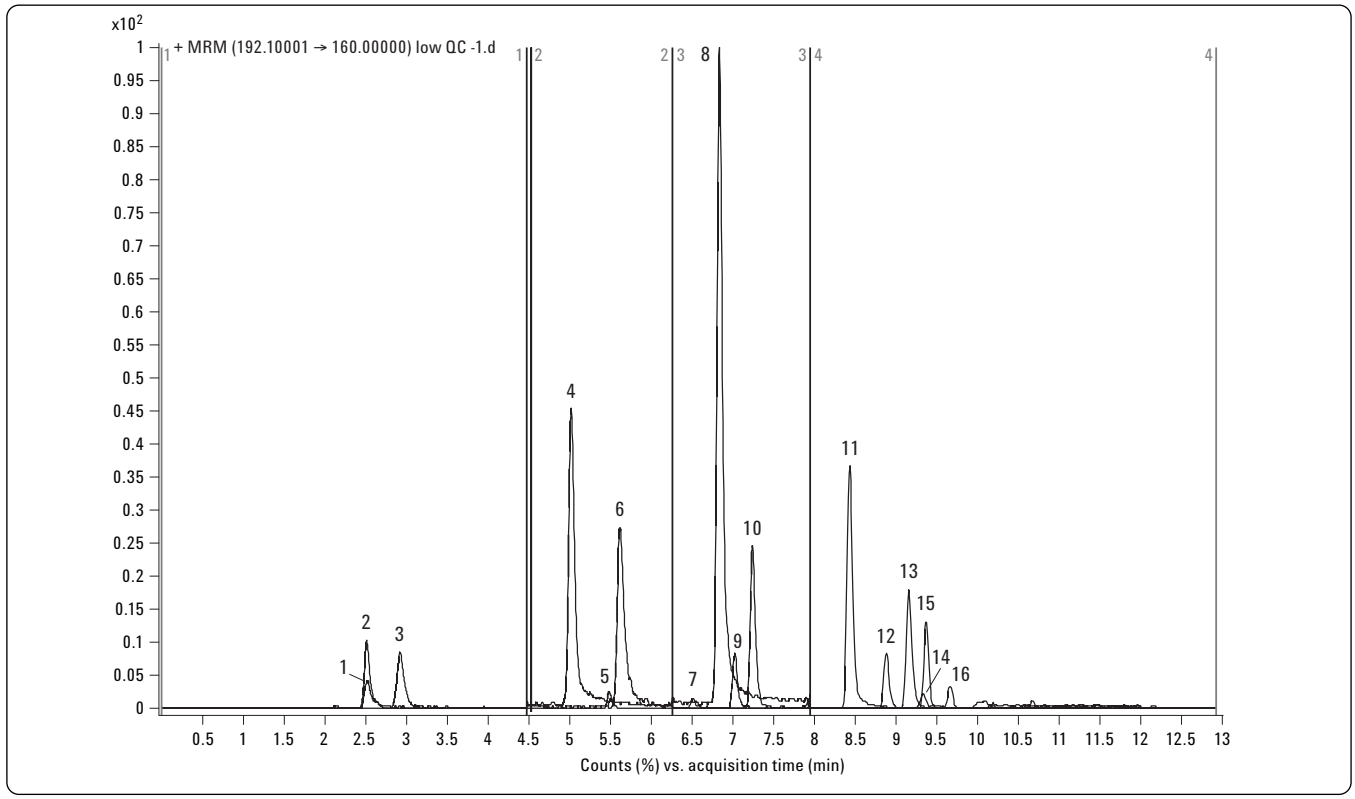


Figure 2b. Chromatogram of 10 ng/g fortified apple extract. Peak identification: 1. Methamidophos, 2. Acephate, 3. Pymetrozine, 4. Carbendazim, 5. Imidacloprid, 6. Thiabendazole, 7. Dichlorvos, 8. Propoxur, 9. Thiophanate methyl, 10. Carbaryl, 11. Ethoprophos, 12. Penconazole, 13. Cyprodinil, 14. Dichlofluanid, 15. Kresoxim methyl, 16, Tolyfluanid.

Linearity and Limit of Quantification (LOQ)

The linear calibration range for all of the pesticides was 5 – 250 ng/g. Since two different dispersive SPE sizes (1 mL and 6 mL sample volume) were used for evaluation and comparison, two sets of calibration curves were generated respectively. Matrix blanks were prepared for each size. Calibration curves, spiked in matrix blanks, were made at levels of 5, 10, 50, 100, 200, and 250 ng/g. The TPP was used as an internal standard (IS) at 100 ng/g level. The calibration curves were generated by plotting the relative responses of analytes (peak area of analyte/peak area of IS) to the relative concentration of analytes (concentration of analyte/concentration of IS). Table 1 shows that the 5 ng/g quantification limits LOQ (5 ppb) established for all of the pesticides is significantly lower than the MRLs of these pesticides in fruit and vegetables. Table 3 shows the regression equation and correlation coefficient (R^2) for both 1 mL and 6 mL dispersive SPE.

Table 3. Linearity of Pesticides in Apple Extract

Analytes	1 mL dispersive SPE Regression equation	R^2	6 mL dispersive SPE Regression equation	R^2
Methamidophos	Y = 0.3203X – 0.0005	0.9972	Y = 0.3255X – 0.0018	0.9957
Acephate	Y = 0.1373X – 0.0021	0.9975	Y = 0.1375X – 0.0010	0.9953
Pymetrozine	Y = 0.4688X – 0.0009	0.9961	Y = 0.3821X + 0.0007	0.9782
Carbendazim	Y = 1.4253X + 0.0126	0.9931	Y = 1.3379X + 0.0045	0.9903
Imidacloprid	Y = 0.0647X – 0.0004	0.9944	Y = 0.0636X – 0.0006	0.9974
Thiabendazole	Y = 0.9014X + 0.0127	0.9922	Y = 0.8600X + 0.0050	0.9942
Dichlorvos	Y = 0.0364X + 0.0002	0.9884	Y = 0.0362X + 0.0002	0.9892
Propoxur	Y = 2.4398X – 0.0001	0.9989	Y = 2.4272X + 0.0029	0.9994
Thiophanate methyl	Y = 0.3171X – 0.0015	0.9965	Y = 0.2869X – 0.0020	0.9904
Carbaryl	Y = 0.6378X + 0.0017	0.9989	Y = 0.6363X + 0.0003	0.9988
Ethoprophos	Y = 1.0897X – 0.0030	0.9984	Y = 1.0628X – 0.0001	0.9992
Penconazole	Y = 0.2334X – 0.0012	0.9978	Y = 0.2186X – 0.0003	0.9979
Cyprodinil	Y = 0.4805X + 0.0008	0.9992	Y = 0.4697X – 0.0017	0.9985
Dichlorfluanid	Y = 0.0552X – 0.0003	0.9970	Y = 0.0562X – 0.0012	0.9946
Kresoxim methyl	Y = 0.2958X – 0.0005	0.9978	Y = 0.2762X – 0.0003	0.9966
Tolyfluanid	Y = 0.0860X – 0.0011	0.9918	Y = 0.0845X – 0.0008	0.9968

Recovery and Reproducibility

The recovery and reproducibility were evaluated by spiking pesticides standards in comminuted apple sample at levels of 10, 50, and 200 ng/g. These QC samples were quantitated against the matrix spiked calibration curve. The analysis was performed in replicates of six (n = 6) at each level. The recovery and reproducibility (RSD) data of 1 mL and 6 mL dispersive SPE are shown in Tables 4 and 5, respectively. It can be seen from the results, that all of the pesticides but pymetrozine give acceptable recoveries (average of 85.7% for 1 mL and 88.2% for 6 mL) and precision (average of 6.0% RSD for 1 mL and 5.7% RSD for 6 mL). The notorious base-sensitive pesticides such as dichlorfluanid and tolyfluanid showed excellent recovery and precision. Pymetrozine, an acid labile pesticide, shows poor recovery using the European method when compared to the AOAC method [2]. With the AOAC method, an average recovery of 88% with 9.4% average RSD for pymetrozine was obtained [8].

Table 4. Recovery and Repeatability of Pesticides in Fortified Apple With 2 mL EN Dispersive SPE Tube (p/n 5982-5021)

	10 ng/g fortified QC		50 ng/g fortified QC		200 ng/g fortified QC	
Analytes	**Recovery**	**RSD (n=6)**	**Recovery**	**RSD (n=6)**	**Recovery**	**RSD (n=6)**
Methamidophos	73.0	5.6	75.6	3.1	84.3	5.3
Acephate	92.8	4.2	87.2	5.6	95.6	5.8
Pymetrozine	27.1	18.2	24.9	10.5	28.1	12.3
Carbendazim	85.1	5.9	89.5	3.4	84.1	4.7
Imidacloprid	91.0	3.3	102.7	5.4	107.2	4.9
Thiabendazole	84.8	6.8	90.4	3.5	86.7	4.0
Dichlorvos	83.1	13.9	92.2	5.2	93.1	4.6
Propoxur	97.8	2.6	100.2	2.9	100.7	3.8
Thiophanate methyl	79.9	8.5	79.9	2.9	85.5	5.7
Carbaryl	89.3	2.8	92.5	3.6	95.8	4.1
Ethoprophos	93.7	1.6	93.5	2.9	95.7	3.4
Penconazole	109.2	6.7	108.1	5.7	110.6	4.4
Cyprodinil	98.9	6.9	101.2	2.7	102.9	4.3
Dichlorfluanid	85.1	7.8	92.2	4.4	99.4	5.5
Kresoxim methyl	90.4	4.8	99.6	3.9	103.7	4.5
Tolyfluanid	98.3	13.7	102.0	4.0	106.0	4.4

Table 5. Recovery and Repeatability of Pesticides in Fortified Apple With 15 mL EN Dispersive SPE Tube (p/n 5982-5056)

Analytes	10 ng/g fortified QC		50 ng/g fortified QC		200 ng/g fortified QC	
	Recovery	RSD (n=6)	Recovery	RSD (n=6)	Recovery	RSD (n=6)
Methamidophos	77.6	4.9	77.8	6.4	81.2	2.1
Acephate	86.6	7.6	87.8	5.5	91.5	1.5
Pymetrozine	28.1	24.3	27.5	12.2	29.1	10.4
Carbendazim	89.9	6.8	88.9	3.3	81.8	3.6
Imidacloprid	105.3	10.8	105.2	4.8	106.6	5.0
Thiabendazole	89.8	4.8	87.6	3.1	84.2	1.4
Dichlorvos	97.8	14.5	98.2	5.6	98.1	2.5
Propoxur	99.5	3.8	104.0	2.6	100.9	3.3
Thiophanate methyl	87.4	5.8	88.3	4.7	89.0	7.6
Carbaryl	92.9	6.1	93.7	3.0	93.6	2.4
Ethoprophos	94.8	5.5	99.2	3.0	98.8	3.8
Penconazole	106.8	4.9	111.2	3.0	109.0	4.1
Cyprodinil	102.7	4.5	105.7	3.5	102.4	2.6
Dichlorfluanid	99.7	18.9	97.4	4.5	98.9	5.5
Kresoxim methyl	102.6	12.0	106.1	2.0	106.1	5.6
Tolyfluanid	92.0	9.3	105.5	3.3	105.1	4.3

Figure 3 shows the recovery and precision results comparison of 1 mL dispersive SPE and 6 mL dispersive SPE. The two different dispersive SPE clean-ups were performed by transferring 1 mL or 6 mL of ACN extract from the sample tube after the extraction step. To simplify the comparison, the average recovery and precision of three fortification concentrations were used for all pesticides. The results of two dispersive SPE clean-up approaches appear to be independent of volume used. There was < 10% difference in recovery and < 5% difference for RSD. Both approaches provided efficient sample clean-up, and generated relatively equivalent results.

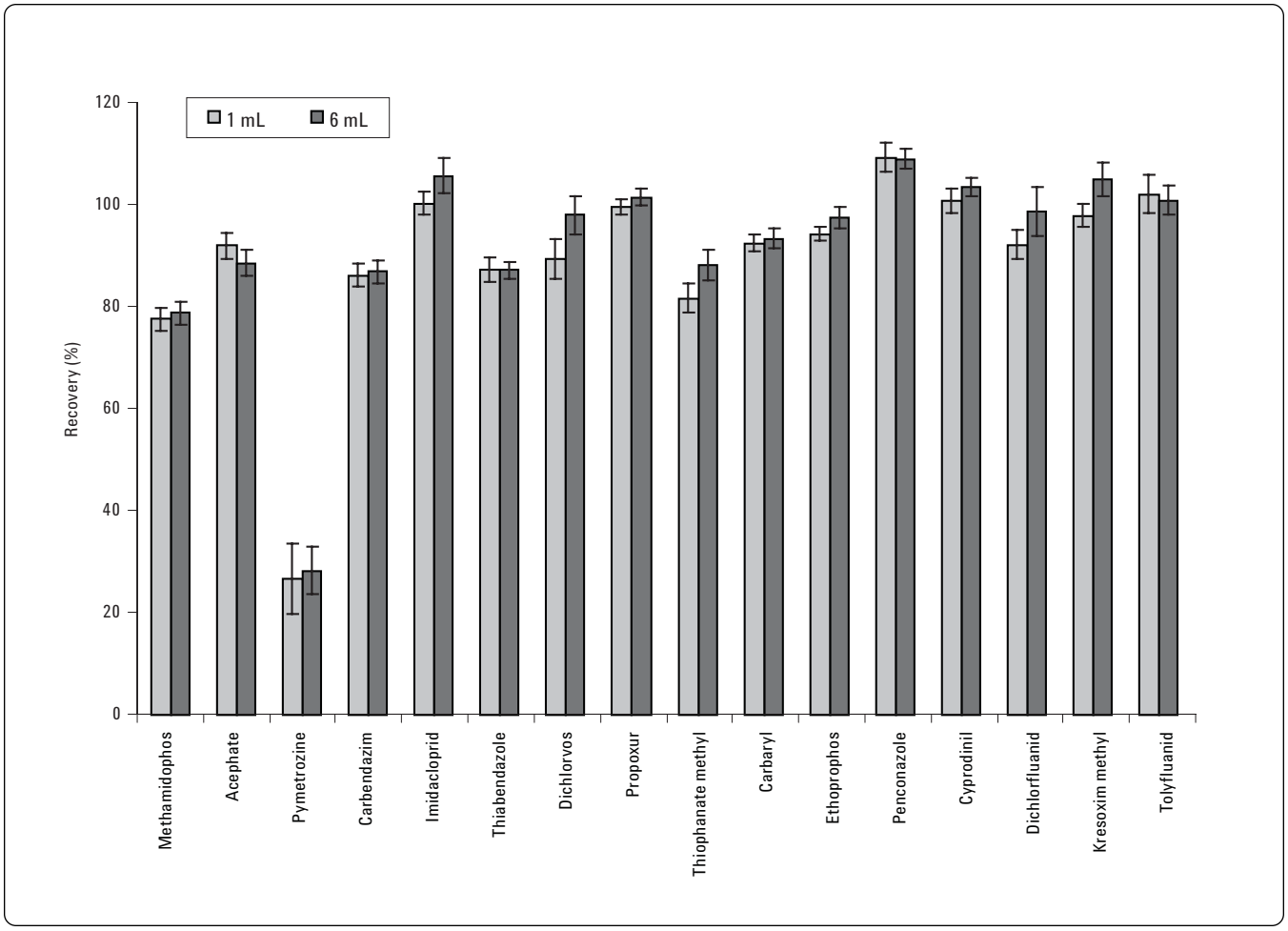


Figure 3. Results comparison of 1-mL dispersive SPE and 6-mL dispersive SPE.

Conclusions

The Agilent SampliQ QuEChERS EN fruit and vegetable kit provides a simple, fast and effective method for the purification and enrichment of selective representative pesticides in apple. The recovery and reproducibility results, based on matrix spiked standards, were acceptable for selected pesticide residue determination in apple. The impurities and matrix effect from apple were minimal and did not interfere with the quantitation of target compounds. The LOQs of the pesticides were lower than their MRLs in fruits and vegetables. As the selected pesticides represented a broad variety of different classes and properties, the Agilent SampliQ QuEChERS EN kit for General Fruits and Vegetables can be used for other pesticides in similar food matrices.

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Determining Malachite Green and Leucomalachite Green in Food by LC/MS/MS

Application

Food Safety

Authors

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Abstract

This application note demonstrates a complete method to rapidly and precisely determine residue levels of malachite green and leucomalachite green in fish with the new Agilent 6410 LC/MS triple quadrupole system. Using positive mode electrospray ionization (ESI+) and multiple reaction monitoring (MRM), qualification and quantification were accomplished without the traditional tedious PbO_2 oxidation process. The LC/MS/MS method's LOQ is 0.01 µg/Kg, which easily meets the import requirement of 2 µg/Kg set by Japan and the EU.

Introduction

Malachite green (MG) is a metallic-looking crystal. It dissolves in water easily as a blue-green solution. It is a toxic chemical primarily used as a dye and has been found very effective in treating parasites, fungal infections, and bacterial infections in fish and fish eggs.[1] On uptake, MG is rapidly reduced into leucomalachite green (LMG) and deposited in the fatty tissue of the fish with little MG remaining.

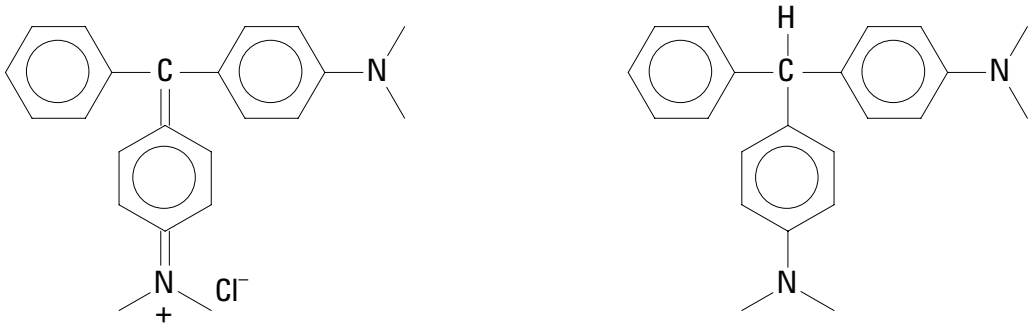
MG can cause significant health risk for humans who eat contaminated fish. For example, it can cause liver tumor formation and is suspected of carcinogenesis.[1] The United States, Japan, China, the European Union, and many other countries have already banned MG in fishery. Due to its low cost and antifungal effectiveness, MG is still being used illegally as indicated in the European Rapid Alert System for Food and Feed.[2]

HPLC with UV detection has been used to analyze MG and LMG. Figure 1 shows the structure of the two compounds. Loss of conjugation by reduction changes the chromaphore of LGM significantly. To obtain the sum of both, the method employs post-column oxidation with PbO_2 to convert LMG to MG, thus providing a sum of both comounds.[3] Most recently, LC/MS has been used to both meet the EU confirmation criteria and provide quantitative results for both compounds without the need for post-column oxidation. In this application, a simple and sensitive method for simultaneously determining MG and LMG is presented.[4, 5] The LC/MS/MS method's LOQ is 0.01 µg/Kg, which easily meets the import requirement set by Japan or the EU.[6]

Experimental

Reagents

MG	Sigma-Aldrich, CAS 569-64-2, USA
LMG	Dr. Ehreastorfer's lab, D-86199, 99% pure, Augsburg, Germany
Acetonitrile	CAS 75-05-8; Burdick & Jackson; Morristown, New Jersey, USA
Acetic acid	Merck, Germany
Ammonium acetate	CAS 631-61-8, Acros Organics, Morris Plains, New Jersey, USA



Malachite green Leucomalachite green

Figure 1. Molecular structure of malachite green and leucomalachite green.

Calibration Solutions

A stock standard solution of MG and LMG in acetonitrile was prepared at 100 µg/mL and stored at −18 °C, avoiding light. The stock solution was diluted in 50:50 acetonitrile:water to make the calibration solutions—10, 50, 100, 500, 1000, 5000, and 10,000 fg/µL.

Sample Preparation

To 5 g tilapia tissue was added 1 mL (0.25 mg/mL) hydroxylamine, 2 mL 1 M toluene sulfonic acid, 2 mL of 0.1 M ammonium acetate buffer (pH 4.5), and 40 mL acetonitrile. The mixture was then homogenized for 2 min. The supernatant was decanted, and to the precipitate was added 20 mL acetonitrile. This was filtered and added to the supernatant. To the combined acetonitrile extracts, 35 mL water and 30 mL methylene chloride were added. The solution was shaken and the methylene chloride layer collected. A second extract of 20 mL methylene chloride was made, and this layer added to the first extract. The methylene chloride was taken to dryness with a gentle stream of nitrogen and the extract reconstituted in 100 µL of acetonitrile

Instrumentation

LC	1100 LC	
Column	C18, 2.1 x 150 mm, 5 µm	
Column temp.	40 °C	
Mobile phase	A − 10 mmol/L ammonium acetate (adjust to pH 4.5 with acetic acid)	
	B − acetonitrile	
Column flow	0.3 mL/min	
Gradient	Time	%B
	0	30
	1	50
	2	95
	8	95
	8.01	30
	13	30
Injection vol.	10 µL	
MS	Agilent 6410 LC/MS Triple Quadrupole	
Ionization	ESI(+)	
Capillary	4000 V	
Nebulizer P.	35 psi	
Drying gas	11 L/min	
Gas temp.	350 °C	
Skimmer	15 V	
OctDc1 (Skim2)	45 V	
Oct RF	500 V	
Q1 resolution	Unit	
Q3 resolution	Unit	
Collision gas	Nitrogen	

The MRM parameters are listed in Table 1.

Table 1. MRM Method Parameters

Time	Compound	Precursor	Product	Dwell (ms)	Fragmentor (V)	Collision Energy (V)
0	MG	329.3	313.3	40	100	40
		329.3	208.2	40	100	40
7	LMG	331.3	316.3	40	100	30
		331.3	239.2	40	100	30

Results and Discussion

To obtain the most sensitive results, optimization of certain fragmentor voltages is important. Figure 2 shows the EICs of both target compounds at fragmentor values of 70 V, 90 V, and 100 V. The results show that the three different fragmentor values have little effect on the intensity of $[M+H]^+$ ions. Thus, 100 V was chosen for this study.

In addition, an optimal collision energy for the MS/MS must be set. Figure 3 shows the MS/MS spectra from three different collisional voltages, (a) 20 V, (b) 30 V, and (c) 40 V. Due to their structural differences, the voltage required for optimum fragmentation of each compound is different. For MG, the optimum fragmentation was observed at 40 V. The ion *m/z* 313 was due to the neutral loss of methane. The ion at *m/z* 208 was due to the neutral loss of N,N-dimethylaniline. For LMG, the optimum fragmentation was observed at 30 V. The ion at *m/z* 316 was due to the loss of a methyl radical. The ion at *m/z* 239 resulted from a subsequent loss of a benzene radical or, more likely, the rearrangement and neutral loss of toluene.

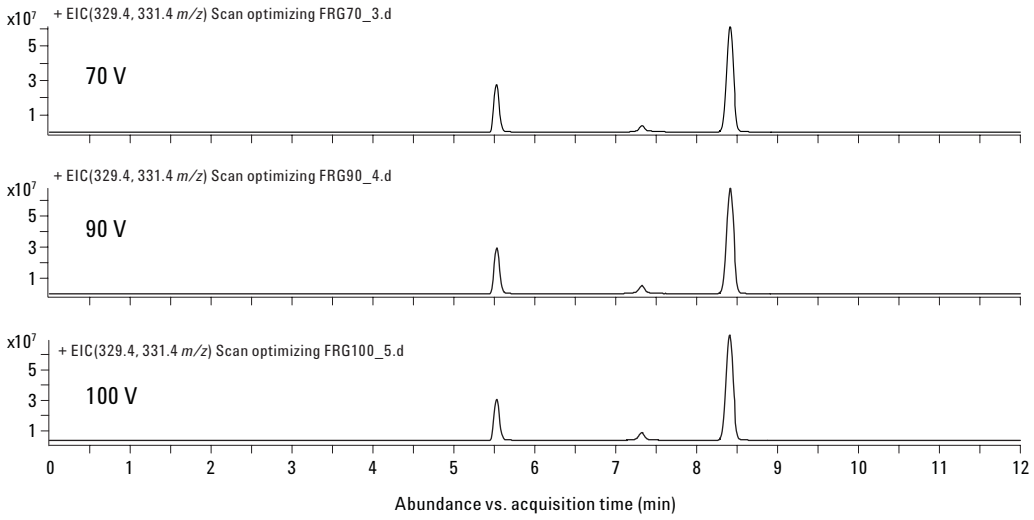


Figure 2. EICs of malachite green and leucomalachite green at fragmentor values of 70 V, 90 V, and 100 V.

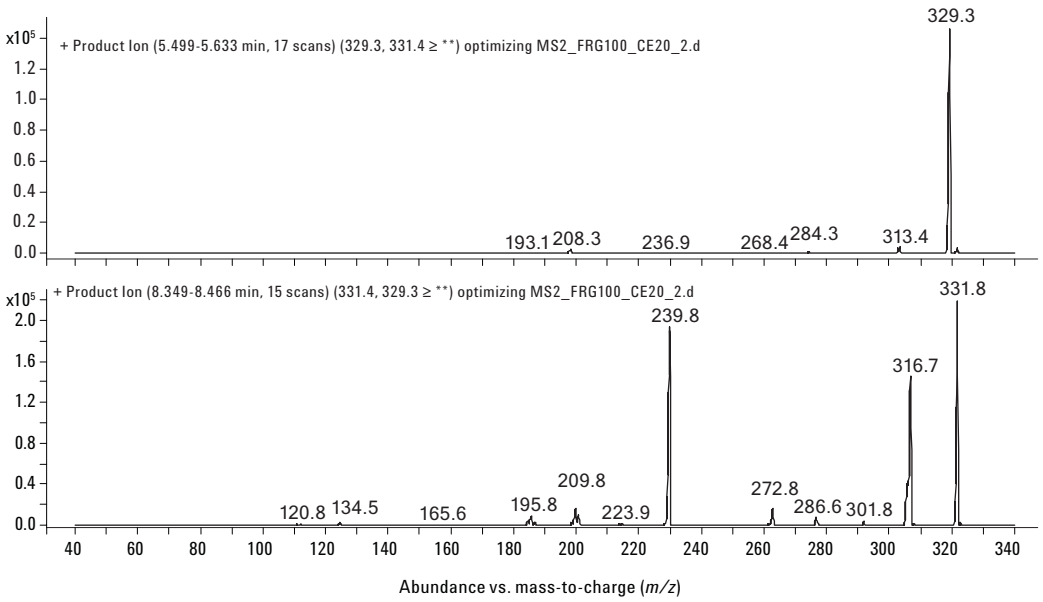


Figure 3a. MS/MS spectra of MG and LMG at collisional voltage of 20 V.

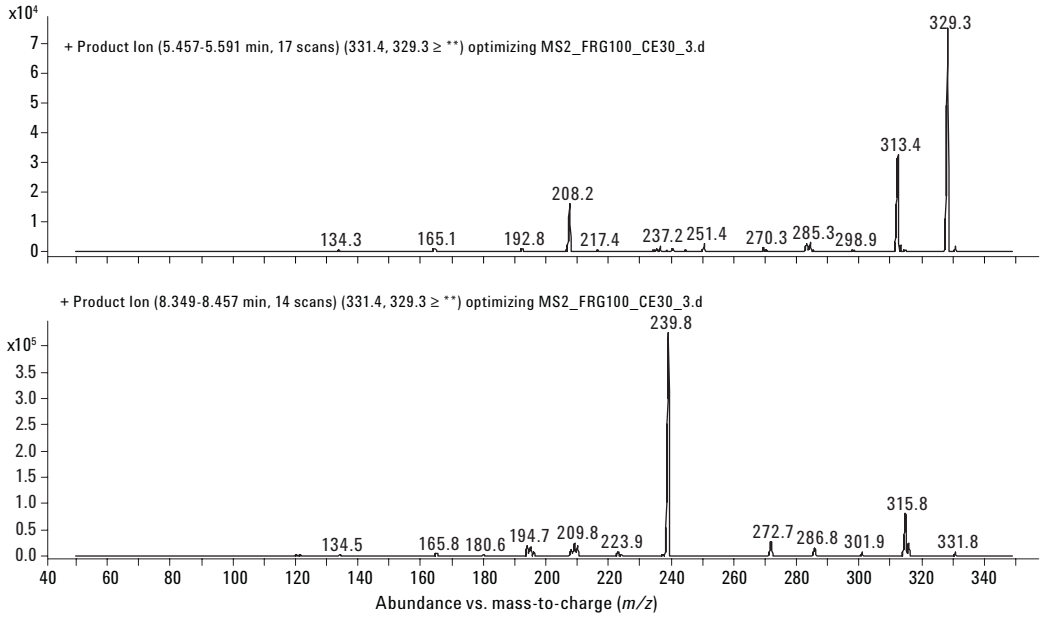


Figure 3b. MS/MS spectra of MG and LMG at collisional voltage of 30 V.

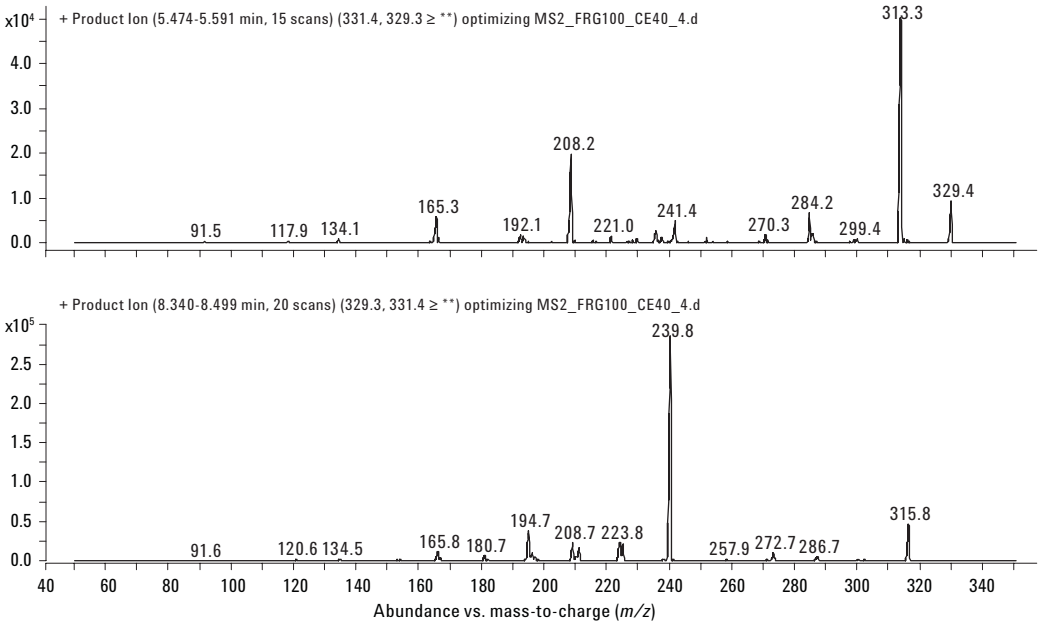


Figure 3c MS/MS spectra of MG and LMG at collisional voltage of 40 V.

Figure 4 shows the calibration curves for both MG (4a) and LMG (4b). Calibration solution concentrations were from 10 to 10,000 fg/µL. The linear calibration range is 100 to 100,000 fg on column for both compounds. The R^2 for both compounds was > 0.999 (origin ignored and no weighting). To demonstrate the sensitivity of the instrument, Figure 5 shows MS/MS spectra of a blank sample extract (5a) and sample extract spiked with 10 ppt of each compound (5b). A sample of tilapia spiked at 100 ppt MG and LMG before extraction was made to demonstrate method performance. The MRM results after extraction and cleanup are shown in Figure 6. The recover-

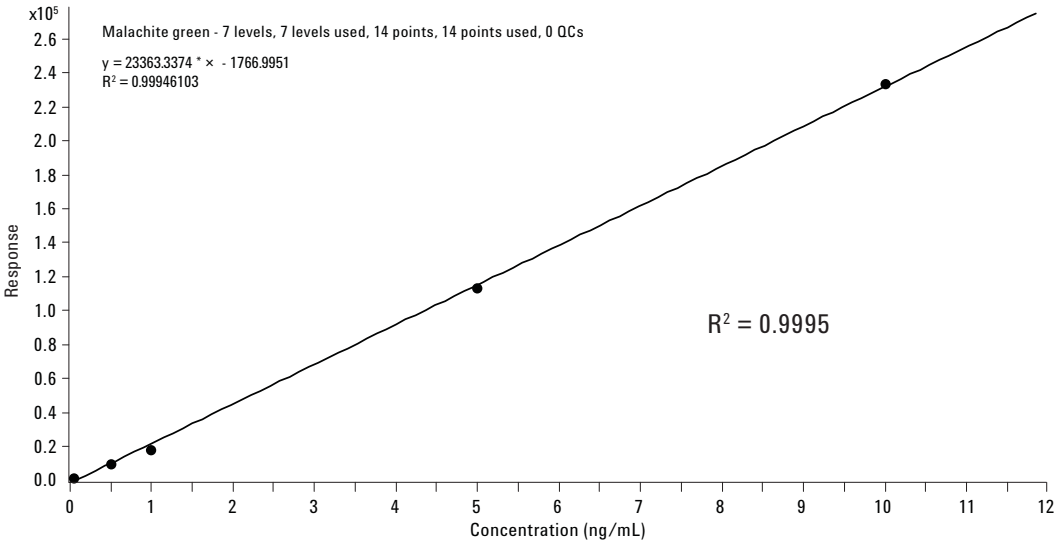


Figure 4a. Calibration curve of malachite green, linear range: 10 ppt to 10 ppb.

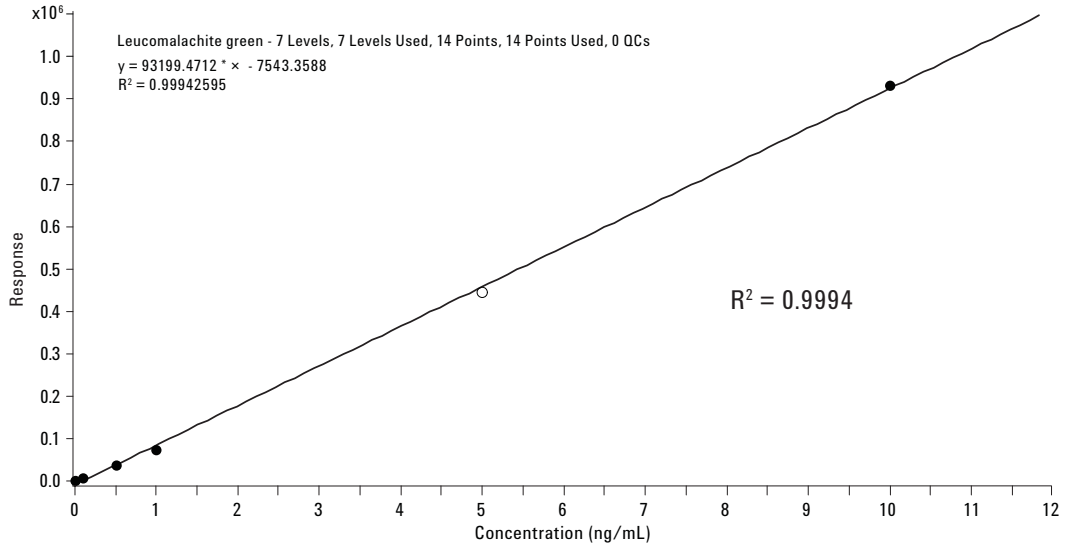


Figure 4b. Calibration curve of leucomalachite green, linear range: 10 ppt to 10 ppb.

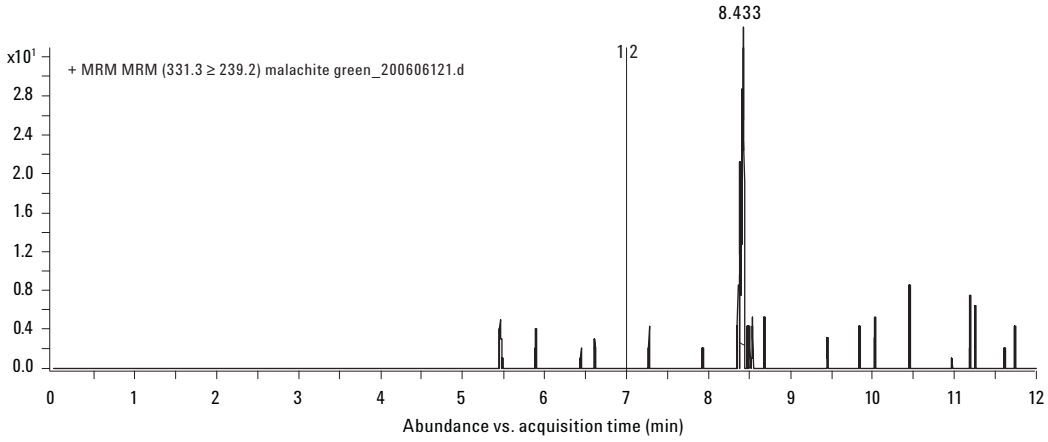
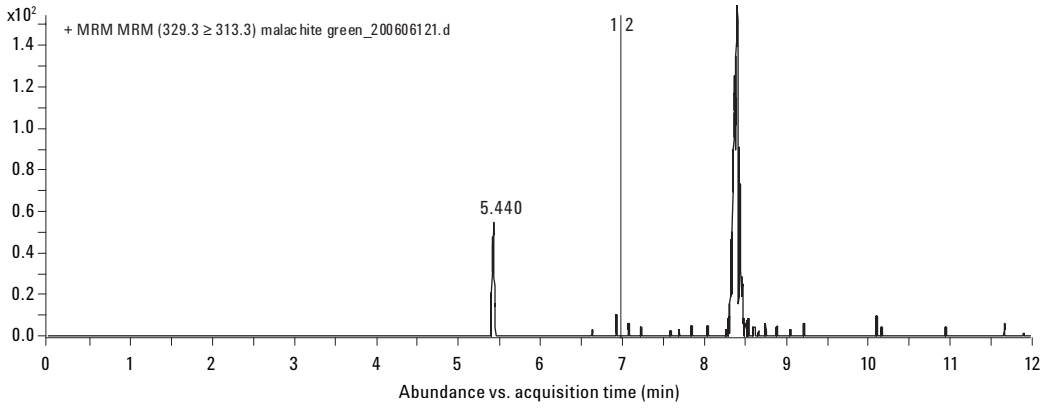


Figure 5a. MG and LMG MRM of a blank sample.



ppt spiked sample.

Figure 5b. MG and LMG MRM of a 10-ppt spiked sample.

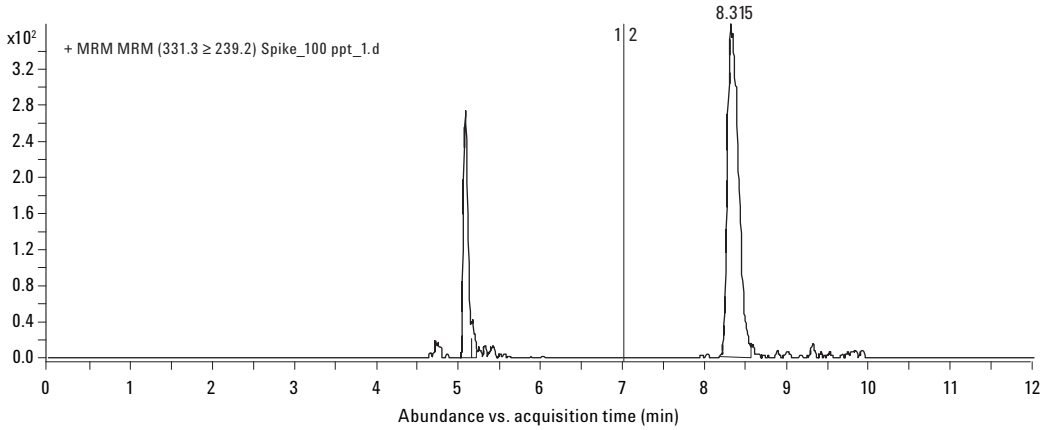


Figure 6. MRM result of talapia extract spiked with 100-ppt MG and LMG.

ies for MG were 48% and 23% for LMG. A mixture of MG and LMG at 100 fg/µL in 50:50 acetonitrile: ammonium acetate was used for the repeatability study for instrument performance. The RSD from eight injections for MG was 3.52% (S/N > 20). The RSD from eight injections for LMG was 2.25% (S/N > 40).

Conclusions

This application note demonstrates a complete method to rapidly and precisely determine residue levels of malachite green and leuco-malachite green in fish. Using positive mode electrospray ionization (ESI+) and multiple reaction monitoring (MRM) technique, the LC/MS/MS method shows detection limit of 10 ppt, which easily meets the import requirement set by Japan or EU.

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Soil Quality & Fertilizer Analysis

As countries worldwide pursue strategies towards more sustainable farming, soil quality and fertilizer analysis become vital in crop yields. Rapid determination of the extractable elements in agricultural soil samples enables assessment of micronutrient content and provides an indication of possible deficiencies. Because fertilizers have a high concentration of active ingredients, and a low concentration of contaminants, monitoring the purity is a difficult application. With MP-AES, ICP-OES and AAS, Agilent offers a comprehensive set of techniques resulting in significantly faster and simpler sample preparation and analysis, requiring only a single analytical system to measure all elements of interest.

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Determination of Mercury in a Certified Reference Sludge Material using the Agilent 710-ES

Application Note

Inductively Coupled Plasma-Optical Emission Spectrometers

Author

Peter Doidge

Introduction

Mercury is an element of great importance because of its toxicity. Over the last century, emissions of mercury due to human activities have resulted in a tripling of the concentration of mercury in the atmosphere and surface oceans [1]. The main present-day sources of the additional man-made burden of environmental mercury are coal combustion, municipal and medical waste incineration, and smelting [2].

Elucidation of the pathways resulting in human exposure to mercury remains a matter of great interest. According to Liebert et al., "there is now [at the time they wrote]..." scientific consensus that "...the most prevalent source of mercury exposure for the general population is from dental amalgam, and chronic inhalation or swallowing of amalgam mercury vapor is the major contributor to the total body burden of mercury in the U.S." [3].

Additional mercury body burdens may be derived from air, water or soils. Sewage sludges and compost from solid waste and sewage sludge often have relatively high mercury concentrations. Such organic wastes are often applied in agriculture as fertilizers, and although the recycling of waste is desirable, waste contamination by mercury may lead to higher concentrations of mercury in the soil to which it is applied, compared to those found when the soil is conventionally fertilized [4, 5].

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Determination of the levels of mercury in solid wastes is therefore a matter of some interest. Concentrations of mercury in sewage sludges are typically in the region of several parts per million (by dry weight) [5]. A concentration of 5.6 µg/g has been reported in Australian urban sewage sludge [6].

Atomic spectrochemical methods such as atomic absorption spectrometry (AAS) and ICP-OES provide the required sensitivity for the measurement of mercury at the concentrations that are usually encountered in environmental analysis. Vapor generation techniques for atomic spectrochemical analysis are widely used because of the excellent sensitivities provided for several elements, including mercury. This application note describes a simple procedure for the determination of mercury in a suitable solid organic reference material (NBS 2781, Domestic Sludge) by vapor generation coupled with ICP-OES.

Experimental

An Agilent VGA-77P (Vapor Generation Accessory) was used with an Agilent 710-ES (ICP-OES) equipped with a megapixel CCD detector. The operating conditions of the ICP instrument and VGA are as shown in Tables 1 and 2. Vapor produced by the VGA was injected into the plasma through the nebulizer and spraychamber. With the VGA in operation, the plasma was run "dry"; accordingly, a lower-than-usual power was set in the method. The VGA was operated under conditions as previously described [7, 8]. Argon was used as the VGA carrier gas. The use of nitrogen as the VGA carrier gas is not recommended on ICP.

The instrument pump rate must be optimized to ensure that the gas-liquid separator of the VGA is pumped effectively and the liquid level does not rise, or fill the gas-liquid separator. A pump rate of 40–45 rpm is usually sufficient for this purpose. This rate must be maintained whenever the VGA is in operation with sample solution being pumped. This pump rate must be incorporated into the ICP method, and can be set as a default on the Ignition page of the Instrument Setup window in the ICP Expert II software, so that as soon as the plasma is turned on, the pump speed changes to the required value. An autosampler was not used in this study.

Table 1. ICP Operating Conditions

Parameter	Setting
Power	0.9 kW
Plasma gas flow	13.5 L/min
Auxiliary gas flow	1.5 L/min
Nebulizer type	Glass concentric
Nebulizer pressure	240 kPa
Spraychamber type	Glass cyclonic (single-pass)
Torch type	Standard axial torch
Pump tubing	Spraychamber waste–blue/blue (1.65 mm ID)
	Gas/Liquid separator waste–purple/black (2.29 mm ID)
Instrument pump rate	45 rpm
Replicate read time (s)	5
Number of replicates	3
Sample delay time (s)	20
Stabilization delay time (s)	30
Fast pump	Off

Table 2. VGA-77P Operating Conditions

Parameter	Setting
Inert gas	Argon
Pump tubing	Sample–purple/black (2.29 mm ID)
	Reductant–black/black (0.76 mm ID)
	Acid/water–black/black (0.76 mm ID)
Solution uptake rate	Sample–8 mL/min
	Reductant–1 mL/min
	Acid/water–1 mL/min

Standard Solutions and Reagents

Working solutions for mercury determination in the micrograms-per-litre range were prepared from a mercury standard (May and Baker). All mercury solutions were stabilized with a 0.01% solution of AIR potassium dichromate, as described by Feldman [9]; this procedure resulted in the stabilization of the mercury solutions for many days. Calibration solutions were prepared at concentrations of 5, 10 and 20 µg/L. Stannous chloride was used as the reducing agent, though sodium borohydride has also been used with the VGA [7,8]. A solution of $SnCl_2$ was prepared as a 25% w/v solution in 20% HCl from AIR grade $SnCl_2$ (BDH) and AIR HCl (BDH). "Conditioning" of the VGA system was carried out according to advice by Moffett [10]. Before conditioning, the response for very low Hg concentrations may be variable. Conditioning of the active sites in the tubing carrying the solutions and reaction products, can be achieved by running the highest concentration Hg calibration standard (in this case 100 µg/L) through the system, until the response for much lower concentrations (for example, 1 µg/L) becomes stable.

Sample Preparation

The standard reference material NBS 2781 (Domestic sludge) was selected for analysis. 3 mL of mixed nitric and hydrochloric acids were added to approximately 0.1 g of the weighed SRM, in the volume ratio 2:1. This was digested on a hot plate for approximately two hours, or until fuming ceased. The solution was cooled, filtered and diluted to 50 mL. A drop of Anti-Foam B (Sigma Chemical Co.) was added to the solution after filtering.

Results and Discussion

Detection Limits

Detection limits determined for a 30-second replicate read time on the Agilent 710-ES instrument are shown in Table 3. (Detection limits (DLs) were measured on two instruments and similar values were obtained for the two.) Under the operating conditions used, it was found that the three lines used gave similar DLs, with slightly better DLs for the 253 nm line. This differs from the typical behavior for Agilent ICP-OES instruments with aqueous sample introduction, for which the 185 nm and 194 nm lines both give slightly better detection limits than does the 253 nm line [11]. This may be a result of the use of lower RF power, a condition which tends to favor the less "hard" spectral lines, as a result of the less efficient ionization and excitation at the lower power.

Table 3. Detection Limits (µg/L) for Hg in Aqueous Solution

Replicate read time (s)	184.887 (nm)	194.164 (nm)	253.652 (nm)
30	0.023	0.022	0.020

Short-term precision, determined from the RSD of replicate measurements of a 5 µg/L standard with 5 second replicate read times, was usually 1–2% RSD.

Analysis of a Certified Reference Material Containing Hg

Certified reference material NBS 2781 (Domestic sludge) was analysed by both "normal" calibration and by the method of additions. No difference was observed in the slopes of the graphs determined by the two methods, indicating no chemical interference. As can be seen from the results in Table 4 for the analysis of eight aliquots of the SRM, the recovery of mercury is within the range of values encompassed by the uncertainty in the concentration of the reference material.

Table 4. Results of Analysis of NBS 2781

	Hg Concentration, mg/kg
Measured value	3.40 ± 0.13
Certified value	3.64 ± 0.25
Recovery %	93

Conclusion

An Agilent VGA-77P has been used with an Agilent 710-ES for the analysis of a certified reference material (NBS 2781). The results obtained agree with certified values, within the combined uncertainties of the two results.

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Rapid Measurement of Major, Minor and Trace Levels in Soils Using the Agilent 730-ES

Application Note

Inductively Coupled Plasma-Optical Emission Spectrometers

Author

Vincent Calderon

Introduction

As part of the global strategy for sustainable farming, considerable emphasis has been placed on the need for fast, accurate and precise determination of elements in agricultural soil. As a result, simultaneous ICP-OES has become a widely used technique for reporting the health of soils in the agricultural industry.

This work describes the preparation and analysis of certified reference soil materials using the Agilent 730-ES simultaneous ICP-OES with CCD detection. The Agilent 730-ES includes a switching valve system that improves the efficiency of sample introduction and washout, providing greater sample throughput and accuracy.

A microwave-assisted acid digestion, based on recommendations given in US EPA method 3051A, was used to rapidly extract the elements from the soil samples. This method is not intended to accomplish total sample decomposition, and sample matrix compounds such as quartz, silicates, titanium dioxide, alumina and other oxides are not easily dissolved. For many environmental monitoring purposes, the concentrations of extractable elements are more important than total concentrations, as bound elements are not considered mobile in the environment [1].

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Instrumentation

An Agilent 730-ES (simultaneous ICP-OES with axially viewed plasma) was used for the analysis.

The Agilent 730-ES features a custom designed CCD detector, which provides true simultaneous measurement and full wavelength coverage from 167 to 785 nm. The patented CCD detector contains continuous angled arrays that are matched exactly to the two-dimensional image from the echelle optics. The thermally stabilized optical system contains no moving parts, ensuring excellent long-term stability.

The Agilent 730-ES also includes the productivity-enhancing Switching Valve System (SVS) that provides more efficient sample introduction and washout than traditional sample introduction systems. The SVS consists of a software-controlled, 4-port switching valve that instantaneously rinses the spray chamber following sample measurement while simultaneously presenting the next sample for measurement.

A Mars % closed vessel, microwave digestion system from CEM was used to digest the solid samples.

Solutions were presented to the spectrometer using the Agilent SPS3, Sample Preparation System.

Table 1 shows the operating parameters used in this work.

Table 1. Operating Parameters

Condition	Setting
Power	1.2 kW
Plasma gas flow	15 L/min
Auxiliary gas flow	1.5 L/min
Spray chamber type	Glass cyclonic (single-pass)
Torch	Standard axial torch
Nebulizer type	Seaspray
Nebulizer flow	0.7 L/min
Pump tubing	Sample: white-white (1.02 mm ID) Waste: blue-blue (1.65 mm ID) Buffer/Reference element: black-black (0.76 mm id)
Pump speed	15 rpm
Total sample usage	1 mL
Replicate read time	3 s
Number of replicates	3
Sample delay time	20 s
Switching valve delay	17 s
Stabilization time	12 s
Rinse time	1 s
Fast pump	On
Background correction	Fitted

Preparation of Calibration Solutions

Calibration solutions were prepared from Inorganic Ventures Inc. custom-grade multi-element solutions (VAR-CAL-1A, VAR-CAL-2A and VAR-MAJOR-1A) and from Spex CertiPrep single element solutions. These solutions contained the following elements:

VAR-CAL-1A (1000 mg/L): Mo, Sb, Sn, and Ti

VAR-CAL-2A (1000 mg/L): Ag, Al, As, Ba, Be, Cd, Co, Cr, Cu, Mn, Ni, Pb, Se, Th, Tl, U, V, and Zn

VAR-MAJOR-1A (5000 ng/L): Ca, Fe, K, Mg, and Na

Spex CertiPrep single element solutions: 1000 mg/L Al
1000 mg/L P
1000 mg/L Sr
1000 mg/L Ti

Tables 2 and 3 list the selected elemental wavelengths and standard concentrations used to calibrate each element. Sensitivity, linear dynamic range and freedom from spectral interferences were taken into consideration during wavelength selection.

Table 2. Calibration Standards for the Major and Minor Elements

Element	Wavelength (nm)	Std 1 (mg/L)	Std2 (mg/L)	Std 3 (mg/L)	Std 4 (mg/L)	Std 5 (mg/L)	Std 6 (mg/L)
Al	396.125	2	10	20	100	200	500
Ca	317.933	1	10	20	100	200	500
Fe	234.350	1	10	20	100	200	500
Mg	285.213	1	10	20	100	200	-
K	766.491	1	10	20	100	–	–
P	178.222	1	10	20	–	–	–
Na	588.995	1	10	20	–	–	–
Ti	336.122	1	10	20	–	–	–

Table 3. Calibration Standards for the Minor and Trace Elements

Element	Wavelength (nm)	Std 1 (µg/L)	Std2 (µg/L)	Std 3 (µg/L)	Std 4 (µg/L)	Std 5 (µg/L)	Std 6 (µg/L)
Cu	327.395	10	50	250	1000	10 000	100 000
Pb	220.353	10	50	250	1000	10 000	100 000
Mn	260.568	10	50	250	1000	10 000	100 000
Zn	206.200	10	50	250	1000	10 000	100 000
As	188.980	10	50	250	1000	10 000	–
Ba	455.403	10	50	250	1000	10 000	–
Cr	267.716	10	50	250	1000	10 000	–
Sr	407.771	20	50	250	1000	10 000	–
Ni	231.604	10	50	250	1000	–	–
V	292.401	10	50	250	1000	–	–
Cd	226.502	10	50	250	–	–	–

The calibration standard and blank solutions were prepared in > 18MΩ/cm^3 deionized water supplied from a Millipore system and stabilized with 5% v/v HNO_3 (Merck Tracepur).

A solution containing 2 mg/L yttrium and 1% w/v $CsNO_3$ in 5% v/v Tracepur HNO_3 was introduced to the sample online via the third channel of the peristaltic pump. Yttrium was used for reference element (internal standard) correction and cesium was used as an ionization buffer to eliminate ionization affects that potentially exist with such matrix types [2-3].

Sample Preparation

A closed-vessel microwave-assisted acid digestion was used to extract the major, minor and trace elements from the soil samples following USEPA method 3051A guidelines. This method is designed to mimic extraction using conventional heating with nitric acid (HNO_3) and hydrochloric acid (HCl) and does not accomplish total decomposition of the sample. Therefore, the extracted analyte concentrations may not reflect the total content in the sample [4]. Certified reference materials NIST SRM 2710 Montana Soil and NIST SRM 2709 San Joaquin Soil were used to validate the method.

The soil samples were prepared by accurately weighing 0.25 g of sample into the microwave digestion vessels and adding 9 mL of 10M HNO_3 (Merck Tracepur) and 3 mL of 10 M HCl (AnalaR). Following digestion, the solutions were cooled, then centrifuged for 30 minutes and transferred to 25.00 mL volumetric flasks. Each solution was diluted to volume with >18MΩ/cm^3 deionized water. Duplicate digestions were carried out.

Table 4 shows the settings used for the temperature dependent, microwave assisted digestion.

Table 4. Settings Required for Microwave Digestion

Stage	Max. power	% power	Ramp (min)	Pressure (PSI)	Temp. (°C)	Hold (min)
1	600W	100	5:00	350	120	0:00
2	600 W	100	5:30	350	175	4:30

Stage 1 was added as a reflux step to remove particulate matter that adhered to the walls of the microwave vessel during sample addition.

The moisture content of each reference material was determined as the certified values are based on dry weights. The samples were oven dried at 110 °C for 2 hours then cooled in a desiccator for 4 hours. The data were adjusted accordingly.

Table 5. Moisture Content

	Measured moisture content	Quoted moisture content range
Montana soil (NIST SRM 2710)	2.3%	1.7%–2.3%
San Joaquin soil (NIST SRM 2709)	2.4%	1.8%– 2.5%

Results and Discussion

The measured concentrations of major, minor and trace elements in the respective soil reference materials are reported in Tables 6–10. Analyses were performed in triplicate and the error reported for each result represents the largest variation from the mean value.

Table 6. Extractable Major Elements in Soil

		Al (Wt%)	Ca (Wt%)	Fe (Wt%)	Mg (Wt%)
NIST SRM 2710 Montana soil	**Reference data**				
	Certified median	1.8	0.41	2.7	0.57
	Certified range	1.2–2.6	0.38–0.48	2.2–3.2	0.43–0.60
	Sample data				
	Digestion 1	2.07 ± 0.01	0.376 ± 0.003	2.50 ± 0.03	0.510 ± 0.016
	Recovery	115	92	93	89
	Duplicate data				
	Digestion 2	2.05 ± 0.004	0.377 ± 0.001	2.50 ± 0.001	0.508 ± 0.005
	Recovery	114	92	93	89
NIST SRM 2709 San Joaquin soil	**Reference data**				
	Certified median	2.6	1.5	3.0	1.4
	Certified range	2.0–3.1	1.4–1.7	2.5–3.3	1.2–1.5
	Sample data				
	Digestion 1	2.00 ± 0.01	1.38 ± 0.01	2.63 ± 0.01	1.15 ± 0.01
	Recovery	77	92	88	82
	Duplicate data				
	Digestion 2	2.54 ± 0.02	1.38 ± 0.01	2.74 ± 0.01	1.21 ± 0.02
	Recovery	98	92	91	86

Table 7. Extractable Major and Minor Elements in Soil

		K (Wt%)	P (Wt%)	Na (Wt%)	Ti (Wt%)
NIST SRM 2710 Montana soil	**Reference data**				
	Certified median	0.45	0.11	0.054	0.10
	Certified range	0.37–0.50	0.106–0.11	0.049–0.062	0.092–0.11
	Sample data				
	Digestion 1	0.497 ± 0.003	0.0677 ± 0.0008	0.0613 ± 0.0003	0.122 ± 0.001
	Recovery	110	62	114	122
	Duplicate data				
	Digestion 2	0.492 ± 0.001	0.0681 ± 0.0001	0.0612 ± 0.0002	0.120 ± 0.0003
	Recovery	109	62	113	120
NIST SRM 2709 San Joaquin soil	**Reference data**				
	Certified median	0.32	0.07	0.068	0.038
	Certified range	0.26–0.37	0.05–0.07	0.063–0.11	0.03–0.04
	Sample data				
	Digestion 1	0.347 ± 0.001	0.0442 ± 0.0003	0.0636 ± 0.0005	0.0234 ± 0.0001
	Recovery	108	63	94	62
	Duplicate data				
	Digestion 2	0.408 ± 0.004	0.0444 ± 0.0004	0.0684 ± 0.0003	0.0545 ± 0.0006
	Recovery	127	63	101	143

Table 8. Extractable Major and Minor Elements in Soil

		Zn (mg/kg)	Mn (mg/kg)	Cu (mg/kg)	Ba (mg/kg)	Sr (mg/kg)
NIST SRM 2710 Montana soil	**Reference data**					
	Certified median	5900	7700	2700	360	100
	Certified range	5200–6900	6200–9000	2400–3400	300–400	94–110
	Sample data					
	Digestion 1	5815 ± 46	7054 ± 86	2426 ± 20	307 ± 4	90.9 ± 1.1
	Recovery	99	92	90	85	91
	Duplicate data					
	Digestion 2	5897 ± 18	7064 ± 10	2436 ± 5	306 ± 1	90.6 ± 0.2
	Recovery	100	92	90	85	91
NIST SRM 2709 San Joaquin soil	**Reference data**					
	Certified median	100	470	32	398	101
	Certified range	87–120	360–600	26–40	392–400	100–112
	Sample data					
	Digestion 1	87.2 ± 0.3	483 ± 3	29.2 ± 0.3	367 ± 1	88.7 ± 0.5
	Recovery	87	103	91	92	88
	Duplicate data					
	Digestion 2	84.2 ± 0.6	485 ± 6	29.3 ± 0.1	377 ± 3	91.4 ± 0.3
	Recovery	84	103	92	95	90

Table 9. Extractable Major, Minor and Trace Elements in Soil

		Pb (mg/kg)	As (mg/kg)	Cr (mg/kg)	Ni (mg/kg)	Co (mg/kg)
NIST SRM 2710 Montana soil	**Reference data**					
	Certified median	5100	590	19	10.1	8.2
	Certified range	4300–7000	490–600	15–23	8.8–15	6.3–12
	Sample data					
	Digestion 1	4433 ± 22	514 ± 4	19.3 ± 0.1	10.4 ± 0.1	8.90 ± 0.06
	Recovery	87	87	102	103	109
	Duplicate data					
	Digestion 2	4484 ± 29	518 ± 1	19.2 ± 0.1	10.3 ± 0.2	8.99 ± 0.05
	Recovery	88	88	101	102	110
NIST SRM 2709 San Joaquin soil	**Reference data**					
	Certified Median median		13	< 20	79	78 12
	Certified Rangerange	12–18	–	60–115	65–90	10–15
	Sample data					
	Digestion 1	10.7 ± 0.1	15.3 ± 0.1	61.8 ± 0.2	67.7 ± 0.6	11.1 ± 0.1
	Recovery	82	–	78	87	93
	Duplicate data					
	Digestion 2	11.0 ± 0.5	15.2 ± 0.6	72.5 ± 0.2	68.2 ± 0.3	11.5 ± 0.1
	Recovery	85	–	92	87	96

Table 10. Extractable Major, Minor and Ttrace Elements in Soil

		Cd (mg/kg)	Mo (mg/kg)	V (mg/kg)
NIST SRM 2710 Montana soil	**Reference data**			
	Certified median	20	20	43
	Certified range	13–26	13–27	37–50
	Sample data			
	Digestion 1	16.4 ± 0.1	14.94 ± 0.1	48.74 ± 0.5
	Recovery	82	75	113
	Duplicate data			
	Digestion 2	16.64 ± 0.1	14.4 ± 0.3	48.64 ± 0.1
	Recovery	83	75	113
NIST SRM 2709 San Joaquin soil	**Reference data**			
	Certified Median median	< 1	< 2	62
	Certified Rangerange	–	–	51–70
	Sample data			
	Digestion 1	< 0.2	1.514 ± 0.03	60.04 ± 0.2
	Recovery	–	–	97
	Duplicate data			
	Digestion 2	< 0.4	1.494 ± 0.05	74.24 ± 0.2
	Recovery	–	–	120

Note: < value indicates an undetected element with the < value expressed as 10 times the standard deviation of background emission.

Although a small amount of undissolved material was observed following microwave digestion, the overall measured concentrations of extractable major, minor and trace elements in the soil samples were in good agreement with the certified leach data.

The major and minor elements Al, Fe, Mg, Na and K were within 15% of the respective certified median values and within the certified range for Montana soil (NIST SRM 2710). The same can be said for San Joaquin soil (NIST SRM 2709) although variation between the original (digestion 1) and duplicate values (digestion 2) were found to be greater with the measured value for magnesium in the original sample and potassium in the duplicate falling just outside the certified range. Good recovery was also achieved for calcium at 92% for both soil samples and duplicates, although the measured concentrations fell just outside the lower end of the certified range. With a measured recovery of 62–63% in both soil sample types, phosphorus did not appear to undergo complete extraction, although the reproducibility of the extraction for P was excellent. On the other hand, titanium produced mixed results suggesting incomplete extraction and in homogeneity.

The majority of the remaining extractable major, minor and trace elements (Zn, Mn, Cu, Ba, Sr, Pb, As, Cr, Ni, Cd, Co, Mo and V) fell within the certified range. Those that did not fall within the certified ranges were within 16% of the certified median value.

Conclusion

Two certified reference soil materials, containing variable levels of major, minor and trace elements were digested following US EPA Method 3051A and analysed on the Agilent 730-ES Simultaneous ICP-OES. Agreement between the measured and certified values was generally very good.

The switching valve, fast rinse accessory was also used allowing more efficient introduction and washout of the sample from the sample introduction system. The determination of 21 elements in a sample took less than 65 seconds and required approximately 1 mL of solution, making the Agilent 730-ES an excellent analytical tool for fast and efficient analysis of soils.

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Analysis of Soil Extracts Using the Agilent 725-ES

Application Note

Inductively Coupled Plasma-Optical Emission Spectrometers

Author

Tran T. Nham

Introduction

In agricultural science, soil samples are routinely analyzed for micronutrient content. Analytical data permits an assessment of the nutrient levels available for plants, and provides an indication of possible nutrient deficiency.

Available metals in soil are extracted with a variety of reagents, for example, diethylene triaminepenta-acetic acid (DTPA), EDTA, water and ammonium acetate, depending on the soil type and the form of the element required [1–4].

Inductively coupled plasma optical emission spectrometry (ICP-OES) is a multi-element analytical technique that offers fast sample throughput, high sensitivity and a wide dynamic range. Soil analysis with this technique is well established[1,3,5–7]. This work describes the use of a radially-viewed simultaneous ICP-OES for the analysis of different soil extracts.

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Instrumentation

An Agilent 725-ES with simultaneous CCD detection was used for the measurements. The Agilent 725-ES features an echelle polychromator equipped with a custom designed and patented CCD detector [8] producing continuous wavelength coverage from 167 to 784 nm. The polychromator can be purged with either argon or nitrogen gas for measurements at low UV wavelengths.

The sample introduction consisted of a one-piece standard quartz torch, V-groove nebuliser and a Sturman-Masters spray chamber. An Agilent SPS3 autosampler was used to introduce the solutions to the ICP.

The operating parameters are listed in Table 1.

Table 1. Instrument Operating Conditions

Condition	Setting
Power	1.2 kW
Plasma gas flow	15 L/min
Auxiliary gas flow	1.5 L/min
Nebulizer flow	0.75 L/min
Pump speed	15 rpm
Integration time	5 s
Points per peak	2
Number of replicates	3
Sample delay time	35 s
Stabilization time	10 s
Background correction	Fitted

Reagents and Standards

All chemicals and reagents used were of analytical reagent grade. All standards and blanks were matrix-matched with the samples. All reagents and standards were prepared or diluted in ultra-pure water (resistivity >18.2 MΩ/cm at 25 °C) supplied from a Millipore water filtration system.

Preparation of Extraction Solutions

0.005 M Diethylene Triaminepenta-Acetic Acid (DTPA) Solution

1.96 g of DTPA, 14.92 g of triethanolamine and 1.47 g of $CaCl_2.2H_2O$ were weighed into a beaker and dissolved in 1000 mL of ultra-pure water. The pH was adjusted to 7.3 with concentrated HCl or triethanolamine.

1 M Ammonium Acetate Solution

77 g of ammonium acetate was weighed and dissolved in 1000 mL of water. The pH was adjusted to 7.0 with ammonia or acetic acid.

0.01 M Calcium Dihydrogen Orthophosphate Solution

1 g of calcium carbonate was weighed into a beaker and 5 mL of ultra-pure water was added. Slowly and with constant stirring, 1.4 mL of 85% orthophosphoric acid was added. Mixing was continued until the calcium carbonate had dissolved. The solution was then made up to 1000 mL with ultra-pure water.

Preparation of Soil Sample Extracts

The soil samples were dried in a 40 °C oven for 24 hours, then finely ground and sieved through a 200 mesh sieve.

DTPA Extraction (for Zn, Fe, Mn, Cu)

10 g of soil sample was weighed into a 125 mL conical flask. 100 mL of 0.005 M DTPA solution was added. The flask was stoppered and shaken for half an hour at 180 oscillations/minute and the mixture was filtered.

Ammonium Acetate Extraction (for Na, K, Ca, Mg)

10 g of soil sample was weighed into a 125 mL conical flask, 100 mL of 1 M ammonium acetate solution at pH 7.0 was then added. It was shaken for half an hour at 180 oscillations/minute and the mixture was filtered.

Phosphate Extraction (for S)

10 g of soil sample was weighed into a 100 mL plastic bottle, 50 mL of 0.01 M calcium dihydrogen orthophosphate solution was added. The bottle was stoppered and placed in a 5 rpm end-to-end tumbler for 16 hours and the mixture was filtered.

Results and Discussion

Wavelength Selection

The selection of wavelengths was based on sensitivity, linear dynamic range and freedom from spectral interferences. The wavelengths used and method detection limits for soil extracts are listed in Table 2.

The ICP Expert II software allows the simultaneous measurement of multiple wavelengths for a given element to extend its calibration range. This important analytical tool called MultiCal can also assist the analyst in confirming the analytical results.

Table 2. Wavelengths and Estimated Detection Limits Obtained at 5 s Integration Time

Stage	Wavelength	Method detection limits (µg/L)
Ca	422.673	15
Ca	317.933	30
Ca	183.738	1000
Ca	220.861	5000
Cu	324.754	10
Cu	327.395	10
Fe	238.204	10
Fe	259.940	25
K	766.491	30
Mg	279.800	100
Mg	279.079	300
Mg	278.142	1000
Mn	257.610	1.5
Mn	259.372	2
Na	588.995	10
S	181.972	150
Zn	213.857	5
Zn	206.200	25

Analysis of DTPA Extract for Zn, Fe, Mn, Cu

The mean results of the analysis of control soil samples 1 and 2 are listed in Table 3. The measured values are in good agreement with the certified values.

Table 3. Results of Cu, Fe, Mn and Zn in DTPA Extract

	Concentration (mg/L)			
	Control sample 1		Control sample 2	
	Measured concentration	Certified range	Measured concentration	Certified range
Cu	0.23 ± 0.01	0.20–0.24	0.130 ± 0.001	0.11–0.13
Fe	13.20 ± 0.03	11.90–15.20	2.49 ± 0.01	2.10–2.60
Mn	3.03 ± 0.02	2.60–3.10	1.410 ± 0.001	1.10–1.40
Zn	0.330 ± 0.001	0.30–0.36	0.042 ± 0.001	0.03–0.04

Analysis of Ammonium Acetate Extract for Na, K, Ca, Mg

The mean results of the analysis of control soil samples 1 and 2 are listed in Table 4. The measured values are in good agreement with the certified values.

Table 4. Results of Ca, K, Mg and Na in Ammonium Acetate Extract

	Concentration (mg/L)			
	Control sample 1		Control sample 2	
	Measured concentration	Certified range	Measured concentration	Certified range
Ca	425 ± 3	430–444	64.7 ± 0.8	67–72
K	11.7 ± 0.9	11.7–12.9	5.6 ± 0.1	5.4–6.2
Mg	76.8 ± 1.2	76–83	13.1 ± 0.5	13.4–14.4
Na	8.0 ± 0.5	7.6–8.3	22.1 ± 0.1	21.6–22.6

Analysis of Phosphate Extract for Sulfur

The primary S 181.972 nm line is recommended over the secondary S 180.669 nm line because of spectral interference from calcium (Ca 180.672 nm) at the S 180.696 line. However, with the use of FACT [9], both lines gave similar results.

FACT is a Fast Automated Curve-fitting Technique that provides real time spectral correction to solve spectral interference by deconvolution. The corrections are done in real time with no time penalty and can be applied retrospectively. Figure 1 shows the spectrum of control soil sample 1 at S 180.669 nm with FACT.

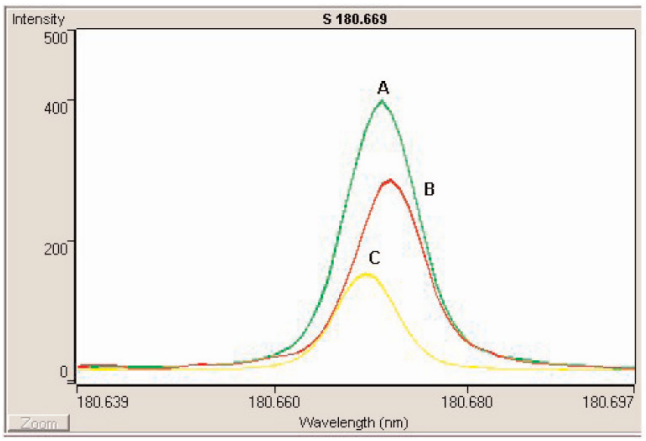


Figure 1. Spectrum of control soil sample 1 at S 180.669 nm.
A is the signal trace of the soil sample.
B is the FACT model of the interference (Ca 180.672 nm).
C is the FACT deconvolution of the S analyte at 180.669 nm.

The mean results of the analysis of control soil samples 1 and 2 are listed in Table 5. The measured values are in good agreement with the certified values.

Table 5. Results of S in Soil Extract

	Concentration (mg/L)			
	Control sample 1		**Control sample 2**	
	Measured concentration	Certified range	Measured concentration	Certified range
S 181.972	2.63 ± 0.05	2.40–3.00	7.58 ± 0.02	6.80–8.00
S 180.669 (with FACT)	2.70 ± 0.01	2.40–3.00	7.68 ± 0.01	6.80–8.00

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The Latest Advances in Axially Viewed Simultaneous ICP-OES for Elemental Analysis

Application Note

Inductively Coupled Plasma-Optical Emmision Spectrometers

Author

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Background

Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) is a popular technique of elemental analysis. ICP is applicable to around 73 elements and provides fast multi-element analysis with superior detection limits to atomic absorption spectrometry (AAS) for many elements.

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Initially ICP-OES systems featured a vertically-oriented plasma. The plasma was "viewed" by the optical system from the side, Figure 1. This configuration is known as "radial viewing" and has the advantage of providing immediate venting of exhaust gases and waste heat to an overhead extraction system. In the mid 1970's workers began to develop end-on or axially viewed plasma systems [1]. The aim of axial viewing is to observe a longer path length in the analyte-rich central channel, while avoiding viewing the surrounding intense argon plasma [2]. This approach provides improved signal to noise ratio and hence better detection limits. Axially and radially viewed plasmas are shown schematically in Figure 1.

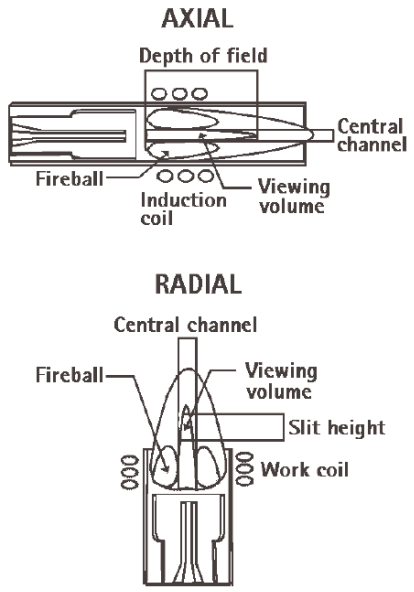


Figure 1. Schematic diagram of radial and axially viewed plasma systems, note the three concentric tubes of the torches, the vertical orientation and side viewing of the "radial" torch and the horizontal orientation and end-on viewing of the "axial" torch.

Axially viewed plasma systems find application where best sensitivity is needed, particularly environmental analyses of waters and wastes. Axially viewed ICP-OES offers a viable and more robust alternative to more expensive ICP-Mass Spectrometry systems while meeting the detection limit requirements of most regulatory bodies. Historically, the performance of axially viewed ICP systems was thought to be limited by injector tube blockage and subsequent signal drift when solutions containing high dissolved solids are aspirated. In addition, the limitations of older ICP detector designs meant that the linear dynamic range of axial ICP was restricted to upper concentration limits of just 10–100 parts-per-million. This linear dynamic range problem spurred interested in "dual viewed" plasmas - a horizontal plasma viewed alternately from the end or from the side. Dual viewed systems however require samples to be analyzed twice - once with each viewing mode, and so productivity is restricted.

The reputation of axial ICP systems has unfortunately been undermined by these historical observations. The purpose of this paper, is to review the latest advances in ICP-OES detectors, software and sample introduction systems which lead to modern simultaneous axially viewed ICPOES systems that overcome these perceptions. Today's modern axially viewed ICP systems provide the productivity of a single analysis with wide dynamic range from one plasma view.

Advances in ICP-OES Detector Design

Early ICP designs used photomultiplier tubes (PMT) to detect light emitted from the plasma. PMTs could be used in either simultaneous ICP systems, with multiple detectors being placed around a Rowland circle or coupled to a sequential scanning monochromator such as a Czerney-Turner design. The disadvantages of each of these approaches is clear - using discrete detectors to measure each wavelength means you have to choose the wavelengths to measure ahead of time - restricting future flexibility. Sequential scanning designs, while more flexible, require more time to complete an analysis.

In the early 1990s various groups developed solid state simultaneous detection systems either based on Charge Coupled Devices (CCD) or Charge Injection Device (CID) designs. These devices differ in the way in which they measure the electronic charge created on the surface of the detector. A useful review of these technologies was provided by Harnly and Fields in 1997 [3]. With both detector types, an array of light sensitive detectors or pixels is used to convert the incoming photons into electrons for measurement.

These detectors are generally used in conjunction with an echelle polychromator which creates a 2 dimensional spectrum from the light emitted by the plasma. The emitted light is split both into its component optical orders (creating a series of "rows" of light) and also into its component wavelengths. One CCD design implemented in the early 1990s positioned the light sensitive pixels at the locations of preferred wavelengths [4]. This design became known as the Segmented

Array CCD detector or SCD. Pixels were created in small linear groups positioned to detect the preferred suite of wavelengths. The SCD used just 6336 pixels, presumably in an attempt to minimize perceived limitations in “readout speed and photometric data quality”. The restricted number of pixels no doubt matched the limited data processing capabilities of the electronics and computers then in use. The SCD is still used today with a current ICP-OES design. The limitations of the SCD are clear - pixel groups are positioned only at locations of preferred wavelengths - thereby imposing the same restrictions of wavelength choice and flexibility as the multiple PMT based designs of the 1970s and 1980s. Barnard et. al. [4] state that the SCD provides only “5.7% coverage of the spectrum from 167 to 782 nm”. Because of this inherent restriction on available wavelengths current ICP designs based on SCD technology cannot take advantage of the improvements in linear dynamic range that can be obtained by using multiwavelength data. As a result, alternative plasma viewing methods, such as the dual view systems, were developed to compensate for this lack of linear dynamic range.

The Vista series of simultaneous ICP-OES spectrometers were introduced in1998. In developing the Vista series, Agilent took advantage of the availability of the next generation of CCD detectors [5]. The VistaChip (Figure 2) CCD features over 70,000 pixels positioned to exactly match the free spectral range of the two dimensional echellogram. Zander et. al. [5] referred to this as "image mapping", with the pixels being positioned so as to match the exact angle and alignment of 70 orders of light coming from the echelle spectrometer. The placement of the pixels in continuous rows provides complete and continuous wavelength coverage of 96% of the analytical spectrum. This approach opens up a major advantage of ICP-OES - using alternative wavelengths to avoid spectral interferences and to extend linear dynamic range by using wavelengths in combination.

In August 2000, the world’s first array CCD detector applied to simultaneous ICP-OES with the Vista- MPX was announced. The MPX detector features over 1.1 million pixels arranged in an X-Y array - again providing up to 96% coverage of the analytical spectrum from a single simultaneous reading. The Vista-MPX achieves the linear dynamic range and flexibility advantages of the Vista image mapped CCD, in an even more affordable package. Both systems provide the advantages of true simultaneous ICP-OES, with simultaneous background correction and internal standardization providing more accurate and precise results. In addition, the systems include no moving optical components, resulting in excellent long term stability and analysis speeds compared to sequential scanning systems. The grating and prism of the echelle spectrometer used in the Vista series are fixed and the optics thermostatted providing long term drift-free performance without the need for correction lamps. With most axial and dual view systems the ICP torch is oriented horizontally and viewed end-on (Figure 1). This orientation is preferred because end-on viewing of a vertical (radially viewed) plasma is more difficult due to hot, corrosive vapors travelling past the viewing optics.

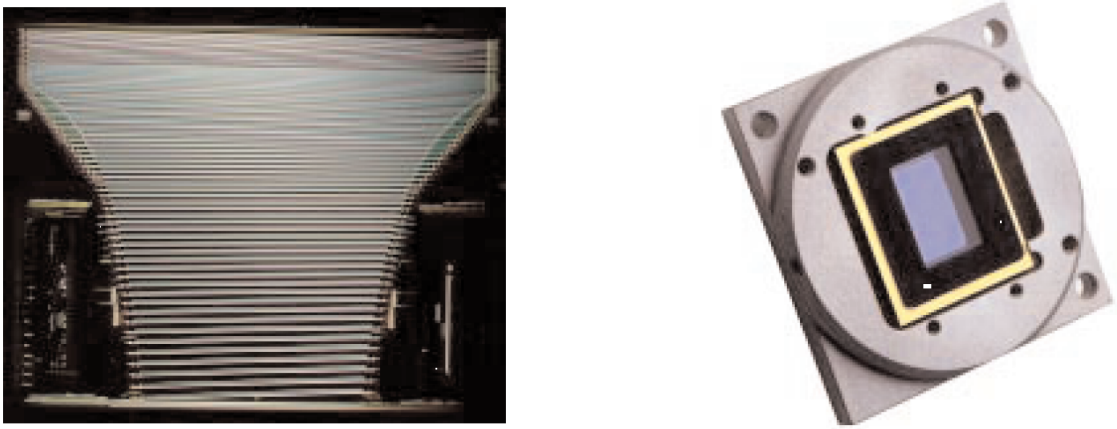


Figure 2. The Agilent VistaChips - the Vista Pro CCD (left) and the Vista-MPX CCD (right). The Vista Pro CCD image shows the continuous lines of pixels (photosensitive detectors) exactly positioned to match the spectral output from the echelle spectrometer. Note the differing slopes and separations of these "diagonal linear arrays" of pixels. The Vista-MPX CCD (right) is an array detector with over 1.1 million pixels in an X-Y grid.

Advances in Axial Viewing Systems

The Horizontal, Axially Viewed Plasma

- Offers 4–10x improvement in sensitivity compared to the radial view, due to longer path length of measurement
- Is ideal for routine analysis of samples containing less than 5% dissolved solids. With simple modifications to the sample introduction system Agilent axial ICP now can analyze 25% dissolved solids solutions directly for 24 hours.
- There are key differences between axially viewed plasmas. Agilent's Cooled Cone Interface (CCI) displaces the cooler tail flame of the plasma away from the optical path, resulting in greater linear dynamic range and a significant reduction in atomization and recombination interferences.
- Agilent's CCI requires only a small counter flow of argon gas, 2.5 L/min. The alternative shear gas approach used on many dual view systems requires very high volumes of gas, from 15-20 L/min, to displace the plasma from the optical path. This gas must be nitrogen to measure below 190 nm.
- Agilent's CCI design makes the axially viewed plasma ideal for analyzing organic solvents.

The axially viewed plasma provides the benefits of improved sensitivity and detection limits and this performance has seen this configuration grow in popularity in the past decade. Table 1 shows the improvement factors in detection limits that can be obtained using axial viewing compared to radial viewing, typically this factor is between 4–10 times. Axially viewed ICP systems achieve detection limits that meet the majority of requirements for drinking water, waste water and other important environmental applications. The axially viewed ICP easily meets the detection limit requirements of the US EPA [6] for example.

Table1. Comparison of 3 σ Detection Limits for Radially and Axially Viewed Vista-Pro ICP. All Data was Collected Using 30 Seconds Integration Times [7]

Element	Wavelength (nm)	3 σ Detection Limits Vista-PRO radial (µg/L)	Vista-PRO axial (µg/L)	Improvement factor
Ag	328.068	1	0.3	3.3
Al	167.016	0.9	0.2	4.5
As	188.979	5	1.5	3.3
Au	267.595	5	1.0	5
B	249.773	0.6	0.1	6
Ba	455.403	0.15	0.03	5
Be	234.861	0.05	0.01	5
Bi	223.061	6	2	3
Ca	396.847	0.06	0.01	6
Cd	214.438	0.6	0.05	12
Ce	418.660	2	2	1
Co	238.892	1	0.2	5
Cr	267.716	0.9	0.15	6
Cu	327.396	1	0.3	3.3
Fe	259.940	0.8	0.1	8
K	766.490	4	0.3	13.3
Li	670.784	1	0.06	16.7
Mg	279.553	0.04	0.01	4
Mn	257.610	0.08	0.03	2.7
Mo	202.030	2	0.5	4
Na	589.592	2	0.15	13.3
Ni	231.604	1.4	0.3	4.7
P	177.432	5	2	2.5
Pb	220.353	5	0.8	6.3
S	181.971	10	5	2
Sb	231.147	5	2	2.5
Se	196.026	6	2	3
Si	251.611	2.2	1.4	1.6
Sr	407.771	0.05	0.01	5
Ti	334.941	0.2	0.1	2
Tl	190.790	6	2	3
V	292.402	0.7	0.2	3.5
W	207.911	3.5	2	1.8
Zn	213.856	0.8	0.2	4
Zr	343.823	0.9	0.3	3

The Agilent axially viewed ICP systems all share the same viewing configuration, shown in Figure 3. The plasma is directed at a Cooled Cone Interface (CCI), consisting of a water cooled nickel cone with a large sampling orifice cut in its tip. A small counter flow of argon gas passing through the cone ensures that the heat and vapors from the plasma cannot compromise the optical system, which is further protected by a quartz window. The CCI displaces the cooler plasma tail away from the optical path of the ICP instrument. It is in this cooler region of the plasma that analyte self-absorption, vaporization and ionization interferences can occur.. Brenner and Zander concluded [8] that "removal of the cool fringe reduces matrix effects due to Easily Ionizable Elements and Ca and extends the linear range of calibration and determination."

High Solids Capabilities

The axially viewed ICP provides excellent long term stability for samples containing up to 5% dissolved solids using standard sample introduction systems. With normal rinsing routines, these samples can be analyzed throughout the day using an axially viewed ICP. Most samples fall below this high dissolved solids limit. For example, if 1g of sample is digested and diluted to 100 mL this represents 1% dissolved solids in the sample. Figure 4 shows the long term stability of the Agilent axially viewed Vista ICP for the continuous analysis of 5% sodium chloride solution [9].

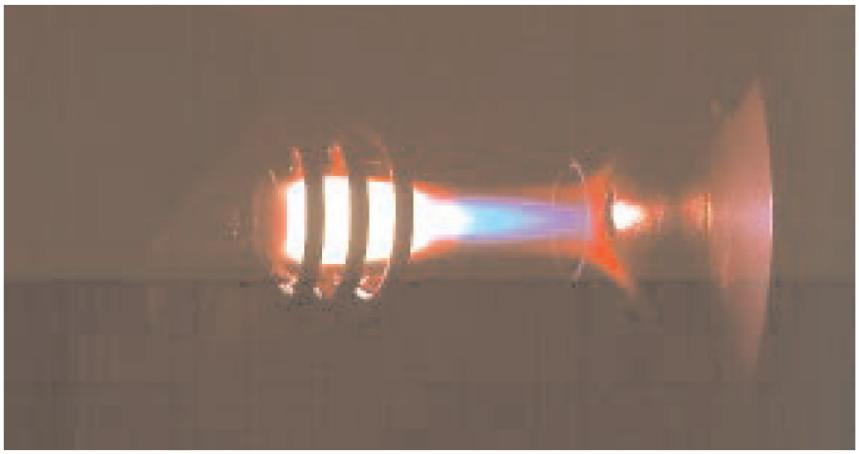


Figure 3. Photo of Agilent's Cooled Cone Interface. The cooler red zone of the plasma can be seen displaced around the outside of the cone, while aspirating Yttrium. The large central hole in the cone allows the optical system to observe the central channel of the plasma. A counter flow of argon gas and a sealed optical window behind the cone, protect the optical system.

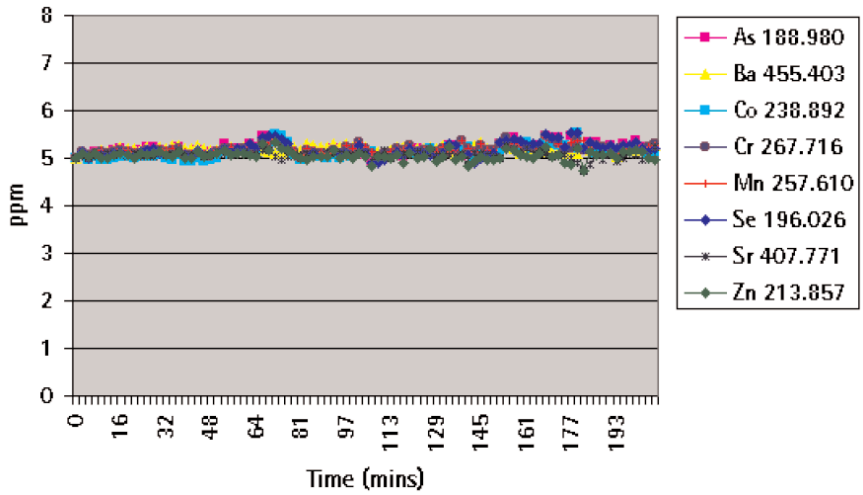


Figure 4. The stability of a range of elements from a continuously aspirated solution of 5% NaCl using the Agilent Vista-MPX simultaneous ICP-OES. Percent standard deviation over a period of 3.5 hours was less than 2.5% in all cases. Note this analysis was conducted without between-sample rinsing which would further extend operation time.

Recently, a range of new spraychamber, nebulizer and torch options has been developed for ICP-OES which allows longer analysis periods for higher levels of dissolved solids. These options include double pass spraychambers, "v-groove" nebulizers, wider bore injector tubes and demountable axial torch designs. By simply selecting appropriate sample introduction system components, long term analysis of high levels of dissolved salts is now obtainable. An example of this work is shown in Figure 5 [10]. Using a modified torch with an axially viewed Vista-MPX simultaneous ICP-OES, the direct analysis of 25% sodium chloride solutions was demonstrated for over 24 hours of continuous aspiration. The precision over 24 hours ranged from 3.3% to 5.2% relative standard deviation. This work shows that the high salts stability of the Agilent axially viewed ICP's is dependent upon the choice of sample introduction system and not on the instrument itself. In this way, the performance of a radially viewed ICP can be obtained from an axially viewed ICP without the need for dual view optics.

Linear Dynamic Range

Linear Dynamic Range (LDR) is an important performance characteristic of ICP spectrometers as it defines the upper and lower limits of analyte concentrations that can be accurately measured. The lower end of this scale is defined by detection limits (see Table 1) or determination limits and the upper end is defined by the limits of calibration linearity. The US EPA [6] uses a 5% calibration accuracy definition to determine this upper concentration limit. LDR limitations are often cited as the need for dual view plasma systems - that is, it is claimed that to measure high levels of analytes (> 100 mg/L) both the radial view and the axial view are required in the one spectrometer. Since both views cannot be measured simultaneously, the dual view approach slows down the productivity of the analysis.

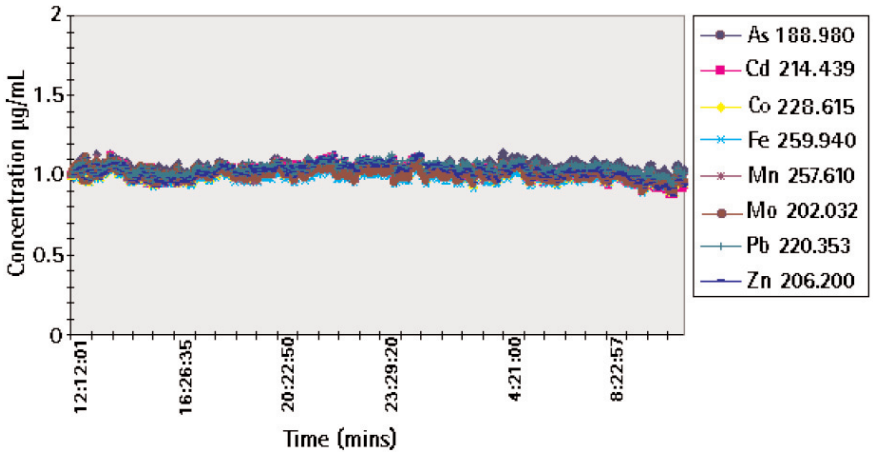


Figure 5. The continuous analysis of 25% sodium chloride solution over 24 hours using a Vista-MPX axially viewed ICP (expected concentrations 1mg/L). The solution was continuously aspirated without rinsing and no internal standard correction was used. The plasma torch was fitted with a high solids injector tube. A Sturman Masters double pass cyclonic spraychamber and v-groove nebulizer were used with an argon saturator accessory (ASA).

As noted earlier, many of the assumptions about the linear dynamic range of ICPOES systems may have been based upon detector and plasma interface design limitations of that era [4]. With the introduction of Agilent's Cooled Cone Interface (CCI) these limitations have been overcome and it is possible to have wide linear dynamic range from one axially viewed plasma system without the need to analyze the sample twice. In the past, the linear dynamic range of axially viewed systems was limited by analyte selfabsorption due to the longer path length of measurement. The Agilent CCI provides extended linear dynamic range by optimizing the observation of the central channel of the plasma and eliminating observation of the cooler plasma tail.

When coupled with the full wavelength coverage of the Agilent ICP-OES systems, the axially viewed configuration can be used from parts-per-billion detection limits up to maximum concentrations of percentage levels. This performance is shown in Table 2 [12], which shows the upper limits of linear dynamic range for elements using the axially viewed, simultaneous Vista-MPX. Comparing these results to the detection limits in Table 1, it can be seen that a very wide dynamic range can be obtained from one axially viewed ICP system with just one sample measurement.

Table 2. Linear Dynamic Range Test Results on the Axially Viewed Vista-MPX with CCI, Showing Accurate Recoveries of Elements up to 500 and 600 mg/L is Easily Possible. Vista CCI Provides Radial View Performance with the Benefits of Axial Sensitivity

Element		LRA (mg/L)	Recovery %
Ag	328.068	10	101
Al	236.705	500	99
Al	308.215	40	100
As	188.980	10	103
Ba	585.367	200	95
Be	313.042	5	101
Ca	315.887	100	99
Ca	370.602	600	97
Cd	226.502	5	99
Co	228.615	50	97
Cr	267.716	20	100
Cu	327.395	30	101
Fe	234.350	100	100
Fe	258.588	500	95
K	404.721	500	104
K	766.491	50	102
K	769.897	200	101
Mg	279.800	600	98
Mg	383.829	600	99
Mn	257.610	50	97
Na	330.237	600	97
Na	589.592	50	98
Ni	231.604	50	97
Pb	220.353	100	101
Sb	206.834	10	103
Se	196.026	100	101
Tl	190.794	100	101
V	311.837	60	99

Advances In Software Design

To take advantage of the full wavelength coverage of Agilent's CCD detector technology, the Vista simultaneous ICP systems feature MultiCal, which automatically assigns each sample to the best wavelength for that result. If a sample result falls within a particular concentration range it is automatically assigned to the wavelength that is most appropriate for that concentration. In this way the Vista series of instruments takes advantage of the availability of all of the wavelengths in the analytical spectrum - automatically combining the most sensitive wavelengths for best detection limits with less sensitive wavelengths for best dynamic range.

Figure 6, shows the MultiCal advantage, with a pair of calibration graphs for calcium, measured on the Vista-MPX. Low concentration results are automatically reported from one calibration graph and high concentration results reported from a second calibration graph - extending the linear dynamic range for this element. Also in this example, the results from the two wavelengths have been mathematically combined into one calcium result. In this case the average of the two calcium results from the two wavelengths has been used, however users may also choose from weighted mean, minimum and median of the results. Only results that fall within the valid calibration range of the wavelengths will be used in these calculations – providing extra surety of data quality. The original wavelength columns can either be displayed (for full information) or hidden (for simplicity and ease of use). By displaying all available wavelength information, the user is given another data quality control check, by comparing the accuracy of the results from two or more wavelengths for the same element.

Conclusions

In this paper we have reviewed the recent developments in the design of axially viewed ICP-OES. Axially viewed ICP-OES has emerged as the preferred viewing technique due to its benefits of enhanced sensitivity and detection. Agilent's Cooled Cone Interface is an optimized design that eliminates chemical and molecular interferences and extends the linear dynamic range of the axial ICP. This axial design combined with Agilent's CCD detector technology provides simultaneous measurement of all wavelengths and further extends the linear dyanamic range of ICP-OES. With simple changes to the sample introduction system, Agilent's axially viewed ICP-OES can analyze high dissolved salt samples continuously with excellent long term stability.

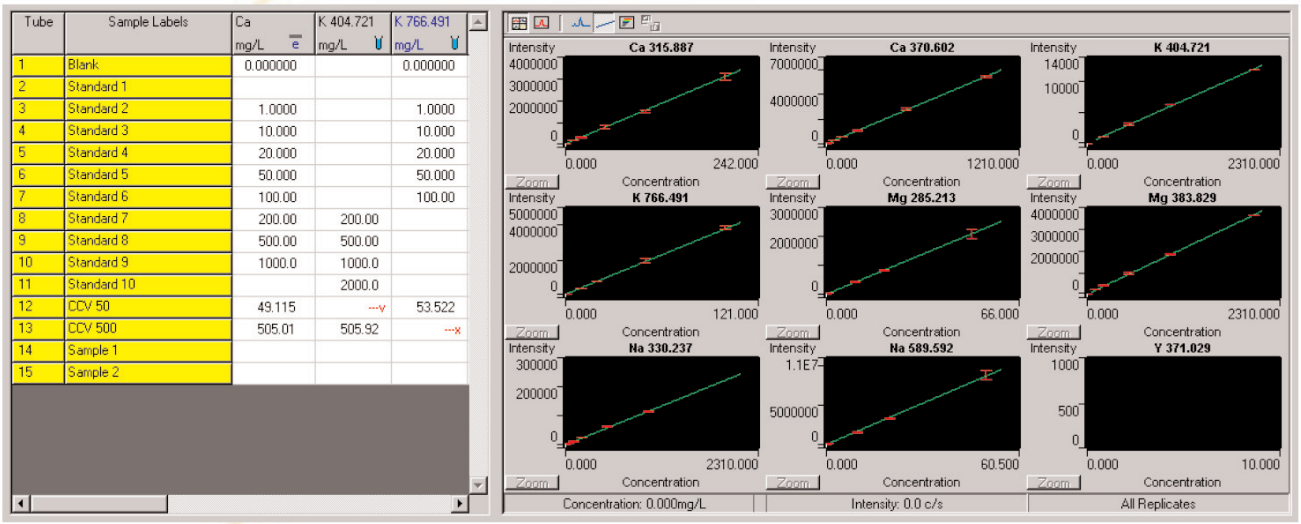


Figure 6. The Agilent MultiCal advantage - the first column shows the mean result from two Ca wavelengths - 315.887 and 370.602 nm, automatically combined by the Vista-PRO software. The combined wavelengths allow calibration to 1000 mg/L from a single analysis on this axially viewed ICP. This extended linear dynamic range would normally only be possible with a radially viewed ICP. With MultiCal, the Vista ICP series provides both the extended linear dynamic range of a radial ICP and the excellent detection limits of an axially viewed ICP with one simple analysis. The accuracy of the results is established from the 50 mg/L and 500 mg/L Continuing Calibration Verification (CCV) results.

The unique MultiCal automates the intelligent assignment of sample results to the most appropriate wavelength. The accurate measurement of high concentration matrix elements can now be performed simultaneously with trace level detection of other analytes of interest from one axial plasma viewing system and one measurement. MultiCal eliminates the need for Dual View optics, enhances productivity and reduces argon consumption.

Agilent's CCI and MultiCal approach offers significant advantages that should see axially viewed ICP-OES remain a popular technique for many years to come.

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Analysis of Plant Materials by VaporGeneration AA

Application Note

Atomic Absorption

Author

Keith G. Brodie

Introduction

Arsenic and antimony are widely used in modern industries although they are toxic to both man and animals. Significant levels of these elements can be found throughout our environment in such diverse sources as soils, plants, fish and animals. Selenium, on the other hand, is an essential trace element but can be toxic at higher levels. The essential role of selenium in animal nutrition was first demonstrated in 1957 [1] but man's requirements are not yet fully defined.

The concentration of these elements in foods is dependent on the soil conditions and ultimately on the methods of preparation of the food. The levels found in animal products is dependent on the plant material or other animal food source.

It is of considerable interest therefore to establish the level of these elements, as well as other toxic metals, in soils, plants and animal tissues.

Arsenic, antimony and selenium have been determined at very low levels by atomic absorption for many years, and vapor generation atomic absorption offers the most sensitive means by which they can be measured.

The results of extensive collaborative studies on acid digestion techniques, and hydride generation AA for the determination of arsenic and selenium in foods have been reported [2,3]. Several laboratories were involved in those reports.

Sample digestion procedures have ranged from ashing in a furnace with a magnesium nitrate solution [4], to hot digestion in acids usually comprising nitric and perchloric mixtures [5]. Agilent instruments have been used for the vapor generation measurement of selenium levels in plant and biological material after suitable acid digestion [5,6].

Many laboratories are reluctant to use perchloric acid with digestions of organic materials because of the potential risks involved [7,8]. For this reason a number of digestion procedures have been devised which have eliminated perchloric acid. Sulphuric acid increases the boiling point of an acid digestion mixture and improves

the action of the other oxidizing acids such as nitric. The only disadvantage of sulphuric acid is the tendency to form insoluble compounds but the presence of nitric acid avoids the precipitation of trace metals with major components of the sample. Digestion mixtures of sulphuric, nitric and hydrogen peroxide have been used successfully for plant materials [7].

The results of the determination of arsenic, antimony and selenium in NBS orchard leaves (No 1571) by the vapor generation technique are reported here. A digestion procedure was prepared which did not contain perchloric acid.

Instrumentation

An Agilent AA-1475 with background correction and the VGA-76 were used in this study. The background signal was examined for all solutions, but no background was found. Measurements were therefore reported with the background corrector off. Hollow cathode lamps were operated at the recommended conditions and an Agilent 9176 strip chart recorder displayed the traces.

For development of this analytical technique samples were presented manually.

Absorbance mode was used and five 3-second integrations were taken.

The printer was an HP 82905A model. The reagents used for the VGA-76 were as follows:

Acid channel	Concentrated hydrochloric acid.
Sodium borohydride channel	0.6% NaBH4in 0.5% NaOH and 10% KI.

Sample Treatment

NBS orchard leaves (No. 1571) were dried at 80 °C and approximately 0.5 g samples were accurately weighed and prepared for analysis. The digestion procedure consisted of the addition of 5 mL concentrated sulphuric with swirling in a beaker. A 5 mL volume of 30% v/v hydrogen peroxide was added slowly and the reaction allowed to proceed. This addition of hydrogen peroxide was repeated, and when the reaction had subsided, the digestion mixture was heated on a hot plate.

After cooling, 2 mL of concentrated nitric acid was added and the mixture heated for about 2 hours to remove excess nitric acid. A further addition of nitric acid with heating may be required until the solution is a light straw color.

The digested mixture was made up to 25 mL to give a final solution in 7 M hydrochloric acid. This stock solution was used directly for the determination of selenium, but was diluted 1 in 10 with 7 M hydrochloric acid for the determination of arsenic and antimony.

Some quantities of powdered orchard leaves were also spiked with known amounts of arsenic, antimony and these samples were taken through the same digestion procedure. A standard additions calibration was thus established. Replicate samples were taken for each. Blank reagents were also prepared.

Samples were prepared in 7 M hydrochloric acid to ensure that any Se^{VI} formed in the digestion procedure, was reduced to the required Se^{IV} prior to analysis. This treatment is commonly used [5,6,9,10].

The reduction of As^{V} (or Sb^{V}) to As^{III} (or Sb^{III}) was achieved by the inclusion of potassium iodide in the sodium borohydride solution [9].

Results and Discussion

Figures 1 and 2 show chart recorder traces for the standard additions measurement of the digests for arsenic and antimony respectively. Approximately 40 seconds are required for the signal to reach an equilibrium value after introduction of the sample. Replicate absorbance readings were taken in the plateau region. It is apparent on Figure 2 that a spike appears on completion of the signal, and this is due to a brief introduction of air before the next solution is pumped. Such an occurrence is not uncommon.

Standard additions calibrations for arsenic and antimony are featured in Figures 3 and 4 respectively. The mean absorbance is shown and two values are represented by the error bars on the Figures.

That the standard additions technique was necessary, is demonstrated by aqueous calibrations for those elements on the same graphs. The calibration slopes are clearly different.

Table 1 shows the results obtained for arsenic and antimony in the orchard leaves. Agreement with the certificate was good.

Table 1 NBS Orchard Leaves No. 1571

Element	Certificate value µg/g	Found* µg/g
As	10 ± 2	11 ± 2
Sb	2.9 ± 0.3	2.7 ± 0.2

* Mean of replicate determinations.

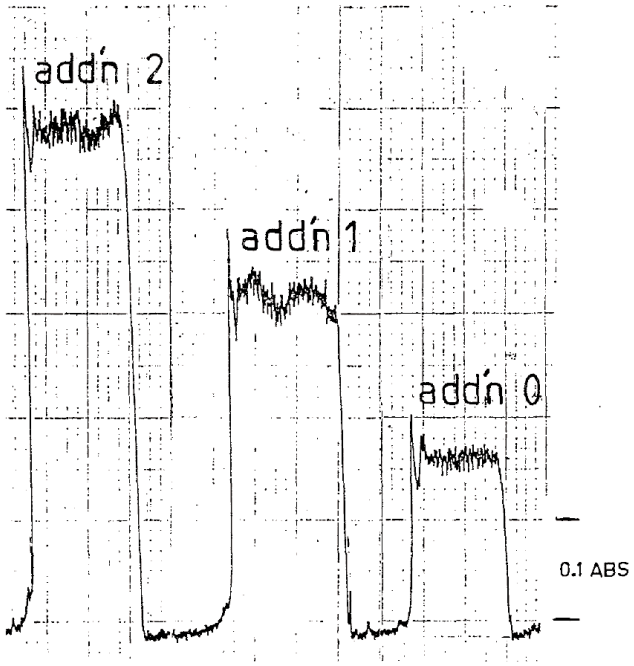


Figure 1. Chart recorder traces for the measurement of arsenic in digested leaves.

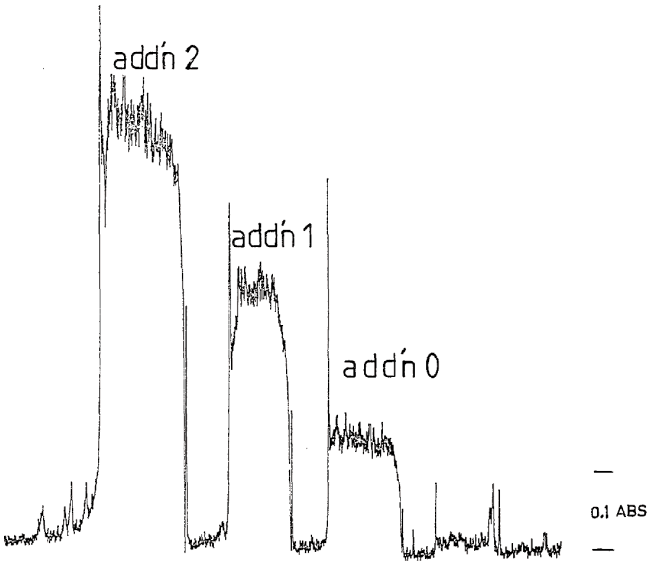


Figure 2. Chart recorder traces for the measurement of antimony in digested leaves.

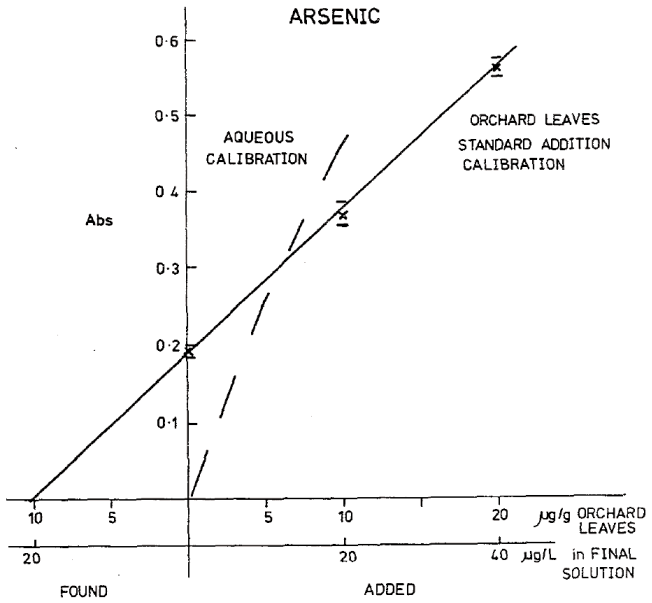


Figure 3. Calibration for the determination of arsenic.

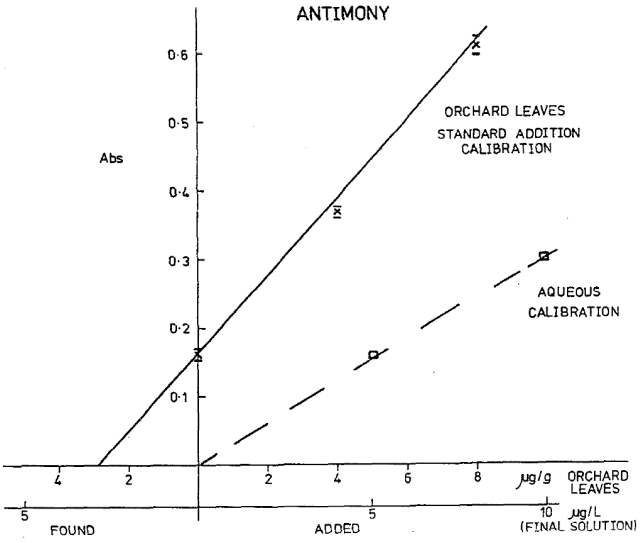


Figure 4. Calibration for the determination of antimony.

The level of selenium in the leaves was quite low (0.08 µg/gm) and a value of 0.05 was obtained from just one measurement by the standard additions technique.

No further study was made on selenium here but details of extensive work on selenium determination in agricultural materials by the VGA-76 has been recently reported [10].

An alternative digestion procedure was also attempted in which nitric and sulphuric acids only were used. The mixture was heated to remove excess nitric acid and the final solution was a light straw color. The recoveries from some samples analysed by this procedure were low, and this procedure was not pursued further.

Summary

The VGA-76 has been used to determine the amount of arsenic and antimony in digested orchard leaves. A digestion procedure was adopted which avoided the use of perchloric acid and it was necessary to use the standard additions calibration procedure.

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Nitrogen Determination in Fertilizer by ICP-OES with an Extended Torch

Application Note

Inductively Coupled Plasma-Optical Emission Spectrometers

Author

Tran T. Nham

For the determination of nitrogen in fertilizer, colorimetry has been widely used because of its high sensitivity. However, sample preparation is very time consuming and tedious for colorimetric measurements, while the ICP-OES technique can analyze the sample solution directly. The purpose of this study was to examine the feasibility of the ICP-OES technique for the measurement of nitrogen in fertilizer.

The emission lines of nitrogen are in the vacuum region (N 174.272 nm and N 174.525 nm), and a vacuum monochromator was used. To prevent the entrainment of air into the plasma, an extended torch was essential.

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Experimental

Instrumental

An Agilent Liberty 220 ICP spectrometer was used for the measurement. The extended torch featured a longer outer tube compared with the standard torch and a slotted cut-out on the side. A higher plasma gas flow of 22.5 L/min was used to form a better shield from the surrounding air.

The operating parameters of the system for the measurement of nitrogen are listed in Table 1.

Table 1. ICP Operating Parameters

Power	1.5 kW
Plasma gas flow	22.5 L/min
Auxiliary gas flow	1.50 L/min
Nebulizer type	Glass concentric
Nebulizer pressure	1 80 kPa
Pump rate	15 rpm
Sample uptake rate	1.8 mL/min
Viewing height	8 mm
PMT voltage	650 V
Integration time	3 sec
Background correction	Dynamic
Snout	High

Sample Preparation

Urea Sample

For ICP analysis: 10 g of sample was accurately weighed and transferred into a 500 mL volumetric flask, then distilled deionized water was added until the mixture was totally dissolved. 20 mL of concentrated HCl was added and made up to volume with distilled deionized water.

For the colorimetric method: 1 g of sample was accurately weighed into a 125 mL Kjeldahl flask, 20 mL of concentrated H2SO4 and 2 Kjeldahl catalyst tablets were added, and then the mixture was digested at 400 oC for 2.5 hours. After cooling, the mixture was transferred to a 500 mL volumetric flask and made up to volume with distilled deionized water.

Ammonium Sulfate Sample

25 g of a ground sample was accurately weighed and transferred into a 400 mL beaker. 150 mL of distilled deionized water and 20 mL of concentrated HCl were added. It was then boiled for 10 minutes, cooled, transferred to a 500 mL volumetric flask and made up to volume with distilled deionized water.

Standard Preparation

All chemicals and reagents used were analytical reagent grade.

Urea and ammonium sulfate were used for the preparation of standards for urea and ammonium sulfate analysis. Both standards and blank were prepared in 0.4 M HCl solution.

Results and Discussion

It was observed that a nitrogen signal was detected in the blank solution. This was because of the presence of dissolved nitrogen in water, nitrogen contamination in the argon gas and residual entrainment from the surrounding air. Figure 1 shows signal traces for a 2 wt% N standard and a blank solution.

However, the background nitrogen peak can be subtracted and corrected for by applying background correction.

Long Term Stability

Long term stability measurement was performed after a calibration with a 1.5 wt% N standard solution and a blank. The standard was then analyzed as a sample every 5 minutes continuously for a period of 90 minutes, as shown in Figure 2. Over the 90 minute period, the reproducibility was 1.44 %RSD.

Results

The N 174.272 nm line, with a detection limit (2 sigma) of 50 mg/L, was selected for the measurement. The results of the N determination by ICP-OES together with that found by colorimetry are listed in Table 2.

Table 2. Results for Nitrogen Determination

Sample	Nitrogen concentration (wt %)	
	ICP-OES	Colorimetry
Urea	46.5 ± 0.2	46.4
Ammonium sulfate	20.1 ± 0.3	20.4

The ICP values are the mean of 3 individual determinations. The precision of the ICP measurement ranged from 0.1–1 %RSD. Typical precision of the colorimetric measurement is about 0.2 %RSD.

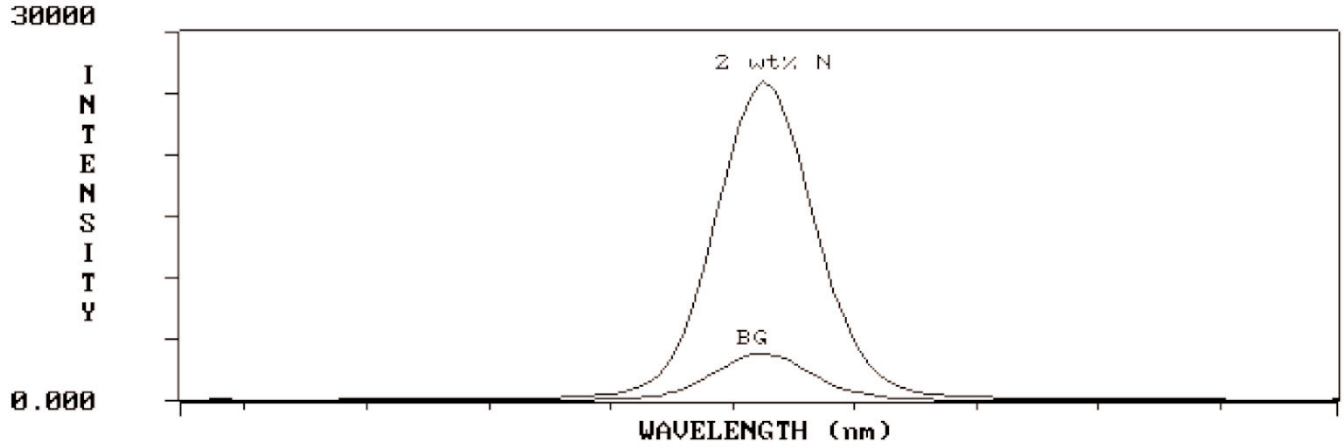


Figure 1. Spectral scan of a 2 wt% N standard solution and a blank at the N 174.272 nm line.

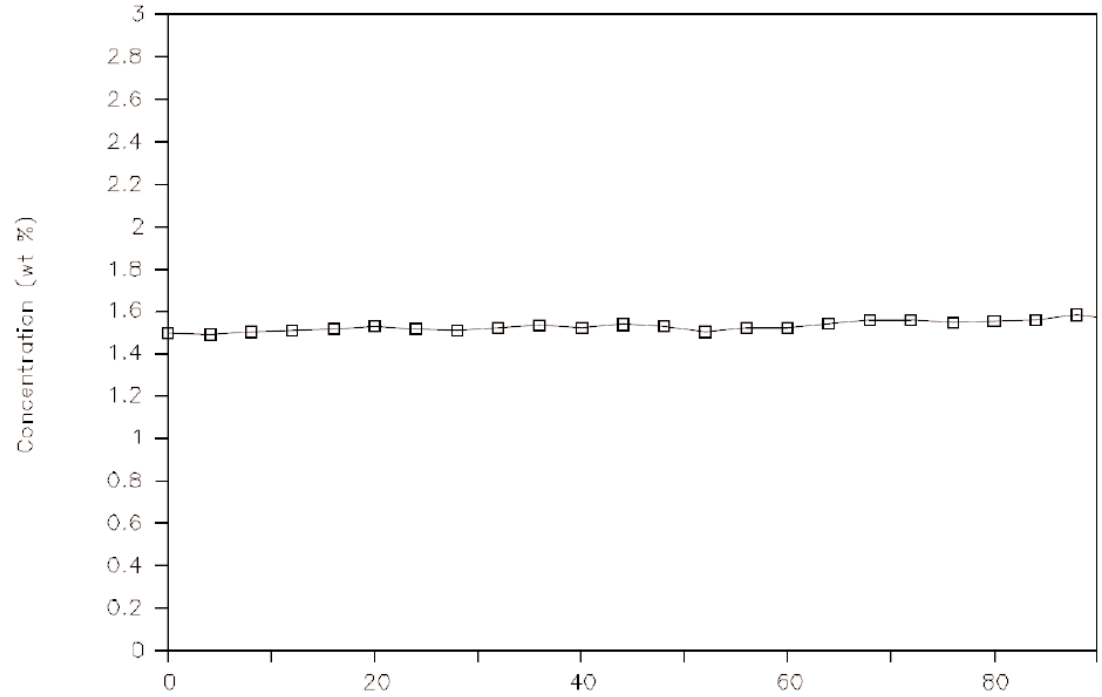


Figure 2. Signal stability over 90 minutes of a 1.5 wt% N standard solution.

Conclusion

Studies have shown that the ICP-OES technique can be applied to the measurement of high level of nitrogen in fertilizer. The ICP values are in good agreement with the colorimetric values. The precision of the ICP measurement ranged from 0.1–1 %RSD.

The sample preparation procedure for colorimetry is time consuming and tedious, especially for the urea sample. It takes about 2.5 hours for complete sample digestion, while a simple dissolution only is required for the ICP-OES technique.

However, the sample weight required is 10 times higher with the ICP technique than for the conventional colorimetric method, although sample availability is rarely a problem. It is noted that for urea analysis, standard solution should be prepared from urea, not ammonium sulfate.

In summary, the Liberty 220 ICP spectrometer with an extended torch can be used to determine nitrogen in fertilizer at wt% level in a fraction of the time taken by the conventional colorimetric method.

Acknowledgement

Thanks are expressed to John L. Lomas of Incitec Pty Ltd, Brisbane for the supply of fertilizer samples and for the results of colorimetric measurements.

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Validation of a leaf reflectance and transmittance model for three agricultural crop species

Application Note

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Abstract

Reflectance and transmittance have been measured for a selection of leaves from three cereal crop species using a laboratory based Cary 500 spectrophotometer. Spectral measurements, along with ancillary data describing chlorophyll, water and dry matter content per unit leaf area, have been used to assess the accuracy of the Prospect model [1] for estimating leaf reflectance and transmittance. An inversion technique has also been applied to the model to derive leaf pigment and leaf water content. This has produced acceptable estimates of the true variation in these parameters. This result indicates that the Prospect model, in conjunction with a suitable method to account for canopy architecture and soil reflectance, has potential to contribute to the generation of crop status information products based on hyperspectral data.

Introduction

Increasingly, surface reflectance estimates derived from hyperspectral data are used to assess spatial variance in crop condition and biomass over large agricultural fields. The distribution and optical properties of individual plant leaves are the primary factors contributing to the reflectance of a mature crop canopy. Consequently, to develop valuable information products from these data sources, an understanding of the reflectance and transmittance of individual plant leaves is essential.

The Prospect leaf reflectance and transmittance model [1] has been developed using contemporary theories of radiative transfer within leaf material. However, the inherent variability in biochemical constituents and leaf structure among plant species requires that accurate laboratory validation of the model be undertaken before it can be used reliably for agricultural crop condition assessment.

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In this experiment, three cereal crop species (barley, oats and wheat) were chosen for analysis. Plants were sown in pots and placed in a greenhouse for four weeks prior to measurement. Etiolates were produced by placing selected plants in complete darkness over the final two weeks to inhibit chlorophyll synthesis. To examine the effect of variations in leaf water content, a selection of wheat leaves were picked 24 hours, 12 hours and 2 hours prior to measurement and placed in a warm oven set at 60 °C.

Healthy and etiolated leaves were picked from each plant an hour prior to spectrophotometric measurement. Replicates of each sample were selected and prepared for destructive analysis to derive values for the Prospect model input parameters; chlorophyll a and b, leaf water and leaf dry matter content per unit area of leaf matter.

Spectral measurements

The reflectance and transmittance of leaf samples were recorded over wavelengths from 350 nm to 2500 nm using a laboratory based Cary 500 spectrophotometer. The instrument was fitted with a 110 mm diameter integrating sphere, permitting the sum of direct and diffuse radiation to be the measured. A total of 28 spectra were recorded, constituting 14 pairs of reflectance and transmittance measurements.

Comparison between healthy and etiolated wheat leaf spectra shows the distinct impact on reflectance and transmittance due to pigment content. As shown in Figure 1, reflectance in the visible red region is reduced considerably in the etiolated leaves due to the inhibition of chlorophyll synthesis. The etiolated leaf spectra still exhibit a significant drop in response in the visible blue region due to the carotenoid group of pigments, which develop in leaves even in the absence of light [2].

The reflectance of a healthy wheat leaf also illustrates the major water absorption bands centered at 1450 and 1940 nm, easily distinguishable in fresh leaf spectra. However, the weaker liquid water absorption bands documented by [3] and centered at 760, 970, 1190, are more difficult to distinguish. The reflectance of a dry wheat leaf in Figure 1 shows a dramatic reduction in the near infrared. Remnant absorption featured across this region is due to biochemicals such as protein and cellulose. Chlorophyll absorption in these dry leaves remains strong due to the rapid nature of the drying process.

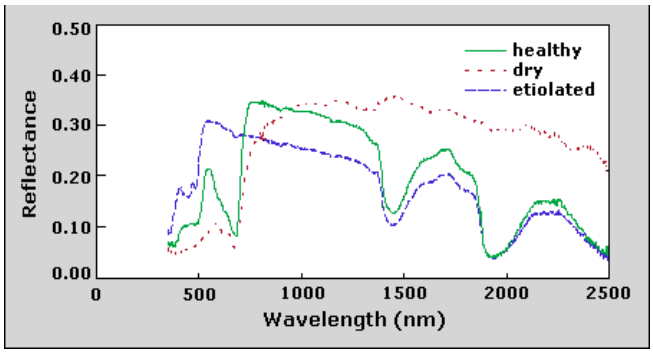


Figure 1. Reflectance spectra of healthy, dry and etiolated wheat leaves measured using the Cary 500 spectrophotometer

Variations in leaf thickness were simulated by stacking multiple leaves over the integrating sphere port. Leaf thickness contributes to the relative proportion of reflected versus transmitted light in each wavelength, altering the number of refractive index changes encountered in a direct path through the leaf, while also increasing the quantity of absorbing material present. This ratio of reflected versus transmitted light is highlighted in Figure 2, where the stacking of multiple leaves has increased the reflectance and decreased the transmittance, particularly in the low absorbing near infrared region.

Ancillary data

Three sets of absorption coefficients [1, 4, 5] have been defined for the Prospect model since its inception. Those used by [5] were selected for this study, as it was more practical to measure dry matter content than the specific biochemicals used by [4]. As a result, the model parameters used to compute leaf reflectance and transmittance comprised the content of chlorophyll, water and dry matter per unit leaf area, as well as a leaf structure parameter, to help define the amount of reflectance versus transmittance.

In order to determine chlorophyll content per unit leaf area, images of each leaf were recorded using a flat bed scanner and leaf area computed using digital image analysis. Each leaf was then ground in acetone to extract the leaf pigments and the solution filtered to remove undissolved leaf matter. Absorption of the dissolved pigment solution was measured using a spectrophotometer and normalized for solvent volume and leaf area. The content of chlorophyll *a* + *b* in the leaves was computed according to the method of [6] and recorded in units of $\mu g/cm^2$ of leaf area.

To derive leaf water and dry matter content, leaf area was again determined using digital image analysis. The fresh leaves were weighed and then placed in a warm oven set at 60 °C, for three days. When removed, a dry weight was recorded and this considered the true weight of dry matter in the fresh leaf. The difference between fresh and dry weights was assumed to account for the weight of water in the fresh leaf. Each weight was normalized for the respective leaf area and recorded in units of g/cm^2.

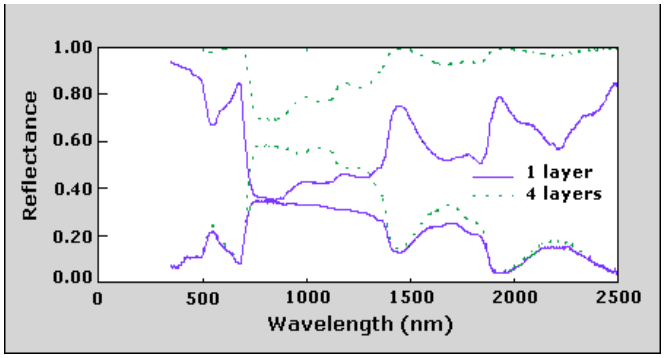


Figure 2. Reflectance (lower curves) and 1-transmittance (upper curves) measured for a single barley leaf layer (solid lines) and for a stack of four barley leaves (dotted lines)

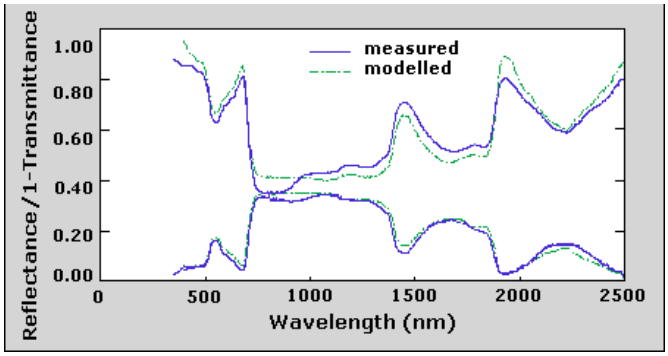


Figure 3. Graphical presentation of measured reflectance and 1-transmittance spectra (solid lines) and those produced using the Prospect model (dashed lines)

Model validation

Figure 3 shows a strong relationship between measured cereal crop leaf reflectance and transmittance spectra to those computed using the Prospect model. Correlation in the visible red chlorophyll absorbing region and the two major water absorption bands in the near infrared are particularly promising for inversion of the model.

Within the theoretical framework of the Prospect model, the leaf is considered to be a series of flat homogeneous plates. The relative proportion of light reflected and transmitted is accounted for by the structure parameter indicating the number of plates in the leaf structure, and the refractive index of these plates. The lowest value accepted by the model for this structure parameter is one. This was the value used for the computation of all spectra, except where multiple leaves were stacked over the integrating sphere port.

One apparent inconsistency between measured and modelled spectra is the general overestimation of reflectance and underestimation of transmittance. This is obvious in Figure 4, which shows the average modelling error for all measured and modelled spectra. This overestimation would be best addressed by reviewing the refractive index used for model calculations. It is assumed that lower refractive index values would allow a more meaningful structure parameter to be set and produce more accurate spectra.

One feature of the measured cereal crop leaf spectra that is not accounted for in the model is the dissimilarity between reflectance and transmittance spectra in the low absorption regions of the near infrared. A substantial increase in the transmittance centered in the region of 800 nm was seen in all fresh leaf spectra. However a corresponding increase is not displayed in the reflectance curves. This causes the underestimation of transmittance in this region seen in Figure 4. A possible reason for this feature is some form of anisotropic scattering causing a dominance of forward scattering. There is no easy way to account for wavelength dependent scattering features in the Prospect model other than to adjust the refractive index in this region. This is another motivation for investigating a possible underestimation of the refractive index in future work.

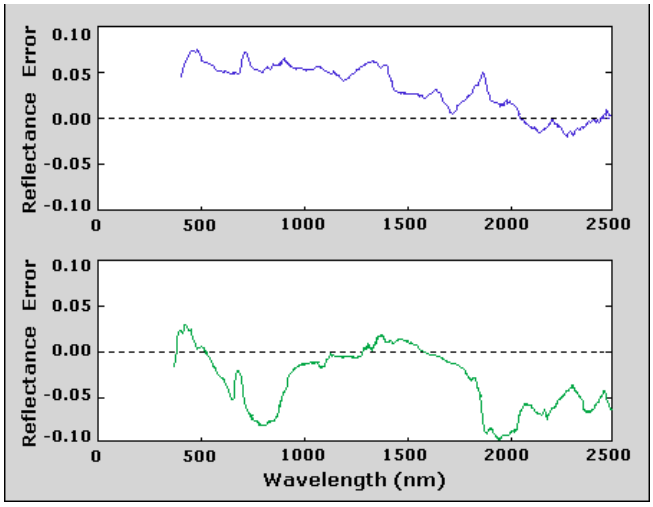


Figure 4. Average modelling error (modelled-measured) for the 14 reflectance and transmittance spectra

Model Inversion

The merit function shown below, defined by [1], was applied to the data to find a set of model parameters that minimize the difference between measured and modeled spectra. In this equation Δ^2 is the function to be minimized, $R_{Cary500}(\lambda)$ and $T_{Cary500}(\lambda)$ and the reflectance and transmittance at wavelength λ measured using the Cary 500, and $R_{Model}(\lambda)$ and $T_{Model}(\lambda)$ are the corresponding reflectance and transmittance estimated using the Prospect model.

$$\Delta^2 = \sum_{\lambda=400}^{2500} \left[\left(R_{Cary\,500}(\lambda) - R_{Model}(\lambda) \right)^2 + \left(T_{Cary\,500}(\lambda) - T_{Model}(\lambda) \right)^2 \right]$$

A generalized reduced gradient (GRG) constrained minimization routine [7] was used to minimise the merit function and derive estimates of leaf structure, leaf pigment, water and dry matter content. The values for chlorophyll and water were compared to measured values (Figure 5). Retrieval of leaf water content per unit leaf area was encouraging with only a slight overestimation shown by the best fit linear coefficient of 1.09824 and an R^2 of 0.9289. Chlorophyll content had a greater tendency for overestimation with a linear coefficient of 1.2399 and a reduced precision with an R^2 of 0.7846. Values retrieved from the inversion process versus their measured value are plotted in Figure 5.

Conclusions

The results of this work have shown that problems exist in the estimation of cereal crop leaf reflectance and transmittance using the Prospect model. This leads to inaccuracies in the retrieval of chlorophyll and leaf water content through model inversion. However, further work to adjust the leaf refractive index may help to address these problems and to achieve better estimates of these parameters.

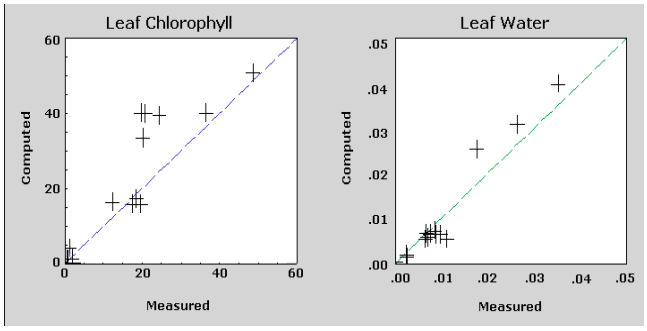


Figure 5. Values retrieved for leaf chlorophyll content and leaf water content plotted against their measured values, along with the line showing the ideal 1 to 1 relationship

With further work to identify its shortcomings specific to cereal crop analysis, the Prospect model, when coupled with a suitable canopy reflectance model, may

prove a valuable tool in the monitoring and management of agricultural crops using hyperspectral data.

Acknowledgments

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Automated nitrate analysis of water

Application Note

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Introduction

Analytical laboratories may analyze thousands of samples a year using UV-Vis spectrophotometers. Before these samples can be analyzed, standards must be prepared and the samples may require one or more reagents to be added. During the analysis of a batch of samples there may be over range samples. If so, the samples must be diluted and re-analyzed.

These tasks can now be completely automated by using the Sample Preparation System (SPS-5).

In this study the following topics are discussed:

- Automated standard and sample preparation versus manual standard and sample preparation
- Accuracy of automated sample analysis versus manual sample analysis
- Accuracy of over range dilutions
- Precision of the SPS-5
- Time required for automated and manual analysis

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Experimental

Equipment

- Cary 1E UV-Vis spectrophotometer
- Routine Sampler Accessory with a 10 mm flow through cell
- SPS-5 with diluter
- Concentration Application software
- Autodilutor Application software
- Type 10, 25, 50 or A type sample racks
- 10, 25, 50 or 150 mL size test tubes

Reagents

- Potassium nitrate (Analytical Reagent)
- 37% m/v Hydrochloric acid (Analytical Reagent)
- Chloroform (Analytical Reagent)
- Water - distilled and de-ionized

Method

For this study, the American Public Health Association (APHA) Nitrate method[1] was used. This method measures the NO_3 content in water samples and is recommended for screening samples that have low organic matter content. The absorbance of the sample is measured at two wavelengths and a simple calculation is performed.

The absorbance is measured at 220 nm and a second reading is taken at 275 nm. This allows correction for the interference due to dissolved organic matter. The difference between the two absorbance measurements is then calculated by the formula:

$$Abs_{220} - 2*(Abs_{275})$$

This is easily performed with the Cary by entering the following ADL command on the *User_Result* line on the Advanced Parameters page:

READ(220)-2*READ(275)

Preparation of stock nitrate solution (10 µg N/mL)

KNO_3 was oven dried at 105 °C for 24 hours and kept in a desiccator. The salt (0.7218 g) was dissolved in approximately 500 mL water. CH_3Cl (2 mL) was added as a preservative. The flask was put in an ultrasonic bath to speed the dissolution process. The solution was diluted to 1000 mL with water.

Preparation of standard nitrate solution (10 µg N/mL)

1. **Manual preparation:** 20.00 mL of the stock nitrate solution was diluted to 200 mL with water.
2. **Automated preparation:** 4.000 mL of the stock nitrate solution was diluted to 40.00 mL with water using the Autodiluter Application Software Samples page.

The stock nitrate standard was placed in the source rack. The destination rack used was a type 25, 50 or 100 mL depending on the volume of standard solution required.

Preparation of 1 N Hydrochloric acid

1. **Manual preparation**: 8.0 mL of HCl was diluted to 100 mL with water.
2. **Automated preparation**: 8.291 mL of HCl was diluted to 100 mL with water using the Autodiluter Application Software Samples page. See Figure 1 for the values used. Care needs to be taken when using concentrated HCl. The SPS-5 was fitted with the front cover and the rear cover/fume hood to minimize dispersal of the acid fumes.

Calibration standards

1. **Manual preparation**:

Table 1. The above dilution scheme was used to prepare the calibration standards

Standard NO_3 (mL)	1 N HCl (mL)	Final Volume (mL)	NO_3 Concentration (µg/mL)
1	2	100	0.10
2	2	100	0.20
6	2	100	0.60
10	2	100	1.00
15	2	100	1.50
30	1	50	6.00

2. Automated preparation: Figure 2 shows the parameters used for the Standard page of the Autodiluter Application to prepare the calibration standards.

The standard nitrate was placed in the source rack. Component 1 was the 1 N HCl.

Sample preparation

1 mL of 1 N HCl was added to 50 mL of sample. The samples used for this evaluation were prepared standards of known values. The report shows the appropriate volume correction (see Figure 3). The volume correction is activated on the Advanced Parameters page of the Cary E software. The factors are entered on the Labels and Corrections page of the Cary 1E software.

If samples require filtering an acid washed, ashless, hard finished filter paper, sufficient enough to remove fine precipitates, is recommended. A Whatman Nº 542 filter paper is adequate for this task.

Instrument parameters

Some operational aspects of the SPS-5 are shown in Figures 1–5.

Figure 1 relates to the automatic preparation of 40 mL of 10 µg/mL standard solutions.

The first line on this page directs the preparation of 40 mL of 10 µg/mL standard solution. The following two lines direct the preparation of 5 and 20 µg/mL standard solutions respectively for the overrange dilution study. The fourth line prepares 1 N HCl. The fifth line was used to add 0.196 mL of 1 N HCl to 9.804 mL of sample (ensuring acid to sample ratio of 1:50) for 100 samples.

A total of 10 different dilutions or reagent additions can be performed using this page.

The program shown in Figure 2 directs the preparation of 20 mL volumes of a blank and six standard solutions[1].

Figure 3 is an example of a nitrate analysis report showing overrange samples and volume correction.

Results

Reproducibility of standard preparation

The results of the SPS-5 prepared standard solution measurements are shown in Figure 4. Absorbances and correlation data obtained for an auto run are also shown.

Figure 5 shows the results and calibration data for the manually prepared standards, together with correlation data from the manual run.

As can be seen from Figures 4 and 5, both calibration graphs have a correlation coefficient (r^2) of 1.000. This shows the accuracy of the SPS-5 standard preparation. The standards prepared with the SPS-5 are in general accordance with those recommended by ASTM. The manually prepared concentrations were restricted to the size of the pipettes available.

The standards were prepared six times by the SPS-5. They were run as samples and the results are shown in Table 2.

Table 2. Accuracy of standards prepared using the SPS-5

Expected Nitrate Conc (µg/mL)	Mean Conc Found (µg/mL)	Std Deviation	%RSD
0.100	0.101	0.006	6.0
0.200	0.198	0.005	5.1
0.600	0.598	0.003	5.0
1.000	1.001	0.006	0.6
1.400	1.398	0.007	0.5

CARY AUTODILUTOR APPLICATION 1.00

Press F1 for HELP, F2 to EDIT

SAMPLES PAGE

Source rack	Source tube	Dest. rack	Dest. tube	Number	Sample mL	Comp 1 mL	Comp 2 mL	Diluent mL	Final mL	Pause	Mix secs
2	1	3	1	1	4.000	0.000	0.000	36.000	40.000	NO	0
2	1	3	2	1	2.000	0.000	0.000	38.000	40.000	NO	0
2	1	3	3	1	8.000	0.000	0.000	32.000	40.000	NO	0
3	10	2	2	1	8.291	0.000	0.000	91.709	100.000	NO	0
1	1	4	1	100	9.804	0.000	0.000	0.196	10.000	NO	0
1	1	1	1	0	0.000	0.000	0.000	10.000	10.000	NO	0
1	1	1	1	0	0.000	0.000	0.000	10.000	10.000	NO	0
1	1	1	1	0	0.000	0.000	0.000	10.000	10.000	NO	0
1	1	1	1	0	0.000	0.000	0.000	10.000	10.000	NO	0
1	1	1	1	0	0.000	0.000	0.000	10.000	10.000	NO	0

Figure 1. The Autodiluter Application Samples page

CARY AUTODILUTOR APPLICATION 1.00

Press F1 for HELP, ESC to continue

STANDARDS PAGE

Standard number	Standard mL	Comp 1 mL	Comp 2 mL	Diluent mL	Final Vol mL	Final Conc ug/mL	Mix secs
Blank		0.400	0.000	19.600	20.000		0
1	0.200	0.400	0.000	19.400	20.000	0.100	0
2	0.400	0.400	0.000	19.200	20.000	0.200	0
3	1.200	0.400	0.000	18.400	20.000	0.600	0
4	2.000	0.400	0.000	17.600	20.000	1.000	0
5	2.800	0.400	0.000	16.800	20.000	1.400	0
6	14.000	0.400	0.000	5.600	20.000	7.000	0
7							
8							
9							
10							

Conc: 10.000 ug/mL Blank: YES Number of Standards: 6

Valid input range is 0.000 to 18.000

Figure 2. The Autodiluter Application Standards page

Method Volume 50.0000 mL
Conc Units Unit

Rack 1 Tube 8
APHA NO3 Sample 1

Sample	Mean	Volume	Dilution Conc
1	1.5749	51.0000	6.9938

Rack 1 Tube 9
APHA NO3 Sample 2

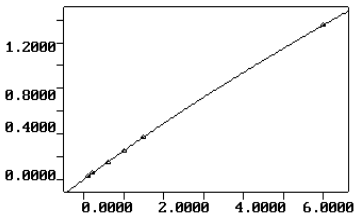
Sample	Mean	Volume	Dilution Conc
2	2.6001	51.0000	12.2255

Figure 3. The Reports Display page

1.0000 Method Name 14 Oct 1992 1.0
ABS(CONC MODE) Gain 100 SBW 2.0 SN
Baseline OFF Page 25

CONCENTRATION APPLICATION

#	Conc	Readings
1	0.100	0.0254
2	0.200	0.0577
3	0.600	0.1527
4	1.000	0.2504
5	1.500	0.3735
6	6.000	1.3561
7		
8		
9		
10		



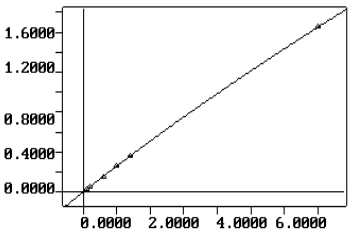
Units Units
Direct Quadratic Fit = 0.00000 y^3+ 0.41423 y^2+ 3.8628 y+ 0.0000
r2 = 1.000 r2 min = 0.000 Instrument Mode Abs

Figure 4. The Concentration Application page for SPS-5 prepared standards

1.0000 Method Name 14 Oct 1992 1.0
ABS(CONC MODE) Gain 100 SBW 2.0 SN
Baseline OFF Page 25

CONCENTRATION APPLICATION

#	Conc	Readings
1	0.100	0.0258
2	0.200	0.0517
3	0.600	0.1570
4	1.000	0.2603
5	1.400	0.3621
6	7.000	1.6555
7		
8		
9		
10		



Units Units
Direct Quadratic Fit = 0.00000 y^3+ 0.27665 y^2+ 3.7703 y+ 0.0000
r2 = 1.000 r2 min = 0.000 Instrument Mode Abs

Figure 5. The Concentration Application page for manually prepared standards

The results show good reproducibility for the automated preparation and automated analysis of standards.

Overrange dilutions

Any sample with a concentration >10 % of the *Ordinate Max* will be diluted. Overrange dilutions will be performed if an overrange solution is in racks 2–5.

A 5 µg/mL nitrate standard was prepared by the SPS-5 and used for calibration. Ten nitrate solutions each of 10 and 25 µg/mL were prepared using the SPS-5 and analyzed as samples. These overrange samples were automatically diluted and the concentrations calculated. The results are shown in Table 3.

Table 3. Overrange dilution accuracy

Expected Conc (µg/mL)	Mean Conc Found (µg/mL)	Std D'vn(n=10)	%RSD	Dilution Error
10.00	10.14	0.05	0.5	1.4
25.00	25.31	0.03	0.1	1.2

One dilution was performed for the 10 µg/mL standard and two dilutions were performed for the 25 µg/mL standard. The mean diluted concentration found reflects the accuracy of the diluter. The 1.4% dilution error for analyzing the standards 10 times shows the excellent accuracy of the SPS-5. The excellent repeatability of the SPS dilution process is also apparent.

Preparation time using the SPS-5

Approximately 2.5 minutes was required to prepare 40 mL of nitrate standard. To prepare six calibration standards, including blank, required 14.5 minutes resulting in a total time of 17 minutes for calibration standard preparation.

There are distinct advantages in automated preparation. These include the following:

1. Once racks, tubes and reagents are loaded, the preparation is fully automatic, allowing the operator to perform other duties.
2. Operator errors in sample preparation are eliminated. Any variations due to different operators preparing standards is also eliminated The SPS-5 consistently prepares accurate standards.
3. The user is not restricted to availability of glass pipettes when using the SPS-5 to prepare standards and an infinite range of concentrations can be prepared.
4. Standards and samples are analyzed in the same vessel in which they were prepared. Any potential cross contamination is therefore eliminated.
5. Cleaning of glass pipettes is eliminated.
6. The time required for the automated standard preparation is constant. The time required for the manual standard preparation will vary and is operator dependent.

The excellent accuracy and precision obtained demonstrates that the built-in mixing routine has excellent agitation for standard and sample preparation.

Analysis time

When using the SPS-5, analyzing a blank, 6 standards and 7 samples requires 14½ minutes.

Once a calibration is performed and stored, recalibration is not usually required.

Using a previously stored calibration, the SPS-5 can analyze 6 samples and a blank in less than 9 minutes.

The analysis time required is greater using the SPS-5 compared to manual analysis but as the operation is unattended, the operator is free to perform other tasks. Productivity can be improved because the sampler may operate unattended outside of normal laboratory working hours.

Conclusion

Standards can be accurately prepared and samples can have reagents automatically added and thoroughly mixed. Up to 500 samples can be automatically analyzed. Any overrange solution which may be present in a batch can be automatically diluted during an analysis using the SPS-5. This will occur accurately and the uncorrected and corrected results are printed.

The SPS-5 with Autodiluter Application software is a powerful stand alone accessory which accurately and precisely prepares standards and samples. When used in conjunction with the Cary it allows analyses of samples and accurate dilutions of overrange samples to be automatically performed.

Analyses with the SPS-5 and Cary can be performed almost continuously throughout the day and night increasing sample throughput, the productivity and profitability of the laboratory.

References

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Agilent Technologies

Determination of phosphorus concentration in hydroponics solution

Application Note

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Introduction

Phosphates exist in many forms in water. They can occur as orthophosphates, condensed phosphates such as pyro, meta and polyphosphates or as organically bound phosphates.[1] The major pollution sources of phosphates in natural waters are detergents used in industry and fertilizers from agricultural farming.

The use of fertilizers is increasing. Phosphates in fertilizer and farm runoffs have to be monitored in order to prevent the possibility of polluting the surrounding environment. The excessive enrichment of natural waters with plant nutrients, known as eutrophication, can result in overabundant growth of undesirable algae and aquatic plants, affecting water quality.[2,3]

Solution spectrophotometry still remains an essential and reliable analytical method for routine water analysis, particularly for the determination of nutrient elements such as nitrogen and phosphorus.

Spectrophotometric methods often require the initial conversion of phosphates to soluble orthophosphates which can then be determined colorimetrically. Orthophosphates can be analyzed quantitatively by three methods:

1. Vanadomolybdophosphoric acid method
2. Stannous chloride method
3. Ascorbic acid method

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Methods 1 and 2 were used in this study to quantitate and confirm an unknown phosphorus concentration in a hydroponics solution. These methods are suitable for determining the level of phosphates such as KH_2PO_4 and $NH_4H_2PO_4$ added into fertilizers and hydroponics as sources of phosphorus. It is also important that the phosphate level conforms to plant requirements. If excess phosphates are used, this could lead to the death of plants as well as excess phosphate in runoffs.

Equipment

Instrument: Cary 1

Concentration Application software

Methodology

Digestion

Pretreatment of the hydroponics sample

The hydroponics sample was digested first, using the potassium persulfate method as described in reference 1. Acid digestion is necessary if total phosphorus is to be determined as phosphorus may be present in combination with organic matter.

Apparatus

1. Hot plate
2. Weighing boats

Reagents

1. Phenolpthalein indicator aqueous solution
2. Sulfuric acid solution (300 mL conc H_2SO_4 in 1 L H_2O)
3. Potassium persulfate $K_2S_2O_8$
4. Sodium hydroxide NaOH, 1 N

Procedure

1. One drop (0.05 mL) phenolpthalein was added to 50 mL of sample. If red color developed H_2SO_4 was added to discharge the color.
2. One mL H_2SO_4 and 0.5 g solid $K_2S_2O_8$ were then added.
3. The solution was boiled gently on a preheated hot plate for 30 to 40 mins or until 10 mL remained.
4. It was then cooled and diluted to 30 mL with distilled H_2O.
5. One drop (0.05 mL) phenolpthalein was added, followed by NaOH until the solution was faintly pink.
6. The solution was then made up to 100 mL with distilled water.

Phosphate analysis

The phosphorus in the sample solution was then determined by Methods 1 and 2.

Separate calibration graphs were constructed using phosphate standards that were also pretreated with persulfate.

Method 1:

Vanadomolybdophosphoric acid method

A wavelength of 470 nm was used. The yellow color was due to the molybdophosphoric acid in the presence of vanadium. The intensity of the yellow color from this complex formation was proportional to the phosphate concentration. Glassware was acid washed before use. The procedure is as described in reference 1.

Reagents

1. Phenolpthalein indicator solution
2. HCL solution (0.5 N)
3. Activated carbon
4. Vanadate-molybdate reagent

Solution A: 2.5 g of ammonium molybdate $(NH_4)_6Mo_7O_{24}.4H_2O$ was dissolved in 30.0 mL of distilled water.

Solution B: 0.125 g of ammonium metavanadate was dissolved by heating to boiling point in 30 mL of distilled water. It was cooled and 33 mL concentrated HCL was added.

Solution C: After cooling to room temperature, Solution A was mixed with Solution B, and diluted to 100 mL.

5. Standard phosphate solutions:

22.068 mg of anhydrous KH_2PO_4 was dissolved in distilled water and diluted to 100 mL. Thus, 1mL = 50.3µg PO_4^{3-}-P.

A series of standards were made: for example, 0.5 mL of stock was put into a 50 mL flask for standard 1, and 5 mLs of Solution C added. Distilled water was used to fill to the 50 mL mark. A calibration graph at 470 nm was obtained on the Cary 1 spectrophotometer using the Concentration Application. A 1 second signal averaging time (SAT) was used throughout.

Sample preparation

Procedures

1. If the digested sample pH was >10, 0.05 mL (1 drop) of phenopthalein was added to 50 mL sample. The pH was lowered with 0.5 N HCl before it was diluted to 100 mL.
2. 200 mg of activated carbon was then added to 50 mL of the sample. It was then shaken for 5 minutes in a flask then filtered to remove the carbon.
3. 35 mL of sample was placed into a 50 mL volumetric flask to which 10 mL of Solution C was added and diluted to the mark with distilled water.
4. The blank was 35 mL distilled water treated as in number 3, in another 50 mL volumetric flask.
5. After 10 minutes, the sample absorbance at 470 nm was measured and compared with that of the blank. Each of the calibration standards was read after a 10 minute delay.

AOAC has described Fe^{2+} and NO^{3-} as interferents. The sample was spiked with ferric nitrate to show the effects of an interferent (Figure 1). The concentration of ferric nitrate added was 27.83 mg/L.

The scan was compared with the unspiked sample scan. The absorbance readings at 470 nm were not affected by this interferent, as the ferrous and nitrate ion concentrations were still <100 and 1000 mg/L respectively, in the spiked sample.[1]

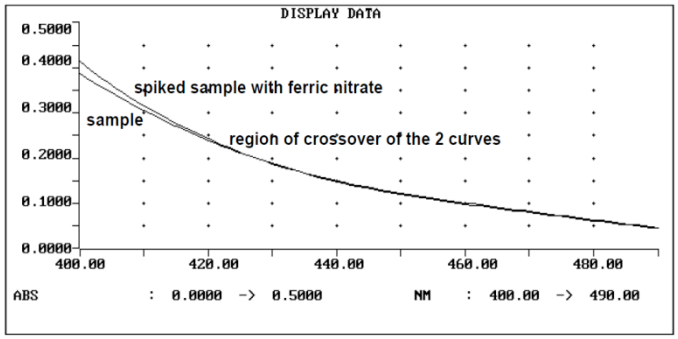


Figure 1. An example of an interferent $Fe(NO_3)_2$ spiking of hydroponics sample

A series of standards was made for calibration at 470 nm. The calibration graph obtained from this method is shown in Figure 2 below:

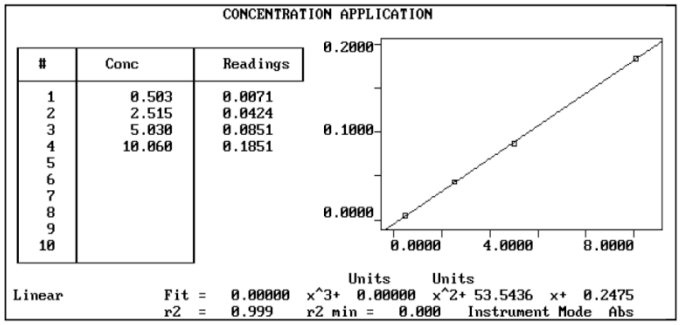


Figure 2. The calibration graph for method 1

The values obtained from the linear calibration graph were then corrected as in reference 1 for dilution factors.

The phosphorus concentration from the Absorbance at 470 nm of 0.08401 Abs was calculated as follows:

(4.745 µg/mL x (50 mL/35 mL))

= 6.78 mg/L

Method 2:

Stannous chloride method

Molybdophosphoric acid reduction by stannous chloride to molybdenum results in the development of a blue color. The procedure used was as described in reference 1. A wavelength of 690 nm was used.

Reagents

1. Phenolpthalein indicator solution
2. Strong acid solution: 300 mL conc H_2SO_4 was added slowly to 600 mL distilled water, cooled, then 4.0 mL conc HNO_3 was added before dilution to 1 L.

Procedure

1. Ammonium molybdate reagent 1: 2.5 g $(NH_4)_6Mo_7O_{24}.4H_2O$ was dissolved in 17.5 mL distilled water. 28 mL of conc H_2SO_4 was added to 40 mL distilled water. The molybdate was then added and diluted to 100 mL.
2. Stannous chloride reagent 1: 2.5 g of $SnCl_2.2H_2O$ was dissolved in 100 mL glycerol. Heat if necessary to aid dissolution.
3. Standard phosphate solutions (as in Method 1).

The samples, standards, and reagents were kept within 2 °C of one another and in the temperature range between 20 to 30 °C. Color measurements were taken 11 minutes after the reagents were added. The color was measured at 690 nm using distilled water as the blank. The light path used was 1 cm. No extraction[1] was required as the amount of phosphorus present in the solution was estimated from the results obtained in Method 1. Increased sensitivity is required only if the concentration is below 100 µg P/L.

The calibration graph for Method 2 is as shown in Figure 3.

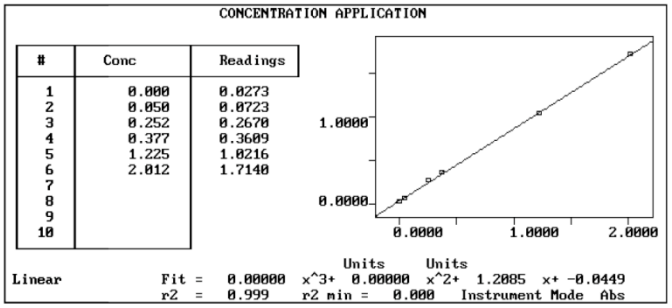


Figure 3. The calibration graph for method 2

Sample: Dilution factors

Forty milliliters of the sample was taken and diluted to 100 mL with distilled water after discharging the pink color with acid. Then 50 mL of this solution was taken and diluted to 100 mL with distilled water.

The calculations include the following dilution factors:

As in reference 1:

µg P/mL = value from line of best fit x 100/40 x 100/50

Thus, the phosphorus concentration measured from Method 2 (which gave an absorbance value of 1.2295 Abs at 690 nm) was calculated as follows:

1.4409 µg/mL x 2.5 x 2

= 7.2 µg/mL

or 7.2 mg/L

Discussion

The concentration of phosphorus in the hydroponics sample was found to be:

Method 1	Method 2
6.8 mg/L	7.2 mg/L

These results give an average of 7 mg/L of phosphorus in the hydroponics sample, and also correspond to the expected value for hydroponics sample. (See Appendix.)

The minimum detectable limit of phosphates using vanadomolybdophosphoric acid method is 200 µg P/L with a 1 cm pathlength cell, while the minimum detection limit using the stannous chloride is lower, about 3 µg P/L.[1] Thus, Method 2 is more sensitive than Method 1.

However, the color generated in Method 1 is stable and its intensity is unaffected by room temperature variations. In Method 2, the time taken to read both standards and samples was critical for the accuracy of the concentration readings.

The temperature of the reactions in Method 2 also affects the phosphate results. However, the choice of methods used to analyze phosphate quantitatively would depend on the interferents present and the detection limits required. The interferents and their levels affecting phosphate analyses are different for Methods 1 and 2.[1]

Increased sensitivity can be achieved in Method 2 by an extraction of molybdophosphoric acid into an organic solvent such as benzene-isobutanol. This additional procedure can improve the detection limit of this method to 3 µg P/L if required.

The concentration levels of various interferents are discussed elsewhere.[1] Clean acid washed glassware is also required for these analyses to prevent phosphate contamination.

Other reducing agents besides stannous chloride can be used to form the blue molybdenum compound. These include ascorbic acid, 1-amino-2-napthol-4 sulfuric acid, hydrazine sulfate and p-methylamino-phenol sulfate. However, stannous chloride (Method 1) and ascorbic acid (Method 3) are the most commonly used reducing agents.

In this hydroponics sample, both results are close, suggesting that both methods are suitable. The results also show that despite the presence of many ions in the sample, these ions have not affected the determination of phosphate. (See Appendix for the composition of the sample.)

These methods can be automated if required for greater sample throughput. Automation is possible when using the Cary 1/3. Automation can increase reliability as well as reproducibility by reducing operator errors that can become significant if too many samples are to be analyzed manually.

Conclusion

The phosphorus content in the hydroponics solution can be accurately measured using the Cary 1 and the Concentration Application software. No interference effects were observed using this methodology.

References

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2. Editors: S.O.Ryding and W.Rast. "The control of eutrophication of lakes and reservoirs." The Parthenon Pubs Group: London; **1989**.
3. E.H. Nicoll. "Small water pollution control works design and practice". Ellis Horwood Ltd: England; **1988**.

Appendix

Table 1. Composition of hydroponics sample

Salts present in solution

KH_2PO_4
$Ca(NO_3)2.H_2O$
$MgSO_4.7H_2O$
KNO_3
K_2SO_4
$FeSO_4.7H_20$
$MnSO_4.4H_2O$
$ZnSO_4.7H_20$
$CuSO_4.5H_20$
$NH_4H_2PO_4$

Theoretical P content of sample

P in KH_2PO_4 = 5.7 mg/L
P in $NH_4H_2PO_4$ = 1.1 mg/L

Therefore:
P content in sample = 6.8 mg/L

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Agilent Technologies

The Investigation of Fertilizer Analyses Using Microwave Digestion and the Agilent 720-ES

Application Note

Inductively Coupled Plasma-Optical Emission Spectrometers

Authors

Christine M. Rivera

Doug Shrader

Introduction

Fertilizers play a vital role in sustaining crop yields by supplying essential plant nutrients such as macronutrients nitrogen (N), phosphorus (P_2O_5) and potassium (K_2O). The optimum "fertilizer ratio" of these three elements can vary according to the type of plant material being fertilized. Secondary nutrients such as calcium, magnesium and sulfur and micronutrients boron, copper, iron, manganese, molybdenum and zinc also play important roles in plant growth.

The purpose of these investigations was to evaluate microwave digestion procedures for two analyses of fertilizer samples. The experiment was divided into two phases.

Phase 1 was to evaluate using microwave digestion to prepare fertilizers for the determination of As, Ca, Cd, Cu, Cr, Fe, K, Na, Mg, Mn, P, Pb, Se and Zn by simultaneous ICP-OES.

Phase 2 was to evaluate using microwave digestion to prepare fertilizers for the determination of available phosphorus (P_2O_5) and potassium (K_2O) by simultaneous ICP-OES. Traditionally, the preparation of samples for phosphorus and potassium is done by extraction with ammonium citrate—EDTA. The samples are placed in a heated water bath and shaken for 1 hour. The reagents are added to warmed samples and the shaking must be continuous. The number of samples typically required for preparation is large and this process is extremely time-consuming.

In this field, phosphorus is traditionally determined using an auto-analyzer, which can be tedious to set up and run. Potassium is typically determined by flame photometry. To simplify sample preparation and analysis, the CEM MARS Xpress microwave digestion system with stirring option and Agilent 720-ES simultaneous ICP-OES were used for both analysis phases.

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Instrumentation

Agilent 720-ES

The Agilent 720-ES with axial torch configuration is a truly simultaneous ICP-OES with solid-state, Charge Couple Device (CCD) detection system. The custom-designed and patented CCD detector incorporates IMAP technology, whereby pixels are arranged in continuous angled arrays matched exactly to the image produced by the echelle optics. This provides true simultaneous measurement and full wavelength coverage from 167 nm to 785 nm.

Microwave Digestion System

CEM, Corp. MARSXpress is an ultra-high throughput microwave digestion system designed to make high-throughput sample preparation and research applications quick and easy.

Forty high-pressure vessels, available in 55 mL or 75 mL sizes, can be processed per run with temperature control of every vessel.

Sample Preparation

Phase 1

To verify the method, two Magruder fertilizer check standards (200204 and 200206), which form part of the association of American Plant Feed Control Officials (AAPFCO) round robin laboratory checks, and a certified reference material, Industrial Sludge (CRM-S-I) from High Purity Standards, were prepared for analysis. Approximately 0.5 g of sample was accurately weighed and transferred to 55 mL MARSXpress vessels. Then, 9 mL of HNO_3 and 1 mL of HCl was added to each sample vessel. Samples were digested in duplicate.

The microwave digestion method is summarized in Table 1.

Table 1. Microwave Digestion Settings

Stages	Max power (W)	% power	Ramp (min.)	Pressure (PSI)	Temp. (°C)	Hold (min)
1	1200	100	15:00	120	200	15:00

The total digestion time was 30 minutes. Figure 1 represents the average digestion temperature over time in the vessels during sample preparation.

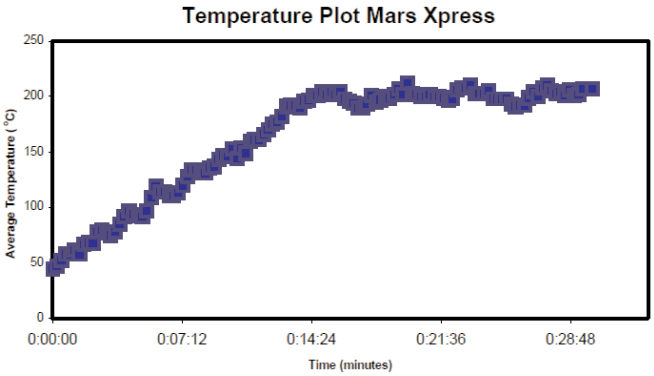


Figure 1. MarsXpress digestion temperature profile.

All samples were diluted to 50.00 mL in plastic disposable tubes and filtered with 2 micron Teflon FilterMate. This high dirt trapping FilterMate is especially suitable for trace level analysis and is supplied with lot certification for trace metals. The filtered samples were transferred directly to the Agilent SPS3 autosampler for analysis on the Agilent 720-ES.

Calibration Solution Preparation

The calibration summary for Phase 1 is listed in Table 2.

Table 2. Phase 1 Calibration Summary

Elements	Concentration (mg/L)
As, Cd, Cr, Cu, Mn, P, Pb, Se, Zn	0.5, 5, 10 and 50
Ca, Fe, K, Na, Mg	5, 10, 100 and 1000

Conditions

Instrument operating conditions are shown in Table 3.

Table 3. Instrument Operating Conditions

Parameter	Setting
Spraychamber type	Double-pass, glass cyclonic
Nebulizer type	SeaSpray
Nebulizer flow	0.75 L/min
RF Power	1.25 kW
Plasma gas flow	15 L/min
Auxiliary gas flow	1.5 L/min
Uptake delay	20 s
Stabilization delay	10 s
Rinse time	15 s
Internal standard	2 mg/L yttrium
Ionization buffer	0.4% caesium
Integration time*	60 s
Replicates	2
Total analysis time#	165 s

*If Se is not required at detection limit concentrations, a 5 s integration time is adequate. # 55 s for sample sets not requiring low concentrations of Se to be measured.

Results and Discussion

Phase 1

The results for Phase 1 are summarized in Table 4. Many of the samples encountered in feed and fertilizer laboratories consist of high concentrations of nutrients. Some of these elements cause ionization interferences while others cause spectral overlap problems, for example, iron. The use of an ionization buffer, for example, 0.4 % caesium minimized the ionization interferences. The spectral overlap can be overcome by the advanced background correction techniques of the ICP Expert II software, such as fitted background correction and FACT (Fast Automated Curve-Fitting Technique) spectral deconvolution.

Table 4. Results Summary

Sample labels units	As 188.980 mg/kg	Expected mg/kg	Ca 370.602 %	Expected %	Cd 214.439 mg/kg	Expected %	Cr 267.716 mg/kg	Expected mg/kg
Sludge B	141	141	0.0233	0.0242	0.64	NA	110	111
Magruder 4B	2.05	1.75	2.71	2.48	12.31	NA	125.2	132.6
Magruder 6B	5.75	5.66	4.93	5.94	1.51	NA	50.88	51.08

Sample labels units	Cu 327.395 %	Expected %	Fe 261.382 %	Expected %	K_2O 404.721 %	Expected % K_2O	Mg 279.078 %	Expected %
Sludge B	0.0407	0.0398	0.012	0.014	NA	NA	12.6	12.2
Magruder 4B	0.0461	0.0307	0.350	0.400	11.02	10.54	1.62	1.64
Magruder 6B	1.010	0.976	0.500	0.500	21.37	20.54	0.53	0.62

Sample labels units	Mn 294.921 %	Expected %	Na 589.592 %	Expected %	P 214.914 % P_2O_5	Expected % P_2O_5
Sludge B	0.51	0.48	0.94	0.94	0.51	0.50
Magruder 4B	0.036	0.039	0.31	0.29	8.1	9.1
Magruder 6B	0.014	0.015	0.57	0.58	9.1	9.9

Sample labels units	Pb 220.353 mg/kg	Expected mg/kg	Se 196.026 mg/kg	Expected mg/kg	Zn 213.857 %	Expected %
Sludge B	6.8	5.7	NA	NA	0.0244	0.0249
Magruder 4B	1.16	2.18	0.43	0.44	0.043	0.048
Magruder 6B	1.88	2.15	0.13	0.12	0.003	0.003

Phase 2 Available K_2O and P_2O_5

The extraction reagent preparation requires 325 g EDTA and 650 g dibasic ammonium citrate dissolved in 19.5 L distilled water. With mixing, 390 mL of a 1:1 solution of NH_4OH:H_2O is then added. When the solution is cooled to room temperature, the pH is carefully adjusted to 7.0 with additional 1:1 solution of NH_4OH:H_2O and the final solution diluted to 26.0 L.

The traditional method requires that 0.25 g of sample undergo extraction in 100 mL ammonium citrate reagent in a Wheaton bath stabilized to 65 °C (the extraction solution is added to the warmed sample). Upon completion of the extraction procedure, the solutions are cooled and diluted to 250 mL with ammonium citrate/EDTA reagent.

The samples are then typically run on the flame photometer for available K_2O and the auto-analyzer for P_2O_5.

The first part of the Phase 2 experiment was to determine if ICP-OES is a viable alternative technique for the determination of K and P. A set of ten fertilizer samples were extracted per the defined method and analyzed by the traditional techniques and ICP-OES.

With the ICP-OES technique, beryllium was selected as the internal standard and 0.8% caesium as an ionization buffer. Optimum instrument conditions were found to be at a power of 1.1 kW and a nebulizer flow of 0.65 L/min. The total sample analysis time was 55 s/sample.

A comparison of the results by technique for the determination of K and P are summarized in Table 5.

Table 5. Traditional Methodology Verses ICP–OES (1 Hour Water Bath Extraction) Results

Calibration solutions (units)	ICP-OES K_2O (769.897 nm) (% w/v)	Flame photometer K_2O (% w/v)	ICP-OES P_2O5 (214.914 nm) (% w/v)	Auto-analyzer P_2O_5 (% w/v)
Blank	0		0	
Std 1			0.1145	
Std 2	0.06023		0.4581	
Std 3			0.68709	
Std 4	0.24038		0.91612	
Std 5			1.3742	

Calibration solutions (units)	ICP-OES K_2O (769.897 nm) (% w/v)	Flame photometer K_2O (% w/v)	ICP-OES P_2O_5 (214.914 nm) (% w/v)	Auto-analyzer P_2O_5 (% w/v)
NIST SRM 200a	34.81	34.64	52.62	52.11
Sample 1	4.51	4.59	21.6	21.59
Sample 2	4.50	4.29, 5.11, 5.19	8.10	8.59
Sample 3	9.22	9.22, 9.30	10.04	10.09
Sample 4	9.89	9.80, 9.71	10.04	9.75, 9.95
Sample 5	1.80	1.48, 2.46, 2.16	19.87	20.07
Sample 6	2.50	2.53, 3.03, 3.15	22.47	22.49, 22.69
Sample 7	10.17	9.88	10.58	10.47
Sample 8	8.56	8.58	4.17	4.06
Sample 9	5.47	5.45	9.71	9.73
Sample 10	18.49	18.00	16.56	16.70, 16.45

Note: No internal standard for K_2O.

The final step of the Phase 2 study was to mimic the extraction process using microwave digestion. The microwave is not used for total digestion of the fertilizer sample, but as a way to consistently heat the extraction to 65 °C.

Three Magruder fertilizers and NIST SRM 200a potassium dihydrogen phosphate (KH_2PO_4) were carefully weighed to 0.1 g into HP 5000 vessels. Using the 75 mL vessels, 75 mL of ammonium citrate/EDTA extraction fluid was added. Stirring bars were added to each sample to simulate the shaking process and the contents were heated to 65 °C for 1 hour.

Table 6 summarizes the results collected by ICP-OES for available K_2O and P_2O_5.

Table 6. Summary of Results for Microwave Extraction and Determination of K_2O and P_2O_5 by ICP–OES

Calibration solutions(units)	K_2O 404.721 (% w/w)	K_2O 769.897 (% w/w)		P_2O_5 185.878 (% w/w)	P_2O_5 214.914 (% w/w)	Calibration
Blank	0	0		0	0	
Std 1				0.01139	0.01139	
Std 2		0.012				
Std 3		0.03038		0.05785	0.05785	
Std 4	0.06022	0.06022		0.11449	0.11449	
Std 5	0.1223	0.1223		0.22881	0.22881	
Std 6	0.2431	0.2431				
Std 7	0.49789					

Samples(units)	K_2O 404.721 (% w/w)	K_2O 769.897 (% w/w)	Expected (% w/w)	P_2O_5 185.878 (% w/w)	P_2O_5 214.914 (% w/w)	Expected (% w/w)
NIST_SRM 200a	34.42	34.58	34.61	52.44	52.56	52.11
NIST SRM duplicate	34.93	34.34		52.70	52.44	
MAG # 4	11.66	10.45	10.54	20.71	20.36	20.82
MAG # 4 duplicate	10.04	10.48		20.90	20.75	
MAG # 6	26.65	25.41	25.42	22.33	22.07	22.54
MAG # 6 duplicate	23.99	24.88		22.25	22.10	
MAG # 9	12.57	13.66	13.00	14.04	13.85	13.00
MAG # 9 duplicate	12.65	13.66		14.09	13.86	

Conclusion

The preparation of fertilizer samples by microwave digestion/extraction for the determination of macro, secondary and micro nutrients by simultaneous ICP-OES was evaluated and found to compare well with more traditional methods. The combination of microwave and ICP-OES techniques resulted in significantly faster and simpler sample preparation and analysis, requiring only a single analytical system to measure all elements of interest.

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Nitrate analysis of water using the quartz fiber optics dip probe on the Cary 50/60 UV-Vis

Application Note

Environmental

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Introduction

Environmental laboratories analyze thousands of water samples a year to determine the concentration levels of heavy metals and other ions, such as nitrates, phosphates and fluorides. To increase sample throughput and efficiency, optical fibers may be used to measure the absorbance of the sample. This allows for analysis on- or off-site, which is more appealing than a conventional cuvette. This paper presents and discusses results obtained from measuring the nitrate content in water using the quartz dip probe on the Cary 50 UV-Vis spectrophotometer. This experiment can also be done on the Cary 60 UV-Vis.

Experimental

Equipment

- Cary 50 (Cary 60)UV-Vis spectrophotometer
- Dip probe fiber optics coupler
- Quartz fiber optic dip probe
- Cary WinUV software

Reagents

- Potassium nitrate (A.R.)
- 37% m/v Hydrochloric acid (A.R.)
- Chloroform (A.R.)
- Water - distilled and de-ionized

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Method

The experimental procedure was taken from *Standard Methods for the Examination of Water and Wastewater*[1] and is also described in *UV Instruments At Work No. 59* [2]. In brief, standard solutions were prepared in the concentration range of 0 - 7mg NO_3^- - N/L and the absorbance measured at 220 and 275 nm. The measurement at two wavelengths allows correction for the interference due to dissolved organic matter, by calculating the difference between both absorbance readings (Equation 1).

Abs(220) - 2xAbs(275) **Equation 1**

The application used was the Cary WinUV Concentration software which evaluates the result of Abs(220)-2xAbs(275) dynamically as a function of concentration. The following instrument settings were used for data collection.

Instrument Settings

User Result	= Read(220)-2*Read(275)
Ordinate Mode	Abs
Ave Time (sec)	1.0000
Replicates	3
Standard/Sample averaging	OFF
Weight and volume corrections	OFF
Fit type	Quadratic
Min R^2	0.95000
Concentration units	mg/L

Results

Figure 1 shows the calibration curve obtained using the Quartz Fiber Optics Dip Probe. The Y axis, Abs, is the resultant from Equation 1 and the X-axis is the concentration of Nitrate Standards in mg/L.

A quadratic function, Equation 2, was fitted to 6 standards giving a correlation coefficient of 0.99931. The raw absorbance data and statistics for the calibration standards are shown in Table 1.

Abs = -0.00017conc^2 + 0.23364conc + 0.01705 **Eq. 2**

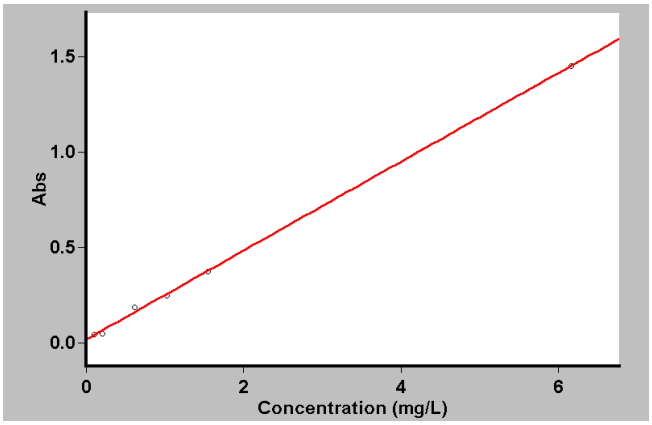


Figure 1. Calibration curve with quadratic fit

Table 1. Nitrate standards data for calibration curve

Std	Conc mg/L	Mean Abs	SD	%RSD	Raw Abs
Std 1	0.103	0.0443	0.0036	8.03	0.0472 0.0404 0.0454
Std 2	0.205	0.0488	0.0002	0.37	0.0488 0.0490 0.0487
Std 3	0.616	0.1856	0.0011	0.57	0.1856 0.1846 0.1867
Std 4	1.027	0.2467	0.0030	1.22	0.2475 0.2492 0.2434
Std 5	1.541	0.3748	0.0006	0.17	0.3741 0.3750 0.3753
Std 6	6.162	1.4506	0.0011	0.07	1.4503 1.4496 1.4517

Two samples of tap water from different sources, A and B, were prepared as described in the reference 1. The absorbance was measured and the concentration of nitrate determined from the calibration curve. The results are shown in Table 2.

Table 2. Raw data and statistics of Water samples

Std	Conc mg/L	Mean Abs	SD	%RSD	Raw Abs
A	0.145	0.0510	0.0009	1.78	0.0520 0.0504 0.0506
B	0.709	0.1825	0.0025	1.36	0.1797 0.1838 0.1841

Discussion

The 3 replicates for each standard and sample, shown in Tables 1 and 2, are reproducible within instrumental uncertainty, which demonstrates the high precision possible using fiber optics on the Cary 50/60. There is negligible solution carry over between samples, washing with only distilled water for approximately 5 seconds.

The time taken to measure 24 solutions of 6 standards and 2 samples, each with 3 replicates, was approximately 5 minutes. This time included washing the probe with de-ionized water in between readings and drying with a tissue. Measurements with the dip probe are significantly faster and easier than using a conventional cuvette.

Conclusion

The quartz fiber optic dip probe on the Cary 50/60 is highly precise and efficient for measuring the nitrate content in water. The time taken to measure 24 solutions is faster than using a cuvette, which makes the technique an attractive alternative for routine analytical measurements.

Reference

1. D. Eaton, L. S. Clesceri and A. E.Greenberg, *Standard Methods for the Examination of Water and Wastewater*, 19th Edition, American Public Health Association, Washington, 1995, p4-85.
2. P. A. Liberatore, *UV-Instrument At Work; Automated nitrate analysis of water*, No. 59, Agilent Australia Pty. Ltd, Australia, 1993.

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Analysis of Shellfish Tissue for Cadmium, Mercury and Nickel

Application Note

Atomic Absorption

Author

Jonathan H. Moffett

Introduction

Huge amounts of toxic effluents are being dumped either directly or indirectly into the world's oceans. The ocean's organisms concentrate these toxic chemicals with unfortunate effects on humans. Since the tragedy at Minamata Bay, Japan, in the 1950s, the levels of toxic metals in seafood have been carefully monitored [1]. This is especially important around coastal industrial and mining areas as well as river mouths.

Shellfish such as oysters and mussels are bottom-dwelling non-mobile filter feeders. These factors mean that shellfish are very good environmental indicators and can locate sources of pollution.

The screening of fish must be rapid and accurate. A method for digesting fish for mercury determinations by cold vapor has been described [1]. As mercury is very volatile, the digestion method should be applicable to other toxic heavy metals.

A freeze dried sample of shellfish tissue was supplied and a method was required for digestion and analysis.

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Experimental

Reagents

All reagents were AR grade. Water was deionized distilled (DD) water (18 M-ohm grade).

Standards

Standards were diluted from 1000 mg/L commercial atomic absorption solutions in deionized distilled (DD) water with dilute nitric acid as stabilizer. Working concentrations used are shown in Table 1.

Table 1. Working Concentration of Standards

Cd	5.00 µg/L in 0.08 M HNO_3
Ni	50.0 µg/L in 0.08 M HNO_3
Hg	0.50 µg/L in 0.3 M HNO_3/0.04% (m/v) $K_2Cr_2O_7$

Modifier

Ammonium dihydrogen orthophosphate ($NH_4H_2PO_4$) was used as a chemical modifier for cadmium [2]. $NH_4H_2PO_4$ (1 g) was dissolved in DD water (100 mL). The solution was found to have an appreciable cadmium signal. Trace metals were removed by using an Agilent Bond Elut SCX (strong cation exchange) column (kit B). Nitric acid (30 mL of 2 M) was drawn through the column using a water vacuum pump. The column was then washed using DD water (30 mL). The column was not allowed to dry out. The modifier solution was allowed to elute through under gravity with the first 2 mL being discarded. The eluant was caught directly in a washed sample vial and placed in the modifier position of the PSD-96 carousel. The overlayed cadmium signals before and after elution can be seen in Figure 1, in which the eluted signal is at baseline level. This method of purification has not yet been fully optimized.

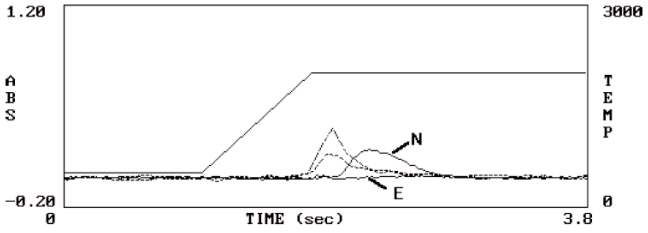


Figure 1. Noneluted (N) and eluted (E) modifier atomic (—) and background (- -) signals.

Reference Sample

A National Institute of Science and Technology (NIST) standard reference material, bovine liver (1577), was used as a reference sample.

Sample Preparation

Two digestion procedures were considered. The first digestion method was shown to be suitable for mercury in fish tissue [1]. In this method a sample (1g) was digested in concentrated nitric acid (10 mL). This method was eventually used for this study.

The second method was used to determine As, Sb and Se in various environmental matrices [3]. This method is a modified version of the first method by including hydrogen peroxide.

NIST 1577

Concentrated nitric acid (10 mL) was added to a known mass of liver (1 g) in a test tube [1]. After heating for three hours at 125 °C, the warm yellow solution was quantitatively transferred to a 50-mL volumetric flask. When made up to the mark with DD water, fat solidified as a precipitate. This did not appear to affect results.

Shellfish Sample

One fish sample was cut into halves using stainless steel scissors. Each half was digested separately. The digestion quantities above were scaled down by a factor of 10 because each half weighed about

0.1 g. Heating times were not changed. The solutions were transferred to 5-mL volumetric flasks. These were subsequently diluted as needed.

All masses and final volumes of the samples are shown in Table 2.

Table 2. Mass and Final Volume of Digested Samples

		Final Volume (mL)	
Sample	**Mass (g)**	**(Cd, Ni)**	**(Hg)**
NIST 1577	1.0026	50	
Shellfish 1	0.1373	100	125
Shellfish 2	0.1026	100	125

Instrumentation

For the determination of nickel and cadmium, an Agilent SpectrAA-300GZ atomic absorption spectrometer with Zeeman background correction, and fitted with a PSD-96 sampler, was used. Atomization for cadmium was from a pyrolytic forked platform. Atomization for nickel was from the wall of a pyrolytic coated partition tube. Argon was the inert gas.

For the determination of mercury, an Agilent SpectrAA-20ABQ atomic absorption spectrometer fitted with a VGA-76 vapor generation accessory and an MCA-90 mercury concentration accessory were used [4].

Lamps were standard Agilent hollow cathode lamps for each of the elements.

Recovery studies for nickel and cadmium were performed using Quality Control Protocol (QCP V2.0). Signal graphics were captured using Signal Graphics Library (SGL V1.01).

Instrument parameters are given in Tables 3a, 3b and 3c.

Results and Discussion

Cadmium

The aqueous calibration graph for cadmium is shown in Figure 2.

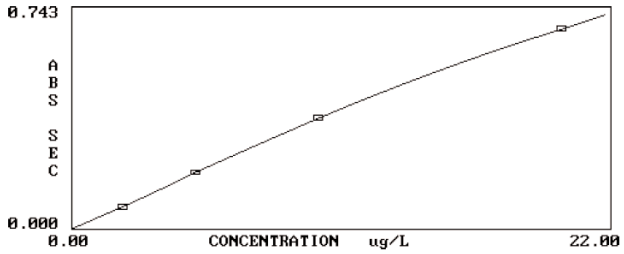


Figure 2. Cadmium calibration graph.

Replicate atomization signal peaks of NIST 1577, shellfish 1 and shellfish 2 for cadmium are shown in Figures 3, 4 and 5 respectively. The peak shapes all compare well. They are well-formed and the peak background signal is well within the capability of the instrument's corrector.

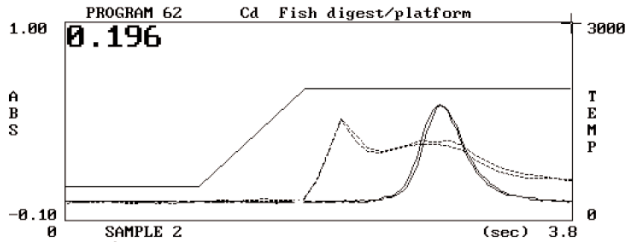


Figure 3. Cadmium signal - NIST 1577.

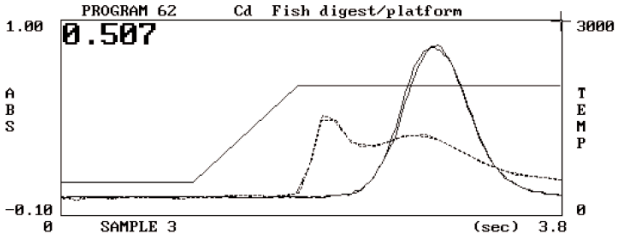


Figure 4. Cadmium signal - shellfish 1.

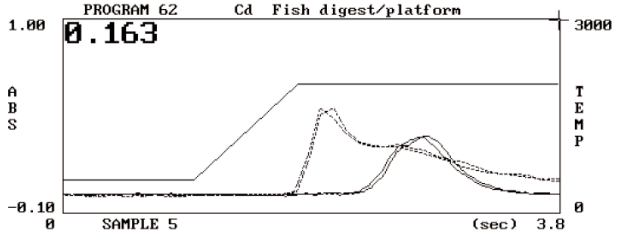


Figure 5. Cadmium signal - shellfish 2.

Table 3a. Instrument Parameters for the Determination of Cadmium

Program 62: Cd fish digest/platform

Instrument mode	Absorbance
Calibration mode	Concentration
Measurement mode	Peak area
Lamp position	3
Lamp current (mA)	4
Slit width (nm)	0.5
Slit height	Normal
Wavelength (nm)	228.8
Sample introduction	Sampler automixing
Time constant	0.05
Measurement time (s)	1.0
Replicates	2
Background correction	On
Maximum absorbance	0.70

Furnace parameters

Step no.	Temperature (C)	Time (s)	Gas flow (L/min)	Gas type	Read command
1	350	20.0	3.0	Normal	No
2	500	15.0	3.0	Normal	No
3	500	10.0	3.0	Normal	No
4	500	1.0	3.0	Normal	No
5	2000	0.8	0.0	Normal	Yes
6	2000	2.0	0.0	Normal	Yes
7	2500	2.0	3.0	Normal	No

Sampler parameters

Volumes (µL)

	Solution	Blank	Modifier
Blank	–	20	5
Standard 1	2	18	5
Standard 2	5	15	5
Standard 3	10	10	5
Standard 4	20	5	
Sample 5	15	5	

		Recalibration rate	0		
		Reslope rate	0		
Multiple inject	No	Hot inject	Yes	Pre inject	No
		Temperature	150		
		Inject rate	5		

Table 3b. Instrument Parameters for the Determination of Nickel

Program 66: Ni fish digest/wall

Instrument mode	Absorbance
Calibration mode	Concentration
Measurement mode	Peak area
Lamp position	2
Lamp current (mA)	6
Slit width (nm)	0.2
Slit height	Normal
Wavelength (nm)	232.0
Sample introduction	Sampler automixing
Time constant	0.05
Measurement time (s)	1.0
Replicates	2
Background correction	On
Maximum absorbance	1.00

Furnace parameters

Step no.	Temperature (C)	Time (s)	Gas flow (L/min)	Gas type	Read command
1	150	20.0	3.0	Normal	No
2	700	15.0	3.0	Normal	No
3	700	10.0	3.0	Normal	No
4	700	1.0	3.0	Normal	No
5	2400	0.9	0.0	Normal	Yes
6	2400	2.0	0.0	Normal	Yes
7	2500	2.0	3.0	Normal	No

Sampler parameters

Volumes (µL)

	Solution	Blank	Modifier
Blank	–	20	
Standard 1	2	18	
Standard 2	5	15	
Standard 3	10	10	
Standard 4	20		
Sample	10	10	

		Recalibration rate	0		
		Reslope rate	0		
Multiple inject	No	Hot inject	Yes	Pre inject	No
		Temperature	120		
		Inject rate	5		

Table 3c. Instrument Parameters for the Determination of Mercury

Program 5: Hg	
Instrument mode	Absorbance
Calibration mode	Concentration
Measurement mode	Peak height
Lamp position	3
Lamp current (mA)	4
Slit width (nm)	0.5
Wavelength (nm)	253.7
Sample introduction	Manual
Delay time	0
Time constant	0.05
Measurement time (s)	40.0
Replicates	1
Background correction	Off
Air flow	0.00
MCA-90 parameters	
Drain (s)	20
Collect (s)	180
Heat (s)	20

The result of the NIST 1577 analysis for cadmium tends to support the validity of the digestion and calibration procedures. Table 4a shows the result is very close to the certified value.

The wide difference between shellfish 1 and shellfish 2 for cadmium cannot be explained from this study alone. The discrepancy is caused either by contamination or by inhomogeneous distribution of cadmium in the original sample, with contamination being the most likely cause.

Nickel

The aqueous calibration graph for nickel is shown in Figure 6.

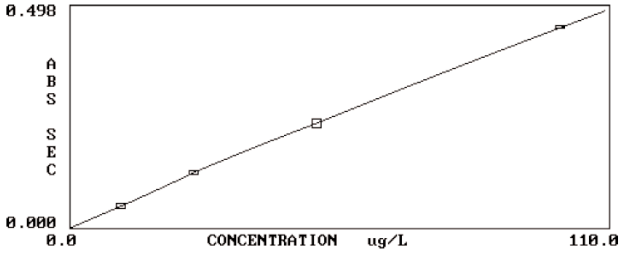


Figure 6. Nickel calibration graph.

Replicate atomization signal peaks of each of the samples for nickel are shown in Figures 7, 8 and 9. As for cadmium, peaks are well-formed, while background is negligible for nickel. NIST 1577 sample has a larger background peak because it was diluted less than the shellfish samples.

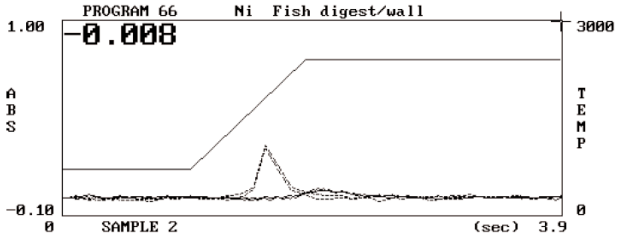


Figure 7. Nickel signal - NIST 1577.

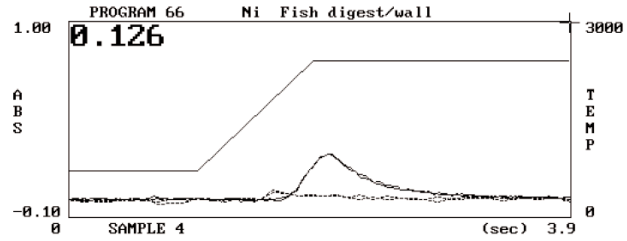


Figure 8. Nickel signal - shellfish 1.

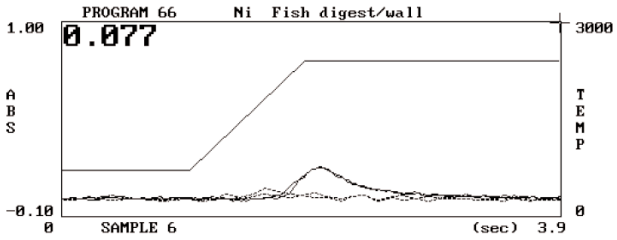


Figure 9. Nickel signal - shellfish 2.

The close result between shellfish 1 and shellfish 2 for nickel indicates homogeneous distribution in the original sample. NIST 1577 does not have a certified level of nickel.

Recovery Studies

QCP software allows a QC spike recovery by spiking the final solution with standard solution during injection into the furnace. The recovery (%R) is calculated as below:

$$\%R = (SSR-SR)/SA \times 100$$

where

SSR = Spiked sample result

SR = Sample result

SA = Spike added

If recovery is between 85% to 115% the aqueous calibration graph is normally considered to be a close match for the sample solution. A recovery of less than 40% usually indicates either a severe matrix effect or else measurement close to or greater than the maximum absorbance of the element using a Zeeman effect instrument. The recovery results for the samples are given in Table 4a, 4b and 4c.

Table 4a. Summary of Analytical Results for Cadmium in Shellfish

Sample	Soln conc (µg/L)	Conc found (µg/L)	Certif value (µg/L)	RSD (%)	recov (%)
NIST 1577	5.17	0.26	0.27 ± 0.04	0.4	94.5
NIST 1577	5.16	0.26		1.5	86.2
shellfish 1	14.08*	10.25*		0.3	28.3
shellfish 1	13.85*	10.09*		1.2	35.8
shellfish 2	4.34	4.23		3.3	85.5
shellfish 2	4.32	4.21		1.8	83.9

* is a software label to indicate the measured absorbance exceeded the maximum recommended Zeeman absorbance.

Table 4b. Summary of Analytical Results for Nickel in Shellfish

Sample	Soln conc (µg/L)	Conc found (µg/L)	Certif value (µg/L)	RSD (%)	recov (%)
NIST 1577	0.5	0.0	not certified	99.9	83.9
NIST 1577	-1.7	-0.1		47.2	102.5
shellfish 1	25.9	18.9		0.3	99.6
shellfish 1	25.2	18.4		1.0	95.1
shellfish 2	15.8	15.4		2.3	91.2
shellfish 2	15.8	15.4		6.7	79.3

Table 4c. Summary of Analytical Results for Mercury in Shellfish

Sample	Soln conc (µg/L)	Conc found (µg/L)	Certified value (µg/L)
NIST 1577	0.332	0.0166	0.016 ± 0.002
shellfish 1	0.344	0.313	
shellfish 2	0.372	0.453	

The recoveries for cadmium are good except for both aliquots of shellfish 1. The instrument software has flagged the results in Table 4a with an asterisk. This indicates that the maximum absorbance (0.7) for cadmium has been exceeded. The unexpectedly high result for shellfish 1 strongly suggested contamination of this sample and further studies (including dilution or reduced sample volumes) were not performed.

The results for shellfish 2 are below the maximum absorbance limit and the recoveries are very good.

The recoveries are also good for nickel except for one of shellfish 2 results. However, a portion of the same solution which immediately preceded this one had an excellent recovery.

A complete recovery study should also include a matrix spike which is the addition of analyte to a sample before digestion. The limited amount of sample available did not permit such study.

Mercury

The low levels of mercury in solution required the use of the MCA-90 [4,5]. This measurement consumes about 20 mg of sample. The amount of sample did not allow spike recovery studies. The close result of NIST 1577 with the certified value and the similar results for shellfish 1 and 2 suggests that the measured results are accurate.

Conclusion

The close agreement of the NIST 1577 results with the certified results indicates that the digestion procedure used was entirely satisfactory for the volatile elements mercury and cadmium. It seems reasonable that the same would be true for the shellfish. The final volumes in Table 2 should be adjusted to measure the expected levels in individual samples. They were suitable for the levels of the metals in NIST 1577 and shellfish 2.

The generally good recovery study results for the shellfish indicate the results are accurate. Certainly the calibration procedure is valid. Confirming the accuracy of any sample analysis requires a considerable amount of work. Procedures should include: long-term trend studies, inter-laboratory studies and using (if available) a certified reference fish sample.

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Using Lead Isotope Ratios to Distinguish between Samples of the Traditional Chinese Medicine Dan-shen

Geological

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Abstract

Quadrupole Inductively Coupled Plasma Mass Spectrometry (ICP-MS) was used to determine lead (Pb) isotope ratios in Dan-shen, a type of herb used in Traditional Chinese Medicines (TCM), and in water and soil samples all taken from the same geographical location. The precision obtained for the $^{208}Pb/^{206}Pb$ ratio, the $^{207}Pb/^{206}Pb$ ratio and the $^{204}Pb/^{206}Pb$ ratio values was considerably lower than 0.5% demonstrating the applicability of the technique for Pb isotope ratio studies. The results show that it is possible to distinguish Dan-shen samples originating from different geographical areas using Pb isotope ratio measurements. As the medicinal effectiveness of a TCM is highly dependant on the source of origin of its herb components, it is useful to have a reliable, routine means of "fingerprinting" the components grown in different habitats.

Introduction

Most Traditional Chinese Medicines (TCMs) are a mixture of several different herbs that undergo special treatment to make them useful. The amounts of the effective components in the plants are influenced by the soil, water, weather conditions etc in the area where they are grown. Experienced practitioners of traditional Chinese healing know that the quality of herb medicines is strongly related to their source of origin. It is therefore important to know the specific location of a TCM herb component so ensure its effectiveness.

Many techniques have been applied to characterize various herb medicines and to correlate them with their place of origin. High Performance Liquid Chromatography, Mass Spectrometry, Nuclear Magnetic Resonance, Infrared Spectroscopy and X-ray Fluorescence have all been used to produce spectra that can be used to "fingerprint" the TCM habitat.

Lead isotope ratio measurements provide analytical information relating to the source of lead contamination in naturally occurring samples. Concentration measurements cannot provide this information. Studies of the isotopic composition of lead are therefore commonly used in environmental science as well as geological and anthropological studies. Among all the naturally occurring lead isotopes, only ^{204}Pb is non-radiogenic, whereas, ^{206}Pb, ^{207}Pb and ^{208}Pb are the daughter products from the radioactive decay of ^{238}U and ^{235}U and ^{232}Th respectively. As a consequence, small Pb isotope abundance variations occur in nature and the isotopic composition of lead in the environment is dependent on local ore deposits. If lead is present in the soil, a plant will take up small amounts and subsequent isotope ratio studies might provide a unique

means of differentiating between different plant sources of origin. Of course, local lead levels may become mixed with external sources of contamination (e.g. automobile exhaust) that vary with time depending on the anthropogenic activity. These mixing processes can be quantified as long as each lead source shows a different lead isotope abundance. The work described in this application note was undertaken to investigate if lead isotope ratios can be used to "fingerprint" the Dan-shen herb grown in different habitats. While isotope ratio studies have traditionally been undertaken with a multicollector mass spectrometer such as Thermal Ionization Mass Spectrometry (TIMS), the technique is relatively slow and the instrumentation is expensive. These experiments were carried using quadrupole Inductively Coupled Plasma Mass Spectrometry (ICP-MS) that provides a fast, convenient and precise method to determine isotope ratios.

The Agilent 7500 Series ICP-MS is an excellent tool for routine isotope ratio measurements. It is easy to operate with its fully automated optimization system (Auto-tune function) and its user-friendly software. Because of these advantages, Agilent ICP-MS systems have been widely used for isotope ratio studies for environmental monitoring applications and nuclear research projects, e.g., U isotope ratios in human urine [2], in the cooling water of nuclear plants etc., the certified values of some certified reference materials such as rock samples [3], biological samples [3,4] by isotope dilution. In Encinar et al.'s work[4,5], an older model Agilent ICP-MS (HP-4500) was used, and the performance was as good as the more expensive multi-collector ICP-MS and double focusing sector field ICP-MS especially for long term stability and isotope dilution applications.

Experimental

Instrumentation

An Agilent 7500i ICP-MS, (Agilent Technologies, Palo Alto, CA, USA), fitted with a PFA micro flow nebulizer (100 uL/min) in self-aspiration mode, a quartz spray chamber and a one-piece quartz torch was used throughout the study.

A MK-II microwave sample digestion system (Shang Hai Xin Ke Factory, China) was used for sample digestion.

Reagents

The following reagents were used during the course of the study:

- SRM981 (National Institute of Standards & Technology, USA) - for mass bias correction.
- HBr, HCl, HF, and HNO_3, (GR grade, after sub-boiling purification) - for sample digestion and dilution.
- Anion exchange resin (DOWEX 1×8, 200-400 mesh, USA), - for separating Pb from the matrix in the samples.
- Ultra Pure Water, supplied by Milli-Q water system (18.2 MOhms)

Sample preparation

Dry samples and filtered water samples were digested using a HNO_3/HF acid mixture in a microwave oven then heated to dryness in a fume cupboard to remove the HF. 1.0 mL HBr (0.5N) was used to redissolve the residue. Samples were then passed through a DOWEX 1×8 column to remove the matrix. The Pb containing eluant was dried and diluted with 3% HNO_3. In order to avoid memory effects and to keep the ICP-MS detector working in pulse counting mode only, the concentrations were controlled between 1 and 80 ug/L.

Optimization of the ICP-MS operation parameters
Since the sample volumes collected after the column pretreatment step were limited, most of them were less than 1 mL, the Agilent PFA micro flow nebulizer (100 uL type) was selected for sample introduction. Self-aspiration mode was used and the carrier gas flow rate was controlled to maintain the sample introduction flow rate at about 50 uL/min. The 1 mL sample was sufficient for 2 or 3 individual measurements so that repeat analyses could be undertaken in case of error during the determination.

Instrument sensitivity and the other parameters, such as oxide level and doubly charged ions, were optimized automatically using the AutoTune feature of ChemStation on a 10 ppb tuning solution containing Li, Y, Ce, Tl. The carrier gas was then set to 0.4 L/min to obtain a sample uptake rate of about 50 uL/min. Finally, the make-up gas was adjusted to produce the best sensitivities and lowest interferences.

Instrument operating parameters are given in Table 1.

Table 1. ICP-MS operating conditions

Plasma Conditions:

RF power:	1250 W
RF Matching:	1.7V
Sample Depth:	7 mm
Torch-H:	0 mm
Torch-V:	0.3 mm
Carrier Gas:	0.4 L/min
Make-up Gas:	0.9 mL/min
Peripump1:	0.1 rps
Spray Chamber Temperature:	2.0 degC

Data Acquisition Parameters:

"Isotope Ratio Analysis" mode, 1000 scan/sec, minimum dwell time 100 us. The integration times were 20sec for ^{204}Pb, 10sec for ^{206}Pb and ^{207}Pb, 5 sec for ^{208}Pb. Each data point is the average of 3 repetitions.

Results

Before beginning the analysis, the instrument was allowed 30 minutes following plasma ignition to reach thermal stability. A 20ppb solution of NIST SRM 981 isotope ratio standard was measured after every 5 unknowns. This data provided the 7500i short-term and long-term stability information and provided a means of making the small mass bias corrections required. The instrument demonstrated excellent precision. See Tables 2 and 3. Note that the instrument consistently delivered %RSD's at less than 0.1% over the short and long term.

Table 2: Short-term Stability of Pb Isotope Ratio Determinations of 20 ppb SRM981 using the Agilent 7500i ICP-MS (20min, 5 measurements)

File Name	PB-206A.D	PB-206B.D	PB-206C.D	PB-206D.D	PB-206E.D	RSD(%)
Acq Date	Jun 9 2001	Jun 9 2001	Jun 9 2001	Jun 9 2001	Jun 9 2001	
Acq Time	3:45 PM	3:50 PM	3:55 PM	4:00 PM	4:06 PM	
204/Total	0.01427	0.01426	0.01426	0.01426	0.01425	0.05
206/Total	0.2413	0.2414	0.2414	0.2415	0.2416	0.05
207/Total	0.2211	0.2208	0.2209	0.2209	0.2209	0.05
208/Total	0.5233	0.5235	0.5235	0.5233	0.5232	0.03
204/206	0.05913	0.05904	0.05907	0.05906	0.05897	0.10
207/206	0.9163	0.9146	0.9149	0.9146	0.9144	0.08
208/206	2.169	2.168	2.169	2.167	2.165	0.08

Table 3. Long-term Stability (10 hours) of Pb Isotope Ratio Determinations of 20 ppb SRM981 using the Agilent 7500i ICP-MS

File Name	Acq Date	Acq Time	206/Total	207/Total	208/Total	207/206	208/206
PB20-A.D	Jun 9 2001	6:14 AM	0.2411	0.2209	0.5235	0.9162	2.171
PB20-B.D	Jun 9 2001	6:26 AM	0.2412	0.2209	0.5236	0.9159	2.171
PB20-C.D	Jun 9 2001	6:33 AM	0.2412	0.2209	0.5235	0.9158	2.170
PB20-D.D	Jun 9 2001	6:40 AM	0.2411	0.2210	0.5236	0.9167	2.172
PB20_1A.D	Jun 9 2001	7:59 AM	0.2411	0.2211	0.5236	0.9172	2.172
Pb-202A.D	Jun 9 2001	9:12 AM	0.2410	0.2211	0.5236	0.9173	2.172
PB_202B.D	Jun 9 2001	9:18 AM	0.2414	0.2210	0.5234	0.9153	2.168
PB-203A.D	Jun 9 2001	11:17 AM	0.2413	0.2211	0.5233	0.9161	2.168
PB-204A.D	Jun 9 2001	12:52 PM	0.2412	0.2212	0.5233	0.9168	2.169
PB-204B.D	Jun 9 2001	12:59 PM	0.2410	0.2214	0.5234	0.9185	2.172
PB_205A.D	Jun 9 2001	2:27 PM	0.2412	0.2210	0.5236	0.9163	2.171
PB_205B.D	Jun 9 2001	2:32 PM	0.2415	0.2210	0.5232	0.9153	2.167
RSD(%)			**0.06**	**0.07**	**0.03**	**0.10**	**0.08**

The excellent results from the NIST quality assurance sample suggest that the data from the unknown samples is also very reliable and can be reported with a high degree of confidence. In this study, three types of samples were measured:

- Four samples of surface water taken from different areas in Zhong-Jiang, Si-Chuan Province
- Soil samples came from Er-Mei Mountain (2), Zhong-Jiang, Si-Chuan Province (5), Jiang-Xi Province (1)
- Dan Shen plant samples were from Zhong-Jiang, Si-Chuan Province (3), Er-Mei Mountain (2), Bei-Jing (1), Jiang-Xi Province (1), Tai Mountain, Shan-Dong Province (1), Shang-Luo, Shan-Xi Province (1), Xin-Jiang Province (1), He-Nan Province (1)

All the samples were taken from different areas even within the same province. The measured results and the standard deviations are listed in Table 4 a-c.

Table 4a: Results of Pb Isotope Ratio Measurements of Dan-shen Plant Samples

Sample Name	$^{207}Pb/^{206}Pb$ ±sd(n=3)	$^{208}Pb/^{206}Pb$ ±sd(n=3)
Jiang-Xi Province	0.8485 ± 0.0005	2.079 ± 0.002
Shan-Luo, Shan-Xi Province	0.8502 ± 0.0014	2.100 ± 0.002
Bei-Jing	0.8674 ± 0.0019	2.159 ± 0.003
Shan Dong Province	0.8570 ± 0.0018	2.107± 0.002
Xin-Jiang Province	0.8495 ± 0.0007	2.095 ± 0.001
He-Nan Province	0.8576 ± 0.0007	2.115 ± 0.002
ErMei Mountain, D1	0.8467 ± 0.0003	2.093 ± 0.001
ErMei Mountain, D2	0.8469 ± 0.0004	2.095 ± 0.004
Zhong-Jiang, D1	0.8529 ± 0.0022	2.105 ± 0.004
Zhong-Jiang, D2	0.8524 ± 0.0012	2.105 ± 0.002
Zhong-Jiang, D3	0.8537± 0.0003	2.103 ± 0.002

Table 4b: Results of Pb Isotope Ratio Measurements of Soil Samples

Sample Name	$^{207}Pb/^{206}Pb$ ±sd(n=3)	$^{208}Pb/^{206}Pb$ ±sd(n=3)
Jiang-Xi Province	0.846 3± 0.0017	2.090 ± 0.001
ErMei Mountain, T1	0.8520 ± 0.0016	2.101 ± 0.004
ErMei Mountain, T2	0.8523 ± 0.0007	2.103 ± 0.001
Zhong-Jiang, T1	0.8395 ± 0.0007	2.084 ± 0.001
Zhong-Jiang, T2	0.8417 ± 0.0004	2.089 ± 0.001
Zhong-Jiang, T3	0.8411 ± 0.0015	2.084 ± 0.002
Zhong-Jiang, T4	0.8400 ± 0.0007	2.090 ± 0.001
Zhong-Jiang, T5	0.8391 ± 0.0014	2.083 ± 0.002

Table 4c: Results of Pb Isotope Ratio Measurements of Water Samples

Sample Name	$^{207}Pb/^{206}Pb$ ±sd(n=3)	$^{208}Pb/^{206}Pb$ ±sd(n=3)
Zhong-Jiang, w1	0.8702 ± 0.0015	2.140 ± 0.003
Zhong-Jiang, w2	0.8735 ± 0.0005	2.137 ± 0.002
Zhong-Jiang, w3	0.8675 ± 0.0002	2.136 ± 0.002
Zhong-Jiang, w4	0.8693 ± 0.0014	2.138 ± 0.003

Discussion

Since ^{206}Pb, ^{207}Pb and ^{208}Pb are the daughter products from the radioactive decay of ^{238}U and ^{235}U and ^{232}Th respectively, the Pb isotope abundance varies in nature. In most of the samples, the $^{208}Pb/^{206}Pb$ ratio values are between 1.95 and 2.15, the $^{207}Pb/^{206}Pb$ ratio values are between 0.78 and 0.86 and the $^{204}Pb/^{206}Pb$ ratio values are between 0.05 and 0.06[1]. In order to distinguish between the different types of samples by their lead isotope ratio, the measurement precision is extremely important, both over the short and long term. The RSD values should be at most 0.5% in order to make a clear differentiation between the samples. As indicated in

Table 4a-c, the SD values for the determinations are considerably better (lower) than 0.5%, so it is possible to use lead isotope ratio measurements to distinguish between the different samples.

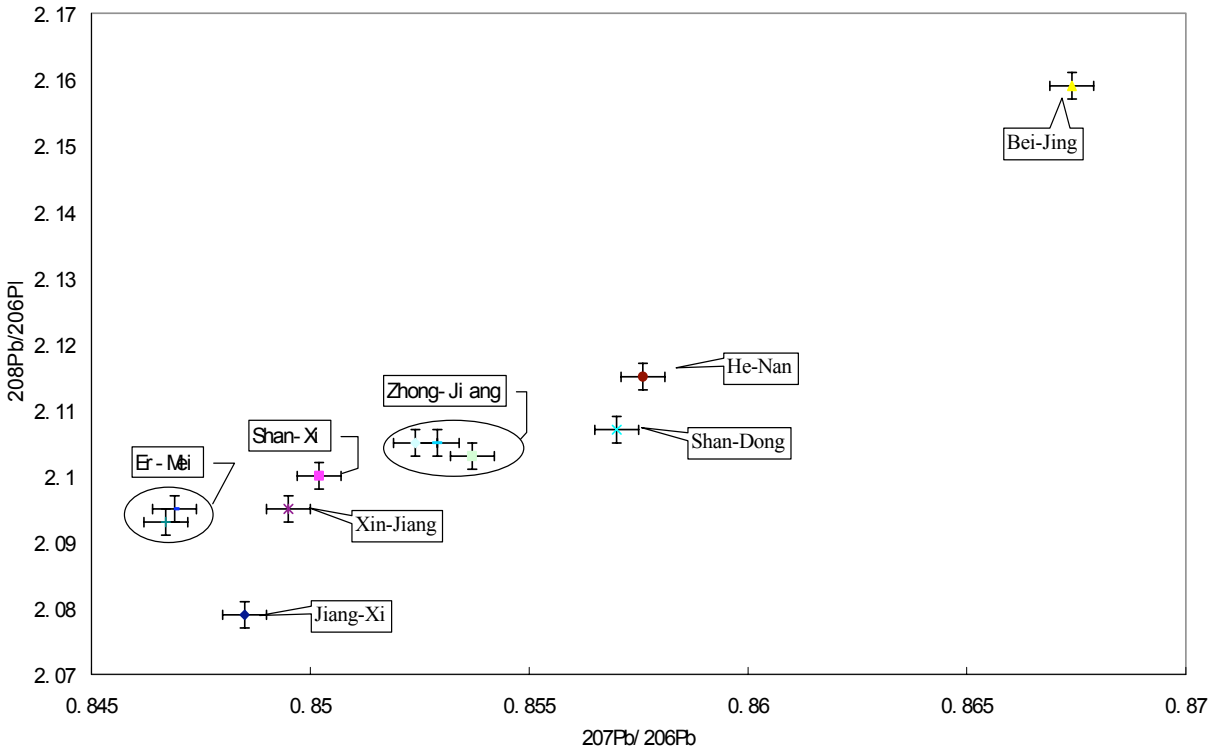


Figure 1: Pb Isotope Ratio Distribution of the Herb Medicine, Dan-Shen, from Different Sources

As a means of looking for variations/similarities in the measurements, the $^{208}Pb/^{206}Pb$ ratio values were plotted on the y-axis vs the $^{207}Pb/^{206}Pb$ ratio values on the x-axis. Figure 1 illustrates the data from the analyses of the Dan-shen plant and shows very interesting results. Obviously, the Pb isotope ratio values show their own special pattern for the Dan-shen samples from different sources. For example, Dan-shen samples grown in the Zhong-Jiang area have similar Pb isotope ratio values, although the samples were taken from different sampling areas. When compared to the Pb isotope values of the Dan-shen samples from the Er-Mei Mountain region (the two samples also have similar Pb isotope ratio values), the difference is distinct. In conclusion, it is possible to distinguish the Dan-shen samples from different sources.

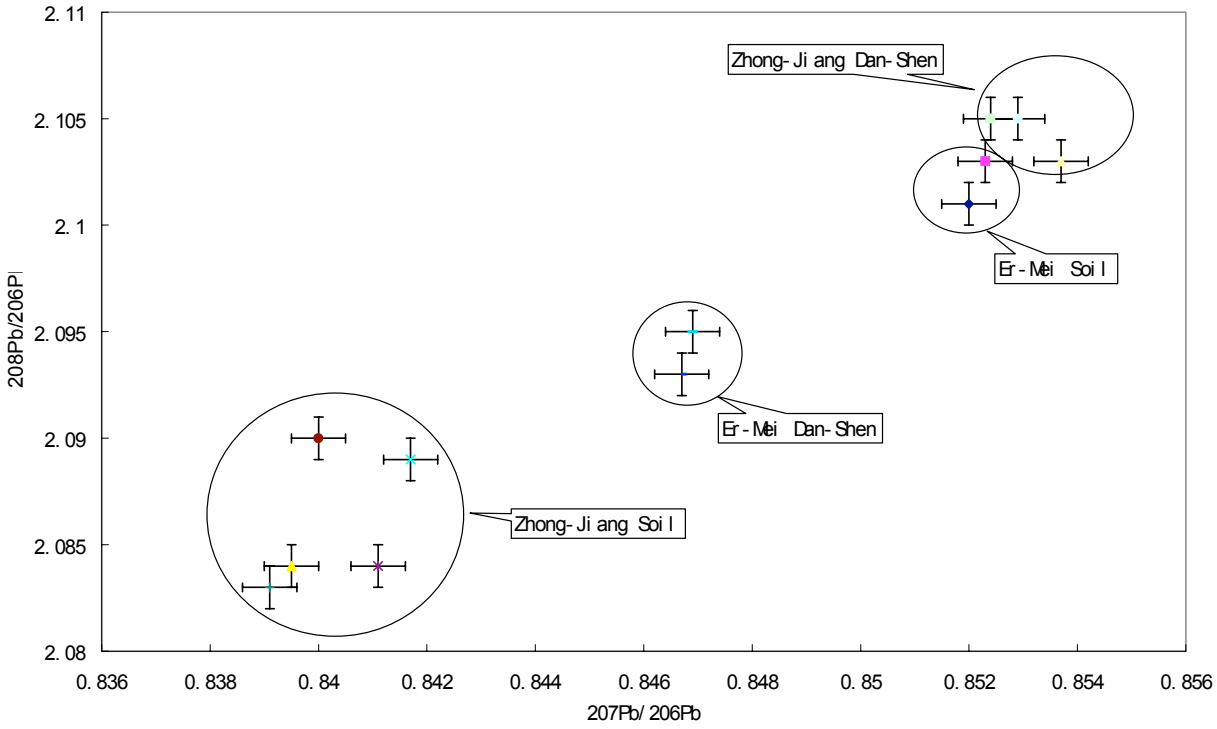


Figure 2: Pb Isotope Ratio Distribution of Soil and Dan-Shen Samples taken from Different Sources

When the soil samples from different sources are considered, it is found that the isotope values of the soil samples from different sources also have their own special pattern, as shown in Figure2. But, interestingly, the isotope values of the soils are different to the Dan-shen samples from the same place. This suggests that soil isn't the only source of lead within the plant.

When the water samples are considered, it is obvious that the Pb isotope ratio values are a combination of those found in the soil and in the water, as shown in Figure 3.

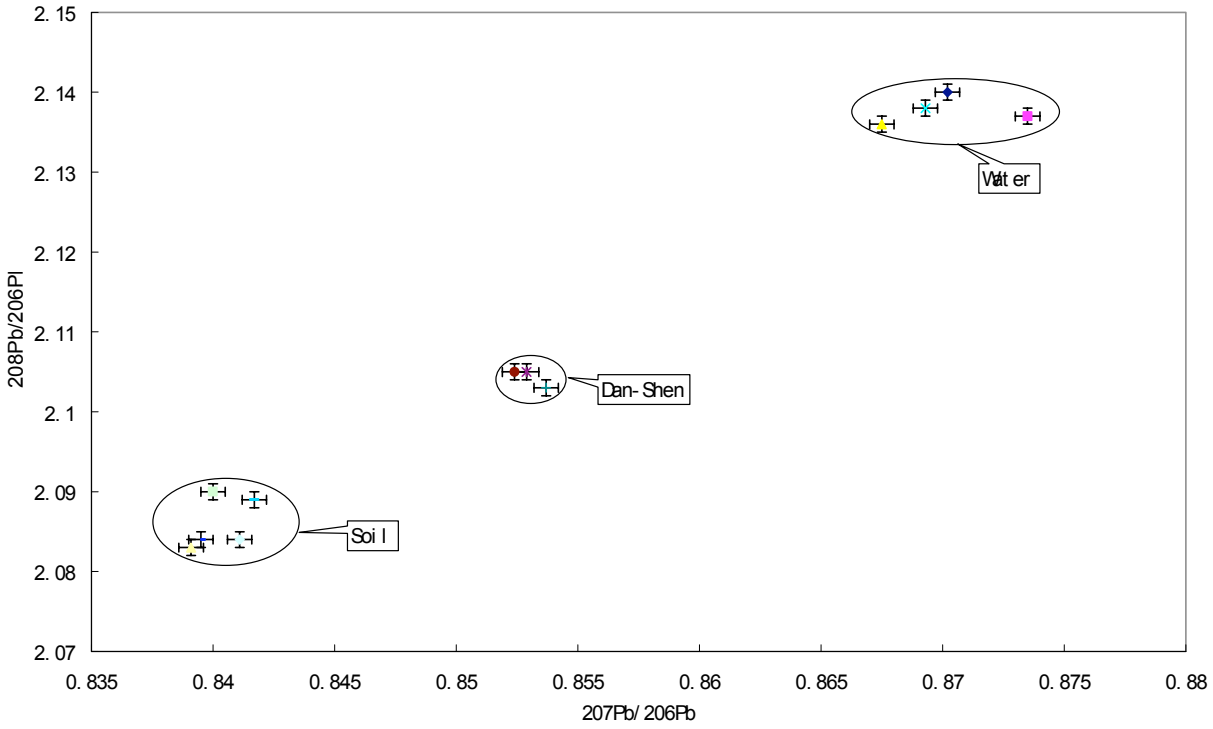


Figure 3: Pb Isotope Ratio Distribution of the Soil, Dan-Shen and Water Samples Taken from similar Sampling Area (Zhong-Jiang, Si-Chuan Province)

Conclusions

The Agilent PFA micro flow nebulizer is a good choice for isotope ratio analysis since it has very good nebulization efficiency and stability, especially when the sample amount is small. Self-aspiration mode avoids small amounts of pulsation from the peristaltic pump that could affect the precision of the isotope ratio analysis.

As this data suggests, the Agilent 7500 Series ICP-MS is well suited for routine isotope ratio analysis. Further improvements in precision may be obtained by modifying the method used in this study slightly. For instance, Tl may be added to the samples as an internal standard for lead isotope ratio analysis[5]. In addition, optimization of the dead-time correction, the stand-by mass selection, may also improve the performance of Agilent 7500 ICP-MS so that the theoretical minimum %RSD of 0.03 can be obtained. Future work at Xiamen University will look in to these potential improvements.

This preliminary research indicates that it is possible to distinguish Dan-shen samples from different areas using Pb isotope ratio measurements. Continuing these studies by running more types of samples will allow a large database to be set-up, and a chemometric model to be built to provide a convenient way to distinguish herb medicines from different sources.

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Fast and Accurate Determination of Arsenobetaine (AsB) in Fish Tissues Using HPLC-ICP-MS

Application

Foods

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Abstract

A high performance liquid chromatography-inductively coupled plasma mass spectrometry method was developed for the fast and accurate analysis of arsenobetaine in fish samples extracted by accelerated solvent extraction. The combined extraction and analysis approach was validated using certified reference materials for arsenobetaine in fish and during a European intercomparison exercise with a blind sample. Up to six species of arsenic can be separated and quantified in the extracts within a 10-minisocratic elution. The method was optimized so as to minimize time-consuming sample preparation steps and to allow for automated extraction and analysis of large sample batches. A comparison of standard addition and external calibration showed no significant difference in the results obtained, which indicates that the liquid chromatography-inductively coupled plasma mass spectrometry method is not influenced by severe matrix effects. The extraction procedure could process up to 24 samples in an automated manner while the robustness of the developed high performance liquid chromatography-inductively coupled plasma mass spectrometry approach is highlighted by the capability to run more than 50 injections per sequence which equates to a total run-time of more than 12 hours. The method can therefore be used to rapidly and accurately assess the proportion of nontoxic arsenobetaine in fish samples with high total arsenic content during toxicological screening studies.

Introduction

The element Arsenic (As) has long been thought of as poisonous and highly toxic. However, it has since been shown that the toxicity of As is largely dependent on the form or “species” the arsenic is in. Arsenic is ubiquitous in the environment due to natural and anthropogenic sources, and the relative contribution of these factors is estimated as roughly 60% and 40% respectively. In the environment, As behaves in similar ways to the Group V elements nitrogen (N) and phosphorus (P). As a result of these similarities, arsenic gets taken into the biochemical pathways of N and P. This results in the formation of compounds such as arsenobetaine (AsB) in fish and arseno-sugars, which are found in marine algae. The toxicity of the inorganic As-species (such as arsenite, As(III) and arsenate, As(V)) is far greater than the organic forms, such as monomethylarsonic and dimethylarsinic acid (MMAA and DMA) and AsB. The International Agency for Research on Cancer (IARC) has classified inorganic arsenic as a human carcinogen, whereas AsB, the predominant form of As in most marine organisms [1], is considered nontoxic to humans. Although AsB is the major form of As in many marine organisms, it is not present in all fish species [2]; therefore, an evaluation of the proportion of AsB to the total As determined can give a useful and rapid estimate of the toxicological significance of a sample. In order to determine the toxicity of seafood, the determination of the total As alone is of limited value, and the different species of As have to be extracted, separated, and determined. Fast, reliable, and practical methods are therefore required that can provide speciation information for the screening of large sample batches.

Aims and Objectives

The aim of this study was to develop a semi-automated analytical method for the extraction and determination of As-species in fish tissues. Requirements for high sample throughput analysis were the automation of the extraction procedure as well as a fully automated separation and detection method capable of analyzing large sample batches (up to 50 injections per run) during overnight runs. In order to streamline the analytical procedure, an attempt was made to develop a method with a minimal number of sample preparation steps. It was intended that the method should be established using calibration by external calibration curves, rather than the lengthy alternative of standard additions. The use of an isocratic liquid chromatography (LC) elution can be favorable in terms of time-efficiency during the liquid chromatography-inductively coupled plasma mass spectrometry (LC-ICP-MS) analysis because it negates the need for column re-equilibration between injections.

Calibration Standards

The following standards were obtained from Fluka (Sigma-Aldrich, Gillingham, UK): di-sodium hydrogen arsenate heptahydrate ($AsHNa_2O_4.7H_2O$) ≥98.5%, sodium (meta)arsenite ($AsNaO_2$) ≥99.0%, and cacodylic acid (dimethylarsinic acid, DMA, $C_2H_2AsO_2$) ≥99.0%; monomethylarsonic acid disodium salt (MMAA, $CH_3AsNa_2O_3$) >98% was obtained from Argus Chemicals (Vernio, Italy). Arsenobetaine (AsB, $C_5H_{11}AsO_2$) was obtained from BCR (Brussels, Belgium) as a solution of AsB in water at 1031 ±6 (95% C.I.) mg/kg (BCR 626).

Extraction

Accelerated solvent extraction (ASE) has been used previously for As-speciation [3, 4] and was chosen as the sample preparation method because it allows for the automated extraction and online filtration of up to 24 samples. In addition, the extraction solution is collected in glass vials, which negates further sample preparation steps such as filtration or centrifuging.

The samples were extracted using a Dionex ASE 200 accelerated solvent extractor. Sample sizes from 0.1–0.3 g were weighed accurately into 11-mL stainless steel extraction cells fitted with filter papers and PTFE liners. The extraction program was set up as shown in Table 1.

Table 1. Extraction Conditions Used for ASE

Instrument	Dionex ASE 200
Preheat	2 min
Heat	5 min
Extraction steps	5 × 2 min
Temperature	100 °C
Pressure	1500 psi
Solvent	Methanol

HPLC-ICP-MS Methodology

The HPLC-ICP-MS instrumentation consisted of an Agilent Technologies 1100 HPLC system coupled to an Agilent Technologies 7500i ICP-MS fitted with a second roughing pump, which enhances sensitivity by increasing ion transmission across the interface. The HPLC system comprised a quaternary pump module, a vacuum degasser, a temperature controlled autosampler, and column compartment. The ICP-MS instrument was tuned for sensitivity, reduced oxides, and doubly charged species prior to connection to the liquid chromatograph by performing a standard instrument tune using a 10 ng/g solution of Li, Y, Ce, and Th in 1% HNO_3. The pulse to analog (P/A) factor was adjusted on a daily basis using a solution containing ~50 ng/g Li, Mg, Mn, Cu, As, Gd, Y, Cd, Pb, and Ba. After this optimization, a 50 ng/g solution of As in 1% HNO_3 was used to specifically optimize the sensitivity for arsenic. The ICP-MS nebulizer was then connected to the HPLC-column using a length of PEEK tubing (yellow, 1/16-inch od, 0.007-inch id). See Table 2 for the ICP-MS conditions used.

Table 2. ICP-MS Conditions Used for HPLC-ICP-MS Determination of As-Species

RF Power	1430–1550 W
RF Matching	1.89–1.92 V
Sampling depth	4.0–4.8 mm
Carrier gas flow	0.89–0.93 L/ min
Make up gas flow	0.10–0.14 L/ min
Optional gas	Oxygen at 5%
Spray-chamber temperature	0 °C
Cones	Platinum
Isotopes monitored	^{75}As ^{103}Rh ^{77}Se ($^{40}Ar^{37}Cl$) to monitor Cl interferences ^{53}Cr ($^{40}Ar^{13}C$) to monitor C interferences
Other parameters	Injector diameter: 2.4 mm Nebulizer 100 µL/min PFA, Two interface pumps used

In order to develop a rapid chromatographic separation of the main As-species in fish tissues, an anion exchange column (Hamilton PRP X-100) was chosen in combination with an isocratic elution profile. Several mobile phases were tested and the best separation of AsB and As(III) as well as DMA and MMAA was achieved within 10 min using 2.2-mM NH_4HCO_3/2.5-mM tartaric acid at pH 8.2 delivered at 1 mL/min isocratic flow. This evaluation was carried out initially using matrix-free calibration standards containing the species of interest and refined using an oyster tissue extract that contained arsenocholine (AsC), two arsenosugars (As-sug. B and As-sug. D), $TMAs^+$ and several unknown species in addition [5]. The injection volume for samples and standards was 50 µL.

In order to enhance the ionization of the As-species [6, 7], methanol was added to the mobile phase at concentrations ranging from 0.5% to 5% v/v. At concentrations above 1%, the chromatographic separation degraded significantly to the degree that base-line resolution between AsB and As(III) was no longer achieved. However, the addition of 1% MeOH to the mobile phase resulted in a significant improvement in the sensitivity (3–4-fold increase in peak height) for all analytes. A chromatogram for a 5-ng/g mixed calibration standard with the final chromatography conditions is shown in Figure 1.

Variations in Signal Response for Different As-Species

The chromatogram shows that the four species analyzed here have very different response factors with this method, even when made up to contain the same concentration of As in solution. This is further illustrated by the calibration curves and their respective slopes, as shown in Figure 2. Such differences in the analyte signal intensity were reported previously in the literature [7] and appear to be due to a combination of the ICP-MS hardware used and the plasma conditions, which are in turn affected by the mobile phase composition. This points to possible differences in the nebulization, transport and/or ionization of different species by such methods. In order to determine whether this effect could be attributed to the coupling of the ICP-MS with a liquid chromatograph, aqueous standards of AsB and As(III) were made up to equivalent concentrations as As and analyzed by direct aspiration without chromatography. This indicated that the signal response of AsB was ~10%–15% higher compared to the inorganic As standard and, therefore, the difference in signal response does not appear to be related to the coupling with a liquid chromatograph.

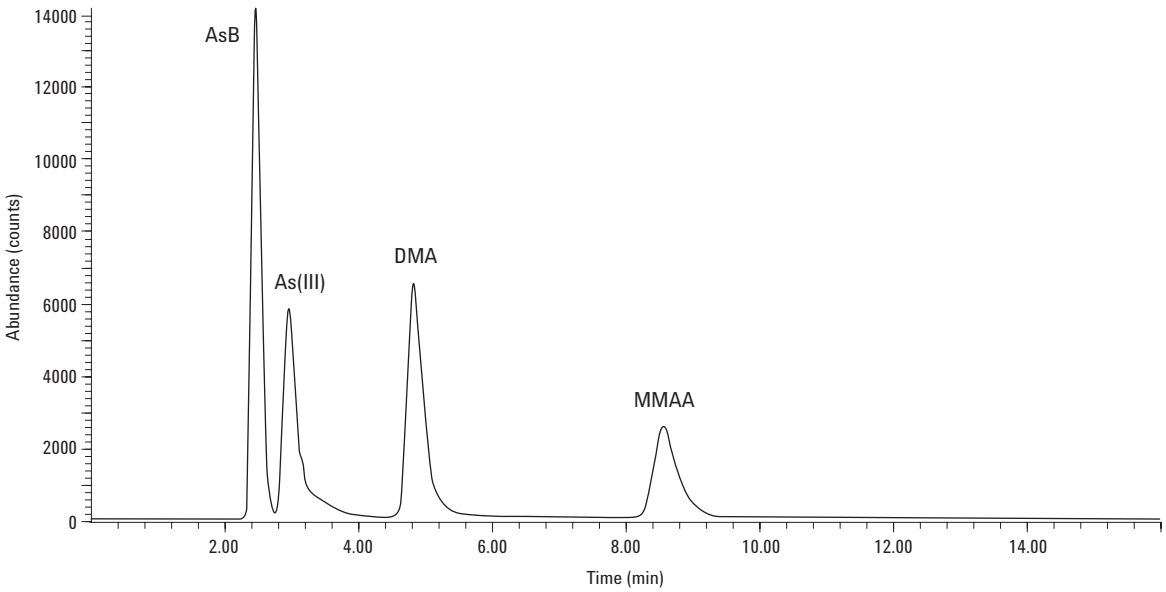


Figure 1. Chromatography A: 2.2-mM NH_4HCO_3 2.5-mM tartaric acid, 1% MeOH, pH 8.2, Hamilton PRP X-100 column. Concentration of standard ~ 5 ng/g as As.

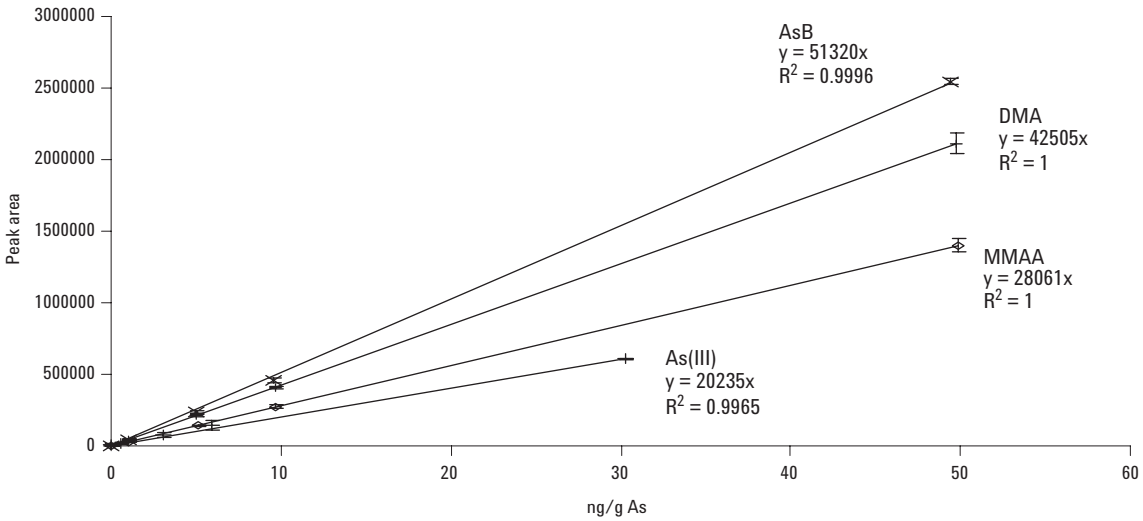


Figure 2. Calibration curves for AsB, DMA, MMAA and As(III) over a range of 0–50 ng/g as As.

In order to increase the signal intensity for species such as As(III) and MMAA by the approach described here, additional MeOH was added via a T-piece post-column so as not to impact on the chromatographic resolution. Although the relative volume of MeOH could be increased by 50%–70% in this way without deteriorating plasma stability, the relative signal responses of the four species were not influenced significantly. Because the relative signal response was stable on a day-to-day basis, no further attempts were made to equalize the signal responses.

The instrumental detection limit for AsB by this method was 0.04 ng/g as As. The linearity obtained, as indicated by the correlation coefficient of the calibration line, was 0.999–1.000 over a calibration range of 0–700 ng/g as As.

Plasma Disturbance Due to Elution of MeOH

During the analysis of fish samples, which had been extracted under the ASE conditions highlighted in Table 1, a disturbance of the plasma was observed between ~2.3 to 4.3 min after injection. This affected all of the isotopes monitored and the effect on ^{75}As and ^{103}Rh is highlighted in Figure 3. As can be seen from the chromatogram, the effect on these two isotopes is nonlinear. The ^{103}Rh signal decreases significantly during this time, whereas the ‘shoulder’ on the tailing side of the AsB peak indicates an increase in the ^{75}As signal.

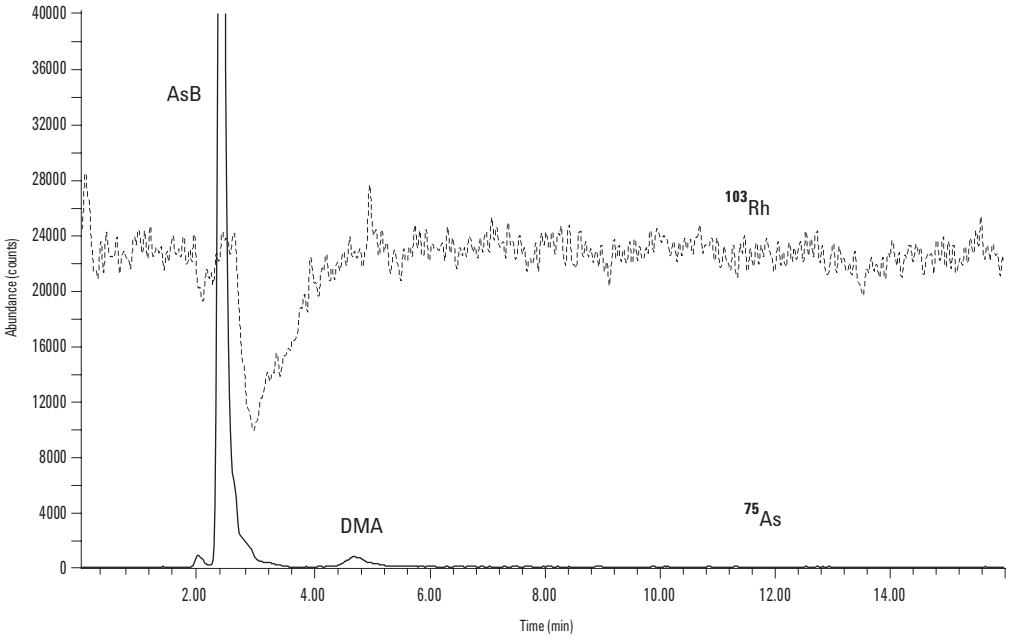


Figure 3. Signals for ^{103}Rh and ^{75}As in an undiluted fish extract. Notice the increase in the ^{75}As signal at the tailing side of the major peak (AsB) coinciding with the decrease in the ^{103}Rh signal.

The observed fluctuation in the signal intensities for the different isotopes coincides with the elution of the organic methanol fraction of the fish extracts from the analytical column. This effect could be reduced slightly by lowering the temperature of the spray-chamber from 5 °C to 0 °C, but the effect was not completely eliminated. During the injection of undiluted sample extracts, the volume of methanol that passes through the column and into the ICP-MS is ~10%. It has already been discussed that the addition of MeOH enhances the ^{75}As signal by increasing the ionization efficiency of this analyte; this effect is observed on a small scale here. Although there is no detectable As(III) in this fish material, the accurate quantitation of this compound (compared to aqueous calibration standards) could obviously lead to an overestimation if the signal of this analyte is enhanced due to the simultaneous elution of MeOH from the column. In this case, a standard addition calibration would represent a more accurate approach for quantitation. However, the spiking of each sample extract at different levels, which is necessary for this type of calibration, would make such an approach less suitable for a high sample-throughput application. In addition, the accurate integration of AsB is influenced by the signal increase on the tailing side of the peak.

In order to eliminate the effect of these signal variations on the accurate quantitation of the As-species in the methanolic extracts, the methanol fraction could either be reduced by evaporation or dilution with water. Dilution was chosen as the preferred option over evaporation in order to avoid possible analyte losses and because of time-efficiency. Whereas evaporation would either involve passing an inert gas over the solution or using rotary evaporation equipment, gravimetric dilutions were easily and quickly achieved by pipetting an aliquot of the extract into a sealed HPLC autosampler vial, weighing, and then adding the appropriate amount of water. In order to observe the effect of different dilution factors on the observed plasma disturbance, a fish extract was diluted 10-, 5-, and 2-fold in water and also injected undiluted. The effects of the different dilutions on the ^{103}Rh signal are shown by the chromatograms in Figure 4.

As demonstrated in Figure 4, a 10-fold dilution is sufficient to eliminate the plasma disturbance sufficiently; therefore, all extracts were diluted 1:10 in water prior to injection.

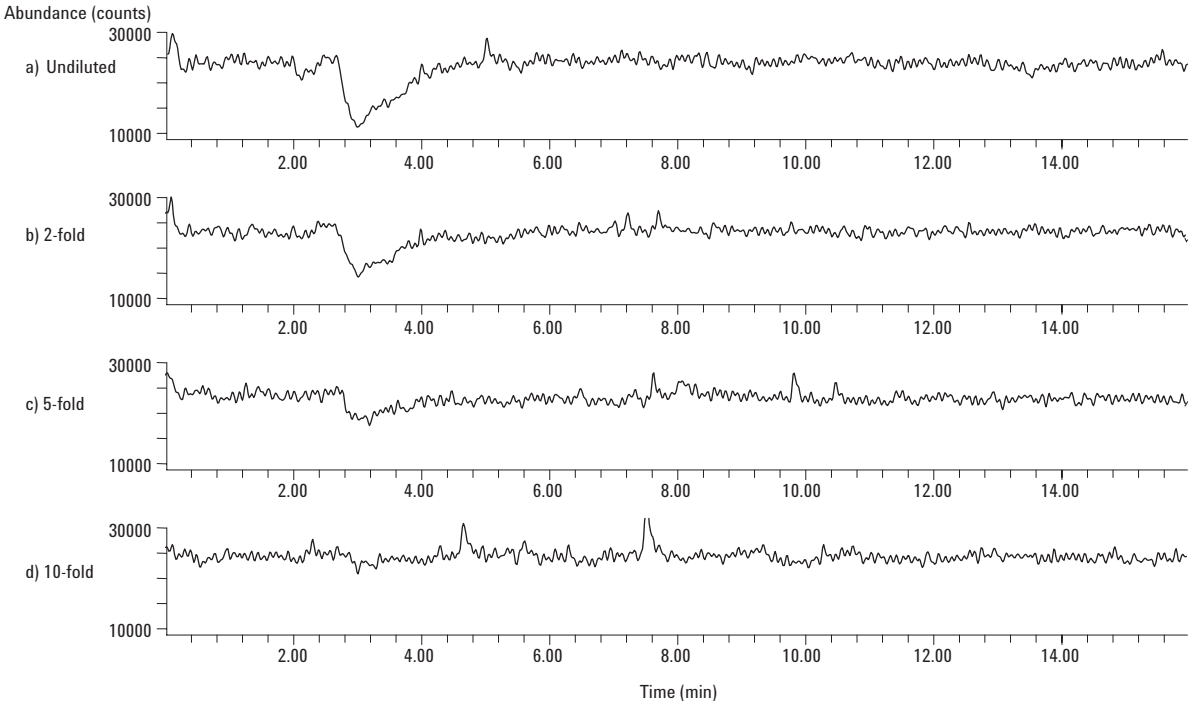


Figure 4. Signal of the internal standard ^{103}Rh, for fish sample extracts a) undiluted and diluted b) 2-fold, c) 5-fold and d) 10-fold.

Comparison of External Calibration and Standard Addition for the Quantitation of AsB in Fish Tissues

Due to the fact that arsenic is mono-isotopic, isotope dilution analysis cannot be used for the high-accuracy quantitation of this compound by LC-ICP-MS. In such circumstances, calibration by standard additions is often used in order to achieve matrix matching of standards and samples. It is also a useful technique in chromatographic applications where the possibility of retention time (RT) shifts of analytes due to matrix components exists. This can result in misidentification, and thus erroneous results. However, standard addition calibration can be very time- consuming because several aliquots of the sample require spiking with different levels of a calibration standard, and at least three levels of standard addition are needed for accurate quantitation of the same sample. External calibration by non-matrix matched standards can be used for applications where the difference in the matrix between samples and standards does not influence the accuracy of the result to a significant extent.

Standard addition calibration and non-matrix matched external calibration were compared for AsB in two certified reference materials (DORM-2, Dogfish muscle, NRC Canada and BCR 627, Tuna Fish, BCR EU) in order to assess whether the calibration technique used significantly influenced the accuracy or precision of the analytical result. The results showed that there was no significant difference in the mean results determined by the different calibration techniques with this method. The mean results for repeat analysis of both materials showed that the difference in the DORM-2 material was less than 1.4% and less than 4.5% for the BCR 627 material. When taking into account the standard deviations (SD) associated with the mean result obtained by each calibration technique, there was no statistically significant difference between the AsB results obtained by either approach in either of the fish tissue certified reference materials (CRMs).

Results of CRM Analysis

In order to test the accuracy of the developed ASE extraction and HPLC-ICP-MS method, a variety of certified and candidate reference materials of marine origin were extracted and analyzed. The samples included the certified fish reference materials DORM-2 and BCR 627, as well as an oyster tissue material (BCR 710)*, which is pending certification.

* The "MULSPOT" project has been financed by the SM&T Program (EU) (Contract SMT4-CT98-2232) and coordinated by ENEA (IT). The Project is at the certification stage and the material is not yet available on the market.

Table 3. Data Obtained for AsB in Two CRMs and a Candidate Reference Material

Expressed as mg/kg As unless otherwise stated	Measured value	Certified value
DORM-2 (Dogfish muscle)	16.3 ±0.9 (±1 SD)	16.4 ±1.1 (±95% C.I.)
BCR 627 (Tuna fish)	3.69 ±0.21 (±1 SD)	3.90 ±0.22 (±95% C.I.)
BCR 710 (Oyster tissue)† (Concentration as species)	31.8 ±1.1 (±1 SD)	32.7 ±5.1 (±1 SD)

† The data shown for this material is based on the consensus mean of the final certification round after the removal of statistical outliers.

Subsamples of the different materials (n = 4–6) were extracted, diluted in water, and analyzed as described above. The data for AsB determined in these samples is shown in Table 3. A chromatogram of the tuna fish material BCR 627 is shown in Figure 5.

The chromatogram indicates that the major species in this sample is AsB with two minor species, which were also extracted and detected. One peak was identified as DMA, and the peak labelled P1 is most likely to be AsC from RT matching. The data in Table 3 shows that the combined ASE/HPLC-ICP-MS methodology is capable of delivering accurate and reproducible results for AsB in these matrices. In addition, the extraction of other minor species, such as DMA and AsC, was achieved in the fish tissues; up to six species were extracted and separated in the oyster material, although none of these (apart from DMA) were quantified during this study. This DMA data for BCR 710 (730 ±30 ng/g DMA) showed a good agreement with the consensus mean value of the certification round (820 ±200 ng/g DMA).

Evaluation of Method Performance During a CRM Feasibility Study

The method performance was assessed in comparison to a number of European expert laboratories during the “SEAS” feasibility study organized by the The University of Plymouth Enterprise Limited and sponsored by the European Union (BCR, EU)‡. A fish material was prepared for this intercomparison by the University of Plymouth and distributed to participating laboratories. Participants were asked to determine AsB in a fish material from two different bottles using a methodology of their choice and making their determinations, at least, in duplicate on separate days.

The developed As-speciation method was used to extract and analyze the fish samples provided. A total of 12 subsamples from the two bottles were

‡The “SEAS” feasibility study was co-ordinated by The University of Plymouth Enterprise Limited (Plymouth, UK) under the EC contract: G6RD CT2001 00473 "SEAS" with the title: 'Feasibility Studies for Speciated CRMs For Arsenic in Chicken, Rice, Fish and Soil and Selenium in Yeast and Cereal'.

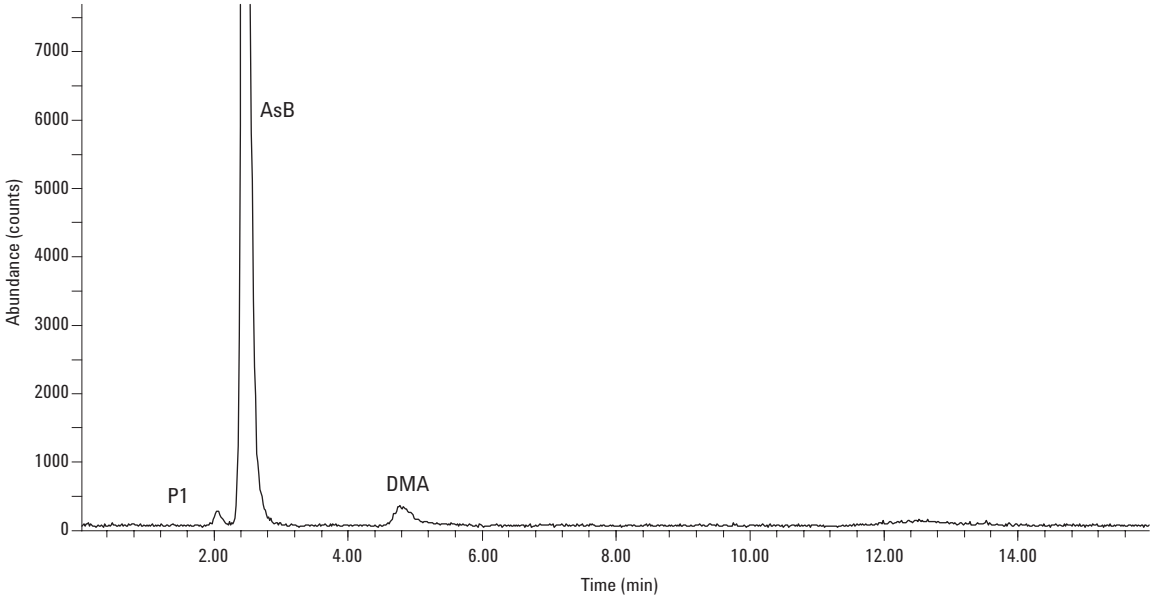


Figure 5. Chromatogram of a tuna fish extract (BCR 627) enlarged to show the detection of minor species in this material.

extracted and analyzed on 3 different days. The data were combined to provide the value labelled "LGC" in Figure 6 below. The error bars indicate the SD of the mean of individual results. The mean of all result (excluding a statistical outlier) together with 1 SD above and below the mean is indicated by the solid and dashed horizontal lines, respectively. The data provided by the combined ASE extraction and developed LC-ICP-MS methodology (94.92 ±3.95 mg/kg AsB) is in very good agreement with the mean result of all labs (95.72 ±7.79 mg/kg AsB, n = 11). The precision achieved was also satisfactory at 4.2% (RSD) for 12 subsamples from different bottles analyzed on 3 separate days. The performance of the method in this international intercomparison is highlighted by the good agreement with data provided by several European expert laboratories with longstanding expertise in As-speciation analysis. It should also be noted that the intercomparison was carried out with a blind sample of unknown concentration, rather than based on the analysis of a CRM with known certified values.

Conclusions

A robust and practical method has been developed based on accelerated solvent extraction and HPLC-ICP-MS analysis for the fast and accurate determination of AsB in fish samples. The benefits of the methods include automated extraction of up to 24 samples, minimal sample preparation steps (dilution only) after extraction, and rapid and automated analysis by HPLC-ICP-MS. The separation of four to six species of toxicological interest is achieved within 10 min using an isocratic elution. This increases the sample throughput by negating the column equilibration period needed with most gradient elution profiles.

The method was validated using commercially available CRMs and during a European intercomparison study with a fish sample of unknown concentration. The performance of the method was very satisfactory in terms of both accuracy and precision compared to several other expert laboratories.

This method can be used to rapidly determine the nontoxic proportion (AsB) in fish samples with high total As content and could therefore be used to determine whether a particular sample poses a toxicological risk in the food chain.

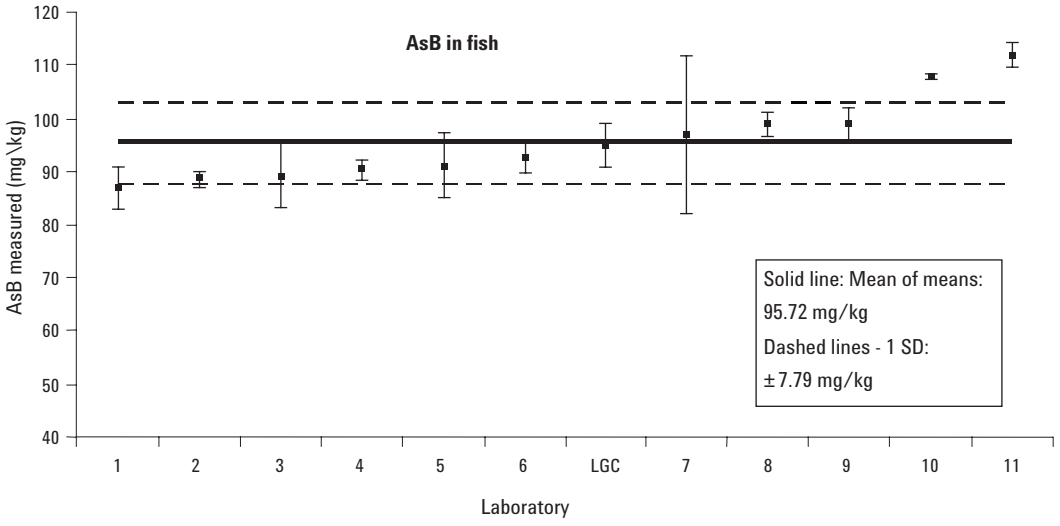


Figure 6. Comparison of data submitted by 12 participants for the determination of AsB in fish during the "SEAS" feasibility study. The error bars associated with the individual data points represent 1 SD of analysis of separate subsamples.

Acknowledgements

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Determination of Mercury in Microwave Digests of Foodstuffs by ICP-MS

Application

Food Safety

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Abstract

The quantitative determination of mercury in foodstuffs is presented using a 7500i ICP-MS. Microwave digests were prepared and then analyzed by ICP-MS. To avoid memory effects often experienced with mercury, gold was added offline to all standards/samples and wash solutions to act as a cleansing agent. The instrumental setup used a second vacuum pump, the integrated sample introduction system in the high sample throughput mode, and a micro-flow concentric nebulizer. This allowed the robust and rapid determination of mercury in the digests at the ppt range. Excellent agreement with the certified value was obtained for two certified reference materials and stability of the system was demonstrated over a 36-hour analytical run.

Introduction

The determination of sub-ppb concentrations of mercury has always been of special importance in the field of trace metal analysis. Even at trace levels, mercury is toxic and causes neurological damage, particularly in fetuses and young children. Anthropogenic sources of mercury in the environment include coal-fired power stations and chlor-alkali works. In the aquatic environment, bacteria convert elemental mercury Hg(0) to methylmercury which is accumulated and passed up the food chain. It has been reported that some whale meat contains 5000 times the Japanese legal limit of 0.4 µg/g. In addition, fish and shellfish are significant contributors to the human diet. Today mercury pollution is a global problem and extensive monitoring of foodstuffs is required. Therefore fast efficient and robust methods are needed. Mercury however is recognized as a problem element. It is known to adsorb onto the walls of storage containers and volatilize as mercury vapor. Additionally, its high first ionization potential and numerous isotopes have limited its sensitivity in ICP-MS analysis. ICP-MS allows the rapid determination of ultratrace levels of metals in food digests, however, extensive washout times have been required to reduce carryover for mercury analysis. Other workers have tried the addition of a number of chemical agents in the past. One of the most effective washout agents is gold chloride. To avoid memory effects and ensure stability, gold chloride (at the 5-ppm level) was added offline to all samples/standards and wash solutions. Extensive washout times were reduced by using by the integrated sample introduction system (ISIS). With the use of the high throughput pump, a large flush volume can be pumped through in a much shorter time. By summing the responses for multiple isotopes (199, 200, 201, and 202) and with the help of a second interface vacuum pump and a micro-flow concentric nebulizer, detection levels of between 10 and 30 ppt were routinely achieved for the digests.

Procedure

Microwave Digestion

Varying aliquots of each sample (generally between 0.2 and 0.6 g, depending on the moisture content of the sample) were weighed to the nearest 0.01 g into the digestion vessels. Wet oxidation was induced using concentrated, ultra-high purity nitric acid (10 mL, from Romil LTD, Cambridge, UK) with the addition of a 0.2 mL of concentrated hydrochloric acid (Romil LTD, Cambridge, UK). Oxidation was carried out in heavy-duty vessels (HDV) using a high-pressure microwave digestion oven (Mars 5 from CEM). Temperature control was used as opposed to pressure control. Samples were ramped to 180 °C over 20 minutes and held at 180 °C for 10 minutes before cooling to below 50 °C before venting the vessel. Both pressure and temperature were monitored by direct measurement throughout the digestion to ensure that samples attained the critical temperature of 180 °C, at which food components, such as fat, are digested. The sample digests were then made up to 100 g using ultra-high purity water (18 mega ohms, from Elga Maxima). The resultant solution was used for determination.

Operating and Acquisition Parameters

Ten milliliter portions of the sample digests were accurately pipetted into sample tubes, and using a micropipette, 20 µL of a 1000-ppm gold chloride solution (Romil LTD, Cambridge, UK) was added. This gives a final gold concentration of 5 ppm in solution. Fifty milliliters each, of blank and four standard solutions covering the range, were prepared from a 100 µg/g stock mercury solution (from SPEX CertiPrep Assurance, Metuchen, New Jersey, USA). Ten percent wt/wt nitric acid containing 5 ppm of gold was used as the wash solution for the autosampler and nebulizer. Gold is thought to have its effect by acting as an oxidizing agent ensuring that mercury stays in an ionized form in solution. Gold was added at elevated levels to ensure that any residual amounts of organic compounds in the digests would not reduce Au(III) to elemental gold and render it ineffective. A 250-ppb Thalium standard was added online as an internal standard (ISTD), using the ISIS system. There was an online dilution factor of 1:20. Gold chloride was also added to the standard solutions at 5 ppm.

Instrument Conditions

Plasma gas flow rate	16 L/min
Carrier gas flow rate	0.85 L/min
Make-up flow	0.14 L/min
RF Power	1400 Watts
Nebulizer	Agilent micro-flow 100 µL
Spray chamber	Glass double pass
Spray chamber temperature	Cooled to 2 °C
ICP Torch injector	2.4 mm
Sample tubing	0.89 mm id
Internal standard tubing	0.19 mm id
Instrument Peri pump	0.1 rps
Sample/Skimmer cones	Nickel
Rotary pumps	2
Autosampler	AX500

Acquisition Parameters

Mass	Element	Integration/Point	Time (s)/Mass
199–201	Hg	3.5	10.5
202	Hg	3.5	10.5
205	Tl	0.05	0.15

Number of points per mass:	3
Acquisition time:	43.79 s
Number of repetitions:	3
Total acquisition time:	131 s

Peristaltic Pump Program

Memory effects arise when the analyte signal is enhanced due to contributions from previous high concentration sample. This is due to adsorption/desorption of mercury in the sample introduction system. As a result, the analyst has to program long washout times. With the use of ISIS this wash-out time can be reduced.

ISIS Peristaltic Pump Program
Analysis Speed : 0.10 rps

Before acquisition	
Uptake speed	0.80 rps
Uptake time	32 s
Stabilization time (undiluted)	150 s
After acquisition (probe rinse)	
Rinse speed	0.80 rps
Rinse time (sample)	8 s
Rinse time (standard)	8 s
After acquisition (rinse)	
Rinse vial	1
Uptake speed	0.8 rps
Uptake time (undiluted)	32 s
Stabilization time	85 s

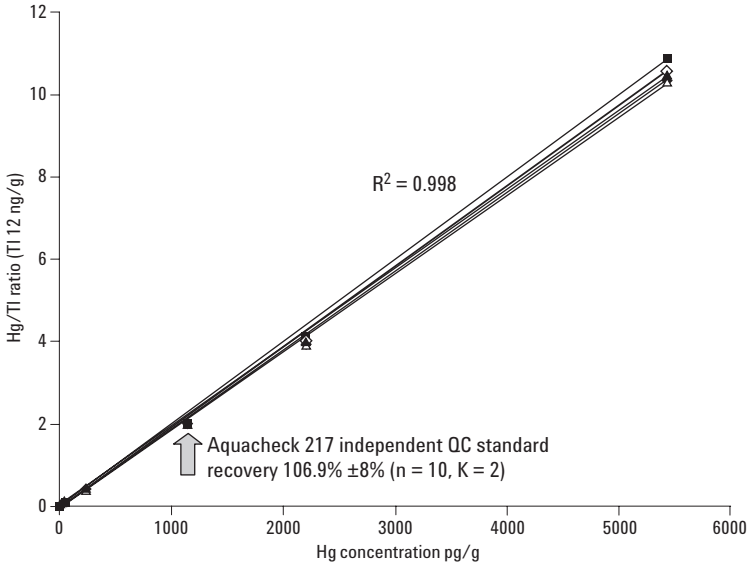


Figure 1. Calibrations over 36 hours continuous operation.

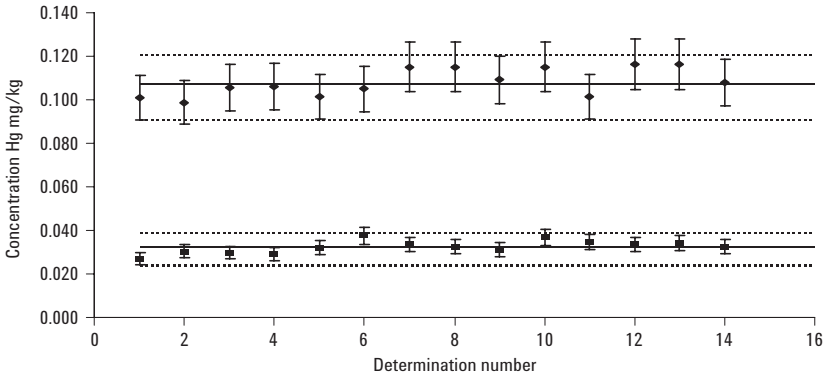
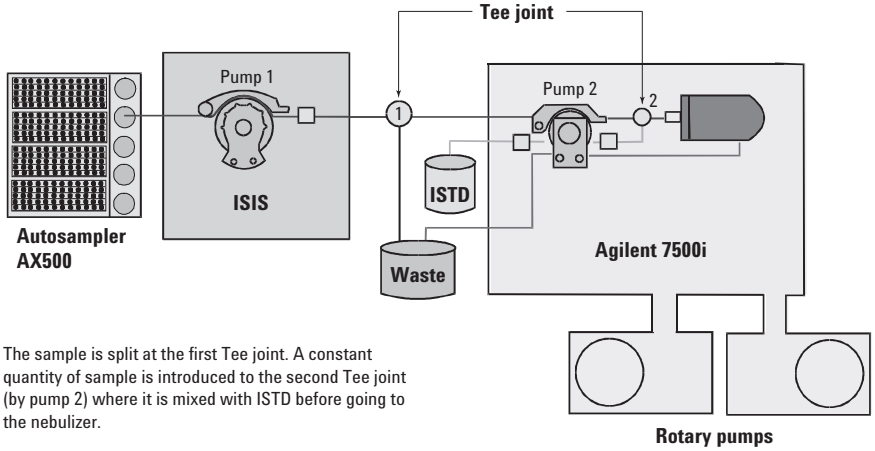


Figure 2. NIST 1547 Peach leaves (bottom trace) and LGC 7160 Crab paste (top trace) were analyzed 14 times over a period of 1 month. Each point represents a separate digest. LGC 7160 certified value 0.096 = ±0.108 mg/kg, NIST 1547 certified value 0.031 = ±0.007 mg/kg.



The sample is split at the first Tee joint. A constant quantity of sample is introduced to the second Tee joint (by pump 2) where it is mixed with ISTD before going to the nebulizer.

When going to the next sample the ISIS pump (pump 1) turns at high speed to shorten the sample and washout transfer times.

Figure 3. Schematic of ISIS in high sample throughput mode.

Results

Five calibration plots over a 36-hour period are shown in Figure 1 demonstrating excellent stability of the system. As can be seen, excellent linearity was achieved over an extended calibration range demonstrating that memory effects had been effectively eliminated. Between the calibrations over 120 various microwave foodstuff digests were analyzed.

Quality Control

An Aquacheck proficiency testing material solution from the Water Research Council (1010 ppt) was analyzed 10 times during the run and a mean recovery of 106.9% (±8%) was achieved (Figure 1). This material was analyzed throughout a run of 120 various foodstuffs samples and acted as a quality control for the quantitation.

Two certified reference materials (CRMs) were analyzed throughout a survey of 500 samples. These acted as quality control materials for the microwave digestion as well as the quantitation. The CRMs used were a crab paste—Metals LGC 7160, 0.096 mg/kg—and peach leaves—NIST 1547, 0.031 mg/kg. Each CRM was analyzed 14 times on different runs during a survey of more that 500 samples of various foodstuffs over a 1-month period. The results can be seen in Figure 2.

Conclusion

This procedure proved robust, as in excess of 500 samples were analyzed in runs lasting in excess of 36 hours without loss of sensitivity.

The 7500i ICP-MS has shown to be a robust and sensitive tool for the analysis of foodstuff for mercury. The system proved stable over an extended time period. The use of the ISIS enables the operator to take advantage of the high sample throughput possible with this technique. The use of Au(III), in particular, shortens washout time considerably, reducing the possibility of carry-over. The sensitivity of the 7500i ICP-MS gives method detection limits at low ppt levels in the foodstuff digests. The extended calibration range reduces the need for dilutions and reruns.

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Measurement of Trace Elements in Malt Spirit Beverages (Whisky) by 7500cx ICP-MS

Application

Food

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Abstract

A method for the measurement of trace elements in malt spirits (whisky) is described with reference to six different samples. An Agilent 7500cx ICP-MS featuring the Octopole Reaction System (ORS) was used for the analysis. The 7500cx ensures simple operation as a single method and a single set of conditions can be used to remove interferences regardless of their source. Excellent spike recoveries were obtained (between 97 and 107%) following a simple dilution of the samples. A 5-hour stability test yielded excellent precision (< 2%) for almost all elements. The study shows that the 7500cx can be used for the routine measurement of trace metals in beverages.

Introduction

The measurement of trace elements in alcoholic beverages is required from a quality control standpoint and also to ensure that the final product complies with any regulatory requirements.

Metal content can originate from the raw ingredients, such as water or grain, as well as during processing, for example, from fermentation or distillation equipment. An example would be high arsenic concentration from distillation vessels manufactured from poor-quality copper. The levels of trace elements can also significantly affect the taste of the whisky. Consequently, there is a requirement to measure elemental concentrations in the final product. While ICP-MS offers high sensitivity and excellent detection limits for many elements, interferences on key elements arising from the alcohol content and required sample preparation can be problematic.

The 7500cx features the Octopole Reaction System (ORS) collision/reaction cell, which removes matrix-based polyatomic interferences using a single set of cell conditions (helium mode). For the analysis of spirits, the major interferences resulting from the sample would be carbon-based (for example, $^{40}Ar^{12}C$ on ^{52}Cr). Many elements are much more stable in a chloride matrix than simple acidification using nitric acid; for this reason, hydrochloric acid (HCl) was added to the samples. New interferences are created by the addition of HCl (for example, $^{35}Cl^{16}O$ on ^{51}V; $^{40}Ar^{35}Cl$ on ^{75}As, etc.) but they are removed by the ORS in helium mode.

An optional cell gas line is available for the 7500cx, enabling operation in hydrogen (H_2) reaction mode, which allows for the measurement of selenium at ultratrace levels. Since several of the solutions contained less than 40 ng/L Se (some significantly lower than this) in solution after dilution, H_2 reaction mode was also used during this study.

Experimental

Sample Preparation & Instrumental Conditions

Four Scottish whiskies (Highland, Speyside, Islay, and a blend), one Irish whisky, one U.S. bourbon –

as well as a further Scottish whisky and a U.S. bourbon that had been stored in lead crystal decanters – were analyzed. The samples were prepared by simple 5x dilution using 1% HNO_3 and 0.5% HCl (v/v). Using an acid mix significantly improves the stability of many elements, particularly Hg and Sn, compared to the use of nitric acid alone. Standards were prepared from 1,000 ppm stock single-element solutions to produce final mixed-element calibration solutions. In order to compensate for sample transport effects and solvent evaporation rates, the alcohol content of the standards was matched to that of the samples by adding 8% ethanol to all standard solutions (equivalent to 5x dilution of the original samples, which contained 40% v/v alcohol). This also compensates for ionization enhancement effects for As and Se in the presence of high carbon concentrations. Gold (400 µg/L) was also added to the standards and samples in order to further improve the stability of Hg.

Table 1 lists the instrumental conditions used for the analysis; sample uptake rate was approximately 150 µL/min and sampling was facilitated by the Agilent ASX-520 autosampler. The solution pump program was optimized using the preemptive rinse function in the ChemStation software in addition to a multichemistry rinse regime [1]. The 7500cx was operated under standard conditions, and internal standards (Ge, Rh, and Ir) were added automatically on line by the system's peristaltic pump. No special precautions were necessary for these sample types.

Table 1. Agilent 7500cx Operating Conditions

RF power	1550 W
Sampling depth	8 mm
Carrier gas flow	0.68 L/min
Makeup gas flow	0.33 L/min
Spray chamber temperature	15 °C
Helium cell gas flow	5.5 mL/min
Hydrogen cell gas flow	4.0 mL/min

Data Acquisition

Data was acquired operating the ORS in helium [He], hydrogen [H_2], and no-gas modes. Helium mode is the default mode of operation of the 7500cx. The inert He cell gas conditions remove interferences based on their ionic cross-section rather than relying on a reactive gas. As almost all interferences in ICP-MS are polyatomic in nature, they possess a greater cross-section than the monatomic analyte at the same mass and therefore undergo a greater number of collisions in the cell. As each collision causes energy loss, the interfering species lose more energy than the analyte and are subsequently filtered from the mass spectrum by discriminating between the two different energies (called energy discrimination). As this process takes place regardless of the analyte-interference combination, a single set of conditions can be used for all analytes.

Selenium was measured in hydrogen mode as the concentration of this element in the diluted sample was at low ppt levels. Although selenium can be measured in helium mode, hydrogen mode removes the Ar-based interference with greater efficiency, improving the detection limit for this element, and is the better option for low-ppt concentrations. Some isotopes were determined in both helium mode and no-gas mode to provide comparative data on cell performance. For routine analysis this would not be necessary, of course. All cell modes were acquired within a single acquisition and sample pass.

Results and Discussion

Table 2 summarizes the detection limits (DLs), background equivalent concentrations (BECs), and calibration regression for the isotopes studied in the different cell modes (default mode is highlighted in bold typeface). For those elements that suffer from interferences in this carbon and chloride matrix, BECs and DLs are severely compromised when operating the instrument in no-gas mode (that is, conventional ICP-MS). This can be clearly observed in the data for chromium: ^{52}Cr BEC without cell gas is 526 µg/L, and in helium mode is 0.07 µg/L. The interference is effectively reduced to background contamination levels as the BECs for both Cr isotopes are very similar. Improvements can also be observed for V, Fe and ^{65}Cu (^{63}Cu does not suffer from interferences in this relatively simple matrix), all of which were acquired in helium mode.

Figures 1 to 3 display the calibration profiles for selected interfered elements with and without cell gas applied; Figures 4 and 5 illustrate the calibration profiles for Be (low mass, difficult to ionize) and Hg (high mass, difficult to ionize, low-abundance isotope). The line does not pass through the origin in the calibrations for those elements that suffer from an interference and this offset can be seen clearly. Be and Hg are also presented to demonstrate the excellent sensitivity for these difficult-to-ionize elements. In order to obtain low detection limits, it is essential to maximize the ion-

ization efficiency of the plasma. This is done through optimization of the sample introduction system (low solution and gas flow rates and wide-bore injector torch) and plasma generator design (27.12 MHz, solid-state fixed frequency, and high-efficiency digital drive). All of these factors combine to increase the effective central channel temperature, improving ionization efficiency. This is allied to an ion lens system designed to improve low-mass ion transmission efficiency, further improving the DL of this important and relatively difficult element.

The elements Ge, Rh, and Ir were used as internal standards and were added on line.

Table 3 displays the quantitative data for all samples, including a spike recovery for the Islay whisky. Data are displayed in the preferred cell gas mode (usually helium). Although some elements were calibrated under gas and no-gas conditions, only the most appropriate cell mode is displayed to simplify the data set. Taking Cr as an example, the data for both isotopes did not match in no-gas mode and were significantly higher than the data obtained in helium mode due to the intensity of the C- and Cl-based interferences. The helium mode data for both Cr isotopes produced comparable results, which is a good indication of the accuracy of the data.

The two samples that had been stored in lead crystal decanters have obviously higher Pb concentration in comparison to the other samples. The mean Pb concentration in the noncrystal samples was about 1.3 µg/L, while the Pb content of the samples stored in the crystal decanters was almost 10x higher. As a comparison, the UK maximum permissible Pb concentration in drinking water is 25 µg/L (at the tap), which means that the Pb concentration is within this guideline; however, in 2013 this level is due to be reduced to 10 µg/L, meaning products stored in crystal would fail to meet drinking water quality standards.

Table 2. Limit of Detection, Background Equivalent Concentrations, and Regression Coefficients for the Studied Isotopes (Data are presented as ng/L [ppt] and are corrected for dilution.)

Element	Mass	Mode	r	DL	BEC
Be	**9**	**No gas**	**1**	**0.5**	**0.3**
V	**51**	**He**	**1**	**26.8**	**12.2**
V	51	No gas	0.9999	495	5220
Cr	**52**	**He**	**1**	**50.4**	**73.7**
Cr	52	No gas	0.985	48600	526000
Cr	**53**	**He**	**1**	**38.6**	**71.3**
Cr	53	No gas	0.9997	2020	52100
Mn	**55**	**He**	**1**	**7.8**	**20.8**
Mn	55	No gas	1	17.5	30.4
Fe	**56**	**He**	**0.9999**	**17.8**	**406**
Fe	56	No gas	1	1160	58300
Co	**59**	**He**	**1**	**0.5**	**3.7**
Co	59	No gas	1	5.6	6.7
Ni	**60**	**He**	**1**	**13**	**38.7**
Ni	60	No gas	1	15.2	73.6
Cu	**63**	**He**	**1**	**10.4**	**41.5**
Cu	63	No gas	1	16.6	59.6
Cu	**65**	**He**	**1**	**18.1**	**36.9**
Cu	65	No gas	1	58.6	230
Zn	**66**	**He**	**1**	**33.9**	**119**
Zn	66	No gas	1	22	171
As	**75**	**He**	**1**	**2.0**	**3.8**
As	75	No gas	1	46.1	382
Se	**78**	**H_2**	**1**	**3.6**	**13**
Se	78	No gas	1	135	1390
Cd	**111**	**He**	**1**	**5.7**	**5.3**
Cd	111	No gas	1	3.3	6.1
Sn	**118**	**No gas**	**1**	**7.8**	**50.5**
Sb	**121**	**No gas**	**1**	**7.1**	**30.8**
Ba	**137**	**No gas**	**1**	**2.8**	**5.3**
Hg	**201**	**No gas**	**1**	**1.7**	**10.7**
Pb	**208**	**No gas**	**1**	**2.1**	**10.2**
U	**238**	**No gas**	**1**	**0.1**	**0.2**

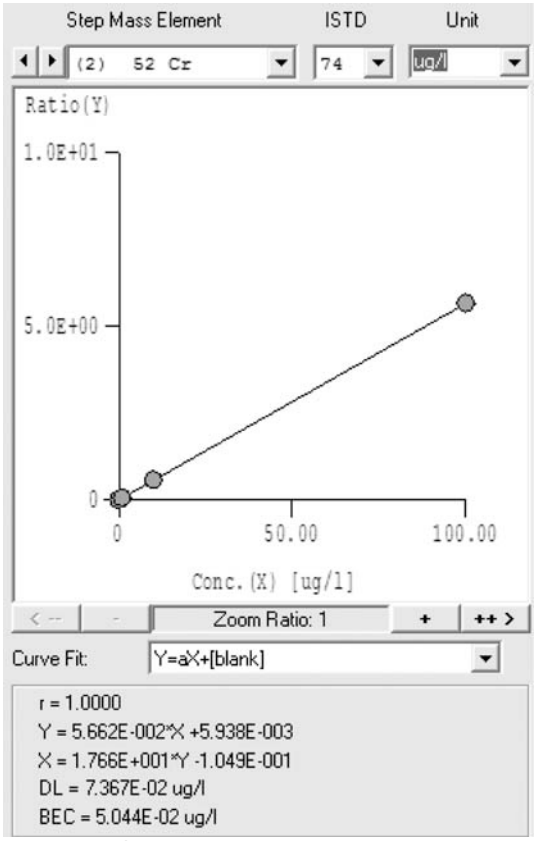


Figure1A. Chromium calibration [He mode]. Note BEC of 0.0504 µg/L.

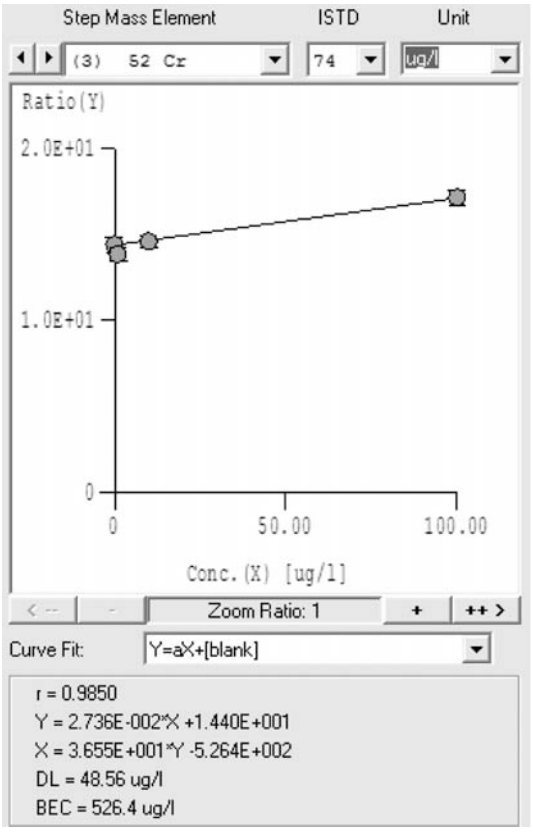


Figure 1B. Chromium calibration [no-gas mode]. Note BEC of 526 µg/L.

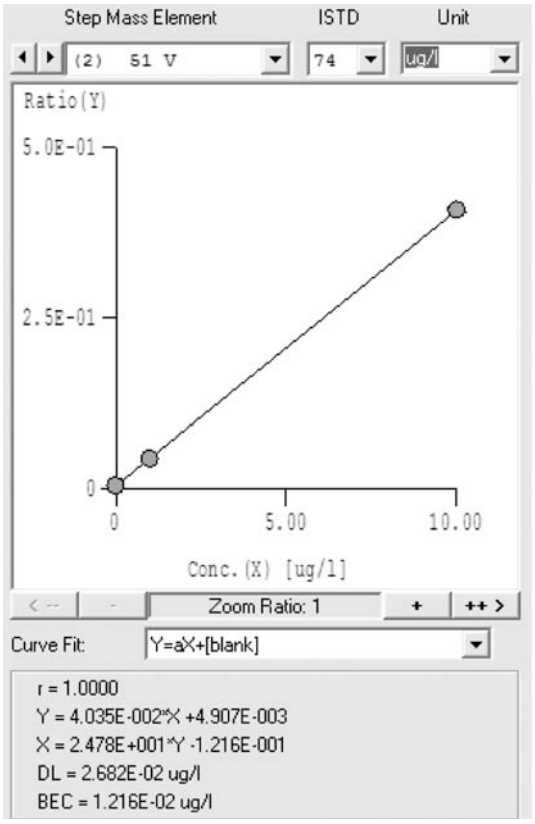


Figure 2A. Vanadium calibration [He mode]. Note BEC of 0.0122 µg/L.

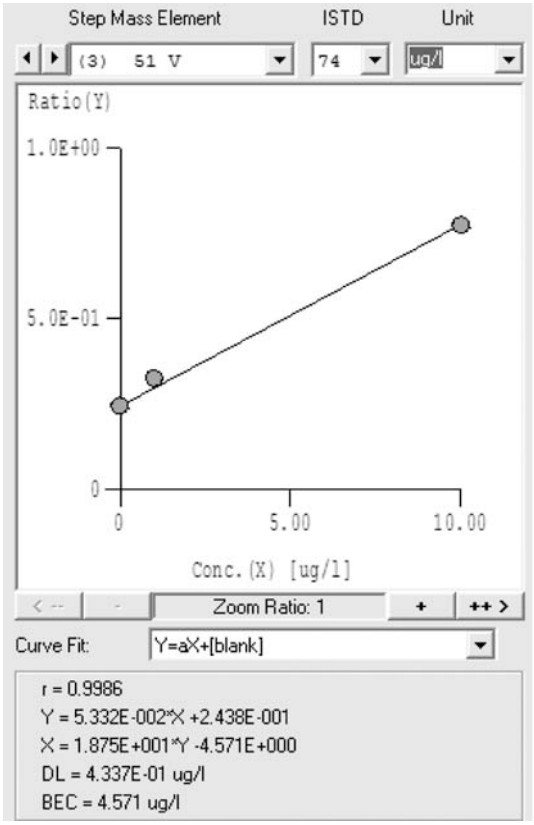


Figure 2B. Vanadium calibration [no-gas mode]. Note BEC of 4.57 µg/L.

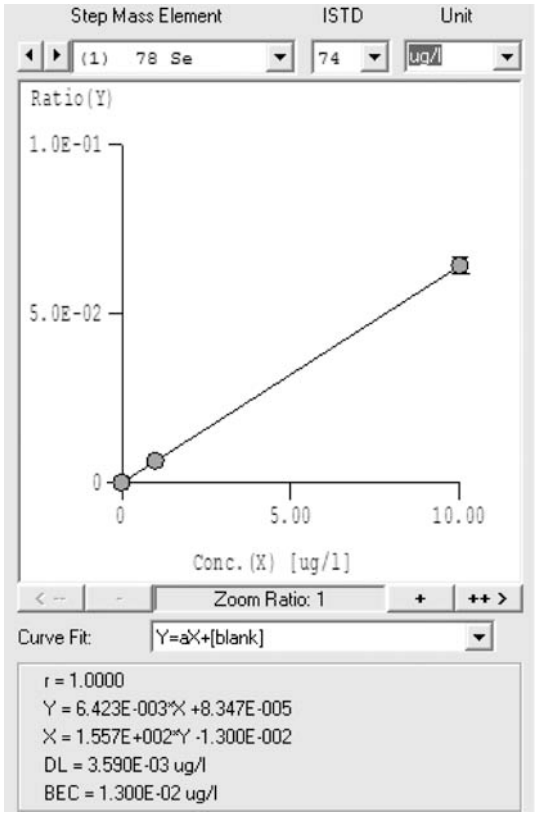


Figure 3A. Selenium calibration [H_2 mode]. Note BEC of 0.013 µg/L.

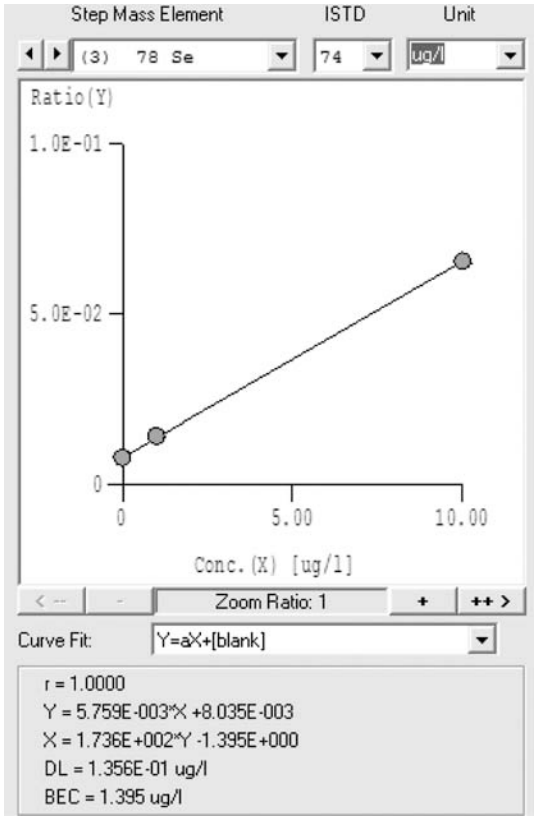


Figure 3B. Selenium calibration [no-gas mode]. Note BEC of 1.4 µg/L.

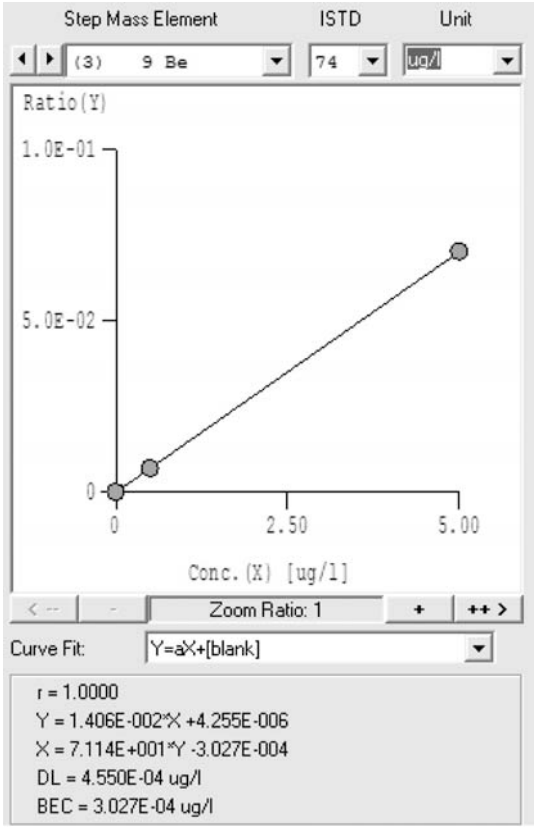


Figure 4. Beryllium calibration [no-gas mode]. Note detection limit of 0.303 µg/L.

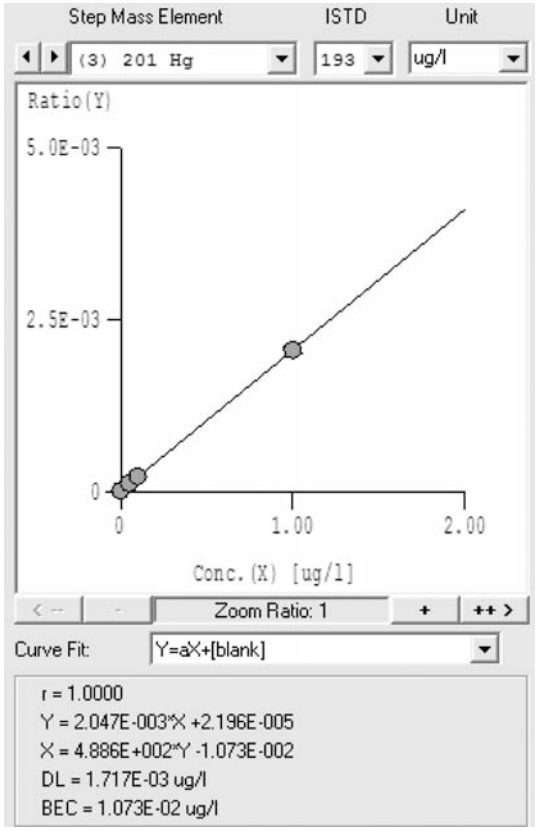


Figure 5. Mercury calibration [no-gas mode]. Note detection limit of 0.0107 µg/L.

Table 3. Quantitative Data Obtained in the Preferred Cell Mode for the Spirit Samples with Spike Recovery for the Islay Sample (Recoveries were generally excellent. All results presented as dilution-corrected µg/L.)

Sample			Highland	Speyside	Islay	Blend	Irish	Bourbon	Bourbon decanter	Whisky decanter	Islay spike	Spike qty	% recovery
Be	9	No gas	0.140	0.052	0.037	0.008	0.035	0.015	0.042	0.048	13.09	12.5	104.4
V	51	He	1.564	0.443	0.344	0.073	0.431	6.321	1.693	0.14	25	25	98.6
Cr	52	He	27.39	14.05	4.064	12.62	22.71	4.077	31.87	4.331	30.95	25	107.5
Cr	53	He	26.15	13.7	3.955	12.57	22.61	3.661	31.52	4.114	30.81	25	107.4
Mn	55	He	54.51	31.76	13.52	12.22	26.95	9.753	90.4	30.54	38.19	25	98.7
Fe	56	He	1125	191.2	99.76	583.8	250.5	131.4	1114	67.03	232.2	125	106.0
Co	59	He	1.097	0.376	0.180	0.130	0.336	0.172	0.323	0.368	12.83	12.5	101.2
Ni	60	He	14.02	3.586	1.442	5.065	3.078	2.274	12.88	1.992	25.91	25	97.9
Cu	63	He	542.9	370.8	454.4	258	38.45	22.2	445.5	367.6	579.8	125	100.3
Cu	65	He	525.5	359.2	441.6	251.4	37.4	21.43	430.8	355.9	568.6	125	101.6
Zn	66	He	21.02	18.54	8.414	14.18	8.149	13.69	68.27	21.9	137.5	125	103.3
As	75	He	0.503	0.427	0.272	0.256	0.164	2.192	0.434	0.424	25.72	25	101.8
Se	78	H_2	0.458	0.357	0.190	0.073	0.045	0.497	0.069	0.293	26.54	25	105.4
Cd	111	He	0.036	0.024	0.012	0.010	0.024	0.036	0.193	0.028	12.55	12.5	100.3
Sn	118	No gas	9.18	14.82	16.68	5.161	2.245	1.681	0.239	15.12	41.3	25	98.5
Sb	121	No gas	0.817	0.514	0.397	0.308	0.311	0.765	0.316	0.188	24.87	25	97.9
Ba	137	No gas	3.282	3.05	1.426	2.001	3.37	3.303	1.396	2.41	25.71	25	97.1
Hg	201	No gas	0.013	0.011	0.010	0.011	0.010	0.018	0.008	0.009	0.252	0.25	97.0
Pb	208	No gas	1.13	0.898	0.903	1.902	1.21	0.912	12.59	11.15	25.33	25	97.7
U	238	No gas	0.295	0.049	0.051	0.026	0.060	0.104	0.028	0.049	24.38	25	97.3

The benefit of operating the instrument in helium mode can clearly be observed for those isotopes suffering from interferences. As helium is a totally inert gas, no side reactions or new product interferences are formed – this lends itself to full mass acquisition allowing interference-free qualitative or semiquantitative analysis. The samples were prepared in an identical way as above (although a separate preparation on a different day) and Table 4 displays the semiquantitative data obtained for the samples analyzed under identical helium cell conditions as with the previous data set.

The full mass spectrum (Figure 6) is from the crystal-stored bourbon sample. The graphic includes an inset, zoomed-in region to demonstrate the excellent isotopic fit for those elements suffering most from interferences. The fit for Cr is particularly important as all three isotopes (50, 52, and 53) demonstrate good agreement with the expected natural ratio in this carbon-based matrix (^{50}Cr suffers interferences from $^{38}Ar^{12}C$, $^{13}C^{37}Cl$, $^{36}Ar^{14}N$, and $^{35}Cl^{15}N$; ^{52}Cr has interferences from $^{36}Ar^{16}O$, $^{40}Ar^{12}C$, $^{35}Cl^{17}O$, and $^{37}Cl^{15}N$; ^{53}Cr has interferences from $^{40}Ar^{13}C$, $^{37}Cl^{16}O$, $^{35}Cl^{18}O$, and $^{35}Cl^{17}O^{1}H$). Several other interferences are also possible, but each is polyatomic in nature and so is removed by the same process and using a single set of helium mode conditions.

To demonstrate instrument stability (Figure 7), 54 separate measurements were made of a spiked Highland malt whisky sample; total measurement time was 5 hours 18 minutes. Stability for the majority of elements was < 2% RSD over the run, indicating applicability of the method to routine analysis.

Table 4. Semiquantitative Data for Spirit Samples Using Helium Mode (Data are presented as µg/L [ppb] unless indicated and are corrected for dilution.)

		Highland	Speyside	Islay	Blend	Irish	Bourbon	Bourbon decanter	Whisky decanter
7	Li	0.24	0.2	0.049	0.084	0.16	0.16	0.34	0.26
9	Be	0.15	N/D	N/D	N/D	N/D	N/D	N/D	N/D
11	B	46	44	42	49	62	69	75	51
12	C	53000 ppm	55000 ppm	55000 ppm	55000 ppm	54000 ppm	76000 ppm	55000 ppm	57000 ppm
23	Na	1400	2100	1600	1600	1000	12000	540	2100
24	Mg	61	48	23	30	63	120	45	48
27	Al	3.9	1.8	1.8	2.3	2.4	1.9	2.5	1.6
29	Si	1300	1400	1300	1300	1300	2000	1500	1400
31	P	13	9.7	6.2	11	86	200	33	18
34	S	100	190	120	240	250	520	210	250
35	Cl	450 ppm	440 ppm	420 ppm	420 ppm	420 ppm	430 ppm	410 ppm	430 ppm
39	K	150	150	100	130	320	470	460	160
43	Ca	60	49	5.5	10	37	18	26	22
47	Ti	0.51	1.6	0.77	0.51	1.5	1.7	0.99	0.8
51	V	0.8	0.54	0.11	0.6	0.6	6.5	1.2	0.21
52	Cr	26	14	5.1	13	23	5.1	27	5.4
55	Mn	54	32	13	14	27	11	90	32
56	Fe	1200	210	100	630	260	150	1100	76
59	Co	1.1	0.47	0.16	0.18	0.36	0.24	0.4	0.4
60	Ni	13	3.5	1.8	5.7	3.4	2.4	13	2.3
63	Cu	530	380	450	260	39	22	440	380
66	Zn	22	18	9	15	8.9	14	71	25
69	Ga	0.65	0.45	0.27	0.39	0.66	0.52	0.3	0.51
75	As	0.44	0.3	0.21	0.2	0.19	2	0.4	0.42
78	Se	N/D	0.48	N/D	N/D	N/D	N/D	N/D	0.47
79	Br	160	150	140	130	120	150	150	150
85	Rb	0.9	1.4	0.92	1	2.2	8.1	6.1	1.5
88	Sr	1.3	0.84	0.22	0.36	1.4	1.7	0.57	0.78
89	Y	0.046	0.03	0.024	0.0084	0.1	0.034	0.0056	0.035
90	Zr	0.18	0.019	0.12	0.064	0.22	0.093	0.069	0.0097
93	Nb	0.0024	0.0076	0.005	0.0051	0.091	0.014	0.0077	0.005
95	Mo	0.7	0.31	0.37	0.41	0.33	1.5	0.76	0.13
101	Ru	0.01	0.02	N/D	0.021	N/D	N/D	0.01	0.02
105	Pd	0.0072	N/D	N/D	0.0075	N/D	0.025	N/D	N/D
107	Ag	0.017	0.027	0.0034	0.0035	0.014	0.0039	0.021	0.01
111	Cd	N/D	0.047	0.047	0.024	0.024	0.1	0.14	0.071
118	Sn	10	18	20	6	3.7	2.2	0.6	17
121	Sb	0.32	0.3	0.27	0.3	0.39	0.8	0.3	0.21
125	Te	N/D	0.38	0.38	0.19	0.19	N/D	N/D	0.18
127	I	0.46	0.42	0.65	0.41	0.5	0.89	0.45	0.47
133	Cs	0.052	0.15	0.026	0.0092	0.063	0.24	0.045	0.15
137	Ba	4.6	3.2	1.3	2.2	3.8	3.8	1.3	2.4
139	La	0.16	0.07	0.063	0.086	0.28	0.15	0.035	0.087
140	Ce	0.47	0.24	0.17	0.11	0.61	0.24	0.04	0.36
141	Pr	0.042	0.028	0.019	0.02	0.063	0.02	0.0044	0.024
146	Nd	0.21	0.12	0.045	N/D	0.3	0.14	0.022	0.14
147	Sm	0.074	N/D	0.01	0.033	0.098	0.037	0.032	0.032
153	Eu	0.0027	0.0083	0.0055	0.011	0.016	0.0063	0.0056	N/D
157	Gd	0.071	0.04	0.048	0.016	0.11	0.056	0.016	0.024
159	Tb	0.0024	0.005	0.0025	N/D	0.012	0.0029	N/D	0.0037
163	Dy	0.071	0.0096	0.024	0.029	0.083	0.028	0.0049	0.029
165	Ho	0.0045	0.0057	0.0057	0.0011	0.012	0.0013	0.0046	0.0034
166	Er	0.1	0.081	0.081	N/D	0.046	0.0075	0.0032	0.078
169	Tm	0.0059	0.003	0.004	0.001	0.0061	0.0046	0.001	0.001
172	Yb	0.055	0.013	0.0043	0.0089	0.013	0.02	0.0044	0.017
175	Lu	0.0018	0.00096	N/D	N/D	N/D	N/D	0.00097	0.00096
178	Hf	0.0096	0.0032	0.0032	0.0033	0.019	N/D	0.0066	0.0032
181	Ta	0.0038	N/D	0.0019	N/D	0.021	0.0011	0.0019	0.00098
182	W	0.11	0.065	0.11	0.13	0.07	0.31	0.07	0.077
185	Re	0.012	0.0049	0.012	0.0051	0.0025	0.0058	0.0076	N/D
189	Os	0.0049	N/D	0.01	0.0051	0.005	0.011	0.005	0.015
195	Pt	0.021	0.026	0.0044	0.018	0.013	0.0051	0.017	0.017
202	Hg	0.057	0.019	0.029	0.029	0.059	0.022	0.039	0.058
205	Tl	0.1	0.084	00.064	0.04	0.041	0.039	0.038	0.052
208	Pb	1.2	0.95	0.94	2	1.3	0.93	12	10
232	Th	0.02	0.0088	0.012	0.0079	0.009	0.027	0.0056	0.0067
238	U	0.26	0.045	0.044	0.023	0.073	0.094	0.032	0.05

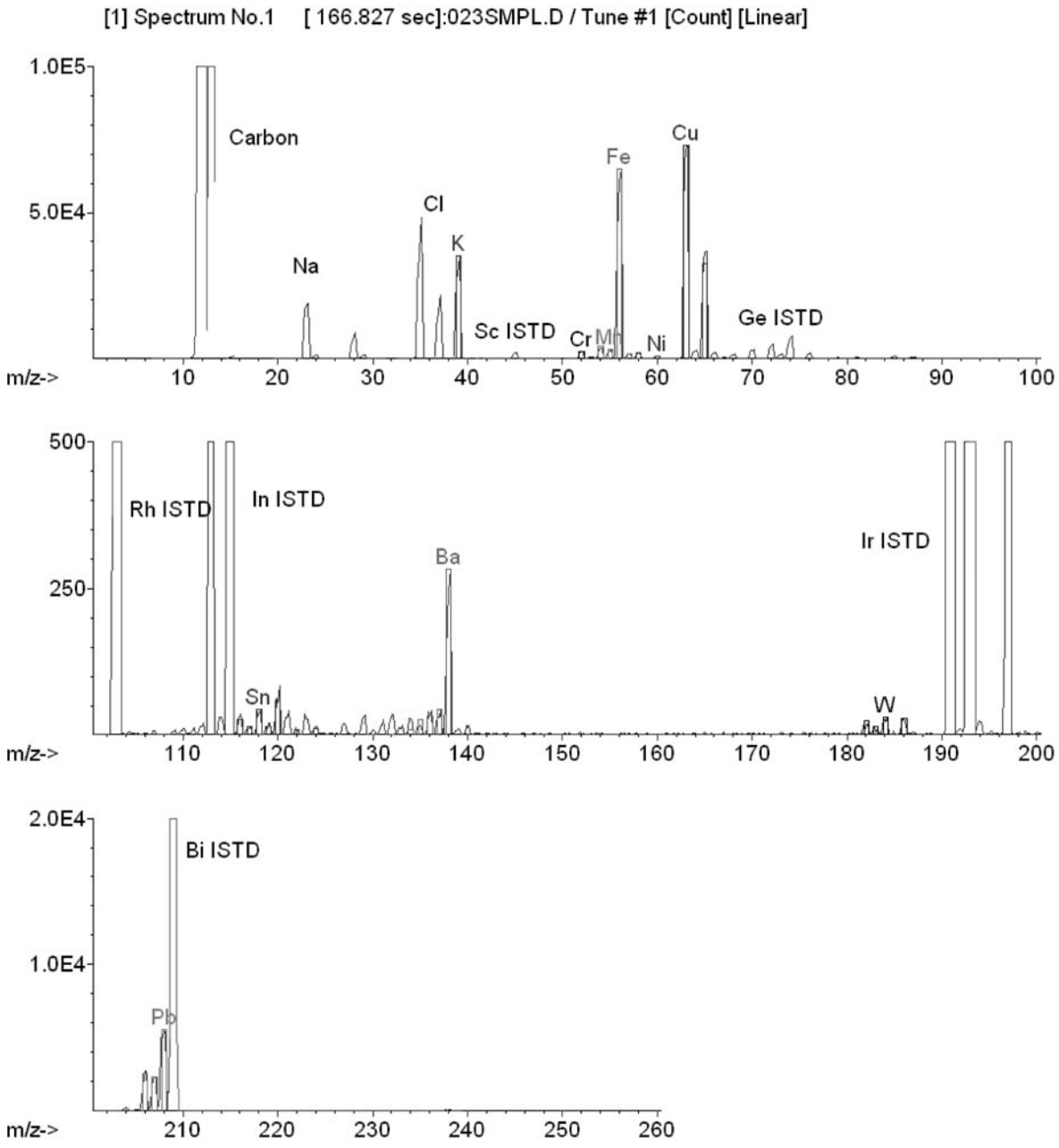


Figure 6a. Full scan (in He mode) of lead-crystal stored bourbon sample. The "major" peaks are indicated, including spectral fit for higher intensity peaks.

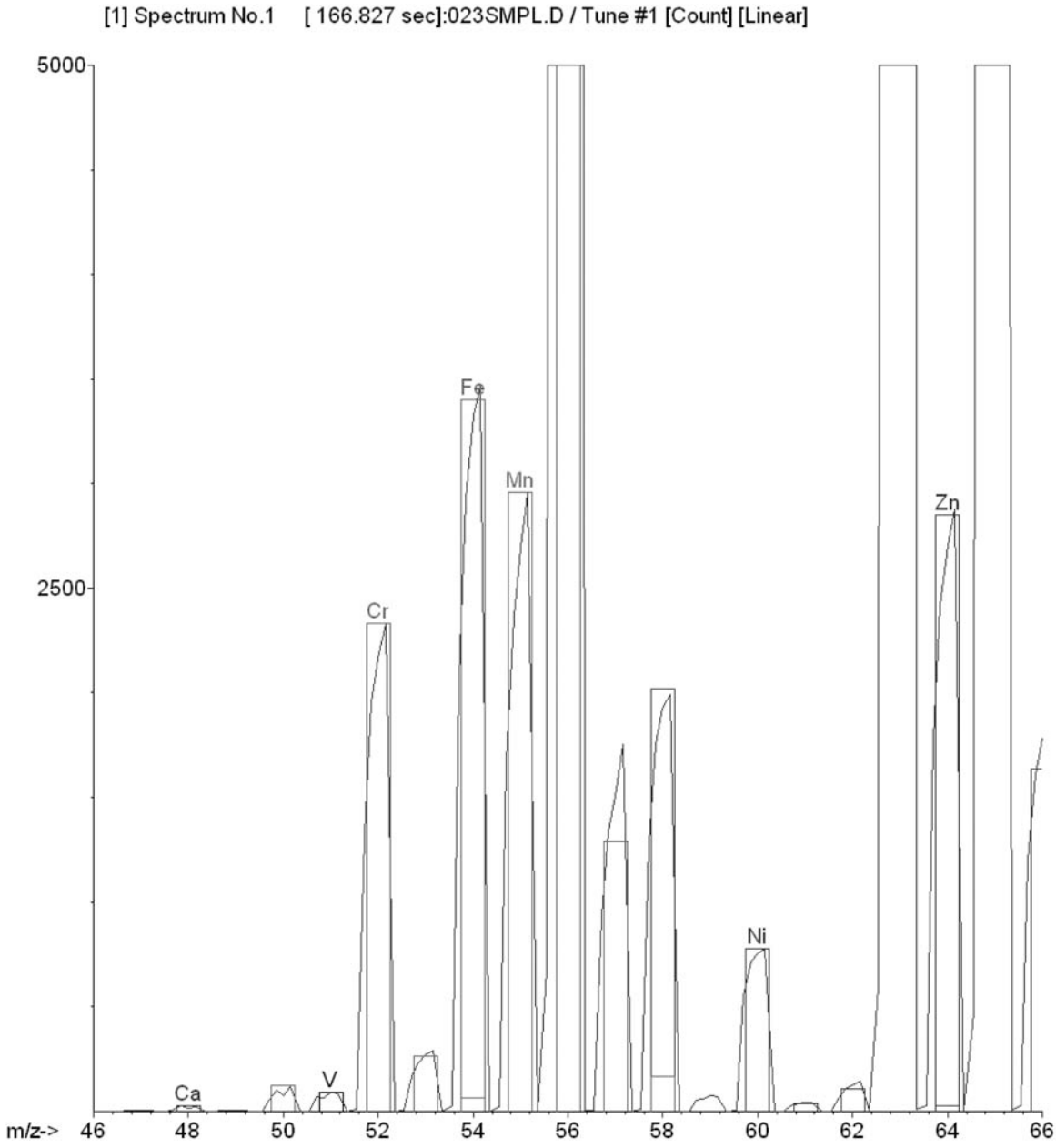


Figure 6b. Zoomed spectrum for those elements suffering from interferences in this matrix. Note good spectral fit, particularly for Cr (suffers from ArO, ArC, and ClO interferences).

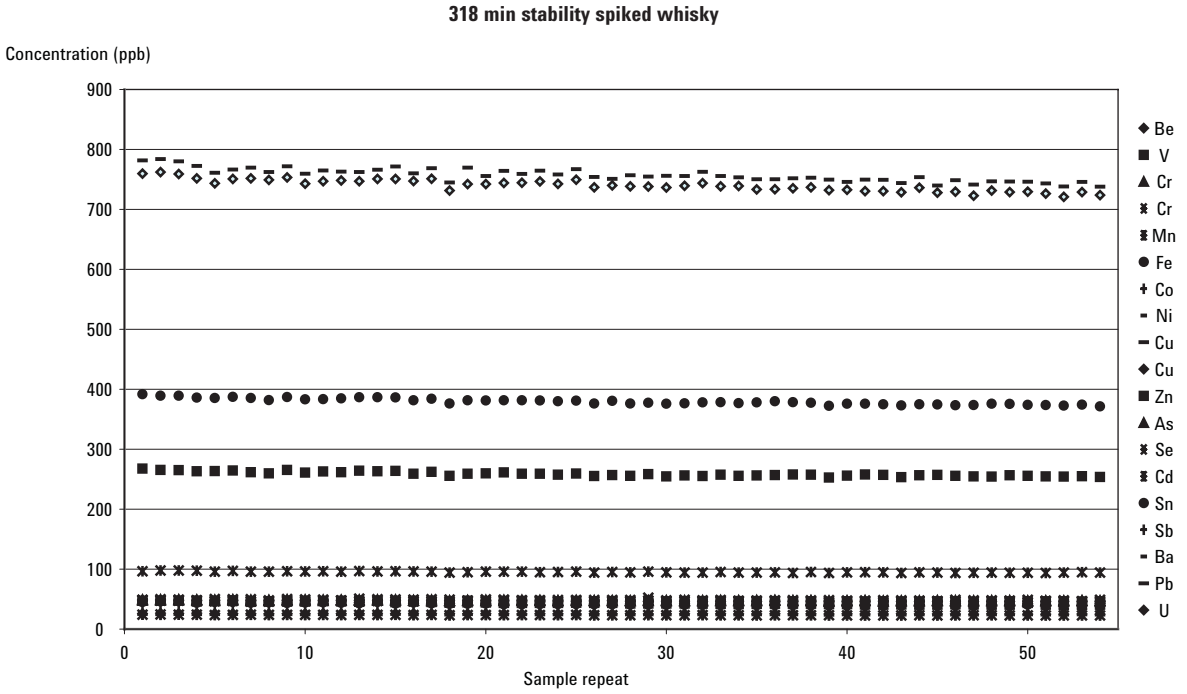


Figure 7. Stability for a spiked Highland malt sample (dilution corrected) taken over 5 hours 18 minutes (54 repeat measurements). Measurement precision was < 2% for almost all elements.

Conclusions

The analysis of high percentage alcoholic beverages using the 7500cx ICP-MS is routine after a simple acidification/dilution. The use of the ORS in the appropriate gas mode efficiently removes the plasma-based and matrix-based interferences, improving detection limits and reliability of the analysis with a simple set of conditions. The use of helium mode also allows interference-free semi-quantitative analysis, permitting greater elemental coverage and rapid screening.

References

1. Achieving Optimum Throughput in ICP-MS Analysis of Environmental Samples with the Agilent 7500ce ICP-MS, Agilent ICP-MS Journal 27, page 4; May 28, 2006, 5989-5132EN

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Speciated Isotope Dilution for the Determination of Methylmercury in Tuna Fish by GC-MS

Application Note

Environmental

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Abstract

A GC-MS with electron impact ionization was used for the development of a speciation method for the determination of methylmercury in fish samples. The method is based on isotope dilution using a spike containing 201Hg-enriched methylmercury. The spike was applied to the determination of methylmercury in tuna samples with excellent results.

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Introduction

Among the various mercury species, methylmercury is the most hazardous because of its accumulative and persistent character in the environment. Sensitive, specific, and precise analytical methods are needed to perform studies at ambient levels. Already the first step in the analysis, the isolation of methylmercury from the sample matrix can be troublesome. Since recovery of the analyte from some matrices is not always quantitative, recovery factors during isolation must be determined. This is usually done by standard addition techniques or recently by using isotope dilution mass spectrometry (IDMS).

Isotope dilution (ID) methodologies provide superior accuracy and precision compared to more common calibration strategies. ID for trace element speciation has been widely applied using ICP-MS and, recently, using GC-MS, a routine technique in testing laboratories.

Experimental

Reagents

Monomethylmercury chloride (96%) was obtained from Aldrich (Steinheim, Germany). Stock solutions were prepared by dissolving the salt in a 3:1 mixture of acetic acid (Merck, Darmstadt, Germany) and methanol (Merck). All standard solutions were kept in the dark at –18 °C and diluted working solutions were prepared by weight daily before the analysis. Acetic acid (Merck) and methanol (Merck) were used for the extraction of the organotin compounds from the solid matrices.

Sodium tetraethyl borate (Galab, Geesthacht, Germany) solutions of 2% (w/v) were prepared daily in 0.1 M sodium hydroxide solution (Merck).

A buffer solution at pH 5.3 was prepared by mixing appropriate volumes of 0.2 M acetic acid (Merck) and 0.2 M sodium acetate (Merck) solutions.

The spike solution (^{201}Hg-enriched monomethylmercury) was obtained from ISC-Science (Oviedo, Spain), diluted by weight with a mixture of methanol and acetic acid (3:1), and stored in the dark at –18 °C. Table 1 shows the isotopic composition as well as the concentration of the butyltin species in the spike solution.

Table 1. Isotopic Composition (Content %) and Concentration of the ^{201}Hg-Enriched Monomethylmercury (Uncertainty Corresponds to 95% Confidence Interval)

Hg-196	**Hg-198**	**Hg-199**	**Hg-200**
<0.01	0.043 (2)	0.109 (5)	0.890 (10)
Hg-201	**Hg-202**	**Hg-204**	
96.495 (29)	2.372 (22)	0.091 (5)	
Concentration: 5.49 ± 0.02 µg g-1 as Hg			

Additional information on www.isc-science.com

Instrumentation

GC/MS: Chromatographic analysis was performed with an Agilent (Agilent Technologies, Santa Clara, CA) gas chromatograph, model 6890N, fitted with a split/splitless injector and an HP-5MS capillary column (cross-linked 5% phenyl methyl siloxane, 30 m × 0.25 mm id × 0.25 µm coating). The gas chromatograph was equipped with an Agilent (Agilent Technologies) mass spectrometric detector, model 5973, network MSD (quadrupole based).

Helium was employed as carrier gas with a constant flow of 1.2 mL min^{-1}. The column temperature was initially held at 60 °C for 1 minute, increased at 30 °C min^{-1} to a final temperature of 300 °C. Injection was performed using a split/splitless injector in splitless mode. The transfer line and ion source temperatures were at 280 and 230 °C, respectively. Electron impact ionization was performed at an electron energy of 70 eV. The measurement of isotope ratios for methylmercury was performed on the molecular ion using 10-ms dwell-time per mass.

The solid-phase microextraction (SPME) device used for manual extraction, a holder assembly and several replaceable divinylbenzene/carboxen/ polydimethylsiloxane (DVB/CAR/PDMS, 50 µm/30 µm) fibers were purchased from Supelco (Madrid, Spain).

Extraction and Derivatization of Methylmercury from Tuna Fish Samples

For extraction, approximately 0.4 g of sample was spiked with a solution of the ^{201}Hg-enriched methylmercury and mixed with 15 mL of a saturated sodium chloride solution and 100 µL of concentrated hydrochloric acid. The mixture was shaken mechanically at room temperature for 5 hours.

Three milliliters of extract was adjusted to pH 5.3 with 3 mL of acetic acid/sodium acetate buffer in SPME glass vials; 1 mL of sodium tetraethyl borate was added and the vial

was then immediately closed with a PTFE-coated silicon rubber septum. The SPME needle pierced the septum and the fiber was exposed to the solution headspace for 15 minutes at room temperature. The solution was intensively stirred with a PTFE-coated magnetic stirring bar with constant velocity. Finally, the fiber was withdrawn into the needle and transferred to the GC injector for thermal desorption for 1 minute at 260 °C. During headspace solid-phase microextraction (HS-SPME), the temperature was controlled by immersing the sample vials in a water bath.

Results and Discussion

Isotope Ratio Measurements by GC-MS

While elemental isotope ratios can be easily obtained with ICP-MS, in GC/MS the isotopic pattern in molecular ions is different from that of the naturally occurring elements due to the contributions from the organic groups attached to the metal because of the presence of ^{13}C. The contribution of ^{13}C to the observed *m+1* ions can be calculated in a fairly straightforward way, by applying equation 1:

$$I_{m+1} = I_m \bullet n x_{13C} \quad (1)$$

where x_{13C} is the relative abundance of ^{13}C with respect to ^{12}C (0.0111/0.9899), n is the number of C atoms in the molecular ion, and I is the intensities of the ions *m* and *m+1*, respectively. The measured signal intensities were corrected by monitoring five molecular clusters, corresponding to the ^{198}Hg, ^{199}Hg, ^{200}Hg, ^{201}Hg, and ^{202}Hg isotopes, taking into account the ^{13}C contributions to *m+1*. The intensity (I) correction equations used were:

$$^{198}Hg = {}^{198}I \quad (2)$$
$$^{199}Hg = {}^{199}I - x(^{198}Hg) \quad (3)$$
$$^{200}Hg = {}^{200}I - x(^{199}Hg) \quad (4)$$
$$^{201}Hg = {}^{201}I - x(^{200}Hg) \quad (5)$$
$$^{202}Hg = {}^{202}I - x(^{201}Hg) \quad (6)$$

where x is the contribution factor *m+1*. The selected molecular clusters for the measurement of methylmercury by GC-MS and the contribution factor x are given in Table 2.

Table 2. Monitored Masses and Contribution Factors for Methylmercury

Corresponding Hg isotopes	*m/z* selected for SIM mode ($MeEtHg^+$)
198	242
199	243
200	244
201	245
202	246

X (*m* + *1*) = 0.034

Analysis of Reference Materials

Methylmercury was determined in the reference material BCR 464 (tuna fish) by the proposed ID procedure. Three independent spiking experiments were made on each certified reference material and each sample was injected three times in GC-MS systems. The overall results obtained for the reference material by GC-MS are given in Table 3.

Table 3. Determination of Methylmercury in BCR 464 Using the 202/201 Isotope Ratio for Quantitation (Data in µg g^{-1} as Hg)

Replicate	Methylmercury
1	5.09 ± 0.06
2	5.02 ± 0.09
3	5.04 ± 0.05
Average	**5.05 ± 0.04**
RSD (%)	0.71
Certified value	***5.12 ± 0.16***

The concentration values obtained for methylmercury in the certified reference material BCR 464 show an excellent agreement between the certified and found values.

Conclusions

A precise and accurate method for the determination of methylmercury in fish samples has been developed. A single injection allows the concentration of methylmercury in the samples to be computed quickly, without the need for time-consuming calibration, standard addition, or recovery correction procedures. The method corrects for all possible errors in the speciation of methylmercury, provides low detection limits, and is fast and simple to apply by untrained personnel.

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Veterinary Drugs

With increased regulations and growing consumer awareness, the need to quickly identify antibiotics, steroids, and other growth hormones present in meat and poultry has never been greater. Agilent has significant experience analyzing for steroids and other drugs in physiological matrices a result of historical involvement with race horse and other sports doping control. Agilent's experience helps you meet the strict surveillance and monitoring guidelines for antibiotics and growth-enhancing drugs.

> Return to Table of Contents
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Transfer and Optimization of Existing Methods for Analysis of Antibiotics in Meat to Agilent Poroshell 120 EC-C18 Columns using MS/MS Detection

Application Note

Food

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Abstract

In this work, a generic gradient method with UV detection is used to evaluate mobile phase choices for fast method optimization of antibiotics analysis in meat, with the ultimate goal of producing a mass spectrometer compatible method. This evaluation included four buffers and two organic choices. The mobile phase combination that yielded the best separation is transferred and optimized to an Agilent Poroshell 120 EC-C18 2.1 mm × 100 mm, 2.7 µm column. Gradient time was decreased from 45 min to 12 min. Time can be further reduced using a 3 mm × 50 mm column, at the cost of some resolution. The method is demonstrated on an Agilent 6410 triple quadrupole LC/MS System coupled with an Agilent 1200 Series Rapid Resolution LC.

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Table 1. Method Parameters for Various Column Dimensions

	4.6 × 50 mm Poroshell 120 EC-C18	3.0 × 50 mM Poroshell 120 EC-C18	2.1 × 100 mM Poroshell 120 EC-C18
Mobile Phase	A: Buffer, varies B: Organic, varies	A: 10 mm ammonium formate pH 3.8 B: Acetonitrile	A: 10 mm ammonium formate pH 3.8 B: Acetonitrile
Gradient	10-40% B	10-40% B	10-40% B
Gradient Time	12 min	12 min	12 min
Flow Rate	2 mL/min	0.85 mL/min	0.42 mL/min
Injection Volume	0.5 µL	5 µL	2.5 µL or 10 µL
Sample	0.1 mg/mL antibiotics	1 µg/mL antibiotics	1 µg/mL or 10 ng/mL antibiotics
TCC Temperature	30 °C	30 °C	30 °C
Detector	DAD: Sig = 270, 4 nm; Ref = 360, 100 nm	MS/MS: See Table 2	MS/MS: See Table 2

Introduction

Administration of antibiotics is a common practice in chicken, pork, beef and fish farming. Many domestic cattle receive various antibiotics in their feed for the prevention and control of disease caused by fungi and bacteria. Many countries regulate acceptable residue levels of these compounds in agricultural and animal products. In this work, an older method is transferred from a 5 µm, 250 mm column to a new superficially porous column to increase the speed of the analysis and change the method of detection from UV to MS/MS. An increase in throughput of 5 to 10 times is demonstrated, while minimally impacting sample preparation. Since the analysis time is shortened dramatically, time is available for optimization of mobile phase selectivity (pH, buffer types and organic modifier).

Transition methods can be developed by modifying an existing method or starting fresh. In this case, the objective was to develop a new MS-compatible separation from an existing UV separation. Consequently, a change in the mobile phase was required because 0.7 % phosphoric acid is not a desirable solvent for MS detection. A generic screening method using 0.1 % formic acid was investigated, but additional MS-compatible solvent systems were also evaluated. In this work a method is developed by first screening different mobile phase combinations using a short Agilent Poroshell 120 column using UV detection, then transferring that method to an Agilent 6410 triple quadrupole LC/MS System. A major advantage of the Agilent Poroshell 120 EC-C18 is that it uses the same 2 µm frit as the original 5 um column, negating the need for sample preparation method development.

Agilent Poroshell 120 EC-C18 4.6 mm × 50 mm, 2.7 µm columns have similar performance to 1.8-µm totally porous Agilent ZORBAX Eclipse Plus C18 columns, but since they use 2-µm column frits similar to those found on 5-µm columns, they require no additional sample preparation. This allows for a more seamless method transfer. While some previous work demonstrates the use of Agilent Poroshell 120 columns on older Agilent 1100 systems, they are ideally used on more modern systems such as the Agilent 1200 or 1260 series UHPLC's.

Table 2. MRM Transitions for Antibiotic Compounds.

Compound name	Precursor ion	Product ion	Fragmentor voltage	Collision energy
Sulfamerazine	265	172	100	25
Sulfamerazine	265	108	100	25
Thiamphenicol	338	308	140	10
Thiamphenicol	338	118	140	50
Sulfamethazine	279	124	100	25
Sulfamethazine	279	108	100	30
Furazolidone	226	137	140	25
Furazolidone	226	122	140	25
Sulfamonomethoxine	281	126	100	25
Sulfamonomethoxine	281	108	100	25
Oxolinic acid	262	160	100	40
Oxolinic acid	262	130	100	45
Pyrimethamine	249	198	140	45
Pyrimethamine	249	128	140	60
Sulfadimethoxine	311	156	140	25
Sulfadimethoxine	311	108	140	55
Sulfaquinoxaline	301	129	100	50
Sulfaquinoxaline	301	108	100	40
Difurazone	361	222	100	15
Difurazone	361	154	100	45

Experimental

Method development is based upon the use of a generic gradient. Using a short 4.6 mm × 50 mm Poroshell 120 EC-C18, 2.7 µm column, several different mobile phases can be quickly evaluated. The generic gradient is run at 2.0 mL/min, starts at 10% and proceeds to 40% organic over 12 min. This gradient is later transferred to 2.1 mm × 100 mm and 3 mm × 50 mm columns by changing the gradient according to Equation 1. The three gradients used are listed in Table 1 with MRM transitions shown in Table 2. MS-compatible mobile phases consisting of volatile buffer components such as formic acid, ammonium formate buffer and ammonium acetate buffer are used.

An Agilent 1200 Rapid Resolution LC (RRLC) system was used for this work:

- G1312B Binary Pump SL.
- G1367C Automatic Liquid Sampler (ALS) SL.
- G1316C Agilent 1290 Infinity Thermostatted Column Compartment (TCC) SL.
- G1315C Agilent Diode Array Detector (DAD) SL using a G1315-60024 micro flow cell (3-mm path, 2-µL volume).
- G6410 Agilent Triple Quadrupole LC/MS System with Electrospray (ESI).
- ChemStation version B.04.01 was used to control the HPLC and process the data. Agilent MassHunter Version 2.0 was also used to control the Agilent 6410 Triple Quadrupole LC/MS System, the Agilent 1200 Rapid Resolution LC (RRLC), and to analyze the data.

Three Agilent Poroshell 120 EC-C18 columns were used in this work:

- 4.6 mm × 50 mm, 2.7 µm p/n 699975-902
- 3.0 mm × 50 mm, 2.7 µm p/n 699975-302
- 2.1 mm × 100 mm, 2.7 µm p/n 695775-902

The compounds of interest are shown in Figure 1, with their respective structures. Compounds were dissolved in water at 1 mg/mL. Equal aliquots were combined to produce a mixed sample. Compounds were purchased from Sigma Aldrich (Bellefonte, PA). Additionally, methanol, acetonitrile, ammonium formate, ammonium acetate, formic acid, and glacial acetic acid were purchased from Sigma Aldrich. Water used was 18 M-Ω Milli-Q water (Bedford, MA).

Buffers used in this work were prepared by dissolving an appropriate amount of the ammonium salt to produce a 10 mM solution, adding 950 mL water and titrating the solution with either formic acid (for the ammonium formate buffers) or glacial acetic acid (for the ammonium acetate buffers). The buffer solutions were then brought to a 1 L volume.

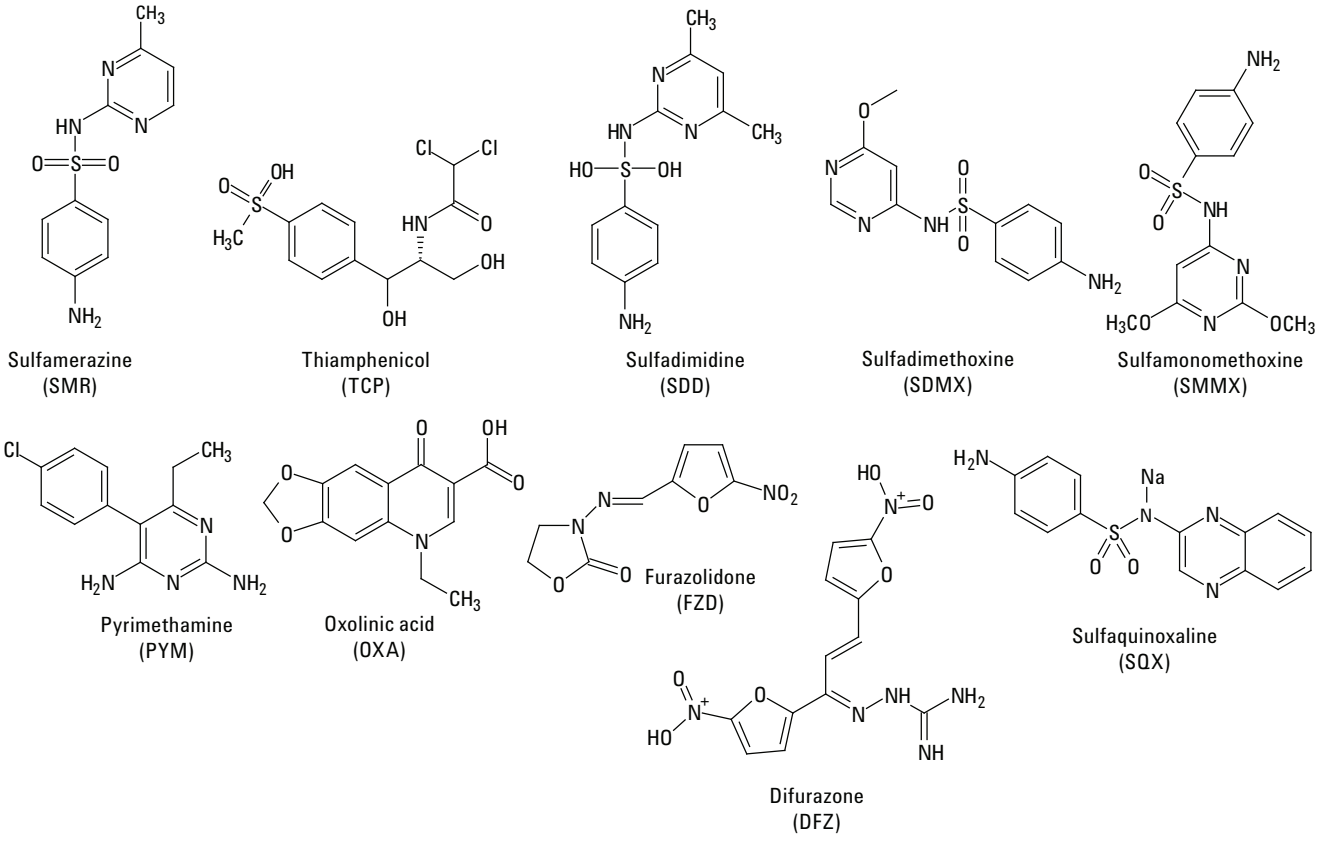


Figure 1. Compounds of interest.

Results and Discussion

The original method published in 2002 by Kumagai and Onigbinde provides an effective method for the analysis of antibiotics in meat using UV detection. As seen in Figure 2, the method separates the analytes in approximately 45 min. However the nonvolatile phosphoric acid in the mobile phase is not compatible with MS detection.

In many cases, simple scaling of a method will allow for a fast method transfer. In this case, however, a change in the mobile phase was required for LC/MS compatibility. The use of short Poroshell 120 EC-18 4.6 mm × 50 mm, 2.7 µm columns for assessing mobile phase changes has several advantages. One advantage is that they allow quick separations without sacrificing resolving power. In addition, since they are used at 2 mL/min with a generic gradient, the solvent is rapidly purged through the system. This ensures that the solvent screening experiment can be quickly performed by changing solvent bottles, with no concerns about residual solvents in the HPLC pump or the degasser. These columns can be used for LC/MS but typically smaller diameter columns such as 3.0 or 2.1 mm columns are used.

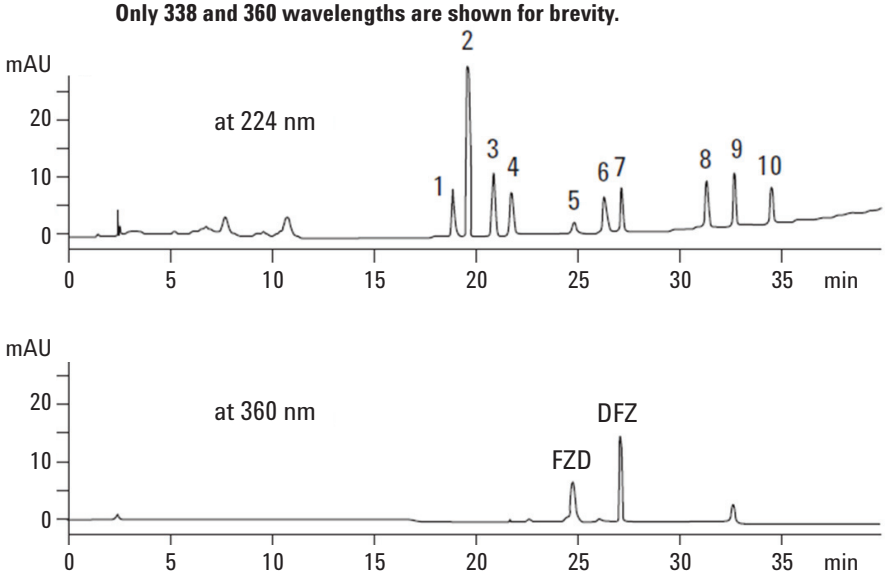
As discussed in reference 5, once a separation has been optimized according to selectivity and retention index, it is possible to further improve the chromatography by varying column length, particle size and flow rate. However the k* value must be maintained, while varying these column conditions so as not to lose selectivity.

Equation 1: $k^* = (t_g F)/(d/2)^2 L(\Delta\%B)$

Where:

t_g is the gradient time
F is the flow rate
L is the column length
d is the column internal diameter
Δ%B is the change in organic content across the gradient segment

Original Method Kumagai and Onigbinde 5988-7135 June 2002
Only 338 and 360 wavelengths are shown for brevity.



1	SMR	6	SMMX
2	PYM	7	DFZ
3	TCP	8	SDMX
4	SDD	9	SQX
5	FZD	10	OXA

Instrument:	Agilent 1100 Series HPLC
Column:	250 mm × 4 mm id, RP-18 Purospher, 5 µm, p/n 79925PU-584
Mobile phase:	A = 0.7% Phosphoric acid, B = CH_3CN
Gradient:	0.0 min 5% B; 10.0 min 5% B; 40.0 min 65% B; 45.0 min 65% B; Post Time 7.0 min 5% B
Flow rate:	1.0 mL/min
Temperature:	40 °C
Injection volume:	20 µL
Diode array detector:	A-338/10 nm, reference wavelength off B-264/8 nm, reference wavelength off C-360/8 nm, reference wavelength off

Figure 2. Original method produces excellent results on a 250 mm column with UV detection.

As illustrated in Figure 3, generic gradients using methanol or acetonitrile are used to separate the compounds of interest. The gradients using methanol generate 50% higher pressure (300 bar instead of 200 bar). While this is not critical when using a 50 mm column, this does become more important as the length of the column is increased to 100 or 150 mm.

With methanol, the last compound elutes later due to the lower solvent strength. Formic acid, while a convenient mobile phase additive, produces less optimal results than 10 mM ammonium formate buffer (pH =3), particularly for pyrimethamine. In addition to peak shape improvements, elution order changes also occur most notably with pyrimethamine.

Many selectivity improvements and changes can be produced by choice of pH or organic modifier. As noted earlier, the peak shape of many basic compounds are improved when using methanol, however Poroshell 120 EC-C18 yields excellent peak shape for all compounds in this study. By adjusting the pH even slightly, both the elution order and peak spacing can be changed. This is most evident in Figure 3, where methanol and pH act to dramatically change the elution order. For the compounds in this study the best mobile phase combination is found at pH 3.8, ammonium formate with acetonitrile.

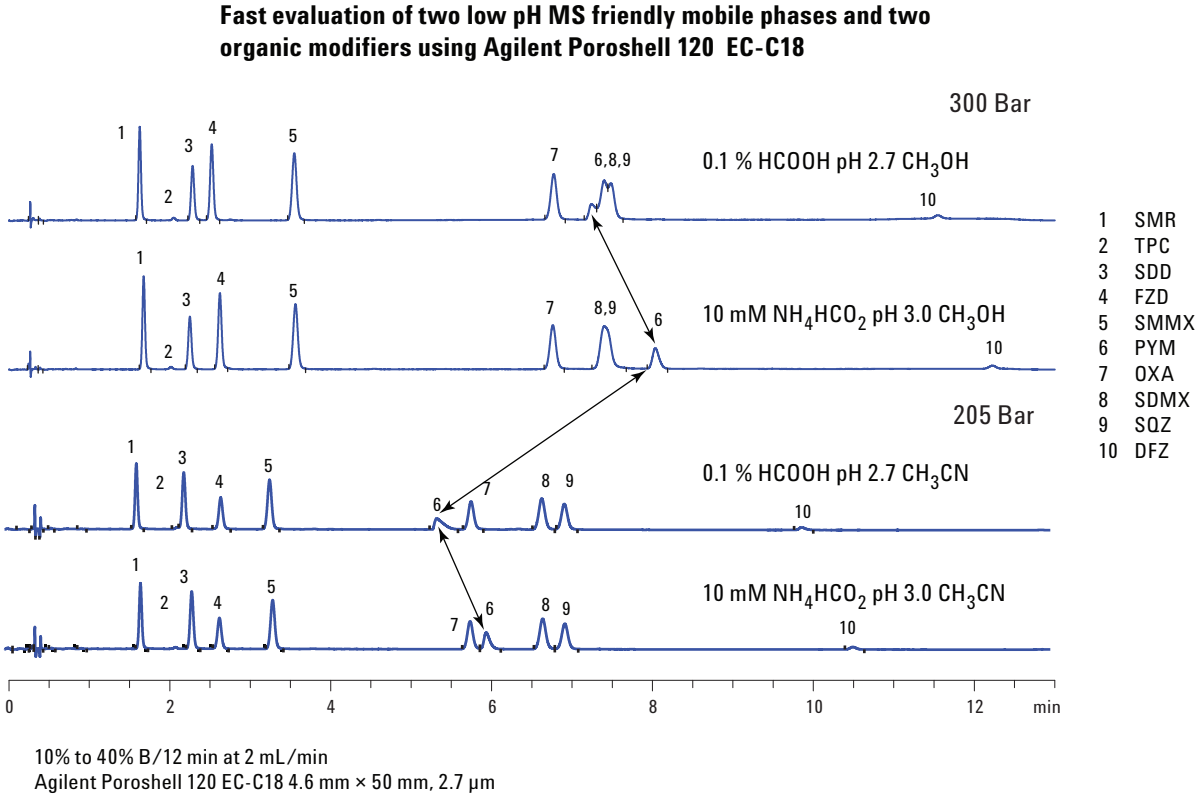


Figure 3. Comparison of chromatographic conditions: buffer, 0.1 % formic acid, CH_3OH, CH_3CN.

Acetonitrile with ammonium formate buffer yields excellent peak shape and selectivity with pH 3.8 being optimal for these analytes

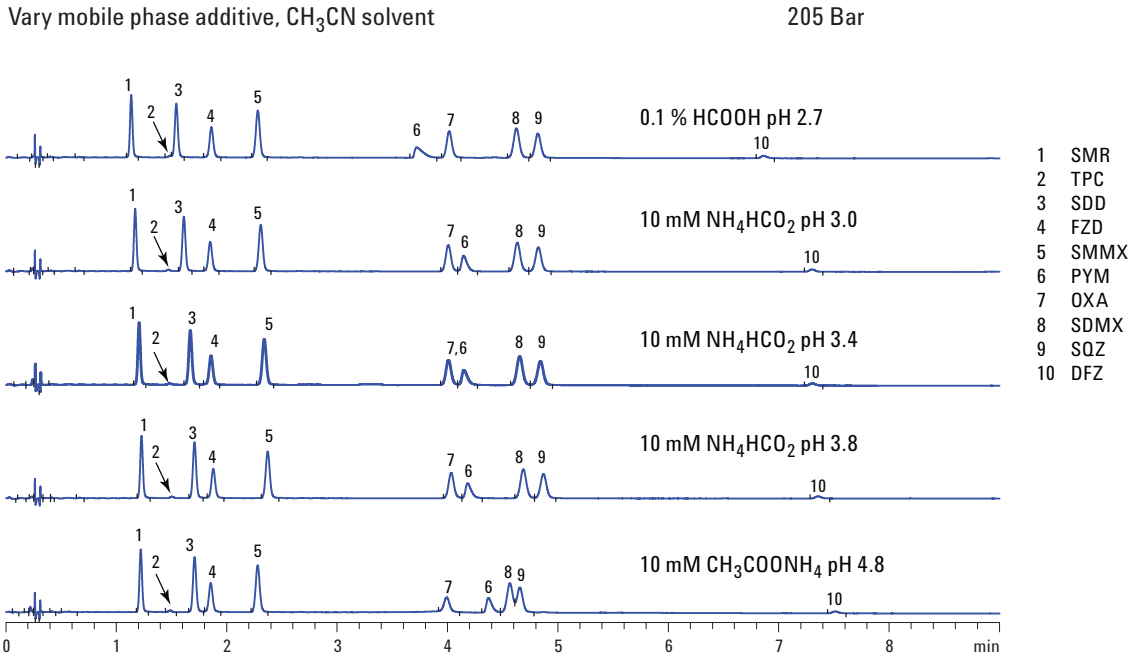
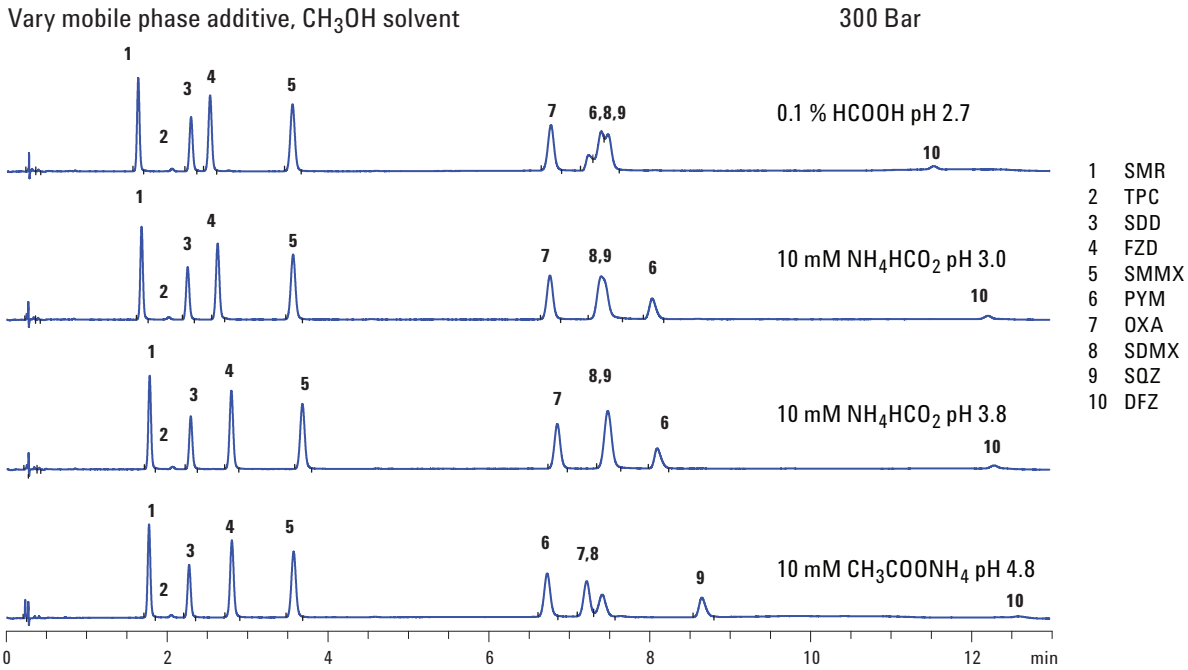


Figure 4. Comparison of buffers with CH_3CN.

Methanol with ammonium acetate buffer yields excellent peak shape with pH 4.8 being optimal for these analytes



10% to 40% B/12 min at 2 mL/min
Agilent Poroshell 120 EC-C18 4.6 mm × 50 mm, 2.7 µm

Figure 5. Comparison of buffers with CH_3OH.

Figure 6 illustrates a total ion chromatogram based on the scouting work shown in Figures 3, 4 and 5. Conditions were scaled according to Equation 1 for the 3.0 mm × 50 mm column. This easy change demonstrates that the 3 mm column can be easily used for both conventional UV and more sensitive MS. In addition, a 2.1 mm × 100 mm column is also used with the same gradient with only the flow rate changed. If the gradient had been exactly scaled, the analysis time would have been twice as long, but as illustrated, the resolution is adequate. Figure 7 shows an MRM chromatogram of the antibiotic mixture. The compounds are sufficiently separated even with a large sample volume injected on-column. Conditions are listed in Tables 1 and 2.

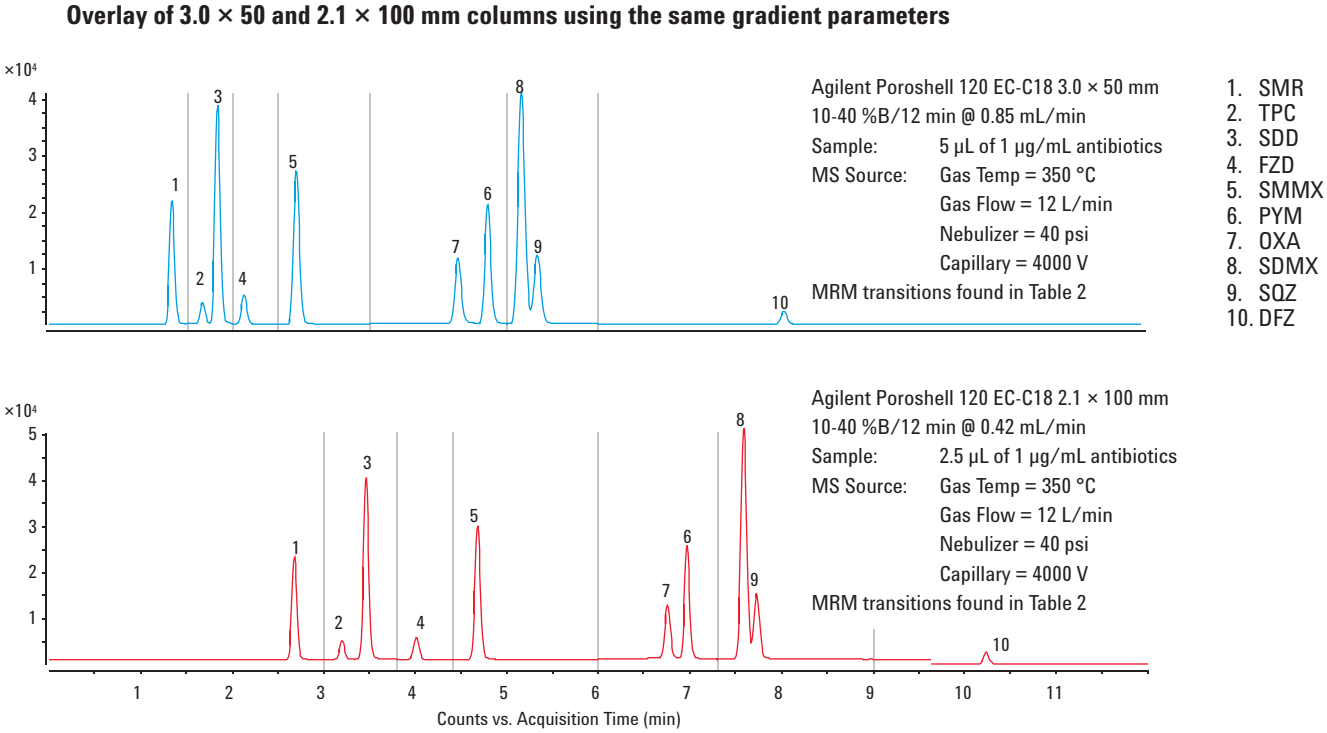
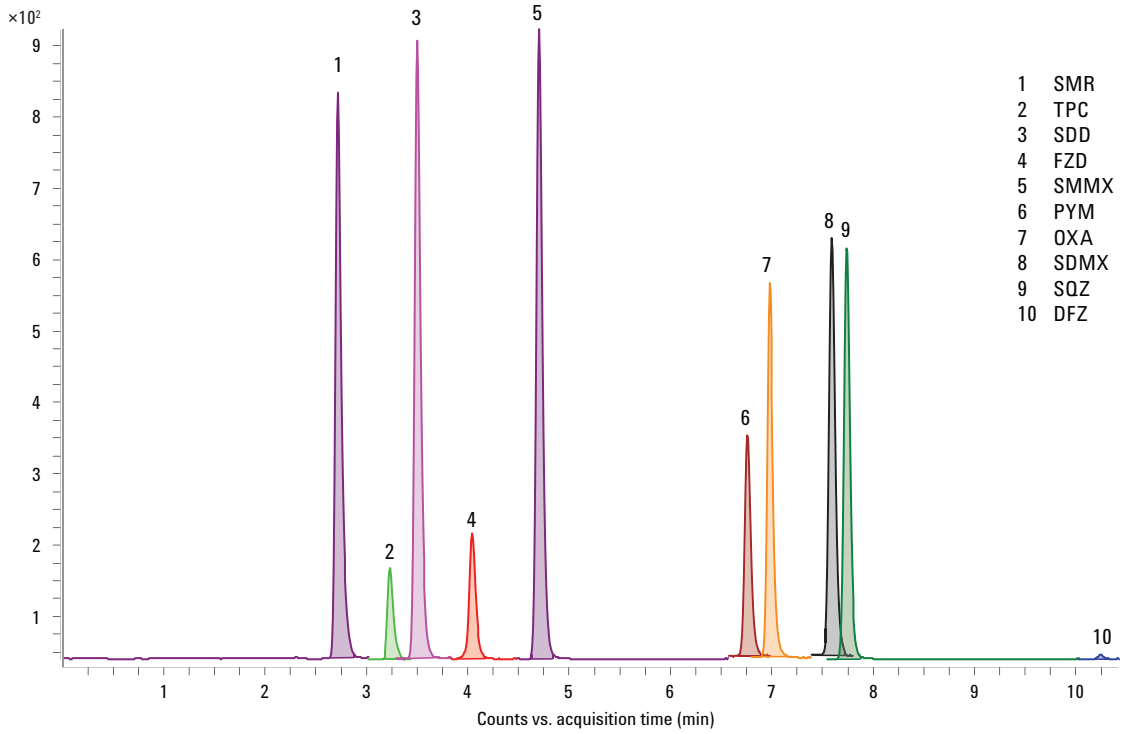


Figure 6. Total ion chromatograms of antibiotic mixture on 3 × 50 mm, and 2.1 × 100 mm Agilent Poroshell 120 EC-C18 columns.

Optimized MRM of 10 antibiotics in less than 11 minutes on Agilent Poroshell 120 EC-C18



Agilent Poroshell 120 EC-C18 2.1 mm × 100 mm, 2.7 µm
10% CH_3CN at t_0, ramp to 40% CH_3CN in 12 min (buffer 10 mM NH_4HCO_2 pH 3.8 adjusted with concentrated formic acid), 0.42 mL/min
Sample: 10 uL of 10 ng/mL antibiotics
using dynamic MRM mode on MS/MS

Figure 7. Dynamic MRM of antibiotic mixture on Agilent Poroshell 120.

Conclusions

This work shows that in method migration, modern colums and fast liquid chromatographs make it easier to start fresh. Using a generic gradient on short columns, 10 mobile phase combinations are quickly evaluated. Following basic scaling equations, a method can easily be transferred to a column of another dimension. By optimizing the mobile phase using a UV detector, the method is partially developed on an instrument that may be commonly used in a lab rather than the more expensive and possibly less available instrument that the method will be transferred to.

Poroshell 120 columns are good to use for LC/MS of complex samples at low pressure. Regardless of the analytical power of the triple quadrupole mass spectrometer, a better separation simplifies data analysis, which may shorten cycle time. Baseline separated compounds also allow the mass spectrometer to maximize dwell time for a given peak to yield more accurate and reproducible results. This ensures the best possible quantitation. Additionally, less chance of ion suppression is possible caused by coeluting compounds.

Several additional factors are also demonstrated. Optimal conditions for this mixture are found using the fast scouting method in acetonitrile ammonium formate buffer pH 3.8 (8 min). The analysis also works in methanol with pH 4.8, ammonium acetate (13 min). This could easily be shortened by changing the gradient to elute the last peak more quickly. For example, ramp organic more quickly at the end with a second step; however this would increase pressure further. The use of a "true buffer" such as 10 mM ammonium formate provides better peak shape for bases than a buffering solution such as 0.1 % formic acid at similar pH. The method as shown is chromatographically optimized and work is in progress to optimize detection conditions.

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Determination of Sulfonamide Residues in Chicken Muscle by Agilent SampliQ QuEChERS AOAC Kit and HPLC-FLD

Application Note

Food

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Abstract

An HPLC-Florescence detection (FLD) method has been developed and validated for the determination of nine sulfonamides in chicken muscle, after precolumn derivatization with fluorescamine. The analyzed drugs include sulfadiazine, sulfathiazole, sulfamerazine, sulfamethazine, sulfamethizole, sulfamethoxypyridazine, sulfachloropyridazine, sulfamethoxazole and sulfadimethoxine with sulfapyridine as an internal standard. The derivatives were separated on an Agilent ZORBAX Eclipse Plus C18 column (4.6 mm × 75 mm, 3.5 µm) using a gradient elution with a binary system of methanol–0.05 M acetate buffer (pH 4.5) and fluorescence detection at excitation and emission wavelengths of 406 and 496 nm, respectively. The method employs a multiresidue sample preparation procedure based on QuEChERS (quick, easy, cheap, effective, rugged and safe) which was adopted from the Association of Analytical Communities (AOAC) Official method 2007.01 for extraction and cleanup. The recoveries ranged from 76.8% to 95.2% with relative standard deviation from 1.5% to 4.7% at the 50, 100 and 150 ng/g fortification levels. The limits of detection and quantification ranged from 0.02 to 0.39 and 0.25 to 1.30 ng/g respectively.

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Introduction

Sulfonamides are a broad spectrum of antimicrobial drugs used mainly in veterinary practice for prophylactic, therapeutic or growth promoting purposes [1]. They are the treatment of choice for disease control of coccidiosis in poultry management [2-3]. Their use in human therapy has since become limited due to the advent of antibiotics [4]. There is a health risk associated with consumption of animal products contaminated with sulfonamide residues. The residues are usually the result of inappropriate administration or withdrawal from these drugs. The presence of sulfonamide residues can trigger adverse side effects such as allergic reactions in hypersensitive individuals and are potential carcinogens in the long term. Furthermore, prolonged exposure to sulfonamide residues may give rise to an increase in drug-resistant bacteria [5]. In order to protect consumers from risks related to the drug residues, maximum residue limits (MRL) have been established by law in many countries. In Europe (EU Regulation 1999), Canada and USA (FDA Regulation 1991) the MRL for the total sulfonamide concentration in edible tissue is 100 µg/kg while it is 20 µg/kg in Japan [6-7].

The AOAC QuEChERS method has been widely applied in the analysis of pesticides in food since it was introduced by USDA scientists [8]. In general, there are two major steps: extraction and dispersive SPE cleanup. The method uses a single-step buffered acetonitrile (1% HOAc) extraction while simultaneously salting out from the aqueous sample using anhydrous magnesium sulphate ($MgSO_4$) to induce liquid-liquid partitioning. For cleanup, a dispersive solid phase extraction (dSPE) step is employed using a combination of primary secondary amine (PSA) to remove fatty acids and other components, and anhydrous $MgSO_4$ to reduce the remaining water in the extract. Other sorbents may be added in this step, such as graphitized carbon black (GCB), to remove pigments and sterol, or C_{18} to remove more lipids and waxes.

This application note presents a method for the determination of sulfonamide drugs in chicken muscle with HPLC-FLD after a precolumn derivatization with fluorescamine, which is a fluorogenic reagent specific for primary aliphatic and aromatic amines [9–10] such as the sulfonamides in the study (Figure 1). The method includes sample preparation with SampliQ AOAC Buffered Extraction kit (p/n 5982-5755) and SampliQ AOAC Fatty Dispersive SPE 15 ml kit (p/n 5982-5158).

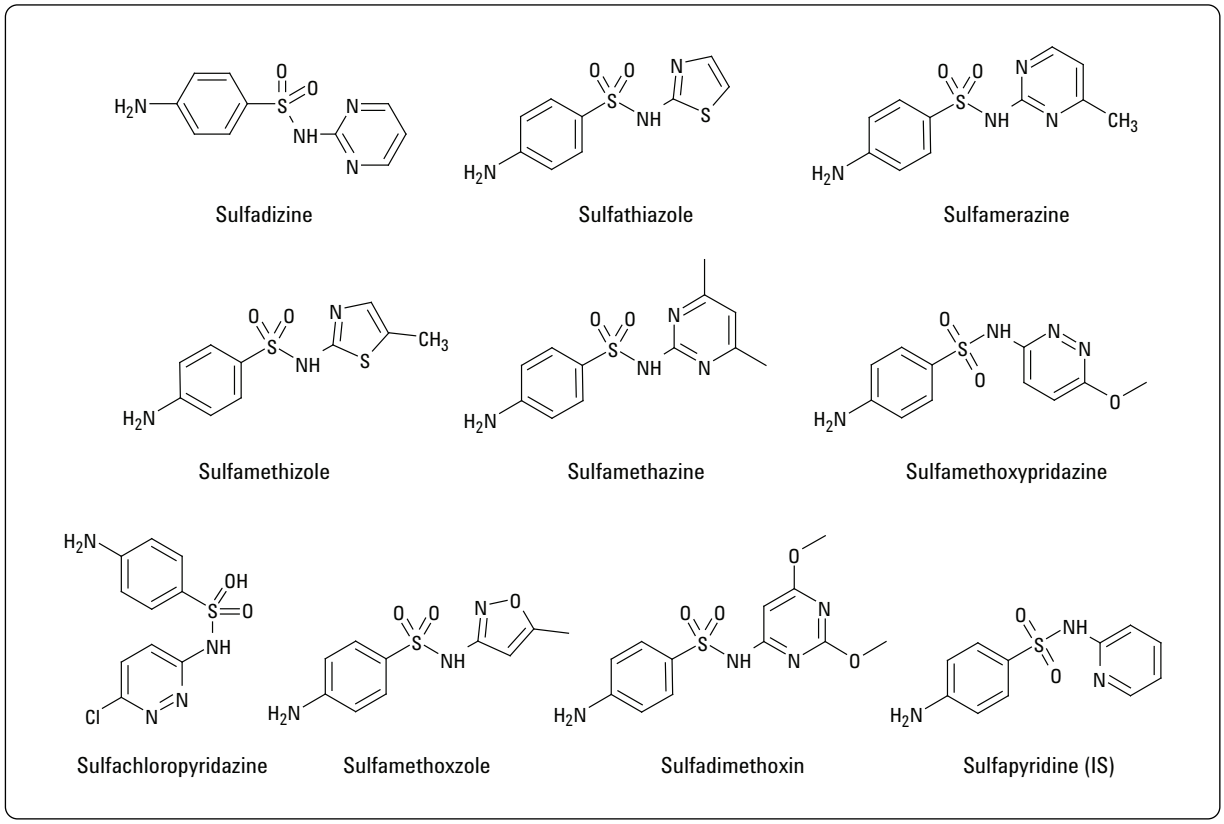


Figure 1. Chemical structures for the sulfonamide drugs used in the study.

Experimental

Reagents and Chemicals

All reagents were analytical or HPLC grade. Methanol (MeOH) was purchased from Merck KGaA (Darmstadt, Germany) while acetonitrile (ACN), acetone and glacial acetic acid (HOAc) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Sodium acetate (NaOAc) was from Saarchem Analytical (Krugersdorp, South Africa). Fluorescamine (98%) and sulfonamide drugs including the internal standard were purchased from Sigma-Aldrich (St. Louis, MO, USA). The water used was from a MilliQ system (Milford, Mass, USA).

Solutions and Standards

A stock solution of 0.05 M sodium acetate was prepared by dissolving 4.1 g NaOAc in 1.0 L of ultrapure water and filtered through a Whatman membrane filter (47 mm diameter and 2 µm pore size). The pH was adjusted using HOAc. Fluorescamine reagent (0.02%) was prepared by dissolving 20 mg Fluram in 10 mL of acetone. The solution was stored at 4 °C. A 1% HOAc in ACN solution was prepared by diluting 10 mL HOAc to 1.0 L with ACN.

Standard and internal standard primary stock solutions (1 mg/mL) were prepared in ACN and stored at –20 °C. From the primary stock solution, 10 µg/mL standard mixtures also in ACN were prepared for the calibration curves. All working solutions were prepared daily by serial dilution in 0.05 M NaOAc (pH 3.5). All the solution vials were wrapped with aluminium foil because some of the sulfonamide drugs are light-sensitive.

Equipment and Material

The analysis was performed on an Agilent 1200 Series HPLC (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with a binary pump and a fluorescence detector (FLD) set at λ_{ex} = 405 nm and λ_{em} = 495 nm. Separation of the compounds was achieved on an Agilent ZORBAX Eclipse Plus C18 column (4.6 mm × 75 mm, 3.5 µm, p/n 959933-902). The data was processed by HPLC 2D Chemstation software.

Extraction and cleanup were carried out with an Agilent SampliQ Buffered QuEChERS AOAC Extraction kit, p/n 5982-5755 and an Agilent SampliQ QuEChERS AOAC Dispersive SPE kit, p/n 5982-5158, (Agilent Technologies).

A Jenway 3510 pH meter (Jenway, London, UK) monitored the pH of the solutions, and a Kenwood grinder (Kenwood, Grahamstown, South Africa) homogenized the chicken sample.

Instrument conditions

HPLC conditions

Table 1. HPLC Conditions Used for Separation and Analysis

Column:	Agilent ZORBAX Eclipse Plus C18 4.6 × 75 mm, 3.5 µm	
Flow rate:	1 mL/min	
Column temperature:	25 °C	
Injection volume:	5 µL	
Mobile phase:	A = 0.05 M Sodium Acetate pH 4.5 B = MeOH	
Gradient:		
	T (min)	% B
	0	35
	35	41
	50	55
Detection:	Ex = 405 nm	Em = 495 nm

Sample preparation

The chicken muscle was purchased from a local food store, minced and deep frozen until analysis.

Extraction

Figure 2 outlines the methodology used in the QuEChERS experiments. A 2-g portion of chicken muscle homogenate was placed into a 50-mL centrifuge tube from the SampliQ QuEChERS AOAC Extraction kit. The tube was centrifuged for 20 s. Samples were then spiked with appropriate spiking solutions to yield 50, 100, and 150 ng/g sample concentrations for recoveries and reproducibility studies. A 100-µL IS spiking solution was added to all the samples except the blank. After shaking vigorously for 1 min, 8 mL Milli-Q water was added followed by further shaking for 30 s. Next, 10 mL 1% HOAc in ACN was added followed by the Agilent SampliQ QuEChERS AOAC Extraction salt packet (p/n 5982-5755). The packet contained 6 g of anhydrous $MgSO_4$ and 1.5 g of anhydrous NaOAc. The sample tubes were hand shaken vigorously for 1 min then further centrifuged at 4000 rpm for 5 min.

Dispersive SPE cleanup

A 6-ml aliquot of the upper ACN layer was transferred into a SampliQ QuEChERS AOAC Dispersive SPE 15 mL tube. This SPE tube contained 400 mg of PSA, 400 mg of C18EC, and 1200 mg of anhydrous $MgSO_4$. The tubes were then centrifuged at 4000 rpm for 5 min. Next, 4 mL of the extract was transferred to a test tube and dried with N_2 gas at 35 °C. Samples (200 µL) were reconstituted into 600 µL of 0.05 M NaOAc (pH 3.5).

Derivatization

Aliquots of 400 µL working standard mixtures of sulfonamides, dissolved in 0.05 M acetate buffer (pH 3.4), were filtered through a 0.45 µm PVDF syringe filter then transferred

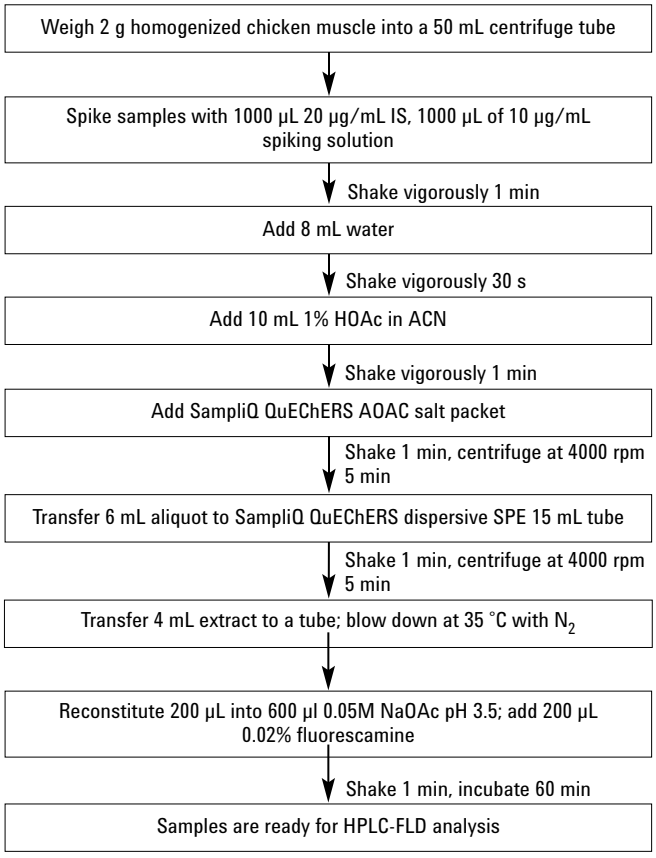


Figure 2. Flow chart for the QuEChERS AOAC sample preparation procedure.

to reaction vials. A 200 µL 0.02% w/v amount of fluorescamine solution in acetone was added. The mixtures were shaken for 1 min and the reaction left to proceed for 60 min at ambient temperature. Aliquots of 10 µL of the derivatized solutions were directly injected into the liquid chromatograph.

Results and Discussion

Derivatization of sulfonamide drugs

Fluorescamine is a fluorogenic reagent specific for primary aliphatic and aromatic amines that produce fluorophors of a high fluorescence yield [9]. This reagent and its hydrolysis products do not fluoresce, which eliminates the extensive cleanup step. Fluorescamine was therefore used in this application note to derivatize sulfonamides in the precolumn mode. The results indicated that the reaction time is the most important factor. The reaction was complete within 60 – 100 min and for reproducibility 60 min was the chosen time. The derivatised sulfonamides were detected with a single pair of wavelengths, λ_{ex} = 405 nm and λ_{em} = 495 nm.

Chromatographic results

The chromatogram of the standard mixture of these sulfonamide derivatives is shown in Figure 3. Figure 4 is the chromatogram for the blank chicken muscle extract, and Figure 5 is that of the spiked chicken muscle. All chromatograms of standards, blanks, and spiked extracts were run using the conditions outlined in Table I.

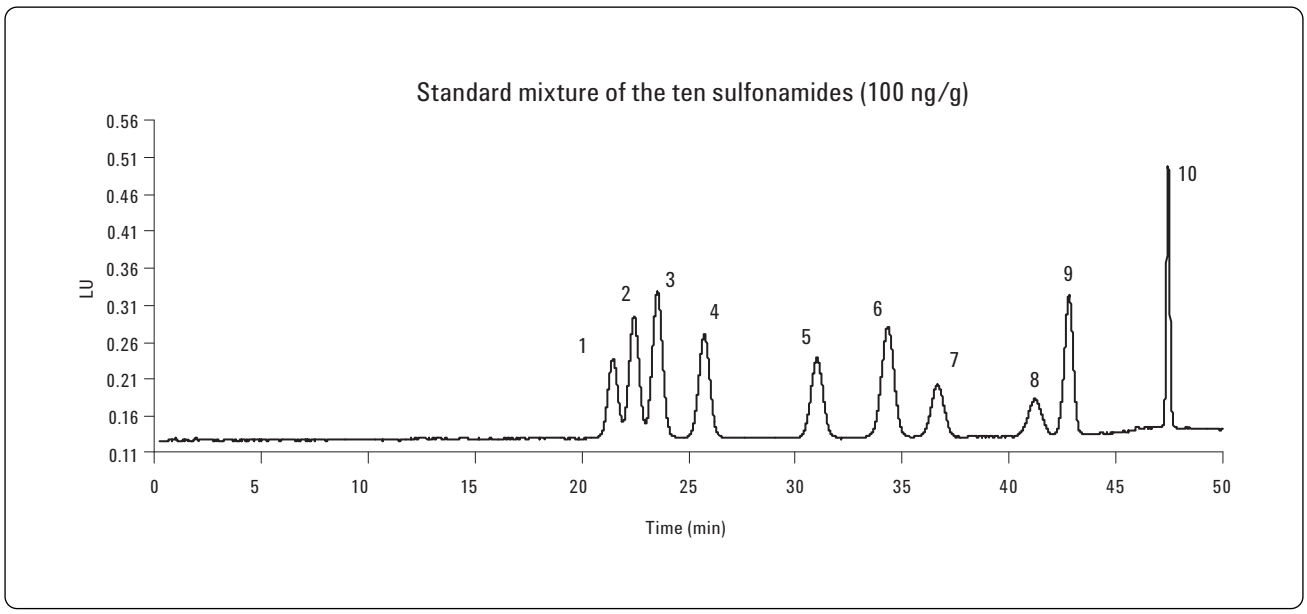


Figure 3. Chromatogram of the standard mixture of the sulfonamides (100 ng/g): 1. Sulfadiazine; 2. Sulfathiazole; 3. Sulfapyridine (IS); 4. Sulfamerazine; 5. Sulfamethazine; 6. Sulfamethizole; 7. Sulfamethoxypyridazine; 8. Sulfachloropyridazine; 9. Sulfamethoxazole; 10. Sulfadimethoxine.

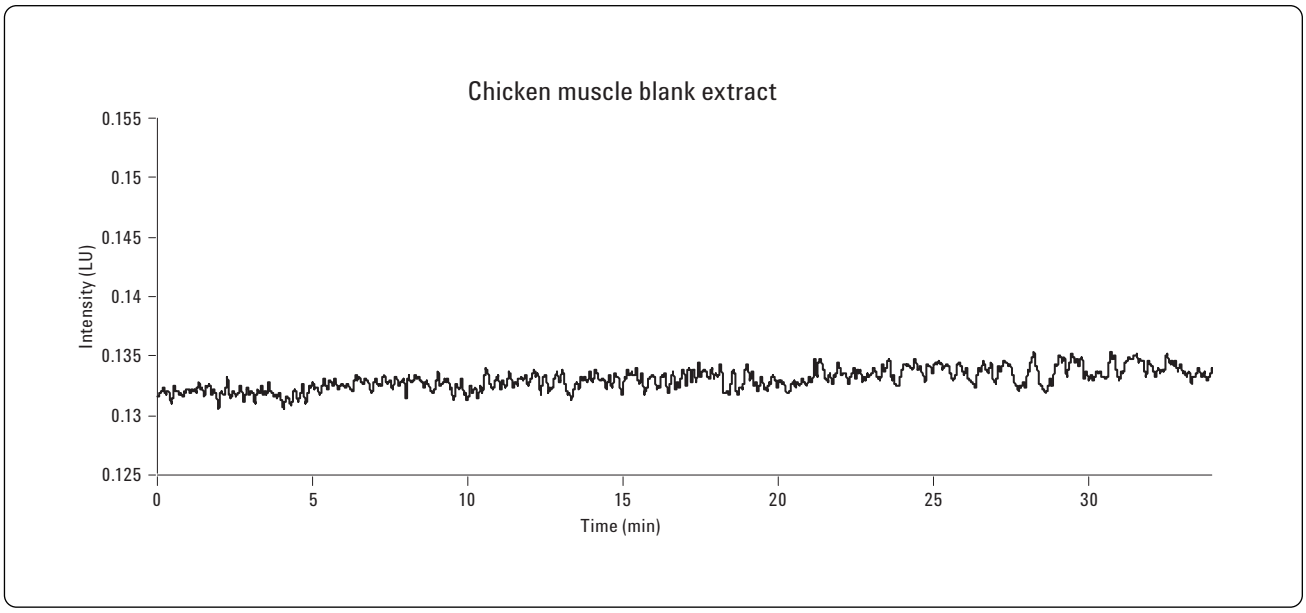


Figure 4. Chromatogram of the blank chicken muscle extract.

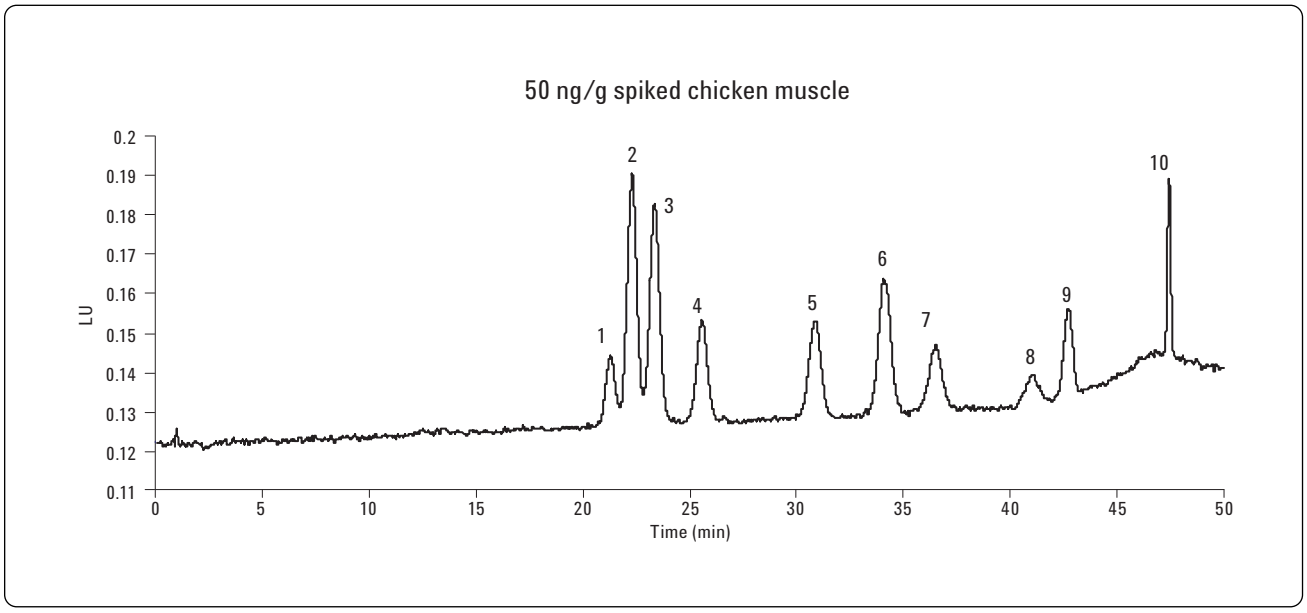


Figure 5. Chromatogram of the spiked chicken muscle extract at 50 ng/g level: 1. Sulfadiazine ; 2. Sulfathiazole; 3. Sulfapyridine (IS); 4. Sulfamerazine; 5. Sulfamethazine; 6. Sulfamethizole; 7. Sulfamethoxypyridazine; 8. Sulfachloropyridazine; 9. Sulfamethoxazole; 10. Sulfadimethoxine.

Linearity, Limit of Detection (LOD) and Limit of Quantification (LOQ)

Linearity

The linear calibration curves were obtained by plotting the relative responses of analytes (peak area of analyte/peak area of IS) verses the relative concentration of analytes (concentration of analyte/concentration of IS). They were generated by spiking the sample blanks at levels of 10, 50, 100, 150, 200, 300 and 400 ng/g.

Limits of Detection and Quantification

The limits of detection and quantification were estimated from the concentration of sulfonamides required to give signal-to-noise ratios of 3 and 10 respectively. Table 2 shows the regression equation, correlation coefficients, and very acceptable limits of detection and quantification.

Recovery and Reproducibility

The recovery and reproducibility (RSD) were evaluated on spiked samples at MRL (100 µg/kg), half MRL (50 µg/kg) and one and a half times the MRL (150 µg/kg). The analysis was performed in replicates of six (n = 6) at each level. Table 3 shows the recoveries and RSD values for the nine sulfonamides.

Table 2. Linearity, LOD and LOQ for the Nine Sulfonamides

Sulfonamide	Regression equation	R^2	LOD ng/g	LOQ ng/g
Sulfadiazine	Y = 0.4154x + 0.0112	0.9995	0.26	0.87
Sulfathiozole	Y = 1.0231x – 0.0757	0.9991	0.02	0.27
Sulfamerazine	Y = 0.6735x + 0.0184	0.9993	0.14	0.46
Sulfamethazine	Y = 0.6735x + 0.0042	0.9996	0.08	0.26
Sulfamethizole	Y = 0.9751x + 0.0115	0.9995	0.30	1.00
Sulfamethoxypyridine	Y = 0.4713x – 0.0069	0.9994	0.24	0.80
Sulfachloropyridazine	Y = 0. 2769x + 0.0190	0.9992	0.33	1.10
Sulfamethoxazole	Y = 0.6996x + 0.0421	0.9991	0.39	1.30
Sulfadimethoxine	Y = 0.5008x + 0.0329	0.9991	0.08	0.25

Table 3. Recovery and Repeatability for Sulfonamides in Spiked Chicken Muscle (n = 6)

Sulfonamide	Level of spiking (ng/g)					
	50		100		150	
	%Recovery	%RSD	%Recovery	%RSD	%Recovery	%RSD
Sulfadiazine	77.8	2.6	78.1	2.1	78	1.9
Sulfathiazole	83.0	3.9	88.2	2.4	85.2	2.2
Sulfamerazine	85.7	2.9	85.5	3.1	88.4	1.6
Sulfamethazine	80.3	3.1	81.3	2.2	80.3	1.5
Sulfamethizole	95.2	4.1	89.5	2.6	87.2	2.5
Sulfamethoxypyridazine	91.4	2.3	90.7	2.1	90.5	1.7
Sulfachloropyridazine	78.1	3.0	89.4	2.6	80.7	2.2
Sulfamethoxazole	76.8	3.5	87.2	2.5	89.1	1.5
Sulfadimethoxine	90.3	4.7	92.8	2.2	89.2	2.0

Conclusions

A simple and fast mulitiresidue method based on SampliQ QuEChERS AOAC Method 2007.01 and HPLC-FLD with pre-column derivatization has been developed for the simultaneous determination of nine sulfonamide residues in spiked chicken muscle. The recoveries were good with excellent RSD and the LOQs were well below the MRL in animal food products. This method can therefore be recommended for residue control purposes.

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Extraction of Sulfa Drugs in Honey with Polymeric SPE Cation Exchange, Bond Elut Plexa PCX

Application Note

Authors

William Hudson and Rich Motyka
Agilent Technologies, Inc.

Introduction

Antibiotics and other drugs in agricultural foods is a recurrent problem that can cause serious harm or death to the allergic or sensitive consumer. In recent years, various sources of honey have been shown to be contaminated with residues of antibiotics and sulfonamides. These contaminants may occur after direct treatment of bacterial diseases of honey bees, such as American or European foulbrood and nosemosis. To address this issue, several countries have regulated use of sulfonamides and require routine testing of honey for their presence.

Honey is a complex matrix consisting of mostly carbohydrates and water. In contrast to less viscous samples, for example milk, in order to utilize SPE with a viscous matrix like honey, it is necessary to take additional steps including sonication and acidification prior to SPE. Herein, we describe a method to extract and analyze sulfa drugs in honey using cation exchange SPE and LC/MS/MS analysis.

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Materials and Methods

Table 1. SPE Reagents and Solutions

4% Phosphoric Acid	Add 40 µL of concentrated H_3PO_4 to 1 mL of DI water
Methanol	Reagent grade or better
2% Formic Acid	Add 20 µL of concentrated formic acid to 1 mL of DI water
Methanol:acetonitrile (1:1, v/v)	Add 1 mL of methanol to 1 mL of acetonitrile
5% NH_4OH Methanol:acetonitrile (1:1, v/v)	Add 50 µL of concentrated ammonium hydroxide to 1 mL of methanol:acetonitrile (1:1, v/v)
Bond Elut Plexa 10 mg 96 well plate (part number A4968010)	

Table 2. SPE Method

Sample Pre-treatment	1.0 g Honey. Add 1 mL 4% H_3PO_4 and sonicate for 20 mins. Dilute with 3 mL of 2% H_3PO_4.
Condition	1. 500 µL CH_3OH 2. 500 µL DI H_2O
Wash 1	500 µL 2% formic acid
Wash 2 (acids, neutral)	500 µL methanol:acetonitrile (1:1, v/v)
Elution (bases)	500 µL 5% NH_3 methanol:acetonitrile

All samples are evaporated to dryness and reconstituted in 100 µL of 80:20 0.1% Aq formic acid: CH_3OH.

Results and Discussion

LC Conditions

Mobile Phase: A: 5 mM Formic acid
B: Methanol

Gradient:
t = 0-0.5 min 90% A : 10% B
t = 5.0-6.0 min 50% A : 50% B
t = 6.01-7.0 min 90% A : 10% B

Column: Pursuit C18 3 µm, 50 x 2.0 mm (part number A3051050X020)

MS Conditions

Transition ions and collision energy were:

Compound	Q1	Q3	CE
Sulfathiazole	256.0	156.0	18.0V
Sulfamethazine	279.0	186.0	21.0V
Sulfaquinoxaline	301.0	156.0	19.5V
Sulfadimethoxine	311.0	92.1	37.0V

Capillary = 70 V, Dry gas temp = 350 °C, 30 psi, CID = Argon

Polarity: Negative

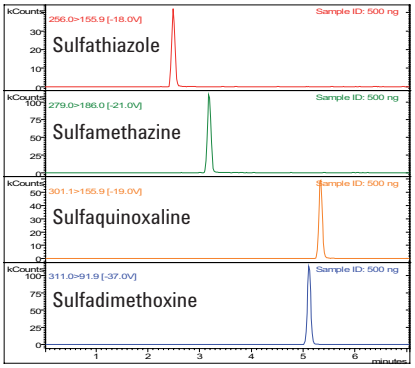


Figure 1. Chromatograms of a 50 ng/mL extract

The procedure describes a method for extracting four sulfonamide antibiotics from honey (Figure 1). The limit of detection (LOD) of the combined solid phase extraction and LC/MS/MS analysis was 25 ng/g. Recoveries were calculated from a 1st order regression with RSD values based on a sampling of n = 6. Excellent absolute recoveries were achieved demonstrating good retention and elution, as well as minimal ion suppression. Response for all the compounds evaluated was linear up to three orders of magnitude from 1.0 ng/mL to 5.0 mg/mL with correlation coefficients all above 0.999. To demonstrate reproducibility, samples were analyzed at two concentrations (n = 6). As shown in Table 3, the extractions produced reproducibly high recoveries.

Table 3. Analyte relative recoveries

Analyte	log P	pKa	% Rec (50 ng/mL)	% RSD	% Rec (500 ng/mL)	% RSD
Sulfathiazole	0.05	7.2	102	6	93	7
Sulfamethazine	0.89	7.4	103	7	87	4
Sulfaquinoxaline	1.68	5.9	107	7	100	7
Sulfadimethoxine	1.63	5.9	100	4	103	5

Conclusions

With excellent flow characteristics Bond Elut Plexa PCX is an ideal choice when working with difficult to process samples such as honey. Similar to other widely used cation exchangers, standard adjustments of pH and organic content with Bond Elut Plexa PCX yields fast and effective analyte recoveries approaching 100% with the four sulfa drugs present in honey. These data suggest Bond Elut Plexa PCX lends itself to the extraction of sulfa and similar drugs from foods and other complex matrices.

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Analysis of tetracyclines by HPLC

Rainer Schuster

Food

Abstract
Tetracyclines are used worldwide as oral or parenteral medication in the form of additives in animal feed. In food-producing animals, these drugs exhibit a high degree of activity toward a wide range of bacteria.[1, 2]

Sample preparation
After homogenization or mincing and addition of mineral acids to dissociate tetracyclines from proteins, the samples were extracted using liquid/liquid extraction followed by degreasing and/or deproteinization, purification, and concentration.[3]

Chromatographic conditions
The HPLC method presented here for the analysis of meat is based on reversed-phase chromatography and UV-visible diode-array detection.
UV spectra were evaluated as an additional identification tool.

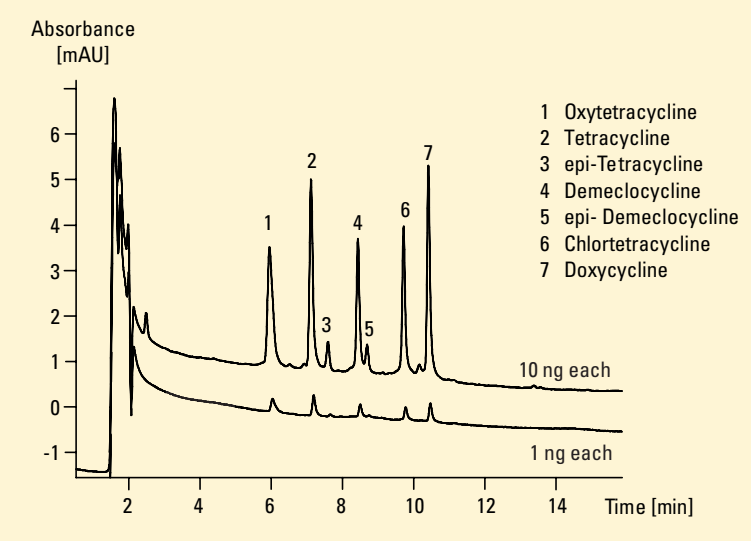


Figure 1
Analysis of tetracyclines by HPLC

Conditions

Column: 100 ˘ 4 mm Hypersil BDS, 3 µm
Mobile phase:
A = water, pH = 2.1 with sulfuric acid
B = ACN
Gradient: start with 15 % B at 10min 60% B
Flow rate: 0.5 ml/min
Column compartment: 25 ºC
Detector:
UV-DAD detection wavelength 355 nm/20 nm, reference wavelength 600/100 nm

Sample preparation

1. 1 g sample was mixed with citric acid (100 mg).
2. add 1 ml nitric acid (30 %) or 0.1 m oxalic acid
3. add 4 ml methanol 5 min in the ultrasonic bath
4. add water up to 10 ml total volume
5. centrifuge
6. inject

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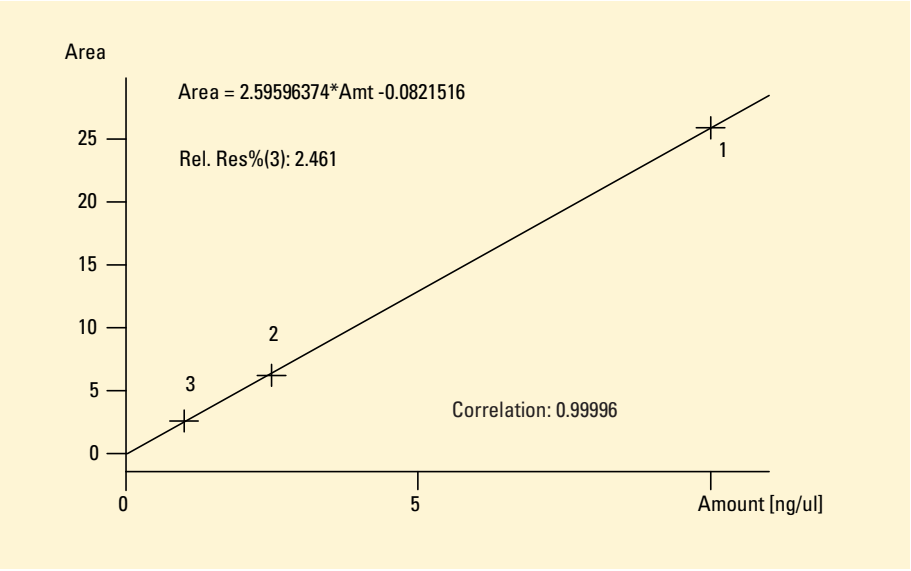


Figure 2
Linearity for oxytetracycline 1-10 ng

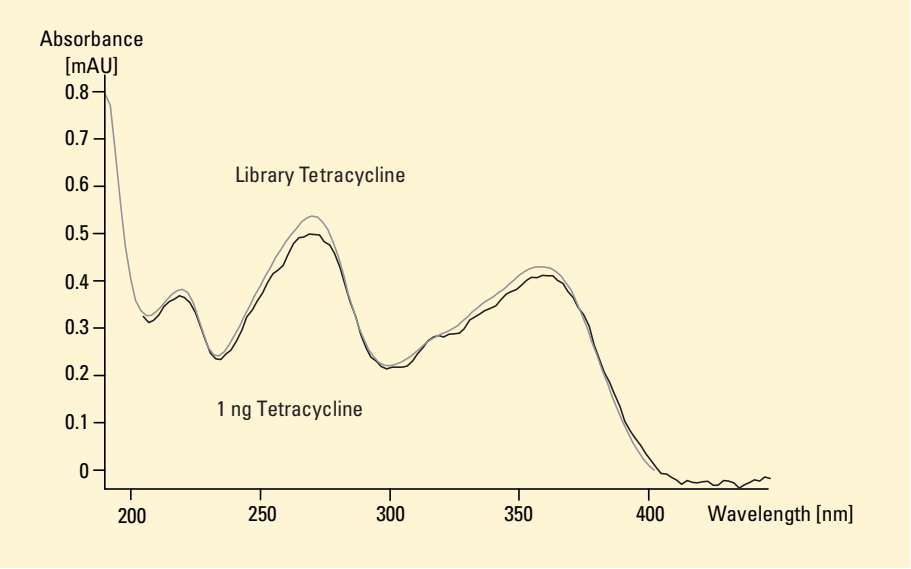


Figure 3
Analysis of tetracyclines at 100 ppb by HPLC

Rainer Schuster is application chemist at Agilent Technologies, Waldbronn, Germany.

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Equipment

Agilent 1100 Series
- vacuum degasser
- quaternary pump
- autosampler
- thermostatted column compartment
- diode array detector, Agilent ChemStation + software

HPLC method performance

Limit of detection
for UV-DAD 100 ppb
Repeatability
of RT over 10 runs <0.2 %
of areas over 10 runs <2 %

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Analysis of Residual Synthetic Antibacterials in Meat by HPLC

Application

Food

Hiroki Kumagai, Adebayo Onigbinde

Many domestic cattle receive various antibacterials in their feed for the prevention and control of disease caused by fungi and bacteria. Residues of antibacterials are found in food made from the meat of these animals. Since many antibiotics are toxic, many countries regulate acceptable residue levels of compounds allowable in agricultural and animal products. Many alkyl-C18 columns tail with basic compounds and have a shorter life time at low pH. Purospher® column separated basic antibacterials with good resolution, peak shape,and efficiency.

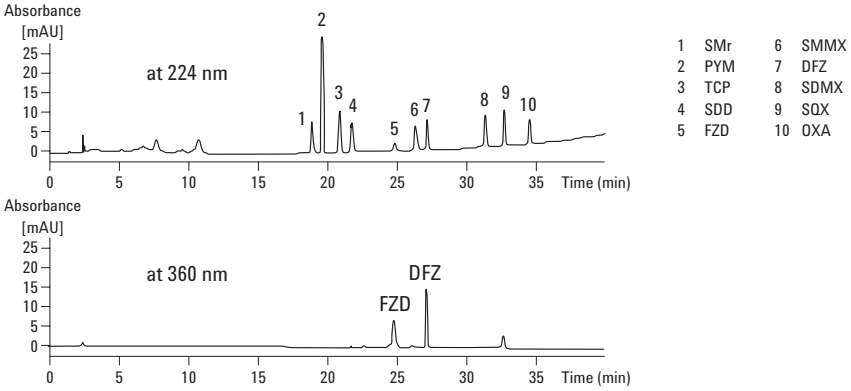


Figure 1. Chromatogram of standard solution, 2 µg/mL each analyte.

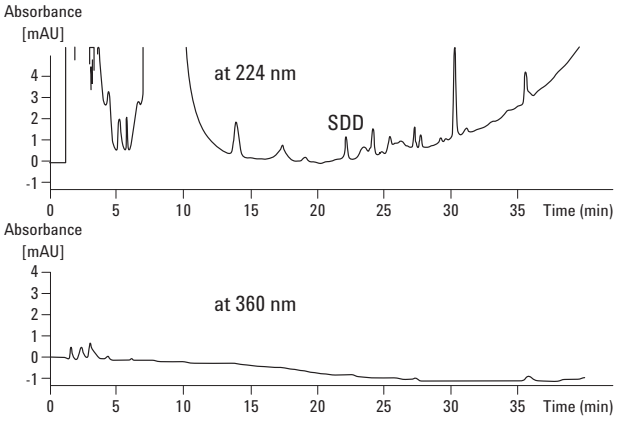


Figure 1. Chromatogram of extract of bovine muscle.

Highlights

- Separation of 10 antibacterials in meat at low pH
- Excellent and rapid resolution of antibacterials at low sample concentration
- Elution of antibacterials from the column with good peak shape and narrow peak width
- Separation of low level amounts of a wide range of pharmaceutical compounds with differing structures in a single analysis by Purospher® column

Analyzed Compounds

- Sulfamerazine (SMR)
- Sulfadimidine (SDD)
- Sulfamonomethoxine (SMMX)
- Sulfadimethoxine (SDMX
- Sulfaquinoxaline (SQX)
- Pyrimethamine (PYM)
- Thiamphenicol (TPC)
- Furazolidone (FZD)
- Difurazone (DFZ)
- Oxolinic acid (OXA)

Sample: Extracts from bovine muscle

Sample preparation: According to the official procedure of the Japanese food sanitation law.

Instrument: Agilent 1100 Series HPLC; **Column:** 250 mm × 4 mm id, RP-18 Purospher®, 5 µm, Part no. 79925PU-584; **Mobile phase:** A = 0.7 % Phosphoric acid, B = CH_3CN; **Gradient:** 0.0 min 5% B; 10.0 min 65% B; 40.0 min 65% B; 45.0 min 65% B; Post Time 7.0 min 5% B; **Flow rate:** 1.0 mL/min; **Temperature:** 40 °C; **Injection volume:** 20 µL; **Diode array detector:** A–338/10 nm, reference wavelength off; B–264/8 nm, reference wavelength off; C–360/8 nm, reference wavelength off

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HPLC Analysis of Antibacterial Drugs with Penicillin-Like Structure

Application

Drug Development

Udo Huber, Adebayo O. Onigbinde

Penicillins can be isolated from the culture medium of certain fungi-producing natural penicillin, such as Penicillium notatumor and P. chrysogenum. Other penicillins can be synthesized semisynthetically or by precursor-indicated biosynthesis. Total synthesis would not be economical.

Penicillin inhibits the polymerization of murin, which is responsible for the stability of the bacteria's cell wall. Because many antibacterials are toxic, various countries regulate the level of antibacterial residues in agricultural, veterinary, dairy, and meat-based food products.

Figure 1 shows the HPLC separation of four common antibacterial drugs with pencillin-like structure (amoxicillin, ampicillin, penicillin G, and penicillin V) on an SB-C18 reversed phase column.

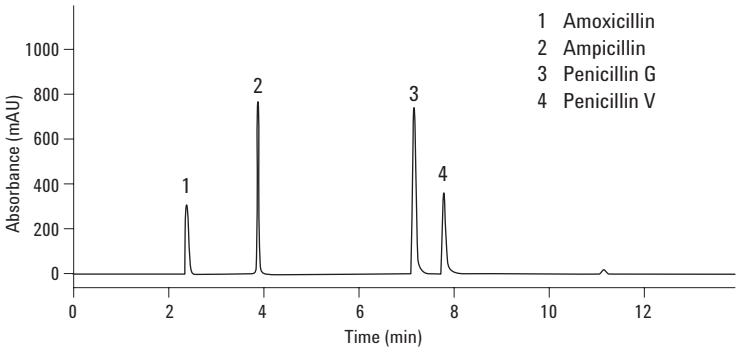


Figure 1. Separation of four penicillin analogs.

Highlights

- There is excellent resolution of penicillin analogs without ion pairing agent.
- There is rapid resolution of the penicillin analogs on the SB-C18 column at low pH and buffer concentration.
- Penicillins are eluted from the column with good and narrow peak shape.
- Extreme stability of sterically protected SB-C18 bonded phases allows for excellent separation at low pH.
- The SB-C18 column provides excellent peak shape and selectivity for antibacterial drugs.
- The HPLC method shows an easy but reliable and precise analysis of the antibacterial drugs.
- The values for limit of detection (LOD), precision of retention time (RT) and area show the good performance of the HPLC analysis.

Experimental Conditions

Equipment: Agilent 1100 Series HPLC; **UV Detector:** Variable wavelength detector, 204 nm, standard cell; **Column:** Zorbax SB-C18, 3. 5 µm, 4.6 × 75 mm (part number 866953-902); **Mobile phase:** A = 0.025 M KH_2PO_4 in water (pH = 3), B = acetonitrile; **Injection volume:** 5 µL; **Temp:** 40 °C; **Flow rate:** 1.0 mL/min; **Gradient:** at 5% B to 60% in 10 min; **Column wash:** 60% B to 5% B in 2 min; **Stop time:** 12 min; **Post time:** 5 min

Table 1. HPLC Method Performance of Antibacterial Drugs with Penicillin-Like Structure

Compound	LOD for S/N = 2 (mg/L)*	Precision of RT (RSD of 10 runs) (100 mg/L)*	Precision of area (RSD of 10 runs) (100 mg/L)*
Ampicillin	1.0	0.32	0.54
Amoxicillin	1.0	0.32	0.55
Penicillin G	1.0	0.32	0.49
Penicillin V	1.0	0.25	0.48

*Injection volume: 5 µL

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HPLC Separation of Antibacterial Drugs with Tetracycline Structure

Application

Drug Development

Udo Huber, Adebayo O. Onigbinde

Tetracyclines occur naturally in some streptomyces species. Besides being used in human and veterinary medicine, they are fed as nutritional antibiotics in pig and poultry farming. Because of their long half-life and resistance, there is a high restriction on their usage in some European countries, such as Germany. Figure 1 shows the HPLC separation of three common tetracycline analogs on a Zorbax SB-C18 reversed phase column.

This application demonstrates separation without ion pairing and the use of an alternative mobile phase to TFA in separating antibacterial drugs.

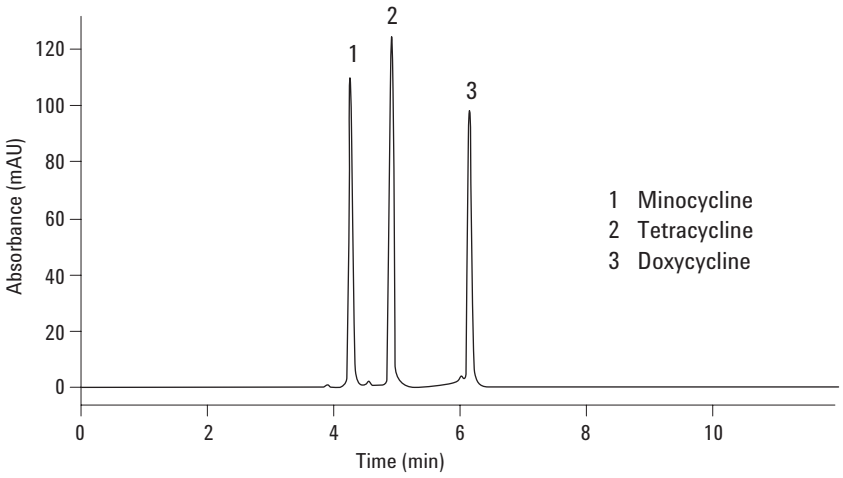


Figure 1. Separation of three antibacterial drugs with tetracycline structure.

Highlights

- The SB-C18 column provides excellent peak shape and selectivity for basic antibacterial drugs.
- The SB-C18 column shows excellent stability at low pH.
- The SB-C18 column shows excellent and rapid resolution of antibiotics at low pH and buffer concentration.
- The HPLC method shows an easy but reliable and precise analysis of the antibacterial drugs.
- The values for limit of detection (LOD), precision of retention time (RT), and area show the good performance of the HPLC analysis.

Experimental Conditions

Equipment: Agilent 1100 Series HPLC; **UV Detector:** Variable wavelength detector, 350 nm, standard cell; **Column:** Zorbax SB-C18, 3. 5 µm, 4.6 × 75 mm (part number 866953-902); **Mobile phase:** A = 0.025 M KH_2PO_4 in water (pH = 3), B = acetonitrile; **Injection volume:** 5 µL; **Temp:** 25 °C; **Flow rate:** 1.0 mL/min; **Gradient:** at 5% B to 60% B in 10 min; **Column wash:** 60% B to 5% B in 2 min

Table 1. HPLC Method Performance of Antibacterial Drugs with Tetracycline Structure

Compound	LOD for S/N = 2 (mg/L)*	Precision of RT (RSD of 10 runs) (100 mg/L)*	Precision of area (RSD of 10 runs) (100 mg/L)*
Minocycline	0.1	0.06	0.14
Tetracycline	0.1	0.05	0.13
Doxycycline	0.1	0.04	0.21

*Injection volume: 5 µL

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Agilent Technologies

Determination of the Metabolites of Nitrofuran Antibacterial Drugs in Chicken Tissue by Liquid Chromatograph-Electrospray Ionization-Mass Spectrometry (LC-ESI-MS)

Application

Food, Environmental

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Abstract

A liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) method was developed for the simultaneous determination of the metabolites of four nitrofuran antibacterial drugs in chicken tissues: furazolidone, furaltadone, nitrofurazone, and nitrofurantoin. Sample clean-up and analyte enrichment were performed by liquid-liquid extraction with ethyl acetate followed by solvent washing, hydrolysis of the protein-bound drug metabolites, and derivatization with 2-nitrobenzaldehyde (2-NBA). ESI parameters were optimized, and the chromatographic separation of all metabolites was examined. Each metabolite produced a simple mass spectrum containing a strong signal corresponding to $[M+H]^+$. Metabolite calibration curves, in the 0.25 to 1 ng/mL range, exhibited correlation coefficients greater than 0.999. The limit of detection (LOD) for each analyte ranged from 0.02 to 0.06 ng/mL.

Introduction

The four drugs shown in Figure 1, furazolidone, furaltadone, nitrofurazone, and nitrofurantoin, belong to the group of nitrofuran antibacterial drugs. These drugs have been widely used as feed additives to prevent bacterial enteritis by *Escherichia coli* and *Salmonella* in cattle, fish, swine, and poultry. The occurrence of furazolidone residue in edible tissue is a major human health concern. Effective June 1995, these drugs were banned from use in food animal production in the European Union (EU) because of concerns about their carcinogenicity and mutagenicity (Commission Regulation 1442/95).

Nitrofuran antibacterial drugs are characterized by their rapid metabolism, with in vivo half-lives of less than a few hours. Therefore, the detection of parent drugs in animal tissue is not practical. Studies using radioactive-labeled furazolidone have shown that protein-bound metabolites are formed in tissues [1-3]. The tissue-bound metabolites are detectable for several weeks after administration. Hence, the analysis of nitrofuran drugs is based on the detection of the tissue-bound metabolites of the parent drugs.

These tissue-bound metabolites are very small molecules which are not UV absorbing, and they elute too quickly out of a column. To induce UV absorption in the molecule and to be reasonably retained on a column, they are derivatized. It is possible to release these metabolites from the proteins under moderately acidic conditions and derivatize the metabolites with 2-nitrobenzaldehyde (2-NBA) to produce 2-NBA-derivatives for liquid chromatography (LC), UV detection, and mass spectrometry (MS) confirmation. The goal of this study is to develop a routine analytical method to simultaneously detect the target nitrofuran metabolites. Because no maximum residue limit (MRL) has

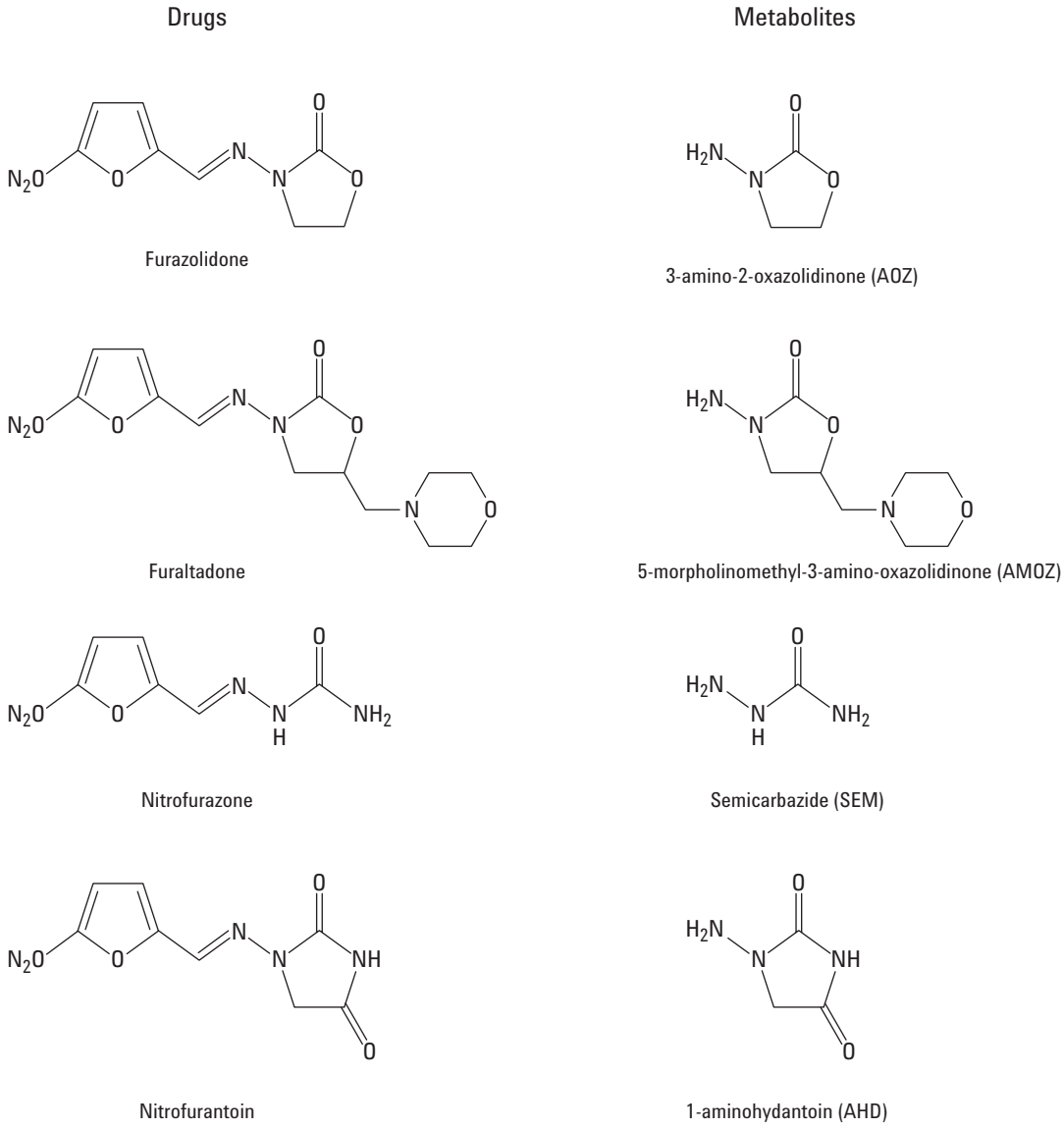


Figure 1. Structure of the nitrofuran antibacterial drugs and their metabolites .

been set by any regulatory agency, the goal of the analytical method was to estimate the lowest possible detection limit.

Experimental

Chemicals and Solvents

Three metabolites: 3-amino-2-oxazolidinone (AOZ), semicarbazide (SEM), and 1-aminohydantoin (AHD) were purchased from Sigma Aldrich Japan (Tokyo, Japan). The purity of these compounds was greater than 99%. The 2-NBA derivatives of these metabolites were prepared by the Livestock Department in Thailand (Palm Thani, Thailand) using the procedure described by Leitner [4]. Stock solutions of these three 2-NBA derivatives were prepared in methanol at 1000 ng/mL and stored in the dark at 4 °C. The stock solution was diluted to the desired concentration just prior to its use for the optimization of ESI parameters.

Acetonitrile, ethyl acetate, formic acid, and dimethyl sulfoxide (DMSO) were supplied by Wako Chemical (Osaka, Japan). Hydrochloric acid and 2-NBA were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Water was purified with a Milli-Q system (Millipore, Tokyo, Japan).

Sample Preparation

Sample preparation procedures included solvent wash and acid extraction by homogenization and derivatization with 2-NBA. Chicken muscle and liver were prepared by the Livestock Department in Thailand.

Calibration curves for the four nitrofuran metabolites (from the Livestock Department) were constructed in the range 0.25 to 1.0 ng/mL. The derivatization and sample preparation procedures used by the Livestock Department are the following:

1. The four metabolite solutions in water, 12.5, 25.0, 37.5, and 50 µL at 100 ng/mL, were transferred to separate 40 mL glass vials with screw caps.
2. A solution of 10 mL HCl (125 mM in water) and 200 µL 2-NBA (50 mM in DMSO) were added to each vial.
3. The reaction mixtures were kept in a water bath at 37 °C for 16 hours.
4. The solutions were cooled to room temperature.
5. The pH was adjusted to about 7.4 by adding 0.1 M aqueous $KHPO_4$ or 0.8 M aqueous NaOH.
6. A 5-mL measure of ethyl acetate was added to each reaction mixture, and shaken for 2 min.
7. Each ethyl acetate phase was transferred to a separate glass vial and evaporated under a stream of nitrogen.
8. Finally, each residue was reconstituted in 5 mL of 1:1 methanol:water (V/V).

The calibration curve was based on the metabolite concentration in clean solvent and derivatization using 2-NBA. Previous studies done by the Livestock Department showed that recovery of all metabolites from chicken extracts was above 80%. Therefore, the amounts of metabolite in chicken extract can be calculated by comparing the responses of 2-NBA derivatives from the samples against the calibration curve.

Instrument and Experimental Conditions

An Agilent 1100 series LC, with a solvent degassing unit, a binary high-pressure gradient pump, an automatic sample injector, and a column thermostat, was used for separation. An 1100 series diode array detector (DAD) was connected in line with an 1100 MSD for detection and confirmation. The column and MS conditions are described in Table 1.

Table 1. Instrument Parameters

LC:	Agilent 1100 series
Column:	Inertsil ODS3, 150 mm × 2.1 mm, 5 µm (GL Science, Tokyo, Japan)
Solvent A:	Acetonitrile
Solvent B:	Aqueous 0.5% formic acid
Gradient:	20/80 A/B to 70/30 A/B in 20 min
Column temp	20 °C
Sample volume	30 µL
Flow rate:	200 µL/min
MS:	Agilent 1100 MSD, SL
Ionization:	ESI (Positive)
Scan range:	100–500 *m/z* for optimization
SIM ion:	Base peak for quantitation
Drying gas:	Nitrogen, 10 L/min at 350 °C
Nebulizer gas:	Nitrogen, 50 psi
Fragmentor:	120 or 140 V
V_{cap}	2000 V

Quantitative analysis was carried out using selective ion monitoring (SIM) of the base peak ions according to the program shown in Table 2. To confirm the presence of the target analytes in chicken extract, the sodium adduct ions (qualifier ions) of all target analytes were also monitored.

Table 2. SIM Program

Group	Time window min	Analyte(s)	Target ion	Qualifier ion	Dwell time msec	Fragmentor voltage, V
1	0–6	2-NBA-AMOZ	335	357	500	140
2	6–12.5	2-NBA-SEM and 2-NBA-AHD	209 and 249	231 and 271	250 and 250	120 and 140
3	12.5–14	2-NBA-AOZ	236	258	500	140

System Optimization

Positive ion mass spectra were acquired over the scan range *m/z* 100–500 using a step size of 0.1 amu and a scan rate of 2 seconds per scan for the optimization of fragmentor voltage. Ion lens voltages in the MS were automatically optimized using a Calibrant Delivery System and the AutoTune program.

Using the analytical column and three 2-NBA derivatives (AOZ, SEM, and AHD) at 100 ng/mL, instrument performance was optimized by adjusting the four major ESI parameters: the capillary voltage, fragmentor voltage, the nebulizer gas pressure, and the drying gas flow rate. However, significant variation in the intensity of analytes was not observed when the drying gas flow rate and nebulizer gas pressure were varied from 4 L/min to 13 L/min and 20 psi to 60 psi, respectively.

Capillary and fragmentor voltages applied to the inlet and exit end of the capillary affected the ion transmission significantly. Fragmentor voltage also affected the fragmentation of sample ions. In general, higher fragmentor voltage helps the transmission of ions through the relatively high-pressure region between the exit of the capillary and the entrance of the skimmer. High fragmentor voltage can cause fragmentation to occur which provides structural information of the ion. For compounds that do not fragment easily, higher fragmentor voltage often results in better ion transmission. Optimal fragmentor voltage is compound dependent. Evaluation of the fragmentor voltages for the three 2-NBA-metabolites was done under the same chromatographic conditions as the analysis. Mass spectra of three 2-NBA-metabolites are shown in Figure 2. Each mass spectrum exhibited $[M+H]^+$ as the base peak. Adducts ions $[M+NH4]^+$ and $[M+Na]^+$ were observed at lower fragmentor voltage (120 V) and some fragment ions (*m/z*=178 and 192) were observed at higher fragmentor voltage (180 V). Interestingly, the $[M+NH_4]^+$ ion was not observed at

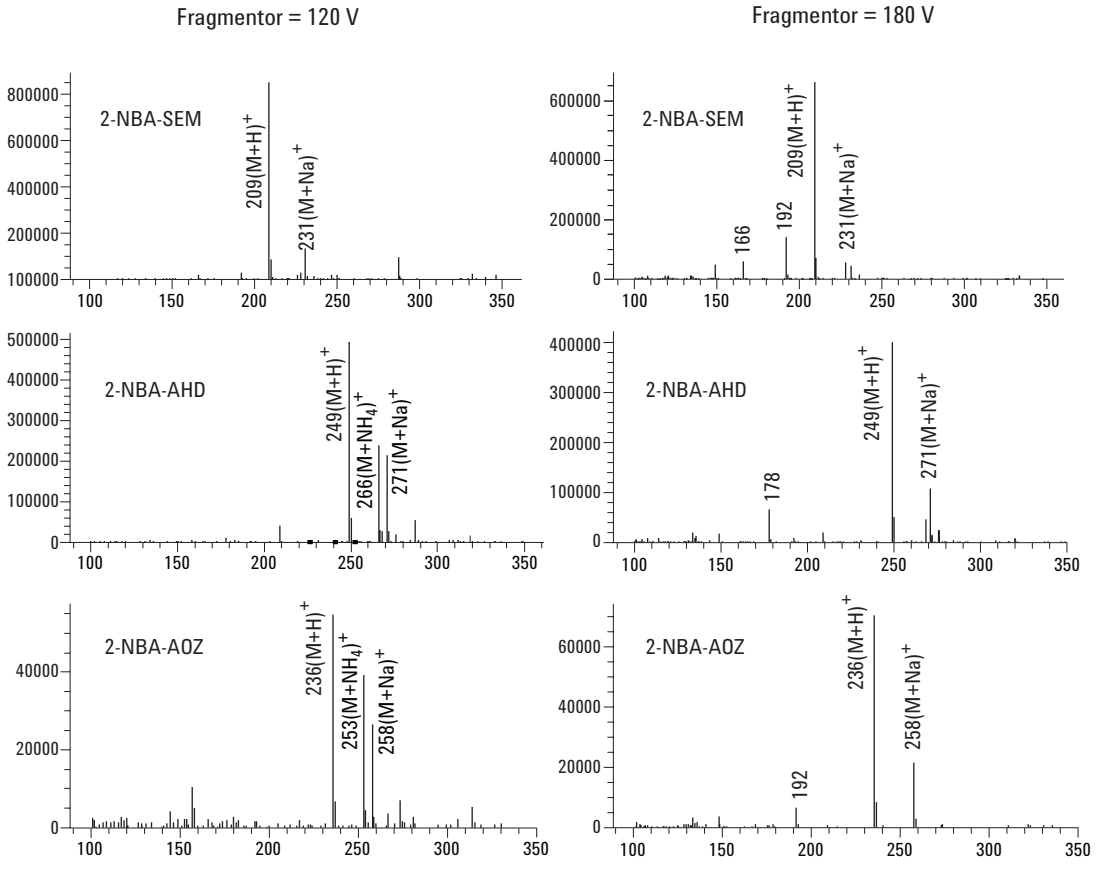


Figure 2. Mass spectra of 2-NBA-SEM, 2-NBA-AHD, and 2-NBA-AOZ from two ESI fragmentor voltages.

180 V fragmentor voltage due to its stability. As seen in Figure 3, in order to ensure the best sensitivity, the fragmentor voltage for 2-NBA-SEM was set to 120 V and that of 2-NBA-AHD and 2-NBA-AOZ was set to 140 V for the analysis. Although 2-NBA-AMOZ was not examined, fragmentor voltage of this compound was set to 140 V because of its structural similarity to 2-NBA-AOZ. For the capillary voltage varied between 1500 and 4500 V, the optimal voltage was found to be 2000 V for all three metabolites.

Linearity, Detection Limits, and Precision

In order to achieve optimal sensitivity, all quantitation experiments were carried out under SIM conditions, and the $[M+H]^+$ ions were monitored for all 2-NBA-metabolites. To evaluate the linearity of the calibration curves, various metabolite solutions ranging from 0.25 ng/mL to 1 ng/mL were derivatized and then analyzed. As shown in Table 3, the linearity was very good for all 2-NBA-metabolites with correlation coefficients (r^2) greater than 0.999.

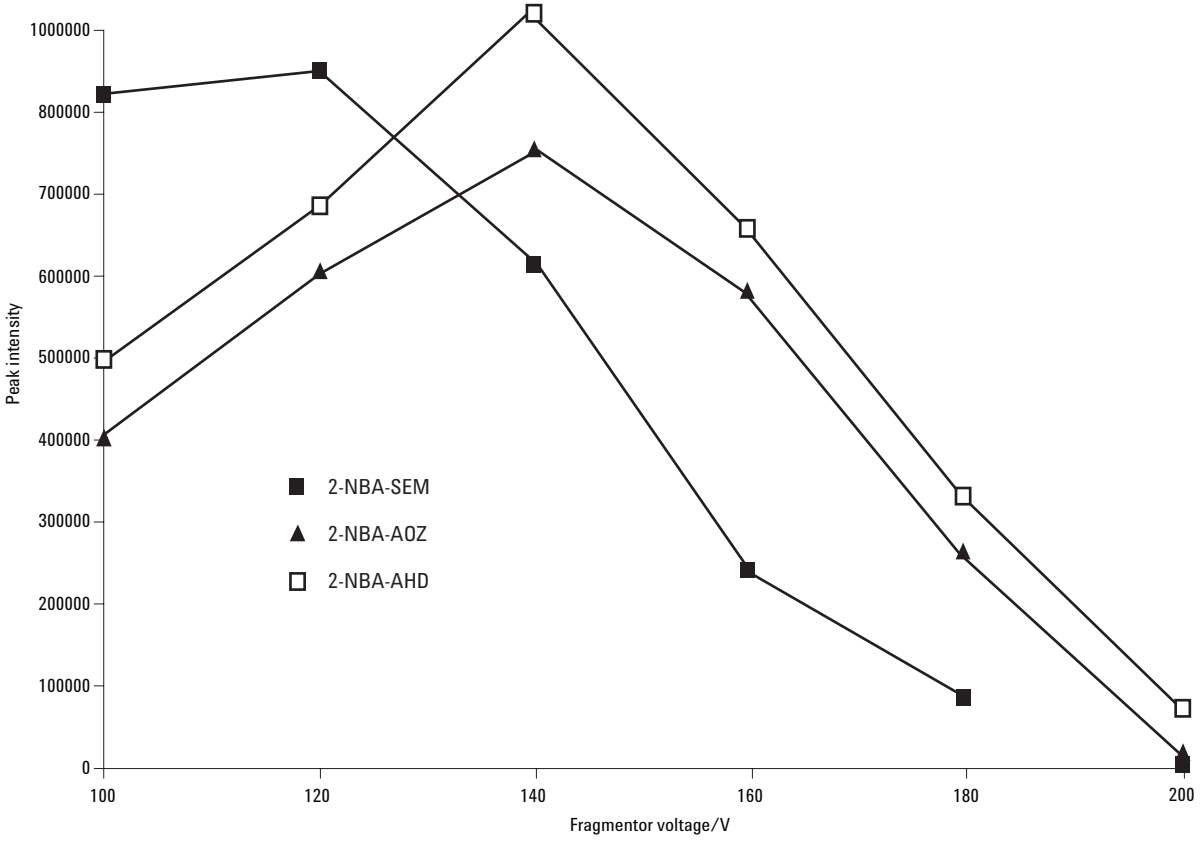


Figure 3. Effect of fragmentor voltage on peak intensity. Mobile phase, 20% acetonitrile/80% water 0.1% formic acid; Analyte concentration, 100 ng/mL.

The LOD for all 2-NBA-metabolites was estimated by extrapolating to a signal-to-noise ratio (S/N) of 3 using the signal from the standard solution at 0.25 ng/mL. These SIM chromatograms are shown in Figure 4. The LODs of the metabolites were in the range of 0.02 ng/mL to 0.06 ng/mL. These LODs were lower than those of the LC/MS/MS method developed by Leitner [4]. The intraday instrument precision (repeatability) was determined by injecting aqueous standard solutions containing all of the 2-NBA-metabolites at 0.5 ng/mL five times during a working day. The interday instrument precision (reproducibility) was evaluated by analyzing the same sample three times over 3 working days. The precision for all analytes ranged from 3.1% to 8.2%, as seen in Table 3.

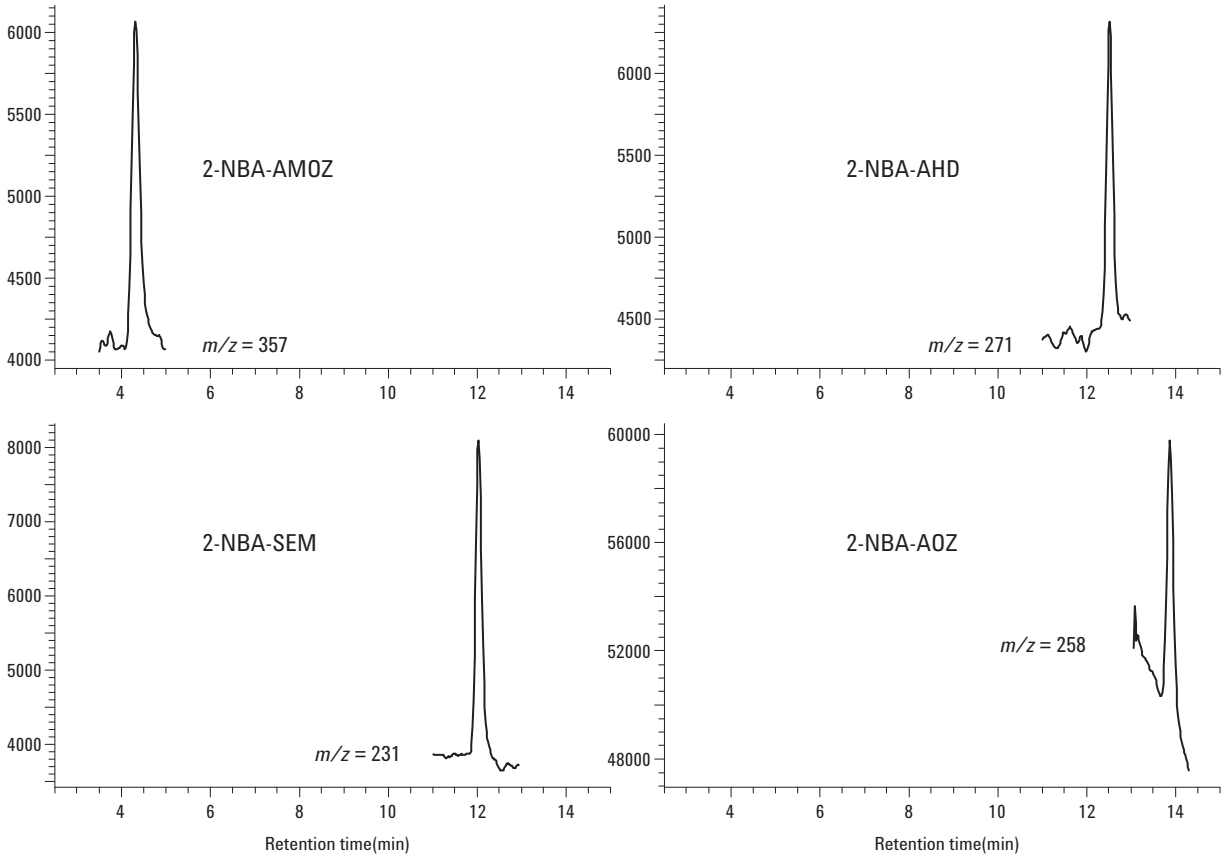


Figure 4. SIM chromatograms of aqueous 2-NBA nitrofuran metabolites solution at 0.25 ng/mL.

Table 3. Linearity, LOD, and Instrument Precision of Metabolites in Aqueous Solutions

			Instrument precision (%RSD)	
Metabolites	**r^2**	**LOD* (ng/mL)**	**Repeatability****	**Reproducibility*****
AMOZ	0.9999	0.04	5.0	7.3
SEM	0.9998	0.02	4.7	8.1
AHD	0.9989	0.06	4.9	7.9
AOZ	0.9997	0.06	3.1	8.2

*Detection limit is LOD defined as S/N = 3 for standard solution at 0.25 ng/mL

**Repeatability was calculated based on five replicates at 0.5 ng/mL within 1 day

***Reproducibility was calculated based on once per day for 3 days at 0.5 ng/mL

Evaluation of Chromatographic Separation

Several reverse-phase columns were evaluated for HPLC performance. In terms of minimizing the inherent matrix suppression effects on the ESI process, Inertsil ODS3 column provided the best separation between analytes and the majority of the matrix components with the given mobile phase. Further, the linear solvent gradient gave the best compromise between short analysis time and sufficient matrix and analytes separation. Figure 5 shows individual SIM chromatograms for the four metabolite derivatives in spiked chicken muscle at 0.2 ng/g. No interference peaks were observed for 2-NBA-AMOZ and 2-NBA-AOZ, but it was difficult to separate 2-NBA-SEM and 2-NBA-AHD from the interfering matrix peaks. However, these peaks could still be identified by comparison with the blank sample, and the analyte amounts could then be calculated.

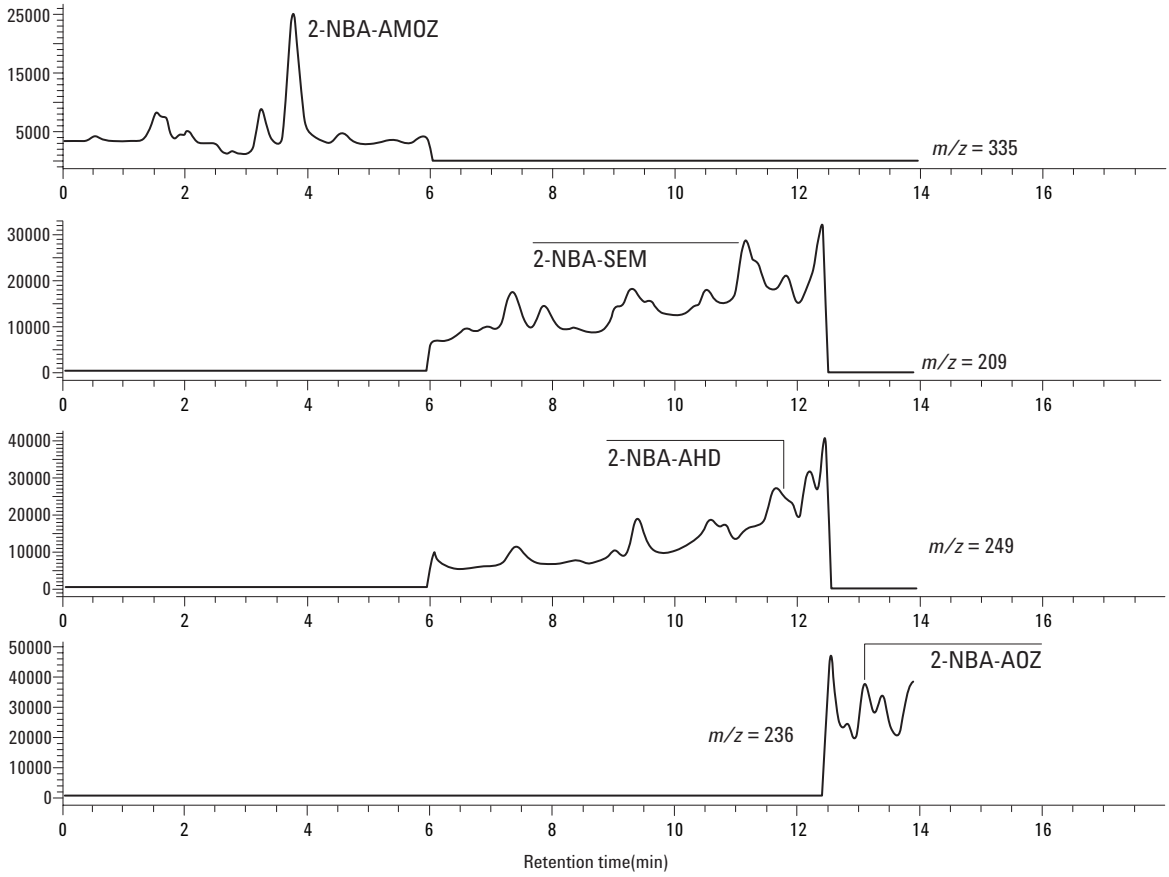


Figure 5. SIM chromatograms of a spiked chicken muscle tissue sample containing 0.2 ng/g of each of the four 2-NBA nitrofuran metabolites.

Application of the Method to Chicken Liver Samples

It has been reported that AOZ concentrations in liver tissue are several times higher than in muscle tissue [1,2]. This indicates that detection of nitrofuran metabolites in liver would be possible over an even longer period of time. Since the nature of the liver matrix is considered to be different from muscle and more difficult to separate target compounds from the interfering matrix, the developed LC/MS muscle method was also tested for the applicability to liver matrix. Figure 6 shows individual SIM chromatograms of the four metabolite derivatives in spiked chicken liver tissue. AMOZ, SEM, and AOZ derivatives were identified unambiguously and quantified down to 0.2 ppb. However, the AHD derivative overlapped with the matrix component and was difficult to quantify.

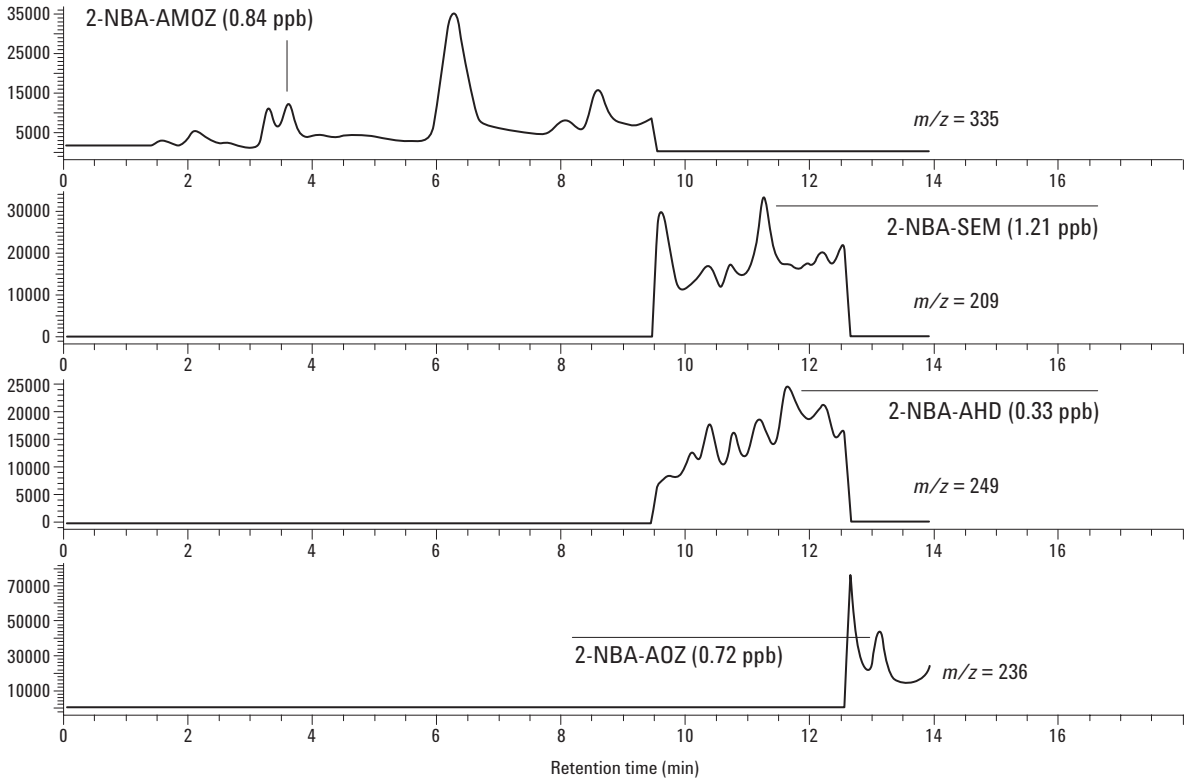


Figure 6. SIM chromatograms of a spiked chicken liver tissue sample containing 0.2 ng/g of each of the four 2-NBA nitrofuran metabolites.

Conclusion

The development of a routine and sensitive LC/MS method allows for the simultaneous detection of four nitrofuran metabolite derivatives. The detection limit of each analyte ranges from 0.05 to 0.2 ng/g in chicken muscle and liver tissues.

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Determination of Chloramphenicol in Fish Meat by Liquid Chromatograph-Atmospheric Pressure Photo Ionization-Mass Spectrometry (LC-APPI-MS)

Application

Foods, Environmental

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Abstract

A liquid chromatography-atmospheric pressure photoionization-mass spectrometry method was developed for the determination of chloramphenicol antibiotics in fish meats. For the optimization of APPI, several ion source parameters were examined. Using the optimized parameters, simple mass spectra and a strong signal corresponding to $[M-H]^-$ was observed. The samples were extracted with ethylacetate and evaporated to dryness followed by a clean-up step using liquid-liquid distribution by acetonitrile and n-hexane. Mean recoveries of chloramphenicol from young yellowtail meat and flatfish meat spiked at 0.1–2 ng/g were 89.3%–102.5% and 87.4%–94.8%, respectively. The limit of detection (signal-to-noise = 3) of the young yellowtail meat and the flatfish meat were 0.27 and 0.10 ng/g.

Introduction

Chloramphenicol (CAP) is a broad-spectrum antibiotic, that exhibits activity against a variety of aerobic and anaerobic microorganisms. Its action works through interference with or inhibition of protein synthesis. However, weeks or months of CAP therapy can result in a well-understood and irreversible type bone marrow depression called aplasia or hypolasia. This, in turn, can lead to aplastic anemia and although uncommon, it is often fatal. Because of these health concerns, a joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives has proclaimed that CAP residues in the human food supply are unacceptable [1]. The use of CAP in food products has been banned in EU and U.S.A. However, CAP's broad-spectrum activity, ready availability, and low cost attract its use by some third world countries. Admittedly, whenever CAP is accessible, indiscriminate and illegal use potentially exists. In fact, the presence of CAP has been detected in shrimp imported from China and Vietnam that was intended for human consumption.

Liquid chromatography/mass spectrometry (LC/MS) methods are very useful in analyzing CAP in food because of the high selectivity and sensitivity of MS detection [2-7]. Atmospheric pressure ionization (API) interfaces, represented by atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI), are commonly used in LC/MS.

Atmospheric pressure photoionization (APPI) is a new ionization technique for LC/MS [8, 9]. The APPI source is based on a high-fluence gas discharge lamp that generates vacuum-ultraviolet (VUV) photons of 10 and 10.6 eV energy. The energy of this discharge lamp is normally greater

than a first ionization potential (IP) of an analyte because many organic compounds have IPs in the range of 7–10 eV. On the other hand, the IPs of the most common LC solvents, which are used as a mobile phase, have higher values (water, IP = 12.6 eV; methanol, IP = 10.8 eV; acetonitrile, IP = 12.2 eV). This provides ionization of many analytes with lower IPs without interference from the mobile phase. To our knowledge, APPI has not yet been applied to residual analysis in food.

This application note describes how parameters affect the ionization efficiency of APPI for the analysis of CAP. In addition, the suitability of LC/MS and liquid-liquid extraction using the APPI technique is evaluated for the determination of CAP in fish meat.

Experimental

Chemicals and Solvents

CAP was purchased from Sigma-Aldrich Japan (Tokyo, Japan). The purity of this compound was greater than 99%. Stock solutions at 1 mg/mL were prepared in methanol, stored in the dark at 4 °C, and diluted to the desired concentrations prior to use. Ammonium acetate, pesticide-grade ethyl acetate, anhydrous sodium sulfate, acetonitrile, HPLC-grade methanol and n-hexane were obtained from Wako Chemical (Osaka, Japan). Water was purified with a Milli-Q system (Millipore, Tokyo, Japan). A nylon-type 0.22 µm centrifuge filter was obtained from Toyo Soda (Tokyo, Japan).

Sample Preparation

The samples analyzed (young yellowtail and flatfish) were obtained from a local market. To a centrifuge tube, 5 g fish meat and 5 g anhydrous sodium sulfate were weighed and 10 mL ethyl acetate was added. The mixture was homogenized for 20 s with an Ultra-Turrax TP 18/10 (Janke & Kunkel KG, Staufen, Germany). After centrifugation for 5 min at 6000 rpm, the supernatant was removed and transferred to a round flask. The extraction step was repeated twice, each with 10 mL ethyl acetate. The combined ethyl acetate extract was then evaporated in a rotary evaporator at 40 °C under vacuum. One mL acetonitrile and 1 mL n-hexane was added to the residue, transferred into a graduated glass stopper reagent bottle, and shaken. The n-hexane phase was discarded. The step was repeated with another 1 mL of n-hexane. Finally, the acetonitrile phase was evaporated to dryness under a stream of dry nitrogen using a heating block at 50 °C, redissolved in 5 mL of a 10% acetonitrile in 10 mM ammonium acetate water solution, and filtered through a 0.22 µm nylon centrifuge filter. The samples were spiked with 0.1–100 ng/mL of CAP after the homogenation step to generate a calibration by LC/APPI-MS selected ion monitoring (SIM).

LC/MS

An Agilent 1100 series LC, consisting of a vacuum solvent degassing unit, a binary high-pressure gradient pump, a standard automatic sample injector, and a column thermostat, was used for the separation. An 1100 series diode array detector (DAD) was connected in line with an 1100 MSD for detection and confirmation. See Table 1. The separation was performed on a 150 × 3 mm id column packed with 5 µm Zorbax Eclipse XDB C18 (Agilent Technologies, Palo Alto, USA). A 15-min linear solvent gradient was used for elution with the mobile phase. Quantitative analysis was carried out using SIM of *m/z* 321 with a dwell time of 500 msec.

The following six parameters were optimized using

Table 1. Instrument Parameters

LC:	**1100 series LC**
Column:	Zorbax Eclipse XDB C18 (150 mm × 3 mm, 5 µm)
Solvent A:	Water with 10 mM ammonium acetate
Solvent B:	Methanol
Dopant:	Acetone at 0.05 mL/min
Gradient:	90/10 A/B 15 min to 70/30 A/B
Column temp:	40 °C
Sample volume:	20 µL
Flow rate:	0.5 mL/min
MS:	**1100 MSD, SL**
Ionization:	APPI (Negative)
Scan range:	*m/z* 100–400 for optimization
SIM ion:	*m/z* 321; $(M\text{-}H)^-$
Drying gas:	Nitrogen, 7 L/min at 350 °C
Nebulizer gas:	Nitrogen, 50 psi
Fragmentor:	120 V
Capillary:	3500 V
Vaporizer temp:	350 °C

the analytical column with CAP at 100 ng/mL: the voltages for in-source-fragmentation (the fragmentor voltage), the capillary voltage (V_{cap}), the drying gas flow rate, the nebulizer pressure, the mobile phase composition, and the mobile phase flow rate. The ion lens voltages in the MS were automatically optimized using a Calibrant Delivery System and the AutoTune program. Negative ion mass spectra were acquired over the scan range *m/z* 100–400 using a step size of 0.1 amu and a scan rate of 2 s per scan for the optimization of fragmentor voltage.

Results and Discussion

Optimization of the APPI Parameters

To optimize the APPI conditions, parameters that influence the ionization efficiency were investigated. The drying gas flow, the nebulizer gas pressure, the vaporizer temperature, the capillary voltage, and the mobile phase composition were evaluated under the chromatographic conditions mentioned in the Experimental section by SIM mode using the *m/z* 321 ion as the target ion. It was found that modification of drying gas flow rate and nebulizer gas pressure did not drastically improve the sensitivity of CAP. In addition, the fragmentor voltage was included in optimization because of its compound dependence and its significant effect on the mass spectral response.

Effect of Capillary Voltage

The capillary voltage is applied to the inlet of the capillary and influences the transmission efficiency of the ions through the capillary sampling orifice. To establish the optimum capillary voltage, this parameter was varied from 1000 to 4000 V. As shown in Figure 1, 1500 V was found optimum. A tremendous effect of this parameter on the intensity of CAP was observed in the case where acetone was not used as the dopant. On the other hand, when acetone was introduced into the APPI source as the dopant, the maximum intensity of the ion was found at 3500 V. The intensity found at 3500 V with the dopant was higher than the maximum intensity without the dopant. Based on the above results, the capillary voltage was set at 3500 V with acetone.

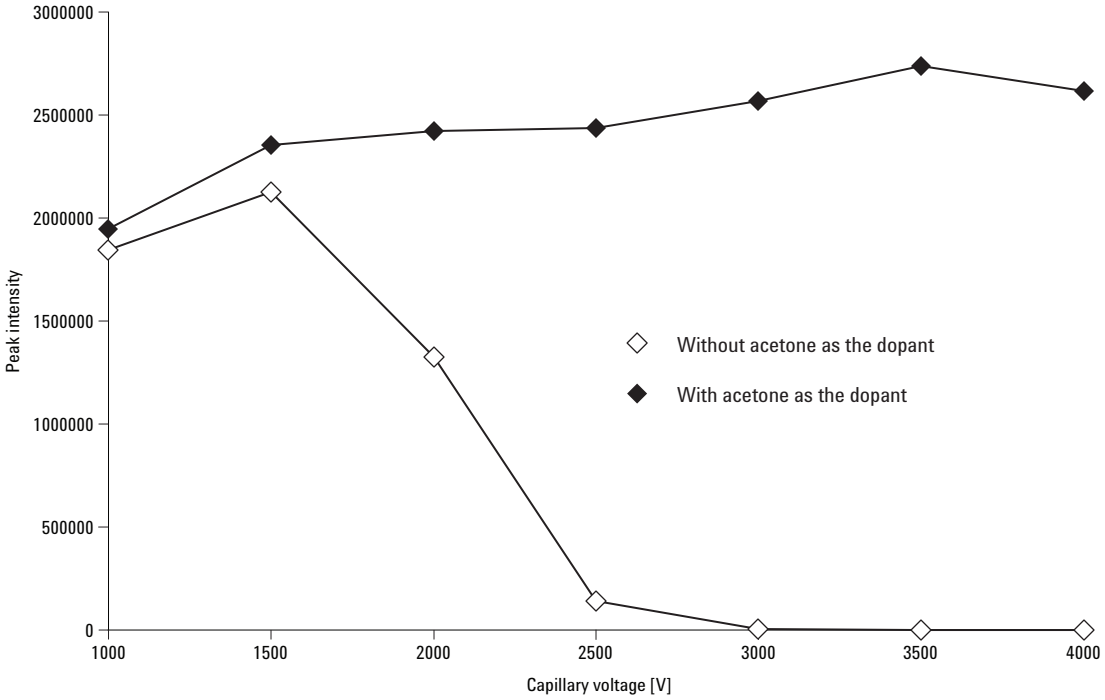


Figure 1. The effect of the capillary voltage on the peak intensity of CAP concentration : 1 ng/mL. For the other conditions, see Experimental section.

Effect of Vaporizer Temperature

In APPI, the vaporizer temperature plays a key role for the complete evaporation of CAP because ionization occurs in the vapor state like APCI. Thus, in the case of using linear gradient elution, this temperature must be kept sufficiently high so that the change of mobile phase composition does not influence the ion intensity of CAP. Under high temperature, however, the risk of thermal degradation occurs. In this study, the vaporizer temperature was modified between 250 and 450 °C to optimize the intensity and the *S/N* ratio. The highest temperature for a maximum intensity and *S/N* ratio of CAP was observed at 350 °C. The intensity of CAP decreased as the vaporizer temperature was increased over 400 °C. In addition, intense fragmentation was observed in the mass spectrum at 400 °C. Therefore, the decrease in intensity above 400 °C seems to be a result of the thermal degradation. Based on the above results, the vaporizer temperature was set at 350 °C.

Optimization of Fragmentor Voltage

The fragmentor voltage is applied to the exit of the capillary and affects the transmission and fragmentation of sample ions between the exit of the capillary and the skimmer at relatively high pressure (3 torr). In general, the higher the fragmentor voltage (which helps the transfer of ions), the more fragmentation will occur. To establish the optimum fragmentor voltage for the analysis of CAP, the intensity of this compound versus the fragmentor voltage was studied in the range from 80 to 200 V. As shown in Figure 2, the optimum fragmentor voltage was found at 120 V, whereas at higher values a significant intensity reduction was observed. Further, the best *S/N* ratio was also observed at 120 V. The mass spectra of CAP at optimal and higher fragmentor voltages are shown in Figure 3. The deprotonated molecule $(M\text{-}H)^-$ was the predominant ion at 120 V, and this included isotopic ions (*m/z* 321, Cl^{35} Cl^{35}; *m/z* 323, Cl^{35} Cl^{37}; *m/z* 325, Cl^{37} Cl^{37}) because CAP includes two

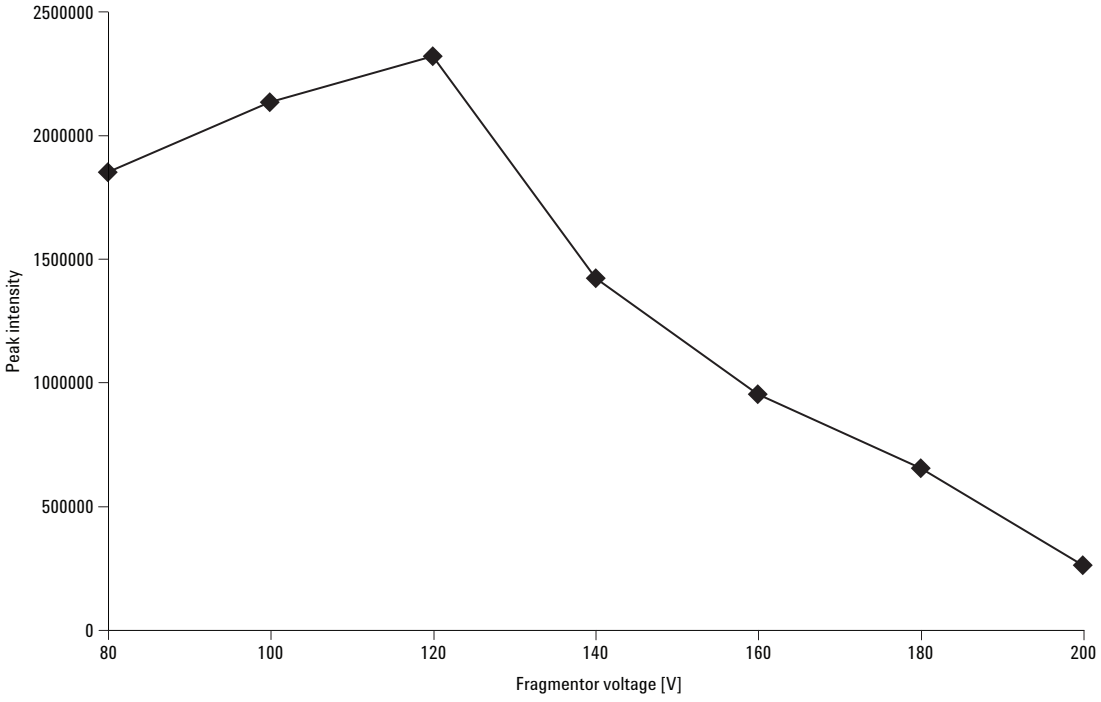


Figure. 2. The effect of the fragmentor voltage on the peak intensity of CAP concentration : 1 ng/mL. For the other conditions, see Experimental section.

chlorines. A higher fragmentor voltage (180 V) generated structurally relevant fragment ions. The *m/z* 152 fragment ion gives the greatest intensity and might be produced by the cleavage of the carbon-carbon bond on the alkyl branch as shown in Figure 3. Other fragment ions are observed at *m/z* 121 and 257. The *m/z* 121 may be the nitrophenyl fragment. The *m/z* 257 fragment might be explained by a charge migration hydrogen shift with a concerted loss of HCl and CO. These observed fragment ions in the APPI source corresponded with the fragment ions in an ESI source and an APCI source. Based on the above results, the fragmentor voltage was set to 120 V.

Optimization of the Chromatographic Conditions

The separation of CAP from sample matrix peaks was optimized using acetonitrile, methanol, water, and ammonium acetate. The combination of methanol and ammonium acetate was found optimum for the separation of CAP. When methanol was replaced with acetonitrile, a significant signal intensity and *S/N* decrease was observed. This result indicates that methanol may be a source of electrons for the hydrogen abstraction from CAP. Therefore, methanol and 10 mM ammonium acetate was used as the mobile phase in this study. The flow rate was set at 0.5 mL/min considering the size of the used column.

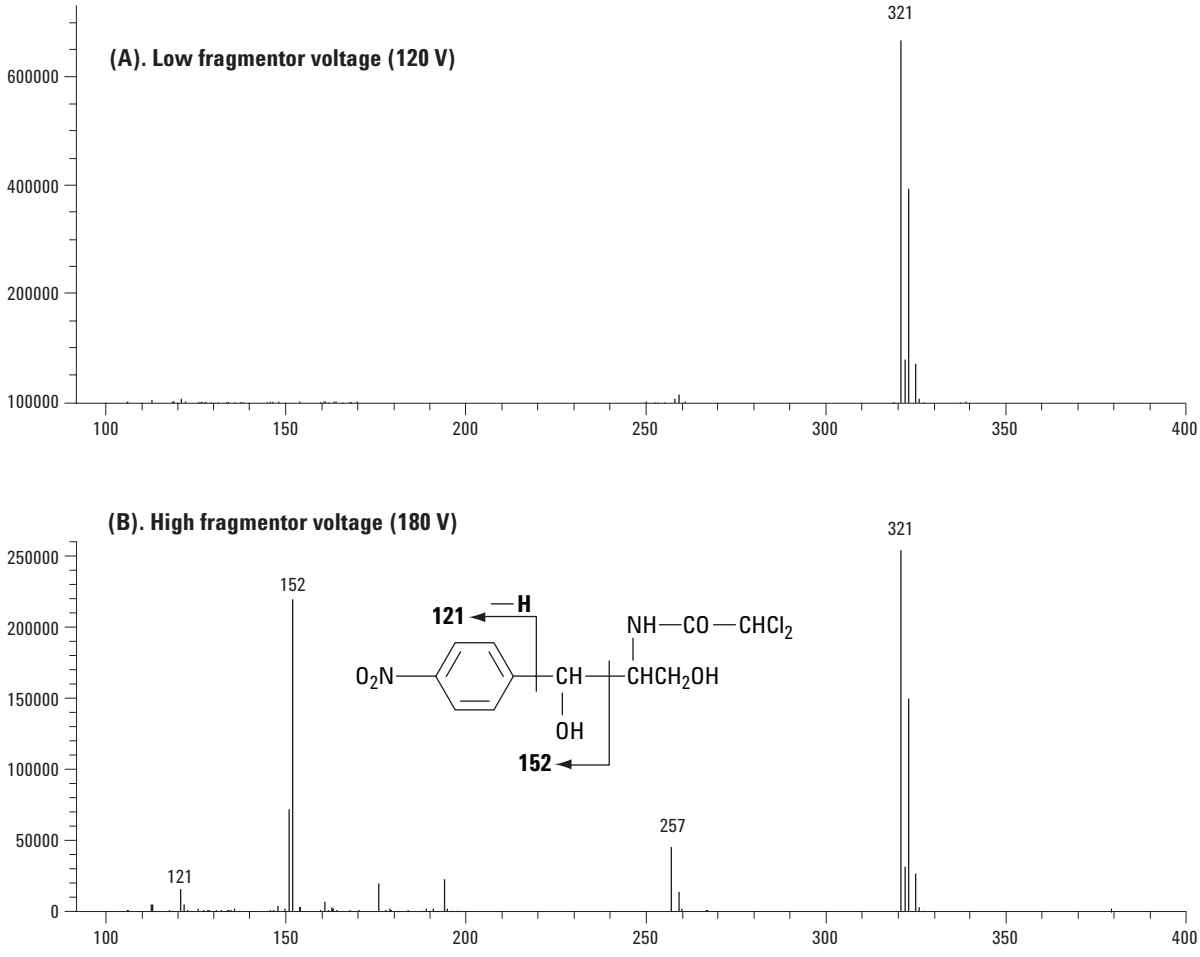


Figure 3. The mass spectra of CAP at two different fragmentor voltages.

Linearity, Detection Limit and Precision of LC/APPI-MS System

The analytical performance characteristics of the optimized LC/APPI-MS were first determined on standard solutions of CAP in pure solvent. See Figure 4. In order to achieve optimum sensitivity, all experiments were carried out under SIM mode using the mass corresponding to the $[M\text{-}H]^-$ ions for CAP. To test the linearity of the calibration curves, various concentrations of CAP ranging from 0.1 to 100 ng/mL were analyzed. The calibration curves of APPI showed good linearity with correlation coefficients (r^2) = 0.9998. The repeatability of APPI for a standard solution was calculated on the basis of five replicates at 0.5 ng/mL in the same day. The limit of detection (LOD) was calculated by using a *S/N* ratio of 3 at 0.1 ng/mL. The SIM chromatogram of CAP with APPI is shown in Figure 4 (the *S/N* ratio of this chromatogram was 4.2); LOD and RSD were 0.07 ng/mL and 2.1%.

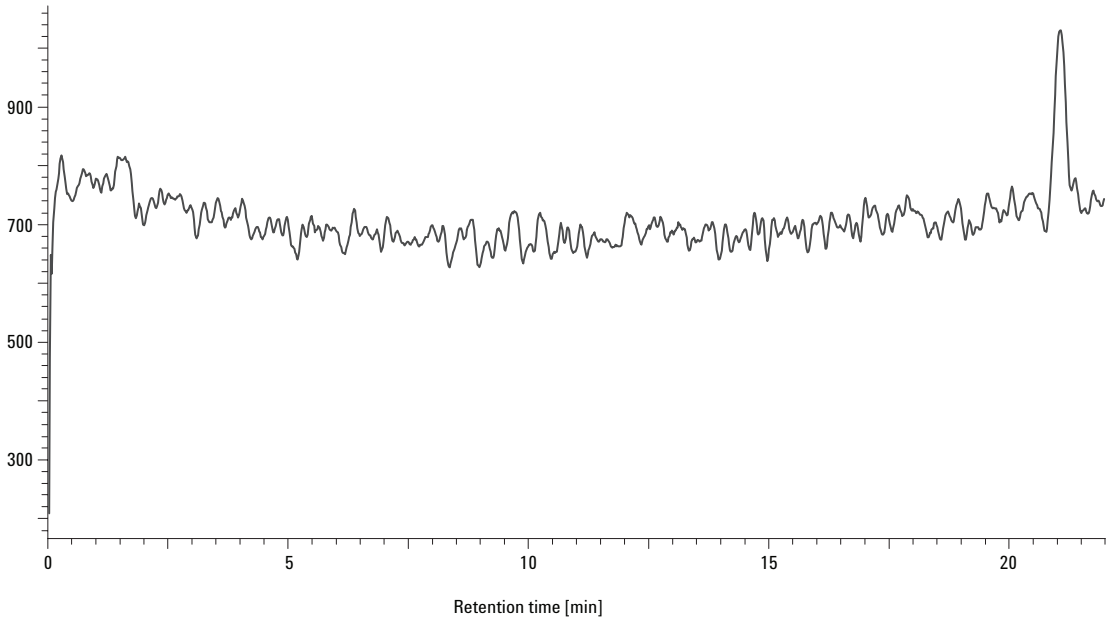


Figure 4. SIM chromatogram of CAP in pure solvent at 0.1 ng/mL with APPI.

APPI Method Evaluation

To evaluate recoveries, the proposed method was applied to the analysis of spiked CAP-free samples of young yellowtail and flatfish meat. Eighteen samples of two different fish were each spiked with CAP and each sample was spiked at three levels. The spiking levels ranged from 0.1 to 2 ng/g. Typical chromatograms from the fish meat extracts spiked at 1 ng/g and 0.1 ng/g are shown in Figure 5.

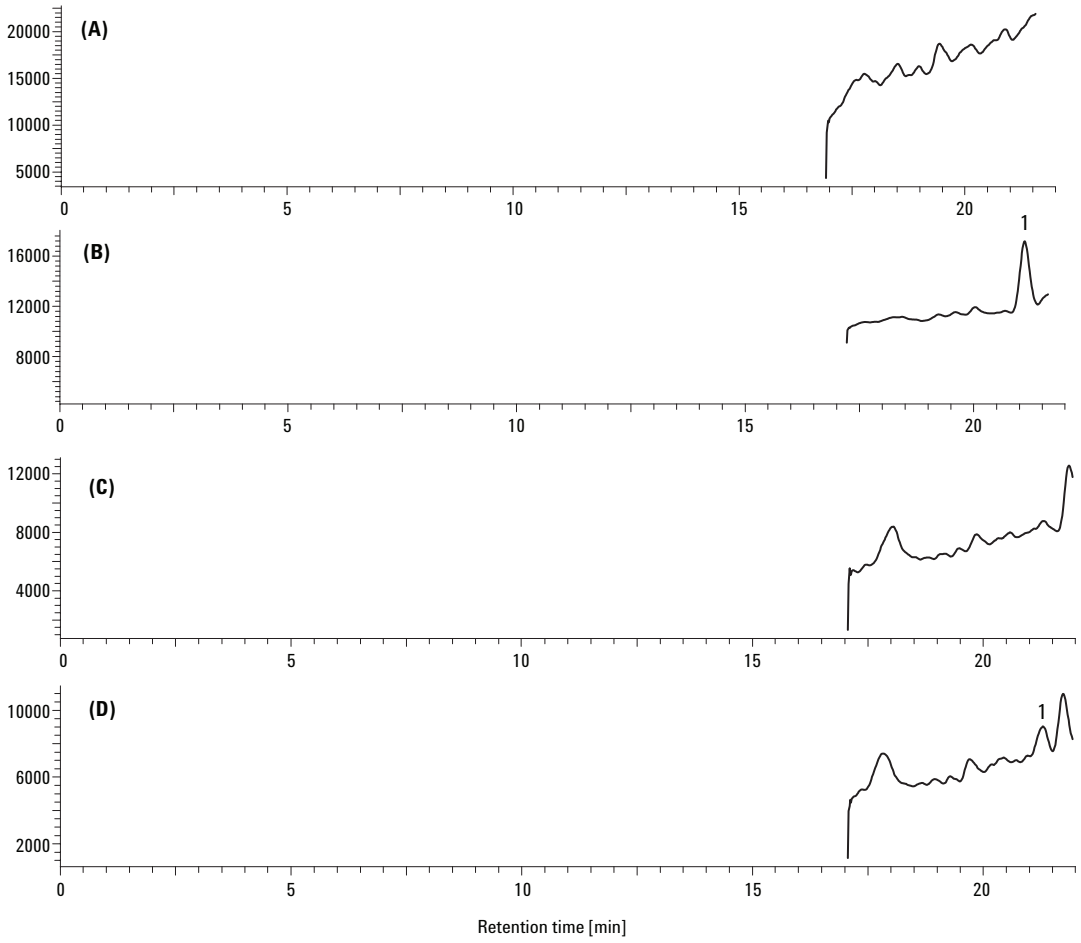


Figure 5. SIM chromatograms of A) Young yellowtail meat, B) Spiked young yellowtail meat at 1 ng/g CAP, C) a flatfish meat, and D) a spiked flatfish meat at 0.1 ng/g CAP.

Data from 18 spiked samples led to recoveries and RSD are summarized in Table 2.

Table 2 Recovery of CAP for Spiked Fish Meat

Spiking levels (ng/g)	Recovery [±RSD (%)]* Young yellowtail	Flatfish
0.1	89.3 ±5.1	87.4 ±6.1
0.5	102.5 ±4.9	94.8 ±6.7
2.0	96.1 ±4.3	91.8 ±4.9

*Three spiked samples at the same amount were analyzed.

Mean recoveries ranged from 87.4% to 102.5% with RSD of 4.3% to 6.7%. The LODs of CAP in fish meats were determined by the signal corresponding to three times the background noise on SIM chromatogram of spiked sample at 1 ng/g and 0.1 ng/g and shown in Table 3. The intraday precision (repeatability) was estimated by injecting the same spiked fish meat extract at 0.1 ng/g five times during a working day. The interday precision (reproducibility) was evaluated by analyzing the same sample over 5 working days. The repeatability and reproducibility for CAP in fish meats were 4.8%, 9.4% and 2.1%, 7.3%, respectively. These results indicate that this LC/APPI-MS method is suitable for the analysis of residues of CAP in fish meats.

Table 3. LODs, Repeatability, and Reproducibility of CAP in Standard Solution Using APPI

Fish meats	LODs* (ng/g)	Repeatability** (RSD, %)	Reproducibility*** (RSD, %)
Young yellowtail	0.27	4.8	9.4
Flatfish	0.10	2.1	7.3

*Detection limit is LOD defined as $S/N = 3$ at 0.1 ng/mL.

**Repeatability was calculated on the basis of five replicates at 0.5 ng/mL within 1 day.

***Reproducibility was calculated by analyzing one fish meat spiked at 0.1 ng mL^{-1} per day during 5 days.

Conclusion

APPI is an ideal ionization technique because of high sensitivity and high selectivity for the determination of CAP in fish meats. An important advantage of using APPI for CAP content of fish meats is that sample matrix did not significantly affect ion intensity of CAP. The data presented here demonstrate that this method is convenient for routine analysis of CAP residues in fish meats at trace levels, as excellent recoveries and precision for different samples were obtained.

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Detection, Confirmation, and Quantification of Chloramphenicol in Honey and Shrimp at Regulatory Levels Using Quadrupole and Ion Trap LC/MS

Application

Foods, Environmental

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Abstract

Methodology capable of meeting regulatory requirements has been developed for the determination of chloramphenicol in honey and shrimp. Samples of the two foodstuffs are extracted with Isolute HN-M cartridges and analyzed with both the Agilent 1100 LC/MSD Trap (SL) and the Agilent 1100 LC/MSD (SL) quadrupole with negative mode electrospray ionization. Using deuterated internal standard and one simple sample extraction procedure, both instruments provide a limit of detection at or below 0.1 ppb in both shrimp and honey. Detection limits are lower using the ion trap for shrimp because of less matrix interference. The Agilent 1100 LC/MSD gives quantitative results and the Agilent 1100 LC/MSD Trap gives full spectrum confirmation.

Introduction

Chloramphenicol is a broad range antibiotic that has found its way into foodstuffs such as honey and shrimp. Because it has displayed significant toxicological effects on humans, it has been banned from foods in the European community and the United States at levels greater than 0.1 ppb. Analytical methods used to determine this limit must achieve both the required sensitivity and maintain sufficient selectively. LC/MS has been demonstrated by the US Food and Drug Administration for these analysis [1-3]. In addition, the Commission of European Communities has issued guidelines stipulating that for mass spectral detection, a molecular ion (or quasimolecular ion) and at least two fragment ions are needed for positive confirmation [4]. For quantitative analysis the Agilent 1100 LC/MSD provides excellent results and can give some confirmation information. The Agilent 1100 LC/MSD Trap gives excellent full spectrum confirmation at the regulated concentration.

Experimental

Reagents and Materials

ISOLUTE HM-N cartridges from IST (Hengoed, UK, Part-nr. 800-1300-FM)

Ethyl acetate from Vel (Merck Eurolab, Leuven, Belgium)

Methanol HPLC-grade from Merck (LiChrosolv, Darmstadt, Germany)

Deuterated (d5) CAP internal standard from Cambridge Isotope Laboratories (CIL, Andover, MA, USA)

Syringe filters (0.2 µm, PTFE) from Alltech Associates Inc. (Lokeren, Belgium)

Sample Preparation

For honey, 5 g of sample is diluted to 20 mL with water and 5 µL of 1 ng/µL internal standard (IS) is added. The solution is loaded on the cartridge and allowed to stand for 5 minutes. Elution is performed with 50 mL ethyl acetate. The eluate is collected and the solvent is evaporated under a nitrogen stream at 40 °C. The residue is redissolved in 1 mL water/methanol (9/1, v/v) and put in an ultrasonic bath for 1 minute. The solution is filtered, using a syringe filter, before injection. No additional clean-up of the sample solution is performed.

For shrimp, a portion of at least 10 g of frozen shrimp is defrosted and mixed in a blender. To 10 g of the mixed shrimp, 30 mL of water and 10 µL of 1 ng/µL IS is added. This portion is centrifuged for 10 minutes (2000 rpm). A 20-mL portion of the supernatant is loaded on the cartridge and allowed to stand for 5 minutes. Elution is performed with 50 mL ethyl acetate. The eluate is collected and the solvent evaporated under a nitrogen stream at 40 °C. The residue is redissolved in 1 mL water/methanol (9/1, v/v) and put in an ultrasonic bath for 1 minute. The solution is filtered before injection.

LC/MS Conditions

The LC/MS systems were the Agilent 1100 LC/MSD quadrupole mass spectrometer and the Agilent 1100 LC/MSD Trap. Both were equipped with Agilent 1100 binary pumps and 1100 well plate autosamplers. See Table 1.

Table 1. LC/MS Conditions

HPLC		
Column	Eclipse XDB C18, 4.6 mm × 150 mm, 5 µm (p/n 993967.902)	
Flow-rate	0.9 mL/min	
Mobile phase	10 mM ammonium acetate in water (solvent A) Methanol/acetonitrile 1/9 (solvent B) both from Merck (LiChrosolv, Darmstadt, Germany)	
Gradient	0–1 min	30% B
	1–8 min	30%–70% B
	8–8.5 min	70%–100% B
	8.5–12 min	100% B
	Post time	4 min at 30% B
Injection	100 µL with needle wash (methanol)	
Injection solvent	Water/methanol (9/1 v/v) for both standards and samples	
Column temperature	30 °C	
MSD source settings		
Source	ESI	
Ion polarity	Negative	
Drying gas temperature	340 °C	
Drying gas flow-rate	11 L/min	
Nebulizer pressure	50 psig	
V_{cap}	3500 V	
Quadrupole MSD		
MSD acquisition on	Between 3 and 7.5 min	
Fragmentor	160 V	
SIM settings	*m/z* 257, 321, 323 (CAP) *m/z* 262, 326, 328 (CAP-d5)	

Table 1. LC/MS Conditions (continued)

Trap MSD	
MSD acquisition on	Between 3 and 7.5 min
Target mass (SPS)	323 *m/z*
Trap parameters	
Max. accumulation time	300 ms
ICC target	30,000
Scan range	160–340
Averaging	2
Fragmentation parameters (MS/MS)	
Smart Frag	On, 30%–200% (default)
Isolation mass	*m/z* 325.0
Isolation width	10.0 *m/z*
Fragmentation amplitude	1.0 V
Fragmentation cutoff	*m/z* 88

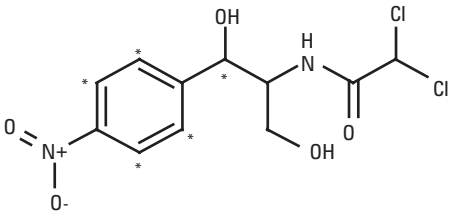
Results and Discussion

Spectral Quality and Sensitivity of Standards

For analysis with the quadrupole LC/MSD, selected ion monitoring (SIM) was used to obtain the required sensitivity. Table 2 shows the structure, fragment ions and identity of CAP and CAP-d5. Figure 1 shows the analysis of a standard mixture containing 2.5 pg/µL CAP and 5 pg/µL CAP-d5. By applying a fragmentor voltage of 160 V, fragment ions at *m/z* 257 and 262 are detected for confirmation purposes. Lowering the fragmentor voltage to optimize for the *m/z* 321 and *m/z* 326 and monitoring those ion alone would obtain greater sensitivity. However, the confirmation of the fragment ions would be lost. For screening analysis without confirmation this would be acceptable and provide a much lower limit of detection (LOD).

Table 2 Structure and Fragment Ions and Identity of CAP and CAP-d5 (* Indicates Deuterated Positions for the CAP-d5 IS)

Chloramphenicol structure



	m/z	Identity
CAP	257	$[M-H-HCOCl]^-$
	249	$[M-H-2HCl]^-$
	194	$[M-H-NH2CoCl2H]^-$
	176	$[M-H-NH2CoCl2H-H2O]^-$
CAP-d5	262	$[M-H-HCOCl]^-$
	254	$[M-H2HCl]^-$
	199	$[M-H-NH_2COCl_2H]^-$
	180	$[M-H-NH_2COCl_2H-HDO]^-$

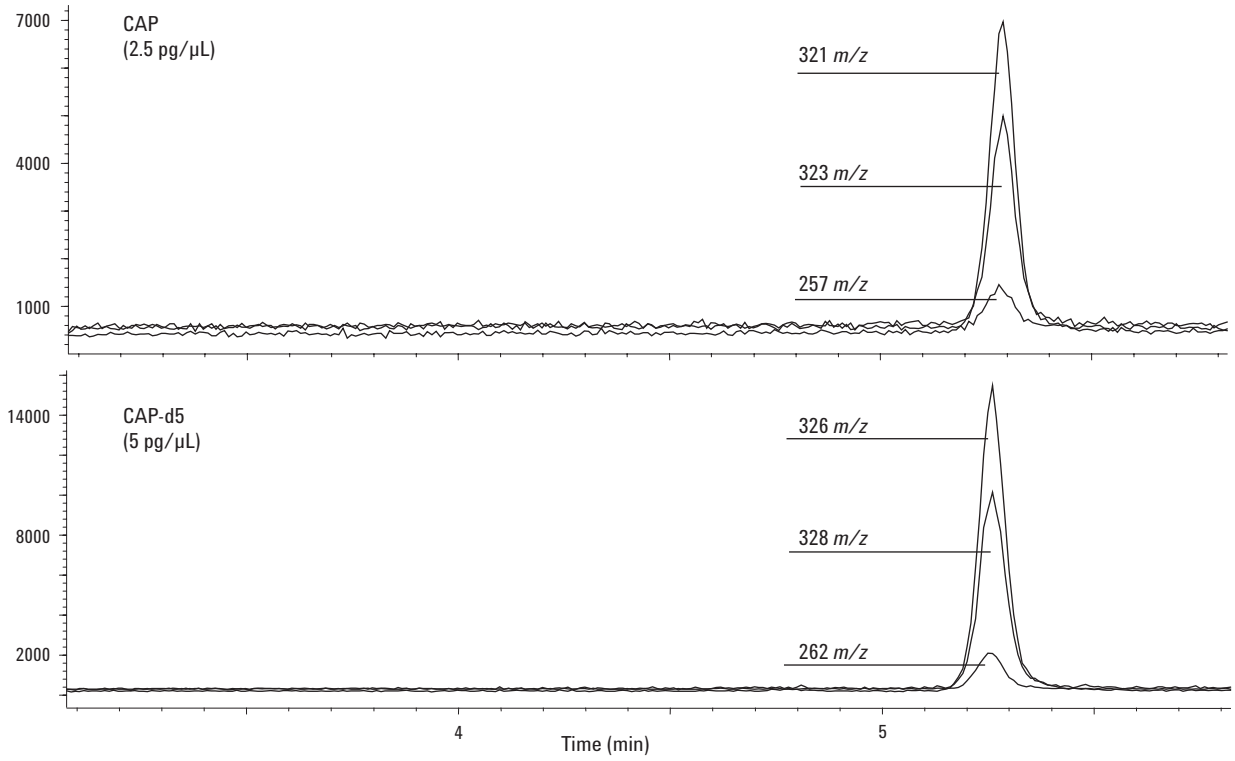


Figure 1. Analysis of a standard solution containing 2.5 ppb of CAP and 5 ppb of CAP-d5 (IS) on the quadrupole MSD. The extracted ion chromatogram for the corresponding ions are shown.

Using the LC/MSD Trap in MS/MS mode both the needed sensitivity (through reduction in chemical noise) and selectivity (for confirmation) is obtained. The compound shows a clear and reproducible fragmentation pattern. An example of the analysis of the standard mixture together with the corresponding MS/MS spectra is shown in Figure 2. Optimizing the fragmentation energy [turning off Smart Frag] and fragmentation cutoff in the ion trap will increase sensitivity even further than shown here. Using an isolation width of 10 *m/z* allows inclusion of the chlorine isotopes in the resulting full scan mass spectra of the analyte and the Cl^{35} isotope of the internal standard. Contact Agilent for more details on these and other ion trap settings.

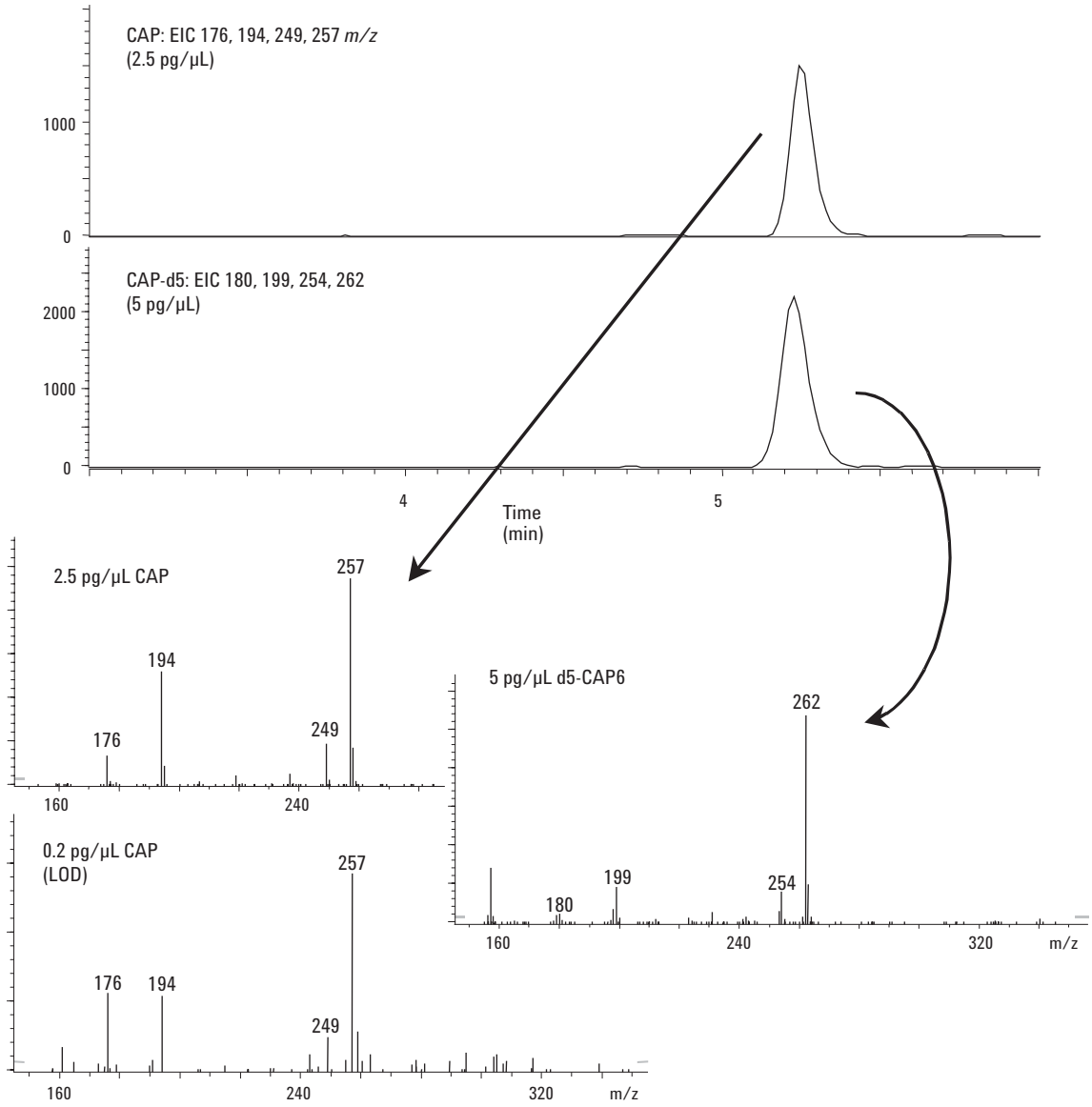


Figure 2. Analysis of a standard solution containing 2.5 pg/µL CAP and 5 pg/µL CAP-d5 (IS) on the LC/MSD Trap together with the corresponding MS/MS spectra and the MS/MS spectrum resulting from an analysis of a standard solution containing 0.2 pg/µL CAP.

Method Performance

Standard solutions of CAP containing 5 pg/µL of CAP-d5 were injected six consecutive times to test repeatability of injection on the mass selective detector (MSD) quadrupole instrument. This was done at two concentration levels. Each time, the response of CAP relative to CAP-d5 was recorded. For a solution containing 0.5 pg/µL CAP the relative standard deviations (RSDs) on the relative response were 5.05%. This 0.5-pg/µL level would correspond to a sample containing approximately 0.1 ppb CAP with the five-fold concentration step. When a solution containing 5 pg/µL CAP was analyzed, RSDs on the relative response were 1.28% for the quadrupole.

A calibration line was constructed by injecting standard solutions of CAP with a concentration of 0 to 25 pg/µL with 5 pg/µL of the IS added to each solution. One injection was performed per concentration. The quadrupole showed a linear response for CAP in this concentration range. Calibration curves and correlation coefficients are shown in Figure 3. The LOD with this method was determined to be ca. 0.2 pg/µL in a standard solution for both mass spectrometers. With the 100-µL injection used, this corresponds with 20 pg on-column.

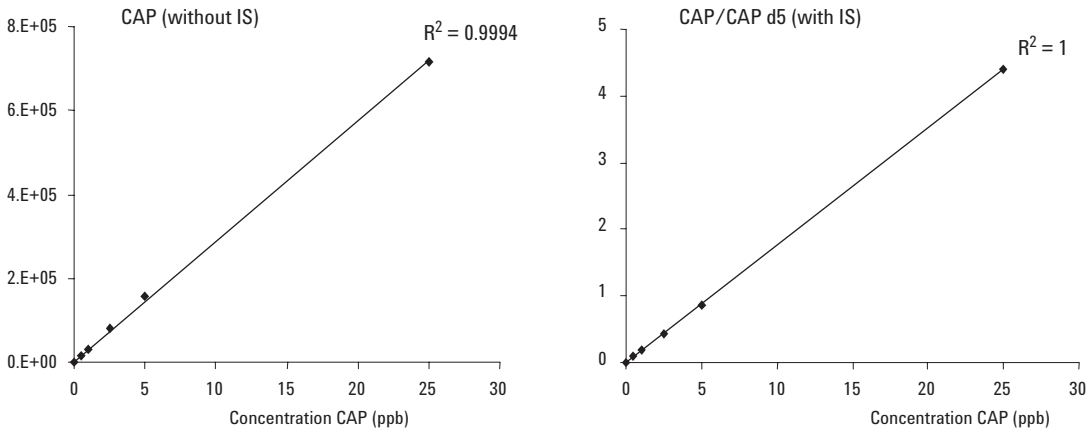


Figure 3. Calibration graphs for standard solutions of CAP on the quadrupole with and without CAP-d5 (IS).

Extraction Recovery and Repeatability of Extraction

The extraction procedure was evaluated on repeatability and linearity with the quadrupole instrument. Blank honey was spiked with 1 ppb CAP and 1 ppb CAP-d5. The extraction procedure was carried out six times and the recovery was calculated. The recovery for CAP varied from 85.31% to 94.94% and the mean recovery was 90.60%. The RSD on the recovery was 4.34% for CAP and 3.39% when the IS was taken into account. An analysis of blank honey spiked only with the IS is shown in Figure 4 run on both instruments. With the quadrupole, LC/MSD matrix interferences are present but chromatographically separated from the CAP signal. The ion trap results show that no matrix interference is present in the isolation window from *m/z* 318 to 328. The data suggest that other endogenous compounds in honey produce fragments at the same *m/z* as CAP. This supports an even lower detection limit for this matrix if a screening analysis were conducted with a lower fragmentor voltage monitoring only the *m/z* 321.

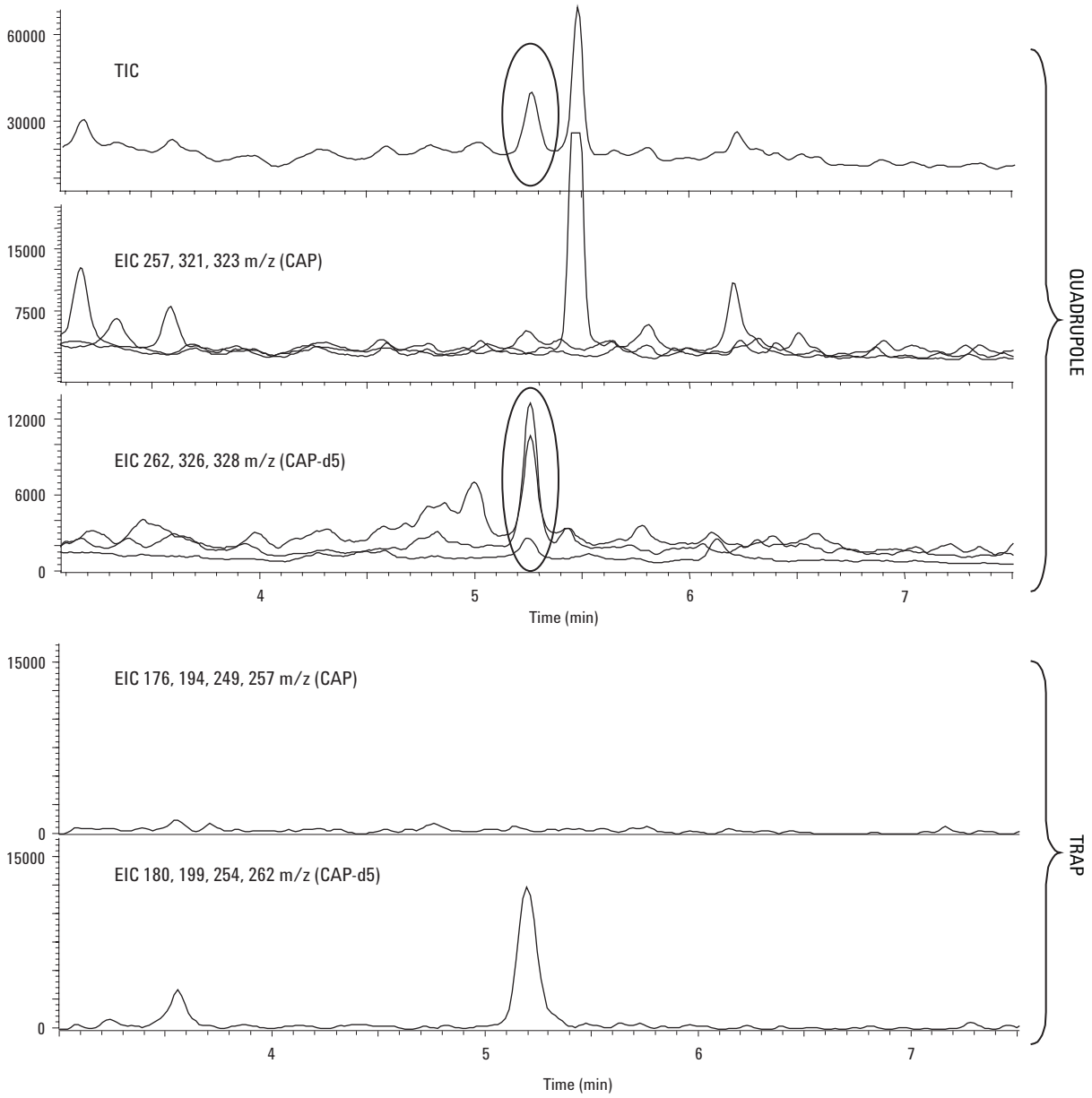


Figure 4. Analysis of a blank honey sample containing 1 ppb CAP-d5.

A calibration curve was constructed with blank honey samples spiked with 0, 0.1, 0.2, 0.5, 1.0, and 2.0 ppb CAP. The samples also contained 1 ppb of the IS. The correlation coefficients were 0.9997 and 0.9998 without and with correction with the IS, respectively. The slope for the calibration curve constructed with these extracts for CAP with correction with the IS was 0.1822. This is in good agreement with the slope obtained with the standard solutions, which is 0.1758 (see Figure 3).

Spectra on the trap were similar for standard solutions and real samples. An example of an MS/MS spectrum of an extract of a honey sample spiked with 0.5 ppb CAP and 1 ppb CAP-d5 is shown in Figure 5. Since the analyte and the IS coelute, a mixed spectrum is obtained. This could be avoided by using a smaller isolation width and the multiple reaction monitoring (MRM) function of the ion trap. Note that the chlorine isotope for $Cl^{35}Cl^{37}$ is not observed for the deuterated internal standard because its precursor ion is at the edge of the isolation width and thus not trapped.

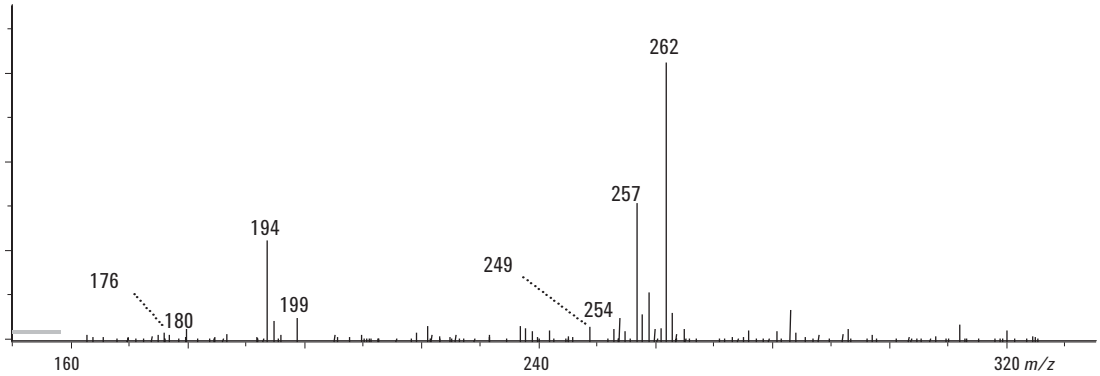


Figure 5. Ion trap MS/MS spectrum from analysis of a honey sample spiked with 0.5 ppb CAP and 1 ppb CAP-d5.

Analysis of Honey

The extraction procedure and LC/MS methods were applied to the analysis of honey samples that were known to contain CAP. Sample results obtained with the quadrupole and trap MSD were compared (Figure 6).

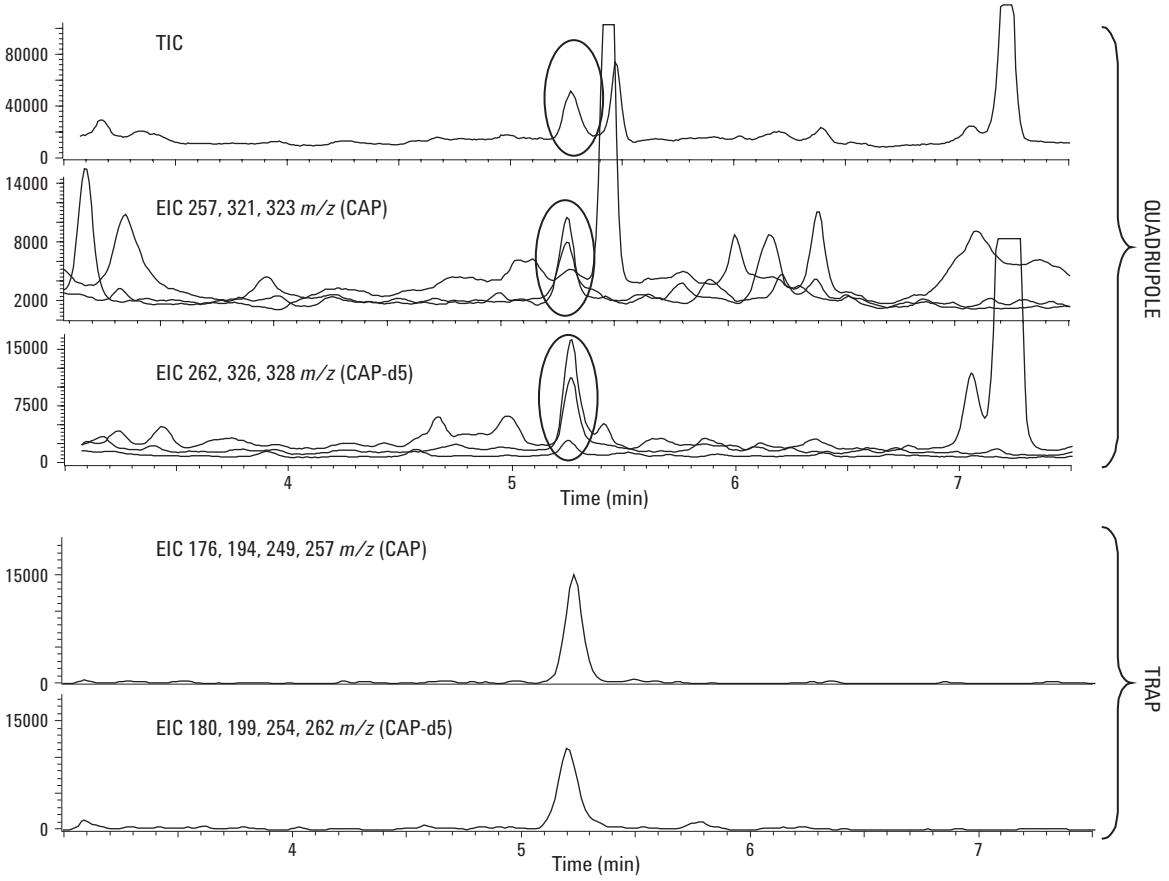


Figure 6. Analysis of a honey sample containing 0.5 ppb CAP and 1 ppb CAP-d5.

The LOD for the honey samples varies between detectors. For the quadrupole, it is found to be 0.5 pg/µL in the analytical solution. This corresponds with 50 pg on-column. Taking into account the sample preparation with a five-fold concentration, samples containing 0.1 ppb CAP can be detected. It is obvious that the sample matrix interferes with the sensitivity (Figures 4 and 6). Due to the increased selectivity using MS/MS in the trap, the LOD with this MS is similar for honey samples as for the standard solutions and is ca. 0.2 pg/µL in the analytical solution. This is equivalent to 0.04 ppb CAP in the sample because of the five-fold concentration step.

Analysis of Shrimp

The same sample preparation method was applied to the analysis of shrimp. The total volume of shrimp and water added was about 40 mL. Taking 20 mL of the 10 g shrimp aliquot for the Isolute sample preparation and reconstituting the dried extract in 1 mL produced a five-fold concentration as with the honey. This sample preparation shows less matrix interference with the analysis compared to honey samples. An example of an analysis of shrimp is shown in Figure 7. Due to the reduced matrix effect, the LOD with the quadrupole is lowered to nearly the same level as for the trap (0.05 ppb in the sample with the five-fold concentration). A concentration of 0.35 ppb was recovered in the shrimp sample by both the quadrupole and the trap MSD. Extraction recovery was approximately 85%.

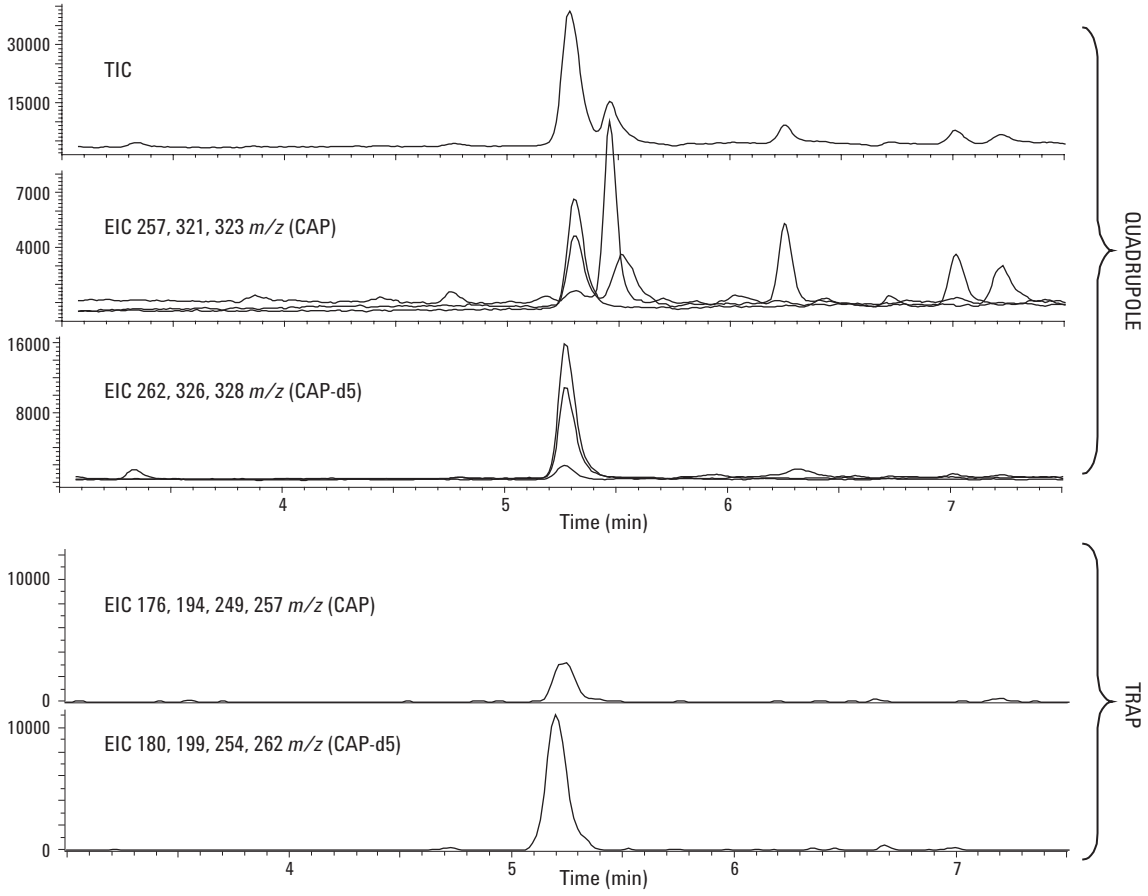


Figure 7. Analysis of a shrimp containing 0.35 ppb CAP and 1 ppb CAP-d5.

Conclusion

Honey and shrimp samples were successfully analyzed for CAP with both the quadrupole and trap MSD. A simple liquid-liquid extraction procedure using ISOLUTE HM-N cartridges was found to perform excellently in view of recovery and repeatability. The LC method used a standard 4.6-mm id column and produced the required sensitivity on both instruments. The LC/MSD quadrupole instrument produced excellent linearity and demonstrated its quantitative ability. The LC/MSD Trap showed the needed sensitivity with excellent full scan capability below the regulated limit in both sample matrices. The use of a broad isolation window for full scan spectra using the ion trap produced more transition ions than required for confirmation.

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A Validated Atmospheric Pressure Chemical Ionization Method for Analyzing Sulfonamides in Pork Muscle

Application

Food

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Abstract

This application note presents a simple method for the analysis of sulfonamide antibiotics in pork muscle. Samples were extracted with acidified methanol, centrifuged, and a portion of the extract was diluted with water. This dilution was analyzed directly by HPLC mass spectrometry using chemical ionization, with all compounds eluting in less than 5 minutes. Using an internal standard, recoveries for seven sulfonamides ranged from 84%–118% at a spiking level of 50 ppb (ng/g). The statistically derived detection limit was 10–25 ppb. A comparison was made to the cleaned extracts using solid phase extraction, as well as a comparison of mass selective detector settings for both screening (maximum sensitivity) and confirmation (greater fragmentation). The enhanced sensitivity of the Agilent quadrupole mass selective detector allows this dilution cleanup technique to be used in labs where high throughput is required.

Introduction

Meat, edible organs, animal feed, and animal waste may contain antibiotics, growth hormones, and other chemicals that can enter the food supply. These compounds are added to maintain animal health, to increase animal growth rate, and to reduce stress. Human exposure can result from eating contaminated meat, or contacting runoff and leaching from manure and compost. Health specialists warn that there may be reduced options for effectively treating disease with antibiotics, such as penicillin and sulfa drugs, since antibiotic-resistant strains of bacteria may develop from the low-level exposure.

Sulfonamides are broad-spectrum antimicrobials used in both humans and animals. The maximum residue limit (MRL) in Canada for sulfonamides in meat is 100 ppb (ng/g), and 10 ppb in milk, while the MRL in the European Union is 100 ppb for both of these matrices. The Canadian Food Inspection Agency method for sulfonamides in meat tissue calls for extraction in ethyl acetate, partitioning with glycine buffer, followed by a pH-adjusted back extraction into methylene chloride [1]. Extracts are evaporated, reconstituted, then separated by thin layer chromatography (TLC), derivatized, and quantitated by densitometry. Alberta Agriculture has improved the quantitative and qualitative aspects by using liquid chromatography/mass spectrometry (LC/MS) with atmospheric pressure chemical ionization (APCI) for the final analysis [2]. There are a number of

extraction steps in the Alberta method, and a faster method would greatly benefit laboratories monitoring the food supply for residues.

The goal of this method was to reliably quantitate the sulfa drugs at one-half of the regulatory limit or lower, with minimal sample preparation, and a maximum injection cycle time of 10 minutes. Maximum sensitivity is generally obtained by forming as many parent ions $[M+H]^+$ as possible and minimizing fragmentation. Due to the operational complexity of triple quadrupole instruments, it is also desirable to confirm positive findings on a single quadrupole. This could be achieved by using collision induced dissociation (CID) to enhance fragment ions characteristic of the compounds.

Experimental

Chemicals and Materials

All sulfonamide standards were purchased from Sigma Aldrich Canada, with a minimum purity of 99%. Stock solutions were prepared at 2 mg/mL in acetone, with the exceptions of sulfadiazine and the sodium salt of sulfaquinoxaline. Three mL of 0.2N NaOH was added in order to completely dissolve these compounds. Standard solutions at different concentrations were prepared for spiking and quantitation by diluting with de-ionized water.

Internal standard (IS): sulfachloropyridazine (SCPD) at 2 mg/mL in de-ionized water.

HPLC-grade methanol and acetonitrile were purchased from Caledon Labs (Georgetown, Ontario).

Formic acid (min. 98%), was purchased from EM Science.

Acidified methanol was prepared by adding about 100 µL of 98% formic acid to 100-mL methanol.

Ultra-Turrax T8 homogenizer with 8-mm diameter dispersing element, 50-mL polypropylene centrifuge tubes, and 13-mm polyvinylidene fluoride (PVDF) syringe filters (0.2 µm), were purchased from VWR Scientific.

Oasis HLB (3 cc, 60 mg) solid phase extraction (SPE) cartridges were purchased from Waters.

Sample Preparation

1. For pork muscle, 3 g samples were weighed directly into 50-mL polypropylene centrifuge tubes.
2. The samples were homogenized for 3 minutes with 10 mL acidified methanol using the Ultra-Turrax homogenizer.
3. The samples were then centrifuged for 10 minutes, and the supernatant decanted into a clean test tube.
4. The samples were then re-extracted with a further 10 mL acidified methanol, and centrifuged again.
5. The supernatants were combined, and 1 mL IS (2 mg) was added to the combined extract.
6. The extract was diluted with de-ionized water 1 in 4 (250 µL extract + 750 µL water), filtered through a 0.2 µm PVDF filter into an autosampler vial, and analyzed directly by LC/MS.

By adding an accurately known amount of IS to the combined extracts, there is no need to measure the final volume of the extract. The IS calculations performed by the ChemStation measure the relative amounts of the analytes and IS. This corrects for any concentration or dilution effects in the samples.

Sample extracts were also taken through SPE cleanup cartridges in order to compare with the dilution-only extracts. The 60-mg Oasis HLB cartridges are prewashed by eluting 1.5 mL acidified methanol, followed by 1.5 mL de-ionized water. The 1 mL extract was diluted to 10 mL with de-ionized water, eluted through the cartridges, and the eluant was discarded. The sulfa drugs were then eluted with 1.5 mL acidified methanol. This eluant was evaporated to near dryness under nitrogen. Samples were reconstituted in 1 mL of 25% methanol in water, filtered, and analyzed by LC/MS.

A further comparison was done by evaporating 1 mL methanol extract to near dryness, and reconstituting it in 1 mL of 25% methanol in water without the SPE cleanup. This gave the sample extract the proper solvent composition for HPLC analysis, but without the dilution step to negatively affect the detection limits (DL) of the compounds.

LC/MS Conditions

The LC/MS system was made up of Agilent Technologies 1100 Series solvent degasser, binary pump, autosampler, column oven, diode array detector, and quadrupole mass selective detector (MSD) (Table 1).

Compound Identification and Confirmation

In general, the goal of a monitoring method for target analytes is to separate the compounds from potential interferences and maximize sensitivity on the instrument. Using mass spectrometry (MS), maximum sensitivity is achieved by the production of a single ion, for example, the protonated parent ion $[M+H]^+$ in LC electrospray ionization (ESI) or APCI in target ion mode. However, once a positive is detected, a confirmation must be made as to whether the suspect peak is actually the target analyte, or simply a co-eluting compound that produces the same ion. There are a number of ways to perform the confirmation: re-extract the sample with a different solvent system; further clean up the sample to a higher final concentration, to allow detection of additional confirmation ions or analysis in scan mode; derivatize and analyze by gas chromatography/mass spectrometry (GC/MS); or re-analyze the extract on a triple quadrupole LC/MS/MS. All of these techniques are useful, but the drawback is the additional time and expense involved, especially with LC/MS/MS.

Table 1. LC/MSD Conditions

HPLC	
Column	Zorbax Eclipse XDB-C8, 150 mm × 4.6 mm, 5 µm (p/n 993967-906)
Solvent A	0.1% Formic acid in water
Solvent B	0.1% Formic acid in acetonitrile
Gradient	t_0 = 20% B t_1 = 20% B t_3 = 90% B $t_{6.5}$ = 90% B Post time = 1.5 min
Flow rate	1.0 mL/min
Injection volume	50 µL
Column temp	30 °C
MSD	
Source	APCI (positive ion mode)
Ion dwell time	8 Ions at 63 ms each
Fragmentor	70 V
Drying gas	6.0 L/min
Nebulizer pressure	60 psi
Drying gas temperature	350 °C
Vaporizer temperature	400 °C
Capillary voltage	3000 V
Corona current	4 µA

The Agilent 1100 MSD has the capability of acquiring up to four separate MS signals during the same run, where each signal can be made up of a number of selected ions (SIM) or a full scan spectrum. For example, Signal 1, with a low fragmentor voltage to maximize parent ion response, can include each of the $[M+H]^+$ ions in the target list, while Signal 2, at higher fragmentor voltages can acquire the confirmatory fragment ions. For analytes expected at higher concentrations, Signal 1 could acquire in SIM mode for quantitation, while Signal 2 could be set for scan mode for identification. Figure 1 demonstrates the former example, with the Fragmentor set to 70 V for Signal 1 (MSD1), and 200 V for Signal 2 (MSD2).

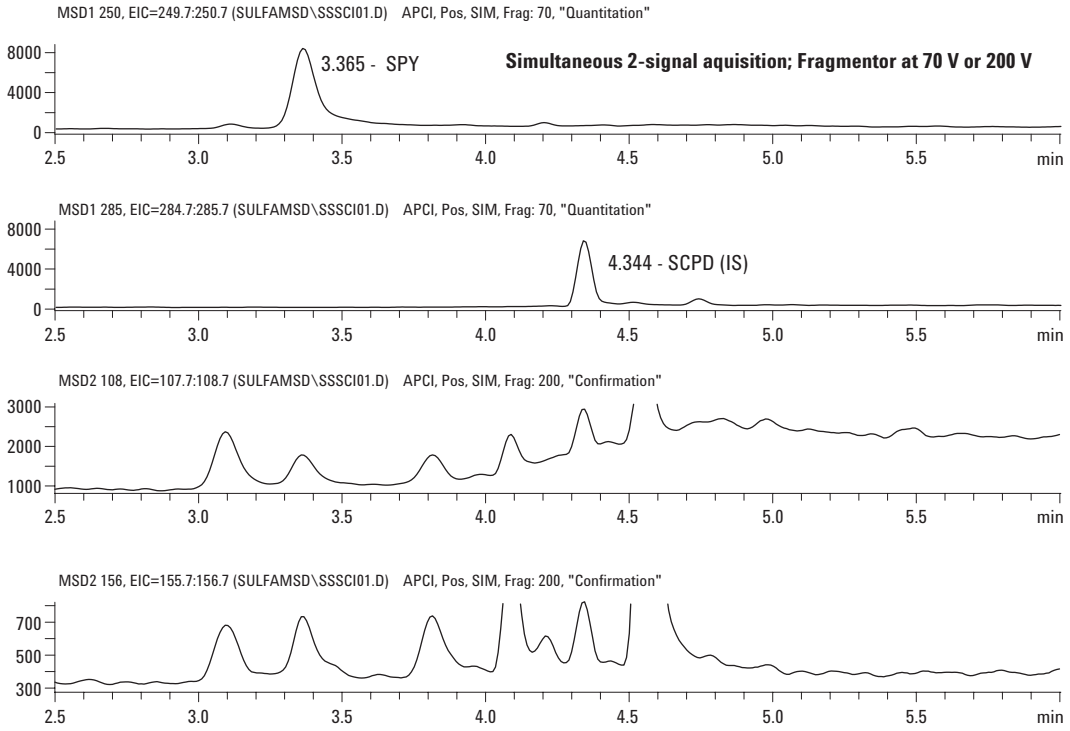


Figure 1. Dual MSD acquisition signals (Masses 108 and 156 are class-specific fragments for sulfonamides).

Table 2 shows the mass spectra for the sulfonamides using various fragmentor voltages. Masses 108 and 156 are class-specific fragments for sulfonamides ($H_2N^+=[C_6H_4]=O$ and $H_2N^+=[C_6H_4]=SO_2$, respectively), and, as such, are very useful diagnostic ions, when acquired along with the protonated molecular ion.

Table 2. APCI Spectra of Sulfonamides, Using Various Fragmentor Voltages

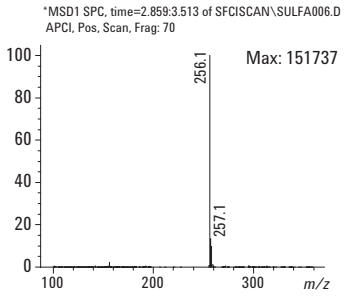
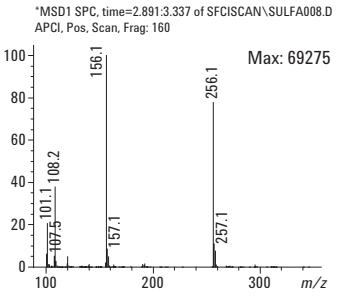
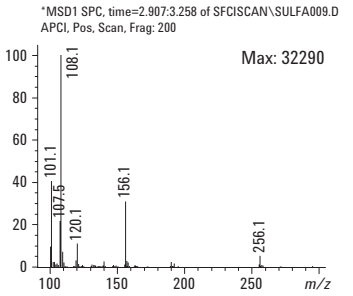
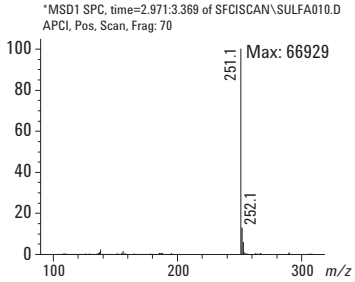
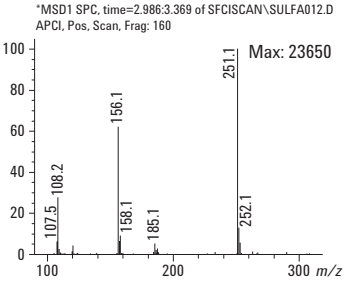
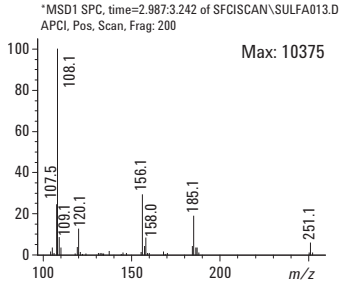
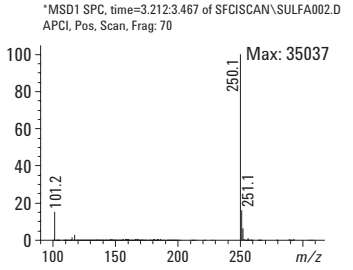
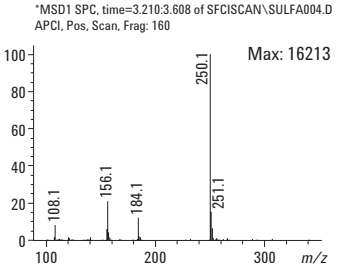
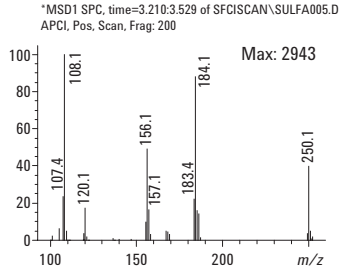
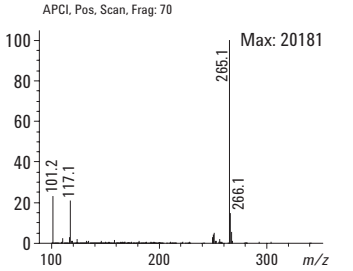
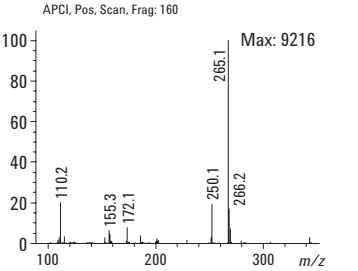
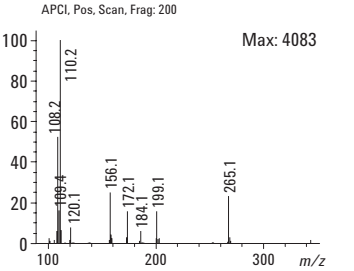
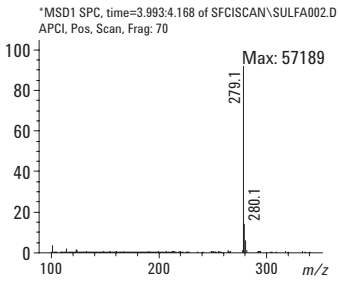
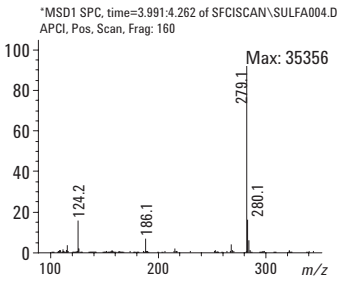
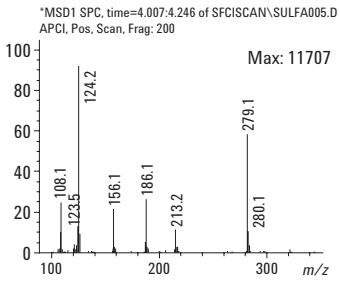
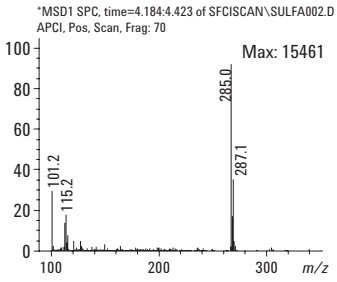
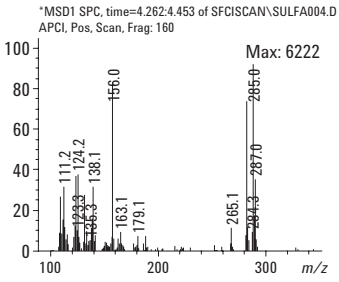
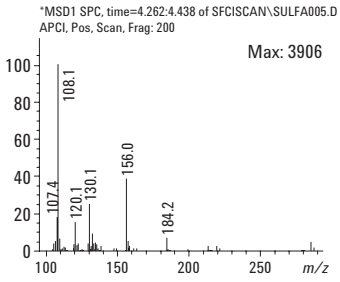
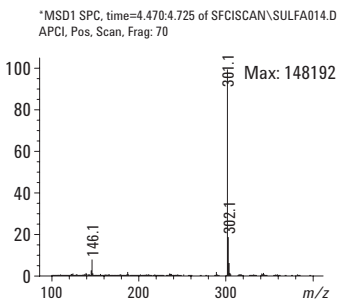
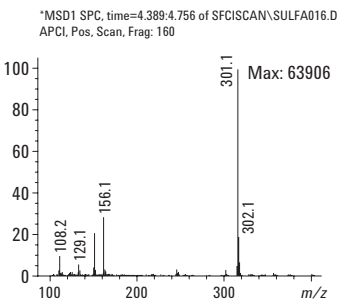
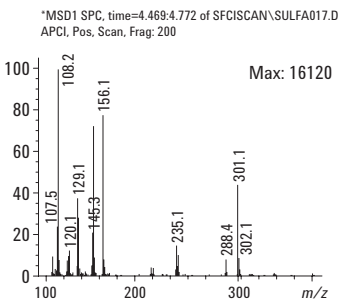
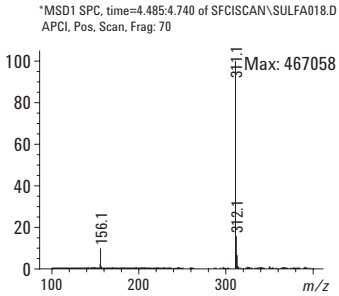
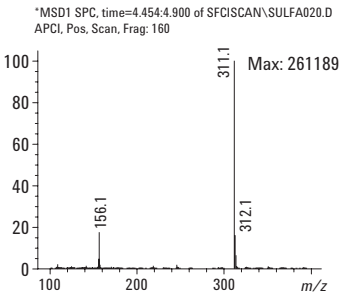
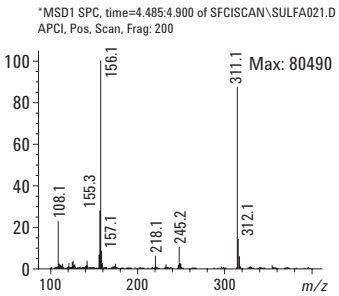
Compound	Fragmentor 70 V	Fragmentor 160 V	Fragmentor 200 V
Sulfathiazole (STZ) $C_9H_9N_3S_2O_2$ MW = 255 RT = 3.05 min			
Sulfadiazine (SDZ) $C_{10}H_{10}N_4SO_2$ MW = 250 RT = 3.09 min			
Sulfapyridine (SPY) $C_{11}H_{11}N_3SO_2$ MW = 249 RT = 3.33 min			
Sulfamerazine (SMR) $C_{11}H_{12}N_4SO_2$ MW = 264 RT = 3.78 min			

Table 2. APCI Spectra of Sulfonamides, Using Various Fragmentor Voltages (Continued)

Compound	Fragmentor 70 V	Fragmentor 160 V	Fragmentor 200 V
Sulfamethazine (SMZ) $C_{12}H_{14}N_4SO_2$ MW = 278 RT = 4.06 min			
Sulfachloropyridazine (SCPD); istd $C_{10}H_9N_4SO_2Cl$ MW = 284 RT = 4.31 min			
Sulfaquinoxaline (SQ) $C_{14}H_{12}N_4SO_2$ MW = 300 RT = 4.51 min			
Sulfadimethoxine (SDMX) $C_{14}H_{12}N_4SO_2$ MW = 310 RT = 4.54 min			

Chromatography

While complete separation of target compounds is not always necessary when using mass spectral detection, it is, however, essential when common ions are present. For example, the protonated molecular ion of SPY is 250 mass units. Due to the naturally-occurring C^{13} isotope, ions 251 coexist with the parent ions 250. Separating SPY from SDZ (m/z = 251) was, therefore, important when trying to optimize the chromatographic conditions, and was achieved as shown in Figure 2. While this results in a slightly longer chromatographic run than would otherwise be necessary, there is more consistent integration of the peaks during data analysis; the chromatogram is easier to interpret; and the amount of SDZ is not underestimated due to co-elution of SPY in the standard mix.

A recently published application shows four sulfonamides were analyzed with an injection cycle time of 1.1 minutes, using a 2-position 10-port valve, two analytical columns in parallel, and a second binary pump [3]. Since most labs do not have such high sample volume requirements, the method described in this application note was developed using more conventional techniques, without the additional hardware costs. Conditions were set up to provide good chromatographic separation in a relatively short time of 6 minutes (total cycle time was 10 minutes).

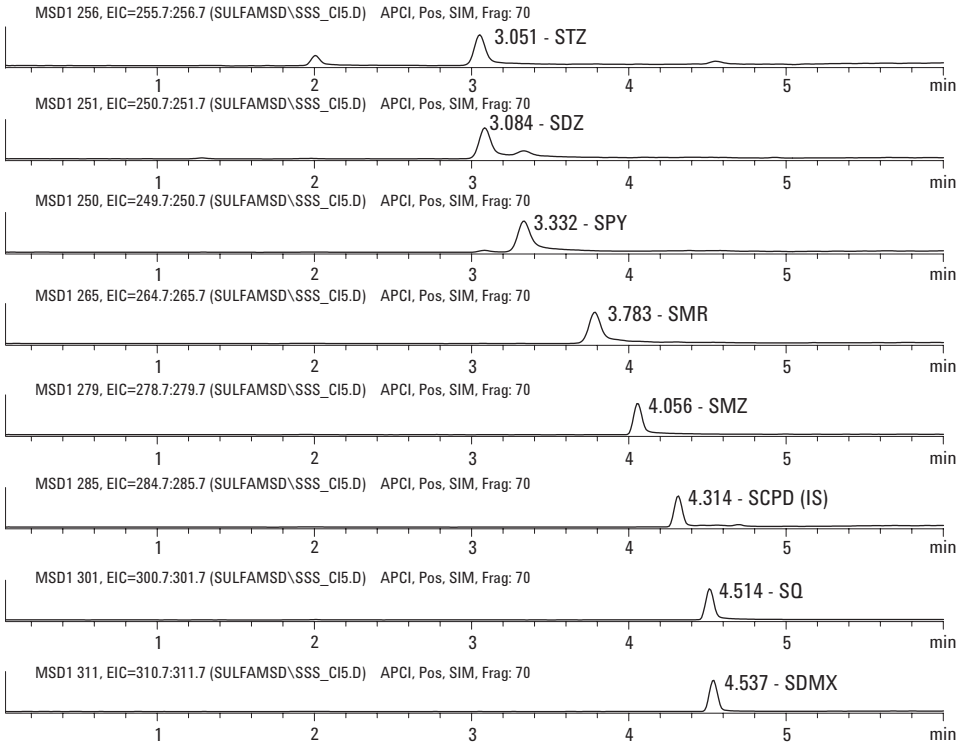


Figure 2. Sulfonamide standard mix, 500 pg each (SIM).

Sample Cleanup

The total ion chromatograms (TIC) in Figure 3 show that there is considerable matrix background from the samples. A simple solvent exchange was performed, where 1 mL of extract was evaporated under nitrogen, and reconstituted in 25% methanol in water. One of the problems with solvent exchange only is the amount of matrix material that is injected onto the HPLC column. Peak shape can be negatively affected by overloading, and eventually the performance of the column will deteriorate. All of this matrix material is also introduced into the MSD. Frequent cleaning and maintenance may be required for the MSD, further reducing productivity.

In order to develop a high-throughput method, keep the number of required steps to a minimum. The Agilent liquid chromatography/mass selective detector (LC/MSD) has enough sensitivity to allow simple dilution of the extracts with water to act as a cleanup technique. This eliminates the need for costly SPE cartridges and analyst time to further prepare the samples. Minimal sample handling can also improve recoveries, since losses are possible at each step.

The third chromatogram in Figure 3 shows how the use of SPE cleanup techniques can remove the majority of co-extracted materials, allowing for a more concentrated final extract and ultimately lower DLs. This also results in a simpler chromatogram for integration and interpretation.

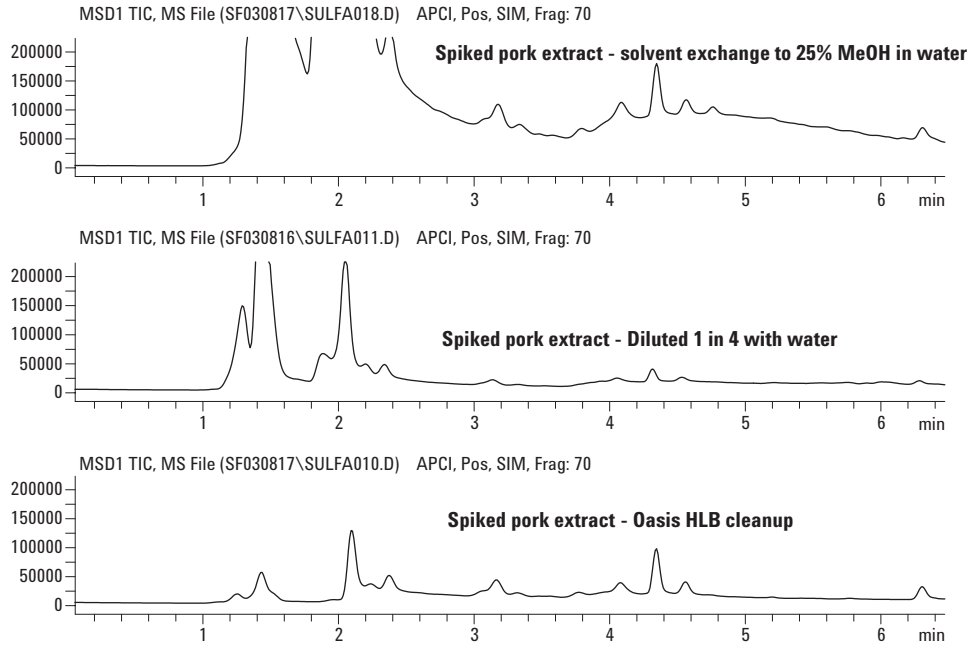


Figure 3. TIC comparisons of various cleanup techniques.

However, where the goal of a method is to screen large numbers of samples to find potential violations of MRLs, a simple dilution technique may be preferred. Dilution could offer enough cleanup for good chromatographic separation, while remaining concentrated enough to meet DL requirements. The second chromatogram in Figure 3 shows a much improved baseline. Figures 4 through 6 show the same analyses with all the target ions in SIM mode.

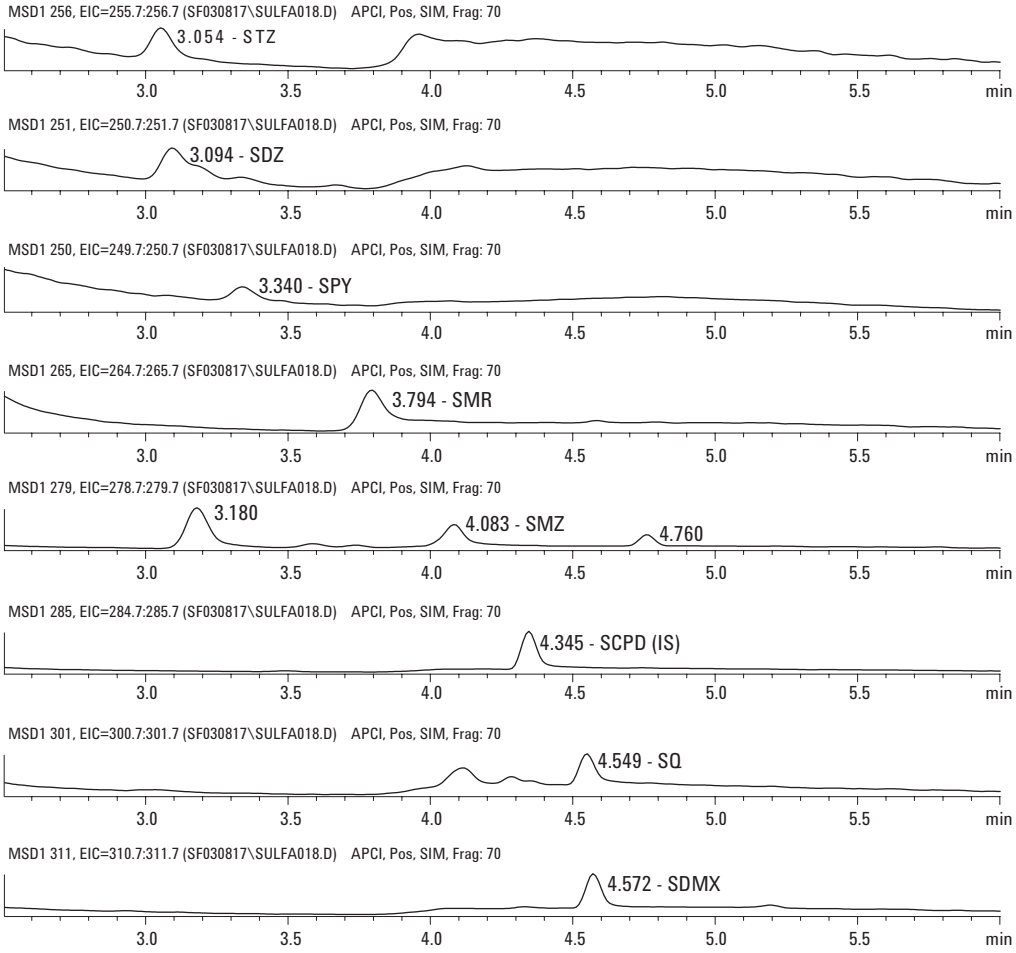


Figure 4. Solvent exchange only (SIM)

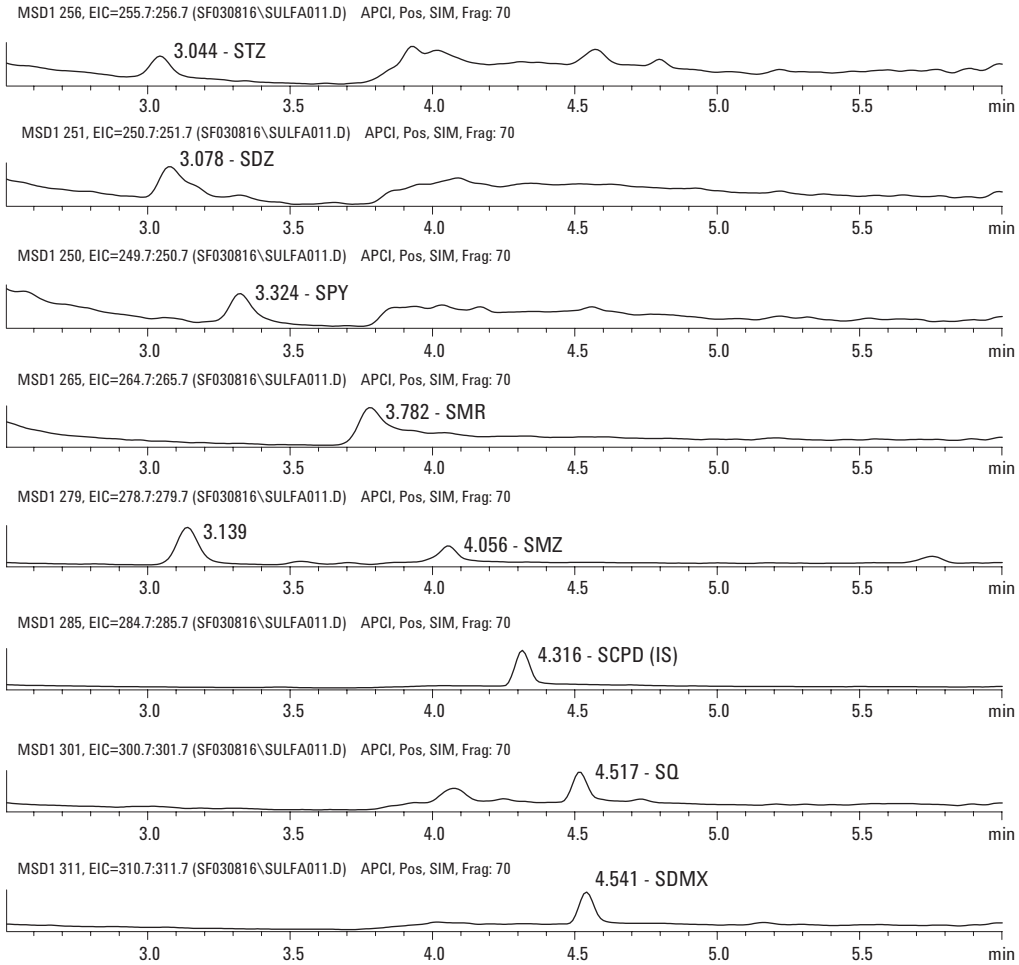


Figure 5. Diluted 1 in 4 with water.

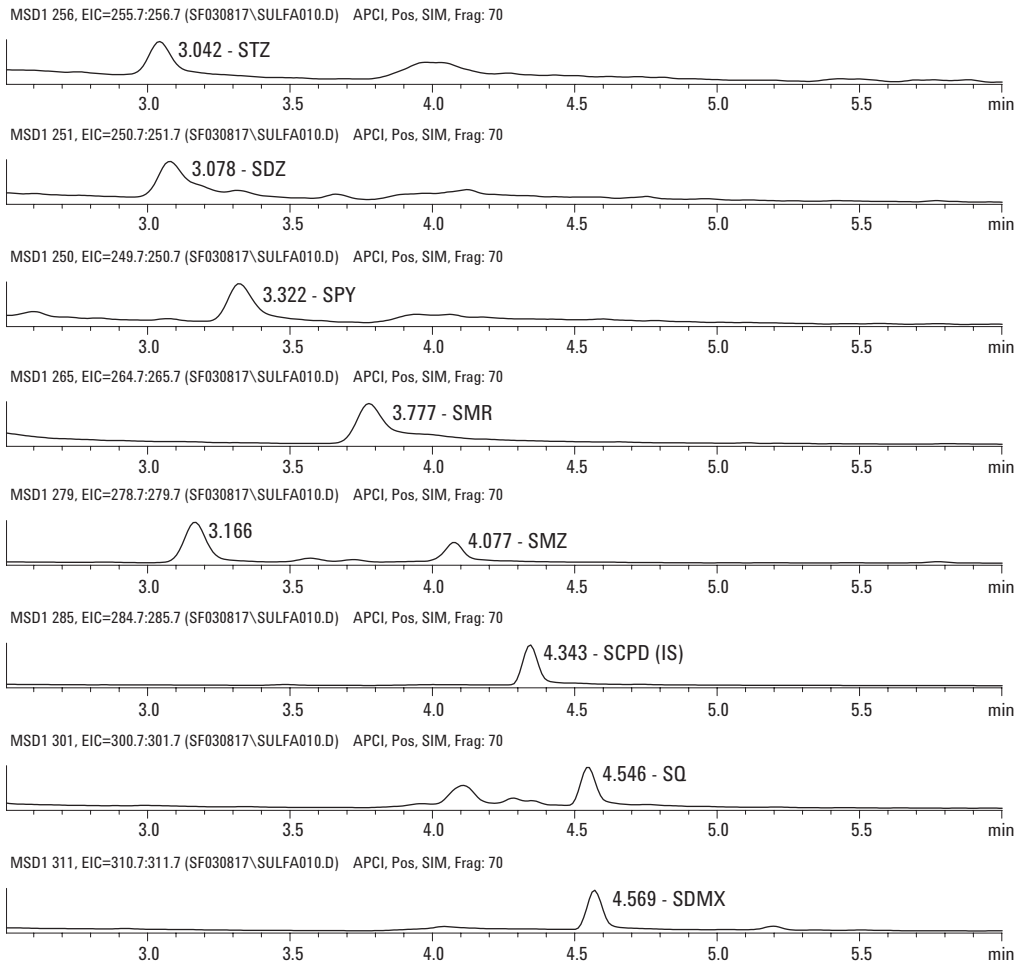


Figure 6. After HLB cleanup (SIM).

Results and Discussion

The recoveries obtained for seven samples spiked at a level of 50 ppb (150 ng of each sulfonamide in 3 g sample) appear in the following tables. The spiking solutions were added before homogenization and allowed to stand for at least 30 minutes before extraction. SMR (sulfamerazine) was added separately at 300 ng per sample before homogenization, and could be used as a surrogate. Results in Table 3 were obtained by simply diluting the extracts 4-fold with water (recovery 84%–118%), while results in Table 4 are from extracts taken through SPE cleanup (recovery 79%–104%).

In both cases, a five-point IS calibration with SCPD was used, with 20 to 200 pg of each target compound injected, plus 2,000 pg SCPD. The five standards were injected both before and after the set of seven spikes, and the curves were created by using the average responses of the two sets of standards. Peak height was used to measure response, as there was less variability compared to peak area, due to the noticeable tailing of these compounds. The linearity results (R^2) are tabulated in Tables 3 and 4.

Table 3. Recoveries of Sulfonamides by Diluting Extracts 1 in 4 with Water

	Amount recovered (ng)							
Description	**STZ**	**SDZ**	**SPY**	**SMR**	**SMZ**	**SCPD(IS)**	**SQ**	**SDMX**
Pork spike 1	167	172	164	317	151	2,000	148	130
Pork spike 2	168	197	68	343	164	2,000	169	137
Pork spike 3	160	183	158	315	157	2,000	133	121
Pork spike 4	158	189	167	336	156	2,000	138	129
Pork spike 5	151	169	154	295	169	2,000	133	129
Pork spike 6	147	161	144	322	143	2,000	120	112
Pork spike 7	144	72	141	272	151	2,000	124	125
Amount spiked (ng)	150	150	150	300	150	2,000	150	150
Mean	156	178	157	314	156	2,000	138	126
SD (Precision)	9	13	11	24	9	–	17	8
MDL (SD × t-stat) ng	29	40	34	77	28	–	53	26
LOQ (SD × 10) ng	94	126	108	245	88	–	167	82
RSD (SD × 100/Mean)	6	7	7	8	6	–	12	7
Accuracy (%)	104	118	104	105	104	100	92	84
Linearity (R^2)	0.9997	0.9996	0.9997	0.9972	0.9996	1.0000	0.9984	0.9992
t-stat (N=7)	3.14	3.14	3.14	3.14	3.14	3.14	3.14	3.14

Table 4. Recoveries of Sulfonamides Using Oasis HLB Cleanup Cartridges

	Amount recovered (ng)							
Description	**STZ**	**SDZ**	**SPY**	**SMR**	**SMZ**	**SCPD(IS)**	**SQ**	**SDMX**
Pork spike 1	161	157	132	273	149	2,000	139	126
Pork spike 2	154	156	132	293	157	2,000	153	131
Pork spike 3	149	158	124	267	155	2,000	132	113
Pork spike 4	145	152	122	279	144	2,000	119	111
Pork spike 5	151	162	127	294	149	2,000	127	121
Pork spike 6	136	147	127	274	136	2,000	116	108
Pork spike 7	148	161	128	275	155	2,000	124	116
Amount spiked (ng)	150	150	150	300	150	2,000	150	150
Mean	149	156	127	279	149	2,000	130	118
SD (Precision)	8	5	4	10	7	–	13	8
MDL (SD × t-stat) ng	24	17	11	33	23	–	40	26
LOQ (SD × 10) ng	76	53	36	104	73	–	128	82
RSD (SD × 100/Mean)	5	3	3	4	5	–	10	7
Accuracy (%)	99	104	85	93	100	100	87	79
Linearity (R^2)	0.9994	0.9994	0.9997	0.9979	0.9998	1.0000	0.9989	0.9989
t-stat (N=7)	3.14	3.14	3.14	3.14	3.14	3.14	3.14	3.14

Table 5 summarizes the comparison of recoveries when diluted with water versus using Oasis HLB cartridge cleanup. Generally there is a greater difference in recoveries for the early eluting compounds, as one might expect. Since the samples are loaded onto the cartridge with a mostly aqueous phase (10% methanol in water), the water-soluble matrix components would tend to pass through the cartridge to waste. Because these early eluting compounds were removed prior to injection on the HPLC column, the chromatograms are cleaner with more reproducible chromatography, as shown by the smaller standard deviations in recoveries. The results from the HLB cleanup exhibited smaller standard deviations and lower minimum detection levels (MDLs).

Conclusion

A fast and sensitive single quadrupole LC/APCI/MS method was developed and validated for detection of sulfonamide residues in pork. The DL ranged from 10 to 25 ng/g of tissue when analyzed by simple dilution of the extracts, and 4 to 13 ng/g when SPE cleanup is used. Instrumental conditions allow injection cycle-time of 10 minutes using typical columns and conditions for most labs.

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Table 5. Comparison of Recoveries Obtained by Dilution vs Oasis HLB Cleanup

Description	STZ	SDZ	SPY	SMR	SMZ	SCPD(IS)	SQ	SDMX
Accuracy % (1 in 4 dilution)	104	118	104	105	104	100	92	84
SD (Precision)	9.4	12.6	10.8	24.5	8.8	–	16.7	8.2
MDL (ng)	29	40	34	77	28	–	53	26
Accuracy % (HLB cleanup)	99	104	85	93	100	100	87	79
SD (Precision)	7.6	5.3	3.6	10.4	7.3	–	12.8	8.2
MDL (ng)	24	17	11	33	23	–	40	26

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The Analysis of Fluoroquinolones in Beef Kidney Using HPLC Electrospray Mass Spectrometry

Application

Food

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Abstract

A fast and simple screening method was validated for the analysis of three fluoroquinolone antibiotics in beef kidney. Samples were extracted with acidified methanol, centrifuged, diluted with water, and filtered. The diluted extract was analyzed directly by HPLC mass spectrometry using electrospray ionization in positive ion mode. Using an internal standard, mean recoveries were 73%–96% at spiking levels of 33 µg/kg (ppb), with statistically derived detection limits of 8–19 µg/kg. This is below the European Union maximum residue limit of 200 µg/kg for enrofloxacin and ciprofloxacin in bovine kidney. The method is evaluated relative to the requirements of the European Commission Decision 2002/657/EC for use as a confirmatory method.

Introduction

Fluoroquinolones are synthetic antibacterial compounds derived from nalidixic acid, and are useful to treat animal infections that are resistant to other antibacterial agents. They have a broad spectrum of activity, acting against both gram-positive and gram-negative bacteria. The maximum residue limit (MRL) for enrofloxacin (as the sum of enrofloxacin and ciprofloxacin) was entered into Annex 1 of Council Regulation (EEC) No. 2377/90 for kidney at 200 µg/kg in bovine and ovine species, and 300 µg/kg for porcine, poultry, and rabbits. For all other food producing species, the MRL is 200 µg/kg in kidney [1].

There are a number of methods describing the analysis of fluoroquinolones in various tissues, with HPLC coupled with fluorescence and mass spectrometric detection being very popular. Most methods involve extraction into acidic or basic organic solvents, followed by some type of cleanup, most notably solid phase extraction (SPE). The Canadian Food Inspection Agency extracts animal tissue with acidic ethanol, followed by strong cation exchange SPE cleanup, and HPLC fluorescence analysis [2]. Chen and Schneider [3] described a screening method for enrofloxacin in chicken, where extracts were detected by fluorescence without cleanup, following extraction and centrifugation.

European Community Commission Decision 2002/657/EC allows the use of HPLC coupled with fluorescence detection [4] for substances in Group B of Annex I to Directive 96/23/EC. Quinolones and other veterinary drugs fall into Group B, where three identification points are required for confirmation by Selected Ion Monitoring (SIM) using mass spectrometry (MS). With low resolution HPLC/MS, one point can be earned for each ion detected, provided that the ion ratios meet relative intensity criteria. Additional requirements of Directive 2002/657/EC, based on spiking levels of 33 µg/kg carried out in this study, are as follows:

- The internal standard (IS) shall be added to the test portion at the beginning of the extraction procedure.
- In order to allow the use of data corrected for mean recovery, the range of recoveries allowed are –20% to +10%.
- The reproducibility of coefficient variation (CV) (%) is expected to be about one-half to two-thirds of the 100 µg/kg CV, which is 23%, at a concentration of half the permitted limit.
- For liquid chromatography/mass spectrometry (LC/MS) procedures, the minimum acceptable retention time (RT) for the analyte under examination is twice the RT corresponding to the void volume of the column.
- The ratio of the chromatographic RT of the analyte to that of the IS, that is, the relative RT of the analyte, shall correspond to that of the calibration solution at a tolerance of 2.5% for LC.
- The molecular ion shall preferably be one of the selected diagnostic ions.
- The maximum permitted tolerances for relative ion intensities shall meet the criteria in the Annex, (in this case, either ±25% or 30%), as reproduced in Table 6.

Experimental

Chemicals and Materials

HPLC-grade methanol and acetonitrile were purchased from Caledon Labs (Georgetown, Ontario).

Formic acid, min. 98%, was purchased from EM Science.

Acidified methanol solution: 30% methanol in pH 3 deionized water (100 µL of formic acid per 100 mL of water).

Acidified methanol was prepared by adding 100 µL of 98% formic acid to 100 mL of methanol.

Acidified deionized water was prepared by adding 100 µL of 98% formic acid to 100 mL deionized water.

Ultra-Turrax T25 homogenizer, 50-mL polypropylene centrifuge tubes, and 13-mm polyvinylidene fluoride (PVDF) syringe filters (0.2 µm), were purchased from VWR Scientific.

All fluoroquinolones, including the IS, were provided as a gift from the Canadian Food Inspection Agency, Calgary, Alberta, Canada, as stock solutions of 100 ng/µL (ppm) in 1% acetic acid in methanol. Solutions were stored at 4 °C. Standard solutions at different concentrations were prepared for spiking by dilution with acidified methanol solution. The analytes ciprofloxacin, enrofloxacin, and sarafloxacin were chosen as targets since these compounds are included in the Canadian Food Inspection Agency's proficiency check samples. The spiking standard for these compounds (1 ng/µL) was prepared by diluting 100 µL of each the stock solutions to a 10-mL volumetric flask, and made to volume with acidified deionized water. A separate IS solution at 1 ng/µL was prepared the same way, except that it only contained norfloxacin and danofloxacin.

Sample Preparation

1. For beef kidney, 3 g samples were weighed directly into 50-mL polypropylene centrifuge tubes.
2. For spiked samples, 100 µL of the 1-ng/µL (100 ng) spiking solution was added, resulting in fortification levels of 33 µg/kg. Samples were allowed to stand for 1 hour before subsequent extraction.
3. For the sample blank, 100 µL of acidified methanol solution was added.
4. For all spiked samples, 100 µL of the 1-ng/µL (100 ng) IS solution was added just prior to extraction. Norfloxacin was included in this solution at the same level, to be used as an alternate IS, if required due to potential interferences for danofloxacin.
5. The samples were homogenized for 2 min with 15 mL of acidified methanol using the Ultra-Turrax homogenizer.
6. The samples were then centrifuged for 10 min, and the supernatant decanted into a clean test tube.

7. The extract was diluted with acidified deionized water 1 in 4 (250 µL of extract + 750 µL of water), filtered through a 0.2-µm PVDF filter into an autosampler vial, and analyzed directly by LC/MS.

By adding an accurately known amount of IS to the initial sample before extraction, there is no need to measure the final volume of the extracts, nor the aliquot to be diluted. The IS calculations, performed by the ChemStation, measure the relative amounts of the analytes and IS. This corrects for any concentration or dilution effects in the samples.

Standard Preparation

A 5-point calibration curve was used for the determination of each of the three target compounds, and a 1-point curve was used for norfloxacin, the alternate IS. Table 1 gives the volumes of the IS and target solutions added (1 ng/µL each) to each of five test tubes. The standards were prepared by adding 250 µL of the blank extract and 750 µL of acidified deionized water to the tubes containing the analytes, after which the solutions were filtered through 0.2-µm PVDF filters.

The final solution of each standard contained 5 ng of IS per mL of diluted extract, or 5 pg/µL. With 50 µL injected, this results in 250 pg injected. The amount of target analyte in each of the five solutions varies to produce the calibration curves, as shown in Table 1.

The correlation coefficient (R^2) for the target analytes ranged from 0.9987 to 0.9992, as shown in Table 4.

Preparation of the standards in this fashion will compensate for any ion suppression or enhancement that may occur, due to the presence of co-eluting material at the MS source, which may not otherwise occur if pure solvents alone are used.

Table 1. Preparation of Analytical Standards (50-µL Injections into LC/MSD)

Standard	IS Volume added (µL)	Target volume added (µL)	IS Amount injected (pg)	Target amount injected (pg)
1	5	1	250	50
2	5	2	250	100
3	5	5	250	250
4	5	10	250	500
5	5	20	250	1,000

LC/MS Conditions

The HPLC system was made up of an Agilent Technologies 1100 series solvent degasser, binary pump, autosampler, column oven, diode array detector (DAD), and quadrupole mass selective detector (MSD) (Table 2).

Table 2. LC/MSD Conditions

HPLC	
Column	Zorbax Eclipse XDB-C8, 150 mm × 4.6 mm, 5 µm (P/N 993967-906)
Solvent A	0.1% Formic acid in water
Solvent B	0.1% Formic acid in acetonitrile
Gradient	t_0 = 20% B t_1 = 20% B t_8 = 90% B t_{15} = 90% B Post time = 2.0 min
Flow rate	0.4 mL/min
Injection volume	50 µL
Column temp	30 °C
MSD	
Source	Electrospray Ionization (ESI) (positive ion mode)
Ion dwell time	14 ions at 40 ms each
Fragmentation	Varies by ion, see Table 3
Drying gas flow	12 L/min
Nebulizer pressure	30 psi
Drying gas temperature	350 °C
Capillary voltage	4000 V

Table 3. Fragmentor Voltages for Acquired Ions in SIM (single acquisition group)

Compound	Ion	Fragmentor (V)
Norfloxacin (IS)	320	120
	302	200
	276	200
Ciprofloxacin	332	120
	314	200
	288	200
Danofloxacin (IS)	358	120
	340	220
Enrofloxacin	360	120
	342	220
	316	220
Sarafloxacin	386	120
	368	220
	342	220

All ions were included in a single acquisition group, which started at injection (time = 0). An alternative approach would be to set the group start time to a value around half a minute before the elution of the first compound, as this will keep the eluant stream diverted to waste as long as possible. This will reduce the amount of co-extracted material being introduced into the source, reducing contamination.

Another alternative is to add an additional time-programmed acquisition group to the method, and only include the ions for compounds eluting within the group times. This will take on more significance as the overall number of compounds in a method increases, and with three ions per compound required for identity confirmation.

Fragmentor voltages were chosen that maximized the response for each selected ion. For each fluoroquinolone, a value of 120 V produced only the protonated parent ion, while higher voltages were required to induce fragmentation to confirmatory ions. The ions monitored corresponded to the neutral losses of water and carbon dioxide in each case.

Note that although mass 342 is acquired for both enrofloxacin and sarafloxacin, it is only added to the MSD acquisition table once.

Chromatography

All compounds eluted between 5 and 9 minutes, however the total run time was set to 15 minutes with 90% organic solvent to allow co-extractives to elute from the column. Otherwise, their eventual elution could interfere with subsequent injections. This is more of a potential problem when methods with abbreviated cleanups, such as dilution-only, are used. The following figures compare the blank beef kidney sample to a sample fortified at 33 µg/kg. In each case, the selected ions are the protonated forms of the parent ion, as well as the protonated ions resulting from the loss of H_2O (M-18) and CO_2 (M-44).

The qualifier ion for danofloxacin, the compound used as the IS for this study, is mass 340. The matrix causes an interference at mass 340. The interference is shown as a small peak in the beef kidney blank as shown in Figure 1. Since a diagnostic qualifier ion is not required for the IS calculations, it had no impact on the results. It does, however, indicate that there is elution of co-extractive material in the samples, and that without further cleanup, ion suppression may result from its presence. All standards were prepared in blank beef kidney extract in order to compensate for these potential effects.

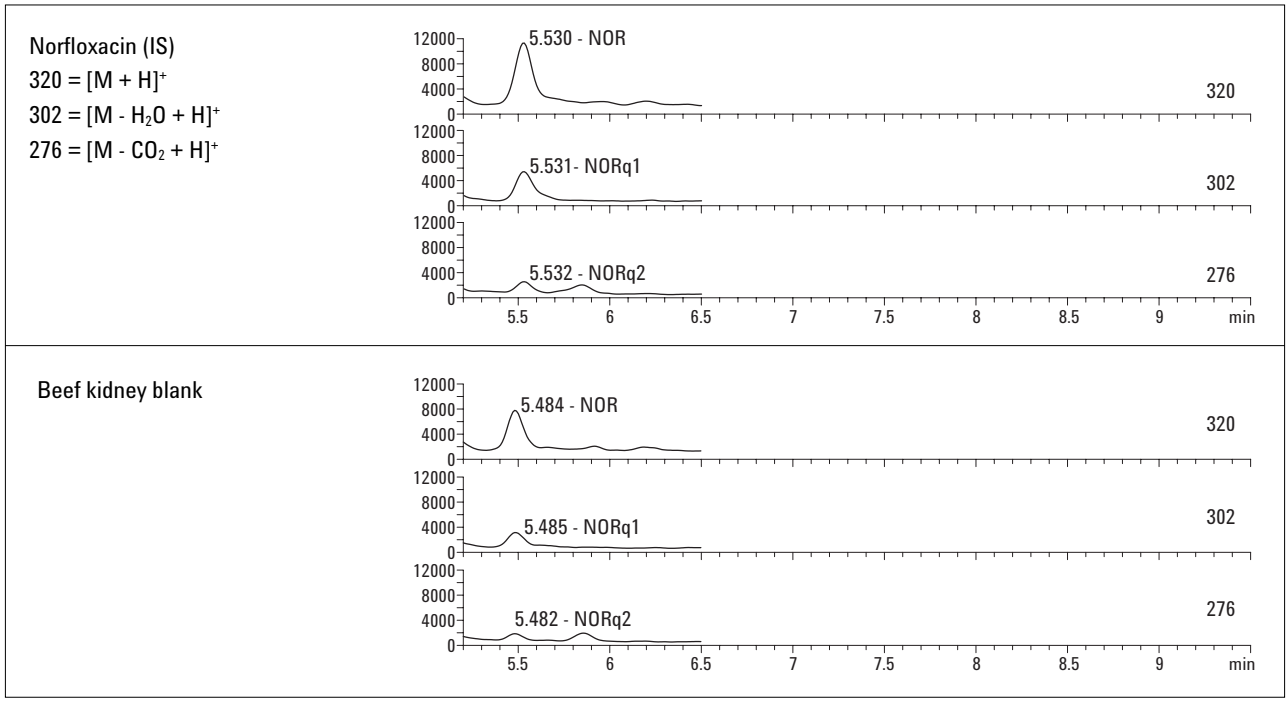
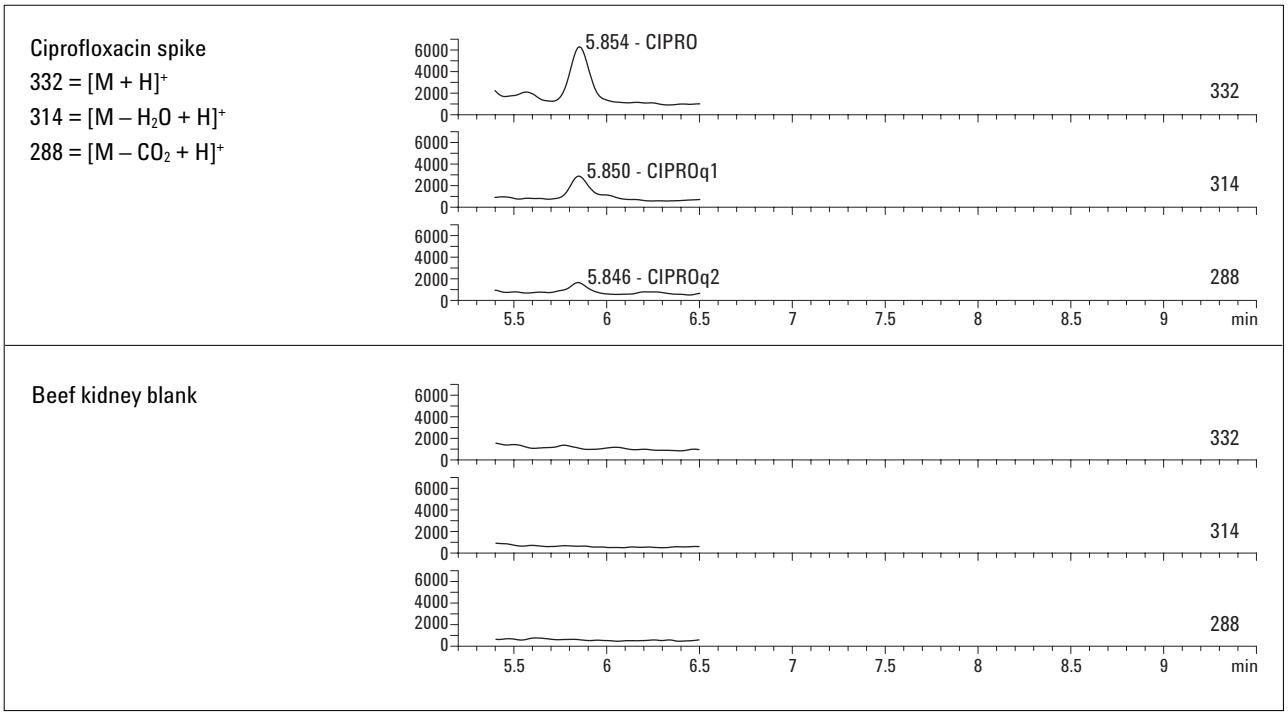


Figure 1. Comparative extracted ion chromatograms for fluoroquinolones spiked into beef kidney.



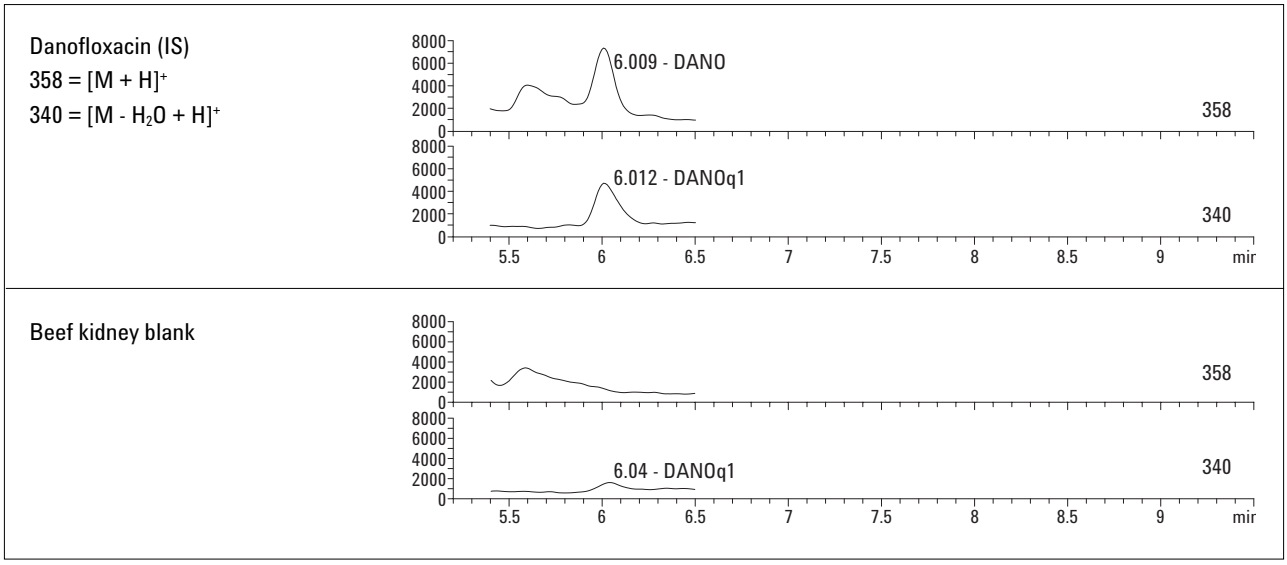
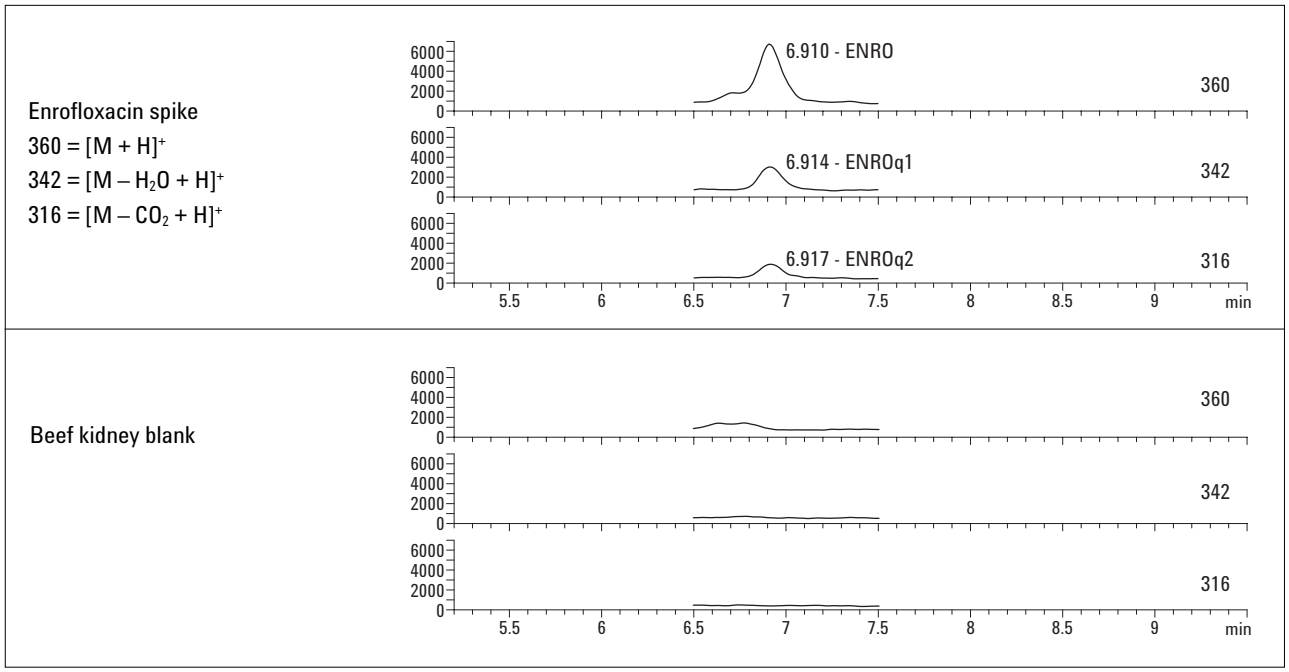


Figure 1. Comparative extracted ion chromatograms for fluoroquinolones spiked into beef kidney (Continued).



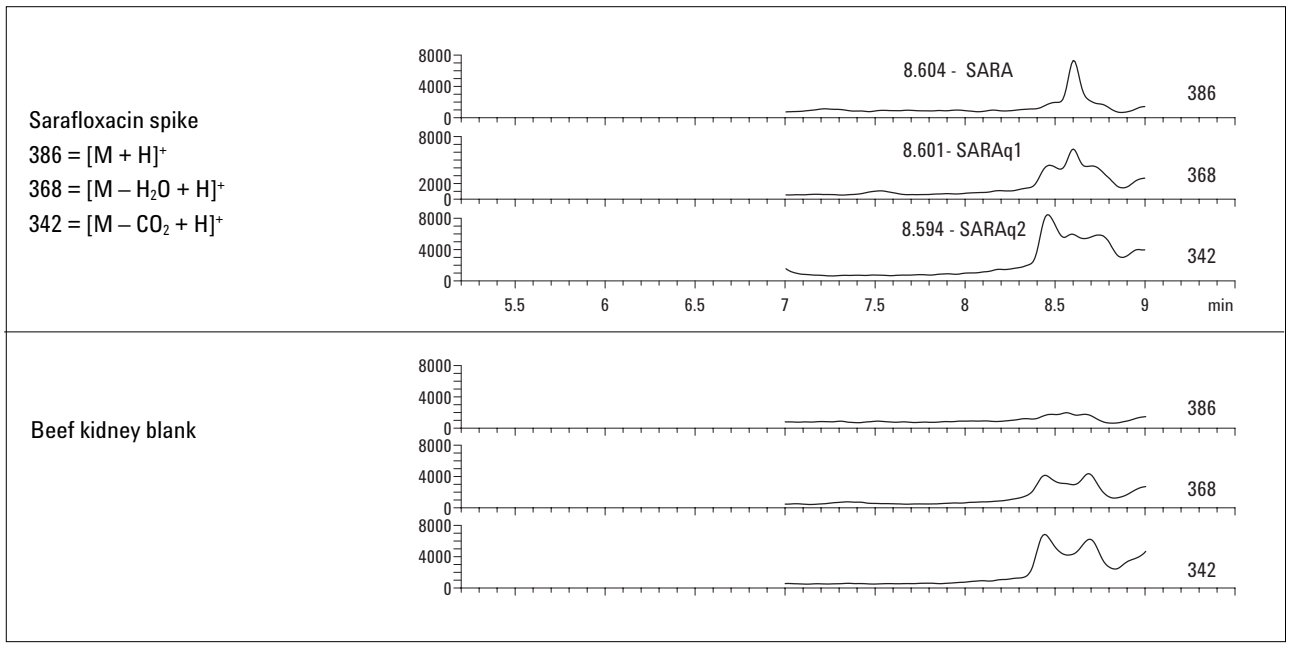


Figure 1. Comparative extracted ion chromatograms for fluoroquinolones spiked into beef kidney (Continued).

Sarafloxacin elutes from the column in the same region as a number of other co-extractives, making identification and quantitation more difficult. However, as shown in Table 7, the qualifier ions still meet the identification criteria for relative responses of the qualifiers, and so further cleanup of the samples may not be necessary. The effect of these co-extractives will also be reduced at higher incurred residue levels, closer to those permitted by the European Union MRL.

Recoveries

In order to allow results to be corrected for recoveries, where the determined incurred levels are divided by the percent recovered from certified reference materials or spiked samples, Table 2 of the Annex requires that the recoveries for analytes at levels greater than 10 µg/kg be within the range of 80% to 110%. Table 4 shows that recoveries for ciprofloxacin and enrofloxacin meet this requirement, with 96.3% and 86.0%, respectively. However, sarafloxacin fails the requirement, with only 72.6% mean recovery. With a CV of only 8% for this compound, it looks as though the method may still produce acceptable results for screening purposes, but some additional work may be required to produce higher recoveries. Since the work presented here involves spiked samples only, recovery-correction calculations do not apply.

Norfloxacin was added along with danofloxacin as an additional IS. However, examination of the blank beef kidney used in this study shows norfloxacin to be present as an incurred residue, at a concentration approximately one half of the spiking level. Assuming a linear response through the origin, this would mean that norfloxacin was detected at approximately 15–20 µg/kg, which is about 10% of the permitted level for enrofloxacin in bovine kidney. Recoveries for norfloxacin are included in Table 4, even though they were calculated with a single point calibration, and not corrected for incurred residues. However, there is some compensation for this since the standards used for calibration were prepared by addition of the targets to the blank extracts.

Table 4. Recoveries of Fluoroquinolones from Beef Kidney

	Amount recovered (ng)			
Description	**Norfloxacin**	**Ciprofloxacin**	**Enrofloxacin**	**Sarafloxacin**
Kidney spike 1	111.8	96.3	84.9	68.5
Kidney spike 2	93.1	94.0	85.6	64.1
Kidney spike 3	88.0	89.6	83.8	77.6
Kidney spike 4	98.9	95.4	86.2	75.2
Kidney spike 5	82.2	93.8	85.4	82.1
Kidney spike 6	143.0	109.3	87.9	72.9
Kidney spike 7	102.6	101.3	83.3	73.0
Kidney spike 8	110.6	90.8	91.3	67.7
Amount spiked (ng)	100.0	100.0	100.0	100.0
Mean	103.8	96.3	86.0	72.6
SD (Precision) ng	18.9	6.3	2.5	5.8
MDL (SD × t-stat) ng	56.7	19.0	7.6	17.4
LOQ (SD × 10) ng	189.1	63.4	25.4	58.1
CV (SD/Mean) %	18.2	6.6	3.0	8.0
Accuracy (%)	103.8	96.3	86.0	72.6
Linearity (R^2)	0.9895	0.9987	0.9992	0.9987
t-stat (N = 8)	3.00	3.00	3.00	3.00

Compound Identification

For chromatographic separation, Section 2.3.3.1 of the Annex to 2002/657/EC requires that the minimum acceptable RT for the analyte under investigation be at least twice the RT corresponding to the void volume of the column (k'=1). The first compound to elute under these conditions is norfloxacin, with a k' of 2.6, therefore this condition is easily met. The second condition is that the ratio of the RT of the analyte to that of the IS, that is the relative RT, shall correspond to that of the calibration solution at a tolerance of ±2.5% for LC. Table 5 shows the RT times of each analyte in the spiked samples, compared to those of the standards, and that they are well within the allowable tolerance.

Table 5. Relative RTs of Analytes in Samples, Compared to Standards

Compound	Average RRT in standards (N = 15)	CV (%) RRT in standards (N = 15)	RRT in samples, relative to standards (N = 8)
Norfloxacin	0.922	0.12%	99.8%–100.1%
Ciprofloxacin	0.975	0.05%	99.9%–100.1%
Enrofloxacin	1.150	0.16%	99.8%–100.2%
Sarafloxacin	1.439	0.47%	99.5%–100.3%

Compound Confirmation

Section 2.3.3.2 of the Annex to 2002/657/EC gives the maximum permitted tolerances for relative ion intensities, which is reproduced in Table 6.

Table 6. Maximum Permitted Tolerances for Relative Ion Intensities Using a Range of Mass Spectrometric Techniques

Relative intensity (% of base peak)	GC/MS(EI) (relative)	GC/MS(CI), GC/MS^n, LC/MS, LC/MS^n (relative)
>50%	±10%	±20%
>20% to 50%	±15%	±25%
>10% to 20%	±20%	±30%
≤10%	±50%	±50%

Note MS^n equals MS/MS if n = 2

Table 7 shows the relative intensities for each of the qualifier ions for the three target compounds, as well as norfloxacin and danofloxacin (one ion). As expected, norfloxacin meets the criteria in each of the eight spiked samples, even though it had incurred residues. The presence of additional norfloxacin should not negatively affect this qualitative aspect of performance, and it does not. Danofloxacin, however, showed an interference for the single qualifier ion monitored, and so the relative amount of this signal would be expected to vary to a larger degree, depending upon the exact amount of blank extract used in preparing the sample dilutions and standards. As previously mentioned, the standards are prepared by accurately measuring the relative amounts of target and IS compounds into a tube or vial, followed by addition of blank kidney extract and water. The exact proportions of extract and water do not have to be known, since the IS calculations uses amount and response ratios, rather than absolute amount and response, in determining concentrations in unknowns. An accurate measurement of extract and water volumes can, however, reduce interference variability.

Table 7. Relative Intensities of Qualifier Ions for Fluoroquinolones in Beef Kidney, Compared to Permitted Tolerances

	Relative intensities (%) of qualifier ions								
	Norfloxacin		**Ciprofloxacin**		**Danofloxacin**	**Enrofloxacin**		**Sarafloxacin**	
Sample	**Q1 = 302**	**Q2 = 276**	**Q1 = 314**	**Q2 = 288**	**Q1 = 340**	**Q1 = 342**	**Q2 = 316**	**Q1 = 368**	**Q2 = 342**
Spike 1	49	15	47	17	64	44	28	50	15
Spike 2	45	15	48	17	58	46	24	41	17
Spike 3	48	16	46	20	63	44	28	45	14
Spike 4	42	15	46	17	60	43	30	43	13
Spike 5	49	17	45	21	65	44	26	45	11
Spike 6	50	19	39	17	72	45	29	43	12
Spike 7	49	17	41	18	65	46	29	46	13
Spike 8	47	17	42	19	62	39	24	46	12
Average for Stds	49	20	44	20	86	43	26	47	15
Std Dev for Stds	2	1	3	2	22	2	2	6	1
Tolerance(Table 7)	25	30	25	30	20	25	25	25	30
Lower Allowable (calculated)	**37**	**14**	**33**	**14**	**69**	**32**	**19**	**35**	**11**
Upper Allowable (calculated)	**62**	**26**	**55**	**25**	**103**	**53**	**32**	**59**	**20**

Conclusion

A fast and sensitive single quadrupole LC/ESI/MS method was validated for the detection of three fluoroquinolone antibiotics (ciprofloxacin, enrofloxacin, and sarafloxacin) in beef kidney. The detection limits ranged from 8 to 19 µg/kg (ppb), with direct analysis of sample extracts after dilution with water. All qualitative requirements were met with respect to the Annex to EU Directive 2002/657/EC for spiked samples, and recoveries of two of the three compounds met the quantitative requirements. Recovery of sarafloxacin was slightly lower than the level required to allow correction for recoveries in reported results.

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Analysis of Nitrofuran Metabolites in Tilapia Using Agilent 6410 Triple Quadrupole

Application

Food Safety

Authors

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Abstract

The metabolites of nitrofuran antibiotics banned in meat and meat products are analyzed by LC/MS/MS with the new Agilent 6410 triple quadrupole. The method is shown to be highly sensitive, to 0.01 ppb (10 ppt), for each of the four analytes. Calibration from 0.1 ppb to 10 ppb is presented with all criteria for confirmation as set by the European Union decisions for analytical method performance. Extracts of tilapia are used to show the performance of the LC/MS/MS method for aquaculture samples.

Introduction

Nitrofurans are inexpensive antibiotics used for Gram positive and Gram negative bacteria. They have been used to treat gastrointestinal and dermatological infections in farm animals and fish. In addition, they have been used to treat bacteria in bees. Because both parent compounds and their metabolites are suspect carcinogens, they have been banned around the world. The Rapid Alert System for Food and Feed Annual report for 2005 [1] shows that these compounds continue to be detected in food samples and remains a major concern for food safety. The four compounds–furazolidone, furaltadone, nitrofurantoin, and nitrofurazone–have been found to metabolize rapidly, and the metabolites bind to muscle tissue. Thus the analytical detection of the metabolites and not the parent compounds are required in samples of animal origin.

The criteria for detection and confirmation of veterinary drugs in animal and animal products established by the European Union (EU) [2] has been accepted in much of the world. This criteria mandates a separation technique combined with a spectrometric technique. For banned substances such as the nitrofurans, no maximum residue limit (MRL) could be set. Therefore a minimum required performance level (MRPL) was set at 1 µg/kg for each metabolite [3]. Only LC/MS could meet these criteria, and very good methods have been reported [4–6]. However, the most widely accepted methodology employs triple quadrupole tandem mass spectrometers. This is the first report showing analysis of these metabolites using the new Agilent triple quadrupole LC/MS system.

Experimental

Chemicals

Derivatized standards of nitrofuran metabolites and all chemicals for sample preparation were received from a food manufacturing company. Acetonitrile was HPLC grade from Merck (Darmstadt, Germany). Formic acid was reagent grade from Merck (Darmstadt, Germany).

Sample Preparation

The accepted procedure for sample preparation was followed. To 2 g of tilapia was added 15 mL 0.125 M HCl and the mixture homogenized.

To this solution, a 50-µL solution of 2-nitrobenzaldehyde (50 mM in DMSO) was added and shaken. The solution was then incubated at 37 °C for 16 hours. This was followed by neutralization to pH ~7 with NaOH and K_2HPO_4. The neutral derivatized sample was then extracted with ethyl acetate, concentrated to dryness, and reconstituted in 100 L of initial LC mobile phase. Standards of the four metabolites were spiked into 0.125 M HCl, derivatized, and extracted for calibration using the same procedure as was used for the samples of tilapia.

LC/MS/MS Method

LC Conditions

Instrument:	Agilent 1100 LC
Column:	C18, 2.1 mm × 150 mm, 3 µm
Column temp.:	40 °C
Mobile phase:	A = 0.1% formic acid in water
	B = acetonitrile
Gradient:	22% B at 0 min
	99% B at 6 min
	99% B at 9 min
Flow rate:	0.3 mL/min
Injection volume:	50 nL

MS Conditions

Instrument:	Agilent 6410 LC/MS Triple Quadrupole
Ionization mode:	Positive ESI
Drying gas flow:	10 L/min
Nebulizer:	35 psig
Drying gas temp.:	350 °C
V_{cap}:	5000 V

Quantitation

Quantitative analysis was done with the first transition listed in the MRM parameter table. The second transition was used as a qualifier ion for confirmation as per the confirmation criteria. Quantitative results were performed with the new MassHunter quantitative analysis software.

Results and Discussion

The instrument sensitivity is an important performance parameter for this analysis when considering the derivatization and extraction needed to meet the required detection limit of 1 ppb for each metabolite, aminohydantoin (AH), 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ), 3-amino-2-oxazolidinone (AOZ), and semicarbazide (SC). To demonstrate this performance, a standard of each 2-nitrobenzaldehyde (2-NBA) derivatized metabolite is shown at 0.01 ppb (10 ppt) in Figure 1. The structure of each derivatized metabolite is given and each is shown with a signal-to-noise ratio of greater than 3:1. Another indicator of performance is linearity. Calibration curves from this concentration (10 ppt) to 10 ppb are displayed in Figure 2 showing the linearity for each compound.

Treatment of fish with nitrofurans is a continual problem for food safety and import into EU member countries. To demonstrate the capability of the Agilent triple quadrupole LC/MS, tilapia samples were spiked with the four metabolites, hydrolyzed, derivatized, and extracted. An analysis of a tilapia extract at 500 ppt is shown in Figure 3 and demonstrates the signal obtained at half the MRPL. In addition to meeting the sensitiv-

MRM Mode Parameters

Compound	Transition	Dwell time (ms)	Fragmentor voltage (V)	Collision energy (V)	MS2 resolution
AMOZ	335.1 → 291.4	60	100	5	Unit
	335.1 → 262.4	60	100	5	Unit
SC	209.1 → 192.3	60	100	5	Unit
	209.1 → 166.3	60	100	5	Unit
AH	249.1 → 134.2	60	100	5	Unit
	249.1 → 104.2	60	100	5	Unit
AOZ	236.0 → 134.1	60	100	5	Unit
	236.0 → 104.1	60	100	5	Unit

ity requirement, the analysis must also meet the confirmation criteria, including both chromatographic retention time match with the standards and measuring a qualifying ion with a relative intensity ratio within a specified tolerance of the quantitation ion. This tolerance is set by the ratio obtained when analyzing standards and increasing as that ratio decreases. This tolerance ranges from 20% for ions with relative ratio intensities above 0.5 and to 50% for ratios below 0.1.

Table 1 shows tilapia samples spiked with the metabolites, derivatized and extracted. The spikes were used as the calibrants, so the final concentration is obtained from the curve. The table is produced as the batch using the MassHunter software results with outliers highlighted in blue (low) and red (high). The table shows that in the blank a peak is found within the tolerance set for the retention time of AMOZ but the qualifier ratio is low. For AOZ and SC, retention times for suspect peaks are below the specified retention in the same blank. For AH, the 0.5 ppb spike, the qualifier ion ratio is outside the 35% tolerance limit set for this ion (again low).

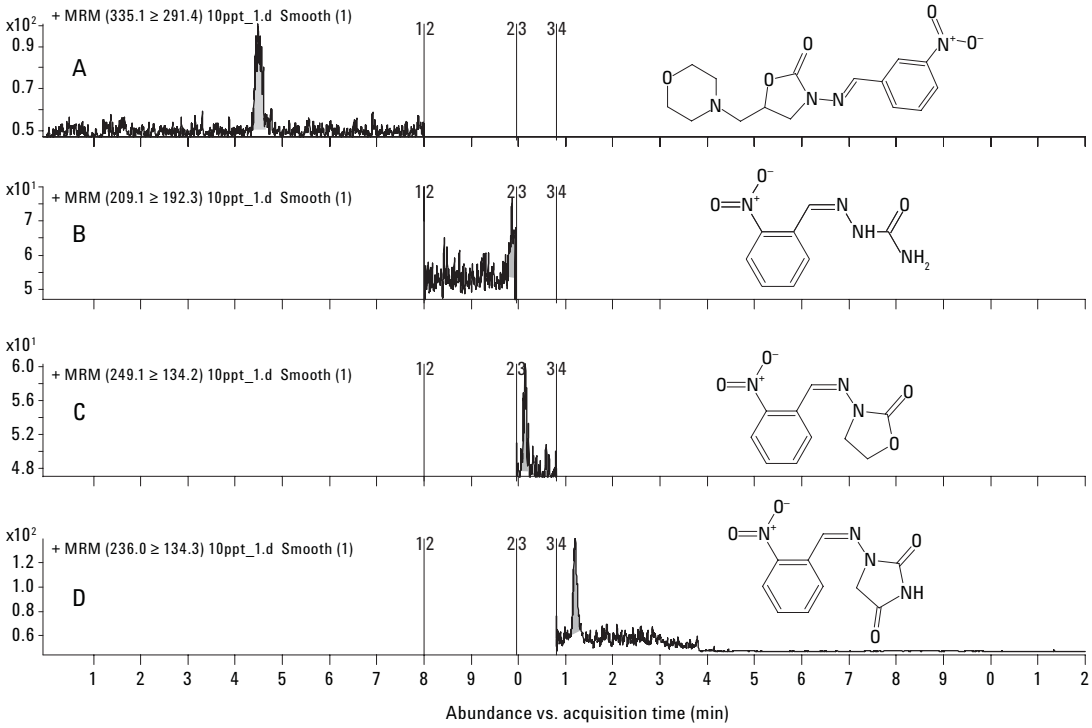


Figure 1. The MRM quant ion chromatogram for each derivatized metabole at 10 ppt of A) 2-NBA AMOZ, B) 2-NBA SC, C) 2-NBA AH, and D) 2-NBA-AOZ

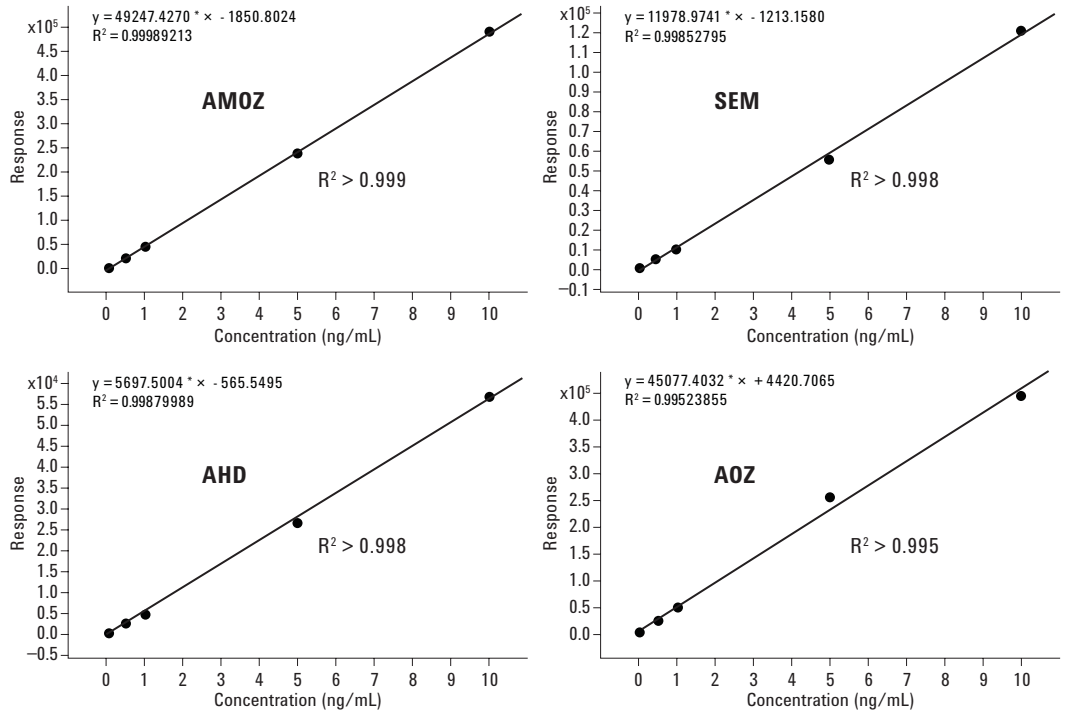


Figure 2. Calibration curves of nitrofuran metabolites linear range from 10 ppt to 10 ppb.

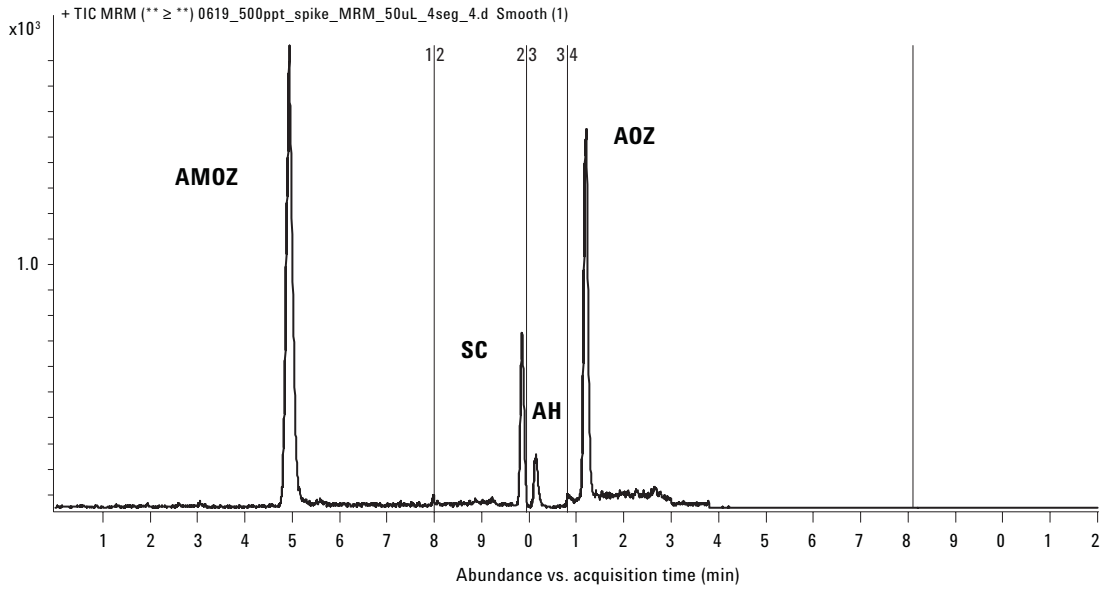


Figure 3. Spiked tilapia sample extract at 500 ppt each metabolite.

Table 1. Analysis of Talapia Spikes Self-Calibrated. Note Qualifier Ratios and Retention Times Reported.

		AH	Qualifier		AMOZ	Qualifier		AOZ	Qualifier		SC	Qualifier
Name	RT	Final Conc.	Ratio	RT	Final Conc.	Ratio	RT	Final Conc.	Ratio	RT	Final Conc.	Ratio
Nitrofuran Blank		0.00		2.72	0.02	8.70	8.71	0.00		7.94	0.00	
Nitrofuran 0.5 ppb	8.66	0.54	6.07	2.62	0.51	17.44	9.19	0.37	7.99	8.44	0.50	65.32
Nitrofuran 1 ppb	8.66	1.07	15.83	2.64	0.92	20.42	9.19	1.06	10.19	8.44	1.05	70.85
Nitrofuran 3 ppb	8.67	3.42	13.78	2.65	3.41	18.67	9.20	3.18	9.51	8.44	2.89	67.47
Nitrofuran 5 ppb	8.66	5.71	14.24	2.66	4.66	19.28	9.19	4.89	8.61	8.44	5.05	69.06

Conclusions

This work shows the high performance of the new Agilent 6410 LC/MS triple quadrupole system for the sensitive analysis of the nitrofuran metabolites in fish samples. The system readily meets the performance requirements and provides advanced quantitation software for calculating and reporting all confirmation parameters specified by the European Commission decision.

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Detection, Confirmation, and Quantification of Chloramphenicol in Honey, Shrimp and Chicken Using the Agilent 6410 LC/MS Triple Quadrupole

Application

Food Safety

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Abstract

This method was developed using the Agilent G6410AA Triple Quadrupole Mass Spectrometer (QQQ) for chloramphenicol in honey, shrimp, and chicken. The sensitivity obtained exceeds the minimum required performance level (MRPL) established by the European Union regulation for food monitoring programs. Using a deuterated internal standard and one simple sample solid phase extraction (SPE) procedure can provide a limit of detection at 10 ppt in sample matrix. The analytical performance of the method was evaluated for three different matrixes and the results show little or no matrix effects. Linearity of response over 2 orders of magnitude was demonstrated (r > 0.99). In addition, good reproducibility of the two required product ion ratios was obtained to meet the EU identification points needed for confirmation. This study is a valuable indicator of the ability of the QQQ for routine quantitative trace analysis of chloramphenicol in honey, shrimp, and chicken.

Introduction

Chloramphenicol (CAP) is a broad-spectrum antibiotic. It was concluded that human exposure to CAP can cause aplastic anemia [1]. Chloramphenicol and other bacterial inhibitors have arguably been the biggest issue facing international seafood trade over the past year. Because chloramphenicol has displayed significant toxicological effects on humans, it has been banned from foods in the European community, Japan, and the United States at levels of 0.3 ppb.

LC/MS has been demonstrated for this analysis by the U.S. Food and Drug Administration[2-4] and others[5]. In this application, the Agilent G6410AA QQQ is used. This method employs negative ion mode with electrospray ionization. An internal standard (IS), CAP-d5, is added at the beginning of the extraction. The use of this IS self-corrects for any extraction variability from sample to sample and response variability caused by the matrix. With the use of this IS, 50 parts per trillion (ppt) CAP levels can be reliably quantified. A solid phase extraction (SPE) procedure is used along with a mobile phase of only methanol and water without salt buffers, which should help minimize MS maintenance.

Experimental

Reagents and Materials

Agilent AccuBond SPE ENV PS DVB Cartridges (P/N 188-3060)
Ethyl acetate from Burdick and Jackson (Morristown, NJ)
Methanol HPLC-Grade from Burdick and Jackson
Water (18 MΩ) from Milli-Q Synthesis System
Chloramphenicol (CAP) from Aldrich Chemical Co. (Milwaulkee, WI)
Deuterated (d5) CAP internal standard from Cambridge Isotope Laboratories (CIL, Andover, MA, U.S.)
Syringe filter (0.2 µm, PTFE) from Agilent (P/N 5185-5843)

Overview of Method

Internal Standard Preparation

1. A 100-µg/mL (100 ppm) stock standard CAP-d5 solution in methanol (MeOH) is purchased from Cambridge Isotope Laboratories, Inc. (Lot SCCE-005)
2. A 1:100 dilution in MeOH of the stock standard gives an intermediate standard concentration of 1 µg/mL (1 ppm) or 1000 ng/mL CAP-d5
3. A 1:100 dilution in MeOH gives a diluent solution (This diluent solution is used to prepare the samples) concentration of 10 ppb.
4. Every 1-g sample is fortified with 25 µL of CAP-d5 diluent solution for a 0.25 ppb IS (internal standard) concentration

Standard Solution Preparation

1. A 100-µg/mL stock standard CAP solution in methanol (MeOH) is prepared by weighing 5.0 mg CAP std into 50 mL methanol.
2. A 1:100 dilution with methanol of the stock standard gives an intermediate standard concentration of 1 µg/mL (1 ppm) or 1000 ng/mL CAP
3. Add 25 µL CAP-d5 diluent solution into each vial.
4. Prepare standard solutions in these vials: 1 ppb, 0.2 ppb, 0.1 ppb, 0.02 ppb, and 0.01 ppb, with IS at 0.25 ppb level.

Sample Preparation

All SPE cartridges are conditioned with 2 mL of water before use.

1. **Honey**, 1 g of sample is diluted to 5 mL with water and 25 µL 10 ppb IS is added. The solution is loaded onto the SPE cartridge and allowed to stand for 5 min. Elution is performed with 10 mL ethyl acetate. The eluate is collected and the solvent is evaporated under a nitrogen stream at 40 °C. The residue is redissolved in 1 mL methanol and put in an ultrasonic bath for 1 min. The solution is filtered, using a syringe filter, before injection. No additional clean-up of the sample solution is performed.
2. **Shrimp**, 1 g of shrimp is defrosted and mixed in a blender. To the 1 g of the mixed shrimp, 3 mL of water and 25 µL 10 ppb IS is added. The portion is centrifuged for 5 min (8,000 rpm). The supernatant is loaded on the cartridge and allowed to stand for 5 min. Elution is performed with 5 mL ethyl acetate. The eluate is collected and the solvent evaporated under a nitrogen stream at 40 °C. The residue is redissolved in 1 mL methanol and put in an ultrasonic bath for 1 min; the solution is filtered before injection.
3. **Chicken**, 1 g of chicken is defrosted and mixed in a blender. To the 1 g of the mixed chicken, 3 mL of water and 25 µL 10 ppb IS is added. The portion is centrifuged for 5 min (8,000 rpm). The supernatant is loaded on the cartridge and allowed to stand for 5 min. Elution is performed with 5 mL ethyl acetate. The eluate is collected and the solvent evaporated under a nitrogen stream at 40 °C. The residue is redissolved in 1 mL methanol and put in an ultrasonic bath for 1 min.; the solution is filtered before injection.

LC/MS conditions

The LC system was the Agilent 1200-SL binary pump with the ALS-SL autosampler. The MS was an Agilent 6410 LC/MS triple quadrupole mass spectrometer. See Table 1 for conditions.

Table1. LC/MS Conditions

HPLC	
Column	ZORBAX SB-C18, 2.1 × 50 mm, 1.8 µm (p/n 827700-902)
Flow rate	0.4 mL/min
Mobile phase	A: water
	B: methanol
Gradient	0-5 min, 30~70% B
	5-6 min, 70~100% B
	8 min, 100% B
Post time	4 min
Temperature	45 °C
Injection	5 µL
MS Source Settings	
Source	ESI
Ion polarity	Negative
Drying gas temperature	350 °C
Drying gas flow rate	10 L/min
Nebulizer	45 psi
V_{cap}	3500 V
Fragmentor	100 V
Collision energy	10 V for *m/z* 257(qualifier ion)
	15 V for *m/z* 152 (quantitation ion)

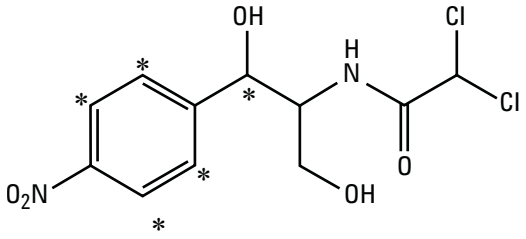
Results and Discussion

Spectral Quality and Sensitivity of Standard

Table 2 lists the structure of the CAP and the fragment ions used for quantitation and confirmation as described by the identification point system.[6] To obtain the most sensitivity, only two or three parameters need to be optimized on this instrument. They are the fragmentor, to provide highest transmission of the precursor ion, the collision energy, to maximize signal for the quantitation and qualifier ion, and possibly the V_{cap} (electrospray voltage), to maximize the number of ions generated.

Table 2. Structure and Fragment Ions of CAP and CAP-d5 (* indicates deuterated positions for the CAP-d5 IS)

Chloramphenicol structure



	m/z	
CAP	257	Qualifier ion
	152	Quantitiation ion
CAP-d5	262	
	157	

Optimization of MS Condition

Figure 1 shows the results of varying the V_{cap}. For this analyte there was little effect from varying this parameter. Only a slight increase in signal is observed at 3,500 V, and this voltage was used. The fragmentor was varied from 90 V to 160 V. Above 120 V, fragment ions are observed and the precursor ion signal drops significantly. At 160 V on the fragmentor almost no *m/z* 321 is observed. This results show that 100 V on the fragmentor provided the highest precursor ion signal. Finally, using a product ion scan of the precursor, m/z 321, the collision energy (CE) was varied from 2 V, 5 V, 8 V, 10 V, 15 V, 18 V to 40 V.

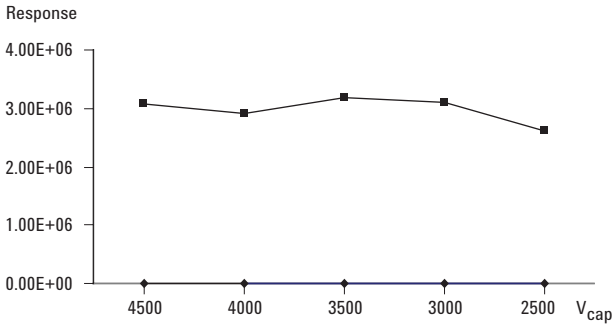


Figure 1. Plot of V_{cap} voltage vs. response of precursor ion at *m/z* 321.

Comparison of extracted ion chromatograms of the quantitation and qualifier ions showed that response maximized at 10 V for *m/z* 257 and at 15 V for *m/z* 152. The product ion spectra for these two collision energy experiments are shown in Figure 2 and Figure 3. As shown in Table 3, the same CE were used for the deuterated internal standard.

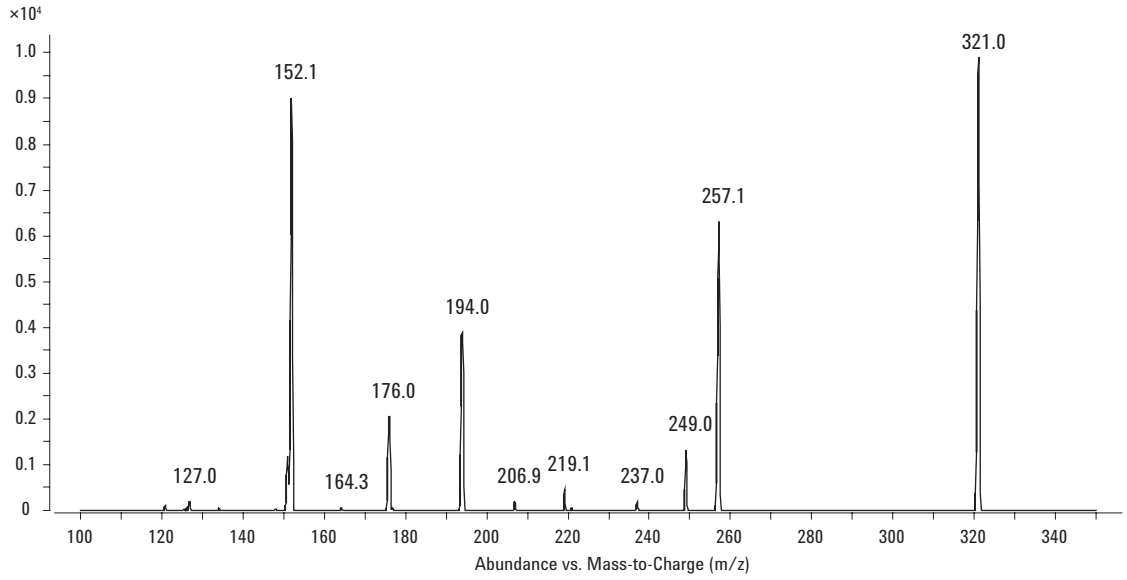


Figure 2. Product ion spectrum of *m/z* 321 at 10 V collision energy.

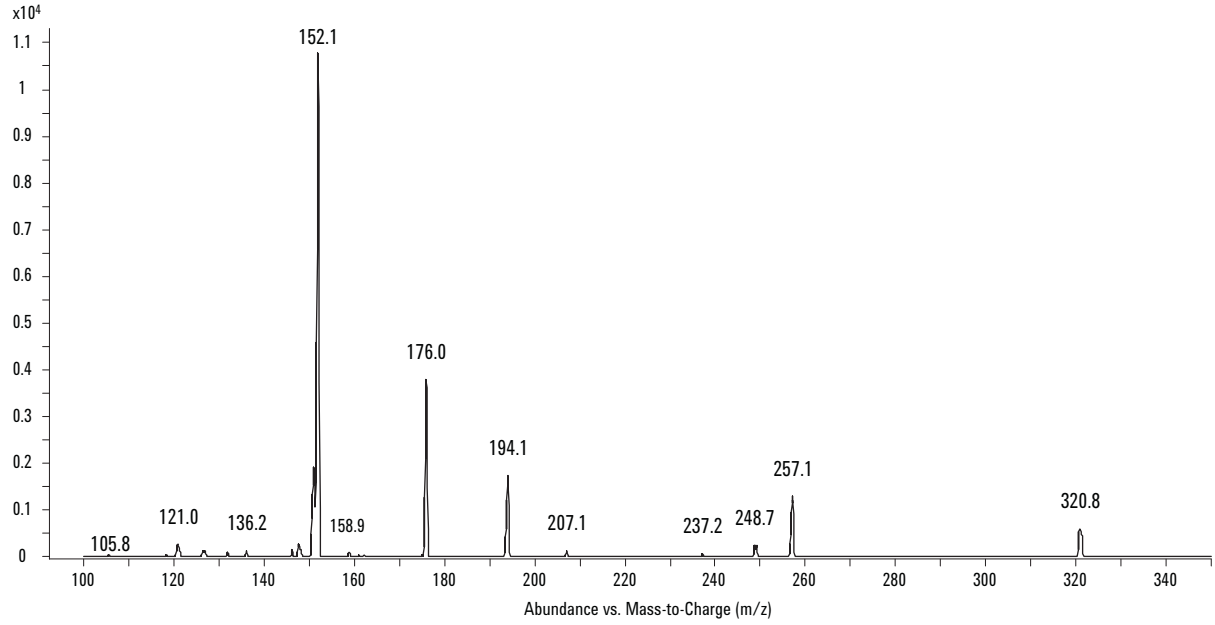


Figure 3. Product ion spectrum of *m/z* 321 at 15 V collision energy.

Table 3. MRM Mode Parameters

Compound	Transition	Dwell time (ms)	Fragmentor Voltage (V)	Collision) Energy (V)	MS2 resolution
CAP	321–257	200	100	10	Unit
	321–152	200	100	15	Unit
CAP-d5	326–262	200	100	10	Unit
	326–157	200	100	15	Unit

Repeatability

Using honey matrix spiked at 0.1 ppb level as an example, the repeatability was tested by running the extract 15 times. Table 4 shows the area of the qualifier and quantitation ions in both the analyte and the IS. On average the areas of each ion vary about 8% and the ratios 5%, well within the 20% required for ratios 50% and above. Masshunter quantitation software tabulates these results and gives a graphic representation as shown in Figure 4.

Table 4. Integrated Areas of the Quantitation Ion and Qualifier Ion and Their Associated Internal Standard Ion

	Chloramphenicol Quantitative ion (321–152)	Qualifier ion (321–257)	Ratio	d5-chloramphenicol Quantitative ion (326–157)	Qualifier ion (326–262)	Ratio
1	350	165	47.1	262	121	50.4
2	346	157	45.2	258	114	55.3
3	346	5	44.6	259	118	49.4
4	313	164	52.3	267	127	47.6
5	301	154	49.5	261	121	46.4
6	313	168	53.6	253	124	49.0
7	320	160	50.1	228	111	48.6
8	326	145	44.5	225	113	50.4
9	317	141	44.5	241	117	48.6
10	290	135	46.6	226	107	47.1
11	300	138	46.2	253	90	45.7
12	281	136	48.4	240	90	47.6
13	303	143	47.3	220	101	45.9
14	290	140	48.3	214	107	49.8
15	261	131	50.3	217	101	46.6
RSD	8.11%	8.30%	5.91%	7.67%	9.99%	4.83%

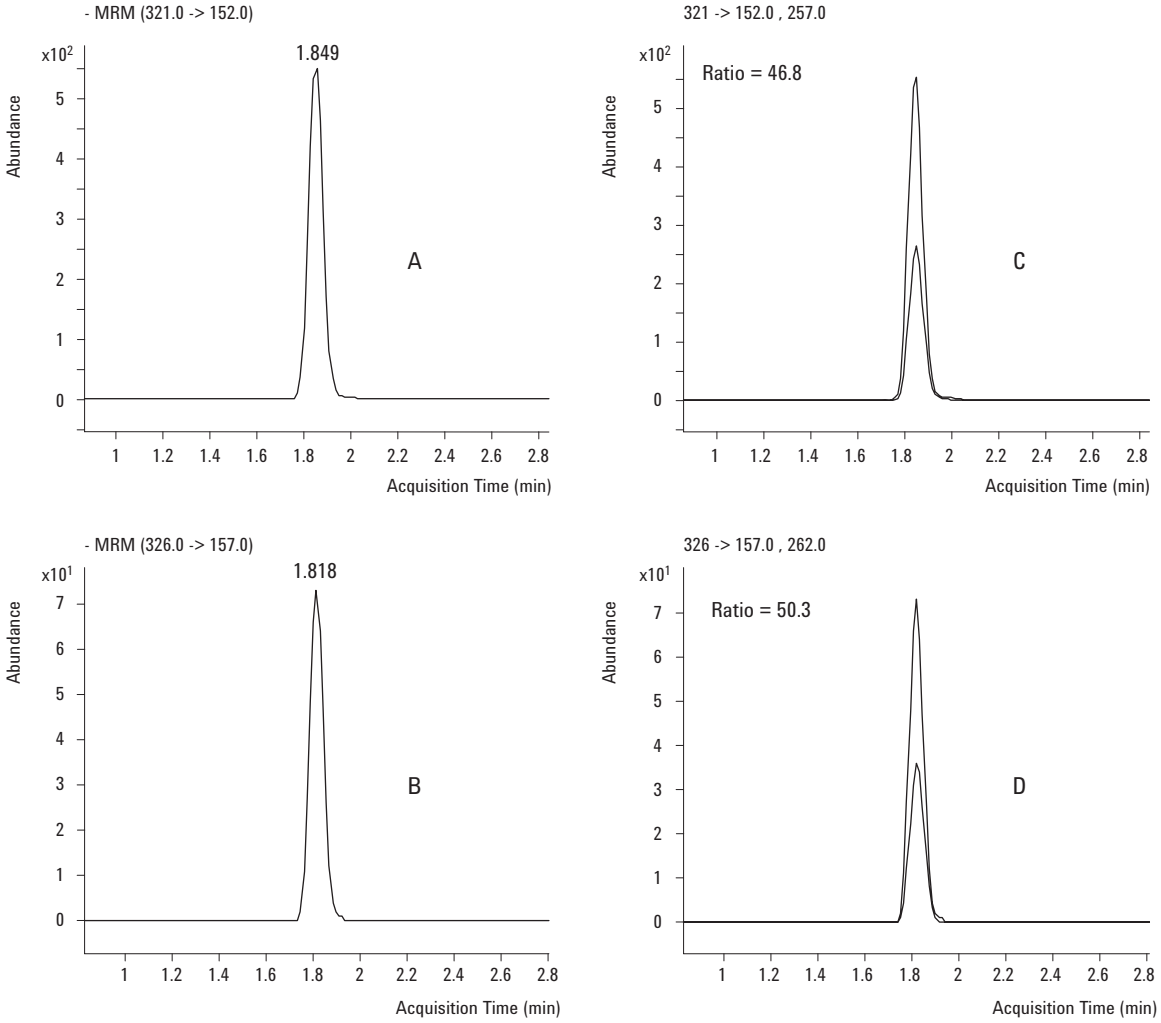


Figure 4. Panels A and B show the CAP and IS peak for the quantitation transition. Panels C and D are the graphic representation of quantitation ion and qualifier ion ratio as shown by MassHunter software.

Linearity

The linearity of the method was determined for CAP in solvent and each of the matrices. This was done from 10 ppt to 1 ppb, well below the minimum required performance level (MRPL) and above that concentration. Figures 5 through 8 show the graphic representation of those results. Each was well above an r^2 value of 0.99.

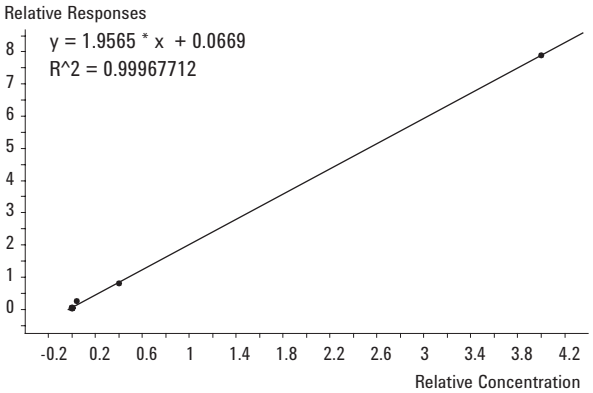


Figure 5. Linearity of CAP in solvent from 10 ppt to 1 ppb.

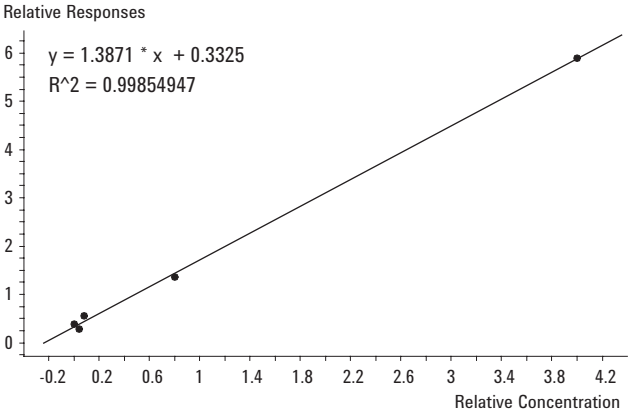


Figure 6. Linearity of CAP in honey from 10 ppt to 1 ppb.

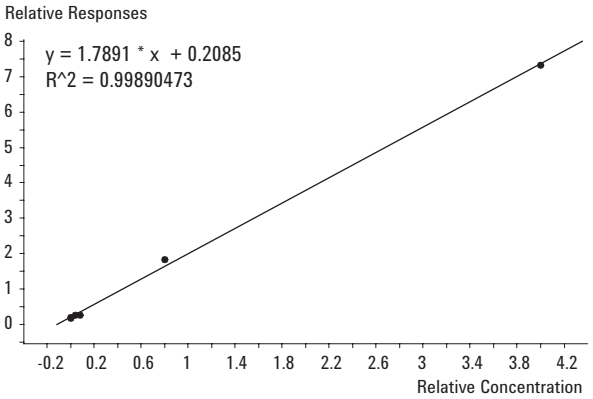


Figure 7. Linearity of CAP in shrimp from 10 ppt to 1 ppb.

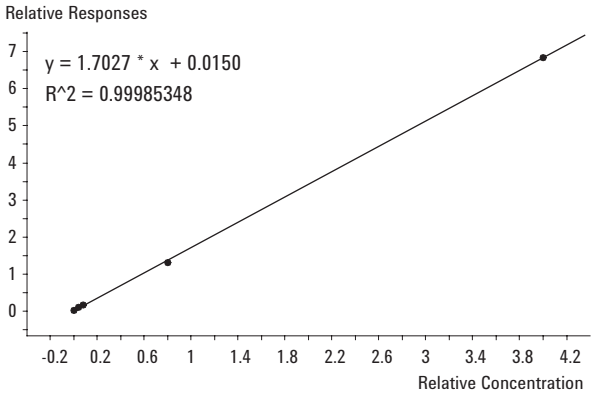


Figure 8. Linearity of CAP in chicken from 10 ppt to 1 ppb.

Sensitivity

The sensitivity of CAP standard in solvent is observed at 10 ppt with an injection volume of 5 µL. The MRM chromatogram is shown in Figure 9. Although this demonstrates the sensitivity of the instrument, it is also important to determine the sensitivity in real sample matrix. This is shown in Figure 10 with a spike concentration of CAP at 10 ppt with a 5-µL injection. Not only is the analyte detectable, but the ratio of the qualifier ion is within the specified tolerance so confirmation can be obtained.

Recovery

Recovery was determined by spiking CAP into three samples of matrix and extracting using the specified SPE. Table 5 shows both the repeatability of extraction and analysis and the mean recovery. Using the internal standard spiked before extraction, recovery is automatically compensated. Thus accuracy of the quantification is very good using this methodology. The recovery results show the overall effectiveness of the method.

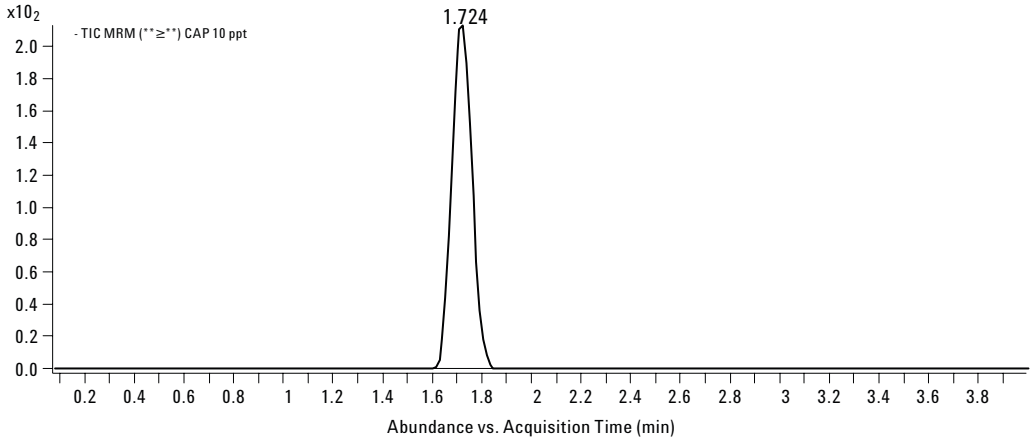


Figure 9. MRM chromatogram of 10 ppt CAP in solvent with injection volume of 5 µL.

Table 5. Recovery of CAP at 0.1 ppb Where Three Sample Aliquots of Each Matrix Were Spiked and Determined

	Honey (n=3)	Shrimp (n=3)	Chicken (n=3)
RSD (%)	6.29	3.93	3.29
Recovery (%)	89.5	85.4	86.4

Conclusions

The method described herein for the analysis of CAP in three important matrices has been shown to be highly effective and meet the criteria for quantitation and confirmation well below the required 0.3 ppb MRPL. Optimization of the method was simple, as few parameters in the mass spectrometer need adjustment. In addition, the requirements for a validated method have been shown. These include sensitivity, repeatability, linearity, and recovery. The Agilent 6410 LC/MS triple quadrupole instrument has been shown to be a highly effective instrument for the analysis of chloramphenicol.

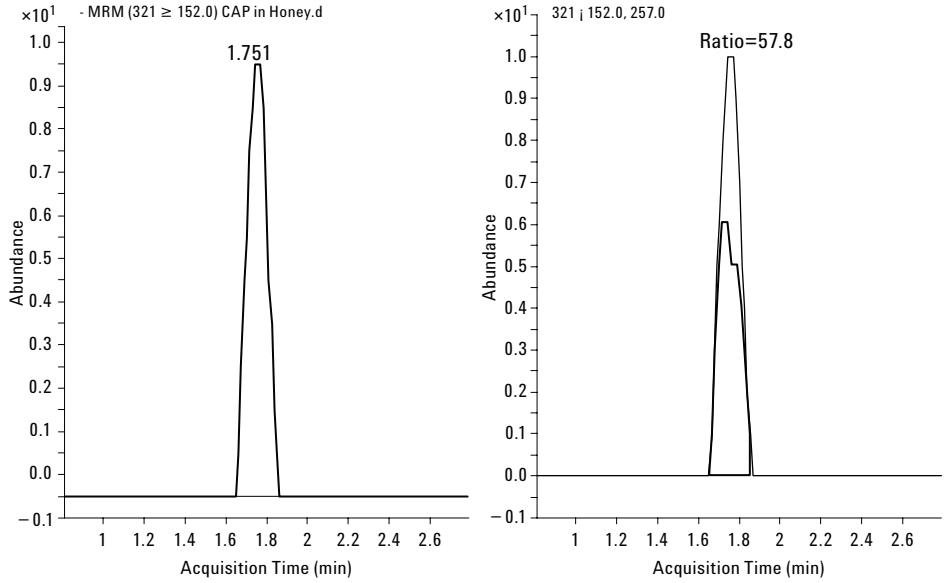


Figure 10. MRM chromatogram of quantitation ion and ratio of qualifier ion for 10 ppt CAP in honey.

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Detection of Recombinant Bovine Somatotropin in Milk by LC-ESI-MS/MS

Application

Food Safety

Authors

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Abstract

Recombinant bovine somatotropin (rbST), also called growth hormone, is a protein hormone used in dairy farming to enhance milk production. A method has been developed for the detection of rbST in milk by ESI(+)-LC-MS/MS. This method allowed a detection limit of 20 pg of tryptic N-terminal peptide rbST in standard solution injected on-column and was successfully applied to extracts obtained from milk samples spiked with 50 ng/mL^{-1} (2.3 pmol/mL^{-1}) rbST.

Introduction

Recombinant bovine somatotropin (rbST), also called growth hormone, is used in lactating cows to increase milk production. Different regulations exist regarding its use, but the lack of confirmatory methods [1] for its detection makes it difficult to apply these regulations. It turns out to be an international issue in terms of animal doping and in food safety as well. Indeed, residues of rbST can be present in the milk of dairy animals treated with this hormone.

In order to detect residues of rbST in milk, the choice has been made to focus the analysis on the tryptic N-terminal peptide of the protein, specifically the difference between the endogenous and recombinant forms. The N-terminal amino acid alanine that is present in the endogenous form is replaced by a methionine in the recombinant one [2].

This application describes a method for the detection of rbST by ESI(+) LC-MS/MS. The method was successfully applied to extracts from milk samples spiked with rbST.

Experimental

Standards of Proteins and Peptides

Protein standards of rbST and recombinant equine somatotropin, reST (EquiGen-5), were obtained from the Harbor-UCLA Medical Center, National Hormone and Pituitary Program (Torrance, CA, USA) and Bresagen Limited (Thebarton, Australia), respectively.

The peptides used as standards, with the following amino acid sequence MFPAMSLSGLFANAVLR (N-terminal tryptic rbST), MFPAMPLSSLFANAVLR (N-terminal tryptic reST), and AFPAMSLSGLFAN-AVLR (N-terminal tryptic bST) were synthesized from Millegen (Labege, France).

Instrumentation

The detail of the instrumentation used for the detection of the N-terminal peptides is described in the following tables.

LC

Instrument	Agilent 1200	
Column	Column Interchrom ModuloCart QS Uptisphere 3HDO 150 mm × 2 mm	
Mobile phase	A: Acetonitrile + 0.1% formic acid B: H_2O + 0.1% formic acid	
Flow rate	0.3 mL/min	
Injection volume	20 µL	
Gradient	Time (min)	%A
	0	10
	5	55
	10	60
	15	100
	17	10
	20	10

MS

Instrument	Agilent 6410 LC/MS Triple Quadrupole
Ionization mode	ESI (+)
Capillary	5000 V
Nebulizer	55 psi
Gas flow	13 L/min
Gas temperature	300 °C

Selected Reaction Monitoring (SRM) Method Parameters

In order to obtain a better specificity, the detection was performed in SRM mode. The transitions monitored are displayed in Table 1.

Table 1. SRM Method Parameters

Compound	RT	Charge	Transitions monitored	Collision energy (V)
Nterm rbST	8.33	z = 2	913.2 → 1047.7	30
			913.2 → 774.1	20
		z = 3	609.3 → 774	10
			609.3 → 643.5	20
Nterm reST	8.39	z = 2	933.2 → 1287.9	30
			933.2 → 794.1	20
Nterm bST	8.20	z = 2	883.2 → 1047.8	20
			883.2 → 774.1	20

Results and Discussion

Application of Triple Quadrupole MS-MS and Electrospray Ionization Mode Methodology

In this method, the choice has been made to use electrospray ionization in positive mode. Indeed, this ionization mode presented as a “soft” ionization technique is optimal for peptides. The ionization of the N-terminal peptide rbST leads to two main forms (z = 2 and z = 3).

This use of a triple quadrupole based methodology enabled very good sensitivity and selectivity and also a possible quantification of the monitored signals.

Separation of the Different Compounds

The detection method was developed for the detection of the tryptic N-terminal peptide of rbST and also for the tryptic N-terminal peptide of endogenous pituitary bovine somatrotropin (bST) and reST as well. Due to the high homology in the amino acid sequence with rbST, reST was used as the internal standard.

The three compounds were separated chromatographically and analyzed utilizing the transitions described in Table 1. The chromatogram corresponding to the injection of 0.2 ng of N-terminal peptide bST, rbST, and reST is shown in Figure 1.

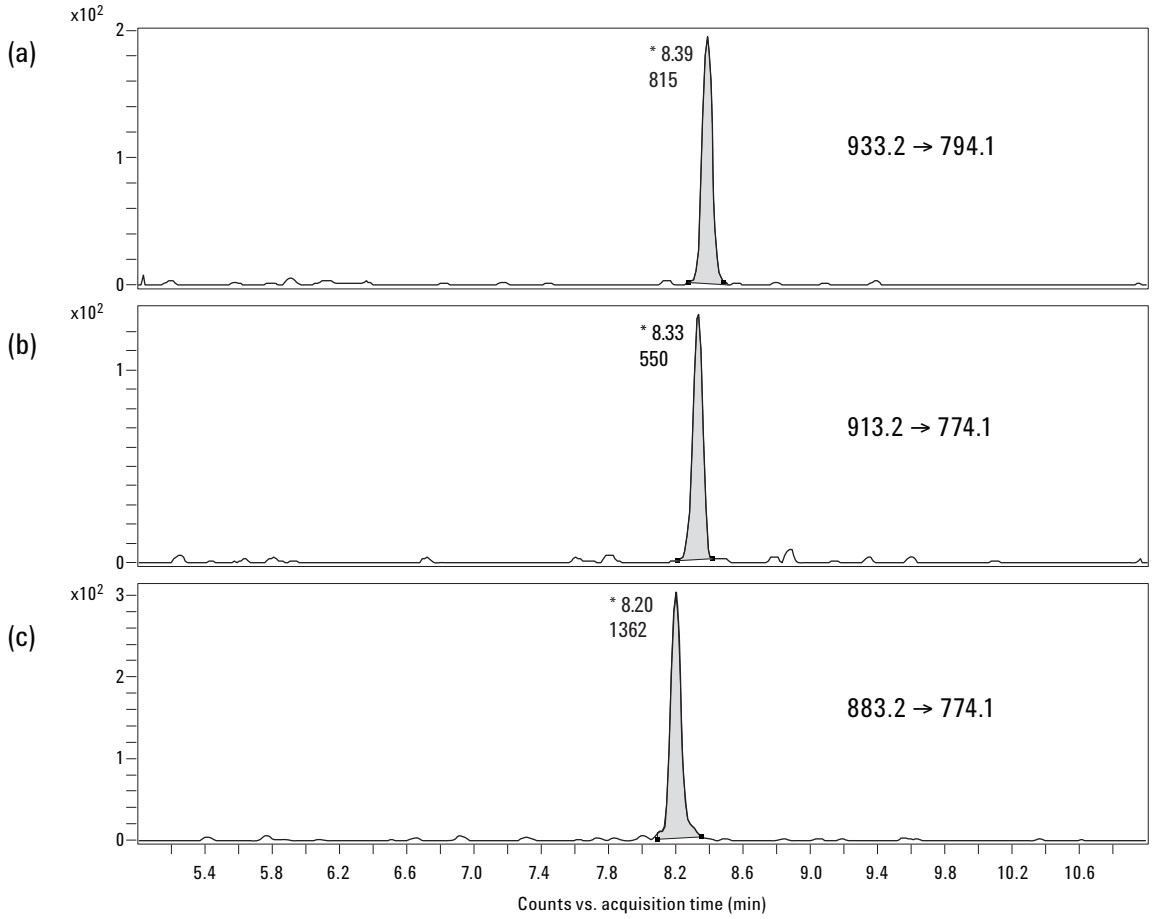


Figure 1. SRM ion chromatograms of standard solutions of tryptic N-terminal peptide of (a) reST, (b) rbST and (c) bST. The injection aliquot used corresponded to 0.2 ng on-column.

Even with an optimized gradient, due to their high homology in terms of sequence, the three compounds eluted with very similar retention times.

Linearity and Sensitivity of the Method

The method described allowed detection of the three peptides with very good sensitivity. A limit of detection of 20 pg injected on-column (~900 femtomole) was reached. Quantification was possible as shown by the good linearity of the calibration curves (Figure 2).

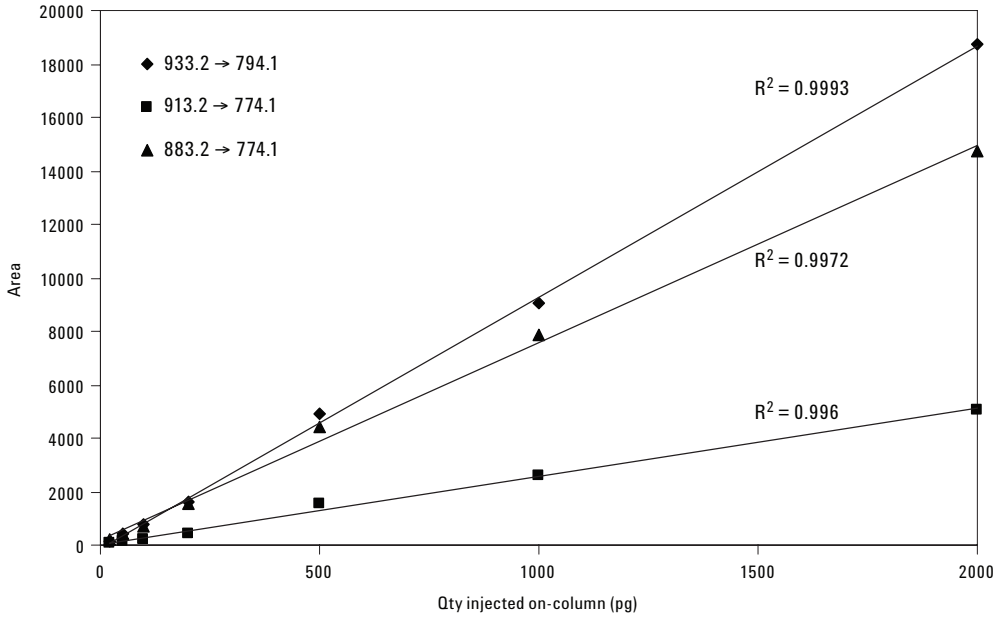


Figure 2. Calibration curve of tryptic N-terminal peptides rbST (913.2 → 774.1), reST (933.2 → 794.1) and bST (883.2 → 774.1).

Results of Spiked Samples

The detection method was applied to extracts obtained from milk samples spiked with rbST. The purification procedure used is described in [3].

Figure 3 shows the chromatogram of a milk sample spiked with 50 ng.mL^{-1} of rbST, in accordance with guidelines for the identification of rbST according to 2002/657 criteria [4]. The chromatogram shows excellent peak shape, and above all, nearly null

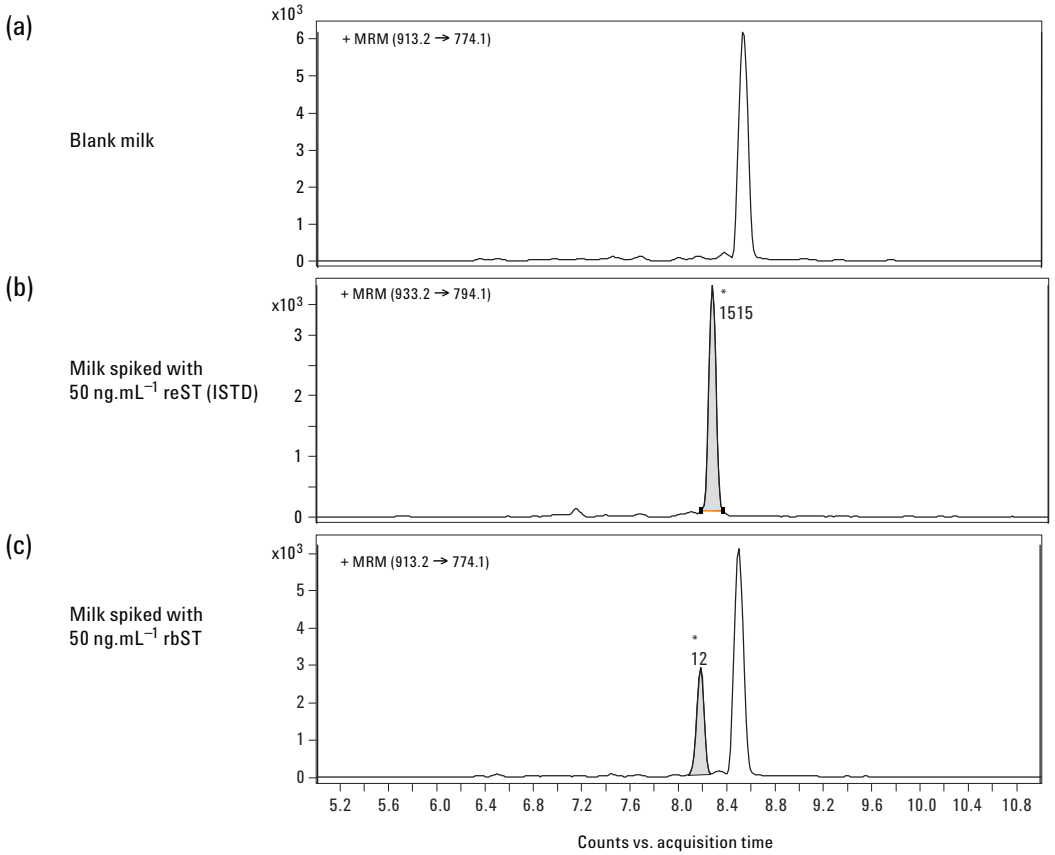


Figure 3. SRM ion chromatograms obtained from milk samples. The different signals correspond to (a) blank milk, (b) the same milk spiked with 50 ng.mL^{-1} reST (internal standard), and (c) 50 ng.mL^{-1} rbST.

background noise, demonstrating the selectivity of the method. The intensity of the signal, although lower than the internal standard, is, however, significant, and shows a clear and distinct signal. The method clearly allows for unambiguous identification of rbST in milk.

Conclusions

The detection of rbST in milk was performed with detection by ESI(+) LC-MS/MS. The method showed very good sensitivity, specificity, and robustness. It was successfully applied to milk samples spiked with rbST at 50 $ng.mL^{-1}$, in accordance with criteria outlined by the 2002/657 Council Directive.

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Development of an LC-MS/MS Method for the Determination of 20-Hydroxyecdysone and Its Metabolites in Calf Urine

Application to the Control of Its Potential Misuse in Cattle

Application Note

Food Safety

Authors

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Abstract

Ecdysteroids, which are steroid hormones present in invertebrates and in plants, could be potentially used as anabolic agents in food-producing animals. The control of ecdysteroid misuse in cattle relies on the development of an efficient method for their detection in biological matrices at trace levels ($\mu g.L^{-1}$). In this context, an analytical procedure dedicated to the identification of 20-hydroxy-ecdysone and its metabolites in urine samples, based on purification on two solid-phase extraction cartridges (SPE C_{18} and SPE SiOH) and LC-(ESI+)-MS/MS measurements has been developed. The performance of tandem quadrupole MS/MS, in terms of sensitivity and specificity, allowed measurements at trace levels in both spiked and incurred samples. Good linearity was observed for all analytes from 0.12 ng to 12 ng on column.

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Introduction

Ecdysteroids are steroid hormones present both in invertebrate species (mainly Arthropods) and plants (belonging to *Asteraceae, Caryophyllaceae,* or *Polypodiaceae*). In arthropods, ecdysteroids act as moulting hormones, whereas these molecules are thought to protect plants against nonadapted phytophagous insects. The archetypal ecdysteroid in both kingdoms is 20-hydroxyecdysone (20E), and several studies have underlined its possible growth-promoting effects in various animal species (rats, mice, and Japanese quail), including humans and cattle [1–3]. Clinical studies demonstrated that 20E is more anabolic than methandrostanolone (dianabol), with no androgenic or other undesirable side effects usually observed with classical steroids [4]. However, despite its growth-promoting properties, only a few methods have been reported for its detection in biological matrices, and no information is available concerning its metabolism in cattle [5]. In this application, the development of a method able to detect and identify 20E and its main metabolites at trace levels (ppb) in calf urine is described [6]. This method was applied to the analysis of calf urine samples after 20E oral administration and used to assess the kinetic of elimination of these substances.

Experimental

Compound Standards

Standard reference 22S,23S-homobrassinolide (belonging to brassinosteroids, vegetable steroid hormones) was from Sigma-Aldrich (St. Quentin Fallavier, France); 20-hydroxyecdysone, 14-deoxy, 20-hydroxyecdysone, and 20,26-dihydroxyecdysone were a kind gift from Pr. Lafont.

Sample Preparation

Twenty-five nanograms of 22S,23S-homobrassinolide were added as internal standard (IS) to 5 mL of calf urine, centrifuged at 3,500 g for 15 min, then purified on SPE C18. The C18-SPE cartridges were conditioned with 10 mL methanol, then 10 mL water, following which the urine samples were applied. The columns were then washed with 6 mL of a water/methanol (80/20) mixture, and the ecdysteroids were subsequently eluted with 10 mL methanol. The eluant was then evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted in 50 µL ethanol and 150 µL cyclohexane before loading onto a SPE SiOH, previously activated with 25 mL cyclohexane. The phase was washed with 6 mL ethyl acetate/cyclohexane (80/20) and the compounds of interest were then eluted with 10 mL of a mixture of chloroform/methanol/acetone (6/2/1). The solvent was evaporated to dryness under nitrogen and the final extract was redissolved in 50 µL of methanol/water (30/70) containing 0.5% acetic acid. From this extract 10 µL was injected onto the HPLC column.

Instrumentation

LC:

Column:	GEMINI C_{18}, Phenomenex (3 µm, 110 Å, 50 × 2 mm)/Agilent equivalent: ZORBAX Extend-C18 3.5 µm, 2.1 mm × 50 mm (p/n 735700-902)	
Column temperature:	40 °C	
Mobile phases:	A: MeOH	
	B: 0.5% acetic acid in water	
Flow rate:	0.3 mL/min	
Gradient:	Time (min)	%B
	0	90
	8	0
	10	0
	12	90
	16	90
Injection volume:	10 µL	
MS:	G 6410A QQQ, Agilent Technologies	
Ionization:	ESI (+)	
Fragmentor:	120 V	
Mass range:	100–500 amu	
Scan time:	300 ms	
Capillary:	4000 V	
Nebulizer:	35 psi	
Drying gas:	11 L/min	
Gas temperature:	325 °C	

The monitored transitions for each target compound are reported in Table 1. The first transition corresponds to the most sensitive signal.

Results and Discussion

Standard solutions of target compounds were analyzed according to the LC-MS/MS parameters described in the Experimental section, which allowed us to obtain the ion chromatograms of 20E, M1, M2, and IS, each at 5 ng on column (Figure 1). All the compounds are eluted within less than 10 min with very good chromatographic resolution and peak shape.

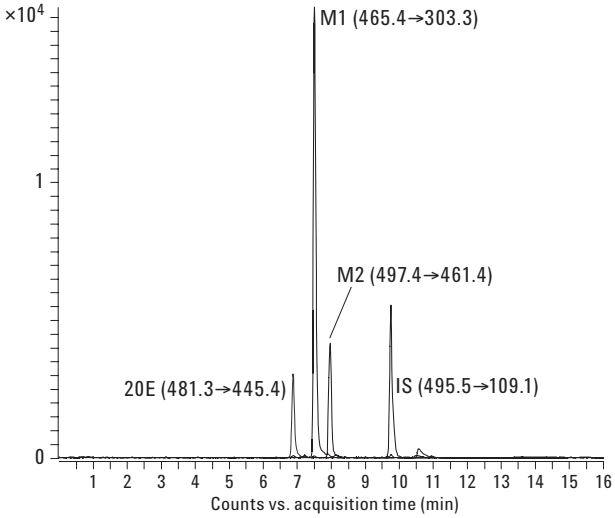


Figure 1. Overlaid extracted ion chromatograms (EICs) for the most sensitive transitions monitored for 20E and its metabolites in positive ion mode.

Table 1. Monitored SRM Transitions for 20E and Its Main Urinary Metabolites and Parameters of Acquisition for Their Analysis by LC-MS/MS (QQQ)

Analytes	Transition 1	Collision energy (eV)	Transition 2	Collision energy (eV)	Transition 3	Collision energy (eV)	RT (min ± 0.2)
22S,23S-homobrassinolide (IS)	495.5→109.1	20	495.5→127.1	10	495.5→459.1	5	9.8
20-hydroxyecdysone	481.3→445.4	10	481.3→371.4	10	481.3→165.1	20	7.5
14-deoxy,20-hydroxyecdysone (M1)	465.4→303.3	20	465.4→285.3	25	–	–	7.9
20,26-dihydroxyecdysone (M2)	497.3→461.4	5	497.3→351.1	15	497.3→371.2	20	6.8

To assess the specificity of the method, a blank urine and a urine sample fortified with 20E (1 µg.L^{-1}) were analyzed. Figure 2 shows the blank traces without any interference at the expected retention time for 20E, demonstrating the good selectivity of the monitored signals. The target analyte 20E was identified in the spiked urine sample with three SRM transitions. The monitored signals are detected with good sensitivity and show high signal-to-noise (s/n) ratios. These results were in accordance with Decision 2002/657/EC criteria, which require more than four identification points [7] in order to validate an identified compound.

The linearity and the repeatability of the method were assessed with the analysis of a pool of urine samples fortified at different concentration levels: the calibration curve was established with five concentration points (0.2, 0.5, 1, 5, and 20 ng.mL^{-1}). The calibration curve correlation coefficients (R^2) were better than 0.99, thus demonstrating the good linearity of the method for 20E.

The method has been successfully applied to incurred calf urine samples after 20E oral administration over four days. 20-hydroxyecdysone was detected in urine as rapidly as 30 minutes after its administration and up until 24 hours after the last administration. 20E metabolism was investigated and two main metabolites, 14-deoxy,20-hydroxyecdysone (M1) and 20,26-dihydroxyecdysone (M2), could be identified [8]. Both M1 and M2 were monitored by LC-MS/MS (Table 1). Figure 3 presents the ion chromatograms for M1 in the urine samples collected before and two days after the last 20E administration.

As can be observed, M1 was not detected in the urine collected before 20E administration, whereas it was throughout the four-day administration period. Furthermore, it could still be detected and identified (in accordance with the four identification points required) two days after the last administration of 20E. This result is of prime interest in the context of potential misuse of ecdysteroids since it offers the longest period for detection, following administration, and therefore enables a more efficient control mechanism.

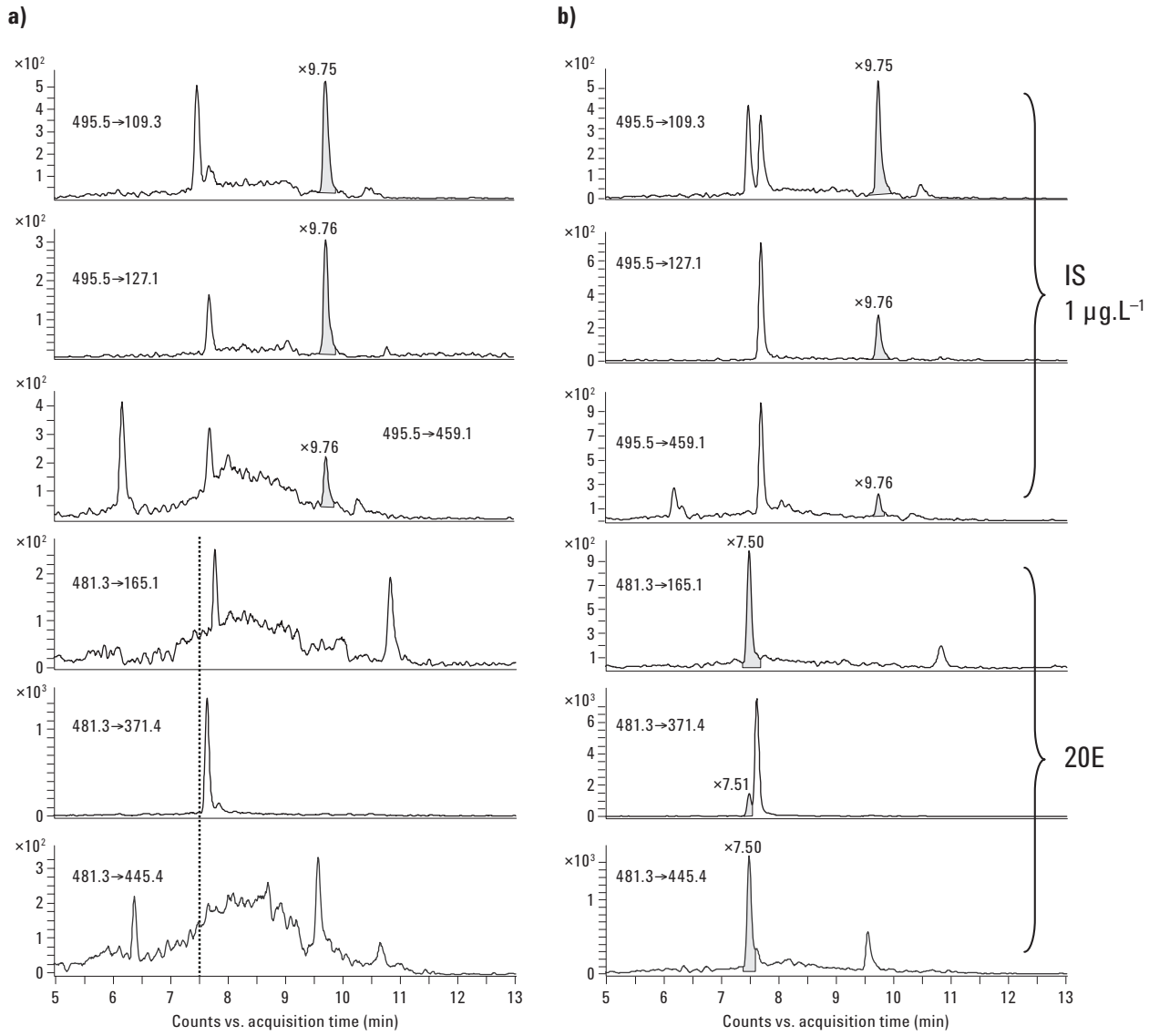


Figure 2. SRM ion chromatograms for a) the blank urine sample and b) the spiked urine sample (1 µg.L-1). LC-(ESI+)-MS/MS measurements.

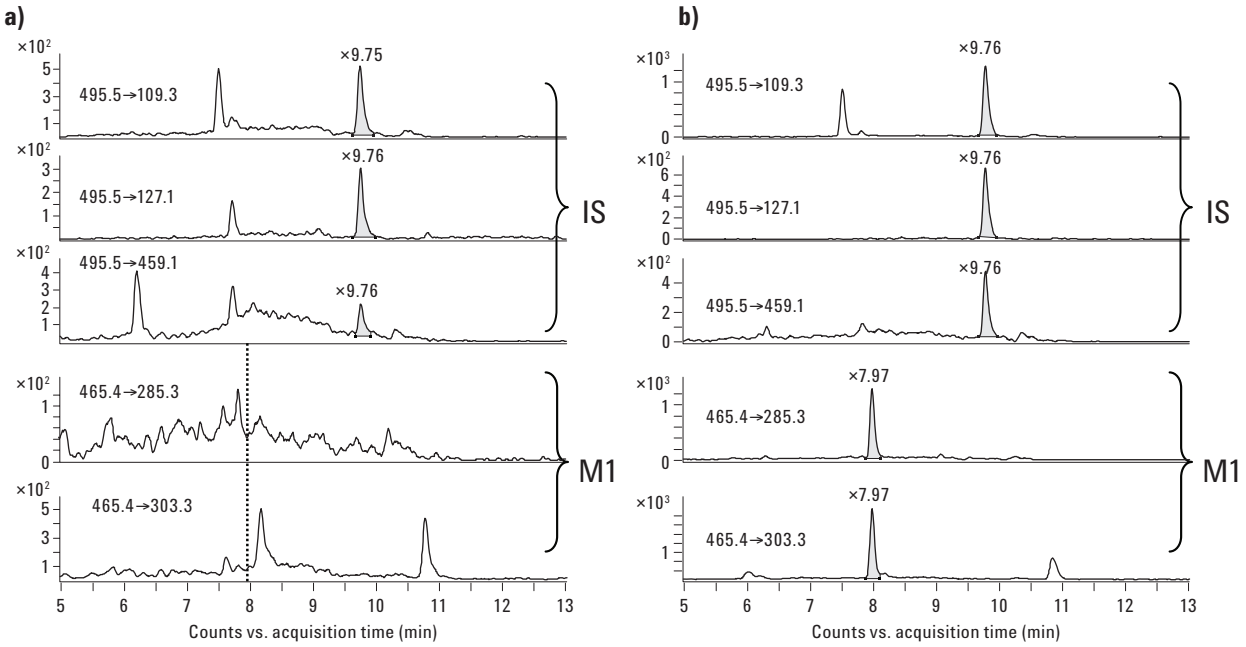


Figure 3. SRM ion chromatograms of IS and M1 in urine sample collected a) before 20E administration and b) two days after the last 20E administration. LC-(ESI+)-MS/MS measurements.

Conclusions

This work demonstrates the performance of LC-MS/MS, which provides efficient identification of 20E and its main metabolites in calf urine. The monitoring of these compounds facilitates the control of the potential misuse of 20E in meat-producing animals. Tandem quadrupole MS/MS is an analytical technique very well suited to this purpose, since it increases confidence in the unambiguous identification of the target compounds, in accordance to the criteria fixed by Decision 2002/657/EC. The successful analysis of the calf urine samples proved the robustness of the developed protocol. Application of this methodology also enabled the determination of the first elimination kinetics and the main metabolites of 20E in calf urine.

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Determination of Tetracyclines in Chicken by Solid-Phase Extraction and High-Performance Liquid Chromatography

Application Note

Food Safety

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Abstract

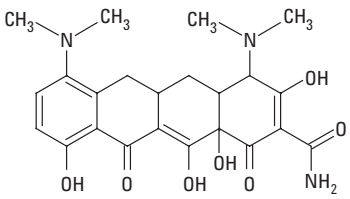
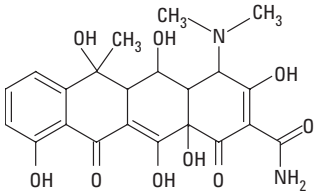
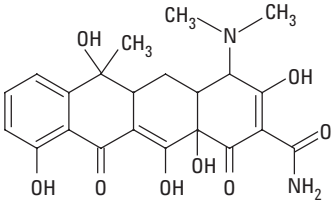
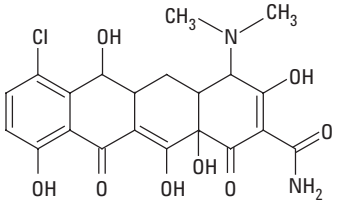
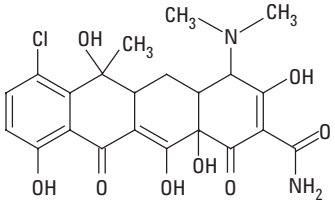
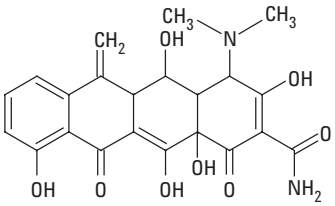
A method for the simultaneous determination of the seven antibiotic residues of minocycline, oxytetracycline, tetracycline, demeclocycline, chlortetracycline, methacycline, and doxycycline in chicken has been developed. In this method, solid-phase extraction (SPE) and HPLC/UV are used consistent with Chinese regulatory methods. Samples are prepared in EDTA-McIlvaine buffer solution (pH 4.0), the clean up is done with an Agilent SampliQ OPT cartridge, and the HPLC separation is performed with an Agilent ZORBAX column (5 µm, 250 mm × 4.6 mm id). The flow rate is 1.5 mL/min, the detector wavelength is 350 nm, and the injection volume is 100 µL. The limits of detection are between 2.5 and 5 µg/kg. Linear calibration curves are obtained over the range of 25 to 500 µg/kg. Overall recoveries range from 59.0 to 99.0%, with RSD values between 1.0 and 6.5%.

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Introduction

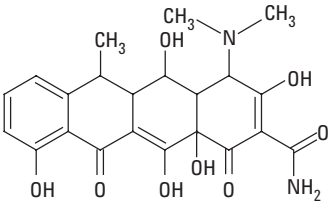
"Tetracyclines" is the common name for a group of antimicrobials with a hydronaphthacene structure (Table 1). Tetracyclines are used against a wide range of gram-negative and gram-positive microorganisms. The Chinese government has set maximum residue limits (MRLs) for tetracyclines in muscle (100 µg/kg) and promulgated a government standard (GB/T 21317-2007) that established a method for the determination of tetracyclines in animal tissues. This application note describes the implementation and optimization of the method described in GB/T 21317-2007 and the results of validation.

Table 1. Tetracyclines Used in This Study

No.	Name	pKa	log P	Structure
1	Minocycline CAS # 10118-90-8	3.3/7.2/9.3	+0.5	
2	Oxytetracycline CAS # 6153-64-6	3.3/7.3/9.1	–0.9	
3	Tetracycline CAS # 60-54-8	3.3/7.7/9.7	–1.3	
4	Demeclocycline CAS # 127-33-3	3.3/7.2/9.3	+0.2	
5	Chlortetracycline CAS # 57-62-5	3.3/7.4/9.3	–0.62	
6	Methacycline CAS # 914-00-1	3.5/7.6/9.2	–0.3	

Continued

Table 1. Tetracyclines Used in This Study (continued)

No.	Name	pKa	log P	Structure
7	Doxycycline CAS# 564-25-0	3.1/7.7/9.3	–0.02	

Experimental

Materials and Chemicals

All reagents and solvents were HPLC or analytical grade. Tetracycline standards were purchased from Sigma-Aldrich or from China's National Institute for the Control of Pharmaceutical and Biological Products (NICPBP).

Stock solution (0.1 mg/mL) was prepared in methanol and kept in the freezer (–20 °C). Working solutions were prepared using the stock solution diluted with a mixture of methanol/10 mmol/L trifluoroacetic acid solution (1/19). The working solutions were prepared daily.

The SPE cartridges were Agilent SampliQ OPT 3 mL, 60 mg (p/n 5982-3036). The analysis was performed on an Agilent 1200 HPLC with DAD. The analytical column was an Agilent ZORBAX SB-C8 5 µm, 250 mm × 4.6 mm id (p/n 880975-906).

McIlvaine buffer, mix 1000 mL 0.1 mol/L citric acid with 625 mL 0.2 M disodium hydrogen phosphate. Adjust pH to 4.0 ± 0.05 with NaOH or HCl as needed.

Na_2EDTA-McIlvaine buffer (0.1 mol/L), mix 60.5g Na_2EDTA. $2H_2O$ into 1625 mL McIlvaine buffer.

HPLC Conditions

Column:	Agilent ZORBAX SB-C8 250 mm × 4.6 mm, 5 µm
Flow rate:	1.5 mL/min
Column temperature:	30 °C
Injection volume:	100 µL
Detector wavelength:	350 nm
Mobile phase:	Methanol-acetonitrile-10 mmol/L TFA solution, gradient elution

Time (minutes)	% methanol	% acetonitrile	% 10 mmol TFA
0	1	4	95
7.5	6	24	70
13.5	7	28	65
15	1	4	95

Sample Preparation

A 200-g sample of chicken was homogenized with a tissue disintegrator, placed in a clean, sealed container, and stored in a freezer below –18 °C.

A 5-g homogeneous sample (accurate to 0.01 g) was placed into a 50-mL polypropylene centrifuge tube with 20 mL 0.1 mol/L Na_2EDTA-McIlvaine buffer solution and vortex mixed for 1 minute followed by a 10-minute ultrasonic extraction in an ice bath. The sample was then centrifuged at a rotate speed of 3,000 r/min for 5 minutes (below 15 °C). The supernatant was removed and saved in a clean tube. The extraction was repeated twice with 20 mL and 10 mL successively. The combined supernatant fluid was brought to 50 mL with buffer, mixed well, centrifuged at a rotate speed of 4,000 r/min for 10 min (below 15 °C), and filtered with fast filter paper.

SPE Purification

The procedure used for the SPE extraction is shown in Figure 1. Agilent SampliQ OPT cartridges were preconditioned with 5 mL of methanol, then 5 mL of a 10 mmol/L TFA solution. A 10-mL extract (equivalent to a 1-g sample) was passed through the SampliQ OPT cartridge at a speed of 1 mL/min. After the sample effused completely, the cartridge was washed with 3 mL of water (pH adjusted to 4.5 with TFA). The entire effluent was discarded. The cartridge was dried under negative pressure below 2.0 kPa for 3 minutes. Finally, the cartridge was eluted with 10 mL of 10 mmol/L oxalic acid in methanol. The eluent was collected and dried under nitrogen below 40 °C. The resulting residue was dissolved and made to a constant volume of 0.5 mL using the methanol/10 mmol/L TFA solution (1/19). Then the residue was filtered through a 0.45-µm filter membrane (p/n 5185-5836) and analyzed.

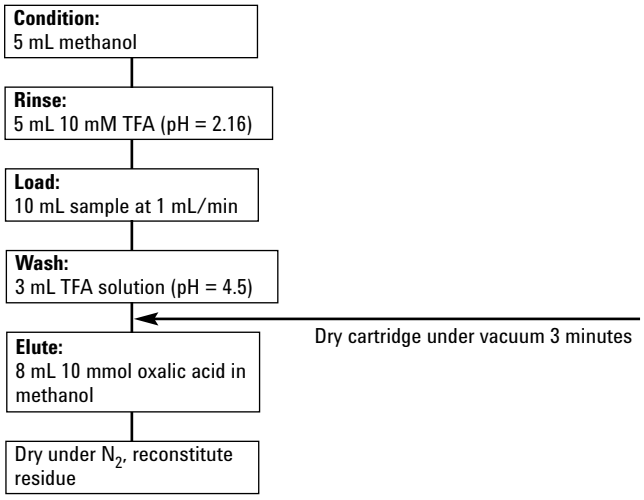


Figure 1. Tetracycline SPE procedure.

Results and Discussion

Linearity, Limits of Detection

Stock solutions were diluted to different concentrations and analyzed by HPLC. Linear regressions were calculated for the tetracyclines using the areas and the solution concentrations. The limit of detection (LOD) was the injection concentration whose signal-to-noise ratio was between 2 and 3. The linear range was between 25 and 500 µg/kg. The linearity and LOD are shown in Table 2.

Table 2. Linearity and LODs of Tetracyclines

Compound	Regression equation	Correlation coefficient	LOD (µg/kg)
Minocycline	Y = 86.313 × –0.1491	0.9996	2.5
Oxytetracycline	Y = 95.965 × +0.0261	0.9999	2.5
Tetracycline	Y = 103.97 × –0.4698	0.9999	2.5
Demeclocycline	Y = 68.659 × –0.1172	0.9998	5
Chlortetracycline	Y = 51.752 × –0.0284	0.9999	5
Methacycline	Y = 98.243 × +1.2567	0.9985	2.5
Doxycycline	Y = 76.408 × +1.0756	0.9987	5

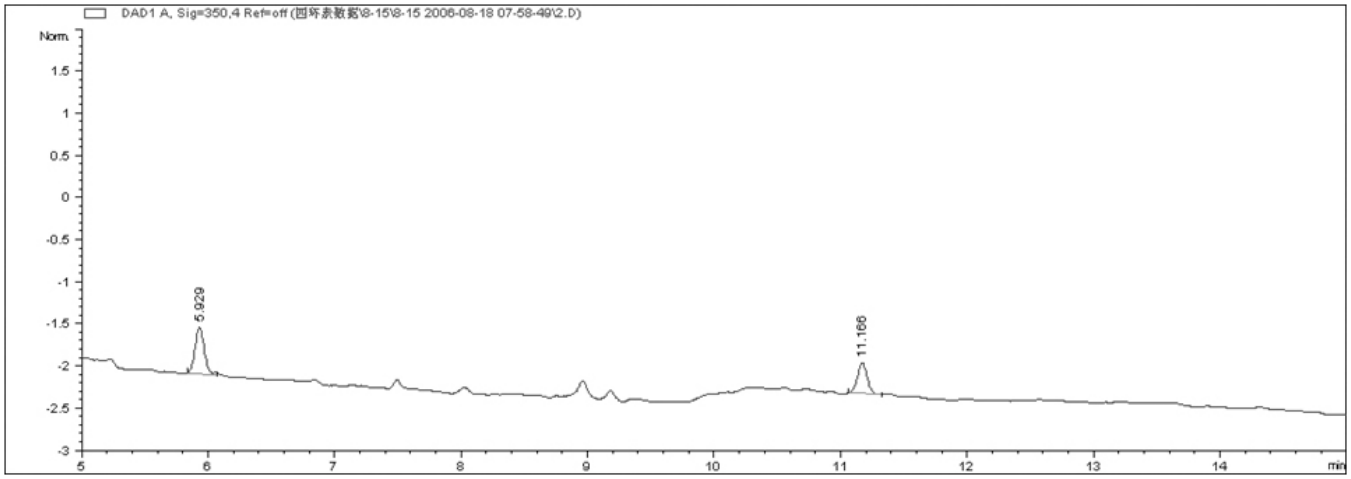


Figure 2. Chromatogram of a chicken blank.

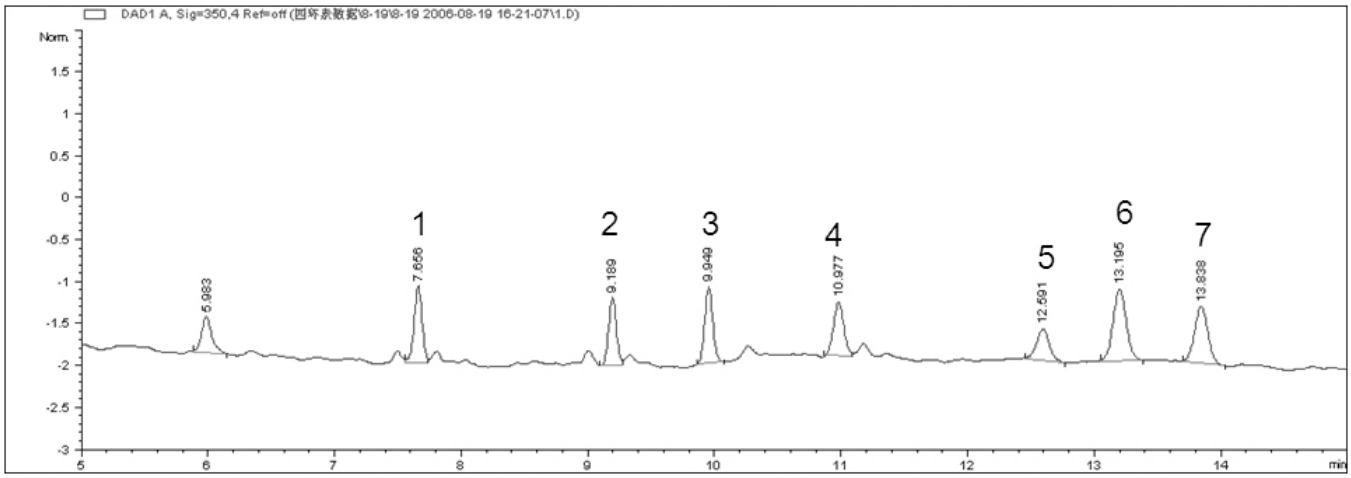


*Figure 3. Chromatogram of a chicken sample spiked at 50 µg/kg. (*1-Minocycline, 2-Oxytetracycline, 3-Tetracycline, 4-Demeclocycline, 5-Chlortetracycline, 6-Methacycline, and 7-Doxycycline)

Recovery and Reproducibility

The precision of the method was determined as recoveries of spiked tetracycline standards in chicken at 50 µg/kg, 100 µg/kg, and 200 µg/kg levels. The analysis was performed in replicates of six at each level. The chromatograms of the blank and spiked standard (50 µg/kg) are shown in Figure 2 and Figure 3. The recovery and reproducibility data are shown in Table 3.

Table 3. Recoveries and RSDs of Tetracyclines in Chicken by SPE

Compound	Spiked level (µg/kg)	Recovery (%)	RSD (%)
Minocycline	50	87.6	4.13
	100	80.8	5.68
	200	81.3	4.19
Oxytetracycline	50	68.8	6.49
	100	63.0	4.87
	200	59.4	4.35
Tetracycline	50	81.0	4.46
	100	70.0	3.47
	200	72.3	4.38
Demeclocycline	50	92.0	2.06
	100	94.8	3.78
	200	92.9	1.92
Chlortetracycline	50	93.3	3.16
	100	92.4	4.01
	200	87.7	2.54
Methacycline	50	93.3	2.89
	100	91.9	2.51
	200	86.6	3.39
Doxycycline	50	95.6	4.38
	100	96.4	1.00
	200	92.0	3.02

Conclusions

Agilent SampliQ provides a simplified and effective single-cartridge method for the purification and enrichment of multiple tetracycline compounds in chicken. The recovery and reproducibility results based on solution standards are acceptable for tetracycline residue determination in chicken under the Chinese regulation. The impurities from chicken were minimal and did not interfere with any of the tetracyclines analyzed. The LODs of the seven tetracyclines were significantly lower than the MRL (of 100 ug/kg).

Part number	Description
5982-3013	OPT Polymer - Box, 100 × 1 mL tubes, 30 mg
5982-3036	OPT Polymer - Box, 50 × 3 mL tubes, 60 mg
5982-3067	OPT Polymer - Box, 30 × 6 mL tubes, 150 mg
5982-3096	OPT Polymer - 96 Well Plate, 10 mg

References

GB/T 21317-2007, Determination of tetracyclines residues in food of animal origin-LC-MS/MS method and HPLC method.

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Determination of Penicillins in Meat by High Performance Liquid Chromatography (HPLC/UV) and HPLC/MS/MS

Application Note

Food

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Abstract

Penicillins are antibiotics widely used to treat diseases in animals. They are occasionally found in animal products destined for human consumption. In this paper, a solid phase extraction method with a high performance liquid chromatograph tandem mass spectrometer (HPLC/MS/MS) is shown for the simultaneous determination of six antibiotic residues: azlocillin, penicillin G, oxacillin, cloxacillin, nafcillin, and dicloxacillin in animal tissues (porcine muscle). In the method, the reversed phase column Agilent ZORBAX Eclipse Plus C18 (3.5 µm, 100 mm × 2. 1 mm) and an Agilent mixed mode polymer solid phase extraction cartridge (Agilent SampliQ OPT) were combined to give a total solution to the analysis of residual penicillins. The performance of the solid phase extraction procedure on trace residues is quantitatively evaluated by HPLC/MS/MS.

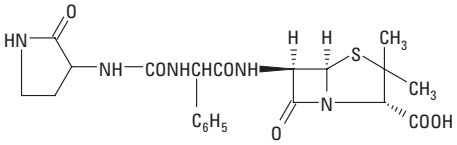
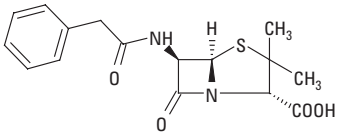
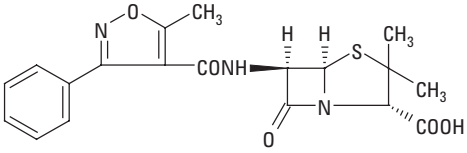
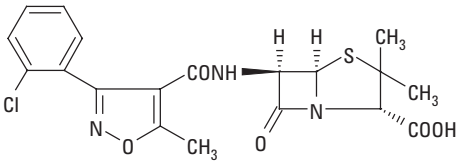
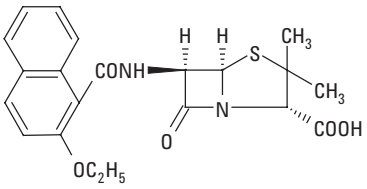
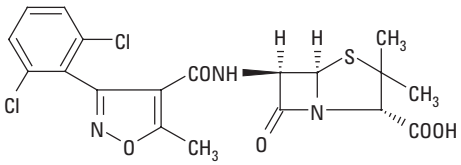
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Introduction

ß-lactam antibiotics (penicillins and cephalosporins) represent some of the most important antibacterial agents used in animals. However, serious reactions are known to occur in some individuals exposed to penicillins and, as a result, these compounds are carefully monitored in foods. Maximum residue limits (MRLs) for pencillins in a variety of foods are established worldwide and are generally in the range of 1ng/g. These regulations require detection and quantification by HPLC/MS/MS. This application will show the development of a sample extraction and cleanup method and the quantification by LC/MS/MS

The structures and chemical constants for the compounds used in this study are shown in Table 1. ß-lactam antibiotics are readily decomposed in acid or base [1] and experimentally show a 20 percent loss in 70 hours at 4 °C (data not shown). Thus, it is necessary to perform the extractions, cleanup, and analysis within 36 hours.

Table 1. The Compounds in This Study

No.	Name	log P.	pKa	Structure
1	Azlocillin	0.2	2.8	
2	Penicillin G	1.5	2.74	
3	Oxacillin	2.4	2.72	
4	Cloxacillin	2.6	2.78	
5	Nafcillin	3.3	2.65	
6	Dicloxacillin	3.7	2.8	

Experimental

Reagents and Chemicals

Water, acetonitrile, and methanol are all HPLC grade (Honeywell Burdick & Jackson). The standards and other chemicals were purchased from Sigma-Aldrich (Saint Louis, MO).

Phosphate buffer (pH = 8.5), 0.05 mol/L: dissolve 8.7 g of potassium phosphate dibasic in HPLC grade water to make 1 L.

Standard stock solutions (1 mg/mL) were made fresh daily in methanol. Spiking solutions were made by appropriate dilution of the stock solutions in phosphate buffer.

Equipment

Agilent 1100 HPLC with diode array detector (Agilent Technologies, Inc., Santa Clara, CA, USA)

Agilent 6410 triple quadrupole LC/MS system with electrospray ionization source (Agilent Technologies, Inc., Santa Clara, CA, USA)

Polytron homogenizer (Brinkman Instruments, Inc., PT10-35, USA)

Refrigerated centrifuge (Sorvall Instruments, RC-5B, rotor SA-600)

Rotary evaporator (BÜCHI, Switzerland/USA)

Sample Preparation

Extraction

Weigh 5 g of raw ground pork (accurate to 0.01 g) into a 50 mL capped polypropylene centrifuge tube, add 15 mL of acetonitrile/water (15:2). Homogenize completely using the Brinkman Polytron (1 minute). Centrifuge at 4,000 rpm and 4 °C for 5 minutes. Save the supernatant. Add 10 mL acetonitrile/water (15:2) to pellet, mix with a spatula to resuspend the ground meat. Homogenize for 1 minute. Centrifuge at 4,000 rpm and 4 °C for five minutes. Combine supernatants. Repeat one additional time.

Take the combined supernatants and place into a round bottom flask and evaporate the acetonitrile at 37 °C. There should be approximately 6 mL of water remaining in the flask. Bring the total volume to 20 mL using pH 8.5 phosphate buffer. Filter with a regenerated cellulose, 25 mm, 45 µm syringe filter (Agilent p/n 5185-5831). Load 10 mL of extract onto the Agilent SampliQ OPT 6 mL/150 mg SPE cartridge (Agilent p/n 5982-3067).

Purification

The procedure for SPE extraction is shown in Figure 1. Load 10 mL of the extract onto the conditioned and equilibrated cartridge. The cartridge is washed with 0.1 percent formic acid in water and then pH 8.5 potassium phosphate buffer. Finally, the sample is eluted with 3 mL acetonitrile. The sample is filtered with a 13 mm, 45 µm PTFE syringe filter (Agilent p/n 5185-5836). The eluent is dried under nitrogen at room temperature. The residue is resuspended in mobile phase to 1.0 mL. The sample is vortexed for 2 minutes and then transferred to a 2 mL autosampler vial (Agilent p/n 5182-0864).

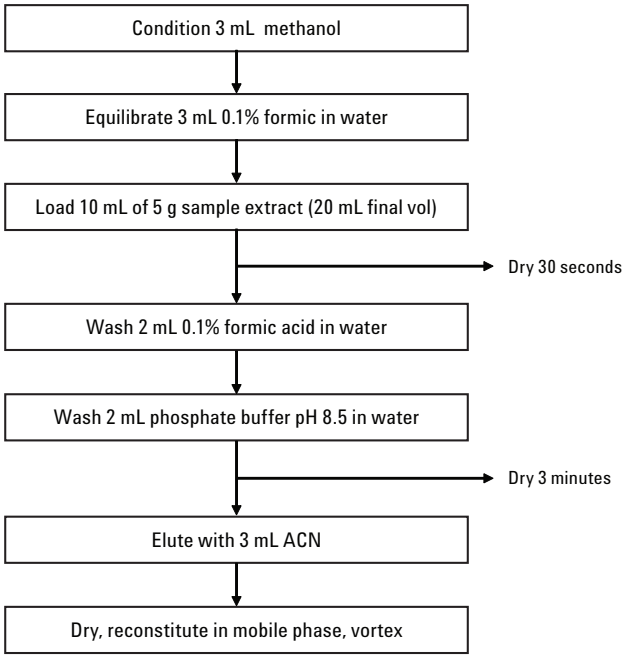


Figure 1. Agilent SampliQ OPT solid phase extraction of penicillins from pork.

Instrument Setting

The HPLC conditions are shown in Tables 2 and 3.

Table 2. HPLC Conditions

HPLC	
Column	Agilent ZORBAX Eclipse Plus, 2.1 mm × 100 mm, 3.5 µm (p/n 959793-902)
Flow rate	0.6 mL/min
Mobile phase	A: water/10 mM ammonium acetate B: acetonitrile
Run time	12 minutes
Post run	3 minutes
Temperature	30 °C
Injection	10 µL

Table 3. HPLC Gradient

Time	%B
0	2
1.2	2
2.0	10
6.0	30
8.0	40
8.5	80
11.9	80
12.0	2

Results and Discussion

Figure 2 shows the chromatograms of the meat spike sample at the limit of quantification (LOQ), 1.0 ng/g (2a), the matrix spiked blank at the LOQ (2b) and the meat extract blank (2c). The cleaned-up pork extract does not show any interferences with the target analytes. HPLC/UV is a significantly less specific detector than HPLC/MS/MS so the impurities remaining after cleanup are more visible using the general UV detector. As shown in Figure 3, the HPLC/UV chromatogram demonstrates that the sample is extremely clean after the sample extraction and SPE cleanup.

Matrix blank material is prepared by taking the meat through the entire extraction and sample cleanup procedure. External standard calibration curves in spiked matrix blanks are made at concentrations of 0.2, 1.0, 10, and 20 ng/g. Table 4 shows the calculated recoveries of spiked meat taken through the entire sample preparation and SPE procedures. All data were calculated automatically with the Agilent MassHunter Quantitative Data Analysis software. Figure 4 shows these results graphically. All of the compounds show acceptable recovery and low relative standard deviation (%RSD).

Conclusions

The results of this study show that the Agilent SampliQ OPT cartridge provides an effective method for cleaning up complex food samples such as porcine muscle. This is demonstrated using penicillins as target compounds. HPLC/UV is

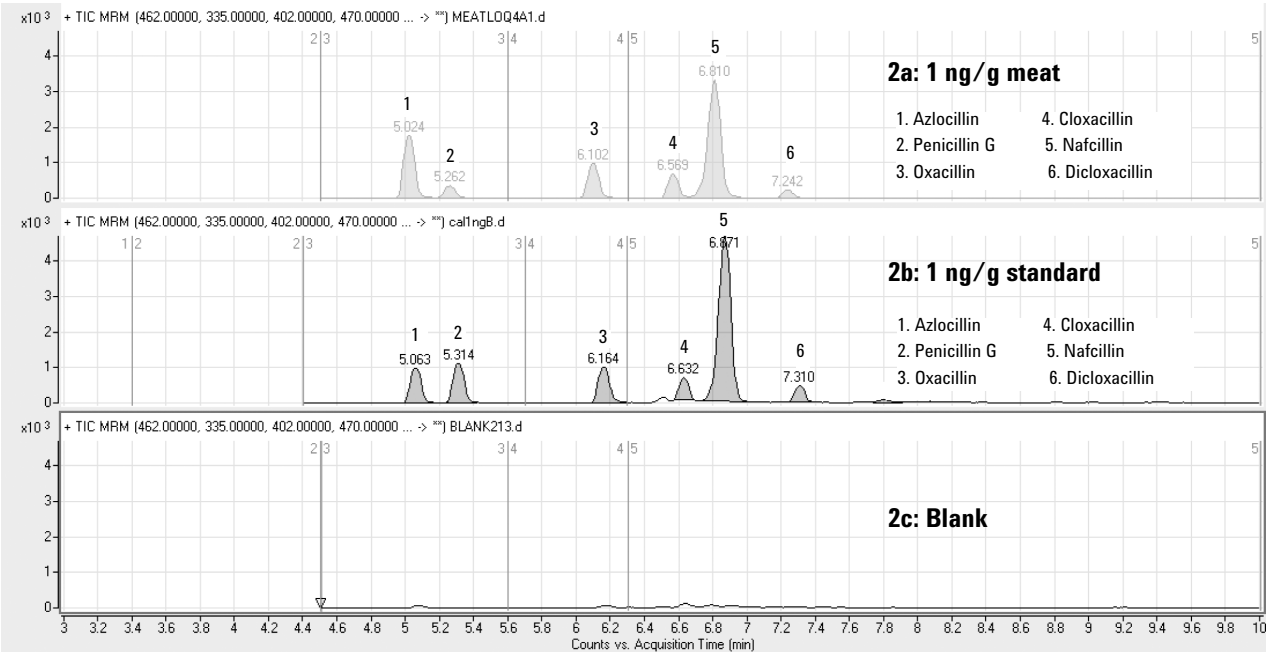


Figure 2. Meat spiked at 1 ng/g taken through extraction and SPE clean-up (2a), meat taken through extraction and clean-up then spiked at 1 ng/g (2b), and unspiked meat taken through extraction and cleanup (2c).

Table 4. Calibration Results for Spiked Meat Blanks

			1.0 ng/g		20 ng/g	
Name	**Linear regression**	**R^2**	**% recovery n = 6**	**%RSD n = 6**	**% recovery n = 6**	**%RSD n = 6**
Azlocillin	y = 6089x –1283	0.9590	77.8	24.2	75.5	6.7
Penicillin G	y = 2690x –513	0.9924	43.6	6.3	48.8	6.8
Oxacillin	y = 2319x +1487	0.9794	96.5	6.0	102.8	8.9
Cloxacillin	y = 2229x +128	0.9848	86.3	7.3	95.3	8.1
Nafcillin	y = 16654x –1264	0.9891	98.5	6.6	101.9	3.1
Dicloxacillin	y = 1899x –113	0.9870	105.7	13.5	103.9	3.6

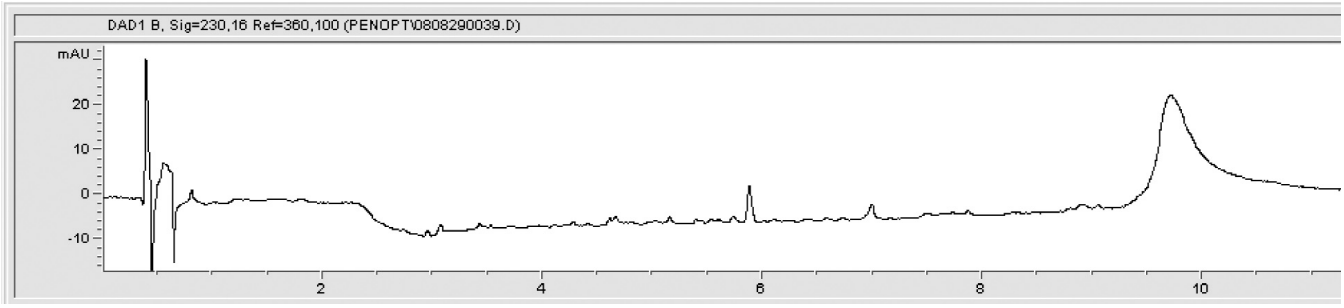


Figure 3. HPLC/UV chromatogram of an unspiked meat sample taken through extraction and SPE cleanup. Wavelength 230 nm.

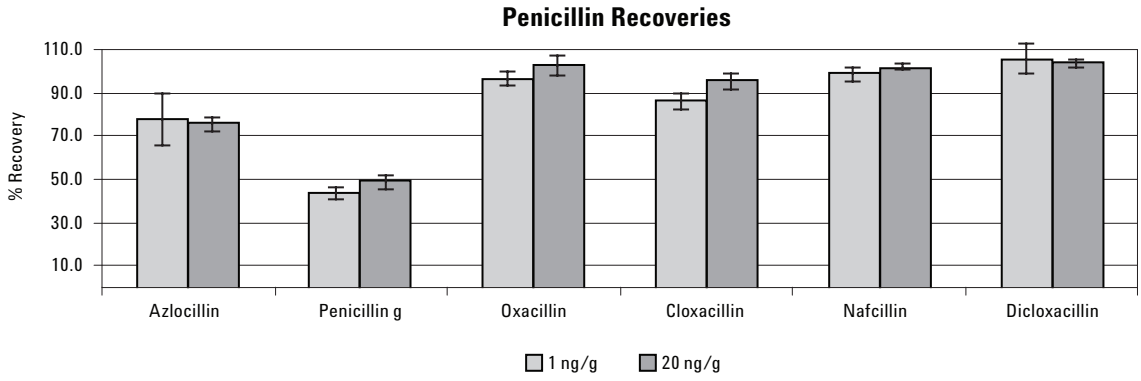


Figure 4. Recovery data for meat extracts at 1.0 and 20 ng/g.

used as a detector to demonstrate the extent of cleanup, which is found to be excellent. The LC/MS/MS is used to demonstrate the recovery of the penicillins at trace level. Even with extensive extraction and SPE cleanup, recoveries are acceptable and reproducibilities are excellent.

Reference

1. Xinbo Lu, Huabin Xing, Baogen Su, and Qilong Ren, Effect of Buffer Solution and Temperature on the Stability of Penicillin G, *J. Chem Eng.* 53 (2), 543–547, 2008

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Determination of Chloramphenicol, Florfenicol, and Thiamphenicol in Honey Using Agilent SampliQ OPT Solid-Phase Extraction Cartridges and Liquid Chromatography-Tandem Mass Spectrometry

Application Note

Food Safety

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Abstract

A method for the simultaneous determination of three antibiotic residues of chloramphenicol (CAP), florfenicol (FF), and thiamphenicol (TAP) in honey has been developed and validated. The analytes are purified by liquid/liquid extraction and solid-phase extraction (SPE) and are quantified by liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) operating in negative ion multiple reaction monitoring (MRM) mode. Chloramphenicol-D_5 is used as the internal standard. The method is validated by achieving reproducible, satisfactory, quantitative results. The method provides a sub-ng/g to ng/g level of limit of quantitation (LOQ) for all three antibiotics in honey. The overall recoveries range from 74.9 to 107% with RSD values between 0.5 and 9.7%. The dynamic calibration ranges for chloramphenicol and florfenicol are obtained over 0.1 to 20.0 ng/g and 1.0 to 20.0 ng/g for thiamphenicol. The method is demonstrated to be fast, simple, and efficient for monitoring chloramphenicol, florfenicol, and thiamphenicol residues in honey.

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Introduction

Chloramphenicol (CAP) is a broad-spectrum bacteriostatic antibiotic, obtained originally from the bacterium *Streptomyces venezuelae*. Due to potential side effects in humans, the drug is not recommended for the treatment of minor diseases, but is reserved for the treatment of serious infections. In veterinary medicine, CAP has been shown to be a highly effective, well-tolerated antibiotic; the potential side effects observed in humans have not been reported in animals. However, because of its toxicity in humans, the use of CAP in animal-derived foods, including honey from honeybees, has been strictly regulated. The European Union (EU) has defined a maximum residue limit (MRL) for CAP in food of animal origin at a level of 0.3 µg/kg [1], while China has an MRL level of 0.5 µg/kg [2]. Thiam-phenicol (TAP) and florfenicol (FF) are the analogue compounds of CAP. They can be used as a replacement veterinary antibiotic for CAP in many countries. The MRLs have been set for TAP (50 ng/g) and FF (100 ng/g) in food to date [3]. Table 1 shows the chemical structure and properties of these three compounds. This application note describes a method for the simultaneous determination of three phenicols in honey, and the results of validation.

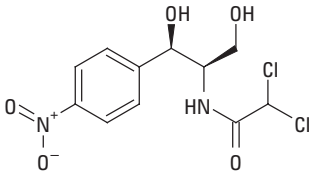
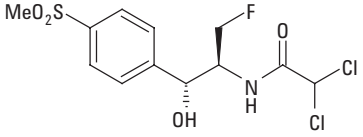
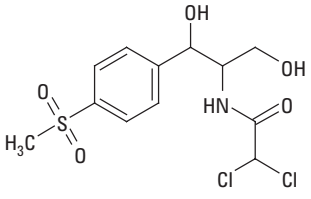
Experimental

Reagents and Chemicals

All reagents and solvents were HPLC or analytical grade. Acetonitrile and methanol were from Honeywell, Burdick & Jackson (Muskegon, MI); ethyl acetate was from J.T.Baker (Phillipsburg, NJ). Dimethyl sulfoxide was from Sigma-Aldrich (St. Louis, MO). The standards and other chemicals were purchased from Sigma-Aldrich.

Water (pH 8.5) was prepared by pH adjustment of Milli-Q water with 0.05% NH_4OH in water solution monitored by a pH meter. A solution of 20:80 methanol/ethyl acetate was prepared by combining 40 mL of methanol and 160 mL of ethyl acetate and mixing well. A solution of 20:80 acetonitrile/H_2O was prepared by adding 40 mL of acetonitrile into 160 mL of Milli-Q water.

Table 1. Chemical Structure and Properties of Target Analytes

Compounds	Log P	pKa	Structure
Chloramphenicol	1.02	9.61	
Florfenicol	–0.12	9.03	
Thiamphenicol	–0.27	9.76	

Standard stock solutions (1.0 mg/mL) were made in dimethyl sulfoxide (DMSO) individually, and stored in the refrigerator at 4 °C. A combined working solution (2,500 ng/mL) was made weekly in 20:80 ACN/H_2O, and also stored at 4 °C. The spiking solutions were then made daily by appropriate dilution of the combined working solution in Milli-Q water or 20:80 ACN/H_2O.

Internal standard (IS) stock solution (0.1 mg/mL) was made in DMSO and stored in the refrigerator at 4 °C. An IS spiking solution (50 ng/mL) was made weekly by appropriate dilution of stock solution into Milli-Q water, and stored at 4 °C.

Equipment and Materials

Agilent 1200 Series HPLC (Agilent Technologies Inc., Santa Clara, CA, USA)

Agilent 6410 Triple Quadrupole LC/MS/MS system with electrospray ionization source (Agilent Technologies Inc., Santa Clara, CA, USA)

Agilent SampliQ OPT solid-phase extraction cartridges, 50 × 3 mL tubes, 60 mg (p/n 5982-3036) (Agilent Technologies Inc., Wilmington, DE, USA)

CentraCL3R centrifuge (Thermo IEC, Needham Heights, MA, USA)

N_2 dryer (Glas-Col, Terre Haute, IN, USA)

Sample Preparation

Liquid-Liquid Extraction

5g of honey (± 0.05 g) was weighed into a 50 mL capped polypropylene tube. 0.5 mL of IS spiking solution (50 ng/mL) was added to the tube and vortexed until mixed. This was followed by the addition of 5 mL of Milli-Q water and vortexing for 3 minutes to mix the sample thoroughly. 5 mL of ethyl acetate was then added, capped tightly, and the tubes shaken for 5 minutes. The tubes were then centrifuged at 3,200 rpm for 5 minutes, before the upper organic layer was carefully transferred to another tube. Ethyl acetate addition, shaking, centrifuging, and organic layer transfer was repeated two more times with all supernatants combined. Samples were evaporated to dryness with a controlled N_2 flow drier at 50 °C before being reconstituted into 5 mL of Milli-Q water, vortexed, and sonicated to completely dissolve the residue. The sample was then ready for SPE purification. Figure 1 shows the extraction procedure flowchart.

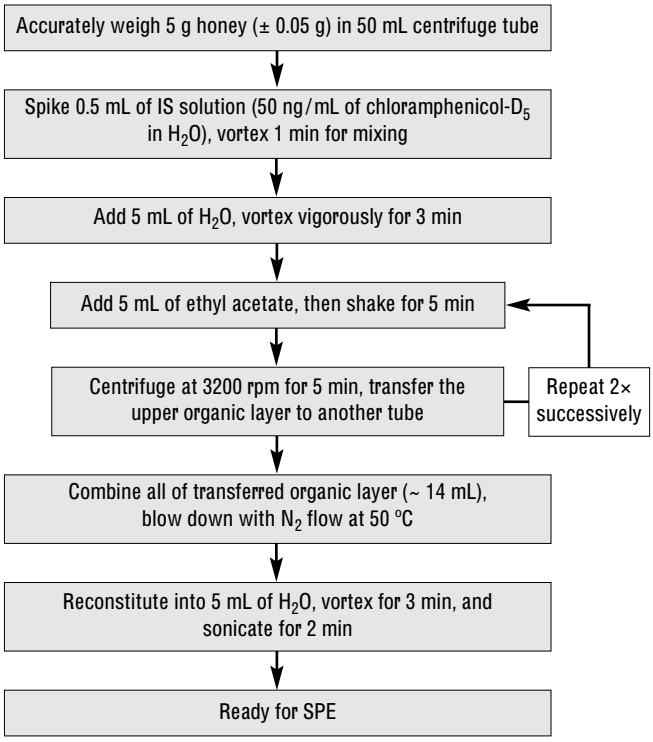


Figure 1. Sample preparation – liquid liquid extraction of phenicols in honey.

Solid-Phase Extraction

The procedure for SPE extraction is shown in Figure 2. Agilent SampliQ OPT cartridges were preconditioned with 3 mL of MeOH, and then equilibrated with 5 mL of water. The 5 mL sample extract was then loaded onto a cartridge and passed through the cartridge slowly by gravity (0.5 mL/min). The tubes were rinsed with 5 mL of Milli-Q water twice. Repeat the above wash procedure once. The entire effluent was discarded. Apply full vacuum to the cartridge for 3 minutes to completely dry the resin. Finally, the compounds were eluted with 5 mL of 20:80 MeOH/ethyl acetate (2.5 mL × 2) at a rate of 1 mL/min. The eluent was collected into clean tubes and dried under N_2 flow at 50 °C. The residue was reconstituted in 0.5 mL of 20:80 AcN/H_2O. The sample was vortexed and sonicated to completely dissolve the residue in the tubes. The sample was transferred to a centrifuge tube and centrifuged at 3,200 rpm for 2 minutes. The samples were then transferred to 2 mL autosampler vials for analysis.

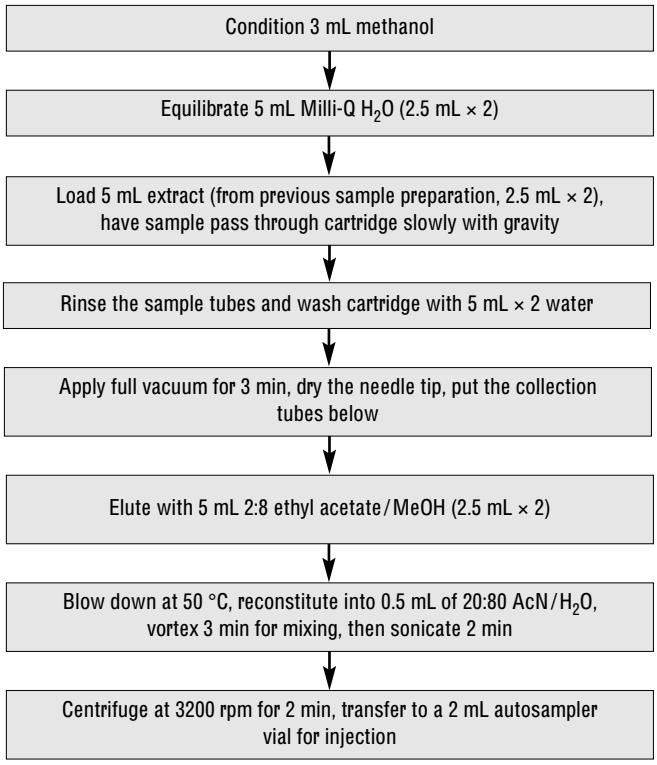


Figure 2. Sample clean-up – Agilent SampliQ solid-phase extraction.

Instrument Conditions

HPLC Conditions

Column:		Agilent ZORBAX Eclipse Plus 150 mm × 2.1 mm, 5 µm (PN: 959701-906)	
Flow rate:		0.3 mL/min	
Column temperature:		30 °C	
Injection volume:		20 µL	
Mobile phase:		pH 8.5 H_2O (A), Acetonitrile (B)	
Gradient:	Time	% Acetonitrile	Flow rate (mL/min)
	0	20	0.3
	0.5	20	0.3
	6.0	80	0.3
	6.01	100	0.5
	6.50	100	0.5
	6.51	20	0.3
	7.00	STOP	

MS Conditions

The three compounds were monitored in the negative ionization mode. The multiple reaction monitoring channels are shown in Table 2.

Table 2. Masses Monitored in the Multiple Reaction Monitoring (MRM) Experiment

Analyte	MRM (*m/z* → *m/z*)	Dwell time (ms)
Thiamphenicol	354.0 → 184.9 (quantifier)	50
	354.0 → 290.0 (qualifier)	25
Florfenicol	355.8 → 185.0 (quantifier)	50
	355.8 → 336.0 (qualifier)	25
Chloramphenicol	320.9 → 152.0 (quantifier)	50
	320.9 → 176.0 (qualifier)	25
Chloramphenicol-D_5 (IS)	325.9 → 156.8	25

Results and Discussion

Linearity, Limit of Detection

The extracted ion chromatograms of fortified honey at a concentration of 0.2 ng/g are shown in Figure 3. The extracted honey blank was clean and free from any analytes, indicating that the cleaned-up honey extract does not contribute any interference with the target analysis.

The concentration ranges studied here are significantly below the limit of quantitation (LOQ) defined by the MRL for TAP (50 ng/g) [3]. In this study the limit of quantitation (LOQ) found for TAP is 1.0 ng/g, and the linear calibration range used for TAP is 1.0 to 20.0 ng/g. The linear calibration range for CAP (LOQ 0.1 ng/g, MRL 0.3 ng/g) and FF (LOQ 0.1 ng/g, MRL 100 ng/g) was 0.1 to 20.0 ng/g.

Calibration curves spiked in matrix blanks were made at levels of 0.1, 0.2, 1.0, 5.0, 10.0, 15.0, and 20.0 ng/g for CAP and FF. While for TAP they were spiked at a level of 1.0, 5.0, 10.0, 15.0, and 20.0 ng/g. The chloramphenicol-D_5 was used as internal standard at 5 ng/g level. The calibration curves were generated by plotting the relative responses of analytes (peak area of analyte/peak area of IS) to the relative concentration of analytes (concentration of analyte/concentration of IS). The limit of detection (LOD) was determined with a signal-to-noise ratio between 4 and 5. Table 3 shows the linearity equation, correlation coefficient (R^2) and LOD. The calibration curve for chloramphenicol is shown in Figure 4.

Table 3. Linearity and LODs of Phenicols

Analytes	Regression equation	R^2	LOD (ng/g)
Chloramphenicol	Y = 0.5643X – 0.0001	0.9957	0.02
Florfenicol	Y = 0.8790X + 0.0006	0.9932	0.02
Thiamphenicol	Y = 0.1510X – 0.0018	0.9953	0.20

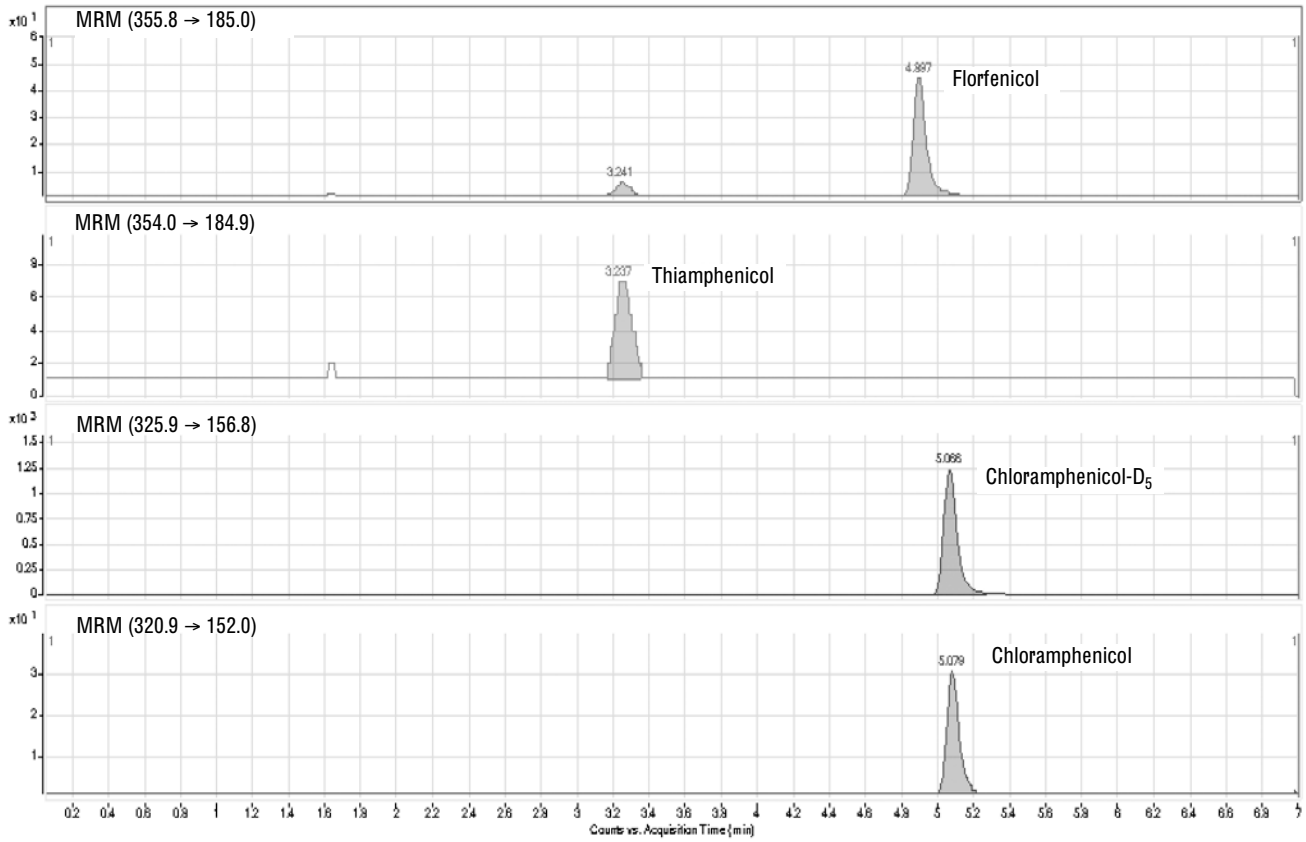


Figure 3. Chromatograms of 0.2 ng/g fortified honey extract.

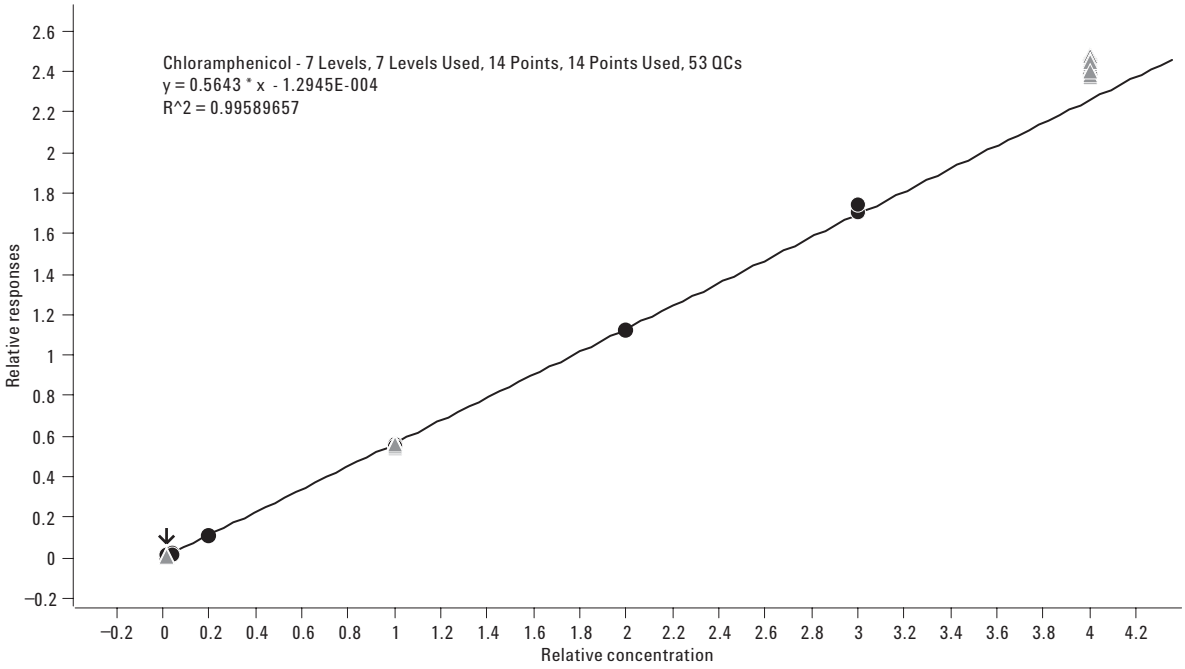


Figure 4. Calibration curve of chloramphenicol (0.1 to 20.0 ng/g). Dots (•) indicate sample results of calibration curve points, and trianges (Δ) indicate sample results of quality controls.

Recovery and Reproducibility

The recovery and reproducibility were evaluated by spiking phenicol standards in honey at levels of 0.1 ng/g (1.0 ng/g for TAP), 5.0 ng/g and 20.0 ng/g as quality control samples (QCs), and quantifying those QCs against the matrix spiked calibration curve. The analysis was performed in replicates of six at each level, except four replicates for TAP low level. The recovery and reproducibility (shown as %RSD) data are shown in Table 4. CAP and FF show excellent recovery and reproducibility at all QC levels. The recovery of TAP is adequate at all concentrations and the reproducibility is excellent.

Table 4. Recoveries and Reproducibility of Phenicols in Fortified Honey

Analytes	Spiking Level (ng/g honey)	Recovery (%)	RSD (%) n = 6
Chloramphenicol	0.10	96.94	3.51
	5.00	98.88	0.87
	20.00	107.32	0.46
Florfenicol	0.10	100.67	9.77
	5.00	100.28	2.84
	20.00	107.49	2.55
Thiamphenicol	1.00	76.00	4.39*
	5.00	74.89	2.34
	20.00	89.81	3.83

* The experiment was done in replicates of four.

Conclusions

Agilent SampliQ OPT SPE cartridges provide a simple and effective method for the purification and enrichment of chloramphenicol, florfenicol, and thiamphenicol in honey. The recovery and reproducibility results based on matrix spiked standards are acceptable for chloramphenicol residue determination in honey under EU or Chinese regulations. The impurities and matrix effect from honey are minimal and do not interfere with the quantitation of any target compound. The LOQs of the three phenicols are significantly lower than the MRLs.

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Determination of Sulfonamides in Milk Using Solid-Phase Extraction and Liquid Chromatography-Tandem Mass Spectrometry

Application Note

Pharmaceuticals

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Abstract

The extraction of trace levels of nine nitrogen-containing sulfa drugs (sulfamethoxazole, sulfadiazine, sulfathiazole, sulfamerazine, sulfamethizole, sulfamethazine, sulfamethoxypyridazine, sulfachloropyridazine, and sulfadimethoxine) in milk samples by solid-phase extraction was studied using Agilent SampliQ polymeric strong cation exchange (SCX) cartridges. An Agilent 6410 triple quadrupole LC/MS-MS System was used for the separation and determination of the sulfa drugs. For reversed-phase chromatography, an Agilent ZORBAX Eclipse Plus column (C18, 3.0 mm × 50 mm, 1.8 µm) with a 0.1% formic acid/acetonitrile gradient was used. Overall recoveries from the milk samples ranged from 73 to 99%, with %RSD values less than 10%. Limits of detection ranged from 0.2 to 2.0 ng/mL in milk (S/N = 3) depending on the sulfa drug, below the U.S. Food and Drug Administration acceptable levels in milk.

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Introduction

Since the discovery of the effective antimicrobial properties of sulfonamides in the early 1900s, they have been used to treat a variety of diseases. In the dairy farming industry, sulfa drugs are administered to dairy cattle to prevent infection. This leads to the possibility of the drugs being excreted in the milk and passed on to the consumer. This ingestion can cause a drug resistance, making the drugs ineffective in later uses to treat illness [1,2].

Sulfonamides, commonly known as sulfa drugs, have proven to be effective antimicrobial agents since their discovery in 1929 by Gerhard Domagk. Today, β-lactam antibiotics are much more commonly used to prevent infection than sulfonamides, but sulfonamides are still routinely used in different parts of the world due to their low cost. Over the years, many microorganisms have become resistant to these compounds. Of growing concern are drug-resistant bacteria that may be passed from animals to humans. One major cause of the resistance to these compounds is that feed animals are being fed antimicrobial drugs at low levels to treat diseases. In the 1990s, the United States Food and Drug Administration (FDA) began conducting tests on several milk supplies [3]. If dairy cattle were given sulfa drugs, low levels of these compounds could be found in the milk, leading to allergic reactions in some consumers, as well as an increase in drug-resistant organisms. This application demonstrates a complete solution to the analysis in milk of nine important sulfa drugs: sulfamethoxazole, sulfadiazine, sulfathiazole, sulfamerazine, sulfamethizole, sulfamethazine, sulfamethoxypyridazine, sulfachloropyridazine, and sulfadimethoxine (see Figure 1 for structures and chemical properties).

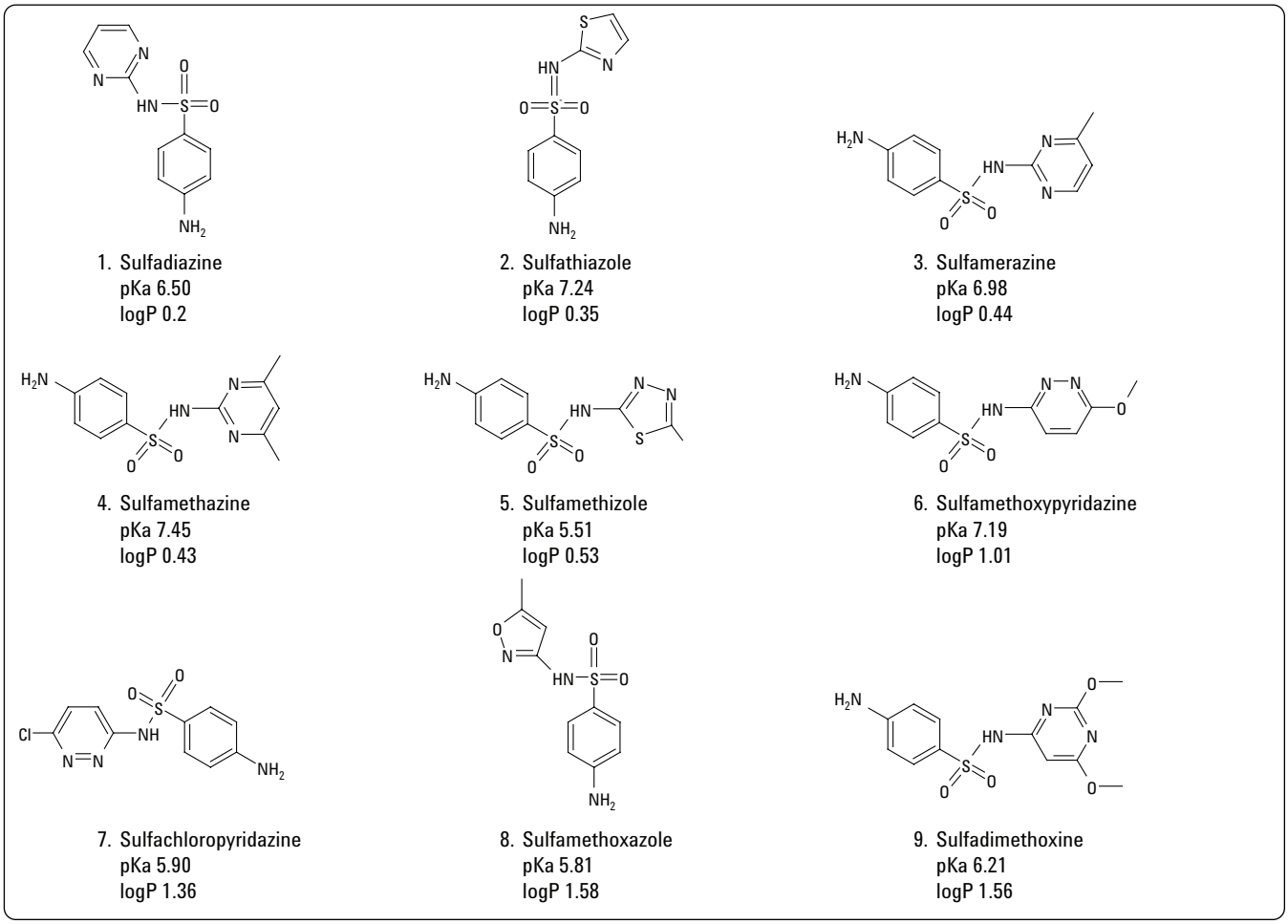


Figure 1. Structures and chemical constants for sulfa drugs used in this study.

Experimental

Materials and Chemicals

Water (EMD Chemicals, Gibbstown, NJ), acetonitrile, and methanol (Burdick and Jackson, Muskegan, MI) were HPLC grade. Sulfa drugs were analytical grade and purchased from Sigma-Aldrich (Saint Louis, MO). The stock solution (~1.19 mg/mL) was prepared in 25 mL of methanol and kept refrigerated for up to 14 days. Working solutions were made daily by dilution of the stock solution in water.

The SPE cartridges were Agilent SampliQ SCX, 3 mL/60 mg p/n 5982-3236), a polymeric cation exchanger with a 30-µm average particle size. The analysis was performed on an Agilent 1200 Series HPLC coupled to a 6410 triple quadrupole mass spectrometer with electrospray ion source. The analytical column was an Agilent ZORBAX Eclipse Plus C18, 3.0 mm × 50 mm, 1.8 µm (p/n 959941-302). Formic acid was purchased from J.T. Baker (Phillipsburg, NJ) (Baker PCS reagent, 90%) for use in mobile phase preparation and for precipitation of proteins and lipids in the milk.

Sample Preparation

20 µL of a 45% solution of formic acid in water (prepared by mixing 10 mL of 90% formic acid with 10 mL of water) solution was added to each 1 mL of whole milk to precipitate proteins and lipids. The milk samples were then centrifuged at 8000 rpm for 10 minutes (Eppendorf 5810R 15 amp, Westbury, NY). Alternatively, the samples may be centrifuged at 3500 rpm for 20 minutes. An aliquot of the supernatant (prepared whole milk extract) was removed and used to load onto SampliQ SCX cartridges.

SPE Purification

After the SPE cartridge was conditioned and equilibrated as described in Figure 2, 5 mL of prepared whole milk extract was loaded onto the column. Care was taken that the flow rate during the load step did not exceed 1.5 mL/min. During both drying steps, the cartridge was dried under vacuum at 15 in Hg for the time indicated. The eluate was dried under nitrogen and then reconstituted in 1 mL of solvent (9:1 water:methanol). The samples were then sonicated (Branson 1200, Danbury, CT) for 5 minutes and analyzed using the Agilent 6410 Triple Quad LC/MS-MS system.

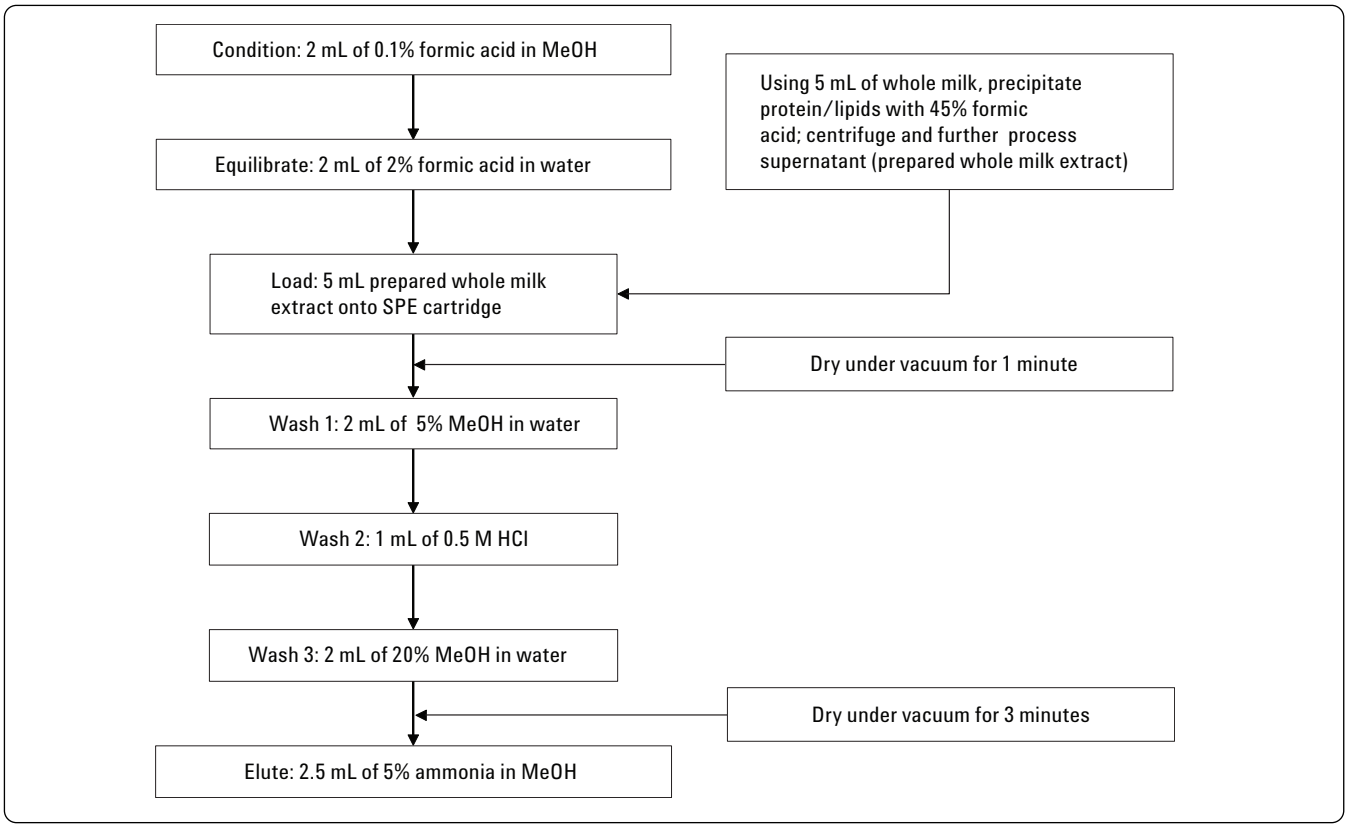


Figure 2. SPE procedure.

Due to the low concentrations of sulfa drugs being analyzed, and the very low LOD that can be achieved with Agilent 6410 Triple Quad LC/MS-MS system, extra care must be taken to keep the SPE manifold system clean to prevent contamination of samples. The manifold system must be thoroughly cleaned between uses or, if the option is available, needle inserts may be newly installed.

Separation and Analysis

The chromatographic and MS/MS experimental setup is shown in Tables 1, 2, and 3.

Table 1. HPLC Setup

Column	Agilent ZORBAX Eclipse Plus C18, 3.0 × 50 mm, 1.8 µm (p/n 959941-302)			
Flow rate	0.42 mL/min			
Column temperature	35 °C			
Injection volume	1.7 µL w/ needle wash; wash for 30 s in flush port with MeOH/H_2O (5:1)			
Mobile phase	A: H_2O/acetonitrile (9:1) w/ 0.1% formic acid B: Acetonitrile w/ 0.1% formic acid			
Run time	8 min			
Post time	3 min			
Gradient	Time	0	3.5	8
	%B	0	0	65

Table 2. MS/MS Conditions

T_R (min)	Compound	Precursor ion	Product ion
1.69	Sulfadiazine	251.2	156.0 108.0
1.93	Sulfathiazole	256.1	156.0 108.0
2.41	Sulfamerazine	265.2	156.0 108.0
3.44	Sulfamethazine	279.2	186.1 124.1
3.89	Sulfamethizole	271.1	156.0 108.0
4.10	Sulfamethoxypyridazine	281.2	156.0 108.0
5.99	Sulfachloropyridazine	285.1	156.0 108.1
6.42	Sulfamethoxazole	254.2	156.1 108.1
7.17	Sulfadimethoxine	311.2	156.0 108.0

Table 3. Conditions for Electrospray Ionization Source

Gas temperature	350 °C
Gas flow	12 L/min
Nebulizer	40 psi
Capillary	4000 V

Results and Discussion

Linearity and Limits of Detection

Solutions used to create external calibration curves were prepared by using a stock solution to spike matrix blanks. Matrix blanks were created by taking the milk through the entire procedure, including the precipitation, centrifugation, and SPE procedures. The results for the calibration curves are summarized in Table 4. The regression results were used to calculate the recoveries. The limits of detection were chosen as the concentration of each drug that gave a signal-to-noise (S/N) ratio greater than 3:1. The limits of detection are given in Table 4.

Table 4. Calibration Curve Regression Analysis for Sulfa Drugs

Compound	Regression equation	R^2	LOD in milk (ng/mL)
Sulfadiazine	y = 282.62x + 225.59	1.0000	0.4
Sulfathiazole	y = 440.38x + 246.43	0.9996	1.0
Sulfamerazine	y = 358.34x + 485.54	0.9998	1.0
Sulfamethazine	y = 539.09x + 576.81	0.9989	1.0
Sulfamethizole	y = 499.57x + 333.03	0.9994	2.0
Sulfamethoxypyridazine	y = 494.61x + 139.66	0.9970	1.0
Sulfachloropyridazine	y = 343.78x + 92.808	0.9999	2.0
Sulfamethoxazole	y = 260.05x – 351.97	0.9901	2.0
Sulfadimethoxine	y = 956.97x + 1420.9	0.9973	0.2

Recovery and Reproducibility

The recoveries and precision for the method were determined at two levels, milk spiked to a concentration of 5 ng/mL and 10 ng/mL. Since the procedure for the SPE used 5 mL of prepared whole milk extract to load the SPE cartridge and the extract was reconstituted in 1 mL of mobile phase, the sample analyzed is five times as concentrated as the milk samples. The analyzed solutions are therefore 25 ng/mL for the samples that were spiked with 5 ng/mL in milk, and 50 ng/mL for the samples that were spiked with 10 ng/mL. The analysis was performed with five replicates at each level. The recovery and reproducibility data are shown in Table 5. The chromatograms for the blank and spiked milk extracts (5 ng/mL) are shown in Figure 3.

Table 5. Recovery and Precision Data for Nine Sulfa Drugs Used in This Study

Compound	Level spiked in milk (ng/mL)	Recovery	RSD (%)
Sulfadiazine	5	74.2	8.3
	10	99.7	5.7
Sulfathiazole	5	76.8	4.4
	10	83.2	4.7
Sulfamerazine	5	73.2	6.3
	10	84.8	0.6
Sulfamethazine	5	78.3	7.5
	10	89.0	3.1
Sulfamethizole	5	78.4	7.0
	10	94.5	5.3
Sulfamethoxypyridazine	5	76.3	6.2
	10	86.9	2.2
Sulfachloropyridazine	5	78.3	9.4
	10	84.3	6.0
Sulfamethoxazole	5	74.0	4.3
	10	87.7	6.4
Sulfadimethoxine	5	75.4	3.1
	10	82.5	5.4

Conclusions

The results of this study show that Agilent SampliQ SCX cartridges can be used as an effective method of purification and enrichment of multiple sulfonamides in complex samples such as whole milk. The impurities remaining after the SPE cleanup step were minimal and did not interfere with the quantitation of the sulfonamides. The levels at which the quantitation was performed are below the levels of sulfonamides that are considered by the FDA as safe in milk for consumption (10 ng/mL). The LOD for the method was also well below these levels (3 ng/mL in milk).

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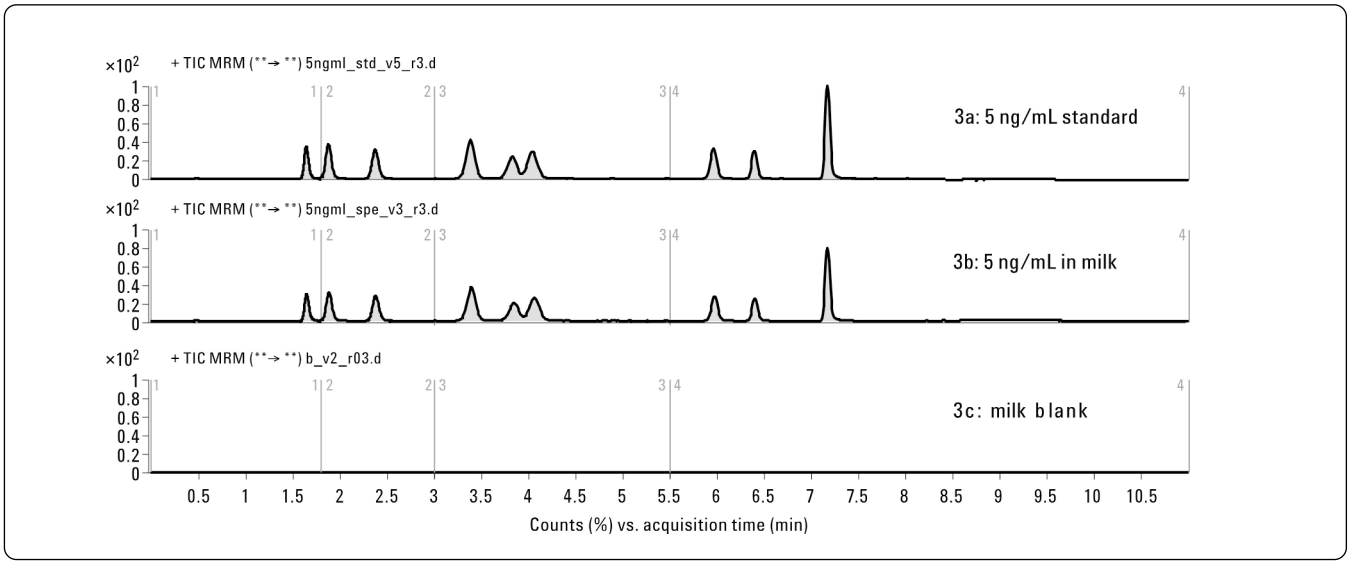


Figure 3. Total ion chromatograms of (3a) milk taken through extraction and cleanup, then spiked with sulfa drugs; (3b) milk spiked at 5 ng/mL, then taken through extraction and SPE cleanup; and (3c) milk blank.

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Determination, Confirmation, and Quantification of Trace ß-Lactam Antibiotics in Milk by LC/MS/MS

Pei-Bin Hu, Xin Ma, and Tao Bo

Application Brief

Food Safety

The ß-lactam antibiotics are widely used in veterinary medicine for the treatment and prevention of disease. This use can result in the presence of residues in milk and edible tissues which can lead to problems in the fermentation processes and to health problems for individuals who are hypersensitive to ß-lactams.

To protect the consumer, MRL values were laid down in EU regulation 2377/90. For milk these values range from 4 µg/kg for penicillin G, ampicillin, and amoxicillin to 30 µg/kg for dicloxacillin, cloxacillin, and oxacillin.

In practice, screening is performed by microbiological and immunological methods. In this way it is possible to give, for a positive sample, the specification of the group to which the residue belongs. Confirmation, however, has to be performed by an independent physicochemical technique. Therefore, a highly sensitive LC/MS/MS method was developed for the detection of penicillins, etc., in milk samples.

Highlights

- Ease of use for method optimization
- Good linearity $R^2 \geq 0.98$ in real milk samples
- Good separation to get almost all the compounds separated well within 9 minutes

Experimental

Sample Preparation

An aliquot of 2 mL of milk (half skimmed consumption milk or raw milk) was mixed with 4 mL of acetonitrile for protein precipitation. The sample was vortexed and then centrifuged for 10 minutes at 3500 rpm. After filtration, 20 µL of the supernatant was injected into the LC/MS/MS system.

LC Conditions

Instrument	Agilent 1200SL
Column	Agilent ZORBAX SB-C18, 2.1 × 150 mm, 3.5 µm (p/n 830990-902)
Mobile phase	A: Water/0.3% acetic acid B: Acetonitrile/0.3% acetic acid
Flow rate	0.3 mL/min
Gradient	0–2 min/A 90%; 2.01–8 min/A 35%; 8.01–9/A 5%
Column compartment temperature	30 °C
Stop time	9 min
Post time	6 min
Injection volume	10 µL

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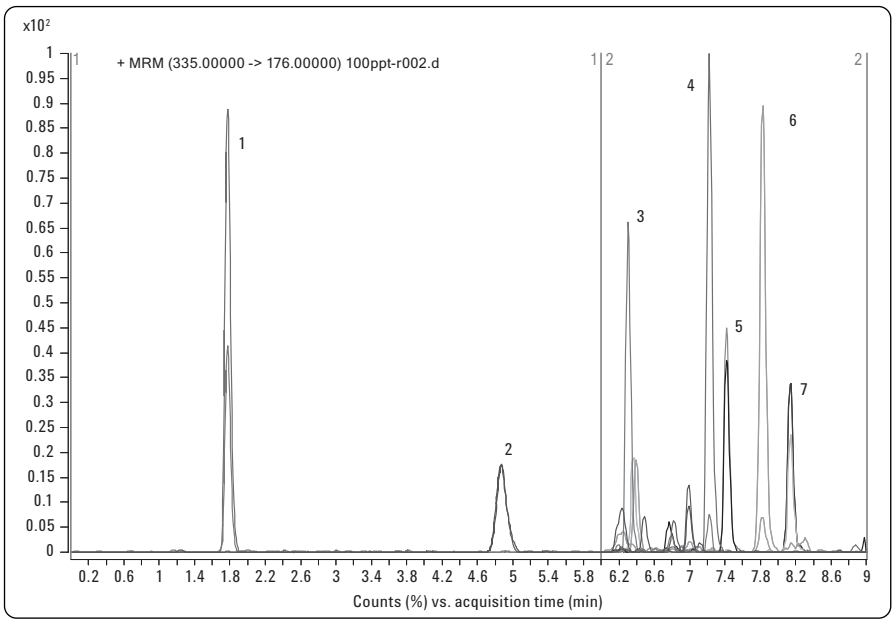
MS Conditions

Instrument	Agilent 6410A triple quadrupole LC/MS system
Source	ESI +
Drying gas temperature	350 °C
Drying gas flow	10 L/min
Nebulizer pressure	45 psi

MRM Setting

TS	Compound	Precursor	Product	Dwell (ms)	Frag (V)	CE (V)
0	Amoxicillin	366	114	100	110	15
			208	100	110	5
	Ampicillin	350	160	100	100	5
			192	100	110	10
6	Dicloxacillin	470	311	70	110	10
			160	70	110	10
	Nafcillin	415	256	70	110	15
			160	70	110	10
	Oxacillin	402	243	70	110	10
			160	70	110	10
	Penicillin V	351	192	70	100	5
			160	70	100	10
	Penicillin G	335	176	70	100	10
			160	70	100	5

Results



1. Amoxicillin
2. Ampicillin
3. Dicloxacillin
4. Nafcillin
5. Oxacillin
6. Penicillin V
7. Penicillin G

Compounds	S/N (Conc = 0.1 pppb)	R^2
Amoxicillin 366-114	224	0.992
Ampicillin 350-160	61.6	0.984
Dicloxacillin 470-160	48.5	0.981
Nafcillin 415-199	52.6	0.998
Oxacillin 402-160	70.9	0.993
Penicillin V 351-160	225.9	0.998
Penicillin G 335-160	33.2	0.981

Pei-Bin Hu is an application engineer based at Agilent Technologies, Chengdu, China. Xi Ma is working in the Training Centre and Tao Bo is an application engineer based at Agilent Technologies, Beijing, China.

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Using LC/MS/MS 6410 for Analysis of Chloramphenicol, Thiamphenicol, and Florfenicol in Fish Samples

Application Brief

Xiaorong Ran, Tao Bo

Chloramphenicol is a banned compound in the EU. It is a zero tolerance compound, and some methods have already been developed for this analysis. The LC/MS/MS is often used for its greater sensitivity and higher selectivity.

In many cases, not only chloramphenicol but also thiamphenicol and florfenicol are also found. This method uses a simple method for detecting all three compounds within less than 6 minutes. Furthermore, the results gave the good results, showing the performance at the negative mode.

Experimental

LC Conditions

Column	Agilent ZORBAX Eclipse Plus, 2.1 mm × 50 mm, 1.8 µm
Mobile phase	A: Water B: Methanol
Flow rate	0.4 mL/min
Gradient	0–2 min/B 10% to 90%; 2–3 min/B 90%; 3.01/B 10%
Stop time	6 min
Column compartment temperature	45 °C
Injection	5 µL

MSD Condition

Instrument	Agilent 6410A triple quadrupole LC/MS system
Source	ESI –

Highlights

- Using a simple RRLC method can separate the compounds well within 6 minutes
- Quite high sensitivity in negative mode
- The ISTD method can remove the matrix effect and minimumize the sample preparation interference

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Sample Preparation

All SPE cartridges are conditioned with 2 mL of water before use.

1. **Honey**, 1 g sample is diluted to 5 mL with water and 25 µL 10 ppb IS is added. The solution is loaded onto the SPE cartridge and allowed to stand for 5 min. Elution is performed with 10 mL ethyl acetate. The eluate is collected and the solvent is evaporated under a nitrogen stream at 40 °C. The residue is redissolved in 1 mL methanol and put in an ultrasonic bath for 1 min. The solution is filtered, using a syringe filter, before injection. No additional cleanup of the sample solution is performed.
2. **Shrimp**, 1 g of shrimp is defrosted and mixed in a blender. To the 1 g of the mixed shrimp, 3 mL of water and 25 µL 10 ppb IS are added. The portion is centrifuged for 5 min (8,000 rpm). The supernatant is loaded on the cartridge and allowed to stand for 5 min. Elution is performed with 5 mL ethyl acetate. The eluate is collected and the solvent evaporated under a nitrogen stream at 40 °C. The residue is redissolved in 1 mL methanol and put in an ultrasonic bath for 1 min; the solution is filtered before injection.
3. **Chicken**, 1 g of chicken is defrosted and mixed in a blender. To the 1 g of the mixed chicken, 3 mL of water and 25 µL 10 ppb IS are added. The portion is centrifuged for 5 min (8,000 rpm). The supernatant is loaded on the cartridge and allowed to stand for 5 min. Elution is performed with 5 mL ethyl acetate. The eluate is collected and the solvent evaporated under a nitrogen stream at 40 °C. The residue is redissolved in 1 mL methanol and put in an ultrasonic bath for 1 min; the solution is filtered before injection.

MRM Setting

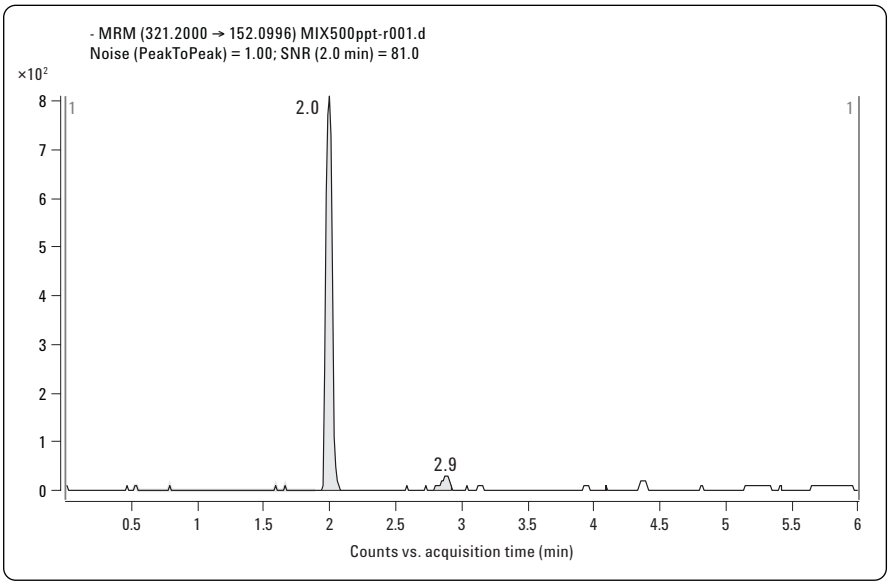
Name	Precursor ion	Product ion	Frag (V)	CE (V)	Dwell (ms)
TAP	354.1	185.1*	120	20	60
	354.1	289.9	120	10	60
FF	356	185.1*	120	20	60
	356	335.8	120	5	60
CAP	321.2	152.1*	120	10	60
	321.2	257.1	120	5	60
D5-CAP (ISTD)	326.2	157.2	130	15	60

Results

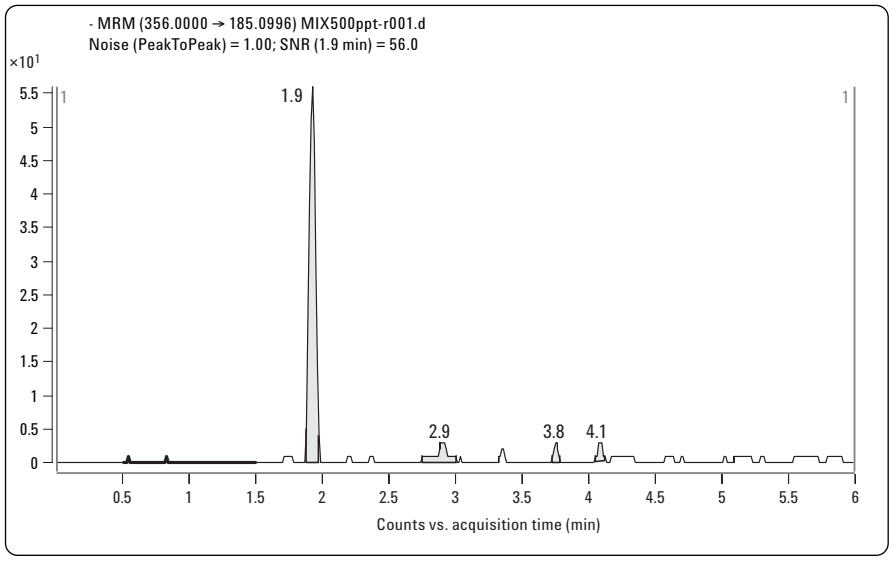
Name	**Linearity** (0.5–20 ppb)
TAP	0.994
FF	0.992
CAP	0.994

Sensitivity

1. CAP: 0.5 ppb S/N = 81



2. FF: 0.5 ppb S/N = 56

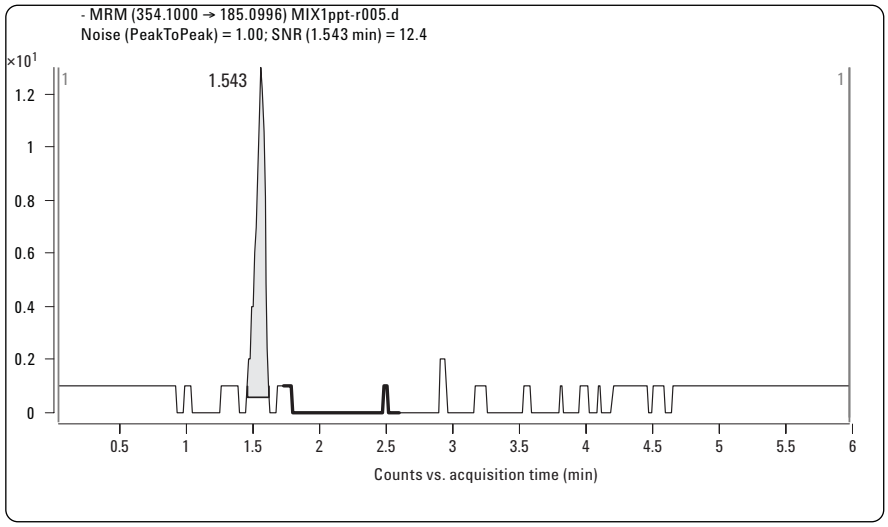


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3. TAP 1 ppb, S/N = 12



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Detection, Monitoring, and Quantitation of Trace Sulfonamides in Pork Muscle Using the Agilent 6410A LC/MS/MS

Application Brief

Jian-qiu Mi

Sulfonamides are the one of the oldest groups of veterinary medicines in use today. All sulfonamide drugs are currently included in Annex 1 of the Council Regulation 2377/90. The existing EU maximum residue level (MRL) for all drugs of the sulfonamide group is 100 µg/kg in all food-producing species.

A variety of methods have been used to measure sulfonamide residue in biological materials, including thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), high-performance capillary electrophoresis (HPCE), gas chromatography (GC), and enzyme-linked immunosorbent assay (ELISA). Now, the LC/MS/MS method is used more widely.

In this study, a multiresidue analysis was performed to simultaneously determine sulfonamides in pork by the Agilent 6410A LC/MS/MS. This multiresidue analysis for sulfonamides can detect different kinds of sulfonamides within one run. Compared with the classic methods, this method can achieve greater sensitivity and be used for screening, confirmation, and quantification.

Highlights

- The pH of the mobile phase played an important role in the LC separation because the retention behaviors of the drugs were dependent on the ionization of the sulfonamides.
- Different kinds of sulfonamide drugs can be analyzed within one run.
- Using the RRLC can get all 14 compounds to elute within 10 minutes.
- High sensitivity easily meets the EU requirements.

Experimental

Sample Preparation

1. **Weigh** – 3-g samples of pork muscle were weighed directly into 50-mL polypropylene centrifuge tubes.
2. **Homogenize** – The samples were homogenized for 3 minutes with 10 mL acidified methanol.
3. **Centrifuge** – The samples were then centrifuged for 10 minutes.
3. **Extract** – 10 mL acidified methanol was extracted, filtered, and injected

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Instrument Settings

LC Conditions

LC	Agilent 1200 Series LC
Column	Agilent ZORBAX SB-C18 (2.1 × 50 mm, 1.8 µm)
Mobile phase	A: 0.1% TFA, B: Acetonitrile 0 min: 5% B 6 min: 23% B 9 min: 23% B 9.01 min: 90% B
Stop time	10 min
Column temperature	30 °C
Injection volume	1 µL
Flow rate	0.3 mL/min

MSD Conditions

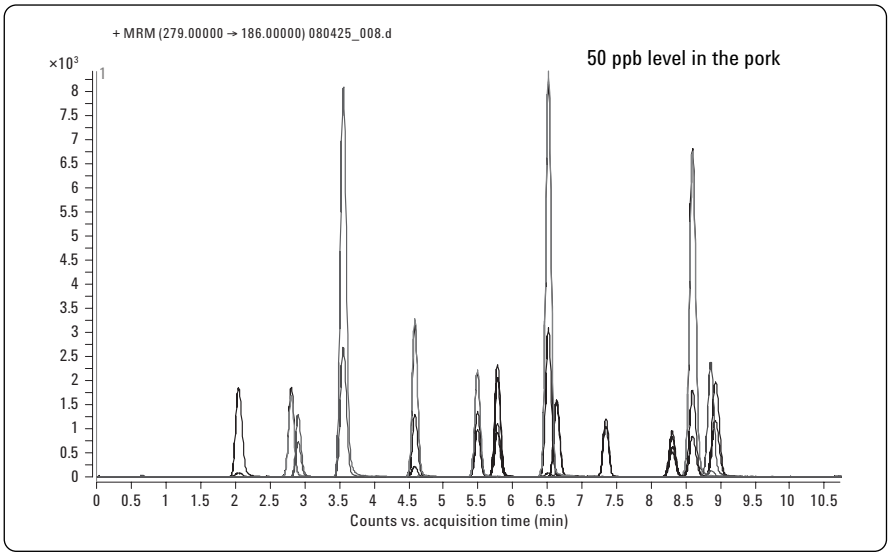
Ionization	ESI (positive)
Scan range	*m/z* 100 to 450
Drying gas	7 L/min at 350 °C
Nebulizer gas	30 psi

MRM setting

Compound	MRM	Frag	CE (V)
Sulfachloropyridazine (SCP)	285–156 285–108	100	15 20
Sulfadiazine (SD)	251–156 251–185	120	10 10
Sulfamethazine (SDM)	311–156 311–218	140	15 15
Sulfamethoxypyridazine (SMP)	281–156 281–215	120	10 15
Sulfamerazine (SM1)	265–156 265–172	120	15 15
Sulfamethazin (SM2)	279–156 279–204	140	15 15
Sulfalmethoxazole (SMZ)	254–156 254–147	120	15 20
Sulfamonomethoxine (SMM)	281–156 281–126	120	10 20
Sulfathiazole (ST)	256–156 256–107	120	15 15
Sulfaquinoxaline (SQX)	301–156 301–208	140	15 15
Sulfadoxine (SDM)	311–156 311–108	140	15 20
Sulfaphenazole (SPP)	315–156 315–160	140	20 20
Sulfaclozine	285–156 285–131	100	15 20
Sulfafurazole (SIZ)	268–156 268–113	120	5 10

Results

Good separation and response



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Determination of Multi-Residue Tetracyclines and their Metabolites in Milk by High Performance Liquid Chromatography - Tandem Mass Spectrometry

Application Note

Food

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Abstract

Tetracyclines are probably the most frequently used antibiotics in animal husbandry. In this paper, a high performance liquid chromatography tandem mass spectrometric (HPLC /MS/MS) method is developed for the simultaneous determination of 10 antibiotic residues: minocycline, 4-epioxytetracycline, 4-epitetracycline, tetracycline, 4-epichlortetracycline, demeclocycline, chlortetracycline, methacycline, doxycycline, oxytetracycline in milk and animal tissues. In the method, Agilent's novel solid phase extraction cartridge and a reversed phase Agilent ZORBAX RX C8 column (5 µm, 150 mm × 2.1 mm) are used for purification and separation. The limit of detection (LOD) is between 0.5 and 10.0 µg/kg and the limit of quantitation (LOQ) is less than 50 µg/kg. The linearity is obtained from 5 to 1000 µg/kg. Overall recoveries are between 76.4% and 101% with a relative standard deviation (RSD, n = 6) less than 8.4%. The method is rapid, sensitive, convenient and robust, and can be used to simultaneously confirm multi-residues of tetracyclines and their metabolites in milk.

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Introduction

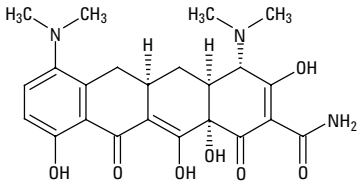
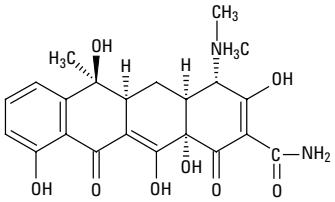
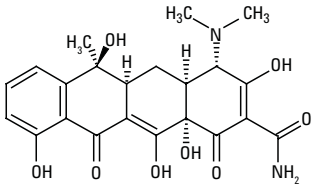
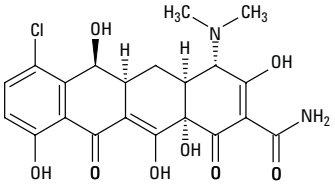
Antibiotics are used worldwide to control bacterial infection and promote healthy farm animals for milk production. Tetracyclines are broad-spectrum antibiotics, so they are widely used. However, it is undesirable to have them in the milk supply.

FDA's regulations for tetracyclines including oxytetracycline and chlortetracycline are set to provide an acceptable daily intake (ADI) and a tolerance for residues in milk. The ADI for total residues of these compounds is 25 micrograms per kilogram of body weight per day. Sixty percent (60 %) of the ADI is reserved for milk and 40 % for edible tissues. Based on the ADI, a tolerance of 300 ppb is set for the sum of residues of the tetracyclines including chlortetracycline, oxytetracycline, and tetracycline in milk. With the establishment of a tolerance of 300 ppb for the sum of residues of tetracyclines, a tolerance of 300 ppb for each of the three tetracyclines is also accepted.

In the EU, the maximum residue limit (MRL) for antibiotics is established according to (EEC) 2377/90, and for tetracyclines in milk is at 100 µg/kg (100 ppb). In China, the Government Standard (GB/T 21317-2007) also establishes the method for determination of these compounds in milk and animal tissues. This regulation took effective April 1, 2008.

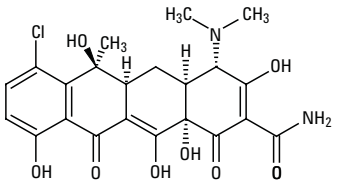
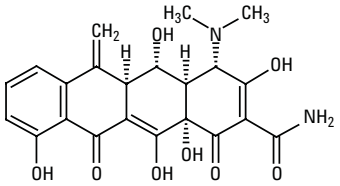
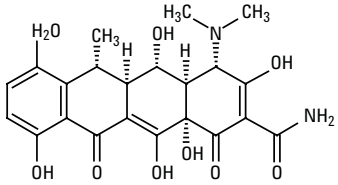
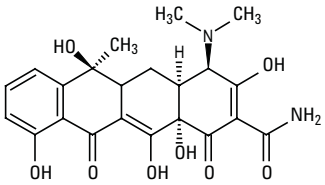
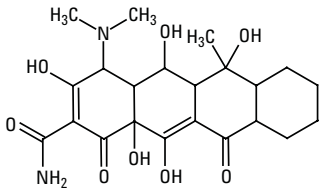
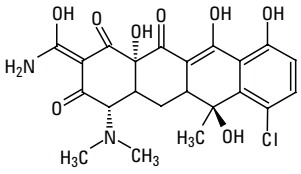
The purpose of this study is to develop a method for the Agilent 6410 LC/MS/MS to determine the presence of tetracyclines and their metabolite residues in milk. The method is rapid and easy to use. The tetracyclines and their metabolites are given in Table 1.

Table 1. The Compounds in this Study

No.	Name	CAS No.	Structure
1	Minocycline	10118-90-8	
2	Oxytetracycline	6153-64-6	
3	Tetracycline	60-54-8	
4	Demeclocycline	127-33-3	

(Continued)

Table 1. The Compounds in this Study

No.	Name	CAS No.	Structure
5	chlortetracycline	57-62-5	
6	methacycline	914-00-1	
7	doxycycline	564-25-0	
8 8	4-epitetracycline	64-75-5	
9	4-epi oxytetracycline	35259-39-3	
10	4-epichlortetracycline	14297-93-9	

Experimental

Reagents and Chemicals

Water and methanol are HPLC grade, and they, along with formic acid were all purchased from Fluka. The standards were purchased from Sigma-Aldrich.

Instrument Settings

Table 2. LC/MS/MS Conditions

HPLC	
Column	ZORBAX RX-C8, 2.1 mm × 150 mm, 5 µm (p/n 883700-906)
Flow rate	0.3 mL/min
Mobile phase	A: Water/ 0.1 % Formic Acid B: Methanol
Gradient	0–10 min, B from 5% to 30% 10–12 min, B from 30% to 40% 12.5–18 min, B 65% 18.5–25 min, B 95% 25.5 min, B 5.0%
Total run	28 min
Post time	5 min
Temp	30 °C
injection	5 µL
MS Source settings	
Source	ESI
Ion polarity	Positive
Drying Gas temp.	350 °C
Drying gas flow rate	10 L/min
Nebulizer	45 psi
V_{cap}	4000V

MRM Setting

Name	Frag.	Precursor ion	Product ion	CE	Rt. (min)
Minocycline	120	458	352	35	
			441	20	8.58
4-Epitetracycline	120	445	410	20	
			427	10	8.60
4-Epioxytetracycline	120	461	426	20	
			444	15	9.47
Tetracycline	120	445	410	20	
			427	15	9.90
Oxytetracycline	120	461	426	20	
			443	10	9.95
Demethylclocycline	120	465	430	25	
			448	15	11.25
4-Epichlortetracycline	120	479	444	22	
			462	15	11.59
Chlortetracycline	120	479	444	22	
			462	15	12.95
Methacycline	120	443	381	25	
			426	15	13.98
Doxycycline	120	445	154	30	
			428	15	14.08

Sample Preparation

Extraction:

1. Weigh a 5 g-milk sample (accurate to 0.01 g) into a 50-mL colorimetric tube, and dissolve with 0.1 mol/L Na_2EDTA-McIlvaine buffer solution and bring volume to 50 mL.
2. Vortex for 1 min and ultrasonicate the extract in an ice water bath for 10 min.
3. Transfer the sample to a 50-mL polypropylene centrifuge tube and cool to 0 °C ~ 4 °C.
4. Centrifuge the sample at a speed of 5000 rpm for 10 min (below 15 °C).
5. Filter with fast filter paper.

Purification:

1. Accurately draw 10 mL of the extract (equivalent to 1 g sample) and put it through the SampliQ OPT cartridge (p/n 5982-3036) at a speed of 1 drop/s.
2. After it elutes completely, clean the cartridge with 3 mL water adjusted to pH 4.5 with trifluoroacetic acid and then discard the entire effluent.
3. Under a negative pressure below 2.0 kPa, drain the cartridge for 5 min.
4. Elute with 10 mL of 10 mmol oxalic acid in methanol.
5. Collect the eluent and dry with nitrogen below 40 °C.
6. Dissolve the residue with 1.0 mL of the initial mobile phase.
7. Filter with a 0.45-µm filter membrane and inject.

Results and Discussion

Optimization and Separation

Fragmentor and Collision Energy (CE) optimization

It is well known that the LC/MS/MS QQQ is the best tool to identify, confirm and quantify target analytes in food matrices. In order to get the best response, only two parameters need to be optimized for each compound on this instrument, the fragmentor and the collision energy. The correct fragmentor voltage allows the highest transmission of the precursor ion into the mass analyzer. The correct collision energy provides the highest intensity of quantitation of the qualifier product ion.

One method of optimization is to inject the sample multiple times at the different fragmentor voltages set within segments of a single run. This is shown in Figure 1 for minocycline. For this compound, there is a small increase in detection as the voltage is increased. Collision energy is optimized in the same way and the results for tetracycline is shown in Figure 2.

Recently, Agilent introduced the "Optimizer" program that automatically determines the optimum fragmentor voltage and collision energy and stores the results in the Optimizer Database. Using this program and flow injection with or without a column, the user enters the compounds to be optimized and their molecular formulas. The nominal mass of the compound is automatically calculated from the formula. The user then specifies the adducts expected for positive and negative modes, the low mass cutoff, any ions to be excluded, and the method to be used (mobile phase conditions etc.). Once started the program will inject the sample, determine the precursor ion, and optimize the fragmentor voltage by stepping through the increments that the user selected for one injection. The program then selects the voltage producing the highest intensity for the precursor ion. For tetracycline, this is shown in Figure 3.

The program then performs a product ion scan on a second injection of the sample, and chooses the four most prevalent product ions. It reinjects the sample again and performs MRMs of each ion collected with collision energies in increments covering the range of voltage selected by the user. The collision energy that generates the maximum signal for each product ion is then automatically determined and can be stored in the database. The data from this collision energy optimization for tetracycline is shown in Figure 4 along with the ion breakdown curve shown in Figure 5. Compounds with product ions can be imported directly into the users' acquisition method.

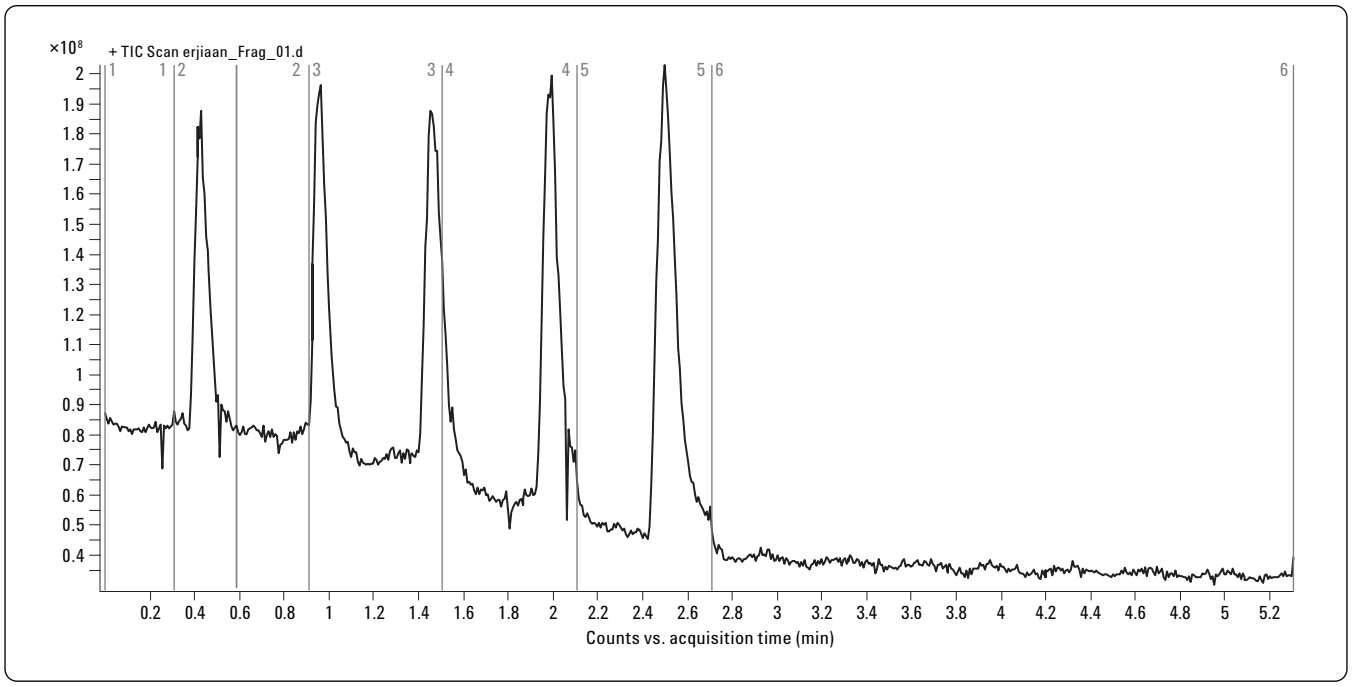


Figure 1. Optimization of fragmentor voltage for minocycline from 60-160 by steps of 20 V.

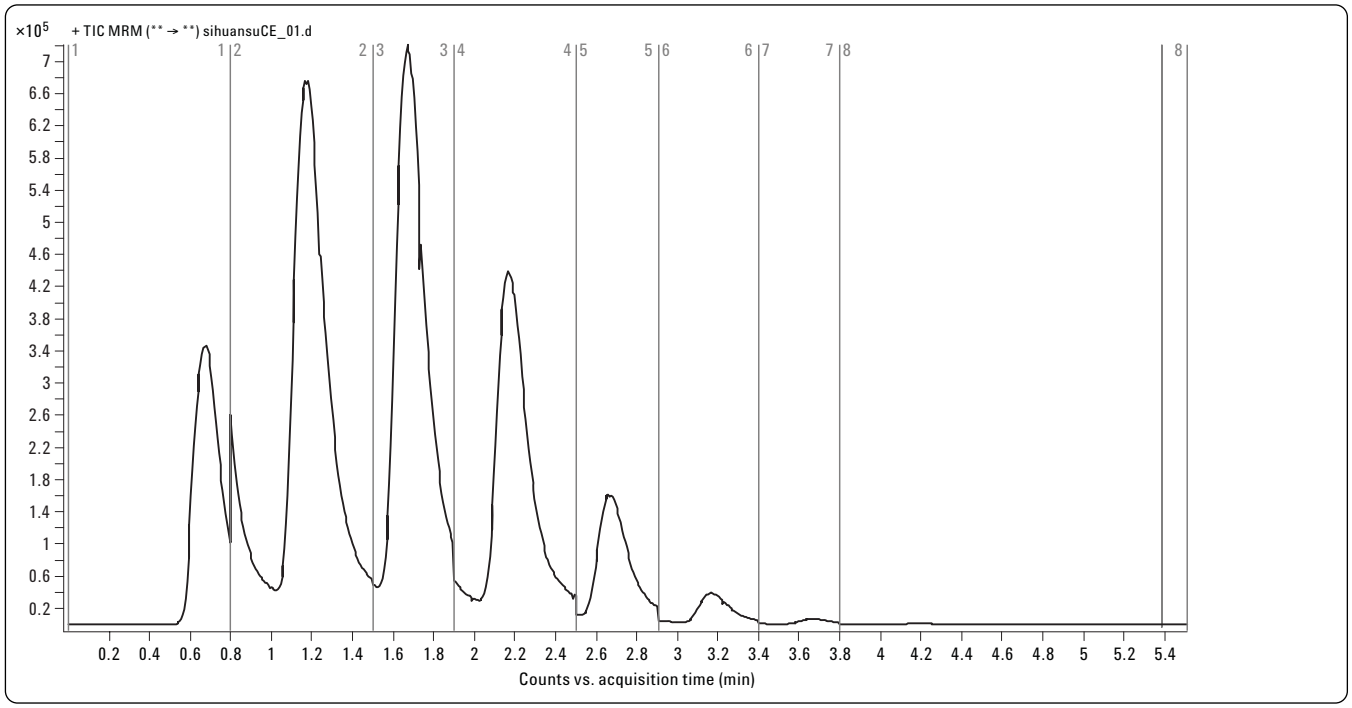


Figure 2. Manual collision energy optimization of tetracycline.

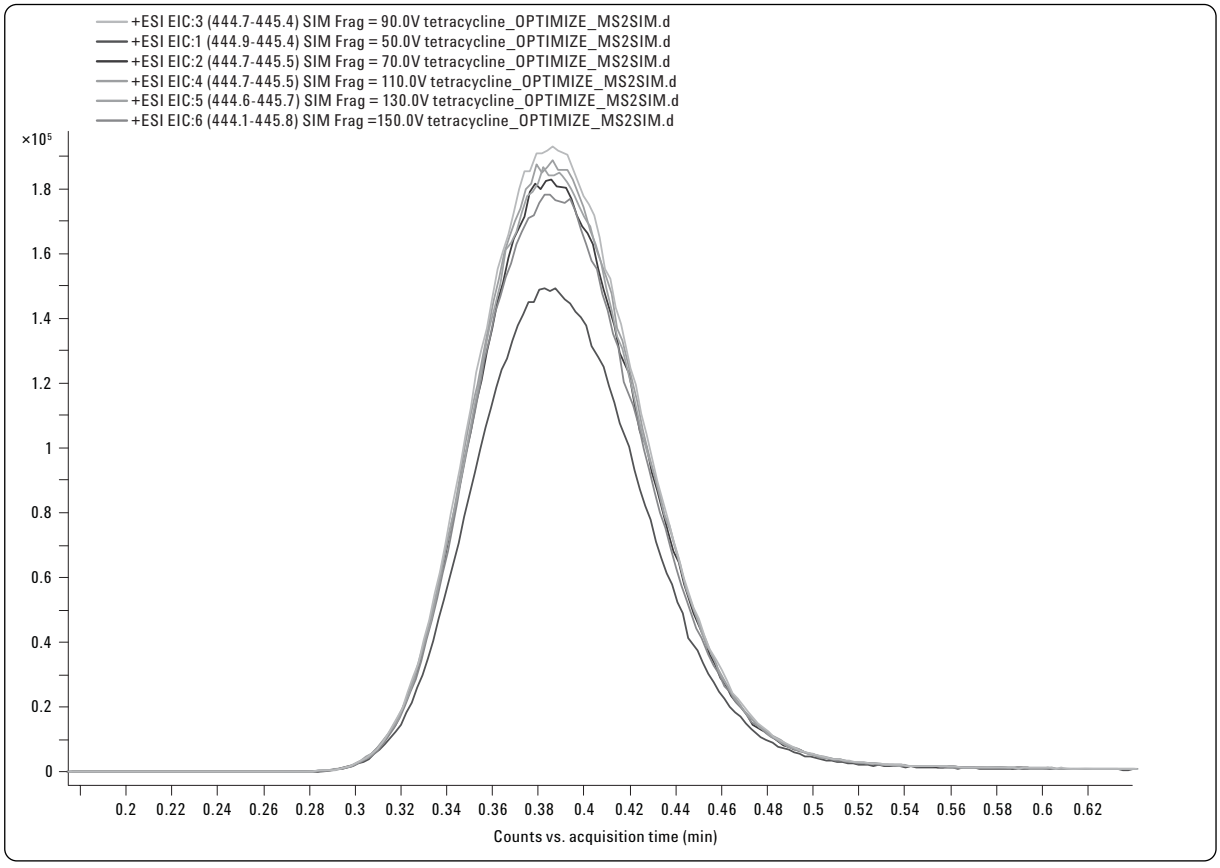


Figure 3. Single injection automatic determination of fragmentor voltage for tetracycline using the Optimizer program.

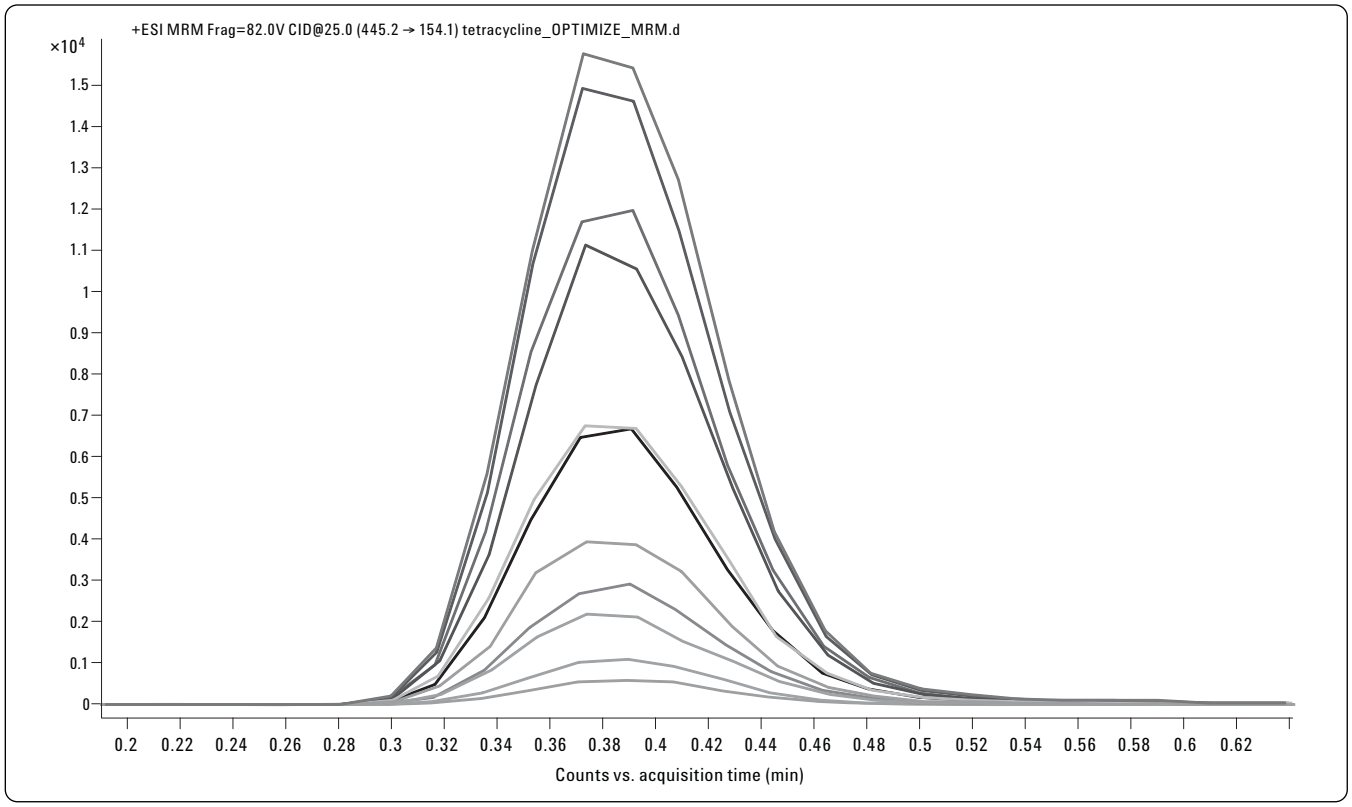


Figure 4. Single injection automated collision energy determination using Optimizer program for tetracycline.

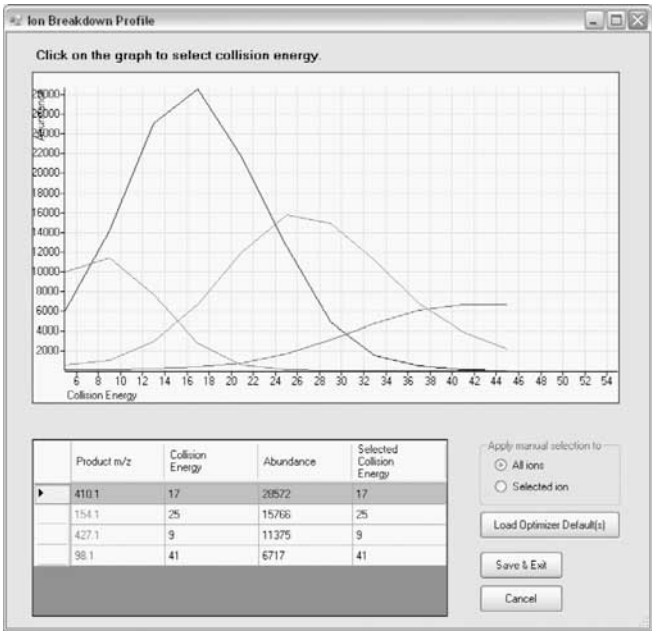


Figure 5. Ion breakdown profile for tetracycline as determined by the Optimizer program.

Separation

Sample preparation and separation of tetracycline, chlortetracyline and oxytetracycline is important. The challenge in separating these kinds of compounds is that they easily degrade under conditions of weak acid, strong acid, strong base, and heat converting the diasteriomer to its diaxial epimer.

The typical process is shown below with tetracycline:

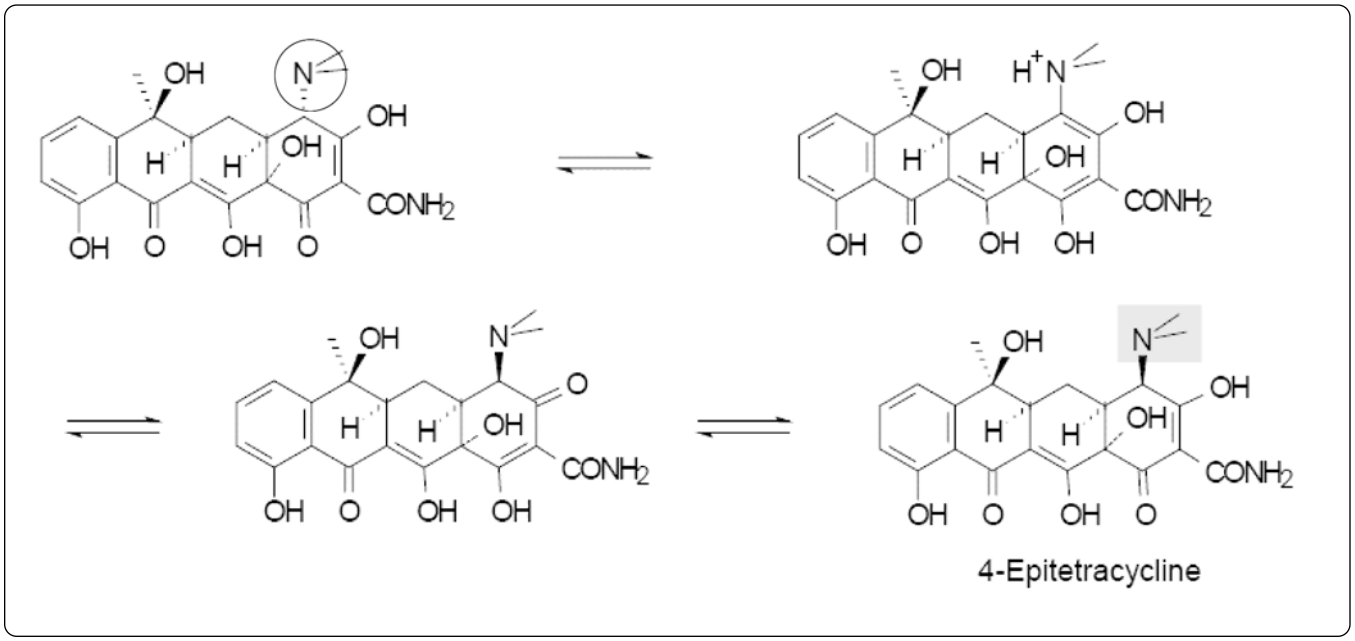


Figure 6. The degradation of tetracycline to 4-epitetracycline.

Tetracyclines and their degradants are diasteriomers with the same formula and the same fragment ions are formed in MS/MS. Therefore, they have the same precursor ions, qualitative ions, and quantitation ions. In order to identify and confirm them in the Rapid Resolution liquid chromatograph (RRLC), separation is important for this analysis. Using the Agilent ZORBAX Rx-C8, 2.1 mm × 150 mm, 5-µm particle size column and a simple gradient, the three epimer pairs of these compounds are well separated. This is shown with the retention times given in Table 2. Figure 7 shows the graphic representation of the separation of tetracycline and its epimer.

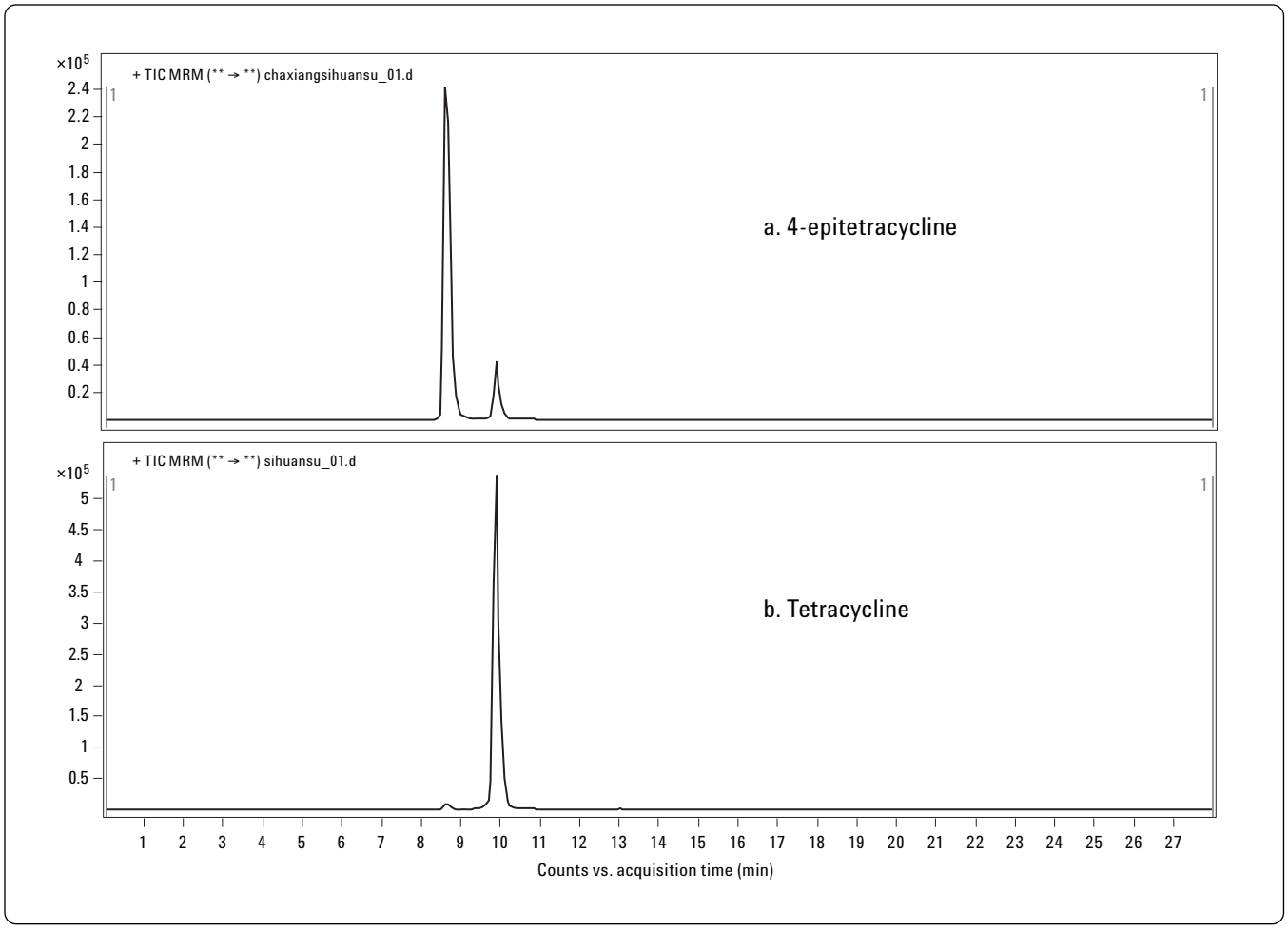


Figure 7. The separation of tetracycline and its degradation product 4-epitetracycline.

Linearity, LOD and LOQ

Linearity, LOD and LOQ were evaluated in both solvent and a milk matrix. The results are given in Table 3 and show that linearity is similar for both solvent and milk matrix and generally provide greater than 0.99 coefficient of variance. The tetracyclines do not ionize well with electrospray but the limits of detection (LOD) for each are still in the low pictograms on-column. The limit of quantitation is typically set at a signal to noise (S/N) of 10:1 but we report twice that in the solvent. The graphic representation of the calibration curve for minocycline is shown in Figure 8.

Table 3. Quantitative Performance of Tetracyclines in Solvent and Milk Matrix

	Standards in solvent*			Standards in Milk matrix*	
Name	**R^2**	**LOQ** (S/N=20) pg on column	**LOD** (S/N=3) pg on column	**R^2**	**LOD** (S/N=3) pg on column
Minocycline	0.999	41.5	6.2	0.990	16.3
4-epitetracycline	0.991	10.8	1.6	0.994	8.7
4-epioxytetracycline	0.996	14.7	2.2	0.996	12.8
Tetracycline	0.998	9.4	1.4	0.994	10.2
Oxytetracycline	0.996	10.7	1.6	0.991	8.6
Demethylclocycline	0.999	22.8	3.4	0.993	8.1
4-epichlortetracycline	0.986	38.2	5.7	0.987	11.9
Chlortetracycline	0.986	8.1	1.2	0.994	7.6
Methacycline	0.999	20.8	3.1	0.994	12.3
Doxycycline	0.999	32.2	4.8	0.995	11.2

Note: *The calibration curve range is from 1 ppb-1 ppm with injection volume of 5 uL

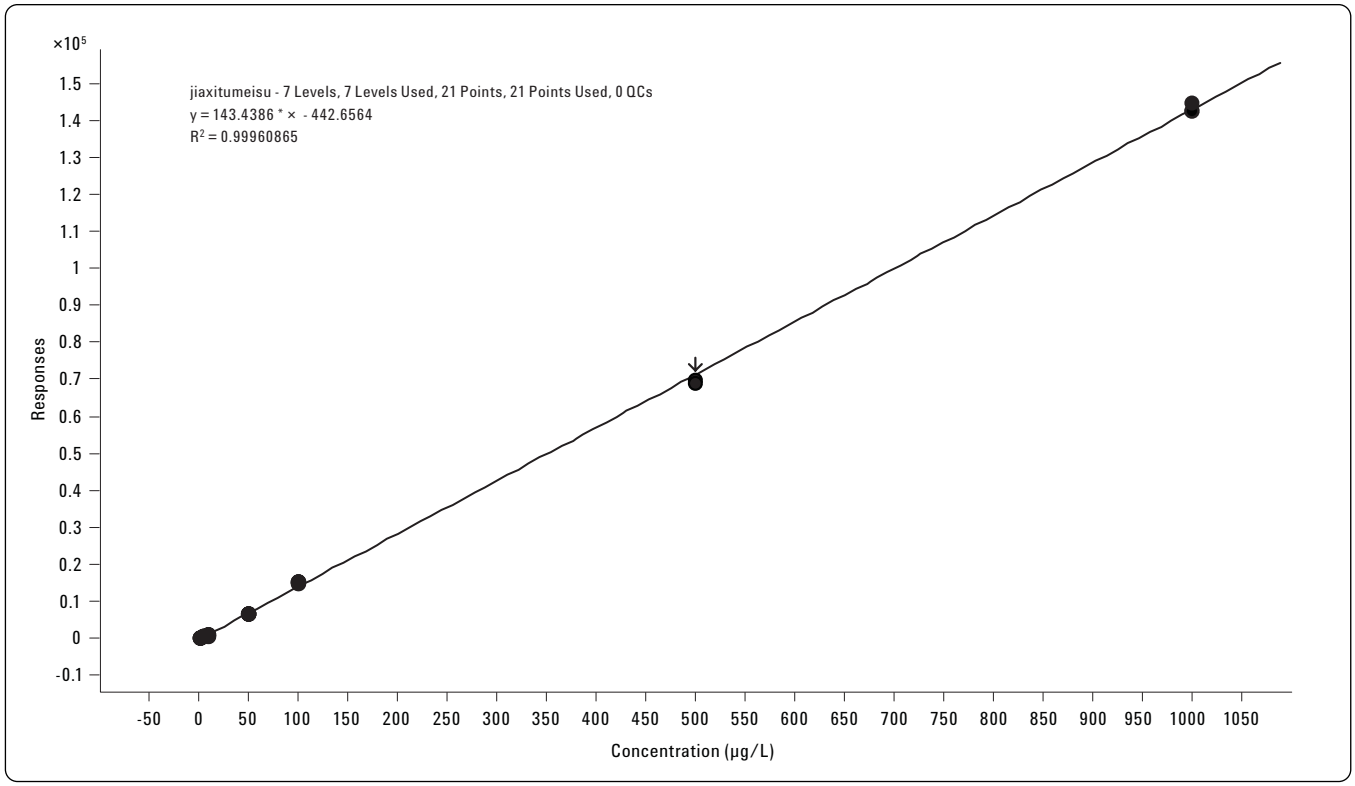


Figure 8. Tetracycline calibration curve from 1 ppb to 1000 ppb.

Recovery and Repeatability

The recovery and repeatability of the method was evaluated and the results shown in Table 4. All recoveries were greater than 80 % which is generally accepted as sufficient. In addition the precision, as shown in the table, is 5 % or better for the lower concentration and less than 2 % for the higher concentration. Ion ratios for confirmation are a very important performance criterion and these results show excellent repeatability. A graphic representation of the ion ratios for methacycline is shown in Figure 9. The ratios combined with matching retention time provide the necessary information for confirmation.

Table 4. Recovery and Repeatability in Milk Matrix

Name	Recovery in milk (Conc. 50 ppb n=6)	RSD % (Signal response n=6)	RSD % (Ion ratio n=6)	Recovery in milk (Conc. 100 ppb n=6)	RSD % (Signal response n=6)	RSD % (Ion ratio n=6)
Minocycline	96.5	4.9	2.1	101.4	1.6	1.0
4-epitetracycline	89.2	3.8	1.5	96.3	1.6	0.9
4-epioxytetracycline	84.4	5.4	1.3	88.2	0.9	0.6
Tetracycline	86.1	2.5	1.2	90.7	1.1	1.2
Oxytetracycline	77.6	3.8	1.6	82.5	1.2	0.9
Demethylclocycline	79.2	2.0	3.1	84.7	0.9	0.6
4-epichlortetracycline	76.4	5.5	5.4	84.3	1.1	0.5
Chlortetracycline	94.3	4.5	1.5	100.9	1.8	1.1
Methacycline	86.3	1.0	1.9	91.2	1.2	0.8
Doxycycline	78.7	3.6	6.7	82.4	1.0	0.8

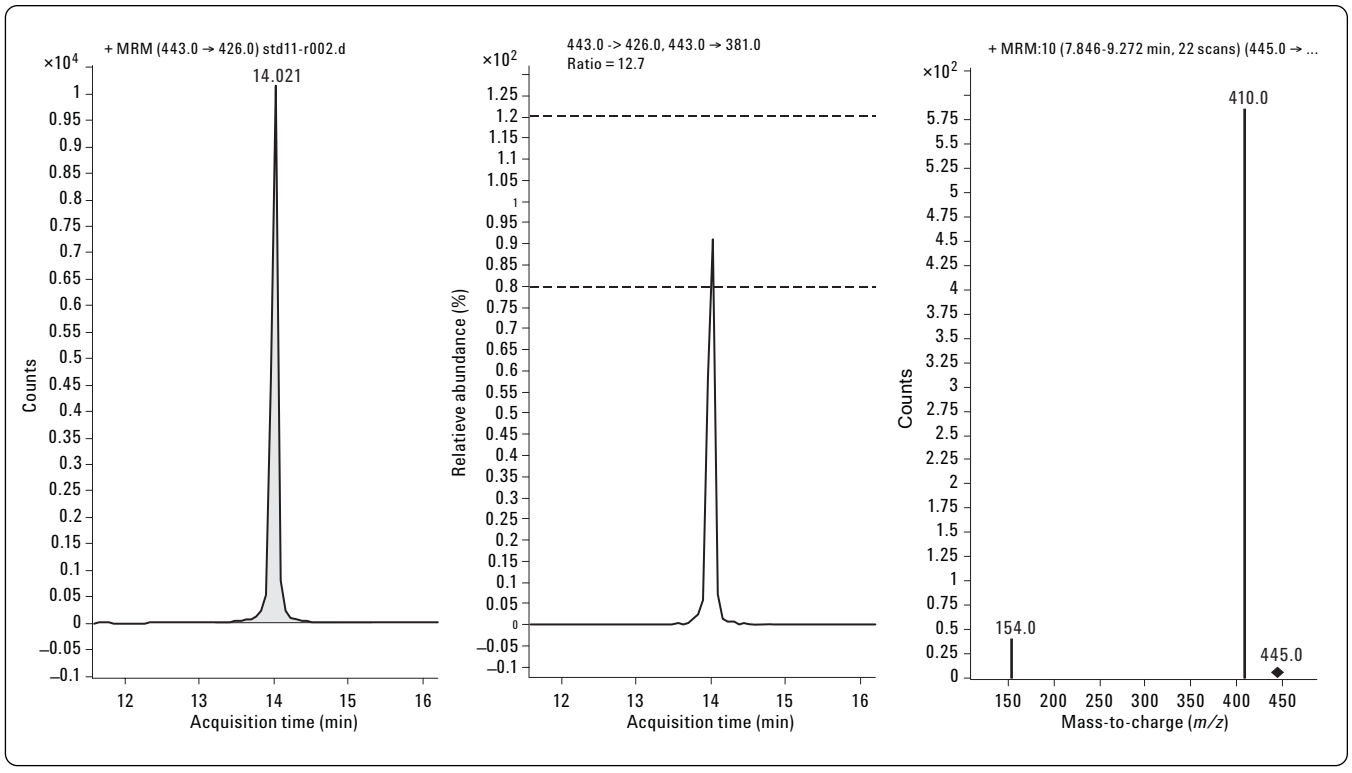


Figure 9. Shows the ion ratios for qualifier ion and the quantitation ion of methacycline.

Study of Ion Suppression

In general, tandem MS can remove chemical noise to get a "clean" spectrum even in dirty and complex food matrices. However, the matrix may contain components that suppress the ionization of the analyte. Figure 10 shows the comparison of the response of methacycline and tetracycline in solvent and milk. The difference in the slope of each curve demonstrates the suppression effect of the milk matrix.

Because of the strong suppression observed, using the external standard method (ESTD) for calibration, matrix matched standards should be prepared in antibiotic-free milk, or milk known to not contain the analytes. In this way, the calibration curve is generated with the same matrix effects as the samples.

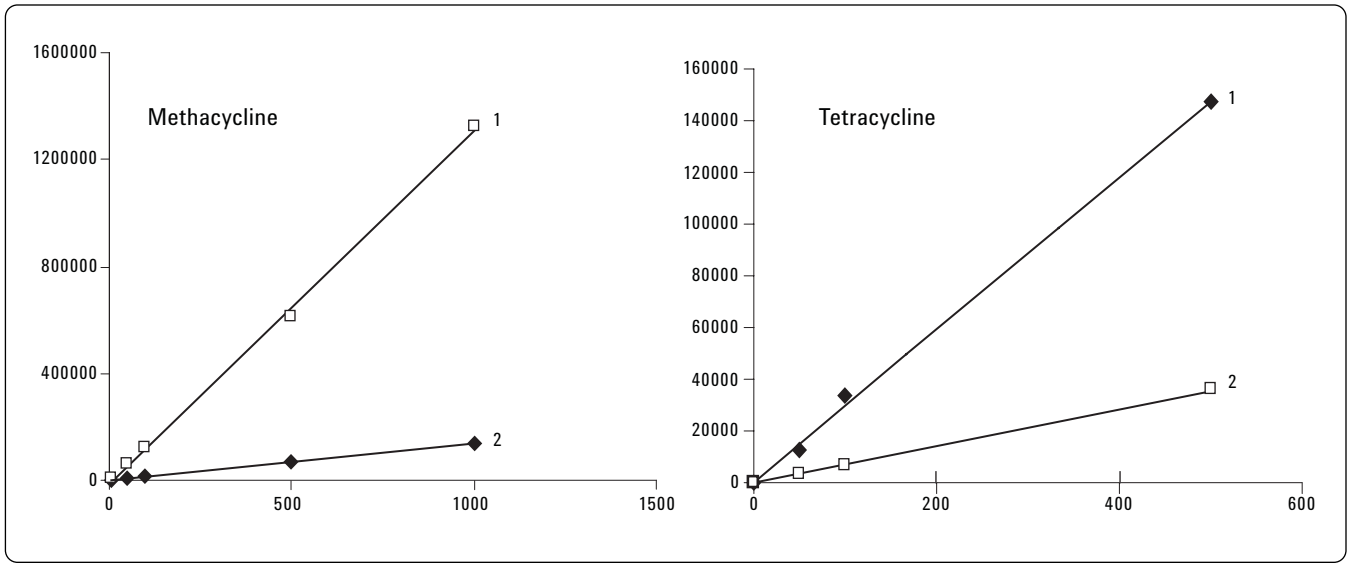


Figure 10. Ion suppression of two of the tetracyclines in milk; 1) response in solvent, 2) response in milk.

Conclusions

The results of this work show that the Agilent 6410 triple quadrupole LC/MS System is a robust, sensitive, and repeatable instrument for the study of tetracyclines residues in a milk matrix. In China, the government standard requirement (GB/T 21317-2007) sets the detection limit at 50 ppb with a 100 µL injection. This method easily meets these requirements. Additionally, these types of antibiotics readily degrade under the conditions of weak acid, base etc. The preparation method used here avoids this reaction, allowing the LC method to separate these isomers for reliable confirmation and quantitation. Finally, ion suppression is considered for the LC/MS/MS method when comparing different compounds in the same matrix to their response in solvent. Using the ESTD method, the preparation of a matrix-matched calibration curve is necessary to obtain accurate results, even though the recoveries measured for the sample preparation are better than 80%.

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Determination of Hormones in Fish (*Carassius Carassius*) by SampliQ-OPT Solid Phase Extraction with High Performance Liquid Chromatography

Application Note

Food Safety

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Abstract

Solid-phase extraction (SPE) coupled with high performance liquid chromatography (HPLC) was optimized for the extraction and determination of sixteen hormones (estriol, prednisolone, hydrocortisone, prednisone, methylprednisolone, betamethasone, dexamethasone, triamcinolone acetate, gestrinone, prednisolone acetate, hydrocortisone acetate, prednisone acetate, estradiol, cortisone acetate, methyltestosterone, estrone) in crucian carp (*Carassius carassius*) meat. Results indicate that SPE using an Agilent SampliQ OPT (60 mg, 3 mL) and HPLC using an Agilent ZORBAX Eclipse Plus C18 column (4.6 mm × 250 mm, 5 µm) is suitable for extraction of these compounds. Recoveries ranged from 76.2 to 106.1 % with relative standard deviations (RSDs) between 1.7 and 8.9 %.

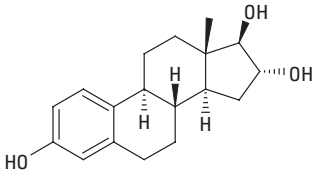
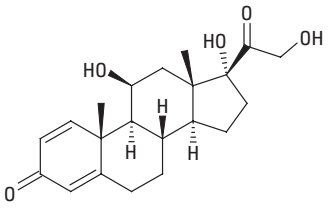
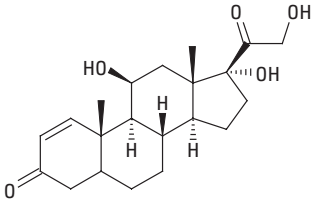
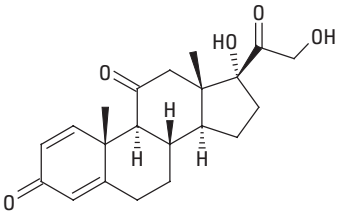
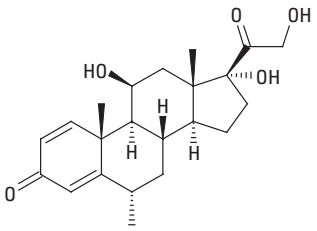
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Introduction

Food safety has increasingly become an important concern of people worldwide. Many chemicals added to food create potential hazards to human health. Hormones are a common food additive. Long-term consumption of glucocorticoid can lead to hyperglycemia, osteoporosis, birth defects, and immune function decline. Other hormones such as estrogen, androgen, and progesterone are carcinogenic and can lead to breast cancer, ovarian cancer and cell carcinoma. Many countries' regulations clearly define residual limits for these compounds in food.

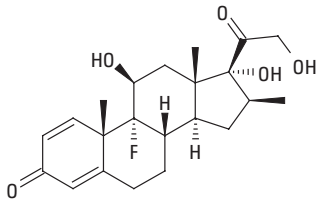
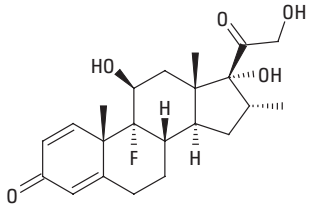
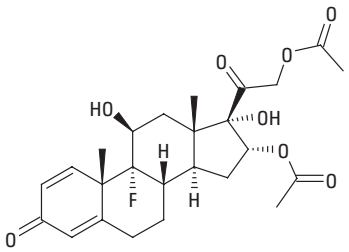
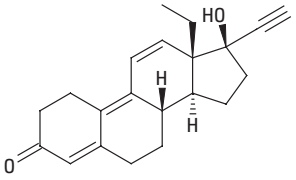
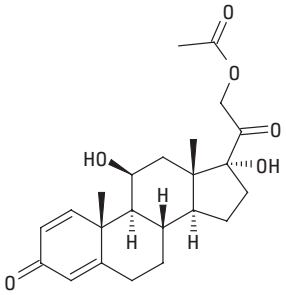
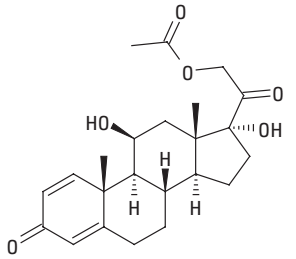
An Agilent SampliQ OPT SPE cartridge was used to extract 16 kinds of hormones (Table 1) from crucian meat and an HPLC method was established to detect these 16 compounds.

Table 1. Hormones Used in this Study

No.	Name	CAS No.	Log P	Structure
1	Estriol	50-27-1	2.45	
2	Prednisolone	50-24-8	1.66	
3	Hydrocortisone	50-23-7	1.79	
4	Prednisone	53-03-2	2.07	
5	Methylprednisolone	83-43-2	2.06	

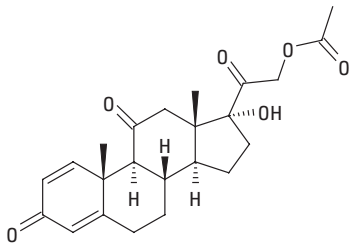
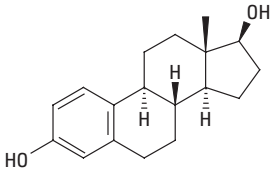
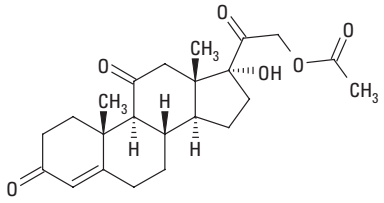
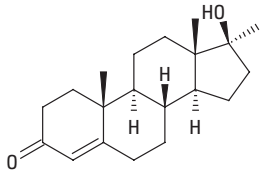
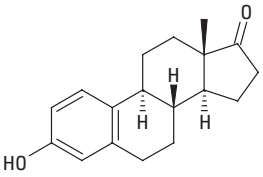
(Continued)

Table 1. Hormones Used in this Study

No.	Name	CAS No.	Log P	Structure
6	Betamethasone	378-44-9	1.93	
7	Dexamethasone	50-02-2	1.93	
8	Triamcinolone acetate	67-78-7	1.9	
9	Gestrinone	16320-04-0	NA	
10	Prednisolone acetate	52-21-1	NA	
11	Methylprednisolone	83-43-2	NA	

(Continued)

Table 1. Hormones Used in this Study

No.	Name	CAS No.	Log P	Structure
12	Prednisone acetate	125-10-0	NA	
13	Estradiol	50-28-2	3.57	
14	Cortisone acetate	50-04-4	2.35	
15	Methylestosterone	58-18-4	NA	
16	Estrone	53-16-7	4.03	

Experimental

Reagents and Chemicals

All reagents and solvents were HPLC or analytical grade. Hormone standards were purchased from NICPBP (National Institute for the Control of Pharmaceutical and Biological Products). Crucian was purchased from a local market.

Stock solutions (1 mg/mL) were prepared in methanol and kept in the freezer (–20 °C). Working solutions were prepared using the stock solution diluted with methanol. The working solutions should be prepared every week and need to be stored below 4 °C.

The SPE cartridges were Agilent SampliQ OPT (3 mL, 60 mg, p/n 5982-3036). The analysis was performed on an Agilent 1200 Series HPLC with a diode array detector (DAD). The analytical column was an Agilent ZORBAX Eclipse Plus C18 (5 µm 250 mm × 4.6 mm id, p/n 959990-902). An Agilent 0.45-µm PTFE Premium Syringe Filter (p/n 5185-5836) was used to filter the sample solution before HPLC.

HPLC conditions

Column:	ZORBAX Eclipse Plus C18 250 mm × 4.6 mm, 5 µm		
Flow rate:	1.0 mL/min		
Injection volume:	5 µL		
Column temperature:	18 °C		
Detection wavelength:	230 nm		
Mobile phase:	Water-Acetonitrile Gradient		
	Time (minutes)	% Water	% Acetonitrile
	0	70	30
	10	65	35
	23	50	50
	30	20	80

Separation

1. Weigh 200 grams of crucian meat, homogenize, and store in a clean, sealed container at –18 °C.
2. Place 1 g of homogeneous sample (accurate to 0.01 g) into a 10-mL polypropylene centrifuge tube with 5 mL of methanol.
3. Vortex for 1 minute.
4. Extract ultrasonically for 10 minutes in an ice bath.
5. Centrifuge the sample at a speed of 4000 r/min for 5 minutes and remove the 3 mL of supernatant.
6. Save in a clean tube and evaporate with N_2 below 40 °C.
7. Reconstitute the residue in 5 mL of 5 % methanol in water.

SPE Purification

The procedure used for the SPE extraction is shown in Figure 1. Agilent SampliQ OPT cartridges are preconditioned with 3 mL of methanol then 5 mL of water. The 5-mL extract (equivalent to 0.6 g sample) is passed through the SampliQ OPT cartridge at a speed of 1 mL/min. After it effuses completely, the cartridge is washed with 5 mL of 30% methanol in water and the entire effluent is discarded. The cartridge is dried under negative pressure (below 2.0 kPa) for 3 minutes. The sample is then eluted with 6 mL of methanol, and the eluent is collected and dried under nitrogen below 40 °C. The residue is dissolved and brought to a constant volume of 1.0 mL using methanol, filtered through a 0.45 µm PTFE filter membrane, and analyzed by HPLC.

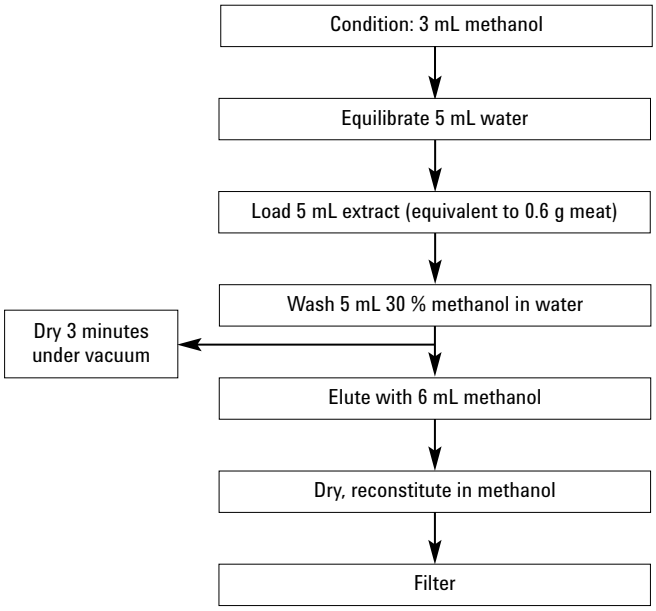


Figure 1. Hormones in crucian meat SPE procedure.

Results and Discussion

Linearity, Limits of Detection

Stock solutions were diluted to different concentrations and analyzed by HPLC. Linear regressions were calculated for the hormones based on the areas and the solution concentrations. Limit of detection (LOD) signifies the injection concentration at which the signal to noise ratio was between 2 and 3. Linear range was between 1–100 mg/kg. The linearity and LOD are shown in Table 2.

Table 2. Linearity and LODs of Hormones.

No.	Compound	Regression equation	Correlation coefficient	LOD (mg/kg)
1	Estriol	Y = 8.096 × –0.824	0.9998	0.5
2	Prednisolone	Y = 17.418 × –2.088	0.9999	0.2
3	Hydrocortisone	Y = 15.746 × –1.518	0.9999	0.3
4	Prednisone	Y = 20.192 × –2.152	0.9998	0.2
5	Methylprednisolone	Y = 16.986 × –1.894	0.9999	0.4
6	Betamethasone	Y = 20.439 × –1.106	0.9997	0.2
7	Dexamethasone	Y = 20.176 × –2.176	0.9999	0.2
8	Triamcinolone acetate	Y = 16.374 × –1.558	0.9997	0.4
9	Gestrinone	Y = 6.370 × –0.668	0.9998	1.0
10	Prednisolone acetate	Y = 15.589 × –1.627	0.9999	0.4
11	Hydrocortisone acetate	Y = 15.051 × –1.584	0.9999	0.4
12	Prednisone acetate	Y = 24.106 × –2.401	0.9997	0.2
13	Estradiol	Y = 8.709 × –0.635	0.9999	0.8
14	Cortisone acetate	Y = 19.826 × –2.336	0.9996	0.4
15	Methyltestosterone	Y = 19.980 × –2.209	0.9996	0.3
16	Estrone	Y = 10.701 × –0.847	0.9999	0.4

Recovery and Repeatability

The precision of the method was determined in terms of the recovery of spiked hormone standards in crucian meat at 2, 5, and 10 mg/kg levels. The analysis was repeated six times at each level. The chromatograms of the blank, the standards, and the spiked standard (2 mg/kg) sample are shown in Figures 2 through 4. The recovery and reproducibility data are shown in Table 3.

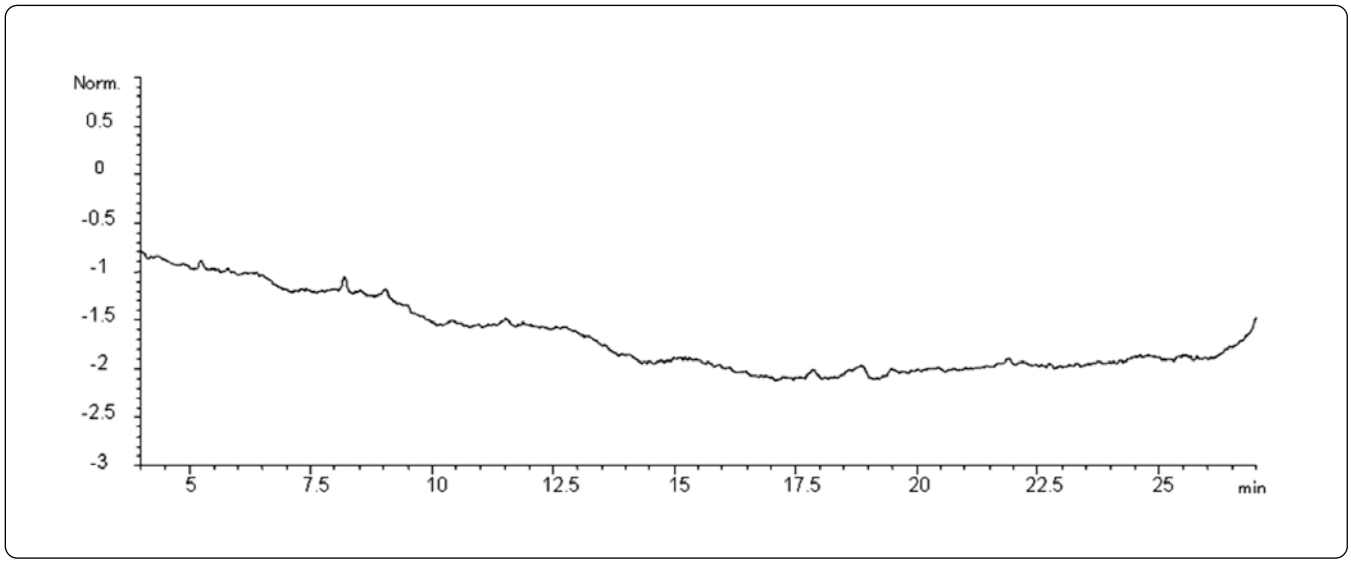
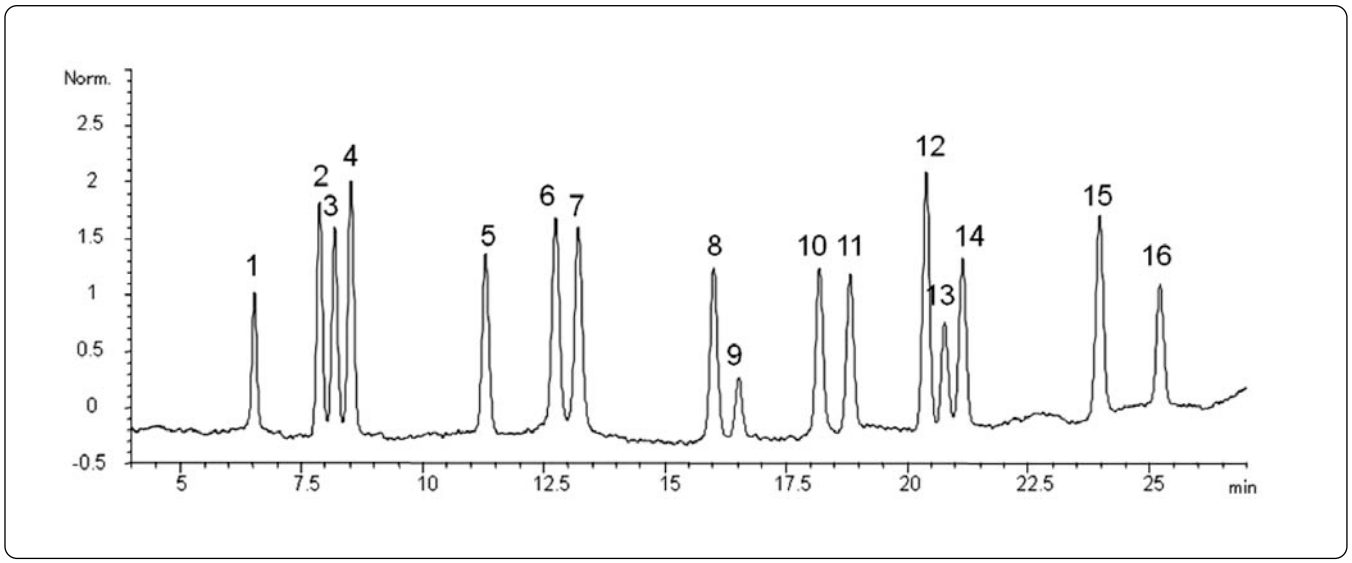
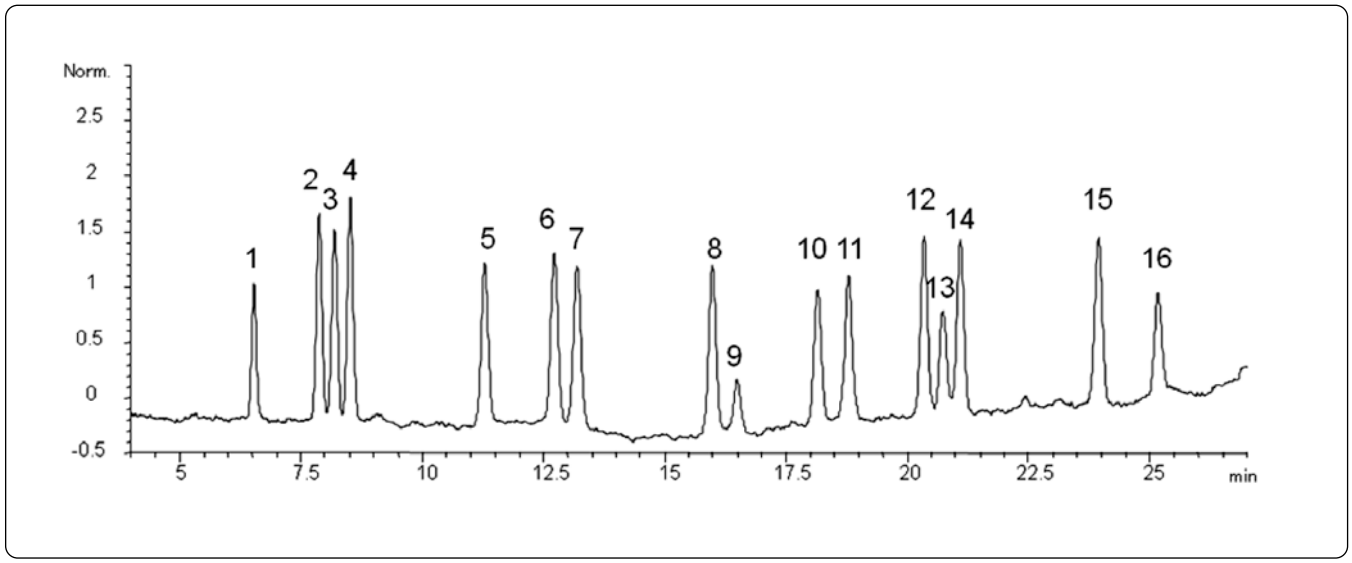


Figure 2. Chromatogram of crucian meat blank.



1	Estriol	5	Methylprednisolone	9	Gestrinone	13	Estradiol
2	Prednisolone	6	Betamethasone	10	Prednisolone acetate	14	Cortisone acetate
3	Hydrocortisone	7	Dexamethasone	11	Hydrocortisone acetate	15	Methyltestosterone
4	Prednisone	8	Triamicinolone acetate	12	Prednisone acetate	16	Estrone

Figure 3. Chromatogram of hormone standards at 2 mg/kg.



1	Estriol	5	Methylprednisolone	9	Gestrinone	13	Estradiol
2	Prednisolone	6	Betamethasone	10	Prednisolone acetate	14	Cortisone acetate
3	Hydrocortisone	7	Dexamethasone	11	Hydrocortisone acetate	15	Methyltestosterone
4	Prednisone	8	Triamicinolone acetate	12	Prednisone acetate	16	Estrone

Figure 4. Chromatogram of crucian meat sample spiked hormone standards at 2 mg/kg.

Table 3. Recoveries and RSDs of Hormones in Crucian Meat by SPE

Compund	Spiked level (mg/kg)	Recovery (%)	RSD (n = 6, %)
Estriol	2	100.4	2.2
	5	106.1	1.9
	10	102.4	4.4
Prednisolone	2	89.4	3.8
	5	90.9	7.6
	10	100.7	2.9
Hydrocortisone	2	85.3	6.7
	5	91.4	7.6
	10	101.4	3.4
Prednisone	2	82.5	7.2
	5	92.1	5.2
	10	100.7	2.9
Methylprednisolone	2	83.2	8.3
	5	93.6	3.2
	10	97.4	1.7
Betamethasone	2	88.3	8.9
	5	99.6	4.9
	10	100.8	3.8
Dexamethasone	2	79.1	4.3
	5	98.4	5.3
	10	98.4	3.9
Triamcinolone acetate	2	86.7	8.4
	5	97.6	5.9
	10	97.9	4.1
Gestrinone	2	78.0	6.6
	5	78.8	8.1
	10	85.3	8.0
Prednisolone acetate	2	86.9	7.3
	5	101.2	4.3
	10	101.9	5.7
Hydrocortisone acetate	2	87.3	6.8
	5	102.7	5.1
	10	101.5	7.9
Prednisone acetate	2	76.7	7.7
	5	94.1	3.5
	10	97.7	4.3
Estradiol	2	78.7	4.2
	5	94.7	3.5
	10	97.4	4.8
Cortisone acetate	2	82.8	6.9
	5	87.8	6.5
	10	94.4	4.1
Methyltestosterone	2	82.9	3.4
	5	91.9	4.9
	10	93.6	4.6
Estrone	2	76.2	6.4
	5	90.0	8.7
	10	93.9	5.9

Conclusions

Agilent's SampliQ OPT, a polymeric sorbent with combined hydrophilic and lipophilic characteristics that allows retention of both polar and non-polar compounds, provides a simplified and effective single cartridge method for the purification and enrichment of multiple hormone compounds in crucian carp. Recovery and reproducibility (routinely below 10%) based on solution standards are acceptable for hormone residue determination in crucian meat. Impurities from crucian were minimal and did not interfere with any of the hormones analyzed.

Product Information

Part number	Description
5982-3013	OPT Polymer - Box, 100x 1 mL tubes, 30 mg
5982-3036	OPT Polymer - Box, 50x 3 mL tubes, 60 mg
5982-3067	OPT Polymer - Box, 30x 6 mL tubes, 150 mg
5982-3096	OPT Polymer - 96 Well Plate, 10 mg
95990-902	Agilent ZORBAX Eclipse Plus C18 250 mm × 4.6 mm, 5 µm
5185-5836	Agilent PTFE 0.45 µm Premium Syringe Filter

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Determination of β2-Agonists in Pork Using Agilent SampliQ SCX Solid-Phase Extraction Cartridges and Liquid Chromatography-Tandem Mass Spectrometry

Application Note

Food Safety

Authors

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Abstract

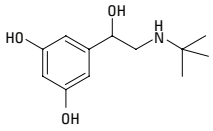
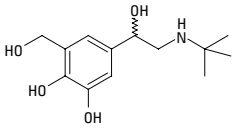
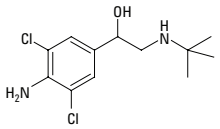
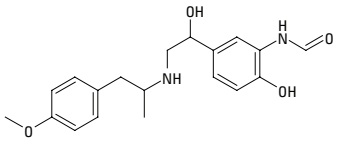
A method for simultaneous determination of four β2-agonist residues of terbutaline, salbutamol, clenbuterol and formoterol in pork has been developed and validated. The analytes are purified by liquid-liquid extraction (LLE) and solid-phase extraction (SPE) and quantified by liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) operating in positive ion multiple reaction monitoring (MRM) mode. The method provides a sub-ng/g level of limit of detection (LOD) for all four β2-agonists in pork. The dynamic calibration ranges for these compounds are obtained from 0.25 to 5 ng/g. The overall recoveries range from 78 to 101% with RSD values between 1.8 and 7.2%.

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Introduction

The β2-agonists have been used worldwide as illegal growth promoters in pork production. Recent incidences of poisoning have occurred due to high levels of the β-agonist (clenbuterol) in pork. This application note used Agilent's new SPE products to extract and enrich four β-agonists from pork and analysis by LC-MS/MS. Table 1 shows the name and structure of the four β-agonist compounds.

Table 1. β2-Agonist Compounds Used in this Study

Compound	Log P	Structure
Terbutaline	0.55	
Salbutamol	0.44	
Clenbuterol	2.94	
Formoterol	1.91	

Experimental

Reagents and Chemicals

All reagents were MS, HPLC or analytical grade.

Acetonitrile and water were from Scharlau. Ethyl acetate and isopropanol were from Fisher. The standards were purchased from National Institute for the Control of Pharmaceutical and Biological Products (NICPBP). Pork was purchased from a local market.

Standard solutions (1.0 mg/mL) were made in methanol individually, and refrigerated at 4 °C. A combined working solution (10 µg/mL) was made in methanol-water (10:90) and also stored at 4 °C. The spiked solutions were then made weekly by appropriately diluting the combined working solution in water.

Equipment and Materials

Agilent 1200 HPLC system

Agilent 6460 Triple Quadrupole LC-MS/MS system

Agilent SamliQ SCX Polymer cartridges, 50 × 3 mL tubes, 60 mg (p/n: 5982-3236)

Agilent ZORBAX Eclipse Plus C18, 50 × 2.1 mm, 1.8 µm (p/n: 959741-906)

Agilent Vaccum Manifold processing station (p/n: 5982-9120)

Sample Preparation

Liquid-Liquid Extraction

A 2 g amount of pork (±0.01 g) was weighed into a 15 mL capped polypropylene tube. To the pork, 8 mL of 0.2 M sodium acetate (pH 5.2) solution were added and mixed in a vortex. Next, 100 µL β-glucuronidase (1000 U/mL) were added and the tube vortexed thoroughly for 2 minutes. The sample was hydrolyzed at 37 °C for 16 hours.

The hydrolysate was shaken for 15 minutes and centrifuged at 4000 rpm for 10 minutes. A 4 mL amount of supernatant was transferred to another centrifuge tube. A 5 mL amount of 0.1 M perchloric acid solution was added and the pH was adjusted to 1 ± 0.3. The tubes were then centrifuged at 4000 rpm for 10 minutes. The supernatant was transferred to another tube, and the pH was adjusted to 11 with 10 M sodium hydroxide.

Ten milliliters each of a saturated sodium chloride solution isopropanol-ethyl acetate (60:40) were added to the tubes. The tubes were shaken for 5 minutes. The tubes were centrifuged at 4000 rpm for 5 minutes before the organic layer was carefully transferred to another tube. Isopropanol-ethyl acetate addition, shaking, centrifuging and organic layer transfer were repeated twice, and all supernants were combined.

Samples were evaporated to dryness with nitrogen at 40 °C. The residue was dissolved in 5 mL of 0.2 M sodium acetate (pH 5.2). The sample was then ready for SPE purification.

Solid-Phase Extraction

The SPE procedure is shown in Figure 1. Agilent SampliQ SCX cartridges were preconditioned with 3 mL of methanol and then equilibrated with 3 mL water. Five milliliters of the sample solution were then loaded onto a cartridge and passed through the cartridge by gravity (about 1 mL/min). The tubes were rinsed with 2 mL of water and 2 mL 2% formic acid in water. The entire effluent was discarded. Full vacuum was

applied to the cartridge for 3 minutes to completely dry the resin. Finally, the compounds were eluted with 5 mL of 5% ammonia solution in methanol at a rate of 1 mL/min. The eluent was dried with nitrogen flow at 40 °C. The residue was reconstituted in 1 mL of 0.1% formic acid in water/acetonitrile (90:10). The sample was vortexed and ultrasonicated to completely dissolve the residue. The sample was transferred to a 1.5 mL tube and centrifuged at 3000 rpm for 5 minutes. The sample was transferred to a 2 mL chromatography vial for analysis.

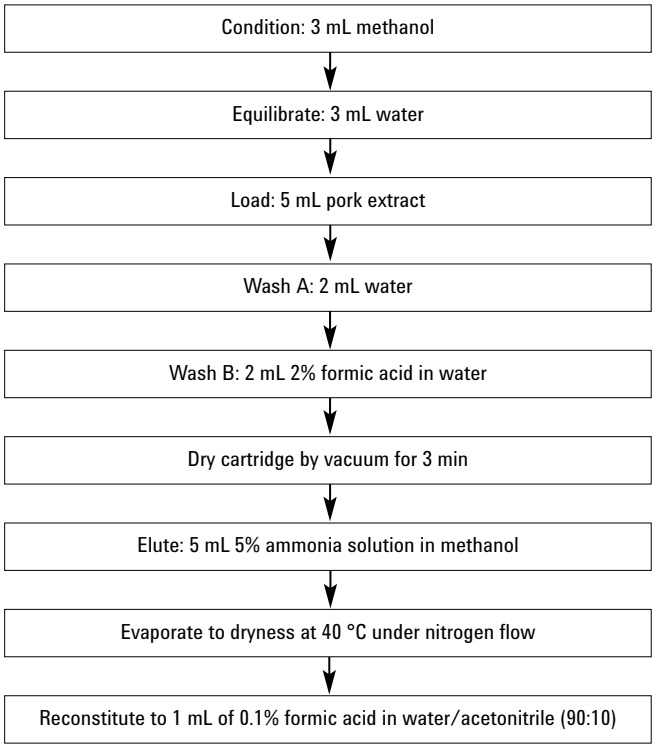


Figure 1. Pork clean up and enrichment – SPE procedure.

Instrument Conditions

HPLC Conditions

Column:	Agilent ZORBAX Eclipse Plus C18 2.1 mm × 50 mm 1.8 µm (p/n: 959741-906)		
Flow rate:	0.4 mL/min		
Column temperature:	40 °C		
Injection volume:	5 µL		
Mobile phase:	Water (0.1% FA+2 mM NH_4Ac, A), Acetonitrile (0.1% FA, B)		
Gradient:	Time (min)	%A	%B
	0	90	10
	0.5	90	10
	1.8	20	80
	2	90	10
	3.5	90	10

MS Conditions

These four compounds were monitored in the positive mode. The source conditions are shown in Figure 2 and the MRM channels are shown in Table 2.

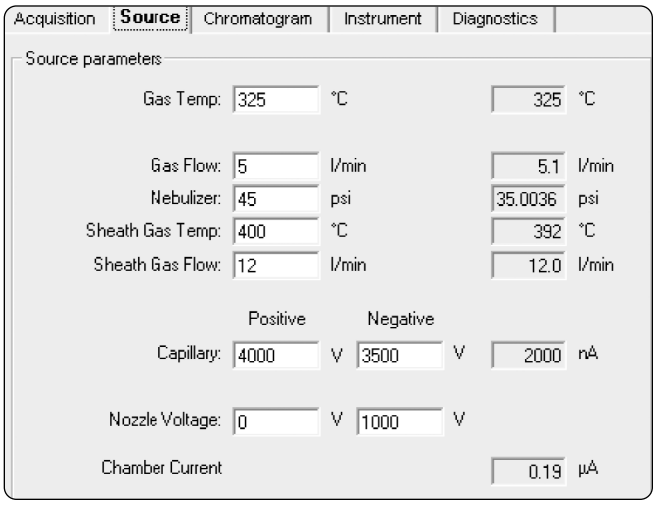


Figure 2. MS source parameters for these four compounds.

Table 2. Masses Monitored in the MRM

Compound	MRM for quantification	MRM for confirmation
Terbutaline	226.1 → 152.1	226.1 → 125
Salbutamol	240.1 → 148.1	240.1 → 222.1
Clenbuterol	227 → 203	227 → 259.1
Formoterol	345.1 → 149.1	345.1 → 327.1

Results and Discussion

Linearity and Limit of Detection

Solutions used to create external calibration curves were prepared by using a combined working solution to spike matrix blanks (0.25, 0.5, 1.0, 2.0 and 5.0 ng/g). Matrix blanks were created by taking pork through the hydrolysis, LLE and SPE procedures. The results for the calibration curves are shown in Table 3. The limits of detection (LOD) were chosen as the concentration of each compound that gave a signal to noise (S/N) ratio greater than 3:1. The LODs are also shown in Table 3.

Table 3. Linearity and LODs of β2-Agonists

Compound	Regression equation	R^2	LOD in pork (ng/g)
Terbutaline	Y = 3470x + 1325.4	0.9972	0.05
Salbutamol	Y = 13099x + 2900.3	0.9921	0.05
Clenbuterol	Y = 27028x + 1143.7	1	0.02
Formoterol	Y = 23251x + 487.44	0.9983	0.02

Recovery and Reproducibility

The recovery and reproducibility of the method were determined at three levels: pork spiked to a concentration of 0.5, 1.0, and 2.0 ng/g. The analysis was performed with six replicates at each level. The recovery and reproducibility data is shown in Table 4. The chromatograms of spiked pork extracts (1.0 ng/g) are shown in Figure 3.

Table 4. Recoveries and Reproducibility of β2-agonists in Pork After SPE Employing Agilent's SampliQ SCX; (p/n: 5982-3236), Recovery 90% and RSD 4.4% on Average

Compound	Spiked level (ng/g pork)	Recovery (%)	RSD (n=6)
Terbutaline	0.5	88.7	5.4
	1	98.0	7.2
	2	100.8	5.9
Salbutamol	0.5	100.6	1.8
	1	92.9	2.1
	2	97.4	3.9
Clenbuterol	0.5	82.3	5.0
	1	91.5	6.3
	2	90.6	4.3
Formoterol	0.5	85.1	1.9
	1	83.0	4.0
	2	77.9	2.5

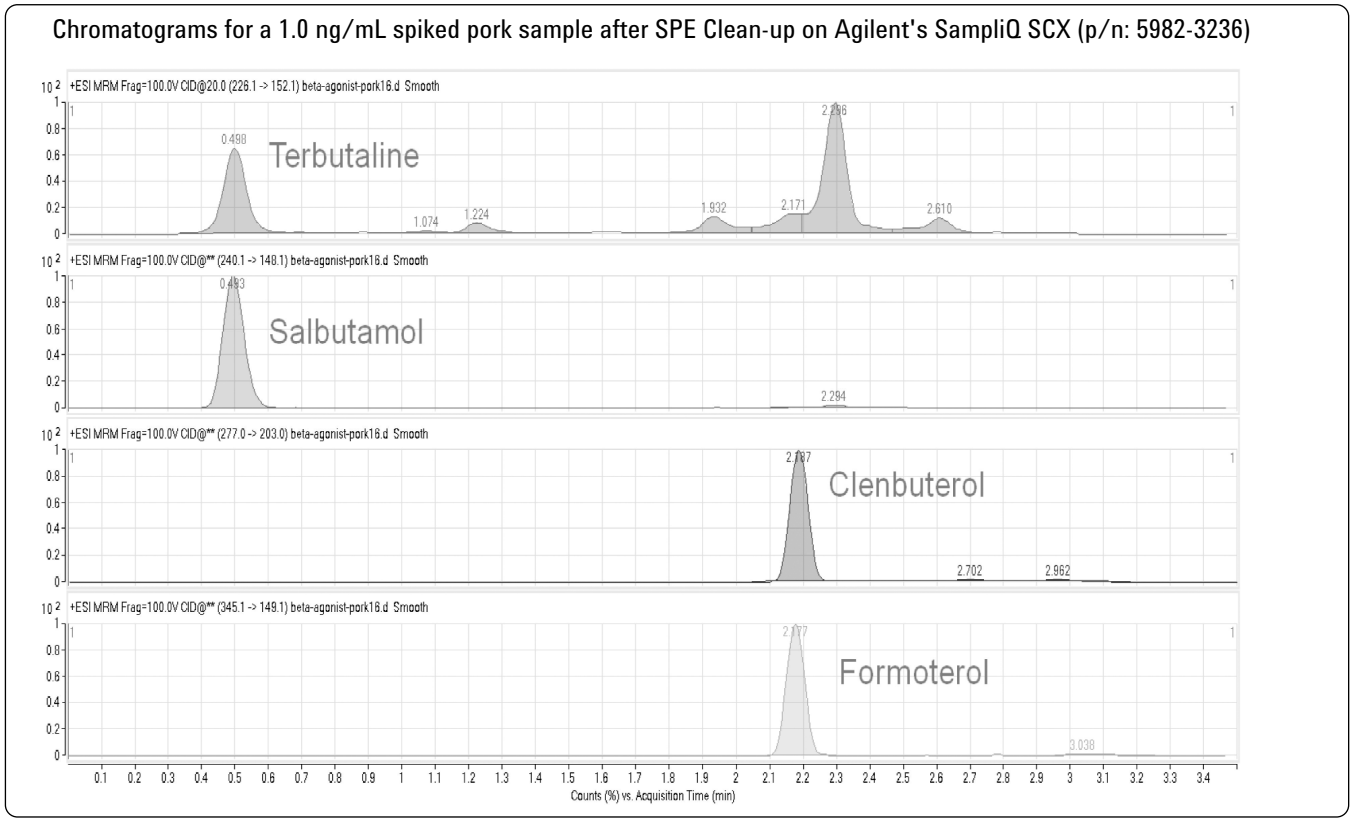


Figure 3. Chromatograms of 1.0 ng/g spiked pork sample extract.

Conclusions

The result of this study show that Agilent SampliQ SCX can be used as an effective method for purification and enrichment of multiple β2-agonists in a complex matrix such as pork. The recovery and reproducibility results based on matrix spiked standards are acceptable for β2-agonists residue determination in pork under Chinese regulations. The impurities and matrix effects are minimal and do not interfere with the quantification of any target compound. The LOQ are significantly lower than the MRLs [1,2].

References

1. GB/T 21313-2007 "Analysis of β2-agonists in Foods of Animal Origin by High Performance Liquid Chromatography Tandem Mass Spectrometry"
2. SN/T 1924-2007 "Determination of Clenbuterol, Ractopamine, Salbutamol and Terbutalin Residues in Foodstuffs of Animal Origin for Import and Export -HPLC-MS/MS Method."

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Profiling medications for decorative or aquarium fish using the Agilent 1290 Infinity LC system and Agilent ZORBAX Poroshell 120 2.7 µm columns

Application Note

Consumer Products

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Abstract

The Agilent 1290 Infinity LC system has significant capabilities for a wide range of HPLC and UHPLC applications. It exhibits a broader power range (for example, the combination of pressure and flow capabilities), and the flexibility to operate a wide range of column dimensions and particle sizes than any other commercially available system. Advanced optical design in the diode array detector allows a wide dynamic range and high sensitivity, both of which are critical in the monitoring of small impurities in fine chemicals.

The combined benefits are demonstrated by a separation of primary components and related impurities including sulfa drugs, nitrofurans and malachite green found in samples of fish medications. A broad range of products for treating ailments in decorative or pet fish are available. Many of these medications are banned or restricted for use in edible fish. If present in edible fish, the levels would be very low or undetectable by HPLC with UV based detection. These examples show a few medications and detail the rapid method development used to establish a rapid MS-compatible separation environment. Many fish medications advertise the use of "pharmaceutical quality" ingredients, and may imply pharmaceutical quality manufacturing and quality control procedures. When profiling these products one should expect to see very low levels of related impurities, consistent with the goals of pharmaceutical quality manufacturing

The high pressure capability of the system allows the use of methanol, and acetonitrile, to explore the selectivity of the two solvents. At 1 mL/min, using a simple 3 minute gradient and a 3.0 mm x 50 mm Poroshell 120 column, the analysis time is only less than 5 minutes including the late eluting phthalate artifact. The separation of the main components of a medicated powder with acetonitrile and methanol is shown in Figure 1, and the extraction of a medicated feed is shown in Figure 2.

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The speed, resolution and flexibility of the system are further demonstrated by a separation of a sulfa standard mix using solvent, gradient and temperature optimization with a 100 mm length Poroshell 120 column (see Figure 3).

After further optimization of the sulfa mix using methanol with the elevated temperature, all of the samples were run with the final method configuration, as shown in Figure 4.

Configuration

- Agilent 1290 Infinity Binary Pump with Integrated Vacuum Degasser (G4220A)
- Agilent 1290 Infinity Autosampler (G4226A)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1200 Diode Array Detector (G1315C)

Conclusion

Taking advantage of flexible solvent and column selection features, and high pressure capability, of the system allows one to use highly efficient columns to rapidly develop separations with remarkable resolution while conserving solvent over the use of 4.6 mm id columns.

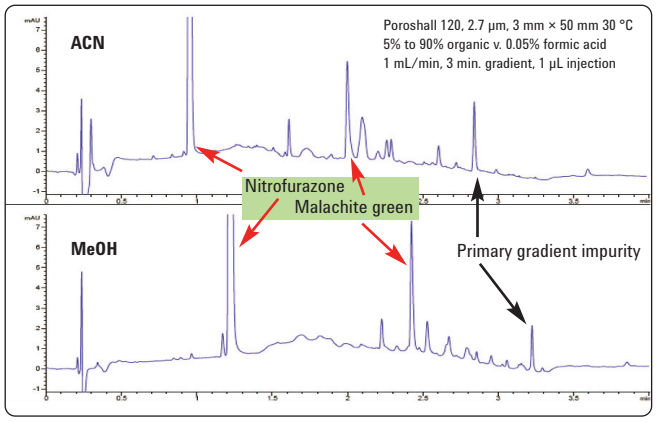


Figure 1
"Super Ick" medicated powder.

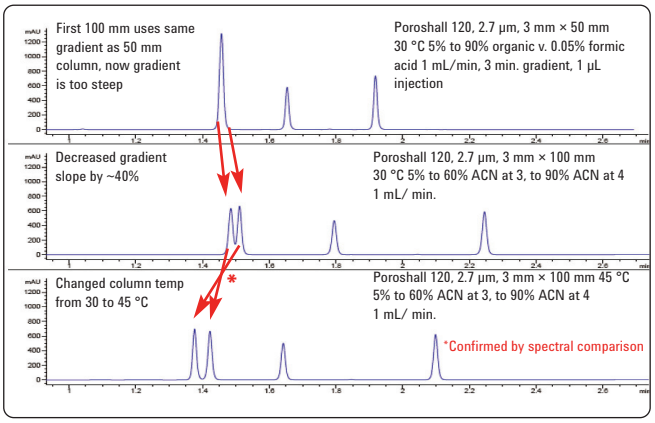


Figure 3
Sulfa standard mixture (Agilent p/n 59987-20033).

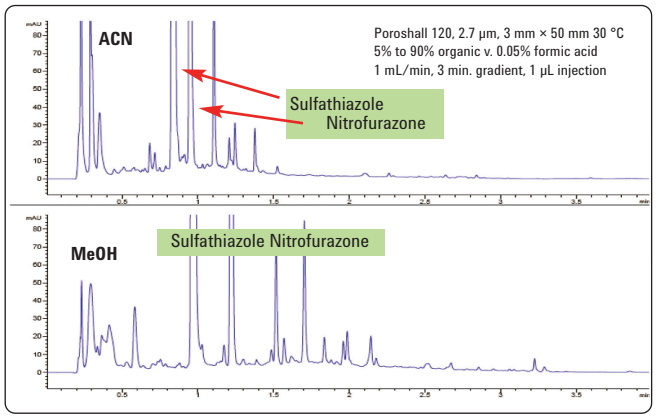


Figure 2
Separation of "Jungle" medicated fish food after methanol/water/formic acid extraction.

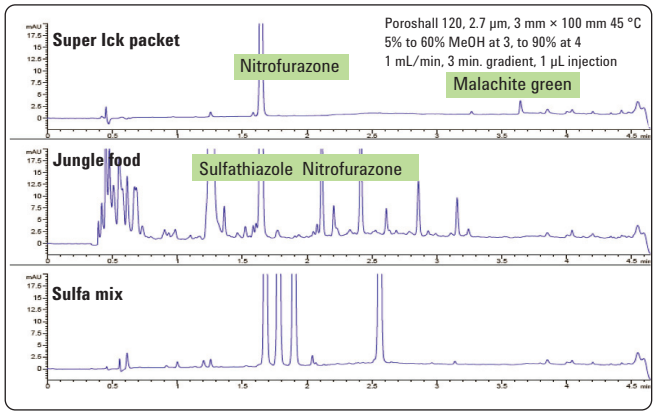


Figure 4
Final method configuration.

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Determination of Sulfonamide Antibiotics in Bovine Liver Using Agilent SampliQ QuEChERS EN Kits by LC/MS/MS

Application Note

Food

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Abstract

This paper presents an analytical method for the determination of nine sulfonamide antibiotic residues in bovine liver: sulfadizine, sulfathiazole, sulfamerazine, sulfamethizole, sulfamethazine, sulfamethoxypyridazine, sulfachloropyridazine, sulfamethoxazole, and sulfadimethoxin. The procedure involves a rapid and efficient pretreatment with SampliQ QuEChERS kits. The homogenized liver sample was initially extracted in a buffered aqueous/1% acetic acid acetonitrile system with an extraction and partitioning step after the addition of salts. Finally, the sample was cleaned up using dispersive solid-phase extraction (dispersive-SPE). The final extracts were analyzed by the sensitive and selective determination of all compounds in a single run using LC-ESI-MS-MS operating in positive ion multiple reaction monitoring (MRM) mode. Sulfapyridine was selected as the internal standard. The accuracy of the method, expressed as recovery, was between 53 and 93%. The precision, expressed as RSD, was between 2.1 and 16.8%. The established 5 ng/g limits of quantification (LOQ) were much lower than the respective Maximum Residue Limit (MRL) for sulfonamide in animal food products (20-100 ng/g).

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Introduction

Sulfonamides (SAs) are a very important class of antibacterial compounds widely used in veterinary practice for therapeutic, prophylactic, and growth-promoting purposes. Residues of SAs may remain in animal tissues if adequate withdrawal time is not observed or if the SAs have been improperly administered. The maximum residue limit (MRL) in the European Union countries and United States for SAs in animal muscle tissue is 100 ng/g, while in Japan it is 20 ng/g. [1]

The QuEChERS EN method is an important variation, which has been officially accepted by the European Committee for Standardization and is widely applied to pesticide analysis in foods of plant origin [2]. The original EN buffered method was designed mostly for multiresidue pesticide analysis in plant food products. In summary, the method uses acetonitrile extraction followed by the salting out of water from the sample using anhydrous magnesium sulfate ($MgSO_4$), NaCl, and buffering citrate salts to induce liquid-liquid partitioning. For cleanup, a dispersive solid-phase extraction (dispersive-SPE) is conducted using a combination of primary secondary amine (PSA) to remove fatty acids from other components and anhydrous $MgSO_4$ to reduce the remaining water in the extract. After mixing and centrifuging, the upper layer is ready for analysis. Fatty dispersive-SPE, which contains 25 mg C18EC per mL of ACN extract, is employed to remove more lipids from the matrix when using fruits and vegetables with fats and waxes.

Food matrices from animal origin contain a substantial amount of proteins and lipids. Therefore, they are very different from food matrices of plant origin such as fruits and vegetables. In this study, a SampliQ QuEChERS EN buffered extraction kit (p/n 5982-5650) and EN fatty dispersive-SPE 15 mL kit (p/n 5982-5165) were tested for the analysis of sulfonamide antibiotics in bovine liver. Because of the differences in food matrices and the chemical properties of the target analytes, modifications to the method were also investigated to achieve efficient extraction and cleanup.

Experimental

Reagents and Chemicals

All reagents and solvents were HPLC or analytical grade. Methanol (MeOH) was from Honeywell (Muskegon, MI, USA). Acetonitrile (ACN), dimethyl sulfoxide (DMSO), and glacial acetic acid (HAc) were from Sigma-Aldrich (St Louis, MO, USA). Ammonium acetate (NH_4OAc) was from Fisher Chemicals (Fair Lawn, NJ, USA). Formic acid (FA) was from Fluka (Sleinheim, Germany). The sulfonamides standards and the internal standard were from Sigma (St Louis, MO, USA).

Solutions and Standards

A 1 M ammonium acetate stock solution was made by dissolving 19.27 g NH_4OAc powder in 250 mL Milli-Q water. The solution was stored at 4 °C. A 5 mM ammonium acetate solution in water, pH 3, was made by adding 5 mL of 1 M ammonium acetate stock solution into 1 L of Milli-Q water. The pH was then adjusted to 3 by adding glacial acetic acid and monitoring with a pH meter. A 1:1 MeOH/ACN solution was made by combining 500 mL of MeOH and ACN, and mixing well. A 1% acetic acid in ACN solution was prepared by adding 5 mL of glacial acetic acid to 500 mL of ACN, and mixing well. A 1:1 ACN/H_2O solution with 0.1% FA was prepared by combining 50 mL of ACN and Milli-Q water, then adding 100 µL of formic acid. A 1:9 MeOH/H_2O solution with 0.1% FA was prepared by combining 10 mL of MeOH and 90 mL of Milli-Q water, then adding 100 µL of formic acid.

Standard and internal standard (IS) stock solutions (1.0 mg/mL for all) were all made in DMSO and stored at 4 °C. Six of the sulfonamides are light-sensitive, so the stock

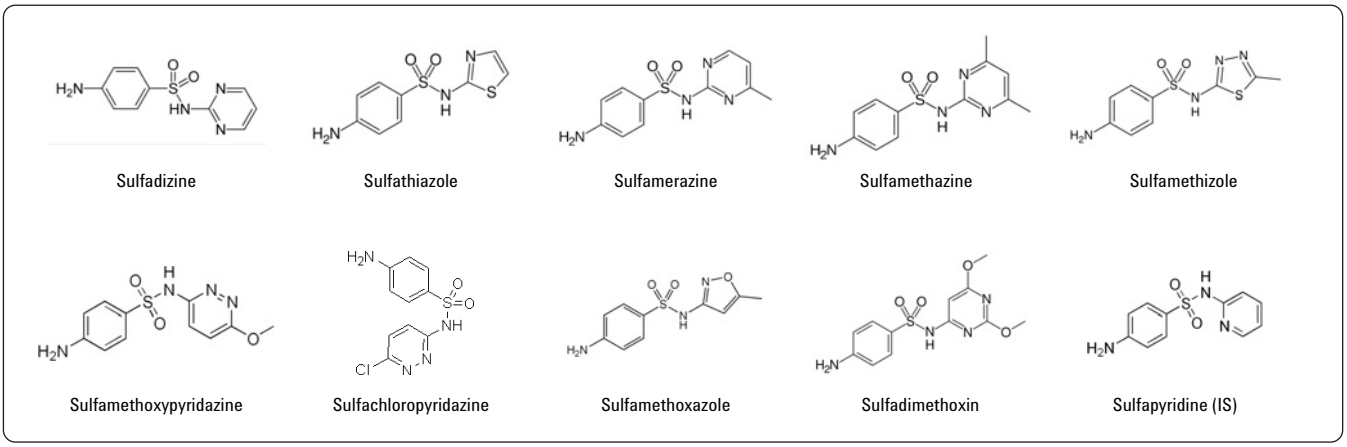


Figure 1. Chemical structures of the quinolone antibiotics investigated in this study.

solutions were kept in amber vials and wrapped in aluminum foil. Three combined QC spiking solutions of 0.2, 4, and 16 µg/mL were made fresh daily in 1:1 ACN/H_2O containing 0.1% FA. A 20 µg/mL standard spiking solution in 1:1 ACN/H_2O containing 0.1% FA was made for the preparation of calibration curves in the matrix blank extract. Due to light sensitivity of certain sulfonamides, all of the combined solutions were kept in amber vials and wrapped in aluminum foil. A 20 µg/mL IS spiking solution of Sulfapyridine was made in 1:1 ACN/H_2O containing 0.1% FA.

Equipment and Material

- Agilent 1200 Series HPLC with Diode Array Detector (Agilent Technologies Inc., CA, USA)
- Agilent 6410 Triple Quadrupole LC/MS system with Electrospray Ionization (Agilent Technologies Inc., CA, USA)
- Agilent SampliQ QuEChERS EN Extraction kit p/n 5982-5650 (Agilent Technologies Inc., DE, USA)
- Agilent SampliQ QuEChERS EN fatty dispersive-SPE kit for 15 mL p/n 5982-5156 (Agilent Technologies Inc., DE, USA)
- CentraCL3R Centrifuge (Thermo IEC, MA, USA)
- Eppendorf microcentrifuge (Brinkmann Instruments, Westbury, NY, USA)
- 2010 Geno Grinder (Spex SamplePrep LLC, Metuchen, NJ, USA)
- Multitube Vortexer (Henry Troemner LLC, Thorofare, NJ, USA)

Instrument conditions

HPLC conditions

Column	Agilent ZORBAX Solvent Saver HT Eclipse Plus C18 50 × 3.0 mm, 1.8 µm (p/n: 959941-302)		
Flow rate	0.3 mL/min		
Column Temperature	30 °C		
Injection volume	10 µL		
Mobile Phase	A: 5 mM ammonium acetate, pH 3.0 in H2O		
	B: 1:1 MeOH/ACN		
Needle wash	1:1:1:1 ACN/ MeOH/ IPA/ H2O with 0.2% FA.		
Gradient	Time	% B	Flow rate (mL/min)
	0	15	0.3
	0.2	15	0.3
	6.0	60	0.3
	6.01	100	0.3
	7.0	STOP	
Post run	3.5 min		
Total cycle time	~11 min.		

MS conditions

Polarity	positive
Gas Temperature	325 °C
Gas Flow	8 L/min
Nebulizer	50 Psi
Capillary	4000 V

Other conditions relating to the analytes are listed in Table 1.

Table 1. Instrument Acquisition Data for the Analysis of 9 Sulfonamide Antibiotics by LC/MS/MS.

Analyte	MRM channels (m/z)	Fragmentor (V)	CE (V)	RT (min)
Sulfadizine	1) 251.1 → 108.0	100	25	2.1
	2) 251.1 → 156.0		13	
Sulfathiazole	1) 256.0 → 156.0	94	13	2.3
	2) 256.0 → 92.1		29	
Sulfamerazine	1) 265.1 → 92.1	125	29	2.9
	2) 265.1 → 108.1		25	
Sulfamethizole	1) 271.0 → 156.0	112	9	3.7
	2) 271.0 → 92.1		29	
Sulfamethazine	1) 279.1 → 124.0	116	21	3.8
	2) 279.1 → 92.1		33	
Sulfamethoxypyridazine	1) 281.1 → 156.0	128	13	3.9
	2) 281.1 → 92.1		29	
Sulfachloropyridazine	1) 285.0 → 156.0	106	9	4.5
	2) 285.0 → 92.1		29	
Sulfamethoxazole	1) 254.1 → 92.1	113	25	4.8
	2) 254.1 → 108.0		21	
Sulfadimethoxin	1) 311.1 → 156.0	141	17	6.0
	2) 311.1 → 92.1		37	
Sulfapyridine (IS)	250.1 → 92.1	113	29	2.7

1) Quantifier transition channel 2) Qualifier transition channel

Sample preparation

Sample preparation includes sample homogenization, extraction and partitioning, and dispersive-SPE cleanup. Since the main focus of existing QuEChERS methodology has been the extraction of pesticides from plant and vegetable matrices, certain modifications were adapted in order to optimize results for the determination of sulfonamides in bovine liver. These modifications will be discussed in detail later.

Bovine liver was purchased from a local grocery store. It was then washed and chopped into small pieces. The chopped liver sample was homogenized thoroughly with a food grinder, then stored at -20 °C. Two gram (±0.05 g) amounts of homogeneous sample were placed into 50 mL centrifuge tubes. Sample tubes were centrifuged 30 s to move any sample sticking to the inside wall of tube to the bottom. Samples were then fortified with appropriate QC spiking solutions (50 µL) when necessary. Then 50 µL of IS spiking solution (20 µg/mL of sulfapyridine) were added. After vortexing sample for 30 s, 8 mL of Milli-Q water were added. Tubes were then vortexed another 10 s for mixing. Ten milliliters of 1% AA in ACN were added to each tube. Tubes were capped and shaken by a 2010 Geno Grinder for 30 s. An Agilent SampliQ QuEChERS EN extraction salt packet (p/n 5982-5650) was added to each tube. Sample tubes were capped tightly and shaken vigorously for 1 min by the 2010 Geno Grinder at 4 °C.

A 6 mL aliquot of the upper ACN layer was transferred into an Agilent SampliQ EN QuEChERS fatty dispersive-SPE 15 mL tube (p/n 5982-5156). This 15 mL dispersive-SPE tube contained 150 mg of PSA, 150 mg of C18EC, and 900 mg of anhydrous $MgSO_4$. The tubes were tightly capped and vortexed for 2 min. The 15 mL tubes were centrifuged at 4000 rpm for 5 min. A 4 mL amount of extract was then transferred into another tube and dried by N_2 flow at 40 °C. Samples were reconstituted into 800 µL of 1:9 MeOH/H_2O solution with 0.1% FA. After vortexing and sonicating for 10 min, the sample was filtered by a 0.22 µm Cellulose Acetate Spin Filter (p/n 5185-5990). The clear filtered sample was transferred into an autosampler vial. The samples were capped and vortexed thoroughly, in preparation for LC/MS/MS analysis.

See Figure 2 for the flow chart of the extraction procedure for a bovine liver sample.

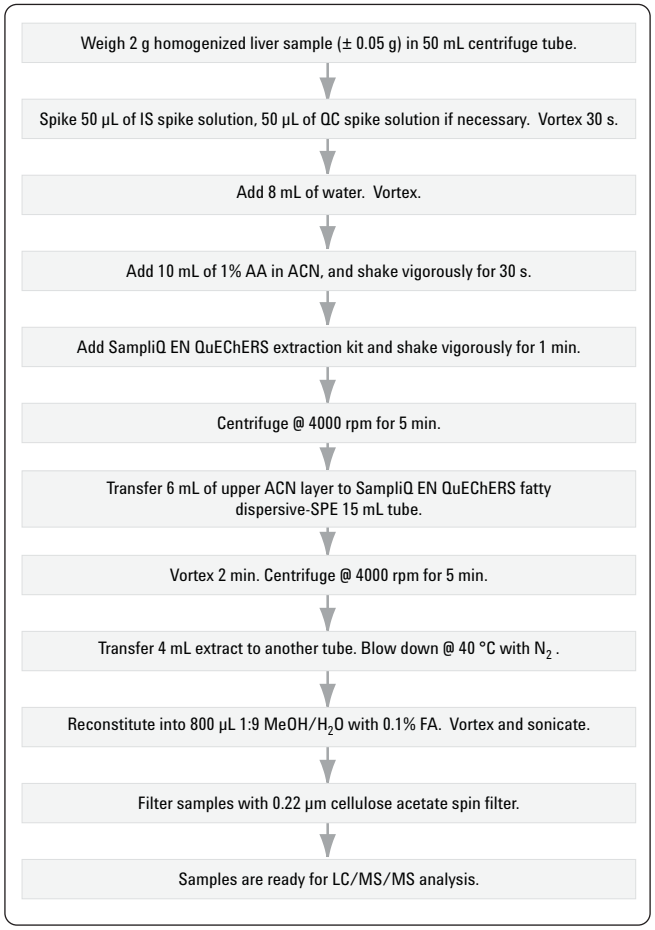


Figure 2. Flow chart of QuEChERS procedure for the determination of sulfonamides in bovine liver.

Results and Discussion

Method Optimization in the Liver Matrix

As mentioned previously, modifications of the QuEChERS EN method were investigated relative to extraction efficiency. An EN buffered extraction system provides a solution with a pH of 5.0 – 5.5, which illicits neutral sulfonamide analytes (pKa ~ 6-7). PSA sorbent used in dispersive-SPE can strongly interact with acid compounds and remove various co-extractive interferences, such as polar organic acids, sugars, and fatty acids. However, it may also interact with target compounds and cause the loss of analytes. Therefore, the QuEChERS fatty EN dispersive-SPE kit with PSA was compared to the dispersive-SPE kit without PSA. A previous study [4] showed that the addition of acid to acetonitrile can inhibit the absorption of PSA, weakening the attraction of PSA to the compounds of interest. Therefore, a 1% AA in ACN was evaluated in addition to standard ACN in the first partitioning step.

To evaluate the original EN and modified method, a 50 ng/g of fortified liver sample was extracted with the following procedures:

1. EN buffered extraction kit with ACN and dispersive-SPE kit with PSA (25 mg PSA and C18EC per mL)
2. EN buffered extraction kit with ACN and dispersive-SPE kit without PSA (25 mg C18EC per mL)
3. EN buffered extraction kit with 1% AA in ACN and dispersive-SPE kit with PSA (25 mg PSA and C18EC per mL).

The corresponding matrix blanks were extracted at the same time, then post-spiked with the same amount of sulfonamide standards. Neat solution post-spiked at the same concentration was also compared to the matrix post-spiked samples.

The results are shown in Table 2. The results of method 1 and method 2 show that PSA does contribute to the matrix cleanup during the dispersive-SPE step. The matrix effect values for all of the analytes in method 1 were lower than those in method 2 indicating that the sample processed by method 1 was cleaner than the sample processed by method 2. This was also demonstrated by the color of the ACN extract. After the extraction step, the ACN extract was a brownish-red color. Using PSA in the dispersive step produced an ACN extract that was light yellow in color. Removing PSA from the dispersive step maintained the previously observed brownish-red ACN extract. Unfortunately, the presence of PSA in the dispersive step also caused the loss of certain analytes and produced very low recovery for sulfachloropyridazine (30%) and sulfamethizole (15%). Method 3 produced substantially better recoveries for all the analytes relative to methods 1 or 2. The matrix effect values show that the sample processed by method 3 is as clean as the sample processed by method 2, but not as clean as the sample processed by method 1. The addition of acid in the ACN partitioning step impedes the performance of PSA in the dispersive step preventing the loss of analytes. It also decreases the interaction of PSA with other matrix interferences, producing a greater matrix effect. This is also shown by the color of the ACN extract. Although PSA was used in the dispersive step, the presence of acidified ACN extract elicited a light brown-red color, rather than the light yellow extract in method 1.

Table 2. Method Optimization Results for the Analysis of Sulfonamides in Bovine Liver

	Method 1		Method 2		Method 3	
Analytes	**Recovery**	**Matrix effect**	**Recovery**	**Matrix effect**	**Recovery**	**Matrix effect**
Sulfadiazine	91.9	-33.0	85.2	-65.2	85.6	-57.9
Sulfathiazole	39.9	-35.9	42.0	-57.3	87.7	-65.7
Sulfamerazine	77.0	-19.3	43.9	-23.9	89.0	-51.7
Sulfamethizole	15.3	-33.6	46.5	-46.9	63.2	-49.8
Sulfamethazine	85.7	-23.1	51.4	-31.6	87.3	-42.0
Sulfamethoxypyridazine	76.6	-32.6	49.0	-31.7	86.1	-49.1
Sulfachloropyridazine	29.6	-38.5	51.1	-42.3	84.8	-50.6
Sulfamethoxazole	60.0	-40.9	53.4	-46.9	87.5	-54.5
Sulfadimethoxin	67.4	-35.3	56.9	-43.0	89.6	-51.9

Method 1 — EN buffered extraction kit + ACN + Fatty dispersive-SPE kit (25 mg PSA + 25 mg C18EC + 150 mg $MgSO_4$ per mL)

Method 2 — EN buffered extraction kit + ACN + Dispersive-SPE kit without PSA (25 mg C18EC + 150 mg $MgSO_4$ per mL)

Method 3 — EN buffered extraction kit + 1% AA ACN + Fatty dispersive-SPE kit (25 mg PSA + 25 mg C18EC + 150 mg $MgSO_4$ per mL)

$$\% \text{ Recovery} = \frac{Response_{\text{extracted sample}}}{Response_{\text{post-extracted spiked sample}}} \times 100$$

$$\% \text{ Matrix Effects} = \left(\frac{Response_{\text{post-extracted spiked sample}}}{Response_{\text{non-extracted neat sample}}} - 1 \right) \times 100$$

Method 3 was selected for the final study. In this study, liver sample was extracted by the EN buffered extraction kit (p/n 5982-5650) with 1% AA in ACN. After centrifuging, the ACN extract was further cleaned by EN fatty dispersive-SPE 15mL tube (p/n 5982-5156). Figure 3 shows the MRM chromatograms of the liver control blank and 100 ng/g fortified liver extract. The liver control blank chromatogram indicated that the target analytes were free from any interference.

Linearity and limit of quantification (LOQ)

The linear calibration range for all the sulfonamide antibiotics was 5 - 400 ng/g. Matrix blanks were prepared for evaluation. Calibration curves, made from spiked matrix blanks, were made at levels of 5, 10, 50, 100, 200, 300 and 400 ng/g for each analyte. The sulfapyridine was used as an internal standard at 200 ng/g. The calibration curves were generated by plotting the relative responses of analytes (peak area of analyte / peak area of IS) versus the relative concentration of analytes (concentration of analyte/concentration of IS). The 5 ng/g limits of quantification (5 ppb) of the sulfonamides is far below the MRLs for residues of these antibiotics in animal food products (20 - 100 ng/g). Table 3 shows the regression equation and correlation coefficient (R^2). Linear regression fit was used with $1/x^2$ weight. Results indicated excellent linearity for all of analytes calibration curves over a broad quantification range.

Table 3. Linearity of Sulfonamide Antibiotics in Bovine Liver

Analytes	Regression Equation	R^2
Sulfadiazine	Y = 1.6585X + 0.0002	0.9963
Sulfathiazole	Y = 1.3899X + 0.0002	0.9942
Sulfamerazine	Y = 3.5019X – 0.0001	0.9962
Sulfamethizole	Y = 2.3064X + 0.0001	0.9963
Sulfamethazine	Y = 4.3780X – 0.0004	0.9977
Sulfamethoxypyridazine	Y = 4.4044X + 0.0003	0.9941
Sulfachloropyridazine	Y = 1.5869X – 0.0005	0.9971
Sulfamethoxazole	Y = 1.9047X – 0.0001	0.9936
Sulfadimethoxin	Y = 4.5106X + 0.0020	0.9922

Recovery and Reproducibility

The recovery and reproducibility were evaluated by fortifying sulfonamides standards in homogenized liver sample at levels of 5, 100, and 400 ng/g. These QC samples were quantified against the matrix spiked calibration curve. The analysis was performed in replicates of six at each level. The recovery and reproducibility (shown as RSD) data are shown in Table 4. The results show that all of the sulfonamides were somewhat low but still at acceptable recoveries (average of 77.8%) and good precision (average of 7.2% RSD). Samples were concentrated during the procedure to optimize sensitivity, which also caused additional matrix effects that possibly contributed to a higher RSD of target compounds at low levels.

Table 4. Recovery and Repeatability of Sulfonamides in Fortified Liver Homogenate

	5 ng/g fortified QC		100 ng/g fortified QC		400 ng/g fortified QC	
Analytes	Recovery	RSD (n=6)	Recovery	RSD (n=6)	Recovery	RSD (n=6)
Sulfadiazine	73.9	15.6	90.0	13.7	81.9	5.3
Sulfathiazole	62.9	16.8	75.3	8.4	67.9	5.8
Sulfamerazine	77.6	11.5	92.8	6.6	82.0	4.2
Sulfamethizole	62.8	4.7	60.7	6.5	53.0	2.1
Sulfamethazine	87.4	6.9	90.0	10.7	83.4	3.4
Sulfamethoxy-pyridazine	81.8	9.4	84.8	8.1	76.4	2.9
Sulfachloro-pyridazine	84.2	10.0	78.6	6.3	73.8	3.6
Sulfamethoxazole	85.9	7.6	82.3	5.9	78.1	3.3
Sulfadimethoxin	77.8	8.4	80.9	4.9	75.6	3.3

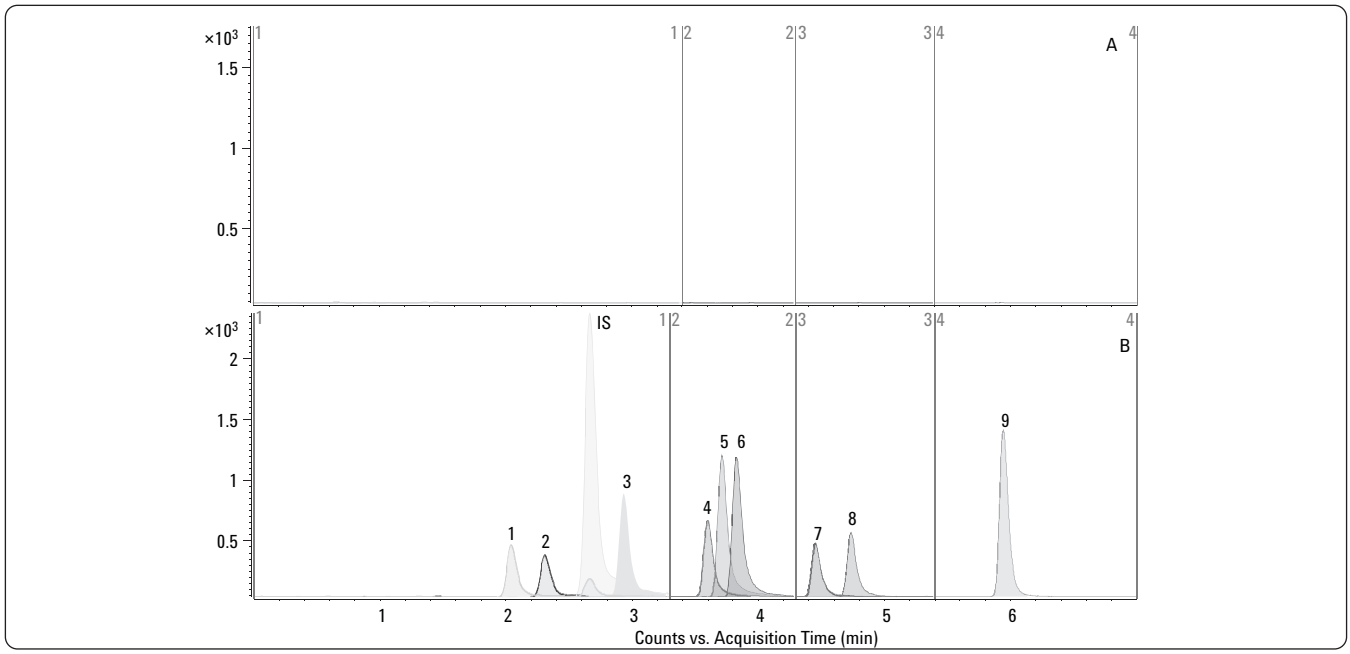


Figure 3. LC/MS/MS Chromatograms of A) liver blank extract, and B) 100 ng/g fortified liver extract. Peaks identification: 1. sulfadizine, 2. sulfathiazole, 3. sulfamerazine, 4. sulfamethizole, 5. sulfamethazine, 6. sulfamethoxypyridazine, 7. sulfachloropyridazine, 8. sulfamethoxazole, 9. sulfadimethoxin, IS (internal standard), sulfapyridine.

Conclusions

The Agilent SampliQ EN Buffered Extraction kit and SampliQ EN fatty dispersive-SPE kit provide a simple, fast, and effective method for the purification of sulfonamide antibiotics in bovine liver. When compared to other sample preparation methods, such as LLE and SPE, QuEChERS methodology is easy, fast, low cost and does not require automation. In addition, it is labor saving and a "greener" technology. The recovery and reproducibility, based on matrix-spiked standards, were acceptable for multiresidue sulfonamide determination in bovine liver. The impurities and matrix effects from liver were minimal and did not interfere with the quantification of any target compound. The LOQs of the quinolones were much lower than their regulated MRLs in animal food products (20-100 ng/g). This modified QuEChERS procedure is a very promising methodology for the quantitative analysis of sulfonamides in food products of animal origin. This application demonstrates great potential of SampliQ QuEChERS extraction and dispersive-SPE kits, and extend far beyond plant matrices to bio-matrices, such as animal food products and bio-fluid.

References:

[1] Sun H., Ai L., Wang F., Quantitative Analysis of Sulfonamide residues in Natural Animal Casings by HPLC, Chromatographia 2007, 66, Sept, (no. 5/6), 333-337.

[2] European Council Regulation 2377/90/EC of 26 June 1990 laying down a community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin, OJ L, 1990, p. 224.

[3] European Committee for Standardization/Technical Committee CEN/TC 275 (2007), Foods of plant origin: Determination of pesticide residues using GC-MS and/or LC-MS/MS following acetonitrile extraction/partitioning and cleanup by dispersive SPE-QuEChERS method. European Committee for Standardization, Brussels.

[4] Zhao L., Stevens J., Determination of quinolone antibiotics in bovine liver using Agilent SampliQ QuEChERS kits by LC/MS/MS, Agilent application note, 5990-5085EN.

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Determination of Quinolone Antibiotics in Bovine Liver Using Agilent SampliQ QuEChERS Kits by LC/MS/MS

Application Note

Food

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Abstract

This paper presents an analytical method which allows the determination of 11 quinolone antibiotic residue in bovine liver: pipemidic acid, ofloxacin, ciprofloxacin, danofloxacin, lomefloxacin, enrofloxacin, sarafloxacin, cinoxacin, oxolinic acid, nalidixic acid, and flumequine.

The procedure involves a rapid and efficient pretreatment by SampliQ QuEChERS kits. The homogenized liver sample was initially extracted in a buffered aqueous, 5% formic acid acetonitrile system. An extraction and partitioning step was performed after the addition of salts. Cleanup was done using dispersive solid phase extraction (dispersive-SPE). The final extracts allowed determination of all compounds in a single run using LC-ESI-MS-MS operating in positive ion multiple reaction monitoring (MRM) mode. Norfloxacin was selected as the internal standard. The accuracy of the method, expressed as recovery, was between 62 and 113%. The precision, expressed as RSD, was between 2.2 and 13.4%. The established limit of quantification (LOQ) was 5 ng/g and is significantly lower than the respective Maximum Residue Limit (MRL) for quinolones in food producing animals.

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Introduction

Quinolones are a family of synthetic broad-spectrum antibiotics. They prevent bacterial DNA from unwinding and duplicating. There is evidence that quinolones in food animals lead to the emergence of quinolone-resistant bacteria in animals. The resistant organisms are transmitted to humans via direct contact with the animal or through the consumption of contaminated food and water. Quinolone-resistant campylobacter is an example of animal-to-human transmission and has been observed in many European countries since the early 1990s [1]. Therefore, public health agencies in many countries such as the EU commission [2], the USA FDA administration [3], and the Chinese Ministry of Agriculture [4] have established maximum residue limits (MRLs) of veterinary drugs in food-producing animals. Given the different drugs in different food origins and in different countries, the MRLs of quinolones in food products of animal origin are usually at the level of 100 µg/kg or higher.

As animal food origins, such as muscle, liver, and eggs, are complicated matrices, it is critical to use an efficient sample pretreatment method for analyte extraction and concentration, and matrix cleanup. The established sample pretreatment methods used for determination of quinolones include traditional solvent extraction, solid phase extraction (SPE), or a combination of both. Although they have been widely used, these traditional methods have inherent limitations. Traditional methods are labor intensive, time consuming, require a large amount of solvent and waste disposal. In 2003, the QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method for pesticide residue analysis in fruit and vegetable matrices was introduced. [5] There are two validated QuEChERS methodologies: the AOAC and EN versions. Both are widely accepted and effective for the multiresidue analysis of pesticides in fruit, vegetables and other plant food matrices. The QuEChERS method contains significant advantages over traditional methods, including high recoveries for a wide range of pesticides, high sample throughput, minimal labor, time savings, limited solvent usage, and low waste. In addition, the method is manually accommodating which has made QuEChERS a very popular methodology for the analysis of pesticide residues in fruits and vegetables in recent years.

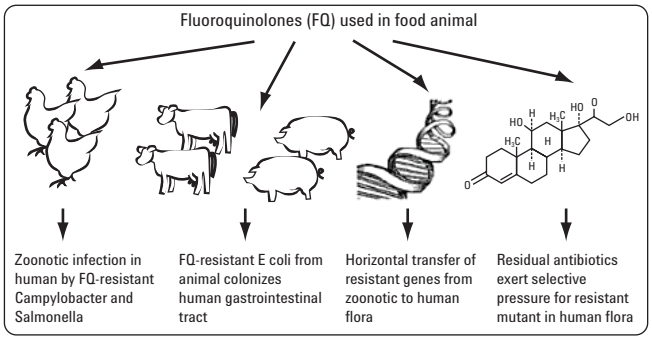


Figure 1. Animal to human transmission of resistant bacteria [1].

Although the current QuEChERS methodology has been designed for removing matrix interferences in food products of plant origin, such as polar organic acids, sugars, and lipids, it also has potential for other food matrices such as meat. Based upon the chemical properties of the compounds of interest and food matrices, some modifications of the original method might be necessary to obtain accurate and precise results. The purpose of this work is to extend the QuEChERS methodology to veterinary drug residues in food-producing animals. Agilent SampliQ QuEChERS EN buffered extraction kits (p/n 5982-5650) and dispersive-SPE 2 mL kits for drug residues in meat (p/n 5982-4921) were used for the analysis of 11 quinolone antibiotics in bovine liver: pipemidic acid, ofloxacin, ciprofloxacin, danofloxacin, lomefloxacin, enrofloxacin, sarafloxacin, cinoxacin, oxolinic acid, nalidixic acid and flumequine (Figure 2). The method was validated in terms of recovery and reproducibility.

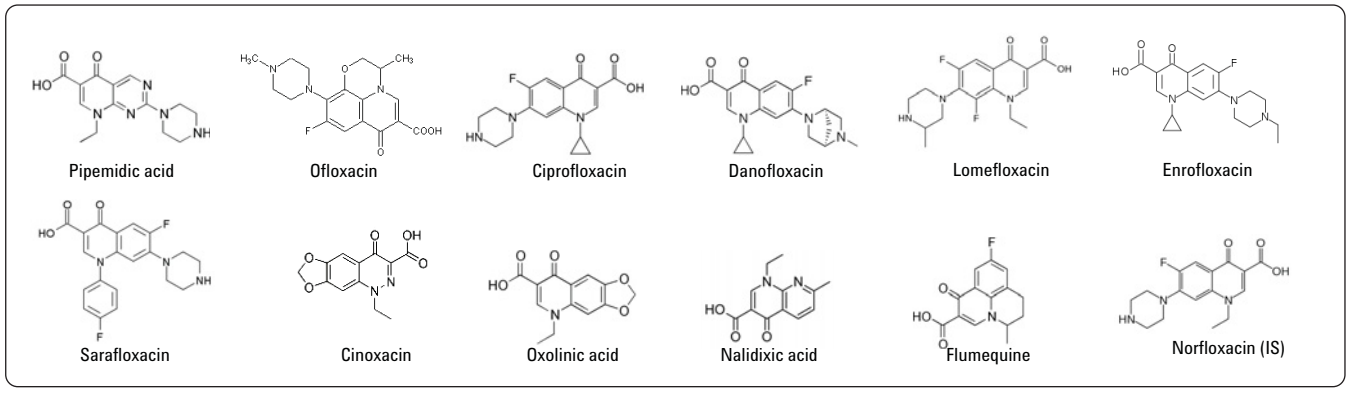


Figure 2. Chemical structures of the quinolone antibiotics investigated in this study.

Experimental

Reagents and Chemicals

All reagents and solvents were HPLC or analytical grade. Methanol (MeOH) was from Honeywell (Muskegon, MI, USA). Acetonitrile (ACN), dimethyl sulfoxide (DMSO) and glacial acetic acid (HAc) were from Sigma-Aldrich (St Louis, MO, USA). Ammonium acetate (NH_4OAc) was from Fisher Chemicals (Fair Lawn, NJ, USA). Formic acid (FA) was from Fluka (Sleinheim, Germany). The quinolone standards and internal standard were purchased from Sigma-Aldrich (St Louis, MO, USA). Potassium phosphate, monobasic (KH_2PO_4), was from J.T. Baker (Phillipsburg, NJ, USA).

Solutions and Standards

1M ammonium acetate stock solution was made by dissolving 19.27 g NH_4OAc powder in 250 mL Milli-Q water. The solution was stored at 4 °C. A 5 mM ammonium acetate in water solution with pH 3 was made by adding 5 mL of 1M ammonium acetate stock solution into 1 L of Milli-Q water, then adjusting the pH to 3 with glacial acetic acid. A 1:1 MeOH/ACN solution was made by combining 500 mL of MeOH and ACN, then mixing well. A 5% formic acid solution in ACN was made fresh daily by adding 10 mL of formic acid to 190 mL of ACN, then mixing well. A 30 mM KH_2PO_4 buffer, pH 7.0, was made by dissolving 4.08 g KH_2PO_4 powder into 1 L Milli-Q water and adjusting the pH to 7.0 with 1 M KOH solution. A 1:1 ACN/H_2O with 0.1% FA was prepared by combining 50 mL of ACN and Milli-Q water, then adding 100 µL of formic acid. A 1:9 MeOH/H_2O solution with 0.1% FA was prepared by combining 10 mL of MeOH and 90 mL of Milli-Q water, then adding 100 µL of formic acid.

Standard and internal standard (IS) stock solutions (1.0 mg/mL for all, except 0.25 mg/mL for ciprofloxacin) were made in DMSO and stored at 4 °C. Due to the solubility of quinolones, it is essential to sonicate stock solutions to ensure they completely dissolve. Three combined QC spiking solutions of 0.2, 8 and 16 µg/mL were made fresh daily in 1:1 ACN/H_2O containing 0.1% FA. A 10 µg/mL standard spiking solution in 1:1 ACN/H_2O containing 0.1% FA was made for the preparation of calibration curves in the matrix blank extract. A 20 µg/mL IS spiking solution of norfloxacin was made in 1:1 ACN/H_2O containing 0.1% FA.

Equipment and Material

- Agilent 1200 Series HPLC with Diode Array Detector (Agilent Technologies Inc., CA, USA).
- Agilent 6410 Series triple quadrupole LC/MS system with Electrospray Ionization (Agilent Technologies Inc., CA, USA).
- Agilent SampliQ QuEChERS EN Extraction kits, p/n 5982-5650, and SampliQ QuEChERS dispersive-SPE kits for Drug Residues in Meat, 2 mL, p/n 5982-4921 (Agilent Technologies Inc., DE, USA).
- CentraCL3R Centrifuge (Thermo IEC, MA, USA)
- Eppendorf microcentrifuge (Brinkmann Instruments, Westbury, NY, USA)
- 2010 Geno Grinder (Spex SamplePrep LLC, Metuchen, NJ, USA)
- Multi-tube Vortexer (Henry Troemner LLC, Thorofare, NJ, USA)

Instrument conditions

HPLC conditions

Column	Agilent ZORBAX Solvent Saver Eclipse Plus Phenyl-Hexyl 150 × 3.0 mm, 3.5 µm (p/n 959963-312)		
Flow rate	0.3 mL/min		
Column Temperature	30 °C		
Injection volume	10 µL		
Mobile Phase	A: 5 mM ammonium acetate, pH 3.0 in H_2O B: 1:1 MeOH/ACN		
Needle wash	1:1:1:1 ACN/ MeOH/ IPA/ H_2O with 0.2% FA.		
Gradient	Time	% B	Flow rate (mL/min)
	0	15	0.3
	0.2	15	0.3
	8.0	75	0.3
	9.0	100	0.3
	11.5	STOP	
Post run	4 min		
Total cycle time	~16 min.		

MS conditions

Polarity	positive
Gas Temp.	325 °C
Gas Flow	8 L/min
Nebulizer	50 Psi
Capillary	4000 V
Solvent cut	5 min

Other conditions relating to the analytes are listed in Table 1.

Table 1. Instrument Acquisition Data for the Analysis of 11 Quinolone Antibiotics by LC/MS/MS

Analyte	MRM channels (m/z)	Fragmentor (V)	CE (V)	RT (min)
Pipemidic acid	1) 304.1 → 286.1	128	17	5.9
	2) 304.1 → 215.1		37	
Ofloxacin	1) 362.2 → 318.1	150	17	6.7
	2) 362.2 → 344.1		21	
Ciprofloxacin	1) 332.1 → 314.1	131	21	6.8
	2) 332.1 → 231.0		41	
Danofloxacin	1) 358.2 → 340.2	159	25	6.9
	2) 358.2 → 82.1		49	
Lomefloxacin	1) 352.2 → 265.2	144	21	7.0
	2) 352.2 → 334.1		21	
Enrofloxacin	1) 360.2 → 342.2	159	21	7.3
	2) 360.2 → 316.2		17	
Sarafloxacin	1) 386.1 → 368.1	144	21	7.9
	2) 386.1 → 348.2		33	
Cinoxacin	1) 263.1 → 217.1	103	21	8.8
	2) 263.1 → 189.0		29	
Oxolinic acid	1) 262.1 → 216.0	106	29	9.2
	2) 262.1 → 160.0		41	
Nalidixic acid	1) 233.1 → 104.1	94	45	10.3
	2) 233.1 → 159.1		33	
Flumequine	1) 262.1 → 202.0	106	33	10.8
	2) 262.1 → 126.0		50	
Norfloxacin (IS)	320.1 → 302.1	134	17	6.6

1) Quantifier transition channel 2) Qualifier transition channel

Sample preparation

The sample preparation procedure includes sample homogenization, extraction/partitioning, and dispersive-SPE cleanup. As mentioned previously the QuEChERS methods were designed for pesticides analysis in fruit and vegetable matrices; therefore modifications were necessary to optimize the results for the determination of quinolones in bovine liver.

Bovine liver was purchased from a local grocery store. It was washed and chopped into small pieces. The chopped liver was homogenized thoroughly with a food grinder and stored at -20 °C. Two-gram (±0.05g) samples of homogenized liver were placed into 50 mL centrifuge tubes. The tubes were centrifuged for 30 s to move the sample from the inside tube wall to the bottom of the tube. Samples were then fortified with appropriate QC spiking solutions (50 µL) when necessary, then 50 µL of IS spiking solution (20 µg/mL of norfloxacin). After vortexing the sample for 30 s, 8 mL of 30 mM KH_2PO_4 buffer, pH 7.0, were added. Tubes were then vortexed for 10 s to mix. A 10 mL volume of 5% FA in ACN was added to each tube. Tubes were capped and shaken by a 2010 Geno Grinder for 30 s. An Agilent SampliQ QuEChERS EN extraction salt packet (p/n 5982-5650) was added to each tube. Sample tubes were capped tightly and shaken vigorously for 1 min by a 2010 Geno Grinder. Tubes were centrifuged at 4,000 rpm for 5 min at 4 °C.

A 1 mL aliquot of the upper ACN layer was transferred into an Agilent SampliQ QuEChERS dispersive-SPE 2 mL tube for Drug Residues in Meat (p/n 5982-4921). This 2 mL dispersive-SPE tube contained 25 mg of C18 and 150 mg of anhydrous $MgSO_4$. The tubes were tightly capped and vortexed for 1 min. The 2 mL tubes were centrifuged with a microcentrifuge at 13,000 rpm for 3 min. An 800 µL volume of extract was transferred into another tube and dried by N_2 flow at 40 °C. Samples were reconstituted into 800 µL of 1:9 MeOH/H_2O with 0.1% FA. After vortexing and sonicating for 10 min, the sample was filtered by a 0.22 µm Cellulose Acetate Spin Filter (p/n 5185-5990). The clear filtered sample was transferred into an autosampler vial. The samples were capped and vortexed thoroughly in preparation for LC/MS/MS analysis. Figure 2 shows the flow chart of the entire extraction procedure for bovine liver sample.

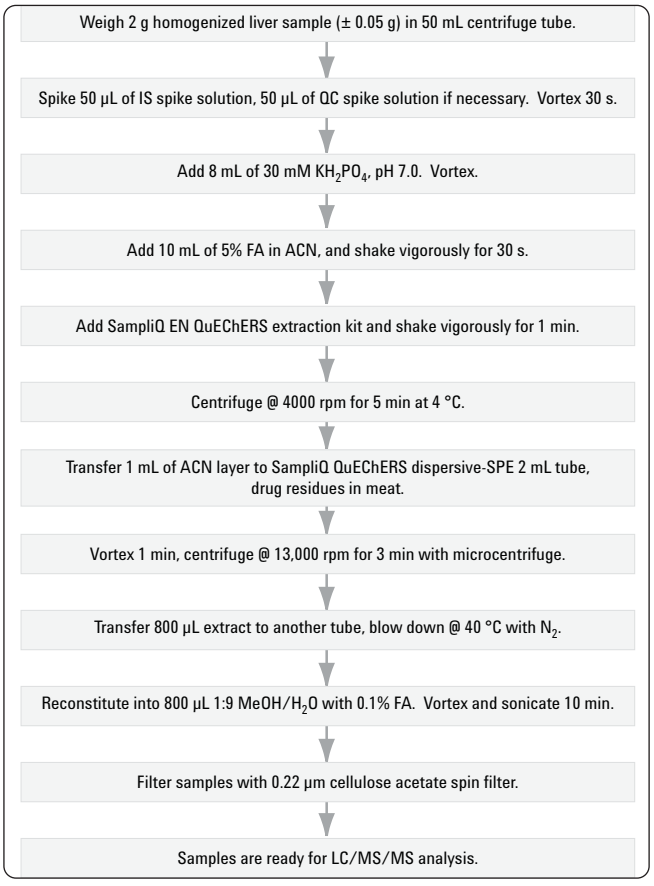


Figure 3. Flow chart of QuEChERS procedure for the determination of quinolones in bovine liver.

Results and Discussion

Feasibility Test

Quinolones are a group of relatively new antibacterials synthesized from 3-quinolone carboxylic acid. As shown in Figure 2, they all contain the carboxylic group, and are weakly acidic (pKa 4-6). Since this is the first time for quinolones determination by the QuEChERS method, the feasibility test was done by extracting 50 ng/mL of neat quinolone solution (prepared in water) with different SampliQ QuEChERS kits, including the SampliQ AOAC extraction kit, SampliQ EN extraction kit, and SampliQ Original extraction kit. In addition, bovine liver is a very different matrix than fruit and vegetables. Therefore, the cleanup was followed by the corresponding fatty dispersive-SPE kit (AOAC and EN fatty dispersive-SPE kit) because these fatty dispersive-SPE kits contain C18 which is critical for removing lipids from liver matrix.

However, the test results were initially very disappointing. All of the analytes had extremely low or nonexistent recoveries. The ACN extracts were tested at two points in the procedure to investigate where the analytes were being lost. The first test was made after the extraction step. The second test was made after both the extraction and the dispersive-SPE steps. Figure 3 shows the chromatogram comparison for the neat ACN extracts after the extraction step using different extraction kits. The ACN extracts using the EN extraction kit (p/n 5982-5650) showed much higher responses than those using the AOAC extraction kit (p/n 5982-5755) and the original extraction kit (p/n 5982-5550). The buffer system in the extraction/partitioning step provided by the addition of salts plays a key role in the extraction efficiency. The pH when the acidic analytes exist in their neutral forms facilitates the extraction. Both the EN and AOAC extraction kits provide a buffer system of approximately pH 5.0 [6, 7], which is the point where most quinolones are neutral. Therefore, these extraction kits generate better extraction efficiency than the original nonbuffered extraction kit. However, it is unknown why the neat extract from the EN extraction buffer system produced higher responses than that from the AOAC extraction buffer system, especially for the early eluted analytes. From these results, the SampliQ EN buffered QuEChERS extraction kit was selected for future work.

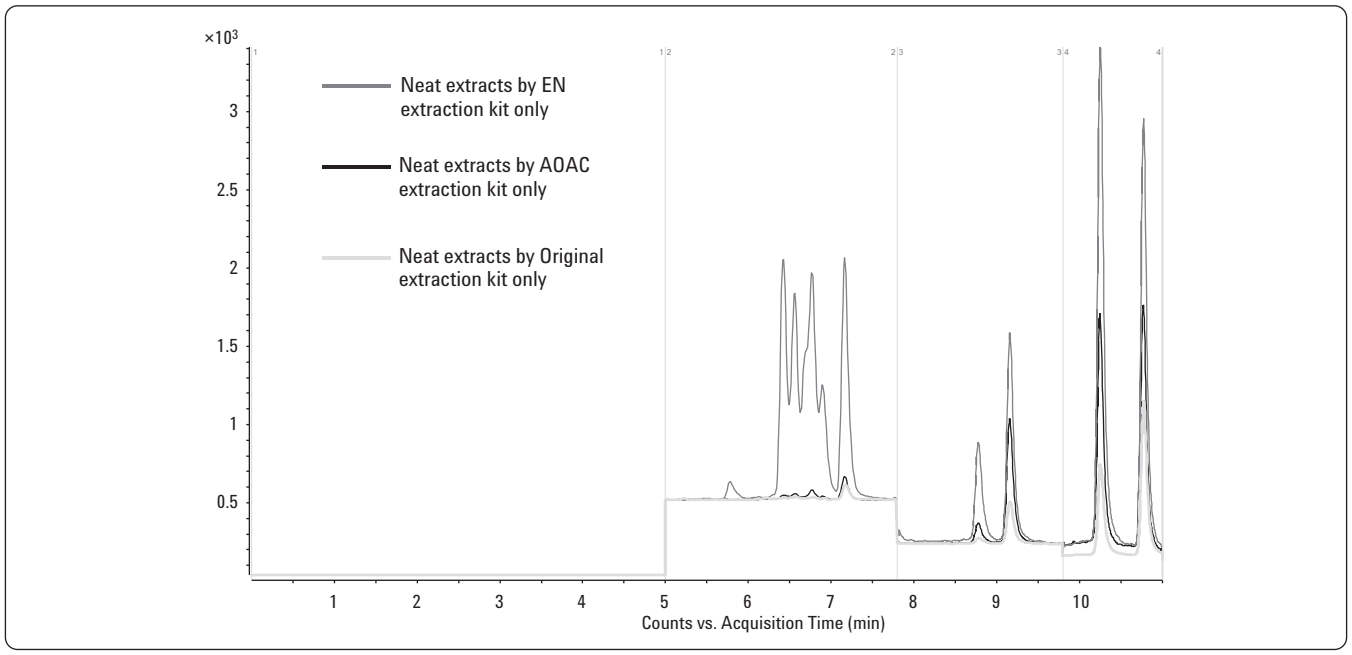


Figure 4. Feasibility test results 1: chromatogram comparison of the neat extracts (no dispersive-SPE) obtained by SampliQ QuEChERS EN extraction kit , AOAC extraction kit, and original extraction kit.

The addition of acid to acetonitrile during the extraction/partitioning step was also investigated. Acetonitrile only, used in the original EN method, and acidified acetonitrile with 5% formic acid were evaluated for their efficiency. As demonstrated in Figure 5 by comparing the results from columns A and D, better analyte recoveries were achieved (10-30% higher) with the acidified acetonitrile. The addition of formic acid into solvent extraction inhibits the acid dissociation for quinolones. Therefore, their protonated neutral form can be extracted easily into the solvent phase [8]. Furthermore, the addition of acid into acetonitrile greatly decreased the negative impact caused by PSA in the dispersive-SPE step (Figure 5, columns C and E). The formic acid in ACN extract interacts with PSA in the dispersive-SPE step, greatly decreasing the binding of PSA with the target quinolones. From these results, 5% (vol/vol) formic acid in acetonitrile was chosen as an extraction solvent for further study.

Although the EN extraction kit generated better recovery, the cleanup using the fatty dispersive-SPE kit in step two significantly lowered extraction efficiency (Figure 5). The selected fatty dispersive-SPE kit contains PSA (primary secondary amine), C18, and $MgSO_4$; however the loss of quinolones was mostly due to the PSA. In the QuEChERS method, PSA is used in all dispersive-SPE kits, because it acts as a weak anion exchanger. It strongly interacts with acidic interferences from fruits and vegetables such as polar organic acids, sugars, and fatty acids. However, it can also strongly interact with the target analytes, the quinolones, leading to the loss of analytes. When acetonitrile was used in the extraction step, PSA from the dispersive-SPE kit caused almost total loss of all of analytes (Figure 5, columns D and E). When acidified acetonitrile was used in the extraction step, the existence of PSA in the dispersive-SPE kit still caused a 10-40% loss of analytes (Figure 5, columns A and C). Because of these results, a brand new SampliQ dispersive-SPE kit for Drug Residues in Meat (p/n 5982-4921) was used for this study. This new SampliQ dispersive-SPE kit contains 25 mg C18 and 150 mg $MgSO_4$ per mL of ACN extract. The new dispersive-SPE kit's effect on the analytes recovery is negligible (Figure 5, columns A and B).

According to the above feasibility test results, a QuEChERS method was developed and applied for the subsequent study in the liver matrix. This method uses the SampliQ EN buffered extraction kit and 5% FA in ACN for the extraction/ partitioning step as well as the new SampliQ dispersive-SPE kit for drug residues in meat for the following cleanup.

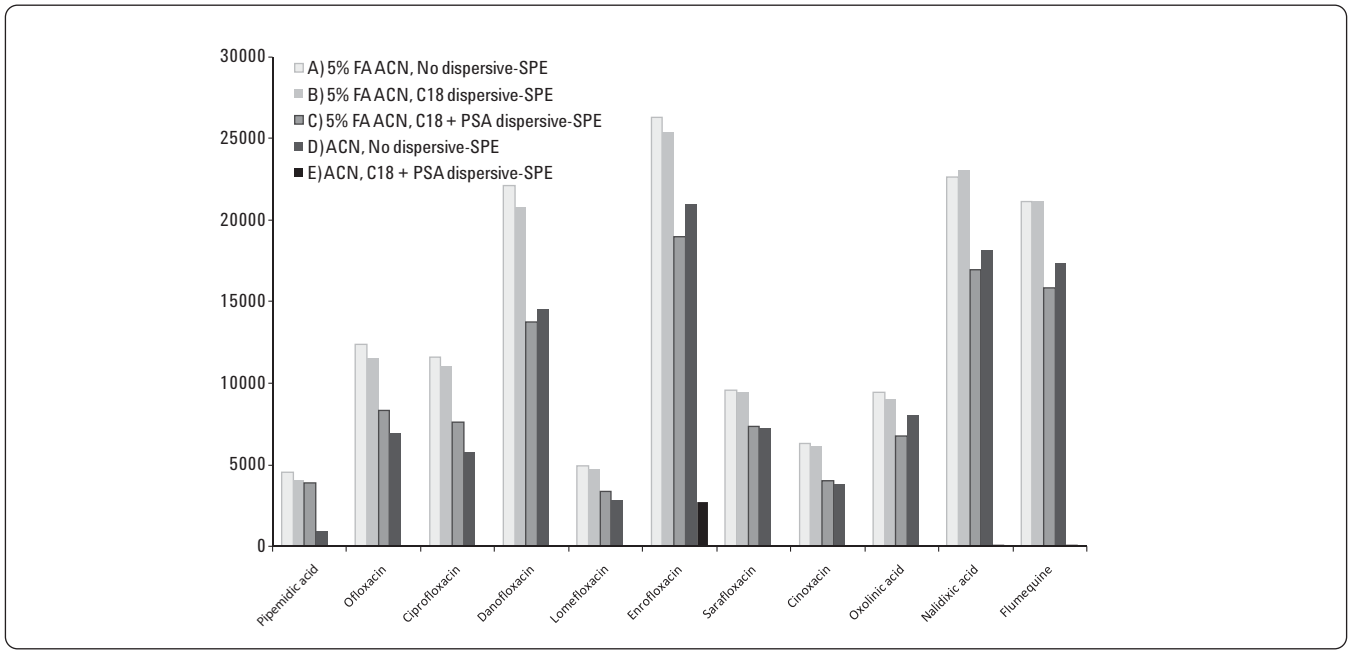


Figure 5. Feasibility test 2. Analytes peak area comparison for the neat extract processed by different procedures. Comparisons include pure ACN and acidified ACN, with and without PSA dispersive-SPE.

Method Optimization in the Liver Matrix

The QuEChERS method established from the results of the feasibility test was applied to the determination of quinolones in bovine liver.

The homogenized liver sample was very thick and could not be used directly for the extraction. Therefore, it was necessary to dilute the liver sample with an aqueous buffer (30 mM KH_2PO_4 in water, pH 7.0) before the extraction. Different sample/buffer ratios including 1:4, 3:7, 1:1, were investigated by adding 8 mL, 7 mL and 5 mL of buffer to 2 g, 3 g, and 5 g of homogeneous liver sample. After dilution, 10 mL of 5% FA in ACN was added. Visually, the more sample used, the more foam was generated during the extraction/partitioning step resulting in a darker red ACN extract. Although more sample should lead to a lower detection limit, it simultaneously introduced more matrix interferences and higher matrix effect. Since the addition of 5% FA ACN to the liver sample is also a protein precipitation procedure, a sample/ACN ratio of 1:4 to 1:5 usually provides the best precipitation effect and sufficient cleanup for proteins. Therefore, a sample/buffer ratio of 1:4 (2 g of liver sample and 8 mL of buffer) was employed.

After the extraction/partitioning step, the sample was centrifuged at 4,000 rpm and 4 °C for 5 min. The low temperature helped to remove lipids from the ACN extracts. After centrifuging, a thin layer of lipids might show up on the surface of the ACN layer. Additional lipids will be removed by C18 in the dispersive-SPE step. A 1 mL amount of ACN extract was transferred into a 2 mL dispersive-SPE tube containing 25 mg C18 and 150 mg $MgSO_4$ for cleanup. An 800 µL amount of upper solvent was transferred into another tube by vortexing and centrifuging. This was the final extract after the QuEChERS extraction and cleanup. It appeared light brown to red in color and was transparent. In order to get sufficient sensitivity and integrity of peak shape, the sample was dried under N_2 flow and reconstituted into 800 µL 1:9 MeOH/H_2O with 0.1% FA. The reconstituted sample was cloudy and filtration was necessary, which was done by a 0.22 µm cellulose acetate spin filter. The sample became colorless and clear after filtering, and was ready for LC/MS/MS injection.

Figure 6 shows the MRM chromatograms of liver control blank and 5 ng/g fortified liver extract (LOQ). The liver control blank chromatogram indicated that it was free from any interference to the target analytes. The 5 ng/g fortified liver extract chromatogram demonstrated that the 5 ng/g limits of quantitation (LOQ) for all of analytes were well established with a signal-to-noise ratio (S/N) greater than 5.

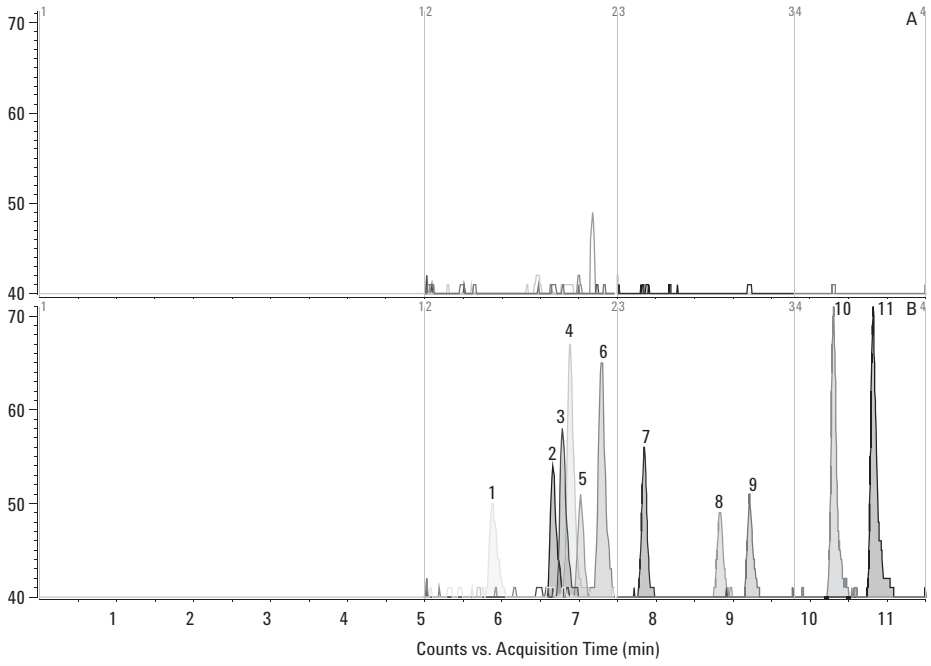


Figure 6. LC/MS/MS chromatograms of A) liver blank extract, and B) 5 ng/g fortified liver extract (LOQ). Peaks identification: 1. Pipemidic acid, 2. Ofloxacin, 3. Ciprofloxacin, 4. Danofloxacin, 5. Lomefloxacin, 6. Enrofloxacin, 7. Sarafloxacin, 8. Cinoxacin, 9. Oxolinoc acid, 10. Nalidixic acid, 11. Flumequine.

Linearity and limit of quantification (LOQ)

The linear calibration range for all of the quinolone antibiotics was 5 – 400 ng/g and matrix blanks were prepared for evaluation. Calibration curves spiked in matrix blanks were made at levels of 5, 10, 50, 100, 200, 300, and 400 ng/g for each analyte. The norfloxacin was used as an internal standard at 200 ng/g. The calibration curves were generated by plotting the relative responses of analytes (peak area of analyte / peak area of IS) to the relative concentration of analytes (concentration of analyte/concentration of IS). The 5 ng/g limit of quantification LOQ (5 ppb) established for all of the quinolones is far below the MRLs for residues of these antibiotics in animal food products. Table 2 shows the regression equation and correlation coefficient (R^2). Linear regression fit was used with $1/x^2$ weight. Results indicated excellent linearity for all of the analytes calibration curves over a broad quantification range.

Table 2. Linearity of Quinolone Antibiotics in Bovine Liver.

Analytes	Regression equation	R^2
Pipemidic acid	Y = 0.2081X – 0.00002	0.9966
Ofloxacin	Y = 0.2221X + 0.00001	0.9964
Ciprofloxacin	Y = 0.2971X – 0.00005	0.9975
Danofloxacin	Y = 0.6861X – 0.0039	0.9957
Lomefloxacin	Y = 0.1702X – 0.00003	0.9958
Enrofloxacin	Y = 0.6530X – 0.0020	0.9962
Sarafloxacin	Y = 0.2132X – 0.0004	0.9937
Cinoxacin	Y = 0.0933X – 0.0004	0.9959
Oxolinic acid	Y = 0.1043X + 0.0003	0.9939
Nalidixic acid	Y = 0.3223X + 0.0005	0.9974
Flumequine	Y = 0.3232X + 0.0003	0.9966

Recovery and Reproducibility

The recovery and reproducibility were evaluated by fortifying quinolone standards in homogenized liver sample at levels of 5, 200 and 400 ng/g. These QC samples were quantified against the matrix spiked calibration curve. The analysis was performed in replicates of six at each level. The recovery and reproducibility (shown as RSD) data are shown in Table 3. It can be seen from the results that all of quinolones except pipemidic acid gave excellent recoveries (average of 95.9%) and precision (average of 6.6% RSD). Pipemidic acid gave lower recovery (average of 66.7%) but great precision (average of 5.7% RSD). Additionally, it still meets the 5 ng/g LOQ requirement. Therefore, the results are acceptable.

Table 3. Recovery and Repeatability of Pesticides in Fortified Liver with 2 mL Dispersive-SPE Tube (p/n 5982-4921)

	5 ng/g fortified QC		200 ng/g fortified QC		400 ng/g fortified QC	
Analytes	Recovery	RSD (n=6)	Recovery	RSD (n=6)	Recovery	RSD (n=6)
Pipemidic acid	71.6	8.1	62.0	6.8	66.4	2.2
Ofloxacin	72.9	9.7	101.0	7.7	102.4	5.7
Ciprofloxacin	108.2	8.3	101.4	4.2	98.9	2.3
Danofloxacin	88.2	7.9	109.3	7.8	114.0	6.1
Lomefloxacin	82.6	13.4	96.8	8.5	97.8	5.3
Enrofloxacin	88.6	7.5	109.5	8.3	113.1	5.8
Sarafloxacin	99.6	9.0	97.7	8.4	97.0	4.6
Cinoxacin	92.3	9.3	95.1	7.9	93.5	2.6
Oxolinic acid	95.1	9.8	92.7	4.3	87.6	2.9
Nalidixic acid	92.7	6.0	90.2	5.3	87.7	3.5
Flumequine	91.6	6.6	93.3	5.3	89.9	2.9

Conclusions

The Agilent SampliQ Buffered Extraction EN kit and the SampliQ dispersive-SPE kit for Drug Residues in Meat provide a simple, fast and effective method for the purification of quinolone antibiotics in bovine liver. Compared to the other sample pretreatment methods, such as LLE and SPE, the QuEChERS method is easier to handle, faster, labor-saving, and cheaper. The recovery and reproducibility, based on matrix spiked standards, were acceptable for multiresidue quinolone determination in bovine liver. The impurities and matrix effects from liver were minimal and did not interfere with the quantification of any target compound. The LOQs of the quinolones were much lower than their regulated MRLs in animal food products. On the whole, the QuEChERS procedures presented here appear to be a promising reference method for the quantitative analysis of quinolones in food products of animal origin. This method also has the potential to extend the applications of SampliQ QuEChERS extraction and dispersive-SPE kits to the quantitative analysis in other bio-matrices, such as animal food products and bio-fluids, rather than just plant matrices.

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Index

Consumer Products

Agilent 1290 Infinity LC with Agilent Poroshell columns for simultaneous determination of eight organic UV filters in under two minutes
Profiling capsaicinoids in spicy foods and food products using the Agilent 1290 Infinity LC system and Agilent ZORBAX Poroshell 120 2.7 columns with UV/VIS diode array and fluorescence detection
Aromatics
Flavoring Agents
Epigallocatechin 3-0-Gallate Extract from Green Tea
Scale-Up of Anthocyanin Separations and Re-Analysis of Collected Fractions on an Agilent Prep-C18 Column
Separation of Paraben Preservatives by Reversed-Phase HPLC
Column Selection for the Analysis of Fatty Acid Methyl Esters
High Throughput Separation of Xanthines by Rapid Resolution HPLC
Analysis of Suspected Flavor and Fragrance Allergens in Cosmetics Using the 7890A GC Capillary Column Backflush
Analysis of Suspected Flavor and Fragrance Allergens in Perfumes Using Two-Dimensional GC with Independent Column Temperature Control Using an LTM Oven Module
Potential Allergens in Aromatherapy Oils by GC/MS Using an Agilent J&W DB-XLB Capillary Column
Determination of Phthalate Concentration in Toys and Children's Products
Analysis of Suncressn Lotion Extract
Reversed Phase HPLC of Fatty Acids
L-Ascorbic Acid Stability in Orange Juice in TetraBrik Cartons
Analysis of ascorbic acid, citric acid and benzoic acid in orange juice
Analysis of fat-soluble vitamins from food matrix for nutrition labeling
Fast analysis of resveratrol in red wine using the Agilent 1290 Infinity LC System
Metabolomic Profiling of Wines using LC/QTOF MS and MassHunter Data Mining and Statistical Tools
A Prediction Model for Determining Wine Variety using the Agilent LC/MS Q-TOF and Agilent Mass Profiler Professional
Direct Analysis of Milk Powder by Axially-Viewed Simultaneous ICP-OES
Rapid Gradient and Elevated Temperature UHPLC of Flavonoids in Citrus Fruit
The Direct Analysis of Milk Powder on the Liberty Series II ICP-OES with the Axially-Viewed Plasma
HPLC Determination of Synthetic Pyrethroid Insecticides in Shampoos and Lotions

Dietary Supplements, Natural Compounds & Additives

Accurate Analysis of Triterpene Glycosides in Black Cohosh by HPLC with ELSD
Analysis of Complex Triglycerides in Starflower Oil from Borage by HPLC using ELSD
Single Run Analysis of St John's Wort Tablets by HPLC with ELSD
Excellent Separation of Ginseng Root Constituents by HPLC with ELSD
Determination of the Limit of Detection for Caffeine by HPLC with ELSD
Fast analysis of fat soluble vitamins using the Agilent 1290 Infinity LC and ZORBAX RRHT and RRHD 1.8 columns
Agilent SampliQ OPT Solid Phase Extraction Sorbent in the Clean-up of Alkaloids in Goldenseal by HPLC-DAD
Extraction and HPLC Analysis of Alkaloids in Goldenseal
Separation of Hypericin in St. John's Wort
Multi-Element Determination of Heavy Metals in Dietary Supplements Using Collision/Reaction Cell ICP-MS

Food Authenticity

Identification of Oxidation Products of L-Ascorbic Acid by HPLC
Accurate analysis of organic acids and alcohols by HPLC
Profiling juice carbohydrates with evaporative light scattering detection
Hi-Plex columns for fingerprinting organic acids in wine
Fast Analysis of Carbohydrates in Chocolate using Liquid Exchange Chromatography with ELSD
Determination of Melamine, Ammeline, Ammelide and Cyanuric Acid in Infant Milk-Based Formula and Other Food and Feed Products Using the Varian 220-MS Ion Trap GC/MS/MS and V:Results GC/MS Software

Index

Food Authenticity (continued)

Authenication of Scotch Whisky by GC Analysis Using an Agilent J&W CP-Wax 57 CB Basic Column
Development of meat speciation assays using the Agilent 2100 bioanalyzer
Analysis of genetically modified soya using the Agilent 2100 bioanalyzer
Detecting genetically modified organisms with the Agilent 2100 bioanalyzer
Characterization of Transgenic Soybean Seedlines by Protein Expression with the Agilent 2100 Bioanalyzer
Nested Multiples Polymerase Chain Reaction for the Determination of DNA From Genetically Modified Corn and Soy Beans Using the Agilent 2100 Bioanalyzer
Determination of PCR-RFLP Profiles for Fish Species Using the Agilent 2100 Bioanalyzer
Agilent 2100 bioanalyzer - Application compendium
Use of the Agilent 2100 Bioanalyzer for Basmati Rice Authenticity Testing
Rapid wheat varietal identification using the Agilent 2100 bioanalyzer and automated pattern-matching
Strawberry and Raspberry Fruit Differentiation Using the Agilent CE 2100 Bioanalyzer
Analysis of the Purity and Storage Stabiltiy of L-Ascorbic Acid Powders
Identification of different meat species by the Agilent Fish ID solution on the Agilent 2100 Bioanalyzer
Discrimination of Different Meat Species by the Agilent Fish ID Solution on the Agilent 2100 Bioanalyzer System
Determination of Melamine Residue in Milk Powder and Egg Using Agilent SampliQ Polymer SCX Solid Phase Extraction and the Agilent 1200 Series HPLC/UV
Quantitative Liquid Chromatography Analysis of Melamine in Dairy Products Using Agilent's 1120 Compact LC and Rapid Resolution LC
A Total Solution for the Analysis of Melamine and Cyanuric Acid in Pet Food by GC/MS and Aqueous Normal-Phase LC/MS/MS
Rapid Screening of Melamine and Cyanuric Acid in Milk Products Using Agilent J&W HP-5ms GC/MSD Column and Agilent 7890A/5975C GC/MSD with Column Backflushing
Rapid Screening and Confirmation of Melamine Residues in Milk and Its Products by Liquid Chromatography Tandem Mass Spectrometry
Trace-Level Analysis of Melamine in Milk Products on Agilent 7890A/5975C GC/MSD Using a New Agilent J&W DB-5ms Ultra Inert Column and SampliQ SCX Cartridges
Rapid Screening and Confirmation of Melamine and its Analogs in Baby Formula and Soy Products Using Triple Quadrupole GC/MS and Backflushing

Food Processing & Packaging

Analysis of elastomers by GPC/SEC
Analysis of polyolefins by GPC/SEC
Analysis of engineering polymers by GPC/SEC
Biodegradable polymers - analysis of biodegradable polymers by GPC/SEC
SEC Analysis of Pectin
SEC Analysis of Dextrans
Identification of Unknown Reaction By-Products and Contaminants in Epoxyphenolic-Based Food Can Coatings by LC/TOF-MS
Identification of Unknown Polyester Oligomers in New Polyester Food Can Coatings by LC/TOF-MS Using Molecular Feature Extraction and Database Searching
LC-TOF-MS As a Tool to Support Can Coating/Food Interaction Studies

Pesticides, Mycotoxins & Other Contaminants

Organophosphorus Pesticides in Apple Matrix by GC/MS/FPD using an Agilent J&W DB-35ms Ultra Inert GC Column
Sensitive Femtogram Determination of Aflatoxins B1, B2, G1 and G2 in Food Matrices using Triple Quadrupole LC/MS
Improved Isolation and Analysis of Mycotoxins from Cereals, Beer and Wine
Fast separation of EU and EPA Regulated PAHs on Agilent J&W FactorFour VF-17ms for PAH
Multi-residue Analysis of PAHs, PCBs and OCPs using an Agilent J&W FactorFour VF-35ms Column
Extra Fast Separation of 16 US EPA 610 Regulated PAHs on Agilent J&W Select PAH
Separation of EU and EPA Regulated PAHs on Agilent J&W FactorFour VF-17ms Column
Benefit of Low Temperature ELSD for the Analysis of Herbicides by HPLC

Index

Pesticides, Mycotoxins & Other Contaminants (continued)

Fast analysis of coal tar polycyclic aromatic hydrocarbons on Agilent J&W Select PAH
Fast Separation of 16 US EPA 610 Regulated PAHs on Agilent J&W Select PAH GC Columns
Fast Separation of EU and US EPA Regulated PAHs on Agilent J&W Select PAH GC Columns
GC Analysis of Halogenated Pesticides using the Agilent J&W FactorFour VF-1701ms with EZ-Guard
Separation of 54 PAHs on an Agilent J&W Select PAH GC Column
GC Analysis of PAHs using an Agilent J&W FactorFour VF-17ms Column with EZ-Guard
Analysis of all 136 tetra- through octa- polychlorinated dibenzo-p-dioxins and dibenzofurans using an Agilent J&W FactorFour VF-Xms GC Column
Fast GC Analysis of EPA and EU Regulated PAHs
New SPE Sorbent for Clean-up of Fusarium Toxin-contaminated Cereals & Cereal-based Foods, Bond Elut Mycotoxin
Multi-Residue Confirmation of Pesticides in Honey using Solid Supported Liquid Extraction
GC/ ECD Analysis and Confirmation of PCBs in Fish Tissue with Agilent J&W DB-35ms and DB-XLB GC Columns
A Low Femtogram Target Screen Method for Perfluorinated Compounds in Food Matrices and Potable Water Using the Agilent 6460 Triple Quadrupole LC/MS System Equipped with Agilent Jet Stream Technology
Analysis of Polycyclic Aromatic Hydrocarbons in Fish with Agilent SampliQ QuEChERS AOAC Kit and HPLC-FLD
Determination of Acrylamide in Cooking Oil by Agilent SampliQ QuEChERS Acrylamide Kit and HPLC-DAD
Analysis of Pesticide Residues in Spinach Using Agilent SampliQ QuEChERS AOAC Kits by GC/MS
PAH Analysis with High Efficiency GC Columns: Column Selection and Best Practices
Anaysis of Pesticide Residues in Spinach Using Agilent SampliQ QuEChERS EN Kit by LC/MS/MS Detection
Anaysis of Pesticide Residues in Spinach Using Agilent SampliQ QuEChERS AOAC Kit by LC/MS/MS Detection
Optimizing Recoveries of Planar Pesticides in Spinach Using Toluene and Agilent SampliQ AOAC QuEChERS Kits with Graphitized Carbon
Analysis of Pesticide Residues in Apple Using Agilent SampliQ QuEChERS EN Kits by GC/MS
Analysis of Pesticide Residues in Apple Using Agilent SampliQ QuEChERS AOAC Kits by GC/MS
Analysis of Pesticide Residues in Apples using Agilent SampliQ QuEChERS AOAC Kit by LC/MS/MS Detection
Highly Sensitive and Rugged GC/MS/MS Tool
A Method for the Trace Analysis of 175 Pesticides Using the Agilent Triple Quadrupole GC/MS/MS
Organophosphorus Pesticides Analysis Using an Agilent J&W DB-5ms Ultra Inert Capillary GC Column
The Analysis of Chlorinated Pesticides and PCBs Using the HP-608 Capillary Column
Analysis of Mycotoxins by HPLC with Automated Confirmation by Spectral Library
Fast Dual-Column GC/ECD Analysis of Chlorinated Pesticides - EPA Methods 608 and 8080
Analysis of Organochlorine Pesticides and PCB Congeners with the Agilent 6890 Micro-ECD
HPLC Analysis of Aflatoxines in Pistachio Nuts using HPLC
Analysis of Pesticides in Salad Samples and Spices using HPLC
Trace Level Pesticide Analysis by GC/MS Using Large-Volume Injection
Analysis of Fumonisin Mycotoxins by LC/MS
Analysis of Poisoned Food by Capillary Electrophoresis
Fast Screening of Pesticide and Endocrine Disrupters Using the Agilent 6890/5973N GC/MSD System, Part 1
Gas Chromatography/Mass Spectrometry Approaches to the Analysis of Acrylamide in Foods
A Blind Study of Pesticide Residues in Spiked and Unspiked Fruit Extracts Using Deconvolution Reporting Software
New Tools for Rapid Pesticide Analysis in High Matrix Samples
Identification and Isolation of DSP-Toxins Using a Combined LC/MS-System for Analytical and Semipreparative Work
Analysis of Terbuthlazine in Olive Oil by LC/Ion Trap MS and LC/Time-of-Flight MS
Separation of Aflatoxins by HPLC
Screening for 926 Pesticides and Endocrine Disruptors by GC/MS with Deconvolution Reporting Software and a New Pesticide Library
Rapid Determination of Hydroxymethlyfurfural in Foods Using Liquid Chromatography-Mass Spectometry
Determination of 44 Pesticides in Foodstuffs by LC/MS/MS
Multiresidue Analysis of 100 Pesticides in Food Samples by LC/Triple Quadrupole Mass Spectrometry

Index

Pesticides, Mycotoxins & Other Contaminants (continued)

Using RTL and 3-Way Splitter to Identify Unknown in Strawberry Extract
Direct Injection of Fish Oil for the GC-ECD Analysis of PCBs: Results Using a Deans Switch With Backflushing
Rapid Analysis of Crude Fungal Extracts for Secondary Metabolites by LC/TOF-MS - A New Approach to Fungal Characterization
Determination of Aflatoxins in Food by LC/MS/MS
Addressing the Challenges of Analyzing Trace Perfluorooctanoic Acid (PFOA) and Perfluorooctane Sulfonate (PFOS) Using LC/QQQ
Anaysis of poisoned food by capillary electrophoresis
Determination of Benzimidazole Fungicides in Apple Juice by SampliQ Polymer SCX Solid-Phase Extraction with High-Performance Liquid Chromatography
An Application Kit for Multi-Residue Screening of Pesticides using LC/TOF or Q-TOF with a Pesticide Personal Compound Database
Multi-Residue Pesticide Analysis with Dynamic Multiple Reaction Monitoring and Triple Quadrupole LC/MS/MS
Analysis of Pesticide Residues in Apple by GC/MS using Agilent SampliQ QuEChERS Kits for Pre-injection Cleanup
Determination of pesticides in baby food by UHPLC/MS/MS using the Agilent 1290 Infinity LC system and the Agilent 6460 triple quadrupole LC/MS
PBDE Analysis Using an Agilent J&W DB-5ms Ultra Inert GC Column
PAH Analysis Using an Agilent J&W DB-5ms Ultra Inert Capillary GC Column
Analysis of Low-level Polycyclic Aromatic Hydrocarbons (PAHs) in Rubber and Plastic Articles Using Agilent J&W DB-EUPAH GC column
EU's Scientific Committee on Food PAH 151 Analysis Using a ZORBAX Eclipse PAH Column
GC-ECD Analysis and Confirmation of Contract Labratory Protocol Pesticides in Olive Oil
Determination of Dioxin-Like and Non-Dioxin-Like Polychlorinated Biphenyl Congeners in Foodstuffs and Animal Feed Using the Agilent 7000 Triple Quadrupole GC/MS System
Determination of Polychlorinated Dibenzo-p-dioxins (PCDD) and Polychlorinated Dibenzofurans (PCDF) in Foodstuffs and Animal Feed using the Agilent 7000 Triple Quadrupole GC/MS System
A reliable and routine GC/MS/MS Method for the Determination of Chemical Contaminants in Marine Shellfish
Agilent 6460 LC-QQQ - Highly Sensitive and Robust Analysis for Lipophilic Marine Toxins in Shellfish
Determination of Chemical Contaminants in Marine Shellfish using the Agilent 7000 Triple Quadrupole GC/MS System
Analysis of Pesticide Residues in Apple using Agilent SampliQ QuEChERS European Standard EN Kits by LC/MS/MS Detection
Determining Malachite Green and Leucomalachite Green in Food by LC/MS/MS

Soil Quality & Fertilizer Analysis

Determination of Mercury in a Certified Reference Sludge Material using the Agilent 710-ES
Rapid Measurement of Major, Minor and Trace Levels in Soils Using the Agilent 730-ES
Analysis of Soil Extracts Using the Agilent 725-ES
The Latest Advances in Axially Viewed Simultaneous ICP-OES for Elemental Analysis
Analysis of Plant Materials by VaporGeneration AA
Nitrogen Determination in Fertilizer by ICP-OES with an Extended Torch
Validation of a leaf reflectance and transmittance model for three agricultural crop species
Automated nitrate analysis of water
Determination of phosphorus concentration in hydroponics solution
The Investigation of Fertilizer Analyses Using Microwave Digestion and the Agilent 720-ES
Nitrate analysis of water using the quartz fiber optics dip probe on the Cary 50/60 UV-Vis

Trace Metals

Direct Analysis of Milk Powder by Axially-Viewed Simultaneous ICP-OES
The Direct Analysis of Milk Powder on the Liberty Series II ICP-OES with the Axially-Viewed Plasma
Analysis of Shellfish Tissue for Cadmium, Mercury and Nickel
Using the Lead Isotope Ratios to Distinguish between Samples of the Traditional Chinese Medicine Dan-shen
Fast and Accurate Determination of Arsenobetaine (AsB) in Fish Tissues Using HPLC-ICP-MS
Determination of Mercury in Microwave Digests of Foodstuffs by ICP-MS

Index

Trace Metals (continued)

Measurement of Trace Elements in Malt Spirit Beverages (Whisky) by 7500cx ICP-MS
Speciated Isotope Dilution for the Determination of Methylmercury in Tuna Fish by GC-MS

Analysis of Plant Materials by VaporGeneration AA

Veterinary Drugs

Transfer and Optimization of Existing Methods for Analysis of Antibiotics in Meat to Agilent Poroshell 120 EC-C18 Columns using MS/MS Detection
Determination of Sulfonamide Residues in Chicken Muscle by Agilent SampliQ QuEChERS AOAC Kit and HPLC-FLD
Extraction of Sulfa Drugs in Honey with Polymeric SPE Cation Exchange, Bond Elut Plexa PCX
Analysis of tetracyclines by HPLC
Analysis of Residual Synthetic Antibacterials in Meat by HPLC
HPLC Analysis of Antibacterial Drugs with Penicillin-Like Structure
HPLC Separation of Antibacterial Drugs with Tetracycline Structure
Determination of the Metabolites of Nitrofuran Antibacterial Drugs in Chicken Tissue by Liquid Chromatograph-Electrospray Ionization-Mass Spectrometry (LC-ESI-MS)
Determination of Chloramphenicol in Fish Meat by Liquid Chromatograph-Atmospheric Pressure Photo Ionization-Mass Spectrometry (LC-APPI-MS)
Detection, Confirmation, and Quantification of Chloramphenicol in Honey and Shrimp at Regulatory Levels Using Quadrupole and Ion Trap LC/MS
A Validated Atmospheric Pressure Chemical Ionization Method for Analyzing Sulfonamides in Pork Muscle
The Analysis of Fluoroquinolones in Beef Kidney Using HPLC Electrospray Mass Spectrometry
Determining Malachite Green and Leucomalachite Green in Food by LC/MS/MS
Analysis of Nitrofuran Metabolites in Tilapia Using Agilent 6410 Triple Quadrupole
Detection, Confirmation, and Quantification of Chloramphenicol in Honey, Shrimp and Chicken Using the Agilent 6410 LC/MS Triple Quadrupole
Detection of Recombinant Bovine Somatotropin in Milk by LC-ESI-MS/MS
Development of an LC-MS/MS Method for the Determination of 20-Hydroxyecdysone and Its Metabolites in Calf Urine
Determination of Tetracyclines in Chicken by Solid-Phase Extraction and High-Performance Liquid Chromatography
Determination of Penicillins in Meat by High Performance Liquid Chromatography (HPLC/UV) and HPLC/MS/MS
Determination of Chloramphenicol, Florfenicol, and Thiamphenicol in Honey Using Agilent SampliQ OPT Solid-Phase Extraction Cartridges and Liquid Chromatography-Tandem Mass Spectrometry
Determination of Sulfonamides in Milk Using Solid-Phase Extraction and Liquid Chromatography-Tandem Mass Spectrometry
Determination, Confirmation, and Quantification of Trace B-Lactam Antibiotics in Milk by LC/MS/MS
Using LC/MS/MS 6410 for Analysis of Chloramphenicol, Thiamphenicol, and Florfenicol in Fish Samples
Detection, Monitoring, and Quantitation of Trace Sulfonamides in Pork Muscle Using the Agilent 6410A LC/MS/MS
Determination of Multi-Residue Tetracyclines and their Metabolites in Milk by High Performance Liquid Chromatography - Tandem Mass Spectrometry
Determination of Hormones in Fish (Carassius Carassius) by SampliQ-OPT Solid Phase Extraction with High Performance Liquid Chromatography
Determination of B2-Agonists in Pork Using Agilent SampliQ SCX Solid-Phase Extraction Cartridges and Liquid Chromatography-Tandem Mass Spectrometry
Profiling medications for decorative or aquarium fish using the Agilent 1290 Infinity LC system and Agilent ZORBAX Poroshell 120 2.7 columns
Determination of Sulfonamide Antibiotics in Bovine Liver Using Agilent SampliQ QuEChERS EN Kits by LC/MS/MS
Determination of Quinolone Antibiotics in Bovine Liver Using Agilent SampliQ QuEChERS Kits by LC/MS/MS