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

Application Note

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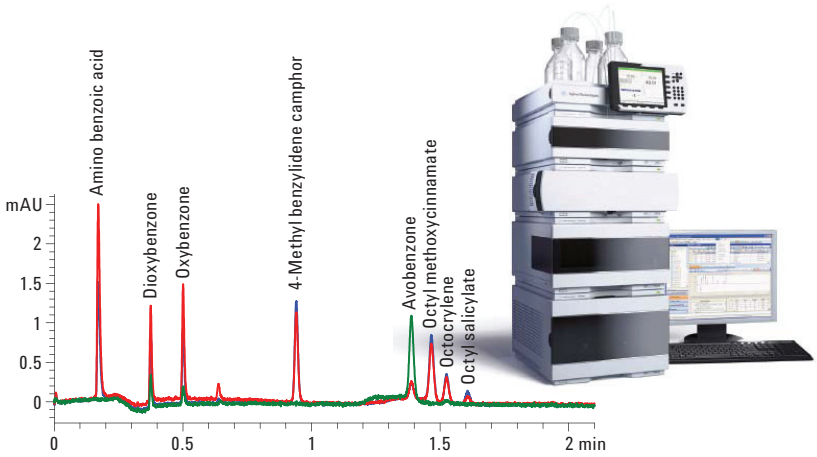

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Abstract

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.

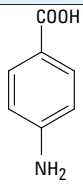
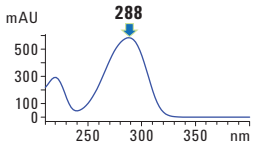
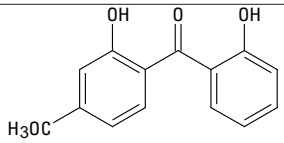
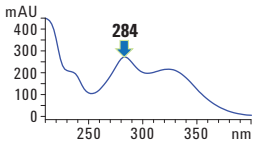
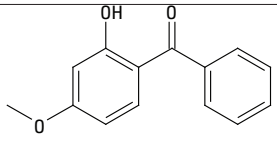
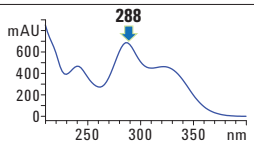
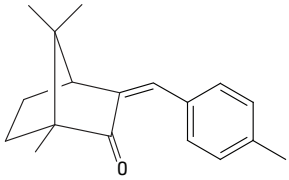
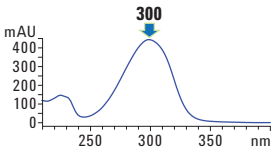
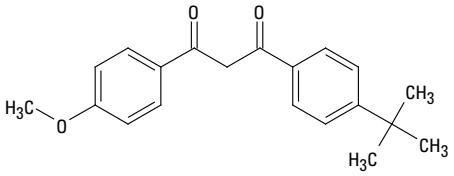
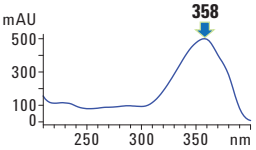
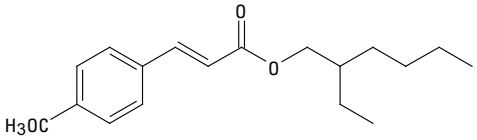
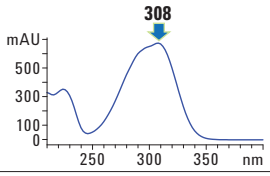
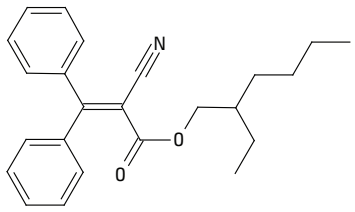
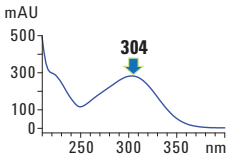
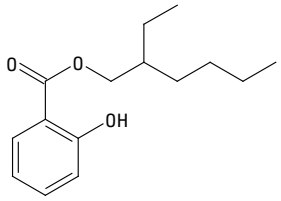
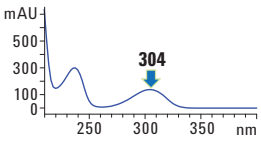
Compound number	Compound	Structure	UV Spectra
1	Name: Aminobenzoic acid CAS No: 150-13-0 Mol Formula: $C_7H_7NO_2$ Mol Wt: 137.14 Detection: 288 nm		
2	Name: Dioxybenzone CAS No: 131-53-3 Mol Formula: $C_{14}H_{12}O_4$ Mol Wt: 244.24 Detection: 288 nm		
3	Name: Oxybenzone CAS No: 131-57-7 Mol Formula: $C_{14}H_{12}O_3$ 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).

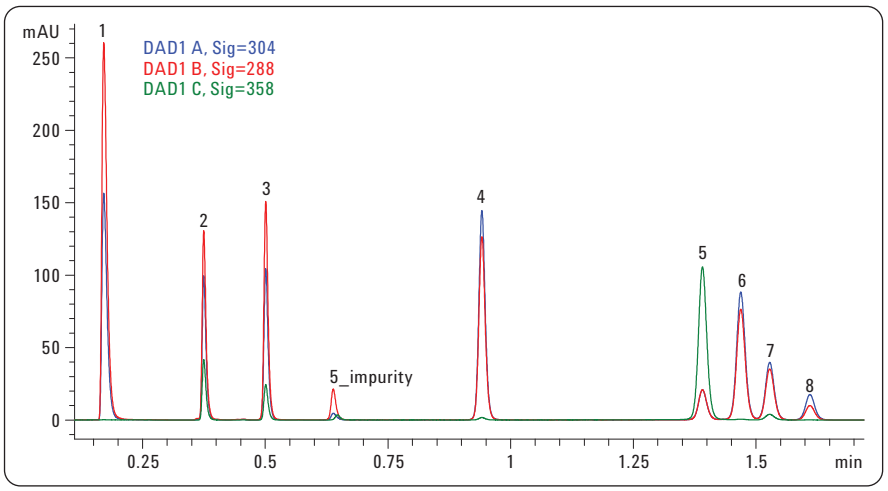


Figure 1
Chromatographic elution profile of eight organic UV filters in 99 seconds using an Agilent 1290 Infinity LC and an Agilent Poroshell 120 EC-C18 column (three different wavelengths are overlaid).

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).

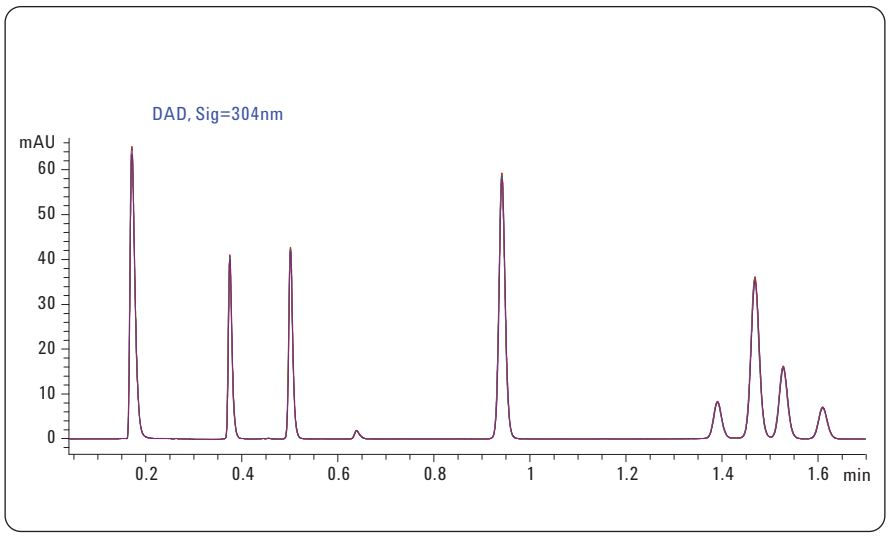


Figure 2
Chromatographic overlay of six replicates of standard mix injections at 10 ng/µL level.

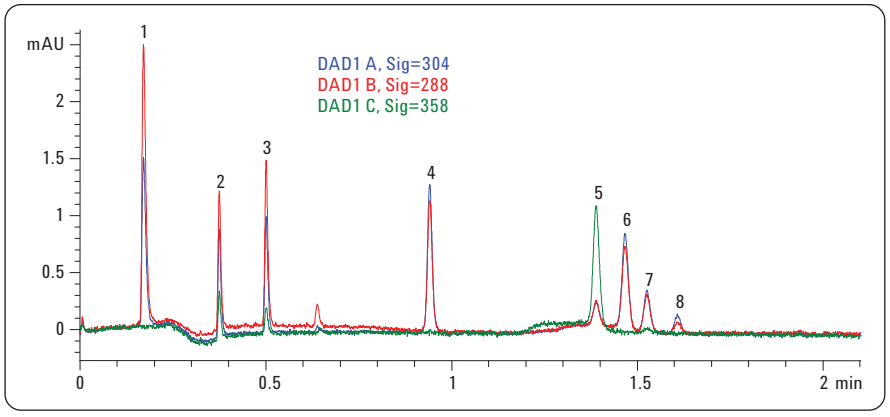


Figure 3
Chromatogram of standard mix where all the analytes were at 0.25 ng on-column concentration (three different wavelengths are overlaid).

	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

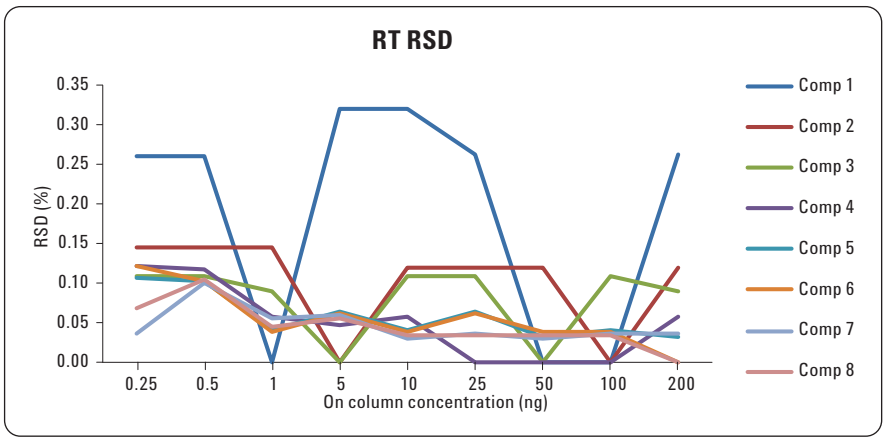


Figure 4
Retention time RSD.

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.

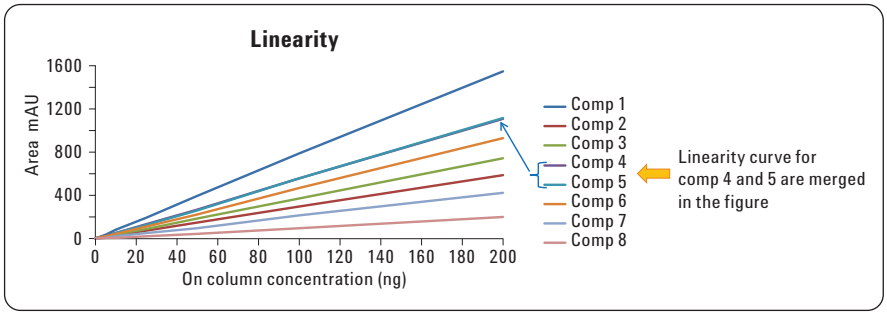


Figure 5
Overlaid linearity curves for all standards.

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.

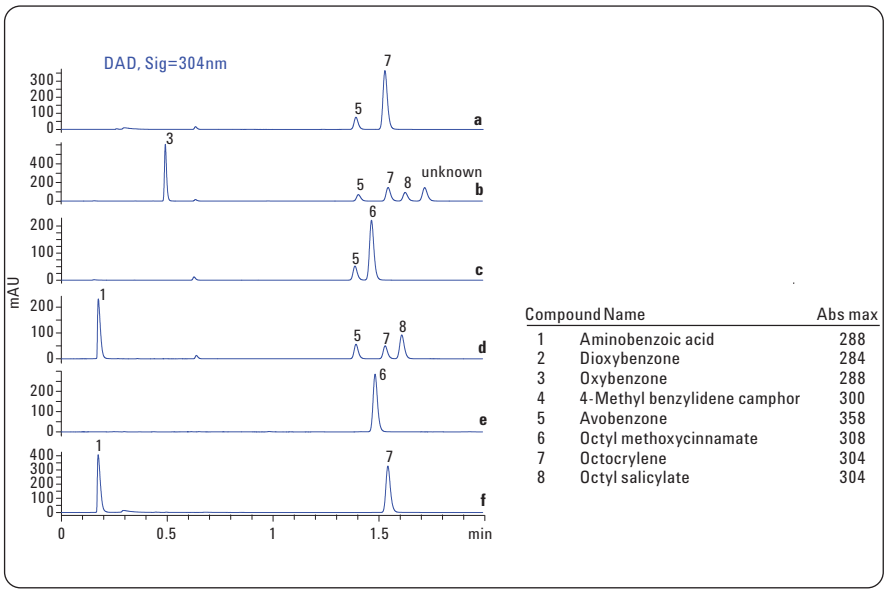


Figure 6
Overlay of chromatogram for all six extracted samples.

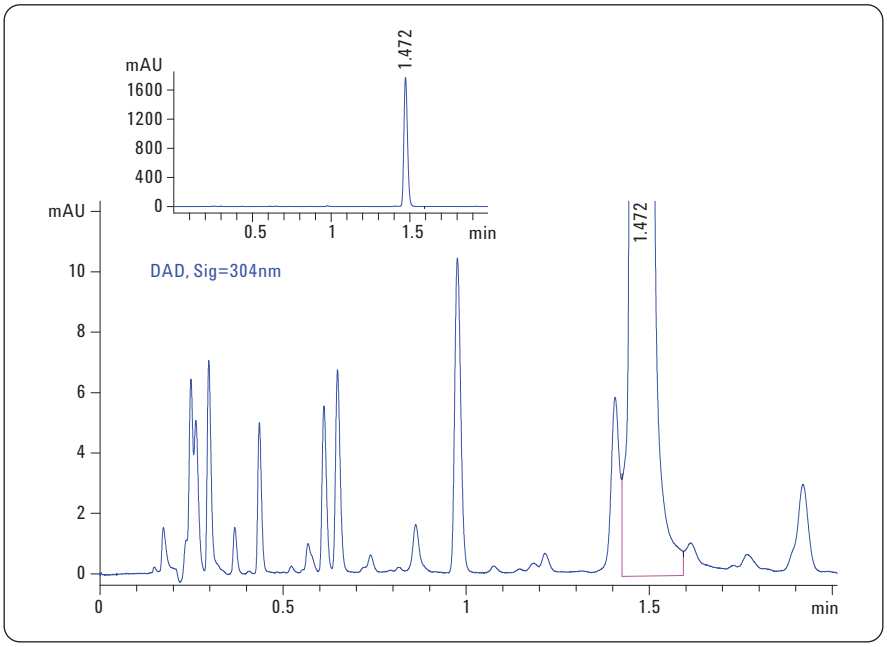


Figure 7
Full scale and zoomed chromatogram of sample "e", which demonstrates well resolved impurity peaks. These minor unidentified peaks may be parabens or other listed ingredients of the formulations and related impurities.

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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.

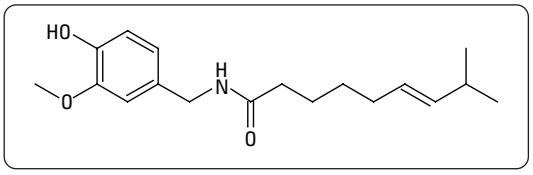

Figure 1
The structure of capsaicin ((E)-N- (4-hydroxy-3-methoxybenzyl) - 8-methylnon-6-enamide).

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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.

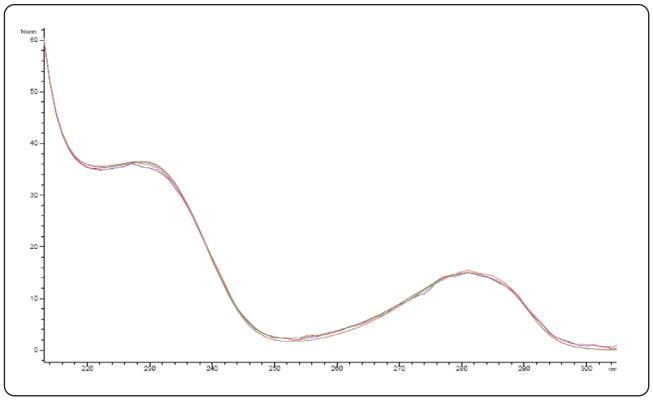


Figure 2
Overlaid extracted UV spectra of capsaicinoid compounds in chili.

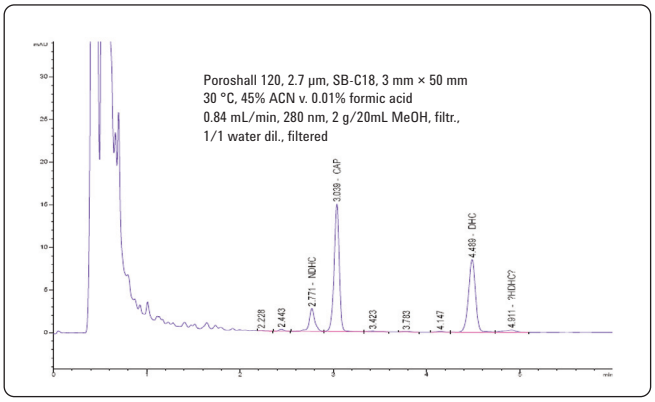
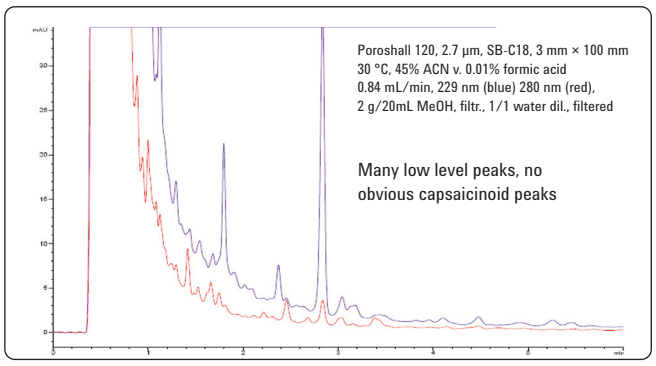


Figure 3
Separation of cayenne dried ground spice (CAP- capsaicin, NDHC – nordihydrocapsaicin, DHC – dihydrocapsaicin).



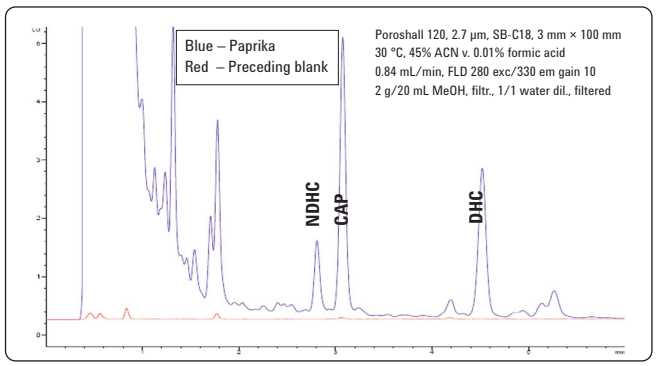


Figure 4
Comparison of UV and fluorescence signals for American Paprika extract (two frames).

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Agilent Technologies

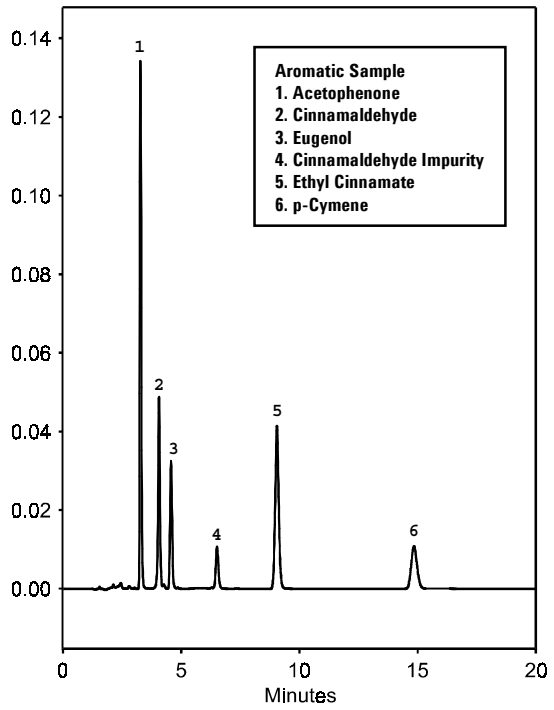
Aromatics

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.*

Agilent Technologies

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5988-6352EN

Agilent Technologies

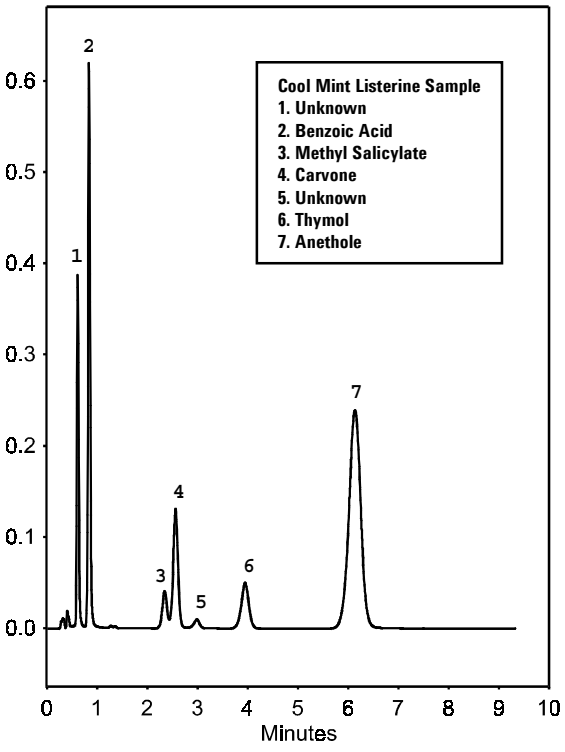
Flavoring Agents

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.*

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Agilent Technologies

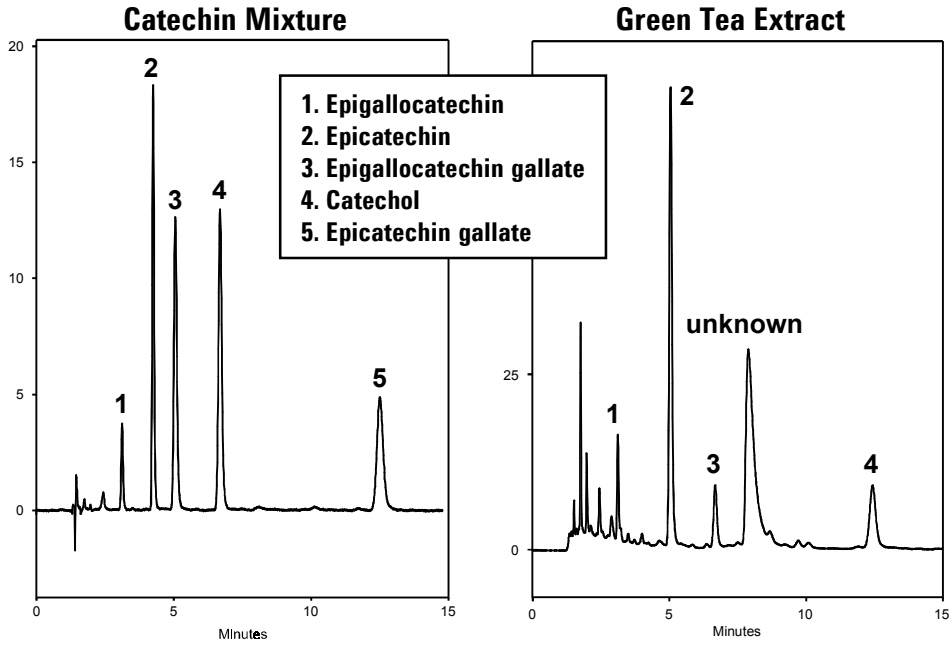
Epigallocatechin 3-O-Gallate Extract from Green Tea

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

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Agilent Technologies

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

Application

Biochemical

Cliff Woodward

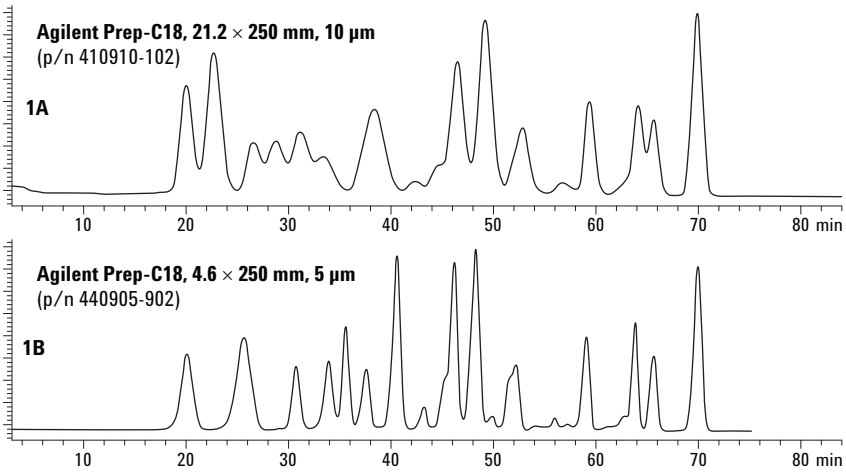
Highlights

- 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.

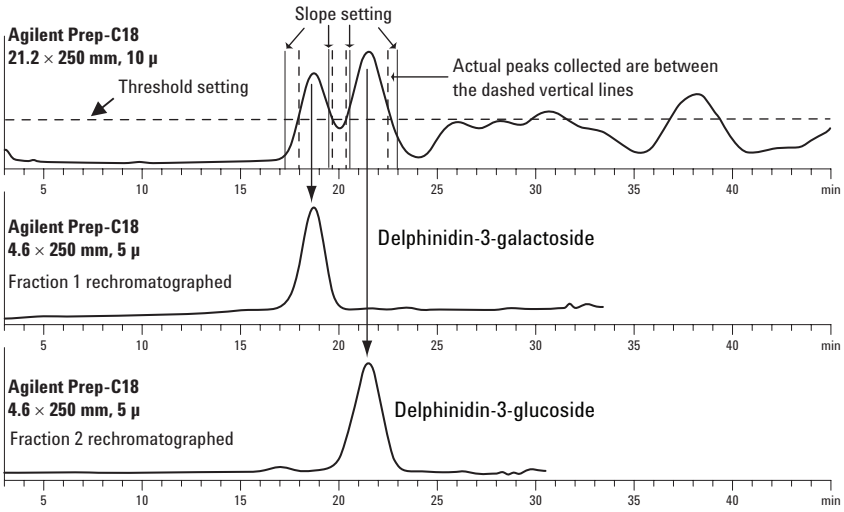


Figure 2. Fraction collection and rechromatography to demonstrate purity.

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Agilent Technologies

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.

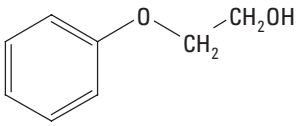
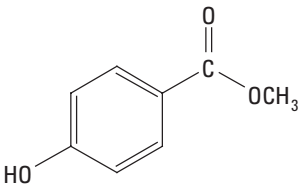
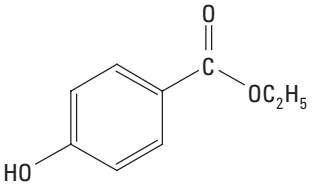
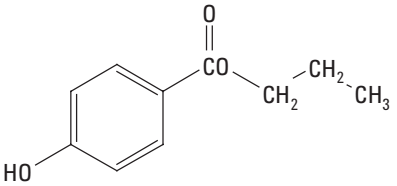
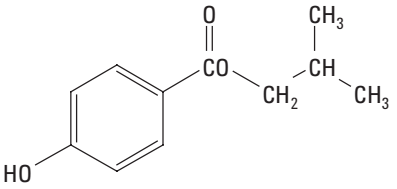
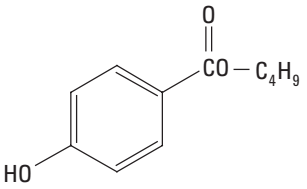
Researchers of the Department of Biology and Biochemistry of Brunel University in the United Kingdom found that the greatest concern regarding parabens focuses on their estrogen-mimicking ability in laboratory animals. In addition, 2-phenoxyethanol (2PX), a chemical substance also used as a preservative 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.

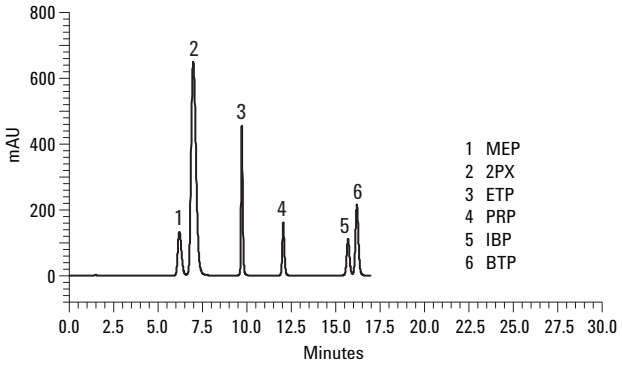


Figure 1. Separation of preservatives by reversed-phase HPLC.

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).

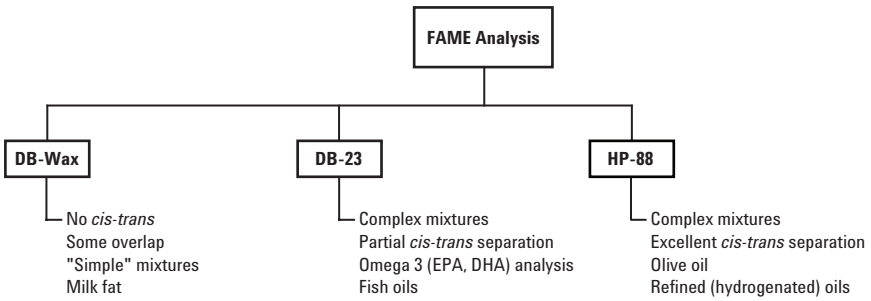

Figure 1. Overview of column selection for FAMEs analysis.

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.

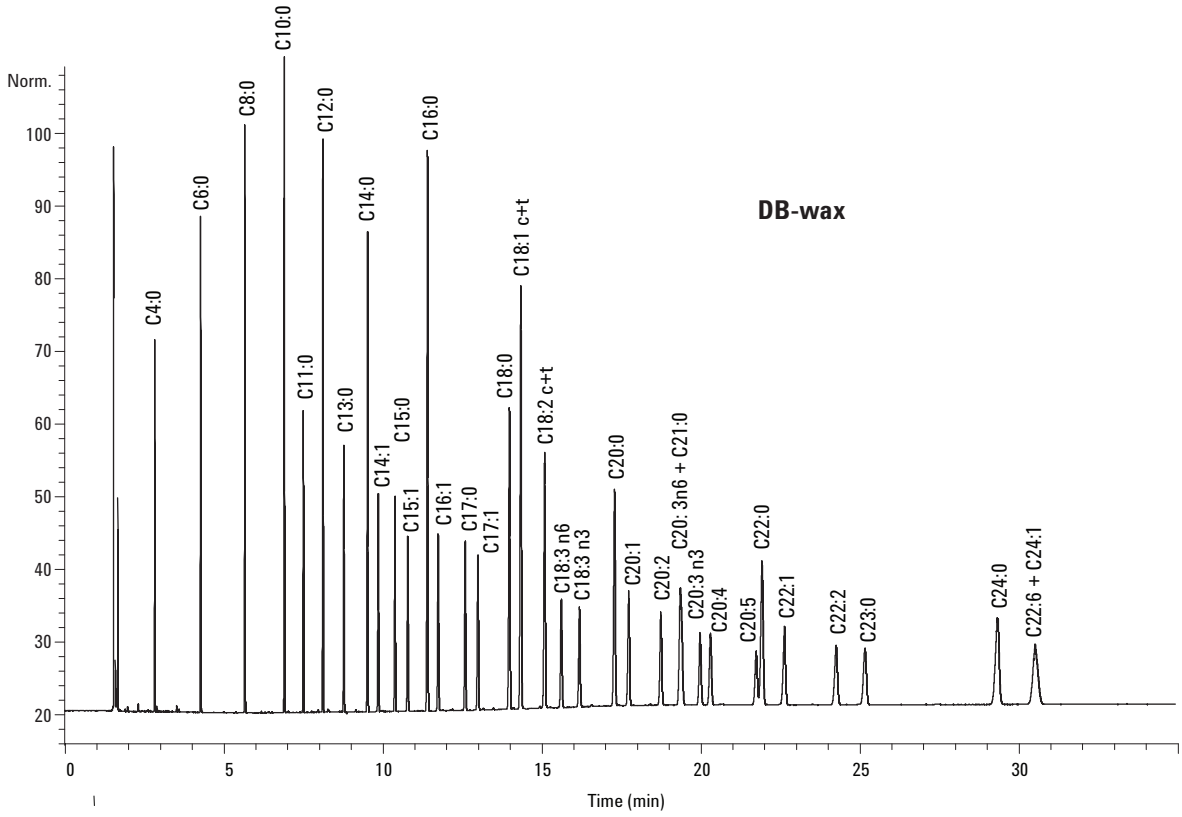

Figure 2. GC-FID analysis of 37-component FAMEs standard mixture on a 30 m × 0.25 mm ID, 0.25 µm DB-Wax column using Method 1. (See Table 2).

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]).

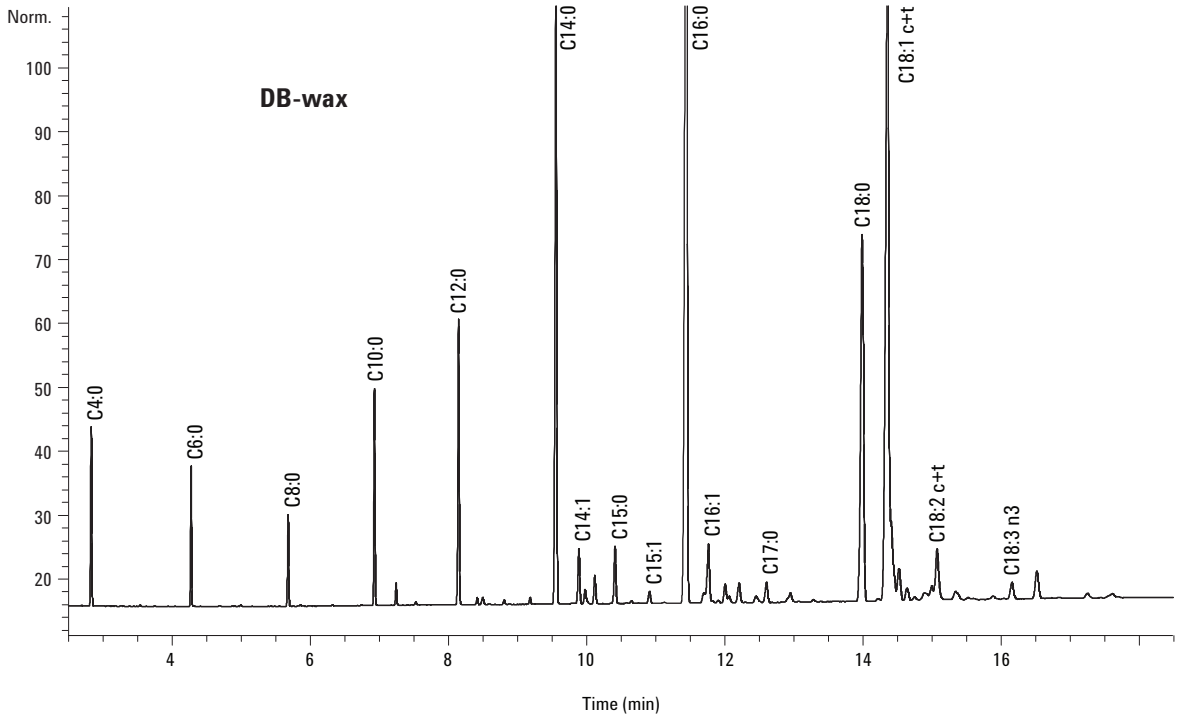

Figure 3. GC-FID analysis of milk fat (CRM 164) fatty acids on a 30 m × 0.25 mm ID, 0.25 µm DB-Wax column using Method 1, Table 2.

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.

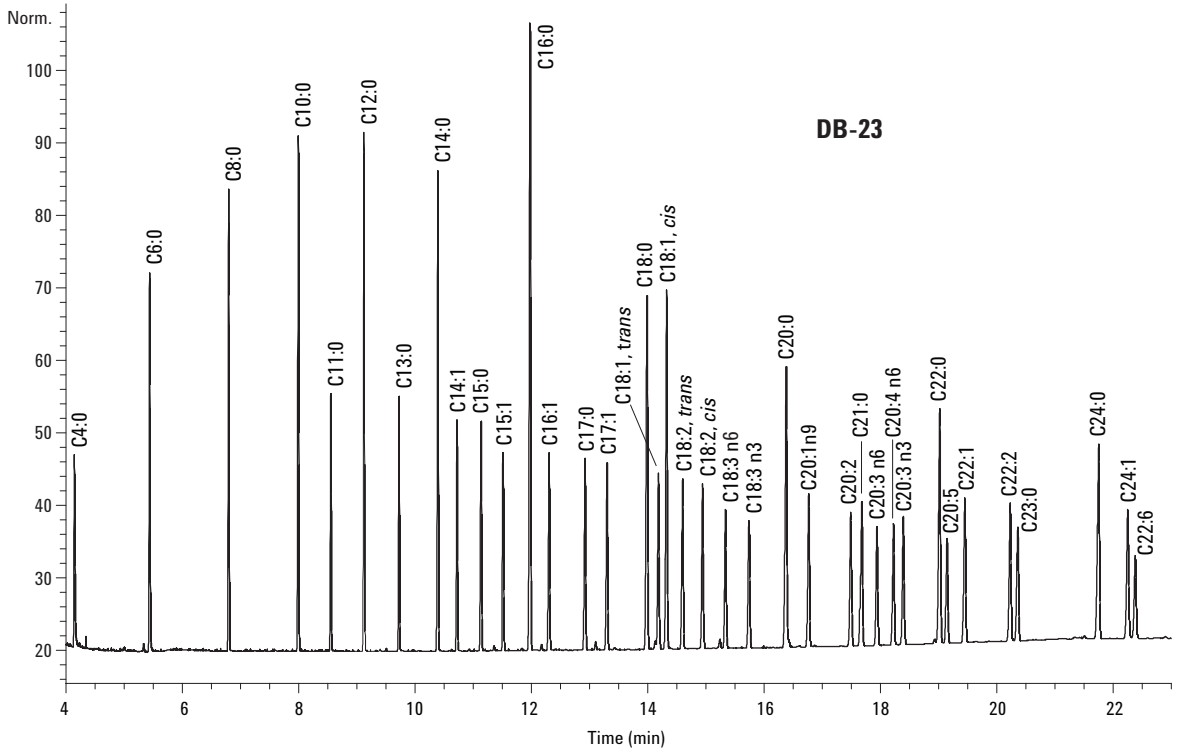

Figure 4. GC-FID analysis of FAMEs standard mixture on a 60 m × 0.25 mm ID, 0.15 µm DB-23 column using Method 2, Table 3.

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.

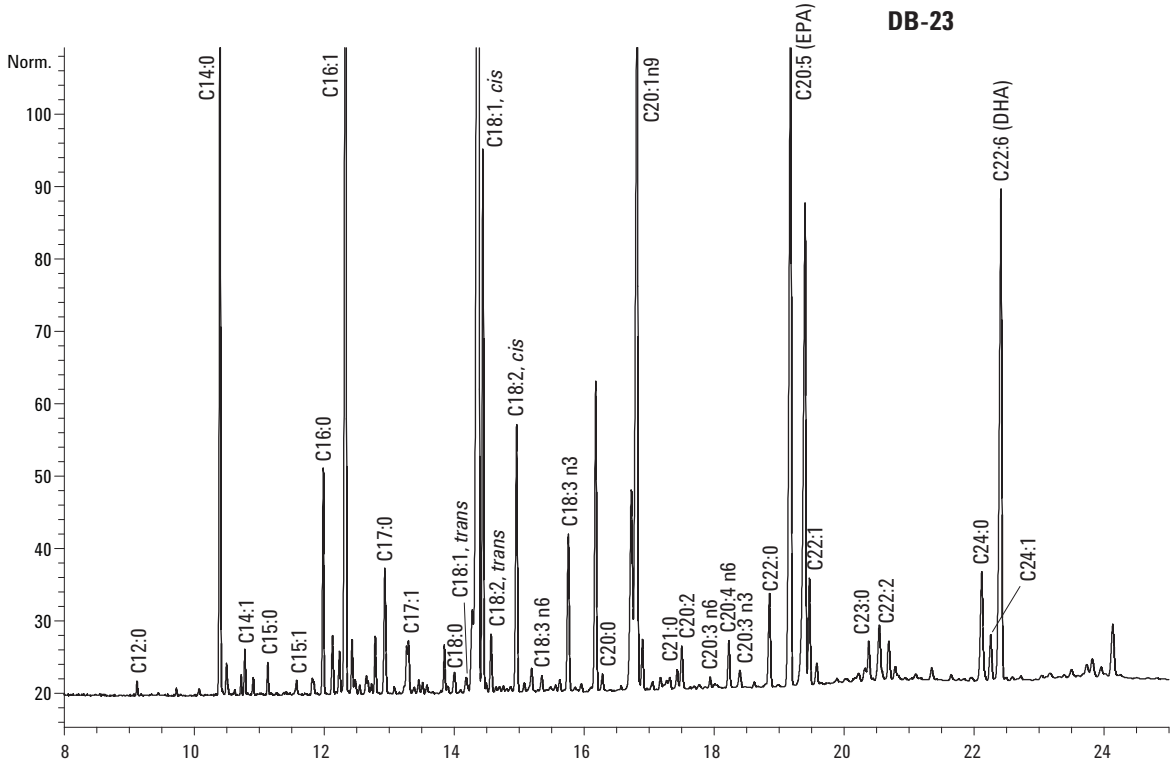

Figure 5. GC-FID analysis of unsaturated fatty acid mixture from marine origin on a 60 m × 0.25 mm ID, 0.15 µm DB-23 column using Method 2, Table 3.

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

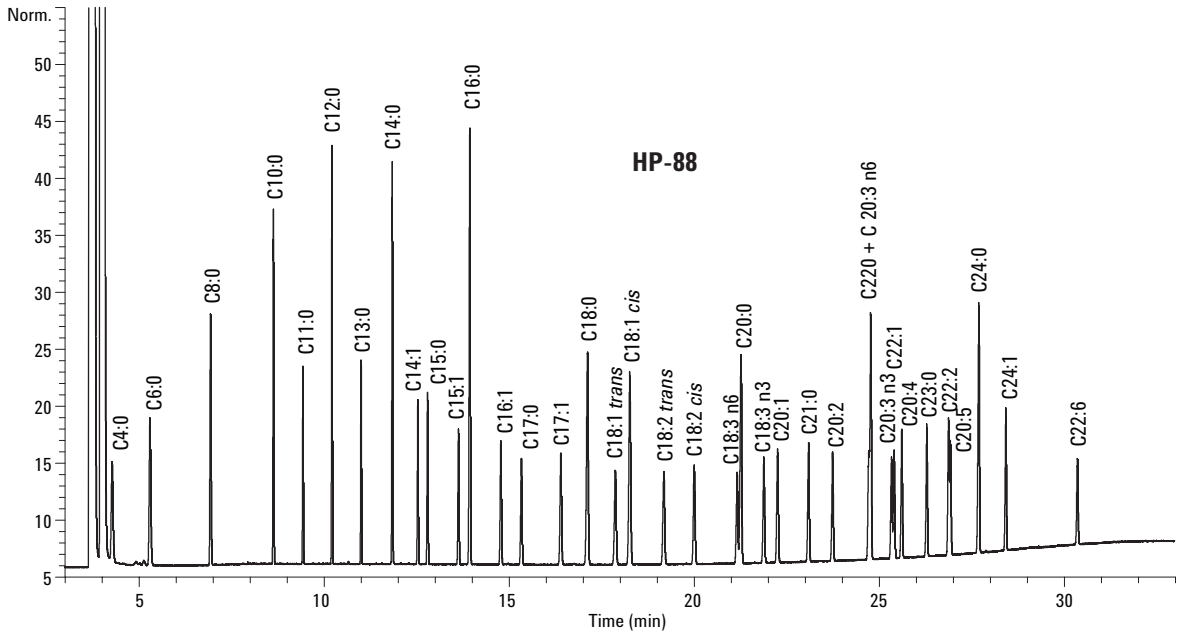

Figure 6. GC-FID analysis of 37-component FAMEs standard mixture on a 100 m × 0.25 mm ID, 0.2 µm HP-88 column using Method 3A, Table 4A.

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.

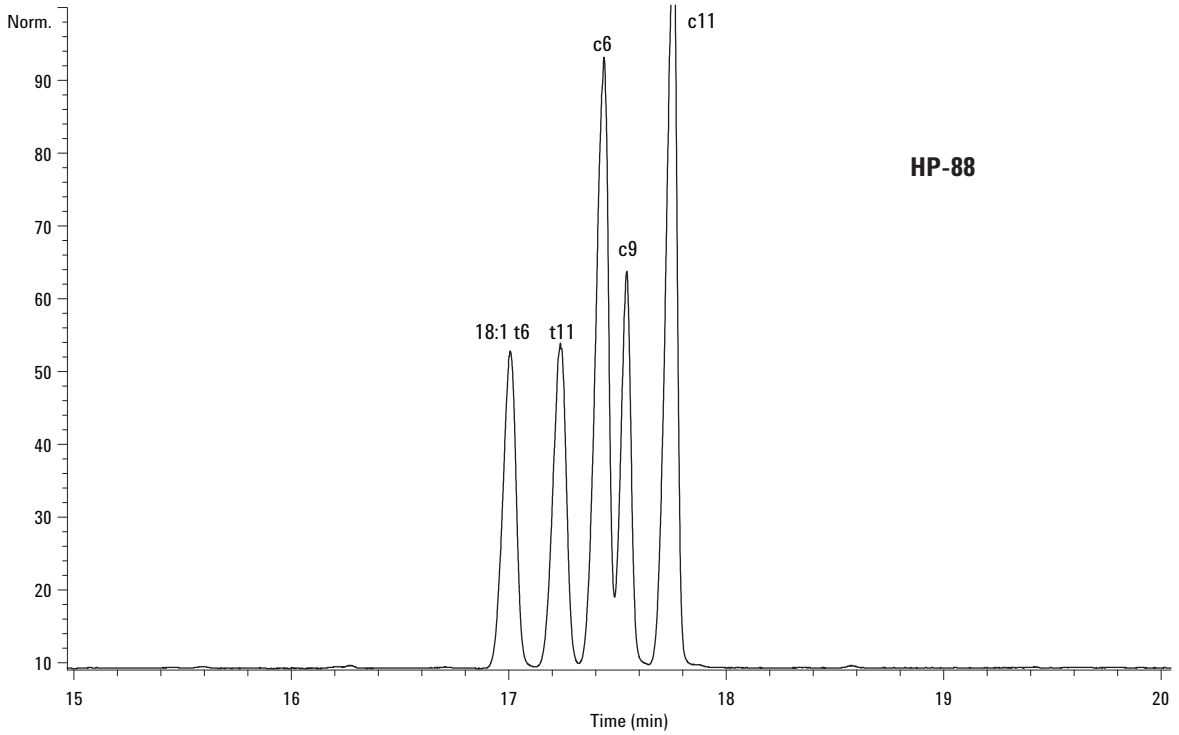

Figure 7. GC-FID analysis of C18:1 isomers on a 100 m × 0.25 mm ID, 0.2 µm HP-88 column using Method 3A, Table 4A.

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

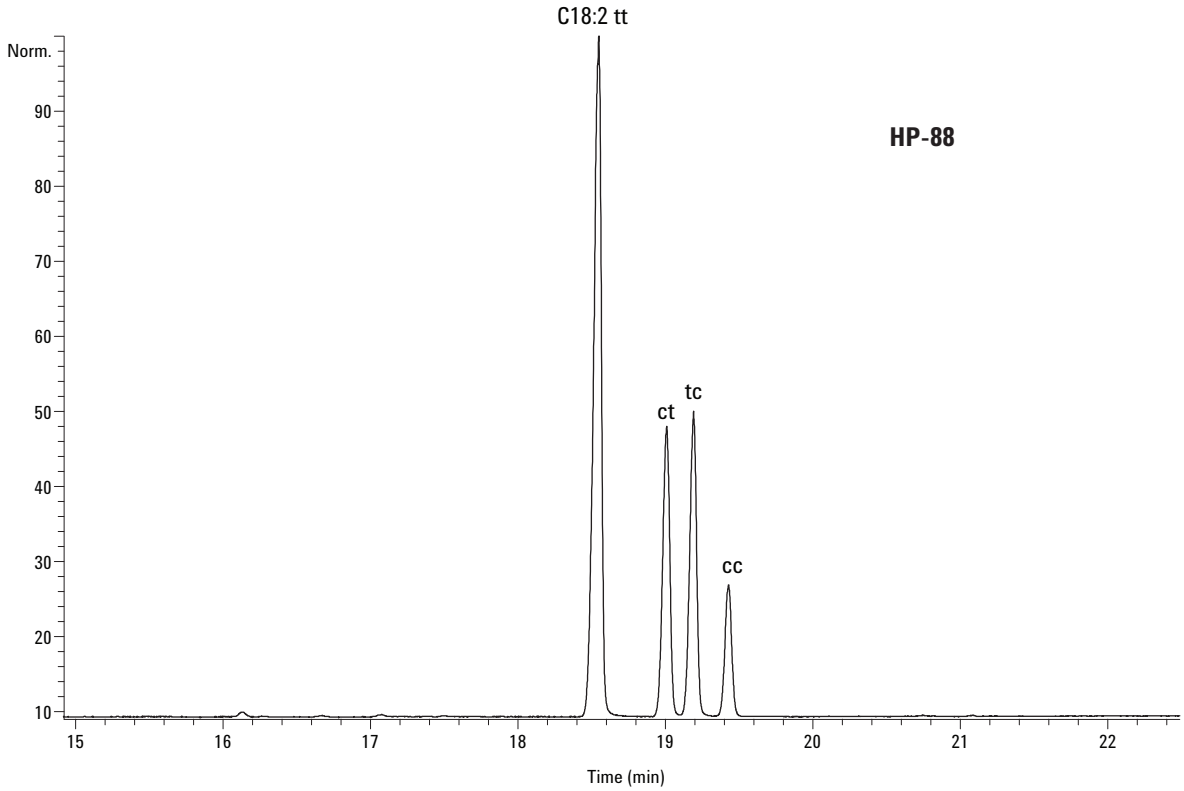

Figure 8. GC-FID analysis of C18:2 isomers on a 100 m × 0.25 mm ID, 0.2 µm HP-88 column using Method 3A, Table 4.

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.

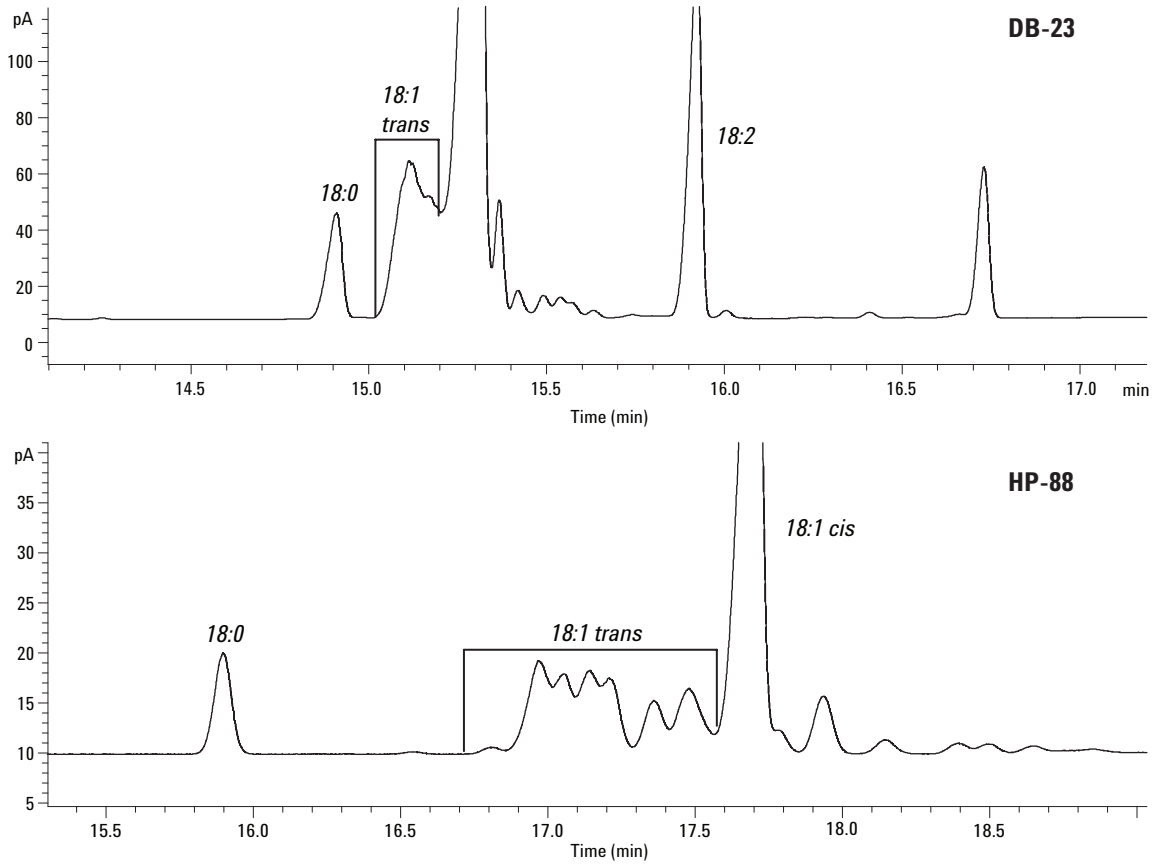

Figure 9. Comparison of the separation of C18:1 isomers from a hydrogenated oil obtained on a 60 m x 0.25 mm ID, 0.15 µm DB-23 column (upper window) and on a 100 m × 0.25 mm ID, 0.2 µm HP-88 column. Both analyses were performed at 180 °C isothermally.

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.

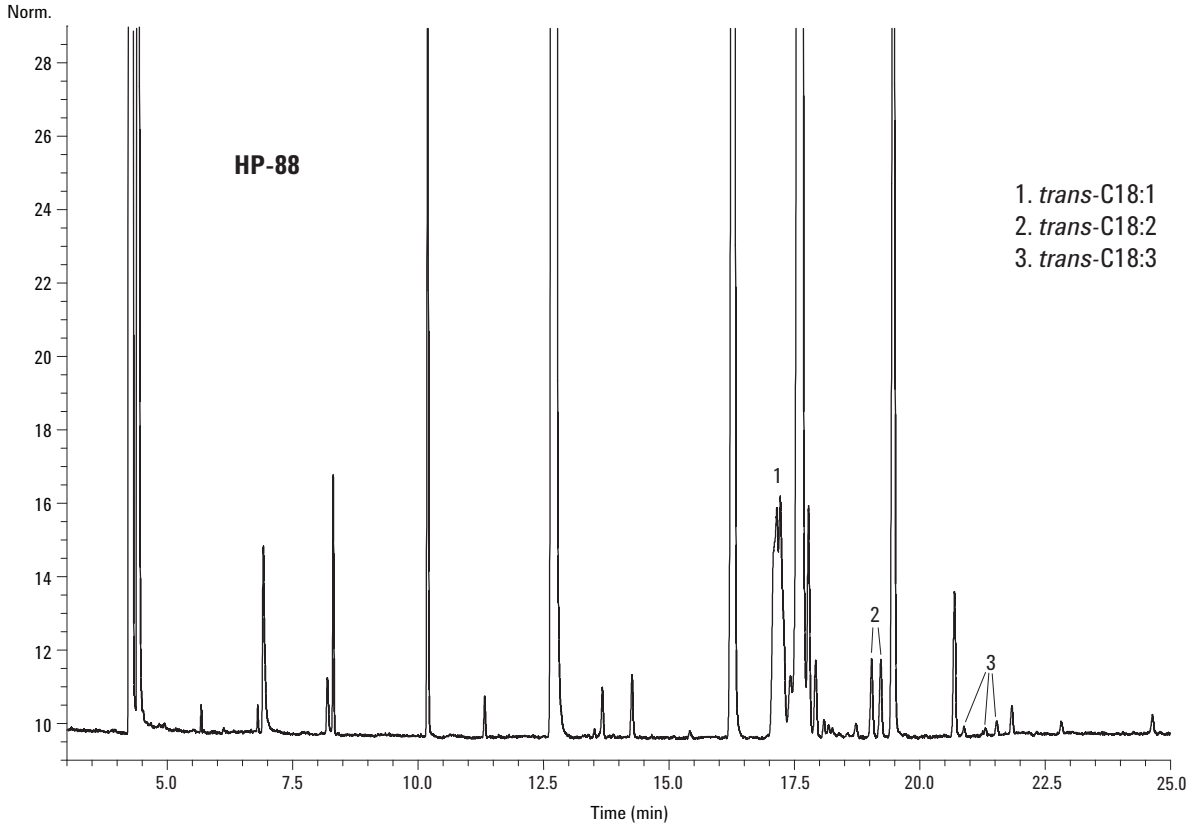

Figure 10. GC-FID analysis of FAMEs from a partially hydrogenated rapeseed oil on a 100 m × 0.25 mm ID, 0.2 µm HP-88 column using Method 3A. (See Table 4).

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].

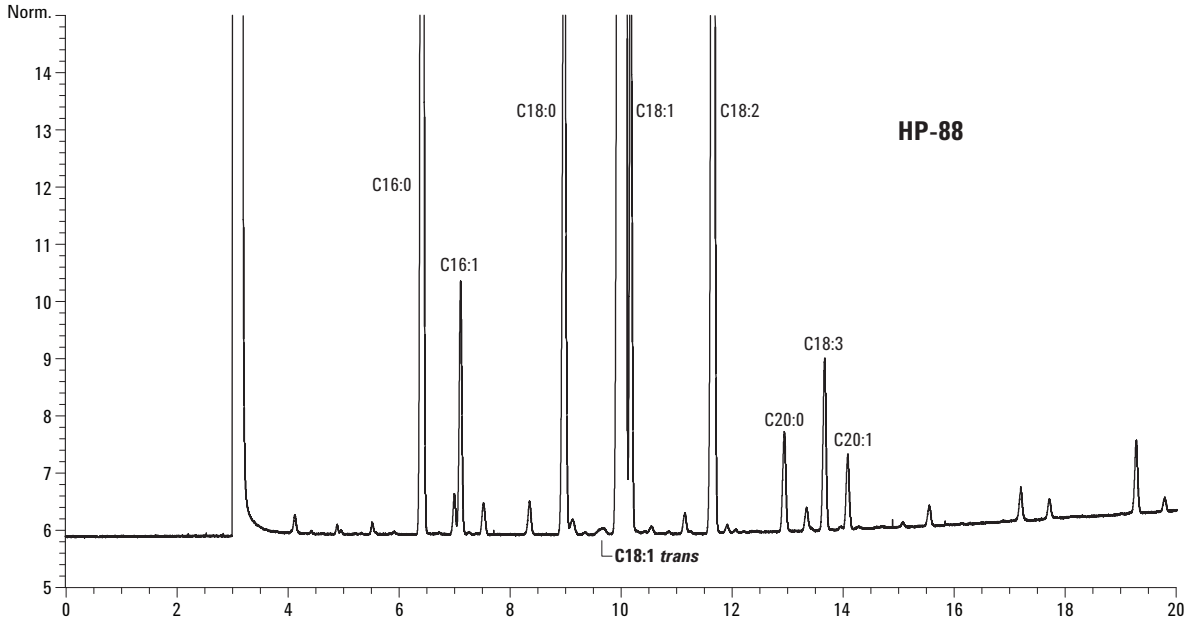

Figure 11. GC-FID analysis of olive oil FAMEs on a 60 m × 0.25 mm ID, 0.2 µm HP-88 column using Method 3B, Table 4.

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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2. AOAC Official Methods of Analysis (2000), method Ce 2–66.
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4. International Standard ISO 15304, version 2003-05-15 (ISO 15304/2002) (can be ordered from www.iso.org)
5. EU regulation 2568/91, *Official Journal EU*, document L248, 5/9/1991.
6. EC, Bureau of Reference (BCR), Brussels, Belgium (see catalog at www.irmm.jrc.be)

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High Throughput Separation of Xanthines by Rapid Resolution HPLC

Application Note

Biochemistry, Food and Beverage, Pharmaceutical

Author

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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.

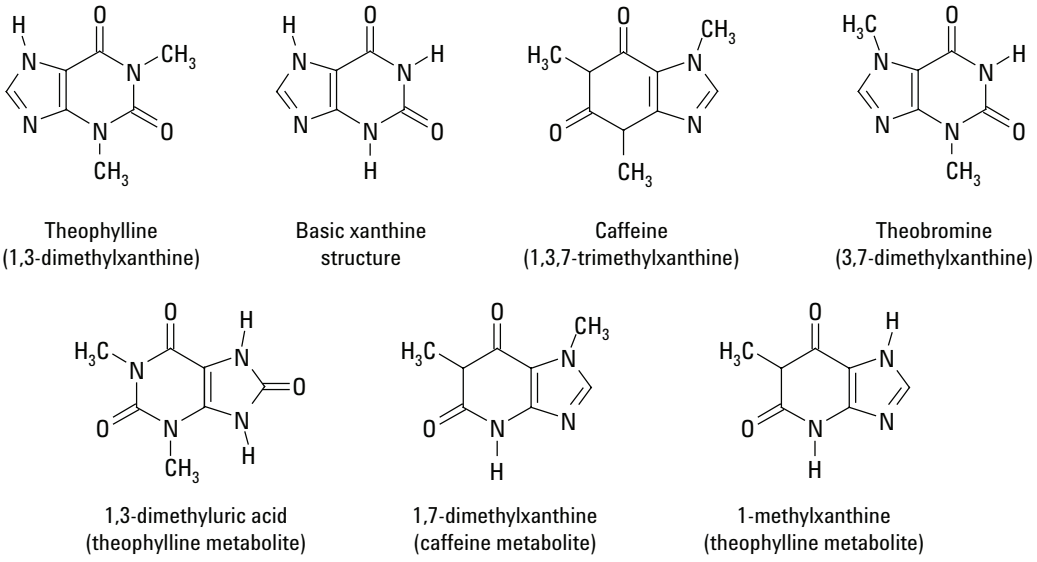


Figure 1. Structures of selected xanthines and metabolites used in this study.

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.

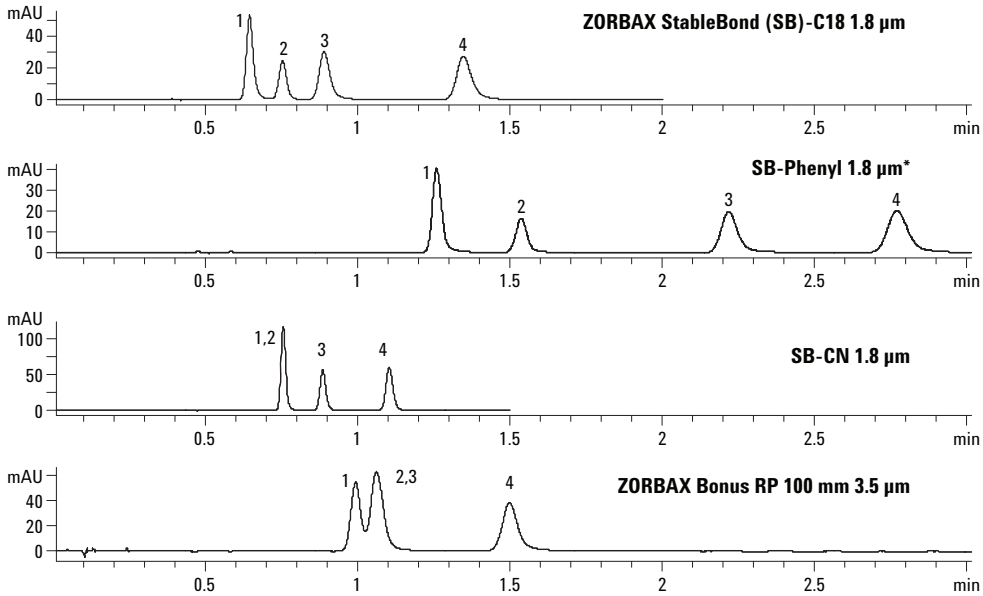


Figure 2. ZORBAX stationary phase selectivity comparisons for xanthines.

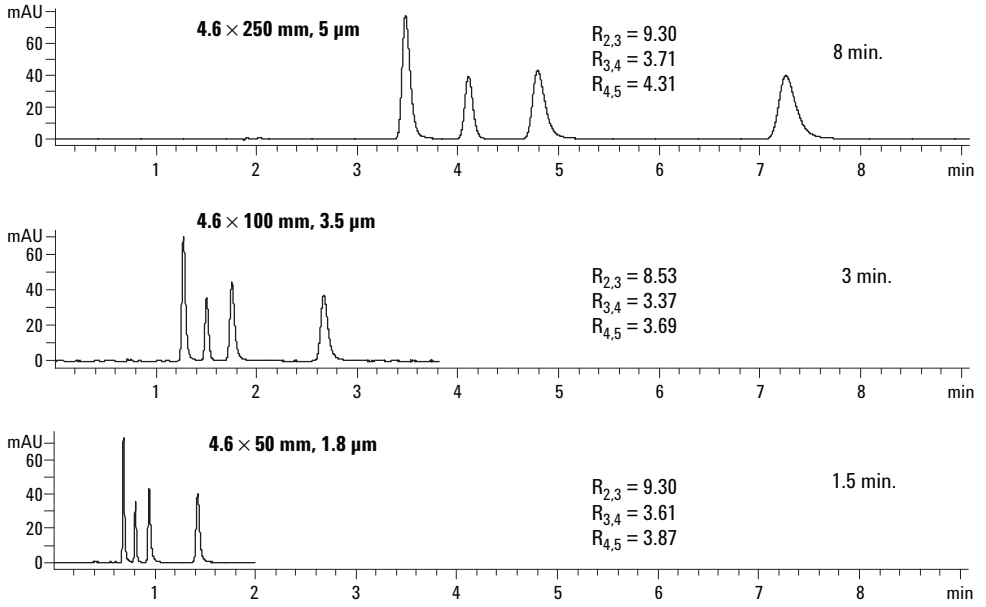
The chromatographic conditions chosen for subsequent experiments appear below:

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.

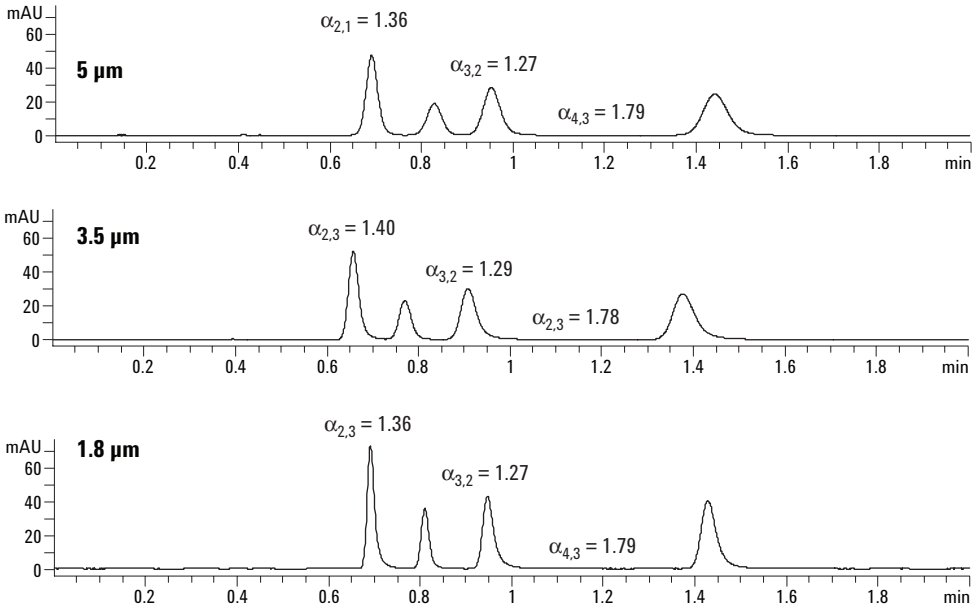
Of course, as one decreases the particle size of a column, the column backpressure increases with the inverse square of the particle diameter. Thus, if the same column length was used, the pressure at the same flow rate (or more correctly the linear velocity), the pressure would go up by a factor of 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.

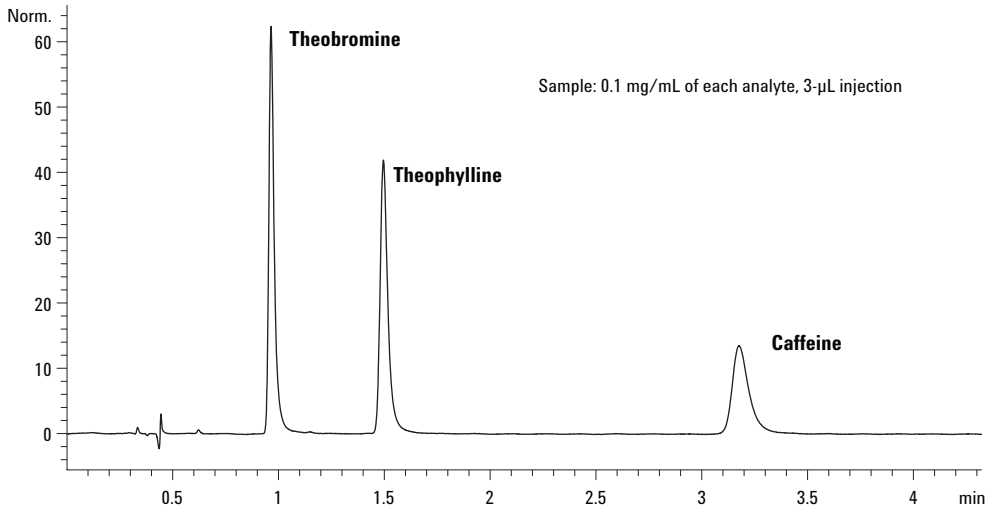
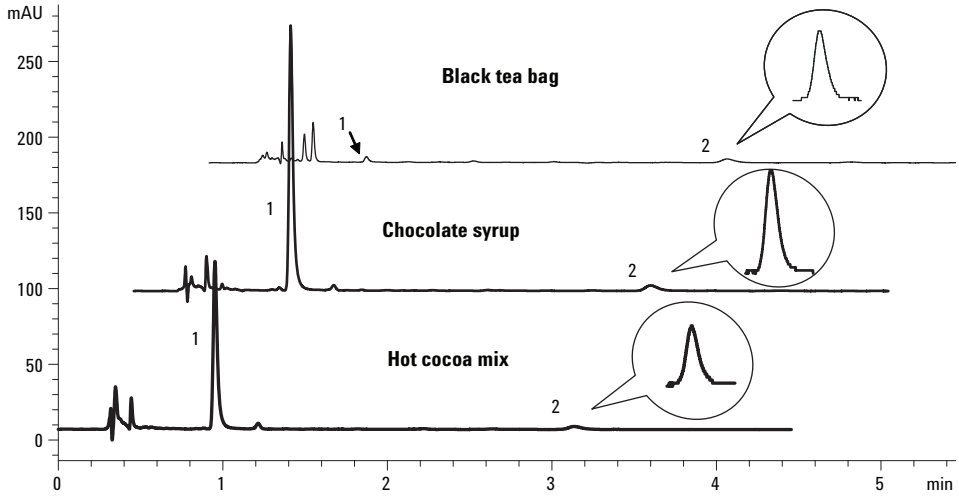


Figure 5. Separation of xanthine standards.



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.

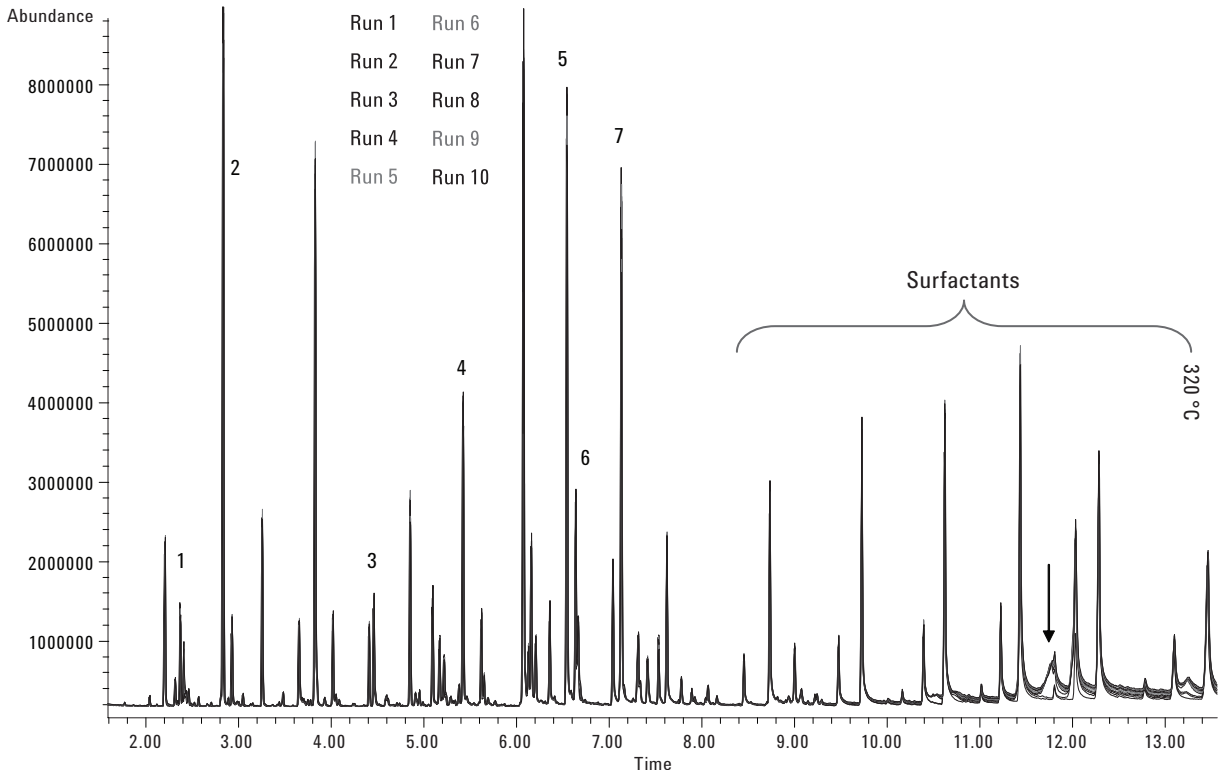

Figure 1. Overlay of 10 consecutive analyses of shampoo extract (oven temperature programmed to 320 °C, no backflush).

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.

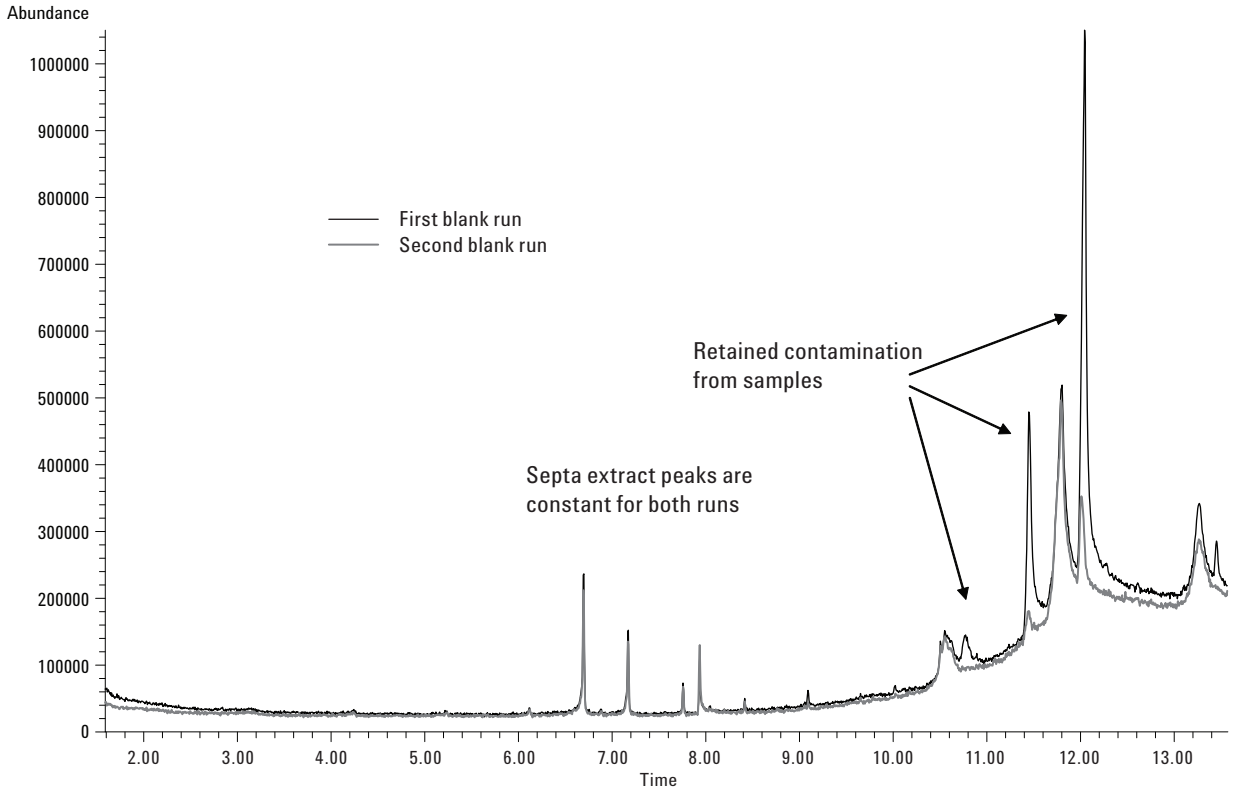

Figure 2. Two consecutive blank runs after analysis of shampoo extract.

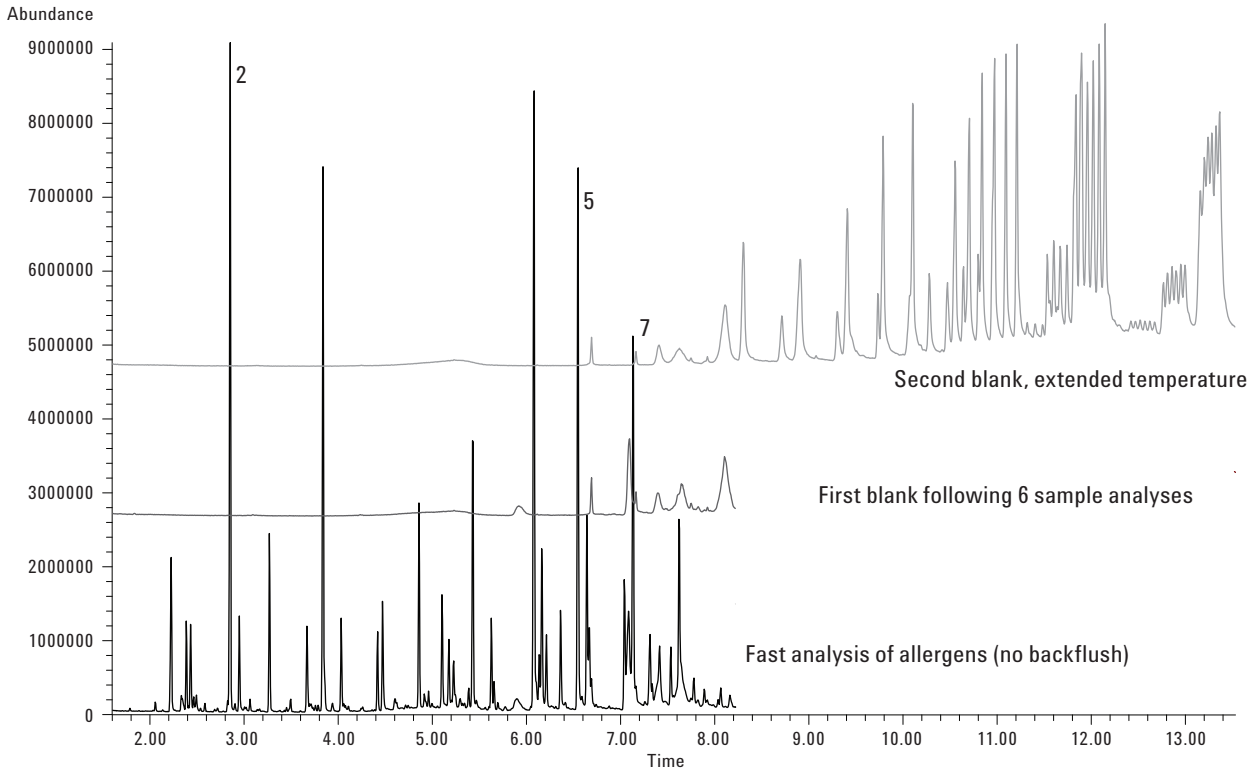

Figure 3. Overlay of sixth analysis of shampoo extract with run stopped at 240 °C (bottom), first blank run to 240 °C (middle), and second blank run to 320 °C (top).

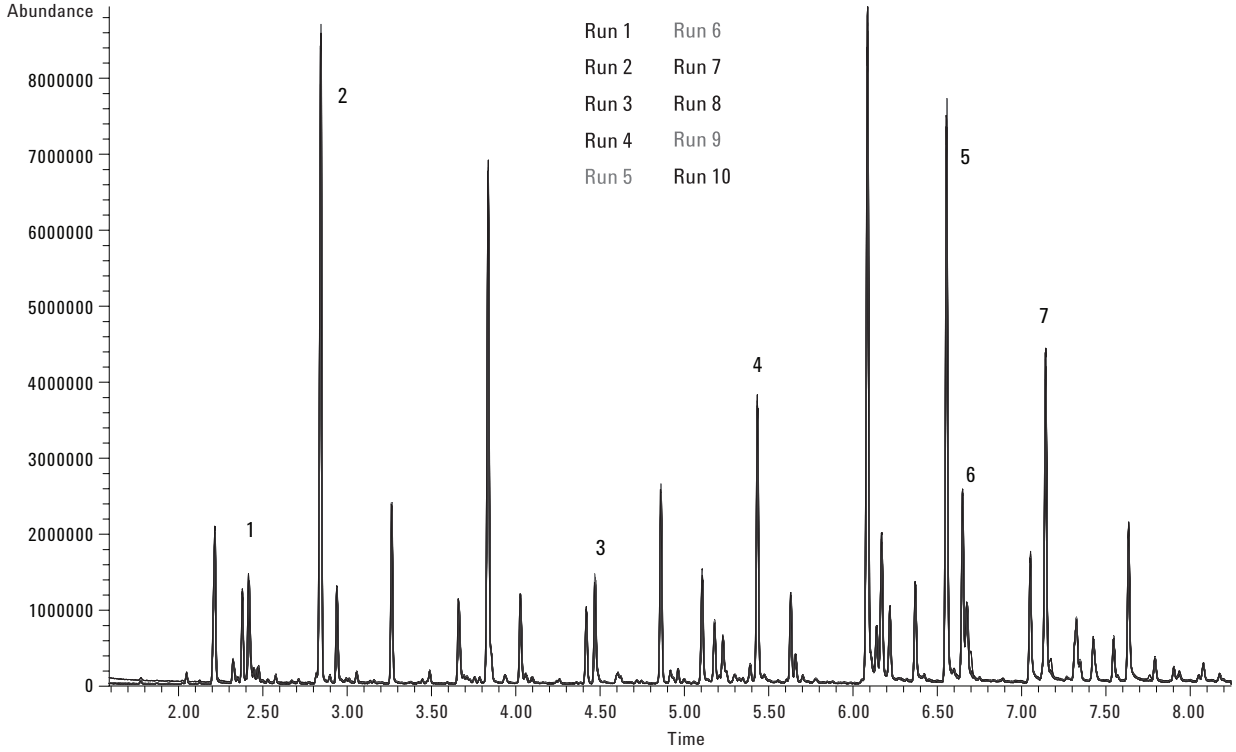

Figure 4. Overlay of 10 consecutive analyses of shampoo extract (oven temperature programmed to 240 °C, with backflush).

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.

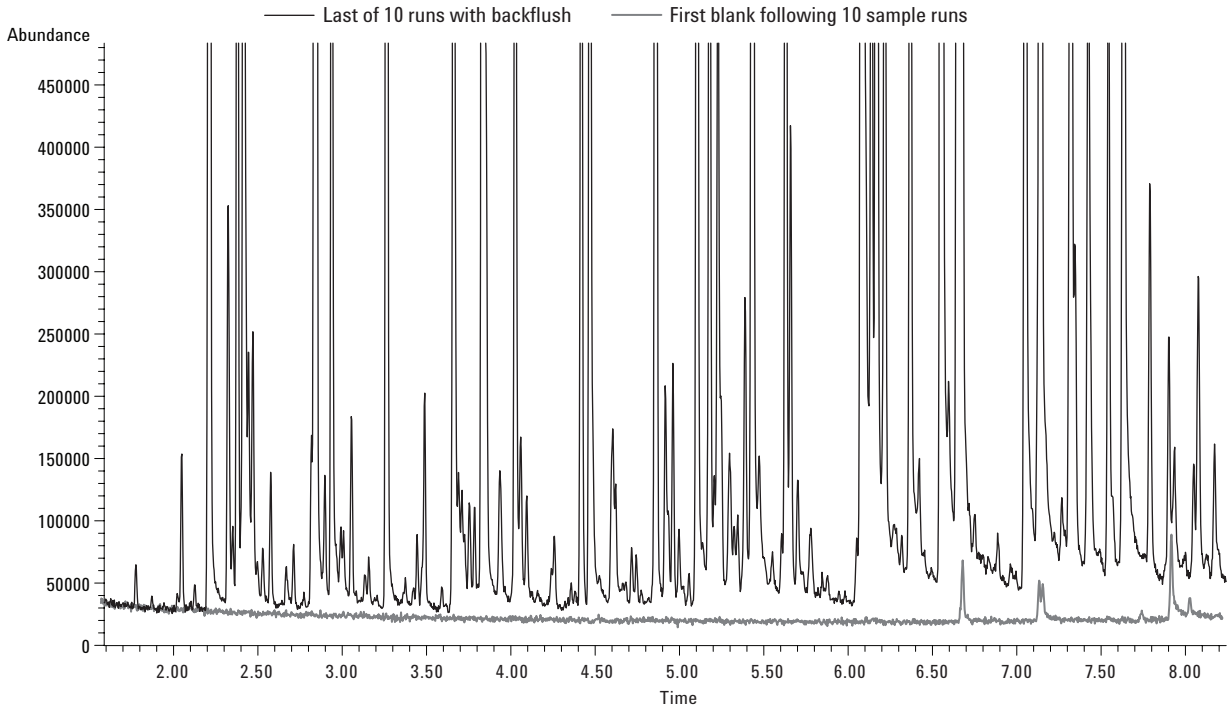

Figure 5. Overlay of 10 analyses of shampoo extract (oven programmed to 240 °C) with backflush (top) and subsequent blank run (bottom).

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.

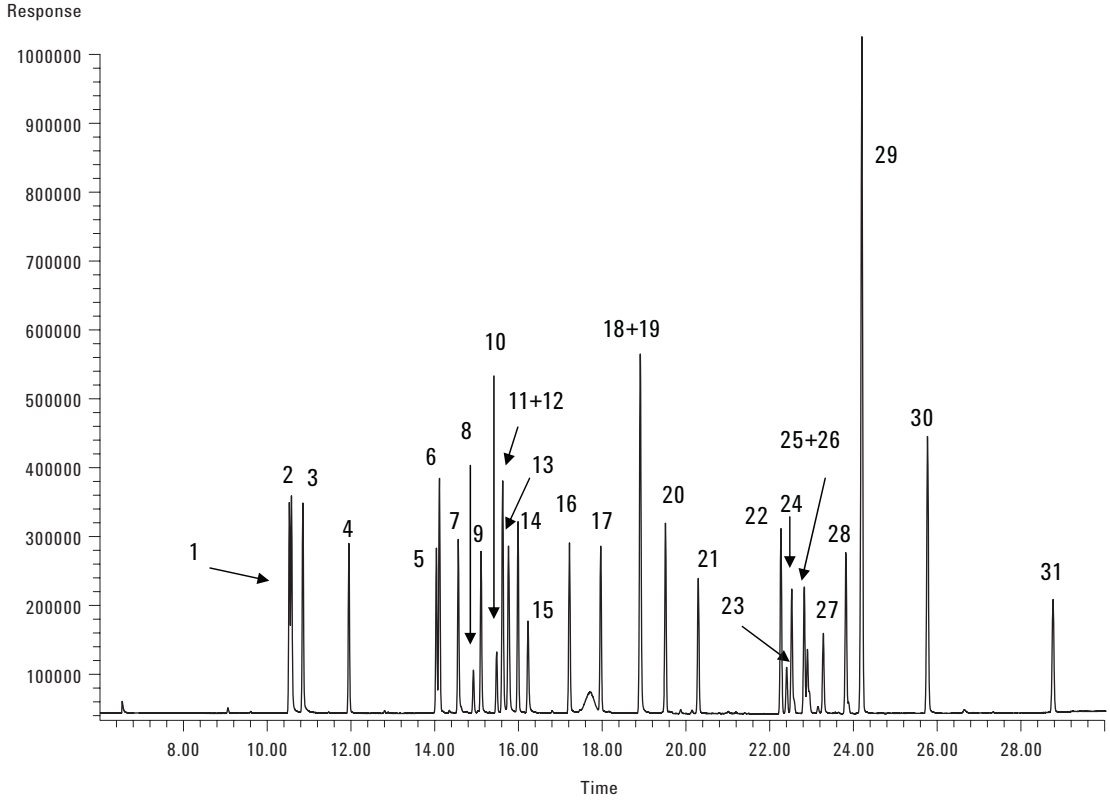

Figure 1. Separation of flavor and fragrance allergen test mixture (100 ppm) on the first dimension column (Agilent J&W HP-5MS) and FID detection. Peak identification is given in Table 1.

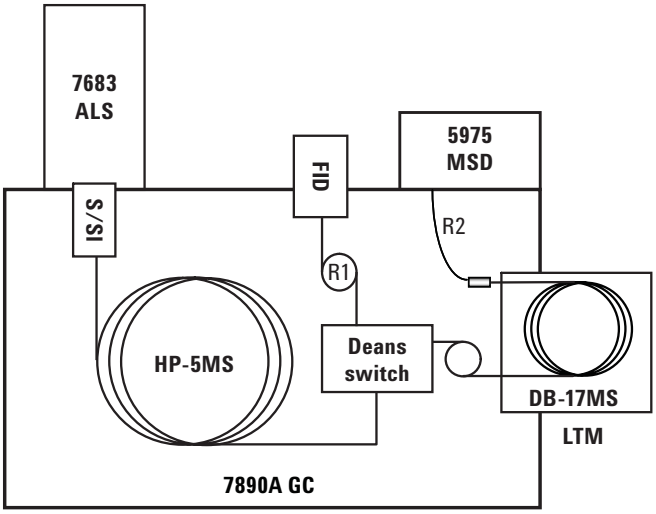

Figure 2. System configuration.

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.

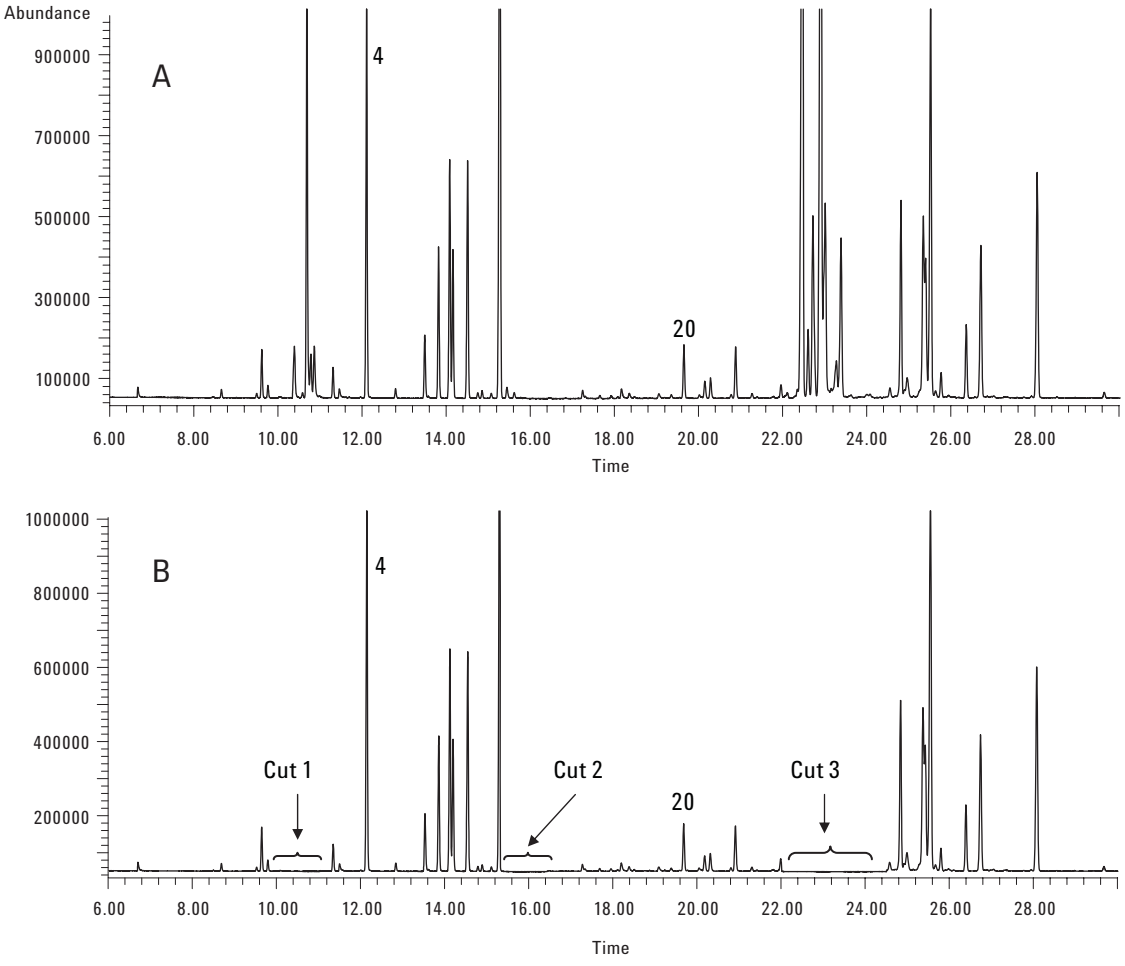

Figure 3. A) Separation of a perfume sample on the first-dimension column (Agilent J&W HP-5MS) using FID detection without heartcutting. Peaks: 4. Linalool; 20. Alpha-isomethyl ionone. B) Separation of a perfume sample on the first-dimension column (Agilent J&W HP-5MS) using FID detection with heartcutting (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.

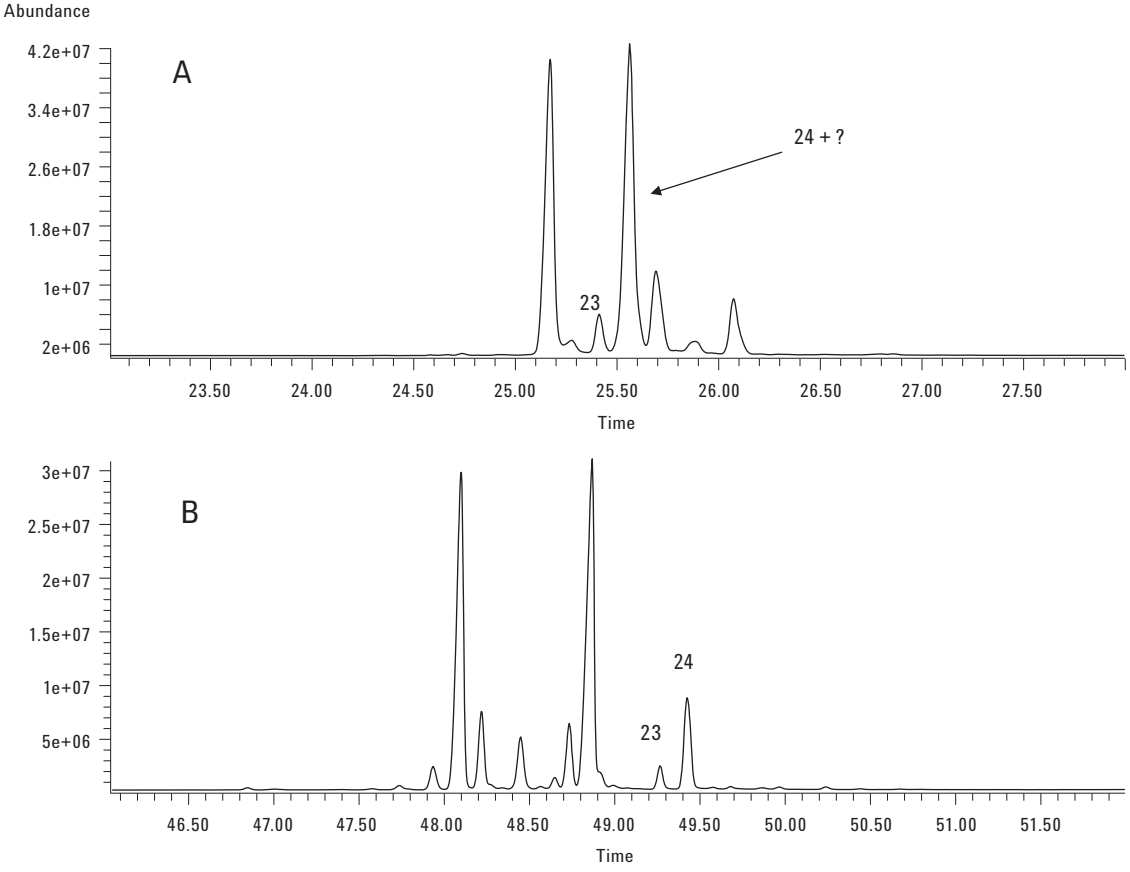

Figure 4. A) Separation of fraction 3 on the second-dimension column (Agilent J&W DB-17ms) using MS detection. Column 1 temperature = column 2 temperature: 50 °C (1 min) → 270 °C at 8 °C/min. Peaks: 23. Lyral 1; 24. Lyral 2. B) Separation of fraction 3 on the second-dimension column (Agilent J&W DB-17ms)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

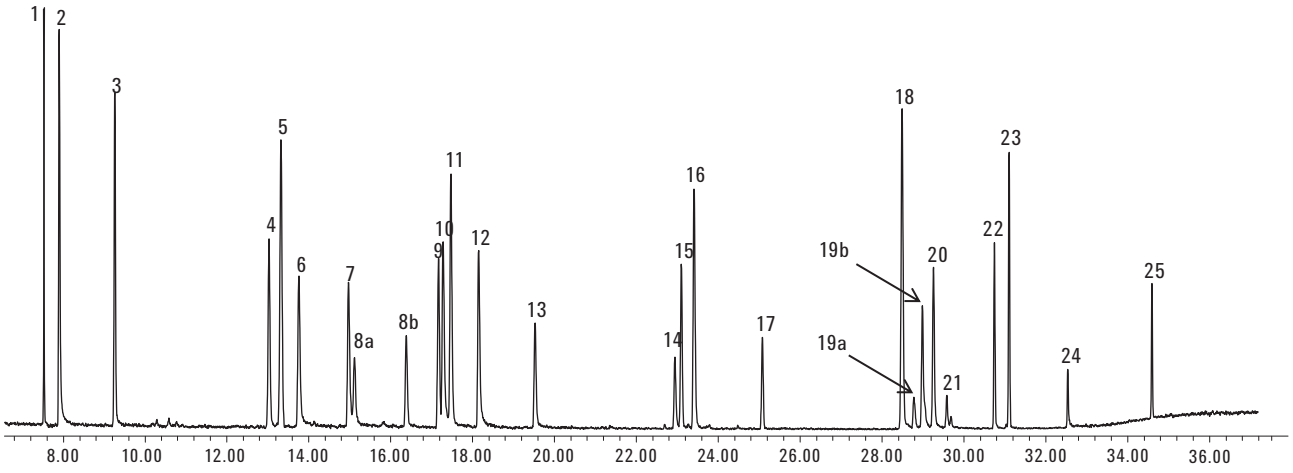
Most of the EU listed allergens fall into two chemical categories; aldehydes and alcohols. Both of these chemical classes of compounds are comprised of active analytes that can often lead to poor chromatographic performance. Using the DB-XLB column, excellent resolution and peak shape was achieved for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

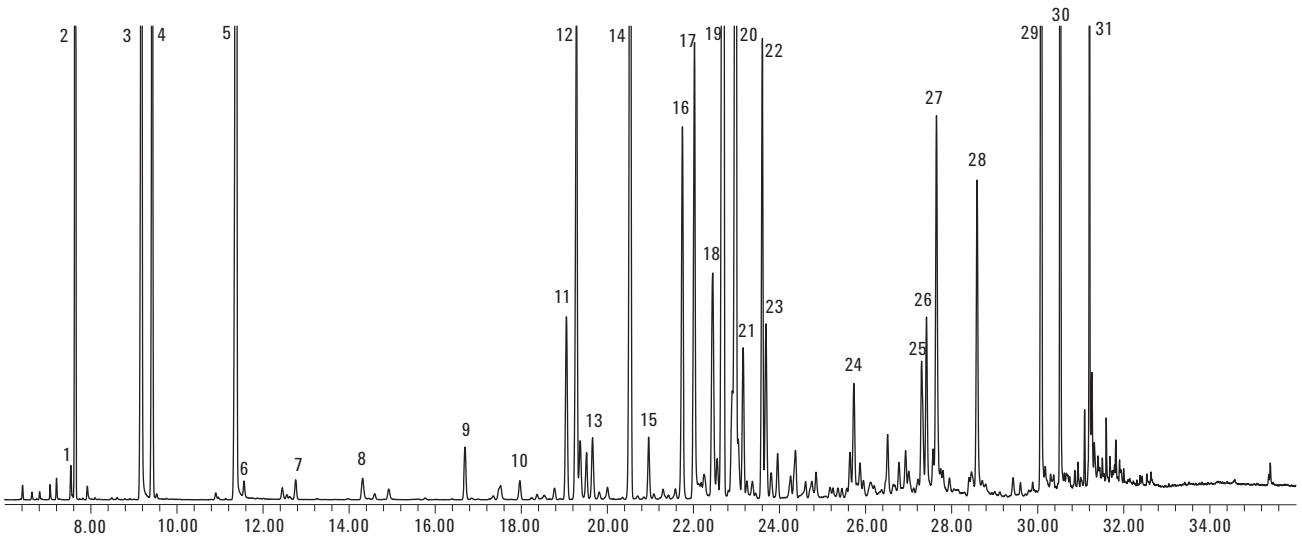


EU Flavor and Fragrance Allergens

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.

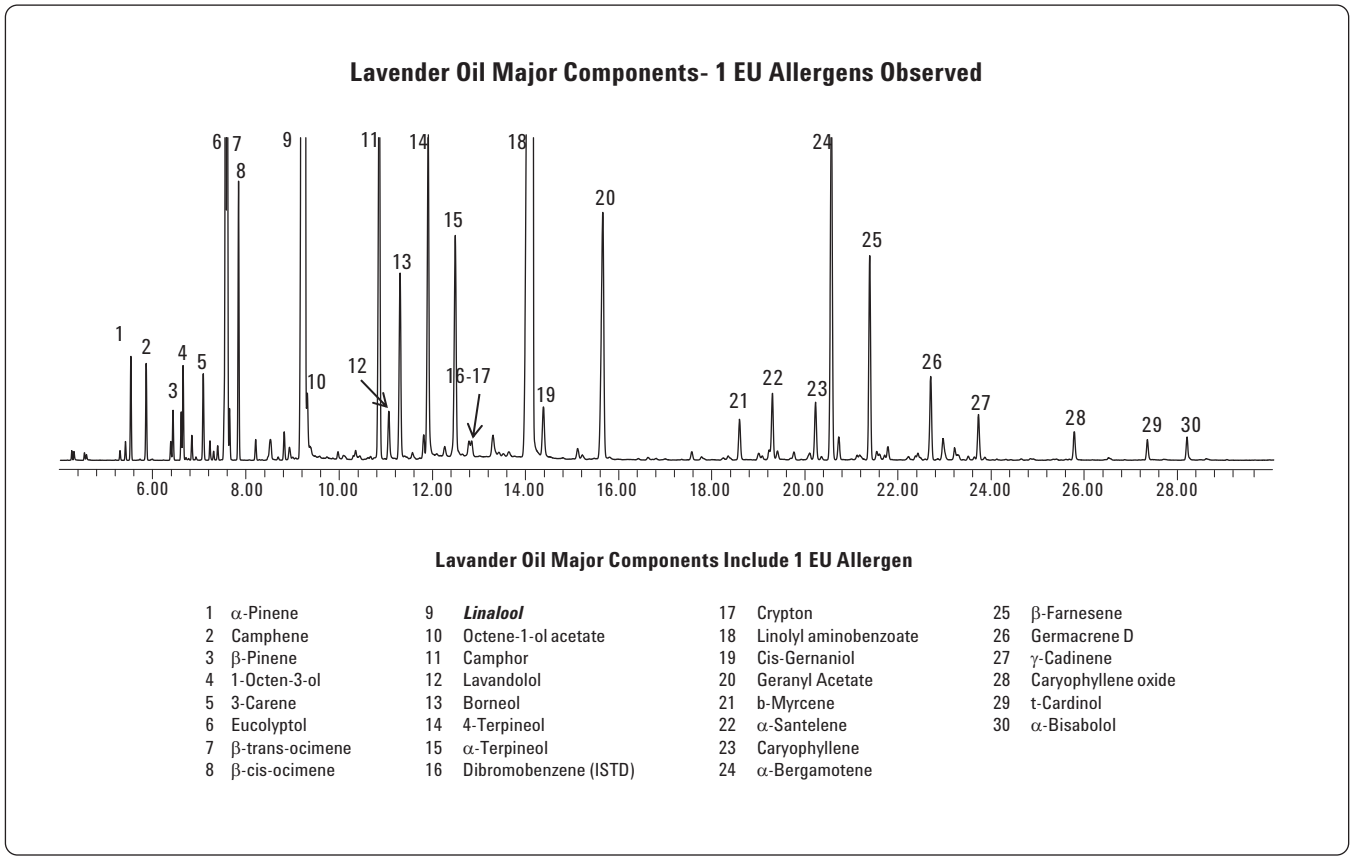


Figure 3. Total ion chromatogram of lavender oil diluted 1:20 in acetonitrile on an Agilent J&W DB-XLB ms 30 m × 0.25 mm × 0.25 µm column (p/n 122-4712). Y scale in this figure ranges from 0 to 5 x 10^6 counts, peak number 16 is the internal standard. GC conditions are in Table 2.

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.

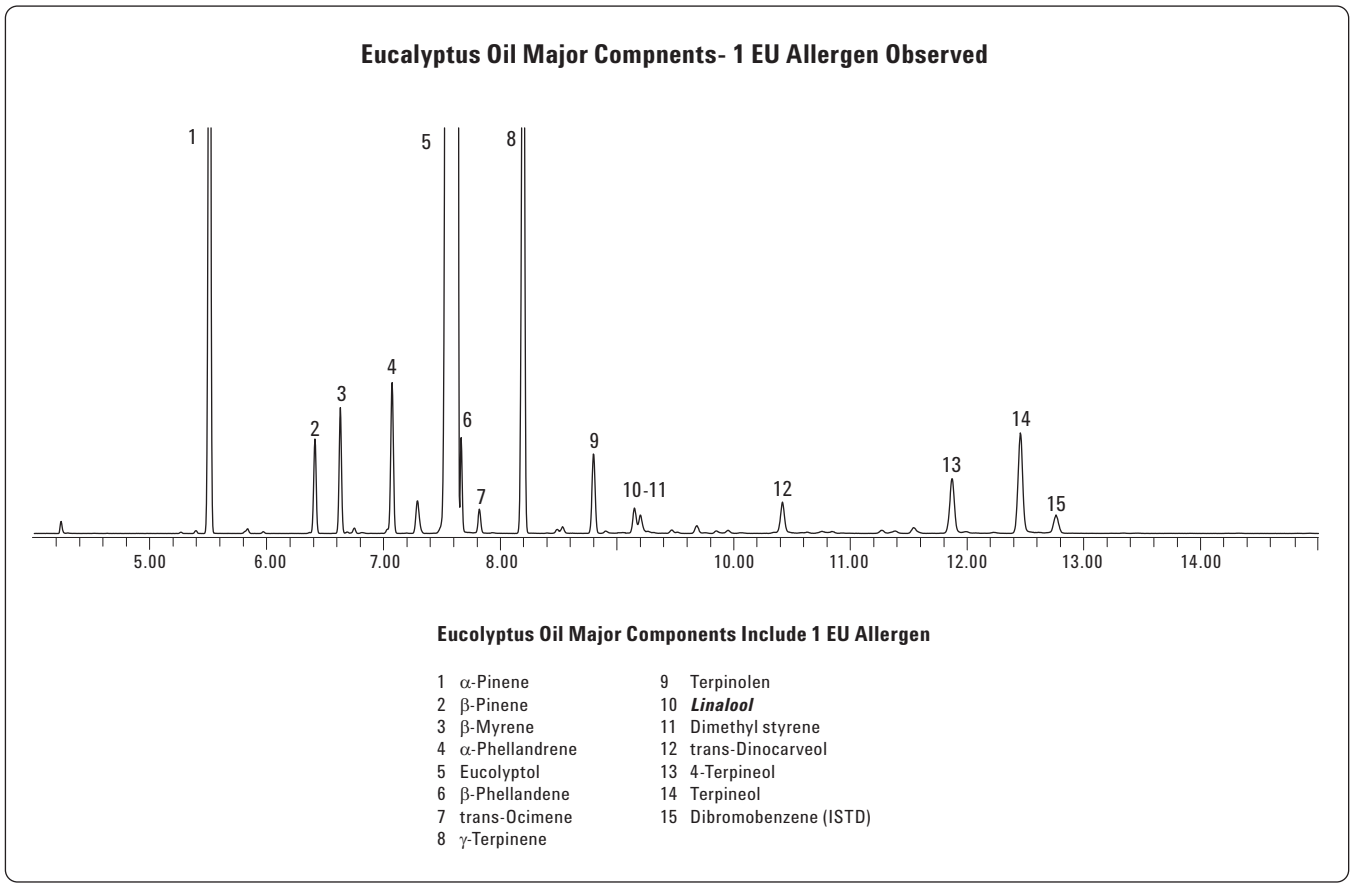


Figure 4. Total ion chromatogram of eucalyptus oil diluted 1:20 in acetonitrile on an Agilent J&W DB-XLB 30 m × 0.25 mm × 0.25 µm column (p/n 122-4712). Y scale in this figure ranges from 0 to 5 x 10^6 counts, peak number 15 is the internal standard. GC conditions are in Table 2.

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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

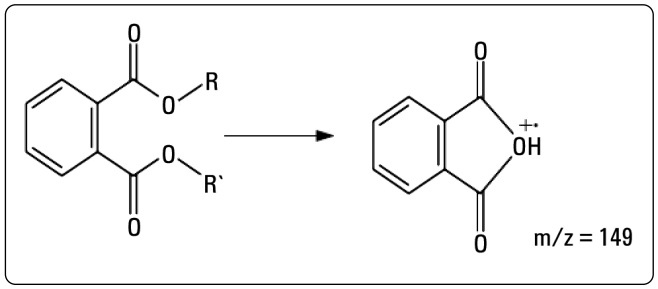

Figure1. Primary structure of phthalates and major fragmentation of phthalate esters in EI ionization (The dominant ion in the EI spectrum is typically m/z 149. The exception is for dimethyl phthalate where both R and R' are CH_3, so the H on the oxygen is replaced by CH_3 and m/z 163 becomes the base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

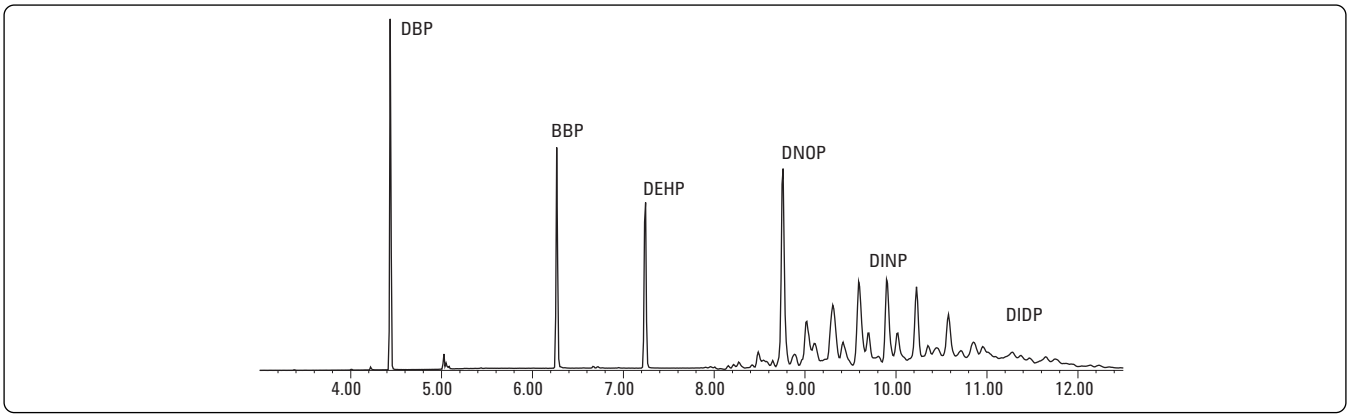

Figure 2. TIC of six regulated phthalates (DBP, BBP, DEHP and DNOP, 5 ppm each; DINP and DIDP 50 ppm each).

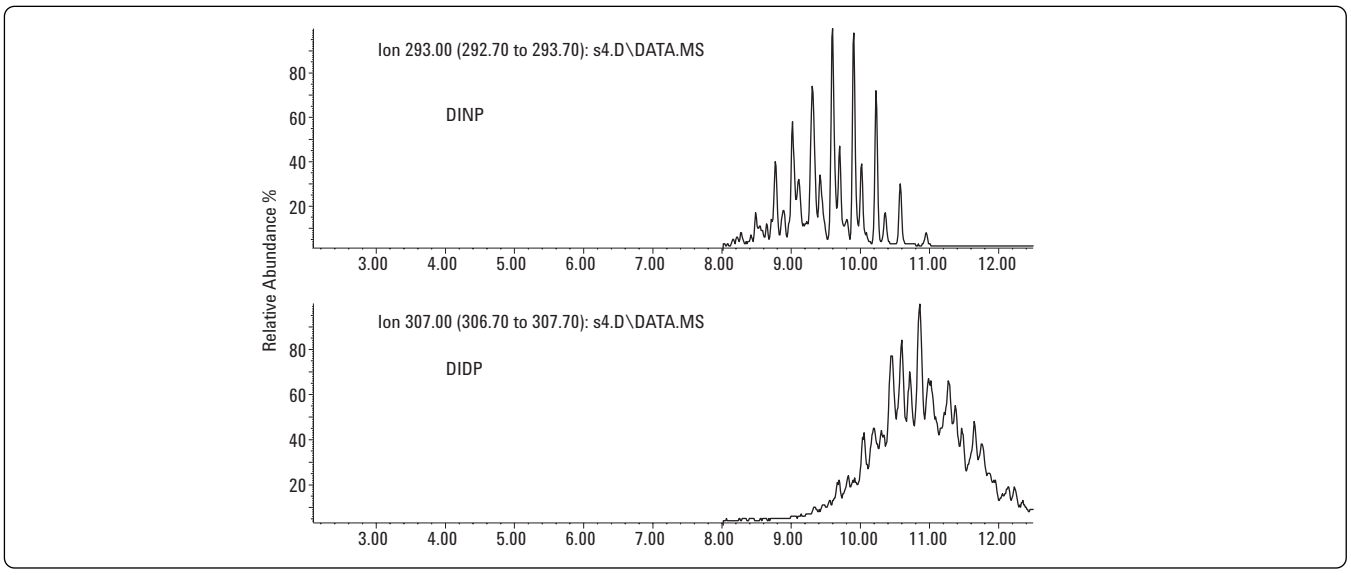

Figure 3. Extracted ion chromatogram of DINP at m/z 293 and DIDP at m/z 307.

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).

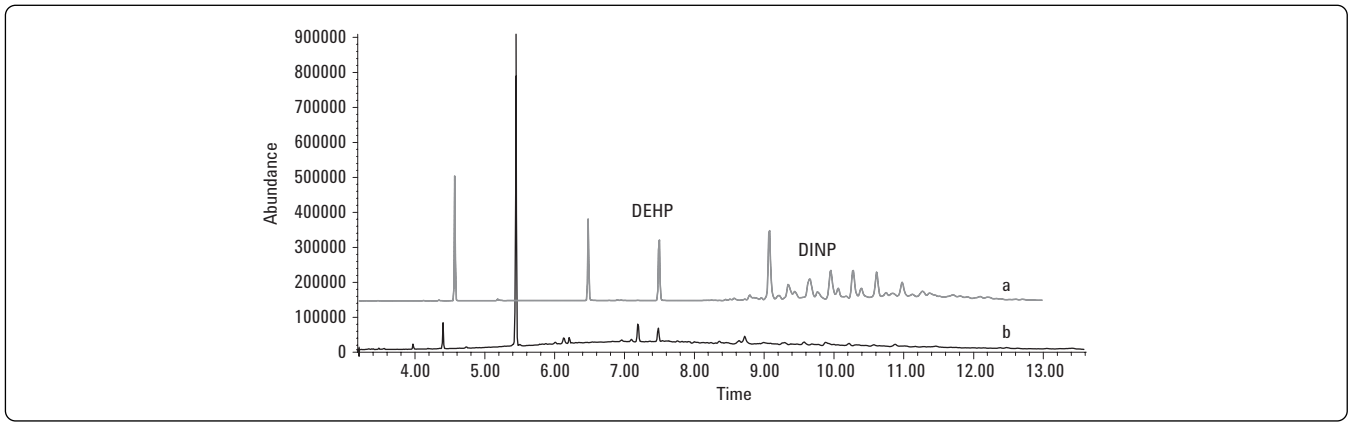

Figure 4. Chromatographic results for the GC/MS analysis of one PVC toy sample. (a) TIC of phthalates standards, (b) TIC of real sample.

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

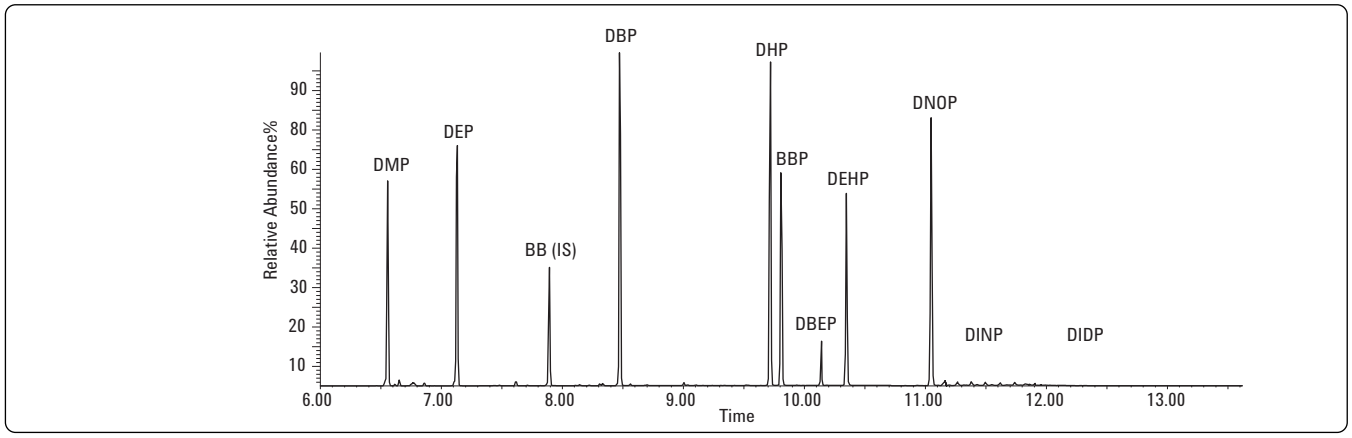

Figure 5. TIC of phthalates at 5 ppm.

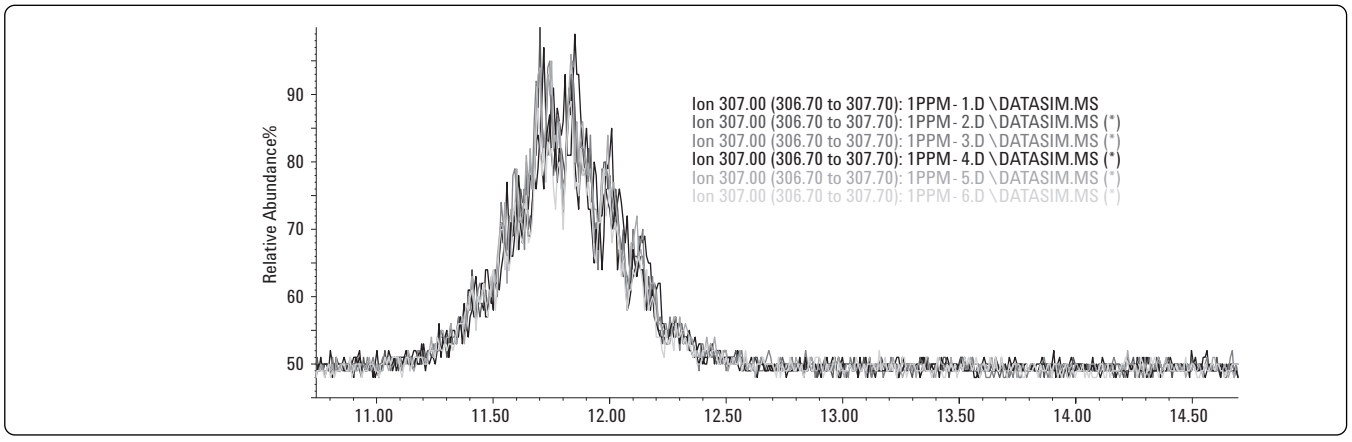

Figure 6. Reproducibility of DIDP from six injections at 1 ppm.

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%.

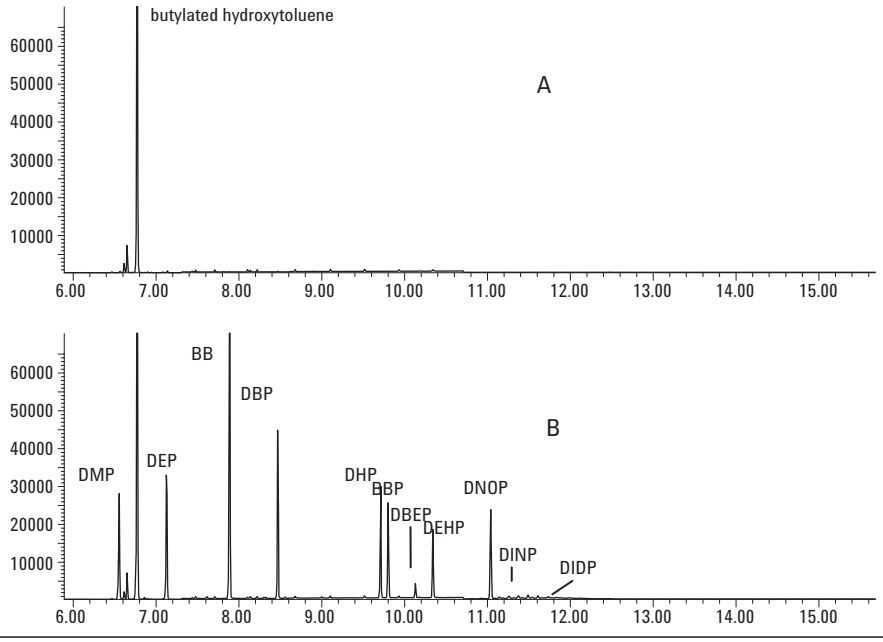

Figure 7. TIC of infant pacifier extract (A) and sample # 2 (pacifier) extract spiked with 2-ppm phthalate mixture (B).

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

www.agilent.com/chem

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October 21, 2009
5990-4863EN

Agilent Technologies

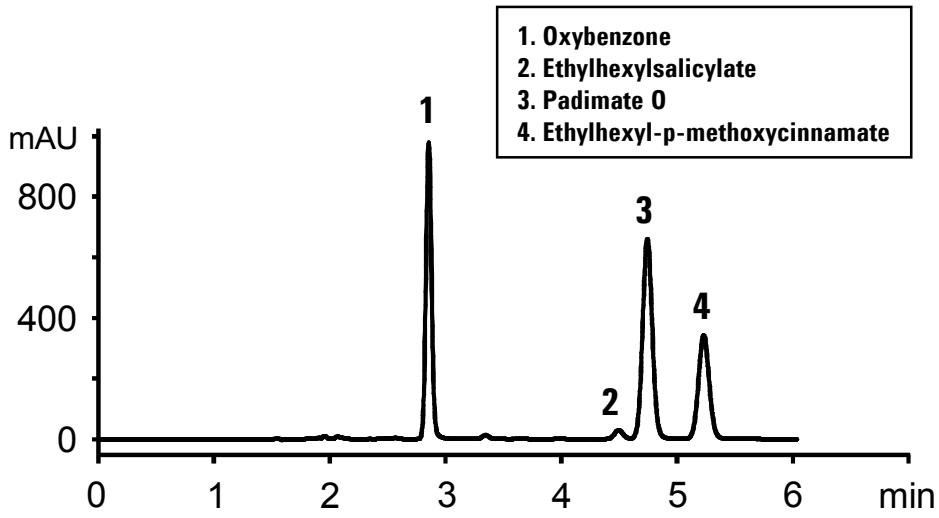
Analysis of Sunscreen Lotion Extract

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.

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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.

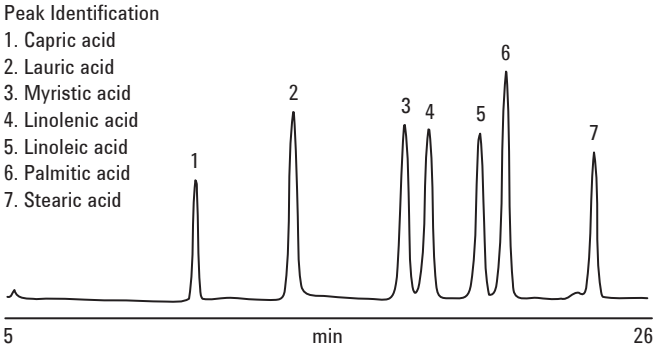

Figure 1. Separation of seven fatty acids using PLRP-S media.

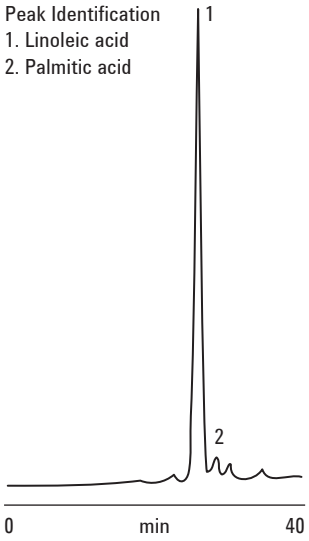

Figure 2. Fatty acid composition of evening primrose oil revealed by PLRP-S.

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).

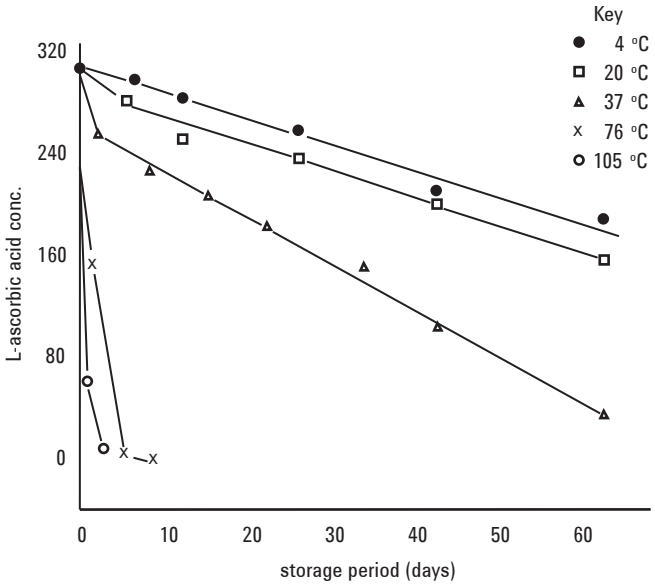

Figure 1. L-ascorbic acid concentration over time in orange juice in TetraBrik cartons at different storage temperatures.

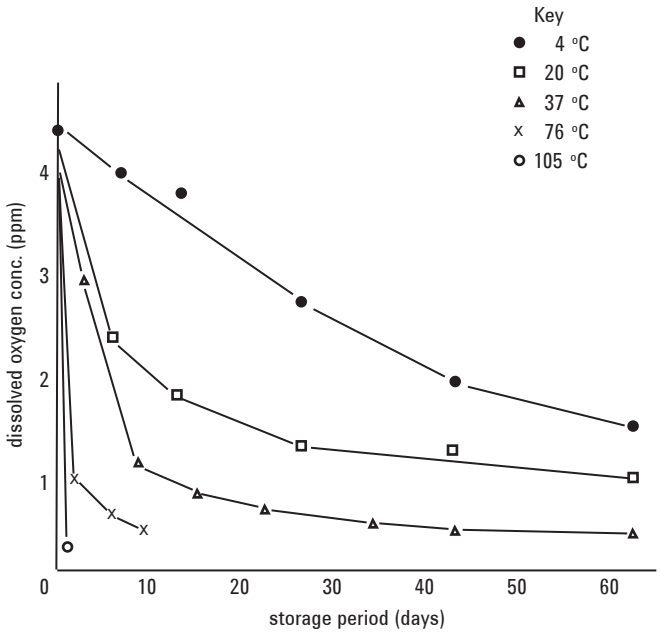

Figure 2. Dissolved oxygen levels vs storage time in TetraBrik cartons at different temperatures.

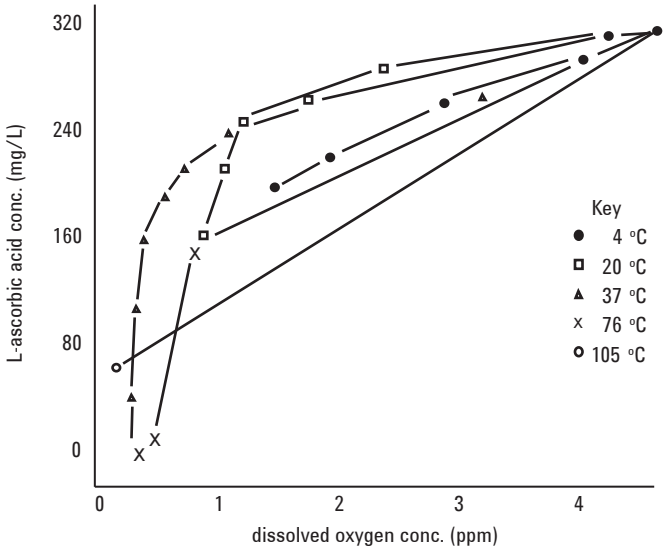

Figure 3. Loss of L-ascorbic acid vs dissolved oxygen level.

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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This information is subject to change without notice.

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Agilent Technologies

Agilent Application Solution

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

Application Note

Food

Author

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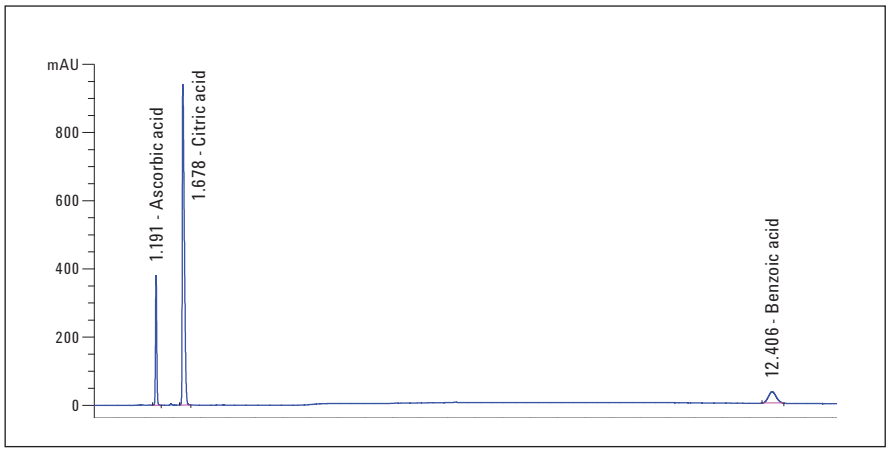

Abstract

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.

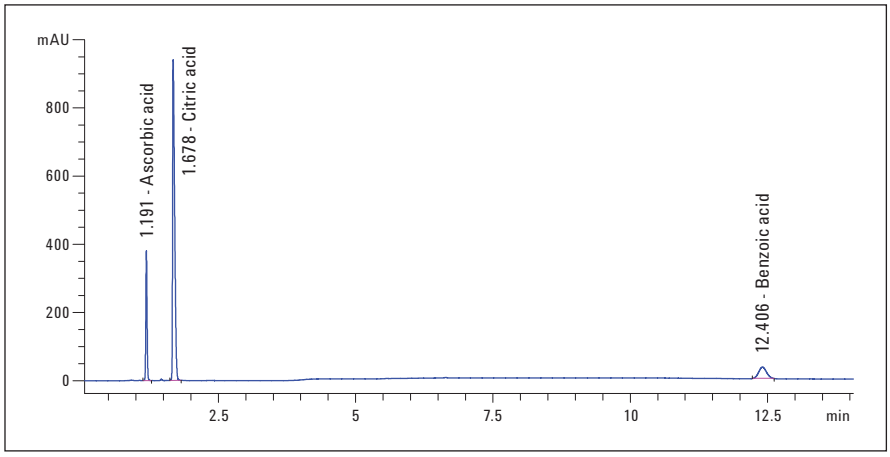

Figure 1
Separation of three standards ascorbic acid, citric acid and benzoic acid using an Agilent Poroshell 120 EC-C18 column. The chromatogram was collected at 230 nm.

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.

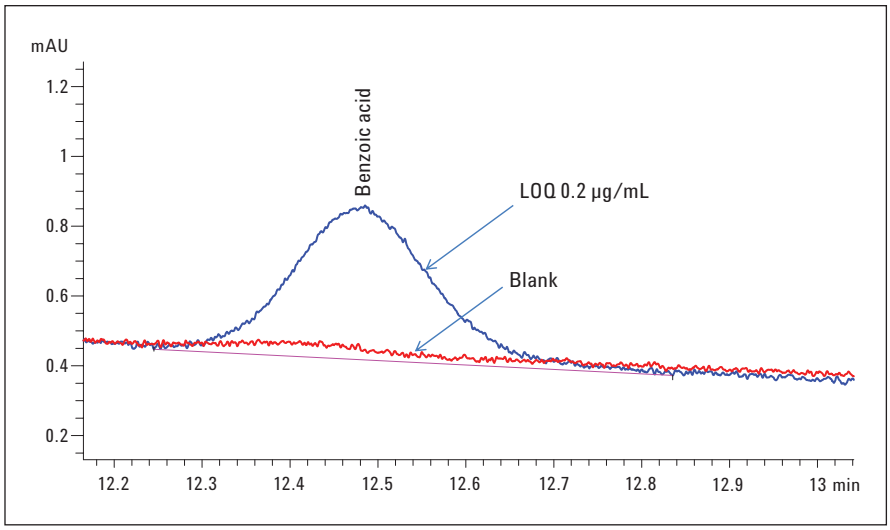

Figure 2
A 0.2 µg/mL (1 ng on column) solution of benzoic acid (at LOQ level) overlaid with blank injection. S/N ratio obtained at this concentration was 16.

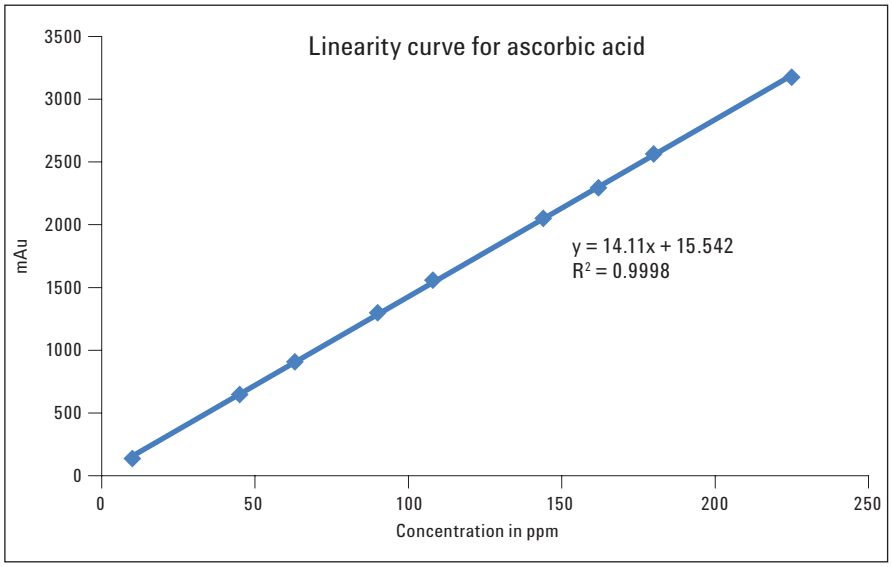

Figure 3
Linearity level of ascorbic acid from 10 µg/mL to 225 µg/mL showing the coefficient value.

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.

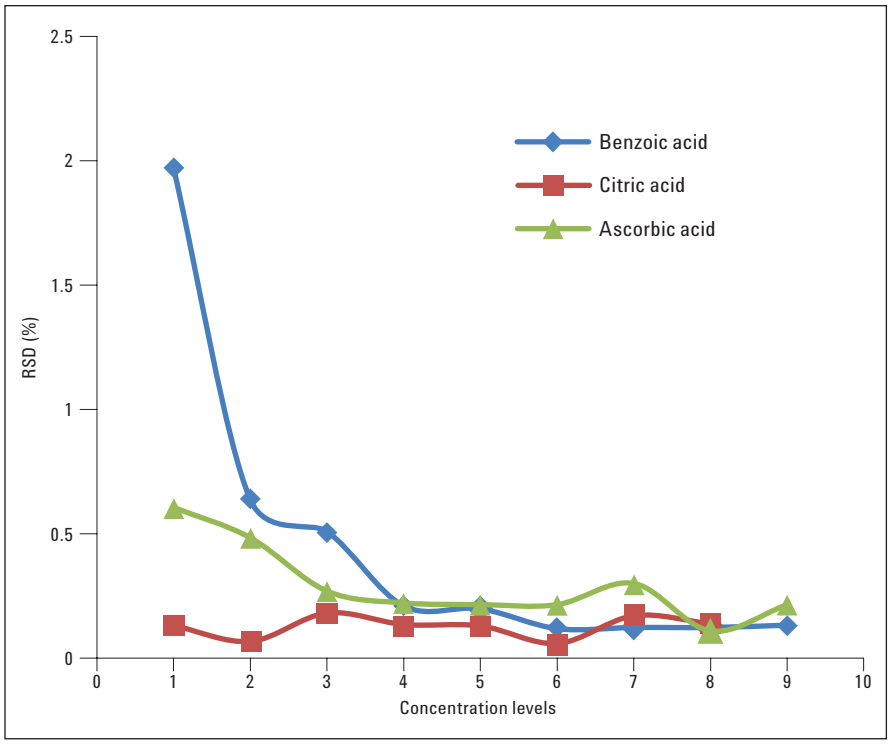

Figure 4
Area precision measured as RSD (%) for six replicates at each concentration level.

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).

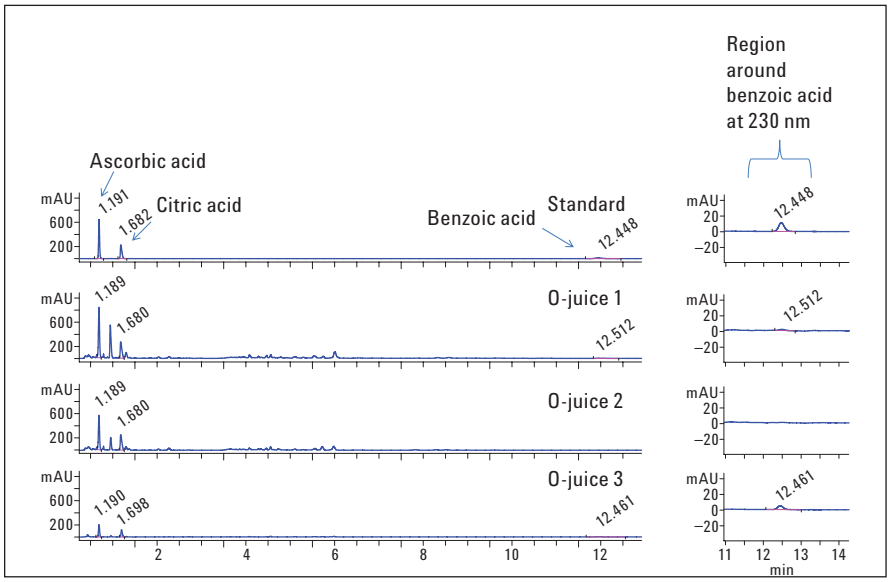

Figure 5
Three different orange juice brands analyzed at 230 nm for benzoic acid.

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.

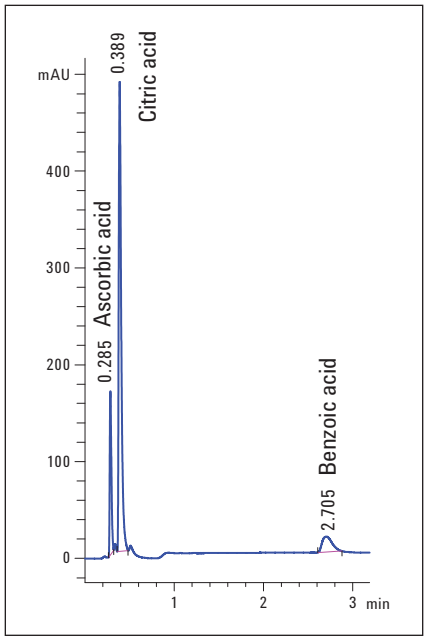

Figure 6
An UHPLC method separating the standards for ascorbic acid, citric acid, and benzoic acid on an Agilent Poroshell 120 EC-C18 column 3.0 × 75 mm, packed with 2.7-µm particles.

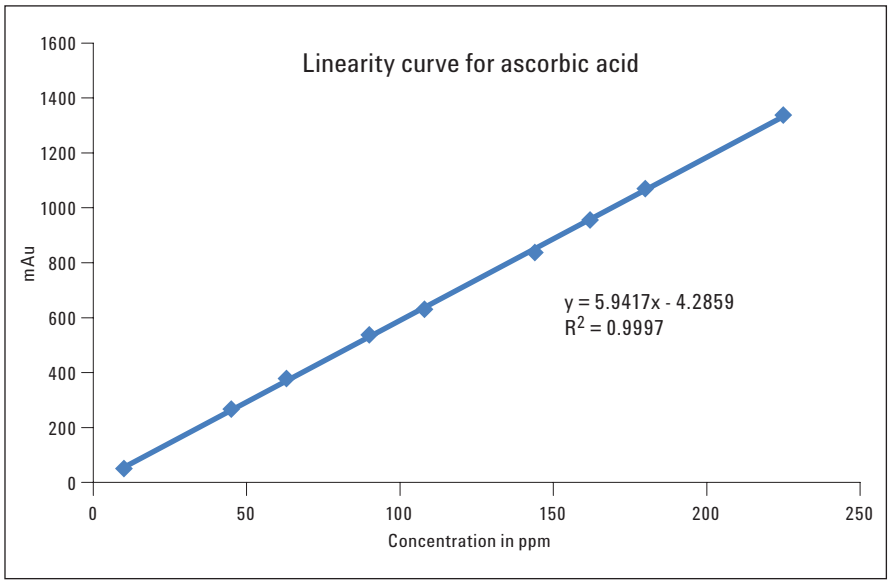

Figure 7
Linearity level of ascorbic acid from 10 µg/mL to 225 µg/mL (50 ng to 1125 ng on column) showing the coefficient value on an Agilent 1290 Infinity LC System.

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.

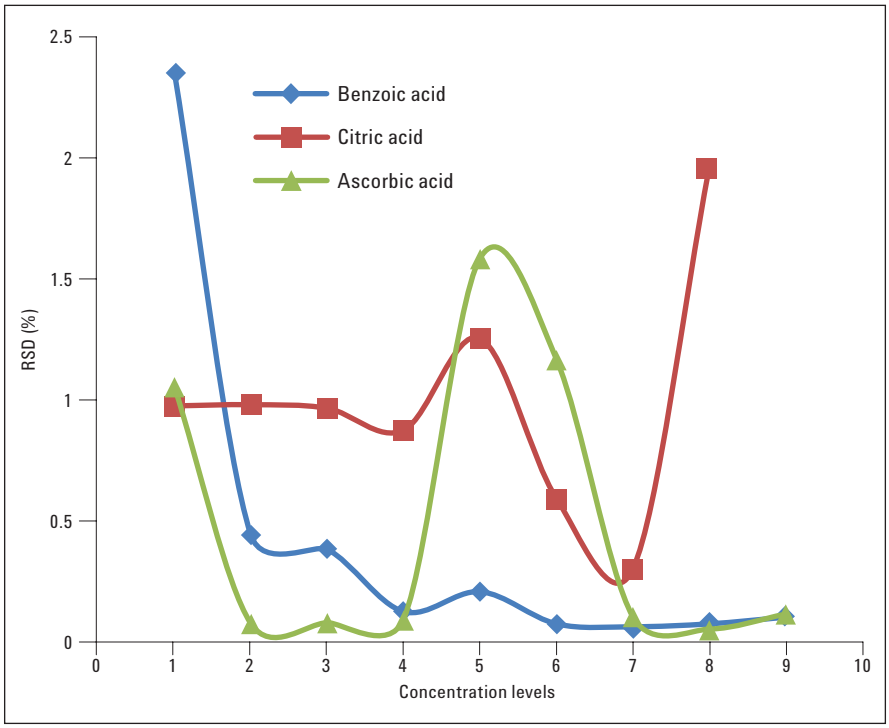

Figure 8
An UHPLC method showing area precision measured as RSD (%) for three different compounds, six replicates for each concentration level.

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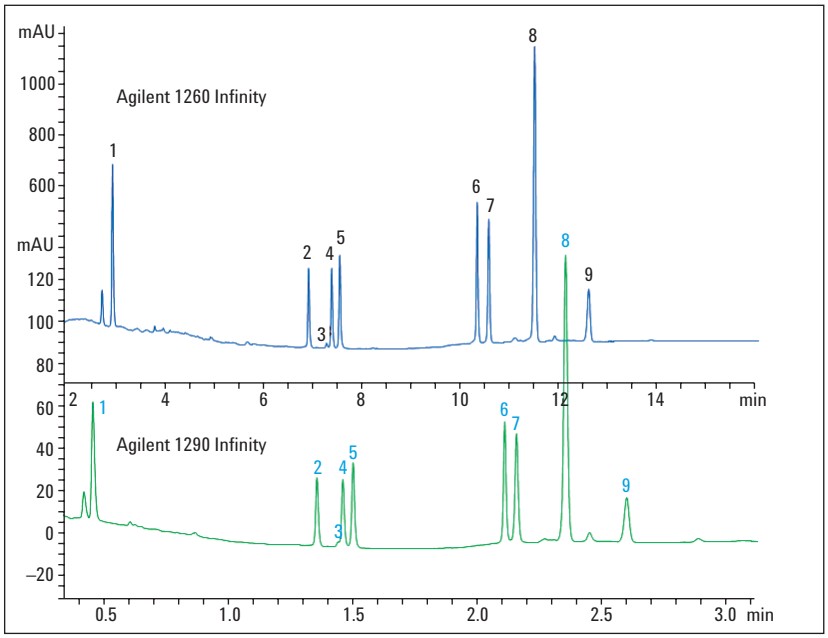
Agilent Technologies

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.

Agilent Technologies

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.

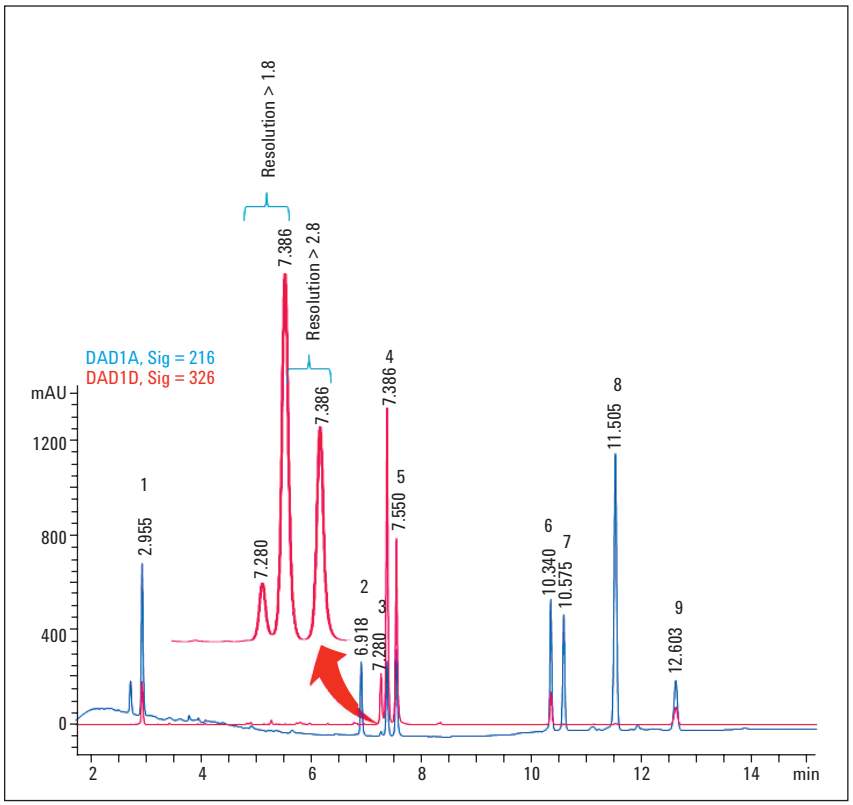

Figure 1
Separation of nine fat soluble vitamins using a 15-cm Agilent Poroshell 120 EC-C18 column. The excellent separation of three different forms of vitamin A is shown in the insert.

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.

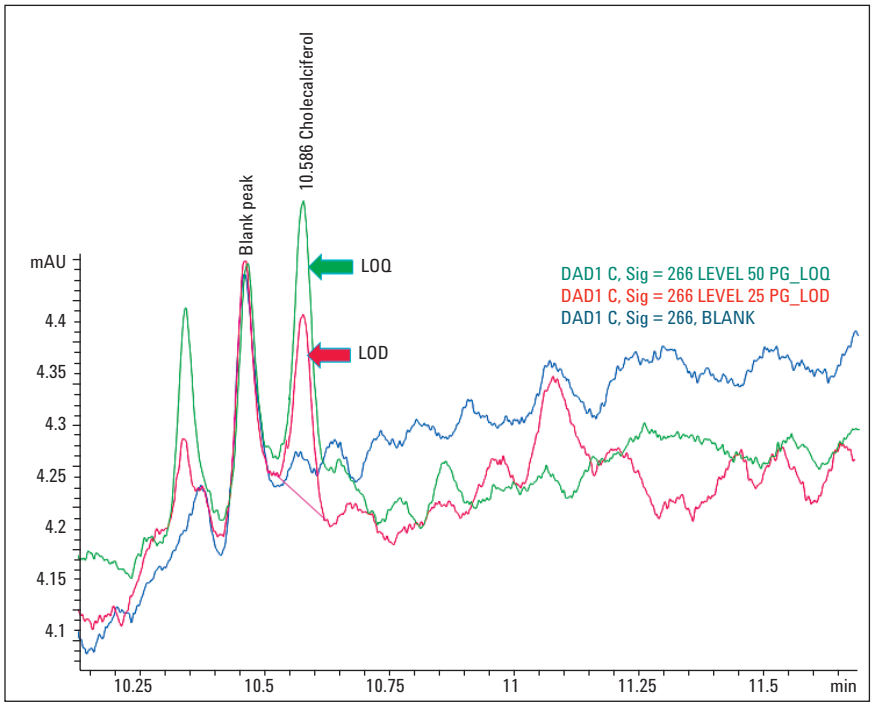

Figure 2
LOD (25 pg) and LOQ (50 pg) chromatograms of cholecalciferol (vitamin D) overlaid with blank.

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.

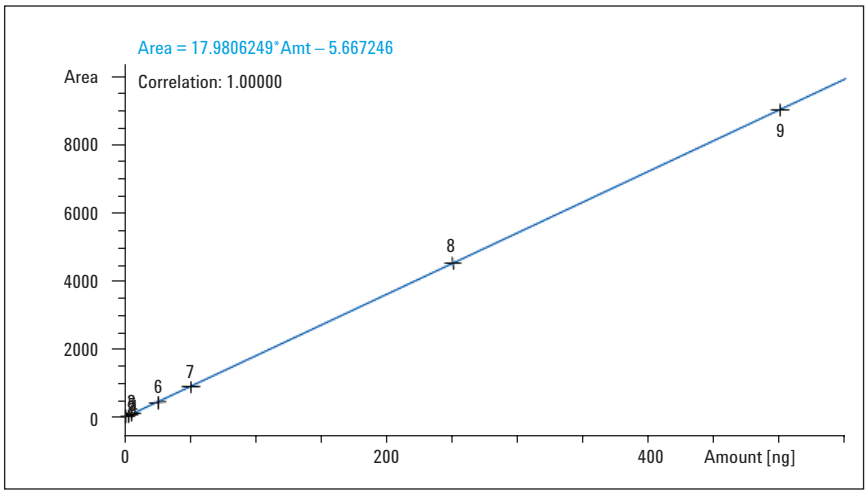

Figure 3
Linearity curve of cholecalciferol from 0.05 ng to 500 ng (on-column concentration) showing excellent coefficient value.

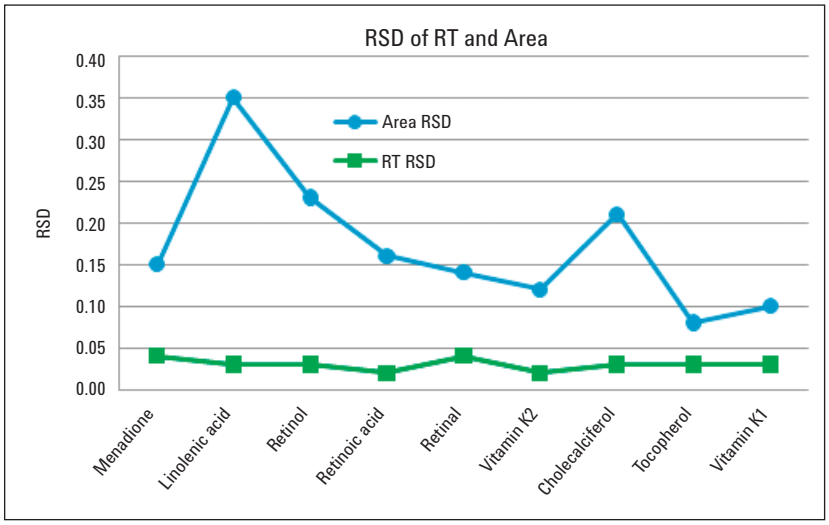

Figure 4
Excellent RT and area RSD values for all vitamins at 50 ng on-column concentration.

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.

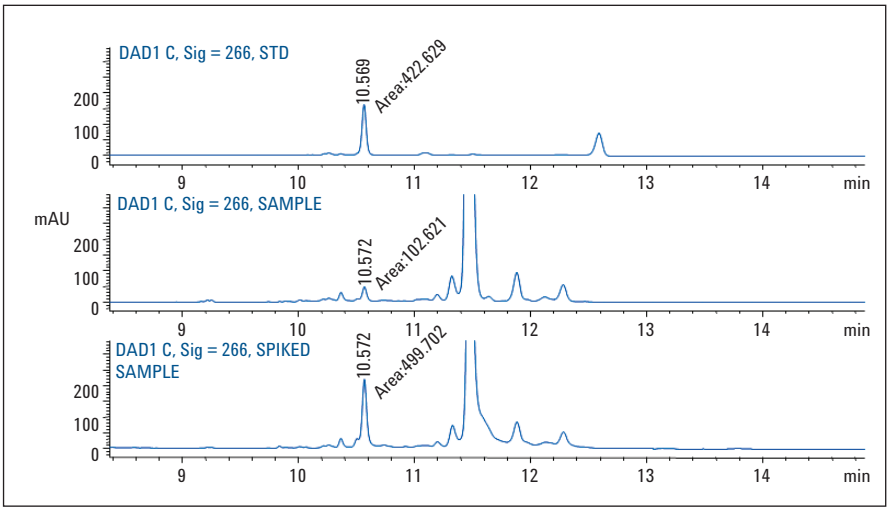

Figure 5
Overlay of spiked, unspiked, and standard chromatograms used for multivitamin tablets.

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.

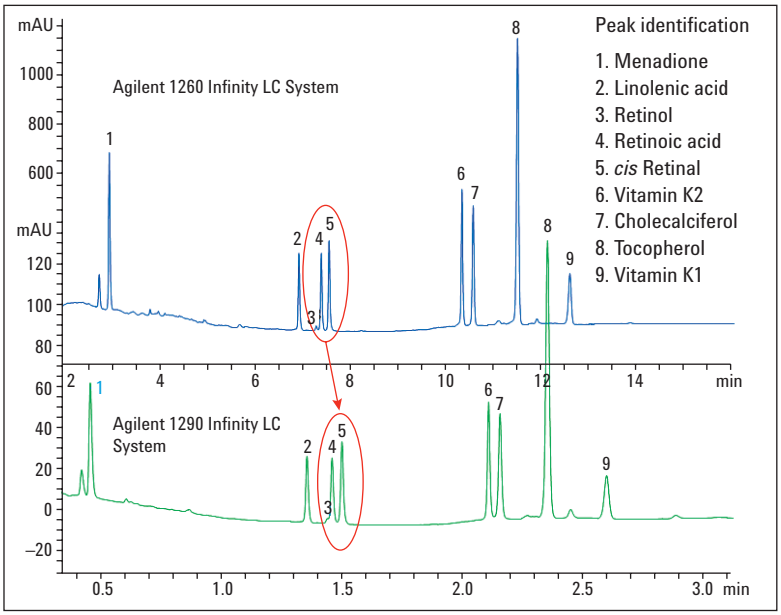

Figure 6
Overlay of separation of nine fat soluble vitamins using HPLC method on Agilent 1260 infinity and UHPLC method on 1290 Infinity LC system.

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.

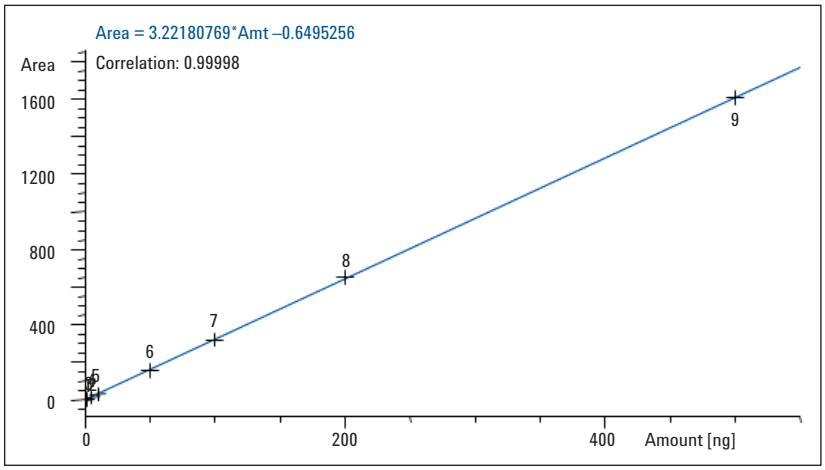

Figure 7
Linearity of vitamin-D from 0.05 ng to 500 ng showing a correlation of 0.99998 (9 levels and six replicates). Injection volume is 1 µL.

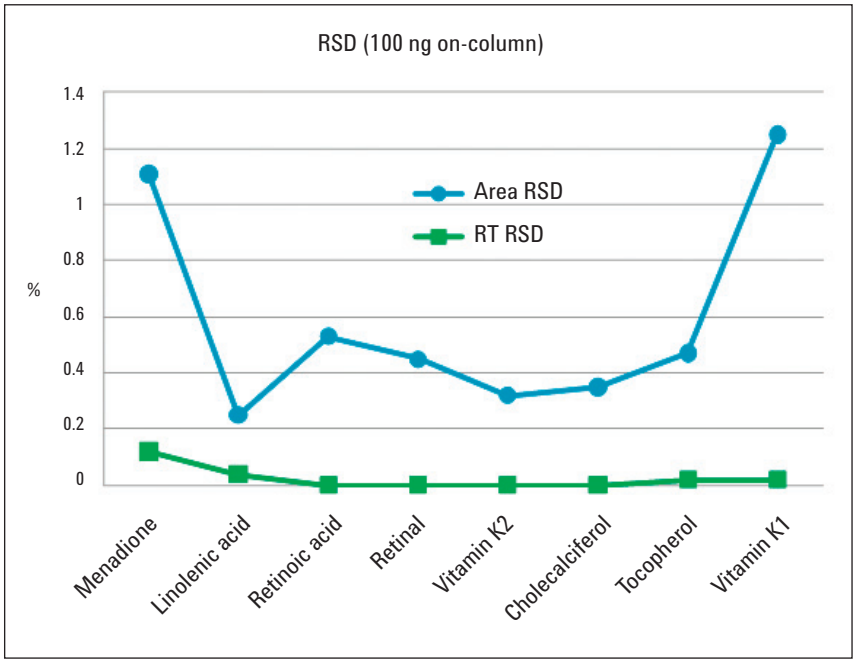

Figure 8
Area and RT RSD values from UHPLC results for all vitamins except retinol at an on-column concentration of 100 ppm. Injection volume is 1 µL and six replicates.

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2.
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3.
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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.

References

1.
"Resveratrol improves health and survival of mice on a high-calorie diet", Joseph A. Baur, Kevin J. Pearson, Nature, Volume 444 Number 7117 pp 337-342, 2006

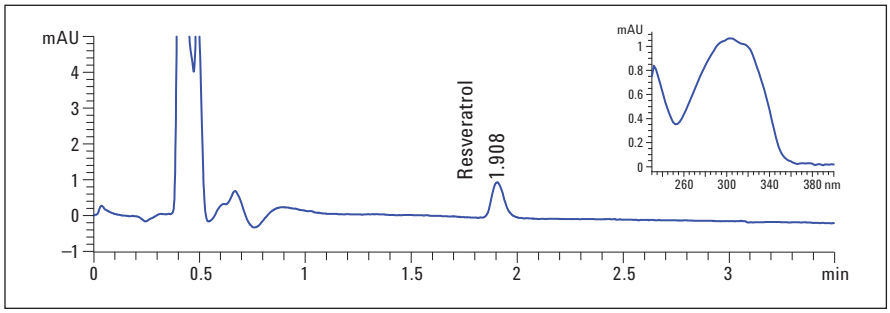

Figure 1
Chromatogram of reference solution (0.01 mg/L) and spectrum of resveratrol.

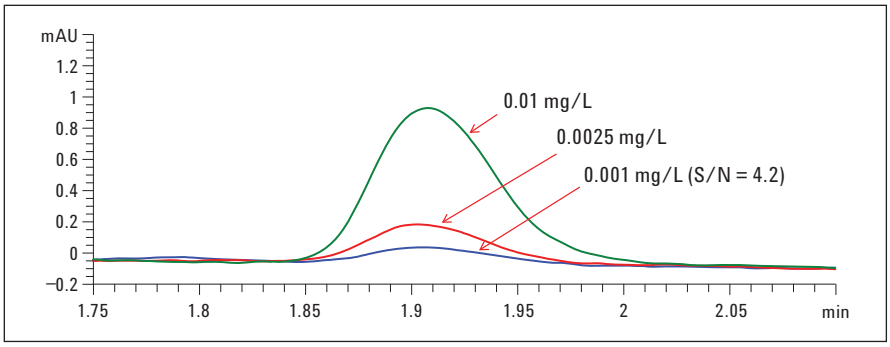

Figure 2
Chromatogram of standard solutions with different concentrations.

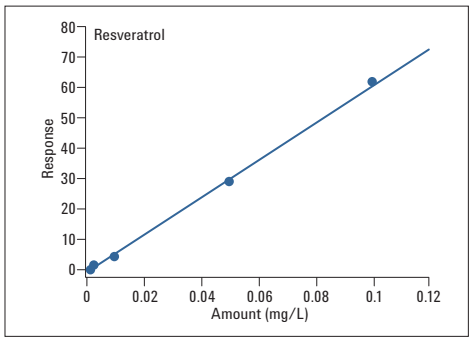

Figure 3
Calibration curve.

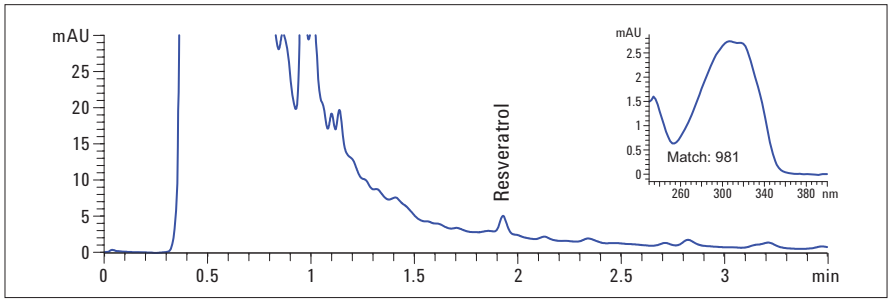

Figure 4
Chromatogram of red wine and spectrum of resveratrol.

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Agilent Technologies

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

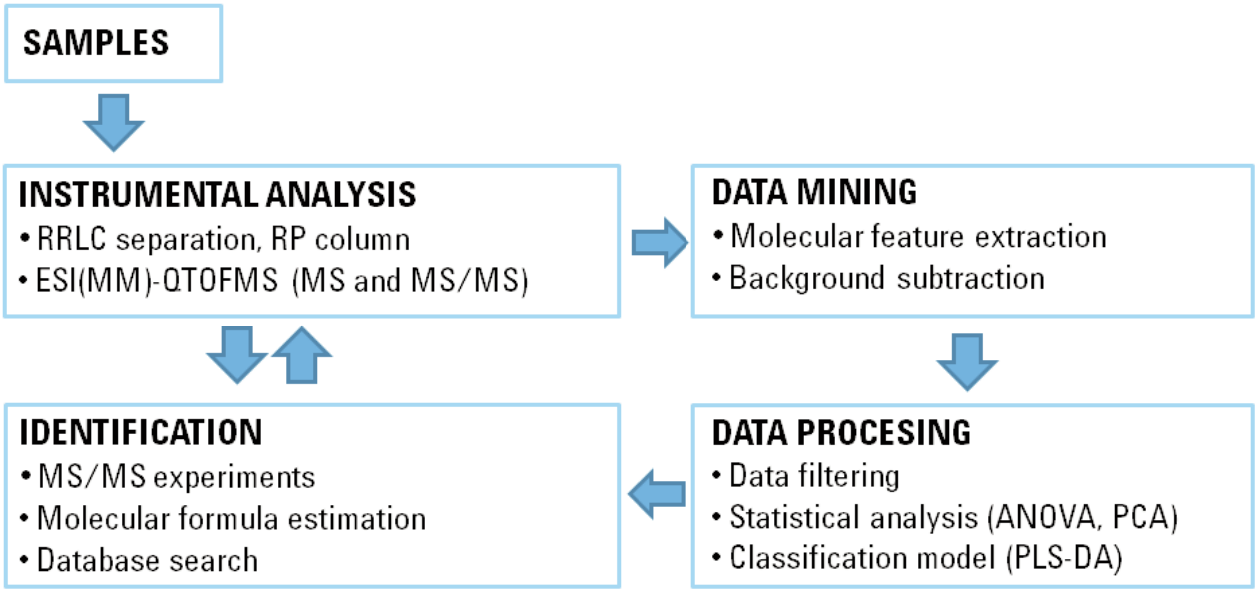

Figure 1. Workflow for a metabolomic study to generate a predictive model for wine classification and identify the marker compounds.

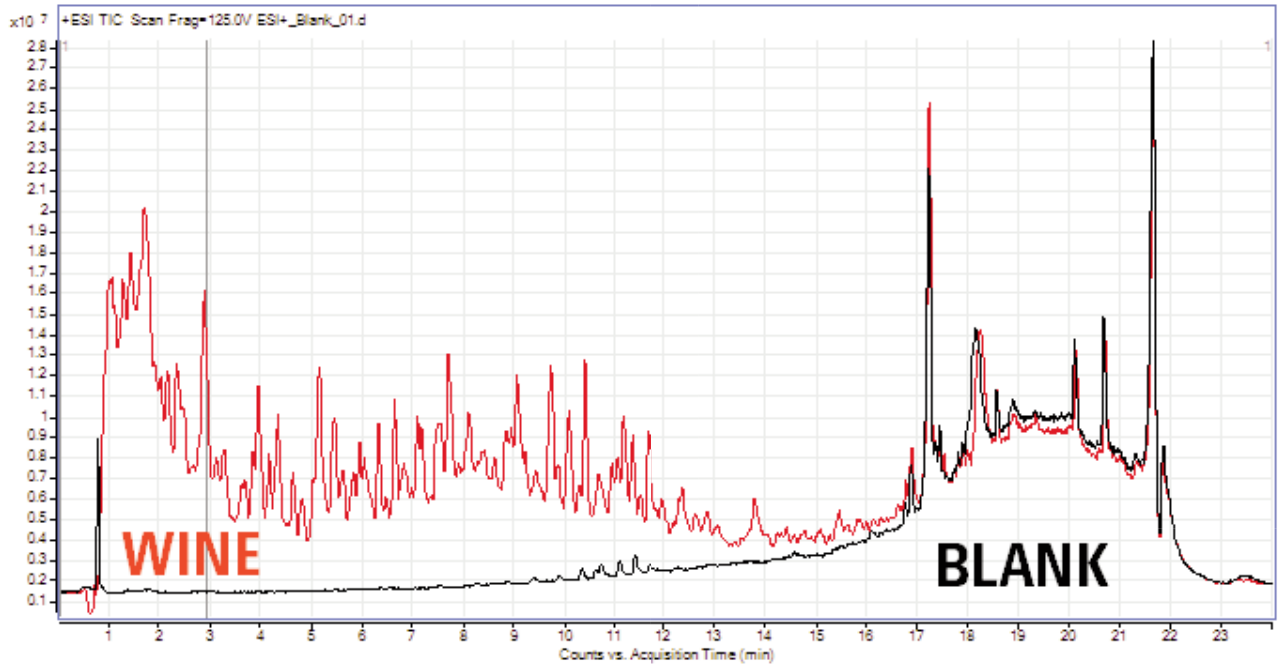

Figure2. Total Ion Current (TIC) chromatogram of a wine sample Ilustrating its complexity. Each peak contains several compounds. The blank must also be subtracted in order to properly interpret the data.

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.

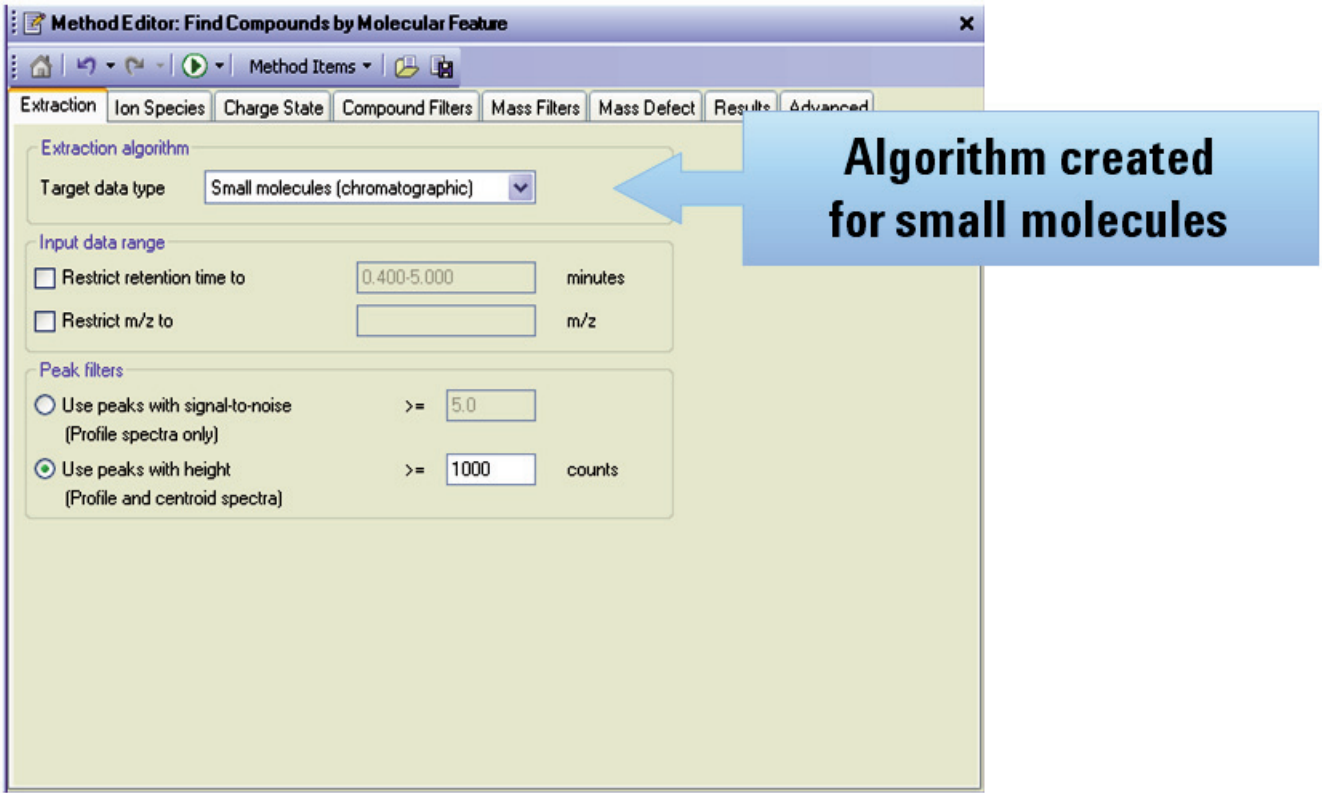

Figure 3. Settings used by the Molecular Feature Extractor (MFE) to extract entities (compounds) from the LC/QTOF MS data.

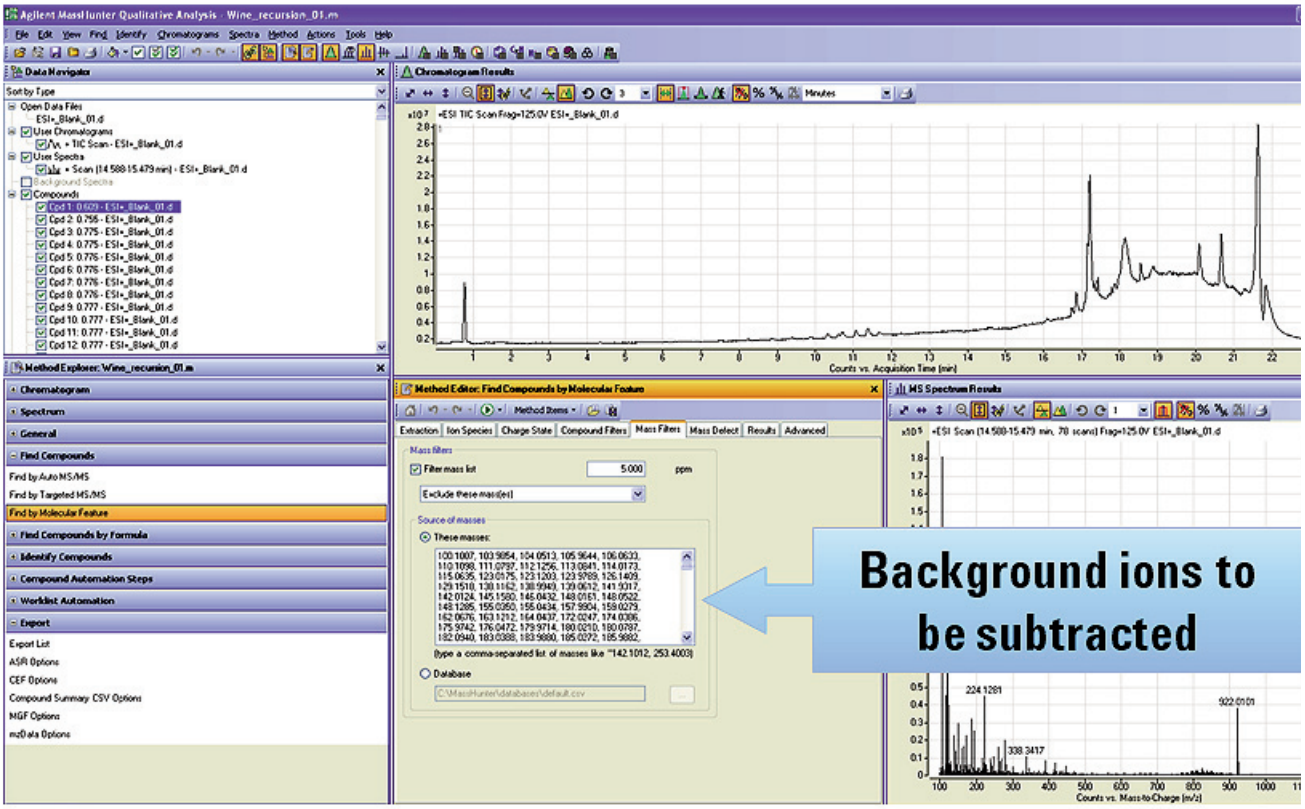

Figure 4. The resulting masses found in the blank can be copied and pasted right into the MFE tab for subtraction from all of the datasets.

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.

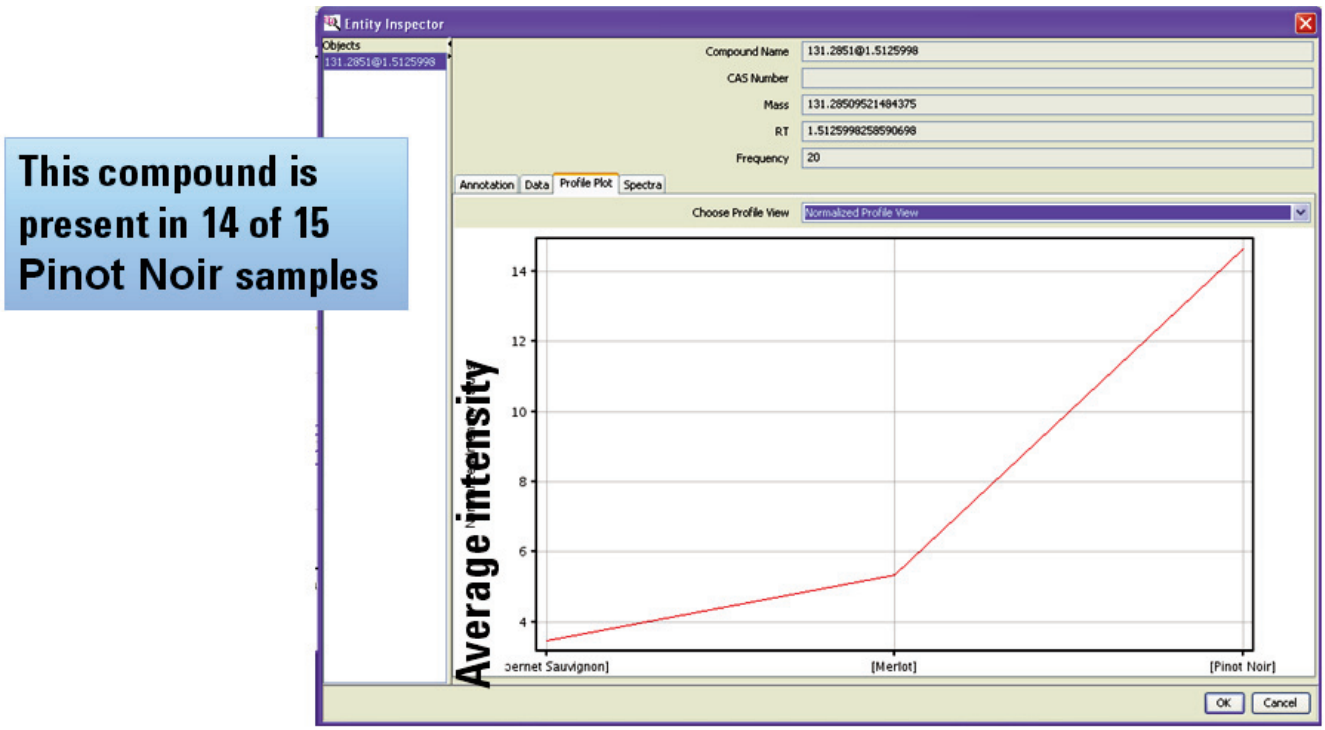

Figure 5. Using a 100% frequency filter can remove useful markers from the dataset, such as this compound that is present in Cabernet Souvignon and Merlot samples at very low frequency, but shows up in 14 of 15 Pinot Noir samples.

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).

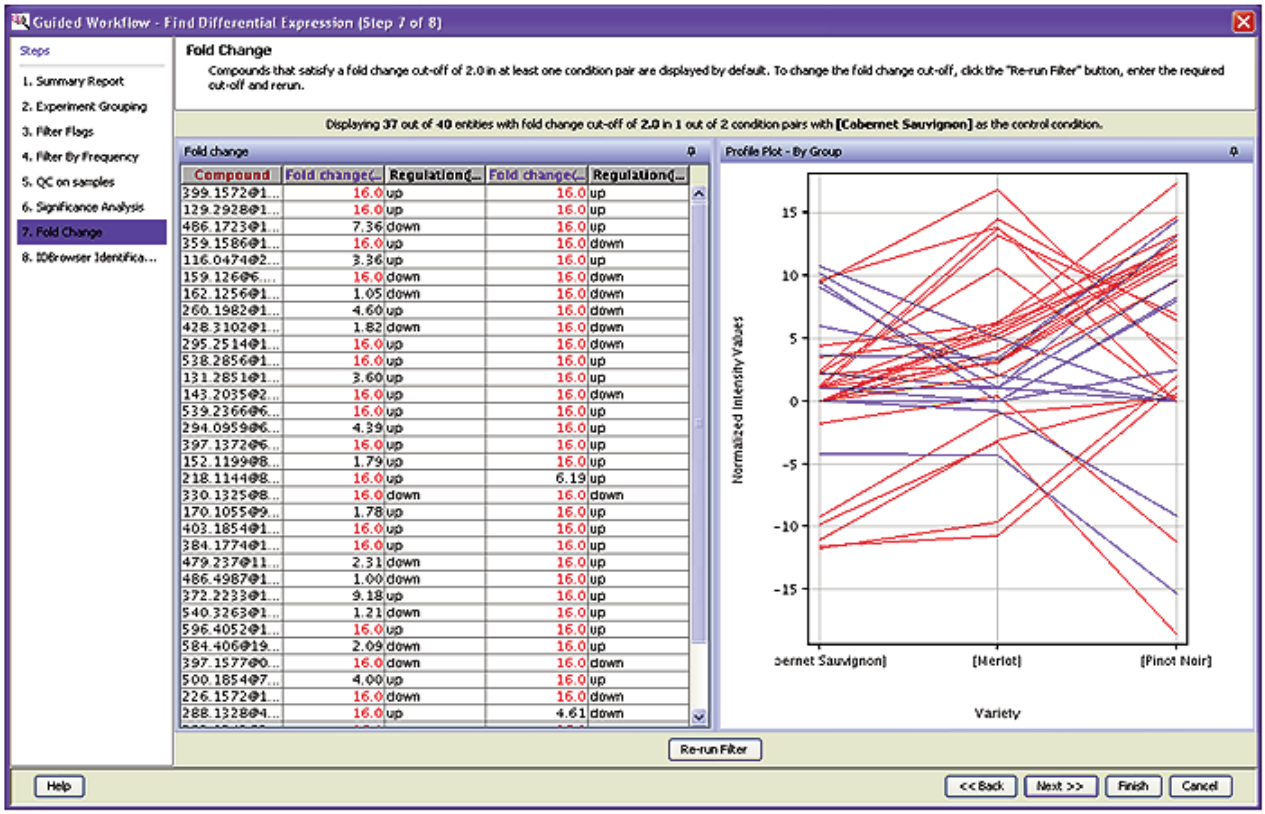

Figure 6. Fold Change Analysis is used to identify entities with significantly different abundance in selected classes. Examining the data at fold change higher than two eliminates possible Cabernet and Merlot markers, so a 2-fold filter was applied. This reduced the data set from 40 to 26 possible markers.

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.

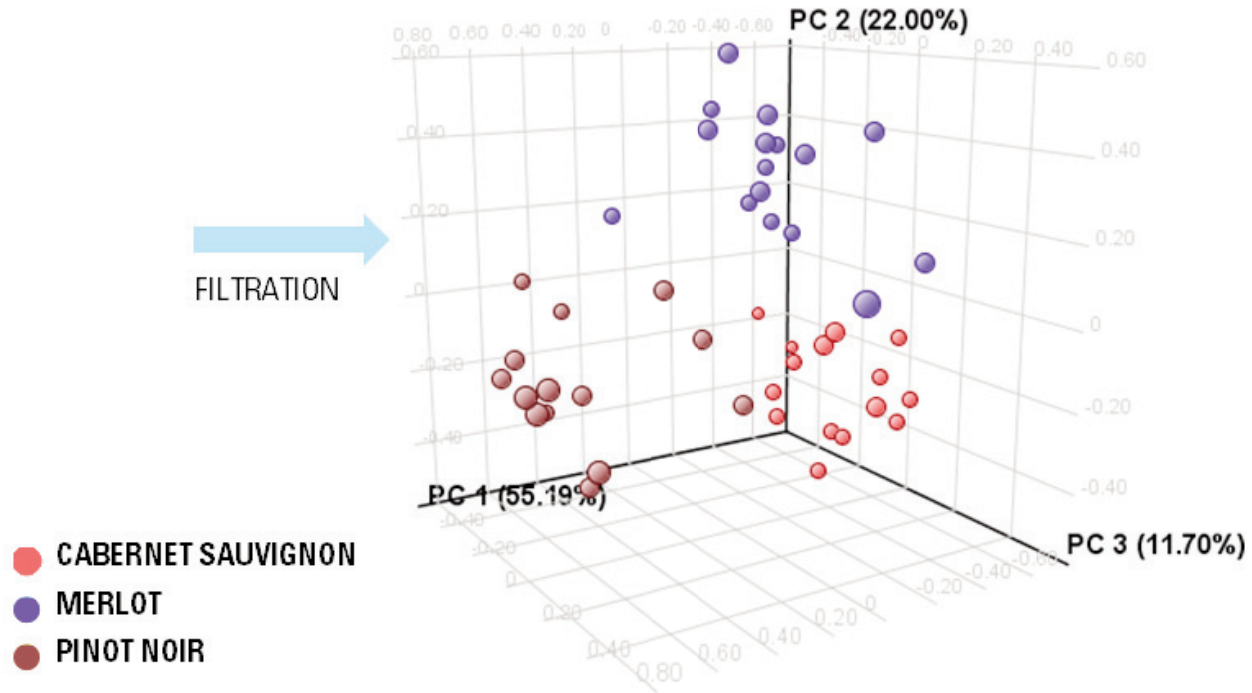

Figure 7. Principle Component Analysis (PCA) of the 26 markers remaining after the ANOVA and Fold Change filters were applied to the dataset. The markers for each of the three wine varieties group together well, indicating their utility for predicting the variety of an unknown wine.

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.

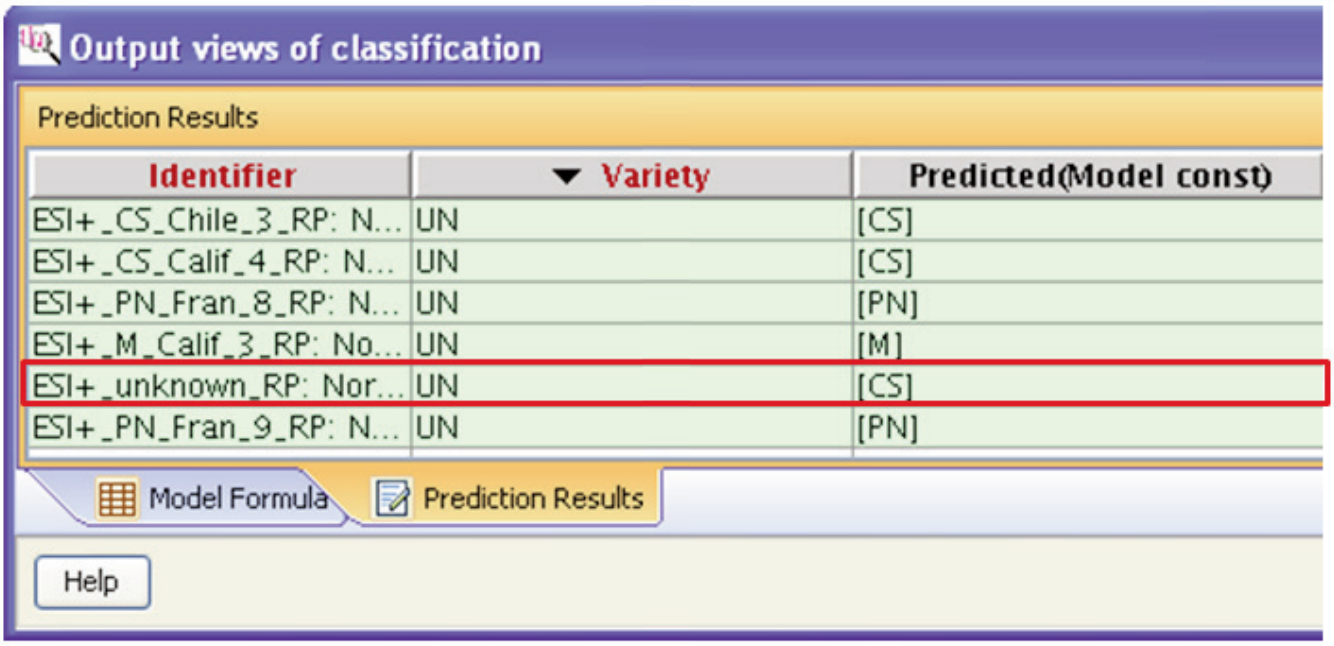

Figure 8. Table showing the results of the predictive model when applied to known wine samples, as well as one unknown. All samples were correctly classified.

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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Agilent Technologies

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.

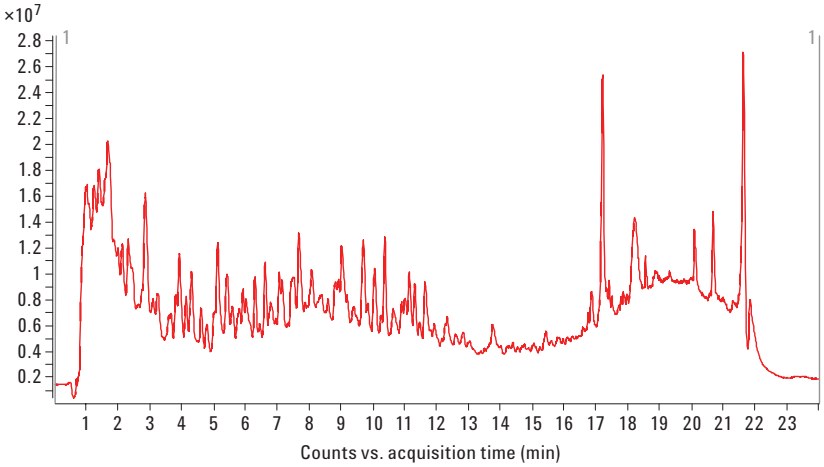
Using the Agilent 6530 Accutate-Mass Q-TOF LC/MS, MS/MS can be performed on the ions shown to correlate specific grapes and identification can be pursued with the excellent accurate mass measurements. It is noted that the identification of true unknowns, compounds not found in any database, is a difficult 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

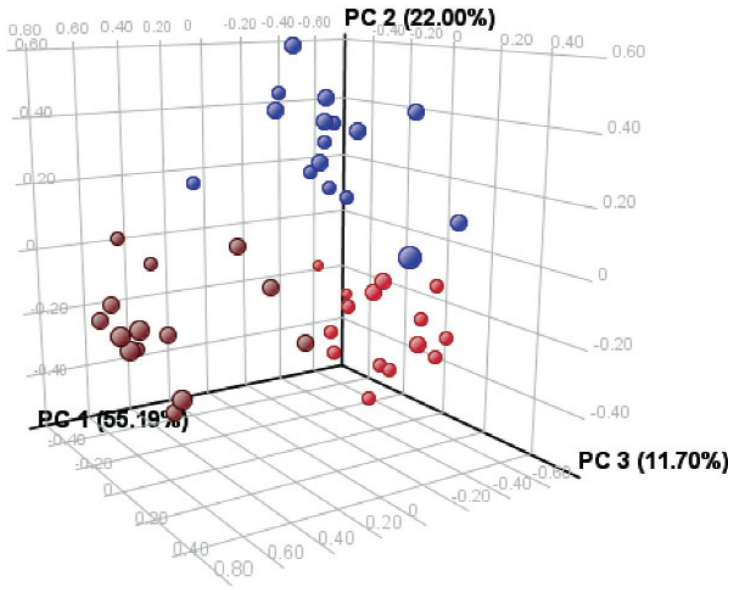
- Agilent 6500 Series Q-TOF LC/MS provides the sensitivity, mass accuracy and resolution needed to separate unique compounds recognizing a wines variety
- Mass Profiler Professional provides the needed multivariate statistics directly on accurate mass spectral data
- Mass Profiler Professional provides 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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Agilent Technologies

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.

Agilent Technologies

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

Authors

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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.

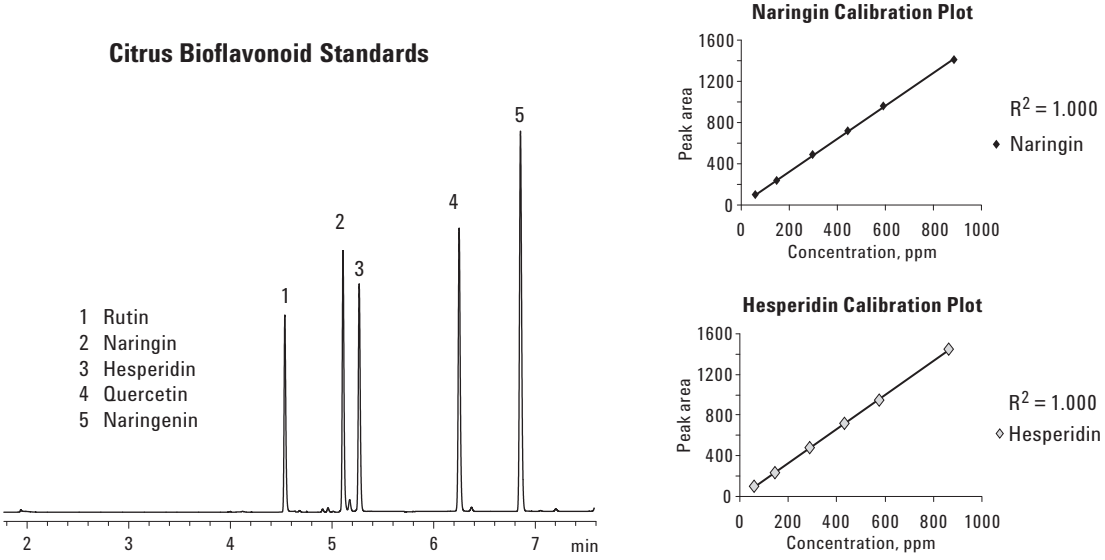

Figure 1. Chromatogram of the flavonoid standard mixture and calibration curves for naringin and hesperidin. Chromatographic conditions are listed in experimental section.

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.

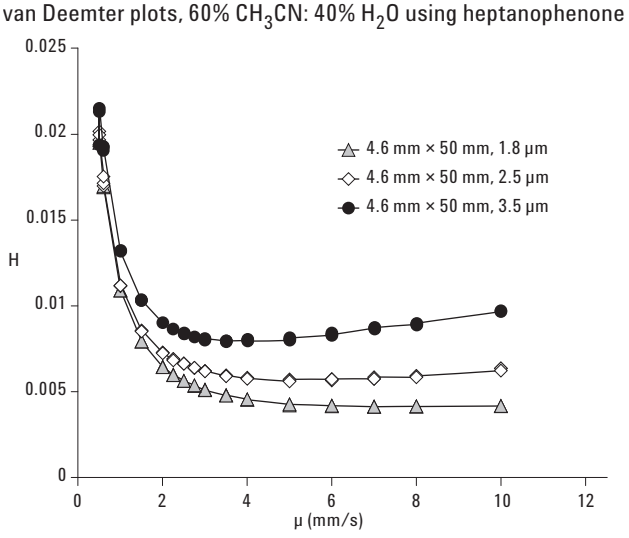

Figure 2 Plots of plate height versus velocity. Plate heights for sub-2-µm columns remain relatively constant compared to larger particle sized columns as mobile phase velocity (flow rate) increases.

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

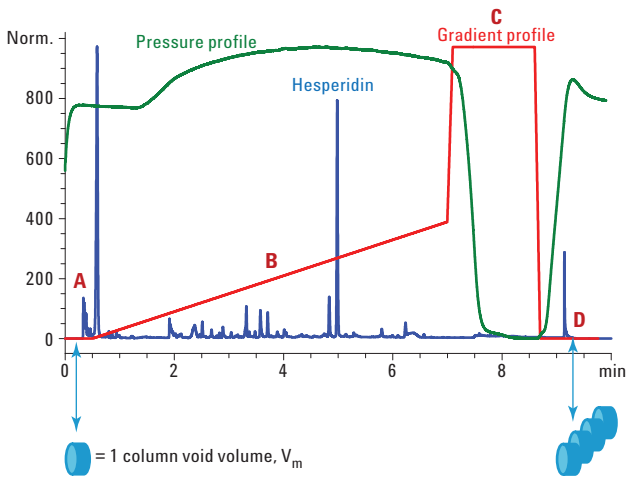

Figure 3. UHPLC of grapefruit juice with pressure and gradient profiles, and column volumes needed for re-equilibration. Segments of the gradient include A: initial isocratic hold, B: linear elution ramp, C: strong solvent column flush, D: re-equilibration.

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

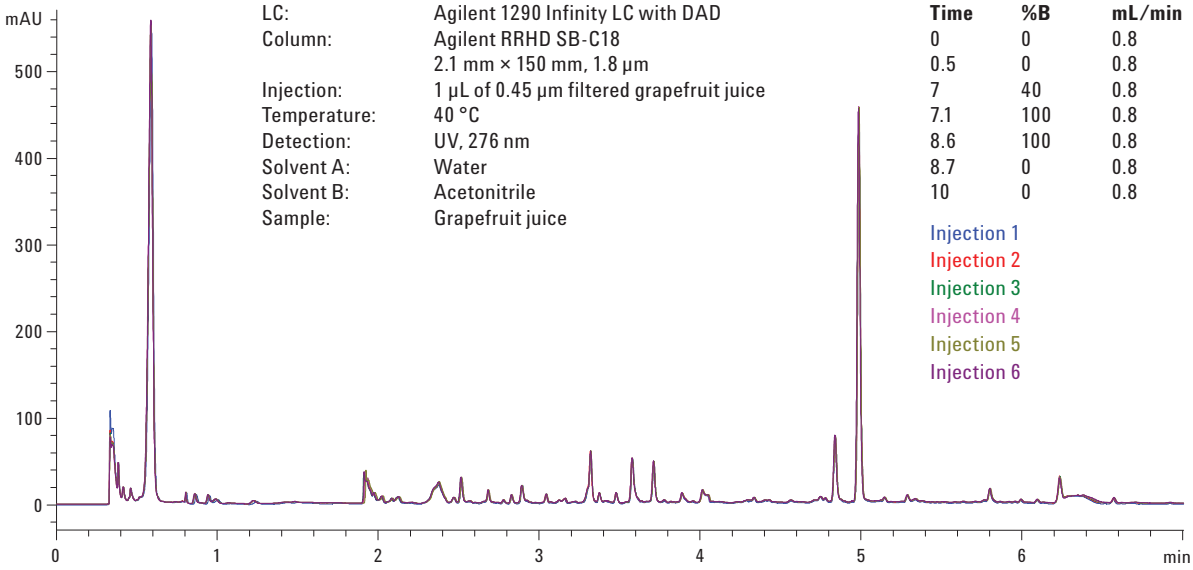

Figure 4. Overlay of six analyses with no variability in retention, selectivity or resolution, indicating sufficient column flushing and re-equilibration.

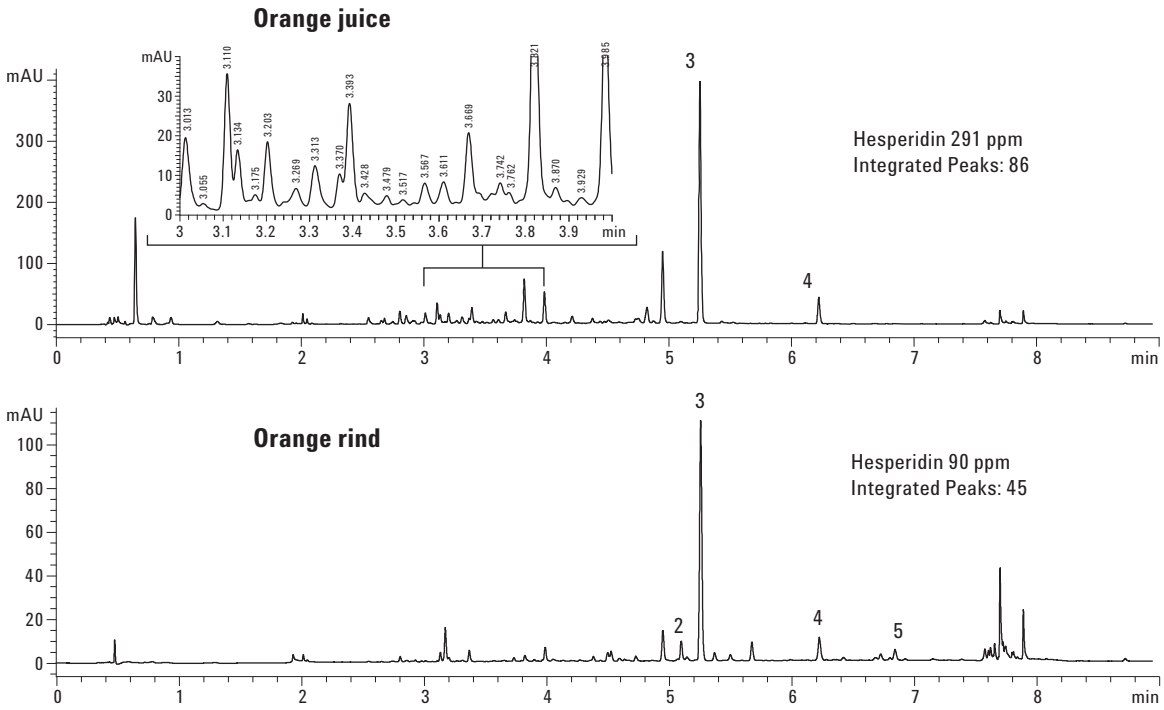

Figure 5. UHPLC fingerprint of orange juice and rind analyzed by an Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 mm × 150 mm (p/n 959759-902). Chromatographic conditions are listed in experimental section.

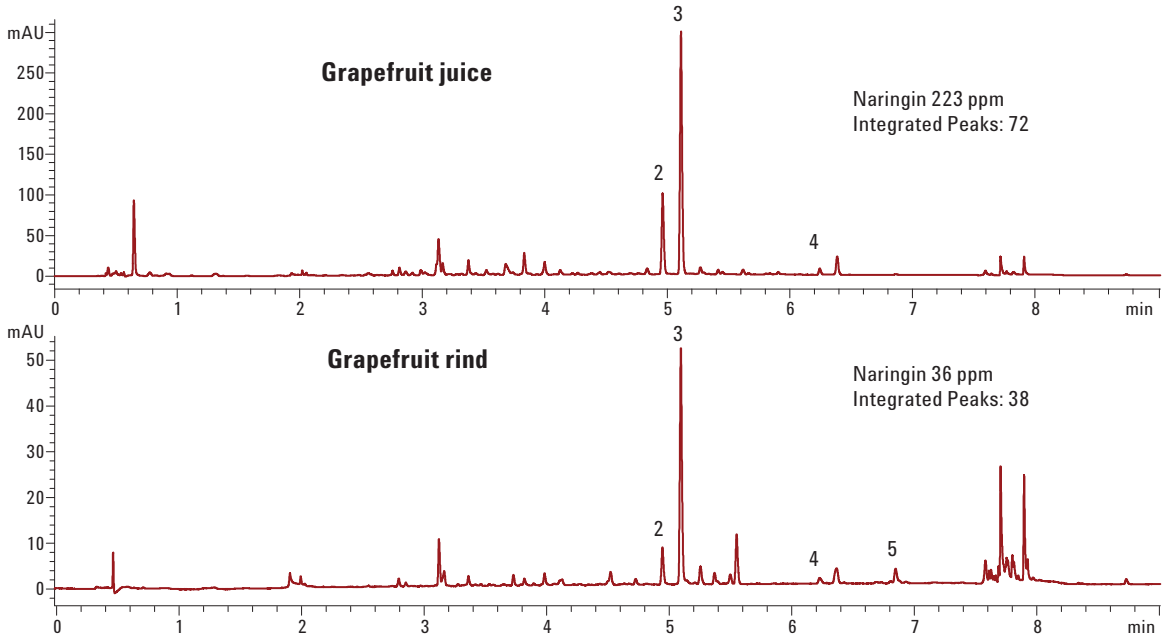

Figure 6. UHPLC fingerprint of grapefruit juice and rind analyzed by an Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 mm × 150 mm (p/n 959759-902). Chromatographic conditions are listed in experimental section.

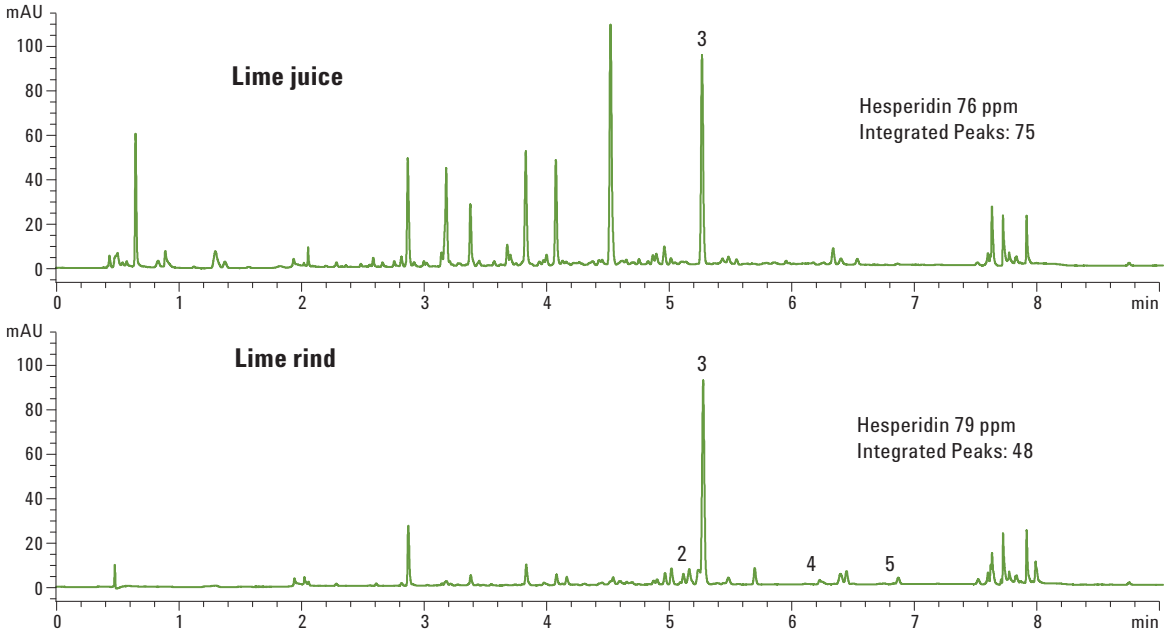

Figure 7. UHPLC fingerprint of lime juice and rind analyzed by an Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 mm × 150 mm (p/n 959759-902). Chromatographic conditions are listed in experimental section.

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

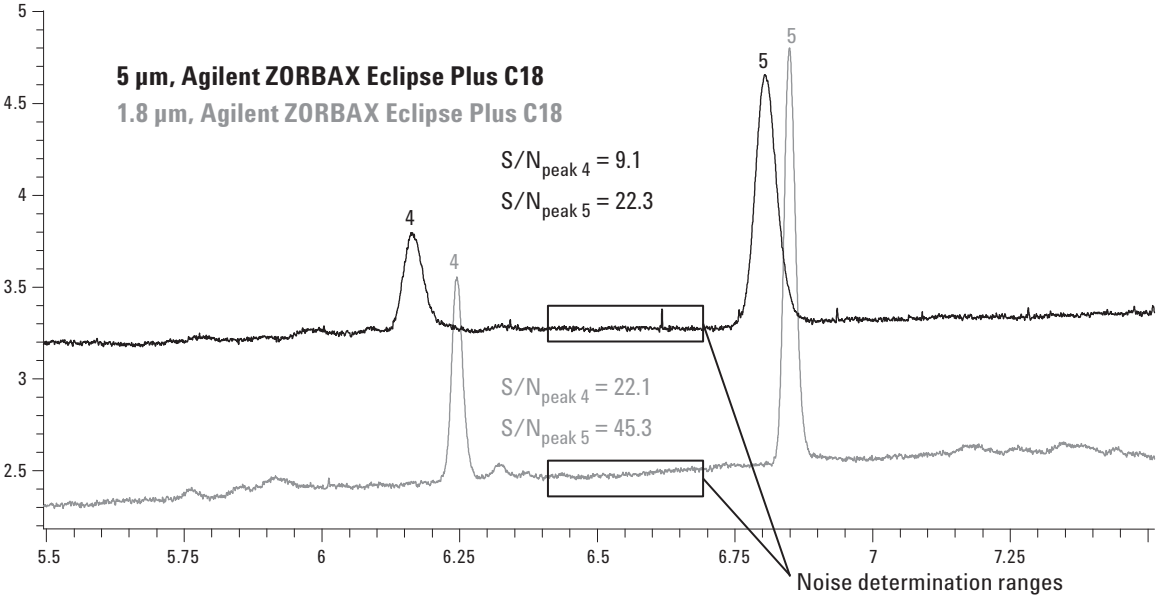

Figure 8. Signal-to-noise comparison of 5 and 1.8 µm Agilent ZORBAX Eclipse Plus C18 columns, 2.1 mm × 150 mm using a 1 ppm each quercetin and naringenin standard. Chromatographic conditions are listed in the experimental section.

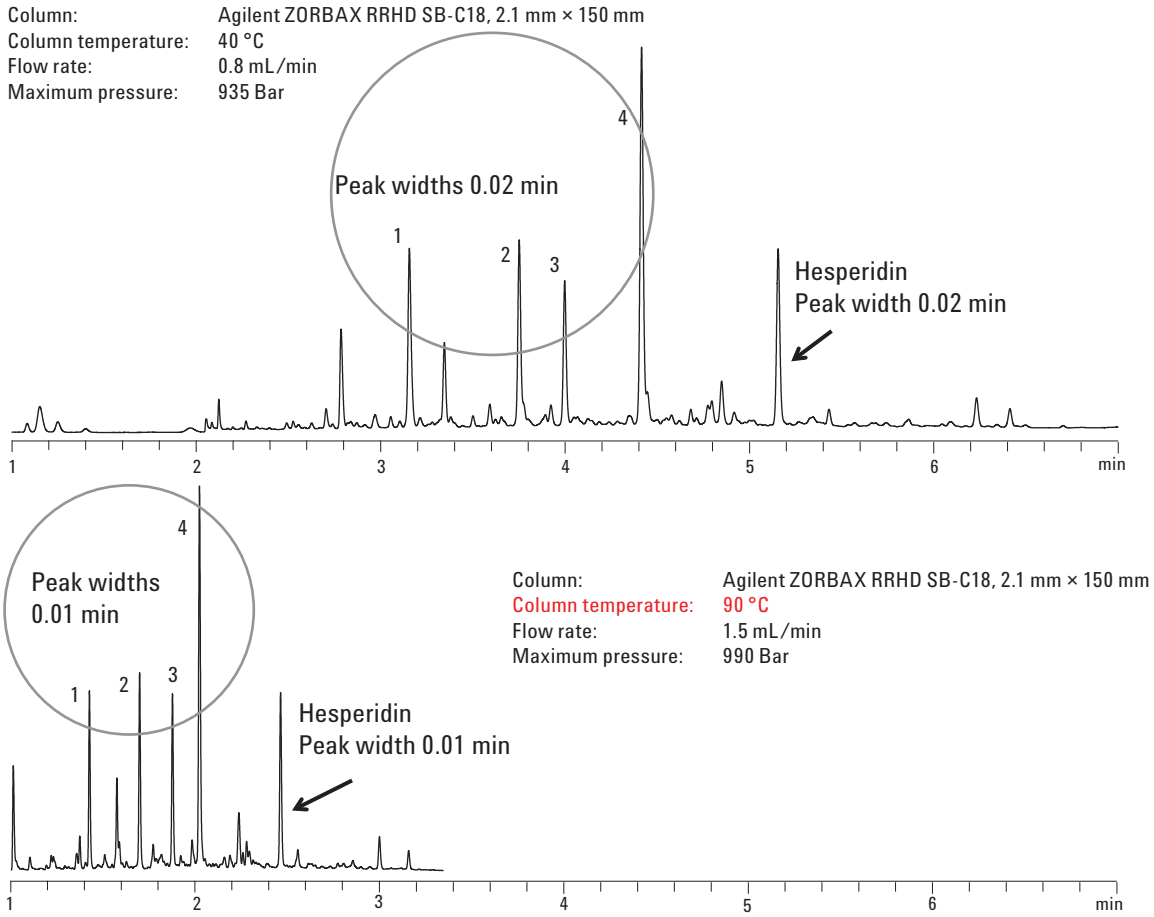

Figure 9. UHPLC of lime juice including high temperature and faster flow rates further reduce analysis time using an Agilent ZORBAX RRHD SB-C18 column. Chromatographic conditions listed in experimental section.

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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Agilent Technologies

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.

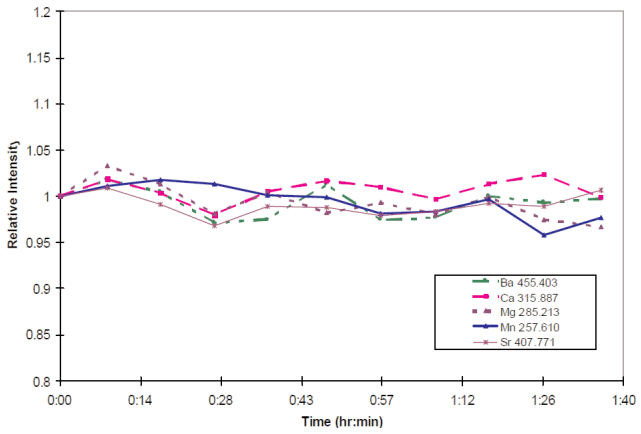

Figure 1. Signal stability over one and a half hours for a 4% full cream milk powder solution with internal standardization.

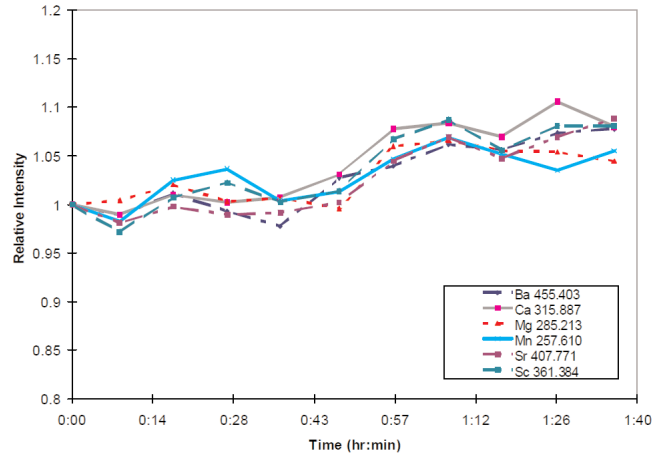

Figure 2. Signal stability over one and a half hours for a 4% full cream milk powder solution without internal standardization.

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.

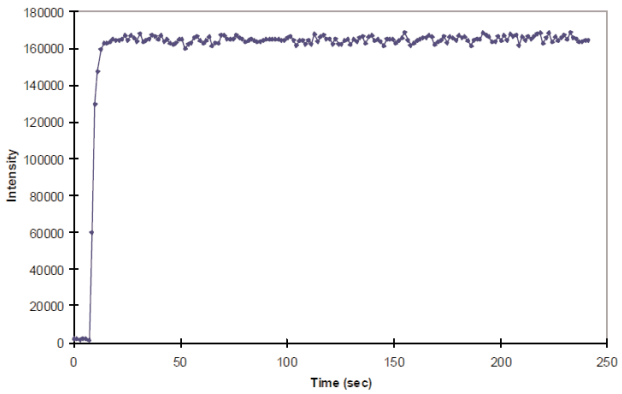

Figure 3. Stabilization profile for a 4% full cream milk powder solution.

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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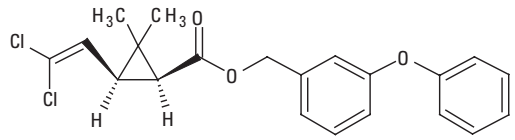
Abstract

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

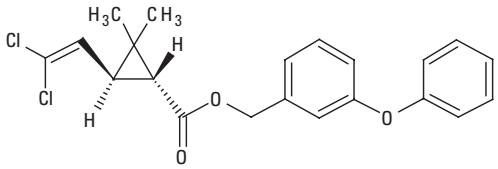
Permethrin (Figures 1A and 1B) is a pyrethroid insecticide that is a chlorinated, synthetic form of pyrethrum, a popular natural insecticide derived from chrysanthemum flowers. Permethrin has multiple uses as an insecticide in household insect foggers, tick and flea spray for yards and pets, as a spray 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



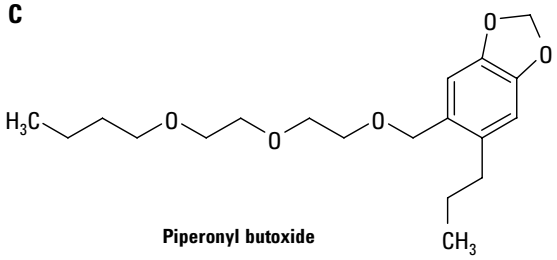
cis-**permethrin**

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.

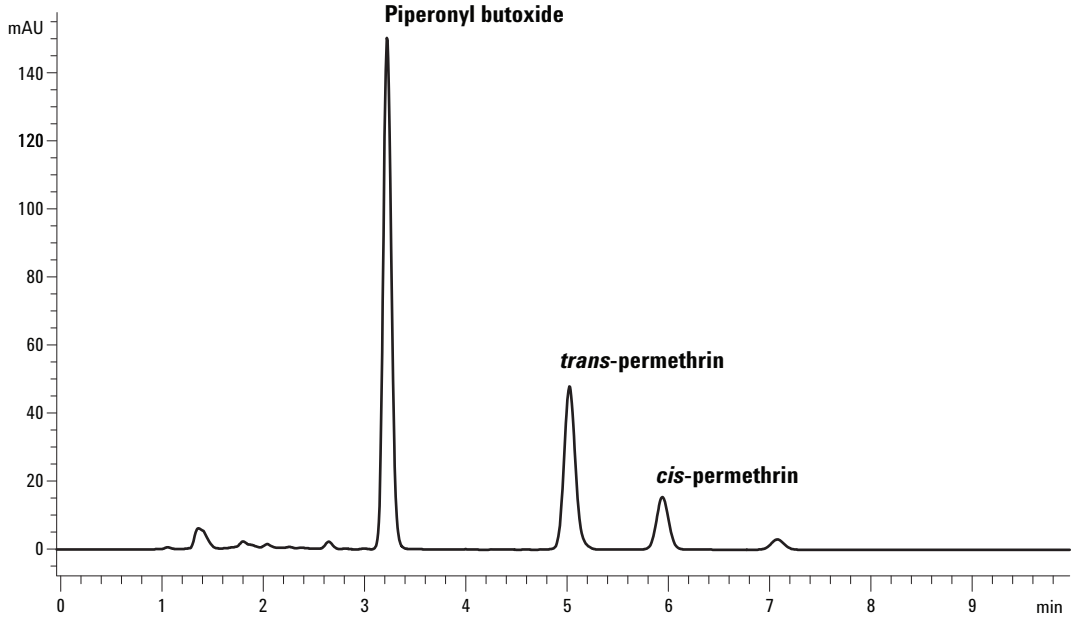


Figure 2. Chromatogram of permethrin and piperonyl butoxide standards.

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.

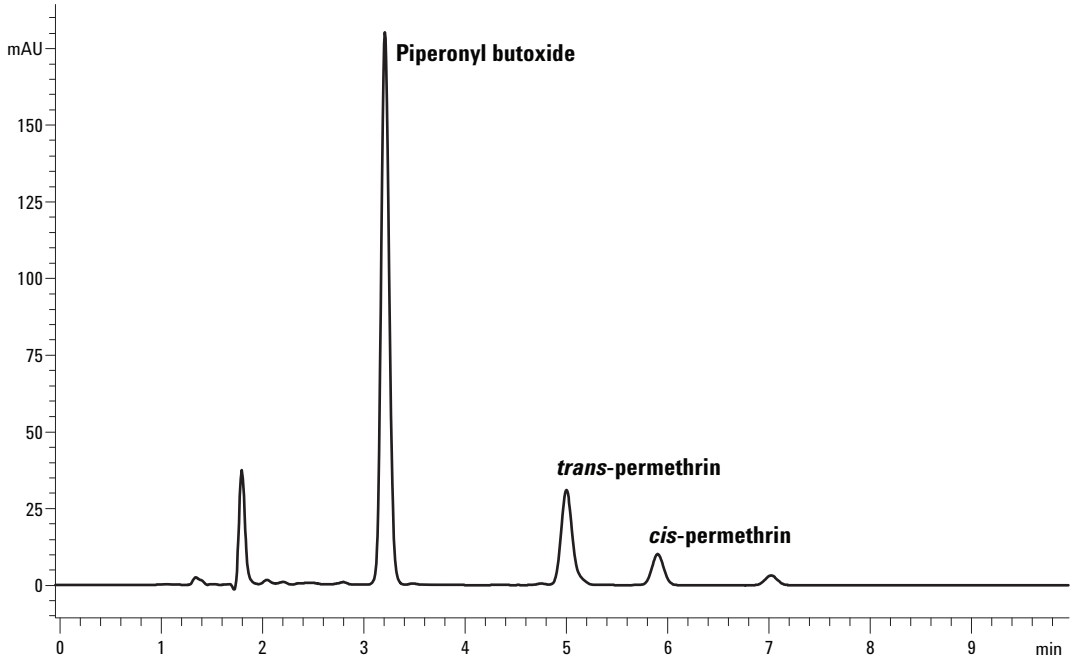


Figure 3. Determination of permethrin and piperonyl butoxide in lotion.

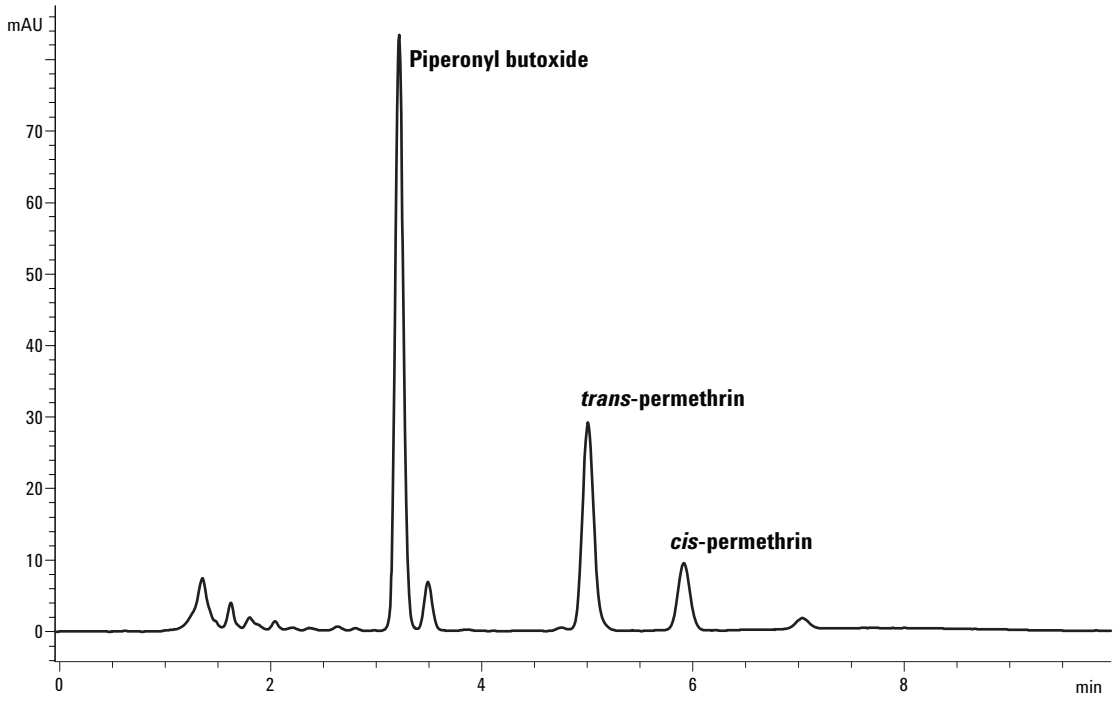


Figure 4. Determination of permethrin and piperonyl butoxide in shampoo.

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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