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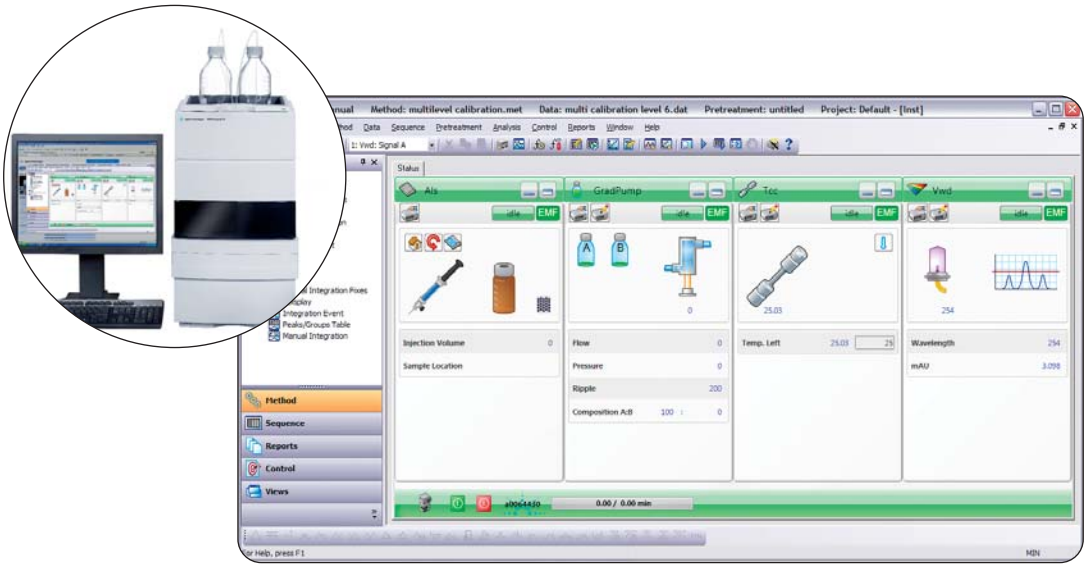
Analysis of sweeteners in food and beverages with the Agilent 1120 Compact LC system

Application Note

Rongjie Fu,
Zhixiu Xu



Abstract

An analysis method for three of the most popular artificial sweeteners was developed in this application, and the sweeteners were analyzed in a food and a beverage. The system suitability results showed that the Agilent 1120 Compact LC is the system of choice for conventional, analytical scale liquid chromatography. This integrated HPLC system was designed for ease of use, performance, and reliability. The quantitative analysis of typical samples is demonstrated in this Application Note.

Agilent Equipment

- Agilent 1120 Compact LC
- EZChrom Elite Compact software

Application Area

- Food and beverage

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Introduction

Artificial sweeteners are widely used all over the world, and some of them have a long history. For example, saccharin was invented nearly 100 years ago. Artificial sweeteners taste similar to cane sugar, but are low-calorie. They benefit overweight people and those who have problems with sugar metabolism. Artificial sweeteners are also cheaper than natural sugar and can reduce the cost for some foods and beverages. However, scientific research has shown that some of them can cause tumors in certain animals, so to prevent potential danger to humans, it is necessary to control the amount of sweeteners in foods and beverages.

Regulations set an upper limit on the concentration of artificial sweeteners in foods and beverages. The labels of foods and beverages should list what kinds of sweeteners are used. Quality control or spot-checking can use a conventional HPLC method to determine the amount of the sweeteners in the samples.

For this application, the most widely used sweeteners were analyzed in samples of yogurt and a beverage. The analysis was performed with the Agilent 1120 Compact LC, which is the system of choice for conventional, analytical scale liquid chromatography. It is an integrated HPLC system designed for ease of use, performance, and reliability. It is ideally suited for routine analyses in the food industry because of its capability to achieve very precise retention times and peak areas, as well as low detection limits for the analyzed compounds.

Experimental

Equipment

- Agilent 1120 Compact LC system with gradient pump (degasser inside), autosampler, column compartment, and variable wavelength detector (VWD)
- EZChrom Elite Compact software

Chemicals and reagents

- Reference standards were purchased from Sinopharm Chemical Reagent Co. Ltd., Shanghai, China.
- Water was obtained from a Millipore water purifier.
- Acetonitrile (Fisher Scientific) was HPLC purity. All other reagents were analytical purity.

Sample preparation

Yogurt: 5 mL was diluted with 5 mL methanol, and then the mixture was stirred and centrifuged. The sample was filtered with a 0.45 µm filter prior to injection.

Diet cola: The sample was treated with an ultrasonic for 10 minutes, and then was filtered with a 0.45 µm filter prior to injection.

Chromatographic conditions

- Column: Agilent TC-C18(2), 4.6 x 250 mm, 5 µm
- Mobile phase: A = 20 mM KH_2PO_4 buffer, pH 3.0; B = acetonitrile
- Gradient: 0 min 15 %B
  5 min 35 %B
  10 min 80 %B
- Flow rate: 1 mL/min
- Wavelength: 214 nm
- Injection volume: 5 µL
- Temperature: 30 °C

Results and discussion

The separation of standards of three sweeteners (acesulfame, saccharin, and aspartame) was done in eight minutes. To make sure the other components of the real sample were eluted from the column, the final method needed 11 minutes runtime.

By overlaying the chromatograms of the standards and the real samples, one can easily find out which kind of sweeteners are used in specific samples. As shown in figure 1, the samples of yogurt and diet cola that were used for this test contained both acesulfame and aspartame, but no saccharin.

The linearity of the compounds was tested within the amount range from 18.75 to 1500 ng, which covers the most likely amounts injected on to the column in real sample analysis. The results are shown in table 1. The data shows that very good regression factors (values of r^2) were achieved for each compound.

The system reproducibility was also tested with the three compounds. The high precision of the retention times gives high confidence when comparing the standards and real samples.

The quantitative results from the two samples are shown in table 3.

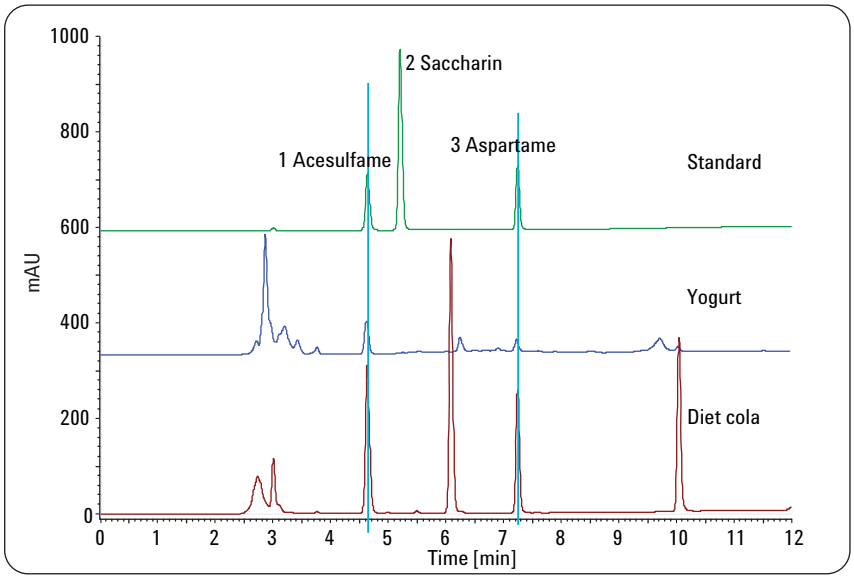

Figure 1
Overlaid chromatograms of sweetener standards and real samples.

Peak	Compound	Range (ng)	r^2
1	Acesulfame	18.75 - 1500	0.99999
2	Saccharin	18.75 - 1500	0.99997
3	Aspartame	18.75 - 1500	0.99998

Table 1
Linearity of the sweetener standards.

Peak	Compound	% RSD retention times	% RSD areas
1	Acesulfame	0.075	0.09
2	Saccharin	0.070	0.24
3	Aspartame	0.033	0.23

Table 2
Reproducibility of the 10 injections of sweetener standards.

	Acesulfame	Aspartame
Yogurt	0.09 mg/mL	0.027 mg/mL
Diet cola	0.205 mg/mL	0.146 mg/mL

Table 3
The amount of sweeteners in the real samples.

Conclusion

The Agilent 1120 Compact LC is ideal for the routine analysis of sweeteners in foods and beverages. Excellent resolution and good separation were achieved, and system suitability experiments showed the robustness and high precision for this kind of application. The high precision of the retention times and peak areas ensures reliable results when quantitation is needed for quality control.

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Rongjie Fu and Zhixiu Xu are Application Chemists at Agilent Technologies in Shanghai, China.

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Agilent Technologies

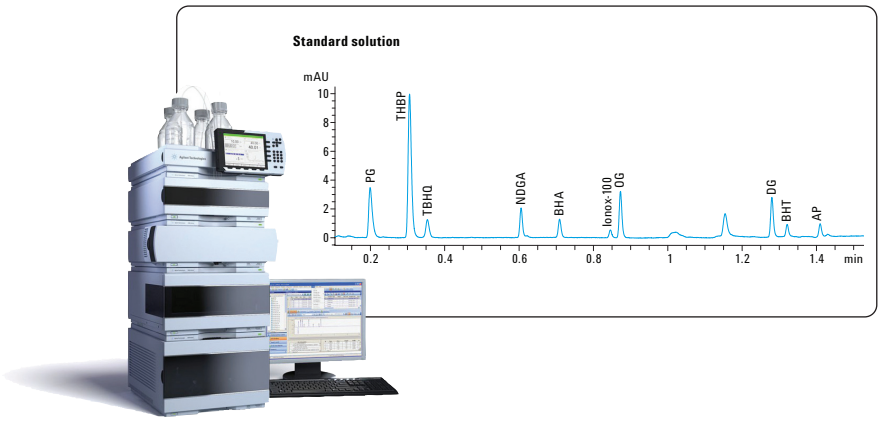
Ultrafast analysis of synthetic antioxidants in vegetable oils using the Agilent 1290 Infinity LC system

Application Note

Food

Authors

Gerd Vanhoenacker, Frank David,
Pat Sandra
Research Institute for Chromatography
Kennedypark 26
B-8500 Kortrijk
Belgium



Abstract

The addition of synthetic antioxidants in edible vegetable oils is regulated in Europe and the US. The official method was translated into an ultrafast LC method using the Agilent 1290 Infinity LC equipped with an Agilent ZORBAX Rapid Resolution High Definition (RRHD) column. High throughput is obtained in 2 min with a backpressure of 1120 bar, which is below the 1200 bar upper limit of the column. Optimization of the mobile phase composition and the temperature are discussed. The figures of merit are illustrated using standard solutions and spiked vegetable oil (sunflower, rapeseed, and olive) extracts. Limits of detection are 1 mg/kg or less in the oil samples. Using a simple methanol extraction, good recovery was obtained for all antioxidants in the oil samples.

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Introduction

Lipid oxidation causes rancidity and odor problems and decreases the nutritional value of food products. Synthetic ascorbyl palmitate and phenolic antioxidants are often added to foods to prevent oxidation of unsaturated fatty acids in oils and fats. Combinations of antioxidants are commonly used to enhance the antioxidative effect. The structures and abbreviations of the investigated antioxidants are shown in Figure 1.

Regulatory agencies in Europe[1] and the US[2] have imposed maximum levels for some antioxidants while the use of others has been forbidden. The determination of antioxidants in foods and food components is therefore an important analysis. The limits are given in Table 1.

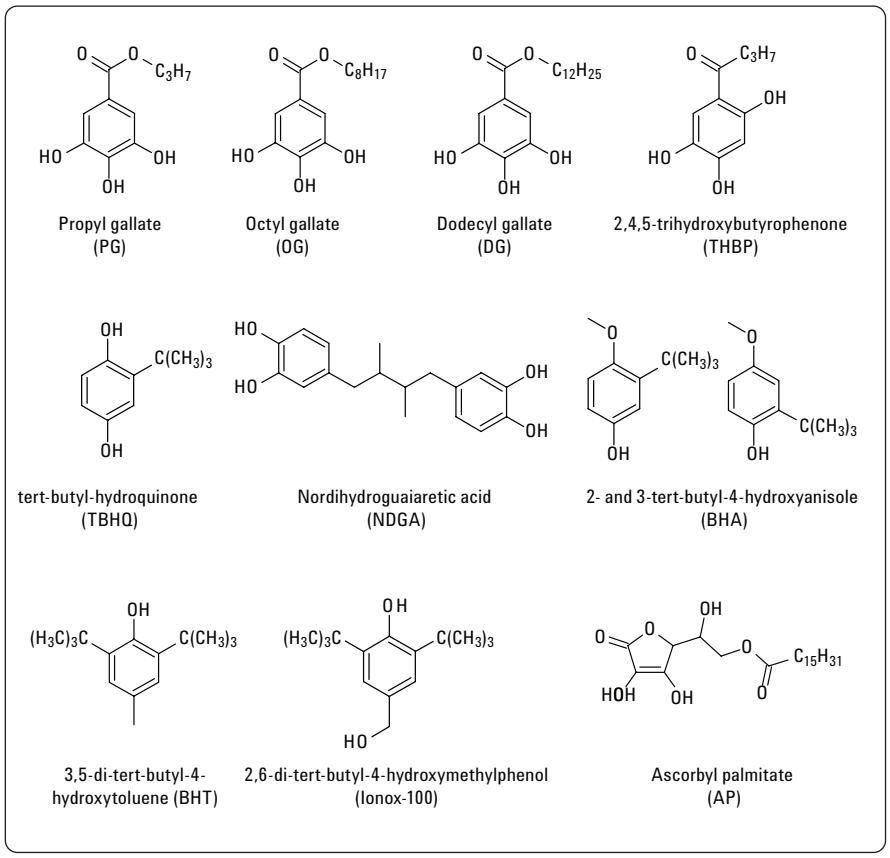

Figure 1
Structures and codes of the investigated antioxidants.

Antioxidant	Europe[1]	US[2]
AP	Quantum satis	No restriction
PG OG DG BHA	≤ 200 mg/kg, individual or combined	≤ 200 mg/kg, individual or combined
BHT	≤ 100 mg/kg	
TBHQ	Not allowed	
THBP	Not allowed	Not allowed
NDGA	Not allowed	Not allowed
Ionox-100	Not allowed	Not allowed

Table 1
Limits for antioxidants in edible oils in Europe and US.

In the official method for the determination of the antioxidants in edible oils, columns of 15 to 25 cm in length with an internal diameter of 4.6 mm, and packed with 5-µm octadecyl silica particles are used.[3] The mobile phase is composed of diluted acetic or phosphoric acid (eluent A) and methanol/acetonitrile 50/50 volume to volume (eluent B). Analysis times are between 15 to 25 min.

There are two reasons for increasing the speed of analysis for this application. First, instability of some of the targets (for example, AP) have been reported and long residence times of samples in an autosampler can already lead to significant degradation of the compounds. Perrin and Meyer could enhance the stability of sample and standard solutions by using citric and isoascorbic acid.[4] They were able to stabilize AP at room temperature for about 7 h. However, QC laboratories in edible oil and fat processing industries have a need for increased analysis speed. The presence or absence, and assay of antioxidants have to be carried out prior to loading or unloading oils and fats. A fast, accurate, and precise result is desirable for economical and practical reasons.

This Application Note describes the analysis of 10 antioxidants in vegetable oils using the Agilent 1290 Infinity LC. The original method was translated into a high throughput method by optimizing the mobile phase composition and the temperature. The figures of merit are presented for vegetable oil and spiked oil extracts.

Experimental

Instrumentation and method

An Agilent 1290 Infinity LC system with the configuration in Table 2 was used:

Solutions and samples

Sample and standard solutions were prepared according to Perrin and Meyer.[4] The solvent for the standards and extraction is a solution of citric acid (1 mg/mL) and isoascorbic acid (1 mg/mL) in methanol. For the spiked samples, a stock solution of the antioxidants in the solvent was added prior to extraction. The extraction was carried out by weighing 1 g of oil and adding 10 mL of the solvent. This mixture was vortexed for 30 s, allowed to stand for 2 min, and vortexed once more for 30 s. The sample was then centrifuged at 5000 x g for 5 min and the supernatant was transferred into an autosampler vial for injection.

Part number	Description
G4220A	Agilent 1290 Infinity Binary Pump with integrated vacuum degasser
G4226A	Agilent 1290 Infinity Autosampler
G1316C	Agilent 1290 Infinity Thermostatted Column Compartment
G4212A	Agilent 1290 Infinity Diode Array Detector

Method parameters:	
Column	ZORBAX RRHD Eclipse Plus C18, 50 mm L × 2.1 mm id, 1.8 µm d_p
Mobile phase	A = 0.02% phosphoric acid in water B = Acetonitrile/methanol 50/50 or 75/25 v/v
Flow rate	Variable
Gradient	Variable
Temperature	Variable
Injection	2 µL
Detection	DAD, 40 or 80 Hz Phenolic antioxidants Signal 280/10 nm, Reference 400/50 nm Ascorbyl palmitate Signal 255/10 nm, Reference 400/50 nm

Table 2
Conditions

Results and Discussion

The analysis was first carried out with the mobile phase used in the official method. The flow rate was set at a moderate 0.4 mL/min. The analysis time was 8 min (see Figure 2A). The synthetic phenolic antioxidants are all detected at 280 nm while for ascorbyl palmitate (AP) 255 nm was used. The eluent B composition was then modified from methanol/acetonitrile 50/50 to 75/25 volume to volume to lower the viscosity and enable a faster separation. The selectivity changed considerably with this mobile phase adaptation and the temperature was optimized to obtain sufficient separation between all target antioxidants. Note that at 45 °C, the elution order of dodecyl gallate (DG) and BHT is reversed compared to Figure 2A at 30 °C. All compounds were stable at 45 °C column temperature.

An additional advantage of the increased temperature is the decrease of the backpressure. When the flow rate was increased to 1.9 mL/min the last peak eluted under 1.5 min and the pressure on the column was 1120 bar (Figure 2C).

	A	B	C
Methanol/Acetonitrile ratio (v/v)	50/50	75/25	75/25
Flow rate	0.4 mL/min	0.4 mL/min	1.9 mL/min
Gradient	0–7.5 min: 35–100% B	0–7.5 min: 30–100% B	0–1.6 min: 30–100% B
Temperature	30 °C	45 °C	45 °C
Detector speed	40 Hz	40 Hz	80 Hz
Maximum pressure	375 bar	270 bar	1120 bar

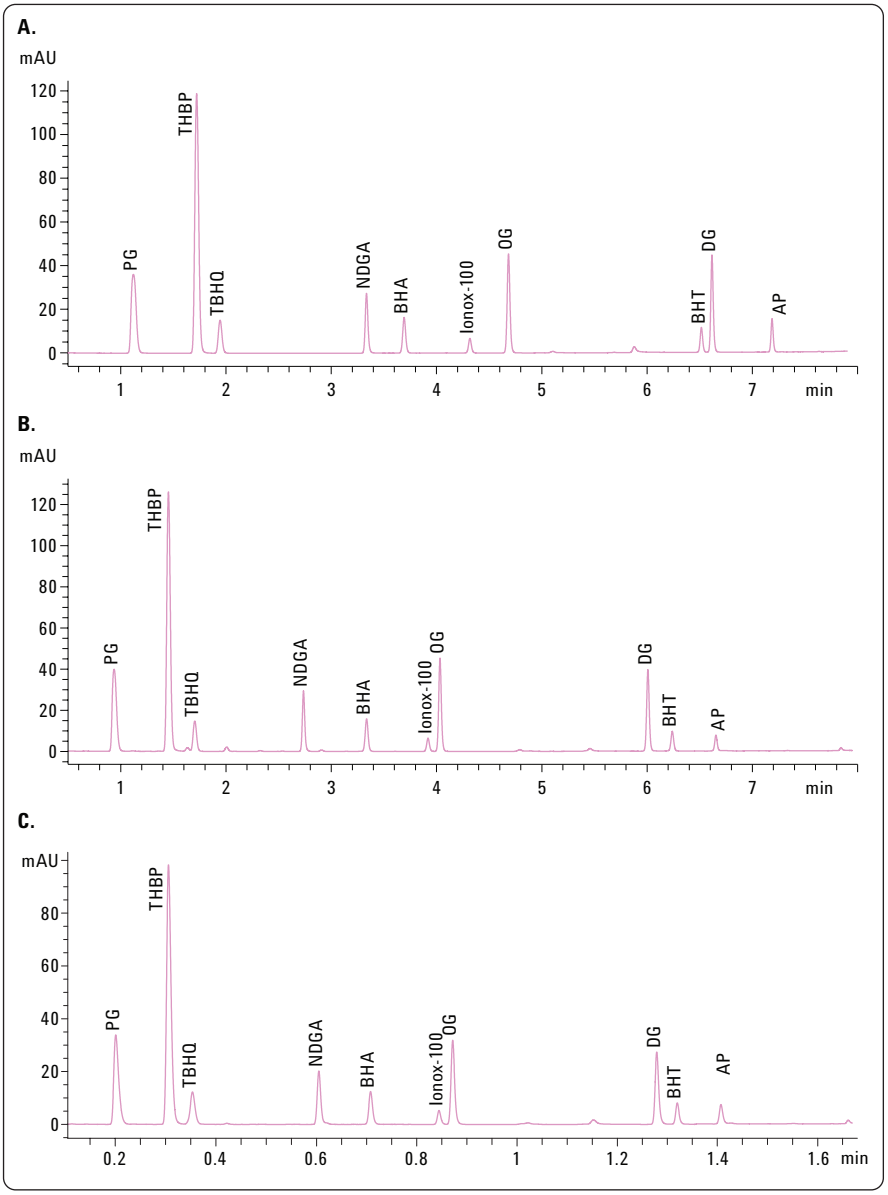

Figure 2
Analysis of 10 µg/mL standard solution under the various conditions.

The performance of the ultrafast method was evaluated and the results are summarized in Table 3. The repeatability and linearity of the method were investigated using standard solutions of the antioxidants. The detection limit was equal to or below 0.1 µg/mL for all antioxidants. This corresponds to approximately 1 mg/kg or lower in an oil or fat sample. Extracts of vegetable oils and spiked oils were analyzed to determine the recovery and accuracy. The oil samples were spiked with 10 or 50 mg/kg of each antioxidant and the detected amounts in the extracts were compared to standard solutions at the same concentration.

	Repeatability (% RSD)[1]	Linearity (R^2)[2]	Recovery 10 mg/kg (%)			Recovery 50 mg/kg (%)		
			Sunflower	Rapeseed	Olive	Sunflower	Rapeseed	Olive
PG	0.27	0.99988	100.1	105.3	95.1	100.0	100.9	98.3
THBP	0.27	0.99983	97.3	99.1	105.9	98.6	99.1	99.4
TBHQ	0.99	0.99933	90.7	89.7	81.2	97.4	95.8	95.6
NDGA	0.16	0.99983	109.3	89.7	93.6	102.2	98.0	98.9
BHA	0.33	0.99983	104.8	107.0	102.0	98.5	96.4	94.4
Ionox-100	0.40	0.99974	90.7	93.8	89.7	97.5	97.5	93.3
OG	0.41	0.99985	99.3	101.0	95.9	99.7	100.3	98.8
DG	0.56	0.99985	97.8	100.1	101.9	98.4	98.9	98.7
BHT	0.54	0.99960	81.0	89.5	74.4	81.6	83.8	79.0
AP	0.67	0.99934	92.6	85.7	75.4	89.5	91.2	83.7

(1) 6 consecutive injections of 10 µg/mL standard solution
(2) 0.1, 0.2, 0.5, 1, 10 µg/mL standard solution, 1 injection/level

Table 3
Method performance data.

The chromatograms for the fast analysis of a standard solution and the spiked oil samples are shown in Figure 3. Additional peaks originating from the oil matrix are visible in the chromatograms but only a few interfere with the analysis. Most interfering peaks are present in the olive oil sample, however, the 10 mg/kg spiked oil can still be differentiated from an unspiked sample and the recovery is satisfactory (Table 3).

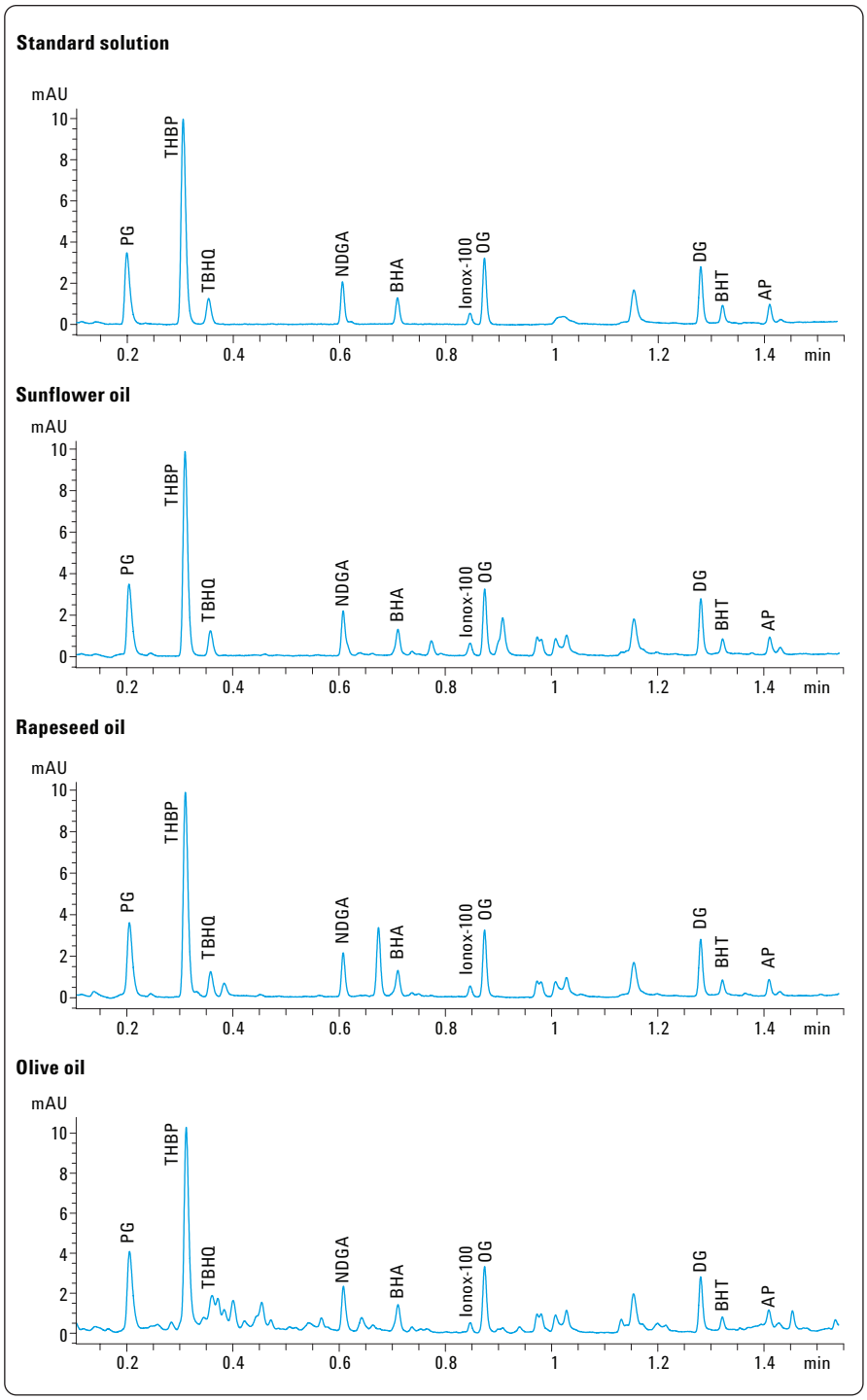

Figure 3
Analysis of standard solution (1 µg/mL) and spiked oil (10 mg/kg) extracts with the fast method.

Conclusion

Using the Agilent 1290 Infinity LC, an ultrafast analytical method could be developed for the determination of antioxidants in vegetable oils. The analysis time could be reduced to less than 2 min with a backpressure of 1120 bar. The performance of the high throughput method (repeatability, linearity, detection limits) was investigated using standard solutions. Oil samples and spiked oil samples were extracted and the recovery of the antioxidants was calculated. Satisfactory recovery was obtained for all antioxidants. The developed method is useful in laboratories where a fast result is mandatory.

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Agilent Technologies

Determination of Allergens in Fragrance Products Using Agilent Deconvolution Reporting Software

Application Brief

Wei Luan, Chris Sandy, and Mike Szelewski

Flavor and Fragrance

Highlights

- Automated deconvolution increases productivity for analysis of complex matrices
- Allergens database is available as a free download from Agilent Technologies, Inc.

The 7th amendment of the European Cosmetics Directive (2003/15/EC) was published in 2003. Manufacturers of cosmetics were required to indicate the presence of 26 fragrance ingredients in finished cosmetic products if they exceed a threshold of 0.01% for rinse-off and 0.001% for leave-on products. Subsequently, the analytical method for 24 allergenic compounds (except two natural extracts – oak moss and tree moss) using GC/MS was published by the International Fragrance Association (IFRA). Natural cosmetics are very complex, containing sterols, waxes, and flavanoids, which can result in poor spectral matches and long data processing time and can generate potential false positive or negative results.

Agilent's deconvolution reporting software (DRS) is designed to automatically deconvolute the spectra from matrices and generate a qualification and quantitation report. DRS integrates information from three processes: MSD ChemStation, automated mass spectral deconvolution and identification system (AMDIS), and NIST Search. DRS increases the confidence in results with complex matrices, and the typical data processing time is about 2 to 3 minutes.

A DRS database of 24 regulated allergenic compounds has been developed (Table 1). Additional compounds can easily be added to the database by the user.

Table 1. Allergens in Fragrance Products

Locked RT	Name	CAS no	Mol form	Mol wt
7.10	Limonene	5989-27-5	$C_{10}H_{16}$	136.1
7.17	Benzyl alcohol	100-51-6	C_7H_8O	108.1
7.41	Phenyl acetaldehyde	122-78-1	$C_8H_8O_2$	120.1
8.47	Linalol	78-70-6	$C_{10}H_{18}O$	154.1
10.29	1,4-Dibromobenzene [ISTD]	106-37-6	$C_6H_4Br_2$	233.9
10.41	Estragole	140-67-0	$C_{10}H_{12}O$	148.2
10.47	Folione	111-12-6	$C_9H_{14}O_2$	154.1
10.94	Citronellol	106-22-9	$C_{10}H_{20}O$	156.2
11.23	Citral (Neral)	106-26-3	$C_{10}H_{16}O$	152.1
11.44	Geraniol	106-24-1	$C_{10}H_{18}O$	154.1
11.77	Citral (geranial)	5392-40-5	$C_{10}H_{16}O$	152.1
11.80	Cinnamaldehyde	104-55-2	C_9H_8O	132.1

Table 1. Allergens in Fragrance Products (Continued)

Locked RT	Name	CAS no	Mol form	Mol wt
12.00	Anisyl alcohol	105-13-5	$C_8H_{10}O_2$	138.1
12.04	Hydroxy citronellal	107-75-5	$C_{10}H_{20}O_2$	172.2
12.33	Methyl octine carbonate	111-80-8	$C_{10}H_{16}O_2$	168.1
12.42	Cinnamic alcohol	104-54-1	$C_9H_{10}O$	134.1
13.37	Eugenol	97-53-0	$C_{10}H_{12}O_2$	164.1
14.13	Methyl eugenol	93-15-2	$C_{11}H_{14}O_2$	178.2
14.82	Coumarin	91-64-5	$C_9H_6O_2$	146.0
14.88	Cinnamyl acetate	103-548	$C_{11}H_{12}O_2$	176.1
14.96	Isoeugenol	97-54-1	$C_{10}H_{12}O_2$	164.1
15.5	Alpha isomethyl ionone	127-51-5	$C_{14}H_{22}O$	206.2
16.26	Lilial	80-54-6	$C_{14}H_{20}O$	204.2
18.14	Amyl cinnamaldehyde	122-40-7	$C_{14}H_{18}O$	202.1
18.27	Lyral 1	31906-04-5	$C_{13}H_{22}O_2$	210.2
18.36	Lyral 2	31906-04-4	$C_{13}H_{22}O_2$	210.2
18.7	Amyl cinnamyl alcohol	101-85-9	$C_{14}H_{20}O$	204.2
18.83	Farnesol 1	100009-91-0	$C_{15}H_{26}O$	222.2
19.18	Farnesol 2	4602-84-0	$C_{15}H_{26}O$	222.2
19.61	Hexyl cinnamaldehyde	101-86-0	$C_{15}H_{20}O$	216.2
19.89	Benzyl benzoate	120-51-4	$C_{14}H_{12}O_2$	212.1
21.36	Benzyl salicylate	118-58-1	$C_{14}H_{12}O_3$	228.1
24.20	Benzyl cinnamate	103-41-3	$C_{16}H_{14}O_2$	238.1

The Agilent Retention Time Locked (RTL) database was developed using the instrument conditions in Table 2, which is locked to alpha-isomethyl ionone in 15.494 min by retention time locking.

Table 2. Gas Chromatograph and Mass Spectrometer Conditions

GC	Agilent Technologies 7890A or 6890N		
Back inlet	Split/splitless		
Injection type	Split		
Inlet temperature	250 °C		
Pressure	11.46 psi		
Split ratio	50:1		
Split flow	68.5 mL/min		
Total flow	72.7 mL/min		
Gas saver	On		
Saver flow	15.0 mL/min		
Saver time	1.00 min		
Gas type	Helium		
4 mm ID, Single Taper Liner			
Oven			
Oven ramp	°C/min	Next °C	Hold min
Initial time		50	1
Ramp rate	8	270	1.5
Total run time	30 min		
Equilibration time	0.5 min		
Column	Agilent Technologies HP-5ms p/n 19091S-433		
Length	30 m		
Diameter	0.25 mm		
Film thickness	0.25 µm		
Mode	Constant pressure		
Pressure	11.46 psi		
Nominal initial flow	1.4 mL/min		
Inlet	Back inlet		
Outlet	MSD		
Outlet pressure	Vacuum		

Table 2. Gas Chromatograph and Mass Spectrometer Conditions (Continued)

RTL	System retention time locked to alpha isomethyl ionone at 15.494 min
Back Injector	
Sample washes	1
Sample pumps	3
Injection volume	1 µL
Syringe size	10 µL
Preinj solvent A washes	3
Preinj solvent B washes	3
Postinj solvent A washes	3
Postinj solvent B washes	3
Viscosity delay	1 second
Plunger speed	Fast
Preinjection dwell	0 minutes
Post-injection dwell	0 minutes
MSD	Agilent Technologies 5975C
Acquistion mode	Scan/SIM
Solvent delay	3 min
Low mass	40
High mass	350
Threshold	20
Sampling	3
Quad temperature	150 °C
Source temperature	230 °C
Transfer line temperature	280 °C
Tune type	Autotune
EM voltage	Atune voltage, 1,023.5 V
MSD-SIM	AutoSIM was used to pick ions, groups, and switching times
Number of groups	17
Ions/group	Varied 4 to 14
Dwell time, msec	10
Cycles/peak	Varied 5.8 to 15.9

A typical total ion chromatogram (TIC) of a fragrance product is displayed in Figure 1.

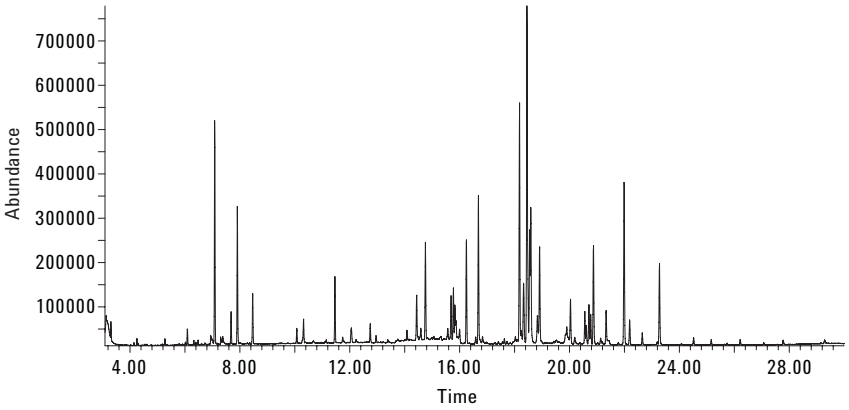

Figure 1. Total ion chromatogram of a fragrance product.

Dirty matrices often disturb the identification of target compounds by high-level chemical noise, resulting in poor library match factors. Background subtraction is both matrix- and operator-dependent and can yield inconsistent results. DRS automatically deconvolutes the signal from the matrix using AMDIS, which identifies target allergens quickly while minimizing false positives and negatives. A typical DRS report is displayed in Figure 2.

MSD Deconvolution Report
Sample Name: Lilac fragrance 10.20mg
Data File: C:\msdchem\1\DATA\Lilac.D
Date/Time: 10:49 AM Thursday, May 8 2008

Adjacent Peak Subtraction = 1
Resolution = Medium
Sensitivity = Medium
Shape Requirements = Medium

The NIST library was searched for the components that were found in the AMDIS target library.

			Amount (ng)		AMDIS		NIST	
R.T.	Cas #	Compound Name	Chem station	AMDIS	Match	R.T. Diff sec.	Reverse Match	Hit Num.
7.0984	5989275	Limonene	0.01		97	-0.1	94	1
7.1792	100516	Benzyl alcohol			84	0.7	86	1
7.3942	122781	Phenyl acetaldehyde			90	-0.3	94	1
8.4788	78706	Linalol	0.09		96	0.7	91	1
12.105	107755	Hydroxy citronellal	0.54		87	4.3	85	1
12.436	104541	Cinnamic alcohol	0.01		89	1.2	76	1
12.4495	111808	Methyl octine carbonate			56	8.0		
12.4495	22771444	cis-p-Mentha-2,8-dien-1-ol					71	1
14.8775	91645	Coumarin			91	3.7	92	1
15.1775	103548	Cinnamyl acetate			44	18.6		
15.1775	103957	3-(4-Isopropylphenyl)-2-methylpropionaldehyde					89	1
18.1555	122407	Amyl cinnamaldehyde			99	1.3	93	1
19.6005	101860	Hexyl cinnamaldehyde			94	0.0	89	1
19.8882	120514	Benzyl benzoate	0.01		72	0.3	91	1
24.1972	103413	Benzyl cinnamate			96	-0.2	90	2
10.296		1,4 Dibromobenzene	1					

Figure 2. Typical DRS report.

Figure 3 shows us the benefit of DRS when analyzing allergens in fragrance products. Cinnamaldehyde was successfully identified by DRS even though it was buried by the coeluting matrix compounds. The upper window is the TIC, the middle window is the raw or dirty spectrum in the scan No. 987 (11.796 min), and the lower window is the comparison of the deconvoluted spectrum (the white plot) with the spectrum of cinnamaldehyde in the allergen RTL library (the black plot). After deconvolution, the spectrum of the scan No. 987 is "clean," and we can easily identify the cinnamaldehyde in the fragrance product.

If your laboratory already has a user library in Agilent format, DRS A.04 software can create the necessary files in AMDIS format for deconvolution. Your laboratory may also have an optimized method or preferred column for allergens analysis. In these cases, DRS A.04 software can still be used with your own retention times and method.

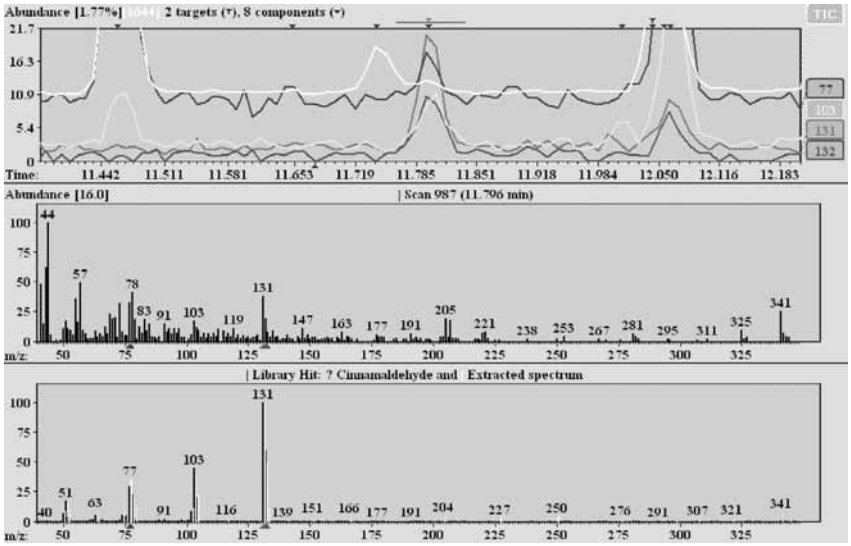

Figure 3. AMDIS display showing: a) the total ion chromatogram of a fragrance product; b) the spectrum where cinnamaldehyde elutes, and c) the deconvoluted spectrum (in white) juxtaposed to the library spectrum for cinnamaldehyde (in black).

Summary

DRS (p/n G1716AA) automatically deconvolutes mass spectra and produces more consistent and reliable identification of compounds in complex matrices. A DRS add-on allergen RTL database has been published on the Agilent Web site and, after registration, can be freely downloaded from http://www.chem.agilent.com/en-US/Support/Downloads/Utilities/RetentionTimeLocking/Pages/default.aspx.

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Wei Luan is an application chemist based at Agilent Technologies, Shanghai, China; Chris Sandy is an application chemist based at Agilent Technologies, UK; and Mike Szelewski is an application chemist based at Agilent Technologies, Wilmington, Delaware, U.S.

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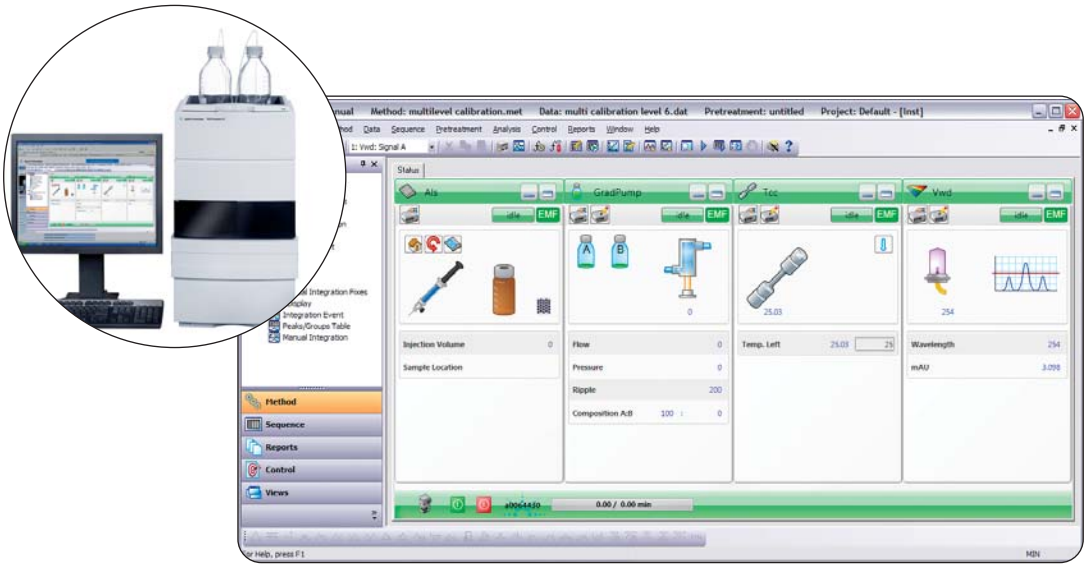
Agilent Technologies

Analysis of preservatives in food and cosmetics with the Agilent 1120 Compact LC system

Application Note

Rongjie Fu,
Zhixiu Xu



Abstract

An HPLC method was developed for simultaneous determination of the nine preservatives most often used in food and cosmetics. The system suitability results showed that the Agilent 1120 Compact LC is the system of choice for conventional, analytical scale liquid chromatography. This integrated HPLC system was designed for ease of use, performance, and reliability. The quantitative analysis of typical samples is demonstrated in this Application Note.

Agilent Equipment

- Agilent 1120 Compact LC
- EZChrom Elite Compact software

Application Area

- Food and cosmetics

Agilent Technologies

Introduction

Preservatives are very popular in the food and cosmetics industries because they prevent these products from degrading within the warranty time. But preservatives are strictly regulated because their overuse can cause some health problems in humans. For example, some preservatives can accumulate in the human body and negatively influence the metabolism process. Today's trends in food and cosmetics increasingly emphasize the concepts of healthy and green. That means use of safer raw materials, as well as fewer preservatives and control of preservatives within a safe limit.

In developing countries like China, the regulation of preservatives in food and cosmetics is approaching the international standards, such as the commonly used regulations adopted by the Codex Alimentarius Commission (CAC). In general, the regulations set the concentration limits on the preservatives in cosmetics and food. With increased research on safety of food and cosmetics, it might be necessary to analyze more preservatives in the future.

Some popular preservatives were analyzed in this application. A face conditioner and glace fruit were selected as typical samples that contain certain kinds of preservatives. The manufacturers of these products need to control the quality of their products before they go to market. The regulatory agencies check these products in the market very carefully to see if the amount of preservative is within the limit.

The experiments in this Application Note were performed with the Agilent 1120 Compact LC, which is the system of choice for conventional, analytical scale liquid chromatography. It is an integrated HPLC system designed for ease of use, performance, and reliability. It is ideally suited for routine analyses in the food and fine chemical industries because of its capability to achieve very precise retention times and peak areas, as well as low detection limits for the analyzed compounds.

Experimental

Equipment

- Agilent 1120 Compact LC system with gradient pump (degasser inside), autosampler, column compartment, and variable wavelength detector (VWD)
- EZChrom Elite Compact software

Chemicals and reagents

- Reference standards were purchased from Sinopharm Chemical Reagent Co. Ltd., Shanghai, China.
- Water was obtained from a Millipore water purifier.
- Acetonitrile (Fisher Scientific) was HPLC purity. All other reagents were analytical purity.

Sample preparation

Glace fruit: The fruit was cut into pieces and 1.5 g was weighed and diluted to 10 mL with water. The mixture was treated with an ultrasonic for 15 minutes, and was filtered with a 0.45 µm filter prior to injection.

Face conditioner: 1 mL was diluted to 10 mL with water, and the sample was filtered with a 0.45 µm filter prior to injection.

Chromatographic conditions

- Column: Agilent HC-C18(2), 4.6 x 250 mm, 5 µm
- Mobile phase: A = 20 mM acetate buffer, pH 4.2; B = acetonitrile
- Gradient: 0 – 25 min, 30 %B – 45 %B
- Flow rate: 1 mL/min
- Wavelength:

0 min	260 nm
5 min	230 nm
5.6 min	260 nm
9.2 min	230 nm
10.2 min	260 nm

- Injection volume: 5 µL
- Temperature: 30 °C

Results and discussion

Development of a method for these preservatives in food and cosmetics must consider the run time and the separation of the nine compounds. The other fact that must be considered is that the matrix of the real samples may influence the separation. The HPLC system needs a column with good efficiency, and for quantitative analysis of the preservatives, it must deliver good precision for retention times and peak areas.

Because the compounds used as preservatives have different ultraviolet (UV) spectra, the wavelength program of the variable wavelength detector was used in this study to detect all the compounds at their most sensitive wavelength.
The overlaid chromatograms of

two real samples and the preservative standards are shown in figure 1. The corresponding peak names are listed in table 1. In glace fruit, the benzoic acid and sorbic acid were found. In the face conditioner sample, only methylparaben was found.

The system reproducibility was tested with the nine compounds (table 1). The high precision of the retention times gives high confidence when comparing the standards and real samples.

The linear range of the standards was tested with this Agilent 1120 Compact LC system. The results are listed in table 2. The data shows that very good regression factors (values of r^2) were achieved for each compound.

The quantitative results from the two samples are shown in table 3.

Conclusion

The Agilent 1120 Compact LC is ideal for the routine analysis of preservatives in food and cosmetics. Excellent resolution and good separation were achieved, and system suitability experiments showed the robustness and high precision for this kind of application. The high precision of the retention times and peak areas ensures reliable results when quantitation is needed for quality control. The variable wavelength detector can be used with programmed wavelength to adjust to the maximum absorbance for all the compounds.

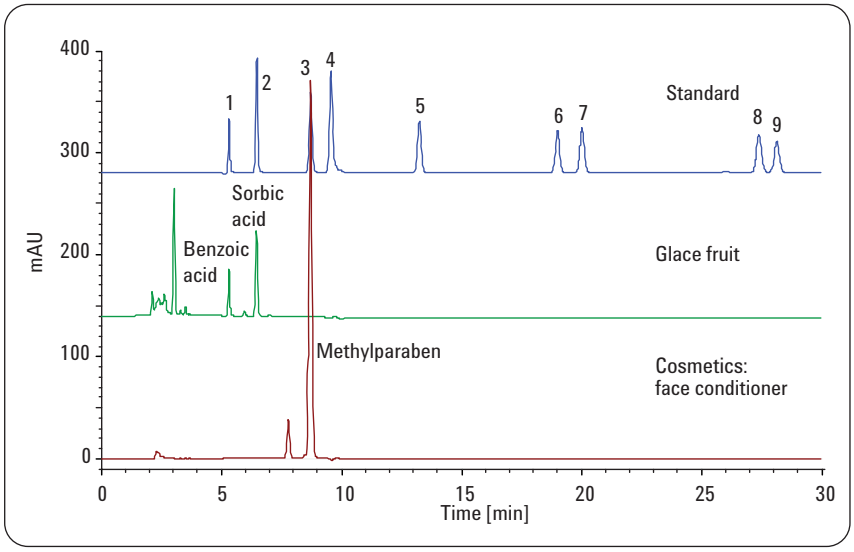


Figure 1
Overlaid chromatograms of preservative standards and real samples.

Peak	Compound	% RSD retention times	% RSD areas
1	Benzoic acid	0.03	1.11
2	Sorbic acid	0.03	0.16
3	Methylparaben	0.03	0.11
4	Dehydroacetic acid (DHA)	0.04	1.05
5	Ethylparaben	0.03	0.10
6	Isopropylparaben	0.03	0.11
7	n-Propylparaben	0.02	0.07
8	Isobutylparaben	0.02	0.10
9	n-Butylparaben	0.02	0.11

Table 1
Reproducibility of six injections of nine preservative standards.

Peak	Compound	Range (ng)	r^2
1	Benzoic acid	45.5 - 455	0.9998
2	Sorbic acid	35 - 350	0.9998
3	Methylparaben	66.5 - 665	0.9998
4	Dehydroacetic acid (DHA)	135 - 1350	0.9991
5	Ethylparaben	64.5 - 645	0.9998
6	Isopropylparaben	67 - 670	0.9998
7	n-Propylparaben	71.5 - 715	0.9998
8	Isobutylparaben	76.5 - 765	0.9998
9	n-Butylparaben	63.5 - 635	0.9998

Table 2
Linearity of the nine preservative standards.

	Benzoic acid	Sorbic acid	Methylparaben
Glace fruit	101.63 mg/Kg	70.59 mg/kg	ND*
Face conditioner	ND	ND	1.07 mg/mL

*ND = not detected

Table 3
The amount of preservatives in the real samples.

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Rongjie Fu and Zhixiu Xu are Application Chemists at Agilent Technologies in Shanghai, China.

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Agilent Technologies

Development of an LC-MS/MS Method for the Determination of 20-Hydroxyecdysone and Its Metabolites in Calf Urine

Application to the Control of Its Potential Misuse in Cattle

Application Note

Food Safety

Authors

Blandine Destrez, Gaud Pinel, Fabrice Monteau, and Bruno Le Bizec
Laboratoire d'Etude des Résidus et Contaminants dans les Aliments (LABERCA), Ecole Nationale Vétérinaire de Nantes (ENVN),
BP 50707, 44307 NANTES, Cedex 3, France

Abstract

Ecdysteroids, which are steroid hormones present in invertebrates and in plants, could be potentially used as anabolic agents in food-producing animals. The control of ecdysteroid misuse in cattle relies on the development of an efficient method for their detection in biological matrices at trace levels ($\mu g.L^{-1}$). In this context, an analytical procedure dedicated to the identification of 20-hydroxy-ecdysone and its metabolites in urine samples, based on purification on two solid-phase extraction cartridges (SPE C_{18} and SPE SiOH) and LC-(ESI+)-MS/MS measurements has been developed. The performance of tandem quadrupole MS/MS, in terms of sensitivity and specificity, allowed measurements at trace levels in both spiked and incurred samples. Good linearity was observed for all analytes from 0.12 ng to 12 ng on column.

Agilent Technologies

Introduction

Ecdysteroids are steroid hormones present both in invertebrate species (mainly Arthropods) and plants (belonging to *Asteraceae, Caryophyllaceae,* or *Polypodiaceae*). In arthropods, ecdysteroids act as moulting hormones, whereas these molecules are thought to protect plants against nonadapted phytophagous insects. The archetypal ecdysteroid in both kingdoms is 20-hydroxyecdysone (20E), and several studies have underlined its possible growth-promoting effects in various animal species (rats, mice, and Japanese quail), including humans and cattle [1–3]. Clinical studies demonstrated that 20E is more anabolic than methandrostanolone (dianabol), with no androgenic or other undesirable side effects usually observed with classical steroids [4]. However, despite its growth-promoting properties, only a few methods have been reported for its detection in biological matrices, and no information is available concerning its metabolism in cattle [5]. In this application, the development of a method able to detect and identify 20E and its main metabolites at trace levels (ppb) in calf urine is described [6]. This method was applied to the analysis of calf urine samples after 20E oral administration and used to assess the kinetic of elimination of these substances.

Experimental

Compound Standards

Standard reference 22S,23S-homobrassinolide (belonging to brassinosteroids, vegetable steroid hormones) was from Sigma-Aldrich (St. Quentin Fallavier, France); 20-hydroxyecdysone, 14-deoxy, 20-hydroxyecdysone, and 20,26-dihydroxyecdysone were a kind gift from Pr. Lafont.

Sample Preparation

Twenty-five nanograms of 22S,23S-homobrassinolide were added as internal standard (IS) to 5 mL of calf urine, centrifuged at 3,500 g for 15 min, then purified on SPE C18. The C18-SPE cartridges were conditioned with 10 mL methanol, then 10 mL water, following which the urine samples were applied. The columns were then washed with 6 mL of a water/methanol (80/20) mixture, and the ecdysteroids were subsequently eluted with 10 mL methanol. The eluant was then evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted in 50 µL ethanol and 150 µL cyclohexane before loading onto a SPE SiOH, previously activated with 25 mL cyclohexane. The phase was washed with 6 mL ethyl acetate/cyclohexane (80/20) and the compounds of interest were then eluted with 10 mL of a mixture of chloroform/methanol/acetone (6/2/1). The solvent was evaporated to dryness under nitrogen and the final extract was redissolved in 50 µL of methanol/water (30/70) containing 0.5% acetic acid. From this extract 10 µL was injected onto the HPLC column.

Instrumentation

LC:

Column:	GEMINI C_{18}, Phenomenex (3 µm, 110 Å, 50 × 2 mm)/Agilent equivalent: ZORBAX Extend-C18 3.5 µm, 2.1 mm × 50 mm (p/n 735700-902)	
Column temperature:	40 °C	
Mobile phases:	A: MeOH B: 0.5% acetic acid in water	
Flow rate:	0.3 mL/min	
Gradient:	Time (min)	%B
	0	90
	8	0
	10	0
	12	90
	16	90
Injection volume:	10 µL	
MS:	G 6410A QQQ, Agilent Technologies	
Ionization:	ESI (+)	
Fragmentor:	120 V	
Mass range:	100–500 amu	
Scan time:	300 ms	
Capillary:	4000 V	
Nebulizer:	35 psi	
Drying gas:	11 L/min	
Gas temperature:	325 °C	

The monitored transitions for each target compound are reported in Table 1. The first transition corresponds to the most sensitive signal.

Results and Discussion

Standard solutions of target compounds were analyzed according to the LC-MS/MS parameters described in the Experimental section, which allowed us to obtain the ion chromatograms of 20E, M1, M2, and IS, each at 5 ng on column (Figure 1). All the compounds are eluted within less than 10 min with very good chromatographic resolution and peak shape.

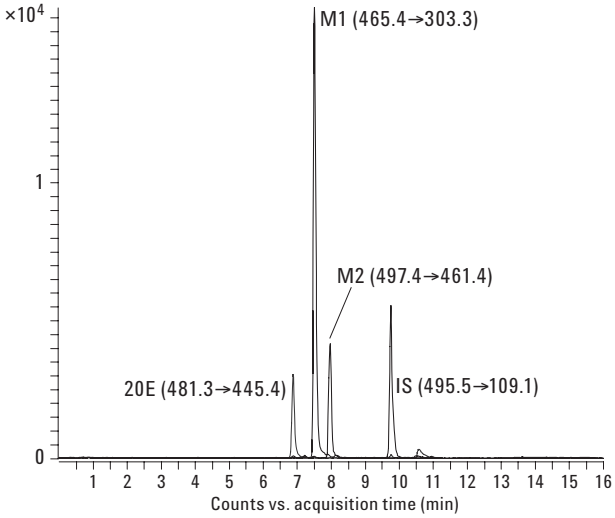

Figure 1. Overlaid extracted ion chromatograms (EICs) for the most sensitive transitions monitored for 20E and its metabolites in positive ion mode.

Table 1. Monitored SRM Transitions for 20E and Its Main Urinary Metabolites and Parameters of Acquisition for Their Analysis by LC-MS/MS (QQQ)

Analytes	Transition 1	Collision energy (eV)	Transition 2	Collision energy (eV)	Transition 3	Collision energy (eV)	RT (min ± 0.2)
22S,23S-homobrassinolide (IS)	495.5→109.1	20	495.5→127.1	10	495.5→459.1	5	9.8
20-hydroxyecdysone	481.3→445.4	10	481.3→371.4	10	481.3→165.1	20	7.5
14-deoxy,20-hydroxyecdysone (M1)	465.4→303.3	20	465.4→285.3	25	–	–	7.9
20,26-dihydroxyecdysone (M2)	497.3→461.4	5	497.3→351.1	15	497.3→371.2	20	6.8

To assess the specificity of the method, a blank urine and a urine sample fortified with 20E (1 $\mu g.L^{-1}$) were analyzed. Figure 2 shows the blank traces without any interference at the expected retention time for 20E, demonstrating the good selectivity of the monitored signals. The target analyte 20E was identified in the spiked urine sample with three SRM transitions. The monitored signals are detected with good sensitivity and show high signal-to-noise (s/n) ratios. These results were in accordance with Decision 2002/657/EC criteria, which require more than four identification points [7] in order to validate an identified compound.

The linearity and the repeatability of the method were assessed with the analysis of a pool of urine samples fortified at different concentration levels: the calibration curve was established with five concentration points (0.2, 0.5, 1, 5, and 20 $ng.mL^{-1}$). The calibration curve correlation coefficients (R^2) were better than 0.99, thus demonstrating the good linearity of the method for 20E.

The method has been successfully applied to incurred calf urine samples after 20E oral administration over four days. 20-hydroxyecdysone was detected in urine as rapidly as 30 minutes after its administration and up until 24 hours after the last administration. 20E metabolism was investigated and two main metabolites, 14-deoxy,20-hydroxyecdysone (M1) and 20,26-dihydroxyecdysone (M2), could be identified [8]. Both M1 and M2 were monitored by LC-MS/MS (Table 1). Figure 3 presents the ion chromatograms for M1 in the urine samples collected before and two days after the last 20E administration.

As can be observed, M1 was not detected in the urine collected before 20E administration, whereas it was throughout the four-day administration period. Furthermore, it could still be detected and identified (in accordance with the four identification points required) two days after the last administration of 20E. This result is of prime interest in the context of potential misuse of ecdysteroids since it offers the longest period for detection, following administration, and therefore enables a more efficient control mechanism.

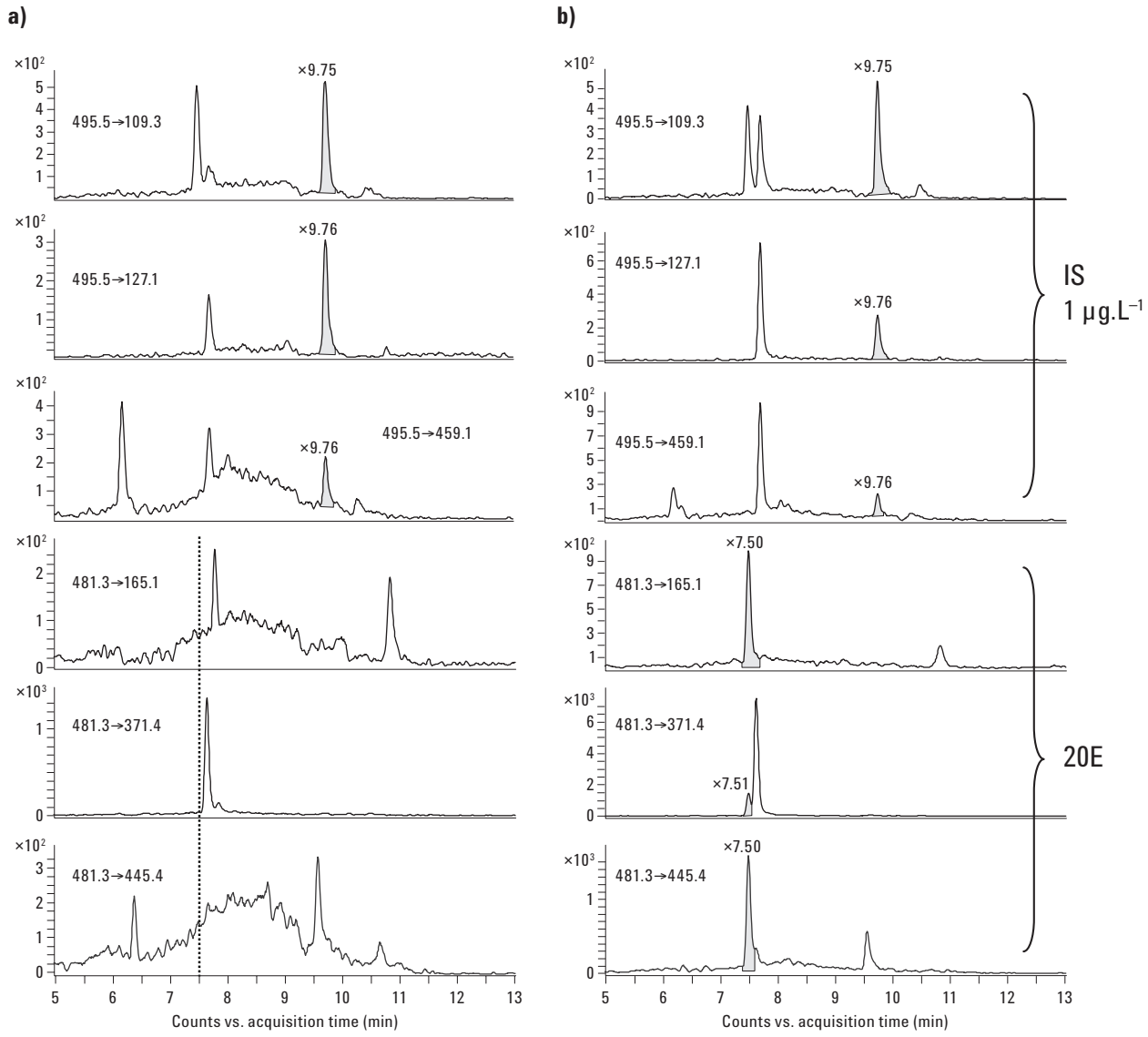

Figure 2. SRM ion chromatograms for a) the blank urine sample and b) the spiked urine sample (1 µg.L-1). LC-(ESI+)-MS/MS measurements.

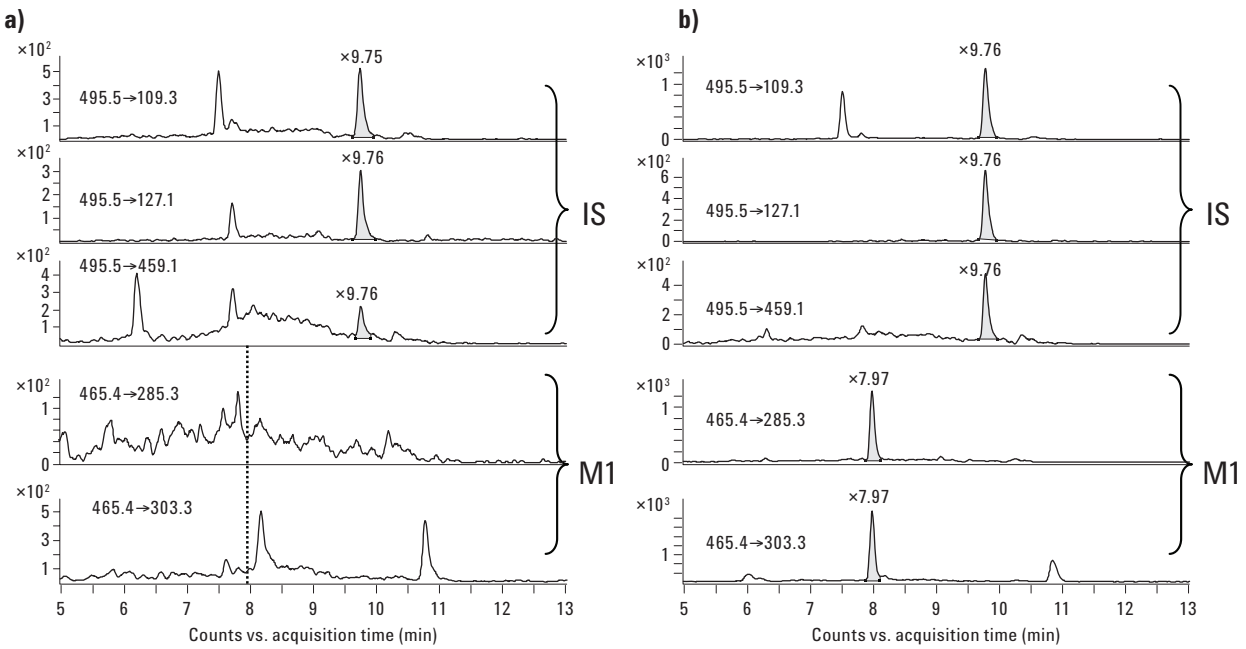

Figure 3. SRM ion chromatograms of IS and M1 in urine sample collected a) before 20E administration and b) two days after the last 20E administration. LC-(ESI+)-MS/MS measurements.

Conclusions

This work demonstrates the performance of LC-MS/MS, which provides efficient identification of 20E and its main metabolites in calf urine. The monitoring of these compounds facilitates the control of the potential misuse of 20E in meat-producing animals. Tandem quadrupole MS/MS is an analytical technique very well suited to this purpose, since it increases confidence in the unambiguous identification of the target compounds, in accordance to the criteria fixed by Decision 2002/657/EC. The successful analysis of the calf urine samples proved the robustness of the developed protocol. Application of this methodology also enabled the determination of the first elimination kinetics and the main metabolites of 20E in calf urine.

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Agilent Technologies

Selective Analysis of Non-Nutritive Food Additives Using Agilent ZORBAX Eclipse Plus C18, Eclipse Plus Phenyl-Hexyl, Eclipse XDB-Phenyl, and StableBond SB-Phenyl Columns

Application Note

Food

Author

Anne E. Brooks and William J. Long
Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE 19808
USA

Abstract

Nine common food additives are examined using 3.5-micron ZORBAX Eclipse Plus C18, Eclipse Plus Phenyl-Hexyl, Eclipse XDB-Phenyl, and StableBond SB-Phenyl Rapid Resolution columns with an isocratic solvent system. The mobile phases used include methanol or acetonitrile with 20 mM phosphate buffer at pH 2.5 or 0.2 percent formic acid. While excellent peak shape is found on all columns examined, selectivity and resolution differences are found when comparing C18 and various phenyl columns, especially the Eclipse Plus Phenyl-Hexyl and StableBond SB-Phenyl columns. In addition, selectivity differences and peak shape are preserved even when using a formic acid mobile phase modifier. The use of formic acid would allow verification of unknown materials using mass spectrometry.

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Introduction

Non-nutritive food additives are often found in food and non-food consumer products; they serve to maintain freshness and enhance flavor. Some of these compounds include preservatives, such as parabens and benzoic acid, which have antibacterial properties, and also acids, such as ascorbic acid and sorbic acid, to act as sacrificial oxidants. Non-nutritive sweeteners, like aspartame and saccharin, are commonly used in products where natural sweeteners would not be welcome, such as diet beverages or dental products. Finally, materials such as caffeine are frequently added to beverages, gums, and candies as mild stimulants. When present in appropriate amounts, each of these additives is nontoxic and serves an important function; however, at higher levels some can evoke allergic reactions or, after prolonged exposure, they can cause sensitization in certain individuals. To prevent these potentially negative side effects, it is important that the levels of additives be regulated and monitored. One common structure found in these additive compounds is their conjugation, which may interact with phenyl stationary phases, resulting in varied selectivity when compared to alkyl and other phenyl phases.

Alkyl columns, such as the Agilent ZORBAX Eclipse Plus C18, are often regarded as an all-purpose column, as they are sufficient for a variety of analyses. Many chromatographers center HPLC method development on C18 columns; however, other columns can offer advantages in selectivity. While the C18 separation is based primarily on hydrophobic interactions, other columns, such as phenyl columns, can also separate based on a degree of aromatic selectivity using π-π interactions. The degree of π-π interactions is based on many factors, including the type of phenyl phase and the type of silica. This has been shown in references 1 through 3. Other examples of applied selectivity with phenyl columns can be found [4–8]; they are shown with 1.8-, 3.5-, and 5.0-µm particles. Agilent offers three phenyl phases in 3.5-micron size, which include the new Eclipse Plus Phenyl-Hexyl, the Eclipse XDB-Phenyl (an ethyl phenyl phase), and the StableBond SB-Phenyl (an ethyl phenyl phase attached to nonendcapped type B silica). The 3.5-micron particles have been previously shown to offer increased efficiency over 5-micron materials. They offer the efficiency of a 25-cm 5-micron column in a 15-cm column length. In many conservative laboratories, changing to these proven columns provides 40 percent or better time and solvent savings. These columns are ideal for use in food laboratories, as they provide advantages of efficiency and robustness.

In this work, a group of non-nutritive food additives will be used to advocate the use of phenyl columns in HPLC method development with conjugated samples. Select consumer product samples will also be analyzed to demonstrate how the level of complexity of a sample might also dictate column choice with respect to resolution and speed of analysis.

Experimental

An Agilent 1200 Rapid Resolution LC (RRLC) system was used for this work:

- G1312B Binary Pump SL with mobile phase A: 20 mM monobasic potassium phosphate in water, pH 2.5 or 0.2% formic acid; B: methanol or acetonitrile. Flow rate was 1.5 mL/min. For methanol mobile phase, the analysis was isocratic with A/B (70:30); when equivalent solvent strength acetonitrile was substituted for methanol, the analysis was isocratic with A/B (79:21).
- G1376C Automatic Liquid Sampler (ALS) SL. Injection volume was 2.0 µL.
- G1316B Thermostated Column Compartment (TCC) SL. Temperature was 25 °C.
- G1315C Diode Array Detector (DAD) SL. Wavelength was 220, 16 nm Ref = 420, 20 nm, with a G1314-60083 semi-micro flow cell (6-mm path, 5-µL volume).
- ChemStation version B.02.01 was used to control the HPLC and process the data.

Four Agilent ZORBAX columns were used in this work:

- ZORBAX Rapid Resolution Eclipse Plus C18, 4.6 mm × 150 mm, 3.5 µm, Agilent p/n 959963-902
- ZORBAX Rapid Resolution Eclipse Plus Phenyl-Hexyl, 4.6 mm × 150 mm, 3.5 µm, Agilent p/n 959963-912
- ZORBAX Rapid Resolution Eclipse XDB-Phenyl, 4.6 mm × 150 mm, 3.5 µm, Agilent p/n 963967-912
- ZORBAX Rapid Resolution StableBond SB-Phenyl, 4.6 mm × 150 mm, 3.5 µm, Agilent p/n 863953-912

Figure 1 shows the compounds that were examined in this work with their respective structures, pKa values, and additive functions. All compounds were dissolved in water to a concentration of about 2 mg/mL. A composite sample was then made by combining equal aliquots of each solution.

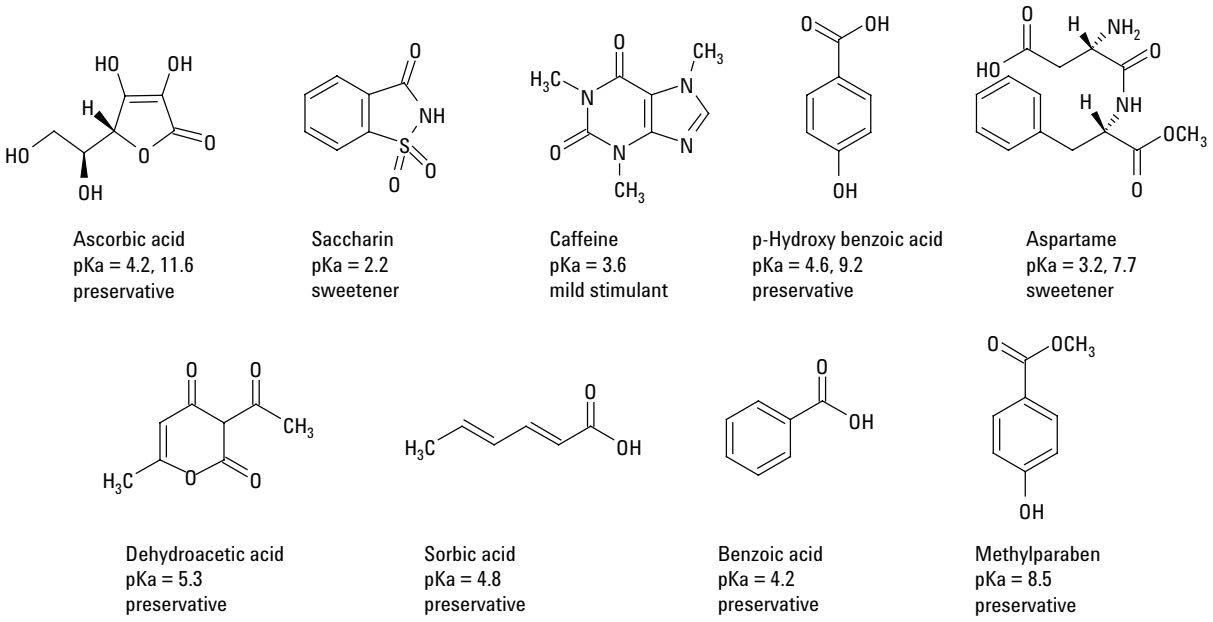


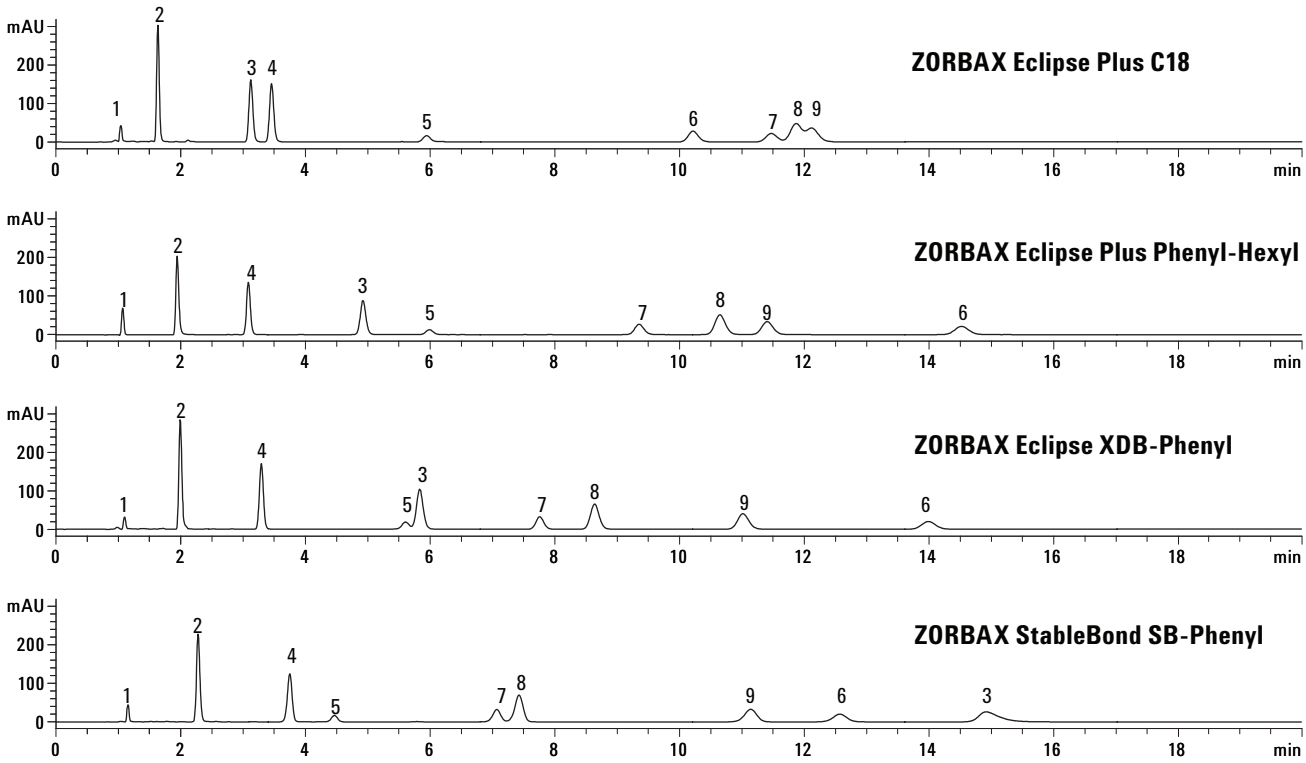
Figure 1. Compounds of interest (non-nutritive additives).

Compounds were purchased from Sigma Aldrich (Bellefonte, PA). In addition, formic acid, phosphoric acid, and monobasic potassium phosphate were also purchased from Sigma Aldrich. Methanol was purchased from Honeywell, Burdick and Jackson (Muskegon, MI). Water used was 18 M-Ω Milli-Q water (Bedford, MA).

Results and Discussion

The compounds shown in Figure 1 are found as ingredients in many food and non-food consumer products. These include, but are not limited to, beverages and dental care products.

In this work we investigated the selectivity between ZORBAX Eclipse Plus C18, Eclipse Plus Phenyl-Hexyl, Eclipse XDB-Phenyl, and StableBond SB-Phenyl. In the chromatograms shown in Figure 2 we compare the separation of these compounds using Rapid Resolution 4.6 mm × 150 mm, 3.5-micron columns.



1) Ascorbic acid
2) Saccharin
3) Caffeine
4) p-Hydroxybenzoic acid
5) Aspartame
6) Dehydroacetic acid
7) Sorbic acid
8) Benzoic acid
9) Methylparaben

Figure 2. Selectivity of additives on C18 and different phenyl columns, using 30% methanol 70% 20 mM phosphate buffer, pH 2.5; flow rate was 1.5 mL/min.

The largest selectivity differences are seen in caffeine (3), dehydroacetic acid (6), sorbic acid (7), and benzoic acid (8). For all columns, there is no significant difference in the elution of ascorbic acid (1), saccharin (2), p-hydroxybenzoic acid (4), aspartame (5), and methylparaben (9). The Eclipse Plus Phenyl-Hexyl column provides the best separation for this application under the given conditions, with a minimum resolution of R = 2.32. The Eclipse Plus C18 column also delivers excellent separation for the first six compounds; however, the separation of sorbic acid (7), benzoic acid (8), and methylparaben (9) is not as good as that shown on the Eclipse Plus Phenyl-Hexyl column. Also, the caffeine (3) and p-hydroxybenzoic acid (4) peaks are in reverse order on the phenyl-hexyl column compared to the C18 column. The StableBond SB-Phenyl column provides the second best separation (minimum resolution, R = 1.51), with substantially different selectivity; most significant is the movement of the caffeine to the end of the chromatogram, preceded by dehydroacetic acid, which elutes in the sixth position on the C18 column. Figure 3 demonstrates the same separation with an equal strength acetonitrile mobile phase compared to the methanol separation shown in Figure 2. What is most notable with acetonitrile is the significant compression of the phenyl chromatograms and the loss of selectivity differences among the C18 and phenyl columns as compared to the methanol separation. It has been theorized that the π-π interactions between the analyte and bonded phase are overwhelmed by the π bonds in acetonitrile [9]. It is evident that the methanol separation is superior to the acetonitrile separation in these cases.

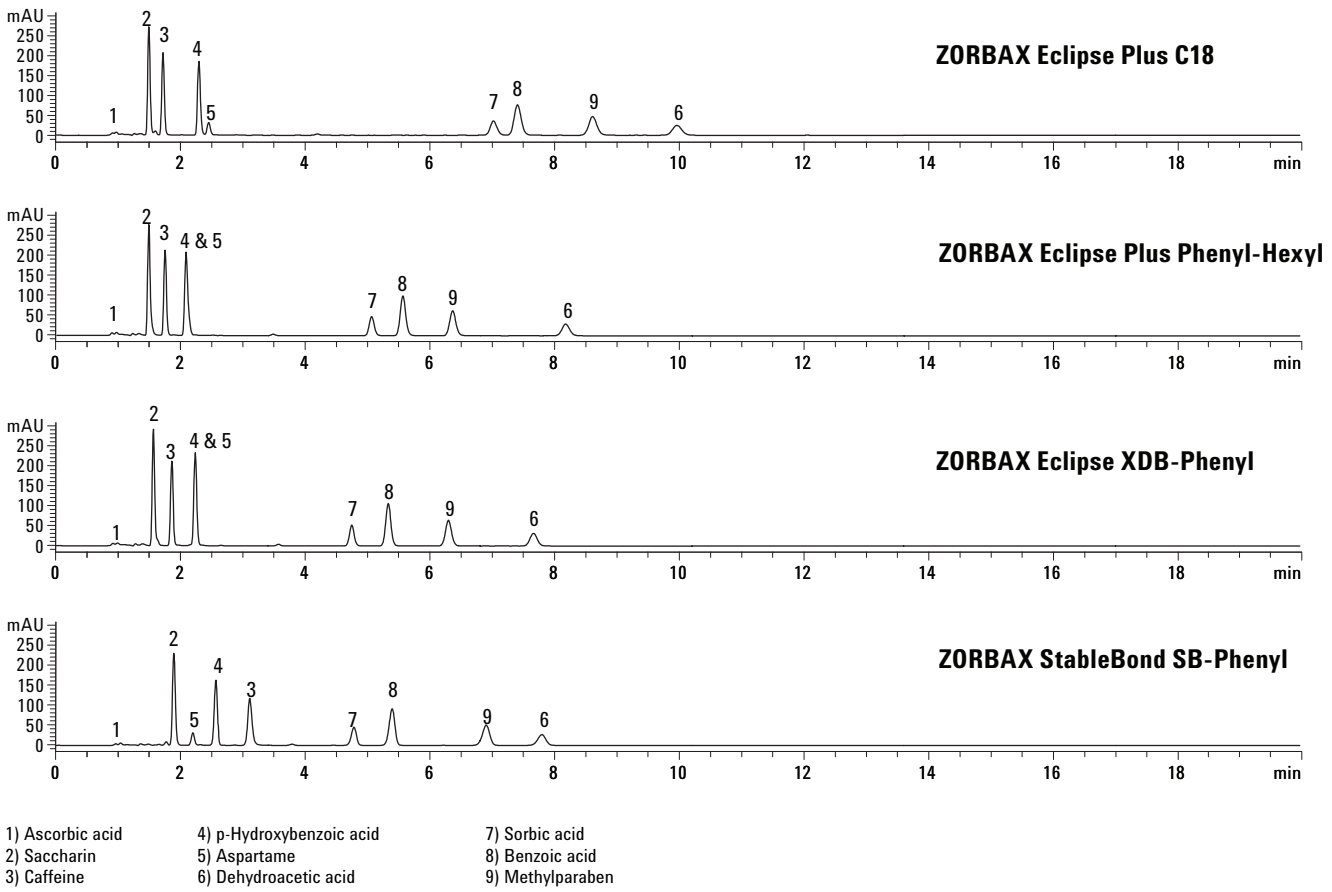
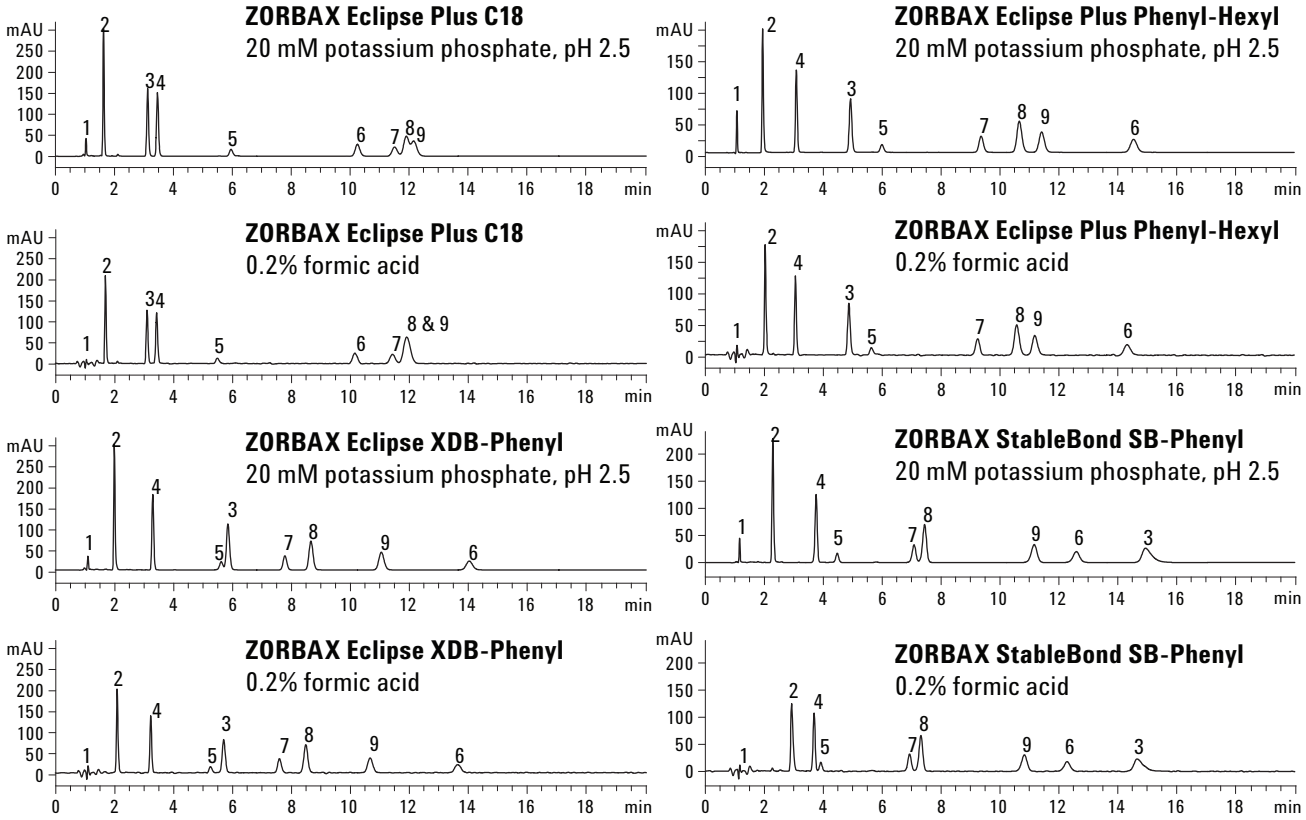


Figure 3. Selectivity of additives on C18 and different phenyl columns, using 21% acetonitrile 79% 20 mM phosphate buffer, pH 2.5; flow rate was 1.5 mL/min.

In many QC laboratories, phosphate buffers are used to maintain pH control of mobile phases. However, due to their lack of volatility, phosphate buffers are not compatible with mass spectrometers. In Figure 4, comparison chromatograms of C18 and phenyl columns are shown using methanol and either phosphate buffer or 0.2 percent formic acid. What is noteworthy is the lack of change in selectivity and elution order when shifting from one solvent to another. This indicates that the formic acid mobile phase can act as a supplementary mobile phase and would be ideal if MS detection were required.

As mentioned earlier, non-nutritive sweeteners and preservatives are found in many beverages and dental products. A simple survey of most prepared beverages will show at least one or two of the compounds in this study. We examined several products including TaB cola (The Coca-Cola Co., Atlanta, GA) and Monster Energy drink (Monster Beverages Co., Corona, CA), as well as Listerine (McNeil-PPC, Inc., Skillman, NJ) and Scope (The Proctor & Gamble Co., Cincinnati, OH) mouthwashes. A list of the ingredients of each product is shown in Table 2. In at least one case an ingredient is listed twice (sodium benzoate and benzoic acid, which are combined by analyzing at low pH, as the acidity of the mobile phase ensures full protonation of the benzoic acid). As can be seen, a great number of other ingredients are not analyzed during this work. In many cases, a second HPLC test or other test might be used, for example LCMS.



1) Ascorbic acid
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3) Caffeine
4) p-Hydroxybenzoic acid
5) Aspartame
6) Dehydroacetic acid
7) Sorbic acid
8) Benzoic acid
9) Methylparaben

Figure 4. Comparison of 70%, 20 mM phosphate buffer, pH 2.5, and 70%, 0.2% formic acid with 30% methanol on food additives separation with C18 and phenyl columns; flow rate was 1.5 mL/min.

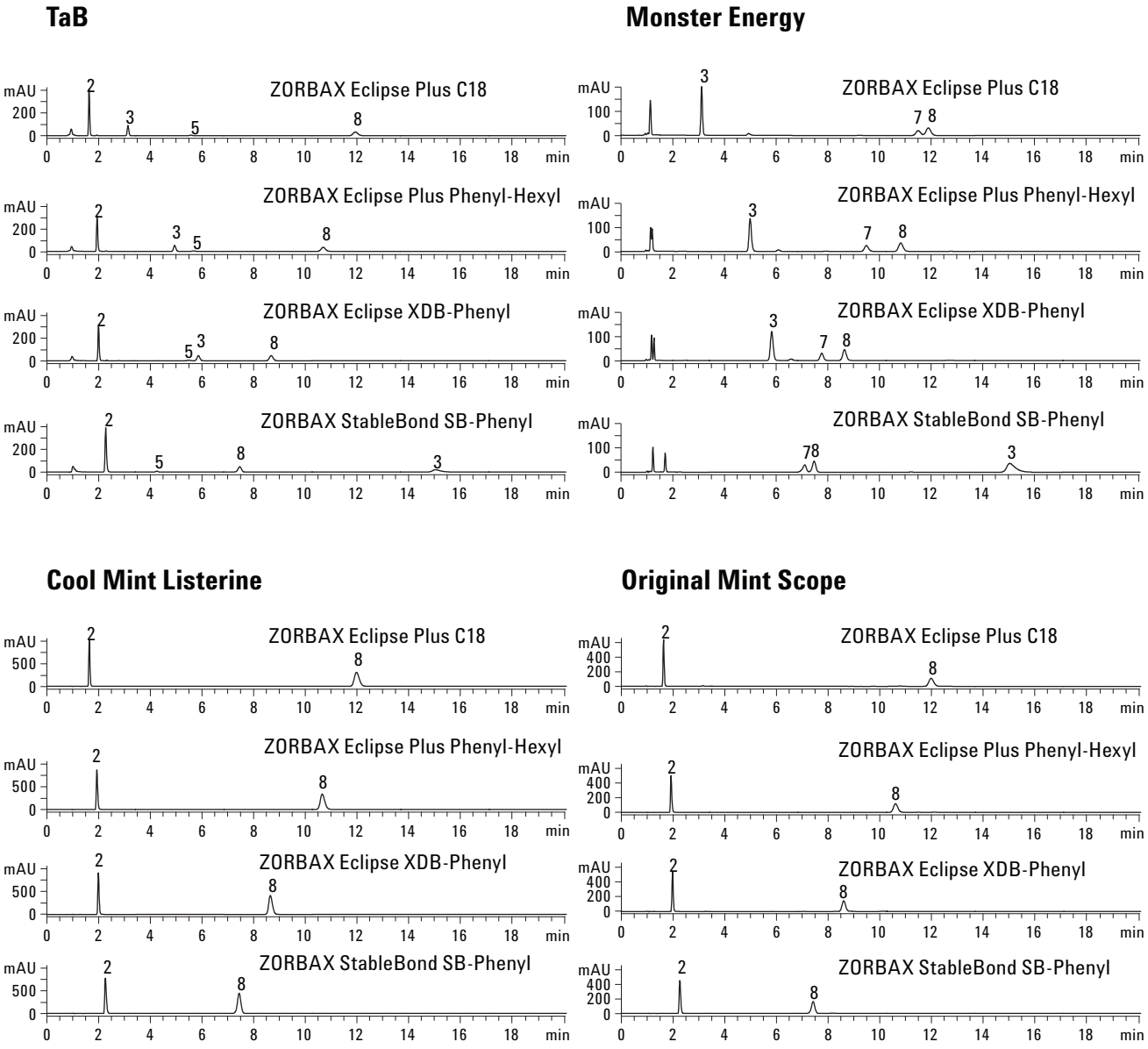
Table 2. Products and Ingredients

Product	Analyzed ingredients	Other ingredients
TaB	Calcium saccharin, potassium benzoate, caffeine, aspartame	Carbonated water, caramel color, natural flavors, phosphoric acid
Monster Energy	Caffeine, sorbic acid, benzoic acid	Carbonated water, sucrose, glucose, citric acid, natural flavors, panax ginseng root extract, L-carnitine, niacinamide, sodium chloride, glucuronolactone, inositol, guarana seed extract, pyridoxine, hydrochloride, sucralose, riboflavin, maltodextrin, cyanobalamin
Listerine	Benzoic acid, sodium saccharin, sodium benzoate	Eucalyptol, menthol, methyl salicylate, thymol Inactive ingredients: water, alcohol (21.6%), sorbitol solution, flavoring, poloxamer 407, FD&C Green no. 3
Scope	Sodium saccharin, sodium benzoate, benzoic acid	Water, alcohol, glycerin, flavor, polysorbate 80, Blue 1, Yellow 5, cetylpyridinium chloride

Equation 1. Resolution Equation

$$R_S = \frac{\sqrt{N}}{4} \bullet \frac{(\alpha - 1)}{\alpha} \bullet \frac{k'}{k' + 1}$$

Theoretical plates | Selectivity | Retention



1) Ascorbic acid
2) Saccharin
3) Caffeine
4) p-Hydroxybenzoic acid
5) Aspartame
6) Dehydroacetic acid
7) Sorbic acid
8) Benzoic acid
9) Methylparaben

Figure 5. Soft drink, energy drink, and mouthwash analysis with varied columns.

As can be seen in Figure 3 the best separation of the nine-compound mixture with this mobile phase is achieved with the Eclipse Plus Phenyl-Hexyl column. However, it is obvious that not all samples contain the same challenging mixture. For the TaB sample shown in Figure 5, it can be seen that the Eclipse Plus C18, Phenyl-Hexyl, and StableBond SB-Phenyl columns all provide, good resolution for all of the analytes in the sample. In the case of the Monster Energy drink, the Eclipse Plus Phenyl-Hexyl column provides excellent resolution of all compounds in this mixture, but the Eclipse XDB-Phenyl column provides similar resolution in less time. Finally, in the analysis of mouthwash samples, the StableBond SB-Phenyl column provides the fastest separation, while maintaining sufficient resolution.

Conclusions

The importance of selectivity in analysis cannot be underestimated. As depicted in the resolution equation (Equation 1), selectivity is the most important factor in this equation. This work has examined selectivity derived from choice of solvent (methanol vs. acetonitrile) and selectivity derived from different columns. The importance of selectivity is further underscored in the examination of real samples, containing only selected components of the mixture, leading to altered analysis requirements, especially when speed of analysis is considered.

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Agilent Technologies

Determination of preservatives in food and drugstore products with the Agilent 1120 Compact LC

Application Note

Food

Authors

Detlef Wilhelm
ANATOX GmbH & Co. KG
Fuerstenwalde
Germany

Abstract

This Application Note describes the analysis of several well known preservatives like benzoic acid, sorbic acid or the esters p-hydroxy benzoic acid (methyl-, ethyl- and propyl-) with the Agilent 1120 Compact LC.

Conventional LC methods are often used in routine analyses to characterize or monitor products for this purpose. The presented data show the determination of these ingredients in several consumer products such as chewing gum, ketchup, and barbecue sauce, which demonstrates the relevance of this method for quality control testing and monitoring of products for consumer protection.

This Application Note shows that the Agilent 1120 Compact LC works as a reliable and highly robust instrument for standard LC. It can be used for routine analyses. The use of methanol for separating the components reduces cost and allows a 15-min determination of all components with an overall resolution of >3. The results of reliability, quality, system suitability and performance testing are shown.

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Introduction

Several consumer products contain preservatives, which can be harmful to some people. The most common preservatives are benzoic or sorbic acid as well as the esters p-hydroxy benzoic acid. These ingredients inhibit microbiological growth in food or drugstore products and are used either alone or as a mixture. During manufacturing, the products are analyzed for quality control and monitored for consumer protection. Standardized conventional LC methods are often used in routine analyses to characterize or monitor products.

There are several requirements for the analytical instrumentation: reliability, flexibility, and ease of use. In addition, the instrument should provide low ownership cost.

This Application Note shows that the Agilent 1120 Compact LC works as a reliable and highly robust instrument for standard LC. It can be used in routine analyses, for preservatives found in several consumer products. These analyses are done in independent control laboratories; therefore the use of methanol for separating the components should be optimized to reduce cost and pollution.

The results of reliability, quality, system suitability and performance tests are shown.

Instrumentation

An Agilent 1120 Compact LC with the following configuration was used:

Configuration of the Agilent 1120 Compact LC
Gradient pump and vacuum degasser
Auto sampler
Column oven
Variable wavelength detector
Software: EZ-Chrom Elite Compact 3.3

Preparation of samples

Reference samples

Dissolve 10 mg of each vitamin in water and dilute to 100 mL with the same solvent. Mix and dilute 1 mL of each solution to 20 mL with mobile phase.

The following common preservatives were checked:
Benzoic acid, sorbic acid, methyl-, ethyl- and propylesters of p-hydroxy benzoic acid (Methyl-PHB, Ethyl-PHB, Propyl-PHB).

Samples from food and drugstores

Dissolve 1g of sample in 10 mL of water. Solution and extraction can be improved by treating for 10 min in a ultrasonic bath. Next, filter the samples with syringe filters; first through a 2-µm filter, followed by a 0.45-µm syringe filter to prepare the clear solution for injection.

Chromatographic conditions		
Column	ZORBAX Eclipse XDB C18, 150 mm × 4.6 mm, 5 µm	
Mobile phases		
Phase A:	Dissolve 6.8 g potassium dihydrogen phosphate in 900 mL water. The pH value should be adjusted to pH = 2.3 with phosphoric acid and then filled to 1000 mL with water.	
Phase B:	Methanol	
Gradient (linear):	Time (min)	
	0	80% A/20% B
	9.6	47% A/53% B
	12	33% A/67% B
	13	33% A/67% B
	13.1	80% A/20% B
Pump settings		
Stop time:	15 min	
Post time:	5 min	
Flow rate:	1.75 mL/min	
Autosampler		
Injection volume:	20 µL	
Thermostatted column compartment		
Temperature:	40 °C	
Detector	14 µL cell, Peak width: >0.05 min, 1 s response time (10 Hz), Signal: 220 nm	

System suitability and performance test:
For system suitability testing, the reference solution with the limits listed below was used. This was in accordance with Q3A(R)- Impurities in New Drug Substances:[2]

- Resolution: minimum 1.5 between each peak
- Precision of areas must be < 2 % RSD.
- Precision of retention times must be < 0.5 % RSD.

With these limits and settings for testing, the samples in Table 1 were prepared and analyzed.

Results and discussion

The separation was achieved with methanol, since it was able to separate the critical pair of benzoic and sorbic acid.[2] The results in Figure 1 show good separation of all preservatives with the Agilent ZORBAX Eclipse XDB C18 material on the Agilent 1120 Compact LC and EZChrom Elite Compact Software.

For the detection of all components, the Agilent 1120 Compact LC variable wavelength detector was set for 260 nm. This setting was chosen because all target analytes except benzoic acid has a maximum adsorption near 260 nm.

Detailed data are listed in Table 2. The first limit for resolution (>1.5) is fulfilled for all peaks. The data for resolution show good selectivity with the ZORBAX Eclipse XDB C18 material.

Sample	Purpose	Number of injections
Blanc solution	Verify baseline stability and identify artifacts	2
Calibration samples	Verify linearity	3 of each level
Control sample	Verify sensitivity and resolution for reference solution	6
Suitability sample	Verify precision of areas and retention times for reference solution	10

Table 1
Setup for testing.

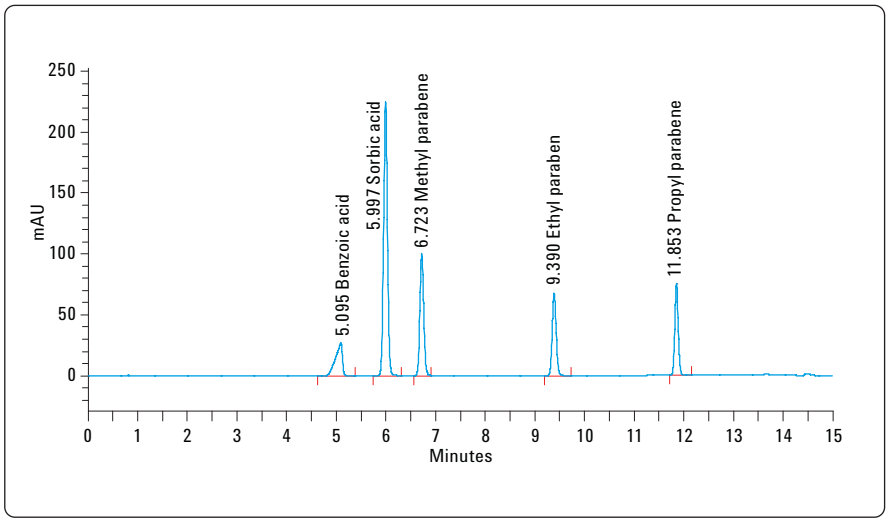

Figure 1
Standard chromatogram of preservatives with the Agilent 1120 Compact LC.

Compound	Retention time (min)	Resolution
Benzoic acid	5.06	–
Ascorbic acid	5.96	4.31
Methyl-PHB	6.69	5.25
Ethyl-PHB	9.39	18.55
Propyl-PHB	11.85	17.83

Table 2
Results for control sample: Retention times and resolution.

The areas and retention time precision results of all compounds of the suitability sample are shown in Table 3. The data demonstrate the high reliability and precision of the Agilent 1120 Compact LC. The data show that the system can be used for QC methods, since the criteria for retention times and areas are fulfilled for all compounds.

The data in Tables 3 and 4 prove high precision and reliability of the autosampler. The correlation coefficient for each calibration curve is very close to 1.0 showing high versatility and quality for QC testing and monitoring.

It can be seen that with the selected column, as well as with the Agilent 1120 Compact LC, characterization and monitoring of products from foods and drugs is possible. The following examples demonstrate the performance of the system:

- The chromatogram of Figure 2 illustrates that the chewing gum is free of all of the preservatives tested.

Compound	Retention time (min)	RSD RT n = 10	RSD Area n = 10	Asymmetry n = 10
Benzoic acid	5.06	0.408	0.188	0.64
Ascorbic acid	5.96	0.153	0.235	1.00
Methyl-PHB	6.69	0.277	0.260	1.09
Ethyl-PHB	9.39	0.039	0.112	1.09
Propyl-PHB	11.85	0.009	0.074	1.10

Table 3
Suitability sample: Precision of retention times and areas for the Agilent 1120 Compact LC.

Compound	m	b	r
Benzoic acid	2151314.8	25187.3	0.999
Ascorbic acid	10188005.0	119381.1	1.0
Methyl-PHB	4459268.1	83135.1	1.0
Ethyl-PHB	3097910.6	57372.8	1.0
Propyl-PHB	3024691.2	24203.6	1.0

Table 4
Calibration data of the Agilent 1120 Comapct LC (Setting "Ignore Origin", Y = mx + b, 1.0 µg/mL–20.0 µg/mL.)

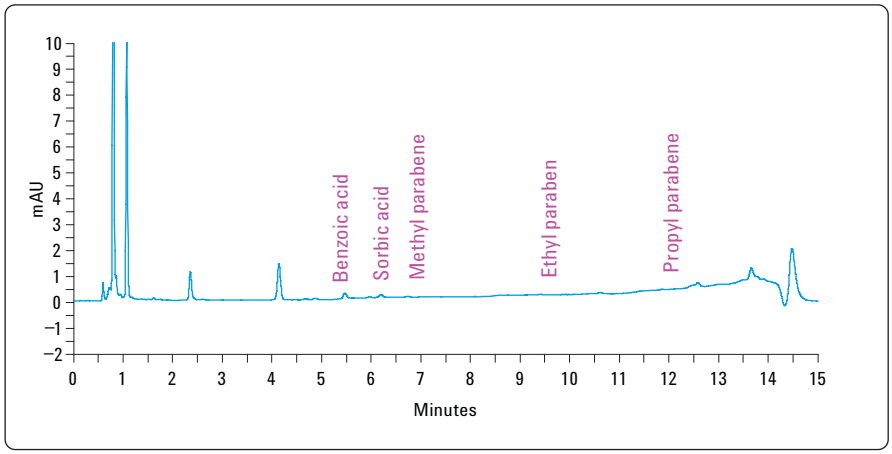

Figure 2
Analysis of preservative "free" chewing gum.

- The chromatogram of Figure 3 shows that the mouthwash tested is free of preservatives tested, but it is possible that different preservatives were used to inhibit microbial growth.
- The chromatograms in Figures 4 and 5 show the analysis of a beauty cream and a toothpaste. The declared preservative (Methyl-PHB) is part of the product. This chromatogram verifies that the method can be used for quality control as well as for monitoring.

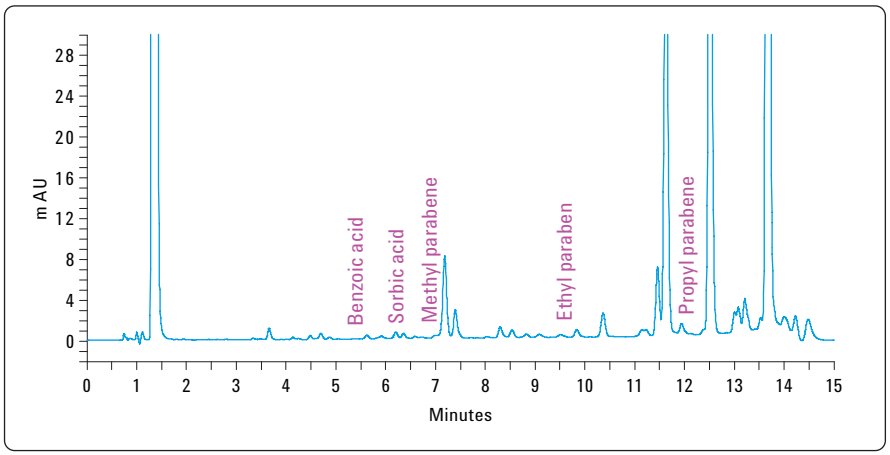

Figure 3
Analysis of mouthwash.

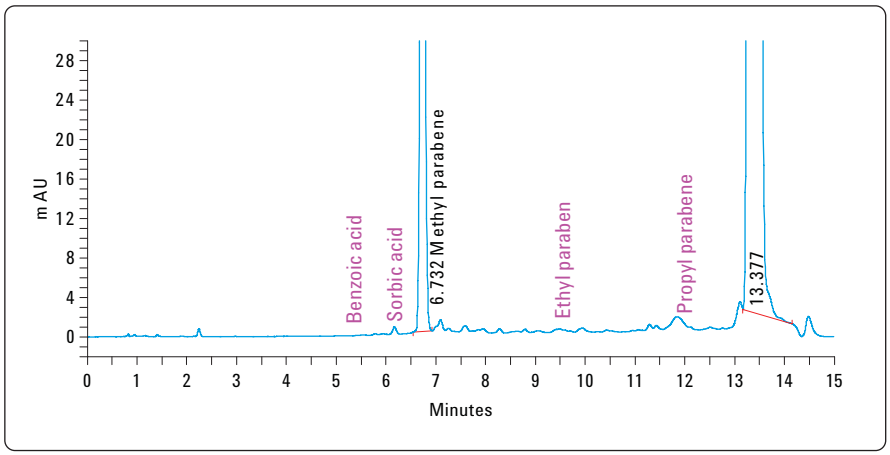

Figure 4
Chromatogram of a beauty cream.

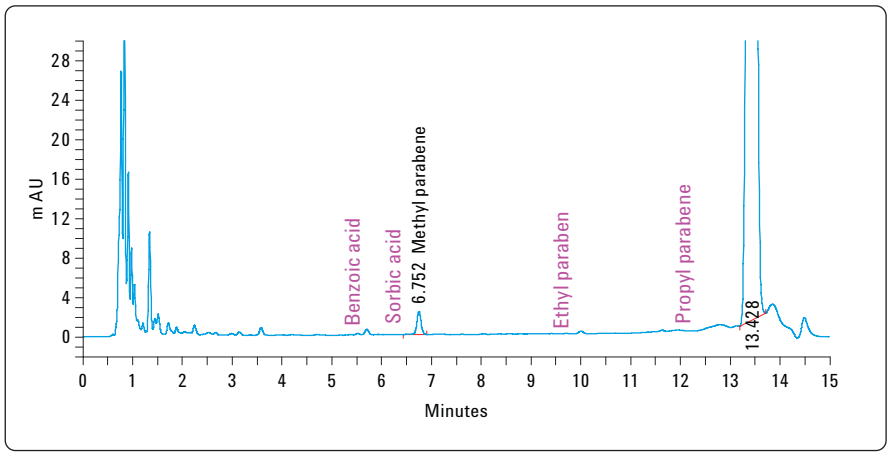

Figure 5
Chromatogram of a toothpaste.

- The chromatograms in Figures 6 and 7 show the method can monitor supermarket products. The preservative-free brand of ketchup is proven to be free of the tested preservatives. The preservative-free barbecue sauce contains some considerable amounts of benzoic acid and methyl-PHB.

All examples show minimal influence of the matrix on the separation. It can be seen that it is easy to detect any of the tested preservatives.

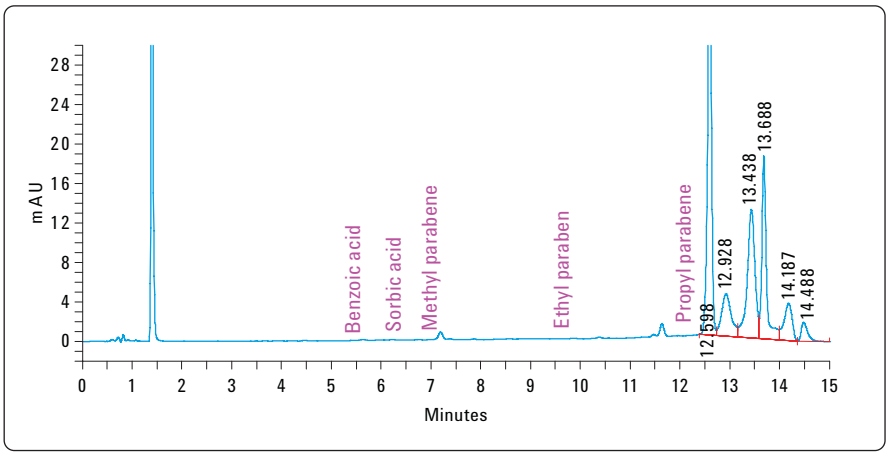

Figure 6
Analysis of a "preservative free" ketchup brand.

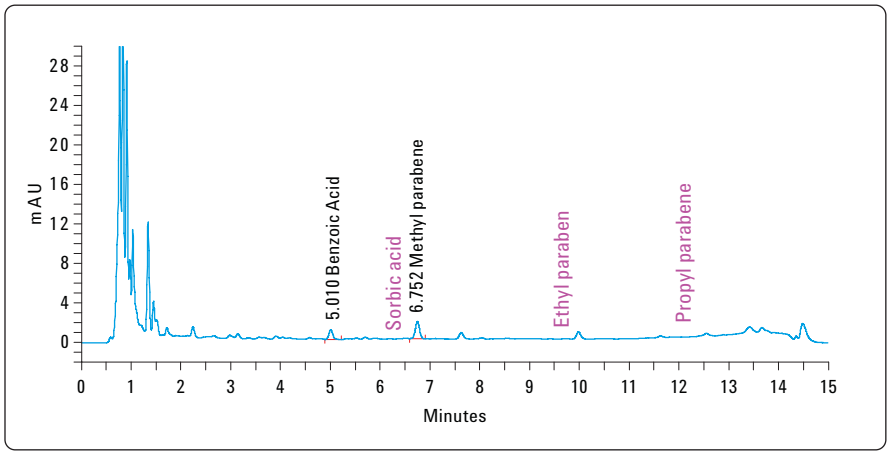

Figure 7
Analysis of a "preservative free" barbecue sauce.

Conclusion

The Agilent 1120 Compact LC is designed for users in independent labs who require LC methodology with reliability, ease-of-use, and low ownership cost, to characterize or monitor products.

This Application Note shows a reliable approach for the determination of preservatives in food or drugstore products. The data prove high precision of retention time, and provide clear chromatographic parameters such as resolution and asymmetry.

As shown in Table 2, the resolution of all main peaks was found to be greater than 3.0. The calibration data of each compound shows that the instrument can be operated as required in a quality control environment. All criteria for precision, such as areas and retention times are fulfilled (see Table 3). The results indicate that the use of the Agilent 1120 Compact LC in QA/QC laboratories to characterize products containing preservatives is appropriate. In addition, it is possible to identify any of the tested preservatives independent of the matrix.

All results explicitly show the applicability of the Agilent 1120 Compact LC for quality control testing with reduced costs per system and improved simplicity of use. In addition to the instrument capabilities, the new version of the EZChrom Elite compact software allows full control of the Agilent 1120 Compact LC with a wide range of features for data analysis and results reporting.

The results for resolution and asymmetry show good selectivity and performance with the ZORBAX Eclipse XDB.

The Agilent 1120 Compact LC is qualified and optimized for everyday productivity and routine analysis.

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www.agilent.com/chem/1120

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Agilent Technologies

Isocratic Stevia Sweetener Analysis using Selective ZORBAX Columns

Application Note

Food

Authors

John W Henderson Jr. and Judy Berry
Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE 19809
USA

Abstract

Two isocratic reversed-phase methods that have distinct selectivity, and use popular UV/MS or UV detection, are presented for analyzing Rebaudioside A and other steviol glycosides. A Rapid Resolution High Throughput (RRHT) Eclipse Plus Phenyl-Hexyl method using UV and MS detection resolved many compounds from a *Stevia rebaudiana Bertoni* plant extract instead of the other approach employing an amino column with a refractive index (RI) or other special detector. The RRHT Eclipse Plus Phenyl-Hexyl method's advantages include high peak capacity, isocratic mobile phase, low acetonitrile consumption, and unique selectivity compared to a ZORBAX Carbohydrate Analysis column method. Three commercially available Stevia sweeteners were analyzed for Rebaudioside A and Stevioside, the two major diterpenoid glycosides present in stevia leaves.

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Introduction

The use of stevia leaf derived sweeteners in food and beverages in the USA will likely increase, because the FDA has no objection to Rebaudioside A having GRAS (generally recognized as safe) status as a general purpose sweetener for food and drink. Stevia sweeteners may contain other steviol glycosides as well, mainly Stevioside, Rebaudioside C and Dulcoside A. The ratio of these stevia components influences the quality, identity and purity of the stevia extracts. Because the FDA GRAS confirmation is only for the use of Rebaudioside A at 95% purity or above in food and beverage products, stevia extracts must be highly purified and characterized prior to use [1-4].

Due to their lack of chromophores (Figure 1) many methods for sugars and similar compounds like steviol glycosides use refractive index, evaporative light scattering, or electrochemically pulsed amperometric detectors. Carbohydrate separation methods often use an amino bonded-silica based column too.

The method presented here takes advantage of the more prevalent UV diode array detector (DAD), and is coupled with an Agilent ZORBAX Eclipse Plus Phenyl-Hexyl Column instead of the more usual amino or carbohydrate specific column. The Eclipse Plus Phenyl-Hexyl method has a high aqueous, low acetonitrile, low UV, MS friendly mobile phase, so hydrolytic deterioration of the amino-silica bonded columns in a high aqueous environment is not a concern [5]. The end result is an uncomplicated, rugged, isocratic LC-UV-MS method for simple and complex stevia extract matrices.

An alternative method using an Agilent ZORBAX Carbohydrate Analysis column was also developed. It features significantly different selectivity compared to the ZORBAX Eclipse Plus Phenyl-Hexyl. This method is recommended as a secondary or confirmatory method for the ZORBAX Eclipse Plus Phenyl-Hexyl column, due to eventual hydrolytic deterioration of the amino silica bond, and also higher acetonitrile consumption. Nonetheless, it is still very useful for its different selectivity.

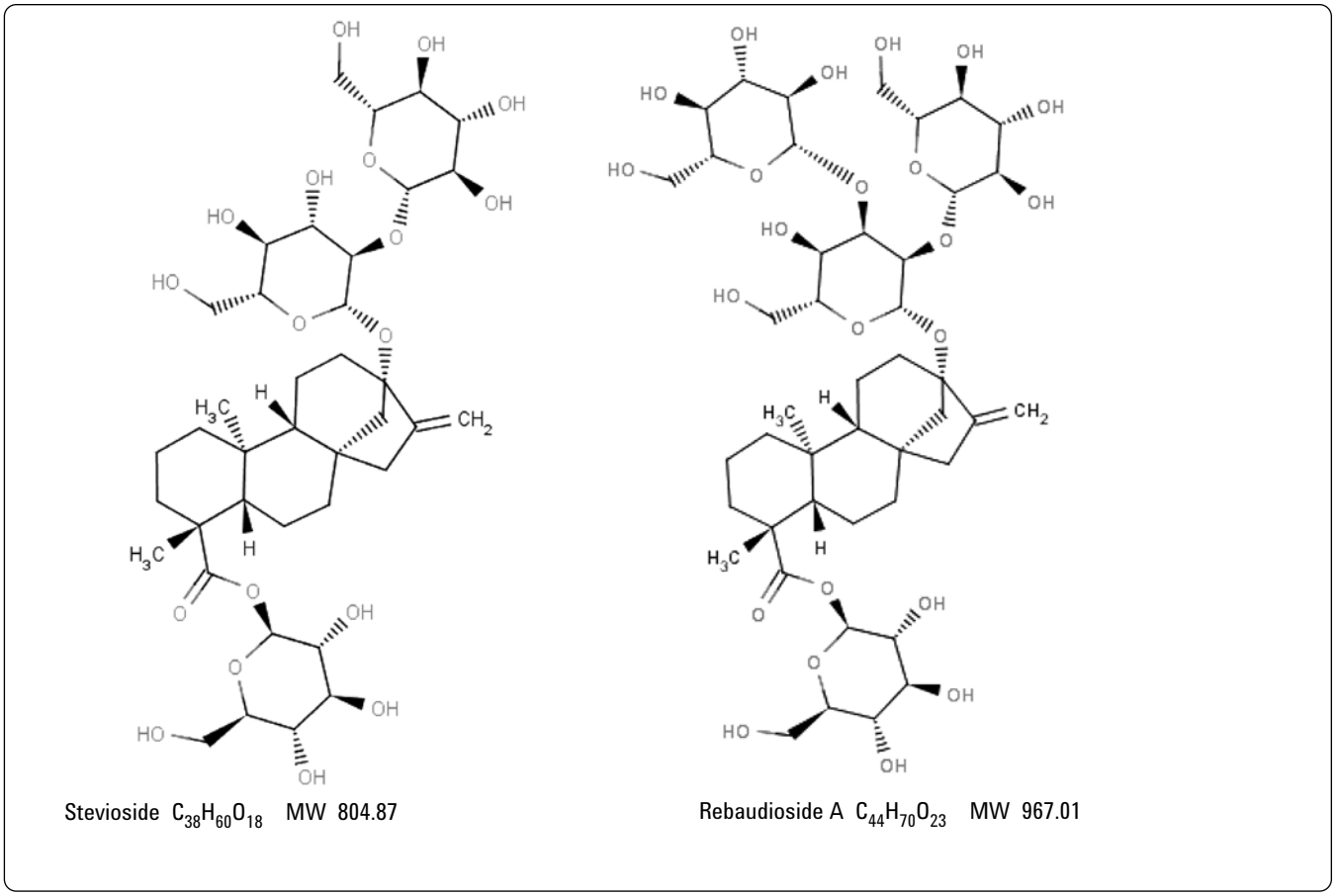

Figure 1. The two major diterpenoid glycosides present in Stevia rebaudiana Bertoni leaves.

Samples: Three retail sweeteners, all labeled as containing "stevia extract" were obtained from a local supermarket and diluted as follows:

1. A single-use powder packet (0.1 g/mL in H_2O)
2. A liquid concentrate (1:10 dilution in H_2O)
3. A stevia leaf powder extract (0.1g/mL in H_2O)

Standards: Stock solutions of Stevioside and Rebaudioside A reagent-grade standards from ChromaDex (Irving, CA) were made in water at concentrations of 2704 µg/mL (Stevioside) and 2867 µg/mL (Rebaudioside A). Further dilutions of the standard stock solutions were made to demonstrate linearity of the method. All samples were syringe filtered (0.2 µm) into autosampler vials for analysis.

ZORBAX Eclipse Plus Phenyl-Hexyl method

LCMS:	Agilent 1200SL with G1312B binary pump, 1316C DAD (diode array detector) and G1956B single quad MSD with electrospray ionization
Column:	ZORBAX Eclipse Plus Phenyl-Hexyl 2.1 mm x 100 mm, 1.8 µm p/n 959764-912
Spray chamber:	Drying Gas: 12 L/min, nebulizer pressure: 1811 torr, drying gas temp: 350 °C, capillary voltage: 3000V
MS parameters:	TIC scan: 300–3000 *m/z*, fragmentor: 70V, peak width 0.1, threshold 150, step size 0.1, gain 1.0
Mobile phase:	[A: B] Water (0.1% formic acid): ACN (0.1% formic acid) (82:18)
Flow rate:	0.42 mL/min
Detection:	204 nm and ESI-MSD
Temperature:	35 °C

ZORBAX Carbohydrate Analysis column method

LC:	Agilent 1200SL with G1312B binary pump and 1316C DAD (diode array detector)
Column:	ZORBAX Carbohydrate Analysis, 4.6 mm × 150 mm, 5 µm p/n 843300-908
Mobile phase:	[A: B] Water: ACN (22:78)
Flow rate:	1.5 mL/min.
Detection:	204 nm
Temperature:	30 °C

Results and Discussion

Two retail food sweeteners, labeled as dietary supplements, were analyzed on an Eclipse Plus Phenyl-Hexyl column. One was a powder packet, similar to sugar packets used for tea and coffee. The other was a stevia extract concentrated in ethanolic water. The ratio of the two major diterpenoid glycosides in the sweetener sources were quite different, with Rebaudioside A being more prevalent in the powdered sweetener, and Stevioside being dominant in the liquid (Figure 2). Rebaudioside A is considered the sweeter and better tasting of the two, although other minor glycosides may play a role in the overall taste quality. Rebaudioside A and Stevioside were identified with commercial standards and calibration curves were constructed, demonstrating linearity from about 100 to 3000 ppm (Figure 3).

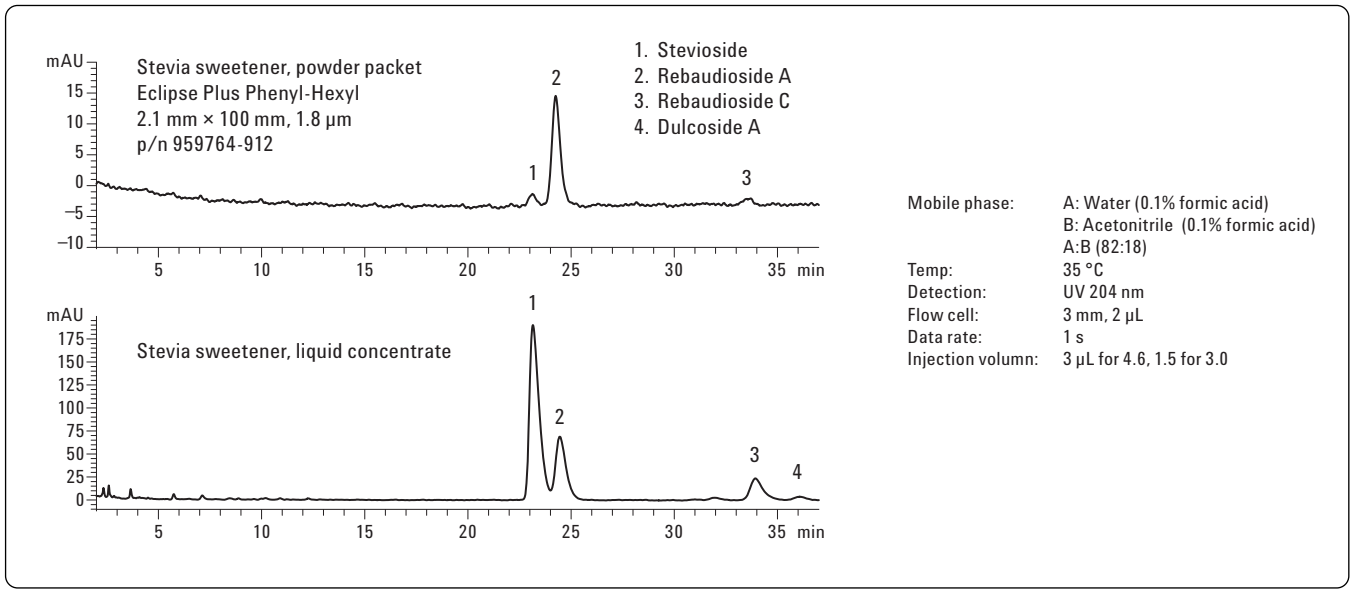

Figure 2. Steviol glycoside ratios differ between stevia sweeteners.

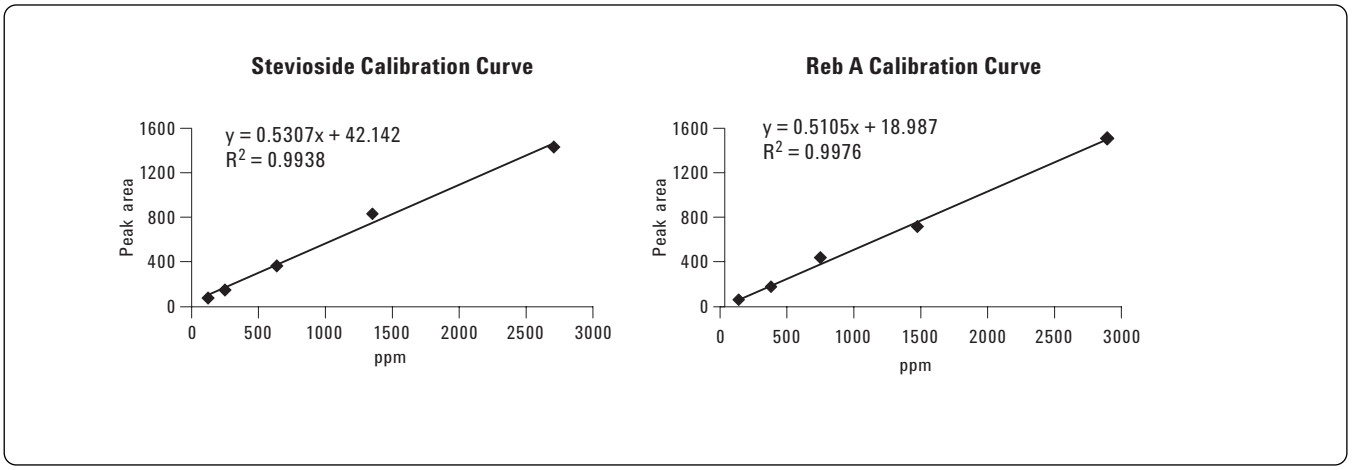

Figure 3. Linearity of Eclipse Plus Phenyl-Hexyl method for Stevia diterpenoid glycosides.

A third stevia extract, in powdered form, was analyzed and found to have many more components compared to the previous two samples. An Agilent 1200 Series LC/MSD SL G1956B Single Quad MSD was connected to the Agilent 1200 Thermostatted Column Compartment SL Plus G1316C DAD detector outlet to identify Dulcoside A and Rebaudioside C in addition to Rebaudioside A and Stevioside (Figure 4). In positive ionization mode, a pseudomolecular ion $[M+23]^+$ (sodium ion adduct) was used to identify peaks, in negative mode, the $[M-1]^-$ ion was used for identification. The negative mode resulted in higher ion abundance than the positive mode. The narrow bore column dimensions and formic acid (0.1% v/v) enhance electrospray ionization, and the isocratic mobile phase was well suited for ESI-MS, eliminating re-equilibration time and baseline drift common with gradients. It is also easy to transfer from one LC to another because delay volume associated with gradients is not a factor. The highly efficient RRHT Eclipse Plus Phenyl-Hexyl column contains 1.8-µm particles, and operated at a pressure of 400 bar under these conditions. This pressure was well within the operating limits of the RRHT column (600 bar) and the 1200SL LC (600 bar).

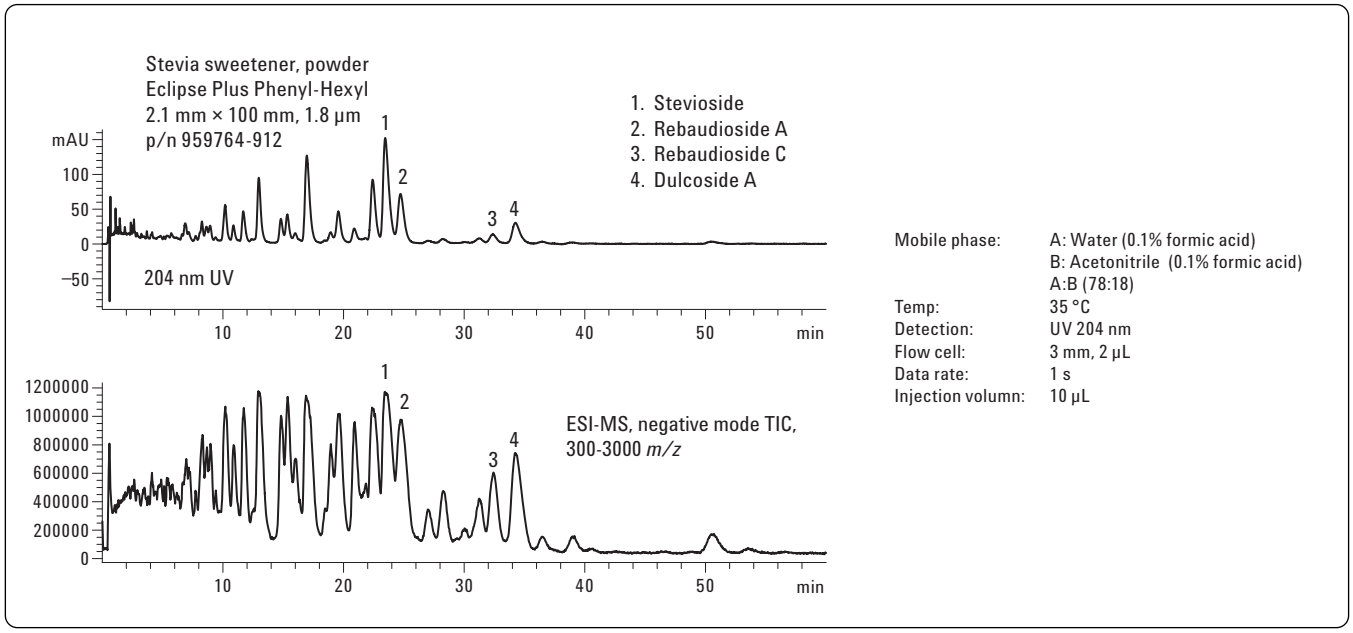

Figure 4. Isocratic analysis of complex Stevia leaf extract.

A supplementary steviol glycoside method was also developed using a ZORBAX Carbohydrate Analysis column with UV detection. The selectivity with this method was notably different compared to the RRHT Eclipse Plus Phenyl-Hexyl column method (Figure 5). The elution order was completely different. Other components of the stevia shown in Figure 3 however, were not as resolved on the Carbohydrate Analysis column, and some appeared to elute in the void volume, indicated by the large peak at t_0. This is likely due to differences in selectivity as well as efficiency between the two columns. The Carbohydrate Analysis column method was linear for Rebaudioside A and Stevioside over a 70–700 ppm range (Figure 6).

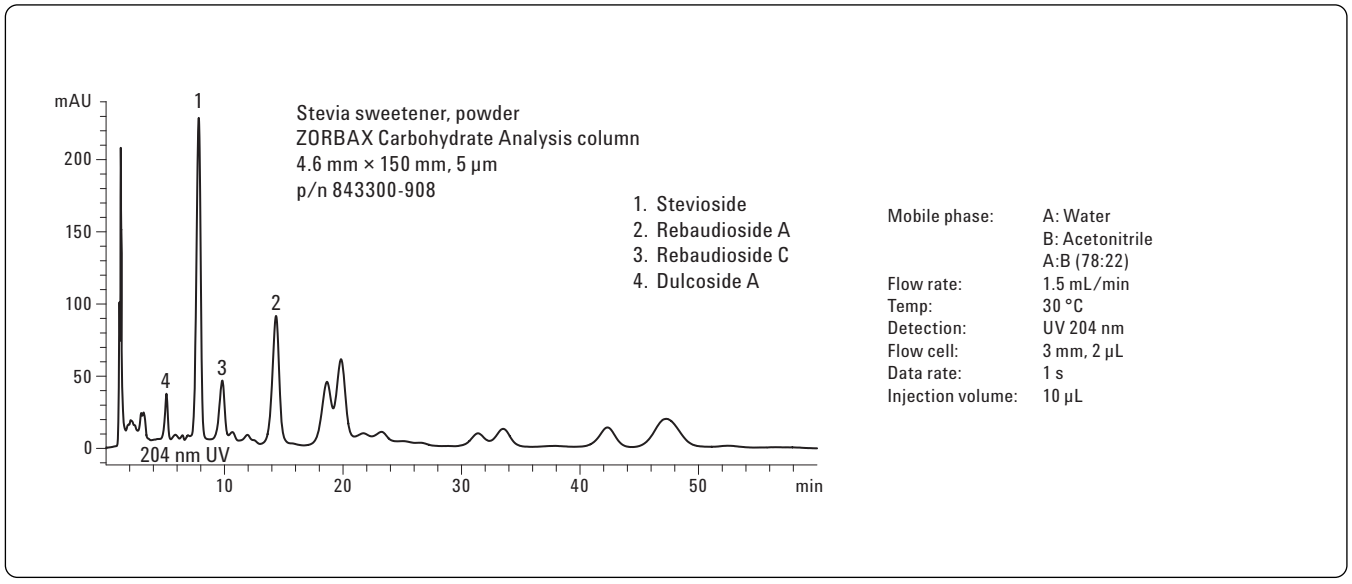

Figure 5. Elution order is completely different using the ZORBAX Carbohydrate Analysis column method.

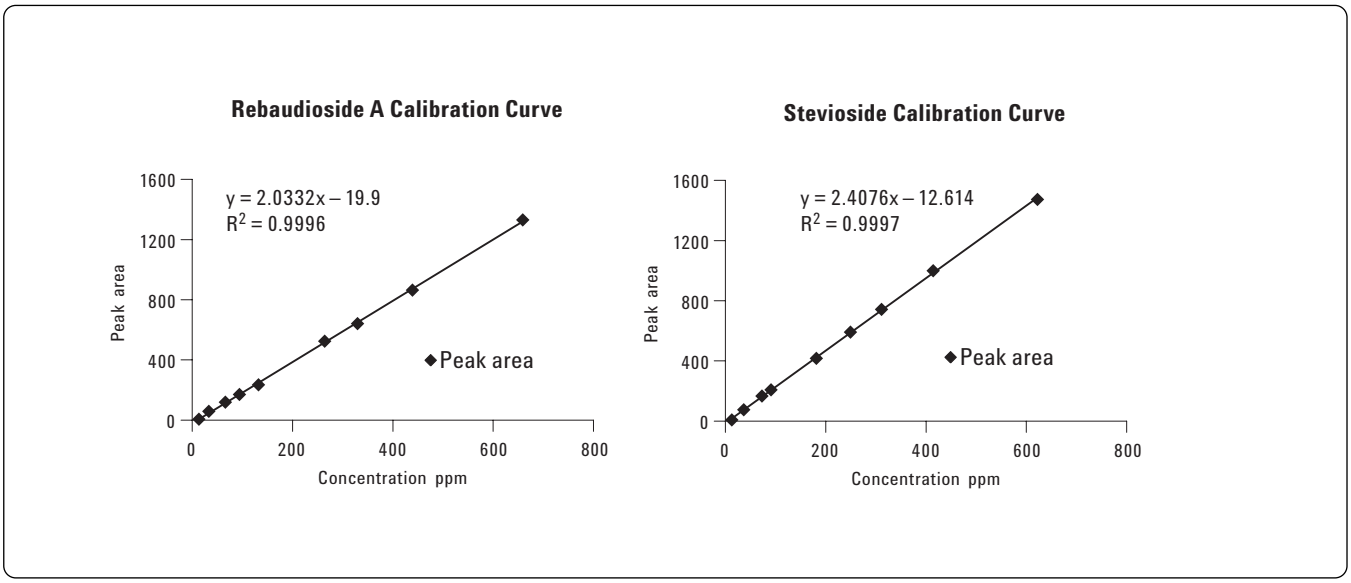

Figure 6. Linearity of ZORBAX Carbohydrate Analysis column method for Stevia diterpenoid glycosides.

Conclusion

The high efficiency and peak capacity of the RRHT Eclipse Plus Phenyl-Hexyl column combined with UV-MS detection suggests this method is useful for simple and complex stevia extracts. Benefits include using a very popular DAD and/or ESI-MS detector, compared to RI and other special detectors. Isocratic mobile phase advantages include no reequilibration time, and there is an ease of method transfer from LC system to LC system. The Carbohydrate Analysis column with UV detection method is a second possibility. The Carbohydrate Analysis column could be useful to confirm peak identity because the two columns have markedly different selectivity. The RRHT Eclipse Plus Phenyl-Hexyl method however, offers higher efficiency and more resolution.

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Agilent Technologies

The Separation of Seven Synthetic/Artificial Food Colors on Agilent HC(2)/TC(2) Reversed Phase Columns

Application

Food and Flavors

Author

Rongjie Fu
Agilent Technologies, Inc.
412 Ying Lun Road
Pu Dong, Shanghai 200131
China

Abstract

Many synthetic or artificial colorants (Red #33, Sunset yellow, etc.) are used in food and beverages to improve product appearance. These compounds can be easily separated by reversed-phase liquid chromatography. [1] A new C18 column was used to separate seven food colorants, using a gradient method with a phosphate buffer-acetonitrile mobile phase. This method is suitable for many samples and is applied here to the analysis of these colorants in beverages.

Introduction

Synthetic or artificial food colorings are almost all water soluble, making them ideal for analysis by HPLC with reversed phase columns. These compounds are generally safe, but there are some possible harmful effects of these compounds, including allergic reactions and hyperactivity in children. Artificial food colorings are restricted in some countries because of these possible effects. The quantity of these compounds in food quality control is becoming more important, as is the need to prove that they meet international food quality control standards. The structures of seven colorants, mostly azo dyes, are shown in Figure 1. These compounds are used in beverages to give them a colorful, attractive appearance.

In this application, we focused on developing a method for separating seven colorants on the Agilent TC-C18(2) column and then applying this method to the analysis of these food colors in beverage samples. This new column is packed with 5-µm high-purity (B type) silica with a surface area of 290 m^2/g and bonded with C18, achieving a carbon load of 12%. This is a moderate carbon load for a column with this surface area and is therefore more hydrophilic than columns with a higher carbon load. The special treatment of the surface of the silica and improved end-capping give this column excellent performance, especially for the separation of polar and basic compounds.

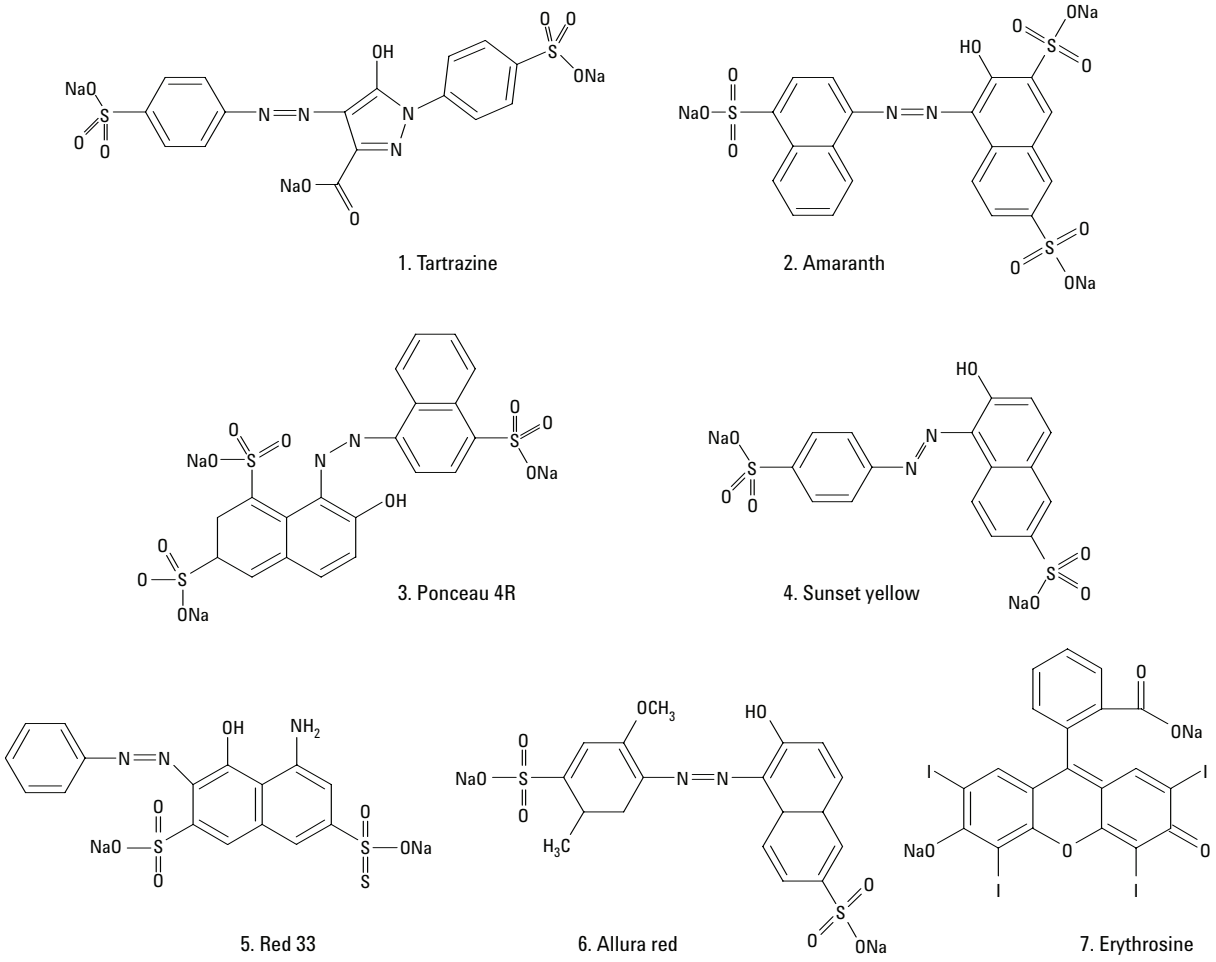

Figure 1. Structures of colorants.

HPLC conditions

Instrument	Agilent 1200SL with DAD
Column	Agilent TC-C18(2), 4.6 mm × 150 mm, 5 µm
Mobile phase	A: 20 mM phosphate buffer, pH 7.0 B: Methanol
Gradient	0–15 min, 10% B – 90% B
Flow rate	1 mL/min
Detector wavelength	254 nm
Temperature	30 °C
Injection volume	5 µL

Seven Food Color Standards Separated on Agilent TC-C18(2)

The compounds shown in Figure 1 are polar, water-soluble compounds with sulfonic acid groups on them, increasing their solubility in water. In fact, many of these compounds are used in the salt form in food. A C18 column was selected to separate these compounds. Two new C18 columns are available from Agilent for routine or QC/QA-type applications. These columns are the Agilent HC-C18(2) column with a carbon load of 17% and the TC-C18(2) column with a carbon load of 12%. The difference in carbon load was designed to provide column choices optimized for the analyses of very polar and very nonpolar analytes. Polar analytes typically require high aqueous mobile phases to be effectively retained. For the artificial food colors, a gradient method starting at low organic in the mobile phase was developed. Starting at very high aqueous mobile phases, the TC-C18(2) column with a carbon load of 12% was the better column for this method development, and can be used for mixtures of polar compounds or for samples of polar and nonpolar compounds.

Figure 2 shows all seven colorants separated on the 4.6 mm × 150 mm, 5 µm Agilent TC-C18(2) column. Using pH 7.0 phosphate buffer-methanol mobile phase, all the compounds are symmetrical with a U.S. Pharmacopeia (USP) tailing factor close to 1.0 and baseline resolution with good reproducibility. Because of their strong acidity, the peak shape and resolution of the compounds are dramatically influenced by the pH selected for the mobile phase. The separation was repeated with mobile phases at three different pH values. The chromatograms for this are shown in Figure 3. The chromatograms clearly show that the peak shape and resolution are influenced by pH; especially the first three components, which are the most acidic and polar. At low pH, pH 2.2, poor resolution was seen between peaks 1 and 2 and peaks 4 and 5; however, pH had less influence on resolution for the later eluting compounds, such as erythrosine (peak 7). Erythrosine is the most hydrophobic of these compounds. The overall elution order of these compounds was tied to their hydrophobic properties, as expected in reversed phase LC.

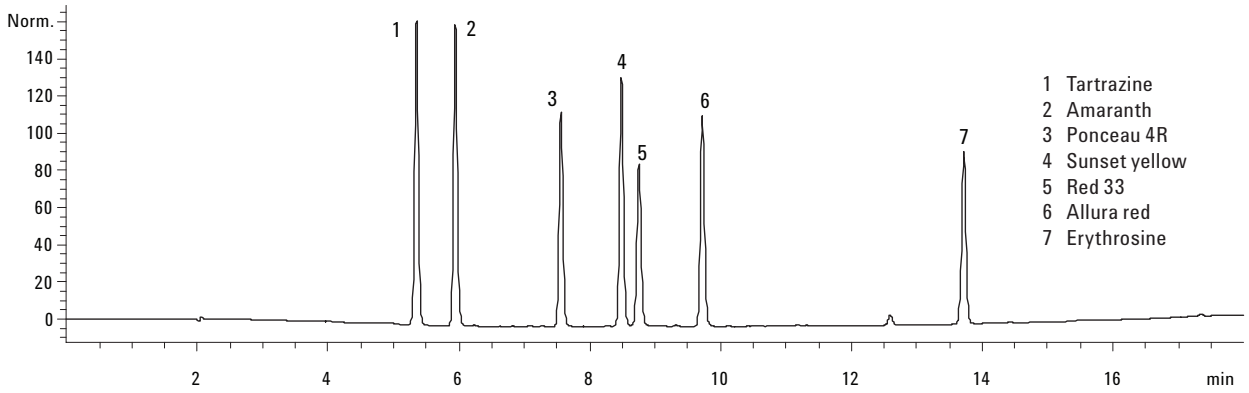

Figure 2. Chromatogram of colorants standards on the Agilent TC-C18(2) column.

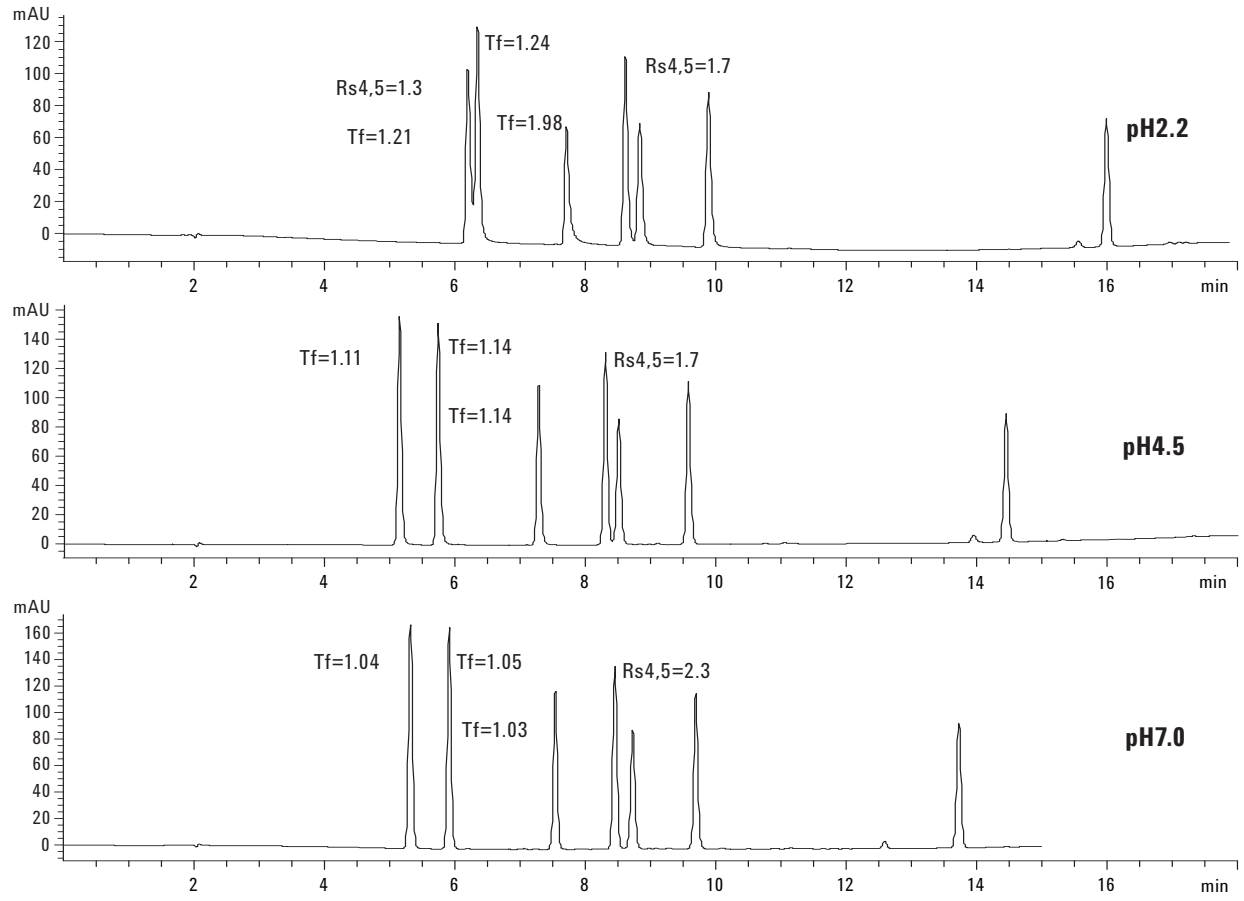

Figure 3. Chromatogram of colorants standards in different pH mobile phase on the Agilent TC-C18(2) column.

Lot-to-Lot Reproducibility

Lot-to-lot reproducibility of columns is important for rugged HPLC methods and routine monitoring. Figure 4 shows the reproducibility test used to evaluate the TC-C18(2) column in this method. Three different batches of columns were used in the test under the same HPLC conditions. The chromatograms show almost no retention time drift and no peak shape variations occurring from column lot-to-lot, which demonstrates the high-quality manufacturing and packing techniques used for these columns.

Analysis of Artificial Food Colors in Beverages

Synthetic or artificial food colorants exist in many common beverages and food, such as fruit-flavored drinks and sodas, cakes, and candy. Some of the colorants in two different beverages were separated in this application. Two popular beverages were selected, an orange flavored soda and a drink powder. For the sample preparation, orange flavored soda was degassed, filtered through a 0.45-µm filter, and then analyzed. The drink powder was dissolved in water, filtered through a 0.45-µm filter, and injected for analysis. The chromatograms from these samples are shown in Figure 5. Three colorants were found and completely separated in the orange flavored soda sample. Based on the standard, the first known peak could be resolved from the unknown peak before it. Two target colorants were found and adequately resolved in the beverage powder sample for effective quantitative analysis.

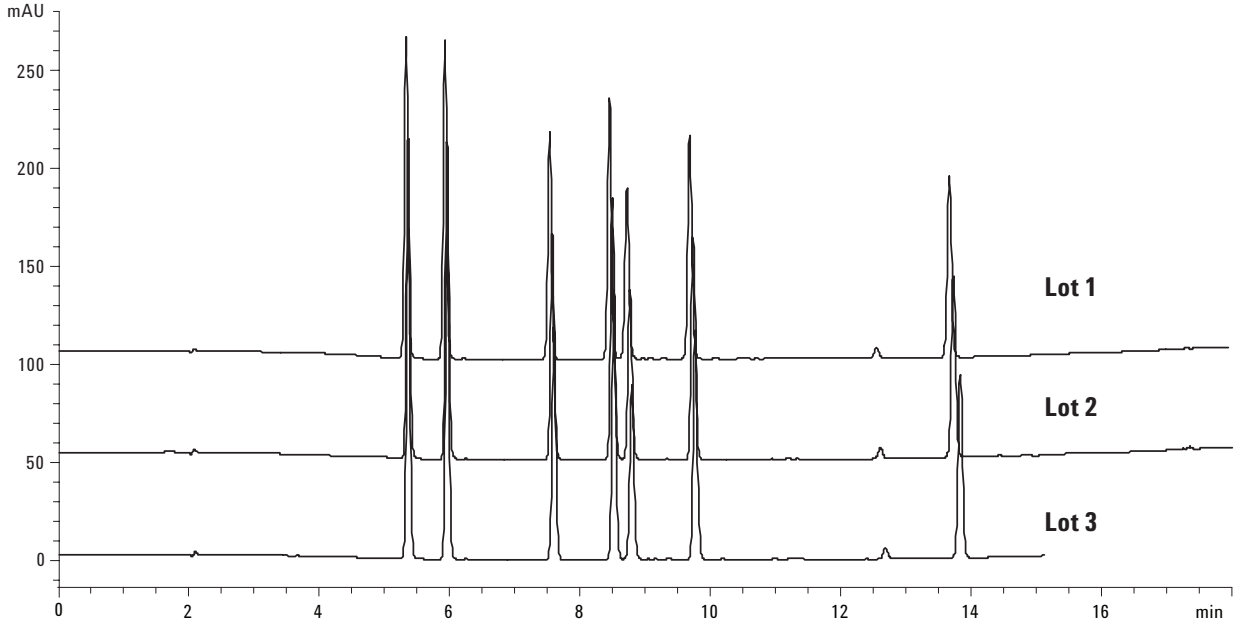

Figure 4. Lot-to-lot reproducibility(TC-C18[2], 4.6 mm × 150 mm, 5 µm).

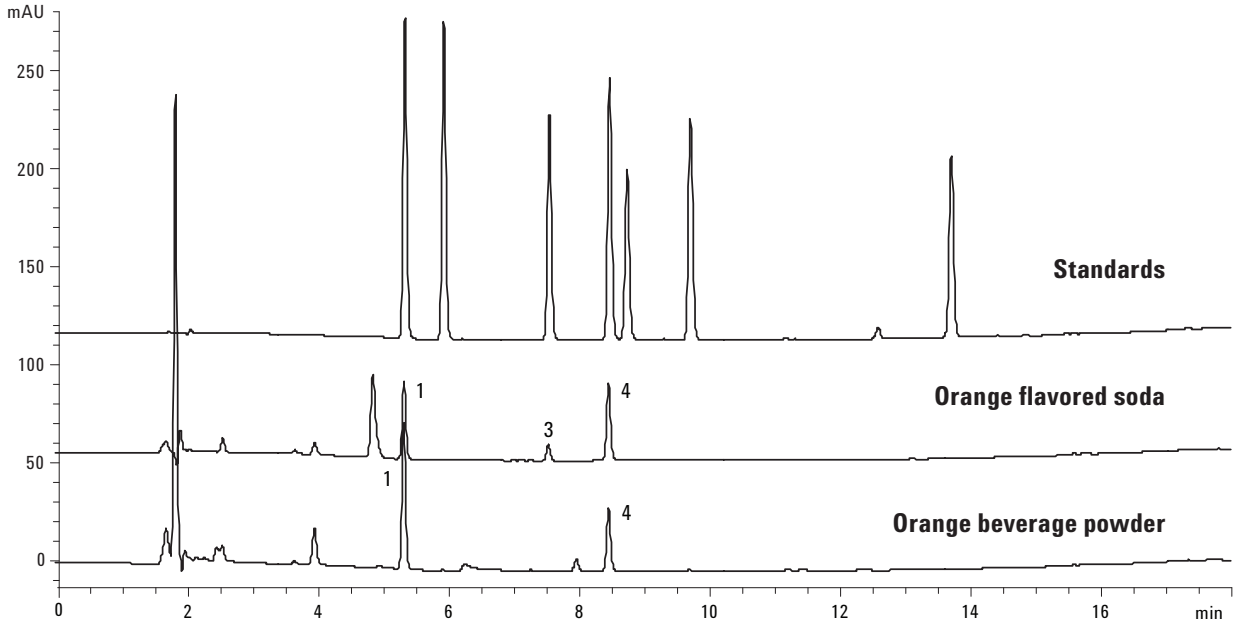

Figure 5. Chromatogram of two samples.

In the beverage powder, there were lower levels of these food colors added. For the low-concentration samples, a large-volume injection is needed to meet the detection and quantitation requirements. Two chromatograms of large-volume 50-µL injections are shown in Figure 6. The separation was still quite good, with symmetrical peaks and little band broadening. For the sample orange flavored soda, peak 1 was completely separated from the unknown compound. All these demonstrate the high capacity of the high surface area TC-C18(2) column.

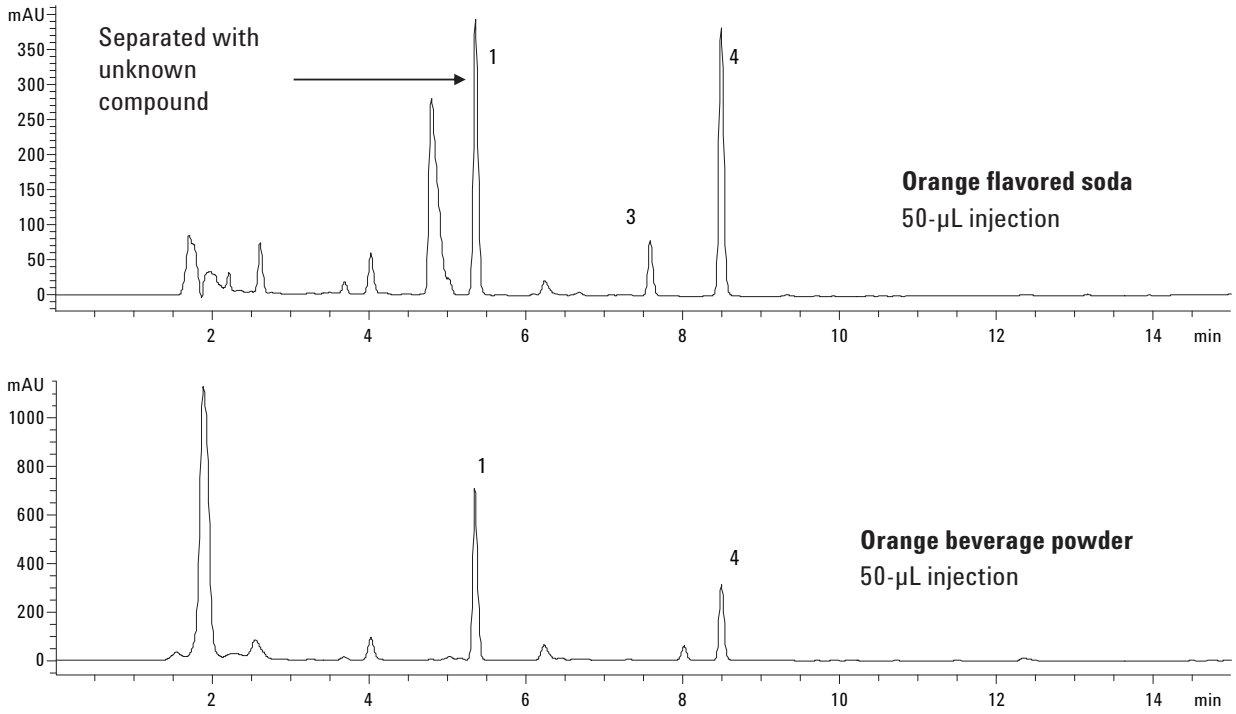

Figure 6. Chromatogram of 50-µL injection.

Selectivity Comparison with HC-C18(2)

As mentioned above, the carbon load of HC-C18(2) is higher than TC-C18(2), which leads to strong retention of nonpolar compounds but not quite so much retention of polar compounds. Figure 7 shows that the HC-C18(2) column retains the more polar food colors slightly less than the TC-C18(2) column does. The change in carbon load also causes a change in selectivity of peaks 4 and 5 between the two columns, which is also shown in the chromatograms. Figure 8 shows chromatograms of orange flavored soda both on TC-C18(2) and HC-C18(2) columns. Peak 1 and the potential interfering compound on the TC-C18(2) column reverse order on the HC-C18(2) column. The possible interfering compound elutes before the target compound 1 on the TC-C18(2) column and has excellent resolution. When the large-volume injection was made, baseline separation could still be achieved; when the elution order was reversed eluted, the two compounds were not baseline separated until the mobile phase composition was adjusted. Because the mobile phase is a low organic (only 10%) starting gradient for this method, we chose the TC-C18(2) column with a lower carbon load for this application. It's important to note that the different selectivity of these two columns provides more column selection opportunities for method development.

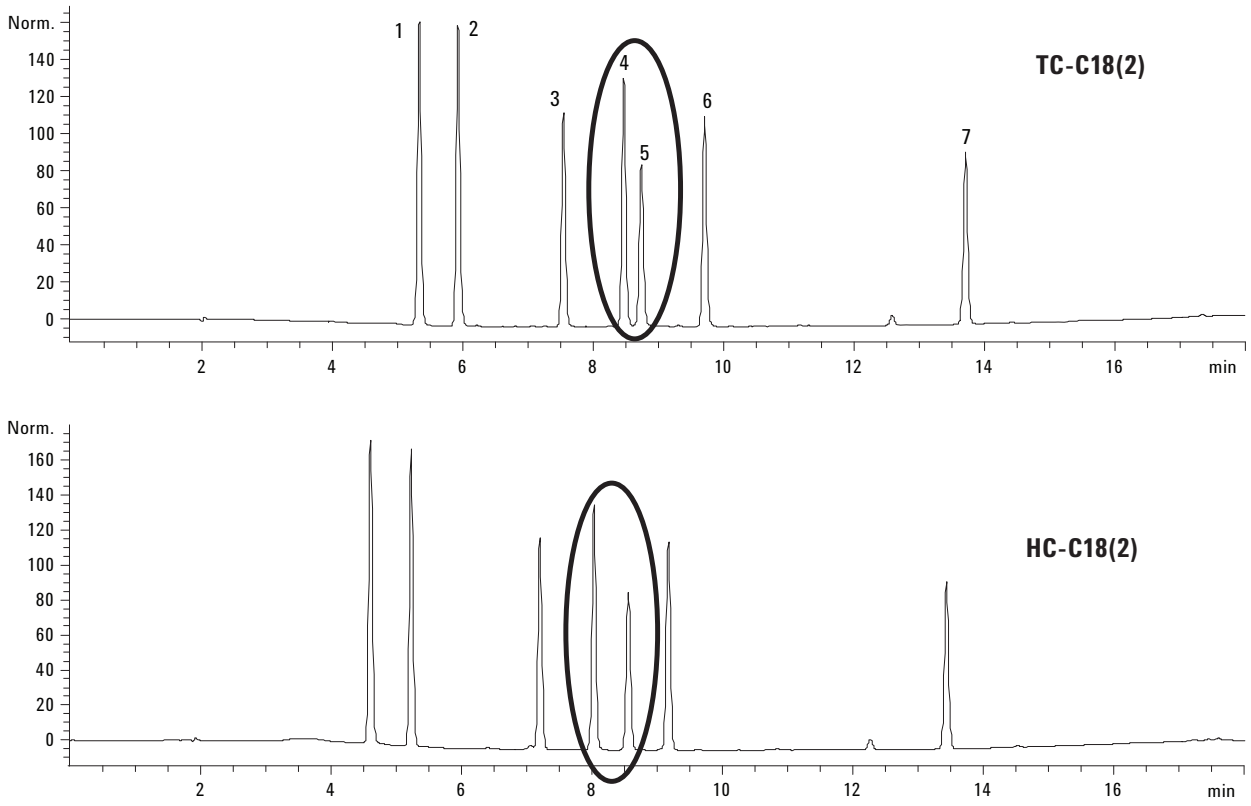

Figure 7. Chromatogram of colorants standards on TC-C18(2) and HC-C18(2).

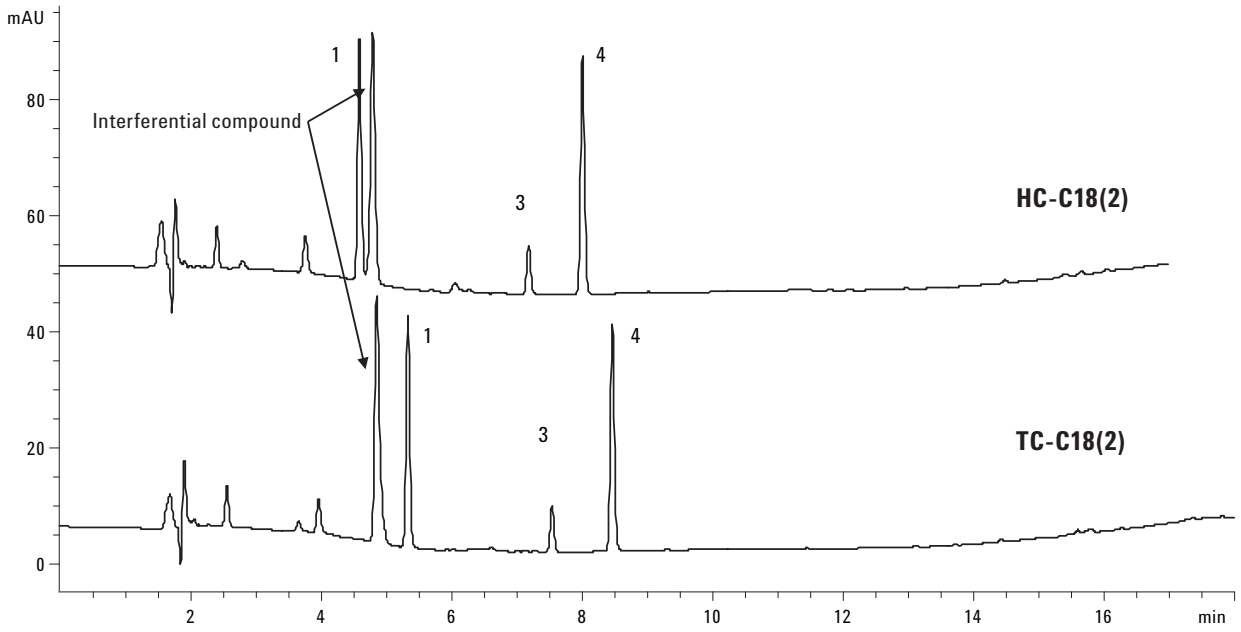

Figure 8. Different selectivity of TC-C18(2) and HC-C18(2).

Conclusions

Using a simple gradient method, many common synthetic colorants can be separated on the Agilent TC-C18(2) column. This method allows baseline separation for many artificial food colors and provides symmetrical peaks and good reproducibility. The Agilent TC-C18(2) column can be used for daily QC analysis of synthetic colorants in foods and beverages with reliable results.

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Agilent Technologies

A Rapid and Sensitive Analysis Method for Sudan Reds in Curry and Chili Powder Using LC/MS/MS

Application

Food Safety

Authors

Yanyan Fang
Agilent Technologies, Inc.
412 Ying Lun Road
Pu Dong, Shanghai 200131
China

Jeffry Keever
Agilent Technologies, Inc.
200 Regency Forest Drive, Suite 330
Cary, NC 27518-8695
USA

Dustin Yaworsky and Jerry Zweigenbaum
Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE 19808-1610
USA

Abstract

A method was developed using the Agilent G6410 Triple Quadrupole LC/MS for Sudan Reds in different matrices, including curry powder, red pepper powder, and chili sources. The analytical performance of the method was evaluated for four different matrices and the results show little or no matrix effects. Linearity of response over three orders of magnitude was demonstrated (r > 0.99). In addition, good reproducibility of the two required product ion ratios was obtained to meet the EU identification points needed for confirmation.

Introduction

The group of color additives known as Sudan dyes consists of a number of red colorants, for example, Sudan I through IV. This group, together with other dyes, such as Para Red, are synthetically produced azo dyes. Because their degradation products are considered to be carcinogens and teratogens, the EU and the U.S. do not permit the use of these colorants as food additives. However, in some countries, these dyes are still occasionally used to intensify the color of bell pepper and chili powders.

The red dyes Sudan I, II, III, and IV are oil-soluble azo dyes used legally in the leather and fabric industries. The International Agency for Research on Cancer (IARC), a part of the World Health Organization, has assessed the Sudan dyes as Group 3 genotoxic carcinogens. [1] The industrial dye Para Red is chemically similar to Sudan I and is also a dye not permitted for use in food.

The EU issued Decision 2003/460/EC requiring as a condition of import that all hot chili and hot chili products be tested for Sudan I. [2] The Decision was amended in January of 2004 (2004/92/EC) to include Sudan II, III, and IV. [3] This requirement remains in effect. The highly selective and sensitive triple quadrupole (QQQ) is used to meet the testing requirement.

Experimental

Reagents and Materials

Ethyl acetate from Burdick and Jackson (Morristown, NJ)

Methanol HPLC-grade from Burdick and Jackson

Water at 18 MΩ from a Milli-Q Synthesis System by Millipore (Billerica, MA)

Syringe filter (0.2 µm, PTFE) from Agilent, p/n 5185-5843

Samples

1. Curry powder (House of Spices [India] Inc.)
2. Red pepper powder (Korean Farm, Inc.)
3. Red pepper powder (Oriental Mascot, Summit Import Corp.)
4. Worcestershire sauce (Lea & Perrins)

Overview of Method

Standard Preparation

1. Standard solution preparation: 10 mg Para Red, Sudan Red I, Sudan Red II, Sudan Red III, and Sudan Red IV were dissolved into 100.0 mL acetontrile/water (90:10) to a final concentration of 100 ppm.
2. Diluents solution preparation: using acetontrile to obtain concentrations at 1 pmm, 0.1 ppm, 10 ppb, 5 ppb, 2 ppb, and 100 ppt.

Sample Preparation

1. ***Curry powder*** was spiked with 10 µL of **standard solution** into 1 g of powder, diluted with 5 mL ethyl acetate, and allowed to ultrasonicate for 10 min. The solution was filtered with a 0.22-µm syringe filter and evaporated to dryness under a nitrogen stream at 40 °C (used below). The residue was reconstituted in 1 mL acetonitrile. This was filtered again before injection. No additional clean up of the sample solution was performed. This gave a spiked sample at a concentration at of 1 ppm. This spiking procedure was repeated to obtain spiked samples at levels of 100, 10, 1, and 0.1 ppb.
2. ***The two red pepper chili powders:*** The procedure used to prepare the curry powder was also used to prepare the spiked samples.
3. ***Worcestershire sauce*** was spiked with 10 µL of standard into 1 mL of sauce. The sauce, at about pH 3, was diluted with 5 mL ethyl acetate and allowed to ultrasonicate for 10 min. The solution was centrifuged for 2 min (8,000 rpm) and the top solvent layer (ethyl acetate) was transferred to a clean tube and evaporated under a nitrogen stream at 40 °C. The residue was redissolved in 1 mL acetonitrile and put in an ultrasonic bath for 1 min. This solution was filtered before injection.

LC/MS/MS Conditions

All analyses were performed with the Agilent 6410 Triple Quadrupole LC/MS equipped with an electrospray ionization source operated in positive mode. The HPLC was the Agilent 1200 LC system equipped with binary pump and well plate autosampler. See Table 1 for the conditions.

Table 1. LC/MS Conditions

HPLC	
Column	ZORBAX XDB C18 2.1 mm × 100 mm, 1.8 µm Agilent p/n: 928700-906
Flow rate	0.4 mL/min
Mobile phases	A: 0.1% formic acid in water B: 0.1% formic acid in acetonitrile
Gradient	0.1–4 min, 70~98% B 4–5 min 98% B 5–7 min 70%
Total run time	10 min including re-equilibration
Temperature	40 °C
Injection	5 µL
MS Source Settings	
Source	ESI
Ion polarity	Positive
Drying gas temp.	350 °C
Drying gas flow rate	10 L/min
Nebulizer	45 psi
V_{cap}	3500 V
MRM parameters	As shown in Table 2

The MS analysis was divided into five segments each containing one of the analytes. The appropriate fragmentor and collision energy for the analyte eluting in that segment was contained therein. Segment 1 started at 0 min, segment 2 at 2.0 min, segment 3 at 2.8 min, segment 4 at 3.6 min, and segment 5 at 4.2 min.

Results and Discussion

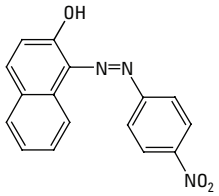
Each of the dyes was analyzed by LC/MS/MS in product ion scan and examined for spectral quality. Appropriate transition ions for quantitative analysis and confirmation were selected. The structure of each of the compounds is shown below. The azo bond cleaves under the collision-induced dissociation conditions and produces unique fragment ions.

Optimization of MS Condition

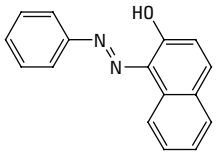
Optimization usually consists of simply finding the fragmentor voltage that maximizes the abundance of the precursor ion and the collision energy that maximizes the abundance of the transition ion. Optimization of the fragmentor was done by stepping through the voltages and recording intensity as shown for Sudan Red I in Figure 1. The other compounds were optimized by the same process. Figure 2 shows the product ion MS/MS spectra for Sudan Red I at two collision energies (CE). It can be seen from the spectra that the transition *m/z* 156 gives a maximum intensity at 15 V and *m/z* 93 at 25 V. Typically the most abundant ion is used for quantitation to maximize precision and accuracy at the lowest levels and the less abundant ion is used as the qualifier for confirmation. However, for the highest selectivity, unique transition ions are sought. In the example of Sudan Red I the transition *m/z* 93 is the aniline radical cation produced by cleavage of the azo bond and hydrogen transposition. [4] This also produces the transition *m/z* 156, both unique. Measuring CE vs intensity for the two most abundant ions of all the compounds resulted in the voltages shown in Table 2 along with the other MS settings.

Dye Structures

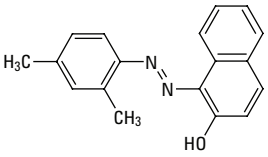
Para red



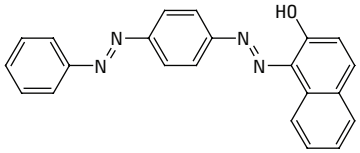
Sudan Red I



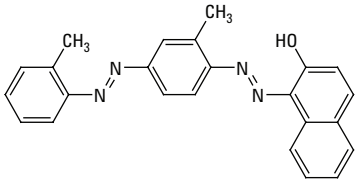
Sudan Red II



Sudan Red III



Sudan Red IV



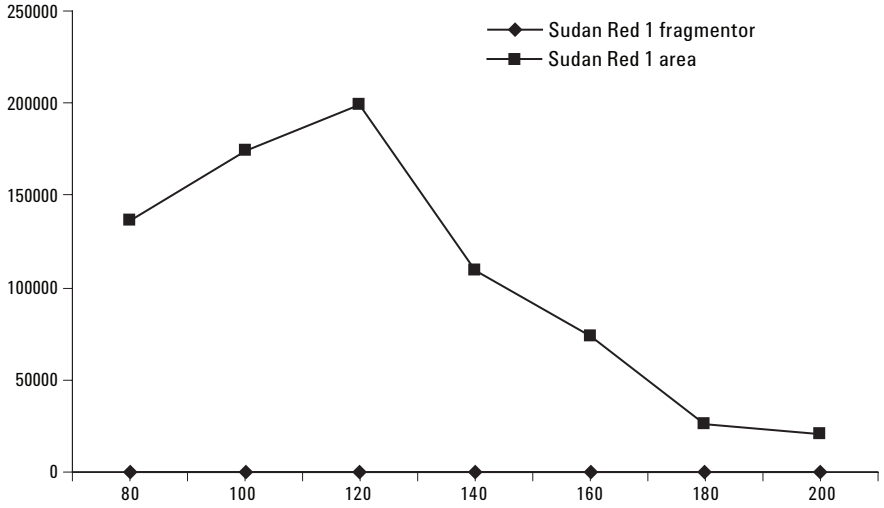


Figure 1. Optimization of fragmentor voltage for Sudan Red I.

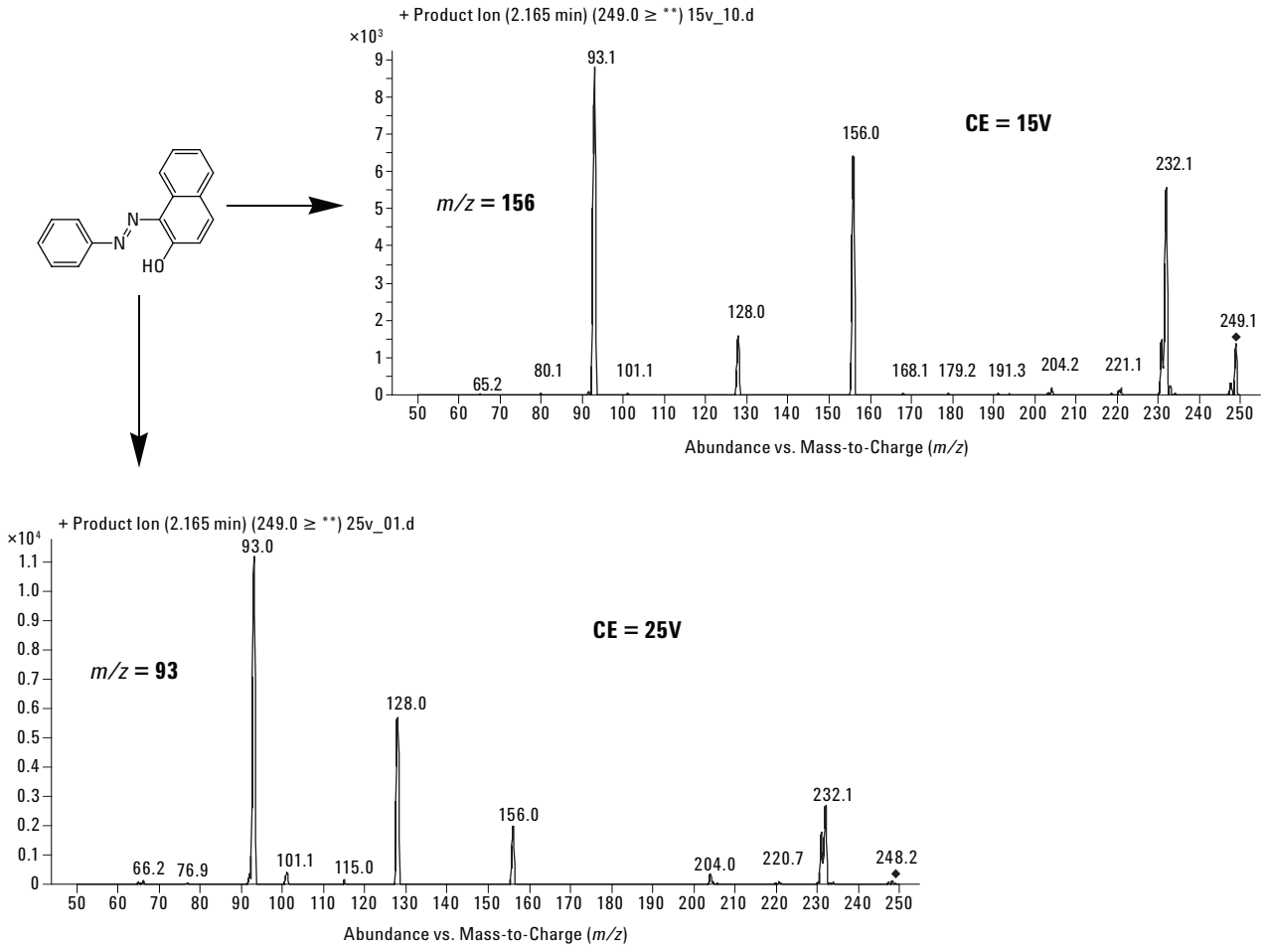


Figure 2. MS/MS spectra of Sudan Red I at collision energies (CE) 15 and 25 V.

Table 2. MRM MS/MS Parameters

Analyte	Transition	Dwell (msec)	Fragmentor voltage	Collision energy	MS2 Res.	Gain
Para Red	294→156	200	120 V	15 V	Unit	1
	294→128	200	120 V	30 V	Unit	1
Sudan Red I	249→156	200	120 V	15 V	Unit	1
	249→93	200	120 V	25 V	Unit	1
Sudan Red II	277→156	200	100 V	10 V	Unit	1
	277→121	200	100 V	20 V	Unit	1
Sudan Red III	353→77	200	120 V	25 V	Unit	1
	353→197	200	120 V	20 V	Unit	1
Sudan Red IV	381→91	200	120 V	30 V	Unit	1
	381→225	200	120 V	15 V	Unit	1

Chromatography and Sensitivity

Figure 3 shows the chromatographic separation achieved for the five analytes allowing the segmentation of the MS/MS conditions. The sensitivity of the methodology is shown with the spike of the chili powder at 1 ppb. This is shown with a blank chili powder (Figure 4) and spike where each transition of the quantifier and qualifier ion for all five analytes is displayed (Figure 5).

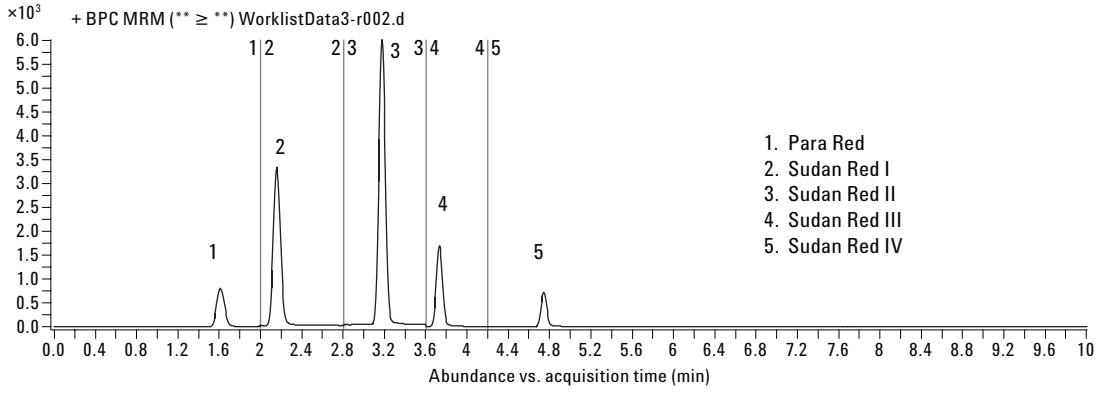


Figure 3. MRM chromatogram of Sudan Reds and Para Red.

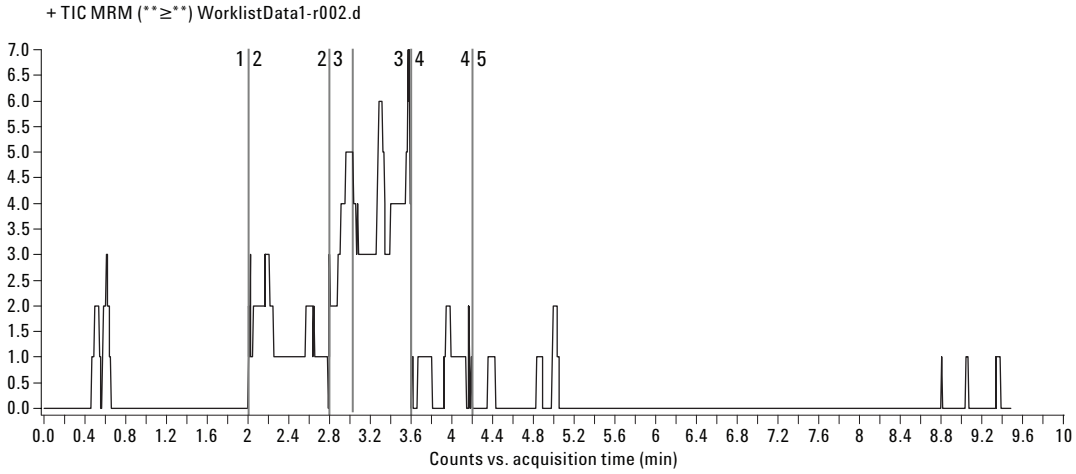


Figure 4. Blank chili pepper extract showing no response for any analyte.

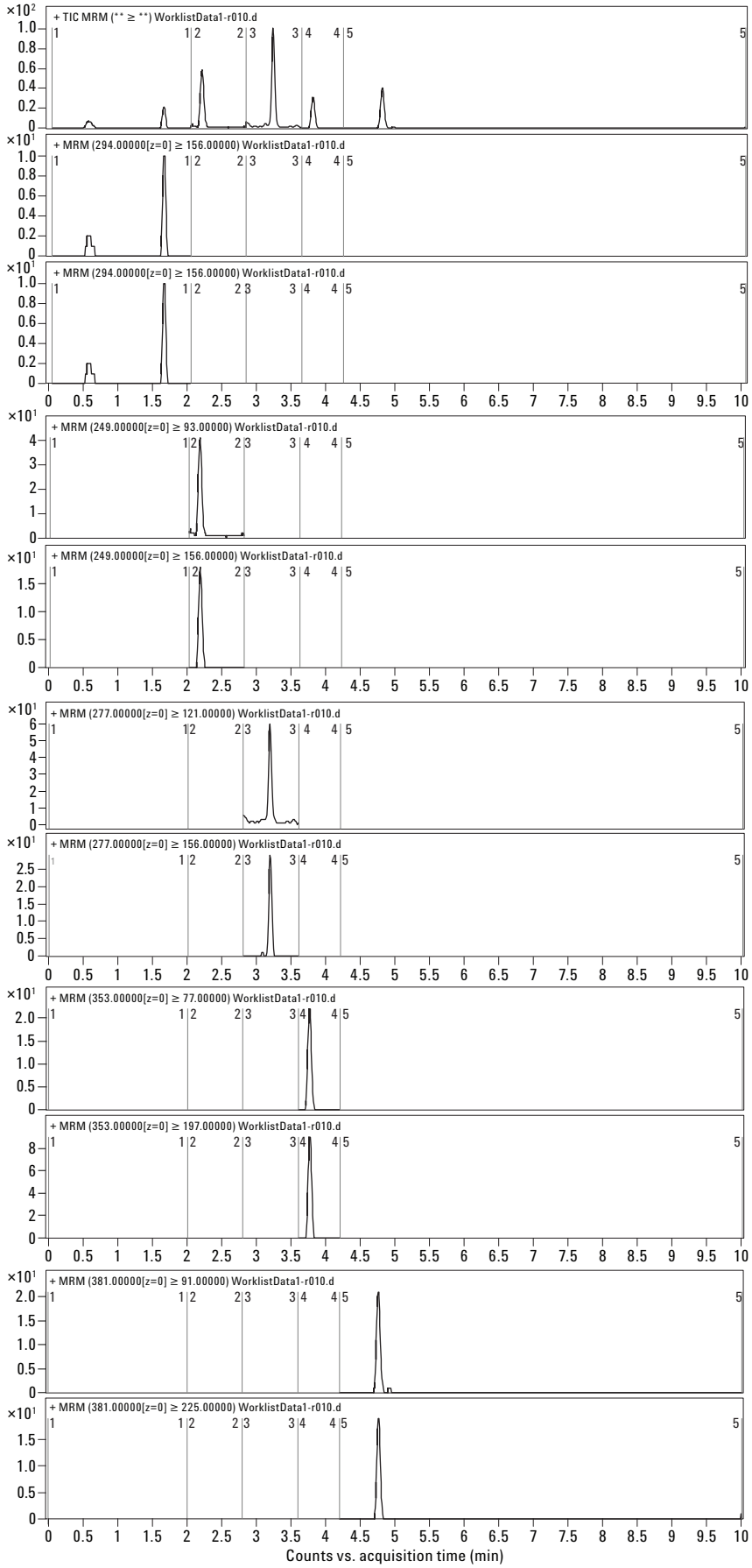


Figure 5. Chili pepper spiked at 1 ppb. Total MRM chromatogram on top and then each transition, quantitation and qualifier of the respective five analytes.

Confirmation by Ion Ratios

The EU Decision 2002/657/EC set confirmation criteria to include two MS/MS transition ions and their relative ratios (to the most abundant of the two). [5] The criteria for the tolerances of those ratios were given by the relative intensity. This is shown in Table 3 and demonstrates that the higher the relative ratio, the tighter the tolerances for acceptance.

We have measured these ratios for each of the analytes in both solvent and matrix and found the matrix has little effect on the measured ratios. Using Sudan Red I as the example, the ratios of the two ions are given in Table 4. In addition, the repeatability of the method was tested using a 1 ppb spike in curry powder. The results of 20 repeats shown in Table 5 demonstrate that the method can perform well within the accepted tolerance of 25%. All of the samples used for the validation study met the relevant identification criteria.

Table 3. Maximum Permitted Tolerances for Relative Ion Intensities Using LC-MS^n

Relative intensity between two transition ions (% of most intensive ion)	Tolerance for LC-MS^n (%)
> 50	± 20
> 20–50	± 25
> 10–20	± 30
≤ 10	± 50

Table 4. Ratio of Quantitation vs Qualifier Transition Ion in Tested Matrices at 100 ppb Spike of Sudan Red I

Matrix	Ratio
Solvent	46.8
Curry powder (Madras)	48.5
Red pepper powder (Korean Farm)	46.5
Red pepper powder (Oriental Mascot)	46.6
Worcestershire sauce (Lea & Perrins)	47.7

Table 5. Repeatability of Sudan Red I Ion Ratios at 1 ppb in Curry Powder

	Quantitation ion (249-93)	Qualifier ion (249-156)	Ratio
1	130	58	45.0
2	141	65	46.0
3	145	71	48.9
4	152	62	41.0
5	135	66	49.1
6	146	66	45.3
7	134	64	47.7
8	139	69	49.5
9	137	71	51.8
10	147	64	43.9
11	143	61	42.4
12	156	65	41.7
13	147	64	43.5
14	144	64	44.7
15	148	65	44.1
16	152	63	41.2
17	143	68	47.7
18	140	66	46.7
19	141	62	44.2
20	142	62	43.8
Average	143.1	64.8	45.4
STD (%)	4.49	4.87	6.55

Linearity, Sensitivity, and Recovery

Pearson's Correlation Coeffcient (R^2) was used as a measure of the standard curve linearity where an R^2 value of at least 0.99 was deemed acceptable. The linearity of the dyes in each of the food matrices was at least 0.99 except where the 1 ppm standard showed saturation, probably of the electrospray current. When that concentration is omitted, all calibrations in all matrices have an $R^2 > 0.99$. An example of this is shown in Figure 6, where red chili pepper is spiked with Sudan Red I.

The detection limit of each of the dyes was estimated by analyzing them in a standard at 0.2 ppb. The total MRM chromatogram is shown in Figure 7. Because each of the signals has little noise, it is difficult to determine with accuracy an s/n of 3:1.

Increasing the gain on the electron multiplier would have given both greater signal and noise and made this determination more accurate. It is reasonable to estimate that the limit of detection (LOD) is close to this concentration.

Finally, recovery of the sample preparation procedure was examined for the Worcestershire sauce since this was a liquid-liquid extraction. This was done with a replicate of three for each level of 100 and 1 ppb. These results are given in Table 6 and show reasonable recoveries.

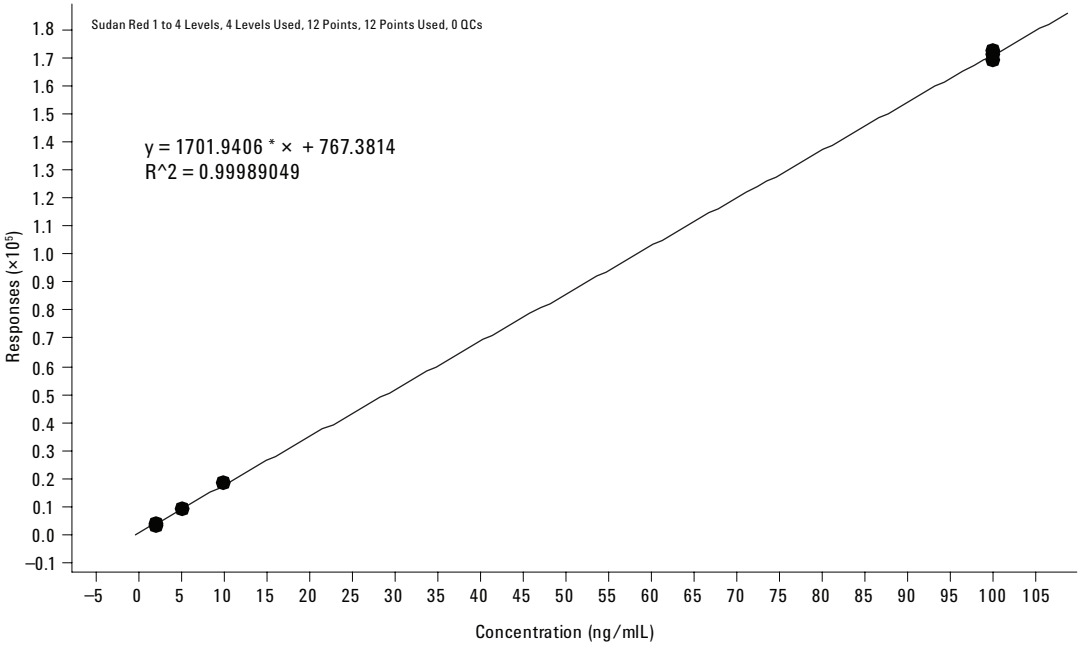


Figure 6. Standard curve of 1 ppb – 100 ppb Sudan Red I in chili powder.

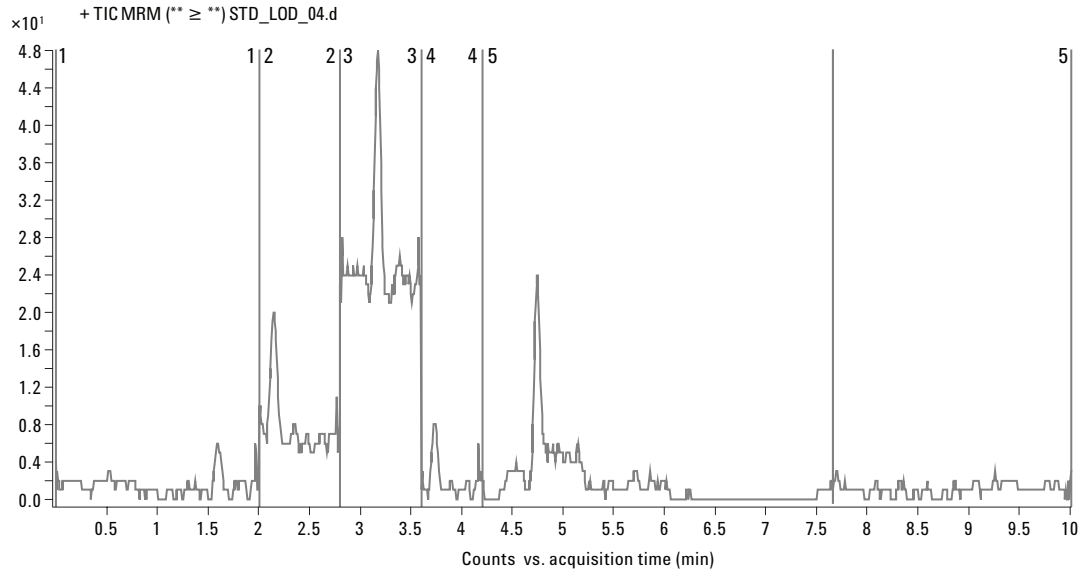


Figure 7. MRM chromatogram of each of the dyes at 0.2 ppb in solvent, representing a response close to the detection limit.

Table 6. Recovery of the Five Dyes in the Worcestershire Sauce

	Para Red (%)	Sudan Red I (%)	Sudan Red II (%)	Sudan Red III (%)	Sudan Red IV (%)
100 ppb recovery (n = 3)	57.8	71.0	63.4	62.1	70.2
1ppb recovery (n = 3)	54.5	66.2	50.1	54.1	51.2

Conclusions

The method described herein for the analysis of four Sudan Red compounds and Para Red in four different matrices has been shown to be highly effective in meeting the criteria for quantitation and confirmation. Optimization of the method was simple, because few parameters in the mass spectrometer need adjustment. In addition, basic requirements for a validated method have been shown. These include sensitivity, repeatability, linearity, and recovery. This shows the Agilent 6410 Triple Quadrupole LC/MS system to be a highly effective instrument for the analysis of Sudan Reds and other azo dyes in food spices.

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

Application

Food

Authors

Frank David
Research Institute for Chromatography
Pres. Kennedypark 26
8500 Kortrijk
Belgium

Matthew S. Klee
Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE 19808-1610
USA

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.

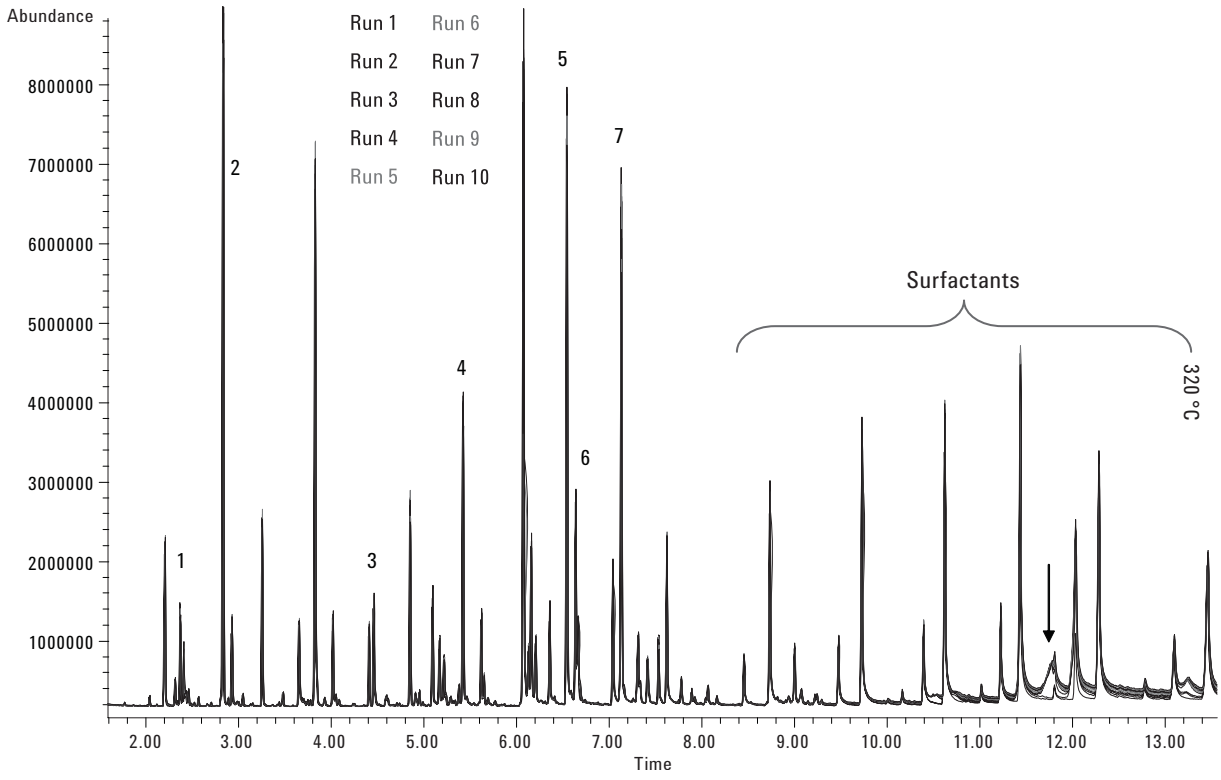

Figure 1. Overlay of 10 consecutive analyses of shampoo extract (oven temperature programmed to 320 °C, no backflush).

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.

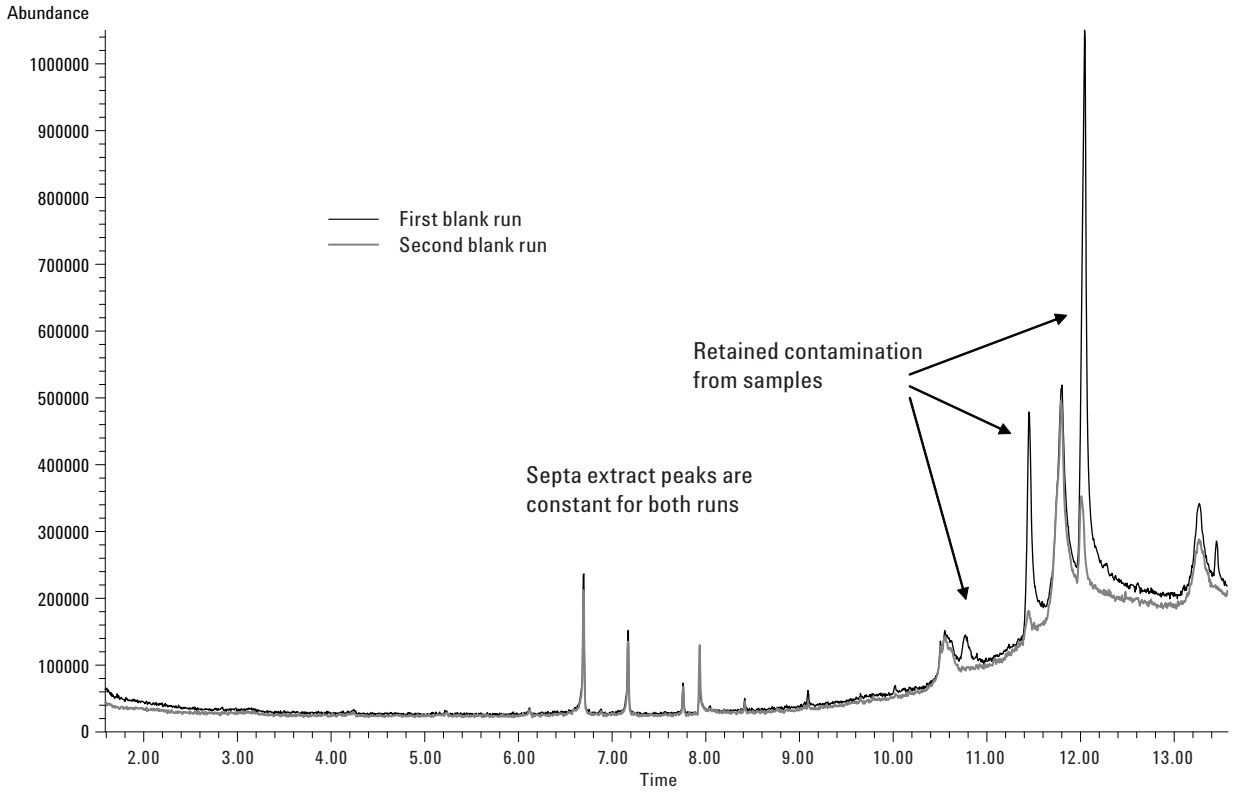

Figure 2. Two consecutive blank runs after analysis of shampoo extract.

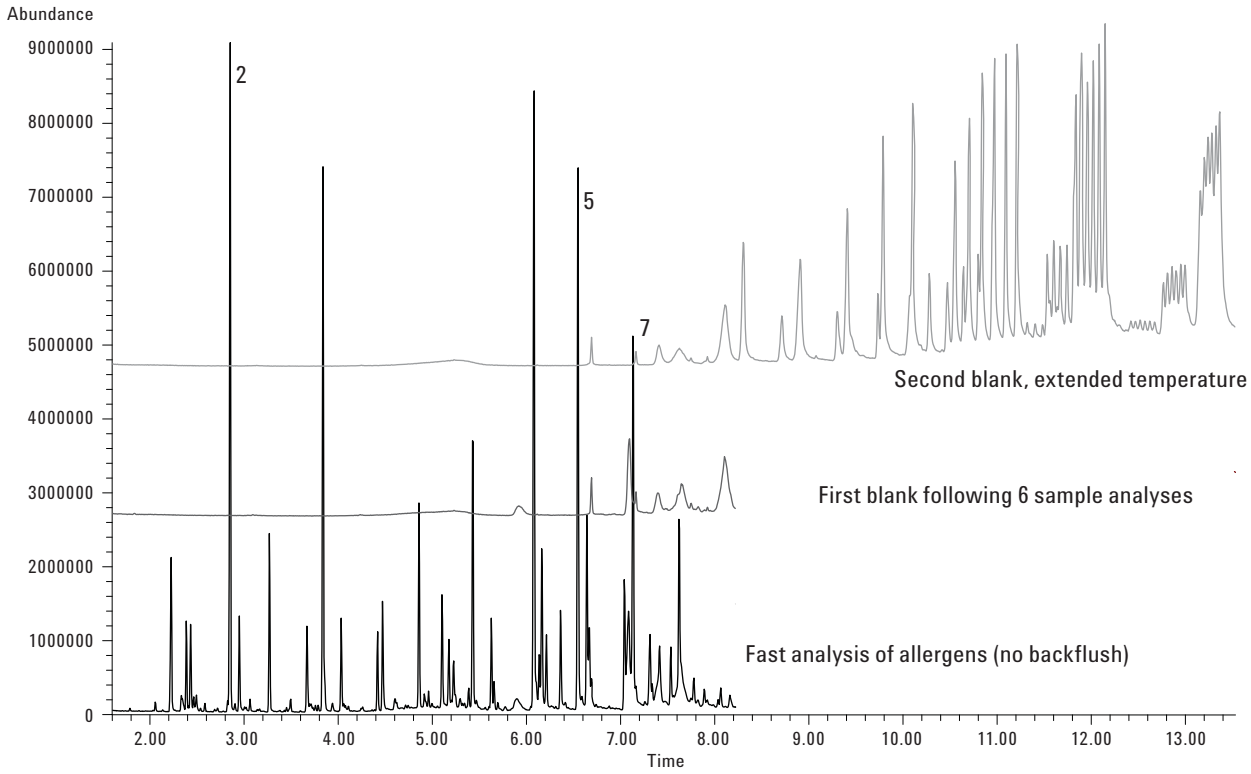

Figure 3. Overlay of sixth analysis of shampoo extract with run stopped at 240 °C (bottom), first blank run to 240 °C (middle), and second blank run to 320 °C (top).

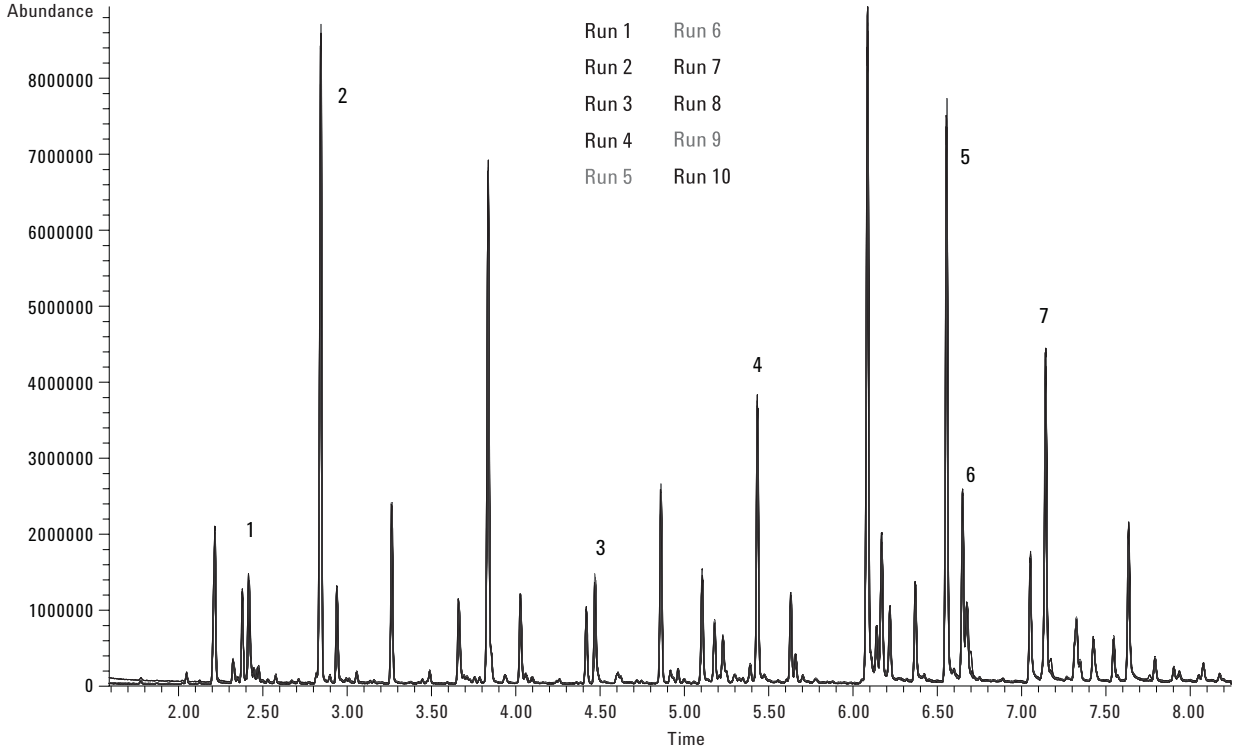

Figure 4. Overlay of 10 consecutive analyses of shampoo extract (oven temperature programmed to 240 °C, with backflush).

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.

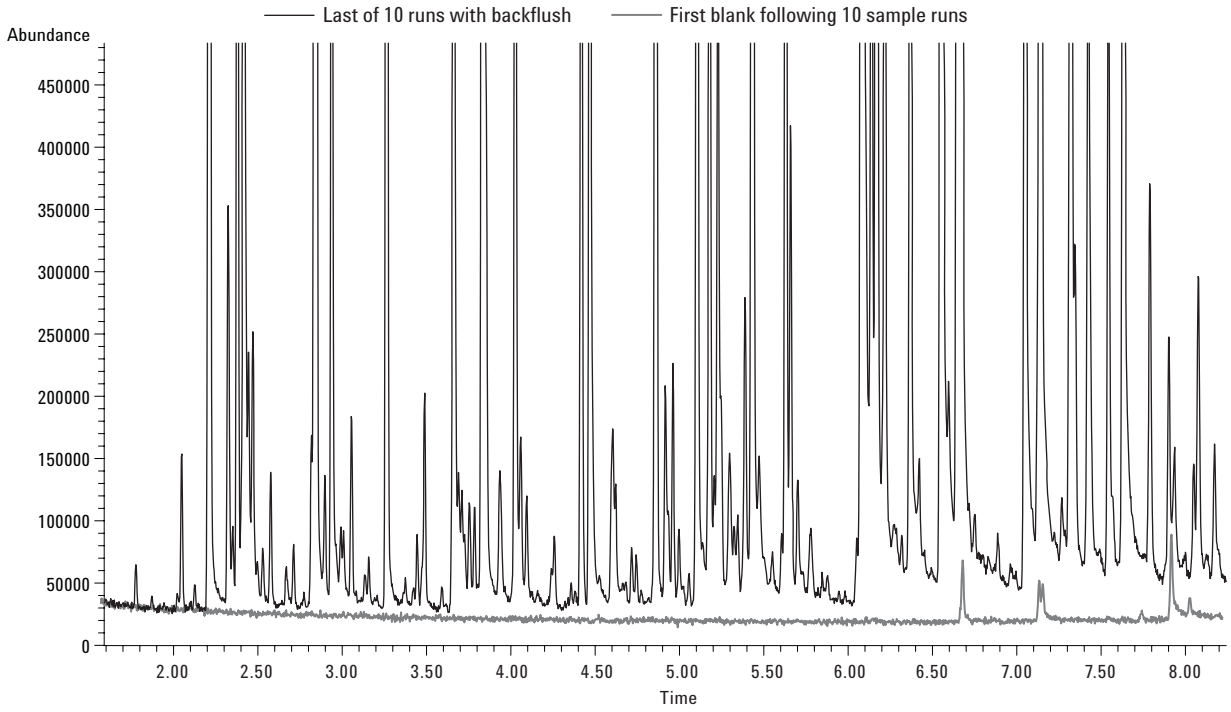

Figure 5. Overlay of 10 analyses of shampoo extract (oven programmed to 240 °C) with backflush (top) and subsequent blank run (bottom).

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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Rapid Screening and Analysis of Components in Nonalcoholic Drinks

Application

Food

Author

Michael Woodman
Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE 19808-1610
USA

Abstract

Soft drinks, juices, and prepared teas are popular drinks that are carefully formulated to provide unique flavor and reliable product stability. Many end-product facilities have the means to monitor critical ingredients, such as sugars, flavors, colorants, and preservatives, but may find their analyses are not fast enough to keep pace during periods of high seasonal demand. Here we take a classical approach to the analysis of additives and sweeteners and convert to a new method that improves sample throughput and resolution while also reducing solvent consumption.

Introduction

Quality control (QC) laboratories are constantly challenged to meet higher sample loads with fewer instruments, reduced staff, and without sacrificing data quality in the process. While some labs have successfully adopted greater automation in sample preparation and data handling, they still find that opportunities exist for increasing their chromatographic throughput. Methods developed on 5- or 10-µm particle size columns may be candidates for modernization by replacing these columns with smaller-dimension columns packed with smaller particle sizes. The greatest opportunities in beverage QC labs exists with the small molecule separations of additives, preservatives, flavorants, and sweeteners. In methods development and research labs, compatibility with mass spectrometer ionization sources has also been a driving force in reducing column size to support low flow-rate operation.

To reduce analysis time without sacrificing resolution, we can reduce column length and packing particle size simultaneously. The balancing effect is remarkably simple if reasonable optimization of the HPLC system, with respect to gradient delay volume and extracolumn dispersion, is practical. For example, a 250-mm long column with 5-µm particles can be replaced by a 150-mm long column packed with 3-µm particles. If the ratio of length to particle size is equal, in other words, resolution is preserved. Solvent consumption is proportionately reduced, and, if an equal mass of analyte can be successfully injected, the detector response should also increase due to reduced dilution of the peak as it travels through a smaller column of equal efficiency.

Liquid chromatography/mass spectrometry (LC/MS) ionization sources, especially the electrospray ionization mode, have demonstrated greater sensitivity at lower flow rates than typically used in normal ultra violet UV/VIS optical detection (LC/UV) methods. In MS detection mode, it is

advantageous to reduce the column volume via reduced internal diameter. Lower flow rates will be required, proportional to the cross-sectional area of the inside diameter of the columns. The relationship of flow rate between different column diameters is shown in Equation 1.

$$\text{Flow}_{\text{col. 1}} \times \left[\frac{\text{Diam.}_{\text{column2}}}{\text{Diam.}_{\text{column1}}}\right]^2 = \text{Flow}_{\text{col. 2}} \quad \text{(eq. 1)}$$

We normally scale the injection mass to the size of the column, and a proportional injection volume would be calculated from the ratio of the void volumes, as shown in Equation 2.

$$\text{Inj. vol.}_{\text{col. 1}} \times \left[\frac{\text{Volume}_{\text{column2}}}{\text{Volume}_{\text{column1}}}\right] = \text{Inj.Vol.}_{\text{col. 2}} \quad \text{(eq. 2)}$$

For isocratic separations, the above conditions will normally result in a successful conversion of the method with little or no change in overall resolution. There are several other parameters that should be considered. The first of these parameters is the column efficiency relative to flow rate, more correctly efficiency to linear velocity, as commonly defined by van Deemter [1] and others, and the second is the often overlooked effect of extracolumn dispersion on the observed or empirical efficiency of the column.

Van Deemter observed and mathematically expressed the relationship of column efficiency to a variety of parameters, but we are most interested here in his observations that there is an optimum linear velocity for any given particle size, in a well-packed HPLC column, and that the optimum linear velocity increases as the particle size decreases. Graphically, it is typically represented as van Deemter plots [2] of plate height versus linear velocity. Generally speaking, a reduction in particle size leads to higher efficiency, at higher linear velocities, and the optimum range of highest efficiency expands to offer a wide flow range where good performance can be expected.

The second important consideration is the often overlooked effect of extracolumn dispersion on the observed or empirical efficiency of the column. As column volume is reduced, peak elution volumes are proportionately reduced. If smaller particle sizes are also employed there is a further reduction in the expected peak volume. Care must be taken by the user to minimize the extracolumn volume and to reduce, where practical, the number of connecting fittings and the volume of injection valves and detector flow cells.

For gradient elution separations, where the mobile phase composition increases through the initial part of the analysis until the analytes of interest have been eluted from the column, successful method conversion to smaller columns requires that the gradient slope be preserved. It is most useful to express the gradient slope as % change per column volume. In this way, the change in column volume during method conversion can be used to accurately render the new gradient condition, regardless of flow rate or column diameter and length. We can express the gradient as shown in Equation 3.

$$\%\ \text{Gradient slope} = \left[\frac{(\text{End\%} - \text{Start\%})}{\#\text{Column volumes}}\right] \quad \text{(eq. 3)}$$

A large value for gradient slope yields very fast gradients with minimal resolution, while lower gradient slopes produce higher resolution at the expense of increased solvent consumption and somewhat reduced sensitivity. Lower gradient slopes are formed by increasing the gradient time or flow rate, or a combination, within an acceptable range of analysis time and operating pressure.

Resolution increases with shallow gradients because the effective capacity factor, k*, is increased. Much like in isocratic separations, where the capacity term is called k', a higher capacity value directly increases resolution. The effect is quite dramatic up to a k value of about 5–10, after which little improvement is observed. In the subsequent examples, we will see the results associated with the calculations discussed above.

Experimental Conditions

System

Agilent 1200 Series Rapid Resolution LC, consisting of:
G1379B micro degasser
G1312B binary pump SL
G1367C HiP ALS autosampler SL, with thermostatic temperature control
G1316B thermostatted column compartment SL
G1315C UV/VIS diode array detector SL, flow cell as indicated in individual chromatograms
ChemStation 32-bit version B.02.01

Columns

Agilent ZORBAX SB-C18, 4.6 mm × 250 mm, 5 µm
Agilent ZORBAX SB-C18, 3.0 mm × 50 mm, 1.8 µm

Mobile phase conditions

Organic solvent:	Acetonitrile (ACN) or ACN containing 0.1% formic acid
Aqueous solvent:	20 mm phosphoric acid in Milli-Q water, pH 3.65 with ammonium hydroxide, or Milli-Q water containing 0.1% formic acid

Gradient conditions

Gradient slope:	2.8% per column volume See individual chromatograms for flow rate and gradient time.

Samples

1. Standard mixture of sodium saccharin, caffeine, aspartame, vanillin, benzoic acid, sorbic acid, benzaldehyde, all 50 µg/mL in 1/1 methanol/water
2. Various soft drinks, decarbonated where applicable

Results

The separation in Figure 1 was performed on a 4.6 × 250 mm, 5-µm ZORBAX SB-C18, column thermostatted to 30 °C using buffered phosphate conditions. At this pH, benzoate and sorbate were adequately resolved on the SB-C18 material with ACN organic modifier. With the formic acid modifier, nominal pH 2.5, poor resolution of these two components was observed, though selectivity and peak shape of other components was comparable. Additionally, on-column degradation of benzaldehyde was observed, presumably to benzoate, at the lower pH. This has been observed in other experiments (data not shown) and is particularly evident as column temperature is increased. Preparing ammonium formate buffer at about the same pH as the phosphate would likely solve both problems and would be MS compatible.

The method was then scaled in flow and time for exact translation to 3.0 × 100 mm, 3.5-µm and 1.8-µm columns (data not shown). The gradient was recalculated for 3.0 × 50 mm, 1.8-µm material (Figure 2). Subsequent gradient calculations yielded higher speed examples (Figures 3 and 4) without resolution loss.

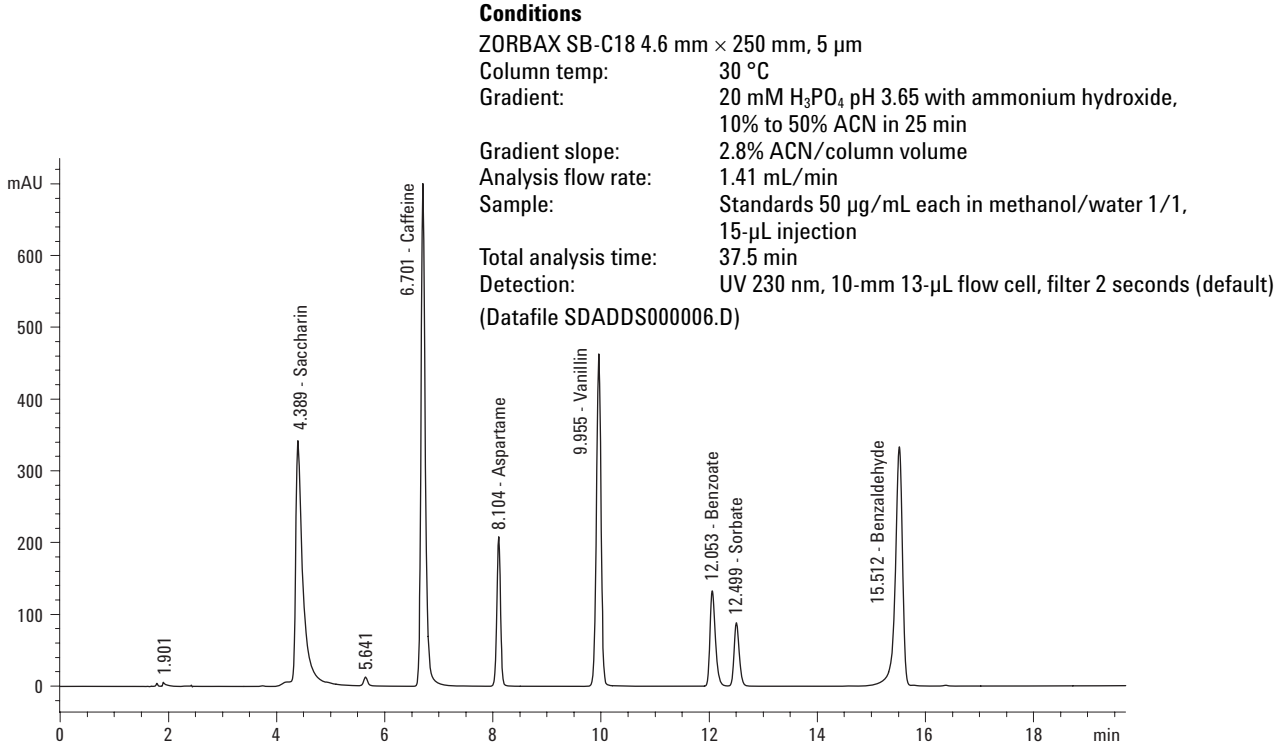
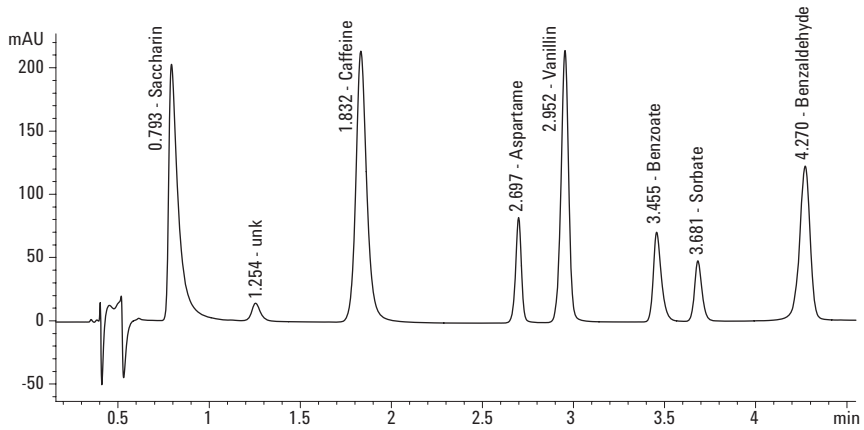


Figure 1 Gradient separation of soft drink additives on 4.6 × 250 mm, 5-µm ZORBAX SB-C18.



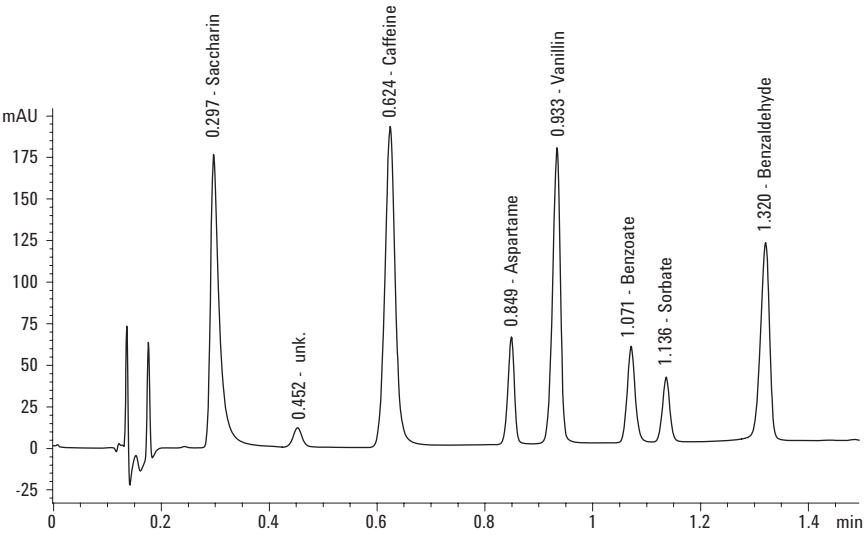
Conditions

ZORBAX SB-C18 3.0 mm × 50 mm, 1.8 µm

Column temp:	45 °C
Gradient:	20 mM H_3PO_4 pH 3.65 with ammonium hydroxide, 10% to 50% ACN in 5 min
Gradient slope:	2.8% ACN/column volume
Analysis flow rate:	0.6 mL/min
Sample:	Standards 50 µg/mL each in methanol/water 1/1, 2.5-µL injection
Total analysis time:	9 min
Detection:	UV 210 nm, 6-mm 5-µL flow cell with 0.12-mm id inlet heat exchanger, filter 0.2 seconds

(Datafile SDADDS_3MM21004.D)

Figure 2. Gradient separation of soft drink additives on 3.0 × 50 mm, 1.8-µm ZORBAX SB-C18.



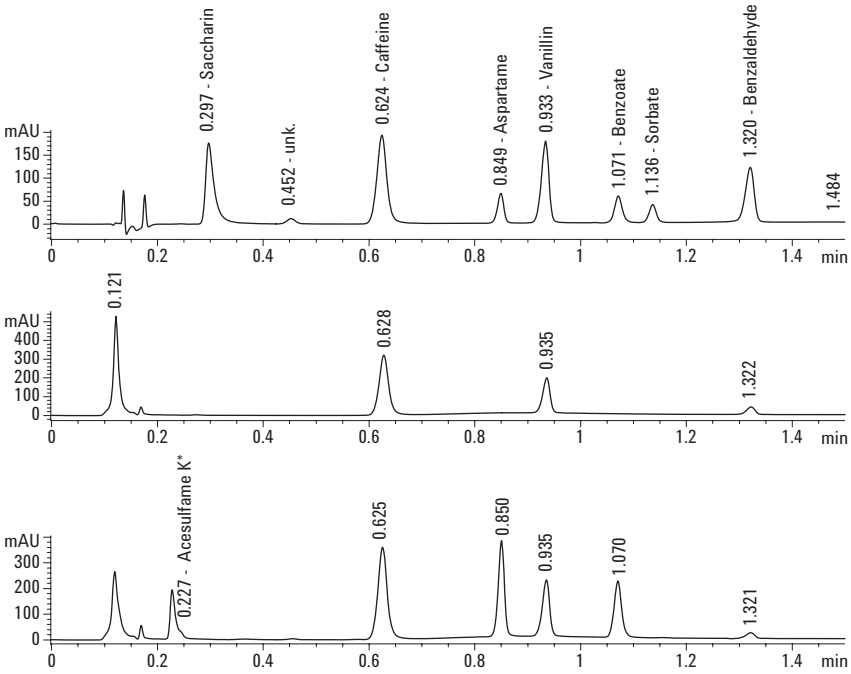
Conditions

ZORBAX SB-C18, 3.0 mm × 50 mm, 1.8 µm

Column temp:	45 °C
Gradient:	20 mM H_3PO_4 pH 3.65 with ammonium hydroxide, 10% to 50% ACN in 1.5 min
Gradient slope:	2.8% ACN/column volume
Analysis flow rate:	2.0 mL/min
Sample:	Standards 50 µg/mL each in methanol/water 1/1, 2.5-µL injection
Total analysis time:	3 min
Detection:	UV 210 nm, 6-mm 5-µL flow cell with 0.12-mm id inlet heat exchanger, filter 0.2 seconds

(Datafile SDADDS_3MM31029.D)

Figure 3. High-speed gradient separation of soft drink additives on 3.0 × 50 mm, 1.8-µm ZORBAX SB-C18.



Conditions

See Figure 3 for chromatographic conditions

*Acesulfame K tentative identification by reference to other samples under similar conditions and product label claim

Sample: Upper panel: mixed standard
Center panel: black cherry vanilla cola, 20-oz polymeric bottle, decarbonated, 2.5-µL injection
Lower panel: diet black cherry vanilla cola, 20-oz polymeric bottle, decarbonated, 2.5-µL injection

(Datafiles SDADDS_3MM31029.D, SDADDS_3MM31043.D, SDADDS_3MM31044.D)

Figure 4. High-speed gradient separation of soft drink additives on 3.0 × 50 mm, 1.8-µm ZORBAX SB-C18.

The conditions in Figures 3 and 4, using a 2.8% slope at increased linear velocity on 3.0 × 50 mm, 1.8-µm material, yield a separation with better resolution than the original 4.6 × 250 mm method. Because the absolute plate count is lower for the 1.8-µm column (12,000 vs. 24,000 predicted for the 250-mm 5-µm column, based on typical calculations), the resolution increase is presumably related to the increased operating temperature, which lowers both solvent viscosity and nonspecific column-analyte interactions, and an improvement in solvent linear velocity relative to the optimum for 1.8-µm materials. With only a 3-minute total analysis time, this is an excellent procedure for high-throughput screening and quantitation of a large number of samples.

Conclusion

Careful translation of gradient slope and the use of optimum linear velocity with sub-2-micron particles can enable users to take advantage of small format columns that yield fast analysis times without compromising resolution. Optimization of extracolumn volume helps minimize resolution losses that unduly degrade column performance, assuring the maximum resolution possible while improving analysis time substantially. We have demonstrated this potential with a complex mixture of typical beverage additives and encourage users to contact us for guidance on this application and when other method translation opportunities are identified.

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1. J. J. van Deemter, F. J. Zuiderweg, A. Klinkenberg, *A. Chemical Engineering Science* 1956, **5**, 271–289

2. The Influence of Sub-Two Micron Particles on HPLC Performance, Agilent Technologies, application note 5989-9251EN, May 2003

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Printe in the USA
August 3, 2006
5989-5178EN

Agilent Technologies

Superior Peak Shape of Xanthines and Metabolites Separated by Eclipse Plus C18

Application Brief

John W. Henderson Jr., William J. Long

Food and Beverage

A new, improved HPLC column technology is shown to provide better peak shape and efficiency. ZORBAX Eclipse Plus columns combine improved ZORBAX Rx-Sil, a high-purity, type B silica that is engineered for the best peak symmetry, with optimized bonding (C18, C8) reagents and bonding processes. The result is superior peak shape and efficiency.

Xanthines are naturally occurring alkaloids, synthesized by certain species of plants. Caffeine is the most widely known, found in coffee beans, tea leaves, and cacao beans. Others found in food and beverages include theophylline and theobromine. Below is an isocratic analysis of xanthines and metabolites on Eclipse Plus and four other C18 columns. While all provide satisfactory peak shape and resolution, to the trained eye, one chromatogram exhibits exceptional chromatographic performance, Eclipse Plus C18 (PN 959993-902). For every peak in this analysis, Eclipse Plus C18 consistently has sharper peaks (higher plates (N) and better peak shape (TF)).

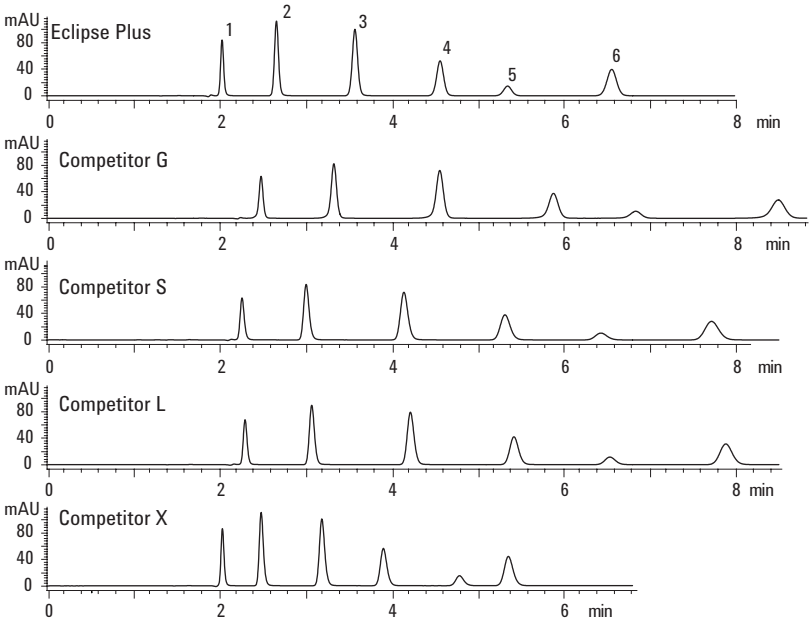

Figure 1. Separation of xanthines on various C18 columns.

Highlights

Advanced HPLC technology incorporated into Eclipse Plus columns delivers superior chromatographic performance.

Zorbax Eclipse Plus C18 columns separate xanthines with similar selectivity to other C18 columns, however with superior peak shape. Sharper peaks yield higher sensitivity.

Efficiency (N) of Eclipse Plus C18 is over 10% higher than any competitor for this analysis.

Tailing factor (TF) of Eclipse Plus C18 is closer to ideal (1.00) for every peak in this analysis compared to the competition.

Figure 1 analyte elution order:

1. 1-Methylxanthine
2. Theobromine
3. Theophylline
4. β-hydroxyethyltheophylline
5. 3-Propylxanthine
6. Caffeine

A: 25 mM Na phosphate pH 7.0
B: MeCN (90:10)
Flow: 1 mL/min
Temperature 40 °C
Columns 4.6 × 150 mm 5 µm

Agilent Technologies

Tables 1 and 2 show the difference in efficiency and peak shape between Eclipse Plus and other C18 columns based on the chromatograms in Figure 1. Clearly, Eclipse Plus C18 has higher efficiency and better peak shape than any of the competitors.

Many factors influencing efficiency and peak shape include: extra column volume, detector data sampling rate, and mobile phase conditions. These factors can be ignored in this example however, because the chromatographic system and method were the same for each analysis. Therefore, the difference in performance is entirely from the column.

Factors attributed to the column that influence efficiency include: silanol interactions with basic analytes such as xanthines (see Figure 2 for chemical structures), column voids inherent in manufacture or from dissolution of silica from mobile phase, and particle size and distribution (column loading). Eclipse Plus' superior peak shape over the others is based on advances in HPLC technology that reduce or eliminate these factors.

Table 1: Theoretical Plates (N) of Xanthines by Various C18 Columns

	Eclipse Plus	G	S	L	X
1-Methylxanthine	14900	13100	10700	12600	15900
Theobromine	15100	13300	10700	12600	13100
Theophylline	15100	13800	10400	13400	12600
β-hydroxyethyltheophylline	15000	13300	10100	13200	12400
3-Propylxanthine	14500	13500	10400	13300	12700
Caffeine	15600	13600	10600	14000	12800

Table 2: Tailing Factor (USP 5%) of Xanthines by Various C18 Columns

	Eclipse Plus	G	S	L	X
1-Methylxanthine	1.11	0.94	1.18	1.13	1.15
Theobromine	1.08	0.95	1.19	1.14	1.18
Theophylline	1.05	0.93	1.15	1.12	1.17
β-hydroxyethyltheophylline	1.02	0.92	1.13	1.10	1.15
3-Propylxanthine	1.01	0.91	1.11	1.09	1.14
Caffeine	1.00	0.91	1.10	1.09	1.14

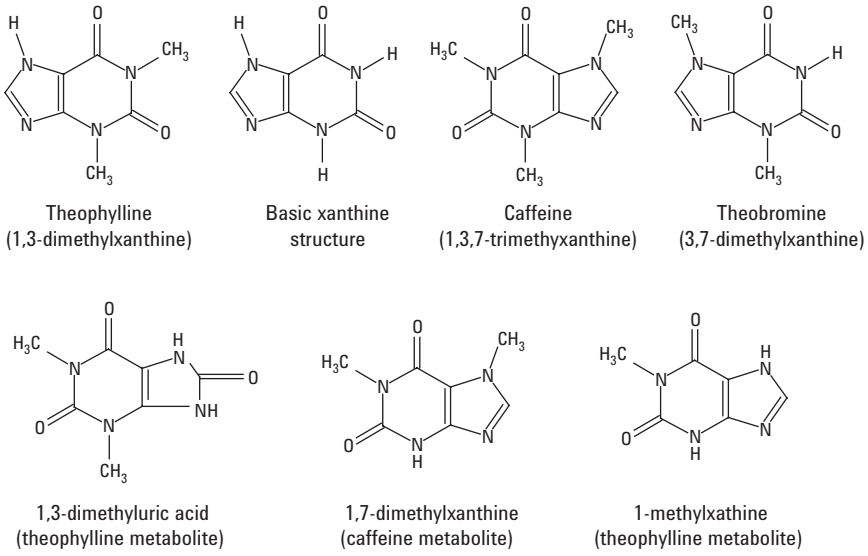

Figure 2. Structures of xanthines and metabolites in this study.

References

"High Throughput Separation of Xanthines by Rapid Resolution HPLC", Agilent publication 5989-4857EN

"New ZORBAX Eclipse Plus LC Columns", Agilent publication 5989-4934EN

John W. Henderson Jr. and William J. Long are application chemists based at Agilent Technologies, Wilmington, Delaware.

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Agilent Technologies

High Throughput Separation of Xanthines by Rapid Resolution HPLC

Application Note

Biochemistry, Food and Beverage, Pharmaceutical

Author

John W. Henderson and Ronald E. Majors
Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE 19808-1610
USA

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.

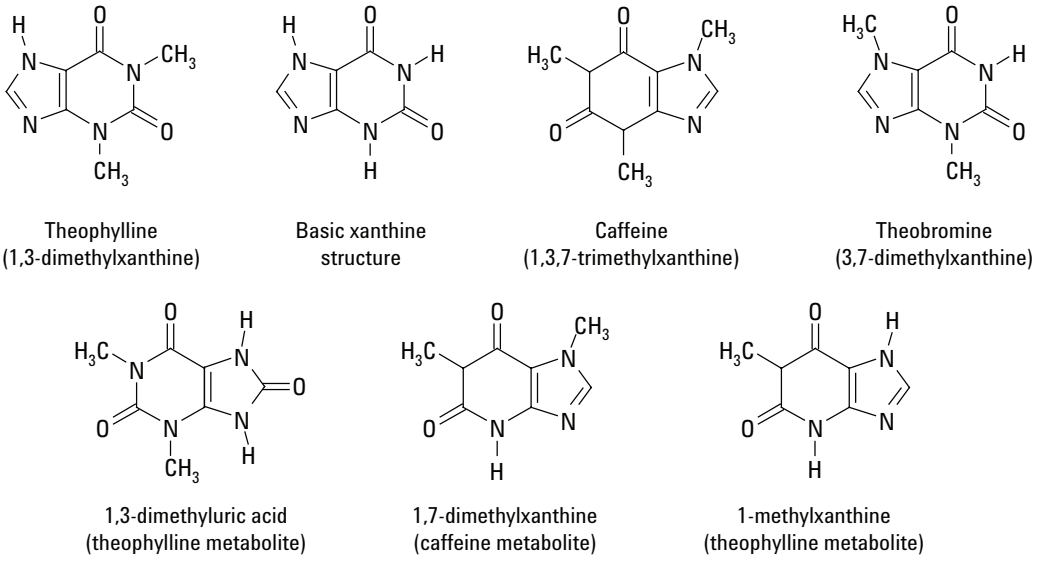


Figure 1. Structures of selected xanthines and metabolites used in this study.

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.

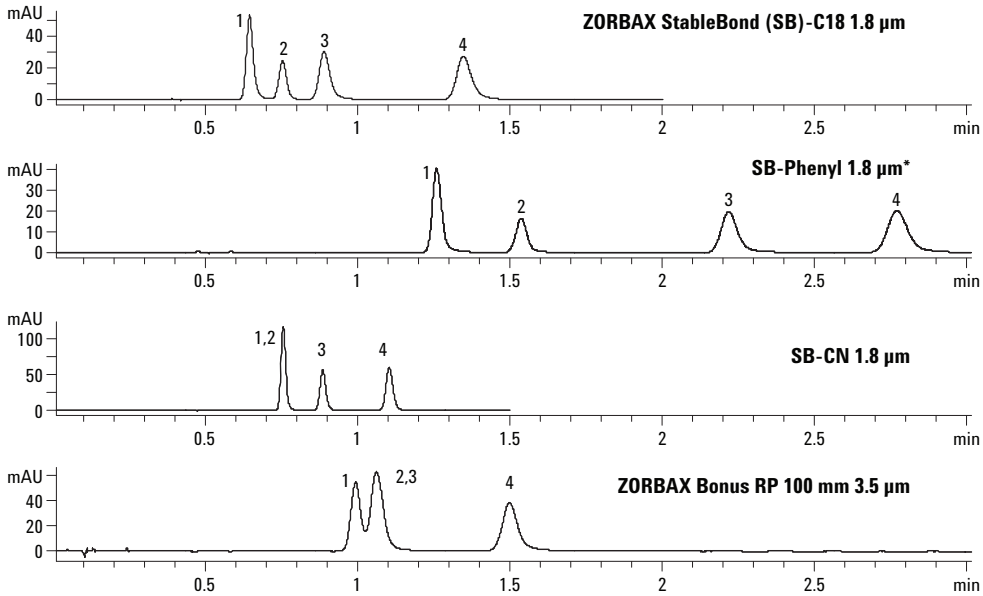


Figure 2. ZORBAX stationary phase selectivity comparisons for xanthines.

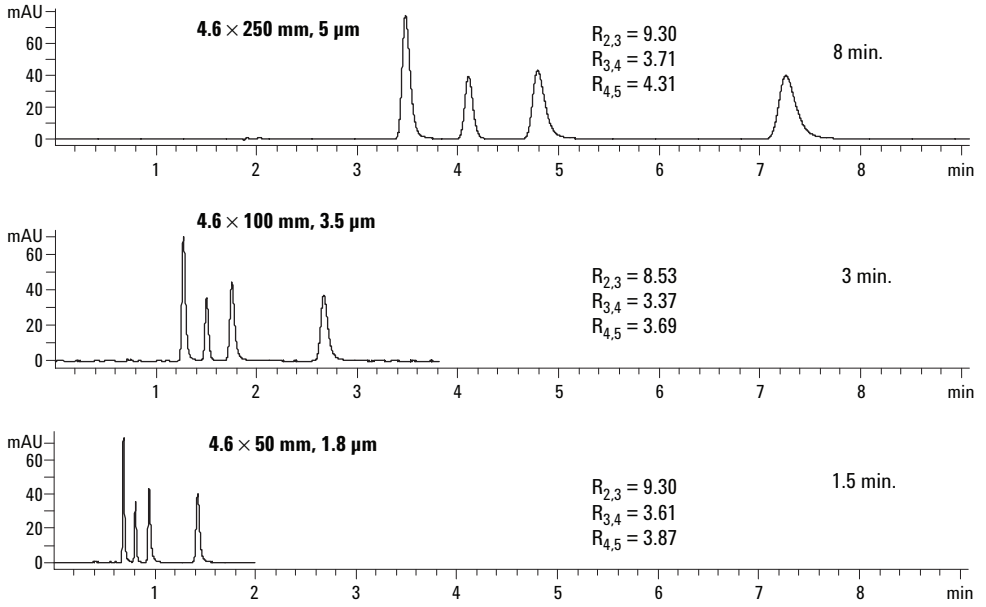
The chromatographic conditions chosen for subsequent experiments appear below:

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.

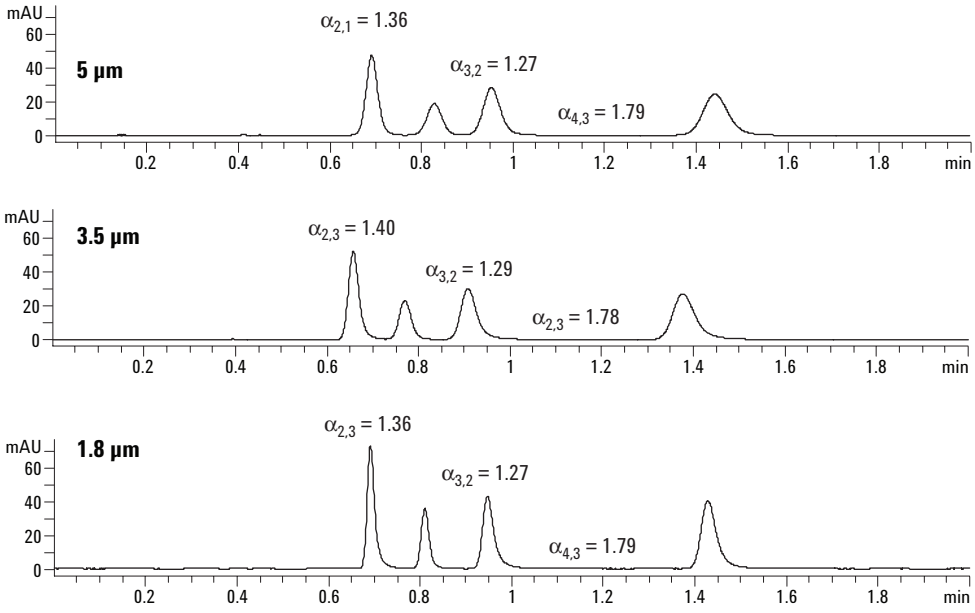
Of course, as one decreases the particle size of a column, the column backpressure increases with the inverse square of the particle diameter. Thus, if the same column length was used, the pressure at the same flow rate (or more correctly the linear velocity), the pressure would go up by a factor of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

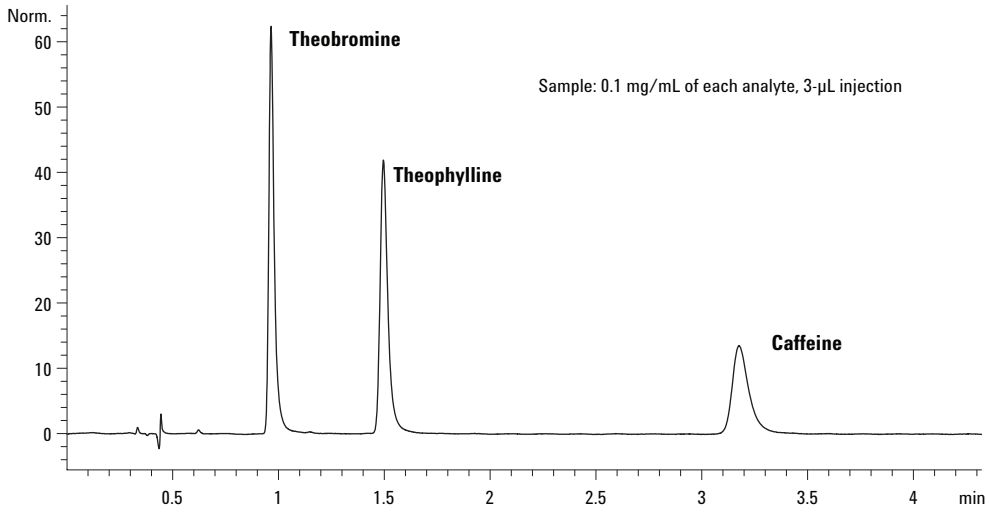
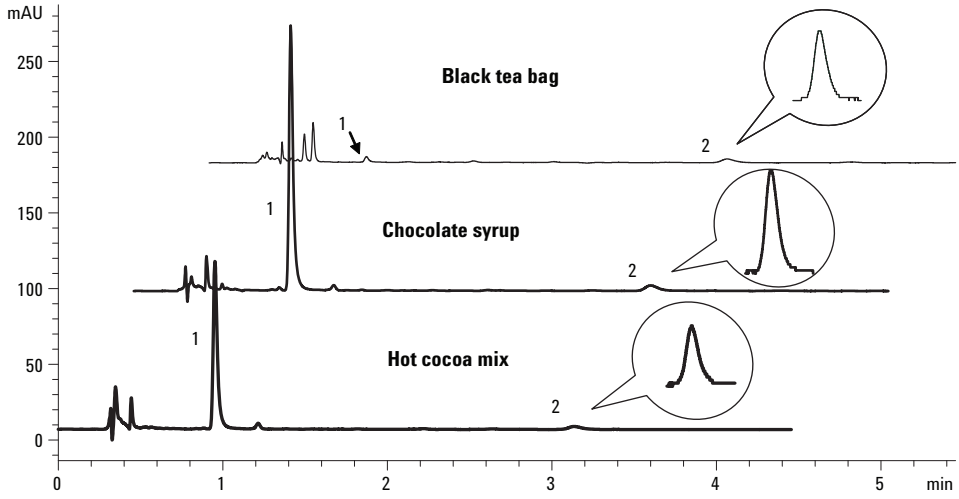


Figure 5. Separation of xanthine standards.



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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Using TOF for Screening and Quantitation of Sudan Red Colorants in Food

Application

Food

Authors

Yanyan Fang
Agilent Technologies, Inc.
412 Ying Lun Road
Pu Dong, Shanghai 200131
China

Michael Zumwalt
Agilent Technologies, Inc.
9780 S Meridian Blvd., MS 1-1E
Englewood, Colorado 80112-5910
USA

Abstract

The Sudan Reds are four commercial dyes, which could increase the risk of cancer if consumed in foods over long periods, or in large quantities. Now, they are banned in most countries. This application note presents the capability of the Agilent Time of Flight Mass Spectrometer (LC/MSD TOF) to be used as a screening, confirmation and quantitation tool-within-one analytical run of 5 min.

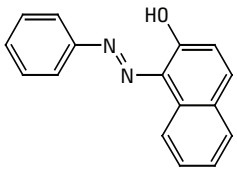
Introduction

Sudan Red is a red dye, traditionally used for coloring solvents, oils, waxes, petrol, and shoe and floor polishes. However, it is prohibited for use by the Regulations Relating to Food Colorants (R.1008) of the Foodstuffs, Cosmetics and Disinfectant Act 54 of 1972. Furthermore, the European Commission extended the scope of the ban on Sudan Red after it was revealed that related chemicals were also being found in chili products adulterated with Sudan Red. These chemical varieties are classified as Sudan Red I, II, III, and IV. All are considered to be carcinogenic.

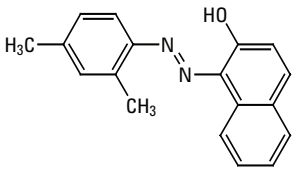
There is now an emergency measure in force dictating that chili and chili products, including curry powder, can only enter an EU country provided it has proof that these illegal chemical dyes are not present. These rulings extend tight measures that have been in place since June 2003, when France alerted the European Commission that traces of Sudan Red I were found in chili and chili powder imports from India. The inclusion of curry powder, which is found extensively in European food products, could lead to a surge in product recalls for the food industry.

In the UK alone, the food industry has recalled for destruction more than 160 products from the supermarket shelves since July 2003 as part of the enforcement of the new measures. Many countries including Canada, South Africa, France, Germany, and China have also discovered traces of Sudan I in their cuisines [1]. Figure 1 shows the structures of the banned dyes.

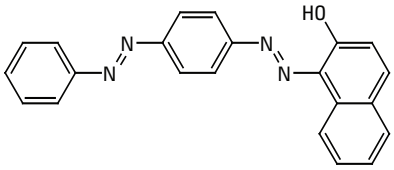
Sudan Red I



Sudan Red II



Sudan Red III



Sudan Red IV

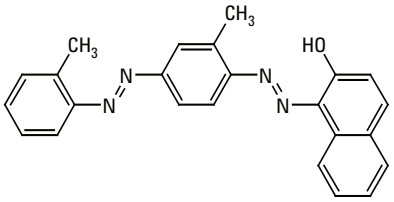


Figure 1. Structures of Sudan Reds.

Some methods exist for Sudan Red analysis. In method GB 19681-2005, the separation is complimented with a solid phase extraction, which simplifies the analysis and concentrates the sample [2].

Accurate mass measurement greatly increases the confidence of identification because it inherently limits the possible number of candidate compounds. That is, the better the precision and accuracy of the mass measurement, the fewer the number of compounds theoretically possible for a given accurate mass. Even without complicated sample preparation, good results can be obtained.

This application note provides an overview of the power of the Agilent TOF mass spectrometer for the screening, confirmation, and quantitation analysis of Sudan Reds. The TOF mass spectrometer provides accurate mass determination (<3 ppm) with good linearity, proving its use as an excellent tool for selectivity, specificity, and sensitivity for detection of analytes in different source matrices. The method used here is not intended to represent one that will determine the lowest possible level of any one particular analyte, or class of analytes, but rather is a procedure that could be expanded to cover a wider range of components used in screening analyses.

Experimental

Instrument

Agilent 1100 Series LC/MSD TOF with Agilent 1100 binary pump and well plate autosampler

LC Conditions

Column	ZORBAX XDB C18, 2.1 mm × 50 mm, 1.8 µm Agilent p/n: 922700-902
Mobile phases	A: H_2O with 5 mM NH_4OAc B: Acetonitrile
Gradient	0–3 min 95% B to 98% B 3–5 min 98% B
Post time	3 min
Flow rate	0.5 mL/min

MS Conditions

Ionization	ESI, Positive
Gas temp	350 °C
Drying gas	8 L/min
Nebulizer pressure	45 psi
Capillary V (+ve)	4000 V

Reference Mass Introduction with LC-TOF

The Agilent TOF MS uses a reference mass for the generation of reliable and high-level accurate mass spectra. The electro-spray ion source for the TOF is a unique dual-spray assembly that allows the simultaneous and constant introduction of reference mass components consisting of ~2-µM purine (*m/z* 121.050873 and HP-0921 (*m/z* 922.009798) calibrant compounds.

Results

Shown in Figure 2 is the total ion chromatogram (TIC) and the corresponding MS spectra for Sudan Red I, Sudan Red II, Sudan Red III, and Sudan Red IV. Note that all the compounds can be separated well within 5 min for a high-throughput analysis, which corresponds to at least half the time seen in most other methods.

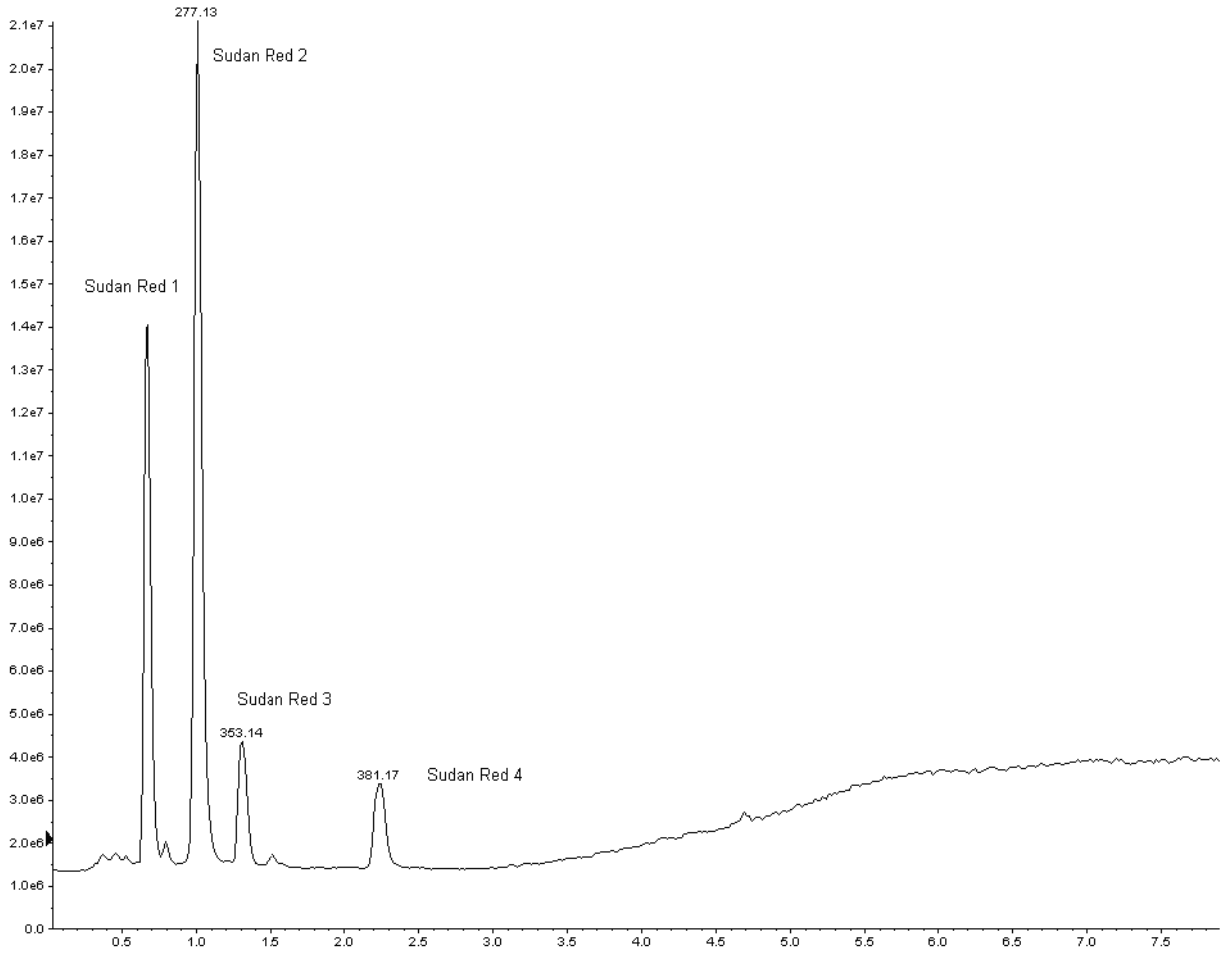


Figure 2. TIC showing the four peaks of the Sudan Red dyes along with the corresponding mass spectum of each peak.

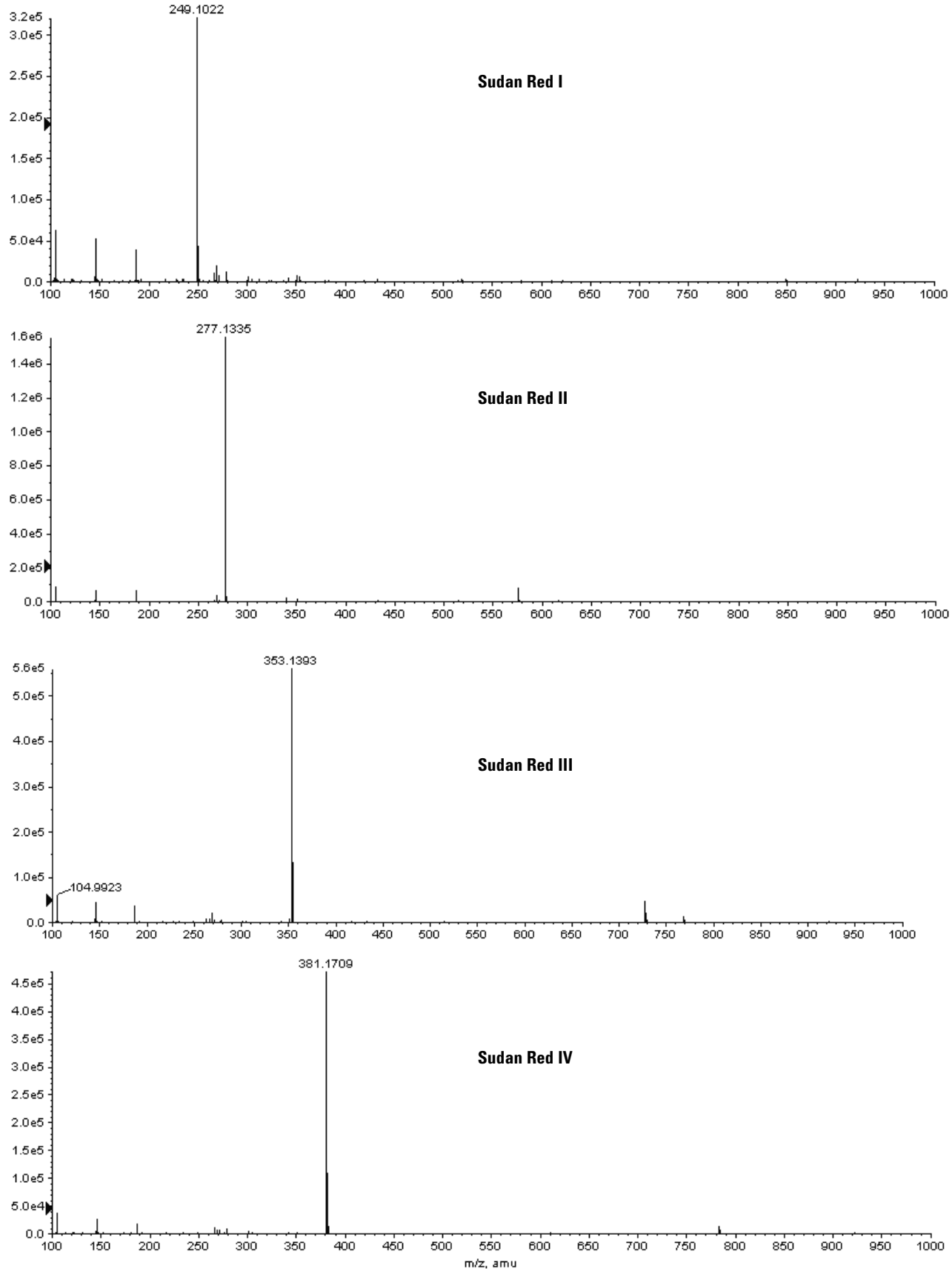


Figure 2. TIC showing the four peaks of the Sudan Red dyes along with the corresponding mass spectum of each peak.

The ability to closely match the expected mass and the observed mass provides the analyst with a higher level of confidence in the assignment given to a chromatographic peak. In the screening for compounds in matrices such as food, this additional confidence is of great importance. This capability also allows the possibility of using this technique as a screening tool for a wide range of components.

To demonstrate the use of high mass accuracy in confirming the presence of compounds like the Sudan Red dyes, the mass of Sudan Red I, from Figure 3, which is 249.1022 is entered into the calculator of the TOF software to determine the empirical formula. (See Table 1.)

So, given the accurate mass of 249.1022, mass accuracy of 3 ppm, and assumption that the compound only contains carbon (C), hydrogen (H), nitrogen (N), and Oxygen (O), only two empirical formulas are determined. However, the second match with only one carbon does not make any sense for the type of compounds being examined here and can be discarded so that only $C_{16}H_{13}N_2O$ is possible, which just happens to be the expected formula for the pseudo-molecular ion, $(M+H)^+$, of Sudan Red I.

Table 1. Theoretical Accurate Mass, Observed Mass and Mass Error

Compound	Monoisotopic mass	Retention time	Adduct	Observed mass	Adduct accurate mass	Mass (ppm)
Sudan Red I	248.0950	0.66	[M+H]+	249.1022	249.1022	–0.1589
Sudan Red II	276.1263	1.01	[M+H]+	277.1340	277.1335	–0.1434
Sudan Red III	352.1318	1.30	[M+H]+	353.1393	353.1390	0.6993
Sudan Red IV	380.4482	2.24	[M+H]+	381.1709	381.1709	–0.2306

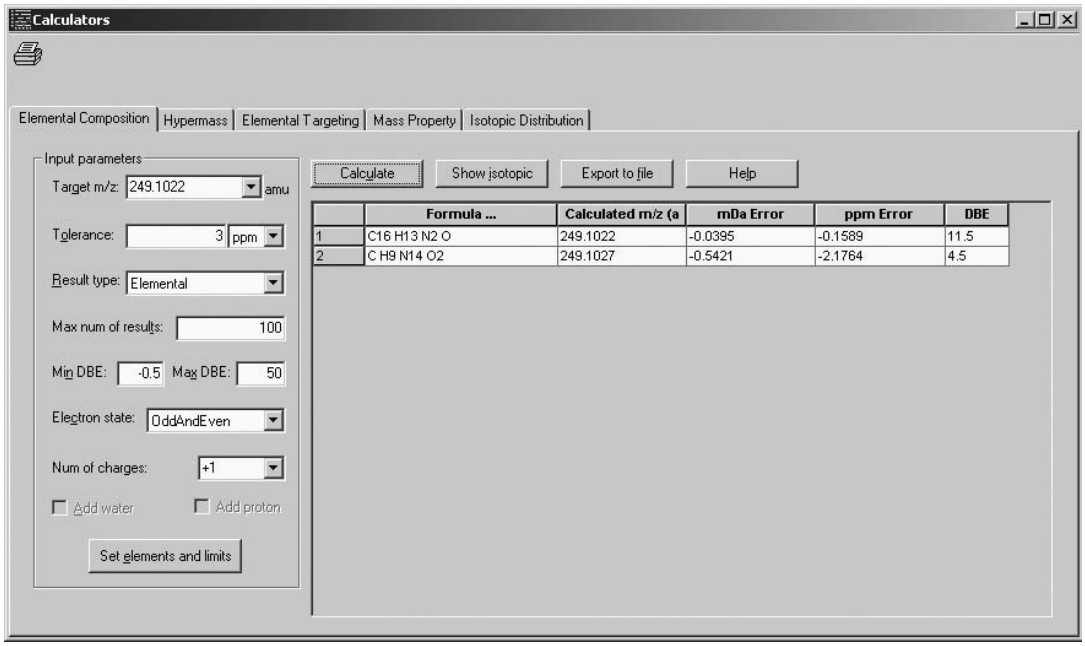


Figure 3. Empirical formula calculator of TOF software determines two possible formulas given the observed mass of 249.1022 and known mass accuracy of <3 ppm.

A greater than two-fold increase in sensitivity for many components is seen with the narrowing of the extracted ion chromatogram (EIC) window. To demonstrate this, Figure 4 shows the reduction in noise that is observed with the extraction of a smaller mass range for Sudan Red I. As a result, the ability of TOF-MS to accurately determine the presence of the components at low level may assist with investigations into reporting the illegal use of the synthetic dyes, and prove to be a critical factor in confirmation when dealing with complex matrixes.

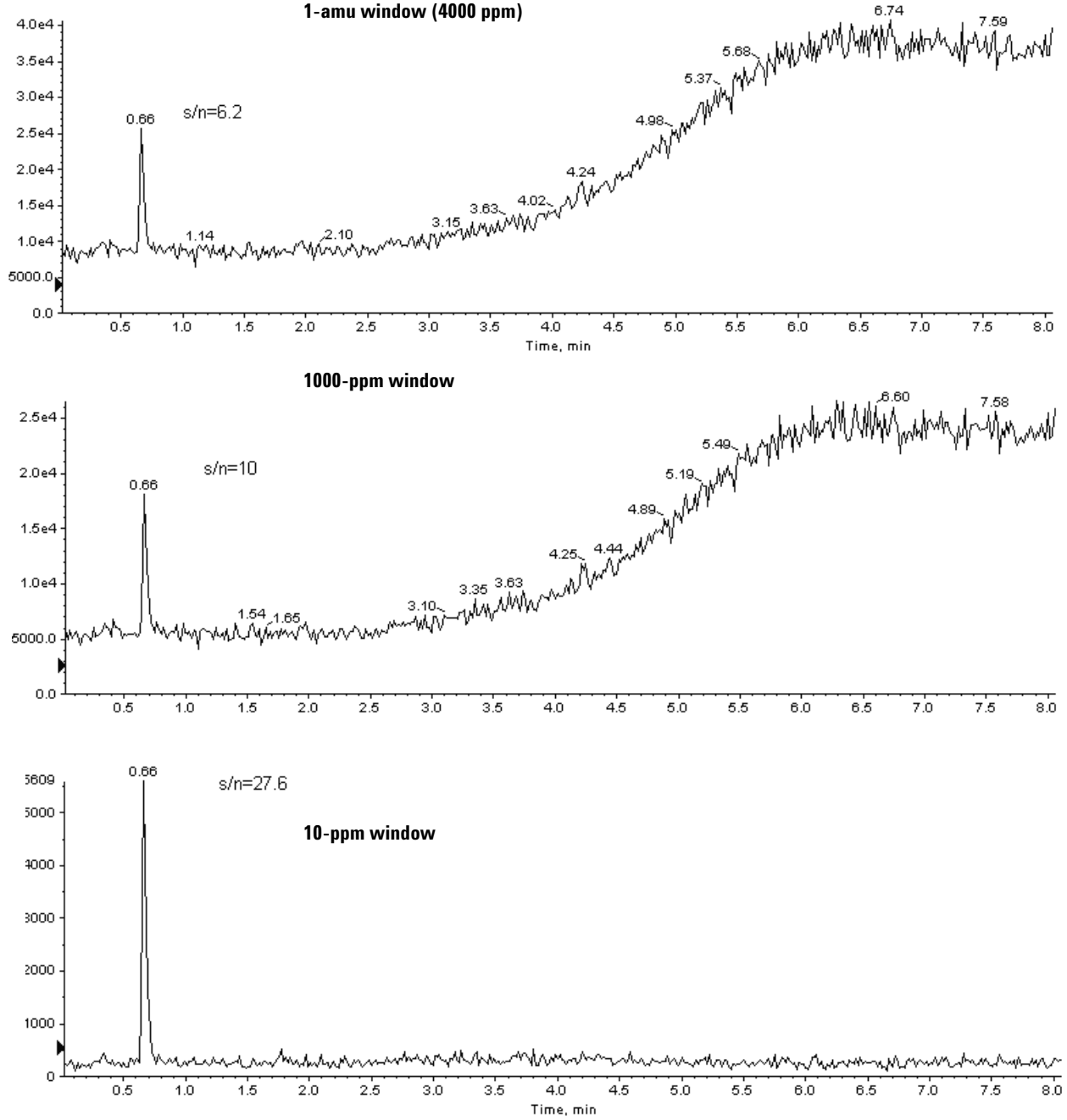
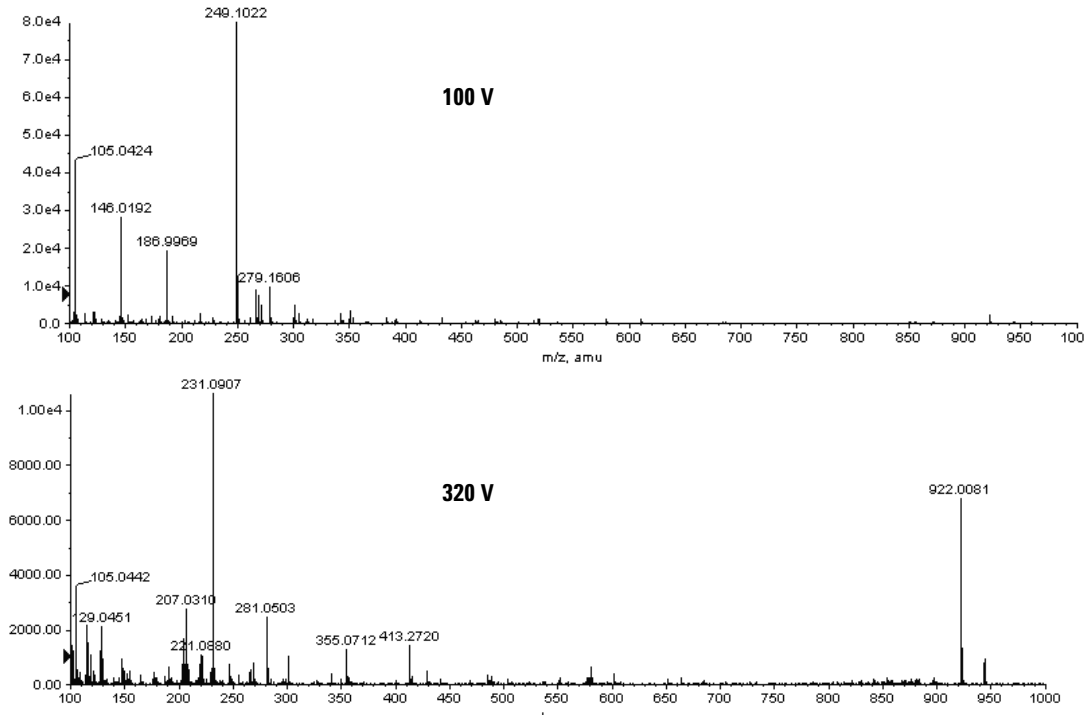


Figure 4. Effect of extracted ion range on noise-Sudan Red I in a real sample of Chinese Source spice oil.

It can be seen from Figure 4 that the ability to narrow the mass extraction window greatly reduces the noise for a given mass, and with the retention time information, can provide a high level of confidence in the assignment of a component.

Furthermore, the TOF can use in-source collision-induced dissociation (CID) to generate fragment ions for confirmation as well. These additional ions can be seen in both the unextracted standards and the compounds in the food matrix. Such spectra obtained with the unextracted standards are shown in Figure 5.

Sudan Red I at different voltages



Sudan Red II at different voltages

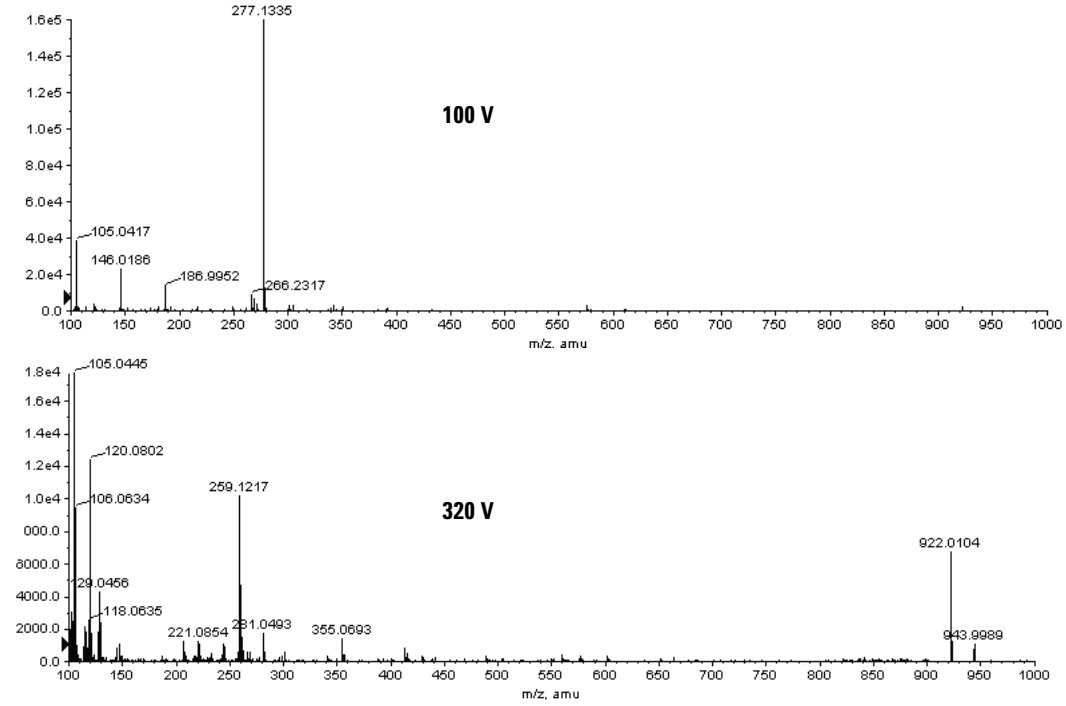
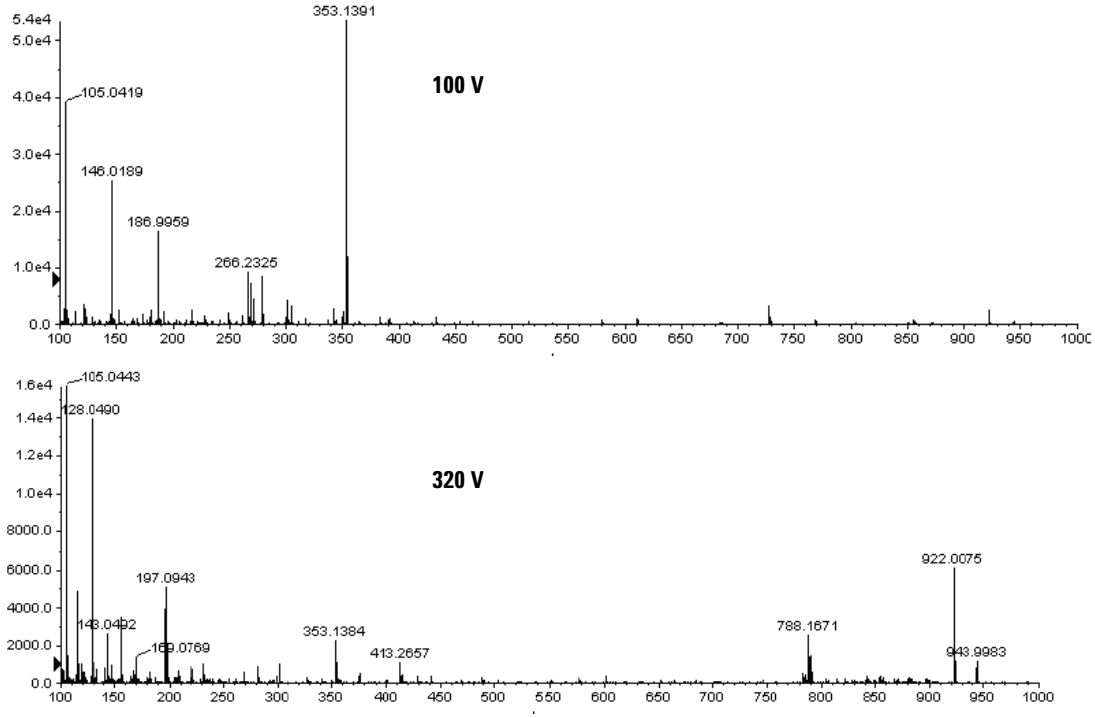


Figure 5. Sudan Red I, II, III, and IV at different in-source CID voltages.

Sudan Red III at different voltages



Sudan Red IV at different voltages

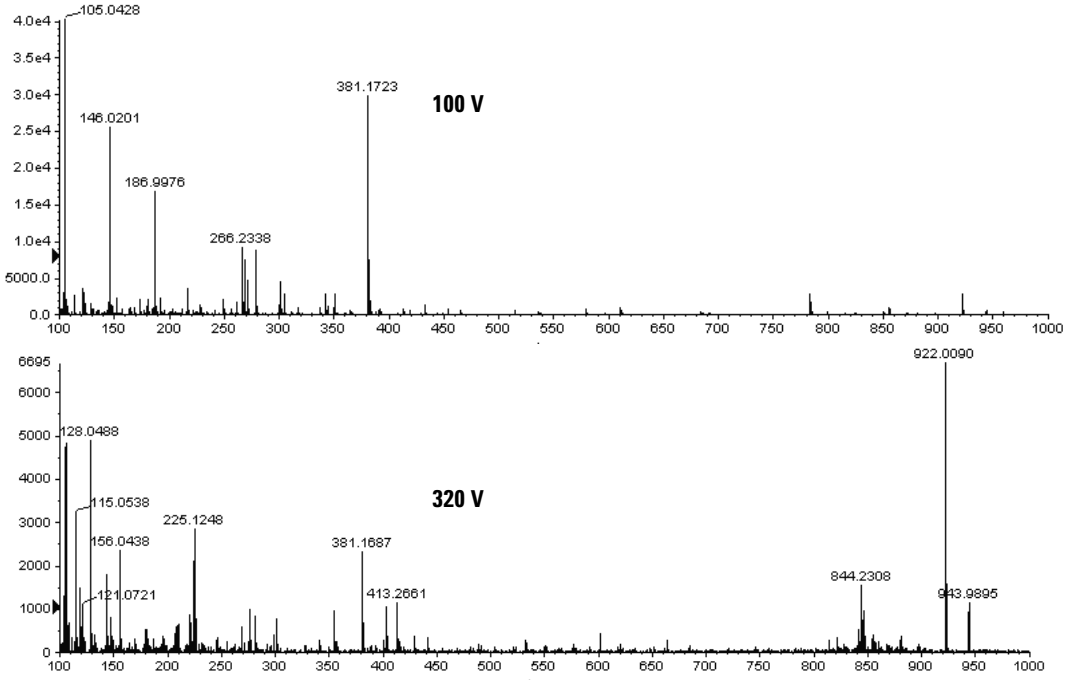


Figure 5. Sudan Red I, II, III, and IV at different in-source CID voltages (continued).

TOF Linearity

The components analyzed by TOF were tested for linearity as part of this study. Figure 6 shows the linearity of these four compounds. The regression values, r^2, of over 0.99 were seen for each of these compounds.

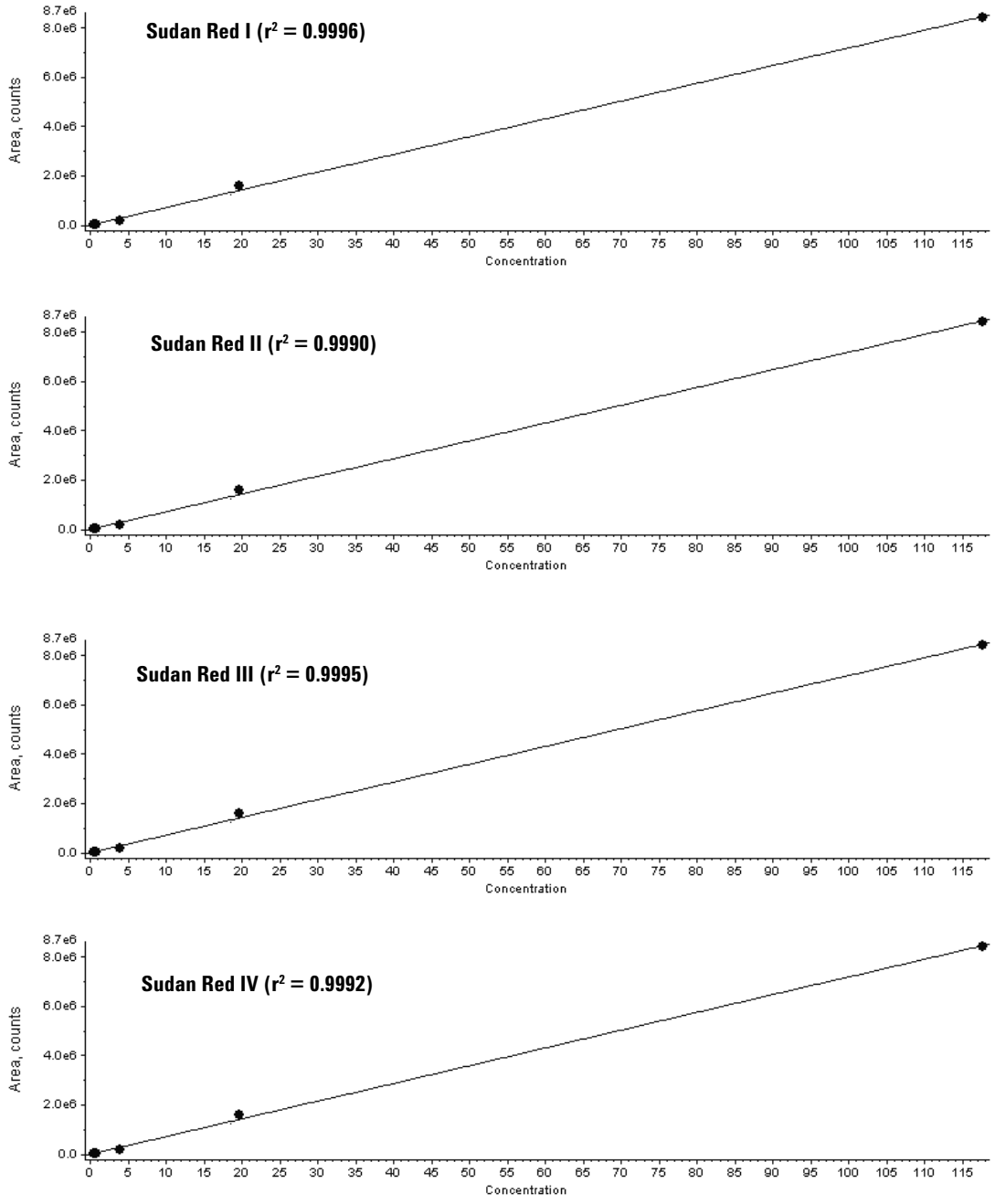


Figure 6. Linearity of the four Sudan Red dyes.

LOD and LOQ

Table 2 summarizes the limits of detection (LODs) and limits of quantitation (LOQs) for each of the components analyzed by this method. As shown in these results, this method is designed to provide a broad screening tool for the analysis of banned colorants.

Table 2. LOD and LOQ for Components by LC/MSD TOF

Component	LOD (ppb) (s/n = 3)	LOQ (ppb) (s/n = 20)
Sudan Red I	3.6	24.4
Sudan Red II	2.4	18.7
Sudan Red III	17.6	126.4
Sudan Red IV	20.4	132.2

An example of the determination for LOD and LOQ is shown for the Sudan Red I dye in Figures 7 and 8, respectively. The upper chromatogram of each figure is the TIC, while the lower chromatogram corresponds to an EIC of *m/z* 249.1022 with a window of 10 ppm. The LOD is calculated based on a signal-to-noise (S/N) value of 3. Figure 7 corresponds to an on-column injection of 4.4 ppb and has a S/N value of 3.7. Therefore, the LOD is calculated as 4.4 ppb * (3/3.7) = 3.6 ppb, which is listed in Table 2. Figure 8 corresponds to an on-column injection of 33.7 ppb. The LOQ is calculated based on a S/N value of 20. Therefore, the LOQ is calculated as 33.7 ppb * (20/27.6) = 24.4 ppb, which is also shown in Table 2. Similar reasoning is used for the other dyes.

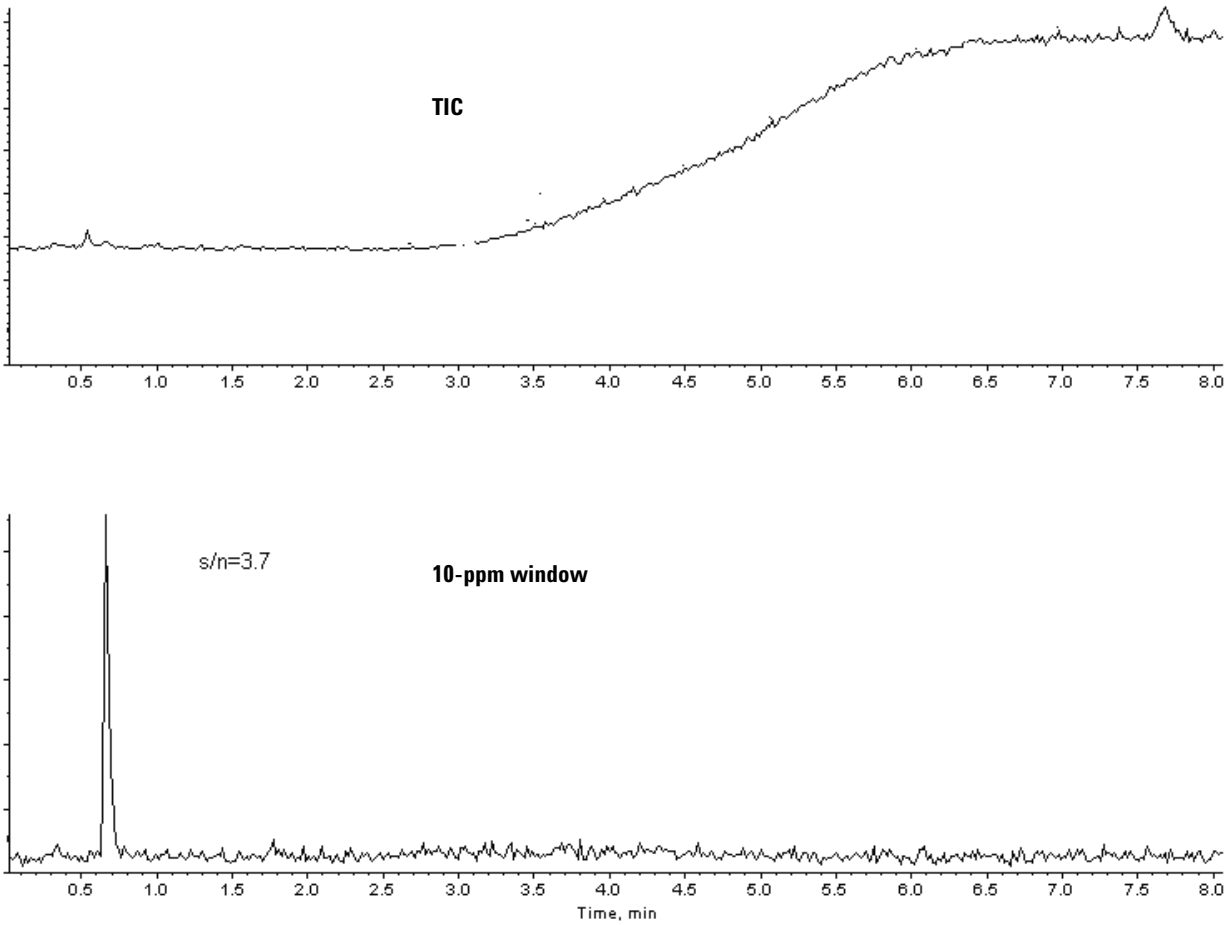


Figure 7. LOD of the Sudan Red I.

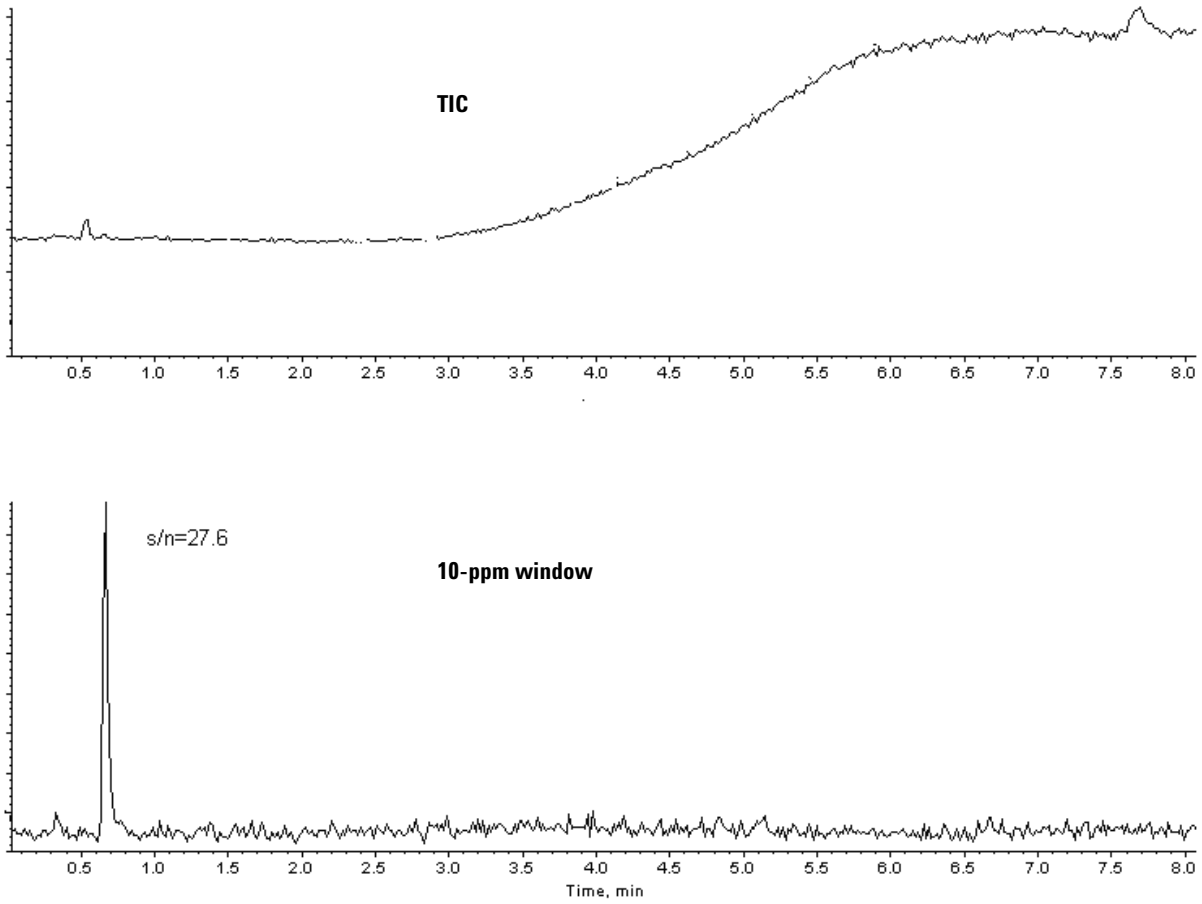


Figure 8. LOQ of the Sudan Red I.

Analysis of Sample

The instrument and software provides the user with the ability to create a database for all components they wish to automatically screen for. The minimum requirement for this database is the empirical formula and name for the component of interest, although the inclusion of a retention time will assist with confidence in the confirmation and reduce analysis time.

Sample Preparation

The sample analyzed in this work is Indian curry powder, but it should be noted that similar results were found in the analysis of Indian, Chinese, and South Korea chili powders. About 5 g of the Indian chili powder is weighed to within 10 mg in a ground Erlenmeyer flask with 100 mL of acetonitrile added using a graduated test tube. Sonicate for 1 hour and filter on filter paper in a ground Erlenmeyer flask. This method is identical to the one recommended by the EU.

Screening and Recovery Results

The prepared sample was spiked with 0.4 g/mL Sudan Red solution. The TIC for this sample is shown in Figure 9. However, also shown is the EIC of the sum of the four *m/z* values for each of the Sudan Red dyes.

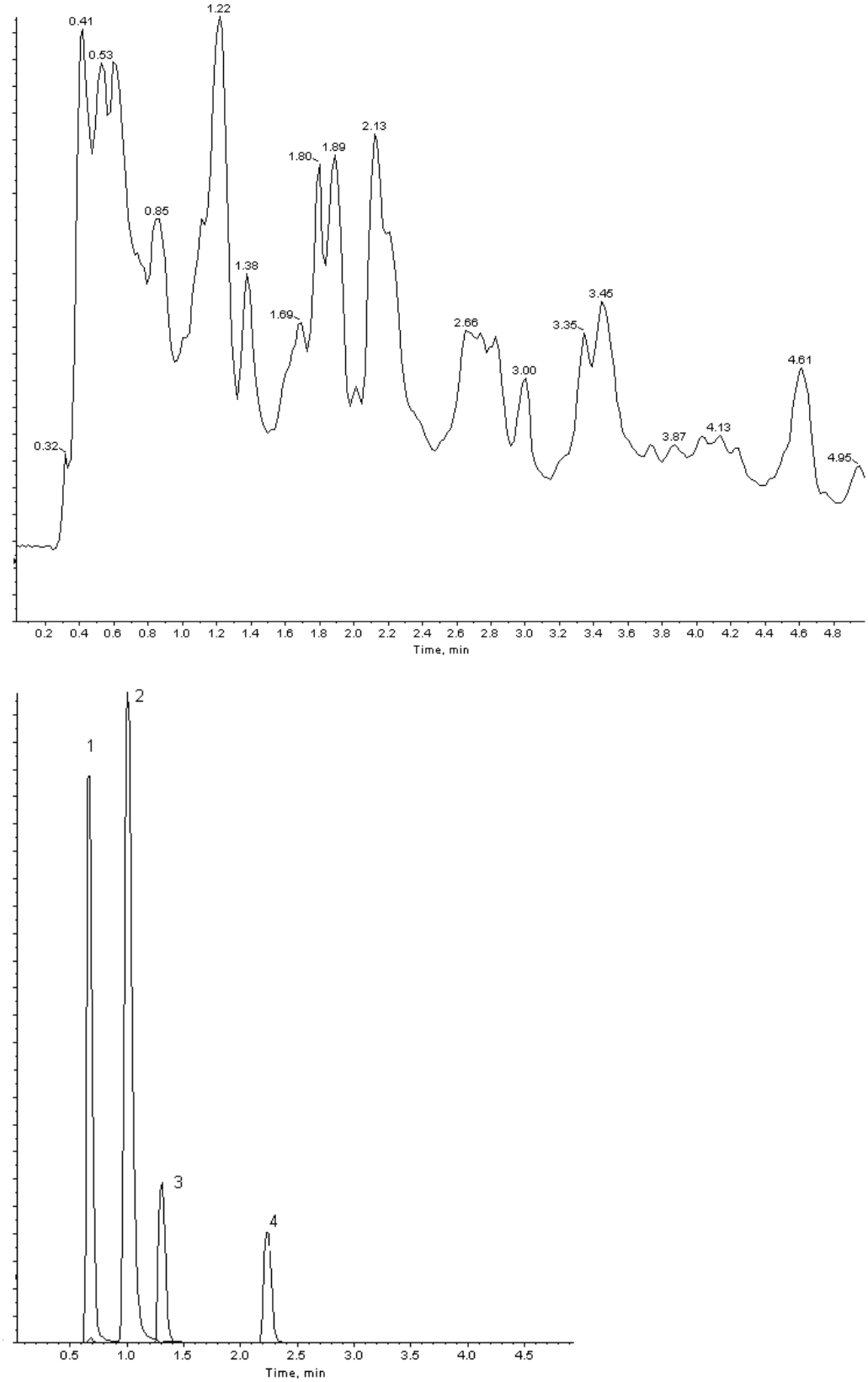


Figure 9. Real sample spectrum (upper); EIC of the 4-Sudan Red colorants (lower).

Table 3 illustrates the excellent quantitation accuracy of the spiked levels as compared to what is expected from the calibration curves. The levels of 0.4 and 0.2 ppm are below the 0.5-ppm cutoffs regulated by the EU but are well quantitated at the 0.4-ppm level with accuracies all close to 100%. At both levels the %RSDs of peak areas are still below 10%.

Table 3. Method Accuracy and Reproducibility for the Four Target Colorants Spiked in Indian Chili

Formula	Compound name	Mass	Peak RT (min)	Peak area	CAS number
$C_{16}H_{12}N_2O$	Sudan Red I	248.09496	0.65	6.6932E5	842-07-9
Species	**Abundance (cts)**	**Ion mass**	**Measured mass**	**Error (mDa)**	**Error (ppm)**
$[M+H]^+$	2.5354 e5	249.10224	249.10249	0.26	1.0

Formula	Compound name	Mass	Peak RT (min)	Peak area	CAS number
$C_{18}H_{16}N_2O$	Sudan Red II	276.12626	1.01	8.8136E5	3118-97-6
Species	**Abundance (cts)**	**Ion mass**	**Measured mass**	**Error (mDa)**	**Error (ppm)**
$[M+H]^+$	2.9543E5	277.13354	277.13372	0.18	0.7

Formula	Compound name	Mass	Peak RT (min)	Peak area	CAS number
$C_{22}H_{16}N_4O$	Sudan Red III	352.13176	1.30	3.2776E5	85-86-9
Species	**Abundance (cts)**	**Ion mass**	**Measured mass**	**Error (mDa)**	**Error (ppm)**
$[M+H]^+$	8.5544E4	353.13904	353.13931	0.27	0.7646

Formula	Compound name	Mass	Peak RT (min)	Peak area	CAS number
$C_{24}H_{20}N_4O$	Sudan Red IV	380.44821	2.24	2.7679E5	85-83-6
Species	**Abundance (cts)**	**Ion mass**	**Measured mass**	**Error (mDa)**	**Error (ppm)**
$[M+H]^+$	7.9608E4	381.17095	381.17098	0.03	0.0787

Reproducibility

	Sudan Red I		Sudan Red II		Sudan Red III		Sudan Red IV	
Standard (ppm)	**RSD (%)**	**Accuracy (%, avg)**	**RSD (%)**	**Accuracy (%, avg)**	**RSD (%)**	**Accuracy (%, avg)**	**RSD (%)**	**Accuracy (%, avg)**
0.2	6.04	97.31	5.76	97.61	7.11	82.3	8.87	87.4
0.4	6.98	101.95	5.64	100.27	8.04	103.12	8.26	104.32
0.8	4.61	104.75	6.12	103.78	8.37	96.23	8.43	102.72
1.6	5.17	102.53	5.99	105.92	7.56	92.79	7.73	95.78
2	6.12	96.77	4.74	94.72	7.31	101.27	6.20	100.79

Conclusions

An overwhelming advantage of using TOF MS for the trace-level detection of any component is the confirmation that is provided through accurate mass. The data shown here demonstrates the ability of LC/MSD TOF to confirm with accurate mass measurement, and quantitate with selective, narrow mass window. High-mass resolution and accuracy (in every spectrum) provides the selectivity that reduces chemical noise for quantitation and confirmation. Also, this method can easily include new components such as other banned or limited use dyes. Furthermore, this application shows the capability of the Agilent LC/MSD TOF as a single tool for both screening and confirmatory analysis, with quantitative information, often at levels below those currently analyzed for today. Using the TOF can save more than 50% the time required as compared to many other methods used today. It provides a strong tool for the large quantity samples for screening.

References

1. ASTA White paper on Sudan and Related Dyes, a publication of the American Spice Trade Association (http://www.astaspice.org/pubs/ASTAWhitePaper.cfm), copyright 2005
2. Solid-phase extraction procedure adapted from People's Republic of China approved national standards method of analysis for sudan dyes, 2005, Standardization Administration of China (http://www.sac.gov.cn/english/home.asp), Standard Number GB/T 19681-2995.

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Separation of Paraben Preservatives by Reversed-Phase HPLC

Application

Foods, Beverages, and Cosmetics

Authors

Coral Barbas and Javier Rupérez
Facultad de CC Experimentales y de la Salud, Universidad San Pablo-CEU
Urbanización Montepríncipe
Boadilla del Monte, 28668 Madrid
Spain

Andre Dams*
Agilent Technologies, Inc.
Amstelveen
The Netherlands

Ronald E. Majors
Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE 19808-1610
USA

*Present address:
Andre Dams
Analytical Consultancy, Amstelveen
The Netherlands

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.

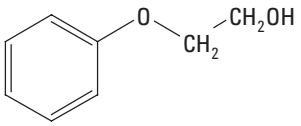
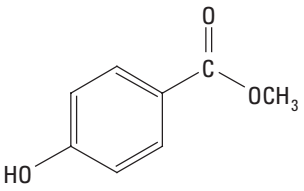
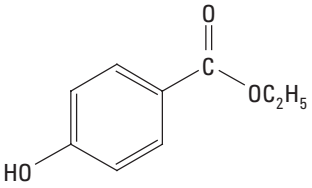
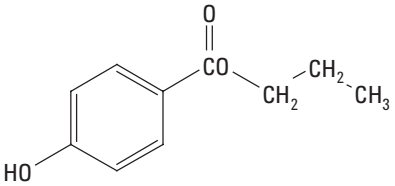
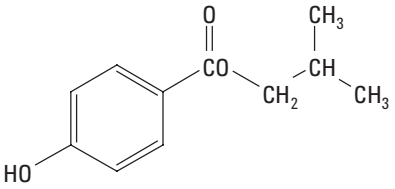
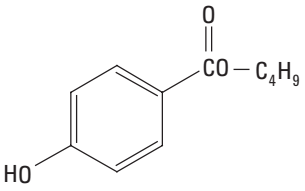
Researchers of the Department of Biology and Biochemistry of Brunel University in the United Kingdom found that the greatest concern regarding parabens focuses on their estrogen-mimicking ability in laboratory animals. In addition, 2-phenoxyethanol (2PX), a chemical substance also used as a preservative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

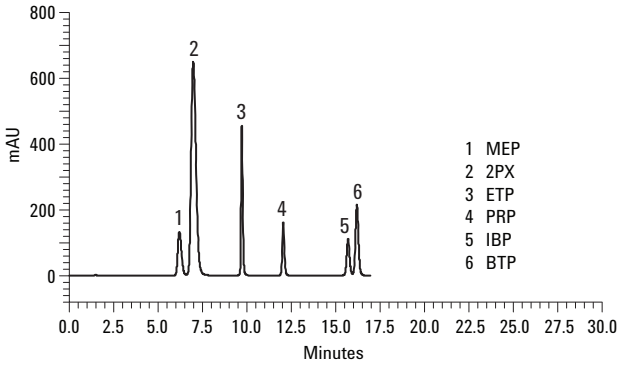


Figure 1. Separation of preservatives by reversed-phase HPLC.

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.

References

1. Robert Ricker, "High-Speed Separation of Parabens", Agilent Technologies, publication 5988-6356EN www agilent.com/chem.
2. M. Borremans, J. van-Loco, P. Roos, and L. Goeyens, (2004) *Chromatographia*., **59**(1–2), 47–53.
3. E. Marengo, M.C. Gennaro, and V. Gianotti, (2001) *J. Chromatogr. Sci.*; **39**(8), 339-344.
4. J. E. Koundourellis, E. T. Malliou, and T. A. Broussali, (2000) *J. Pharm. Biomed. Anal.,* **23**(2–3), 469–475.

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Agilent Technologies

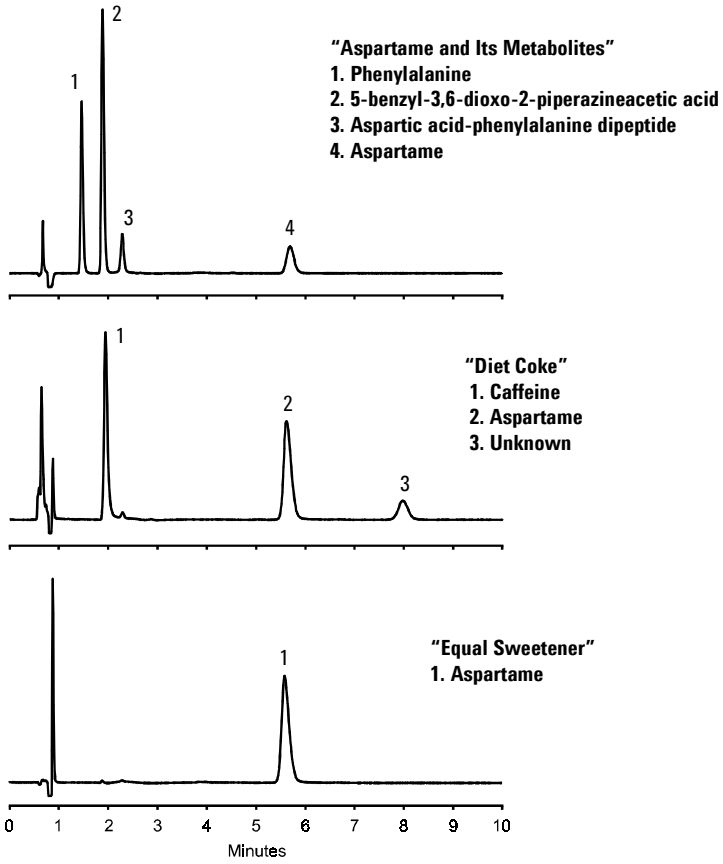
Aspartame: Metabolites and Applications

Application

Food Analysis

Robert Ricker

Aspartame is of interest to the food industry as an artificial sweetener in diet drinks and "light" foodstuffs. It is also used as a sugar substitute and does not have the bitter aftertaste of saccharin and cyclamates. Speedy analysis is of particular interest to quality control labs which perform routine testing for aspartame and its metabolites in dietary food products.



Conditions:
ZORBAX SB-C18 (4.6 x 75 mm, 3.5 µm) (Agilent P/N: 866953-902)
Mobile Phase: 85:15, 0.1% TFA:ACN
1 mL/min, 35°C, Detect. UV (210 nm)

Highlights

- *Rapid analysis (while maintaining resolution of aspartame and its metabolites) is achieved with shorter columns (75 mm) and smaller particle-size (3.5 µm) packings.*
- *Sample preparation is minimal for liquid samples which can be diluted and injected directly onto the column. Solid samples are extracted with water and then injected onto the column.*

Agilent Technologies

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

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Agilent Technologies

FD&C Colors

Application

Food Analysis

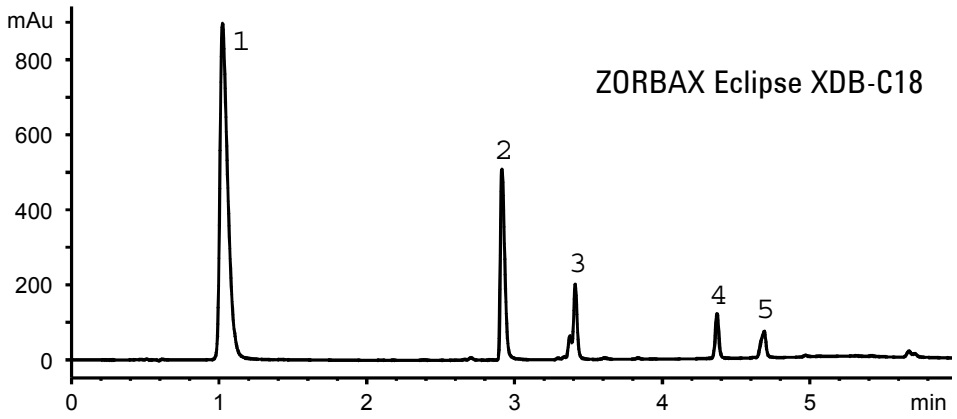
Robert Ricker

FD&C colorants are commonly used in foods and cosmetics. Propylparaben is a preservative found in the mixture of food colorings used for this analysis. Green food coloring is comprised of yellow # 5 and blue # 1. A combination of ion-pairing and gradient HPLC is used because of the wide chemical differences of compounds. Note the molecular formulas.

Operating Conditions:

HPLC System:	Agilent 1100 with quaternary pump
Column:	ZORBAX Eclipse XDB-C18 Rapid-Resolution (3.5 µm), 4.6 x 50 mm Agilent Part No. 935967-902
Mobile Phase:	A: 0.1% TFA, pH to 4.4 with TEA, B: MeOH
Gradient:	17 to 100% B / 4 min
Detection:	UV 254 nm
Flow:	1 mL/ min.
Temperature:	ambient

1. Yellow # 5	$C_{16}H_9N_4Na_3O_9S_2$	MW= 534
2. Red # 40	$C_{18}H_{14}N_2Na_2O_8S_2$	MW= 496
3. Blue # 1	$C_{37}H_{34}N_2Na_2O_9S_3$	MW= 760
4. Propylparaben	$C_{10}H_{12}O_3$	MW= 180
5. Red # 3	$C_{20}H_4I_4Na_2O_5$	MW= 878



Highlights

- *ZORBAX Eclipse-XDB is designed for extended column life at intermediate pH.*
- *Reducing column length and particle size simultaneously can:*
  - *Reduce analysis time*
  - *Maintain resolution*
  - *Reduce solvent use*

Agilent Technologies

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

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Agilent Technologies

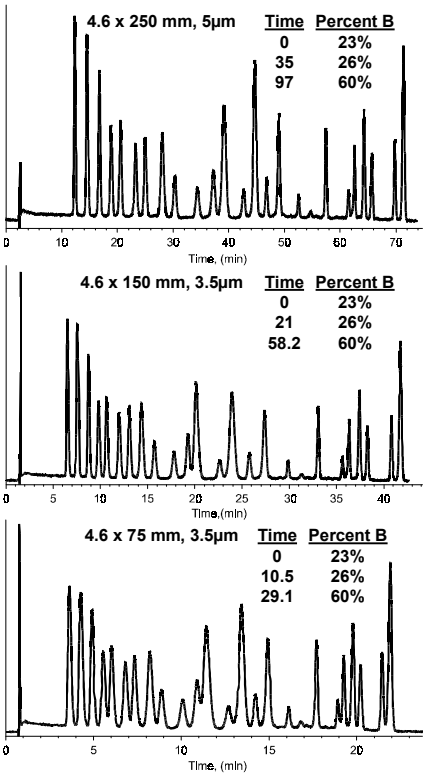
High-Efficiency and High-Speed Separation of Natural Anthocyanins

Application

Food Analysis

Robert Ricker

Anthocyanins are natural pigments responsible for the brilliant red and blue (and purple) colors found in many fruits and flowers. The colors of blueberries are the result of many different anthocyanins being present in the fruit. Qualitative and quantitative analysis of anthocyanins can be used to distinguish between different cultivars of blueberry plants and determine their quality. Therefore the chromatographic separation of anthocyanins is of increasing importance to the agricultural and wine industries. Recent interest in medicinal use of anthocyanins, as antioxidants/anticancer agents, has also stimulated interest in their chromatographic separation.



Conditions:
ZORBAX SB-C18 (4.6 x 250 mm, 5 µm; 4.6 x 150 mm, 3.5 µm; 4.6 x 75 mm; 3.5 µm)
(Agilent P/N: 880975-902, 863953-902, 866953-902, respectively)
Mobile Phase: A:3% Phosphoric acid B: 100% MeOH, Gradient: (see individual chromatogram)
Inj.: 20 µL, 1 mL/min, 30°C, Detect. UV(525 nm), Sample preparation: (see back)

Highlights

- *Traditionally, a low-pH mobile phase (containing formic acid) in these types of separations has caused degradation of the column and change in the separation (Goiffon, J.-P., Brun, M., and Bourrier, M.-J., J. Chromatogr. 537 101-121, 1991). ZORBAX StableBond SB-C18 columns provide the chromatographer with long-term stability for reverse-phase separations requiring very low pH.*

- *In these experiments, phosphoric acid has replaced the traditional formic acid in the mobile phase (Gao, L. and Mazza, G., J. Liq. Chromatogr., 18(2) 245-259, 1995), resulting in a superior separation of >25 anthocyanins on the ZORBAX SB-C18 column.*

- *As a result of reproducible products and smaller chromatographic particles (3.5µm) of narow size-distribution, the analyst can systematically change column configuration to save both time and solvent.*

Agilent Technologies

Method of Preparing Blueberry Extracts*

Begin my mixing: 10 g. Blueberries
10mL Solvent (70:28:2, MeOH:H2O, Formic acid)
Blend for 10 minutes on ice.
Filter through glass wool in a 10 mL syringe.
Allow filtrate to sit for 1 hour.
Filter through a 0.2 µm filter.
Inject 50 µL immediately for HPLC analysis.

Method of Extraction and blueberry extracts were kindly provided by Drs. Willy Kalt and Jane McDonald, Agriculture and Agri-Food Canada, Kentville, Nova Scotia, Canada

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

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application

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Innovating the HP Way

LC/MS | Natural Food Colorants

Analysis of Natural Food Colorants by Electrospray and Atmospheric Pressure Chemical Ionization LC/MS

Introduction

Many kinds of natural colors are used in beverages, jellies, and candies. In many countries, food regulations have been recently revised to cover natural colorants to the same degree as synthetic ones. Accordingly, it has become necessary to develop reliable analytical methods for various natural colorants in food. In this study, LC/MS methods using electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) were developed to identify major pigments in four natural colorants: red cabbage, paprika, Monascus, and lac.

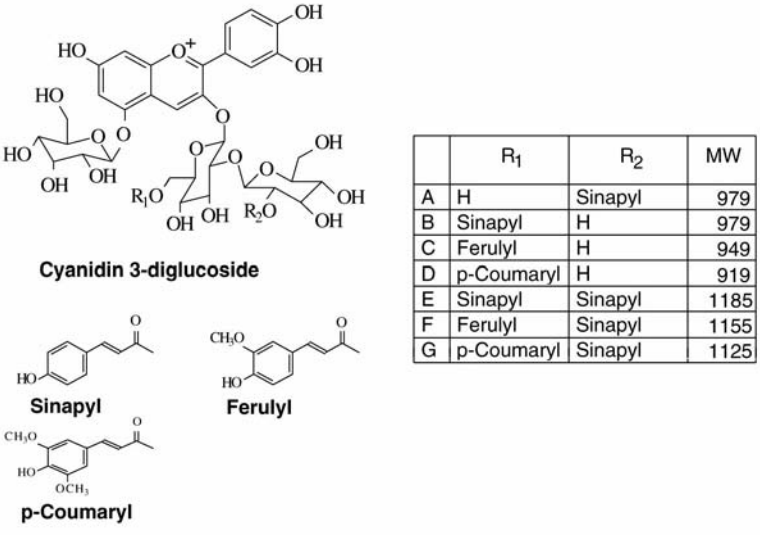
Experimental

Paprika and Monascus colorants were dissolved in acetone; the other colorants were dissolved in deionized water. Eachcolorant was filtered through a 0.2-µm filter. A 10-µl portion was injected into the system, which consisted of an Agilent 1100 Series binary pump, thermostatted column compartment, vacuum degasser, autosampler, and LC/MSD. The LC/MSD used either an ESI or APCI source. Complete system control and data handling were done on the Agilent ChemStation for LC/MS. Operating conditions were optimized for each sample.

Results and discussion

Red cabbage colorant.

Figure 1 shows the structure of seven major pigments of red cabbage. The pigments share the basic cyanidin 3-diglucoside structure with differing R1 and R2 groups. Figure 2 shows the total ion chromatogram (TIC) and extracted ion chromatograms (EIC) of red cabbage pigments. Although every major pigment can be chromatographically separated using 10% formic acid in the mobile phase, the high acid concentration reduces sensitivity. Therefore, 1% formic acid was used in this study. The EICs show the separation of the pigments based on their main ion (base peak).



	R_1	R_2	MW
A	H	Sinapyl	979
B	Sinapyl	H	979
C	Ferulyl	H	949
D	p-Coumaryl	H	919
E	Sinapyl	Sinapyl	1185
F	Ferulyl	Sinapyl	1155
G	p-Coumaryl	Sinapyl	1125

Figure 1. The structure of major pigments in red cabbage colorant.

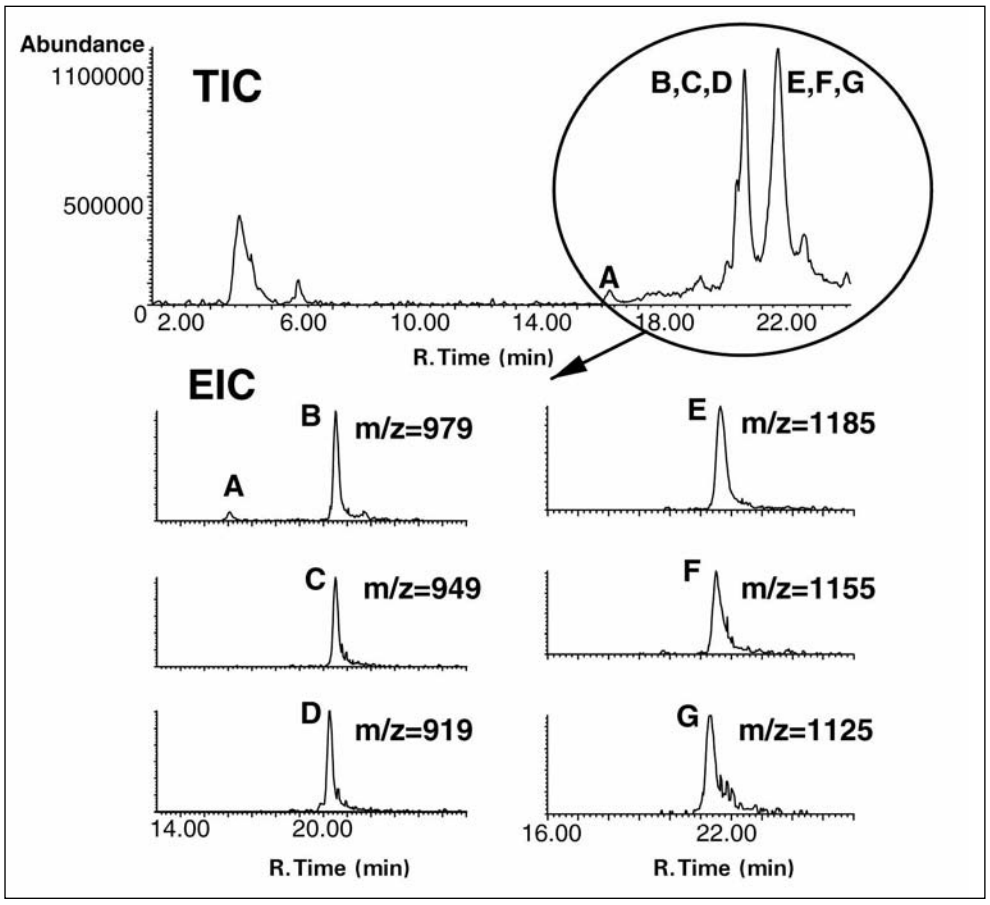


Figure 2. Total and extracted ion chromatograms of red cabbage colorant.

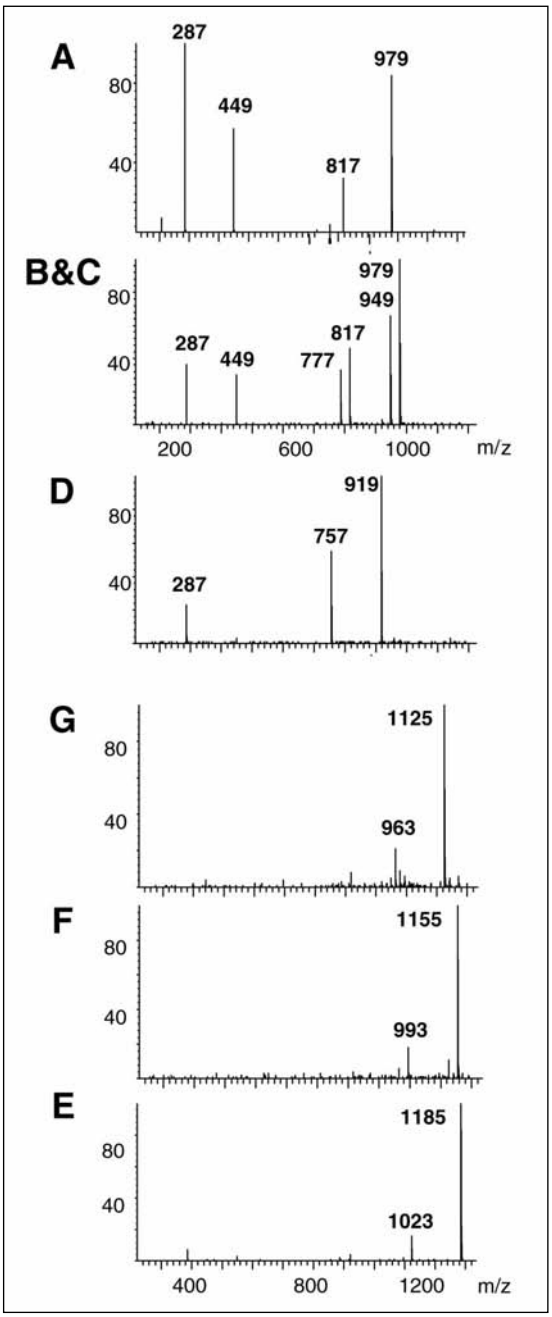


Figure 3. Mass spectra of major pigments in red cabbage colorant.

LC conditions	
Column:	250 x 2.1 mm Inertsil ODS3, 5 μm
Mobile phase:	A = 1% formic acid B = acetonitrile
Gradient:	Start with 5% B At 30 min 50% B
Flow rate:	0.2 ml/min
Column temp:	40° C
Injection vol:	10 μl
MS Conditions	
Source:	ESI
Ion mode:	positive
Vcap Voltage:	4000 V
Nebulizer:	50 psig
Drying gas flow:	10 l/min
Drying gas temp:	350° C
Corona:	4 μA
Vaporizer temp:	350° C
Scan range:	100-1200 amu
Step size:	0.1
Peak width:	0.15 min
Time filter:	on
Fragmentor:	200 V

Figure 3 shows the mass spectra of the seven major pigments in red cabbage colorant. For these pigments, the singly charged molecular ion is observed rather than the more typical $[M+H]^+$ ion, because the cyanidin group already has a positive charge on an oxygen. In-source collision-induced dissociation (CID) can be used to generate fragment ions to provide structural confirmation. Using CID, mass spectra of these pigments show common fragments corresponding to the loss of a glucose, as well as cyanidin (m/z 287) and cyanidin 3-glucoside (m/z 449) ions.

Monascus colorant

Monascus contains six major pigments; their structures are shown in Figure 4. Four pigments were identified from the mass spectra of major peaks in the TIC. See Figure 5.

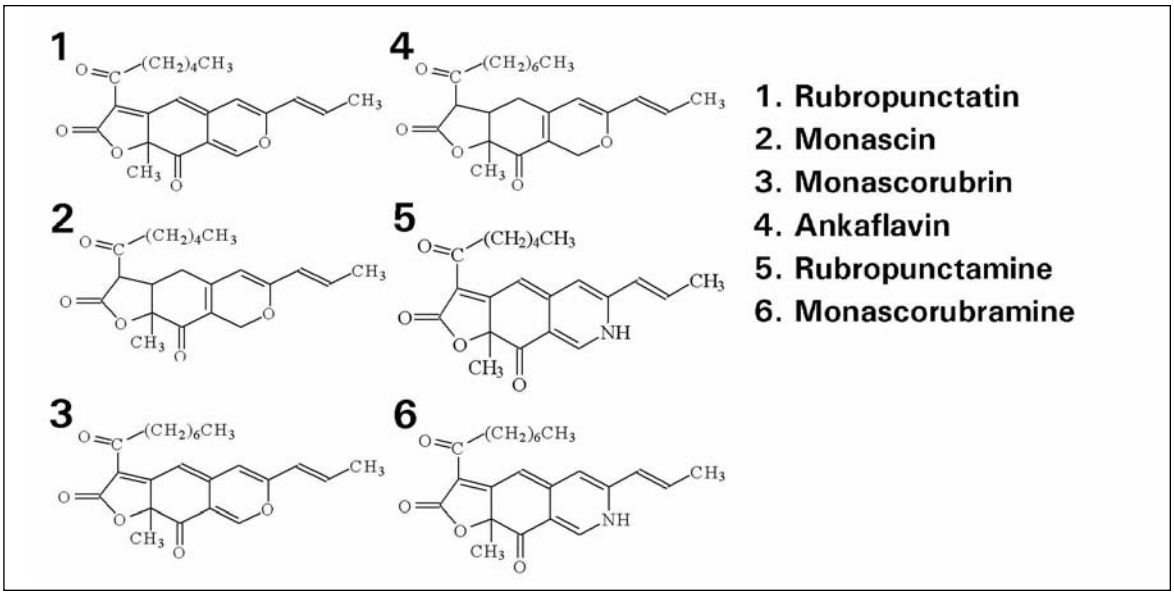


Figure 4. The major pigments of Monascus colorant.

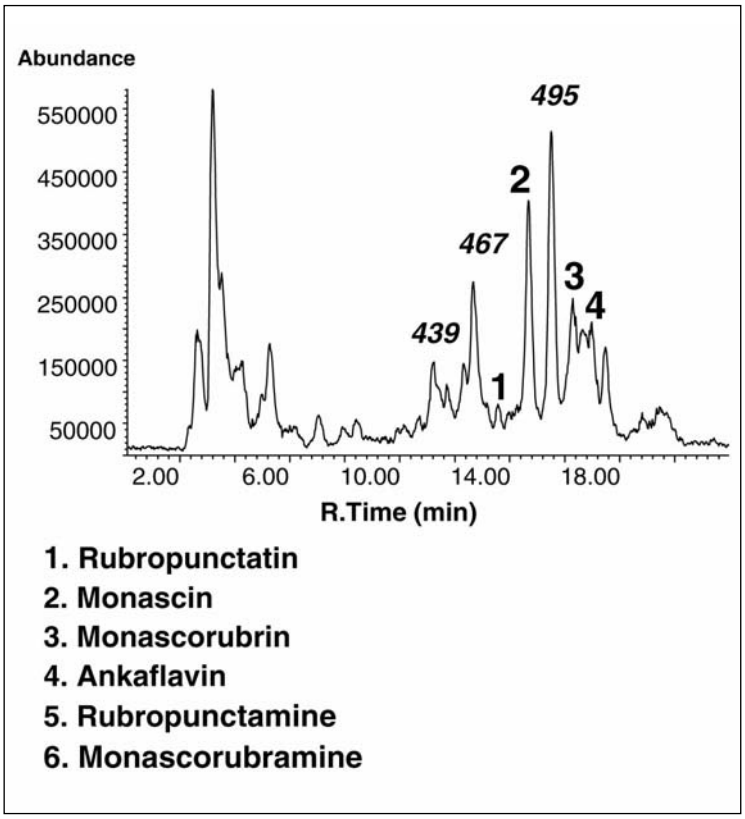


Figure 5. The total ion chromatogram of Monascus colorant.

LC conditions	
Column:	250 x 2.1 mm Inertsil ODS3, 5 μm
Mobile phase:	A = 1% formic acid B = acetonitrile
Gradient:	Start with 50% B At 10 min 90% B
Flow rate:	0.2 ml/min
Column temp:	40° C
Injection vol:	10 μl
MS Conditions	
Source:	ESI
Ion mode:	positive
Vcap voltage:	4000 V
Nebulizer:	50 psig
Drying gas flow:	10 l/min
Drying gas temp:	350° C
Corona:	4 μA
Vaporizer temp :	350° C
Scan range:	100-1200 amu
Step size:	0.1
Peak width:	0.15 min
Time filter:	on
Fragmentor:	100 V

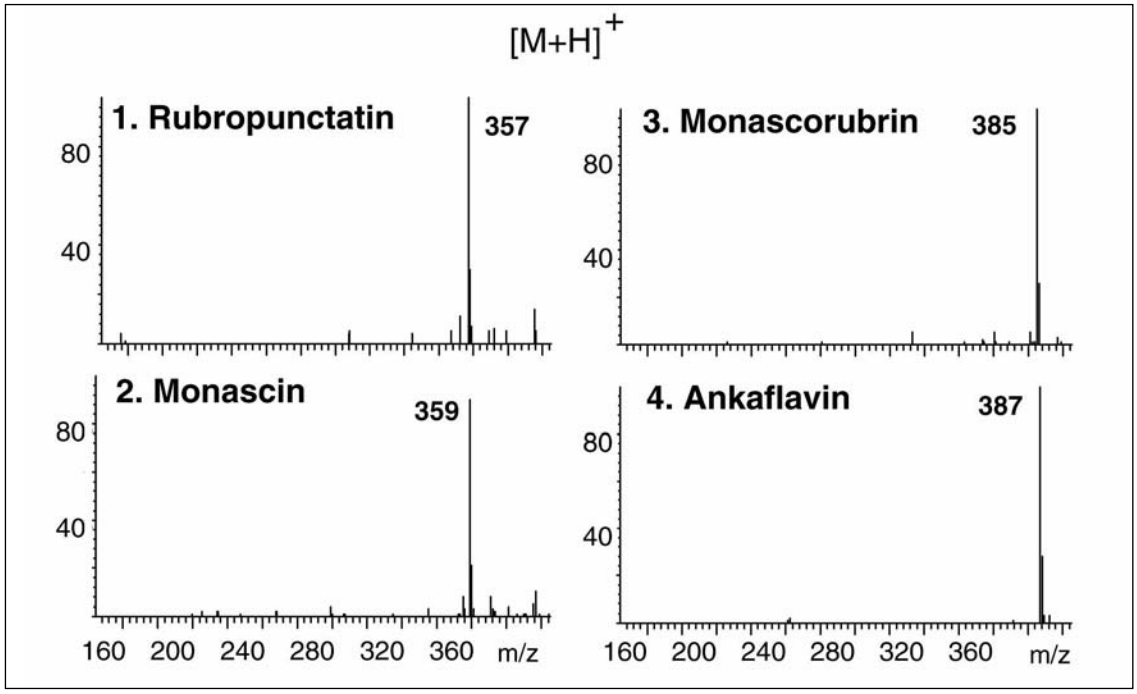


Figure 6. Mass spectra of major pigments in Monascus colorant.

Three major peaks with base peaks at m/z 439, 467, and 495 were not identified. Figure 6 shows the mass spectra of the identified pigments. Protonated molecular ions $[M+H]^+$ were observed for the four identified pigments.

Paprika color

Capsanthin and the mono- and di- esters of capsanthin with fatty acids are known as the major pigments in paprika colorant. See Figure 7. Two monoesters and five diesters of capsanthin were identified in the paprika colorant analyzed in this study. See Figure 8.

The protonated molecular ions $[M+H]^+$ were observed for every major pigment. See Figure 9. However, with the exception of capsanthin monoeicosanoate, the intensity of these ions was very low. Except for capsanthin monoeicosanoate, the pigments show fragment ions resulting from the loss of one or two fatty acid fragments. A common fragment ion was observed at m/z 567 in the mass spectra of these pigments.

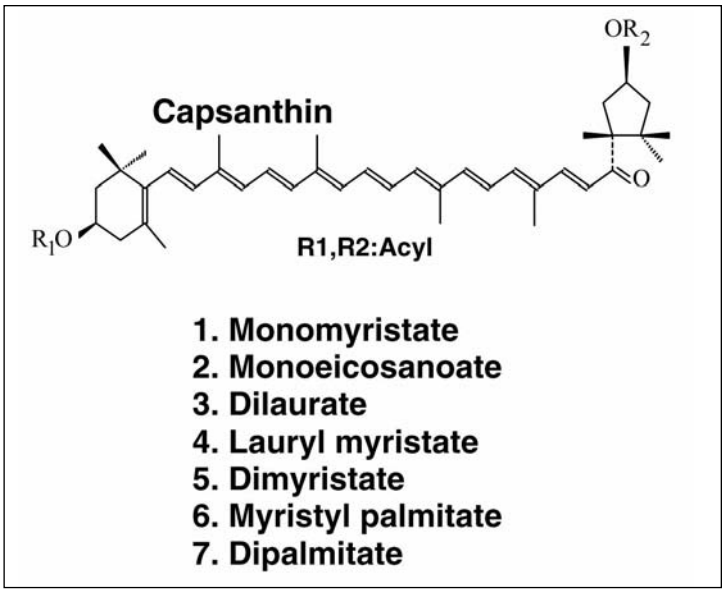


Figure 7. The structure of major pigments of paprika colorant.

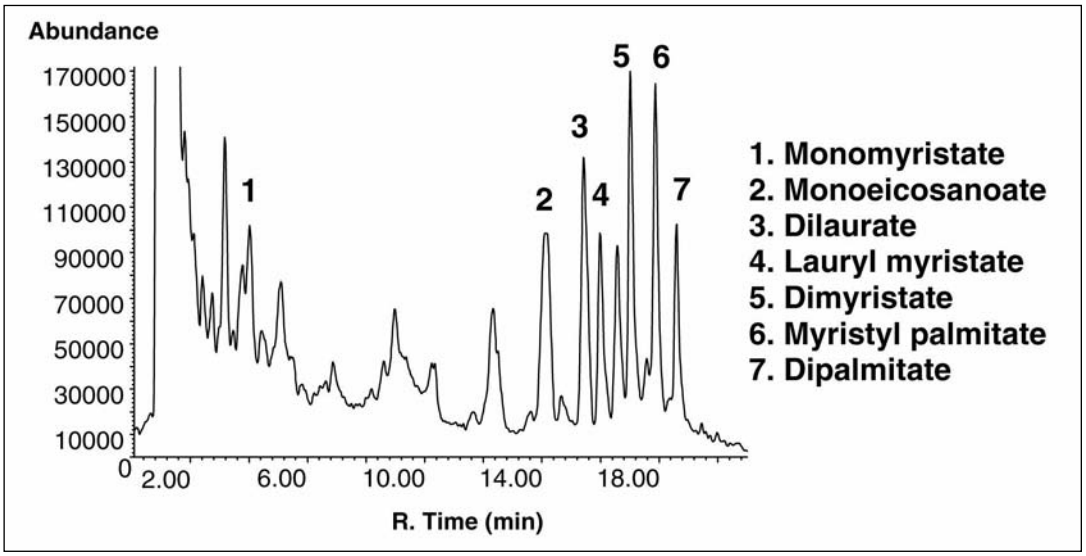


Figure 8. The total ion chromatogram of paprika colorant.

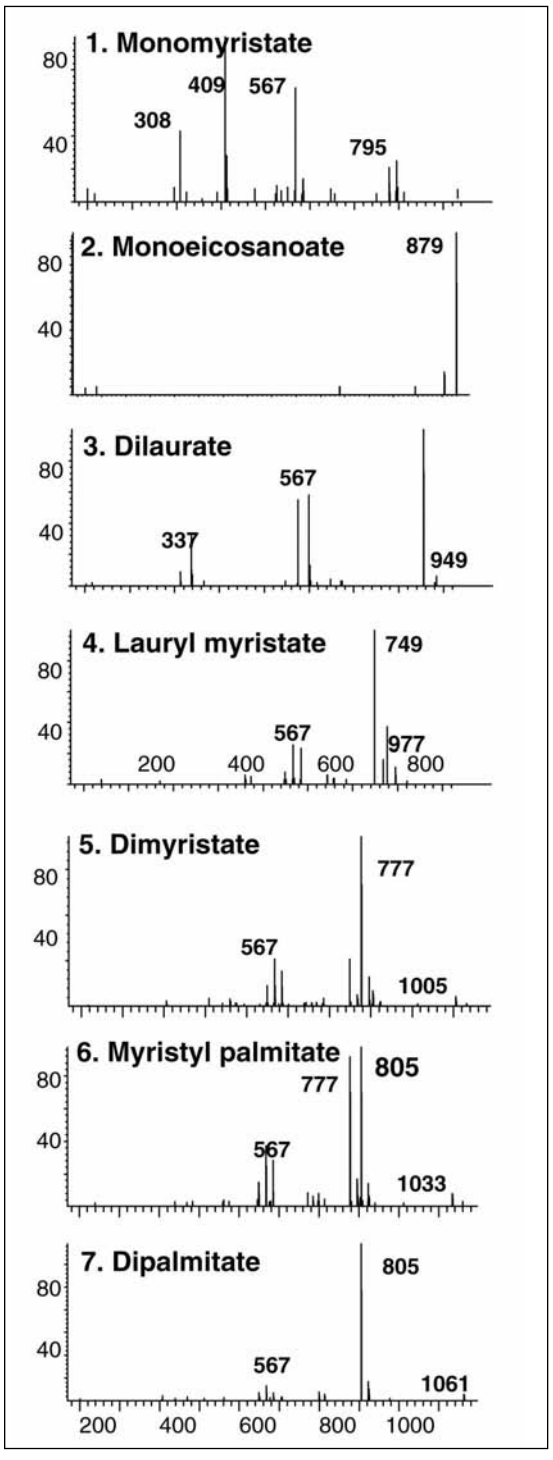


Figure 9. Mass spectra of major pigments in paprika colorant.

LC conditions	
Column:	
	250 x 2.1 mm Inertsil ODS3, 5 μm
Mobile phase:	A = acetone B = methanol
Gradient:	Start with 10% B At 10 min 90% B
Flow rate:	0.2 ml/min
Column Temp:	40° C
Injection vol:	10 μl
MS Conditions	
Source:	APCI
Ion mode:	Positive
Vcap voltage:	4000 V
Nebulizer:	50 psig
Drying gas flow:	5 l/min
Drying gas temp:	350° C
Corona:	4 μA
Vaporizer temp:	350° C
Scan range:	100-1200 amu
Step size:	0.1
Peak width:	0.15 min
Time filter:	On

Lac colorant

Figure 10 shows the structure of the major pigments in lac colorant. Laccaic acids A, B, C are known as the major pigments in lac colorant. These compounds have the same basic anthraquinone structure but with different R groups. Three major peaks were detected in the TIC. See Figure 11. Although laccaic acids A, B, and C were identified, A and B could not be separated.

Figure 12 shows the mass spectra of two peaks, laccaic acid C and a combination of laccaic acids A and B. The deprotonated molecular ions were observed at m/z 495, 536, and 538. Fragment ions resulting from the loss of carbon dioxide were observed at m/z 451, 492, and 494.

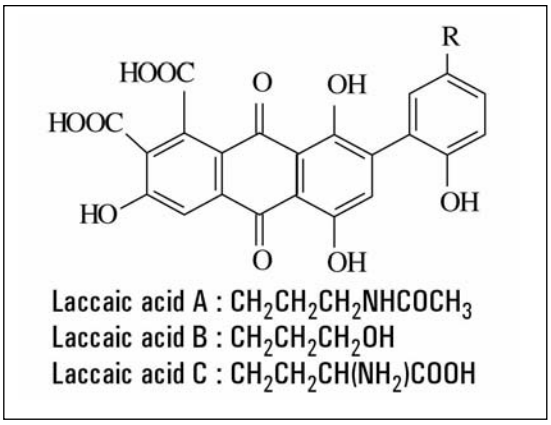


Figure 10. The strucuture of major pigments of lac colorant.

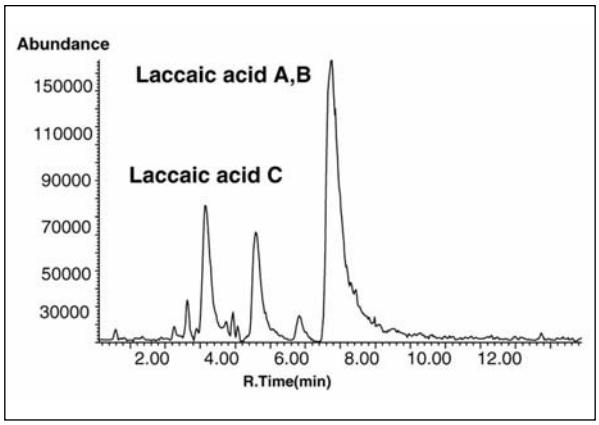


Figure 11. The total ion chromatogram of lac colorant.

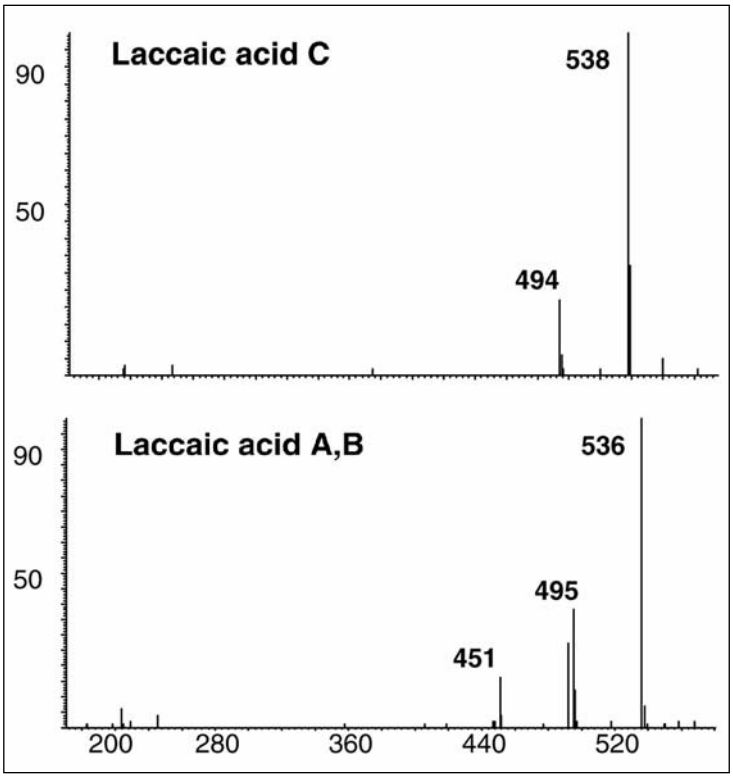


Figure 12. Mass spectra of the major pigments in lac colorant.

LC conditions	
Column:	250 x 2.1 mm Inertsil ODS3, 5 μm
Mobile phase:	30% acetonitrile in 5 mM dibutylamine, isocratic
Flow rate:	0.2 ml/min
Column Temp:	40° C
Injection vol:	10 μl
MS Conditions	
Source:	ESI
Ion mode:	Negative
Vcap voltage:	4000 V
Nebulizer:	50 psig
Drying gas flow:	10 l/min
Drying gas temp:	350° C
Scan range:	100-1200 amu
Step size:	0.1
Peak width:	0.15 min
Time filter:	On
Fragmentor:	100 V

Conclusion

Four commercial natural colorants were analyzed using ESI and APCI-LC/MS. The MS data provided molecular weight information and some structural information for the major pigments.

Masahiko Takino is an applications chemist at Yokogawa Analytical Systems, Inc.

**Printed in the U.S.A. April 2000
(23) 5968-2979E**

Analysis of Acidulants in White Wine using HPLC

Angelika Gratzfeld-Huesgen

Food

Abstract

Sorbic acid and citric acids are commonly used as acidulants[1] and/or as preservatives. Acetic, propionic, succinic, adipic, lactic, fumaric, malic, tartaric, and phosphoric acids can serve as acidulants as well. Acidulants are used for various purposes in modern food processing. For example, citric acid adds a fresh, acidic flavor, whereas succinic acid gives food a more salty, bitter taste. In addition to rendering foods more palatable and stimulating, acidulants act as

- flavoring agents to intensify certain tastes and mask undesirable aftertastes
- buffering agents to control the pH during food processing and of the finished products
- preservatives to prevent growth of microorganisms
- synergists to antioxidants to prevent rancidity and browning
- viscosity modifiers in baked goods
- melting modifiers in cheese spreads and hard candy
- meat curing agents to enhance color and flavor

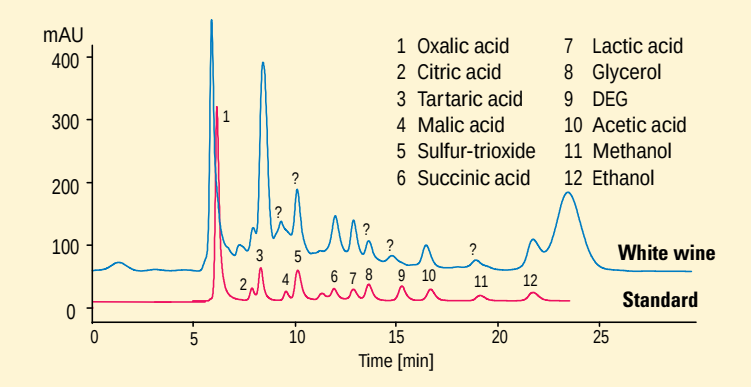


Figure 1
Analysis of acidulants in white wine

Conditions

Column
300 ˜ 7.8 mm BioRad HPX 87-H, 9 µm
Mobile phase
0.0035M H_2SO_4 isocratic
Flow rate 0.6 ml/min
Column compartment 65 °C
Injection vol 10 µl
Detector
UV-VWD detection wavelength
192 nm or 210 nm

Sample preparation
Filtration

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Sample preparation

Sample preparation depends strongly on the matrix to be analyzed, but in general steam distillation and solid-phase extraction techniques can be used.

Chromatographic conditions

High-performance liquid chromatography (HPLC) with UV-visible diode-array detection (UV-DAD) has been applied in the analysis of citric acid in wine and in a vodka mixed drink. Retention time and spectral data were used as identification tools.

HPLC method performance

Limit of detection
100ng injected amount,
S/N = 2 equivalent to 2 ppm
with 50 µl injected volume

Repeatability of
RT over 10 runs <0.1%
areas over 10 runs <3 %

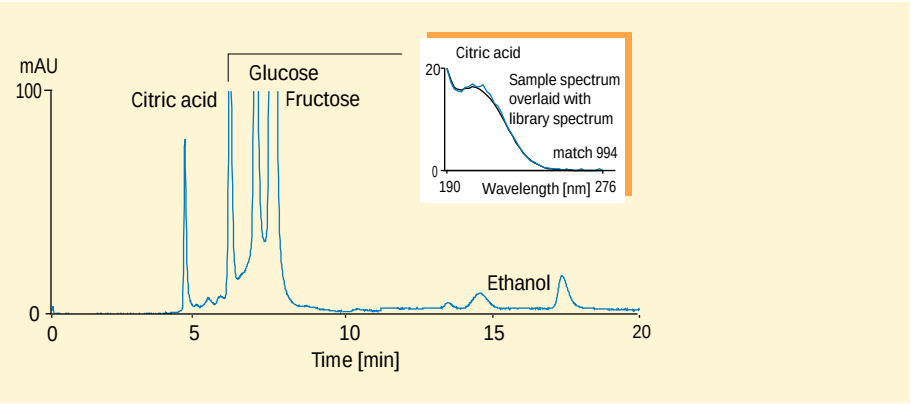

Figure 2
Analysis of citric acid in vodka

References

1.
Official Methods of Analysis, Food Compositions; Additives, Natural Contaminants, 15th ed; AOAC: Arlington, VA, 1990, Vol. 2.; Official Method AOAC 986.13: quinic, malic, citric acid in cranberry juice cocktail and apple juice.

Conditions

Sample preparation filtration
Column
300 ˘ 7.8 mm BioRad HPX 87-H, 9 µm
Mobile phase
0.007M H_2SO_4 isocratic
Flow rate
0.6 ml/min
Column compartment
65 °C
Injection vol
10 µl
Detector
UV-DAD

Equipment

Agilent 1100 Series
- degasser
- isocratic pump
- autosampler
- thermostatted column compartment
- diode array detector, variable wavelength detector or refractive index

Agilent ChemStation + software

Angelika Gratzfeld-Huesgen is application chemist at Agilent Technologies, Waldbronn, Germany.

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Agilent Technologies
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Analysis of Antioxidants in Chewing Gum using HPLC

Angelika Gratzfeld-Huesgen

Food

Abstract

The following compounds are used as antioxidants in food products:[1]

Natural antioxidants:
- vitamin C
- vitamin E

Synthetic antioxidants:
- BHT butylated hydroxytoluene
- BHA butylated hydroxyanisole
- TBHQ mono-tert-butylhy-droquinone
- THBP 2,4,5-trihydroxybuty-rophenone
- PG propyl gallate
- OG octyl gallate
- DG dodecyl gallate
- Ionox-100 4-hydroxymethyl-2,6-di(tert-butyl) phenol
- NDGA nordihydroguaiaretic acid
- TDPA 3,3'-thiodipropionic acid
- ACP ascorbyl-palmitate

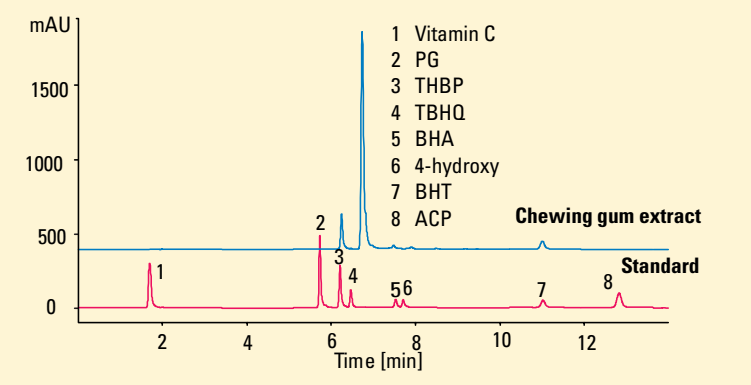

Figure 1
Analysis of antioxidants in chewing gum

Conditions

Column 100 × 4 mm BDS, 3 µm
Mobile phase
A = water + 0.2 ml H_2SO_4, pH = 2.54
B = ACN
Gradient
start with 10% B; at 3 min 60% B
at 4 min 80% B; at 11 min 90% B
Flow rate 0.5 ml/min
Post time 4 min
Column compartment 30 °C
Injection vol 5 µl
Detector
UV-DAD detection wavelength 260/40 nm, reference wavelength 600/100 nm

Sample preparation
Ultrasonic liquid extraction with acetonitrile (ACN)

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Antioxidants may be naturally present in food, or they may be formed by processes such as smoking. Examples of natural antioxidants include tocopherols (vitamin E) and acsorbic acid (vitamin C). A second category of antioxidants comprises the wholly synthetic antioxidants. When these antioxidants are added to foodstuffs, they retard the onset of rancidity by preventing the oxidative degradation of lipids. In most countries where antioxidants are permitted either singly or as combinations in foodstuffs, maximum levels for these compounds have been set.[2]

Sample preparation

Sample preparation depends strongly on the matrix to be analyzed. For samples low in fat, liquid extraction with ultrasonic bath stimulation can be used. For samples with more complex matrices, solid-phase extraction, liquid/liquid extraction, or steam distillation may be necessary.

Chromatographic conditions

HPLC and UV-visible diode-array detection have been applied in the analysis of antioxidants in chewing gum. Spectral information and retention times were used for identification.

HPLC method performance

Limit of detection
0.1–2 ng (injected amount),
S/N = 2

Repeatability of
RT over 10 runs <0.2 %
areas over 10 runs <1 %

References

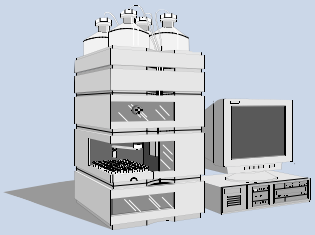
1.
Official Methods of Analysis, Food Compositions; Additives, Natural Contaminants, 15th ed; AOAC: Arlington, VA, **1990**, Vol. 2.; AOAC Official Method 983.15: Antioxidants in oils and fats.

2.
M. Rothaupt, "Food Analysis, Introduction and Applications", *Agilent Technologies Publication Number* 5963-2317E, **1994**.

Equipment

Agilent 1100 Series
- vacuum degasser
- quaternary pump
- autosampler
- thermostatted column compartment
- diode array detector,

Agilent ChemStation + software



Angelika Gratzfeld-Huesgen is application chemist at Agilent Technologies, Waldbronn, Germany.

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Analysis of aspartame using HPLC

Rainer Schuster

Food

Abstract

The following compounds are used as artificial sweeteners in food products:
• acesulfam • aspartame • saccharin[1]
Nowadays, low-calorie sweeteners are widely used in foods and soft drinks. Investigations of the toxicity of these compounds have raised questions as to whether they are safe to consume. As a result, their concentration in foods and beverages is regulated through legislation in order to prevent excessive intake.

Sample preparation

Sample preparation depends strongly on the matrix to be analyzed. For sample low in fat, liquid extraction at low pH with ultrasonic bath stimulation can be used. For samples with more complex matrices, solid-phase extraction, liquid/liquid extraction, or steam distillation may be necessary.

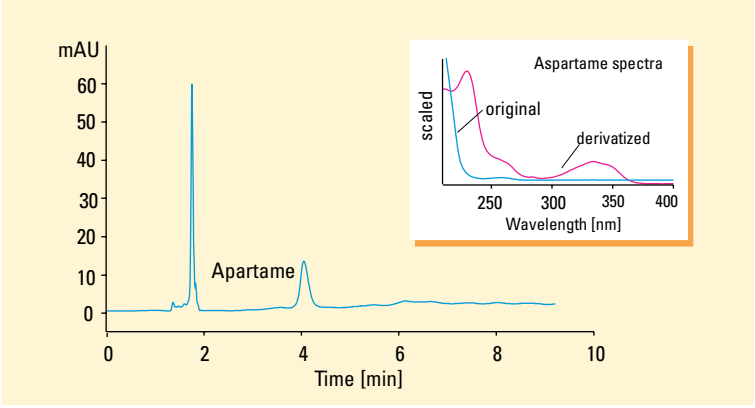

Figure 1
Determination of the quality of vanillin extract

Conditions

Derivatization agent
o-phthalaldehyde (OPA)
mercapto-propionic acid (MPA)
Column
100 ˘ 2.1 mm Hypersil ODS, 5µm
Mobile phase
A = 0.01 mM sodium acetate
B = methanol
Gradient
start with 5% B; at 5 min 25% B
at 10 min 35% B; at 13 min 55% B
at 18 min 80% B; at 20 min 95% B
Flow rate 0.35 ml/min
Post time 5 min
Column compartment 40 °C
Injection vol 1 µl
Injector program for online derivatization
1. Draw 5.0 µl from vial 3 (borate buffer)
2. Draw 0.0 µl from vial 0 (water)
3. Draw 1.0 µl from vial 1 (OPA/MPA)
4. Draw 0.0 µl from vial 0 (water)
5. Draw 1.0 µl from sample
6. Mix 7 µl (6 cycles)
7. Inject
Detectors
UV-DAD detection wavelength 338/20 nm or fluorescence: excitation wavelength 230 nm, emission wavelength 445 nm

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Chromatographic conditions

The HPLC method presented here for the analysis of aspartame is based on automated on-column derivatization and reversed-phase chromatography. UV spectra were evaluated as an additional identification tool.[2]

HPLC method performance

Limit of detection
for fluorescence 200 pg (injected amount), S/N = 2

for DAD 1 ng (injected amount), S/N = 2

Repeatability
of RT over 10 runs <0.1 %
of areas over 10 runs <5 %

References

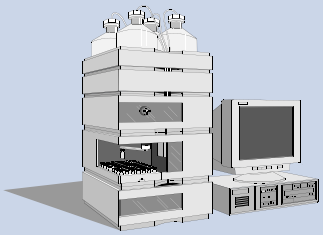
1.
Official Methods of Analysis, Food Compositions; Additives, Natural Contaminants, 15th ed; AOAC: Arlington, VA, **1990**, Vol. 2.; Official Method AOAC 979.08: Benzoate, caffeine, saccharin in soda beverages.

2.
A.M. Di Pietra et al., "HPLC analysis of aspartame and saccharin in pharmaceutical and dietary formulations"; *Chromatographia*, **1990**, 30, 215–219.

Equipment

Agilent 1100 Series
- vacuum degasser
- quaternary pump
- autosampler
- thermostatted column compartment
- diode array detector, fluorescence detector

Agilent ChemStation + software



Rainer Schuster is application chemist at Agilent Technologies, Waldbronn, Germany.

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Agilent Technologies
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Analysis of Preservatives in White Wine and Salad Dressing using HPLC

Angelika Gratzfeld-Huesgen

Food

Abstract

The following compounds are used as preservatives in food products:

- benzoic acid
- sorbic acid
- propionic acid
- methyl-, ethyl-, and propylesters of p-hydroxy benzoic acid (PHB-methyl, PHB-ethyl, and PHB-propyl, respectively).[1]

Preservatives inhibit microbial growth in foods and beverages. Various compound classes of preservatives are used, depending on the food product and the expected microorganism. PHBs are the most common preservatives in food products. In fruit juices, in addition to sulfur dioxide, sorbic and benzoic acid are used as preservatives, either individually or as a mixture.

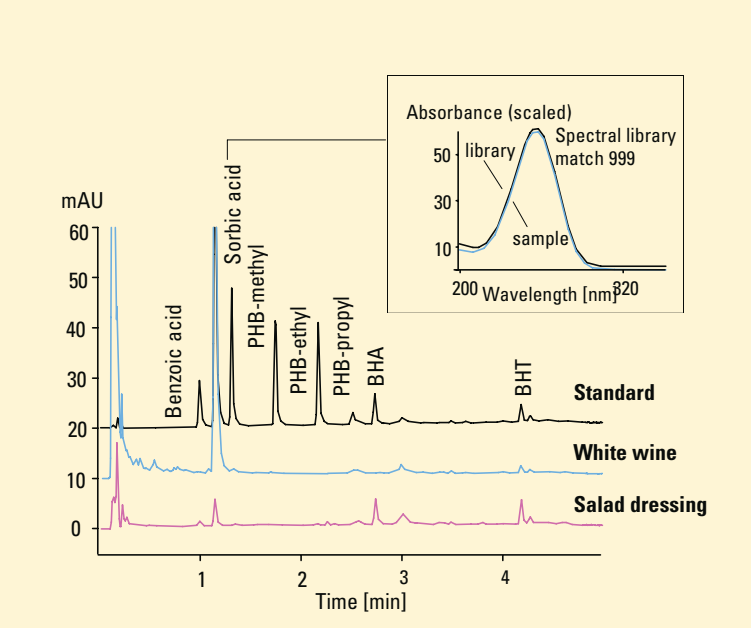

Figure 1
Analysis of antioxidants in chewing gum

Conditions

Column 125 ˘ 4 mm Hypersil BDS, 5 µm
Mobile phase
A = water + 0.2 ml H_2SO_4, pH = 2.3
B = ACN
Gradient start with 10% B
at 3 min 60% B; at 4 min 80% B
at 6 min 90% B; at 7 min 10% B
Flow rate 2 ml/min
Post time 1 min
Column compartment 40 °C
Injection vol 2 µl
Detector
UV-DADdetection wavelength 260/40 nm

Sample preparation
Carrez clearing and filtration for the salad dressing. None for white wine.

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Sample preparation

Sample preparation depends strongly on the matrix to be analyzed. For samples low in fat, liquid extraction with ultrasonic bath stimulation can be used. For samples with more complex matrices, solid-phase extraction, liquid/liquid extraction, or steam distillation may be necessary.

Chromatographic conditions

HPLC and UV-visible diode-array detection have been applied in the analysis of preservatives in white wine and salad dressing. Spectral information and retention times were used for identification.

HPLC method performance

Limit of detection
10 ppm, S/N = 2

Repeatability of
RT over 10 runs <0.1 %
areas over 10 runs <3 %

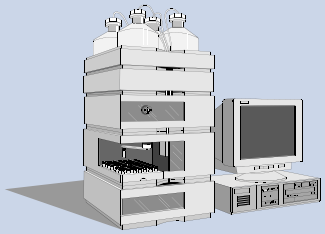
References

1.
Official Methods of Analysis, Food Compositions; Additives, Natural Contaminants, 15th ed; AOAC: Arlington, VA, **1990**, Vol. 2.; AOAC Official Method 979.08: Benzoate, caffeine, saccharine in carbonated beverages.

Equipment

Agilent 1100 Series
- vacuum degasser
- quaternary pump
- autosampler
- thermostatted column compartment
- diode array detector,

Agilent ChemStation + software



Angelika Gratzfeld-Huesgen is application chemist at Agilent Technologies, Waldbronn, Germany.

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Agilent Technologies
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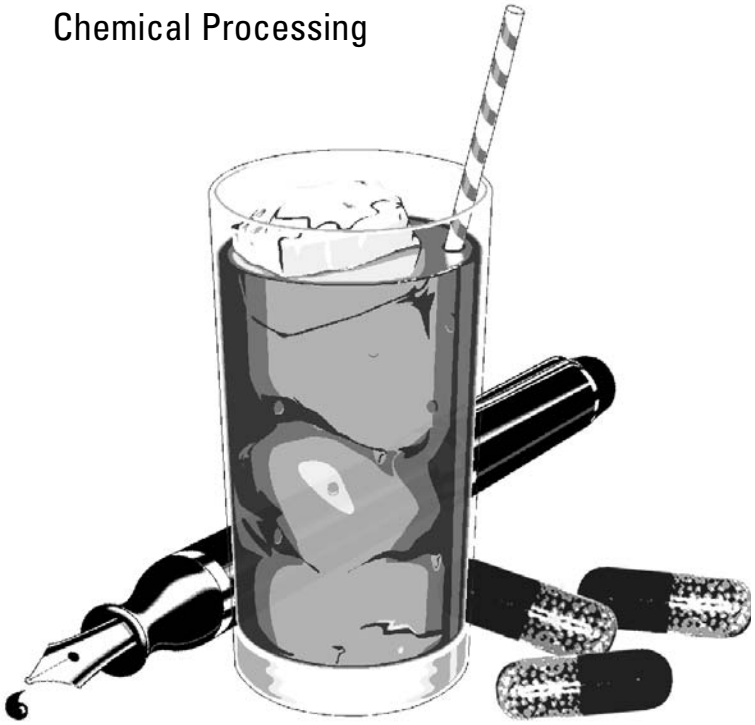
Sensitive Analysis of Synthetic Colors using HPLC and Diode- Array Detection at 190–950 nm

Application Note

Food and Flavors
Pharmaceutical
Chemical Processing

Angelika Gratzfeld-Hüsgen
Rainer Schuster



In this study synthetic dyes were analyzed using ion-pair, reversed phase chromatography on a special base-deactivated HPLC column. This separation mechanism was chosen to reduce tailing effects of highly polar colors. Detection was performed using a new design of diode-array detector based on two lamps — a deuterium lamp and a tungsten lamp. This ensured highest light output at 190 – 950 nm, which resulted in lowest detection limits over the entire wavelength range. It was possible to analyze blue, black or green colors in the low ng range at their absorption maxima of 600 – 700 nm. Complete spectra, feasible from 190 – 950 nm, facilitated identification with an automated library search.

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Introduction

Synthetic colors are widely used in the food, pharmaceutical and chemical industries.

The regulation of colors and the need for quality control relating to traces of starting products and by-products have forced the development of analytical methods. Nowadays various HPLC methods are in use, based either in ion-pair reversed phase chromatography or ion-exchange chromatography. UV absorption is the detection method of choice. The UV absorption maxima of colors are very characteristic, starting with maxima at about 400 nm for yellow colors, 500 nm for red colors and at 600 – 700 nm for green, blue and black colors. To analyze all different colors at maximum sensitivity and selectivity, the light output from the detector lamp should be high across the complete wavelength range. For black, green and blue colors, which show absorption maxima at or above 600 nm, this is not possible with conventional UV-Visible detectors based on a one-lamp design. For example, deuterium lamps have their maximum output in the UV range, whereas the visible range shows low light output.

A further analytical problem is the tailing-free separation of dyes. Figure 1 shows that some colors are of more polar nature, which sometimes causes problems even on reversed phases depending on the strength of polarity tailing. This results in worse detection limits and integration problems.

In the following study we evaluated the influence of:

- special deactivated columns on the separation of colors using different mobile phases,
- a newly designed diode-array detection system on limit of detection for colors which absorb at above 500 nm, and
- low noise and complete spectra on identification of sample compounds using automated library search in the low mAU range.

Experimental

For the experiments the Agilent 1100 Series HPLC system was used. The system comprised a low-pressure quaternary gradient pump with vacuum degasser, autosampler, Peltier-regulated column compartment, and diode-array detector with a wavelength range of 190 – 950nm. System control and data evaluation was done through an Agilent ChemStation for HPLC.

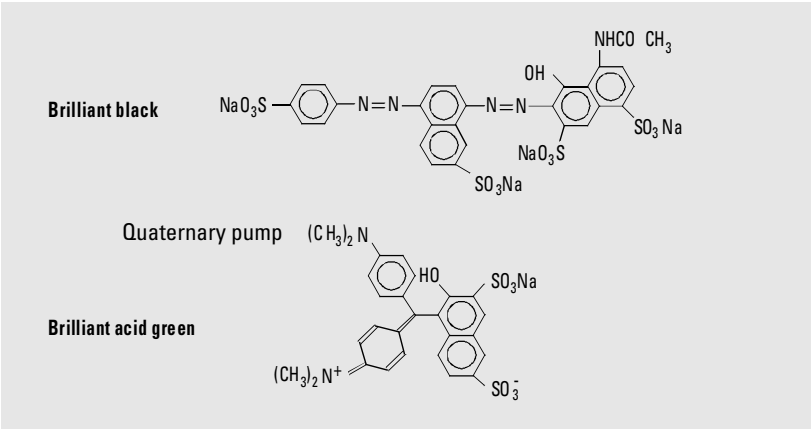

Figure 1
Chemical structure of brilliant black and brilliant green BS

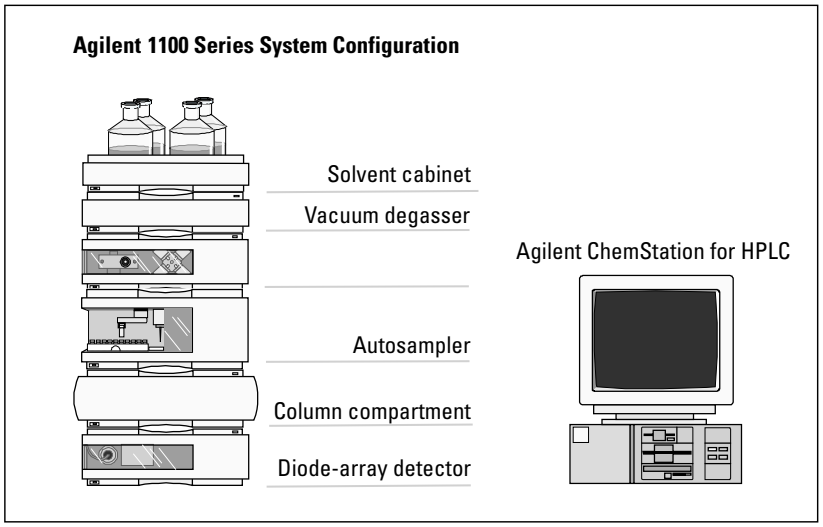

Figure 2
HPLC system used

Results and discussion

Separation of synthetic colors
During method development a special base-deactivated column and four different mobile phases were evaluated. Two mobile phases were simple buffer systems and the other two phases contained ion-pairing reagents (see figure 3). A 125 x 3 mm Hypersil BDS, 3-µm column (Agilent part number 79926BD-363) was used. The use of 3-mm id. and 3-µm material allowed optimum flow rates below 1 ml/min. This saved purchasing disposal costs of solvents. Mobile phases C and D showed tailing for compounds with sulfonic groups. Using ion-pairing chromatography (see figure 3, examples A and B), the separation of colors with different functional groups and different chemical structure was achieved with minimum or no tailing for all different color classes. As a conclusion, we recommend using mobile phases from A.

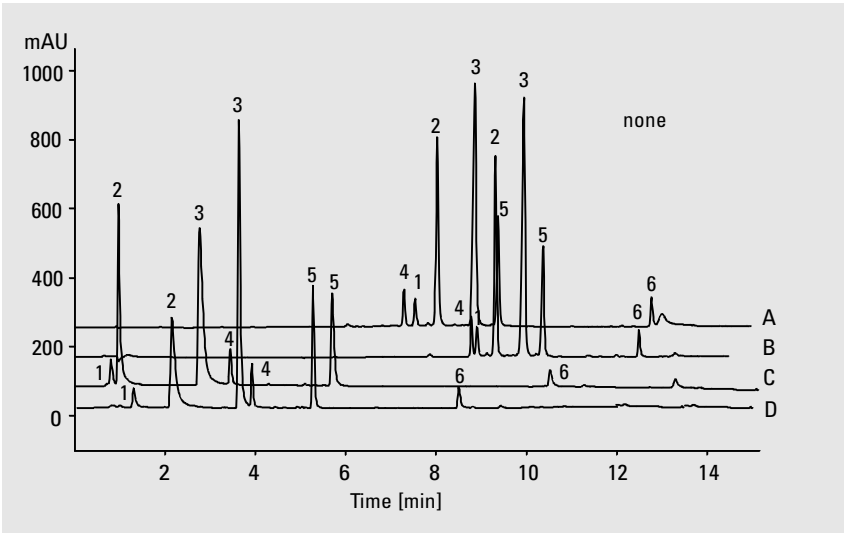

Figure 3
Separation of synthetic colors on BDS column using different mobile phases

Peak	Compound name	Number of sulfonic group
1	E102 Tartrazine	2
2	E123 Amaranth	3
3	E124 Ponceau 4R	3
4	E110 Sunset yellow	2
5	E125 Scarlet red	none
6	E127 Erythrosine	

Table 1
Content of sulfonic groups for colors shown in figure 3

Chromatographic conditions

For the application examples that follow we used mobile phases from A.

Column : 125 × 3 mm Hypersil BDS, 3 µm (HP part number 79926BD-363)

Mobile phase A: A = 0.01 M NaH_2PO_4 + 0.001 M Tetrabutylammonium-dihydrogenphosphate pH 4.2
B = acetonitrile (ACN)

Gradient for A: start with 15 %, in 10 min to 40 %, in 14 min to 90 %, until 19 min at 90 %, in 20 min to 15 % ACN

Mobile phase B A = 0.01 M NaH_2PO_4 + 0.001 M Tetrabutyl ammoniumhydrogen sulfate pH 4.8
B = acetonitrile (ACN)

Gradient for B: start with 15 %, in 10 min to 40 %, in 14 min to 90 %, until 19 min at 90 %, in 20 min to 15 % ACN

Mobile phase C A = 0.01 M ammoniumacetate pH = 4.9, B = ACN

Gradient for C: start with 7 %, in 10 min to 40 %, in 14 min to 90 %, until 19 min at 90 %, in 20 min to 7 % ACN

Mobile phase D A = 0.01 M NaH2P04, pH = 4.3, B = acetronitrile (ACN)

Gradient for D: start with 5 %, in 10 min to 60 %, in 14 min to 90 %, until 19 min at 90 % in 20 min to 5 % ACN

Stop time 20 min
Post time 4 min
Flow rate 0.8 ml/min
Col. temp. 40 °C
Inject. vol. 1 µl
Detector signal A: 254 nm/50 nm (for optimization of separation)
B: 350 nm/20nm,
C: 465 nm/30 nm,
D: 600 nm/40 nm
E: 750/40

Detection of synthetic colors using diode-array UV-visible absorption detector

The diode-array detector used here was equipped with two lamps, a deuterium lamp and a tungsten lamp. This ensured highest light output from 190 to 950 nm and therefore lowest detection limits over the entire wavelength range. The use of 1024 diodes and a programmable slit ensured highest spectral resolution. This gave the following advantages for the analysis of colors:

- acquisition of five signals simulataneously,
- highest sensitivity and selectivity even for blue, green and black colors with absorption maxima above 500 nm,
- complete spectral data up to 950 nm, and
- optimization of signal to noise ratio using different slit width without the need to exchange optical slits mechanically.

Figure 4 shows the complete spectra of a yellow, red and two blue colors.

For each of the analyzed colors characteristic absorption maxima were obtained. The yellow color tartrazine had its maxima at around 400 nm, the red color amaranth absorbed best at about 500 nm, the blue color patent blue had its maxima around 600 nm whereas the darker blue color brilliant blue showed its maxima at 740 nm. This clearly demonstrated that several signals had to be acquired for optimum sensitivity and selectivity for all colors.

The spectra of pure compounds can be stored in spectral libraries and used for later identification of colors in food, paints or pharmaceutical preparations.

That sensitivity is of utmost importance is shown in figure 8. The quality of inks is often determined by the content of traces of other colors, which may be unwanted by-products from production process or which are added to influence the nuance of a color. Therefore the quantitation and identification of trace compounds is as important as the determination of main compounds.

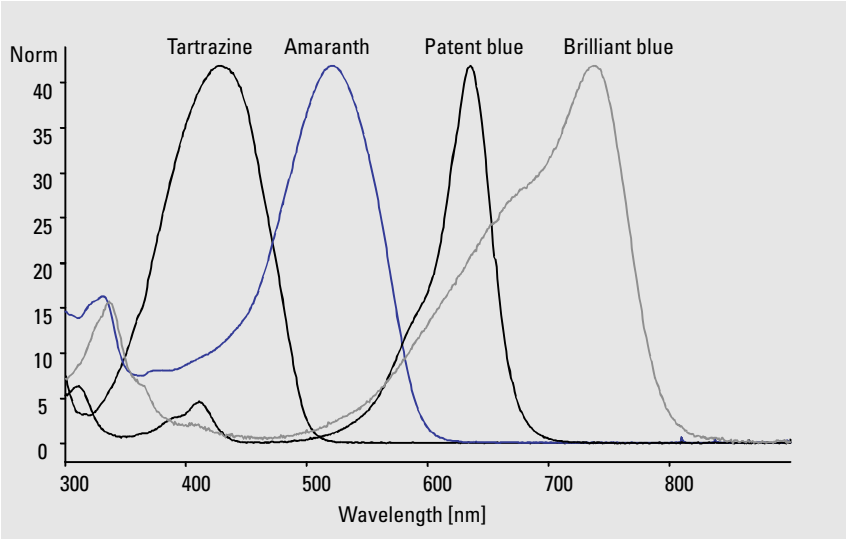

Figure 4
Spectra of different colors

As already demonstrated colors have very characteristic spectra which can be used to identify peaks not only by retention times but also by spectral data. The detector used here allowed identification using spectral data, see figure 5, even in the low mAU absorption range.

The *detection limit* for the evaluated colors, measured at their specific absorption maximum, was in the low ng range.

The *repeatability* of the HPLC method used here was measured using the standard mixture of figure 3. The relative standard deviation for retention times measured over 10 runs was below 0.2 %. For the areas the relative standard deviation was below 1 %.

The *linearity* was evaluated using blue ink color measured at 600 nm. Linearity was given from the low ng up to the low µg range.

Application Examples

Food colors

Colors are vital constituents of foods and probably the first characteristic perceived by the human senses. Today synthetic dyes have widely replaced natural colors. Table 2 lists some of the most frequently used food colors.

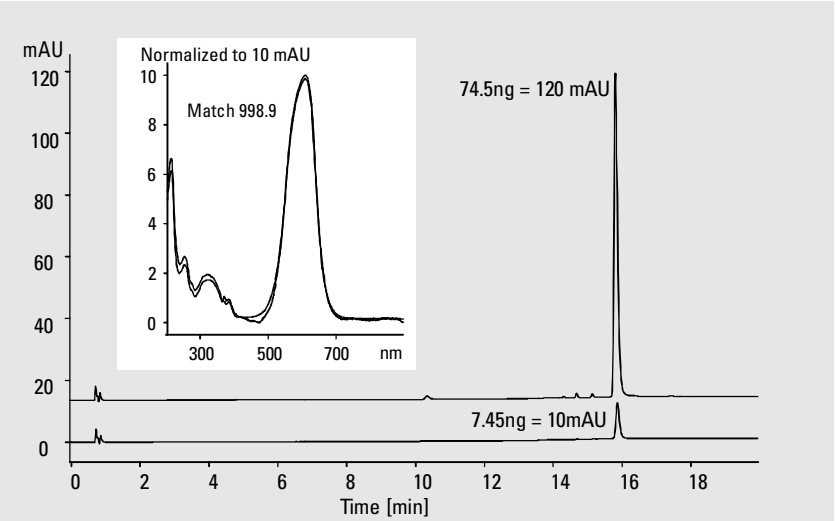

Figure 5
Identification of spectra in the low mAU absorption range for a blue ink color through overlay of trace level sample spectrum with library spectrum

EC	Name	Food, Drug & Cosmetics	CI
E102	Tartrazine	FD&C Yellow No. 5	19140
E103	Chryosine		14270
E104	Quinoline yellow	FD&C Yellow No. 10	47005
E105	Yellow		13015
E110	Sunset yellow	FCF FD&C Yellow No. 6	15985
E111	Sunset yellow	FD&C Orange No. 2	15980
E122	Azorubine	Carmoisine	14720
E123	Amaranth FD&C	Red No. 2	16185
E124	Ponceau 4R	Ponceau 4R	16255
E125	Scarlet red	Scarlet red	14815
E126	Ponceau 6R		16290
E127	Erythrosine	FD&C Red No. 3	45430
E131	Patent blue V	FD&C Violet No. 1	42051
E132	Indigo carmine	FD&C Blue No. 2	73015
E142	Acid brilliant green	FD&C Green	No. 3
E151	Black PN		28440
	Ponceau SX	FD&C Red No. 4	

Table 2
List of commonly-used colorings in EC and US classifications with color index numbers (CI

These dyes are used to supplement and enhance the natural colors destroyed during processing or storage, and substantially increase the appeal and acceptability of foodstuffs where no natural colors exist, for example, soft drinks or ice cream.

The usage of synthetic colors is well regulated worldwide, but the regulations differ from one country to the next. To ensure compliance with regulatory requirements, the used colors have to be identified and qualified according to national directives.

As an example of the determination of colors in foodstuffs we analyzed the synthetic colors used for a green carbonated drink — *a woodruff-ade*. The sample was injected directly and the compounds were identified using a library search (figure 6).

The green color was produced by a mixture of quinoline yellow and patent blue. The yellow color quinoline yellow split into four peaks showing an absorption maximum at 410 nm. The blue color patent blue had its maxima at 600 nm.

Colors for pharmaceutical preparations

The pharmaceutical industry uses colors, for example, for tablets, capsules and syrups. Here the intention is not only to improve the optical appearance but also to give more safety to the consumer. For example, different colors may help to avoid errors for a patient who has to take several medicaments.

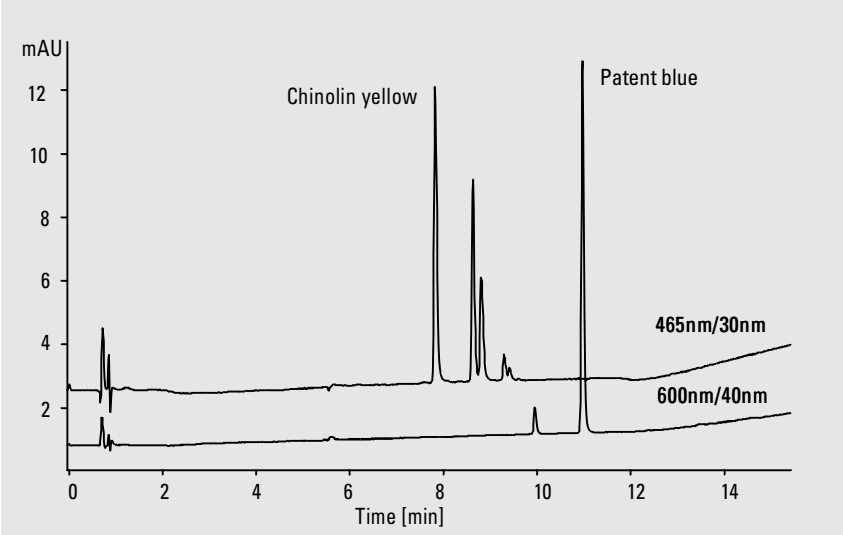

Figure 6
Chromatogram from the analysis of woodruff lemondade

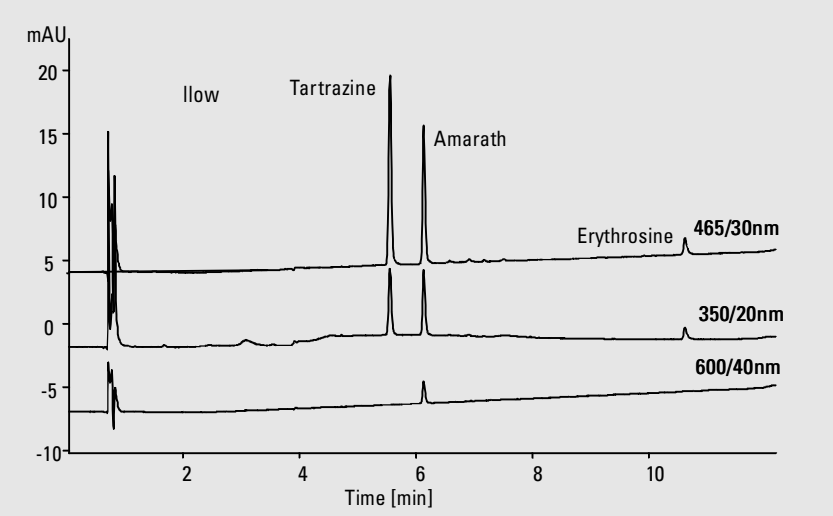

Figure 7
Chromatogram from the analysis of a red capsule

Figure 7 shows the analysis of tablet capsules which were dissolved in water, filtered and injected directly.

The red coloring comprised two red colors erythrosine and amaranth and a yellow color tartrazine.

Ink colors

The quality of ink colors relating to color brightness, stability against light and reproducibility of the same colors nuance for years, is determined by the accurate composition of different dyes in different concentrations. Here the right concentration of color traces is as important as the concentration of the main color compounds.

In figure 8 the chromatograms of a blue and a black ink color are overlaid showing that both inks do not only differ in one main compound but also in some trace compounds.

The *blue* color compound in the black ink is producing the main difference between black and blue.

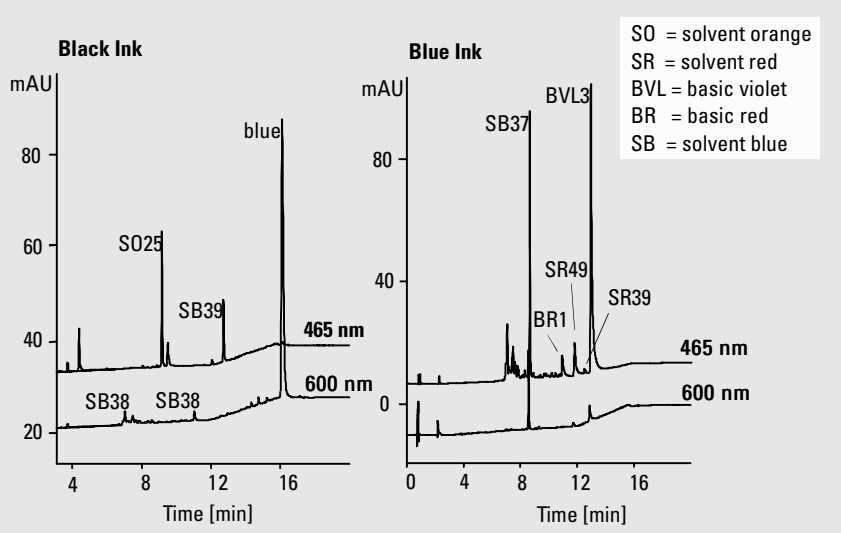

Figure 8
Comparison between a black and a blue ink

Conclusion

In this study we have demonstrated that the separation and detection of synthetic colors can be improved.

Ion-pairing reversed phase chromatography on a special deactivated column allowed the separation of dyes of different polarity with no or only slight tailing.

The UV-visible detection, especially from 400 to 950 nm, gained sensitivity by increasing the light output with a tungsten lamp in addition to a deuterium lamp.

Angelika Gratzfeld-Hüsgen and Rainer Schuster are application chemists based at Agilent Technologies, Waldbronn Analytical Division, Germany.

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Analysis of synthetic dyes in food samples by capillary zone electrophoresis

Application Note

Foods and Flavors

Authors

Rainer Schuster,
Angelika Gratzfeld-Hüsgen
Agilent Technologies,
Waldbronn, Germany

Abstract

Synthetic food dyes were separated by capillary zone electrophoresis using an alkaline phosphate buffer as background electrolyte. The precision had a relative standard deviation of less than 0.5 % for the run-to-run migration times and 2 % for the peak areas with buffer replenishment after each run. The detection limit for the individual dyes was about 1 ng using a 50-µm internal diameter Agilent extended path length capillary. Compounds were detected at different wavelengths— 215, 410, 520 and 598 nm—and the identities of the individual dyes were confirmed using peak-purity routines and a UV-visible spectral library.

Agilent Technologies

Introduction

Color is a vital constituent of foods and probably the first characteristic perceived by the human senses. Almost all foods—from raw agricultural commodities to finished products—have an associated color. Further, many tests have shown that color can organoleptically dominate the flavor of a food.

Colors have been added to foods since ancient times and include chlorophylls, carotinoids, flavonoids and anthocyans extracted from different plants.

Today synthetic dyes have widely replaced natural colors. These dyes are used to supplement and enhance the natural colors destroyed during processing or storage, and substantially increase the appeal and acceptability of foodstuffs where no natural colors exist, for example, soft drinks or ice cream.

But, synthetic dyes are also used to mask decay, to redye food, to mask aging effects or to disguise poor products.

Colors permitted for food use can be divided in three categories:

1. synthetic dyes—described in this study,
2. natural colors (for example, caramel or beetroot) and naturally identical colors (canthaxanthine), and
3. inorganic pigments.

Synthetic colors in food products are predominantly azo and triarylmethane dyes. These are mostly acidic or anionic dyes containing carboxylic acid, sulfonic acid or hydroxy groups, which form negatively-charged colored ions at basic pH ranges. Capillary zone electrophoresis (CZE) is an ideal tool because it can separate all the different functional groups in one analysis within a short run time. CZE separates compounds based on charge and size.

The range of color shades covered by the azo group includes red, orange, yellow, brown and blue-black. These dyes are prepared by coupling diazotized sulfanilic acid to a phenolic sulfonic acid moiety that often contains unwanted byproducts from corresponding impurities. Triarylmethane colors are distinguished by their brilliance of color and high tinc- torial strength, but have poor light stability. The United States (US) and the European Community (EC) are the two major geographical areas where color regulations are enforced. The lists of admitted colors are updated continuously because of suspected carcinogenicity.
For example:

- amaranth (E123, FD&C Red No. 2)—one of the most widely-used red color—was delisted in the US in 1970,
- indigo carmine (E132, FD&C Blue No. 2) was delisted in 1980,
- tartrazine (E102, FD&C Yellow No. 5) was subjected to rigorous tests, and
- in 1990 the use of erythrosine (E127, FD&C Red No. 3) was discontinued.

Figure 1 shows the chemical structures of some common synthetic dyes.

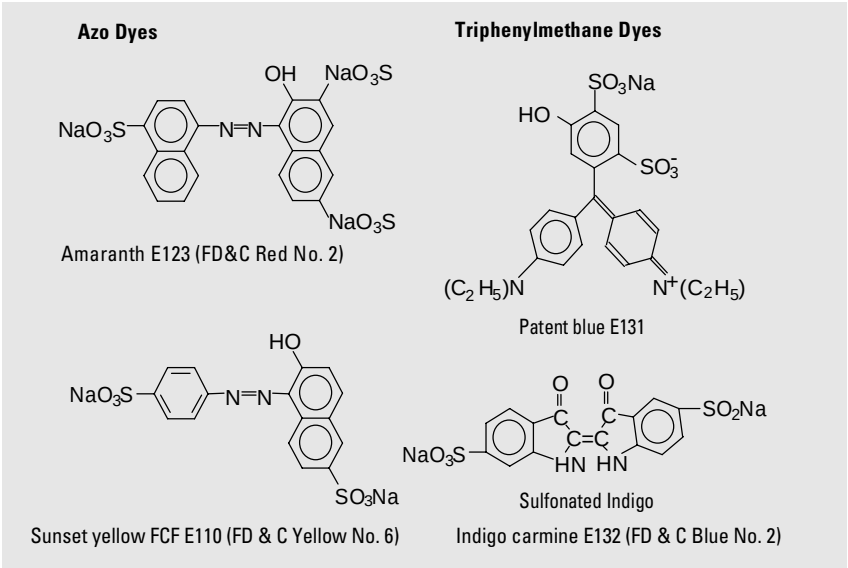


Figure 1
Chemical structures of some common synthetic dyes

In the EC colors are regulated by council directives on coloring matters. Of relevant interest are the proposals from 1985 and 1992 in foodstuff directives for international harmonization of colorant regulations. Table 1 shows some of the most widely-used synthetic dyes with their EC name (E-number), the US name (FD&C) and the color index number (CI).

To ensure compliance with regulatory requirements, analytical methods are required to determine nature and concentration of a colorant in a food product. Paper chromatography, thin-layer chromatography, and more recently high-performance liquid chromatography (HPLC) have helped the analyst in the examination of synthetic colors.[1,2,3]

Conventional HPLC separations use reversed-phase ion-pair chromatography with silica ODS or RP-2 material and a quaternary ammonium salt as the counter-ion.[4,5]

Several workers have adopted this approach for different applications using different sample-preparation techniques.[6,7,8,9] For example, in the wool-fiber method the sample is extracted in a tartaric acid solution and a wool fiber is used to absorb the colors of interest.[8] After washing with water and methanol the colors are washed off with a diluted ammonia solution, evaporated to dryness, and dissolved in water or ethanol.

The polyamide method involves extracting the sample with acetic acid and polyamide powder, whereby a chromatographic column is filled with polyamide and washed with water and methanol.[9] The colors are extracted with an ammonia solution, evaporated to dryness, and dissolved in water or ethanol.

Both wool-fiber and polyamide extraction are official methods in Germany and described in detail in §35 LMBG.[10] In the US individual methods for colors in food are described in the handbook of official methods of analysis of AOAC.[11] Ion pair extraction with tetrabutyl-ammonium phosphate and back extraction using perchlorate has been reported by Puttemans et al.[12]

EC	Name	Food, Drug & Cosmetics	CI
E102	Tartrazine	FD&C Yellow No. 5	19140
E103	Chryosine		14270
E104	Quinoline yellow	FD&C Yellow No. 10	47005
E105	Yellow		13015
E110	Sunset yellow FCF	FD&C Yellow No. 6	15985
E111	Sunset yellow	FD&C Orange No. 2	15980
E122	Azorubine	Carmoisine	14720
E123	Amaranth	FD&C Red No. 2	16185
E124	Ponceau 4R	Ponceau 4R	16255
E125	Scarlet red	Scarlet red	14815
E126	Ponceau 6R		16290
E127	Erythrosine	FD&C Red No. 3	45430
E131	Patent blue V	FD&C Violet No. 1	42051
E132	Indigo carmine	FD&C Blue No. 2	73015
E142	Acid brilliant green	FD&C Green No. 3	
E151	Black PN		28440
	Ponceau SX	FD&C Red No. 4	14700

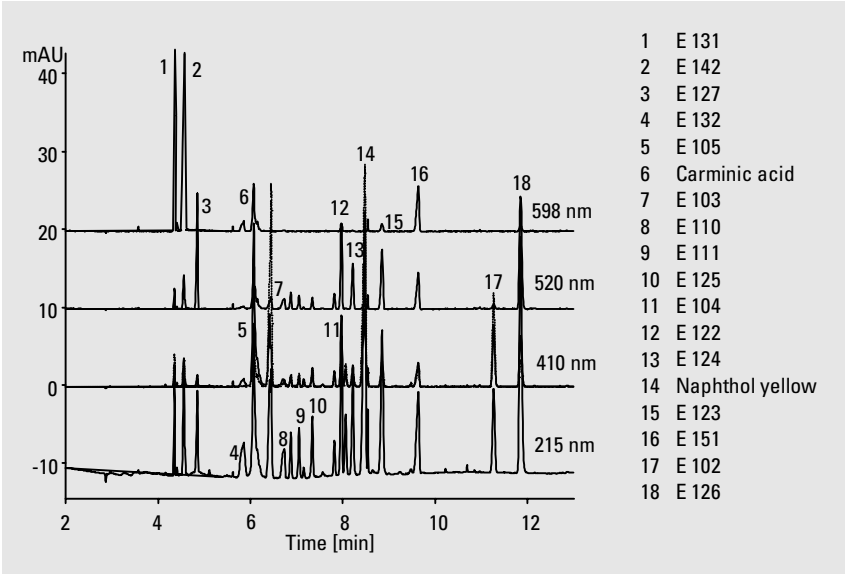
Table 1
List of commonly-used colorings in EC and US classifications with color index numbers (CI)

Experimental

Separations were performed using an Agilent CE system with a built-in diode-array detector and Agilent CE ChemStation software. Separations were achieved with fused-silica 50-µm id capillaries (64.5 cm total length, 56 cm effective length) with an extended path length or bubble cell at the detector end. All separations were performed at 30°C using a 10-mM sodium phosphate with 5-mM sodium hydrogen carbonate buffer at pH 10.5. Capillaries were preconditioned by flushing with 1-M sodium hydroxide for 3 minutes followed by running buffer for 10 minutes.

Samples were introduced hydrodynamically with 100 and 200 mbars followed by a 200 mbars buffer plug. The samples were analyzed with an applied voltage of 30 kV and detected at 215/50 nm, 520/60 nm, 410/60 nm and 598/4 nm. After each run the inlet and outlet vials were replenished and the column was rinsed with the separation buffer for 1 minute. A voltage ramp from 0 to 30 kV within 0.5 minutes was performed to avoid possible thermal expansion and loss of sample.

Details of the separation conditions are listed alongside Figure 2 which shows the separation of common synthetic dyes: patent blue E131, acid brilliant green E142, erythrosine E127, indigo carmine E132, chryosine E103, sunset yellow FCF E110, sunset yellow E111, scarlet red E125, quinoline yellow E104, azorubine E122, ponceau 4R E124, amaranth E123, black PN E151, tartrazine E102 and ponceau 6R E126. The E numbers correspond to the EC regulations for additives in food.



Buffer	10-mM sodium borate with 5-mM sodium hydrogen carbonate at pH 10.5
Electric field	465 V/cm
Capillary	l = 56 cm, L = 64.5 cm, id = 50 µm
Injection	100 mbars
Temperature	30 °C
Detection	
Signal	215/50 nm 520/60 nm red 410/60 nm yellow 598/4 nm blue
Reference	off

Figure 2
Electropherogram of a standard sample

Results and discussion

Classical methods still used for determination of synthetic dyes are thin-layer chromatography (TLC), paper chromatography, and HPLC together with diode-array detection (DAD) system.

In HPLC separation is based on ion-exchange or reversed phase using ion-pairing reagents—separation modes which require time (ion exchange) or chromatographic skill (ion pairing). Further HPLC can only analyze some of the existing colors.

The use of CZE with a diode-array detector enabled us to separate most of the synthetic dyes—legal and illegal ones—in one run and detect them at their individual wavelength: 410 nm for yellow, 520 nm for red, and 598 nm for blue.

A wavelength of 215 nm was used as a pilot wavelength for spectral acquisition and as a universal monitoring wavelength (for example, sweeteners and preservatives).

The spectral information of the compound of interest was compared with the spectra in a library—compounds were analyzed not only by migration time but also by spectral comparison—and a peak-purity check was performed by overlaying spectra taken in the peak. With the new software generation just one step was required to combine all these capabilities (migration time, spectral identification and peak purity) to produce quantitative reports based on three-dimensional data.

Table 2 shows a report based on the analysis of Figure 2 with the corresponding library search and relative standard deviation data (%RSD).

Depending on the functional groups—mostly sulfonic acid groups as shown in Figure 1—a pH in the range 8–11 was chosen for method development to separate colors as anions by CZE. Different buffers, such as borate, CAPS (3-cyclohexylalmino-1-propane sulfonic acid) and phospate were tested at different pH values and concentrations. The optimum buffer was found to be 10 mM phospate at pH 10.5.

Library search mode: Automatic library search
Signal 1: DAD1 A, Sig=215,50 Ref=off
Meas. Library CalTbl %RSD (10 runs)

Time [min]	Timet [min]	Time [min]	Sig	Amount ppm/100m	Library		Match	Name	100 mbs 60-260ng/µl Mt	Area	200 mbs 6-26ng/µl Mt	Area
4.71	4.79	4.79	1	91.069	-	1	999	erythrosine	E127	0.2	3.3	0.08	5.0
6.45	6.71	6.71	1	84.340	-	1	975	sunset yellow	E110	0.6	2.4	0.05	2.9
6.93	7.19	7.19	1	138.535	-	1	998	scarlet red	E125	0.5	2.7	0.06	3.3
7.71	8.00	8.00	1	69.244	-	1	1000	ponceau 4R	E124	0.6	2.7	0.1	1.9
8.15	8.54	8.54	1	31.708	-	1	1000	amaranth	E123	0.8	3.1	0.07	3.2
10.23	10.81	10.81	1	84.758	-	1	1000	tartrazine	E102	1.2	3.7	0.1	5.9

Table 2
Report and standard deviation on migration time and area for some color compounds

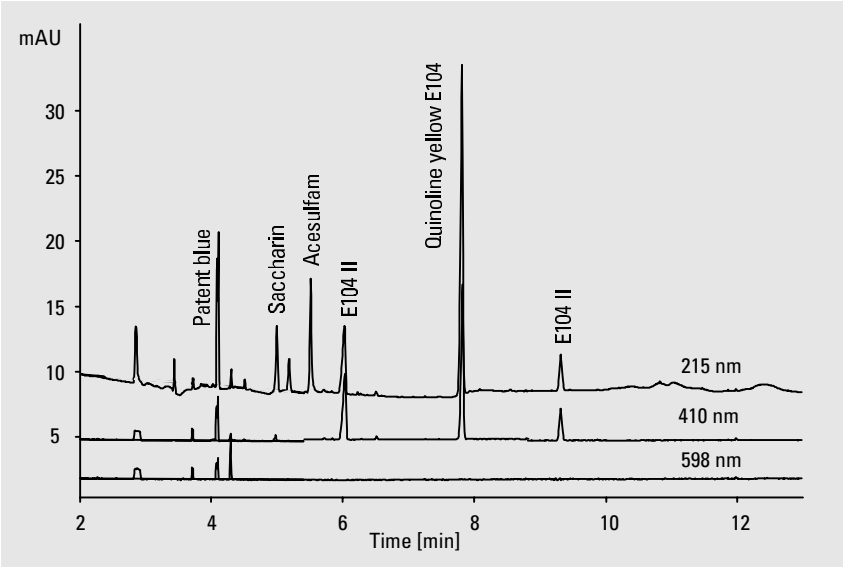


Figure 3
Electropherogram of a carbonated drink containing colors and artificial sweeteners

However, the reliability data showed a clear trend to shorter migration times depending on the pH changes of the buffer even when replenishment of the two vials was used. This was mainly due to the instable pH of this buffer system. According to literature data we found at this pH sodium hydrogen carbonate was a stable alternative—a buffer system which did not give a ideal separation of the colors.

Finally a mix of 10 mM phosphate and 5 mM sodium hydrogen carbonate adjusted to pH 10.5 with sodium hydroxide gave optimum conditions for separation of all colors, see figure 2. The buffer was filtered through a 0.45-µm filter and its pH value had to be checked when used over several days. The migration order of the two colors azorubine E122 and quinoline yellow E104 was reversed using different pH values (for example, 10.5–10.2) whereas migration times of other compounds were more or less stable.

Application

The method was applied to different matrices: carbonated beverages, pasta, glacé cherries, taramasalata extract and tablet capsules. The method was also tested to control quality of the colors themselves, for example, quinoline yellow.

The carbonated drink—a woodruff-ade—was injected directly and the compounds were identified using a library search, see figure 3. The “mint” impression (green color) was produced by a mix of quinoline yellow E104 monitored at 410 nm with patent blue E131 monitored at 598 nm. Other compounds such as the sweeteners labeled on the bottle could also be quantified using a library search (acesulfam and saccharine).

Figure 4 shows the electropherogram of a pasta extract containing quinoline yellow E104 with impurities (see figure 9), and sunset yellow FCF E110 to simulate the use of eggs. Both yellow compounds were selectively detected at 410 nm.

Figure 5 shows the analysis of glacé cherries containing ponceau 4R E124 which gives the intensive red color monitored at 520 nm.

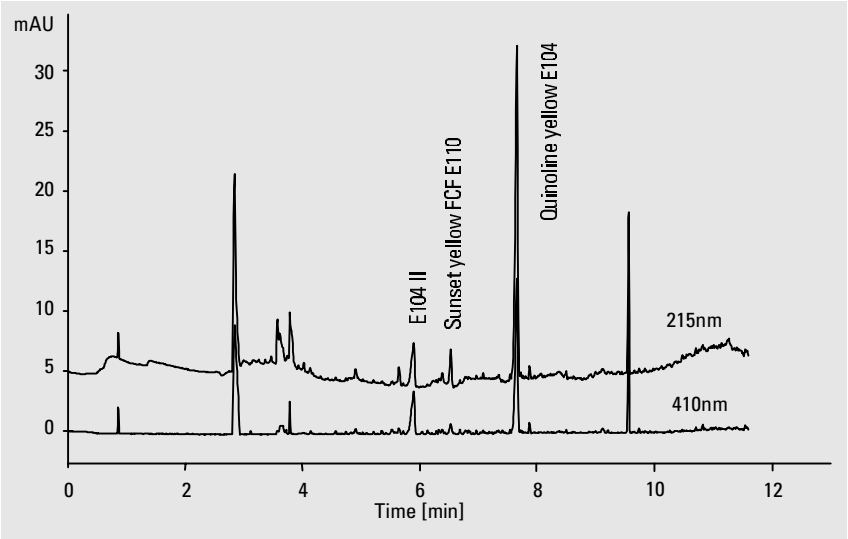


Figure 4
Electropherogram of pasta extract using wool-fiber sample preparation method

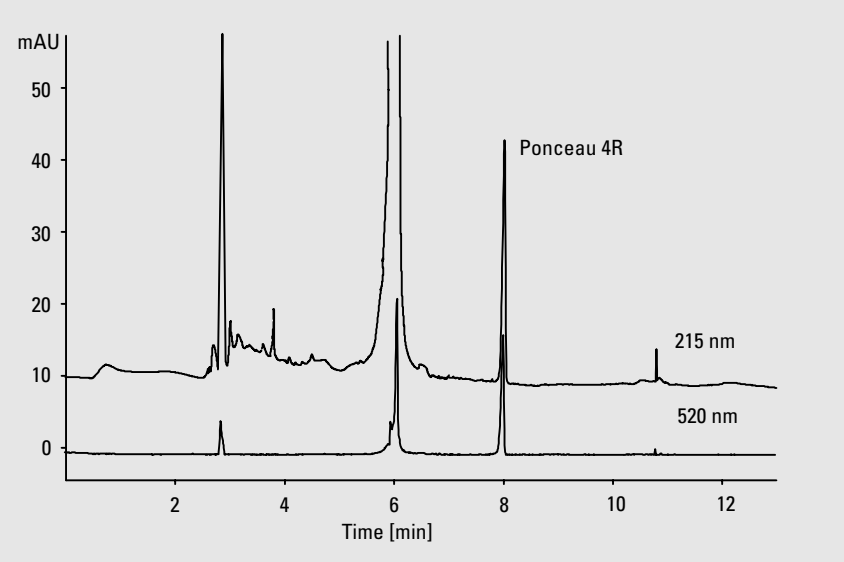
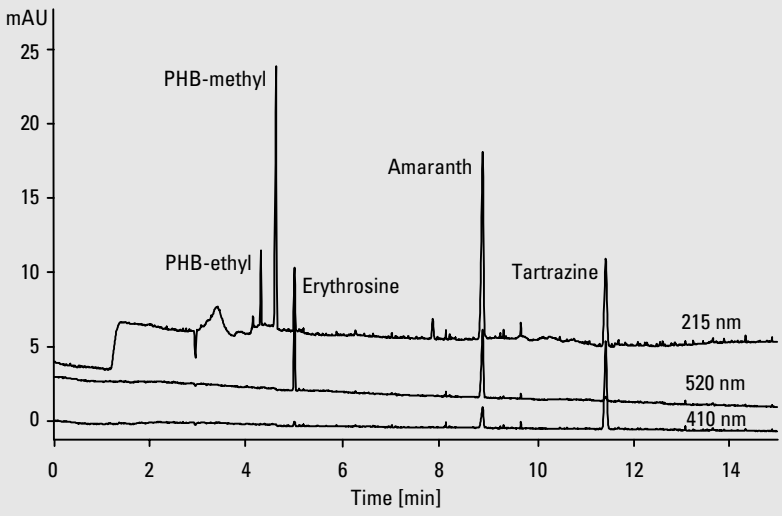


Figure 5
Electropherogram of glacé cherries colored with E124



Meas Mt [min]	Library Mt [min]	CalTbl Mt [min]	Sig	Amount ppm/100m bar*s	Library Match Factor	Name
4.99	4.79	4.79	1	11.100	998	erythrosin E127
8.89	8.54	8.54	1	21.133	980	amaranth E123
11.43	10.81	10.81	1	20.978	971	tartrazine E102

Uncalibrated compounds:

Meas Mt [min]	Library Mt [min]	CalTbl Mt [min]	Sig	Amount ppm/100m bar*s	Library Match Factor	Name
4.14	4.13	-	1	-	983	PHB-propyl
4.29	4.25	-	1	-	1000	PHB-ethyl
4.61	4.41	-	1	-	1000	PHB-methyl

Figure 6
Electropherogram of a tablet capsule and corresponding report based on a library search

Figure 6 shows the analysis of tablet capsules which were dissolved in water and injected directly. The red coloring of the capsule comprised two red colors erythrosine E127 and amaranth E123 (monitored at 520 nm), and a yellow color tartrazine E102 (monitored at 410 nm). The preservatives PHB-propyl, -ethyl and -methyl (215 nm) could be identified using a library search. It was found that under these conditions some of the sweeteners and preservatives could also be determined by this method.[13]

In addition to classical food applications this method could be applied to the quality control of the individual colors where the use of intermediates, containing impurities and coupling reactions, results in the formation of unwanted products.

For example, figure 7 shows the analysis of quinoline yellow E104 which contained seven impurities. After spectral analysis two different types of yellow were identified—type I with a wavelength maximum at 422 nm and type II with a maximum at 387 nm. All other compounds could be associated with either of these two spectral types. This was also observed in all samples containing quinoline yellow E104, see figure 3. Similar impurities were found in the analysis of quinoline yellow FCF E110 (FD&C Yellow No. 6), tartrazine E102 (FD&C Yellow No. 5) and FD&C Red No. 40 (allura red is not used in Europe).[11]

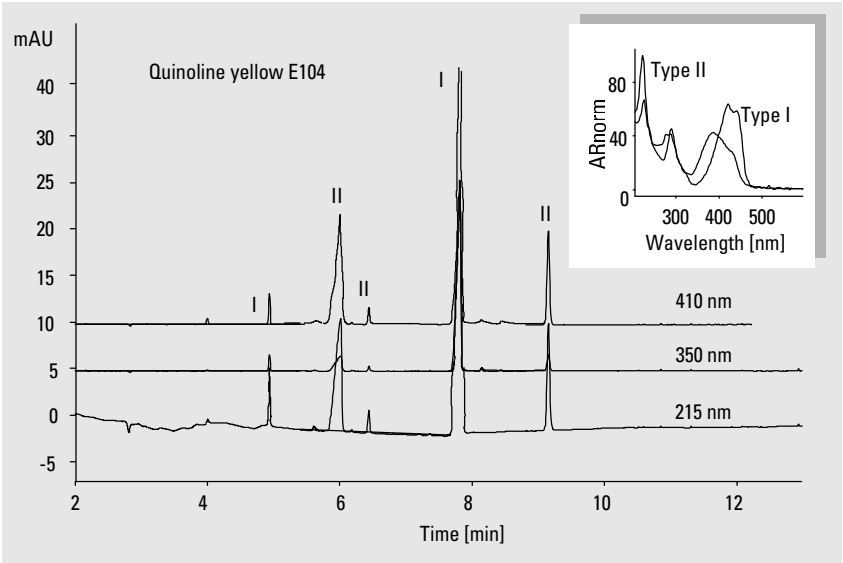


Figure 7
Electropherogram of quinoline yellow E104

In recent years concern has been expressed about the safety of certain synthetic dyes and this has prompted increased consideration for the use of natural colorants in food samples. Natural and naturally-identical colors are usually mixtures of several colored as well as non-colored compounds.

Figure 8 shows the analysis of an aqueous beetroot extract with two red compounds (520 nm).

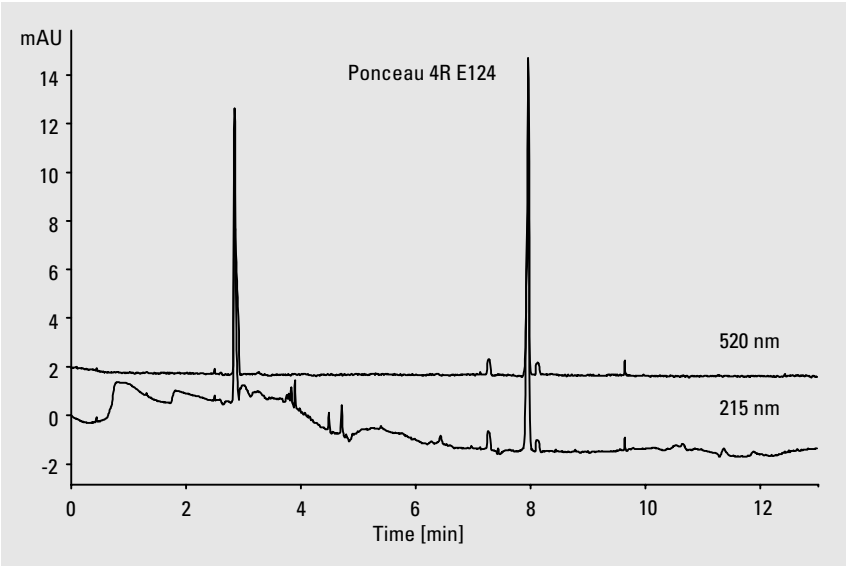
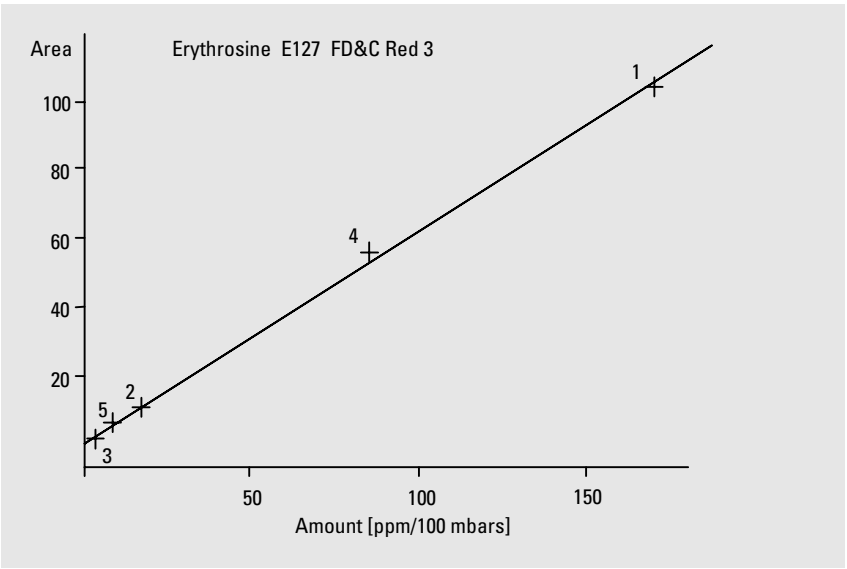


Figure 8
Electropherogram of an aqueous beetroot extract



Correlation	0.99920
Formula	y = mx + b
m	6.1747e-1
b	1.06058
x	amount
y	area

Figure 9
Linearity curve for erythrosine E127

Reproducibility and Linearity

The migration-time precision calulated as %RSD for all compounds was better than 0.5 % and the peak-area precision was between 2–4 %. The calculation was based on ten runs with amounts of 60–260 ng injected at 100 mbars and 6–26 ng injected at 200 mbars, see table 2. To achieve such excellent reproducibility, buffer replenishment after each run was necessary. The method was nearly linear over a range from low nanogram amounts to about 0.5 µg with variable injection volumes, see figure 9.

Conclusion

We have shown that CZE is well suited for control of synthetic dyes in food samples and for some sweeteners and preservatives. The combination of this separation method with library searches and peak-purity checks enabled separation, identification and identity confirmation of the analytes in a single run. Diode-array detection enabled selective monitoring of individual color group at their appropriate wavelength. The detection limit for most of the compounds was found to be in the low nanogram range.

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Rainer Schuster and Angelika Gratzfeld-Hüsgen are application chemists at Agilent Technologies, Waldbronn, Analytical Division, Germany.

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Publication Number 5964-0280E

Agilent Technologies
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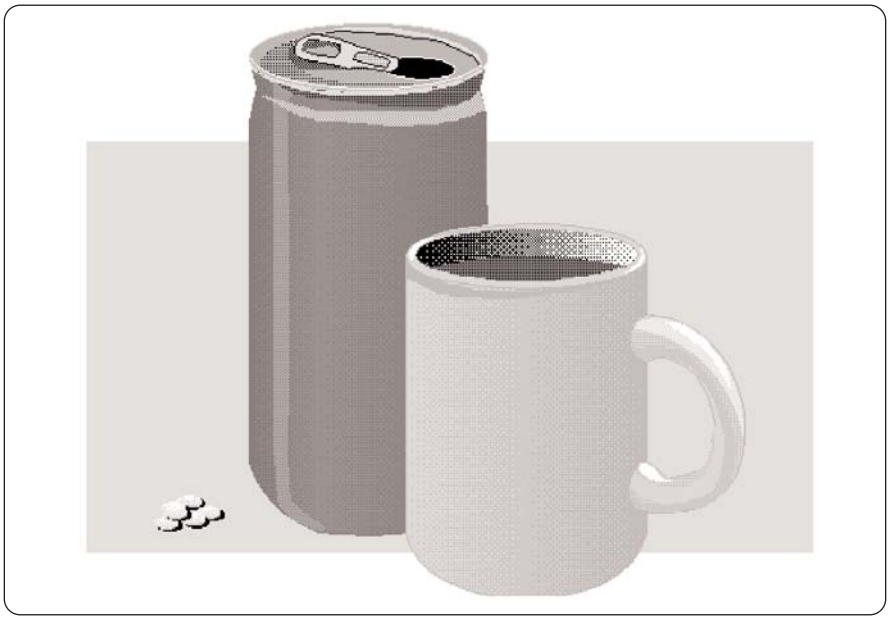
CZE analysis of artificial sweeteners and preservatives in drinks

Application Note

Foods and Flavors



Authors

Rainer Schuster,
Angelika Gratzfeld-Hüsgen
Agilent Technologies
Waldbronn, Germany

Abstract

Capillary electrophoresis can reduce the complexity associated with the analysis of sweeteners in drinks.The method described here has been successfully applied to both beverages and tablet formulations. A single run on an 50-µm id Agilent Extended Path Length capillary at 192 nm, with simultaneous UV-visible absorbance spectral library and peak purity routines, detects and confirms most compounds in the low nanogram range. With buffer replenishment every five injections, repeatability is better than 0.15 % for migration times and approximately 2 % for areas.

Agilent Technologies

Introduction

Sweetening agents can be classified as belonging to one of two main groups: caloric, or nutritive, and noncaloric or non-nutritive compounds. Nutritive sweeteners are carbohydrates (or their derivatives such as glucose, fructose and maltose) or products hydrolyzed from starch. Non-nutritive sweeteners do not belong to any particular chemical group. Synthetic sweeteners are steadily increasing in importance with increased public awareness of diabetes and its special dietary requirements, and more consumers becoming concerned about obesity and dental caries. The most frequently used synthetic sweeteners are: saccharin, cyclamate, aspartame and acesulfame, see figure 1.

To date, artificial sweeteners (table 1) have been determined by HPLC with reversed phase chromatography using different buffer systems, ion pairing reagents and specific derivatization procedures (aspartame with *o*-phthalaldehyde [OPA]; cyclamate with 4-fluoro-7-nitrobenzofurazone [NBDF]). Derivatization overcomes detection limitations for these compounds in the low UV range.

A number of methods have been published [1, 2] for simultaneous determination of aspartame and saccharin. Conditions are shown in table 1.[1]

	Saccharin	Saccharin Aspartame Dulcin	Acesulfame-K	Cyclamate NBDF-Derivative
Column	RP-18	ODS	ODS	RP-18
Mobile phase	0.05 mM H_2PO_4– acetonitrile, 9:1	0.01 mM KH_2PO_4 pH 3.5-acetonitrile, 85:15	0.01 mM TBAHS– H_2O, acetonitrile, 55:45 methanol, 9:1	
Detector	230/260 nm	216 nm	227 nm	490 nm Fluorescence λ_{ex}485nm, λ_{em}530 nm

Table 1
HPLC conditions for determination of sweeteners

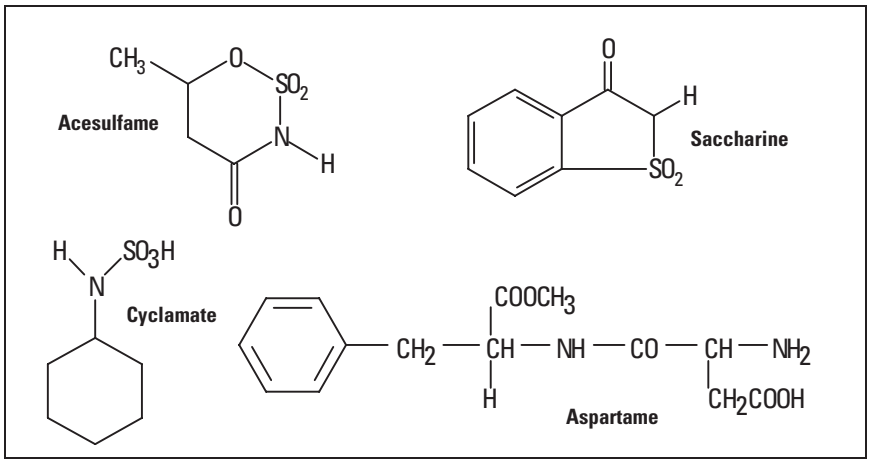


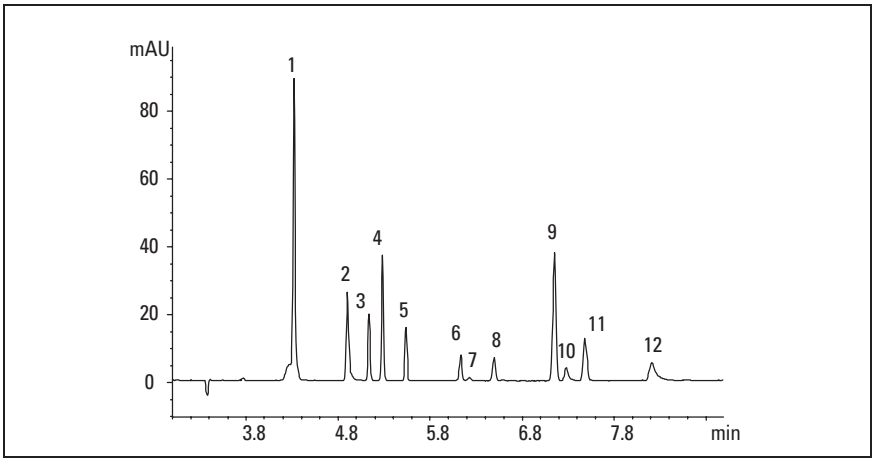
Figure 1
Chemical structure of the common artificial sweeteners

Hermann and coworkers [3] reported on a method for the detection of aspartame, cyclamate, dulcin, and saccharin using an ion-pair HPLC separation with indirect photometric detection.

Toxicological data has led to the use of some artificial sweeteners being controlled, for example cyclamate is banned in the United States, the United Kingdom and Japan. Aspartame is metabolized to aspartic acid, methanol, and *phenylalanine* a substance critical to persons who suffer from phenylketonuria (PKU). Reliable means of obtaining analytical data are required for food samples containing these compounds. However, such varied methods with their differing derivatization protocols make the analysis of artificial sweeteners time consuming and labor intensive. An alternative to HPLC is capillary zone electrophoresis (CZE). All compounds can be separated sufficiently in one run.

Experimental

CZE separations were performed using the Agilent CE system with a built-in diode-array detector and Agilent CE ChemStation (DOS Series) software. Separations were achieved with fused-silica 50-µm id capillaries (64.5 cm total length, 56 cm effective length) with an extended path length or bubble cell at the detector end. All separations were performed at 25°C using a 20-mM sodium tetraborate buffer at pH 9.4. New capillaries were preconditioned by flushing with 1M sodium hydroxide for 3 minutes followed by running buffer for 10 minutes.



Buffer	20mM borate pH 9.4
E	465 V/cm
Effective capillary length	56 cm
Total capillary length	64.5 cm
id 50 µm	
Injection	100 mbars
Temperature	25°C
Detection	
Signal	192/2 nm
Reference	450/100 nm

Key

1	phenylalanine	7	cyclamate
2	aspartame	8	sorbic acid
3	PHB propyl	9	benzoic acid
4	PHB ethyl	10	aspartic acid
5	PHB methyl	11	saccharine
6	Dehydroacetic acid	12	acesulfame

Figure 2
Electropherogram of a standard sample

Meas. Library CalTbl

Time [min]	Time [min]	Time [min]	Sig	Amount [ng/µl]	Purity Factor	#	Library Match		Name	%RSD- (n=5) MT	AREA
4.21	4.10	4.20	1	177.884	1000	1	998		phenylalanine	0.02	9.4
4.78	4.67	4.78	1	87.063	1000	1	999		aspartame	0.006	1.1
5.00	4.90	4.99	1	62.506	1000	1	999		PHB-propyl	0.141	6.0
5.00	5.10	5.12	1	57.134	1000	1	998	?	PHB-ethyl		
5.13	5.10	5.12	1	109.938	1000	1	999		PHB-ethyl	0.03	2.8
5.37	5.30	5.36	1	45.242	1000	1	999		PHB-methyl	0.03	1.4
5.95	5.85	5.94	1	50.572	1000	1	998		dehydroacetic acid	0.02	0.22
6.04	6.00	6.03	1	245.684	-	1	621	x	cyclamate	0.03	7.0
6.29	6.20	6.29	2	69.561	1000	1	998		sorbic acid	0.026	2.3
6.91	6.90	6.89	1	45.588	1000	1	998		benzoic acid	0.03	1.9
6.99	6.86	6.96	1	157.647	1000	1	955		aspartic acid	0.045	0.9
7.22	7.20	7.20	1	53.055	1000	1	1000		saccharine	0.035	1.4
7.88	7.80	7.87	2	136.327	969	1	923	x	acesulfame	0.04	1.7

Table 2
Report of figure 2 for artificial sweeteners and preservatives

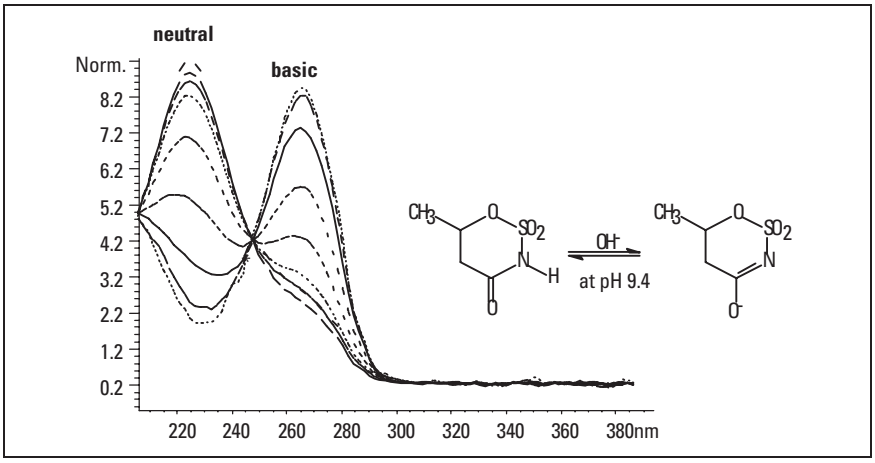
Samples were introduced hydrodynamically in 2 s at 50 mbar and analyzed with an applied voltage of 30 kV and detected at 191 nm (2-nm bandwidth). After each run the column was rinsed with the separation buffer for 2 minutes. Detailed separation conditions are listed alongside figure 2, the separation of common artificial sweeteners: aspartame (and its decomposition products phenylalanine and aspartic acid, cyclamate, saccharine and acesulfame together with the normally occuring preservatives PHB-esters (propyl-, ethyl-, and methyl), sorbic acid and benzoic acid.

Results and discussion

Cyclamate and aspartame lack chromophores and require detection wavelengths in the low UV-range below 200 nm, that part of the spectrum where certain ion pairing reagents also absorb. Monitoring with the CE system's built-in diode-array detector permits detection at 192 nm and simultaneous acquisition of spectra. This spectral information compared to spectra in a library stored on the ChemStation can confirm that the response is indeed from the compound of interest and not from interfering matrix compounds. Peak purity analysis can be achieved by overlaying spectra taken in the peak. The system's software performs all three actions (migration time report, libraray search and peak purity) in one step, producing quantitative reports based on three-dimensional data. Table 2 shows a report based on the analysis of figure 2 with the corresponding library search and peak purity data.

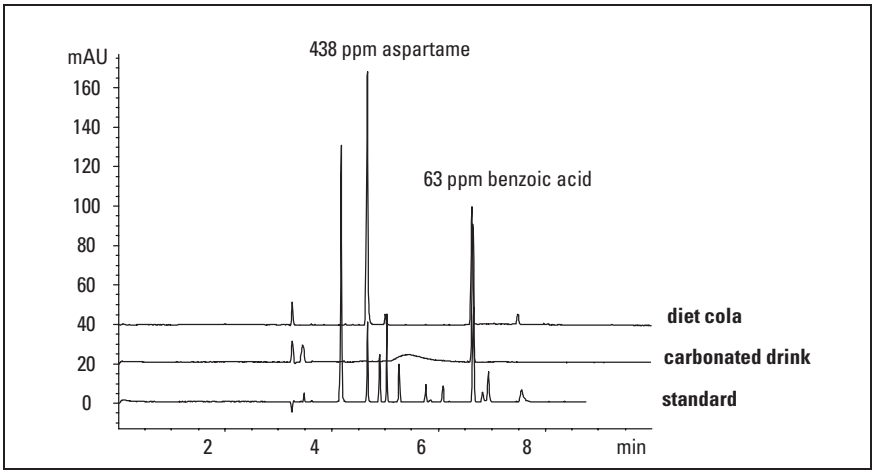
Concentrations injected were in the range 50–200 ppm. The *x* flag in the report shows that acesulfame might contain an impurity: its library match factor of 923 and purity factor of 969 are lower than could be expected for a pure peak. Although flagged, cyclamate concentration is too low, even at 192 nm, to make any conclusive judgements. A spectral overlay of spectra taken over the peak migrating at 7.88 (all spectra in peak) show a rather interesting aspect, see figure 3.

The overlay reveals an isosbestic point (245 nm) —acesulfame exists in a tautomeric equilibrium in that buffer, stable at this pH value.



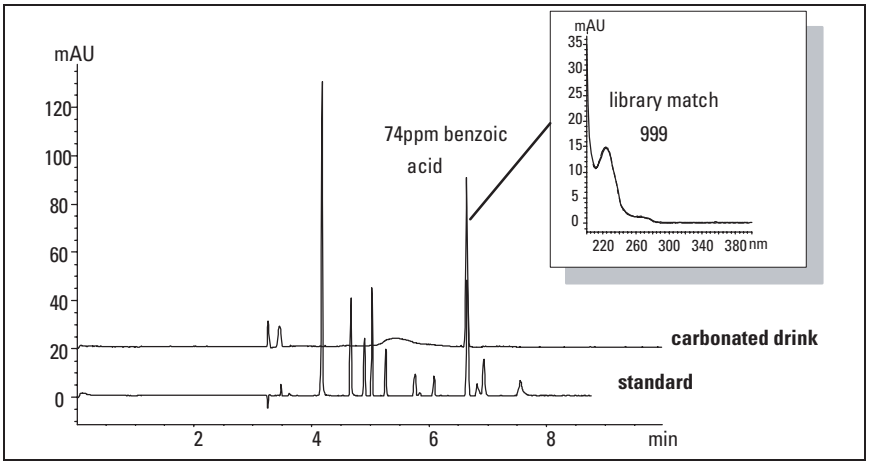
Buffer	20 mM borate pH 9.4
E	465 V/cm
Effective capillary length	56 cm
Total capillary length	64.5 cm
id	50 µm
Injection	100 mbars
Temperature	25 °C
Detection	
Signal	192/2 nm
Reference	450/100 nm

Figure 3
Overlay of spectra taken from acesulfame



Buffer	20 mM borate pH 9.4
E	465 V/cm
Effective capillary length	56 cm
Total capillary length	64.5 cm
id	50 µm
Injection	100 mbars
Temperature	25 °C
Detection	
Signal	192/2 nm
Reference	450/100 nm

Figure 4
Electropherogram of a diet cola containing aspartame and benzoic acid and a carbonated drink containing benzoic acid



Buffer	20 mM borate pH 9.4
E	465 V/cm
Effective capillary length	56 cm
Total capillary length	64.5 cm
id	50 µm
Injection	100 mbars
Temperature	25 °C
Detection	
Signal	192/2 nm
Reference	450/100 nm

Figure 5
Electropherogram of a carbonated drink with benzoic acid and spectral overlay

Application

The method has been applied to different matrices: beverages, such as diet cola and coffee, and tablets. All compounds have been identified with library search see figure 4.

Reproducibility

The repeatability for all compounds was better than 0.15 % for retention time and between 1 and 7 % (9 % for phenylalanine due to an impurity) for peak area. The calculation was based on five runs with injected amounds of 50 to 250 ng absolute, see table 2 and figure 7. Buffer replenishment after five injections is necessary for highest reproducibility.

CZE is known to have linearity characteristics half that of HPLC, nevertheless the equipment used here is linear up to 600 mAU.[4]

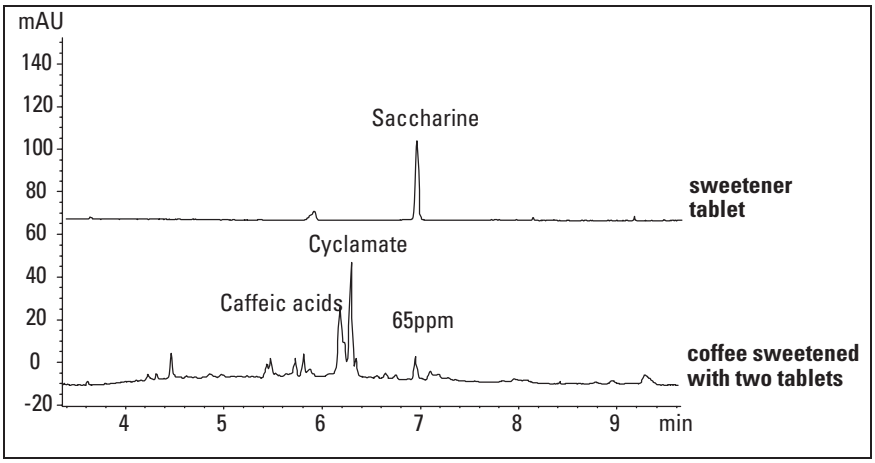


Figure 6
Electropherogram of analysis of a sweetener tablet overlaid with the electropherogram of directly injected coffee sweetened with two such tablets

Buffer	20 mM borate pH 9.4
E	465 V/cm
Effective capillary length	56 cm
Total capillary length	64.5 cm
id	50 µm
Injection	100 mbars
Temperature	25 °C

Detection

Signal	192/2 nm
Reference	450/100 nm

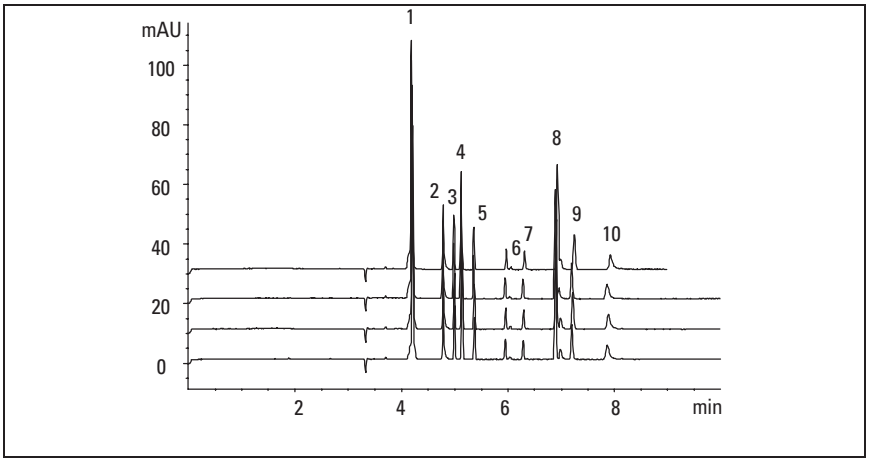


Figure 7
Overlay of five artificial sweetener standards

Buffer	20 mM borate pH 9.4
E	465 V/cm
Effective capillary length	56 cm
Total capillary length	64.5 cm
id	50 µm
Injection	100 mbars
Temperature	25 °C

Detection

Signal	192/2 nm
Reference	450/100 nm

Key

1	phenylalanine	6	cyclamide
2	aspartame	7	sorbic acid
3	PHB propyl	8	benzoic acid
4	PHB ethyl	9	saccharine
5	PHB methyl	10	acesulfame

Conclusion

We have been able to show that capillary electrophoresis and UV-visible absorbance spectral library search is well suited for controlling food samples for artificial sweeteners and preservatives. Due to the transparency of the borate buffer detection could be done at 192 nm. Detection limit for most of the compounds is in the low nanogram range. Peak purity control for identification and confirmation of the compounds is possible in the same single run.

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Rainer Schuster and Angelika Gratzfeld-Hüsgen are HPLC application chemists based at Agilent Technologies Waldbronn Analytical Division, Germany.

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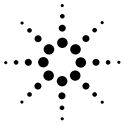

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Natural Compounds & Additives

Amino Acids

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High-Speed Amino Acid Analysis (AAA) on 1.8 µm Reversed-Phase (RP) Columns

Application

Pharmaceuticals and Foods

Authors

Cliff Woodward and John W. Henderson Jr.
Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE 19808
USA

Todd Wielgos
Baxter Healthcare Corp.
Round Lake, IL 60073
USA

Abstract

Amino acid analysis (AAA) is commonly used in proteomics and food quality testing as a tool to discover the precise amino acid (AA) makeup of samples. In order to do this in a timely fashion a short turnaround time is needed; to do it with limited sample amounts, a highly sensitive method is needed; and to maximize laboratory productivity, an automated method is desirable. This paper describes a new methodology using recently developed columns with an engineered particle size of 1.8 µm. These patented particles are specifically designed to create less backpressure than other sub-two-micron particles on the market; therefore, they can be used on 400 bar HPLC systems as well as higher pressure limit instruments. We take advantage of the OPA/FMOC chemistry first introduced by Hewlett-Packard/Agilent in 1988 and improve on the precision, run time, column longevity, and ruggedness of the previous methods [1—4].

Introduction

The detection and quantitation of amino acids has been a large part of protein and food analysis since Moore and Stein developed the first analyzer in 1951 utilizing ion exchange chromatography to separate underivatized amino acids (AAs) followed by post-column derivatization with ninhydrin and detection in the visible region [5]. They were able to develop a completely automated analyzer, introduced commercially by Beckman in 1958. This was a revolution in the analysis of proteins and strongly contributed to the winning of the 1972 Nobel Prize in Chemistry for their work on ribonuclease. A single analysis had previously taken weeks to complete but was now accomplished in less than a day. Subsequent work reduced the analysis time to approximately 110 minutes on the last revision of the Beckman Amino Acid Analyzer produced, but the sensitivity was still a problem using ninhydrin.

Post-column reaction with o-phthalaldehyde (OPA) and mercaptoethanol offered some promise, but only primary AAs were detected (see Figure 1a). In 1971 the first precolumn derivatization of AAs with OPA was published [6]. The derivatives were rather unstable but the process could be automated. Sensitivity was enhanced, particularly when fluorescence detection (FLD) was used. While the OPA is nonfluorescent, the derivatives are highly fluorescent; however, the secondary AAs are still not detected. Another derivatizing agent, 9-fluorenylmethyl chloroformate (FMOC), formed stable derivatives and derivatized both pri-

mary and secondary AAs but was itself strongly UV absorbing and highly fluorescent (see Figure 1b) [7]. An extraction step or reaction with very hydrophobic amine at the end was needed to remove the excess FMOC and its reaction byproducts [8]. This latter methodology was also automated as a precolumn method [9] but had problems, in commercial versions, of having higher relative standard deviations (RSDs) than systems using no back extractions.

By combining these two chemistries in a sequential way, in 1986 Hewlett-Packard/Agilent was able to fully automate the derivatization, chromatography, detection, and reporting of AAs from protein hydrolysates without back extraction [1]. A commercial analyzer was developed and sold by 1988 that had a total turnaround time of 36 minutes with femtomole sensitivity. This was definitely a step in the right direction for biotech companies and university researchers, who were sample limited, particularly in the early R&D phases of drug discovery.

A few chemical innovations were needed in order to make this commercially viable: OPA derivatives were made more stable by changing the source of incorporated thiol to 3-mercaptopropionic acid (MPA); all primary AAs were reacted first with OPA to quantitatively remove them from further reaction; then FMOC was introduced to react only with the secondary AAs. Since FMOC, its derivatives, and reaction byproducts were all more hydrophobic than any of the OPA derivatives, they did not interfere with any primary AA detection. Because there were only a few FMOC derivatives, it was possible, using a simple two-segment gradient, to adequately separate those AAs from the reaction byproducts and FMOC [1]. This is summarized in Figures 1 and 2. The names corresponding to the peak numbers are given in Table 1.

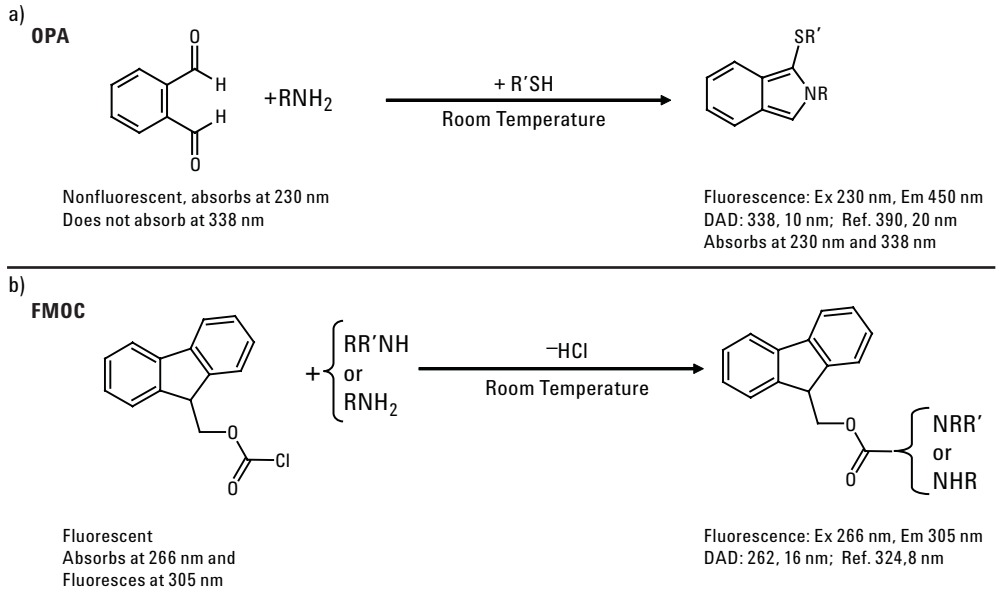

Figure 1. o-Phthalaldehyde (OPA) and 9-Fluorenylmethyl chloroformate (FMOC) reactions with amines.

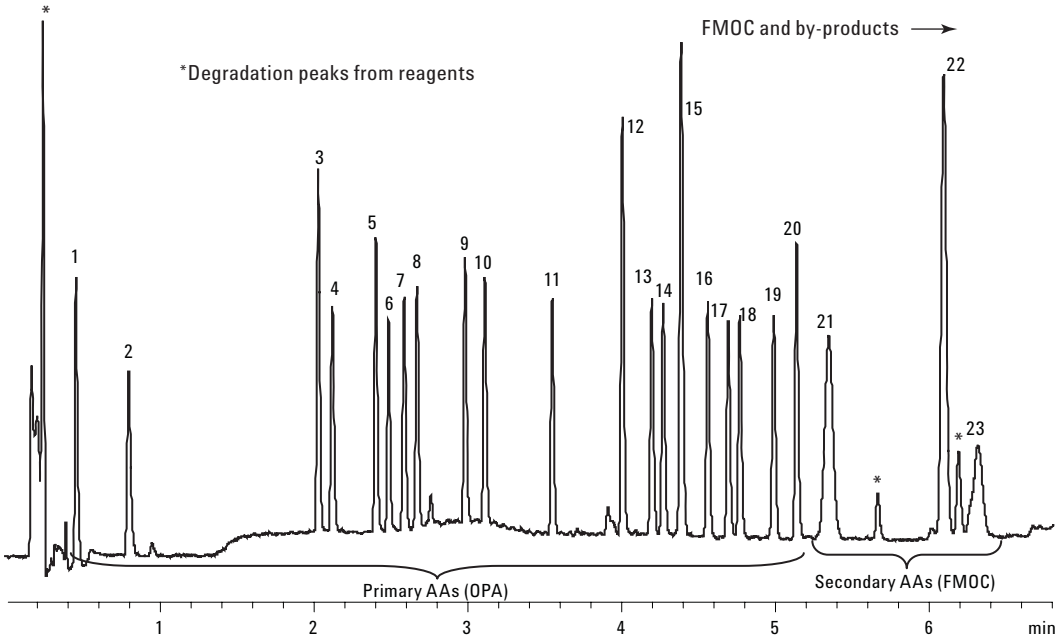

Figure 2. Amino acid analysis on Rapid Resolution HT Eclipse Plus C18, 4.6 x 50 mm, 1.8-µm column: DAD 125 pMoles on column.

Table 1. Names and Order of Elution for OPA and FMOC Derivatives of Amino Acids

Peak #	AA name	AA abbreviation	Derivative type
1	Aspartic Acid	ASP	OPA
2	Glutamic Acid	GLU	OPA
3	Asparagine	ASN	OPA
4	Serine	SER	OPA
5	Glutamine	GLN	OPA
6	Histidine	HIS	OPA
7	Glycine	GLY	OPA
8	Threonine	THR	OPA
9	Arginine	ARG	OPA
10	Alanine	ALA	OPA
11	Tyrosine	TYR	OPA
12	Cystine	CYS-CYS	OPA
13	Valine	VAL	OPA
14	Methionine	MET	OPA
15	Norvaline†	NVA	OPA
16	Tryptophan	TRP	OPA
17	Phenylalanine	PHE	OPA
18	Isoleucine	ILE	OPA
19	Leucine	LEU	OPA
20	Lysine	LYS	OPA
21	Hydroxyproline	HYP	FMOC
22	Sarcosine†	SAR	FMOC
23	Proline	PRO	FMOC

† Internal Standard

Experimental

Instrument

The results obtained in this application were all performed on an Agilent 1200SL HPLC system consisting of the following components:

G1312B, binary pump SL
G1379B, micro degasser
G1367C, high-performance well plate autosampler (WPS) configured with 54 x 2 mL sample tray in front and 15 x 6 mL tray in back
G1316B, thermostatted column compartment SL (TCC) with low dispersion kit installed
G1315C, diode array detector SL (DAD) with semi-micro flow cell
G1321A, fluorescence detector (FLD)

All tubing used throughout the instrument is 0.12 mm id (0.005 inch).

It is likely that older instruments, such as 1100 binary systems, and possibly 1100 and 1200 quaternary pump-based systems can run this analysis on 4.6 mm id columns, though this has not been tested. The use of 3.0 mm id and 2.1 mm id is not recommended on those systems without extensive testing due to the larger delay volume encountered using those systems; this may cause anomalies in some of the earlier eluting peaks due to dwell volume effects.

Instrument Configuration

Pump Parameters: mixer and pulse dampener bypassed with 100 mm long tubing (0.12 mm id), bypass programmed (at 0.1 min after inject command; see WPS parameters below), compressibility settings used: A = 35, B = 80

Flow: 0.42 mL/min for 2.1 mm id; 0.85 mL/min for 3.0 mm id; 2.00 mL/min for 4.6 mm id

Gradient Timetable:	Time (min)	%B
	0.0	2.0
	1.0	2.0
	7.0	57.0
	7.1	100.0
	8.4	100.0
	8.6	2.0
	Stop time 8.7	

DAD: PW 0.01 min; slit 4 nM; Stop time 7 min (adjust as needed); Cell volume = 5 µl, 6 mm flow path (Agilent P/N G1315-60025)
Signal A: 338, 10 nm; Ref 390, 20 nm
Signal B: 262, 16 nm; Ref 324, 8 nm
Signal C: 338, 10 nm; Ref 390, 20 nm
Signal D: 230, 16 nm; Ref 360, 100 nmM
Timetable Signal C): 0.00 min 338, 10 nm; Ref 390, 20 nm; 5.53 min 262, 16 nm; Ref 324, 8 nm (adjust as needed; 3.0 mm id transition at approximately 5.45 min, 4.6 mm id transition at approximately 5.4 min)

Table 2. Preparation of Low-Sensitivity Amino Acid Standard Solutions (Prepare the three low-sensitivity standards by mixing together stock solutions in the volumes shown.)

	Concentration of final AA solutions (pMoles/µL)		
	900	**225**	**90**
Take 5 mL 18 nMoles EAA	5 mL	5 mL	5 mL
Dilute with 0.1 N HCl		15 mL	45 mL
Diluted EAA mix	**5 mL**	**20 mL**	**50 mL**
Take 5 mL diluted EAA mix	5 mL	5 mL	5 mL
Add 10 nMoles ISs solution	5 mL	5 mL	5 mL
EAA-ISs mix	**10 mL**	**10 mL**	**10 mL**
Take 100 µL EAA-ISs mix	100 µL	100 µL	100 µL
Add 1000 pMoles AA standard	900 µL	—	—
Add 250 pMoles AA standard	—	900 µL	—
Add 100 pMoles AA standard	—	—	900 µL
Final AA solution with EAA and 500 pMoles/µL ISs	**1 mL**	**1 mL**	**1 mL**

FLD: PW 0.01 min; Stop time 7 min (adjust as needed), never use this detector in series before another due to fragility of flow cell Ex 230 nm; Em 450 nm; Filter 295 nm (Default filter)
Timetable Signal: 0.00 min Ex 230 nm, Em 450 nm; PMT Gain 9 (as needed) 5.53 min Ex 266 nm, Em 305 nm; PMT Gain 9 (as needed; wave length transition times as for DAD + approximately 0.03 min)

TCC: used with low dispersion kit installed; T = 40 °C for column side, 30 °C for exit side. Low dispersion kit used on both sides.

WPS: Default volume set to 0.5 µl, default speed used throughout injector program is 200 µl/min

Injector program
1) Draw 2.5 µl from Borate vial (Agilent P/N 5061-3339)
2) Draw 0.5 µl from Sample vial
3) Mix 3 µl in washport 5X
4) Wait 0.2 min
5) Draw 0.5 µl from OPA vial (Agilent P/N 5061-3335)
6) Mix 3.5 µl in washport 6X
7) Draw 0.4 µl from FMOC vial (Agilent P/N 5061-3337)
8) Mix in 3.9 µl in washport 10X
9) Draw 32 µl from Injection Diluent vial
10) Mix 20 µl in washport 8X
11) Inject
12) Wait 0.10 min
13) Valve bypass

Amino Acid Mix for Calibration Curves

For the construction of calibration tables and curves, 17 amino acids, plus the 4 extended amino acids, are combined at various concentrations with fixed amounts of internal standards. The internal standards (ISs) (norvaline and sarcosine) are part of the supplemental amino acid kit (P/N 5062-2478). The remaining amino acids in this kit (glutamine [GLN], asparagine [ASN], tryptophan [TRP], and hydroxyproline [HYP]) form the extended amino acids (EAA). To make the appropriate solutions, refer to Tables 2 and 3 for low- and high-sensitivity standards, respectively.

Table 3. Preparation of High-Sensitivity Amino Acid Standard Solutions (Prepare the three high-sensitivity standards by mixing together stock solutions in the volumes shown.)

	Concentration of final AA solutions (pMoles/µL)		
	90	**22.5**	**9**
Take 5 mL 1.8 nMoles EAA	5 mL	5 mL	5 mL
Dilute with 0.1 N HCl	—	15 mL	45 mL
Diluted EAA mix	**5 mL**	**20 mL**	**50 mL**
Take 5 mL diluted EAA mix	5 mL	5 mL	5 mL
Add 1.0 nMoles ISs solution	5 mL	5 mL	5 mL
EAA-ISs mix	**10 mL**	**10 mL**	**10 mL**
Take 100 µL EAA-ISs mix	100 µL	100 µL	100 µL
Add 100 pMoles AA standard	900 µL	—	—
Add 25 pMoles AA standard	—	900 µL	—
Add 10 pMoles AA standard	—	—	900 µL
Final AA solution with EAA and 50 pMoles/µL ISs	**1 mL**	**1 mL**	**1 mL**

Amino Acid Standards (10 pMoles/µL to 1 nMoles/µL): Divide each 1-mL ampoule of standards (P/N 5061-3330 through 5061-3334) into 100-µL portions in conical vial inserts, cap, and refrigerate aliquots at 4 °C. Calibration curves may be made using from two to five standards, depending on experimental need.

Extended Amino Acid (EAA) Stock Solution: This solution is made using four of the six amino acids in the supplemental amino acid kit (P/N 5062-2478). For use with low-sensitivity standards (Table 2), make a 25-mL solution containing 18 nMoles/µL of glutamine, asparagine, tryptophan, and 4-hydroxy-proline in deionized water. Sonicate the solution until dissolved. Store the solution at 4 °C. For use with high-sensitivity standards (Table 3), make a 1.8 nMoles/µL solution by diluting 5 mL of the 18 nMoles/µL standard with 45 mL deionized H_2O.

ISs Stock Solution: These solutions are made using two of the six amino acids in the supplemental amino acid kit (P/N 5062-2478). For use with low-sensitivity standards (Table 2), make a 25-mL solution containing 10 nMoles/µL of norvaline and sarcosine in deionized water. Sonicate the solution until dissolved. Store in refrigerator (4 °C). For use with high-sensitivity standards (Table 3), make a 1 nMoles/µL solution by diluting 5 mL of the 10 nMoles/µL standard with 45 mL deionized H_2O. Store at 4 °C.

HPLC Columns

ZORBAX Rapid Resolution HT Eclipse Plus C18, 2.1 x 50 mm, 1.8 µm, P/N 959741-902
ZORBAX Rapid Resolution HT Eclipse Plus C18, 3.0 x 50 mm, 1.8 µm, P/N 959941-302
ZORBAX Rapid Resolution HT Eclipse Plus C18, 4.6 x 50 mm, 1.8 µm, P/N 959941-902

Mobile Phase and Injection Diluent

Mobile phase A: 10 mM Na_2HPO_4: 10 mM $Na_2B_4O_7$, pH 8.2: 0.5 mM NaN_3 (5.6 gm anhydrous Na_2HPO_4 + 15.2 gm $Na_2B_4O_7 \cdot 10H_2O$ in 4 L water + 32 mg NaN_3). Adjust to approximately pH 9 with 6 mL concentrated HCl and then small drops until pH 8.2. ***Be cautious with strong acids***. Filter through 0.45 µm regenerated cellulose membranes (Agilent P/N 3150-0576). Stable for approximately 1.5 weeks at room temperature.

Mobile phase B: Acetonitrile methanol: water (45:45:10 by volume). All mobile-phase solvents must be HPLC grade.

Injection Diluent: 100 ml mobile phase A + 1,500 µL concentrated H_3PO_4 in a 100-mL bottle. Keep at 4 °C. Dispense into 6-mL vials before use. ***Be cautious with strong acids.***

See *Ordering Information* for descriptions and part numbers of all supplies available for AAA.

Derivatization Reagents

Borate Buffer: Agilent P/N 5061-3339
Solution is 0.4 N in water, pH 10.2. Keep refrigerated (4 °C). Dispense as necessary.

FMOC Reagent: Agilent P/N 5061-3337
Pipette 200-µL aliquots of the 1-mL FMOC reagent into conical inserts, cap immediately, and refrigerate (4 °C); solution is useable for 7 to 10 days, maximum, after dispensing if kept at 4 °C; useable for 1 day if kept at room temperature.

OPA Reagent: Agilent P/N 5061-3335
Pipette 200-µL aliquots of the 1-mL OPA reagent into conical inserts, cap immediately, and refrigerate (4 °C); solution is useable for 7 to 10 days, maximum, after dispensing, if kept at 4 °C; useable for 1 day if kept at room temperature.

Water: Deionized water, HPLC Grade

See *Ordering Information* for descriptions and part numbers of all supplies available for AAA.

Sample Preparation

The various bottled beer samples analyzed in this paper were obtained locally. The only preparation needed was degassing, which was accomplished quite simply by sonication. Caution must be used because sonication causes instantaneous, explosive degassing of carbonated beverages. It is recommended that the beer be poured into a wide-mouth beaker of greater volume than the beer, to allow for the large amount of foam generated.

Results and Discussion

Separation Options

In this application we demonstrate that the new method allows AAA to be done on a wider variety of columns than in previous versions. We report the results run on Rapid Resolution HT Eclipse Plus C18 columns, all of which were made with 1.8-µm particles in 4.6-, 3.0-, or 2.1-mm id and 50-mm length. The only differences in each method are the flow rate, so that a constant column flow velocity is maintained, and detector wavelength switching time, so that secondary AAs can be included on the same chromatogram.

We have also run this separation on Rapid Resolution HT Eclipse Plus C8 columns and on Rapid Resolution HT Eclipse XDB-C18 and -C8 columns, packed with 1.8-µm particles and the same variety of dimensions (data not shown). All columns gave very similar results to those obtained on the Rapid Resolution HT Eclipse Plus C18 (data not shown). The comparative separations obtained on different diameter Rapid Resolution HT Eclipse Plus C18 columns are shown in Figures 3 and 4 for DAD and FLD, respectively. As may be seen, there are only slight shifts in retention times for all chromatograms no matter what diameter is used. The shifts are caused by the slight changes in delay times among the three different diameter columns owing to the fact that the delay volume is constant; thus, the delay times are less for higher flow rates.

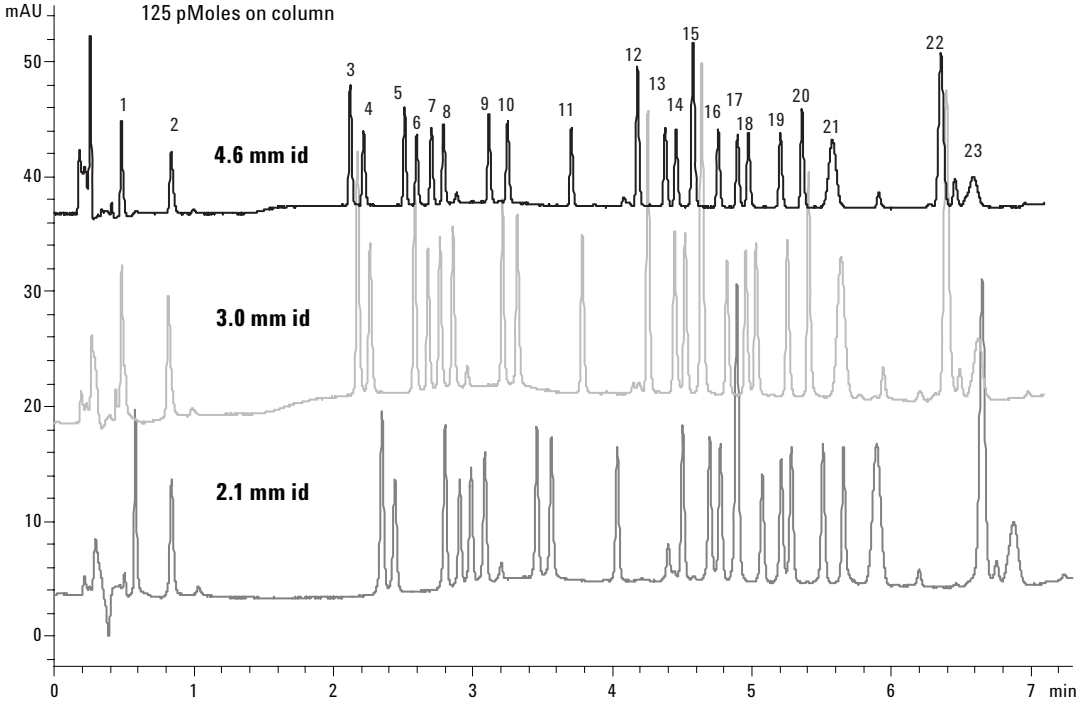

Figure 3. Scalability of AAA on various Rapid Resolution HT Eclipse Plus C18, 50 mm long, 1.8-µm columns, DAD.

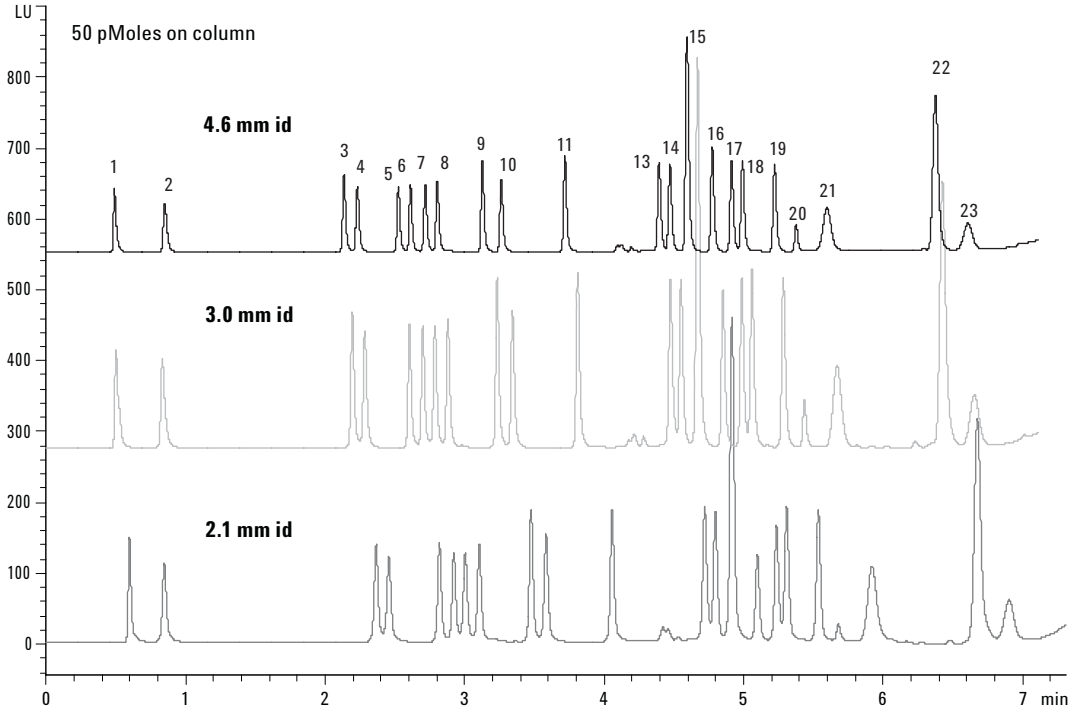

Figure 4. Scalability of AAA on various Rapid Resolution HT Eclipse Plus C18, 50 mm long, 1.8-µm columns, FLD.

Reproducibility and Linearity

The reproducibility of the secondary AAs in previous versions of this analysis was always less than that of the primaries [1−4]. With the current methodology we have improved the secondary AAs' reproducibility to equality with the primary AAs'. This data is shown in Tables 4 and 5 for ZORBAX Eclipse Plus-C18, 2.1-mm id columns, without ISs and in Table 6 for 4.6-mm id columns with ISs. The absolute peak area reproducibilities in Table 4 are superior. The average RSD is below 2% and only two are above 3% for all the AAs. Note that the linearities shown in Tables 5 and 6 are also excellent for all the AAs, being very close to 1.00 for all with a small edge to the linearities obtained with ISs. The results without ISs may be seen graphically in Figures 5 and 6 for Eclipse Plus-C18, 3.0-mm id columns. The chromatographic reproducibility of low-level AAA on the Eclipse Plus-C18, 2.1-mm id column may be seen in Figure 7 for 5.0 pMoles on column. The staggered overlay allows the retention time reproducibility and peak sizes to be easily compared. This is raw data with no corrections and no ISs.

Table 4. Absolute Peak Area Reproducibility on Rapid Resolution HT Eclipse Plus C18, 2.1 x 50 mm, 1.8-µm Columns Using an Agilent 1200SL HPLC System, no ISs

Detector reproducibility
50 pMoles FLD; 125 pMoles DAD; raw data areas

AA name	Abbreviation	FLD RSD (n = 10)	DAD RSD (n = 12)
Aspartic acid	ASP	1.1	0.8
Glutamic acid	GLU	0.8	2.3
Asparagine	ASN†	1†	1.6†
Serine	SER	0.9	1
Glutamine	GLN*	2.9*	2.1*
Histidine	HIS	0.8	2.1
Glycine	GLY	1	1.4
Threonine	THR	1.2	0.9
Arginine	ARG	1	1.9
Alanine	ALA	0.9	.3
Tyrosine	TYR	1	1
Cystine	CYS-CYS	NA	1.1
Valine	VAL	1.5	0.9
Methionine	MET	2.7	0.8
Norvaline	NVA	3.3	2.3
Tryptophan	TRP	1.3	1.1
Phenylalanine	PHE	1	0.9
Isoleucine	ILE	0.9	1.5
Leucine	LEU	2.1	0.7
Lysine	LYS	4.4	0.8
Hydroxyproline	HPA	1.8	1.7
Sarcosine	SAR	1.9	2.3
Proline	PRO	3.0	2.2
Average RSD =		1.7	1.4

† Asparagine is somewhat unstable in solution
* Glutamine is very unstable in solution

Table 5. Linearity of AAA on Rapid Resolution HT Eclipse Plus C18, 2.1 x 50 mm, 1.8-µm Columns Using an Agilent 1200SL HPLC System, no ISs

	Using raw data areas	
Amino acid	DAD coefficients of linearity (r^2)	FLD coefficients of linearity (r^2)
ASP	0.9949	0.9992
GLU	0.9976	0.9995
ASN†	0.9928†	1.0000†
SER	0.9933	0.9997
GLN*	0.9966*	0.9937*
HIS	0.9951	0.9996
GLY	0.9931	0.9996
THR	0.9942	0.9996
ARG	0.9960	0.9998
ALA	0.9965	0.9996
TYR	0.9936	0.9997
CYS-CYS	0.9818	NA
VAL	0.9952	0.9998
MET	0.9944	0.9997
NVA	0.9989	0.9991
TRP	0.9923	0.9987
PHE	0.9925	0.9996
ILE	0.9934	0.9993
LEU	0.9944	0.9994
LYS	0.9857	0.9957
HYP	0.9980	0.9924
SAR	0.9975	0.9960
PRO	0.9981	0.9969

† Asparagine is somewhat unstable in solution.
* Glutamine is very unstable in solution.

Table 6. Linearity of AAA on Rapid Resolution HT Eclipse Plus C18, 4.6 x 50 mm, 1.8-µm Columns Using an Agilent 1200SL HPLC System with ISs

Amino acid	DAD coefficients of linearity (r^2)	FLD coefficients of linearity (r^2)
ASP	0.99995	0.99887
GLU	0.99916	0.99879
ASN†	1.00000†	0.99581†
SER	0.99982	0.99880
GLN*	0.99996*	0.99549*
HIS	0.99978	0.99993
GLY	0.99982	0.99681
THR	0.99996	0.99941
ARG	0.99993	0.99946
ALA	0.99984	0.99924
TYR	0.99991	0.99946
CYS-CYS	0.99992	NA
VAL	0.99992	0.99886
MET	0.99992	0.99996
NVA	IS+	IS+
TRP	0.99997	0.99687
PHE	0.99988	0.99943
ILE	0.99973	0.99910
LEU	0.99986	0.99932
LYS	0.99956	0.99979
HYP	0.99991	0.99751
SAR	IS+	IS+
PRO	0.99998	0.99979

† Asparagine is somewhat unstable in solution.
* Glutamine is very unstable in solution.
+ IS at 250 pMoles on column for DAD and 25 pMoles on column for FLD

Extended Amino Acids (EAAs) and Internal Standards (ISs)

There are several amino acids that are destroyed during acid hydrolysis of proteins (asparagine, glutamine and tryptophan) and one that is rarely seen anywhere but in structural proteins (hydroxyproline). Additionally, many analysts prefer to use ISs in their sample in an effort to improve accuracy and reproducibility. It is necessary to choose the ISs carefully in order to obtain the best results. There are two common ways these are used:

1. Addition to the sample before any sample manipulations at all (i.e., before acid hydrolysis). This corrects for all variations, providing, of course, that the ISs are stable to hydrolysis and have the same derivatization reactivity as the other components in the sample. In order to properly use this mode the standards should also undergo the hydrolysis process; but this usually leads to more variation due to the different stabilities of various amino acids. An example is serine, which is destroyed progressively, dependent on hydrolysis time. This brings us to the most useful way of running with ISs.
2. Addition just before analysis, which corrects for volumetric pipetting errors and autosampler variations and also assumes that the reactivities of ISs and sample components are the same. This is the method used in this application.

For the OPA/FMOC analysis demonstrated here, there are two ISs available: norvaline for primary amino acids and sarcosine for secondary amino acids. These AAs may be used for both methods of using ISs (unpublished data); but method 2 is definitely the preferred way. When the four amino acids listed above (EAAs) are included in the standards using the Agilent Amino Acids Supplement

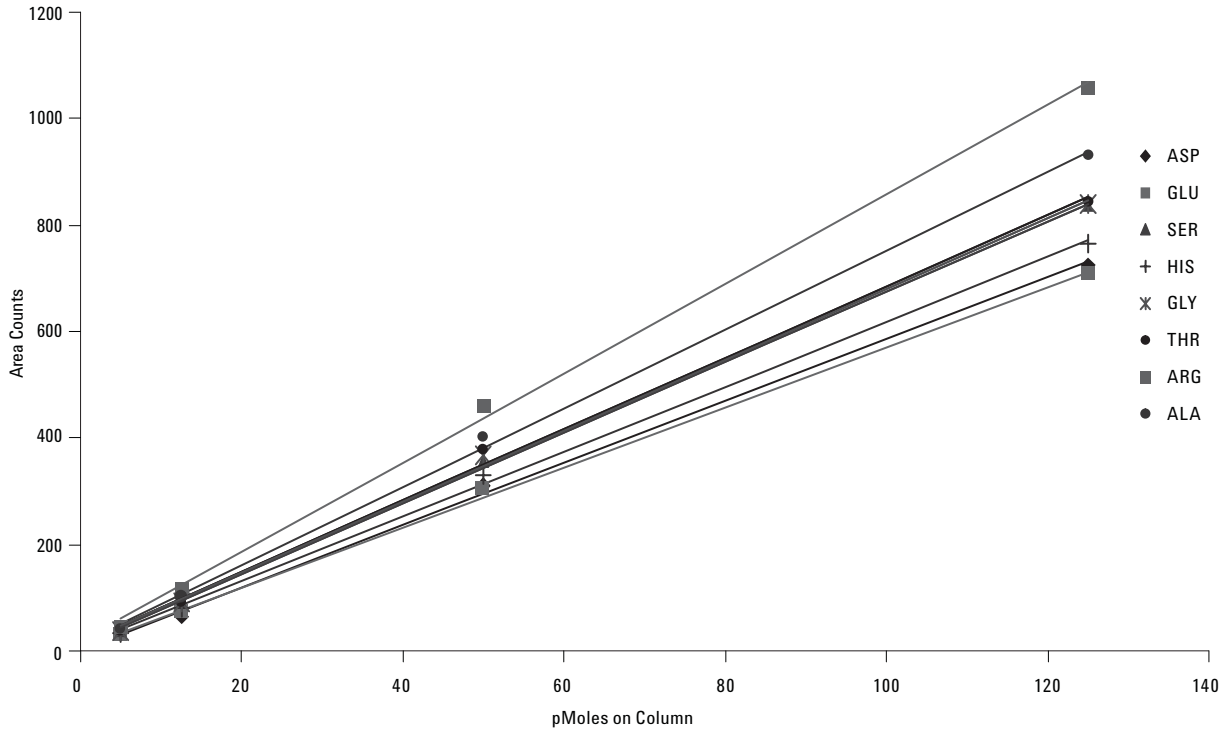

Figure 5. Amino acid linearities by FLD on Rapid Resolution HT Eclipse Plus C18, 3.0 x 50 mm, 1.8 µm.

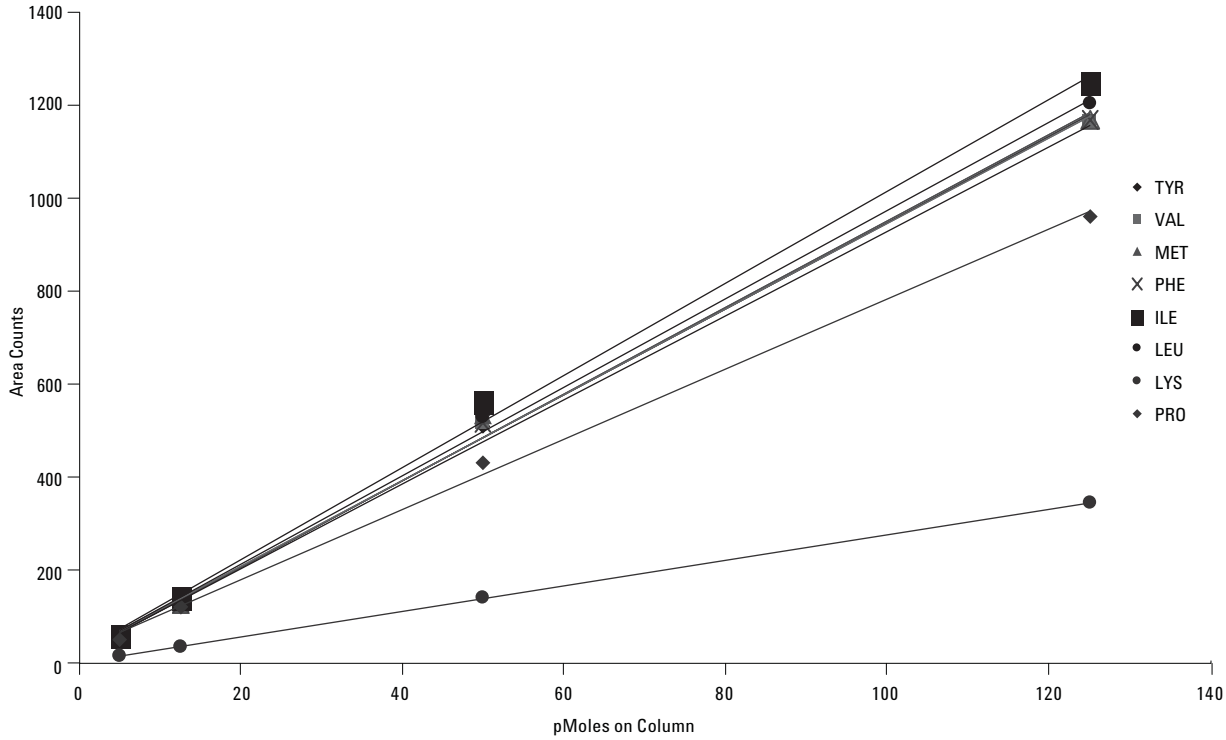

Figure 6. Amino acid linearities by FLD on Rapid Resolution HT Eclipse Plus C18, 3.0 x 50 mm, 1.8 µm.

kit (P/N 5062-2478), all are well separated in this new version of AAA. This has been shown in Figures 3 and 4 already on a Rapid Resolution HT Eclipse Plus C18, 2.1, 3.0, or 4.6 x 50 mm, 1.8-µm columns. In these two figures the ISs, norvaline and sarcosine, are added at 100 pMoles on column for FLD chromatograms and at 250 pMoles on column for DAD simply to demonstrate where they appear in the chromatogram. The original process for the addition of the EAAs and ISs has been described previously [4]. This procedure is used to produce an IS-based calibration table for the quantitation needed for real-world samples. The process is described in the Experimental section and is shown in Tables 2 and 3.

Real samples

The result of a comparison of a variety of lager beers is shown in Figure 8. Each beer has been spiked with ISs at a level of 500 pMoles/µL each so that a comparison of the relative AA content

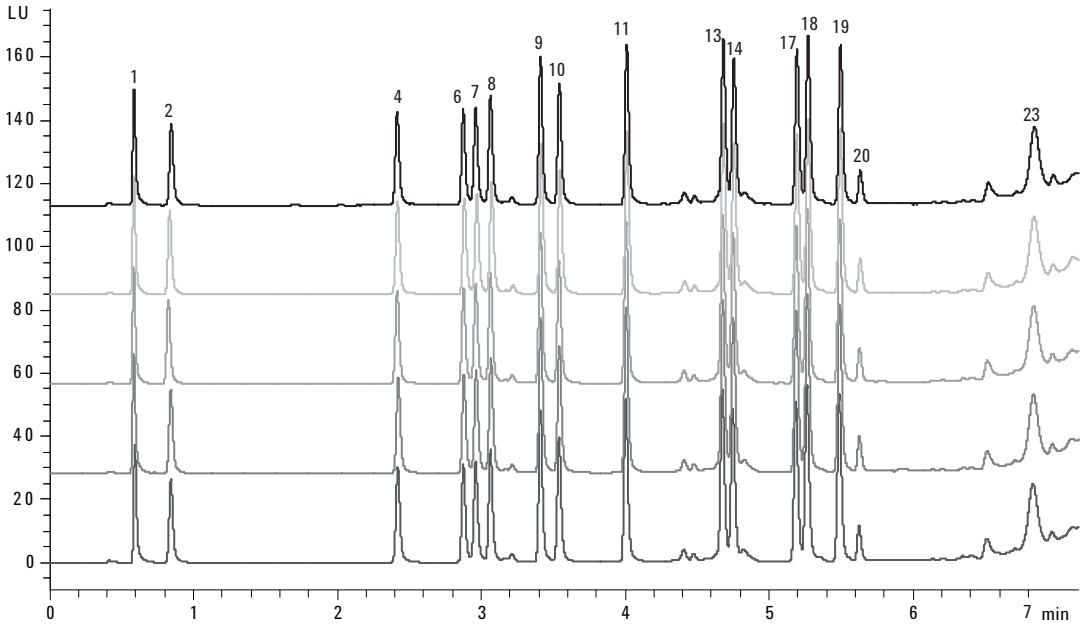

Figure 7. Reproducible AAA of 5.0 pMoles on column using a Rapid Resolution HT Eclipse Plus C18, 2.1 x 50 mm, 1.8 µm, FLD, no ISs.

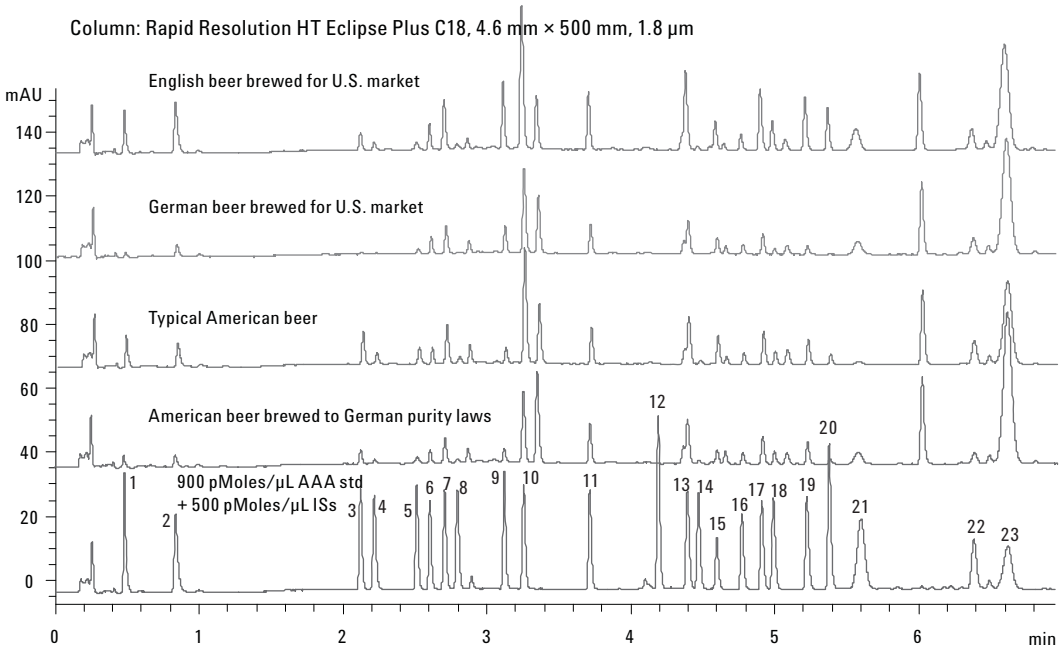

Figure 8. Comparison of a variety of beers available in the USA using the 1200SL, DAD and ISs.

could be calculated. This comparison shows that the relative AA content of these beers is not consistent with many peoples' perception—that "typical" American beers have lower AA content than do traditional German beers. The results indicate that at least some German and English beer manufacturers now brew their beers sold in the U.S. for American taste. It may be that the micro-brew from the U.S. is more consistent with a German beer brewed for German distribution, since it is brewed in compliance with German purity laws and therefore, more representative of "traditional" German brewing. The results are tabulated in Table 7.

Acid hydrolysis and subsequent AAA are commonly used in the analysis of proteins. Cell culture media are also monitored, both in the laboratory and in the new Process Analytical Technology (PAT) initiative now being implemented throughout biotechnology and pharmaceutical companies. AAA on these subjects will be presented in subsequent papers.

Table 7. Comparison of the Amino Acid Content of Various Beers

Amino acid	Typical American beer µMoles /mL	American beer brewed to German purity laws µMoles /mL	German beer brewed for U.S. market µMoles /mL	English beer brewed for U.S. market µMoles /mL
ASP	1.06	0.73	0.26	1.15
GLU	1.07	0.93	0.80	1.98
ASN	1.19	0.98	0.14	0.54
SER	0.45	0.40	0.12	0.26
GLN	0.67	0.76	0.38	0.33
HIS	0.68	1.00	1.07	0.89
GLY	1.54	2.00	1.61	1.57
THR	0.30	0.34	0.74	0.20
ARG	0.48	0.94	1.29	1.83
ALA	4.28	5.14	4.56	4.60
TYR	1.36	2.86	1.65	1.77
CYS-CYS	0.08	0.10	0.08	0.05
VAL	1.92	3.35	1.93	2.83
MET	0.17	0.27	0.11	0.16
NVA	IS	IS	IS	IS
TRP	0.58	1.07	0.80	0.67
PHE	1.33	2.21	1.23	2.07
ILE	0.54	0.97	0.34	0.94
LEU	1.02	1.71	0.55	1.75
LYS	0.30	0.32	0.08	0.90
HYP	0.20	1.74	1.17	1.26
SAR	IS	IS	IS	IS
PRO	8.65	34.84	15.88	10.29
Total =	27.9	62.7	34.8	36.0

Conclusions

All aspects of the AAA have been improved by this work. The use of 1.8-µm sized particle columns has enabled the cycle time of the analysis to be more than cut in half, from approximately 35 minutes to approximately 13.5 minutes. The peak shapes of the early eluting aspartic acid and glutamic acid have been improved. The reproducibility of the secondary AAs has been improved, as has that of the slowest AA to react, lysine. The linearity is excellent (nearly 1); and the average peak area reproducibility is below 2%. Use of the new Rapid Resolution HT Eclipse Plus C18, 1.8-µm columns of any diameter from 2.1 to 4.6 mm has been demonstrated. The new mobile phase and injector conditions have made it very simple to convert from 2.1- to 3.0- or 4.6-mm id columns by simply changing flow rate. It is also possible to use Rapid Resolution HT Eclipse Plus C8 or Rapid Resolution HT Eclipse XDB-C18 or -C8 columns as well (data not shown).

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ZORBAX Rapid Resolution HT Eclipse Plus C18 HPLC Columns

Description	Size (mm)	Particle Size (µm)	Agilent Part No.
Eclipse Plus C18	4.6 x 50 mm	1.8 µm	959941-902
Eclipse Plus C18	3.0 x 50 mm	1.8 µm	959941-302
Eclipse Plus C18	2.1 x 50 mm	1.8 µm	959741-902

Derivatization Reagents

Description	Agilent Part No.
Borate Buffer: 0.4 M in water, pH 10.2, 100 mL	5061-3339
FMOC Reagent, 2.5 mg/mL in ACN, 10 x 1 mL ampoules	5061-3337
OPA Reagent, 10 mg/mL in 0.4 M borate buffer and 3-mercaptoproprionic acid, 6 x 1 mL ampoules	5061-3335
DTDPA Reagent for analysis of cysteine, 5 g	5062-2479

Mobile Phase and Injection Diluent Components

Description	Manufacturer	Manufacturer Part No.
Na_2HPO_4, Sodium Phosphate, Dibasic, Anhydrous	Sigma	71639
$Na_2B_4O_7.10H_2O$, Sodium Tetraborate Decahydrate	Sigma	S 9640
NaN_3, Sodium Azide	Sigma	S 2002
H_3PO_4, ortho Phosphoric Acid	Sigma	79617

Vials

Description	Agilent Part No.
100-µL conical insert with polymer feet, 100/pk	5181-1270
Amber, wide-opening, write-on, screw-cap vial, 2 mL, 100/pk	5182-0716
Blue polypropylene cap, PTFE/silicone septum, 100/pk	5182-0721
Clear glass screw-cap vial, 6 mL, 16 mm cap size, 100/pk	9301-1377
Screw caps, 16 mm, 100/pk	9301-1379
PTFE/silicone septa, 16 mm, 100/pk	9301-1378

Standards

Description	Agilent Part No.
Amino Acid Standards in 0.1 M HCl, 10 x 1 mL ampoules	
1 nMoles/µL	5061-3330
250 pMoles/µL	5061-3331
100 pMoles/µL	5061-3332
25 pMoles/µL	5061-3333
10 pMoles/µL	5061-3334
Supplemental Amino Acids: Nva, Sar, Asn, Gln, Trp, Hyp, 1 g each	5062-2478

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Measurement of Macro and Trace Elements in Plant Digests Using the 7500c ICP-MS System

Application

Food

Author

Kazuo Yamanaka
Agilent Technologies
Nakacho 1-15-5
Musashino-shi
Tokyo 180-8543
Japan

Fred Fryer
Agilent Technologies
12/2 Eden Park Drive,
North Ryde NSW 2113
Australia

Abstract

Inductively coupled plasma mass spectrometry is a powerful tool for the investigation of many materials. The Agilent 7500c with Octopole Reaction System was used to analyze major, minor and trace elements in two standard reference plant materials. The data obtained using the 7500c is compared to the certificate reference values and to results that were generated using inductively coupled plasma optical emission spectroscopy. Results for all elements obtained using the 7500c agree with the certified values.

Introduction

The reliable measurement of trace elements in food is becoming more important as information is revealing that over-dependence on processed grains such as wheat and rice is resulting in a nutritionally poor diet. Micronutrient [1] malnutrition is an identified problem that has coincided with the rapid adoption of modern cereal cropping systems. Profitable and sustainable agriculture depends on the understanding of the nutrients required and available for plant growth, as well as the nutrients for a balanced human diet.

"World food production will need to double over the next 30 years to keep pace with increasing demands from both industrialized and rapidly developing countries. As well as the need to increase production, there will be an increase in demand for higher quality and healthier food products as developing countries become more affluent."

Taken from the Commonwealth Scientific and Industrial Research Organisation (CSIRO) website: http://www.csiro.gov.au (select: Agribusiness/Field Crops/Field Crops & Australia)

Human dietary micronutrients are required by humans in very small amounts. They include at least 14 trace elements (As, B, Cr, Cu, F, I, Fe, Mn, Mo, Ni, Se, Si, V, Zn) as well as 13 vitamins (thiamin, riboflavin, niacin, pantothenic acid, biotin, folic acid, vitamins B6, B12, C, A, D, E, K)

The recommended daily intake of the micronutrient trace elements is of the order of:

- mg per day for B, Cu, F, Fe, Mn, Zn
- µg per day for As, Cr, I, Mo, Ni, Se, Si, V

Accurate determination of these trace elements in food materials is useful in ensuring that dietary intake is providing adequate levels of micronutrient elements. Due to the very low concentrations that must be measured and, in many cases, the high and variable sample matrix in which the measurements must be made, this analysis has proved challenging for elemental analysis instrumentation. Traditionally, a combination of techniques was required for a complete analysis of the plant digest—typically Graphite Furnace Atomic Absorption Spectroscopy (GFAAS), Hydride-Atomic Absorption Spectroscopy (HG-AAS) and Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES).

Such is the performance and elemental coverage of modern inductively coupled plasma mass spectrometry (ICP-MS) instrumentation, in many cases (metals analysis in drinking water, for example) a single ICP-MS has replaced all of the above mentioned techniques, enabling all analytes to be determined in a single measurement. The analysis of plant and food digests for nutritional studies is more challenging. In ICP-MS, isobaric interferences arise from the argon used to sustain the plasma and from the reagents used for sample preparation. Table 1 summarizes some well-known interfering species. In biological sample analysis, there are well-documented interferences for ICP-MS that can bias the measurement of Fe, Cr, V, As and Se at trace levels, with the result that ICP-MS has not yet been widely adopted by the foods industry.

Table 1. Examples of Potential Interferences in Biological/Clinical Matrices

Element	Mass	Molecular interference
Cr	52; 53	$^{40}Ar^{12}C$, $^{36}Ar^{16}O$, $^{35}Cl^{16}O^{1}H$; $^{37}Cl^{16}O$
V	51	$^{35}Cl^{16}O$
Fe	56	$^{40}Ar^{16}O$
Cu	63	$^{40}Ar^{23}Na$
As	75	$^{40}Ar^{35}Cl$
Se	77; 78; 80	$^{40}Ar^{37}Cl$; $^{40}Ar^{38}Ar$; $^{40}Ar^{40}Ar$;

One obvious way to remove interferences is to eliminate the source of the interfering species. Traditionally plant materials are digested on a hot plate using a mixture of nitric and perchloric acids. Chloride-based mass spectral interferences are introduced by this method. An alternative sample preparation method is available using microwave digestion with hydrogen peroxide and nitric acid. This digestion media does not generate additional interferences for ICP-MS and is a complete digest. However, for high sample numbers, the traditional hot plate digest offers higher sample throughput than closed vessel microwave digestion [2].

Recently, the advent of collision/reaction cells has improved the detection capability of quadrupole ICP-MS (ICP-QMS) by removing spectral interferences on analytes such as Fe, Cr, V, As and Se. The Agilent 7500c ICP-MS features an Octopole Reaction System (ORS) for highly efficient removal of multiple interferences arising from complex sample matrices. The ORS removes interferences by either reacting a gas with the interference or by preventing the interfering species from entering the analyzer stage using a process called energy discrimination. The 7500c exhibits highly efficient interference removal. The Ar_2 overlap on Se at mass 80 is virtually eliminated, reducing the background equivalent concentration from 100's of ppb to <10 ppt. Moreover, the 7500c was designed specifically to handle complex matrices such as plant and food digests.

The key to the successful multi-element determination of trace elements in complex samples is a combination of matrix tolerance and efficient interference removal. Matrix tolerance is mainly determined by the "plasma efficiency", which must be optimized to ensure efficient sample decomposition, and is monitored by the CeO/Ce ratio. An efficient plasma minimizes the formation of plasma- and matrix-based interferences, while maximizing the conversion of analyte atoms into ions.

The importance of matrix tolerance of any ICP-MS system should not be underestimated, as this leads to improved analytical accuracy, better tolerance to matrix changes and reduced requirements to carry out routine maintenance of the vacuum, ion lens and pump components.

All of these aspects contribute to the usability of the analytical instrument, as routine maintenance contributes far more to the down-time of a modern, reliable ICP-MS instrument than hardware breakdowns. The unique capability of the Agilent 7500 Series lies in the mode of operation of the plasma source, which decomposes sample matrices five to 10 times more efficiently than is typical for other ICP-MS instruments.

The 7500c was designed specifically to handle complex, high matrix samples. A robust 27.12-MHz plasma, low sample uptake rate, cooled spray chamber and proven small orifice interface protect the ORS from contamination by undissociated sample matrix. A novel ion optic, mounted outside the high vacuum region for easy access, further protects the reaction cell, which features an octopole for optimum ion transmission. The octopole is mounted off-axis to minimize random background levels. A schematic of the 7500c is shown in Figure 1.

Some of the important instrument parameters that contribute to good matrix decomposition are:

- The standard low sample flow rate (100 to 400 µL/min) and Peltier-cooled spray chamber reduce the sample and water vapor loading on the plasma, which leads to a hotter plasma central channel.
- The 7500 Series uses a high efficiency, solid state 27.12-MHz plasma RF generator, ensuring good energy transfer into the plasma central channel.
- The unique wide internal diameter plasma torch design ensures that the sample aerosol is resident in the plasma for sufficient time to ensure complete matrix decomposition, leading to exceptionally good matrix decomposition (low CeO/Ce ratio).

The optimized interface design, which uses the smallest skimmer cone orifice of any commercial ICP-MS instrument, ensures that minimal sample matrix is passed into the high-vacuum part of the instrument, dramatically reducing the requirement for routine maintenance of the interface cones, the ion lenses and the collision cell.

In summary, as the complexity of the sample matrix increases, the benefit of minimized interference levels becomes more significant. Because modern analytical laboratories rarely have the luxury of pre-analyzing samples to identify the matrix, it is impractical to rely on matrix matching of the samples or data correction using complicated interference equations.

Sample Preparation and Analysis

About 800 mg of sample was accurately weighed and carefully heated with 10 mL nitric acid (70%), followed by gentle heating with the addition of 8 mL perchloric acid (70%) until colorless. After cooling, 30 mL water was added and heating resumed for 10 min. Finally, the solutions were cooled, then made to 100 mL volume with water.

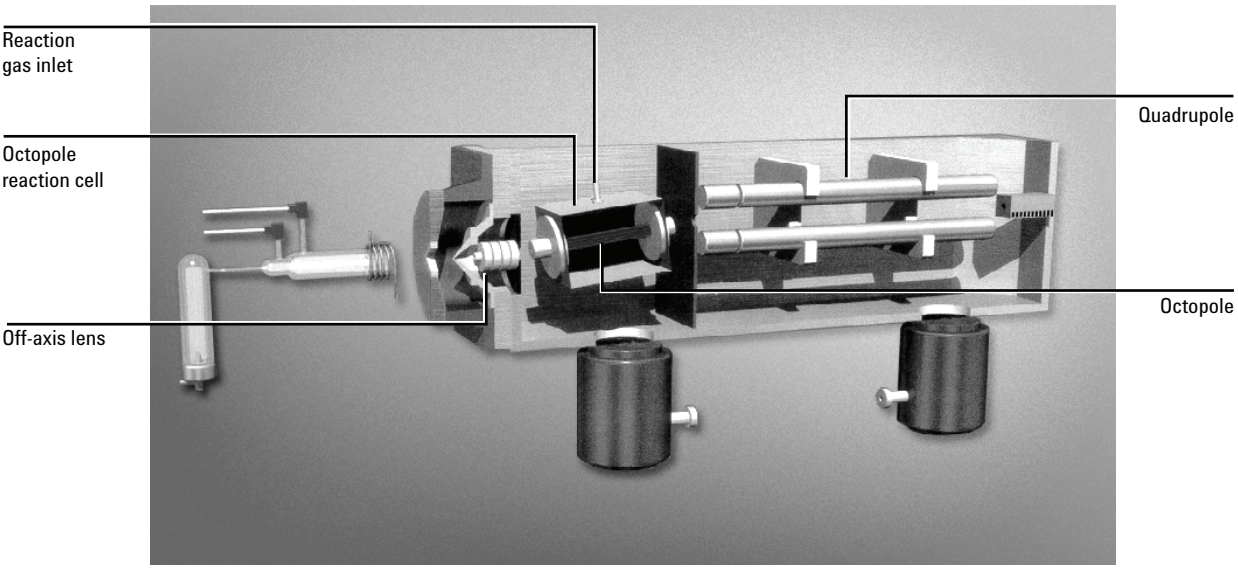

Figure 1: Schematic diagram of the Agilent 7500c Octopole Reaction System.

The instrument was tuned and optimized as detailed in Table 2. Calibrations were performed using external standards prepared from 1000 ppm single element stock, made up as appropriate with 2% nitric acid.

Table 2. Agilent 7500c Operating Conditions

Plasma RF power	1500 W
Sample depth	9.5 mm from load coil
Carrier gas flow	1.1 L/min
Spray chamber temperature	2 °C
Sample flow rate	240 µL/min
Nebulizer	Agilent microflow (PFA)
Interface	Nickel sample and skimmer cones

The external calibrations were run in the same analytical sequence as the samples. Sample concentration was calculated using the internal standard method. Table 3 summarizes the element and relevant internal standard information.

Table 3. Reaction Gases and Internal Standards Used

Measured element	Reaction gas	Internal standard
Potassium	Helium	Scandium
Calcium	Helium	Scandium
Chromium	Helium	Gallium
Iron	Helium	Gallium
Copper	Helium	Cobalt
Zinc	Helium	Cobalt
Arsenic	Helium	Yttrium
Selenium	Hydrogen	Indium (115)
Cadmium	Hydrogen	Indium (115)

Results and Discussion

The practical effect of the 7500c's unique combination of matrix tolerance and interference removal is that complex and variable samples can be measured with a simple quantification procedure using external standard calibration and internal standard correction for all masses. As and Se were accurately quantified at sub-ppb levels, even in a matrix containing 8% perchloric acid. Tables 4 and 5 summarize the results obtained in a blind analysis of plant digests using the 7500c, comparing the results with both the certified values and data obtained from analysis by ICP-OES.

Table 4. NIST 1573a (Tomato Leaves, Blank Corrected)

Name	Certified (mg/kg)	ICPOES (mg/kg)	7500c (mg/kg)
43 Ca	5.05%	5.00%	5.08%
39 K	2.70%	2.72%	2.62%
52 Cr	1.99	1.7, 1.8	1.60
53 Cr	1.99	1.7, 1.8	1.63
54 Fe	368	342, 347	368
56 Fe	368	342, 347	368
63 Cu	4.7	2.49, 2.40	4.43
65 Cu	4.7	2.49, 2.40	4.47
75 As	0.112	5.7, 6.6	0.175
78 Se	0.054	0.1, 0.8	0.061
111 Cd	1.52	5.5, 5.9	1.32

Table 5. NIST 1570a (Spinach, Blank Corrected)

Name	Certified (mg/kg)	Reference 2: (mg/kg)	7500c (mg/kg)
39 K	2.90%	2.63%	2.56%
43 Ca	1.53%	1.32%	1.39%
52 Cr	-	-	1.24
53 Cr		-	1.29
54 Fe	-	252	248
56 Fe		252	250
63 Cu	12.20	11.6	10.48
65 Cu	12.20	11.6	10.51
75 As	0.07	-	0.062
78 Se	0.12	-	0.09
111 Cd	2.89	-	2.33
54 Fe	-	252	248
56 Fe		252	250
63 Cu	12.20	11.6	10.48
65 Cu	12.20	11.6	10.51
75 As	0.07	-	0.062
78 Se	0.12	-	0.09
111 Cd	2.89	-	2.33

Measurements of Cr, Fe and Cu were made on two separate isotopes for each element. Because molecular interferences will, in many cases, only affect one of the analyte isotopes, the presence of an interference can cause a large discrepancy between results for different isotopes of the same element. An example of this is the measurement of Cu in a high Na matrix, where $^{40}Ar^{23}$ Na gives an overlap on ^{63}Cu, but no interference on ^{65}Cu. As the results indicate, the 7500c obtained excellent agreement for all the pairs of isotopes, highlighting the capabilities of the ORS in reducing interfering molecular species that, until now, have prevented the accurate trace analysis of transition metals in complex matrices by ICP-QMS.

Values for major and trace element concentrations agreed both with the expected value and the results obtained from ICP-OES. In the cases where the trace values for some elements were below the detection limit of the ICP-OES, the 7500c returned results in excellent agreement with the certified value. This data illustrates the wide dynamic range of the system and demonstrates its advantages as a replacement for traditional techniques such as ICP-OES.

The quantitative analysis of the NIST SRM samples also demonstrates that both the 7500c and the operating conditions are robust and tolerant of the changing matrix composition found in plant digests.

Conclusions

The trace analysis of plant digests is an application that can be suitably addressed by the 7500c. Advances in technology now allow the determination of multiple elements in complex sample matrices, with efficient interference removal and, in the case of the 7500c, with the excellent matrix tolerance for which the 7500 Series is renowned. Accurate quantification of As and Se at low and even sub-ppb levels in plant digests is possible, even where high concentrations of perchloric acid have been added during the sample preparation stage.

Acknowledgement

The ICP-OES measurements and NIST sample preparation were performed at the University of Queensland, School of Land and Food Sciences, Australia.

References

1. Ross M. Welch; USDA-ARS, US plant and soil and nutrition laboratory, Towner Road, Ithaca, NY 14853, USA, “Micronutrients, Agriculture and Nutrition: Linkages for Improved Health and Wellbeing”
2. Da-Hai Sun; Waters, J. K.; Mawhinney, T. P. “Determination of thirteen common elements in food samples by inductively coupled plasma atomic emission spectrometry: comparison of five digestion methods.”; Journal of AOAC International 2000, 83 (5) 1218-1224

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5988-4450EN

Agilent Technologies

Rapid Screening of Amino Acids in Food by CE-ESI-MS

Tomoyoshi Soga and Maria Serwe

Food Industry

Abstract

In the food industry, amino acids are measured to correlate flavor trends, monitor fermentation and assess the quality of the final product. Although amino acids are most commonly analyzed by HPLC with pre- or post column derivatization, capillary electrophoresis coupled to electrospray-mass spectrometry (CE-ESI-MS) is ideally suited for the rapid screening of free amino acids at low mg/L levels in complex matrices.

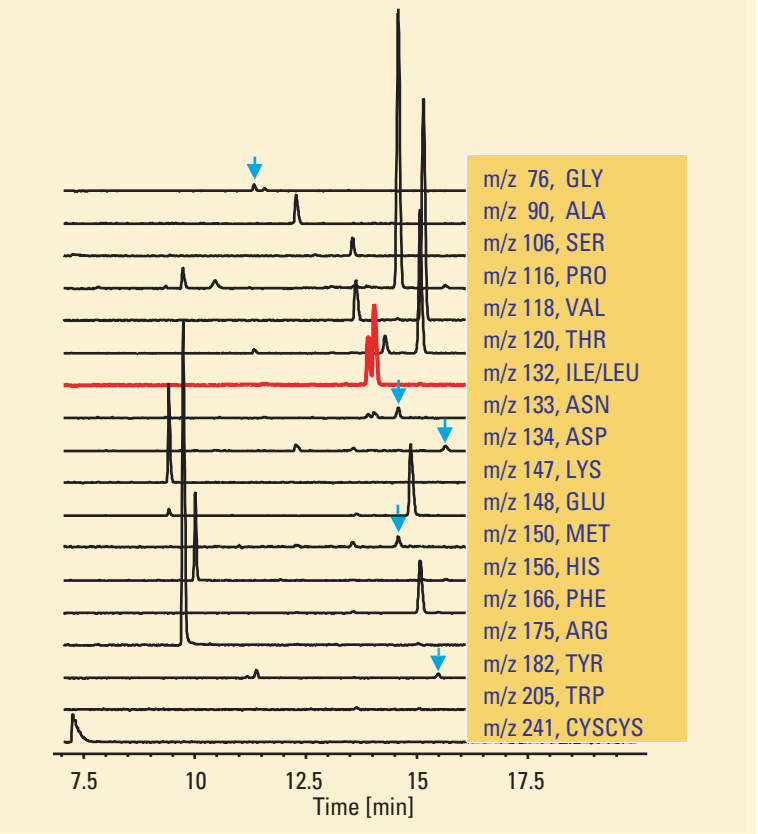

Figure 1
Amino acid analysis in soy sauce (selected ion traces).

Conditions

Sample
soy sauce, diluted 1:100 and ultrafiltrated
Injection
3 sec @ 50 mbar
Capillary
bare fused silica
L= 100 cm, 50 µm id
Buffer
1 M formic acid
Voltage
30 kV
Temperature 20°C
Preconditioning
4 min flush with run buffer at 1 bar
Sheath liquid
5 mM ammonium acetate in 50 % aqueous methanol, 10 µL/min
Nebulizing gas nitrogen, 10 psi
Drying gas nitrogen, 10 L/min, 300 °C
Acquisition
positive mode, Vcap -4 kV, fragmentor 70 V
Scan range
50–350 m/z

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Experimental

CE-ESI-MS analysis was performed using the Agilent Capillary Electrophoresis system with CE-MS capillary cassette coupled to the Agilent 1100 LC/MSD equipped with electrospray source and orthogonal sprayer for CE-MS. Agilent ChemStation software was used for instrument control. For method parameters see figure 1. A new capillary was flushed with run buffer for 20 minutes.

A 17-amino acid standard (2.5 mmol/L except Cys at 1.25 mmol/L in 0.1 N HCl, not containing Gln) was obtained from Pierce (Rockford, IL, USA). Asp and Trp were purchased from Wako (Osaka, Japan) and prepared at a concentration of 2.5 mmol/L in 0.1 N NaOH. Amino acid standards were mixed and diluted with deionized water to a final concentration of 250 µmol/L (Cys 125 µmol/L).

Sample preparation was simple and consisted of dilution with deionized water and ultrafiltration through a 30 kDa cutoff filter to remove peptides and proteins.

Results

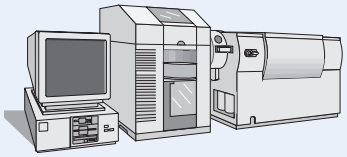
An optimized method for the rapid analysis of amino acids by CE-ESI-MS has been developed. Using an electrolyte with a pH value below the analyte's isoelectric point (< 2.77) allows the simultaneous determination of basic and acidic amino acids as positive molecular ions. In this study an ion at m/z 122 for Cys could not be observed. However, an ion at m/z 241 for Cys-Cys indicated oxidation by exposure to air.

When analyzing a standard composed of 19 amino acids, the assay was linear from 10–500 µmol/L. Detection limits ranged from 0.3 - 1.1 µmol/L for basic amino acids to 6–11 µmol/L for acidic amino acids. Migration time reproducibility was < 1.2 % RSD (n = 8), RSD for peak area was 2.0–4.7 % (except for Met, which gave decreasing peak areas due to oxidation).

The analysis of amino acids in food is important. Their monitoring can help track fermentation metabolites and is used to correlate flavor trends. Figure 1 shows the analysis of soy sauce by CE-ESI-MS. In addition to amino acids, soy sauce contains a great number of organic compounds with amino-functional groups. Using methods with derivatization can cause very complex separations. Without derivatization, as shown here, well-defined extracted ion traces can be obtained, allowing detection of amino acids in less than 13 minutes. Migration time reproducibility was < 0.4 % RSD (n = 5). Area reproducibility was 1.1–6.0 % RSD except for Met. This method was further applied to the analysis of amino acids in beer and sake.

Equipment

- Agilent Capillary Electrophoresis system
- Agilent CE-MS Adapter Kit
- Agilent 1100 Series LC/MSD module with API Electrospray Source
- Agilent CE-ESI-MS Sprayer Kit
- Agilent ChemStation and CE-MS add-on software



References

1
Soga, T., Heiger, D., J. Anal. Chem.2000, 72, 1236-1241.

Tomoyoshi Soga is an application chemist at Yokogawa Analytical Systems, Inc. Tokyo, Japan.
Maria Serwe is an application chemist at Agilent Technologies, Waldbronn, Germany.

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Analysis of Amino Acids in Beer using HPLC with Online Derivatization

Rainer Schuster

Food

Abstract

Both primary and secondary amino acids were analyzed in one run.

The amino acid composition of proteins can be used to determine the origin of meat products and thus to detect adulteration of foodstuffs. Detection of potentially toxic amino acids is also possible through such analysis. Through the use of chiral stationary phases as column material, D and L forms of amino acids can be separated and quantified.

HPLC in combination with automated online derivatization is now a well-accepted method for detecting amino acids owing to its short analysis time and relatively simple sample preparation.

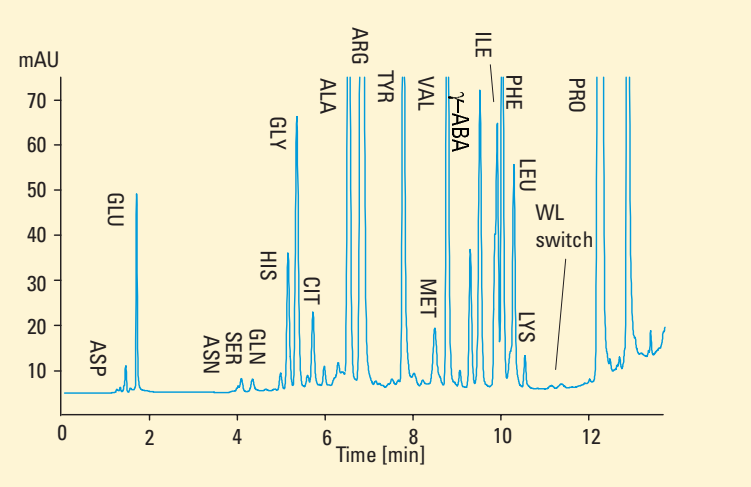

Figure 1
Analysis of amino acids in beer after online derivatization

Conditions

Column 200 ˘ 2.1 mm Hypersil ODS, 5 µm
Mobile phase A = 0.03M sodium acetate pH = 7.2 + 0.5% THF
B = 0.1M sodium acetate/ ACN (1:4)
Gradient
at 0 min 0% B at 0.45 ml/min flow rate
at 9 min 30% B
at 11 min 50% B at 0.8 ml/min flow rate
at 13 min 50% B
at 14 min 100% B at 0.45 ml/min flow rate
at 14.1 min at 0.45 ml/min flow rate
at 14.2 min at 0.8 ml/min flow rate
at 17.9 min at 0.8 ml/min flow rate
at 18.0 min at 0.45 ml/min flow rate
at 18 min 100% B; at 19 min 0% B
Post time 4 min
Flow rate 0.45 ml/min
Column compartment 40 ºC
Injection vol 1 µl standard
Detector UV -DAD 338 nm and 266 nm
Fluorescence
Excitation wavelength: 230 nm
Emission wavelength: 450 nm at 11.5 min
Excitation wavelength: 266 nm
Emission wavelength: 310 nm
Slit width excitation: 2 mm (25 nm)
Slit width emission 1: 4 mm (50 nm)
Slit width emission 2: 4 mm (50 nm)
Photomultiplier gain: 12
Cut-off filter: 280 nm
Lamp: 55 Hz (always on)
Response time: 4 s

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Sample preparation

Hydrolyzation with HCl or enzymatic hydrolysis is used to break protein bonds.

Chromatographic conditions

The HPLC method presented here was used in the analysis of secondary and primary amino acids in beer with precolumn derivatization and fluorescence detection.[1]

HPLC method performance

Limit of detection
5 pmol

Repeatability of
RT over 6 runs <1 %
areas over 6 runs <5 %

Linearity
1 pmol to 4 nmol

References

1.
R. Schuster, "Determination of amino acids in biological, pharmaceutical, plant and food samples by automated precolumn derivatization and HPLC", *J. Chromatogr.*, **1988**, 431, 271–284.

Conditions

Injector program for online derivatization

1. Draw 3.0 µl from vial 2 (borate buffer)
2. Draw 1.0 µl from vial 0 (OPA reagent)
3. Draw 0.0 µl from vial 100 (water)
4. Draw 1.0 µl from sample
5. Draw 0.0 µl from vial 100 (water)
6. Mix 7.0 µl (6 cycles)
7. Draw 1.0 from vial 1 FMOC reagent
8. Draw 0.0 µl from vial 100 (water)
9. Mix 8.0 µl (3 cycles)
10. Inject

Equipment

Agilent 1100 Series

- vacuum degasser
- quaternary pump
- autosampler
- thermostatted column compartment
- diode array detector
- fluorescence detector

Agilent ChemStation + software

Rainer Schuster is application chemist at Agilent Technologies, Waldbronn, Germany.

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Analysis of Vanillin Extract Quality using HPLC

Angelika Gratzfeld-Huesgen

Food

Abstract

The following compounds are examples of flavoring agents used in food products:

- lupulon and humulon (hop bittering compounds)
- vanillin
- naringenin and hesperidin (bittering compounds)

Three major classes of compounds are used as flavoring agents: essential oils, bitter compounds, and pungency compounds. Although the resolution afforded by gas chromatography (GC) for the separation of flavor compounds remains unsurpassed, HPLC is the method of choice if the compound to be analyzed is low volatile or thermally unstable.

Sample preparation

Turbid samples require filtration, whereas solid samples must be extracted with ethanol. Afterfiltration, the solution can be injected directly into the HPLC instrument.

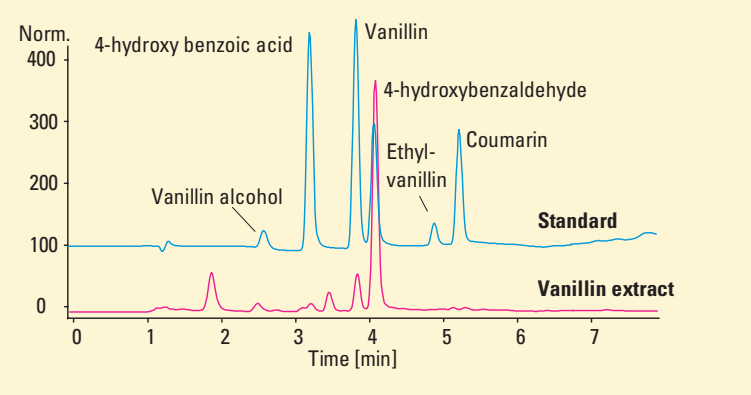

Figure 1
Determination of the quality of vanillin extract

Conditions

Column 100 ˘ 4 mm Hypersil BDS, 3 µm
Mobile phase
A = water + 0.15ml H_2SO_4 (conc.), pH = 2.3
B = ACN
Gradient
start with 10% B
at 3 min 40% B; at 4 min 40% B
at 6 min 80% B; at 7 min 90% B
Flow rate 0.8 ml/min
Post time 3 min
Column compartment 30 °C
Injection vol 5 µl
Detector
UV-DADdetection wavelength 280/80 nm, reference wavelength 360/100 nm

Sample preparation
Injection without further preparation

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Chromatographic conditions

The HPLC method presented here for the analysis of vanillin is based on reversed-phase chromatography. UV spectra were evaluated as an additional identification tool.[1]

HPLC method performance

Limit of detection
0.2–5 ng (injected amount)
S/N = 2

Repeatability
of RT over 10 runs <0.2 %
of areas over 10 runs <1 %

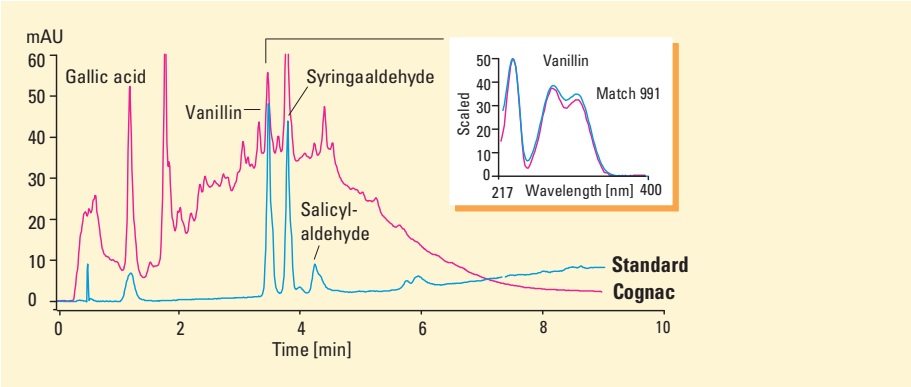


Figure 2
Analysis of vanillin in cognac. Identification of vanillin through spectra comparison

References

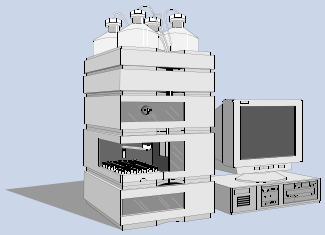
1.
Herrmann, A, et al.;,"Rapid control of vanilla-containing products using HPLC"; *J. Chromatogr.*, **1982**, 246, 313–316.

Conditions

Conditions as above, except
Column
100 ˘ 2.1 mm Hypersil ODS, 5 µm
Mobile phase
A = water + 5 mM NaH_2PO_4
B = methanol
Gradient
at 10 min 70% B
Flow rate
0.4 ml/min

Equipment

Agilent 1100 Series
- vacuum degasser
- quaternary pump
- autosampler
- thermostatted column compartment
- diode array detector, Agilent ChemStation + software



Angelika Gratzfeld-Huesgen is application chemist at Agilent Technologies, Waldbronn, Germany.

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Natural Compounds & Additives

Carbohydrates

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Simultaneous analysis of inorganic anions, organic acids, amino acids and carbohydrates using the Agilent Basic Anion Buffer

Application Note

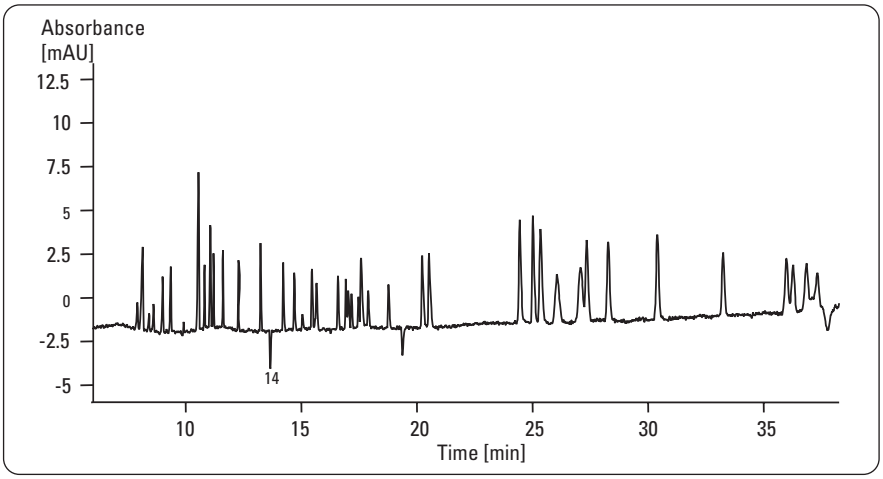
Food

Author

Tomoyoshi Soga

Institute for Advanced Biosciences,

Keio University, Japan



Abstract

This Application Note describes extended applications of the Agilent Basic Anion Buffer. It facilitates the analysis of many anions including inorganic anions, organic acids, amino acids and carbohydrates. The method described here is useful for the screening analysis of anions in food and beverage samples. To separate these anions simultaneously, a highly alkaline pH condition is used to confer a negative charge not only on inorganic and organic anions but also on amino acids and carbohydrates, and promote their migration towards the anode.

Electroosmotic flow (EOF) is reversed in the direction of the anode by adding quaternary ammonium salt to the electrolyte. This is necessary to have both anion migration and EOF in the same direction (towards the anode) and ensure anion migration past the detector. In this method, indirect UV detection is employed to visualize anions which have little or no chromophore. The Agilent Basic Anion Buffer is pre-made with the pH already adjusted, therefore no further preparation is required. Detailed method parameters and some typical electropherograms are shown.

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Necessary supplies

The following parts are necessary for the simultaneous analysis of anions:

Component	Quantity	Part No.
Agilent Basic Anion Buffer*	50 mL	5064-8209
Fused silica capillary (id = 50 µm, l=104 cm L=112.5 cm)	1 pk	G1600-64211
CE buffer vials 2 mL (glass**)	100/pk	5182-9697
CE sample vials PUR, 100 µL (polypropylene)	1000/pk	9301-0978
CE caps (polyurethane)	100/pk	5181-1512
CE water	500 mL	5062-8578

*** For this buffer, only 50-µm id straight capillaries are usable.**

**** It is recommended to use glass vials rather than polypropylene vials.**

Procedures

Buffer preparation

The Basic Anion Buffer is premade and ready to use.

Do not leave the solution uncapped since the buffer is a highly alkaline solution and will readily absorb gaseous carbonate from the atmosphere. This will cause its pH to drop immediately. Make certain the bottle is capped after use.

Do not store opened bottles which are a third or less full. These should be discarded because gaseous carbonate in the bottle can be adsorbed and will reduce the pH of the residual buffer.

The buffer should be stored at room temperature (not less than 20 °C, since some buffer components may crystallize at lower temperatures).

Buffer and waste vials

Prepare three vials (one flushing vial and two home vials). When using 2-mL glass vials, fill each vial with 1.4 mL of the buffer. Also prepare a waste vial (filled with 300-mL CE water or deionized water).

Since buffers used for indirect UV detection have limited buffering capacity, the buffer should be replaced every 8 runs when using 2-mL glass vials.

For this application, do not use the replenishment system for buffer replacement. The level of dissolved carbonate may increase due to pressurization of the buffer bottle, causing a pH drop. Also, crystallization of the buffer might block the tubes.

Standard preparation

Individual stock solutions of inorganic and organic anions should be prepared from their sodium salts or free acids. Since carbohydrates are unstable, they should be prepared shortly before use.

For amino acids, individual stock solution of Tyr, Cys-Cys, Asp, Trp, Leu, Ile and Phe should be prepared at a concentration of 10 g/L in 0.1 M NaOH. Other amino acids should be prepared in 0.01 M HCl. The working mixture standard should be prepared by diluting stock solutions with deionized water.

If a commercially available amino acid standard mixture in 0.1 M HCl is used, Arg may not be detected since the pKa value of Arg is 10.76. Arg is positively charged in 0.1 M HCl and migrates toward the opposite direction of the detector. In this case, the standard solution should be diluted with 0.01 M NaOH.

The concentration of the standard mixture should be in the range of 50 to 1000 mg/L to obtain good peak shape and sensitivity.

Sample preparation

For actual samples, dilution with deionized water is necessary in order to reduce the conductivity of the samples, for example, 1:50 dilution for soy sauce.

If the sample contains proteins and the migration times increase from run to run, removal of the proteins is recommended by using centrifugal filtering through a 30-kDa cutoff filter.

Capillary

Only 50-µm id straight capillaries are suitable for this method. Baseline noise is markedly increased if a 75-µm id capillary is used due to the high UV absorptivity of the buffer. Neither bubble cell capillaries nor the High Sensitivity Detection Cell should be used. A 50-µm id capillary (L=112.5 cm, l=104 cm) is recommended.

Capillary conditioning

Avoid capillary conditioning with sodium hydroxide since this degrades the performance of this application.

Prior to first use, a new capillary should be flushed only with the run buffer for 15 minutes.

Between analyses it is recommended that the capillary be flushed for 4 minutes with buffer from the flushing vial.

Capillary storage

If the capillary is removed from the instrument it should be washed for 10 minutes with deionized water and then flushed with air for 10 minutes. When the capillary is to be reinstalled, it is necessary to flush with the run buffer for at least 15 minutes.

Method summary

The following method can be used to separate most inorganic anions, organic acids, amino acids and carbohydrates simultaneously. Below are the general analytical conditions.

Chromatographic conditions	
Capillary:	Fused silica id = 50 µm, l=104 cm, L=112.5 cm (Agilent part number G1600-64211)
Injection:	1. Pressure: 50 mbar for 6 seconds from sample vial 2. Post-injection of buffer from InHome vial, 50 mbar for 4 seconds
Applied voltage:	- 30 kV
Capillary temperature:	15 °C
Detection wavelength:	Signal 350/20 nm, reference 230/10 nm
Preconditioning:	Buffer flush for 4 minutes at 1 bar prior to each run

The method as it should be entered into the Agilent ChemStation is as follows:

HPCE mode	CE	
Home values		
Lift Offset	4	
Cassette Temperature	15.00 °C	
Inlet Home Vial	10	*Place the buffer vials at position 10 and 11.*
Outlet Home Vial	11	*Vial locations are exemplary only.*
Replenishment and Preconditioning		
Serial processing		
Replenishment Entries		
No Replenishment used		*Do not use replenishment. The level of dissolved carbonate might increase due to pressurization of the buffer bottle causing a pH drop.*
Preconditioning Entries		
Function Parameter		
1 Flush 4.00 min, I: 9, O:1		*Place flushing vial at 9 and waste vial at 1. Remember to monitor the waste vial volume for overflow.*
Postcondition Entries		
No Postconditioning used		
Injection Table Entries		
Function Parameter		
1 PRESSURE 50.0 mbar, 6.0 sec, I: InjectVial, O: OutHomeVial		*May be increased or decreased depending on sample concentration.*
2 PRESSURE 50.0 mbar, 4.0 sec, I: InHomeVial, O:OutHomeVial		*The post injection plug helps to minimize sample loss upon application of voltage. A voltage ramp is used for the same purose.*

Electric		
Electric	On	
Polarity	Negative	*Negative polarity is used since EOF is reversed.*
Voltage	30.0 kV	
Current	150.0 mA	*A current limit is not necessary but may be used to prevent excessive current generation in case the wrong vial is used.*
Power	System Limit	
Low Current Limit	2 µA	
Store Data		
Collect voltage	Yes	
Collect current	*Yes*	*It is recommended to store the current for every analysis. Current in this method shows from -30 to -40 µA.*
Collect power	No	
Collect pressure	No	
Collect temperature	Yes	
Time entries		
Stop time	40.00 min	*Adjust as needed when running actual sample.*
Post time	Off	
Time Table is empty.		

Diode array detector

Settings		
Stop Time	as HPCE: 40.00 min	
Post Time	Off	
Response Time	1.3 sec	*This is recommended to reduce baseline noise.*
Peak width	> 0.1 min	
Prerun Autobalance	On	
Postrun Autobalance	Off	
Spectrum		
Store	None	
Signals		
Store	Reference	
Signal, Bw	Reference, Bw [nm]	
A: Yes 350/20	230/10	

Results and discussion

Simultaneous analysis of inorganic anions, organic acids, amino acids and carbohydrates

Figure 1 shows a typical electropherogram of a 43-component anion sample including seven inorganic anions, five organic acids, 16 amino acids and 15 carbohydrates using the standard method. If the results are not similar to these please refer to the section *Troubleshooting* in this note.

In this method most inorganic anions and carbohydrates can be separated. However, migration times of several organic acids such as tartarate, succinate, malate and -ketoglutarate are close. This separation can be improved by using the Organic Acids Solutions kit from Agilent Technologies (Agilent part number 5063-6510).

With respect to amino acids, Leu and Ile cannot be resolved. Although Arg is not observed in figure 1, Arg can be detected at approximately 35 minutes if the sample is dissolved in an alkaline

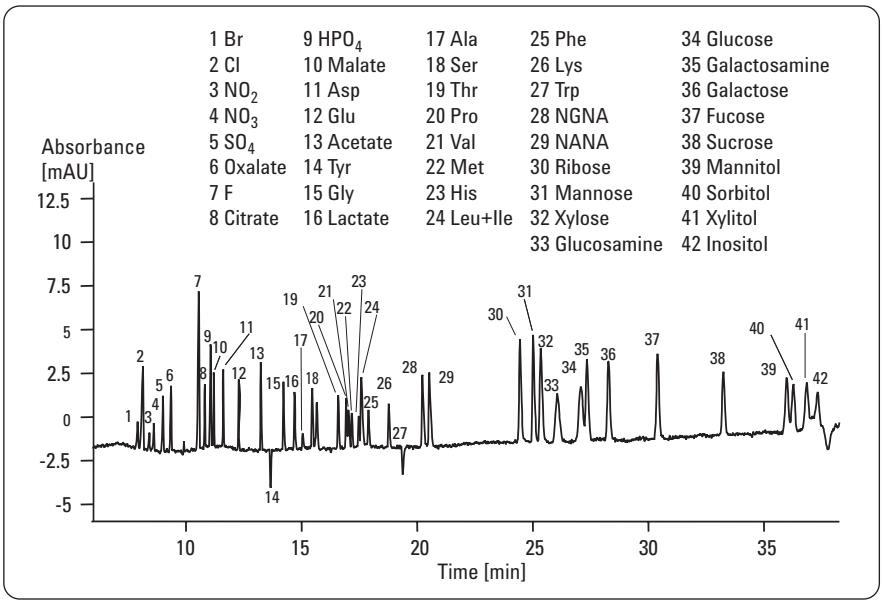

Figure 1
Analysis of 43-component anion standard mixture.

Chromatographic conditions

Buffer:	Agilent Basic Anion Buffer (Agilent part number 5064-8209)
Sample:	Cl 110 mg/L, carbohydrates, 200 mg/L each, others 50 mg/L each
Capillary:	Fused silica, l=104 cm, L=112.5 cm, id= 50 µm (Agilent part number G1600-64211)
Injection:	300 mbar x s
Temperature:	15 °C
Voltage:	-30 kV
Detection:	Signal 350/20 nm, reference 230/10 nm

Compound	Mobility ($X10^{-4}$ cm^{-2}/Vs)	Compound	Mobility ($X10^{-4}$ cm^{-2}/Vs)	Compound	Mobility ($X10^{-4}$ cm^{-2}/Vs)	Compound	Mobility ($X10^{-4}$ cm^{-2}/Vs)
Bromide	-7.181	Acetate	-3.589	n-Hexanoate	-2.385	Glucosamine	-0.846
Chloride	-6.983	Pyruvate	-3.540	Galacturonic	-2.337	Mannosamine	-0.832
Nitrite	-6.648	CysCys	-3.514	His	-2.310	Lactose	-0.774
Nitrate	-6.442	Glycolate	-3.495	Leu	-2.300	Arabinose	-0.764
Sulfate	-6.140	Tyr	-3.493	Ile	-2.300	Glucose	-0.761
Oxalate	-5.784	Gly	-3.260	Phe	-2.220	Maltose	-0.731
Ascorbate	-5.409	n-Propionate	-3.111	n-Heptanoate	-2.149	Galactosamine	-0.725
Malonate	-5.093	Lactate	-3.041	Gluconate	-2.060	Lactulose	-0.676
Fluoride	-4.990	Borate	-2.963	Lys	-2.026	Galactose	-0.659
Formate	-4.911	Ala	-2.858	Trp	-1.910	Fucose	-0.485
Citrate	-4.775	Ser	-2.795	NGNA	-1.719	Sucrose	-0.291
Pyrophosphate	-4.760	n-Butyrate	-2.781	n-Octanoate	-1.707	Raffinose	-0.284
Phosphate	-4.677	Levulinate	-2.729	NANA	-1.675	Mannitol	-0.134
Tartarate	-4.584	Mannuronic	-2.674	ManNAc	-1.221	Trehalose	-0.121
Succinate	-4.565	Pyroglutamate	-2.631	Ribose	-1.037	Sorbitol	-0.118
Malate	-4.520	n-Pentanoate	-2.578	Fructose	-0.983	Galactitol	-0.104
a-Ketoglutarate	-4.513	Thr	-2.542	GlcNAc	-0.975	Xylitol	-0.086
Asp	-4.418	Glucuronic	-2.497	Mannose	-0.966	Erythritol	-0.076
Glutarate	-4.196	Pro	-2.450	Xylose	-0.935	Inositol	-0.064
Glu	-4.084	Val	-2.444	GalNAc	-0.919		
Adipate	-3.934	Met	-2.389	Rhamnose	-0.904		

Table 1
Electrophoretic mobilities of anions at 20 °C.

solution. Since Tyr and Trp have UV absorbance at 230 nm, they are recorded as negative peaks. Ser migrates just before a system peak.

Electrophoretic mobilities at 20 °C

The effective mobilities of 82 compounds including nine inorganic anions, 23 organic acids, 18 amino acids and 32 carbohydrates were determined by this method at 20 °C and are listed in table 1. If the mobilities of the compounds of interest are close, they are difficult to separate. In this case investigating a change of temperature or pH is recommended.

Applications

Soy sauce analysis

Figure 2 shows the analysis of a soy sauce. The sample was diluted 1:50 with CE water. Centrifugal filtering through a 30 kDa cutoff filter was applied to remove proteins and peptides. A well-defined electropherogram was obtained without interference from other matrix compounds. Satisfactory reproducibilities were obtained for all compounds with RSD values (n=5) for migration times better than 0.3 % and for peak areas between 0.6 and 5.4 %.

Pineapple analysis

This method was applied to the analysis of organic acids and carbohydrates in pineapple. In the agriculture industry, technical experts are trying to develop a new crossbreed of fruits. Since the content of organic acids and carbohydrates determines the taste of the pineapple juice, their analysis can help to characterize the product. If citrate and malate concentrations are high, the taste tends to be sour. If the carbohydrate concentration is high, the taste issweet. Determination of these compounds is traditionally performed using two HPLC methods.

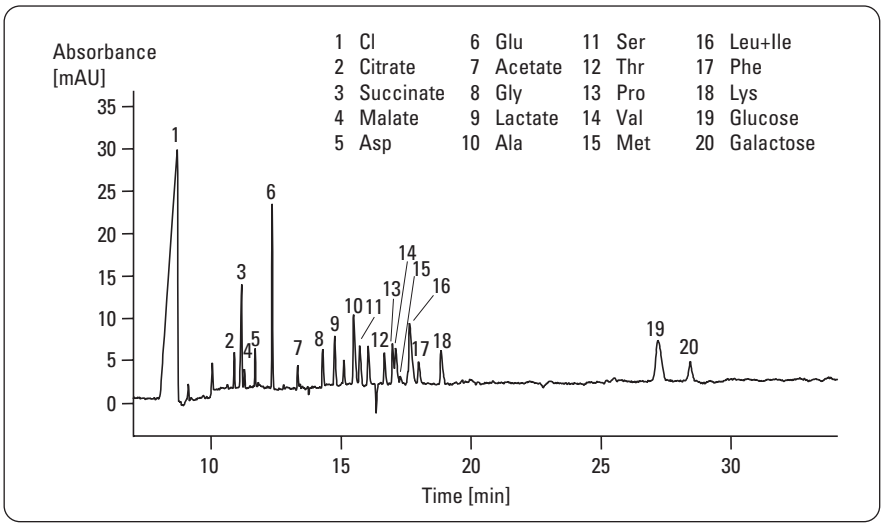

Figure 2
Analysis of soy sauce.

Chromatographic conditions

Buffer:	Agilent Basic Anion Buffer (Agilent part number 5064-8209)
Sample:	Soy sauce, 1:50 diluted with water, ultrafiltration with 30-kDa cutoff filter
Preconditioning:	4 min with run buffer
Capillary:	Fused silica, l=104 cm, L=112.5 cm, id= 50 µm, Injection 300 mbar x s (Agilent part number G1600-64211)
Temperature:	15 °C
Voltage:	-30 kV
Detection signal:	350/20 nm, reference 230/10 nm

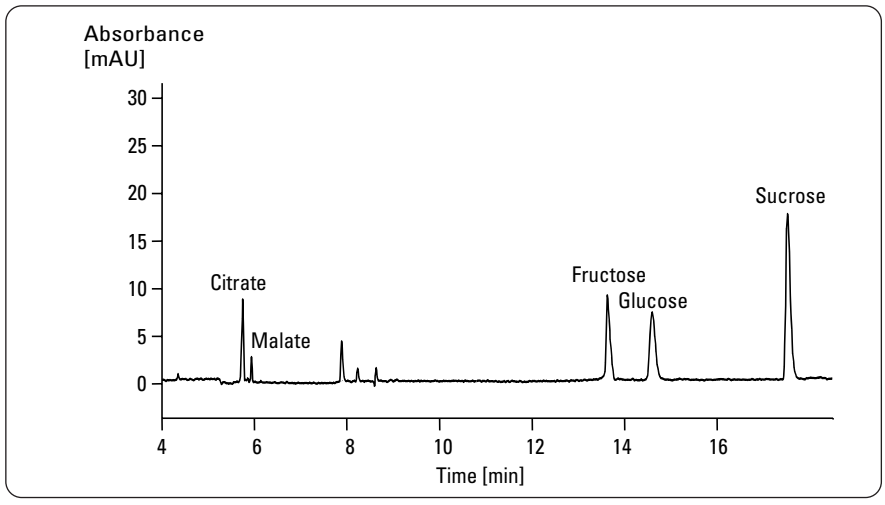

Figure 3
Analysis of pineapple.

Chromatographic conditions

Buffer:	Agilent Basic Anion Buffer (Agilent part number 5064-8209)
Sample:	Pineapple, 1:50 diluted with water,
Capillary:	Fused silica, l=72 cm, L=80.5 cm, id= 50 µm (Agilent part number G1600-62211)
Injection:	300 mbar x s
Temperature:	20 °C
Voltage:	-25 kV (reversed polarity)
Detection signal:	350/20 nm, reference 275/10 nm

However, the method described here enables the simultaneous analysis of both organic acids and carbohydrates in a much shorter time (less than 18 minutes) and in a single run. In order to reduce the analysis time, a shorter length capillary was used for this sample. Squeezed pineapple juice was diluted 50-fold with CE water prior to injection. Figure 3 shows a result of the analysis.

Troubleshooting

Problem	Possible Cause	Solution
Poor resolution or broad/split peaks	Buffer is old	Use new buffer
	Capillary damaged	Replace capillary
	Sample overloaded	Dilute sample
	Capillary too short	Replace capillary
Peak leading	Buffer absorbed carbonate	Use new buffer
No signal	Sample not injected	Verify no air bubble trapped in bottom of sample vial Verify inlet capillary set correctly
	Capillary damaged	Replace capillary
	Wrong buffer used	Verify buffer
	Wrong setting of power supply polarity	Check that polarity is negative
	Detection wavelength incorrect	Verify signal: 350/20, reference: 230/10 nm
Noisy baseline	Wrong setting of response time	Verify response time 1.3 seconds DAD
	Capillary window not adjusted	Examine capillary window
	Capillary window dirty	Examine and clean with lint-free paper/MeOH
	Lamp is old	Replace lamp
	Buffer pH higher than 12.3	Verify buffer pH
Poor reproducibility	Buffer overused	Replace buffer
Unstable current	Capillary broken	Replace capillary
	Capillary not filled with buffer	Increase flush time

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1.
Soga, T. and Ross, G. A. "Capillary Electrophoretic Determination of Inorganic and Organic Anions using 2,6-pyridinedicarboxylic Acid: Effect of Electrolyte's Complexing Ability", *J. Chromatogr. A, 767, 223-230,* **1997.**

2.
Soga, T. and Heiger, D. N. "Simultaneous Determination of Monosaccharides in Glycoproteins by Capillary Electrophoresis", *Anal. Biochem., 261, 73-78,* **1998.**

3.
Soga, T. and Ross, G. A. "Simultaneous Determination of Inorganic Anions, Organic Acids, Amino Acids and Carbohydrates by Capillary Electrophoresis" *J. Chromatogr., 837, 231-239,* **1999.**

www.agilent.com/chem/ce

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Agilent Technologies

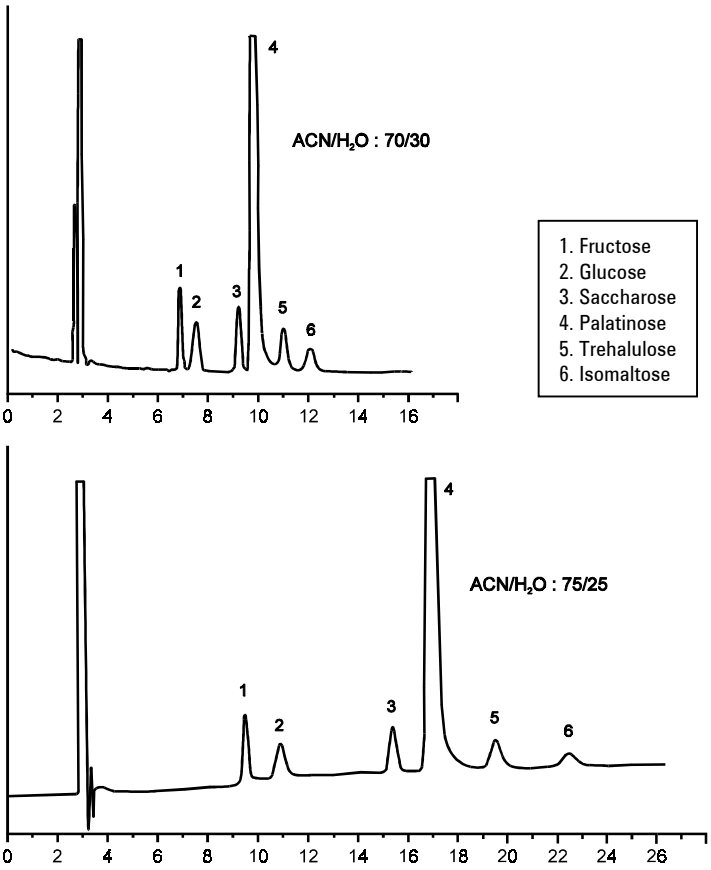
Effect of Mobile-Phase Strength in Separations of Mono- and Disaccharides

Application

Food Analysis

Robert Ricker

Sugars are important component of many foodstuffs derived from plant and animal sources Sugars are also of great clinical importance. They have traditionally separated by normal- phase on NH_2 bonded phases. Effect of mobile-phase solvent strength in these separations is demonstrated for various mono- and disaccharides.



1. Fructose
2. Glucose
3. Saccharose
4. Palatinose
5. Trehalulose
6. Isomaltose

Conditions:
ZORBAX NH_2 (4.6 x 250 mm) (Agilent P/N: 880952-708)
Mobile Phase: ACN : H_2O, as indicated
1 mL/min, Detect. = Refractive Index

Highlights

- *Good resolution of various mono- and disaccharides is obtained using a ZORBAX NH2 column.*
- *Retention of sugars increases with increasing organic content in the mobile phase.*

Agilent Technologies

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

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5988-6360EN

Agilent Technologies

High-Performance Carbohydrate Analysis

Application

Food Analysis

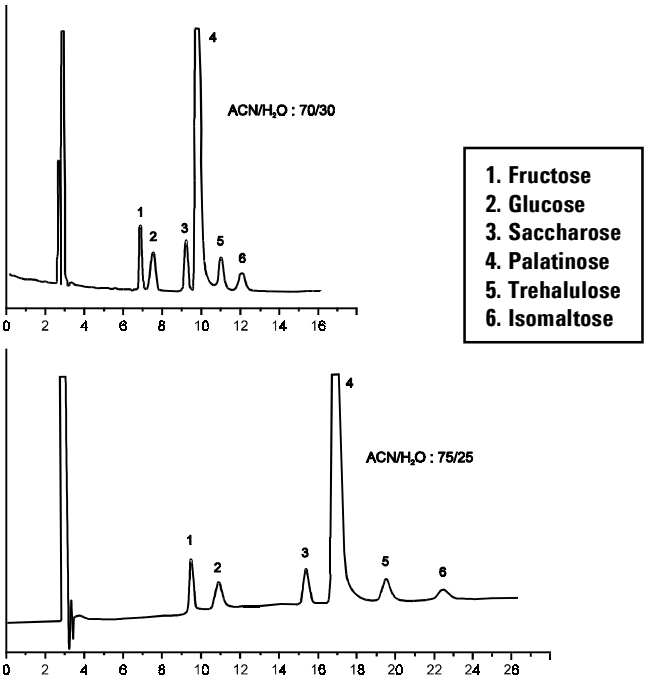
Robert Ricker

Analysis of mono and disaccharides (simple sugars) in foods is required when their presence is greater than 1%, according to the Food and Drug Administration's new policy. Furthermore, sugar content must be included on the food label.

When sugar analyses are required, HPLC provides speed and ease of use. In fact, the Association of Official Analytical Chemists (AOAC) has specified HPLC and a propylamine column as an official method for several matrices (e.g., pre-sweetened cereals). The high performance ZORBAX NH_2 columns (5µm) are an optimal choice for these applications as well as for analysis of some high-fructose corn syrups which are added to foods instead of cane sugar. The Agilent ZORBAX NH_2 column provides higher resolution than many so-called 10µm, "sugar analysis specified" columns.

Highlights

- *Good resolution of various mono- and disaccharides is obtained using a ZORBAX NH_2 column.*
- *Retention of sugars increases with increasing organic content in the mobile phase.*



1. Fructose
2. Glucose
3. Saccharose
4. Palatinose
5. Trehalulose
6. Isomaltose

ZORBAX NH_2 (4.6 x 250 mm) (Agilent Part No. 880952-708)
Mobile Phase: ACN : H_2O, as indicated
1 mL/min, Detect. = Refractive Index

Agilent Technologies

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

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Agilent Technologies

Application of Liquid Chromatography/ Mass Spectrometry to the Analysis of Sugars and Sugar-Alcohol

Application

Food

Author

Hiroki Kumaguai

Abstract

Sugars and sugar alcohols were successfully analyzed by liquid chromatography/mass spectrometry using atmospheric pressure chemical ionization in negative ion mode.

Background

Sugars, quantitatively the largest organic compound group on earth, are widely distributed among both flora and fauna. Higher classes of vegetation and algae contain large quantities of sugars, and the shells of arthropods, represented by crabs and shrimp, are made of chitin, which are polysaccharides.

Although sugars represent a huge biomass; they also exist in very small amounts within individual living organisms. Various kinds of sugars and compound sugars are involved in bodily functions, and as sources of energy. Sugars are used as raw materials within the textile, food processing and pharmaceutical industries.

Sugars have been analyzed by various methods:

- Gas chromatorgraphy/mass spectrometry (GC/MS) methods require preliminary derivatization to increase sugar's volatility.
- High-performance liquid chromatography (HPLC) methods have limitations, including detector sensitivity. Commonly, one of two different detectors is used.
  - differential refraction detector
  - ultraviolet (UV)/fluorescence detector, following application of pre- or post-column derivatization; without derivatization, sugars are not UV detectable
- Liquid chromatography/mass spectrometry (LC/MS) methods using electrospray ionization (ESI) also require pre- or post-column derivatization to obtain a high level of sensitivity.
- LC/MS method, described here, using atmospheric pressure chemical ionization (APCI) does not require pre- or post-column derivatization to attain high sensitivity. However, this method does require $CHCl_3$ and CH_2Cl_2 to be added in the post-column stage.

Methods using derivatization are the most sensitive, with a minimum detectability of several to tens of picograms (pg). Although the APCI method, employing post-column addition, has a lower sensitivity (several hundred picograms), it is an easy and versatile method of analysis, with superior ionization repeatability, generated by the $[M+Cl]^-$ ion.

Method

- Instrument: HP 1100[1] LC/MS with APCI
  Negative ion mode
  Drying gas: N_2 13 L/min and 350 °C
  Nebulizer: N_2 40 psi
  Fragmentor: 20 V
  Corona current: 30 µA
  Mass range: 100–500 *m/z*
  Mode: SIM (*m/z*) negative

  Sorbitol 217, 219
  Glucose, Fructose 215, 217
  Xylitol 187, 189
  Sucrose 377, 379
- LC Conditions:
  Mobile phase: CH_3CN/H_2O (75/25) at 0.2 mL/min
  Oven temperature: 40 °C
  Injection volume: 10 µL
  Post-column addition: $CH_3CN/CHCl_3$ (50/50) at 0.2 mL/min
- Column: Asahipak NH_2-50 2D, 2.0 mm id × 150 mm long

[1]Now available as the Agilent 1100 LC/MS from Agilent Technologies, Inc.

Sample Analysis

Selected sugars and sugar alcohols in some standards and common beverages were measured using this LC/MS method with APCI in negative ion mode. The three figures below illustrate both the sensitivity and applicability of this method.

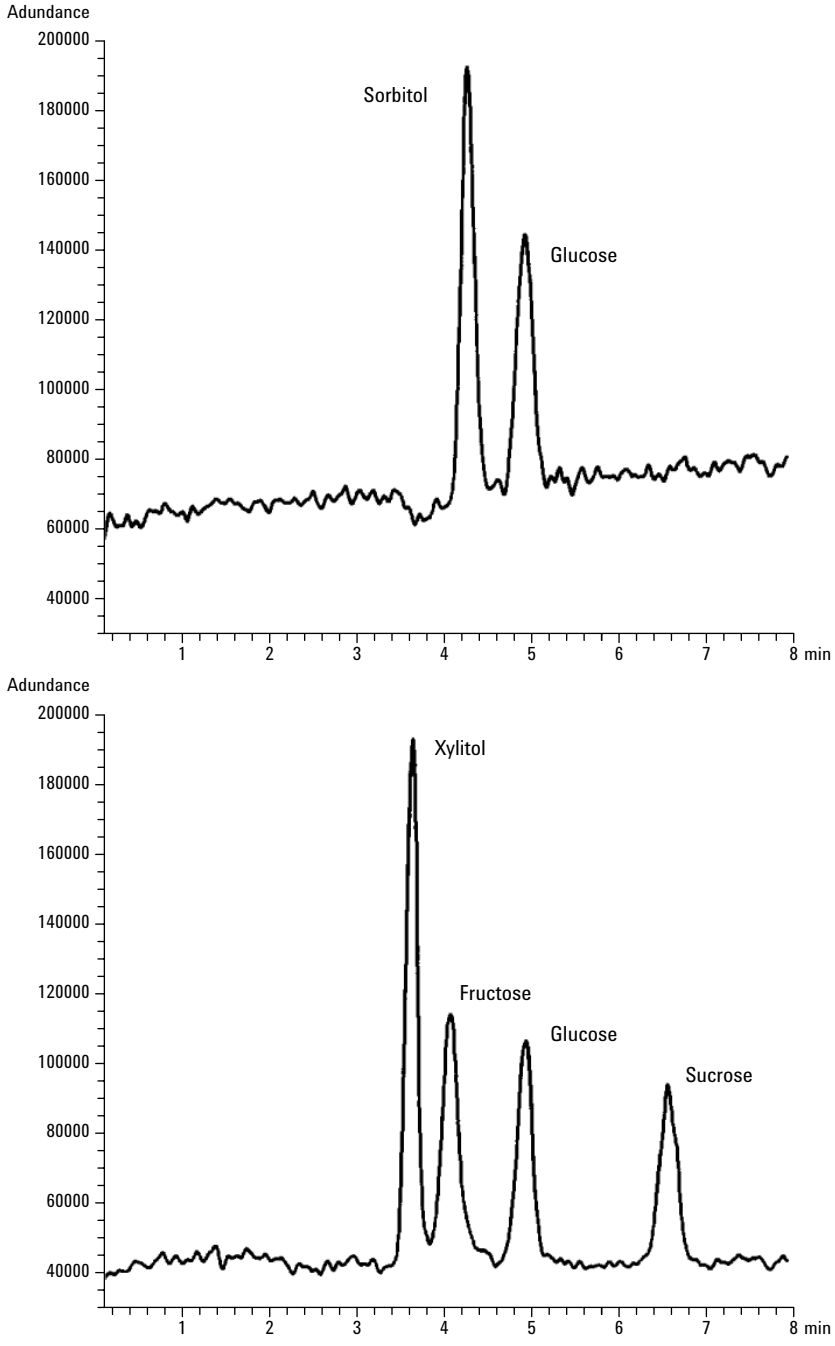

Figure 1. Stacked SIM chromatograms of sugar standards at 1 µg/mL each.

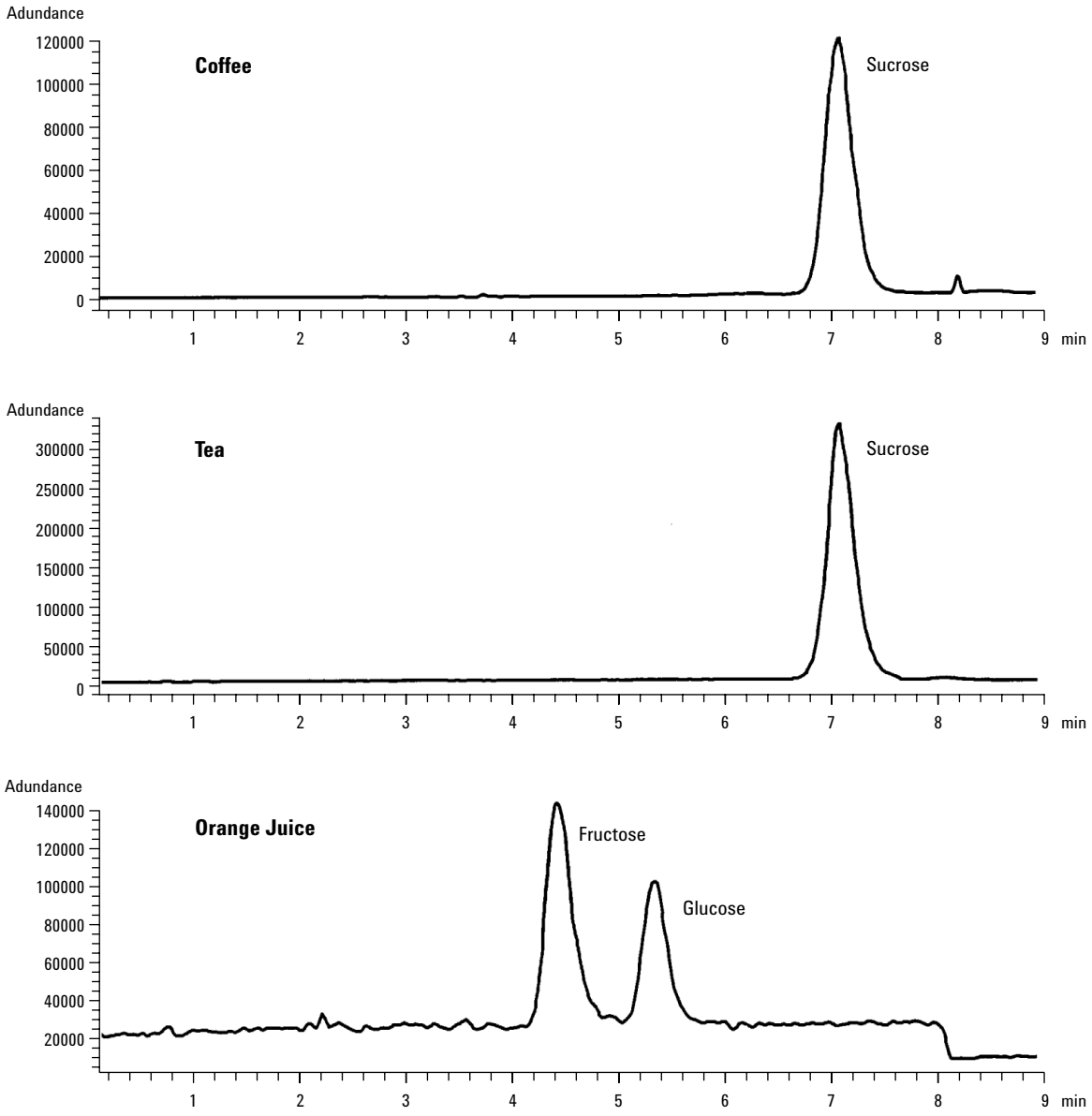

Figure 2. Stacked SIM chromatograms for typical sugars in three common beverages.

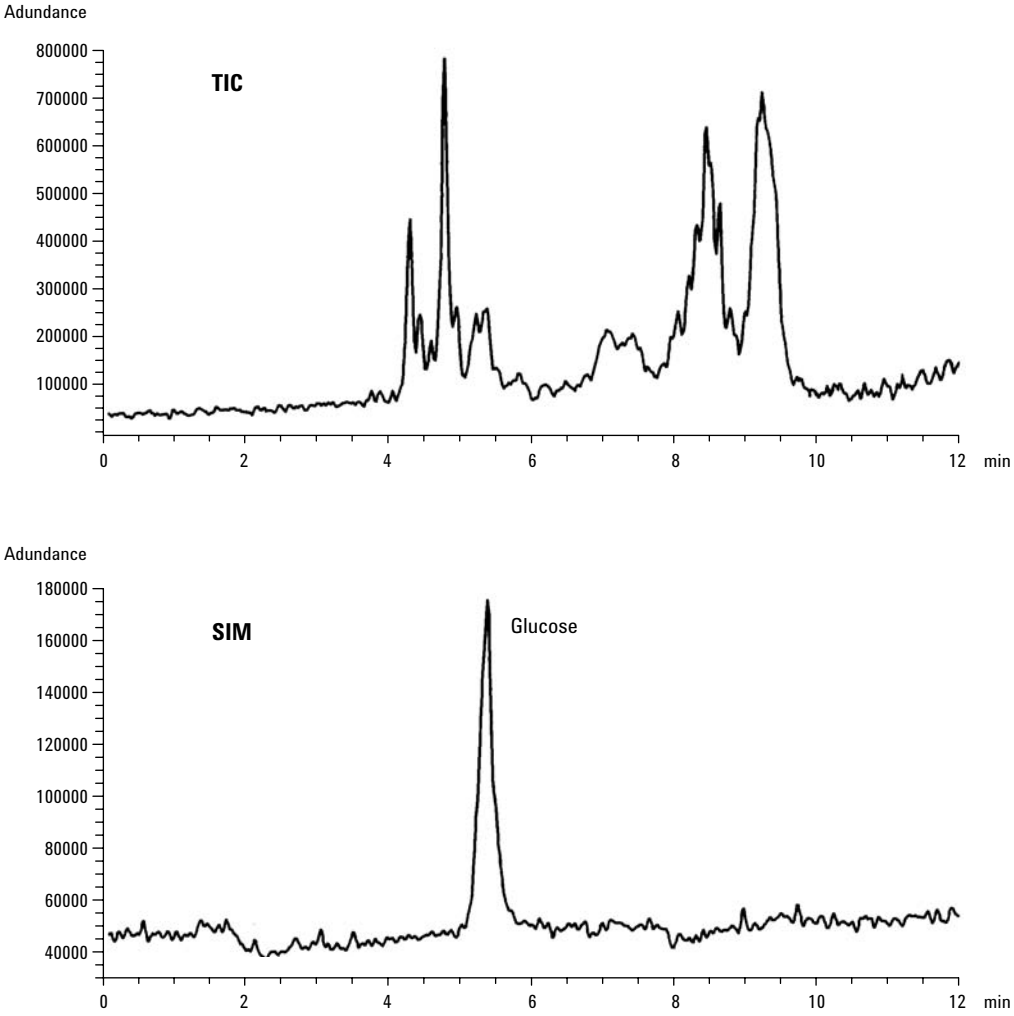

Figure 3. Stacked TIC and SIM chromatograms for Sake (Japanese rice wine).

Conclusion

Underivatized sugars and sugar alcohols can be successfully analyzed by LC/MS using atmospheric pressure chemical ionization (APCI) in negative ion mode.

Hiroki Kumagai is an application chemist at Agilent/Yokogawa Analytical Systems, Tokyo, Japan.

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Agilent Technologies

Process control of starch

Application

Heinz Goetz
and Peter Kilz

Starch is a polysaccharide composed of two components with different molecular weight–about 20–30 % amylose and about 80–70 % amylopectin. Starch is a very important part of the diet for both humans and animals. For example, it is available in potatoes, flour and bread. Further areas of use are in the production of paste, adhesive, glucose, dextrin and beer. Figure 1 shows an overlay of three chromatograms obtained under the same conditions. The chromatogram of the

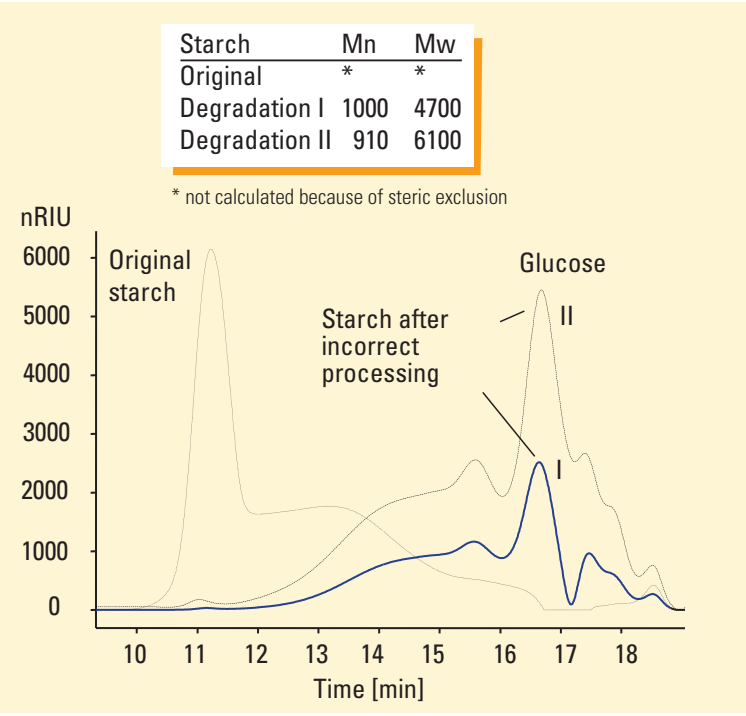

Figure 1
Overlay of chromatograms of original starch and starch after incorrect processing

Conditions

Sample preparation
Sample was dissolved in 1 mL eluent at 20 °C (concentration 0.1 % w/w). Dextran standards from Polymer Standards Services (PSS) were used for narrow standard calibration.

Column
PSS Suprema 100 + 1000 in series, 10 µm, 300 × 8 mm

Mobile phase
0.1 M sodium nitrate

Flow rate
1 ml/min

Column compartment temperature
25° C

Injection volume
100 µl

Detector
refractive index detector

original starch shows a big peak of the polysaccharide eluting at a retention time of approximately 11 minutes. This peak almost disappears in the chromatograms of the degraded starch (chromatograms I and II). New peaks from degradation products elute between 12 and 17 minutes, especially from the degradation end product glucose. Degradation of the original starch is also shown by the molecular weight data (table in figure 1). Both the number (M_n) and the weight average molecular weight (M_w) of the degraded products are significantly below 10000 dalton while starch in it's original state gives data of 300000 to 2000000 dalton.

The GPC-SEC method described here shows an easy but reliable analysis for process and quality control of starch.

Equipment

Agilent 1100 Series GPC-SEC system
consisting of

- vacuum degasser for efficient degassing of the mobile phase
- isocratic pump with large solvent cabinet
- autosampler with single valve design
- thermostatted column compartment for precise column temperatures
- refractive index detector with automatic recycle valve
- ChemStation Plus with GPC-SEC data analysis software

Columns supplier:
Polymer Standards Service, Mainz, Germany

Heinz Goetz is an application chemist at Agilent Technologies, Waldbronn, Germany. Peter Kilz is Managing Director at Polymer Standards Service, Mainz, Germany

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Publication Number 5988-0117EN

Agilent Technologies
Innovating the HP Way

Rapid monitoring of carbohydrates in food with capillary electrophoresis

Application Note

Food

Authors

Tomoyoshi Soga
Institute for Advanced Biosciences,
Keio University, Japan

Maria Serwe
Agilent Technologies
Waldbronn, Germany

Abstract

Processed food often contains additives such as sweeteners or preservatives. For regulatory compliance the concentration of these substances needs to be monitored. This is important to ensure the consumer's protection and also has economic implications. In Japan, for example, the tax rate of imported juice depends on the sucrose content. Non-derivatized carbohydrates can rapidly be determined using capillary electrophoresis with indirect UV-detection. The method's simplicity and stability make it suitable for the application in routine labs.

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Experimental

Carbohydrate analysis was performed using the Agilent Capillary Electrophoresis system equipped with DAD detection and Agilent ChemStation software. The method is based on the use of the Agilent Basic Anion Buffer (Agilent part number 5064-8209) together with a standard bare fused silica capillary (Agilent part number G1600-62211).

Prior to first use, a new capillary was flushed with run buffer for 15 minutes (at 1 bar). Between analyses the capillary was flushed 4 minutes from an extra buffer vial into waste. Sample preparation was simple and consisted, even in the case of yogurt, of only dilution and ultrafiltration for protein removal.

Equipment

- Agilent Capillary Electrophoresis system
- Agilent ChemStation
- Agilent Basic Anion Buffer

Results and discussion

Figure 1 shows the analysis of orange juice and yogurt in comparison with a carbohydrate standard. The standard assay was linear over the range 50–10 000 ppm with $r^2 > 0.999$. Method detection limits were 7–15 ppm. Sucrose levels were 43 g/L in orange juice and 109 g/L in yogurt. Fructose and glucose in orange juice were determined as 23 g/L and 25 g/L, respectively. The yogurt contained 26 g/L lactose. Repeatability was < 0.16 % RSD for migration times and < 2.3 % RSD for peak areas (n = 6).

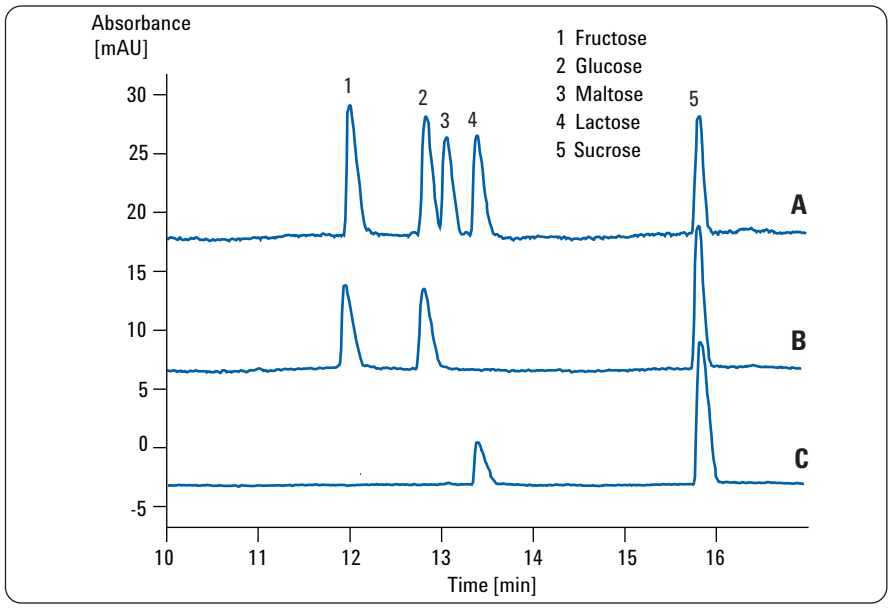

Figure 1
Analysis of sugars in orange juice and yogurt.

Chromatographic conditions

Sample:	A Carbohydrate standard (1000 ppm each)
	B Orange juice (diluted with H_2O 1:20, filtered through 0.22-µm filter)
	C Yogurt (diluted with H_2O 1:20, filtered through 30 kDa cut-off filter)
Injection:	6 seconds at 50 mbar
Capillary:	Bare fused silica capillary total length 80.5 cm, effective length 72 cm, internal diameter 50 µm (Agilent part number G1600-62211)
Buffer:	Agilent Basic Anion Buffer (Agilent part number 5064-8209)
Voltage:	-25 kV
Temperature:	15 °C

Detection signal: 350/20 nm, reference 275/10 nm

High performance liquid chromatography (HPLC) with refractive index detection is routinely used for the analysis of carbohydrates in food, yielding low ppm detection limits when modern equipment is used. However, capillary electrophoresis with indirect UV-detection, providing the same sensitivity at short overall run times, can be a worthy complement. This is especially true for complex sample matrices, which present challenges for the classical stationary phases used for carbohydrate analysis by HPLC. This makes the method applicable to a wide range of food with complex matrices.

In Japan, the method presented here is routinely applied as a quick screening for carbohydrates in fruit juices, whiskey or rice plant sap.

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Agilent Technologies

Analysis of sugars in foods and beverages by HPLC with pulsed amperometric detector

Hiroki Kumagi and Ikuko Tajima

Food

Abstract

Sugars are generally analyzed by HPLC with refractive index detection. UV or fluorescence detection are also frequently used, coupled with pre or post derivatization for high sensitive analysis. However, the methods mentioned above have some limitations, such as the difficulty of simultaneously analyzing various sugars, or the tedious procedure of derivatization. The pulsed amperometric detector (PAD) is a common method for sugar analysis. The coupling of the PAD and post-column pH–changing offers a simple, easy and highly sensitive way of detecting sugars.

This brief demonstrates the analysis of various sugars in candy and juice using PAD with post-column pH–changing. The sugars are separated by both ligand exchange and size exclusion mode.

Analyzed Compounds

Sucrose, glucose, galactose, mannose, fructose, mannitole, and sorbitol.

Sample

Candy and apple juice.

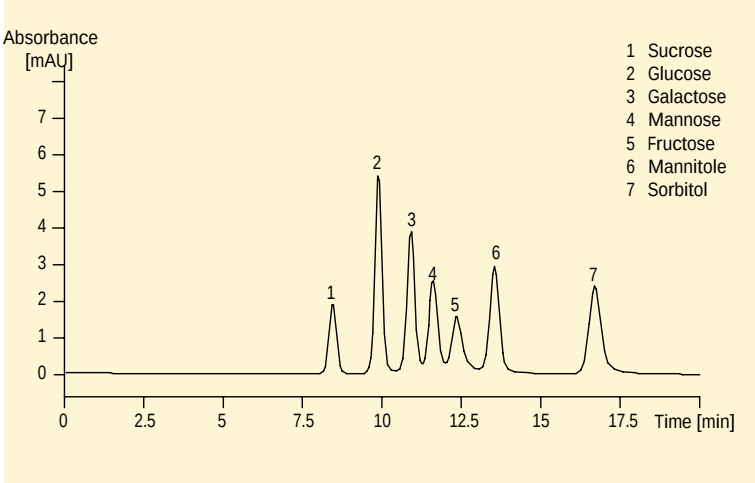

Figure 1
Chromatogram of standard solution, 50 mgl/l each

Conditions

Column 300 x 7.8 mm Excelpak CHA-L31 (This column is same as SPELCOGEL C-611)
Mobile phase
0.1 mM NaOH, 0.75 ml/min
Post column reagent
400 mM NaOH, 0.6 ml/min
Column temp 80 °C
Injection vol 20 µl
Detector
Agilent 1049 Electrochemical detctor
Mode; Pulse mode
Working electrode; gold
Applied Potential; Pot1 = 0.65 V, Pot2 = -0.95 V, Pot3 = 0.15 V

Sample preparation
Candy was dissolved in water and filtrated Juice was diluted and filtrated.

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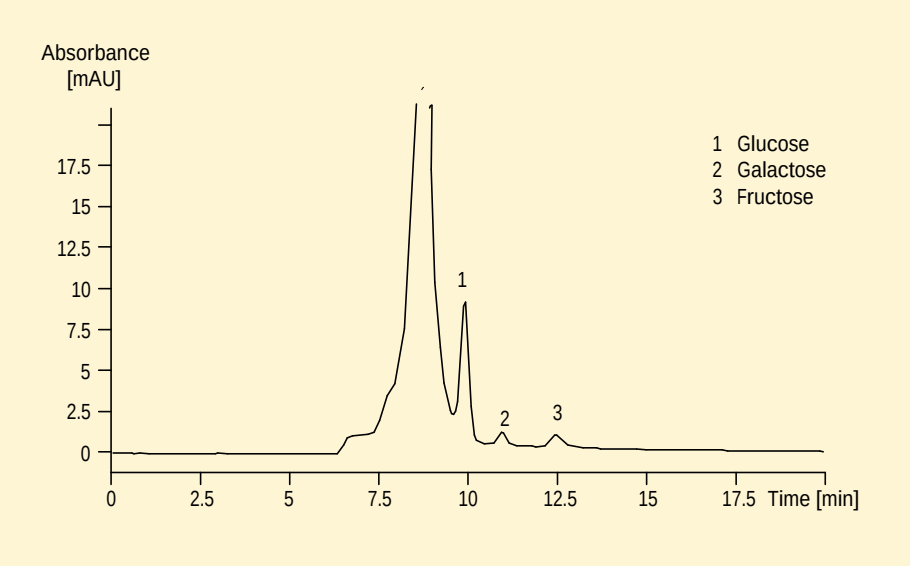


Figure 2
Chromatogram of sugars in candy

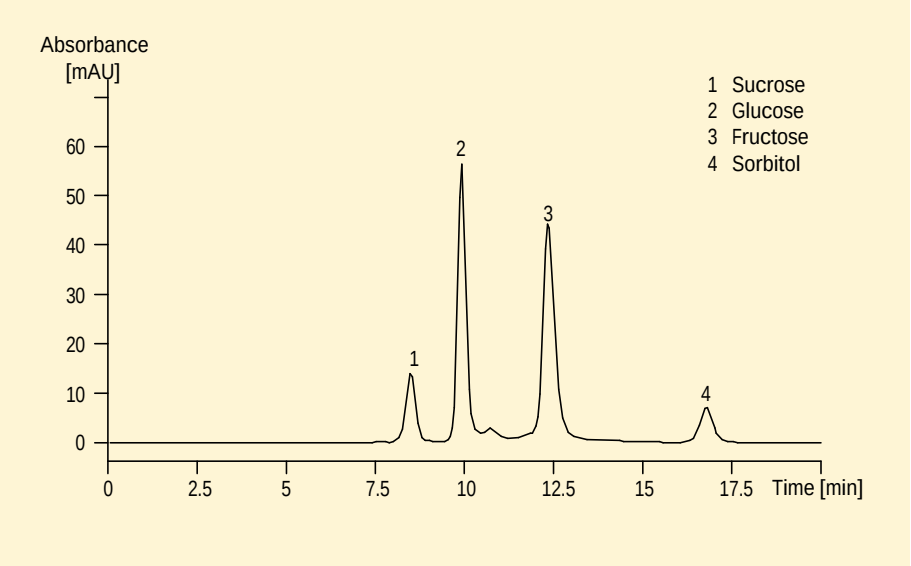


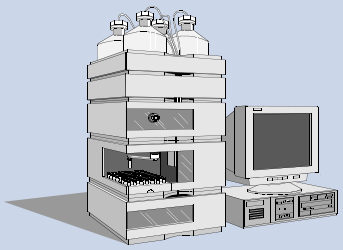
Figure 3
Chromatogram of sugars in apple juice

Equipment

Agilent 1100 Series

- 2 isocratic pumps with
- vacuum degasser
- autosampler
- thermostatted column compartment
- programmable electro chemical detector

Agilent ChemStation + software



Method Performance

Limit of detection
0.23~0.95 mg/L (S/N = 3)
RSD of peak area 1.0~4.0 %
RSD of retention time
0.1~0.2 %

Hiroki Kumagi and Ikuko Tajima are application engineers at Yokogawa Analytical Systems Inc., Tokyo, Japan.

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Agilent Technologies
Innovating the HP Way

Analysis of Carbohydrates in Lemonade using HPLC

Angelika Gratzfeld-Huesgen

Food

Abstract

The following carbohydrates have been analyzed: glucose, galactose, raffinose, fructose, mannitol, sorbitol, lactose, maltose, cellobiose, and sucrose. Food carbohydrates are characterized by a wide range of chemical reactivity and molecular size. Because carbohydrates do not possess chromophores or fluorophores, they cannot be detected with UV-visible or fluorescence techniques. Nowadays, however, refractive index detection can be used to detect concentrations in the low parts per million (ppm) range and above, whereas electrochemical detection is used in the analysis of sugars in the low parts per billion (ppb) range.

Sample preparation

Degassed drinks can be injected directly after filtration. More complex samples require more extensive treatment, such as fat extraction and deproteination. Sample cleanup to remove less polar impurities can be done through solid-phase extraction on C18 columns.

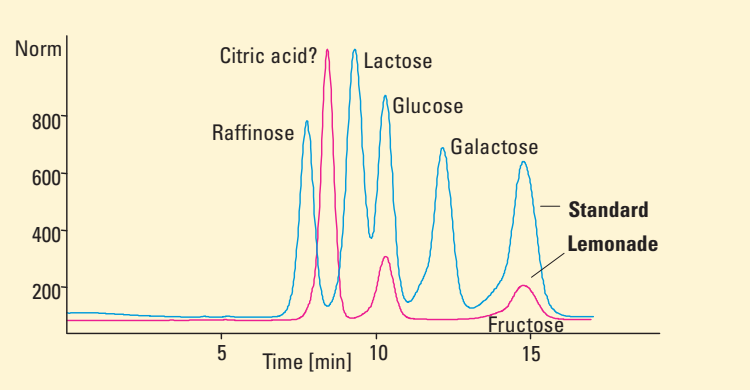

Figure 1
Analysis of carbohydrates in lemonade

Conditions

Column
300 ˜ 7.8 mm Bio-Rad HPXP, 9 µm
Mobile phase water
Column compartment 80 ºC
Flow rate 0.7 ml/min
Detector refractive index

Sample preparation
Samples were directly injected.

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Innovating the HP Way

Chromatographic conditions

The HPLC method presented here was used to analyze mono-, di-, and trisaccharides as well as sugar alcohols.

HPLC method performance

Limit of detection
<80 ng with S/N = 2

Repeatability of
RT over 10 runs <0.05 %
areas over 10 ru ns 2 %

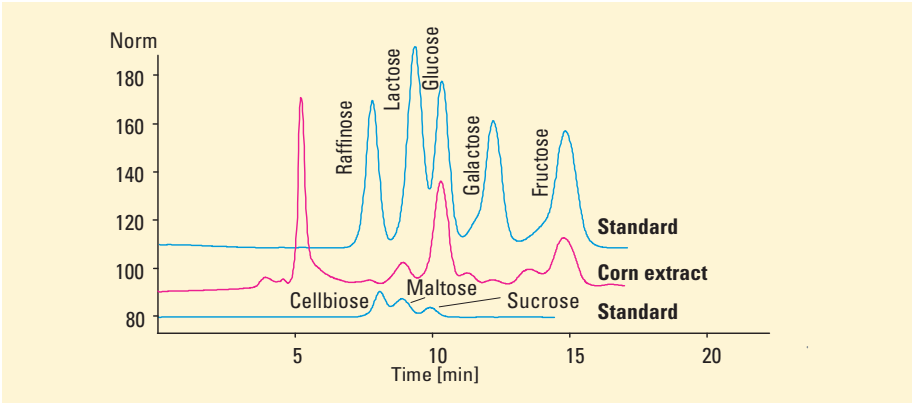

Figure 2
Analysis of carbohydrates in corn extract

References

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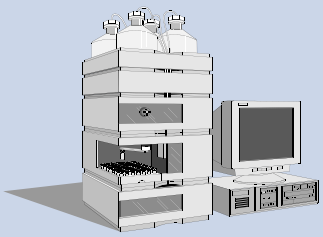
Equipment

Agilent 1100 Series
- degasser
- isocratic pump
- autosampler
- thermostatted column compartment
- refractive index detector

Agilent ChemStation + software



Angelika Gratzfeld-Huesgen is application chemist at Agilent Technologies, Waldbronn, Germany.

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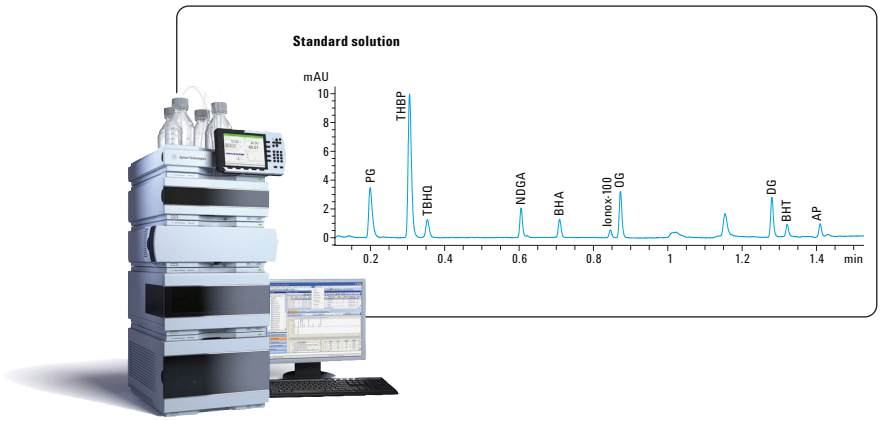
Ultrafast analysis of synthetic antioxidants in vegetable oils using the Agilent 1290 Infinity LC system

Application Note

Food

Authors

Gerd Vanhoenacker, Frank David,
Pat Sandra
Research Institute for Chromatography
Kennedypark 26
B-8500 Kortrijk
Belgium



Abstract

The addition of synthetic antioxidants in edible vegetable oils is regulated in Europe and the US. The official method was translated into an ultrafast LC method using the Agilent 1290 Infinity LC equipped with an Agilent ZORBAX Rapid Resolution High Definition (RRHD) column. High throughput is obtained in 2 min with a backpressure of 1120 bar, which is below the 1200 bar upper limit of the column. Optimization of the mobile phase composition and the temperature are discussed. The figures of merit are illustrated using standard solutions and spiked vegetable oil (sunflower, rapeseed, and olive) extracts. Limits of detection are 1 mg/kg or less in the oil samples. Using a simple methanol extraction, good recovery was obtained for all antioxidants in the oil samples.

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Introduction

Lipid oxidation causes rancidity and odor problems and decreases the nutritional value of food products. Synthetic ascorbyl palmitate and phenolic antioxidants are often added to foods to prevent oxidation of unsaturated fatty acids in oils and fats. Combinations of antioxidants are commonly used to enhance the antioxidative effect. The structures and abbreviations of the investigated antioxidants are shown in Figure 1.

Regulatory agencies in Europe[1] and the US[2] have imposed maximum levels for some antioxidants while the use of others has been forbidden. The determination of antioxidants in foods and food components is therefore an important analysis. The limits are given in Table 1.

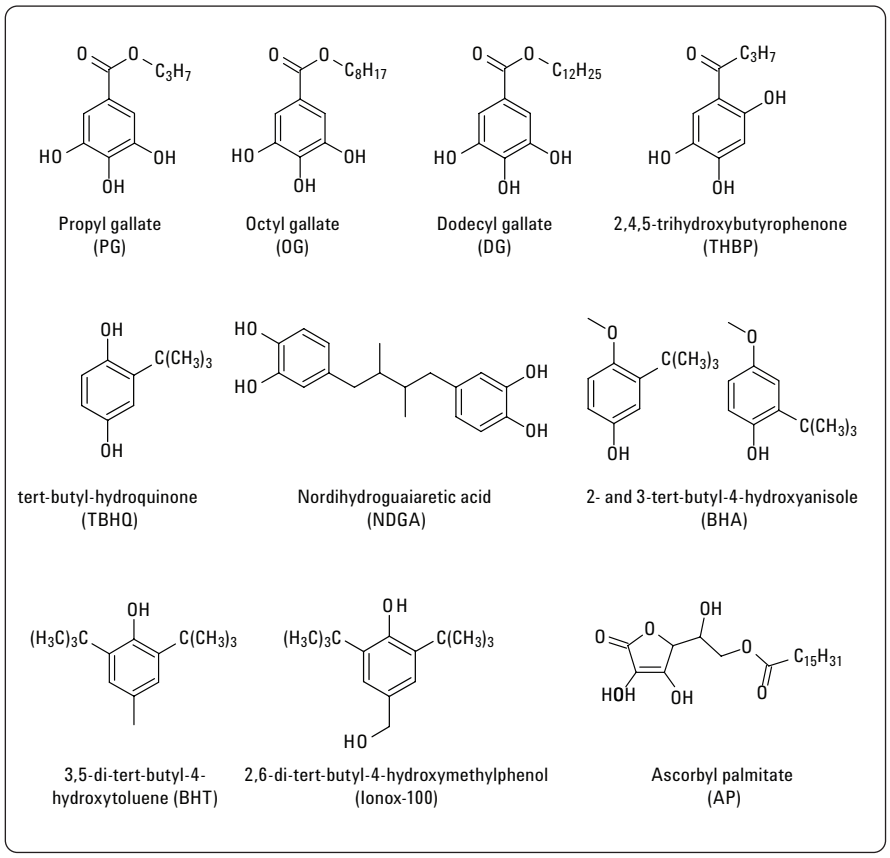

Figure 1
Structures and codes of the investigated antioxidants.

Antioxidant	Europe[1]	US[2]
AP	Quantum satis	No restriction
PG OG DG BHA	≤ 200 mg/kg, individual or combined	≤ 200 mg/kg, individual or combined
BHT	≤ 100 mg/kg	
TBHQ	Not allowed	
THBP	Not allowed	Not allowed
NDGA	Not allowed	Not allowed
Ionox-100	Not allowed	Not allowed

Table 1
Limits for antioxidants in edible oils in Europe and US.

In the official method for the determination of the antioxidants in edible oils, columns of 15 to 25 cm in length with an internal diameter of 4.6 mm, and packed with 5-µm octadecyl silica particles are used.[3] The mobile phase is composed of diluted acetic or phosphoric acid (eluent A) and methanol/acetonitrile 50/50 volume to volume (eluent B). Analysis times are between 15 to 25 min.

There are two reasons for increasing the speed of analysis for this application. First, instability of some of the targets (for example, AP) have been reported and long residence times of samples in an autosampler can already lead to significant degradation of the compounds. Perrin and Meyer could enhance the stability of sample and standard solutions by using citric and isoascorbic acid.[4] They were able to stabilize AP at room temperature for about 7 h. However, QC laboratories in edible oil and fat processing industries have a need for increased analysis speed. The presence or absence, and assay of antioxidants have to be carried out prior to loading or unloading oils and fats. A fast, accurate, and precise result is desirable for economical and practical reasons.

This Application Note describes the analysis of 10 antioxidants in vegetable oils using the Agilent 1290 Infinity LC. The original method was translated into a high throughput method by optimizing the mobile phase composition and the temperature. The figures of merit are presented for vegetable oil and spiked oil extracts.

Experimental

Instrumentation and method

An Agilent 1290 Infinity LC system with the configuration in Table 2 was used:

Solutions and samples

Sample and standard solutions were prepared according to Perrin and Meyer.[4] The solvent for the standards and extraction is a solution of citric acid (1 mg/mL) and isoascorbic acid (1 mg/mL) in methanol. For the spiked samples, a stock solution of the antioxidants in the solvent was added prior to extraction. The extraction was carried out by weighing 1 g of oil and adding 10 mL of the solvent. This mixture was vortexed for 30 s, allowed to stand for 2 min, and vortexed once more for 30 s. The sample was then centrifuged at 5000 x g for 5 min and the supernatant was transferred into an autosampler vial for injection.

Part number	Description
G4220A	Agilent 1290 Infinity Binary Pump with integrated vacuum degasser
G4226A	Agilent 1290 Infinity Autosampler
G1316C	Agilent 1290 Infinity Thermostatted Column Compartment
G4212A	Agilent 1290 Infinity Diode Array Detector

Method parameters:	
Column	ZORBAX RRHD Eclipse Plus C18, 50 mm L × 2.1 mm id, 1.8 µm d_p
Mobile phase	A = 0.02% phosphoric acid in water B = Acetonitrile/methanol 50/50 or 75/25 v/v
Flow rate	Variable
Gradient	Variable
Temperature	Variable
Injection	2 µL
Detection	DAD, 40 or 80 Hz Phenolic antioxidants Signal 280/10 nm, Reference 400/50 nm Ascorbyl palmitate Signal 255/10 nm, Reference 400/50 nm

Table 2
Conditions

Results and Discussion

The analysis was first carried out with the mobile phase used in the official method. The flow rate was set at a moderate 0.4 mL/min. The analysis time was 8 min (see Figure 2A). The synthetic phenolic antioxidants are all detected at 280 nm while for ascorbyl palmitate (AP) 255 nm was used. The eluent B composition was then modified from methanol/acetonitrile 50/50 to 75/25 volume to volume to lower the viscosity and enable a faster separation. The selectivity changed considerably with this mobile phase adaptation and the temperature was optimized to obtain sufficient separation between all target antioxidants. Note that at 45 °C, the elution order of dodecyl gallate (DG) and BHT is reversed compared to Figure 2A at 30 °C. All compounds were stable at 45 °C column temperature.

An additional advantage of the increased temperature is the decrease of the backpressure. When the flow rate was increased to 1.9 mL/min the last peak eluted under 1.5 min and the pressure on the column was 1120 bar (Figure 2C).

	A	B	C
Methanol/Acetonitrile ratio (v/v)	50/50	75/25	75/25
Flow rate	0.4 mL/min	0.4 mL/min	1.9 mL/min
Gradient	0–7.5 min: 35–100% B	0–7.5 min: 30–100% B	0–1.6 min: 30–100% B
Temperature	30 °C	45 °C	45 °C
Detector speed	40 Hz	40 Hz	80 Hz
Maximum pressure	375 bar	270 bar	1120 bar

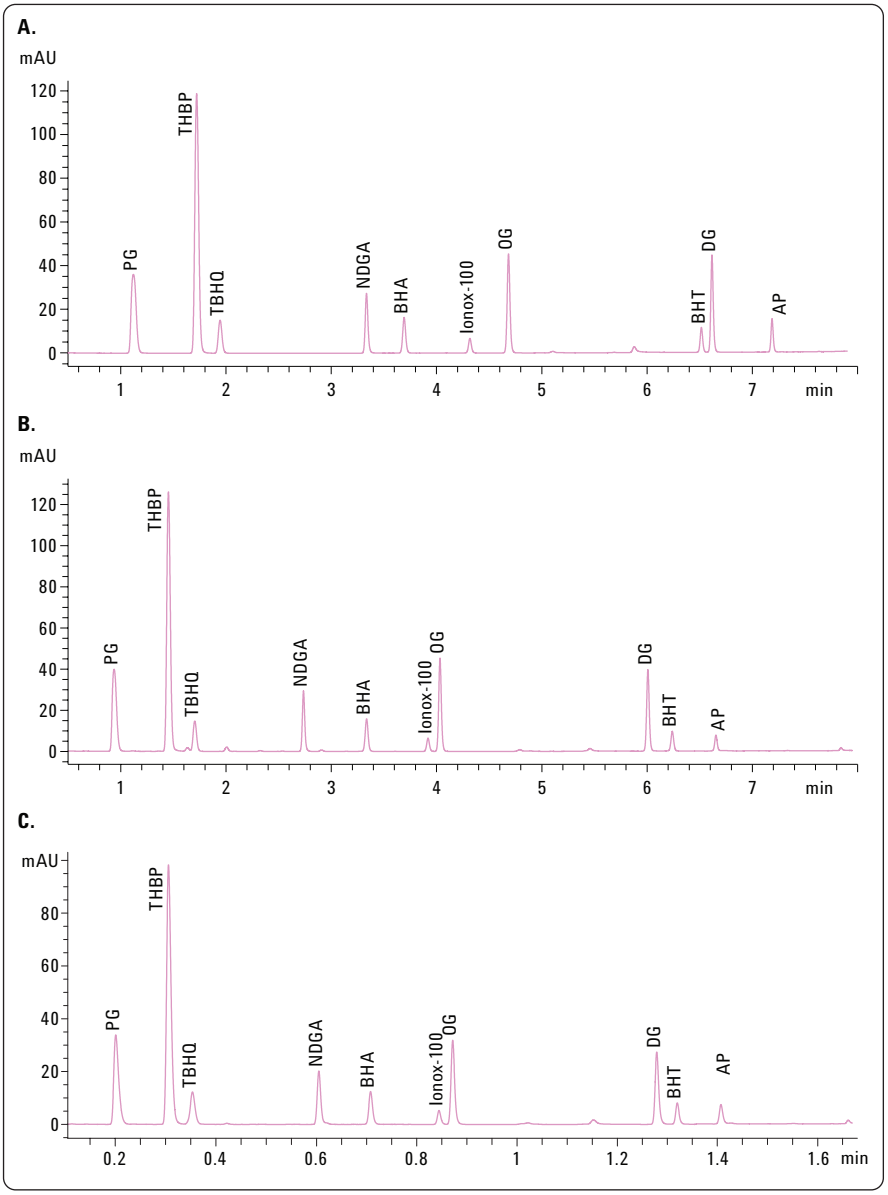

Figure 2
Analysis of 10 µg/mL standard solution under the various conditions.

The performance of the ultrafast method was evaluated and the results are summarized in Table 3. The repeatability and linearity of the method were investigated using standard solutions of the antioxidants. The detection limit was equal to or below 0.1 µg/mL for all antioxidants. This corresponds to approximately 1 mg/kg or lower in an oil or fat sample. Extracts of vegetable oils and spiked oils were analyzed to determine the recovery and accuracy. The oil samples were spiked with 10 or 50 mg/kg of each antioxidant and the detected amounts in the extracts were compared to standard solutions at the same concentration.

	Repeatability (% RSD)[1]	Linearity (R^2)[2]	Recovery 10 mg/kg (%)			Recovery 50 mg/kg (%)		
			Sunflower	Rapeseed	Olive	Sunflower	Rapeseed	Olive
PG	0.27	0.99988	100.1	105.3	95.1	100.0	100.9	98.3
THBP	0.27	0.99983	97.3	99.1	105.9	98.6	99.1	99.4
TBHQ	0.99	0.99933	90.7	89.7	81.2	97.4	95.8	95.6
NDGA	0.16	0.99983	109.3	89.7	93.6	102.2	98.0	98.9
BHA	0.33	0.99983	104.8	107.0	102.0	98.5	96.4	94.4
Ionox-100	0.40	0.99974	90.7	93.8	89.7	97.5	97.5	93.3
OG	0.41	0.99985	99.3	101.0	95.9	99.7	100.3	98.8
DG	0.56	0.99985	97.8	100.1	101.9	98.4	98.9	98.7
BHT	0.54	0.99960	81.0	89.5	74.4	81.6	83.8	79.0
AP	0.67	0.99934	92.6	85.7	75.4	89.5	91.2	83.7

(1) 6 consecutive injections of 10 µg/mL standard solution
(2) 0.1, 0.2, 0.5, 1, 10 µg/mL standard solution, 1 injection/level

Table 3
Method performance data.

The chromatograms for the fast analysis of a standard solution and the spiked oil samples are shown in Figure 3. Additional peaks originating from the oil matrix are visible in the chromatograms but only a few interfere with the analysis. Most interfering peaks are present in the olive oil sample, however, the 10 mg/kg spiked oil can still be differentiated from an unspiked sample and the recovery is satisfactory (Table 3).

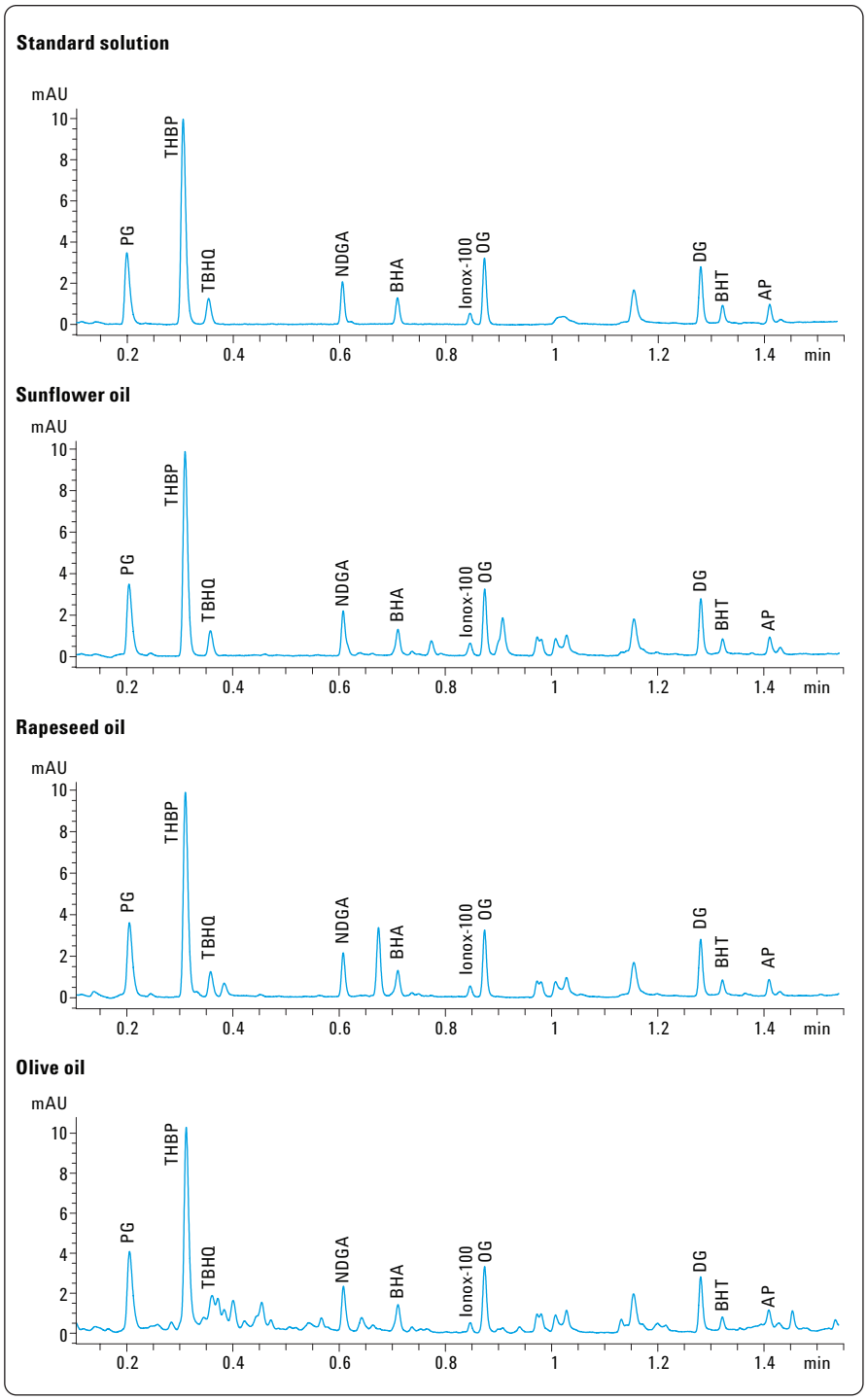

Figure 3
Analysis of standard solution (1 µg/mL) and spiked oil (10 mg/kg) extracts with the fast method.

Conclusion

Using the Agilent 1290 Infinity LC, an ultrafast analytical method could be developed for the determination of antioxidants in vegetable oils. The analysis time could be reduced to less than 2 min with a backpressure of 1120 bar. The performance of the high throughput method (repeatability, linearity, detection limits) was investigated using standard solutions. Oil samples and spiked oil samples were extracted and the recovery of the antioxidants was calculated. Satisfactory recovery was obtained for all antioxidants. The developed method is useful in laboratories where a fast result is mandatory.

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Agilent Technologies

High-Resolution Analysis of Intact Triglycerides by Reversed Phase HPLC Using the Agilent 1290 Infinity LC UHPLC System

Application Note

Food, Hydrocarbon Processing

Authors

Michael Woodman
Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE 19808
USA

Abstract

The Agilent 1290 Infinity LC System with ultraviolet/visible (UV/VIS) Diode Array detection (DAD) is used to analyze triglycerides in soybean oil under non-aqueous reversed phase gradient conditions. The Agilent 1290 Infinity LC System was used for the chromatographic separation of the sample on 3.0 and 2.1 mm id C18 columns, of various lengths, with 1.8-µm packing materials prepared in 600 bar (9000 psi) or special 1200 bar (18,000 psi) configurations. The ability of the Agilent 1290 Infinity LC System to operate with long, high resolution columns is demonstrated with isopropanol (IPA) or methyl tert butyl ether (MTBE) as the strong solvent and acetonitrile as the weak component of the mobile phase mixture.

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Introduction

The analysis of intact triglycerides from animal or vegetable sources has many practical uses including understanding the chemical composition of the triglyceride, assessing fuel potential, and understanding lipid metabolism and behavior in living systems. The general conditions for successful analysis of these components by high-performance liquid chromatograph (HPLC) include gradient elution and low-wavelength monitoring of the overall separation. Because triglycerides have relatively few chromophores it is also beneficial to use evaporative light scattering detectors (ELSD) or mass spectrometers to facilitate other views of the separation.

During the development of this application, we analyzed a number of vegetable oils from various sources including soy, corn, rice bran, safflower, grape seed, olive, and palm oil. Because of the wide abundance of soybean oil in the United States and its growing significance in the production of biofuels, most of this work was standardized on maximizing the resolution of soybean oil triglycerides. These general conditions, however, are also suitable for a wide variety of samples including samples from animal lipid sources.

Intact triglycerides generally have very low water solubility and as such are commonly separated by normal phase chromatography, which separates species largely based on differences in polar functional groups, or by reversed phase chromatography operating in a non-aqueous mode of separation, which has more selectivity for small differences on carbon character such as chain length or unsaturation.

According to information published by Perkins [1] the predominant fatty acids, which are the building blocks of triglycerides on a glycerol backbone, found in soybean oil are myristic (14:0), palmitic (16:0), oleic (18:1), linoleic (18:2) and linolenic (18:3). Many other minor fatty acids are also present and because all of the fatty acids are randomly constructed into triglycerides, an extensive permutation of fatty acid substructure is obviously possible. Because the predominant difference between fatty acids consists of carbon chain length and number of double bonds, most of the diversity in triglycerides is found in the rather non-polar organic structural features. As a result, reversed phase chromatography is most useful for this application. Triglycerides have extremely poor solubility in water so one normally chooses either a high organic starting position, with respect to the aqueous content or, as in this work, a completely non-aqueous separation environment.

The typical structure of a triglyceride is shown in Figure 1. [2] In this example, from top to bottom, palmitic acid (C16:0), oleic acid (C18:1), alpha-linolenic acid (C18:3) are shown with respect to chain length and degree of unsaturation. The chemical formula is $C_{55}H_{98}O_6$.

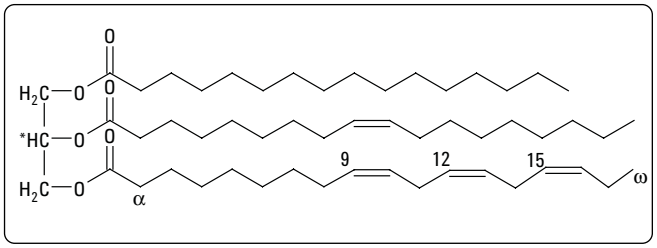

Figure 1. Typical triglyceride structure.

Experimental

Sample Preparation

The primary solution was prepared at a concentration of 10 mg/mL, in 2-propanol or 2:1 volume to volume MeOH/MTBE, and subsequently diluted to lower concentrations as needed. Injection volumes of 0.2-2 µL were made into the LC/DAD system.

LC Method Details

LC Conditions

Agilent 1290 Infinity LC System binary pump G4220A,
Agilent 1290 Infinity LC System autosampler G4226A
Agilent Thermostatted Column Compartment G1316C with switching valve
Agilent 1290 Infinity LC System diode array UV/VIS detector G4212A with 10 mm path fiber optic flow cell

Columns:	(See individual figures for specific usage) Agilent ZORBAX SB-C18 RRHT, 3 mm × 150 mm, 1.8 µm 600 bar, p/n 829975-302 Agilent ZORBAX SB-C18 RRHD, 2.1 mm × 100 mm, 1.8 µm 1200 bar, p/n 858700-902 Agilent ZORBAX SB-C18 RRHD, 2.1 mm × 150 mm, 1.8 µm 1200 bar, p/n 859700-902 In some cases, columns were coupled to extend the length and resolution.
Column temp:	20 °C or 30 °C
Mobile phase:	A = acetonitrile B = isopropanol (IPA) or tert butyl methyl ether (MTBE) (See individual figures)
Flow rate:	See individual figures
Gradient:	The gradient conditions were either 20% to 60% IPA or 10% to 40% MTBE, based on the strong eluting strength of MTBE when compared to IPA. The gradient slope was maintained at 2.6% organic phase increase per column volume for IPA gradients and 2.0% with MTBE, altering gradient time and flow rate accordingly. This was determined by calculations using a modification of the Agilent Method Translator. [3]

UV Conditions

Monitoring 210, 220 and 230 nm, bandwidth 4 nm, reference wavelength off

Results and Discussion

A typical gradient separation of triglycerides using acetonitrile IPA gradient is shown in Figure 2.

Some general comments are appropriate about the conditions and chromatographic profile shown in Figure 2. While it would be ideal to consider less expensive methanol as the weak eluent, introduction of methanol or denatured ethanol containing methanol has consistently shown a dramatic reduction in the overall resolution of the triglycerides. The significant increase in operating pressure, when running the gradient from acetonitrile to IPA, is clearly limiting and undesirable. Increasing the operating temperature of the column as a means of reducing solvent viscosity has proven to be undesirable because the chromatographic resolution tends to collapse as temperature increases.

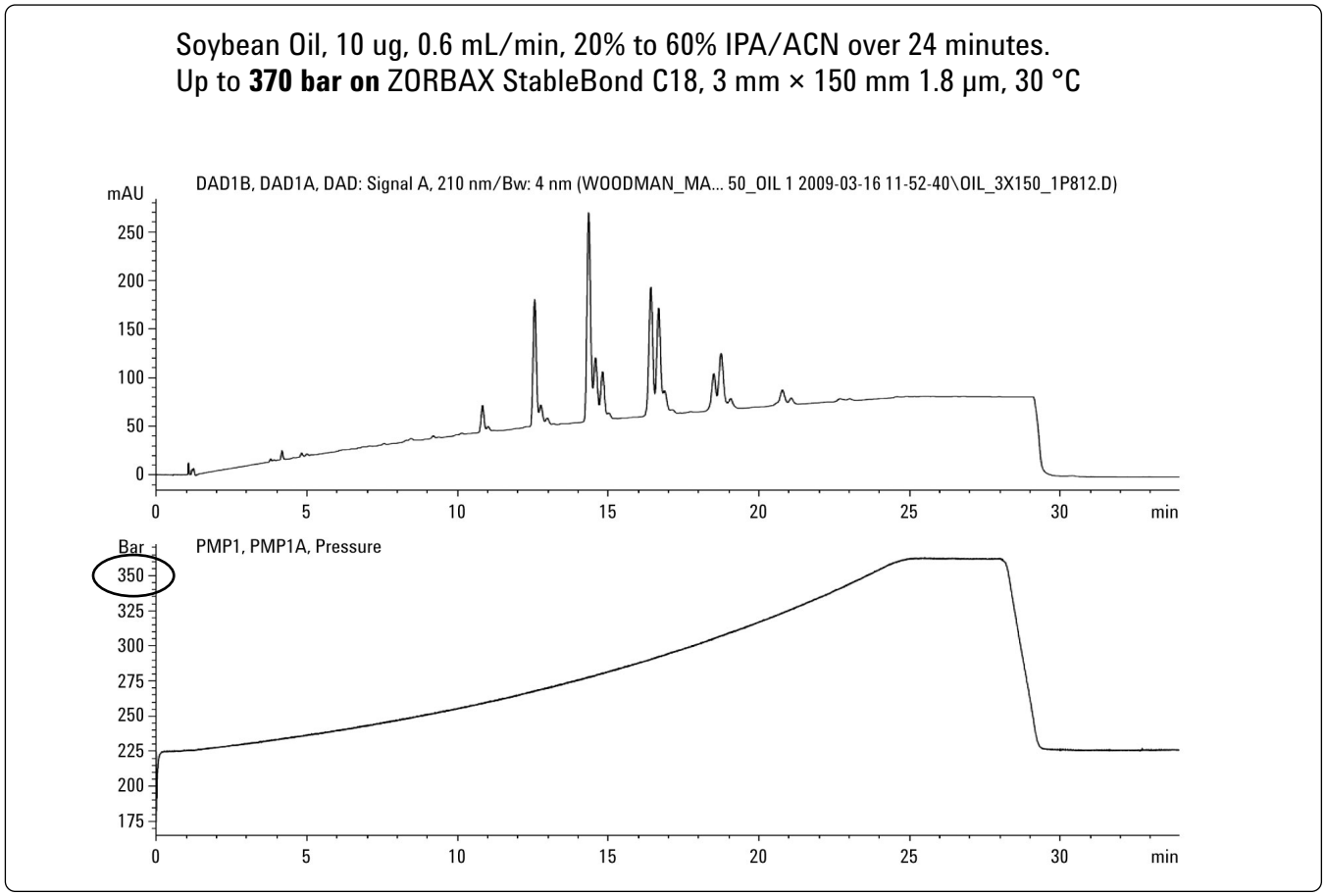

Figure 2. A 210-nm UV chromatogram of soybean oil sample on a 3 mm × 150 mm ZORBAX Rapid Resolution High Throughput (RRHT), 1.8 µm column, upper panel. System pressure trace showing the general progress of the gradient elution, lower panel. Flow rate 0.6 mL/min, gradient time 24 min. Strong solvent, isopropanol. The chromatogram demonstrates the typical difficulty encountered with this type of separation, which is small clusters of chromatographically similar triglycerides. These clusters are not positional isomers of the same carbon number and degree of unsaturation, rather a mixture of various chain lengths and number of double bonds as shown by mass spectrometric evaluation.

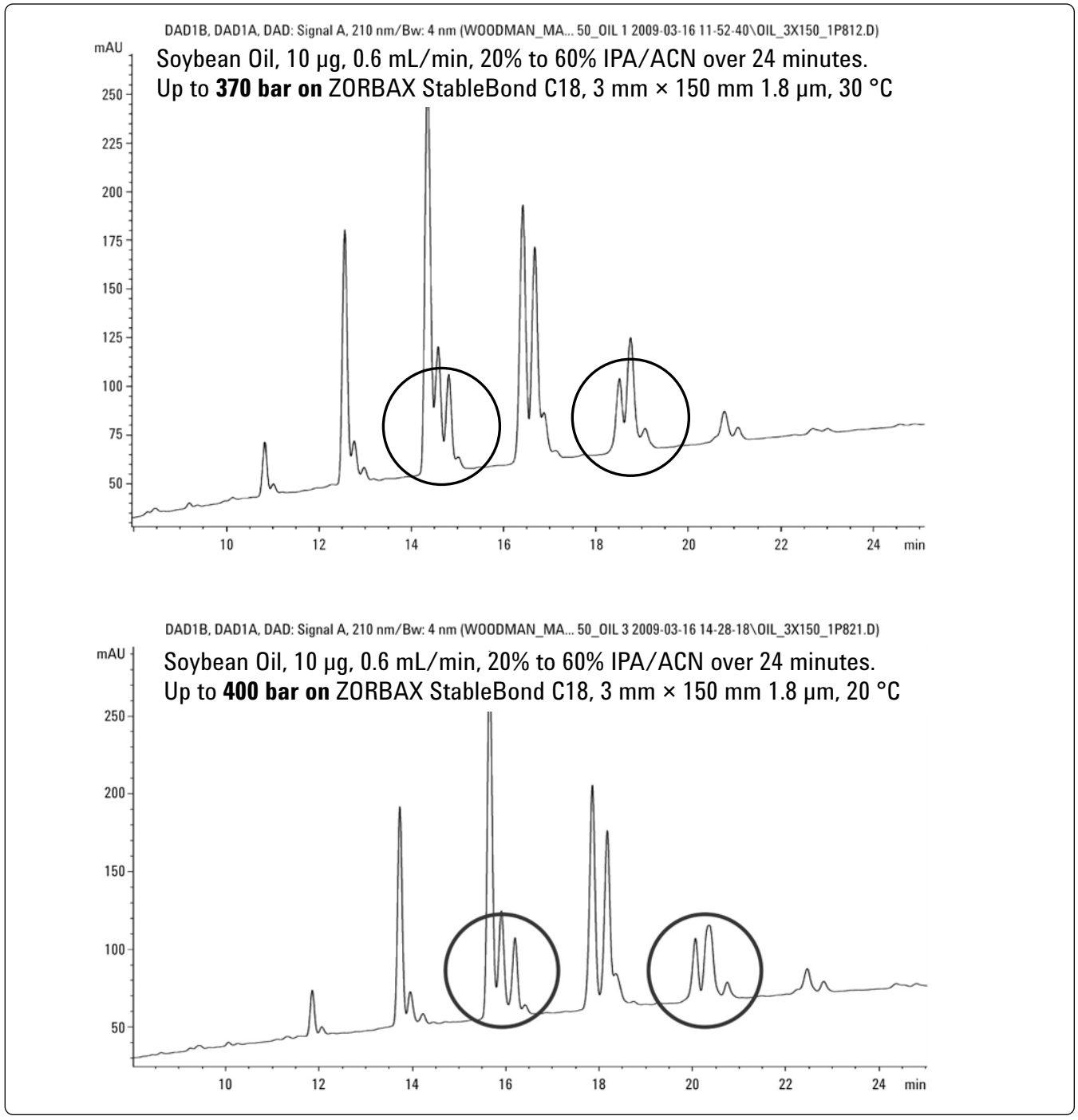

Figure 3. An expanded presentation of the chromatogram shown in Figure 2 at 30 °C, upper panel, compared with the same conditions in Figure 2 operating the column at 20 °C.

In Figure 3 we see the improvement achieved by operating the separation at 20 °C rather than 30 °C. The operating pressure increase is approximately 10% at the lower temperature. While many of our separations have been performed at 30 °C as a compromise between separation and operating pressure, the availability of the Agilent 1290 Infinity LC System with increased operating pressure capability has allowed us to reduce the temperature to 20 °C and demonstrate a usable improvement in separation.

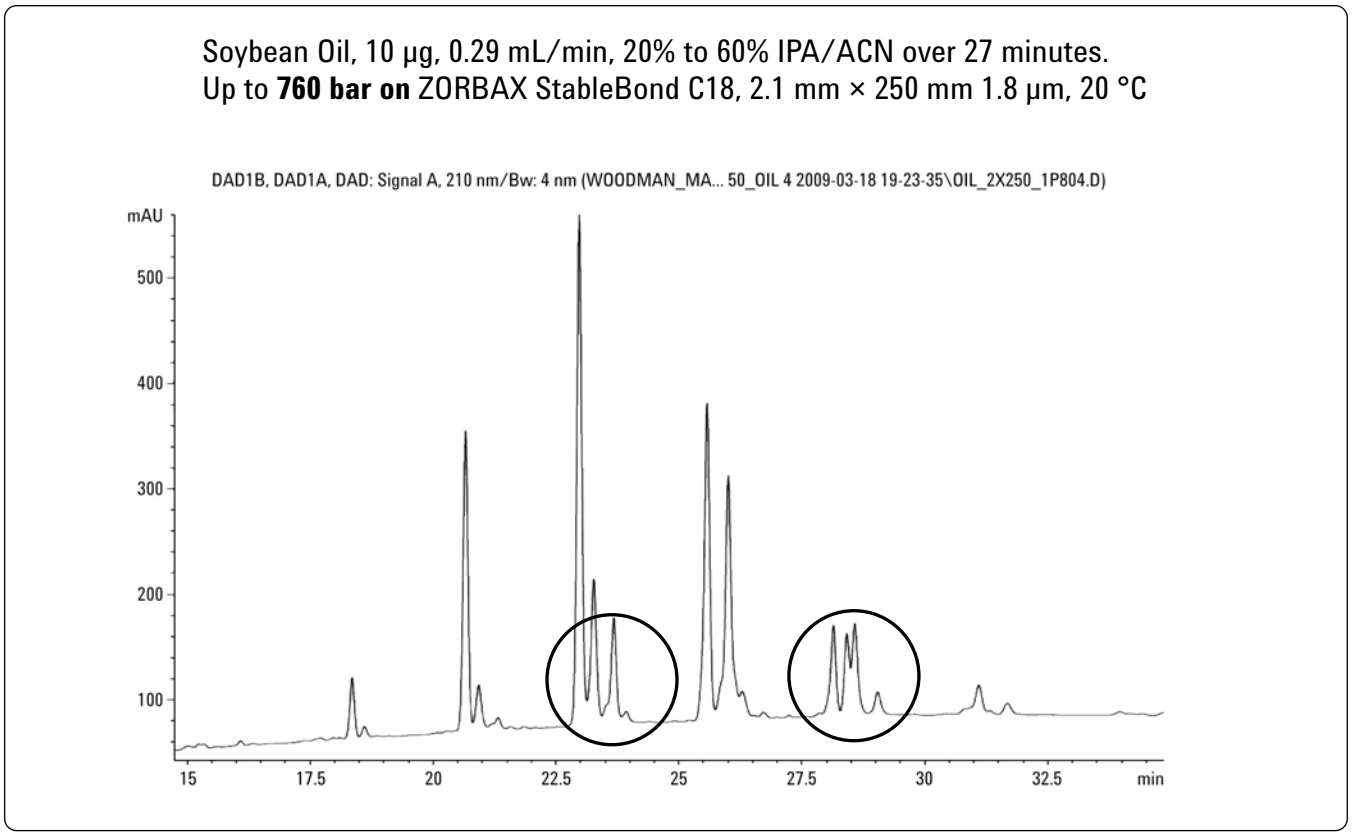

Figure 4. Analysis of the soybean oil sample on an Agilent ZORBAX StableBond C18 column, 2.1 mm × 250 mm, 1.8 µm, (150 mm in series with 100 mm) prepared for operation at 1200 bar pressure limit. Flow rate 0.29 mL/min, gradient time 27 min. Maximum observed pressure 760 bar.

In Figure 4, we see that increasing the length of the column has resulted in a significant increase in the resolution of some of the observed components. To further increase resolution, it would be practical to explore longer columns or explore alternative mobile phase or column chemistries. As with most very high performance separations, rate-limiting features tend to include operating pressure, operating temperature, and maximum flow rate. The triglyceride separations evaluated thus far have not been receptive to operation at higher column temperatures or higher flow rates, presumably because of their relatively high molecular weight and flexible organic structure. Even when gradient slope translations are carefully made to ensure organic strength consistency from method to method, operating at higher flow rates has consistently shown degradation of the overall separation. Because the isopropanol has significantly high viscosity and high pressure, it seemed appropriate to consider other non-polar solvents that are miscible with acetonitrile and friendly to low UV detection, as a substitute for isopropanol.

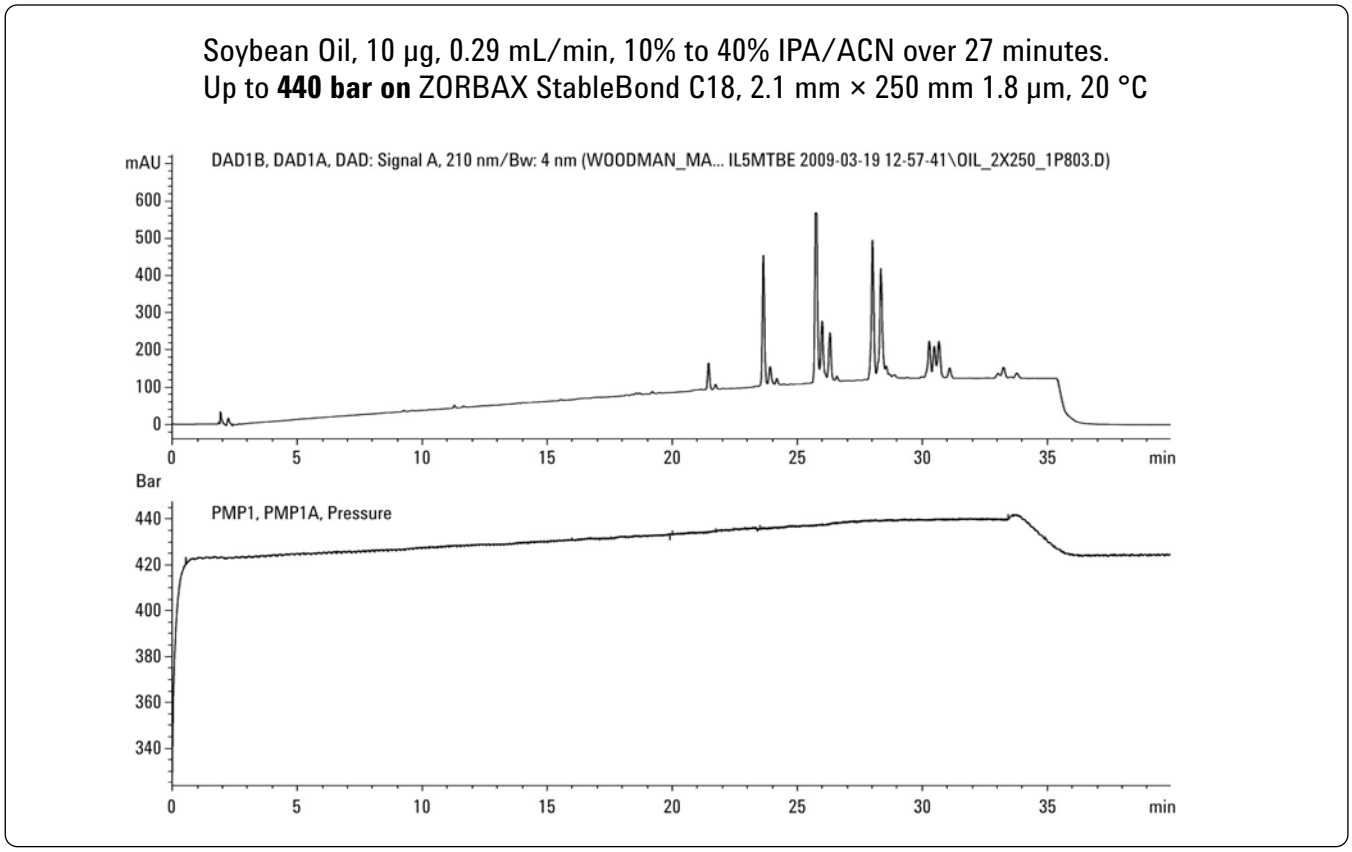

Figure 5. By substituting MTBE for isopropanol with otherwise the same conditions as Figure 4, and then re-optimizing the gradient for the significant increase in eluting strength of MTBE, we arrive at a new set of operating conditions where there is only a small difference in operating pressure over the gradient run. Flow rate 0.29 mL/min, gradient 27 min for 10% to 40% MTBE, maximum observed pressure 440 bar.

In Figure 5, the change to MTBE and subsequent readjustment of the gradient resulted in a separation that was very comparable to the original isopropanol separation, however at a much lower maximum operating pressure. In view of the prior evidence and comments regarding increased temperature or flow rate resulting in degraded separation, it seemed that the most appropriate way to take advantage of the new operating pressure capability of the Agilent 1290 Infinity LC System was to continue to increase the column length. The Agilent 1290 Infinity LC System and associated ZORBAX chemistries are capable of operating pressures up to 1200 bar, or approximately 18,000 psi. To ensure robust and rugged system operation many users typically specify the upper pressure limit for a method at a value less than 80% of the rated operating pressure.

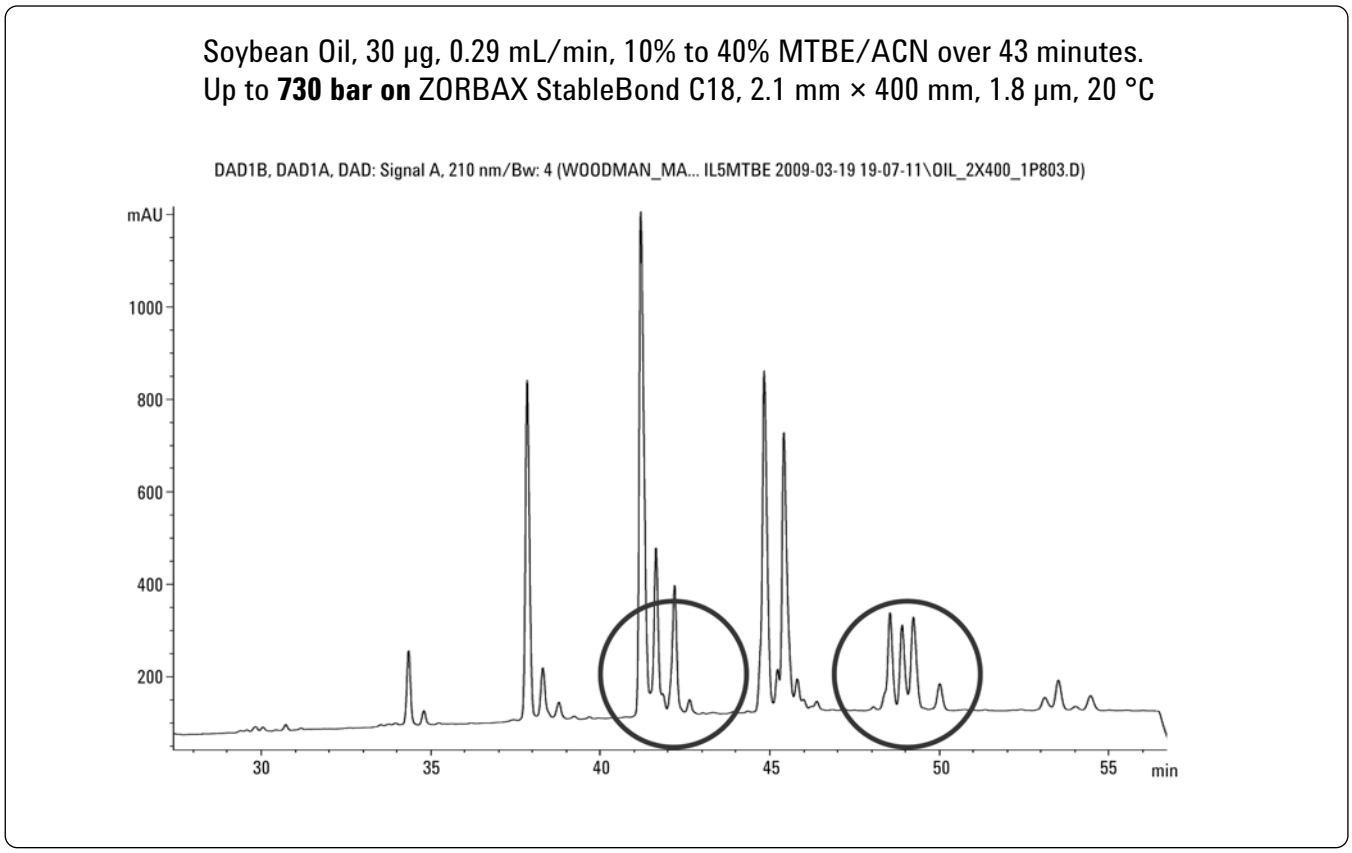

Figure 6. Separation of the soybean oil sample on a 2.1 mm × 400 mm ZORBAX StableBond C18, 1.8 µm 1200 bar columns (150 mm + 150 mm + 100 mm in series). Flow rate 0.29 mL/min gradient time 43 min, for a gradient of 10% to 40% MTBE. Maximum operating pressure 730 bar at 20 °C.

As shown in Figure 6, having previously optimized the column temperature, operating flow rate and gradient slope for the best possible balance between resolution and analysis time, and after investigating a variety of solvents as candidates for both the weak solvent and strong solvent choice, we are left with an ultimate opportunity to operate on a very long column set of 1.8 µm particle size columns under conditions ideal for the separation of this group of triglycerides. With an operating pressure of only 730 bar, which is about 60% of the rated capability of the Agilent 1290 Infinity LC System, it is clearly possible to consider even longer column lengths or a further reduction in the operating temperature as both of these seem promising in terms of delivering even higher resolution out of the mixture.

The separation with MTBE or isopropanol can be adapted for use with a mass spectrometer as one of the detectors. In previous studies (see www.Agilent.com/chem ASMS 2009 for a poster on this subject) we have been able to demonstrate the capability of quickly and confidently identifying the composition of many of the triglycerides found in this and other samples. For optimum electrospray performance in the non-aqueous, non-buffered environment it was useful to do post UV detector addition of a mixture of methanol and water with ammonium formate buffer to enhance ionization and to ensure a consistent ability to preserve the molecular ion into the mass spectrometer. It has been shown by McIntyre [4] that the presence of ammonium formate in the mobile phase significantly improves the probability that a molecular ion will be formed and preserved in the mass analyzer portion of a mass spectrometer.

Conclusions

Using the Agilent 1290 Infinity LC System, we were able to easily demonstrate UHPLC capabilities well within the operating range of the instrument. The significantly enhanced resolution afforded by long sub-2 micron particle size columns in the sub-ambient column compartment environment will contribute significantly to our understanding of the major and minor composition of this sample and other similar materials. This should significantly enhance the contribution of liquid chromatography to the understanding of seed oil composition, the role of triglycerides in metabolism, and the area of lipidomics where great interest has been directed on the LC separation coupled to time-of-flight high-resolution mass spectrometry (LC/TOF).

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Analysis of Triglycerides by Liquid Chromatography/Mass Spectrometry

Application

Food

Author

Hiroki Kumagai

Abstract

Triglycerides were readily analyzed using liquid chromatography/mass spectrometry with atmospheric pressure chemical ionization in positive ion mode.

Background

Oil is a primary component of many foods, such as cooking oils and dairy products. Consequently, it is desirable to conduct compositional analyses of oil-based fatty acid components in food. Such analyses may use either gas chromatography (GC) or high-performance liquid chromatography (HPLC), but the GC methods have problems with the complexity of sample preprocessing, which requires saponification.

With HPLC a direct analysis is possible; however, these methods are low in sensitivity because the compounds of interest do not absorb ultraviolet and separation is barely adequate.

In this study, six triglycerides (Table 1) with identical fatty acid compositions were analyzed using liquid chromatography/mass spectrometry (LC/MS) and atmospheric pressure chemical ionization (APCI) as the ionization mode.

Table 1. Chemical Structure of Triglycerides

$CH_2 - OCO - R_1$
$CH - OCO - R_2$
$CH_2 - OCO - R_3$

Triglyceride	$R_1=R_2=R_3$
Trilaurin	C_{11}
Trimyristin	C_{13}
Tripalmitin	C_{15}
Tristearin	C_{18}
Triolein	$C_{18:1}$
Trilinolein	$C_{18:2}$

Method

- Instrument: Agilent 1100 LC/MS with APCI in positive mode

  Mass range: 100 to 1000 *m/z*

  Drying gas: N_2 4 L/min, 350 °C

  Nebulizer: N_2 50 psi

  Fragmentor: 160 V

  Vaporizer: 400 °C

- LC Conditions:

  Mobile phase: $(CH_3)2CO/H_2O$ (98/2)

  Flow rate: 1.0 mL/min

  Oven temperature: 40 °C

  Injection volume: 15 µL

- Column: Develosil ODS-UG3, 4.6 mm id × 75 mm long

Results

The following figures show total ion chromatogram (TIC) and selected ion mode (SIM) chromatograms, and mass spectra for the selected triglyceride standards.

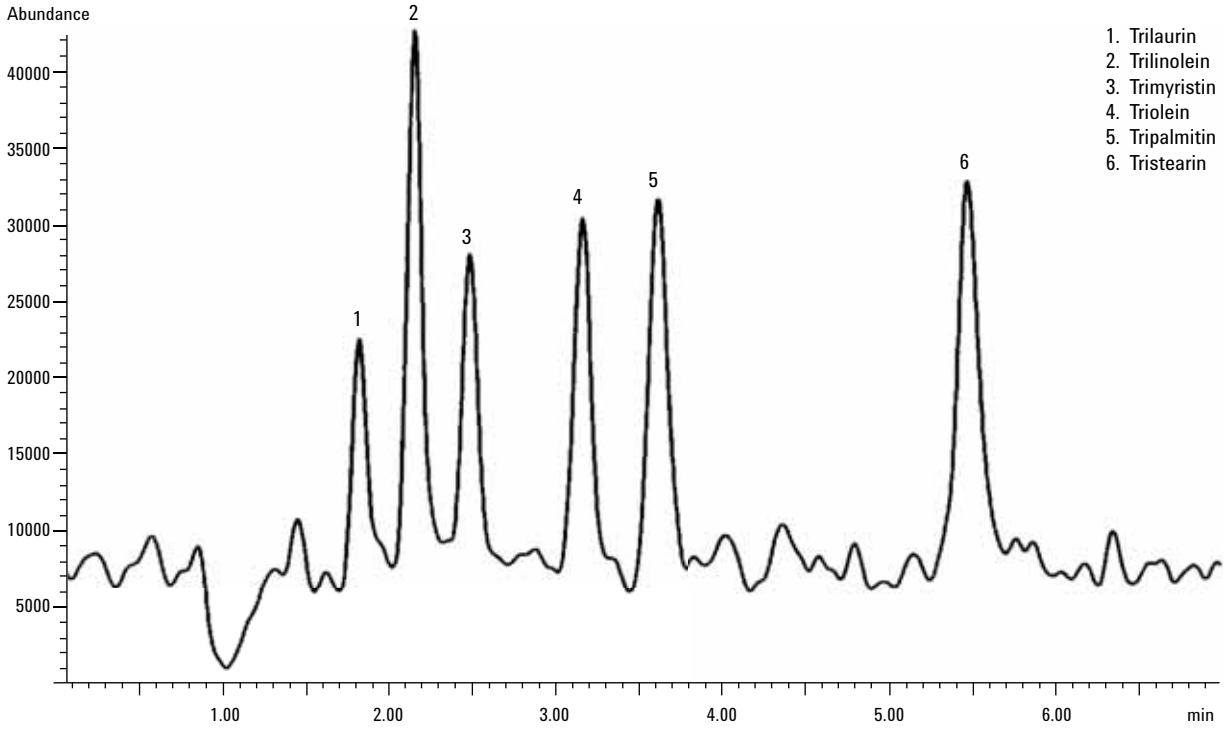

Figure 1. TIC of triglyceride standards, each at 0.2 ppm.

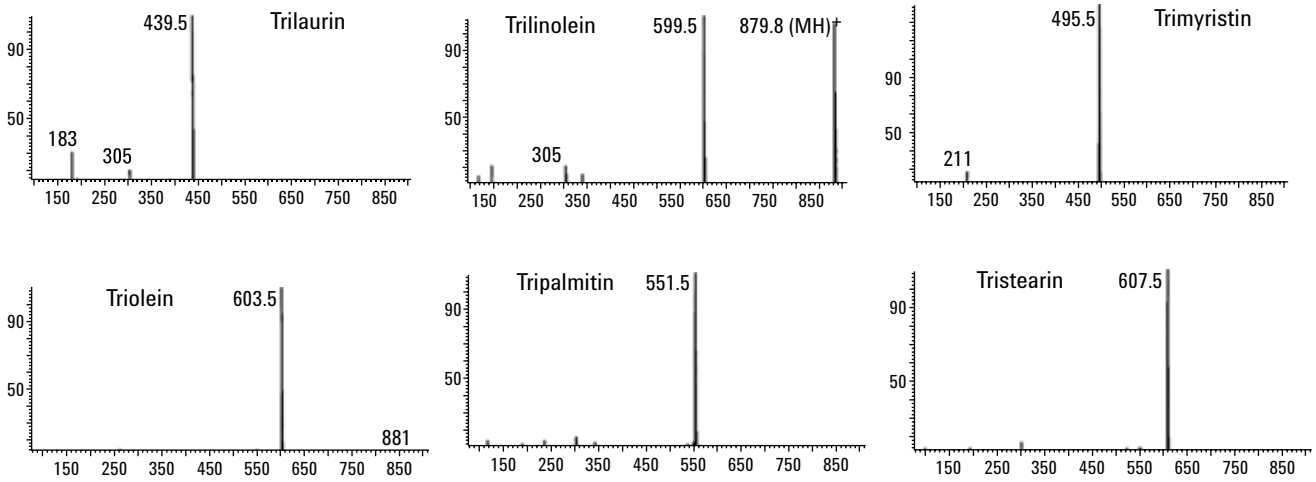

Figure 2. Mass spectra of individual triglyceride standards at 0.2 ppm.

The tallest peak in each case represents the $[MH\text{-}HOOCR]^+$ ion created after the loss of a fatty acid group from the protonated psuedomolecular ion. With the exception of Trilinolein, the protonated psuedomolecular ion was not observed.

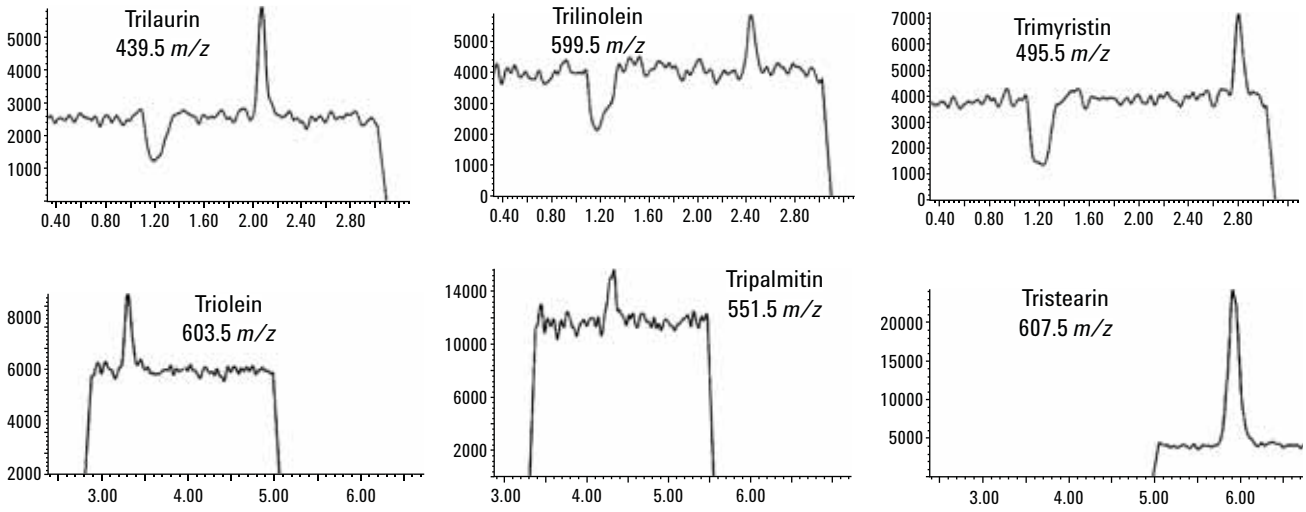

Figure 3. SIM chromatograms of individual triglyceride standards at 1 ppb.

The protonated molecular ions were rarely observed—trilinolein in the scan mode is the exception—and the base peaks consisted of fragmented ions from which fatty acid had been detached.

Sensitivity was favorable, extending down to 0.2 ppm in TIC mode. In the SIM mode, measurements at 1 ppb were possible by selecting the base peak in the mass spectrum of each composition of monitored ions. With this technique, it is possible to measure fat-soluble substances such as triglycerides with a high degree of sensitivity.

Conclusions

LC/MS, with APCI in positive ion mode, readily detected selected triglyceride standards with high sensitivity.

The analytical peaks were the positvely charged fragmented ions from which fatty acid had been detached.

SIM mode yielded sensitivity to 1 ppb.

Hiroki Kumagai is an application chemist at Agilent/Yokogawa Analytical Systems, Tokyo, Japan.

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Column Selection for the Analysis of Fatty Acid Methyl Esters

Application

Food Analysis

Authors

Frank David
Research Institute for Chromatography
President Kennedy Park 20
B-8500 Kortrijk,
Belgium

Pat Sandra
University of Gent
Krijgslaan 281 S4,
B-9000 Gent
Belgium

Allen K. Vickers
Agilent Technologies, Inc.
91 Blue Ravine Road
Folsom, CA 95630-4714
USA

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).

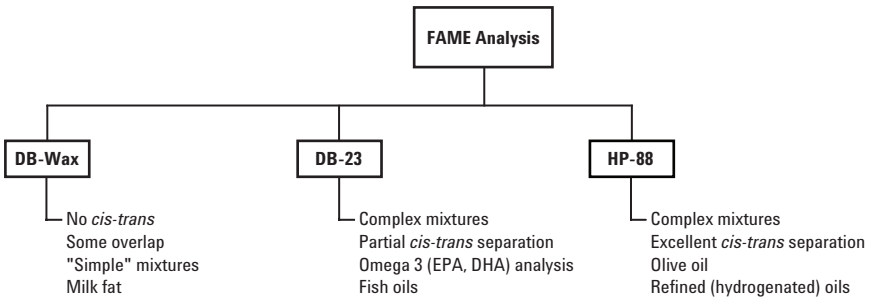

Figure 1. Overview of column selection for FAMEs analysis.

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.

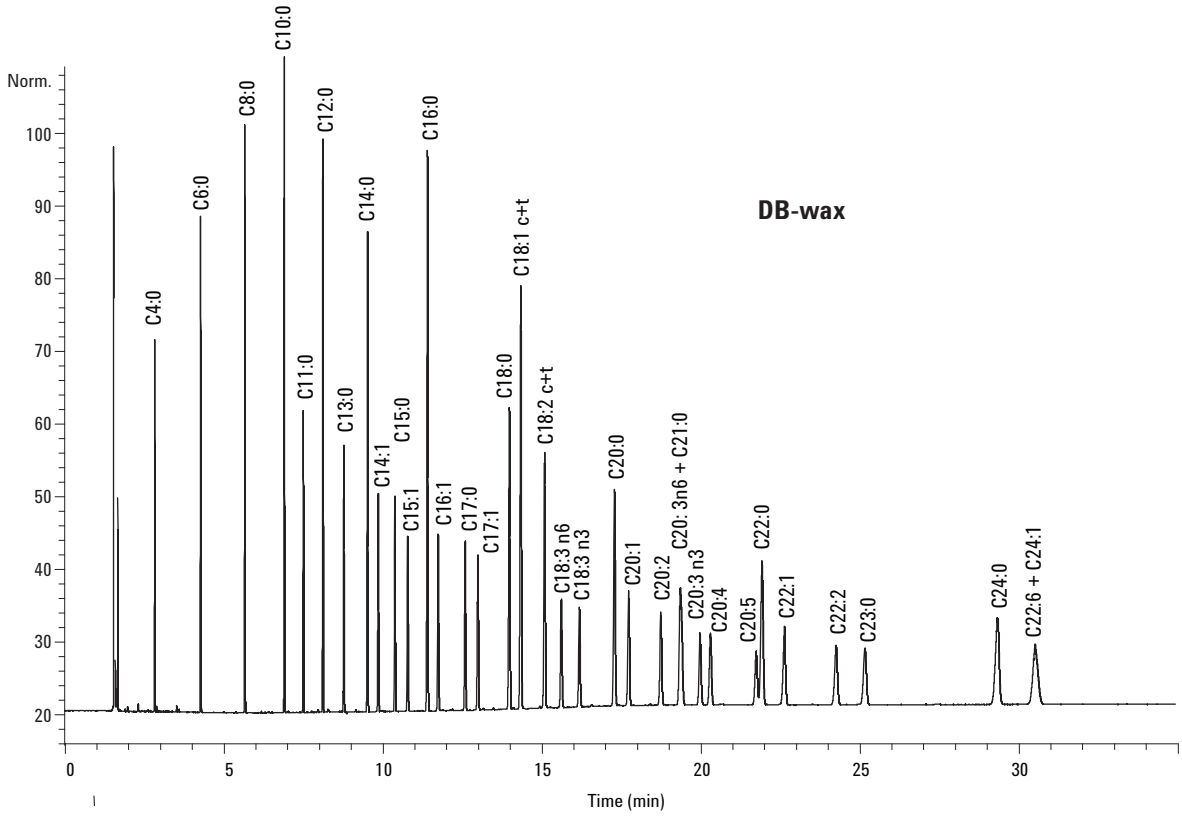

Figure 2. GC-FID analysis of 37-component FAMEs standard mixture on a 30 m × 0.25 mm ID, 0.25 µm DB-Wax column using Method 1. (See Table 2).

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]).

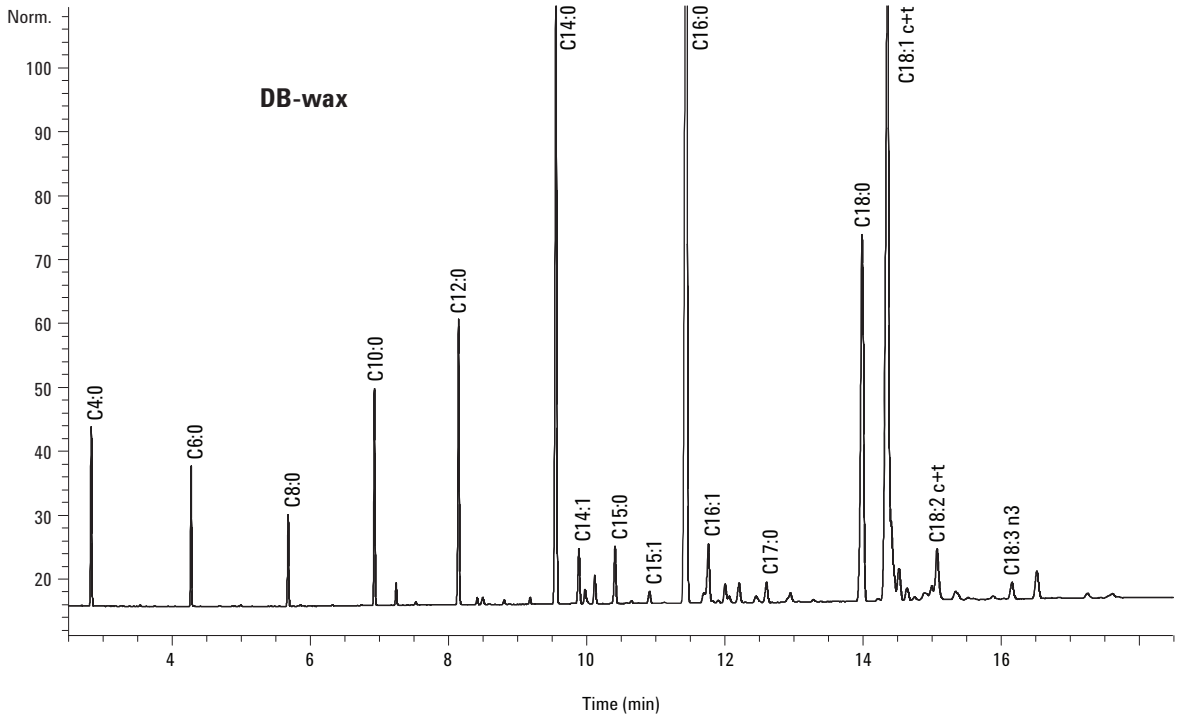

Figure 3. GC-FID analysis of milk fat (CRM 164) fatty acids on a 30 m × 0.25 mm ID, 0.25 µm DB-Wax column using Method 1, Table 2.

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.

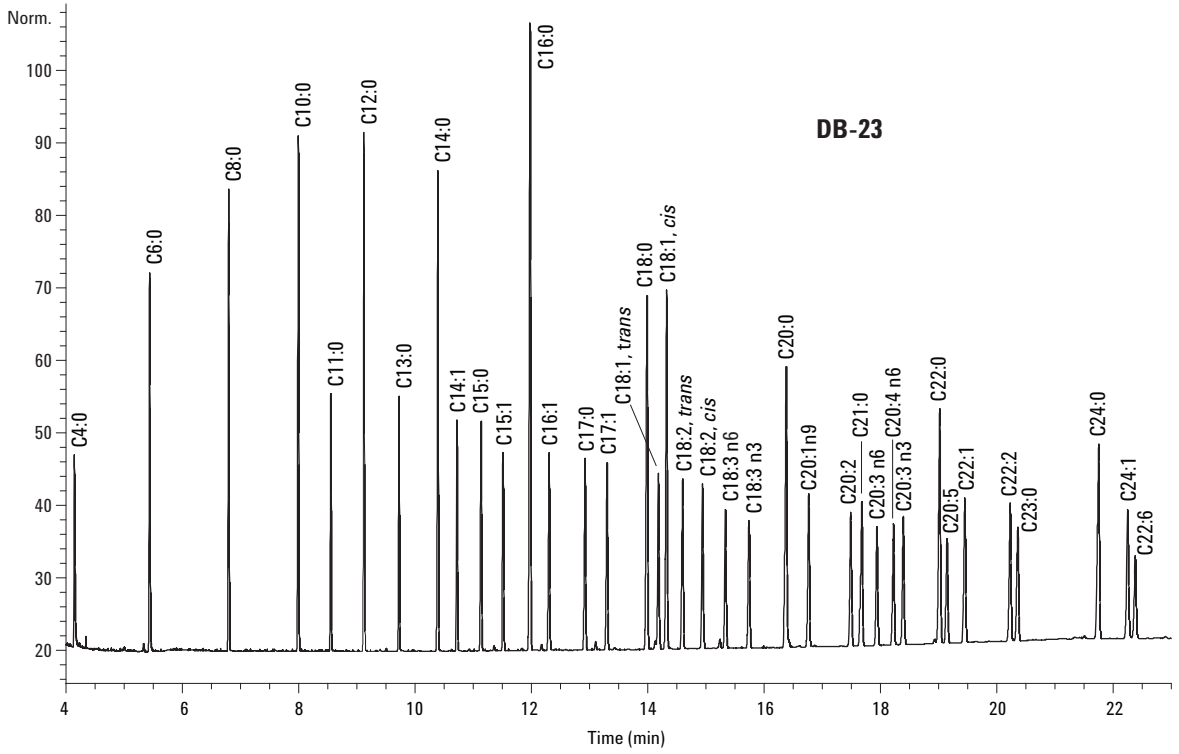

Figure 4. GC-FID analysis of FAMEs standard mixture on a 60 m × 0.25 mm ID, 0.15 µm DB-23 column using Method 2, Table 3.

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.

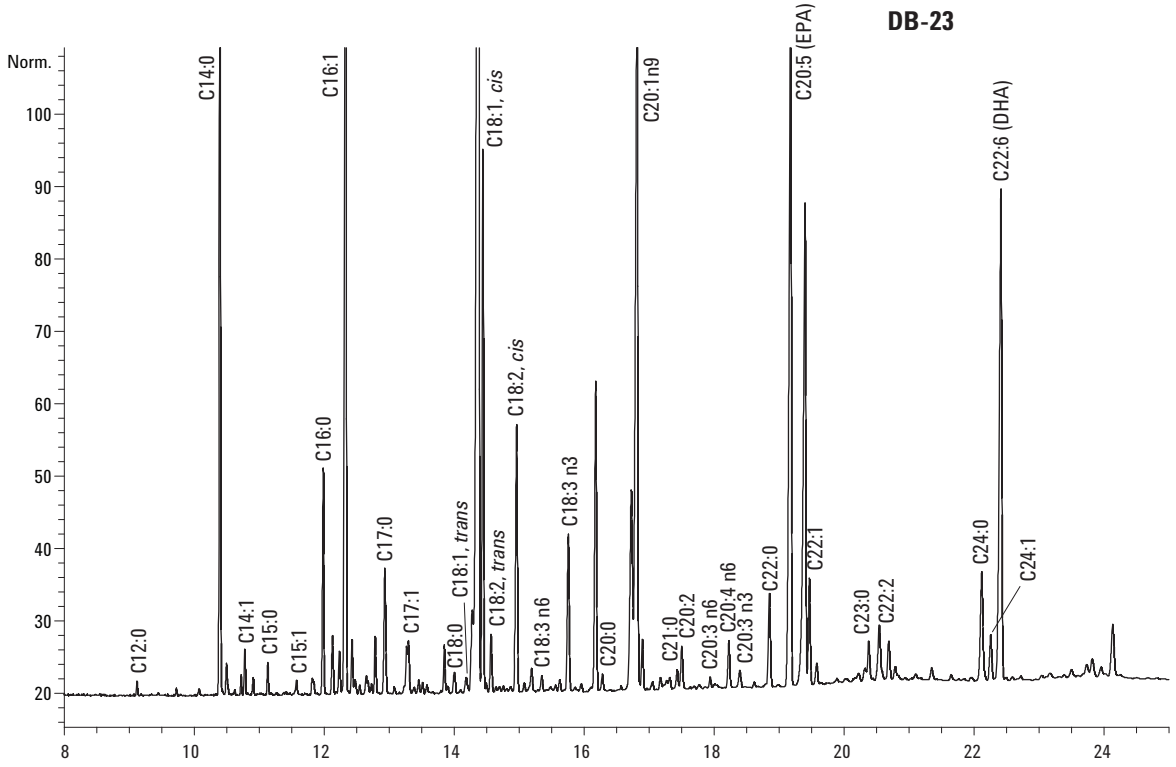

Figure 5. GC-FID analysis of unsaturated fatty acid mixture from marine origin on a 60 m × 0.25 mm ID, 0.15 µm DB-23 column using Method 2, Table 3.

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

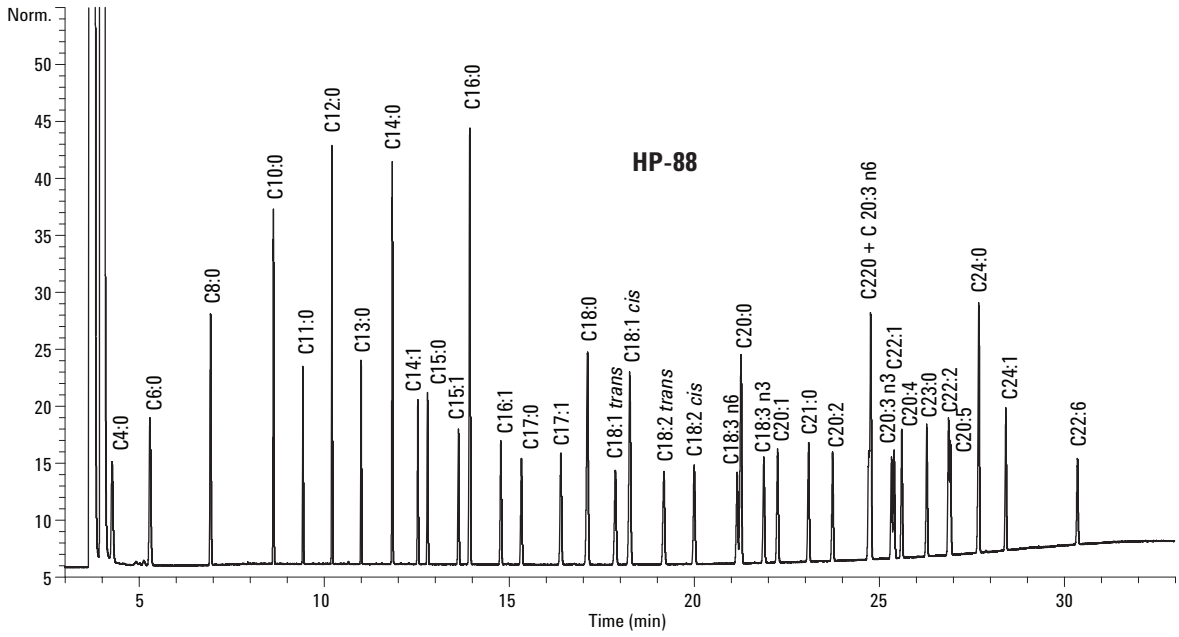

Figure 6. GC-FID analysis of 37-component FAMEs standard mixture on a 100 m × 0.25 mm ID, 0.2 µm HP-88 column using Method 3A, Table 4A.

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.

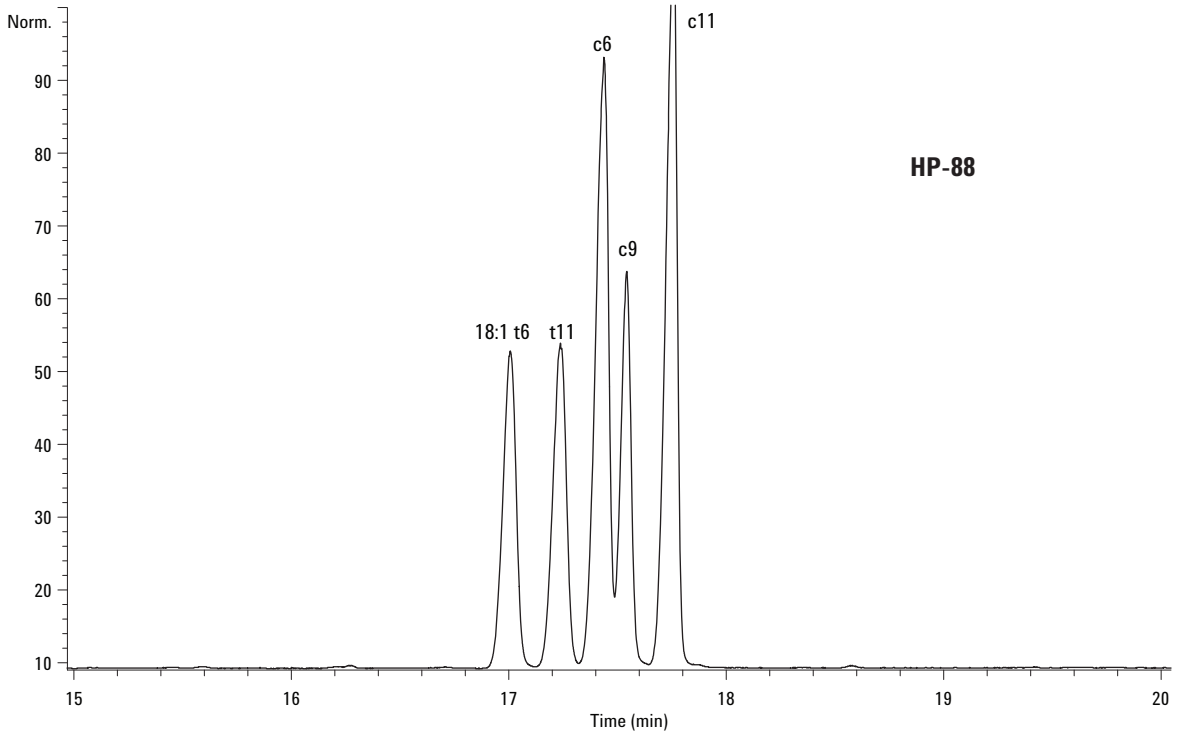

Figure 7. GC-FID analysis of C18:1 isomers on a 100 m × 0.25 mm ID, 0.2 µm HP-88 column using Method 3A, Table 4A.

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

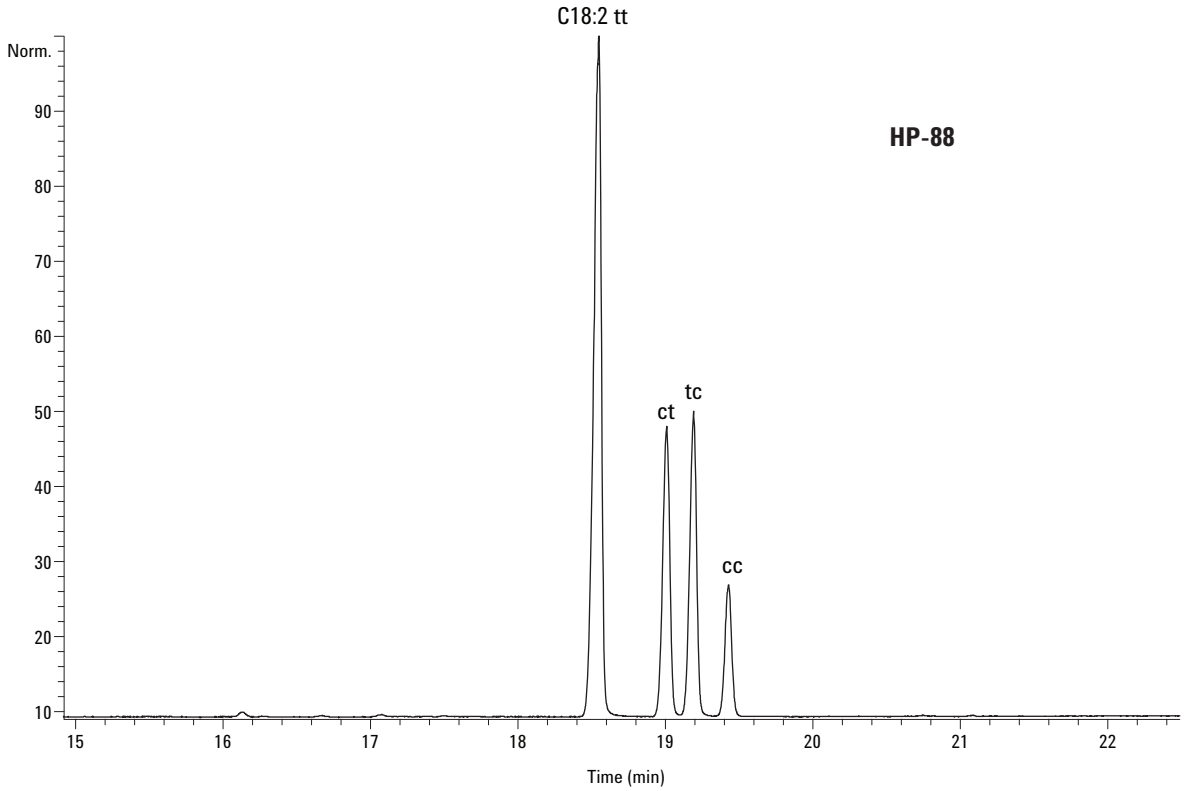

Figure 8. GC-FID analysis of C18:2 isomers on a 100 m × 0.25 mm ID, 0.2 µm HP-88 column using Method 3A, Table 4.

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.

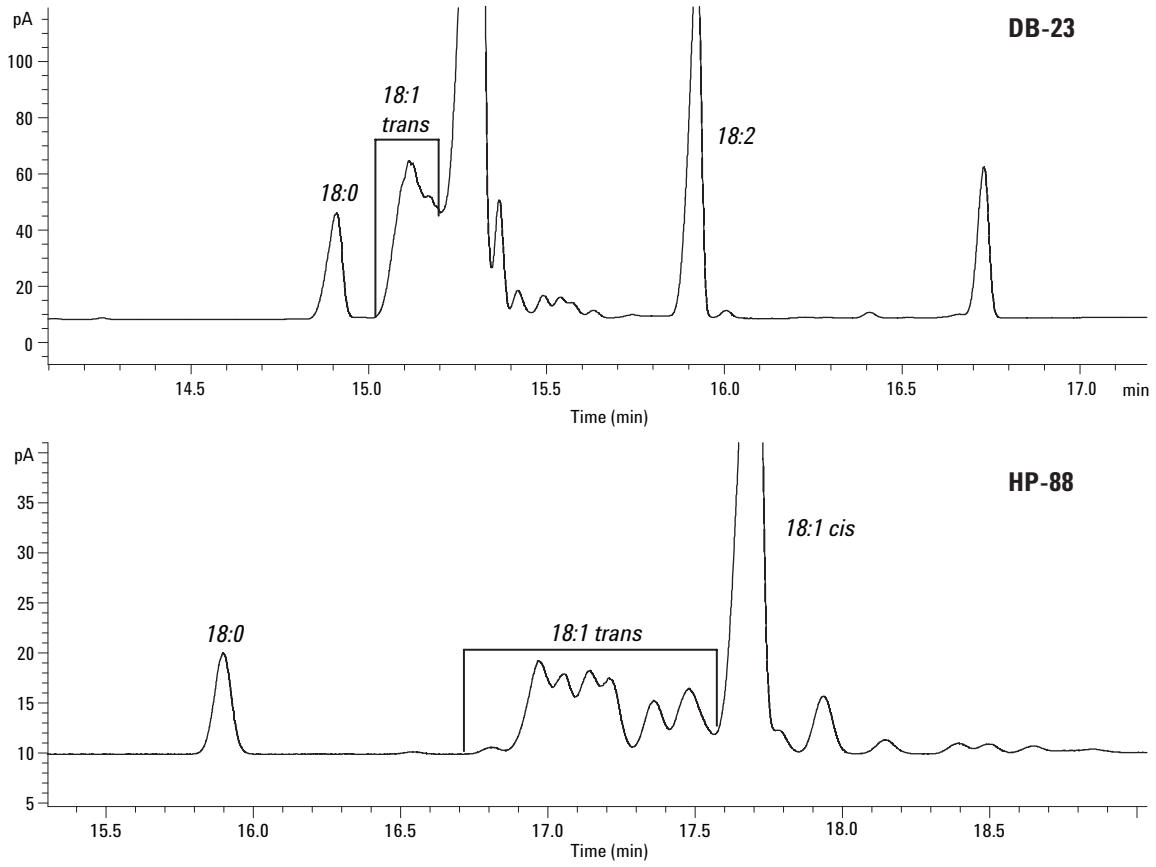

Figure 9. Comparison of the separation of C18:1 isomers from a hydrogenated oil obtained on a 60 m x 0.25 mm ID, 0.15 µm DB-23 column (upper window) and on a 100 m × 0.25 mm ID, 0.2 µm HP-88 column. Both analyses were performed at 180 °C isothermally.

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.

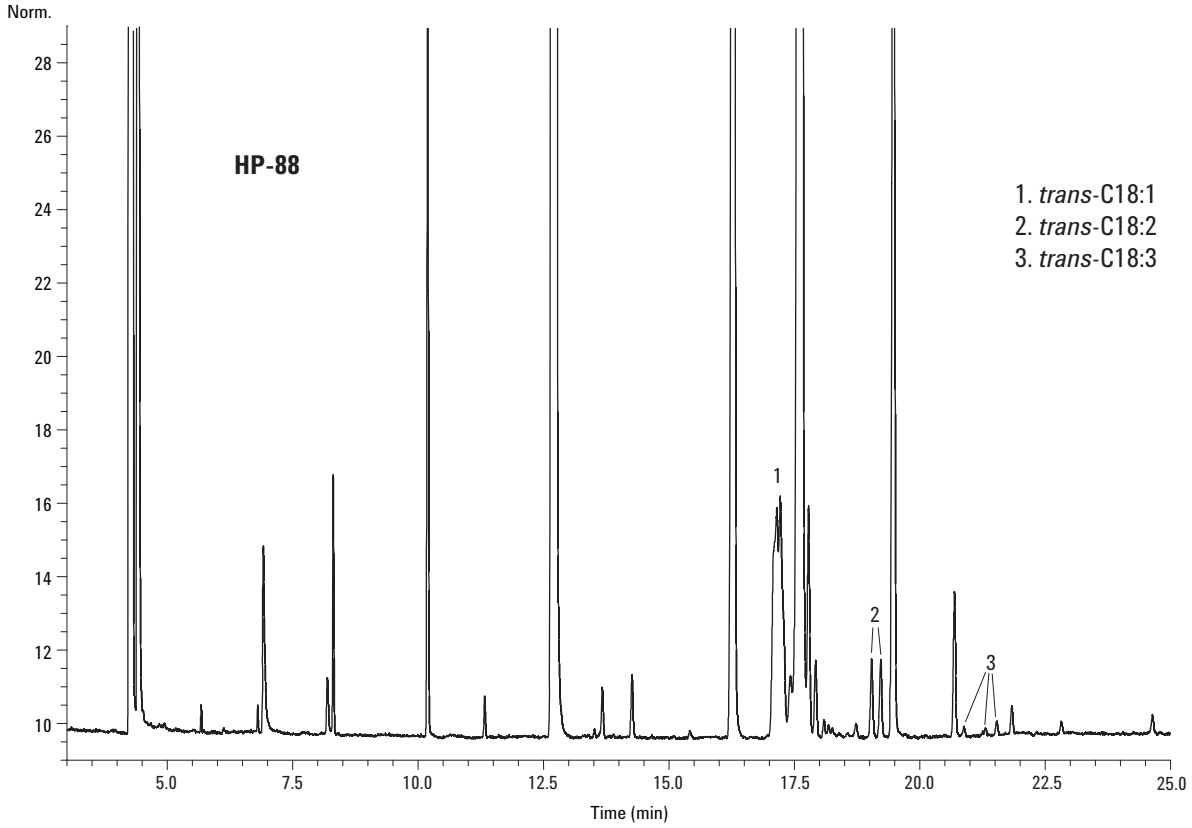

Figure 10. GC-FID analysis of FAMEs from a partially hydrogenated rapeseed oil on a 100 m × 0.25 mm ID, 0.2 µm HP-88 column using Method 3A. (See Table 4).

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].

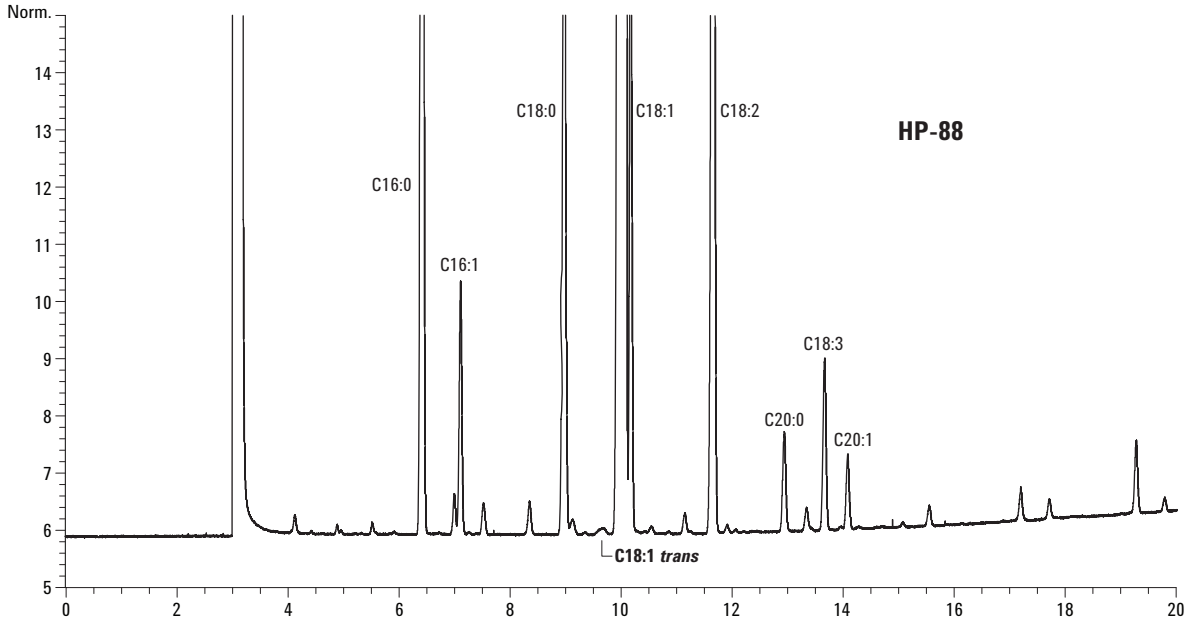

Figure 11. GC-FID analysis of olive oil FAMEs on a 60 m × 0.25 mm ID, 0.2 µm HP-88 column using Method 3B, Table 4.

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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The HPLC Preparative Scale-Up of Soybean Phospholipids

Application

Food and Flavors

Authors

Cliff Woodward and Ron Majors
Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE 19808-1610
USA

Abstract

Classes of major soybean phospholipids can be separated from a crude mixture, under both analytical and preparative conditions, using a matched set of columns, normal phase conditions, and a small amount of volatile salt added to the mobile phase. At higher concentrations of phosphatidylcholine, however, two distinct peaks, whose areas changed with the mass injected, were observed. These two peaks were determined by mass spectrometry to be the same or, at least, very similar.

Introduction

Phospholipids have an important role in animal and plant systems since they are commonly found in cellular and intracellular membranes where they fulfill the role of primary structural components. Phospholipids are also widely used in the food, cosmetic and industrial manufacturing industries and account for 80% of worldwide plant oil production. The structure of a typical phospholipid is depicted in Figure 1. Phospholipids are the major components of soybean lipids and are often desired in a pure form as standards for chromatography and other studies. Preparative amounts are therefore needed and HPLC is often the first choice for this purpose. This application note describes both the development of an analytical normal phase HPLC separation of the most common phospholipids found in crude soy lecithin extracts and the possibility of performing a linear scale-up to preparative columns filled with the same packing.

Properties of Phospholipids

From a chromatographic viewpoint, phospholipids are in a class of polar lipids and are amphipathic. They contain an ionic portion due to the phosphate moiety and they may contain additional hydrophilic or ionic portions due to the presence of choline, serine, ethanolamine, myo-inositol and/or glycerol functionality. All phospholipids contain a hydrophobic portion due to the presence of the fatty acid moieties and their complexity results from the two fatty acids on the 1,2-diacylglycerol portion. These fatty acid moieties may be the same or different. The main phospholipids present in soybeans along with their abbreviations are shown in Table 1. In a typical soybean extract, about half of the extractables are phospholipids with neutral lipids, mainly triglycerides, and carbohydrates making up the remainder. Since phospholipids are generally very complex and difficult to prepare in a pure form, most commercially prepared mixtures contain varying amounts of these extractables.

Phospholipids generally have low water solubility and can be dissolved in nonpolar solvents. Since their ultraviolet (UV) absorbance arises only from the double bond in the fatty acid portion, they provide low response with UV detectors. Refractive index-, evaporative light scattering- or mass spectrometric-detectors are, therefore, more frequently used.

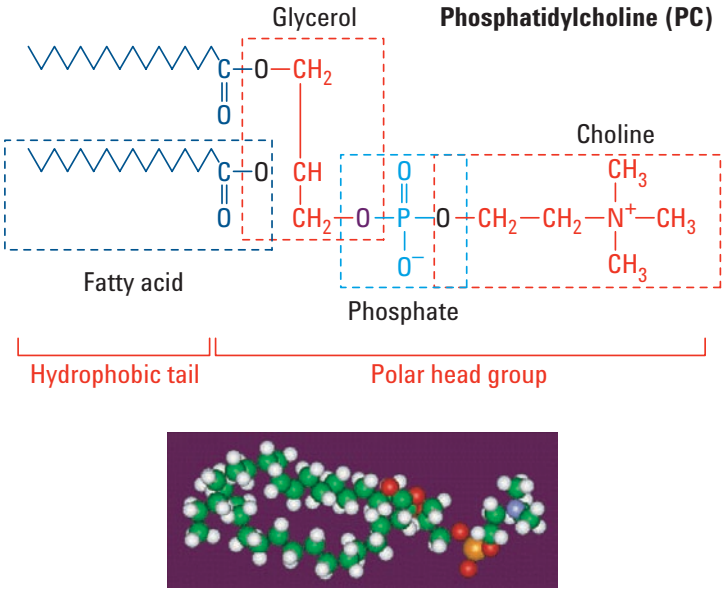

Figure 1. Structure of typical phospholipid.

Table 1. Important Phospholipids in Commercial Plant Oil Extracts

Phospholipid	Abbreviation
Phosphatidylethanolamine	PE
Phosphatidylserine	PS
Phosphatidylinositol	PI
Phosphatidylcholine	PC

Chromatographic Separation of Phospholipids

Because of their chemical properties, phospholipids may be separated by both normal phase and reversed-phase chromatography. For preparative scale-up from analytical dimensions, the use of silica normal phase chromatography has advantages when compared to reverse phase chromatography (RPC): 1) The mobile phases are more volatile; thus, collected fractions can easily be recovered. 2) The viscosity of the common normal phase solvents is low, allowing higher flow rates to be employed, thereby improving throughput. 3) Using the adsorption mechanism, class separations of major phospholipids are easier to perform compared to RPC. 4) In normal phase work, since phospholipids absorb poorly in the UV, detection is better accomplished using the evaporative light scattering detector (ELSD) that allows gradient systems to be employed and provides excellent sensitivity in organic mobile phases.

Row and Kang demonstrated successful separation of phospholipids using normal phase chromatography but they operated at 208- or 210-nm in the UV [1]. In their work, they used a ternary gradient system. However, since our preparative chromatograph employed a binary pumping system, we had to adapt our gradient conditions for both the analytical and preparative chromatography accordingly.

Evaporative Light Scattering as a Detection Principle

The ELSD detects compounds based somewhat on a compound's mass, and not on its spectroscopic characteristics. It is nearly universal and it responds to all compounds as long as they are less volatile than the mobile phase. As seen in Figure 2, the chromatographic flow stream enters the heated nebulizer and is nebulized using a stream of dry nitrogen; the resultant mist containing the analyte is sequentially directed through the heated evaporator where is loses the solvent and becomes a particle, which is then directed through the polychromatic light beam. The particles cause photon scattering as they traverse the path of the light beam. The signal generated is proportional to the analyte particle size present. The solvent is evaporated and does not interfere with the analyte measurement. The presence or absence of chromophores, fluorophores, or electroactive groups has no impact on the signal. The ELSD can be optimized for analyte sensitivity and baseline stability by adjustment and optimization of evaporation and nebulization temperatures. For preparative applications, the upper flow rate capability of the ELSD is 4 mL/min, and thus requires post-column splitting of the chromatographic effluent.

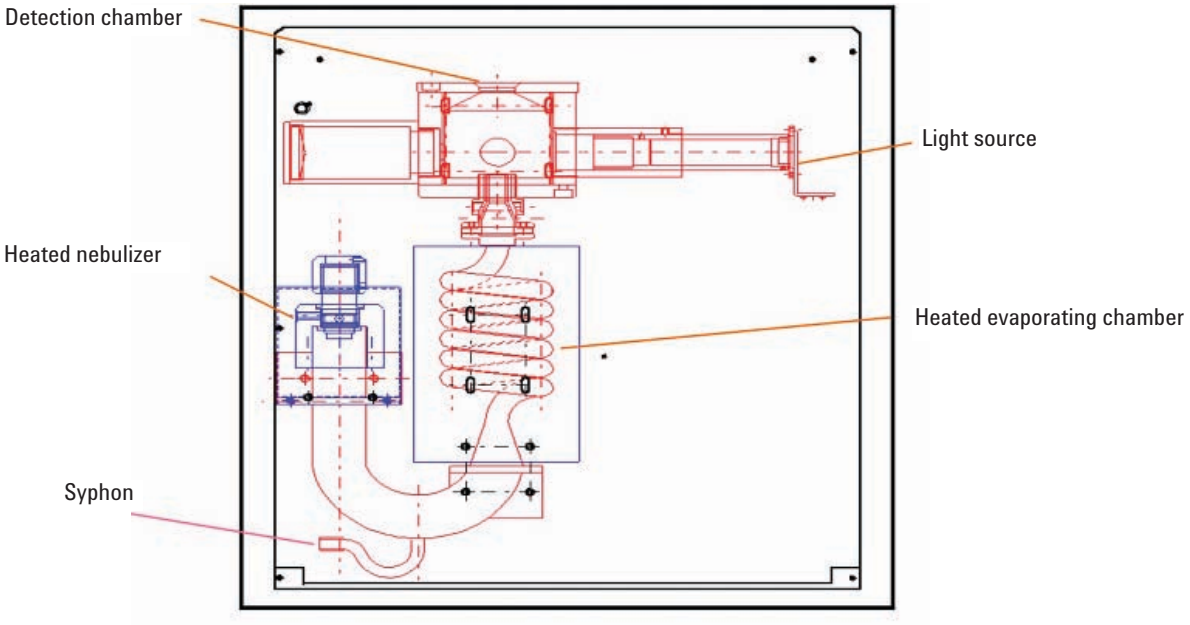

Figure 2. Schematic of ESA Chromachem ELSD.

Scaling Up from Analytical to Preparative Chromatography

In scaling up to a preparative separation, it is recommended that initial method development use an analytical-sized column. Since flow rates and sample injection sizes are lower than for prep, solvent costs are lowered and less sample is used, which is important when sample mass is limited or for samples that are relatively expensive. Once the separation is optimized using an analytical (or scalar) column, linear scale-up to a preparative column of the same materials is easily achieved.

Initially, we adjusted our gradient to approximate the one used by Row and Kang [1]. Using 2.5% methanol (MeOH), 2.5% isopropanol (IPA), and 95% hexane as solvent A, and using 40% IPA and 60% MeOH as solvent B, we achieved a successful separation of the four standard phospholipids but found that our retention times often varied from run to run. In earlier normal phase work [2], Christie found that the addition of a small amount of ionic material to the most polar solvent, in his case, isopropanol-water, gave better resolution and extended the column life. However, several of his suggested ionic additives were nonvolatile. Since the ELSD requires the use of a volatile mobile phase, we chose a low concentration of ammonium acetate (10-mM) but added it to both mobile phase constituents. Higher concentrations could be used to give improved retention behavior, but at >10-mM levels there is danger of salt precipitation, and should therefore be avoided.

Using this hexane-methanol-isopropanol-ammonium acetate (NH_4Ac) gradient system allowed us to fine tune the separation, especially relative to isocratic methods of the past. The final set of chromatographic conditions are depicted in Table 2. Figure 3 shows the optimized separation on a silica-gel scalar column as a function of the injected amount of phospholipid standards: PE, PS, PI, and PC. The small amount of NH_4Ac, a volatile salt, gave much better retention behavior for PC, a zwitterion with a quaternary amine functionality (see Figure 1). The salt addition also assured minimal retention variation with load at low injection mass. Surprisingly, at higher injection masses, we observed two peaks for PC.

Table 2. Final Set of Experimental Conditions

Chromatography		
Analytical runs:		
Column:	Agilent Prep SIL Scalar Column, 4.6 × 150 mm, 10 µm	
Mobile phase:	A = 95:2.5:2.5% hexane-isopropanol-methanol B = 40:60% isopropanol-methanol Both solvents contain 10-mM ammonium acetate	
Flow rate:	1.5 mL/min	
Gradient:	Time, min	%B
	0	0
	20	18.7
	20.2	100
	25	100
	25.1	0
Temperature:	Ambient	
Preparative runs:		
Column:	Agilent Prep SIL Column, 21.2- × 150 mm, 10 µm	
Mobile phase:	Same as above	
Flow rate:	31.9 mL/min	
Gradient:	Same as above	
Temperature:	Ambient	
Detection		
Detector:	Chromachem ELSD, (ESA, Cambridge, MA)	
Temp. nebulizer:	45 °C	
Temp. evaporator:	65 °C	
Filter:	High	
Gas pressure:	23 psi	
Attenuation:	1	
Sample		
Dissolved in:	$CHCl_3$:Hexane 2:1	
Analytical:	5 mg/mL	
Prep:	25 mg/mL	

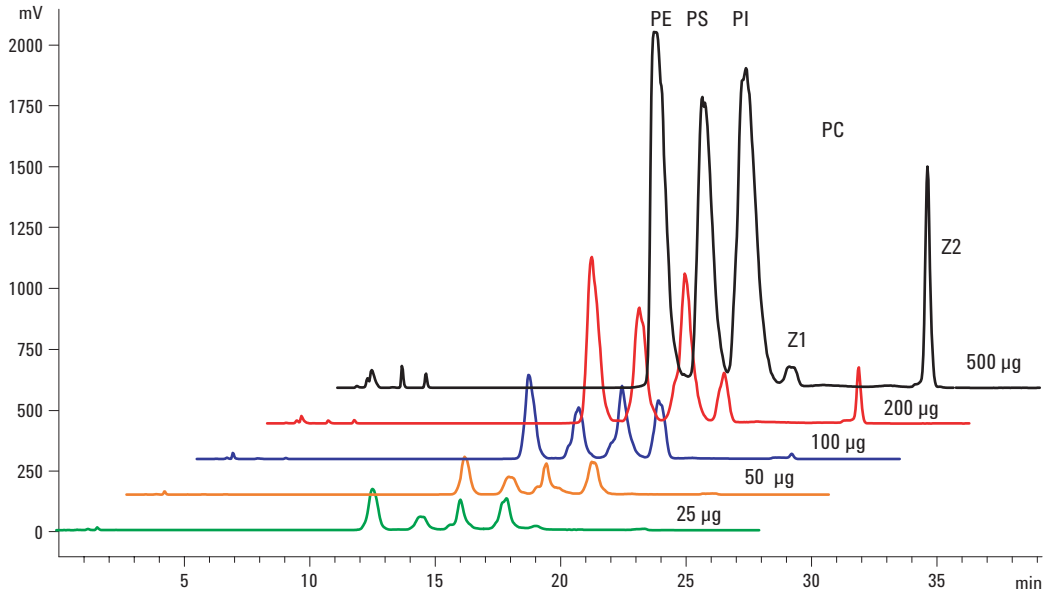

Figure 3. Agilent prep 4.6 × 150 mm, 10 µm – Mixture of soy phospholipid standards. - Loadability.

Study of PC Elution Behavior

As stated above, at higher injection masses, in Figure 3, we observed two peaks for PC, one eluting at a 16.1 minute (PC-Z1) and the second 21.8 minutes (PC-Z2). As sample mass increased (above 75 µg on the 4.6 × 150-mm column), the latter peak increased while the former decreased but the total area increased as expected. As can be seen in Figure 4, with increasing sample mass, the peak areas for all of the phospholipids, including PC, increased. Typical of the ELSD, response was nonlinear, especially at the lowest concentrations (less than 50 µg); however, since the double peak phenomenon occurred with PC only, we suspected that it had something to do with the zwitterion functionality. We, therefore, investigated this aspect more thoroughly.

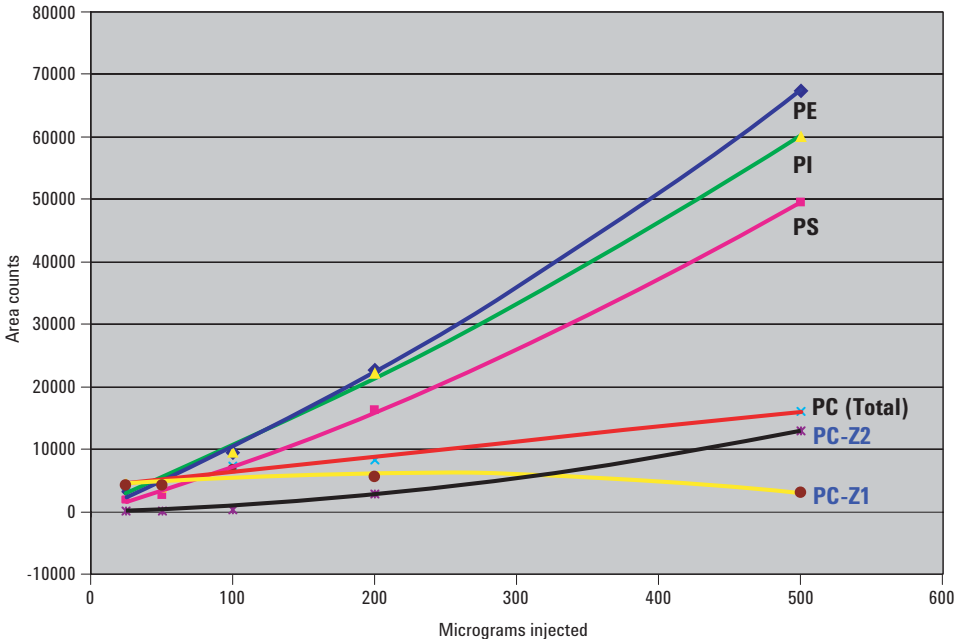

Figure 4. Area counts for ELSD versus micrograms injected (analytical column, 4.6 × 150 mm).

Using liquid chromatography/mass spectrometry (LC/MS), we attempted to determine the structure of the two peaks observed with PC. Since in normal phase chromatography ionization could be a problem, we first investigated MS ionization approaches. MS sensitivity is related to the formation of stable ions in the source. Using electrospray ionization, a minimal signal was observed; so, we resorted to atmospheric pressure photometric ionization (APPI), a more useful ionization technique for neutral organic compounds. Since PC is zwitterionic, its natural ionic character allowed it to be detected in either positive or negative APPI. To enhance sensitivity, acetone and toluene dopants were added post-column as a 50:50 mixture. From other studies [3] it is known that soybean PC consists of multiple phospholipid entities with varying fatty acid content. We were able to assign certain *m/z* values to individual PCs on the basis of the fatty acid portion of the 1,2-diacylglycerol functionality. Figure 5 shows the raw, unsmoothed mass spectral data of the two PC peaks (PC-Z1 and PC-Z2).

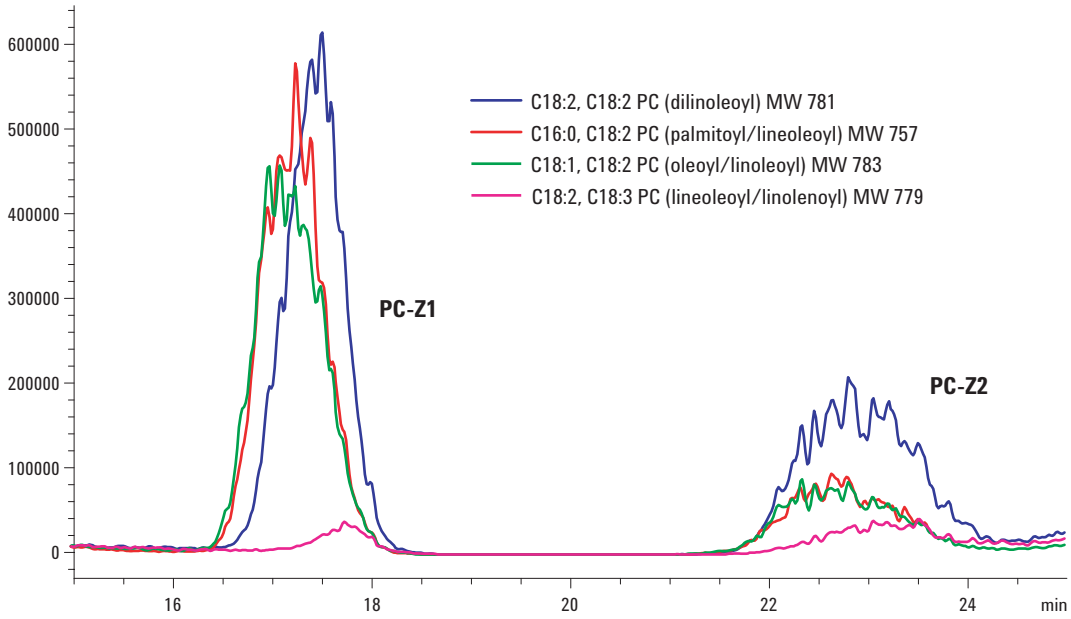

Figure 5. Overlay of the most common phosphatidylcholine (lecithin) (PC) species present in crude soy lecithin – Extracted ions from LC/MS (unsmoothed data).

Clearly, one can determine that there was partial resolution by the type of fatty acid moiety present. Note that the same species are present in both peaks PC-Z1 and PC-Z2. Furthermore, as can be seen in Table 3, if one explores the peak height ratios of the individual PCs within the PC-Z1 and PC-Z2 umbrellas, they are similar enough to deduce that the peaks are the same. In addition, based on fatty acid ratio studies of the individual PCs in purified PC as determined by gas chromatography [3], for a first approximation, the fatty acid composition of the PCs depicted in Figure 5 are similar. This observation also led us to believe that these two PC peaks were the same compound (Table 3). Our speculation is that the two peaks are a result of the competitive silanol binding between the positively charged ammonium ion in the mobile phase modifier and the positively charged portion of PC. At low PC concentrations, the ammonium binds more to the silica silanol groups and the concentration of unbound PC-Z1 is insufficient to displace it. As the concentration of PC increases, however, some of the PC adsorbs to the silanol sites in competition with the ammonium ion. This strongly held PC (identified as PC-Z2) elutes only at very high concentrations of IPA-MeOH. As larger amounts of crude soy lecithin are injected, the available PC increases so that more binds to the surface, resulting in an increase in its elution peak, PC-Z2. If one estimates the total amount of available silanol groups (assuming 8-µmoles/m^2 for a totally hydroxylated surface) on the surface of the packing inside of the column, the number of µmoles of PC potentially bound to the surface at saturation (around 600 µmoles) is consistent with the calculated level of binding sites available.

Preparative Studies of Crude Soy Lecithin

Figure 6 depicts the preparative scale-up of the same phospholipid standard to a 21.2-mm × 150-mm column packed with the same material as the scalar column. The flow rate was increased to 31.88 mL/min to achieve the same linear velocity and separation time as under the analytical conditions. The same relative amounts injected onto the preparative column allowed as much as 20 mg of total phospholipid before the ELSD detector showed signs of overload as noted in the upper chromatogram. Note that the same PC-Z1 and PC-Z2 peaks can be observed in the chromatogram of Figure 6.

Table 3. Table of Relative Extracted Ion Currents

Fatty acid composition	Weight,% typical soybean*	Relative weight.%	Relative height PC-Z1	Relative height PC-Z2
C18:2/ C18:2 PC (dilinoleoyl)	47.5	50	39	45
C16:0/ C18:2 PC (palmitoyl/ lineoleoyl)	30.0	31	31	24
C18:1/ C18:2 PC (oleoyl/ linoleoyl)	10.5	13	28	21
C18:2/ C18:3 PC (lineoleoyl/ linolenoyl)	6.1	6	3	9

*B. Herslof, U. Olsson, and P. Tingvall in I. Hanin and G. Pepeu,Eds. (1990). Phospholipids: Biochemical, Pharmaceutical, and Analytical Considerations, Plenum Press, New York, pp. 295–298.

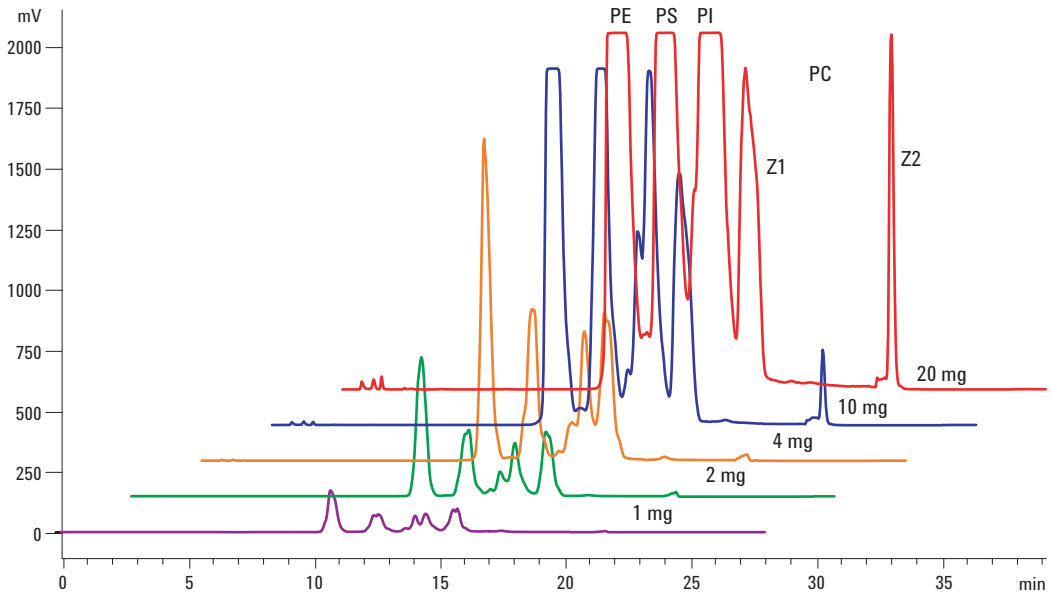

Figure 6. Agilent prep 21.2 × 150 mm, 10-µm – Mixture of soy phospholipid standards. - Loadability.

Finally, in Figure 7, we investigated the scale-up of a 20-mg injection of a crude soy lecithin sample which, by overlaying the standards run under the same conditions, revealed the presence of three phospholipids, including PE, PI, and PC. No PS is seen in this crude extract.

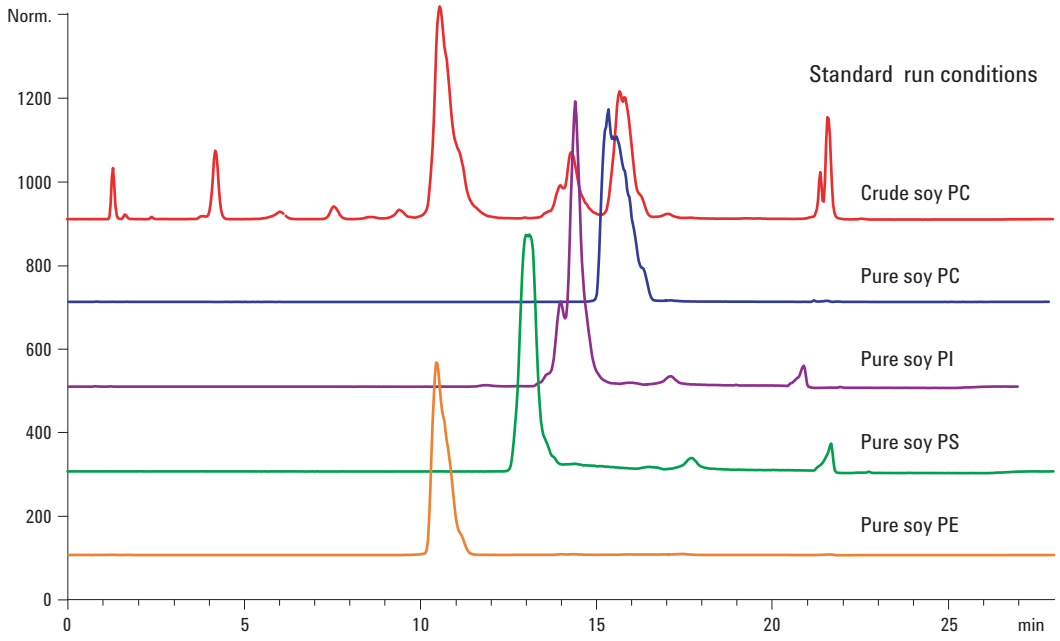

Figure 7. Agilent prep – SIL, 21.2 × 150 mm,10 µm – Crude soy lecithin (PC) and pure individual standards.

Conclusion

These experiments show that the classes of major soybean phospholipids can be successfully separated from a crude mixture, under both analytical and preparative conditions, using a matching set of columns, normal phase conditions, and a small amount of volatile salt added to the mobile phase to cut down on possible electrostatic interactions. At higher concentrations of PC, however, two distinct peaks, whose areas change with mass injected, are observed. By mass spectrometric measurements of the eluted peaks, these two peaks were determined to be the same or, at least, very similar. It is suspected that PC is strongly binding to the silica silanol group in competition with the ammonium acetate, a mobile phase modifier, and is eluting at high gradient solvent polarity.

Acknowledgement

We would like to thank ESA for the loan of the Chromachem ELSD detector for the duration of this study.

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Improving the Analysis of Fatty Acid Methyl Esters Using Retention Time Locked Methods and Retention Time Databases

Application

Food

Authors

Frank David
Research Institute for Chromatography
Pres. Kennedypark 20, B-8500 Kortrijk, Belgium

Pat Sandra
University of Gent
Krijgslaan 281 S4, B-9000 Gent, Belgium

Philip L. Wylie
Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE 19808-1610
USA

Abstract

The analysis of fatty acid methyl esters is a very important application in food analysis. Fatty acid methyl 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 carbon number and according to the degree of unsaturation.

Two retention time locked methods are presented in this application note. In the first method, the analysis of fatty acid methyl esters is performed on a DB-Wax column using gas chromatography/flame ionization detector or gas chromatography/mass spectrometry. In the second method, a DB-23 column is used. Retention time and mass spectral libraries are available for both methods. Retention time locking allows easy peak identification, easy exchange of data between instruments (gas chromatography/flame ionization detector, gas chromatography/mass spectrometry, different labs), and avoids the need to modify the retention times in calibration tables after column maintenance or column change.

Introduction

The analysis of fatty acid methyl esters (FAMEs) is used for the characterization of the lipid fraction in foods and is one of the most important applications in food analysis. Lipids mainly consist of triglycerides, which are esters of one glycerol molecule and three fatty acids. Most edible fats and oils are composed largely of 12- to 20-carbon fatty acids [lauric acid (dodecanoic acid) to arachidic acid (eicosanoic acid)]. Besides linear saturated fatty acids, branched, mono-unsaturated, di-unsaturated, and higher unsaturated fatty acids can occur. An overview of the most important fatty acids and their common abbreviations appears in Table 1.

Table 1. Fatty Acids, Common Names and Abbreviation Used

Fatty acid	Common Name	Simplified Abbreviation[1]	Abbreviation specifying *cis* and *trans* bonds[1]
Butanoic acid	Butyric acid	4:0	4:0
Decanoic acid	Caproic acid	10:0	10:0
Dodecanoic acid	Lauric acid	12:0	12:0
Tetradecanoic acid	Myristic acid	14:0	14:0
Hexadecanoic acid	Palmitic acid	16:0	16:0
Hexadecenoic acid	Palmitoleic acid	16:1 n-7	9*c*-16:1
Octadecanoic acid	Stearic acid	18:0	18:0
cis-9-Octadecenoic acid	Oleic acid	18:1 n-9	9*c*-18:1
trans-9-Octadecenoic acid	Elaidic acid	*t*18:1 n-9	9*t*-18:1
all *cis*-9,12-Octadecadienoic acid	Linoleic acid	18:2 n-6	9*c*12*c*-18:2
ll *trans*-9,12-Octadecadienoic acid	Linolelaidic acid	*t*18:2 n-6	9*t*12*t*-18:2
all *cis*-9,12,15-Octadecatrienoic acid	alpha-Linolenic acid	18:3 n-3	9*c*12*c*15*c*-18:3
all *cis* -6,9,12-Octadecatrienoic acid	gamma-Linolenic acid	18:3 n-6	6*c*9*c*12*c*-18:3
Eicosanoic acid	Arachidic acid	20:0	20:0
cis-11-Eicosenoic acid		20:1 n-9	11*c*-20:1
all *cis* -11,14-Eicosadienoic acid		20:2 n-6	11*c*14*c*-20:2
all *cis* -11,14,17-Eicosatrienoic acid		20:3 n-3	11*c*14*c*17*c*-20:3
all *cis* -8,11,14-Eicosatrienoic acid	Dihomogammalinolenic acid	20:3 n-6	8*c*11*c*14*c*-20:3
all *cis* -5,8,11,14-Eicosatetraenoic acid	Arachidonic acid	20:4 n-6	5*c*8*c*11*c*14*c*-20:4
all *cis* 5,8,11,14,17-Eicosapentaenoic acid	EPA	20:5 n-3	5*c*8*c*11*c*14*c*17*c*-20:5
Docosanoic acid	Behenic acid	22:0	22:0
cis -13-Docosenoic acid	Erucic acid	22:1 n-9	13*c*-22:1
all *cis* -7,10,13,16-Docosatetraenoic acid		22:4 n-6	7*c*10*c*13*c*16*c*-22:4
all *cis* 4,7,10,13,16,19-Docosahexaenoic acid	DHA	22:6 n-3	4*c*7*c*10*c*13*c*16c19*c*-22:6
Tetracosanoic acid	Lignoceric acid	24:0	24:0
cis -15-tetracosenoic acid	Nervonic acid	24:1 n-9	15*c*-24:1

[1]Several different versions of fatty acid nomenclature and structural abbreviation have been used in the past. For discussions of past and currently-accepted nomenclature, the following web sites are recommended:
http://www.ajcn.org/misc/lipid.shtml
http://www.chem.qmul.ac.uk/iupac/lipid/
http://www.aocs.org/member/division/analytic/fanames.htm
http://www.cyberlipid.org/index.htm

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 a FFAP column), more robust and reproducible chromatographic data are obtained if the fatty acids are derivatized to the FAMEs. Several methods are available for derivatization, which requires hydrolysis of the glycerides and methylation of the resulting fatty acids [1]. These easy-to-use methods do not require expensive reagents or equipment. Two useful methods are described in the Experimental section.

After preparation of the FAMEs, the FAMEs are separated according to carbon number (number of carbon atoms in the fatty acid chain, not including the methyl ester carbon) and according to 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, two methods are described for the GC analysis of FAMEs. The method choice depends both on sample complexity and the degree of fatty acid characterization that is required (Figure 1). Method 1 uses a DB-Wax column that separates FAMEs from C4 (butyric acid) to C24 (lignoceric acid) according to carbon number and unsaturation. On this column, no separation of *cis* and *trans* isomers is obtained, and for complex mixtures (such as fish oils), some FAMEs are not resolved. However, the separation of FAMEs on polyethylene glycol columns is widely used and can be applied to the characterization of "classical" samples such as vegetable oils (corn oil, maize oil, olive oil, soybean oil, and so on). For certain applications, animal fats can also be analyzed using the Agilent DB-Wax column. An important application, for instance, is the analysis of butyric

acid in milk fat. The concentration of butyric acid in milk is an important indicator of its quality. This determination is very important in milk and dairy analysis and in the analysis of chocolate products. All these applications can be performed on the Agilent DB-Wax column using method 1.

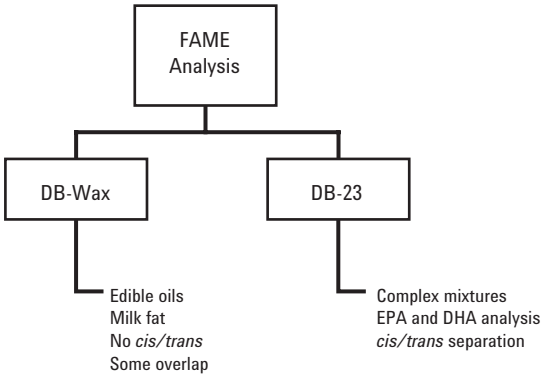

Figure 1. Overview of method selection for FAME analysis.

For the analysis of more complex samples, such as fish oils, additional resolution of FAMEs is obtained using a capillary column coated with a cyanopropyl stationary phase (method 2). On an Agilent DB-23, highly unsaturated fatty acids, such as all *cis* 5,8,11,14,17-eicosapentenoic acid methyl ester (EPA, 20:5 n-3) and all *cis* 4,7,10,13,16, 19-Docosahexenoic acid methyl ester (DHA, 22:6 n-3), are separated from other FAMEs. Separation of *cis* and *trans* isomers is also possible on the cyanopropyl column due to the stronger interaction of *cis* isomers with the cyano-dipole. This causes the *trans* isomers to elute before the *cis* isomers. An increasingly important food analysis is the determination of *trans* fatty acids, which can be performed using the DB-23 column with the conditions described in method 2.

Both methods are retention time locked using methyl stearate as the locking compound. Retention time locking (RTL) allows the analyst to obtain virtually identical retention times on any GC, independent of the inlet, injection technique, or detector used [2, 3, 4]. Because RTL reproduces retention times so accurately, FAME identification can be done based on absolute retention times. It is unnecessary to have all of the FAME standards available because peak identification is possible using Agilent's published retention time database. An additional benefit of RTL is that retention times in the calibration table remain unchanged even after column maintenance or column change (after re-locking the method). Moreover, gas chromatography/flame ionization detection (GC/FID) and gas chromatography/mass spectrometry (GC/MS) methods can be scaled [2, 3], so that retention times obtained on the two different systems match very closely. For GC/MS, a custom spectral library was created so that data files can be screened using spectra together with their locked retention times [4].

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 to 0.1 % (w/v) concentration. For method and instrument check-out, a 37-component mixture (Supelco number 18919) was used. The mixture was purchased as a 100-mg neat mixture, containing C4 to C24 FAMEs (2 to 4% relative concentration). The whole sample was diluted in 10 mL of hexane (final concentration = 0.2 to 0.4 mg/mL per FAME).

Oil and fat samples can be prepared using any one of several different methods [1]. The following sample preparation methods were tested.

Sample preparation method 1 [5] : Weigh 100-mg sample in a 20-mL test tube (with screw cap) or a reaction vial. Dissolve the sample in 10 mL of hexane. Add 100 µL of 2N potassium hydroxide in methanol (11.2 g in 100 mL). Close the tube or vial, and vortex for 30 seconds. Centrifuge. Transfer the clear supernatant to a 2-mL autosampler vial.

Sample preparation method 2 [6]: Weigh 50-mg sample in a 20-mL test tube (with screw cap) or a reaction vial. Add 2 mL of 2N sodium hydroxide in methanol (8 g in 100 mL). Close the tube or vial, and heat at 80 °C for 1 hour. Allow to cool. Add 2 mL of a 25% borontrifluoride solution in methanol (Sigma-Aldrich, cat. no. 13,482-1, 50% solution in methanol, to be diluted to 25% in methanol). Close the tube or vial, and heat again for 1 hour at 80 °C. Allow to cool. Add 5 mL of water and 5 mL of hexane. Shake well. Allow the phases to separate (or centrifuge). Transfer the clear supernatant to a 2-mL autosampler vial.

Both methods performed equally well.

Analytical Conditions

The analyses were performed on an Agilent 6890 GC equipped with an FID or on an Agilent 6890/5973 GC/MSD system. Automated split injection was performed using an Agilent 7683 automatic sampler. The instrumental configuration and analytical conditions are summarized in Table 2 (DB-Wax column) and Table 3 (DB-23 column). For both methods, methyl stearate was used as the locking standard. The retention time for methyl stearate was locked at 14.000 min. When duplicating this method, the column head pressure can be set to the pressures indicated in Tables 2 and 3 (nominal pressure). Then the RTL calibration runs can be performed (at -20%, -10%, +10% and +20% of the nominal pressure)[4]. The retention time versus head pressure curve is then automatically calculated and stored in the method.

Table 2. DB-Wax Method

Instrumentation		
Chromatographic system	Agilent 6890 GC	
Inlet	Split/splitless	
Detector	FID or Agilent 5973 MSD	
Automatic sampler	Agilent 7683	
Liner	Split liner (part no. 5183-4647)	
Column	30-m × 0.25-mm id × 0.25-µm DB-Wax (part no. 122-7032)	
Experimental conditions GC/FID		
Inlet temperature	250 °C	
Injection volume	1 µL	
Split ratio	1/50	
Carrier gas	Hydrogen	
Head pressure	Methyl stearate is retention time locked to 14.000 min Constant Pressure mode (pressure approximately 53 kPa at 50 °C, 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: Air: Helium make-up gas:	40 mL/min 450 mL/min 30 mL/min
Experimental conditions GC/MS		
Inlet temperature	250 °C	
Injection volume	1 µL	
Split ratio	1/50	
Carrier gas	Helium	
Head pressure	Methyl stearate is retention time locked to 14.000 min Constant Pressure mode (pressure approximately 55 kPa at 50 °C, 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	
MSD Parameters	Scan (40 to 500 amu), threshold 100 MS quad 150 °C MS source 230 °C Solvent delay: 2 min	

Table 3. DB-23 Method

Instrumentation		
Chromatographic system	Agilent 6890 GC	
Inlet	Split/splitless	
Detector	FID or Agilent 5973 MSD	
Automatic sampler	Agilent 7683	
Liner	Split liner (part no. 5183-4647)	
Column	60-m × 0.25-mm id × 0.15-µm DB-23 (part no. 122-2361)	
Experimental conditions GC/FID		
Inlet temperature	250 °C	
Injection volume	1 µL	
Split ratio	1/50	
Carrier gas	Helium	
Head pressure	Methyl stearate is retention time locked to 14.000 min Constant Pressure mode (pressure approximately 230 kPa at 50 °C, 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: Air: Helium make-up gas:	40 mL/min 450 mL/min 30 mL/min
Experimental conditions GC/MS		
Inlet temperature	250 °C	
Injection volume	1 µL	
Split ratio	1/50	
Carrier gas	Helium	
Head pressure	Methyl stearate is retention time locked to 14.000 min Constant Pressure mode (pressure approximately 180 kPa at 50 °C, 33 cm/s at 50 °C)	
Oven temperature	50 °C, 1 min, 25 °C/min to 175 °C,4 °C/min to 235 °C, 5 min	
Transfer line	250 °C	
MSD parameters	Scan (40 to 500 amu), threshold 100 MS quad 150 °C, MS source 230 °C Solvent delay: 3.5 min	

Results and Discussion

A typical chromatogram for the analysis of the FAME reference standard (obtained on the DB-Wax column using method 1) is shown in Figure 2. A good separation is obtained, except for the following compounds: *cis* and *trans* 18:1 co-elute at 14.38 min, *cis* and *trans* 18:2 co-elute at 15.13 min, 20:3 n-6 and 21:0 co-elute at 19.44 min, and 22:6 and 24:1 co-elute at 30.73 min. However, this separation is sufficient for most classical oil and fat characterization. Butyric acid elutes at 2.85 min and can be determined in milk fat using the same method. Because the GC/MS and GC/FID instruments were locked, virtually identical chromatograms were obtained on both systems. A comparison between the GC/FID and GC/MS profiles is shown in Figure 3. Although different outlet pressures (ambient versus vacuum) and different carrier gases (hydrogen in GC/FID and helium for GC/MS) were used, the correspondence between the two chromatograms was very good. Peaks detected in the GC/FID trace can easily be located in the GC/MS trace and identification is possible based on retention times alone or in combination with mass spectra.

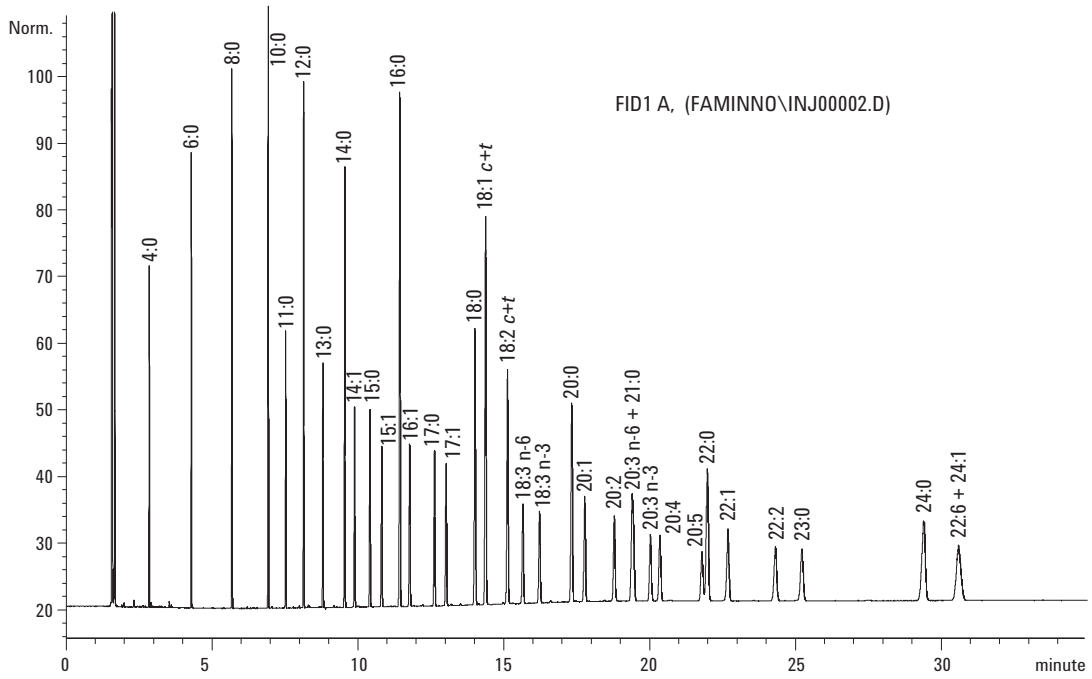

Figure 2. GC/FID analysis of FAMEs standard mixture on a 30-m × 0.25-mm id 0.25-µm DB-Wax column (part no. 122-7032) using method 1 (see Table 2).

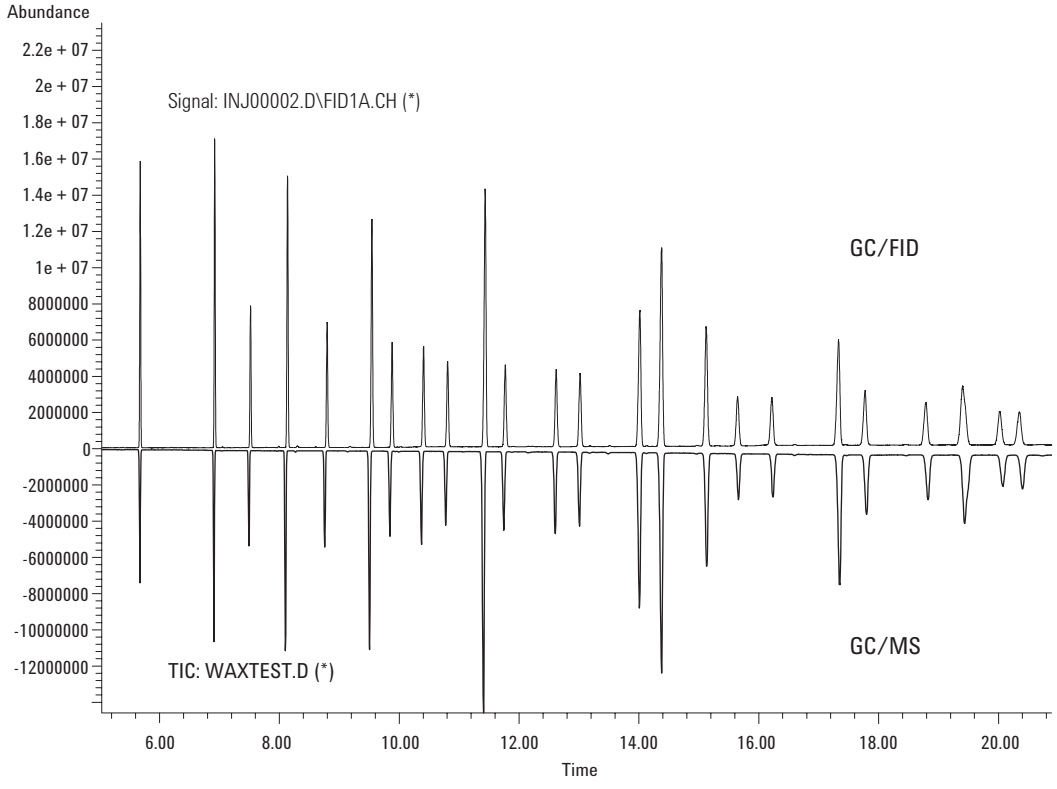

Figure 3. Comparison of GC/FID and GC/MS chromatograms obtained on a 30-m × 0.25-mm id × 0.25-µm DB-Wax column (part no. 122-7032) using method 1 (see Table 2).

The retention time locked method on the DB-Wax column was applied to the analysis of two certified reference samples (CRM 162, a soy-maize blend oil and CRM 164, a milk fat)[7]. Both samples were prepared according to sample preparation method 2. The resulting chromatograms are shown in Figure 4 (soy-maize oil blend) and in Figure 5 (milk fat). The peaks were automatically identified using the RTL FAMEs retention time database. The quantitative results are summarized in Tables 4 and 5.

Figure 4 shows a classical fatty acid profile normally obtained for edible oils. Figure 5 shows the fatty acid profile typical for milk fat. Butyric acid elutes at 2.85 min and is easily detected. Very good reproducibility is obtained. The standard deviation of the relative areas is smaller than 1% in all cases. Also the correspondence between the measured fatty acid composition and the certified values is good.

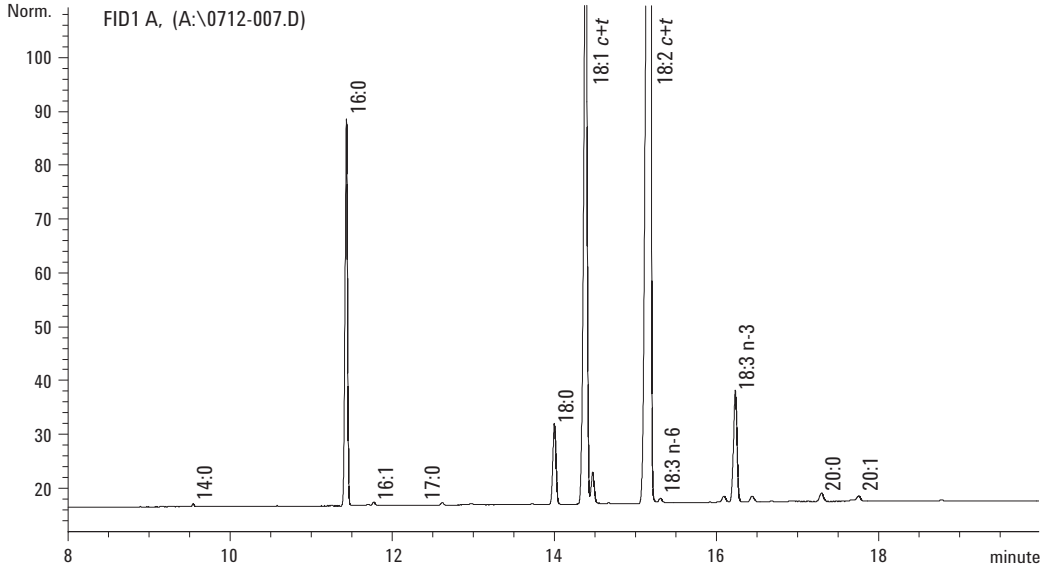

Figure 4. GC/FID analysis of soy-maize oil (CRM 162) FAMEs on a 30-m × 0.25-mm id × 0.25-µm DB-Wax column (part no. 122-7032) using method 1 (see Table 2).

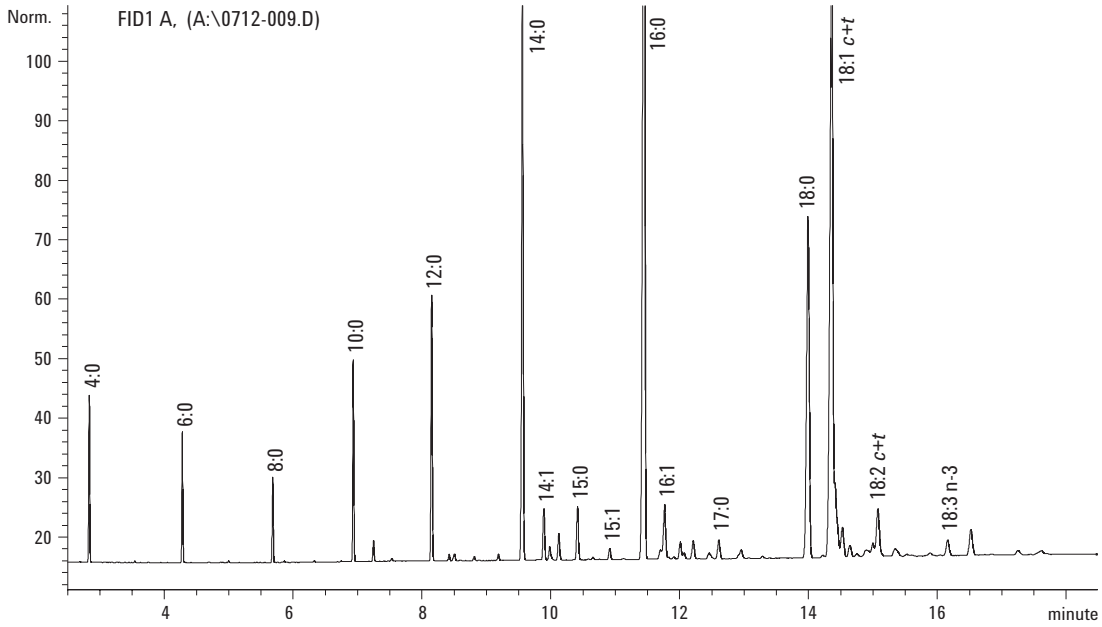

Figure 5. GC/FID analysis of milk fat (CRM 164) FAMEs on a 30-m × 0.25-mm id × 0.25-µm DB-Wax column (part no. 122-7032) using method 1 (see Table 2).

Table 4: Quantitative Data Obtained Using RTL Method 1 for CRM 162

Fatty acid	Measured concentration (g/100 g)	Standard deviation	Certified concentration (g/100 g) [7]	Uncertainty (**)
16:0	10.607	0.003	10.65	0.17
18:0	2.917	0.005	2.87	0.07
18:1	24.461	0.013	24.14	0.28
18:2	57.051	0.017	56.66	0.54
18:3	4.286	0.017	4.68	0.21
20:0	0.368	0.003	(0.3) (*)	
20:1	0.246	0.003	(0.2) (*)	

(*) Indicative values, not certified

(**) Uncertainty is taken as half-width of the 95% confidence interval of the certified mean value.

Table 5: Quantitative Data Obtained Using RTL Method 1 for CRM 164

Fatty acid	Measured concentration (g/100 g)	Standard deviation	Certified concentration (g/100 g) [7]	Uncertainty (*)
4:0	3.522	0.012	3.49	0.06
6:0	2.318	0.003	2.36	0.19
8:0	1.420	0.002	1.36	0.10
10:0	3.010	0.006	2.89	0.12
12:0	3.907	0.008	4.03	0.10
14:0	11.383	0.014	10.79	0.35
16:0	27.693	0.005	26.91	0.84
18:0	10.882	0.009	10.51	0.40
18:1	24.832	0.009	24.82	0.61
18:2 (Σ)	2.844	0.012	2.68	0.40
18:3	0.604	0.005	0.51	0.04

(*) Uncertainty is taken as half-width of the 95% confidence interval of the certified mean value.

The separation for the 37-component standard mixture on the 60-m × 0.25-mm id 0.15-µm DB-23 column using method 2 is shown in Figure 6. Using these conditions, all compounds in the standard mixture are resolved. Important is the separation of the *cis*/*trans* isomers and the separation of EPA (20:5, 19.15 min) and DHA (22:6, 22.38 min). This method is very useful for the analysis of fatty acid profiles in complex mixtures. An example of the separation obtained for a mixture of polyunsaturated FAMEs from a marine source appears in Figure 7. The identifications shown on the chromatogram were done by using a classical calibration table with a ±5% retention time window for identification and quantitation (the default setting). Using this setting, the last three peaks (at 22.122, 22.262, and 22.414 min) were identified as 24:0, 24:1, and 22:6, respectively. The result from the peak identification using the locked retention time database appears in Table 6. RTL library searching shows that the initial identification (using the calibration table) was wrong. The correct identification was: 22.122 min = unknown (no fatty acid methyl ester), 22.262 min = 24:1, and 22.414 min = 22:6. These identifications could be confirmed easily by GC/MS. The profiles obtained by GC/FID and GC/MS for the same sample of marine FAMEs appears in Figure 8. An excellent retention time correlation was obtained.

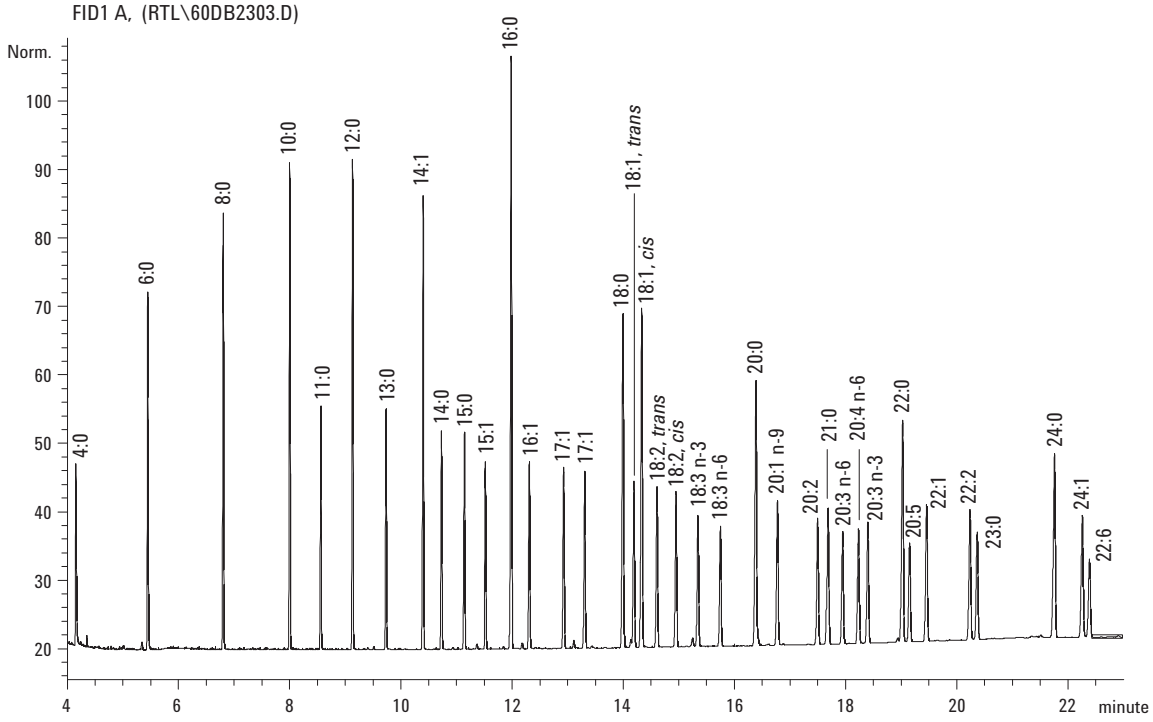

Figure 6. GC/FID analysis of FAMEs standard mixture on a 60-m × 0.25-mm id × 0.15-µm DB-23 column (part no. 122-2361) using method 2 (see Table 3).

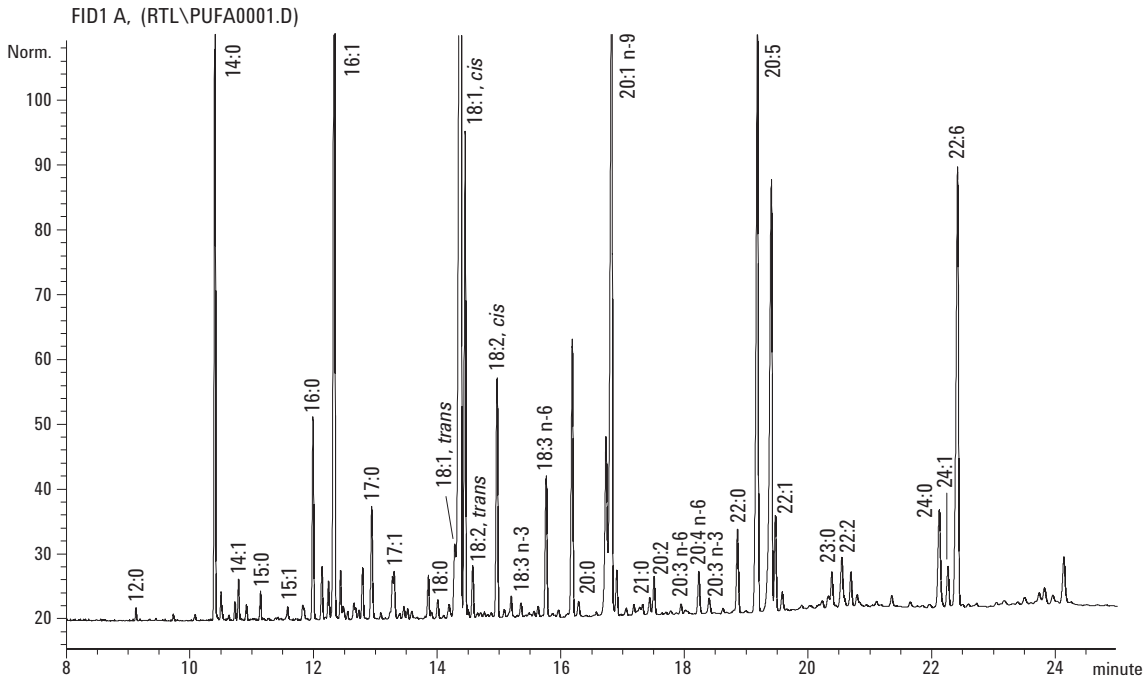

Figure 7. GC/FID analysis of unsaturated FAME mixture of marine origin on a 60-m × 0.25-mm id × 0.15-µm DB-23 column (part no. 122-2361) using method 2 (see Table 3). The peak at 22.122 min was initially identified as 24:0 using a normal calibration table with the default retention time window. However, RTL library searching proved that this compound was, instead, an unknown.

Table 6. GC/FID Peak Identifications Using the FAMEs Retention Time Database

Data file:	D:\HPCHEM\2\DATA\RTL\PUFA0001.D		
Sample name:	PUFA 1		
Instrument 2:	2/15/02 2:40:50 PM		
Injection date:	2/14/02 5:04:32 PM	Seq. Line :	1
Sample name:	PUFA 1	Vial:	01
Acq. operator:	VH	Inj:	1
Acq. method:	RTLDB23.M		

Results of RT table search

Search results for 22.122 ±0.100 minutes

Contains elements:	{No restriction}
Does not contain elements:	{No restriction}
RTT file searched:	D:\HPCHEM\RTL\FAMDB23.RTT

No matches found.

Search results for 22.262 ±0.100 minutes

Contains elements:	{No restriction}
Does not contain elements:	{No restriction}
RTT file searched:	D:\HPCHEM\RTL\FAMDB23.RTT

RT	Compound
22.254	Nervonic acid methyl ester
	Code 24:1

Search results for 22.414 ±0.100 minutes

Contains elements:	{No restriction}
Does not contain elements:	{No restriction}
RTT file searched:	D:\HPCHEM\RTL\FAMDB23.RTT

RT	Compound
22.382	*cis*-4, 7, 10, 13, 16, 19-docosahexaenoic acid methyl ester
	Code 22:6

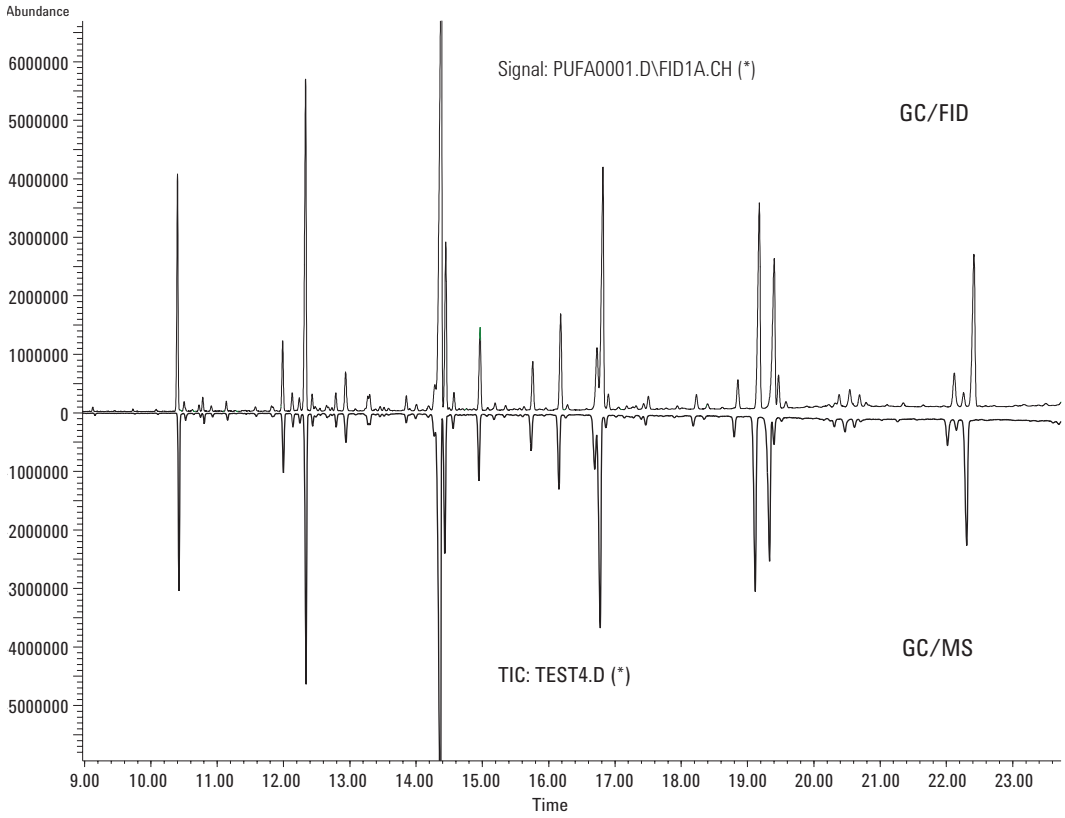

Figure 8. Comparison of GC/FID and GC/MS chromatograms obtained for the unsaturated FAME mixture of marine origin on a 60-m × 0.25-mm id × 0.15-µm DB-23 column (part no. 122-2361) using method 2 (see Table 3).

Conclusions

Two methods are described for the analysis of FAMEs. Method 1, using an Agilent DB-Wax column, is useful for the analysis of classical edible oils and fats, including the determination of butyric acid in milk fat. Method 2, applying an Agilent DB-23 cyanopropyl column, can be used for the analysis of more complex samples, including fish oils and hydrogenated fats, for the determination of EPA and DHA and for *cis*/*trans* determination. Using retention time locking, GC/FID and GC/MS retention times can be closely matched for easy correlation of chromatograms between the instruments. RTL database searching makes peak identification more accurate.

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Agilent Technologies

High-Speed Separation of Parabens

Application

Food Analysis

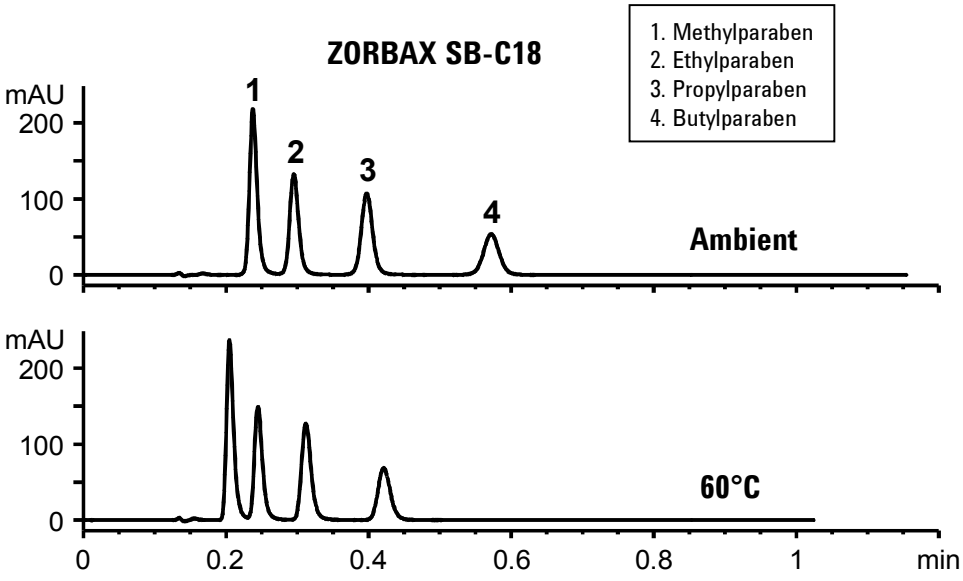
Robert Ricker

Parabens, or para-hydroxy benzoic acid alkyl esters are popular preservatives used in the cosmetic and food industry to battle microbe degradation. Analysis time of less than one minute is accomplished with a Rapid Resolution SB-C18, 4.6 x 30 mm column. Columns with particle sizes under 5 µm and dimensions up to ten-times smaller than traditional analytical-size columns are ideal for high-speed methods. If desired, increasing temperature cuts analysis time further.

Extra-column volume is a crucial factor in chromatographic performance. Here, no modification to the modern instrument is necessary for optimal resolution.

Operating Conditions:

HPLC System:	Agilent 1100 with quaternary pump
Column:	ZORBAX StableBond-C18 Rapid-Resolution (3.5 µm) Cartridge-Column, 4.6 x 30 mm
	Agilent Part No. 833975-902
Mobile Phase:	0.1% H_3PO_4: ACN, (50:50)
Detection:	UV 254 nm with standard flow cell (13 µL)
Flow:	2 mL/ min.
Inj. Volume:	1 µL
Temperature:	top: ambient, bottom: 60°C



Highlights

- *Reducing column length and particle size simultaneously can:*
  - *Reduce analysis time*
  - *Maintain resolution*
  - *Reduce solvent use*
- *Elevated operating temperature is effective in reducing run time.*
- *ZORBAX StableBond SB-C18 can operate at higher temperatures and lower pH than other commercial reversed-phase columns.*

Agilent Technologies

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

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The Analysis of Triglycerides in Edible Oils by APCI LC/MS

Doug McIntyre

Application Note

Food

Abstract

Triglycerides are the major components in edible oils. This note describes a method for analyzing intact triglycerides by LC/MS and the type of information available.

Introduction

Triglycerides are found in both plant oils and animal fats. Their characterization is important for nutritional reasons, where the amount of unsaturation must be known as well as for determining other characteristics of the oil such as product purity, suitability for deep-frying, etc.

Traditionally, they have been analyzed by hydrolyzing the triglycerides to yield the fatty acids, which are then derivatized and analyzed as methyl esters by GC/MS.[1] This offers only indirect analysis of the original triglycerides. Triglycerides can be chromatographed intact by HPLC but their lack of a chromophore unless conjugated makes detection difficult.[2] In addition, in order to identify each individual triglyceride in an oil, a larger number of standards than is practical would be required and the analysis time would be quite lengthy.

LC/MS analysis offered the ease of an LC separation plus the specificity of mass detection. Due to the non-polar nature of the molecules, atmospheric pressure chemical ionization (APCI) was employed.

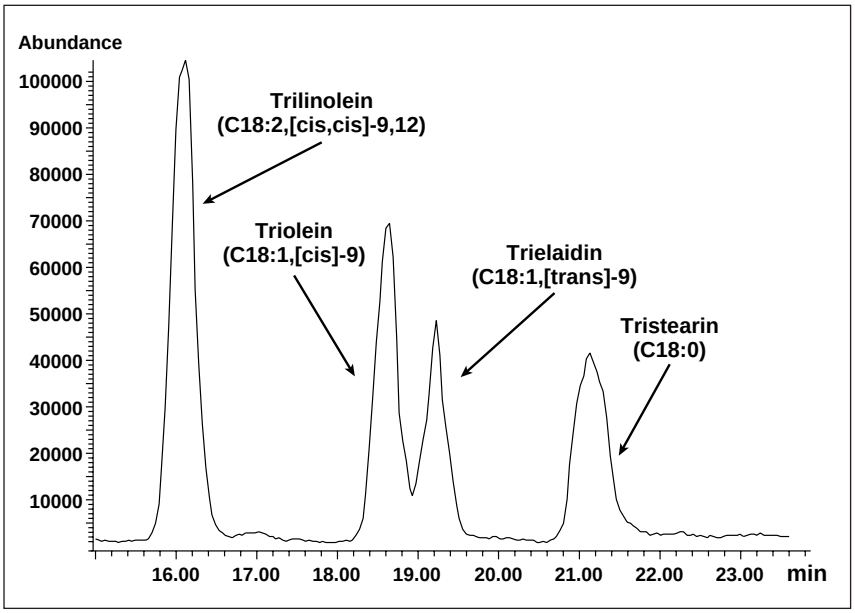

Figure 1. TIC showing separation obtained for four C18 triglyceride standards. Note that cis and trans isomers are well resolved.

Chromatographic Conditions	
Column:	200 × 2.1 mm Hypersil MOS, 5 µm
Mobile phase:	A = 60:40 water isopropanol + 25 mM ammonium formate B = 10:10:80 water:isopropanol:n-butanol + 25 mM ammonium formate
Gradient:	Start with 30% B at 1.5 min 30% B at 25 min 60% B at 28 min 100% B
Flow rate:	0.25 ml/min
Column temp	50°C
Injection vol:	1.5 µl
MS Conditions	
Source:	APCI
Ion mode:	Positive
Vcap:	4000 V
Nebulizer:	50 psig
Drying gas flow:	4 l/min
Drying gas temp:	325 ¡C
Corona	4 µA
Vaporizer:	300 ¡C
Scan range:	300–1100 m/z
Stepsize:	0.1 m/z
Peakwidth:	0.3 min
Time filter:	On
Fragmentor	80 V

Experimental

The system was comprised of an 1100 Series binary pump, vacuum degasser, autosampler, thermostatted column compartment, and LC/MSD. The LC/MSD was used with the atmospheric pressure chemical ionization (APCI) source. Complete system control and data evaluation was done on the ChemStation for LC/MS. Earlier work to demonstrate feasibility was done on the 1090 HPLC and 5989B MS Engine equipped with the 5987A Electrospray Accessory and the G1075A Atmospheric Pressure Chemical Ionization source.

A variety of vegetable oils as well as animal fat products such as butter were purchased at local supermarkets. All products were dissolved in isopropanol at a concentration of 20 µl of oil per 10 ml of IPA or 20 mg of fat per 10 ml IPA. Triglyceride standards were purchased from Sigma and prepared by dissolving 10 mg of standard in 5 ml chloroform then further diluted to the desired concentration with IPA.

Results and Discussion

Separation and Ionization

In order to have the non-polar triglycerides elute in a reasonable mobile phase composition; a Hypersil MOS (C8) column was used. Even this column required using isopropanol and n-butanol to elute the larger triglycerides. The final gradient was able to resolve cis and trans isomers of C18:1 triglyceride standards as shown in Figure 1.

It was found that the addition of ammonium formate to the mobile phase allowed the formation of an ammonium adduct [M+18]+ which was more stable than the protonated [M+1]+ species formed without the ammonium formate. The effect of adding ammonium formate is shown in Figure 2. Additionally, the vaporizer had to be lowered to 300°C to minimize fragmentation and enhance the molecular signal. If structural information was desired, in-source collision induced dissociation (CID) was employed by raising the fragmentor from 80 volts to 150 volts.

Qualitative Spectral Information

Under these analytical conditions, each triglyceride shows predominantly an M+18 adduct ion and few other major fragments. Since triglycerides in plants are made up predominantly of fatty acids with even numbers of carbons (C12, C14, etc) and either 0, 1 or 2 double bonds per acid, much can be determined from this single adduct ion. A spreadsheet was created to determine the possible combinations of double bonds and carbon

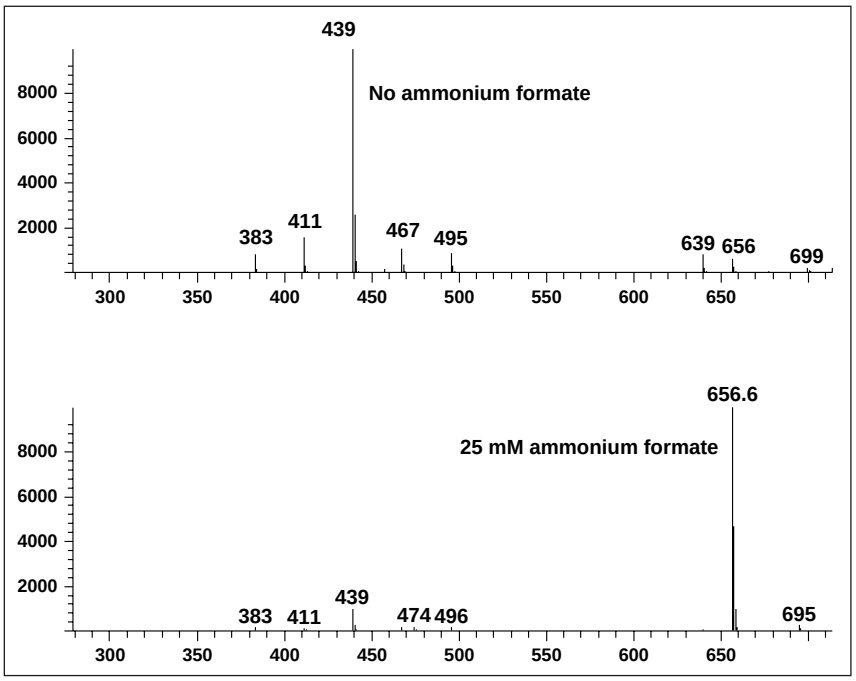

Figure 2. Effect of ammonium formate on the spectrum of trilaurin from coconut oil.

Chromatographic Conditions	
Column:	200 × 2.1 mm Hypersil MOS, 5 µm
Mobile phase:	A = 60:40 water isopropanol + 25 mM ammonium formate B = 10:10:80 water:isopropanol:n-butanol + 25 mM ammonium formate
Gradient:	Start with 30% B at 1.5 min 30% B at 25 min 60% B at 28 min 100% B
Flow rate:	0.25 ml/min
Column temp	50°C
Injection vol:	1.5 µl
MS Conditions	
Source:	APCI
Ion mode:	Positive
Vcap:	4000 V
Nebulizer:	50 psig
Drying gas flow:	4 l/min
Drying gas temp:	325 ¡C
Corona	4 µA
Vaporizer:	300 ¡C
Scan range:	300–1100 m/z
Stepsize:	0.1 m/z
Peakwidth:	0.3 min
Time filter:	On
Fragmentor	80 V

chain length. This was determined from the basic formula:

$M = 218.03 + 28.03*m + 26.02*n$

Where M is the mass of the observed adduct ion, m is the number of ethylene ($–CH_2–CH_2–$) groups and n is the number of ethenyl (–CH=CH–) groups. Since only integer solutions are allowed, the number of possible answers is typically very short. The choice is reduced even further if you assume fatty acids of the same length and unsaturation as the most likely solution, followed by only small differences in the fatty acids. For example, an unsaturated butyric acid with two oleic acids is not a likely solution. Figure 3 shows a typical TIC trace for coconut oil with the masses of the base ion annotated over each peak. Inserting these masses into the spreadsheet produces the results shown in Table 1. Further confirmation, if needed, can be

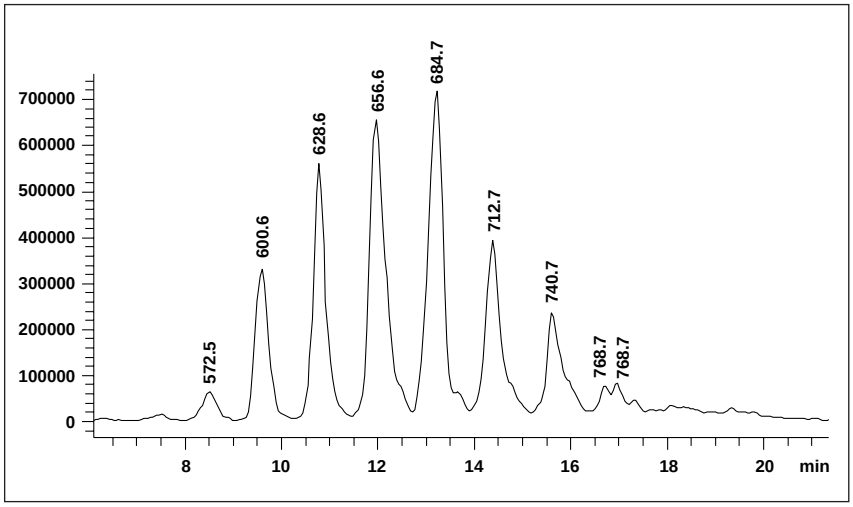

Figure 3. TIC showing mass of the base peak of the spectrum from peak apex. Peaks differ by m/z 28 corresponding to difference in the number of $–(CH_2CH_2)–$ groups on sidechains.

Chromatographic Conditions

Column:	200 × 2.1 mm Hypersil MOS, 5 µm
Mobile phase:	A = 60:40 water isopropanol + 25 mM ammonium formate B = 10:10:80 water:isopropanol:n-butanol + 25 mM ammonium formate
Gradient:	Start with 30% B at 1.5 min 30% B at 25 min 60% B at 28 min 100% B
Flow rate:	0.25 ml/min
Column temp	50°C
Injection vol:	1.5 µl

MS Conditions

Source:	APCI
Ion mode:	Positive
Vcap:	4000 V
Nebulizer:	50 psig
Drying gas flow:	4 l/min
Drying gas temp:	325 ¡C
Corona	4 µA
Vaporizer:	300 ¡C
Scan range:	300–1100 m/z
Stepsize:	0.1 m/z
Peakwidth:	0.3 min
Time filter:	On
Fragmentor	80 V

Table 1. Structure calculations for the major peaks in coconut oil.

Observed Ion	Calc. MW	Backbone Removed	m	n	Possible Sidechains	# Double Bonds
516.50	498.52	280.42	10	0	C8,C8,C10	0
544.55	526.52	308.42	11	0	C8,C10,C10 or C8,C8,C12	0
572.55	554.52	336.42	12	0	3 X C10	0
600.55	582.52	364.44	13	0	C10,C10,C12	0
628.55	610.52	392.44	14	0	C10,C12,C12 or C10,C10,C14	0
656.70	638.67	420.59	15	0	3 X C12	0
684.75	666.72	448.64	16	0	C12,C12,C14	0
712.75	694.72	476.64	17	0	C12,C14,C14 or C12,C12,C16	0
740.80	722.77	504.69	18	0	3 X C14	0
768.75	750.72	532.64	19	0	C14,C14,C16	0
796.75	778.72	560.64	20	0	C14,C16,C16 or C14,C14,C18	0

obtained using in-source CID. This is shown in Figure 4.

Most oils do not produce as simple a TIC trace as coconut oil. Figure 5 shows a trace for two grades of olive oil. Some differences are visible in the TIC but not much useful information is apparent. However, by looking at extracted ion chromatograms (EICs), much more can be determined. Figure 6 shows the results from extracting the signals for m/z 908.8, 906.8, 904.8, 902.8, 900.8 and 888.8. These correspond to triglycerides made up from 3 C18 fatty acids with 0, 1, 2, 3, 4 and 5 double bonds respectively. Not surprisingly, the dominant ion is at m/z 902.8, which corresponds to the ammonium adduct ion for triolein (3 C18:1). Oleic acid is the major fatty acid component in olive oil. Using EICs in this way, different oils can be compared to see how they differ in degree of unsaturation.

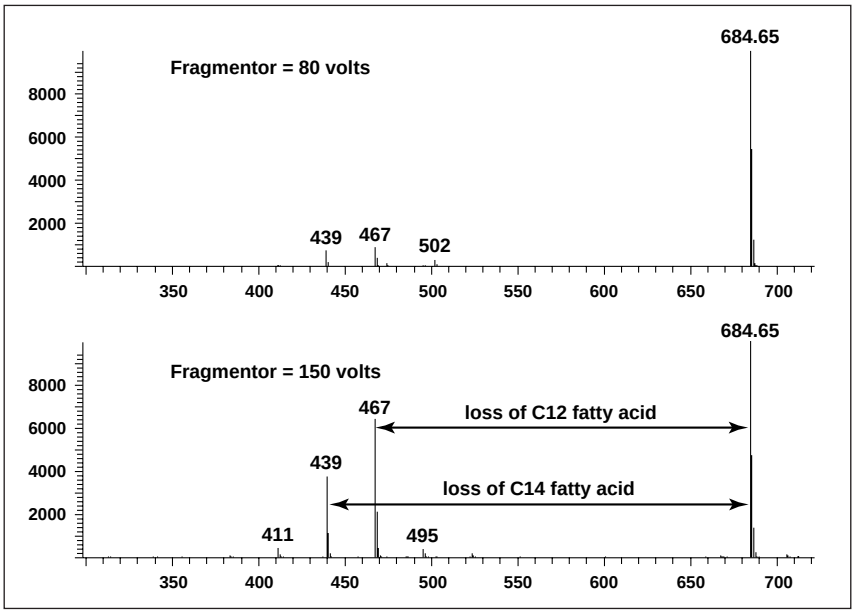

Figure 4. The effect of raising the Fragmentor voltage on a mixed triglyceride in coconut oil. The fragment masses and ratios are consistent with two C12 fatty acids and one C14.

Chromatographic Conditions

Column:	200 × 2.1 mm Hypersil MOS, 5 µm
Mobile phase:	A = 60:40 water isopropanol + 25 mM ammonium formate B = 10:10:80 water:isopropanol:n-butanol + 25 mM ammonium formate
Gradient:	Start with 30% B at 1.5 min 30% B at 25 min 60% B at 28 min 100% B
Flow rate:	0.25 ml/min
Column temp	50°C
Injection vol:	1.5 µl

MS Conditions

Source:	APCI
Ion mode:	Positive
Vcap:	4000 V
Nebulizer:	50 psig
Drying gas flow:	4 l/min
Drying gas temp:	325 ¡C
Corona	4 µA
Vaporizer:	300 ¡C
Scan range:	300–1100 m/z
Stepsize:	0.1 m/z
Peakwidth:	0.3 min
Time filter:	On
Fragmentor	80 V

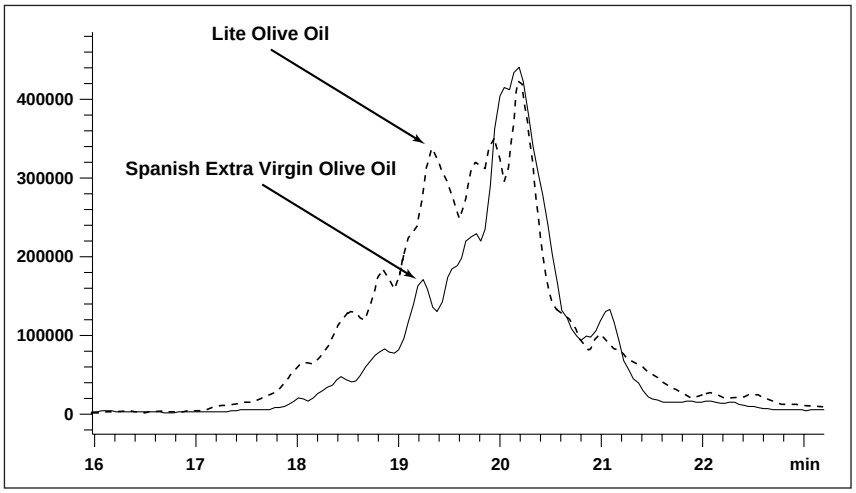

Figure 5. The TIC of extra lite and spanish virgin olive oils. The data indicates lite oil is more unsaturated or has shorter fatty acids. Further examination of EICs indicate more unsaturation.

Chromatographic Conditions

Column:	200 × 2.1 mm Hypersil MOS, 5 µm
Mobile phase:	A = 60:40 water isopropanol + 25 mM ammonium formate B = 10:10:80 water:isopropanol:n-butanol + 25 mM ammonium formate
Gradient:	Start with 30% B at 1.5 min 30% B at 25 min 60% B at 28 min 100% B
Flow rate:	0.25 ml/min
Column temp	50°C
Injection vol:	1.5 µl

MS Conditions

Source:	APCI
Ion mode:	Positive
Vcap:	4000 V
Nebulizer:	50 psig
Drying gas flow:	4 l/min
Drying gas temp:	325 ¡C
Corona	4 µA
Vaporizer:	300 ¡C
Scan range:	300–1100 m/z
Stepsize:	0.1 m/z
Peakwidth:	0.3 min
Time filter:	On
Fragmentor	80 V

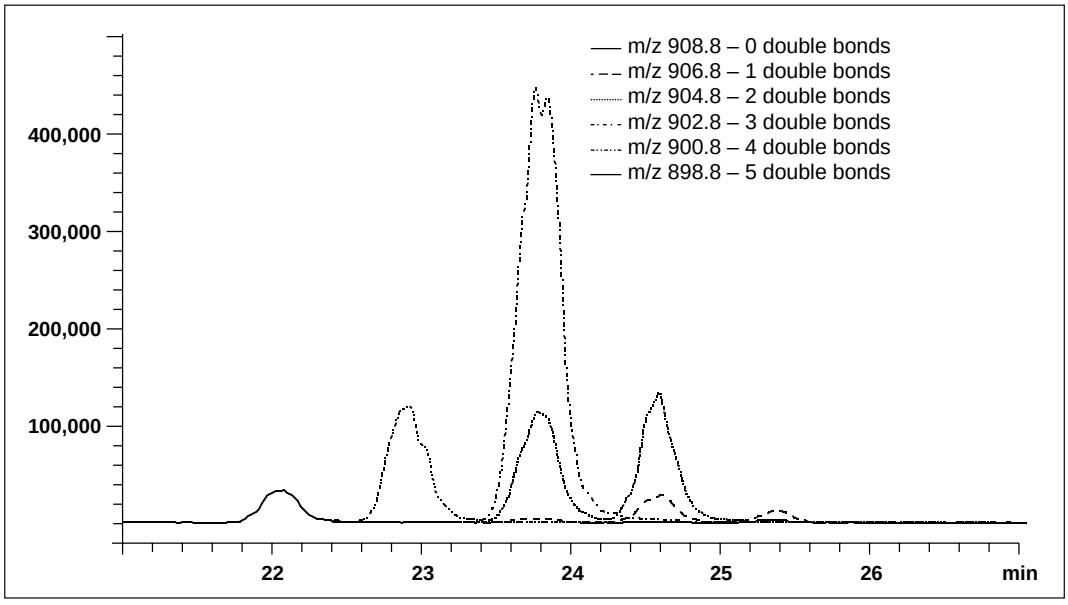

Figure 6. Extracted ion chromatograms (EICs) for olive oil corresponding to the 3 C18 series. Triolein is the major component.

Conclusion

LC/MS using APCI as an ionization process provides a simple way to analyze oils and fats for triglycerides. Information can be obtained directly rather than indirectly on the type of fatty acids including the degree of unsaturation. This information can be used for determining nutritional value and other physical properties of the oil or fat. It also has the potential of determining the amount of processing the oil has undergone (extra virgin versus pure). Little or no sample cleanup is required, allowing for a rapid analysis.

References

1. M. M. Mossoba and D. Firestone, *Food Testing and Analysis*, **1996**, 2 (2), 24–32.
2. R. Schuster, "Multicomponent Analyses of Fats and oils using Diode-Array Detection," *Hewlett-Packard Application Note*, **1987**, publication number 12-5954-6269.

Doug McIntyre is an applications chemist at Agilent Technologies

**Printed in the U.S.A. May, 2000
(23) 5968-0878E**

Analysis of Triglycerides in Olive Oil and Rape Oil using HPLC

Angelika Gratzfeld-Huesgen

Food

Abstract

Unsaturated triglycerides in olive oil have very characteristic patterns. Other fats and oils are also complex mixtures of triglycerides but with different patterns.

Sample preparation

Triglycerides can be extracted from homogenized samples with petrol ether. Fats and oils can be dissolved in tetrahydrofurane.

Chromatographic conditions

The presented HPLC method was used to analyze the unsaturated triglycerides, LnLnLn, LLL, and OOO.[1]

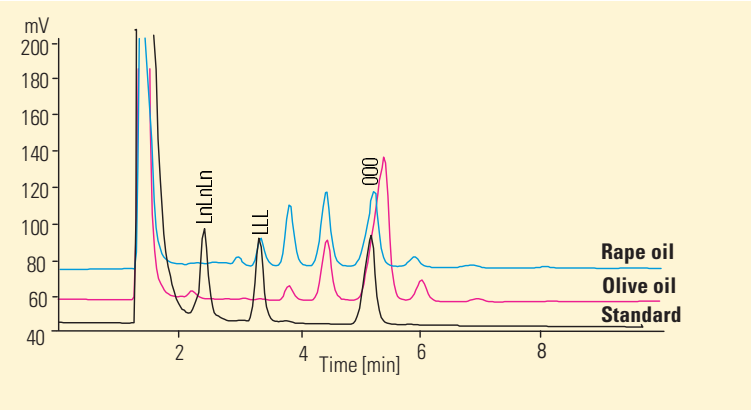

Figure 1
Analysis of the triglyceride pattern of olive and rape oil

Conditions

Column
200 ˘ 2.1 mm Hypersil MOS, 5 µm
Mobile phase
acetone/ACN (30:70)
Flow rate
0.5 ml/min
Column compartment
30 °C
Injection vol
2 µl
Detector
refractive index

Sample preparation
Samples were dissolved in tetrahydrofurane.

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HPLC method performance

Limit of detection for ECD
50 µg/l with S/N = 2

Repeatability of

RT over 10 runs	<0.3 %
areas over 10 runs	5 %

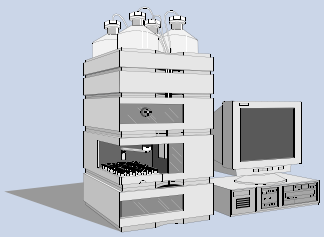
References

1.
“Determination of triglycerides in vegetable oils”, EC Regulation No. L248, 28ff.

Equipment

Agilent 1100 Series
- degasser
- isocratic pump
- autosampler
- thermostatted column compartment
- refractive index detector

Agilent ChemStation + software



Angelika Gratzfeld-Huesgen is application chemist at Agilent Technologies, Waldbronn, Germany.

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Publication Number 5966-0634E

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Analysis of Unsaturated Triglycerides using HPLC

Rainer Schuster

Food

Abstract

The HPLC method presented here was used to analyze triglycerides, hydroperoxides, sterols, and vitamins with UV-visible diode-array detection (UV-DAD). Spectra were evaluated in order to trace hydroperoxides and to differentiate saturated from unsaturated triglycerides. Unsaturated triglycerides in olive oil have a very distinctive pattern. Other fats and oils are also complex mixtures of triglycerides but exhibit an entirely different pattern. Adulteration with foreign fats and the use of refined triglycerides in olive oil also can be detected through triglyceride analysis.

Sample preparation

Triglycerides can be extracted from homogenized samples with petrol ether. Fats and oils can be dissolved in tetrahydrofuran.[1]

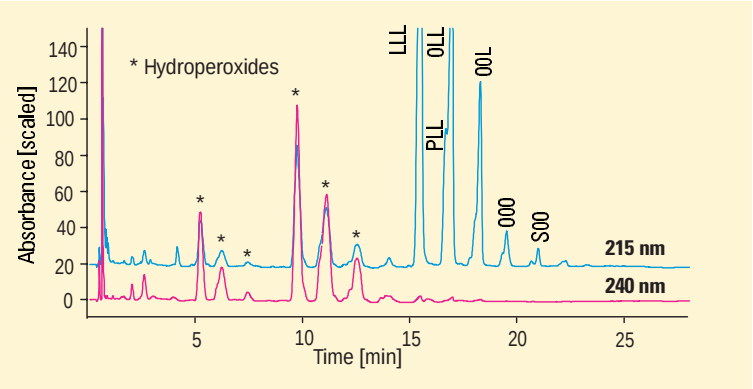

Figure 1
Triglyceride pattern of aged sunflower oil. The increased response at 240 nm indicates hydroperoxides

Conditions

Column
200 ˜ 2.1 mm Hypersil MOS, 5 µm
Mobile phase
A = water
B = ACN/methyl-tert.butylether (9:1)
Gradient
at 0 min 87% B; at 25 min 100% B
Post time 4 min
Flow rate 0.8 ml/min
Column compartment 60 °C
Injection vol 1 µl standard
UV absorbance
200 nm and 215 nm to detect triglycerides 240 nm to detect hydroperoxides 280 nm to detect tocopherols and decomposed triglycerides (fatty acids with three conjugated double bonds)

Sample preparation
Samples were dissolved in tetrahydrofuran (THF).

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HPLC method performance

Limit of detection
for saturated triglycerides
>10 µg

for unsaturated triglycerides
fatty acids with 1 double bond >150 ng
fatty acids with 2 double bonds >25 ng
fatty acids with 3 double bonds <10 ng

Repeatability of
RT over 10 runs <0.7 %
areas over 10 runs <6 %

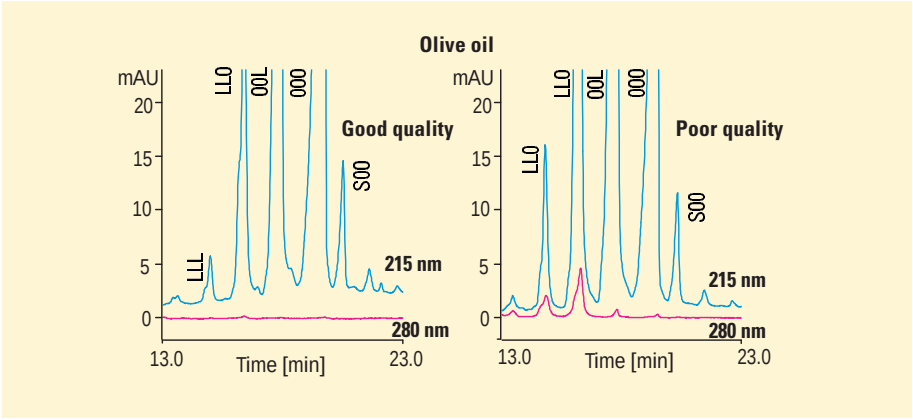


Figure 2
Analysis of olive oil. The response at 280 nm indicates a conjugated double bond and therefore poor oil quality

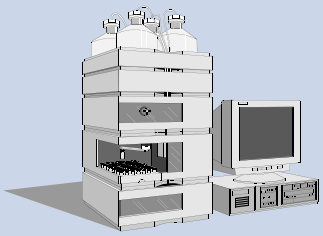
References

1.
"Determination of triglycerides in vegetable oils", EC Regulation No. L248, 28ff.

Equipment

Agilent 1100 Series
- vacuum degasser
- quaternary pump
- autosampler
- thermostatted column compartment
- diode array detector,

Agilent ChemStation + software



Rainer Schuster is application chemist at Agilent Technologies, Waldbronn, Germany.

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Analysis of Hydrolized Fatty Acids in Dietary Fat using HPLC

Angelika Gratzfeld-Huesgen

Food

Abstract

Saturated and unsaturated fatty acids from C4 through C22 have been analyzed. Fatty acids are the primary components of oils and fats and form a distinctive pattern in each of these compounds. For example, butter and margarines can be differentiated by the percentage of butyric acid in the triglycerides. To determine the fatty acid pattern of a fat or oil, free fatty acids first are obtained through hydrolysis. Derivatization is then performed to introduce a chromophore, which enables analysis of the fatty acids using HPLC and UV-visible detection.

Sample preparation

The triglycerides were hydrolyzed using hot methanol and KOH, followed by derivatization.

Chromatographic conditions

The HPLC method presented here was used in the analysis of the fatty acid pattern of dietary fat. The method involves hydrolysis with hot KOH/methanol and online derivatization with bromophenacyl bromide.

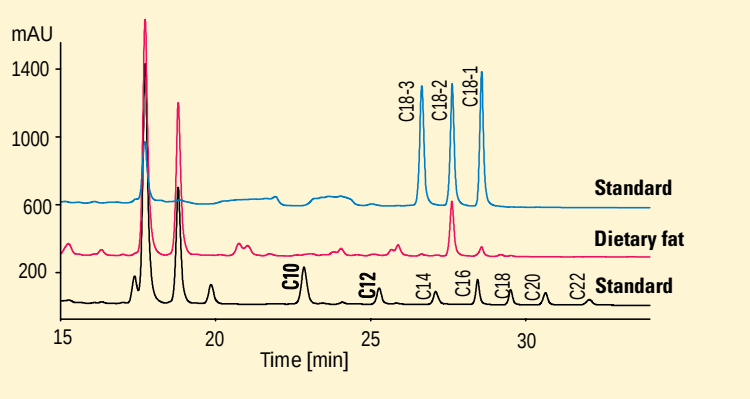

Figure 1
Analysis of a dietary fat triglyceride pattern. Overlay of one sample and two standard chromatograms

Conditions

Column 200 ˘ 2.1 mm, MOS, 5 µm
Mobile phase
A = water (70 %)
B = ACN + 1% THF (30%)
Gradient
at 5 min 30% B; at 15 min 70% B
at 17 min 70% B; at 25 min 98% B
Flow rate 0.3 ml/min
Column compartment 50 °C
Detector variable wavelength, 258 nm
Derivatization
60 mg/ml bromophenacyl bromide was dissolved in ACN

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HPLC method performance

Limit of detection
200 pg injected amount, S/N = 2

Repeatability of
RT over 10 runs <0.1 %
areas over 10 runs 5 %

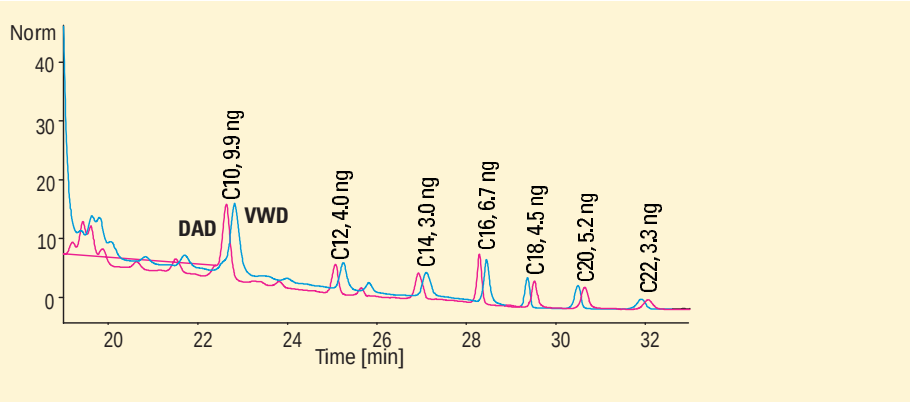

Figure 2
Trace analysis of triglycerides with a diode-array and a variable wavelength detector in series

Conditions

Injector program for online derivatization

1. Draw 2.0 µl from vial 2 (ACN)
2. Draw 1.0 µl from air
3. Draw 1.0 µl from vial 3 (derivatization agent)
4. Draw 0.0 µl from vial 4 (wash bottle) (ACN/THF, 50:50)
5. Draw 1.0 µl from sample
6. Draw 0.0 µl from vial 4 (wash bottle)
7. Draw 1.0 µl from vial 3 (derivatization agent)
8. Draw 0.0 µl from vial 4 (wash bottle)
9. Draw 1.0 µl from vial 5 (acetonitrile + 5 % TEA)
10. Draw 0.0 µl from vial 4 (wash bottle)
11. Mix 9 µl in air, 30 µl/min speed, 10 times
12. Wait 2.0 min
13. Inject

Sample preparation

0.215 g fat was hydrolyzed with 500 µl MEOH/ KOH at 80 °C for 40 min in a thermomixer. After cooling 1.5 ml ACN/THF (1:1) was added, and the mixture was shaken for 5 min. The mixture was then filtered through a 0.45-µm Minisart RNML from Satorius.

Equipment

Agilent 1100 Series
- vacuum degasser
- quaternary pump
- autosampler
- thermostatted column compartment
- variable wavelength detector

Agilent ChemStation + software

Angelika Gratzfeld-Huesgen is application chemist at Agilent Technologies, Waldbronn, Germany.

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Natural Compounds & Additives

Flavors and Fragrances

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Determination of Allergens in Fragrance Products Using Agilent Deconvolution Reporting Software

Application Brief

Wei Luan, Chris Sandy, and Mike Szelewski

Flavor and Fragrance

Highlights

- Automated deconvolution increases productivity for analysis of complex matrices
- Allergens database is available as a free download from Agilent Technologies, Inc.

The 7th amendment of the European Cosmetics Directive (2003/15/EC) was published in 2003. Manufacturers of cosmetics were required to indicate the presence of 26 fragrance ingredients in finished cosmetic products if they exceed a threshold of 0.01% for rinse-off and 0.001% for leave-on products. Subsequently, the analytical method for 24 allergenic compounds (except two natural extracts – oak moss and tree moss) using GC/MS was published by the International Fragrance Association (IFRA). Natural cosmetics are very complex, containing sterols, waxes, and flavanoids, which can result in poor spectral matches and long data processing time and can generate potential false positive or negative results.

Agilent's deconvolution reporting software (DRS) is designed to automatically deconvolute the spectra from matrices and generate a qualification and quantitation report. DRS integrates information from three processes: MSD ChemStation, automated mass spectral deconvolution and identification system (AMDIS), and NIST Search. DRS increases the confidence in results with complex matrices, and the typical data processing time is about 2 to 3 minutes.

A DRS database of 24 regulated allergenic compounds has been developed (Table 1). Additional compounds can easily be added to the database by the user.

Table 1. Allergens in Fragrance Products

Locked RT	Name	CAS no	Mol form	Mol wt
7.10	Limonene	5989-27-5	$C_{10}H_{16}$	136.1
7.17	Benzyl alcohol	100-51-6	C_7H_8O	108.1
7.41	Phenyl acetaldehyde	122-78-1	$C_8H_8O_2$	120.1
8.47	Linalol	78-70-6	$C_{10}H_{18}O$	154.1
10.29	1,4-Dibromobenzene [ISTD]	106-37-6	$C_6H_4Br_2$	233.9
10.41	Estragole	140-67-0	$C_{10}H_{12}O$	148.2
10.47	Folione	111-12-6	$C_9H_{14}O_2$	154.1
10.94	Citronellol	106-22-9	$C_{10}H_{20}O$	156.2
11.23	Citral (Neral)	106-26-3	$C_{10}H_{16}O$	152.1
11.44	Geraniol	106-24-1	$C_{10}H_{18}O$	154.1
11.77	Citral (geranial)	5392-40-5	$C_{10}H_{16}O$	152.1
11.80	Cinnamaldehyde	104-55-2	C_9H_8O	132.1

Table 1. Allergens in Fragrance Products (Continued)

Locked RT	Name	CAS no	Mol form	Mol wt
12.00	Anisyl alcohol	105-13-5	$C_8H_{10}O_2$	138.1
12.04	Hydroxy citronellal	107-75-5	$C_{10}H_{20}O_2$	172.2
12.33	Methyl octine carbonate	111-80-8	$C_{10}H_{16}O_2$	168.1
12.42	Cinnamic alcohol	104-54-1	$C_9H_{10}O$	134.1
13.37	Eugenol	97-53-0	$C_{10}H_{12}O_2$	164.1
14.13	Methyl eugenol	93-15-2	$C_{11}H_{14}O_2$	178.2
14.82	Coumarin	91-64-5	$C_9H_6O_2$	146.0
14.88	Cinnamyl acetate	103-548	$C_{11}H_{12}O_2$	176.1
14.96	Isoeugenol	97-54-1	$C_{10}H_{12}O_2$	164.1
15.5	Alpha isomethyl ionone	127-51-5	$C_{14}H_{22}O$	206.2
16.26	Lilial	80-54-6	$C_{14}H_{20}O$	204.2
18.14	Amyl cinnamaldehyde	122-40-7	$C_{14}H_{18}O$	202.1
18.27	Lyral 1	31906-04-5	$C_{13}H_{22}O_2$	210.2
18.36	Lyral 2	31906-04-4	$C_{13}H_{22}O_2$	210.2
18.7	Amyl cinnamyl alcohol	101-85-9	$C_{14}H_{20}O$	204.2
18.83	Farnesol 1	100009-91-0	$C_{15}H_{26}O$	222.2
19.18	Farnesol 2	4602-84-0	$C_{15}H_{26}O$	222.2
19.61	Hexyl cinnamaldehyde	101-86-0	$C_{15}H_{20}O$	216.2
19.89	Benzyl benzoate	120-51-4	$C_{14}H_{12}O_2$	212.1
21.36	Benzyl salicylate	118-58-1	$C_{14}H_{12}O_3$	228.1
24.20	Benzyl cinnamate	103-41-3	$C_{16}H_{14}O_2$	238.1

The Agilent Retention Time Locked (RTL) database was developed using the instrument conditions in Table 2, which is locked to alpha-isomethyl ionone in 15.494 min by retention time locking.

Table 2. Gas Chromatograph and Mass Spectrometer Conditions

GC	Agilent Technologies 7890A or 6890N		
Back inlet	Split/splitless		
Injection type	Split		
Inlet temperature	250 °C		
Pressure	11.46 psi		
Split ratio	50:1		
Split flow	68.5 mL/min		
Total flow	72.7 mL/min		
Gas saver	On		
Saver flow	15.0 mL/min		
Saver time	1.00 min		
Gas type	Helium		
4 mm ID, Single Taper Liner			
Oven			
Oven ramp	°C/min	Next °C	Hold min
Initial time		50	1
Ramp rate	8	270	1.5
Total run time	30 min		
Equilibration time	0.5 min		
Column	Agilent Technologies HP-5ms p/n 19091S-433		
Length	30 m		
Diameter	0.25 mm		
Film thickness	0.25 µm		
Mode	Constant pressure		
Pressure	11.46 psi		
Nominal initial flow	1.4 mL/min		
Inlet	Back inlet		
Outlet	MSD		
Outlet pressure	Vacuum		

Table 2. Gas Chromatograph and Mass Spectrometer Conditions (Continued)

RTL	System retention time locked to alpha isomethyl ionone at 15.494 min
Back Injector	
Sample washes	1
Sample pumps	3
Injection volume	1 µL
Syringe size	10 µL
Preinj solvent A washes	3
Preinj solvent B washes	3
Postinj solvent A washes	3
Postinj solvent B washes	3
Viscosity delay	1 second
Plunger speed	Fast
Preinjection dwell	0 minutes
Post-injection dwell	0 minutes
MSD	Agilent Technologies 5975C
Acquistion mode	Scan/SIM
Solvent delay	3 min
Low mass	40
High mass	350
Threshold	20
Sampling	3
Quad temperature	150 °C
Source temperature	230 °C
Transfer line temperature	280 °C
Tune type	Autotune
EM voltage	Atune voltage, 1,023.5 V
MSD-SIM	AutoSIM was used to pick ions, groups, and switching times
Number of groups	17
Ions/group	Varied 4 to 14
Dwell time, msec	10
Cycles/peak	Varied 5.8 to 15.9

A typical total ion chromatogram (TIC) of a fragrance product is displayed in Figure 1.

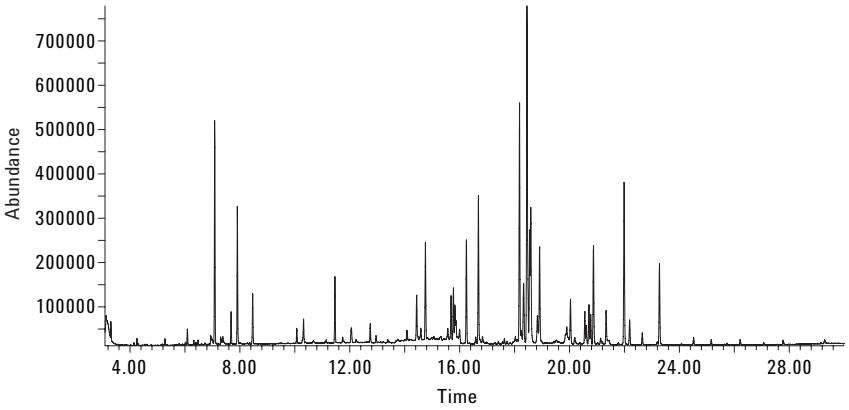

Figure 1. Total ion chromatogram of a fragrance product.

Dirty matrices often disturb the identification of target compounds by high-level chemical noise, resulting in poor library match factors. Background subtraction is both matrix- and operator-dependent and can yield inconsistent results. DRS automatically deconvolutes the signal from the matrix using AMDIS, which identifies target allergens quickly while minimizing false positives and negatives. A typical DRS report is displayed in Figure 2.

MSD Deconvolution Report
Sample Name: Lilac fragrance 10.20mg
Data File: C:\msdchem\1\DATA\Lilac.D
Date/Time: 10:49 AM Thursday, May 8 2008

Adjacent Peak Subtraction = 1
Resolution = Medium
Sensitivity = Medium
Shape Requirements = Medium

The NIST library was searched for the components that were found in the AMDIS target library.

			Amount (ng)		AMDIS		NIST	
R.T.	Cas #	Compound Name	Chem station	AMDIS	Match	R.T. Diff sec.	Reverse Match	Hit Num.
7.0984	5989275	Limonene	0.01		97	-0.1	94	1
7.1792	100516	Benzyl alcohol			84	0.7	86	1
7.3942	122781	Phenyl acetaldehyde			90	-0.3	94	1
8.4788	78706	Linalol	0.09		96	0.7	91	1
12.105	107755	Hydroxy citronellal	0.54		87	4.3	85	1
12.436	104541	Cinnamic alcohol	0.01		89	1.2	76	1
12.4495	111808	Methyl octine carbonate			56	8.0		
12.4495	22771444	cis-p-Mentha-2,8-dien-1-ol					71	1
14.8775	91645	Coumarin			91	3.7	92	1
15.1775	103548	Cinnamyl acetate			44	18.6		
15.1775	103957	3-(4-Isopropylphenyl)-2-methylpropionaldehyde					89	1
18.1555	122407	Amyl cinnamaldehyde			99	1.3	93	1
19.6005	101860	Hexyl cinnamaldehyde			94	0.0	89	1
19.8882	120514	Benzyl benzoate	0.01		72	0.3	91	1
24.1972	103413	Benzyl cinnamate			96	-0.2	90	2
10.296		1,4 Dibromobenzene	1					

Figure 2. Typical DRS report.

Figure 3 shows us the benefit of DRS when analyzing allergens in fragrance products. Cinnamaldehyde was successfully identified by DRS even though it was buried by the coeluting matrix compounds. The upper window is the TIC, the middle window is the raw or dirty spectrum in the scan No. 987 (11.796 min), and the lower window is the comparison of the deconvoluted spectrum (the white plot) with the spectrum of cinnamaldehyde in the allergen RTL library (the black plot). After deconvolution, the spectrum of the scan No. 987 is "clean," and we can easily identify the cinnamaldehyde in the fragrance product.

If your laboratory already has a user library in Agilent format, DRS A.04 software can create the necessary files in AMDIS format for deconvolution. Your laboratory may also have an optimized method or preferred column for allergens analysis. In these cases, DRS A.04 software can still be used with your own retention times and method.

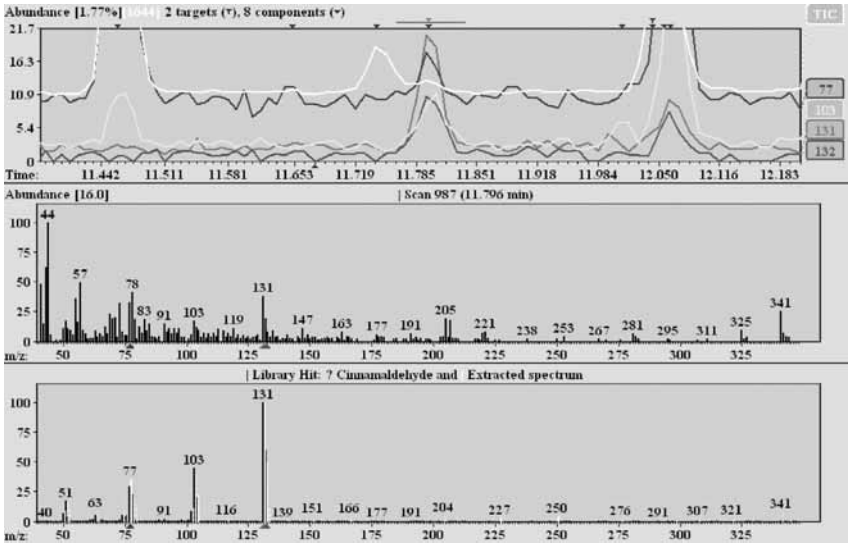

Figure 3. AMDIS display showing: a) the total ion chromatogram of a fragrance product; b) the spectrum where cinnamaldehyde elutes, and c) the deconvoluted spectrum (in white) juxtaposed to the library spectrum for cinnamaldehyde (in black).

Summary

DRS (p/n G1716AA) automatically deconvolutes mass spectra and produces more consistent and reliable identification of compounds in complex matrices. A DRS add-on allergen RTL database has been published on the Agilent Web site and, after registration, can be freely downloaded from http://www.chem.agilent.com/en-US/Support/Downloads/Utilities/RetentionTimeLocking/Pages/default.aspx.

References

1. "GC/MS Quantitation of Potential Fragrance Allergens in Fragrance Compounds," Version 3, September 12, 2007, International Fragrance Association
2. "Data Treatment Strategy to Analyze Allergens in Complex Samples Using the IFRA Procedure," Version 3, September 12, 2007, IInternational Fragrance Association

Wei Luan is an application chemist based at Agilent Technologies, Shanghai, China; Chris Sandy is an application chemist based at Agilent Technologies, UK; and Mike Szelewski is an application chemist based at Agilent Technologies, Wilmington, Delaware, U.S.

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Developing a green LC method for the determination of the furocoumarins 5-MOP and 8-MOP in citrus oils using the Agilent 1290 Infinity LC

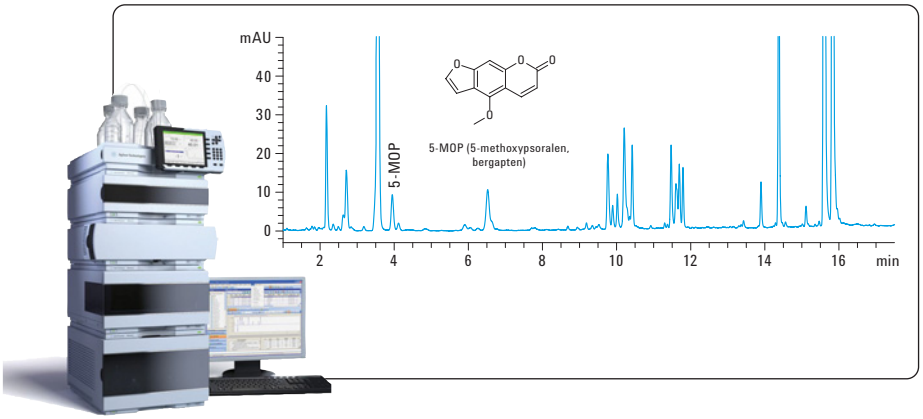
Application Note

Natural Products, Fragrances

Authors

Gerd Vanhoenacker, Frank David, and Pat Sandra
Research Institute for Chromatography
Kennedypark 26
B-8500 Kortrijk
Belgium

Bernd Glatz and Edgar Naegele
Agilent Technologies R & D and Marketing GmbH and Co. KG
Hewlett-Packard-Str. 8
76337 Waldbronn
Germany



Abstract

Lemon and orange oils were analyzed for the presence of the furocoumarins 5-methoxypsoralen (5-MOP) and 8-methoxypsoralen (8-MOP) with the Agilent 1290 Infinity LC system. At 1000 bar, the total analysis time in reversed-phase (RP) liquid chromatography (LC) with acetonitrile and water as mobile phase components could be reduced to 4 min. In the frame work of green chromatography, and the present acetonitrile shortage, mobile phases constituted of water/methanol and water/ethanol were compared to water/acetonitrile. The influence of the nature of organic modifiers on selectivity and peak capacity was investigated. The figures of merit of the different methods were compared for the determination of 5-MOP in a lemon oil sample containing approximately 50 mg/kg. 8-MOP was not detected at the ppm level. The detection limit for both furocoumarins was approximately 1 mg/kg citrus oil.

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Introduction

Furocoumarins are natural products that may be present in plant extracts and essential oils used in fragranced cosmetic products. The furocoumarins have been identified as photomutagenic and photocarcinogenic products. The International Agency for Research on Cancer (IARC) has classified 5-MOP (5-methoxypsoralen, bergapten) and 8-MOP (8-methoxypsoralen, xanthotoxin) when combined with UV radiation as group 2A (probably carcinogenic to humans) and as group 1 (carcinogenic to humans) risk carcinogens, respectively.

On that basis, limits have been defined for the presence of psoralens in cosmetics. The Commission Directive 95/34/DC of 1995 states that furocoumarins should be below 1 mg/kg (1 ppm) in sun protection and in bronzing products.[1] There is an ongoing debate to extend this 1 ppm limit to all finished cosmetic products.[2] Therefore, fast analysis of furocoumarins in cosmetics and in the essential oils used in cosmetics is of utmost importance.

The Agilent 1290 Infinity LC system in combination with UV detection was evaluated for the determination of 5-MOP and 8-MOP (Figure 1) in a lemon and orange oil sample. Typical analysis times for such samples using standard LC instrumentation are 30 min.[3] The possibility of increasing the analysis speed by employing the high pressure capabilities of the Agilent 1290 Infinity LC was investigated. Mobile phases for these analyses are commonly composed of water and acetonitrile. Different organic modifiers (acetonitrile, methanol, and ethanol) were compared and a green method with biodegradable ethanol as the mobile phase constituent was developed.

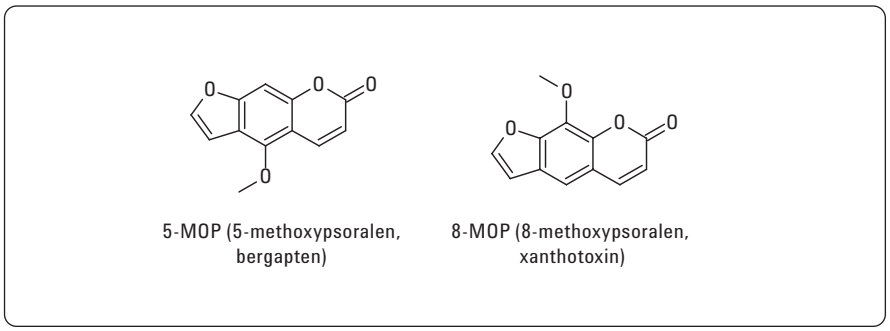

Figure 1
Structures of 5-MOP and 8-MOP.

The features of diode array detection (DAD) and mass spectrometer (MS) detection for trace analysis of furocoumarins using the Agilent 1290 Infinity LC will be described elsewhere.[4]

Experimental

Instrumentation and method

An Agilent 1290 Infinity LC system with the following configuration was used:

Part number	Description
G4220A	Agilent 1290 Infinity Binary Pump with integrated vacuum degasser
G4226A	Agilent 1290 Infinity Autosampler
G1316C	Agilent 1290 Infinity Thermostatted Column Compartment
G4212A	Agilent 1290 Infinity Diode Array Detector

Method parameters:	
Column	C18 150 mm L × 2.1 mm id, 1.7 µm d_p C18 100 mm L × 2.1 mm id, 1.7 µm d_p
Mobile phase	A = Water B = Acetonitrile, methanol or ethanol
Flow rate	Variable
Gradient	Variable
Temperature	80 °C
Injection	1 µL
Detection	DAD, Signal 315/4 nm, Reference 500/60 nm, 40 Hz

Solutions and samples

Stock solutions of 500 µg/mL of 5-MOP and 8-MOP standards were prepared in ethanol. The solutions were further diluted in ethanol prior to injection. Samples of lemon and orange oil were used. The oils were analyzed separately, as well as mixed, and they were diluted 1/10, volume to volume, in ethanol prior to injection.

Results and Discussion

Increase speed

The high pressure capabilities of the Agilent 1290 Infinity LC system were utilized to increase speed of analysis. Starting from a RP-LC method with a water/acetonitrile gradient, the flow rate and gradient slope were increased proportionally to each other while the isocratic hold time at the beginning of the analysis was reduced to maintain the elution profile. The flow rate was increased from 0.45 to 0.6, 0.9, and 1.2 mL/min resulting in pressure drops up to 1090 bar on the 150 mm long column. The analysis time was more than 2.5 times faster and could be obtained without sacrificing resolution.
Figures 2A, B, and C show some typical profiles for the mixed oil sample. Only 5-MOP was detected in the sample. Only use the analysis conditions in Figure 2C if your column and hardware is rated above 1000 bar.

A further increase in speed could be obtained by reducing the column length to 100 mm and increasing the flow rate to 1.45 mL/min (Figure 2D). The result is that the analysis time is approximately 4 min with a pressure of 1000 bar. The drawback of using a shorter column for high speed analysis is that the resolution decreases as well. However, the separation is still sufficient to detect 5-MOP in the sample.

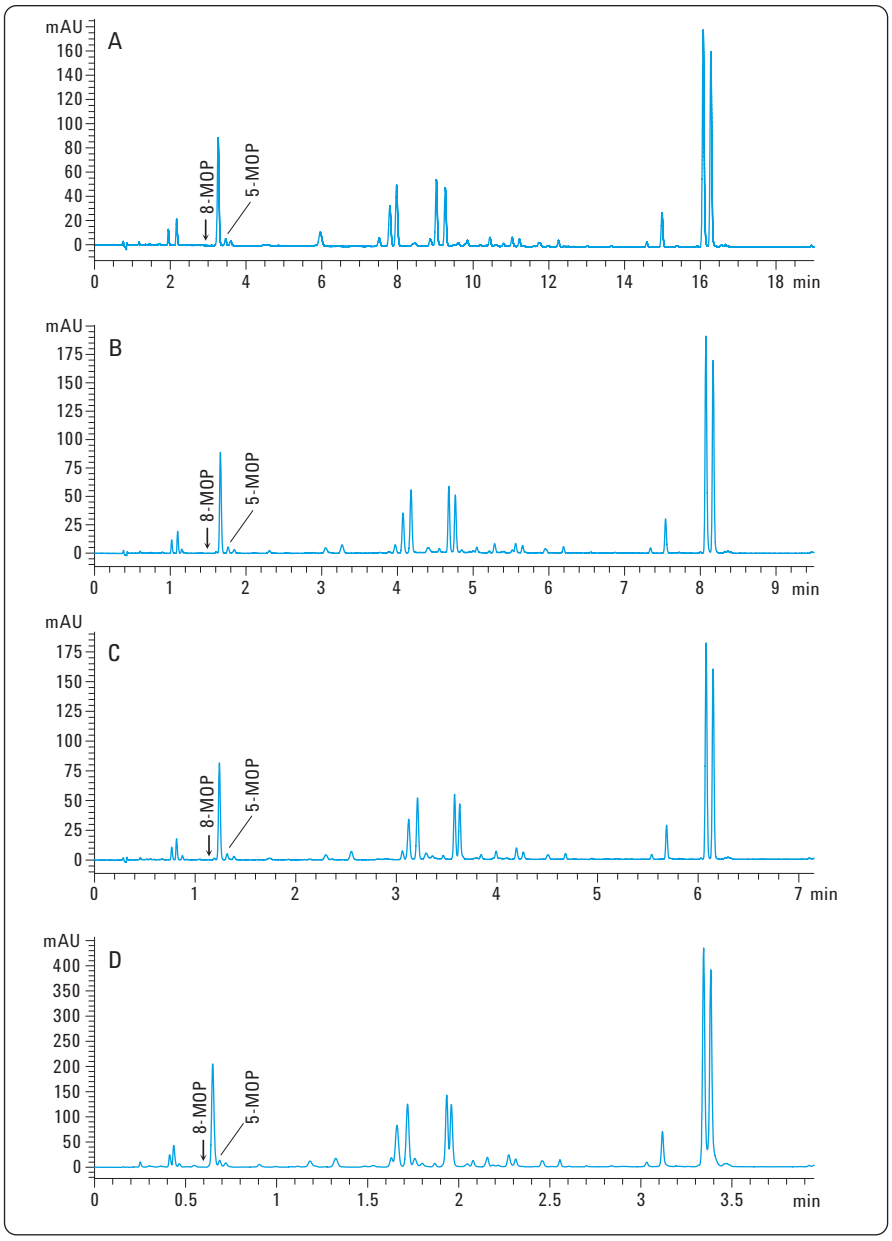

Figure 2
Chromatograms for the analysis of the mixed oil sample with different flow rates and columns. Flow rate/column length: 0.45 mL/min/150 mm (A), 0.9 mL/min/150 mm (B), 1.2 mL/min/150 mm (C), and 1.45 mL/min/100 mm (D). Mobile phase: water-acetonitrile.

Conditions Figure 2	A	B	C	D
Column length	**150 mm**	**150 mm**	**150 mm**	**100 mm**
Flow rate	**0.45 mL/min**	**0.9 mL/min**	**1.2 mL/min**	**1.45 mL/min**
Gradient	0–5 min: 30% B isocratic 5–25 min: 30–100% B	0–2.5 min: 30% B isocratic 2.5–12.5 min: 30–100% B	0–1.9 min: 30% B isocratic 1.9–9.4 min: 30–100% B	0–1 min: 30% B isocratic 1–5.2 min: 30–100% B
Maximum pressure	**440 bar**	**840 bar**	**1090 bar**	**1000 bar**

Green chromatography

In a second application, the Agilent 1290 Infinity LC system was used to develop a green LC method. The citrus oil was analyzed with three different organic modifiers. Solvent composition and gradients had to be adapted for each combination to obtain a similar elution window. The results are shown in Figure 3. The elution profile showed significant differences with the sample analyzed in section 1 (mixed oil sample). The reason is that the orange oil contains mainly polymethoxylated flavanoids while the lemon oil is mostly composed of psoralene derivatives.

Analyzing samples with only water and ethanol as mobile phase components is green chromatography. This approach is interesting ecologically as well as economically due to the present acetonitrile shortage. The drawback of using water/ethanol mobile phases is the high backpressure that is generated. For this particular analysis the pressure reached 770 bar with ethanol while only 440 and 590 bar for acetonitrile and methanol, respectively. The Agilent 1290 Infinity LC is rated to 1200 bar and has no problem with these high backpressures.

The presence of 5-MOP could be elucidated in the 3 chromatograms. This indicates that in the mixed sample, 5-MOP was originating from the lemon oil sample and not from the orange oil sample. Note that, identification and quantification of all furocoumarins at trace levels is best performed by using mass spectrometry.[4]

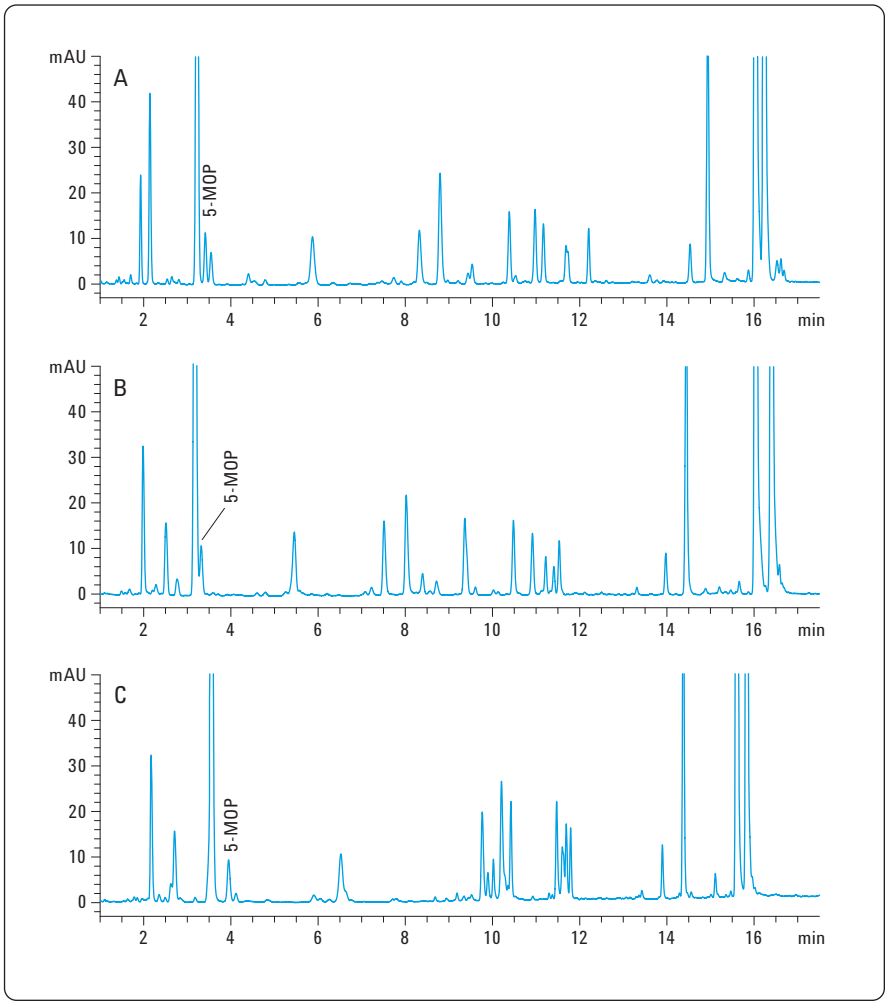

Figure 3
Chromatograms for the analysis of a lemon oil sample with 3 different modifiers. Acetonitrile (A), methanol (B), and ethanol (C). Flow rate: 0.45 mL/min.

Conditions Figure 3	A	B	C
Modifier	**Acetonitrile**	**Methanol**	**Ethanol**
Gradient	0-5 min: 30% B isocratic 5–25 min: 30-100% B	0–3.5 min: 40% B isocratic 3.5–23.5 min: 40-100% B	0–5 min: 23% B isocratic 5–25 min: 23–100% B
Maximum pressure	**440 bar**	**590 bar**	**770 bar**

Quantitative and performance data

The different methods were applied to perform the quantitative analysis of 5-MOP in the lemon oil sample and their performances were compared (Table 1). Calibration lines were created by single consecutive injections of standard solutions containing 0.1, 0.5, 1, 5, and 10 µg/mL of 5-MOP. The data are summarized in Table 1.

In order to compare the resolving power of the different methods the peak capacity was calculated for each. This was done by dividing the gradient time with the average peak width at the base (4σ) determined for the 5 µg/mL 5-MOP solution. As expected, the resolving power on the shorter column decreased compared to the 150-mm column. It is noteworthy that the peak capacity with the water/acetonitrile was not affected by the increased flow rate while peak capacities for acetonitrile and ethanol were similar.

The assay of 5-MOP was very similar with all methods and the relative standard deviation (RSD) was 1.98%. In the sample solution an average concentration of 5.46 µg/mL was detected. This corresponds to 51 mg/kg 5-MOP in the original lemon oil.

Mobile phase	Column length (mm)	Flow rate (mL/min)	Maximum pressure (bar)	Peak capacity	Linearity (R^2)	Assay 5-MOP (µg/mL)*
Acetonitrile	150	0.45	440	219	>0.9999	5.39
Methanol	150	0.45	590	192	>0.9999	5.60
Ethanol	150	0.45	770	215	>0.9999	5.30
Acetonitrile	150	0.90	850	218	>0.9999	5.53
Acetonitrile	150	1.20	1090	213	>0.9999	5.44
Acetonitrile	100	1.45	1000	152	>0.9999	5.52
					Average	**5.46**
					RSD (%)	**1.98**

*Concentration in sample solution. The oil is diluted 1/10 v/v in ethanol prior to analysis.

Table 1.
Comparison of the different methods. Assay was performed on the lemon oil sample.

Conclusion

The Agilent 1290 Infinity LC system was used to analyze furocoumarins in citrus oil samples. The performance of the original method with a water/acetonitrile mobile phase was compared to green chromatography methods where the acetonitrile was replaced with methanol or with ethanol. Additionally, the performance of a high speed method was evaluated. This Application Note demonstrates that the high pressure capabilities and the high detector acquisition rate of the Agilent 1290 Infinity LC system are very useful tools for increasing analysis speed. In addition, it shows that the Agilent 1290 Infinity LC system can be used for methods that are less toxic and more environmentally friendly than existing methods.

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Analysis of Polar Compounds Using 100% Aqueous Mobile Phases with Agilent ZORBAX Eclipse Plus Phenyl-Hexyl and Other ZORBAX Phenyl Columns

Application Note

Pharmaceuticals and Food

Authors

William J. Long, Anne E. Brooks, and William Biazzo
Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE 19808
USA

Abstract

The analysis of polar compounds, such as acids and bases, typically requires ion pairing reagents or Hydrophobic Interaction Chromatography (HILIC) columns in HPLC. In this work, the utility of phenyl columns is demonstrated using 100% aqueous mobile phase, without the inconvenience of phase collapse. Selectivity of different Agilent ZORBAX phenyl phases (StableBond SB-Phenyl, Eclipse XDB-Phenyl and Eclipse Plus Phenyl-Hexyl) is shown with both aliphatic acids and catecholamines (bases). The selectivity of these compounds is further shown to be a function of mobile phase additive.

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Introduction

Polar compounds, like acids and bases, are generally difficult to separate using reversed phase liquid chromatography. Bases can be more easily retained at higher pH where the compounds are not charged. However, in some cases, other compounds may be in the sample that will not be separable at high pH. Another possible solution is to use an ion pair reagent to increase retention. In general, ion pair reagents are not compatible with mass spectrometry. Acidic compounds are also noted as difficult samples to separate or even retain. In general, it is necessary to work below the pKa of the compound where it will be fully protonated (not charged) and decrease the organic content of the mobile phase [1]. A problem that can occur with alkyl columns such as C8 or C18 phases is poor retention or reproducibility of retention in low organic mobile phase. One of the unique properties of phenyl columns is their resistance to "dewetting", or what is sometimes referred to as phase collapse [2]. Phenyl columns may be a good choice when 100% aqueous mobile phase will be utilized. This work will show separation of both basic and acidic compounds using 100% aqueous conditions.

Catecholamines are basic compounds that act as neurotransmitters. Control of many body functions can be altered by a lack or overabundance of these materials. Changes in catecholamine levels have been shown to be useful in the diagnosis of several pathological states [3].

Acids and their salts serve a variety of functions in foods, including the following: flavoring to provide a desired taste, controlling the pH to retard the growth of microorganisms, chelating of metal ions that can cause lipid oxidation (Cu, Fe), reducing color and texture changes in some fruits and vegetables, and changing the texture of foods by modifying gels made from pectin or proteins [4].

Experimental

HPLC analysis was performed with the Agilent 1200 Series Rapid Resolution LC (RRLC) system:

- G1312B binary pump SL, mobile phase Channel A only various mobile phase additives in water, 1 mL/min
- 1376C automatic liquid sampler (ALS) SL, injection volume was 5 µL
- G1316B Thermally Controlled Column (TCC) Compartment SL, temperature was 25 °C
- G1316C diode array detector (DAD), wavelength settings were 268,4 and 360,50 nm for the catecholamines and 220,4 and 360,50 nm for the acidic compounds, with a G1315-60024 micro flow cell (5 mm path, 6 µL volume)

ZORBAX Columns

- Eclipse Plus Phenyl-Hexyl 4.6 mm × 100 mm, 5 µm (p/n 959996-912)
- Eclipse Phenyl 4.6 mm × 100 mm, 5 µm (custom)
- StableBond Phenyl 4.6 mm × 100 mm, 5 µm (custom)
- Eclipse Plus Phenyl-Hexyl 4.6 mm × 150 mm, 3.5 µm (p/n 959961-912)
- Eclipse Phenyl 4.6 mm × 150 mm, 3.5 µm (p/n 963967-912)
- StableBond Phenyl 4.6 mm × 150 mm, 3.5 µm (p/n 863953-912)

Chemicals

The 18 MΩ Milli-Q water was produced on site. Formic acid, trifluoroacetic acid (TFA) and acetic acids were purchased from Sigma Aldrich (Bellfonte, PA). Catecholamines ephedrine, norephedrine, dopamine, levadopa, and tyrosine were also purchased from Sigma Aldrich. They were prepared in water at 1 mg/mL and mixed together to produce a final concentration of 0.2 mg/mL. L-(+)-tartaric acid, DL-malic acid, DL-lactic acid, acetic acid, citric acid, and propionic acid were dissolved in water at 1 mg/mL, and mixed to a final concentration of 0.2 mg/mL. All compounds were also injected separately in order to identify the peak and any impurities. Structures and pKa values for these chemicals are shown in Figure 1.

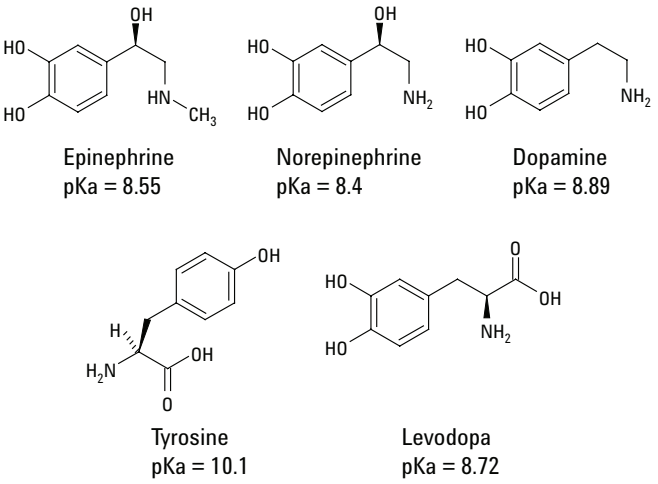


Figure 1a. Structures and pKa of catecholamines.

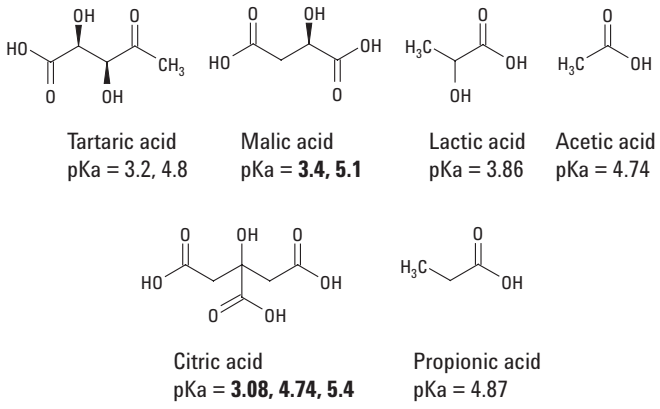


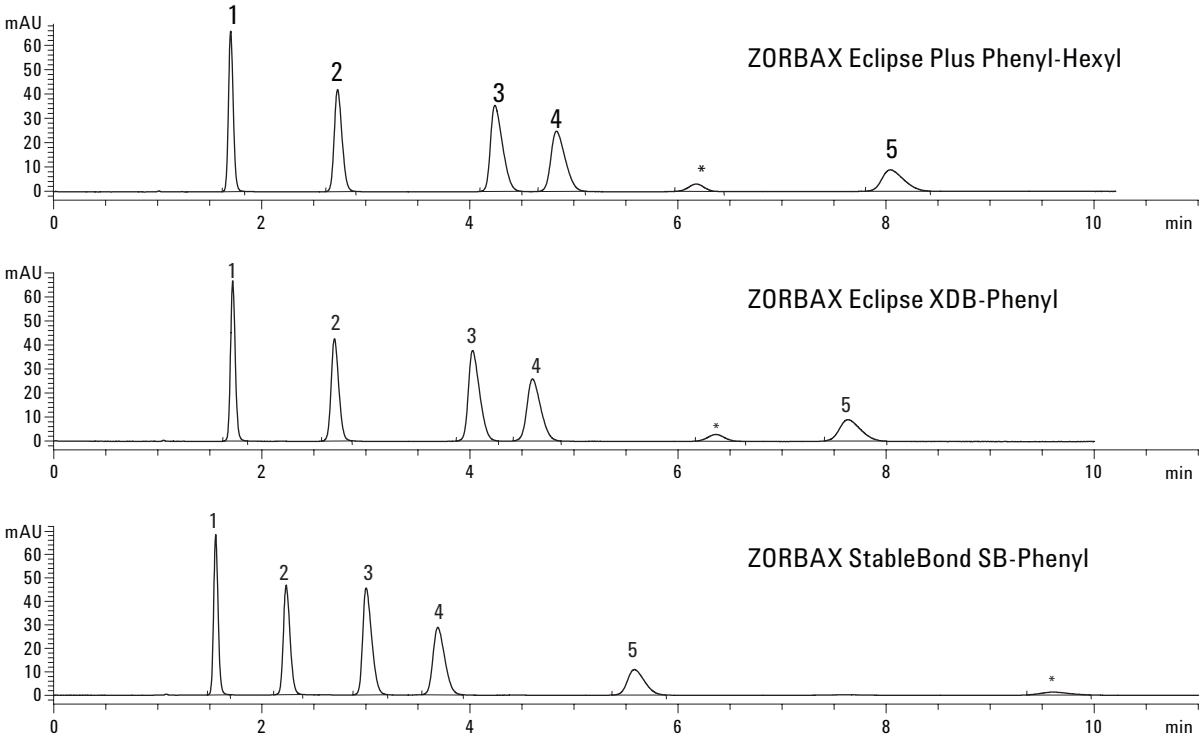
Figure 1b. Structures and pKa of aliphatic acids.

Results and Discussion

The ZORBAX Eclipse Plus Phenyl-Hexyl and Eclipse XDB-Phenyl (an ethyl phenyl phase) columns provide very similar separations (see Figure 2). For most of the components in the mixture, the separation appears more similar than different. The most notable exception appears to be tyrosine, which is only slightly more retained on the phenyl-hexyl column, perhaps due to its longer attaching group or higher carbon load (9.5 vs. 7%). StableBond SB-Phenyl, with its exposed silica silanol groups but larger protecting groups, leads to an alternative selectivity in which most of the peaks elute in the same order, with the exception of the impurity peak and the tyrosine, which have exchanged positions. The more highly retained and later-eluting impurity peak is in an excellent position for isolation. However, in general, the peaks in the StableBond SB-Phenyl separation are not as highly retained as those in the endcapped ethyl phenyl, Eclipse XDB-Phenyl separation.

Figure 3 shows the Eclipse Plus Phenyl-Hexyl column run with different acid mobile phases. The pKa's of these compounds are listed in Figure 1. As these compounds are all amines it is interesting to see how alternative mobile phases will affect this separation. Even though all of these compounds are weak bases, it can be noted that the strongest retention is found with the higher concentration of trifluoroacetic acid (TFA) mobile phase.

The varied retention shown in Figure 3 can be explained in many ways. If we are working close to the pKa of a group of compounds, we will typically see more retention if the charge

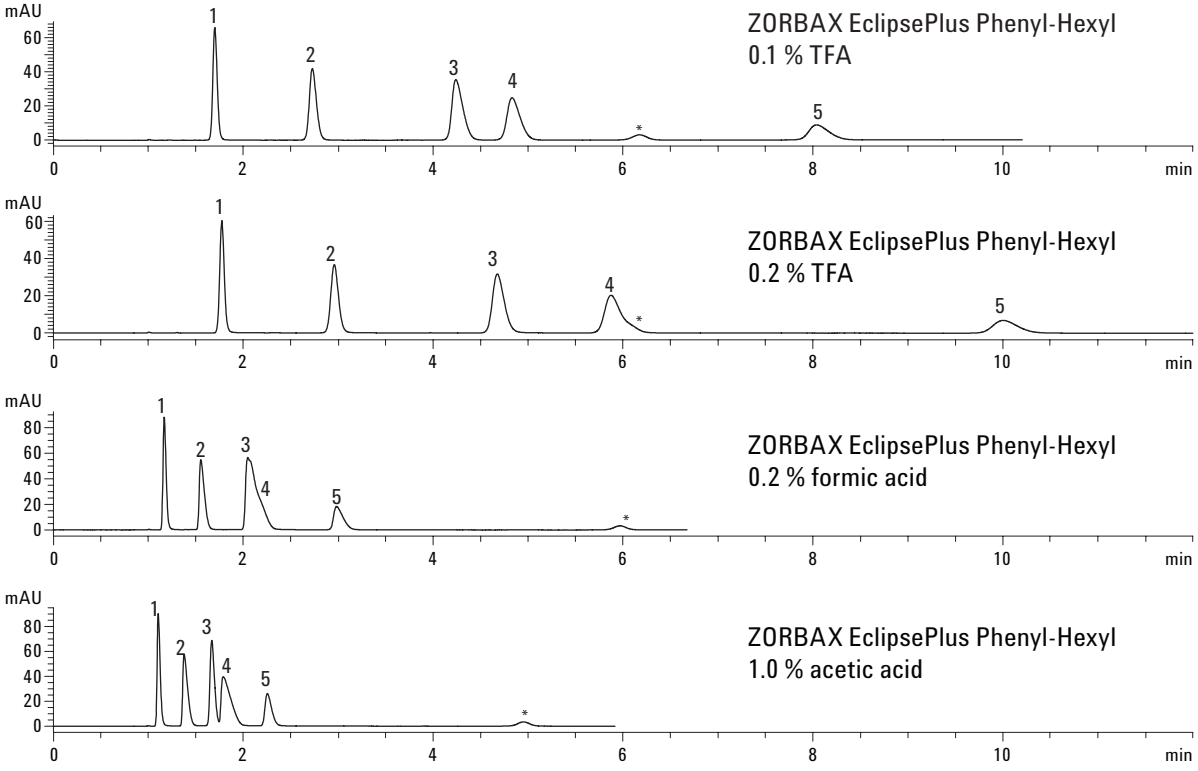


Norepinephrine, epinephrine, dopamine, levodopa, impurity*, tyrosine 0.2 mg/mL each 5 µL 4.6 mm × 100 mm, 5 µm columns.
Mobile phase = 0.1% TFA in water, 1 mL /min, 265 nm.

Figure 2. Comparison of ZORBAX phenyl column selectivity using 0.1% TFA mobile phase.

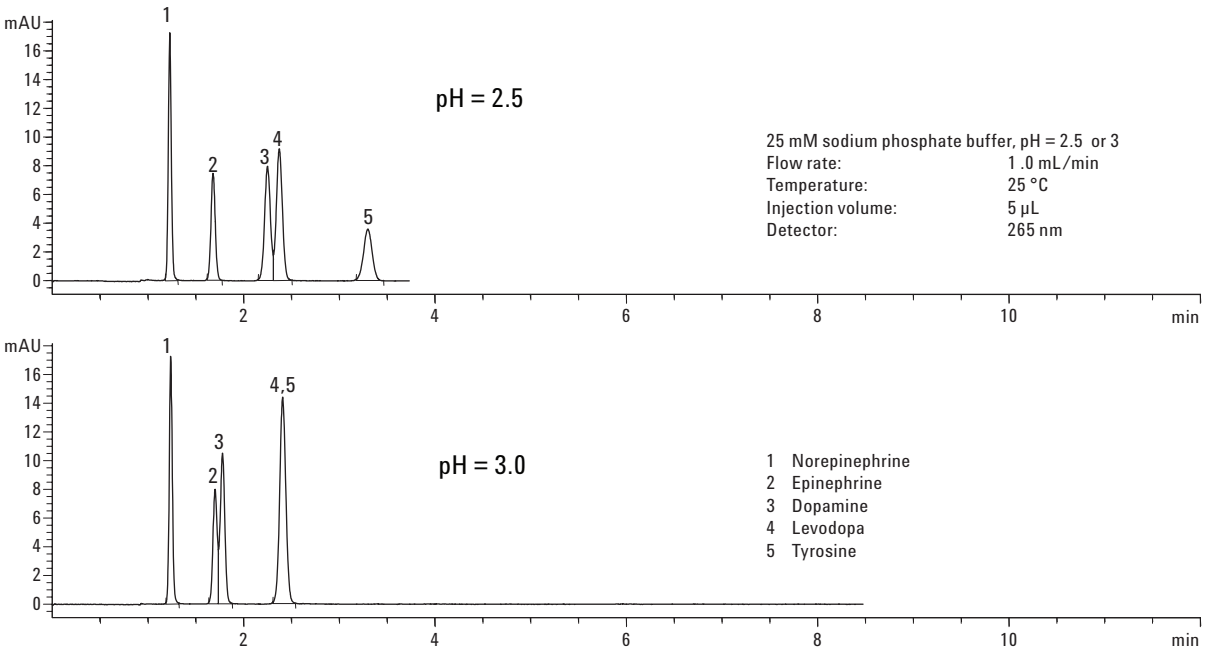
on the compounds is neutralized. Uncharged compounds are more highly retained than charged compounds [5,6]. An alternative explanation is ion pairing. TFA and formic acid are commonly referred to as ion pairing reagents, while acetic acid is not. As can be seen, retention is increased with additional TFA. A further experiment with two phosphate buffers in Figure 4 shows better retention at lower pH, but not as good as that using the TFA mobile phase [7,8].

In Figure 5, these aliphatic acids are well resolved using phenyl-hexyl and phenyl-ethyl columns. This suggests that phenyl columns may be good choices when separating highly polar compounds. The ZORBAX Eclipse Plus Phenyl-Hexyl column, with its longer alkyl linking group, is more lipophilic in nature than the ethyl phenyl Eclipse XDB-Phenyl or StableBond SB-Phenyl columns. Furthermore, by working well below the pKa's of these aliphatic acids, we can fully separate these aliphatic compounds. The acidic compounds are best retained on the more lipophilic Eclipse Plus Phenyl-Hexyl column; however, the column with the best separation, the separation of all compounds, is actually the Eclipse XDB-Phenyl (see Figure 5). This can be seen in the lack of overlap with any compound as shown in Figure 5.



Norepinephrine, epinephrine, dopamine, levodopa, impurity*, tyrosine 0.2 mg/mL each 5 µL 4.6 mm × 100 mm, 5 µm columns.
Mobile phase = 0.1% TFA in water, 1 mL /min, 265 nm.

Figure 3. Comparison of mobile phase selectivity using ZORBAX Eclipse Plus Phenyl-Hexyl column.



Norepinephrine, epinephrine, dopamine, levodopa, tyrosine 0.2 mg/mL each 5 µL 4.6 mm × 100 mm, 5 µm columns.
Mobile phase = Sodium phosphate buffer 25 mM, 1 mL /min, 265 nm.

Figure 4. Comparison of mobile phase selectivity using ZORBAX Eclipse Plus Phenyl-Hexyl (non-ion pair).

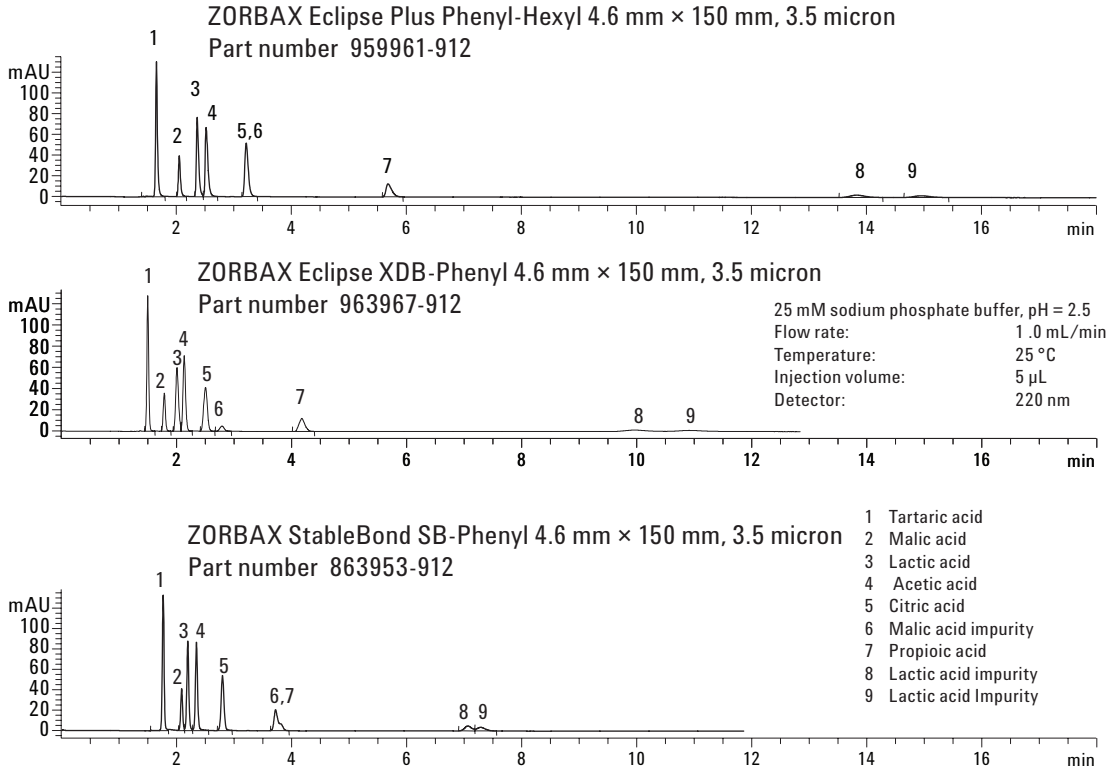
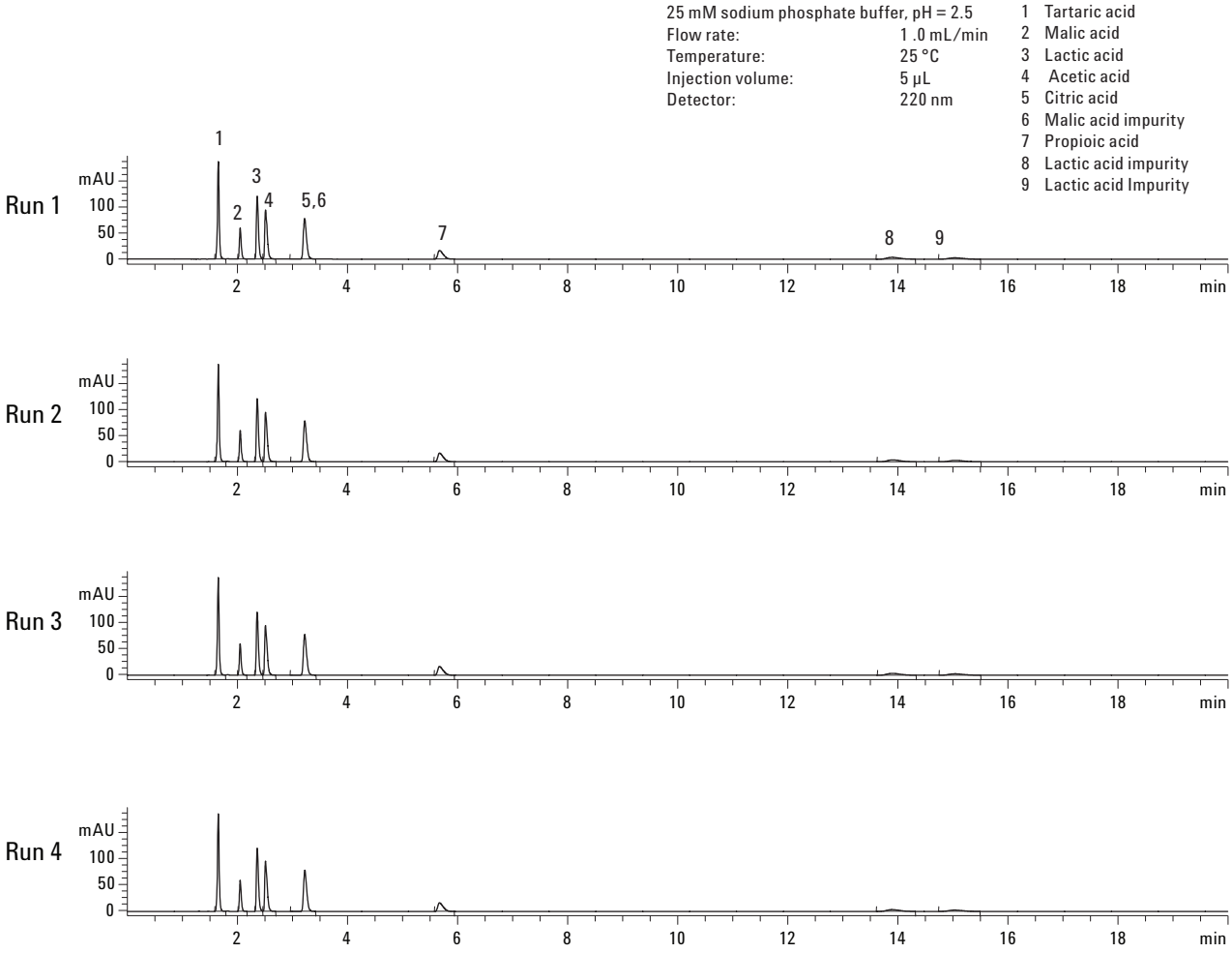


Figure 5. Comparison of mobile phase selectivity using ZORBAX Eclipse Plus Phenyl-Hexyl aliphatic acids 25 mM Na phosphate buffer.

This work has shown separations using 100% aqueous mobile phase. One of the less known features of ZORBAX phenyl columns is their ability to work while using high aqueous mobile phases. In some applications it is necessary to retain and separate polar analytes using a nonpolar C18 stationary phase. This often requires a high aqueous or a buffered-water mobile phase to achieve the desired separation. Many polar analytes are not well retained on a reversed-phase column except when the organic content of the eluent is very low (< 5%).

The bonding on a C18 reversed-phase column gives it a highly hydrophobic surface. Decreasing the organic content of the mobile phase to the levels previously described may lead to a loss in analyte retention over time (or instantaneously, with flow stoppage). This phenomenon is best described as dewetting, retention loss or, more commonly, phase collapse [2]. The onset of dewetting is unpredictable, but stopping the flow of eluent through the column is known to initiate this effect. Figure 6 shows the effect of repeatedly stopping the flow for 30 minutes between injections. The Eclipse Plus Phenyl-Hexyl column surpasses this test with ease. As can be seen in the four stop-flow test cycles, no significant changes are observed in the retention time or shape of peaks. Work reported in reference 2 showed very good performance for StableBond SB-Phenyl using water-soluble vitamins (niacin) as a probe.



1 and 2) Run successively with no pause. 3) Pump stopped 30 minutes and restarted. 4) Pump stopped 30 minutes and restarted ZORBAX Eclipse Plus Phenyl-Hexyl 4.6 mm × 150 mm 3.5 micron, p/n 959961–912.

Figure 6. Resistance to dewetting.

Conclusions

The separation provided on ZORBAX Eclipse Plus Phenyl-Hexyl, Eclipse XDB-Phenyl, and StableBond SB-Phenyl columns can be performed on most commercial HPLC systems. The methods shown here can be easily modified to fit most researchers' needs. They are easily implemented and do not require complicated mobile phase compositions. These phenyl columns show varied selectivity and retention with acids and bases without the added complication of phase collapse.

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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

Authors

Frank David
Research Institute for Chromatography
Pres. Kennedypark 26, B-8500 Kortrijk
Belgium

Matthew S. Klee
Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE 19808-1610
USA

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.

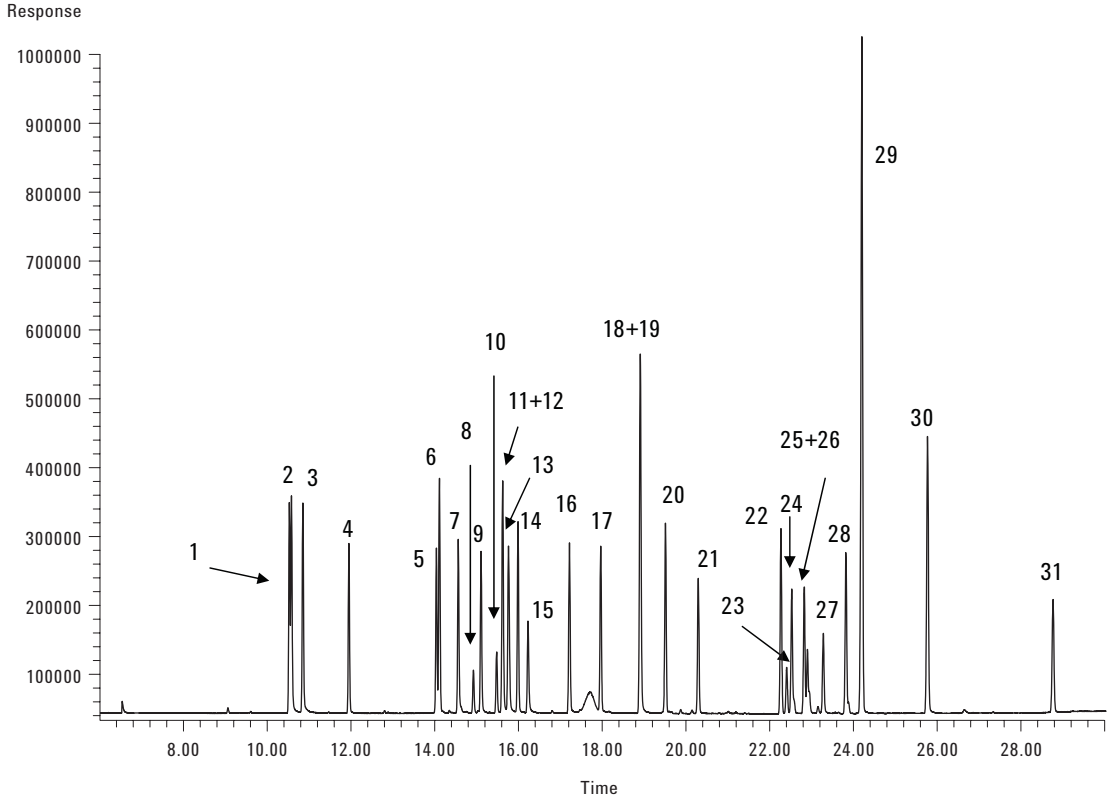

Figure 1. Separation of flavor and fragrance allergen test mixture (100 ppm) on the first dimension column (Agilent J&W HP-5MS) and FID detection. Peak identification is given in Table 1.

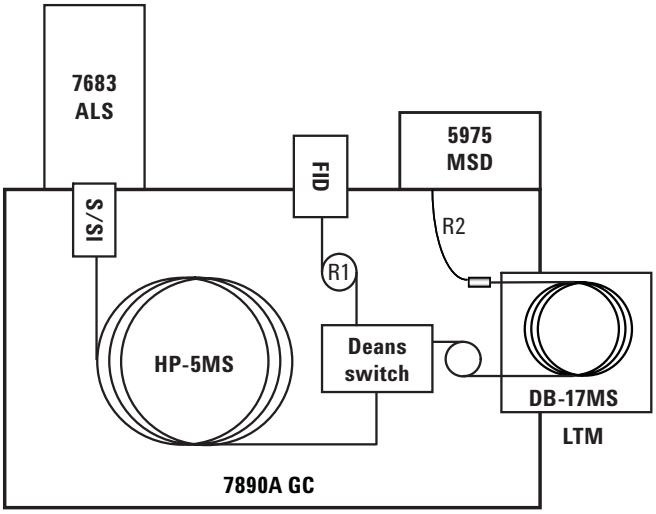

Figure 2. System configuration.

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/SI, 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.

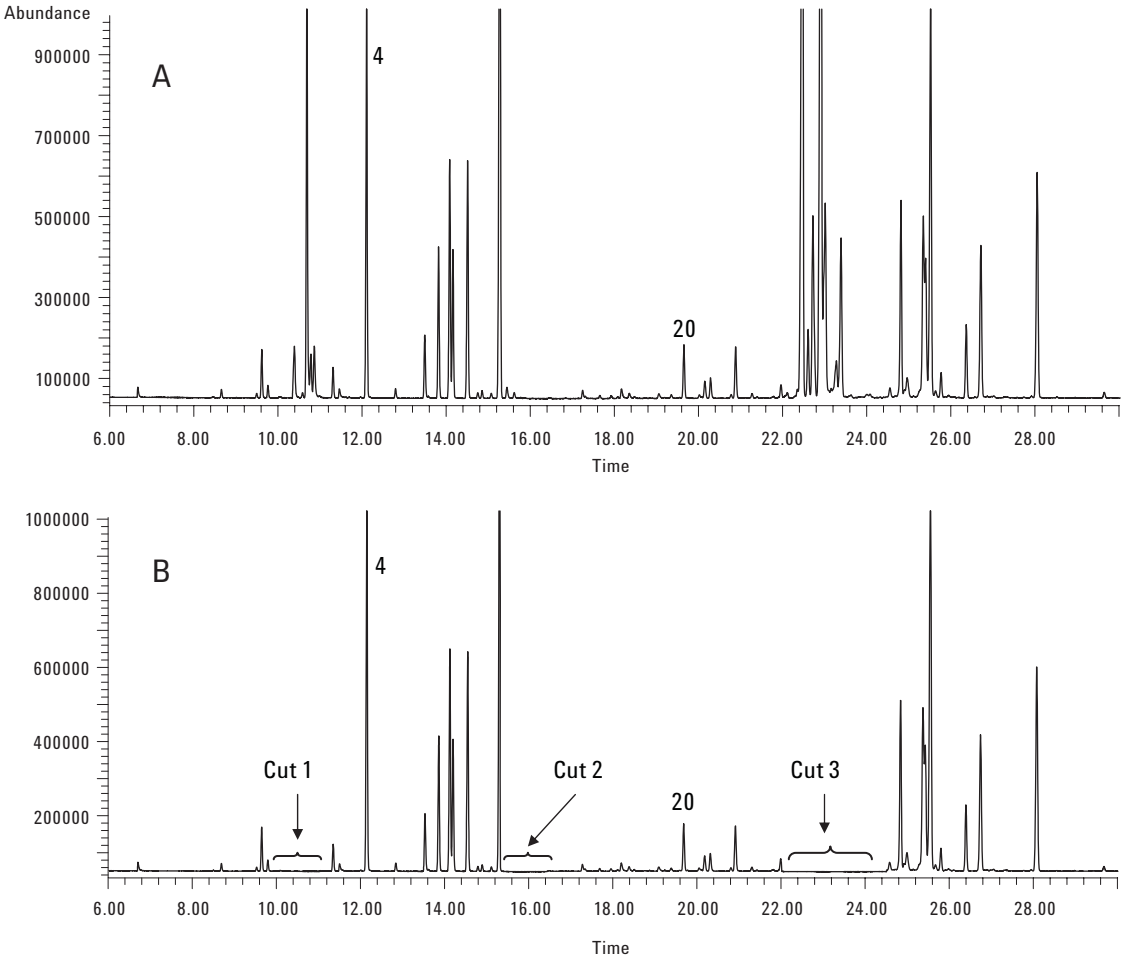

Figure 3. A) Separation of a perfume sample on the first-dimension column (Agilent J&W HP-5MS) using FID detection without heartcutting. Peaks: 4. Linalool; 20. Alpha-isomethyl ionone. B) Separation of a perfume sample on the first-dimension column (Agilent J&W HP-5MS) using FID detection with heartcutting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

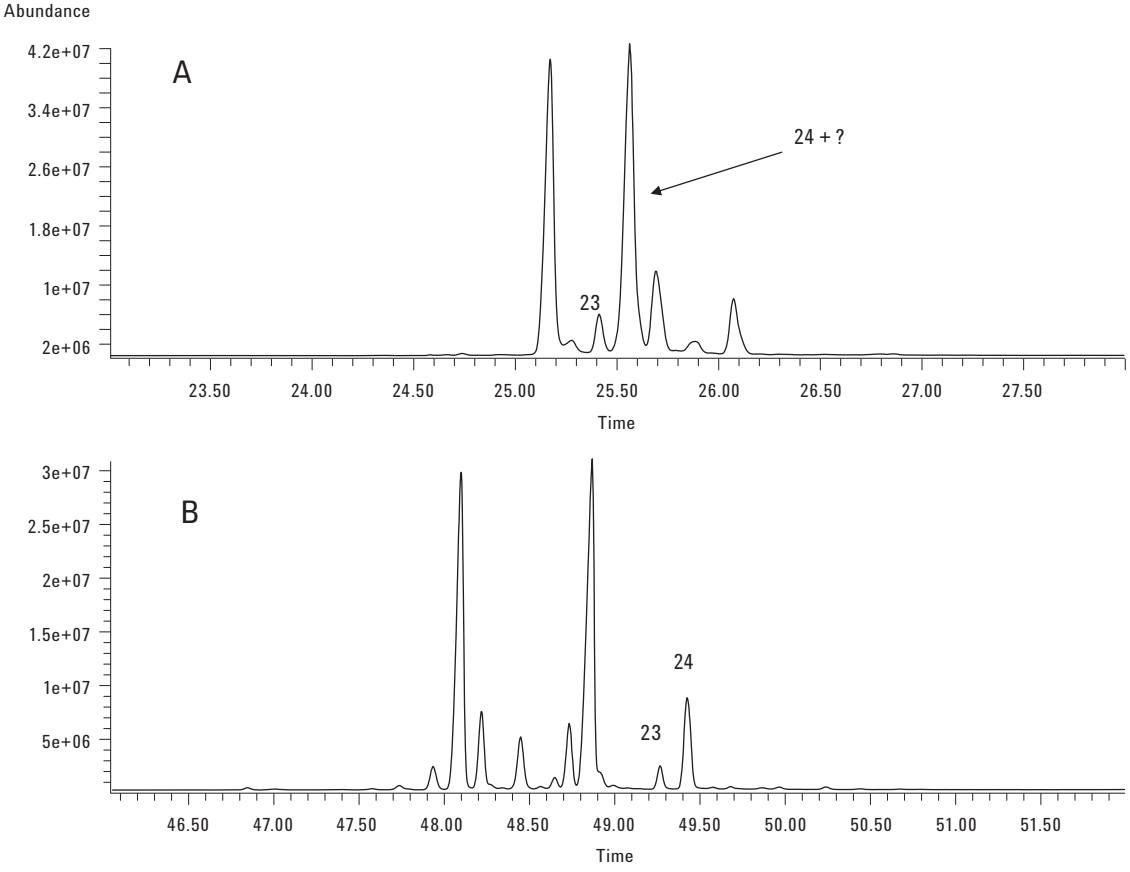

Figure 4. A) Separation of fraction 3 on the second-dimension column (Agilent J&W DB-17ms) using MS detection. Column 1 temperature = column 2 temperature: 50 °C (1 min) → 270 °C at 8 °C/min. Peaks: 23. Lyral 1; 24. Lyral 2. B) Separation of fraction 3 on the second-dimension column (Agilent J&W DB-17ms)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

Application

Food, Flavors and Fragrances

Authors

Mark Sinnott and Simon Jones
Agilent Technologies, Inc.
91 Blue Raving Road
Folsom, CA 95630-4714
USA

Abstract

An analysis of various essential oils/flavors was performed using both polar and nonpolar high-efficiency 0.18-mm id GC columns. Agilent's GC method translation software was used to translate existing 0.25-mm id column methods to 0.18-mm id columns. The ease with which this software can be used allowed for simple method development. Elution times were compared between the standard 0.25-mm id columns and the high-efficiency 0.18-mm id columns. The benefits of using hydrogen carrier gas for shorter analysis time will also be illustrated.

Introduction

There are many misconceptions about what it means to perform fast gas chromatography (GC) and what the term fast GC implies. Fast GC is often associated with the use of hydrogen as a carrier gas, and although this is certainly a good approach, it is not always necessary in order to shorten the analysis time. A second misconception is that changing column dimension results in time-consuming method development. Utilizing 0.18 mm id high-efficiency GC columns can greatly reduce the analysis time. When coupled with Agilent's GC method translation software, the time spent on method development can be greatly minimized.

Efficiency is often related to the number of theoretical plates (n) that a column has and is expressed as plates per meter. It follows that the longer the column, the more plates you have, and thus the more efficient the column. One way to measure column efficiency is to calculate the height equivalent of a theoretical plate (HETP = h). Following Equation 1, the lower the value of h is, the greater the value of n and therefore the efficiency. The shorter the plate is, the larger the number of plates that can be "stacked" in a given length of column. By reducing the column id, the plate height is reduced, which results in more plates per meter (see Equation 2). A more efficient, smaller id column can be used to obtain the same number of plates in a shorter length of column. The shorter the column, the less time the analytes take to travel that length of column, which equates to shorter analysis times without the loss of efficiency or resolution.

$$h = \frac{L}{n}$$

Equation 1: Height Equivalent to a Theoretical Plate

h = height equivalent to a theoretical plate
L = length of the column in millimeters
n = number of theoretical plates

Equation 2: Height Equivalent to a Theoretical Plate in Relation to Column Diameter

$$h_{\min} = r\sqrt{\frac{(11k^2 + 6k + 1)}{3(1+k)^2}}$$

h_{min} = height equivalent to a theoretical plate
r = radius of column
k = capacity factor (partition coefficient) of an analyte

The high-efficiency GC columns are designed to maintain the same phase ratio as the more commonly used 0.25-mm id columns, making for easy method translation, as will be illustrated. Phase ratio is a unitless measure of the relationship between the column radius and the stationary phase thickness. If this calculated number changes when changing from one dimension column to another, there is a change in the retention of a particular solute (k). Equation 3 illustrates that even though a shorter column means a shorter time that the analyte takes to elute from the column, k will remain constant because the unretained compound will also elute more quickly.

Equation 3: Partition Ratio

$$k = \frac{t_r - t_o}{t_o}$$

k = partition coefficient of an analyte
t_r = retention time of analyte
t_o = retention time of an unretained compound

During the chromatographic process, the resulting chromatogram and its associated resolution are the product of the thermodynamics of the system. If the dimensions of the column are changed, then the thermodynamics of the system also change. A new temperature program must be developed to match the new column dimensions. This is why most analysts avoid trying to go faster; the time and energy that goes into developing a new method just isn't worth it. One solution to this problem is to utilize the GC method translation software that is available online at the Agilent Web site http://www.chem.agilent.com/cag/servsup/usersoft/files/GCTS.htm (see Figure 1). This free software takes the guesswork out of developing a new temperature program. This assumes that the same column phase type and same phase ratio are being used between the two methods. It is not imperative to use the same phase ratio; however, if the phase ratio is not maintained, the elution order should be confirmed. An additional option for faster analysis is to utilize a more efficient carrier gas. When changing carrier gas types from one to another, the method translation software takes into account the efficiencies of the four most commonly used carrier gases (argon, nitrogen, helium, and hydrogen) and adjusts the method parameters accordingly.

In this application the benefits of using high-efficiency columns to shorten run times will be illustrated. Two commonly used columns for food/fragrance analysis are the DB-1 and DB-WAX. A comparative analysis will be performed between a more commonly used dimension (30 m × 0.25 mm × 0.25 µm) and that of the high-efficiency column dimension (20 m × 0.18 mm × 0.18 µm). In addition, the ability of the GC method translator to minimize time spent performing method development will be demonstrated. The benefits of using hydrogen as a carrier gas in conjunction with the high-efficiency columns will also be addressed.

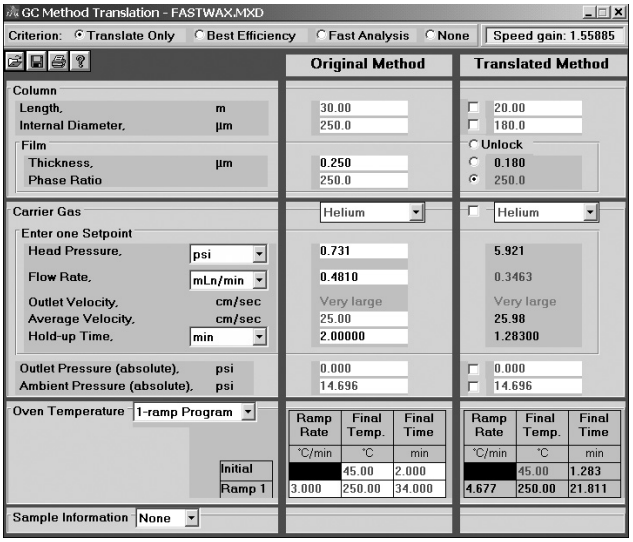

Figure 1. Method translation input screen.

Experimental

All analyses were performed using an Agilent 6890 GC with a 5973 MSD, equipped with a split/splitless inlet. The analytical conditions are summarized in Table 1 (DB-1 columns) and Table 2 (DB-WAX columns). Original method parameters were not optimized for each compound, but rather developed to accommodate a wide range of essential oils and fragrances. Method parameters used for the high-efficiency columns were translated directly from the Agilent method translation software. Spearmint and ylang-ylang samples were prepared by dilution of neat oils with acetone at roughly 40:1.

Table 1. Method Conditions for DB-1 Columns

Method A	
Column	30 m × 0.25 mm × 0.25 µm DB-1 p/n 122-1032
Carrier	Helium 25 cm/sec measured at 40 °C
Injector	250 °C, Split 40:1, 1-µL injection
Oven	40 °C hold 1 min
	5 °C/min to 290 °C
Method B	
Column	20 m × 0.18 mm × 0.18 µm DB-1 p/n 121-1022
Carrier	Helium 26 cm/sec measured at 40 °C
Oven	40 °C hold 0.64 min
	4.67 °C/min to 290 °C
Method C	
Column	20 m × 0.18 mm × 0.18 µm DB-1 p/n 121-1022
Carrier	Hydrogen 47 cm/sec measured at 40 °C
Oven	40 °C hold 0.38 min
	13 °C/min to 290 °C hold 13.09 min

Table 2. Method Conditions for DB-WAX columns

Method A	
Column	30 m × 0.25 mm × 0.25 µm DB-WAX p/n 122-7032
Carrier	Helium 25.4 cm/sec measured at 45 °C
Injector	250 °C, Split 30:1, 1-µL injection
Oven	45 °C hold 2 min
	3 °C/min to 250 °C hold 34 min
Method B	
Column	20 m × 0.18 mm × 0.18 µm DB-WAX p/n 121-7022
Carrier	Helium 26.3 cm/sec measured at 45 °C
Oven	45 °C hold 1.28 min
	4.68 °C/min to 250 °C hold 21.81 min
Method C	
Column	20 m × 0.18 mm × 0.18 µm DB-WAX p/n 121-7022
Carrier	Hydrogen 44.3 cm/sec measured at 45 °C
Oven	45 °C hold 0.77 min
	7.79 °C/min to 250 °C hold 13.09 min

Results and Discussion

Typical chromatograms are presented here for all three GC methods on both the DB-1 and DB-WAX columns. Peak identities can be found in Tables 3A and 3B. Significant speed gain is achieved by simply switching to the high-efficiency column and continuing to use helium as the carrier, without a loss of resolution. Spearmint was tested on the DB-1 column. The elution time for the last-eluting compound for spearmint is viridiflorol, which decreased from 27.41 minutes to 17.73 minutes, as illustrated in Figures 1 and 2. This represents a speed gain of approximately 35%. Resolution between three close-eluting compounds remained nearly identical as is illustrated in Table 4. Elution time of the last compound for ylang-ylang oil tested on the DB-WAX column, benzyl salicylate, decreased from 63.47 to 41.07 minutes. This is illustrated in Figures 3 and 4. This represents a speed gain of approximately 35%. Resolution between two pairs of compounds remained essentially unchanged (see Table 5).

Using hydrogen as a carrier gas in conjunction with the high-efficiency columns resulted in additional speed gains. Due to its small molecular size, hydrogen can be used at higher velocities without loss of efficiency. These additional benefits are illustrated in Figures 5 and 6 as well as in Tables 4 and 5. The overall speed gain from the original method was found to be 61% for both the DB-1 and DB-WAX methods. The method translation software allowed for essentially plug-and-play method development. The results that were obtained were used without modification to the values provided by the translation software.

Table 3A. Component List for DB-1 Chromatograms

	Compound List for Spearmint Oil Chromatogram				
1	α-Pinene	12	γ-Terpinene	23	cis-Carvyl acetate
2	Sabinene	13	trans-Sabinene hydrate	24	cis-Jasmone
3	ß-Pinene	14	Terpinolene	25	ß-Bourbonene
4	3-Octanol	15	Linalool	26	α-Bourbonene
5	Myrcene	16	3-Octyl acetate	27	ß-Caryophylene
6	α-Terpinene	17	Isomenthone	28	α-Copaene
7	ρ-Cymene	18	Terpinen-4-ol	29	trans-ß-Farnesene
8	1,8-Cineol	19	Dihydrocarvone	30	Germacrene-d
9	Limonene	20	trans-Carveol	31	Viridiflorol
10	cis-Ocimene	21	l-Carvone		
11	trans-Ocimene	22	trans-Dihydrocarveol acetate		

Table 3B. Component List for DB-WAX Chromatograms

	Compound List for Ylang-Ylang Oil Chromatogram				
1	α-Pinene	7	Methyl benzoate	13	Geranial acetate
2	Methyl-ρ-cresol	8	α-Caryophyllene	14	trans-Cinnamyl acetate
3	α-Copaene	9	Germacrene-d	15	ß-Bisbolene
4	α-Gurjunene	10	Benzyl acetate	16	Farnesyl acetate
5	Linalool	11	Farnescene	17	Benzyl benzoate
6	ß-Caryophyllene	12	δ-Cadinene	18	Benzyl salycilate

Table 4. Resolution of Closely Eluting Compounds by Column ID and Carrier Gas

	DB-1 ID and Carrier Gas Type		
Compound(s)	0.25 mm Helium	0.18 mm Helium	0.18 mm Hydrogen
Sabinene β-Pinene	1.52	1.59	1.56
α-Terpinene p-Cymene	1.61	1.73	1.86
Speed gain	N/A	35%	61%

Table 5. Resolution of Closely Eluting Compounds by Column ID and Carrier Gas

	DB-WAX ID and Carrier Gas Type		
Compound(s)	0.25 mm Helium	0.18 mm Helium	0.18 mm Hydrogen
α-Farnesene δ-Cadinene	2.16	2.14	2.13
δ-Cadinene Geranial acetate	1.67	1.66	1.64
Speed gain	N/A	35%	61%

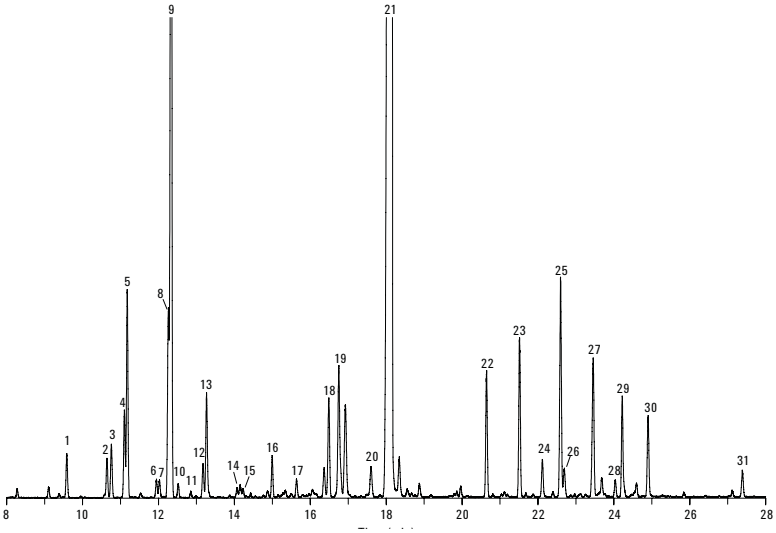

Figure 1. Spearmint oil sample on a DB-1, 30 m x 0.25 mm x 0.25 µm column and He carrier. (See Table 1, Method A, for experimental parameters.)

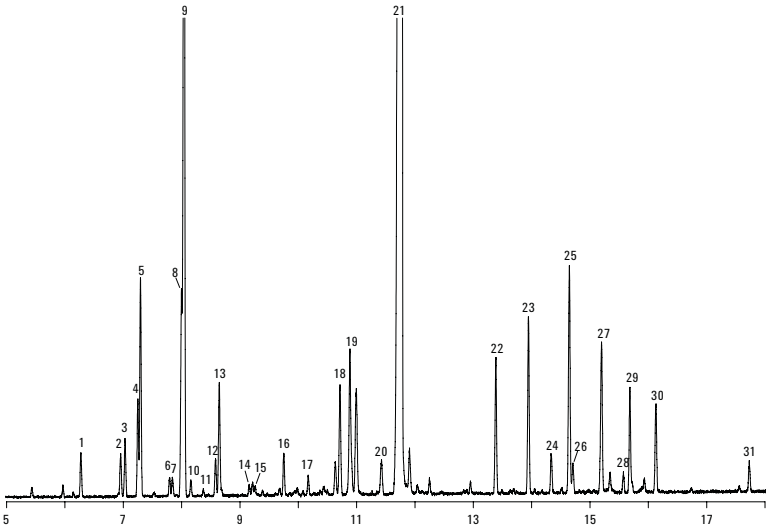

Figure 2. Spearmint oil sample on DB-1, 20 m x 0.18 mm x 0.18 µm column, He carrier. (See Table 1, Method B, for experimental parameters.)

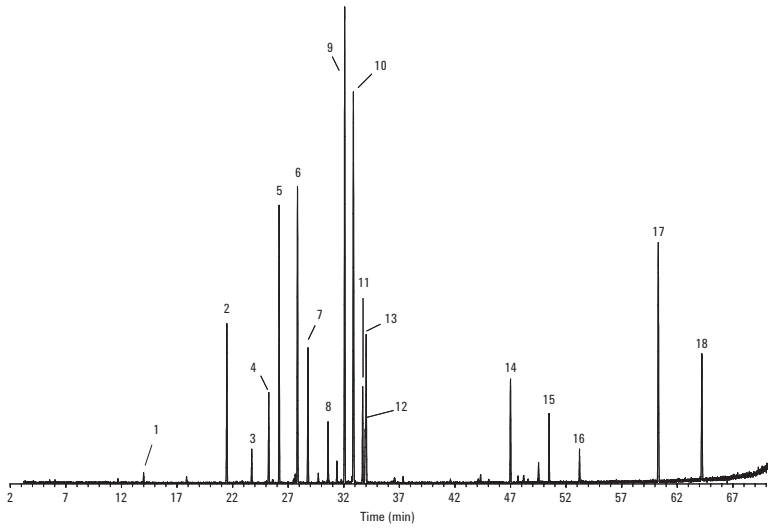

Figure 3. Ylang-ylang oil sample on a DB-WAX, 30 m x 0.25 mm x 0.25 µm column and He carrier. (See Table 2, Method A, for experimental parameters.)

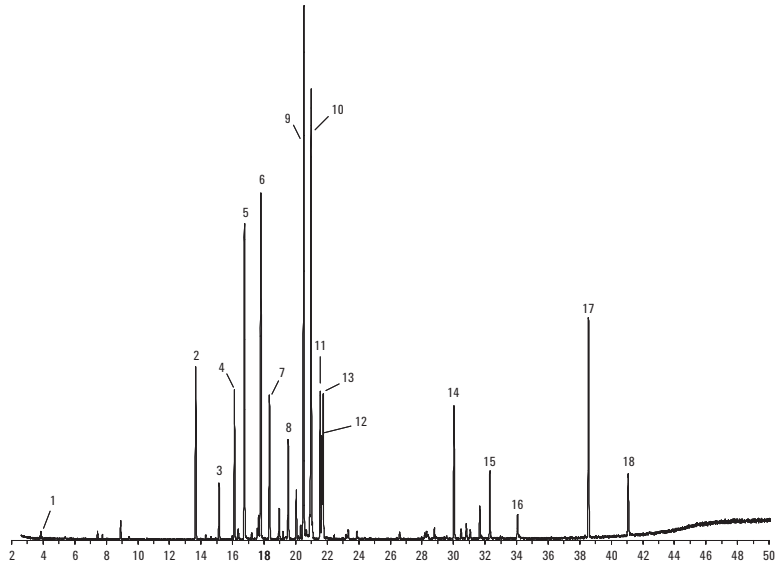

Figure 4. Ylang-ylang oil on DB-WAX, 20 m x 0.18 mm x 0.18 µm column, He carrier. (See Table 2, Method B, for experimental parameters.)

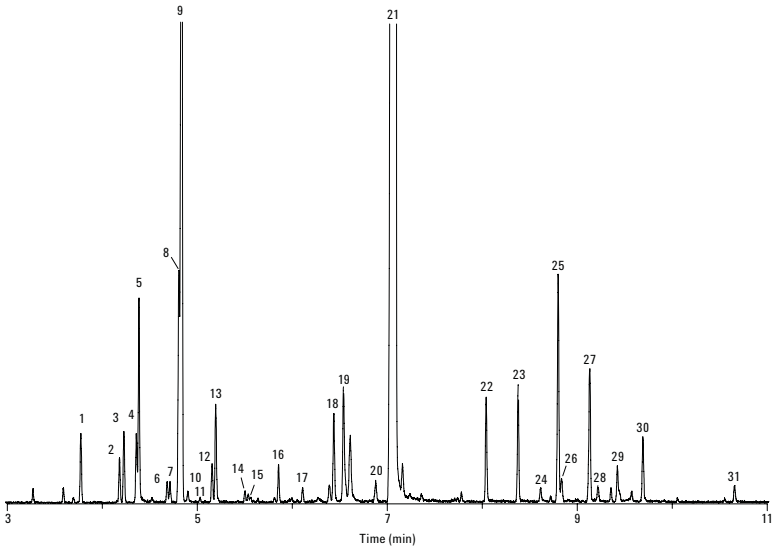

Figure 5. Spearmint oil sample on DB-1, 20 m x 0.18 mm x 0.18 µm with H_2 carrier. (See Table 1, Method C, for method parameters.)

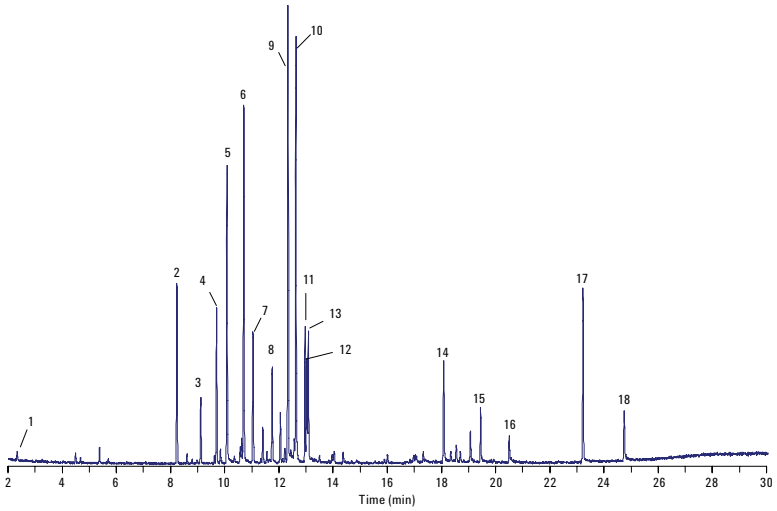

Figure 6. Ylang-ylang oil sample on a DB-WAX, 20 m x 0.18 mm x 0.18 µm column with H_2 carrier. (See Table 2, Method C, for method parameters.)

Conclusions

The use of high-efficiency columns has many benefits, as illustrated here. Shorter analysis times were achieved without significant loss of resolution. Time spent in method development was kept to a minimum through the use of the GC method translation software and the fact that the high-efficiency columns were phase ratio matched. While there are additional benefits for using hydrogen as the carrier gas, significant speed gain can be realized by simply using the high-efficiency columns while maintaining helium as the carrier.

*Although this application only depicts two oils, several additional flavors/fragrances were analyzed. Please contact Agilent Technologies Application Support for additional information.

Reference

D. Rood, *The Practical Guide to the Care, Maintenance and Troubleshooting of Capillary Gas Chromatographic Systems*, Huthig, Heidelberg, 1991

For More Information

For more information on our products and services, visit our Web site at www.agilent.com/chem.

For more information about the high-efficiency GC columns, visit our Web site at http://www.chem.agilent.com/scripts/PDS.asp?1Page=60005.

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Agilent Technologies

Comprehensive GC System Based on Flow Modulation for the 7890A GC

Application Brief

Introduction

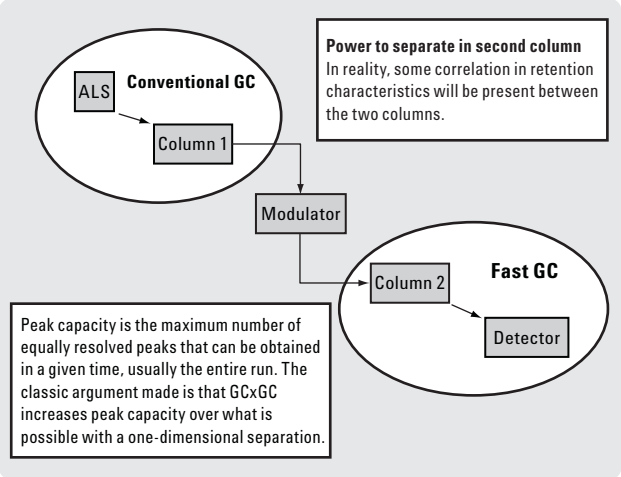
A hardware solution is available on the 7890A for the practice of comprehensive GC. The system uses a capillary flow modulator controlled by the 7890A GC. The system is offered with factory checkout using an FID detector. Other detectors, preferably those operating at 50 Hz or greater, can be used.

Comprehensive two-dimensional (2D) GC, or GCxGC, is a powerful technique that can be used to separate very complex mixtures, such as those found in the hydrocarbon processing, environmental, and food/fragrance industries.

The method uses two columns, typically of very different polarities, installed in series with a modulator in between. The second column is much shorter than the first column to effect a fast separation. The entire assembly is located inside the GC oven.

The modulator performs three functions:

1. It collects effluent from the first column for a fraction of the time equal to peak width. For example, if a peak from column one is six seconds wide, the modulator will accumulate material every two to three seconds, thereby dividing the peak from the first column into two or three "cuts."
2. It focuses the material collected from each cut into a very narrow band through flow compression.
3. It introduces the bands sequentially onto the second column, resulting in additional separation for each band injected onto the second column.



Comprehensive 2D GC uses a primary column (conventional separation), a flow modulator, a second column (very fast separation), and a fast detector.

This technique provides a second dimension of information that can increase the peak resolution and capacity.

A number of different modulator designs have been described in the literature, most relying on thermal cycling to focus the bands from the first column and release them into the second column. Some disadvantages to this approach are:

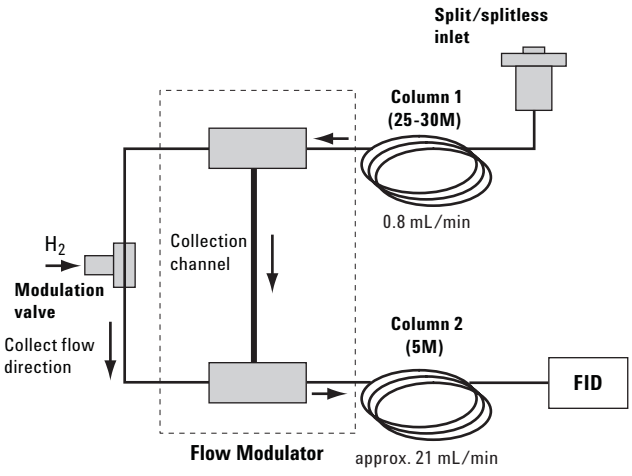
- Large usage of expensive cryogenic gases leading to a high cost of analysis
- Complexity of the hardware
- Longer analysis times

Agilent's proprietary Capillary Flow Technology and fourth-generation Electronic Pneumatics Control (EPC) enable the use of a differential flow modulator to conduct comprehensive 2D-GC without the use of cryogenic gases or complex hardware.

The key to operation is the flow differential (typically 20 to 1) between the second and first columns, respectively. This compresses and focuses the analytes present in any given modulation "inject" pulse into the second column. Precise timing of the modulator is made possible by installing a driver board in the Aux det 2 detector slot of the 7890A mainframe.

The Capillary Flow Technology modulator uses a deactivated, stainless steel structure with all flow splitters and the collector channel incorporated internally in the device. It has low thermal mass so it can track the oven temperature very closely, and its GC oven location allows precise temperature control without lag during programmed runs. All external connections are made using Agilent's Ultimate Union technology for leak-free operation and extremely small, well-swept volumes. A micro three-way solenoid valve, installed on the side of the gas chromatograph, connects to a pneumatics control module (PCM) to accurately and precisely control the flows through the modulator.

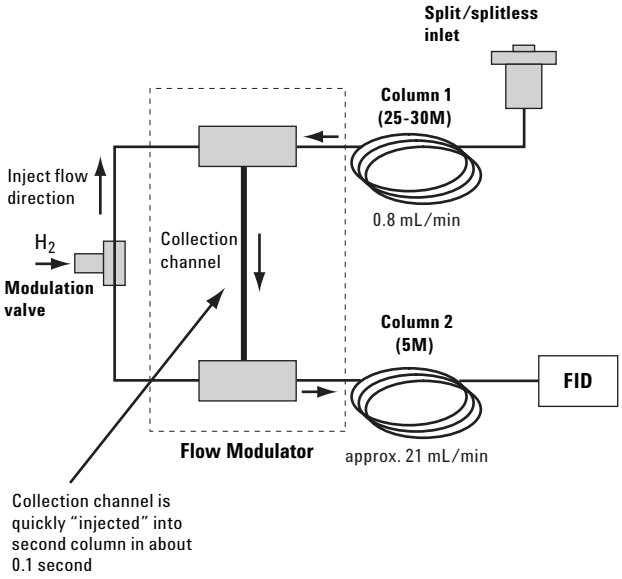
The figures below illustrate the modulator. A three-way solenoid valve receives a controlled supply of hydrogen gas from a PCM. The periodic switching of this three-way valve drives the modulator. The precisely timed and synchronized switching between the collect and inject states directs discrete sample pulses continuously to the second column for additional fast separation throughout the chromatographic run. Both columns are run in constant flow mode. For optimal performance, injection size and split ratio should be carefully adjusted to avoid overloading, which can lead to excessive peak tailing.



Flow rates and flow directions during the load or collect portion of the modulation cycle

Load or collect state (above): At the beginning of this state, the collection channel is filled with hydrogen gas from a previous injection cycle flush.

The primary column effluent enters the modulator's top tee connection and flows into the collection channel. The analytes from this column enter one end of the collection channel. Hydrogen flow from the PCM/three-way micro valve exits the modulator at the bottom tee and is sent to the second column.



Flow rates and flow directions during the transfer or inject portion of the modulation cycle

Inject or flush state (above): Hydrogen gas flow from the three-way solenoid valve is directed to the top tee. A high flow of typically 20 mL/min for about 0.1 second rapidly flushes the collection channel, transferring material in a very narrow band onto the second column where any analytes collected in the channel undergo rapid separation.

What is required:

- Agilent 7890A GC with firmware version A.04.06 or higher
- FID with 200 Hz data collection rate or other fast detector
- Split/splitless inlet
- Capillary Flow Technology modulator option or accessory
- Capillary Flow Technology modulator checkout kit
- Pneumatics control module (PCM)
- Agilent GC ChemStation B.03.02 or other data collection and analysis system that can control the flow modulator cycle
- 30-m × 0.25-mm × 0.25-µm DB-5ms column (included with option or accessory)
- 5-m × 0.25-mm × 0.15-µm INNOWax column (included with option or accessory)
- 2D data analysis software, GC Image recommended (not provided by Agilent)
- Internal column nuts and SilTite ferrules

Ordering Information

Description	Part number
7890A GC with Capillary Flow Technology Modulator (requires checkout kit)	G3440A Option 887 or accessory G3486A
7890A GC with 200 Hz FID	G3440A Option 211 or accessory G3462A
7890A GC with split/splitless inlet	G3440A Option 112 or accessory G3452A
Capillary Flow Technology modulator checkout kit	G3487A
PCM for 7890A GC	G3440A Option 309 or accessory G3471A
SilTite metal ferrules, 1/16-in × 0.4-mm id, 10/pk, includes 2 column nuts	5184-3569
Agilent 32-bit ChemStation for 1 GC	G2070BA
Agilent 32-bit ChemStation Bundle for 1 GC includes: – G2070BA 32-bit ChemStation software – Computer with monitor and Windows operating system – Printer	G1875BA
2D GC software Recommend GC Image software, which can be purchased from Zoex Corporation	www.zoex.com

Application Examples

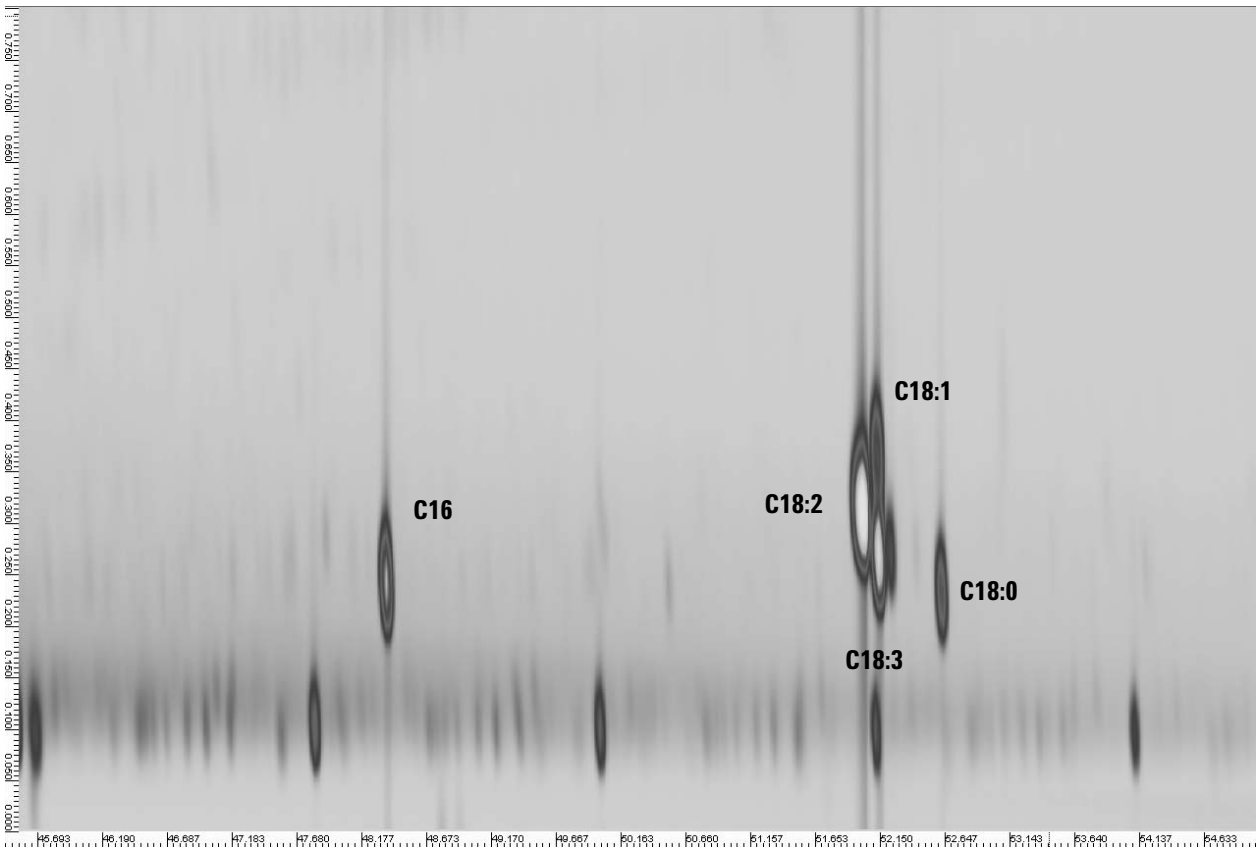
Several applications are shown. Note that primary column lengths have been chosen to give optimal results. While the 30M column that is shipped with the system is an excellent choice for a wide range of applications, other lengths can be used to optimize a given separation. Various columns have been used in these examples to illustrate some of the possibilities. The GC Image software package was used for processing the ChemStation data.

1. B20 biodiesel based on soy FAMES. Section of the 2D image showing the C16 and C18 FAMES is shown.

   Column 1: 60 m × 0.25 mm × 0.10 µm DB-5ms

   Column 2: 5 m × 0.25 mm × 0.15 µm INNOWax

   Modulation: 1.40 s load, 0.10 s inject

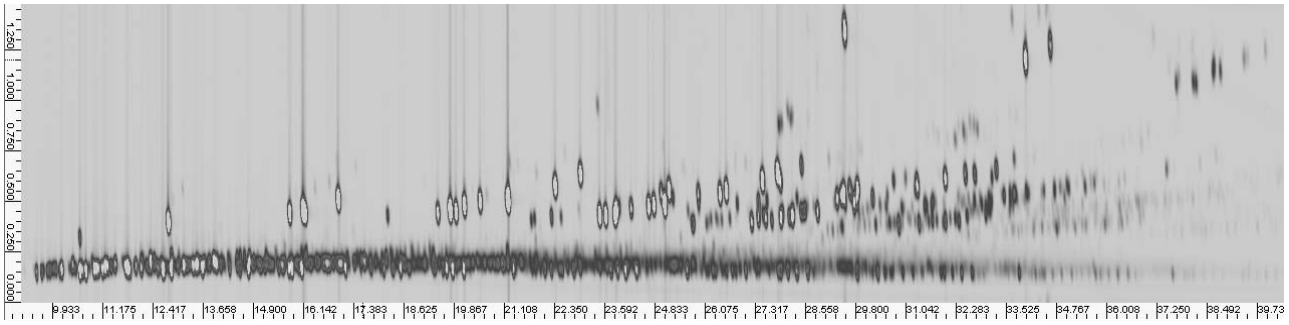


2. Complete 2D image of a sample of heavy gasoline. Each series of substituted 1-ring aromatics is well separated, making hydrocarbon class grouping possible.

   Column 1: 60 m × 0.25 mm × 0.10 µm DB-5ms

   Column 2: 5 m × 0.25 mm × 0.15 µm INNOWax

   Modulation: 1.40 s load, 0.10 s inject

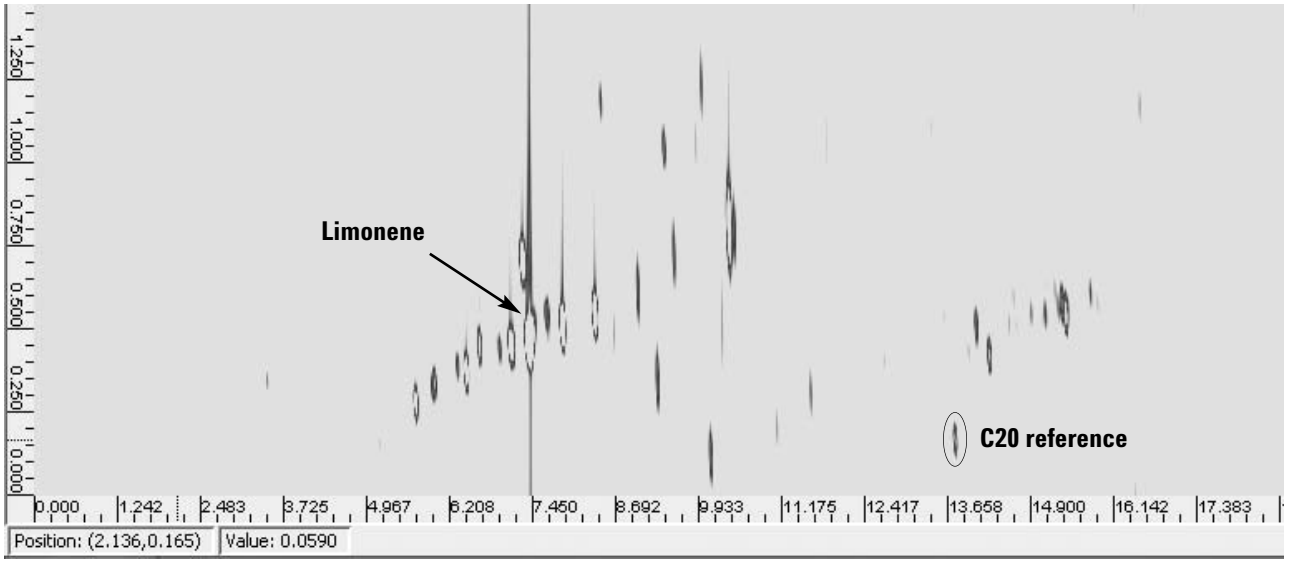


3. Lime oil 2D image.

   Column 1: 15 m × 0.25 m × 0.25 µm DB-5ms

   Column 2: 5 m × 0.25 mm × 0.15 µm DB-17HT

   Modulation: 1.40 s load, 0.10 s inject



Thermal vs. Flow Modulation

Since competitors offer only systems based on thermal modulation, the following table summarizes the key points about the respective approaches of thermal vs. flow modulation.

Thermal modulation	Differential Flow modulation
Cryo-focusing provides potentially narrower peaks in second dimension	Peak widths comparable to thermal. Usually no more than 20% wider. Many users want to sum regions of peaks where peak width is not as critical
Lower flows – Can be used with high-vacuum detectors (TOF)	MSD can be used with a splitter over limited scan range
Large consumption of cryogen	No cryogen required
Complex hardware design, set-up, and maintenance	Simple, reliable Capillary Flow Technology based hardware; small thermal foot print
Long chromatographic runs required for best performance	Run times comparable to a 1D separation
System price (estimate) $60 to $70K	Agilent system approximately $60K (list)

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Agilent Technologies

Rapid Analysis of Food and Fragrances Using High-Efficiency Capillary GC Columns

Application

Food, Flavors and Fragrances

Authors

Mark Sinnott and Simon Jones
Agilent Technologies, Inc.
91 Blue Raving Road
Folsom, CA 95630-4714
USA

Abstract

An analysis of various essential oils/flavors was performed using both polar and nonpolar high-efficiency 0.18-mm id GC columns. Agilent's GC method translation software was used to translate existing 0.25-mm id column methods to 0.18-mm id columns. The ease with which this software can be used allowed for simple method development. Elution times were compared between the standard 0.25-mm id columns and the high-efficiency 0.18-mm id columns. The benefits of using hydrogen carrier gas for shorter analysis time will also be illustrated.

Introduction

There are many misconceptions about what it means to perform fast gas chromatography (GC) and what the term fast GC implies. Fast GC is often associated with the use of hydrogen as a carrier gas, and although this is certainly a good approach, it is not always necessary in order to shorten the analysis time. A second misconception is that changing column dimension results in time-consuming method development. Utilizing 0.18 mm id high-efficiency GC columns can greatly reduce the analysis time. When coupled with Agilent's GC method translation software, the time spent on method development can be greatly minimized.

Efficiency is often related to the number of theoretical plates (n) that a column has and is expressed as plates per meter. It follows that the longer the column, the more plates you have, and thus the more efficient the column. One way to measure column efficiency is to calculate the height equivalent of a theoretical plate (HETP = h). Following Equation 1, the lower the value of h is, the greater the value of n and therefore the efficiency. The shorter the plate is, the larger the number of plates that can be "stacked" in a given length of column. By reducing the column id, the plate height is reduced, which results in more plates per meter (see Equation 2). A more efficient, smaller id column can be used to obtain the same number of plates in a shorter length of column. The shorter the column, the less time the analytes take to travel that length of column, which equates to shorter analysis times without the loss of efficiency or resolution.

$$h = \frac{L}{n}$$

Equation 1: Height Equivalent to a Theoretical Plate

h = height equivalent to a theoretical plate
L = length of the column in millimeters
n = number of theoretical plates

Equation 2: Height Equivalent to a Theoretical Plate in Relation to Column Diameter

$$h_{\min} = r\sqrt{\frac{(11k^2 + 6k + 1)}{3(1+k)^2}}$$

h_{min} = height equivalent to a theoretical plate
r = radius of column
k = capacity factor (partition coefficient) of an analyte

The high-efficiency GC columns are designed to maintain the same phase ratio as the more commonly used 0.25-mm id columns, making for easy method translation, as will be illustrated. Phase ratio is a unitless measure of the relationship between the column radius and the stationary phase thickness. If this calculated number changes when changing from one dimension column to another, there is a change in the retention of a particular solute (k). Equation 3 illustrates that even though a shorter column means a shorter time that the analyte takes to elute from the column, k will remain constant because the unretained compound will also elute more quickly.

Equation 3: Partition Ratio

$$k = \frac{t_r - t_o}{t_o}$$

k = partition coefficient of an analyte
t_r = retention time of analyte
t_o = retention time of an unretained compound

During the chromatographic process, the resulting chromatogram and its associated resolution are the product of the thermodynamics of the system. If the dimensions of the column are changed, then the thermodynamics of the system also change. A new temperature program must be developed to match the new column dimensions. This is why most analysts avoid trying to go faster; the time and energy that goes into developing a new method just isn't worth it. One solution to this problem is to utilize the GC method translation software that is available online at the Agilent Web site http://www.chem.agilent.com/cag/servsup/usersoft/files/GCTS.htm (see Figure 1). This free software takes the guesswork out of developing a new temperature program. This assumes that the same column phase type and same phase ratio are being used between the two methods. It is not imperative to use the same phase ratio; however, if the phase ratio is not maintained, the elution order should be confirmed. An additional option for faster analysis is to utilize a more efficient carrier gas. When changing carrier gas types from one to another, the method translation software takes into account the efficiencies of the four most commonly used carrier gases (argon, nitrogen, helium, and hydrogen) and adjusts the method parameters accordingly.

In this application the benefits of using high-efficiency columns to shorten run times will be illustrated. Two commonly used columns for food/fragrance analysis are the DB-1 and DB-WAX. A comparative analysis will be performed between a more commonly used dimension (30 m × 0.25 mm × 0.25 µm) and that of the high-efficiency column dimension (20 m × 0.18 mm × 0.18 µm). In addition, the ability of the GC method translator to minimize time spent performing method development will be demonstrated. The benefits of using hydrogen as a carrier gas in conjunction with the high-efficiency columns will also be addressed.

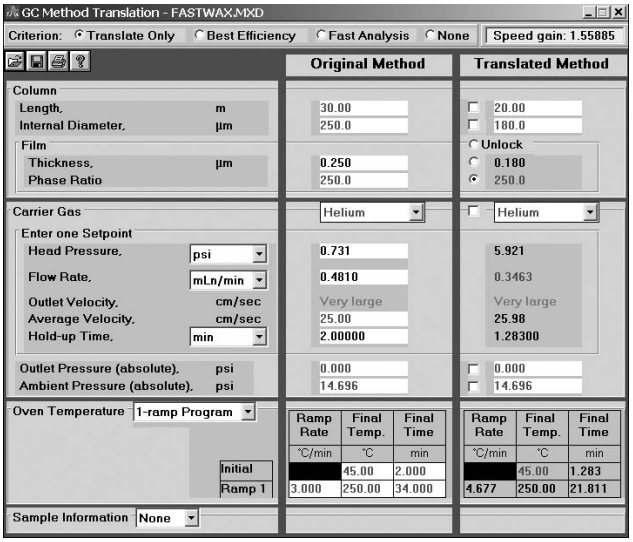

Figure 1. Method translation input screen.

Experimental

All analyses were performed using an Agilent 6890 GC with a 5973 MSD, equipped with a split/splitless inlet. The analytical conditions are summarized in Table 1 (DB-1 columns) and Table 2 (DB-WAX columns). Original method parameters were not optimized for each compound, but rather developed to accommodate a wide range of essential oils and fragrances. Method parameters used for the high-efficiency columns were translated directly from the Agilent method translation software. Spearmint and ylang-ylang samples were prepared by dilution of neat oils with acetone at roughly 40:1.

Table 1. Method Conditions for DB-1 Columns

Method A	
Column	30 m × 0.25 mm × 0.25 µm DB-1 p/n 122-1032
Carrier	Helium 25 cm/sec measured at 40 °C
Injector	250 °C, Split 40:1, 1-µL injection
Oven	40 °C hold 1 min
	5 °C/min to 290 °C
Method B	
Column	20 m × 0.18 mm × 0.18 µm DB-1 p/n 121-1022
Carrier	Helium 26 cm/sec measured at 40 °C
Oven	40 °C hold 0.64 min
	4.67 °C/min to 290 °C
Method C	
Column	20 m × 0.18 mm × 0.18 µm DB-1 p/n 121-1022
Carrier	Hydrogen 47 cm/sec measured at 40 °C
Oven	40 °C hold 0.38 min
	13 °C/min to 290 °C hold 13.09 min

Table 2. Method Conditions for DB-WAX columns

Method A	
Column	30 m × 0.25 mm × 0.25 µm DB-WAX p/n 122-7032
Carrier	Helium 25.4 cm/sec measured at 45 °C
Injector	250 °C, Split 30:1, 1-µL injection
Oven	45 °C hold 2 min
	3 °C/min to 250 °C hold 34 min
Method B	
Column	20 m × 0.18 mm × 0.18 µm DB-WAX p/n 121-7022
Carrier	Helium 26.3 cm/sec measured at 45 °C
Oven	45 °C hold 1.28 min
	4.68 °C/min to 250 °C hold 21.81 min
Method C	
Column	20 m × 0.18 mm × 0.18 µm DB-WAX p/n 121-7022
Carrier	Hydrogen 44.3 cm/sec measured at 45 °C
Oven	45 °C hold 0.77 min
	7.79 °C/min to 250 °C hold 13.09 min

Results and Discussion

Typical chromatograms are presented here for all three GC methods on both the DB-1 and DB-WAX columns. Peak identities can be found in Tables 3A and 3B. Significant speed gain is achieved by simply switching to the high-efficiency column and continuing to use helium as the carrier, without a loss of resolution. Spearmint was tested on the DB-1 column. The elution time for the last-eluting compound for spearmint is viridiflorol, which decreased from 27.41 minutes to 17.73 minutes, as illustrated in Figures 1 and 2. This represents a speed gain of approximately 35%. Resolution between three close-eluting compounds remained nearly identical as is illustrated in Table 4. Elution time of the last compound for ylang-ylang oil tested on the DB-WAX column, benzyl salicylate, decreased from 63.47 to 41.07 minutes. This is illustrated in Figures 3 and 4. This represents a speed gain of approximately 35%. Resolution between two pairs of compounds remained essentially unchanged (see Table 5).

Using hydrogen as a carrier gas in conjunction with the high-efficiency columns resulted in additional speed gains. Due to its small molecular size, hydrogen can be used at higher velocities without loss of efficiency. These additional benefits are illustrated in Figures 5 and 6 as well as in Tables 4 and 5. The overall speed gain from the original method was found to be 61% for both the DB-1 and DB-WAX methods. The method translation software allowed for essentially plug-and-play method development. The results that were obtained were used without modification to the values provided by the translation software.

Table 3A. Component List for DB-1 Chromatograms

	Compound List for Spearmint Oil Chromatogram				
1	α-Pinene	12	γ-Terpinene	23	cis-Carvyl acetate
2	Sabinene	13	trans-Sabinene hydrate	24	cis-Jasmone
3	ß-Pinene	14	Terpinolene	25	ß-Bourbonene
4	3-Octanol	15	Linalool	26	α-Bourbonene
5	Myrcene	16	3-Octyl acetate	27	ß-Caryophylene
6	α-Terpinene	17	Isomenthone	28	α-Copaene
7	ρ-Cymene	18	Terpinen-4-ol	29	trans-ß-Farnesene
8	1,8-Cineol	19	Dihydrocarvone	30	Germacrene-d
9	Limonene	20	trans-Carveol	31	Viridiflorol
10	cis-Ocimene	21	l-Carvone		
11	trans-Ocimene	22	trans-Dihydrocarveol acetate		

Table 3B. Component List for DB-WAX Chromatograms

	Compound List for Ylang-Ylang Oil Chromatogram				
1	α-Pinene	7	Methyl benzoate	13	Geranial acetate
2	Methyl-ρ-cresol	8	α-Caryophyllene	14	trans-Cinnamyl acetate
3	α-Copaene	9	Germacrene-d	15	ß-Bisbolene
4	α-Gurjunene	10	Benzyl acetate	16	Farnesyl acetate
5	Linalool	11	Farnescene	17	Benzyl benzoate
6	ß-Caryophyllene	12	δ-Cadinene	18	Benzyl salycilate

Table 4. Resolution of Closely Eluting Compounds by Column ID and Carrier Gas

	DB-1 ID and Carrier Gas Type		
Compound(s)	0.25 mm Helium	0.18 mm Helium	0.18 mm Hydrogen
Sabinene β-Pinene	1.52	1.59	1.56
α-Terpinene p-Cymene	1.61	1.73	1.86
Speed gain	N/A	35%	61%

Table 5. Resolution of Closely Eluting Compounds by Column ID and Carrier Gas

	DB-WAX ID and Carrier Gas Type		
Compound(s)	0.25 mm Helium	0.18 mm Helium	0.18 mm Hydrogen
α-Farnesene δ-Cadinene	2.16	2.14	2.13
δ-Cadinene Geranial acetate	1.67	1.66	1.64
Speed gain	N/A	35%	61%

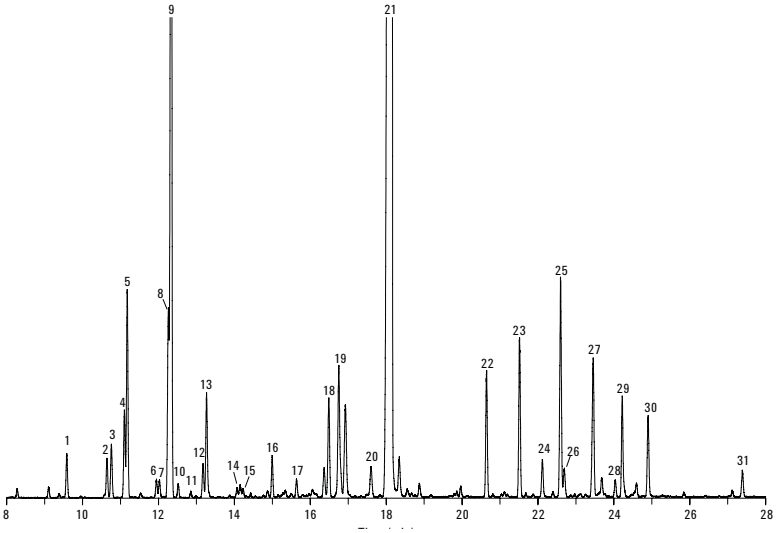

Figure 1. Spearmint oil sample on a DB-1, 30 m x 0.25 mm x 0.25 µm column and He carrier. (See Table 1, Method A, for experimental parameters.)

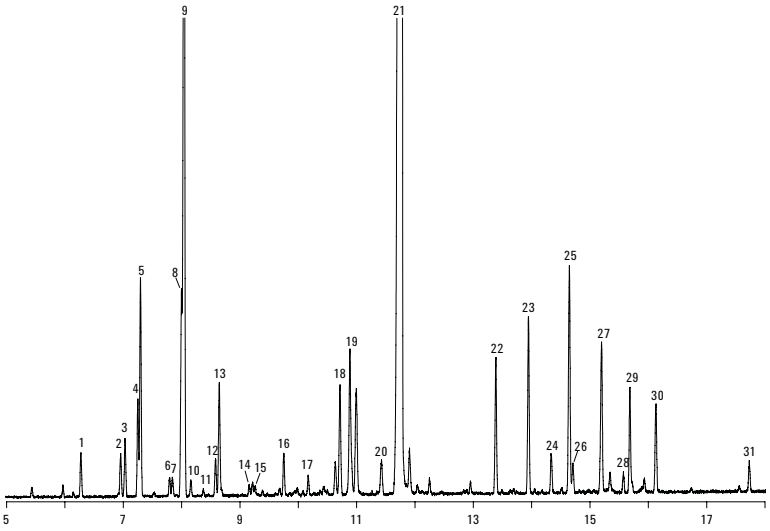

Figure 2. Spearmint oil sample on DB-1, 20 m x 0.18 mm x 0.18 µm column, He carrier. (See Table 1, Method B, for experimental parameters.)

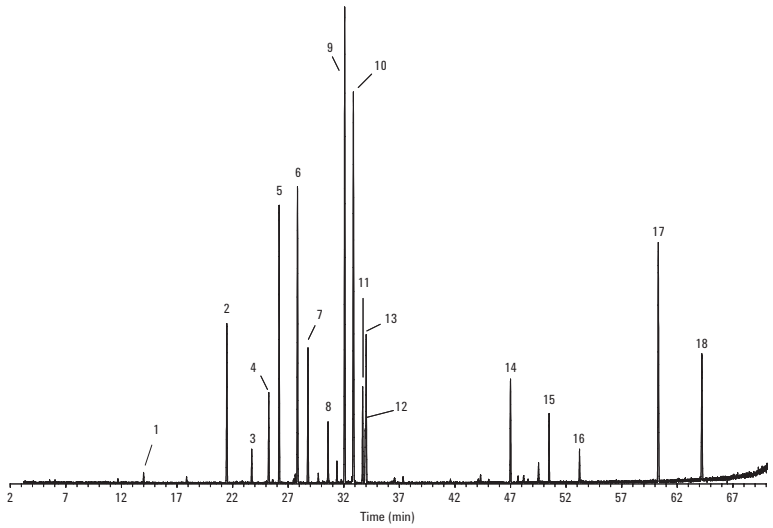

Figure 3. Ylang-ylang oil sample on a DB-WAX, 30 m x 0.25 mm x 0.25 µm column and He carrier. (See Table 2, Method A, for experimental parameters.)

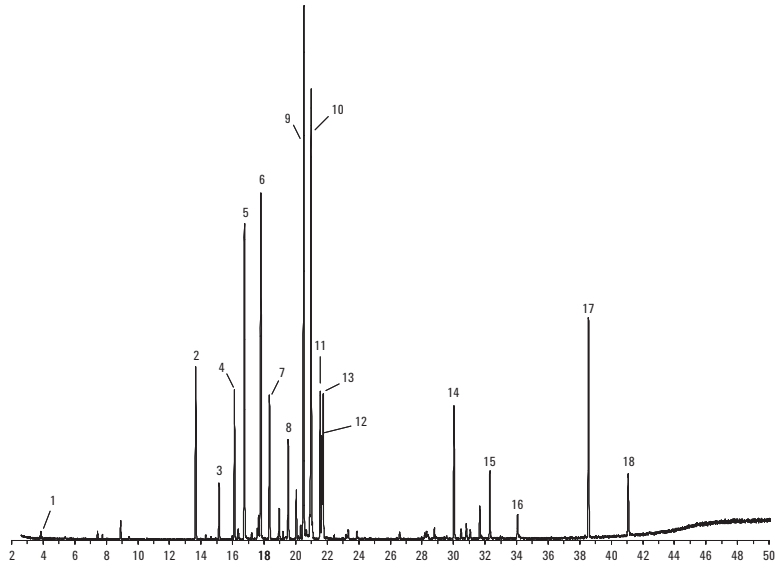

Figure 4. Ylang-ylang oil on DB-WAX, 20 m x 0.18 mm x 0.18 µm column, He carrier. (See Table 2, Method B, for experimental parameters.)

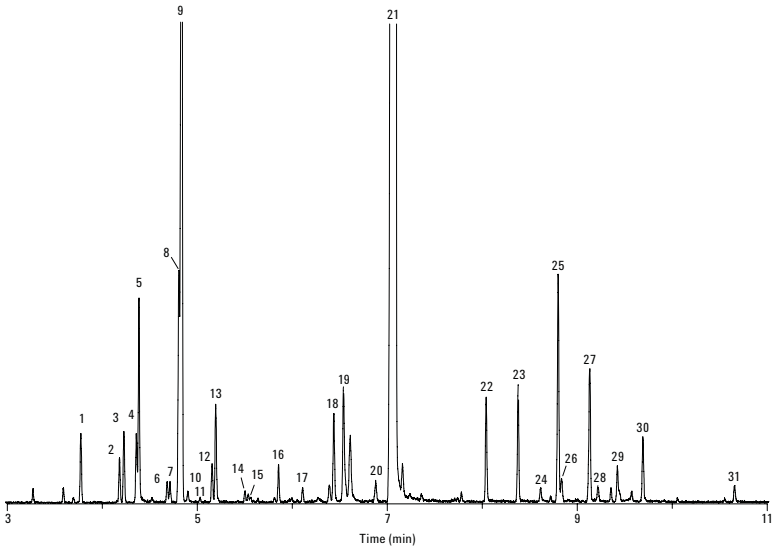

Figure 5. Spearmint oil sample on DB-1, 20 m x 0.18 mm x 0.18 µm with H_2 carrier. (See Table 1, Method C, for method parameters.)

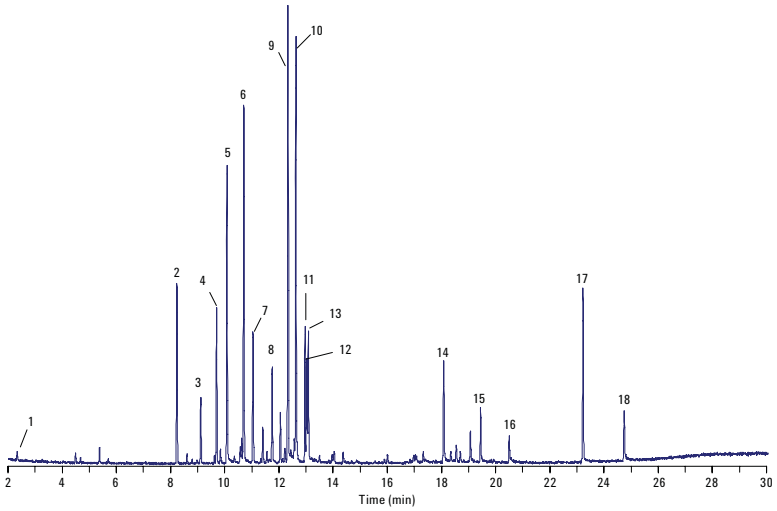

Figure 6. Ylang-ylang oil sample on a DB-WAX, 20 m x 0.18 mm x 0.18 µm column with H_2 carrier. (See Table 2, Method C, for method parameters.)

Conclusions

The use of high-efficiency columns has many benefits, as illustrated here. Shorter analysis times were achieved without significant loss of resolution. Time spent in method development was kept to a minimum through the use of the GC method translation software and the fact that the high-efficiency columns were phase ratio matched. While there are additional benefits for using hydrogen as the carrier gas, significant speed gain can be realized by simply using the high-efficiency columns while maintaining helium as the carrier.

*Although this application only depicts two oils, several additional flavors/fragrances were analyzed. Please contact Agilent Technologies Application Support for additional information.

Reference

D. Rood, *The Practical Guide to the Care, Maintenance and Troubleshooting of Capillary Gas Chromatographic Systems*, Huthig, Heidelberg, 1991

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The High-Resolution Reversed-Phase HPLC Separation of Licorice Root Extracts Using Long Rapid Resolution HT 1.8-µm Columns

Application

Food Additive, Natural Products, and Pharmaceuticals

Author

Bernard Permar and Ronald E. Majors
Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE 19808-1610
USA

Abstract

High-resolution reversed-phase HPLC analytical studies using licorice, a licorice hydrolysis product, and commercial licorice samples, showed that resolution and throughput using a ZORBAX 1.8-µm column greatly exceeded that obtained using the conventional 5.0-µm column.

Introduction

Licorice is derived from the root of the *Glycyrrhiza glabra* plant, a 4- to 5-foot woody shrub that grows in Europe, the Middle East and Western Asia. The root of the plant is known to contain about 4% glycyrrhizin, the potassium or calcium salt of glycyrrhizinic acid. The latter is a glycoside of a pentacyclic triterpine carboxylic acid (18-ß-glycyrrhetic acid) with two molecules of glucuronic acid (Figure 1).

Glycyrrhizin is about 50 times sweeter than sucrose (cane sugar) but at high dosage is known to have toxicity. Upon hydrolysis, the glycoside loses its sweet taste and is converted to the aglycone glycyrrhetinic acid plus two molecules of glucuronic acid.

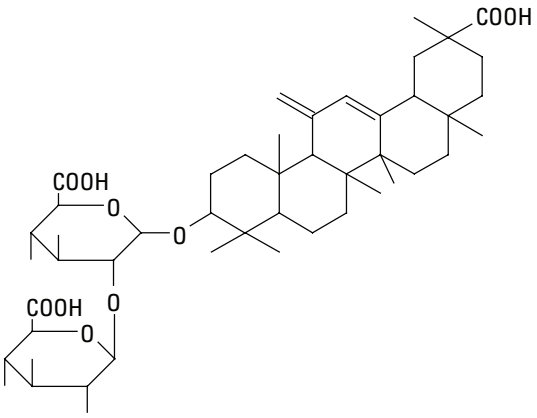


Figure 1. Structure of glycyrrhizinic acid.

Extractions from any of the many species of this plant will yield a complex mixture containing more than 100 compounds. Several of these compounds are used as additives in candy as sweeteners, in cough syrup as flavoring agents, and in drugs to mask a bitter taste, or for their therapeutic qualities, mainly in Traditional Chinese Medicines (TCMs). The medicinal properties of licorice have been known for several centuries in China, as well as India, Egypt, Greece, and Rome. Uses included cough suppressant, laxative, and treatments for gastric ulcer, early Addison disease, and liver disease. Most recently, glycyrrhizin has been shown to have antiviral activity against DNA and RNA viruses (influenza A and B, HIV, VZV, Hepatitis B and C) [1]. Licorice has also been used in topical cosmetic applications.

The abundance of certain compounds of interest will vary greatly according to the species of the plant, the time of harvest, and the method of extraction. Thus, analytical methods to follow the active ingredients are required. Gradient elution reversed-phase HPLC has been found to be an effective method for separating some of the important compounds in licorice [2]. This application note compares the traditional HPLC methodology and the newer Rapid Resolution high-throughput (RRHT) columns. We will apply these HPLC techniques to investigate the differences between two commercially available licorice root extracts.

Experimental

Two reversed-phase (RP) columns were used in this study:

- Conventional ZORBAX StableBond (SB)-C18, 4.6 mm × 250 mm, 5 µm
- ZORBAX SB-C18 RRHT, 4.6 mm × 150 mm, 1.8 µm

The smaller particle size of the RRHT column allows use of a shorter column to achieve the same resolution as the longer conventional column, and also allows more rapid separations.

HPLC conditions

Instrument:	Agilent 1200 Series Rapid Resolution System
Detector:	Multiple wavelength detector (MWD), 254 nm/100 BW, 450 nm reference
Mobile phase:	A = 1% Acetic acid in water B = 1% Acetic acid in acetonitrile

Gradient conditions for ZORBAX SB-C18 columns:

Conventional:	4.6 mm × 250 mm, 5 µm 5% to 100% B in 50 minutes
RRHT:	4.6 mm × 150 mm, 1.8 µm 5% to 100% B in 30 minutes
Flow:	1.0 mL/min
Temperature:	Ambient

Standards: Purchased from Sigma Aldrich

- (G) 0.1-mg glycyrrhizic acid ammonium salt, ~75 %, dissolved in 0.5-mL mobile phase B, then brought to 1.0 mL by adding 0.5-mL mobile phase A
- (GA) 0.1-mg 18-beta-glycyrrhetinic acid, 97%, dissolved in 0.5-mL mobile phase B, then brought to 1.0 mL by adding 0.5-mL mobile phase A

Samples:

- Licorice root extract A (HERB FARM brand)
- Licorice root extract B (Newark Natural Foods)

Both extracts should be vortexed, then filtered (0.2 micron) prior to injection.

Injection volume: 5 µL of extract

Results

The most important compound found in a typical licorice extract is G and to a lesser extent, its hydrolysis product, GA. These substances can be purchased commercially. Although some of the other components of licorice have been identified and are available commercially, they are quite expensive. Since our main objective was to demonstrate the advantage of using shorter, high-resolution HPLC columns, we used only two standards (G and GA) to develop the initial method. Figure 2a shows the gradient separation of G and GA on the conventional column (ZORBAX SB-C18, 4.6 mm × 250 mm, 5 µm) using gradient elution. Since the licorice extract to be examined was quite complex, isocratic conditions were not usable to separate all of the components. The G being more polar by virtue of the additional sugar moieties eluted first while the GA came off the column much later. Using this gradient, the GA eluted in just under 42 minutes. By switching to the shorter ZORBAX SB-C18 RRHT column (4.6 × 150 mm, 1.8 µm), the separation was virtually the same, as can be seen in Figure 2b. However, the separation time was now just over 25 min, a savings of about 40% in time.

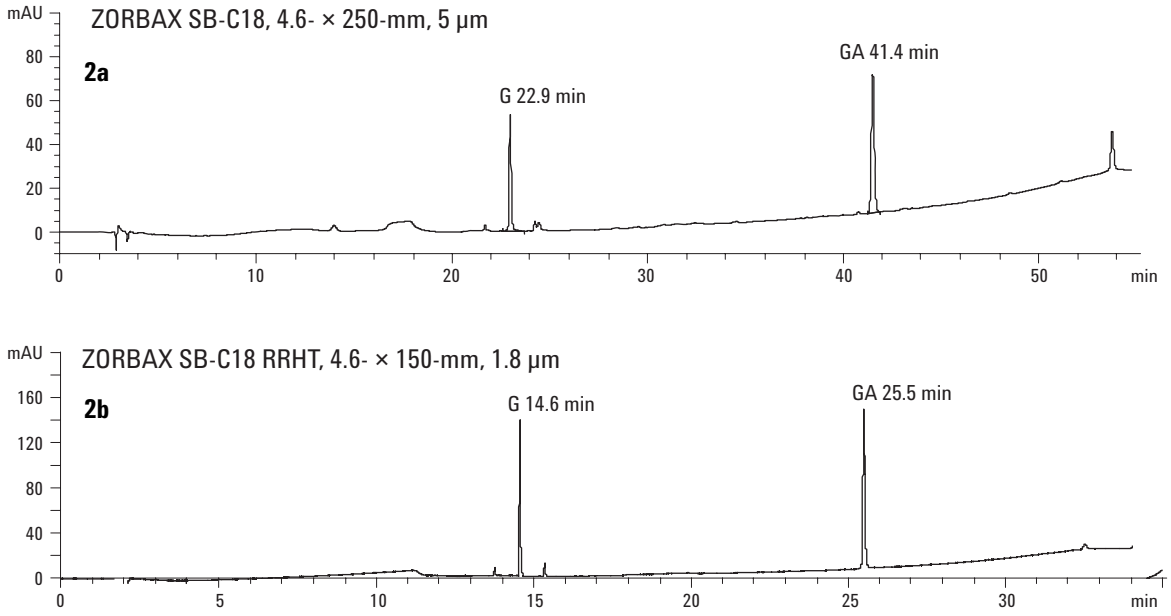


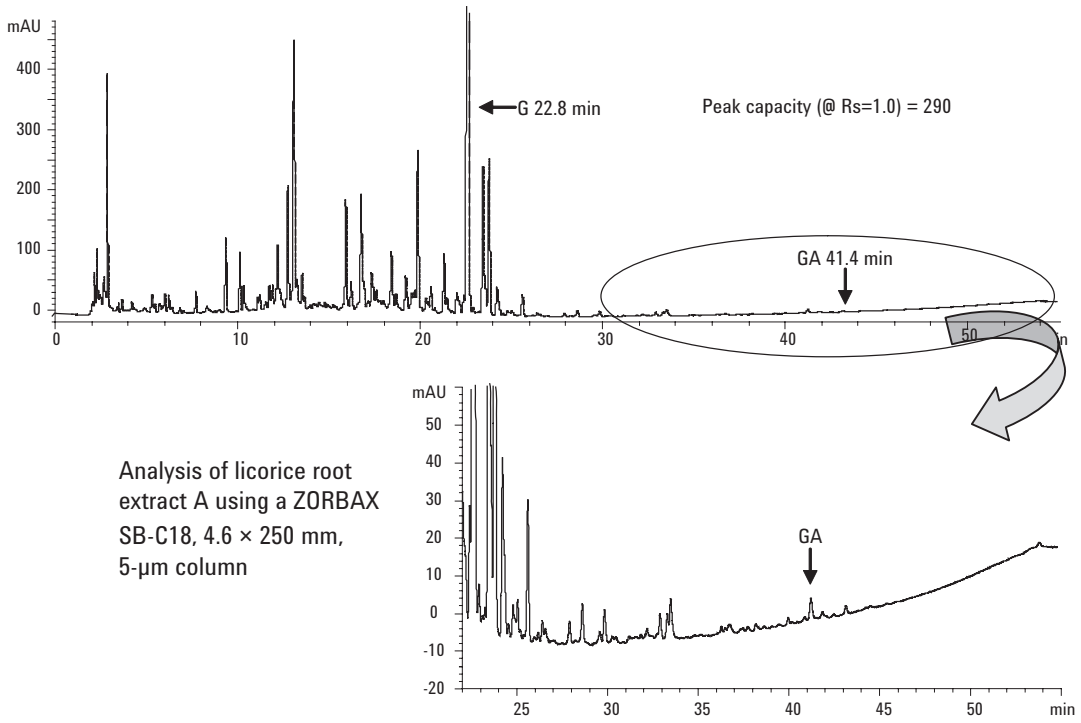
Figure 2. Gradient reversed-phase separation of G and GA on the test 5.0- and 1.8-µm columns.

To investigate the use of these columns for the separation of actual licorice root extracts, Figures 3 and 4 depict the complex chromatograms observed by injection of filtered extracts, identified in the Experimental section. Figure 3a shows the complex chromatogram obtained using the 5-µm 250-mm column. The cut-away shows the small amount of GA that was present in the extract. Since GA is a hydrolysis product of G, it should be at a much lower concentration in a licorice extract, unless the extract was treated to enhance the concentration of the hydrolyzed product. Based on the area count, the GA concentration was less than 0.5% of the G concentration in extract A.

Although the actual peaks were not counted, the calculated peak capacity (3) for the 5-µm column was determined to be 290 (resolution: 1.0). Running the same extract A on the 1.8-µm 150-mm column, one can see the finer structured (that is, higher resolution) chromatogram that results (Figure 3b). The calculated peak capacity for this higher efficiency column was determined to be 442, over 50% higher than by using the longer 5-µm column. Thus, it would be easier to determine minor components on this shorter rapid resolution column. The peaks per unit time (Resolution = 1.0) was calculated to be 17.7 peaks/min for the 1.8-µm column versus 7.1 for the 5-µm column.

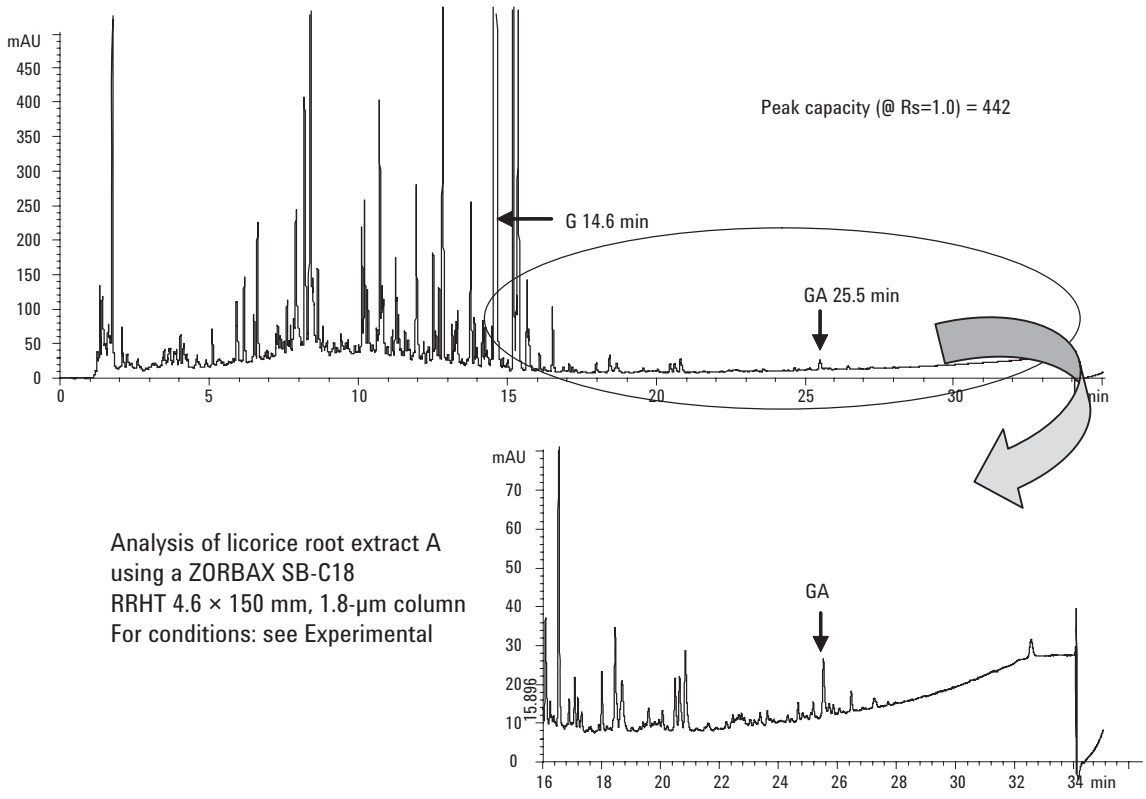
Figures 4a and 4b show similar runs using extract B. This extract was even more complex than extract A which is borne out by comparing the high resolution chromatograms of Figure 3b versus Figure 4b. Again, the calculated peaks per minute for the 1.8-µm column greatly exceeded that of the 5-µm column (17 versus 7.5 respectively). Based on the peak area counts for GA, it was roughly 1% of the concentration of G in extract B.

(A)



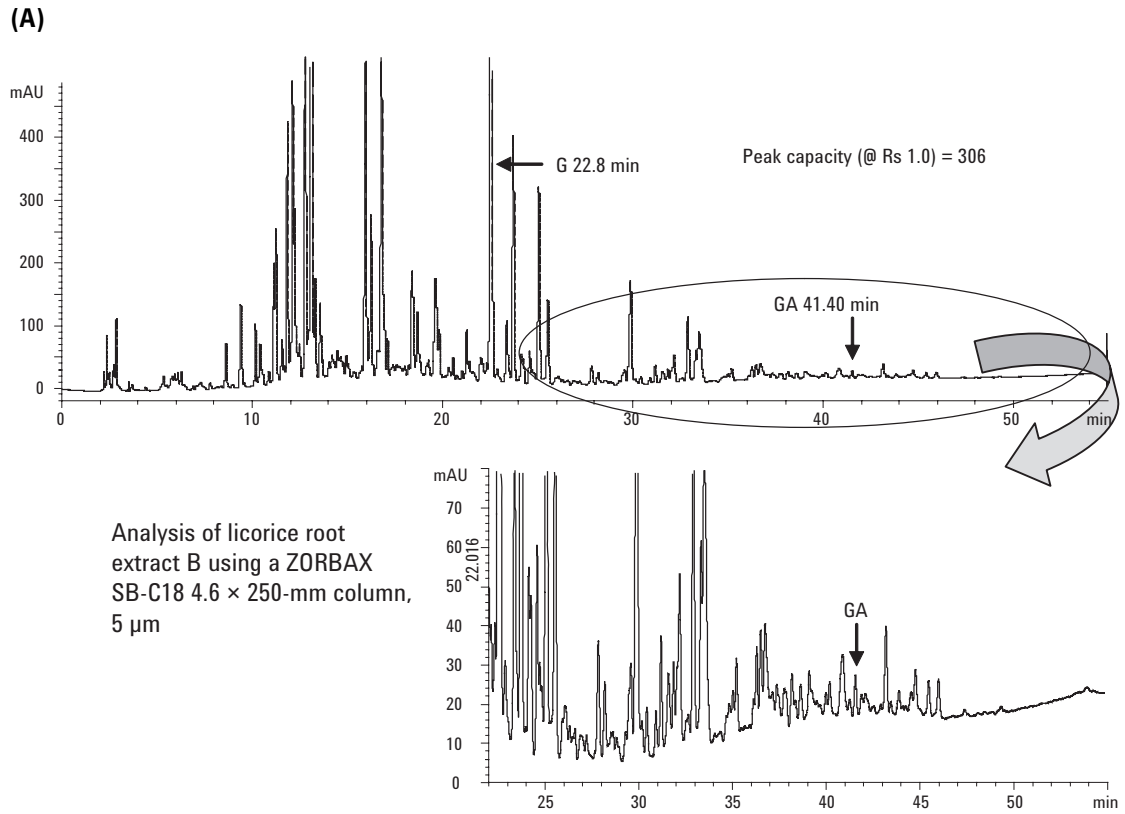
Analysis of licorice root extract A using a ZORBAX SB-C18, 4.6 × 250 mm, 5-µm column

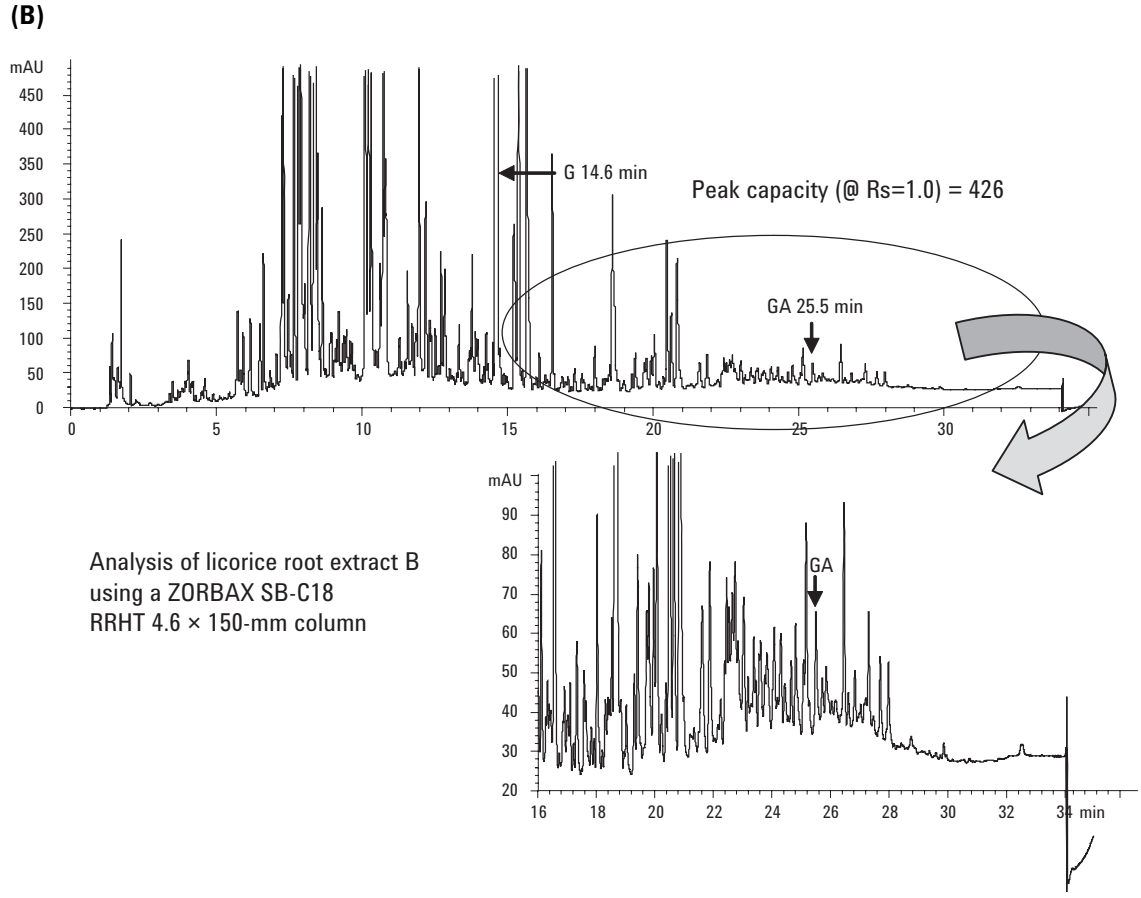
(B)



Analysis of licorice root extract A using a ZORBAX SB-C18 RRHT 4.6 × 150 mm, 1.8-µm column For conditions: see Experimental

Figure 3a and 3b. The gradient reversed-phase separation of licorice root extract A on the 5.0-µm column (A) and on the 1.8-µm column (B).





Figures 4a and 4b. Gradient reversed-phase separation of licorice root extract B on the 5.0-µm column (A) and on the 1.8-µm column (B).

Conclusions

No attempt was made to perform quantitative analysis on the components of the licorice extracts. From our studies, it was obvious that resolution and throughput using the 1.8-µm column greatly exceeded that obtained using the 5.0-µm conventional column. As more complex samples of natural products are encountered and researchers require more detailed component analyses, the use of high resolution, small particle columns should grow. In the investigation of licorice root, other natural products, and TCMs, it is necessary to have gradient capability and sensitive detection.

References

1. S. Fanali, Z. Aturki, G. D'Orazio, M. A. Raggi, M. G. Quaglia, C. Sabbioni, and A. Rocco, (2005) *J. Sep. Sci.*, **28**, 982–986.

2. I. Kitagawa, (2002) *Pure Appl. Chem.* **74** (7), 1189–1198.

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Complete Separation and Quantitation of Fusel Oils by Capillary GC

Application

Authors

Eberhardt R. Kuhn and Allen K. Vickers
Agilent Technologies, Inc.
Life Sciences and Chemical Analysis
91 Blue Ravine Road
Folsom, CA 95630

Susan E. Ebeler, Dawn M. Ahlgren, and John H. Thorngate
University of California, Davis
Department of Enology and Viticulture

Abstract

Fusel oils are of great importance in the alcoholic beverages industry, since they affect the flavor and aroma of the beverage. Thus, their accurate quantitation is essential in assuring consistent quality of alcoholic beverages. Traditionally, packed GC columns were used for this analysis. Capillary columns do not offer the specific stationary phases that were available, and necessary, for packed columns. This application note describes the successful separation of all fusel oils, including other compounds typically found in alcoholic beverages, in a single run on a common stationary phase. In particular, baseline separation of methanol/acetaldehyde and isoamyl/active amyl alcohol was achieved. Examples include standards as well as real samples of fermented and distilled spirits, with quantitative data provided for a number of spirits.

Introduction

Fusel oils are small alcohols that typically include 1-propanol, 1-butanol, isobutanol, as well as isoamyl alcohol (3-methyl-1-butanol) and active amyl alcohol (2-methyl-1-butanol). They are formed through transamination of carbohydrates by amino acids:

Glucose → α-Keto-Acids → Decarboxylation and Reduction → Alcohols

Amino acid	α-Keto acid	Fusel alcohol
Leucine	α-Isocaproate	3-Methyl-1-butanol
Isoleucine	α-Keto- β-methyl valerate	2-Methyl-1-butanol
Valine	α-Ketoisovalerate	2-Methyl-1-butanol
Threonine	α-Ketobutyrate	1-Propanol
2-Phenylalanine	α-Phenyl-2-ketopropionate	2-Phenylethanol

Fusel oils are important flavor constituents in alcoholic beverages. As a group they contribute "fusel/diesel-like" character. Individual aromas range from "banana" (isoamyl acetate) to "rose-like" (phenylethanol). At high levels they are considered undesirable; however, low to moderate levels contribute to the complexity of the beverage. Analysis of fusel oils is used to monitor distillation processes, malfunctions in distillation apparatuses, as well as confirming fermentation substrate authenticity. Thus, their accurate quantitation is essential in assuring consistent quality of alcoholic beverages.

Separation of all fusel oils and the low boiling components on a single capillary column can be problematic. In particular, baseline separation of isoamyl alcohol (3-methyl-1-butanol) and active amyl alcohol (2-methyl-1-butanol) present some challenges. Traditionally, packed GC columns were used for this analysis. Capillary columns do not offer the plethora of different stationary phases that were available, and necessary, for packed columns to accomplish specific separations. In general, isoamyl- and active amyl alcohol will not

separate on polar columns typically used for separation of alcohols (Figure 1). By contrast, their separation is easily achieved on a DB-MTBE (Figure 2), one of the least polar columns with respect to polar analytes. Unfortunately, resolution of other analytes typically found in alcoholic beverages, such as methanol and acetaldehyde, behaves in just the opposite manner (Figures 1 and 2). It would then stand to reason that a compromise of the two, that is, a mid-polarity column, should separate all 4 compounds. Complete baseline resolution of all fusel oils in about 13 minutes (Figure 3) was achieved with a DB-624.

Materials and Methods

Samples

All alcoholic beverage samples were purchased from commercial sources.

Standards

All standards were purchased commercially and were of the highest grade available. A list of analytes is given in Table 1. Standard solutions containing 0, 10, 50, 100, 250, and 500 ppm (vol/vol) of each analyte were prepared in 20% (vol/vol) aqueous ethanol.

Internal Standards

Two internal standards were evaluated, 2-propanol and 3-pentanol. Separate solution of each internal standard were prepared by diluting 25 mL of the neat IS component to a final volume of 250 mL with absolute ethanol.

GC Conditions

GC:	Agilent 6890 Gas Chromatograph;
	ChemStation Software
Autosampler:	Gerstel Model MPS2
Column:	DB 624 60 m × 0.25 mm id × 1.4 µm
Carrier gas:	Helium at 35 cm/s at 40 °C (1.9 mL/min)
Oven:	40 °C for 5 min;
	10 °C/min to 250 °C
Injector:	250 °C
	Splitless; Split vent open at 5.00 min
	at 17.7 mL/min
Detector:	Agilent 5973 MSD; Interface 280 °C

SPME Fibers

Polyacrylate 85um (Supelco, Inc.)
Carbowax-Divinylbenzene 65 um (Supelco, Inc.)

Sampling Conditions

Beverage samples were diluted to 20% ethanol with deionized water prior to sampling. A 10 mL aliquot of standard or diluted sample was placed into a 20 mL headspace vial and 100 mL of IS was added prior to closing with a Teflon lined crimp top seal. The SPME fiber was inserted into the headspace and allowed to equilibrate at 25 °C for 30 min. The fiber was then inserted into the GC inlet and desorbed at 250 °C for 5 min.

Statistical Analyses

All samples and standards were analyzed a minimum of two times. Means, standard deviations, and relative standard deviations (%RSD) were calculated where appropriate. Linear calibration curves were prepared and used for quantitation of unknown samples.

Results and Discussion

SPME Fiber Choice

Two different fibers were evaluated for their response to the analytes monitored. Peak areas for early eluting analytes (methanol, acetaldehyde, ethanol, 2-propanol) were approximately two times greater using the carbowax-divinylbenzene fiber compared to the polyacrylate fiber. Response of the later eluting analytes (amyl alcohols, 1-hexanol, phenylethanol) was slightly higher using the polyacrylate fiber compared to the Carbowax fiber. The Carbowax fiber was used for all quantitative analyses.

Internal Standards

Both 2-propanol and 3-pentanol were evaluated for use as internal standards. The retention time for 2-propanol was close to that of ethanol. 3-Pentanol was well resolved from all other analytes (Table 1; Figure 3). Standard curves calculated using peak area ratios for both internal standards gave similar results in terms of linearity and reproducibility. 3-Pentanol was chosen as the internal standard for quantitation in this study.

Standard Curves

Table 1. Standard Curve Equations. Analyte Concentration vs. Peak Area Ratio for Analyte and Internal Standard

Analyte	Retention time (min)	Range (ppm)	Equation	r^2
Acetaldehyde	4.6	10–500	Y = 6x 10–5(x) + 0.0048	0.9954
Methanol	4.9	10–500	Y = 0.0001(x) – 0.0033	0.9938
Acetone	7.3	10–500	Y = 0.0007(x) – 0.0006	0.9994
1-Propanol	9.7	10–500	Y = 0.0013(x) + 0.05	0.9637
Ethylacetate	10.7	10–500	Y = 0.0049(x) + 0.0412	0.9983
2-Methyl-1-propanol	11.9	10–500	Y = 0.0037(x) + 0.0728	0.9716
1-Butanol	13.1	10–500	Y = 0.0038(x) + 0.0184	0.9772
3-Pentanol (IS)	14.0			
3-Methyl-1-butanol	15.2	10–500	Y = 0.0094(x) + 0.0607	0.9994
2-Methyl-1-butanol	15.3	10–500	Y = 0.013(x) + 0.2127	0.9928
1-Hexanol	18.5	10–500	Y = 0.029(x) + 0.7569	0.9907
Phenyethanol	24.5	10–500	Y = 0.0497(x) + 1.5034	0.9918

All standards prepared in 20% (vol/vol) ethanol.

Precision

Table 2. Relative Sstandard Deviation (%) for Replicate Analyses (n>3) at 5 Concentrations on Different Days

Analyte	500 ppm	250 ppm	100 ppm	50 ppm	10 ppm
Acetaldehyde	9.5	15.9	7.3	14.6	22.0
Methanol	6.9	13.2	20.9	4.6	18.6
Acetone	8.2	15.4	8.4	15.1	11.9
1-Propanol	13.5	11.7	15.7	12.2	22.3
Ethylacetate	5.3	12.4	3.3	11.7	10.2
2-methyl-1-propanol	4.6	3.0	9.6	6.6	10.8
1-Butanol	2.4	4.0	8.8	12.5	17.6
3-Methyl-1-butanol	7.1	3.0	7.8	10.3	11.2
2-Methyl-1-butanol	4.6	4.5	8.4	8.1	12.1
1-Hexanol	7.2	8.3	13.4	7.0	10.9
Phenylethanol	6.7	10.9	11.0	9.6	15.9

Note that %RSD is slightly greater at the low concentrations, particularly for the low molecular weight analytes. Volatilization from the standards between days and during analysis can occur and further work is needed to minimize this variation at low concentrations.

Analysis of Alcoholic Beverages

Chromatograms of selected alcoholic beverages are presented in Figures 4–6. Quantitative results for individual analytes are given in Table 3. Analyte concentrations are typical of those reported for these beverages (Nykanen, 1986).

Table 3. Fusel Alcohol, Methanol, Acetaldehyde, Acetone, and Ethylacetate Concentrations (ppm) in Commercial Beverages*

Analyte	Brandy A (80 proof)	Brandy B (80 proof)	Vodka (80 proof)	Gin (80 proof)	Scotch (80 proof)
Acetaldehyde	14.7	58.9	<10	85.6	<10
Methanol	<10	<10	<10	<10	<10
Acetone	<10	<10	<10	<10	<10
1-Propanol	54.4	74.5	<10	<10	107.9
Ethylacetate	78.2	44.4	<10	<10	74.3
2-methyl-1-propanol	142.8	192.4	<10	<10	261.4
1-Butanol	<10	<10	<10	<10	<10
3-Methyl-1-butanol	<10	<10	<10	<10	<10
2-Methyl-1-butanol	571.9	764.8	<10	<10	87.2
1-Hexanol	153.3	215.9	<10	<10	40.9
Phenylethanol	<10	<10	<10	<10	<10

*Concentrations reported in ppm (vol/vol).

Summary

Resolution of all analytes of interest was achieved using a mid-polarity stationary phase (DB-624). Further optimization of column length and film thickness provided baseline resolution in under 30 minutes for all components quantified. Use of SPME headspace sampling combined with either FID or MSD detection was easy, rapid (~30 min per sample) and readily automated. Volatility of the low molecular weight analytes requires careful sample preparation and temperature control to ensure reproducible results between days. This technique holds promise for the routine analysis of alcoholic beverages in order to monitor distillation processes, malfunctions in still operations, and fermentation substrate authenticity.

References

L. Nykanen, Am. J. Enol. Vitic., 37, 84 (1986).

Column: **DB-WAX**
30 m × 0.25 mm id, 0.25 µm
P/N: **122-7032**
Carrier: Hydrogen at 45 cm/s
Oven: 30 °C for 5 min
30–80 °C at 1°/min
Injector: Split 1:50, 1.5 µL
Detector: FID, Nitrogen makeup gas
at 30 mL/min

1 Acetaldehyde
2 Ethyl acetate
3 Methanol
4 Ethanol
5 1-Propanol
6 3-Pentanol
7 2-Methyl-1-butanol
8 3-Methyl-1-butanol

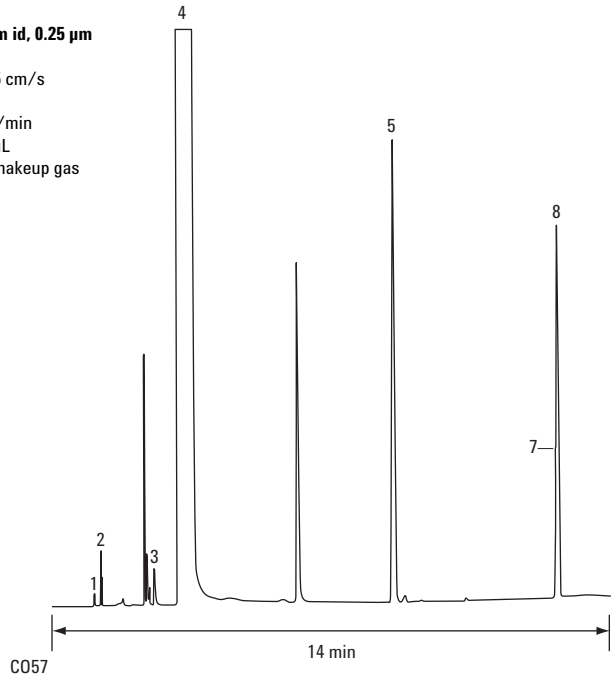

Figure 1. Scotch whiskey.

Column: **DB-MTBE**
30 m × .45 mm id × 2.55 µm
Inlet: 250 °C, split
FID: 300 °C
Carrier: H2, 50 cm/s
Oven: 40 °C for 5 min.
10 °C/min to 250 °C

1 Acetaldehyde
2 Methanol
3 3-Methyl-butanol (isoamyl alcohol)
4 2-Methyl-butanol (active amyl alcohol)

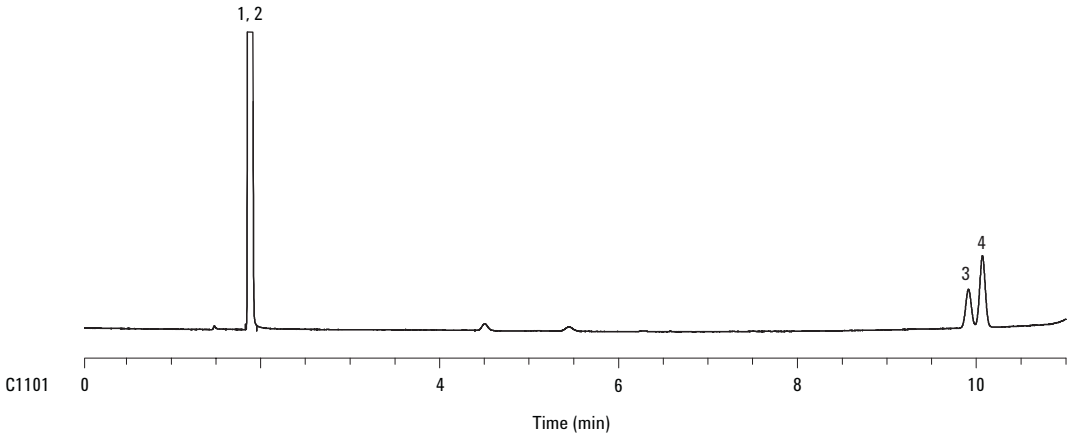

Figure 2. Fusel oil simple standard.

Column:	**DB-624**
	60 m × .25 mm id × 1.4 µm
Inlet:	250 °C, split
FID:	300 °C
Carrier:	H2, 50 cm/s
Oven:	40 °C for 5 min.
	10 °C/min to 250 °C

1	Acetaldehyde
2	Methanol
3	Ethanol
4	Acetone
5	1-Propanol
6	Ethyl acetate
7	Isobutanol
8	1-Butanol
9	3-Pentanol (IS)
10	3-Methyl-butanol (isoamyl alcohol)
11	2-Methyl-butanol (active amyl alcohol)
12	Hexanol
13	Phenylethanol

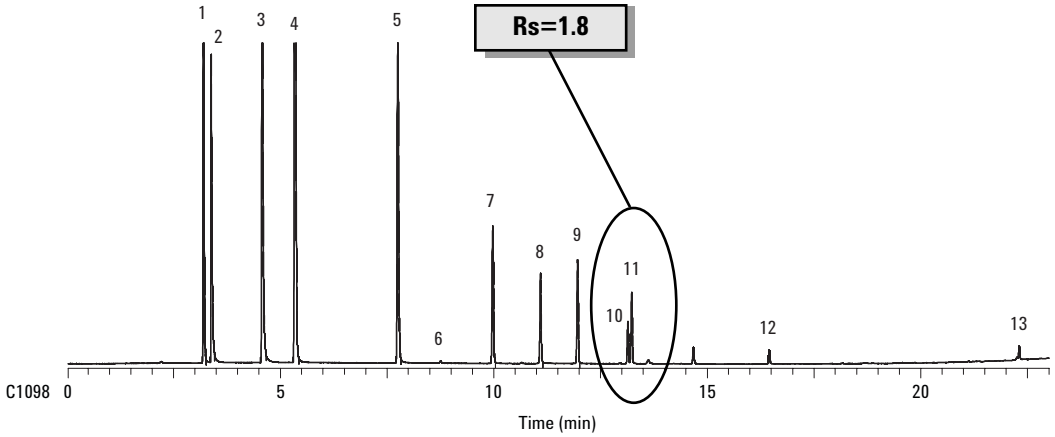

Figure 3. Fusel oil standard.

Column:	**DB-624**
	60 m × .25 mm id × 1.4 µm
Inlet:	250 °C, split
FID:	300 °C
Carrier:	H2, 50 cm/s
Oven:	40 °C for 5 min.
	10 °C/min to 250 °C

1	Acetaldehyde
2	Methanol
3	Ethanol
4	Acetone
5	1-Propanol
6	Ethyl acetate
7	Isobutanol
8	1-Butanol
9	3-Pentanol (IS)
10	3-Methyl-butanol (isoamyl alcohol)
11	2-Methyl-butanol (active amyl alcohol)
12	Hexanol
13	Phenylethanol
14	Acetic acid

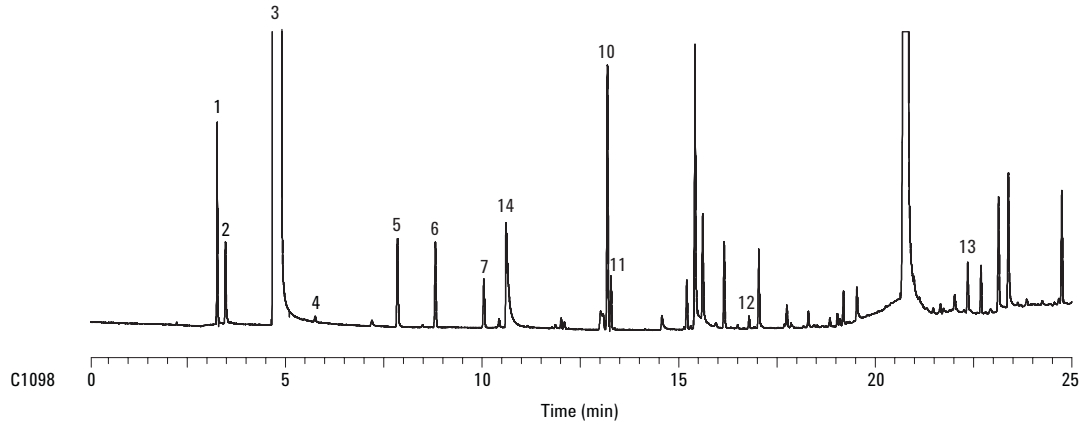

Figure 4. Sherry sample.

Column:	**DB-624**
	60 m × .25 mm id × 1.4 µm
Inlet:	250 °C, split
FID:	300 °C
Carrier:	H2, 50 cm/s
Oven:	40 °C for 5 min.
	10 °C/min to 250 °C

1	Acetaldehyde	8	1-Butanol
2	Methanol	9	3-Pentanol (IS)
3	Ethanol	10	3-Methyl-butanol (isoamyl alcohol)
4	Acetone	11	2-Methyl-butanol (active amyl alcohol)
5	1-Propanol	12	Hexanol
6	Ethyl acetate	13	Phenylethanol
7	Isobutanol		

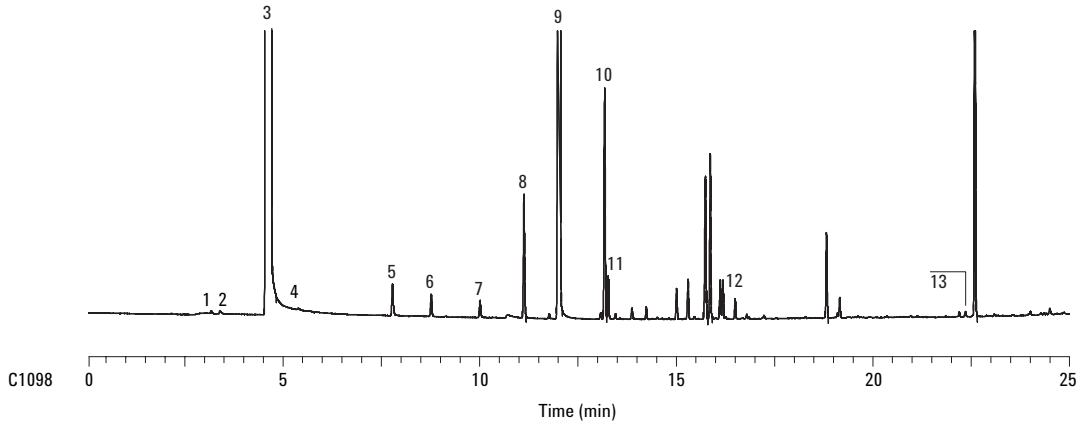

Figure 5. Brandy sample (SPME).

Column:	**DB-624**
	60 m × .25 mm id × 1.4 µm
Inlet:	250 °C, split
FID:	300 °C
Carrier:	H2, 50 cm/s
Oven:	40 °C for 5 min.
	10 °C/min to 250 °C

1	Acetaldehyde	8	1-Butanol
2	Methanol	9	3-Pentanol (IS)
3	Ethanol	10	3-Methyl-butanol (isoamyl alcohol)
4	Acetone	11	2-Methyl-butanol (active amyl alcohol)
5	1-Propanol	12	Hexanol
6	Ethyl acetate	13	Phenylethanol
7	Isobutanol		

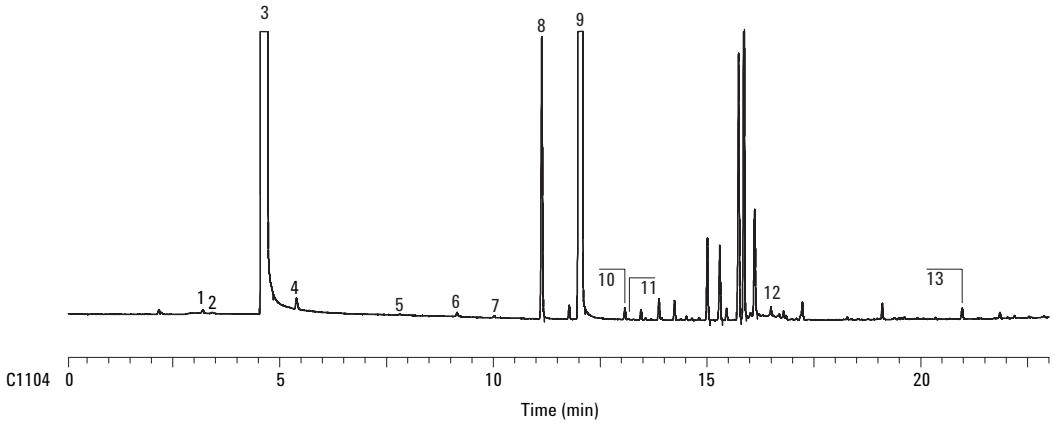

Figure 6. Vodka sample (SPME).

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Analysis of Essential Oil Compounds Using Retention Time Locked Methods and Retention Time Databases

Application

Food and Flavors

Author

Frank David
Research Institute for Chromatography
Pres. Kennedypark 20, B-8500 Kortrijk
Belgium

Francis Scanlan
Quest International
Naarden, The Netherlands

Pat Sandra
Laboratory of Organic Chemistry
University of Gent
Krijgslaan 281, B-9000 Gent
Belgium

Michael Szelewski
Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE 19808-1610
USA

Abstract

Two retention time locked methods for the analysis of essential oil compounds are described. The first method is a gas chromatography/flame ionization detector method using a 50 m × 320 µm id × 1.05 µm HP-5 column. The second method is a gas chromatography/mass spectrometry method using a 30 m × 250 µm id × 0.25 µm HP-5MS column. Retention times of approximately 400 essential compounds were measured using both methods, and two retention time databases were created. Flavor compounds in food extracts or essential oil constituents can be automatically searched based on retention times and/or mass spectra. Finally, transformation of existing retention index libraries into locked retention time databases is discussed.

Introduction

Capillary gas chromatography (GC) has been for many years the method of choice for the analysis of essential oils [1]. The constituents of essential oils are identified using a combination of different GC techniques, including GC with flame ionization detection (FID) and determination of retention indices, GC with olfactometric detection (sniffing), GC in combination with mass spectrometry (GC/MS) and GC with element-selective detection (flame photometric detection, nitrogen phosphorous detection, atomic emission detection, etc). Although GC/MS is probably the most powerful technique, and extended mass spectral libraries are available, it does not allow complete identification. Essential oils are complex mixtures of monoterpenes, monoterpenoids, sesquiterpenes, sesquiterpenoids, diterpenes, and diterpenoids. No single capillary column can resolve all possible compounds, and spectral data are not always conclusive because isomers give similar spectra. In flavor and fragrance quality control, retention indices are still frequently used as a complementary technique to GC/MS. Several libraries are

available with retention indices for many flavor and fragrance compounds [2–4]. Retention indices are less dependent on operational parameters than absolute retention times, but they still depend significantly on the column type (stationary phase and supplier), on the temperature program, and to a lesser extent, on the carrier gas velocity. Therefore, it is sometimes difficult to reproduce published retention indices in different laboratories. Moreover, most companies in the flavor and fragrance industry are still using in-house methods based on historical choices of columns and conditions. Finally, the use of retention indices (with n-alkanes as reference compounds) is not possible with element-selective detectors. A peak detected at a certain retention time using a sulphur selective detector, for instance, might be difficult to locate in an FID or MS chromatogram.

Recent developments in GC have led to the ability of locking and matching retention times for a given application [5-6]. Using retention time locking, it is no longer necessary to calculate the retention index, but the absolute retention time can be used as an identification tool. Of course, retention times are still dependent on operating conditions, but small differences in carrier gas velocity and column length are compensated by re-locking the GC method by adjustment of the column head-pressure. After re-locking, elution temperatures of the solutes are also constant. Moreover, retention time locking can also be used in combination with different detectors, and exact scaling of GC/FID, GC/sniffing, GC/MS, and GC/AED chromatograms is possible [6]. Retention time locking and retention time databases are, therefore, excellent tools in essential oil and in flavor QA/QC analysis.

In this paper, two retention time locked methods are presented. For each method, a retention time database is available containing approximately 400 flavor compounds and essential oil constituents. The first method is a GC/FID method. A long, thick film column is used in combination with a slow temperature program. These conditions are frequently used in QA/QC analysis in the flavor and essential oil industry because a high sample capacity is combined with a high resolving power, resulting in a detailed picture of the samples. The second method is a GC/MS method. For this method, a 30 m × 0.25 mm id × 0.25 µm HP-5MS column was selected because this is the most frequently used column in GC/MS analysis. While the resolution and sample capacity are lower on this column, the GC/MS analysis mainly focuses on identification of analytes. For this method, a combined retention time and mass spectral library Screener Database is available.

Because a lot of retention data are already available as retention indices, it was also evaluated if these data could be transferred into absolute retention times that match with locked retention times. It was shown that retention indices from existing retention index libraries can be recalculated as absolute retention times that match with experimental data.

Experimental

GC/FID analyses were performed on an Agilent 6980 gas chromatograph equipped with a split/splitless inlet. Separation was done on a 50 m × 0.32 mm id × 1.05 µm HP-5 column (β=72) (Agilent part number 19091J-215). The analytical conditions are summarized in Table 1. Helium at approximately 85 kPa (12.33 psi) constant pressure was used as carrier gas. The inlet pressure was adjusted to give a retention time of 70.000 min for n-pentadecane. This is done by retention time locking (RTL), using five runs at different pressures (respectively 70, 80, 90, 100, and 110 kPa), and plotting the retention time of n-pentadecane as a function of the inlet pressure [5]. From this curve, the inlet pressure can be calculated to adjust the retention time of n-pentadecane to exactly 70.000 min. The analytical conditions in this GC/FID method are typical conditions used in quality control of essential oils. The column choice and the slow temperature program offer high resolution and a detailed sample profiling. The column also offers high sample capacity, which is also important in essential oil profiling, because important trace constituents can be present and elute close to major constituents. On columns with restricted sample capacity, overloading and, consequently, peak leading is frequently observed for the main constituents.

Table 1. GC/FID Conditions

Column	50 m × 0.32 mm id × 1.05 µm HP-5 (β=72) (Agilent part number 19091J-215)
Injection	Split, split ratio 25:1, 250 °C, 1 µL injection volume
Carrier	Helium (approximately 85 kPa) (12.33 psi), constant pressure
RTL	The inlet pressure is adjusted to give a retention time of 70.000 min for n-pentadecane
Oven program	50 °C to 280 °C at 2 °C/min (110 min analysis time)
Detection	FID, 300 °C

Table 2. GC/MS Conditions

Column	30 m × 0.25 mm id × 0.25 µm HP-5MS (β=72) (Agilent part number 19091S-433)
Injection	Split, split ratio 25:1, 250 °C, 1 µL injection volume
Carrier	Helium (approximately 65 kPa) (9.43 psi), constant pressure
RTL	The inlet pressure is adjusted to give a retention time of 27.500 min for n-pentadecane
Oven program	60 °C to 240 °C at 3 °C/min (60 min analysis time)
Detection	MS in scan mode (40–400 amu); solvent delay: 2 min; transfer line: 300 °C

The second method is a GC/MS method. GC/MS analyses were performed on an Agilent 6980 gas chromatograph equipped with a split/splitless inlet in combination with an Agilent 5973N MSD. Separation was done on a 30 m × 0.25 mm id × 0.25 µm HP-5MS column (β=250) (Agilent part number 19091S-433). The analytical conditions are summarized in Table 2. Helium at approximately 65 kPa (9.43 psi) constant pressure was used as carrier gas. The inlet pressure was adjusted to give a retention time of 27.500 min for n-pentadecane. This is done by retention time locking, using five runs at different pressures, and plotting the retention time of n-pentadecane as a function of the inlet pressure. This is automatically done by starting the "acquire RTL calibration runs" command in the GC/MS instrument control. From this curve the inlet pressure can be calculated to adjust the retention time of n-pentadecane to exactly 27.500 min. These analytical conditions can be used to screen essential oils using GC/MS. Essential oil constituents can be identified based on the mass spectral data, and on retention times, using a screener library. The operational conditions are identical to the conditions used by Adams [4]. Spectra and retention data published in this reference are also valid for this method.

Test mixtures of flavor compounds and n-alkanes were prepared from pure chemicals at 0.1% concentration in carbon tetrachloride or chloroform. Essential oil mixtures are diluted in carbon tetrachloride or chloroform at a 5% level (50 mg/mL).

Results and Discussion

GC/FID Method

The described GC/FID method is used for quality control of essential oil mixtures. The long, thick film column results in high resolution and high sample capacity. Traces of important compounds can be detected besides the main constituents. A typical separation obtained by this method appears in Figure 1, showing the analysis of a Spanish orange oil. The chromatogram shows a detailed picture of the main compounds and of minor constituents. This type of analysis, giving both qualitative and quantitative information, is used for quality control of essential oils. This GC/FID method was locked to n-pentadecane (t_R = 70.000 min). Under these locked conditions, n-decane elutes at 31.640 min and n-eicosane at 99.557 min. Using these conditions, a retention time locked database was created containing approximately 400 compounds that are important in quality control of essential oil mixtures. Using this database and the GC ChemStation RTL option, peaks in the GC chromatogram can be identified based on a retention time search in a given retention time window (for instance ±0.2 min). For the 10 main peaks of the Spanish orange oil, the results of such a retention time search are given in Table 3. It is clear, that in some cases, several compounds elute in the 0.4-min window and further identification is needed. However, this tool already allows an excellent profiling of samples.

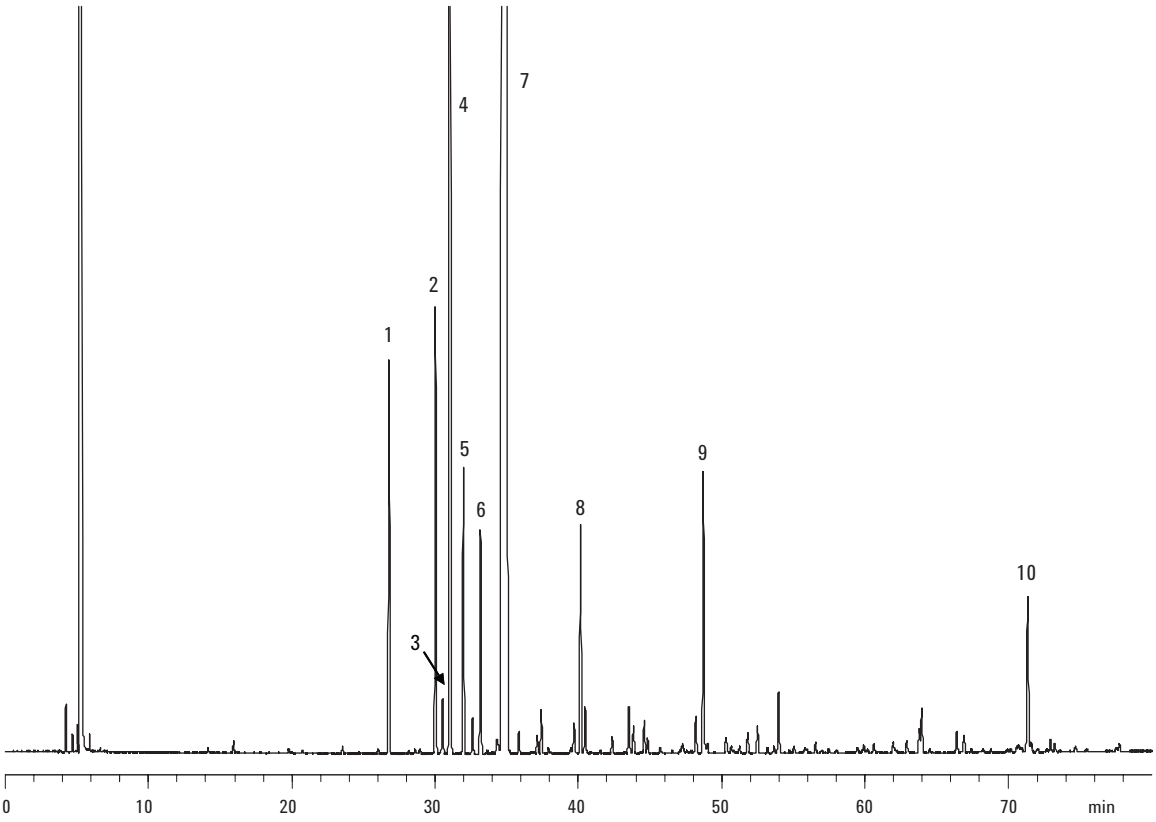

Figure 1. GC/FID chromatogram of Spanish orange oil. (Conditions: Table 1, peak identification: Table 3)

Table 3 Identification of main compounds in Spanish orange oil using a retention time database and a combined mass spectral and retention time identification.

Peak number	GC/FID* t_R (min)	t_R identification	GC/MS t_R (min)	MS + t_R identification
1	26.793	α-pinene	5.172	α-pinene
2	30.042	1-octen-3-ol 3-(methylthio)-1-propanol sabinene	6.181	sabinene
3	30.539	hexanoic acid β-pinene 6-methyl-5-hepten-2-one	6.282	β-pinene
4	31.053	2-octanone myrcene furfuryl acetate	6.658	myrcene
5	31.987	octanal	6.987	octanal
6	33.190	*trans*-2-hexenoic acid Δ-3-carene	7.267	Δ-3-carene
7	35.001	limonene benzylalcohol ocimene	8.130	limonene

Table 3 Continued

Peak number	GC/FID* t_R (min)	t_R identification	GC/MS t_R (min)	MS + t_R identification
8	40.162	n-undecane *cis*-3-hexenylpropionate δ-hexalactone 1-methyl-2,3-cyclohexadione linalool methyl benzoate	10.391	linalool
9	48.728	dihydrocarveol methyl salicylate estragole n-decanal octylacetate	14.750	n-decanal
10	71.366	anisylproprionate valencene piperonyl acetate	27.134	valencene

* For the GC/FID retention time identification, a 0.4-min window was used (±0.2 min)

Another example of the GC/FID method appears in Figure 2, showing the analysis of an Indonesian nutmeg oil. Again a very detailed chromatogram is obtained. Using the retention time locked database, most constituents are identified. The small peak eluting at 67.468 min is, for instance, identified as isoeugenol. This is an important flavor compound.

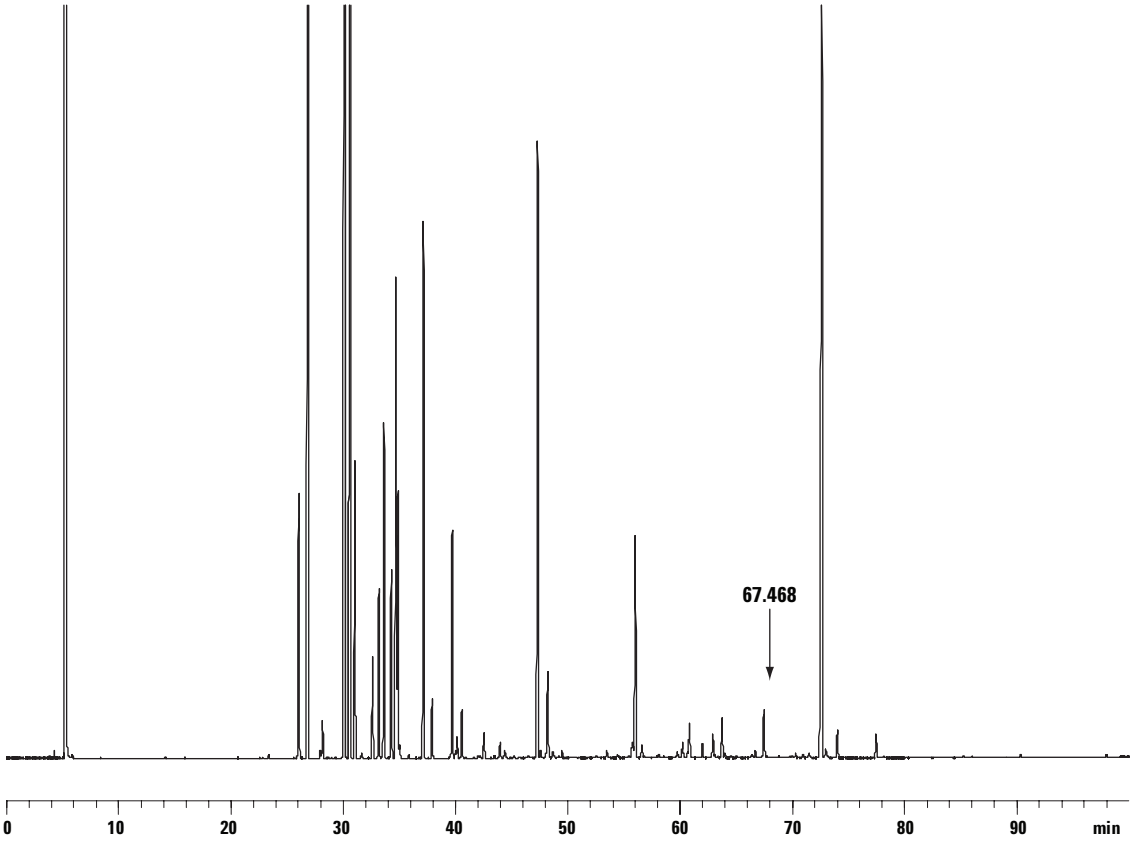

Figure 2. GC/FID chromatogram of Indonesian nutmeg oil. (Conditions: Table 1)

GC/MS Method

For confirmation of solute identities and for the identification of unknown peaks, the essential oils are analyzed by GC/MS. The described method uses a standard column and a faster temperature program. These conditions are similar to the method published by Adams [4]. The chromatogram obtained for the Spanish orange oil is given in Figure 3. A similar separation is obtained as in Figure 1, but the resolution is lower due to the lower column plate number. Moreover, some peak overloading can be observed. Due to the fact that a different temperature program is used, the compounds also elute at different temperatures (method not translated) and, therefore, also some differences in relative elution profiles are observed (see, for instance, relative elution of peaks 2, 3, and 4). Nevertheless, this method can be used for detailed identification of the essential oil constituents. For this GC/MS method, a mass spectral library was created containing approximately 400 compounds, together with the retention times. With this library, identification is possible based on mass spectra AND on retention time. The identification of the 10 main compounds (same as labelled in Figure 1) using the Results Screener is included in Table 3. The combination of mass spectral and retention time data allows complete identification. It is also important to notice that the correct compound was, in all cases, already selected in the GC/FID retention time window.

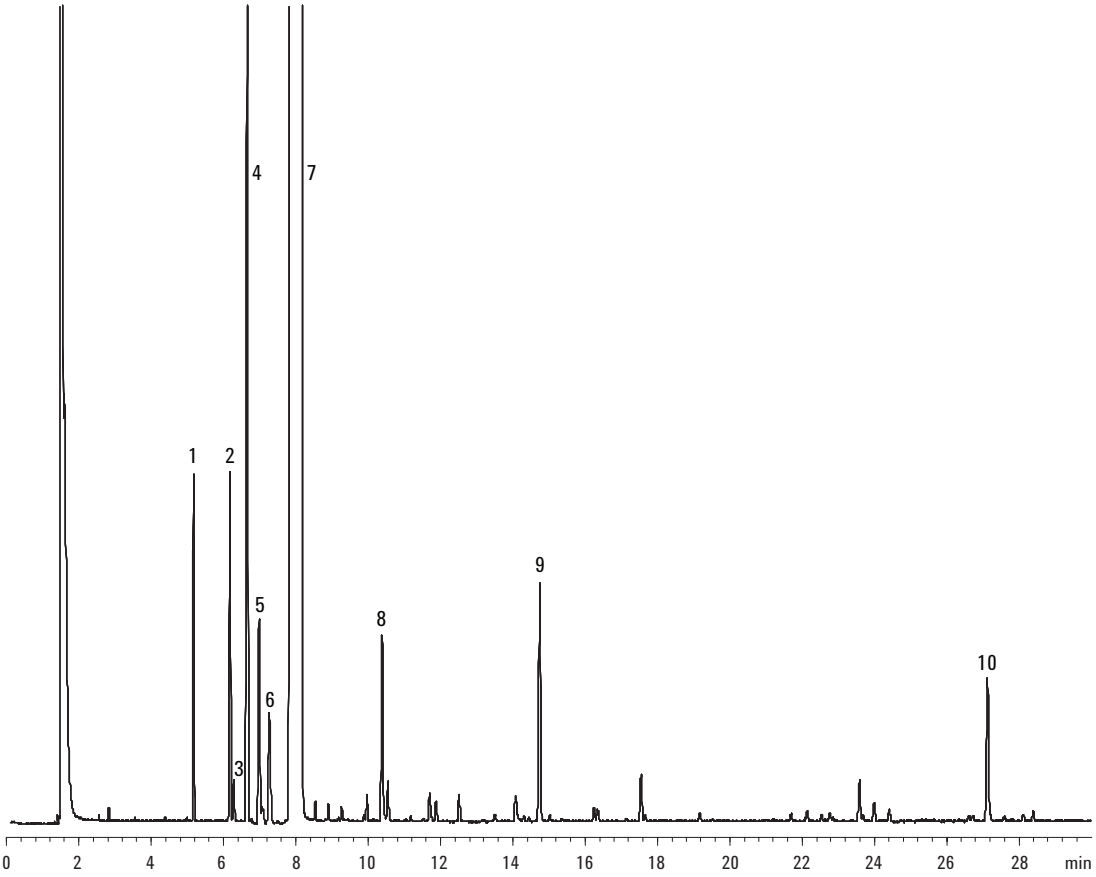

Figure 3. GC/MS chromatogram of Spanish orange oil. (Conditions: Table 2, peak identification: Table 3)

The Indonesian nutmeg oil was also analyzed by this GC/MS method. The chromatogram is given in Figure 4. A classical library search using the standard NIST mass spectral library of the peak at 25.304 min gave isoeugenol as the first hit (probability 96%) and eugenol as the second hit (probability 94%). The spectra of both compounds are very similar (Figure 5). Using the Results Screener, the compound was identified unequivocally as isoeugenol (retention time plus mass spectral match). This example clearly demonstrates the power of combined retention time and mass spectral search.

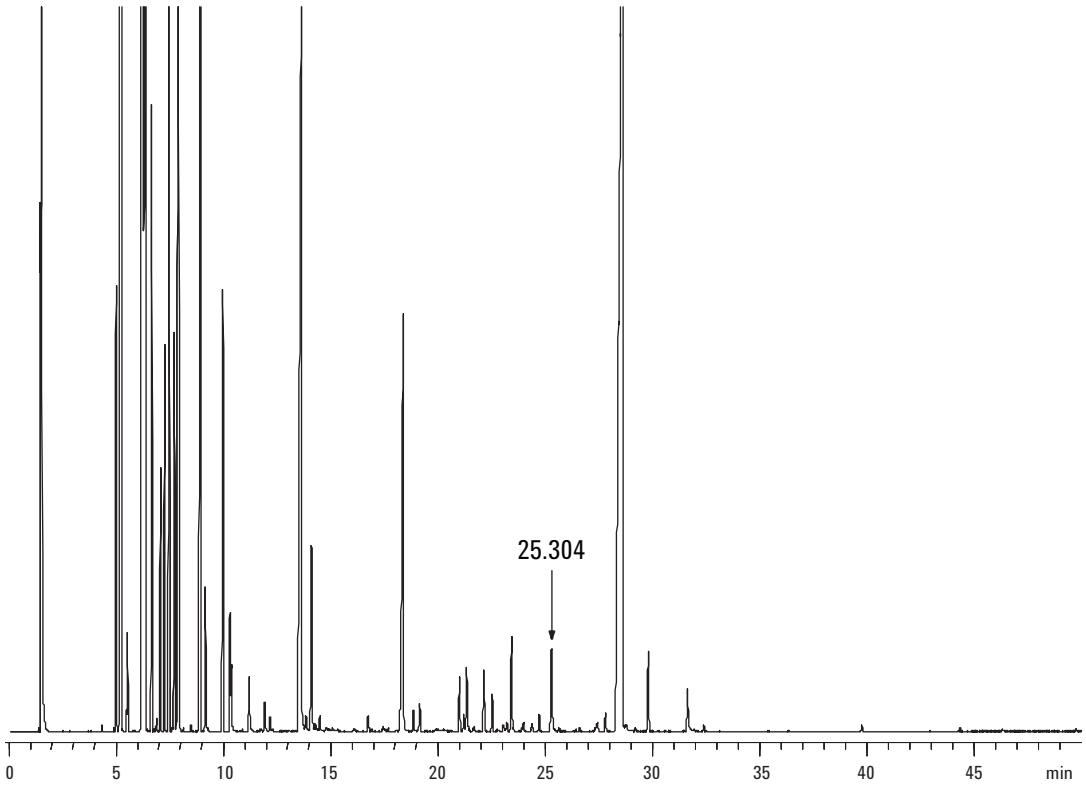

Figure 4. GC/MS chromatogram of Indonesian nutmeg oil. (Conditions: Table 2)

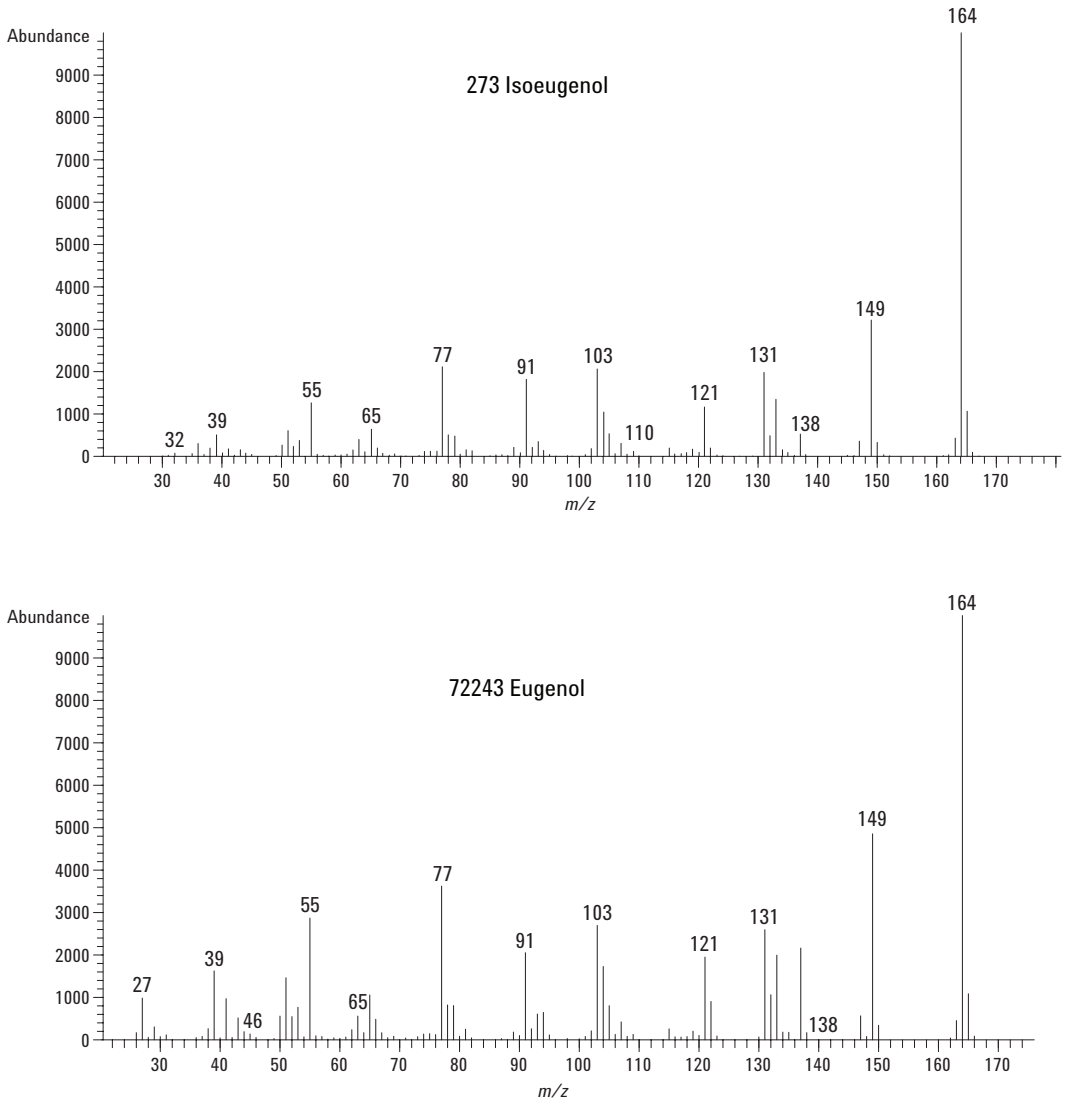

Figure 5. Comparison of mass spectra of eugenol and isoeugenol.

Transformation of Retention Indices

Further it was evaluated if published retention indices could be transferred into retention times and if these calculated retention times match with experimental data. A total of 34 test solutes were analyzed. The compounds are listed in Table 4, together with the FEMA code, the retention index in an existing database (RI) [7], and the measured retention time ($t_{R\,exp}$) under retention time locked conditions. From the retention index, the absolute retention time was calculated using the retention times of n-alkanes as reference compounds. The calculated values are also listed in Table 4 ($t_{R\,calc}$). Thus, these retention times are not the original retention times used for the retention index calculation, but calculated values. In the last column, the difference between calculated and experimental retention times are also given. From these data, it is clear that the calculated and experimental retention times match very well (within ±0.2 min). This means that retention times can be calculated from the retention indices present in an existing database using the locked retention times for n-alkanes if the column dimensions and the temperature program are the same. This is also valid for the GC/MS method as shown in the following example. Isoeugenol is present in the database of

Table 4. FEMA names, FEMA codes, retention indices, calculated retention times, experimental retention times, and retention time differences for test solutes.

Compound	FEMA name	FEMA code	RI	$t_{R\ calc}$ (min)	$t_{R\ exp}$ (min)	$t_{r\ diff}$ (min)
1	Acetal	2002	725.1	11.836	11.836	0.000
2	Amyl alcohol	2056	760.0	13.844	13.833	-0.011
3	Hexyl alcohol	2567	865.8	21.068	20.941	-0.127
4	Anisole	2097	923.0	25.529	25.403	-0.126
5	Ethyl acetoacetate	2415	944.4	27.300	27.319	0.019
6	Heptyl alcohol	2548	968.4	29.285	29.180	-0.105
7	Octanal	2797	1003.8	32.218	32.215	-0.003
8	Methyl 3-(methylthio)propionate	2720	1026.8	34.140	34.121	-0.019
9	Benzyl alcohol	2137	1036.9	34.984	35.011	0.027
10	Isoamyl butyrate	2060	1055.5	36.539	36.524	-0.015
11	Octyl alcohol	2800	1069.7	37.726	37.663	-0.063
12	Acetophenone	2009	1072.4	37.952	38.119	0.167
13	Benzylformate	2145	1081.2	38.688	38.851	0.163
14	Benzyl acetate	2135	1169.1	45.848	45,915	0.067
15	Allylheptanoate	2031	1180.0	46.729	46.664	-0.065
16	Decanal	2362	1207.4	48.917	48.970	0.053
17	Benzyl propionate	2150	1266.2	53.444	53.378	-0.066
18	1-Decanol	2365	1272.1	53.899	53.904	0.005
19	Anisyl alcohol	2099	1295.0	55.662	55.799	0.137
20	Isobornyl acetate	2160	1301.7	56.171	56.252	0.081
21	Benzyl isobutyrate	2141	1305.0	56.411	56.452	0.041
22	Undecanal	3092	1310.6	56.819	56.798	-0.021
23	Triacetin	2007	1344.4	59.279	59.192	-0.087
24	Benzyl butyrate	2140	1354.5	60.014	60.049	0.035
25	Acetanisole	2005	1369.0	61.070	60.960	-0.110
26	gamma-Nonalactone	2781	1373.4	61.390	61.273	-0.117
27	Anisyl acetate	2098	1426.0	65.116	65.203	0.087
28	Allyl cyclohexylpropionate	2026	1435.0	65.735	65.747	0.012
29	Lauryl alcohol	2617	1475.0	68.489	68.531	0.042
30	Isoamyl octanoate	2080	1487.0	69.315	69.230	-0.085
31	Isoamyl phenylacetate	2081	1503.0	70.405	70.387	-0.018
32	Ethyl-methylphenylglycidate	2444	1517.0	71.317	71.447	0.130
33	Ethyl-3-phenylglycidate	2454	1529.0	72.099	72.063	-0.036
34	gamma-Undecalactone	3091	1589.0	76.007	75.955	-0.052

Adams [4] with a retention index of 1447. This retention index can be transferred into an absolute retention time using the following formula:

$$\left[\frac{[RI - (Z \times 100)]}{100} \times \left(t_{R_{Z+1}} - t_{R_Z}\right)\right] + t_{R_Z} = t_{R_X}$$

whereby: RI = retention index (from existing data base),
Z = carbon number of preceding n-alkane,
t_{RZ+1} and t_{RZ} = retention times of following and preceding n-alkanes (in RTL method) and
t_{RX} = retention time of solute in RTL method

For isoeugenol, with an RI = 1447, and the preceding tetradecane (Z=14) eluting at 23.26 min (t_{RZ}), and the following n-pentadecane (Z+1) eluting at 27.50 min (t_{RZ+1}) using the retention time locked GC/MS method, the calculated retention time is 25.25 min. This corresponds well (within ±0.2 min) with the measured retention time (25.30 min). Using these calculations, compounds can also be added to the RTL databases.

Conclusion

Two methods were developed for the analysis of essential oils. The first method is used for quality control analysis. The method is locked using n-pentadecane as locking standard. A retention time locked database, containing approximately

400 compounds was created. This database can be used to identify constituents based on their absolute retention time under the locked conditions. The locked method also guarantees retention time stability in function of time, between columns and between instruments.

Secondly, a GC/MS method was developed. This method can be used for identification of essential oil constituents. Identification is done based on the combination of retention time and mass spectral matching.

Finally, it is shown that retention indices for flavor compounds measured under specific operational conditions can be transferred into locked retention times using the locked retention times of n-alkanes. Thus, existing retention index databases can be translated into locked retention time databases. Moreover, a retention time locked database is not restricted to the use of one (FID) detector, but compounds detected by any GC detector can be searched if the locked method is used.

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Agilent Technologies

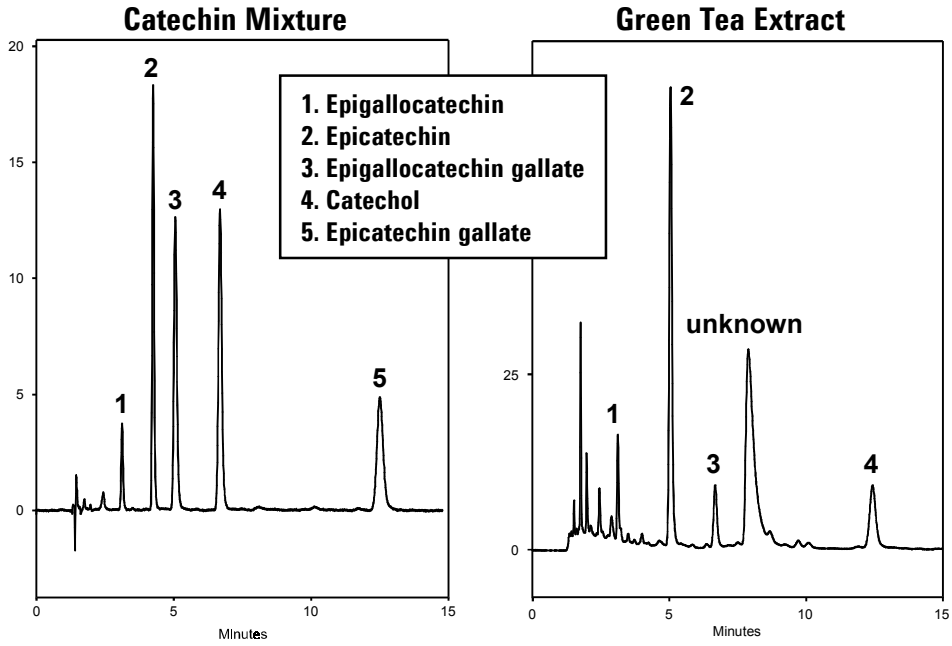
Epigallocatechin 3-O-Gallate Extract from Green Tea

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

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

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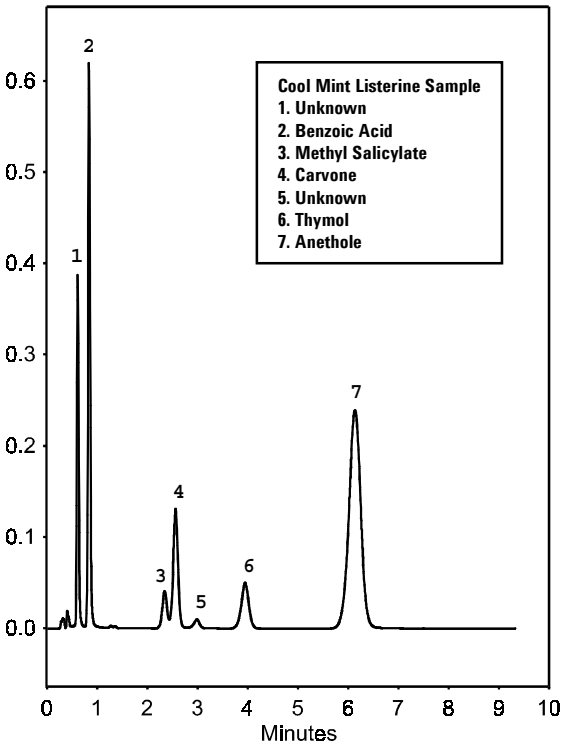
Flavoring Agents

Application

Food Analysis

Robert Ricker

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



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

Highlights

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

Agilent Technologies

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

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April 25, 2002
5988-6353EN

Agilent Technologies

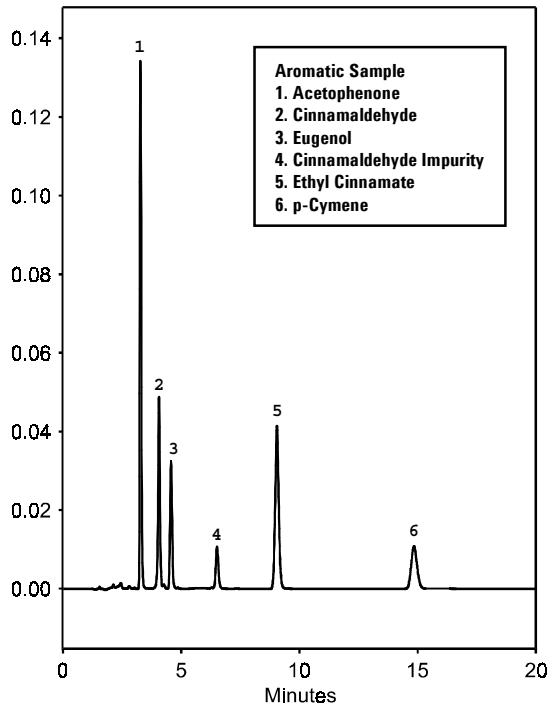
Aromatics

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

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

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Agilent Technologies

Analysis of flavonoids in plant extracts by CE-MS

Application Note

Foods and Flavors

Authors
Martin Greiner,
Gordon Ross
Agilent Technologies,
Waldbronn, Germany

Abstract

Complex extracts of plant compounds often need a very effective separation mechanism for a clear compound identification. MS detection can provide a specific identification of the analyte in such complex and difficult matrices. Here we show the analysis of flavonoids from extracts of oranges, illustrating the specificity and sensitivity of an electrospray mass spectrometer (ESI-MS) which can add a new dimension to capillary electrophoresis separations.

Hesperidine, a flavone glucoside, is present in the peel of green oranges while naringin, a rhamnoglucoside flavanone, is a bitter component of the orange peel. If not controlled properly, the high squeeze pressure applied to oranges during production of orange juice yields a high content of these flavonoids which gives it a bitter taste. Monitoring the presence of these in orange juice provides a way of monitoring the quality of the product.

CE was the separation technique of choice to allow a fast and efficient separation and additionally to reduce sample preparation to centrifugation and filtering of the juice.

Agilent Technologies

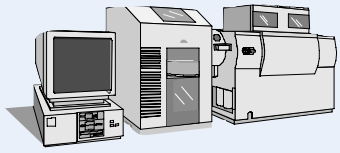
Experimental

All experiments were performed using a Agilent Capillary Electrophoresis system equipped with DAD detection and controlled via Agilent CE ChemStation software. The Agilent capillaries for CE-MS were 50 µm id with a total length of 75 cm. These capillaries have a UV detection window at 22 cm. The buffer used was 50 mM borate, pH 9.3 diluted tenfold by Agilent CE-grade water (all Agilent Technologies, Waldbronn, Germany). Sheath flow (4 µl/min) was delivered by an Agilent 1100 isocratic HPLC pump running at 400 µl/min split 1:100 by an Agilent splitting device. Sheath liquid was 1 % formic acid in 50 % methanol for positive ion detection.

Figure 1 shows hesperetin (a glycon of the hesperidine), found at m/z value 325 (the Na adduct mass). Naringin was found under these conditions as the protonated adduct at m/z 581. Besides the qualitative aspects of identifying compounds by mass spectra CE-MS can also provide quantitative information. The calibration curve shown in figure 2 resulted from a naringin standard diluted in a range from 200 to 2 µg/ml. The lowest amount used here still gave a reasonable signal larger than 5:1 s/n. Figure 3 shows the MS trace of 2 µg/ml naringin.

Equipment

- Agilent Capillary Electrophoresis system
- Agilent CE-MS Adapter Kit
- Agilent 1100 Series LC/MSD module with API Electrospray Source
- Agilent CE-ESI-MS Sprayer Kit
- Agilent ChemStation +CE-MS Add-on software



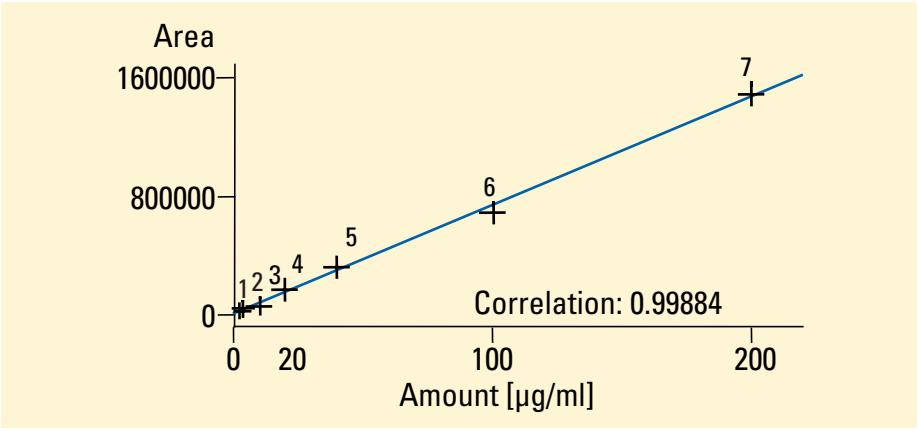


Figure 2
Linearity of naringin detection with MS

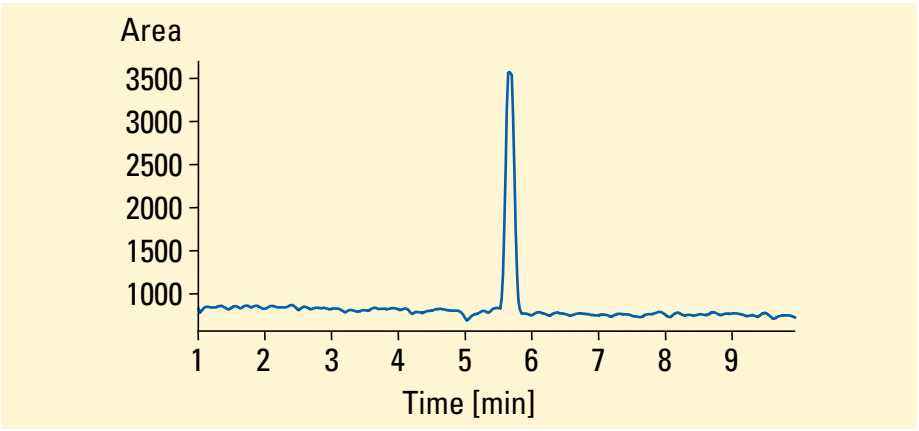


Figure 3
Analysis of 2 µg/ml naringin

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Publication Number 5968-5729E

Agilent Technologies
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Analysis of Bitter Compounds, in Orange Juice using HPLC

Angelika Gratzfeld-Huesgen

Food

Abstract

The following compounds are examples of flavoring agents used in food products:

- lupulon and humulon (hop bittering compounds)
- vanillin
- naringenin and hesperidin (bittering compounds)

Three major classes of compounds are used as flavoring agents: essential oils, bitter compounds, and pungency compounds. Although the resolution afforded by gas chromatography (GC) for the separation of flavor compounds remains unsurpassed, HPLC is the method of choice if the compound to be analyzed is low volatile or thermally unstable.

Sample preparation for bitter compounds in orange juice[1]

The samples were prepared according to Carrez 1 and 2. This method uses potassium ferrocyanide and zinc sulfate for protein precipitation.

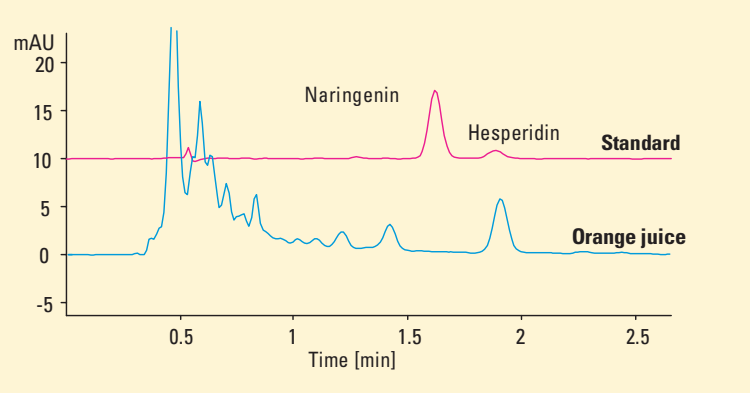

Figure 1
Analysis of bitter ccmpounds in orange juices

Conditions

Column 125 ˘ 4 mm Hypersil BDS, 5 µm
Mobile phase
A = water + 0.15ml/l H_2SO_4 (conc.), pH = 2.4
B = ACN
Gradient
start with 20% B; at 3 min 20% B
at 5 min 90% B; at 6 min 20% B
Flow rate 2 ml/min
Post time 1 min
Column compartment 40 °C
Injection vol 1 µl
Detector
UV-DAD detection wavelength 260/80 nm, reference wavelength 380/80 nm
Sample preparation
The orange juice was prepared according to Carrez 1 and 2

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Chromatographic conditions

The HPLC method presented here for the analysis of hesperidin and naringenin is based on reversed-phase chromatography. UV spectra were evaluated as an additional identification tool.

HPLC method performance

Limit of detection
1 ng (injected amount),
for DAD S/N = 2

Repeatability
of RT over 10 runs <0.2 %
of areas over 10 runs <1 %

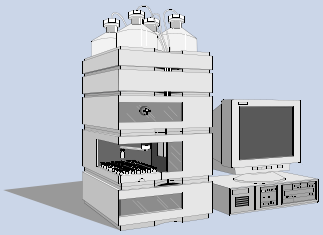
References

1.
Official Methods of Analysis; Horwitz, W., Ed.; 14th ed.; AOAC: Arlington, VA, **1984**; secs 12.018–12.021.

Equipment

Agilent 1100 Series
- vacuum degasser
- quaternary pump
- autosampler
- thermostatted column compartment
- diode array detector,

Agilent ChemStation + software



Angelika Gratzfeld-Huesgen is application chemist at Agilent Technologies, Waldbronn, Germany.

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Released 09/97
Publication Number 5966-0631E

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Miscellaneous

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Analysis of Saponins in Spine Date Seed using Agilent ZORBAX Solvent Saver HT (1.8 µm) Columns

Application Note

Food and Traditional Chinese Medicine

Author

Rongjie Fu
Agilent Technologies, Inc.
412 Ying Lun Road
Pu Dong, Shanghai 200131
China

Abstract

The two main active compounds in spine date seed, the saponins Jujuboside A and B, were extracted and analyzed by high performance liquid chromatography (HPLC) with Agilent ZORBAX Eclipse Plus C18 LC columns. The method was first developed on a traditional 4.6 mm × 150 mm, 5 µm column and then transferred to a ZORBAX Eclipse Plus Solvent Saver High Throughput (1.8 µm) C18 column, 3.0 mm × 50 mm, 1.8 µm. The advantages of the Solvent Saver HT column include low solvent consumption and faster analysis. An evaporative light scattering detector (ELSD) was used due to the lack of chromophores in the two compounds. This provided higher sensitivity and a cleaner baseline compared with UV detection.

Agilent Technologies

Introduction

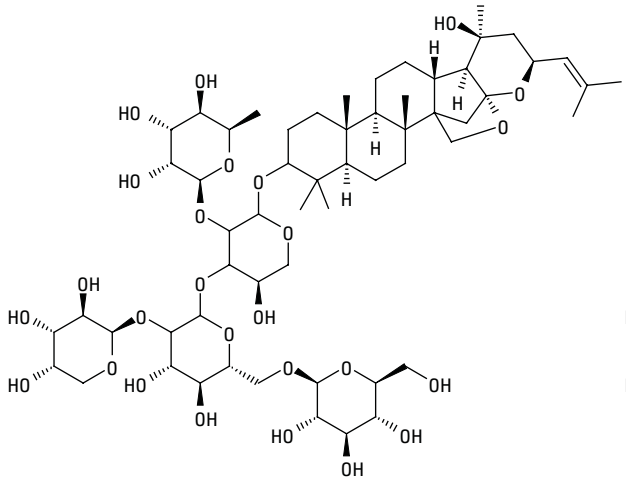
Jujube has been a part of food and Chinese medicines for at least 2,500 years. The Chinese used it as a Qi tonic to strengthen liver function. Jujube has a pleasant taste as well as a high nutritional value. The fruit contains vitamins A, B2, and C, and saponins, flavonoids, sugars, mucilage, calcium, phosphorus, and iron. Europeans have recognized its usefulness as a tonic for all parts of the body, especially the lungs and kidneys even though since the 17th century, the West dismissed Jujube as not having any medicinal qualities.[1]

Jujube seeds, or the English name spine date seeds, are used for medicinal purposes. Saponin compounds are also present in many plants such as Ginseng and Notoginseng, and have been the subject of research for a long time. The saponin compounds have a triterpenes structure, and many other triterpenes have been proven to have therapeutic effects.

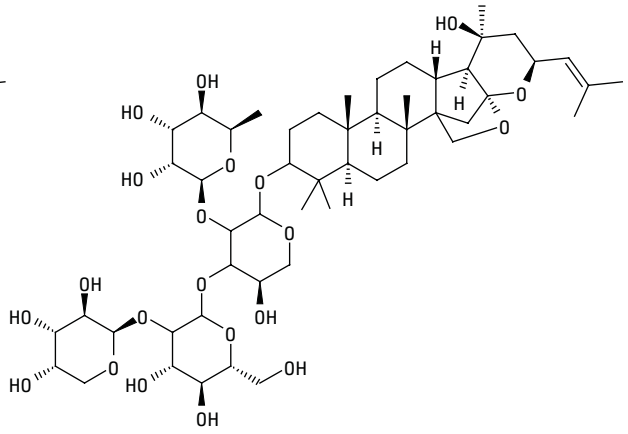
Scientific studies with animals have shown Jujubosides to have anti-anxiety and hypnotic effects, causing a reduction in the speed of conditioned reflexes, a reduction in hyperactivity, and a lowering of blood pressure. [1] Therefore, they can be used as a remedy for irritability, insomnia, anxiety, oedema, congestive heart failure, asthma and throat diseases.

The structures of the two main saponin compounds in spine date seeds, Jujubosides A and B, are shown in Figure 1. Since these compounds lack a chromophore and do not produce a sufficient signal with an ultraviolet (UV) detector, the ELSD which is a universal detector, was used in this method.

In this application note, the separation of the Jujobosides on the ZORBAX Solvent Saver RRHT column (3.0 mm × 50 mm, 1.8 µm) was compared to the separation on a traditional ZORBAX column (4.6 mm × 150 mm, 5 µm).



Jujuboside A MW 1207.36



Jujuboside B MW 1045.22

Figure 1. Structures of Jujubosides.

Experimental

Sample preparation

Spine date seeds were purchased at a pharmacy (Qun-Li TCM Pharmacy). The sample was treated as follows to extract the target compounds for analysis. [2]

- Weigh 5.0 g of the dried powder.
- Degrease with Soxhlet extractor using 50 mL ethyl ether for 4 h.
- Filter after cooling down and discard the liquid phase, then dry the residues.
- Put the residues in a 250-mL round-bottom flask, add 50 mL of methanol and extract for 2 h.
- Repeat and mix the extracts.
- Recover the methanol under vacuum and add 20 mL of water to the residue to dissolve.
- Extract using 15 mL of water saturated n-Butanol 3 times, then mix the extracts and evaporate under vacuum.
- Dissolve the residue with up to 5.0 mL methanol. Filter this final sample with a 0.45-µm Regenerated Cellulose Membrane filter (p/n: 5064-8221) before injecting into HPLC for analysis.

HPLC conditions

The HPLC analysis was performed with the Agilent 1200 Series Rapid Resolution LC (RRLC) system including a G1312B binary pump SL, G1376C automatic liquid sampler SL (ALS), G1316B Thermostated Column Compartment SL (TCC), G1316C Diode Array Detector SL (DAD) and G4218A Evaporative Light Scattering Detector (ELSD).

Mobile Phase:	35% water, 65% methanol
Flow rate:	1mL/min for 4.6 mm × 150 mm, 5 µm; 0.4 mL/min for 3.0 mm × 50 mm, 1.8 µm
Injection volume:	5 µL for 4.6 mm × 150 mm, 5 µm; 2 µL for 3.0 mm × 50 mm, 1.8 µm
Column:	ZORBAX Eclipse Plus C18, 4.6 mm × 150 mm, 5 µm (p/n: 959993-902) and 3.0 mm × 50 mm, 1.8 µm (p/n: 959941-302)
UV:	210 nm
TCC temp:	30 °C
ELSD temp:	40 °C
ELSD pressure:	3.5 bar
ELSD gain:	7
ELSD filter:	5s for 4.6 mm × 150 mm and 2s for 3.0 mm × 50 mm

Results and Discussion

The two compounds were separated well on the ZORBAX Eclipse Plus C18 column (4.6 mm × 150 mm, 5 µm) with excellent peak shape using ELSD detection as shown in Figure 2. For this complex sample matrix, many of the potential compounds have absorption below 210 nm, which made it difficult to get a clean baseline. The two target peaks detected at UV 210 nm were not well resolved from matrix compounds, which results in a potential error in quantitative results. The ELSD gave higher sensitivity compared to UV detection and a cleaner baseline with fewer matrix analytes. The two peaks of interest were baseline resolved.

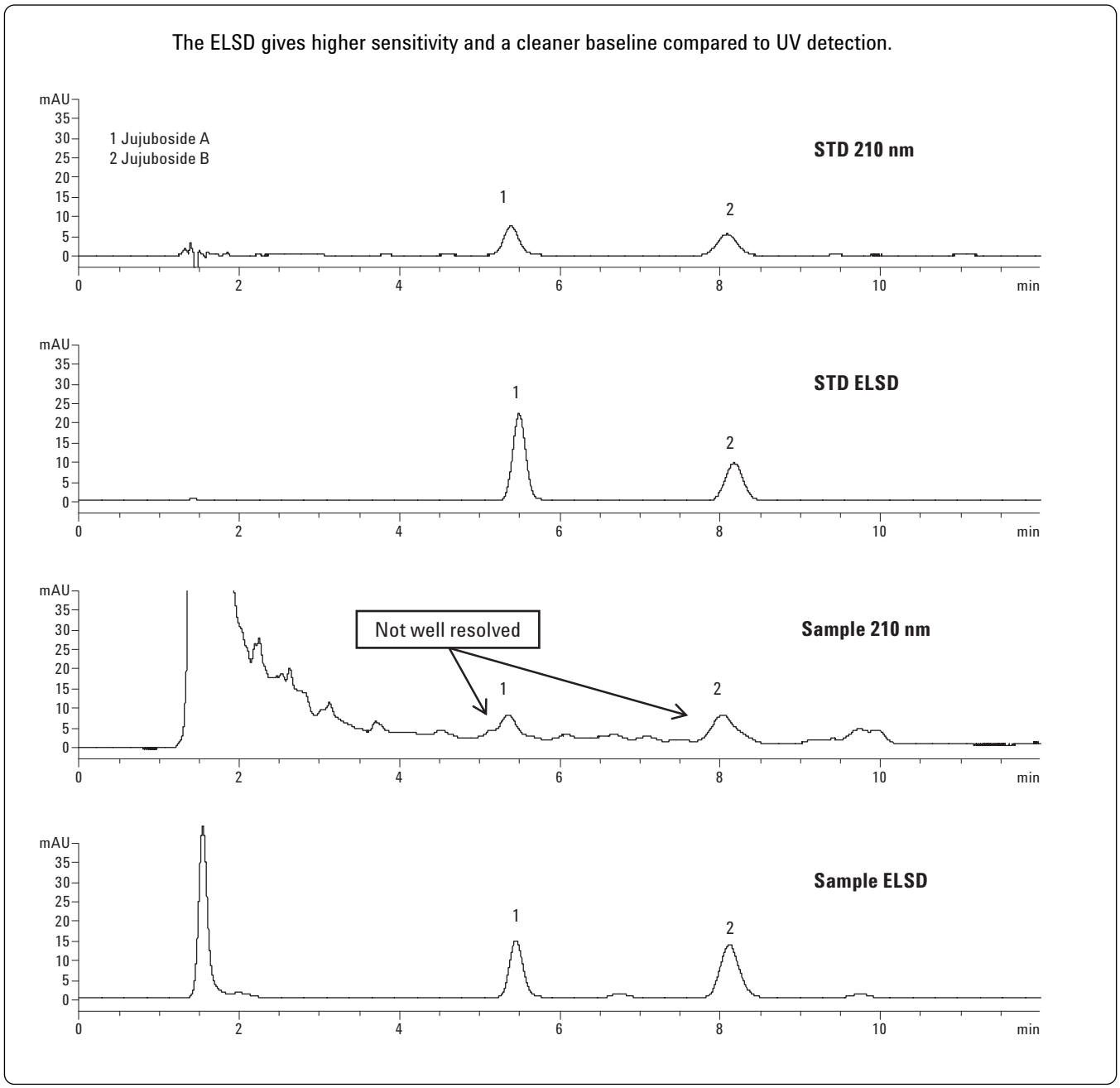


Figure 2. Standards and sample chromatograms by UV 210 nm/ELSD on Agilent ZORBAX Eclipse Plus C18, 4.6 mm × 150 mm, 5 µm.

The method developed in this analysis is a conventional method with about a 12-min analysis time and 12 mL of solvent consumption in one injection. The method can be easily adapted to a Solvent Saver HT column to achieve a savings in time and the amount of solvent used (Figure 3).

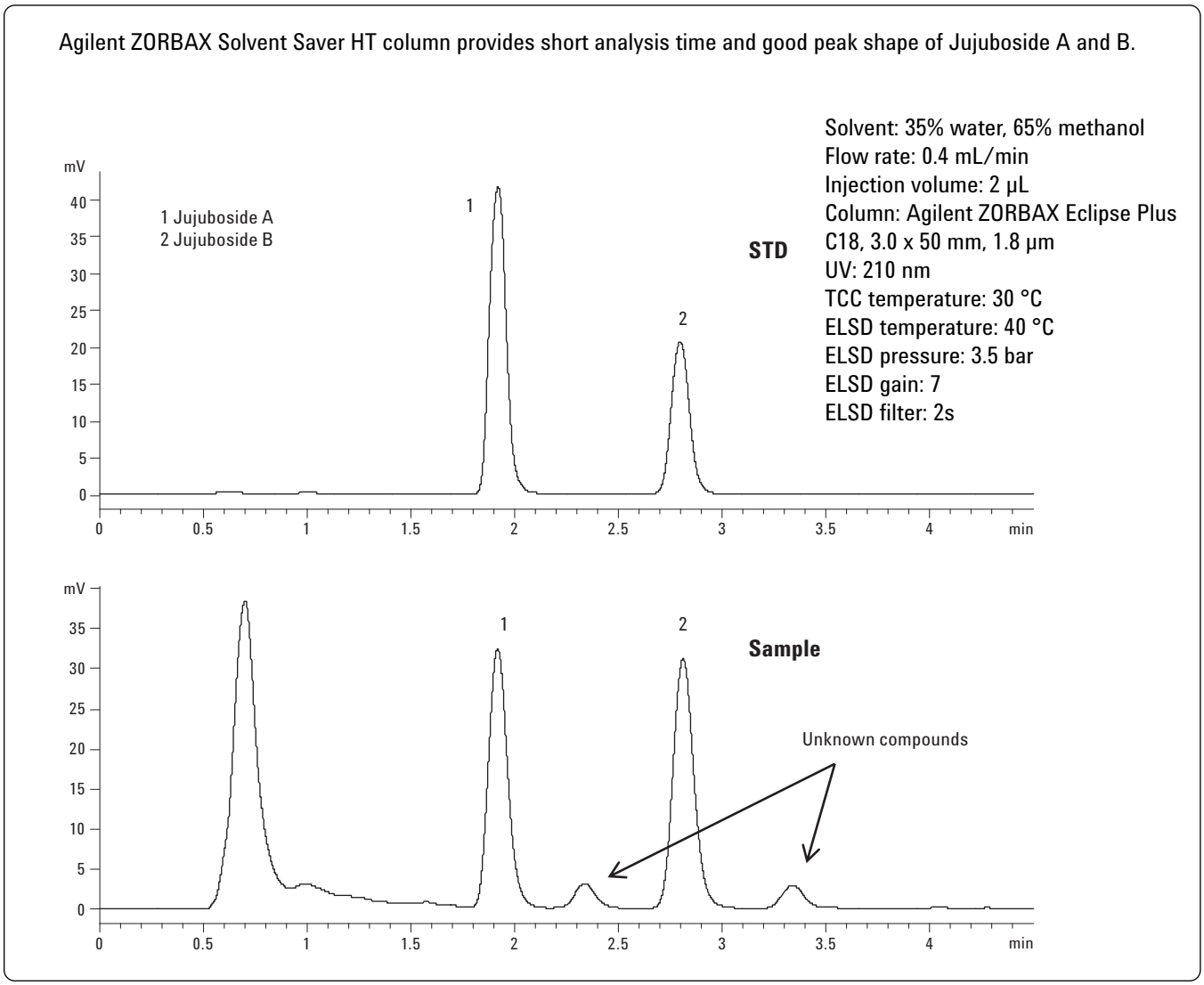


Figure 3. Standards and sample chromatograms by ELSD on Agilent ZORBAX Eclipse Plus C18, 3.0 mm × 50 mm, 1.8 µm.

Figure 4 shows the comparison of analysis time and solvent consumption between 4.6 mm × 150 mm and 3.0 mm × 50 mm columns. Changing from a 4.6 mm × 150 mm column with a 5 µm particle size to 3.0 mm × 50 mm column with a 1.8-µm particle size allows a savings of 87 percent of the solvent and 67 percent of the time. The peaks 1 and 2 show the same resolution with unknown sample compounds.

The method on 3.0 mm × 50 mm column can still be run on a standard LC instrument [3], but optimization may be required to minimize extra column volume. The ELSD detector parameters must also be optimized. When the column in the method was first switched to 3.0 mm × 50 mm, 1.8 µm column, all the parameters of the ELSD remained the same. However, the heights of the two target peaks on the 3.0 mm × 50 mm column were not consistent with the peak height obtained on the 4.6 mm × 150 mm 5 µm column. This resulted in lower theoretical plates of over two thousand for peak 1 and over four thousand for peak 2. Two parameters of the ELSD were optimized. When the sampling rate of ELSD was changed from 2.5 Hz to 30 Hz there was minimal improvement. However, when the filter value was adjusted between OFF, 2, 5 and 8, the theoretical plates increased dramatically as the filter value decreased. Baseline noise also increased simultaneously. In order to determine the ideal signal to noise value, the filter value was set to 2, which provided theoretical plates of over five thousand for peak 1 and over eight thousand for peak 2. The height of the two peaks was then consistent between the two columns, as shown in Figure 4.

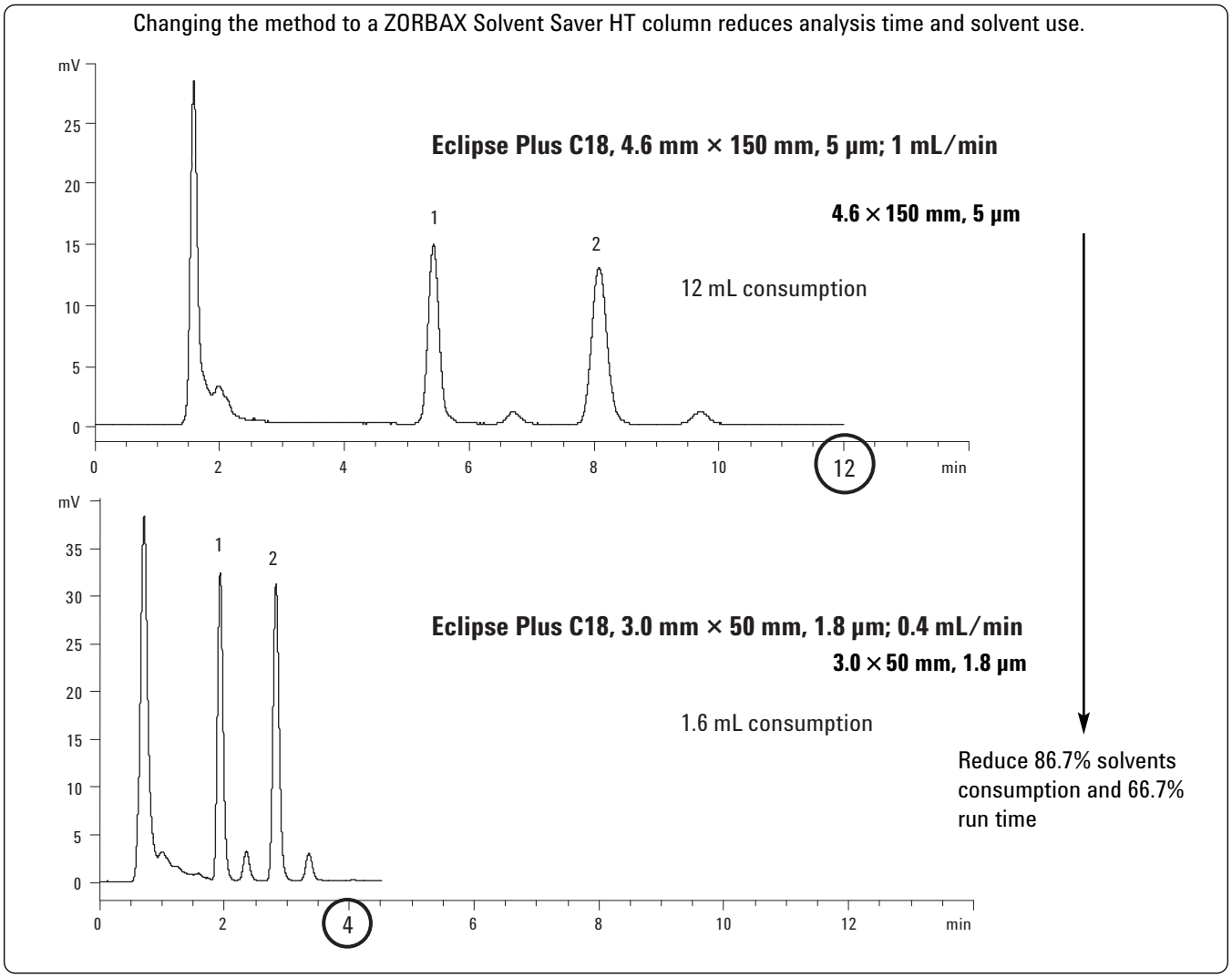


Figure 4. Comparison of the methods developed on Eclipse Plus C18 4.6 mm × 150 mm, 5 µm and Eclipse Plus C18 3.0 mm × 50 mm, 1.8 µm.

Conclusion

ZORBAX Eclipse Plus C18 can easily separate Jujoboside A and B, the two saponins of interest in spine date seed, with high efficiency and symmetrical peaks. The method developed on a 3.0 mm id Solvent Saver column dramatically reduced the solvent used and the analysis time, increasing sample throughput. An ELSD gives a cleaner baseline and higher sensitivity when separating these saponins, which makes it a better detector for these kinds of compounds in complex matrices such as traditional Chinese medicine.

Reference

1. http://www.mdidea.com/products/new/new029.html, spine date seed
2. Li Huijun and Li Ping, "Determination of Jujuboside A and Jujuboside B in Spine Date Seed by HPLC-ELSD," *Chinese Journal of Pharmaceutical Analysis*, 2000,20(2): 82-84.
3. "LC Column for Reducing Solvent Use and Waste," Agilent Technical Overview, 5990-3972EN

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June 16, 2009
5990-4176EN

Agilent Technologies

Determination of Hormones in Fish (*Carassius Carassius*) by SampliQ-OPT Solid Phase Extraction with High Performance Liquid Chromatography

Application Note

Food Safety

Authors

Chen-Hao Zhai and Yun Zou
Agilent Technologies Co. Ltd.
412 Ying Lun Road
Waigaoqiao Free Trade Zone
Shanghai 200131
China

Rou-Nan Jin
Second Military Medical University
259 Changhai Road
Shanghai 200093
China

Abstract

Solid-phase extraction (SPE) coupled with high performance liquid chromatography (HPLC) was optimized for the extraction and determination of sixteen hormones (estriol, prednisolone, hydrocortisone, prednisone, methylprednisolone, betamethasone, dexamethasone, triamcinolone acetate, gestrinone, prednisolone acetate, hydrocortisone acetate, prednisone acetate, estradiol, cortisone acetate, methyltestosterone, estrone) in crucian carp (*Carassius carassius*) meat. Results indicate that SPE using an Agilent SampliQ OPT (60 mg, 3 mL) and HPLC using an Agilent ZORBAX Eclipse Plus C18 column (4.6 mm × 250 mm, 5 µm) is suitable for extraction of these compounds. Recoveries ranged from 76.2 to 106.1 % with relative standard deviations (RSDs) between 1.7 and 8.9 %.

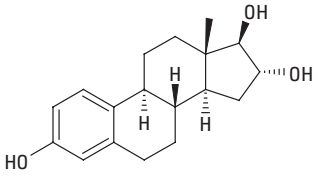
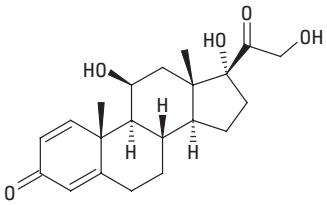
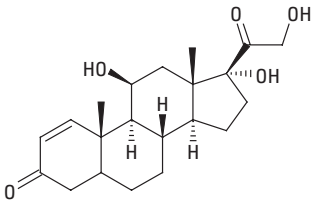
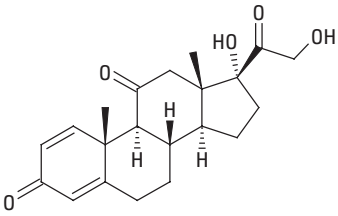
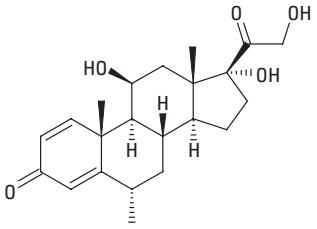
Agilent Technologies

Introduction

Food safety has increasingly become an important concern of people worldwide. Many chemicals added to food create potential hazards to human health. Hormones are a common food additive. Long-term consumption of glucocorticoid can lead to hyperglycemia, osteoporosis, birth defects, and immune function decline. Other hormones such as estrogen, androgen, and progesterone are carcinogenic and can lead to breast cancer, ovarian cancer and cell carcinoma. Many countries' regulations clearly define residual limits for these compounds in food.

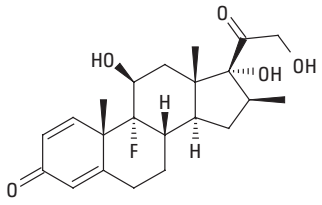
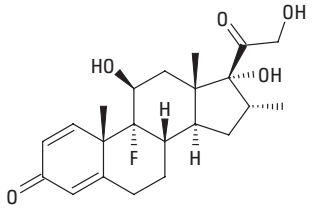
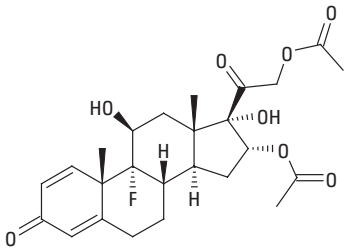
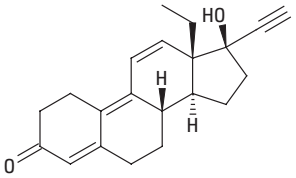
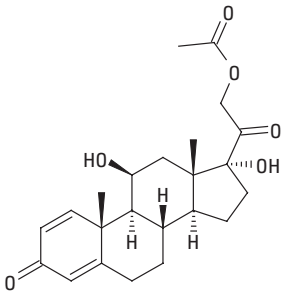
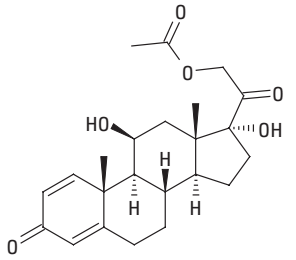
An Agilent SampliQ OPT SPE cartridge was used to extract 16 kinds of hormones (Table 1) from crucian meat and an HPLC method was established to detect these 16 compounds.

Table 1. Hormones Used in this Study

No.	Name	CAS No.	Log P	Structure
1	Estriol	50-27-1	2.45	
2	Prednisolone	50-24-8	1.66	
3	Hydrocortisone	50-23-7	1.79	
4	Prednisone	53-03-2	2.07	
5	Methylprednisolone	83-43-2	2.06	

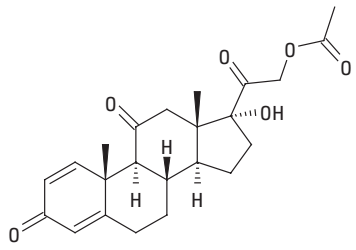
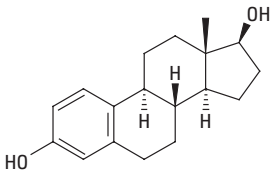
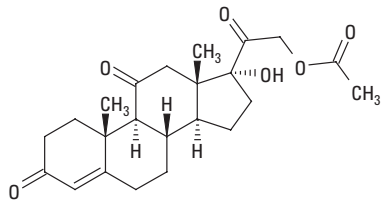
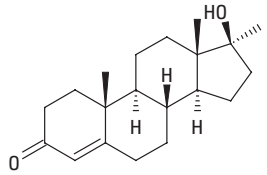
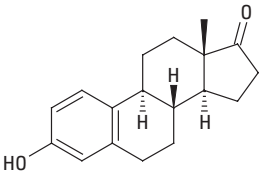
(Continued)

Table 1. Hormones Used in this Study

No.	Name	CAS No.	Log P	Structure
6	Betamethasone	378-44-9	1.93	
7	Dexamethasone	50-02-2	1.93	
8	Triamcinolone acetate	67-78-7	1.9	
9	Gestrinone	16320-04-0	NA	
10	Prednisolone acetate	52-21-1	NA	
11	Methylprednisolone	83-43-2	NA	

(Continued)

Table 1. Hormones Used in this Study

No.	Name	CAS No.	Log P	Structure
12	Prednisone acetate	125-10-0	NA	
13	Estradiol	50-28-2	3.57	
14	Cortisone acetate	50-04-4	2.35	
15	Methylestosterone	58-18-4	NA	
16	Estrone	53-16-7	4.03	

Experimental

Reagents and Chemicals

All reagents and solvents were HPLC or analytical grade. Hormone standards were purchased from NICPBP (National Institute for the Control of Pharmaceutical and Biological Products). Crucian was purchased from a local market.

Stock solutions (1 mg/mL) were prepared in methanol and kept in the freezer (–20 °C). Working solutions were prepared using the stock solution diluted with methanol. The working solutions should be prepared every week and need to be stored below 4 °C.

The SPE cartridges were Agilent SampliQ OPT (3 mL, 60 mg, p/n 5982-3036). The analysis was performed on an Agilent 1200 Series HPLC with a diode array detector (DAD). The analytical column was an Agilent ZORBAX Eclipse Plus C18 (5 µm 250 mm × 4.6 mm id, p/n 959990-902). An Agilent 0.45-µm PTFE Premium Syringe Filter (p/n 5185-5836) was used to filter the sample solution before HPLC.

HPLC conditions

Column:	ZORBAX Eclipse Plus C18 250 mm × 4.6 mm, 5 µm		
Flow rate:	1.0 mL/min		
Injection volume:	5 µL		
Column temperature:	18 °C		
Detection wavelength:	230 nm		
Mobile phase:	Water-Acetonitrile Gradient		
	Time (minutes)	% Water	% Acetonitrile
	0	70	30
	10	65	35
	23	50	50
	30	20	80

Separation

1. Weigh 200 grams of crucian meat, homogenize, and store in a clean, sealed container at –18 °C.
2. Place 1 g of homogeneous sample (accurate to 0.01 g) into a 10-mL polypropylene centrifuge tube with 5 mL of methanol.
3. Vortex for 1 minute.
4. Extract ultrasonically for 10 minutes in an ice bath.
5. Centrifuge the sample at a speed of 4000 r/min for 5 minutes and remove the 3 mL of supernatant.
6. Save in a clean tube and evaporate with N_2 below 40 °C.
7. Reconstitute the residue in 5 mL of 5 % methanol in water.

SPE Purification

The procedure used for the SPE extraction is shown in Figure 1. Agilent SampliQ OPT cartridges are preconditioned with 3 mL of methanol then 5 mL of water. The 5-mL extract (equivalent to 0.6 g sample) is passed through the SampliQ OPT cartridge at a speed of 1 mL/min. After it effuses completely, the cartridge is washed with 5 mL of 30% methanol in water and the entire effluent is discarded. The cartridge is dried under negative pressure (below 2.0 kPa) for 3 minutes. The sample is then eluted with 6 mL of methanol, and the eluent is collected and dried under nitrogen below 40 °C. The residue is dissolved and brought to a constant volume of 1.0 mL using methanol, filtered through a 0.45 µm PTFE filter membrane, and analyzed by HPLC.

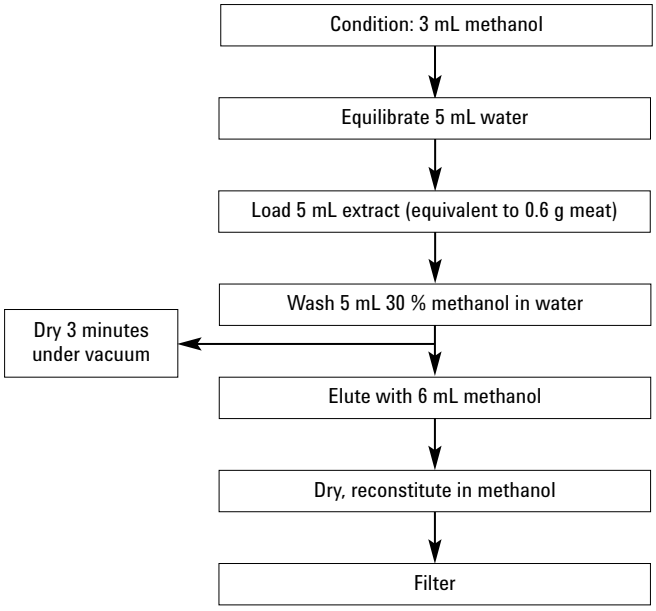


Figure 1. Hormones in crucian meat SPE procedure.

Results and Discussion

Linearity, Limits of Detection

Stock solutions were diluted to different concentrations and analyzed by HPLC. Linear regressions were calculated for the hormones based on the areas and the solution concentrations. Limit of detection (LOD) signifies the injection concentration at which the signal to noise ratio was between 2 and 3. Linear range was between 1–100 mg/kg. The linearity and LOD are shown in Table 2.

Table 2. Linearity and LODs of Hormones.

No.	Compound	Regression equation	Correlation coefficient	LOD (mg/kg)
1	Estriol	Y = 8.096 × –0.824	0.9998	0.5
2	Prednisolone	Y = 17.418 × –2.088	0.9999	0.2
3	Hydrocortisone	Y = 15.746 × –1.518	0.9999	0.3
4	Prednisone	Y = 20.192 × –2.152	0.9998	0.2
5	Methylprednisolone	Y = 16.986 × –1.894	0.9999	0.4
6	Betamethasone	Y = 20.439 × –1.106	0.9997	0.2
7	Dexamethasone	Y = 20.176 × –2.176	0.9999	0.2
8	Triamcinolone acetate	Y = 16.374 × –1.558	0.9997	0.4
9	Gestrinone	Y = 6.370 × –0.668	0.9998	1.0
10	Prednisolone acetate	Y = 15.589 × –1.627	0.9999	0.4
11	Hydrocortisone acetate	Y = 15.051 × –1.584	0.9999	0.4
12	Prednisone acetate	Y = 24.106 × –2.401	0.9997	0.2
13	Estradiol	Y = 8.709 × –0.635	0.9999	0.8
14	Cortisone acetate	Y = 19.826 × –2.336	0.9996	0.4
15	Methyltestosterone	Y = 19.980 × –2.209	0.9996	0.3
16	Estrone	Y = 10.701 × –0.847	0.9999	0.4

Recovery and Repeatability

The precision of the method was determined in terms of the recovery of spiked hormone standards in crucian meat at 2, 5, and 10 mg/kg levels. The analysis was repeated six times at each level. The chromatograms of the blank, the standards, and the spiked standard (2 mg/kg) sample are shown in Figures 2 through 4. The recovery and reproducibility data are shown in Table 3.

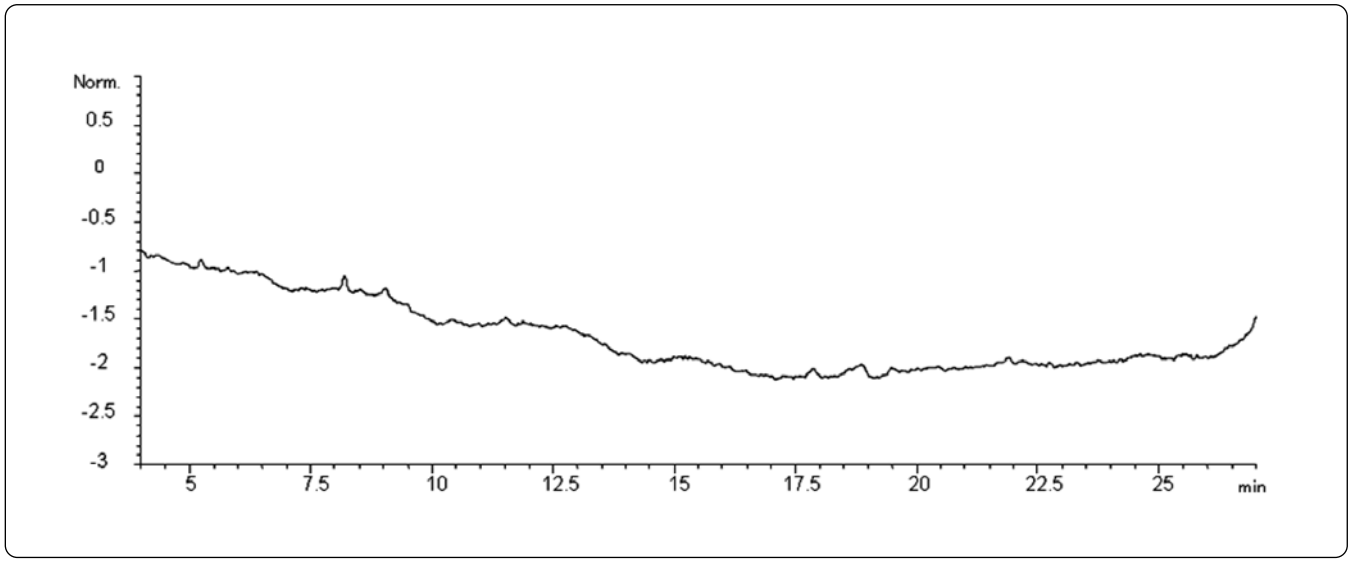
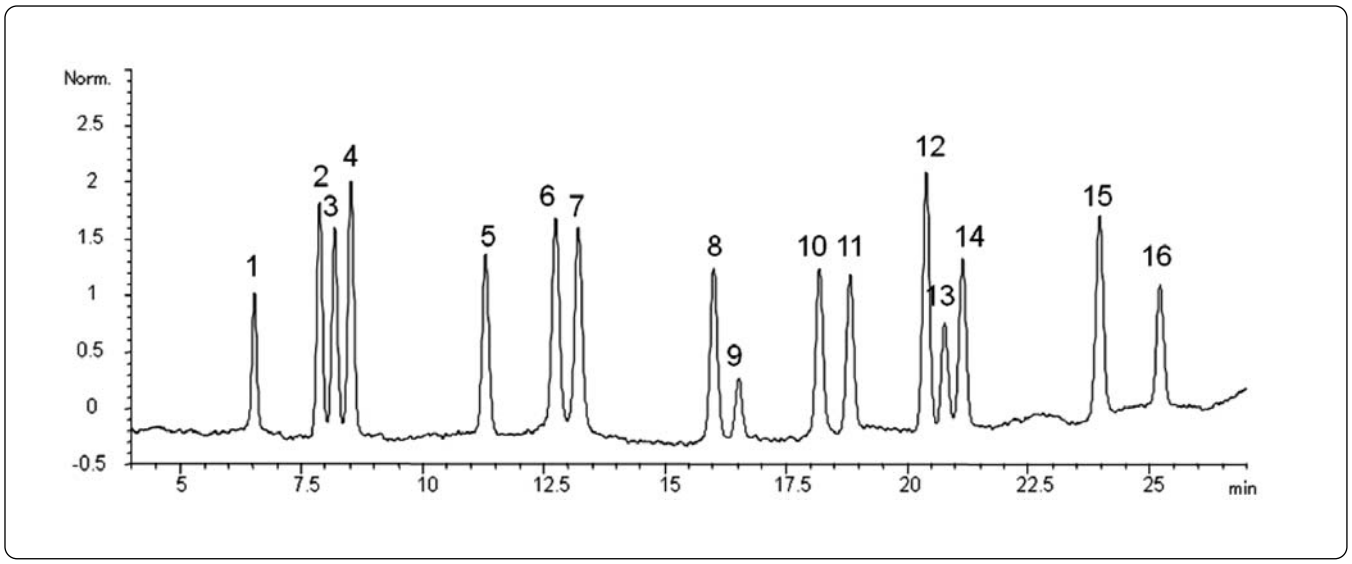
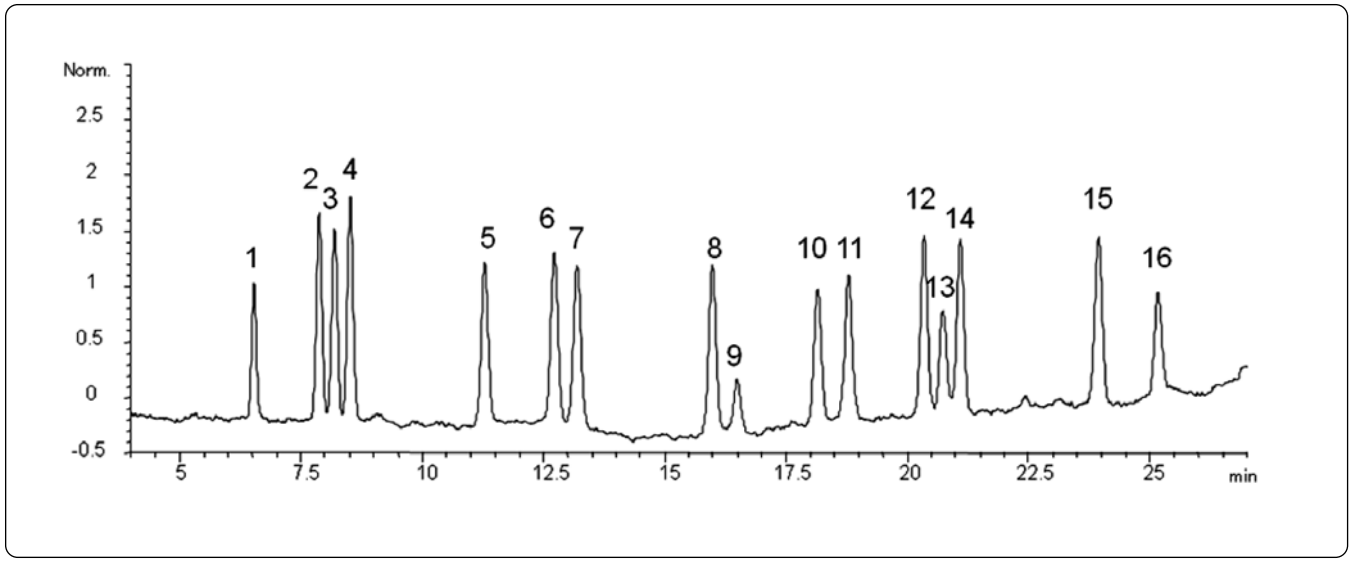


Figure 2. Chromatogram of crucian meat blank.



1 Estriol	5 Methylprednisolone	9 Gestrinone	13 Estradiol
2 Prednisolone	6 Betamethasone	10 Prednisolone acetate	14 Cortisone acetate
3 Hydrocortisone	7 Dexamethasone	11 Hydrocortisone acetate	15 Methyltestosterone
4 Prednisone	8 Triamicinolone acetate	12 Prednisone acetate	16 Estrone

Figure 3. Chromatogram of hormone standards at 2 mg/kg.



1 Estriol	5 Methylprednisolone	9 Gestrinone	13 Estradiol
2 Prednisolone	6 Betamethasone	10 Prednisolone acetate	14 Cortisone acetate
3 Hydrocortisone	7 Dexamethasone	11 Hydrocortisone acetate	15 Methyltestosterone
4 Prednisone	8 Triamicinolone acetate	12 Prednisone acetate	16 Estrone

Figure 4. Chromatogram of crucian meat sample spiked hormone standards at 2 mg/kg.

Table 3. Recoveries and RSDs of Hormones in Crucian Meat by SPE

Compund	Spiked level (mg/kg)	Recovery (%)	RSD (n = 6, %)
Estriol	2	100.4	2.2
	5	106.1	1.9
	10	102.4	4.4
Prednisolone	2	89.4	3.8
	5	90.9	7.6
	10	100.7	2.9
Hydrocortisone	2	85.3	6.7
	5	91.4	7.6
	10	101.4	3.4
Prednisone	2	82.5	7.2
	5	92.1	5.2
	10	100.7	2.9
Methylprednisolone	2	83.2	8.3
	5	93.6	3.2
	10	97.4	1.7
Betamethasone	2	88.3	8.9
	5	99.6	4.9
	10	100.8	3.8
Dexamethasone	2	79.1	4.3
	5	98.4	5.3
	10	98.4	3.9
Triamcinolone acetate	2	86.7	8.4
	5	97.6	5.9
	10	97.9	4.1
Gestrinone	2	78.0	6.6
	5	78.8	8.1
	10	85.3	8.0
Prednisolone acetate	2	86.9	7.3
	5	101.2	4.3
	10	101.9	5.7
Hydrocortisone acetate	2	87.3	6.8
	5	102.7	5.1
	10	101.5	7.9
Prednisone acetate	2	76.7	7.7
	5	94.1	3.5
	10	97.7	4.3
Estradiol	2	78.7	4.2
	5	94.7	3.5
	10	97.4	4.8
Cortisone acetate	2	82.8	6.9
	5	87.8	6.5
	10	94.4	4.1
Methyltestosterone	2	82.9	3.4
	5	91.9	4.9
	10	93.6	4.6
Estrone	2	76.2	6.4
	5	90.0	8.7
	10	93.9	5.9

Conclusions

Agilent's SampliQ OPT, a polymeric sorbent with combined hydrophilic and lipophilic characteristics that allows retention of both polar and non-polar compounds, provides a simplified and effective single cartridge method for the purification and enrichment of multiple hormone compounds in crucian carp. Recovery and reproducibility (routinely below 10%) based on solution standards are acceptable for hormone residue determination in crucian meat. Impurities from crucian were minimal and did not interfere with any of the hormones analyzed.

Product Information

Part number	Description
5982-3013	OPT Polymer - Box, 100x 1 mL tubes, 30 mg
5982-3036	OPT Polymer - Box, 50x 3 mL tubes, 60 mg
5982-3067	OPT Polymer - Box, 30x 6 mL tubes, 150 mg
5982-3096	OPT Polymer - 96 Well Plate, 10 mg
95990-902	Agilent ZORBAX Eclipse Plus C18 250 mm × 4.6 mm, 5 µm
5185-5836	Agilent PTFE 0.45 µm Premium Syringe Filter

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Agilent Technologies

Development of an LC-MS/MS Method for the Determination of 20-Hydroxyecdysone and Its Metabolites in Calf Urine

Application to the Control of Its Potential Misuse in Cattle

Application Note

Food Safety

Authors

Blandine Destrez, Gaud Pinel, Fabrice Monteau, and Bruno Le Bizec
Laboratoire d'Etude des Résidus et Contaminants dans les Aliments (LABERCA), Ecole Nationale Vétérinaire de Nantes (ENVN),
BP 50707, 44307 NANTES, Cedex 3, France

Abstract

Ecdysteroids, which are steroid hormones present in invertebrates and in plants, could be potentially used as anabolic agents in food-producing animals. The control of ecdysteroid misuse in cattle relies on the development of an efficient method for their detection in biological matrices at trace levels ($\mu g.L^{-1}$). In this context, an analytical procedure dedicated to the identification of 20-hydroxy-ecdysone and its metabolites in urine samples, based on purification on two solid-phase extraction cartridges (SPE C_{18} and SPE SiOH) and LC-(ESI+)-MS/MS measurements has been developed. The performance of tandem quadrupole MS/MS, in terms of sensitivity and specificity, allowed measurements at trace levels in both spiked and incurred samples. Good linearity was observed for all analytes from 0.12 ng to 12 ng on column.

Agilent Technologies

Introduction

Ecdysteroids are steroid hormones present both in invertebrate species (mainly Arthropods) and plants (belonging to *Asteraceae, Caryophyllaceae,* or *Polypodiaceae*). In arthropods, ecdysteroids act as moulting hormones, whereas these molecules are thought to protect plants against nonadapted phytophagous insects. The archetypal ecdysteroid in both kingdoms is 20-hydroxyecdysone (20E), and several studies have underlined its possible growth-promoting effects in various animal species (rats, mice, and Japanese quail), including humans and cattle [1–3]. Clinical studies demonstrated that 20E is more anabolic than methandrostanolone (dianabol), with no androgenic or other undesirable side effects usually observed with classical steroids [4]. However, despite its growth-promoting properties, only a few methods have been reported for its detection in biological matrices, and no information is available concerning its metabolism in cattle [5]. In this application, the development of a method able to detect and identify 20E and its main metabolites at trace levels (ppb) in calf urine is described [6]. This method was applied to the analysis of calf urine samples after 20E oral administration and used to assess the kinetic of elimination of these substances.

Experimental

Compound Standards

Standard reference 22S,23S-homobrassinolide (belonging to brassinosteroids, vegetable steroid hormones) was from Sigma-Aldrich (St. Quentin Fallavier, France); 20-hydroxyecdysone, 14-deoxy, 20-hydroxyecdysone, and 20,26-dihydroxyecdysone were a kind gift from Pr. Lafont.

Sample Preparation

Twenty-five nanograms of 22S,23S-homobrassinolide were added as internal standard (IS) to 5 mL of calf urine, centrifuged at 3,500 g for 15 min, then purified on SPE C18. The C18-SPE cartridges were conditioned with 10 mL methanol, then 10 mL water, following which the urine samples were applied. The columns were then washed with 6 mL of a water/methanol (80/20) mixture, and the ecdysteroids were subsequently eluted with 10 mL methanol. The eluant was then evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted in 50 µL ethanol and 150 µL cyclohexane before loading onto a SPE SiOH, previously activated with 25 mL cyclohexane. The phase was washed with 6 mL ethyl acetate/cyclohexane (80/20) and the compounds of interest were then eluted with 10 mL of a mixture of chloroform/methanol/acetone (6/2/1). The solvent was evaporated to dryness under nitrogen and the final extract was redissolved in 50 µL of methanol/water (30/70) containing 0.5% acetic acid. From this extract 10 µL was injected onto the HPLC column.

Instrumentation

LC:

Column:	GEMINI C_{18}, Phenomenex (3 µm, 110 Å, 50 × 2 mm)/Agilent equivalent: ZORBAX Extend-C18 3.5 µm, 2.1 mm × 50 mm (p/n 735700-902)	
Column temperature:	40 °C	
Mobile phases:	A: MeOH B: 0.5% acetic acid in water	
Flow rate:	0.3 mL/min	
Gradient:	Time (min)	%B
	0	90
	8	0
	10	0
	12	90
	16	90
Injection volume:	10 µL	
MS:	G 6410A QQQ, Agilent Technologies	
Ionization:	ESI (+)	
Fragmentor:	120 V	
Mass range:	100–500 amu	
Scan time:	300 ms	
Capillary:	4000 V	
Nebulizer:	35 psi	
Drying gas:	11 L/min	
Gas temperature:	325 °C	

The monitored transitions for each target compound are reported in Table 1. The first transition corresponds to the most sensitive signal.

Results and Discussion

Standard solutions of target compounds were analyzed according to the LC-MS/MS parameters described in the Experimental section, which allowed us to obtain the ion chromatograms of 20E, M1, M2, and IS, each at 5 ng on column (Figure 1). All the compounds are eluted within less than 10 min with very good chromatographic resolution and peak shape.

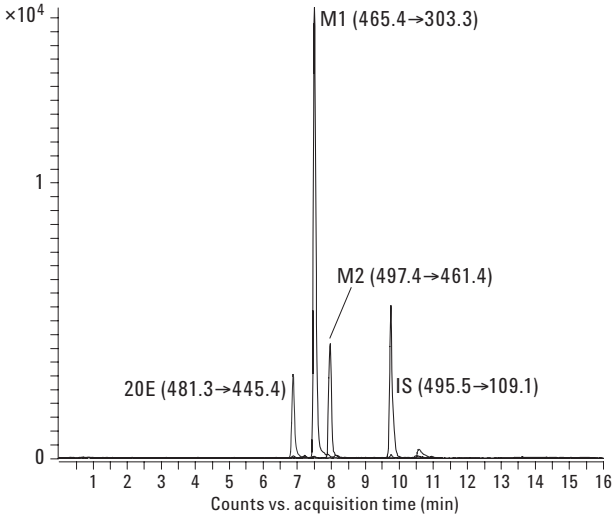

Figure 1. Overlaid extracted ion chromatograms (EICs) for the most sensitive transitions monitored for 20E and its metabolites in positive ion mode.

Table 1. Monitored SRM Transitions for 20E and Its Main Urinary Metabolites and Parameters of Acquisition for Their Analysis by LC-MS/MS (QQQ)

Analytes	Transition 1	Collision energy (eV)	Transition 2	Collision energy (eV)	Transition 3	Collision energy (eV)	RT (min ± 0.2)
22S,23S-homobrassinolide (IS)	495.5→109.1	20	495.5→127.1	10	495.5→459.1	5	9.8
20-hydroxyecdysone	481.3→445.4	10	481.3→371.4	10	481.3→165.1	20	7.5
14-deoxy,20-hydroxyecdysone (M1)	465.4→303.3	20	465.4→285.3	25	–	–	7.9
20,26-dihydroxyecdysone (M2)	497.3→461.4	5	497.3→351.1	15	497.3→371.2	20	6.8

To assess the specificity of the method, a blank urine and a urine sample fortified with 20E (1 $\mu g.L^{-1}$) were analyzed. Figure 2 shows the blank traces without any interference at the expected retention time for 20E, demonstrating the good selectivity of the monitored signals. The target analyte 20E was identified in the spiked urine sample with three SRM transitions. The monitored signals are detected with good sensitivity and show high signal-to-noise (s/n) ratios. These results were in accordance with Decision 2002/657/EC criteria, which require more than four identification points [7] in order to validate an identified compound.

The linearity and the repeatability of the method were assessed with the analysis of a pool of urine samples fortified at different concentration levels: the calibration curve was established with five concentration points (0.2, 0.5, 1, 5, and 20 $ng.mL^{-1}$). The calibration curve correlation coefficients (R^2) were better than 0.99, thus demonstrating the good linearity of the method for 20E.

The method has been successfully applied to incurred calf urine samples after 20E oral administration over four days. 20-hydroxyecdysone was detected in urine as rapidly as 30 minutes after its administration and up until 24 hours after the last administration. 20E metabolism was investigated and two main metabolites, 14-deoxy,20-hydroxyecdysone (M1) and 20,26-dihydroxyecdysone (M2), could be identified [8]. Both M1 and M2 were monitored by LC-MS/MS (Table 1). Figure 3 presents the ion chromatograms for M1 in the urine samples collected before and two days after the last 20E administration.

As can be observed, M1 was not detected in the urine collected before 20E administration, whereas it was throughout the four-day administration period. Furthermore, it could still be detected and identified (in accordance with the four identification points required) two days after the last administration of 20E. This result is of prime interest in the context of potential misuse of ecdysteroids since it offers the longest period for detection, following administration, and therefore enables a more efficient control mechanism.

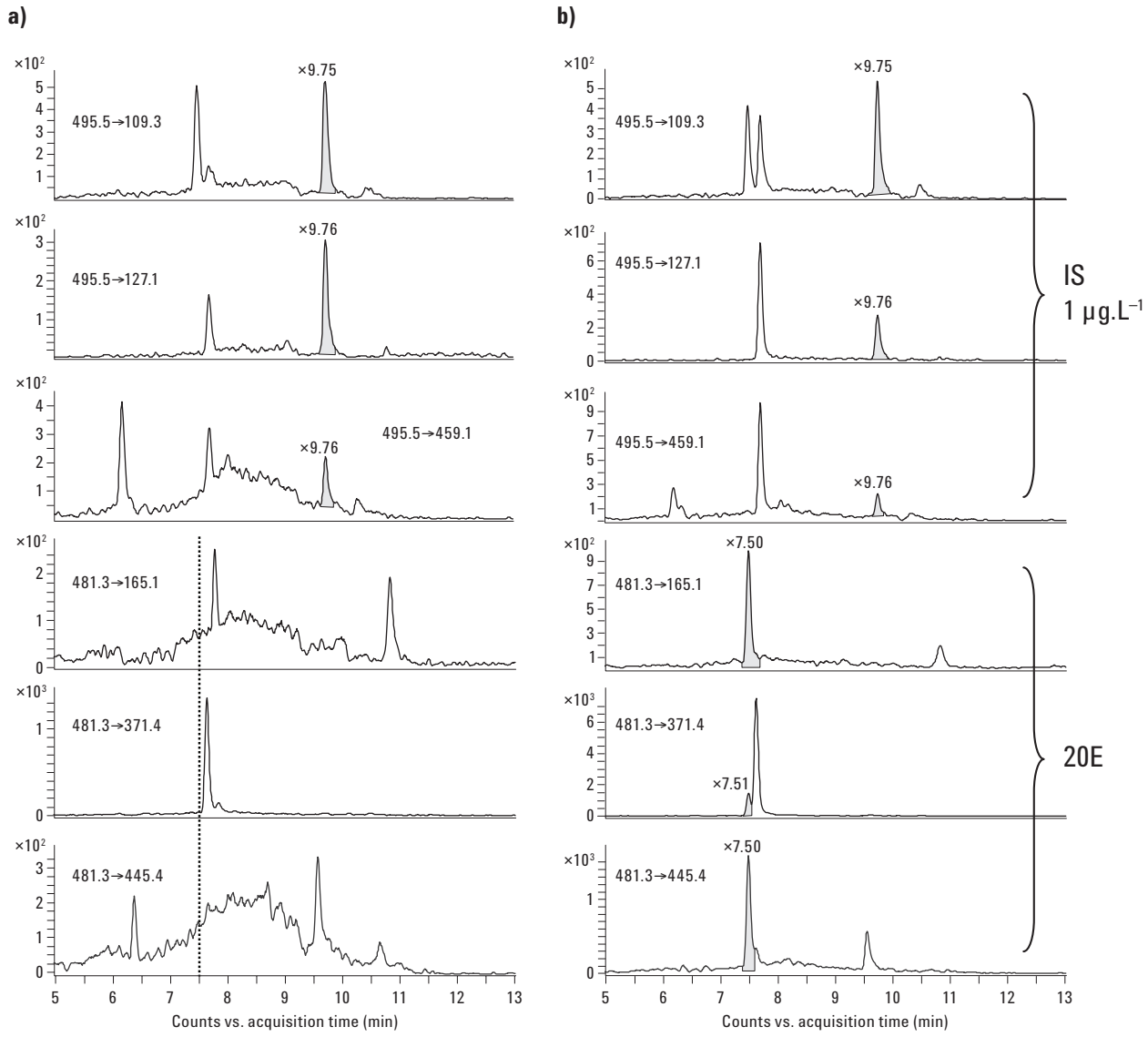

Figure 2. SRM ion chromatograms for a) the blank urine sample and b) the spiked urine sample (1 µg.L-1). LC-(ESI+)-MS/MS measurements.

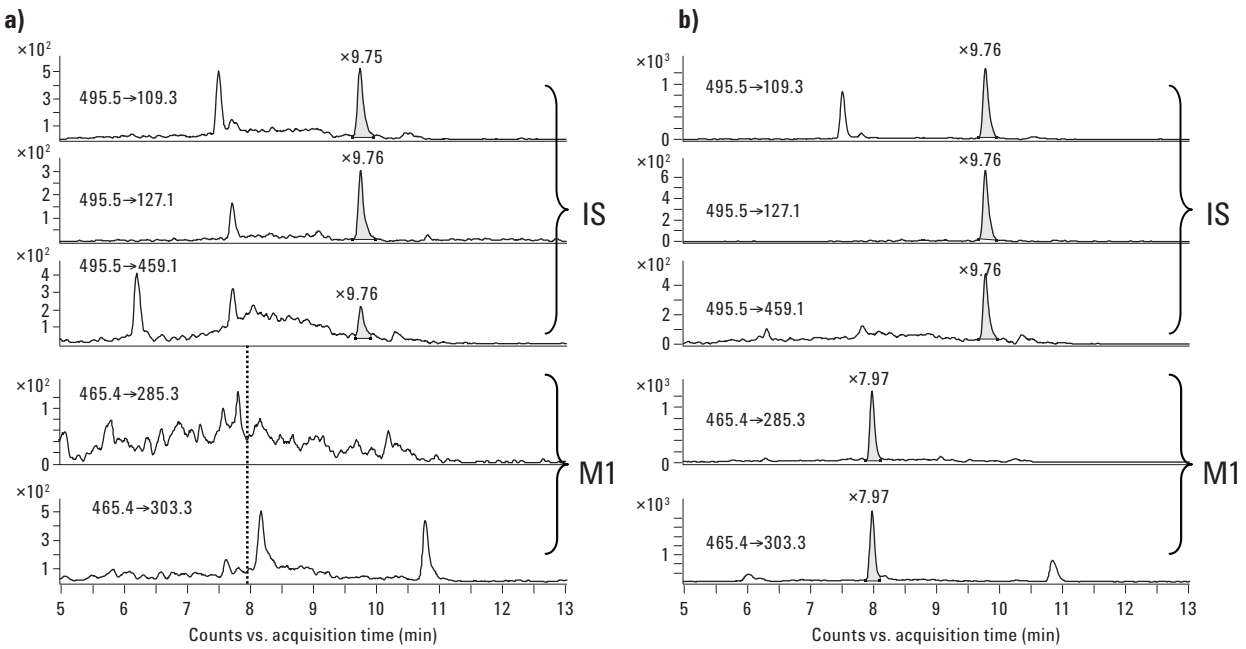

Figure 3. SRM ion chromatograms of IS and M1 in urine sample collected a) before 20E administration and b) two days after the last 20E administration. LC-(ESI+)-MS/MS measurements.

Conclusions

This work demonstrates the performance of LC-MS/MS, which provides efficient identification of 20E and its main metabolites in calf urine. The monitoring of these compounds facilitates the control of the potential misuse of 20E in meat-producing animals. Tandem quadrupole MS/MS is an analytical technique very well suited to this purpose, since it increases confidence in the unambiguous identification of the target compounds, in accordance to the criteria fixed by Decision 2002/657/EC. The successful analysis of the calf urine samples proved the robustness of the developed protocol. Application of this methodology also enabled the determination of the first elimination kinetics and the main metabolites of 20E in calf urine.

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Agilent Technologies

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

Application

Biochemical

Cliff Woodward

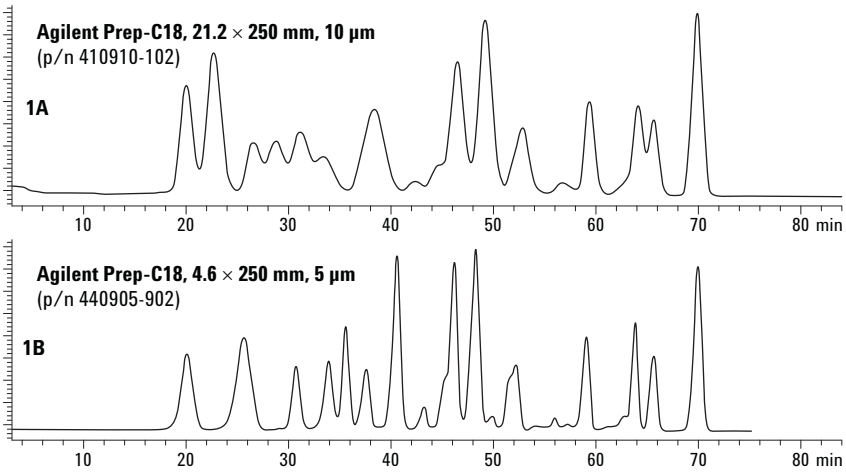
Highlights

- 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.

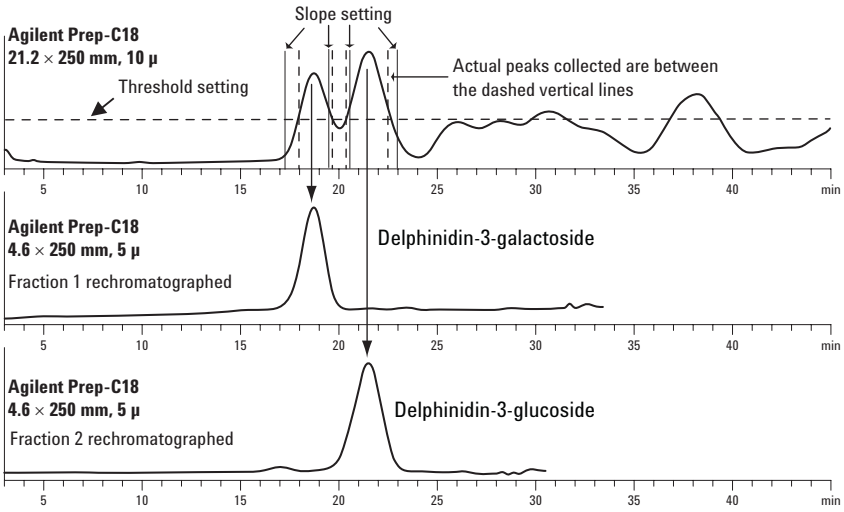


Figure 2. Fraction collection and rechromatography to demonstrate purity.

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The author, Cliff Woodward is an Application Biochemist based at Agilent Technologies, Wilmington, Delaware.

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Agilent Technologies

Using Lead Isotope Ratios to Distinguish between Samples of the Traditional Chinese Medicine Dan-shen

Geological

Authors

Dengyun Chen
Agilent Technologies Co., Ltd
China

Chaoyong Yang, Zhiyong Huang & Xiaoru Wang
Analytical Sciences Laboratory
Xiamen University
Xiamen 361005
China

Abstract

Quadrupole Inductively Coupled Plasma Mass Spectrometry (ICP-MS) was used to determine lead (Pb) isotope ratios in Dan-shen, a type of herb used in Traditional Chinese Medicines (TCM), and in water and soil samples all taken from the same geographical location. The precision obtained for the $^{208}Pb/^{206}Pb$ ratio, the $^{207}Pb/^{206}Pb$ ratio and the $^{204}Pb/^{206}Pb$ ratio values was considerably lower than 0.5% demonstrating the applicability of the technique for Pb isotope ratio studies. The results show that it is possible to distinguish Dan-shen samples originating from different geographical areas using Pb isotope ratio measurements. As the medicinal effectiveness of a TCM is highly dependant on the source of origin of its herb components, it is useful to have a reliable, routine means of "fingerprinting" the components grown in different habitats.

Introduction

Most Traditional Chinese Medicines (TCMs) are a mixture of several different herbs that undergo special treatment to make them useful. The amounts of the effective components in the plants are influenced by the soil, water, weather conditions etc in the area where they are grown. Experienced practitioners of traditional Chinese healing know that the quality of herb medicines is strongly related to their source of origin. It is therefore important to know the specific location of a TCM herb component so ensure its effectiveness.

Many techniques have been applied to characterize various herb medicines and to correlate them with their place of origin. High Performance Liquid Chromatography, Mass Spectrometry, Nuclear Magnetic Resonance, Infrared Spectroscopy and X-ray Fluorescence have all been used to produce spectra that can be used to "fingerprint" the TCM habitat.

Lead isotope ratio measurements provide analytical information relating to the source of lead contamination in naturally occurring samples. Concentration measurements cannot provide this information. Studies of the isotopic composition of lead are therefore commonly used in environmental science as well as geological and anthropological studies. Among all the naturally occurring lead isotopes, only ^{204}Pb is non-radiogenic, whereas, ^{206}Pb, ^{207}Pb and ^{208}Pb are the daughter products from the radioactive decay of ^{238}U and ^{235}U and ^{232}Th respectively. As a consequence, small Pb isotope abundance variations occur in nature and the isotopic composition of lead in the environment is dependent on local ore deposits. If lead is present in the soil, a plant will take up small amounts and subsequent isotope ratio studies might provide a unique

means of differentiating between different plant sources of origin. Of course, local lead levels may become mixed with external sources of contamination (e.g. automobile exhaust) that vary with time depending on the anthropogenic activity. These mixing processes can be quantified as long as each lead source shows a different lead isotope abundance. The work described in this application note was undertaken to investigate if lead isotope ratios can be used to "fingerprint" the Dan-shen herb grown in different habitats. While isotope ratio studies have traditionally been undertaken with a multicollector mass spectrometer such as Thermal Ionization Mass Spectrometry (TIMS), the technique is relatively slow and the instrumentation is expensive. These experiments were carried using quadrupole Inductively Coupled Plasma Mass Spectrometry (ICP-MS) that provides a fast, convenient and precise method to determine isotope ratios.

The Agilent 7500 Series ICP-MS is an excellent tool for routine isotope ratio measurements. It is easy to operate with its fully automated optimization system (Auto-tune function) and its user-friendly software. Because of these advantages, Agilent ICP-MS systems have been widely used for isotope ratio studies for environmental monitoring applications and nuclear research projects, e.g., U isotope ratios in human urine [2], in the cooling water of nuclear plants etc., the certified values of some certified reference materials such as rock samples [3], biological samples [3,4] by isotope dilution. In Encinar et al.'s work[4,5], an older model Agilent ICP-MS (HP-4500) was used, and the performance was as good as the more expensive multi-collector ICP-MS and double focusing sector field ICP-MS especially for long term stability and isotope dilution applications.

Experimental

Instrumentation

An Agilent 7500i ICP-MS, (Agilent Technologies, Palo Alto, CA, USA), fitted with a PFA micro flow nebulizer (100 uL/min) in self-aspiration mode, a quartz spray chamber and a one-piece quartz torch was used throughout the study.

A MK-II microwave sample digestion system (Shang Hai Xin Ke Factory, China) was used for sample digestion.

Reagents

The following reagents were used during the course of the study:

- SRM981 (National Institute of Standards & Technology, USA) - for mass bias correction.
- HBr, HCl, HF, and HNO_3, (GR grade, after sub-boiling purification) - for sample digestion and dilution.
- Anion exchange resin (DOWEX 1×8, 200-400 mesh, USA), - for separating Pb from the matrix in the samples.
- Ultra Pure Water, supplied by Milli-Q water system (18.2 MOhms)

Sample preparation

Dry samples and filtered water samples were digested using a HNO_3/HF acid mixture in a microwave oven then heated to dryness in a fume cupboard to remove the HF. 1.0 mL HBr (0.5N) was used to redissolve the residue. Samples were then passed through a DOWEX 1×8 column to remove the matrix. The Pb containing eluant was dried and diluted with 3% HNO_3. In order to avoid memory effects and to keep the ICP-MS detector working in pulse counting mode only, the concentrations were controlled between 1 and 80 ug/L.

Optimization of the ICP-MS operation parameters
Since the sample volumes collected after the column pretreatment step were limited, most of them were less than 1 mL, the Agilent PFA micro flow nebulizer (100 uL type) was selected for sample introduction. Self-aspiration mode was used and the carrier gas flow rate was controlled to maintain the sample introduction flow rate at about 50 uL/min. The 1 mL sample was sufficient for 2 or 3 individual measurements so that repeat analyses could be undertaken in case of error during the determination.

Instrument sensitivity and the other parameters, such as oxide level and doubly charged ions, were optimized automatically using the AutoTune feature of ChemStation on a 10 ppb tuning solution containing Li, Y, Ce, Tl. The carrier gas was then set to 0.4 L/min to obtain a sample uptake rate of about 50 uL/min. Finally, the make-up gas was adjusted to produce the best sensitivities and lowest interferences.

Instrument operating parameters are given in Table 1.

Table 1. ICP-MS operating conditions

Plasma Conditions:

RF power:	1250 W
RF Matching:	1.7V
Sample Depth:	7 mm
Torch-H:	0 mm
Torch-V:	0.3 mm
Carrier Gas:	0.4 L/min
Make-up Gas:	0.9 mL/min
Peripump1:	0.1 rps
Spray Chamber Temperature:	2.0 degC

Data Acquisition Parameters:

"Isotope Ratio Analysis" mode, 1000 scan/sec, minimum dwell time 100 us. The integration times were 20sec for ^{204}Pb, 10sec for ^{206}Pb and ^{207}Pb, 5 sec for ^{208}Pb. Each data point is the average of 3 repetitions.

Results

Before beginning the analysis, the instrument was allowed 30 minutes following plasma ignition to reach thermal stability. A 20ppb solution of NIST SRM 981 isotope ratio standard was measured after every 5 unknowns. This data provided the 7500i short-term and long-term stability information and provided a means of making the small mass bias corrections required. The instrument demonstrated excellent precision. See Tables 2 and 3. Note that the instrument consistently delivered %RSD's at less than 0.1% over the short and long term.

Table 2: Short-term Stability of Pb Isotope Ratio Determinations of 20 ppb SRM981 using the Agilent 7500i ICP-MS (20min, 5 measurements)

File Name	PB-206A.D	PB-206B.D	PB-206C.D	PB-206D.D	PB-206E.D	RSD(%)
Acq Date	Jun 9 2001	Jun 9 2001	Jun 9 2001	Jun 9 2001	Jun 9 2001	
Acq Time	3:45 PM	3:50 PM	3:55 PM	4:00 PM	4:06 PM	
204/Total	0.01427	0.01426	0.01426	0.01426	0.01425	0.05
206/Total	0.2413	0.2414	0.2414	0.2415	0.2416	0.05
207/Total	0.2211	0.2208	0.2209	0.2209	0.2209	0.05
208/Total	0.5233	0.5235	0.5235	0.5233	0.5232	0.03
204/206	0.05913	0.05904	0.05907	0.05906	0.05897	0.10
207/206	0.9163	0.9146	0.9149	0.9146	0.9144	0.08
208/206	2.169	2.168	2.169	2.167	2.165	0.08

Table 3. Long-term Stability (10 hours) of Pb Isotope Ratio Determinations of 20 ppb SRM981 using the Agilent 7500i ICP-MS

File Name	Acq Date	Acq Time	206/Total	207/Total	208/Total	207/206	208/206
PB20-A.D	Jun 9 2001	6:14 AM	0.2411	0.2209	0.5235	0.9162	2.171
PB20-B.D	Jun 9 2001	6:26 AM	0.2412	0.2209	0.5236	0.9159	2.171
PB20-C.D	Jun 9 2001	6:33 AM	0.2412	0.2209	0.5235	0.9158	2.170
PB20-D.D	Jun 9 2001	6:40 AM	0.2411	0.2210	0.5236	0.9167	2.172
PB20_1A.D	Jun 9 2001	7:59 AM	0.2411	0.2211	0.5236	0.9172	2.172
Pb-202A.D	Jun 9 2001	9:12 AM	0.2410	0.2211	0.5236	0.9173	2.172
PB_202B.D	Jun 9 2001	9:18 AM	0.2414	0.2210	0.5234	0.9153	2.168
PB-203A.D	Jun 9 2001	11:17 AM	0.2413	0.2211	0.5233	0.9161	2.168
PB-204A.D	Jun 9 2001	12:52 PM	0.2412	0.2212	0.5233	0.9168	2.169
PB-204B.D	Jun 9 2001	12:59 PM	0.2410	0.2214	0.5234	0.9185	2.172
PB_205A.D	Jun 9 2001	2:27 PM	0.2412	0.2210	0.5236	0.9163	2.171
PB_205B.D	Jun 9 2001	2:32 PM	0.2415	0.2210	0.5232	0.9153	2.167
RSD(%)			**0.06**	**0.07**	**0.03**	**0.10**	**0.08**

The excellent results from the NIST quality assurance sample suggest that the data from the unknown samples is also very reliable and can be reported with a high degree of confidence. In this study, three types of samples were measured:

- Four samples of surface water taken from different areas in Zhong-Jiang, Si-Chuan Province
- Soil samples came from Er-Mei Mountain (2), Zhong-Jiang, Si-Chuan Province (5), Jiang-Xi Province (1)
- Dan Shen plant samples were from Zhong-Jiang, Si-Chuan Province (3), Er-Mei Mountain (2), Bei-Jing (1), Jiang-Xi Province (1), Tai Mountain, Shan-Dong Province (1), Shang-Luo, Shan-Xi Province (1), Xin-Jiang Province (1), He-Nan Province (1)

All the samples were taken from different areas even within the same province. The measured results and the standard deviations are listed in Table 4 a-c.

Table 4a: Results of Pb Isotope Ratio Measurements of Dan-shen Plant Samples

Sample Name	$^{207}Pb/^{206}Pb$ ±sd(n=3)	$^{208}Pb/^{206}Pb$ ±sd(n=3)
Jiang-Xi Province	0.8485 ± 0.0005	2.079 ± 0.002
Shan-Luo, Shan-Xi Province	0.8502 ± 0.0014	2.100 ± 0.002
Bei-Jing	0.8674 ± 0.0019	2.159 ± 0.003
Shan Dong Province	0.8570 ± 0.0018	2.107± 0.002
Xin-Jiang Province	0.8495 ± 0.0007	2.095 ± 0.001
He-Nan Province	0.8576 ± 0.0007	2.115 ± 0.002
ErMei Mountain, D1	0.8467 ± 0.0003	2.093 ± 0.001
ErMei Mountain, D2	0.8469 ± 0.0004	2.095 ± 0.004
Zhong-Jiang, D1	0.8529 ± 0.0022	2.105 ± 0.004
Zhong-Jiang, D2	0.8524 ± 0.0012	2.105 ± 0.002
Zhong-Jiang, D3	0.8537± 0.0003	2.103 ± 0.002

Table 4b: Results of Pb Isotope Ratio Measurements of Soil Samples

Sample Name	$^{207}Pb/^{206}Pb$ ±sd(n=3)	$^{208}Pb/^{206}Pb$ ±sd(n=3)
Jiang-Xi Province	0.846 3± 0.0017	2.090 ± 0.001
ErMei Mountain, T1	0.8520 ± 0.0016	2.101 ± 0.004
ErMei Mountain, T2	0.8523 ± 0.0007	2.103 ± 0.001
Zhong-Jiang, T1	0.8395 ± 0.0007	2.084 ± 0.001
Zhong-Jiang, T2	0.8417 ± 0.0004	2.089 ± 0.001
Zhong-Jiang, T3	0.8411 ± 0.0015	2.084 ± 0.002
Zhong-Jiang, T4	0.8400 ± 0.0007	2.090 ± 0.001
Zhong-Jiang, T5	0.8391 ± 0.0014	2.083 ± 0.002

Table 4c: Results of Pb Isotope Ratio Measurements of Water Samples

Sample Name	$^{207}Pb/^{206}Pb$ ±sd(n=3)	$^{208}Pb/^{206}Pb$ ±sd(n=3)
Zhong-Jiang, w1	0.8702 ± 0.0015	2.140 ± 0.003
Zhong-Jiang, w2	0.8735 ± 0.0005	2.137 ± 0.002
Zhong-Jiang, w3	0.8675 ± 0.0002	2.136 ± 0.002
Zhong-Jiang, w4	0.8693 ± 0.0014	2.138 ± 0.003

Discussion

Since ^{206}Pb, ^{207}Pb and ^{208}Pb are the daughter products from the radioactive decay of ^{238}U and ^{235}U and ^{232}Th respectively, the Pb isotope abundance varies in nature. In most of the samples, the $^{208}Pb/^{206}Pb$ ratio values are between 1.95 and 2.15, the $^{207}Pb/^{206}Pb$ ratio values are between 0.78 and 0.86 and the $^{204}Pb/^{206}Pb$ ratio values are between 0.05 and 0.06[1]. In order to distinguish between the different types of samples by their lead isotope ratio, the measurement precision is extremely important, both over the short and long term. The RSD values should be at most 0.5% in order to make a clear differentiation between the samples. As indicated in

Table 4a-c, the SD values for the determinations are considerably better (lower) than 0.5%, so it is possible to use lead isotope ratio measurements to distinguish between the different samples.

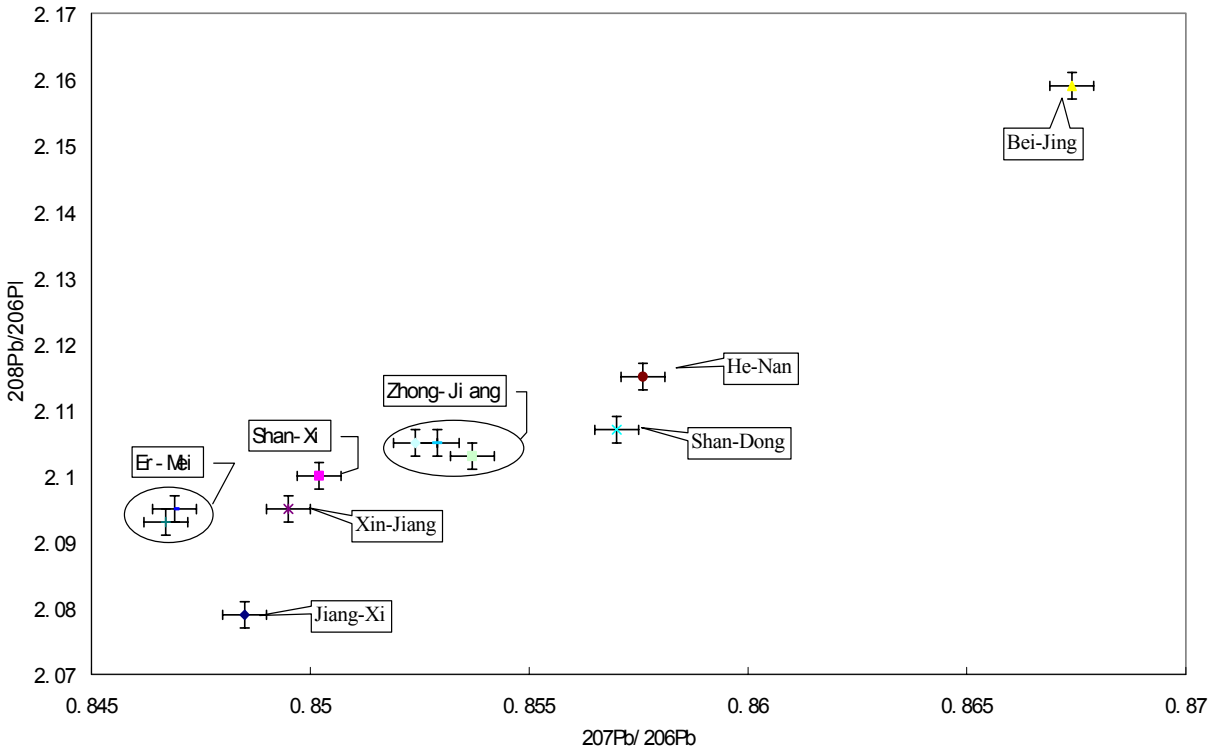

Figure 1: Pb Isotope Ratio Distribution of the Herb Medicine, Dan-Shen, from Different Sources

As a means of looking for variations/similarities in the measurements, the $^{208}Pb/^{206}Pb$ ratio values were plotted on the y-axis vs the $^{207}Pb/^{206}Pb$ ratio values on the x-axis. Figure 1 illustrates the data from the analyses of the Dan-shen plant and shows very interesting results. Obviously, the Pb isotope ratio values show their own special pattern for the Dan-shen samples from different sources. For example, Dan-shen samples grown in the Zhong-Jiang area have similar Pb isotope ratio values, although the samples were taken from different sampling areas. When compared to the Pb isotope values of the Dan-shen samples from the Er-Mei Mountain region (the two samples also have similar Pb isotope ratio values), the difference is distinct. In conclusion, it is possible to distinguish the Dan-shen samples from different sources.

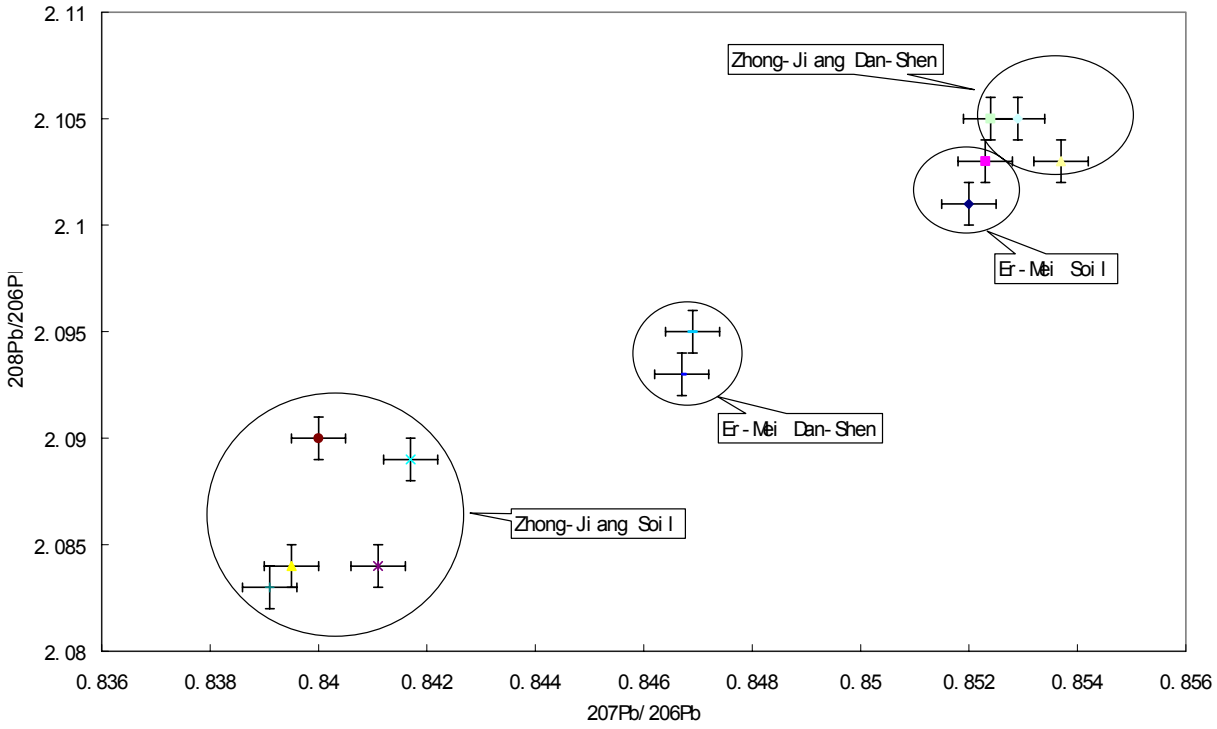

Figure 2: Pb Isotope Ratio Distribution of Soil and Dan-Shen Samples taken from Different Sources

When the soil samples from different sources are considered, it is found that the isotope values of the soil samples from different sources also have their own special pattern, as shown in Figure2. But, interestingly, the isotope values of the soils are different to the Dan-shen samples from the same place. This suggests that soil isn't the only source of lead within the plant.

When the water samples are considered, it is obvious that the Pb isotope ratio values are a combination of those found in the soil and in the water, as shown in Figure 3.

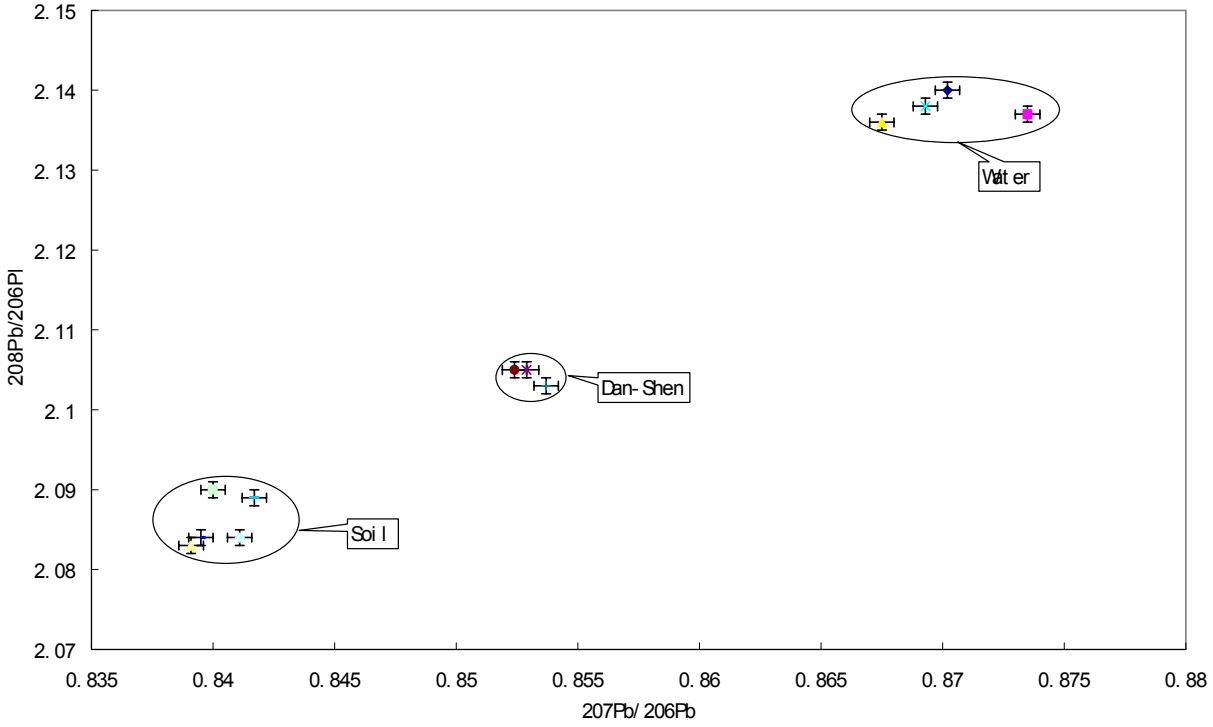

Figure 3: Pb Isotope Ratio Distribution of the Soil, Dan-Shen and Water Samples Taken from similar Sampling Area (Zhong-Jiang, Si-Chuan Province)

Conclusions

The Agilent PFA micro flow nebulizer is a good choice for isotope ratio analysis since it has very good nebulization efficiency and stability, especially when the sample amount is small. Self-aspiration mode avoids small amounts of pulsation from the peristaltic pump that could affect the precision of the isotope ratio analysis.

As this data suggests, the Agilent 7500 Series ICP-MS is well suited for routine isotope ratio analysis. Further improvements in precision may be obtained by modifying the method used in this study slightly. For instance, Tl may be added to the samples as an internal standard for lead isotope ratio analysis[5]. In addition, optimization of the dead-time correction, the stand-by mass selection, may also improve the performance of Agilent 7500 ICP-MS so that the theoretical minimum %RSD of 0.03 can be obtained. Future work at Xiamen University will look in to these potential improvements.

This preliminary research indicates that it is possible to distinguish Dan-shen samples from different areas using Pb isotope ratio measurements. Continuing these studies by running more types of samples will allow a large database to be set-up, and a chemometric model to be built to provide a convenient way to distinguish herb medicines from different sources.

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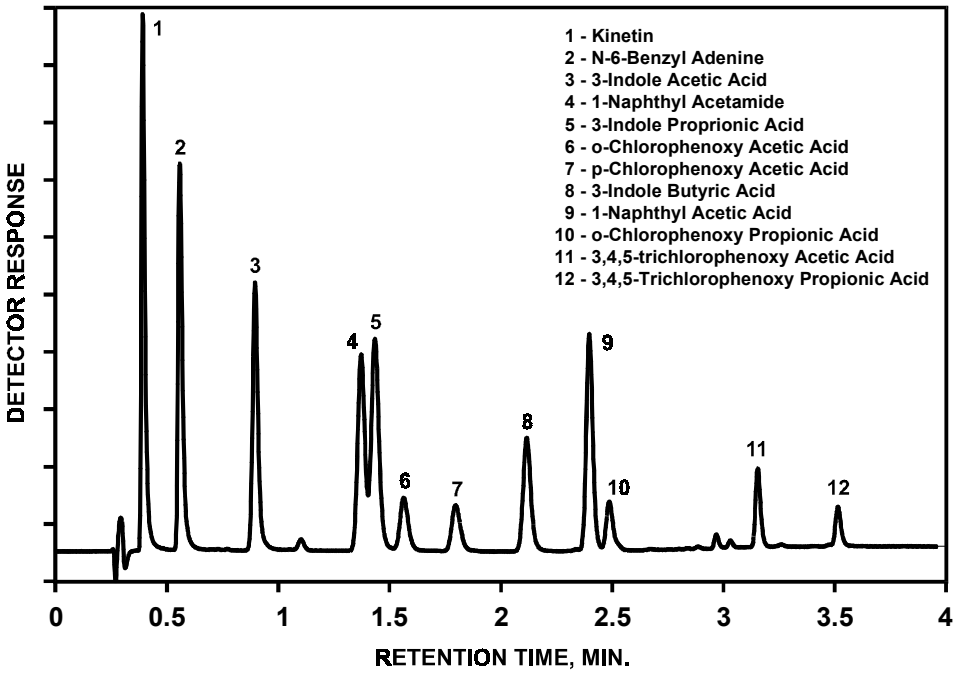
Plant Hormones
Rapid Gradient Elution Separation

Application

Agrichemical

Robert D. Ricker

Plant hormones play an essential role in the growth and development of plant species. Rapid analysis of these compounds by HPLC may be used for general research into metabolism and the study of plant disease mechanisms. HPLC may also be used in studies of plant control, whether that is stimulation of growth for the purpose of larger and sturdier plants, or for stopping growth, as in plant pesticides (herbicides). The wide variety and structure of these molecules makes them a candidate for gradient HPLC, to reduce runtime and increase throughput.



Conditions: LC: Hewlett Packard 1050
Column: ZORBAX SB-C8, 4.6 x 75 mm (Agilent Part No. 866953-906)
Mobile Phase:
A Solvent: Water with 0.1% TFA
B Solvent: Acetonitrile with 0.1% TFA
Gradient: 22% B to 50% B in 1.5 min.
UV: 254 nm; Flow: 3.0 mL / min.; 60°C

Highlights

- *These sterically protected bonded phases provide long column life and good peak shape at low pH (0.1% TFA)*
- *Use of temperature (60°C) with short column length (75mm) permits operation with reasonable back pressure at higher flow rates (e.g. 3.0mL/min).*
- *Agilent ZORBAX 3.5 µm particles can be used in columns to provide an excellent balance in speed of separation and resolution.*

Agilent Technologies

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

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application

Agilent Technologies
Innovating the HP Way

Structural Determination of Ginsenosides Using MS^n Analysis

Linda L. Lopez

Introduction

Ginseng root, a traditional Chinese herbal remedy, contains more than a dozen biologically active saponins called ginsenosides. This class of natural products is believed to play an important role in the treatment and prevention of a number of diseases including atherosclerosis, arthritis, asthma, diabetes, stroke, multiple sclerosis, and endotoxin liver injury.[1–3] Ginsenosides are among a growing class of herbal and vitamin products know as nutraceuticals, that is, food products that have pharmacological benefits to human health because of their therapeutic properties. With an estimated 15 million patients at risk of potentially adverse drug-herb interactions,[4] there is renewed interest in the isolation and characterization of these compounds.

Ginsenosides are structurally described as glycosides consisting of an aglycone moiety, which is typically a triterpenoid or steroid, and one or more covalently linked sugar monomers. Since most ginsenosides contain multiple oligosaccharide chains at different positions in the molecule, structural elucidation of these compounds can be quite complicated. Tandem mass spectrometric methods have been developed for the characterization of ginsenosides contained in ginseng extracts.[5] However, MS/MS experiments carried out on a triple quadrupole mass spectrometer using a collision cell typically generate complex product ion spectra that are often difficult to interpret. This is because first-stage product ions tend to undergo further collisions with the background gas to yield second and third generation fragments that cannot be easily distinguished from first-stage MS/MS product ions.

MS^n analysis in an ion trap mass spectrometer permits multiple isolation and fragmentation stages, ensuring that product ions in each stage are specifically related to the precursor ion from that particular stage. This type of stepwise fragmentation can be quite advantageous because it allows product ion origins to be unambiguously assigned, making MS/MS spectra simpler to interpret and permitting individual fragmentation pathways to be followed.

This note demonstrates the power of MS^n analysis for the structural determination of ginsenosides from a ginseng root extract.

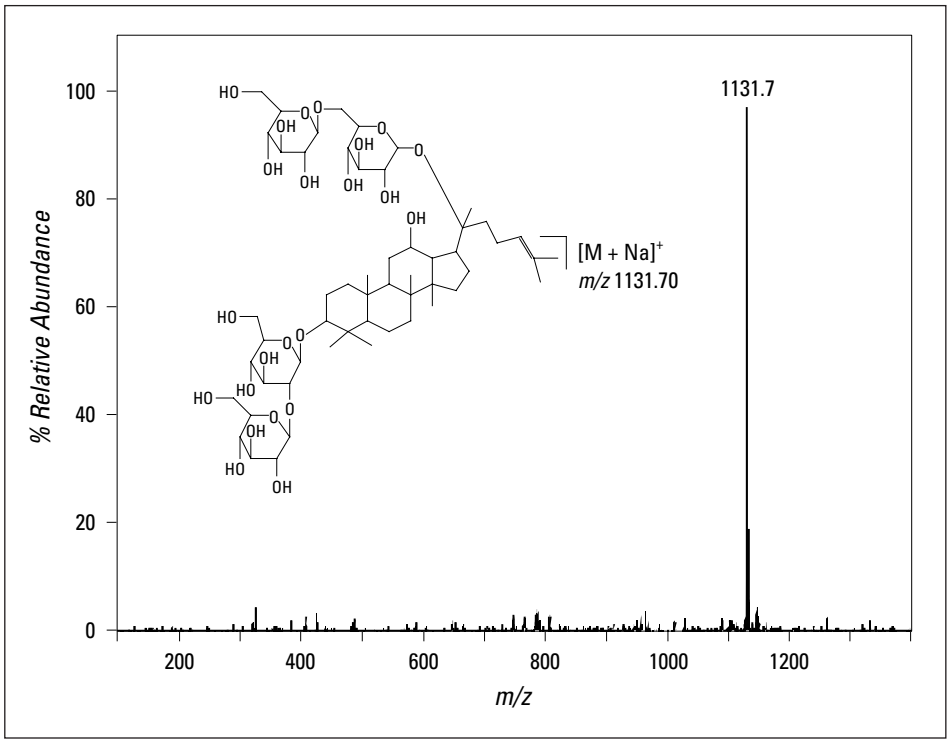
Experimental

All experiments were done using an Agilent 1100 Series LC/MSD Trap system composed of a binary pump, vacuum degasser, autosampler, and thermostatted column compartment with column-switching valve. The system was operated with the electrospray ionization (ESI) source in the positive ion mode.

Reagent grade chemicals and HPLC grade solvents were used in preparing mobile phases and standards.

Results and Discussion

Figures 1a–c show the full scan MS, MS/MS and MS^3 spectra from direct infusion of the Rb1 ginsenoside standard, along with proposed origins of the observed product ions. The mass spectrum of Rb1 (Figure 1a) shows predominantly the intact $[M+Na]^+$ pseudomolecular ion at *m/z* 1131.7, with little or no decomposition of the labile ginsenoside adduct under typical ESI interface conditions using a drying gas temperature of 350°C. This is in contrast to previous studies in which a room temperature API interface was required to observe an intact molecular ion,[5] and emphasizes the gentle nature of the orthogonal spray ion source on the LC/MSD Trap.



Analysis method:

Direct infusion
Flow rate: 5 mL/min

MS Conditions

Source:	ESI
Drying gas flow:	6 l/min
Nebulizer:	10 psig
Drying gas temperature:	300°C
Skim 1:	25.0 V
Cap exit offset:	50.0 V
Averages:	5 V
ICC:	On
Max accu time:	200 ms
Target:	40000
Ion mode:	Positive

Figure 1a. Full scan mass spectrum of ginsenoside Rb1 standard.

MS/MS of the *m/z* 1131.7 sodium adduct shown in Figure 1b yields a product ion at *m/z* 789.6 corresponding to cleavage of a single glycosidic bond. For the case of the ginsenoside Rb1, which contains two isomeric oligosaccharide chains, cleavage at either glycosidic linkage would result in isobaric fragment ions at *m/z* 789.6. Subsequent isolation and fragmentation of *m/z* 789.6 (Figure 1c) yields two products: (1) a more abundant ion corresponding to loss of the oligosaccharide chain (–Glc^2–Glc) at *m/z* 365.1 and (2) loss of a deoxyhexose sugar at *m/z* 627.5. The stepwise fragmentation observed in the ion trap provides specific information on molecular structure; however, in this case it is not possible to establish the initial site of glycosidic bond cleavage in the molecule.

For use as a reference in the interpretation of ginsenoside Rb1 fragmentation spectra, several analogs of ginsenoside Rb1 were analyzed by ion trap LC/MS^n. In this manner an MS/MS comparative method was devised based on the premise that ginsenoside Rb1 would be expected to retain substructures of the related ginsenosides. For example, Figure 2 shows the total ion chromatogram generated from online LC/MS^3 analysis, as well as extracted ion chromatograms of the pseudomolecular ions of each chromatographic peak generated from a water extract of American ginseng root. Figures 3a and 3b show the online product ion spectra of the ginsenoside Rb2 which contains two different oligosaccharide chains. Isolation and fragmentation of the pseudomolecular ion at *m/z* 1101.6 yields a single ion at *m/z* 789.7 resulting from cleavage of the –Glc^6–Ara(p) dissacharide linkage. Comparison of the product ion spectra of ginsenosides Rb1 with Rb2 shows isobaric product ions (*m/z* 789.7), thus supporting the proposed fragmentation pattern for ginsenoside Rb1 shown in Figure 1. Furthermore, *m/z* 789.7 was a major fragment ion observed in the product ion spectra of all the ginsenosides analyzed, providing a substructural template that supports the fragmentation patterns proposed for Rb1 and Rb2.

Subsequent isolation of the *m/z* 789.7 precursor generates a full scan MS^3 spectrum, which is shown in Figure 3b along with proposed origins of the observed product ions.

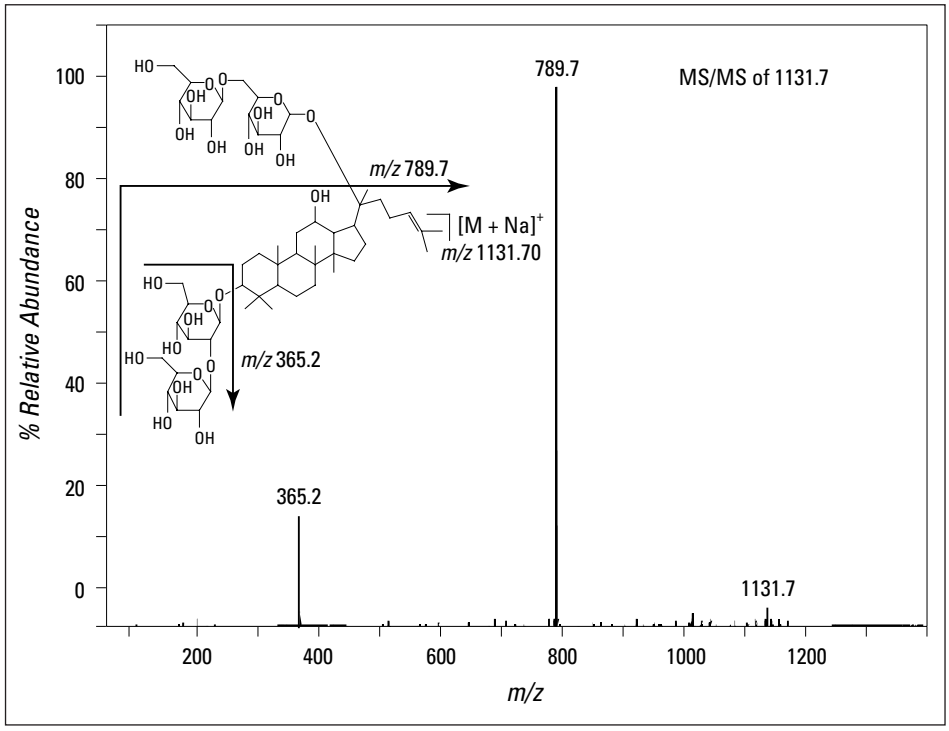


Figure 1b. Product ion spectrum generated from infusion of ginsenoside Rb1 standard by MS/MS analysis of *m/z* 1131.7 ([M+Na]$^+$).

ANALYSIS METHOD:

Direct infusion
Flow rate: 5 mL/min

MS Conditions

Source:	ESI
Drying gas flow:	6 l/min
Nebulizer:	10 psig
Drying gas temperature:	300°C
Skim 1:	25.0 V
Cap exit offset:	50.0 V
Averages:	5 V
ICC:	On
Max accu time:	200 ms
Target:	40000
Ion mode:	Positive

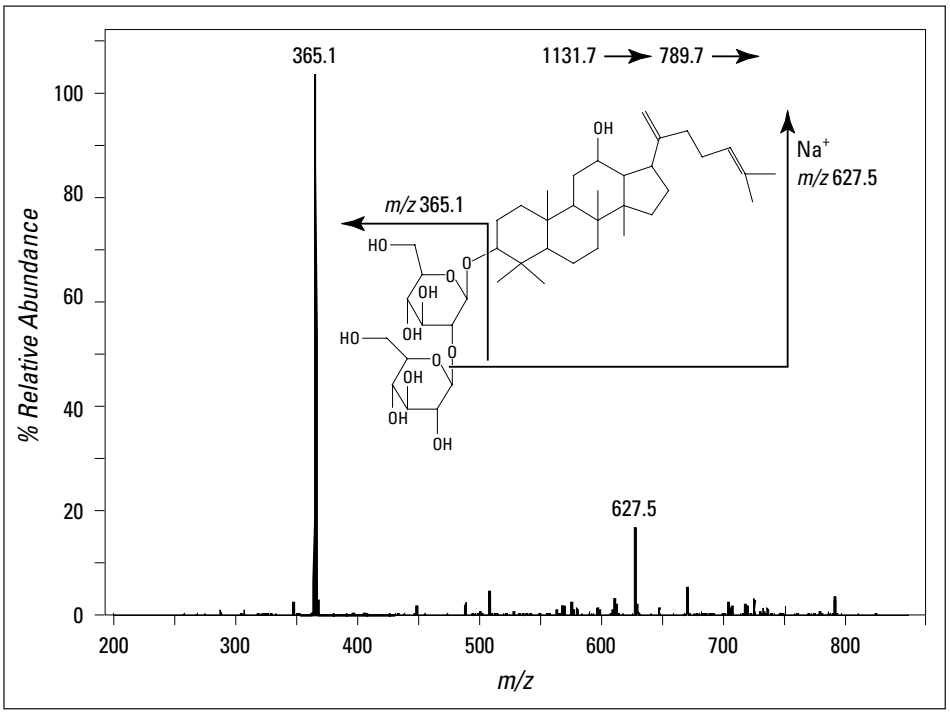


Figure 1c. Product ion spectrum generated from infusion of ginsenoside Rb1 standard by MS^3 analysis of *m/z* 789.7 (from *m/z* 1131.7).

ANALYSIS METHOD:

Direct infusion
Flow rate: 5 mL/min

MS Conditions

Source:	ESI
Drying gas flow:	6 l/min
Nebulizer:	10 psig
Drying gas temperature:	300°C
Skim 1:	25.0 V
Cap exit offset:	50.0 V
Averages:	5 V
ICC:	On
Max accu time:	200 ms
Target:	40000
Ion mode:	Positive

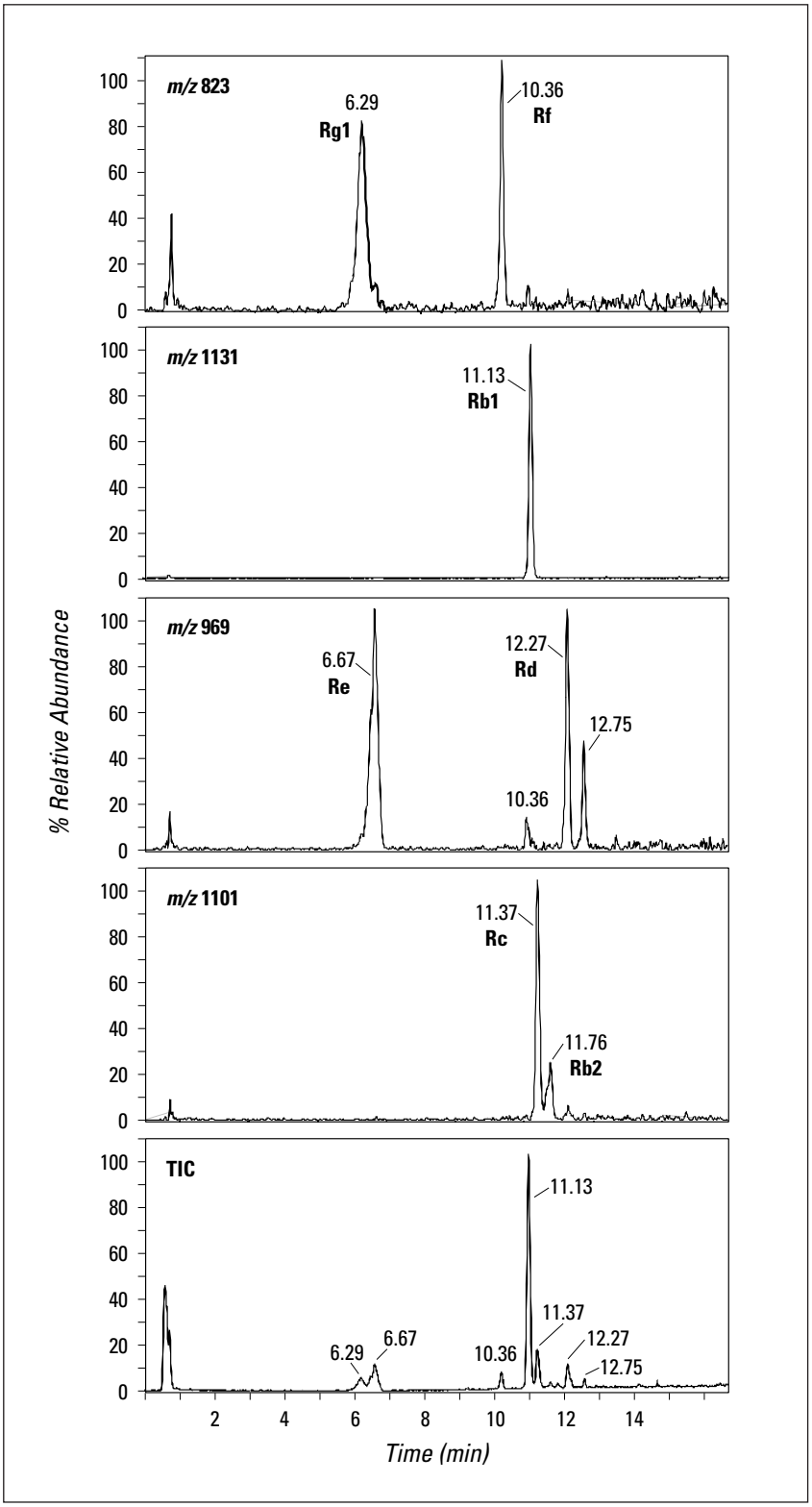


Figure 2. Total ion chromatogram (TIC) and extracted ginsenoside pseudomolecular ion chromatograms generated from LC/MS^n analysis of ginseng root extract.

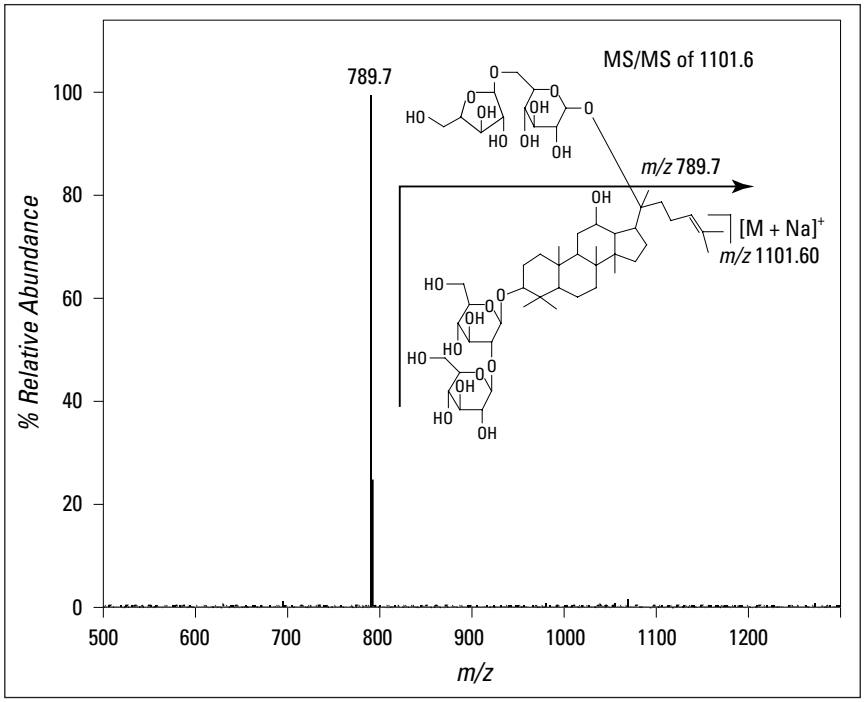


Figure 3a. Product ion spectrum generated from ginseng root extract by LC/MS^n analysis of *m/z* 1101.6 ([M+Na]$^+$).

ANALYSIS METHOD:

Chromatographic Conditions

Column:	2 × 50 mm Zorbax® SB-C18, 3.5 µm (p/n 863954-302)
Mobile phase:	A = 0.01% acetic acid in water; B = acetonitrile
Gradient:	start with 20% B at 1 min 20% B at 11 min 95% B
Flow rate:	0.3 ml/min
Injection volume:	10 µl

MS Conditions

Source:	ESI
Drying gas flow:	7 l/min
Nebulizer:	30 psig
Drying gas temperature:	300°C
Skim 1:	25.0 V
Cap exit offset:	50.0 V
Averages:	5 V
ICC:	On
Max accu time:	200 ms
Target:	40000
Ion mode:	Positive

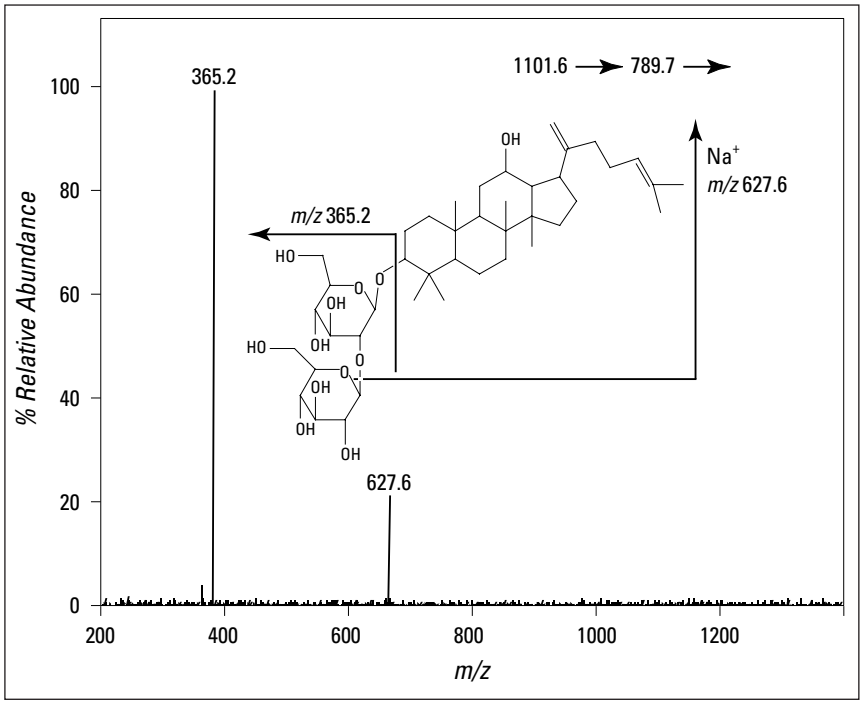


Figure 3b. Product ion spectrum generated from ginseng root extract by LC/MS^n analysis of *m/z* 789.7 (from *m/z* 1101.6).

ANALYSIS METHOD:

Chromatographic Conditions

Column:	2 × 50 mm Zorbax® SB-C18, 3.5 µm (p/n 863954-302)
Mobile phase:	A = 0.01% acetic acid in water; B = acetonitrile
Gradient:	start with 20% B at 1 min 20% B at 11 min 95% B
Flow rate:	0.3 ml/min
Injection volume:	10 µl

MS Conditions

Source:	ESI
Drying gas flow:	7 l/min
Nebulizer:	30 psig
Drying gas temperature:	300°C
Skim 1:	25.0 V
Cap exit offset:	50.0 V
Averages:	5 V
ICC:	On
Max accu time:	200 ms
Target:	40000
Ion mode:	Positive

Conclusions

MS^n analysis using an ion trap mass spectrometer specifically selects the desired precursor ion and dissociates it to produce a specific fragmentation pattern in individual stages. As a result, it is a powerful analytical tool for deducing molecular structure. Electrospray ionization provides a soft ionization technique for generating predominantly intact molecular or pseudomolecular ions with little or no structurally relevant fragment ions in the mass spectra. MS/MS fragmentation in an ion trap mass spectrometer is useful because the product ions generated are derived only from the original molecular ion and are not the result of any additional fragmentation, as can be the case with collision induced dissociation (CID) in a collision cell. Additional MS stages tend to show stepwise fragmentations in which all or most of the ion current is localized in a single product ion, greatly facilitating interpretation of the spectra.

References

1. Kim Y. C., Kim S. R., Markelonis G. J., Oh T. H., *J. Neurosci. Res.* **53**, 1998, 426–32.
2. Kim H. S., Hong Y. T., Jang C. G., *J. Pharm. Pharmacol.* **50**, 1998, 555–60.
3. Yokozawa T.; Liu Z. W.; Dong E., *Nephron.* **78**, 1998, 201–6.
4. Smolinske S. C., *J. Am. Med. Womens Assoc.* **54**, 1999, 191–2.
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Author

Linda L. Lopez is an applications chemist at Agilent Technologies in Palo Alto, CA.

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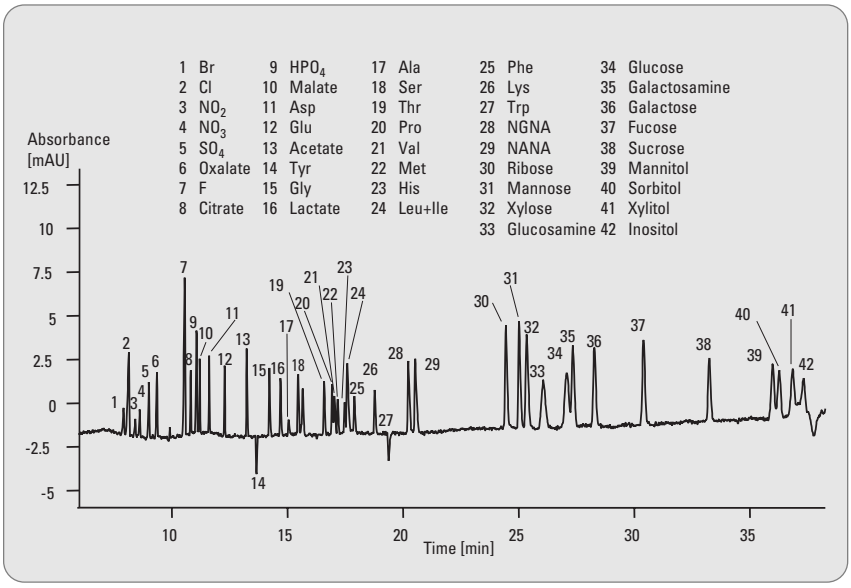
Simultaneous analysis of inorganic anions, organic acids, amino acids and carbohydrates using the Agilent Basic Anion Buffer

Application Note

Food

Tomoyoshi Soga



Introduction

This application note describes extended applications of the Agilent Basic Anion Buffer. It facilitates the analysis of many anions including inorganic anions, organic acids, amino acids and carbohydrates. The method described here is useful for the screening analysis of anions in food and beverage samples.

To separate these anions simultaneously, a highly alkaline pH condition is used to confer a negative charge not only on inorganic and organic anions but also on amino acids and carbohydrates, and promote their migration towards the anode. Electroosmotic flow (EOF) is reversed in the direction of the anode by adding quaternary ammonium salt to the electrolyte. This is necessary to have both anion migration and EOF in the same direction (towards the anode) and ensure anion migration past the detector.

In this method, indirect UV detection is employed to visualize anions which have little or no chromophore. The Agilent Basic Anion Buffer is pre-made with the pH already adjusted, therefore no further preparation is required. Detailed method parameters and some typical electropherograms are shown.

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Necessary supplies

The following parts are necessary for the simultaneous analysis of anions:

Component	Quantity	Part No.
Agilent Basic Anion Buffer*	50 ml	5064-8209
Product literaure	1	5968-7715E

* For this buffer, only 50-µm id straight capillaries are usable.

The following Agilent parts should be ordered separately when used with the Agilent CE system:

Component	Quantity	Part No.
Fused silica capillary (id = 50 µm, l=104 cm L=112.5 cm)	1 pk	G1600-64211
CE buffer vials 2 ml (glass*)	100/pk	5182-9697
CE sample vials, 100 µl (polypropylene)	1000/pk	9301-0978
CE caps (polyurethane)	100/pk	5181-1512
CE water	500 ml	5062-8578

* It is recommended to use glass vials rather than polypropylene vials.

Procedures

Buffer preparation

The Basic Anion Buffer is premade and ready to use.

Do not leave the solution uncapped since the buffer is a highly alkaline solution and will readily absorb gaseous carbonate from the atmosphere. This will cause its pH to drop immediately. Make certain the bottle is capped after use.

Do not store opened bottles which are a third or less full. These should be discarded because gaseous carbonate in the bottle can be adsorbed and will reduce the pH of the residual buffer.

The buffer should be stored at room temperature (not less than 20 °C, since some buffer components may crystallize at lower temperatures).

Buffer and waste vials

Prepare 3 vials (one flushing vial and two home vials). When using 2-ml glass vials (PN 5182-9697), fill each vial with 1.4 ml of the buffer. Also prepare a waste vial (filled with 300-ml CE water or deionized water).

Since buffers used for indirect UV detection have limited buffering capacity, the buffer should be replaced every 8 runs when using 2-ml glass vials (PN 5182-9697).

For this application, do not use the replenishment system for buffer replacement. The level of dissolved carbonate may increase due to pressurization of the buffer bottle, causing a pH drop. Also, crystallization of the buffer might block the tubes.

Standard Preparation

Individual stock solutions of inorganic and organic anions should be prepared from their sodium salts or free acids. Since carbohydrates are unstable, they should be prepared shortly before use.

For amino acids, individual stock solution of Tyr, Cys-Cys, Asp, Trp, Leu, Ile and Phe should be prepared at a concentration of 10 g/l in 0.1 M NaOH. Other amino acids should be prepared in 0.01 M HCl. The working mixture standard should be prepared by diluting stock solutions with deionized water.

If a commercially available amino acid standard mixture in 0.1 M HCl is used, Arg may not be detected since the pKa value of Arg is 10.76. Arg is positively charged in 0.1 M HCl and migrates

toward the opposite direction of the detector. In this case, the standard solution should be diluted with 0.01 M NaOH.

The concentration of the standard mixture should be in the range of 50 to 1000 mg/l to obtain good peak shape and sensitivity.

Sample Preparation

For actual samples, dilution with deionized water is necessary in order to reduce the conductivity of the samples, for example, 1:50 dilution for soy sauce.

If the sample contains proteins and the migration times increase from run to run, removal of the proteins is recommended by using centrifugal filtering through a 30-kDa cutoff filter.

Capillary

Only 50-µm id straight capillaries are suitable for this method. Baseline noise is markedly increased if a 75-µm id capillary is used due to the high UV absorptivity of the buffer. Neither bubble cell capillaries nor the High Sensitivity Detection Cell should be used. A 50-µm id capillary (L=112.5 cm, l=104 cm) is recommended.

Capillary conditioning

Avoid capillary conditioning with sodium hydroxide since this degrades the performance of this application.

Prior to first use, a new capillary should be flushed only with the run buffer for 15 minutes.

Between analyses it is recommended that the capillary be flushed for 4 minutes with buffer from the flushing vial.

Capillary storage

If the capillary is removed from the instrument it should be washed for 10 minutes with deionized water and then flushed with air for 10 minutes. When the capillary is to be reinstalled, it is necessary to flush with the run buffer for at least 15 minutes.

Method summary

The following method can be used to separate most inorganic anions, organic acids, amino acids and carbohydrates simultaneously. Below are the general analytical conditions. The method as it should be entered into the Agilent ChemStation is shown on the following page.

Capillary	Fused silica id = 50 µm, l=104 cm, L=112.5 cm (G1600-64211)
Injection	1. Pressure: 50 mbar for 6 seconds from sample vial 2. Post-injection of buffer from InHome vial, 50 mbar for 4 s
Applied voltage:	- 30 kV
Capillary temperature	15 °C
Detection wavelength	Signal 350/20 nm, reference 230/10 nm
Preconditioning	Buffer flush for 4 min at 1 bar prior to each run

Programming the method

HPCE mode: CE

Home values:

Lift Offset	4	
Cassette Temperature	15.00 °C	
Inlet Home Vial	10	*Place the buffer vials at position 10 and 11.*
Outlet Home Vial	11	*Vial locations are exemplary only.*

Replenishment and Preconditioning:

Serial processing

Replenishment Entries:

No Replenishment used	*Do not use replenishment. The level of dissolved carbonate might increase due to pressurization of the buffer bottle causing a pH drop.*

Preconditioning Entries:

Function	Parameter	
1 Flush	4.00 min, I: 9, O:1	*Place flushing vial at 9 and waste vial at 1. Remember to monitor the waste vial volume for overflow.*

Postcondition Entries:

No Postconditioning used

Injection Table Entries:

Function	Parameter	
1 PRESSURE	50.0 mbar, 6.0 sec, I: InjectVial, O:OutHomeVial	*May be increased or decreased depending on sample concentration.*
2 PRESSURE	50.0 mbar, 4.0 sec, I: InHomeVial, O:OutHomeVial	*The post injection plug helps to minimize sample loss upon application of voltage. A voltage ramp is used for the same purpose.*

Electric:

Electric	On	
Polarity	Negative	*Negative polarity is used since EOF is reversed.*
Voltage	30.0 kV	
Current	150.0 mA	*A current limit is not necessary but may be used to prevent excessive current generation in case the wrong vial is used.*
Power		System Limit
Low Current Limit		2 µA

Store Data:

Collect voltage	Yes	
Collect current	Yes	*It is recommended to store the current for every analysis. Current in this method shows from -30 to -40 µA.*
Collect power		No
Collect pressure		No
Collect temperature		Yes

Time entries:

Stop time	40.00 min *Adjust as needed when running actual sample.*
Post time	Off

Time Table is empty.

Diode array detector

Settings:

Stop Time	as HPCE: 40.00 min
Post Time	Off
Response Time 1.3 sec	*This is recommended to reduce baseline noise.*
Peak width >	0.1 min
Prerun Autobalance	On
Postrun Autobalance	Off

Spectrum:

Store	None

Signals:

Store	Reference
Signal, Bw	Reference, Bw [nm]
A: Yes 350/20	230/10

Results and discussion

Simultaneous analysis of inorganic anions, organic acids, amino acids and carbohydrates

Figure 1 shows a typical electropherogram of a 43-component anion sample including seven inorganic anions, five organic acids, 16 amino acids and 15 carbohydrates using the standard method. If the results are not similar to these please refer to the section *Troubleshooting* in this note.

In this method most inorganic anions and carbohydrates can be separated. However, migration times of several organic acids such as tartarate, succinate, malate and α-ketoglutarate are close. This separation can be improved by using the Organic Acids Analysis Kit from Agilent Technologies (PN 5063-6510).

With respect to amino acids, Leu and Ile cannot be resolved. Although Arg is not observed in figure 1, Arg can be detected at approximately 35 minutes if the sample is dissolved in an alkaline solution. Since Tyr and Trp have UV absorbance at 230 nm, they are recorded as negative peaks. Ser migrates just before a system peak.

Electrophoretic mobilities at 20 °C

The effective mobilities of 82 compounds including nine inorganic anions, 23 organic acids, 18 amino acids and 32 carbohydrates were determined by this method at 20 °C and are listed in table 1. If the mobilities of the compounds of interest are close, they are difficult to separate. In this case investigating a change of temperature or pH is recommended.

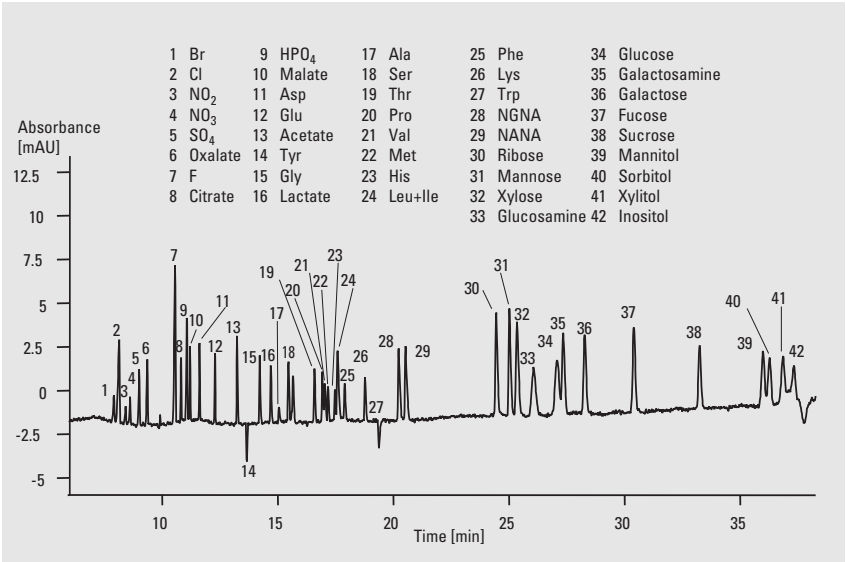


Figure 1
Analysis of 43-component anion standard mixture

Buffer	Agilent Basic Anion Buffer (PN 5064-8209)
Sample	Cl 110 mg/l, carbohydrates 200 mg/l each, others 50 mg/l each
Capillary	fused silica, l=104 cm, L=112.5 cm, id= 50 µm
Injection	300·mbar·s
Temperature	15 °C
Voltage	-30 kV
Detection	signal 350/20 nm, reference 230/10 nm

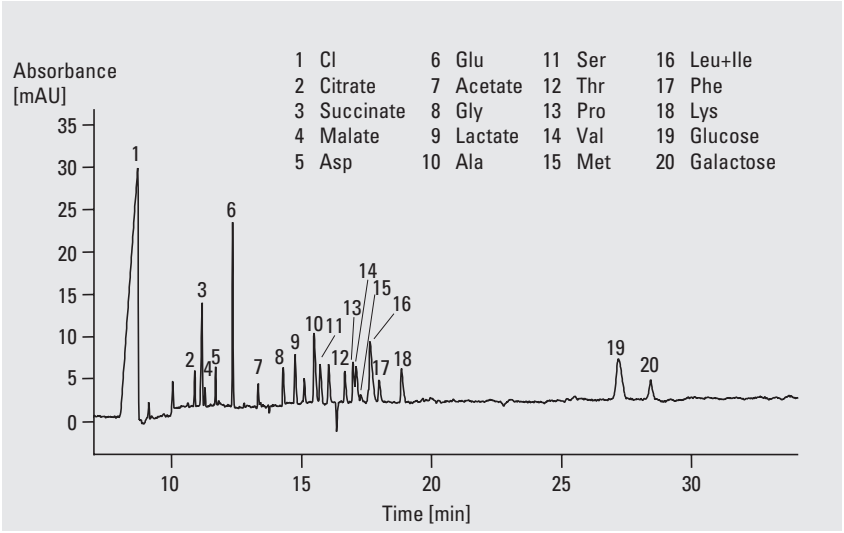
Compound	Mobility (X10-4 cm2/Vs)	Compound	Mobility (X10-4 cm2/Vs)	Compound	Mobility (X10-4 cm2/Vs)	Compound	Mobility (X10-4 cm2/Vs)
Bromide	-7.181	Acetate	-3.589	n-Hexanoate	-2.385	Glucosamine	-0.846
Chloride	-6.983	Pyruvate	-3.540	Galacturonic	-2.337	Mannosamine	-0.832
Nitrite	-6.648	CysCys	-3.514	His	-2.310	Lactose	-0.774
Nitrate	-6.442	Glycolate	-3.495	Leu	-2.300	Arabinose	-0.764
Sulfate	-6.140	Tyr	-3.493	Ile	-2.300	Glucose	-0.761
Oxalate	-5.784	Gly	-3.260	Phe	-2.220	Maltose	-0.731
Ascorbate	-5.409	n-Propionate	-3.111	n-Heptanoate	-2.149	Galactosamine	-0.725
Malonate	-5.093	Lactate	-3.041	Gluconate	-2.060	Lactulose	-0.676
Fluoride	-4.990	Borate	-2.963	Lys	-2.026	Galactose	-0.659
Formate	-4.911	Ala	-2.858	Trp	-1.910	Fucose	-0.485
Citrate	-4.775	Ser	-2.795	NGNA	-1.719	Sucrose	-0.291
Pyrophosphate	-4.760	n-Butyrate	-2.781	n-Octanoate	-1.707	Raffinose	-0.284
Phosphate	-4.677	Levulinate	-2.729	NANA	-1.675	Mannitol	-0.134
Tartarate	-4.584	Mannuronic	-2.674	ManNAc	-1.221	Trehalose	-0.121
Succinate	-4.565	Pyroglutamate	-2.631	Ribose	-1.037	Sorbitol	-0.118
Malate	-4.520	n-Pentanoate	-2.578	Fructose	-0.983	Galactitol	-0.104
a-Ketoglutarate	-4.513	Thr	-2.542	GlcNAc	-0.975	Xylitol	-0.086
Asp	-4.418	Glucuronic	-2.497	Mannose	-0.966	Erythritol	-0.076
Glutarate	-4.196	Pro	-2.450	Xylose	-0.935	Inositol	-0.064
Glu	-4.084	Val	-2.444	GalNAc	-0.919		
Adipate	-3.934	Met	-2.389	Rhamnose	-0.904		

Table 1
Electrophoretic mobilities of anions at 20 °C

Applications

Soy sauce analysis

Figure 2 shows the analysis of a soy sauce. The sample was diluted 1:50 with CE water. Centrifugal filtering through a 30 kDa cutoff filter was applied to remove proteins and peptides. A well-defined electropherogram was obtained without interference from other matrix compounds. Satisfactory reproducibilities were obtained for all compounds with RSD values (n=5) for migration times better than 0.3 % and for peak areas between 0.6 and 5.4 %.



Buffer	Agilent Basic Anion Buffer (PN 5064-8209)
Sample	soy sauce, 1:50 diluted with water, ultrafiltration with 30-kDa cutoff filter
Preconditioning	4 min with run buffer
Capillary	fused silica, l=104 cm, L=112.5 cm, id= 50 µm
Injection	300 mbar·s
Temperature	15 °C
Voltage	-30 kV
Detection	signal 350/20 nm, reference 230/10 nm

Figure 2
Analysis of soy sauce

Pineapple analysis

This method was applied to the analysis of organic acids and carbohydrates in pineapple. In the agriculture industry, technical experts are trying to develop a new crossbreed of fruits. Since the content of organic acids and carbohydrates determines the taste of the pineapple juice, their analysis can help to characterize the product.

If citrate and malate concentrations are high, the taste tends to be sour. If the carbohydrate concentration is high, the taste is sweet. Determination of these compounds is traditionally performed using two HPLC methods. However, the method described here enables the simultaneous analysis of both organic acids and carbohydrates in a much shorter time (less than 18 min) and in a single run. In order to reduce the analysis time, a shorter length capillary was used for this sample. Squeezed pineapple juice was diluted 50-fold with CE water prior to injection. Figure 3 shows a result of the analysis.

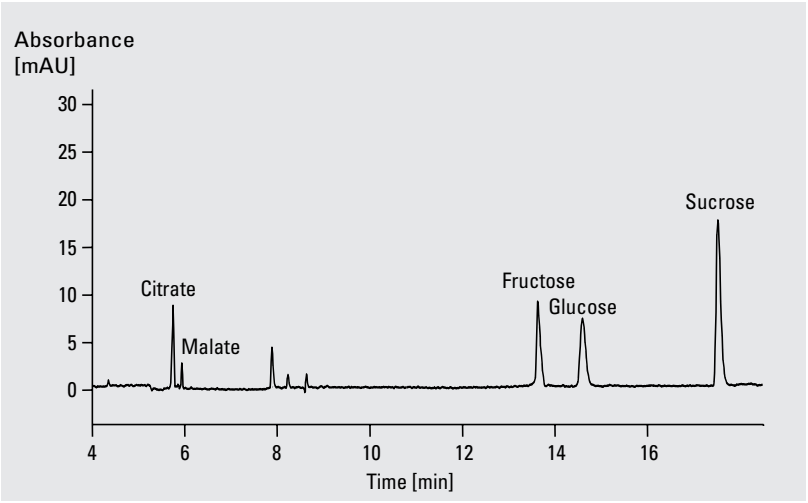


Figure 3
Analysis of pineapple

Buffer	Agilent Basic Anion Buffer (PN 5064-8209)
Sample	pineapple, 1:50 diluted with water,
Capillary	fused silica, l=72 cm, L=80.5 cm, id= 50 µm
Injection	300 mbar·s
Temperature	20 °C
Voltage	-25 kV (reversed polarity)
Detection	signal 350/20 nm, reference 275/10 nm

Troubleshooting

Problem	Possible Cause	Solution
Poor resolution or broad/split peaks	Buffer is old	Use new buffer
	Capillary damaged	Replace capillary
	Sample overloaded	Dilute sample
	Capillary too short	Replace capillary
Peak leading	Buffer absorbed carbonate	Use new buffer
No signal	Sample not injected	Verify no air bubble trapped in bottom of sample vial
		Verify inlet capillary set correctly
	Capillary damaged	Replace capillary
	Wrong buffer used	Verify buffer
	Wrong setting of power supply polarity	Check that polarity is negative
	Detection wavelength incorrect	Verify signal: 350/20, reference: 230/10 nm
Noisy baseline	Wrong setting of response time	Verify response time 1.3 sec DAD
	Capillary window not adjusted	Examine capillary window
	Capillary window dirty	Examine and clean with lint-free paper/MeOH
	Lamp is old	Replace lamp
	Buffer pH higher than 12.3	Verify buffer pH
Poor reproducibility	Buffer overused	Replace buffer
Unstable current	Capillary broken	Replace capillary
	Capillary not filled with buffer	Increase flush time

References

1.
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Tomoyoshi Soga is application chemist at Yokogawa Analytical Systems, Japan. Agilent Technolgies recognizes his efforts in the development of this work.

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Analysis of catechins in tea by HPLC with electrochemical detector

Kuniko Koizumi and Hiroki Kumagai

Food

Abstract

Catechin (C), epicatechin (EC), epigallocatechin (EGC) and epigallocatechingallate (EGCG)—collectively called catechins, have many phenolic hydroxyl groups in the molecule. It is known that catechins have various physiological benefits such as anti-oxidation, anti-bacterium, and anti-tumor effects, as well as the suppression of the increase of cholesterol concentration in blood. Catechins also effect the flavor of foods such as tea, wine, and beer.

This brief demonstrates the analysis of catechins in tea using the Agilent 1100 Series modules and systems for LC with an electrochemical detector.

Analyzed Compounds

(+)-catechin,
(-)-epicatechin
(-)-epigallocatechin
(-)-epigallocatechingallate

Sample

Green tea, tea and woolong tea

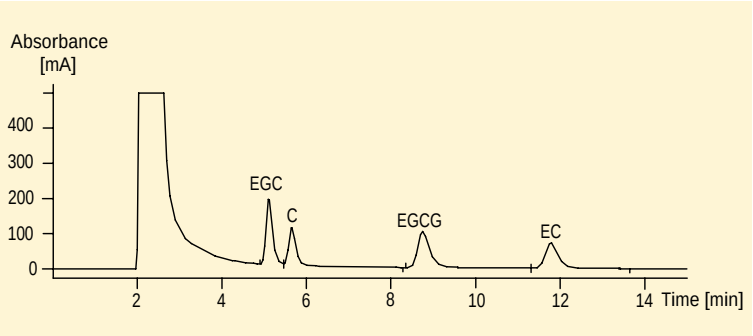

Figure 1
Chromatogram of standard solution, 2 mg/L each

Conditions

Column
250 x 4.0 mm Hypersil BDS
(Agilent Parts No: 79926 OB-584)
Mobile phase
Methanol: (2g $NaNO_3$ + 0.05 g H_2SO_4 in 1l Water) = 22:78
Column temp 25 °C
Injection vol 20 µl
Detector
Agilent 1049 Electrochemical detector
Mode
Amperometry Working electrode; glassy carbon. Applied Potential:0.850 V

Sample preparation
Green tea, tea and woolong tea were diluted with water and filtrated.

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Method Performance

Limit of detection
0.26–2.00 ng (S/N = 3)

RSD of peak area
0.73–1.14 %

RSD of retention time
0.09–0.14 %

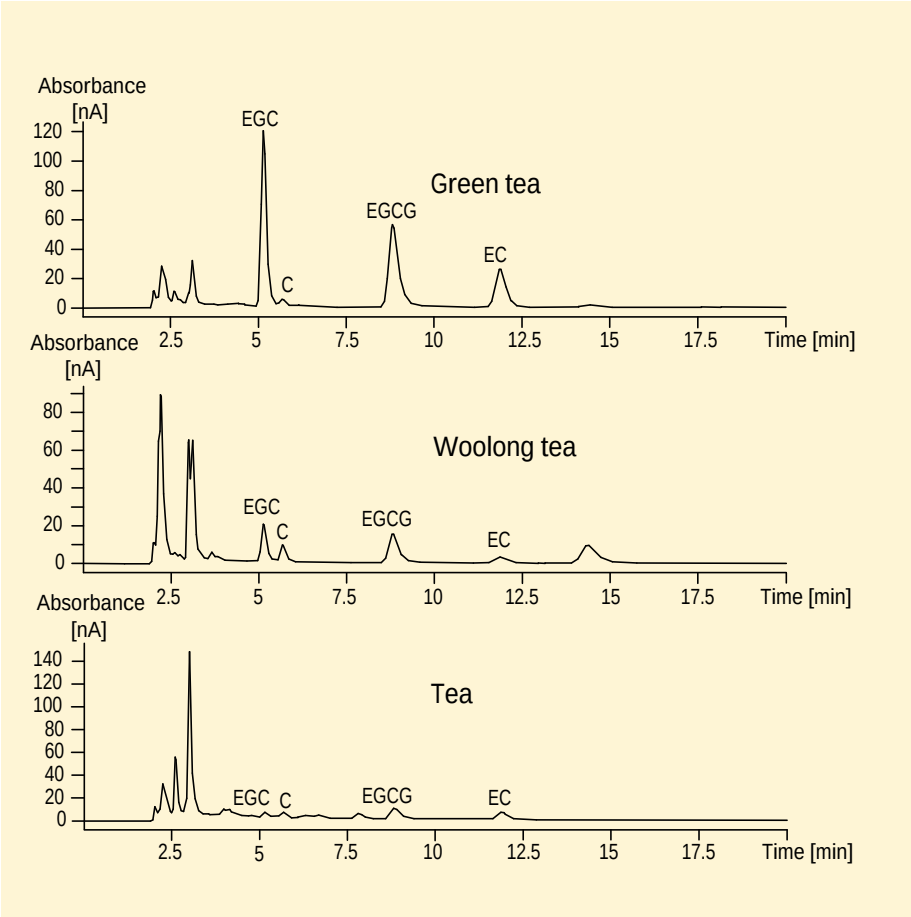
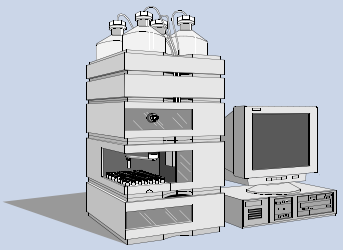


Figure 2
Chromatogram of catechins in various teas

Equipment

Agilent 1100 Series
- binary pump with
- vacuum degasser
- autosampler
- thermostatted column compartment
- programmable electro chemical detector
- diode array detector

Agilent ChemStation + software



Kuniko Koizumi and Hiroki Kumagai are application engineers based at Yokogawa Analytical Systems Inc., Tokyo, Japan.

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Released 10/97
Publication Number 5965-9802E

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Normal Phase Analysis of Tocopherols in Margarine using HPLC

Angelika Gratzfeld-Huesgen

Food

Abstract

Tocopherols cannot be separated completely using reversed-phase chromatography. However, normal-phase chromatography can separate isocratically all eight tocopherols (T) and tocotrienols (T_3) naturally occurring in fats, oils, and other foodstuffs. Fluorescence detection is recommended for the analysis of total lipid extraction because UV absorbance detection is not selective enough to prevent detection of coeluting peaks.

Chromatographic conditions

The HPLC method presented here was used in the analysis of margarine.

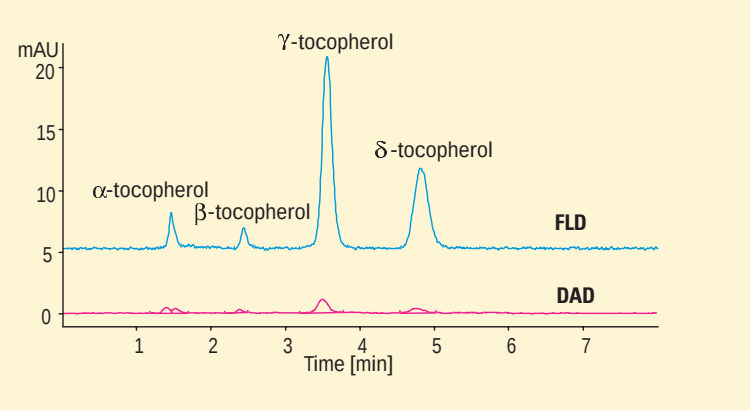

Figure 1
Analysis of tocopherols on normal phase using UV and fluorescence detection

Conditions

Column
100 ˘ 2.1 mm Hypersil SI 100, 5 µm
Mobile phase
hexane + 2 % isopropanol
Stop time 8 min
Flow rate 0.3 ml/min
Column compartment 25 °C
Injection vol 0.5 µl
Detector UV-DAD 295/80 nm
Fluorescence
excitation wavelength 295 nm, emission wavelength 330 nm

Sample preparation
20 g sample dissolved in 15 ml hexane

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HPLC method performance

Limit of detection for diode-array
10–20 ng, S/N = 2

Limit of detection for fluorescence
0.5–2 ng S/N = 2

Repeatability of
RT over 10 runs <2 %
areas over 10 runs <2 %

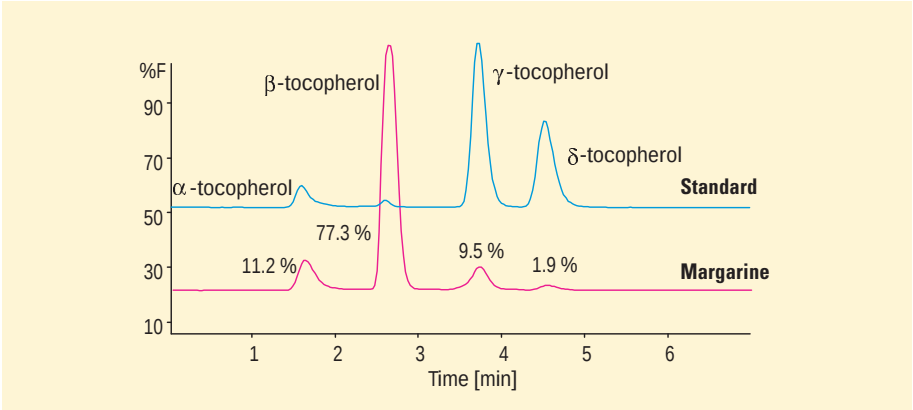
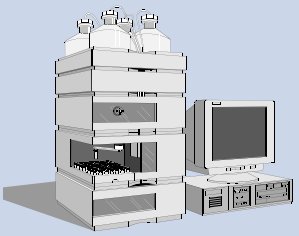


Figure 2
Analysis of tocopherol concentration in margarine fat extract with fluorescence detection

Equipment

Agilent 1100 Series
- vacuum degasser
- isocratic pump
- autosampler
- thermostatted column compartment
- electrochemical detector

Agilent ChemStation + software



Angelika Gratzfeld-Huesgen is application chemist at Agilent Technologies, Waldbronn, Germany.

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Released 09/97
Publication Number 5966-0641E

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HEWLETT PACKARD

Computer-Aided Development of a Reversed-Phase HPLC Separation of Acids in Coffee

Application Note 228-207

Food

October 1992

Authors

Valerie L. Vaughn
Hewlett-Packard Co.
Midwestern Sales Region
Kansas City, MO 64133

Hans-Jürgen P. Sievert
and Clifford B. Woodward
Hewlett-Packard Co.
Analytical Marketing Center
Avondale, PA 19311

Abstract

This application note describes the use of Hewlett-Packard's Interactive Computer-Aided Optimization Software (ICOS) for HPLC separations for the development of a reversed-phase separation. The elution strength search module of the software was used to determine the concentrations of two binary solvent blends that would elute the sample in a predetermined time. The ternary solvent space between the binary blends was then explored using the lattice search module of the software. Finally, the retention modeling module was applied to predict future optimization steps.

Introduction

Green coffee beans have no desirable flavor or aroma of their own; these aspects of coffee are derived from compositional changes that occur in the bean during the roasting process. Among the coffee components that undergo the greatest compositional change during roasting are the chlorogenic acid isomers. These isomers, a group of phenolic acids found in coffee and other plant materials, are commonly referred to simply as "chlorogenic acid". The major component of chlorogenic acid has been designated 5-caffeoyl-quinic acid by IUPAC (1976) nomenclature.

Destruction of chlorogenic acid occurs in a progressive and extensive manner during the roasting process, with losses sometimes exceeding 90%[1]. The degree of roast may have an influence on the final beverage flavor because the individual isomers appear to have different sensory qualities and are not necessarily destroyed at the same rate[2].

Several analytical methods have been developed that monitor roast severity by comparing the relative loss of chlorogenic acid to that of caffeine, a component of coffee found to be essentially stable to the roasting process, including an HPLC separation that correlates the 5-caffeoyl-quinic acid/caffeine ratio to the degree of coffee roast[3,4].

HPLC offers a simple, fast, analytical method capable of separating and quantitating the 3-, 4-, and 5-caffeoyl-quinic acid isomers of chlorogenic acid.

Experimental

Chromatographic Conditions

Column:	ODS Hypersil, 100 x 2.1 mm, 5 µm particle size (HP p/n 79916SI-552)
Mobile phase:	20-mm citric acid buffer, pH 2.25, ACN, MeOH; variable organic modifier ratio
Flow rate:	0.45 ml/min
Column temperature:	40°C
Injection volume:	15 µl
Detector:	Diode array, at 290 and 320 nm with 8 nm bandwidth; reference at 420 nm with 20 nm bandwidth

Instrumentation

Chromatography was conducted using an HP 1090M Series II liquid chromatograph equipped with a ternary DR5 solvent delivery system, an automatic sampler, an automatic injector, and a diode array detector. Data were acquired using an HP 79994A Pascal HPLC ChemStation with HP 79988A Operating Software, revision 5.22, and the HP 79979A Interactive Computer Optimization Software (ICOS) for HPLC separations, revision 1.0.

Reagents

Citric acid was purchased from Fisher Scientific (Fair Lawn, NJ). All chromatography solvents were HPLC-grade and were obtained from Burdick and Jackson (a division of Baxter Health Care, Muskegon, MI). Deionized HPLC-grade water was obtained using a Hewlett-Packard model 661A water purifier. Chlorogenic acid, caffeine, and 5-hydroxymethylfurfural were obtained from Sigma Chemical (St Louis, MO).

Sample and Standard Preparation

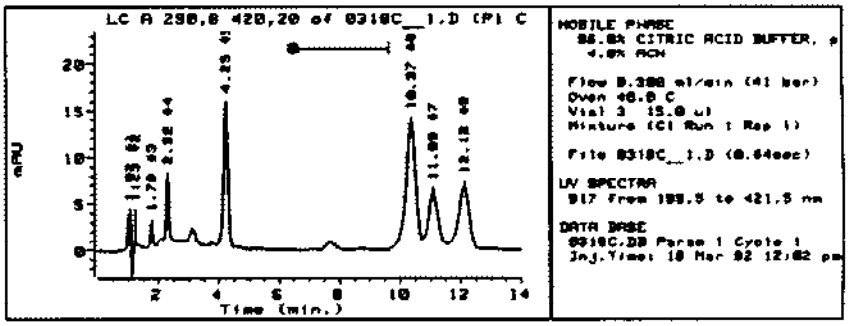
The chlorogenic acid isomers were extracted from a coffee matrix by adding 0.50 g of coffee to 25 ml of boiling HPLC-grade water and diluting to 50 ml with water. Six ml of the extract were then diluted to 50 ml with water and an aliquot injected for chromatography. The chlorogenic acid isomers were prepared by adding several drops of concentrated ammonium hydroxide (Fisher Scientific) to an aqueous solution of the chlorogenic acid standard[5]. The caffeine and 5-hydroxymethylfurfural standards were dissolved in HPLC-grade water for chromatography.

Results and Discussion

A low pH buffer was chosen for initial starting conditions, and the elution strength search module of the software was used to determine an elution concentration for acetonitrile. Starting with a concentration sufficiently high to elute all analytes in a short time period, the software automatically and iteratively reduced the acetonitrile concentration to fulfill a preset analysis time requirement of 12 minutes. The final concentration of acetonitrile determined in this fashion was 4%.

Isoelutropic theory was then used to calculate a comparable methanol elution concentration.

Although either binary solvent system, buffer/acetonitrile (ACN or buffer/methanol (MeOH), provided sufficient separation and resolution of all components in the sample mixture, the two organic binary mixtures were incorporated into a solvent lattice search in one dimension with two limits. This search moved from a binary buffer ACN mix through ternary mixes of buffer/ACN/MeOH to a binary system of buffer/MeOH in four steps (analyses). Figure 1 is an example of a typical ICOS lattice search report. The report shown is the result of the first step of the lattice search defined above.



Peak#	time (min)	k'	ln(k')	area	TotArea (%)	MaxArea (%)	height	sigma (sec)	symmetry	plates	Rs	S	
1	1.073	0.46	-0.784	69	7.4	22.0	6.4	3.82	4.480	284	—	—	
2	1.231	0.67	-0.399	27	2.9	8.5	2.6	3.80	0.610	578	*0.62	0.0686	
3	1.782	1.42	0.350	12	1.2	3.7	2.4	1.83	0.710	3396	2.94	0.1829	
4	2.318	2.15	0.764	48	5.2	15.3	7.1	2.47	1.290	3166	3.73	0.1307	
5	4.246	4.76	1.561	149	16.1	47.6	15.1	2.82	1.100	4442	9.19	0.2937	
6	10.373	13.08	2.571	313	33.9	100.0	15.6	8.77	1.190	5041	14.60	0.4191	
7	11.092	14.06	2.643	139	15.0	44.4	6.0	8.84	0.850	5665	1.23	0.0335	
8	12.121	15.45	2.738	169	18.2	53.8	6.7	9.73	1.210	5582	1.66	0.0443	
	5.530			925		313		5.39	1.430	3494	1862.75	0.0000	product
											0.0294	0.0808	relative
#peaks : 8			analysis time : 12.875 min			RsMin : 0.62				(T-Zero : 0.737 min)			

Figure 1. Plot and report for a single analysis in a lattice search.

During this search, spectral information was acquired from 198.5 to 421.5 nm using a diode array detector. The lattice search module of the software offers the user the option of reporting chromatographic and spectral information automatically as displayed in figure 2. Spectra that have been acquired at the inflection points and the apex were normalized and overlaid, and a peak purity factor was calculated. The spectral information and peak purity factor are presented for each peak of interest in separate windows below the chromatogram. For example, peak 1, which is not as well resolved as the other peaks, has a peak purity factor of 974.35. A peak with a purity factor less than 990 is typically considered impure.

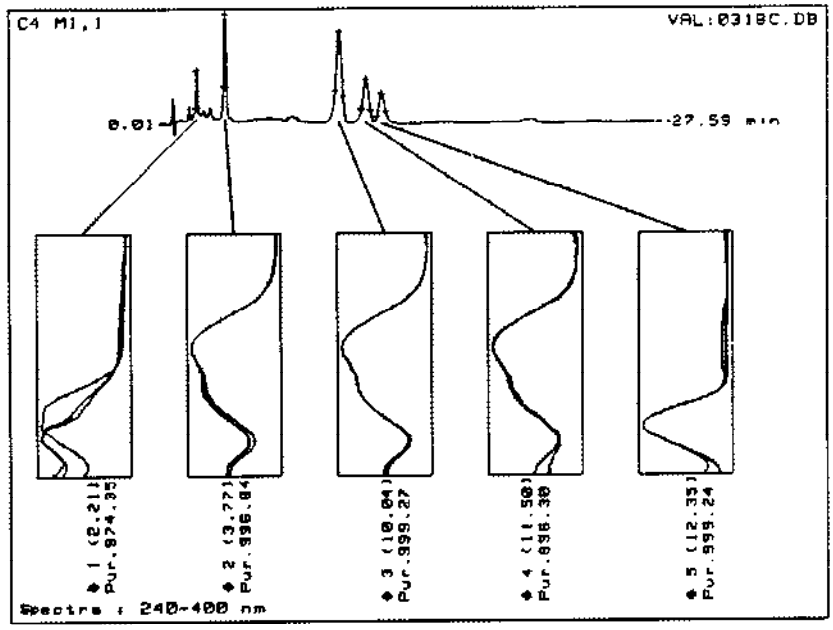


Figure 2. Plot of chromatographic signal and peak spectra for purity analysis.

A retention model generated from the data obtained during the lattice search is shown in the upper part of figure 3. The analyses incorporated into the retention model are marked by asterisks on the right side of the retention model window; the two binary limit analyses are also indicated by percentage composition given as text, and the two ternary steps are indicated by asterisks alone.

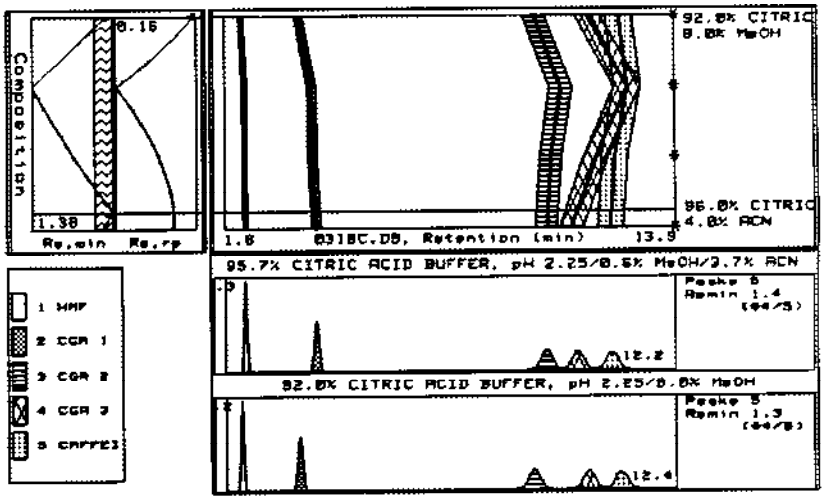


Figure 3. Retention model based on three analyses.

The retention model automatically calculates separation criteria. "Rs,min" gives the resolution of the least separated pair. "Rs,rp" the relative resolution product, describes how evenly the chromatographic peaks are distributed. Both measures are emphasized in the two simulated chromatograms displayed below the retention model in figure 3. The top chromatogram (95.7% 20 mm citric acid buffer pH 2.25/0.6% MeOH/ 3.7% ACN) shows a simulated separation that should provide the best resolution of the least separated pair of peaks in the chromatogram. In the lower chromatogram, the predicted separation that would provide the best "Rs,rp" value for this analysis (92.0% 20 mm citric acid buffer, pH 2.25/8.0% MeOH) is displayed.

Separation of the five components in this sample can be obtained using either a binary buffer/ACN or buffer/MeOH mixture; however, a solvent lattice search was done to examine the use of a ternary solvent system. A retention model was created based on the lattice search data base with intent to further optimize the separation. In this instance, however, the separation criteria "Rs,min" and "Rs,rp" indicate that a simple binary blend provides adequate separation of all five peaks and that moving to a ternary solvent system does not enhance separation.

If an appropriate separation had not been identified through a simple lattice search, the retention modeling portion of the software could have been redefined iteratively to aid the chromatographer in optimizing the separation in a minimum number of analyses (see Product Brief, publication no. 12-5091-0325E).

Conclusion

ICOS solvent optimization software can be used to develop a reversed-phase HPLC separation of acid componenets extracted from coffee in a minimum number of analyses.

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5. Turgo, L. C. and Macrae, R., *Analyst*, 1984, **109**, 263.

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Analysis of Selected Anions with HPLC and Electrochemical Detection

Application Note

Food

Angelika Gratzfeld-Huesgen
and Rainer Schuster

Traditionally, conductivity is the standard detection method for ion analysis. During the last 10 years, however, there has been increasing interest in electrochemical detection (ECD) for the analysis of anions. This is due to the high sensitivity and selectivity of this type of detection.

Figure 1 shows how certain anions can be selectively detected in the presence of several other anions. In total, 16 anions were injected. Four that can be oxidized: iodide, nitrite, bromide, thiocyanide, and twelve that cannot be oxidized: fluoride, chloride, azide, nitrate, phosphite, sulfate, molybdate, hydrogencarbonate, perchlorite, phosphate, selenate and perchlorate.

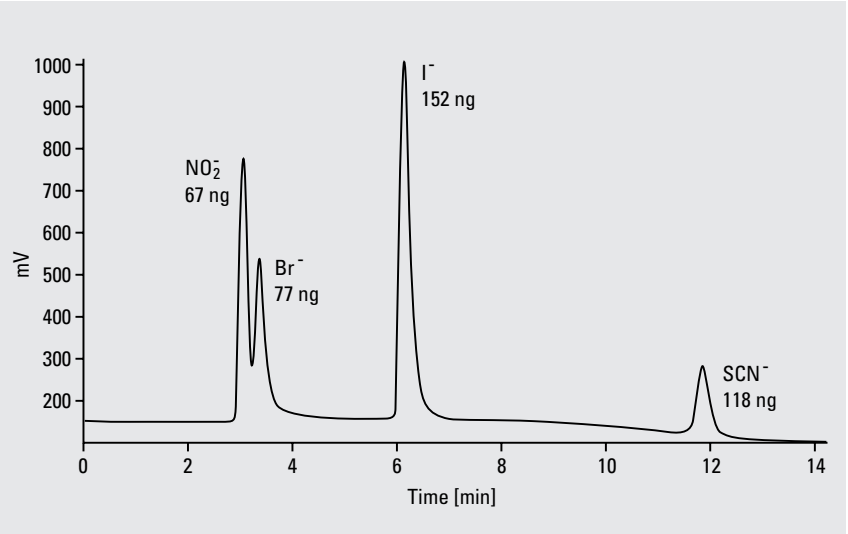

Figure 1
Analysis of standard

Chromatographic conditions

Column:	200 x 4 mm, Spherisorb ODS2, 5 µm
Mobile phase:	water with 5.2 g/l K_2HPO_4 + 3.6 g/l KH_2PO_4 + 3 g/l $TBAHSO_4$*/ACN=85:15
Flow rate:	1 ml/min
Inj. volume.:	0.1 µl
Potential:	1 V
Mode:	amperometry
Electrode:	glassy carbon

* Tetrabutylammoniumdihydrogensulphate

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In order to demonstrate the selectivity and sensitivity, iodide in table salt was analyzed. There is currently a great deal of interest in the determination of iodide in various foodstuffs, because some data indicates that the consumption of iodide has increased, leading to a potential rise in thyroid disorders. Table salt is almost 100% sodium chloride and with conductivity detection a large chloride peak may overlap with the iodide. Figure 2 shows the selective detection of iodide in a large excess of cloride.

Detection potential is of great importance for selectivity and sensitivity and must be determined for each set of analysis parameters.

The Agilent 1049A ECD in combination with an auto-sampler provides a time-saving way to find the optimum potential for several compounds through its auto-increment mode. The potential is automatically increased within a series of runs and the best detection potential can be selected from the chromatogram plots.

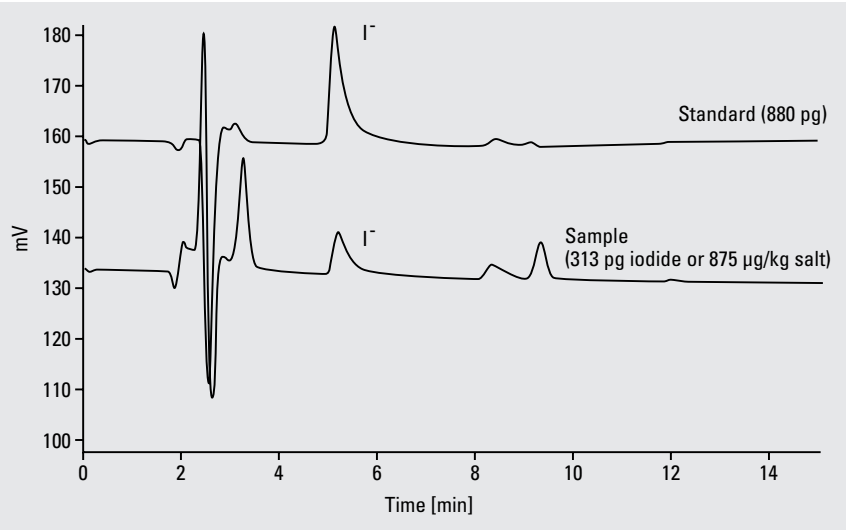

Figure 2
Analysis of iodide in table salt, dissolved in water

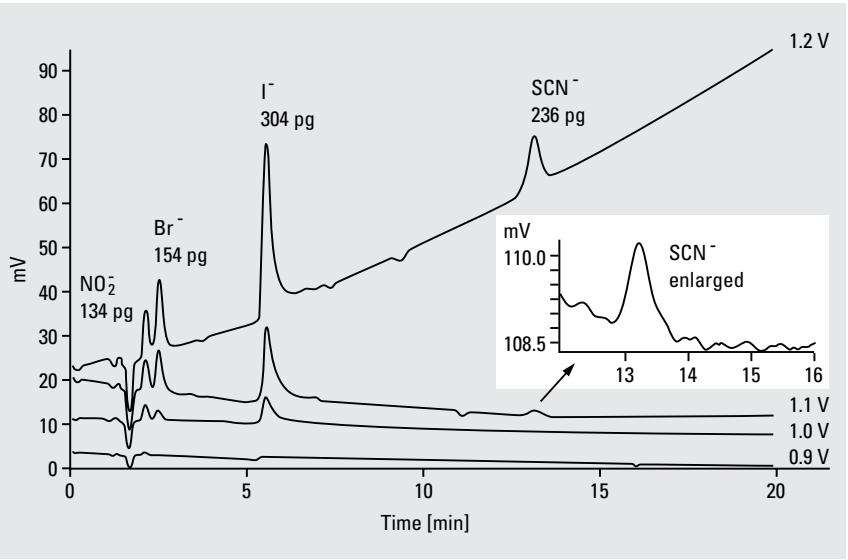

Figure 3
Optimizing detection potential using the 'auto-increment' mode

Chromatographic conditions

Column:	200 x 4 mm, Spherisorb ODS2, 5 µm
Mobile phase:	water with 5.2 g/l K_2HPO_4 + 3.6 g/l KH_2PO_4 + 3 g/l $TBAHSO_4$*/ACN=85:15
Flow rate:	1 ml/min
Inj. volume.:	0.1 µl
Potential: 1 V	
Mode:	amperometry
Electrode:	glassy carbon

* Tetrabutylammoniumdihydrogensulphate

An example is given in figure 3. The optimum potential was 1.1 V. At lower potentials the response was insufficient, at higher potentials the detector drift was unacceptable.

The minimum detectable level for iodide was about 40 µg iodide per kilogram table salt. The relative standard deviation for peak heights over 75 runs was 3% for 300 pg of iodide and an injection volume of 10 µl.

Angelika Gratzfeld-Huesgen and Rainer Schuster are applications chemists based at Agilent Technologies, Waldbronn, Germany

Publication Number 5091-1815E

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Natural Compounds & Additives

Proteins

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Detection of Recombinant Bovine Somatotropin in Milk by LC-ESI-MS/MS

Application

Food Safety

Authors

Marie-Hélène Le Breton, Sandrine Rochereau-Roulet, Gaud Pinel, Fabrice Monteau, and Bruno Le Bizec
Laboratoire d'Etude des Résidus et Contaminants dans les Aliments (LABERCA)
Ecole Nationale Vétérinaire de Nantes (ENVN)
BP 50707, 44307, Nantes
France

Marie-Hélène Le Breton
2 Nestlé Research Center
Nestec Ltd., P.O. Box 44
CH-1000 Lausanne 26
Switzerland

Abstract

Recombinant bovine somatotropin (rbST), also called growth hormone, is a protein hormone used in dairy farming to enhance milk production. A method has been developed for the detection of rbST in milk by ESI(+)-LC-MS/MS. This method allowed a detection limit of 20 pg of tryptic N-terminal peptide rbST in standard solution injected on-column and was successfully applied to extracts obtained from milk samples spiked with 50 ng/mL^{-1} (2.3 pmol/mL^{-1}) rbST.

Introduction

Recombinant bovine somatotropin (rbST), also called growth hormone, is used in lactating cows to increase milk production. Different regulations exist regarding its use, but the lack of confirmatory methods [1] for its detection makes it difficult to apply these regulations. It turns out to be an international issue in terms of animal doping and in food safety as well. Indeed, residues of rbST can be present in the milk of dairy animals treated with this hormone.

In order to detect residues of rbST in milk, the choice has been made to focus the analysis on the tryptic N-terminal peptide of the protein, specifically the difference between the endogenous and recombinant forms. The N-terminal amino acid alanine that is present in the endogenous form is replaced by a methionine in the recombinant one [2].

This application describes a method for the detection of rbST by ESI(+) LC-MS/MS. The method was successfully applied to extracts from milk samples spiked with rbST.

Experimental

Standards of Proteins and Peptides

Protein standards of rbST and recombinant equine somatotropin, reST (EquiGen-5), were obtained from the Harbor-UCLA Medical Center, National Hormone and Pituitary Program (Torrance, CA, USA) and Bresagen Limited (Thebarton, Australia), respectively.

Reversed-Phase HPLC Separation of Water-Soluble Vitamins on Agilent ZORBAX Eclipse Plus Columns

Application

Pharmaceutical, Food/Beverage

Authors

Anna Glinko, Michael J. Bozym, Michelle L. Owens, and Karyn M. Usher
Department of Chemistry
West Chester University
West Chester, PA 19383
USA

Ronald E. Majors
Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE, 19808
USA

Abstract

A mixture of six B vitamins and vitamin C were separated on the same Agilent ZORBAX Eclipse Plus C-18 stationary phase in three different particle sizes and column dimensions: 5 µm (4.6 × by 150 mm); Rapid Resolution (RR), 3.5 µm (4.6 × 100 mm), and RR High Throughput (RRHT), 1.8-µm (4.6 × 50 mm). A simple phosphate buffer-methanol mobile phase gradient was used. The method was designed to supplement United States Pharmacopeia (USP) methods in which these vitamins are separated singly on different columns by different methods, some involving complex ion-pair reagents. The shorter columns provided more rapid separations with little change in resolution. All compounds could be separated on the 1.8-µm column in 3.5 minutes. A gain in sensitivity was noted when the column length and particle size were reduced. The HPLC method was applied to the analysis of vitamins in a variety of commercial products, including multivitamins and various soft drinks. The columns chosen allowed for good separation, but the RRHT column also allowed quick method development without giving up the resolution and speed requirements for the method. The ZORBAX Eclipse Plus columns were shown to have high efficiency and superior performance characteristics with minimal tailing.

Introduction

Vitamins are essential nutrients for the proper functioning of the human body. The daily requirements are small, ranging from 2.4 µg to 90 mg [1]. Inadequate amounts of vitamins can cause numerous health problems, such as adrenal impairment (vitamin B) or scurvy or a compromised immune system (vitamin C). With the exception of pyridoxine and cyanocobalamin, water-soluble vitamins are not stored in the body [1]. Taking a multivitamin daily can ensure one is getting the proper amount of vitamins if diet alone cannot provide them.

Many vitamins easily degrade upon exposure to heat, light, and oxygen. Ascorbic acid is known to degrade quickly and easily in aqueous solutions. This degradation has been studied in solutions of different pH and in both aerobic and anaerobic conditions [2]. It has also been documented that the inside surface of glassware may contain materials that measurably degrade ascorbic acid within a short period of time. According to a study published by Margolis and Park, this is particularly true for autosampler vials used to hold samples for chromatography. Their paper gives several suggestions for preventing this decomposition [3]. In our study, vitamin samples were prepared every four

hours as needed. Although the percent relative standard deviation (%RSD) values given for ascorbic acid are very good, these injections were done immediately after the solution was made. Within a couple hours of preparing the solutions, we could see the peak areas for ascorbic acid decreasing with consecutive injections.

Currently, the United States Pharmacopeia (USP) has standard methods for the analysis of the vitamins used in our study; however, there is no USP method for the separation of all eight of the water-soluble vitamins in the mixture used. Some of the USP methods are complicated and involved the use of ion pair reagents (see Table 1). Ion pair reagents can often be used to improve resolution, but may result in irreversible changes in column performance [4]. The USP method for ascorbic acid is not currently a chromatographic method. Furthermore, these USP methods do not allow for the use of some newer column technologies, such as sub-2-micron particle sizes in shorter dimensions.

The main goal of the project was to create a single high-performance liquid chromatographic method that would allow separation of the following water-soluble vitamins: ascorbic acid, biotin, cyanocobolamin, niacinamide, panthothenic acid, pyridoxine, riboflavin, and thiamin. See Figure 1 for the structures of these vitamins. A secondary goal was to demonstrate the effect of decreasing particle size on resolution, separation time, and system pressure.

Experimental

Chromatographic experiments were conducted using an Agilent 1200 SL Rapid Resolution liquid chromatograph (LC) equipped with an autosampler and an 80-Hz diode array detector (Agilent Technologies, Inc., Santa Rosa, CA). Both isocratic and binary gradients were generated using this system. Columns used in the study were: 5-µm Agilent ZORBAX Eclipse Plus C-18 stationary phase in 4.6 mm x 150 mm (P/N: 959993-902), 3.5-m ZORBAX Eclipse Plus C-18 stationary phase in 4.6 mm x 100 mm (P/N: 959961-902), and 1.8-µm ZORBAX Eclipse Plus C-18 stationary phase in 4.6 mm x 50 mm (P/N: 959941-902) formats.

Vitamin standards were obtained from Sigma Aldrich (Milwaukee, WI). Identification of the vitamins in the standard mixture, supplements, and vitamin drinks was confirmed using the diode array detector with high-speed full-spectral UV-VIS detection.

Table 1. Reagents and Requirements for USP Methods (USP31-NF 26, May 2008. Ref. 5)

Vitamin	Reagents needed	Tailing Factor (TF)	%RSD t_R
Pyridoxine	Glacial acetic acid, sodium 1-hexanesulfonate, methanol, water	–	< 3%
Thiamine	Sodium 1-hexanesulfonate, phosphoric acid, hydrochloric acid, acetonitrile, water	< 2	< 3%
Cyanocobalamin	Methanol, water	–	–
Pantothenic acid	Monobasic potassium phosphate, phosphoric acid, methanol, water	–	< 2%
Biotin	Sodium perchlorate, phosphoric acid, dimethyl sulfoxide, acetonitrile, water	–	< 3%
Niacin	Sodium 1-hexanesulfonate, methanol, acetonitrile, glacial acetic acid, water	< 2	< 2%
Riboflavin	Glacial acetic acid, edetate disodium, sodium acetate, triethylamine, methanol, water	–	< 2%

Mobile phase channel A was 25 mM NaH_2PO_4 (pH = 2.5), mobile phase channel B was methanol. The flow rate was 1.0 mL/min. The complete chromatographic conditions are depicted in Table 2.

The mobile phase gradient was changed proportionally to match the column length to keep solvent-strength selectivity (k*) the same (see Table 3).

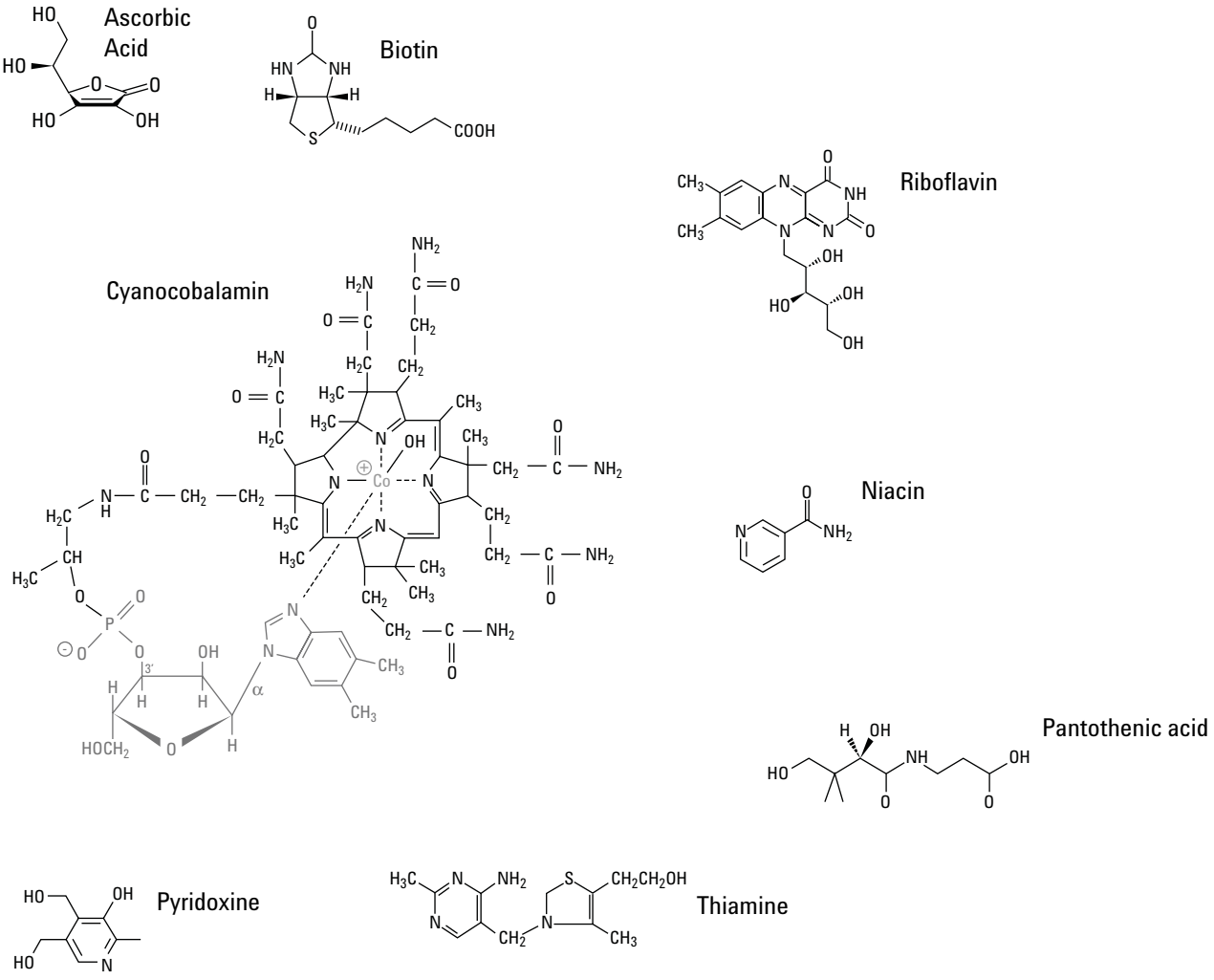

Figure 1. Structures of water-soluble vitamins used in this experiment.

Table 2. Chromatographic Conditions

LC	Agilent 1200 SL
Mobile phase A	25 mM NaH_2PO_4 pH = 2.5
Mobile phase B	Methanol
Flow rate	1.00 mL/min
Column compartment temperature	35 °C
Detection	220 nm, no Reference
Response time	0.05 s
Injection volume	Adjusted for column size: 5 µm, 5 µL 3.5 µm, 3.3 µL 1.8 µm, 1,7 µL
Detector flow cell	Micro flow cell (2 µL)

Table 3. Gradients for Equivalent k*

%B	5 µm	3.5 µm	1.8 µm
1	0.00 min →	0.00 min →	0.00 min
12	1.50 min	1.00 min	0.50 min
30	1.53 min	1.03 min	0.51 min

The individual standards and standard mixture were prepared by weighing out the appropriate masses of each vitamin using an analytical balance or microbalance, as needed, and dissolving in 10 mL of water. All samples that were analyzed for vitamins were purchased locally. The Vitaminwater (Glaceau, Flushing, NY) was injected without dilution. For sample preparation, the chewable vitamin tablets (Berkley & Jensen Children's Chewable Complete Multi Vitamins and Minerals Supplement, Natick, MA) were powdered and then dissolved in 100 mL of water. The adult tablet (Equate Adult Multivitamin, Walmart, Bentonville, AR) was treated in a similar manner. Table 4 outlines all of the final concentrations for standards and vitamin tablets. Prior to injection, all samples were filtered using Agilent syringe Econofilter, regenerated cellulose, 25-mm diameter, 0.20-µm porosity (P/N: 5185-5830).

Results and Discussion

Chromatographic Reproducibilty

The %RSDs for replicate injections of each vitamin are given in Table 5. These values were calculated based on seven replicate injections of each vitamin. In Table 5, the concentrations of each vitamin in the standard solution used to obtain reproducibility data on the 1.8-µm column is reported along with the %RSD values for retention time, area, and tailing factor (at 5 percent height). Some of the USP methods required that the %RSD for retention times be less than 2 or 3 percent (see Table 1); the method for cyanocobalamin had no such requirement. Table 5 shows that all the %RSD values were within the required range, and were actually much lower than the stated limits.

Table 4. Peak Identifications and Concentrations of Vitamins

Pk #	Vitamin	Conc. (g/L) in standard	Conc. (g/L) in soln. of adult vitamin tablet	Conc. (g/L) in soln. of chewable supplement
1	Ascorbic acid	8×10^{-2}	9×10^{-1}	6×10^{-1}
2	Niacin	4.5×10^{-2}	1.5×10^{-1}	2×10^{-1}
3	Pyridoxine	9×10^{-2}	5×10^{-2}	2×10^{-2}
4	Pantothenic acid	2.4×10^{-1}	1×10^{-1}	1×10^{-1}
5	Cyanocobalamin	9×10^{-2}	2×10^{-5}	6×10^{-5}
6	Biotin	Saturated	3×10^{-4}	4×10^{-4}
7	Riboflavin	1.5×10^{-1}	3.2×10^{-2}	1.7×10^{-2}

Table 5. %RSD on 1.8-µm Column

Vitamin	Conc. g/L	%RSD t_R	%RSD area	%RSD TF
Thiamine	1.20	0.7	0.3	1.3
Ascorbic acid	0.11	0.2	0.9	0.6
Niacin	0.15	0.5	0.3	0.9
Pyridoxine	0.09	0.3	1.8	1.9
Pantothenic acid	0.24	0.0	0.4	0.7
Cyanocobalamin	0.18	1.4	0.9	0.4
Biotin	Saturated	0.2	2.9	4.2
Riboflavin	0.15	0.2	2.3	0.9

Comparison of Tailing Factors at 5% Peak Height

Table 6 gives the retention times and tailing factors for each solute on the three ZORBAX Eclipse Plus columns used for these experiments. These columns have a very inert surface, which displays very little tailing for these water-soluble compounds. Note that the average tailing factors for all the vitamins studied were less than 1.2 on all the columns used. This is well within the range of acceptable tailing factors included in the USP methods (see Table 1).

Table 6. Comparison of Retention Times and Tailing Factors at 5% for Each Solute on the Three Agilent ZORBAX Eclipse Plus Columns

	5 µm		3.5 µm		1.8 µm	
Vitamin	Ret. time	Tailing factor	Ret. time	Tailing factor	Ret. time	Tailing factor
Ascorbic acid	2.10	1.1	1.41	1.2	0.76	1.2
Niacin	2.62	1.1	1.87	1.2	1.01	1.2
Pyridoxine	3.03	1.1	2.25	1.3	1.31	1.3
Pantothenic acid	4.02	1.1	2.95	1.1	1.68	1.2
Cyanocobalamin	4.47	1.1	3.33	1.1	1.98	1.2
Biotin	6.33	1.1	4.69	1.1	2.63	1.0
Riboflavin	7.24	1.1	5.40	1.1	3.04	1.1
Average (Tf)	–	1.1	–	1.2	–	1.2

Scalability from One Column Configuration to Another

The three column configurations gave a similar elution pattern as shown in Figure 2. The ZORBAX particles allowed for straightforward scalability from longer columns packed with 5-µm and 3.5-µm particles to the shortest column, packed with 1.8-µm particles. The same mixture was injected onto all three of the columns used. The gradient time was adjusted as described in Table 3 to give equivalent k* values.

Gain in Sensitivity When Comparing Three Different Column Configurations

Table 7 shows the gain in sensitivity for cyanocobalamin in the standard vitamin mix injected into the three different columns. Since the injection volume was scaled proportionally to column length, the peak heights cannot be compared directly. Instead, the amount injected is normalized, which allows the sensitivity to be compared. After the normalization, the ratio of the peak areas to injected amounts is the same. However, the data shows that there is a significant difference in the ratio of the heights and the injected amounts. There is a significant increase in the sensitivity of the 3.5-µm column compared to the 5-µm column, and another significant increase in the sensitivity of the 1.8-µm column. Table 7 shows the gain in sensitivity in comparison to the 5-µm column. The gain in sensitivity when the column is changed from a 5-µm column to a 3.5-µm column is 34 percent, while the gain in sensitivity when the column is changed from a 5-µm column to a 1.8-µm column is 52 percent. This gain in sensitivity is due to the higher efficiency (that is, lower dilution factors) of the smaller particles, not simply because the analytes spend less time in the column.

Table 7. Comparing the Sensitivity of Three Column Configurations (Cyanocobalamin)

Column	Normalized injection amount	Area	% area	Height	% height	% gain in sensitivity
5 µm	100	269	–	49	–	–
3.5 µm	66	171	64	48	98	34
1.8 µm	33	91	34	42	86	52

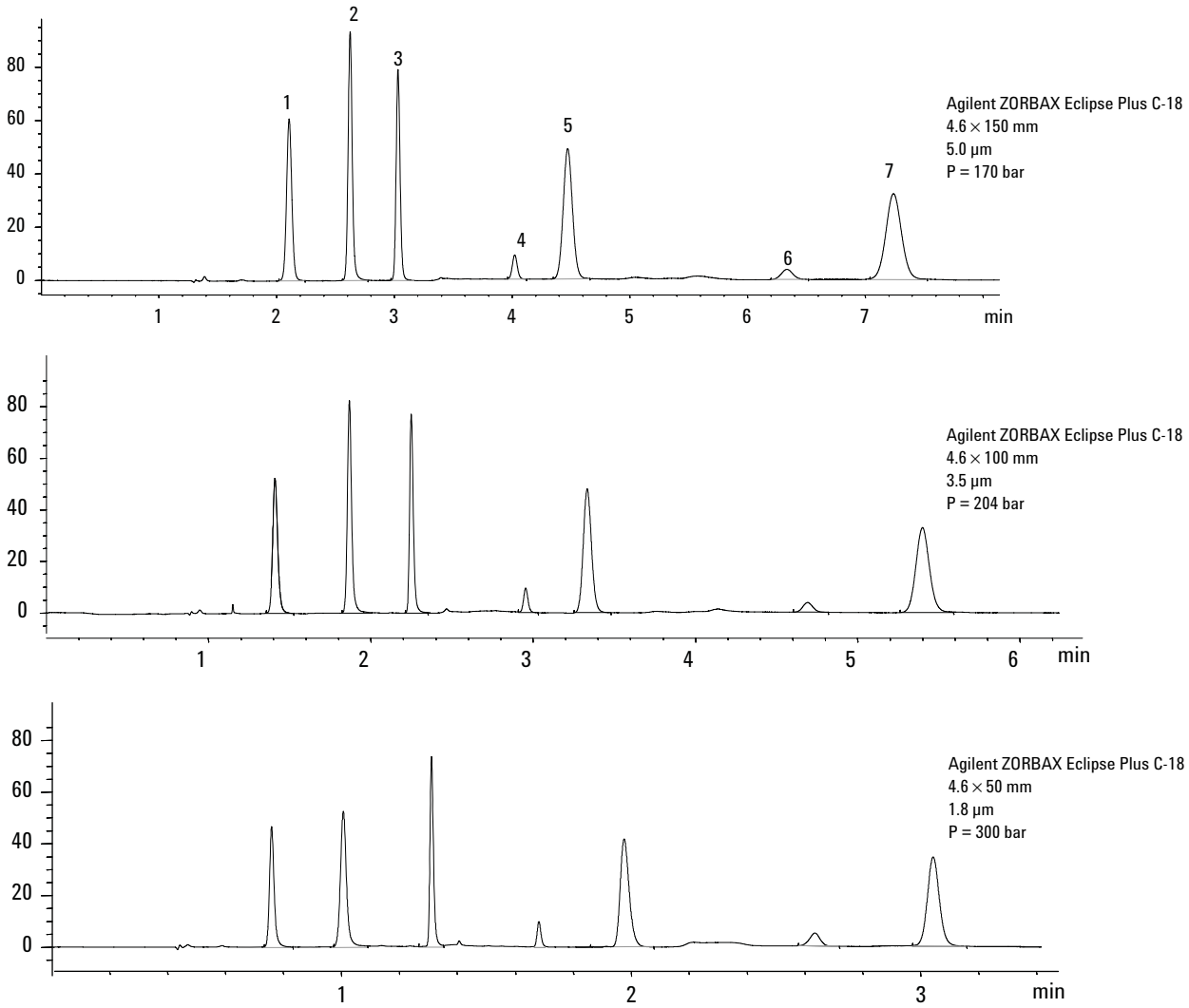

Figure 2. Scalability of Agilent ZORBAX Eclipse Plus C-18 column dimensions and the effect on analysis time and pressure (conditions shown in Table 2).

Comparing Speed and Pressure in Three Different Column Configurations

Figure 2 also shows the difference in separation time performed on the different columns. The separation was shortened from an 8-minute run time on the 4.6 x 150 mm, 5-µm column to a 3.5-minute run on the RRHT, 4.6 x 50 mm, 1.8-µm column. The time could have been shortened further by using a higher flow rate. With the good peak resolution shown in Figure 2 it would have been possible to increase the flow rate. However, when this was attempted, some of the peaks in the commercially available vitamins and drinks could not be identified due to the presence of other overlapping unidentified ingredients. As expected, the pressure increased when the particle size of the column packing was reduced. Figure 2 indicates the system pressures (P), in bar, for the three columns. The highest system pressure reached was 300 bar, when using the 1.8-µm column (RRHT). This is well within the operating range for both the Agilent 1100 (max. 400 bar) and the 1200 SL (max. 600 bar) systems. These results are summarized in Table 8.

Table 8. Gradient Separation Results of the Column Configurations, Holding k* Constant

Column Configuration			Separation Attributes	
Length (mm)	**Particle (m)**	**Flow (mL/min)**	**Gradient time (min)**	**Pressure (bar)**
150	5	1	8	170
100	3.5	1	6	204
50	1.8	1	3.5	300

Identification and Quantitation of Water-Soluble Vitamins in Vitamin Supplements

Figure 3 shows chromatograms of two different vitamin supplements. The upper chromatogram is for the Equate Adult Multivitamin, and the lower chromatogram is for the Berkley & Jensen Children's Chewable Multivitamin. The water-soluble vitamins that were positively identified in the sample are indicated on Figure 3 with the peak numbers assigned in Table 4. The primary identification was based on retention time, but a secondary positive identification was made for each vitamin using UV spectra. Although the standard mixture included seven of the water-soluble vitamins, an eighth water-soluble vitamin, thiamine, was added later to allow its identification and quantitation in the vitamin supplements.

In the adult vitamin, five of the seven vitamins that were in the stock solution were identified. Biotin was also identified, but neither an accurate retention time nor an area count could be recorded due to the very low concentration in the sample. In the children's chewable vitamin, six of the seven vitamins used in the stock solution were identified. In addition to these vitamins that were identified, in both the vitamins tested, the first peak that came out after the void was identified as thiamine. Each of the vitamins is needed in the body in different amounts. Due to the large range of concentrations in these samples, we were unable to get a chromatogram that showed good peaks for all the vitamins using UV-VIS detection. For example, ascorbic acid, niacin, and pyridoxine are needed in larger amounts and therefore are at higher concentrations in the vitamin tablets. Thus, the peaks are very large in the chromatograms. For the chromatograms depicted in Figure 3, the injection volume was changed to 15 µL as opposed to the 1.7 µL injection volume that was used for the standard mixture. These volumes were chosen in order to observe the peaks for the vitamins that were present in lower concentrations. Cyanocobalamin was not identified in any of the chromatograms of the vitamins, because it is only needed in very small amounts in the body, and only a few micrograms are present in the vitamin samples. Table 9 shows the quantitation data for thiamine, niacin, and pyridoxine in the adult and children's multivitamins. The measured values agree quite well with that expected from the label of the tablet bottles.

Some popular vitamin and energy drinks were also analyzed. These were sugar-free Red Bull (Red Bull, N.A., Inc., Santa Monica, CA) and strawberry-kiwi Vitaminwater. In these drinks, it was typically possible to identify ascorbic acid, niacin, and pyridoxine, but even when it was known to be added, a peak for cyanocobalamin did not appear in the chromatogram. This lack of signal was due to the low concentration of this vitamin in the drinks. Table 10 shows the quantitation data for niacin and pyridoxine in the sugar- free Red Bull and strawberry-kiwi Vitaminwater.

Table 9. Quantitation of Vitamins in Children's and Adult Multivitamins

Vitamin	Supplement	Expected value (mg/tab)	Measured value (mg/tab)
Thiamine	Adult multivitamin	1.5	–
Thiamine	Children's multivitamin	1.5	1.8
Niacin	Adult multivitamin	20	22.6
Niacin	Children's multivitamin	20	22.2
Pyridoxine	Adult multivitamin	2	2.0
Pyridoxine	Children's multivitamin	2	2.5

Table 10. Quantitation of Vitamins in Popular Drinks

Vitamin	Supplement	Expected value (mg)	Measured value (mg)
Niacin	Vitaminwater	12.5	12.1
Niacin	Red Bull, sugar free	21.2	30
Pyridoxine	Vitaminwater	5.0	5.8
Pyridoxine	Red Bull, sugar free	2.1	2.4

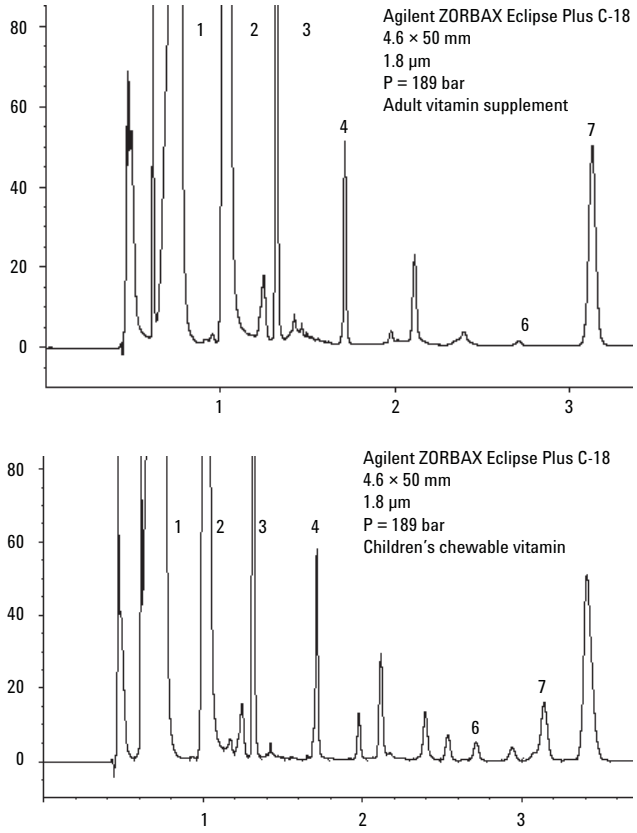

Figure 3. Chromatograms of solutions of an adult multivitamin supplement and a children's chewable vitamin tablet under the same conditions. Conditions are the same as in Figure 2 and positive identification of vitamins are as numbered in Table 4.

Conclusions

The chromatographic method allowed for the separation of water-soluble vitamins in a standard solution without the use of ion pair reagents. Six of the eight vitamins of interest were positively identified using retention times and UV spectra in an adult vitamin supplement. Seven of the eight vitamins of interest were positively identified by the same methods in a children's chewable multivitamin. Cyanocobalamin could not be identified in either tablet since it is found in very small amounts in these tablets, and the concentration in the vitamin drinks was below the limit of detection for this method. Quantitation of thiamine, niacin, and pyridoxine was performed for the two commercial vitamin tablets. Identification and quantitation of niacin and pyridoxine were also done for two popular energy/vitamin drinks. In this way, the method was proven suitable for the identification of vitamins in a variety of commercially available products.

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The method also showed that ZORBAX Eclipse Plus columns, which are available in a large variety of column dimensions and particle sizes, including RR formats, are useful for the separation and identification of water-soluble vitamins. These columns offer high efficiencies and display a very inert surface that gives superior performance with low tailing for these types of separations. The column dimensions chosen depend on the specific use objectives for the data being obtained. Shorter columns with smaller particles offer much shorter run times, which allow faster method development and higher throughput while still maintaining sufficient resolution.

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The peptides used as standards, with the following amino acid sequence MFPAMSLSGLFANAVLR (N-terminal tryptic rbST), MFPAMPLSSLFANAVLR (N-terminal tryptic reST), and AFPAMSLSGLFAN-AVLR (N-terminal tryptic bST) were synthesized from Millegen (Labege, France).

Instrumentation

The detail of the instrumentation used for the detection of the N-terminal peptides is described in the following tables.

LC

Instrument	Agilent 1200	
Column	Column Interchrom ModuloCart QS Uptisphere 3HDO 150 mm × 2 mm	
Mobile phase	A: Acetonitrile + 0.1% formic acid B: H_2O + 0.1% formic acid	
Flow rate	0.3 mL/min	
Injection volume	20 µL	
Gradient	Time (min)	%A
	0	10
	5	55
	10	60
	15	100
	17	10
	20	10

MS

Instrument	Agilent 6410 LC/MS Triple Quadrupole
Ionization mode	ESI (+)
Capillary	5000 V
Nebulizer	55 psi
Gas flow	13 L/min
Gas temperature	300 °C

Selected Reaction Monitoring (SRM) Method Parameters

In order to obtain a better specificity, the detection was performed in SRM mode. The transitions monitored are displayed in Table 1.

Table 1. SRM Method Parameters

Compound	RT	Charge	Transitions monitored	Collision energy (V)
Nterm rbST	8.33	z = 2	913.2 → 1047.7	30
			913.2 → 774.1	20
		z = 3	609.3 → 774	10
			609.3 → 643.5	20
Nterm reST	8.39	z = 2	933.2 → 1287.9	30
			933.2 → 794.1	20
Nterm bST	8.20	z = 2	883.2 → 1047.8	20
			883.2 → 774.1	20

Results and Discussion

Application of Triple Quadrupole MS-MS and Electrospray Ionization Mode Methodology

In this method, the choice has been made to use electrospray ionization in positive mode. Indeed, this ionization mode presented as a “soft” ionization technique is optimal for peptides. The ionization of the N-terminal peptide rbST leads to two main forms (z = 2 and z = 3).

This use of a triple quadrupole based methodology enabled very good sensitivity and selectivity and also a possible quantification of the monitored signals.

Separation of the Different Compounds

The detection method was developed for the detection of the tryptic N-terminal peptide of rbST and also for the tryptic N-terminal peptide of endogenous pituitary bovine somatrotropin (bST) and reST as well. Due to the high homology in the amino acid sequence with rbST, reST was used as the internal standard.

The three compounds were separated chromatographically and analyzed utilizing the transitions described in Table 1. The chromatogram corresponding to the injection of 0.2 ng of N-terminal peptide bST, rbST, and reST is shown in Figure 1.

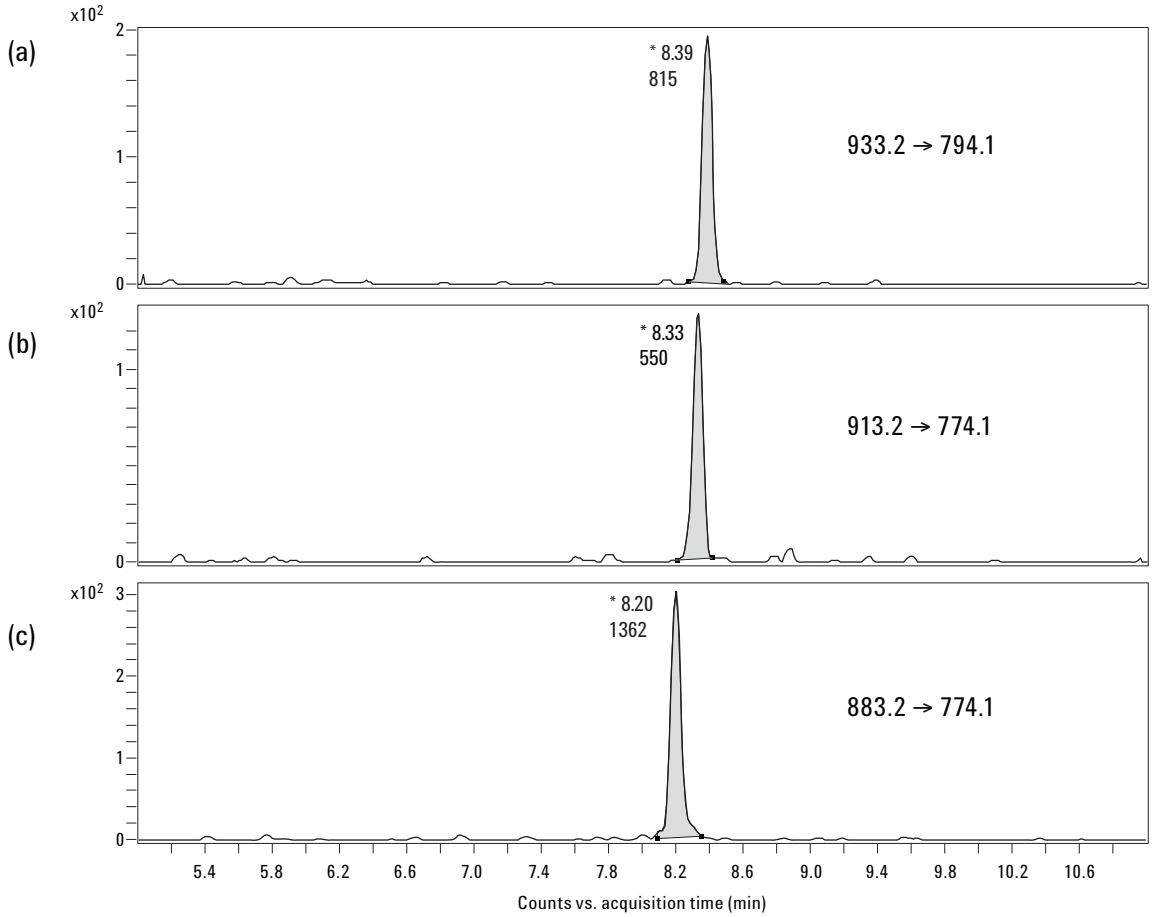

Figure 1. SRM ion chromatograms of standard solutions of tryptic N-terminal peptide of (a) reST, (b) rbST and (c) bST. The injection aliquot used corresponded to 0.2 ng on-column.

Even with an optimized gradient, due to their high homology in terms of sequence, the three compounds eluted with very similar retention times.

Linearity and Sensitivity of the Method

The method described allowed detection of the three peptides with very good sensitivity. A limit of detection of 20 pg injected on-column (~900 femtomole) was reached. Quantification was possible as shown by the good linearity of the calibration curves (Figure 2).

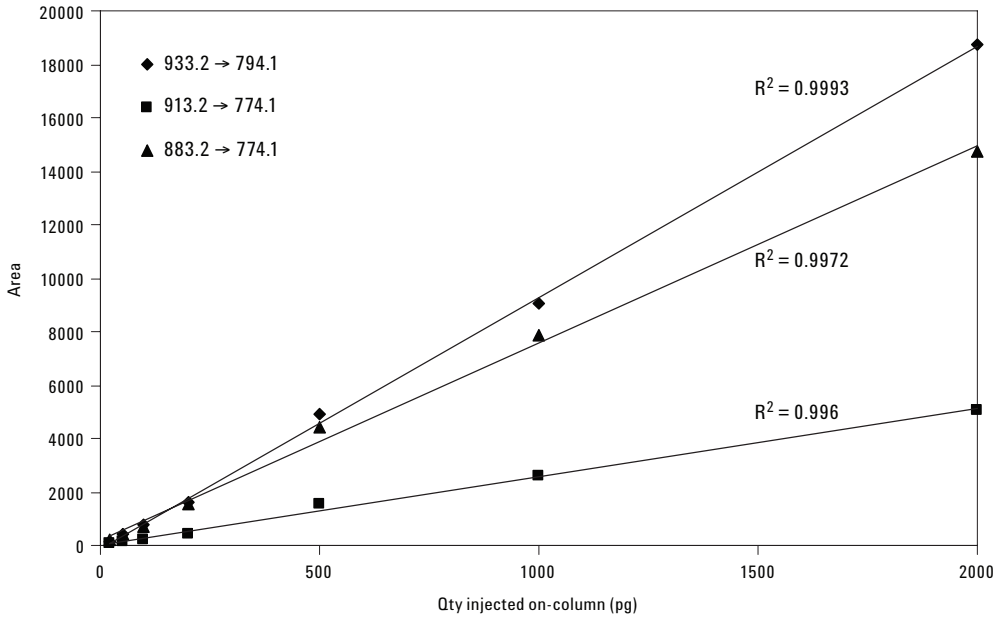

Figure 2. Calibration curve of tryptic N-terminal peptides rbST (913.2 → 774.1), reST (933.2 → 794.1) and bST (883.2 → 774.1).

Results of Spiked Samples

The detection method was applied to extracts obtained from milk samples spiked with rbST. The purification procedure used is described in [3].

Figure 3 shows the chromatogram of a milk sample spiked with 50 ng.mL^{-1} of rbST, in accordance with guidelines for the identification of rbST according to 2002/657 criteria [4]. The chromatogram shows excellent peak shape, and above all, nearly null

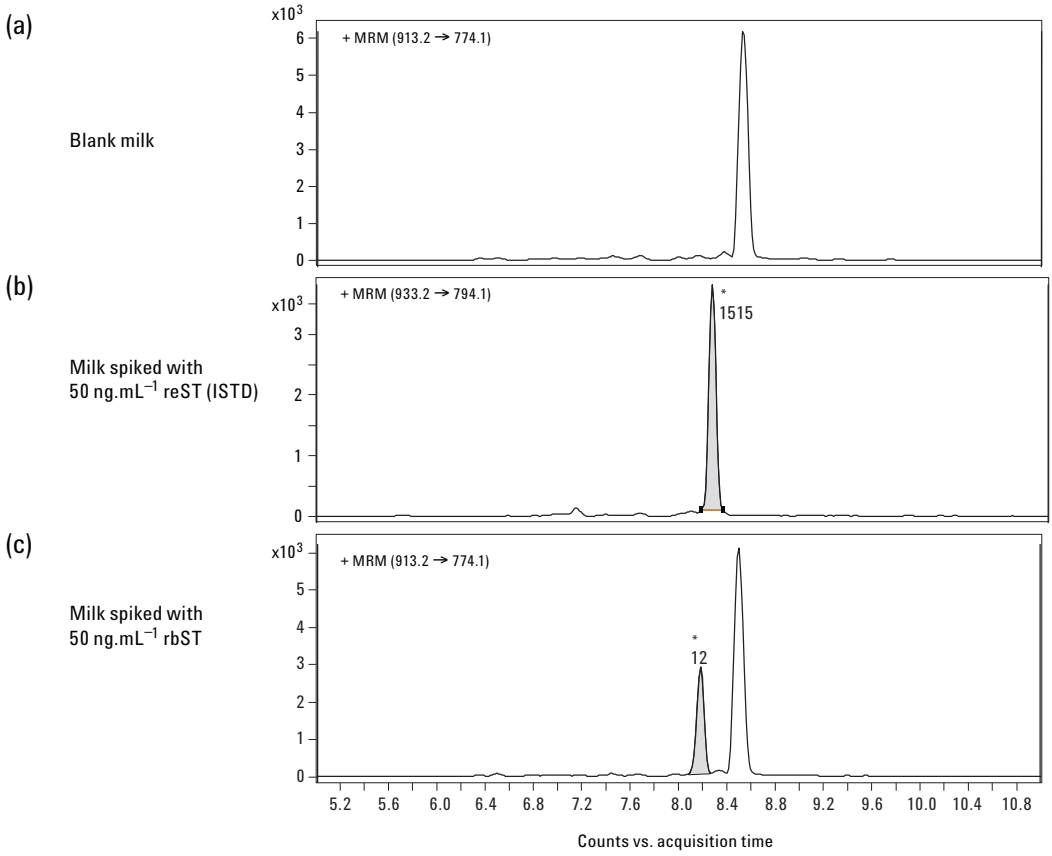

Figure 3. SRM ion chromatograms obtained from milk samples. The different signals correspond to (a) blank milk, (b) the same milk spiked with 50 ng.mL^{-1} reST (internal standard), and (c) 50 ng.mL^{-1} rbST.

background noise, demonstrating the selectivity of the method. The intensity of the signal, although lower than the internal standard, is, however, significant, and shows a clear and distinct signal. The method clearly allows for unambiguous identification of rbST in milk.

Conclusions

The detection of rbST in milk was performed with detection by ESI(+) LC-MS/MS. The method showed very good sensitivity, specificity, and robustness. It was successfully applied to milk samples spiked with rbST at 50 $ng.mL^{-1}$, in accordance with criteria outlined by the 2002/657 Council Directive.

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For more information on this application, you may also contact Agilent Technologies, Inc., Attention: Lea Bonnington, European Field Support Centre, Hewlett-Packard Strasse 8, 76337 Waldbronn, Germany.

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Characterization of Transgenic Soybean Seedlines by Protein Expression with the Agilent 2100 Bioanalyzer

Application

Food

Authors

Debra Dempsey
E.I. DuPont de Nemours
DuPont Experimental Station
Wilmington, DE 19880
USA

Mark Jensen
Agilent Technologies
2850 Centerville Road
Wilmington, DE 19808-1610
USA

Abstract

This application note describes how the Agilent Technologies 2100 Bioanalyzer can be used to analyze protein extracts from transgenic seedlines. Accuracy and precision in the determination of protein size and concentration was sufficiently good to allow for the characterization of experimental seedlines based solely on expressed protein profiles.

Introduction

ß-conglycinin(7S) and glycininin(11S) are the primary seed storage proteins in soybean, comprising about 70% of the total storage proteins. Because these proteins make up such a large portion of soya protein, they are of critical economic importance. Characterizing the expression of these proteins in various soybean seed lines is also essential in expanding the range of soy protein applications in food. The relative levels of these two proteins have been shown to significantly impact the nutrition, taste, and texture of food products derived from soy protein extracts [1]. For this reason, soybean lines that preferentially express the 11S or 7S proteins continue to be an active target in the efforts to improve seed quality.

Both conglycinin and glycinin are complex aggregates made of smaller protein subunits. β-conglycinin is a 7S protein with a trimeric structure and is composed of 53, 70, and 76 kDa units. Glycinin is an 11S hexameric protein consisting of six monomer units, where each monomer is made up of 40 and 20 kDa subunits [2].

Given the sizes of protein subunits, it is relatively straightforward to characterize the levels of these two proteins by electrophoresis. The Agilent 2100 Bioanalyzer, an automated microfluidic electrophoresis platform, is well suited for the analysis of proteins in this size range. The Protein 200 LabChip has a size range of 14-200 kDa. Samples of soy protein isolate can be loaded, separated, and analyzed for relative protein composition in less than 45 minutes. In this application we describe the use of the Agilent 2100 Bioanalyzer in the analysis of soybeans, to determine the level of expression of 7S and 11S proteins.

Experimental

Extraction Protocol

Grind the seed into a fine powder. Place a 30 mg sample in an Eppendorf® tube and add 1000 µL of extraction buffer (50 mM Tris [pH 7.5], 10 mM 2-mercapto-ethanol, 0.1% SDS). Agitate the mixture on a rotary shaker for 30 minutes and then centrifuge at 15,000 g for 10 minutes. Remove the supernatant and introduce the extract directly into the Protein 200 LabChip to begin the assay protocol.

If the extracts contain an excessive amount of oil, the supernatant may be removed and further diluted prior to beginning the Protein 200 protocol.

Methodology

To determine if a transgenic line of soybeans preferentially expressed the β-conglycinin or glycinin protein groups, seed extracts were compared to extracts made from wild-type seed lines that strongly expressed either the 7S or the 11S group. Twenty extracts from the unknown seedline were prepared as described above. The protein profiles were determined by separating the proteins in the Agilent 2100 Bioanalyzer. Because of the size range of the proteins, the Protein 200 Plus chip (14-200 kDa) was used for the separation. The concentration of the individual proteins was determined by a comparison with an internal concentration marker (myosin). The ratio of 7S/11S proteins was then calculated from those values and the ratio was compared to the ratio of wild-type seedlines that preferentially expressed either 7S or 11S protein groups. Figure 1 shows the electropherogram for a control seedline, a 7S wild-type, an 11S wild type, and a representative sample from the unknown transgenic seedline. A simulated gel image of the same traces is shown in Figure 2.

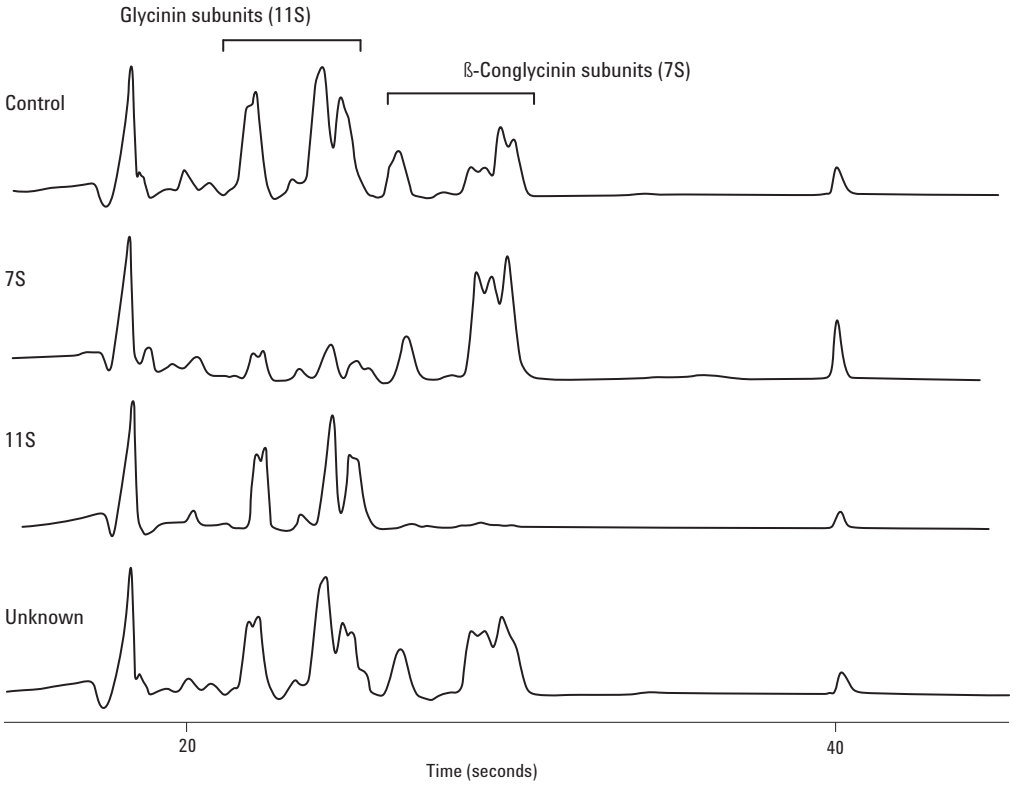

Figure 1. Electropherograms of soya protein extracts.

The ratio of 7S to 11S for the 20 sample extracts was calculated by integrating the individual components comprising the 7S and 11S groups and then determining the summed areas of the two groups. The levels of extracted protein and the 7S/11S ratios for the control 7S, 11S, and unknown groups are summarized in Table 1.

The ratios determined for the high 11S and high 7S seedlines indicate the range of expected 7S/11S ratios should fall between 0.04-3.4. The ratios determined for both the controls and unknown extracts both fall within this range. All 20 unknown samples showed a higher 7S/11S ratio than the control. Average ratio values for 20 unknown extracts and 5 control extracts was 0.72 and 0.39, respectively. Measurement precision was excellent for both sample sets.

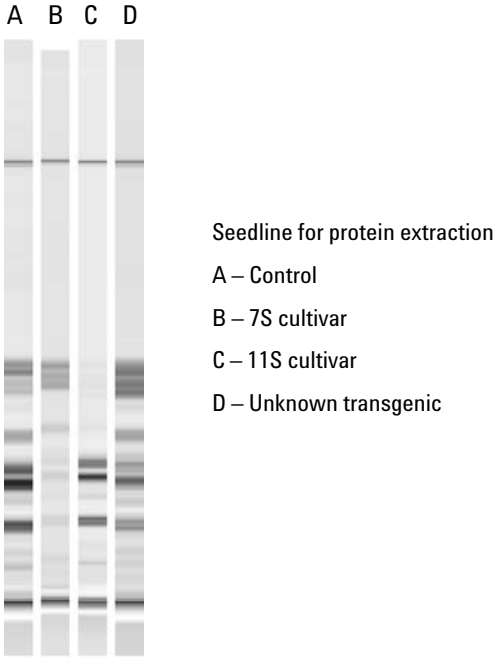

Figure 2. Gel simulation of electropherograms for soya protein extracts.

Table 1. Summary of Extracted Protein Levels and 7S/11S Protein Ratios

SeedLine	Extracted protein level µg/mL	7S/11S Ratio
Control	14,000	0.39 ±0.004 (n=5)
7S	5,200	3.4
11S	14,000	0.04
Unknowns	13,000	0.72 ±0.1 (n=20)

Conclusions

This application note describes the use of the Agilent 2100 Bioanalyzer and the Protein 200 LabChip Kit for evaluating the relative expression of β-conglycinin and glycininin in unknown seedlines. In the 20 protein extracts taken from the unknown seedline, the average ratio was 0.72 ±0.1. This ratio lays in range that is characteristic of high 7S expression seedlines. Given the precision of the ratio determination, it is clearly apparent that the assignment of this unknown seedline to the high 7S group is statistically significant. This conclusion is further supported by a comparison to a normal control seedline where the ratio of 7S/11S is 0.39 ±0.004.

The Agilent 2100 Bioanalyzer together with the Protein 200 LabChip Kit are quick and efficient tools for the determination of relative protein expression. The resulting protein expression profiles are in turn a highly effective means for the characterization of new transgenic seedlines.

References:

1. Kitamura, K., (1995) "Genetic Improvement of Nutritional and Food Processing Quality in Soybean," *Jpn. Agric. Res. Quart.*, **29(1)**, 1-8.
2. Yaklich, R. W., (2001) "-Conglycinin and Glycinin in High-Protein Soybean Seeds," *J. Agric. Food Chem.* **49**, 729-735.

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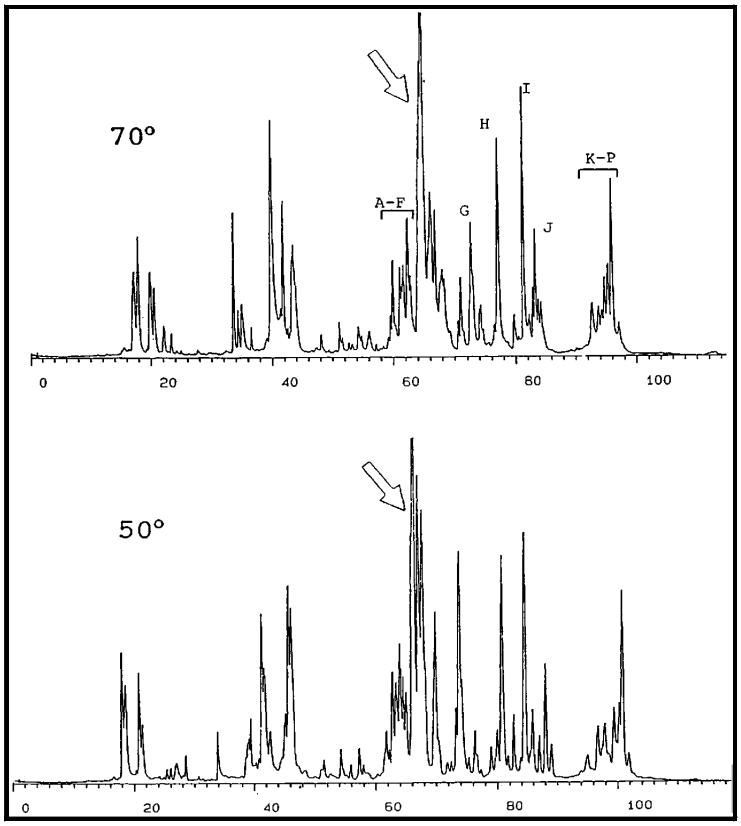
Effect of Temperature on Separation of a Wheat Protein

Application

Food Analysis

Robert Ricker



Conditions:
ZORBAX 300 SB-C8, 4.6 x 150 mm (Agilent P/N: 883995-906)
1 ml/min., 15 µl of Neepawa Wheat Extract
Abs. 210 nm 23-48% B in 120 min.
A=0.1% TFA/water, B=0.1% TFA/ACN

Highlights

- *Wide-pore ZORBAX 300 SB-C8 demonstrates high peak capacity for complex samples of plant protein.*
- *Temperature can be effectively used to increase resolution in reversed-phase separations of complex samples.*

Agilent Technologies

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

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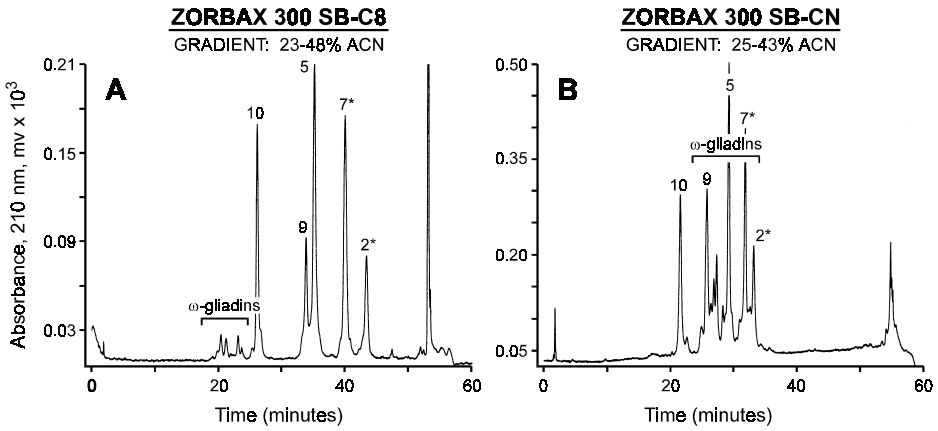
Agilent Technologies

Stationary-Phase Selectivity Comparison: High-Molecular-Weight, Alkylated, Wheat Glutenin, Protein Subunits

Application

Food Analysis

Robert Ricker



Conditions:
ZORBAX 300SB-C8, 300SB-CN (4.6 x 150 mm) Agilent P/N: (883995-906) (883995-905)
Gradient: 60 min., Mobile Phase (as above) with 0.1% TFA, 1.0 mL/min., 50°C

Highlights

- *Improved separations are achieved by taking advantage of different bonded-phase chemistries.*
- *Sterically protected SB bonded phases are hydrolytically stable, especially at low pH, and can be relied upon for stable, reproducible separations.*

Agilent Technologies

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

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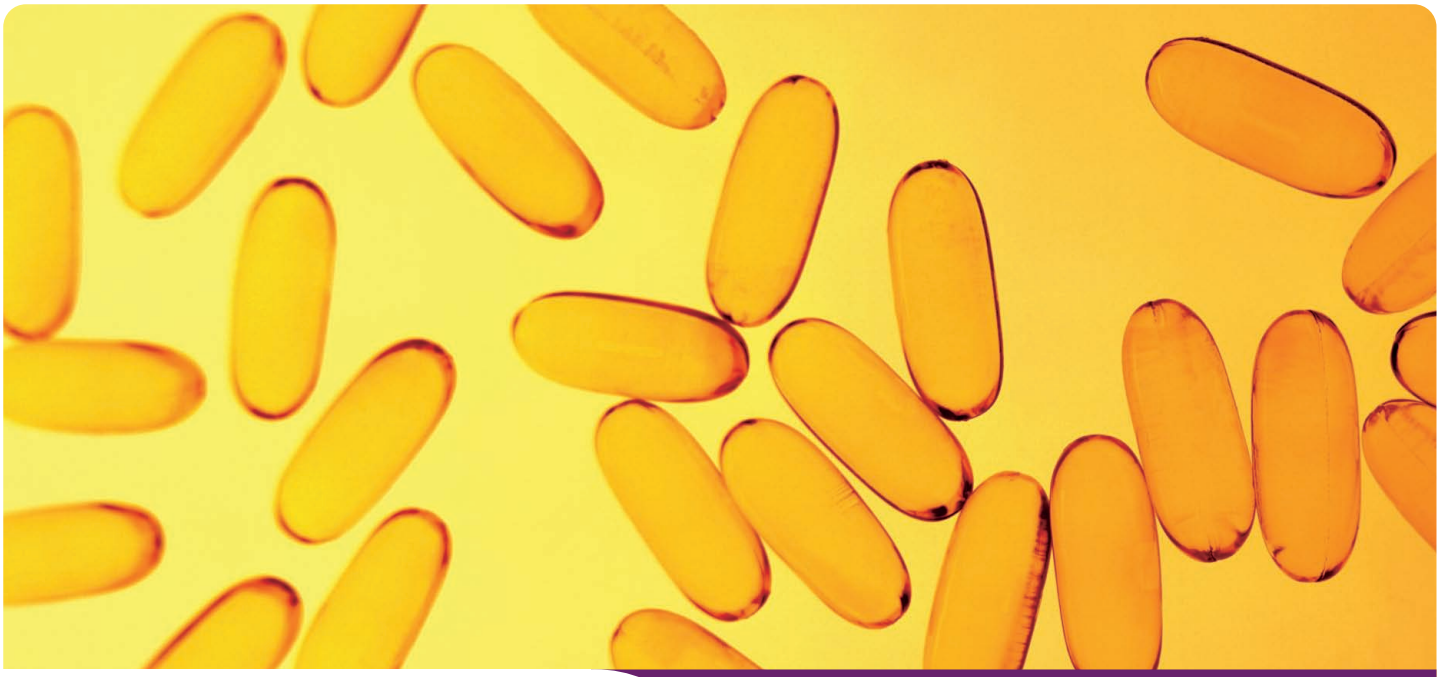

Natural Compounds & Additives

Vitamins

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Reversed-Phase HPLC Separation of Water-Soluble Vitamins on Agilent ZORBAX Eclipse Plus Columns

Application

Pharmaceutical, Food/Beverage

Authors

Anna Glinko, Michael J. Bozym, Michelle L. Owens, and Karyn M. Usher
Department of Chemistry
West Chester University
West Chester, PA 19383
USA

Ronald E. Majors
Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE, 19808
USA

Abstract

A mixture of six B vitamins and vitamin C were separated on the same Agilent ZORBAX Eclipse Plus C-18 stationary phase in three different particle sizes and column dimensions: 5 µm (4.6 × by 150 mm); Rapid Resolution (RR), 3.5 µm (4.6 × 100 mm), and RR High Throughput (RRHT), 1.8-µm (4.6 × 50 mm). A simple phosphate buffer-methanol mobile phase gradient was used. The method was designed to supplement United States Pharmacopeia (USP) methods in which these vitamins are separated singly on different columns by different methods, some involving complex ion-pair reagents. The shorter columns provided more rapid separations with little change in resolution. All compounds could be separated on the 1.8-µm column in 3.5 minutes. A gain in sensitivity was noted when the column length and particle size were reduced. The HPLC method was applied to the analysis of vitamins in a variety of commercial products, including multivitamins and various soft drinks. The columns chosen allowed for good separation, but the RRHT column also allowed quick method development without giving up the resolution and speed requirements for the method. The ZORBAX Eclipse Plus columns were shown to have high efficiency and superior performance characteristics with minimal tailing.

Introduction

Vitamins are essential nutrients for the proper functioning of the human body. The daily requirements are small, ranging from 2.4 µg to 90 mg [1]. Inadequate amounts of vitamins can cause numerous health problems, such as adrenal impairment (vitamin B) or scurvy or a compromised immune system (vitamin C). With the exception of pyridoxine and cyanocobalamin, water-soluble vitamins are not stored in the body [1]. Taking a multivitamin daily can ensure one is getting the proper amount of vitamins if diet alone cannot provide them.

Many vitamins easily degrade upon exposure to heat, light, and oxygen. Ascorbic acid is known to degrade quickly and easily in aqueous solutions. This degradation has been studied in solutions of different pH and in both aerobic and anaerobic conditions [2]. It has also been documented that the inside surface of glassware may contain materials that measurably degrade ascorbic acid within a short period of time. According to a study published by Margolis and Park, this is particularly true for autosampler vials used to hold samples for chromatography. Their paper gives several suggestions for preventing this decomposition [3]. In our study, vitamin samples were prepared every four

hours as needed. Although the percent relative standard deviation (%RSD) values given for ascorbic acid are very good, these injections were done immediately after the solution was made. Within a couple hours of preparing the solutions, we could see the peak areas for ascorbic acid decreasing with consecutive injections.

Currently, the United States Pharmacopeia (USP) has standard methods for the analysis of the vitamins used in our study; however, there is no USP method for the separation of all eight of the water-soluble vitamins in the mixture used. Some of the USP methods are complicated and involved the use of ion pair reagents (see Table 1). Ion pair reagents can often be used to improve resolution, but may result in irreversible changes in column performance [4]. The USP method for ascorbic acid is not currently a chromatographic method. Furthermore, these USP methods do not allow for the use of some newer column technologies, such as sub-2-micron particle sizes in shorter dimensions.

The main goal of the project was to create a single high-performance liquid chromatographic method that would allow separation of the following water-soluble vitamins: ascorbic acid, biotin, cyanocobolamin, niacinamide, panthothenic acid, pyridoxine, riboflavin, and thiamin. See Figure 1 for the structures of these vitamins. A secondary goal was to demonstrate the effect of decreasing particle size on resolution, separation time, and system pressure.

Experimental

Chromatographic experiments were conducted using an Agilent 1200 SL Rapid Resolution liquid chromatograph (LC) equipped with an autosampler and an 80-Hz diode array detector (Agilent Technologies, Inc., Santa Rosa, CA). Both isocratic and binary gradients were generated using this system. Columns used in the study were: 5-µm Agilent ZORBAX Eclipse Plus C-18 stationary phase in 4.6 mm x 150 mm (P/N: 959993-902), 3.5-m ZORBAX Eclipse Plus C-18 stationary phase in 4.6 mm x 100 mm (P/N: 959961-902), and 1.8-µm ZORBAX Eclipse Plus C-18 stationary phase in 4.6 mm x 50 mm (P/N: 959941-902) formats.

Vitamin standards were obtained from Sigma Aldrich (Milwaukee, WI). Identification of the vitamins in the standard mixture, supplements, and vitamin drinks was confirmed using the diode array detector with high-speed full-spectral UV-VIS detection.

Table 1. Reagents and Requirements for USP Methods (USP31-NF 26, May 2008. Ref. 5)

Vitamin	Reagents needed	Tailing Factor (TF)	%RSD t_R
Pyridoxine	Glacial acetic acid, sodium 1-hexanesulfonate, methanol, water	–	< 3%
Thiamine	Sodium 1-hexanesulfonate, phosphoric acid, hydrochloric acid, acetonitrile, water	< 2	< 3%
Cyanocobalamin	Methanol, water	–	–
Pantothenic acid	Monobasic potassium phosphate, phosphoric acid, methanol, water	–	< 2%
Biotin	Sodium perchlorate, phosphoric acid, dimethyl sulfoxide, acetonitrile, water	–	< 3%
Niacin	Sodium 1-hexanesulfonate, methanol, acetonitrile, glacial acetic acid, water	< 2	< 2%
Riboflavin	Glacial acetic acid, edetate disodium, sodium acetate, triethylamine, methanol, water	–	< 2%

Mobile phase channel A was 25 mM NaH_2PO_4 (pH = 2.5), mobile phase channel B was methanol. The flow rate was 1.0 mL/min. The complete chromatographic conditions are depicted in Table 2.

The mobile phase gradient was changed proportionally to match the column length to keep solvent-strength selectivity (k*) the same (see Table 3).

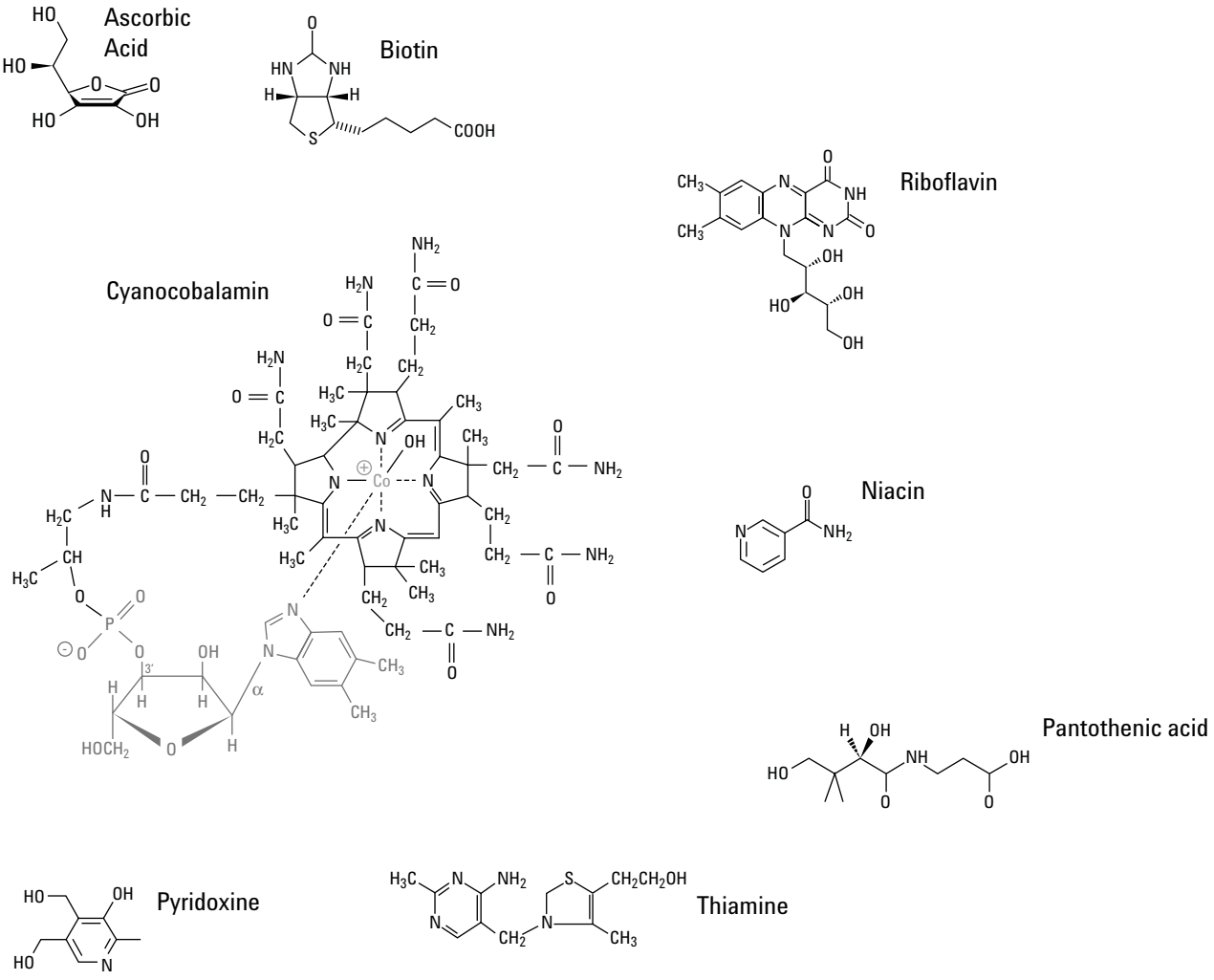

Figure 1. Structures of water-soluble vitamins used in this experiment.

Table 2. Chromatographic Conditions

LC	Agilent 1200 SL
Mobile phase A	25 mM NaH_2PO_4 pH = 2.5
Mobile phase B	Methanol
Flow rate	1.00 mL/min
Column compartment temperature	35 °C
Detection	220 nm, no Reference
Response time	0.05 s
Injection volume	Adjusted for column size: 5 µm, 5 µL 3.5 µm, 3.3 µL 1.8 µm, 1,7 µL
Detector flow cell	Micro flow cell (2 µL)

Table 3. Gradients for Equivalent k*

%B	5 µm	3.5 µm	1.8 µm
1	0.00 min →	0.00 min →	0.00 min
12	1.50 min	1.00 min	0.50 min
30	1.53 min	1.03 min	0.51 min

The individual standards and standard mixture were prepared by weighing out the appropriate masses of each vitamin using an analytical balance or microbalance, as needed, and dissolving in 10 mL of water. All samples that were analyzed for vitamins were purchased locally. The Vitaminwater (Glaceau, Flushing, NY) was injected without dilution. For sample preparation, the chewable vitamin tablets (Berkley & Jensen Children's Chewable Complete Multi Vitamins and Minerals Supplement, Natick, MA) were powdered and then dissolved in 100 mL of water. The adult tablet (Equate Adult Multivitamin, Walmart, Bentonville, AR) was treated in a similar manner. Table 4 outlines all of the final concentrations for standards and vitamin tablets. Prior to injection, all samples were filtered using Agilent syringe Econofilter, regenerated cellulose, 25-mm diameter, 0.20-µm porosity (P/N: 5185-5830).

Results and Discussion

Chromatographic Reproducibilty

The %RSDs for replicate injections of each vitamin are given in Table 5. These values were calculated based on seven replicate injections of each vitamin. In Table 5, the concentrations of each vitamin in the standard solution used to obtain reproducibility data on the 1.8-µm column is reported along with the %RSD values for retention time, area, and tailing factor (at 5 percent height). Some of the USP methods required that the %RSD for retention times be less than 2 or 3 percent (see Table 1); the method for cyanocobalamin had no such requirement. Table 5 shows that all the %RSD values were within the required range, and were actually much lower than the stated limits.

Table 4. Peak Identifications and Concentrations of Vitamins

Pk #	Vitamin	Conc. (g/L) in standard	Conc. (g/L) in soln. of adult vitamin tablet	Conc. (g/L) in soln. of chewable supplement
1	Ascorbic acid	8×10^{-2}	9×10^{-1}	6×10^{-1}
2	Niacin	4.5×10^{-2}	1.5×10^{-1}	2×10^{-1}
3	Pyridoxine	9×10^{-2}	5×10^{-2}	2×10^{-2}
4	Pantothenic acid	2.4×10^{-1}	1×10^{-1}	1×10^{-1}
5	Cyanocobalamin	9×10^{-2}	2×10^{-5}	6×10^{-5}
6	Biotin	Saturated	3×10^{-4}	4×10^{-4}
7	Riboflavin	1.5×10^{-1}	3.2×10^{-2}	1.7×10^{-2}

Table 5. %RSD on 1.8-µm Column

Vitamin	Conc. g/L	%RSD t_R	%RSD area	%RSD TF
Thiamine	1.20	0.7	0.3	1.3
Ascorbic acid	0.11	0.2	0.9	0.6
Niacin	0.15	0.5	0.3	0.9
Pyridoxine	0.09	0.3	1.8	1.9
Pantothenic acid	0.24	0.0	0.4	0.7
Cyanocobalamin	0.18	1.4	0.9	0.4
Biotin	Saturated	0.2	2.9	4.2
Riboflavin	0.15	0.2	2.3	0.9

Comparison of Tailing Factors at 5% Peak Height

Table 6 gives the retention times and tailing factors for each solute on the three ZORBAX Eclipse Plus columns used for these experiments. These columns have a very inert surface, which displays very little tailing for these water-soluble compounds. Note that the average tailing factors for all the vitamins studied were less than 1.2 on all the columns used. This is well within the range of acceptable tailing factors included in the USP methods (see Table 1).

Table 6. Comparison of Retention Times and Tailing Factors at 5% for Each Solute on the Three Agilent ZORBAX Eclipse Plus Columns

	5 µm		3.5 µm		1.8 µm	
Vitamin	Ret. time	Tailing factor	Ret. time	Tailing factor	Ret. time	Tailing factor
Ascorbic acid	2.10	1.1	1.41	1.2	0.76	1.2
Niacin	2.62	1.1	1.87	1.2	1.01	1.2
Pyridoxine	3.03	1.1	2.25	1.3	1.31	1.3
Pantothenic acid	4.02	1.1	2.95	1.1	1.68	1.2
Cyanocobalamin	4.47	1.1	3.33	1.1	1.98	1.2
Biotin	6.33	1.1	4.69	1.1	2.63	1.0
Riboflavin	7.24	1.1	5.40	1.1	3.04	1.1
Average (Tf)	–	1.1	–	1.2	–	1.2

Scalability from One Column Configuration to Another

The three column configurations gave a similar elution pattern as shown in Figure 2. The ZORBAX particles allowed for straightforward scalability from longer columns packed with 5-µm and 3.5-µm particles to the shortest column, packed with 1.8-µm particles. The same mixture was injected onto all three of the columns used. The gradient time was adjusted as described in Table 3 to give equivalent k* values.

Gain in Sensitivity When Comparing Three Different Column Configurations

Table 7 shows the gain in sensitivity for cyanocobalamin in the standard vitamin mix injected into the three different columns. Since the injection volume was scaled proportionally to column length, the peak heights cannot be compared directly. Instead, the amount injected is normalized, which allows the sensitivity to be compared. After the normalization, the ratio of the peak areas to injected amounts is the same. However, the data shows that there is a significant difference in the ratio of the heights and the injected amounts. There is a significant increase in the sensitivity of the 3.5-µm column compared to the 5-µm column, and another significant increase in the sensitivity of the 1.8-µm column. Table 7 shows the gain in sensitivity in comparison to the 5-µm column. The gain in sensitivity when the column is changed from a 5-µm column to a 3.5-µm column is 34 percent, while the gain in sensitivity when the column is changed from a 5-µm column to a 1.8-µm column is 52 percent. This gain in sensitivity is due to the higher efficiency (that is, lower dilution factors) of the smaller particles, not simply because the analytes spend less time in the column.

Table 7. Comparing the Sensitivity of Three Column Configurations (Cyanocobalamin)

Column	Normalized injection amount	Area	% area	Height	% height	% gain in sensitivity
5 µm	100	269	–	49	–	–
3.5 µm	66	171	64	48	98	34
1.8 µm	33	91	34	42	86	52

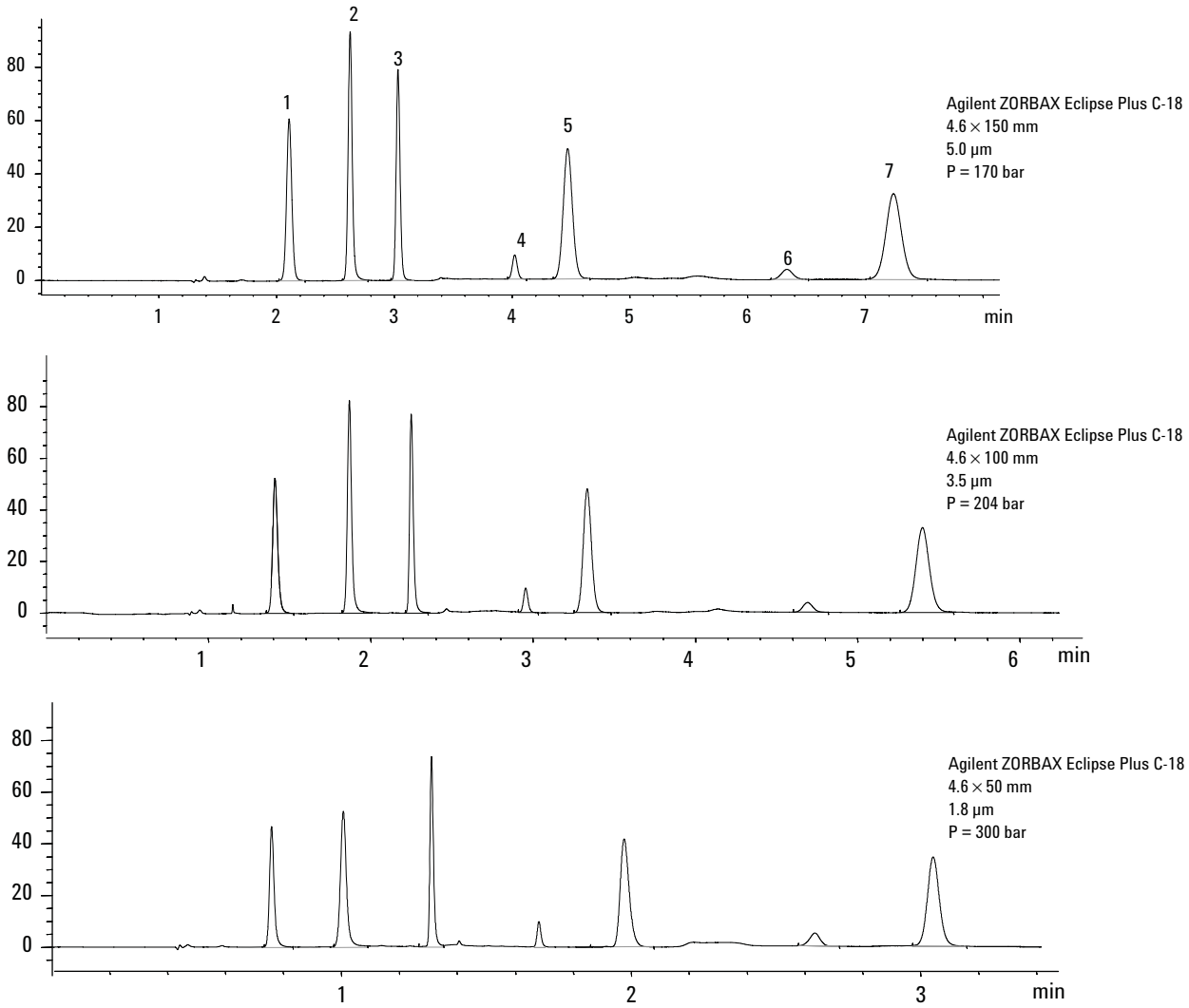

Figure 2. Scalability of Agilent ZORBAX Eclipse Plus C-18 column dimensions and the effect on analysis time and pressure (conditions shown in Table 2).

Comparing Speed and Pressure in Three Different Column Configurations

Figure 2 also shows the difference in separation time performed on the different columns. The separation was shortened from an 8-minute run time on the 4.6 x 150 mm, 5-µm column to a 3.5-minute run on the RRHT, 4.6 x 50 mm, 1.8-µm column. The time could have been shortened further by using a higher flow rate. With the good peak resolution shown in Figure 2 it would have been possible to increase the flow rate. However, when this was attempted, some of the peaks in the commercially available vitamins and drinks could not be identified due to the presence of other overlapping unidentified ingredients. As expected, the pressure increased when the particle size of the column packing was reduced. Figure 2 indicates the system pressures (P), in bar, for the three columns. The highest system pressure reached was 300 bar, when using the 1.8-µm column (RRHT). This is well within the operating range for both the Agilent 1100 (max. 400 bar) and the 1200 SL (max. 600 bar) systems. These results are summarized in Table 8.

Table 8. Gradient Separation Results of the Column Configurations, Holding k* Constant

Column Configuration			Separation Attributes	
Length (mm)	**Particle (m)**	**Flow (mL/min)**	**Gradient time (min)**	**Pressure (bar)**
150	5	1	8	170
100	3.5	1	6	204
50	1.8	1	3.5	300

Identification and Quantitation of Water-Soluble Vitamins in Vitamin Supplements

Figure 3 shows chromatograms of two different vitamin supplements. The upper chromatogram is for the Equate Adult Multivitamin, and the lower chromatogram is for the Berkley & Jensen Children's Chewable Multivitamin. The water-soluble vitamins that were positively identified in the sample are indicated on Figure 3 with the peak numbers assigned in Table 4. The primary identification was based on retention time, but a secondary positive identification was made for each vitamin using UV spectra. Although the standard mixture included seven of the water-soluble vitamins, an eighth water-soluble vitamin, thiamine, was added later to allow its identification and quantitation in the vitamin supplements.

In the adult vitamin, five of the seven vitamins that were in the stock solution were identified. Biotin was also identified, but neither an accurate retention time nor an area count could be recorded due to the very low concentration in the sample. In the children's chewable vitamin, six of the seven vitamins used in the stock solution were identified. In addition to these vitamins that were identified, in both the vitamins tested, the first peak that came out after the void was identified as thiamine. Each of the vitamins is needed in the body in different amounts. Due to the large range of concentrations in these samples, we were unable to get a chromatogram that showed good peaks for all the vitamins using UV-VIS detection. For example, ascorbic acid, niacin, and pyridoxine are needed in larger amounts and therefore are at higher concentrations in the vitamin tablets. Thus, the peaks are very large in the chromatograms. For the chromatograms depicted in Figure 3, the injection volume was changed to 15 µL as opposed to the 1.7 µL injection volume that was used for the standard mixture. These volumes were chosen in order to observe the peaks for the vitamins that were present in lower concentrations. Cyanocobalamin was not identified in any of the chromatograms of the vitamins, because it is only needed in very small amounts in the body, and only a few micrograms are present in the vitamin samples. Table 9 shows the quantitation data for thiamine, niacin, and pyridoxine in the adult and children's multivitamins. The measured values agree quite well with that expected from the label of the tablet bottles.

Some popular vitamin and energy drinks were also analyzed. These were sugar-free Red Bull (Red Bull, N.A., Inc., Santa Monica, CA) and strawberry-kiwi Vitaminwater. In these drinks, it was typically possible to identify ascorbic acid, niacin, and pyridoxine, but even when it was known to be added, a peak for cyanocobalamin did not appear in the chromatogram. This lack of signal was due to the low concentration of this vitamin in the drinks. Table 10 shows the quantitation data for niacin and pyridoxine in the sugar- free Red Bull and strawberry-kiwi Vitaminwater.

Table 9. Quantitation of Vitamins in Children's and Adult Multivitamins

Vitamin	Supplement	Expected value (mg/tab)	Measured value (mg/tab)
Thiamine	Adult multivitamin	1.5	–
Thiamine	Children's multivitamin	1.5	1.8
Niacin	Adult multivitamin	20	22.6
Niacin	Children's multivitamin	20	22.2
Pyridoxine	Adult multivitamin	2	2.0
Pyridoxine	Children's multivitamin	2	2.5

Table 10. Quantitation of Vitamins in Popular Drinks

Vitamin	Supplement	Expected value (mg)	Measured value (mg)
Niacin	Vitaminwater	12.5	12.1
Niacin	Red Bull, sugar free	21.2	30
Pyridoxine	Vitaminwater	5.0	5.8
Pyridoxine	Red Bull, sugar free	2.1	2.4

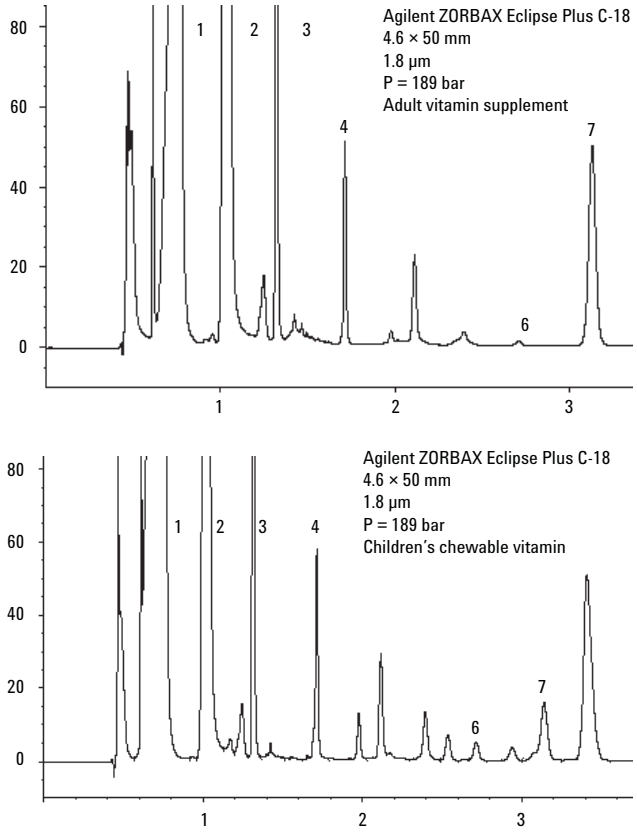

Figure 3. Chromatograms of solutions of an adult multivitamin supplement and a children's chewable vitamin tablet under the same conditions. Conditions are the same as in Figure 2 and positive identification of vitamins are as numbered in Table 4.

Conclusions

The chromatographic method allowed for the separation of water-soluble vitamins in a standard solution without the use of ion pair reagents. Six of the eight vitamins of interest were positively identified using retention times and UV spectra in an adult vitamin supplement. Seven of the eight vitamins of interest were positively identified by the same methods in a children's chewable multivitamin. Cyanocobalamin could not be identified in either tablet since it is found in very small amounts in these tablets, and the concentration in the vitamin drinks was below the limit of detection for this method. Quantitation of thiamine, niacin, and pyridoxine was performed for the two commercial vitamin tablets. Identification and quantitation of niacin and pyridoxine were also done for two popular energy/vitamin drinks. In this way, the method was proven suitable for the identification of vitamins in a variety of commercially available products.

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The method also showed that ZORBAX Eclipse Plus columns, which are available in a large variety of column dimensions and particle sizes, including RR formats, are useful for the separation and identification of water-soluble vitamins. These columns offer high efficiencies and display a very inert surface that gives superior performance with low tailing for these types of separations. The column dimensions chosen depend on the specific use objectives for the data being obtained. Shorter columns with smaller particles offer much shorter run times, which allow faster method development and higher throughput while still maintaining sufficient resolution.

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Agilent Technologies

Determination of water soluble vitamins with the Agilent 1120 Compact LC after method development with the Agilent 1200 Series Rapid Resolution LC system and back transfer

Application Note

Food

Authors

Detlef Wilhelm
ANATOX GmbH & Co. KG
Fuerstenwalde
Germany

Abstract

Quality control to characterize products is often done with standardized liquid chromatography (LC) methods. The needed analytical instrumentation requires optimal cost-of-ownership instruments with high reliability, high flexibility, and ease of use.

The analysis of several water soluble vitamins with the Agilent 1120 Compact LC is described. The preceding method development was done with Agilent 1200 Series Rapid Resolution (RR). This Application Note starts with the final results of that method development. The transfer of those results by the Method Translator Software to conventional LC parameters and columns shows that this method can be used with standard LC equipment.

This Application Note shows that the Agilent 1120 Compact LC works as a reliable and highly robust instrument for routine LC analyses and everyday quality control testing. Full separation was achieved by using an ion pairing eluent and Agilent ZORBAX Eclipse Plus material. The analysis of a typical nutritional mixture of vitamins shows no disturbances by other peaks. The results for reliability and quality testing, system suitability, and performance are shown.

Agilent Technologies

Introduction

Quality control is a main consideration in the field of standard LC product analyses. The analytical instrumentation has several requirements, such as optimal cost-of-ownership, high reliability, high flexibility, and ease of use.

This Application Note targets routine quality control testing, and shows that the Agilent 1120 Compact LC is a reliable and highly robust instrument for standard methodology. The Agilent 1120 Compact LC can also be used for determinations after method development with an Agilent 1200 Series RRLC system. Back transfer from the RR separation to conventional columns can be achieved by the Method Translator Software, and the results of reliabiltiy, quality, system suitability, and performance testing are shown.

Instrumentation

An Agilent 1200 Series Rapid Resolution LC system and an Agilent 1120 Compact LC were used for the method development. Table 1 lists the configurations used for each instrument.

Configuration of the Agilent 1200 LC Series	Configuration of the Agilent 1120 Compact LC
Binary pump and vacuum degasser	Gradient pump and vacuum degasser
Well-plate autosampler	Autosampler
Column compartment	Column oven
Diode array detector	Variable wavelength Detector
Software: Chemstation B.04.01	Software: EZ-Chrom Elite Compact 3.3

Table 1
Instrumentation configurations used for method development

Preparation of samples

Reference samples

Dissolve 10 mg of each vitamin in water and dilute to 100 mL with the same solvent. Dilute 1 mL of the solution to 20 mL with the mobile phase. Figure 1 illustrates the substances to check.

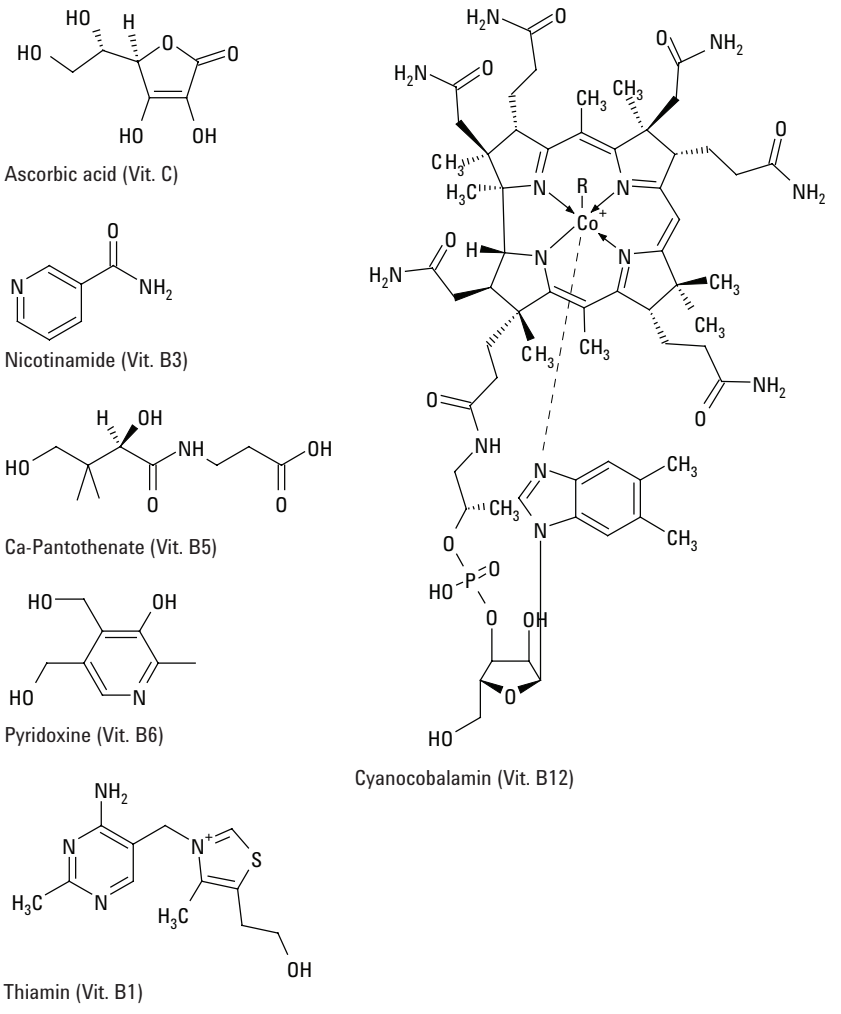

Figure 1
Substances to check.

Chromatographic conditions	
Column	
For method development:	ZORBAX Eclipse Plus C18, 50 mm × 2.1 mm, 1.8 µm
For routine testing:	ZORBAX Eclipse Plus C18, 150 mm × 4.6 mm, 5 µm
Mobile phases	
Phase A:	Dissolve 1.03 g hexane sulfonic acid and 6.8 g potassium dihydrogene phosphate in 1000 mL water. The pH value should be adjusted to pH = 2.3 with phosphoric acid.
Phase B:	Acetonitrile
Gradient (linear):	Time (min)
	0 100% A/0% B
	6 80% A/20% B
	9 50% A/50% B
	10 100% A/0% B
Pump settings	
Stop time:	10 min
Post time:	5 min
Flow rate:	1.0 mL/min
Autosampler	
Injection volume:	30 µL
Thermostatted column compartment	
Temperature:	40 °C
Detector	14-µL cell for the Agilent 1120 LC system, Peak width: >0.05 min, 1 s response time (10Hz), Signal: 220 nm 13-µL cell for the Agilent 1200 LC system, Peak width: >0.05 min, 1 s response time (10Hz), Signal: 220 nm

System suitability and performance test:

For system suitability testing the reference solution with the following limits was used:

- Resolution: minimum 1.5 between peaks.
- Precision of areas must be < 2 % RSD.
- Precision of retention times must be < 0.5 % RSD.

With these limits and settings for testing, the samples in Table 2 were prepared and analyzed.

Sample	Purpose	Number of injections
Blanc solution	Verify baseline stability and identify artifacts	2
Calibration samples	Verify linearity	3 of each level
Control sample	Verify sensitivity and resolution for reference solution	6
Suitability sample	Verify precision of areas and retention times for reference solution	10

Table 2
Setup for testing

Results and discussion

Hexanesulfonic acid was chosen as the ion-pairing agent to achieve the best separations for all peaks. The results in Figure 2 show good separation of all vitamins with the ZORBAX Eclipse Plus C18 material on an Agilent 1200 Series RRLC system.

The parameters found during method development with the Agilent 1200 Series RRLC system were transferred to parameters suitable for using 5-µm columns with conventional HPLC systems with the Agilent Method Translator Software (see Figure 3).

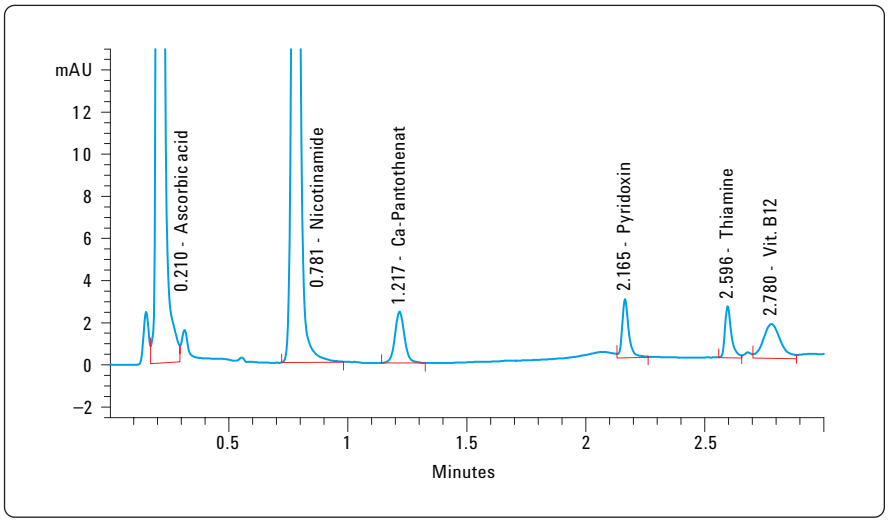

Figure 2
Example chromatogram of water soluble vitamins with the Agilent 1200 Series RRLC system.

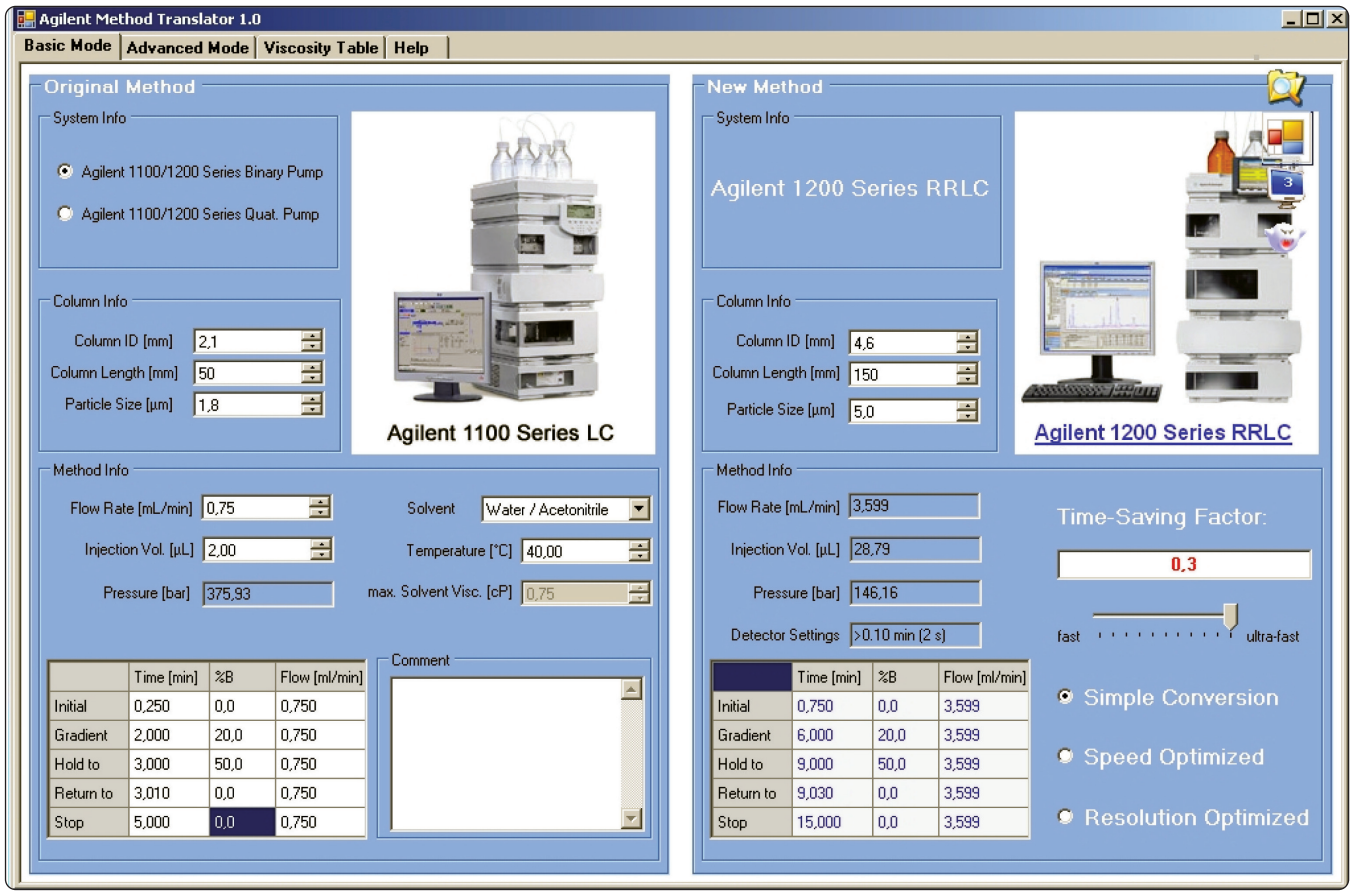

Figure 3
Conversion of LC parameters found by Rapid Resolution to parameters suitable for conventional HPLC with the Method Translator software.

Because Ca-pantothenate has no significant absorbance at wavelengths >230 nm, a 220 nm setting on the Agilent 1120 Compact LC wavelength detector was selected to detect all components. Figure 4 shows a chromatogram, achieved after transfer of the parameters from the 50 mm × 2.1 mm Rapid Resolution column to a 150 mm × 4.6 mm (5-µm material) column with the Agilent 1120 Compact LC and EZ-Chrom Elite Compact Software.

As Figure 4 shows, the elution order is the same, which proves that the selectivity does not change with the particle size. Only small differences can be seen in the resolution between nicotinamide and Ca-pantothenate, but full baseline separation still exists. Detailed data are listed in Table 3. As a main result it can be emphasized that it is possible to transfer LC parameters from Rapid Resolution to conventional LC systems and columns with 5-µm particles.

The only difference in parameters between the Agilent 1200 Series binary system and the Agilent 1120 Compact LC is that the 1120 LC separation resulted in slightly later eluting peaks, as seen in Figures 2 and 3. The reason can be found in the difference of gradient mixing. With the Agilent 1120 Compact LC, the gradient is mixed at the low pressure site whereas with the Agilent 1200 binary system the mixing is done at the high pressure site. The different mixing volumes (delay volumes) are responsible for the different raise of the gradients in the columns.

However, as seen in Figure 3 the resolution of all peaks is unaffected by the different gradient mixing. In addition, all other impurities in the fine chemicals are separated.

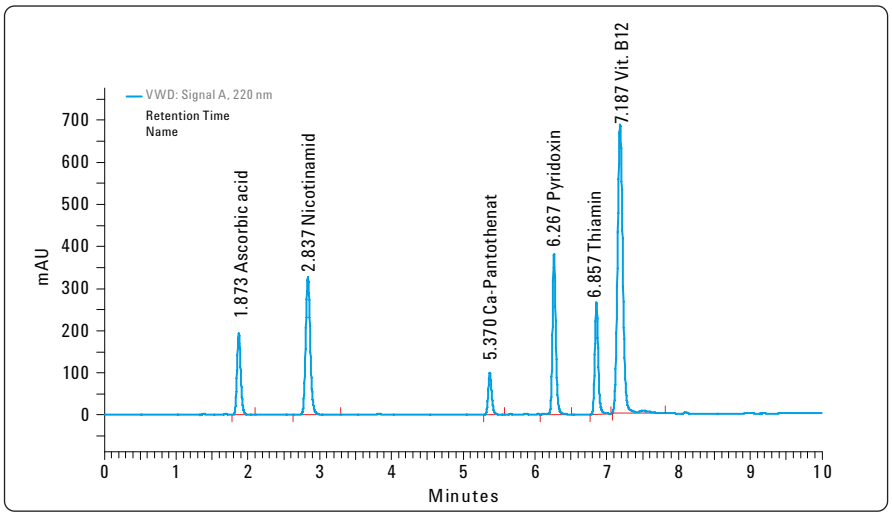

Figure 4
Example chromatogram of water soluble vitamins with the Agilent 1120 Compact LC.

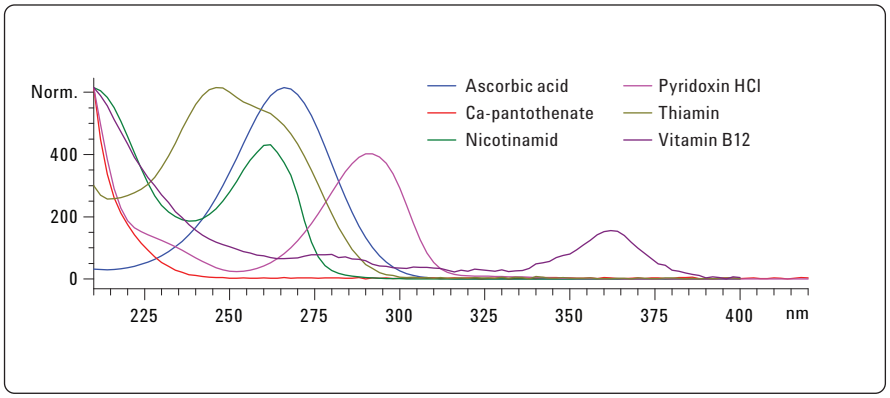

Figure 5
UV spectra of all vitamins aquired by the Agilent 1200 Series Diode Array Detector.

Compound	Retention time 1200er (min)	Retention time 1120 (min)	Resolution 1200er	Resolution 1120
Ascorbic acid	0.210	1.873	–	–
Nicotinamide	0.781	2.837	12.78	9.24
Ca-Pantothenate	1.217	5.370	7.27	23.18
Pyridoxine	2.165	6.267	16.68	10.10
Thiamine	2.596	6.857	9.76	6.83
Vitamin B12	2.780	7.187	2.24	3.38

Table 3
Results for control sample: Retention times and resolution.

The results shown in Table 3 illustrate good separation achieved with the ZORBAX Eclipse Plus C18 material. The high resolution (every peak >2) for both systems shows that this column material is highly suitable for this determination, because of its good performance (see data for asymmetry in Table 4).

Table 4 shows the areas and retention time precision results of all compounds in the suitability sample. The reliability and precision of the Agilent 1120 Compact LC is demonstrated. The criteria (see "System suitabiltiy and performance tests") of precision of retention times and areas are fulfilled for all compounds. This shows that the system can be used for QC methods.

The high precision and reliability of the autosampler is best shown by the data in Tables 4 and 5. The correlation coefficient for each calibration curve is very close to 1.0 showing high versatility and quality for QC testing.

The chromatogram in Figure 6, shows the determination of a nutritional mixture of vitamins with great differences in concentration of each vitamin, which illustrates the capabilities of the method.

Compound	Retention time (min)	RSD RT n = 10	RSD Area n = 10	Asymmetry 1120
Ascorbic acid	1.873	0.225	0.234	1.186
Nicotinamide	2.837	0.398	0.236	1.185
Ca-Pantothenate	5.370	0.277	0.508	1.238
Pyridoxine	6.267	0.323	0.325	1.178
Thiamine	6.857	0.407	0.374	1.193
Vitamin B12	7.187	0.145	0.652	1.116

Table 4
Suitability sample: Precision of Retention times and areas for the Agilent 1120 Compact LC.

Compound	m	b	r
Ascorbic acid	1601035.9	48890.7	0.999
Nicotinamide	17812069.2	17303.5	1.0
Ca-Pantothenate	2231477.8	3546.1	1.0
Pyridoxine	35097452.7	75231.6	0.999
Thiamine	26988637.8	40978.7	0.999
Vitamin B12	104653435.3	19666.3	0.999

Table 5
Calibration data of the Agilent 1120 Comapct LC (Setting "Ignore Origin", Y = mx + b, 1.6–16.1 µg/mL for ascorbic acid and 0.11–1.12 mg/L for the other vitamins).

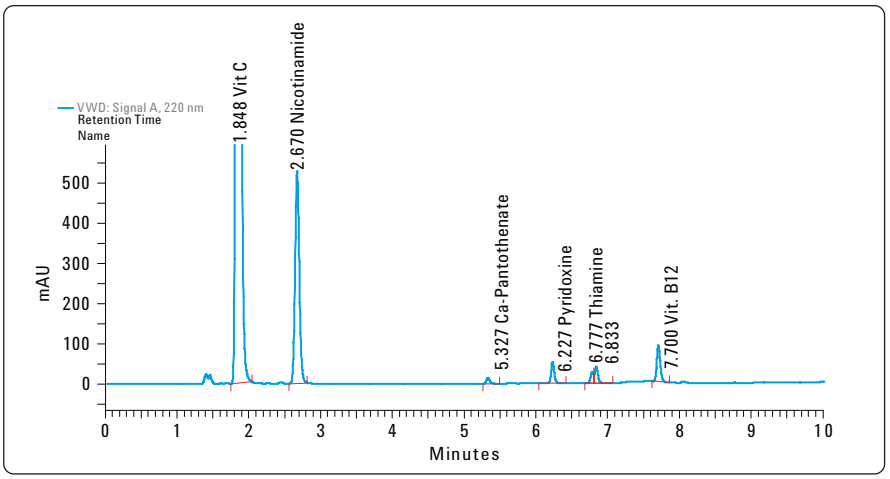

Figure 6
Chromatogram of a typical nutritional mixture of vitamins.

Conclusion

The Agilent 1120 Compact LC is a good choice for medium to small sized company users who need high reliability, ease-of-use, and lowest cost-of-ownership for standard QA/QC LC methodology.

This Application Note shows the setup for the determination of water soluble vitamins after method development with rapid resolution. This reliable approach is proven with precision of areas and retention time data, as well as chromatographic parameters such as resolution and asymmetry. The results from a system optimized for everyday productivity and calibration meet the requirements for routine analysis.

As shown in Table 3 the resolution of all peaks was found to be greater than 3.0 with values near 1.0 for the asymmetry of all main peaks. It is concluded from the calibration data that the instrument can be operated in a quality control environment.

The results in Table 4 show that all criteria for the determination of precision (areas, retention times) are fulfilled. From these results, it can be concluded that the Agilent 1120 Compact LC can be used in QA/QC laboratories to determine water soluble vitamins in samples for nutritional purposes.

All results show the applicability of the Agilent 1120 Compact LC for quality control testing by reduced costs per system and improved simplicity of use. In addition to the instrument capabilities, the new version of the EZChrom Elite compact software allows full control of the Agilent 1120 Compact LC with a wide range of features for data analysis and results reporting.

The results for resolution and asymmetry show good selectivity and performance of the ZORBAX Eclipse Plus material, independent of the particle size. The data also show good flow design of the LC systems ensuring that no band broadening and peak distortion will occur during method transfer. In summary, this Application Note shows that fast method development can be achieved with an Agilent 1200 Series Rapid Resolution LC system and the results can be back transferred to a conventional HPLC such as the Agilent 1120 Compact LC. This approach will meet the highest requirements for everyday productivity.

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Agilent Technologies

Simultaneous analysis of watersoluble vitamins using capillary electrophoresis

Application Note

Foods and Flavors

Authors

Tomoyoshi Soga

Institute for Advanced Biosciences,

Keio University, Japan

Abstract

This application note describes the development of an MEKC method, its reproducibility, its sensitivity for vitamins analysis and its use for the analysis of vitamin-enriched drinks. Water-soluble vitamins including B_1, B_2 phosphate, B_3 (nicotinamide), B6 and caffeine were analyzed simultaneously by capillary electrophoresis (CE). The vitamins were separated with high resolution by using MEKC and were detected using a diode-array detector. The relative standard deviation (RSD) for migration time was between 0.3 % and 0.7 % and for peak area the RSD was better than 1.8 %. The minimum detectable level for vitamins ranged from 160 to 660 µg/L.

Introduction

Vitamins play an important role for healthy growth and development of many organisms. Today, multivitamin products are becoming more and more widespread. Consequently, a rapid, easy and reliable method for vitamin analysis is required by the food and pharmaceutical industries. In general, water-soluble vitamins are determined by reversed-phase high-performance liquid chromatography (RP-HPLC). However, it is not easy to analyze all vitamins simultaneously by RP-HPLC, because vitamin B_1 and B_6 are strongly ionic compounds, whereas vitamin B_2 phosphate is a hydrophobic species. In order to analyze these vitamins simultaneously with RP-HPLC, an ion-pair technique,[1,2] or gradient elution,[3] is needed. Both these techniques have limitations. Ionpair chromatography suffers from poor reproducibility and limited column lifetime. With gradient elutions, a complicated system and long analysis times are required. High performance capillary electrophoresis (HPCE) has the advantages of high efficiency and resolution, automation, and rapid analysis times. Another advantage of HPCE is its unique selectivity. Capillary zone electrophoresis (CZE) separates compounds based on their charge and size. However, neutral species cannot be

separated by capillary zone electrophoresis (CZE). Micellar electrokinetic chromatography (MEKC)[5,6] was used for this analysis because water-soluble vitamins contain both ionic and neutral compounds. In MEKC, ions migrate depending upon their mobility, while neutral species are separated by hydrophobic interaction with the micelles.

Experimental

All experiments were performed using an Agilent Capillary Electrophoresis system. The system comprises a CE unit with built-in diode-array detector and an Agilent ChemStation for system control, data collection and data analysis. Vitamin B_2 phosphate was obtained from Tokyo Kasei Kogyo (Tokyo, Japan) and sodium dodecyl sulfate (SDS) in an electrophoresis purity reagent grade purchased from Bio-Rad (Richmond, CA, USA). All other reagents were obtained from Wako (Osaka, Japan). Water was purified with a Milli-Q purification system from Millipore (Bedford, MA, USA).

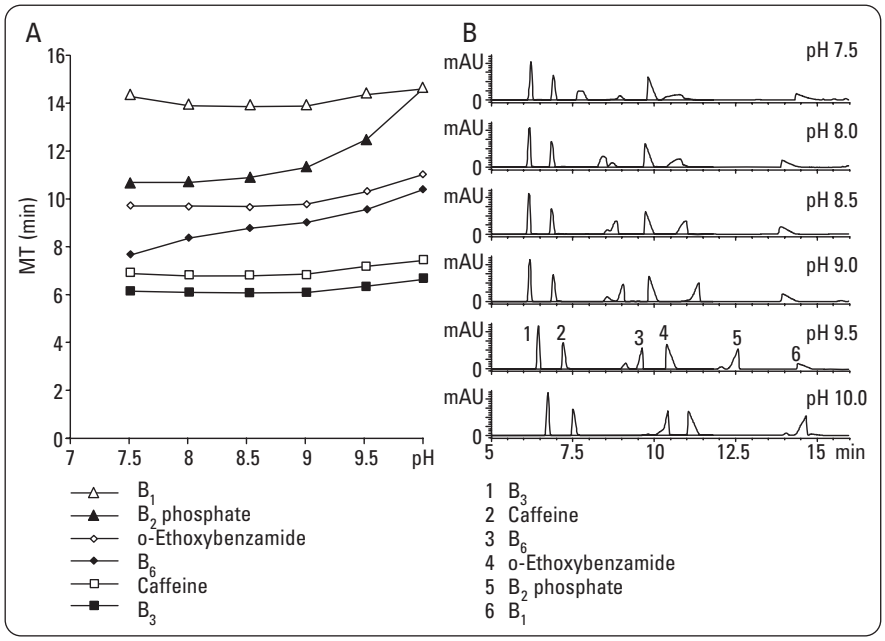

Figure 1
Influence of buffer pH on water-soluble vitamins separation
A) migration time of vitamins versus buffer pH
B) electropherogram at the indicated pH.

Compound	RSD % (n=5)		Linearity	Detection limit
	Migration time	Peak area	Correlation γ	(µg/L)
B_3	0.5	1.6	0.99998	240
Caffeine	0.7	1.7	0.99998	160
B_6	0.4	1.0	0.99980	190
B_2 phosphate	0.3	1.3	0.99995	650
B_1	0.3	1.8	0.99968	660

Table 1
Reproducibility, linearity and sensitivity of vitamin standards.

Results and discussion

Separation of vitamins using MEKC

MEKC has been used for the separation of water-soluble vitamins using borate buffer and SDS micelles. 30 mM SDS in 20 mM tetraborate was chosen for this study. The influence of buffer pH on vitamin separation is shown in figure 1. At pH values lower than 8.5, broadening peaks of B6 and B2 phosphate were observed. This is because the hydroxy groups of B6 and B2 phosphate form borate complexes in tetraborate buffer around pH 9. Lower than pH 8.5, they form partial complexes, therefore the peaks were boardened. At values higher than pH 8.5, they form borate complexes completely. Consequently, the peak shapes are improved. Between pH 9.0 and 9.5, good separation was obtained. At a pH of 10.0, the migration time of B_2 phosphate increased and it eluted at the same time as B_1. This coelution occurs because the B_2 phosphate acquires a negative charge due to phosphate dissociation and is attracted to the anode, thereby increasing its elution time.

Reproducibility, linearity and sensitivity

Usually, detection in HPCE is carried out at 200 nm or below, but in this work 220 nm was selected because the sensitivity to B_2 phosphate is about three times better than at 200 nm. The electropherogram of 200 mg/L of vitamin B_1, B2 phosphate, B_3, B_6, caffeine and o-ethoxybenzamide (internal standard) is shown in figure 2. Table 1 shows that satisfactory reproducibilities were obtained, as reflected by the relative standard deviations (RSD). Also, the calibration graphs for all the water-soluble vitamins (10 to 1000 mg/L) were linear. The detection limits for vitamins were between 160 and 660 µg/L at a signal-to-noise ratio of three.

Vitamin-enriched drink analysis

This method was applied to the analysis of water-soluble vitamins in a vitamin-enriched drink. The drink contains several vitamins at a concentration that ranged from 50 to 300 mg/L. The sample was diluted with water (1:5) before injection. Figure 3 shows the result of the vitamin-enriched drink analysis. Although the sample contai-

ned other compounds, a well-defined electropherogram was obtained without matrix interference. Each vitamin in the sample was identified by matching the migration time with the standard and by using a spectral library search.

An example of this identification is shown in figure 4. The RSD values (n=5) of the sample migration times were better than 1.1 % and peak areas were 0.5, 0.9, 1.4 and 0.7 % for B_3, caffeine, B_2 phosphate and B_1, respectively. The amounts of vitamins were in good agreement with the content listed in the product description.

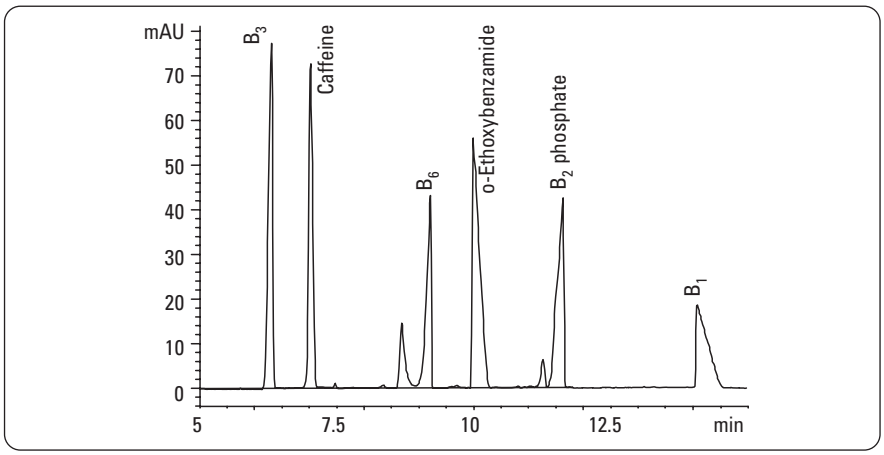

Figure 2
Separation of a standard mixture of vitamins.

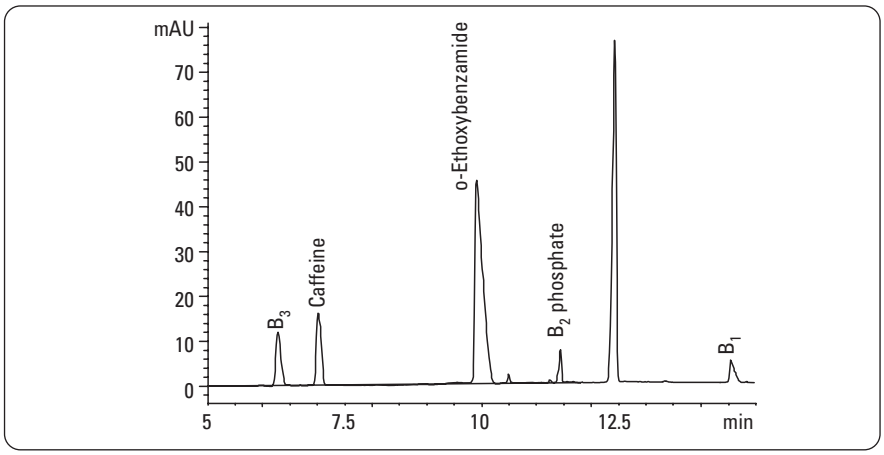

Figure 3
Analysis of water-soluble vitamins in a vitamin-enriched drink.

Conclusions

A method for the determination of water-soluble vitamins using MEKC has been developed. The method was applied to the analysis of a vitamin-enriched drink and good results were obtained. It was concluded that this method has advantages with respect to resolution, selectivity, analysis time and simplicity, when compared with existing HPLC methods.

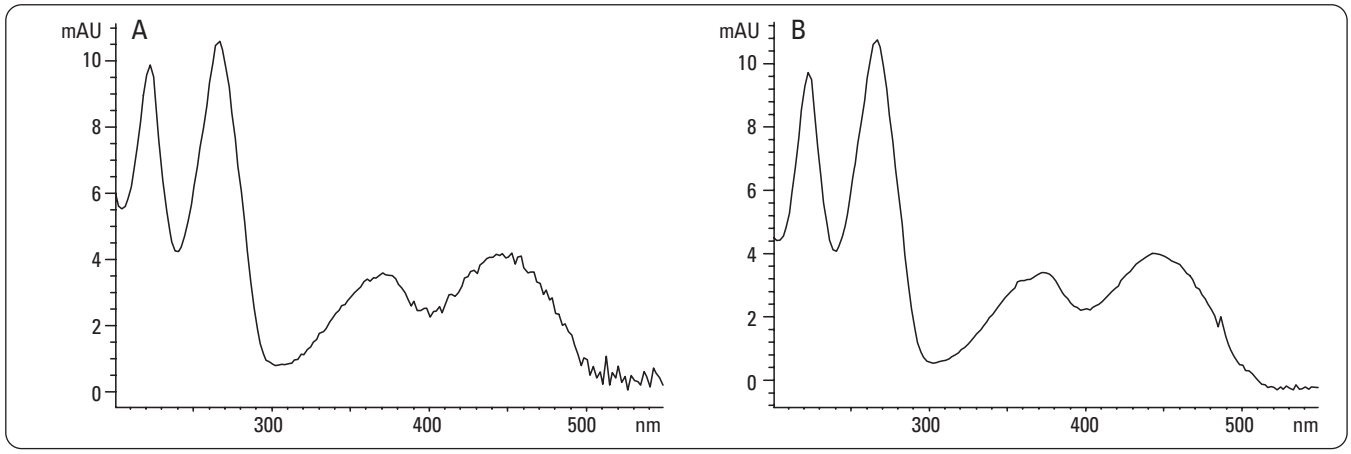

Figure 4
Spectral identification of B_2 phosphate A) spectra of peak eluting after 13 minutes in a vitamin-enriched drink B) spectra of B_2 phosphate standard.

Chromatographic conditions for figures 2, 3, 4

Running buffer.	20 mM tetraborate, 30 mM SDS, pH 9.0
Effective capillary length.	56 cm
Internal diameter:	75 µgm
Voltage:	15 kV
Injection:	200 mbar x s
Detection wavelength:	220 nm
Temperature:	35° C

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Agilent Technologies

Quantitative Analysis of Water-Soluble B-Vitamins in Cereal Using Rapid Resolution LC/MS/MS

Application

Food Analysis

Authors

Sheher Mohsin
Agilent Technologies, Inc.
Schaumburg, Il
USA

Michael Zumwalt
Agilent Technologies, Inc.
9780 S. Meridian Blvd.
Englewood, CO 80112-5910
USA

Indarpal Singh
ConAgra Foods, Inc.
Omaha, NE 68102
USA

Abstract

An Agilent 6410 Triple Quadrupole Mass Spectrometer (QQQ) is used to analyze several water-soluble B-vitamin compounds in breakfast cereal. A simple gradient elution is carried out on a Rapid Resolution High Throughput SB-Aq column (particle size 1.8 µm). All compounds elute in less than 7.5 minutes, and with the exception of pyridoxine, good linearity over more than three orders of magnitude, from 0.5 to 500 ppb, is demonstrated with good peak area reproducibility at the 0.5 ppb level, which is the lowest level of quantitation considered.

Introduction

Water-soluble vitamins are very polar and have poor retention on reverse-phase columns. The presence of ion pair reagents such as heptafluorobutyric acid in the mobile phase has been shown to improve the separation and retention of these compounds. However, the drawback of using such ion pair reagents is the high background levels that are generated inside the mass spectrometer. Therefore, we have developed a rapid and sensitive method with ammonium formate in the mobile phase solvent using a column with a bonded phase designed to retain hydrophilic compounds.

The Agilent 1200 Series liquid chromatography (LC) system used in this work was designed to take advantage of sub-2-micron particle columns for rapid, high-resolution separations. Included in the LC design were decreased delay volume, increased pressure range, and increased column temperature. This LC system was coupled to the Agilent 6410 Triple Quadrupole Mass Spectrometer (QQQ) by way of the G1948B electrospray ionization source. Target compound separation was achieved on a ZORBAX AQ 1.8-micron column using a water and methanol gradient with ammonium formate.

Typical LC/MS methods for water-soluble vitamins have shown analysis times as high as 30 minutes with heptafluorabutyric acid ion pairing reagent in the mobile solvent. We have developed a rapid and sensitive method for the LC/MS/MS analysis of water-soluble vitamins by employing a high-efficiency 1.8-micron column in a low-dispersion, 600-bar LC/MS configuration that allowed screening and quantitation with a run-to-run cycle time as low as 10 minutes. Linearity of the mass spec-

trometer response was observed over three orders of magnitude with limits of quantitation in the 0.5 pg/µL range for all of the analytes except for pyridoxine. In the case of pyridoxine, good sensitivity was demonstrated, but good linearity was limited to just under three orders of magnitude. Calibration curves and chromatograms for the vitamins between 0.5 and 500 pg/µL were generated for all compounds with the exception of pyridoxine, which was between 0.5 and 250 pg/µL.

The structures of the B vitamins are shown below.

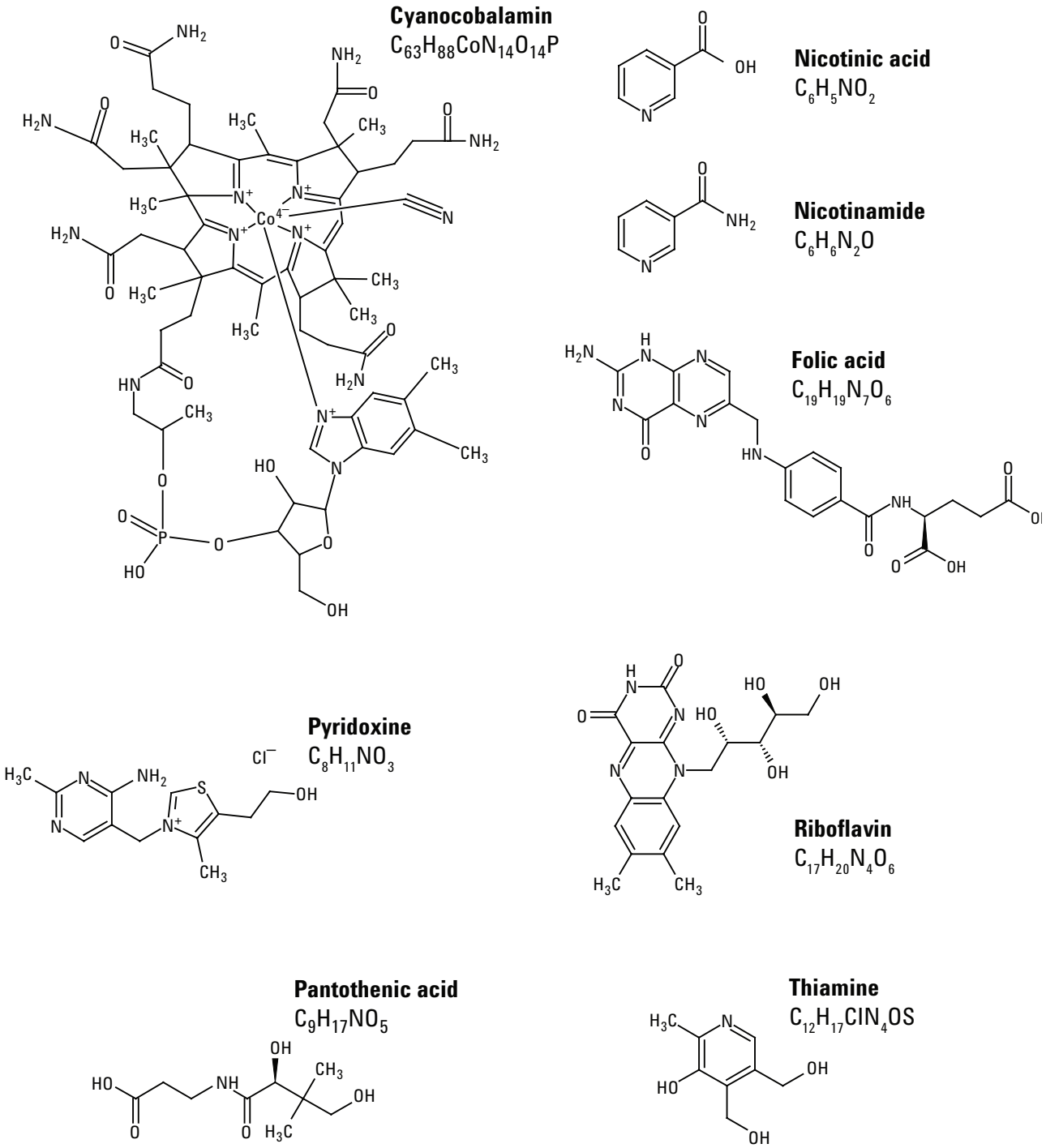

Figure 1. Structures of B vitamins analyzed in this work.

Experimental

Sample Preparation

A standard mix containing all eight compounds in methanol was provided by ConAgra Foods and diluted in 90% water/10% methanol with 20 mM ammonium formate and 0.1% formic acid to the following concentrations: 500, 250, 100, 50, 5, and 0.5 pg/µL. These dilutions were used for the quantitation of unknown samples.

One B-vitamin-fortified sample was also provided using the following sample preparation procedure:

1. Grind and homogenize breakfast cereal in blender
2. Weigh 1 gram of homogenized sample into a 50-mL vial
3. Add 25 mL of 0.1M HCl and heat in water bath at 100 °C for 20 min. This solubilizes the vitamins.
4. Cool to ambient temperature
5. Adjust volume to 1 L with deionized water
6. Filter with 0.45-µm glass microfiber membrane.

It should be noted that the provided fortified sample was created for testing the sensitivity of the instrument for customer demonstration purposes. A typical unfortified sample extraction consists of 1 g homogenized sample treated with enzymatic digestion, to release naturally occurring vitamins from their conjugated forms, and volume adjusted to 10 mL, which is 1/100th the volume used in the fortified sample analyzed in this work. At the higher concentration, salts and other matrix contributions are seen to cause interference in the analysis of some vitamins. As a result, further dilution may be used to accommodate the matrix effect in these samples.

Table 1. MRM Mode Parameters

Segment	Compound	Transition	Fragmentor (V)	Collision Energy (V)
1	Thiamine	265.2 > 122.0	85	10
	Pantothenic acid	220.2 > 90.0	110	13
	Pyridoxine	170.1 > 152.1	100	10
	Nicotinic acid	124.1 > 80.0	100	27
	Nicotinamide	123.1 > 80.0	100	25
2	Cyanocobalamin	678.6 > 146.7	130	35
	Folic acid	442.2 > 295.1	120	10
	Riboflavin	377.2 > 243.1	110	25

LC/MS Method Details

LC Conditions

Agilent 1100 Series binary pump, degasser, wellplate sampler, and thermostatted column compartment

Column:	Agilent ZORBAX RRHT SB-Aq, 3.0 mm × 100 mm, 1.8 µm (PN: 828975-314)		
Column temperature:	35 °C		
Mobile phase:	A = 20 mM ammonium formate and 0.1% formic acid in water		
	B = 20 mM ammonium formate and 0.1% formic acid in methanol		
Flow rate:	0.5 mL/min		
Injection volume:	10 µL		
Gradient:	Time (min)	%B	
	0.0	10	
	8.0	55	Stop time: 10 min
	8.1	10	
Needle wash:	75:25 methanol/water (flush port 20 seconds)		

MS Conditions

Mode:	Positive ESI using the Agilent G1948B ionization source
Nebulizer:	30 psig
Drying gas flow:	10 L/min
Drying gas temperture:	350 °C
V_{cap}:	1850 V
Resolution (FWHM):	Q1 = low res; Q2 = low res
Dwell time for all MRM transitions	200 msec

The precursor ion mass for cyanocobalomin (*m/z* 678.6) is about half of the expected value in which the empirical formula for this compound is $C_{63}H_{88}CoN_{14}O_{14}P$, as denoted in Figure 1. It is believed that the structure of this compound is not very stable and breaks apart during the ionization process.

Results and Discussion

The calibration curves for all eight compounds are shown in Figures 2A through 2H. Only for the compound pyridoxine is the 500 ppb level needed to be removed for good linearity. No internal standard is included.

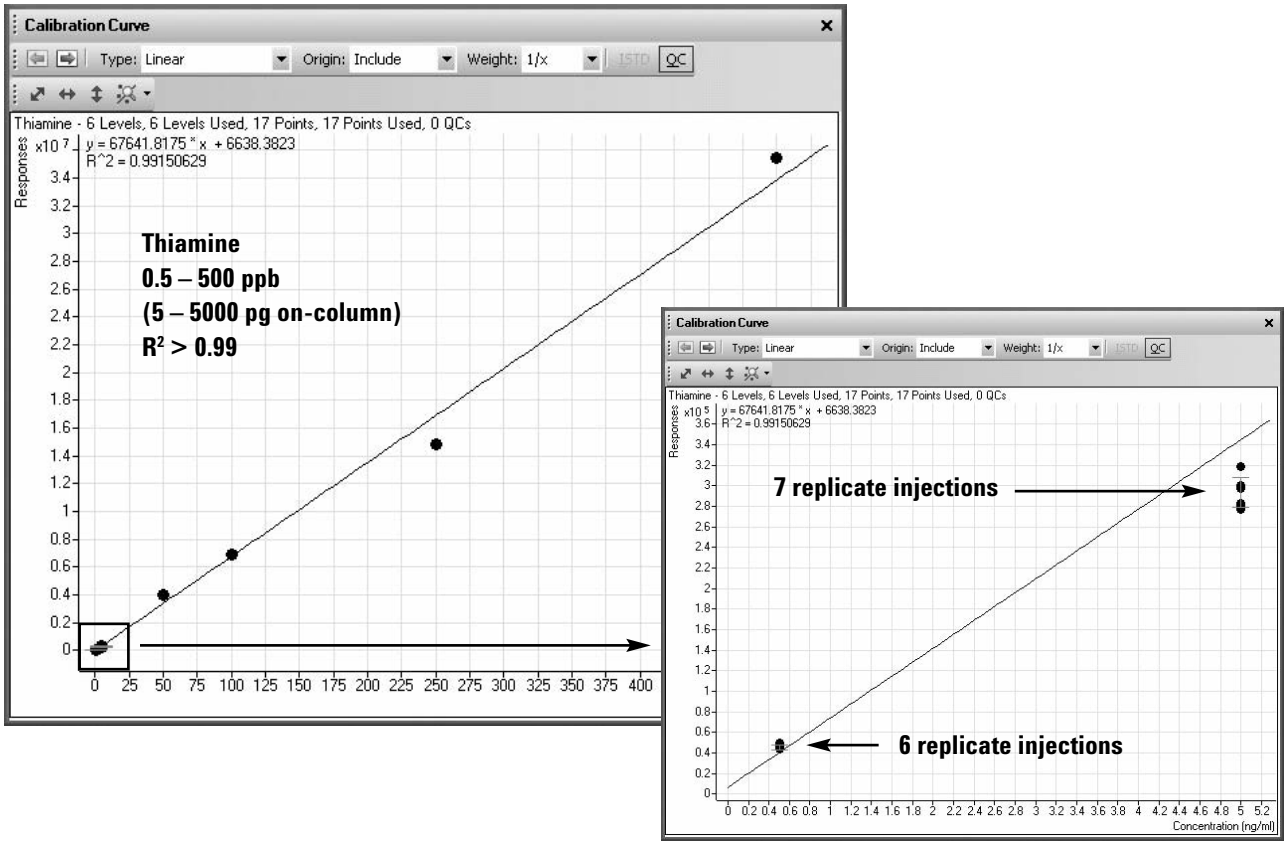

Figure 2A. Linearity of thiamine over three orders of magnitude.

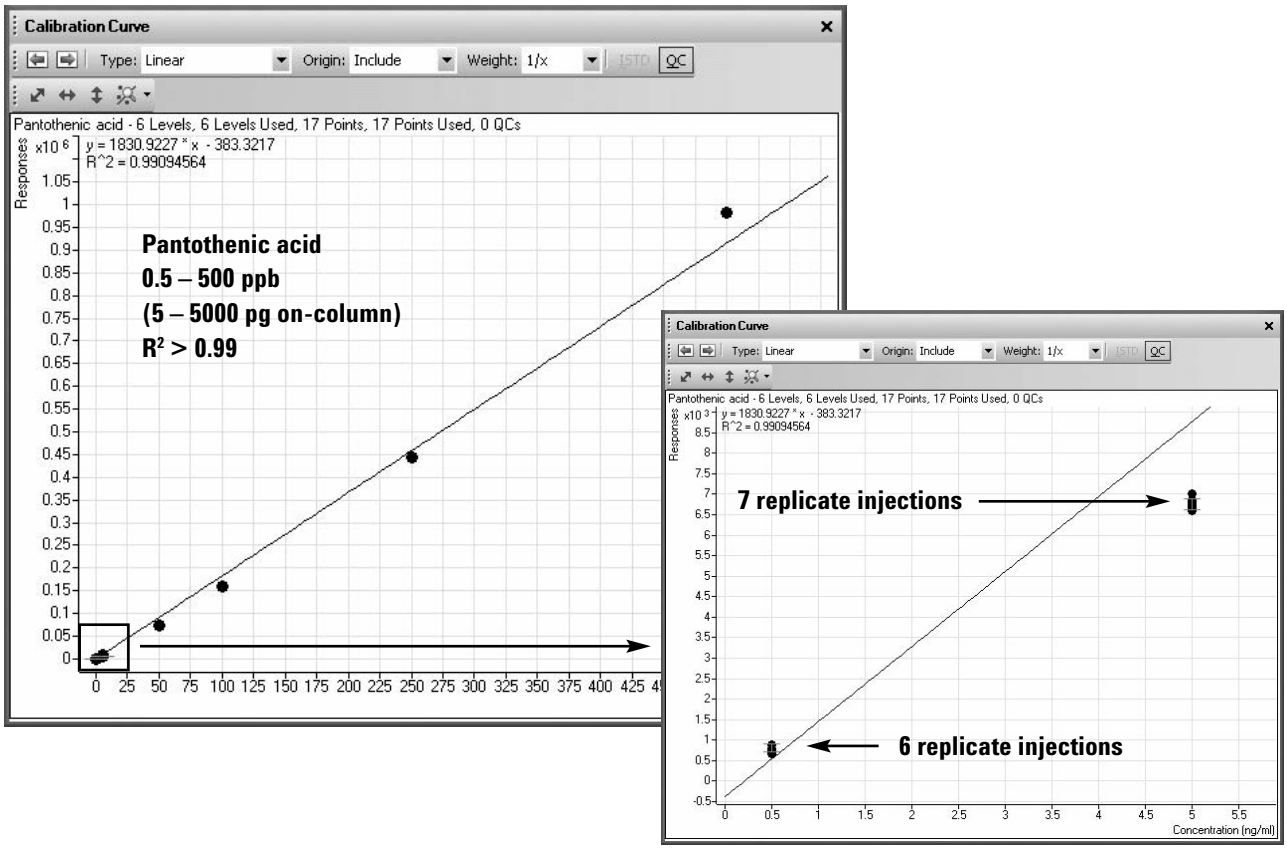

Figure 2B. Linearity of pantothenic acid over three orders of magnitude.

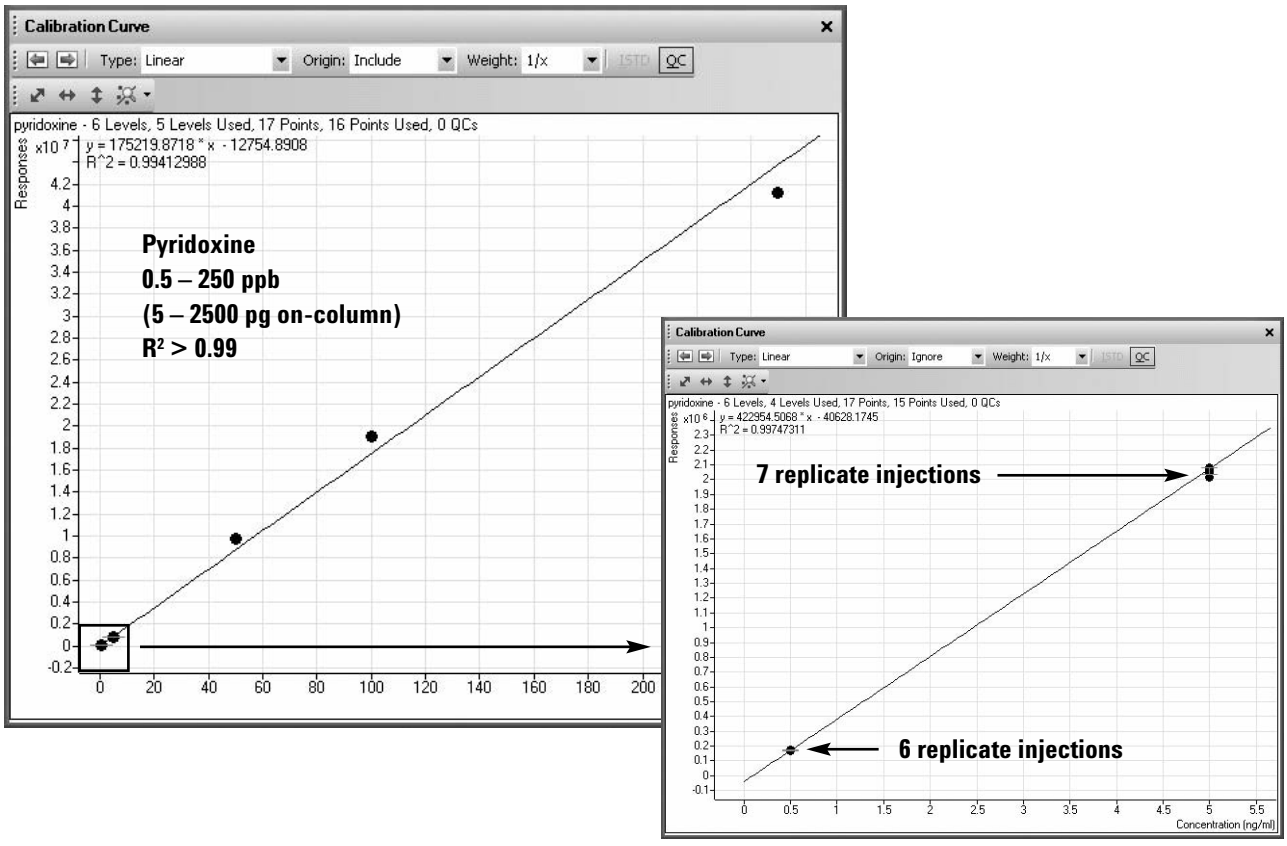

Figure 2C. Linearity of pyridoxine acid over nearly three orders of magnitude.

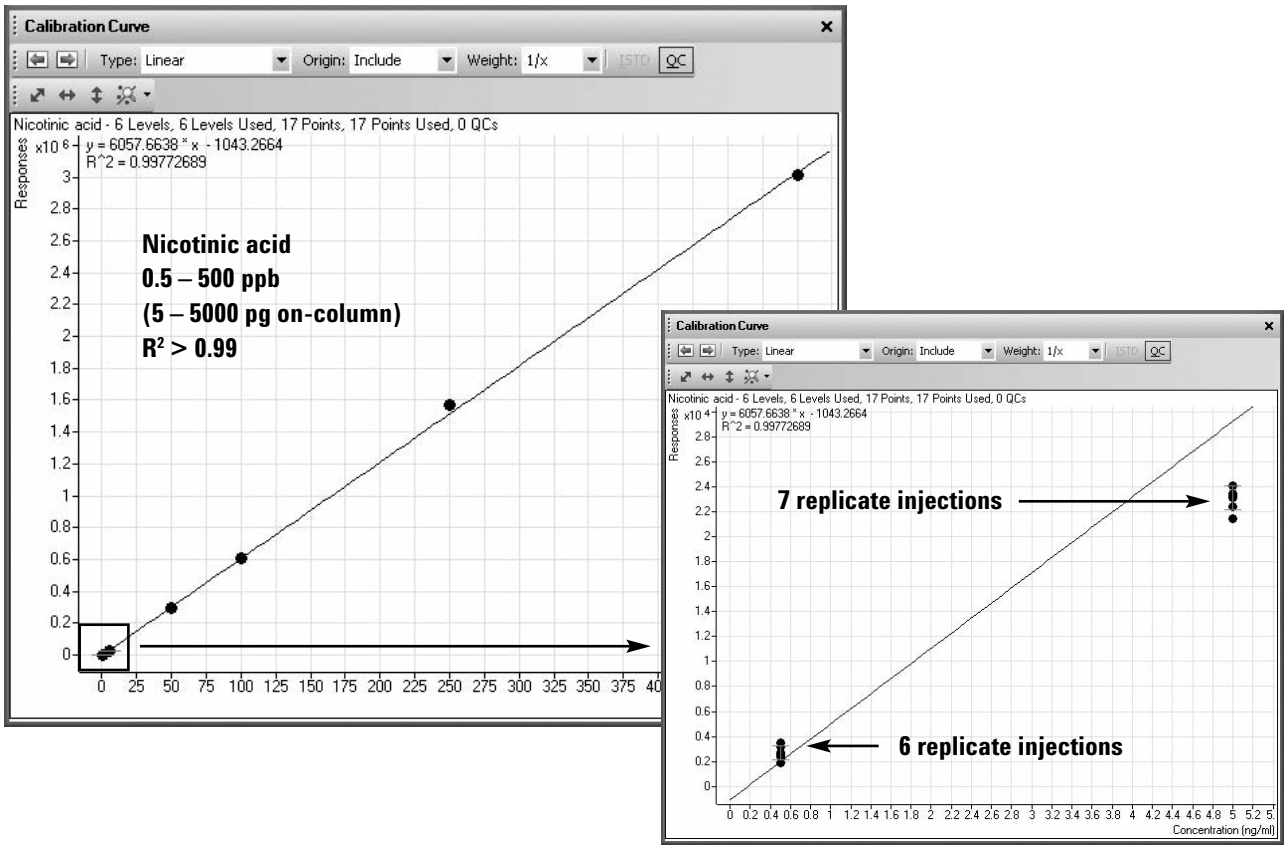

Figure 2D. Linearity of nicotinic acid over three orders of magnitude.

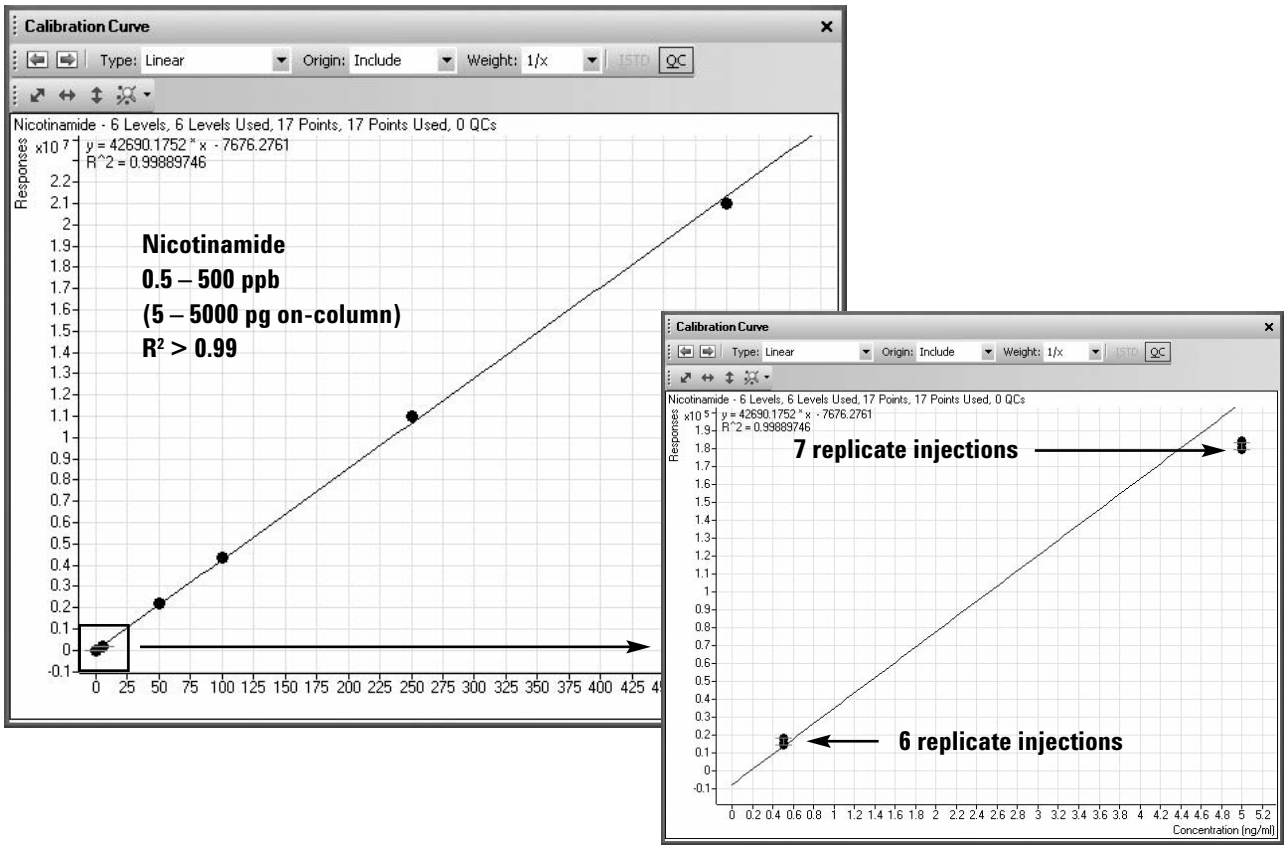

Figure 2E. Linearity of nicotinamide acid over three orders of magnitude.

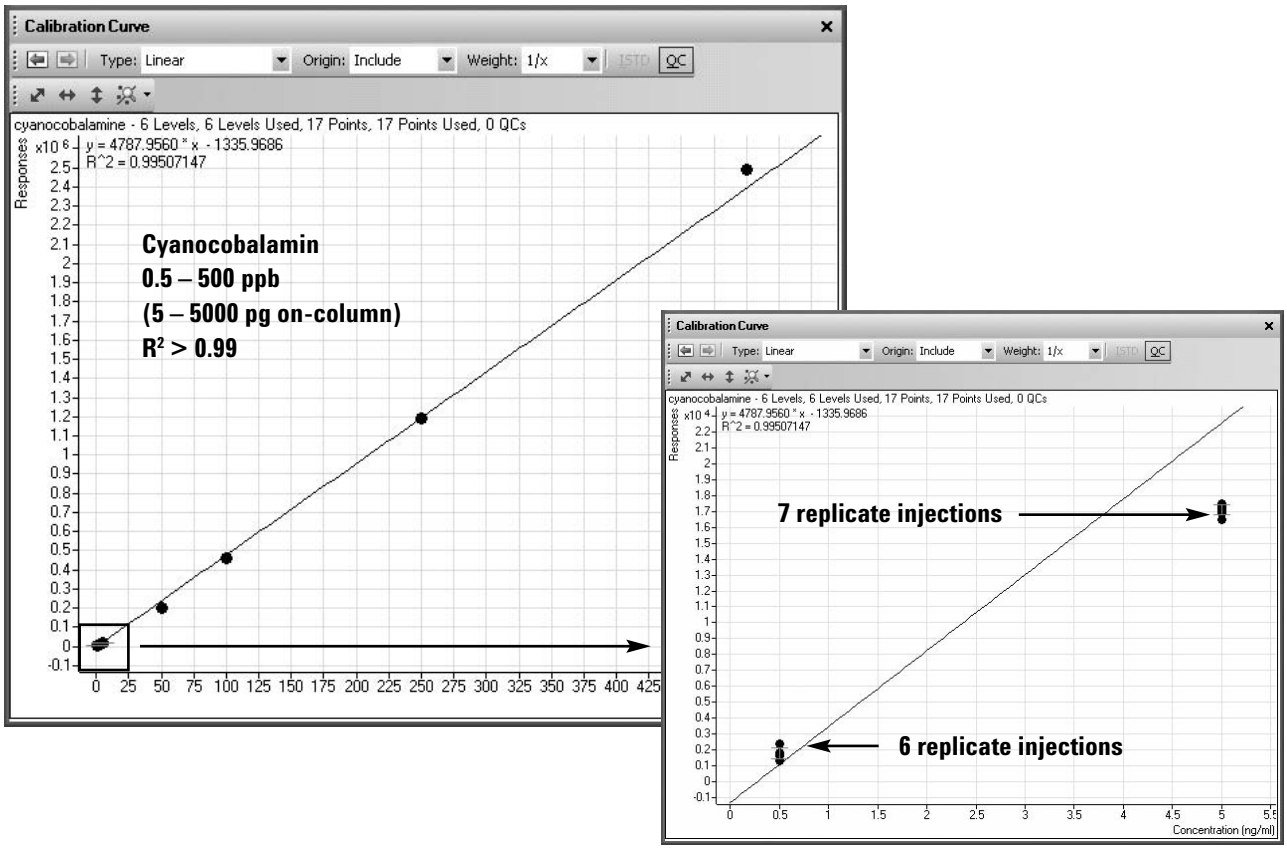

Figure 2F. Linearity of cyanocobalamin over three orders of magnitude.

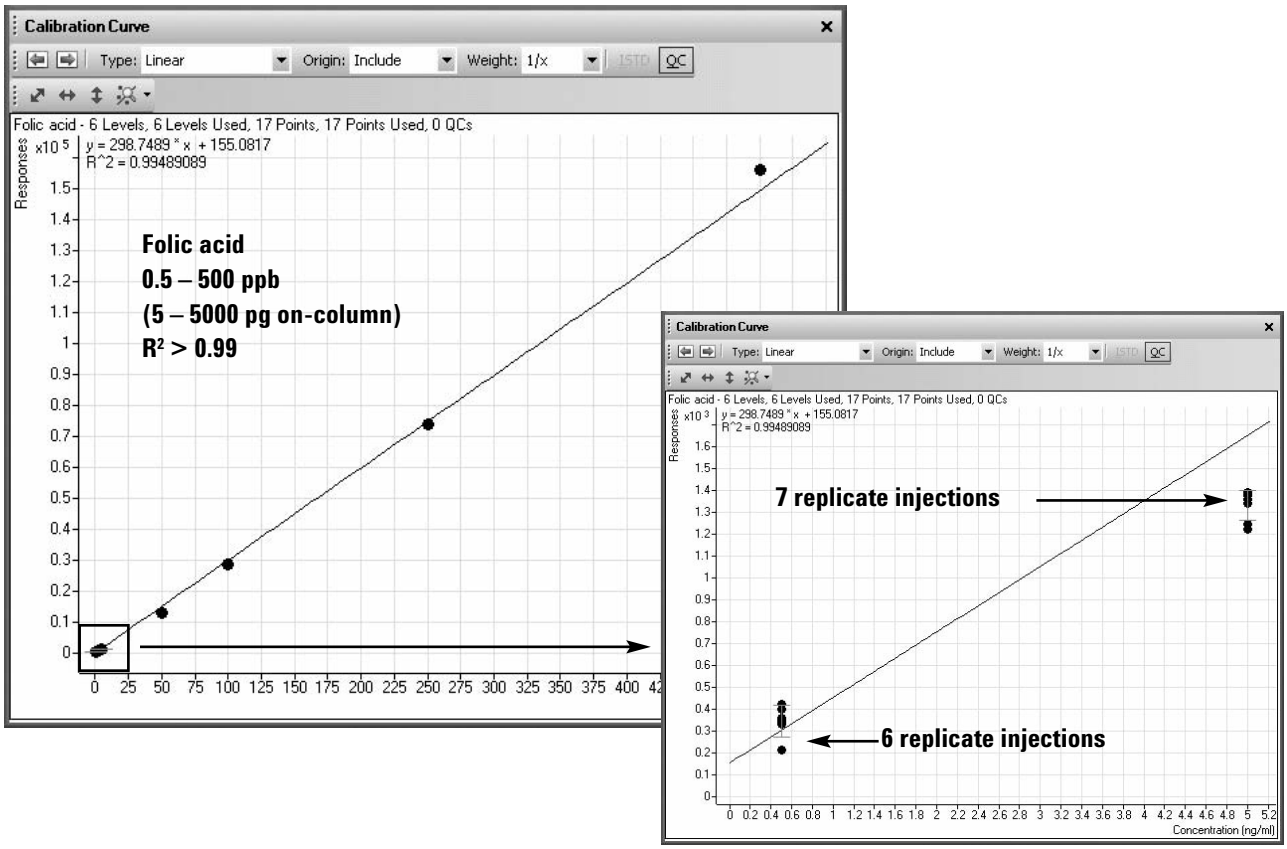

Figure 2G. Linearity of folic acid over nearly three orders of magnitude.

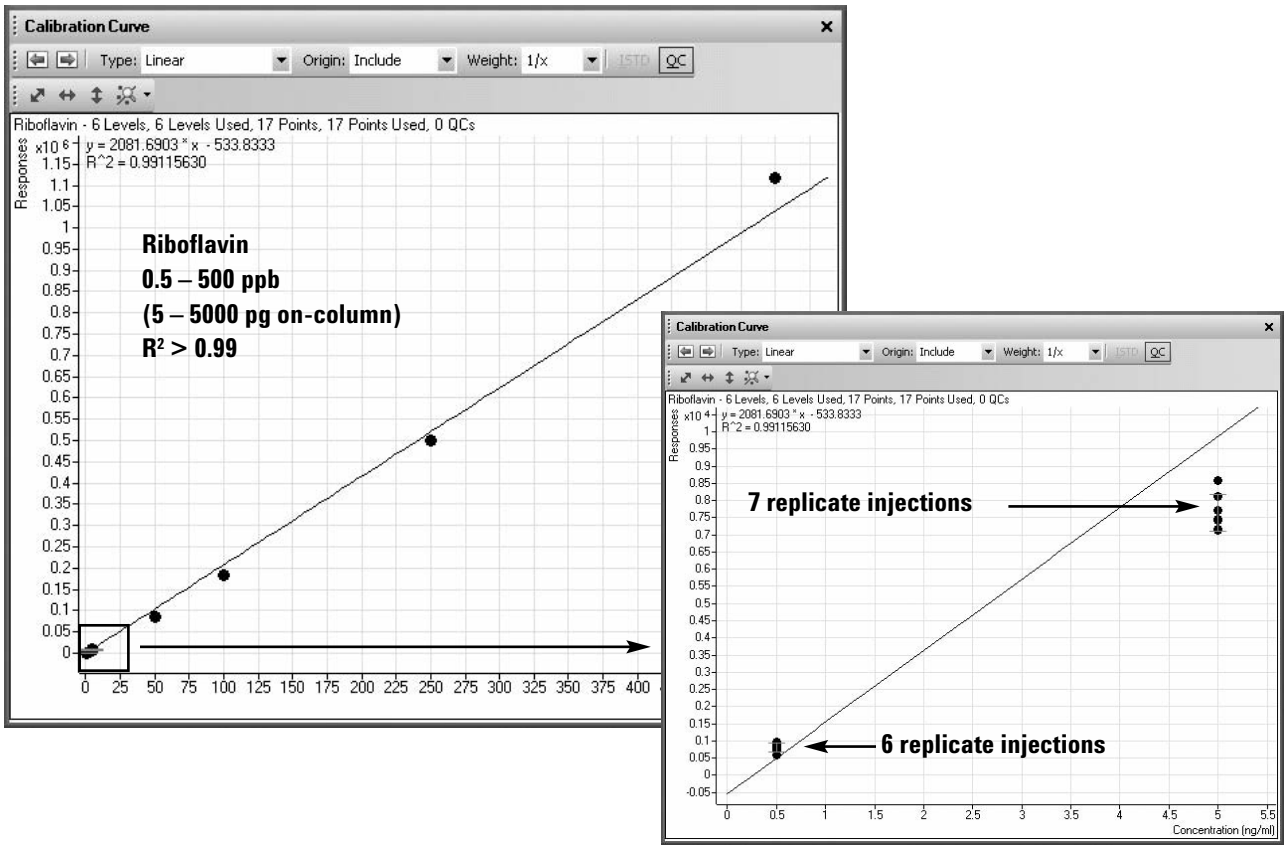

Figure 2H. Linearity of riboflavin acid over three orders of magnitude.

All line fits to data are carried out as linear with the origin ignored and a 1/x weighting.

An example of reproducibility at the 0.5 ppb level for pyridoxine is shown in Figure 3. The peak area %RSD values for all compounds at the 0.5 ppb level are given in Table 2.

Table 2. Peak Area Reproducibility for Each Compound at the Lowest Level of Quantitation, Which Is 0.5 ppb

Compound	Peak area %RSD
Thiamine	5.6
Pantothenic acid	11.6
Pyridoxine	2.6
Nicotinic acid	12.9
Nicotinamide	10.1
Cyanocobalamin	19.4
Folic acid	13.4
Riboflavin	17.1

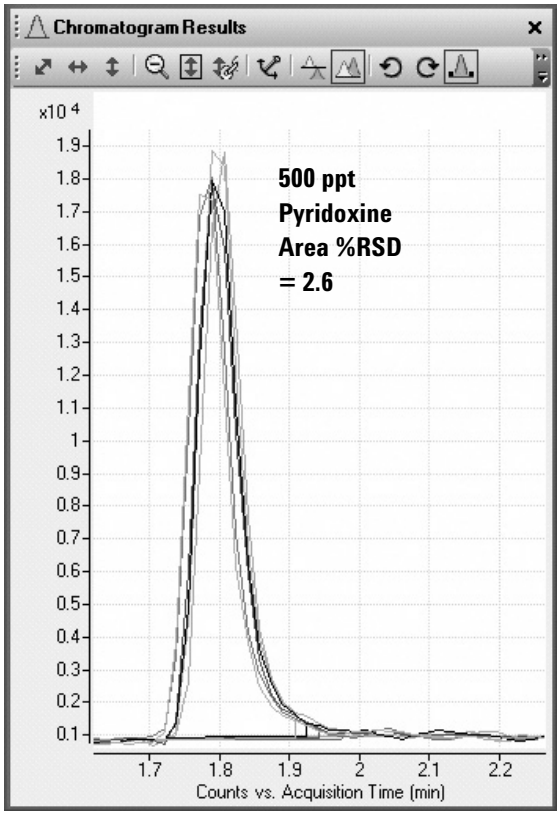

Figure 3. Peak area reproducibility of pyridoxine at 500 ppt level, 6 injections.

A fortified cereal extract is also analyzed and quantitated using the diluted standard mix already mentioned. An example of the batch results using the MassHunter Quantitative Analysis is shown in Figure 4. The concentration of nicotinamide present in the sample is calculated to be 111.6 pg/µL.

The concentrations calculated for all compounds in the fortified extract are given in Table 3.

The corresponding chromatographic elution of the eight compounds detected in the fortified extract is shown in Figure 5.

Table 3. Calculated Concentrations for Each Compound in Fortified Cereal Extract

Compound	Calculated concentration (pg/µL)
Thiamine	24.0
Pantothenic acid	1.2
Pyridoxine	15.5
Nicotinic acid	43.5
Nicotinamide	111.6
Cyanocobalamin	0.4*
Folic acid	2.6
Riboflavin	8.6

* Outside quantitation limits.

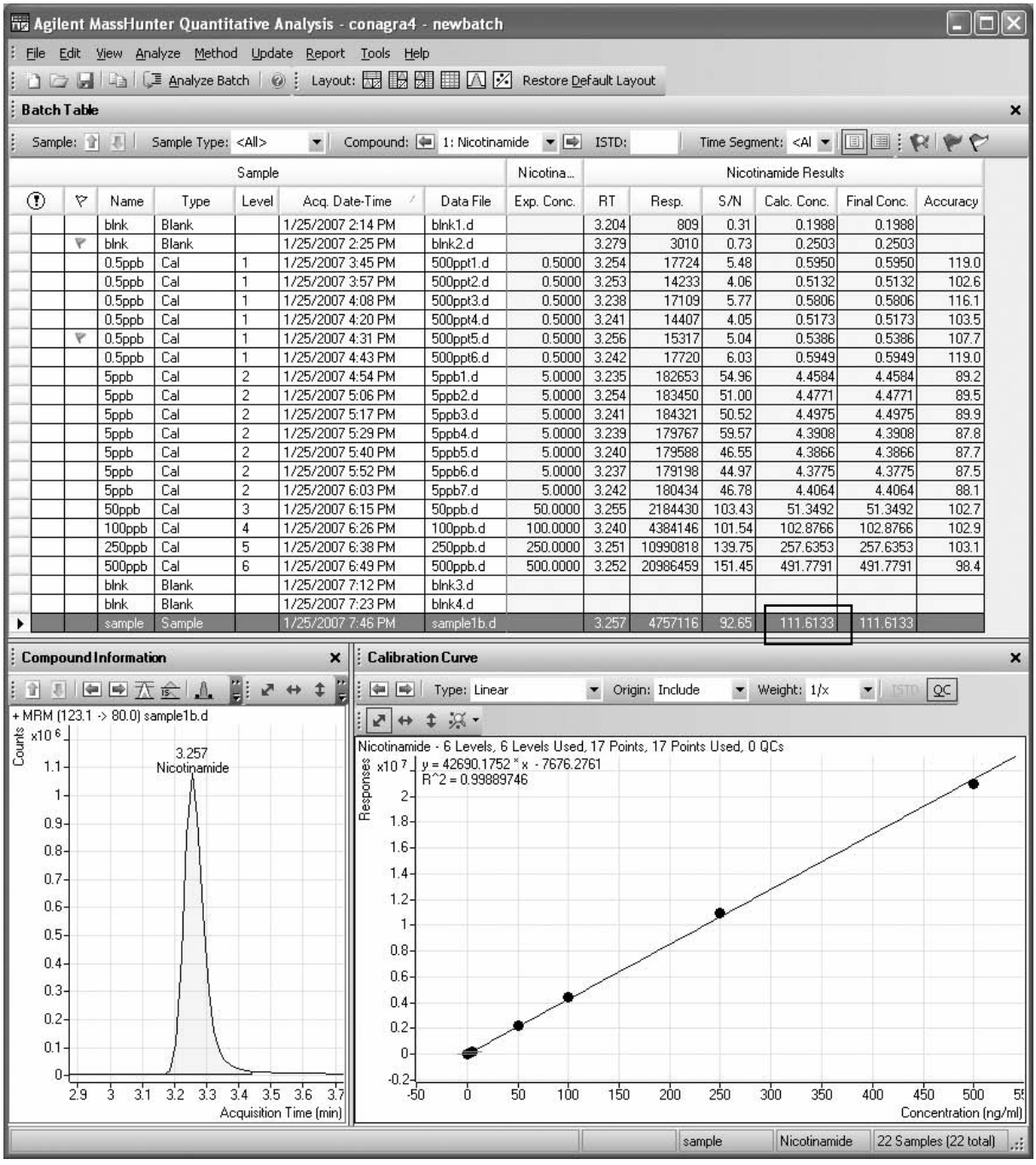

Figure 4. Quantitation batch results for nicotinamide in sample. Concentration calculated to be 111.6 pg/µL (highlighted).

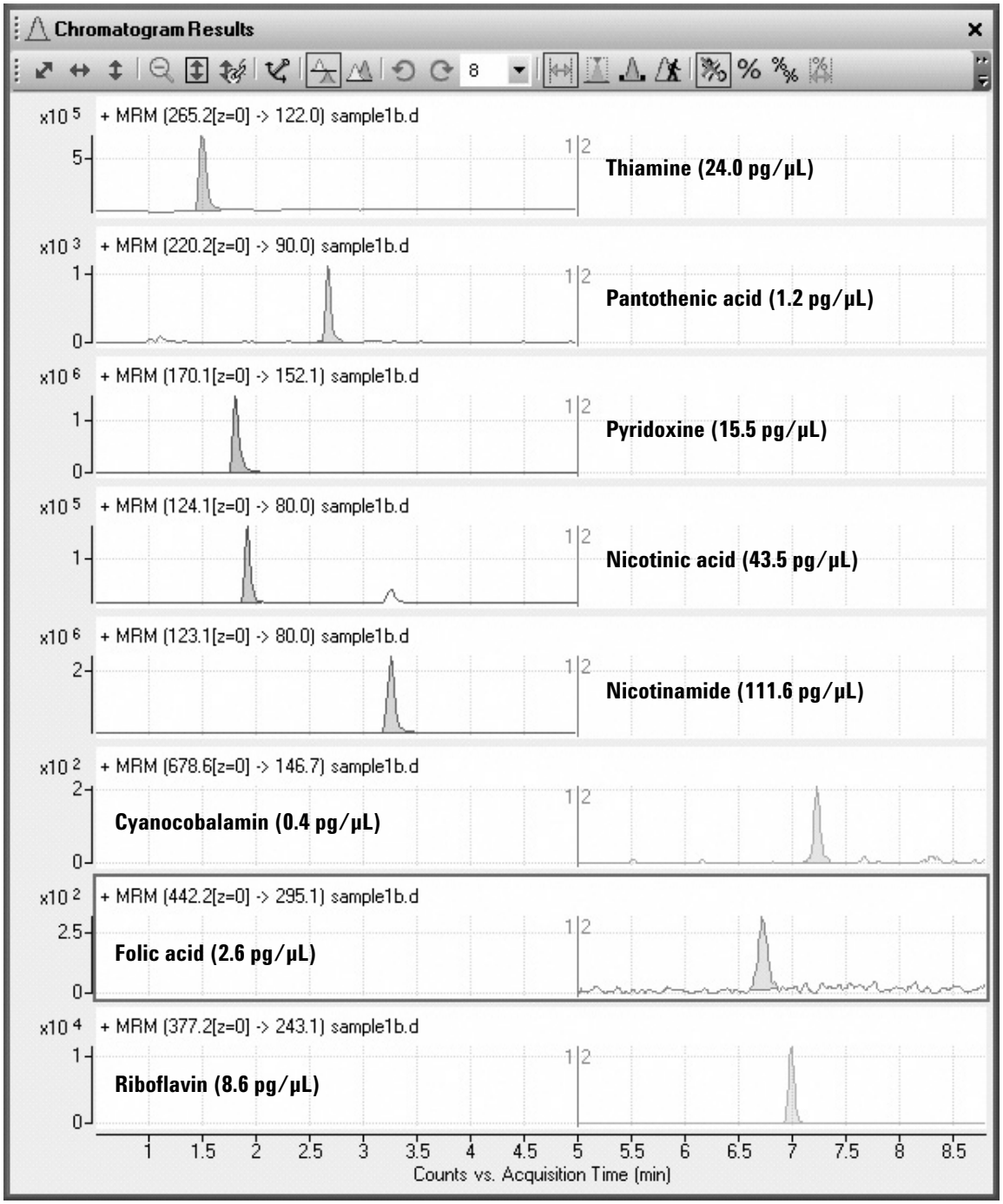

Figure 5. Chromatogram of compounds in fortified extract and calculated concentrations.

Conclusions

The water-soluble B vitamins are successfully analyzed using LC/MS/MS. Good linearity with $R^2 > 0.99$ is demonstrated over three orders of magnitude for all compounds, with reproducibility as low as 2.5 %RSD at the lowest level of quantitation for pyridoxine. An extracted fortified sample is successfully analyzed with only the cyanocobalamin concentration falling below the quantitation limit.

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Direct Analysis of Folic Acid in Digestive Juices by LC/TOF-MS

Application

Food Safety

Authors

M. H. Blokland, P. W. Zoontjes, S. S. Sterk, and L. A. van Ginkel,
National Institute of Public Health and the Environment (RIVM)
Laboratory for Food and Residue Analysis
Bilthoven
The Netherlands

Jerry Zweigenbaum
Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE
USA

Abstract

By use of the high resolving power and accurate mass measurement of the LC/TOF-MS it is possible to measure folic acid without cleanup directly in gastrointestinal juices. The samples are obtained from an *in vitro* digestion model. The method is validated for measuring uptake in this type of model.

Introduction

Health benefits associated with the intake of folic acid are well known. Increasing the daily intake can be achieved through medication or through the consumption of enriched food products. In the latter case, it is important to know the fraction of the total intake that is accessible in the gastric tract. To study the accessibility, an *in vitro* digestion model can be used.

Due to the complex nature of gastrointestinal juices (for example, saliva, stomach juice, and chyme), analysis of folic acid in these juices generally requires an extensive cleanup procedure. Traditionally, cleanup procedures consist of immuno affinity chromatography or solid phase extraction.

Using the high-resolution capabilities and unique accurate mass measurement of LC/TOF-MS, these cleanup steps can be eliminated and direct detection of folic acid in gastrointestinal juices is possible. By doing so, the risk of degradation of folic acid during cleanup is minimized and high-throughput analysis becomes possible. This application describes the direct measurement of folic acid in gastrointestinal juices.

Experimental

Cleanup Procedure

Saliva, stomach juice, and chyme were prepared as described elsewhere.[1] Sample preparation is straightforward; an aliquot of the juices is taken and directly injected in the LC/TOF-MS system.

LC/MS Method Details

LC Conditions

Instrument:	Agilent LC 1200 SL
Column:	ZORBAX SB-C8, 100 mm x 2.1 mm, 1.8 µm (p/n 828700-906)
Column temp:	80 °C
Mobile phase:	A: 1% formic acid in water B: 26/60/14 v/v acetonitrile/water/methanol
Gradient:	5% B at 0 min 5% B at 4 min 40% B at 8 min 40% B at 8.1 min 5% B at 9 min
Flow rate:	0.5 mL/min
Injection volume:	10 µL

MS Conditions

Instrument:	Agilent 6210 LC/TOF-MS
Source:	Positive ESI
Drying gas flow:	9 L/min
Nebulizer:	60 psig
Drying gas temp:	350 °C
V_{cap}:	4000 V
Fragmentor:	150 V
Skimmer:	60 V
Scan mode:	*m/z* 110—940, 12419 transients/scan, 1.5 scan/sec
Reference mass solution:	100 mg/L solutions of purine 2 ml/L and HP-921 1 ml/L in acetonitrile infused into second sprayer at constant rate
Reference mass:	*m/z* 121.020873 and 922.009798

Results and Discussion

Calibration Standards

The structure of folic acid is shown in Figure 1. Because sensitivity is not a concern in this analysis conditions were only optimized for accurate mass measurement and linearity of response within the concentrations expected for the samples. In Figure 2 a typical chromatogram is shown of a standard of 1 µg/mL (expressed as concentration in matrix). The S/N for this concentration is typically greater then 500.

Calibration curves are produced from seven calibration points in the range between 1 µg/mL to 100 µg/mL. It is demonstrated that the LC/TOF-MS has the necessary linear dynamic range.

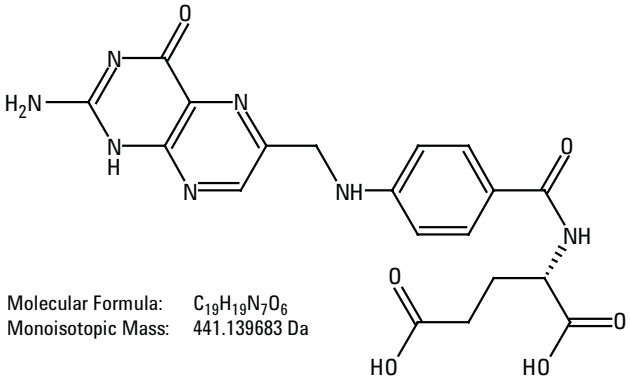

Figure 1. Structure of folic acid, its exact molecular weight, and its empirical formula.

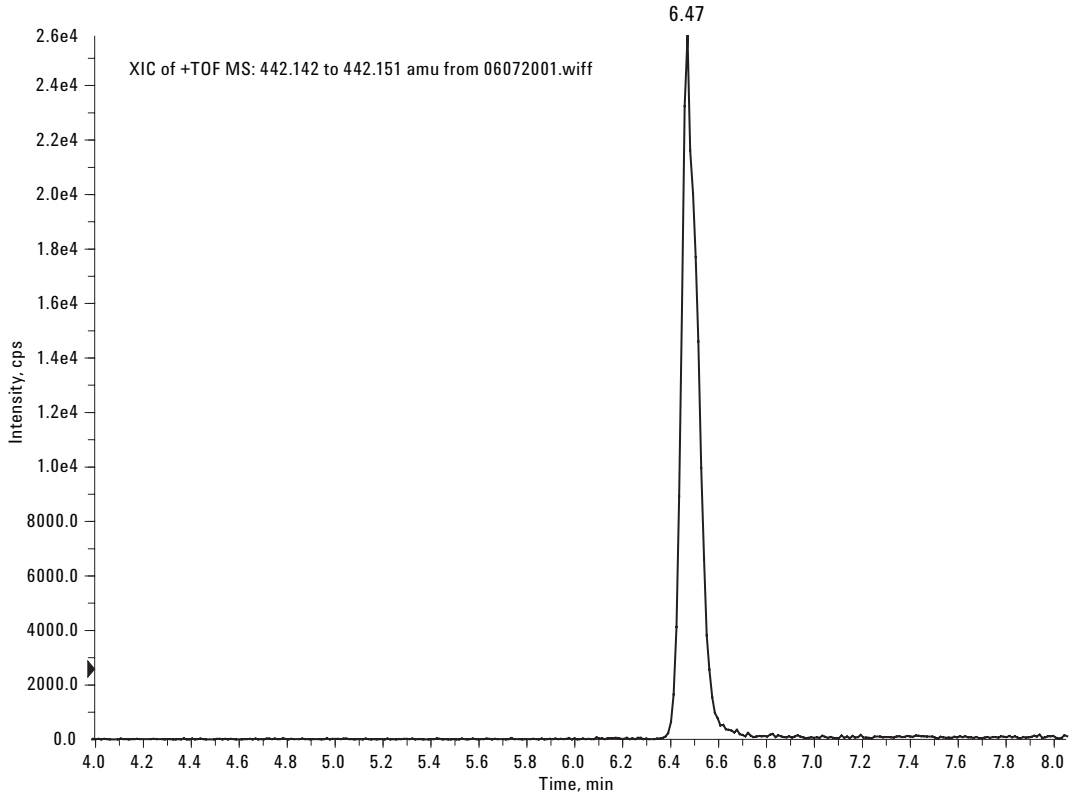

Figure 2. Extracted ion chromatogram of a standard of folic acid at 1 µg/mL (ion extraction window of ± 10 ppm of exact mass of M+H ion).

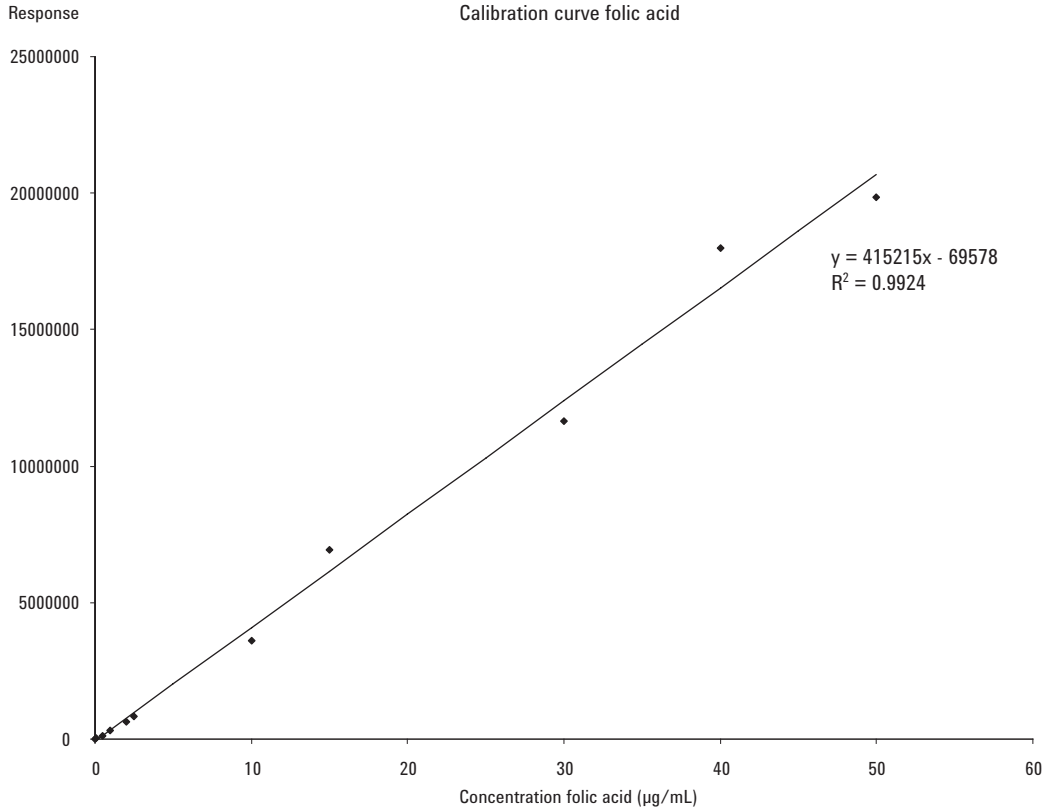

Figure 3. Calibration curve of folic acid standards from 1 to 100 µg/mL.

Validation of the Method

To obtain reliable validation results it is important to obtain clean chromatograms without matrix interference. Figures 4 to 6 show examples of total ion chromatograms, extracted ion chromatograms (EIC), and spectra of spiked gastrointestinal juices (1 µg/mL). In all samples mass accuracy was better then 3 ppm. EIC of each were extracted with a mass window of 442.1425 to 442.1515 (± 10 ppm) to obtain chromatograms with no matrix interference.

Validation of the Sample Data

Validation of the method was done by spiking saliva, gastric juice, and chyme at the following concentrations: 1, 2.5, 5, 10, 25, 50, 75, and 100 µg/ml. The results would indicate whether there were ion-suppression effects in these matrices. Figure 7 shows the response for each of these matrices. The data show that chyme produces the least effects, whereas saliva and gastric juice produce slightly higher ion suppression effects.

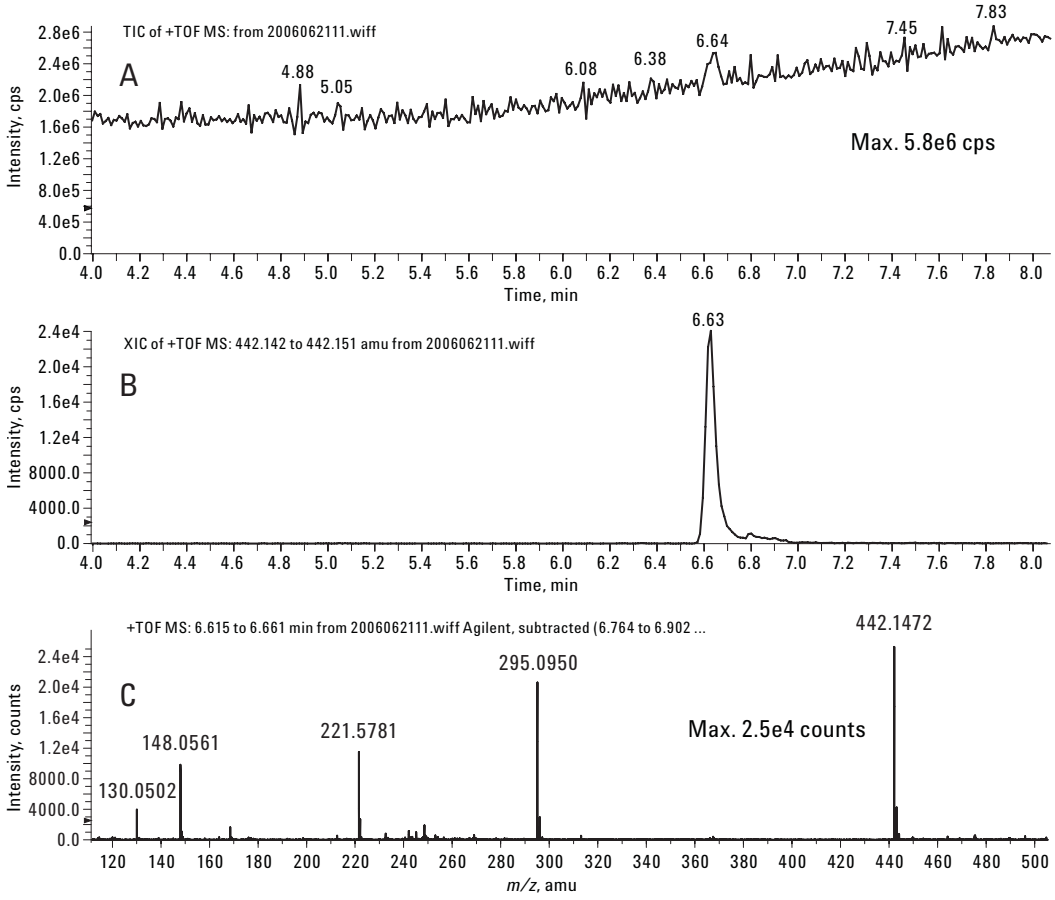

Figure 4. TIC (A), EIC (B), and spectra (C) for folic acid in saliva spiked at 1µg/mL.

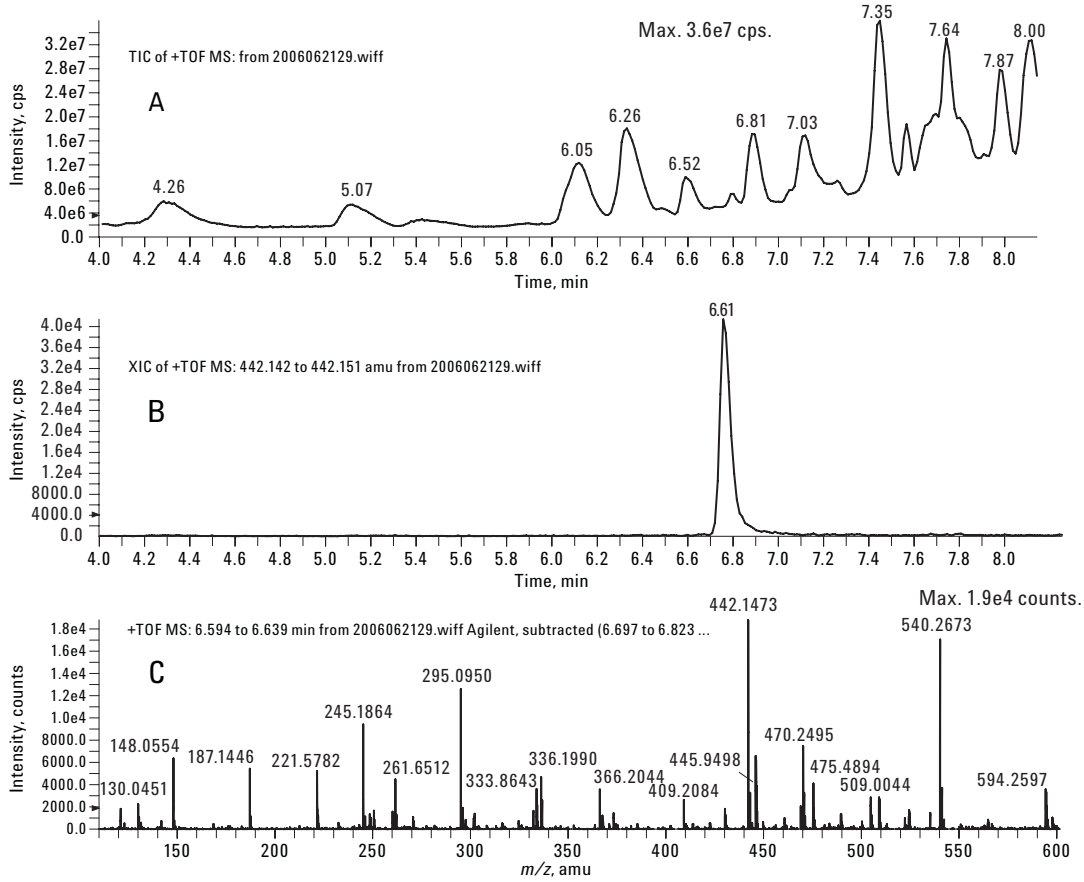

Figure 5. TIC (A), EIC (B), and spectra (C) for folic acid in gastric juice spiked at 1 µg/mL.

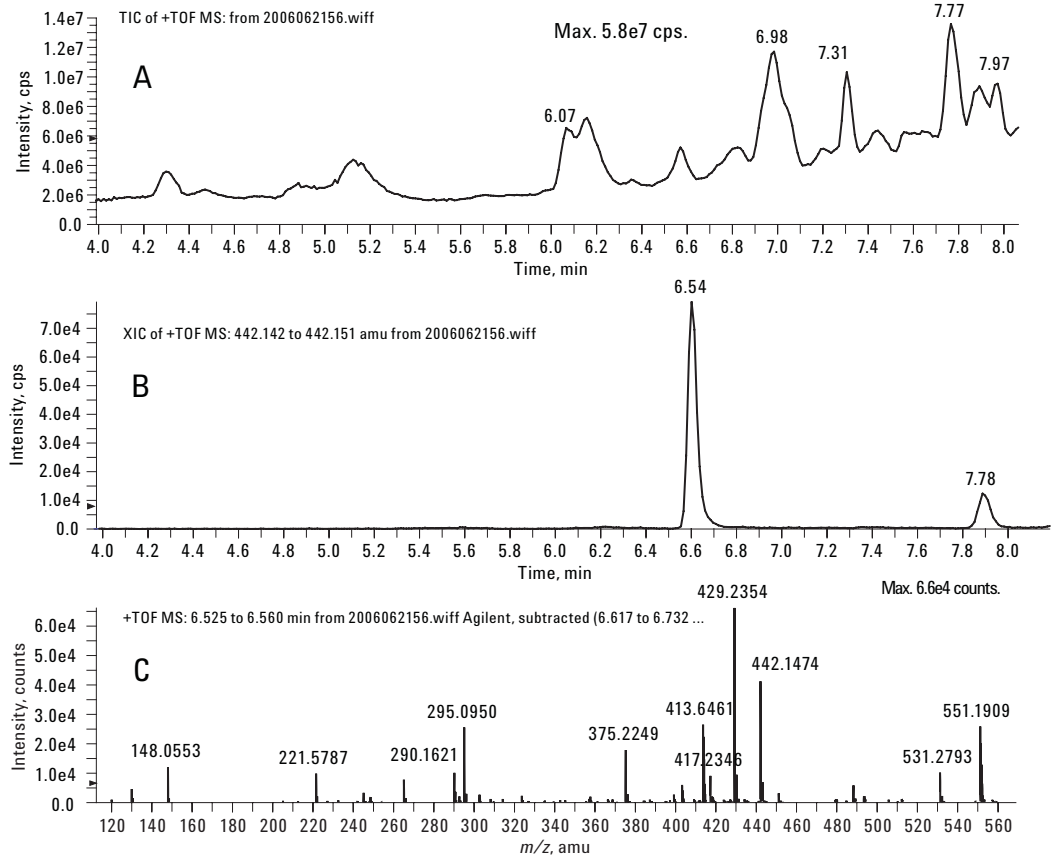

Figure 6. TIC (A), EIC (B), and spectra (C) for folic acid in chyme spiked at 1µg/mL.

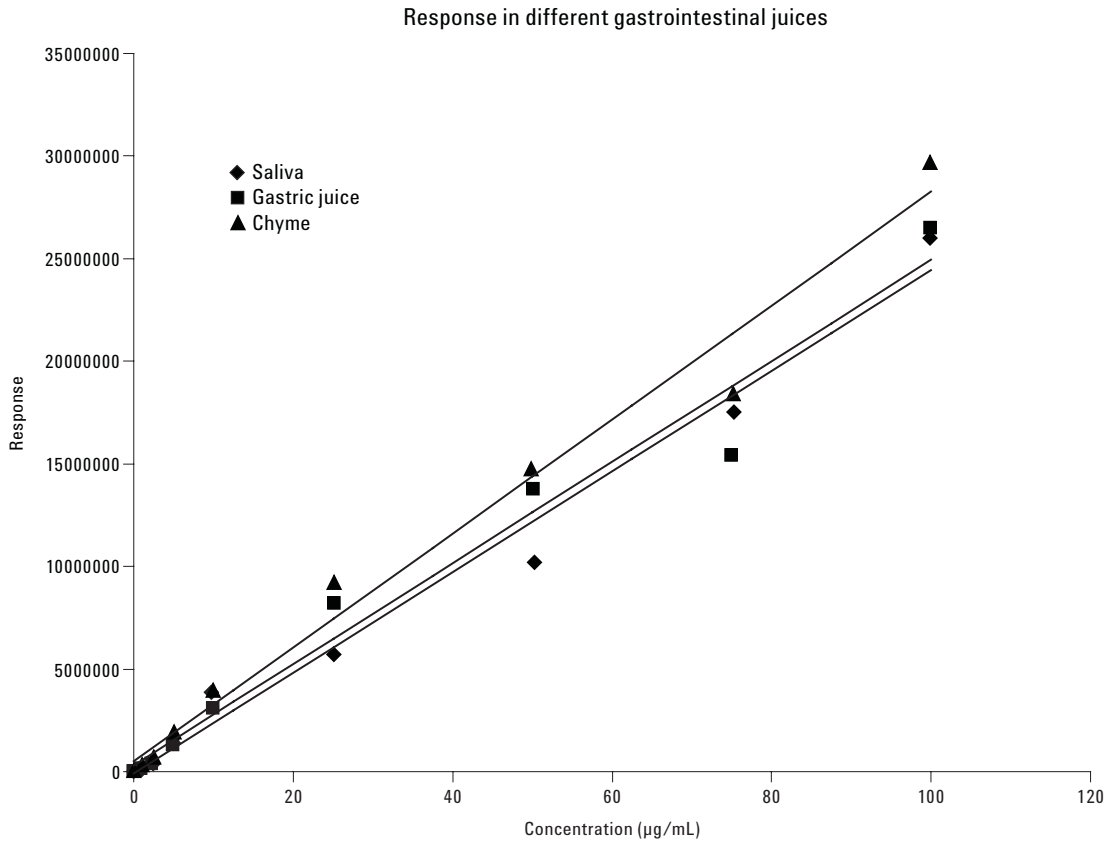

Figure 7. Calibration curve of folic acid spiked in different juices.

To determine the accuracy and covariance of the measurement, gastric juice and chyme were spiked (n = 4) at 50 µg/mL and analyzed on four separate days, with a one-week interval. These results are shown in Table 1.

The Limit of Detection (LOD) was determined by preparing a standard curve in saliva, gastric juice, and bile. From this calibration curve the Y-intercept and slope were calculated. The LOD is the corresponding concentration at the Y-intercept plus three times the standard deviation of the Y-intercept. The results of these determinations are given in Table 2.

Table 1. Accuracy (%) and Covariance (%)

	Week 1		Week 2		Week 3		Week 4
	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)
Gastric	91.9	1.2	99.3	4.2	82.9	6.0	91.7
Chyme	99.9	2.1	102.5	3.7	91.4	4.2	93.3

Table 2. Determination of the Limit of Detection (LOD)

	Saliva (µg/mL)	Stomach (µg/mL)	Chyme (µg/mL)
LOD	0.4	0.7	0.6

Conclusions

The aim of this work was to develop a method with a minimal cleanup to detect folic acid in gastrointestinal juices. By using the high resolving power of the Agilent LC-TOF, it was possible to measure directly in these juices without any cleanup. The measurements proved to be reproducible with a high accuracy in complex matrices. The response was linear for the different digestive fluids tested with little matrix effects, and the method was validated within the concentrations required for folic acid uptake studies.

Reference

1. C. H. Versantvoort, A. G. Oomen, E. Van de Kamp, C. J. Rompelberg, and A. J. Sips. "Applicability of an *in vitro* digestion model in assessing the bioaccessibility of mycotoxins from food." *Food Chem Toxicol,* 2005 Jan, 43(1).

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Improved and Simplified Liquid Chromatography/Atmospheric Pressure Chemical Ionization Mass Spectrometry Method for the Analysis of Underivatized Free Amino Acids in Various Foods

Application

Food

Authors

Süreyya Özcan
Food Engineering Department
Hacettepe University
06532 Beytepe, Ankara
Turkey

Hamide Z. Şenyuva
Ankara Test and Analysis Laboratory
Scientific and Technical Research Council of Turkey
Ankara 06330
Turkey

Abstract

An improved analytical method that offers rapid, accurate determination and identification of 22 amino acids in a variety of matrixes is reported. The amino acids were extracted from the matrixes using acidified water. Simultaneous determination of 22 underivatized amino acids was carried out by liquid chromatography–mass spectrometry (LC/MS). A narrow-bore column allowed rapid screening and quantitative analysis by LC/atmospheric pressure chemical ionization (APCI) MS in positive ion mode.

Introduction

There are a total of 22 amino acids classified by their functional group, or their "R" group. Of those 22 amino acids, eight are histidine, lysine, phenylalanine, methionine, leucine, isoleucine, valine, and threonine considered essential, which means that humans need to get a certain amount of them in their diet to function properly. The determination of amino acids is of great importance to the food industry because of nutritional labeling requirements.

There are several approaches to amino acids analysis based on the pre- or post-column derivatization techniques, which have demonstrated good results but have drawbacks of very long analysis time or poor repeatability. Most of the other methods used for amino acid analysis involve a chromatographic separation following or preceding the derivatization of amino acids with UV absorptive or fluorescent functional group detection. Although these methods are often faster than the classical ion-exchange method, which involves quantification after ninhydrin and OPA derivatization and offers lower detection limits, these methods are still very time consuming, taking 20 to 75 min, are not repeatable, and are difficult to undertake.

The objectives of the present study were 1) to develop an analytical method for the simple, rapid, accurate, and repeatable determination of 22 free amino acids in various foods using an improved LC/APCI-MS method, 2) to develop an easy aqueous extraction of amino acids without clean-up, and 3) to perform screening and both qualitative and quantitative analysis on a narrow-bore column with a short run time.

Method

LC Conditions

Column:	ZORBAX Bonus-RP, narrow-bore (100 mm × 2.1 mm, 3.5 µm)
Flow rate:	0.2 mL/min
Mobile phase:	0.01 mM acetic acid in 0.2% aqueous solution of formic acid
Injection:	20 µL out of 1000 µL

MS Conditions

Ionization mode:	Positive APCI
Nebulizer pressure:	55 psi
Drying gas flow:	4 L/min
Drying gas temperature:	320 °C
Vaporizer temperature:	425 °C
Skimmer:	20 V
Capillary voltage:	3 kV
Fragmentor voltage:	55 V
Dwell time:	27 ms

Experimental

LC/MS experiments were performed using an Agilent 1100 Series HPLC system consisting of a binary pump, an autosampler, and a temperature-controlled column oven, coupled to an Agilent 1100 MS detector equipped with an atmospheric pressure chemical ionization (APCI) interface.

Data acquisition was performed in SIM mode using the interface parameters: drying gas (N_2, 100 psig) flow of 4 L/min, nebulizer pressure of 55 psig, drying gas temperature of 320 °C, vaporizer temperature of 425 °C, capillary voltage of 3 kV, corona current of 8 µA, fragmentor voltage of 55 V, and dwell time of 27 msec. Ions monitored for 22 underivatized amino acids are given in Table1.

The chromatographic separations were performed on a ZORBAX Bonus-RP, narrow-bore (100 mm × 2.1 mm, 3.5 µm) using the isocratic mixture of 0.01 mM acetic acid in a 0.2% aqueous solution of formic acid at a flow rate of 0.2 mL/min at 400 °C.

Sample preparation

According to the sample matrix, the sample was ground (by using a blender, mesh size 2 mm) or mixed (by Ultra Turrax). Dry samples like baby foods, breakfast cereal, and cookies were milled, and soft samples, like tomato, and liquid samples, like juices, were mixed with an Ultra Turrax blender. The pH of each homogenized sample was measured before sample preparation. Subsamples of the homogenate were stored at –20 °C in high-density polyethylene bottles with plastic screw-cap lids. Finely ground or homogenized sample (1 g) was weighed (fresh weight [FW]) into a 10 mL glass centrifuge tube with cap. After mixing in a vortex mixer for 2 min, the mixture was centrifuged at 5,000 rpm for 10 min at –5 °C. The clear supernatant was quantitatively transferred into a vial, avoiding the top oil layer if present. The supernatant was filtered through 0.45-µm nylon syringe filter prior to LC/MS analysis.

Table 1. Characteristic Fragments of 22 Amino Acids and Ions Used in SIM Mode for Quantification

ID	Fragment ions *m/z*	Selected ion *m/z*
Alanine (ala)	90, 73	90
Arginine (arg)	175, 129	175
Asparagine (asn)	133, 116, 87, 74	133
Aspartic acid (asp)	134, 116, 88	134
Cysteine (cys)	122, 105, 87, 73	122
Cystine(cys-cys)	241, 122	241
Glutamic acid (glu)	148, 130, 102	148
Glutamine (gln)	147, 130, 101	147
Glycine (gly)	76, 59	76
Histidine (his)	156, 110, 96, 73, 59	156
Hydroxyproline (hyp)	132, 86	132
Leucine-isoleucine (leu-ile)	132, 86	132
Lysine (lys)	147, 130, 84	147
Methionine (met)	150, 133, 104	150
Phenylalanine (phe)	166, 149, 120	166
Proline (pro)	116, 70	116
Serine (ser)	106, 88, 60	106
Threonine (thr)	120, 102, 74	120
Tyrosine (tyr)	182, 165, 136, 123	182
Tryptophan (trp)	205, 188, 130	188
Valine (val)	118, 72	118

Results and Discussion

Optimization of MS fragmentation and chromatographic conditions

Fragmentation patterns were reproducible in both modes, and quantification could be performed on single *m/z* fragments. In-source collision induced dissociation at 55, 70, and 100 V was applied for all amino acids, and optimum conditions were found to be 55 V and positive polarity. All the most abundant fragmentations were present, with optimization of the MS parameters. Under the positive APCI conditions applied here, SIM-MS analysis showed excellent identification and quantification conditions within the 7.5 min total run time.

Specificity

MS detection has the advantage of providing structural information about the eluted compounds. Moreover, resolution of co-eluting compounds can be achieved by selecting different *m/z* ions for monitoring. In this study, a simple, rapid, and economic sample preparation method was used.

During the extraction with a 0.2 mM acetic acid solution, colloids soluble in water (starch) were simultaneously precipitated by centrifugation. Fat was separated by cold centrifugation, if present in the food sample. Figure 1 shows the MS signals for specific *m/z* ions of 22 amino acids in green peas using a single injection.

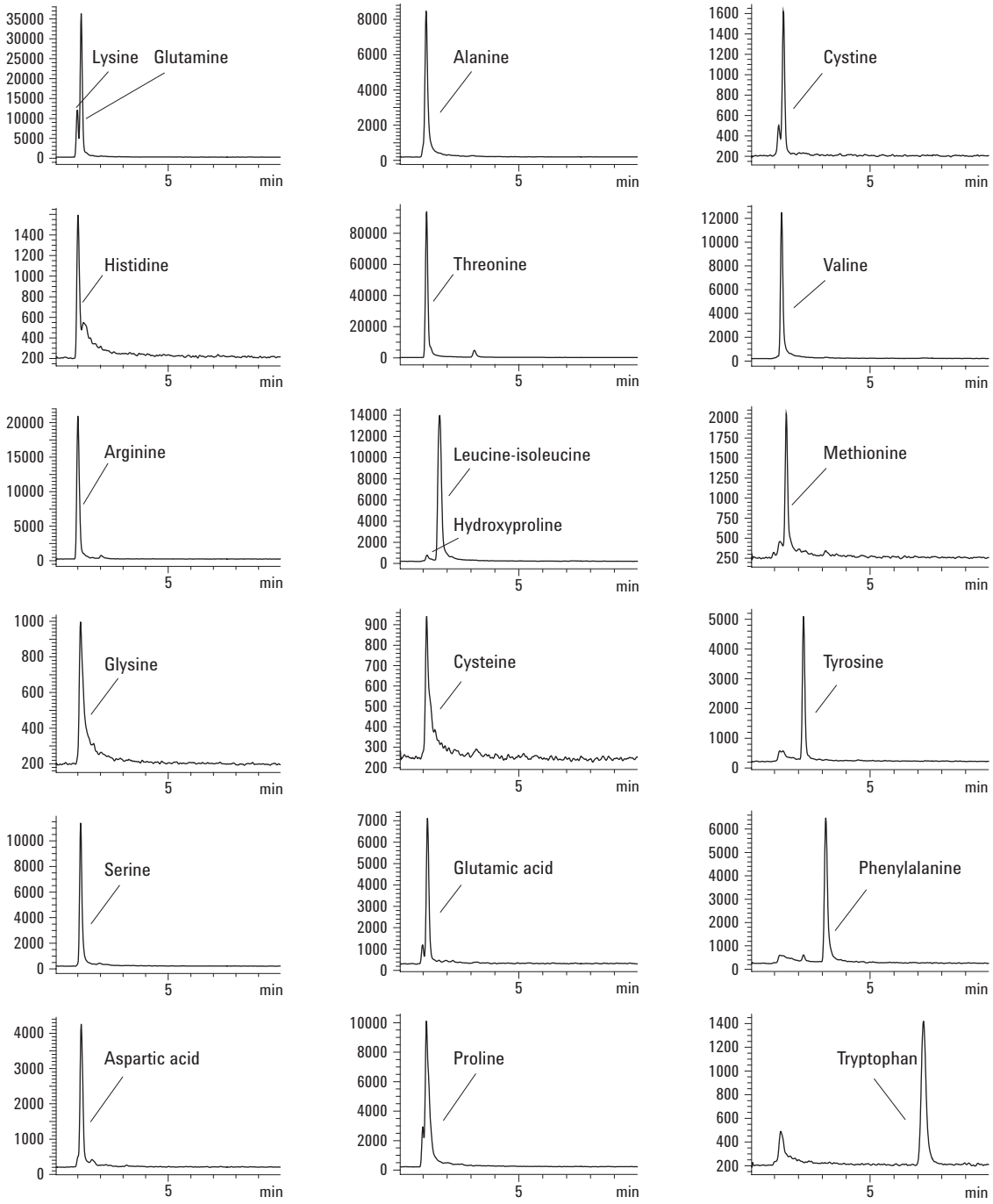

Figure 1. Determination of underivatized free amino acids using LC/APCI-MS. LC conditions: column, ZORBAX Bonus RP (2.1 mm × 100 mm, 3.5 µm); flow rate, 0.2 mL min^{-1}; mobile phase composition, 0.01 mM acetic acid in a 0.2% aqueous solution of formic acid; and MS signals for specific *m/z* ions of 22 amino acids in green pea.

Food samples

In this study, 22 food samples were analyzed for their amino acid content. The food groups are baby foods, vegetables, fruits, juices, nuts, wine, beer, milk, chicken, and honey. The mean concentrations of amino acids in each food are given in Table 2.

Table 2. The Results of 22 Free Amino Acids in Various Food, as mg/100 g FW

Food Types	Ala	Arg	Asn	Asp	Cys	Cys-Cys	Glu	Gln	Gly	His	Hyp
Baby food	3.59	1.25	1.07	0.50	0.17	9.77	0.37	1.06	0.52	0.30	1.42
Tomato	10.07	3.94	11.02	36.86	1.31	1.52	85.94	49.64	2.03	0.34	0.73
Pea	69.84	37.26	112.10	30.22	6.96	3.62	15.78	57.49	7.92	3.27	1.17
Pear	< LOD	1.81	45.27	17.01	0.60	13.58	8.19	86.33	1.14	< LOD	< LOD
Apple paste	< LOD	1.40	3.80	3.56	0.30	16.04	2.18	23.46	< LOD	< LOD	< LOD
Apple juice	2.03	1.79	12.80	10.58	0.98	12.52	4.82	39.89	< LOD	0.13	0.43
Sour cherry juice	1.53	1.62	24.10	7.30	0.38	10.95	7.11	52.45	< LOD	1.01	0.32
Orange juice	3.95	8.30	8.08	15.15	0.70	9.99	5.10	3.36	1.38	1.11	0.53
Pomegranate juice	15.66	1.99	13.58	17.33	0.27	16.53	17.23	42.35	< LOD	0.17	0.44
Peach juice	3.38	1.64	78.57	21.52	0.84	12.26	3.38	10.05	1.44	0.14	0.35
White grape juice	6.54	2.18	10.50	16.08	0.50	16.06	8.34	41.50	1.26	0.11	0.43
Red grape juice	8.58	0.60	5.32	5.54	0.74	17.60	7.98	4.17	1.52	1.46	0.52
Beer	4.00	0.88	2.98	1.15	< LOD	5.33	1.89	1.54	1.48	0.26	8.59
Milk	7.02	16.50	8.99	4.00	0.36	16.12	9.06	4.85	1.38	0.75	0.36
Wine	4.49	1.35	0.94	0.55	0.66	0.92	0.73	0.42	1.69	0.46	0.24
Honey	12.23	< LOD	4.16	8.43	< LOD	203.22	7.93	18.90	2.93	1.26	0.76
Green coffee	41.06	2.38	31.36	70.26	43.19	14.82	57.18	3.89	3.43	3.27	< LOD
Hazelnut	12.98	1.28	2.21	13.05	< LOD	6.02	21.01	0.51	2.57	< LOD	< LOD
Walnut	6.56	2.71	0.71	7.42	< LOD	4.90	46.80	3.55	1.72	0.38	0.74
Almond	11.39	4.14	57.51	44.92	0.73	4.50	3100	6.31	3.76	0.34	0.46
Pistachio	25.15	7.58	14.45	27.40	1.36	5.82	35.64	39.28	2.88	< LOD	7.26

Food Types	Leu-ile	Lys	Met	Phe	Pro	Ser	Thr	Tyr	Trp	Val
Baby food	0.30	< LOD	0.20	0.26	0.78	0.40	1.66	0.50	0.12	1.21
Tomato	0.96	< LOD	0.63	3.25	6.30	3.38	2.70	2.05	0.74	2.43
Pea	26.84	17.41	4.44	13.52	21.50	55.52	0.03	15.45	3.31	38.60
Pear	1.38	< LOD	< LOD	0.44	17.92	3.86	1.23	0.68	0.48	5.25
Apple paste	< LOD	< LOD	< LOD	< LOD	< LOD	0.57	< LOD	< LOD	< LOD	< LOD
Apple juice	0.32	< LOD	< LOD	< LOD	4.48	0.78	0.33	< LOD	< LOD	1.70
Sour cherry juice	0.23	< LOD	4.52	0.14	6.52	1.29	0.53	0.25	< LOD	2.62
Orange juice	0.37	1.76	4.72	0.99	30.55	6.07	1.13	0.99	0.58	3.64
Pomegranate juice	1.37	< LOD	0.53	0.94	8.52	3.01	1.94	1.76	0.64	4.62
Peach juice	< LOD	< LOD	< LOD	0.45	12.89	2.48	1.17	0.33	< LOD	2.18
White grape juice	1.54	1.21	0.89	0.81	8.94	2.80	1.75	1.80	0.64	5.10
Red grape juice	1.80	1.39	0.64	1.88	14.05	4.30	5.33	3.90	0.75	5.03
Beer	< LOD	1.17	0.30	0.15	0.84	< LOD	0.32	0.27	< LOD	3.84
Milk	2.04	1.43	1.80	0.61	11.61	3.14	3.12	2.33	0.65	5.40
Wine	0.23	1.28	0.25	0.25	30.50	0.36	0.31	2.37	1.86	0.88
Honey	2.06	4.80	< LOD	11.18	81.32	6.31	2.65	8.63	< LOD	6.34
Green coffee	6.37	4.35	1.66	14.31	32.30	20.48	3.63	13.58	7.66	8.61
Hazelnut	3.24	2.06	< LOD	2.41	5.38	3.84	2.38	3.69	2.16	4.08
Walnut	1.81	1.64	0.52	1.09	5.50	1.44	1.12	1.71	1.75	3.01
Almond	7.21	4.93	1.93	6.08	33.65	7.82	3.34	4.07	2.28	10.33
Pistachio	2.75	3.64	1.43	381	55.98	3.80	3.71	6.00	1.51	5.73

There was a great variation in the amino acid levels in each food. The variations in amino acid concentrations might be due to the differences in the composition of formulas and/or the differences in processing conditions. Also the protein composition can give rise to slight differences in the free amino acid profiles of foods. The pH of the samples was measured before extraction and found to range from 2 to 3 for juices, beer, and wine; about pH 4 for tomato; pH 6 for peas; and pH 7 for milk. It is very well known that the measurement of amino acids is very important in various areas, such as:

1) The determination of the geographical sources of honey from the ratios between concentrations of amino acids

2) Nutritional analysis as the amino acid supply in an infant's first month of life must be sufficient in quantity and quality to fulfill the needs of this critical period.

3) Some of the important free amino acids, such as asparagine, glutamine, aspartic acid, and glutamic acid, are important precursors for acrylamide in various products.

Conclusion

A simple, reliable, and rapid LC/MS method for the determination of 22 free amino acids in foods was developed in this study. The method was found to be applicable for a wide variety of foods. Our goal was to obtain a single-run LC/APCI-MS analysis of underivatized amino acids exhibiting sensitivity in positive mode, and this was improved by the use of further acidic LC/MS mobile phase to achieve a very short run time, ca. 7.5 min. Sample preparation and the subsequent chromatographic run took < 25 min to complete. Previous studies, which used conventional LC columns, long extractions with clean-up, and subsequent derivatization were expected to take one day to analyze five samples, whereas with this new, simplified, faster method, a batch of 20 samples can be completed in the same time.

Reference

S. Özcan, H. Şenyuva, Improved and simplified liquid chromatography/atmospheric pressure chemical ionization mass spectrometry method for the analysis of underivatized free amino acids in various foods, *J. Chromatogr. A* (2006), Doi:10.1016/j.chroma.2006.09.039

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Agilent Technologies

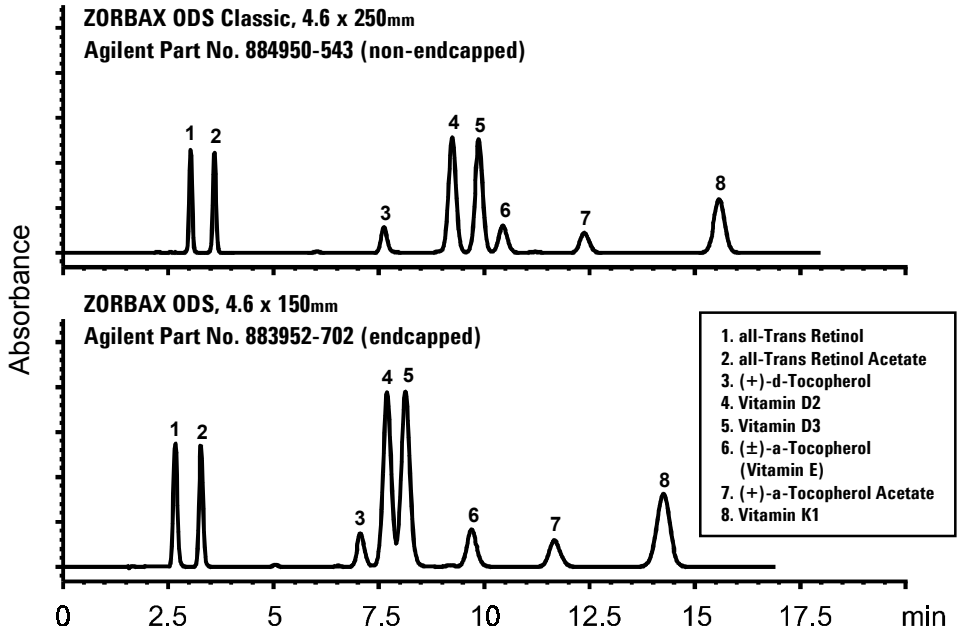
Separation of Vitamin D2 from D3 and Other Fat-Soluble Vitamins

Application

Food Analysis

Robert D. Ricker

Analysis of fat-soluble vitamins is of widespread importance in clinical diagnosis, general research, and food product analysis. Separation of vitamins D2 and D3 is particularly difficult. Change in retention and resolution of these D vitamins and others is shown for both a classic, non-endcapped Agilent ZORBAX ODS column and its endcapped counterpart. Endcapping of bonded phases is performed in an effort to minimize secondary interaction of polar sample components with the column packing.



Conditions:
LC: Hewlett Packard HP 1050
Mobile Phase: 75% ACN : 25% MeOH
Det.: UV: 325 nm for 4 min., 280 nm after
Flow: 1.0 mL/min.; 40°C
Inj Vol: 5 µL (10 µg / µL)

Highlights

- *The non-endcapped ZORBAX ODS column outperforms the endcapped ODS in obtaining resolution of vitamins D2 and D3.*
- *Endcapped and non-endcapped versions of the ZORBAX ODS column are both able to separate a wide variety of fat-soluble vitamins; although, with differing selectivities (see peak pairs 3-4 and 5-6).*

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

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Agilent Technologies

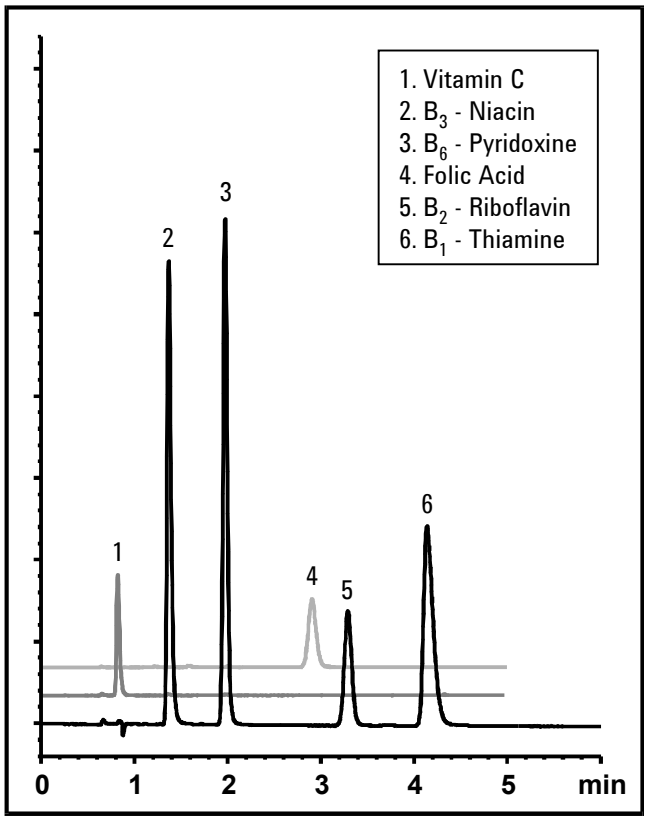
High-Speed Separation of Water-Soluble Vitamins with Ion-Pairing Chromatography

Application

Food Analysis

Robert Ricker

The rapid separation and detection of vitamins is becoming increasingly important to both food and pharmaceutical industries, as well as basic research. Water-soluble vitamins can be easily separated using reverse-phase ion-pairing chromatography. Four B vitamins, folic acid and vitamin C were analyzed in less than 5 minutes using a 75 mm SB-C8 column.



Conditions:
ZORBAX SB-C8 (4.6 x 75 mm, 3.5µm) (Agilent P/N: 866953-906)
Mobile Phase: 10mM Hexane Sulfonate with 0.1% Phosphoric Acid:MeOH (74:26)
Injection volume 10 µL, 1 mL/min, Ambient, Detect: UV(245 nm)

Highlights

- *Short run times and excellent peak shape of water-soluble vitamins are achieved using a low-pH mobile phase and a short (75 mm) ZORBAX SB-C8 column with small-particle packing (3.5 µm).*
- *ZORBAX StableBond technology offers reproducibility and column stability even at low pH (0.1% phosphoric acid) and with ion-pairing agents.*

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

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Agilent Technologies

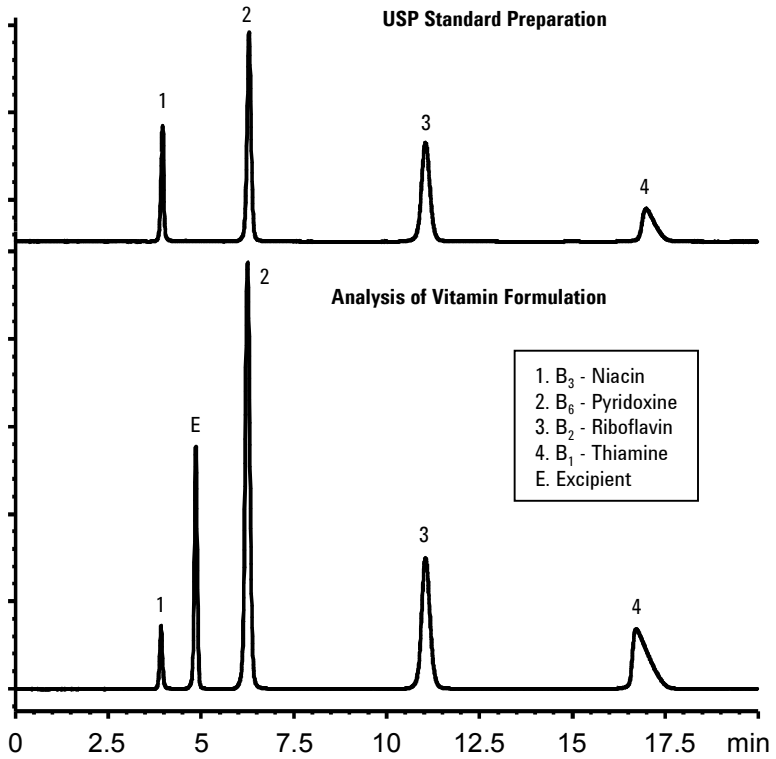
Separation of Water-Soluble Vitamins Using the USP 23 Method

Application

Food Analysis

Robert Ricker

Recently, USP-recommended methods for vitamin analysis have become increasingly important. Analysis and quantitation of water-soluble vitamins using USP-recommended chromatographic methods can become difficult when column performance is questionable. Routine analyses performed by analysts in the pharmaceutical industry require the use of reproducible and stable columns.



Conditions:
ZORBAX SB-C18 (L1 packing) (4.6 x 250 mm) (Agilent P/N: 880975-902)
Mobile Phase: 7.2 mM Hexane Sulfonate:MeOH:Acetic Acid (73:27:1) (ratio to 101%)
Injection: 20 µL, 1 mL/min, 30°C, Detect. UV(280 nm)

Highlights

- *StableBond columns provide analysts with long-term stability and reliability for the demands of USP-generated methods.*
- *Lot-to-lot reproducibility of StableBond column packings assures the chromatographer of consistency in USP-generated methods.*
- *Details of the USP Method appear on page 2168 of USP 23.*

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

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Agilent Technologies

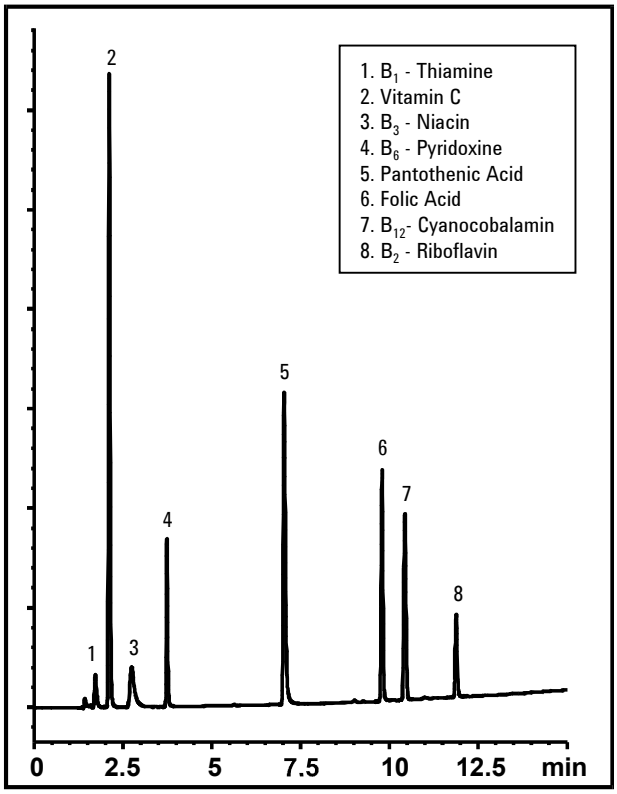
Separation of Water-Soluble Vitamins Using Reversed-Phase Chromatography

Application

Food Analysis

Robert Ricker

The analysis and quantitation of water-soluble vitamins has recently become an area of interest in the pharmaceutical industry. An alternative to routine isocratic separation, the use of a reversed-phase gradient (without ion pairing), allows analysis of an 8-component sample containing B vitamins, pantothenic acid, folic acid, and vitamin C. A ZORBAX SB-C8 column was used for the analysis.



Conditions:
ZORBAX SB-C8 (4.6 x 150 mm) (Agilent P/N: 883975-906)
Mobile Phase: A=50mM Sodium Phosphate, pH 2.5:MeOH (90:10)
B=50mM Sodium Phosphate, pH 2.5:MeOH (10:90); Gradient 0-70% B/18 min.
Injection: 10 µL, 1 mL/min, Ambient, Detect. UV(245 nm)

Highlights

- *High-speed gradient analysis of 8 water-soluble vitamins is achieved using a short (150 mm) ZORBAX SB-C8 column and a low-pH mobile phase.*
- *Gradient reversed-phase separations produce good peak shape for vitamin B_{12} and pantothenic acid in addition to typical water-soluble vitamins.*
- *StableBond packings offer reproducibility and stability at low pH.*

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Agilent Technologies

Fat-Soluble Vitamins on ZORBAX XDB-C8

Application

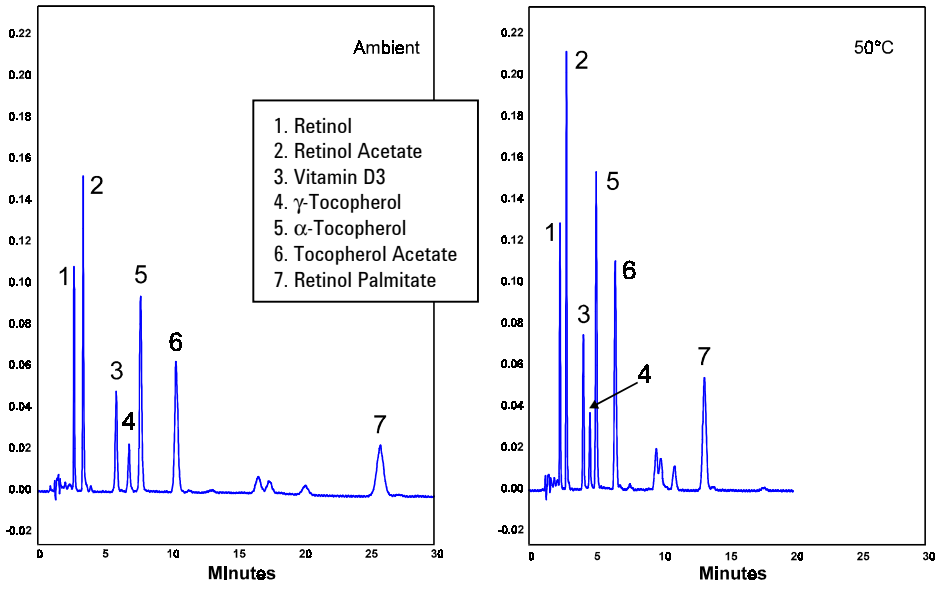
Food Analysis

Robert Ricker

The fat-soluble vitamins are comprised of vitamins A,D, E and K. Margarine, milk products, breakfast cereals and infant formulas are fortified with vitamins A, D and E; Vitamin K is fortified in infant formulas, only. Assays are needed to conform with nutrient-labeling regulations and to study changes in vitamin content due to storage, packaging, and processing. Normal-phase chromatography has been the chromatographic mode of choice. This application demonstrates a reversed-phase separation using high- organic concentration (>90% methanol) on a ZORBAX XDB-C8 column. By using reversed-phase chromatography for water-soluble and fat-soluble vitamins, labs requiring both assays can now minimize changeover time. The extra-dense bonding and double endcapping of ZORBAX Eclipse packings (e.g., XDB- C8) shield ionized silanols that may interact with analytes during high-organic, reversed-phase separations.

Highlights

- *Methods for normal-phase chromatography of fat-soluble vitamins can be replaced by high-organic, reversed-phase separation.*
- *Operation at high temperature results in reduced elution times, peak widths, and run-times.*



Conditions:
ZORBAX XDB-C8 (4.6 x 150mm) (Agilent P/N: 993967-906)
Mobile Phase: (5:95) water : MeOH
Injection 5µL, 1 mL/min, Detect. UV (280 nm)

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Agilent Technologies

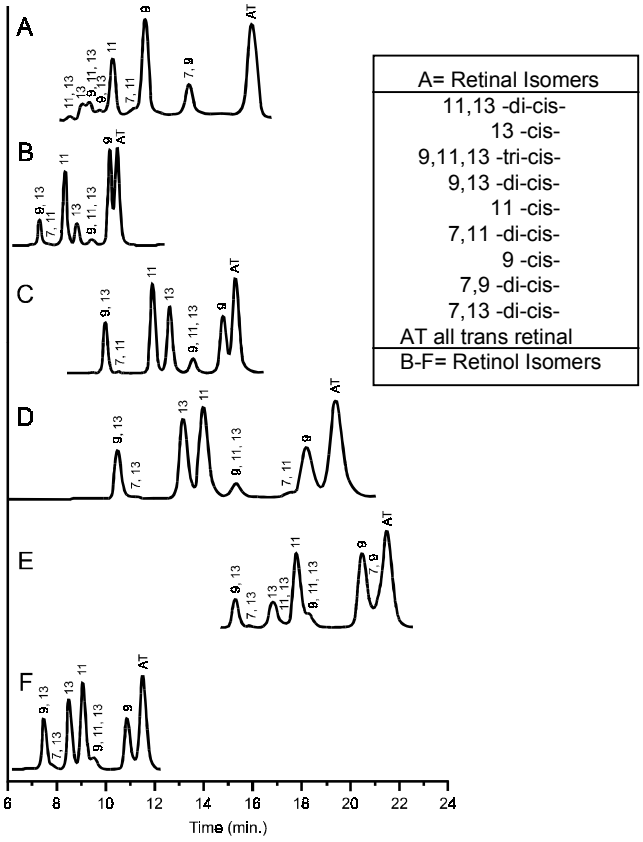
Separation of Retinal (Vitamin A) Isomers: Comparison of Mobile-Phase Composition

Application

Food Analysis

Robert Ricker

HPLC separation of vitamin A isomers (retinal isomers) is important in the analysis of various food stuffs, metabolism of visual pigments, liver function, and the corpus luteum. HPLC has traditionally been carried out using 1,4-dioxane. Efforts here were to find a more friendly solvent and a column that provides good chromatographic performance.



Courtesy of Dr. G. Nöll Physiologisches Inst. -- Justus Liebig Uni. Giessen

Conditions:
ZORBAX Sil (4.6 x 250 mm) (Agilent P/N: 880952-701)
Injection volume 20µL, 25°C

Highlights

- *Various solvent combinations can be used in conjunction with ZORBAX Sil to provide adequate resolution of Vitamin A isomers.*
- *ZORBAX Sil silica provides good peak shape for these retinoids.*

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Chromatogram	Mobile-Phase	Ratio (V/V)	Flow Rate mL/min	Pressure (bar)	λ nm	All Trans at min
A. retinal	n-hexane/t-BME	97:3	2	58	371	15.94
B. retinol	n-hexane/1,4-dioxane	93:7	2	54	320	10.07
C. retinol	n-hexane/1,4-dioxane	94:6	2	67	325	14.90
D. retinol	n-hexane/BME	93:7	4	118	325	19.29
E. retinol	n-heptane/BME	94:6	3	140	325	21.50
F. retinol	n-heptane/BME	93:7	4	187	325	11.25

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

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Agilent Technologies

Rapid Analysis of Water-Soluble Vitamins with Extraction from Cat Food

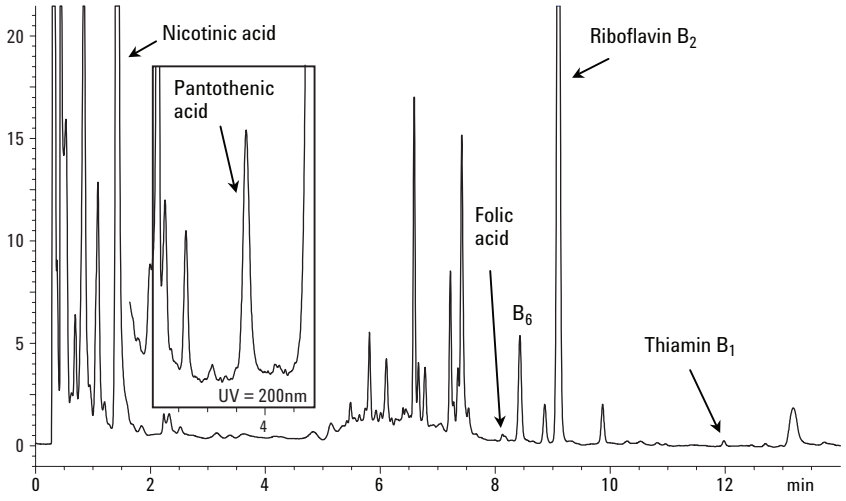
Application

Food and Flavors

Robert D. Ricker*
Thomas Schellenberger**

The qualitative and quantitative analysis of vitamins is of increasing importance in both the medical and food-related sciences. Such analyses may be carried out for vitamins found in a variety of matrices, including tablets, feed and foodstuffs, pharmaceuticals and cosmetics, and beverages. The analysis of water-soluble vitamins in pet foods is important for ensuring that products have met internal (corporate) as well as government quality standards. The following work describes the analysis of water-soluble vitamins in cat food but is applicable to analysis of these vitamins from many sources.

The goal was to develop a high-throughput analysis of essential water-soluble vitamins in cat food.



Conditions

Instrument	Agilent 1100 3D HPLC System
Column	Agilent Zorbax SB-C18, 4.6 × 75 mm, 3.5 µm particle size, part no: 866953-902
Flow	1.9 mL/min
Temperature	Ambient
Injection volume	20 µL
Detection	UV at 275 nm plus spectra

Highlights

- Six water-soluble vitamins can be analyzed and sensitively detected within their background matrix after extraction from cat food, within 20 minutes.
- Agilent Zorbax 3.5 µm packing materials in shorter columns (75 mm were used here) provide rapid, high-resolution analysis.
- Shorter columns allow high flow rates that result in rapid isocratic or gradient analysis.

Mobile Phase

A = 1L H_2O, 2.5 g hexane sulfonic acid, 2.5 mL HOAc, 4 g NaH_2PO_4, 50 µL triethylamine

B = 600 mL H_2O, 2.5 g hexane sulfonic acid, 2.5 mL HOAc, 50 µL triethylamine, 400 mL ACN

Gradient Timetable

Time (min)	% Solvent B
0.00	0.0
3.60	0.0
6.00	20.0
13.50	55.0
14.40	90.0
16.50	90.0
16.80	0.0
20.00	0.0

Sample Preparation

The total content of one can (125 g) of cat food is homogenized using a kitchen mixer or by mashing. Of the total sample, 10.0 g are transferred into a 250 mL brown glass flask. Approximately 120 mL saturated Titriplex-V solution (Titriplex-V dissolved in MeOH/Water, HPLC-grade) is added and the sample is stirred for exactly 5 minutes in a water bath at 90°C. The measuring flask is filled nearly to the mark with saturated Titriplex-V solution and cooled to room temperature. After filling to the mark with saturated Titriplex-V solution, the sample is mixed and filtered using a syringe-filter (for example, hydrophilic Teflon membrane, pore size 0.45 µm). An aliquot is placed in the Agilent 1100 autosampler using appropriate vials and 20 µL are injected.

Water-Soluble Vitamins Detected

1. Nicotinic acid
2. Pantothenic acid
3. Folic acid
4. Pyridoxine B_6
5. Riboflavin B_2
6. Thiamine B_1

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

** Thomas Schellenberger is a manager of the analytical laboratory at the fine chemical division at BASF AG, Ludwigshafen, Germany.

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Agilent Technologies

Analysis of Fat-Soluble Vitamins by HPLC

Udo Huber

Pharmaceutical

Vitamins are compounds, which are necessary to maintain a healthy and properly functioning human organism. A few milligrams a day are enough to regulate the utilization of the nutrients, such as carbohydrates, fats, proteins and minerals. Usually vitamins are supplied in food.

Figure 1 shows the separation of the three fat-soluble vitamins retinol (A), cholecalciferol (D_3) and α-tocopherol (E) using gradient analysis on a reversed phase column and UV detection. The autosampler temperature was set to 4 °C to avoid decomposition of the samples.

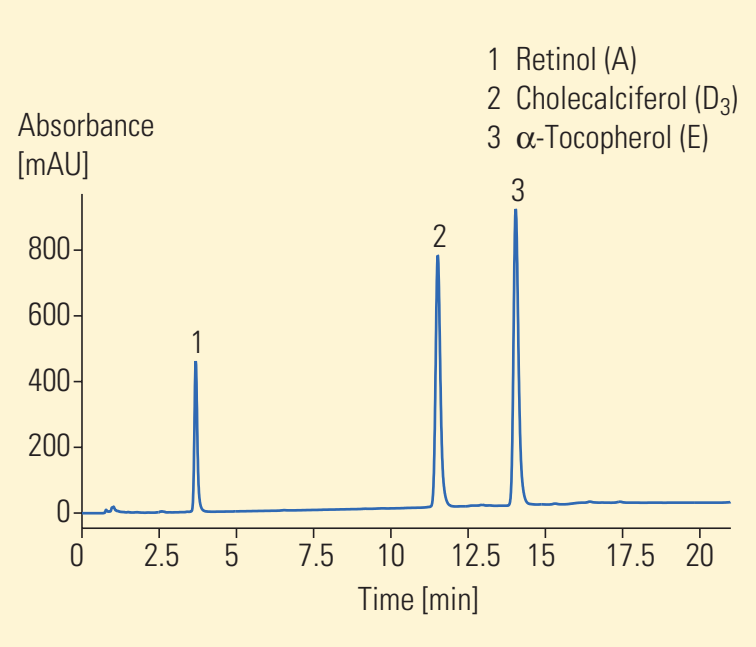

Figure 1
Analysis of three fat-soluble vitamins

Conditions

Column
4.6 x 75 mm Zorbax Eclipse XDB-C18, 3.5 µm
Mobile phase
A = water, B = methanol
Flow rate
1.0 ml/min
Gradient
at 0 min 90 % B
at 15 min 100 % B
at 20 min 100 % B
Column wash
at 21 min 90 % B
UV detector
variable wavelength detector
210 nm, standard cell
Column compartment temperature
20 °C
Stop time
21 min
Post time
5 min
Injection volume
5 µl

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HPLC Performance

Compound	LOD for S/N=2 (mg/l)*	Precision of RT (RSD of 10 runs) (1000 mg/l)*	Precision of Area (RSD of 10 runs) (1000 mg/l)*
Retinol	4.0	0.10	0.14
Cholecalciferol	2.5	0.04	0.16
α-Tocopherol	2.0	0.04	0.20

* Injection volume: 5 µl

The performance of the HPLC method is shown in the table above.

The HPLC method presented here shows an easy but reliable and precise analysis of the fat-soluble vitamins retinol (A), cholecalciferol (D_3) and α-tocopherol (E). The values for LOD, precision of RT and precision of area show the good performance of the analysis.

Equipment

Agilent 1100 Series

- Quaternary pump (includes vacuum degasser)
- Thermostatted autosampler
- Thermostatted column compartment
- Variable wavelength detector, standard flow cell 10-mm path length, 13-µl cell volume

Alternative:

- Binary pump
- Vacuum degasser
- Diode array detector standard flow cell 10-mm path length, 13-µl cell volume
- Agilent ChemStation + 3D software

Columns

- Zorbax Eclipse XDB, 3. 5 µm, 4.6 x 75 mm (Agilent part number 7995118-344)
- *Recommended:* Guard cartridges Zorbax Eclipse XDB-C18, 5 µm, 4 x 4 mm (Agilent part number 7995118-504, 10/pk)

Note:

Since the method was specifically developed on the Agilent 1100 Series system you might not be able to reproduce this analysis on an older system or even on a new system with lower performance. To avoid sample decomposition it is necessary to use a cooled autosampler, for example, the Agilent 1100 Series thermostatted autosampler.

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HPLC Analysis of Vitamins in Tablets using HPLC

Angelika Gratzfeld-Huesgen

Food

Abstract

Fat-soluble vitamins, such as vitamins E, D, and A, and water-soluble vitamins, such as vitamins C, B_6, B_2, B_1, and B_{12}, have been analyzed.

Vitamins are biologically active compounds that act as controlling agents for an organism's normal health and growth. The level of vitamins in food may be as low as a few micrograms per 100 g. Vitamins often are accompanied by an excess of compounds with similar chemical properties. Thus not only quantification but also identification is mandatory for the detection of vitamins in food. Vitamins generally are labile compounds that should not exposed to high temperatures, light, or oxygen. HPLC separates and detects these compounds at room temperature and blocks oxygen and light.[1] Through the use of spectral information, UV-visible diode-array detection yields qualitative as well as quantitative data. Another highly sensitive and selective HPLC method for detecting vitamins is electrochemical detection.

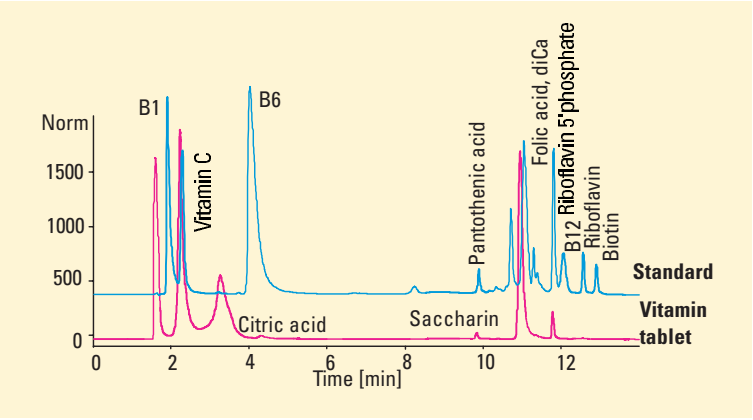

Figure 1
Analysis of water-soluble vitamins in a vitamin tablet

Conditions

Column 100 ˘ 4 mm Hypersil BDS, 3 µm
Mobile phase
A= water with pH = 2.1 (H_2SO_4) = 99%
B = ACN + 10% A = 1 %
Gradient
at 3.5 min 1% B; at 11 min 25% B
at 19 min 90% B
Post time 6 min
Flow rate 0.5 ml/min
Column compartment 30 ºC
Injection vol 2–5 µl
Detector
UV-DAD detection wavelength 220/30 nm, reference wavelength 400/100 nm

Sample preparation
Filtration

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Sample preparation

Different food matrices require different extraction procedures.[1] For simple matrices, such as vitamin tablets, water-soluble vitamins can be extracted with water in an ultrasonic bath after homogenization of the food sample.

Chromatographic conditions

The HPLC method presented here was used to analysis vitamins in a vitamin drink.

HPLC method performance

Limit of detection
<500 pg (injected amount),
S/N = 2

Repeatability of
RT over 10 runs <0.2 %
areas over 10 runs <2 %

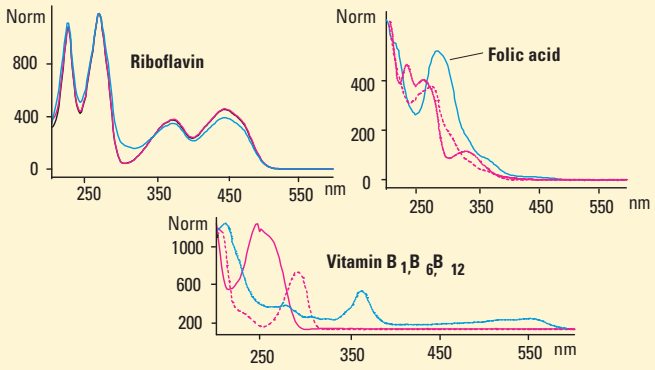

Figure 2
Analysis of carbohydrates in corn extract

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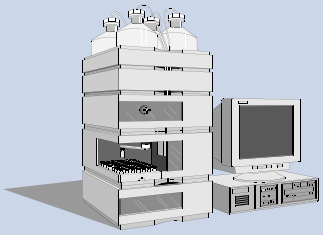
Equipment

Agilent 1100 Series
- vacuum degasser
- quaternary pump
- autosampler
- thermostatted column compartment
- diode array detector

Agilent ChemStation + software



Rainer Schuster is application chemist at Agilent Technologies, Waldbronn, Germany.

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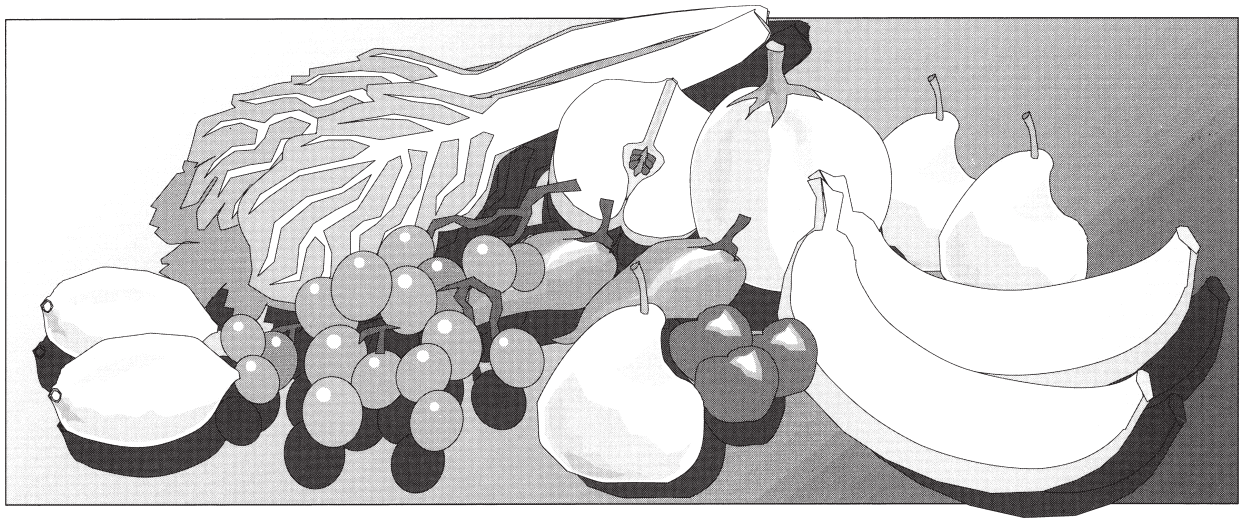
Analysis of Selected Vitamins with HPLC and Electrochemical Detection

Application Note

Food and Clinical

Angelika Gratzfeld-Hüsgen,
Rainer Schuster,
Wolfgang Haecker



Several oxidizable vitamins (vitamin A, B_6, C, D_3 and E) were analyzed using HPLC and electrochemical detection. Excellent sensitivity was achieved with all vitamins. The minimum detectable level for vitamin A-palmitate was about 80 pg, for β-carotene about 50 pg, for vitamin B_6 about 30 pg, for vitamin C below 1 pg, for vitamin D_3 about 70 pg and for vitamin E about 30 pg. The optimum potentials were evaluated using an automated increment for the potentials. The reproducibility was tested using vitamin B_6 and was found to be 3% RSD of peak height for 100 runs. Due to the high selectivity of electrochemical detection, it was possible to keep sample preparation very simple.

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Introduction

In the last decade, liquid chromatography (LC) with electrochemical detection (ECD) has been used more and more for the determination of electroactive compounds at trace levels and in complex matrices.

For the analysis of electroactive vitamins in food, pharmaceutical preparations, tissue and body fluids, the combination of HPLC and ECD provides high sensitivity and excellent selectivity [1,2,7,8,9,11]. Electroactive vitamins can be determined in the low pg range and moreover, fewer matrix effects are encountered. This enables sample preparation and enrichment to be simplified and reduced [11].

Despite the high sensitivity and selectivity of electrochemical detection for vitamin analysis, it is not widely used. It has a reputation for low response stability due to the fact that the electroactive surface of the detector cell is in direct contact with the mobile phase and all the compounds and products of the oxidative and reductive reaction. This can result in contamination of the working electrode and an unstable response. The response instability can be minimized by using modern electrochemical detectors with electrochemical self-cleaning routines [19].

This note describes a method for analyzing oxidizable vitamins with high sensitivity and reproducibility using a modern electrochemical detector.

Experimental

We used Agilent 1049A electrochemical detector with HP 1050 Series isocratic pump and autosampler. The chromatographic conditions for each experiment are listed with the appropriate figures.

Results and discussion

Analysis of vitamin A (retinol), provitamin A (β-carotene) in fruit juice

Vitamin A is one of the fat-soluble vitamins and is of importance for normal growth and development of the human body.
Malabsorbtion results in disease of the liver [1] and an insufficiency results in extreme dryness of the skin and nightblindness [12]. Sources of vitamin A are liver, fish oil, milk, butter and the yolk of eggs. A further source is provitamin A (β-carotene), which is present in many plants and is converted in the human body to retinol (vitamin A_1).

The analysis of vitamin A is of interest for quality control of food and pharmaceutical products and for diagnosis and therapeutic reasons in clinical routine testing and medical research.

Measurement of this vitamin is nowadays based on HPLC methods [10] using UV-Visible, fluorescence or electrochemical detection. The analysis of vitamin A and/or β-carotene with electrochemical detection has several advantages over UV-Visible or fluorescence detection. UV detection shows a lack of sensitivity and selectivity [1, 2, 4], whereas fluorescence detection normally offers sufficient sensitivity but sometimes (depending on the matrix) insufficient selectivity [1,3].

Using an electrochemical detector, the minimum detectable level for vitamin A-palmitate was about 80 pg and for β-carotene about 50 pg with a signal-to-noise ratio of 2, (figure 1).

In figure 1 the analysis of vitamin A-palmitate and β-carotene is shown. 0.5 µl of a standard mixture was injected and the minimum detectable level was calculated. In order to measure at optimum sensitivity or at the highest possible selectivity, it is necessary to evaluate the optimum detection potential for each compound of interest. This can be done using built-in software routines (auto-increment mode).

The sample is injected as often as specified between various selected potentials. The voltage difference between these potentials can also be determined by the user.

Column	125 x 4 mm Lichrospher RP18, 5 µ (Part No. 799250-564)
Mobile phase	Methanol + 5 g/l lithiumperchlorate + 1 g/l acetic acid
Flow rate	1.5 ml/min
Oven temperature	30 °C
Injection volume	0.5 µl

Detector parameters

Operation mode	Amperometry
Potential	1 V
Range	0.5 µA
Reference electrode	AgCl/KCl
Response time	8 s.

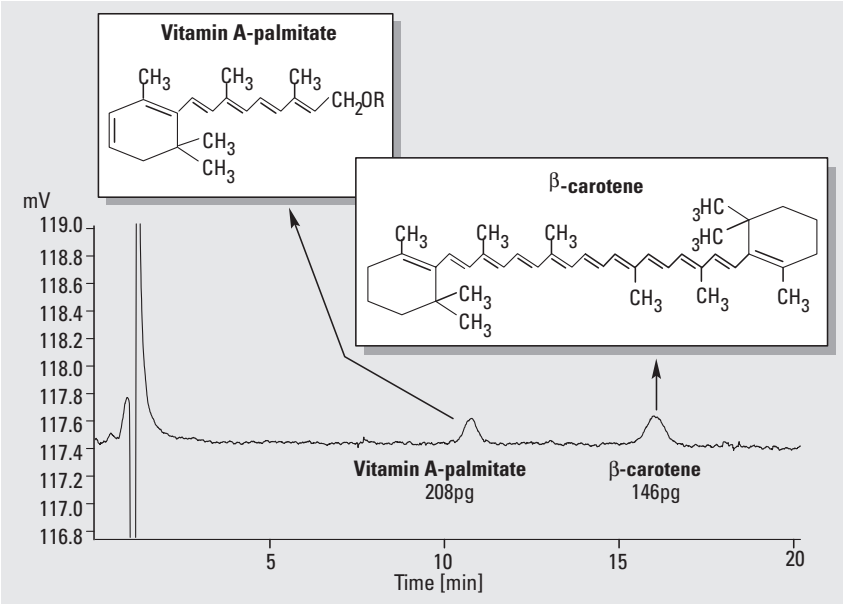


Figure 1
Sensitivity of electrochemical detection with vitamin A and β-carotene

Figure 2 shows the result of the automated optimization procedure. At 1 V the optimum signal-to-noise ratio was obtained. Further increase of the oxidative potential did not increase the signal height but produced more noise and drift, and so no better signal-to-noise ratio could be achieved.

The optimization procedure also showed how easily β-carotene can be oxidized. Oxidation started at 0.5 V. This can be of advantage when high selectivity is more important than high sensitivity.

For the analysis of vitamins in food, the main problem is often not only sensitivity but also selectivity [13-17]. The high selectivity of electrochemical detection means that problems due to the matrix or coeluting peaks can be avoided without time-consuming sample preparation. For example, β-carotene in fruit juice can be analyzed by direct injection into an HPLC instrument, figure 3.

Figure 3 shows that, with 1 µl injection volume and no sample preparation, β-carotene in fruit juice was easily determined. The concentration of β-carotene was found to be 2.7 mg/100 ml fruit juice.

Column	125 x 4 mm Lichrospher RP18, 5 µ (Part No. 799250-564)
Mobile phase	Methanol + 5 g/l lithiumperchlorate + 1 g/l acetic acid
Flow rate	1.5 ml/min
Oven temperature	30 °C
Injection volume	3 µl
Detector parameters	
Auto-increment parameters	
Start potential	0.4 V
End potential	1.1 V
Increment	0.1 V

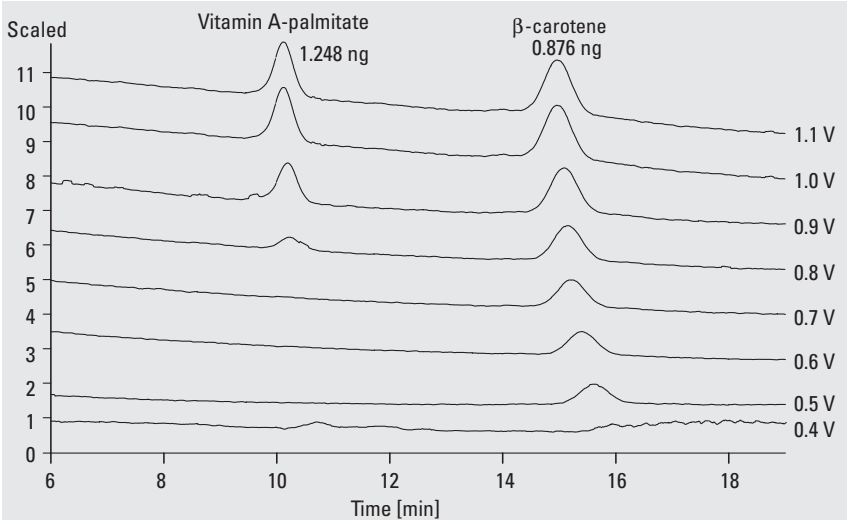


Figure 2
Optimization of detection potentials

Column	125 x 4 mm Lichrospher RP18, 5 µ (Part No. 799250-564)
Mobile phase	Methanol + 5 g/l lithiumperchlorate + 1 g/l acetic acid
Flow rate	1.5 ml/min
Oven temperature	30 °C
Injection volume	1 µl
Detector parameters	
Operation mode	Amperometry
Potential	1 V
Range	0.5 µA
Reference electrode	AgCl/KCl
Response time	8 s.

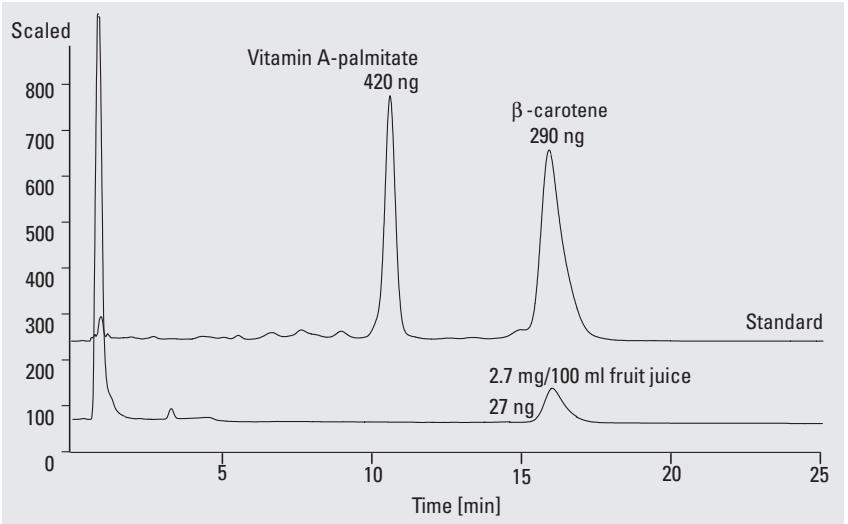


Figure 3
Analysis of β-carotene in fruit juice

Analysis of vitamin B_6 (pyridoxine hydrochloride) in a pharmaceutical vitamin preparation

Vitamin B_6 is a water-soluble vitamin and consists of three interconvertible forms (pyridoxine hydrochloride, pyridoxamine dihydrochloride and pyridoxal hydrochloride). Vitamin B_6 is of great importance for the nervous system [5, 6]. In food, it is present in liver, bananas, wheat and vegetables.

Whereas other methods show lack of sensitivity and selectivity, e.g. the minimum detectable level with HPLC and fluorescence detection is in the low ng range [5], a combination of HPLC and electrochemical detection enables the analysis of vitamin B_6 in the pg range (30 pg with a signal-to-noise ratio of 2), figure 4.

Figure 4 shows, that the determination of vitamin B_6 is possible even when vitamin C is present in large quantities. The sample preparation was kept very simple: 10 µl of the vitamin preparation was diluted with 1 ml distilled water and 0.5 µl of the liquid was injected. The vitamin concentration was 804.16 mg/l vitamin preparation.

In order to evaluate the reproducibility of this type of analysis, 5 µl (450 pg) of the standard sample were injected. The reproducibility was about r.s.d. = 3% for peak heights and about 5% for area counts over 100 runs.

Column	125 x 4 mm Lichrospher RP18, 5 µ (Part No. 799250-564)
Mobile phase	Water + 0.02M KH_2PO_4 + 0.03M tetrabutylammoniumhydrogensulfate + 0.03M heptanesulfonic acid + 2% acetonitrile
Flow rate	0.8 ml/min
Oven temperature	30 °C
Injection volume	Standard 1µl Sample 0.5 µl
Detector parameters	
Working electrode	Glassy carbon
Operation mode	Amperometry
Potential	1.2 V
Range	0.5 µA
Reference electrode	AgCl/KCl
Response time	1 s

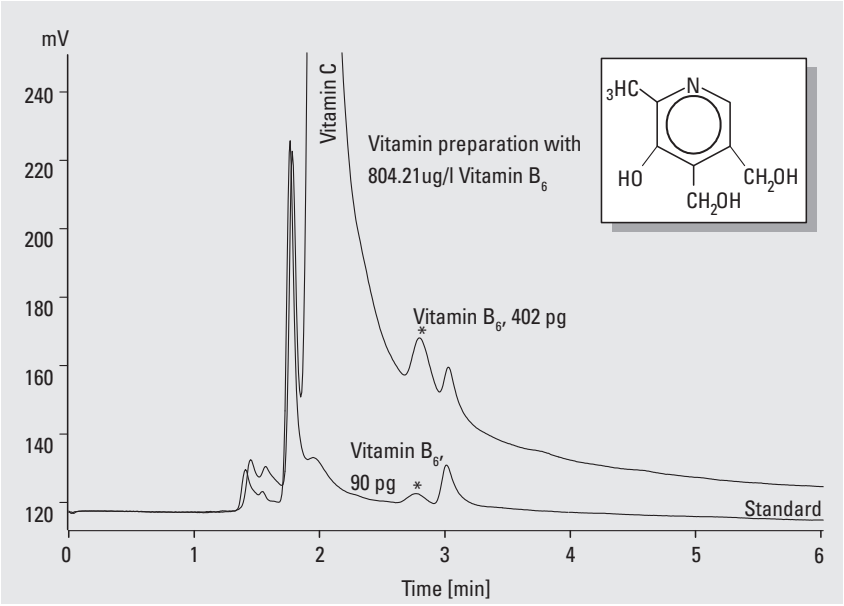


Figure 4
Analysis of vitamin B_6 in a vitamin preparation for chicken feed

Analysis of vitamin C (ascorbic acid) in fruit juice

Vitamin C belongs to the water-soluble vitamins and is of vital importance for mammalian health. Lack of this vitamin is responsible for scorbutus (scurvy) and in former times many people (specially sailors) died because of this deficiency disease. Vitamin C is present in many fruits and vegetables [12].

The analysis of vitamin C in food is mainly performed for quality control purposes. Determination of this vitamin in tissue and body fluids is mainly carried out to increase knowledge of its physiological role in mammalians [7].

There are several methods available for the analysis of vitamin C, but most of them show lack of sensitivity and/or selectivity. Tedious sample preparation steps are needed to remove interfering compounds [7,11,18]. HPLC, in combination with UV-Visible detection for example, shows insufficient sensitivity for the analysis of vitamin C in tissue and body fluids [7,11]. The minimum detectable level for this method is about 4 ng.

Using HPLC and electrochemical detection, the minimum detectable level for vitamin C is below 1 pg. (Due to the low stability of vitamin C, the determination of the minimum detectable level must be done directly after dilution of freshly prepared stock solutions.) The analysis of vitamin C in food such as fruit and fruit juices, is mainly done to control the freshness and proper storage of these products.

Figure 5 shows the chromatogram of a fruit juice which contained 43.16 mg vitamin C in 100 ml fruit juice. The fruit juice was prefiltered, 10 times diluted and 1 µl of the diluted sample was injected. The standard solution contained 1 mg vitamin C in 100 ml water.

Column	100 x 4.6 mm ODS Hypersil, 5 µ (Part No. 79916OD-554)
Mobile phase	Water + 5.4 g/l Na acetate-trihydrate + 3g/l tetrabutylammonium-hydrogensulfate + 3 ml KCl solution (3.3 m), pH 5 with acetic acid.
Flow rate	0.8 ml/min
Oven temperature	30 °C
Injection volume	1 µl
Detector parameters	
Working electrode	Glassy carbon
Operation mode	Amperometry
Potential	0.6 V
Range	0.5 µA
Reference electrode	in-situ reference electrode AgCl/KCl in mobile phase
Response time	8 s

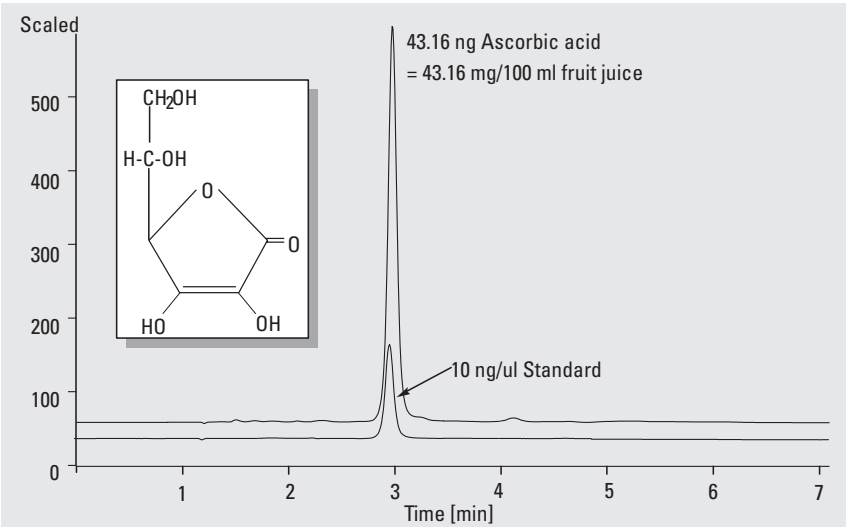


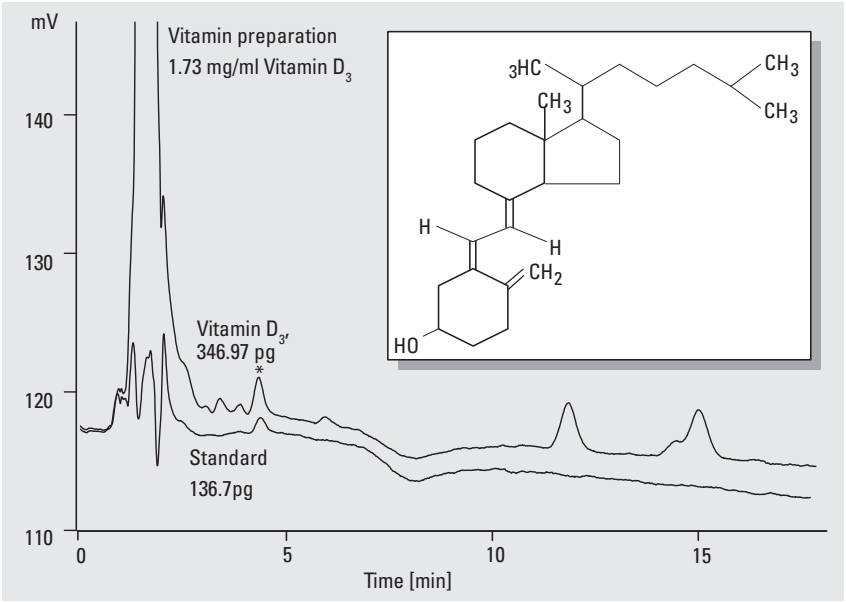
Figure 5
Analysis of vitamin C (ascorbic acid) in fruit juice

Analysis of vitamin D_3 (colcalciferol) in a multi-vitamin preparation

Vitamin D_3 belongs to the fat-soluble vitamins and is of great importance for the metabolism of bone. Lack of this vitamin results in bone deformation from rachitis (rickets).

The determination of vitamin D_3 in pharmaceutical preparations, tissue and body fluids plays an important role in quality assurance procedures and in the understanding of bone metabolism [8]. The methods in use are generally time-consuming, involving several sample preparation steps and some of them are not sufficiently sensitive or selective [8]. The combination of HPLC and electrochemical detection offers excellent sensitivity. The minimum detectable level is about 70 pg with a signal-to-noise ratio of 2.

Figure 6 shows the analysis of vitamin D_3 in a highly viscous liquid multivitamin solution, which is used in poultry farms. Due to the excellent selectivity of electrochemical detections, sample preparation could be kept very simple. 40 µl of the vitamin liquid was shaken with 1 ml of cyclohexane for 1 min and 5 µl of the supernatant liquid were injected. The vitamin D_3 concentration in this preparation was found to be 1,73 mg/l.



Column	125 x 4 mm Lichrospher
RP18, 5 µ	
	(Part No. 799250-564)
Mobile phase	Methanol + 5 g/l lithiumperchlorate + 1 g/l acetic acid
Flow rate	1 ml/min
Oven temperature	30 °C
Injection volume	5 µl

Detector parameters

Working electrode	Glassy carbon
Operation mode	Amperometry
Potential	1.3 V
Range	0.5 µA
Reference electrode	AgCl/KCl
Response time	8 s

Figure 6
Analysis of vitamin D3 in a pharmaceutical multivitamin preparation

Analysis of vitamin E (α-tocopherol) in vitamin E capsules

Vitamin E belongs to the fat-soluble vitamins. In mammalians it fulfills the function of a lipid-soluble antioxidant and is necessary for the maintenance and protection of neuronal, muscular and reproductive tissue [9].

The analysis of vitamin E is of interest in the food and cosmetic industry, see for example, the analysis of this vitamin in butter or milkpowder [10] or in beauty creams, where it is used as an antioxidant [12]. The analysis of vitamin E in tissue has become more and more important, because there is a possibility that vitamins A and E may act as cancer-chemopreventive agents [4].

Measurements of plasma vitamin A and E provides an indication of the proper absorbtion of these two compounds. Malabsorbtion of these vitamins occurs in cystic fibrosis and in cholestative liver diseases [1].

The analysis of tocopherols with HPLC and electrochemical detection also offers more sensitivity and selectivity compared with HPLC and UV-Visible or fluorescence detection, figure 7 [9].

A standard solution of 100 pg in 1 µl (figure 7) was injected and the minimum detectable limit was calculated. It was found to be about 30 pg for a signal-to-noise ratio of 2.

Column	125 x 4 mm Lichrospher RP18, 5 µ (Part No. 799250-564)
Mobile phase	Methanol + 5 g/l lithiumperchlorate + 1 g/l acetic acid
Flow rate	1 ml/min
Oven temperature	30 °C
Injection volume	1 µl

Detector parameters

Working electrode	Glassy carbon
Operation mode	Amperometry
Potential	0.9 V
Range	0.5 µA
Reference electrode	AgCl/KCl
Response time	8 s

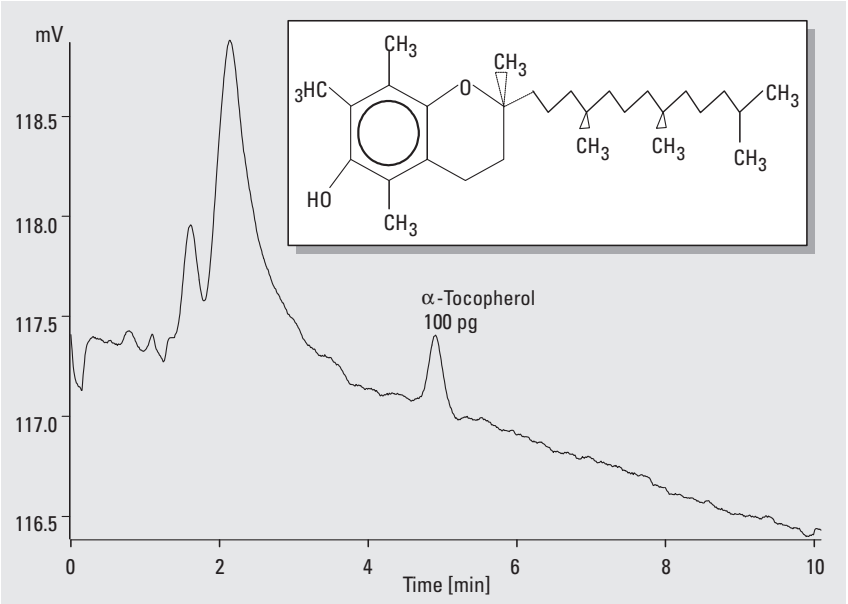


Figure 7
Sensitivity of tocopherol analysis with electrochemical detection

In figure 8, the analysis of α-tocopherol and traces of other tocopherol isomers in a pharmaceutical preparation is shown.

For sample preparation the capsule was cut and extracted with 5 ml of methanol in an ultrasonic bath for 10 minutes. 3 µl of the supernatant liquid was injected. The concentration of a-tocopherol was found to be 270 mg/capsule.

The chromatogram shows that not only α-tocopherol, but also traces of other tocopherol isomers, such as beta, delta and/or gamma tocopherol, were present. Under the chromatographic conditions used, gamma and delta tocopherol could not be separated.

Column	125 x 4 mm Lichrospher RP18, 5 µ (Part No. 799250-564)
Mobile phase	Methanol + 5 g/l lithiumperchlorate + 1 g/l acetic acid
Flow rate	1 ml/min
Oven temperature	30 °C
Injection volume	3 µl
Detector parameters	
Working electrode	Glassy carbon
Operation mode	Amperometry
Potential	0.9 V
Range	0.5 µA
Reference electrode	AgCl/KCl
Response time	8 s

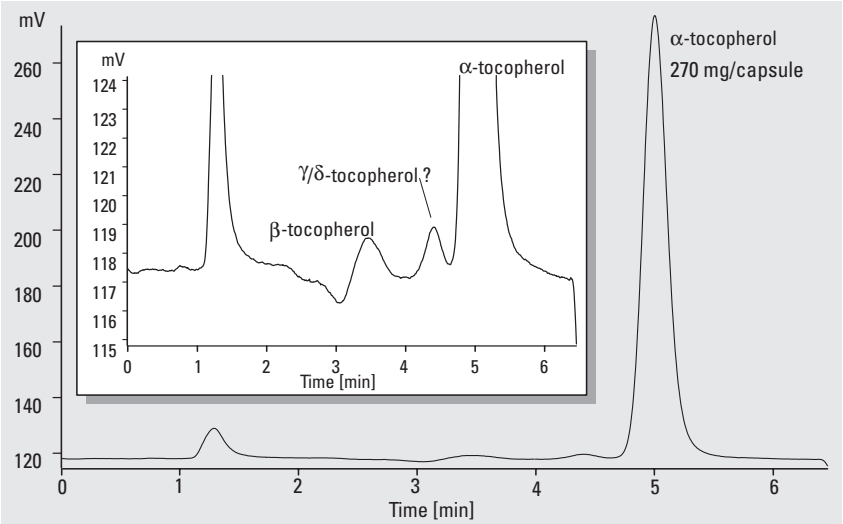


Figure 8
Analysis of tocopherols in vitamin E capsules

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Angelika Gratzfeld-Hüsgen, Rainer Schuster and Wolfgang Häcker are based at Agilent Technologies, Waldbronn, Germany.

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