ACQUITY UPC^2

FOOD APPLICATION NOTEBOOK

Expanding analytical capability of food testing laboratories

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THE EASE OF REVERSED PHASE MEETS THE POWER OF NORMAL PHASE LC

CONVERGENCE CHROMATOGRAPHY

Waters® UltraPerformance Convergence Chromatography™ (UPC²®) is a holistically designed chromatographic system that leverages the proven benefits of UPLC® Technology to modernize the traditional supercritical fluid chromatography technique.

UPC² utilizes sub-2 micron particle columns and compressed CO_2 mobile phase along with organic co-solvent and additives to achieve unparalleled resolving power and selectivity in chromatographic separation. By using CO_2, as the primary mobile phase, UPC² significantly reduces the amount of organic solvent waste in labs in support of green initiatives.

The ACQUITY UPC² System brings together the ease-of-use of reversed-phase LC and the selectivity of normal-phase LC. The miscibility of CO_2 with a variety of polar and non-polar organic solvents has made UPC² versatile enough to separate a much wider range of compounds than reversed-phase LC. UPC² enables food scientists to separate, detect, and quantify a broad range of compounds, including fat-soluble vitamins, lipids and fatty acids, structural analogs and isomers, in complex matrices with unequaled speed and confidence.

ACQUITY UPC² Technology expands the capability of food testing labs in addressing both complex and routine separation challenges.

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FAT-SOLUBLE VITAMINS

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Simultaneous Analysis of Vitamin A and D_3 in Vitamin Premixes and Concentrates by Convergence Chromatography/PDA Detection

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APPLICATION BENEFITS

- Simultaneous determination of vitamin A and D_3 in premixes and concentrates.
- Simplified sample preparation – no purification or dilution in sample preparation.
- Direct injection of sample extract – no reconstitution.
- Much lower solvent waste compared to LC.

WATERS SOLUTIONS

ACQUITY UPC2® System with PDA detection

Empower 3 Chromatography Data Software

ACQUITY UPC2 BEH Column

KEY WORDS

UPC,[2] Convergence Chromatography, fat-soluble vitamin, vitamin premix, vitamin A, vitamin D_3, retinyl acetate, cholecalciferol, ergocalciferol

INTRODUCTION

Simultaneous analysis of fat-soluble vitamins in foods is challenging due to their different properties and concentrations. A typical method involves extraction and saponification of fat, followed by high performance liquid chromatography (HPLC) with UV/Vis detection. After saponification the extracts can be analyzed for vitamin A directly, but they have to be diluted due to high abundance of the vitamin A in foods and its high molar extinction. Unfortunately, the dilution makes it impossible to detect vitamin D_3 in the same solution. To measure vitamin D_3, the extracts have to be cleaned on a semi-preparative chromatograph and concentrated. For these reasons vitamins A and D_3 had to be analyzed separately. The HPLC of these compounds suffers from a long runtime, slow equilibration, and poor reproducibility.

As the extraction and saponification of separate vitamin A and D_3 methods are identical, we investigated whether it would be possible to apply Waters® UltraPerformance Convergence Chromatography™ (UPC^2®) to analyze extract for vitamin A and D_3 in a single chromatographic run.

UPC^2 is a separation technique that uses compressed carbon dioxide as the primary mobile phase. It takes advantage of sub-2 µm particle chromatography columns, the low viscosity of CO_2, and an advanced chromatography system. This differs from traditional HPLC and improves the sensitivity of this assay. UPC^2 also generates much less solvent waste compared to conventional liquid chromatography.[1] In this application note, we report a method for analysis of vitamin A and D_3 in vitamin premixes and concentrates in one analytical run without purification or dilution. The metrological properties of the UPC^2 and the advantages of the method compared to the HPLC are also discussed.

EXPERIMENTAL

UPC² conditions

System:	ACQUITY UPC²
Detector:	PDA
Software:	Empower 3
Column:	ACQUITY UPC² BEH
	3.0 mm x 100 mm, 1.7 µm
Mobile phase A:	Compressed CO_2
Mobile phase B:	isopropanol
Wash solvent:	Methanol
Flow rate:	1.7 mL/min
APBR:	2,000 psi
Column temp.:	55 °C
Sample temp.:	10 °C
Injection volume:	7.0 µL
Detection:	UV 260 nm
Gradient:	0.5% to 20% B in 9.9 min Hold at 20% for 2 min, re-equilibrate for 3 min

Sample description

Retinyl acetate (vitamin A acetate) was purchased from Sigma-Aldrich, cholecalciferol (vitamin D_3) and ergocalciferol (vitamin D_2) were purchased from the US pharmacopeia. The vitamin premix was from DSM Nutritional Products, Switzerland. Figure 1 shows the structures of relevant compounds used in this study.

Sample preparation

1.5 g vitamin raw sample was weighed and spiked with 1 mL of vitamin D_2 solution (internal standard) into a 250-mL Erlenmeyer flask. 50 mL (±10%) ethanol was added, along with 10 mL of 50% KOH, and 2 mL of 33% sodium ascorbate. The samples were saponified for about 1 hour at 80 °C to 85 °C in a water bath. After saponification, the samples were cooled down to room temperature and extracted with a n-hexane diethyl ether mixture. The extract was washed with de-ionized water until neutral, and the extracts were made up to 50-mL with n-hexane. The solution was filtered through a 0.2 µm syringe filter into 2-mL autosampler vials and analyzed by UPC²/PDA.

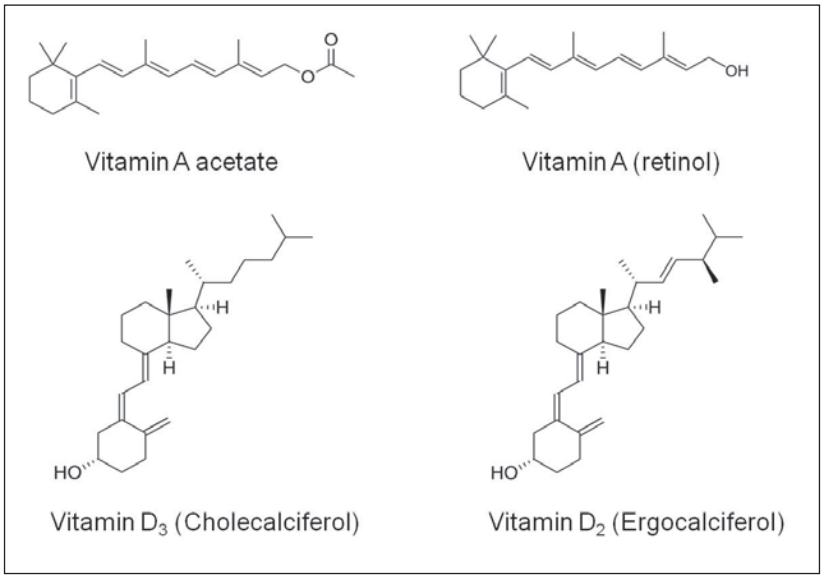

Figure 1. Structures of retinyl acetate, retinol, cholecalciferol, and ergocalciferol.

RESULTS AND DISCUSSION

Due to the fact that the molar absorbance of vitamin D_3 is low and its concentration in samples is ten times lower than vitamin A, both vitamins A and D_3 were quantified at the wavelength of the maximum absorbance of D_3. The typical chromatograms obtained at 260 nm UV wavelengths for vitamin standards in solvent are shown in Figure 2. Chromatograms of the sample extract are shown in Figure 3.

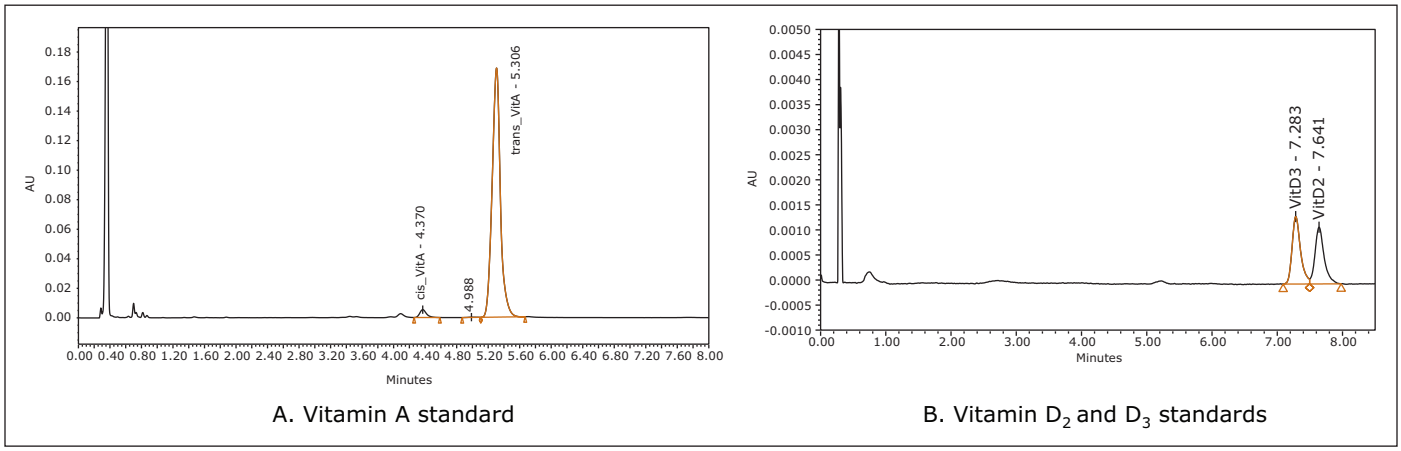

Figure 2. Chromatograms for the vitamin standard solutions at 260 nm.

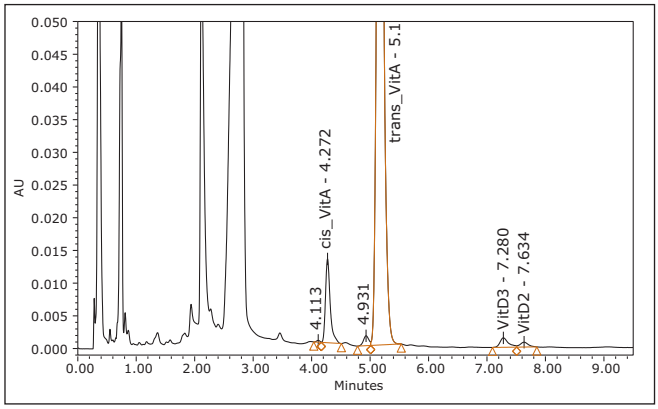

Figure 3. Chromatogram for the sample extract at 260 nm.

The linearity of the assay was investigated using standard solutions. The average peak areas of triplicate injections of seven concentrations were used. Vitamin D_2 was used as the internal standard for the quantification of vitamin D_3; there was no internal standard used for vitamin A. The coefficient of the determinations R^2 for vitamins A and D_3 were 0.9998 and 1.0000, respectively. The intermediate precision and reproducibility of the assay over a three-month period are shown in Table 1. The intermediate precision of the method for vitamin A was 5.1% and 5.7% for vitamin D_3, both falling well within the requirement of ≤8%.

Analyte	Intermediate precision, %	Requirement %,[2,3]	Product range, IU/g
Vitamin A	5.1	≤8	38,000 to 55,800
Vitamin D3	5.7	≤8	2,280 to 4,230

Table 1. Metrological properties of the method evaluated over three-month period.

To evaluate ruggedness, changes were made to the flow rate, system pressure, and column temperature to observe their effects on the results. There was no statistically significant difference to the ±2% changes on the results, and the chromatographic resolution of the critical D_2/D_3 pair was maintained above 1.6 (requirement for R ≥1.2).

The utilization of supercritical fluid chromatography using CO_2 combined with separation on sub 2-µm particle size columns provided fast and sufficient separation of the vitamins A and D within the same chromatographic run in a broad range of concentrations. The excellent selectivity of UPC^2 provided baseline separation of analytes from interfering impurity peaks and allowed quantification of the cis- and trans- retinols and the vitamin Ds at 260 nm UV in the same analytical run. Vitamins D_3 and D_2 (internal standard) were separated from each other, and eluted before the matrix peak, in contrast to our traditional method. The extracts in n-hexane can be injected directly into the system, which eliminates the solvent exchange step previously required in our laboratory.

CONCLUSIONS

A simple and cost-effective assay to analyze vitamin A and D_3 in different vitamin raw materials, premixes, and concentrates using UPC^2 has been developed and validated. The UPC^2/PDA method demonstrates excellent linearity, resolution, and repeatability. The intermediate precision of the method calculated over a three-month period was less than 6%. The introduction of the ACQUITY UPC^2 System significantly reduced the consumption of HPLC solvents. Beside CO_2, the only other solvent required was isopropanol. The consumption of isopropanol was 0.12 mL per test. The laboratory has therefore significantly reduced the consumption of HPLC solvents as well as the disposal of waste solvents. By simplifying the procedures and eliminating the repartitioning steps, the reduction of solvent usage has streamlined our workflow and decreased the potential exposure of both laboratory staff and the environment to harmful solvents.

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1. Aubin A, Analysis of Fat Soluble Vitamin Capsules using UltraPerformance Convergence Chromatograqphy UPC.^{2} Waters Application Note No. 720004394EN (2012).
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3. Standard Method Performance Requirements for Vitamin D3 in Pre-Blends, Pre-Mixes, and Pure Materials – AOAC SMPR 2012.004.

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Rapid Separation of Vitamin K_1 Isomers and Vitamin K_2 in Dietary Supplements Using UltraPerformance Convergence Chromatography with a C_{18} Column

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APPLICATION BENEFITS

- Fast and reliable separation of vitamin K_1 *trans* and *cis* isomers and MK-4 in less than three minutes.
- Separation is achieved on a C_{18} column; no special C_{30} column is needed.
- The use of carbon dioxide as the primary mobile phase minimizes organic solvent waste.

WATERS SOLUTIONS

ACQUITY UPC^2® System with the ACQUITY UPC^2 PDA Detector

ACQUITY UPC^2 HSS C_{18} SB Column

Empower® 3 CDS Software

KEY WORDS

Vitamin K_1 isomers, phylloquinone, menaquinone, menatetrenone, MK-4, UPC^2

INTRODUCTION

Vitamin K_1 (phylloquinone) is an essential human nutrient produced in plants, especially green leafy vegetables. The vitamin K_1 in natural products exists mainly as the *trans* form, while the vitamin K_1 used in food supplementation is often synthetic K_1, which may contain appreciable amounts of the *cis* form. The *trans*-vitamin K_1 is bioactive, while the *cis*-K_1 is not. It is highly desirable to separate the *trans*- and the *cis*-vitamin K_1 isomers to truly evaluate the nutritional value of the supplement ingredient. Available HPLC methods for the separation of vitamin K_1 isomers require C_{30} columns. Their typical run time is about 20 minutes, and chlorinated solvents are used in some of the methods.[1-3]

UltraPerformance Convergence Chromatography™ (UPC^2) is a separation technique that leverages the unique properties (*i.e.*, low viscosity and high diffusivity) of compressed CO_2 at or near its supercritical state, as well as sub-2 micron particle packed columns to significantly improve the separation efficiency, speed, and selectivity.[4] This application note demonstrates a fast separation of vitamin K_1 *trans* and *cis* isomers and menatetrenone (MK-4), a common form of vitamin K_2, by UPC^2 in less than three minutes on an ACQUITY UPC^2 HSS C_{18} SB Column. Figure 1 shows the structures of vitamin K_1 isomers and MK-4. Comparing to current LC-based vitamin K_1 *trans* and *cis* isomers analysis methods, this UPC^2 method is faster, simpler (no need to use a C_{30} column), and it uses less organic solvent.

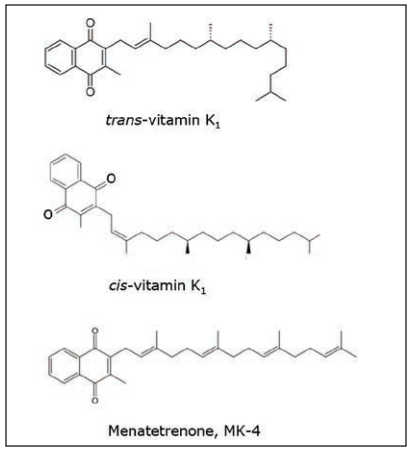

Figure 1. Structures of trans- and cis-vitamin K_1 and menatetrenone.

EXPERIMENTAL

Sample preparation

Vitamin K_1 (Sigma-Aldrich) and MK-4 (Sigma-Aldrich) were weighed and dissolved in iso-octane (ReagentPlus, Sigma-Aldrich) to obtain a stock solution at 1 mg/mL. Intermediate and working standard solutions were obtained by serial dilution of the stock solution with iso-octane. Vitamin K_1 supplement tablets were purchased from a local store and were ground into a powder and extracted with iso-octane. The supernatant was filtered with a 0.45-µm PTFE syringe filter and diluted before injection.

Conditions

UPC² conditions

System:	ACQUITY UPC² with ACQUITY UPC² PDA Detector
Software:	Empower 3
Detection:	UV at 243 nm (compensation reference 400 to 500 nm, res. 6 nm)
Column:	ACQUITY UPC² HSS C_{18} SB 3.0 x 100 mm, 1.8 µm
Column temp.:	50 °C
Sample temp.:	10 °C
Injection volume:	20 µL (Full loop)
Flow rate:	3.00 mL/min
Mobile phase A:	Compressed CO_2
Mobile phase B:	Acetonitrile/methanol mixture (50/50 v/v)
Run time:	4 min
ABPR pressure:	1500 psi
Gradient:	0.5% B for 2 min, ramp to 20% B in 1.5 min, hold at 20% B for 0.5 min

RESULTS AND DISCUSSION

Vitamin K_1 *cis* and *trans* isomers and MK-4 were baseline separated in less than three minutes by UPC² using a single UPC² HSS C_{18} SB Column (3.0 x 100 mm, 1.8 µm). The *cis* form eluted first, followed by the *trans* form, then the MK-4, as shown in Figure 2. The USP resolution between the critical pair, the *cis*- and the *trans*-K_1, was 1.7 (Table 1). In the gradient program, the initial two-minute isocratic elution at 0.5% B was necessary for the baseline separation of the *cis*- and the *trans*-vitamin K_1. Precise control of the mobile phase B delivery volume at 0.5% is critical for the critical pair separation. The ACQUITY UPC² System is the only SFC system on the market that can provide this level of precision control. Following the isocratic hold, a generic gradient from 0.5% to 20% B was used in the study. This gradient range could be modified in applications depending on the retention of the actual vitamin K_2 homologues of interest. MK-4 was included in this study because it is a common form of vitamin K_2, and it is structurally the closest vitamin K_2 to K_1. Other forms of vitamin K_2, such as MK-7, have longer side chains, and tend to be retained longer at column. They can therefore be easily separated from vitamin K_1. The total run time was four minutes, which was at least five times faster than the typical run time for HPLC methods using C_{30} columns. The organic solvent consumption was less than 1 mL per injection, which is only a fraction of the typical 15 to 30 mL of solvent used in LC methods.

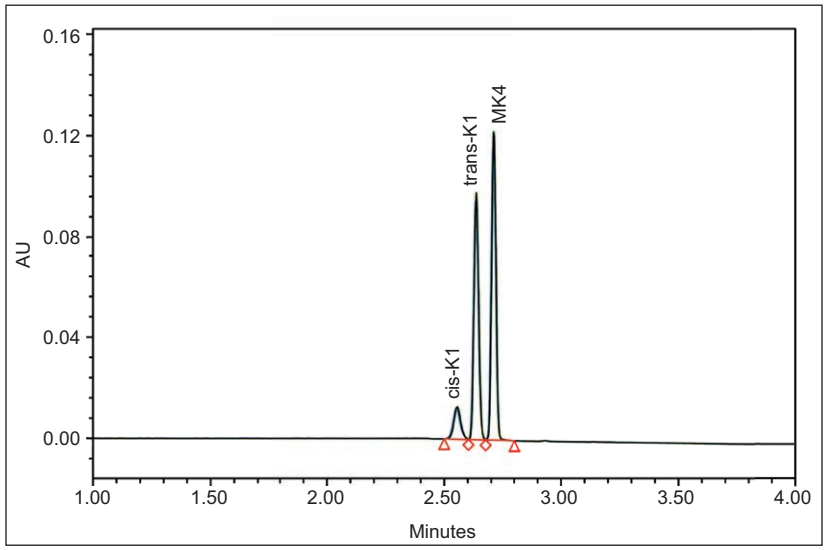

Figure 2. Chromatogram overlay of vitamin K_1 isomers and MK-4 standard mixture (n=10).

	RT (min)	RT RSD	Peak area RSD	Resolution	Resolution RSD
cis-vitamin K1	2.553	0.08%	0.6%	–	–
trans-vitamin K1	2.636	0.05%	0.2%	1.7	1.1%
MK-4	2.710	0.05%	0.2%	2.0	0.9%

Table 1. Results of replicate analysis of vitamin K standard mixture (n=10).

Ten replicate analyses of a standard mixture demonstrated excellent repeatability (Table 1). The limits of quantitation (LOQ), estimated at a signal-to-noise ratio at 10, were 0.06, 0.06, and 0.04 µg/mL for the *cis*-vitamin K_1, the *trans*-vitamin K_1 and the MK-4, respectively (Table 2). Excellent linearity (R^2>0.998) was obtained for these compounds (Table 2). Analysis of a commercial vitamin K supplement product also showed excellent repeatability and resolution (Figure3). In this product, the *cis*-K_1 was found to account for 11.2% of the total vitamin K_1 (Table 3).

Parameters	*cis*-vitamin K_1	*trans*-vitamin K_1	MK-4
Range (µg/mL)	0.03 to 1.5	0.02 to 8.5	0.02 to 10
Regression (R^2)	0.9980	0.9997	0.9999
Slopes (mV sec mL/µg)	17.7	16.3	16.0
LOQ (µg/mL)	0.06	0.06	0.04

Table 2. LOQ and linearity.

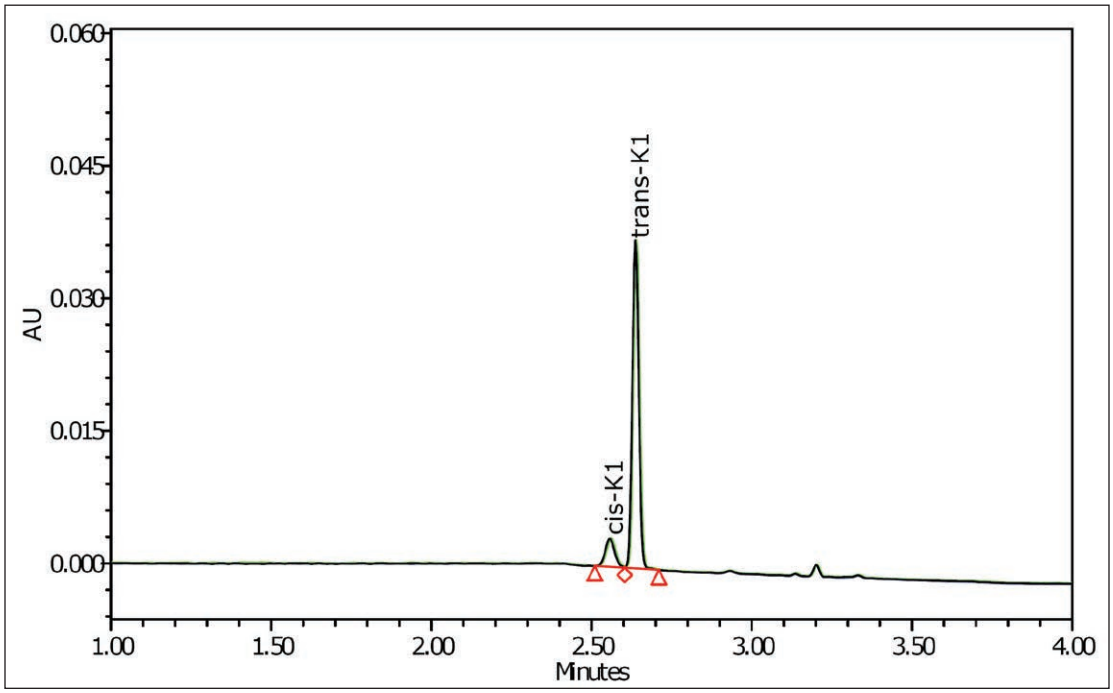

Figure 3. Chromatogram overlay of replicate analysis of vitamin K tablet (n=3).

	RT		Conc.		
	Mean (Min)	RSD (%)	Mean (µg/mL)	RSD (%)	% of total K_1 Conc.
cis-vitamin K_1	2.558	0.09	0.38	2.1	11.2
trans-vitamin K_1	2.638	0.06	3.20	0.3	88.8

Table 3. Results of replicate analysis of vitamin K supplement tablet (n=3).

CONCLUSIONS

UPC^2 Technology enables a rapid separation of the *cis*- and the *trans*-vitamin K_1 isomers and MK-4 on an ACQUITY UPC^2 HSS C_{18} SB Column in less than three minutes. The analysis time is at least five times faster than the current available HPLC methods, and no special C_{30} column is needed. This UPC^2 method has excellent separation selectivity, resolution, sensitivity, repeatability, and it uses much less solvent than HPLC methods. UPC^2 can potentially be used by food ingredient testing labs for routine vitamin K analysis with significant increases in throughput and decreases in operating cost.

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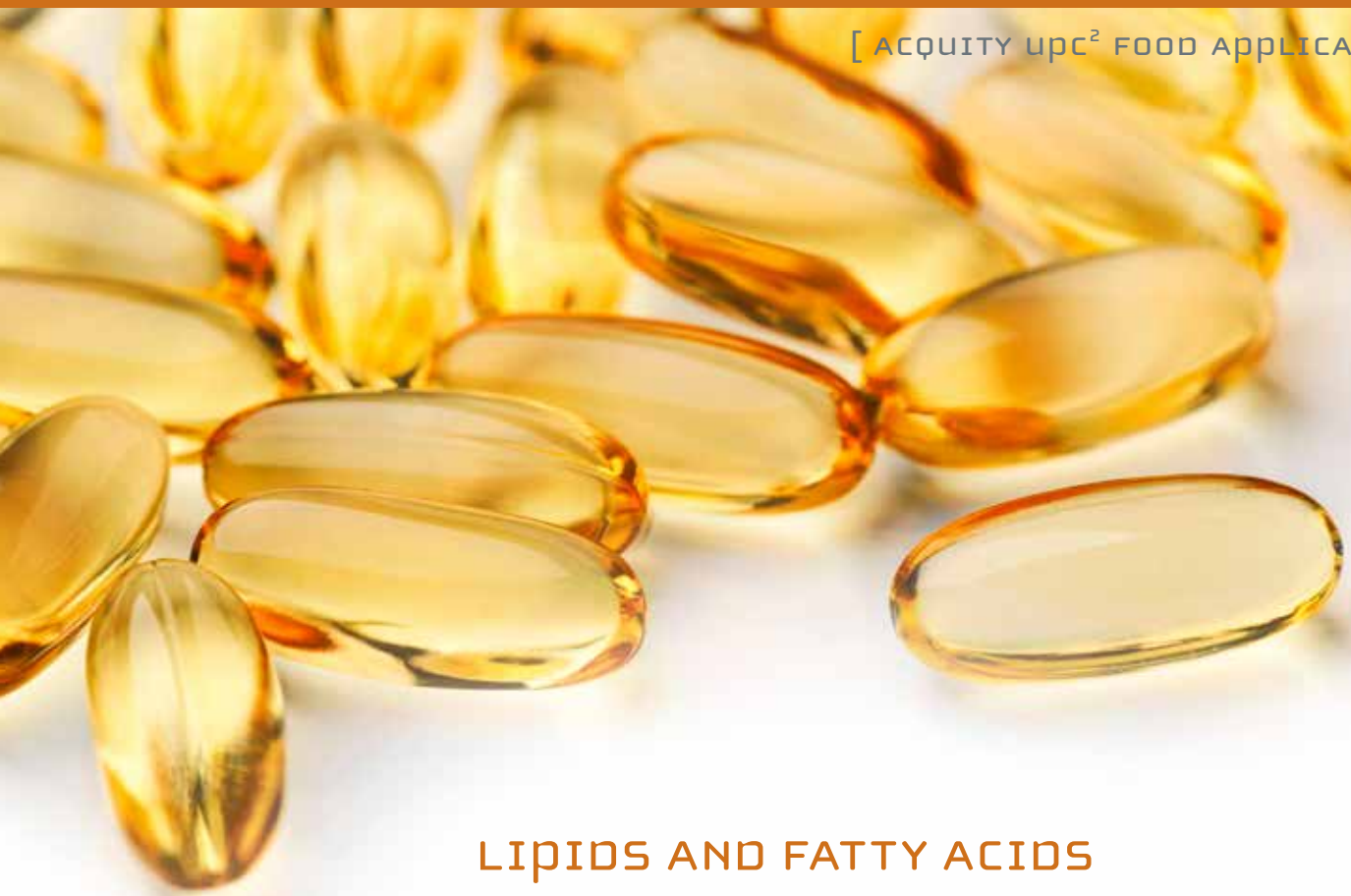

LIPIDS AND FATTY ACIDS

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Fast Separation of Triacylglycerols in Oils using UltraPerformance Convergence Chromatography (UPC^2)

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[2] Waters Corporation, Milford, MA

APPLICATION BENEFITS

- No analyte pre-column derivatization
- No column coupling
- No mobile phase additives
- Fast analysis time of 10 minutes

WATERS SOLUTIONS

ACQUITY UPC^2™ System

ACQUITY UPC^2 PDA Detector

ACQUITY UPLC® ELS Detector

Xevo® G2 Q-Tof™ MS

ACQUITY UPC^2 HSS C_{18} SB Column

KEY WORDS

SFC, convergence chromatography, CC, UPC,^{2} triacylglycerols, TAG, oils, tobacco, edible oils, ELS, MS, Q-Tof, UV

INTRODUCTION

UltraPerformance Convergence Chromatography™ (UPC$^{2®}$) is a novel technology that applies the performance advantages of UPLC to supercritical fluid chromatography (SFC). Combining the use of supercritical CO_2 with sub-2-µm particle columns, UPC^2 represents an analysis technique that is orthogonal to reversed-phase LC and can be used to solve many troublesome separations that challenge conventional LC or GC analyses. It also generates less solvent waste as compared to liquid chromatography. These benefits have led to interest in applying this technology to various industrial analytical areas. The established UPC^2/MS approach has potential application in lipidomics as a complementary method alongside LC/MS and GC/MS, as it can separate both polar and non-polar lipids, and in many cases does not require derivatization of lipids to improve detection limits and peak shape. Studies using UPC^2 coupled with ultraviolet (UV) detection, mass spectrometry (MS), and evaporative light scattering (ELS) detection for the separation of triacylglycerols in tobacco, corn, sesame, and soybean seed oils are presented in this application note. A single unendcapped C_{18} column with acetonitrile-modified CO_2 was used for the separation of all seed oils.

EXPERIMENTAL

Sample preparation

Tobacco seed oil was obtained from R.J. Reynolds Tobacco Co. (Winston-Salem, NC); soybean oil, corn oil, and sesame seed oil were obtained from Sigma Aldrich (St. Louis, MO). 5% of the different oils were dissolved in dichloromethane/methanol (1/1) for UV and ELS and 0.1% for the Xevo G2 Q-Tof MS.

Method conditions

System:	ACQUITY UPC^2
Column:	ACQUITY UPC^2 HSS C_{18} SB, 1.8 µm, 3.0 x 150 mm
ABPR:	1500 psi
Column temperature:	25 °C
Injection volume:	2-8 µL (UV and ELSD), 0.5 µL (MS)
Sample solvent:	Dichloromethane/methanol (1/1)
Flow rate:	1-2 mL/min
Mobile phase A:	Compressed CO_2
Mobile phase B:	Acetonitrile or 90/10 acetonitrile/MeOH
Make up solvent:	IPA for ELSD and MeOH in 10 mM ammonium acetate for MS
Make up flow rate:	0.2 mL/min
Gradient:	A- 98/2 to 80/20 in 18 min for ELSD/UV and 18 min for MS B- 90/10 to 50/50 in 10 minutes for MS
Detectors:	ACQUITY UPC^2 PDA 200-400 nm, Ref. 400-500 nm ACQUITY ELS Nebulizer: Cooling, drift tube: 50 °C, Gas pressure: 40 psi and Gain 10

MS conditions

MS:	Xevo G2 Q-Tof
Ionization mode:	ESI positive
Capillary voltage:	3.0 kV
Cone voltage:	30 V
Source temperature:	150 °C
Desolvation temp.:	500 °C
Cone gas flow:	10 L/h
Desolvation gas flow:	600 L/h
Acquisition range:	40 to 1200 *m/z*

RESULTS AND DISCUSSION

From the perspective of current applied lipid research, UPC^2 is a complementary and perhaps preferred technique to gas chromatography (GC) and high performance liquid chromatography (HPLC) for metabolic profiling of lipids.[1] The use of UPC^2/MS allows for high throughput and exhaustive analysis of diverse lipids, leading to potential application in lipidomics. Currently, there is much interest in rapid characterization of triacylglycerols (TAGs). TAGs are natural compounds produced by the esterification of glycerol with fatty acids. In humans, TAGs serve as a source of energy stored in fat tissues and they form a thermal and mechanical protective layer around important organs.[2] Furthermore, TAGs are the source of essential fatty acids such as linoleic and linolenic acids.

Figure 1 shows UV, ELSD, and MS chromatograms for the separation of TAGs in soybean oil. All of the effluent first passes through the PDA flow cell and then to the back pressure regulator and the ELSD using the PEEK ELSD splitter kit (205001048). Each gradient separation was performed at 25 °C with an ACQUITY UPC^2 HSS C_{18} SB Column and a mobile phase of acetonitrile-modified CO_2. Near baseline peak resolution was observed on a single ACQUITY UPC^2 HSS C_{18} SB, 1.8 µm, 3.0 x 150 mm Column in approximately 16 minutes. Baseline stability was excellent, even under gradient conditions, demonstrating the stability of the system and allowing reproducible detection and possible quantitation of lower level peaks in the samples.

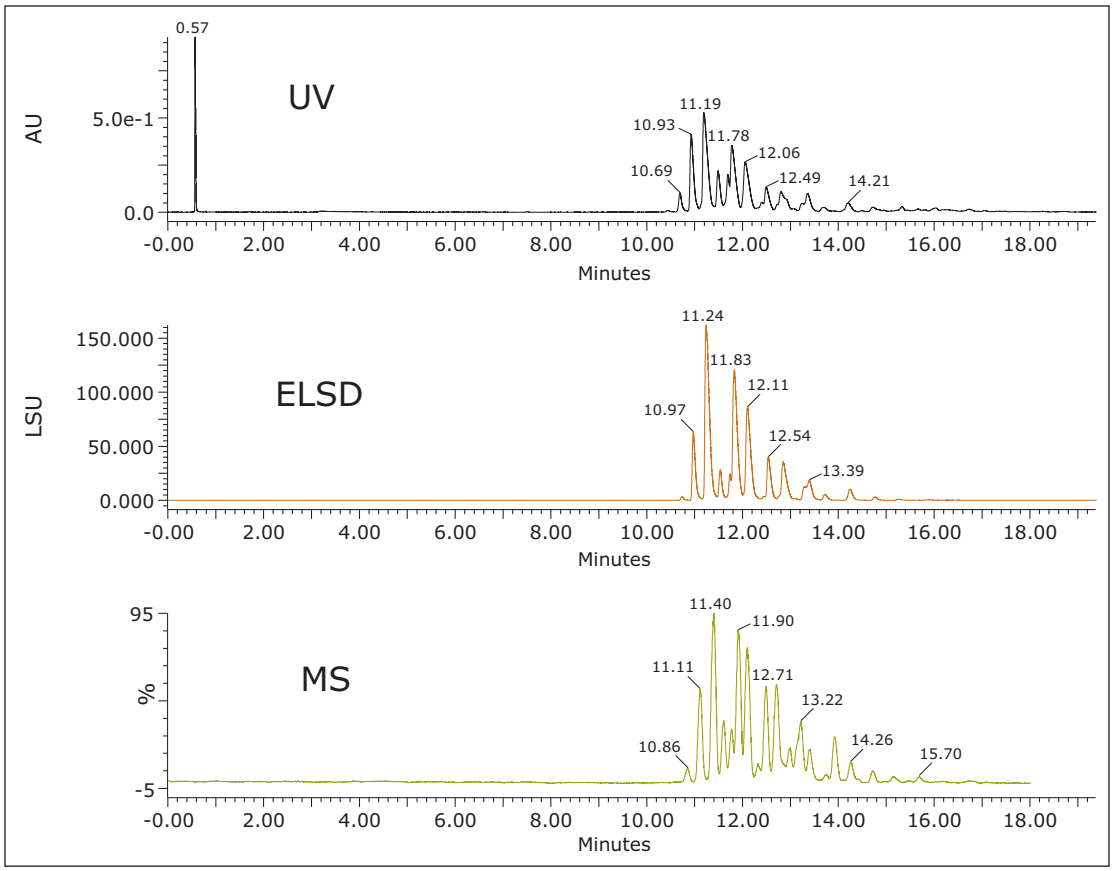

Figure 1. Analysis of soybean oil using UPC^2 with CH_3CN as the modifier. Chromatography conditions: Gradient elution: 2 to 20% CH_3CN in 18 min, Flow: 1.5 mL/min, Column temp.: 25 °C.

The conditions from Figure 1 were used to separate and profile TAGs in tobacco seed oil, soybean oil, corn oil, and sesame seed oil. Data were acquired in UPC^2/MS^E mode, an unbiased Tof acquisition method in which the mass spectrometer switches between low and high energy on alternate scans for structural elucidation and identification. In all cases, distinct profiles and excellent separations were obtained for all oil types when using UPC^2 with both MS and ELS detection. Figure 2 shows the UPC^2/MS separation and detection of different TAGs in different oils using UPC^2/MS^E. TAGs were identified using accurate mass spectra collected by QTof MS with MS^E and Waters TransOmics™ Informatics. In positive ion mode MS^E low energy, TAGs produce intact ammonium adduct $[M+NH_4]^+$ precursor exact mass when ammonium acetate is present in the make-up solvent. In MS^E high energy, abundant fragment ions are produced corresponding to the neutral loss of one of the sn-1, sn-2, or sn-3 fatty acids plus ammonia.

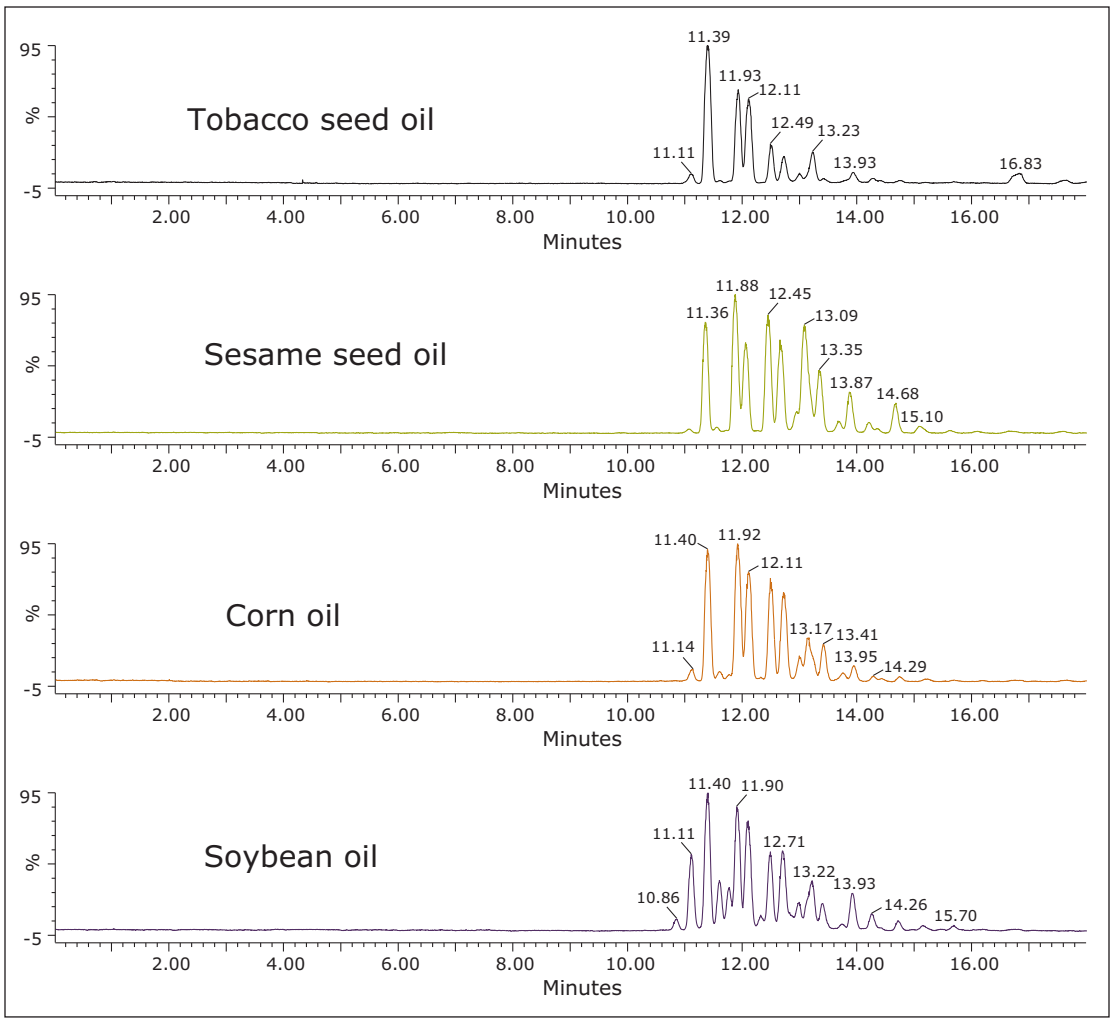

Figure 2. UPC^2-MS analysis of different oils using CH_3CN as a modifier. Chromatography conditions: Gradient elution: 2 to 20% CH_3CN in 18 min, Flow: 1.5 mL/min, Column temp.: 25 °C.

For example, Figure 3 shows the ion at *m/z* 874.7823 corresponding to 52:3 TAG (calculated fatty acid carbon atom: total number of double bonds), which can be identified as POL due to the presence of abundant fragment ions at *m/z* 575.5038, 577.5201, and 601.5186, corresponding to the neutral loss of fatty acyl groups P, O, and L plus ammonia, respectively.[4] Table 1 shows a list of all identified TAGs in the different oils based on the low energy precursor exact mass and corresponding high energy fragment ions.

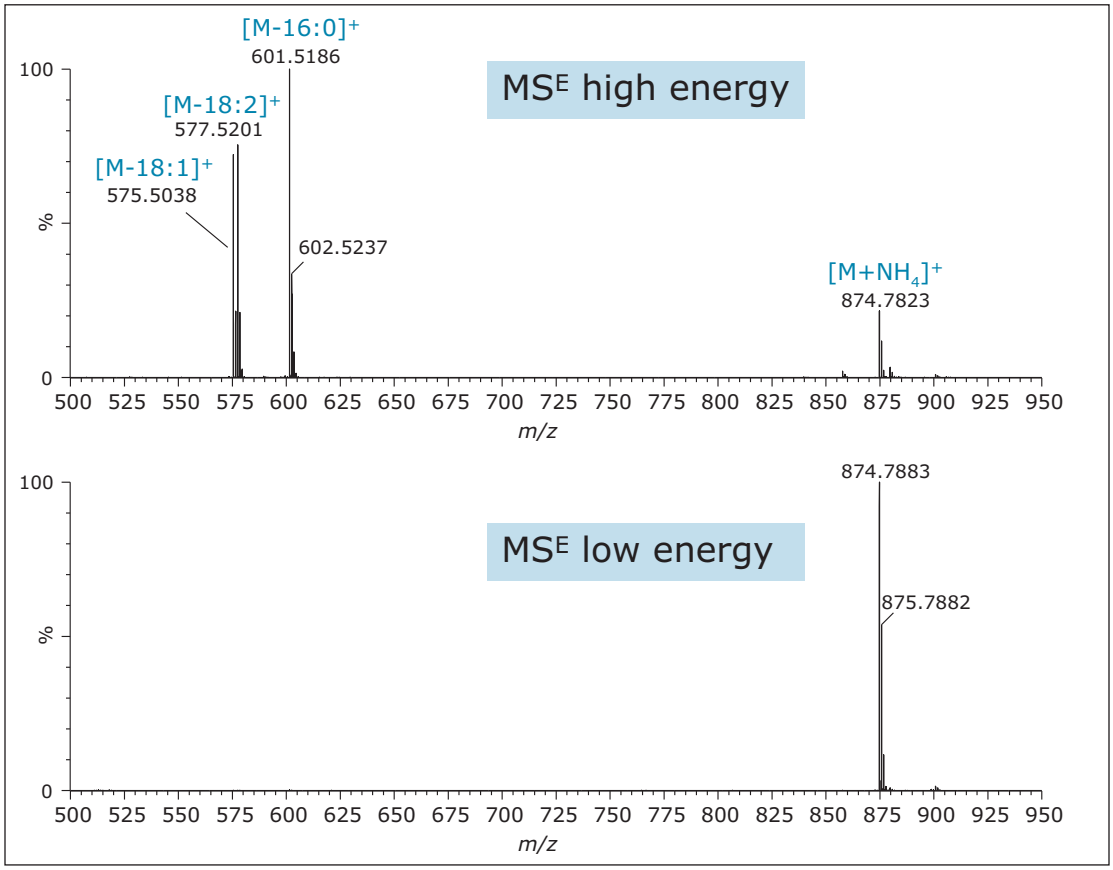

Figure 3. Example MS^E spectra of TAG species identified as POL, which elutes at 12.71 min using the conditions in Figures 1 and 2.

Ret. time, min.	Soybean oil	Corn oil	Sesame oil	Tobacco seed oil
10.86	LnLnL	—	—	—
11.11	LnLL	LnLL	LnLL	LnLL
11.40	LLL	LLL	LLL	LLL
11.60	OLLn	OLLn	OLLn	—
11.78	PLLn	—	—	
11.90	OLL	OLL	OLL	OLL
12.11	PLL	PLL	PLL	PLL
12.32	POLn	POLn	POLn	—
12.49	OOL	OOL	OOL	OOL
12.71	POL	POL	POL	POL
13.00	PPL	PPL	PPL	PPL
13.08	OOO	OOO	OOO	—
13.22	SLL	SLL	SLL	SLL
13.40	POO	POO	—	POO
13.93	SOL	SOL	SOL	SOL
14.26	PSL	PSL	PSL	PSL
14.73	SSL	SSL	SSL	SSL
15.14	PSO	PSO	PSO	—
15.70	PSA	PSA	PSA	—

Table 1. List of detected triacylglycerols in each seed oil with corresponding retention time via UPC^2-MS (using conditions from Figures 1 and 2)

In an attempt to reduce the analysis time of the TAGs, a faster gradient elution was used. Figure 4 shows the separation of soybean oil using two different gradient profiles with acetonitrile as a modifier. With the faster gradient elution (Figure 4A), all the components were eluted in less than nine minutes. With slower gradient elution (Figure 4B), all components eluted in less than 16 minutes. Little resolution was lost when the faster gradient was used, thus increasing the throughput of the UPC^2/MS method.

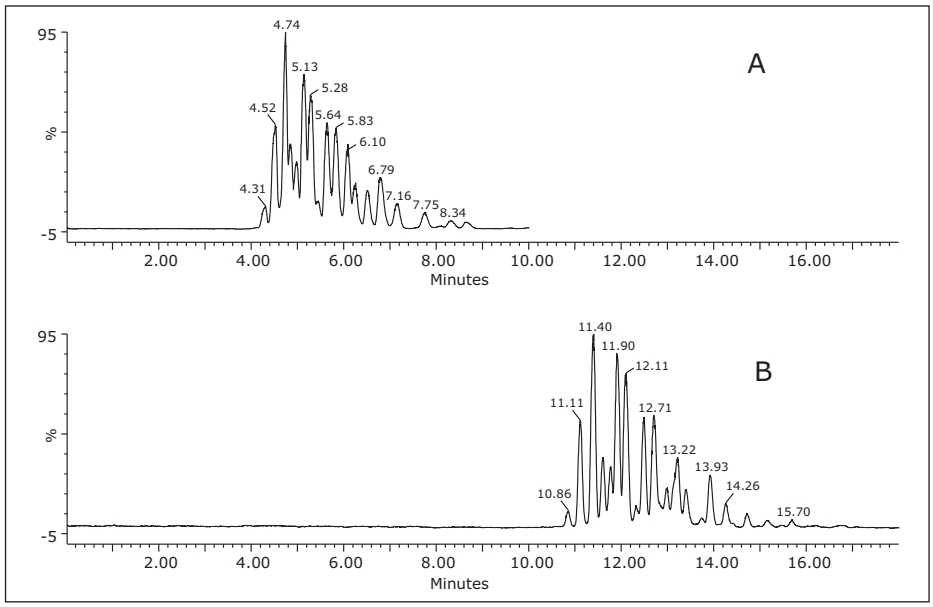

Figure 4. Analysis of soybean oil at 25 °C using UPC^2-MS with CH_3CN as the modifier. Chromatography conditions A: 2 to 20% CH_3CN in 18 min, Flow: 1.5 mL/min Chromatography conditions B: 10 to 50% CH_3CN in 10 min Flow: 1.2 mL/min

Also, a mobile phase modifier of acetonitrile and methanol (9:1) was tested under the same chromatographic conditions.

Overall retention time decreased approximately 5 minutes with minimal loss in peak resolution (Figure 5). This data indicates that changing the gradient profile, flow rate, and mobile phase modifier can be performed to optimize the separation based on the type of TAG analysis required. All of these parameters are compatible with MS detection, thus allowing positive identification of all TAG species in oils.

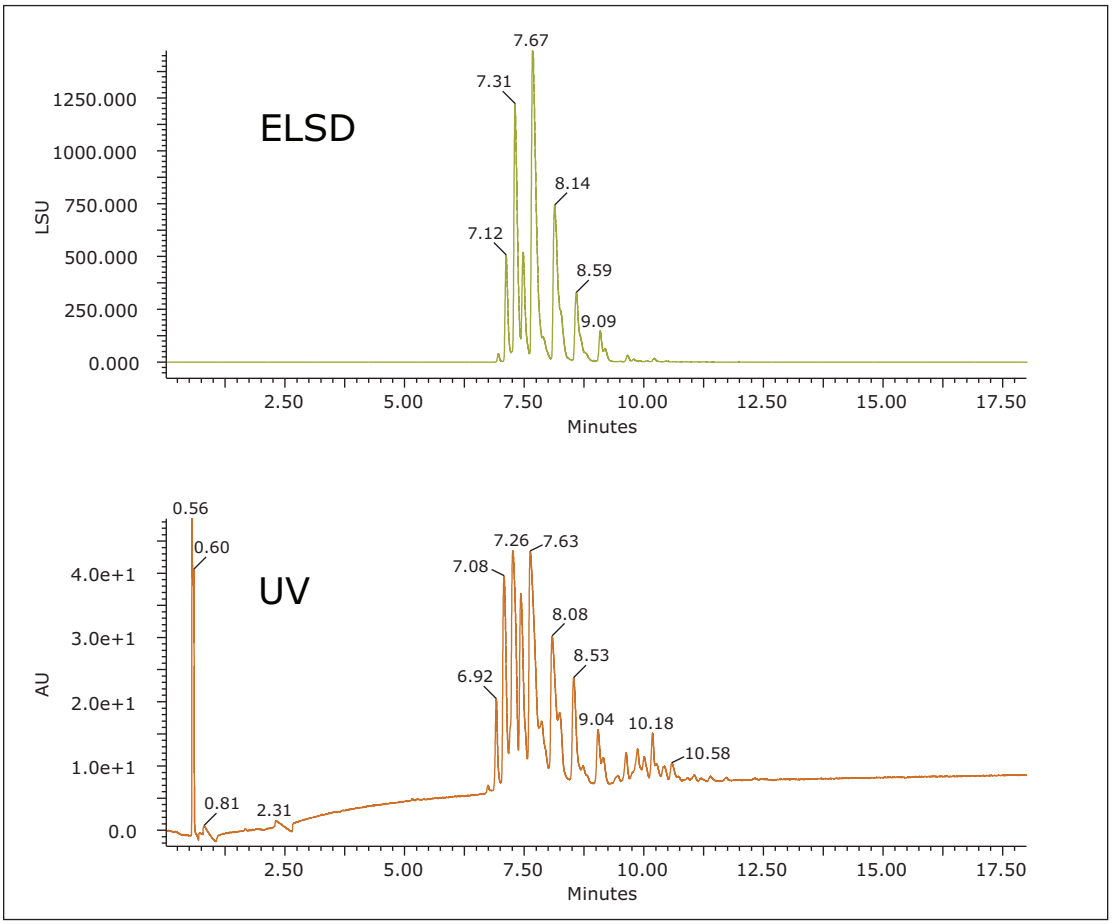

Figure 5. Analysis of soybean oil using UPC^2 with CH_3CN/MeOH (90:10) as the modifier.
Chromatography conditions: 2 to 20% CH_3CN/MeOH in 18 min, Flow: 1.5 mL/min, Column temp: 25 °C.

CONCLUSIONS

UltraPerformance Convergence Chromatography, in combination with sub-2-µm particles and UV, ELS, and MS detection, is a valuable technique for the determination of triacylglycerol composition in a variety of seed oils. Excellent resolution on a single column in as little as 10 minutes serves as an improvement on past generations of SFC instrumentation. This methodology can be used as a tool for rapid characterization and profiling a suite of acylglycerols from different oil sources.

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Fast and Simple Free Fatty Acids Analysis Using UPC²/MS

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APPLICATION BENEFITS

- Demonstrates the separation of free fatty acid (FFA) species based on chain length and number of double bonds
- No derivatization is required, which results in easier and fast sample preparation and eliminates artifact formation
- Organic phase lipid extract can be directly injected onto the system, saving time and reducing cost per analysis
- Less than three-minute chromatographic separation is up to 10X faster compared to GC/MS
- Unlike GC/MS, low volatile and very long chain fatty acids (>24 carbon atoms) can be easily analyzed with UPC²®

WATERS SOLUTIONS

ACQUITY UPC²® System

TransOmics™ Informatics

Xevo® G2 QTof Mass Spectrometer

ACQUITY UPC² HSS Column

KEY WORDS

Free fatty acids, UltraPerformance Convergence Chromatography™, UPC², TransOmics, time-of-flight mass spectrometry, UPC²/MS/MS

INTRODUCTION

Fatty acids, both free and as part of complex lipids, play a number of key roles in metabolism – as major metabolic fuel (storage and transport of energy), as essential components of all membranes, and as gene regulators. In addition, dietary lipids provide polyunsaturated fatty acids that are precursors of powerful locally acting metabolites, e.g., eicosanoids.

The common fatty acids of animal and plant origin have even-numbered chains of 16 to 24 carbon atoms with 0 to 6 double bonds. Nature provides countless exceptions, however, and odd- and even-numbered fatty acids with up to nearly 100 carbon atoms exist. In addition, double bonds can be of the *cis* (Z) and *trans* (E) configuration and there can be innumerable other structural features, including branch points, rings, oxygenated functions, and many more.

Fatty acid chains may contain one or more double bonds at specific positions (unsaturated and poly unsaturated with *cis* (Z) or *trans* (E) configuration) or they may be fully saturated. The LIPIDMAPS systematic nomenclature for fatty acids indicates the location of double bonds with reference to the carboxyl group with "Δ".[1] Fatty acid structures also contain a methyl group at one end of the molecule (designated omega, ω) and a carboxyl group at the other end. The carbon atom next to the carboxyl group is called α carbon and the subsequent one the β carbon. The letter "n" is also often used instead of ω to indicate the position of the double bond closest to the methyl end.[2] Figure 1 outlines the structures of different straight chain fatty acids.

The isolation of free fatty acids (FFA) from biological materials is a complex task and precautions should be taken at all times to prevent or minimize the effects of hydrolyzing enzymes. After isolation, the typical chromatographic methods for analyzing fatty acids include gas chromatography/mass spectroscopy (GC/MS) and liquid chromatography-tandem mass spectrometry (LC/MS/MS). However, there are shortcomings associated with each of these methods.

For example, GC methods require derivatization of the fatty acids to hydrolyze and convert to methyl esters, which is time-consuming and risks re-arrangement of the fatty acids during derivatization, leaving doubt as to whether the esters formed are from FFA or intact complex lipids. Moreover, the GC/MS analysis of low volatile, very-long-chain fatty acids with high molecular weight (>C24) is a problem even after fatty acid methyl ester (FAME) derivatization.

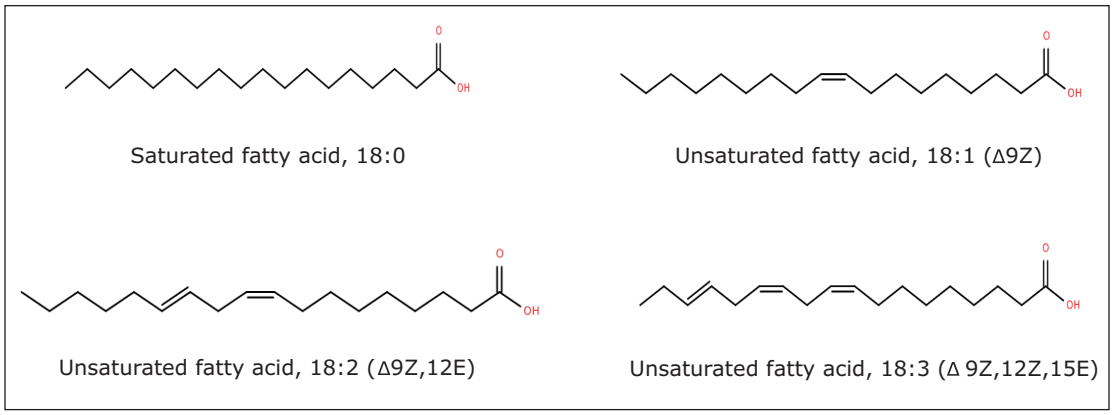

Figure 1. Structure and nomenclature of different straight chain fatty acids with a methyl and a carboxyl (acidic) end. Fatty acids may be named according to systematic or trivial nomenclature. One systematic way to describe the position of double bonds is in relation to the acidic end of the fatty acids; symbolized as Δ (Greek delta) followed with numbers. All unsaturated fatty acids are shown with cis (Z) or trans (E) configuration of the double bonds.

In LC/MS methods, although no sample derivatization is required, the runs typically involve labor-intensive and time-consuming sample preparation, and utilize toxic organic solvents, which are expensive to purchase and dispose. In a typical reversed phase (RP) LC/MS analysis, the organic extracts containing all the lipids have to be evaporated and re-constituted in a more compatible injection solvent.

Thus, it would be beneficial to have streamlined methods for the separation and determination of fatty acids. Here, we present a rapid, high-throughput and efficient method for the separation and analysis of FFA using UltraPerformance Convergence Chromatography (UPCC, or UPC^2) with mass spectrometry.

UPC^2 is a complementary, orthogonal separation technology that is taking its place alongside LC and GC. While all three use a stationary phase to interact with compounds of interest and a mobile phase to move compounds through the stationary phase and achieve separation, the techniques differ mainly by the mobile phases used.

GC is defined by using a gas as its mobile phase, LC is defined by using liquids as its mobile phase, and CC is defined by using both gas and liquids. It is this convergence of mobile phases in combination with a far greater choice of stationary phases that makes CC a powerful additional choice for laboratory scientists. Because UPC^2 can receive samples in organic solvents such as hexane and chloroform, it significantly simplifies the requirements for sample preparation, while maintaining all the advantages of RPLC.

Here, the analysis of fatty acids in the free form instead of FAME derivatives results in easier and faster sample preparation. The organic phase extract containing all the FFA can be injected directly into the system, which results in significant savings in sample preparation and analysis time, solvent costs, and solvent waste disposal. Additionally, artifact formation that can result from a derivatization procedure is eliminated.

EXPERIMENTAL

Method Conditions

UPC² conditions

System:	ACQUITY UPC²
Columns:	ACQUITY UPC² HSS C_{18} SB 1.8 µm, 2.1 x 150 mm
Column temp.:	50 °C
Sample vial:	Total Recovery Vial (p/n 186000385C)
Sample temp.:	10 °C
Injection volume:	0.5 µL
Flow rate:	0.6 mL/min
Mobile phase A:	CO_2
Mobile phase B:	Methanol in 0.1% formic acid
Make up:	Methanol in 0.1% NH_4OH (0.2 mL/min)
Splitter:	Upchurch cross 1/16 PEEK

Gradient

Time (min)	%A (CO_2)	%B	Curve
0.0	95	5	Initial
5.0	75	25	6
5.1	50	50	1
6.0	50	50	11
8.0	95	5	1

MS conditions

Mass spectrometer:	Xevo G2 QTof
Ionization mode:	ESI negative
Capillary voltage:	1.0 kV
Cone voltage:	30 V
Source temp.:	100 °C
Desolvation temp.:	500 °C
Cone gas flow:	10 L/h
Desolvation gas flow:	600 L/h
Acquisition range:	50 to 600 *m/z*

Sample preparation

FFA standard mixtures

Individual saturated FFA standards containing even carbon number C_8 to C_{24} were purchased from Sigma. A complex model mixture of different FFA standards (GLC-85 in FFA form) was purchased from Nu-Chek Prep (Elysian, MN, USA). The list of FFA standards analyzed and other detailed information is provided in Table 1. A 1 mg/mL stock solution was prepared in chloroform, and 0.1 mg/mL working lipid mixtures were prepared in chloroform, then injected onto the UPC²/MS system.

Algae and algaenan produced oils

Oil produced from hydrous pyrolysis of algae and algaenan at low and high pyrolysis temperature were provided from Old Dominion University (Norfolk, VA, USA). Algae 1 and algaenan 1 were treated at a pyrolysis temperature of (310 °C); and Algae 2 and algaenan 2 were treated at a pyrolysis temperature of (360 °C).

Extraction of algaenan was performed by a modified extraction procedure. Briefly, lipids were removed from the algae by Soxhlet extraction with 1:1 (v/v) benzene/methanol solvent mixture for 24 hours. The residue was treated with 2N sodium hydroxide at 60 °C for two hours. The remaining residue was then washed excessively with deionized water, followed by treatment with Dowex 50W-x8 cation exchange resin to exchange any residual sodium. Finally, the solid was rinsed with deionized water. The oil samples were diluted 10 times in dichloromethane, and 1 µL was injected onto the UPC²/MS system.

Data acquisition and processing

When using multivariate data analysis for sample comparison, it is crucial that each sample is randomized and injected a minimum of three times to ensure that the data analysis is statistically valid. For this study, five replicates of each algae and algaenan oil extracts were acquired in MS^E mode, an unbiased Tof acquisition method in which the mass spectrometer switches between low and elevated collision energy on alternate scans. Data analysis and FFA identification were performed using TransOmics Informatics for Metabolomics and Lipidomics (TOIML).

Compound	Formula	Neutral Mass	$[M-H]^-$	Retention time (min)	Common Name	Description
1	$C_4H_8O_2$	88.052429	87.045153	0.89	Butyric acid	C4:0
2	$C_6H_{12}O_2$	116.083730	115.076453	0.96	Caproic acid	C6:0
3	$C_8H_{16}O_2$	144.115030	143.107753	1.06	Caprylic acid	C8:0
4	$C_{10}H_{20}O_2$	172.146330	171.139053	1.17	Capric acid	C10:0
5	$C_{11}H_{22}O_2$	186.161980	185.154704	1.23	Undecylic acid	C11:0
6	$C_{12}H_{24}O_2$	200.177630	199.170354	1.31	Lauric acid	C12:0
7	$C_{13}H_{26}O_2$	214.193280	213.186004	1.41	Tridecylic acid	C13:0
8	$C_{14}H_{28}O_2$	228.208930	227.201654	1.54	Myrislc acid	C14:0
9	$C_{15}H_{30}O_2$	242.224580	241.217304	1.67	Pentadecylic acid	C15:0
10	$C_{16}H_{32}O_2$	256.240230	255.232954	1.80	Palmilc acid	C16:0
11	$C_{17}H_{34}O_2$	270.255880	269.248604	1.97	Margaric acid	C17:0
12	$C_{18}H_{36}O_2$	284.271530	283.264254	2.11	Stearic acid	C18:0
13	$C_{20}H_{40}O_2$	312.302831	311.295554	2.41	Arachidic acid	C20:0
14	$C_{22}H_{44}O_2$	340.334131	339.326854	2.70	Behenic acid	C22:0
15	$C_{14}H_{26}O_2$	226.193280	225.186004	1.45	Physeteric acid	C14:1
16	$C_{15}H_{28}O_2$	240.208930	239.201654	1.57		C15:1
17	$C_{16}H_{30}O_2$	254.224580	253.217304	1.67	Palmitoleic acid	16:1
18	$C_{17}H_{32}O_2$	268.240230	267.232954	1.81	10-HEPTADECENOIC Acid	C17:1 (Δ10)
19	$C_{18}H_{30}O_2$	278.224580	277.217304	1.76	Gamma Linolenic Acid	C18:3 (Δ6,9,12)
20	$C_{18}H_{30}O_2$	278.224580	277.217304	1.86	Linolenic Acid	C18:3(Δ9,12,15)
21	$C_{18}H_{32}O_2$	280.240230	279.232954	1.88	Linoleic Acid	C18:2
22	$C_{18}H_{34}O_2$	282.255880	281.248604	1.98	Oleic Acid	C18:1
23	$C_{18}H_{34}O_2$	282.255880	281.248604	1.98	Elaidic Acid	C18:1T
24	$C_{20}H_{32}O_2$	304.240230	303.232954	1.93	Arachidonic acid	C20:4
25	$C_{20}H_{34}O_2$	306.255880	305.248604	2.04	HOMOGAMMA LINOLENIC Acid	C20:3 (Δ8,11,14)
26	$C_{20}H_{34}O_2$	306.255880	305.248604	2.14	11-14-17-EICOSATRIENOIC Acid	C20:3 (Δ11,14,17)
27	$C_{20}H_{36}O_2$	308.271530	307.264254	2.17	11-14-EICOSADIENOIC Acid	C20:2 (Δ11, 14)
28	$C_{20}H_{38}O_2$	310.287180	309.279904	2.24	11-EICOSENOIC Acid	C20:1 (Δ11)
29	$C_{22}H_{32}O_2$	328.240230	327.232954	2.09	Docosahexaenoic Acid	C22:6
30	$C_{22}H_{40}O_2$	336.302831	335.295554	2.46	Docosadienoic Acid	C22:2
31	$C_{22}H_{42}O_2$	338.318481	337.311204	2.54	Erucic Acid	C22:1
32	$C_{24}H_{46}O_2$	366.349781	365.342504	2.83	Nervonic acid	C24:1

RESULTS AND DISCUSSION

Analysis of saturated FFA standards

Figure 2 shows the separation of saturated FFA with carbon chain length C_8 to C_{24}. The ACQUITY UPC^2 High Strength Silica (HSS) C_{18} SB 1.8 µm, 2.1 x 150 mm Column provides an RP-like separation that results in effective separation of the different FFA species. The gradient is run under acidic conditions using a small percentage of formic acid (0.1% v/v in methanol) to improve the peak shape and decrease peak tailing.

The ACQUITY UPC^2 method is 10X faster (only a three-minute run) than GC/MS and RPLC methods, and uses less toxic and cheaper CO_2 as a solvent. A typical lipidomics study involves the analysis of thousands of biological samples, and the additional speed allows for large sample sets to be analyzed efficiently, improving the overall power of the experiment.

The FFA lipid molecular species separation mechanism is mainly based on hydrophobic interaction of the FFA carbon numbers and number of double bonds with the HSS C_{18} SB material. Therefore, the elution order of the FFA species depends on the length and the number of double bonds on the fatty acid chain. Thus, the longer and the more saturated the acyl chain length the longer the retention time.

The co-solvent mobile phase B (methanol in 0.1% formic acid) can be optimized to increase the chromatographic resolution and peak capacity. The higher the percentage of the co-solvent, the shorter the retention time and the narrower the peaks. However, when analyzing a complex biological sample containing saturated and unsaturated FFA species with different carbon chain length, peak capacity is important in order to reduce coeluting lipid species. The co-solvent gradient 5% to 25% methanol in 0.1% formic acid was used for further analysis.

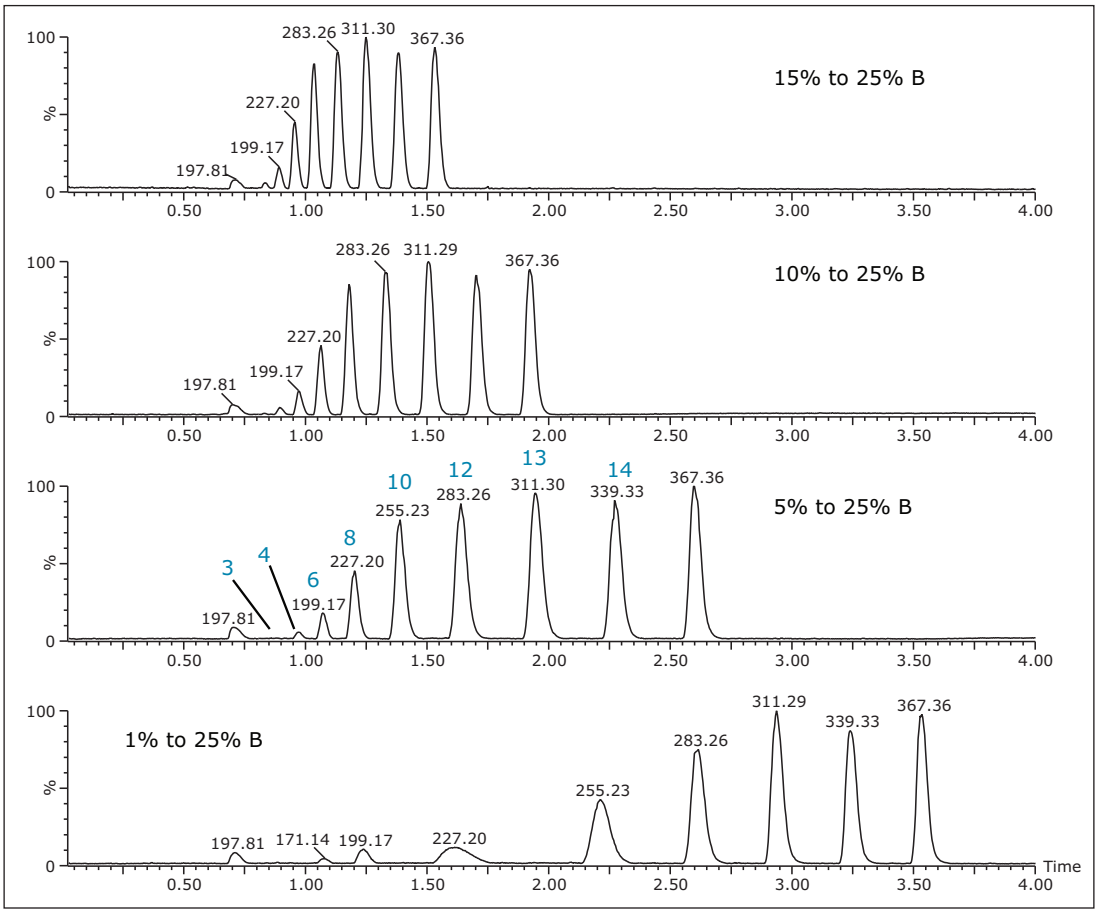

Figure 2. The separation of saturated FFA with carbon chain length C8-C24 with various co-solvent gradient. For the lipid ID, see Table 1.

Analysis of complex saturated and unsaturated FFA standards GLC-85

Reversed-phase chromatography separates lipids according to both chain-length and degree of unsaturation. The problem lies in the fact that the dual nature of the reversed-phase separation process (a double bond in the fatty acyl chain reduces the retention time and the fatty acyl chain length increases the retention time) can hamper the analysis of real samples; the number of components is often so great that identification becomes difficult due to coelution (Figures 3A and B).

On the other hand, by using the precursor exact mass, corresponding product ion information and ion mobility (separation of lipid ions in the gas phase according to their size and molecular shape), each coeluting peak can be extracted and identified.

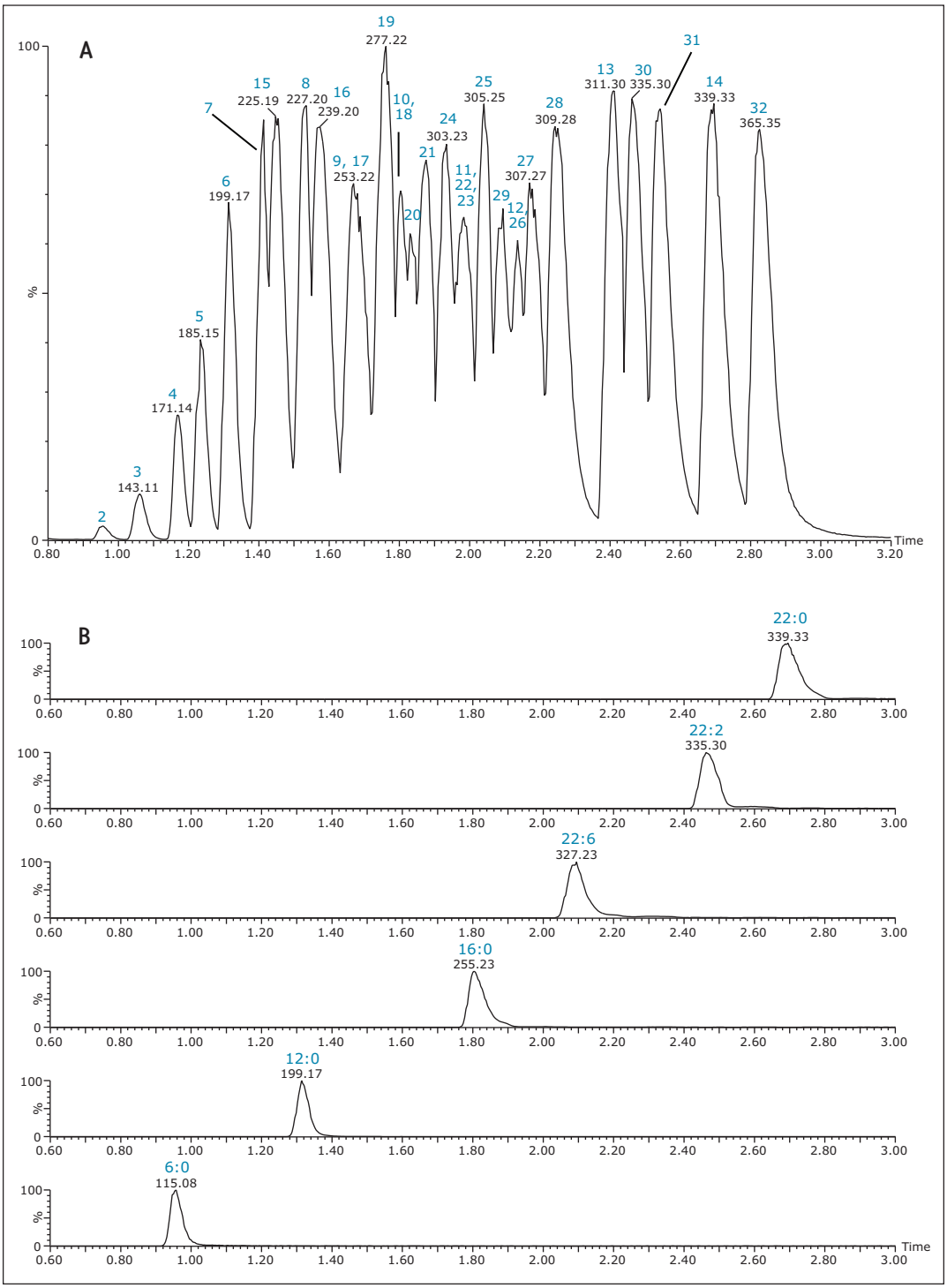

Figure 3. A) The separation of complex standard mixture that contains saturated, unsaturated, short and long chain 32 different FFA species. B) The separation depends on both chain length and degree of unsaturation. In an RP separation, the fatty acyl chain length increases the retention time and the number of double bonds in the fatty acyl chain decreases the retention time. For the lipid ID, see Table 1.

Another benefit of the method is the ability to separate between lipid isomers. FFA can have different biological functions based on the double bond position (e.g., omega-3 and omega-6). Figure 4 shows the separation of FFA isomers based on the position of the double bond. The separation of 18:3 (Δ6,9,12) and 18:3 (Δ9,12,15); and 20:3 (Δ8,11,14) and 20:3 (Δ11,14,17) isomers have been observed.

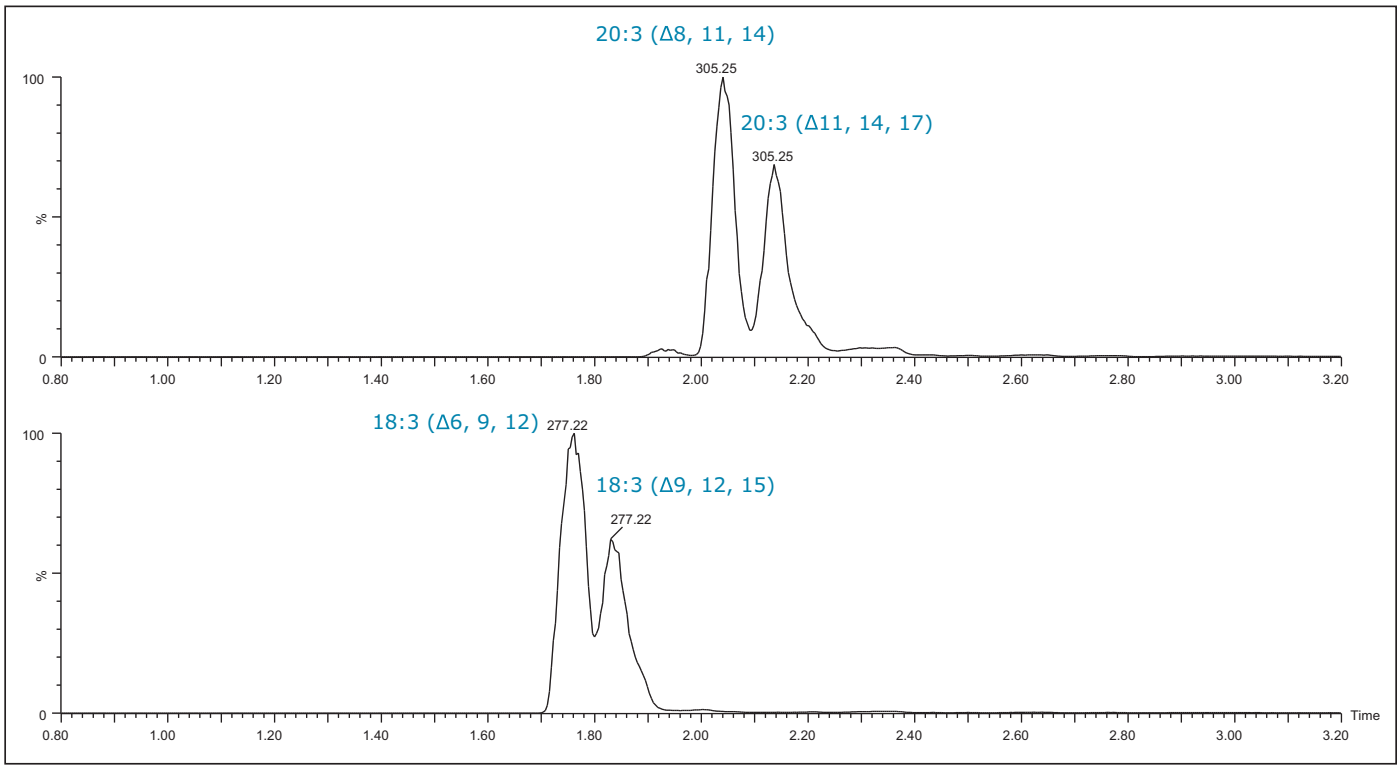

Figure 4. Extracted ion chromatogram (from figure 3) showing the separation of isobaric lipid species based on the position of the double bond.

Biological application and data analysis using TransOmics

The developed UPC^2/Xevo G2 QTof MS method was applied with minor modifications for the profile of FFA in algae and algaenan extracts treated at low (310 °C) and high (360 °C) pyrolysis temperatures.

Algaenan is a non-hydrolyzable, insoluble biopolymer in the cell walls of several green freshwater and marine microalgae.[3] Figure 5 shows a representative chromatogram from algaenan 1 with the UPC^2 conditions used for the analysis. For complete analysis of the data, set the gradient 1% to 10% co-solvent mobile phase B (methanol in 0.1% formic acid) in 10 minutes was used.

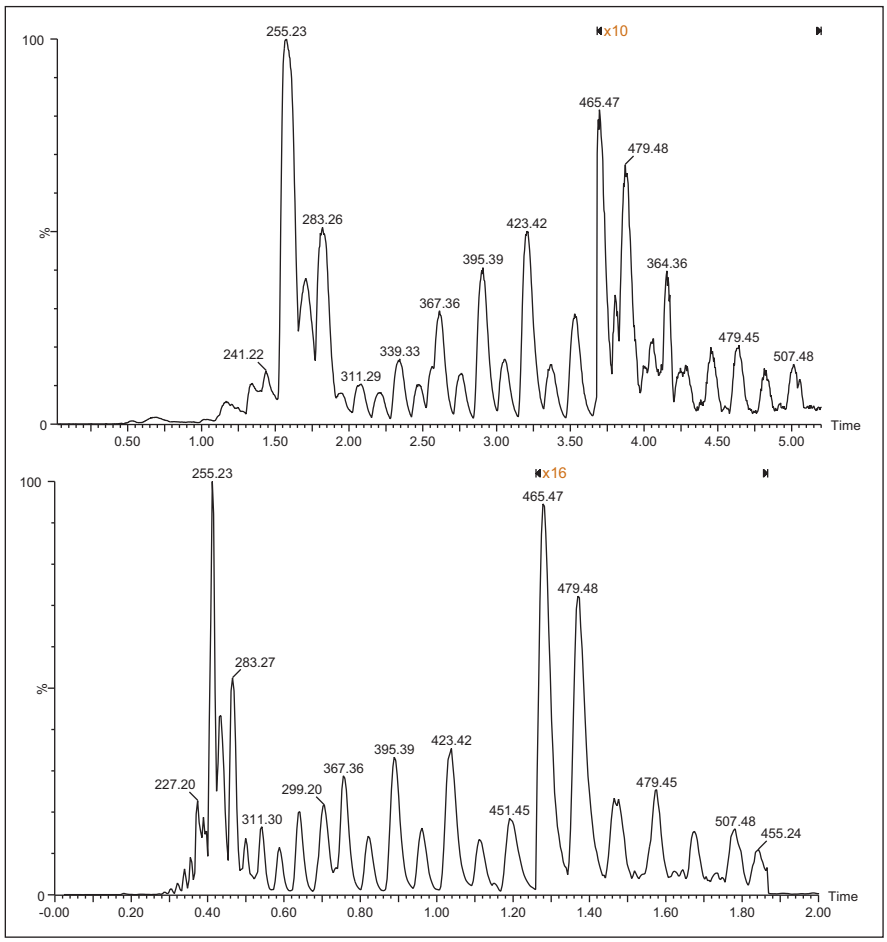

Figure 5. Representative chromatogram from algaenan 1 with various co-solvent gradients (top 1% to 10% methanol in 10 minutes, lower 5% to 20% methanol in 10 minutes). (UPC^2 conditions: HSS C_{18} SB (2.1 x 100 mm), flow rate= 1.5 mL/min. The other UPC^2 conditions are described in the method conditions).

The lipid profiles of the algae and algaenan oil were investigated using TransOmics (TOIML) Software to determine the pattern and composition of FFA at two different pyrolysis temperatures. Differential analysis of results across different treatments can quickly be performed, thereby facilitating identification and quantitation of potential biomarkers. The software adopts an intuitive workflow approach to performing comparative UPC^2/Xevo G2 QTof MS metabolomics and lipidomics data analysis.

The workflow starts with UPC^2/MS raw data file loading, then retention time alignment and deconvolution, followed by analysis that creates a list of features. The features are then identified with compound searches and explored using multivariate statistical methods.

Principal component analysis (PCA) was used in the first instance to identify the combination of the FFA species that best describe the maximum variance between algae 1, algae 2, algaenan 1, and algaenan 2 oils (Figure 6). The PCA plot showed excellent technical UPC^2/MS measurements. The PCA plot effectively displays the inter-sample relationships in multi-dimensional hyperspace, with more similar samples clustering together and dissimilar samples separated.[4]

The clustering in Figure 6 indicates that algae 1 and algaenan 1 are different, but algae 2 and algaenan 2 have more similarity in their FFA compositions after high pyrolysis temperature treatment. Orthogonal projections latent structure discriminant analysis (OPLS-DA) binary comparison can be performed between the different sample groups (algae 1 vs. algae 2, algaenan 1 vs. algaenan 2, algae 1 vs. algaenan 1, and algae 2 vs. algaenan 2) to find out the features that change between the two groups.

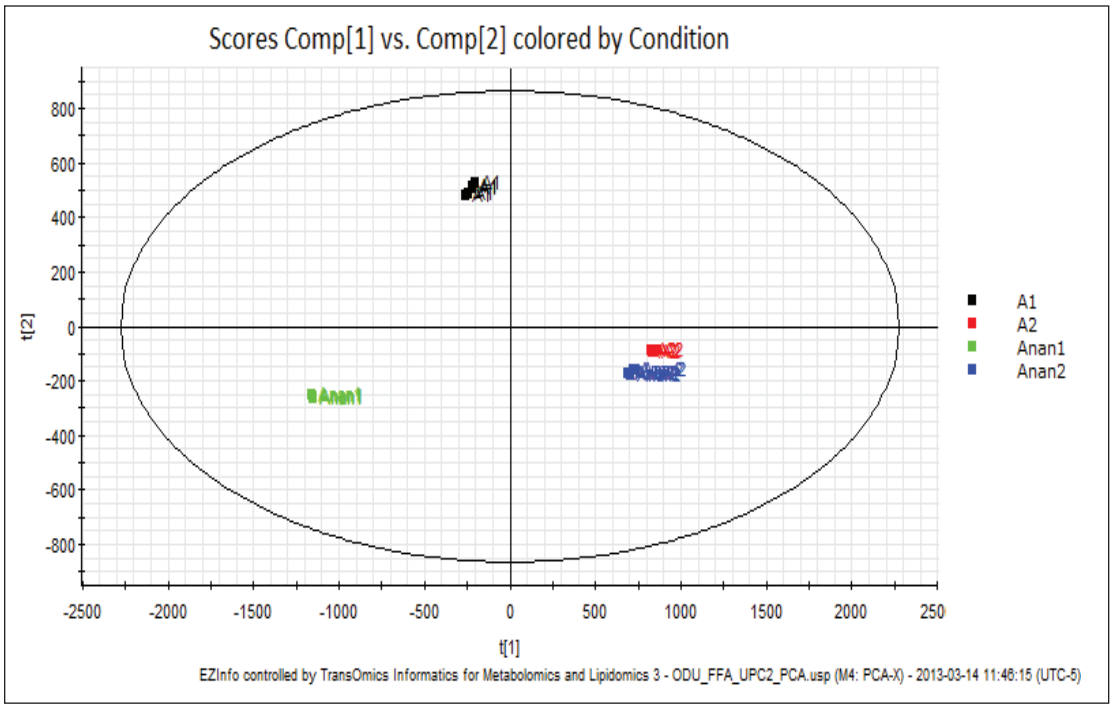

Figure 6. Principal component analysis of algae and algaenan oil extracts treated at low and high pyrolysis temperature. (A1= algae at low pyrolysis temperature A2= algae at high pyrolysis temperature Anan1= algaenan at low pyrolysis temperature Anan2= algaenan at high pyrolysis temperature).

As an example, the OPLS-DA binary comparison between algae 1 vs. algae 2 is shown in Figure 7A. As shown in the S-plot, the features that contribute most to the variance between the two groups are those farthest from the origin of the plot, highlighted in red (Figure 7B). These selected features can be exported to TransOmics for further identification. This helps the researcher focus on the features/compounds that change between samples instead of spending time on the whole data set.

Figures 7C and 7D show representative trend plots that change most between algae 1 and algae 2. Figure 8A shows the ion map, mass spectrum, and chromatogram across all the runs for FFA 29:0. This view allows to review compound measurements such as peak picking and alignment to ensure they are valid across all the runs. Figure 8B shows the normalized abundance of FFA 29:0 across all the conditions. FFA 29:0 is elevated in algeanan 1 compared to algae 1, algae 2, and algeanan 2; however, there is no significant difference between algae 2 and algeanan 2. Detailed investigation and comparison between algae 1 and algae 2 showed that algae 1 contains elevated levels of short (C9:0 to C13:0) and long (C31:0 to C37:0) chain FFA, whereas algae 2 contains elevated levels of medium (C14:0-C29:0) chain FFA. Similarly, the comparison between algaenan 1 and algaenan 2 showed that algaenan 1 contains elevated levels of long (C28:0 to C37:0) chain FFA, whereas algaenan 2 contains elevated levels of short and medium (C9:0 to C27:0) chain FFA.

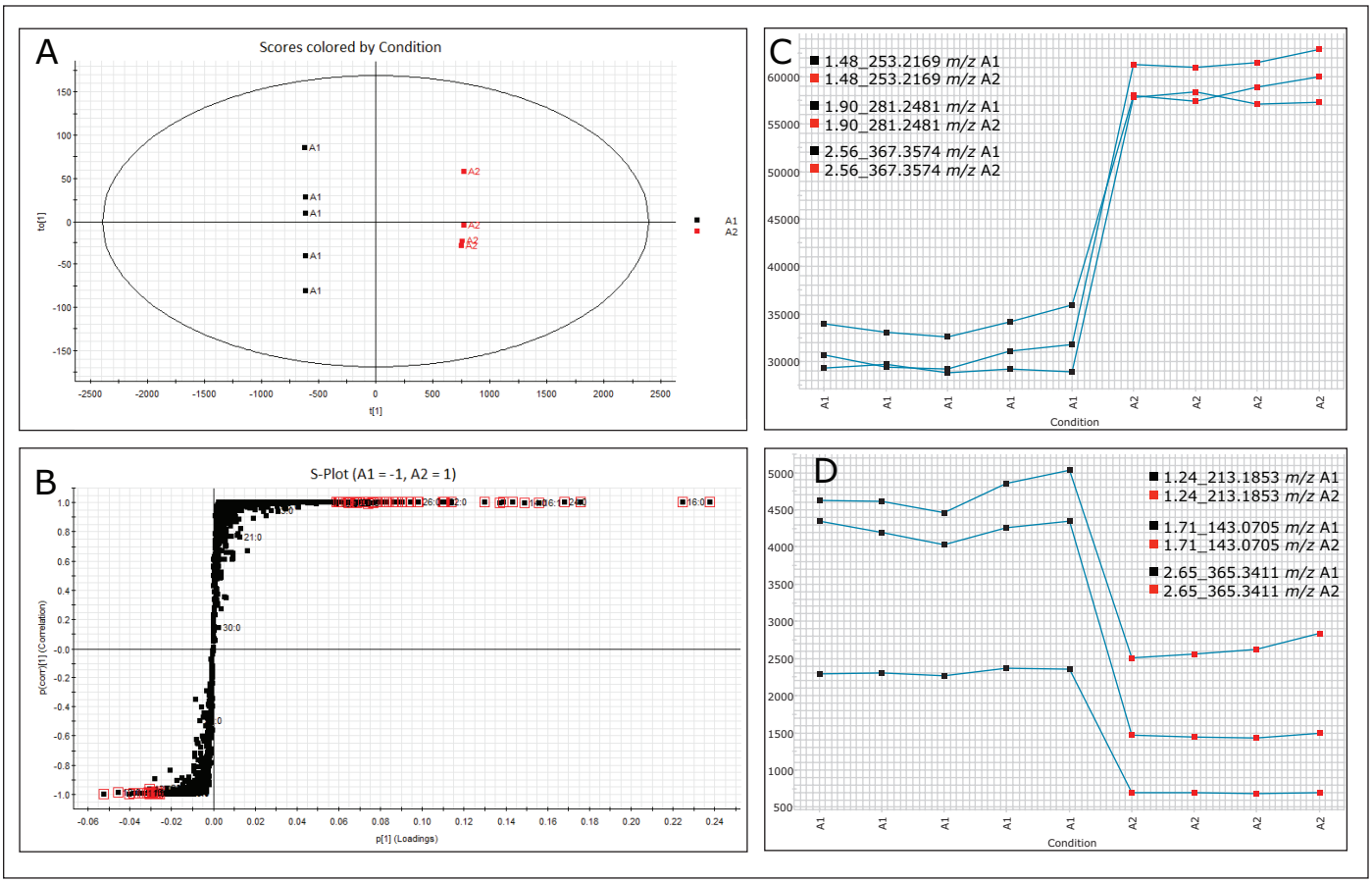

Figure 7. (A) OPLS DA plot between algae 1 and algae 2 group difference. (B) S-plot indicating the major features (highlighted in red) that contribute to the group difference between algae 1 and algae 2. (C) Representative trend plot showing the major up-regulated 16:1, 18:1, and 24:0 FFA in A1 (D) Representative trend plot showing the major up-regulated 8:0, 13:0, and 24:1 FFA in A2. (A1= algae at low pyrolysis temperature A2= algae at high pyrolysis temperature).

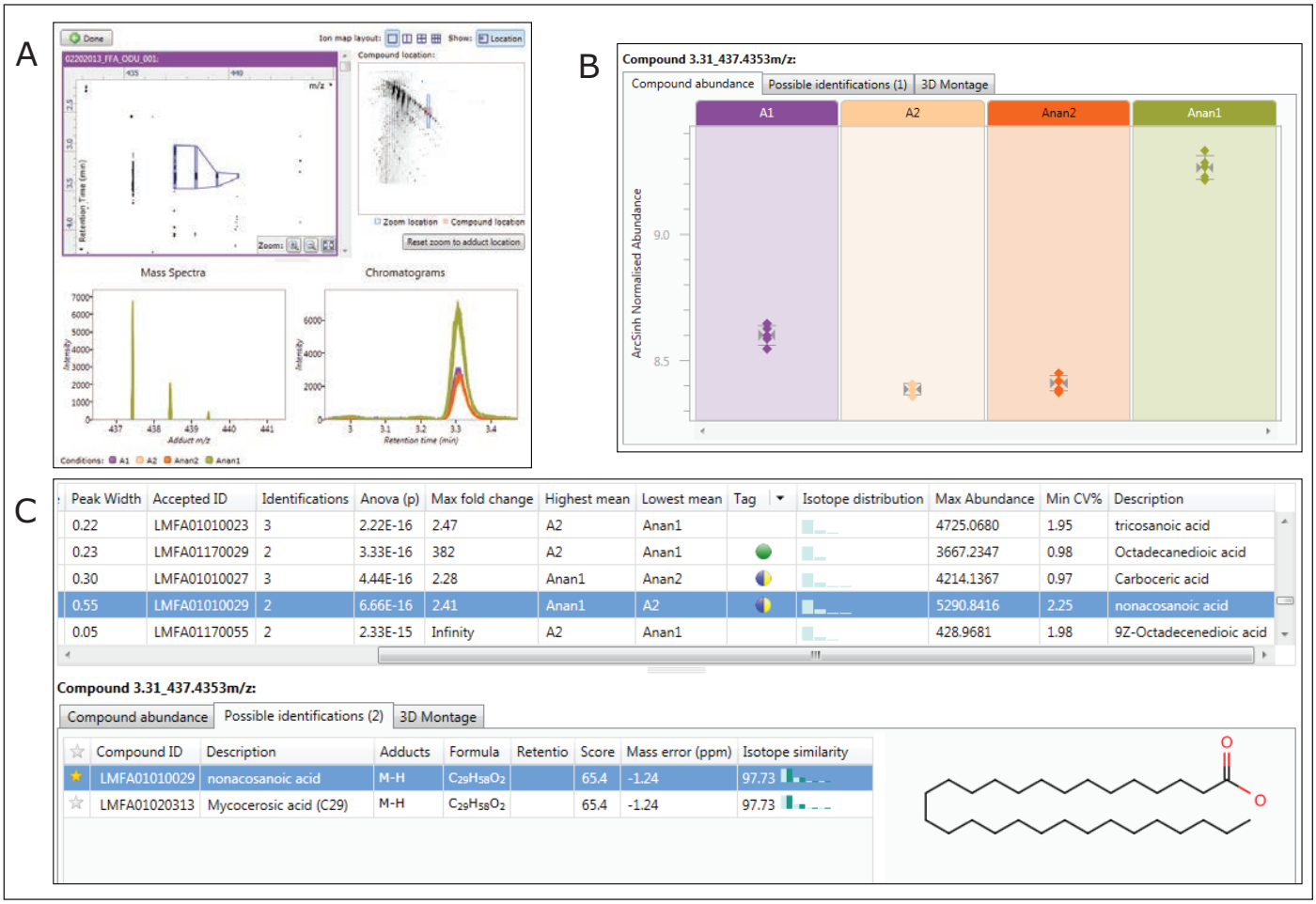

Figure 8. (A) Selected FFA 29:0 showing its ion map, mass spectrum, and chromatogram across all the runs. (B) Normalized abundance of FFA 29:0 across all the conditions. (C) Identification can be performed by means of local or web-based database search. In this example, the feature with retention time and exact mass pair 3.31_437.4353 is identified as nonacosanoic acid (29:0 FFA). (A1= algae at low pyrolysis temperature, A2= algae at high pyrolysis temperature; Anan1= algaenan at low pyrolysis temperature Anan2= algaenan at high pyrolysis temperature).

Identification can be performed by means of local or web-based (such as LIPID MAPS, HMDB, and METLIN) compound searches based on retention time, low energy exact mass, high energy fragment ion, theoretical isotope pattern distribution, and collision cross section area (CCS) (Figure 8C). In this example, the feature with retention time and exact mass 3.31_437.4353 is identified as nonacosanoic acid (29:0 FFA) based on retention time, low energy exact mass, and theoretical isotope pattern distribution. Figure 9 shows the expression and abundance profile of selected features according to their relative similarity between the different groups.

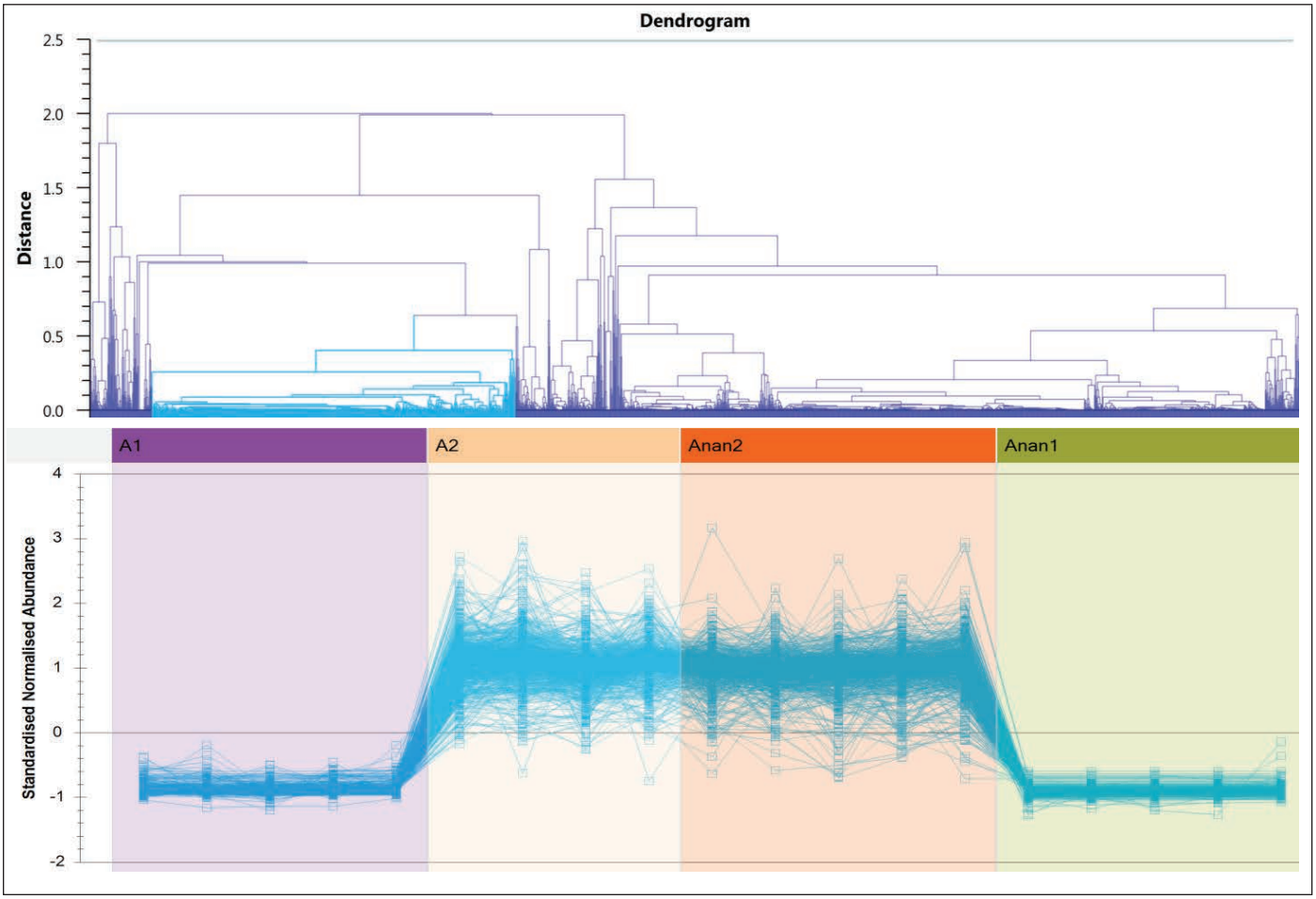

Figure 9. Expression and abundance profile of selected features according to their relative similarity between the different groups.

CONCLUSIONS

The UPC^2/MS FFA analysis described provides a simple and fast method with a significant reduction in analysis time compared to alternative techniques such as GC/MS, which requires FAME derivatization. In addition, the organic layer extract containing the lipids can be injected directly into the system, omitting the need for solvent exchange for compatibility with reversed-phase LC methods.

Saturated and unsaturated FFA containing C_8 to C_{36} carbons were separated and determined, including low volatile very long chain fatty acids (>24 carbon atoms) that have challenged GC/MS even after FAME derivatization. Data analysis and FFA identification was facilitated using TransOmics for Metabolomics and Lipidomics Software that adopts an intuitive workflow approach to performing comparative ACQUITY UPC^2/Xevo G2 QTof MS metabolomics and lipidomics data analysis.

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APPLICATION HIGHLIGHT:
Analysis of Steroids by UltraPerformance Convergence Chromatography

Christopher J. Hudalla and Kenneth J. Fountain/Waters Corporation, Milford, MA, USA

INTRODUCTION

Steroid biosynthesis is a complex metabolic pathway utilizing simple precursors to synthesize multiple steroidal forms. This biosynthetic pathway is unique to animals and provides a common target for antibiotics and other anti-infective drugs. Precise and accurate steroid analysis is critical for the development of steroid-based therapeutics. Typical analysis methods utilize GC/MS, which require sample derivatization and lengthy analysis times (~25 minutes), or LC/MS with typical analysis times of four to 12 minutes. Many of the steroid structures are closely related making their analysis challenging even when using the selectivity of mass spectrometric detection. Chromatographic separation is, therefore, essential for analysis of steroids and steroid derivatives resulting in long analysis times. Convergence chromatography, with CO_2 as the primary mobile phase, presents a unique opportunity to provide rapid and precise analyses for these structurally related compounds, as shown in Figure 1.

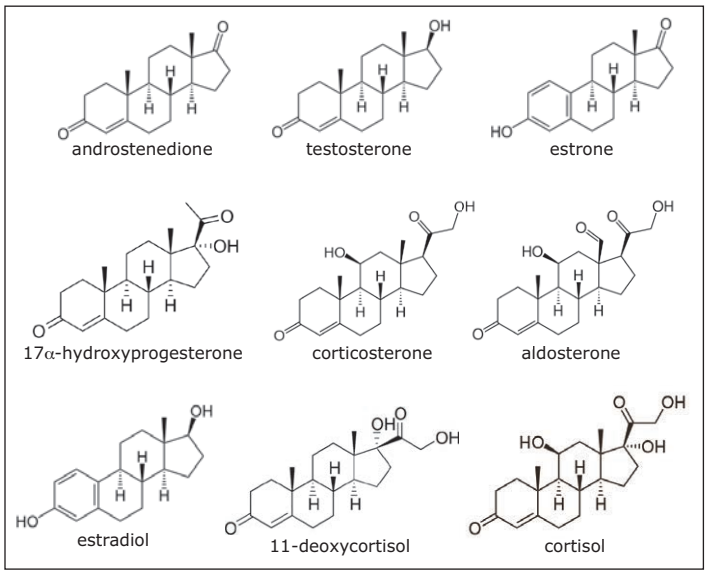

Figure 1. Steroid structures for the current investigation.

EXPERIMENTAL CONDITIONS

A mixture of nine steroids was prepared at a concentration of 0.2 mg/mL each, using a diluent of 88:12 methanol/ethanol. Steroids used included the following: androstenedione, estrone, 17α-hydroxyprogesterone, testosterone, 11-deoxycortisol, estradiol, corticosterone, aldosterone, and cortisol.

All data was collected on an ACQUITY® UltraPerformance Convergence™ Chromatography (UPC²™) System with photodiode array (PDA) detection. The steroid sample was screened on three different ACQUITY UPC²™ column chemistries including: BEH, BEH 2-EP, and CSH Fluoro-Phenyl, using a 1.7 µm particle size in a 3.0 x 50.0 mm column dimension. The mobile phases were CO_2 with methanol as a co-solvent. A two-minute screening gradient was used from 2% to 17% methanol at a flow rate of 3.65 mL/min, and a temperature of 40 °C. The Automatic Back Pressure Regulator (ABPR) was set to 1800 psi. Data was collected at 220 nm (compensated for 380 to 480 nm). The injection volume was 1 µL.

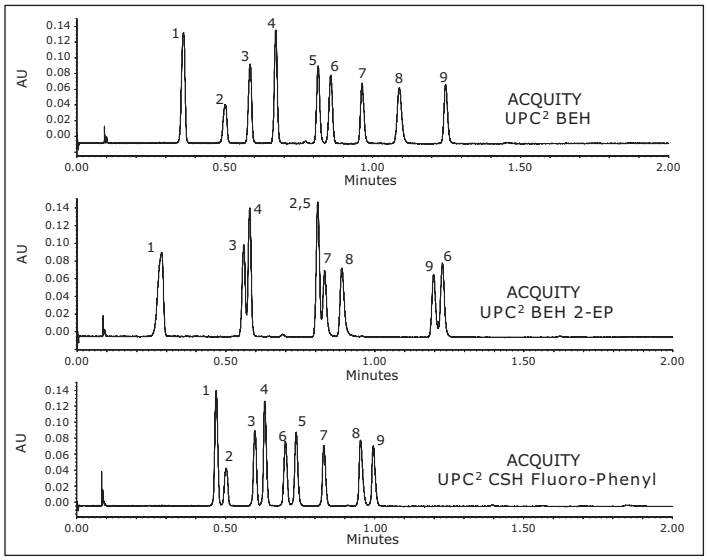

Figure 2. UPC² separations (with UV detection) of steroid standards on the following ACQUITY UPC² columns: BEH (top), BEH 2-EP (middle), and CSH Fluoro-Phenyl (bottom). All columns were 1.7 µm, 3.0 x 50.0 mm configurations. Steroid compounds are (1) androstenedione, (2) estrone, (3) 17a-hydroxyprogesterone, (4) testosterone, (5) 11-deoxycortisol, (6) estradiol, (7) corticosterone, (8) aldosterone, and (9) cortisol.

CONCLUSIONS

The chromatograms shown in Figure 2 demonstrate the selectivity differences of the ACQUITY UPC^2 stationary phases, as well as the inherent speed of this chromatographic technique, with a significant reduction in analysis times compared to alternative techniques. Without the need for derivitization (required for GC analysis), samples can be analyzed directly in organic extraction solvents, omitting the need for diluent exchange for compatibility with reversed-phase LC methods. These factors combined yield a streamlined workflow with significant savings in analysis and sample prep time, solvent costs, and solvent waste disposal.

ORDERING INFORMATION

Columns	Part Number
ACQUITY UPC^2 BEH 3.0 x 50.0 mm, 1.7 µm Column	186006562
ACQUITY UPC^2 BEH 2-EP 3.0 x 50.0 mm, 1.7 µm Column	186006580
ACQUITY UPC^2 CSH Fluoro-Phenyl 3.0 x 50.0 mm, 1.7 µm Column	186006571

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Qualitative and Quantitative Analysis of β-carotene Using UPC²

Jacquelyn Runco, Lakshmi Subbarao, and Rui Chen
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- A fast UPC²™ method to separate the three most common carotenoids that minimizes the risk of degradation.
- The UPC² method is four times faster than the traditional methods of analysis, thereby, reducing organic solvent consumption by 85%.
- For the trageted analysis, the β-carotene extract in MTBE can be directly injected onto an ACQUITY UPC²™ System for analysis without the need for time-consuming evaporation and reconstitution steps.

WATERS SOLUTIONS

ACQUITY UPC² System with a photodiode array (PDA) detector

MassLynx® Software

ACQUITY UPC² HSS C_{18} SB Column

KEY WORDS

Carotenoids, lutein, β-carotene, lycopene, fat-soluble, vitamins, convergence chromatography, UPC²

INTRODUCTION

Carotenoids are natural pigments synthesized by plants and some microorganisms. For animals and humans, carotenoids play an important role in vision. Carotenoids also act as important antioxidants with a preventative effect for various diseases.[1-2] Since carotenoids cannot be synthesized *de novo* in the human body, humans need to acquire them through diet and supplements. In 2010, the market value of commercially used carotenoids was estimated to be $1.2 billion and projected to grow to $1.4 billion by 2018.[3] As more stringent legislation for regulatory compliance of micronutrients in fortified food products and dietary supplements is being enacted or contemplated, there is an increasing demand for rapid and reliable analytical methods for the analysis and quantification of carotenoids in a variety of matrices.[4] The speed of analysis is of particular importance because regulatory compliance monitoring often requires a large number of assays. In addition, many carotenoids are thermal- or photo-sensitive and highly susceptible to isomerization and chemical degradation. Prolonged analysis time could lead to inaccurate quantification results.

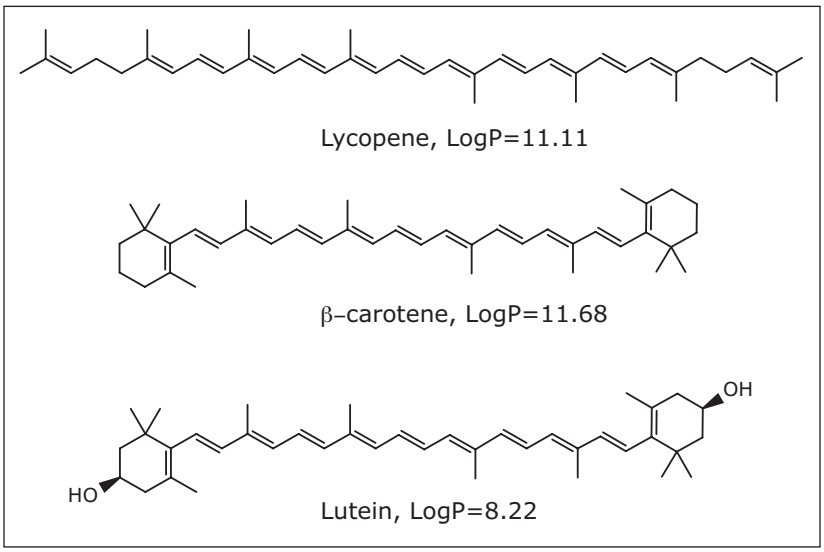

Figure 1. Chemical structures and LogP values of three carotenoids used in this study.

EXPERIMENTAL

UPC² conditions for column screening

System:	ACQUITY UPC²
Detection:	PDA detector
Flow rate:	1.5 mL/min
Mobile phase A:	CO_2
Mobile phase B:	Ethanol
Column:	ACQUITY UPC² BEH, CSH™ Fluoro-Phenyl, BEH 2-EP (3.0 x 100 mm, 1.7 µm), and HSS C_{18} SB (3.0 x 100 mm, 1.8 µm)
Back pressure:	2190 psi
SM temp.:	5 °C
Temp.:	40 °C
Sample diluent:	MTBE
Injection volume:	1 µL
Vials:	Waters® Amber Glass 12 x 32 mm Screw Neck Vial, 2 mL
PDA scan range:	220 to 600 nm
Data management:	MassLynx Software

Gradient:

Time (min)	B%
0	5
5	20
7	20
8	5
10	5

The central part of the carotenoid structure is the long polyene chain of alternating double and single bonds, as shown in Figure 1. Consequently, the carotenoids often possess high hydrophobicity, especially those that do not contain any hetero-atoms, such as lycopene and β-carotene. High-performance liquid chromatography (HPLC) with various absorbance detectors is the most commonly used analytical technique for determining carotenoids qualitatively and quantitatively.[1-2, 6-10] Due to their high hydrophobicity, separation of carotenoids by RPLC often results in lengthy analysis times. Furthermore, all RPLC-based methodologies generally suffer from the low solubility of carotenoids in the mobile phase. Non-aqueous reversed phase (NARP) LC has been employed to reduce the run time by using semi-aqueous or non-aqueous mobile phases. However, the NARP approach often involves the use of complex mixtures of organic solvents as the mobile phase. For example, in the official AOAC method for β-carotene in supplements and raw material,[11] a mixture of butylated hydroxytoluene (BHT), isopropanol, *N*-ethyldiisopropylamine, ammonium acetate, acetonitrile, and methanol is used as the mobile phase.

The separation of carotenoids has long been the subject of supercritical fluid chromatography (SFC)[12-18] studies since its inception.[12] The primary component of the mobile phase in SFC, CO_2, offers superior solubility for carotenoids and promotes non-polar interactions between carotenoids and the mobile phase, thereby reducing the retention time.[17] In addition to high chromatographic efficiency rendered by the high diffusivity of CO_2, the mild temperatures used in SFC are advantageous by avoiding thermal degradation of carotenoids.

UltraPerformance Convergence Chromatography™ (UPC²) is a new category of separation science that marries the merits of both SFC and UPLC. While adhering to the basic principles of SFC, UPC² leverages the reduced system volume of UPLC, and more importantly, the exceptional separation power of sub-2-µm particle packed columns, thereby, resulting in a greatly reduced run time, improved resolution, and increased detection sensitivity.

In this application note, we describe fast separations of three common carotenoids by UPC² in less than 2 minutes. A quantitative analysis of β-carotene dietary supplement capsules is also demonstrated.

EXPERIMENTAL

Optimized UPC^2 conditions for β-carotene extract analysis

Flow rate:	1.5 mL/min
Mobile phase:	75:25 CO_2/ethanol, isocratic
Column:	ACQUITY UPC^2 HSS C_{18} SB 3.0 x 100 mm, 1.8 µm
Back pressure:	2190 psi
SM temp.:	5 °C
Temp.:	40 °C
Sample diluent:	MTBE
Injection volume:	1 µL
Vials:	Waters Amber Glass 12 x 32 mm Screw Neck Vial, 2 mL
PDA scan range:	350 to 600 nm
Wavelength compensation:	440 nm with a reference wavelength 550 to 600 nm

Sample description

All sample preparation was performed in an environment with subdued lighting. For the column screening and subsequent optimization, 1 mg each of lycopene, β-carotene, and lutein was dissolved in 10 mL of methyl tert-butyl ether (MTBE) to make a 0.1 mg/mL (each) stock solution.

Calibration curve: A serial dilution of a stock solution of β-carotene (0.1 mg/mL in MTBE) was performed. The average peak area of three replicate injections at each concentration was used for each data point.

Capsule analysis: Three β-carotene capsules with a label claim of 15 mg/capsule were prepared by cutting them open and dissolving the contents in 250 mL of MTBE with slight perturbation. For each assay, six replicate injections were performed, and the average peak area was used for calculating β-carotene content in the capsules.

RESULTS AND DISCUSSION

Lutein, lycopene, and β-carotene are the three most common carotenoids found in the North American diet. Preliminary screening work revealed that methanol as mobile phase B (co-solvent) resulted in poor peak shape due to the low solubility of carotenoids in methanol, while isopropanol as a co-solvent led to broader peaks. Ethanol was, therefore, chosen as the co-solvent in all experiments. Figure 2 shows the UPC^2/UV chromatograms of the carotenoids mixture from the column screening. The peak identities were confirmed by injecting individual standard using the same condition. While the ACQUITY UPC^2 C_{18} SB Column yielded baseline resolution of all three carotenoids, another relatively non-polar column, CSH Fluoro-Phenyl, also provided partial separation between lycopene and β-carotene. No separation between lycopene and β-carotene was observed with either BEH or BEH 2-Ethyl Pyridine columns. Despite the similarities in structure and polarity between lycopene and β-carotene, the octadecyl carbon chains on the ACQUITY UPC^2 C_{18} SB stationary phase offered sufficient resolution to differentiate the two analytes in UPC^2.

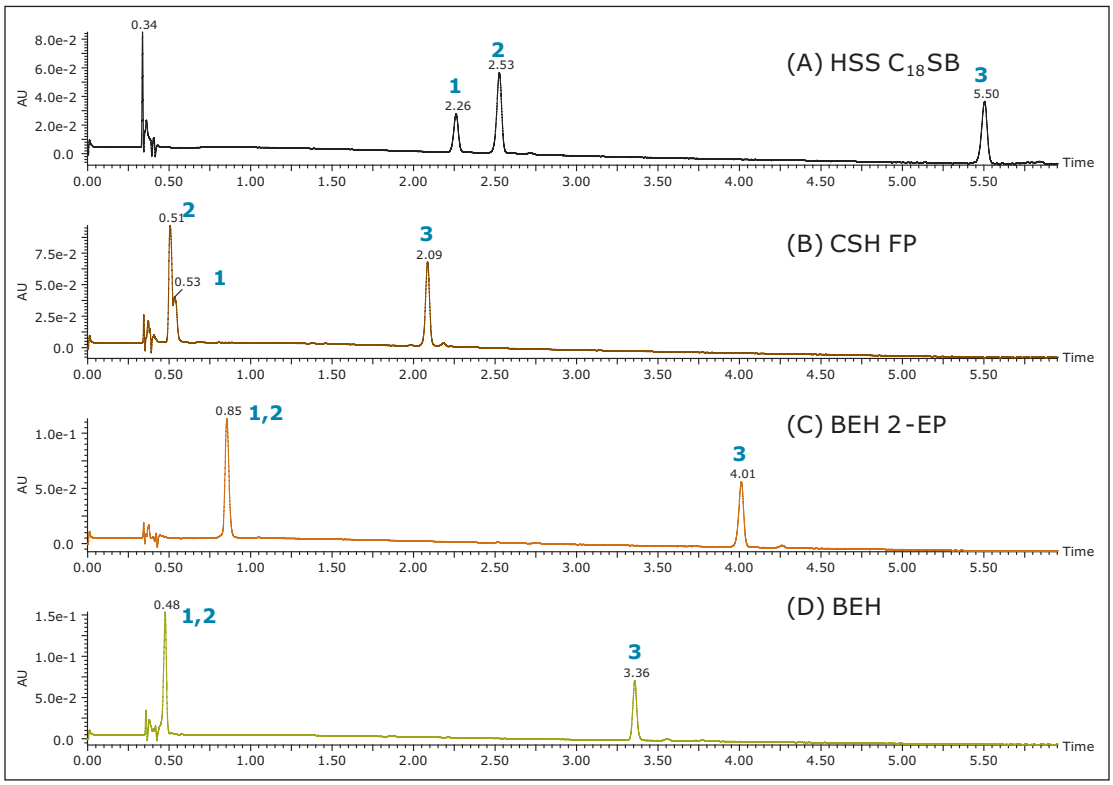

Figure 2. UPC^2/UV chromatograms of a mixture of lycopene, β-carotene, and lutein using the following different columns: (A) HSS C_{18} SB, (B) CSH Fluoro-Phenyl, (C) BEH 2-EP, and (D) BEH. The identities of the peaks are as follows: 1. Lycopene, 2. β-carotene, and 3. Lutein.

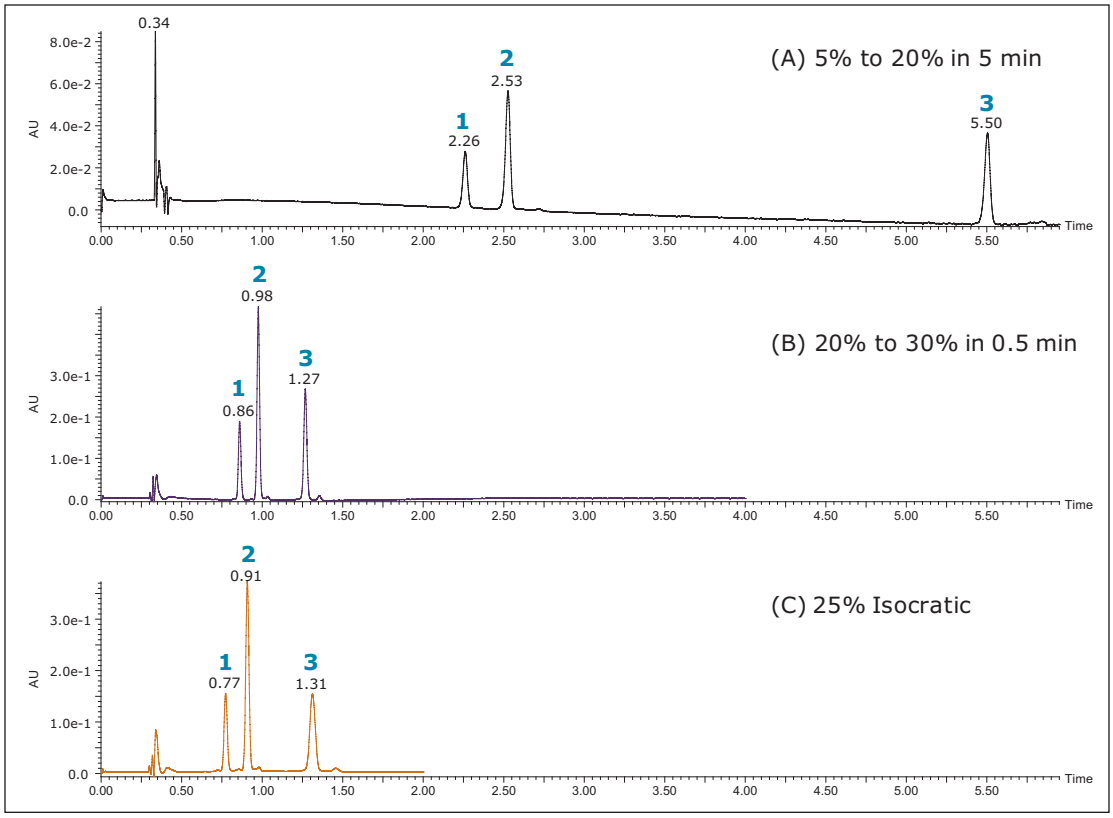

Figure 3. UPC²/UV chromatograms obtained using an ACQUITY UPC² HSS C_{18} SB Column under different gradient/isocratic conditions including: (A) the initial screening condition: 5% to 20% in 5 min, (B) 20% to 30% in 0.5 min, and (C) 25% isocratic. The identities of the peaks are: 1. Lycopene, 2. β-carotene, and 3. Lutein.

Next, an optimization step was performed to shorten the run time. A ballistic gradient of 20% B/min, shown in Figure 3B, and an isocratic method at 25% B, as seen in Figure 3C, both offered sufficient resolution for all three carotenoids with a run time of less than 2 min. The late-eluting peak (lutein) from the isocratic method has a slightly wider peak width than that from the gradient method, but the isocratic method generated a smoother baseline that can be beneficial for low level detection. The isocratic method was, therefore, chosen for ensuing quantitative analyses. The optimized method is four times faster than traditional methods of analysis.5 As a result, the organic solvent consumption was reduced by ~85%. It is also important to note that for SFC using C18 columns, retention of non-polar analytes, such as carotenoids, decreases with the analytes' solubility in the mobile phase.[17] Since compressed CO_2 offers superior solubility for non-polar analytes, UPC² is inherently more compatible and faster for carotenoids analyses than RPLC.

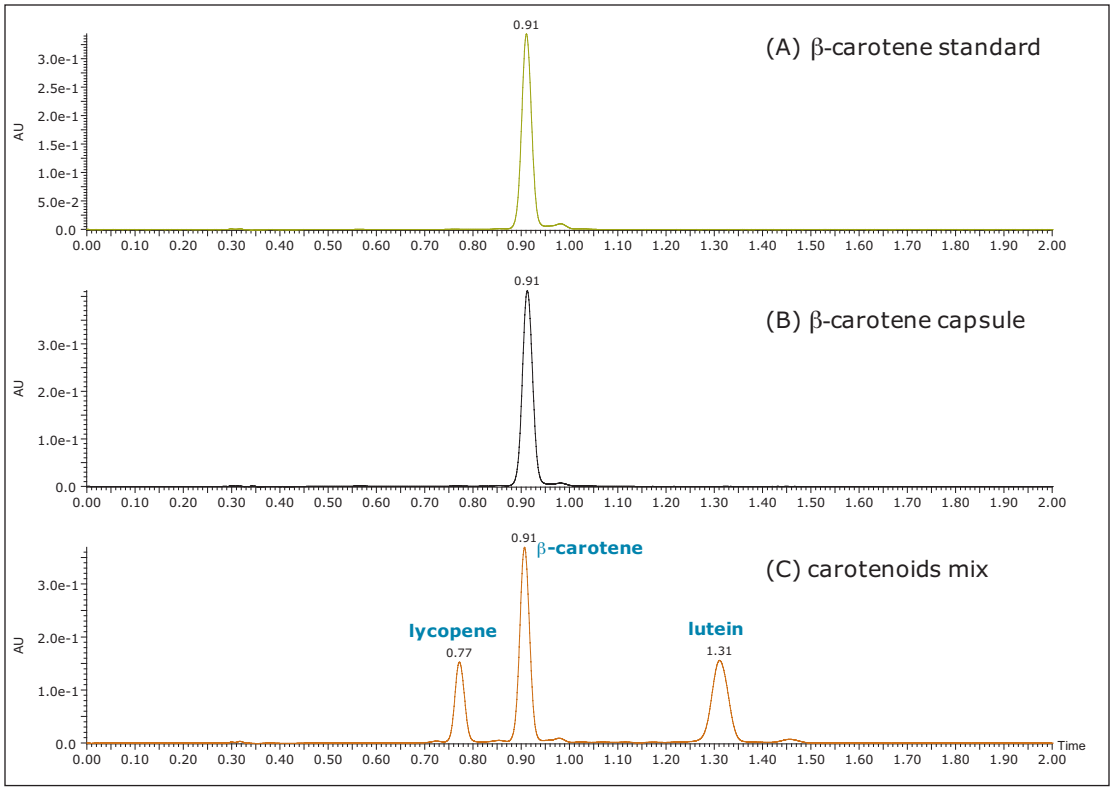

Figure 4. UPC^2 chromatograms of (A) β-carotene standard, (B) β-carotene extract from capsules, and (C) three carotenoids mixture under optimal chromatographic conditions.

For quantification, the β-carotene content of a commercially available capsule formulation was simply dissolved in MTBE, and the resulting extract was directly injected onto an ACQUITY UPC2 System for analysis using the optimized method, shown in Figure 3C. A representative chromatogram of the resulting β-carotene extract is shown in Figure 4B. The simple sample preparation exemplifies another advantage of using UPC2 for low polarity sample analysis. Dissolving low polarity samples often requires the use of low polarity solvents, such as MTBE and hexane, which are inherently compatible with UPC2. In contrast, RPLC requires that samples dissolved in low polarity organic solvents be evaporated and reconstituted into suitable diluents prior to analysis.

Figure 5 shows a calibration curve for β-carotene in MTBE with concentrations ranging from 0.0001 to 0.1 mg/mL. The linearity range spans three orders of magnitude with R2 > 0.99. The limit of detection (LOD, defined as S/N >3) and the limit of quantitation (LOQ, defined as S/N >10) are 50 and 100 ng/mL, respectively. These values are equivalent or better than those reported using HPLC.6-8 The high detection sensitivity can be attributed to, in part, the inherent compatibility between carotene analysis and UPC2. The non-polar interaction between CO2 and β-carotene greatly reduces its retention, thus results in an early eluting sharp peak for improved detection sensitivity.

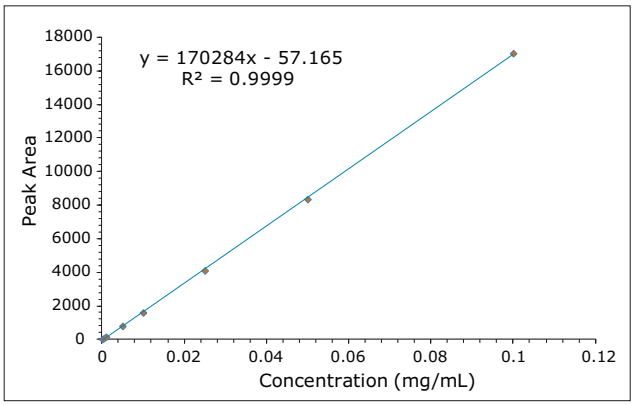

Figure 5. Calibration curve for β-carotene in MTBE with a concentration range of 0.0001 to 0.1 mg/mL in MTBE.

Tables 1 and 2 summarize the β-carotene capsule analyses. Excellent inter- and intra-assay reproducibility in both retention time and peak area was achieved. Overall assays also yielded good accuracy against the label claim. The sample preparation was simple and straightforward, and the chromatographic analyses using UPC2 were fast and reproducible.

Injection	Peak area	Retention time (min)
1	10348	0.91
2	10291	0.91
3	10382	0.91
4	10330	0.91
5	10313	0.91
6	10293	0.91
Average	10326.17	0.91
RSD%	0.34	0

Table 1. Reproducibility of a β-carotene capsule assay with six replicate injections.

Label Claim: 15 mg/capsule				
Assay #1	Assay #2	Assay #3	Average	RSD%
mg/capsule				
15.13	15.39	15.24	15.25	0.84%

Table 2. Quantification of β-carotene in three capsules.

CONCLUSIONS

In summary, a UPC^2 method was successfully developed to separate the three most common carotenoids in less than two minutes. The method is four times faster than traditional methods of analysis, thereby reducing organic solvent consumption by 85%. The short analysis time also minimizes the risk of on-column degradation of the analytes. The improved speed of analysis is attributed to the inherent compatibility between UPC^2 and low polarity analytes. The UPC^2 method uses ethanol as the co-solvent instead of mixtures of organic solvents often used in HPLC methods. Thus, the UPC^2 method is a much more environmentally sustainable method.

A targeted 1.5-minute UPC^2 method was developed for the quantitative analysis of β-carotene in dietary supplement capsules. The dynamic range spans three orders of magnitude, with an LOD and an LOQ of 50 ng/mL and 100 ng/mL, respectively. Using MTBE as the extraction solvent, the resulting β-carotene extract can be directly injected onto an ACQUITY UPC^2 System for analysis without the need for time-consuming evaporation and reconstitution steps often associated with RPLC-based methodology. Excellent reproducibility and accuracy were also demonstrated for dietary supplement capsule analysis. The high-throughput UPC^2 method is ideally suited for laboratories routinely performing quality control and regulatory compliance monitoring where a large number of assays are required.

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Quantitative Analysis of Astaxanthin in Dietary Supplements by UltraPerformance Convergence Chromatography (UPC²)

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APPLICATION BENEFITS

- Due to the non-polar nature of astaxanthin, the UPC²® method offers reduced analysis time due to superior solubility in the supercritical CO_2 mobile phase.
- UPC² employs sub-2-µm particle packed columns resulting in a higher efficiency separation.
- The UPC² method uses a simple CO_2/methanol mobile phase and gradient for astaxanthin analysis, in comparison to the complex solvent scheme currently in use in HPLC, reducing solvent costs and improving safety.
- The excellent precision (RSD <1.5%) and the experimentally determined label claim agreement (within 5%) proves the UPC² astaxanthin analysis can be easily adapted to the current workflow.
- The proposed 5-min UPC² method can improve productivity for laboratories routinely performing quality control and regulatory compliance monitoring where a large number of assays are required.

WATERS SOLUTIONS

ACQUITY UPC2® System

ACQUITY UPC² PDA Detector

ACQUITY UPLC® HSS C_{18} column (3 x 150mm, 1.8 µm)

MassLynx® v4.1

KEY WORDS

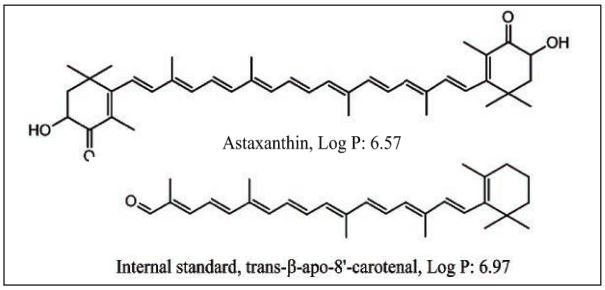
Astaxanthin, carotenoid, dietary supplement, ACQUITY UPC², SFC, label claim, quantitative analysis, food, natural product, nutraceuticals

INTRODUCTION

In recent years, carotenoids have received considerable attention for their antioxidant activity and potential clinical uses.[1] They are widely used in various industries including food, dietary supplements, aquaculture, pharmaceutical, and cosmetics.[2] In particular, astaxanthin (Figure 1) is a carotenoid known for its anti-inflammatory effects and strong antioxidant activity (superior to β-carotene and Vitamin C).[3] Found in large quantities primarily in Haematococcus pluvialis algae, astaxanthin is responsible for the familiar red color of salmon, shrimp, and lobster.[4]

Astaxanthin supplements are produced commercially by many manufacturers.[4] As regulatory compliance monitoring of nutraceuticals becomes more stringent, rapid and reliable analytical methods for quantitation become increasingly necessary. Currently, astaxanthin quantitation is done by two methods: spectrophotometrically, and chromatographically by HPLC. The spectophotometric method suffers from a lack of specificity between the astaxanthin and other carotenoids, resulting in an overestimation of the astaxanthin content, sometimes by as much as 20%.[4] The standard HPLC method has long analysis times, and involves unfriendly, complex (three component normal phase) solvent schemes due to the non-polar properties of the analytes.[5]

Naturally-derived astaxanthin is present primarily as a mixture of fatty acid esters. Many commercial supplements are kept in this form for stability reasons. Therefore, the esterified astaxanthin must first be hydrolyzed (de-esterified) to yield free astaxanthin prior to analysis. An internal standard, *trans*-β-apo-8'-carotenal (Figure 1) is used for quantitation in order to account for any variation in the assays.[6]



EXPERIMENTAL

UPC^2 conditions

All experiments were performed on a Waters ACQUITY UPC^2 system, equipped with an ACQUITY UPC^2 PDA Detector, and controlled by MassLynx software. Following an initial screen of five columns, the ACQUITY UPLC HSS C_{18} (1.8 µm, 3 x 150 mm) Column was selected for method optimization and all quantitative experiments. Table 1 contains the optimized UPC^2 method parameters.

Mobile phase A:	CO_2
Mobile phase B:	Methanol
Flow rate:	1.0 mL/min
Backpressure:	200 Bar
Temperature:	30 °C
Injection volume:	2 µL
Column:	ACQUITY UPLC HSS C_{18}
PDA detector:	Compensated: 457 nm Reference: 530–600 nm

Gradient:	Time (min)	%B
	0	5
	2	15
	3	15
	4	5
	5	5

Table 1. UPC^2 method parameters for astaxanthin analysis.

In UltraPerformance Convergence Chromatography™ (UPC^2) the primary component of the mobile phase, CO_2, has lower viscosity, allowing for faster flow rates and the use of smaller particle sizes, which increases separation efficiency. The efficiency combined with the higher solubility of the non-polar analytes in CO_2 results in faster run times. Here, a fast 5-minute method was developed for astaxanthin quantitation. The method was applied to confirm the label claim for three commercially available astaxanthin supplements.

Standards

For Standard A (Std A) 2.50 mg of *trans*-astaxanthin (Alexis Biochemicals, Farmingdale, NY, USA) was dissolved in 100 mL acetone and then diluted 1:10 for a final concentration of 2.50 µg/mL. The internal standard (I.S.) was prepared by dissolving ~ 3.75 mg 20% oil suspension of *trans*-β-apo-8-carotenal (Sigma-Aldrich, Allentown, PA, USA) in 100 mL acetone for a final concentration of ~7.50 µg/mL. Standard B (Std B) is a mixture of 7.50 µg/mL astaxanthin and ~7.50 µg/mL I.S. The samples were kept in the refrigerator, protected from light, to minimize acetone evaporation and possible photo-degradation of the analytes.

Supplement assay solutions

Three astaxanthin supplement formulations were obtained from commercial sources. The content of one capsule from each supplement was dissolved in 100 mL acetone. The aliquots were further diluted by 1:10 (v/v) in acetone to make the Assay A solution. For each brand of supplement, samples were prepared in triplicate using 2 mL of Assay A solution and 1 mL of I.S. solution. The samples were hydrolyzed by enzymatic de-esterification using cholesterol esterase (following the Fuji methodology).[6] The resulting solution was extracted with 2 mL hexane and centrifuged. The top hexane layer was transferred to another test tube, dried down by nitrogen and reconstituted in 1 mL acetone (Assay B solution). For method development purposes, a hydrolyzed sample (Assay B solution) was spiked with the non-hydrolyzed esters (Assay A solution) to ensure the sample contained both free astaxanthin and its fatty acid esters.

Calculations

The calculations used to determine astaxanthin concentration and % label claim are displayed in Figure 2. All injections were done in triplicate and average areas were used. Due to the unavailability of 9-*cis* and 13-*cis* standards, previously established response factors of 1.1 and 1.3 were used respectively in the peak ratio calculation. Std B injections were performed and the Std B peak ratio (RstdB) was calculated for each supplement. Using the concentration of astaxanthin in Std B, the RstdB value, and the peak ratio for the Assay B solution (RAstx), the astaxanthin concentration in Assay B could be determined. A simple back calculation was done to account for dilution, and compared against the label claim (%Label claim).

Peak ratio (R) of total astaxanthin to I.S:

$$R = \frac{A_{trans} + 1.1A_{9-cis} + 1.3A_{13-cis}}{A_{I.S.}}$$

A = Peak Area

Astaxanthin concentration (C_{Astx}) in Assay B:

$$C_{Astx} = \frac{C_{stdB} \times R_{stdB}}{R_{Astx}}$$

C_{stdB} = Concentration of *trans* - astaxanthin in Std B (7.5 µg/mL)

R_{stdB} = Peak ratio for Std B

R_{Astx} = Peak ratio for Assay B

% Label claim:

$$= \frac{C_{Astx(mg/mL)} \times \frac{1mL\ Assay\ B}{2mL\ Assay\ A} \times 1000\ mL\ dilution}{Label\ claim\ (mg/capsule)} \times 100$$

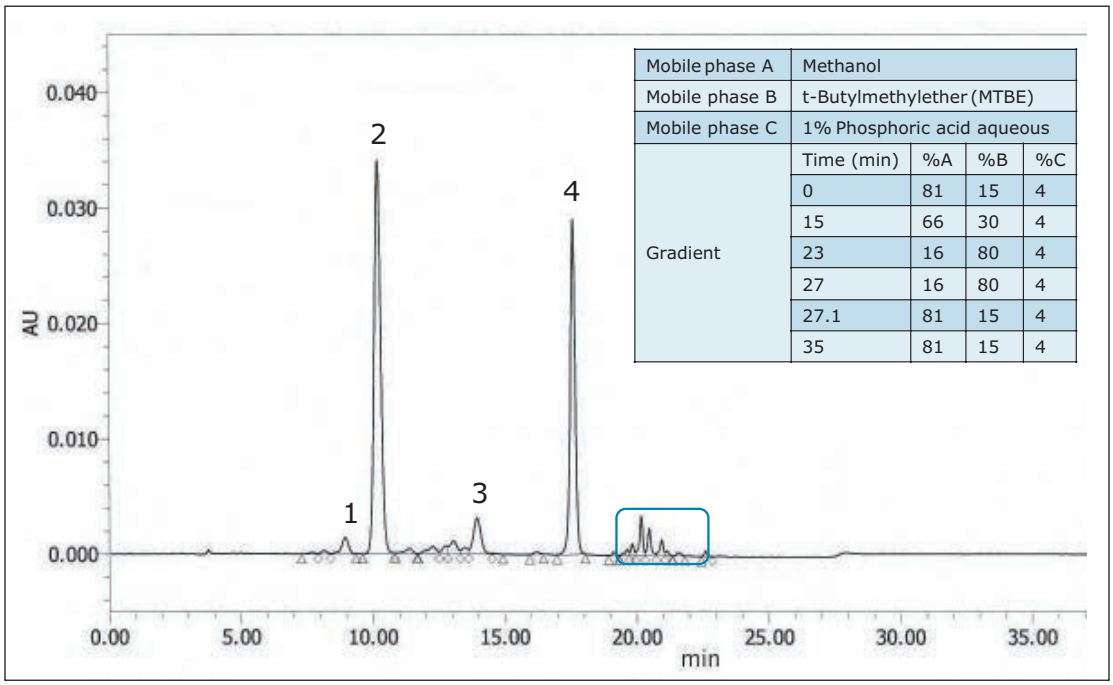
Figure 2. Calculations used to determine astaxanthin concentration and % label claim.

RESULTS AND DISCUSSION

HPLC vs. UPC^2 methods

There are multiple chromatographic challenges associated with astaxanthin quantitation. Free astaxanthin is a mixture of geometric *trans, 9-cis* and *13-cis isomers*; *trans* being the most dominant form. Due to the difference in UV absorption coefficients, an accurate quantitation requires the separation of all three isomers and the internal standard. Also, in the event of incomplete hydrolysis, the astaxanthin esters must be resolved from the rest of the analytes to avoid interfering with the peak areas.

The HPLC method is currently accepted as the standard for astaxanthin quantitation (Figure 3). The method exhibits good separation, but suffers challenges mostly due to the non-polar nature of the analytes. A complex three component mobile phase is required, employing methanol, t-butylmethylether, and an aqueous phosphoric acid solution in a relatively lengthy 35-minute gradient method.



Mobile phase A	Methanol			
Mobile phase B	t-Butylmethylether (MTBE)			
Mobile phase C	1% Phosphoric acid aqueous			
Gradient	Time (min)	%A	%B	%C
	0	81	15	4
	15	66	30	4
	23	16	80	4
	27	16	80	4
	27.1	81	15	4
	35	81	15	4

Figure 3. Astaxanthin separation under the standard HPLC gradient conditions. The peaks are: (1) 13-cis-astaxanthin; (2) trans-astaxanthin; (3) 9-cis -astaxanthin; and (4) I.S. The esters are indicated by the blue rectangle.

In contrast to HPLC, UPC^2 employs supercritical CO_2 as the main component of the mobile phase, offering superior solubility for non-polar analytes. The UPC^2 method uses a simple CO_2/methanol mobile phase and 5-minute gradient method to achieve separation in a little over 2 minutes (a 10-fold improvement over the HPLC method).

In figure 4, three chromatograms are shown. The first (A) displays a sample containing unhydrolyzed esters used for method development to ensure resolution of the esters from the analytes. Figure 4(B) shows Std B (*trans*-astaxanthin standard and I.S.) used to determine the peak ratio (R_{stdB}). Lastly, a fully hydrolyzed supplement (Assay B) is shown in Figure 4(C). The internal standard and astaxanthin peaks were confirmed by MS (not shown), and the geometric isomers were distinguished by their UV spectra, where the 13-*cis* isomer has a characteristic dual maximum.[1]

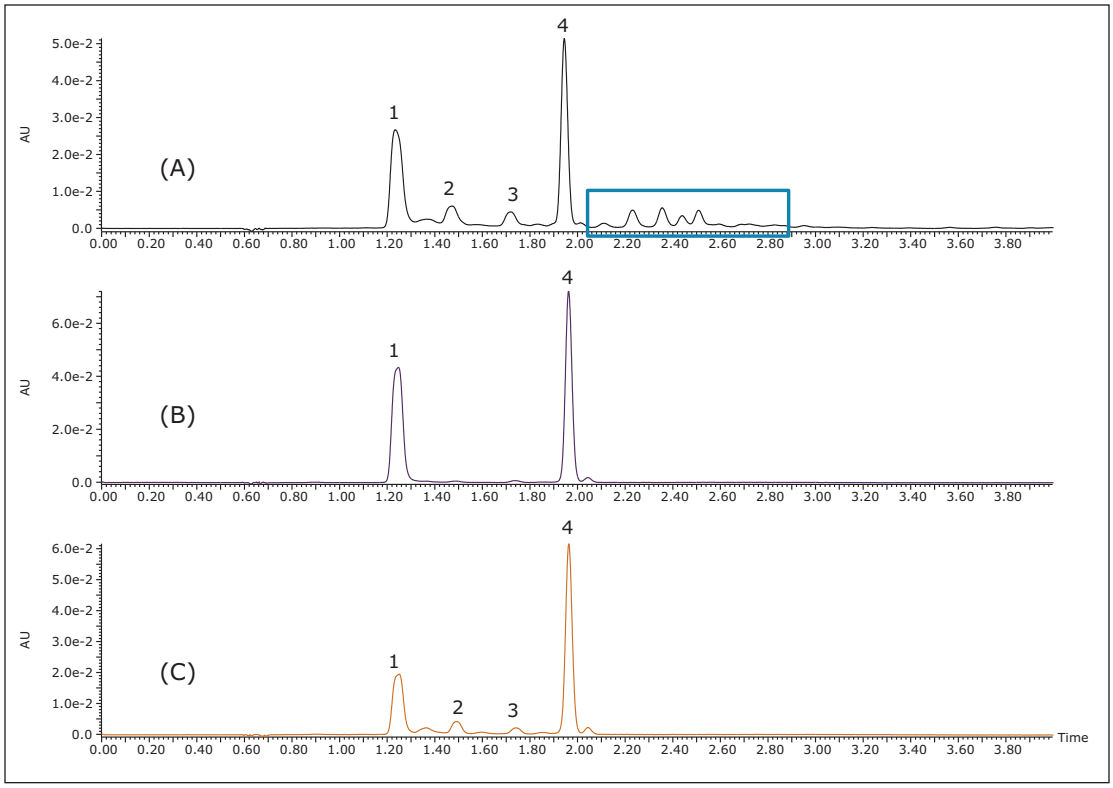


Figure 4. UPC²-UV chromatograms of (A) sample containing esters, (B) Std B, (C) hydrolyzed supplement (Assay B). The peaks are: (1) trans-astaxanthin; (2) 9-cis-astaxanthin; (3) 13-cis -astaxanthin; and (4) I.S. The esters are indicated by the blue rectangle.

Repeatability

Intra- and inter-day experiments were performed using one of the Assay B solutions and the corresponding %RSD was calculated. Six replicate injections were done for the intra-day experiment while the inter-day experiments were carried out over 3 days (6 replicate injections each day). The results are summarized in Table 2.

For intra-day repeatability, the 13-*cis*-astaxanthin exhibits the highest %RSD, possibly due to its relatively small peak area. For inter-day assays, the %RSD values are slightly elevated. This can be ascribed to the propensity of these analytes for degradation in the presence of light and oxygen, and the tendency for isomeric conversion between the *cis* and *trans* isomers. Nevertheless, satisfactory RSDs (<5%) were obtained for both inter- and intra-day assays.

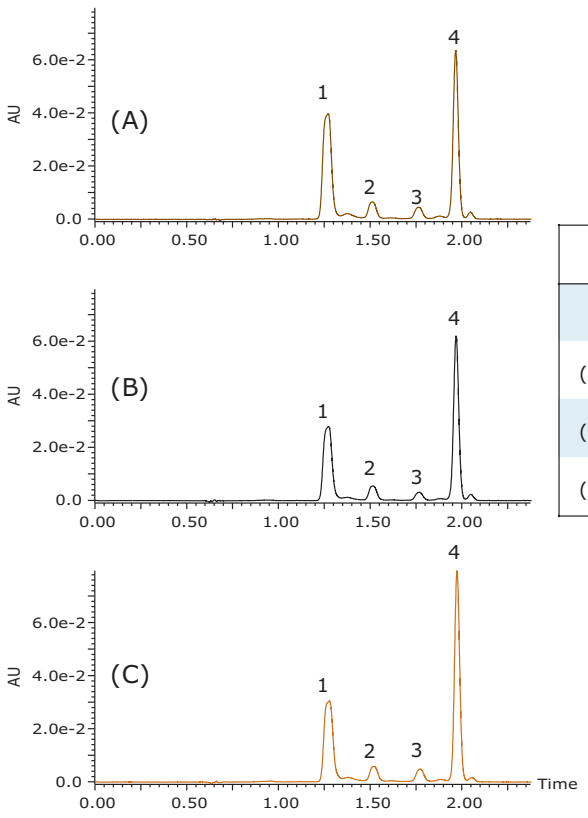
	Intra-day					Inter-day			
Inj #	***Trans***	**9-*cis***	**13-*cis***	**I.S.**	**Day**	***Trans***	**9-*cis***	**13-*cis***	**I.S.**
1	1997	320	133	2199	**1**	1957	320.7	126.7	2156
2	1982	330	131	2155	**2**	1872	325.8	139.3	2233
3	1963	323	128	2154	**3**	1813	315.3	133.7	2249
4	1943	318	123	2146					
5	1937	318	122	2143					
6	1921	315	123	2139					
%RSD	**1.47**	**1.65**	**3.69**	**1.02**	**%RSD**	**3.86**	**1.64**	**4.76**	**2.25**

Table 2. Calculated % RSDs for intraday and inter-day area results.

Supplement analysis

The described UPC^2 method for quantitative analysis of astaxanthin was utilized to confirm the label claim for three commercially available supplements. Example chromatograms for the three dietary supplement assays are displayed in Figure 5 and exhibit similar profiles. No astaxanthin esters were detected, indicating complete hydrolysis.

Each supplement was assayed in triplicate and injected in triplicate. Average areas were used to calculate the % label claims presented. For each supplement, excellent repeatability (%RSD <1.5) was attained, and the experimentally determined content agreed well with the label claim.



	Label	% Label Claim (Avg.)			%RSD
		Assay 1	Assay 2	Assay 3	
(A)	5 mg	101.1	100.4	102.7	1.18
(B)	4 mg	97.74	98.64	99.72	1.00
(C)	4 mg	104.6	101.8	102.5	1.42

Figure 5. UPC^2-UV chromatograms of the hydrolyzed astaxanthin supplements and the calculated %label claim results. The peaks are: (1) trans-astaxanthin, (2) 9-cis-astaxanthin, (3) 13-cis-astaxanthin, and (4) I.S.

CONCLUSIONS

- In UPC^2, supercritical CO_2 offers superior solubility for astaxanthin, resulting in a 10-fold reduction in analysis time when compared to HPLC.
- The optimized UPC^2 method is superior to the standard HPLC method, achieving good resolution with a simpler and faster gradient and mobile phase.
- The method was repeatable, which meant it could be successfully applied to the quantitation of three commercially available astaxanthin dietary supplements.
- Excellent precision was attained for the assays, and the experimentally determined content agreed well with the label claims proving it could be easily adapted into the currently accepted process.
- The UPC^2 method was demonstrated to be rapid and reliable, meeting the requirements necessary in an increasingly regulated and growing market.

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FLAVOR

Authentication of Vanilla Extracts by Convergence Chromatography

Paula Hong, Michael Jones, and Patricia McConville

GOAL

Fast, sensitive analysis for screening and authentication of vanilla extracts with minimal sample preparation, reduced solvent usage, and an orthogonal separation compared to reversed-phase chromatography.

UPC²® Technology provides greater retention of highly polar, secondary components of vanillin while providing adequate retention and identification of potentially harmful, non-polar adulterants.

BACKGROUND

To reduce the cost of vanilla extract, some manufacturers use synthetic or artificial flavorings in place of more expensive pure vanilla. In many instances, these cheaper alternatives include synthetic components, such as ethyl vanillin. However, some extracts contain potentially harmful adulterants, including coumarin, a fragrance derived from tonka beans. This particular adulterant is a suspected carcinogen, and can interact with blood-thinning medications. While coumarin is banned in the United States for use as a food ingredient, in recent years its prevalence in vanilla extracts has led to consumer warnings from the FDA (2009).[1]

A number of reversed-phase liquid chromatography (RPLC) methods have been developed for analyses to determine the actual components in vanilla extract.[2-4] These methods screen for both synthetic and artificial flavorings as well as secondary vanillin components, the latter of which are indicative of authentic extract from vanilla beans. While these methods can provide high-throughput analyses,[4] an orthogonal separation can provide benefits in terms of different selectivity. For example, in reversed-phase separations, some vanillin secondary compounds, such as vanillic acid, are poorly retained, making separation of these polar components challenging.[5] In convergence chromatography, the elution of components is reversed, allowing for greater retention and resolution of highly polar compounds.

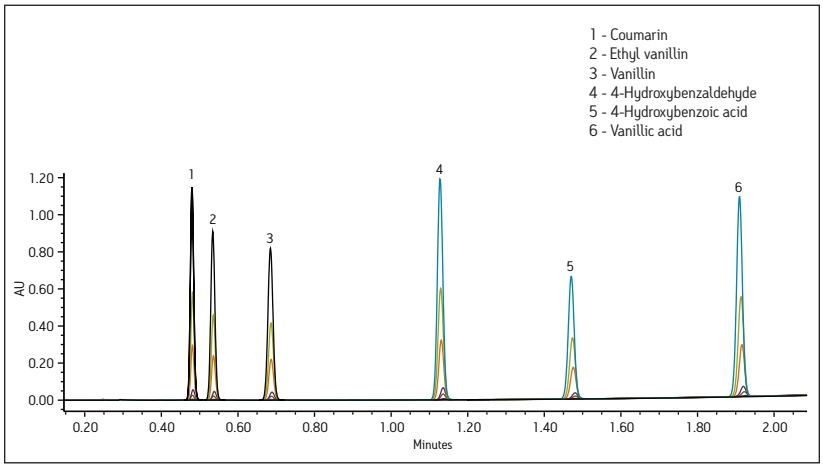

Figure 1. Overlay of linearity calibration standards from 1.25 to 500 µg/mL. Injection n=5 at each level. Wavelength: 260 nm, compensated.

THE SOLUTION

Method development was performed using a standard containing flavor components from vanilla pods (vanillin, 4-hydroxybenzoic acid, 4-hydroxybenzaldehyde and vanillic acid), a synthetic vanillin (ethyl vanillin), and coumarin, a banned adulterant. The standard was prepared in 2-propanol. A 2.5-minute method was developed using an ACQUITY UPC²™ BEH 2- Ethylpyridine 130Å 3.0 x 100 mm, 1.7 µm Column, as shown in Figure 1. The UV method conditions used 20 mM citric acid in methanol as a modifier/additive to improve peak shape for the acidic components.

The UV method was evaluated for repeatability (Table 1) and linearity (Table 2). Standards were prepared from 0.250 to 500 µg/mL. Retention time repeatability (n=5) at 12.5 ug/mL was ≤0.10 %RSD and peak area repeatability for the same standard injections was <1.80 %RSD. Linearity was demonstrated between two to three orders of magnitude, analyte-specific, with R^2 values >0.999 (Table 1). The limit of quantitation (LOQ) for the tested analytes ranged from 0.250 to 1.25 µg/mL. Given that the analysis of vanilla extracts requires dilution of the sample, the sensitivity requirements for this particular assay were met using the UV method.

To test for adulteration, the method was used to screen vanilla extracts including those labeled both pure and imitation, from different geographical regions (Figure 3). The vanilla extracts were diluted 10X in ethanol (for sample miscibility), and filtered prior to analysis. Analysis of the imitation vanilla extract from the United States (A) showed the presence of both synthetic vanillin (ethyl vanillin) and vanillin. The absence of other natural flavor components in this sample indicated that the vanillin was likely from a synthetic source. A known imitation vanilla extract purchased outside the United States (B) contained both the adulterant coumarin as well as vanillin, again likely from a synthetic source due to the absence of the secondary vanilla components. Lastly, analysis of a labeled "pure" vanilla extract (C) confirmed its

Compound	% RSD peak retention time	% RSD peak area
Coumarin	0.093	1.78
Ethyl vanillin	0.10	0.45
Vanillin	0.10	0.53
4-Hydroxybenzaldehyde	0.074	0.26
4-Hydroxybenzoic acid	0.088	0.61
Vanillic acid	0.070	0.66

Table 1. Repeatability data for vanilla extract standards (12.5 µg/mL). Injection n=5.

Compound	R^2	Linearity range
Coumarin	0.999915	0.25 to 500 µg/mL
Ethyl vanillin	0.999970	1.25 to 500 µg/mL
Vanillin	0.999961	1.25 to 500 µg/mL
4-Hydroxybenzaldehyde	0.999970	0.25 to 500 µg/mL
4-Hydroxybenzoic acid	0.999882	1.25 to 500 µg/mL
Vanillic acid	0.999954	1.25 to 500 µg/mL

Table 2. Linearity data for compounds by UPC².

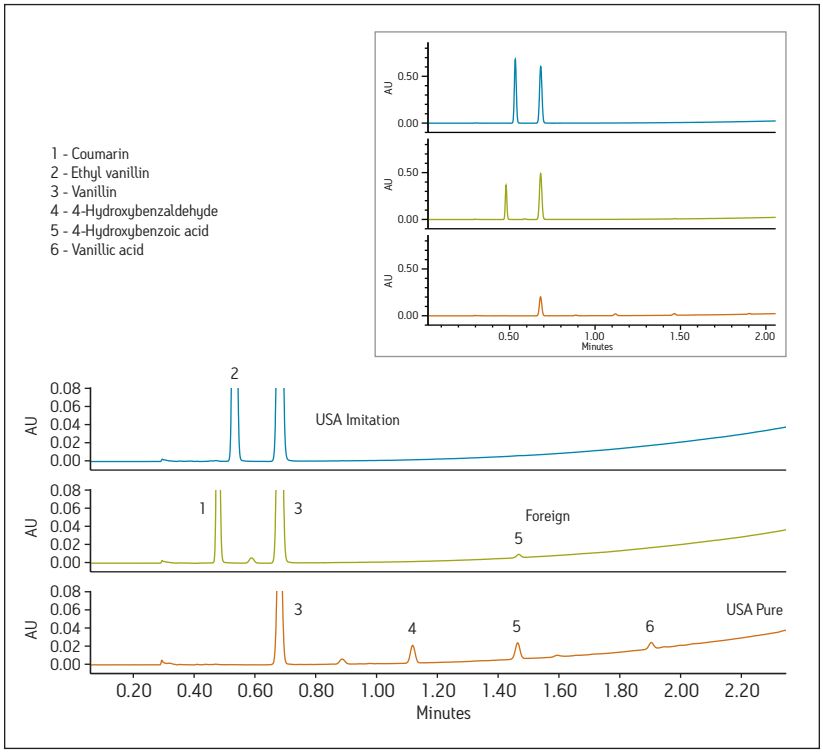

Figure 2. UV chromatograms of vanilla extracts analyzed by UPC². Samples were diluted 10X in ethanol. Wavelength: 260 nm, compensated.

Extract	Pure/ artificial	Country	Amount coumarin	Amount ethyl vanillin	Amount vanillin	Amount 4-hydroxybenzaldehyde	Amount 4-hydroxybenzoic acid	Amount vanillic acid
A	Artificial	USA	n/d	403.5 (0.41)	397.7 (0.41)	n/d	n/d	n/d
B	Artificial	Foreign	164.3 (0.70)	4.5 (0.63)	321.9 (0.50)	n/d	n/d	n/d
C	Pure	USA	n/d	n/d	136.0 (0.40)	9.0 (0.32)	14.5 (1.2)	3.7 (0.43)

Table 3. Quantitation data measured in µg/mL of diluted (10X) commercial vanilla extracts. Five replicate injections were performed. Relative Standard Deviations are in parentheses.

authenticity. Vanillin, as well as secondary natural flavor components, were identified and quantified in this sample. In addition, the ratio of vanillin to 4-hydroxybenzaldehye (14.9) was within the previously indicated range for authentic vanilla extracts (Table 3).[2]

SUMMARY

The Waters® ACQUITY UPC^2 System utilizes CO_2 mobile phases along with organic co-solvent and additives to provide orthogonal selectivity to that of RPLC. For the analysis of vanilla extracts, this separation technique provides greater retention of highly polar, secondary components of vanillin while providing adequate retention and identification of non-polar adulterants. In addition, this chromatographic technique allows for improved efficiency and lower solvent usage than traditional RPLC methods, while providing a high-throughput, sensitive screening method for the analysis of vanilla extracts.

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Enantiomeric and diastereomeric separations of fragrance and essential oil components using the ACQUITY UPC^2 System with ACQUITY UPC^2 Trefoil Columns

John P. McCauley and Rui Chen
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Shorter analysis times compared to chiral GC.
- The 2.5-µm particle chiral stationary phases provide high efficiency enantiomeric separations for fragrance compounds.
- The low system volume and extra-column volume of the ACQUITY UPC^2 System enables superior, faster, and more efficient enantiomeric separations of fragrance compounds compared to traditional SFC.
- UPC^2 solvents are more compatible with mass spectrometry, compared to those used in normal-phase chiral HPLC, enabling superior real time peak identification.

WATERS SOLUTIONS

ACQUITY UPC2® Trefoil™ AMY1 and CEL1 2.5 µm Columns

ACQUITY UPC2 System with ACQUITY UPC2 PDA Detector and ACQUITY® TQ Detector

MassLynx® Software

KEY WORDS

Enantiomers, chiral stationary phase, fragrance, essential oils, UltraPerformance Convergence Chromatography (UPC^2), convergence chromatography (CC), Trefoil

INTRODUCTION

Perception of aroma occurs at the olfactory membrane. This membrane is comprised in part of proteins and carbohydrates, which are chiral in nature. This makes it possible for the olfactory receptors to distinguish between enantiomers. Many enantiomers of fragrance molecules are perceived differently by our sense of smell.[1] For example, carvone is a chiral terpenoid where the R enantiomer smells like spearmint while the S enantiomer has the distinct odor of caraway seed.[2]

Chiral composition of fragrance molecules is important for many industries, including food, cosmetics, and consumer products, in controlling the olfactory perception of products.[1] In addition, chiral analyses are routinely performed to authenticate the natural sources of essential oils. Since naturally chiral sources of essential oils are generally more costly and have a greater perceived health benefit than their synthetic counterparts, rapid chiral analysis allows manufacturers to quickly exclude adulterated products containing inexpensive racemic synthetic materials at the time of purchase.[3]

Historically, chiral separations of fragrance compounds have primarily been carried out using chiral stationary phases (CSPs) in capillary gas chromatography (GC).[2,3,4] The analysis time often ranges from 15 to 50 minutes.[3] More recently, supercritical fluid chromatography (SFC) with CSPs has been applied to these separations, often resulting in comparable resolution and reduced run time.[5,6] Despite the great success in chiral separation by SFC, the associated instrumentation and CSPs have been slow to tap into the technology advancements that have taken place in the HPLC field. For example, one of most significant breakthroughs in HPLC in the past decade is the advent of Waters® UPLC® Technology, which utilizes sub-2-µm particles. ACQUITY UPLC® Systems retain the practicality and principles of HPLC while increasing the overall interlaced attributes of speed, sensitivity, and resolution. Until very recently, the standard particle size for commercially available CSPs has remained 5 µm.

Convergence chromatography is the next evolution in SFC. The Waters ACQUITY UPC^2 System is a holistically designed system that has similar selectivity to normal-phase chromatography and is built upon proven UPLC technology.

EXPERIMENTAL

Instrumentation

All experiments were performed on an ACQUITY UPC^2 System equipped with an ACQUITY UPC^2 PDA Detector and an ACQUITY TQ Detector. The system is controlled by MassLynx Software.

Samples

The standard samples used in this study were purchased from TCI Americas, with their structures shown in Figure 1. Essential oils were purchased from various commercial sources. All samples were dissolved in tert-butyl methyl ether (TBME) for the analyses.

UPC^2 conditions

Column: ACQUITY UPC^2 Trefoil AMY1 or CEL1 (2.5 µm, 3.0 x 150 mm)

Backpressure: 1740 psi

Temperature: 40 °C

Mobile phase A: CO_2

Mobile phase B: Isopropanol.

MS: APCI positive mode.

Other key parameters are listed in their respective figure captions.

UltraPerformance Convergence Chromatography™ (UPC$^{2®}$) offers minimized system and dwell volume, enabling users to leverage the superior separation power inherent to smaller particle sizes.

We present herein the enantiomeric and diastereomeric separations of four fragrance compounds using Waters ACQUITY UPC^2 Trefoil AMY1 and CEL1 Columns on an ACQUITY UPC^2 System. Compared to the traditional method of analysis by GC, UPC^2 offered similarly high resolution with significantly shorter run times, resulting in improved productivity.

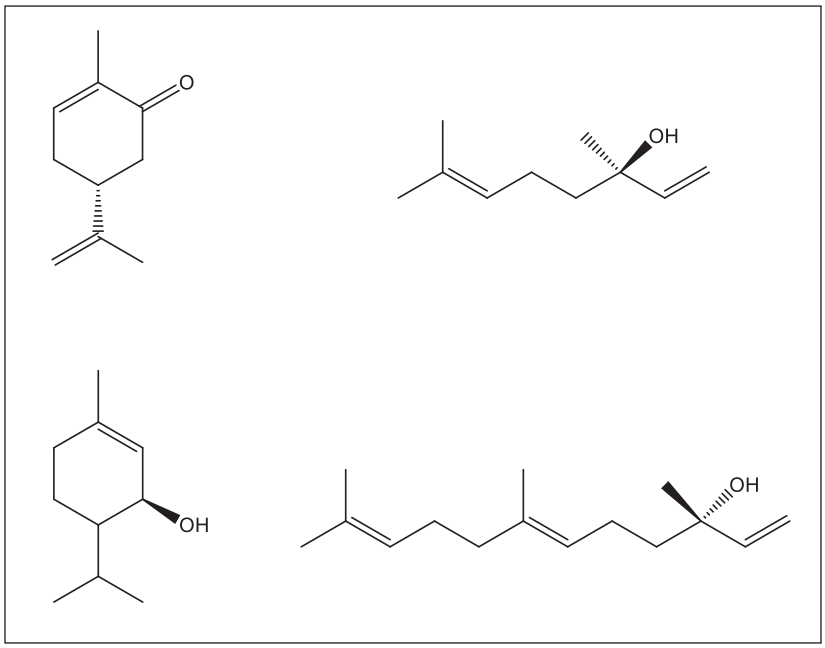

Figure 1. Structures of the four fragrance compounds presented in this study.

RESULTS AND DISCUSSION

Figure 2 shows the UPC^2-UV chromatogram of carvone enantiomers on an ACQUITY UPC^2 Trefoil CEL1 Column. The enantiomeric pair was baseline resolved in less than 2.5 min (Figure 2C). The peak widths at half-height are 2-3 s. It is also interesting to note that there are detectable antipodes present in both single enantiomer standards (Figures 2A and 2B). In both cases, the minor peaks account for approximately 1% of the main peaks, resulting in a 98% enantiomeric excess (e. e.). This example clearly demonstrates a high efficiency chiral separation enabled by a 2.5-µm CSP on an ACQUITY UPC^2 System, resulting in short analysis time, sharp peaks, and improved detection sensitivity.

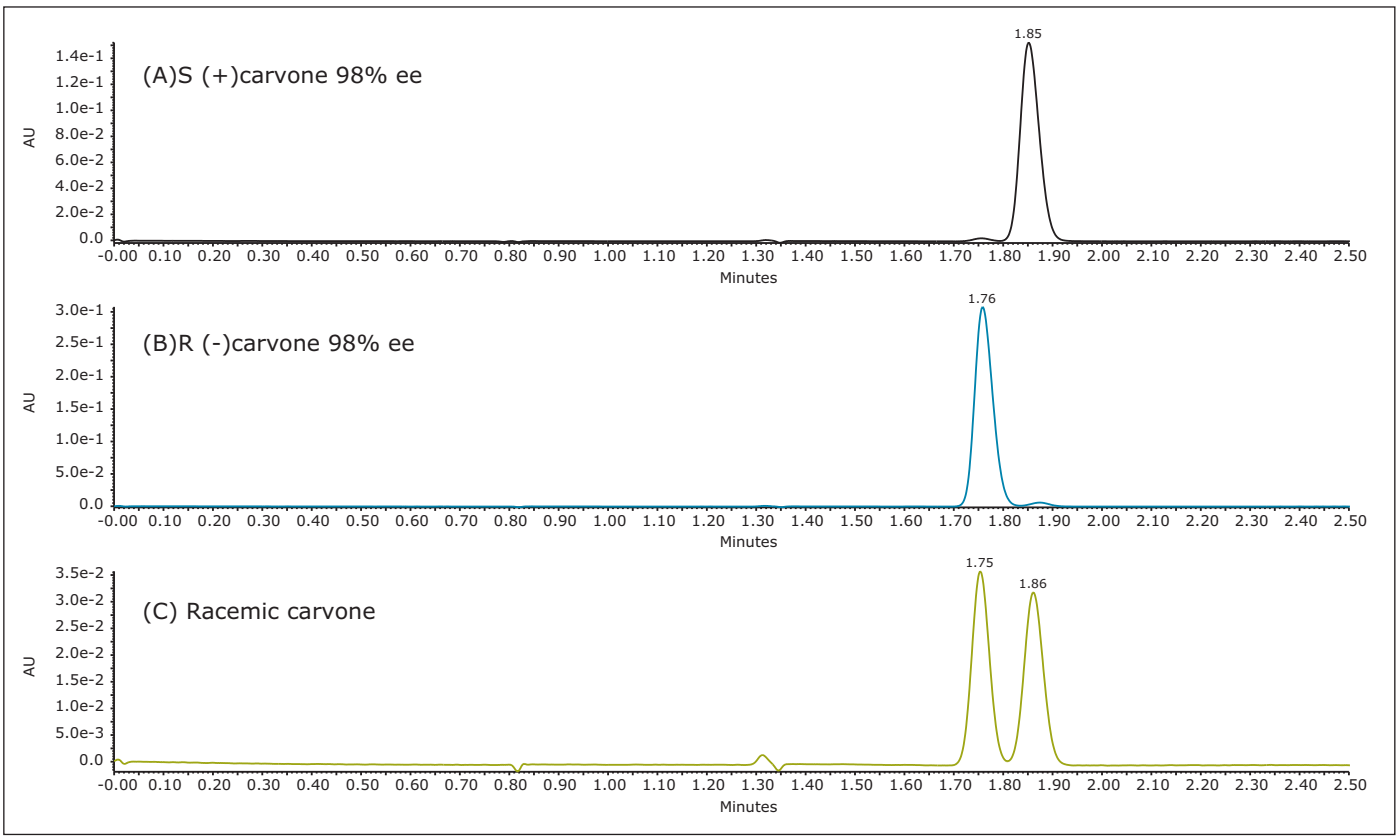

Figure 2. UPC^2-UV chromatograms of the enantiomeric separation of carvone on an ACQUITY UPC^2 Trefoil CEL1 Column: (A) S (+) carvone; (B) R (-) carvone; and (C) racemic carvone. An isocratic method with 4% isopropanol was used. The flow rate was 0.9 mL/min.

Linalool is a terpene alcohol with a soft floral odor, and can be found in different plant extracts. Figure 3A shows the enantiomeric resolution of the linalool standard on an ACQUITY UPC^2 Trefoil AMY1 Column. It is noted that the linalool standard was non-racemic (Figure 3A), suggesting the standard was derived from a natural source. The e. e. was estimated to be 40% in favor of the late eluting isomer. Figure 3B is the UPC^2-UV chromatogram of a commercially available lavender essential oil obtained under the same condition. The two linalool enantiomers were identified by both retention time and corresponding mass spectra (results not shown). It is noted that MS plays a critical role for the positive identification of the target analytes in a complex matrix. While bearing a similar selectivity to normal-phase LC, UPC^2 is inherently advantageous in incorporating MS detection due to its MS-friendly mobile phase. The linalool in this lavender essential oil exhibited a 92% e. e. in favor of the later eluting peak at 2.07 min.

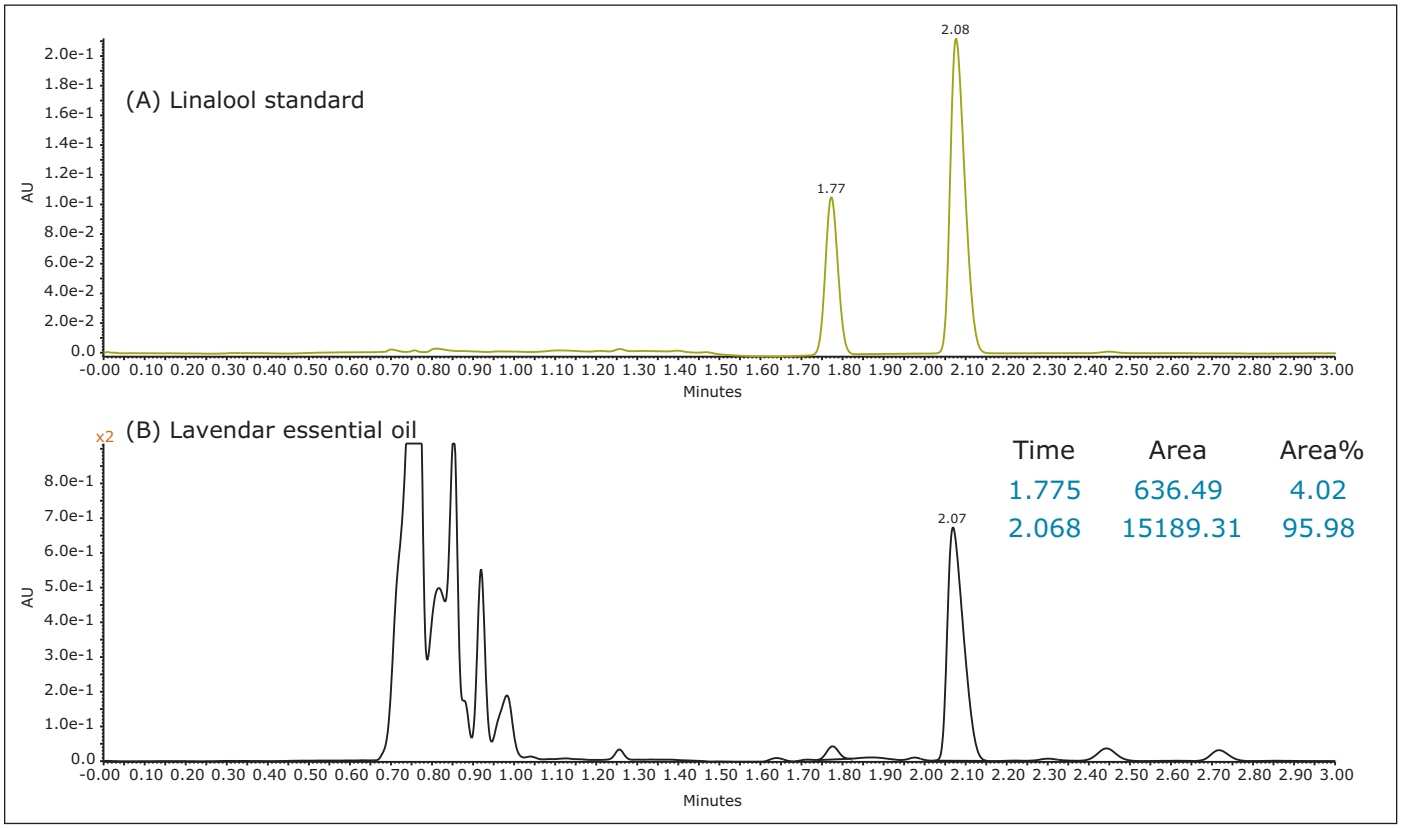

Figure 3. UPC^2-UV chromatograms of (A) linalool standard (B) lavender essential oil on an ACQUITY UPC^2 Trefoil AMY1 Column. An isocratic method with 3% isopropanol was used for linalool. The flow rate was 1.0 mL/min.

Similarly, terpinen-4-ol is a terpene with a pleasant conifer odor, and is a major constituent of tea tree oil. Figure 4A shows the enantiomeric resolution of the two isomers of a terpinen-4-ol standard on an ACQUITY UPC^2 Trefoil™ AMY1 Column. The terpinen-4-ol standard was nearly racemic (Figure 4A), suggesting its synthetic origin. Examination of a tea tree essential oil (Figure 4B) revealed that the terpinen-4-ol exhibited a 37% e. e. in favor of the early eluting isomer at 1.95 min.

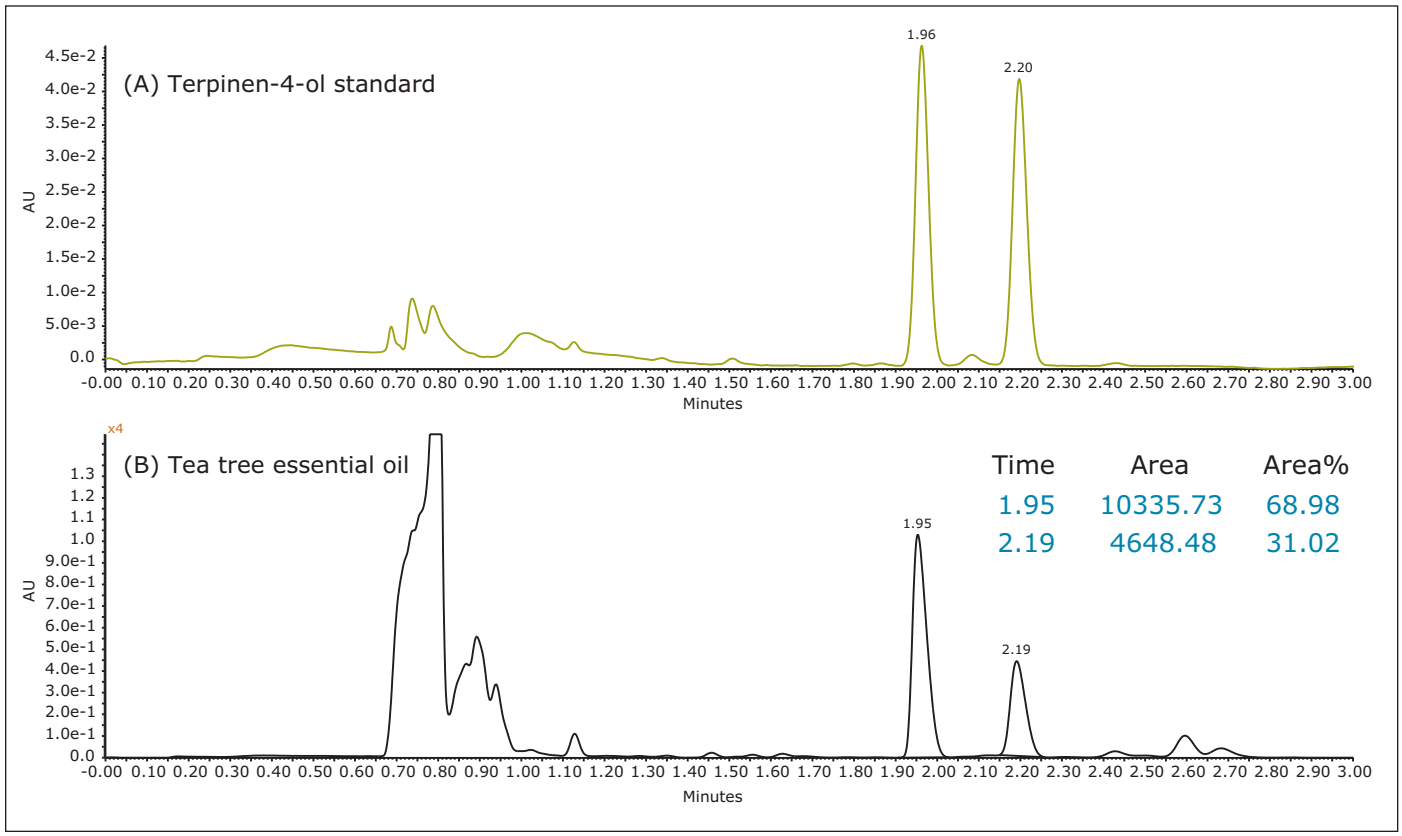

Figure 4. UPC^2-UV chromatograms of (A) Terpinen-4-ol standard and (B) Tea Tree essential oil on an ACQUITY UPC^2 Trefoil AMY1 column. An isocratic method with 5% isopropanol was used. The flow rate was 1.0 mL/min.

Nerolidol, which can be found in the neroli essential oil derived from the bitter orange plant, is a sesquiterpene with a pleasant woody odor reminiscent of fresh bark. The nerolidol molecule (Figure 1) contains a chiral center and a double bond generating cis/trans isomerism, resulting in four possible stereoisomers in a mixture. Figure 5 shows the simultaneous separation of all four nerolidol isomers on an ACQUITY UPC^2 Trefoil AMY1 column in less than 3 min. Figure 5B is the selected ion recording (SIR) for the isomeric mixture at *m/z* 205.2, corresponding to the $[(M+H)-H_2O]^+$ of nerolidol. The observation of the base peak of nerolidol resulting from the loss of water is consistent with other reports.[7]

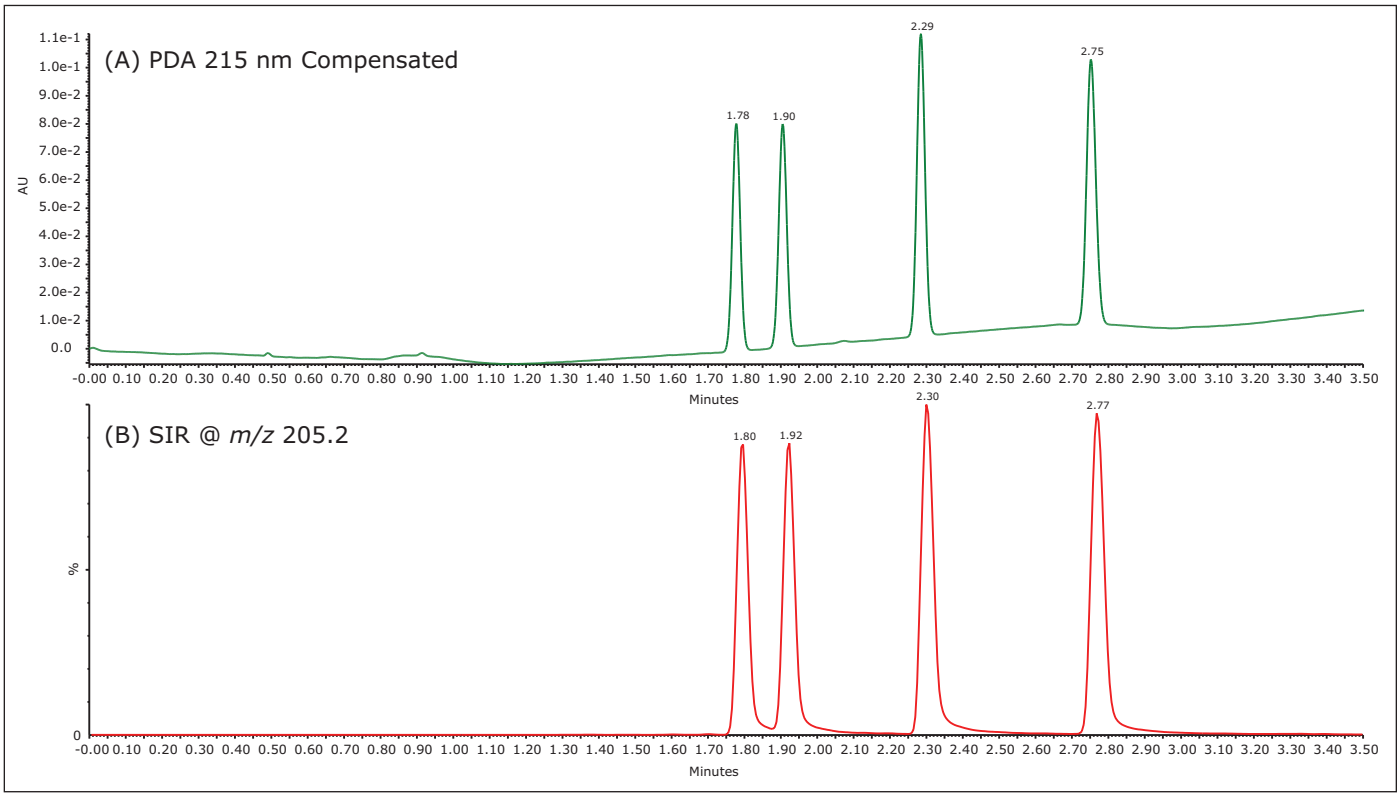

Figure 5. UPC^2 chromatograms of a nerolidol standard separated on an ACQUITY UPC^2 Trefoil AMY1 Column: (A) UV at 215 nm with a compensation wavelength of 260-310 nm; and (B) SIR at m/z 205.2. The flow rate was 1.5 mL/min. A gradient of 2-7% isopropanol in 3.5 min was used.

CONCLUSIONS

In this application note, we have demonstrated the successful chiral separations of fragrance compounds on ACQUITY UPC^2 Trefoil AMY1 and CEL1 Columns using an ACQUITY UPC^2 System. The low system volume and extra-column volume of the UPC^2, combined with the reduced particle size of the ACQUITY UPC^2 Trefoil AMY1 and CEL1 Columns, enable superior, faster, and more efficient separations compared with traditional SFC and GC. The demonstrated analysis times range from 2 to 3 minutes, significantly shorter than the 15-50 minute analysis time typical for chiral GC,[3] allows for a fast authentication of the natural sources of essential oils. In all cases, the closely eluting isomers were baseline resolved. For the essential oil analysis, the oil samples were diluted and directly injected onto an ACQUITY UPC^2 System without tedious sample preparation. Furthermore, the inherent compatibility between UPC^2 and MS offered an unambiguous identification of the target analytes in a complex sample matrix. The high efficiency, short analysis time, and simple sample workup demonstrated in this study should be welcomed by industries where chiral analyses of fragrance compounds are routinely performed.

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PREPARATIVE SFC

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UPC² Strategy for Scaling from Analytical to Preparative SFC Separations

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Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

Scaling strategy based on density modulation can be used to efficiently transfer methods across different SFC system/column configurations.

Density modulation can be used to offset changes in density caused by changes in mobile phase flow rate.

Density modulation enables the fast method development of SFC applications on the analytical scale using UPC²® with subsequent transfer to preparative SFC while maintaining chromatographic integrity.

WATERS SOLUTIONS

ACQUITY UPC²™ System

Waters Prep 100q SFC

ACQUITY UPC² Columns

Viridis® SFC Columns

LCMS Certified Max Recovery Vials

KEY WORDS

UPC², SFC, prep, scaling, method transfer, method development, density modulation

INTRODUCTION

Scaling of chromatographic methods is a routine necessity to utilize different instrumentation or column configurations for a successful separation method. A common example of this would be the fast development of a chromatographic method on the analytical scale with the goal of transferring the separation to the preparative scale. For liquid chromatography (LC) applications, this scaling methodology is well understood and the guidelines for transferring methods are straight forward. For chromatographic methods using CO_2 as the principal component of the mobile phase, the scaling process is not as well understood. This is due to the high compressibility of the CO_2 mobile phase which makes many of the scaling methodologies developed for LC invalid. Therefore, most of the current scaling strategies used for supercritical fluid chromatography (SFC) are based on empirical observations and often times require additional method manipulation on the transferred system.

It is well known that for separations using CO_2 as the principal mobile phase component, analyte retention factors are influenced largely by the mobile phase density and temperature. Because of the high compressibility of CO_2 under standard operating conditions, the density can change significantly with changes in pressure (under isothermal conditions), with retention factors increasing with decreasing mobile phase density (pressure). In addition, the selectivity and resolution of the analytes may be impacted as they respond differently to the same changes in mobile phase density. This can present a challenge when attempting to transfer a method between different column configurations that involve changes in column length or stationary phase particle size, which in turn alters the pressure (density) profile along the column. This is best exemplified when analytical scale separations, developed using UltraPerformance Convergence Chromatography™ (UPC²) on sub-2-µm stationary phases, are scaled up for preparative SFC conditions using 5 µm particle size stationary phases. The difference in the density profiles across the column, between the analytical and the preparative system, may lead to very different chromatography unless the scale-up procedure is guided by a systematic approach.

EXPERIMENTAL

Sample preparation

For development of the scaling strategy, a standard sample mix was prepared with caffeine (1), carbamazepinel (2), uracil (3), hydrocortisone (4), prednisolone (5), and sulfanilamide (6), using methanol as diluent. For the analytical evaluations, the concentration of analytes in the mixture was 0.2 mg/mL each. For preparative scale separations the concentration of analytes was 3.75 mg/mL each. The numbers in parentheses are used in labeling all chromatograms presented in this application note.

Method conditions

UPC^2 conditions

System:	ACQUITY UPC^2 with PDA Detector
Columns:	ACQUITY UPC^2 BEH 2-Ethylpyridine, 1.7 µm, 2.1 x 150 mm column (P/N:186006579); ACQUITY UPC^2 BEH 2-Ethylpyridine, 1.7 µm, 3.0 x 50 mm column (P/N:186006580); Viridis BEH 2-Ethylpyridine, 5 µm, 2.1 x 150 mm column (P/N:186006545); Viridis BEH 2-Ethylpyridine OBD™ Prep, 5 µm, 19 x 150 mm column (P/N:186005764)
Mobile phase A:	CO_2 (tank, medical grade)
Mobile phase B:	Methanol
Column temp.:	40 °C
ABPR:	Varied (noted in each figure)
UV detection:	254 nm (compensated 380–480 nm) [40 pts/sec]
Injection volume:	1.5 µL
Strong needle wash:	2-Propanol (IPA)
Weak needle wash:	2-Propanol (IPA)
Seal wash:	2-Propanol (IPA)
Vials:	LCMS Certified Max Recovery Vials (P/N: 600000749CV)

Preparative SFC conditions

System:	Waters Prep 100q SFC system with PDA detection
Columns:	Viridis BEH 2-Ethylpyridine OBD Prep, 5 µm, 19 x 150 mm, (P/N:186005764)
Mobile phase A:	CO_2 (house CO_2 delivery system)
Mobile phase B:	Methanol
Column temp.:	40 °C (unless otherwise noted)
ABPR:	Varied (noted in each figure)
UV detection:	254 nm
Injection volume:	240 µL
Wash solvent:	Methanol

Here we present a strategy for scaling SFC separations between various system, condition, and column configurations by employing density modulation to maintain similar average density profiles between separations. The ability to scale methods efficiently enables the rapid screening of methods on the faster analytical scale (using UPC^2), with the direct transfer of the final method to preparative chromatography while maintaining chromatographic integrity between separations. The net result is a scalable, predictable separation with significant savings in time and mobile phase costs (raw materials and disposal of waste).

RESULTS AND DISCUSSION

It has been well established that mobile phase density plays a predominant role in the retention mechanisms governing analyte retention in SFC. The importance of understanding this behavior for development of a scaling strategy is demonstrated in Figure 1 for the separation of a standard mix on analytical columns of the same chemistry and dimension, with different particle sizes (1.7 µm and 5 µm). While the separation configurations differ only by the stationary phase particle size, the resulting chromatography is significantly different, with changes in selectivity and resolution for the analytes. The difference in retention factors and resolution can be attributed to the higher density mobile phase resulting from the increased pressure (increased resistance to flow) with the smaller 1.7 µm particle size (flow rate and temperature were kept constant). To understand this better, it is necessary to understand the density drop across the column which can be calculated with available chromatographic method and system parameters (*e.g.*, modifier, temperature, pressure).

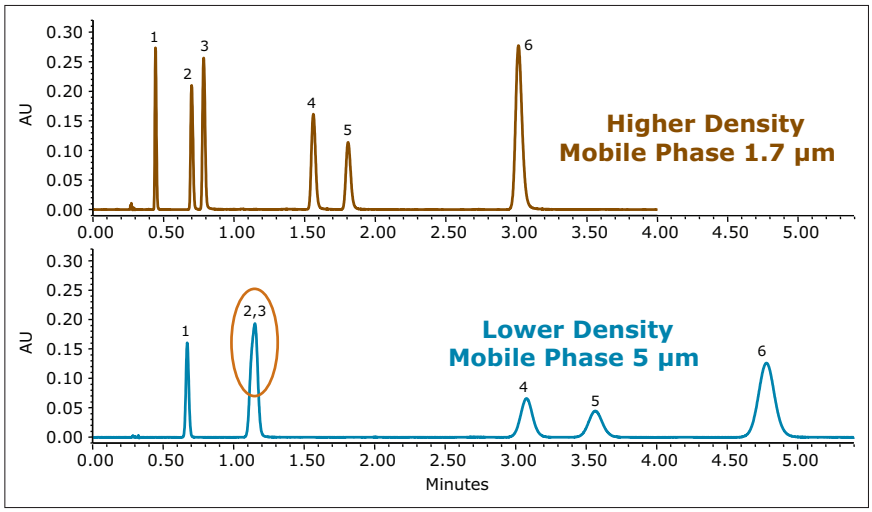

Figure 1. Isocratic separations of a standard mix on the ACQUITY UPC^2 BEH 2-EP, 1.7 µm Column (top) and on a Viridis BEH 2-EP, 5 µm Column (bottom). Both separations were performed using a 2.1 x 150 mm column dimension under isocratic conditions with 10% methanol modifier at 1.4 mL/min. The temperature was 40 °C and the Automated Back Pressure Regulator (ABPR) setting was 1500 psi.

Density Simulations

The simulation of the density profiles presented here were conducted based on the assumption that the variation of pressure profile along the column is linear, which is true for most of the experimental operating conditions used in SFC.[1,2] The densities of the CO_2/methanol mixtures were calculated using the REFPROP software from NIST.[3] REFPROP calculates the neat CO_2 density following the Span and Wagner equation of state (EOS) and calculates the CO_2/MeOH mixture density using the Kunz and Wagner model.[4,5] Under typical SFC operating conditions, the errors in the estimation of CO_2 density using Span and Wagner EOS range between 0.03 and 0.05% for CO_2 pressures up to 4,350 psi and temperatures up to 250.°C.[3] For methanol, the errors on the values provided by REFPROP are 1% for the density of the dilute gas and between 0.6 and 3% for that of the liquid at pressures up to 14,500 psi and temperatures between 0 and 70.°C.[3] No specific information regarding the estimation of errors made by the Kunz and Wagner mixing rule is available.

For the chromatography shown in Figure 1, the density profile simulations were performed and are shown below in Figure 2 (left). From these simulations, it is clear that the analytes experience different average mobile phase density during the separations. Using density calculations, appropriate chromatographic conditions can be determined to modulate the density profile inside the column in such a way that the analytes experience nearly the same average mobile phase density (Figure 2, right).

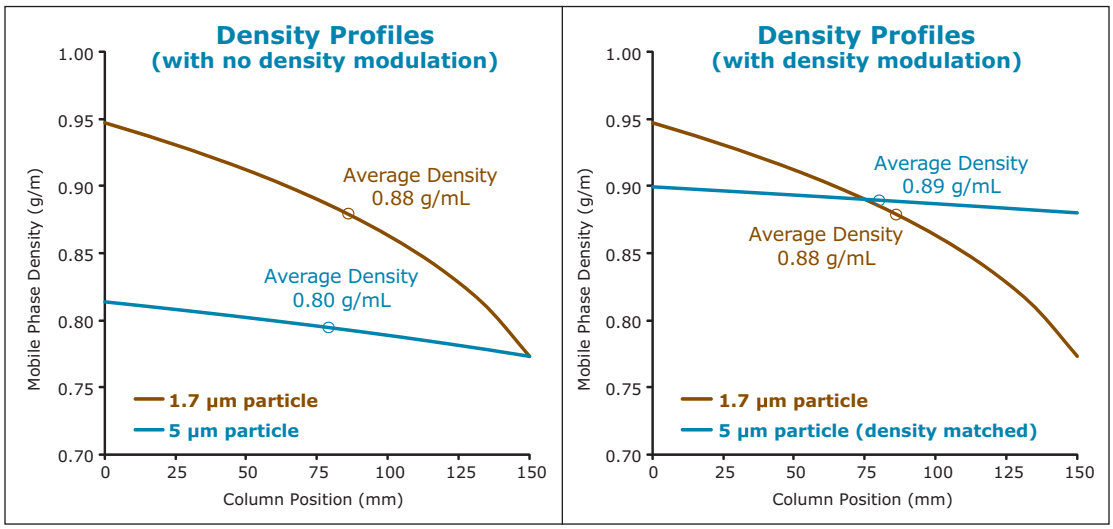

Figure 2. Density simulations showing the mobile phase density drop across the 150 mm columns. The left figure represents the calculations for the separations shown in Figure 1 with no density modulation. The figure on the right includes the same calculations for the 1.7 µm particle, but uses density modulation for the 5 µm particle to achieve approximately the same average density for the two separations.

Implementation of the density modulation as shown in Figure 2 (right) yields the chromatography shown below in Figure 3 (bottom chromatogram). Minor chromatographic differences can be attributed to the approach of using density profile averages instead of exact density profiles, which would be difficult, if not impossible, to achieve. Despite the minor differences, the overall chromatographic integrity of the initial separation (top chromatogram) is nearly preserved, with similar retention and resolution obtained on both particle sizes, contrary to the example without density modulation.

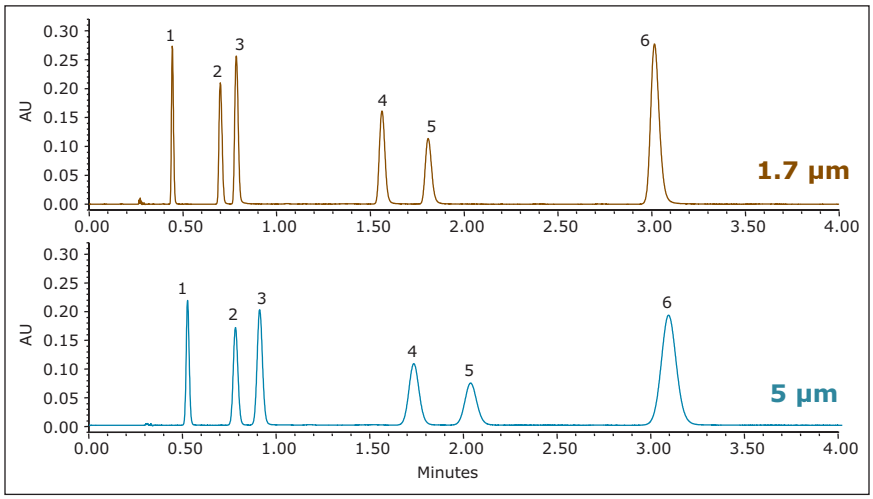

Figure 3. Comparison of the original 1.7 µm separation (top), from Figure 1, with the separation on the 5 µm particle size column (bottom), with density modulated to approximate the same average density calculated for the 1.7 µm separation. The ABPR settings were 1500 and 3390 psi, respectively.

Variations in Flow Rate

This strategy can be applied more broadly to deal with any system or method alteration that has a direct impact to the density profile of a separation. One example would be an alteration of flow rate. At flow rates faster than the optimum linear velocity, chromatographic efficiency decreases in SFC, similar to what is observed for LC applications. But often times this decrease in efficiency is an acceptable trade-off for the decrease in run time. However, for SFC applications, any alteration in flow rate will alter the pressure, and therefore the density profile of the separation, potentially altering the resulting chromatography. The use of density modulation to match the density profile averages can be used to mitigate chromatographic changes, as demonstrated in Figure 4.

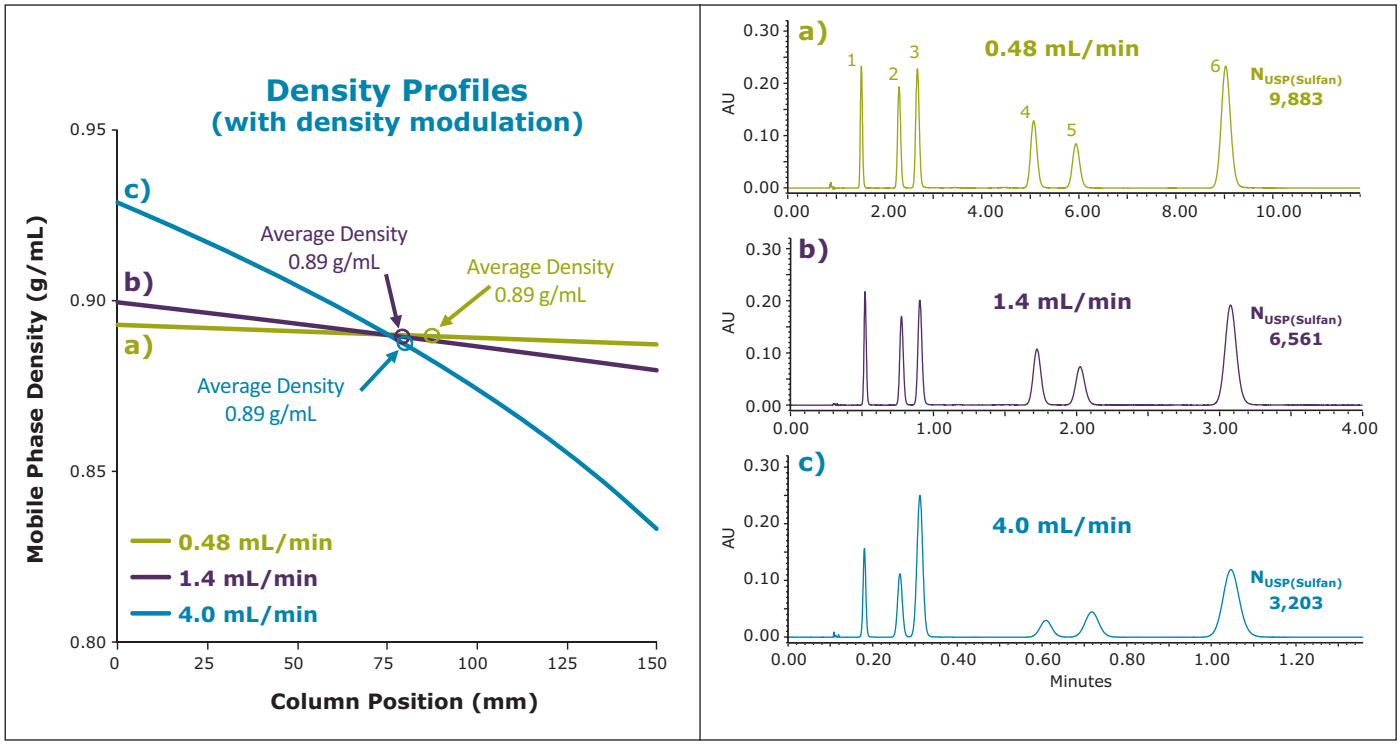

Figure 4. Isocratic separations of the standard mix on the Viridis BEH 2-EP, 5 µm, 2.1 x 150 mm Column under isocratic conditions of 10% methanol at flow rates of 0.48 mL/min (a), 1.4 mL/min (b), and 4.0 mL/min (c). For each separation, the density was modulated so that the mobile phase density profile for each separation had approximately the same average density (0.89 g/mL). The ABPR settings were 3600, 3390, and 2311 psi, respectively.

For the 5 µm SFC particle size, in a 2.1 x 150 mm column configuration, the optimum linear velocity is achieved at a flow rate of approximately 0.48 mL/min (Figure 4a). As the flow rate is increased to 1.4 and 4.0 mL/min (Figures 4b and 4c), the decrease in chromatographic efficiency is obvious, but expected due to the predominant mass transfer term of the van Deemter equation at the faster flow rates. This example demonstrates the utility of this approach to maintain chromatographic selectivity of a separation in the presence of configuration/method alterations that have a direct impact on the density profile of a separation.

Scaling from analytical to preparative conditions

One of the most obvious and beneficial applications of this strategy would be to the scale-up of analytical applications to preparative chromatography. Figure 5 demonstrates the initial separation, developed on the 1.7 µm particle size column, with subsequent transfer to analytical 5 µm and preparative 5 µm columns with density modulation to match the density profile averages. The flow rates for the 5 µm separations were decreased for the lower optimum linear velocity of the larger particle.

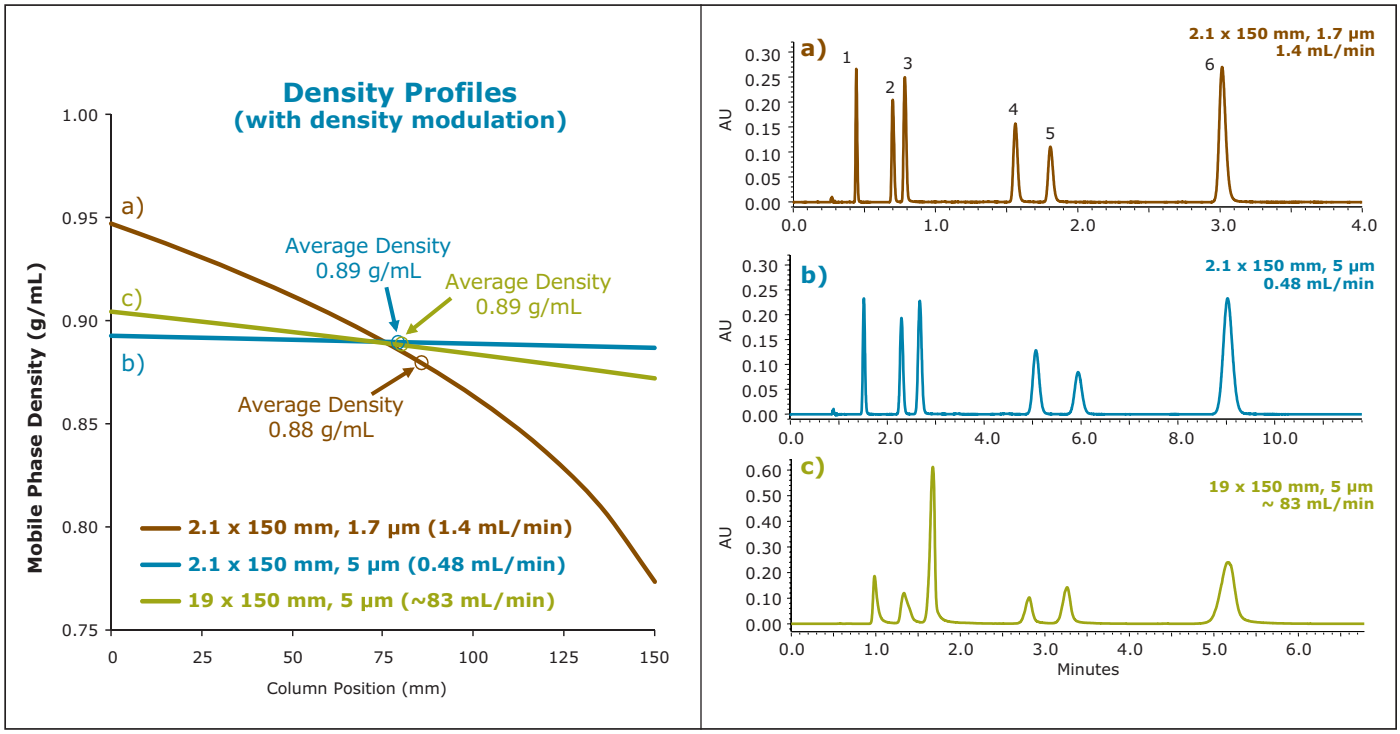

Figure 5. Isocratic separations of the standard mix on the ACQUITY UPC² BEH 2-EP, 1.7 µm Column (a), and on Viridis BEH 2-EP, 5 µm Columns in analytical (b) and preparative (c) configurations. Density modulation was used for the isocratic separations with 10% methanol at flow rates of 1.40 mL/min, 0.48 mL/min, and 83.00 mL/min. The ABPR settings were 1500, 3600, and 3191 psi, respectively.

For the preparative example, a 240 µL injection volume was used with analytes at 3.75 mg/mL each. With approximately 1 mg each on column, the effects of the higher loading can be seen in the chromatography, but selectivity is maintained.

Another common scaling strategy involves maintaining the ratio of column length to particle size (L/d_p) between separations. This approach can be used in combination with density modulation as shown below in Figure 6 with the direct transfer of a method from a 3.0 x 50 mm, 1.7 µm column to a preparative 19 x 150 mm, 5 µm column ($L/d_p \sim 30{,}000$).

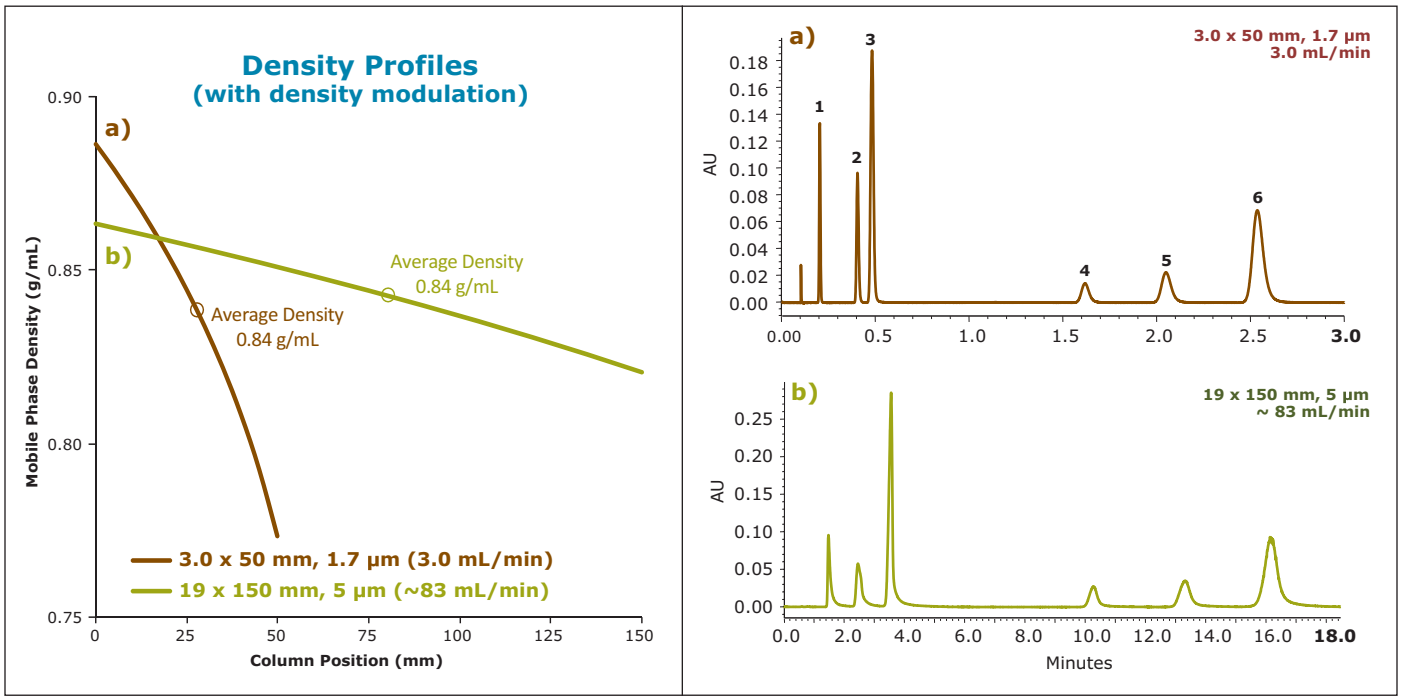

Figure 6. Isocratic separations of the standard mix on the ACQUITY UPC² BEH 2-EP, 1.7 µm Column (a), and on Viridis BEH 2-EP, 5 µm OBD Prep Column (b) configurations. Density modulation was used for the direct scale up from analytical to preparative conditions, using 6% methanol at flow rates of 3 mL/min and 83 mL/min. The ABPR settings were 1500, 3600, and 3191 psi, respectively.

Gradient Methods

For the previous isocratic examples, although the density varied across the column, the variation was static because the composition of the mobile phase did not change. For gradient methods, the situation is more complicated because the density not only varies along the column, but also changes with time. The increasing modifier concentration during the gradient results in increases in mobile phase viscosity and therefore pressure, which in turn impacts the mobile phase density profile. For gradient separations, the density modulation strategy should consider the changing density profile as the modifier transitions from lower to higher concentrations. Figure 7 (left) shows the results from density simulations at the column inlet and outlet for both the analytical and preparative configurations. For the preparative configuration, the density was modulated to yield approximately the same average density profile as for the analytical configuration. As in the previous isocratic examples, density modulation under gradient conditions yields similar chromatography for the analytical and preparative separations, with similar resolution and selectivity for the individual compounds. Differences observed between the two examples can be attributed to differences in system dwell volume or injection mode (modifier stream vs. mixed stream injection), neither of which were considered for these experiments.

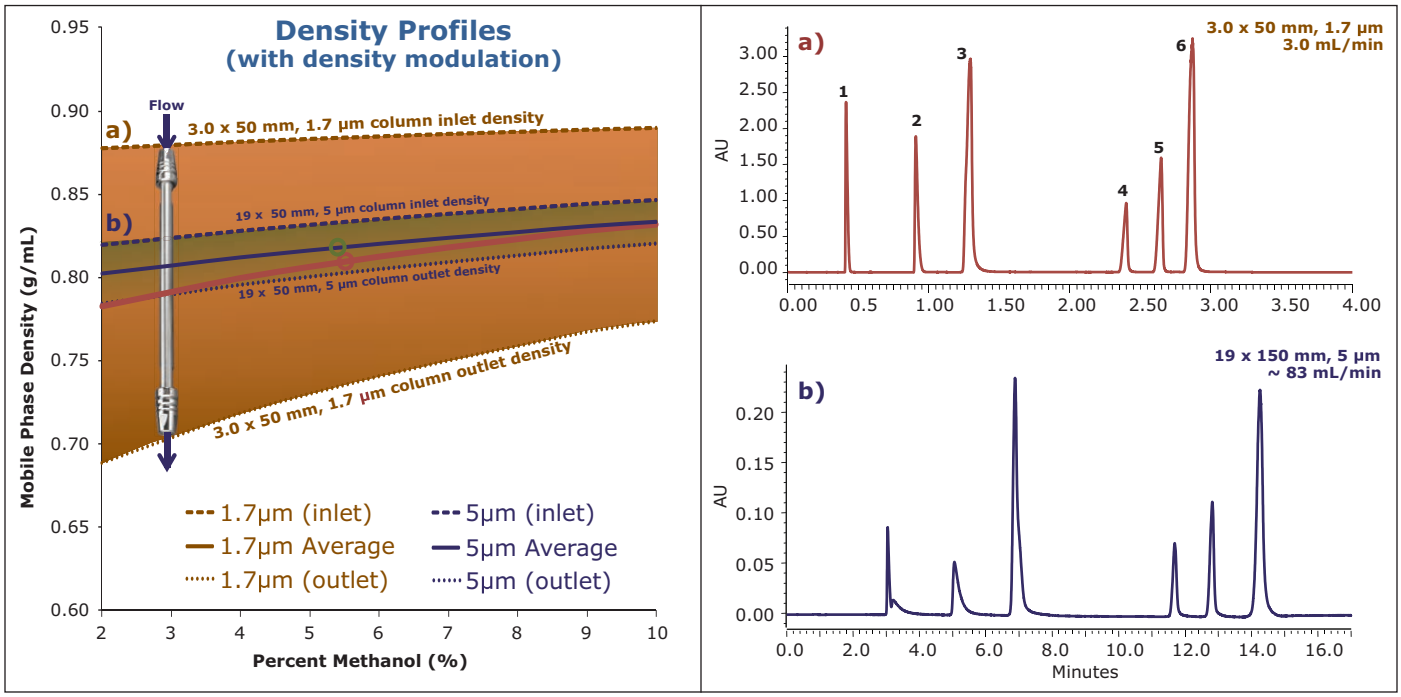

Figure 7. Gradient separations of the standard mix on the ACQUITY UPC² BEH 2-EP, 1.7 µm Column (a), and on Viridis BEH 2-EP, 5 µm OBD Prep Column (b) configurations. Density modulation was used for the direct scale up from analytical to preparative conditions, using a gradient from 2-10% methanol at flow rates of 3 mL/min and 83 mL/min. The gradient times (3.0 minutes and 13.5 minutes) were scaled to yield the same number of column volumes for each gradient separation. The ABPR settings were 1500 and 2103 psi, respectively.

CONCLUSIONS

The application of a systematic approach using density modulation facilitates the efficient transfer of SFC methods between different column configurations (length and/or particle size). In addition, this approach provides more flexibility in manipulating method conditions to explore broader flow rate regimes while maintaining chromatographic integrity. With application to both isocratic and gradient methods, this methodology is suitable for both chiral and achiral separations. This is especially pertinent for chiral method development in which multiple chiral columns may be screened against multiple modifier combinations using analytical configurations in a very short period of time. The resulting method can then be scaled up efficiently for preparative purifications, yielding predictable, reproducible chromatography. The ability to perform method development on the analytical scale before scaling directly to preparative chromatography represents a substantial savings in time and resources.

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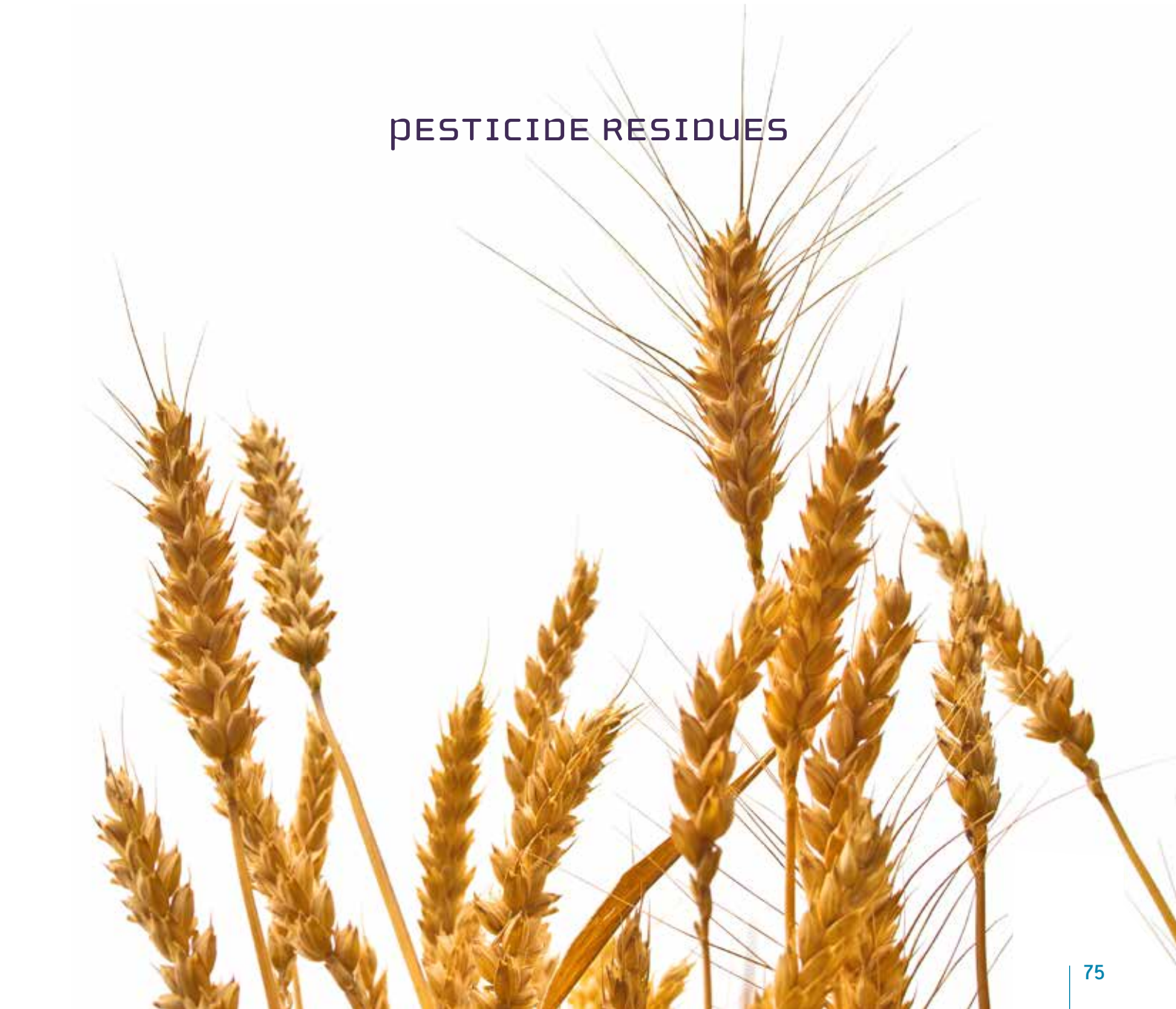

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Enantioseparation and Detection of Triazole Fungicides in Wheat Grain and Wheat Straw using the ACQUITY UPC^2 System and Xevo TQ-S

Marian Twohig, Michael O'Leary, and Claude Mallet
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Enantiomeric detection and quantitation of triazole fungicides at parts per trillion levels (ppt).
- Improved enantiomeric resolution and shorter analysis times using SFC compared with normal-phase HPLC chiral separations resulting in higher sample throughput.
- Reliable and reproducible measurement of the enantiomer ratios for use in enantioselective degradation studies.

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ACQUITY® UPC2® System

Xevo® TQ-S

Oasis® Sample Extraction Products

DisQuE™ Dispersive Sample Preparation

Sep-Pak SPE

MassLynx® Software

KEY WORDS

Chiral quantitation of pesticides, enantiomer, chiral separation, uniconazole, tebuconazole, diniconazole, flutriafol, fenbuconazole, triazole fungicide, SFC, supercritical fluid chromatography, UPC,^{2} enantioselective degradation studies.

INTRODUCTION

The safe use of crop protection products is of paramount importance to the agricultural chemicals manufacturing industry. Extensive studies and trials are carried out in support of product registration. These studies ensure that any risks associated with using the product are characterized and properly understood so that it can be safely applied to the field. When a crop protection active ingredient (AI) contains one or more stereogenic centers in its structure the enantioselective behavior must be studied, since it is known that enantiomers can exhibit different bioactivities.[1,2] Analytical methods used to evaluate the influence of stereochemistry on the degradation dynamics, environmental fate, and final residue levels help to establish a more accurate risk assessment of crop protection products.

Liquid chromatography (LC) on chiral stationary phases (CSPs), such as polysaccharide stationary phases including amylose and cellulose, has been the most commonly used chiral separation technique.[3-6] More recently, there has been an increasing adoption of using supercritical fluid chromatography (SFC) on CSPs for chiral separation.[7,8] The properties of a supercritical fluid, its high diffusivity and low viscosity in particular, enable high efficiency chiral separations with shorter run times. For example, triazole fungicides, such as tebuconazole, structure shown in Figure 1, are a commonly used group of pesticides due to their potent activity against a broad spectrum of crop diseases. Using HPLC, the analysis times for the enantiomeric resolution of tebuconazole ranged from 13 to 45 min.[3-6] Similar resolutions were achieved for tebuconazole using SFC, but the analysis times were reduced to 10 min.[8]

UltraPerformance Convergence Chromatography™ (UPC^2) applies the performance advantages of UPLC® to SFC, combining the use of supercritical CO_2 with sub-2-µm particle columns.[9,10] UPC^2 is an orthogonal analytical technique to reversed-phase LC and can be used to solve complex separations challenges. The detection sensitivity and specificity offered by tandem MS/MS is advantageous for determining trace levels of pesticides in complex matrices like field crops or soil.[11-14]

EXPERIMENTAL

SFC conditions

SFC system:	ACQUITY UPC^2
Chiral separation:	Diniconazole, fenbuconazole, flutriafol, tebuconazole
Column:	Chiralpak IA-3, 4.6 x 150 mm, 3.0 µm
Co-solvent (B):	Methanol with 2% water and 0.1% formic acid
ABPR:	1990 psi/137 bar
Flow rate:	2.5 mL/min
Column temp.:	40 °C
Injection volume:	4 µL
UPC^2 conditions:	0 min 20% B, 2.5 min 20% B, 2.6 min 30% B, 5 min 30% B, return to initial conditions.
Chiral separation:	Uniconazole
Column:	Chiralpak IA-3 4.6 x 150 mm, 3.0 µm
Co-solvent (B):	50:50 2-propanol/ethanol with 2% water and 0.1% formic acid
ABPR:	1990 psi/137 bar
Flow rate:	2.5 mL/min
Column temp.:	15 °C
Injection volume:	4 µL
UPC^2 conditions:	0 min 15% B, 4 min 15% B, 4.1 min 35% B, 5 min 35% B, return to initial conditions.

MS conditions

MS system:	Xevo TQ-S
Ionization mode:	ESI+
Capillary voltage:	2.8 kV
Cone voltage:	See Table 1
Desolvation temp.:	600 °C
Source temp.:	150 °C
Collision energy (eV):	See Table 1
MS scan range:	100 to 800 *m/z*

An AgileSLEEVE™ 30 cm x 1/16" I.D. tubing heater (Analytical Sales and Services Inc. Pompton Plains, NJ) set to 65 °C was used to heat the transfer line to the MS system. All compounds were automatically tuned by direct infusion using IntelliStart™ prior to the analysis. A summary of the optimized MRM transitions and voltages is shown in Table 1.

In this application note, ACQUITY UPC^2 and tandem quadrupole mass spectrometry were used for the trace level enantioanalysis of five triazole fungicides (Figure 1) in wheat grain and/or wheat straw. A QuEChERS (quick easy cheap effective rugged and safe) extraction modified for dry commodities was performed followed by solid phase extraction using Oasis MCX. Chiral separations using a 3.0 µm chiral CSP followed by multiple reaction monitoring (MRM) detection allowed concentrations of part per trillion (ppt) levels to be reproducibly detected and quantified.

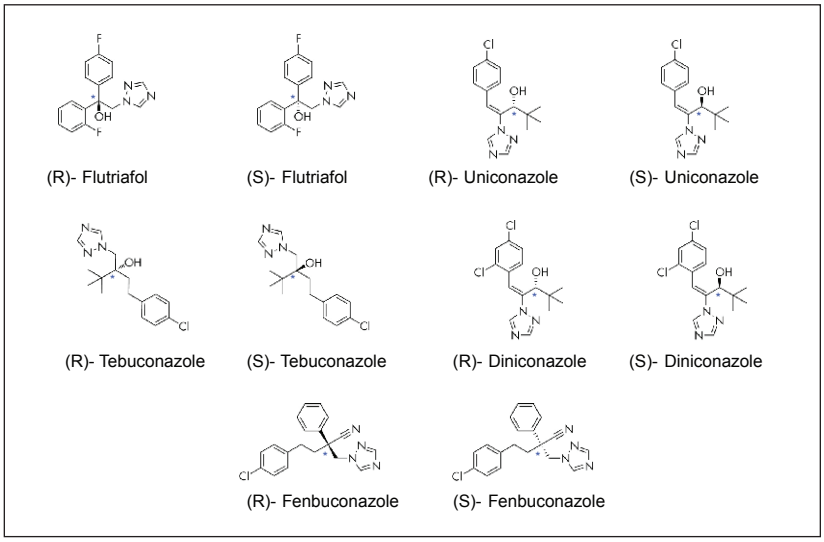

Figure 1. Structures of the target triazole fungicide enantiomers. The asterisks denote the stereogenic centers.

Instrumentation

All separations were performed on a Waters® ACQUITY UPC^2 System. Detection was by positive ion electrospray mass spectrometry (MS) using a Xevo TQ-S tandem quadrupole mass spectrometer. MassLynx Software was used for data acquisition, and TargetLynx Application Manager was used for data processing.

Sample preparation

Initial extraction (QuEChERS):

Triturated wheat straw (1 g) or wheat grain (5 g) were placed in a 50-mL polypropylene centrifuge tube. The volume of water added to the wheat straw was 9 mL with 5 mL of water added to the wheat grain, followed by phosphoric acid (100 µL) and acetonitrile (10 mL). The mixture was shaken for 20 minutes. A DisQuE Pouch for the European Committee for Standardization (CEN) QuEChERS method (Part No. 186006813) was added to the tube and shaken vigorously for 1 minute. Centrifugation at 4000 rpm followed to produce a liquid partition with a clear acetonitrile top layer. The top acetonitrile layer (5 mL) was transferred to a clean 50-mL centrifuge tube and diluted with water (45 mL) for cleanup using an Oasis MCX 3 cc, 60 mg Cartridge (Part No. 186000254).

Oasis MCX sample cleanup

Oasis MCX 3 cc, 60 mg Cartridges were conditioned with 3 mL of methanol and equilibrated with 3 mL of water. The samples were loaded in reverse phase mode into Sep-Pak 60 cc Reservoirs (Part No. 186005587) at a flow rate of 1 to 3 mL/min. After sample loading was completed the cartridge was washed with 2% formic acid in water (3 mL) followed by 100% methanol (3 mL). A collection vessel was installed and elution was achieved using 2 mL 2% ammonium hydroxide in methanol. The base containing eluent from the elution step was blown down to dryness and reconstituted in neat methanol (5 mL).

Standard and sample preparation

Working standard solutions were prepared by sequential dilution of the stock solution using acetonitrile. The working standards were spiked (in triplicate) on to the dry wheat straw/wheat grain at levels of 1, 5, and 10 ng/g. The samples were allowed to equilibrate for 30 min prior to extraction. Matrix-matched standard curves were prepared with blank matrix extracted using the same protocol.

Wheat straw and wheat grain samples were obtained from online vendors.

Table 1. MRM transitions and instrument settings for the analysis of the triazole fungicides. The primary quantitation transition is listed (top) with the confirmatory transition (bottom).

Analyte	MRM transitions	Cone voltage (V)	Collision energy (eV)
Diniconazole	326>70	16	20
	326>159	16	30
Fenbuconazole	337>70	14	18
	337>125	14	26
Flutriafol	302>70	16	16
	302>123	16	26
Tebuconazole	308>70	40	18
	308>125	40	36
Uniconazole	292>70	20	22
	292>125	20	24

RESULTS AND DISCUSSION

Enantioseparation of the five triazole fungicides

A Chiralpak IA-3, 4.6 x 150 mm, 3.0 µm was used to perform enantioseparation of the five triazole fungicides. Resolution was achieved for diniconazole, fenbuconazole, flutriafol, and tebuconazole using methanol as the co-solvent; while the chiral resolution of uniconazole was improved using a mixture of ethanol and 2-propanol (50:50 v/v). Water (2%) and formic acid (0.1%) were added directly to the co-solvents to promote ionization. A chromatogram of wheat grain directly spiked at a level of 1 ng/g and extracted using QuEChERS followed by sample cleanup using Oasis MCX is shown in Figure 2. All triazole AI's were enantiomerically resolved in less than 3.5 minutes. The United States Pharmacopeia (USP) resolution (Rs) ranged from 1.73 to 6.83.

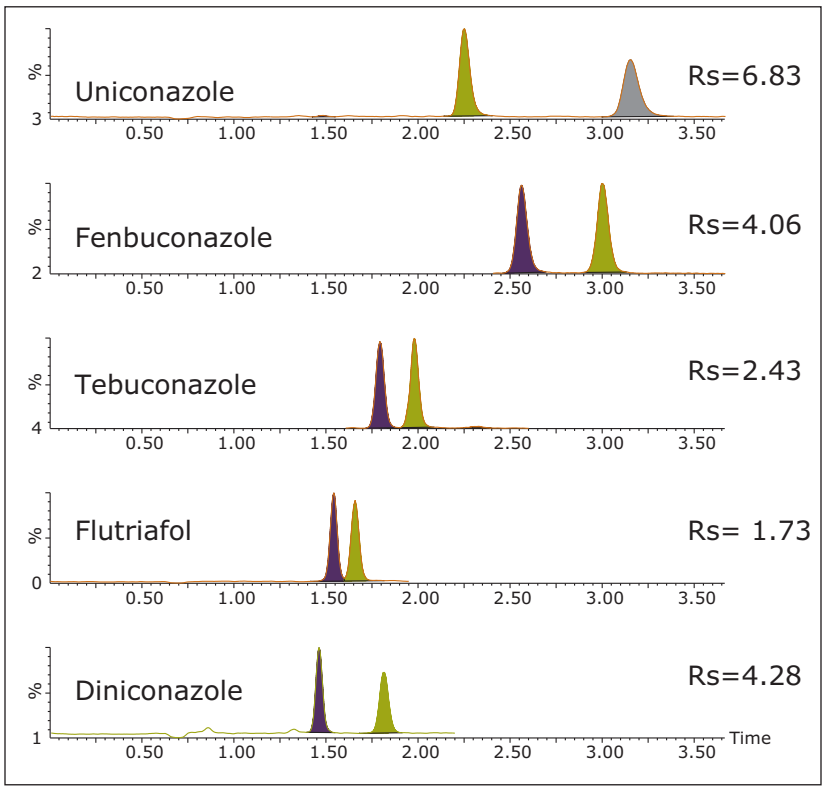

Figure 2. ACQUITY UPC²-MRM chromatograms showing the enantioseparation of five triazole fungicides pre-spiked onto wheat grain at a level of 1 ng/g and extracted using QuEChERS with Oasis MCX sample cleanup.

Linearity, accuracy, and sensitivity

The five triazole fungicides were post spiked into the wheat grain and/or wheat straw extracts. The spiked extracts were sequentially diluted with blank matrix extract to produce a series of matrix-matched curves and QC samples ranging in concentration from 0.005 to 50 ng/mL. Examples of the quantitation curves for each flutriafol enantiomer spiked into blank wheat grain extract, and for the fenbuconazole enantiomers spiked into blank wheat straw extract are shown in Figures 3 and 4 respectively. Linear calibration curves (R^2 >0.998) for each enantiomer of the target fungicides were obtained.

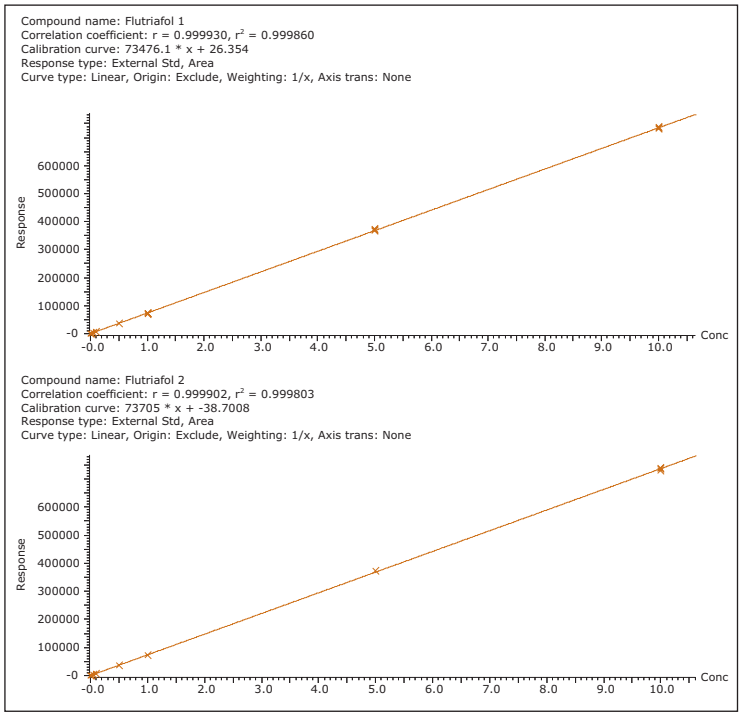

Figure 3. Wheat grain matrix-matched quantitation curves for each flutriafol enantiomer analyzed in triplicate 0.005 to 10 ng/mL.

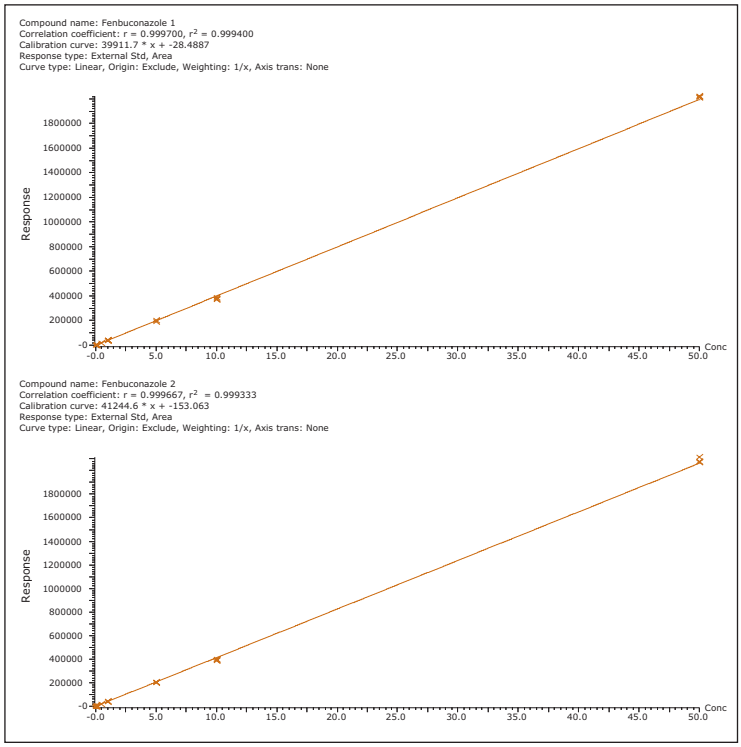

Figure 4. Wheat straw matrix-matched quantitation curves for each fenbuconazole enantiomer analyzed in triplicate 0.01 to 50 ng/mL.

To assess the accuracy of the method, quality control (QC) samples were made up in the blank extracted matrices at four concentration levels: 0.016, 0.16, 1.66, and 16.66 ng/mL. Three concentration levels were analyzed against the curves. The calculated concentrations for the QC samples were within +/- 15% of the known concentration for each enantiomer in both the wheat and straw matrices.

Examples of the blank, 0.01 ng/mL and 0.05 ng/mL level for all compounds spiked into extracted wheat grain matrix are shown in Figure 5.

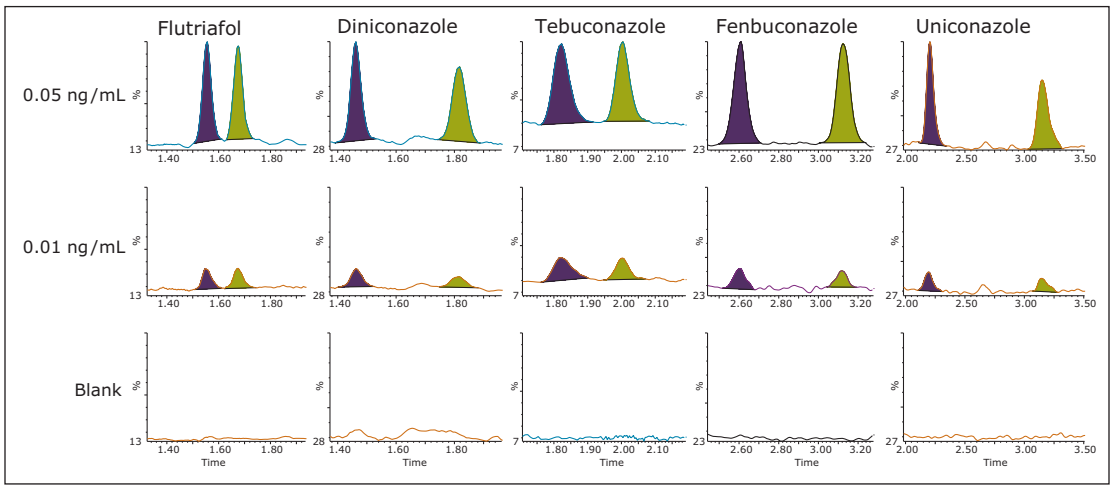

Figure 5. ACQUITY UPC²-MRM chromatograms showing the enantioseparation of five triazole fungicides spiked into blank wheat grain matrix extracted using QuEChERS, followed by cleanup using Oasis MCX. The blank, 0.01 ng/mL, and 0.05 ng/mL levels (equivalent to 0.02 ng/g and 0.1 ng/g if spiked directly on to the wheat grain pre-extraction) are shown; 4 µL injection.

Reproducibility

The precision of the technique was determined by a repeatability study (n=4) using four concentration levels 0.05 ng/mL, 1 ng/mL, 5 ng/mL, and 10 ng/mL of matrix-matched wheat grain standards, as shown in Table 2.

Table 2. Table shows the %RSD (n=4) for each enantiomer of the triazole fungicides at four concentration levels 0.05, 1, 5, and 10 ng/mL (4 µL injection).

		%RSD (n=4)			
	Enantiomer	0.05 ng/mL	1 ng/mL	5 ng/mL	10 ng/mL
Flutriafol	Peak 1	1.2	1.5	1.0	1.2
	Peak 2	2.9	1.9	0.6	2.5
Tebuconazole	Peak 1	1.3	1.3	2.8	2.0
	Peak 2	1.8	2.2	1.7	2.0
Fenbuconazole	Peak 1	1.9	0.8	2.5	1.3
	Peak 2	2.5	1.3	1.7	1.0
Diniconazole	Peak 1	2.4	2.9	2.1	1.1
	Peak 2	2.6	3.9	2.9	2.8
Uniconazole	Peak 1	1.5	1.3	1.9	1.7
	Peak 2	2.6	1.1	1.5	1.5

Internal standards were not available for the study; however the RSD's ranged from 0.6% to 3.9%. These results illustrate the reliability of the method reproducibility over a range of concentration levels.

Matrix effects and recovery

A series of standard solutions was prepared in methanol at the same concentration levels as the matrix-matched curves. The analyte response and slopes from both curves were compared. The matrix effects were calculated to within +/- 10% for each enantiomer of the target fungicides.

The average extraction recoveries from three samples fortified at 1 ng/g, 5 ng/ g, and 10 ng/g (n=3) in wheat were calculated. Recoveries in excess of 75% were obtained for each enantiomer of the pesticides analyzed in the study.

Simultaneous qualitative tools: RADAR™ and PICs

Depending on the chromatographic conditions target analytes can co-elute with endogenous matrix components which can lead to matrix effects and decreased method robustness. The Xevo TQ-S employs a proprietary scanning technology known as RADAR from which full scan (MS) and MRM (MS/MS) data can be acquired simultaneously. RADAR provides a convenient way to monitor the background matrix using its full-scan MS function. Co-eluting components can be identified at an earlier stage of the method development process.

In addition Product Ion Confirmation Scan (PICs) can be activated, which facilitate the collection high quality full-scan spectra during MRM acquisition, and provide an additional means of chromatographic peak identification based on MS or MS/MS spectra. Activated by a single check box in the method editor, PICS automatically triggers a product ion scan when a peak is detected by MRM.

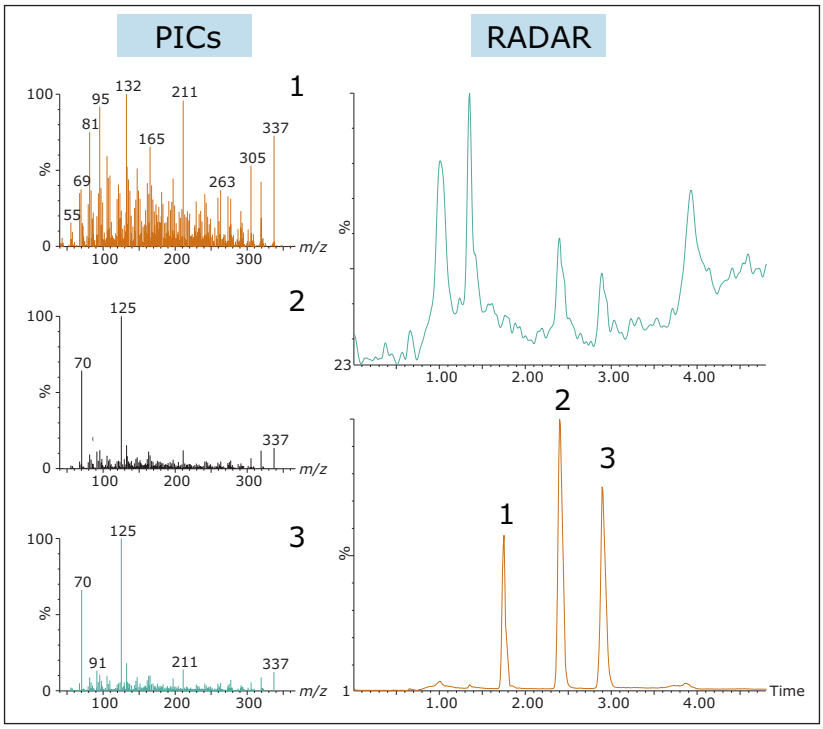

Figure 6. ACQUITY UPC²-MRM (lower trace) and RADAR chromatograms using gradient elution showing the resolution of fenbuconazole enantiomers pre-spiked onto wheat straw.

During method development for the analysis of fenbuconazole enantiomers in wheat straw the RADAR and PICs chromatograms shown in Figure 6 were acquired simultaneously with the MRM function. Peak 1 in the MRM chromatogram was isobaric (*m/z* 337) with fenbuconazole and shared a common fragment. Enantiomers were differentiated using the MS/MS PIC spectrum. The RADAR data acquired simultaneously identified components eluting closely to the analytes (Peaks 2 and 3). Changes were made to the SPE cleanup and isocratic elution was employed, which resulted in a lower spectral background and the removal of closely eluting matrix components.

CONCLUSIONS

The study of pesticide enantiomers is important as they can exhibit different bioactivities. Analytical methods that can rapidly provide information about each enantiomer at trace concentration levels can lead to a more accurate assessment of the influence of stereochemistry on the degradation dynamics, environmental fate, and final residue levels of crop protection chemicals.

In this study, the enantioseparation of five triazole fungicides was performed in less than 3.5 minutes. The Xevo TQ-S was used for detection of the rac-triazole fungicides in wheat grain and wheat straw. The results from the chiral UPC^2-MRM analysis show that trace level detection (ppt) can be achieved with good precision and accuracy over at least 3.5 orders of magnitude using this technique.

The use of RADAR, where full-scan data can be acquired simultaneously with MRM data can help identify co-eluting components that could potentially decrease the assay's robustness.

When complex matrices are analyzed, despite the specificity of MRM, matrix components give rise to signals that can be misidentified as an analyte peaks. PICs data provides an added qualitative element to the acquisition, which is useful for achieving higher selectivity, and greater confidence for peak assignment and confirmation.

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