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Includes sample preparation, chromatography, and mass spectrometry conditions

Explosives

Illicit Drugs

Natural Compounds

Perfluorinated Compounds (PFCs)

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Application Note: 51879

Simultaneous UHPLC/MS Analyses of Explosive Compounds

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Key Words

- MSQ Plus Mass Detector
- Explosives
- Library Spectra
- Sensitivity
- UHPLC

Introduction

Explosive compounds, which are recognized as four major categories, nitroaromatics, nitroamines, nitrate esters and peroxides according to their chemical structures, are widely used in warfare, mining industries, terrorist attacks and civil constructions. Explosive contaminated soils are mostly found on firing points, impact areas and training ranges. Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is a primary explosive found on the training ranges, as well as 2,4,6-trinitrotoluene (TNT), 2,6-dinitrotoluene (2,6-DNT) and 2,4-dinitrotoluene (2,4-DNT). The explosive contaminates in soil are possible sources for surface and ground water contaminations, posing the environmental and public health risks due to the compounds' toxicity, carcinogenicity and mutagenicity.[1,2] The increased terrorism activities have brought the world's attention on explosive compounds, especially peroxide explosives. Triacetone triperoxide (TATP) became a well known peroxide explosive after its use by a terrorist in 2001. The analyses of explosive compounds are demanded by the environmental monitoring and protection agencies, crime scene investigations and homeland securities. Explosive analyses are challenging processes because most of the explosive materials degrade quickly after their explosion and the sample matrices vary from one to the other. Furthermore, the peroxide explosives are not suitable for UV detection because of their lack of chromophores and their instability under the illumination of UV light.

The U.S. Environmental Protection Agency (USEPA) method 8330 is the current standard method for the identification of explosive compounds, which uses HPLC separation and UV detection of nitroaromatic and nitroamine compounds. However, the lack of selectivity of UV detection makes compound identification in complicate matrices ambiguous. Mass spectrometry has been employed in TATP detection with Agilent LC/MSD TOF instrument; however, the Agilent instrument and method demonstrated poor sensitivity with limit of quantitation (LOQ) at 1 mg/L.[3]

In this application, we developed an ultra high performance liquid chromatography/mass spectrometry (UHPLC/MS) method to efficiently separate, detect and quantitate all four classes of explosive compounds, including eight nitroaromatics, two nitroamines, five nitrate esters and two peroxides. The explosives were separated on a Thermo Scientific Hypersil GOLD PFP, 1.9 µm, 2.1 x 100 mm column and detected by selected ion monitoring (SIM) on an Thermo Scientific MSQ Plus Mass Detector – a fast scanning, single-quadrupole mass spectrometer.

Experimental Conditions

Standard Preparation

Hexamethylenetriperoxidediamine (HMTD), octohydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), ethylene glycol dinitrate (EGDN), diethylene glycol dinitrate (DEGDN), 1,3,5-trinitrobenzene (1,3,5-TNB), 1,3-dinitrobenzene (1,3-DNB), methyl-2,4,6-trinitrophenylnitramine (Tetryl), 4-amino-2,6-dinitrotoluene (4A-DNT), 2-amino-4,6-dinitrotoluene (2A-DNT), nitroglycerin (NG), 2,4,6-trinitrotoluene (TNT), 2,6-dinitrotoluene (2,6-DNT), 2,4-dinitrotoluene (2,4-DNT), pentaerythritol tetranitrate (PETN), trimethylolethane trinitrate (TMETN), and triacetone triperoxide (TATP) were purchased from AccuStandard® (New Heaven, CT, USA) as 100 mg/L standard solution in acetonitrile or in solid form. The stock solutions of 1000 mg/L of RDX, TNT, Tetryl and PETN standard were prepared by dissolving accurately weighed solids in acetonitrile or methanol. The calibration standards were prepared by diluting the 100 mg/L stock solutions with water to 0.010, 0.032, 0.160, 0.800, 4.00, and 20.00 mg/L.

Sample Preparation

Blank soil sample (San Jose, CA) was dried and homogenized. Each 2.0 g of the dried blank soil sample was amended with 0.04 µL, 0.2 µL, 1 µL, 2 µL and 10 µL standard solution containing 100 mg/L RDX, TNT, Tetryl and PETN, which corresponded to 2, 10, 50, 100 and 500 µg/kg for each analyte in soil. The amended soil samples (2.0 g) were added to 5 mL of acetonitrile. The solutions were capped and sonicated for 15 min. The supernatants (3.5 mL) were transferred to a clean vial, evaporated at 37 °C to dryness under nitrogen. The residues were reconstituted with 200 µL acetonitrile as samples for LC/MS analyses.

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Chromatographic Conditions

Instruments:	Thermo Scientific Accela pump Thermo Scientific Accela Autosampler			
Columns:	Hypersil GOLD PFP, 1.9 µm, 100 x 2.1 mm			
Flow Rate:	0.5 mL/min			
Mobile Phase:	A: water, 1 mM ammonium formate B: methanol			
Gradients:	Time (min)	A(%)	B(%)	µL/min
	0.0	80.0	20.0	500
	10.0	45.0	55.0	500
	12.0	20.0	80.0	500
	12.1	5.0	95.0	500
	12.9	5.0	95.0	500
	13.0	80.0	20.0	500
	15.0	80.0	20.0	500
Injection Volume:	2 µL partial loop injection, 25 µL loop size			

Mass Spectrometer Conditions

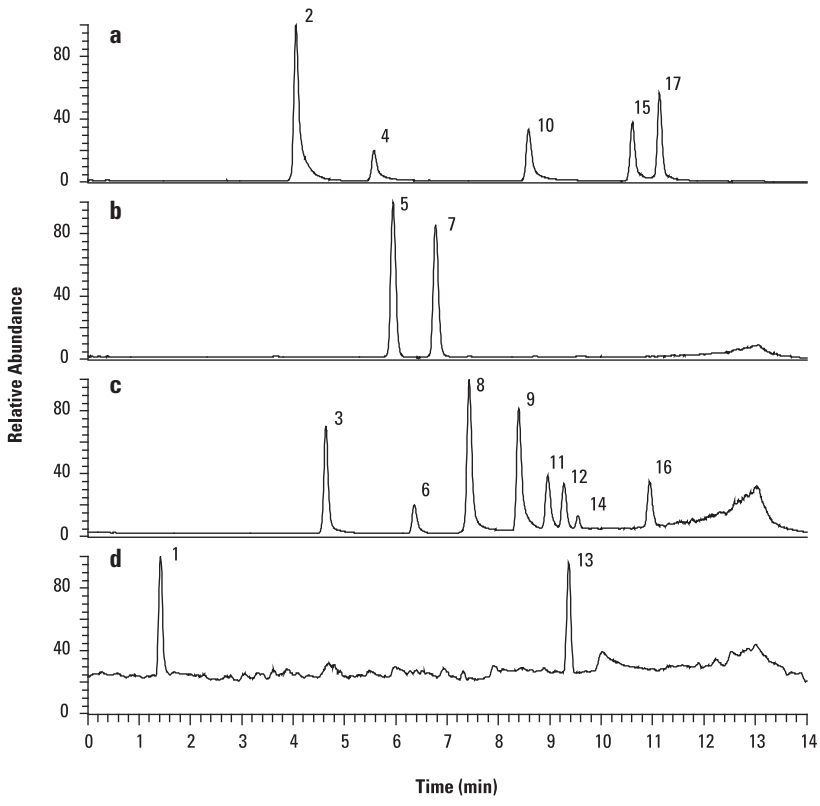
Instrument:	MSQ Plus Mass Detector
Ionization:	Atmospheric Pressure Chemical Ionization (APCI)
Polarity:	Positive and Negative
Probe Temperature:	350 °C
Cone Voltage:	60.0 V
Scan Mode:	Full scan with mass range of 50-400 amu or selected ion monitoring (SIM)
Corona Current:	30 µA
Scan Time:	0.5 s for full scan, 0.25 s for SIM

Results and Discussion

UHPLC Separation and MS Detection

USEPA 8330 method provides sensitive UV detection for nitroaromatic and nitroamine explosives. However, two analytical columns with different stationary phases are required to separate and identify the isomers, 2,4-DNT and 2,6-DNT, 4-A-2,6-DNT and 2-A-4,6-DNT, which make this method time consuming and results in low sample throughput.

The simultaneous separation and detection of seventeen explosive compounds was achieved through UHPLC/MS, using the Thermo Scientific Accela system with a fast scanning, single quadrupole mass spectrometer (Figure 1). Water and methanol were used as the mobile phases and the optimized gradient is shown in the Chromatographic Conditions. The elution order of the compounds and their retention times are shown in Figure 1. Hypersil GOLD™ PFP has a fluorinated phenyl group in the stationary phase which improves selectivity towards aromatic compounds. It also provides better resolutions for polar compounds containing hydroxyl, carboxyl, nitro or other polar groups. Eight nitroaromatic compounds, two nitroamine compounds, five nitrate ester compounds and two peroxides were separated with baseline resolution on a Hypersil GOLD PFP, 1.9 µm, 100 x 2.1 mm column. The isomer pairs, 2,4-DNT and 2,6-DNT , 4-A-2,6-DNT and 2-A-4,6-DNT, were separated with the peak resolution of 2.8 and 7.3 respectively (Peaks 9 and 11, 12 and 16).



Peak	Compound	Retention Time (min)
1	HMTD	1.42
2	EGDN	4.06
3	TNB	4.64
4	DEGDN	5.58
5	HMX	5.95
6	1,3-DNB	6.36
7	RDX	6.77
8	TNT	7.43
9	2,6-DNT	8.40
10	NG	8.58
11	2,4-DNT	8.96
12	4-A-2,6-DNT	9.28
13	TATP	9.37
14	TETRYL	9.55
15	TMETN	10.60
16	2-A-4,6-DNT	10.94
17	PETN	11.13

Figure 1: UHPLC/MS separation and detection of the 17 explosives standard with negative APCI (a-c) and positive APCI (d) ionizations. a) Extracted ion chromatogram at *m/z* of 61.96; b) Extracted ion chromatogram at *m/z* of 102.05; c) Extracted ion chromatogram at *m/z* of 213.02, 168.09, 227.01, 182.07, 197.04 and 241.02; d) Extracted ion chromatogram at *m/z* of 209.04 and 348.08.

The MSQ™ Plus Mass Detector was employed for the detection of the explosive compounds. Full scan mode with a mass range of 50-400 amu was employed for the compound identification and confirmation, while SIM mode was used for the sensitivity and quantitation studies.

The mass spectra for some explosive compounds are difficult to be predicted because of their reactivity. An array of the ions, such as additive adducts and decomposing ions, is observed in the LC/MS analyses of explosives.[4] The observed ion signals vary depending on many factors, for example, the ionization sources, analytes concentrations, additive concentrations, impurities in the mobile phases and the contaminations of the LC/MS system.

APCI was used in the MS detection of the explosives because it gave better sensitivities than ESI. Nitroaromatics, nitroamines and nitrate esters were detected using APCI negative mode, while peroxides were detected using APCI positive ionization (Figure 2). Some explosive standards, including TNB, 1,3-DNB, TNT, 2,6-DNT, 2,4-DNT, 4-A-2,6-DNT and 2-A-4,6-DNT, showed both molecular ion signals ($[M]^-$ or $[M-H]^-$) and decomposing ions ($[M-30]^-$ and/or $[M-17]^-$) in their MS spectra. Other explosive standards showed only decomposing ions: the nitrate esters, including EGDN, DEGDN, NG, TMETN and PETN, showed decomposing ions of $[NO_3]^-$ at *m/z* 61.95; the nitroamines, including RDX and HMX, showed decomposing ions at *m/z* 102.05 and 129.16. TATP formed adduct ions with its decomposing ions and ammonium, $[M+NH_4 + H(OOC(CH_3)_2OOH]^+$ at *m/z* of 348.08. In this case, the addition of 1 mM ammonium acetate in the mobile phase A was critical, providing the sources of ammonium ions to facilitate the formation of the ammonium adduct.

The two isomer pairs, 2,6-DNT and 2,4-DNT, 4-A-2,6-DNT and 2-A-4,6-DNT, demonstrated significant differences in their fragmentation MS spectra with the source induced fragmentation (SID) of the MSQ Plus Mass Detector. The spectrum of the 2,6-DNT showed one major fragmentation ion $[M-30]^-$ at *m/z* 152.10, while 2,4-DNT gave two major fragmentation ions $[M-30]^-$ at *m/z* 152.11 and $[M-17]^-$ at *m/z* 165.15. 4-A-2,6-DNT showed one major fragmentation ion $[M-30]^-$ at *m/z* 167.09, while 2-A-4,6-DNT gave two major fragmentation ions $[M-30]^-$ at *m/z* 167.10 and $[M-17]^-$ at *m/z* 180.16. Thus, the identification of these isomers was strengthened with the single quadrupole MS detector.

The identification of the explosive compounds with EPA 8330 method is based solely on the retention times of LC separations. The interference of the sample matrices alters the retention times of target compounds and causes false identifications. With the current UHPLC/MS method, target compounds are identified and confirmed by matching the APCI mass spectra against the MS spectra library. Figure 3A showed a total ion chromatogram (TIC) of a customer sample collected by this method. TNT and 2,4-DNT were easily identified by library spectra search against more than 20 explosive compounds (Figure 3). The Thermo Scientific Xcalibur software displayed the searching result with a list of compounds ranked by their matching scores. The implementation of the MS spectra library in compound identification provided more confirmative results compared to EPA 8330 method.

Detection Linearity and Sensitivity

The detection linearity of the UHPLC/MS system was investigated using the explosives standard. Calibration curves of seventeen standards were constructed over a concentration range of 10-100,000 ng/mL (ppb). Correlation coefficients of 0.999 or better were achieved for most of the standards (Table 1). The calibration curves for TNB, TNT, 2,6-DNT, 2,4-DNT and TETRYL showed linearity over four orders of magnitude working ranges (Table 1).

Improved sensitivities were observed by high throughput UHPLC because of the sharper and taller peaks produced by the sub-2 µm particle columns. The SIM mode of the MSQ Plus Mass Detector further extended the detection sensitivity compared to the traditional UV detector. The limit of quantitation (LOQ) and the limit of detection (LOD) for seventeen standard explosive compounds were examined. The sensitivities were achieved at ppb level for TNB, 1,3-DNB, TNT, 2,6-DNT, 2,4-DNT, TATP and TETRYL (Table 1). This represents a thirty-five times improvement in the detection sensitivity for TATP relative to the detection sensitivity of the Agilent instrument and method. The detection sensitivities obtained by the UHPLC/MS method with library matching of APCI mass spectra was more than tenfold versus the EPA 8330 method.

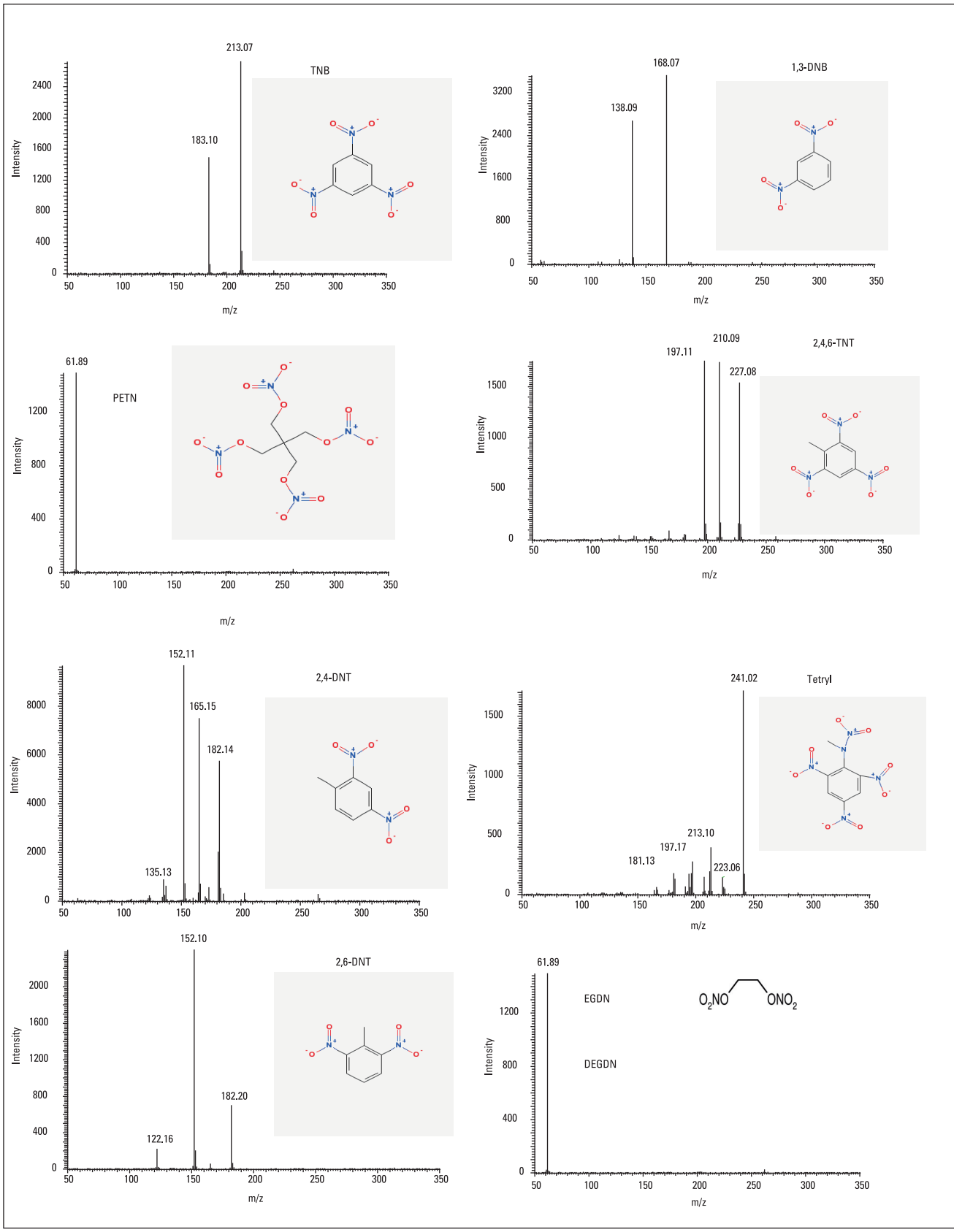
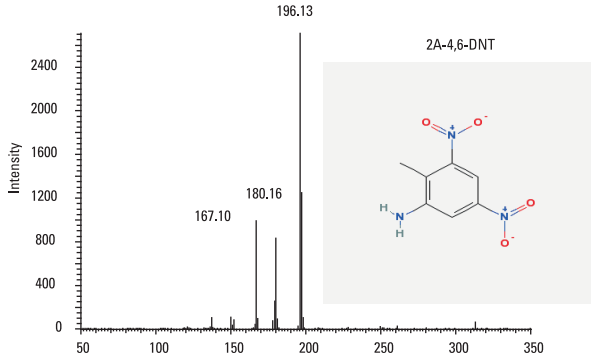
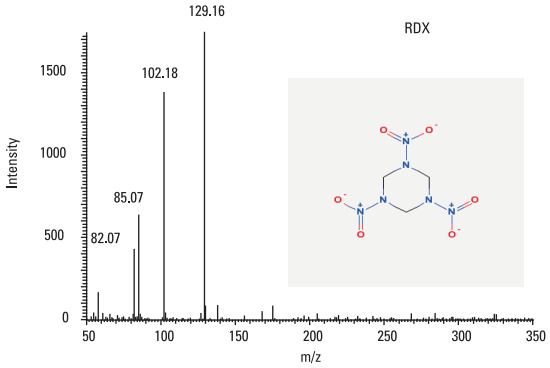
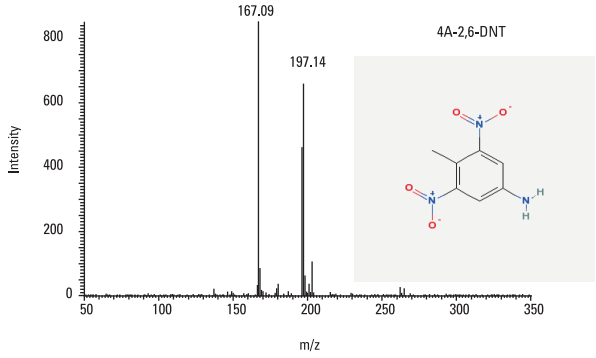
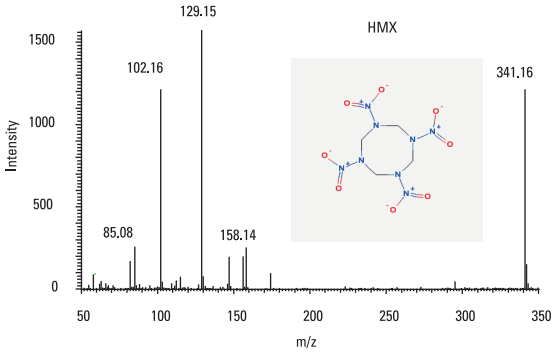


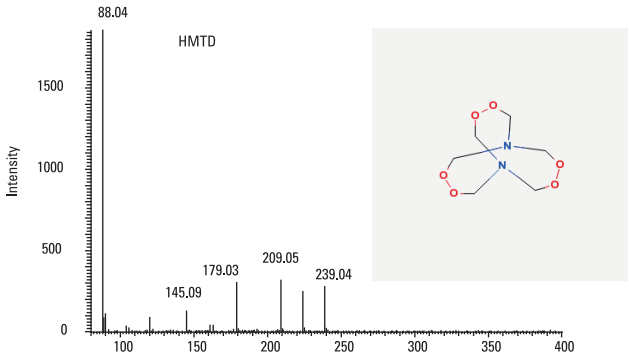
Figure 2: The MS spectra of the 17 explosive standards

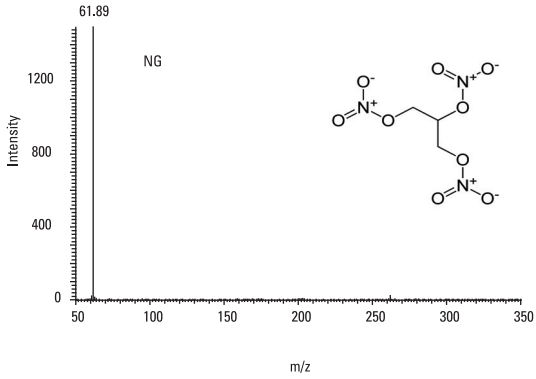

2A-4,6-DNT
196.13
180.16
167.10
Intensity

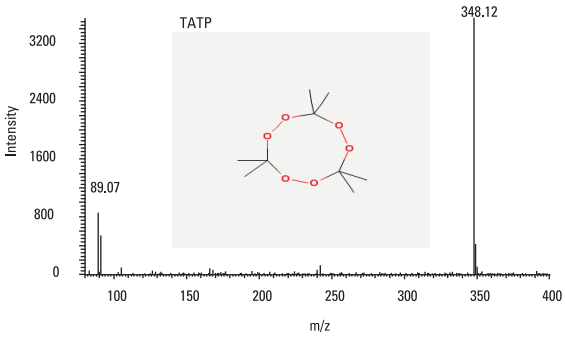

RDX
129.16
102.18
85.07
82.07
Intensity
m/z

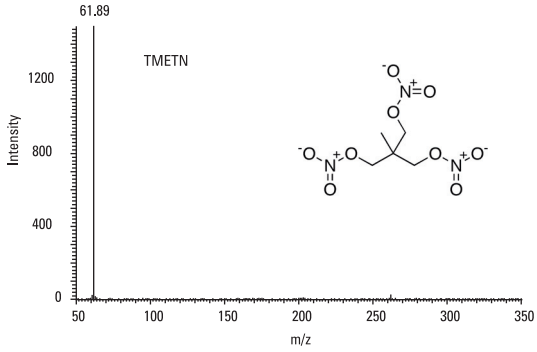

4A-2,6-DNT
167.09
197.14
Intensity
m/z

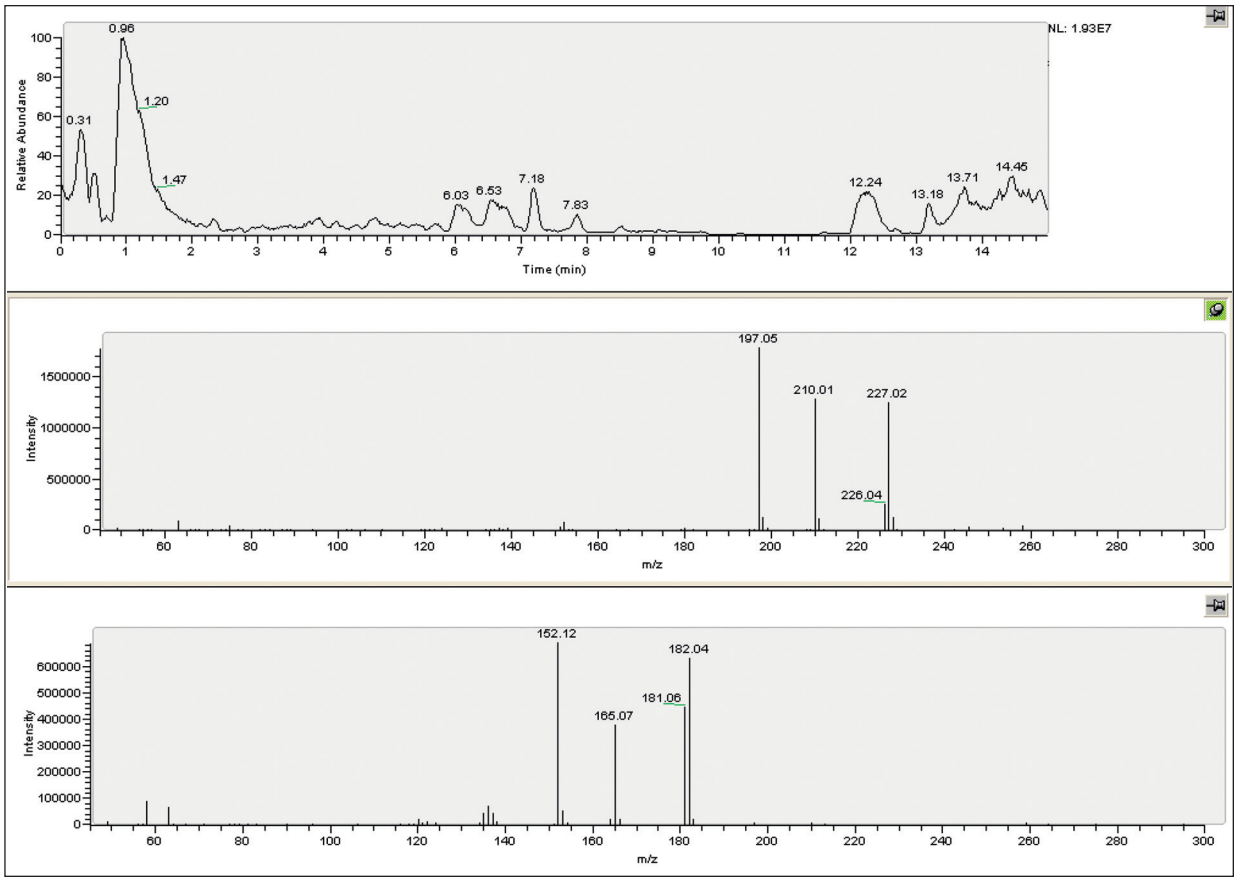

HMX
129.15
102.16
341.16
85.08
158.14
Intensity
m/z


HMTD
88.04
179.03
209.05
239.04
145.09
Intensity

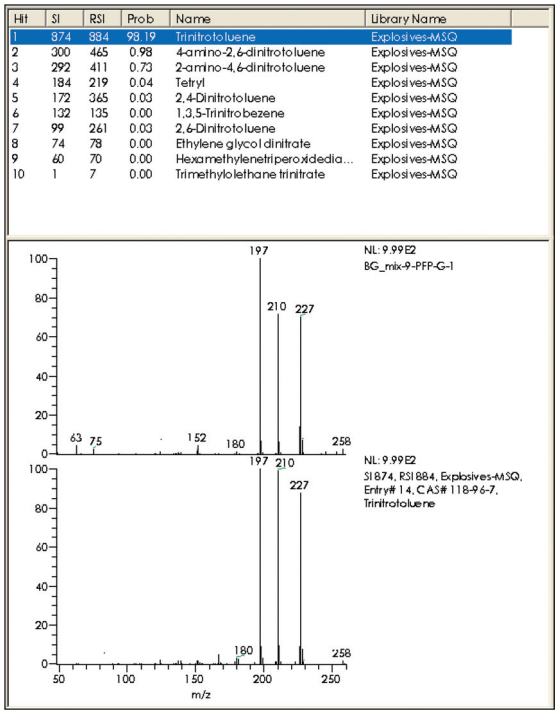

NG
61.89
Intensity
m/z


TATP
348.12
89.07
Intensity
m/z

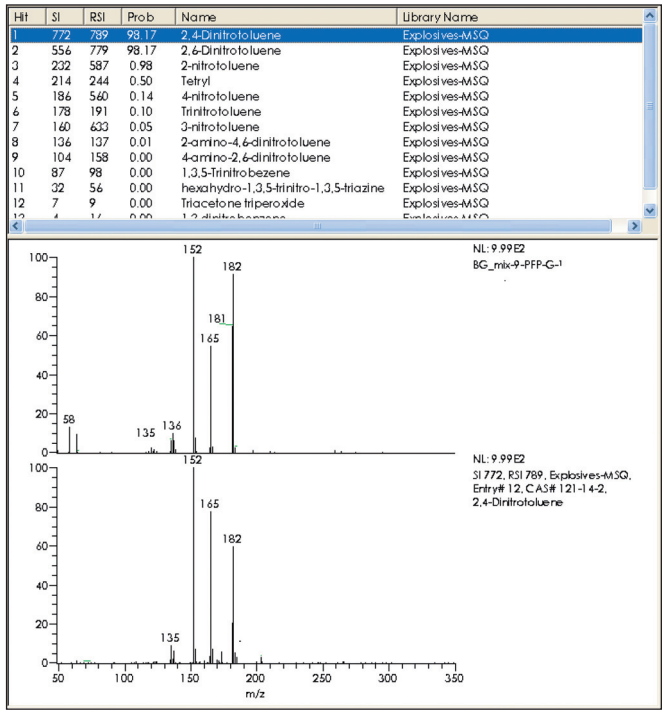

TMETN
61.89
Intensity
m/z



3a



3b



3c

Figure 3: The identifications of the explosives in customer sample using library spectra search: a) Total ion chromatography of the customer sample and the two MS spectra at 7.18 and 7.83 minute, respectively; b) The MS library search result for peak at 7.18 minute; c) The MS library search result for peak at 7.83 minute.

Compound	Monoisotopic Mass	Observed Mass	Linearity Range ng/mL	Correlation Coefficients	LOQ ng/mL	LOD ng/mL
HMTD	208.07	209.04	1000-100,000	0.9915	1136	341
EGDN	152.01	61.96	200-100,000	0.9997	79	24
TNB	213.00	213.00	10-100,000	0.9971	8	2
DEGDN	196.12	61.96	200-100,000	0.9991	617	185
HMX	296.05	102.05	225-100,000	0.9990	55	16
1,3-DNB	168.02	168.09	32-100,000	0.9950	16	5
RDX	222.03	102.05	225-100,000	0.9990	89	27
TNT	227.02	227.01	10-100,000	0.9977	8	2
2,6-DNT	182.03	152.07	10-100,000	0.9996	3	1
TATP	222.11	348.08	100-100,000	0.9964	28	8
NG	227.00	61.95	200-100,000	0.9994	265	79
2,4-DNT	182.03	152.07	10-100,000	0.9995	7	2
4-A-2,6-DNT	197.04	197.04	160-100,000	0.9998	91	27
TETRYL	287.01	241.02	10-100,000	0.9924	10	3
TMETN	255.14	61.95	200-100,000	0.9990	110	33
2-A-4,6-DNT	197.04	196.04	160-100,000	0.9965	75	22
PETN	316.01	61.95	200-100,000	0.9994	76	23

Table 1: LOQ and LOD of seventeen standard compounds

Analyses of Explosive Compounds in Soil Matrices

The explosive compounds, extracted from soil sample with acetonitrile, were analyzed using the UHPLC/MS method. Figure 4 showed the chromatography traces of RDX, TNT, Tetryl and PETN at 500 µg/kg, 10 µg/kg and the solvent extraction blank. The sample extraction recoveries from the soil matrices were evaluated. Four compounds, RDX, TNT, Tetryl and PETN, were tested at 500 µg/kg and 10 µg/kg levels (Table 2). Greater than 94% extraction recovery at 500 µg/kg level and more than 82% recovery at 10 µg/kg level were achieved for all the compounds tested. The method linearity and sensitivity were investigated for those compounds in soil matrices in the range of 2 to 500 µg/kg. Linear correlation coefficients of 0.996 or better were obtained (Figure 5). LOD of 0.2 to 0.6 µg/kg were achieved for TNT, Tetryl and PETN in soil matrices (Table 3).

	Extraction Recovery %	
Compound	10 µg/kg	500 µg/kg
RDX	89.7	96.2
TNT	92.1	98.5
Tetryl	90.6	95.4
PETN	82.3	94.3

Table 2: Extraction recoveries in soil matrices

	LOQ µg/kg	LOD µg/kg
RDX	16.5	5.0
TNT	0.7	0.2
Tetryl	1.8	0.6
PETN	2.0	0.6

Table 3: The method LOQ and LOD for compounds in soil matrices

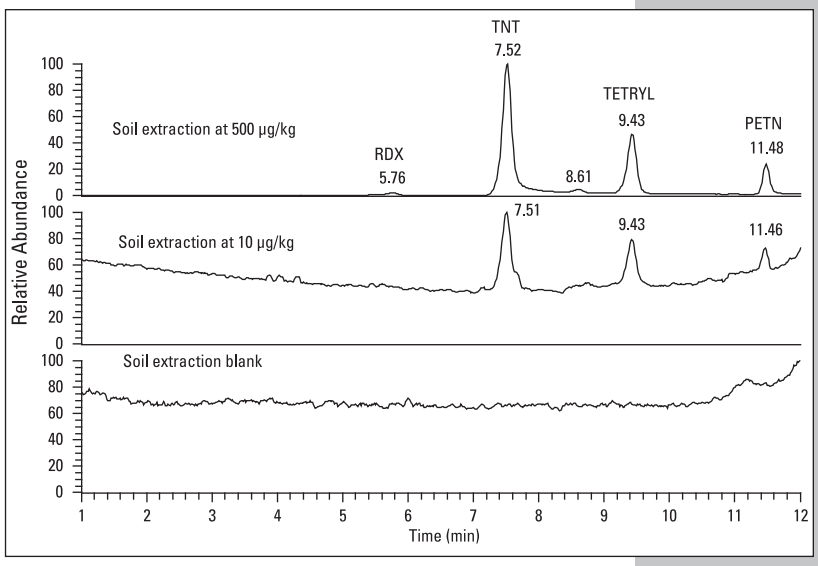


Figure 4: The UHPLC/MS analyses of the explosives in soil matrices

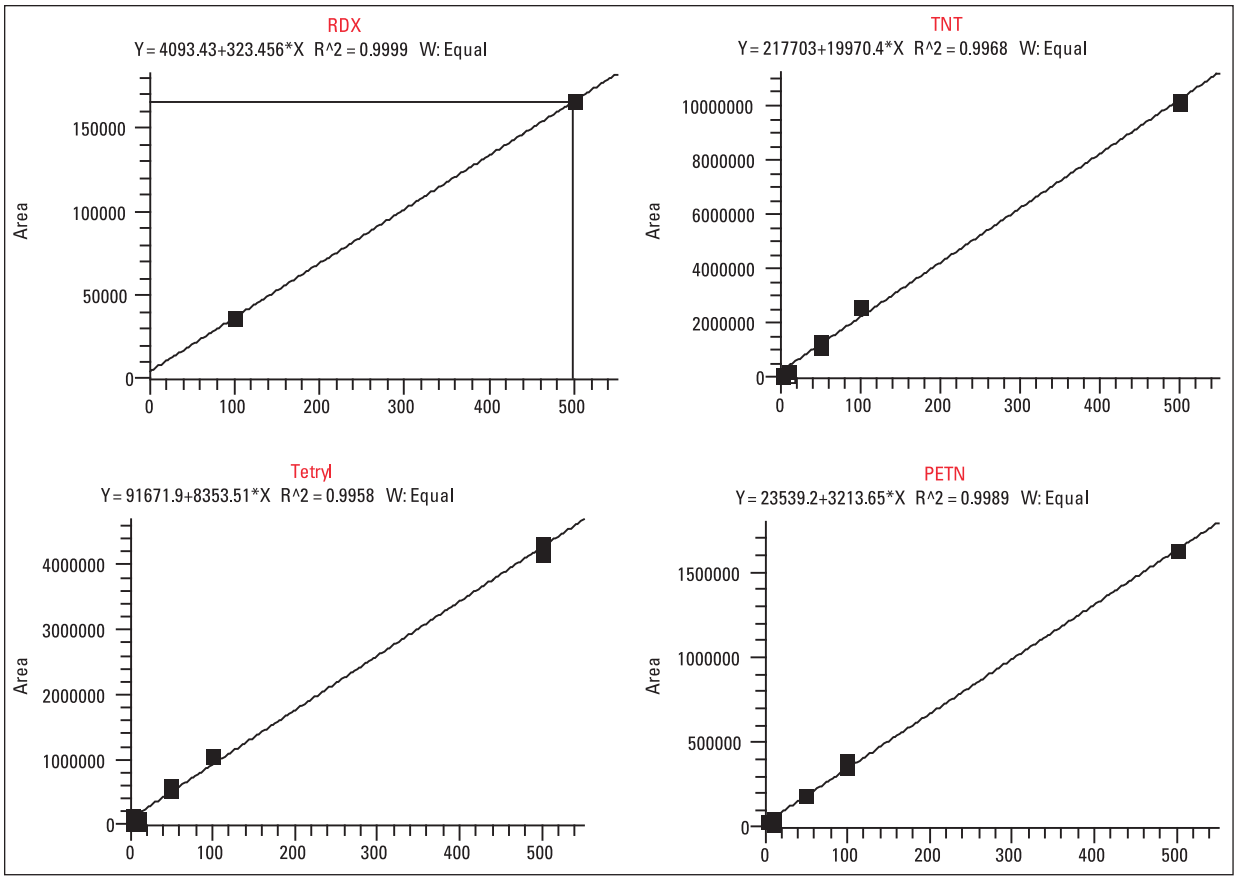


Figure 5: Linearity of the UHPLC/MS method for the analyses of explosives compounds in soil matrices

Conclusions

The simultaneous analyses of nitroamines, nitroaromatics, nitrate esters, and peroxide explosives by UHPLC/MS were accomplished. The UHPLC method, utilizing sub-2 µm particles, improved the separation efficiencies and resolutions. The MS detection method offered improved sensitivities, good selectivity and additional MS confirmations. The detection sensitivities were further increased by the pre-concentration step implemented in the sample preparation process. The more confirmative identifications of explosives were achieved by comparing of the collected APCI mass spectra to the comprehensive MS spectra library of the explosive residues. We demonstrated the improved separation performance, increased detection sensitivity and better selectivity, compared to the current USEPA 8330 method. We also achieved 35 times detection sensitivity for TATP compared to the Agilent instrument and method.

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AN51879_E 03/10M

Application Note: 433

Identification of Cannabinoids in Baked Goods by UHPLC/MS

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Guifeng Jiang, Thermo Fisher Scientific, San Jose, CA, USA

Key Words

- Accela™ UHPLC
- Hypersil GOLD™ PFP
- MSQ Plus™
- Δ^9-tetrahydro-cannabinol (THC)
- Forensic Analysis

Goal

Positively identify trace amounts of cannabinoids in a complex food matrix quickly, with minimal sample preparation and no chemical derivatization.

Introduction

Marijuana is the most common illegal drug in the United States, and each year U.S. law enforcement agencies seize more than two million pounds of marijuana in various forms. Seized evidence submitted to forensic laboratories is screened for marijuana by microscopic inspection and simple chemical tests such as the Duquenois-Levine test. Presumptive positive results are confirmed by using gas chromatography-mass spectrometry (GC/MS) to positively identify cannabinoids including Δ^9-tetrahydrocannabinol (THC, the main psychoactive component), cannabinol (the main degradation product of THC) and cannabidiol. This traditional approach works fairly well for leaf marijuana, hashish, hash oil and residue collected from smoking paraphernalia.

GC/MS is less useful for confirming the presence of marijuana in complex food matrices such as baked goods. Simple sample preparation procedures using methanol or methylene chloride coextract many small molecules found in baked goods that can coelute with the target cannabinoids. Cholesterol, fatty acids, and caffeine can contaminate the gas chromatograph, forcing the analyst to clean the instrument and rerun all subsequent samples. More extensive sample preparation methods are time-consuming and often require greater amounts of the controlled substance than are present in the evidence.

An alternative method to positively identify marijuana cannabinoids in complex food matrices is to use ultra high performance liquid chromatography with mass spectrometry detection (UHPLC/MS). UHPLC/MS offers a threefold benefit compared to GC/MS; simpler sample preparation, no derivatization, and less instrument clean up time. This application note demonstrates how a working forensic laboratory uses UHPLC/MS to analyze baked goods for three cannabinoids of forensic importance. The cannabinoids are extracted, separated within 8 minutes on a Hypersil GOLD PFP 1.9 µm, 100 x 2.1 mm column and detected by a fast scanning single quadrupole mass spectrometer.

Experimental Conditions

1. Standard and Sample Preparation

The standard compounds THC, cannabidiol and cannabinol were purchased from Alltech (State College, PA, USA) and used as received. These compounds were mixed and diluted to about 10 ppm with methanol to prepare a stock standard solution.

Brownie and cookie samples were obtained from evidence archived after adjudication. Two (2) mL methanol was added to 25 mg of baked-good material. This mixture was vortexed for 30 seconds, allowed to settle for 2 min, and the supernatant was filtered through a cotton-plugged Pasteur pipette. The filtrate was centrifuged at 12,000 rpm for 90 seconds, and filtered again. The second filtrate was diluted 50 fold with methanol prior to analysis.

2. Chromatographic Conditions

Chromatographic analyses were performed using the Accela UHPLC system (Thermo Fisher Scientific, San Jose, CA). The chromatographic conditions were as follows:

Column:	Hypersil GOLD PFP (perfluorinated phenyl) 1.9 µm, 100 x 2.1 mm			
Flow Rate:	1 mL/min			
Mobile Phase:	A: Water with 0.06 % acetic acid B: Acetonitrile (ACN) with 0.06% acetic acid C: Methanol with 0.06% acetic acid			
Gradient:	T (min)	A%	B%	C%
	0.00	95.0	0.0	5.0
	1.00	60.0	32.5	7.5
	2.00	50.0	40.0	10.0
	5.00	45.0	45.0	10.0
	6.00	25.0	60.0	15.0
	6.50	5.0	0.0	95.0
	7.50	5.0	0.0	95.0
	7.51	95.0	0.0	5.0
	8.00	95.0	0.0	5.0
Column Temperature:	45 °C			
Injection:	2 µL partial loop injection, 25 µL loop size Syringe Speed: 8 µL/sec Flush Speed: 100 µL/sec Flush Volume: 400 µL Wash Volume: 100 µL Flush/Wash Source: Bottle with methanol			

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3. Mass Spectrometer Conditions

MS analysis was carried out on a MSQ Plus single quadrupole LC/MS detector with Xcalibur 2.05 (Thermo Fisher Scientific, San Jose, CA). The MS conditions were as follows:

Ionization:	Electrospray (ESI)
Polarity:	Positive
Probe Temperature:	500 °C
Cone Voltage:	90 V
Scan Mode:	Full scan with mass range of 50-500 *m/z*
ESI Voltage:	3.5 kV
Scan Time:	0.2 s

Results

The cannabinoid standards elute with good resolution at 4.1 min (cannabidiol), 5.1 min (THC) and 5.4 min (cannabinol). The cannabinoids were detected by using full scans (50-500 *m/z*) of the single quadrupole mass spectrometer, and the extracted ion chromatograms from *m/z* 310.5-311.5 + 314.5-315.5 are displayed in Figure 1A. Molecular ions of each compound (*m/z* 315 for cannabidiol and THC and *m/z* 311 for cannabinol) are observed (Figure 2A-C).

The brownie sample, which was taken from an adjudicated case and was known to contain THC, tested positive for THC (Figure 1B, 2D), demonstrating that the sample preparation required for this LC/MS method is simpler, faster and requires less sample than the GC/MS method employed for the original casework.

After ten years in the forensic laboratory's training vault, cannabinoids in the cookie sample had degraded significantly, but by increasing the sample injection from 2 µL to 10 µL, THC was detected with good signal-to-noise (Figure 1C, 2E).

Solvent blanks were analyzed after each sample run, with no apparent carryover from one run to the next (results not shown).

Conclusion

Cannabinoids in baked goods can be identified using UHPLC/MS with minimal sample preparation. The preparation time (10 min) and run time (8 min) make this a very efficient analytical method.

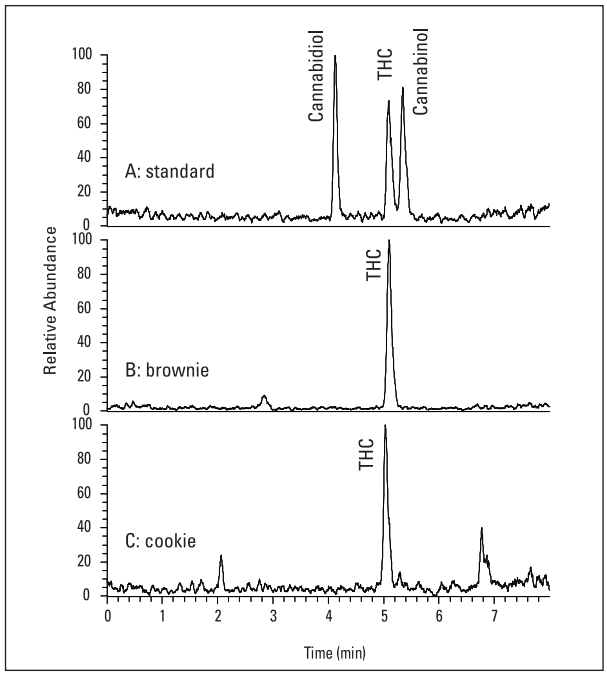

Figure 1: Extracted ion chromatograms (*m/z*, 310.5-311.5, 314.5-315.5) of cannabinoid standards (A) and extracts from brownie (B) and cookie (C) by UHPLC/MS

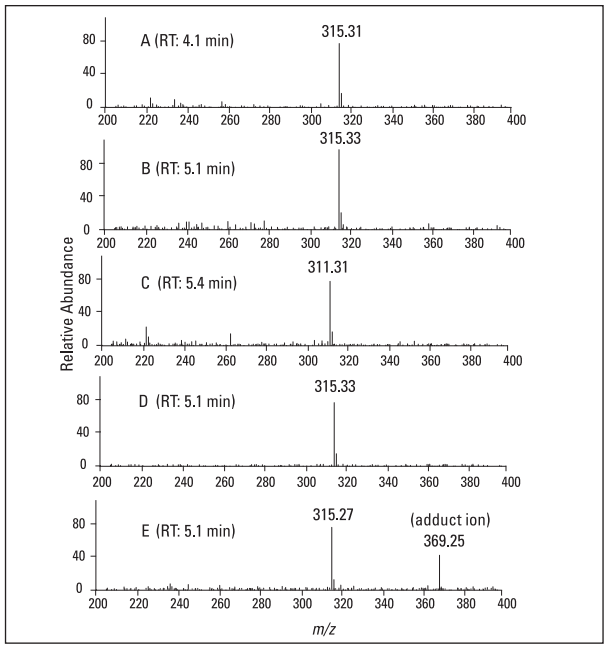

Figure 2: MS spectra of cannabinoid standards, cannabidiol (A), THC (B), cannabinol (C), eluted at 4.1 min, 5.1 min and 5.4 min respectively, and extracts from brownie (D) and cookie (E), eluted at 5.1 min

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AN62876_E 09/08M

Application Note: 432

Identification of Lysergic Acid Diethylamide (LSD) in Candy by UHPLC/MS

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Key Words

- Accela™ UHPLC
- MSQ Plus™
- Hypersil GOLD™ PFP
- Forensic Analysis
- LSD

Goal

Positively identify trace amounts of lysergic acid diethylamide (LSD) in sugar candy quickly, with minimal sample preparation and no chemical derivatization.

Introduction

Lysergic acid diethylamide (LSD) is a controlled substance in forensic chemistry that is notorious for being difficult to identify. Its myriad evidentiary forms include paper tabs, eye drops, sugar cubes and small sugary candies such as sweet tarts, valentine hearts or mints. Because it is such a potent hallucinogen, typical street doses require only 40 to 120 µg of LSD. The small personal-use amounts seized by state and local law enforcement often lack sufficient drug to allow both forensic analysis by traditional means and archiving of some of the evidence for follow-up testing.

Most forensic laboratories confirm the presence of LSD by using gas chromatography with mass spectrometry (GC/MS). LSD is extracted from the evidence with an organic solvent, derivatized, and determined by GC/MS. GC/MS resolves LSD from other compounds and provides structural information that can be compared to reference spectra in a searchable library.

The disadvantages of GC/MS are its requirements for extensive sample preparation, including chemical derivatization of LSD to a more volatile form, and its impaired performance with analytes that are polar, thermally labile, or nonvolatile. LSD has a high affinity for active sites in liners that can spoil chromatographic resolution. LSD-doped sugar cubes or candy can foul the GC with sugars, increasing the burden of instrument maintenance and hindering throughput.

An alternative method to positively identify LSD in complex food matrices is to use ultra high performance liquid chromatography with mass spectrometric detection (UHPLC/MS). UHPLC/MS offers a threefold benefit compared to GC/MS; simpler sample preparation, no derivatization, and less time wasted baking out or cleaning the instrument. This application note demonstrates how a working forensic laboratory uses UHPLC/MS to analyze sugar candies for LSD. LSD in doped sugar cubes and candy hearts is simply extracted, separated within 5 minutes on a Hypersil GOLD PFP 1.9 µm column, and confirmed by a fast scanning single quadrupole mass spectrometer.

Experimental Conditions

1. Standard and Sample Preparation

A 1000 mg/L solution of LSD in methanol was purchased from Alltech (State College, PA, USA) and diluted to about 5 mg/L with methanol.

The sugar cubes and candy hearts were purchased from a local grocery store. The candy hearts and sugar cubes were treated with 3-5 drops of this LSD solution and allowed to stand for 24 hours prior to use. Ten (10) mg scrapings from the sugar cube or candy heart were added to 2 mL methanol. This mixture was vortexed for 30 sec, allowed to settle for 1 min, and the supernatant was filtered through a cotton-plugged Pasteur pipette. The filtrate was centrifuged for 90 sec, and the supernatant was filtered through a second cotton-plugged Pasteur pipette and transferred to the autosampler vial.

2. Chromatographic Conditions

Chromatographic analyses were performed using the Accela UHPLC system (Thermo Fisher Scientific, San Jose, CA). The chromatographic conditions were as follows:

Column:	Hypersil GOLD PFP (perfluorinated phenyl) 1.9 µm, 100 x 2.1 mm			
Flow Rate:	1 mL/min			
Mobile Phase:	A: Water with 0.06 % acetic acid B: Acetonitrile (ACN) with 0.06% acetic acid C: Methanol with 0.06% acetic acid			
Gradient:	T (min)	A%	B%	C%
	0.00	95.0	0.0	5.0
	1.00	95.0	0.0	5.0
	1.50	90.0	5.0	5.0
	2.70	70.0	10.0	20.0
	3.00	5.0	15.0	80.0
	7.00	5.0	0.0	95.0
	7.10	95.0	0.0	5.0
	8.00	95.0	0.0	5.0
Column Temperature:	45 °C			
Injection:	2 µL partial loop injection, 25 µL loop size Syringe Speed: 8 µL/sec Flush Speed: 100 µL/sec Flush Volume: 400 µL Wash Volume: 100 µL Flush/Wash source: Bottle with methanol			

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3. Mass Spectrometer Conditions

MS analysis was carried out on a MSQ Plus single quadrupole LC/MS detector (Thermo Fisher Scientific, San Jose, CA). The MS conditions were as follows:

Ionization:	Electrospray (ESI)
Polarity:	Positive
Probe Temperature:	500 °C
Cone Voltage:	90 V
Scan Mode:	Full scan with mass range of *m/z* = 125-425 amu
ESI Voltage:	3.5 kV
Scan Time:	0.2 s

Results

LSD elutes at 4.49 min and is detected by using full scans (*m/z* = 125 - 425) of the single quadrupole mass spectrometer. The extracted ion chromatograms from *m/z* 324 ± 0.5 are displayed in Figure 1A. The MS spectrum of the LSD standard shows two molecular ion signals: $[M+H]^+$ at *m/z* 324.1, and $[M+ACN+Na]^+$ at *m/z* 387.1. The MS spectrum of LSD also shows two fragment ion signals at *m/z* 223.3 and 281.3 (Figure 2A).

The methanol extracts from the candy hearts and sugar cubes, doped with LSD, were analyzed using the same UHPLC/MS method as for the standard LSD (Figure 1B, 1C). Positive confirmation of LSD in the samples is assured both by retention time matching and MS spectra matching of the samples (Figure 2B-C) with the LSD standard.

Conclusion

UHPLC/MS can positively identify trace amounts of lysergic acid diethylamide (LSD) in sugar candy in 8 min, after a simple 10 min sample prep involving no chemical derivatization.

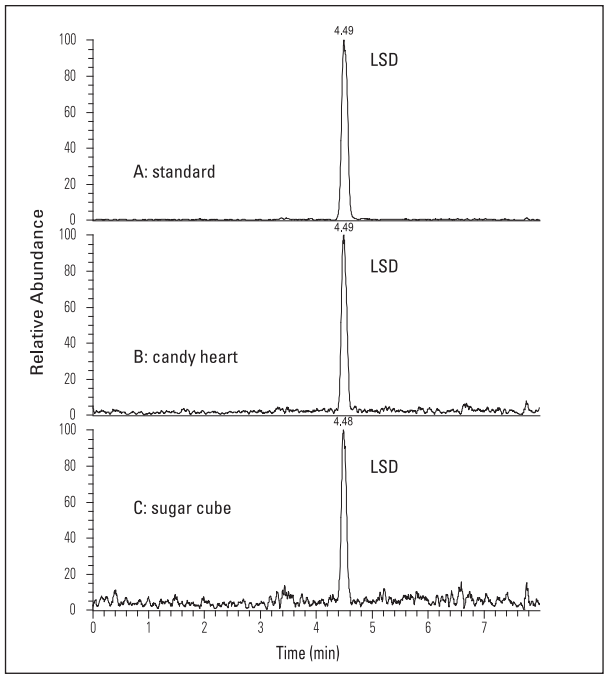

Figure 1: Extracted ion chromatograms at *m/z* = 324 ± 0.5 amu obtained by UHPLC/MS on a Hypersil GOLD PFP column: (A) LSD standard; (B) methanol extract of LSD-doped candy heart; (C) methanol extract of LSD-doped sugar cube. See text for details.

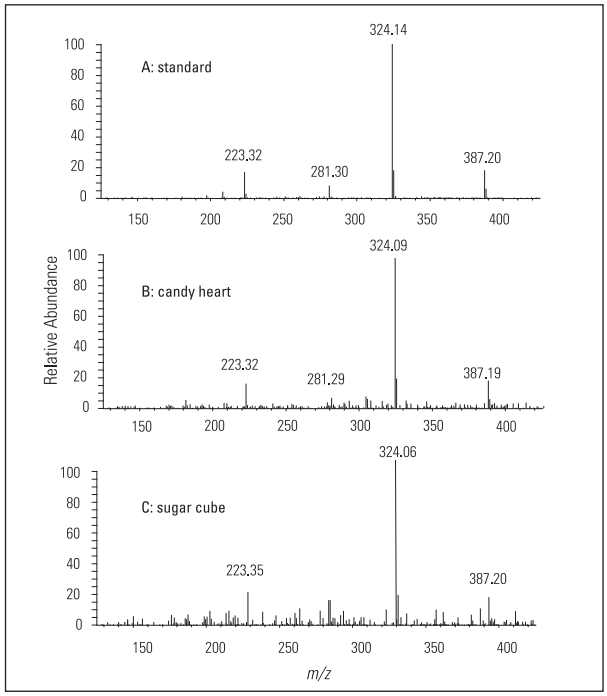

Figure 2: MS spectra of LSD obtained by UHPLC/MS on a Hypersil GOLD PFP column: (A) LSD standard; (B) methanol extract of LSD-doped candy heart; (C) methanol extract of LSD-doped sugar cube. See text for details.

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Application Note: 428

Identification of Psychotropic Substances in Mushrooms and Chocolate by UHPLC/MS

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Guifeng Jiang, Chris Loran, Thermo Fisher Scientific, San Jose, CA, USA

Key Words

- Accela UHPLC System
- MSQ Plus MS Detector
- Magic Mushroom
- Toxicology: Forensics
- Psilocybin: Psilocin

Goal

Develop a UHPLC/MS method for the detection, separation and confirmation of psilocybin and psilocin in mushrooms and chocolate matrices.

Introduction

The identification of controlled substances in food matrices is a challenge for forensic laboratories. Classical techniques, such as color tests and thin-layer chromatography, do not provide molecular structural information and cannot be used as a principal means of identification. Infrared spectroscopy (IR) and gas chromatography/mass spectrometry (GC/MS) can identify controlled substances, but suffer from several shortcomings. For example, IR analysis requires that the substances be pure for identification of the controlled substances; clean-up methods are cumbersome and demand excess sample, especially in light of the legal requirement to leave more than half of the exhibit available for subsequent testing. GC/MS methods, the typical screening approach for controlled substances, suffer from coelution of small molecules present in food and beverage matrices. Worse yet, a sample with a high concentration of cholesterol, fatty acids, or caffeine can contaminate the GC/MS instrument, forcing the analyst to clean the instrument and reanalyze all subsequent samples. Some compounds, such as psilocybin, a component of the 'magic mushroom' commonly found in food matrices, are thermally labile and do not survive the conditions of GC/MS intact.

To ameliorate these shortcomings, extensive wet-chemistry preparation methods have been developed; unfortunately, they are time-consuming and often require greater amounts of the controlled substances than are present in the evidence. These preparation schemes also do not eliminate some of the more problematic small molecules, and can exclude controlled substances during the course of the separation.

Given the increasing emphasis on instrumental methods of analysis and the limitations of the traditional instruments in the field of forensics, another solution is necessary. LC/MS holds several advantages over the traditional methods of analysis. For example, psilocybin does not decompose at the lower temperatures used in HPLC. The low concentrations of psilocybin and psilocin are not an issue due to increased sensitivity of this technique. Many matrix components that interfere in GC/MS methods do not interfere in HPLC methods because of greater differences in analyte solubility as compared to analyte volatility. However, the employment of traditional HPLC has been limited by lack of resolving power compared to capillary GC. Ultra high performance liquid chromatography (UHPLC) performs separations with improved efficiency and resolution with the use of sub-2 µm diameter particles. The 1-2 second peak widths and high resolving power are more competitive with capillary GC. In this application note, we identify, separate and confirm psilocybin and psilocin in mushroom and chocolate matrices using UHPLC/MS. The psychotropic substances are separated within 5 minutes on a Hypersil GOLD™ PFP 1.9 µm, 100 x 2.1 mm column and detected by a fast scanning single quadrupole mass spectrometer.

Experimental Conditions

1. Sample Preparation

Psilocybin (Lot #284) and psilocin (Lot #383) standards were purchased from Alltech (State College, PA, USA) and used as received. These standard compounds were mixed and diluted to about 50 ppb with methanol as stock standard solution. The psilocybin mushrooms were taken from a training sample and the chocolates were from returned evidence after adjudication.

Two (2) mL methanol was added to 10 mg dried magic mushroom. This mixture was vortexed for 30 seconds before the supernatant was filtered through a cotton-plugged Pasteur pipette, centrifuged for 90 seconds, and filtered again. The supernatant was further diluted 50-fold with methanol prior to analysis.

The chocolate samples were prepared using 10 mg of chocolate material, and the same extraction as for the mushroom material.

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2. Chromatographic Conditions

Chromatographic analyzes were performed using the Accela™ UHPLC system (Thermo Scientific, San Jose, CA). The chromatographic conditions were as follows:

Column:	Hypersil GOLD PFP (perfluorinated phenyl) 1.9 µm, 100 x 2.1 mm			
Flow Rate:	1 mL/min			
Mobile Phase:	A: Water with 0.06 % acetic acid B: Acetonitrile with 0.06% acetic acid C: Methanol with 0.06% acetic acid			
Gradient:	T (min)	A%	B%	C%
	0	95	0	5
	3.0	5	5	90
	4.0	5	5	90
	4.1	95	0	5
	5.0	95	0	5
Injection Volume:	2 µL partial loop injection, 25 µL loop size			
Column Temp:	45 °C			

3. Mass Spectrometer Conditions

MS analysis was carried out on a MSQ Plus single quadrupole LC/MS detector (Thermo Scientific, San Jose, CA). The MS conditions were as follows:

Ionization:	Electrospray (ESI)
Polarity:	Positive
Probe Temp:	500 °C
Cone Voltage:	90 V
Scan Mode:	Full scan with mass range of 100-400 *m/z*
ESI Voltage:	3.5 kV
Scan Time:	0.2 s

Results

Psilocybin and psilocin standards elute rapidly at 0.65 min and 2.25 min respectively with excellent resolution (Figure 1). Detection was carried out using full scans (100-400 *m/z*) of the single quadrupole mass spectrometer. Molecular ions of each compound (*m/z* 285 and *m/z* 205 for psilocybin and psilocin respectively) were observed. Authentic mushroom samples, taken from a training sample known to contain psilocybin and psilocin, were prepared by a series of extractions and filtrations as described above. These mushroom samples were analyzed using the same method developed for the standards and showed peaks with retention times consistent with the psilocybin and psilocin standards (Figure 2). The identities of peaks at 0.64 min and 2.22 min in figure 2 were also confirmed as psilocybin and psilocin by MS detection. The chocolate samples, which were taken from an adjudicated case and were known to contain psilocybin and/or psilocin, also showed the presence of both psilocybin and psilocin (Figure 3). Blanks were run successively after each run, with no apparent carryover from one run to the next.

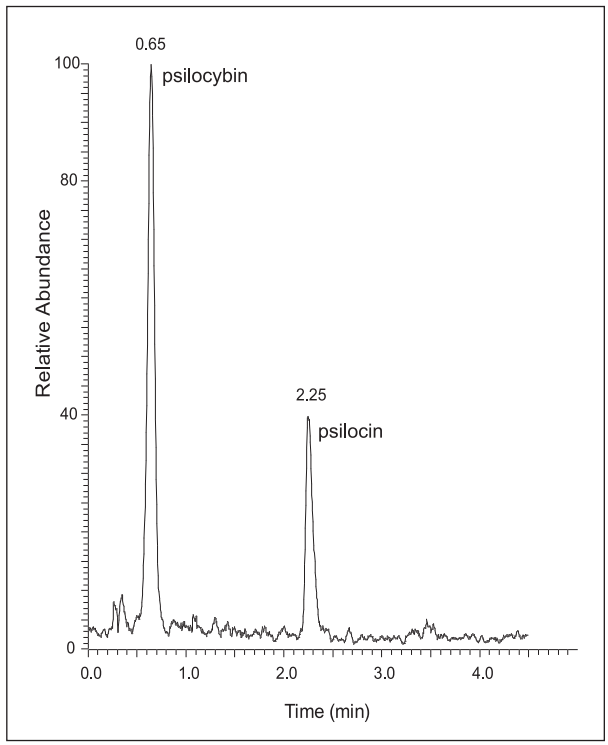

Figure 1: Psilocybin and psilocin standards

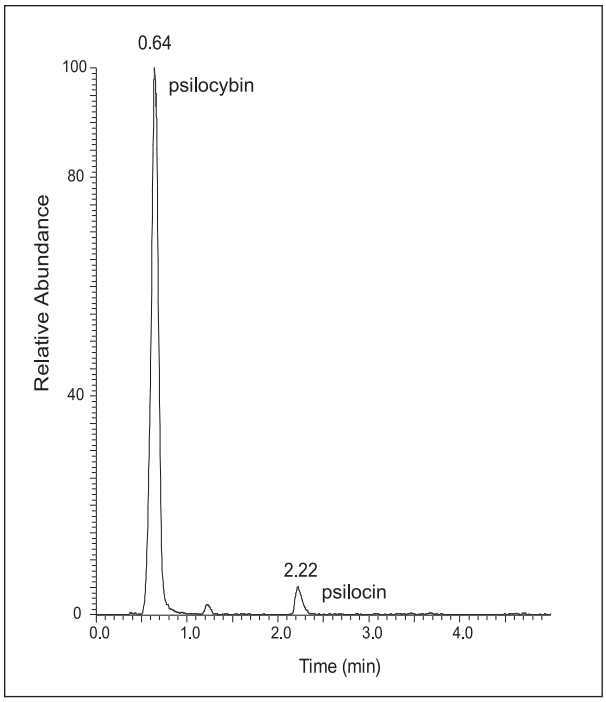

Figure 2: Methanol extract of raw mushroom material

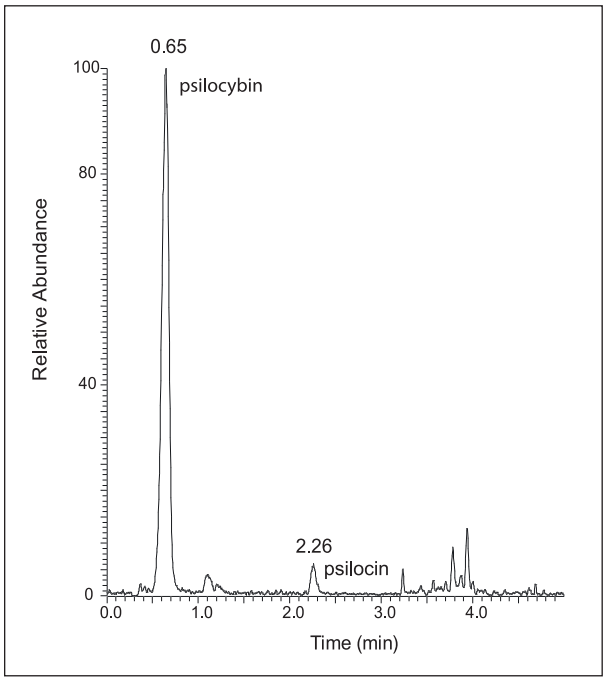

Figure 3: Methanol extract of chocolates containing psilocybin and psilocin

Conclusion

UHPLC/MS is an excellent means of identifying psilocybin and psilocin in both raw mushrooms and chocolates. The preparation time (10 minutes) and run time (10 minutes including blank) make this a very efficient analytical method.

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Application Note: 30173

Direct Analysis of Red Wine Using Ultra-Fast Chromatography and High Resolution Mass Spectrometry

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Key Words
- LTQ Orbitrap™
- U-HPLC
- Flavonoids
- Red Wine

Overview
Red wine is a very complex mixture and a rich source of beneficial anti-oxidants. Identification and quantitation of these natural products is challenging. Ultra High Pressure Liquid Chromatography (U-HPLC) coupled to the Thermo Scientific LTQ Orbitrap XL mass spectrometer was used for analysis of French red wine, which enabled simultaneous detection and relative quantitation of the wine's anti-oxidant constituents. The phenolic compounds (such as quercetin) responsible for most of the health benefits associated with the consumption of red wine were identified and their variable content across two different harvest years was observed. Direct wine analysis approach was then applied to monitor the progressive changes in red wine after its exposure to air. This work demonstrated the feasibility of analyzing complex mixtures without any prior sample preparation by making use of the high resolving power of both U-HPLC and the Orbitrap™ mass analyzer detector.

Introduction
Free radicals derived from molecular oxygen are considered major causative agents of tissue damage.[1,2] Both recent and historical evidence suggests that regular drinking of wine ***in moderation*** has a positive impact on human health thanks to its high content of anti-oxidants.[3,4] Red wine in particular contains a complex mixture of phenolic compounds which are important contributors to the organoleptic quality of wines as well as essential components in the evolution of wine. Quercetin is of special interest for its commercial use as an anti-oxidant food supplement with a proven record of promoting vascular relaxation, inhibiting human platelet aggregation *in vitro*, and modulating eicosanoid synthesis towards a pattern likely to be protective against coronary heart disease.[5]

Reversed-phase HPLC is well established for the analysis of flavonoids in red wine, including quantitative analysis.[6,7,8] Coupling reversed-phase HPLC to a mass spectrometer adds considerable benefits such as the ability to:

1) analyze complex mixtures without much sample fractionation
2) monitor hundreds of compounds in a single analysis over a wide dynamic range of concentrations
3) provide an unambiguous identification and structural characterization of the compounds based on accurate mass measurement and informative fragmentation spectra.

Recent advances in both HPLC and mass spectrometry techniques are having a significant impact on the analyses of complex mixtures such as those represented by food and agricultural products. First, the use of small particles (< 2 µm) in HPLC columns can provide remarkable increase in speed of analysis while maintaining or even improving the separation efficiency. Second, the new generation of powerful but easy-to-use hybrid mass spectrometers, like the LTQ Orbitrap XL, combines extremely high mass accuracy and resolution with the capability of multiple levels of fragmentation.[9] The combination of these powerful techniques provides a robust and confident means of profiling complex mixtures as well as successful identification and advanced structural characterization of detected compounds. As a result, we are seeing rapidly growing interest in the area of metabolomic analysis being applied in nutrition and health research.[10,11]

We investigated the potential of a direct analysis of red wine using U-HPLC coupled to a linear ion trap – Orbitrap hybrid mass spectrometer. Of particular interest was the ability of the designed workflow to pinpoint statistically significant differences between individual harvest years for wines of a specific origin (area, label). In addition to that, we used the developed methodology to monitor the trend in oxidative changes of red wine after exposure to air.

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Methods

Two bottles of French red wine Les Charmes de Kirwan, Margaux (cuvee, Bordeaux region, France), years 2003 and 2005, were obtained from a specialized wine merchant. The wine was stored at room temperature in the dark until analyzed. Immediately after opening the bottle, a glass vial (20 mL) was filled with the wine to the very top, quickly closed to ensure minimum oxidation, and stored at 4°C in the dark. This sample was collected just in case there was a need for repeated analysis of the profiling experiments or structural elucidation studies. A second 20 mL aliquot of wine was poured from the original bottle into a glass beaker. From this beaker a sample vial was immediately filled to the rim and placed in the chilled (4° C) Thermo Scientific Accela autosampler tray, awaiting analysis. For a wine oxidation trend analysis, further samples were taken from this open beaker 1, 5 and 24 hours after the bottle opening.

Chromatography was performed using an Accela U-HPLC injecting 20 µL sample from a cooled tray (4°C) directly onto a Thermo Scientific Hypersil GOLD column (2.1 mm x 100 mm, 1.9 µm particles, equilibrated in 95% solvent A (0.1% aqueous solution of formic acid), 5% solvent B (acetonitrile containing 0.1% formic acid). The compounds were eluted using flow rate 300 µL/min by linearly increasing solvent B concentration from 5% to final 40% over 15 min, and from 40% to 95% over 1 min. The column was then washed with 95% solvent B (2 min) and re-equilibrated in 95% solvent A, 5% solvent B. The total run time, including column wash and equilibration, was 20 min.

A Thermo Scientific LTQ Orbitrap XL mass spectrometer was operated in positive ion mode at 30,000 resolving power (defined as FWHM @ *m/z* 400) for full scan analysis (mass range 150 – 1500 u) followed by data dependent MS/MS on the most intense ion from the full scan at 7,500 resolving power (~0.3 sec per scan). The measurements were done in triplicate with external calibration. The settings for the higher energy collisional dissociation (HCD) fragmentation mode were 65% normalized collision energy, isolation width 3 u.

Thermo Scientific SIEVE 1.2 software was used for comparative and trend analyses. The software allows for processing a large number of samples, presenting the statistically significant differences between populations and various time points. Data were normalized on total spectral ion current. Results were filtered using pValue < 0.001 and at the same time requiring a minimum 2-fold change in peak height.

The results from SIEVE™ were further subjected to multivariate analysis with SIMCA P+™, version 11 (Umetrics, Umea, Sweden).

Mass Frontier™ (HighChem, Slovakia) software was used to confirm a suggested compound identity and structure based on observed fragmentation patterns.

Results

Due to the large number and the chemical complexity of phenolic compounds in wine matrix, analytical methods in the past involved sometimes difficult and complicated traditional chromatographic techniques. One of the major problems underlying separation of the phenolic compounds is their similarity in chemical characteristics. As many phenolics show similar UV spectra with maxima in a narrow range of 280-320 nm, extensive fractionation steps might be needed prior to HPLC analysis. Rather large initial volumes required and variable losses occuring due to incomplete extraction or oxidation can be an issue. The use of modern chromatographic techniques coupled to mass spectrometric detection can alleviate these problems.

Our approach avoids entirely the sample fractionation step: red wine is injected directly on the reverse phase column. Moreover, the use of small particles (< 2 µm) and relatively high flow rates (300 µL/min) enable swift analysis with excellent chromatographic resolution. The observed peak width for individual compounds was, on average, 7 sec, back pressure not exceeding 350 bar. With 20 min total cycle time per injection, this setup allows for high throughput analysis while the total sample consumption remains negligible (20 µL per injection). U-HPLC coupled to the LTQ Orbitrap XL proved to be very robust, allowing for an uninterrupted analysis of 24 untreated red wine samples which corresponds to an 8-hour continuous analysis without any requirement for a system cleanup or column change.

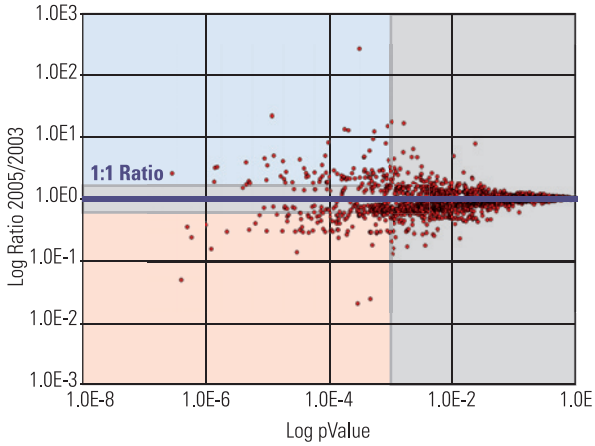

Figure 1: Overview of differences between harvest years 2003 and 2005. The result from differential analysis software (SIEVE 1.2) highlights the compounds having at least two-fold higher concentration in year 2005 compared to year 2003 (blue shaded area) and compounds whose concentration in year 2005 was less than a half of that in year 2003 (red shaded area). The purple horizontal line represents 1:1 ratio between concentrations in the year 2005 and 2003. The grayed area covers the features with less pronounced concentration difference and those with low statistical significance, i.e. pValue > 0.001.

Variables like wine varietals, soil composition, and harvest year will play an important role by providing the basic pool of compounds for these biotransformations. With accurate mass acting as a highly selective filter we could monitor hundreds of compounds across multiple samples, enabling advanced comparative studies and trend analyses. Initially, we were interested in comparing the wine of the same origin (area, label) but harvested in different years.

Our differential analysis of the Les Charmes de Kirwan, Margaux, contrasted wine from production years 2003 and 2005 using SIEVE software. The features (peaks) were filtered for their statistical significance (pValue < 0.001) and significant change defined as a minimum 2-fold concentration difference between the two harvest years (Figure 1). We observed 75 individual compounds which showed at least 2-fold higher content in year 2005 compared to year 2003 (blue shaded area in Figure 1). Kaempferol and quercetin concentration increased 25- and 8-fold, respectively, in year 2005 compared to 2003 (Figure 2). On the other hand, there were 36 other compounds whose concentration in the 2005 sample was significantly less than in the 2003 sample (red shaded area in Figure 1). Some flavonoids (myricetin) showed no change in concentration between the two harvest years.

Total anti-oxidant status refers to overall antioxidant properties of wine, and can be largely ascribed to a group of compounds comprising vanillic acid, *trans*-polydatin, catechin, *m*-coumaric acid, epicatechin, quercetin, *cis*-polydatin and *trans*-resveratrol.[12] In our analysis we detected vanillic acid, (epi)catechin, coumaric acid, and quercetin. When compared to wine produced in 2003, the wine produced in 2005 contained 50, 40 and 20% less coumaric acid, vanillic acid and (epi)catechin, respectively, while the amount of quercetin increased 8-fold (Table 1).

Calc *m/z*	Formula MW	Name	Change 24h/0h	Change 2003/2005
165.0546	$C_9H_8O_3$	Coumaric acid	0.32	0.51
169.0495	$C_8H_8O_4$	Vanillic acid	0.40	0.61
199.0601	$C_9H_{10}O_5$	Syringic acid	0.59	0.92
391.1387	$C_{20}H_{22}O_8$	Polydatin	Not found	Not found
229.0859	$C_{14}H_{12}O_3$	Resveratrol	Not found	Not found
291.0863	$C_{15}H_{14}O_6$	(Epi)catechin	0.66	0.82
303.0499	$C_{15}H_{10}O_7$	Quercetin	0.78	7.96
319.0448	$C_{15}H_{10}O_8$	Myricetin	0.69	1.00
287.0550	$C_{15}H_{10}O_6$	Kaempferol	1.00	20.96

Table 1: Overview of some compounds of interest and the changes in their content between year 2003 and 2005 (column **Change 2003/2005**), and after 24 hours following exposure to air (column **Change 24h/0h**). The compounds highlighted are the major contributors to the total anti-oxidant status.[12]

The remarkable difference in the content of quercetin between the two harvest years is interesting. Quercetin is one of the most abundant natural flavonoids found in fruits, vegetables and wine.

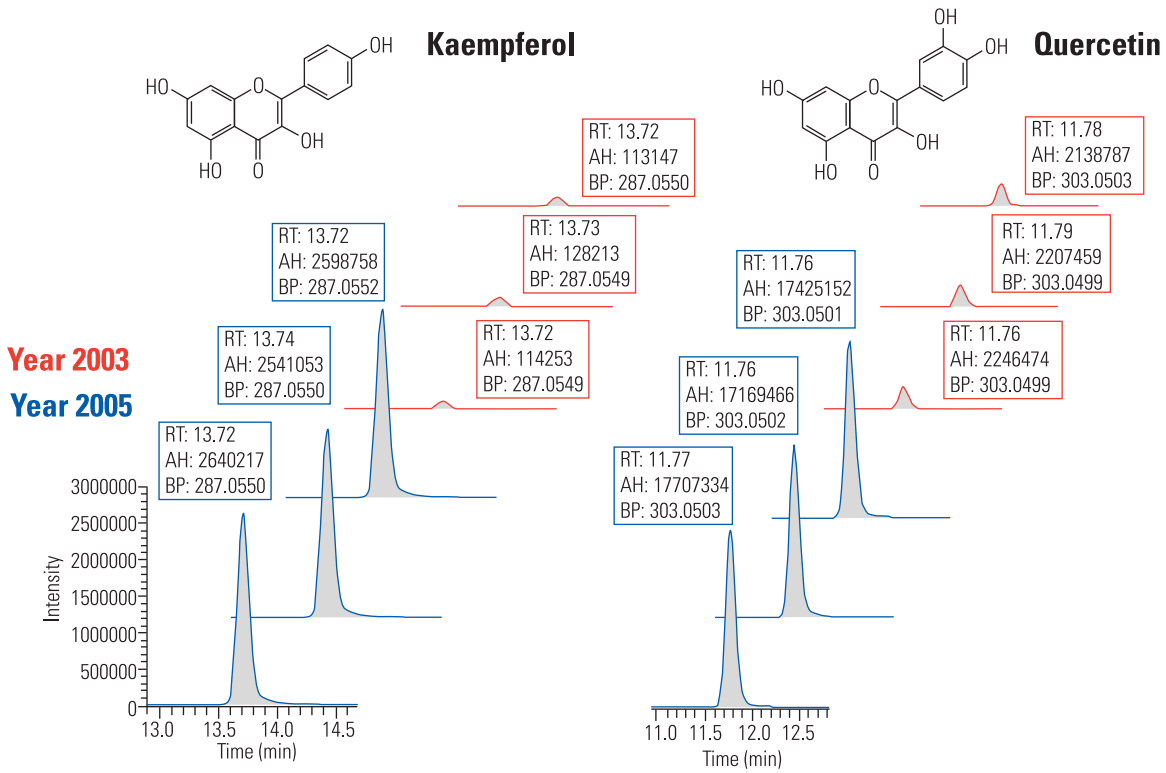

Figure 2: Extracted ion chromatogram for kaempferol and quercetin (left and right pane, respectively; 3 injections each) shows remarkable difference in concentration of these compound in wine harvested in year 2005 (blue trace) and 2003 (red trace). The mass deviation did not exceed 0.7 ppm for kaempherol (calculated *m/z* = 287.0550) and 1.3 ppm for quercetin (calculated *m/z* = 303.0499). Note the reproducibility of the retention time (RT) values and peak height calculations (AH) for 3 replicate injections.

At present, labeling requirements for red wine are far from comprehensive, basically limited to listing the total alcohol content and the comment that it contains sulfites. Including more specific information about compounds with strong anti-oxidant properties would improve a general public awareness and be helpful in the current climate of debate on healthy balanced diet. A fast but highly informative analysis of wines as described herein can thus help maintain consistency and quality, and provide useful information about product's nutritional value.

Reliable accurate mass measurements over a broad dynamic range of concentration are helpful for unambiguous identification of compounds of interest. The mass deviation of our measurements did not exceed 2 ppm using external calibration. Such an accuracy supported by reliably measured isotope abundancies in the LTQ Orbitrap XL enabled a confident assignment of elemental composition to individual peaks.

For confident identification of a compound, the elemental composition suggestions based on mass accuracy need to be complemented with the evidence from the fragmentation spectra. Our method was set up to collect higher energy collision dissociation (HCD) spectra. On average 700 such spectra were collected during each 20-minute LC-MS run. The MS/MS spectrum acquired in the multipole collision cell of the LTQ Orbitrap XL serves for confirming identity of a known compound or even determining identity of an unknown. Such an approach was demonstrated for the analysis of antioxidant compounds in olive oil.[13] Rich fragmentation, accurate mass measurement of both parent and fragment ions, and spectrum interpretation provided by Mass Frontier software were all crucial for this challenging task (Figure 3).

The anti-oxidant properties of wine are clearly beneficial to a consumer. On the other hand, wines with higher polyphenolic concentration are more susceptible to oxidation. We were interested to observe a trend of changes in the wine samples over the period of 24 hours after opening the bottle. The groups of samples from time points 0, 1, 5, and 24 hours (triplicate injections) were processed with SIEVE and further subjected to principal component analysis. The progressive changes caused by exposure to air are well observable and statistically significant (Figure 4).

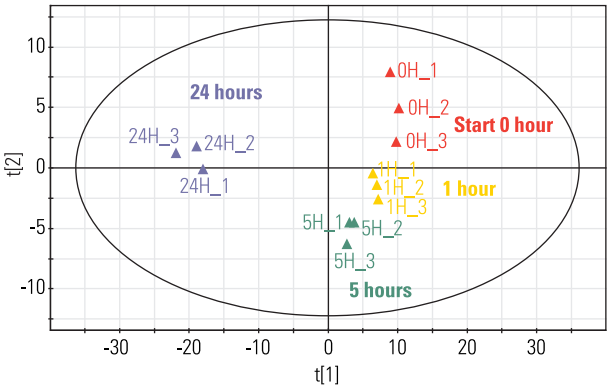

Figure 4: Wine sampled in triplicate at 0, 1, 5, and 24 hours after exposure to air. The sample groups are easily separated by the first two principal components.

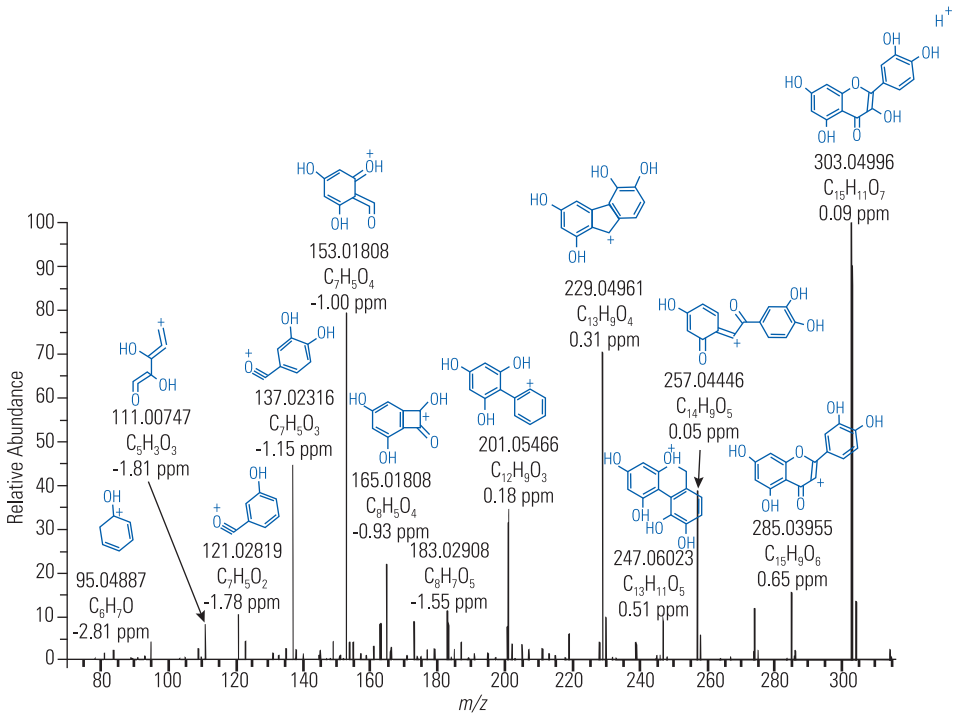

Figure 3: Confirmation and structural characterization of quercetin. Assignment of fragments in HCD spectrum using the Mass Frontier software relying on its extensive database of fragmentation mechanisms.

At this point, a potential effect of evaporation of more volatile constituents of wine has to be considered. In general, the partial pressure for compounds with molecular weight 300 and higher is considered negligible – such compounds should not be lost to evaporation at room temperature. Kaempferol (MW 286), (epi)catechin (MW 290), myricetin (MW 318) and quercetin (MW 302) would fall into such a category. Kaempferol showed no change over this period. Thus the decrease of 20% for quercetin and 30% for (epi)catechin and myricetin observed over the period of 24 hours following the bottle opening could be confidently ascribed to oxidation. For coumaric (MW 164), vanillic (MW 168) and syringic (MW 198) acids we observed a more pronounced drop in concentration (60, 70 and 40%, respectively) after 24 hours following the exposure to air (Figure 5). It might prove difficult, however, to distinguish between the effect of evaporation and oxidation under the employed experimental conditions.

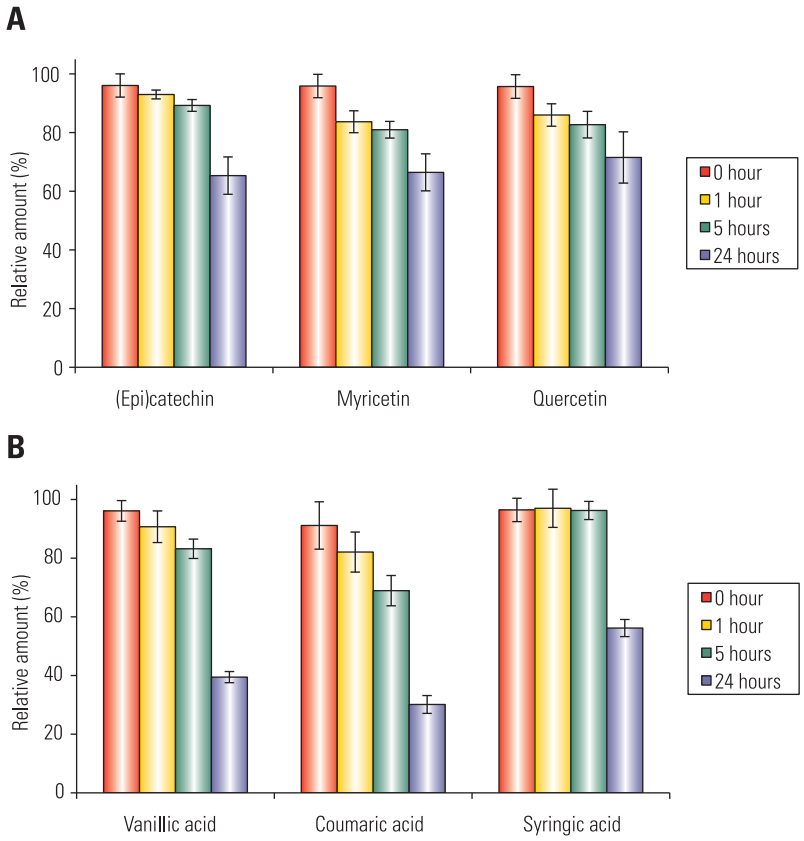

Figure 5: Changes over a 24-hour period of air exposure. The amount of a given compound at time 0 h defined as 100%. Panel A shows decrease in content of higher molecular weight compounds such as (epi)catechin (MW 290), myricetin (MW 318) and quercetin (MW 302). Lower molecular weight components including coumaric (MW 164), vanillic (MW 168) and syringic (MW 198) acid display much higher rate of disappearance (panel B). Error bars show the standard deviation for three repetitive analyses of samples at each time point.

Conclusions

As consumers are becoming increasingly aware of the harmful as well as helpful content of what they eat and drink, modern powerful analytical tools will undoubtedly play a crucial role to supply that information more accurately and quickly. Albeit a very complex mixture, red wine is perfectly suitable for mass spectrometric supported by SIEVE differential expression software. Such 'fingerprinting' analysis can be applied in quality control and process monitoring, and for highlighting relevant nutritional value to consumers.

- U-HPLC affords fast analysis times while maintaining very high chromatographic resolution (peak width 7 seconds at half height).
- The mass deviation of the LTQ Orbitrap XL measurements was always smaller than 2 ppm using external calibration up to one day old.
- Higher collision energy dissociation MS/MS spectra confirm the identity and structure of compounds in complex mixtures.
- Accurate mass measurements also significantly improve the precision of quantitation by eliminating nearly isobaric interferences. This is a particularly important aspect for complex mixture analyses, which red wine undoubtedly is.
- The methodology described here is extremely robust, allowing for an uninterrupted analysis of 24 untreated red wine samples (continued analysis over an 8-hour period).

Acknowledgements

The authors are grateful to David Kusel for insightful comments to the manuscript.

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Application Note: 420

Metabolomic Analysis of Green and Black Tea Extracts Using an LTQ Orbitrap XL Hybrid Linear Ion Trap Mass Spectrometer

Donna L. Wilson and Charles Yang; Thermo Fisher Scientific, San Jose, CA, USA

Key Words

- Accela™ High Speed UHPLC
- SIEVE™ Software
- Higher Energy Collisional Dissociation (HCD)
- Mass Frontier™ Software
- Natural Product Analysis

Overview

Purpose: To show a complete analytical metabolomic workflow including (1) data acquisition using a high resolution accurate mass instrument that is equipped with a Higher Energy Collisional Dissociation (HCD) cell and coupled to a high pressure LC (Figure 1), (2) metabolite differential abundance analysis, and (3) structural elucidation of relevant metabolites using accurate mass and HCD fragmentation information to highlight the component differences between green and black tea.

Methods: Green and black tea extracts were analyzed using an LTQ Orbitrap XL™ with an HCD cell. Chromatography was performed using an Accela High Speed LC equipped with a 2.1 mm ID Hypersil GOLD™ column packed with 1.9 µm particles. Data Dependent™ analysis was performed using an LTQ Orbitrap XL with full scan data acquired at a resolving power of 30,000 and MS^n data acquired at a resolving power of 7500 following HCD fragmentation.

Results: The study included a comparative analysis of green and black tea using differential analysis software to identify compositional variations between the two tea samples. Using a UHPLC coupled with a small particle column afforded a fast analysis time while maintaining very high chromatographic resolution. The high mass accuracy data (better than 3 ppm with external calibration) was used to determine elemental composition and for tentative identification of compounds via database searching. HCD fragmentation facilitated structural identification and confirmation. This was demonstrated with the example of epigallocatechin gallate (EGCG).

Introduction

Metabolomics, the comprehensive and quantitative analysis of wide arrays of metabolites in biological samples, marks promising new research territory. The numerous analytes in these samples have diverse chemistries and polarities. In addition, metabolites occur at a range of concentrations within a particular sample. Consequently, comprehensive metabolomics investigations create many analytical challenges that can be addressed using LC-MS/MS.

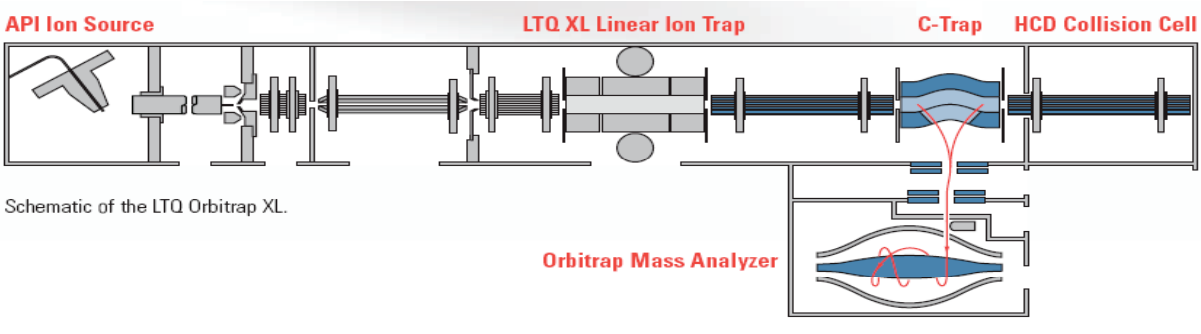
Tea contains a wide range of components including vitamins, amino acids, and polyphenols, many of which are structurally similar and may differ only in the type and location of a side chain. The use of high resolution chromatography is essential for the separation of such a complex mixture. Furthermore, acquisition of accurate mass data in both full scan and MS^n modes enables complete structural characterization.

Here, we highlight an untargeted metabolomic workflow from data acquisition through metabolite ID. The study included differential and structural characterization of polyphenolic catechin (flavan-3-ol) derivatives and theaflavin components of green tea and black tea.

Methods

Samples

Green tea and black tea aqueous extracts were examined without any pre-treatment. Each sample was analyzed in quadruplicate.



Schematic of the LTQ Orbitrap XL.

Figure 1: Schematic of the LTQ Orbitrap XL mass spectrometer with an HCD collision cell. The LTQ Orbitrap XL features an HCD collision cell to provide additional flexibility for low level components in complex mixtures. Ions can be selected in the linear ion trap and fragmented either in the ion trap (CID) or in the HCD collision cell. For HCD, ions are passed through the C-trap into the gas-filled collision cell, providing high sensitivity and high signal-to-noise fragmentation.

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Chromatography Conditions

Chromatographic separation was performed using the Accela UHPLC system (Thermo Fisher Scientific, San Jose, CA). The LC conditions were as follows:

Column: Hypersil GOLD, 100 × 2.1 mm, 1.9 µm particle size (Thermo Fisher Scientific, Bellefonte, PA)

Mobile phase: (A) water with 0.1% formic acid; (B) acetonitrile with 0.1% formic acid

Flow rate: 500 µL/min

Injection volume: 10 µL

Gradient: Linear gradient of 100%–1% A over 20 minutes

Mass Spectrometry Conditions

MS analysis was carried out using an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA). The MS conditions were as follows:

Positive electrospray ion source voltage: 5.0 kV

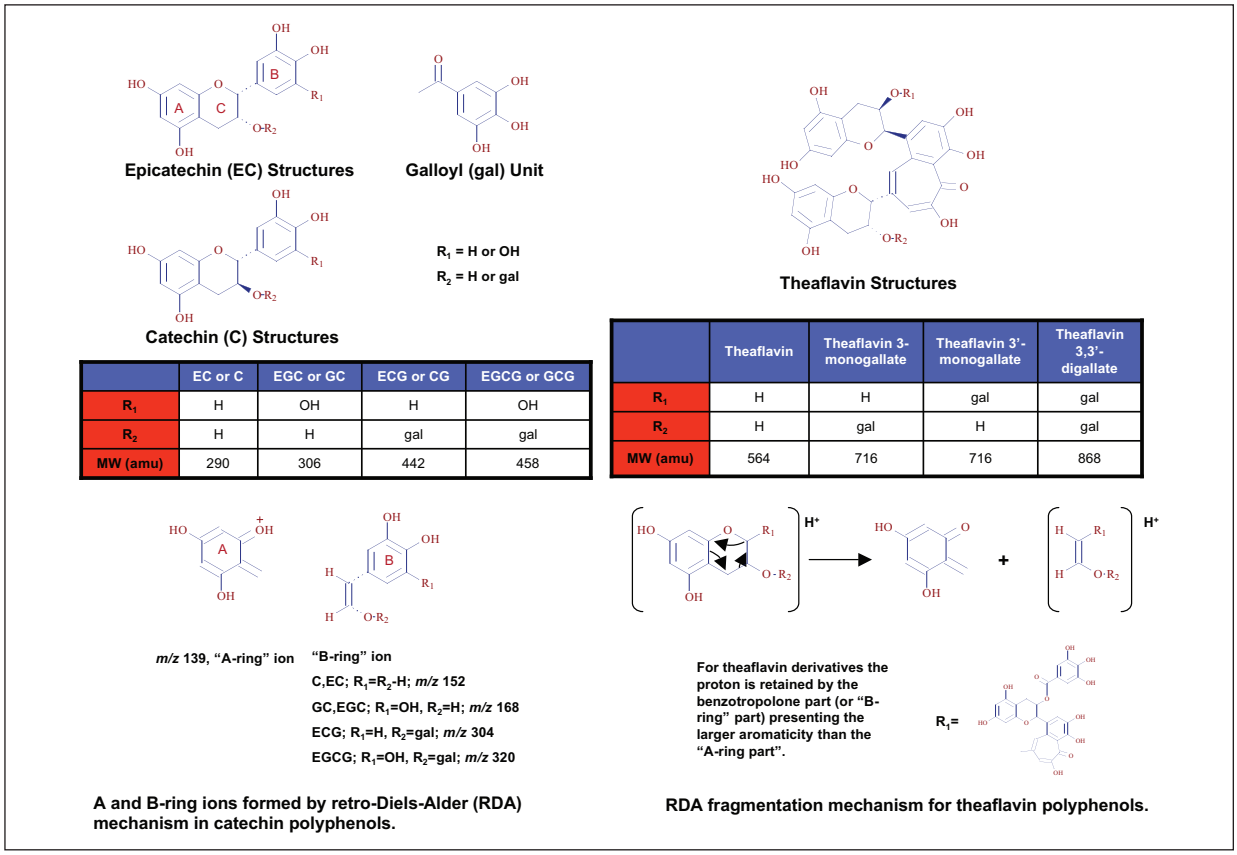
All methods: Full scan MS in the Orbitrap with a mass resolution of 30,000. Data Dependent MS/MS in the Orbitrap on the top three most intense ions from the full scan at a mass resolution of 7500.

Results

Considerable interest has developed in the potential health benefits of teas, particularly in the antioxidant and other properties of some of the polyphenolic catechins and theaflavins (Figure 2). The analysis described here focused on detection, relative quantitation, and identification of these low molecular weight components in green and black tea samples.

The HPLC separation of tea samples shows excellent peak separation and low noise, with most components eluting in less than 10 min. High resolution full scan spectra were acquired at a mass accuracy of better than 3 ppm.

After data acquisition, SIEVE software was used to determine statistically significant differences in the metabolite profiles of green and black tea samples (Figure 3). By comparing peak intensities between the two chromatographically aligned samples, metabolites present at different levels in the two teas were identified.



	EC or C	EGC or GC	ECG or CG	EGCG or GCG
R_1	H	OH	H	OH
R_2	H	H	gal	gal
MW (amu)	290	306	442	458

	Theaflavin	Theaflavin 3-monogallate	Theaflavin 3'-monogallate	Theaflavin 3,3'-digallate
R_1	H	H	gal	gal
R_2	H	gal	H	gal
MW (amu)	564	716	716	868

Figure 2: Three types of tea are produced from *Camellia sinensis* leaves: green tea (nonfermented), oolong tea (semi-fermented), and black tea (fermented). Catechins are polyphenolic antioxidant plant metabolites of the class flavanoids called flavan-3-ols and are highly present in tea plants. Fermentation induces enzymatic oxidation of flavan-3-ols and leads to the formation of two major pigments in black tea, theaflavins (TFs) and thearubigns (TRs). Catechins are expected to be more abundant in green tea and theaflavins more abundant in black tea. The proposed fragmentation pathway for these compounds proceeds via a Retro-Diels-Alder rearrangement as outlined here.

After differentially abundant metabolites of interest were detected, the accurate mass and the MS^n data were used for structural identification. The elemental formula, as determined by the accurate mass data, and the accurate mass value itself were used for metabolite database searching. The EGCG metabolite was tentatively assigned using this combination of tools.

Further metabolite characterization was accomplished using MS^n spectra and Mass Frontier software. Mass Frontier allowed confident metabolite identification using its comprehensive spectral library and predictive fragmentation algorithms to facilitate structural elucidation (Figure 4). The compounds in Figure 3 were identified using this workflow.

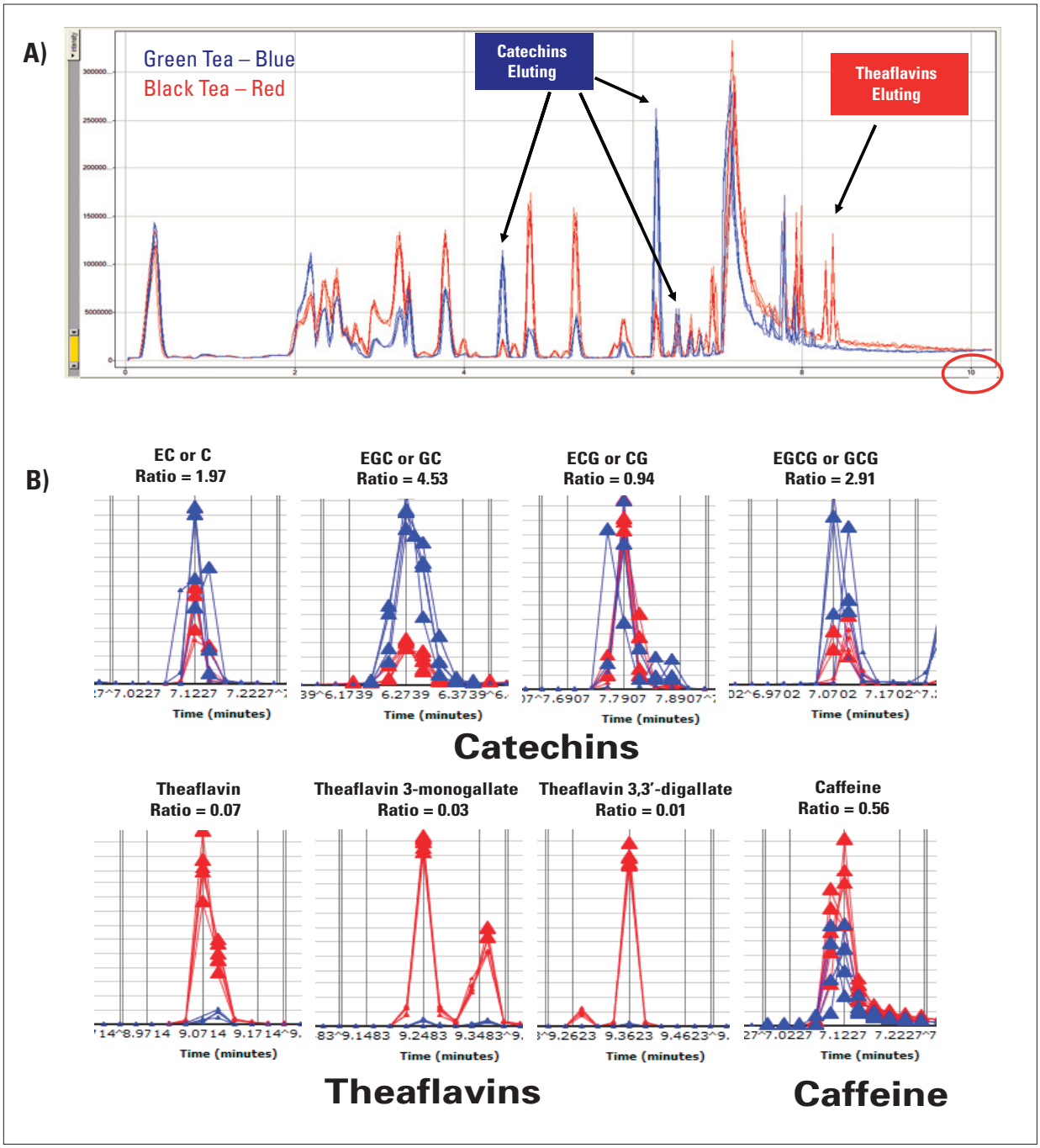


Figure 3: Differential metabolite abundance analysis with SIEVE software. A) Chromatographic alignment of the various LC sample traces is the first step in the SIEVE process. Differences between the green tea samples (Blue) and the black tea samples (Red) can be identified. The Accela UHPLC provided highly resolved chromatographic peaks and high signal-to-noise ratios. B) After alignment, the corresponding peak intensities are compared for green tea (Blue) and black tea (Red). The relative abundances of several compounds of interest are shown with their abundance ratios. These metabolites were identified using a combination of accurate mass database searching and MS^n spectra interpretation via Mass Frontier software.

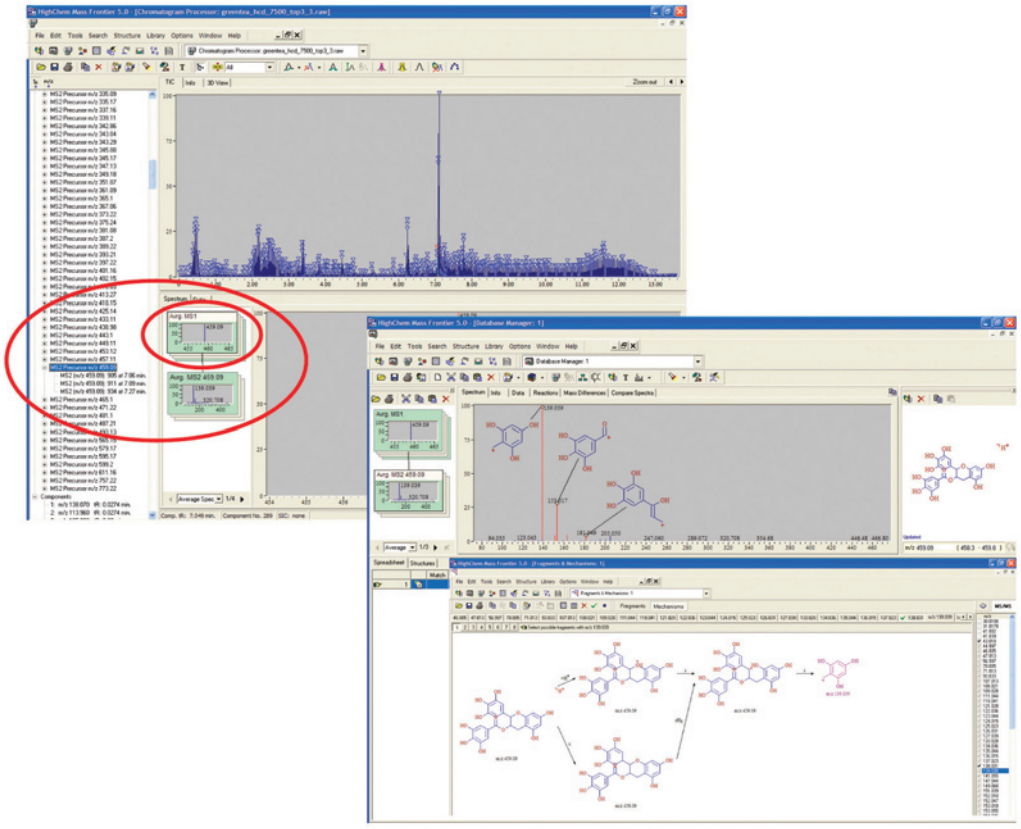


Figure 4: A metabolite of interest from Figure 3 was chosen for further characterization. This ion was present at levels ~3 times that of black tea and was identified as the potent antioxidant, EGCG. Accurate mass was used to identify the metabolite via database searching, and Mass Frontier software was used to confirm the EGCG identification by using the fragmentation spectra of the parent ion.

Conclusions

The analytical metabolomic workflow described here encompasses data acquisition, discovery of differentially abundant metabolites, and metabolite identification. The LTQ Orbitrap XL coupled to an Accela U-HPLC system afforded fast analysis times while maintaining high chromatographic resolution. Accurate mass measurements increased the confidence in elemental composition assignments and ultimately metabolite identification. SIEVE differential analysis software enabled large-scale evaluation of multiple complex LC-MS data and comparison of metabolite profiles between green and black tea samples. The spectral database and fragmentation algorithms of Mass Frontier software facilitated structural assignments of metabolites of interest utilizing MS^n fragmentation spectra.

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Application Note: 52038

Quantitative Analysis of Catechins in Tea by UHPLC/UV

Guifeng Jiang, Terry Zhang, Thermo Fisher Scientific, San Jose, CA, USA

Key Words

- Accela
- Catechins
- Food Analysis
- Phenols
- UHPLC

Goal

Develop and validate robust UHPLC/UV methods for high-throughput screening and quantitation of catechins, and demonstrate applicability of these methods to the analysis of tea matrices.

Introduction

Tea, derived from the leaves of the *Camellia sinensis* plant, is the world's second most popular beverage after water.[1] The health benefits associated with tea are primarily attributed to its catechin constituents. These polyphenols are found in many food products but are present at particularly high concentrations in green tea, which is unfermented. Existing evidence indicates that tea catechins, notably epigallocatechin-3-gallate, are pharmacologically active. Their chemopreventive and cardioprotective effects have been demonstrated in numerous animal studies and some, but not all, human studies.[1,2] Several catechins exhibit exceptional antimicrobial properties at low nanomolar levels and may prove beneficial in applications such as food protection, the prevention and treatment of infection, and the promotion of oral and digestive health.[3,4] Furthermore, several studies show that green tea catechins stimulate thermogenesis and fat oxidation, which may have implications for the prevention of obesity.[5,6] Catechins likely exert their biological effects through multiple and complementary mechanisms of action, including antioxidation, pro-oxidation, and the modulation of cell signaling pathways and transcription factors. Determination of the effects of catechins and tea consumption on human health requires sensitive and robust methods for analysis and quantitation of polyphenols in a range of matrices. Sensitive, rapid and robust analytical methods are also necessary for routine screening and quality assurance of catechin-rich foods.

Tea is a complex matrix and its components are generally analyzed by high performance liquid chromatography (HPLC) coupled with UV detection. The main drawback of conventional HPLC methods for the analysis of tea catechins is the compromise between speed and resolution, resulting in typical analysis times of 20 minutes or longer. Ultra high performance liquid chromatography (UHPLC) enables faster separations and higher resolution through the use of sub-2 µm diameter particles.

The Thermo Scientific Accela UHPLC system offers the flexibility of performing both HPLC and UHPLC separations on a single platform. The Accela™ 1250 quaternary pump delivers accurate and precise flows and gradients over a wide range of flow rates (0.1–2 mL/min) and pressures (1–1250 bar) to accelerate method development and maximize method flexibility. Thermo Scientific 1.9 µm Hypersil GOLD PFP (perfluorophenyl) columns enhance retention and selectivity for UHPLC separations of substituted aromatic compounds. In this application note, we demonstrate fast and robust separation, detection and quantitation of sub-ppm levels of catechins and other phenolic compounds using the Accela LC platform and high performance sub-2 µm columns.

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Materials and Methods

Preparation of Catechin Standard Solutions

Catechin/phenol standards (1 mg/mL) were purchased from Sigma-Aldrich (St. Louis, USA). Stock solution of the standard mixture at 125 mg/L were prepared by mixing the 11 standards, diluting with 60:40 methanol:water (v/v) and used for methods development. Calibration standards at 0.0125, 0.05, 0.125, 0.5, 1.25, 5.0, 12.5, 50 and 125 mg/L levels were prepared by serial dilution of the stock mixture solution.

Preparation of Tea Samples

Two commercially available teas, jasmine green tea and black tea, were examined for catechin content. For each tea, 1 g of tea leaves was placed in 10 mL of 80:20 methanol:water for an hour. The supernatant was diluted 1/10 with 50:50 methanol:water and analyzed directly by LC.

LC Instrumentation

LC separations were performed on an Accela UHPLC system with a 1250 UHPLC pump, an autosampler (Thermo Fisher Scientific, San Jose, CA, USA) and an 80 Hz Accela PDA detector (Thermo Fisher Scientific, San Jose, CA, USA).

Results and Discussion

UHPLC Separation of Catechin Standards

The use of sub-2 µm particle columns facilitates rapid analysis of complex samples by improving chromatographic resolution, speed and sensitivity. Columns with perfluorophenyl functionalities help to enhance retention and improve resolution when separating difficult or complex mixtures of substituted aromatic compounds. Using the Accela UHPLC system, a Hypersil GOLD PFP column (1.9 µm, 2.1 × 100 mm) and a simple water/methanol gradient (Gradient I), a standard mixture containing five catechins and six other phenolic compounds was separated and detected under 7 minutes (Figure 1). With the exception of catechin gallate and anisic acid, all analytes were baseline-resolved with an elution order of gallic acid, 3,5-dihydroxybenzoic acid, epigallocatechin, caffeine, epicatechin, epigallocatechin gallate, epicatechin gallate, anisic acid, catechin gallate, 3,4,5-trimethoxycinnamic acid, and myricetin. As the majority of aqueous and matrix contaminants usually elute early at void volume, elution of the first catechin, epigallocatechin at 3.86 minute ensures a robust quantitation method. For this analysis, a flow rate of 420 µL/min was used, generating a backpressure of 600 bar.

LC Parameters

Column	Hypersil GOLD™ C18 PFP columns: 2.1 × 50 mm, 1.9 µm particle size 2.1 × 100 mm, 1.9 µm particle size 2.1 × 150 mm, 1.9 µm particle size 2.1 × 200 mm, 1.9 µm particle size
Mobile Phase	A: Water 0.1 % Formic acid B: Methanol 0.1% Formic acid
Column Temperature	30 °C
Sample Injection Volume	2 µL

Gradient I — Column: HG PFP 2.1 × 100 mm, 1.9 µm

Time	A %	B %	µL/min
0.00	98.0	2.0	420
0.10	98.0	2.0	420
7.10	35.0	65.0	420
7.20	5.0	95.0	420
7.90	5.0	95.0	420
8.00	98.0	2.0	420
10.00	98.0	2.0	420

Gradient II — Column: HG PFP 2.1 × 100 mm, 1.9 µm

Time	A %	B %	µL/min
0.00	98.0	2.0	900
0.10	98.0	2.0	900
2.80	35.0	65.0	900
3.10	5.0	95.0	900
3.50	5.0	95.0	900
3.51	98.0	2.0	900
5.00	98.0	2.0	900

Gradient III — Column: HG PFP 2.1 × 50 mm, 1.9 µm

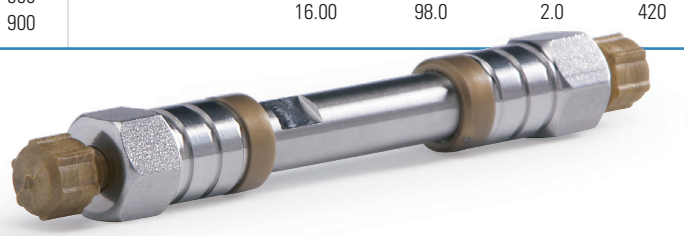
Time	A %	B %	µL/min
0.00	98.0	2.0	420
0.10	98.0	2.0	420
3.60	35.0	65.0	420
3.70	5.0	95.0	420
4.50	5.0	95.0	420
4.60	98.0	2.0	420
6.00	98.0	2.0	420

Gradient IV — Column: HG PFP 2.1 × 150 mm, 1.9 µm

Time	A %	B %	µL/min
0.00	98.0	2.0	420
0.10	98.0	2.0	420
6.60	35.0	65.0	420
6.70	5.0	95.0	420
7.20	5.0	95.0	420
7.30	98.0	2.0	420
9.00	98.0	2.0	420

Gradient V — Column: HG PFP 2.1 × 200 mm, 1.9 µm

Time	A %	B %	µL/min
0.00	98.0	2.0	420
0.10	98.0	2.0	420
11.10	35.0	65.0	420
11.20	5.0	95.0	420
13.00	5.0	95.0	420
13.10	98.0	2.0	420
16.00	98.0	2.0	420



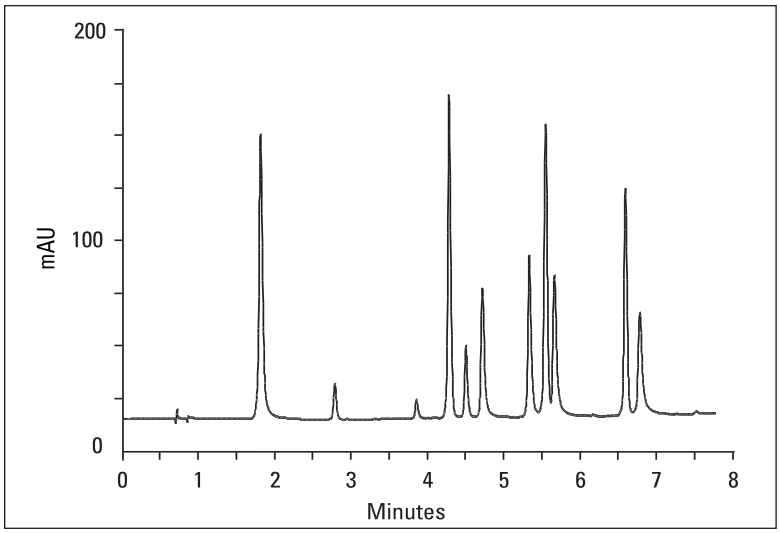


Figure 1: UHPLC separation of a mixture of phenolic compounds containing catechin standards (125 ppm). Column: 1.9 µm Hypersil GOLD PFP, 100 × 2.1 mm. Mobile phase: A – Water and 0.1% formic acid; B – Methanol and 0.1% formic acid. Flow rate: 420 µL/min. UV detection: 275 nm.

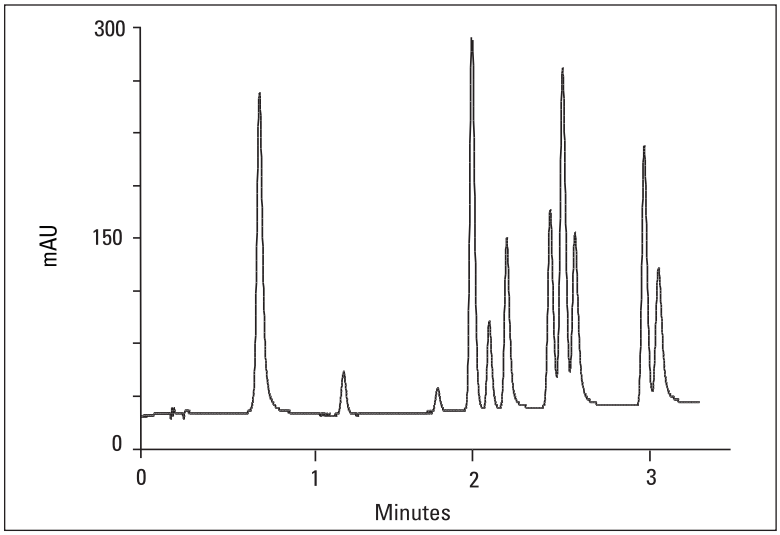


Figure 2: Effect of increasing flow rate. Column: 1.9 µm Hypersil GOLD PFP, 100 × 2.1 mm. Mobile phase: A – Water and 0.1% formic acid; B – Methanol and 0.1% formic acid. Flow rate: 900 µL/min. Pressure: 1200 bar. UV detection: 275 nm.

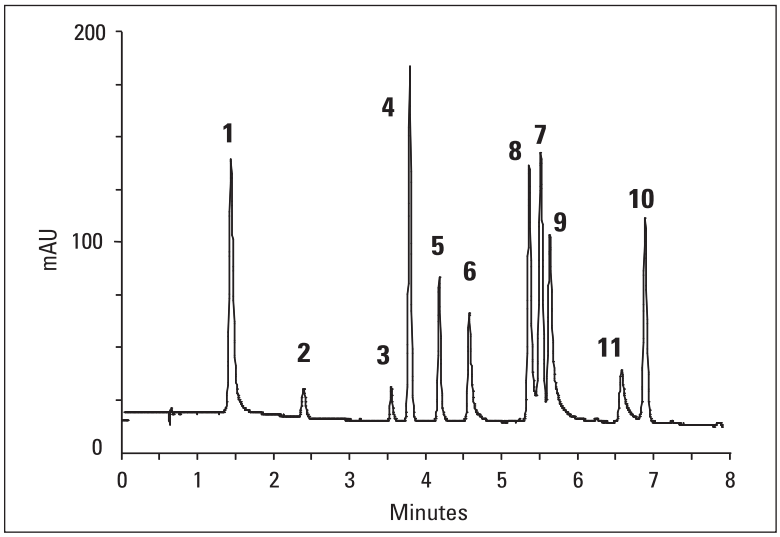


Figure 3: UHPLC separation of a mixture containing catechin standards (125 ppm) using a water/acetonitrile gradient. Column: 1.9 µm Hypersil GOLD PFP, 100 × 2.1 mm. Flow rate: 420 µL/min. UV detection: 275 nm.

Effect of Flow Rate

Compared to larger (5 and 3.5 µm) particles, sub-2 µm particles are less affected by flow rate, therefore faster flow rates, within the pressure limit of the LC system, may be used to increase sample throughput without detrimental effects on peak efficiency. Figure 2 demonstrates that increasing the flow rate to 900 µL/min decreases the analytical run time from approximately 7 minutes to under 3 minutes (Gradient II). At this flow rate, backpressures as high as 1200 bar were generated. While this 3-minute separation is ideal for high-throughput analysis, some degradation in resolution is observed; hence the lower flow rate (420 µL/min) separation was selected as the method for robust quantitation.

Effect of Mobile Phase

The mobile phase composition has a significant effect on analyte retention and the quality of separations. Specifically, the influence of methanol and acetonitrile mobile phases on the UHPLC separation was investigated. Baseline resolution and excellent peak shapes were achieved with a water/methanol mobile phase (Figure 1). In contrast, a water/acetonitrile mobile phase resulted in asymmetrical peakshapes for some compounds (Figure 3) Elution orders of the compound 7, 8 and 10 and 11 (listed in table 1) are reversed. While water/methanol is clearly the superior mobile phase for this separation, its 1.5- to 2-fold higher viscosity compared to water/acetonitrile mixtures results in significantly higher backpressures, which is an important consideration in LC method development.

Effect of Column Length

Increasing the length of the column increases the number of plates and enhances resolution (Figure 4), but also increases analysis times and column backpressures (Figure 5). Unlike some LC systems, the Accela platform provides the same high level of performance at 300 bar as at 1200 bar. As shown in Figure 5, sub-2 µm columns of 20 cm in length can be used with highly viscous water/methanol mobile phases without exceeding the upper pressure limits of the Accela 1250 pump. The 10 cm-long column was chosen for this application because it provides both the necessary separation speed and resolution required for high-throughput screening and quantitative analysis.

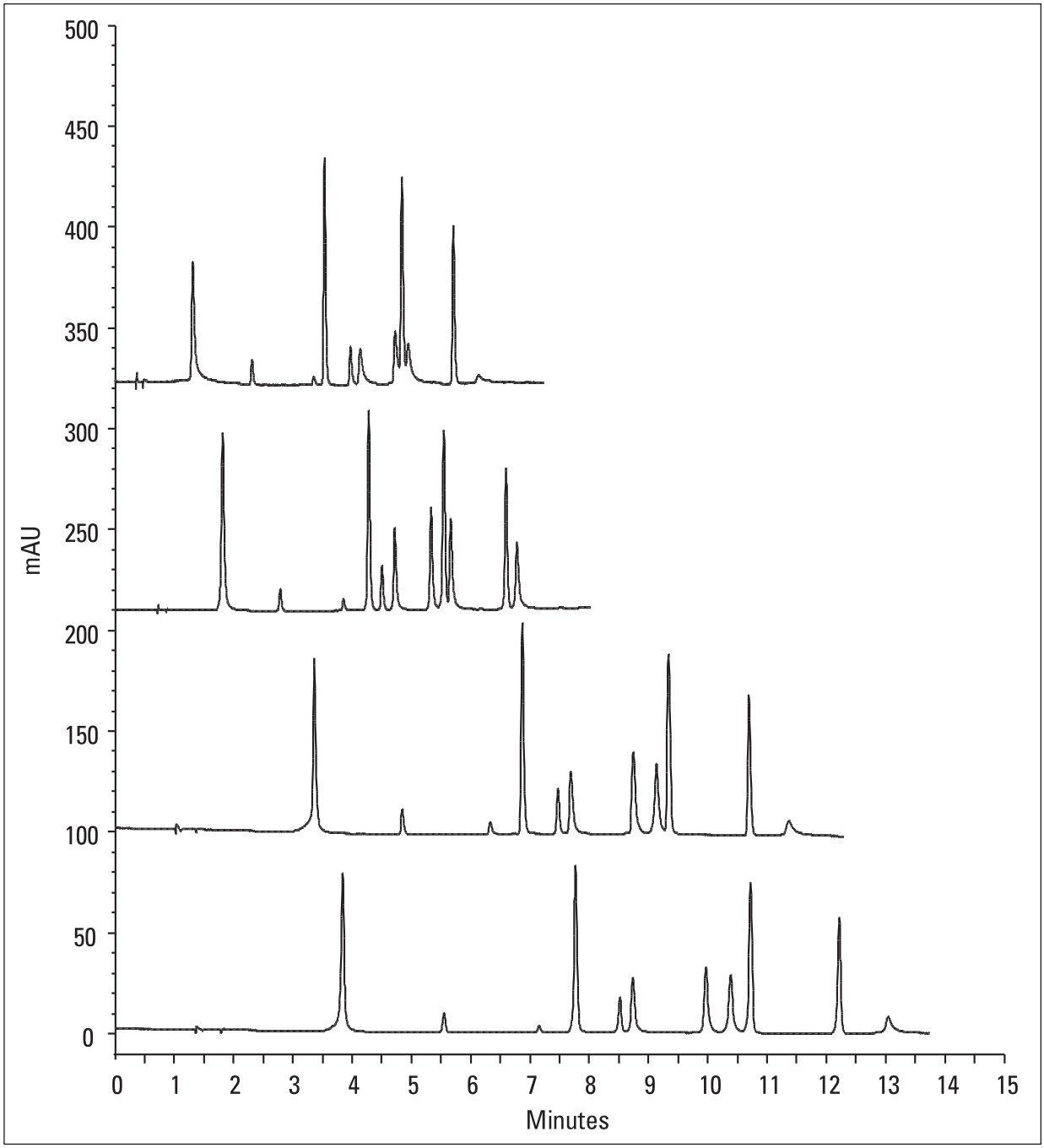


Figure 4: The effect of column length on the UHPLC separation of a mixture containing catechin standards.
Mobile phase: Column: 1.9 µm Hypersil GOLD PFP. Flow rate: 420 µL/min. UV detection: 275 nm.

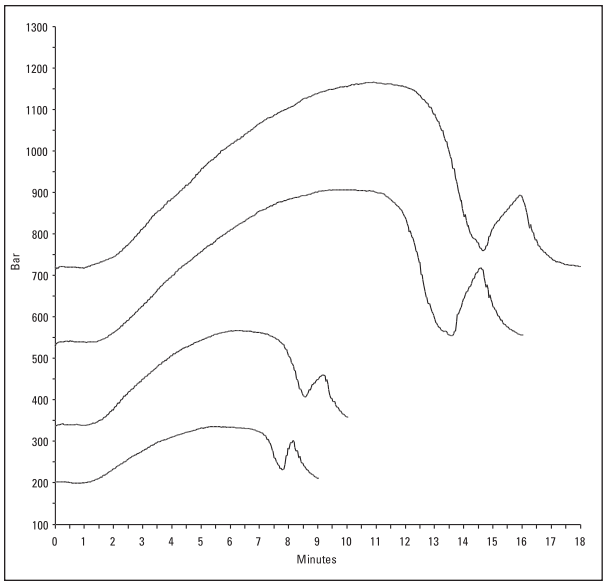


Figure 5: The generated backpressure as a function of column length. Flow rate: 420 µL/min.

Validation of UHPLC High-throughput and Quantitation Methods

Reproducibility of the high-throughput method (900 µL/min, 3 minute analytical run) was investigated by analyzing six replicate injections of each analyte (Figure 6). Retention time RSDs ranged from 0.04–0.18% while peak area RSDs ranged from 0.20–0.91% (Table 1), indicating excellent method reproducibility at 1200 bar, particularly of the UHPLC pump.

Peak Name	RSD% RT	RSD% Area
1. Gallic Acid	0.18	0.66
2. 3,5-dihydroxybenzoic acid	0.14	0.57
3. Epigallocatechin	0.05	0.91
4. Caffeine	0.06	0.20
5. Epicatechin	0.05	0.31
6. Epigallocatechin Gallate	0.07	0.20
7. Epicatechin Gallate	0.07	0.47
8. Anisic Acid	0.07	0.31
9. Catechin Gallate	0.08	0.49
10. 3,4,5-Trimethoxycinnamic Acid	0.04	0.77
11. Myricetin	0.06	0.99

Table 1: Reproducibility of the High-throughput UHPLC Method

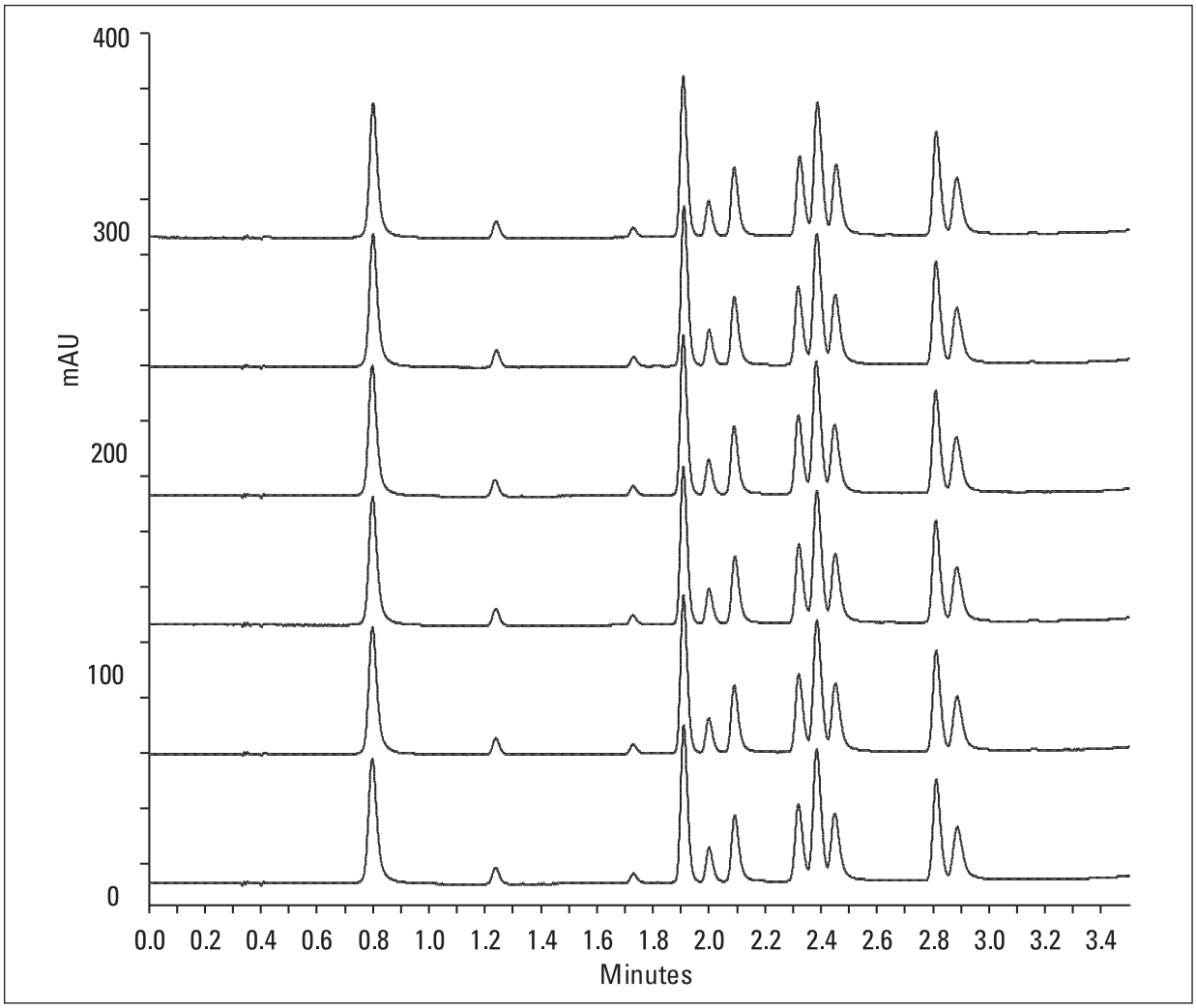


Figure 6: Reproducibility of the high-throughput UHPLC method (900 µL/min) was assessed by analyzing six replicate injections of each analyte. Column: 1.9 µm Hypersil GOLD PFP, 100 × 2.1mm. Mobile phase: A – Water and 0.1% formic acid; B – Methanol and 0.1% formic acid. Flow rate: 900 µL/min. Pressure: 1200 bar. UV detection: 275 nm.

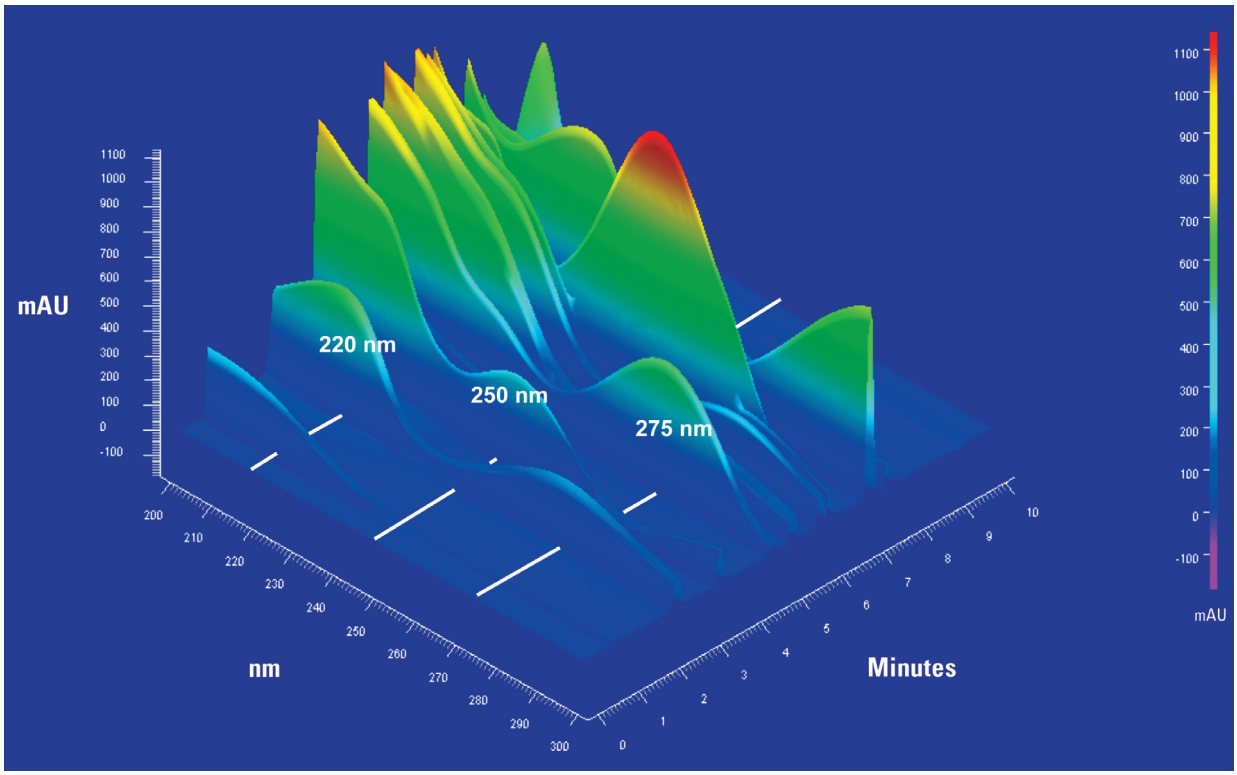


Figure 7: Selection of the wavelength for quantitation was based on the best S/N for all analytes rather than on the most intense wavelength (λmax) for most analytes.

Quantitative Analysis

The optimal wavelength for quantitation, 275 nm, was determined based on the best signal-to-noise ratio (S/N) for all analytes, and not on the most intense wavelength, in order to ensure the lowest limits of detection (LODs) and limits of quantitation (LOQs) (Figure 7). The UHPLC quantitation method (Gradient I) exhibited excellent reproducibility, with peak area RSDs in the range 0.20-0.91%. (Table 2). Excellent linearity in detector response was observed over three orders for catechin and the other phenolic standards, with correlation coefficients greater than 0.99 for all analytes (Table 2). LODs and LOQs, defined as S/N ratio of 3 and 10, respectively, are shown in Table 2. LODs ranged from 0.11 to 9 mg/L, and LOQs ranged from 0.34 to 28 mg/L.

Compound	RSD% Area at 50 ppm	Linearity Range mg/L	Correlation Coefficients	LOQ mg/L	LOQ mg/L
Gallic Acid	0.39	0.5–125	0.9993	0.1	0.4
3,5-Dihydroxybenzoic Acid	0.51	1.25–125	0.9999	0.5	1.6
Epigallocatechin	0.79	12.5–1000	0.9999	9.0	28.0
Caffeine	0.17	0.125–125	0.9999	0.1	0.2
Epicatechin	0.13	0.5–125	0.9983	0.5	1.6
Epigallocatechin Gallate	0.29	0.5–125	0.9954	0.2	0.6
Epicatechin Gallate	0.73	0.5–125	0.9984	0.2	0.5
Catechin Gallate	0.97	0.5–125	0.9993	0.2	0.6
Anisic Acid	0.30	0.125–125	0.9999	0.1	0.2
3,4,5-Trimethoxycinnamic Acid	0.06	0.125–125	0.9999	0.1	0.3
Myricetin	0.54	5–125	0.9993	0.5	1.7

Table 2: Validation parameters of the UHPLC quantitation method. Column: 1.9 µm Hypersil GOLD PFP, 100 × 2.1mm.
Mobile phase: A – Water and 0.1% formic acid; B – Methanol and 0.1% formic acid. Flow rate: 420 µL/min. Pressure: 600 bar. UV detection: 275 nm.

Compound	Jasmine (mg/g)	RSD% (n=3)	Black Tea (mg/g)	RSD% (n=3)
Gallic Acid	0.7	0.6	0.8	0.8
3,5-Dihydroxybenzoic Acid	N.D.	-	N.D.	-
Epigallocatechin	51.3	0.5	17.5	1.9
Caffeine	15.7	0.3	10.6	0.7
Epicatechin	4.1	0.4	1.0	1.8
Epigallocatechin Gallate	13.7	0.4	2.0	0.1
Epicatechin Gallate	15.1	0.4	2.7	0.5
Catechin Gallate	0.3	0.4	N.D.	-
Anisic Acid	0.06	0.1	N.D.	-
3,4,5-Trimethoxycinnamic Acid	0.3	0.2	0.7	0.2
Myricetin	0.9	0.4	N.D.	-

Table 3: Quantitation of catechins and other phenols in tea extracts. Column: 1.9 µm Hypersil GOLD PFP, 100 × 2.1 mm.
Mobile phase: A – Water and 0.1% formic acid; B – Methanol and 0.1% formic acid. Flow rate: 420 µL/min. Pressure: 600 bar. UV detection: 275 nm.

Determination of Catechin Content in Tea Matrices

The UHPLC methods developed and validated for high-throughput screening and quantitation of phenolic compounds were used to analyze and quantitate catechin content in two commercially available teas. Figure 8 shows the UHPLC separations of catechins and others phenols in standard solution and in jasmine and black tea matrices using the high-throughput (900 µL/min) method. The overlaid pressure traces of these three samples demonstrate the exceptional stability of the Accela pump at 1200 bar.

Table 3 summarizes the concentrations of catechins and other phenols detected in the jasmine and black tea samples using the quantitation (420 µL/min) method. Most analytes were detected at sub-ppm levels, and reproducibility, as determined from three replicate injections, was excellent, with RSDs generally below 1%. All five catechins (catechin gallate, epigallocatechin, epicatechin, epigallocatechin gallate, and epicatechin gallate) were present in jasmine green tea, whereas only four were detected in black tea. In general, jasmine green tea contained significantly higher concentrations of catechins and other phenolic compounds compared to black tea. These differences are likely attributed to differences in the way green and black teas are processed. Unlike green tea, black tea is fermented, a process that causes oxidative degradation of catechins and other phenols.

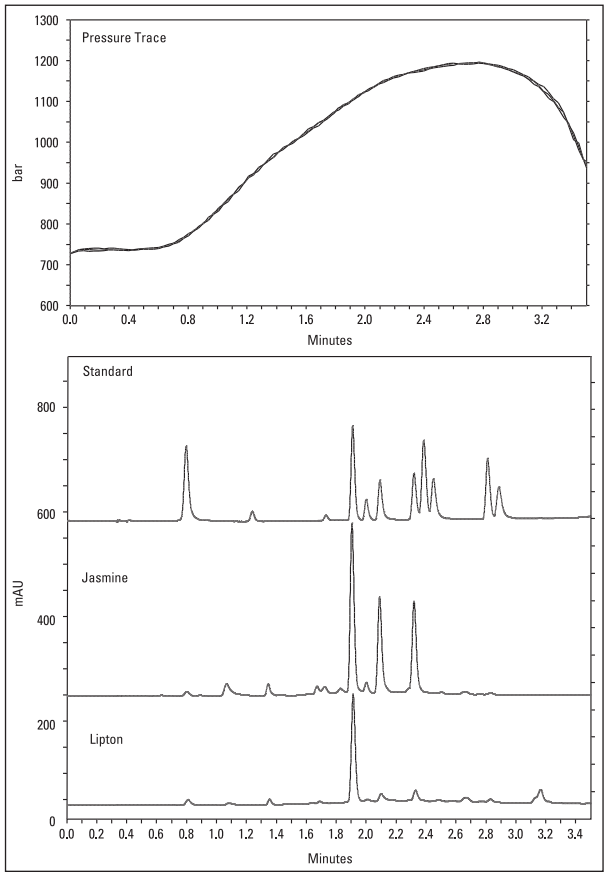


Figure 8: UHPLC separation and detection of catechins and other phenolic compounds in a standard solution and in jasmine and Lipton tea matrices.
Column: 1.9 µm Hypersil GOLD PFP, 100 × 2.1 mm.
Mobile phase: A – Water and 0.1% formic acid;
B – Methanol and 0.1% formic acid.
Flow rate: 900 µL/min. Pressure: 1200 bar. UV detection: 275 nm.

Conclusion

Robust UHPLC methods for high-throughput screening and quantitation of sub-ppm levels of catechins and other phenolic compounds were developed using the Accela UHPLC system. Several method parameters – mobile phase composition, flow rate and column length – were investigated in order to optimize separation speed and resolution for high-throughput and quantitation applications. Using these UHPLC methods, significant differences in catechin and phenolic content in green and black tea samples were found. Although these methods were applied for the analysis of catechins and phenols in tea, they can be adapted for analysis of other foodstuffs as well as for use in studies examining the effects of catechin intake on human health.

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Application Note: 543

Analysis of Perfluoroalkyl Acids in Wastewater, Sludge, and Liver Extracts Using High-Resolution, Accurate Mass LC-MS

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Key Words

- Exactive
- Orbitrap technology
- Environmental application
- PFAAs
- PFOS

Introduction

Perfluoroalkyl acids (PFAAs) are global pollutants and have been shown to bioaccumulate in the food chain. PFAAs have been detected in livers of fish, birds, and marine mammals from Greenland and the Faroe Islands.[1] Biomagnification of perfluorooctane sulfonate (PFOS), the predominant fluorochemical detected, was observed along the marine food chain (Figure 1).

The performance of the Thermo Scientific Exactive mass spectrometer equipped with Orbitrap™ technology has been evaluated for the analysis of ten selected perfluoroalkyl acids in pooled extracts from environmental samples. The following PFAAs were analyzed: perfluoroheptanoic acid (PFHpA), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnA), perfluorododecanoic acid (PFDoA), perfluorotridecanoate acid (PFTrA), perfluorohexane sulfonate (PFHxS), perfluorooctane sulfonate (PFOS) and perfluorooctane sulfonamide (PFOSA) (Figure 2).

The sample extracts were chosen to represent both high and low levels of the analytes in complex matrices. Low levels were expected in liver extracts from Antarctic seals. Medium and high levels were expected in Arctic seals, influent water, and sludge from a wastewater treatment plant. The performance has been evaluated in terms of linearity (range 0.1-50 µg/kg), specificity, and sensitivity.

Goal

To demonstrate the performance of the Exactive™ high-resolution, accurate mass benchtop liquid chromatography-mass spectrometry (LC-MS) system in the analysis of ten selected perfluoroalkyl acids.

Experimental Conditions

Sample Preparation

The sample preparation process is illustrated in Figure 3. Liver samples were extracted by ion pairing with tetrabutylammonium hydrogen sulfate (TBAS) and methyl tertiary butyl ether (MTBE). Sludge samples were extracted by sonication with methanol followed by solid phase extraction (SPE). Effluent water samples were extracted by SPE on C18 columns.

HPLC

Chromatographic analysis was performed using a Thermo Scientific Accela autosampler and pump. The chromatography conditions were as follows:

HPLC column:	Thermo Scientific Hypersil GOLD, 50 mm x 2.1 mm, 1.9 µm
Pre-column:	Thermo Scientific Hypercarb, 100 mm x 2.1 mm, 5 µm
Column temperature:	40 °C
Mobile phase C:	Ammonium acetate (2 mM)
Mobile phase D:	90% water, 10% ammonium acetate

A trapping column placed in line with the Accela™ pump and autosampler enabled less contamination of perfluorinated compounds (PFC) into the system, thus achieving a lower background.

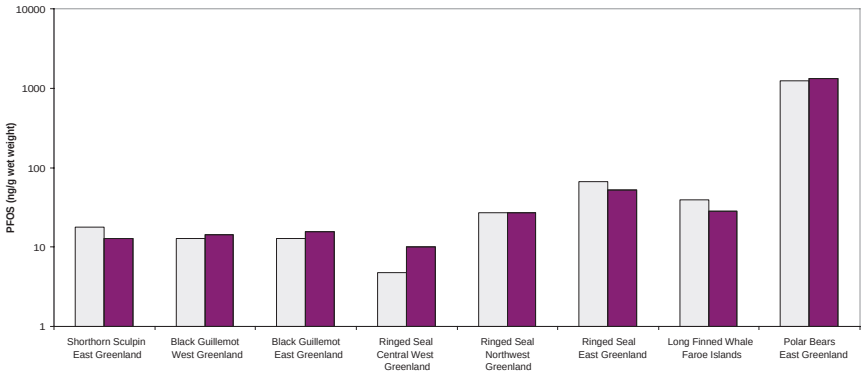

Figure 1. PFOS concentrations (analysis of two samples) in Arctic mammals, birds, and fish [Bossi et al. (2005)]

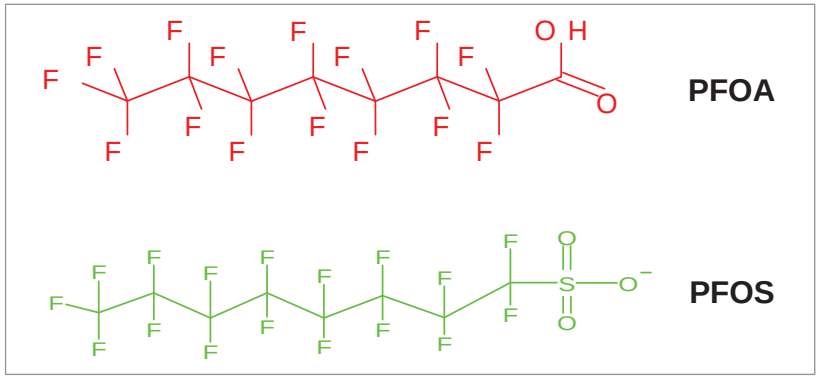

Figure 2. Examples of PFAA target compounds – PFOA and PFOS

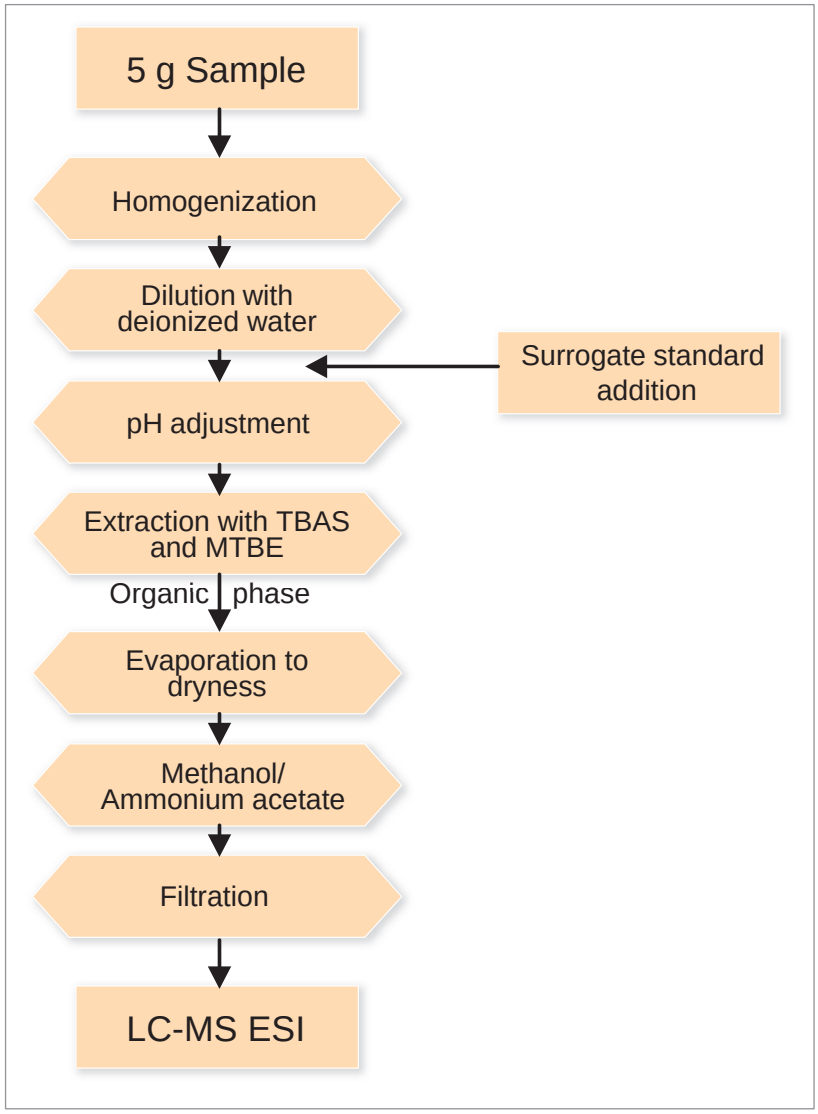

Figure 3. Sample preparation process

Mass Spectrometry

MS analysis was carried out on an Exactive high performance benchtop LC-MS with an electrospray ionization (ESI) source in negative ion mode. Full scan data with a resolution of 50,000 FWHM at *m/z* 200-800 was acquired. No lock mass was applied.

Results and Discussion

The high mass resolution (50,000 FWHM) and mass accuracy (1 ppm) of the Exactive high-resolution, accurate mass system provide efficient peak confirmation and decrease the effects of matrix peaks (Figure 4). The concentration of the target compound in the liver samples was quantified by linear calibration. The use of lock masses could enhance the mass accuracy; however, the Orbitrap instrument was stable for the duration of the sample analysis.

In Figure 5, the extracted ion chromatograms, normalized to the response, are shown. The blank (a) shows very little background and no significant signal at the appropriate retention time. The 0.1 µg/kg standard (b) has good signal for all compounds, as does the sample of extracted Arctic seal liver (c).

For the calibration curves, three standards per level (0.1, 1, 5, 10, and 50 µg/kg) were run. The calibration curve for PFOA is shown in Figure 6.

The results of the analysis are displayed in Table 1. The extracted liver from Antarctic seals showed significantly lower concentrations of PFAAs than the extracted liver from Arctic seals.

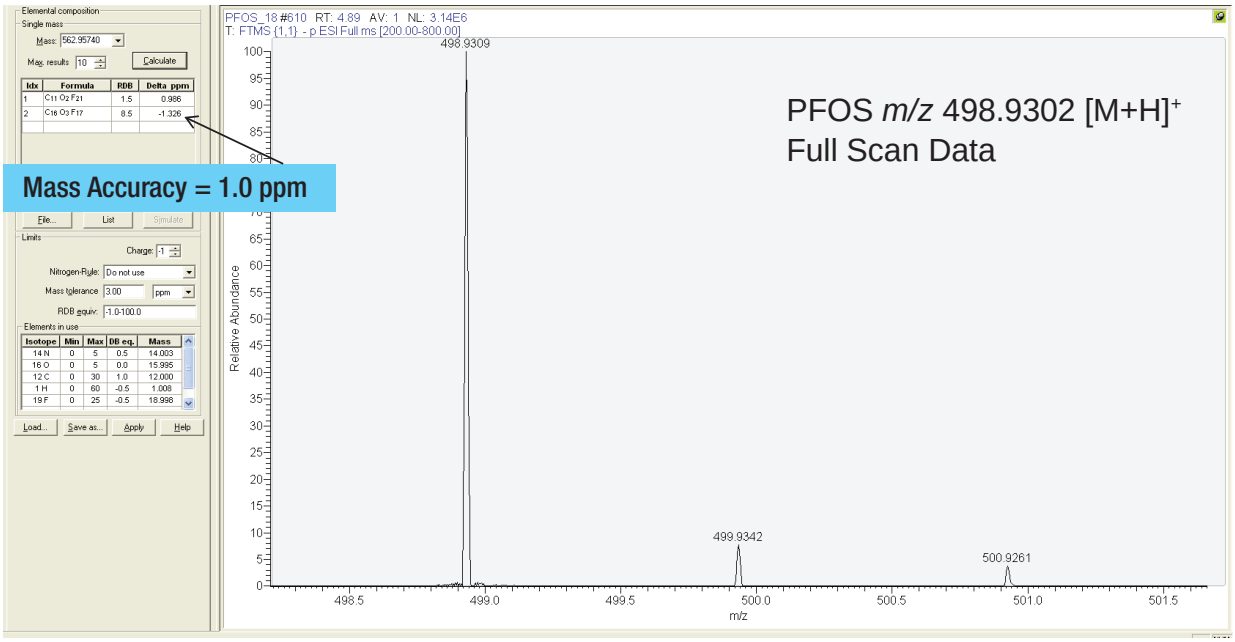

Figure 4. Mass accuracy of PFOS

Table 1. PFAAs in Antarctic and Arctic seal liver

	Antarctic Seal µg/kg	Arctic Seal µg/kg	Arctic Seal µg/kg	Arctic Seal µg/kg
PFHpA	–	–	0.08	1.05
PFHxS	–	0.21	–	0.21
PFOA	0.25	0.35	2.28	4.37
PFNA	0.07	4.78	1.72	1.76
PFOS	–	22.95	17.79	2.28
PFDA	–	2.82	12.59	1.09
PFOSA	–	0.14	–	–
PFUnA	–	5.45	0.44	–
PFDoA	0.22	0.87	–	–
PFTrA	–	1.97	–	–

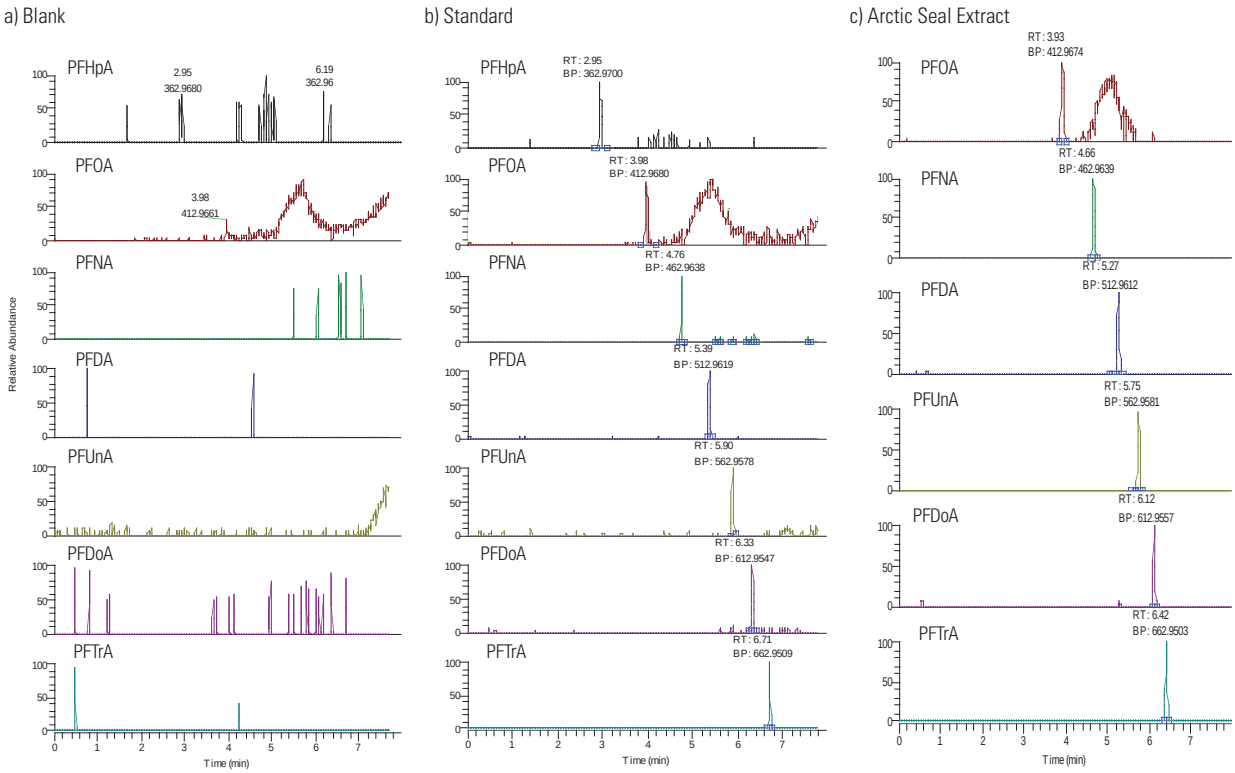

Figure 5. Extracted chromatograms: (a) Blank, (b) Standard, (c) Arctic seal extract

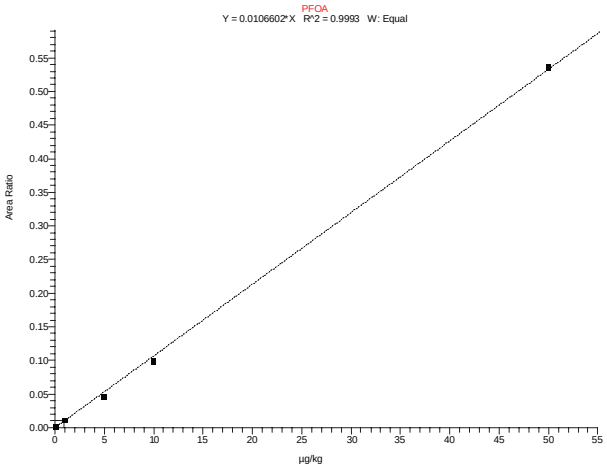

Figure 6. Calibration curve for PFOA

Conclusion

Full scan in negative mode LC-MS acquisition on the Exactive LC-MS system with Orbitrap technology is suitable for quantification of PFAAs at low concentrations ranging from 0.1 µg/kg to 50 µg/kg in complex matrix extracts. No tuning or fragment determination is required and there is less background with high-resolution, accurate mass acquisition.

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Application Note: 51936

Sensitive and Accurate Quantitation of Perfluorinated Compounds in Human Breast Milk using Selected Reaction Monitoring Assays by LC/MS/MS

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Key Words

- PFC-free Accela
- TSQ Vantage
- High Resolution MS
- H-SRM
- Perfluorinated Compounds

Overview

Perfluorinated compounds (PFCs) are ubiquitous and persistent pollutants that bioaccumulate in animals and humans. The potential toxicity of these chemicals has fueled efforts to develop robust analytical techniques for measuring low levels of PFCs in human matrices. Quantitative selected reaction monitoring (SRM) assays were developed for six PFCs using the Thermo Scientific TSQ Vantage triple-stage quadrupole mass spectrometer (MS) coupled to a PFC-free Thermo Scientific Accela LC system. Using this method, PFCs were accurately and reproducibly detected at ppt concentrations in neat solution and in human milk matrix. Exceptionally sensitive and accurate, this integrated LC-MS platform is ideally suited for robust ultra-trace analysis of PFCs in a wide range of matrices.

Introduction

The unique water-, oil-, grease-, stain- and heat-resistant properties of perfluorinated compounds (PFCs) have led to their widespread use in diverse industrial applications and multiple consumer products for over fifty years. Resistant to degradation, many of these synthetic compounds have become persistent and ubiquitous environmental pollutants. Bioaccumulation of PFCs in wildlife and in humans as well as studies linking some of these chemicals to developmental, reproductive and systemic toxicity in laboratory animals have led to efforts to regulate these compounds and have prompted the need for PFC monitoring and risk assessment in humans.[1,2] PFCs are detectable in human serum and breast milk and have even been found to be present in the blood of newborns, possibly through lactational transfer from mothers.[3] Determination of exposure pathways and health outcomes requires sensitive and accurate methods for trace-level analysis of PFCs in a range of human and environmental matrices.

PFCs encompass neutral and ionic species that contain the perfluorinated alkyl moiety. While gas chromatography-mass spectrometry (GC/MS) methods have been used to analyze volatile neutral PFCs and derivatives of ionic PFCs, many of these chemicals are more amenable to other analytical techniques. Liquid chromatography-tandem mass spectrometry (LC/MS/MS) is the method of choice for the analysis of ionic PFCs in a variety of matrices, but accurate quantification has proven to be difficult using this technique due to background PFC contamination and matrix interferences. These analytical challenges underscore the need for a high performance LC-MS platform capable of exceptional sensitivity, selectivity and accuracy.

The TSQ Vantage™ triple-stage quadrupole MS coupled to the Accela™ high speed LC system enables rapid, accurate and robust LC/MS/MS analysis of small molecules and biomolecules. Delivering up to a 10-fold improvement in signal-to-noise ratio compared to existing triple-quadrupole MS systems, the TSQ Vantage mass spectrometer facilitates high-sensitivity quantitation in matrix-rich samples and enhances analytical accuracy and precision. The instrument is capable of high resolution (0.2 Da. FWHM) selection of precursor ions, enabling highly selective reaction monitoring (H-SRM) for greater analytical selectivity and accuracy. The Accela system, together with 1.9 µm particle columns, enables fast and efficient chromatographic separations over an expansive range of flow rates and pressures.

In this note, we demonstrate highly sensitive, accurate and reproducible analysis and quantitation of six PFCs in neat solution and in human breast milk matrix using selected reaction monitoring (SRM) and H-SRM on the integrated UHPLC Accela-TSQ Vantage LC-MS platform. Elimination of PFC contamination from the analytical system was achieved by using a PFC-free Accela pump with a pre-cleaned PFC-free degasser and replacing Teflon® tubing with PEEK tubing. The excellent sensitivity and selectivity afforded by SRM on the TSQ Vantage system obviated the need for any further modifications of the LC configuration, a distinct advantage over other commercial platforms that require the use of in-line contaminant traps or column-switching methods for PFC analysis.

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Materials and Methods

Sample Preparation

PFC Standard Solutions

Standards for perfluoro-1-butanesulfonate (PFBS), perfluoro-1-hexanesulfonate (PFHxS), perfluoro-n-heptanoic acid (PFHpA), perfuoro-1-decanesulfonate (PFDS), perfluoro-n-undecanoic acid (PFUnA), and perfluoro-n-dodecanoic acid (PFDoA) were obtained from a proprietary source. A stock solution of a mixture of these six PFCs was prepared at a concentration of 1 mg/L. Calibration solutions, with concentrations of 0.04-2.5 ng/mL (ppb), were prepared by serial dilution of the stock solution in 60:40 (v/v) methanol/water. Two internal standards, m-PFUnA and m-PFHxS, were added into each calibration solution and sample at 2 ng/mL (ppb) concentration.

Milk Matrix A

A 2 g human breast milk sample, obtained from a proprietary source, was diluted in acetonitrile to precipitate proteins. Weak anion exchange solid-phase extraction was performed and the resulting PFC extract was eluted using 2% ammonium hydroxide in methanol, evaporated to dryness and reconstituted in 60% methanol/water (0.6 mL).

Milk Matrix B

The six PFCs were spiked into Matrix A at concentrations of 0.1 ng/mL to generate Matrix B.

Milk Matrix C

To generate Matrix C, six PFCs were spiked into Matrix A at concentrations of 0.3 ng/mL.

Milk Matrix D

Matrix D was prepared by spiking the six PFCs into Matrix A at concentrations of 1.0 ng/mL.

LC/MS Analysis

Instrumentation

LC/MS analysis was performed on a PFC-free Accela 600 LC system and PAL autosampler coupled to a TSQ Vantage triple-stage quadrupole mass spectrometer. The PFC-free Accela pump was equipped with a pre-cleaned PFC-free degasser and all Teflon tubing was replaced with PEEK tubing.

LC Parameters

Column:	Thermo Fisher Scientific Hypersil GOLD PFP column (100 x 3 mm, 1.9 µm particle size)			
Mobile Phase:	A: 5 mM ammonium acetate and 10% methanol/water B: 2 mM ammonium acetate/99% methanol			
Flow Rate:	see gradient			
Column Temperature:	ambient			
Sample Injection Volume:	10 µL			
Gradient:	Time (min)	A%	B%	Flow rate (µL/min)
	0.0	70	30	400
	0.5	70	30	400
	1.0	54	46	400
	4.0	30	70	400
	9.0	12	88	400
	9.4	12	88	400
	9.6	0	100	400
	9.7	0	100	500
	11.0	0	100	500
	11.1	70	30	500
	14.5	70	30	500
	15.0	70	30	400

MS Parameters

Negative Ion Mode Ionization with HESI Probe	
Heated Electrospray Ionization Source Conditions:	
Spray Voltage:	3500 V
Capillary Temperature:	300 °C
Sheath Gas:	60 au
Auxiliary Gas:	15 au
Vaporizer Temperature:	400 °C
Resolution for SRM Setup:	Q1, Q3 = Unit [0.7 Da. FWHM]
Resolution for H-SRM Setup:	Q1 = 0.2 Da. FWHM; Q3 = 0.7 Da. FWHM

#	Parent	Product	Collision Energy	RT Start	RT End	S-Lens	Name
1	299.0	80.2	43	3.15	4.15	115	PFBS
2	299.0	99.2	34	3.15	4.15	115	PFBS
3	299.0	169.0	23	3.15	4.15	115	PFBS
4	399.0	80.2	45	4.7	5.7	89	PFHxS
5	399.0	99.2	35	4.7	5.7	89	PFHxS
6	399.0	169.1	29	4.7	5.7	89	PFHxS
7	403.0	84.2	43	4.7	5.7	89	m-PFHxS
8	403.0	103.2	37	4.7	5.7	89	m-PFHxS
9	363.0	169.0	10	5.0	6.0	51	PFHpA
10	363.0	319.0	17	5.0	6.0	51	PFHpA
11	598.9	99.1	47	7.1	8.1	128	PFDS
12	598.9	230.1	50	7.1	8.1	128	PFDS
13	598.9	80.3	47	7.1	8.1	128	PFDS
14	562.9	269.0	18	7.75	8.75	62	PFUnA
15	562.9	519.0	12	7.75	8.75	62	PFUnA
16	564.9	520.0	18	7.75	8.75	64	m-PFUnA
17	612.9	169.0	25	8.4	9.4	78	PFDoA
18	612.9	569.0	12	8.4	9.4	78	PFDoA

Table 1: SRM transitions monitored for the detection of PFBS, PFHxS, PFHpA, PFDS, PFUnA and PFDoA.

Results and Discussion

Separation of PFC Standards

A total of fifteen unique SRM transitions were monitored for PFBS, PFHxS, PFHpA, PFDS, PFUnA and PFDoA, and three were monitored for the internal standards m-PFHxS and m-PFUnA (Table 1). Using the modified PFC-free LC-MS platform, a mixture of the six PFC standards was separated and detected under 10 minutes (Figure 1). All of the compounds were baseline resolved with the elution order of PFBS, PFHxS (m-PFHxS), PFHpA, PFDS, PFUnA (m-PFUnA) and PFDoA. As the majority of interferences from matrices elute early at void volume, elution of the first compound at 3.64 min ensured a robust quantitation method.

Linearity and Sensitivity

Excellent linearity in detector response was observed over the range of 0.04-2.5 ppb, with correlation coefficients greater than 0.999 for all transitions. Representative calibration curves for PFBS and PFUnA, obtained using the internal standard method, are shown in Figure 2, with coefficients of 0.9997 and 0.9996 respectively.

The sensitivity of the method is dependent on the levels of interferences that are present in the blank and in the solvents used. Limits of detection (LODs) and limits of quantitation (LODs), defined as S/N ratio of 3 and 10, respectively, are shown in Table 2. LODs ranged from 2–174 ppt, and LOQs ranged from 5–756 ppt. PFBS and PFDS were detectable at 2 ppt and quantifiable at 5 ppt. Figure 3 shows the separation and detection of 10 ppt PFBS and 10 ppt PFDS at different SRM transitions, and the corresponding blanks as comparisons. The higher LOD and LOQ values observed for PFHpA, PFUnA and PFDoA may be attributed to interferences present in the blank and mobile phases.

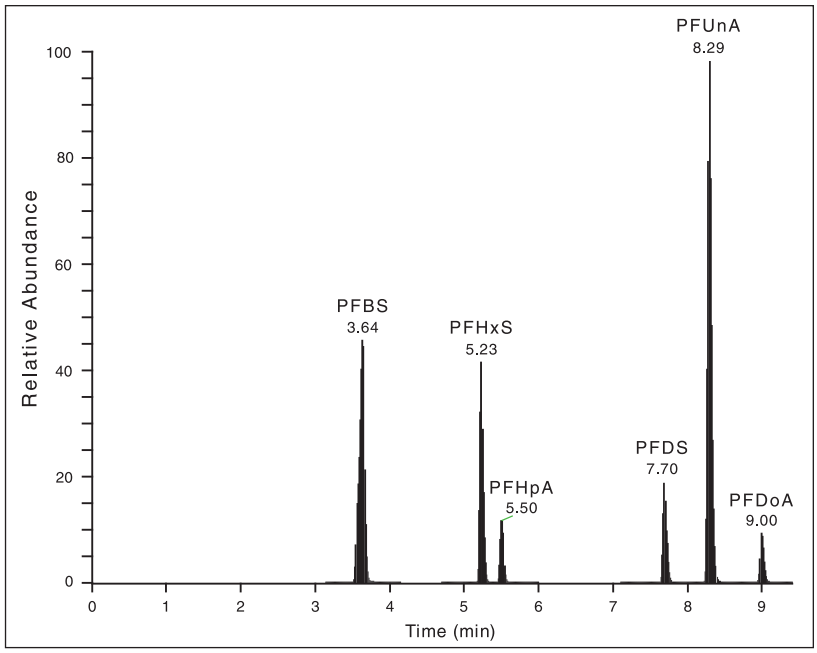

Figure 1: Separation and detection of six PFC standards at 2.5 ppb concentrations.

Compounds	SRM	LOD (ppt)	LOQ (ppt)		
PFBS	298.9 > 80.2	2	5	SRM	
	298.9 > 99.2	5	12	SRM	
PFHxS	398.9 > 80.2	21	83	SRM	
	398.9 > 99.2	12	66	SRM	
PFHpA	362.9 > 169.0	174	756	SRM	Blank Contamination
	362.9 > 319.0	120	457	SRM	Blank Contamination
PFDS	598.9 > 80.2	2	7	SRM	
	598.9 > 99.2	3	9	SRM	
PFUnA	562.9 > 269.0	35	156	SRM	
	562.9 > 519.0	52	235	SRM	
PFDoA	612.9 > 169.0	59	296	SRM	
	612.9 > 569.0	64	295	H-SRM	

Table 2: LODs and LOQs of the PFC standards. LOQs were estimated from triplicate injections (CV < 15%) of standard solutions at concentration levels corresponding to a signal-to-noise ratio of 10.

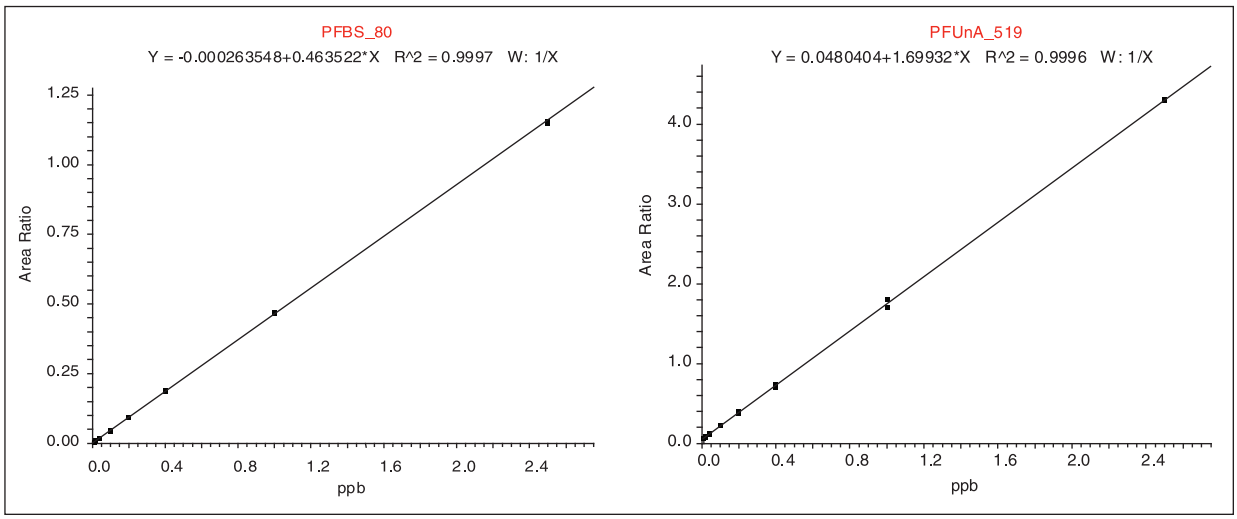

Figure 2: Representative calibration curves of PFBS and PFUnA standards.

Significant background interference was observed for the SRM transition 613 > 569 of PFDoA at Q1 resolution of 0.7 Da. FWHM, therefore H-SRM was employed. As shown in Figure 4, using the higher Q1 resolution of 0.2 Da. FWHM removed the matrix interference without compromising sensitivity. Moreover, sensitive and unambiguous PFC detection was achieved without the use of in-line trapping or column switching.

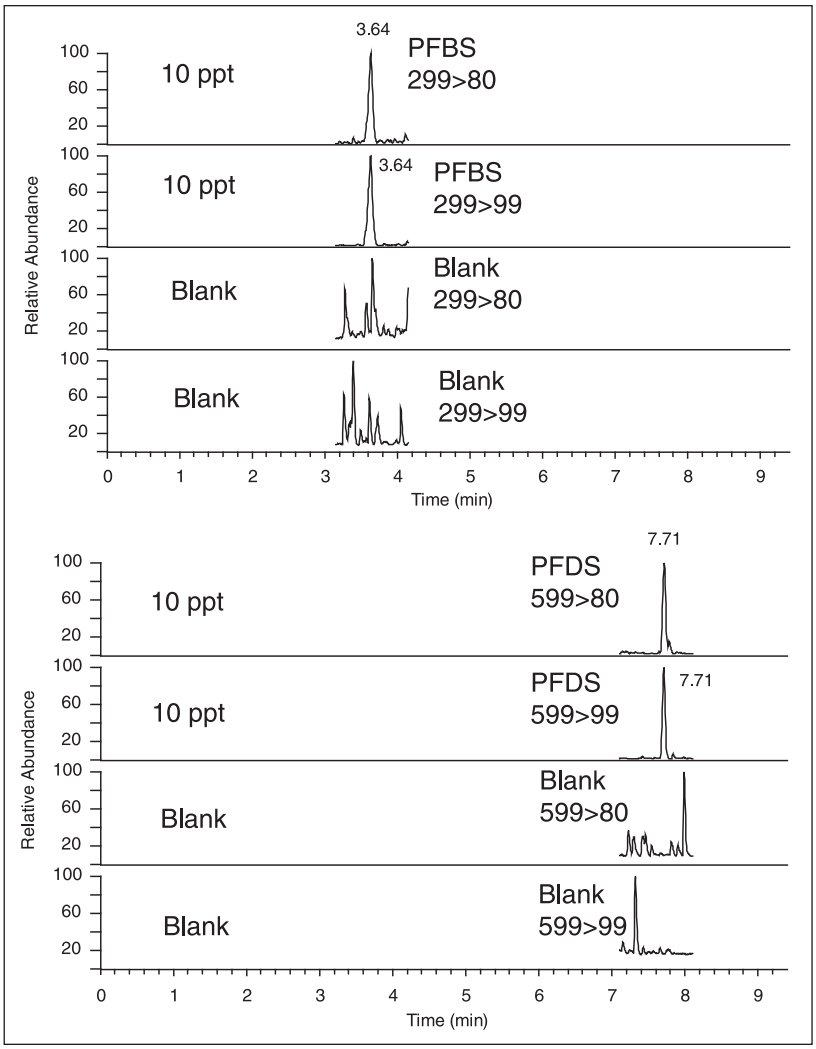

Figure 3. Separation and detection of 10 ppt of PFBA and PFDS.

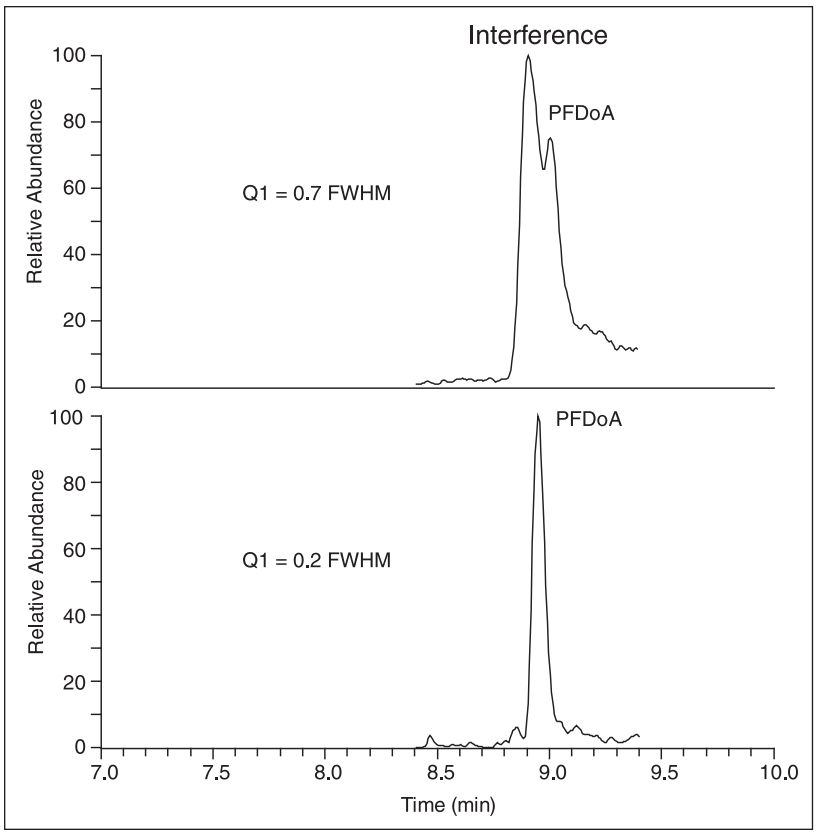

Figure 4: H-SRM eliminates interference peaks without any change in signal intensity.

Analysis of PFCs in Human Milk Matrix

To evaluate the applicability of this technique to complex matrices, the SRM assays were used to analyze and quantitate PFCs in human breast milk. UHPLC separation of the six PFC analytes in a spiked milk matrix was achieved within 9 minutes (Figure 5). All analytes were baseline resolved using the optimized LC method.

Reproducibility was investigated by analyzing fifteen replicate injections of a spiked matrix (Table 3). Peak area RSDs for compounds and internal standards were 10.8% and 11.0% respectively, the response ratio RSD was 1.29%, and retention time RSD was 0.29%, indicating excellent method and system reproducibility, particularly of the LC pump.

File Name	Peak Area	ISTD Area	Response Ratio	RT (Min)
Mark D_0 17	149 369	8 268 9	1.806	8.29
Mark D_0 18	147 075	8 081 9	1.820	8.27
Mark D_0 19	145 882	8 127 6	1.795	8.29
Mark D_0 20	146 012	7 990 7	1.827	8.29
Mark D_0 21	143 987	8 071 2	1.784	8.27
Mark D_0 22	143 095	8 011 6	1.786	8.25
Mark D_0 23	140 298	7 802 3	1.798	8.25
Mark D_0 67	121 597	6 929 2	1.755	8.25
Mark D_0 68	119 763	6 776 4	1.767	8.29
Mark D_0 69	119 149	6 654 3	1.791	8.27
Mark D_0 70	121 775	6 647 6	1.832	8.32
Mark D_0 71	113 885	6 376 6	1.786	8.27
Mark D_0 72	115 138	6 271 2	1.836	8.31
Mark D_0 73	116 884	6 561 6	1.781	8.24
Mark D_0 74	114 601	6 358 6	1.802	8.31
RSD%	**11**	**10.8**	**1.29**	**0.29**

Table 3: Reproducibility (RSD) of instrument performance for fifteen replicate injections of Matrix D. Peak area is the LC peak area response for fifteen injections. Peak area was used for quantitation, both for the internal standard method and external standard method. ISTD area = peak area of the internal standard. Response ratio is the peak area of the compounds over the peak area of the internal standard, and was used for quantitation with the internal standard method. RT = retention time.

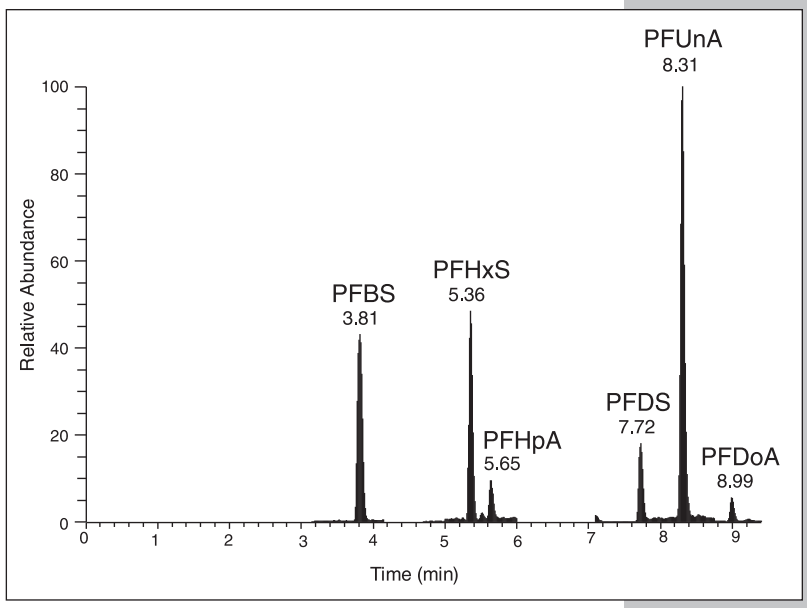

Figure 5: The separation and detection of the PFCs in human milk matries C

Table 4 summarizes the concentrations of the PFCs detected in a human milk sample (Matrix A). PFBS, PFHxS, PFHpA, PFDS, and PFUnA were detected at concentrations of less than 60 ppt, while PFDoA was not found to be present in the sample. Assay accuracy was investigated using spiked milk matrices B, C, and D and internal and external standards (Table 3). For PFHxS and PFUnA, the two PFCs for which internal standards were available, using the internal standard method was significantly more accurate (98–110%) than the external standard method (81–144%) in the concentration range 0.1–1.0 ng/mL. While internal standards eliminate the matrix effect to facilitate greater quantitative accuracy, they are expensive and may be difficult to obtain. Using the external standard method, the accuracy of all PFC analytes was 81–144% in the concentration range 0.1–1.0 ng/mL.

		PFBS	PFHxS	PFHpA	PFDS	PFUnA	PFDoA
Matrix A (unknown)	Measured value with IS (ppt)		48.0			12.0	
	Measured value with ES (ppt)	10.0	40.0	50.0	50.0	35.0	0.0
Matrix B (Matrix A + spiked 100 ppt)	Measured value with IS (ppt)		152			115	
	Measured value with ES (ppt)	110	145	185	150	195	130
	Method Accuracy with IS (%)		103			103	
	Method Accuracy with ES (%)	100	104	123	100	144	130
Matrix C (Matrix A + spiked 300 ppt)	Measured value with IS (ppt)		382			340	
	Measured value with ES (ppt)	260	290	365	285	420	280
	Method Accuracy with IS (%)		110			109	
	Method Accuracy with ES (%)	84	85	104	81	125	93
Matrix D (Matrix A + spiked 1000 ppt)	Measured value with IS (ppt)		1023			1042	
	Measured value with ES (ppt)	930	945	1255	935	1495	985
	Method Accuracy with IS (%)		98			103	
	Method Accuracy with ES (%)	92	91	120	89	144	99

Table 4: PFC concentrations (ppt) in human milk matrix A and spiked milk matrices B, C, and D. Note: The method accuracy was calculated with the formula of 100 x measure value/(measure value of Matrix A + spiked value).

Conclusion

A highly sensitive, accurate and robust SRM-based approach for PFC analysis was developed on a PFC-free Accela-TSQ Vantage LC-MS platform. PFCs were accurately and reproducibly detected at ppt levels in neat solution and in human milk. The unique H-SRM capability of the TSQ Vantage instrument removed interference peaks and significantly improved selectivity. Furthermore, unlike other approaches, this platform does not require trapping or column switching techniques to ensure exceptional sensitivity in high chemical backgrounds.

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