REPORTER

Applications Newsletter Volume 32.1

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pg. 3

Faster Protein and Peptide Liquid Chromatography

pg. 12

FAST EXTRACTION AND LOW LEVEL DETECTION OF CHLORAMPHENICOL IN SHRIMP

pg. 22

ONLINE SPE/LC-MS METHOD FOR THE RAPID ANALYSIS OF THYROID HORMONES IN PLASMA

SIGMA-ALDRICH®

Reporter
Volume 32.1

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Table of Contents

Pharmaceutical

Faster Protein and Peptide Liquid Chromatography (FP2LC): Introducing BIOshell™ Fused-Core® U/HPLC Columns 3

Food and Beverage Analysis

Extraction and Analysis of PAHs from Grilled Hamburger.................... 7

Analysis of Enantiomers in Caraway Essential Oils......................... 10

Fast Extraction and Low Level Detection of Chloramphenicol in Shrimp 12

Selective Extraction and Cleanup of Patulin Mycotoxin from Apple Juice Samples 14

Clinical/Bioanalytical

Method Optimization for LC-MS Analysis of Vitamin D Metabolite Critical Pairs in Serum............................... 16

Developing Robust UHPLC Methods for Clinical, Forensic and Bioanalytical Samples............................ 20

Online SPE/LC-MS Method for the Rapid Analysis of Thyroid Hormones in Plasma........................... 22

Environmental

Extraction and Analysis of Neonicotinoid Pesticides from Flower Blossoms..... 24

Simple Collection and 5-minute UHPLC Assay for 11 Isocyanates 26

Standards

Elevate Your Enjoyment.............. 27

Cover Photo:
Getting more work done in the same amount of time makes companies more successful, but in the end, it is the patient who benefits from faster protein and peptide separations on BIOshell Fused-Core HPLC columns.

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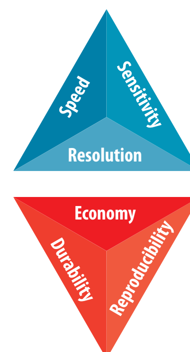
Our Commitment to Serving All Sides of the Triangles

Paul Ross, Ph.D.
Director of Marketing and R&D
Analytical Separations

Dear Colleague:

Supelco's product offering includes approximately 40,000 unique part numbers, with typically several hundred more introduced each quarter. With so large a number, it's easy to overlook why we have them and what they all have in common. Everything we develop and introduce to the market is tied to these customer needs:



- **Speed** – Throughput, getting more done, processing more samples in the increasingly compressed available time
- **Sensitivity** – Seeing low levels of target analytes, often in complex matrices
- **Resolution** – Being able to resolve analytes, again, often in complex matrices

This is the chromatographer's triangle, with which we are all familiar. However, we consider the sides of a different triangle, one that considers how practical our products are, and how users can implement them in all laboratory situations. So we also look for ways to maximize:

- **Economy** – The technology should improve your ROI and reduce operating expenses
- **Durability** – Longer instrument lifetime, less change-out of consumables
- **Reproducibility** – Reliability, ensuring the method gives the same results now and into the future, at sites across the globe

Our recent innovations featured in this Reporter relate directly to the six factors in the Chromatographer's and Practicality triangles:

- **Titan™ UHPLC Columns** deliver leading UHPLC column performance, at half the cost.
- **BIOshell™ Fused-Core® U/HPLC Columns** provide rapid, high efficiency separations of proteins and other biopolymers.
- **HybridSPE®-PLus** plates remove phospholipids and proteins for accurate and reproducible LC-MS analysis of analytes in biological fluids, while increasing column lifetime.
- **ASSET™ EZ4-NCO Dry Sampler** gives ultimate sensitivity in vapor phase and particulate isocyanates in the most challenging atmospheres.
- **SupelMIP® SPE - Patulin** tubes save time, reduce cost and provide lower detection limits via robust and rapid methodology.
- **Supel™ QuE Z-Sep and Z-Sep+** take QuEChERS technology to a new level by removing lipid and pigment interferences from challenging matrices.

These are just a few products that highlight our ongoing commitment to giving you effective tools to solve your most challenging analytical methods.

Best regards,

Ron.

Paul Ross, Ph.D.
Director of Marketing and R&D
Analytical Separations

Faster Protein and Peptide Liquid Chromatography (FP2LC): Introducing BIOshell™ Fused-Core® U/HPLC Columns

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Abstract

A new and broad product line for the analysis of peptides and proteins by reversed-phase high performance liquid chromatography (RP-HPLC) is introduced under the name BIOshell Fused-Core U/HPLC columns. As the Ascentis® Express column line for the analysis of small molecular mass compounds, BIOshell columns outperform fully porous silica-based columns of the same particle size in terms of one or more important chromatographic attributes; such as efficiency, resolution, analysis time (speed) and back pressure. By providing faster peptide and protein liquid chromatography (FP2LC), the benefits of porous-shell columns are now also available to scientists who are tasked to advance our knowledge about the living world.

Background and Characteristics

The development of Fused-Core U/HPLC columns is based on the pellicular silica technology developed by J. J. Kirkland at E. I. du Pont de Nemours and Company in the late 1960s during the formative years of HPLC, and applied in this century to create very efficient particles in the range of 2.7 to 5 micron.[1,2] The latest innovations derived from this technology are now available as BIOshell Fused-Core columns for faster protein and peptide liquid chromatography (FP2LC) separations. BIOshell columns for peptide analysis are either packed with 2.7 or 5 micron particles containing 160 Å pores and bonded with a C18 alkyl or alkyl cyano functionality, while protein separations are best performed with 3.4 micron particles containing 400 Å pores bonded with C4 alkyl functional groups. The complete list of available BIOshell columns is provided at the end of this article.

In 2007 Supelco introduced the groundbreaking Ascentis Express Fused-Core column line for the analysis of compounds with low molecular mass. In the succeeding years it has become clear that (a) columns packed with 2.7 micron, 90 Å pore size, porous-shell particles provide a dramatic and unexpected improvement in column efficiency, rivaling the efficiency of columns packed with fully porous 1.7 micron particles, and (b) a paradigm shift had to take place in the minds of theoreticians to adjust the theory of chromatographic band broadening to account for column efficiencies that were, until then, considered outside the realm of possibility.[3,4]

Like the particles in Ascentis Express columns, the particles in BIOshell columns are composed of a spherical solid glass core surrounded by a thin layer of nano-size silica particles. **Table 1** summarizes the characteristics and maximum operating conditions of the new BIOshell porous-shell columns.

As shown in **Table 1**, to accommodate the larger size of biopolymers, the particles in BIOshell columns either feature160 Å pores for unhindered access by peptides up to a mass of about 20 kDa, or 400 Å pores for proteins with a molecular mass up to about 500 kDa. Note that the thickness of the porous shell varies as does the percentage of the surface area when compared to a fully porous particle of the same size. Thus, the 0.2 micron shell for the 3.4 micron particles results in a surface area that is 31% of a fully porous particle of that size. Similarly, the 0.5 micron shell for the 2.7 micron particle provides 75% of the maximum surface area, while the 0.6 micron shell for the nominally 5 micron but actually 4.7 micron particles contains 59% of the surface area of a fully porous particle. Although the loss of surface area may seem a cause of concern, Unger et al., first demonstrated that even nonporous matrices provide sufficient retention for larger biomolecules, while Gritti and Guiochon showed that the capacity of core-shell particles is not substantially reduced even for large biopolymers, a conclusion that was recently confirmed in a paper from Guillaume et al., who found that the loading capacity of a wide pore core-shell column was 2–3 fold less than that of a fully porous column.[5-7]

Table 1 also shows the bonded phase characteristics, recommended pH range, and the highest values for back pressure and temperature at which each particle type was tested. While a maximum pressure of 600 bar or 9,000 psi may seem modest in current day UHPLC systems, a maximum operating temperature of 80–100 °C provides a degree of freedom that can be very beneficial, particularly when working with more hydrophobic proteins, including monoclonal antibodies, as will be illustrated in the following proof statements.

Table 1. Characteristics and Operating Conditions of BIOshell Fused-Core Columns

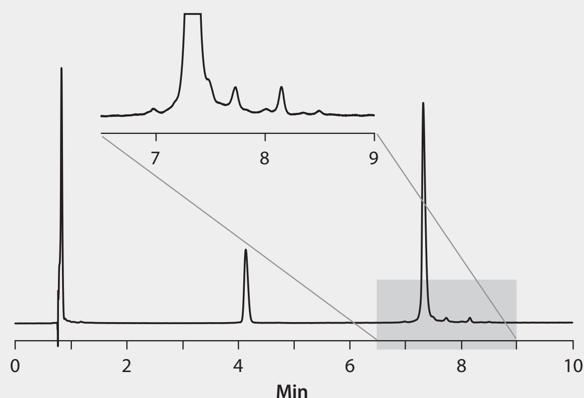
	Nominal Values						Phase Characteristics and Operating Conditions					
Physical Parameter	Dp (µm)	Core (µm)	Shell (µm)	Pore Size (Å)	SBET (m²/g)	Capacity vs. Porous	Bonded Phase Ligand	End Capped	Tmax °C	pH Range	Pmax (bar)	Frit (µm)
2.7 µm A160 Peptide C18	2.7	1.7	0.5	160	80	75%	di-isobutyl-octadecylsilane	No	100	1–8	600	2
2.7 µm A160 Peptide CN	2.7	1.7	0.5	160	80	75%	di-isopropyl-cyanopropylsilane	Yes	80	1–8	600	2
5 µm A160 Peptide C18	4.7	3.5	0.6	160	60	59%	di-isobutyl-octadecylsilane	No	100	1–8	600	2
5 µm A160 Peptide CN	4.7	3.5	0.6	160	60	59%	di-isopropyl-cyanopropylsilane	Yes	90	1–8	600	2
3.4 µm A400 Protein C4	3.4	3.0	0.2	400	15	31%	dimethylbutylsilane	Yes	90	2–9	600	2

(continued on next page)

The chromatogram in **Figure 1** shows the analysis of IgG2-B subtype monoclonal antibody following solubilization and reduction of the sulfide bonds with 100 mM dithiothreitol (DTT) in 8 M guanidine HCl at 50 °C for 35 minutes. The main components in **Figure 1** are the light (LC) and heavy (HC) chains of IgG2-B. The inset clearly shows several expected variants near the heavy chain peak, although the identity of the minor peaks was not confirmed. The analysis was performed at 80 °C based on the results from Dillon and co-workers at Amgen, which were more recently confirmed by other groups.[8-11]

Figure 1. High Temperature Analysis of IgG2-B Antibody Fragments Using a 10 cm x 2.1 mm I.D. BIOshell™ A400 Protein C4 Column

column: BIOshell A400 Protein C4, 10 cm x 2.1 mm I.D., 3.4 µm (66825-U)
mobile phase: A: water:0.1% TFA, B: acetonitrile:water:0.1% TFA, 80:20
gradient: 33-40% B in 10 min
flow rate: 0.25 mL/min
temp: 80 °C
detection: 280 nm
injection: 1 µL
sample: 0.5 mg/mL IgG2-B treated with 100 mM DDT in 8 M guanidine HCl at 50 °C for 35 min
instrument: Shimadzu™ Nexera®



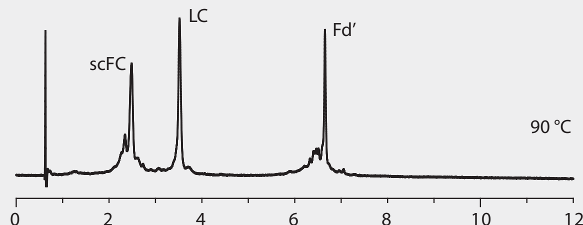
Another example of the need to operate reversed-phase columns at high temperature is provided in **Figure 2**, in which a so-called middle-down, as opposed to a top-down or bottom-up, approach was used to analyze the structure of a monoclonal antibody. In a middle-down approach IgG is digested into a few large fragments prior to further characterization. The novel proteolytic enzyme IdeS, commercially available as FabRICATOR®, cleaves an antibody into $F(ab')_2$ and scFc fragments, which have approximate molecular masses of 100 kDa and 25 kDa respectively. Using disulfide reduction, the $F(ab')_2$ fragment can be further resolved into its light-chain (LC) and F(ab) components. Each of the components in the resulting mixture has a molecular mass of about 25 kDa.

Visual inspection of the results shown in **Figure 2** reveals that when run at 90 °C, the BIOshell A400 Protein C4 column (A) provides narrower peak widths and better peak shape than a competitive column packed with fully porous 3.5 micron particles (B). The same conclusion can be drawn from the results for two other competitive columns (C and D) which were operated at 80 °C, the highest temperature recommended by their manufacturer. The ability to operate BIOshell A400 Protein C4 columns at 90 °C provides a beneficial degree of flexibility for biochemists involved in the development and analysis of antibody-based biotherapeutic drugs.

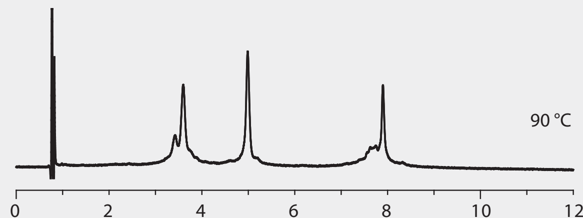
Figure 2. Analysis of Antibody Fragments on Wide Pore Reversed Phase Columns Operated at Maximum Recommended Temperature

column: as indicated, 10 cm x 2.1 mm
mobile phase A: 80:20, (water, 0.1% TFA) : (acetonitrile, 0.1% TFA)
mobile phase B: 50:50, (water, 0.1% TFA) : (acetonitrile, 0.1% TFA)
gradient: 30 to 70% B in 12 min
flow: 0.3 mL/min
column temp.: as indicated
detection: UV, 215 nm
injection: 1 µL, after sample diluted in mobile phase A

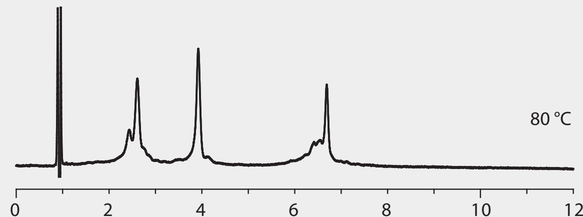
A. BIOshell A400 Protein C4, 3.4 µm



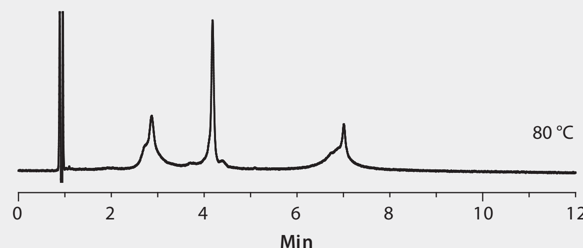
B. Zorbax® 300SB-C18, 3.5 µm



C. XBridge® BEH300 C4, 3.5 µm



D. AcQuity® UPLC® BEH300 C4, 1.7 µm



To determine the stability of columns packed with large pore Fused-Core particles with continued operation at high temperature and flow rate, five globular protein standards were repeatedly injected on a 10 cm x 2.1 mm I.D. BIOshell A400 Protein C4 column. The results are shown in **Figure 3** for the first and last injection of an experiment in which the column was exposed to repeated 10 minute gradients from 25 to 40% acetonitrile/0.1% trifluoroacetic acid (TFA) in water at a flow rate of 0.5 mL/min and a temperature of 90 °C. A noticeable but small loss of retention was observed for all proteins after the column had been in contact with almost 15,000 gradient column

volumes (1 CV ~ 0.2 mL) at 90 °C under acidic (pH ~ 2.0) conditions. Note that the peak shape and peak widths of the test proteins did not change during the experiment, indicating excellent physical stability of BIOshell Fused-Core® columns.

Figure 3. BIOshell A400 Protein C4: Column Stability

column: BIOshell A400, 10 cm x 2.1 mm 3.4 µm (66825-U)
mobile phase A: water:0.1% TFA
mobile phase B: acetonitrile:0.1% TFA
gradient: 25-40% B in 10 min
flow rate: 0.5 mL/min
column temp: 90 °C
detection: 215 nm
injection: 1.0 µL
instrument: Shimadzu Nexera

1. Cytochrome c (12.4 kDa)
2. Lysozyme (14.3 kDa)
3. Apomyoglobin (17 kDa)
4. Catalase (250 kDa total; tetramer of ~60 kDa each)
5. Enolase (93 kDa total; dimer of 46.7 kDa each)

Black trace = injection 1
Red trace = injection 385 or after 14,620 column volumes

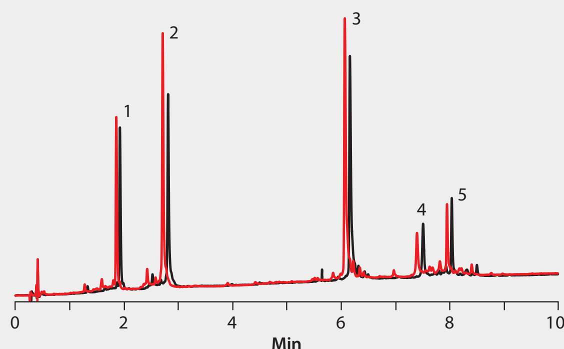


Figure 4. Peak Capacity of a Commercial Fused-Core and Fully Porous Reversed Column for a Peptide Digest Mixture

columns: as indicated, 15 cm x 2.1 mm
mobile phase A: 0.1 % formic acid
mobile phase B: 25:75, (0.4 % formic acid):acetonitrile
gradient: as indicated (in terms of column volumes)
flow rate: as indicated
column temp.: 35 °C
det.: ESI(+)-TOF
injection: 2 µL
sample: 10 pmol / µL

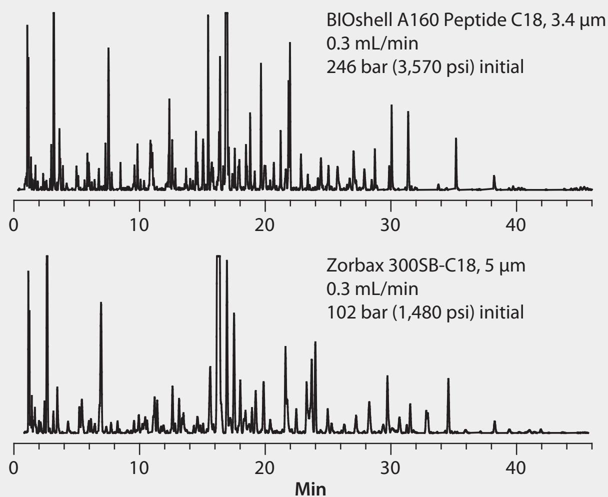


Table 2. Calculated Peak Capacities

Column	t_g*	w_{ave}	P_c
BIOshell A160 Peptide C18	41.3	0.1213	340
Zorbax® SB300-C18	47.2	0.1951	242

* Recall that the gradient volume is scaled to the same number of column volumes. t_g and w are units of minutes

In a final example, **Figure 4** shows chromatograms of a mixture of 5 different tryptic digests (cytochrome c, beta-lactoglobulin, glutamate dehydrogenase, carbonic anhydrase, myoglobin) run on both a 2.7 micron BIOshell A160 Peptide C18 column and a fully porous 5 micron C18 column of the same dimensions. The analyses were run at the same flow rate of 0.3 mL/min and with the same starting and final gradient mobile phase composition. However, to account for the smaller pore volume of the Fused-Core particles in the BIOshell column, the gradient times were adjusted so that each column was exposed to the same number of gradient column volumes, 46 in this case. About 20 peaks from across the chromatograms (**Figure 4**) were extracted from the MS data to serve as a representative sample in the calculation of the peak capacity (Pc) for each column, using the accepted definition Pc = gradient time/ average peak width.Peak widths were calculated at baseline for each of the 20 selected peptides. **Table 2** lists the experimental values for the gradient time (t_g) and the average peptide peak width (w) as well as the calculated peak capacity for this particular separation on each column.

The 2.7 µm BIOshell column showed a 40% increase in peak capacity over the 5 micron Zorbax 300SB-C18 porous column which is in agreement with the results obtained by Gilar et al. who confirmed results from earlier workers that peak capacity is proportional to the square root of the plate number.[12]

Conclusions

The advances brought about by using porous shell particles with narrow particle size distribution for the analysis of small molecular weight compounds, commercially available as Ascentis® Express columns, have been expanded to the reversed-phase U/HPLC analysis of peptides and proteins. As expected and demonstrated, larger pore size BIOshell Fused-Core columns provide benefits in terms of efficiency per unit pressure drop, which can either be taken advantage of by increasing peak capacity or traded in to reduce analysis time, benefits that are captured in the acronym FP2LC, or Faster Peptide and Protein Liquid Chromatography.

(continued on next page)

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Acknowledgment: The authors thank Stephanie A. Schuster and Robert E. Moran from Applied Materials Technologies, Inc. for contributing Figures 1 and 3.

Featured Products

Pore Size	Particle Size	I.D. (mm)	L (cm)	C4	C18	CN
BIOshell Fused-Core Peptide and Protein Columns						
400 Å	3.4 µm	2.1	5	66824-U	—	—
400 Å	3.4 µm	2.1	10	66825-U	—	—
400 Å	3.4 µm	2.1	15	66826-U	—	—
400 Å	3.4 µm	4.6	5	66827-U	—	—
400 Å	3.4 µm	4.6	10	66828-U	—	—
400 Å	3.4 µm	4.6	15	66829-U	—	—
160 Å	2.7 µm	2.1	3	—	66901-U	66965-U
160 Å	2.7 µm	2.1	5	—	66902-U	66966-U
160 Å	2.7 µm	2.1	7.5	—	66903-U	66967-U
160 Å	2.7 µm	2.1	10	—	66904-U	66968-U
160 Å	2.7 µm	2.1	15	—	66905-U	66969-U
160 Å	2.7 µm	3.0	3	—	66906-U	66970-U
160 Å	2.7 µm	3.0	5	—	66907-U	66971-U
160 Å	2.7 µm	3.0	10	—	66908-U	66972-U
160 Å	2.7 µm	3.0	15	—	66909-U	66973-U
160 Å	2.7 µm	4.6	5	—	66913-U	66974-U
160 Å	2.7 µm	4.6	10	—	66915-U	66975-U
160 Å	2.7 µm	4.6	15	—	66917-U	66976-U
160 Å	5 µm	2.1	3	—	67001-U	67061-U
160 Å	5 µm	2.1	5	—	67002-U	67062-U
160 Å	5 µm	2.1	7.5	—	67003-U	67063-U
160 Å	5 µm	2.1	10	—	67004-U	67064-U
160 Å	5 µm	2.1	15	—	67006-U	67065-U
160 Å	5 µm	3.0	3	—	67007-U	67066-U
160 Å	5 µm	3.0	5	—	67008-U	67067-U
160 Å	5 µm	3.0	10	—	67011-U	67068-U
160 Å	5 µm	3.0	15	—	67012-U	67069-U
160 Å	5 µm	4.6	5	—	67013-U	67071-U
160 Å	5 µm	4.6	10	—	67014-U	67080-U
160 Å	5 µm	4.6	15	—	67015-U	67081-U
BIOshell Fused-Core Peptide and Protein Guard Columns, pk. of 3						
400 Å	3.4 µm	2.1	0.5	66830-U	—	—
400 Å	3.4 µm	4.6	0.5	66831-U	—	—
160 Å	2.7 µm	2.1	0.5	—	66918-U	66977-U
160 Å	2.7 µm	3.0	0.5	—	66919-U	66978-U
160 Å	2.7 µm	4.6	0.5	—	66921-U	66979-U
160 Å	5 µm	2.1	0.5	—	67016-U	67082-U
160 Å	5 µm	3.0	0.5	—	67017-U	67083-U
160 Å	5 µm	4.6	0.5	—	67018-U	67084-U

Related Product

Description	Cat. No.
BIOshell Guard Cartridge Holder	66841-U

Extraction and Analysis of PAHs from Grilled Hamburger

Using Supel QuE Z-Sep QuEChERS Sorbents and SPB-608 Capillary GC Columns

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Introduction

Polynuclear aromatic hydrocarbons (PAHs) are compounds consisting of two or more fused aromatic rings. These compounds are lipophilic in nature, and are ubiquitous in the environment as a result of the incomplete combustion of organic matter. Those with five or more rings in their structure are usually referred to as "heavy" PAHs. These heavy PAHs are often the focus of toxicity studies, and some have been found to be carcinogenic and mutagenic.[1] In the case of grilled meats, PAHs are formed and adhere to the meat when fat and juices come into contact with an open flame.[2] In the United States, there are currently no guidelines regarding the consumption of foods containing PAHs,[2] however in 2011 the European Union (EU) adopted regulation 835/2011 that established the maximum allowable levels in various foodstuffs. In heat treated meats, this maximum was set at 5 µg/kg of benzo[a]pyrene, and 30 µg/kg total as a sum of four PAHs: benzo[a]pyrene, benzo[a]anthracene, benzo[b]fluoranthene and chrysene.[3]

Since PAHs are lipophilic, extraction from fatty samples such as hamburger meat will subsequently co-extract a significant amount of matrix. If GC-MS analysis is used, fatty matrix will prematurely foul the inlet, column and detector. Backflush of heavy constituents can be used to prevent contamination of the GC column and detector, but it will not prevent residue buildup in the inlet liner. Consequently, sample cleanup is imperative if GC analysis is to be performed. Traditional methods of cleanup include gel permeation chromatography (GPC), and fractionation on large columns containing silica gel, alumina, or Florisil®. In this application, a simple extraction and cleanup method was developed for 28 different PAHs from grilled hamburger meat using the "**Qu**ick, **E**asy, **Ch**eap, **E**ffective, **R**ugged and **S**afe" (QuEChERS) approach. Subsequent cleanup was done using several different sorbents, including new zirconia-based Z-Sep and Z-Sep+ materials. Analysis was performed by GC-MS in selected ion mode (SIM) using an SPB®-608 capillary column. This column provided the selectivity and efficiency necessary to resolve several isomeric sets, including benzo[b], [j] and [k] fluoranthene. A shorter, narrow-bore dimension was chosen to reduce analysis time while maintaining high efficiency.

Experimental

Hamburger grilled to well-done was ground and spiked at 100 ng/g with a mixture of PAHs. An internal standard solution containing naphthalene-d_8, fluoranthene-d_{10} and perylene-d_{12} was then added. After 10 minutes, the mixture was subjected to the extraction and cleanup procedure outlined in **Table 1**. Acetonitrile was chosen as the extraction solvent because it presented a good balance between maximizing extraction of the PAHs and minimizing extraction of the fatty matrix. For cleanup, four different sorbents were evaluated: (1) Z-Sep+, 500 mg (2) Z-Sep+/PSA, 500 mg/400 mg (3) Z-Sep, 500 mg and (4) PSA/C18, 400 mg/150 mg.

Three replicate spikes and an unspiked hamburger sample were processed using each cleanup. GC/MS analysis was done in selection ion mode (SIM) using the conditions described in **Table 2**. Quantitation was done against a 5-point calibration curve prepared in acetonitrile.

Table 1. Extraction and Cleanup Procedure for Grilled Hamburger

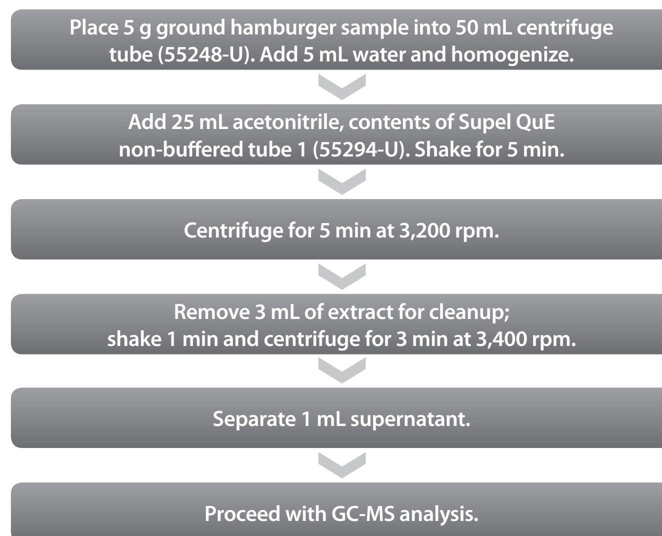


Table 2. GC-MS Analysis Conditions

column:	SPB-608, 20 m x 0.18 mm I.D., 0.18 µm
inj. temp.:	265 °C
oven:	60 °C (1 min), 25 °C/min to 275 °C, 10 °C/min to 300 °C (13 min)
flow rate:	1.5 mL/min constant
injection:	1 µL, splitless (1 min)
liner:	4 mm FocusLiner™ w/taper
ms tune:	ATUNE
ms temps.:	quads = 160 °C, source = 240 °C, transfer line = 300 °C

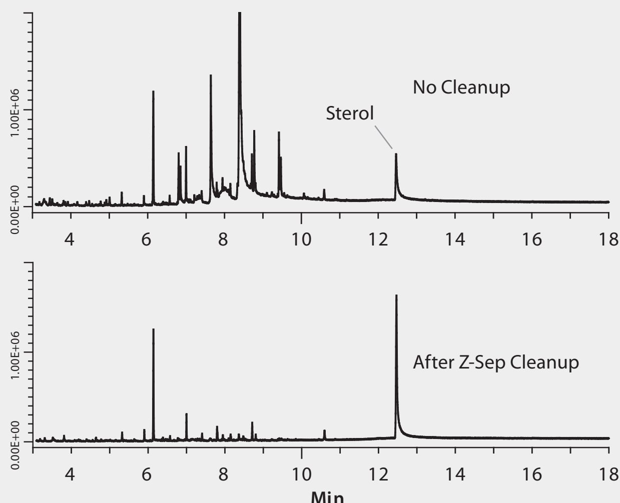
Results and Discussion

Background Removal

Background reduction was evaluated by analysis of the cleaned extracts by GC-MS in full scan mode. The Z-Sep cleaned extract had the lowest background (**Figure 1**). The large peak eluting between 12 and 13 minutes was identified as a sterol. In SIM mode, it did not interfere with the ions used for quantitation of the PAHs eluting in that region. The chromatogram of PAHs in grilled hamburger after cleanup with Z-Sep is shown in **Figure 2**.

(continued on next page)

Figure 1. GC-MS Background Before and After Cleanup with Z-Sep



PAH Recoveries

A summary of the recoveries after cleanup is presented in Figure 3. PAHs were detected in the unspiked hamburger, and these values were subtracted when determining recoveries for the spiked samples. %RSD values were below 10 in most cases, indicating good reproducibility for the extraction method. The best overall recoveries were obtained using Z-Sep cleanup. The PAHs from pyrene onward showed lower recoveries after cleanup with sorbents containing C18, including Z-Sep+. Z-Sep is a zirconia coated silica (zirconia on silica) and does not contain any C18, while Z-Sep+ is a dual bonded C18 and zirconia silica.

There was some matrix interference observed in the first 1/3 of the GC-MS analysis. This prevented accurate integration of the first internal standard, naphthalene-d_8, so it was not used for quantitation. Instead, only fluoranthene-d_{10} and perylene-d_{12} were used. The SPB-608 column was able to resolve all isomeric sets except triphenylene and chrysene; thus they are reported together.

Figure 2. GC-MS/SIM Analysis of Grilled Hamburger, Spiked at 100 ng/g with PAHs, After Cleanup with Z-Sep

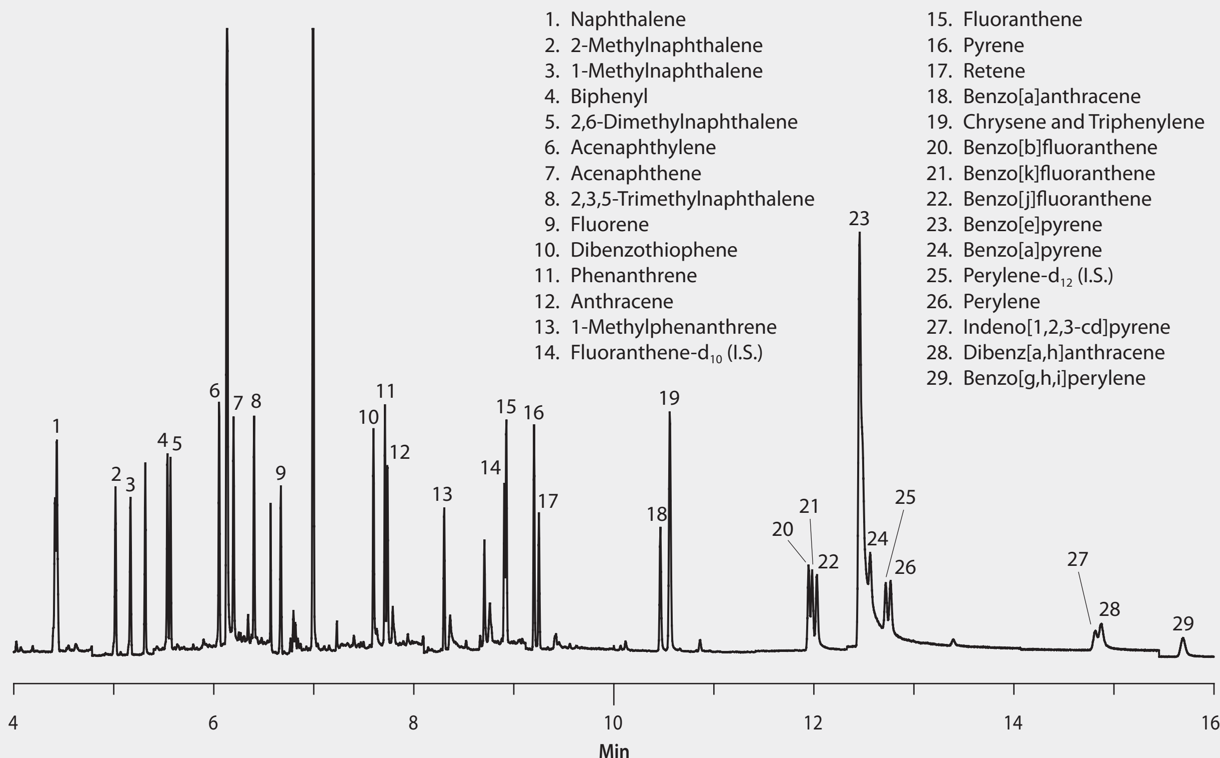
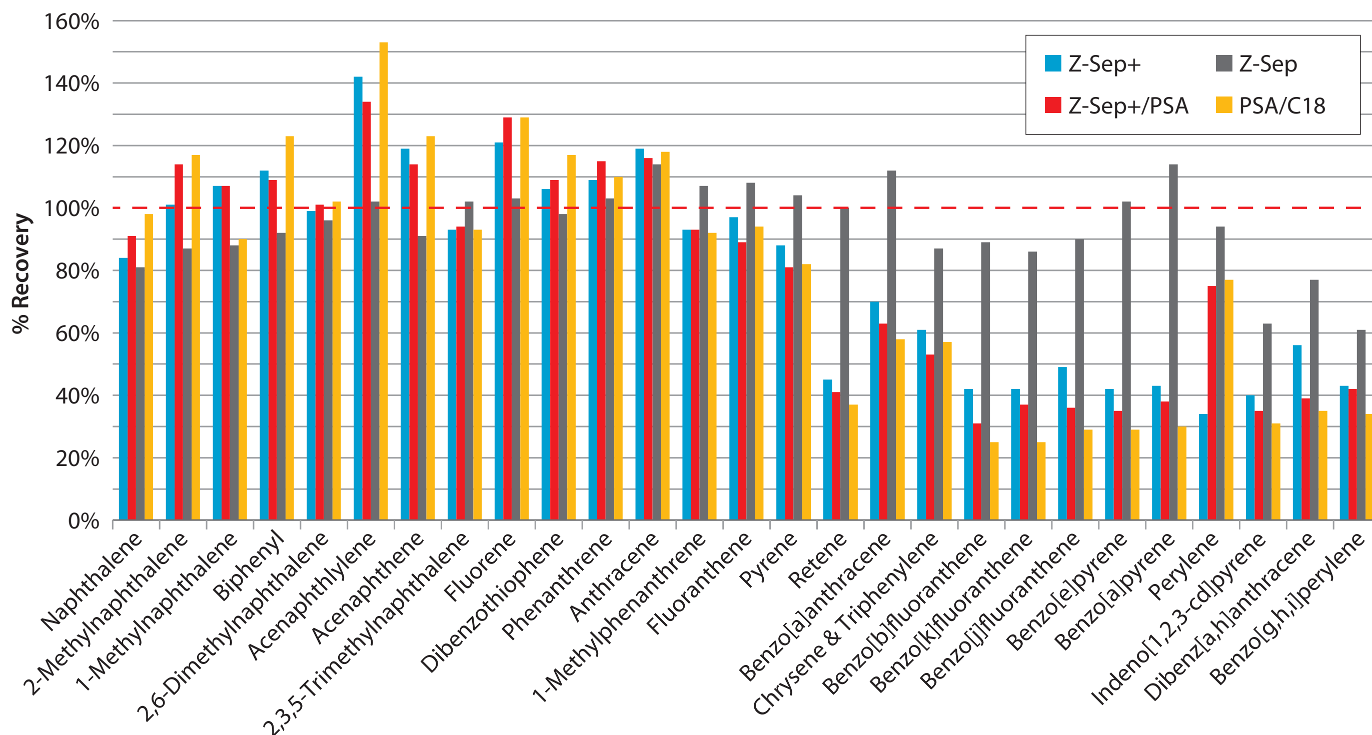


Figure 3. Average PAH Recoveries from Grilled Hamburger After Cleanup with Different Sorbents, Spike Level of 100 ng/g



Conclusions

A QuEChERS approach was effectively used to extract PAHs from grilled hamburger meat. For extract cleanup, it was found that sorbents containing C18, including Z-Sep+, gave reduced recoveries for PAHs with >4 rings in their structures. Z-Sep, a zirconia coated silica which does not contain C18, yielded the highest average recoveries. Z-Sep also produced extracts with the lowest GC-MS background. The SPB-608 column provided the selectivity necessary to resolve all but one isomeric set. The dimensions used allowed the GC run to be completed in <20 minutes.

References

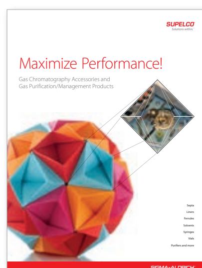
1. Wenzl, T., Simon, R.,Kleiner, J., Anklam, E., Analytical methods for Polycyclic Aromatic Hydrocarbons (PAHs) in Food and the Environment Needed For New Food Legislation in the European Union. *Trends Anal. Chem.*, **2006**, *25(7)*, 716-725.
2. National Cancer institute website: www.cancer.gov/cancertopics/factsheet/Risk/cooked-meats. (accessed 8/28/2013)
3. *EU Commission Regulation No. 835/2011*; Official Journal of the European Union; August 20, 2011.

Featured Products

Description	Cat. No.
Supel QuE QuEChERS Products	
Supel QuE Z-Sep Tube, 500 mg/12 mL, 50 ea	55403-U
Supel QuE Z-Sep+ Tube, 500 mg/12 mL, 50 ea	55296-U
Supel QuE Non-Buffered Tube 1, 12 mL, 50 ea	55294-U
Capillary GC Column	
SPB-608, 20 m x 0.18 mm I.D., 0.18 µm	custom
Analytical Solvent	
Acetonitrile CHROMASOLV®, gradient grade for HPLC	34851

Related Information

To learn more about Supel QuE QuEChERS products, visit
sigma-aldrich.com/food-pesticides



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Analysis of Enantiomers in Caraway Essential Oils

Using Astec® CHIRALDEX® G-TA Capillary GC Columns

Leonard M. Sidisky, R&D Manager
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Introduction

D-carvone and L-carvone are enantiomeric compounds that have very different smells. These mirror image compounds provide very different aroma notes. D-carvone is the major compound found in caraway seed essential oil, and a large component of dill seed essential oil. L-carvone is one of the major compounds found in spearmint essential oils. Resolving the enantiomers of carvone is a difficult application that requires the use of a chiral capillary GC column to perform the separation. In this study we demonstrate the separation of D- and L-carvone enantiomers in samples of caraway seed, dill seed, native spearmint and scotch spearmint essential oils using an Astec CHIRALDEX G-TA capillary GC column.

The Astec CHIRALDEX G-TA is a 2,6-di-o-pentyl-3-trifluoroacetyl derivatized gamma cyclodextrin capillary GC column that has the highest selectivity for oxygen containing compounds like alcohols, ketones, acids, aldehydes and lactones. This stationary phase is the first choice of the Group 1 chiral stationary phases that typically utilize surface interactions as one of the primary separation mechanisms.

Experimental

A mixture of D- and L-carvone, with the L-enantiomer in excess concentration, was first analyzed on the Astec CHIRALDEX G-TA column to determine the elution pattern of the compounds. The GC analysis conditions are summarized in **Table 1**. Peak identification of the carvone enantiomers was done by injecting neat individual D- and L-carvone samples.

Table 1. GC Conditions

column:	Astec CHIRALDEX G-TA, 30 m x 0.25 mm I.D., 0.12 µm (73033AST)
oven:	40 °C (1 min), 2 °C/min to 170 °C (15 min)
inj. temp.:	250 °C
detector:	FID, 250 °C
carrier gas:	helium, 25 cm/sec (set @ 110 °C)
injection:	wet needle, 100:1 split

Caraway seed, dill seed, native spearmint and scotch spearmint essential oil samples were each evaluated as neat oils to determine their fingerprint profiles and to identify the native carvone enantiomer peak. Caraway seed and dill seed essential oils were then spiked with L-carvone, and both spearmint essential oils were then spiked with D-carvone. All four spiked samples were reevaluated to determine if the spiked enantiomer could be resolved from the natural occurring carvone enantiomer present in the oil.

Results and Discussion

Figure 1 demonstrates that the D-carvone enantiomer elutes before L-carvone. **Figures 2, 4, 6 and 8** show the chromatograms resulting from analysis of neat samples of caraway seed, dill seed, native spearmint and scotch spearmint essential oils. Spiked sample chromatograms are shown in **Figures 3, 5, 7 and 9**, and show that the spiked enantiomer is resolved and can be identified. This demonstrates the chiral selectivity of the Astec CHIRALDEX G-TA capillary GC column.

Figure 1. Standard Containing D- and L-Carvone

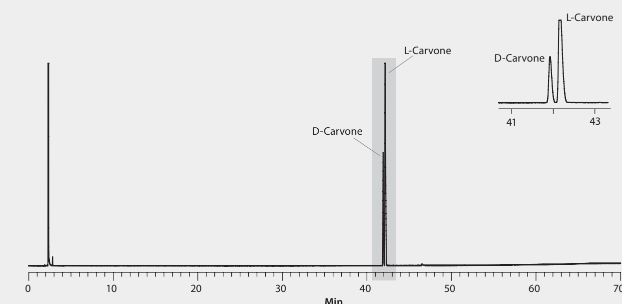


Figure 2. Caraway Seed Essential Oil

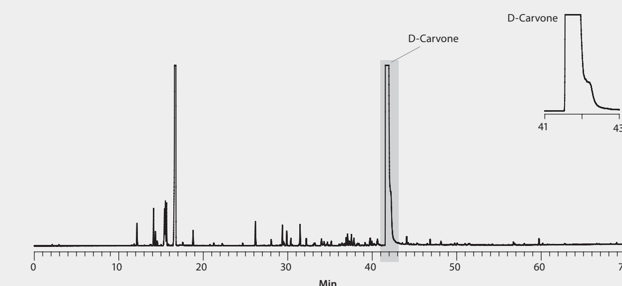


Figure 3. Caraway Seed Essential Oil (Spiked with L-Carvone)

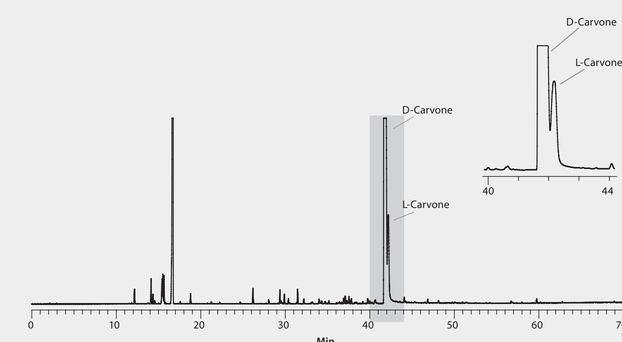


Figure 4. Dill Seed Essential Oil

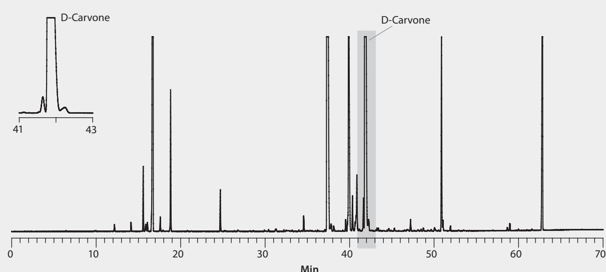


Figure 5. Dill Seed Essential Oil (Spiked with L-Carvone)

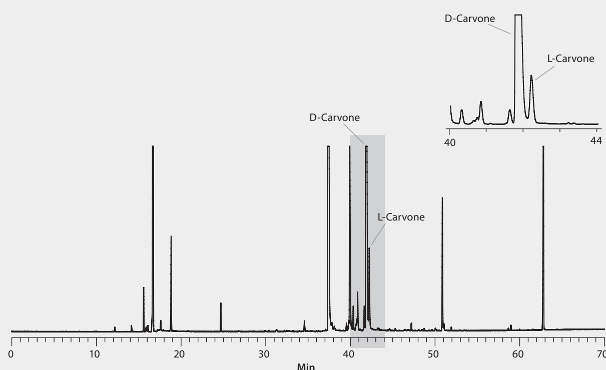


Figure 6. Native Spearmint Essential Oil

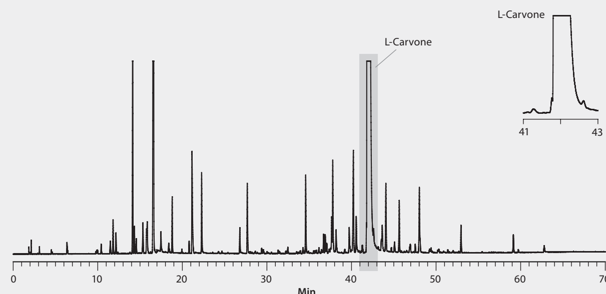


Figure 7. Native Spearmint Essential Oil (Spiked with D-Carvone)

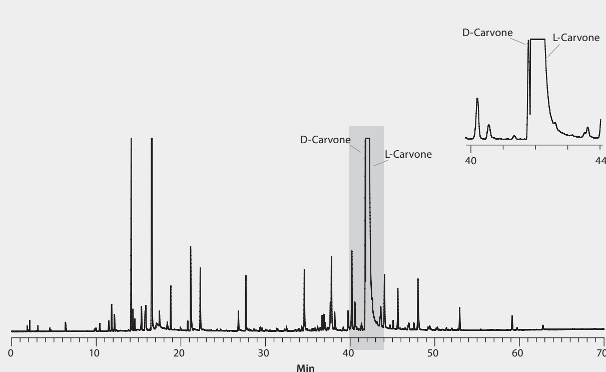


Figure 8. Scotch Spearmint Essential Oil

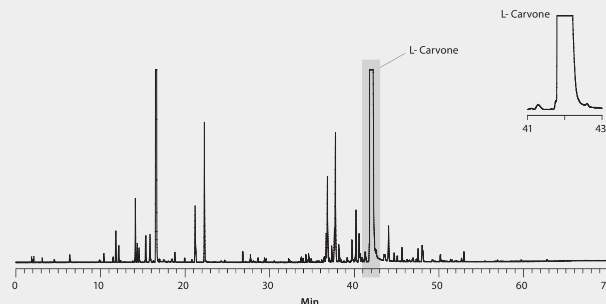
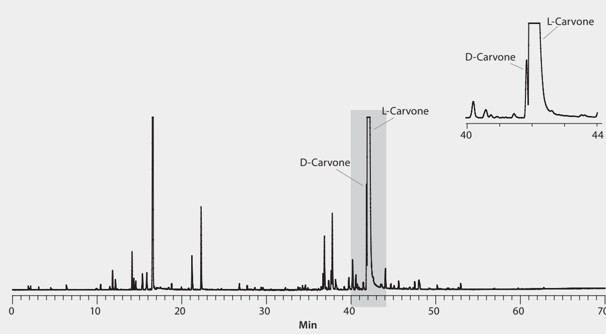


Figure 9. Scotch Spearmint Essential Oil (Spiked with D-Carvone)



Conclusion

The data presented here demonstrates the 30 m x 0.25 mm I.D., 0.12 µm Astec CHIRALDEX G-TA capillary GC column can be used to resolve D- and L-carvone enantiomers in neat essential oils with the D-carvone enantiomer eluting prior to the L-carvone enantiomer.

Reference

1. Ravid, U.; Putievsky, E.; Katzir, I.; Weinstein, V.; Ikan, R., Chiral GC analysis of (S)(+)- and (R)(-)-carvone with high enantiometric purity in caraway, dill and spearmint oils. *Flavour and Fragrance Journal* **1992**, *7*, 280-292.

Featured Products

Description	Cat. No.
Astec CHIRALDEX G-TA, 30 m x 0.25 mm I.D., 0.12 µm	73033AST
S-(+)-Carvone (D-Carvone), Analytical Standard	22070
R-(-)-Carvone (L-Carvone), Analytical Standard	22060

Related Product

Description	Cat. No.
Astec CHIRALDEX G-TA, 10 m x 0.25 mm I.D., 0.12 µm	73031AST
Astec CHIRALDEX G-TA, 20 m x 0.25 mm I.D., 0.12 µm	73032AST
Astec CHIRALDEX G-TA, 40 m x 0.25 mm I.D., 0.12 µm	73034AST
Astec CHIRALDEX G-TA, 50 m x 0.25 mm I.D., 0.12 µm	73035AST

Fast Extraction and Low Level Detection of Chloramphenicol in Shrimp

Using Z-Sep+ Sorbent with QuEChERS Sample Preparation and Analysis by Ascentis Express C18 LC-MS/MS

Emily Barrey, Senior R&D Scientist; Olga Shimelis, Principal R&D Scientist; Michael Halpenny, R&D Technician; and Jennifer Claus, Product Manager

jennifer.claus@sial.com

Introduction

Chloramphenicol is a broad spectrum antibiotic that has been determined as a causative agent of aplastic anemia and possible carcinogen in humans. These health concerns prompted a ban on the use of chloramphenicol in animal products including farmed fish and shrimps. As of today, a "zero" tolerance level has been established for this antibiotic; however, the drug is still widely available in developing countries, and no "safe" residue levels have been determined in food.[1] Therefore, a highly selective and sensitive analytical assay to control and monitor chloramphenicol residues in difficult matrices, such as food stuffs, is needed.

The QuEChERS (**Qu**ick, **E**asy, **Ch**eap, **E**fficient, **R**ugged and **S**afe) technique is rapidly becoming a universal sample preparation approach for a variety of analyte classes and a variety of matrices.[2-4] In this study, several different QuEChERS sorbents were used in the sample cleanup procedure of shrimp samples prior to chloramphenicol analysis. Analyte recovery, reproducibility and ionization effects were compared.

Experimental

Frozen shrimp samples were homogenized, and 1 gram samples were extracted with 9 mL of acetonitrile. Samples were shaken and centrifuged. The supernatant was decanted, 90 µL of formic acid was added and the solution was mixed with 500 mg of appropriate dispersive sorbent: Z-Sep+, Z-Sep, C18 or no cleanup sorbent. Samples were shaken and centrifuged, then 8 mL of supernatant was evaporated to dryness and reconstituted with water.

Multiple replicates of both unspiked and spiked extracts at 0.3 ppb were processed. All samples were analyzed by LC-MS/MS using negative electrospray ionization (ESI). Resulting samples were quantified with the use of solvent calibration standards and a matrix-matched calibration curve to identify matrix effects.

Results

Figures 1a and 1b demonstrate the differences in pigment removal for the various sorbents. In **Figure 1a**, the lack of color in the extracts shows that Z-Sep+, C18 and Z-Sep all effectively removed pigment from the shrimp matrix. Also, as seen in **Figure 1b**, samples that were extracted without the application of a cleanup sorbent produced precipitation over time. Therefore, the use of QuEChERS sorbents is essential for the removal of both interfering pigments as well as solid matrix components in shrimp samples.

Figure 1a. Final Shrimp Extracts Using QuEChERS Cleanup

Figure 1b. Final Shrimp Extracts Without QuEChERS Cleanup

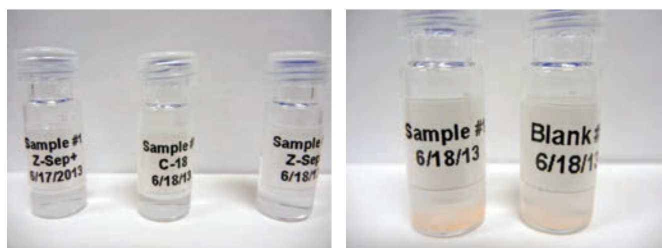


Figure 2 charts the comparison of analyte recovery and percent relative standard deviation (%RSD) using matrix matched calibration standards and external solvent calibration standards. As illustrated, the overall chloramphenicol recoveries were within an acceptable range (greater than 97%) for the variety of cleanup methods used. However, as indicated by the error bars, Z-Sep+ gave more reproducible results than the other dispersive sorbents.

Figure 2. Analyte Recovery and %RSD Using Matrix Matched Calibration Standards and Solvent Curve (n = 4)

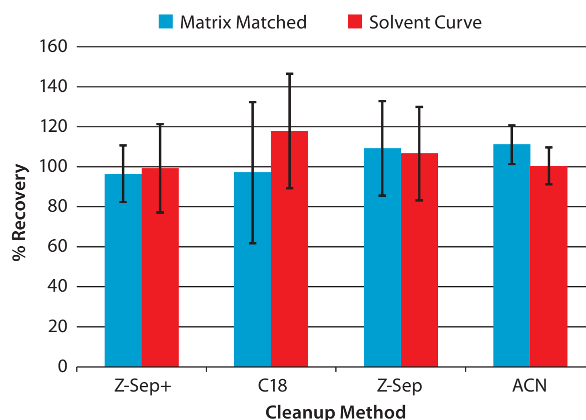


Figure 3 demonstrates the ionization effects for each cleanup procedure by deviation from the solvent calibration curve. Matrix suppression was evident for the acetonitrile extraction where QuEChERS cleanup was not performed. Matrix enhancement was observed for the C18 cleanup method. A chromatogram of a 0.3 ng/g chloramphenicol spiked shrimp sample is shown in **Figure 4**.

Figure 3. Matrix Ionization Effects by Deviation from Solvent Curve

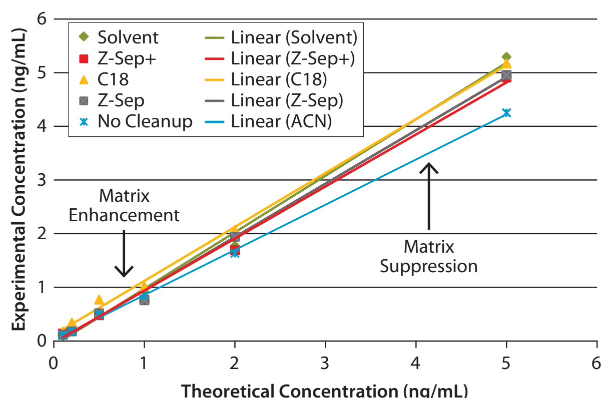
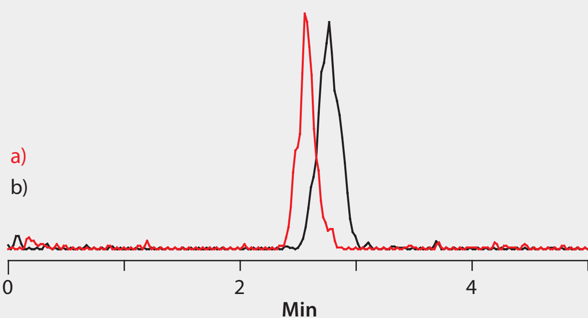


Figure 4. LC-MS/MS Extracted-Ion Chromatogram (XIC) for a) Chloramphenicol in Shrimp Extract (0.3 ng/g) and b) Internal Standard in Shrimp Extract (0.6 ng/g)

mobile phase: (A) water; (B) acetonitrile
gradient: 15% B for 0.1 min, 15–80% in 1.9 min, held at 80% for 1.5 min, 80–15% in 0.5 min, at 15% for 3 min
flow rate: 0.5 mL/min
pressure: 190 bar
detector: MS/MS, ESI(+), MRM, 320.9/151.9, 326/157
injection: 10 µL
sample: chloramphenicol or internal standard spiked at 0.3 ng/g in shrimp extract



Conclusion

A highly selective and sensitive analytical method was developed for chloramphenicol using a QuEChERS type sample preparation approach and LC-MS/MS detection. Samples extracted using the QuEChERS cleanup sorbents gave no ion suppression or sample precipitation over time. The QuEChERS cleanup using the Z-Sep+ sorbent produced acceptable recoveries and provided more reproducible results in comparison to that using Z-Sep or C18 sorbents. Sufficient resolution and retention was achieved using a Fused-Core® Ascentis® Express C18 column with a very short run time. Therefore, the combination of the new cleanup sorbent Supel™ QuE Z-Sep+, Fused-Core technology and LC-MS/MS detection provided a fast and sensitive method for the low level detection of chloramphenicol in shrimp.

References

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2. Lehotay, S. J. Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) Approach for the Determination of Pesticide Residues. Proceedings AOAC Annual Meeting, St. Louis, MO USA, 2004.
3. AOAC Official Method 2007.01, Pesticide Residues in Foods by Acetonitrile Extraction and Partitioning with Magnesium Sulfate.
4. EN15662:2008, Foods of plant origin - Determination of pesticide residues using GC-MS and/or LC-MS/MS following acetonitrile extraction/partitioning and cleanup by dispersive SPE - QuEChERS-method.

Featured Products

Description	Cat. No.
Supel QuE QuEChERS Products	
Empty Centrifuge Tube, 50 mL, 50 ea	55248-U
Z-Sep+ Cleanup Tube, 12 mL, 50 ea	55296-U
Z-Sep Cleanup Tube, 12 mL, 50 ea	55403-U
Discovery® DSC18, bulk sorbent, 200 g	52600-U
Ascentis Express HPLC Column	
C18, 5 cm x 2.1 mm I.D., 2.7 µm particles	53822-U
Analytical Solvents	
Acetonitrile, LC-MS CHROMASOLV®, >99.9%	34967
Water, LC-MS CHROMASOLV, >99.9%	39253
Analytical Standards	
Chloramphenicol, ≥98%	C-0378
DL-*threo*-Chloramphenicol-d_5, ≥97.0%	41724

Related Products

Description	Cat. No.
Supel QuE QuEChERS Products	
Citrate Extraction Tube, 12 mL, 50 ea	55227-U
Acetate Extraction Tube, 12 mL, 50 ea	55234-U
Z-Sep/C18 Cleanup Tube, 12 mL, 50 ea	55401-U
Ascentis Express HPLC Column	
C18, 3 cm x 2.1 mm I.D., 2.7 µm particles	53802-U
C18, 10 cm x 2.1 mm I.D., 2.7 µm particles	53823-U
Vials	
Certified Vial Kit, Low Adsorption (LA), 2 mL	29652-U

Related Information

To learn more about Supel QuE QuEChERS products, visit
sigma-aldrich.com/quechers

To learn more about Ascentis Express HPLC columns, visit
sigma-aldrich.com/express

To learn more about analytical standards, visit
sigma-aldrich.com/standards

To learn more about analytical reagents and solvents, visit
sigma-aldrich.com/analytical_reagents

Selective Extraction and Cleanup of Patulin Mycotoxin from Apple Juice Samples

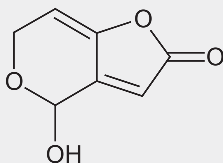
Using Molecularly Imprinted Polymer SPE and Ascentis Express HPLC-UV Analysis

Olga Shimelis, Principal R&D Scientist; K. G. Espenschied, R&D Technician; and Jennifer Claus, Product Manager
jennifer.claus@sial.com

Introduction

Patulin (**Figure 1**) is a mycotoxin produced by a number of fungal species. The mold Penicillium Expansum, in particular, is known as the main source of patulin contamination.[1] Patulin occurrence is found most often in rotting apples and products made from spoiled apples. Included among these products is apple juice and apple puree.[2]

Figure 1. Structure of Patulin



Because studies have shown that patulin possesses immunotoxic and genotoxic properties, regulatory limits for patulin have been set in 50 countries, including USA and European Union. In Europe, the maximum limit recommended for patulin in fruit juices is 50 µg/kg. The maximum patulin level permitted by the US FDA in apple juice is 50 µg/L.[2-4]

Historically, analytical methods for patulin have employed liquid-liquid extraction (LLE) followed by HPLC separation with UV detection at 276 nm.[1,5] Researchers have highlighted problems with these methodologies, including:

- Tedious sample preparation associated with liquid-liquid extraction
- Patulin instability in alkaline conditions resulting from sodium carbonate cleanup
- The requirement of extra cleanup or chromatographic method development to prevent the coelution of patulin and interfering matrix component 5-hydroxymethylfurfural (HMF).[1,6]

Therefore, a quick, simple and robust sample preparation method for patulin analysis is needed.

Molecularly Imprinted Polymers

SupelMIP® SPE – Patulin, a sample cleanup product used in this application, is made using the technology of molecularly imprinted polymers (MIPs). During MIP synthesis, a polymerization takes place around a template molecule that is similar to the analyte of interest. This template guides the formation of specific cavities within the polymer that are sterically and chemically complimentary to the target analyte. The subsequent removal of the template results in a polymer that is selectively retentive for the specific analyte of interest.

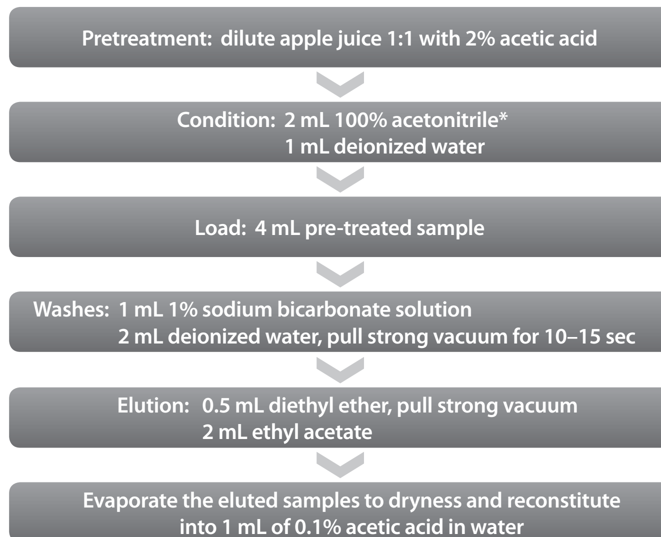
During sample preparation, the MIP phase extracts the target analyte with a high degree of selectivity. The use of vigorous washes during the SPE procedure results in superior cleanup, producing a highly pure, concentrated sample for analysis.

In this application, the use of SupelMIP SPE – Patulin is demonstrated for the cleanup of patulin in apple juice. Please note that other apple-containing products and matrices can be analyzed using similar methodology.

Experimental

The extraction process for patulin from apple juice is described in **Figure 2**. The SPE drop rate was controlled at 1–2 drops per second. The experiment was done with a replicate of 6 apple juice spikes. Analysis was performed by HPLC-UV with an Ascentis® Express C18 column. Recovery was calculated and corrected for patulin found in the unspiked apple juice.

Figure 2. SPE Procedure for Patulin Extraction and Cleanup



*Flow rates were carefully maintained at 1-2 drops/second throughout the method

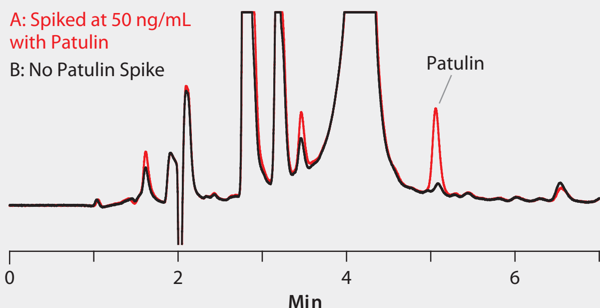
Results and Discussion

This MIP phase SPE procedure yielded high analyte recovery with excellent reproducibility. The average recovery of patulin was calculated to be 84% with a relative standard deviation (RSD) of 2%.

As seen in **Figure 3**, chromatographic analysis showed that no direct interferences with patulin detection were observed. Unlike LLE procedures, the cleanup procedure using SupelMIP SPE – Patulin successfully removed HMF and other common interfering components from the final extract.

Figure 3. Chromatograms of Apple Juice after SPE Cleanup

column: Ascentis Express C18, 15 cm x 2.1 mm, 2.7 µm particles (53825-U)
mobile phase: (A) 95:5 water:acetonitrile (B) 100% acetonitrile
gradient: hold at 100% A for 6 min; 0% to 80% B in 0.1 min; hold at 80% B for 3 min, 80% to 0% B in 0.1 min, hold at 100% A for 13 min
flow rate: 0.2 mL/min
temp.: 30 °C
detector: UV, 276 nm
injection: 10 µL



The procedure provided a stable solution for patulin analysis. Although the sodium bicarbonate wash was necessary to perform during the SPE protocol, the final extract was acidified, contributing to patulin stability during analysis.

Due to the nature of the MIP phase extraction procedure, a highly concentrated patulin sample was produced for LC-UV analysis. As a result, patulin was easily detectable in apple juice at concentrations of 50 ng/mL. A concentration of 16 ng/mL of incurred patulin was quantified in the blank sample.

Conclusion

The study successfully demonstrated the use of SupelMIP SPE – Patulin for the cleanup and pre-concentration of patulin from apple juice. By selectively extracting patulin with the use of molecularly imprinted polymers and effectively removing interfering matrix compounds during the SPE washing procedure, HPLC-UV analysis of patulin was made possible. The MIP procedure also allowed trace amounts of patulin to be easily detected in apple juice with high analyte recovery and reproducibility. In conclusion, the use of SupelMIP SPE – Patulin for sample cleanup eliminates the aforementioned problems associated with liquid-liquid extraction and provides a fast, easy and reliable sample preparation method for patulin analysis in apple juice.

References

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3. Commision Regulation (EC) 1881/2006. *Official Journal of Europian Union*, **2006**, *L364*, 5-24.
4. *Apple Juice, Apple Juice Concentrates and Apple Juice Products - Adulteration with Patulin*; FDA CPG Sec.510.150; Food and Drug Administration, issued 10/22/2001, updated 11/29/2005.
5. Roach, J. A. et al. HPLC Detection of Patulin in Apple Juice with GC/MS Confirmation of Patulin Identity *Adv. Exp. Med. Biol.*, **2002**, *504*, 135-140.
6. Sargenti, S. and Almeida, C.A.A. Determination of Patulin in Apple Juice by HPLC Using a Simple and Fast Sample Preparation Method *Ecl. Quím.*, **2010**, *35*, 14-21.

Featured Products

Description	Cat. No.
SupelMIP® SPE Product	
Patulin Tube, 3 mL, 50 ea	52776-U
Ascentis Express HPLC Column	
C18, 15 cm x 2.1 mm I.D., 2.7 µm particles	53825-U
Analytical Solvents	
Acetonitrile, CHROMASOLV® Plus, for HPLC, ≥99.9%	34998
Diethyl Ether, CHROMASOLV, for HPLC, ≥99.9%	309966
Ethyl Acetate, CHROMASOLV Plus, for HPLC, ≥99.9%	650528
Analytical Standards	
Patulin, 100 µg/mL in acetonitrile	34127-2ML

Related Products

Description	Cat. No.
Supel™ Tox SPE Cartridges	
AflaZea SPE Cartridge, 6 mL, 30 ea	55314-U
DON SPE Cartridge, 6 mL, 30 ea	55316-U
Tricho SPE Cartridge, 6 mL, 30 ea	55308-U
TrichoBind SPE Cartridge, LRC, 25 ea	55307-U
FumoniBind SPE Cartridge, LRC, 25 ea	55315-U
OchraBind SPE Cartridge, LRC, 25 ea	55318-U
Ascentis® Express C18 HPLC Columns	
10 cm x 2.1 mm I.D., 2.7 µm particle size	53823-U
Vials	
Certified Vial Kit, Low Adsorption (LA), 2 mL	29652-U

Related Information

To learn more about SupelMIP products, visit
sigma-aldrich.com/supelmip

To learn more about Ascentis Express HPLC columns, visit
sigma-aldrich.com/express

Did you know . . .

Supelco/Sigma-Aldrich has a line of SPE products to provide sample cleanup for mycotoxin analysis?

For more information, visit
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Method Optimization for LC-MS Analysis of Vitamin D Metabolite Critical Pairs in Serum

Using HybridSPE-Phospholipid, Ascentis Express, and Other LC-MS Workflow Components

Craig Aurand, Senior Application Chemist and Hugh Cramer, Associate Scientist
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The unique combination of column selectivity using Ascentis Express F5 and phospholipid removal using HybridSPE-PLus provides a robust and accurate LC-MS method for accurate and sensitive analysis of vitamin D metabolites, including isobaric critical pairs.

Introduction: Interest in Vitamin D

Analysis of vitamin D metabolites has continued to be a topic of interest in recent publications, primarily as biomarkers for possible disease states and vitamin deficiency. While vitamin D is present in two forms, vitamin D_3 and vitamin D_2, current ELISA methods demonstrate different cross-reactivities and cannot distinguish between D_2 and D_3 forms of the vitamin metabolites resulting in erroneous reporting of total 25-hydroxyvitamin D concentrations. Further, there is interest in an analytical means to differentiate the D_2 and D_3 forms from the D_2 and D_3 epimers because of their different degrees of bioactivity.

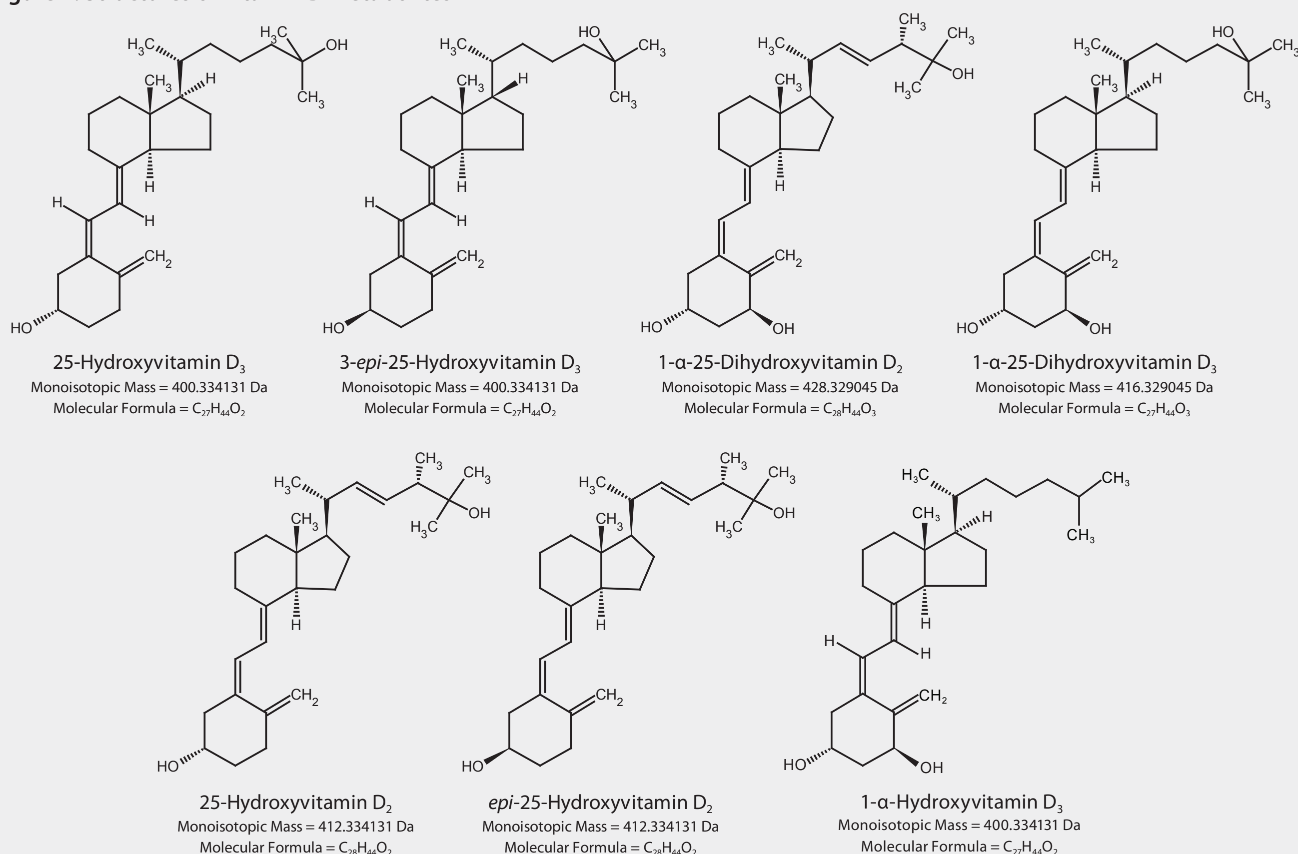
Study Aim and Overview

In this study, chromatographic screening studies for the analysis of vitamin D metabolites were expanded to include dihydroxy metabolites along with 25-*epi*-homologs (**Figure 1**). Screening consisted of evaluating various column stationary phases, organic modifiers and buffers for the resolution of critical pair isobaric compounds. The goal was to develop chromatographic conditions for the quantitation of hydroxy and dihydroxy vitamin D_2 and D_3 metabolites, including the isobaric epimers, from human serum samples. In addition to chromatographic method optimization, sample preparation techniques were evaluated to determine the impact of biological matrix on the sensitivity and accuracy of the LC-MS method.

Analytical Challenges with Vitamin D Metabolites

The particular difficulty in vitamin D metabolite analysis is the differentiation between the epimer isoform of the D_2 and D_3 metabolites. The epimers are isobaric to the D_2 and D_3 metabolites

Figure 1. Structures of Vitamin D Metabolites



rendering them indistinguishable in the MS and thus requiring chromatographic separation to resolve them.

Chromatographic Method Development

Traditional reversed-phase chemistries, e.g., C18, do not resolve the critical epimeric pairs of vitamin D metabolites. Therefore, the initial phase of chromatographic method development consisted of screening Ascentis Express cyano, phenyl hexyl and pentafluorophenyl (F5) stationary phases for the efficient resolution of 1-α-25-dihydroxyvitamin D_3, 1-α-25-dihydroxyvitamin D_2, 25-hydroxyvitamin D_3, 25-hydroxyvitamin D_2, 3-*epi*-25-hydroxyvitamin D_3, 3-*epi*-25-hydroxyvitamin D_2 and 1-α-hydroxyvitamin D_3. Gradient elution was conducted using several organic modifiers (**Table 1**). Initial organic modifier concentrations were adjusted to provide equivalent eluotropic strength.

The column screening criteria was used to determine which stationary phase/organic modifier combination produced the highest resolution of the critical isobaric pairs 25-hydroxyvitamin D_2 and *epi*-25-hydroxyvitamin D_2 (observed mass m/z 413.33) and 25-hydroxyvitamin D_3 and *epi*-25-hydroxyvitamin D_3 (observed mass m/z 401.33). **Table 2** presents the minimum resolution table of these isobaric pairs. Results indicated that methanol produced the highest resolution between isobaric pairs on all stationary phases compared to acetonitrile and ethanol. While both the cyano and F5 stationary phases demonstrated some resolution of the isobaric pairs, baseline resolution was only achieved on the F5.

Table 1. Gradient Screening Conditions for Analysis of Vitamin D Metabolites

column: Ascentis Express, various phases, 10 cm x 3.0 mm I.D., 2.7 µm particles
flow rate: 0.6 mL/min
column temp: 35 °C
injection: 5.0 µL
mobile phase: (A) 1% formic acid; (B) water; (C) as listed below

gradient acetonitrile (C): 10% A constant; 50% B, 40% C held for 1 min; to 0% B, 90% C, in 10 min; 0% B, 90% C, held for 3 min
gradient methanol (C): 10% A constant; 40% B, 50% C held for 1 min; to 0% B, 90% C, in 8 min; 0% B, 90% C, held for 3 min
gradient ethanol (C): 10% A constant; 50% B, 40% C held for 1 min; to 0% B, 90% C, in 10 min; 0% B, 90% C, held for 3 min

Table 2. Summary of Minimum Isobaric Resolution by Column Phase and Organic Modifier

Ascentis Express Column	Organic Modifier	Minimum Resolution Between Isobars
F5	Methanol	2
ES-CN	Methanol	1.2
F5	Acetonitrile	1.2
F5	Ethanol	1.1
ES-CN	Acetonitrile	0.8
ES-CN	Ethanol	0.5
Phenyl-Hexyl	Ethanol	0.3
Phenyl-Hexyl	Acetonitrile	0
Phenyl-Hexyl	Ethanol	0

Note: Minimum resolution is defined as resolution between two sets of isobaric compounds
25-hydroxyvitamin D_2 and *epi*-25-hydroxyvitamin D_2, 413.33 m/z
25-hydroxyvitamin D_3 and dpi-25-hydroxyvitamin D_3, 401.33 m/z

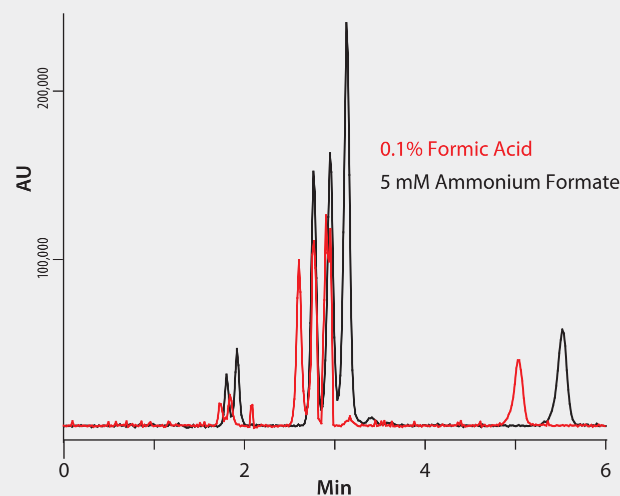
Sensitivity Optimization and Buffer Selection

The second phase of the study focused on conversion of the optimum screening method into an isocratic method that could ultimately be used for the analysis of human serum samples. The F5 column was selected based on its ability to provide the highest minimum resolution of isobaric pairs using methanol as the organic modifier in the mobile phase. At this stage, the impact of the buffer modifier was evaluated for the analyte responses. Because the vitamin D metabolites are neutral compounds, it was anticipated that the buffer would have minimal impact on selectivity, although it might impact ionization in the LC-MS source.

Figure 2 and **Table 3** demonstrate the impact of mobile phase buffer on the ionization of the vitamin D metabolites when a 300 ng/mL standard of vitamin D metabolites is injected onto the F5 column. **Figure 2** depicts the full scan comparison of analyte response differences between analysis conducted using the ammonium formate buffer and the formic acid modified mobile phase. As can be observed in **Table 3**, using ammonium formate as the mobile phase buffer nearly doubles the response of the vitamin D metabolites over the formic acid additive. The final optimized chromatographic method using ammonium formate buffer and the Ascentis Express F5 stationary phase is shown in **Figure 3**. Note that baseline resolution of isobaric vitamin D metabolites is achieved in less than 6 minutes.

Figure 2. Comparison of Buffer Effect on Vitamin D Metabolite Response on Ascentis Express F5

column: Ascentis Express F5, 15 cm x 2.1 mm I.D., 2.7 µm particles (53571-U)
mobile phase: buffer as listed, (A) water, (B) methanol, (25:75, A:B)
flow rate: 0.4 mL/min
temp: 40 °C
injection: 2.0 µL
detection: ESI+, 100-1000m/z
instrument: Agilent 1290, 6210 TOF



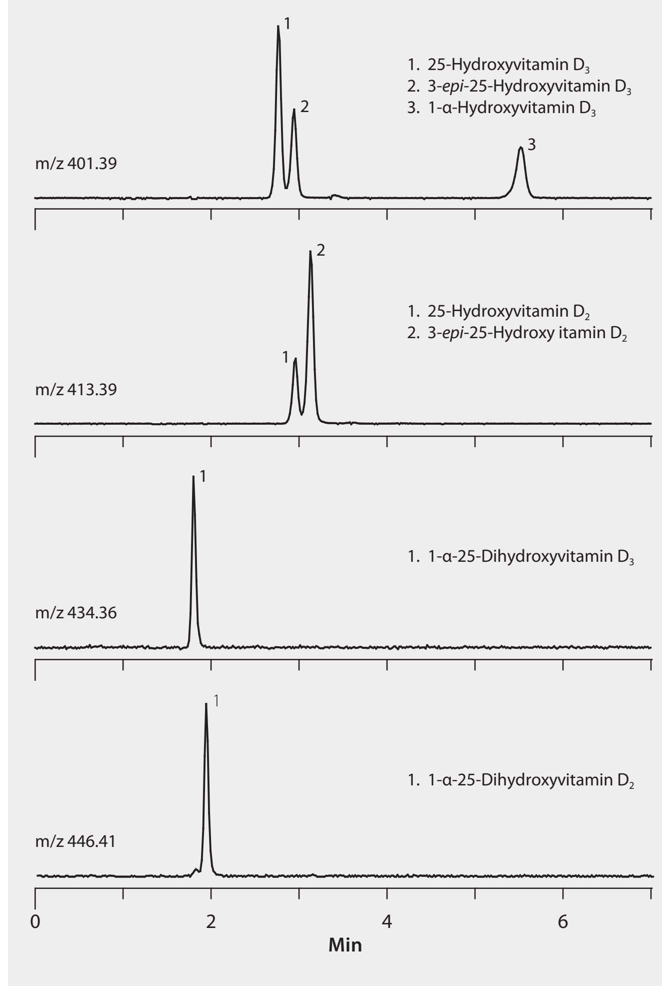
(continued on next page)

Table 3. Comparison of Buffer Effect on Vitamin D Metabolite Response on Ascentis Express F5

Analyte	5 mM Ammonium Formate Peak Area	0.1% Formic Acid Peak Area
1-α-25-Dihydroxyvitamin D_3	95414	25517
1-α-25-Dihydroxyvitamin D_2	181755	59337
25-Hydroxyvitamin D_3	659087	344920
3-*epi*-25-Hydroxyvitamin D_3	351227	178107
25-Hydroxyvitamin D_2	495520	238007
3-*epi*-25-Hydroxyvitamin D_2	1331699	724588
1-α-Hydroxyvitamin D_3	351885	234494

Figure 3. Separation of Vitamin D Metabolites on Ascentis Express F5

Conditions as in Figure 2.
5 mM ammonium formate



Comparison of Sample Prep Techniques to Reduce Serum Matrix Effect

The final phase of the study involved implementing the optimized chromatographic method for the analysis of spiked human serum samples. Particular attention was paid toward sample preparation and its impact on analytical results. Serum sample preparation requires protein precipitation with organic solvents or strong acids. This approach results in gross depletion of proteins from the sample, but leaves high levels of matrix interference from coextracted phospholipids. Coextracted phospholipids can cause quantitative irregularities and decrease sample throughput due to gradient column washing requirements.

Human serum samples were spiked with vitamin D metabolites and processed using two sample preparation techniques: standard protein precipitation alone and standard protein precipitation followed by phospholipid depletion with HybridSPE-PLus plates. (Note that protein precipitation can also be performed directly in the HybridSPE plates for a truly one-step sample prep method. However, users may prefer to carry out the protein precipitation as a separate step.) Results obtained from sample processed using the two sample preparation techniques were evaluated to determine analytical impact of phospholipid matrix interferences, both in terms of chromatographic overlap and impact on analyte response.

Sample Prep Methodologies

Human serum samples were spiked at 25 ng/mL with vitamin D metabolites. Protein precipitation was performed offline by adding 100 µL of spiked serum followed by 300 µL of 1% formic acid acetonitrile to a 96-well collection plate. Samples were thoroughly mixed by performing five 300 µL draw/dispense cycles using a digital pipette. Samples were then left to sit for 5 minutes before transferring 200 µL of the resulting supernatant into a HybridSPE-PLus 96-well plate. Samples were passed through the HybridSPE-PLus plate by applying 10" Hg vacuum for 4 minutes and analyzing the resulting filtrate directly. As a comparison, spiked human serum was also processed using standard protein precipitation alone by adding 100 µL of serum followed by 300 µL of 1% formic acid acetonitrile into 2 mL centrifuge vials. Samples were then vortexed and centrifuged, and the resulting supernatant was analyzed directly.

Improved Analyte Response after Phospholipid Removal

Figure 4 depicts the phospholipid selected ion chromatograms from samples processed via standard protein precipitation (red trace) and samples processed using the HybridSPE-PLus plate (black trace). A significant amount of phospholipid matrix from samples processed via protein precipitation alone was observed eluting in the 2–4 minute window where several vitamin D metabolites also elute. Conversely, samples processed by the HybridSPE-PLus plate displayed no detectable phospholipid matrix. Phospholipid co-elution with analytes of interest has the potential to cause sensitivity and reproducibility issues resulting in irregularities in quantitation. Table 4 compares analyte recovery of the two sample preparation techniques. The direct overlap in the elution windows of vitamin D metabolites and the phospholipid matrix interference for samples

processed with standard protein precipitation resulted in a 40% reduction in signal response for several of the metabolites. As a result, samples processed using the HybridSPE-PLus plate, which demonstrated no co-eluting matrix interference, demonstrated a higher analyte response.

Figure 4. Phospholipid Matrix Monitoring (selected ion)

Conditions as in Figure 2.
5 mM ammonium formate

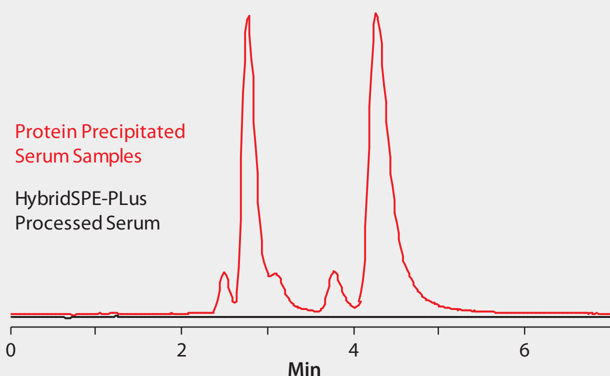


Table 4. Vitamin D Metabolite Recovery

Sample	HybridSPE-PLus Processed Serum 25 ng/mL Average n=16	Protein Precipitated Serum 25 ng/mL Average n=8
1-α-25-Dihydroxyvitamin D_3	21.1	17.1
1-α-25-Dihydroxyvitamin D_2	19.7	19.0
25-Hydroxyvitamin D_3	24.3	15.5
3-*epi*-25-Hydroxyvitamin D_3	21.3	15.3
25-Hydroxyvitamin D_2	29.8	21.4
3-*epi*-25-Hydroxyvitamin D_2	24.5	23.0
1-α-Hydroxyvitamin D_3	27.7	21.0

Summary

Chromatographic resolution still plays an important role in LC-MS applications when dealing with isobaric compounds. The unique selectivity of the Ascentis Express F5 column gave a fast and efficient analytical method for 25-hydroxyvitamin D and related forms from serum samples. In addition, phospholipid depletion using the HybridSPE-PLus 96-well plate enabled efficient sample cleanup increasing method reproducibility and accuracy. As demonstrated in this study, this novel sample prep technique coupled with the unique selectivity of the Ascentis Express F5 column enables a fast and simplified bioanalytical method for associated vitamin D metabolites. Likewise, this approach demonstrates how selectivity, in both chromatographic and sample preparation steps, allows for efficient analysis that would otherwise be unattainable with traditional reversed-phase approaches.

Featured Products

Description	Cat. No.
HPLC Column	
Ascentis Express F5, 15 cm x 2.1 mm, 2.7 µm particles	53571-U
Sample Prep Plates & Accessories	
HybridSPE-PLus 96-Well Plates, bed wt. 50 mg	575659-U
Round Well Cap Mat, Pierceable for HybridSPE-PLus	575680-U
96 Round/Deep-Well Collection Plate, polypropylene	Z717266
96 Well-Plate Pre-cut Sealing Films	Z721581
Supelco PlatePrep Vacuum Manifold	57192-U
Standards & Reagents	
Water LC-MS Ultra CHROMASOLV, tested for UHPLC-MS	14263
Methanol LC-MS Ultra CHROMASOLV, tested for UHPLC-MS	14262
Ammonium formate, eluent additive for LC-MS	70221
1-α-25-Dihydroxyvitamin D_3 (Cerilliant Certified Reference Material)	H-089
1-α-25-Dihydroxyvitamin D_2 (Cerilliant Certified Reference Material)	H-090
25-Hydroxyvitamin D_3 (Cerilliant Certified Reference Material)	H-083
25-Hydroxyvitamin D_2 (Cerilliant Certified Reference Material)	H-073
3-*epi*-25-Hydroxyvitamin D_3	705993
3-*epi*-25-Hydroxyvitamin D_2	753556
1-α-Hydroxyvitamin D_3	17946

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Developing Robust UHPLC Methods for Clinical, Forensic and Bioanalytical Samples

Using HybridSPE, Titan C18 UHPLC Columns and Other Products for the LC-MS Workflow

Craig Aurand, Senior Application Chemist and Jennifer Claus, Product Manager
craig.aurand@sial.com

HybridSPE-PLus plates remove endogenous phospholipids for consistently high analyte response compared to protein precipitation plates. Combined with Titan UHPLC columns, they provide a powerful analytical pair for robust UHPLC-MS of biological samples.

Introduction

As the limits of method speed, sensitivity and specificity are pushed using modern UHPLC systems and mass spectrometric detection, compromise of data quality and throughput from sample matrix contamination cannot be tolerated. Researchers and analysts looking for small molecule analytes in biological sample matrices face challenges from matrix interferences that can impact the robustness, accuracy and sample throughput of their analytical method.

Goal of the Study

The purpose of this study was to evaluate the improvement in method robustness when utilizing the HybridSPE®-PLus phospholipid depletion plate in a realistic bioanalytical application with a Titan UHPLC sub-2.0 µm particle column. The goal was to document control response variability and demonstrate the impact of the sample preparation technique on method performance when using the electrospray ionization (ESI+) source of the LC-MS system. Monitoring the control response is also useful for determining maintenance intervals for cleaning of the ESI source.

Rationale for Using MS Response Over Column Pressure to Assess Performance

Though monitoring column pressure is one common means of assessing the impact of sample cleanup technique on column performance, extracted sample matrix can also have a dramatic impact on performance of the electrospray ionization (ESI) source. Sample sprayed within the desolvation chamber causes deposition of sample matrix onto ionization source surfaces, potentially decreasing the efficiency of the source. This can be further compounded by ionizable species that compete with target analytes during the ionization process, thus forming a charge competition within the source. Problems associated with this type of matrix interference are further amplified as a result of concentration variability of the competing ionizable species within the sample population.

Experimental

Fluoxetine and tamoxifen (**Figure 1**) were chosen as controls to monitor ESI source changes throughout the spiked plasma sample series. Two 96-well sample prep methods were explored, protein precipitation plates and HybridSPE-PLus phospholipid depletion plates. Titan C18 UHPLC columns were used for efficient and rapid separation. Detection was via TOF-MS in ESI(+) mode. System performance was established initially, and then at intervals of 300, 600 and 1,000 injected samples. Plasma samples were first precipitated using 1% formic acid:acetonitrile at a ratio of 3:1 solvent to plasma. Samples were then vortex agitated and centrifuged for 4 minutes at 1,000 rpm. A 300 µL aliquot of the sample supernatant was applied to a 96-well plate, either protein precipitation (Seahorse 400 µL, 1.0 µm GF) or HybridSPE-PLus. (Note that protein precipitation can also be performed in the HybridSPE plates for a truly one-step sample prep method. However, users often prefer to carry out the protein precipitation as a separate step.) Vacuum was applied and the resulting eluent was collected in a 96-well collection plate and injected directly into the LC-MS system. Chromatographic conditions are listed in **Figure 2**.

Figure 1. Structure of Control Analytes

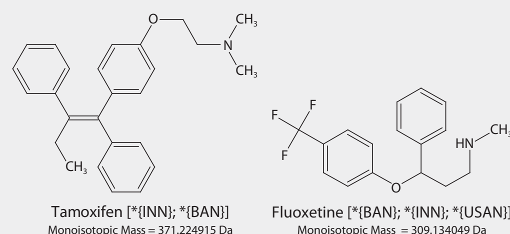
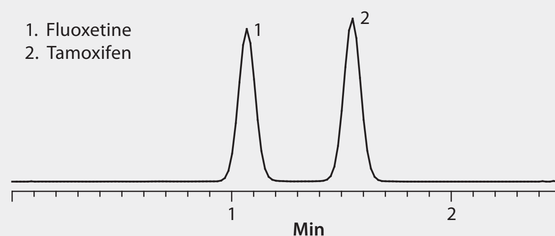


Figure 2. Gradient Analysis of Fluoxetine and Tamoxifen on Titan™ C18 1.9 µm

system: Agilent 1290, 6210 TOF/MS
column: Titan C18, 5 cm x 2.1 mm I.D., 1.9 µm particles (577122-U)
mobile phase: (A) 5 mM ammonium formate water
(B) 5 mM ammonium formate (90:10 acetonitrile:water)
gradient: 35% to 90% B in 1.5 min, hold 90% B for 0.5 min
flow rate: 0.4 mL/min
column temp.: 35 °C
injection: 1.0 µL
sample: 300 ng/mL
detection: ESI+, 100-1000m/z



Comparing Effectiveness of Sample Prep Methods

System pressure was consistent across the study, even with the protein precipitation plates, with less than 7 bar increase across the 1,000 sample series. This demonstrates the ability of the Titan UHPLC columns to stand up to bioanalytical samples, irrespective of the sample prep method. However, baseline and analyte response

was affected by the sample prep method. Column and system performance data are presented in **Figures 3 through 5**.

Improvement in Analyte Response

Using the HybridSPE-PLus plates, 300 ng/mL control response samples had a variability of less than 10% over the 1,000 samples (**Figure 3**). This consistency is due to the ability of the HybridSPE-PLus plate to deplete the phospholipid matrix from the samples that would otherwise interfere with analyte response.

In contrast, with the protein precipitation plates the response of the control samples dropped to less than 70 ng/mL by the 300 sample series (**Figure 4**). Tamoxifen control values continued to drop over the injection series, with a final value of less than 40 ng/mL at the 1,000 sample series. This dramatic drop in tamoxifen control response after only a few hundred samples would signal a need to halt the analysis, clean the source and recalibrate the instrument, dramatically impacting sample throughput. Although the control response for fluoxetine did not decrease, it was more erratic throughout the 1,000 sample series when compared to samples processed using the protein precipitation technique alone versus samples processed using the HybridSPE-PLus phospholipid depletion plate. This further exemplifies the need for sufficient sample preparation to avoid sample matrix interference which is both varied and compound dependent.

Figure 3. Control Response Over 1,000 Injections for Samples Processed with HybridSPE-PLus Phospholipid Depletion Plates

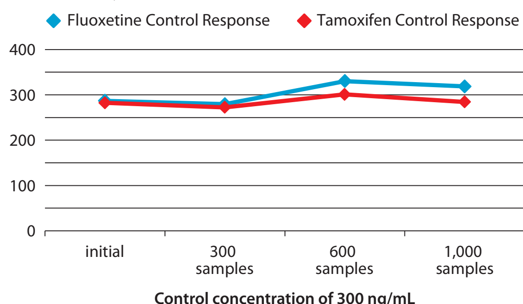
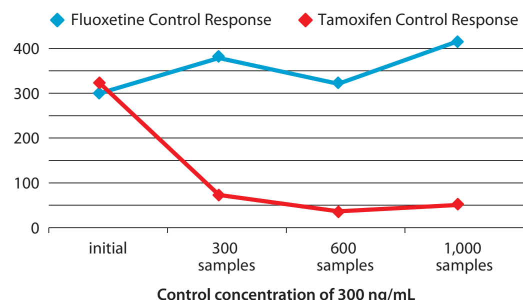


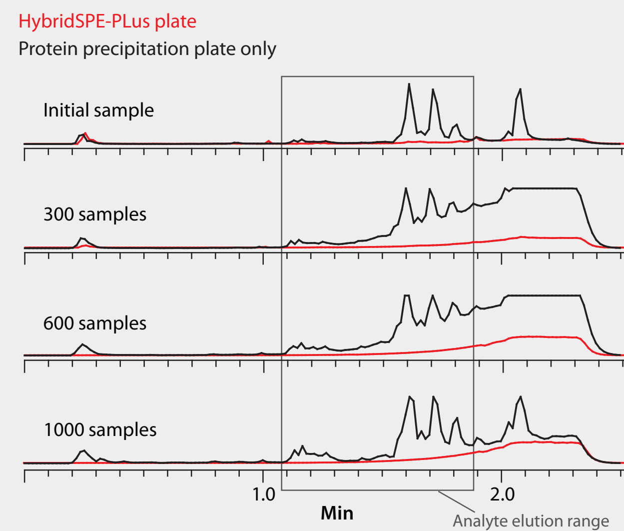
Figure 4. Control Response Over 1,000 Injections for Samples Processed with Protein Precipitation Alone



The results can be visualized in **Figure 5** which compares the monitoring of the phospholipid matrix after the HybridSPE-PLus and protein precipitation techniques across the 1,000 sample series. Samples processed using the HybridSPE-PLus plates presented a clean and consistent background over the 1,000 samples studied. However, the protein precipitation plates provided an initially well-defined elution region for several of the phospholipid species. As the number of injected samples increased, the total amount of phospholipid background continued to rise. Instead of a defined phospholipid peak, a broader total background increase was observed. As previously mentioned, a dramatic decrease in tamoxifen response was coincident with this phospholipid background increase over the 1,000 sample series for samples processed using only the protein precipitation technique.

Figure 5. Phospholipid Matrix for Samples Processed with Protein Precipitation Plates vs. HybridSPE-PLus Plates

Conditions as in Figure 2



Summary

This study demonstrates that monitoring column pressure is not always a sufficient assessment of analytical assay robustness. Rather, specifically for LC-MS applications, routine monitoring of control sample response provides an effective means for establishing the long-term accuracy of a method. Likewise, taking steps to ensure sufficient sample cleanup, not just particulate removal, can greatly aid in accurate sample assessment and decreased instrument downtime to improve sample throughput and assay robustness.

Featured Products

Description	Cat. No.
UHPLC Column	
Titan C18, 5 cm x 2.1 mm I.D., 1.9 µm particles	577122-U
Sample Prep Plates & Accessories	
HybridSPE-PLus 96-Well Plates, bed wt. 50 mg	575659-U
Round Well Cap Mat, Pierceable for HybridSPE-PLus	575680-U
96 Round/Deep-Well Collection Plate, polypropylene	Z717266
96 Well-Plate Pre-cut Sealing Films	Z721581
Supelco PlatePrep Vacuum Manifold	57192-U
Standards and Reagents	
Water LC-MS Ultra CHROMASOLV, tested for UHPLC-MS	14263
Acetonitrile LC-MS Ultra CHROMASOLV, tested for UHPLC-MS	14261
Ammonium formate, eluent additive for LC-MS	70221
Tamoxifen, analytical standard (Fluka)	85256
Fluoxetine, 1 mg/mL as free base in methanol (Cerilliant Certified Reference Material)	F-918

Online SPE/LC-MS Method for the Rapid Analysis of Thyroid Hormones in Plasma

Using Ascentis Express Fused-Core Columns for Trapping and Separation

Xiaoning Lu, Senior R&D Chemist and David Bell, Manager
Pharmaceutical and Bioanalytical Research
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The power of bonded phase selectivity in both sample prep and separation is demonstrated here. The Ascentis Express RP-Amide was shown to be a better trapping phase than conventional C8, and the Ascentis Express Phenyl-Hexyl phase provided baseline resolution of the closely-related compounds. The technique uses standard hardware and is a viable approach to solve other clinical and bioanalytical challenges.

Introduction

Thyroid hormones play critical roles in the regulation of biological processes such as growth, metabolism, protein synthesis and brain development. Specifically, thyroid hormones 3,3′,5,5′-tetraiodo-L-thyronine (thyroxine or T4) and 3,3′,5-triiodo-L-thyronine (triiodothyronine or T3) are essential for development and maintenance of normal physiological functions. For a clinical laboratory, measurements of total T4 and total T3, along with estimates of free T4 (FT4) and free T3 (FT3) are important for the diagnosis and monitoring of thyroid diseases. The physiological role and need for testing of 3,3´,5´-triiodo-L-thyronine (reverse triiodothyronine or rT3) is debated in clinical research circles.

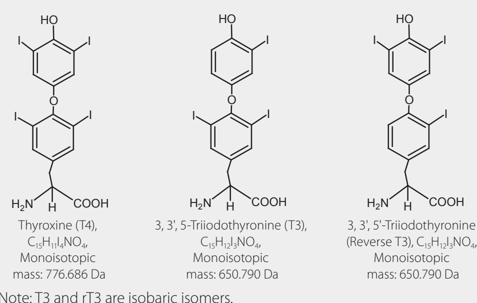
Analytical Challenges of Thyroid Hormone Measurement

Thyroid hormones are typically measured by an immunoassay technique that is subject to assay interference and easily perturbed by changes in protein levels that alter the free hormone availability.[1] Liquid chromatography with mass spectrometric detection (LC-MS) has been reported to offer superior specificity and speed over immunoassays for determination of thyroid hormones in biological matrices, such as serum, plasma and tissue. Nevertheless, the reported sample preparation procedures, typically by liquid-liquid extraction followed by solid phase extraction (SPE), involve multiple time-consuming steps, and are less compatible with automation.[2-3]

Study Goal and Overview of Approach

The present work exploited online SPE with LC-MS for rapid determination of T4, T3 and rT3 from plasma. Structures of these compounds appear in **Figure 1**. A 100 µL aliquot of rabbit plasma was spiked with T4, T3 and rT3. Protein was precipitated by addition of 25 µL $ZnCl_2$ and 200 µL methanol, vortex agitation for 1 minute and centrifugation at 9,000 *g* for 3 minutes. The resulting supernatant was collected and injected into the online SPE/LC-MS system described below. The LC-MS conditions are described in **Table 1**.

Figure 1. Chemical Structures of Thyroid Hormone Analytes



Note: T3 and rT3 are isobaric isomers.

Table 1. Online SPE LC-MS Method

instrument:	Shimadzu LCMS-8030
trapping column:	Ascentis Express RP-Amide (53514-U) or Ascentis Express C8 guard cartridges (53509-U), both 5 mm x 2.1 mm I.D., 2.7 µm particles
HPLC column:	Ascentis Express Phenyl, 5 cm x 2.1 mm I.D., 2.7 µm particles (53334-U)
mobile phase:	(A) water; (B) methanol, both with 0.1% acetic acid
column pressure:	1800 psi (88 bar)
injection vol:	5 µL
detection:	MS, ESI(+), MRM mode (777.7/731.8 for T4; 651.8/605.5 for T3 or rT3)

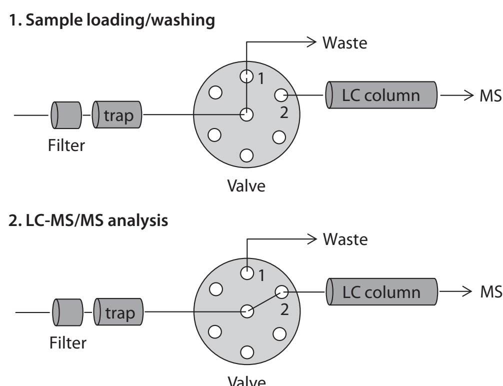
Online SPE/LC-MS Program

The online SPE/LC-MS system shown in **Figure 2** comprises a trapping column, a switching valve and an LC-MS instrument. The program consists of three distinct steps outlined in **Table 2**. The valve switches between position 1 for loading/washing (Step 1) and equilibration (Step 3), and position 2 for LC-MS analysis (Step 2). In Step 1 samples are loaded onto the trap and washed with mobile phase containing low percent organic and high flow rate to remove salts and other interferences which are directed to the waste. In Step 2 the analytes are eluted from the trap, separated and detected by the LC-MS at optimum flow for both chromatographic separation and MS signals. In Step 3, the system returns back to valve position 1 for re-equilibration under the sample loading/washing conditions.

Table 2. Online SPE/LC-MS Program

Final online SPE/LC-MS Program:	min	Flow (mL/min)	Valve	Pump B%
1. Sample loading/washing	0.00	0.4	1	5
	2.79	0.4	1	5
	2.80	0.25	1	5
2. LC-MS/MS	3.00	0.25	2	5
	5.00	0.25	2	70
	8.00	0.25	2	70
3. Equilibration	8.01	0.25	1	70
	8.09	0.25	1	70
	8.10	0.4	1	5
	12.0	0.4	1	5

Figure 2. Configuration of the LC-MS System and Valve Positions for Online SPE/LC-MS

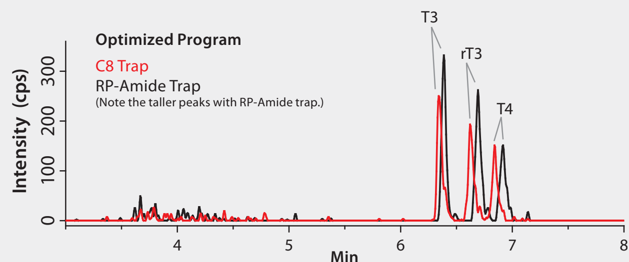


Choosing the Trapping Column

The choice of the right trapping column is critical for successful online SPE/LC-MS method development as it affects both trapping efficiency (e.g., the recovery of the analytes) and the downstream LC separation. As a rule of thumb, the trapping column should be less retentive than the analytical column to avoid possible peak broadening. Two guard cartridges (traps), Ascentis Express Fused-Core RP-Amide and C8, of the same dimensions were evaluated. As can be seen in **Figure 3**, both trapping columns led to comparable sharp peak shape (high separation efficiency). However, the RP-Amide delivered higher peak responses than the C8 trap under the trapping conditions with 5% methanol (top panel) as the washing solvent. Further increase of the methanol content to 20% in the washing solvent (**Table 3**) resulted in a 50% signal decrease on the C8 traps, but almost no change in signals with the RP-Amide traps (**Figure 3**, bottom panel). These results indicate better washing can be achieved with minimal sample loss with RP-Amide traps.

Figure 3. Comparison of the LC-MS Chromatograms with Online Trapping with C8 and RP-Amide Guard Cartridges

Online SPE/LC-MS conditions as in Tables 1 and 2.



Online SPE/LC-MS conditions as in Tables 1 and 3.

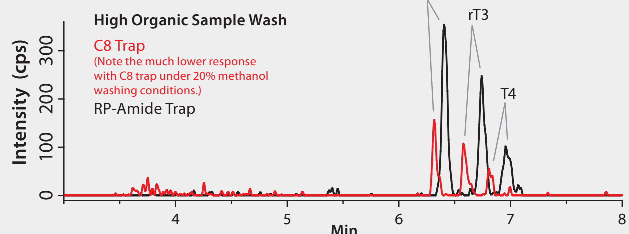


Table 3. Online SPE/LC-MS Conditions (high organic sample wash)

LC Program:	min	Flow (mL/min)	Valve	Pump B%
1. Sample loading/washing	0	0.4	1	5
	0.5	0.4	1	20
	2.8	0.25	1	20
2. LC-MS/MS	3.0	0.25	2	20
	5.0	0.25	2	70
	8.0	0.25	2	70
3. Equilibration	8.0	0.25	1	70
	8.1	0.4	1	5
	12.0	0.4	1	5

Summary

An online SPE/LC-MS method was developed and described here for the determination of the thyroid hormones T4, T3 and rT3 from plasma. The method uses an Ascentis Express RP-Amide cartridge to trap the analytes, and an Ascentis Express Phenyl-Hexyl column to resolve them. Compared to the commonly used C8 trap, the RP-Amide trap gave higher analyte signals and was compatible with 20% methanol washing. Another advantage of the RP-Amide traps is their compatibility with 100% aqueous mobile phases and samples. This is due to the hydrophilic nature of the amide-embedded phase, which makes it very useful for polar analytes.

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2. Taia, S.S.; Sniegoski, L.T.; Welch, M.J.; Candidate Reference Method for Total Thyroxine in Human Serum Use of Isotope-Dilution Liquid Chromatography-Mass Spectrometry with Electrospray Ionization. *Clinical Chemistry* **2002**; *48(4)*, 637-642.
3. Wang, D. and Stapleton, H.M.; Analysis of thyroid hormones in serum by liquid chromatography-tandem mass spectrometry. *Anal Bioanal Chem.* **2010**, *397(5)*, 1831-1839.

Featured Products

Description	Cat. No.
HPLC Column	
Ascentis Express Phenyl-Hexyl, 5 cm x 2.1 mm I.D., 2.7 µm particles	53334-U
Trap Columns	
Ascentis Express RP-Amide Guard Cartridge, 5 mm x 2.1 mm I.D., 2.7 µm particles	53514-U
Ascentis Express C8 Guard Cartridge, 5 mm x 2.1 mm I.D., 2.7 µm particles	53509-U
Accessories	
Ascentis Express Guard Cartridge Holder	53500-U
OPTI-SOLV EXP® Pre-Column Filter Cartridges	51166-U
EXP® Pre-Column Filter holder with EXP titanium hybrid ferrule	51163-U
Standards & Reagents	
Water LC-MS Ultra CHROMASOLV, tested for UHPLC-MS	14263
Methanol LC-MS Ultra CHROMASOLV, tested for UHPLC-MS	14262
Acetic acid, eluent additive for LC-MS	49199
L-Thyroxine (T4), 100 µg/mL (Cerilliant Certified Reference Material)	T-073
3,3',5-Triiodo-L-thyronine (T3); 100 µg/mL (Cerilliant Certified Reference Material)	T-074
3,3',5'-Triiodo-L-thyronine (Reverse T3); 100 µg/mL (Cerilliant Certified Reference Material)	T-075

Extraction and Analysis of Neonicotinoid Pesticides from Flower Blossoms

Using Supel QuE and Ascentis Express

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Neonicotinoids are systemic insecticides that are toxic to insects but do not affect mammals. They are water soluble and have the ability to migrate from treated soil into plant tissue, including fluids such as the nectar in flowers. These characteristics make them very useful for crop protection.[1] One neonicotinoid, imidacloprid, is currently the most widely used pesticide in the world.[2] The advent of the die-off of honey bees due to colony collapse disorder (CCD) has spawned investigation into pesticide exposure as the cause. Recent studies have indicated neonicotinoid pesticides as having detrimental effects on the brain cells of honey bees.[3] Bees can come into contact with these pesticides in several ways. Seeds that are treated with these pesticides can generate contaminated dust when using equipment that incorporates air for planting.[4] Also, some crops directly pollinated by honeybees such as cantaloupe and cucumber are treated with these pesticides.[5] Recently the European Union adopted a regulation restricting the use of three of these pesticides: clothianidin, thiamethoxam and imidacloprid.[6]

In this application, the **Qu**ick, **E**asy, **Ch**eap, **E**ffective, **R**ugged, **S**afe (QuEChERS) approach was used to develop an extraction and cleanup method for the analysis of seven neonicotinoid pesticides in flower blossoms. Analysis of the final extracts was done by LC-MS/MS on an Ascentis® Express C18 column. This column was chosen due to the high efficiency associated with its Fused-Core® particle structure, and its relatively low backpressure as compared to sub-2 micron UHPLC columns. This allowed the column to be used in a standard pressure HPLC system with a relatively high concentration of aqueous at the start of the mobile phase gradient.

Experimental

The blossoms used represent several types pollinated by honeybees: dandelion, sweet cherry and crab apple. The blossoms were picked fresh and then frozen prior to use. Prior to extraction, they were pulverized and spiked at 50 ng/g with a mixture of neonicotinoid pesticides. After 1 hour of wait time, extraction and cleanup proceeded as described in **Table 1**. Three replicate spikes and one unspiked sample were processed for each blossom type.

LC-MS/MS Analysis was performed using the conditions in **Table 2**. The MRMs used for each pesticide are listed in **Table 3**. Quantitation was performed against a matrix-matched calibration curve. Separate curves were prepared for each blossom type.

Results and Discussion

For QuEChERS extraction, sodium acetate (NAOAc) was chosen as the salt based on previously published findings.[5] For cleanup, several different sorbents were evaluated. It was found that PSA/C18 yielded the best recoveries while still reducing pigmentation in the sample extract. PSA/C18/ENVI-Carb™ yielded the cleanest extracts (no pigmentation), but very poor recovery for all the target compounds. The zirconia-based sorbents Z-Sep and Z-Sep+ were found to be incompatible with these pesticides, yielding very low recoveries. PSA alone yielded an extract with more pigmentation than PSA/C18, and recoveries were very poor.

Table 1. Extraction and Cleanup Procedure

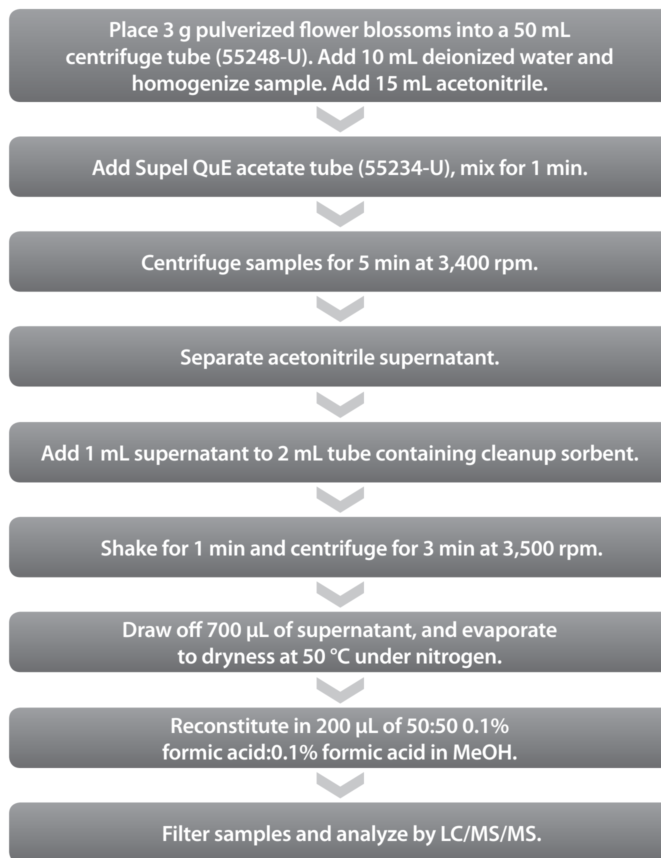


Table 2. LC-MS/MS Analysis Conditions

column:	Ascentis Express C18, 10 cm x 3.0 mm I.D., 2.7 µm (53814-U)
mobile phase:	(A) 0.1% formic acid in water; (B) 0.1% formic acid in methanol
gradient:	30% B from 0 to 5 min; to 100% B in 0.2 min; held at 100% B for 5.3 min; to 30% B in 0.5 min; held at 30% B for 5 min
flow rate:	0.5 mL/min
column temp.:	ambient
detector:	MS, ESI(+), MRM, see Table 3
injection:	3 µL

Table 3. MRMs Used

Name	m/z
Acetamiprid	223.2/126.0
Clothianidin	250.0/132.0
Dinotefuran	203.2/129.2
Imadicloprid	256.0/175.2
Nitenpyram	271.2/225.0
Thiacloprid	253.0/125.8
Thiamethoxam	292.1/211.0

A summary of the recoveries from the three blossom types obtained after extraction and PSA/C18 cleanup is summarized in **Table 4**. None of the target pesticides were detected in the unspiked blossoms. Excellent recoveries and low %RSD values were obtained for each target compound for all blossom types. The extract color after cleanup appeared slightly yellow for the dandelion and crab apple, and pale green for the cherry, and all were significantly lighter in color than uncleaned extract. The TICs appeared very clean for dandelion and crab apple (**Figures 1 and 2**). The cherry had the most matrix present (**Figure 3**), although it did not interfere with quantitation. The use of blossom specific matrix-matched standards compensated for any additional ion suppression that may have occurred.

Table 4. Average Percent Recoveries from Flower Blossoms Spiked at 50 ng/g, QuEChERS Extraction and Cleanup with PSA/C18.

n=3	Dandelion	Sweet Cherry	Crab Apple
Acetamiprid	99 (1)	96 (2)	105 (4)
Clothianidin	99 (6)	106 (2)	106 (7)
Dinotefuran	101 (2)	103 (4)	89 (4)
Imadicloprid	93 (4)	97 (4)	90 (3)
Nitenpyram	105 (1)	107 (2)	97 (6)
Thiacloprid	94 (2)	99 (1)	104 (2)
Thiamethoxam	98 (1)	99 (3)	93 (8)

Figure 1. TIC of Dandelion Extract, Spiked at 50 ng/g Pesticides; PSA/C18 Cleanup

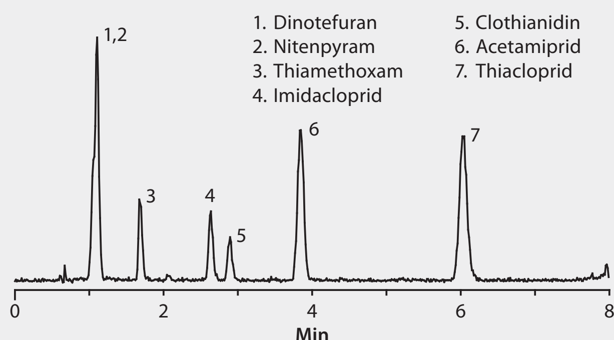


Figure 2. TIC of Crab Apple Extract, Spiked at 50 ng/g Pesticides, PSA/C18 Cleanup

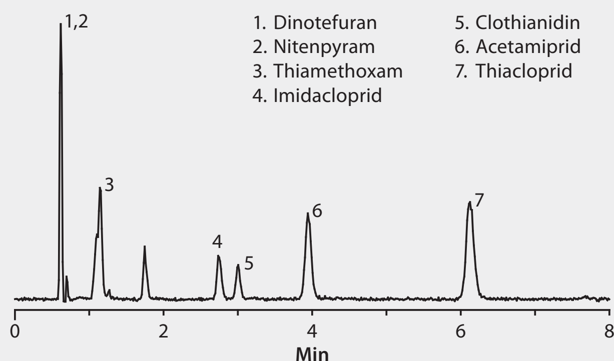
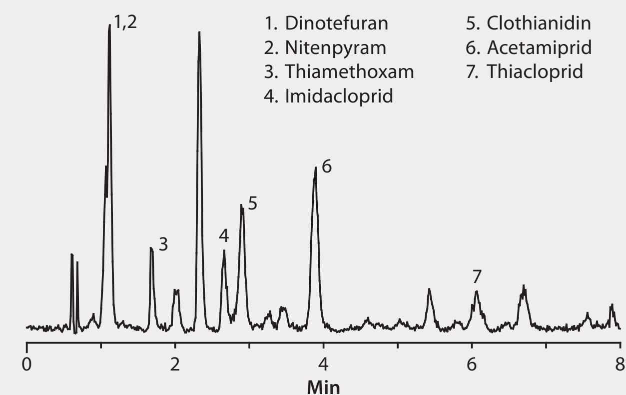


Figure 3. TIC of Sweet Cherry Blossom Extract, Spiked at 50 ng/g Pesticides, PSA/C18 Cleanup



Conclusions

The QuEChERS approach can be used in the extraction of neonicotinoid pesticides from various species of flower blossoms. For sample cleanup, this same approach can also be used. Of the various cleanup sorbents evaluated, PSA/C18 was found to yield the best recoveries for these pesticides, while providing adequate matrix removal. The Ascentis Express C18 column provided for an efficient separation with backpressure compatible with a standard HPLC system.

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6. *EU Commission Regulation No. 485/2013*; Official Journal of the European Union; May 5, 2013.

Featured Products

Description	Cat. No.
Centrifuge Tube, 50 mL	55248-U
Acetonitrile, CHROMASOLV® Plus for HPLC	34998
Formic Acid, Eluent Additive for LC-MS	14265
Methanol with 0.1% Formic Acid, LC-MS CHROMASOLV	34671
Supel™ QuE Acetate Tube, 12 mL, pk. of 50	55234-U
Supel QuE PSA/C18 Tube, 2 mL, pk. of 100	55288-U
Ascentis Express C18 Column, 10 cm x 3.0 mm, 2.7 µm	53814-U
Low Adsorption QSertVial, 300 µL, with PTFE/silicone septa (w/slit)	29662-U
Acetamiprid, PESTANAL®	33674
Thiacloprid, PESTANAL	37905
Imidacloprid, PESTANAL	37894
Clothianidin, PESTANAL	33589
Nitenpyram, PESTANAL	46077
Dinotefuran, PESTANAL	32499
Thiamethoxam, PESTANAL	37924

Simple Collection and 5-minute UHPLC Assay for 11 Isocyanates

Using ASSET EZ4-NCO Samplers and Titan C18 UHPLC Columns

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wayne.way@sial.com

Isocyanates are highly reactive, low molecular weight chemicals. They are widely used in the manufacture of flexible and rigid foams, fibers, coatings such as paints and varnishes, and elastomers. Spray-on polyurethane products containing isocyanates have been developed for a wide range of retail, commercial and industrial uses to protect cement, wood, fiberglass, steel and aluminum, including protective coatings for truck beds, trailers, boats, foundations and decks.

Isocyanates are powerful irritants to the mucous membranes of the eyes and gastrointestinal and respiratory tracts. Isocyanates can also sensitize workers, making them subject to severe asthma attacks if they are exposed again. Preventing exposure to isocyanates is a critical step in eliminating the health hazard. Engineering controls such as closed systems and ventilation should be the principal method for minimizing isocyanate exposure in the workplace. Other controls, such as worker isolation and personal protective clothing and equipment may also be necessary. Monitoring exposure is equally critical in the health and well-being of workers.

With that in mind, a simple collection tube for personal monitoring and associated analytical assay has been developed to support the industrial hygiene market. The ASSET™ EZ4-NCO Dry Sampler (**Figure 1**) for isocyanates is safe for the worker to use for personal sampling because it requires no field addition of reagents, no field extraction and no special storage requirements. In addition, it is the only sampling device that achieves reliable low detection limits compared to existing commercially available devices. The ASSET EZ4-NCO sampling device is a unique dry sampler based on derivatization of isocyanate groups with di-*n*-butylamine (DBA). The sampler consists of a denuder and a filter, both impregnated with DBA. The extract is analyzed using LC-MS/MS offering the ultimate sensitivity and low detection limits not achievable with other methods.

The design offers several advantages over existing devices such as:

- The ability to achieve low detection limits for both vapor and particulate phase isocyanates
- Sampling from 5 minutes to 8 hours
- Fast derivatization reactions into stable derivatives
- No storage limits before and after sampling
- High capacity
- No interferences
- No breakthrough
- No field extraction

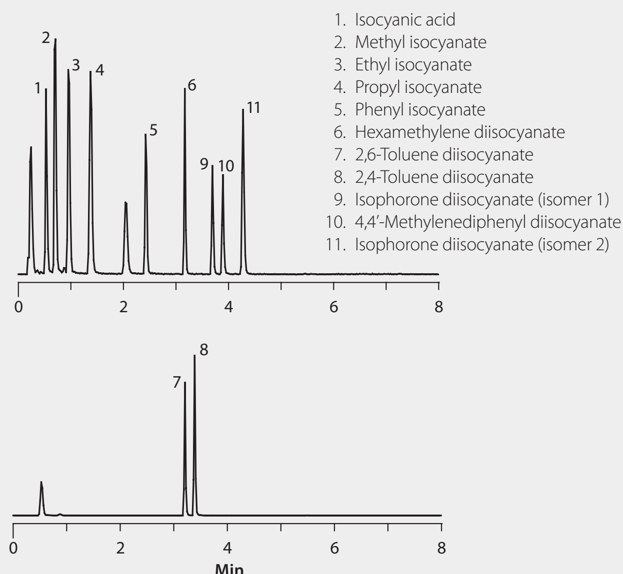
Discussion

Figure 2 shows the chromatogram of a series of 11 common isocyanates. This isocyanate test mix is available as a standard, Cat. No. 40141-U. The gradient mobile phase employs LC-MS suitable solvents and buffers consisting of water, acetonitrile and formic acid. All peaks are well resolved, exhibiting baseline resolution. Peak shapes are symmetrical with minimal tailing. The 5 cm × 2.1 mm Titan™ UHPLC column. Cat. No. 577122-U, used in **Figure 2** is a new offering from Supelco. Titan UHPLC columns are the outcome of the patent pending Ecoporous™ process, an economical route to UHPLC grade silica.

Figure 1. Photograph of the ASSET Isocyanate Field Sampler

Figure 2. Chromatogram of 11 Common Isocyanates with MS Detection

column: Titan C18, 5 cm x 2.1 mm, 1.9 µm (577122-U)
mobile phase: (A) 0.05% formic acid in 5:95 acetonitrile:water; (B) 0.05% formic acid in 95:5 acetonitrile:water
flow rate: 0.5 mL/min
pressure: 270 bar
column temp.: 30 °C
detector: MS, ESI (+), MRM
inj.: 2 µL
sample: isocyanates in acetonitrile, 0.06 µg/mL



For further information on the ASSET field sampler, visit **sigma-aldrich.com/asset**

For further information on the Titan UHPLC, visit **sigma-aldrich.com/titan**

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NEW Flavor & Fragrance Standards for the Food and Cosmetic Industry

Eva Katharina Richter, Product Manager Analytical Standards
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In the food industry, flavorings and fragrances are extensively used either to add taste and/or scent to food lacking aroma, to cover a bad smell, or to maintain the stability of the original flavor of the food. Also used in the cosmetic industry, these compounds are applied to neutralize unpleasant odors that may be in the chemicals or to enhance the individuality and appeal of products.

The analysis of flavorings and fragrances is crucial for product development as well as for quality control in the food and cosmetic industry. However, taste and odor do not usually originate from single compounds, but rather from complex blends of aroma compounds. This makes the analysis of flavorings and fragrances a challenging task and requires a suitable analytical method as well as reliable analytical reference materials for improved confidence in results and instrumentation. Therefore, in 2012, Sigma-Aldrich launched a NEW Flavor and Fragrance standards portfolio with more than 300 analytical neat and standard solutions for precise quality control. The latest product additions are mostly analytical reference materials, which are certified by quantitative nuclear magnetic resonance (qNMR). The qNMR value is measured under ISO/IEC 17025 accreditation and the result is traceable to NIST standard reference materials. These standards allow us to fulfill the needs of many accredited laboratories.

In fact, some flavor and fragrance substances show allergenic effects and are therefore banned in many countries.[1] To assure that both the raw and final products are free of harmful substances, our assortment also includes allergenic compounds for precise quality control.

Each product has been carefully formulated to meet the needs of analysts in the food and beverage and the cosmetic industries.

Reference

1. Scientific Committee on Consumer Safety. Opinion on Fragrance Allergens in Cosmetic Products. SCCS/1459 (2011)

To find an up-to-date product list of all Flavor & Fragrance Standards by their occurrence in food and beverages, by substance classification, and in alphabetical order, visit
sigma-aldrich.com/flavor

Featured Products

Description	Cat. No.
Analytical Reference Materials	
(-)-Methyl L-lactate, 1 mL	90867
2,3-Diethylpyrazine, 1 mL	16877
2,3-Dimethylpyrazine, 1 mL	07187
2-Dodecanone, 1 mL, 5 mL	03411
Ethyl-3,5(6)-dimethylpyrazine, mixture of isomers, 100 mg	43236
2-Ethyl-3-methylpyrazine, 1 mL	16315
3-Mercapto-3-methylbutyl formate, 100 mg	38229
4-Pentenyl acetate, 250 mg	40631
5-Methylfurfural, 1 mL	91307
Allyl phenoxyacetate, 1 mL	16760
Atranol, 10 mg	77236
Benzyl cinnamate, 100 mg	69139
Butylated hydroxyanisole, 500 mg	78943
D-Camphor, 100 mg	50843
Ethyl 2-methylpentanoate, 1 mL	68104
Ethyl hexanoate, 1 mL	08375
Ethyl octanoate, 1 mL, 5 mL	44879
Furfural, 1 mL	04623
Furfuryl acetate, 1 mL	02369
γ-Butyrolactone, 1 mL, 5 mL	90970
γ-Decalactone, 1 mL	93738
γ-Nonalactone, 1 mL	44542
Hexyl 2-methylbutanoate, 1 mL	53184
L-Glutamic acid monosodium salt hydrate, 1 g	01495
Methyl 2-nonynoate, 1 mL	50921
Naringin, 500 mg	91842
Nerolidol, mixture of *cis* and *trans*, 1 mL	00459
Analytical Standards	
2-Furanmethanthiol, 100 mg	67319
3,7-Dimethyl-3-octanol, 1 mL	52125
4-Ethylguaiacol, 1 mL, 5 mL	39774
4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone, 100 mg	44869
4-Methoxybenzyl alcohol, 100 mg	50138
DL-Malic acid, 100 mg	94916
Isobutyraldehyde, 1 mL, 5 mL	68529
Pyrazine, 100 mg	65693
Tyramine, 100 mg	80345

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