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Chromatography Applications Newsletter Volume 58

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Volume 58

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Cover Photo:
Ascentis Express 2.0 µm columns are the best Fused-Core UHPLC columns -- an optimized solution for high throughput small molecule analysis.

No Time or Budget to Attend an Off-Site Seminar? Try a Cost-Free On-Demand Webinar!

Errol Fernandes, PhD

Dear Colleague:

Scientists use a variety of means to stay on top of the latest developments in their field and to build knowledge or expertise within a specific area. They subscribe to relevant journals and other publications, and they periodically attend conferences, workshops and seminars.

An increase in demand for workplace efficiency, coupled with budgetary limitations, often make it difficult to justify the travel time and expense to attend an educational seminar or workshop that satisfies a professional development goal but does not align with the needs of the organization. To help overcome this challenge, Supelco is pleased to offer a collection of (cost-free) chromatography webinars, which can be accessed online and at your convenience.

For the past two years, Supelco scientists and their research collaborators have partnered with a handful of leading online information and learning resources – notably, Separation Science, Bioanalysis Zone, LCGC and Lab Manager – to deliver globally broadcasted webinars pertaining to the fundamentals, best practices, troubleshooting, technology advances and applications in chromatography.

Through some of our recently completed webinars, you can learn about:

- Achieving higher efficiency UHPLC performance for small-molecule separation with the new line of Ascentis® Express columns based on 2-µm Fused-Core® particles, which also happens to be the featured topic of this Reporter edition
- UHPLC and HPLC reversed-phase separation of biomolecules using new BIOshell™ columns
- Applications for ionic liquid GC columns, demonstrating their unique selectivity benefits
- GC column selection and method development basics; also, the principles of fast GC
- SPE fundamentals and recent developments, including novel phases for difficult sample matrices

Each presentation is given by an (academic, agency or industry) expert in the field. The full-length recording is available to you with the same on-demand ease of watching a video from the convenience of your desk or the comfort of your home; most importantly, without the travel time, expense and effort involved with attending an off-site seminar or workshop.

I urge you to access our useful webinar resources to help broaden your chromatography knowledge, to keep up with separation technology trends, and to seek solutions for challenging applications in the clinical, pharmaceutical, food and environmental fields.

To access the Analytical Video & Webinar portal, visit **sigma-aldrich.com/videos**

Best regards,

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Ascentis Express UHPLC Columns Now Available with 2.0 µm Fused-Core Particles

Richard A. Henry, Technical Advisor to Supelco, Division of Sigma-Aldrich
Gaurang Parmar, HPLC Product Manager
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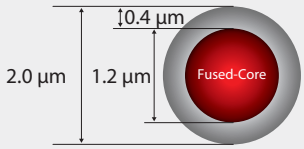
- Proven column performance and ruggedness using the latest Fused-Core® particles
- Lower operating pressure than typical sub-2 µm particle columns
- For selectivity choices C18 and F5 (penta fluorophenyl phase) phases available; other phases in preparation

Column Performance

The new Supelco Ascentis® Express column line has been designed specifically for UHPLC instruments with 2.0 µm Fused-Core silica, as diagrammed in **Figure1**. Ascentis Express 2.0 µm UHPLC columns are optimized to preserve efficiency advantages of using sub-2 µm particles while minimizing pressure disadvantages. As shown in **Figure 2**, the plot of reduced plate height vs flow velocity for Fused-Core 2.0 µm particle columns matches a Fused-Core 2.7 µm particle column by delivering h values that are consistently below 2 (typically ~ 1.7). This is significantly lower than typical commercial sub-2 µm UHPLC columns that employ either porous or core-type particles.

Figure 1. Ascentis Express Fused-Core Silicas for Small Molecule Separations



Columns with lower reduced plate heights demonstrate higher efficiency for a given particle size. Ascentis Express columns with 2.0 µm particles show comparable efficiency at significantly lower pressure than competitor columns that use smaller particles. Achieving high performance at lower pressures can extend useful column life and minimize selectivity changes created by frictional heating that creates temperature variation inside the column bed. Ascentis Express 2.0 µm columns will show highest performance when used in Waters ACQUITY, Agilent Infinity, Thermo Scientific™ Dionex UltiMate™ and Vanquish™, Shimadzu® Nexera, and other modern UHPLC instruments. **Figure 3** plots pressure drop vs flow rate for the columns compared in **Figure 2. Figure 4** shows a chromatographic QC chromatogram for Ascentis Express 10 cm x 2.1 mm, 2.0 µm column, and **Table 2** (page 6) compares test results to other Supelco and competitor columns. The bar chart in **Figure 5** illustrates column performance in plates per pressure unit. Note that Supelco Ascentis Express columns easily outperform typical commercial C18 columns when ranked by a plates/pressure measurement. The only other column that compares favorably to Ascentis Express C18, 2.0 µm in plates/pressure is the new Supelco Titan C18, 1.9 µm column which features a similar, narrow particle distribution.

Figure 2. Reduced Plate Height van Deemter Plots Showing Performance of Ascentis Express Columns with 2.0 µm and 2.7 µm Fused-Core Particles Compared to Columns with sub-2 µm Particles at Various Flow Velocities

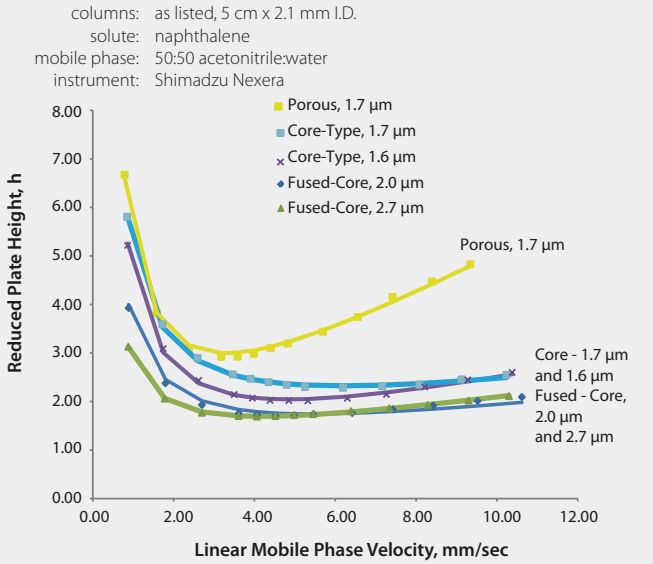


Figure 3. Pressure Drop (flow resistance) for Ascentis Express 5 cm x 2.1 mm Columns with 2.0 µm and 2.7 µm Fused-Core Particles Compared to sub-2 µm Columns at Various Flow Rates

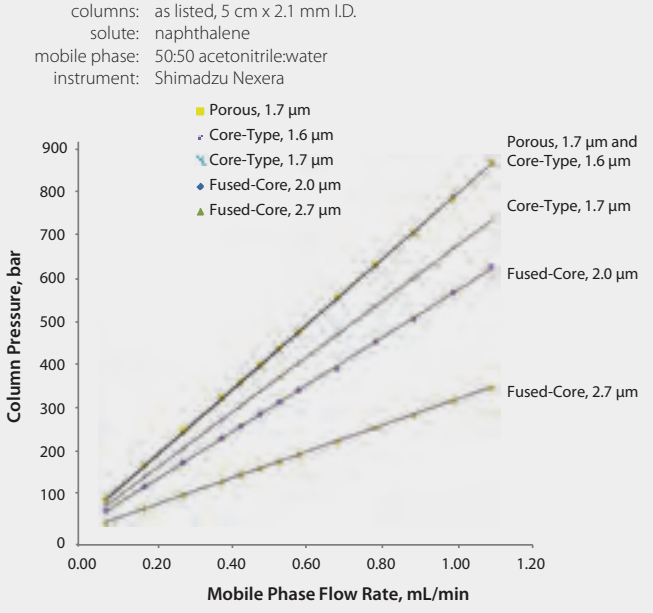


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Figure 4. Suitability Test Chromatogram

columns: Ascentis Express C18, 10 cm x 2.1 mm I.D., 2.0 µm (50813-U)
mobile phase: (A) water, (B) acetonitrile, (50:50, A:B)
flow rate: 0.4 mL/min
pressure and performance: see Table 2
system background pressure: 370 psi
column temp.: 35 °C
detector: UV, 250 nm
injection: 0.5 µL
sample: 75:25 water:acetonitrile
Instrument: Dionex UltiMate 3000

1. Uracil
2. Acetophenone
3. Benzene
4. Toluene
5. Ethylbenzene

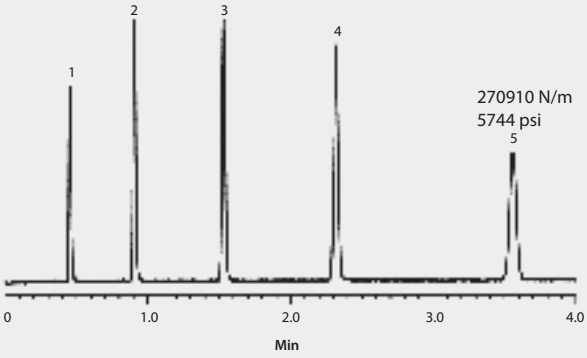


Figure 5. Performance Ranking for Modern UHPLC Columns in Plates per Pressure Unit

columns: as listed, 10 cm x 2.1 mm I.D.
mobile phase: (A) water, (B) acetonitrile, (50:50, A:B)
flow rate: 0.4 mL/min
pressure and performance: see Table 2
system background pressure: 370 psi
column temp.: 35 °C
detector: UV, 250 nm
injection: 0.5 µL
sample: 75:25 water:acetonitrile
Instrument: Dionex UltiMate 3000

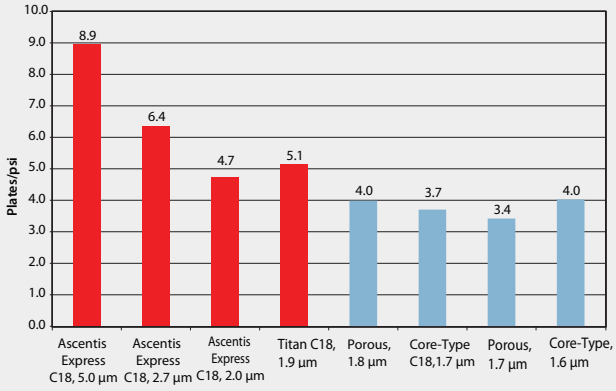
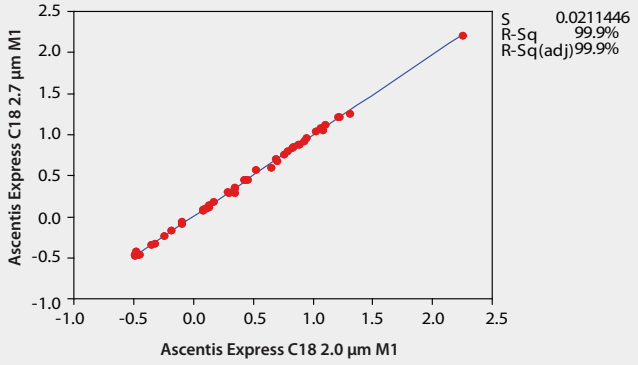
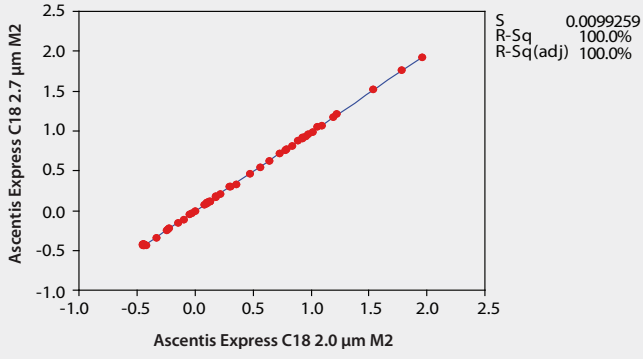
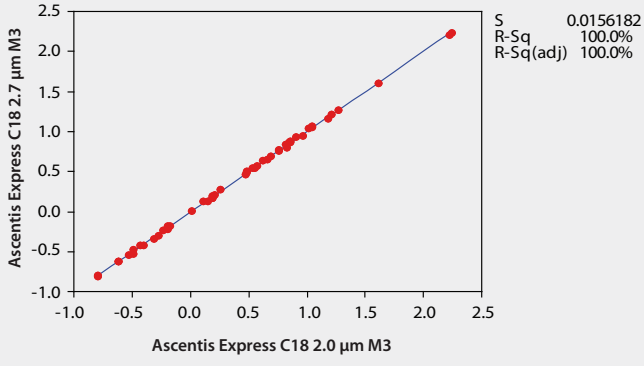


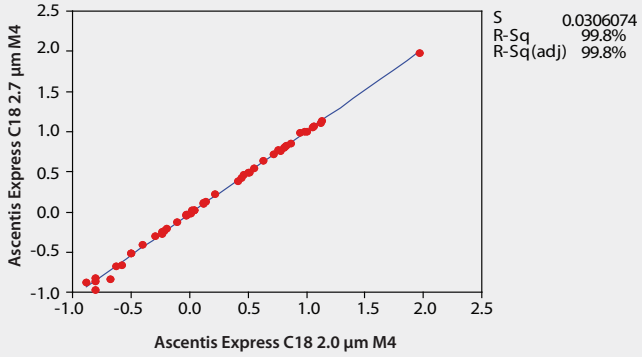
Figure 6. Log k Plots Showing Equivalent Selectivity for Ascentis Express C18, 2.0 and 2.7 µm Columns at Two Different Solvent and pH Conditions

columns: Ascentis Express C18 as indicated, 10cm x 2.1mm I.D.
mobile phase 1 (M1): 10 mM ammonium formate (70:30 water:acetonitrile), pH 7.0
mobile phase 2 (M2): 10 mM ammonium formate (70:30 water:acetonitrile), pH 4.0
mobile phase 3 (M3): 10 mM ammonium formate (50:50 water:methanol), pH 7.0
mobile phase 4 (M4): 10 mM ammonium formate (50:50 water:methanol), pH 4.0
flow rate: 0.3 mL/min
column temp: 35° C
injection: 2 µL
instrument: Agilent 1290 Infinity with 6210 TOF MS in ESI+ mode, 100-1000m/z









Column Selectivity

Ascentis Express C18 and F5 (pentafluoropropyl or PFP) columns are currently available in the 2.0 µm particle size, with a complete range of phases under development (for latest details, refer to **sigma-aldrich.com/express**. C18 phases with flexible alkyl chains are extremely versatile because solutes are retained by a simple partition mechanism where dispersive interactions and solubility in organic solvent controls retention and selectivity. Ascentis Express F5 phase behaves differently because sample components can interact not only by dispersive forces but also with the aromatic ring that becomes highly polarized by five fluorine substituent groups. C18 and PFP phases typically show very large selectivity differences so they are complementary choices for method development.

Different particle sizes in the Ascentis Express 90 Å Fused-Core column family are designed to have the same retention and selectivity for easy method development or method transfer. Log k values for a 60-component sample containing acidic, basic and neutral pharmaceuticals are compared in **Figure 6** between the new Ascentis Express C18, 2.0 µm column and the popular Ascentis Express C18, 2.7 µm column that has become well-established in many HPLC methods; acetonitrile and methanol conditions were investigated at pH 4.0 and 7.0. Note correlation is essentially 100% for all four conditions, indicating that a C18 phase bonded to either the 2.0 µm or 2.7 µm Fused-Core particle will produce the same selectivity factor ($\alpha = k2/k1$) for a wide range of different chemical structures. Note also the slopes for each log k plot are essentially unity, indicating that two C18 columns will also match one another in retention factor (k). This identical selectivity and retention performance is demonstrated in **Figures 7A** and **7B** for Ascentis Express C18, 2.0 µm and 2.7 µm columns using a mixture of sulfa drugs. Performance is also compared to a commercial core-type C18, 1.7 µm column **(Figure 7C)** to demonstrate that Ascentis Express columns have similar retention and selectivity to other C18 columns regardless of particle size or design. Note that the Ascentis Express C18 2.0 µm column matches the sub-2 µm C18 core-type in efficiency, but operates at lower pressure.

Table 1. Fused-Core Silica Specifications

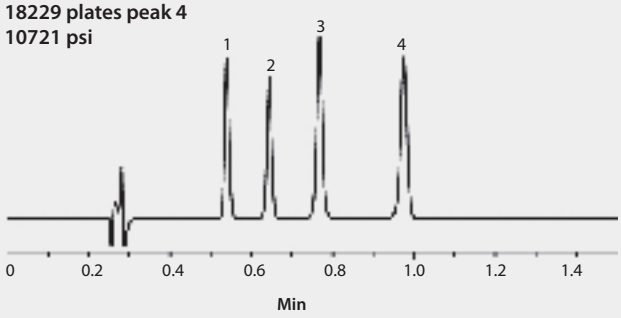
Particle Size	2.0 µm
Pore Size	90 Å
Surface Area m^2/g	120
Max Temp	60 °C
Pressure	1000 bar (14500 psi)
Operating pH	2-9
Other Features	End-capped phases unless specified
Frit Size	1 µm

Figure 7. Sulfa Drug Comparison

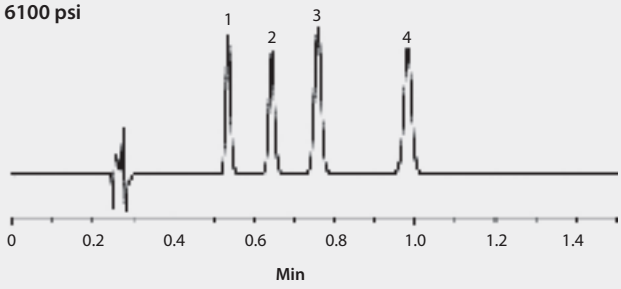
column:	as listed, 10 cm x 2.1 mm I.D.
mobile phase:	(A) water with 0.1% acetic acid; (B) acetonitrile with 0.1% acetic acid, (90:1 A:B)
flow rate:	0.8 mL/min
pressure:	see below
column temp.:	45 °C
detector:	UV, 250 nm
injection:	0.5 µL
sample:	25 µg/mL in 90:10 water:methanol
instrument:	Agilent 1200

1. Sulfacetamide
2. Sulfadiazine
3. Sulfathiazole
4. Sulfamerazine

7A. Ascentis Express C18, 2.0 µm
18229 plates peak 4
10721 psi



7B. Ascentis Express C18, 2.7 µm
11772 plates peak 4
6100 psi



7C. Core-Type C18, 1.7 µm
18096 plates peak 4
12490 psi

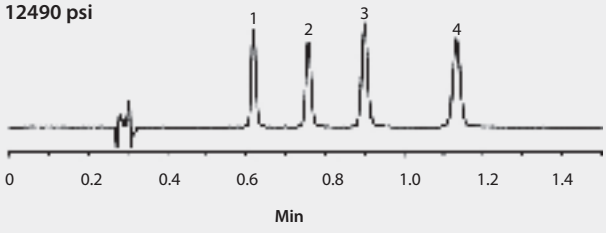


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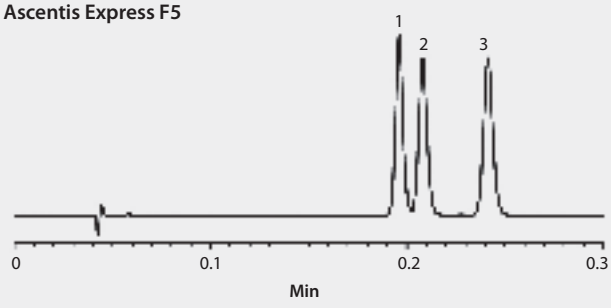
Figure 8 illustrates the selectivity difference between Ascentis Express C18 and F5 columns using a steroid example. Hydrocortisone and prednisolone differ by only one double bond and have nearly identical solubility. Since the main difference between these rigid solutes is shape rather than solubility, C18 columns typically show poor selectivity because alkyl chains can readily change conformation to provide nearly equivalent amounts of solute interaction. For rigid isomers, the PFP column can provide additional selectivity because one isomer may approach the planar aromatic ring differently and interact more strongly. Aromatic phases, such as PFP, often show different, enhanced selectivity over C18 between closely-related, rigid compounds in any sample.

Figure 8. Steroid Comparison

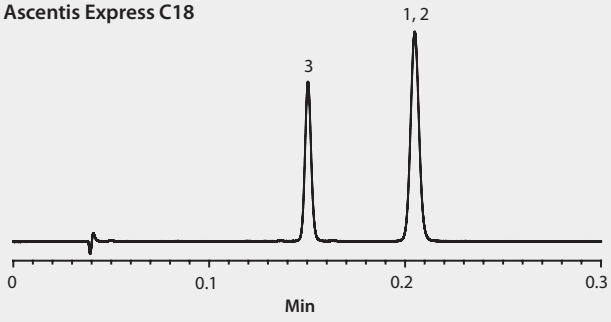
column:	as indicated, 10 cm x 2.1 mm I.D., 2.0 µm
mobile phase:	(A) water, (B) methanol (50:50, A:B)
flow rate:	0.5 mL/min
pressure:	11430 psi (788 bar)
system background pressure:	420 psi
column temp.:	35 ° C
detector:	UV, 240 nm
injection:	0.5 µL
sample:	50 µg/mL in 85:15, water:methanol)
instrument:	Dionex UltiMate 3000

1. Hydrocortisone
2. Prednisolone
3. Prednisone

Ascentis Express F5



Ascentis Express C18



Conclusion

- Fused-Core particles have revolutionized HPLC/UHPLC performance. Ascentis Express 2.0 µm Fused-Core particles are designed to provide UHPLC performance for practical small molecule analysis.
- Ascentis Express column line now features three 90 Å Fused-Core particle sizes for high speed separation of small molecules.
- Ascentis Express 2.0, 2.7 and 5 µm Fused-Core columns represent the best selection of phases and geometries for every analytical application and instrument.

Table 2. Comparison of Theoretical Plates and Pressure for Ascentis Express C18 Columns Compared to other Commercial C18 sub-2 µm Columns

columns:	as listed, 10 cm x 2.1 mm
mobile phase:	(A) water, (B) acetonitrile, (50:50, A:B)
flow rate:	0.4 mL/min
pressure and performance:	as listed in Table 2
system background pressure:	370 psi
column temp.:	35 °C
detector:	UV, 250 nm
injection:	0.5 µL
sample:	75:25 water:acetonitrile
Instrument:	Dionex UltiMate 3000

Column	Particle (µm)	Uracil Rt (min)	Toluene Rt (min)	Toluene k	Toluene As	Toluene Efficiency (N)	Backpressure (psi)	Plates/psi
Ascentis Express C18	5.0	0.447	1.251	1.80	1.01	12822	1433	8.9
Ascentis Express C18	2.7	0.438	2.292	4.23	1.06	20149	3165	6.4
Ascentis Express C18	2.0	0.453	2.319	4.12	1.03	27091	5744	4.7
Titan C18	1.9	0.502	3.689	6.35	1.00	24034	4677	5.1
Porous C18	1.8	0.506	2.987	4.90	1.06	22259	5585	4.0
Core-Type C18	1.7	0.469	2.087	3.45	1.04	24849	6712	3.7
Porous C18	1.7	0.536	2.583	3.82	1.06	23092	6751	3.4
Core-Type C18	1.6	0.460	2.230	3.85	1.07	31643	7860	4.0

Featured Products

Particle Size	ID	Length	Cat. No. C18	Cat. No. F5
Ascentis Express HPLC Columns*				
2.0 µm	2.1 mm	2 cm	50805-U	50857-U
2.0 µm	2.1 mm	3 cm	50809-U	50858-U
2.0 µm	2.1 mm	5 cm	50811-U	50859-U
2.0 µm	2.1 mm	7.5 cm	50812-U	50861-U
2.0 µm	2.1 mm	10 cm	50813-U	50863-U
2.0 µm	2.1 mm	15 cm	50814-U	50867-U
2.0 µm	3.0 mm	3 cm	50815-U	50869-U
2.0 µm	3.0 mm	5 cm	50816-U	50871-U
2.0 µm	3.0 mm	7.5 cm	50817-U	50876-U
2.0 µm	3.0 mm	10 cm	50819-U	50879-U
2.0 µm	3.0 mm	15 cm	50821-U	50881-U
Ascentis Express Guard Cartridges, Package of 3				
2.0 µm	2.1 mm	0.5 cm	50822-U	50884-U
2.0 µm	3.0 mm	0.5 cm	50823-U	50886-U

***New release:** Ascentis Express 2.0µm phases OH5 and HILIC.

For further information, visit
sigma-aldrich.com/express

Re-equilibration in HILIC Chromatography

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Introduction

Interest in chromatography using hydrophilic interaction liquid chromatography (HILIC) has continued to build in recent years. Full adoption of the technique, however, has been slowed by experiences of poor reproducibility. Reproducibility issues generally stem from a lack of understanding of the controls one sets up in a chromatographic system. Re-equilibration times in HILIC, for example, have been reported as being exceptionally long as compared to reversed-phase chromatography. Is it possible, then, that some reproducibility issues are a result of improper re-equilibration settings?

In this study, re-equilibration times in HILIC, for both aqueous:organic gradients and buffer gradients are systematically explored. Repeatability of retention and selectivity was studied as a function of equilibration times following aqueous:organic gradients.

The impact of the use of buffer gradients and subsequent equilibration procedures on retention times were also investigated using several HILIC stationary phases and probes. The results not only promise to improve method development practices, but also provide valuable insight into HILIC retention mechanisms across a diverse set of polar stationary phases.

Experimental

Organic: Aqueous Gradient Study

Using a bare silica (Ascentis Express HILIC, 10 cm x 3.0 mm, 2.7 µm) and a pentahydroxy phase (Ascentis Express OH5, 10 cm x 3.0 mm, 2.7 µm), a set of neutral molecules was run employing a gradient from 5% aqueous to 50% aqueous with varied equilibration times. The same study was also performed using a gradient from 5% to 25% of the aqueous component. Mobile phase A was 5 mM ammonium acetate in 95% acetonitrile while mobile phase B was 5 mM ammonium acetate in 50% acetonitrile. Mobile phase C was 5 mM ammonium acetate in 75% acetonitrile. Gradient 1 ran from A to B in 10 minutes. Gradient 2 ran from A to C in 10 minutes. Re-equilibration times were set at 2, 5, 10, 15 and 20 minutes. The column temperature was held at 35 ºC, flow rate at 0.5 mL/min, injection volume at 2 µL and detection by UV absorbance at 254 nm. Initially, a set of neutral polar analytes (adenosine and cytosine) were injected in triplicate. To represent polar basic molecules, metanephrine and normetanephrine were also tested.

Buffer Gradient Study

The buffer gradient study was run identically to the organic:aqueous study except that mobile phase A consisted of 2 mM ammonium acetate in 90% acetonitrile, pH* adjusted to 7.0 (+/- 0.05) with acetic acid and mobile phase B consisted of 10 mM ammonium acetate in 90% acetonitrile, pH* adjusted to 7.0 (+/- 0.05) with acetic acid. The gradient was run from 100% A to 100% B in 10 minutes. In addition to the polar neutral mix and the polar basic mix, an additional nonpolar basic mix was included.

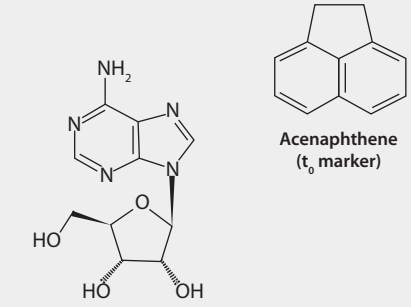
*pH measured in the presence of organic and calibrated using aqueous standards.

Results and Discussion

Figure 1 shows the structures of the test probes used throughout the study. Adenosine and cytosine represent polar neutral molecules that should only interact with the stationary phase via partition or polar (dipole type) interactions. The metanephrines are both polar and positively charged under the conditions of the study and thus can interact via partitioning, polar and ionic mechanisms. Lastly, the tricyclic antidepressants (TCAs) amitriptyline and nortriptyline are ionized, but relatively nonpolar. The TCAs, then, are expected to retain via ion-exchange mechanisms and perhaps some polar interactions, but not via partition.

Figure 1. Structures of the Study Probe Molecules

Polar Neutral

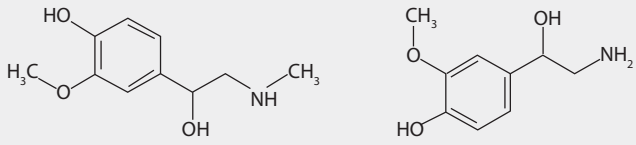


Adenosine [*{BAN}; *{USAN}]

Acenaphthene (t_0 marker)

Cytosine

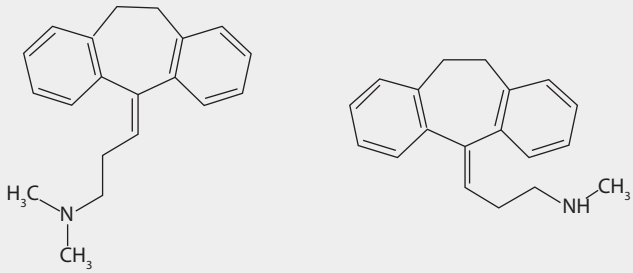
Polar Basic



Metanephrine

Normetanephrine

Nonpolar Basic



Amitriptyline

Nortriptyline

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Plots of retention time versus organic-aqueous re-equilibration time are shown in **Figure 2** for the neutral probes. For the steeper gradient (to 50% aqueous) there exists some slight irreproducibility at 2 minutes re-equilibration time; however, at 5 minutes and above, highly reproducible results are obtained. For the shallower gradient just two minutes appears to be sufficient to generate reproducible results. In both plots the retention drifts upwards as re-equilibration times increase; however, at each given time point, reproducible results are obtained.

Figure 2. Retention as a Function of Re-equilibration Time

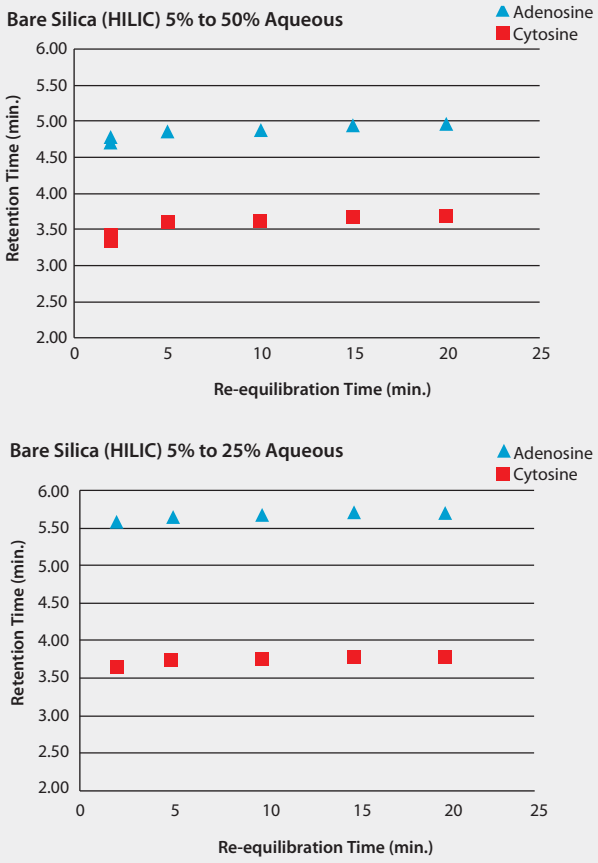


Figure 3. Retention as a Function of Re-equilibration Time

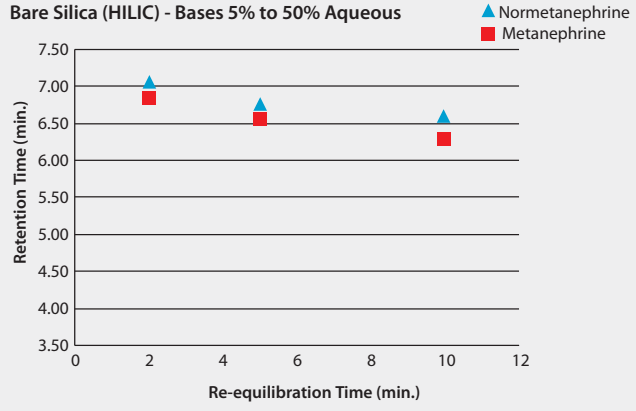
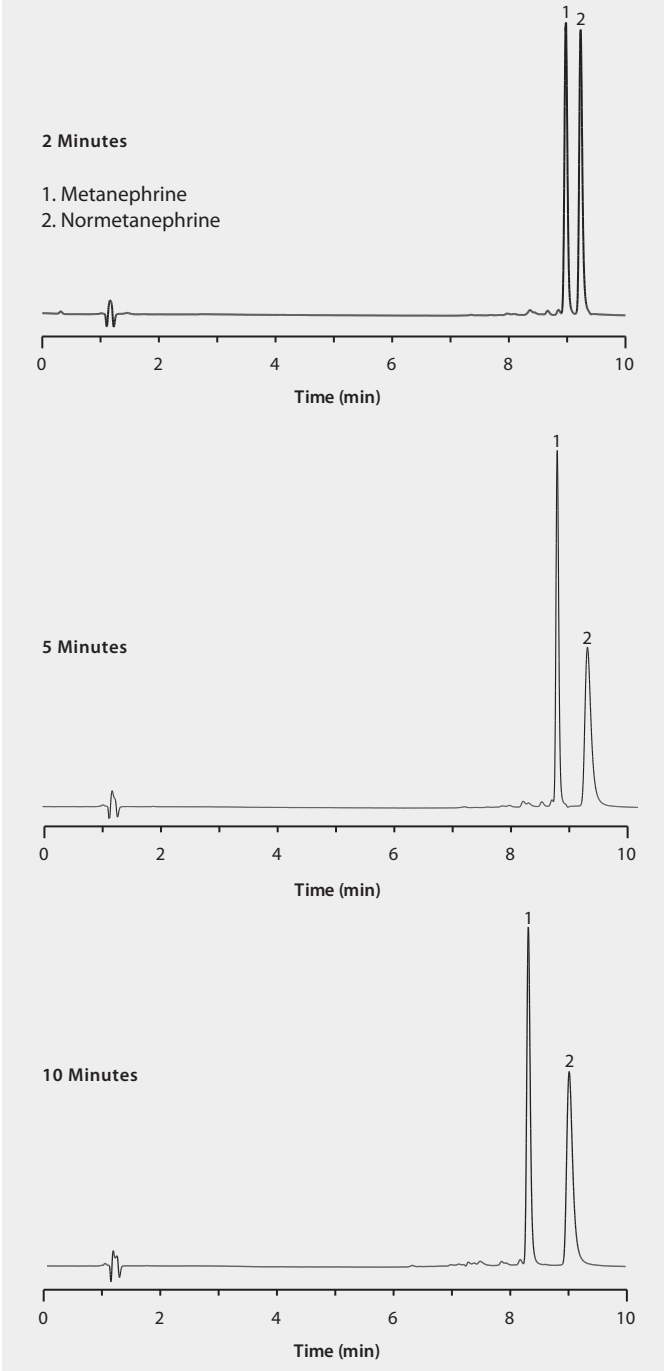


Figure 3 shows the results from the polar basic probes using the steep gradient on the HILIC phase. Again the retention times are shown to be highly reproducible at a given re-equilibration time. In the case of the polar bases the retention drifts downward as a function of re-equilibration time. In addition, as shown in **Figure 4**, selectivity and peak shape for the polar bases are significantly impacted by the re-equilibration time on the bare silica phase.

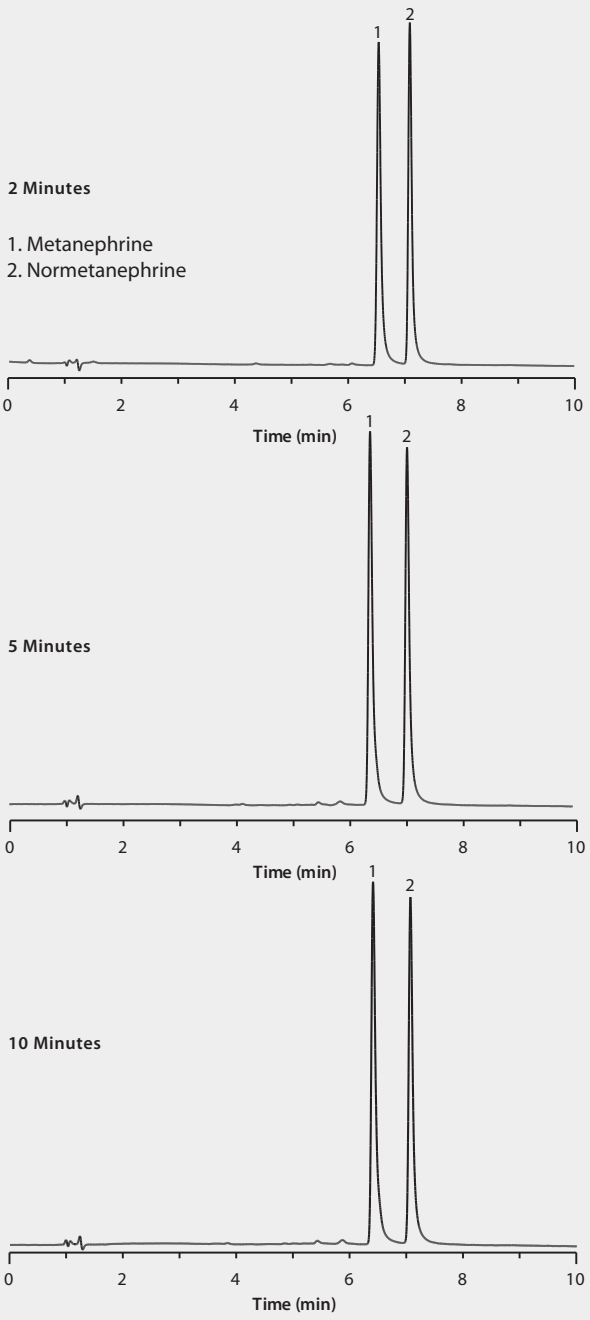
Figure 4. Chromatographic Traces of Polar Basic Analytes on Bare Silica (HILIC) Phase at Different Re-equilibration Times



The chromatographic traces obtained for the polar bases on the OH5 phase are shown in **Figure 5**. In this case, the re-equilibration times do not appear to significantly impact the retention, selectivity or peak shape.

In previous studies, the OH5 phase has shown less ion-exchange character as compared to bare silica HILIC phases.[1] This observation coupled with the faster equilibration of the basic analytes suggests that ion-exchange mechanisms are the main cause of slow equilibration on the bare silica phase. To investigate this further, buffer gradients were run on both phases and an additional set of nonpolar, basic probes was added.

Figure 5. Chromatographic Traces of Nonpolar Basic Analytes on Pentahydroxy (OH5) Phase at Different Re-equilibration Times



Figures 6 and **7** show the plots of retention times versus buffer gradient re-equilibration times for each set of test probes on the bare silica and pentahydroxy phases, respectively. On both phases, the neutral polar molecules are barely affected. For both the polar and nonpolar bases, retention increases as re-equilibration time increased. The magnitude of the increase on the OH5 phase, however, is greatly attenuated as compared to the bare silica phase for both sets of basic compounds. It is also important to note that reproducibility at any given re-equilibration time on both phases was observed.

Figure 6. Retention as a Function of Re-equilibration Time

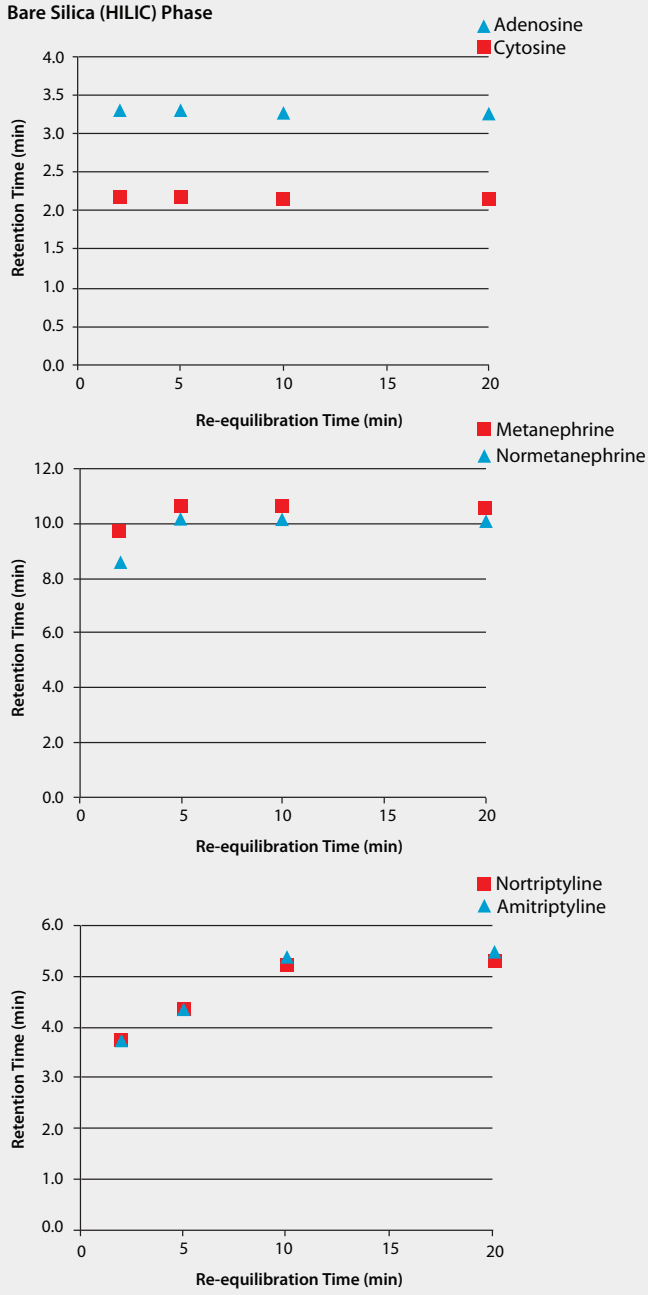


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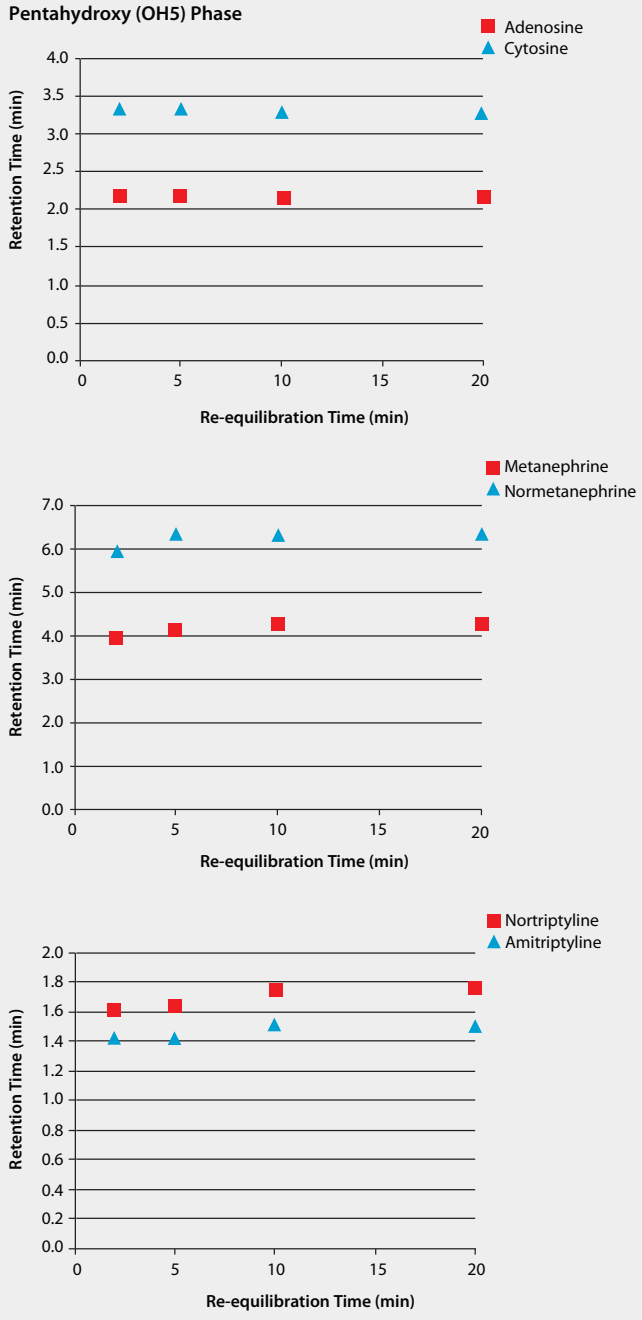
Figure 7. Retention as a Function of Re-equilibration Time



Conclusions

HILIC gradient methods are often run to levels of the aqueous component that is well outside the normal HILIC percentages. An initial assumption regarding reproducibility of gradient HILIC methods was that running gradients past the 70% acetonitrile level would have significant impact on the overall reproducibility. The present study shows that running to even 50% aqueous had only a small impact on reproducibility. Interestingly, when re-equilibration times were held constant, reproducible retention, selectivity and peak shapes were observed. Marked changes however, were observed upon extending re-equilibration times, especially for basic analytes on the bare silica stationary phase. Each of the experiments suggests that the presence of ion-exchange mechanisms results in slower overall equilibration. The use of bonded HILIC phases such as the OH5 may provide additional control of retention times when ion-exchange is present.

During the development of reversed-phase methods it is often assumed that if one allows the system to equilibrate for an extensive amount of time, the results would be the same as if the system were allowed to equilibrate for "just enough" time. The results of this study show that a gradient method in HILIC mode may appear equilibrated by virtue of reproducible retention times, however with extended re-equilibration time, may change. Therefore, it is important that gradient HILIC methods specify the re-equilibration times in order to ensure reproducible and reliable results.

Featured Products

Description	Cat. No.
Ascentis Express HPLC Columns	
HILIC, 10 cm x 3.0 mm I.D., 2.7 µm particle size	53970-U
OH5, 10 cm x 3.0 mm I.D., 2.7 µm particle size	53769-U

Reference

1. Bell, D. HILIC Chromatography: Theory and Method Development Practices. http://view6.workcast.net/register?pak=5061956691401502 (accessed Nov 2014)

Note: See also the Ascentis Express 2.0 µm with 2 new phases: OH5 and HILIC.

For further information, visit
sigma-aldrich.com/express

Sample Prep Method Development and Optimization for the LC/MS/MS Analysis of Steroid Hormones in Plasma

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HybridSPE®-Phospholipid plates provided excellent analyte recovery of these difficult compounds from plasma samples with subsequent LC/MS/MS analysis on Ascentis® Express Fused-Core® C18 columns. The extracts were free of endogenous phospholipids that can interfere with quantitation, decrease sample throughput and reduce column lifetime.

Introduction

There is a growing trend toward converting some clinical methods from immunoassay to LC/MS/MS for a variety of reasons. LC/MS/MS improves assay selectivity, is not limited by antibody availability, and allows multiplexed analyte assays to be conducted simultaneously. However, LC/MS/MS is not without its limitations, most notably interferences from endogenous sample matrix, which can result in seemingly random and arbitrary discrimination in analyte response, among other effects.[1]

Objectives of the Study

The goal of this study was two-fold. First, to develop a simple LC/MS/MS method, including sample preparation using HybridSPE-Phospholipid plates, for the direct analysis of the steroid hormones progesterone, aldosterone, corticosterone, deoxycorticosterone, testosterone, and 17α-methyltestosterone from blood plasma. Second, to compare the background from sample matrix between the resultant sample prep method to standard protein precipitation.

Experimental

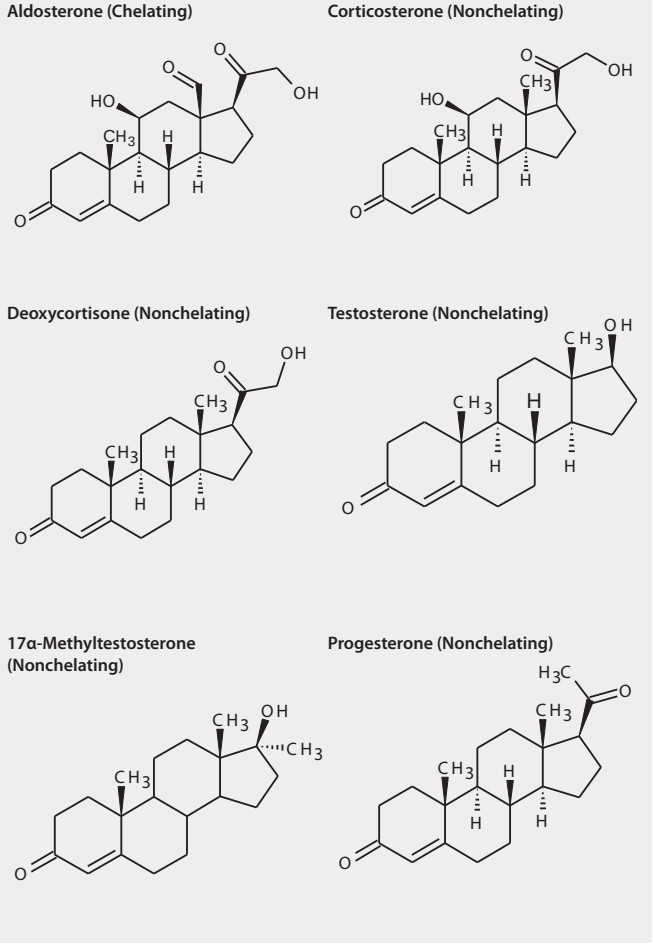
Chromatographic (LC/MS/MS) Conditions

The chemical structures of the steroid hormones are shown in **Figure 1**. Initial evaluation was conducted using a mixture of steroid hormones to establish chromatographic conditions on Ascentis Express C18 column. The gradient profile was extended so matrix monitoring could be conducted for the processed plasma samples.

HybridSPE-Phospholipid Operating Principles

HybridSPE-Phospholipid technology combines simple, standardized methodology of protein precipitation with the selectivity of solid phase extraction (SPE) for the simultaneous removal of proteins and phospholipids from biological samples. The technology is based on hybrid zirconia-silica particles for targeted isolation of phospholipids, while PTFE frit materials act as a depth filter for efficient removal of precipitated protein particles. The zirconia portion of the hybrid particle behaves as a Lewis acid (electron acceptor) which interacts strongly with Lewis bases (electron donors), like the phosphate moiety of phospholipids. This technology allows for highly selective phospholipid matrix removal while remaining non-selective towards a broad range of analytes.[2]

Figure 1. Steroid Hormones



Sample Prep Method Development: Factors that Impact Analyte Recovery

Sample prep method development consisted of establishing recovery of standard compounds from the HybridSPE-Phospholipid 96-well plates, then transferring those conditions for use with spiked plasma samples. Method optimization consisted of evaluating several precipitation solvent systems. Once sufficient analyte recovery was established for the plasma samples, the final portion of the study was to compare the HybridSPE-Phospholipid technique with the commonly accepted protein precipitation for phospholipid matrix removal.

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Recovery of Analytes from Standard Solution using HybridSPE-Phospholipid

Method development with the HybridSPE-Phospholipid 96-well plate began with establishing recovery of the six steroids from standard solutions. External standard calibration curves from 10 to 300 ng/mL of each compound in 1% (v/v) formic acid acetonitrile:water (3:1) were developed. A 300 µL aliquot of standard solution (50 ng/mL) was added to the HybridSPE-Phospholipid 96-well plate. Vacuum was applied for 4 minutes at 10" Hg. Samples were then analyzed directly.

Recovery of Analytes from Plasma using HybridSPE-Phospholipid

The primary solvent used with the HybridSPE-Phospholipid for precipitation of plasma samples is 1% (v/v) formic acid in acetonitrile. Used in a 3:1 ratio with the plasma sample, this solution effectively precipitates plasma proteins. Formic acid also disrupts binding of target analytes with proteins, providing increased sensitivity. However, with analytes with chelating properties, the use of 0.5% (w/v) citric acid in acetonitrile as the precipitation additive and in a preconditioning step greatly increases their recovery from the zirconia-silica particles.

Formic acid system: 1% (v/v) formic acid in acetonitrile:water (3:1)

Rat plasma stabilized with K_2EDTA (Lampire Biological Laboratories, Pipersville PA) was spiked with the analytes from a standard solution. A 100 µL aliquot was added to the HybridSPE-Phospholipid plate followed by 300 µL of 1% formic acid acetonitrile precipitation solvent. The plate was agitated via vortex for 4 minutes, placed on vacuum manifold and subjected to 10" Hg vacuum for 4 minutes. The filtrate was collected and analyzed directly. The concentration of steroid hormones in the final sample work up was equivalent to 50 ng/mL. Recovery was based on interpolation of a standard curve of analytes in buffer.

Citric acid system: 0.5% (w/v) citric acid in acetonitrile, for chelating compounds

The citric acid procedure was the same as the formic acid procedure described above, except for these two steps: The precipitation solvent was 0.5% (w/v) citric acid in acetonitrile.
The HybridSPE-Phospholipid plate was first preconditioned with 300 µL/well of 0.5% (w/v) citric acid in acetonitrile.

Monitoring Phospholipids from the Plasma Sample Matrix

Spiked plasma samples were precipitated using 0.5% citric acid acetonitrile and analyzed directly. Phospholipid monitoring was conducted using Q3 full scan along with precursor ion scan of 104 m/z. Gradient chromatographic conditions utilized for the assay enable elution of phospholipids under high organic conditions.

Results and Discussion

The gradient elution of the six steroid hormones on the Ascentis Express C18 with LC/MS/MS detection is shown in **Figure 2**.

Analyte Recovery

Table1 presents the recovery data of the steroid compounds extracted by the HybridSPE-Phospholipid plates from a standard solution using the formic acid system, and from spiked plasma using the formic acid and citric acid systems. The table reports average observed concentration, standard deviation and coefficient of variation (% c.v.) of replicate samples (n=8).

Figure 2. LC/MS/MS Analysis of Steroid Hormones

column: Ascentis Express C18, 10 cm x 2.1 mm I.D., 2.7 µm particle size (53823-U)
mobile phase: (A) 5 mM ammonium formate, pH 4.0 with formic acid; (B) methanol
gradient: 60% B for 3 min, to 95% B in 5 min, held at 95% B for 2 min
flow rate: 0.3 mL/min
column temp: 50 °C
pressure: 90 bar
detector: ESI(+), MRM
injection: 2 µL
sample: 50 ng/mL of each compound
system: Shimadzu LC-30AD, LCMS8030

	MRM Transition
1. Aldosterone	361.0/343.1
2. Corticosterone	347.6/109.0
3. 11-Deoxycorticosterone	331.1/109.0
4. Testosterone	289.0/109.0
5. 17α-Methyltestosterone	303.1/97.0
6. Progesterone	315.0/109.1

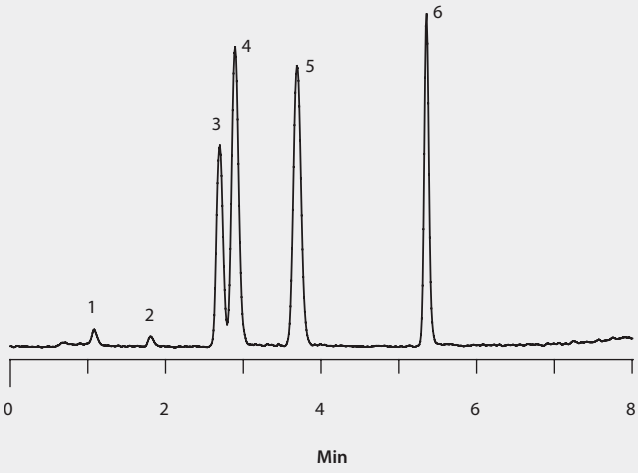


Table 1. Analyte Recovery from HybridSPE-Phospholipid Plates

Analytical conditions appear in Figure 2. Note higher recovery and reduced variability using the citric acid system

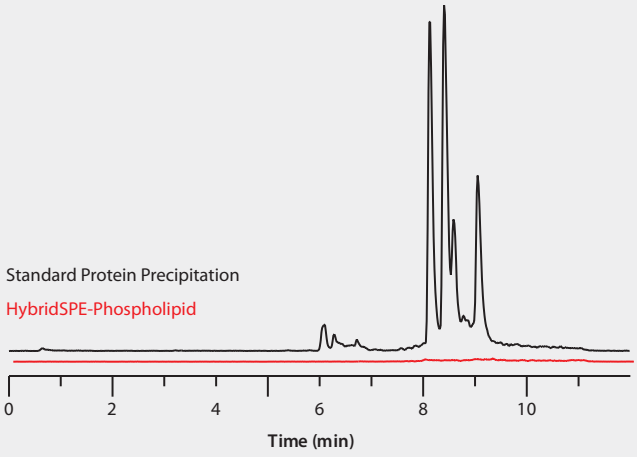
Analyte	Observed Concentration	Std. Dev. (n=8)	% cv.
A. Standards (50 ng/mL) in 1% formic acid acetonitrile			
Aldosterone	2.70	1.83	67.75
Deoxycorticosterone	25.13	1.04	4.15
Cortocosterone	14.76	2.46	16.65
Progesterone	57.27	0.95	1.66
Testosterone	59.23	1.16	1.96
17α-Methyltestosterone	57.15	1.21	2.11
B. Spiked plasma (50 ng/mL) extracted using formic acid system			
Aldosterone	1.87	0.83	44.39
Deoxycorticosterone	54.58	1.10	2.02
Cortocosterone	54.31	3.86	7.11
Progesterone	54.09	0.99	1.83
Testosterone	55.75	0.76	1.36
17α-Methyltestosterone	54.30	0.93	1.71
C. Spiked plasma (50 ng/mL) extracted using citric acid system			
Aldosterone	45.03	1.89	7.55
Deoxycorticosterone	54.32	2.55	4.10
Cortocosterone	56.23	3.91	5.47
Progesterone	51.40	1.58	4.30
Testosterone	51.66	1.28	3.62
17α-Methyltestosterone	61.68	1.53	3.76

In the formic acid system, with the standard solutions, poor recovery and high variability of aldosterone, corticosterone and deoxycorticosterone were observed. This is due to chelation of combined carbonyl/carboxylic acid site on these molecules with the zirconia-silica surface of the HybridSPE- Phospholipid particles. The structures of aldosterone and progesterone can be compared in **Figure 1**. Note the position of the carbonyl and hydroxyl groups that give aldosterone its chelation tendency. It was anticipated that analyte recoveries from the HybridSPE-Phospholipid would be higher when extracted from serum or plasma compared to standard solutions. This is because phospholipids are in high abundance in the plasma sample and they interact strongly with Lewis acid zirconia molecules on the HybridSPE-Phospholipid particles, thereby, masking these sites from sensitive analytes. **Table 1B** supports this premise; recovery and variability of corticosterone and deoxycorticosterone extracted from plasma were dramatically improved over extraction of aqueous standards. However, aldosterone recovery from plasma was still very poor in the formic acid system.

By replacing the formic acid with citric acid, the chelation phenomenon is overcome. Citric acid behaves as a chelating agent, and when utilized as an additive for the HybridSPE-Phospholipid it acts to tie up the zirconia surface. Preconditioning the plates with citric acid prevents the retention of chelating compounds, but does not impede the selective retention of the phospholipids. **Table 1C** shows that the use of citric acid as the precipitation additive along with the preconditions step greatly increases recovery and reproducibility of chelating compounds from the HybridSPE-Phospholipid particle.

Figure 3. Monitoring Co-extracted Phospholipid Matrix

column:	Ascentis Express C18, 10 cm x 2.1 mm I.D., 2.7 µm particle size (53823-U)
mobile phase:	(A) 5 mM ammonium formate, pH 4.0 with formic acid; (B) methanol
gradient:	60% B for 3 min, to 95% B in 5 min, held at 95% B for 2 min
flow rate:	0.3 mL/min
column temp:	50 °C
pressure:	90 bar
detector:	ESI(+), MRM
injection:	2 µL
sample:	50 ng/mL of each compound
system:	Shimadzu LC-30AD, LCMS8030



Monitoring Coextracted Phospholipid Matrix

Figure 3 presents the matrix monitoring chromatograms for both the HybridSPE-Phospholipid technique and protein precipitation. Plasma samples processed using the HybridSPE-Phospholipid had no detectable matrix interference compared to the standard protein precipitation technique. Although the phospholipid matrix from the standard protein precipitation technique did not directly co-elute with the target analytes, this would still be a concern for fouling of the MS source. The phospholipid matrix would result in chromatographic carryover into the subsequent samples resulting in random interference and potential column failure.

Summary

The 0.5% citric acid acetonitrile system on HybridSPE-Phospholipid plates gave excellent analyte recovery and high efficiency phospholipid removal from the plasma samples. This study shows that there are simple and effective approaches to take when faced with particularly difficult analytes. In the example presented here, initially poor recovery of chelating steroid compounds in the formic acid system was overcome using a citric acid system that eliminated chelation interactions. The method gave high recovery and low variability of all tested steroids from both standard solutions and spiked plasma samples. The citric acid system was also very effective in removal of endogenous phospholipids from the matrix. In conclusion, with slight modification of the generic protocol, HybridSPE-Phospholipid was shown to be effective at matrix reduction while providing excellent recovery of difficult, chelating analytes. HybridSPE-Phospholipid is a simple and effective LC/MS sample prep technique for reducing matrix effects in LC/MS analysis of biological samples.

References

1. Aurand, C., *Understanding the Impact of Phospholipid-Induced Ion Suppression in LC-MS;* Supelco Reporter 52: 10-12. http://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Supelco/The_Reporter/1/T212002.pdf
2. HybridSPE-Phospholipid Technology Home Page. sigma-aldrich.com/hybridspe-pl

Featured Products

Description	Cat. No.
HPLC Column	
Ascentis Express C18, 10 cm x 2.1 mm I.D., 2.7 µm particle size	53823-U
Sample Prep Plates and Accessories	
HybridSPE-Phospholipid 96-Well Plates, bed wt. 50 mg, volume 2 mL	575656-U
HybridSPE-PLus Plate Essentials Kit (Includes HybridSPE-PLus 96-well plate (575659-U), one plate cap mat (as in 575680-U), one sealing film (Z721581) and one collection plate (Z717266)	52818-U
96 Round/Deep-Well Collection Plate, polypropylene	Z717266
96 Well-Plate Pre-cut Sealing Films, pk of 100	Z721581
Supelco PlatePrep Vacuum Manifold	57192-U
Mobile Phase Components	
Water LC/MS Ultra CHROMASOLV®, tested for UHPLC/MS	14263
Acetonitrile LC/MS Ultra CHROMASOLV, tested for UHPLC/MS	14261
Methanol LC/MS Ultra CHROMASOLV, tested for UHPLC/MS	14262
Formic acid, eluent additive for LC/MS	56302
Ammonium formate, LC/MS Ultra eluent additive	14266
Cerilliant Certified Reference Materials	
Aldosterone, 100 µg/mL in acetonitrile	A-096
Corticosterone, 1.0 mg/mL in methanol	C-117
11-Deoxycorticosterone, 100 µg/mL in methanol	D-105
Testosterone, 1.0 mg/mL in acetonitrile	T-037
17α-Methyltestosterone, 1.0 mg/mL in 1,2-dimethoxyethane	M-906
Progesterone, 1.0 mg/mL in acetonitrile	P-069

Aminoglycoside Analysis in Pork Muscle using Molecularly Imprinted Polymer Cleanup and LC/MS/MS Detection

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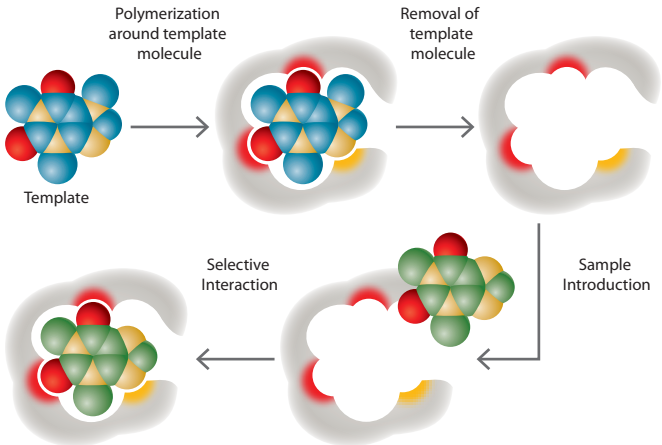
Introduction

Aminoglycosides are a well-known class of antibiotics that are routinely monitored in animal-derived foods. Many countries have instituted regulatory limits for aminoglycosides. There is growing concern of the impact these microbial resistant compounds have on human health and development. A sensitive and robust analytical method is required in order to enforce the regulations and ensure the safety and quality of the food supply.

The focus of this study is the extraction and analysis of ten aminoglycosides from porcine tissues using Molecularly Imprinted Polymer (MIP) solid phase extraction with LC/MS/MS detection. The current USDA method does not reliably quantify levels of these analytes in porcine tissues.[1] This study utilized the unique extraction capabilities of MIPs to successfully quantitate ten aminoglycosides by LC/MS/MS at 100 ng/g (400 ng/g for neomycin) with recoveries ≥70%.

Molecularly Imprinted Polymers (MIPs) are solid phase extraction phases that are prepared by polymerizing either preformed or self-assembled monomer template complexes together with a cross-linking monomer. After removal of the template molecule, a polymer with binding sites for the template/analyte(s) of interest is obtained. MIPs exhibit selective target recognition and can be described as artificial receptors. Selectivity is predetermined by the template for a particular analyte or group of analytes. **Figure 1** illustrates a generalized synthesis and MIP interaction.

Figure 1. A Generalized Diagram of MIP Synthesis and Selective Analyte Interaction



Experimental

Muscle Preparation

Locally obtained pork muscle was homogenized with a commercially purchased food processor. Samples (2 g) were weighed into 50 mL polypropylene centrifuge tubes.* Samples were fortified using a mixed aminoglycoside standard prepared at 10 µg/mL from 1 mg/mL individual stock solution standards. The stock solutions were prepared from neat materials obtained from Sigma-Aldrich®. Ten milliliters of the extraction solvent, 10 mM of potassium phosphate monobasic (KH_2PO_4) with 0.4 mM ethylenediaminetetraacetic acid (EDTA) and 2% trichloroacetic acid (TCA) were added, and samples shaken for 2 min. Samples were then centrifuged for 5 min. at 3200 rpm, and the supernatant was decanted into a clean 50 mL polypropylene centrifuge tube. An additional 10 mL of extraction solvent was added to the pork muscle sample, and the shaking and centrifuge steps were repeated. The supernatant was combined with the previous supernatant, and then 20 mL of 50 mM potassium phosphate in water (pH = 7.8) was added. The pH of the sample was adjusted to 7.5 with concentrated ammonium hydroxide.

MIP SPE Sample Cleanup

The SupelMIP® SPE-Aminoglycoside cartridge was conditioned with 1 mL of methanol followed by 1 mL of 50 mM potassium phosphate in water (pH = 7.8). Three milliliters of extracted pork sample were passed through the cartridge. The cartridge was then washed with 3 mL of water, dried on high vacuum for 2-3 min, washed with 1 mL of 60:40 water:acetonitrile (v/v) and dried with slight vacuum for 10 sec. The cartridge was finally washed with 1 mL of 50:50 dichloromethane:methanol (v/v) and dried with slight vacuum for 10 sec. Analytes were eluted with 1 mL of 1% formic acid containing 5 mM heptafluorobutyric acid (HFBA) in 80:20 acetonitrile:water (v/v).

Sample Analysis

Eluted samples were vortexed and transferred to 750 µL polypropylene HPLC vials. Analytical separation was performed using an Ascentis® Express C18, 10 cm x 2.1 mm I.D., 2.7 µm HPLC column. **Figure 2** depicts chromatograms of the analytes in pork muscle extracts. Quantitation was performed using matrix matched calibration standards, ranging from concentrations of 10 ng/mL to 1000 ng/mL.

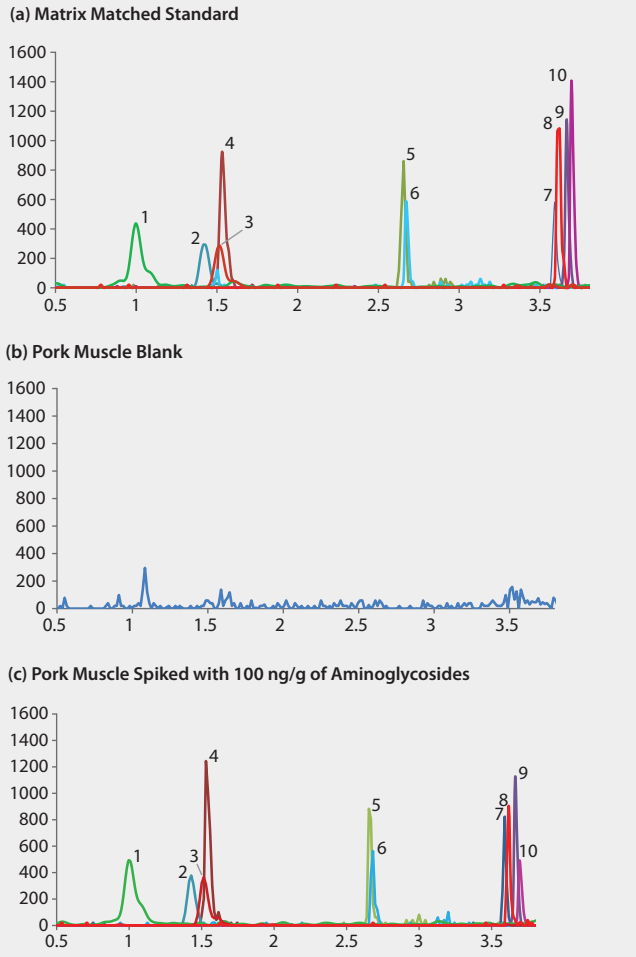
* Because aminoglycosides can bind to untreated glass surfaces, polypropylene vessels were used throughout the procedure.

Figure 2. LC/MS/MS Analysis of Aminoglycosides after SupelMIP SPE Cleanup

column: Ascentis Express C18, 10 cm x 2.1 mm I.D., 2.7 µm (53823-U)
mobile phase: (A) 5 mM HFBA in water; (B) 5 mM HFBA in acetonitrile
gradient: 20 to 90% B in 3.0 min; held at 90% B for 1 min; 90 to 20% B in 0.1 min; held at 20% B for 5.9 min
flow rate: 0.4 mL/min
column temp.: 40 °C
detector: MS/MS, ESI(+), MRM
injection: 10 µL

Analyte	Precursor	Product
Gentamicin C1	478.1	157.2
Streptomycin	582.1	263.2
Neomycin	615.0	161.1
Kanamycin	485.2	163.1
Tobramycin	468.1	163.1
Amikacin	586.2	163.1
Hygromycin B	528.1	177.1
Spectinomycin	351.1	333.1
Dihydrostreptomycin	584.2	263.1
Apramycin	540.2	217.1

1. Spectinomycin
2. Hygromycin B
3. Streptomycin
4. Dihydrostreptomycin
5. Amikacin
6. Kanamycin
7. Apramycin
8. Tobramycin
9. Gentamicin
10. Neomycin



Results and Discussion

Recoveries for the 10 aminoglycosides are given in **Figure 3**. Most of the analyte recoveries were ≥70%, except for neomycin and tobramycin. Low recoveries for neomycin and tobramycin may be attributed to insufficient elution of the analyte from the SPE material. Both of these analytes have a higher number of amino groups which could lead to stronger binding of the analyte to the MIP sorbent.

The use of the SupelMIP SPE-Aminoglycoside cartridge kept the analytes bound to the sorbent while a series of aggressive washes were applied to the sorbent to eliminate matrix interferences. The absence of matrix effects may be an indication of superior sample cleanup. The resulting matrix effects were evaluated by comparing solvent prepared standards to matrix-matched standards. The matrix factor was calculated for each analyte in **Figure 4**. Matrix factors close to 1.0 indicate little to no matrix influence on analyte detection. Values significantly greater than 1.0 suggest matrix enhancement on the analyte and values less than 1.0 are considered to be the result of matrix suppression. For these analytes, significant matrix enhancement was observed for neomycin and gentamicin with matrix factors greater than 2.

Figure 3. Aminoglycoside Recoveries in Pork Muscle Fortified at 100 ppb (400 ppb*)

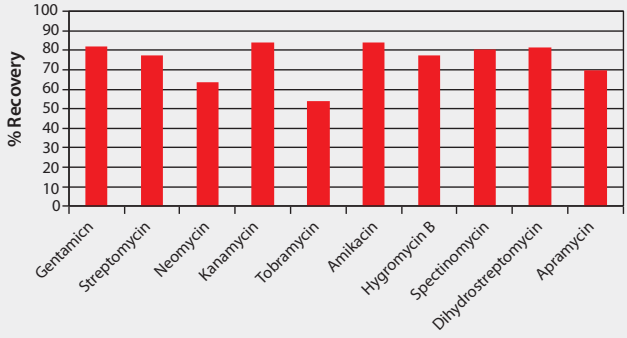


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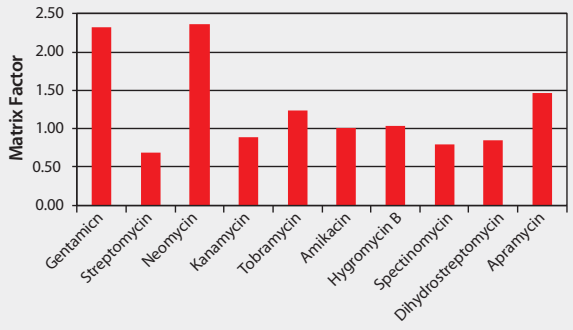
Figure 4. Matrix Factor for Aminoglycosides in Pork Muscle

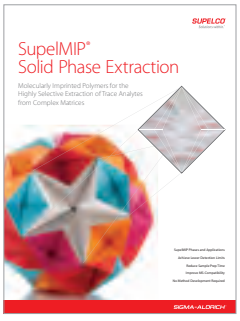


Conclusion

A simple and sensitive method for the cleanup, analysis and quantification of aminoglycosides in pork muscle has been developed using SupelMIP SPE-Aminoglycosides and LC/MS/MS analysis. This method was able to successfully obtain recoveries ≥70% for most analytes including amikacin; whereas, the current USDA method does not reliably confirm amikacin in porcine tissues.[1] The unique features of the MIP material afforded the ability to wash additional matrix interferences off of the cartridge prior to eluting the analytes of interest.

Reference

1. Confirmation of Aminoglycosides by HPLC-MS/MS (Revision 03). United States Department of Agriculture Food Safety and Inspection Service, Office of Public Health Science **www.fsis.usda.gov**



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sigma-aldrich.com/supelmip

Featured Products

Description	Cat. No.
SupelMIP Aminoglycosides SPE Cartridge	
3 mL, 50 ea	52777-U
Ascentis Express C18 HPLC Column	
10 cm x 2.1 mm I.D., 2.7 µm particle size	53823-U
Analytical Standards and Reagents	
Dihydrostreptomycin sesquisulfate	D7253
Streptomycin	S1277
Tobramycin sulfate	T1783
Kanamycin	K4000
Amikacin	A3650
Gentamicin sulfate	G1914
Neomycin	N1876
Apramycin	A2024
Spectinomycin	S2647
Hygromycin B	H3274

Related Products

Description	Cat. No.
Ascentis Express C18 HPLC Columns	
10 cm x 2.1 mm I.D., 2.7 µm particle size	53823-U
15 cm x 2.1 mm I.D., 2.7 µm particle size	53825-U

For more information on the Molecularly Imprinted Polymer SPE products or to request your **Free Sample**, visit us at
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Improved Recoveries and Lower Background for the Analysis of PAHs in Olive Oil using a Novel SPE Cartridge

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Background

Polynuclear aromatic hydrocarbons (PAHs) are a class of compounds found in the environment as a result of natural and man-made process, such as petroleum processing and incomplete combustion of fossil fuels. Some, such as benzo[*a*]pyrene, have been found to have carcinogenic properties in laboratory animals.[1] PAHs can find their way into the human body through direct contact with contaminated surfaces, breathing contaminated air (such as cigarette smoke), and through some foods. Due to the lipophilic nature of PAHs, they can easily migrate into edible oils such as olive oil. Contamination of olives and/or olive-based products can occur during growing, harvesting, storage, and production, with routes of exposure suspected to be due to industrial process and possibly even automobile exhaust.[2]

Spain and Italy are the largest consumers of olive oil, followed by the United States.[3] Olive oil is an essential part of the "Mediterranean Diet," which research has associated with a reduced risk of heart disease, cancer, Alzheimer's and Parkinson's diseases.[4] Concern over exposure to these compounds has resulted in some countries within the European Union to set limits on PAH content in olive oil. In 2011, European Union Commission Regulation No. 835/2011 set a maximum limit for PAHs in various foodstuffs, including edible oils. The limits for oils and fats (excluding cocoa butter and coconut oil) are 2 ng/g of benzo[*a*]pyrene individually, and 10 ng/g combined of benzo[*a*]pyrene, benzo[*b*]fluoranthene, chrysene and benzo[*a*]anthracene.[5]

Analysis of PAHs in olive oil poses an analytical challenge due to the problem of separating the lipophilic PAHs from fatty matrix. Traditional methods of extraction and cleanup have involved liquid/liquid extraction followed by gel permeation chromatography (GPC), or normal phase SPE on large columns. While GPC provides a very good cleanup technique, it requires the use of special instrumentation and often adds an extra day to the sample preparation time. Extraction and cleanup using normal phase SPE often requires the use of large glass columns, which are expensive, and require large volumes of solvent. In this study, a new approach for the extraction and cleanup of PAHs from olive oil was evaluated. The method developed uses Supelclean™ EZ-POP NP, a dual-layer SPE cartridge packed with Florisil® as the top sorbent bed, and a mixture of Z-Sep/C18 as the bottom bed. Florisil retains compounds with polar functionality, such as fatty acids. Z-Sep/C18 acts to retain fatty components by Lewis acid/base interaction (Z-Sep) and hydrophobic interaction (C18). In this way, lipids are preferentially retained, while the PAHs are eluted with acetonitrile. The resulting extract is then suitable for either HPLC/FLD or GC/MS analysis.

In this study, the EZ-POP NP was compared with a conventional silica gel SPE procedure for the extraction of PAHs from spiked olive oil. Background, as detected by GC/MS analysis, as well as recoveries and reproducibility were compared between the two techniques for PAHs containing from 2-6 rings in their structures.

Experimental

Extra-light olive oil was spiked at 20 ng/g with PAHs, and allowed to sit for several days prior to analysis. Replicate samples were extracted using EZ-POP NP SPE, and normal phase SPE on 5 g/20 mL silica gel cartridges from two different sources. The sample preparation procedures are listed in **Table 1**. Analysis was performed by GC/MS in selected ion mode (SIM) using the conditions listed in **Figure 1a**. Conditions were optimized for response and peak shape of the PAHs, especially those with 5 and 6 rings. Quantitation was performed using a 5-point calibration curve prepared in unspiked olive oil extract.

Figure 1a. GC/MS Analysis of Olive Oil Extracts for PAH quantitation

column:	SLB®-5ms, 20 m x 0.18 mm I.D., 0.18 µm (28564-U)
oven:	60 °C (1 min.), 15 °C/min. to 250 °C, 8 °C/min. to 330 °C (7 min.)
inj. temp.:	300 °C
MS interface:	330 °C
MS source temp.:	250 °C
MS quad. temp.:	200 °C
injection:	1 µL, pulsed splitless (50 psi until 0.75 min, splitter open at 0.75 min.)
carrier gas:	helium, 1 mL/min constant flow
liner:	4 mm ID FocusLiner™ with taper and quartz wool
GC/MS:	Agilent® 7890/5975, selected ion mode (SIM)

Figure 1b. Total Ion Chromatogram (TIC) of Extracts (same y-scale) for background analysis.

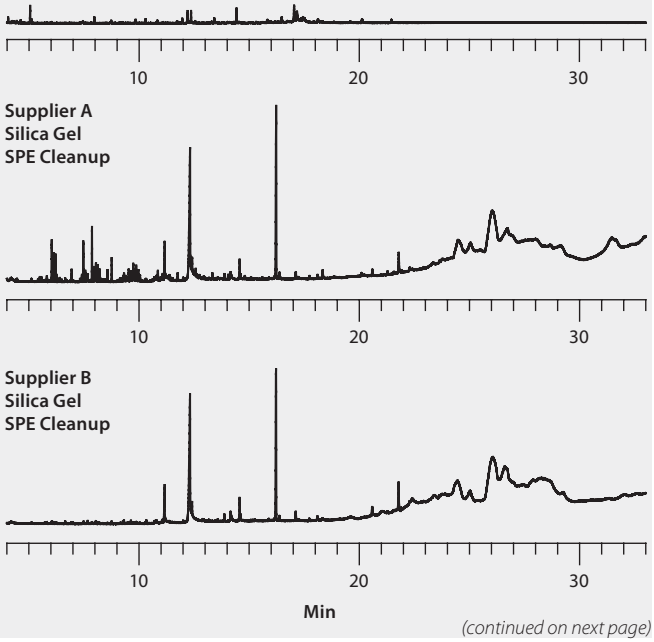

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Table 1. SPE Procedures

	Silica Gel SPE, 5 g/20 mL	EZ-POP NP
Condition	20 mL of hexane (gravity)	10 mL of acetone (gravity), followed by drying at -10 to -15" Hg for 10 min.
Load	1 mL of 5 g oil diluted to final volume of 10 mL in hexane	0.5 mL of oil, weighed directly onto cartridge
Wash	3 mL of hexane:methylene chloride (70:30)	None
Elute	8 mL of hexane:methylene chloride (70:30)	15 mL of acetonitrile, applied in 2 x 7.5 mL volumes
Flow Rate	Gravity	Approx. 1 drop / second (using vacuum)
Concentration	Final volume = 1 mL, 40 °C under nitrogen	Final volume = 1 mL, 40 °C under nitrogen

Results and Discussion

Olive oil extracts were analyzed by GC/MS in full scan mode. Comparisons of the total ion chromatograms (TICs) generated from both extractions (same Y-scale) are presented in **Figure 1b**. Much higher background was observed for both silica gel extracts compared to EZ-POP NP. During actual sample analysis, the late eluting background present in the silica gel extracts contaminated the GC/MS system, and required extensive bake out to remove. This heavy background also caused retention time shifts of the last 6 eluting PAH peaks, making identification difficult. In the case of brand B silica gel, the last internal standard, perylene-d_{12}, could not be detected.

Recoveries and Reproducibility

A graphical representation of the average recoveries obtained with each extraction is presented in **Figure 2**. The error bars represent the reproducibility (as %RSD) for each n=3 data set. Overall, recoveries and reproducibilities were better using EZ-POP NP than silica gel. As noted previously, with the silica gel prepared extracts, specific matrix effect problems were encountered with the heavier PAHs. In addition, background made integration of the lighter PAHs difficult, which affected recovery values. Response of some PAHs in the silica gel extracts dropped as the sequence of runs progressed. This was probably due to contamination of the GC system. In comparison, the extracts prepared using EZ-POP NP did not exhibit these issues.

Detection of PAHs at Lower Levels

Using a single quadrupole GC/MS system for detection of PAHs at the 2 ng/g level designated in EU Commission Regulation 835/2011 requires some modifications to the method described earlier. Specifically, it may be necessary to concentrate the samples to a lower final volume in order to increase the mass injected into the instrument. A second set of olive oil extracts was prepared using EZ-POP NP. This set was spiked at the EU limit of 2 ng/g with benzo[*a*]pyrene. The extraction was performed as described in **Table 1**, except the extract was concentrated to a final volume of 0.2 mL prior to the GC/MS analysis. Quantitation was done using a calibration curve prepared in unspiked olive oil extracts that were also concentrated to this lower final volume.

In addition, these minor changes were made to the GC run conditions to enhance response:

- Substitution of 2 mm I.D. FocusLiner with taper for the 4 mm I.D. liner
- Reduction of the injection volume from 1 µL to 0.5 µL

The smaller ID liner increased response of the heaviest PAHs, and reduction of the injection volume was necessary to ensure that the vapor cloud resulting from the acetonitrile injection did not exceed the liner's capacity.

The resulting recovery is summarized in **Table 2**. For comparison, the recovery obtained at 20 ng/g in the previous experiment is included. Recovery of benzo[*a*]pyrene at the lower spiking level was comparable to the 20 ng/g spikes. In addition, reproducibility was still acceptable, with an RSD value of 17%.

Table 2. Recovery of Benzo[*a*]pyrene Spike from Spiked Olive Oil using EZ-POP NP with (%RSD)

	2 ng/g Spike Level, n=4	20 ng/g Spike Level, n=3
Benzo[*a*]pyrene	91 (17)	91 (20)

GC/MS Method Ruggedness

In GC analysis, a loss of response can occur as a result of residue buildup in the inlet liner and column, and contamination of the detector. In order for the SPE method used to be acceptable, it must produce an extract that will not cause rapid fouling of the GC or detector. The cleanliness of the olive oil extract produced using EZ-POP NP was evaluated by repeated injections (98 total) of a 20 ng/g spiked extract into the GC/MS system. Absolute response of the PAHs was monitored. It was found that response for the PAHs was fairly stable (RSD <10%) up to 45 injections. After this point, response declined gradually. The total decrease in response after 98 injections was generally in the range of 20-30% (**Figure 3**); however, all PAHs were easily detected at the last injection. After completion of the testing sequence, the liner was removed from the GC system, and found to be free of visible residue (**Figure 4**).

Conclusions

The combination of Florisil and Z-Sep/C18 mixture in the 2-bed EZ-POP NP SPE cartridge was able to extract a full range of PAHs (2-6 rings) from olive oil. For the PAHs studied, the recoveries and reproducibility obtained were better than normal phase SPE using silica gel, and the extract was substantially cleaner. Low level analysis was possible, as demonstrated with benzo[*a*]pyrene at the EU regulated level of 2 ng/g. The EZ-POP NP method contains minimal steps, uses less solvent than silica gel SPE, and produces a final extract in acetonitrile, which is amenable to either HPLC/FLD or GC/MS analysis. In the latter case, the cleanup is sufficient for analysis on single quadrupole MS instrumentation, without the need for MS/MS detection or hardware modifications to the GC system. Ruggedness testing with repeated injections of an olive oil extract did not prematurely foul the GC/MS system, with all PAHs still easily detected after 98 total runs.

Figure 2. Recoveries and Reproducibility of PAHs Extracted from Olive Oil using Silica Gel and Supelclean EZ-POP NP SPE (n=3 for each data set). Spiking Level of 20 ng/g

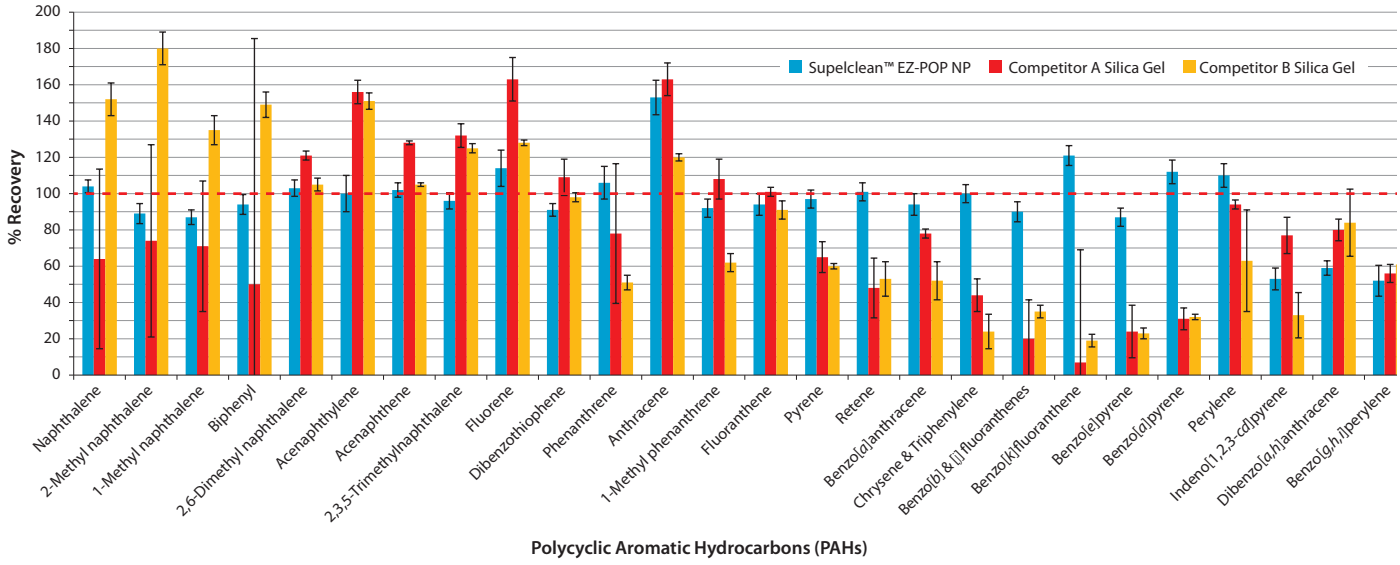

Figure 3. Change in Absolute Response of PAHs in Olive Oil Extract Produced using the Supelclean EZ-POP NP after 98 Injections into GC/MS System

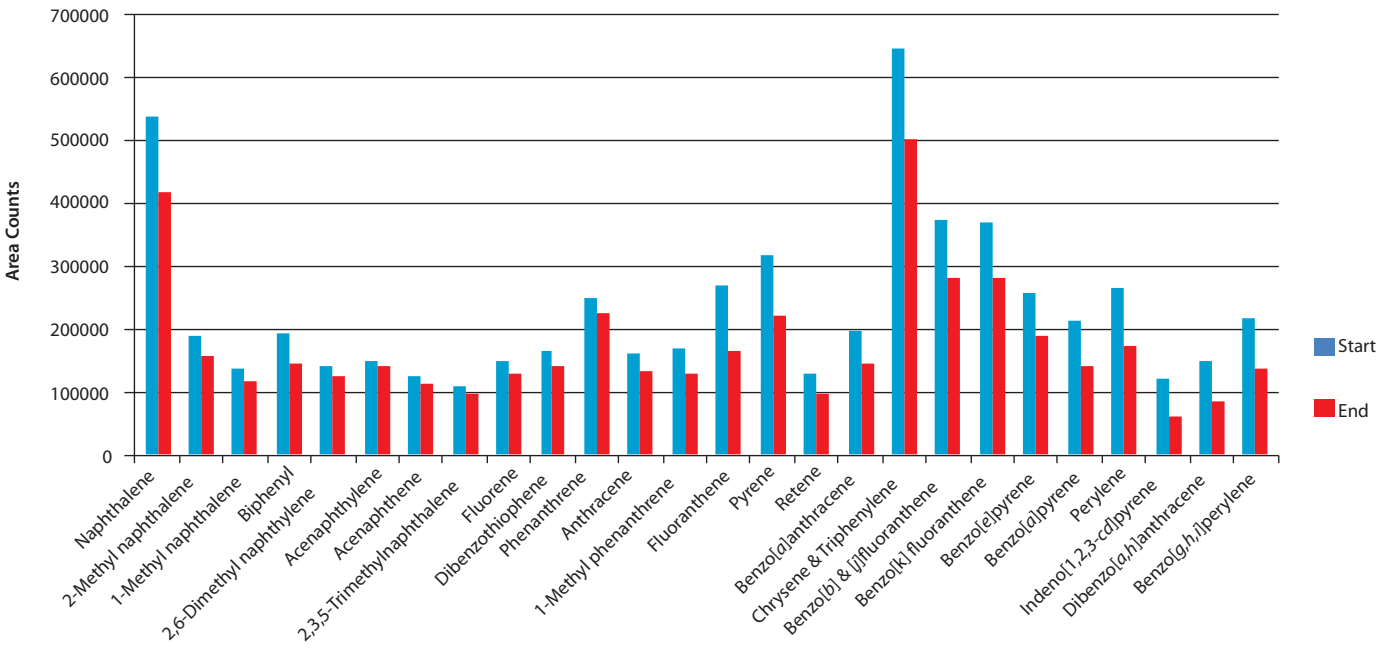

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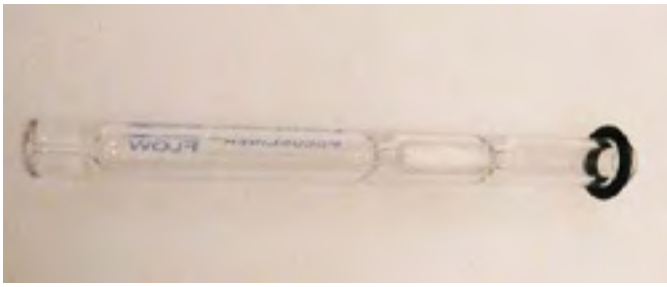
Figure 4. GC Inlet Liner after Ruggedness Testing with Olive Oil Extract Prepared using Supelclean EZ-POP NP



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Featured Products

Description	Cat. No.
Supelclean SPE Cartridge	
EZ-POP NP, 12 mL, 20 ea	54341-U
SLB-5ms Capillary GC Column	
20 m x 0.18 mm I.D., 0.18 µm	28564-U
Analytical Standards	
Polynuclear Aromatic Hydrocarbons Mix, TraceCERT® CRM – 2000 µg/mL each component in methylene chloride:benzene (1:1)	CRM48905
Naphthalene-d_8 solution – 2000 µg/mL in methylene chloride	48715-U
Fluoranthene-d_{10} – 50 mg ampul	442843
Perylene-d_{12} solution – 2000 µg/mL in methylene chloride	48081
Solvent	
Acetonitrile, LC-MS CHROMASOLV®	34967

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Determination of Triclosan in Environmental Waters using Polymeric SPE Cleanup and HPLC with Mass Spectrometric Detection

Olga Shimelis, Principal R&D Scientist; Michael Halpenny, R&D Technician; Ken Espenschied, R&D Technician; and Kristen Schultz, Air Sampling Product Manager
kristen.schultz@sial.com

Introduction

Triclosan is a chemical that has been used as an antibacterial agent in many soaps and a variety of other consumer and personal care products for many years. Because of its widespread use and high production volume (exceeding 1 million pounds annually based on a 1998 U.S. EPA assessment), the US FDA is involved in "scientific and regulatory review of this ingredient". In 2003-2004, the US Centers for Disease Control detected the chemical in the urine of 75% of people tested.[2] Recent animal studies have shown that triclosan is a potential endocrine disruptor. With regard to the impact of triclosan on the environment, monitoring programs found the chemical in many tested streams, where it is introduced through the discharge of wastewater treatment plants.[3] Triclosan was included in EPA Method 1694 developed for the measurement of pharmaceutical and personal care products by LC/MS/MS.[4]

In this study, triclosan was detected in the environmental samples of local water using Supel™-Select HLB polymeric SPE for cleanup and an Ascentis® Express C18 HPLC column for detection.

Experimental

The water samples were collected from the discharge of the local wastewater treatment plant. The samples were also collected from the stream about 360 m below the discharge location.

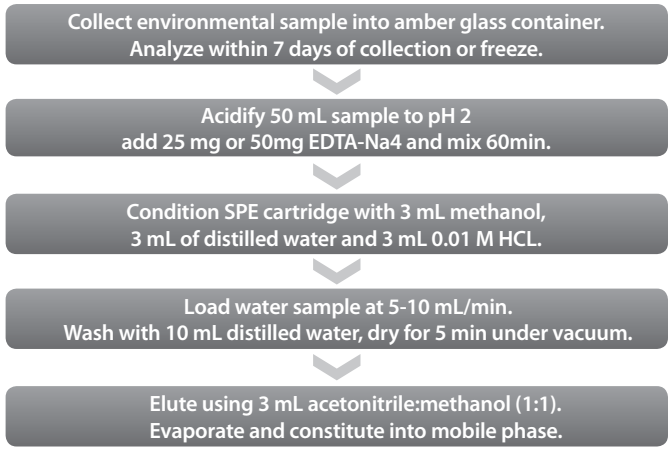
The method was developed by adapting standard EPA method 1694 for analysis of triclosan and using Supel-Select HLB SPE cartridges. The modifications to method 1694 included introduction of an alternate elution solvent -- 1:1 acetonitrile:methanol. It was better suited for elution of the analyte from the HLB cartridge. The sample loading volume was reduced to 50 mL from 500 mL and the elution volume was reduced to 3 mL instead of 6 mL used in the standard method. The final methodology for detection of triclosan is presented in **Figure 1**.

Both UV and MS/MS detection methods were tested for the environmental water samples. Higher concentration of triclosan in the wastewater plant eluent enabled its detection by UV while lower concentration of triclosan in the stream required MS/MS detection for accurate quantitation. The HPLC method was developed using Ascentis Express C18 HPLC column. The Fused-Core® technology provided good resolution of triclosan peak from the background peaks in the samples, resulting in sharper peaks and lower limits of detections for triclosan. The Ascentis Express columns allowed the separation to be run at low backpressure using existing HPLC instruments (Agilent® 1200 and Agilent 1100 stacks were used).

Results

Figure 2 shows the resulting UV chromatograms of water samples. **Figure 3** shows the MS/MS chromatograms for the same water samples.

Figure 1. Sample Preparation Method for Analysis of Water Samples for Triclosan using Supel-Select HLB SPE 60 mg/3 mL (54182-U)



Discussion

Limit of detection for triclosan by UV method was estimated at 100 ng/L when using 50 mL loading water sample. EPA method 1694 specified the detection limit for triclosan as 94 ng/L using 500 mL loading SPE volume and MS/MS detection. The use of Ascentis Express column resulted in sharper peaks and allowed the low detection limit to be met using less sensitive UV detection technique.

The concentration of triclosan in the sample from wastewater plant was found to be high at about 1,000 ng/L. This concentration was easily detectable by UV method. MS/MS method confirmed that the UV method provided correct concentration result.

In the samples taken 360 m downstream, the triclosan levels were further diluted by the stream water, producing results that were below the detection limits for the UV method. The MS/MS methodology allowed the detection of triclosan in the stream below the discharge from wastewater treatment plant. The detection limit for triclosan by MS/MS method was estimated to be 50 ng/L when using 50 mL water sample.

Table 1. Results of Method Validation of Triclosan in Water (n=3)

Sample	Conc. By UV	Recovery
Distilled water, spiked at 4000 ng/L	3500 ng/L	88%
Stream water, spiked at 4000 ng/L	3300 ng/L	82%

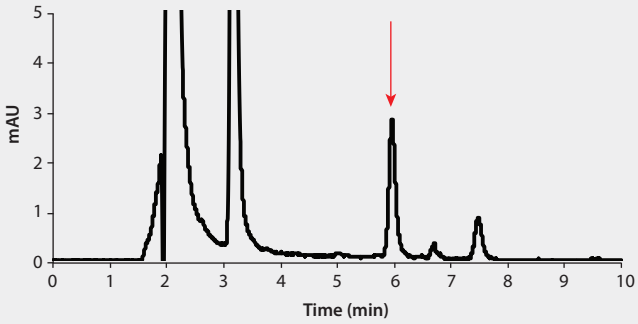
Table 2. Results for Triclosan Identification in Environmental Waters (n=3)

Sample	Water Conc. by UV (ng/L)	Water Conc. by MS-MS (ng/L)
Effluent from wastewater plant	943 (6%)	1100 (13%)
Stream water	Below LOD	63 (9%)

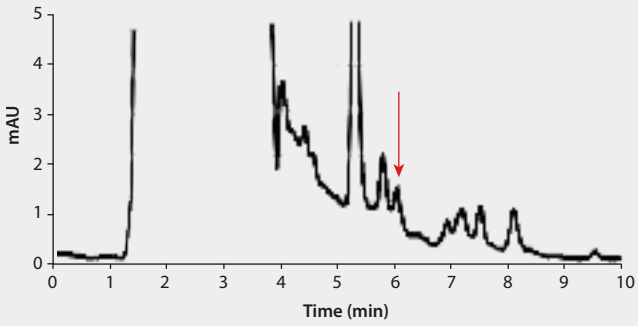
Figure 2. UV Chromatograms of Triclosan in Water at 280 nm

column: Ascentis Express C18, 15 x 2.1 mm I.D., 2.7 µm (53825-U)
mobile phase: 40% methanol, 40% acetonitrile, 20% potassium phosphate tribasic, pH 2.5
flow rate: 0.150 mL/min
temp 30 °C
detector: UV 280 nm
column pressure: 112 bar
injector volume: 10.0 µL
instrument: Agilent 1200 Series HPLC-UV

2a. Spiked Into Distilled Water at 5000 ng/L



2b. In the Wastewater Plant Eluent



Conclusions

A method for detection of triclosan in environmental water samples was developed using Supel Select SPE cleanup and convenient 50 mL loading sample volume. A Fused-Core Ascentis Express C18 HPLC column allowed sharper peaks, lower detection limits with low backpressure and was used in existing HPLC systems. The triclosan was detected in the wastewater plant discharge by both UV and MS/MS methods at about 1,000 ng/L. Triclosan was present in stream water at 63 ng/L and at this level required MS/MS detection.

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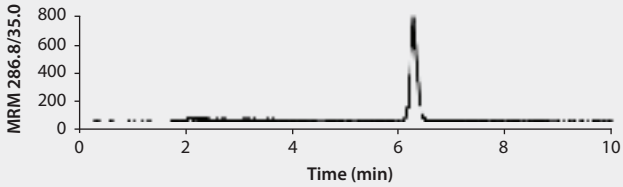
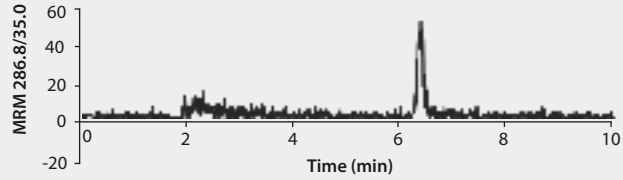
Figure 3. LC/MS/MS Chromatograms of Triclosan in Water

column: Ascentis Express C18, 15 cm x 2.1 mm I.D., 2.7 µm (53825-U)
mobile phase: 40% methanol, 40% acetonitrile, 20% aqueous 0.1% formic acid
flow rate: 0.150 mL/min
column temp: 30 °C
injection: 10.0 µL
detector: Q1/Q3 286.8/35.0 ESI negative
instrument: Agilent 1100/1200 HPLC and AB SCIEX QTRAP® 3200

3a. In Wastewater Plant Eluent



3b. In Stream Water 360 m downstream



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3. http://www.epa.gov/oppsrrd1/REDs/factsheets/triclosan_fs.htm
4. US EPA Method 1694 "Pharmaceutical and personal care products in water, soil, sediment and biosolids by HPLC/MS/MS" http://water.epa.gov/scitech/methods/cwa/bioindicators/upload/2008_01_03_methods_method_1694.pdf

Featured Products

Description	Cat. No.
Supel-Select HLB SPE Tube	
60 mg/3 mL	54182-U
Ascentis Express HPLC Column	
C18, 15 cm x 2.1 mm I.D., 2.7 µm particle size	53825-U
Analytical Solvents	
Acetonitrile, CHROMASOLV® Plus, for HPLC, ≥99.9%	34998
Methanol, CHROMASOLV Plus, for HPLC, ≥99.9%	34860
Acetonitrile, LC-MS Ultra CHROMASOLV	14261
Methanol, LC-MS Ultra CHROMASOLV	14262
Water with 0.1% formic acid, LC-MS CHROMASOLV	34673
Analytical Standard	
Triclosan, 5 g	72779-5G-F

Related Products

Description	Cat. No.
Vials	
Certified Vial Kit, Low Adsorption (LA), 2 mL, pk of 100	29652-U

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Solid Phase Microextraction (SPME) Method for 4-Methyl-1-cyclohexanemethanol (MCHM) in Water

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Introduction

4-Methylcyclohexanemethanol (MCHM) is a chemical used in the coal cleaning process. On January 9, 2014 there was an accidental release of an estimated 100,000 gallons of a chemical mixture containing MCHM into the Elk River in West Virginia. According to the owners of the tank, the mix contained 88.5% MCHM, 7.3% PPH stripped basic, and 4.2% water. PPH stripped basic consists of a mixture of propylene glycol phenyl ether (PPH) and dipropylene glycol phenyl ether. Consequently, drinking water for the local community of Charleston became contaminated, with the MCHM imparting a "licorice-like" odor to it. The Center for Disease Control (CDC) has set advisory limits in drinking water of 1 ppm maximum for MCHM and 1.2 ppm for PPH. [1,2] Consequently, this initiated an immediate need for water testing. Since there was no established test method for MCHM, labs had to develop their own within a short amount of time. Solid phase microextraction (SPME) is a widely accepted technique for trace level determination of a variety of compounds in many different matrices. Method development can often be more rapid than other techniques such as solid phase extraction (SPE) and liquid-liquid extraction (LLE). In direct comparison to these techniques, it is easier to automate and requires fewer laboratory consumables such as solvents and SPE cartridges. In this application, a rapid extraction method for MCHM from drinking water and surface water was developed. The method, which was a headspace extraction, required a total time of 10 minutes, including equilibration and extraction. Combined with GC/MS-SIM analysis, detection of MCHM was achieved at low part per billion levels. This method was used to analyze samples of surface and tap water collected locally, as well as a sample of tap water from Charleston, WV which was collected several months after the spill incident. PPH was included in the SPME method; however, as will be shown, there were stability issues for this compound in the presence of chlorine.

Experimental

The SPME and GC/MS analysis conditions are summarized in **Tables 1** and **2**. Quantitation was done using an external standard 5-point calibration curve from 0.3 µg/L to 10 µg/L, prepared in deionized water and analyzed with each set of samples. MCHM elutes as two isomers. The relative ratio of the isomers (1:3) was determined by liquid injection of the standard into the GC/MS system. This ratio was used to calculate the amounts of each isomer in the calibration standards. Quantitation was then done separately for each MCHM isomer. Calibration was linear for both MCHM isomers and PPH, with r2 values of >0.995.

For quantitation of MCHM, m/z=110 was used. The most abundant ions in the MS spectrum of MCHM are m/z=55 and m/z=97 (**Figure 1**); however, there was an interference coeluting with isomer #1 detected in blank samples, which contained these two ions.

For determination of accuracy and reproducibility, samples of drinking water and surface water were collected locally, and spiked with MCHM and PPH at 1 µg/L. A method detection limit study was conducted using deionized water spiked at 1 µg/L.

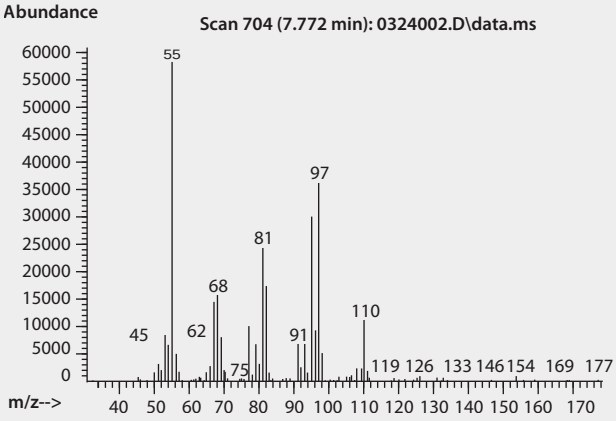
Table 1. SPME Conditions

Fiber	100 µm PDMS (57301)
Extraction	Headspace, 5 min equilibration, 5 min extraction @ 50 °C, agitation 250 rpm
Desorption Process	270 °C, 2 min (splitter open at 0.75 min)
Fiber Post Bake	5 min, 270 °C
Sample/Matrix	6 mL + 1.5 g NaCl (25%) in 10 mL headspace vial

Table 2. GC/MS Conditions

GC Column	VOCOL, 20 m x 0.18 mm I.D., 1.0 µm (28463-U)
Oven	50 °C (1 min), 15°C/min to 220 °C (5 min)
Carrier Gas	helium, 1 mL/min
Liner	0.75 mm I.D. SPME liner
MS Conditions	aux at 220 °C, source at 230 °C, quads at 150 °C, SIM: m/z= 110 (quant), 97 for MCHM; m/z=94 (quant), 152 for PPH

Figure 1. MS spectra of MCHM



Results

Accuracy and Reproducibility. A summary of the amounts measured in spiked local water samples are presented in **Table 3**. No MCHM or PPH were detected in the unspiked samples. Accuracy and reproducibility (indicated by %RSD values in parentheses) were very good, with the exception of PPH in the tap water sample. Since this compound was detected easily in the spiked private well and stream water samples, it is believed that the presence of chlorine in the tap water may have degraded the PPH.

A representative chromatogram obtained from the GC/MS-SIM analysis of the stream water is presented in **Figure 2.** The MCHM and PPH peaks were easily detectable above the sample background. The addition of salt to the samples prior to extraction was found to increase response by 10 times, and thus, is critical to achieving a low detection level. However, data generated during analysis of the

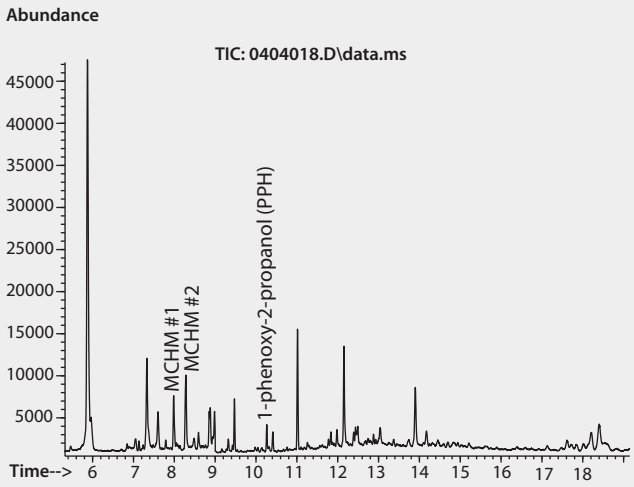
method detection limit samples indicates that it is detrimental to the determination of PPH if samples are prepared in advance of analysis.

Table 3. Accuracy and Reproducibility Results Using SPME Method, n=4

	Local Private Well Water	Local Stream Water	Local Municipal Tap Water
Spiking L evel	1 µg/L*	1 µg/L*	1 µg/L*
amt. measured MCHM isomer #1	0.27 (6)	0.30 (6)	0.25 (3)
amt. measured MCHM isomer #2	0.84 (9)	0.91 (11)	0.84 (6)
MCHM total (two isomers)	1.11 µg/L	1.21 µg/L	1.12 µg/L
amt. measured PPH	0.94 (1)	1.16 (6)	ND

Note: amt. measured in µg/L; *total MCHM

Figure 2. TIC of local stream water, spiked at 1 ppb; extracted using SPME



Method Detection Limit Study. A set of 7 deionized water samples, spiked at 0.5 ug/L total MCHM, were analyzed and used to calculate a method detection limit (MDL). The method used for calculating MDL was as described by EPA in 40 CFR Part 136, Appendix B rev. The results of this study are summarized in **Table 4**. The MDL for MCHM was calculated to be 0.08 µg/L for isomer #1 and 0.33 µg/L for isomer #2. The limits of quantitation (LOQ) for each isomer were 0.25 and 1.0 µg/L, respectively. PPH results were low with regards to amount measured vs. spiked, and highly variable within the sample set, thus an MDL and LOQ could not be calculated. It is believed that the presence of the salt added for the SPME procedure generated chlorine over time, which resulted in decomposition of the PPH, similar to what was observed in the chlorinated tap water samples.

Table 4. Results of Method Detection Limit (MDL) Study (n=7); spiking level 0.5 µg/L total MCHM

	Mean	Std. Dev	MDL (µg/L)	LOQ (µg/L)
MCHM isomer #1	0.10 µg/L	0.03	0.08	0.25
MCHM isomer #2	0.34 µg/L	0.11	0.33	1.0
PPH	Not determined			

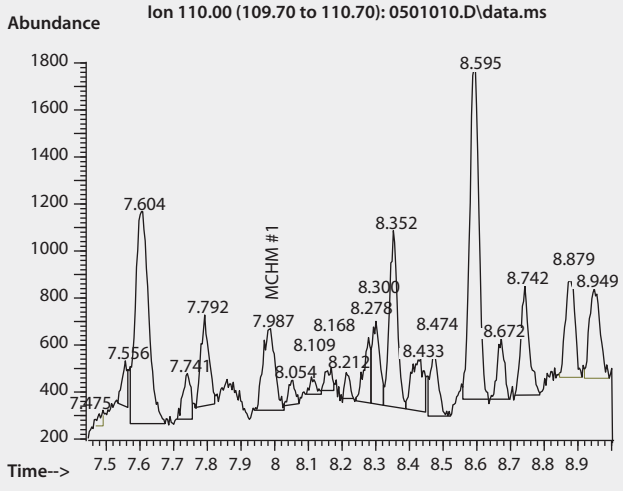
WV Tap Water Sample. A sample of tap water from Charleston, WV collected approximately 3 months after the spill was tested using the SPME method. Three replicates of sample were analyzed without spiking. Two additional samples were spiked at 1 µg/L of total MCHM and run as matrix spikes (MS/MSD). Spiked deionized water was also analyzed as a laboratory control standard (LCS). The MS/MSD and LCS showed good accuracy for determination of the MCHM isomers at the spiking levels indicated (**Table 5**). There was no recovery of the PPH in the matrix spikes, confirming the findings of the effect of chlorination seen with the previous local tap water. A peak corresponding to MCHM isomer #1 was detected in the unspiked WV tap water samples (**Figure 3**); however, the level calculated was below the LOQ for the method. Isomer #2 was not detected above the MDL.

Table 5. Analysis Results of Charleston, WV Tap Water Sample

	WV Tap Water	MS	MSD	LCS
Spiking Level	unspiked	1 µg/L*	1 µg/L*	1 µg/L*
MCHM isomer #1; average of n=3	0.09 µg/L (9)	0.29	0.28	0.20
MCHM isomer #2	<0.33 µg/L	0.67	0.67	0.64
PPH	ND	ND	ND	ND

*Spiking levels of individual MCHM isomers: MCHM #1–0.25 µg/L and MCHM #2–0.75 µg/L.

Figure 3. Peak corresponding to MCHM isomer #1 in municipal tap water collected in Charleston, WV. The peak was calculated to be above the method detection limit but below the limit of quantitation



Conclusion

Solid phase microextraction can be used to determine MCHM at low level from water. The detection level for the method described was calculated to be 0.08 µg/L for isomer #1 and 0.33 µg/L for isomer #2. The limits of quantitation were 0.25 and 1.0 µg/L, respectively, for the two isomers. Lower limits may be achievable with further optimization of the GC/MS instrument, allowing use of the more abundant ions in the spectrum of MCHM for detection and quantitation.

PPH appears to be unstable in the presence of chlorine, making reliable detection difficult. It could not be recovered from chlorinated tap water, or deionized water that had been treated with sodium chloride and allowed to sit for a prolonged time prior to analysis.

References

1. http://www.cnn.com/2014/01/09/us/west-virginia-contaminated-water/index.htmlCNN.com
2. http://emergency.cdc.gov/chemical/MCHM/westvirginia2014/index.asp
3. 40 CFR Appendix B to Part 136. Definition and Procedure for the Determination of the Method Detection Limit – Rev. 1.11

Featured Products

Description	Qty.	Cat. No.
SPME Assembly 100 µm, PDMS FS 24GA Auto (RED)	3	57301
VOCOL, 20 m x 0.18 mm I.D., 1.0 µm	1	28463-U

Variables that Impact Immunosuppressant LC/MS/MS Analysis

Extracted from a paper by Josh Cooper, Beth Marek, Isil Dilek, Uma Sreenivasan; Cerilliant®

Maximilian Magana, Technical Marketing Specialist
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ISO GUIDE 34
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GMP/GLP

Immunosuppressants such as everolimus, sirolimus, tacrolimus, and cyclosporin A are routinely used to prevent organ rejection in transplant patients. These drugs require clinical monitoring since they act within a narrow therapeutic range of concentrations and show large differences among individual patients in drug handling and efficacy.

With improved sensitivity and selectivity compared to immunoassay methods, LC-MS/MS techniques have become a clinical laboratory's method of choice to analyze immunosuppressant levels in patient whole-blood samples. Clinical analysis of immunosuppressant drug levels by LC-MS/MS can be difficult, because the whole-blood matrix requires extensive sample preparation. Additionally, the large size of immunosuppressant molecules makes obtaining reasonable peak shape chromatographically challenging.

LC-MS/MS method development revealed extensive sample-to-sample variability for multicomponent spiking solutions of everolimus, sirolimus, and tacrolimus. Several parameters such as sample preparation, MS interference, and surface interaction between compounds and the glass sample containers were investigated to determine the cause of the observed analytical variability.

An exhaustive investigation revealed that fill volume and to a lesser extent compound-to-compound interactions had contributed to the high sample-to-sample variability observed in the multi-component solutions. Special consideration should be applied during preparation and storage of spiking solutions used in the preparation of matrix calibrators, especially immunosuppressant solutions in low volumes and low concentrations.

These immunosuppressant standards are part of a large catalog of solution certified reference materials (CRMs) developed by Cerilliant to address the stringent and complex requirements of therapeutic drug monitoring and other clinical applications.

Cerilliant's extensive catalog of certified reference standards can be viewed at **sigma-aldrich.com/cerilliant**

To receive notifications of newly developed reference standards, register at **sigma-aldrich.com/registercerilliant**

Featured Products

Cat. No.	Description	Package Size
A-094	Ascomycin	1.0 mg/mL in acetonitrile
C-093	Cyclosporin A	1.0 mg/mL in acetonitrile
C-104	Cyclosporin A	100 µg/mL in acetonitrile
C-108	Cyclosporin D	1.0 mg/mL in acetonitrile
E-068	Everolimus	1.0 mg/mL in acetonitrile
E-070	Everolimus-D_4	100 µg/mL in acetonitrile
M-106	Mycophenolic acid	1.0 mg/mL in acetonitrile
M-135	Mycophenolic acid-β-D-glucuronide	1.0 mg/mL in acetonitrile
M-137	Mycophenolic acid-D_3	100 µg/mL in acetonitrile
M-180	Mycophenolic acid-D_3	1.0 mg/mL in acetonitrile
S-015	Sirolimus (Rapamycin)	1.0 mg/mL in acetonitrile
T-049	Tacrolimus	1.0 mg/mL in acetonitrile

A copy of the full paper including mass spectra can be obtained from: **cerilliant.com/immuno.aspx**

Combating the Rise of New Designer Drugs: Improved GC/MS Derivatization Techniques

For Analysis of Methylone, Ethylone, Butylone, Mephedrone and Methedrone

Extracted from a paper by Ryan Carrell, Isil Dilek, Ning Chang, Uma Sreenivasan, Yunming Ying, Greg Kirkovits; Cerilliant

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jaspreet.kaur-nandra@sial.com

Cathinone-based stimulants emerged as a regulatory and social threat in the European Union, Asia, and US in late 2010. Marketed as "bath salts" at head shops and on the internet, these synthetic cathinones offer recreational highs that mimic the effects of illegal drugs such as cocaine, methamphetamine and LSD. Packaging labels list the compounds as "not for human consumption," a tactic used to circumvent regulatory control and maintain legal status.

Drugs such as methylone, ethylone, mephedrone, butylone and other methcathinone analogues are commonplace and as they have increased in popularity, toxicologists require certified native and labeled reference materials to accurately identify and quantify the new compounds. There have been problems with use of deuterated internal standards of these drugs due to loss of label in the GC/MS fragmentation of the derivatized compound with PFPA and BSTFA such that an alternative method was required. By freeing the base with 0.1 M sodium bicarbonate and derivatizing withTrifluoroacetic anhydride (TFAA) in controlled temperature conditions, the decomposition of the α-amino ketones was prevented. It also enables butylone and ethylone to be readily distinguished and retains the deuterium label in the fragment ions.

The reaction scheme for this acylation step is shown in **Figure 1.** A great advantage of using GC/MS for this assay is the adaptability of the derivatisation method to many other related compounds of clinical / toxicological interest, such as amphetamines and MDA/MDMA/MDEA or any primary or secondary amine.

- Thermally stable derivatives, reproducible derivatisation
- Increased mass spectral abundances for molecular and fragment ions
- Labeled analogues retain the deuterium label in the fragment ions
- Butylone and ethylone are readily distinguished by the different relative abundance of two common fragment ions.

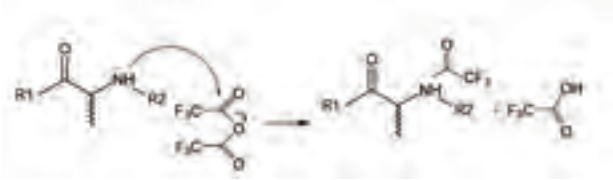

Figure 1. Acylation of Amines and the paragraph highlighted on page 27.

These standards are part of a range of ready-to-use solution-based certified reference standards developed by Cerilliant that are packaged in a format that preserves concentration and integrity of the material. Time saving to the toxicologist, they provide a convenient, consistent and cost-effective alternative to the individual preparation of working reference standard solutions in the analytical lab.

The synthesis of both native and deuterated cathinones was developed specifically for the assay of methcathinones; they are suitable for both GC/MS and LC/MS applications. An HPLC method is also available in the Cathinone brochure featured on page 28.

These CRMs are just a small part of a range of solution standards for forensic and toxicology applications that is continually being updated.

To receive notifications of newly developed standards, register at
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The current range can also be viewed on
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A copy of the full paper including mass spectra can be obtained from
sigma-aldrich.com/cathinone-poster-2011

Previously presented at the Society of Forensic Toxicologists (SOFT)/ The International Association of Forensic Toxicologists (TIAFT), San Francisco, CA, September 2011.

Featured Products

Cat. No.	Description	Package Size
B-045	Butylone HCl	1.0 mg/mL (as free base) in MeOH
B-046	Butylone-D_3 HCl	100 µg/mL (as free base) in MeOH
E-071	Ethylone HCl	1.0 mg/mL (as free base) in MeOH
E-072	Ethylone- D_5 HCl	100 µg/mL (as free base) in MeOH
M-138	Mephedrone HCl	1.0 mg/mL (as free base) in MeOH
M-139	Mephedrone-D_3 HCl	100 µg/mL (as free base) in MeOH
M-140	Methylone HCl,	1.0 mg/mL (as free base) in MeOH
M-141	Methylone-D_3 HCl	100 µg/mL (as free base) in MeOH
M-147	Methedrone HCl	1.0 mg/mL (as free base) in MeOH

Related Products

Cat. No.	Description	Package Size
91719	Trifluoroacetic anhydride for GC Drivatisation	10x1mL, 10mL, 50mL

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Supelco SLB-IL60 Ionic Liquid GC Columns: Unique Selectivity & Alternative to PEG/"Wax" Columns

Leonard M. Sidisky, R&D Manager; Katherine K. Stenerson, Principal Scientist; and Michael D. Buchanan, Product Manager
mike.buchanan@sial.com

The SLB®-IL60 gas chromatography (GC) column is based on the ionic liquid stationary phase platform, and displays desirable features that existing non-ionic liquid columns do not. This is the first of several Reporter articles which explore various aspects of this column.

The SLB-IL60 column is able to undergo the same analyte-phase interactions as polyethylene glycol (PEG) columns, but at different relative amounts. Based on its unique phase structure, the SLB-IL60 column is also able to undergo additional interactions that PEG columns cannot.

- With PEG columns, possible interactions appear to be dispersive, hydrogen bonding, and acid-base interactions
- With the SLB-IL60 column, possible interactions appear to be dispersive, dipole-dipole, dipole-induced dipole, π-π, hydrogen bonding, and acid-base interactions

As such, the SLB-IL60 column will retain some polar and polarizable analytes relatively longer, and some non-polar analytes relatively less. This results in unique and alternative selectivity compared to PEG columns.

The SLB-IL60 column was compared directly to five popular commercially available PEG columns, each from a different manufacturer. All columns were 30 m x 0.25 mm I.D., 0.25 µm dimensions, except the SLB-IL60 column, which has a 0.20 µm film thickness. **Table 1** shows the maximum temperature limits for all columns tested. Complete specifications of SLB-IL60 columns are shown in **Table 2.**

Table 1. Maximum Temperature Limits*

Column	Isothermal	Programmed
PEG 1	280 °C	280 °C
PEG 2	260 °C	270 °C
PEG 3	250 °C	260 °C
PEG 4	250 °C	260 °C
PEG 5	280 °C	300 °C
SLB-IL60	300 °C	300 °C

* Obtained from paperwork included with commercial columns.

Table 2. SLB-IL60 Column Specifications

Application	The IL60 is a Modified (deactivated) version of SLB-IL59 that provides better inertness. Selectivity more polar than PEG/wax phases, resulting in unique elution patterns. Higher maximum temperature than PEG/wax columns (300 °C compared to 270-280 °C). Excellent alternative to existing PEG/wax columns. Also a good GCxGC column choice.
USP Code	None
Phase	Non-bonded; 1,12-Di(tripropylphosphonium)dodecane bis(trifluoromethylsulfonyl)imide
Temp. Limits	35 °C to 300 °C (isothermal or programmed)

Esters and Ethers

A 12-component esters and ethers mix was analyzed on each column under identical conditions. All five PEG columns produced almost identical chromatography. **Figure 1** shows chromatograms obtained from the PEG 1 and SLB-IL60 columns. Observations are that the SLB-IL60 column provides:

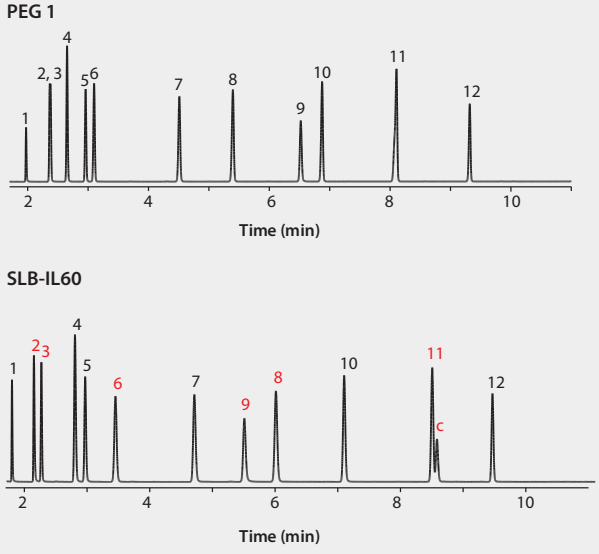
- Resolution of ethyl formate (peak 2) and methyl acetate (peak 3)
- Longer relative retention of isopropyl acetate (peak 6)
- Longer relative retention of isobutyl acetate (peak 8) and shorter relative retention of 1,4-dioxane (peak 9), resulting in an elution order change
- Resolution of a contaminant peak (peak c), suspected to be 2-methylbutyl acetate, from isoamyl acetate (peak 11)

Figure 1. Esters and Ethers

columns: PEG 1, 30 m x 0.25 mm I.D., 0.25 µm
SLB-IL60, 30 m x 0.25 mm I.D., 0.20 µm (29505-U)
oven: 40 °C (4 min), 8 °C/min to 200 °C (5 min)
inj. temp.: 250 °C
carrier gas: helium, 30 cm/sec
detector: FID, 250 °C
injection: 1 µL, 100:1 split
liner: 4 mm I.D., split/splitless type, single taper wool packed FocusLiner™ design
sample: 12-component esters and ethers mix, each analyte at 0.2 % (v/v) in pentane

1. Methyl formate
2. Ethyl formate
3. Methyl acetate
4. Tetrahydrofuran
5. Ethyl acetate
6. Isopropyl acetate
7. *n*-Propyl acetate
8. Isobutyl acetate
9. 1,4-Dioxane
10. *n*-Butyl acetate
11. Isoamyl acetate
12. *n*-Amyl acetate

c: Contaminant (2-Methylbutyl acetate)



(continued on next page)

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Chlorinated Hydrocarbons

A 12-component chlorinated hydrocarbon mix was analyzed on each column under identical conditions. All five PEG columns produced very similar chromatography, with the PEG 2 column producing the best overall resolution. Several PEG columns were unable to provide resolution of tetrachloroethene (peak 8) and chloroform (peak 9). **Figure 2** shows chromatograms obtained from the PEG 2 and SLB-IL60 columns. Observations are that the SLB-IL60 column provides:

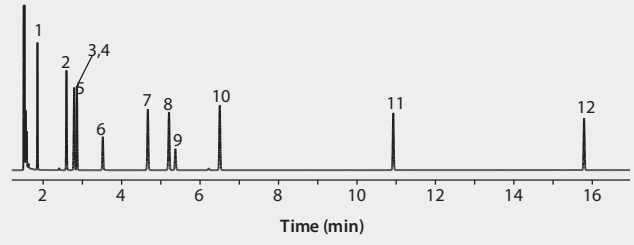
- Significantly less retention (11 minutes compared to 16 minutes)
- Many elution order differences (peaks 3-10)
- Resolution of carbon tetrachloride (peak 3) and 1,1,1-trichloroethane (peak 4)

Figure 2. Chlorinated Solvents

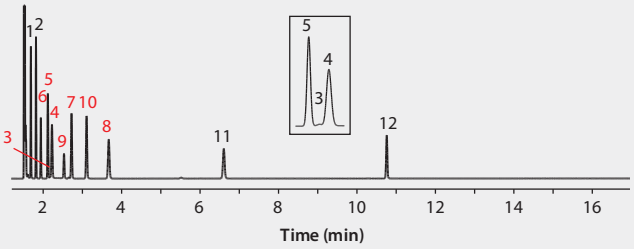
columns:	PEG 2, 30 m x 0.25 mm I.D., 0.25 µm SLB-IL60, 30 m x 0.25 mm I.D., 0.20 µm (29505-U)
oven:	40 °C (4 min), 8 °C/min to 200 °C (5 min)
inj. temp.:	250 °C
carrier gas:	helium, 30 cm/sec
detector:	FID, 250 °C
injection:	1 µL, 100:1 split
liner:	4 mm I.D., split/splitless type, single taper wool packed FocusLiner™ design
sample:	12-component chlorinated solvent mix, each analyte at 0.2 % (v/v) in pentane

1. 1,1-Dichloroethylene
2. *trans*-1,2-Dichloroethylene
3. Carbon tetrachloride
4. 1,1,1-Trichloroethane
5. 1,1-Dichloroethane
6. Methylene chloride
7. Trichloroethylene
8. Tetrachloroethene
9. Chloroform
10. 1,2-Dichloroethane
11. 1,1,1,2-Tetrachloroethane
12. 1,1,2,2-Tetrachloroethane

PEG 2



SLB-IL60



Fatty Acid Methyl Esters (FAMEs)

A 38-component FAME mix was analyzed on each column under identical conditions. All five PEG columns produced very similar chromatography, with the PEG 3 column producing the best overall resolution. **Figure 3** shows chromatograms obtained from the PEG 3 and SLB-IL60 columns. Observations are that the SLB-IL60 column provides:

- An overall faster elution (36 minutes compared to 52 minutes)
- Resolution of C18:1n9c (peak 17) and C18:1n9t (peak 18)
- Elution of *trans* isomers (peaks 18 and 20) before *cis* isomers (peaks 17 and 19)
- Less relative retention of C20:3n3 (peak 28) resulting in an elution order change
- Elution of C22:6n3 (peak 37) before C22:5n3 (peak 35)

Conclusion

Columns based on polyethylene glycol phase chemistry are widely used for a variety of applications (such as solvents and FAMEs). However, modification of PEG phase chemistry to affect selectivity is very limited. The SLB-IL60 column is able to undergo many of the same analyte-phase interactions as PEG columns, plus some additional interactions. This results in the SLB-IL60 column being similar enough to PEG columns to make it useful for many of the same applications, but different enough to impart unique selectivity which can be leveraged to change elution patterns and/or improve resolution.

Featured Products

Description	Cat. No.
SLB-IL60 GC Columns	
15 m x 0.10 mm I.D., 0.08 µm	29503-U
20 m x 0.18 mm I.D., 0.14 µm	29504-U
30 m x 0.25 mm I.D., 0.20 µm	29505-U
60 m x 0.25 mm I.D., 0.20 µm	29506-U
30 m x 0.32 mm I.D., 0.26 µm	29508-U
60 m x 0.32 mm I.D., 0.26 µm	29509-U

Related Information

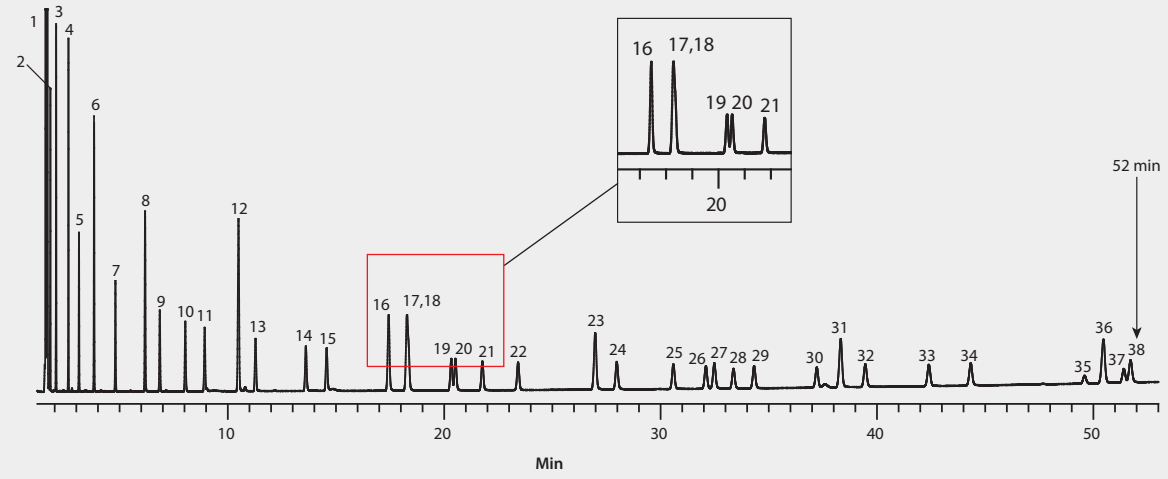
For more information on the SLB-IL60 and other ionic liquid columns, visit **sigma-aldrich.com/il-gc**

Figure 3. FAMEs

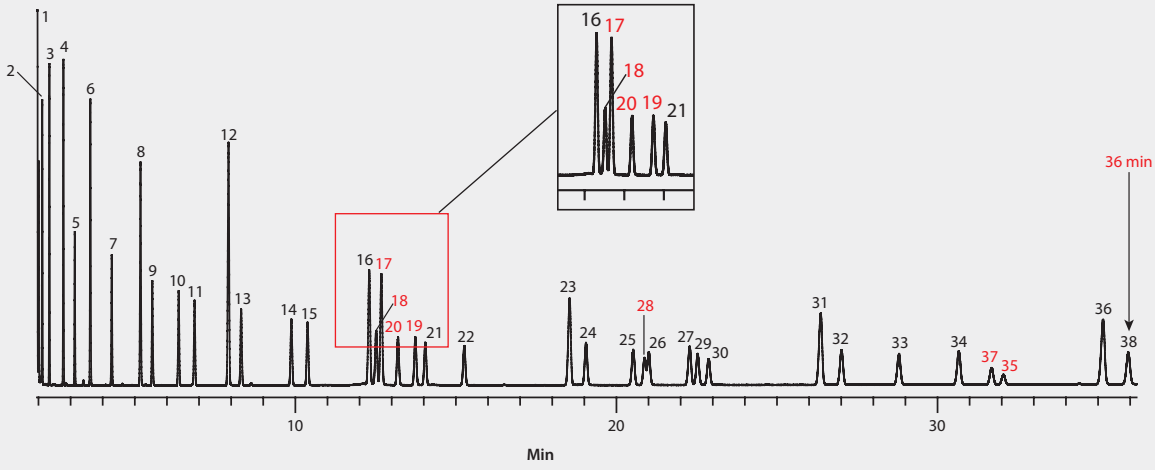
columns: PEG 3, 30 m x 0.25 mm I.D., 0.25 µm
SLB-IL60, 30 m x 0.25 mm I.D., 0.20 µm (29505-U)
oven: 170 °C, 1 °C/min to 225 °C
inj. temp.: 250 °C
carrier gas: helium, 1.2 mL/min
det.: FID, 260 °C
injection: 1 µL, 100:1 split
liner: 4 mm I.D., split/splitless type, single taper wool packed FocusLiner™ design
sample: Supelco 37-Component FAME Mix (CRM47885) + C22:5n3, in methylene chloride

1. C4:0
2. C6:0
3. C8:0
4. C10:0
5. C11:0
6. C12:0
7. C13:0
8. C14:0
9. C14:1
10. C15:0
11. C15:1
12. C16:0
13. C16:1
14. C17:0
15. C17:1
16. C18:0
17. C18:1n9c
18. C18:1n9t
19. C18:2n6c
20. C18:2n6t
21. C18:3n6
22. C18:3n3
23. C20:0
24. C20:1n9
25. C20:2
26. C20:3n6
27. C21:0
28. C20:3n3
29. C20:4n6
30. C20:5n3
31. C22:0
32. C22:1n9
33. C22:2
34. C23:0
35. C22:5n3
36. C24:0
37. C22:6n3
38. C24:1n9

PEG 3



SLB-IL60



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