Dear Colleague:

Upon the birth of gas chromatography (GC) in 1952, the user was required to make their packed columns as well as their GC instrument. Over the next few years, column and instrument manufacturing was commercialized. Supelco was founded in 1966 and became one of the pioneers in the manufacture and supply of highly-reproducible packed GC columns and components (empty columns, packings, stationary phases, and supports).

In 1979, fused silica capillary columns were invented. Within a few years, the GC column market became dominated by fused silica capillary columns providing great efficiency. Supelco evolved to offer:

- **Capillary GC columns**, many specialty made and tested for specific applications
- **GC accessories** (septa, liners, ferrules, syringes, vials, etc.) to properly maintain GC systems
- **Calibration standards**, including multi-component mixes, single-component mixes, and neat materials
- **Gas purification/management products** (purifiers, generators, tubing, etc.) to ensure gas streams of an adequate quality

The microchip age heralded in practical and inexpensive MS detection, broadening the use of GC to new fields. The expanded use due to GC/MS and other hyphenated techniques is still evident.

Supelco joined the Sigma-Aldrich family in 1993, increasing our offering of GC-related products to include:

- **Solvents** in GC-grades, eliminating variability and impurities
- **Derivatization** reagents for increasing volatility or response of analytes, or for suppressing activity

Today, GC remains relevant as a very powerful analytical tool, able to resolve multiple analytes in a single run. In fact, no other chromatographic technique is able to achieve the same level of resolution with short analysis times in such a cost-effective manner. Two newer areas, GCxGC and Fast GC, are expected to see major advances as current barriers are overcome (costs and user acceptance, respectively). Both of these areas use the fundamental strengths of GC, superior efficiency and resolution.

Another area of interest is the evolution of columns that incorporate ionic liquid stationary phases. These phases are unique because they remain liquid over wide temperature ranges while displaying very low volatility. Their highly polar nature (expanding the polarity scale upwards) and novel selectivities (allowing new or improved separations) increase their applicability.

Most recently, we created a GC Learning Center (sigma-aldrich.com/gc-learning) as a repository of our top technical content to help users get the most from their system. Included at this convenient location are column selection criteria, chromatogram searches, multiple presentations, several on-demand webcasts, and relevant brochures and bulletins.

We are very excited about the possibilities the future holds, and look forward to bringing you many innovations in the coming years.

Best regards,

Michael D. Buchanan

Product Manager, Gas Separations
mike.buchanan@sial.com
Principal Component Analysis (PCA) Evaluation of Seven Commercial Ionic Liquid Capillary GC Columns

Contributed Article

The following was generated with the assistance of an outside source using Sigma-Aldrich® products. Technical content was generated and provided by:

S. Rodríguez-Sánchez, P. Galindo-Iranzo, A.C. Soria, M.L. Sanz, J.E. Quintanilla-López, and R. Lebrón-Aguilar

1Instituto de Química Orgánica General (CSIC), Madrid, Spain
2Instituto de Química-Física “Rocasolano” (CSIC), Madrid, Spain
mike.buchanan@sial.com

Introduction

Ionic liquids are organic salts utilized for various industrial applications. Their unique and tunable physicochemical properties are unlike any other solvent. They typically remain liquid over wide temperature ranges, and tend to exhibit low melting points, good thermal stability, negligible vapor pressure, and high viscosity. These properties also make them ideal candidates as stationary phases for gas chromatography (GC). In fact, a quick literature search reveals a great deal of development aimed at preparing columns using ionic liquid stationary phases.

While monocationic ionic liquids can be employed for industrial applications, it was discovered that dicationic and polycationic ionic liquids make suitable GC stationary phases. Currently, there are seven different commercialized capillary GC columns which use ionic liquid stationary phases. Their main advantage is that they offer different separation properties compared to columns prepared with polysiloxane polymer and polyethylene glycol stationary phases. They also exhibit lower column bleed, higher thermal stability, greater resistance to damage from moisture and oxygen, and longer life time when compared to columns of similar polarity.

McReynolds and Abraham Methods

The polarity of a GC stationary phase can be estimated using the McReynolds method, in which retention indices (I) are determined for five test probes, representing different compound classes. The relationships of probes to compound classes are summarized in Table 1. Each probe relates to a set of solute–stationary phase interactions.

Combining the five retention indices can then be used to determine the polarity of the stationary phase. However, this approach cannot fully differentiate individual interactions since the retention of probes is not driven by a single interaction, but is most often due to several simultaneous interactions. Thus, the imprecision of ‘polarity’ alone is probably not sufficient to characterize GC stationary phases.

In contrast, the solvation parameter model (SPM) can quantitatively evaluate the individual intermolecular interactions between a substance and the stationary phase. Used for many years to characterize HPLC phases, the SPM is also applicable for characterizing GC stationary phases. This model is described for GC by the Abraham equation:

\[
\log k = c + eE + sS + aA + bB + lI
\]

where \(k\) is the retention factor of a solute on the stationary phase at a specific temperature; \(c\) is the model intercept; the capital letters \((E, S, A, B, \text{ and } I)\) represent the solute descriptors that are probe-specific parameters determined for many substances; and the lowercase letters \((e, s, a, b, \text{ and } l)\) are referred to as the system constants, in which all information concerning the solvation properties of the stationary phase is represented. Table 2 lists the correlation between system constants and the capacity of the stationary phase for various interactions.

Table 1. Relationship of McReynolds Probes to Compound Classes

<table>
<thead>
<tr>
<th>Probe</th>
<th>Characteristic Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene</td>
<td>aromatics, olefins</td>
</tr>
<tr>
<td>Butanol</td>
<td>alcohols, nitriles, carboxylic acids, diols</td>
</tr>
<tr>
<td>2-Pentanone</td>
<td>ketones, ethers, aldehydes, esters, epoxides, dimethylamino derivatives</td>
</tr>
<tr>
<td>Nitropropane</td>
<td>nitro and nitrile derivatives</td>
</tr>
<tr>
<td>Pyridine</td>
<td>aromatic bases</td>
</tr>
</tbody>
</table>

Table 2. Correlation of Abraham System Constants to Stationary Phase Interactions

<table>
<thead>
<tr>
<th>System Constant</th>
<th>Defines Capability of Phase for</th>
</tr>
</thead>
<tbody>
<tr>
<td>(e)</td>
<td>(n-n) and (n-n) interactions</td>
</tr>
<tr>
<td>(s)</td>
<td>dipole-type interactions</td>
</tr>
<tr>
<td>(a)</td>
<td>hydrogen-bond basicity</td>
</tr>
<tr>
<td>(b)</td>
<td>hydrogen-bond acidity</td>
</tr>
<tr>
<td>(l)</td>
<td>overall dispersive-type interactions</td>
</tr>
</tbody>
</table>

Experimental

In order to cover a broad range of possible solute-stationary phase interactions, 95 solutes with varied functional groups were selected to perform stationary phase characterization by the SPM. The group of columns included seven ionic liquid columns and 45 non-ionic liquid columns. The solutes selected were grouped into different mixtures, and then chromatographed isothermally at several temperatures to obtain their \(k\) values. The following were then generated:

- A table of system constants with standard deviations and goodness-of-fit statistics at each isothermal temperature
- Radar plots of system constants, which proved a useful tool to display multivariate observations in a two-dimensional chart
- A 3D plot of a principal component analysis (PCA) using data from 120 °C isothermal runs, providing a valuable overview of selectivity

Additional details of the experimental design, the table of system constants and further discussion, several radar plots and discussion, and the 3D PCA plot can be found in reference 8.

(continued on next page)
**PCA Results and Discussion**

PCA is a statistical procedure that rotates and transforms the original axes, each representing an original variable (system constants in our case), into new axes called principal components (PCs) that are linear combinations of the original variables and account for most of the variance in the data. PCA can reveal those variables, or combination of variables, which describe some inherent structure in the data and these may be interpreted in chemical or physicochemical terms.\(^5\)

The 3D score plot shown in Figure 1 is an update of a plot previously reported.\(^6\) The update is the inclusion of data for three additional ionic liquid columns. As shown, the first three principal components explain 99.2% of the variance, representing the whole column selectivity space quite well. Principal components 1 and 2 take into account contributions from the \(a\) and \(s\) system constants, while principal component 3 is mainly related to the \(b\) and \(e\) system constants. Dashed lines indicate the distribution trend of polysiloxane polymer stationary phases substituted with trifluoropropyl (line 1), phenyl (line 2), and cyanopropyl (line 3) groups. There are two other clearly differentiated groups, corresponding to polyethylene glycol (cluster I) and ionic liquid (cluster II) stationary phases.

The PCA score plot clearly reveals that most of the ionic liquid stationary phases possess separation characteristics unlike any non-ionic liquid stationary phase. They are the only ones that have high values of both PC1 and PC3. That is, among all the stationary phases characterized, only the ionic liquids are capable of simultaneously providing intense H-acceptor plus dipolar interactions (high \(a + s\) value [high PC1]) and intense H-donor plus \(\pi-\pi\) interactions (high \(b + e\) value [high PC3]).

The solvation characteristics of the SLB-IL61 column were found to be more similar to cyanopropyl substituted polysiloxane polymer phases (line 3) than to the other ionic liquid phases (cluster II). This is due to the trifluoromethylsulfonate anion incorporated as part of the phase, which decreases the hydrogen-bond acidity of the column.

One of the most outstanding characteristics observed for the ionic liquid stationary phases was the non-zero value for the \(b\) system constant (hydrogen-bond acidity). This inherent acidity contributes toward imparting distinct selectivity to these phases. This is clearly shown by principal component analysis. However, it should be noted that they also present a low inertness to compounds with a high capability to interact through hydrogen-bonding, often observed as poor peak shapes. This makes it difficult to use these columns to quantify low levels of such compounds.

**Conclusion**

In this work, seven commercial capillary columns that utilize ionic liquid stationary phases were thoroughly studied in order to better understand their retention mechanisms. According to the SPM results, dipole-type interactions (s) and hydrogen-bond basicity interactions (a) were the dominant contributions to retention, hydrogen-bond acidity interactions (b) were moderate, and \(\pi-\pi\) and \(n-\pi\) interactions (e) were barely significant. It was also revealed they provide a low separation for members of a homologous series, defined as overall dispersive-type (I) interactions.

A 3D PCA plot visually showed that ionic liquid columns fill an empty area of the available selectivity space, which should clearly enhance the separation capacity of the GC technique. It is believed that as a result of the different retention mechanisms involved, the ionic liquid columns studied are highly versatile and have a noteworthy capacity to resolve complex mixtures.

**References**


**Did you know...**

Three informative ionic liquid literature pieces (Introduction to the Technology, Applications, and Bibliography) can be viewed and downloaded at no-charge from [sigma-aldrich.com/il-gc-lit](sigma-aldrich.com/analytical)
Supelco SLB-IL60 Ionic Liquid GC Columns: Improved Resolution

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 an ionic liquid stationary phase platform and displays desirable features that existing non-ionic liquid columns do not. This is the second of several Reporter articles which explore various aspects of this column.1

The variety of analyte-phase interactions possible with the SLB-IL60 column can be leveraged to provide improved separations. This is especially true for complex mixtures comprised of compounds with varying functionality. Polyethylene glycol (PEG) columns rate at 50-52 on the GC Column Polarity Scale, whereas the SLB-IL60 column has a 60 rating. Therefore, the SLB-IL60 column will exhibit ‘wax-like’ polarity for many compound classes. However, the higher rating (60 compared to 50-52) does create subtle selectivity differences which is advantageous for many applications, such as multi-component industrial solvent mixtures.

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*

<table>
<thead>
<tr>
<th>Column</th>
<th>Isothermal</th>
<th>Programmed</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG 1</td>
<td>280 °C</td>
<td>280 °C</td>
</tr>
<tr>
<td>PEG 2</td>
<td>260 °C</td>
<td>270 °C</td>
</tr>
<tr>
<td>PEG 3</td>
<td>250 °C</td>
<td>260 °C</td>
</tr>
<tr>
<td>PEG 4</td>
<td>250 °C</td>
<td>260 °C</td>
</tr>
<tr>
<td>PEG 5</td>
<td>280 °C</td>
<td>300 °C</td>
</tr>
<tr>
<td>SLB-IL60</td>
<td>300 °C</td>
<td>300 °C</td>
</tr>
</tbody>
</table>

* Obtained from paperwork included with commercial 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 2. SLB-IL60 Column Specifications

| Application | Modified (deactivated) version of SLB-IL59 provides better inertness. More polar than PEG/wax phases, resulting in unique selectivity/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. Launched in 2012.
| USP Code    | None
| Phase       | Non-bonded, 1,12-Di(tripropylphosphonium)dodecane bis(trifluormethylsulfonyl)imide
| Temp. Limits| 35 °C to 300 °C (isothermal or programmed)

Industrial Solvents

A 56-component industrial solvent mix that contained alcohols, aldehydes, aromatics, chlorinated hydrocarbons, esters, ethers, ketones, and nitrogen-containing compounds was analyzed on each column under identical conditions. All five PEG columns produced almost identical chromatography. Figure 1 and Figure 2 show the chromatograms obtained from the PEG 1 and SLB-IL60 columns, respectively. Observations show the SLB-IL60 column provides:

• An overall faster analysis (22 minutes compared to 26 minutes)
• A vastly different elution pattern
• Resolution of more analytes; three co-elutions (6 compounds total) compared to six co-elutions (12 compounds total)
• Resolution of a contaminant peak (peak c), suspected to be 2-methylbutyl acetate, from isoamyl acetate (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. It is advantageous to possess columns with alternative selectivity, because resolution can be greatly affected by selectivity. 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.

Reference


Related Information

For more information on the SLB-IL60 and other ionic liquid columns, visit sigma-aldrich.com/il-gc
Figure 1. Industrial Solvents on PEG 1

column: PEG 1, 30 m x 0.25 mm I.D., 0.25 µm
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
liner: 4 mm I.D., split/splitless type, single taper wool packed, FocusLiner design
sample: 56-component industrial solvent mix, each analyte at 0.2 % (v/v) in pentane

1. Hexane
2. 1,1-Dichloroethylene
3. Methyl formate
4. Acetone
5. Ethyl formate
6. Methyl acetate
7. trans-1,2-Dichloroethylene
8. Tetrahydrofuran
9. Carbon tetrachloride
10. 1,1,1-Trichloroethane
11. 1,1-Dichloroethane
12. Ethyl acetate
13. Methanol
14. Isopropyl acetate
15. 2-Butanone
16. 2-Propanol
17. Methylene chloride
18. Ethanol
19. Benzene
20. n-Propyl acetate
21. Trichloroethylene
22. 4-Methyl-2-pentanone
23. Isobutyl acetate
24. Tetrachloroethene
25. Chloroform
26. sec-Butanol
27. Toluene
28. n-Propanol
29. 1,4-Dioxane
30. 1,2-Dichloroethane
31. n-Butyl acetate
32. 2-Butanone
33. Isobutanol
34. Nitropropane
35. Isomyl acetate
36. Ethylbenzene
37. Mesityl oxide
38. p-Xylene
39. m-Xylene
40. 5-Methyl-2-hexanone
41. n-Butanol
42. n-Amyl acetate
43. o-Xylene
44. Isoamyl alcohol
45. Chlorobenzene
46. Styrene
47. 1,1,1,2-Tetrachloroethane
48. Dimethylformamide
49. Diacetone alcohol
50. Cyclohexanol
51. 2-Butoxyethanol (Butyl Cellosolve®)
52. 1,4-Dichlorobenzene
53. 1,1,2,2-Tetrachloroethane
54. 2-Methylphenol
55. 3-Methylphenol
56. 4-Methylphenol

Figure 2. Industrial Solvents on the SLB-IL60

column: 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
liner: 4 mm I.D., split/splitless type, single taper wool packed, FocusLiner design
sample: 56-component industrial solvent mix, each analyte at 0.2 % (v/v) in pentane

1. Hexane
2. 1,1-Dichloroethylene
3. Methyl formate
4. Acetone
5. Ethyl formate
6. Methyl acetate
7. trans-1,2-Dichloroethylene
8. Tetrahydrofuran
9. Carbon tetrachloride
10. 1,1,1-Trichloroethane
11. 1,1-Dichloroethane
12. Ethyl acetate
13. Methanol
14. Isopropyl acetate
15. 2-Butanone
16. 2-Propanol
17. Methylene chloride
18. Ethanol
19. Benzene
20. n-Propyl acetate
21. Trichloroethylene
22. 4-Methyl-2-pentanone
23. Isobutyl acetate
24. Tetrachloroethene
25. Chloroform
26. sec-Butanol
27. Toluene
28. n-Propanol
29. 1,4-Dioxane
30. 1,2-Dichloroethane
31. n-Butyl acetate
32. 2-Butanone
33. Isobutanol
34. Nitropropane
35. Isomyl acetate
36. Ethylbenzene
37. Mesityl oxide
38. p-Xylene
39. m-Xylene
40. 5-Methyl-2-hexanone
41. n-Butanol
42. n-Amyl acetate
43. o-Xylene
44. Isoamyl alcohol
45. Chlorobenzene
46. Styrene
47. 1,1,1,2-Tetrachloroethane
48. Dimethylformamide
49. Diacetone alcohol
50. Cyclohexanol
51. 2-Butoxyethanol (Butyl Cellosolve®)
52. 1,4-Dichlorobenzene
53. 1,1,2,2-Tetrachloroethane
54. 2-Methylphenol
55. 3-Methylphenol
56. 4-Methylphenol

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Quantifying Picogram Concentrations of Aflatoxin M₁ in Liquid Milk using SPE Cleanup and LC/MS Analysis

K. G. Espenschied, R&D Technician; Emily Barrey, Senior R&D Scientist; and Michael Ye, Senior R&D Manager

Jennifer.claus@sial.com

Introduction

Aflatoxins are produced by fungi of the Aspergillus genus. Of several aflatoxin producing species, two are widely studied due to their importance to agriculture and medicine: Aspergillus flavus and Aspergillus parasiticus. These species infect important cereal grains, including maize, as well as ground (peanuts) and tree nuts. When infected, such commodities may become contaminated with aflatoxins and may subsequently be introduced into products routinely used in human and animal nutrition. When ingested, these toxins have the potential to impact animal, and especially, human health. They have been classified by the International Agency for Research on Cancer (IARC) as group 1 carcinogens, that is, compounds known to be carcinogenic in humans. Their target organ is the liver where they are thought to interfere with regulatory elements of DNA function. The most biologically active aflatoxin is aflatoxin B₁ (AFB₁). A recent risk assessment determined that aflatoxins may be implicated in 4.6-28.2% of hepatocellular carcinomas (HCC) worldwide.

The focus of this study is the metabolite of AFB₁, aflatoxin M₁ (AFM₁). AFM₁ can be present in the milk of lactating dairy animals that have consumed aflatoxin contaminated feeds. Although this aflatoxin is less biologically active than its precursor, it is also listed by the IARC as a group 1 human carcinogen. According to one understanding of aflatoxin toxicity, it is generally felt that there is no threshold dose below which no tumor formation would occur... only a zero level of exposure will result in no risk... Because of its potential as a carcinogen, and because milk is widely used as a food source, particularly for children and infants, the concentration of AFM₁ in milk is widely regulated.

The European Union (EU), along with Israel, has imposed the most stringent limits for the presence of AFM₁ in raw milk and milk used for the manufacture of milk-based products. Currently, EU and Israeli permissible level of AFM₁ in milk-based products is 0.050 µg/kg, while the regulatory specification for AFM₁ levels in infant formula, follow-on milk and other specified infant products, is 0.025 µg/kg. Feasibility for this LC/MS analysis was initially investigated using protein precipitation with milk samples and FLD detection. Samples that were spiked at 0.5 ng/mL (0.5 µg/kg) and analyzed by HPLC, demonstrated that cleanup was sufficient to permit detection of AFM₁ with a recovery of 88%. This study further develops the AFM₁ sample cleanup and analysis method to detect and quantify aflatoxin M₁ at concentrations corresponding to the lowest EU regulatory limit. A successful method will encompass regulatory limits for AFM₁ in milk as determined by the U.S. and China.

Experimental

Milk Preparation

Locally obtained 2% milk (45 grams) was weighed into 50 mL polypropylene centrifuge tubes* and centrifuged for 60 minutes at 5000 revolutions per minute (rpm), with a relative centrifugal force (RCF) of 4863 x g. Following centrifugation, milk was chilled at 0 °C for 30 minutes. After cooling, the top (fat) layer was removed using a laboratory spatula, and then 20 mL of serum milk (middle layer) was transferred to a collection flask.

Aflatoxin Spike

A 0.5 µg/mL AFM₁ reference standard was diluted to 0.05 µg/mL by transferring 150 µL of the standard into 1350 µL of acetonitrile. Prepared milk (80 mL) was transferred into each of two 200 mL silane treated Erlenmeyer flasks.* Either 40 or 400 µL of the 50 ng/mL aflatoxin spiking solution was added to 80 mL of milk, resulting in 25 and 250 pg/mL concentrations of AFM₁ in the respective spikes. The milk was spiked and gently swirled for two minutes prior to extraction.

Protein Precipitation and AFM₁ Extraction

Acetonitrile (20 mL) was pipetted into clean 50 mL polypropylene centrifuge tubes. AFM₁ spiked milk (20 mL) was added to each tube in four 5 mL aliquots, inducing protein precipitation. The mixture was shaken for 5 seconds by hand. A Supel™ QuE non-buffered tube 2 (Cat. No. 55295-U) was added to the liquid and the suspension was immediately shaken vigorously to disperse the salts. The centrifuge tubes were then placed on a laboratory shaker for 5 minutes. After shaking, the tubes were centrifuged at 5000 rpm (RCF 4863 x g) for 20 minutes. AFM₁ was extracted into the organic (top) layer of the preparation (Figure 1a).

Figure 1a.

1a. Milk Extraction after Centrifugation

1b. Evaporated Milk Samples With and Without SPE Cleanup

Top Layer (Organic)

Nonfat Milk Solids

Bottom Layer (Water + Salts)

Top Layer No SPE

Top Layer AflaZea SPE

(continued on next page)
Sample Cleanup
Supel Tox AflaZea SPE cartridges (Cat. No. 55314-U) were mounted on a vacuum manifold fitted with a rack containing 16 x 125 mm silane-treated glass culture cartridges. The vacuum was set to -5” Hg and 10.0 mL of sample from the top layer of each extract was loaded onto a corresponding SPE cartridge. Immediately after the cartridges were drained, 100% acetonitrile (2 x 2 mL aliquots) was passed through the cartridges to wash any remaining sample into the collection vials.

Sample Evaporation, Reconstitution, and Analysis
Collected samples were evaporated at 70 °C under a 10 psi nitrogen stream. The nitrogen stream was initiated at 3 psi, increased to 6 psi, and finally increased to 9 psi at 5 minute intervals. Samples were reconstituted by pipetting 500 μL of 20:80 acetonitrile:water onto the dried residue, followed by vortex mixing for 30 seconds. The dissolved residue was then transferred by Pasteur pipette into 1 mL syringes, tipped with 13 mm, 0.2 µm polyvinylidine fluoride (PVDF) filters, and pushed into 750 µL polypropylene vials. Quantitation was performed using matrix matched calibration standards ranging from extracted concentrations of 12.5 pg/mL to 500 pg/mL (absolute concentrations of 250 pg/mL to 10,000 pg/mL).

Results and Discussion
Milk was initially centrifuged to remove fats and nonfat milk solids (NFMS). For n=16, an average 2.6% of fats and 0.7% of NFMS by weight of milk was removed.

The SPE portion of the sample cleanup was simple and fast (less than 1 min/sample, n=3). During the SPE cleanup, the unwanted interferences from the milk remained bound to the cartridge while the analytes of interest were eluted. Compared to other SPE methods in which the analytes of interest bind to the cartridge and are subsequently eluted after various wash steps, this “interference removal” strategy required fewer steps and resulted in a sample preparation time savings. The evaporation time that elapsed was approximately 30 minutes. As seen in Figure 1b, the AflaZea purified sample appeared visually cleaner in terms of remaining residues and sample tint than the raw sample. The chromatograms in Figure 2 indicate that the AFM1 peak was highly discernable from the background with no matrix interferences at either of the concentrations. Also, Table 1 illustrates that AFM1 was detected and quantified in both 25 pg and 250 pg concentrations with acceptable recoveries (102 % and 76%, respectively) and good reproducibility (10.2% RSD and 8.6% RSD, respectively). Thus, this SPE method demonstrated sufficient sample cleanup for the identification and quantification of AFM1 in milk at both concentrations.

Table 1. Analyte Recovery of AFM1 at 25 pg/mL and 250 pg/mL Spiking Levels (n=3)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Average Percent AFM1 Recovery</th>
<th>Percent RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spiked Milk, 25 pg/mL</td>
<td>102</td>
<td>10</td>
</tr>
<tr>
<td>Spiked Milk, 250 pg/mL</td>
<td>76</td>
<td>9</td>
</tr>
</tbody>
</table>
Conclusion
A fast method for the cleanup, analysis, and quantification of aflatoxin M1 in milk samples to EU regulatory standards (0.025 µg/kg) has been developed using Supel Tox AflaZea SPE and LC/MS analysis. Unlike the multiple step “bind and elute” strategy implemented by other SPE methods, the Supel Tox AflaZea employed “interference removal,” producing time savings via elimination of wash steps. This SPE method served to remove problematic matrix interferences associated with milk in a timely fashion, while also attaining high analyte recoveries and good reproducibility. Early feasibility studies also suggest that this sample preparation method may be transferred to an HPLC/FLD method if working with AFM1 samples at higher concentrations.

References

For more information on the SPE cleanup for mycotoxin analysis, visit sigma-aldrich.com/supeltox

Visit our Food and Beverage/Toxin resources, sigma-aldrich.com/food-toxins

To see our full offering of Mycotoxin Standards and download our Standards brochure visit us at sigma-aldrich.com/mycotoxins
HPLC Analysis of Water-Soluble Vitamins Using Titan C18 Column

Olga Shimelis, Principal R&D Scientist
olga.shimelis@sial.com

Introduction
Vitamins are important compounds and are essential for normal metabolism. They are naturally found in many foods and are often added to processed food products. Water-soluble vitamins include such compounds as thiamine (B₁), riboflavin (B₂), niacin (B₃), pyridoxine (B₆), pantothenic acid (B₅), biotin (B₇), folic acid (B₉), and cyanocobalamine (B₁₂). Qualitative and quantitative analysis of vitamins is a routine but challenging task since vitamins are relatively unstable and affected by a number of factors such as heat, light, air, and other food components. In this report a Titan C18 UHPLC column was used to analyze B vitamins in both standard mixture and in vitamin water.

Experimental
An Agilent® 1290 UHPLC system was used for separations. The UV detection wavelength was set to 220 nm. The column used in this study was a Titan C18, 5 cm x 2.1 mm I.D., packed with monodisperse 1.9 μm particles. The column temperature was controlled at 35 °C. The B vitamins are very hydrophilic, consequently a gradient with a low concentration of methanol and acidic mobile phase was used for elution under the reversed-phase conditions.

Water-soluble vitamins were obtained from Sigma-Aldrich. Most B vitamins were dissolved in water. Riboflavin and biotin were dissolved in 1 M KOH and their solutions were prepared daily. Orange-flavored vitamin water was purchased in the grocery store. It was filtered through the 0.2 μm syringe filter for sample preparation.

Results and Discussion
B vitamins can be eluted from reversed-phase columns using low concentrations of aqueous acetonitrile or methanol. At pH 3 few compounds (thiamine, pyridoxine, niacinamide) were very weakly retained on the C18 Titan column and required only 0.5% of methanol for elution. Biotin, cyanocobalamine, and riboflavin vitamins are more hydrophobic and were retained more strongly. Gradient elution required an increase of methanol concentration to 30%. The Titan C18 column exhibited good retention for all nine vitamins and baseline separation for all vitamin peaks was easily achieved (Figure 1). All vitamins were eluted within 2.5 minutes with good peak shapes. Although the Titan C18 column contained sub-2 micron particles, the total backpressure was not excessive when using the aqueous methanol mobile phase; which is known to be more viscous and results in backpressures higher than those using aqueous acetonitrile mobile phases. The Titan column backpressure peaked at 550 bar during the separation. The smaller particle size allowed for faster separation.

The developed HPLC method was applied to the analysis of B vitamins in vitamin water. The drink was filtered prior to injection to HPLC and no dilution was required. The peaks for four B vitamins were identified by comparison to the standard mixture (Figure 2).

Conclusions
The use of a Titan C18 column for analysis resulted in excellent resolution of the mixture of nine water-soluble vitamins in 2.5 minutes. The retention of polar compounds was adequate on the C18 column when using an aqueous methanol gradient and an acidic pH of the mobile phase. The method was applied to the analysis of the vitamin water and four B vitamins added to the water were identified.
Figure 2. Analysis of Vitamin Water Using Titan C18 Column

- Column: Titan C18, 5 cm x 2.1 mm I.D., 1.9 μm (577122-U)
- Mobile phase: [A] 20 mM potassium phosphate, pH 3.0, [B] methanol
- Gradient: 0.5% B for 0.5 min, increase to 30% B in 1.3 min, hold at 30% B for 1 min, re-equilibrate at 0.5% B for 0.8 min
- Flow rate: 0.5 mL/min (backpressure not more than 550 bar)
- Detector: UV, 220 nm
- Injection: 0.5 μL

1. Pyridoxine
2. Niacinamide
3. Calcium pantothenate
4. Cyanocobalamin

Featured Products

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1.9 μm UHPLC Columns

The Titan™ C18 UHPLC columns are based on 1.9 μm totally porous, monodisperse silica particles. These particles are the result of a newly developed, patent-pending, Ecoporous™ silica manufacturing process.

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Aminoglycoside Analysis in Honey Using Molecularly Imprinted Polymer Cleanup and LC/MS/MS Detection

Emily Barrey, Senior R&D Scientist and Olga Shimelis, Principal R&D Scientist
emily.barrey@sial.com

Introduction
Streptomycin and related compounds belong to a well-known class of antibiotics, the aminoglycosides. They are widely used to control bacterial and fungal diseases of fruit trees, seeds, and ornamental crops. The use of streptomycin to control fireblight on apples and pears accounts for 58% of its total usage in agriculture. Honeybees can become exposed to streptomycin when pollinating the fruit trees. The honeybees can then leave traces of antibiotics in honey and other bee products. There is growing concern of the impact that these antimicrobial 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.1

The focus of this study is the extraction and analysis of six aminoglycosides from honey using molecularly imprinted polymer (MIP) solid phase extraction (SPE) with LC/MS/MS detection. This study utilized the unique extraction capabilities of MIPs to successfully quantitate six aminoglycosides by LC/MS/MS at 50 ng/g.

Molecularly imprinted polymers are SPE 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 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).

Experimental

Honey Preparation
The stock solutions were prepared from neat materials obtained from Sigma-Aldrich. Samples were fortified using a mixed aminoglycoside standard prepared at 10 µg/mL from 1 mg/mL individual stock solution standards. Locally obtained honey (2 gram samples) were weighed into 50 mL polypropylene centrifuge tubes.* Three milliliters of 50 mM potassium phosphate in water (pH = 7.8) was added and samples were vortexed for ~2 min. The pH of the sample was adjusted to ~7.5 with concentrated ammonium hydroxide.

MIP SPE Sample Cleanup
The SupelMIP® Aminoglycoside SPE cartridge (Cat. No. 52777-U) was conditioned with 1 mL of methanol followed by 1 mL of 50 mM potassium phosphate in water (pH = 7.8). The prepared honey sample was passed through the cartridge. The cartridge was washed with 3 mL of water and 1 mL of 0.1% ammonium hydroxide and then dried on high vacuum for ~2-3 min. The cartridge was then washed with 1 mL of 40:60 acetonitrile:water (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. The cartridge was eluted with 1 mL of 1% formic acid containing 5 mM heptafluorobutyric acid (HFBA) in 20:80 water:acetonitrile (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, 27 µm HPLC column (Cat. No. 53823-U). Figures 2a, 2b, 2c depict chromatograms of the analytes in honey 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.
Results and Discussion

Recoveries for the 6 aminoglycosides are given in Figure 3. Most of the analytes had recoveries ≥ 60%, except for apramycin. It was determined that low recoveries can be attributed to insufficient elution of the analyte off of the SPE material. Analytes having a higher number of amino groups could lead to stronger binding of the analyte to the MIP sorbent.

Figure 3. Aminoglycoside Recoveries in Honey

The use of the SupelMIP Aminoglycoside SPE cartridge kept the analyte bound to the sorbent while a series of aggressive washes were applied to the sorbent to eliminate matrix interferences. The absence of matrix effects can be an indicator 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 for 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, no significant matrix effects were observed.

Figure 4. Matrix Factors for Aminoglycosides in Honey

(continued on next page)
Conclusion
A simple and sensitive method for the cleanup, analysis, and quantification of aminoglycosides in honey has been developed using SupelMIP SPE - Aminoglycosides and LC/MS/MS analysis. This method was able to successfully obtain recoveries >60% for most analytes. The unique features of the MIP material afforded the ability to wash additional matrix interferences off the cartridge prior to eluting our analytes of interest.

References

Related Products

### Description

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

**Visiprep™ DL SPE Vacuum Manifold**
- DL (Disposable Liner), 12-port model
  - Cat. No. 57044
- DL (Disposable Liner), 24-port model
  - Cat. No. 57265

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Extraction of Permethrin Pesticides from Spinach Using QuEChERS Methodology with Automated Shaking

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

Introduction
Sample preparation procedures for pesticide analysis in fruits and vegetables were simplified with the introduction of QuEChERS methodology in 2003. Multi-residue pesticide extraction was accomplished through a 1 minute shaking procedure using acetonitrile as an extraction solvent followed by salt partitioning and dispersive solid phase extraction (SPE) cleanup. This extraction procedure has been successfully applied to a variety of fruits and vegetables. The QuEChERS procedure has grown in popularity and has gained worldwide acceptance during the past 10 years.

It is common to evaluate and validate pesticide residue methods by spiking representative blank matrix samples with a standard mix of pesticides. Pesticide-free produce samples, such as organically grown produce, are often used for this purpose. The pesticides are typically added to a matrix sample 1-2 hours prior to testing in order to allow pesticide incursion. They are then easily extracted using the 1 minute shaking protocol. However, recently, it was noted that existing residues may not be readily accessible for extraction as they can be ‘enclosed into cells or wax particles’ of the produce. The 1 minute shaking time specified in the QuEChERS protocol was not sufficient to fully extract existing pesticide residues in approximately 50% of tests conducted. It was later observed that a 10-15 minute shaking time with the use of an automated shaker was required to obtain acceptable extraction efficiencies.

Recently the BenchMixer™ shaker was added to the Supelco/Sigma-Aldrich product listing to compliment the Supel QuE product line. Spinach, a vitamin and mineral-rich green leafy vegetable, was recently cited in the Environmental Working Group’s “Dirty Dozen 2014” for having high levels of pesticide contamination. For this study, the BenchMixer shaker was used for the extraction and cleanup of existing pesticide residues from non-organically grown spinach. The results were compared to a standard QuEChERS protocol involving the manual shaking of the spinach extracts for 1 minute.

Experimental
Spinach was extracted with acetonitrile using 1 minute manual shaking (by hand) or 10 minute automated shaking using the BenchMixer vortex shaker. Previous work showed that using the BenchMixer for 1 minute and 5 minutes resulted in incomplete extractions. A minimum of 10 minutes shaking time at the 2250 rpm setting was required for full extraction of existing pesticides. The extraction was performed in Supel QuE 50 mL centrifuge tubes. For each pesticide extraction, 5 replicates were completed. The extraction procedure is presented in Figure 1.

The samples were then analyzed by GC/MS/SIM, using m/z 183 for quantitation, and m/z 163 as a qualifier. Calibration was done using matrix-matched standards. Extracts of organically grown spinach were obtained and were used to construct the calibration curve. The calibration curve standards ranged from 25 ng/mL to 5,000 ng/mL.

Results and Discussion
Two permethrin isomers were resolved chromatographically (Figure 2) and quantitated separately. As illustrated in Table 2, the yield of permethrin isomer 1 was found to be 4.18 ppm using the manual method, but 4.76 ppm using the shaker method. Permethrin isomer 2 gave a level of 0.90 ppm from the hand-shaken samples, but a level of 1.03 ppm from BenchMixer shaken samples. For both isomers, the manual method extracted 14% less permethrin than the shaker method. This difference is significant. Similar results were observed in an experiment by Anastassiades et al. when 10-15 minutes of automated shaking was employed for the initial acetonitrile extraction step in the QuEChERS procedure. The reproducibility of this work was similar for both shaking techniques (2-5%) when hand-shaking was performed by an experienced laboratory technician.

The use of the shaker was convenient as the QuEChERS horizontal rack can hold seven 50 mL tubes during the extraction step, and the rack for 15-mL tubes can hold up to 50 tubes for the cleanup step. Both 50 mL extraction tubes and the new Supel QuE 15 mL cleanup tubes were compatible with the shaker. In addition to producing higher yields of existing pesticides, use of the BenchMixer shaker may also decrease the physical strain experienced by laboratory personnel when performing multiple daily QuEChERS extractions.

Figure 1. QuEChERS Procedure Used for the Extraction of Pesticides from Spinach

Add 10 mL acetonitrile to 10 g homogenized fresh spinach in 50 mL tube (Cat. No. 55248-U)
Shake 10 min using BenchMixer (Cat. No. 55278-U) at 2,250 rpm OR shake 1 min by hand
Add Supel QuE Acetate Extraction salts (Cat. No. 55234-U) Shake 1 min by hand or 10 min using shaker
Centrifuge for 5 minutes at 4000 rpm
Transfer 5 mL of the extract to the PSA/C18/ENVI-Carb™ 15 mL cleanup tube (Cat. No. 55474-U) and shake for 1 min
Centrifuge for 3 min at 3000 rpm, transfer to sample vial

Table 2

<table>
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<th>Pesticide</th>
<th>Manual Method</th>
<th>Shaker Method</th>
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<td>Permethrin 1</td>
<td>4.18 ppm</td>
<td>4.76 ppm</td>
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<tr>
<td>Permethrin 2</td>
<td>0.90 ppm</td>
<td>1.03 ppm</td>
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</table>

(continued on next page)
**Figure 2. GC/MS of Permethrin Pesticide Isomers Extracted from Spinach Using the BenchMixer Shaker**

- **column:** SLB-5ms, 20 m x 0.18 mm I.D., 0.18 µm (28564-U)
- **oven:** 120 °C (1 min), 10 °C/min to 330 °C (3 min)
- **inj. temp.:** 250 °C
- **carrier gas:** helium, 0.7 mL/min
- **detector:** Agilent GC 7890 with 5975 MS Detector, selected ion mode (SIM) m/z = 163 (quant), 183 (qual)
- **MSD interface:** 325 °C, source = 250 °C, quad = 200 °C
- **injection:** 1 µL
- **liner:** 2 mm I.D., split/splitless type, single taper wool packed FocusLiner™ design (2879525-U)

### Table 2. Detected Permethrin Level and Reproducibility of Two Shaking Methods for Pesticide Extraction (n=5)

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<th>Permethrin Isomer 2</th>
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<td>Manual, 1 min</td>
<td>4.18</td>
<td>2%</td>
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<tr>
<td>Shaker, 10 min</td>
<td>4.76</td>
<td>3%</td>
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**Conclusion**

Introduction of the BenchMixer shaker into the QuEChERS protocol resulted in a 14% increase in the observed yield of permethrin residues existing in commercially bought spinach. The shaker can be successfully used for analysis of pesticide residues when an extended shaking time of 10-15 minutes is required to achieve better extraction efficiencies.

**References**

Two Distinct Sample Prep Approaches to Overcome Matrix Effect in LC/MS of Serum or Plasma Samples

This article highlights the impact that sample matrix effects can have on LC/MS response and discusses two novel approaches to reduce it.

Craig R. Aurand, Technology Program Manager
craig.aurand@sial.com

Introduction

Advancements in electrospray ionization mass spectrometry (ESI-MS) have changed the way identification and quantification of small molecules in biological fluids is conducted. Systems are capable of routinely detecting sub-femtogram levels of analytes. Although these improvements enable low-level detection, ESI-MS is not without limitations, the phenomenon of matrix ionization suppression being a major one. Matrix ionization suppression results from charge competition in the ESI source between components in the sample matrix and the target analytes, and causes diminished, augmented, and irreproducible analyte response.

The Negative Impact of Sample Matrix on LC/MS

Of particular concern with clinical, forensic, and bioanalytical methods is the impact of phospholipids from the matrix. Phospholipids are a major component of cell membranes and notorious for fouling the MS source (Figure 1). They are a major contributor to matrix-induced ionization suppression because they usually co-extract with analytes during sample prep that involves protein precipitation with traditional solid phase extraction (SPE). Additionally, phospholipids often elute in the same timeframe as the analytes from the HPLC column. This co-elution decreases sensitivity which results in increased limits of quantitation and decreased method precision and accuracy. Phospholipids reduce HPLC column lifetime, and their buildup on the column is a problem because they elute erratically, thereby reducing the reproducibility of the method. Excessive gradient elution is needed to wash the HPLC column and the system, which ultimately decreases the sample throughput. The impact of phospholipids on LC/MS experiments has been discussed at length in previous Reporter articles. 

Approaches to Reducing Matrix Effect

In this article, two novel techniques to mitigate matrix effects will be discussed. The techniques differ in their approach to discriminate target analytes from the sample matrix. The first technique focuses on the isolation of the sample matrix while allowing the target analytes to remain in the sample solution. The second technique is similar to traditional SPE where target analytes are isolated from the sample matrix. In both cases, the focus will be on matrix reduction from blood plasma or serum samples where phospholipids are the endogenous component of primary concern.

![Figure 1. MS Source Fouled with Phospholipid Residue from Injecting Serum or Plasma Samples](image1)

![Figure 2. Effectiveness of HybridSPE®-Phospholipid Technique for the Targeted Matrix Isolation Approach to Interference Removal](image2)
Approach 1: Targeted Matrix Isolation

This approach to matrix reduction targets specific components of the sample matrix. In this example, a targeted phospholipid depletion technology (HybridSPE-Phospholipid, also called HybridSPE-PLus) is used for the selective isolation of phospholipids from serum or plasma. HybridSPE-Phospholipid comprises a hybrid zirconia-silica particle packed into 96-well plate or SPE cartridge formats. The electron-deficient empty d-orbitals of the zirconia atoms bond with the electron-rich phosphate groups of the phospholipids via Lewis acid/base interaction. The result is highly efficient phospholipid isolation. Proteins are also removed by precipitation directly in the well plate or tube. Plasma or serum samples are added to the HybridSPE-Phospholipid plate or tube followed by a 3:1 ratio of precipitation solvent. Samples are mixed via draw-dispense or vortex agitation to fully precipitate sample proteins.

Figure 2 shows the same plasma sample processed using standard protein precipitation (top) or phospholipid depletion (bottom) using HybridSPE-Phospholipid. Notice the direct overlap of phospholipids with the target analytes in the protein precipitation sample and concurrent decrease in analyte response compared to the samples processed with the HybridSPE-Phospholipid plate. HybridSPE depleted the phospholipids from the sample, eliminating matrix interference, giving a dramatic increase in analyte response.

Figure 3 shows that the sample processed using protein precipitation resulted in a 75% reduction in response for propranolol due to phospholipid matrix interference. Under these conditions, propranolol eluted in the same region that a large portion of the phospholipids eluted. The error bar for propranolol is much larger due to the irreproducible suppression of the phospholipids. Conversely, samples processed using HybridSPE-Phospholipid demonstrated improved response for propranolol along with much smaller error bars. By selectively isolating the phospholipids, no matrix was introduced onto the analytical column resulting in a more accurate and precise method.

Approach 2: Targeted Analyte Isolation

The second approach to reduce matrix interference is to target the isolation of analytes while excluding components of the sample matrix. This approach is common in traditional SPE where analyte binding and matrix washing procedures are followed. A continuation of the analyte binding strategy is applied to solid phase micro extraction (SPME) using biocompatible phase chemistries. Figure 4 shows two configurations of biocompatible SPME (bioSPME) fibers currently available from Supelco.

SPME is based upon an equilibrium distribution of the analytes between the solution (serum or plasma, in this case) and the phase layer on the fiber. BioSPME fibers comprise C18-modified silica particles embedded in biocompatible binder. Analytes are desorbed from the fiber using traditional reversed-phase HPLC solvents and analyzed directly by LC/MS. An interesting property of the bioSPME fiber is that the particle binder shields larger biomolecules from adhering to the fiber. This permits the concentration of target analytes on the fiber without coextraction of the sample matrix. The result is a very unique process, whereby sample concentration and sample cleanup are conducted simultaneously. Because bioSPME is not destructive, multiple extractions of the same sample can be performed.

Figure 5 shows a plasma sample spiked with nine cathinone compounds. In the chromatogram on the top, sample prep consisted of standard protein precipitation. In comparison, the chromatogram on the bottom shows the same sample extracted with the bioSPME fiber. Notice the difference in signal response for both analytes and phospholipids between the two techniques: The bioSPME method gave over twice the analyte response but one-tenth the phospholipid response of the protein precipitation method. This demonstrates the ability of the bioSPME fiber to concentrate analytes from biological samples without interference from matrix components.
Reducing matrix effect is an important consideration when developing an LC/MS method for serum or plasma samples. This brief report described two distinct sample prep techniques to reduce matrix effect: targeted phospholipid depletion using HybridSPE-Phospholipid and analyte enrichment using biocompatible SPME. Both methods provide effective means to reduce matrix interference that can rob the method of sensitivity, reproducibility, precision, and accuracy.

**Figure 5. Effectiveness of Biocompatible SPME Technique for the Targeted Analyte Isolation Approach to Interference Removal**

- **Column:** Ascentis Express HILIC, 10 cm × 2.1 mm I.D., 2.7 µm (53939-U)
- **Mobile Phase:** 5 mM ammonium formate in 98:2 (v/v) acetonitrile:water
- **Flow Rate:** 0.6 mL/min
- **Pressure:** 1842 psi (127 bar)
- **Column Temp.:** 35 °C
- **Detector:** MS, ESI(+) TOF, m/z = 100-1000
- **Injection:** 1 µL

**System:** Agilent 1290 Infinity; 6210 Time of Flight (TOF) MS

**Red traces:** Analyte monitoring

**Blue traces:** Phospholipid monitoring

**Featured Products**

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**References**

1. Aurand, Craig. Understanding, Visualizing and Reducing the Impact of Phospholipid-Induced Ion Suppression in LC/MS; Supelco Reporter Europe Volume 52: 10 - 12.
3. Aurand, Craig. Improvement in LC-MS/MS Analysis of Vitamin D Metabolites in Serum by leveraging Column Selectivity and Effective Sample Prep.; Supelco Reporter Europe Volume 54: 17.

**Summary**

Reducing matrix effect is an important consideration when developing an LC/MS method for serum or plasma samples. This brief report described two distinct sample prep techniques to reduce matrix effect: targeted phospholipid depletion using HybridSPE-Phospholipid and analyte enrichment using biocompatible SPME. Both methods provide effective means to reduce matrix interference that can rob the method of sensitivity, reproducibility, precision, and accuracy.
A Fast and Robust HPLC Separation of Bile Acids and Their Conjugates with Ascentis Express C18
Helping to Elucidate the Role of Plasma Bile Acid Signalling in Humans

Clare Glicksman¹, Michael Wright¹, Dave Bell¹ and Anders Fridström²
¹ Biochemistry department, King’s College Hospital, London, UK
² Biochemistry department, Cambridge University Hospital Trust, Cambridge, UK
¹ Supelco®, Sigma-Aldrich

Introduction
Bile acids are increasingly recognized as playing an important signaling role in the control of immune response and energy metabolism. Recently, there has been interest in measuring bile acid concentrations in plasma and their impact on hormone concentrations, for example as affected by bariatric surgery (Figure 1).

Primary bile acids are synthesized in the liver from cholesterol. The majority are conjugated, increasing hydrophilicity, with either glycine or taurine. They are stored in the gall bladder and released into the small intestine on food consumption. Bacteria in the gut can convert primary bile acids to secondary bile acids which are then reabsorbed and taken back to the liver as part of enterohepatic circulation. Here secondary bile acids can also be glycine or taurine conjugated. Spill-over from this system results in bile acids in the peripheral circulation. There are potentially 15 species which can be measured in a peripheral plasma sample (Figure 2).

Methodology
The quantitation of plasma bile acids by tandem mass spectrometry MS/MS presents an analytical challenge. When employing collision-induced dissociation, bile acids will either form no useful fragments for quantitation (unconjugated), or produce a charged fragment originating from a conjugation group (conjugated taurine and glycine salts, prefixed T or G respectively, in figure 2).

As three of the bile acids have the same molecular weight and elemental composition, they cannot be differentiated by MS/MS alone and require chromatographic separation of the group in both their unconjugated and conjugated forms. To enable separation, several orthogonal HPLC stationary phases were screened for selectivity. The best separation was achieved with an Ascentis Express C18 analytical column based on Fused-Core® technology.

Figure 1. Roux-en-Y Gastric Bypass and Gastric Banding
These are two possible options for reducing dietary food intake. Both procedures create a small pouch at the start of the stomach by either restricting the stomach with a gastric band or by surgically creating a separate pouch from the stomach and then attaching it to the remainder of the GI tract.

Figure 2. Structural Overview of Bile Acids and Conjugated Salts with Corresponding m/z Values

<table>
<thead>
<tr>
<th>Bile Acid(s)</th>
<th>m/z transition</th>
<th>Bile Acid(s)</th>
<th>m/z transition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unconjugated</td>
<td>X = OH</td>
<td>TDC, TCDC, TUDC</td>
<td>498.2/80.0</td>
</tr>
<tr>
<td>Taur (T)</td>
<td>X = NHCH₂CH₂SO₃H</td>
<td>TCA</td>
<td>514.0/80.0</td>
</tr>
<tr>
<td>Glyco (G)</td>
<td>X = NHCH₂COOH</td>
<td>TLC</td>
<td>482.2/80.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>d₄-DC</td>
<td>395.2/395.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>d₄-GDC</td>
<td>452.1/74.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>d₄-TDC</td>
<td>502.2/80.0</td>
</tr>
</tbody>
</table>

Note: Acronym explanation can be found in the featured product listing “Standards” on p.22.
**Sample Preparation**

Plasma proteins were removed with addition of 900 µL of acetonitrile containing deuterated internal standards, to 250 µL of human EDTA plasma. The mixture was vortexed, centrifuged, and the supernatant evaporated before being reconstituted in a 50:50 solution of methanol and water. A 10 µL aliquot (corresponding to 8.57 µL of plasma) was injected into the HPLC.

**Results and Conclusion**

The resulting chromatogram showing the fifteen bile acid species separated on an Ascentis Express C18 Fused-Core column is shown in Figure 3. The method is fast and robust and allowed the compounds to be measured individually rather than the alternative measurement of a total concentration by colorimetric kinetic enzyme assays. This is particularly useful in research into the role of individual bile acids as signaling molecules. The method is suitable for clinical laboratories and is currently being used to investigate potential mechanisms linked to gut hormone profiles and glycemic control.

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**Figure 3. A Typical Chromatogram Showing all Analytes and Internal Standards with Full Resolution of all Derivatives with Similar m/z Values**

- **column**: Ascentis Express C18, 15 cm x 4.6 mm I.D., 2.7 µm (53829-U)
- **mobile phase**: 5 mM ammonium acetate, 0.012% formic acid in [A] water; [B] methanol
- **gradient**: 70 to 95% B in 10 min; held at 95% B for 1 min
- **flow rate**: 0.6 mL/min
- **column temp.**: 40 °C
- **detector**: ESI(-), MRM mode (m/z shown in Figure 2)
- **injection**: 10 µL (corresponding to 8.57 µL of plasma)
- **sample**: protein-precipitated human plasma

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Featured Products

**Description** | **Cat. No.**
--- | ---
HPLC Column |  
Ascentis Express C18, 15 cm x 4.6 mm I.D., 2.7 µm particle size | S3829-U

**Mobile Phase Solvents and Additives**

<table>
<thead>
<tr>
<th>Description</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetonitrile, LC-MS Ultra CHROMASOLV, tested for UHPLC-MS, 1 L, 2 L</td>
<td>14261</td>
</tr>
<tr>
<td>Water, LC-MS Ultra CHROMASOLV, tested for UHPLC-MS, 1 L, 2 L</td>
<td>14263</td>
</tr>
<tr>
<td>Formic acid, LC-MS Ultra eluent additive, 1 mL, 2 mL</td>
<td>14265</td>
</tr>
<tr>
<td>Ammonium acetate, LC-MS Ultra eluent additive, 25 g</td>
<td>14267</td>
</tr>
</tbody>
</table>

**Standards**

<table>
<thead>
<tr>
<th>Description</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conjugated bile acids kit (contains GCDC, GCA, TCDC, TCA, TDC)</td>
<td>CBA-1KT</td>
</tr>
<tr>
<td>Chenodeoxycholic acid (CDC)</td>
<td>C9377</td>
</tr>
<tr>
<td>Cholic acid (CA)</td>
<td>C1129</td>
</tr>
<tr>
<td>Deoxycholic acid (DC)</td>
<td>D2510</td>
</tr>
<tr>
<td>Glycochenodeoxycholic acid (GCDC)</td>
<td>G0759</td>
</tr>
<tr>
<td>Glycocholic acid (GCA)</td>
<td>G2878</td>
</tr>
<tr>
<td>Glycoursodeoxycholic acid (GUDC)</td>
<td>06863</td>
</tr>
<tr>
<td>Lithocholic acid (LC)</td>
<td>L6250</td>
</tr>
<tr>
<td>Taurochenodeoxycholic acid (TCDC)</td>
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<tr>
<td>Taurocholic acid (TCA)</td>
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<tr>
<td>Taurodeoxycholic acid (TDC)</td>
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<td>Taurolithocholic acid (TLC)</td>
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<tr>
<td>Taouroursodeoxycholic acid (TUDC)</td>
<td>T0266</td>
</tr>
<tr>
<td>Ursodeoxycholic acid (UDC)</td>
<td>U5127</td>
</tr>
</tbody>
</table>

**References**


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- Microbiology and Reference Materials
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- Recent Guidance for Reference Material Users
- Confidence in Identification for Preparation of Biological-Matrix Reference Materials
- Nanoparticles in Environmental, Food, and Medical Samples
- Speciation Analysis and Reference Materials
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Symposium Chairman:
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Sigma-Aldrich Contacts:
Americas & Asia Pacific:
Alan Nichols
e-mail: alan.nichols@sial.com
Phone: +1 814-359-5496

Europe, Middle East & Africa:
Peter Jenks
e-mail: peter.jenks@sial.com
Phone: +44 1747 833377

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