Our customers represent an astounding diversity in geography, markets, samples, job functions, whether they come from academia, industry, government, and do pure or applied research.
Dear Colleague:

It is truly an Analytical World we serve. Our customers represent an astounding diversity in geography, markets, samples, job functions, whether they come from academia, industry, government, and do pure or applied research.

From my vantage point as Marketing and R&D Director, this means that as we develop new products, programs, and campaigns, and even when we consider product packaging, we have to stay abreast of what’s going on in our customers’ world. The more we stay in touch with you, the more we can develop relevant products to help you solve the problems and challenges you face.

Here are just a few examples of how our innovative products have recently been used:

- **Food Safety:** Ascentis® Express Fused-Core columns for bisphenol A and related compounds in canned food, and the plasticizer DEPH in sports drink bottles; SPME for rapid detection of PAHs in seafood after the Gulf Oil Spill.
- **Biomarker Discovery:** Engineered carbons for early detection of cancer and other diseases; Ag-Ion SPE tubes for detection of invasive mollusks in waterways.
- **Clinical and Forensics:** Ascentis Express F5 for 3-epi separation of 25-hydroxy vitamin D to avoid over quantitation of vitamin D, especially young children; SPME for forensics in a high profile murder trial in the US.
- **Pharmaceutical Drug Discovery:** Ascentis Express for helping researchers improve throughput, get drugs to market faster; Astec® HPLC columns and HybridSPE-Phospholipid plates for chiral LC-MS in bioanalysis.
- **Consumer Product Safety:** Radiello® sampler for detection of low levels of H2S from Chinese drywall in indoor air.
- **Environmental:** SPME to measure the uptake of groundwater contaminants by trees; Radiello passive sampling devices to survey street-level air pollution in the five boroughs of New York City.
- **Nutritional Labeling:** Ionic liquid GC column SLB-IL111 provides alternative selectivity and improved resolution of fatty acid methyl esters (FAMES), relevant to trans-fat content of foods.

You’ll find more examples in the following pages of this edition of the Reporter.

The members of our R&D and Marketing teams that I have the pleasure to work with have backgrounds as diverse as our customers. We go to the same trade shows and read the same trade journals as you do. We try to keep abreast of current events that have analytical implications: from food poisoning to oil spills. We are here to serve you, the Analytical World. Please let us know how we can help.

Paul Ross, Ph.D.
Director of Marketing & R&D
Analytical Separations
paul.ross@sigma-aldrich.com

ORDER: 800-247-6628 (US ONLY) / 814-359-3441

Paul Ross
Director of Marketing & R&D
Analytical Separations
paul.ross@sial.com

---

**Table of Contents**

**Liquid Chromatography**
- Fast LC-MS-MS Analysis of 25-Hydroxyvitamin D2 and 25-Hydroxyvitamin D3 .............................3
- LC-MS Nitrogen: Generation and Purification ...............................................................6
- Derivatization and Improved Detection of Estradiol with ESI-MS ..............................18
- Introduction of Novel Performance-Tested Solvents for UHPLC Applications ........22
- High-Purity Headspace Grade Solvents ...................................................23

**Sample Preparation**
- Highly Selective Separation of Vitamin D epi-Metabolites Using HybridSPE-Phospholipid.........................14
- Aldehydes and Ketones in Indoor Air using a Low Background LpDNPH Solvent Desorption Tube and Fused-Core® HPLC ............................................16

**Gas Chromatography**
- EN 14103 FAMEs in B100 Biodiesel on the Omegawax™ ........................................10
- FAMEs in B20 Biodiesel on the SLB™-IL111 ............................................................12

**Standards**
- Withania somnifera Analytical Standards and an Improved HPLC Method .................20

**Chiral Chromatography**
- Chiral HPLC Analysis of Underivatized Amino Acid Enantiomers ...............................8
Fast LC-MS-MS Analysis of 25-Hydroxyvitamin D2 and 25-Hydroxyvitamin D3

**Contributed Article**

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

Jesse C. Seegmiller and Keith J. Goodman
AB SCIEX Framingham, MA
wayne.way@sial.com

**Abstract**

A robust method for analyzing 25-hydroxyvitamin D3 and 25-hydroxyvitamin D2 in serum using a QTRAP® 5500 and an MPX™-2 High Throughput multiplexed LC-MS-MS system is presented. Sample preparation was simplified in order to accommodate automated liquid handling systems and to minimize the time commitment needed by clinical staff. With multiplexing, sample results were achieved in less than 1.5 minutes per sample. Accuracy and linearity was demonstrated for 25-hydroxyvitamin D3 and 25-hydroxyvitamin D2 over a concentration range 0.5-100 ng/mL in serum. This dynamic range was achieved without the use of solid phase extraction, online extraction, or any other type of sample concentration steps.

**Introduction**

Heightened interest in vitamin D is related to an observed trend of increased vitamin D insufficiency and its role in human health (1). Vitamin D is derived in vivo primarily from UVB radiation impacting the skin where 7-dehydrocholesterol (vitamin D3) is converted through a photolytic, nonenzymatic reaction. Smaller amounts of vitamin D are acquired through diet, including vitamin D3 (cholecalciferol) and vitamin D2 (ergocalciferol) from animal and plant sources, respectively. Vitamin D3 is metabolized to 25-hydroxyvitamin D3 in the liver and then to the active form 1,25-dihydroxyvitamin D3 in the kidney. Circulating levels of 1,25-dihydroxyvitamin D are tightly controlled and serve to modulate expression of specific genes through the vitamin D receptor.

Vitamin D acts to promote intestinal calcium and phosphate absorption and also controls their liberation from bone. In addition to rickets and osteomalacia, low serum 25-hydroxyvitamin D3 and D2, has been linked to hypertension, autoimmune diseases, and cancer (2,3).

The most abundant metabolite of vitamin D is 25-hydroxyvitamin D and it is considered one of the best indicators of vitamin D status. Accurately measuring 25-hydroxyvitamin D is necessary to adequately assess an individual’s vitamin D levels and to help determine the role of vitamin D in various diseases. Immunoassays suffer from high reagent cost, narrow dynamic range, and poor selectivity (inability to distinguish 25-hydroxyvitamin D3 from 25-hydroxyvitamin D2) (4). These inadequacies of immunoassays make them unattractive for vitamin D analysis in patient samples.

Liquid chromatography tandem mass spectrometry (LC-MS-MS) is a highly sensitive and specific technique for the analysis of a wide range of compounds contained in biological matrices. The current generation of this technology is capable of reliably determining 25-hydroxyvitamin D concentrations across the entire clinical range. Coupled with liquid chromatography, accurate and precise measurements of both 25-hydroxyvitamin D3 and 25-hydroxyvitamin D2 can be routinely achieved using LC-MS-MS. The primary goal of this method was to use a simple sample preparation step while accommodating high throughput 25-hydroxyvitamin D runs, ~500/day, on a single LC-MS-MS system.

**Sample Preparation**

Protein precipitation consisted of combining 50 μL of serum with 100 μL acetonitrile in a 1.5 mL centrifuge tube. The sample was then centrifuged at 5,000 g for 5 minutes before transferring the supernatant into a sample vial for analysis.

**MS/MS Conditions**

The multiplexed LC system, MPX™, was coupled to an AB SCIEX QTRAP® 5500 LC-MS-MS system with a Turbo V™ source and Atmospheric Pressure Chemical Ionization (APCI) probe in positive ion mode. A total of 5 Multiple Reaction Monitoring (MRM) transitions were monitored, including 2 per compound (quantifier and qualifier) and one for the internal standard, 25-hydroxyvitamin D3-d6. Chromatographic conditions are listed in Figures 1 and 2.

Figure 1. Initial Serum Extracted Sample of 350 Injections

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>%A</th>
<th>%B</th>
<th>Flow Rate (mL/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>50.00</td>
<td>50.00</td>
<td>1.00</td>
</tr>
<tr>
<td>0.85</td>
<td>7.50</td>
<td>92.50</td>
<td>1.00</td>
</tr>
<tr>
<td>0.90</td>
<td>5.00</td>
<td>95.00</td>
<td>1.50</td>
</tr>
<tr>
<td>1.00</td>
<td>0.00</td>
<td>100.00</td>
<td>1.50</td>
</tr>
<tr>
<td>1.45</td>
<td>0.00</td>
<td>100.00</td>
<td>1.50</td>
</tr>
<tr>
<td>1.50</td>
<td>0.00</td>
<td>100.00</td>
<td>1.00</td>
</tr>
<tr>
<td>1.60</td>
<td>50.00</td>
<td>50.00</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>50.00</td>
<td>50.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>

flow rate: see flow program in gradient table
temp: 40 °C
det: MS, APCI (+) mode
inj: 5 μL
sample: as indicated in method
Results and Discussion

A method suitable for quantification of 25-hydroxyvitamin D2 and D3 required only two minutes, prior to multiplexing. Figure 1 shows the separation of 25-hydroxyvitamin D2 and D3 and the internal standard. This is the first injection of an extracted serum sample on the Ascentis Express C18 column. Figure 2 shows the result of re-injecting this first sample after 350 serum extracted samples were run. Note that peak shape, retention, and signal response have not changed after the 350 injections. This indicates a very robust method which employed simple sample preparation (protein precipitation) prior to analysis.

Quantitative results for serum samples are presented in Tables 1 and 2. These results show satisfactory precision and accuracy for a high-throughput method requiring minimal sample preparation.

Conclusion

A robust and reliable assay for 25-hydroxyvitamin D capable of handling high sample throughput observed in a clinical research laboratory was presented. Through the use of the AB SCIEX MPX™ system and the QTRAP® 5500 LC-MS-MS system in combination with Supelco’s Ascentis Express C18 column, laboratories can achieve 25-hydroxyvitamin D sample analysis times of <1.5 minutes per sample. This streamlined method can be implemented without the need for UHPLC systems or advanced knowledge of separation chemistry and achieves Limits of Quantitation (LOQ) of 1 ng/mL for vitamin D2 and D3.

References


The new Aldrich Handbook contains the widest selection of chemistry and materials science products and is your resource for chemical structures, literature references, and extensive chemical and physical data. Our complimentary catalog includes new and innovative reagents and building blocks, plus a focused line of Labware products to support your chemistry needs.

The Aldrich Handbook’s portfolio supports the research community with:

- More than 40,000 research chemicals
- Over 4,000 new products
- 10,000 chemical structures
- 8,500 updated literature citations
- Extensive chemical and physical data

For reliable, high-quality chemicals you can trust, add your free copy of the Aldrich Handbook to your laboratory by visiting:

Aldrich.com/aldrichcatalogs
LC-MS Nitrogen: Generation and Purification

Robert F. Wallace and Michael D. Buchanan
bob.wallace@sial.com

The current trends in High Performance Liquid Chromatography (HPLC) are switching to more efficient columns, and applications that require higher sensitivity and higher selectivity. Mass Spectrometry (MS) is a technique being used to help achieve some of these targets. An important consideration for analytical accuracy is an uninterrupted supply of a relatively large volume of high-purity nitrogen to the MS unit.

Many laboratories that utilize LC-MS may purchase their nitrogen in high-priced certified gas cylinders in an effort to ensure gas purity. A better strategy is to generate and purify sufficient volumes of nitrogen to the high-grade necessary for LC-MS use. This can be accomplished by installing an on-site nitrogen generator coupled to contaminant-specific purifiers that are installed downstream.

Nitrogen Generation

Parker® offers several nitrogen generators that are specifically designed to meet the gas flow, purity, and pressure requirement needed to supply single or multiple LC-MS instruments. These units are compatible with both atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) interfaces. Oil-free compressed air is first filtered then passed through a bed of carbon molecular sieve which selectively removes oxygen and other contaminants. Some models are offered with an integral oil-free air compressor, while other models require a separate oil-free air compressor to be installed upstream. Pressure swing adsorption technology is then employed to produce a continuous supply of high-purity nitrogen. This technology works by separating gas species in a mixture under pressure according to their molecular characteristics and their affinity for an adsorbent material operating near ambient temperatures. Two adsorbent beds alternate between purification and regeneration modes to ensure a continuous supply of nitrogen at the specified purity levels.

Laboratory gas generators are a great alternative to gas cylinders. In addition to being a much more sensible source of gas from a cost standpoint, generators take up less space, are safer, and do not require the labor needed to transport bulky cylinders into the lab.

All generator models offered by Parker are available through Sigma-Aldrich. Simply contact one of our Technical Service chemists at techservice@sial.com, specifying the make, model, and electrical requirements.

Nitrogen Purification

Whether using nitrogen gas cylinders or nitrogen generators, removing trace contaminants is critical to the proper operation of an LC-MS instrument. We recommend Super Clean™ LC-MS purifiers for this purpose. These two-cartridge base-plate systems are specifically designed to meet the high-flow and high-purity requirements of LC-MS units. Two versions are available, one for hydrocarbon removal, and one for moisture removal.

Independent of the original gas quality, these purifiers can reduce contaminants to reach nitrogen purity greater than 99.9999% quality. Cartridges are inert, comprised of an adsorbent in a non-diffusive glass tube, a strong polycarbonate shell, and an inert stainless steel fitting. Base plates are a special two-position design with an inert gas path that is split equally between the two cartridges and rejoined afterwards before leaving the base plate. Therefore, each cartridge handles half the flow, providing longer contact with the gas stream. This special design is what allows these purifiers to produce such high-quality gas at flow rates up to 20 L/min and pressures up to 150 psi (11 bar). Note that each base plate must contain the same type cartridge (either both hydrocarbon removal, or both moisture removal).

Some other features/benefits of Super Clean LC-MS purifiers are:

- Permanent connections
  - After installation, connections to the gas line never need to be broken
  - Reduces the risk of leaks from kinked tubing
- Continuous operation
  - Needle valves instantly close to provide a diffusion-proof seal when cartridges are removed
  - Eliminates the need to suspend operation during cartridge change-out
- Quick cartridge change-out and no tools required
  - Install in seconds without exposing the gas lines to room air
  - Only needs held in place on the base-plate while the retaining ring is hand-tightened
- Vertical design requires very little bench space
- Indicator capability for moisture

As a safeguard, it is recommended to install Super Clean LC-MS purifiers just before the gas line enters the LC-MS unit. This will protect against contaminants that may have entered through fittings.
Conclusion

A nitrogen generator is a great alternative for supplying gas to the LC-MS instrument. If you are unsure of the economic advantage of replacing gas cylinders with gas generators, look at the convenience, safety, and reliability. Generators eliminate the labor involved and the need to shut down the entire system when changing empty cylinders. Additionally, installing gas purifiers downstream of the nitrogen source helps guarantee that the quality of the gas going to the LC-MS instrument will be free of contaminants, allowing the highest quality analytical results to be achieved.

Featured Products

<table>
<thead>
<tr>
<th>Description</th>
<th>Qty.</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC-MS High-Flow Base-Plate Design Kits</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrocarbon Removal Kit (SU861029 + 28879-U)</td>
<td>1 kit</td>
<td>SU861046</td>
</tr>
<tr>
<td>Moisture Removal Kit (SU861028 + 28879-U)</td>
<td>1 kit</td>
<td>SU861045</td>
</tr>
<tr>
<td>LC-MS High-Flow Base-Plate Design Replacement Items</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrocarbon Removal Cartridges, w/out indicator</td>
<td>2 ea</td>
<td>SU861029</td>
</tr>
<tr>
<td>Moisture Removal Cartridges, w/indicator</td>
<td>2 ea</td>
<td>SU861028</td>
</tr>
<tr>
<td>LC-MS High-Flow Two Position Base Plate</td>
<td>1 ea</td>
<td>28879-U</td>
</tr>
<tr>
<td>Replacement O-Ring Set (10 small and 10 large)</td>
<td>1 ea</td>
<td>SU861050</td>
</tr>
<tr>
<td>Base-Plate Wall Mounting Bracket</td>
<td>1 ea</td>
<td>SU861016</td>
</tr>
</tbody>
</table>

Related Information

Additional information regarding generators and purifiers for nitrogen can be found at sigma-aldrich.com/hplc

Maximize System Performance with Easy-to-Use HPLC Accessories

With current trends in achieving faster analyses and throughput, this new HPLC accessories brochure makes product selection easier.

HPLC Accessories

- for high speed and high sensitivity analytical applications
- to maximize the efficiency and reliability of the analysis
- to help protect your column investment

To view or download a copy of this new brochure, please visit our web at sigma-aldrich.com/hplc or request a copy to be sent to you (NNX) by returning the enclosed postcard.
Chiral HPLC Analysis of Underivatized Amino Acid Enantiomers

Jennifer E. Claus
jennifer.claus@sial.com

In this brief article, we report a single, simple LC-MS-compatible mobile phase system that resolves the enantiomers of the common underivatized amino acids on an Astec CHIROBIOTIC® T column. Some experiments to provide insight into the variables that control retention and selectivity will also be touched upon.

Although L-amino acids dominate in nature, D-amino acids have been found in almost all species of bacteria, plants, and animals. Their presence has implications in physiology, nutrition, pharmacology, and toxicology that have spawned development of chromatographic methods to resolve in order to identify and quantify amino acid enantiomers (1,2). Resolving the enantiomers on polysaccharide-based chiral stationary phases (CSPs) is a challenge because native (underivatized) amino acids are zwitterionic and poorly soluble in non-polar solvents. Derivatization prior to separation can be used to improve solubility, or to create diastereomers that are resolvable by achiral HPLC (3). Derivatization however, adds an additional step and potential impurities. Direct analysis is preferred, and possible on macrocyclic glycopeptide-based CSPs.

Unlike polysaccharide-based CSPs, macrocyclic glycopeptides possess ionic groups (4) and are compatible with both organic and aqueous mobile phases. This makes them ideal CSPs for separating enantiomers of polar and ionic compounds, like amino acids. One such CSP that is particularly successful for resolving the enantiomers of underivatized amino acids is Astec CHIROBIOTIC T, which employs the macrocyclic glycopeptide teicoplanin as the chiral selector (5,6). The goal of this study was to develop a single mobile phase system that would resolve the majority of common amino acids on Astec CHIROBIOTIC T.

Approach

The retention of four representative amino acids, DL-arginine (positively charged), DL-aspartic acid (negatively charged), DL-threonine (polar, uncharged), and DL-tyrosine (hydrophobic) versus percentage of methanol in a water:methanol:formic acid mobile phase was measured and plotted (Figure 1). The mobile phase composition that gave the best overall enantioselectivity was applied to the remaining amino acids.

Results

A simple mobile phase comprising water:methanol:formic acid (30:70:0.02) gave baseline resolution of most of the twenty chiral amino acids enantiomers on the Astec CHIROBIOTIC T column (Table 1). The small amount of formic acid was necessary to produce elution of the charged acidic and basic amino acids. Enantiomers of histidine, cysteine, and proline were not resolved under these conditions, but could be resolved on the same column under slightly different conditions (see Table 1 footnote). Representative chromatograms of DL-tryptophan and DL-methionine appear in Figures 2 and 3, respectively.

### Table 1. Screen of Underivatized Amino Acids Under Optimized Mobile Phase Conditions

<table>
<thead>
<tr>
<th>Amino Acid*</th>
<th>Rt1 (min.)</th>
<th>Rt2 (min.)</th>
<th>Selectivity</th>
<th>Resolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>DL-Arginine</td>
<td>7.367</td>
<td>9.623</td>
<td>1.31</td>
<td>3.78</td>
</tr>
<tr>
<td>DL-Histidine</td>
<td>9.132</td>
<td>9.737</td>
<td>1.07</td>
<td>0.67</td>
</tr>
<tr>
<td>DL-Aspartic Acid</td>
<td>4.477</td>
<td>5.165</td>
<td>1.15</td>
<td>2.68</td>
</tr>
<tr>
<td>DL-Glutamic Acid</td>
<td>4.340</td>
<td>5.321</td>
<td>1.23</td>
<td>4.59</td>
</tr>
<tr>
<td>DL-Serine</td>
<td>4.441</td>
<td>4.896</td>
<td>1.10</td>
<td>2.05</td>
</tr>
<tr>
<td>DL-Threonine</td>
<td>4.234</td>
<td>4.619</td>
<td>1.09</td>
<td>2.09</td>
</tr>
<tr>
<td>DL-Asparagine</td>
<td>5.267</td>
<td>6.835</td>
<td>1.30</td>
<td>4.64</td>
</tr>
<tr>
<td>DL-Glutamine</td>
<td>4.934</td>
<td>6.033</td>
<td>1.22</td>
<td>4.51</td>
</tr>
<tr>
<td>DL-Alanine</td>
<td>4.744</td>
<td>6.156</td>
<td>1.30</td>
<td>5.54</td>
</tr>
<tr>
<td>DL-Isoleucine</td>
<td>4.349</td>
<td>5.662</td>
<td>1.30</td>
<td>5.83</td>
</tr>
<tr>
<td>DL-Leucine</td>
<td>4.421</td>
<td>5.938</td>
<td>1.34</td>
<td>6.39</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>4.811</td>
<td>6.674</td>
<td>1.39</td>
<td>3.65</td>
</tr>
<tr>
<td>DL-Phenylalanine</td>
<td>4.994</td>
<td>6.170</td>
<td>1.24</td>
<td>6.17</td>
</tr>
<tr>
<td>DL-Tryptophan</td>
<td>5.095</td>
<td>6.275</td>
<td>1.23</td>
<td>3.90</td>
</tr>
<tr>
<td>DL-Tyrosine</td>
<td>4.578</td>
<td>5.594</td>
<td>1.88</td>
<td>4.25</td>
</tr>
<tr>
<td>DL-Valine</td>
<td>4.472</td>
<td>5.385</td>
<td>1.20</td>
<td>3.93</td>
</tr>
</tbody>
</table>

* Optimized conditions for histidine, cysteine, and proline on Astec CHIROBIOTIC T:
  - DL-Histidine: 160 mM sodium phosphate-ethanol, pH 4.5 (40:60)
  - DL-Cysteine: water-acetonitrile (30:70)
  - DL-Proline: water-acetonitrile (95:5)

For all amino acids tested, enantioselectivity increased with organic modifier concentration. Retention versus organic modifier concentration exhibited a U-shaped profile (Figure 1). This observation has been well documented in small and large molecule achiral separations, and has also been reported for chiral compounds in methanol and acetonitrile on teicoplanin-based CSPs. It is likely due to the combined effects of analyte solubility and conformational changes in the CSP as a function of organic modifier content (7). The effect has also been reported on cyclodextrin (CD)-based CSPs, but only in acetonitrile. It is thought to be related to the accessibility of the CD cavity by the acetonitrile molecule (8).

It is interesting to note that the D enantiomer is always more strongly retained than the corresponding L enantiomer on macrocyclic glycopeptide CSPs. This is no coincidence since these molecules exert their antibiotic activity by interacting with terminal D-alanyl-D-alanine residues in bacterial cell membrane peptides (9).
Figure 1. Effect of Organic Modifier Concentration on Amino Acid Retention

- **Column:** Astec CHIROBIOTIC T, 25 cm x 4.6 mm I.D., 5 μm particles (12024AST)
- **Mobile Phase:** Methanol:Water:Formic Acid (20:70:0.02)
- **Flow Rate:** 1.0 mL/min
- **Temp.:** 25 °C
- **Det.:** UV at 205 nm
- **Injection:** 10 μL
- **Sample:** 0.2 mg/mL in 50:50 Water:Ethanol

*Figure 2. Representative Chromatogram of DL-Tryptophan on Astec CHIROBIOTIC T*

- Conditions same as Figure 1 except:
  - **Mobile Phase:** Water:Methanol:Formic Acid (30:70:0.02)

*Figure 3. Representative Chromatogram of DL-Methionine on Astec CHIROBIOTIC T*

- Conditions same as Figure 1 except:
  - **Mobile Phase:** Water:Methanol:Formic Acid (30:70:0.02)

**Conclusion**

Because Astec CHIROBIOTIC T columns possess ionic functional groups and operate in mobile phase systems that favor polar analyte solubility, they are uniquely able to separate underivatized D- and L-amino acids. A simple LC-MS-compatible mobile phase system was found that resolved most amino acid pairs, and provides a foundation for future studies in this area.

**References**

Introduction

Biodiesel is a renewable, alternative diesel fuel produced from vegetable oils, animal fats, or recycled restaurant grease. This non-toxic, biodegradable liquid fuel consists of mono-alkyl esters of long chain fatty acids (also known as fatty acid methyl esters, or FAMEs) and may be used alone or blended with petroleum-based diesel fuels. The most common process for producing biodiesel involves two steps:

- Through the transesterification reaction, triglycerides (i.e. oils or fats) are chemically reacted with an alcohol, usually methanol, in the presence of a catalyst, like sodium or potassium hydroxide, yielding fatty acid methyl esters (FAMEs) and glycerin by-product.
- The FAMEs and glycerin by-product are then separated and purified. Biodiesel is the name given to the FAME fraction retained for use as fuel. The glycerin fraction is sold for use in soaps and other products.

The resulting biodiesel contains little-to-no sulfur or fossil fuel aromatics. Biodiesel is almost 10% oxygen, making it an oxygenated fuel, which aids combustion in fuel-rich circumstances. Biodiesel can be used pure (B100 biodiesel = 100% biodiesel) or blended (for example, B20 biodiesel = 20% biodiesel and 80% petroleum diesel).

FAME Profile as a Measure of Purity

Biodiesel is produced around the world using a variety of biomass starting materials, including oils from canola, palm, soy, and tallow. The biomass starting material used by a given manufacturing facility depends on how plentiful it is in their region of the world. As an indicator of the amount of useable fuel in the final B100 biodiesel product, many bulk biodiesel producers measure the FAME profile. DIN Method EN 14103 specifies a gas chromatography (GC) procedure for determining the FAME profile in B100 biodiesel samples. (1)
GC Analysis

Four B100 biodiesel samples, manufactured from various biomass starting materials, were each diluted to 50 mg/mL in n-heptane. Additionally, heptadecanoic acid methyl ester was added to a level of 1 mg/mL in each for use as an internal standard. Each mix was then analyzed on an Omegawax™ capillary GC column. The resulting chromatograms are shown in Figure 1 on the same time scale for ease of comparing patterns.

The "soy source" mix was analyzed five months prior to the analyses of the other mixes. The slightly longer retention times for this run can be attributed to small differences in the carrier gas linear velocity and/or the fact the column was slightly longer (it was subsequently trimmed several times prior to the later analyses). FAME peak identifications were done by comparing retention times to Characterized Reference Oils as well as AOCS Animal and Vegetable Reference Mixes that were also analyzed on the Omegawax column under identical conditions during each period of analysis.

Results and Discussions

As expected, the starting biomass material used influences the FAME profile of the final product. Pattern recognition (a useful QA/QC tool) can be performed, as each mix exhibits its own unique FAME profile. The Omegawax column separates unsaturated FAMEs primarily by degree of unsaturation, with no separation by the cis/trans orientation of the double bonds. This results in a fairly clean chromatogram (not cluttered with numerous cis/trans peaks), making the Omegawax column ideal for this application.

Conclusion

The Omegawax column was originally designed for, and is specifically tested for, the analysis of omega 3 and omega 6 fatty acids (as methyl esters). As shown here, it can also be used for determining the FAME profile of B100 biodiesel samples.

References

1. DIN EN 14103, “Fat and Oil Derivates - Fatty Acid Methylesters (FAME) - Determination of Ester and Linolenic Acid Methyl Ester Contents”

Related Products

<table>
<thead>
<tr>
<th>Description</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Omegawax, 15 m x 0.10 mm I.D., 0.10 μm</td>
<td>23399-U</td>
</tr>
<tr>
<td>Omegawax, 30 m x 0.32 mm I.D., 0.25 μm</td>
<td>24152</td>
</tr>
<tr>
<td>Omegawax, 30 m x 0.53 mm I.D., 0.50 μm</td>
<td>25374</td>
</tr>
</tbody>
</table>

Characterized Reference Oils

Characterized Reference Oils are useful to assist in identifying biomass starting materials, and to identify if pure or blended materials were used. Each standard is packaged in an amber ampul under nitrogen. A Certificate of Composition is provided with each.

<table>
<thead>
<tr>
<th>Description</th>
<th>Qty.</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canola oil</td>
<td>1 g</td>
<td>46961</td>
</tr>
<tr>
<td>Coconut oil</td>
<td>1 g</td>
<td>46949</td>
</tr>
<tr>
<td>Corn oil</td>
<td>1 g</td>
<td>47112-U</td>
</tr>
<tr>
<td>Cottonseed oil</td>
<td>1 g</td>
<td>47113</td>
</tr>
<tr>
<td>Lard oil</td>
<td>1 g</td>
<td>47115-U</td>
</tr>
<tr>
<td>Linseed (Flaxseed) oil</td>
<td>1 g</td>
<td>47599-U</td>
</tr>
<tr>
<td>Menhaden fish oil</td>
<td>1 g</td>
<td>47116</td>
</tr>
<tr>
<td>Olive oil</td>
<td>1 g</td>
<td>47118</td>
</tr>
<tr>
<td>Palm oil</td>
<td>1 g</td>
<td>46962</td>
</tr>
<tr>
<td>Peanut oil</td>
<td>1 g</td>
<td>47119</td>
</tr>
<tr>
<td>Safflower oil</td>
<td>1 g</td>
<td>47120-U</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>1 g</td>
<td>47122</td>
</tr>
<tr>
<td>Sunflower oil</td>
<td>1 g</td>
<td>47123</td>
</tr>
</tbody>
</table>

AOCS animal and vegetable reference mixes

AOCS animal and vegetable reference mixes are also available. Each quantitative mix is similar to the fatty acid distribution of specific oils and conforms to the requirements of AOCS Method Ce 1–62. (2) A lot-specific Certificate of Analysis is supplied with each mix. Compositional specifications for each mix is available at sigma-aldrich.com/fame.

<table>
<thead>
<tr>
<th>Description</th>
<th>Qty.</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>AOCS No. 1</td>
<td>100 mg</td>
<td>O7006-1AMP</td>
</tr>
<tr>
<td>Corn, cottonseed, kapek, poppyseed, rice, safflower, soybean, sunflower, and walnut</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AOCS No. 2</td>
<td>100 mg</td>
<td>O7131-1AMP</td>
</tr>
<tr>
<td>Hempseed, linseed, perilla, and rubberseed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AOCS No. 3</td>
<td>100 mg</td>
<td>O7256-1AMP</td>
</tr>
<tr>
<td>Mustard seed, peanut, and rapeseed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AOCS No. 4</td>
<td>100 mg</td>
<td>O7381-1AMP</td>
</tr>
<tr>
<td>Neatsfoot, olive, and teaseed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AOCS No. 5</td>
<td>100 mg</td>
<td>O7506-1AMP</td>
</tr>
<tr>
<td>Babassu, coconut oil, ouri-curi, and palm kernel</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AOCS No. 6</td>
<td>100 mg</td>
<td>O7631-1AMP</td>
</tr>
<tr>
<td>Lard, beef tallow, mutton tallow and palm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AOCS for Low Erucic Rapeseed Oil</td>
<td>100 mg</td>
<td>O7756-1AMP</td>
</tr>
</tbody>
</table>
FAMEs in B20 Biodiesel on the SLB-IL111

Katherine K. Stenerson and Michael D. Buchanan
mike.buchanan@sial.com

B100 Biodiesel vs. B20 Biodiesel
In its undiluted, just-manufactured state, biodiesel is known as B100 biodiesel. Following several tests to determine purity and the absence of impurities, this material is typically blended with petroleum-based diesel prior to consumer use. A common blend is B20 biodiesel, which contains 20% biomass-based diesel and 80% petroleum-based diesel.

One test performed on biodiesel is to measure its FAME profile as a measure of purity. As shown in the previous article, an Omegawax column is suitable for performing this analysis with B100 biodiesel samples. Is it possible to also perform this test with a B20 biodiesel sample, maybe as a stability check? Will the petroleum-based biodiesel component interfere with the analysis?

GC Analysis, Results, and Discussion
A B20 biodiesel sample was made by mixing biomass-based diesel (soy source) and petroleum-based diesel to a 20:80 ratio. This mixture was diluted 1:20 with hexane prior to analyses on Omegawax and SLB-IL111 columns. Resulting chromatograms are shown in Figure 1, displayed using the same time scale for ease of comparison.

The Omegawax uses a polar phase, poly(ethylene glycol), as the stationary phase. This column was originally designed for, and is specifically tested for, the analysis of omega 3 and omega 6 fatty acids (as methyl esters). As evident, there is some overlap of the n-alkane and FAME fractions. Specifically, the C16:0 FAME (the first major FAME in the sample) elutes after the C22 n-alkane. Modifications to these analysis conditions did not result in a decrease in the overlap of the n-alkane and FAME fractions.

The SLB-IL111 uses an extremely polar ionic liquid as the stationary phase. Due to its higher polarity, it was predicted that this column would exhibit less overlap of the n-alkane and FAME fractions, compared to the Omegawax. Analysis confirmed this. Specifically, the C16:0 FAME elutes after the C25 n-alkane (the last significant n-alkane in the sample). These chromatograms also demonstrate the phenomenon that analytes will elute at lower temperatures on a more polar phase than on a less polar phase. The benefit is that the SLB-IL111 not only has the selectivity necessary to separate these n-alkanes and FAMEs by class, it also does it quicker (11 minutes compared to 16 minutes).

Conclusion
Bulk biodiesel producers regularly determine the FAME profile of their final product as a measure of purity. As B100 biodiesel, the Omegawax column is well-suited to perform this application. However, once blended, the SLB-IL111 is better able to perform this analysis. It results in better class separation in addition to an overall shorter analysis.
Get More from Your Money at

\textit{sigma-aldrich.com/gc-offers}

In today’s economy, budgets are tight and money may be scarce at many GC labs. Sigma-Aldrich can help! We run Promotional Offers on our newest products, as well as on many of the products that you use daily. The savings can really add up. Present/planned offers include these items:

- Capillary GC Columns
- GC Accessories (GC septa, inlet liners, ferrules, etc.)
- Gas Purification/Management (purifiers, tubing, fittings, etc.)

Be sure to visit \textit{sigma-aldrich.com/gc-offers} often, as offers are updated throughout the year.

---

**Featured Products**

<table>
<thead>
<tr>
<th>Description</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLB-IL111, 30 m x 0.25 mm I.D., 0.20 μm</td>
<td>28927-U</td>
</tr>
<tr>
<td>Omegawax, 30 m x 0.25 mm I.D., 0.25 μm</td>
<td>24136</td>
</tr>
</tbody>
</table>

**Introductory Offer:**

\textbf{40% off SLB-IL111 Columns}

Use \textbf{Promo Code 962} when placing your order. Offer expires December 31, 2011. Offer not valid in Argentina, Brazil, China, India, and Japan.

**Related Products**

<table>
<thead>
<tr>
<th>Description</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLB-IL111, 15 m x 0.10 mm I.D., 0.08 μm</td>
<td>28925-U</td>
</tr>
<tr>
<td>SLB-IL111, 100 m x 0.25 mm I.D., 0.20 μm</td>
<td>29647-U</td>
</tr>
</tbody>
</table>

**Related Information**

Additional information regarding the SLB-IL111, or any of our ionic liquid GC columns, can be found at our website: \textit{sigma-aldrich.com/il-gc}
Highly Selective Separation of Vitamin D epi-Metabolites Using HybridSPE-Phospholipid

Craig Aurand, David Bell, and Anders Fridstrom
emily.barrey@sial.com

Introduction
Vitamin D deficiency has become a topic of interest in recent publications (1-3). Vitamin D, along with calcium, promotes proper bone growth in children and aids in the prevention of osteoporosis in older adults. Vitamin D is present in two forms, Vitamin D3 and Vitamin D2. Both D2 and D3 vitamins are metabolized in the liver to form 25-hydroxyvitamin D2 (25-OH D2) and 25-hydroxyvitamin D3 (25-OH D3), respectively. In addition, biologically inactive 3-epi analogs of 25-OH D2 and 25-OH D3 have been reported, especially in young children (3). Recent studies have indicated that separation from the inactive 3-epi analogs may provide more accurate information for treatment and prevention. Analytical methods that can accurately quantitate both of the 25-hydroxyvitamin D analytes in the presence of 3-epi analogs may become essential for diagnosis and monitoring of patients with vitamin D disorders. The structures of the vitamin D analytes are shown in Figure 1.

Figure 1. Vitamin D Metabolite Structures
A. 25-Hydroxyvitamin D3
Monoisotopic Mass = 400.334131 Da; Molecular Formula = C27H44O2
B. 3-epi-25-Hydroxyvitamin D3
Monoisotopic Mass = 400.334131 Da; Molecular Formula = C27H44O2
C. 25-Hydroxyvitamin D2
Monoisotopic Mass = 412.334131 Da; Molecular Formula = C28H44O2

HPLC analysis of 25-OH D2 and 25-OH D3 is traditionally performed using C18 stationary phases. Under such conditions, the 3-epi analogs are poorly resolved, and thus are included in the overall reported value. Recently, Phinney, et al., reported the use of a cyano column for the effective separation of the 25-OH and the 3-epi forms for use in reference measurement procedures (1). Although effective, the conditions necessitate a run time greater than 40 minutes limiting its utility for routine high throughput analyses. LC-MS analysis methodologies for 25-hydroxyvitamin D3 are not without disadvantages as well. Sample preparation from serum requires protein precipitation with organic solvents or strong acids. This technique results in gross depletion levels of proteins from the sample, but also results in high levels of matrix interference from the still present phospholipids. LC-MS methods using standard protein precipitation are susceptible to ion suppression caused by the PLs or require gradient elution to wash this co-extracted matrix from the column resulting in longer cycle times for sample analysis.

Discussion
This study continues to detail new methodology for the analysis of Vitamin D metabolites which was introduced in a previous Reporter (4). In this study, the HybridSPE-Phospholipid was utilized to selectively extract the phospholipids from the serum sample. This technique combines the simplicity of standard protein precipitation with the added benefit of additional matrix removal. The combination of this novel sample preparation technique along with the unique selectivity of the Ascentis Express F5 HPLC column provides a fast and simplified bioanalytical method for the associated Vitamin D metabolites.

Rat serum purchased from Lampire Biological Laboratories (Pipersville PA) was spiked at 300 ng/mL with 25-hydroxyvitamin D2, 25-hydroxyvitamin D3 and epi-25-hydroxyvitamin D3. An internal standard was not included as part of this method. Protein precipitation was performed offline by adding 100 μL of spiked serum into a 500 μL 96-well collection plate followed by 300 μL of 1% formic acid acetonitrile. Samples were mixed by performing five 300 μL draw/aspiration cycles using a digital pipetter, then set for 5 minutes before transferring 200 μL of precipitate into the HybridSPE-Phospholipid 96-well plate. Samples were passed through the HybridSPE-Phospholipid plate by applying 10” Hg vacuum for 4 minutes, the resulting filtrate was analyzed directly.

As a comparison, spiked rat serum was also processed using standard protein precipitation by adding 100 μL of serum into 2 mL centrifuge vials followed by 300 μL of 1% formic acid acetonitrile. Samples were vortexed and centrifuged, and the resulting supernatant was collected and analyzed directly.

Figure 2 depicts the phospholipid monitoring chromatograms of the resulting co-extracted matrix from standard protein precipitation and from using the HybridSPE-Phospholipid technique. The HybridSPE-Phospholipid selectively depleted the
phospholipid matrix resulting in no matrix interference from this source. The standard protein precipitation technique shows a large amount of co-extracted phospholipids resulting in interferences that co-elute in the retention range of 25-hydroxyvitamin D2, 25-hydroxyvitamin D3 and epi-25-hydroxyvitamin D3. This co-elution has the potential to cause sensitivity loss and reproducibility issues resulting in irregularities in quantitation.

Figures 3a and 3b demonstrate the selectivity of the Ascentis Express F5 phase enabling isocratic resolution of 25-OH D3, 25-OH D2 and epi-25-OH D3 in less than four minutes, enabling and allowing for quantitation of all three components in one chromatographic analysis. Table 1 details a comparison of the average analyte response after HybridSPE-Phospholipid sample preparation for the three Vitamin D metabolites versus protein precipitation. The responses for the metabolites were 10-70% greater using HybridSPE-Phospholipid and a significant improvement was observed with the analysis of 25-OH D2 in which more reproducible results were achieved. By removing the interfering phospholipid matrix, enhanced sensitivity and precision were demonstrated for the three Vitamin D metabolites.

Summary
Separation of the biologically inactive 3-epi analog serves to provide improved data in support of vitamin D related clinical diagnostics and treatment. The pentafluorophenyl stationary phase has been shown to provide superior selectivity for the separation of the closely related 25-OH D3 and 3-epi-25-OH D3 as compared to methods reported in the literature. The unique selectivity of the Ascentis Express F5 combined with the selective phospholipid depletion of the HybridSPE-Phospholipid 96-well plate enable a fast and efficient analysis of 25-hydroxyvitamin D and related forms, that would otherwise be unattainable with traditional sample prep and reversed-phase HPLC approaches.

References

Table 1. Average Vitamin D Peak Areas for Rat Plasma Spiked at 300 ng/mL

<table>
<thead>
<tr>
<th></th>
<th>25-OH Vit D3 Results</th>
<th>epi-25-OH Vit D3 Results</th>
<th>25-OH Vit D2 Results</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HybridSPE-Phospholipid</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average Area (cps)</td>
<td>12729</td>
<td>19875</td>
<td>4466</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>1045</td>
<td>1146</td>
<td>542</td>
</tr>
<tr>
<td>Relative Standard Deviation (%)</td>
<td>8.2</td>
<td>5.8</td>
<td>12.1</td>
</tr>
<tr>
<td><strong>Protein Precipitation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average Area (cps)</td>
<td>9583</td>
<td>16965</td>
<td>2638</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>1160</td>
<td>1346</td>
<td>3130</td>
</tr>
<tr>
<td>Relative Standard Deviation (%)</td>
<td>12.1</td>
<td>7.9</td>
<td>118.6</td>
</tr>
</tbody>
</table>

Figure 3. Analysis of 25-Hydroxyvitamin D Using Ascentis Express F5

- Column: Ascentis Express F5, 10 cm x 2.1 mm I.D., 2.7 μm (53569-U)
- Mobile phase A: 5 mM ammonium formate-water
- Mobile phase B: 5 mM ammonium formate-methanol
- Mixing ratio: A:B = 25:75
- Flow: 0.4 mL/min.
- Temp: 40 °C
- Injection: 1 μL
- Ms detection: 100-1000 m/z

1. 25-hydroxyvitamin D3 (400.33 Da) 2.57 min
2. epi-25-hydroxyvitamin D3 (400.33 Da) 2.76 min
3. 25-hydroxyvitamin D2 (412.33 Da) 2.77 min

+ Featured Products

<table>
<thead>
<tr>
<th>Description</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>96-well Plate, 50 mg/well</td>
<td>575656-U</td>
</tr>
<tr>
<td>Ascentis Express F5, 10 cm x 2.1 mm I.D., 2.7 μm</td>
<td>53569-U</td>
</tr>
</tbody>
</table>

+ Related Products

<table>
<thead>
<tr>
<th>Description</th>
<th>Qty.</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>96-well Plate, 15 mg/well</td>
<td>1</td>
<td>52794-U</td>
</tr>
<tr>
<td>SPE Tubes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 mg/1 mL</td>
<td>100</td>
<td>55261-U</td>
</tr>
<tr>
<td>500 mg/6 mL</td>
<td>30</td>
<td>55267-U</td>
</tr>
<tr>
<td>SPE Tube for In-tube Precipitation</td>
<td>100</td>
<td>55269-U</td>
</tr>
</tbody>
</table>

Figure 3a. 25-Hydroxyvitamin D3 and epi-25-Hydroxyvitamin D2

Figure 3b. 25-Hydroxyvitamin D3 and 25-Hydroxyvitamin D2

G005310

G005311
Aldehydes and Ketones in Indoor Air using a Low Background LpDNPH Solvent Desorption Tube and Fused-Core HPLC

James Desorcie and Kristen Schultz  
kristen.schultz@sial.com

Introduction

Aldehydes are well-known and routinely measured pollutants due to their toxic properties and their role as ozone precursors. Human exposure to aldehydes in both commercial and residential environments continues to be a hot topic of interest for regulatory agencies such as the OSHA, NIOSH, EPA, and ASTM. Materials testing have also become an important factor for Green Building Council Initiatives (GBCI) and Leadership in Energy and Environmental Design (LEED) Programs whose goal is to create a system to promote sustainable building practices. The demand for low aldehyde background and detection limits is important for successful sampling protocols in both indoor and ambient environments.

A widely accepted method for capturing airborne aldehydes and ketones is via their reaction with 2,4-dinitro-phenylhydrazine (DNPH) coated on a silica support. The reaction produces non-volatile, hydrazone derivatives as depicted in Figure 1. These products are readily measured using HPLC analysis.

Figure 1. DNPH Derivatization Process

![Figure 1. DNPH Derivatization Process](image)

Experimental

Indoor laboratory air was sampled using an LpDNPH Cartridge (21014) with a DNPH loading of 1 mg and a PAS-500 Micro Air Sampling Pump (24865) powered by a 9-volt replaceable battery. Air was sampled at a rate of 200 mL/min for 20 hours. Following sampling, the cartridge was gravity eluted with acetonitrile (3 mL). HPLC analysis was used to measure the amounts of hydrazone derivatives in the eluent solution.

**Did you know...**

Sigma-Aldrich/Supelco has the widest selection of air sampling media available for sampling aldehydes – 12 different products in all, suitable for OSHA, NIOSH, EPA and ASTM methods. For more information, go to sigma-aldrich.com/air_monitoring
Results and Discussion

Chromatograms from the elution of a blank LpDNPH cartridge, the elution blank spiked with DNPH derivative standards, and elution of an LpDNPH cartridge used to sample laboratory air are shown in Figure 2. The low background of the LpDNPH S10 cartridge (clean blank extract) permits measurement of airborne aldehydes and ketones at low concentrations. The standards are used to identify and quantify the DNPH derivatives in the sample chromatogram. The measured airborne concentrations were formaldehyde: 3.8 μg/m³ (3.1 ppb), acetaldehyde: 3.2 μg/m³ (1.8 ppb) and acetone: 16.8 μg/m³ (7.1 ppb).

The Ascentis Express C18 HPLC column utilizes innovative Fused-Core particle technology. Compared to conventional HPLC columns with 5 μm particle silica, the Ascentis Express shows much higher column efficiency, allowing the use of shorter columns and still achieve similar or better separations. To maintain similar DNPH derivative retention times, lower flow rates than traditional methods can be applied (0.5 mL/min flow rate instead of the 1.2 mL/min) resulting in significant mobile phase savings. Because the column backpressure is similar to that achieved with 5 μm silica columns at higher flow rates (approx. 1,500 psi), the Ascentis Express column can be readily used with conventional HPLC instrumentation. It should be noted that another method modification is the need to reduce the injection volume from 20 to 10 μL in order to avoid column overload. However, there is no sacrifice in method sensitivity due to the inherent higher chromatographic efficiency of the Fused-Core particles.

Summary

Low background LpDNPH S10 air sampling cartridges are ideally suited for measuring low concentrations of airborne aldehydes and ketones. The Ascentis Express C18 column maintains the quality characteristics of the HPLC analysis while providing significant cost and environmental savings through the reduction of mobile phase consumption.
Steroid hormones are derivatives of cholesterol and play an important role in a large variety of organisms, as they can have a direct control on the gene expression. 17β-Estradiol (E2) controls the growth and the function of female secondary sexual characteristics. High blood concentrations inhibit the formation of further regulatory factors responsible for ovulation and pregnancy. E2 and its derivatives, e.g. ethinyl estradiol, are part of combined contraceptive pharmaceuticals, which have become widespread and common in use, thus leading so far to an unconsidered environmental problem: increased concentrations of estradiol and its metabolites in waste water (1-2). Now both, clinical and environmental laboratories have a vital interest in finding the most sensitive method for analysis of E2 and other steroid hormones mostly in matrices, which are difficult to remove. E2 is a nonpolar compound and hard to detect by ESI.

Fortunately, the analyte can be extracted very efficiently with solvents like methylene chloride or acetone. Additionally, this procedure reduces negative effects of the matrix, e.g. signal suppression by alkali salts. But only the introduction of ionizable moieties by derivatization can enhance the detection limits significantly. Dansyl chloride is the most common agent and reacts selectively and quantitatively with E2, testosteron and their derivatives (3-4). The detection is limited to APCI and APPI sources, which have some disadvantages regarding availability, dopant usage and lower sensitivity of the APCI source (Figure 1). Only a short pre-column (Ascentis Express C18, 5 cm x 2.1 mm, 2.7 μm) is necessary to separate the analyte from excessive reagent and byproducts (BPC, magenta). The MS/MS spectra results in a large number of fragments and a lower sensitivity on the quantifier.

A more sensitive and versatile derivatization agent for ESI sources is 4-(Dimethylamino)benzoyl chloride (DMABC Cat. No. 67954-1G). The reagent can be dissolved in acetone and applied on the dried residue of the sample extract. An adjustment of the pH is not necessary, only an anhydrous reaction medium is needed. At a high E2 level of 5 ppm only 0.2 % (rel. area fraction) of DAMBC react with the 2nd hydroxyl moiety (2:1 adduct). At 5 ppb E2 concentration the 2:1 adduct is below the detection limit. The high purity of DMABC guarantees a good solubility, very selective and quantitative reaction at a moderate temperature between 55-60 °C (5 min). The reagent and possible byproducts can be separated from the analytes by a standard reversed-phase HPLC column and detected down to very low concentrations (Figure 3). The MS/MS
Figure 3. Injection of 5 pg DMAB-E2 derivative and separation on a UHPLC system

- Column: Ascentis Express C18, 5 cm x 2.1 mm I.D., 2.7 μm (53822-U)
- Mobile phase: Water:formic acid:acetonitrile, 30:0.01:70
- Flow rate: 0.4 mL/min.

Spectrum (inset) shows only 4 major peaks, which is ideal for the quantification and identification using triple quadrupole mass spectrometer.

References

Superior Derivatization Reagents

Broad offering for accurate analysis on GC, HPLC or TLC

Over 400 Derivatizing Reagents
- Silylation, acylation and alkylation reagents for GC
- UV/VIS, fluorescent and electrochemical derivatives for HPLC
- Optically pure derivatizing reagent for chiral

- Derivatizing reagents for TLC applications
- Accessories for derivatizing reaction

To order your free copy of the New Derivatization Guide, and for product and ordering information, visit sigma-aldrich.com/derivatization
New *Withania somnifera* Analytical Standards and an Improved HPLC Method

Matthias Nold, David S. Bell and Hugh Cramer

 Sigma-Aldrich recently added several standards of *W. somnifera* constituents to our herbal medicinal product portfolio for the characterization and quantification of Ashwagandha. An HPLC column was also identified for providing a fast and efficient separation of the key constituents.

**Withania Constituents**

The characteristic constituents belong to a group of steroidal lactones consisting of a steroidal scaffold attached to a six membered lactone ring. The structures are shown in Figure 1.

**HPLC Analysis of Withania Constituents Using Ascentis Express Columns**

As part of a large number of natural health product studies recently conducted, many of the main constituents of Withania were screened on several modern Fused-Core stationary phases. The results from the screening effort showed that the Ascentis Express F5 and the Ascentis Express Phenyl-Hexyl phases provided improved selectivity over the C18 stationary phase. Both phases, presumably due to their intrinsic rigidity, are known to provide enhanced shape selectivity. The shape selectivity component is often found useful for the separation of closely related compounds with rigid structures.

Figure 2 shows a comparison of Withania standard constituents separated using the USP method to an optimized separation using Ascentis Express Phenyl-Hexyl. The USP method calls for a long, 40-minute gradient and the use of a 25 cm x 4.6 mm C18 column. Even with this lengthy system, only marginal separation of Withanolide A and Withanone is obtained. Conversely, a 15-minute gradient utilizing a shorter 10 cm x 2.1 mm phenyl-hexyl phase provides baseline separation of all components.

Figure 3 shows the separation of Ashwagandha extract constituents using both systems. The use of the phenyl hexyl column is again shown to provide improved resolution in a shorter period of time and with approximately 3X greater sensitivity. Note that only those components that could be confidently identified are noted. Similar results were obtained utilizing the Ascentis Express F5 stationary phase (data not shown).

**Conclusions**

Analyses of complex matrices such as the assay of natural product components may be greatly facilitated through the availability and use of quality standards and modern analytical separation tools. In this case, a fast method with full resolution of 7 constituents from the Ashwagandha root was achieved on an Ascentis Express Phenyl-Hexyl Fused Core particle column.
Figure 2. Comparison of Withania Standard Separation Using a Standard C18 vs Optimized Ascentis Express Phenyl-Hexyl Method

Discovery C18

Ascentis Express Phenyl-Hexyl

Discovery C18 Conditions for Figures 2 and 3
- Column: Discovery C18, 25 cm x 4.6 mm I.D., 5 μm (504971)
- Mobile phase A: phosphate buffer*
- Mobile phase B: acetonitrile
- Gradient:
  - Min %A %B
  - 0.0 95.0 5.0
  - 18.0 55.0 45.0
  - 25.0 20.0 80.0
  - 28.0 20.0 80.0
  - 30.0 95.0 5.0
  - 40.0 95.0 5.0
- Flow rate: 1.5 mL/min
- Temp.: 27 °C
- Det.: 227 nm
- Injection: 20 μL
- Samples: Standard: 20 μg/mL each in 80:20 water:methanol
- Extract: As per USP

Ascentis Express Phenyl-Hexyl Conditions for Figures 2 and 3
- Column: Ascentis Express Phenyl-Hexyl, 10 cm x 2.1 mm I.D., 2.7 μm (53336-U)
- Mobile phase A: water
- Mobile phase B: acetonitrile
- Gradient:
  - Min %A %B
  - 0.0 80.0 20.0
  - 10.0 0.0 100.0
  - 10.5 0.0 100.0
  - 11.0 20.0 80.0
  - 15.0 20.0 80.0
- Flow rate: 0.3 mL/min
- Temp.: 35 °C
- Det.: 227 nm
- Injection: 5 μL
- Samples: same as Discovery C18

Acknowledgement
Photographs of *Withania somnifera* were provided by Dr. Amit Agarwal, Director, Natural Remedies Pvt. Ltd., Bangalore, India.

References
1. USP 34, Dietary Supplements, Ashwagandha 1079.
2. BP 2011, Withania somnifera roots for THMP, 3674
3. A. Agarwal, B. Murali; “Quality Assessment of Selected Indian Medicinal Plants”; Volume 1.

Featured Products

<table>
<thead>
<tr>
<th>Description</th>
<th>Pkg. Size</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>12-Deoxywithastramonolide</td>
<td>10 mg</td>
<td>94187</td>
</tr>
<tr>
<td>Withaferin A</td>
<td>10 mg</td>
<td>89910</td>
</tr>
<tr>
<td>Withanolide A</td>
<td>10 mg</td>
<td>74776</td>
</tr>
<tr>
<td>Withanolide B</td>
<td>10 mg</td>
<td>94284</td>
</tr>
<tr>
<td>Withanone</td>
<td>10 mg</td>
<td>90896</td>
</tr>
<tr>
<td>Withanoside IV</td>
<td>10 mg</td>
<td>94186</td>
</tr>
<tr>
<td>Withanoside V</td>
<td>10 mg</td>
<td>66042</td>
</tr>
<tr>
<td>Ascentis Express Phenyl-Hexyl Column</td>
<td>10 cm x 2.1 mm I.D., 2.7 μm</td>
<td>53336-U</td>
</tr>
</tbody>
</table>

Related Information
Visit sigma-aldrich.com/medicinalplants to view our current offering of herbal medicinal analytical standards.
Introduction of Novel Performance-Tested Solvents for UHPLC Applications

New LC-MS Ultra CHROMASOLV® Grade Solvents and Additives

Rudolf Köehling
rudolf.koeihling@sial.com

Recent innovations in HPLC and mass spectrometry (MS), including Fused-Core® particles and ultra high performance/pressure liquid chromatography (UHPLC) systems, have pushed the limits of speed (throughput), efficiency and sensitivity. They have greatly increased the amount and quality of data obtained from HPLC and LC/MS experiments. While the technical “buzz” is usually around columns and instruments, the solvents used for mobile phases, sample preparation, and sample dissolution are also critical components of the system. Their influence on reducing background noise and baseline instability, extending column lifetime, and maintaining system integrity cannot be underestimated nor overlooked.

Solvent-derived impurities are one major issue facing chromatographers today. Background noise is typically caused by impurities that enter the system from the sample, by leaching from system components (bottles, tubes, valves, vials, etc.), and from solvents and additives. To reduce the latter contribution, Sigma-Aldrich developed and recently introduced Fluka®-brand LC-MS Ultra CHROMASOLV grade solvents and additives. During their development, several factors were taken into consideration when looking into UHPLC applications:

1. Impurities (types and concentrations)
2. Concentration of impurities under weak gradient conditions, with resulting elution later in the chromatogram or in subsequent runs
3. Baseline under gradient elution
4. Reduction of system down-time for cleaning

This new product line is tested specifically for UHPLC performance, including special designed gradient tests for UV and MS UHPLC separations with short columns, typically 5 cm to 10 cm in length, gradient applications. High flow rates under these condition types permit detection of as many impurities as possible in short run times and MS experiments are performed in both ESI(-) and ESI(+) modes. These detection limits differ considerably from standard HPLC gradient or LC-MS grade solvents, and pay tribute to the higher demands of UHPLC. In particular, the improvements in detection systems (UV, MS) reveal the smallest amounts of impurities. In order to obtain very low detection limits, reliable instrument performance and to lessen the amount of system maintenance, it is important to always use the highest quality solvent. The LC-MS Ultra CHROMASOLV solvents meet and exceed these criteria for UHPLC gradient separations in UV, as well as positive and negative ion mode MS detection.

In conclusion, the advent of UHPLC and other high-efficiency HPLC and LC-MS techniques have set new standards for sensitivity, efficiency, and throughput. In order for the UHPLC system to provide this reliable data and high performance and eliminate system down-time, it is critical to use solvents that are as carefully developed, prepared and tested as other components of the system. The solvents must be tested in situ under demanding UHPLC conditions and under various detection modes. Fluka’s new LC-MS Ultra CHROMASOLV grade solvents fulfil these requirements. For more information, visit us at sigma-aldrich.com/lc-ms.

**Featured Products**

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Package Size</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetonitrile</td>
<td>LC-MS Ultra CHROMASOLV, ≥99.9%, gradient tested for UHPLC, UV &amp; MS</td>
<td>1 L, 2 L</td>
<td>14261</td>
</tr>
<tr>
<td>Methanol</td>
<td>LC-MS Ultra CHROMASOLV, ≥99.9%, gradient tested for UHPLC, UV &amp; MS</td>
<td>1 L, 2 L</td>
<td>14262</td>
</tr>
<tr>
<td>Water</td>
<td>LC-MS Ultra CHROMASOLV, gradient tested for UHPLC</td>
<td>1 L, 2 L</td>
<td>14263</td>
</tr>
<tr>
<td>Trifluoroacetic acid</td>
<td>LC-MS Ultra eluent additive, ≥ 99% suitable for UHPLC-MS</td>
<td>1 mL, 2 mL</td>
<td>14264</td>
</tr>
<tr>
<td>Formic acid LC-MS</td>
<td>Ultra eluent additive, ≥ 98% suitable for UHPLC-MS</td>
<td>1 mL, 2 mL</td>
<td>14265</td>
</tr>
<tr>
<td>Ammonium formate</td>
<td>LC-MS Ultra eluent additive, suitable for UHPLC-MS</td>
<td>25 g</td>
<td>14266</td>
</tr>
<tr>
<td>Ammonium acetate</td>
<td>LC-MS Ultra eluent additive, suitable for UHPLC-MS</td>
<td>25 g</td>
<td>14267</td>
</tr>
</tbody>
</table>
New High-Purity Headspace Grade Solvents

Michael Kiselewsky
michael.kiselewsky@sial.com

When developing a GC-HS method, parameters such as sample solvent, extraction temperature, extraction time, sample volume and headspace volume are optimized. Sigma-Aldrich has developed solvents specifically for GC-HS applications. The purity and handling specifications for these solvents meet the requirements of the European Pharmacopoeia (Ph.Eur.) and United States Pharmacopeia (USP), as well as ICH guidelines. The new GC-HS product line includes water and three of the most commonly used organic solvents: dimethyl sulfoxide (DMSO), N,N-dimethylformamide, and N,N-dimethylacetamide. N,N-dimethylformamide and dimethyl sulfoxide are specified in Ph.Eur. and USP for water-insoluble substances. Water is the preferred solvent for water-soluble solutions, as described in Ph.Eur. and USP monographs. In addition, Sigma-Aldrich has now expanded its GC-HS portfolio with two new products, GC-HS Cyclohexanone and 1-Methyl-2-pyrrolidinone.

All solvents are microfiltered at 0.2 μm and packed under inert gas for extended shelf life.

For more information, visit us at sigma-aldrich.com/gc-hs

Literature


Product name Abbreviation BP Package Size Cat. No.
--- --- --- --- ---
Cyclohexanone, for GC-HS - 155 °C 1 L 68809
N,N-Dimethylacetamide, for GC-HS DMA 166 °C 1 L 44901
N,N-Dimethylformamide, for GC-HS DMF 153 °C 1 L 51781
1,3-Dimethyl-2-imidazolidinone, for GC-HS DMI 225 °C 100 mL, 1 L 67484
Dimethyl sulfoxide, for GC-HS DMSO 189 °C 1 L 51779
1-Methyl-2-pyrrolidinone, for GC-HS NMP 202 °C 1 L 69337
Water, for GC-HS - 100 °C 1 L 53463

Innovation Seminars Coming to Your Area

Supelco and Fluka – Sigma-Aldrich’s Analytical & Chromatography brands – invite you to one of our remaining 2011 complimentary half-day, open seminars in your region. Delivered by our technical experts and special guest speakers, the seminars show how our practical, innovative products and technologies can help you today.

Date Area
October 18-20 New England and New York City
November 29-30 Southeast (AL, TN, AR)
December 13-15 Southeast (TN, SC, GA)

For details and to register for seminars, visit, sigma-aldrich.com/events or email ac_seminars@sial.com.

Conferences and Exhibitions

Visit us at the following exhibitions to see the latest products and applications for analytical and chromatography.

<table>
<thead>
<tr>
<th>Conference</th>
<th>Date</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCC 2011</td>
<td>Oct. 11-12, 2011</td>
<td>Galveston, TX</td>
</tr>
<tr>
<td>ISPPP</td>
<td>Oct. 23-26, 2011</td>
<td>Alexandria, VA</td>
</tr>
<tr>
<td>EAS 2011</td>
<td>Nov. 14-17, 2011</td>
<td>Somerset, NJ</td>
</tr>
<tr>
<td>PITTCON 2012</td>
<td>March 11-15, 2012</td>
<td>Orlando, FL</td>
</tr>
</tbody>
</table>

For more information, visit sigma-aldrich.com/lc-ms.