THE APPLICATION NOTEBOOK – FEBRUARY 2019

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Cover Photography: Getty Images
Utilizing Automated SPE to Improve the Recovery of Semivolatile Compounds in Compliance with EPA Method 8270

Michael Ebitson, Stephen Panos, and Andrew Taylor, Biotage

Groundwater is one of our most precious, yet easily contaminated resources. Contaminants can originate from a multitude of sources. Industrial sources include underground storage tanks, septic systems, farms that have been treated with pesticides, and hazardous waste sites. Smaller contributors, such as individual households, can pollute local groundwater with items such as household cleaners, disinfectants, laundry detergents, stain removers, motor oil, gasoline, paint, and paint removers. Even an inkjet printer can be a source of groundwater contamination.

Consuming contaminated drinking water is a well-known public health risk (1), and can produce effects ranging from mild illnesses to serious diseases, and even death. Effects can be challenging to predict with 100% accuracy as they depend on a number of factors, including the chemical properties of the contaminants, and the degree and length of exposure, on top of factors such as the person's age and whether he or she has pre-existing health conditions. The key to ensuring our safety is to minimize our exposure to those contaminants with known health hazards.

The U.S. EPA monitors a variety of compounds that pose public health risks when they are present in our air, soil, or water. The 8000 Series EPA Methods outline the quantification of contaminants in groundwater, and Method 8270 specifically addresses semivolatile compounds (2). The method outlines the extraction of over 200 semivolatile compounds that fall into a range of compound classes, including; nitrosamines, pesticides, ethers, ketones, phenols, polyaromatic hydrocarbons (PAHs), anilines, pyridines, and aldehydes. While most laboratories do not quantify all 243 compounds outlined in the method, they often measure at least 100 compounds from several different compound classes and in a variety of challenging sample matrices.

This applicate note presents the use of an automated solid-phase extraction (SPE) system to improve the accuracy and precision of semivolatile extractions, in compliance with EPA Method 8270. In this work, a Biotage® Horizon 5000 automated SPE system was used to extract 114 semivolatile organic compounds from groundwater samples. The extraction system was fitted with an Atlantic® One-Pass SPE Disk, combined with a One-Pass Carbon Cartridge, to streamline the extraction and reduce losses due to precipitation or volatilization. The extracts were concentrated using a DryVap® Concentrator before being transferred to a GC–MS instrument for measurement. Results for a pair of matrix spike (MS) and matrix spike duplicate (MSD) samples are presented in Table I. Data for each analyte is presented as an average spike recovery and as a relative percent difference (RPD) between the two spiked samples. For simplicity, only a subset of the measured analytes is presented.

As indicated in Table I, the extraction and concentration workflow is ideally suited for extracting a wide range of semivolatile organic compounds with precision and accuracy that is compliant with EPA Method 8270E. With the exception of challenging compounds such as pyridine, NDMA, hexachlorocyclopentadiene, and 2-picoline, all matrix spikes were recovered between 50–150% of their spiked concentration and relative percent differences were below 20%, in compliance with Method 8270E requirements.

References


Table I: Spike recovery results for matrix spike (MS) and matrix spike duplicate (MSD) samples

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<thead>
<tr>
<th>Analyte</th>
<th>Results for Matrix Spike 1</th>
<th>Results for Matrix Spike 2</th>
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<tr>
<td></td>
<td>Avg. Spike Recovery (%)</td>
<td>RPD (%)</td>
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<tr>
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<td>Avg. Spike Recovery (%)</td>
<td>RPD (%)</td>
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<td>3.2</td>
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<td>2,3,4,6-Tetrachlorophenol</td>
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<td>1-Naphthylamine</td>
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<tr>
<td>2-Methylphthalalene</td>
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<td>3-Nitroaniline</td>
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<td>5.6</td>
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<td>4-Bromophenyl phenyl ether</td>
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<td>Anthracene</td>
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<td>Benzo(a)pyrene</td>
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<td>Dibenzo furan</td>
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<tr>
<td>Saffrole</td>
<td>86.0</td>
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16 Northwestern Drive, Salem, NH 03079
tel. (603) 893-3663, fax (603) 893-4994
Website: www.biotage.com
Analysis of Amino Acids in Oxidized and Unoxidized Feed Samples

Maria Ofitserova, PhD, Pickering Laboratories, Inc.

Commission Regulation (EC) No 152-2009, published in The Official Journal of European Union, laid down the methods of sampling and analysis for the official control of feed. The regulation describes methods of analysis to control the composition of feed materials and compound feed products. Establishing the amino acids profile is an important way to control quality and nutritional value of feeds. This regulation specifies HPLC with post-column derivatization with ninhydrin reagent as the method of analysis for total and free amino acids. Pickering Laboratories developed an analytical method to comply with all the chromatographic requirements of Commission Regulation (EC) No 152-2009. The same method is used to analyze oxidized and unoxidized feed samples.

Method

Analytical Conditions

Column: High-efficiency sodium cation-exchange column, 4.0 x 150 mm, Catalog Number 1154150T

Guard: Cation-exchange GARD™, Catalog Number 1700-3102

Flow rate: 0.4 mL/min

Mobile Phases: Na270, Na740, RG011.

Injection Volume: 10 uL

Table 1: HPLC Program

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<th>Time, min</th>
<th>Na270, %</th>
<th>Na740, %</th>
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Table 2: Column oven program

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<td>41</td>
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<td>42</td>
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Table 2: Column oven program

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<tbody>
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<td>41</td>
<td>65</td>
</tr>
<tr>
<td>42</td>
<td>55</td>
</tr>
</tbody>
</table>

Figure 1: Chromatogram of a standard solution of amino acids
**Post-Column Conditions**

- **Post-Column System:** Pinnacle PCX
- **Reactor volume:** 0.5 mL
- **Reagent:** Trione®
- **Column Temperature:** See method in Table II
- **Reactor Temperature:** 130 °C
- **Flow rate:** 0.25 mL/min
- **Detection:** UV-VIS 570 nm for primary amino acids, 440 nm for secondary amino acids

---

**Figure 2:** Chromatogram of an oxidized feed sample

**Figure 3:** Chromatogram of a non-oxidized feed sample

---

**Pickering Laboratories**

1280 Space Park Way, Mountain View, CA 94043
tel. (800) 654-3330, (650) 694-6700, fax: (650) 968-0749
Website: www.pickeringlabs.com
LC–MS/MS Analysis of Mycotoxins in Peanut Powder in 5.5 Min

Restek Corporation

- Fast analysis for higher sample throughput
- Excellent separation improves accuracy for 12 regulated mycotoxins
- Quick and easy sample preparation (dilute-filter-shoot)

Certain fungi that can grow on agricultural products produce toxic metabolites known as mycotoxins. Modern food processing procedures cannot completely remove these compounds if they are present, so strict monitoring protocols have been established. Although a universal method for the analysis of mycotoxins would allow highly efficient screening, it is very challenging to develop such a method due to differences in physiochemical properties of mycotoxins, extraction efficiencies, and matrix effects. Zhang and associates published a multi-lab study (1) aimed at providing labs with an analytical procedure that could be broadly applied to the analysis of a variety of mycotoxins in many different matrices. Using that work as inspiration, we developed the following LC–MS/MS method that resolves 12 FDA regulated mycotoxins within the pressure limits of traditional HPLC instruments.

In this example, mycotoxins were analyzed in a peanut powder matrix. The use of a relatively short column format, the selectivity of the Biphenyl stationary phase, and the efficiency of 2.7-μm Raptor supercritically porous particles provided excellent separations in a fast 5.5-min analysis (total cycle time of 7 min). A coeluting matrix compound that shared the most abundant MRM transition for mycotoxin HT-2 (447.3-285.3) was observed, so a less abundant transition (447.3-345.3) was selected for quantitation. To increase sensitivity, an ammonium buffer was used to promote better ionization of mycotoxins. The Raptor Biphenyl column worked very well for the 12 mycotoxins studied in the cited work, but for longer compound lists containing isobaric mycotoxins with similar structures, the Raptor FluoroPhenyl phase may be necessary to provide adequate chromatographic resolution. The selectivity of the Raptor FluoroPhenyl column is demonstrated in an analysis of 20 mycotoxins that can be found by visiting www.restek.com and entering LC_FS0511 in the search.

This method showed excellent precision and accuracy for the 12 FDA regulated mycotoxins that were evaluated during a validation study that covered a variety of matrices (including multiple sources of cornmeal and brown rice flour, in addition to the peanut powder example shown here).

Restek would like to thank Dr. Zhang for his technical support during this project.

\[\text{Matrix interference was observed for HT-2 transition 447.3-285.3 in peanut powder. Therefore, 447.3-345.3 was chosen for quantification.}\]

\[\text{Columns: Raptor Biphenyl (cat. # 9309A352); Dimensions: 50 mm x 2.1 mm ID; Particle Size: 2.7 μm; Flow Rate: 0.3 mL/min;} \]

\[\text{Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column Column 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Raptor Biphenyl LC Columns (USP L11)

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Reference

**Why are C18s So Different?**

**A Focus on Contributions of Steric Selectivity**

Robert Puryear*, Piotr Macech*, Dustin Austin*, Hiroshi Tachikawa†, and Itaru Yazawa†, *Imtakt USA, Portland, OR, Imtakt Corp., †Kyoto, Japan

One of the fundamental tenets of chromatography is the interaction between an analyte and stationary phase. We often assume that this should be consistent between different columns with the same stationary phase, but this is not always the case. With C18 columns, this highlights the contribution of factors other than hydrophobicity, such as electrostatic interaction, when a column has exposed unreacted surface silanols, or steric selectivity, which is related to surface ODS density (Figure 1).

**Experimental Conditions** are shown in each figure.

**Result and Discussion**

Figure 2 shows that the columns tested had significantly different separation profiles for o-terphenyl and triphenylene, despite having nearly identical hydrophobic resolution ($\alpha = 1.4–1.5$, data not shown). This comparison shows how steric selectivity can contribute to variation, with Brand XB ($\alpha=1.4$) having the least resolution, followed by Cadenza CD-C18 ($\alpha=1.6$), then Cadenza CX-C18 ($\alpha=2.2$), with Dacapo DX-C18 ($\alpha=2.5$) having the highest.

**Conclusion**

We first discovered how to improve steric selectivity with the development of our Cadenza CD-C18 column. In Figure 1 you can see that, by having a higher ligand density, this creates a narrower space between the C18 chains, which presents a different geometry of interaction for molecules that are more planar. We have developed this technology further with Cadenza CX-C18 and Dacapo DX-C18, improving steric selectivity to $\alpha$ values of 2.2 and 2.5, respectively. The advantage of high steric selectivity can be seen in Figure 3, where four steroids with only minor differences were baseline resolved. In this case, the differences were minute, as with hydrocortisone vs. prednisolone, where a single bond is replaced by a double bond, and in cortisone vs. hydrocortisone, where a ketone is replaced by an alcohol.

In summary, we have shown here that four ODS columns can have very different retention characteristics, despite all having the same ligand, with steric selectivity significantly contributing to this variation. We have also shown the importance of steric selectivity in providing separation of compounds that are very similar to each other. This suggests that the use of a high steric selectivity ODS column should be considered when separating compounds such as isomers or impurities, which may have very little difference between them.
Separation of Five Steroids on a Hamilton PRP™-C18 Reversed-Phase HPLC Column

Steroids represent a chemically distinct class of hormones with wide-ranging biological functions. Synthetic derivatives of endogenous steroid prototypes are used medically in birth control and in the treatment of asthma, arthritis, inflammation, and osteoporosis.

Steroids share a characteristic polycyclic structure, and have varying degrees of lipophilicity (log P). In this study, a reversed-phase HPLC method was developed for separation of five steroid hormones with partition coefficients ranging from 1.47 (cortisone) to 4.5 (pregnenolone) on a Hamilton PRP-C18 HPLC column.

For more information on Hamilton HPLC columns and accessories or to order a product, please visit www.hamiltoncompany.com or call (800) 648-5950 in the US or +41-81-660-60-60 in Europe.
Monoclonal antibodies (mAbs) comprise the largest class of glycosylated protein therapeutics currently on the market, and glycosylation is known to be a major source of mAb heterogeneity. N-glycosylation of IgG-Fc of mAbs is known to impact drug therapeutic mechanism of action (MOA), thus monitoring glycan critical quality attributes (CQAs) is an essential part of biopharmaceutical development. Glycosylation is a critical factor in drug product solubility, kinetics, stability, efficacy, and immunogenicity. Analytical methods utilize a suite of chromatographic modes using high performance liquid chromatography (HPLC) to analyze glycosylation of both intact and digested protein molecules.

The TSKgel FcR-IIIA-NPR column is a high performance affinity chromatography column for the analysis of IgG glycoforms. The stationary phase utilizes a recombinant FcR-IIIA protein bound to a nonporous polymethacrylate polymer. The retention mechanism is based on the interaction between the FcR ligand and the sugar moieties attached to the ASN amino acid in the conserved region of the monoclonal antibody. The resulting elution profile of the glycoprotein mimics ADCC activity, which is correlated to the composition of the N-glycans.

The purpose of this study is to demonstrate the use of mass spectrometry to characterize the elution profile of a typical IgG molecule separation on a TSKgel FcR-IIIA-NPR column, and verify the observations that certain glycan structures impart higher activity to the monoclonal antibody, especially as it relates to the presence of terminal galactose sugars.

**Experimental HPLC Conditions**

**TSKgel FcR-IIIA-NPR Separation**

- **Column:** TSKgel FcR-IIIA-NPR, 5 μm, 4.6 mm ID × 7.5 cm
- **Instrument:** Agilent 1200
- **Mobile phase:** A: 50 mmol/L Na citrate, pH 6.5
  B: 50 mmol/L Na citrate, pH 4.5
- **Gradient:** 0 min: 0% B, 20 min: 100% B, 30 min: 100% B
- **Flow rate:** 0.85 mL/min
- **Detection:** UV @ 280 nm, 25 Hz
- **Temperature:** 15 °C
- **Injection vol.:** 5 μL
- **Sample:** NIST mAb fractions; 5 mg/mL in mobile phase A

**Top Down MS Characterization**

- **Column:** TSKgel Protein C 4-300, 3 μm, 2.0 mm ID × 15 cm
- **Instrument:** Shimadzu Nexera® XR
- **Mobile phase:** A: 0.1% formic acid in water
  B: 0.1% formic acid in acetonitrile
- **Gradient:** 0 min: 10% B, 40 min: 95% B, 50 min: 95% B
- **Flow rate:** 0.2 mL/min
- **Detection:** Sciex X500B Q-TOF, ESI positive, m/z 900-4000
- **Temperature:** 50 °C
- **Injection vol.:** 5 μL
- **Sample:** NIST mAb fractions; 100 μg/mL in LC–MS water

**MS Conditions:**

<table>
<thead>
<tr>
<th>Source gas 1</th>
<th>50 psi</th>
<th>Spray voltage</th>
<th>5000 eV</th>
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<tr>
<td>Source gas 2</td>
<td>50 psi</td>
<td>Declustering potential</td>
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<tr>
<td>Curtain gas</td>
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<td>DP spread</td>
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<tr>
<td>CAD gas</td>
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<td>Source temp</td>
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<td>CE spread</td>
<td>0 eV</td>
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<tr>
<td>Accumulation time</td>
<td>1 sec</td>
<td>Bins to sum</td>
<td>80</td>
</tr>
</tbody>
</table>
Results and Discussion

Figure 1 demonstrates the separation of NIST mAb on the TSKgel FcR-IIIA-NPR column. IgG₁ molecules yield this typical type of elution profile based on glycoform composition that is consistent with ADCC activity. This offers a fast orthogonal chromatographic method for determination of antibody activity and comparisons of antibody heterogeneity.

The three largest eluting peaks were collected and analyzed by offline mass spectrometry. Peak fractions were pooled from successive 25 μg on column injections, concentrated, and buffer exchanged to LC/MS grade water.

Figures 2 and 3 illustrate analysis of the NIST mAb standard compared against the collected peak fractions. It is observed that each peak has a unique composition of intact mAb glycoforms and that the selectivity of the stationary phase is based on the amount of terminal galactose units on the glycans moiety. This conclusion agrees with studies that show antibodies with higher amounts of G1- and G2-containing sugars show greater ADCC activity. Because of some peak overlap in the initial separation, there is some overlap of different galactose-containing species in the MS profile, though the general trend between galactose and activity has been confirmed.

Conclusions

The separation of an IgG₁ molecule was demonstrated using the TSKgel FcR-IITA-NPR column and peaks from that separation were characterized by high resolution mass spectrometry. The results support that the stationary phase selectivity is based on the same Fc-glycan/Fc receptor interaction as ADCC activity. The glycoform composition of each peak is consistent with previous published observations on the activity of N-glycan sugars with higher amounts of terminal galactose.

This application demonstrates the efficacy of this approach and characterization data that demonstrate the proof of concept of this chromatographic technique for a fast orthogonal analysis to evaluate mAb ADCC activity, potentially for early cell line development, bioreactor modeling and lot-to-lot comparability of therapeutic antibodies.
Figure 3: Reconstructed spectra for each of the isolated peak fractions, indicating that later eluting fractions have a greater proportion of terminal galactose glycan sugars, consistent with observations of antibody activity and percentage of galactose.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Mass</th>
<th>Glycoform</th>
<th>Peak</th>
<th>Mass</th>
<th>Glycoform</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>147620</td>
<td>G0F/G0F (-2GlcNAc)</td>
<td>7</td>
<td>148292</td>
<td>G0F/G1F (Adduct)</td>
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<td>2</td>
<td>147756</td>
<td>G0F/G0F (-GlcNAc)</td>
<td>8</td>
<td>148362</td>
<td>G1F/G1F</td>
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<tr>
<td>3</td>
<td>147966</td>
<td>G0F/G1F (-GlcNAc)</td>
<td>9</td>
<td>148455</td>
<td>G1F/G1F (Adduct)</td>
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<td>4</td>
<td>148038</td>
<td>G0F/G0F</td>
<td>10</td>
<td>148524</td>
<td>G1F/G2F (+Hex)</td>
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<tr>
<td>5</td>
<td>148129</td>
<td>G0F/G0F (Adduct)</td>
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<td>148684</td>
<td>G2F/G2F (+Hex)</td>
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<tr>
<td>6</td>
<td>148200</td>
<td>G0F/G1F (Adduct)</td>
<td>12</td>
<td>148843</td>
<td>G2F/G2F</td>
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</tbody>
</table>

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Extraction of Loperamide and N-Desmethyl Loperamide from Blood Followed by LC–MS/MS Analysis

Tina Fanning, UCT, LLC

Loperamide is an over-the-counter antidiarrheal drug that has been considered to be safe when used as directed. However, as the opioid epidemic continues to ravage our nation, there has been an increasing number of reported cases of loperamide overdose. This application note outlines a simple three-step method for the extraction of loperamide and its main metabolite, N-desmethyl loperamide, from blood. UCT’s Clean Screen® XCEL I column provides users with the same level of sample clean-up as traditional SPE, while allowing the elimination of timely conditioning and wash steps.

**Extraction/Analytical Materials**

<table>
<thead>
<tr>
<th>Material</th>
<th>Details</th>
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</thead>
<tbody>
<tr>
<td>CSXCE106</td>
<td>Clean Screen XCEL I 130 mg / 6 mL SPE Cartridge</td>
</tr>
<tr>
<td>SLC-18100ID21-18UM</td>
<td>Selectra® C18 HPLC 100 x 2.1 mm, 1.8 μm</td>
</tr>
</tbody>
</table>

**Procedure:**

**Sample Pretreatment:**

1. To 1 mL blood sample add 3 mL Acetate Buffer pH 5 and appropriate amount of internal standard
2. Vortex Samples for 30 s to mix

**SPE Procedure:**

1. Apply samples to SPE tubes without any preconditioning
2. Allow samples to flow through the column at a rate of 1–2 mL/min.
3. Wash samples with 2 mL of D.I. H₂O
4. Wash SPE column with 2 mL of 98:2 MeOH:Glacial Acetic Acid
5. Dry column for 5 min at full vacuum or pressure
6. Wash samples with 2 mL of Hexane
7. Dry column for 10 min at full vacuum or pressure
8. Elute compounds with 2 mL of 78:20:2 DCM:IPA:NH₄OH
9. Collect eluate at a rate of 1–2 mL/min
10. Evaporate to dryness at < 50 °C
11. Reconstitute sample in mobile phase

**Instrumental:**

**LC–MS/MS:** Thermo Scientific™ Dionex™ 3000 (LC) TSQ Vantage™ (MS/MS)
**Column:** UCT Selectra DA HPLC Column 100 x 2.1 mm, 1.8 μm

**Results:**

**MRM transitions (ESI+, 50 ms dwell time)**

<table>
<thead>
<tr>
<th>Compound</th>
<th>t_R (min)</th>
<th>Q1 ion</th>
<th>Q3 ion 1</th>
<th>Q3 ion 2</th>
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</thead>
<tbody>
<tr>
<td>N-Desmethyl-loperamide</td>
<td>2.83</td>
<td>463.1</td>
<td>252.06</td>
<td>196.03</td>
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<tr>
<td>loperamide</td>
<td>2.95</td>
<td>477.1</td>
<td>266.05</td>
<td>209.98</td>
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</table>

**Recovery (%) from Blood (n=3)**

<table>
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<tr>
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<th>5 ng/mL</th>
<th>10 ng/mL</th>
<th>50 ng/mL</th>
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<tbody>
<tr>
<td>N-desmethyl-loperamide</td>
<td>106</td>
<td>97</td>
<td>96</td>
</tr>
<tr>
<td>loperamide</td>
<td>102</td>
<td>95</td>
<td>93</td>
</tr>
<tr>
<td>Average recovery</td>
<td>104</td>
<td>96</td>
<td>94.5</td>
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</table>

**Conclusion:**

This application note effectively outlines a simple three-step method for the extraction of loperamide and its main metabolite, N-desmethyl loperamide, from blood. UCT’s Clean Screen XCEL I column provides users with the same level of exceptional sample clean-up as traditional SPE while allowing the elimination of timely conditioning and wash steps in addition to a concentration step. Sample extracts are analyzed via UHPLC–MS/MS utilizing UCT’s Selectra C18 1.8 μm analytical column.

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Absolute Molar Mass Analysis of Chitosans

Wyatt Technology

Chitosans are analyzed by SEC-MALS to determine molar mass moments and distributions.

Chitin is one of the most abundant biopolymers on earth (the other is cellulose). Chitin, or poly-N-acetylglucosamine, is the major polymer in the exoskeleton of marine arthropods and can also be found in fungi and yeasts.

Chitosan is deacetylated chitin. It can be obtained from shrimp or crab shells. Its applications vary from the therapeutic, such as wound healing, to cosmetics and dietary supplements.

For many of these applications, it is useful to fully characterize the molar mass moments and distributions of chitosan products. Size-exclusion chromatography, in combination with multi angle light scattering detection (SEC-MALS), provides an easy method to obtain these properties in an absolute manner, free of molecular references. In this note, we describe the results for two chitosan samples analyzed by SEC-MALS.

**Experimental Conditions**

A DAWN® MALS detector (Wyatt Technology, Santa Barbara) and an Optilab® differential refractive index (dRI) detector (Wyatt Technology) were plumbed downstream of the GPC column. Data collection and analysis were performed in the ASTRA® software (Wyatt Technology) using empirically determined differential refractive index increments (dn/dc). Polymer molar mass $M$ was calculated at each elution volume using signals from the two detectors.

The differential refractive index is a property of the polymer–solvent system. It is measured by injecting a series of known concentrations into the Optilab, using solutions that are often prepared by the dry weight method for accuracy, and the GPC mobile phase as solvent. ASTRA collects and analyzes the results to determine $dn/dc$.

**Results**

The molar masses of two chitosan samples are plotted as a function of elution volume in Figure 1. The molar mass decreases logarithmically, indicating that chromatographic conditions were optimal. The offset in elution volume for a given molar mass may be a result of conformation (short-chain branching, SCB) or non-ideal analyte–column interaction. SCB may be further investigated by making use of simultaneous size (radius of gyration, $R_g$) and molar mass analysis by MALS.

A cumulative molar mass distribution plot, depicted in Figure 2, clearly differentiates the two chitosan samples. ASTRA software can also report weight fractions above, below, or between the molar masses of interest. As an example, the weight fractions of molar masses below 50 kDa and above 500 kDa for these samples are given in the table in Figure 2. These calculations and the cumulative molar mass distribution plot are ideal for quality control applications.

**Conclusions**

The results described herein show that MALS detection combined with SEC provides a useful tool for biopolymer characterization. Absolute molar mass and molar mass distribution can be readily obtained without the use of any standards or empirical relations, simplifying QC and routine analyses while enhancing in-depth analysis with absolute measurements of size as well as molar mass.
Develosil UHPLC C18 and C30 for Oligonucleotide Analysis

Satoshi Horikiri, Develosil USA

Reversed-phase HPLC is common for analysis of small to midsize molecules, but careful experimental design and selection of columns are both required for successful separation.

Here we applied our newly developed UHPLC C18 and C30 columns to oligonucleotide analysis. This new type of column is designed and optimized for the rapid separation of small to midsize molecules, utilizing 1.6-μm diameter particles with 11-nm diameter pores to achieve higher resolution. Theoretically, this pore size should allow separation of molecules of up to 25,000 kDa with this column.

**Experimental Condition**

Table I shows the analytical conditions. For Develosil 1.6 μm UHPLC columns, C18 has a higher density of alkyl chains on the silica gel surface than C30. This results in the C18 columns having higher retention of hydrophobic compounds. The lower density of alkyl chains in the C30 column allows the mobile phase more access to the endcapped silica, which can influence the separation of hydrophilic compounds. Because of this, modulation of the pH and organic solvent content of the mobile phase can result in larger changes in retention of hydrophilic species such as oligonucleotides on C30 when compared to C18.

**Results and Discussion**

Figure 1 shows an oligodeoxythymidine ladder standard mixture (DNA ladder standard, oligos at 15, 20, 25, 30, 35, and 40 mer) separated using Develosil UHPLC C18 1.6 μm. The sample has higher concentrations at each 5-mer increment from 15-mer and up. This chromatogram shows each additional individual nucleotide length is a clearly separated peak.

We then applied Develosil UHPLC C30 1.6 μm to this analysis (Figure 2). Compared to C18, the C30 column retains nucleotides more strongly, leading to longer retention times under the same conditions. Increasing the final concentration of mobile phase B by 0.5% results in similar retention times to that of the C18 column. During the process of optimizing the mobile phase, we also found that small differences in mobile phase composition will affect retention time on C30 much more so than on C18.

**Conclusion**

Using the 1.6 μm particle of Develosil UHPLC C18 and C30 showed clear and sharp peaks of oligonucleotides up to 40 mer. The resolution was higher on C30. This indicates that choosing different column lengths or mobile phase conditions can enable analysis of oligonucleotides 40 nucleotides or even longer.
Add More Confidence to Your UHPLC–MS Analysis

Rudolf Köhling, PhD, MilliporeSigma

For your highly sensitive UHPLC–MS analyses, how can you reduce noise and additional signals to a minimum? Our new high-end UHPLC-MS solvents raise the standard for low baseline noise and clean mass spectra.

Our new range of advanced UHPLC–MS LiChrosolv® solvents have been developed to exceed all expectations, providing rapid and reliable results in both ESI/APCI positive and negative ionization modes.

Thanks to their lowest level of background noise and ion suppression, this quality ensures the optimum ionization efficiency to enable the highest sensitivity. With these features, use of these solvents can also help to extend column lifetime.

To ensure that you have confidence in your results, we specify the lowest possible limit of polyethylene glycol (PEG) impurities in all our UHPLC-MS solvents.

Our advanced UHPLC–MS LiChrosolv solvents have been designed to meet the highest requirements of UHPLC–MS in research and quality control, including proteomics and metabolomics, as well as environmental, clinical, food, or industrial testing applications.

A new standard for the unlimited application of ultra high-pressure chromatography has been set.

Features and Benefits

- Suitability tested and specified for UHPLC-MS and UHPLC-UV: for analytical flexibility
- Specified quality in positive and negative ESI and APCI MS for lowest detection limits and confidence in analyses in all important MS modes (Test 1)
  - ESI or APCI (+) < 2 ppb
  - ESI or APCI (-) < 10 ppb
- Lowest impurity profile: for interference-free baselines (Test 2)
- Microfiltration through 0.2 μm filter (Test 3)
  - Prolonged lifetime of filters and mechanical parts in HPLC systems
  - Reduced risk of column clogging
- Packaged in borosilicate glass bottles: minimized contamination with metal ions
- Lowest levels of trace metal impurities: for minimized metal ion adduct formation
  - <5 ppb
- Lowest level of polyethylene glycol (PEG) impurities in our entire UHPLC–MS solvent lineup to give you confidence in your results (PEG S/N signal-to-noise-ratio < 50)

LiChrosolv Methanol for UHPLC–MS shows a flat baseline and by far the lowest impurity profile compared to the competition. Both competitor’s high purity UHPLC-MS products A and B show a baseline drift and significant impurity peaks.

Test 1: UHPLC-MS gradient run with LiChrosolv acetonitrile for UHPLC–MS shows a clear detection and identification of 1 ppb reserpine, 500 ppt propazine, and 4 ppb prednisolone, with very low background interferences.

Test 2: Comparison of LiChrosolv methanol for UHPLC–MS (blue line) with two competitor UHPLC-MS products.

To read the rest of this application please visit SigmaAldrich.com/UHPLC–MS.
Simultaneous Analysis of Ten Water-Soluble Vitamins Using a Polymer-Based Reversed-Phase Column—Shodex™ RSpak DE-413L

Showa Denko America, Inc.

Vitamins are micronutrients essential for the metabolism of living organisms. Since humans cannot produce vitamins, the intake of vitamins must be part of their intake. There are many commercial foods and drinks supplemented with vitamins for nutrient enhancement purposes, including most processed foods.

Methods using microbiological assays, absorption spectrophotometry, and HPLC have been used to analyze vitamins, creating a long process. A typical HPLC method to separate and quantify vitamins can use an ODS column with an addition of ion-pair reagent. However, the ion-pair reagent tends to remain on the column and the flow-lines, resulting in increased background level and lowers the sensitivity.

Therefore, in this application, a simple method to simultaneously analyze various water-soluble vitamins was developed. Shodex DE-413L, a polymer-based reversed phase column, without the use of an ion-pair reagent. We further applied the developed method to quantify vitamins in a commercial multi-vitamin supplement.

Experimental

Ten vitamins (thiamin HCl, pyridoxine HCl, nicotinamide, ascorbic acid, nicotinic acid, calcium pantothenate, cyanocobalamin, folic acid, riboflavin, and biotin) were used as standards. A 4-mM standard solution was used for biotin and 2-mM standard solutions were used for other vitamins. More standards were dissolved in 250-mM phosphoric acid.

Five levels of multi-vitamin calibration standards were prepared using standard solutions and 250-mM phosphoric acid. We used 250-mM of phosphoric acid to prevent the oxidation of ascorbic acid.

A Shodex RSpak DE-413L column (4.6 mm I.D. × 250 mm, 4 μm) was used with a PDA detector (190–400 nm). The eluent conditions were as follows: (A) 10 mM H3PO4 aq. / (B) CH³CN, linear gradient (high pressure); (B%) 0% (0 min) → 30% (5-10 min) → 0% (10.1–20 min). The column was kept at 50 ºC and the flow rate was 1.0 mL/min.

Results and Discussion

Figure 1 shows the UV chromatograms of the standards. Peaks of the ten vitamins were fully resolved using the developed method. The UV absorbance was measured at 254 nm. However, since the absorbance of pantothenic acid and biotin at 254 nm were low, 210 nm was used for the measurement.

This simple method using phosphoric acid and acetonitrile as the eluents demonstrated a successful simulated analysis of ten water-soluble vitamins in 20 min, including the column equilibration time.

We analyzed the extract of a commercial multi-vitamin supplement. We used a guard column (Shodex RSpak DE-G 4A) during the sample analysis (Figure 2).

A method for simultaneous analysis of ten water-soluble vitamins was developed using the Shodex RSpak DE-413L column. The eluents used consisted of a mixture of an acid and acetonitrile. One sample measurement completes in 20 min.

The Shodex RSpak DE series provide a stable analysis even under highly aqueous eluent conditions, without the concern of column deterioration due to the polymer-based packing materials compared to using silica-based material.

References


Shodex™/Showa Denko America, Inc.
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Website: www.shodexHPLC.com
Thermal Extraction of Phthalates in Polymers Based on IEC Method 62321-8 Using a Pyroprobe

Karen Sam, CDS Analytical

This application note presents calibration plots, RSDs, and MDLs for IEC Method 62321-8 using a CDS Model 6150.

Certain phthalate additives are known to be harmful to humans, resulting in regulations regarding their use. With growing environmental awareness and perceptions, the use of phthalates has been restricted in many countries, including the European Union and the United States of America. As a result, a few international standards and conformity assessment bodies such as the International Electrotechnical Commission (IEC) and the American Society for Testing and Materials (ASTM) have published standards for determining certain phthalates in polymeric materials. The recent IEC Method 62321-8 defines approaches to determine di-isobutyl phthalate (DIBP), di-n-butyl phthalate (DBP), benzyl butyl phthalate (BBP), bis-2-ethyl hexyl phthalate (DEHP), di-n-octyl phthalate (DNOP), di-isononyl phthalate (DINP), and di-iso-decyl phthalate (DIDP) in electronics using GC–MS and TD–GC–MS.

Experimental Conditions

The samples were pyrolyzed in a CDS Pyroprobe 6150 with an autosampler, equipped with Drop-In-Sample-Chamber (DISC) technology. A DISC tube was used as the sample vessel.

Pyro Chamber

Ramp 1: 200 to 300 °C at 20 °C/min
Ramp 2: 300 to 340 °C at 5 °C/min
IsoZones: 300 °C

GC-MS

Column: 5% phenyl (30 m × 0.25 mm)
Carrier: Helium, 50:1 split
Injector: 320 °C
Oven: 80 °C for 13 min
20 °C/min to 300 °C
Mass Range: 50–1000 amu

Results

Thermal extraction of additives is a straightforward approach involving only a few steps, and therefore the possibility of greater recovery when compared to solvent extraction techniques exists. Sample is simply placed in a sample tube, and dropped into the DISC of a 6150 Pyroprobe. The Pyroprobe thermally extracts the sample using two sequential temperature ramps as defined in the experimental conditions, straight to a single quadrupole GC–MS instrument. Resulting chromatograms closely match the chromatograms in Annex C.2 of the IEC Method (Figure 1). Calibration curves based on a one-point calibration (as indicated in the method) are also shown in Figure 1.

The statistical measures related to reproducibility depend on temperature precision, along with sample related issues like homogeneity and sample preparation. Eight replicates a 500 ng phthalate standard provided area RSDs of around 3% for most of the phthalates. Furthermore, when method detection limits were studied in accordance with the IEC method, seven replicates produced calculated MDLs ranging from 9.4 to 21.7 mg/kg, 78–91% lower than the 100 mg/kg requirement (Table I).

Conclusion

The latest version of the Pyroprobe from CDS Analytical ensures repeatable, reliable results for thermal extraction of phthalates in accordance with standard methods, like IEC Method 6321-8 for determination of phthalates in electrotechnical products.

Table I: Area RSDs and calculated MDLs of regulated phthalates

<table>
<thead>
<tr>
<th>Phthalate</th>
<th>Quant. Ion</th>
<th>RSD (%)</th>
<th>MDL (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIBP</td>
<td>223</td>
<td>3.2</td>
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<td>DBP</td>
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<td>DIDP</td>
<td>307</td>
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</table>

Figure 1: Calibration curve and chromatograms (TIC and EIC) of phthalates.