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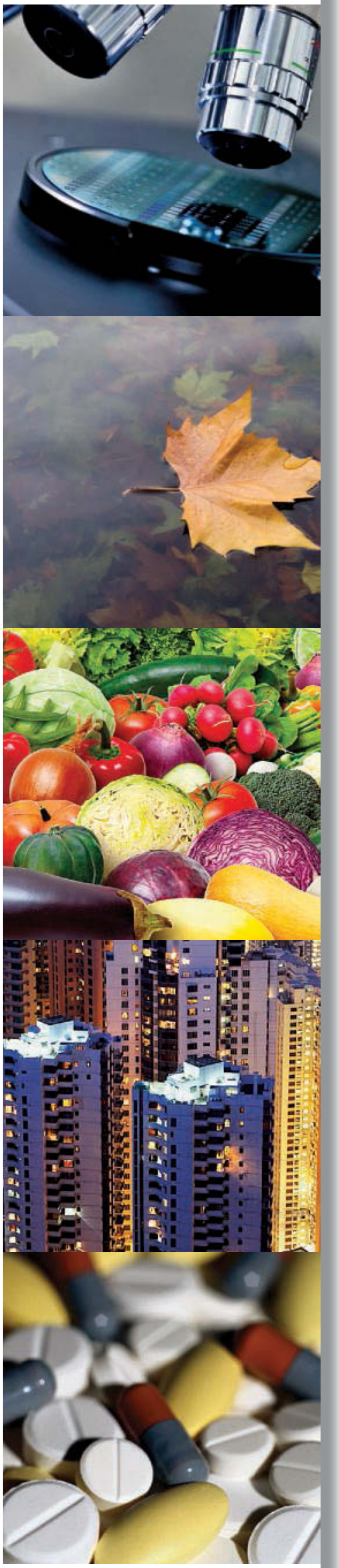

THE APPLICATION NOTEBOOK

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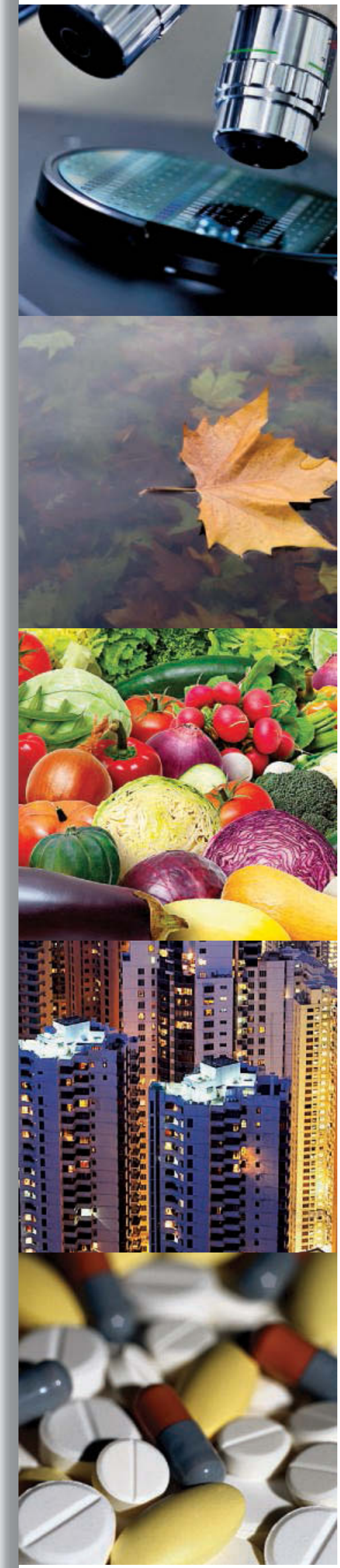

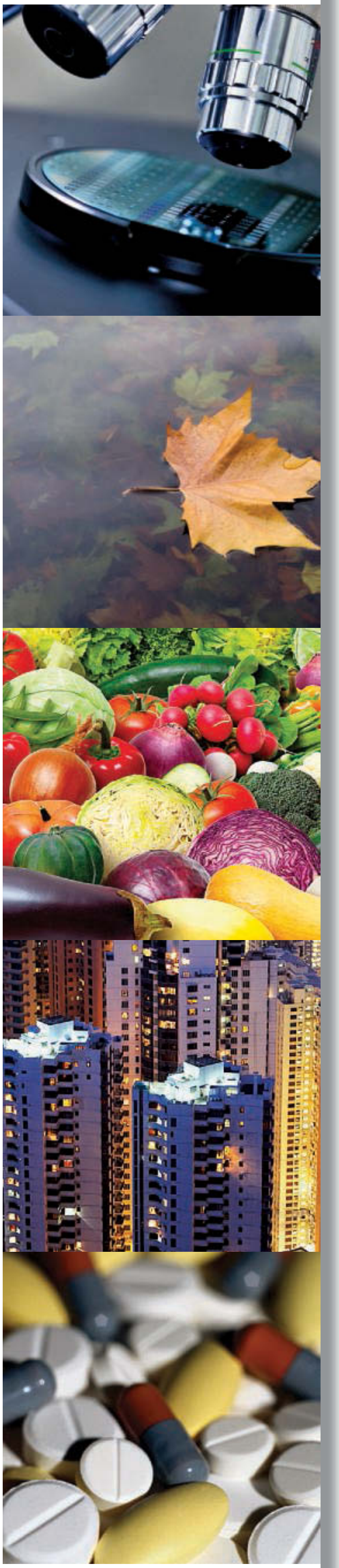

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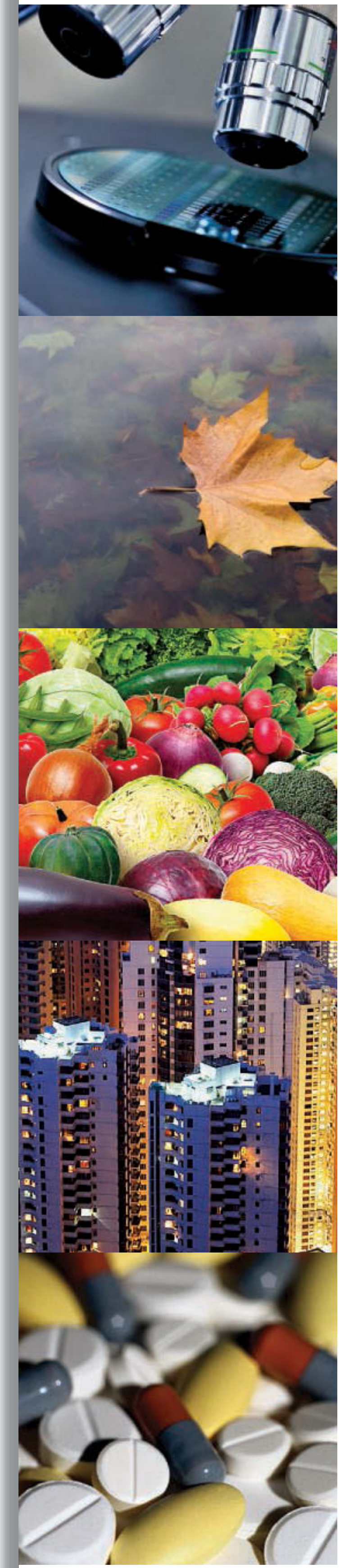

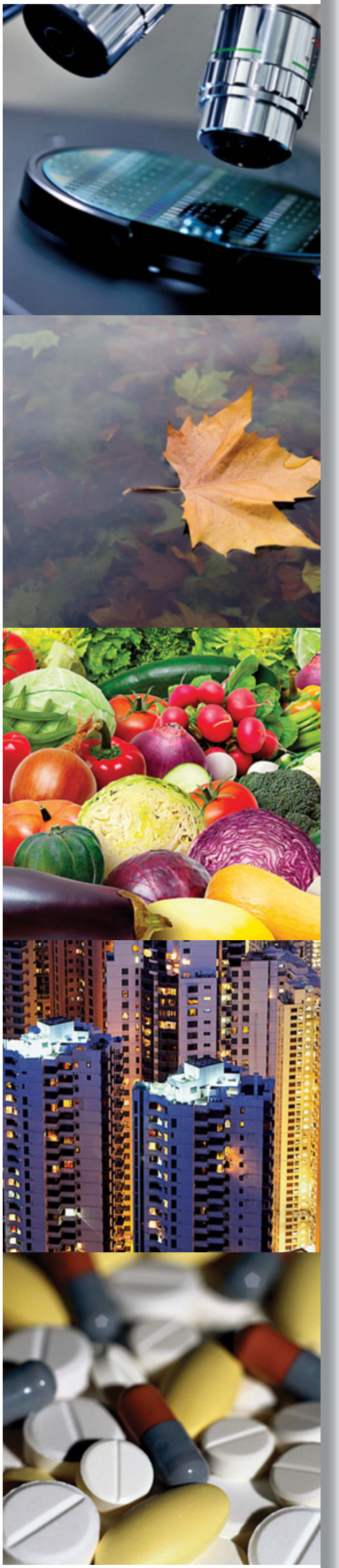

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FROM the **PUBLISHER**

The Application Notebook: A More Critical Resource Than Ever

To the members of *LCGC's* readership, the science behind the headlines is always apparent. Separation science in general, and chromatography in particular, find application in nearly every facet of everyday life, from pharmaceutical analysis to the biomedical field to environmental testing, and this *Application Notebook* is a tribute to just how thoroughly separation science permeates our daily lives.

However, as this, the September edition of *The Application Notebook*, reaches *LCGC's* readership, separation science has come to the forefront of the mass media like few other times before. With the exception of PED testing and the baby formula/pet food scares of recent years, perhaps no incident has brought the application of chromatography to the forefront of the world's consciousness like the Gulf Oil Spill. With the combined threats of food and air contamination being addressed with various chromatographic techniques, this tragic occurrence has brought chromatography into mainstream news articles and television stories once again.

Here at *LCGC*, we believe this demonstrates the need for a supplement such as this one more than ever. As scientists grapple with life-affecting real world crises such as this one, with potential consequences as serious as they have ever been, having a one-of-a-kind resource at your fingertips becomes critical. "Solutions for separation scientists" is the *LCGC* motto, and a dire occasion such as this one illustrates how important it is to have the latest cutting-edge applications research on-hand in the lab.

We hope you find this issue useful and informative, and as always, feel free to contact us at the addresses below or at the phone numbers listed in the masthead with your feedback.

It is always welcome.

Michael J. Tessalone
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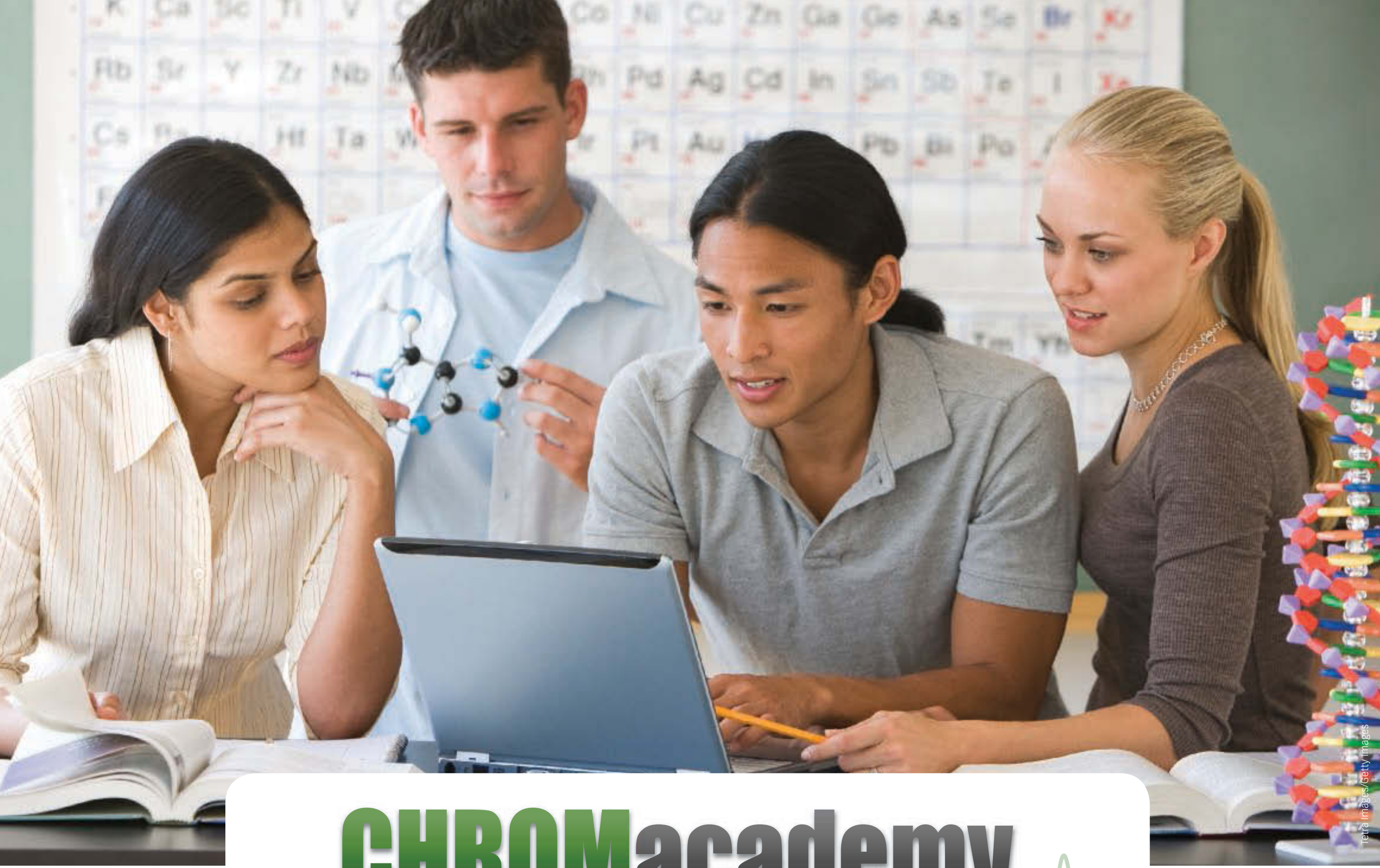

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Agilent Technologies

Peptide Mapping of a Monoclonal Antibody Using Poroshell 120

Susanne C. Moyer, Agilent Technologies, Inc.

Protein-based pharmaceutical drugs, such as fusion proteins and monoclonal antibodies (mAb), are playing an increasing role in modern therapeutics. Analytical characterization of protein therapeutics is very complex and time consuming. One type of analysis that is undertaken to determine the primary structure of the protein is peptide mapping. In peptide mapping analysis, it is imperative that 100% sequence coverage be achieved and that modifications to the molecule are identified. Typical LC–MS peptide mapping protocols can have LC gradients in excess of 100 min. Obviously, such lengthy run times adversely affect throughput capabilities and methods for reducing these run times while achieving analytical goals are needed. The Agilent Poroshell 120-SB C18 column was used for the LC–MS analysis of a tryptic digest of a monoclonal antibody standard to demonstrate a greatly reduced analysis time of 20 min while achieving 100% sequence coverage of both the heavy and the light chains.

Experimental Conditions

A 3.0 × 150 mm Agilent Poroshell 120 SB-C18 column (PN 683975-302) was used with an Agilent 1200 LC and 6520 QTOF for peptide mapping experiments. Mobile phase A: 0.1% formic acid in water and mobile phase B: 0.1% formic acid in acetonitrile. The flow rate of the column was 0.3 mL/min and the column temperature was maintained at 40 °C. The LC gradient was: 0–3 min 2%B, 3–13 min 2%–45%B, 13–15 min 45%–65%B, 15–15.1 min 65%–90%B, 15.1–17 min 90%B, 17–18 min 90%–2%B, stop time 20 min.

Results and Discussion

Complex tryptic digests of mAbs employ shallow gradients, often stepped, to separate the many peptide fragments into discreet peaks. Often tryptic maps take 2 h to complete using 2.1 × 250 mm, 5 μm particle-sized columns, significantly affecting laboratory productivity. Sample degradation also may occur

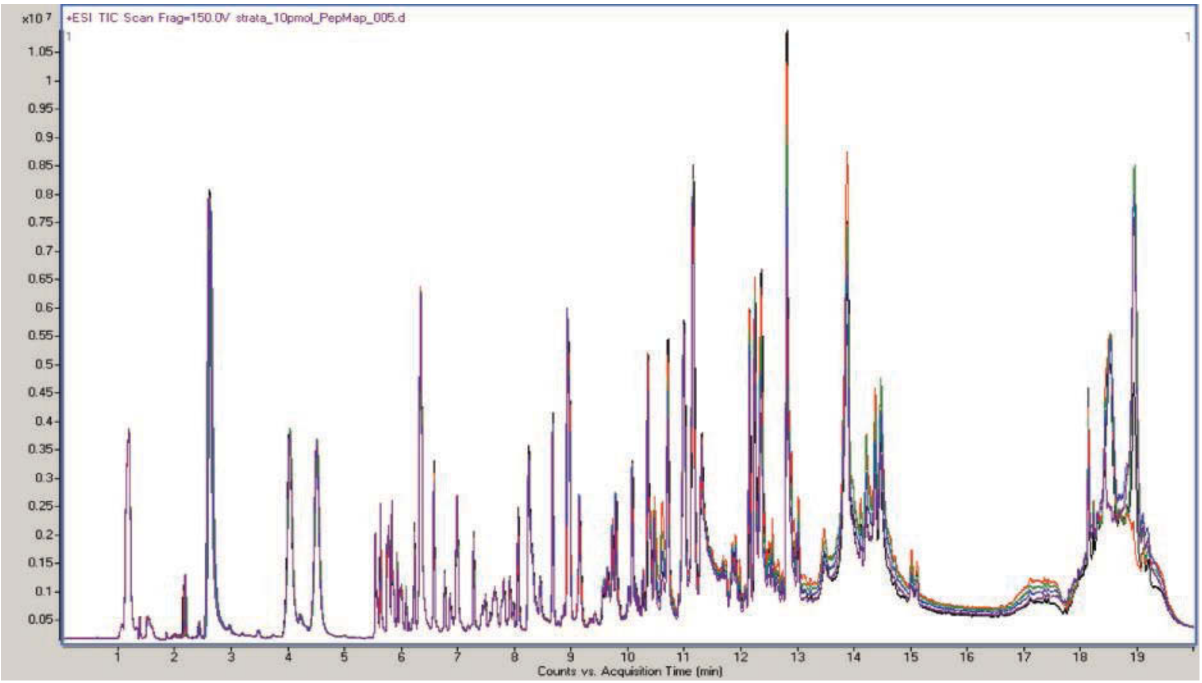

Figure 1: Rapid analysis of an mAb peptide map using a Poroshell 120, 3.0 × 150 mm (PN683975-302).

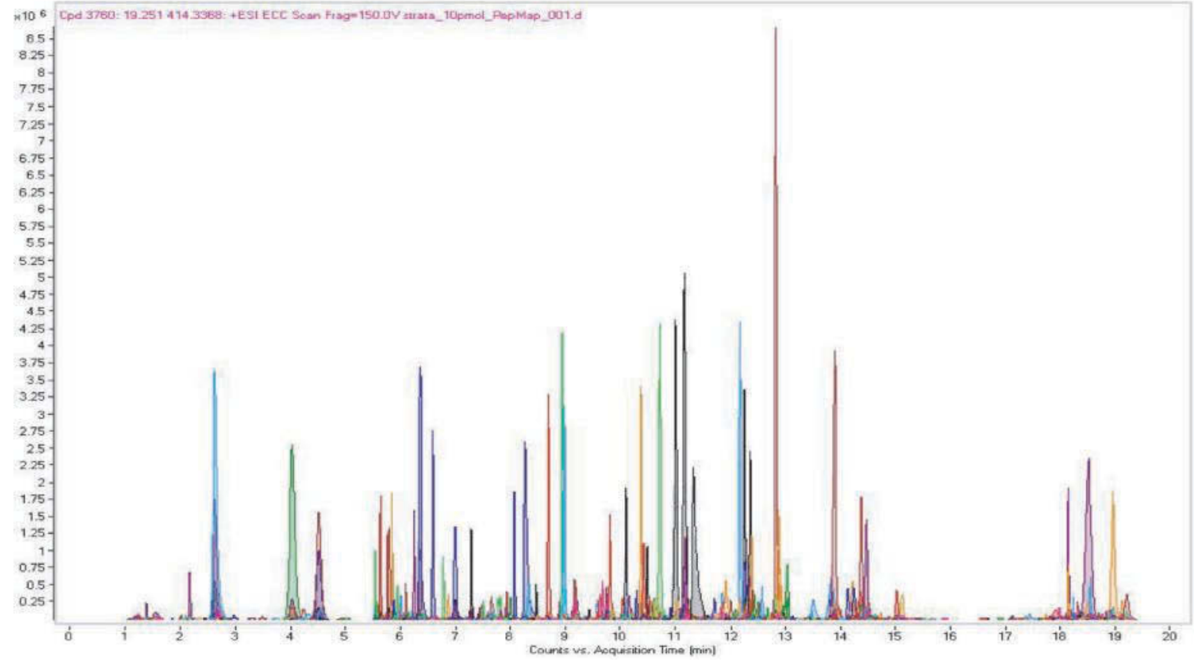

Figure 2: 100% sequence coverage achieved of mAb with many unique compounds identified using MassHunter.

between long injection cycles so any reduction in analysis time is welcome.

One way to achieve shorter analysis times is to use Poroshell 120 columns. These columns contain 2.7 µm superficially porous particles that provide almost equivalent efficiency to UHPLC (sub-two micron totally porous) columns. Thus, more efficient Poroshell 120 particles packed in a shorter column length can provide similar chromatographic results to their longer 5 µm predecessors in a fraction of the time.

A tryptic digestion of a standard 10 pmol mAb was injected five times onto a Poroshell 120 column. Total analysis time including gradient re-equilibration was only 20 min. Figure 1 shows the five overlays of the total ion chromatograms (TIC). Good retention time, reproducibility, and peak-to-peak resolution indicate a stable instrument, column, and mobile phase gradient and re-equilibration, and is the first indicator of a robust method for validation. Also, Poroshell 120 columns have larger frit pores compared to smaller sub-two micron column frits, to resist plugging from “dirty” or complex samples such as protein hydrolysates.

The faster analysis gained from Poroshell 120 did not compromise the chromatographic map of the mAb. Figure 2 shows the results from Agilent MassHunter Molecular Feature Extractor (MFE). MFE is an algorithm that uses the LC retention time and the QTOF accurate mass to extract unique compounds from the data set. These MFE compound results are then matched back to the heavy chain and light chain protein sequences from the mAb standard. This 20 min Poroshell-120 method resulted in 100% sequence coverage for both the heavy and light chains of the mAb.

Conclusion

The Poroshell 120 SB-C18 column can greatly reduce the analysis time of an LC–MS peptide mapping experiment, while achieving high-performance and highly reproducible peptide separations. The combination of highly efficient 2.7 µm superficially porous particles and relatively large pored frits make Poroshell 120 ideal for analysis if complex samples such as mAb digests.

References

(1) L.W. Dick Jr. et al., *J. Chromatogr. B*, **877** 230–236, (2009).

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High Resolution Analysis of an Intact Antibody and Its Component Light Chains

Darwin Asa, Bruker Daltonics, Inc.

Modern analytical applications often demand definitive tandem MS results on ever more complex samples utilizing fast separation techniques. maXis™ is the only mass spectrometer able to deliver the maximum MS performance specification at the very highest speeds delivered by modern ultra performance liquid chromatography (UPLC) and capillary electrophoresis (CE). The maXis is specially designed to deliver excellent results in many applications including:

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With resolution in excess of 40,000 and MS and MS-MS mass accuracy typically between 600–800 ppb at speeds of up to 20 full spectra per s simultaneously, no other mass spectrometer is better equipped to deliver definitive data on complex samples in proteomics, metabolomics, and small molecule identification challenges.

Typical Results from the maXis:

20 Hz speed of acquisition at high resolution for high-speed chromatography
40 k+ resolution in both MS and MS-MS mode
Wide dynamic range of 5 orders of magnitude for trace detection in complex mixtures
Sub-ppm mass accuracy in both MS and MS-MS mode for high confidence IDs

Experimental Conditions

HPLC: Agilent 1200, binary pump
Column: Zorbax SBC8, Rapid Resolution Cartridge, 2.1 × 30 mm, 3.5 µm
Solvent A: 0.1% Formic Acid in water
Solvent B: 0.1 % Formic Acid in Acetonitrile
Gradient: 0 min-0 % B; 3 min-0 % B; 10 min-100 % B; 13 min-100 % B; 13.1 min-0 % B; 18 min-0 % B
Flow rate: 300 µL/min
Column oven: 70 °C
Injection of: Recombinant IgG1 from Chinese Hamster Ovary (CHO) cells

In this study, the maXis was challenged to directly analyze an intact recombinant IgG (MW~149 kDa) and its component light chains (MW~24.4 kDa). As can be seen from the above data, the maXis was readily able to analyze a protein as large as an IgG with the necessary resolution to discriminate discrete changes in the glycosylation patterns of a molecule this large.

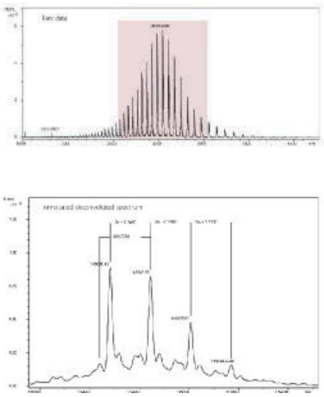

Figure 1: Raw and deconvoluted spectra of an intact IgG.

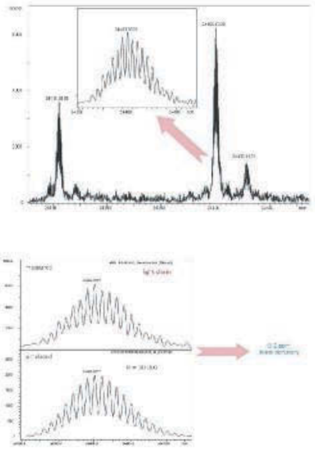

Figure 2: Analysis of IgG reduced and alkylated light chain with 0.2 ppm mass accuracy.

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A New High-Resolution MAbPac SCX Column for Characterization of Monoclonal Antibody Heterogeneity

Srinivasa Rao, Yuanxue Hou, Yury Agroskin, and Chris Pohl, Dionex Corporation

The new MAbPac™ SCX-10 column is based on 10 µm nonporous, highly cross-linked styrene-type polymeric media with a proprietary uniform hydrophilic coating that is grafted with sulfonic acid functional groups. An ATRP-based grafting approach is used to control functional group charge density and degree of polymerization. In addition, these particles exhibit a wide range of pH stability with high selectivity and minimal band spreading. The column is available in two formats: 4 × 250 mm analytical column and 4 × 50 mm guard column.

Experimental

A Dionex ICS-3000 HPLC system with a DP pump, VWD absorbance detector, autosampler, and a TCC-100 thermostatted column compartment was used.

Chromatography was controlled by Chromeleon® Chromatography Data System software (Dionex).

Materials

The monoclonal antibody sample was a gift from a biotech company. Other analytical grade chemicals were obtained from Sigma.

Results and Discussion

MAbPac SCX-10 is a strong cation-exchange column specifically developed for characterization of heterogeneity and analysis of monoclonal antibodies (MAbs) (1). MAb heterogeneity can be attributed to glycosylation, oxidation, mutation, phosphorylation, amino terminal modifications, incomplete processing of the C-terminus, and asparagine deamidation. These variations in composition occur in MAbs and many other types of proteins and can impact their efficacy, stability, and safety. Monitoring such variations of therapeutic proteins is required by the FDA and other regulatory agencies. Figure 1 shows the MAb separation using the MAbPac SCX column. Acidic variants, lysine truncation, and other basic variants could be resolved. An example separation of the C-terminus lysine truncation variants with and without carboxypeptidase treatment is shown.

Conclusion

The MAbPac SCX-10 column provides high resolution separation of monoclonal antibody variants in characterization of MAb heterogeneity. This new column is a complimentary addition to the existing ProPac® WCX-10 columns, providing high resolution and orthogonal selectivity for various proteins and MAb charge variant characterization.

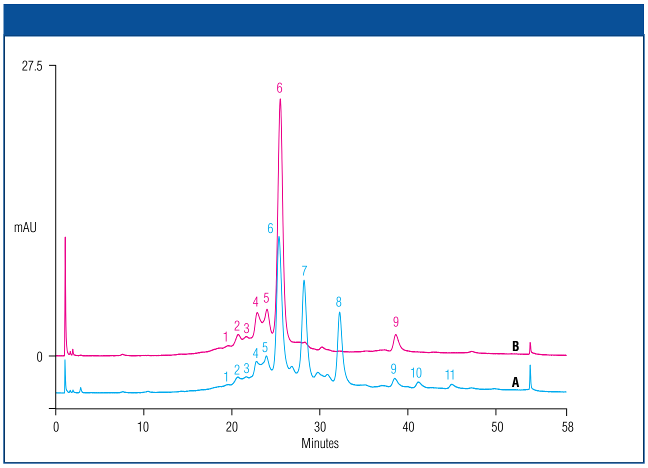

Figure 1: Characterization of acidic, lysine truncation, and other basic variants of a MAb using MAbPac SCX-10 column. Eluents: A: 20 mM MES (pH 5.6) + 60 mM NaCl; B: 20 mM MES (pH 5.6) + 300 mM NaCl; Gradient: 15–36.44%B in 50 min; Flow Rate = 1 mL/min; Injection Volume = 5 µL; detection; UV at 280 nm; Temperature = 30 °C.
Samples: A. MAb 900 µg in 100 µL (no carboxypeptidase). Peaks 1–5: Acidic variants; Peaks 6, 7, 8: C-terminal Lys truncation variants of main peak; Peaks 9, 10, 11: C-terminal Lys truncation variants of a minor variant peak. B. MAb 900 µg in 100 µL+ carboxypeptidase (CBP) 50 µg in 10 µL (incubation for 3 h at 37 °C). Lysine truncation variant peaks 7 and 8 lose their terminal lysine and combine with peak 6 which has no lysine after CBP treatment. Similarly, minor variant lysine truncation peaks 10 and 11 combine with peak 9 after CBP treatment.

References

(1) MAbPac SCX-10 manual and data sheets are available at www.dionex.com.

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A New High-Resolution Size Exclusion Chromatography Column for MAb Aggregate Analysis

Srinivasa Rao, Xiaodong Liu, and Chris Pohl, Dionex Corporation

The new MAbPac™ size exclusion (SEC) column is based on high-purity, spherical, porous (300 Å), 5 µm silica covalently modified with a proprietary diol hydrophilic layer. The column is packed into nonmetallic, biocompatible, PEEK™ column housing to eliminate compromised chromatography due to the presence of metal from the column hardware. This offering results in minimal nondesired interactions between the biomolecules and the stationary phase. Stable surface bonding leads to low column bleed and enhances compatibility with MS, ELSD, and Corona® CAD® detection. The column is available as 4 × 300 mm analytical and 4 × 50 guard column formats.

Experimental

A Dionex ICS 3000 HPLC system with a DP pump, VWD absorbance detector, auto-sampler, and a TC thermostatted column compartment was used.

Chromatography was controlled by Chromeleon® Chromatography Data System software (Dionex).

Monoclonal antibody was a gift from a local biotech company. Other analytical grade chemicals used in the buffers were obtained from Sigma. Super SW3000 SEC column was obtained from Tosoh Bioscience.

Results and Discussion

MAbPac SEC-1 is a size exclusion chromatography (SEC) column specifically developed for characterization and analysis of monoclonal antibody (MAb) aggregates, papain, or other enzyme digested fragments and for other size based separation applications (1). The diol stationary phase is designed to minimize undesired interactions to handle both high and low salt mobile phase eluents and mass spectrometry compatible volatile eluents.

Size-exclusion chromatography (SEC) is a well-accepted technique for the detection and accurate quantification of protein aggregates in MAbs and other biological drug products. An example separation of MAb aggregation using MAbPac SEC-1 is shown in Figure 1. A comparison of MAb analysis on MAbPac SEC-1 and Super SW3000 using mass spectrometry compatible mobile phase (100 mM ammonium acetate) is shown in Figure 2. The MAbPac SEC-1 column provided superior efficiency, peak shape and asymmetry, sensitivity, and better recovery when compared with Tosoh's Super SW3000 SEC column.

References

(1) MAbPac SEC-1 manual and data sheets are available at www.dionex.com

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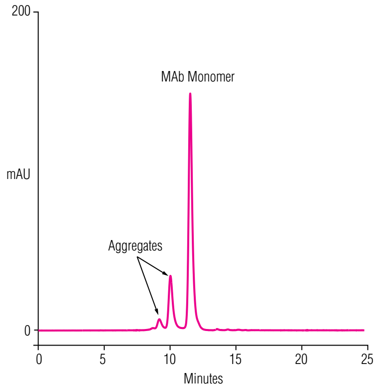

Figure 1: MAb aggregate analysis using MAbPac SEC-1 column. Mobile Phase: 300 mM NaCl in 50 mM phosphate buffer (pH 6.8); Flow Rate = 0.2 mL/min; Sample = MAb 10 mg/mL; Injection volume= 2 µL; detection; UV at 280 nm; Temperature = 30 °C.

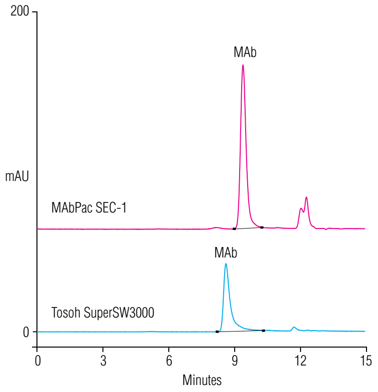

Figure 2: Comparison of MAbPac SEC-1 with Tosoh's Super SW 3000 using volatile eluents. Mobile Phase: 100 mM NH_4OAC (pH 5.0); Sample = MAb 1 mg/ mL; Flow Rate = 0.25 mL/min (Dionex); 0.33 mL/min (Tosoh); Injection volume = 2 µL (Dionex); 2.5 µL (Tosoh); detection: UV at 280 nm; Temperature = 30 °C.

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Glycosylation Analysis by Dedicated Sample Preparation and Complementary nano-LC (HILIC/RP)/ESI-MS

Jessica Wohlgemuth[1], Wen Jiang[2], Egidijus Machtejevas[1], and Sven Andrecht[1],
[1]Merck KGaA, Darmstadt, Germany and [2]Merck SeQuant AB, Umea, Sweden

Site-specific analysis of protein glycosylation has become a very important issue in pharmaceutical/biotech industries, requiring specific analytical tools and methodologies. Common techniques for glycosylation analysis on the peptide level use LC–MS. However, analysis is challenging due to low abundance of glycopeptides in complex protein digests. Improved chromatographic resolution and detection of low abundant glycoforms was achieved through complementary HILIC- and RP-LC coupled to MS after quantitative enrichment of N- and O-glycopeptides.

Experimental

IgG was digested with trypsin and enriched using the ProteoExtract® Glycopeptide Enrichment Kit (PN#72103, EMD Chemicals Inc.). LC-ESI-MS analysis was performed using an UltiMate 3000 HPLC (Dionex GmbH) equipped with either Chromolith® CapRod® RP-18e HR monolithic silica capillaries (150 × 0.1 mm, Merck KGaA, Germany) or 300 × 0.1 mm ZIC®-HILIC modified monolithic silica capillaries (research sample, Merck-SeQuant AB, Sweden) coupled to an Esquire 3000 Plus™ ion trap MS (Bruker Daltonics Inc.) operated in positive ion mode.

Results

ZIC® Glycocapture Resin allows for specific and quantitative enrichment of N- and O-linked glycopeptides without preference for particular glycan structures or information loss. RP-18 and ZIC®-HILIC chromatography are orthogonal and complementary for separation of glycopeptides. RP-18 facilitated the separation by peptide sequence and degree of sialylation whereas a separation by the composition of glycan moiety was facilitated by ZIC®-HILIC. Furthermore, ZIC®-HILIC separation of glycopeptides can be efficiently tuned by altered solvent conditions (e.g., ± salt or FA). The use of both stationary phases in LC–MS setups results in the detection of low abundant glycoforms, which cannot be identified if only one stationary phase is employed.

Conclusion

The combination of all described methods enables detection of low abundant and new glycoforms and delivers deeper insight into site-specific protein glycosylation.

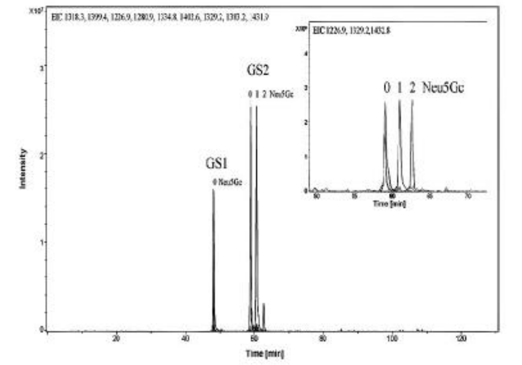

Figure 1: Separation by peptide sequence and degree of sialylation. Glycopeptides of an mAb with two glycosylation sites (GS) were enriched and analyzed by nano-LC-ESI-MS using a Chromolith® CapRod® RP-18e HR monolithic silica capillary (150 × 0.1 mm) at 500 nL/min. Gradient: 2%–32% ACN (+ 0.1% FA) in 90 min. (Adapted from *J.Sep.Sci.*, 33, 2010.)

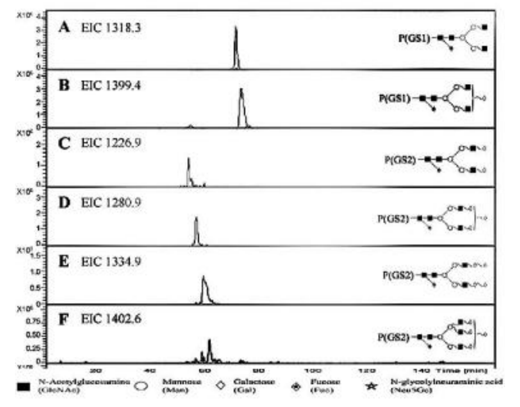

Figure 2: Separation by composition of glycan moiety. Glycopeptides of an mAb with two glycosylation sites (GS) were enriched and analyzed by nano-LC-ESI-MS using a ZIC®-HILIC monolithic silica capillary (300 × 0.1 mm), flow rate 500 nL/min. Gradient: 80%–50% ACN (+ 0.1% FA) in 130 min. (Adapted from *J.Sep. Sci.*, 33, 2010.)

Reference

(1) J. Wohlgemuth et. al., *J.Sep.Sci.* 2010 Mar 10; **33**(6–7):880–890.

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Determining Saccharidic Tracers in Atmospheric Aerosols

S. Rick[1], A. Wille[2], and A. Steinbach[2],
[1]Leibniz Center for Tropical Marine Ecology (ZMT) and [2]Metrohm International Headquarters

Levoglucosan is a combustion product of cellulose and, as such, widely used as a unique tracer for biomass burning sources in atmospheric aerosols. Similarly, the sugar alcohols arabitol and mannitol are tracers for primary bioparticles, such as fungal spores, in aerosols. Therefore, determination of these polar oxygenated organic compounds is of high interest.

Glass fiber filters containing winter and summer aerosol fractions were extracted with water and analyzed for levoglucosan, mannosan, galactosan, and the sugar alcohols arabitol and mannitol. The analytical method couples ion chromatography with a highly sensitive pulsed amperometric detector (IC-PAD) providing a simple alternative to GC–MS, which requires laborious sample extraction and derivatization. A Metrosep A Supp 15 – 150/4.0 was coupled to a Metrosep Carb 1 – 150/4.0 column. Separation between the levoglucosan and mannosan peaks can be further improved by using the longer Metrosep Carb 1 – 250/4.6 column. The calibration for the anhydrosugars and sugar alcohols is linear over a wide concentration range and thus copes with the strongly varying tracer concentrations in atmospheric aerosols. Less than 17 min were needed to achieve separation with a detection limit for levoglucosan of approximately 5 ng/m³.

Aerosol concentrations of the isomeric anhydrosugars made up the largest fraction of identified carbohydrates in both winter and summer. Owing to elevated wood burning during the winter season, concentrations of the anhydrosugars were clearly higher in winter, while biogenic sugar alcohol and glucose contributions were more prevalent during the summer season.

The presented methodology allows the sensitive and robust determination of saccharidic tracers in atmospheric aerosols enabling a deeper understanding of biomass burning and primary biogenic aerosols.

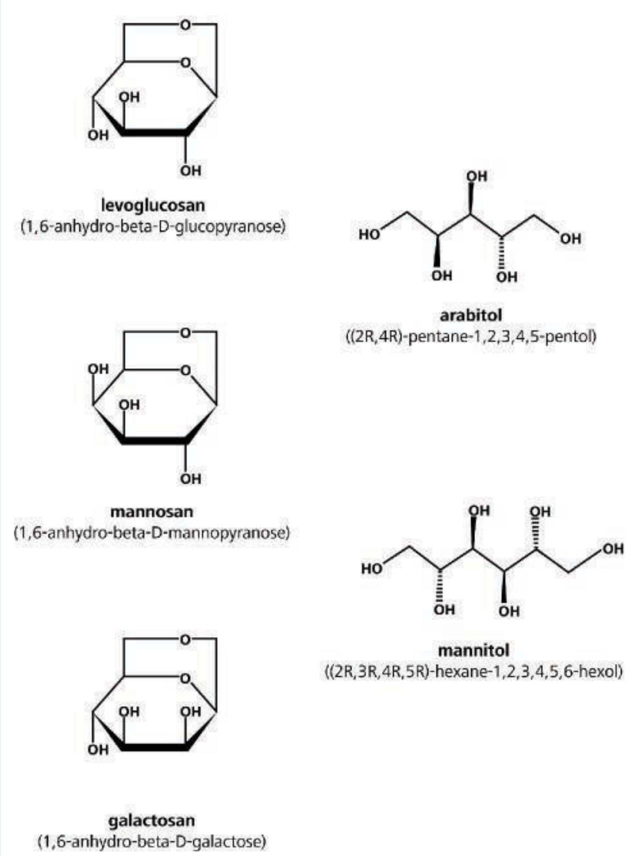
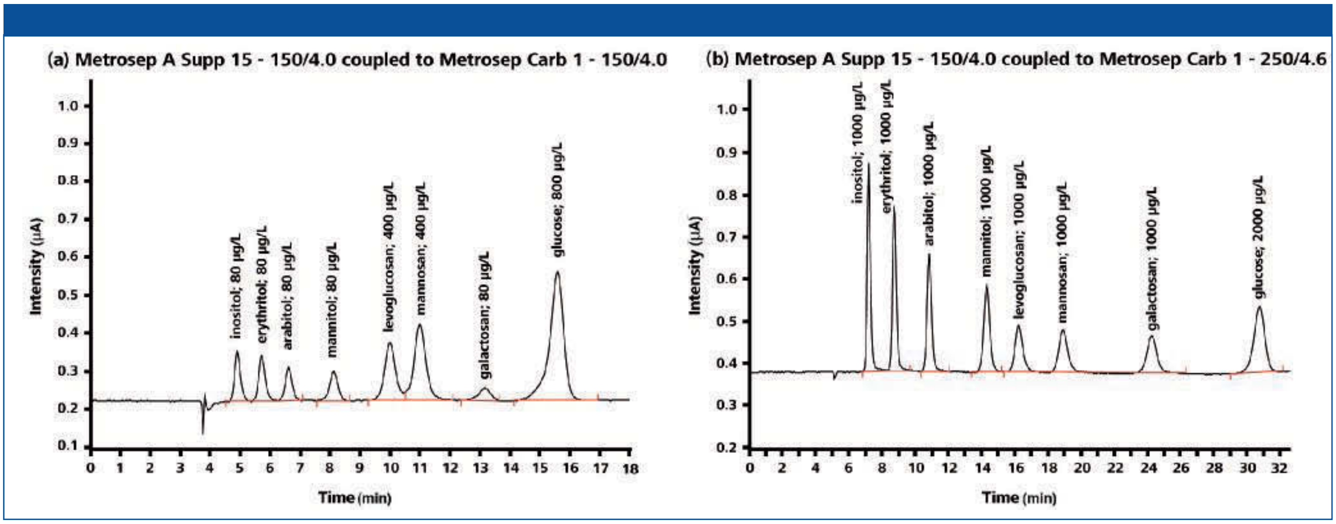


Figure 1: Chemical structures of the anhydrosugars levoglucosan, mannosan, and galactosan as well as of the sugar alcohols arabitol and mannitol.

Besides vehicle emissions and industrial sources, biomass burning is one of the largest sources of air pollution. Approximately 3 billion metric tons of biomass are annually burned. Main contributions derive from residential wood burning, the burning of leaves, grass, and trash as well as from wildfires. The evolving atmospheric aerosol particles — suspensions of fine solid particles or liquid droplets in a gas — can persist for days to weeks in the troposphere and thus significantly contribute to long-range particle transport. Most of the released smoke aerosol particles are smaller than 10 microns in size (PM_{10}) and can easily enter the human lung and cause adverse health effects. Moreover, smoke aerosol particles affect cloud formation and precipitation and thus the climate in general. For this reason, properties and chemical composition of aerosol particulate matter have to be investigated.

Aerosols consist of a complex mixture of inorganic and organic compounds. Especially, the water-soluble organic compounds (WSOC) make up a major fraction of the atmospheric aerosol. Within the WSOC, the monosaccharide anhydrides levoglucosan


(a) Metrosep A Supp 15 - 150/4.0 coupled to Metrosep Carb 1 - 150/4.0

(b) Metrosep A Supp 15 - 150/4.0 coupled to Metrosep Carb 1 - 250/4.6

Figure 2: PAD chromatograms of an 8-component carbohydrate standard using a Metrosep A Supp 15 – 150/4.0 column in series with a Metrosep Carb 1 – 150/4.0 (a) and a Metrosep Carb 1 – 250/4.6 (b). The following determinations were performed with the shorter Metrosep Carb 1 – 150/4.0 column; Table I summarizes the chromatographic parameters and the PAD settings of the method.

(1,6-anhydro-beta-D-glucopyranose) and, to a lesser extent, its stereoisomers mannosan (1,6-anhydro-beta-D-mannopyranose) and galactosan (1,6-anhydrobeta-D-galactose) can be determined as thermal degradation products of structural polysaccharides present in biomass. While levoglucosan arises from the pyrolysis of cellulose, the main building material of wood, its stereoisomers mannosan and galactosan stem from the pyrolysis of hemicellulose. During the combustion of fossil fuels no anhydrosugars are released. Levoglucosan fulfils all important requirements of an ideal molecular marker necessary for source apportionment: it is abundant in wood smoke, highly source specific, and relatively stable in the atmosphere. In contrast, monosaccharides are released by microrganisms, plants, and animals; sugar alcohols are characteristic for fungi, lichens, and bacteria. In particular, arabitol and mannitol, which are storage substances in fungal spores, can be used as valuable tracers for quantifying airborne fungal spores (1).

Anhydrosugars are typically extracted into an organic solvent, derivatized with a silylation reagent, and most commonly determined via gas chromatographic (GC) separation with mass spectrometric detection (MS). Due to their polar nature, these compounds are challenging to analyse by GC–MS. A faster and simpler method, which does not require relatively sophisticated and expensive analytical instrumentation, organic solvents, sample workup, and derivatization, is ion chromatography (IC) followed by pulsed amperometric detection (PAD). PAD, the oxidation of electrochemical active molecule groups at a gold electrode, works well for carbohydrates and combines the advantages of an excellent sensitivity and good selectivity over other classes of compounds. Moreover, IC-PAD allows a straightforward water extraction of the large WSOC fraction of aerosols and thus excels by the possibility to handle wet samples, such as rain, fog, or cloud water (2, 3). Characterization of these samples is important for understanding the role of aerosols in cloud formation and precipitation.

This study presents a straightforward ion chromatographic method with amperometric detection for the simultaneous determination of the biomass burning tracer levoglucosan, its stereoisomeric anhydrosugars mannosan and galactosan as well as the sugar alcohols arabitol and mannitol (Figure 1).

Materials and Methods

Instrumentation

871 Advanced Bioscan
838 Advanced IC Sample Processor
818 Advanced IC Pump

Reagents and Eluents

Levoglucosan, the sugar alcohols, and glucose were reagent grade and purchased from Fluka (Sigma Aldrich, Buchs, Switzerland). Mannosan and galactosan were obtained from Carbosynth Ltd (Berkshire, UK). All standard solutions and eluents were prepared with deionized water having a specific resistance higher than 18 MΩ·cm. A carbohydrate standard mix covering the concentration range 80–800 µg/L served to determine the system characteristics. An air monitoring agency provided the aerosol winter and summer filter subsamples for method development.

Separation, Calibration, and Precision

A problem frequently encountered in the analysis of atmospherically relevant saccharidic compounds in aerosols is the poor chromatographic separation of the two compound pairs arabitol/levoglucosan and mannitol/mannosan (4). Because both pairs represent different compound classes and are important tracers for different aerosol sources, chromatographic separation is crucial. To overcome this drawback, the optimized IC-PAD method uses two columns in series, a Metrosep Carb 1 – 150/4.0 and a Metrosep A Supp 15 – 150/4.0, and thus achieves an excellent separation of anhydrosugars and sugar alcohols in a single isocratic run in less than 17 min [Figure 2(a)]. Separation, especially between levoglucosan and mannosan, can be further improved by using a weaker eluent or a longer carbohydrate separation column [250 mm instead of 150 mm, Figure 2(b)].

The saccharidic tracers are determined by applying a triple-step potential waveform: a positive potential E1 to determine the carbohydrates followed by a stronger pulse E2 for oxidative desorption of adsorbed species and finally a third, negative potential E3 to reactivate the electrode's surface (Table I). The entire sequence lasts 1 s and is continuously repeated. PAD detection is highly sensitive and as shown in Figure 3, calibration curves are linear over a wide concentration range. Calibration curves for the

Table I: Chromatographic parameters and PAD settings of carbohydrate determinations

Column:	Metrosep Carb 1 – Guard/4.0	Potential E1/time t1	+50 mV/400 ms
	Metrosep A Supp 15 – 150/4.0	Potential E2/time t2	+750 mV/200 ms
	Metrosep Carb 1 – 150/4.0	Potential E3/time t3	−150 mV/400 ms
Column temperature:	32 °C		
Eluent:	70 mmol/L sodium hydroxide	Working electrode	Gold
Flow rate:	0.65 mL/min	Sample time	100 ms
Loop:	100 µL	Range:	10 µA

Table II: Linearity and precision of the developed method

		Carbohydrate							
		Inositol	Erythritol	Arabitol	Mannitol	Levoglucosan	Mannosan	Galactosan	Glucose[2]
	Calibration range (µg/L)	10–1500	10–1500	10–1500	10–1500	50–7500	50–7500	10–1500	100–15000
Linearity	Correlation coefficient[1]	0.99999	0.99998	0.99983	0.99992	0.99998	0.99999	0.99986	0.99998
	RSD	0.409%	0.878%	2.548%	2.995%	0.882%	0.449%	2.169%	0.964%
Precision	Concentration (µg/L)	100	100	100	100	500	500	100	1000
	Height (µA)	0.15441	0.45026	0.04212	0.13889	0.11011	0.10391	0.19673	0.24058
	RSD[3]	0.9	0.4	1.9	1.4	2.0	1.8	0.8	0.3

[1]each calibration curve is based on ten different standard concentrations, [2]regression graph not shown, [3]n = 6

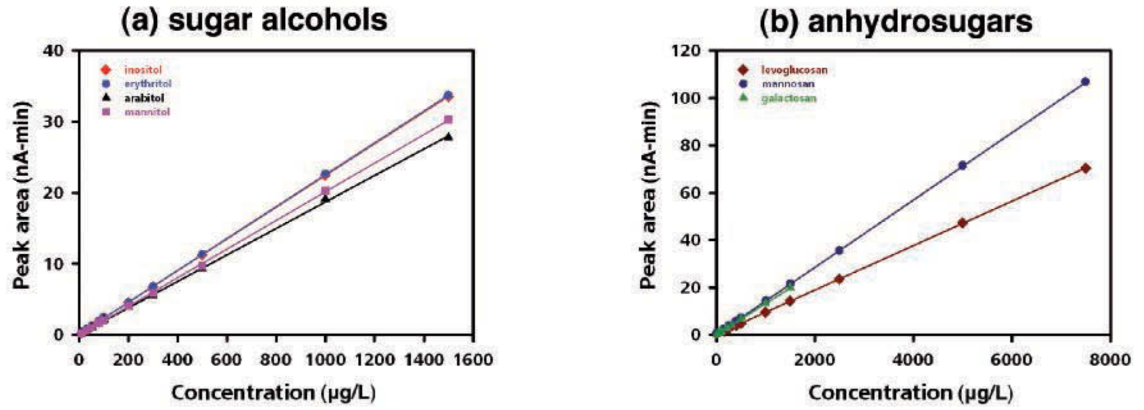


Figure 3: Calibration curves of (a) the sugar alcohols inositol, erythritol, arabitol, and mannitol as well as of the (b) anhydrosugars levoglucosan, mannosan, and galactosan using linear regression.

atmospherically important saccharidic compounds have correlation coefficients (R) better than 0.9998 (Table II). The reproducibility of the IC-PAD method was evaluated for the peak height of each carbohydrate with RSDs equal to or less than 2%. The detection limit for levoglucosan was estimated to be below 10 µg/m^3 using a 100 µL loop. With an extraction volume of 4 mL, 5 ng/m^3 levoglucosan aerosol concentration can be determined. These data show the suitability of IC-PAD to accurately quantify water-extractable saccharidic compounds in atmospheric aerosols.

Filter Extraction

Contribution of biomass smoke and biogenic sources to ambient aerosol levels is determined by collecting the aerosol fraction of a defined air volume on a filter. Before extraction, the 15 cm diameter glass fiber filter, through which 80 m^3 air was drawn, was subdivided into 4.7 cm diameter filter samples. After addition of 14 mL ultrapure water, the filters were treated by ultrasonic extraction for about 30 min. Before injection, samples were filtrated through a 0.45 µm syringe filter. Alternatively, Metrohm's Inline Ultrafiltration was used. In case concentrations on the filters are very low, the extraction volume may be reduced to 4 mL.

Results

Seasonal Variations in Summer and Winter Samples

Table III shows the results of the determined carbohydrate concentrations of three analyzed winter and four summer filter extracts. Figure 4 contains the chromatograms of an exemplary winter (ID 13) and summer sample (ID 408); corresponding aerosol concentrations in ng/m^3 are plotted in the bar chart of Figure 5.

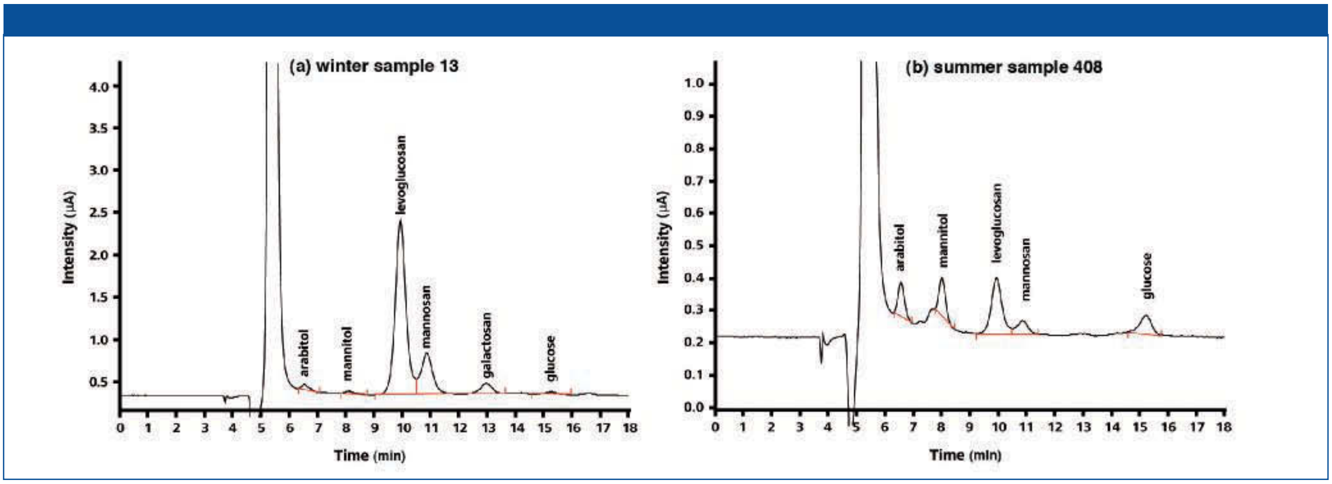


Figure 4: PAD chromatogram obtained from the analysis of (a) the winter sample 13 and (b) the summer sample 408.

Table III: Overview of analyzed glass fiber winter and summer samples and corresponding carbohydrate concentrations

		Arabitol		Mannitol		Levoglucosan		Mannosan		Galactosan		Glucose	
	ID	(µg/L)	(ng/m³)	(µg/L)	(ng/m³)	(µg/L)	(ng/m³)	(µg/L)	(ng/m³)	(µg/L)	(ng/m³)	(µg/L)	(ng/m³)
Winter	20	57.0	101.8	43.0	76.7	7470.8	13335.6	1524.9	2722.0	382.3	682.5	127.8	228.1
	13	57.5	103.3	42.6	76.5	5772.9	10363.6	1032.5	1853.6	279.5	501.7	82.5	148.0
	1	23.6	42.1	20.9	37.2	1875.6	3345.6	306.4	546.6	93.8	167.4	47.9	85.4
Summer	306	62.5	111.4	75.3	134.3	485.3	865.1	73.4	130.9	n.d.	n.d.	71.9	128.1
	307	51.2	91.7	50.6	90.8	284.6	510.3	66.1	118.5	n.d.	n.d.	88.8	159.3
	108	96.0	171.1	78.6	140.2	903.9	1611.1	163.6	291.6	17.9	31.9	136.8	243.8
	408	90.6	163.1	99.3	178.6	479.3	862.2	86.1	154.9	n.d.	n.d.	139.9	251.7

(µg/L) × a mL / 7.8542 m³ = (ng/m³), the extraction volume a was approximately 14 mL

The anhydrosugars levoglucosan and mannosan as well as the sugar alcohols arabitol and mannitol and the monosaccharide glucose were detected in each filter. Galactosan was only quantified in one summer filter in the low µg/L range. In both summer and winter samples, levoglucosan was the most dominant identified carbohydrate. The highest levoglucosan concentration, approximately ten times higher than that detected in summer, was observed in winter, when residential wood burning for heating purposes was most intense.

In summer, concentrations of combustion-derived anhydrosugars strongly decreased whereas the contributions of biogenic sugars and sugar alcohols increased. Arabitol and mannitol summer aerosol concentrations even achieved mannosan levels. These results are consistent with the fact that in summer the biological activity and thus the emission of biogenic volatile organic compounds such as fungal spores and plant pollen is at their maximum.

Conclusions

Ion chromatographic determination coupled with pulsed amperometric detection is highly sensitive and selective to saccharidic aerosol tracers. After straightforward water extraction of the filters, atmospheric tracers for biomass combustion and for biogenic sources can be determined at the ng/m³ scale. The method requires no complex sample pretreatment or derivatization and is well suited to handle a large number of samples. The IC-PAD method is an invaluable tool for the distinction between anthropogenic and natural aerosol emissions and offers novel insights for elucidating the impact of atmospheric aerosols on the climate.

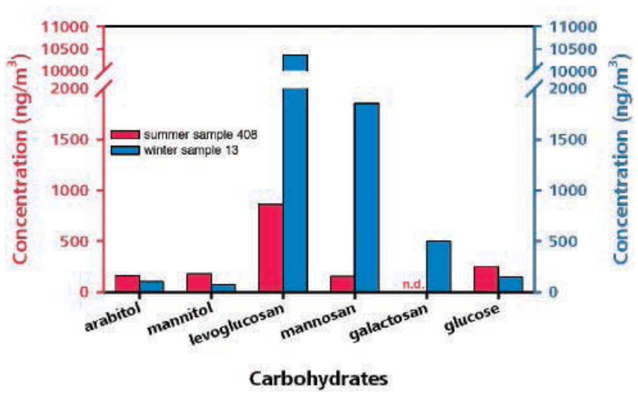


Figure 5: Bar chart comparing the detected carbohydrate concentrations in (a) the winter sample 13 and (b) the summer sample 408.

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Aggregation Analysis of Biosimilar EPO (Erythropoietin) Using a BioSep 2000 GFC Column

Michael McGinley, Phenomenex, Inc.

Gel filtration chromatography is the primary method used to analyze the amount of aggregate and dimer present in a therapeutic protein sample. A BioSep 2000 column is used to analyze EPO samples to determine difference in the amounts of aggregate present.

Next to insulin, erythropoietin is probably the most widely manufactured recombinant biosimilar protein with several companies throughout the world currently developing their version of the recombinant protein. Since protein aggregate is a major concern in the manufacture of any recombinant therapeutic, many groups are currently developing new methods for quantitating dimer and aggregate for EPO and other biosimilar proteins.

Materials and Methods

A BioSep-SEC-S 2000 column (300 × 4.6 mm dimension) was used for all GFC separations (Phenomenex, Torrance, CA). All samples were analyzed on an Agilent 1100 HPLC (Palo Alto, CA) with an autosampler and variable wavelength detector set at 220 nm; data was collected using Chemstation software (Agilent). Mobile phase was 50 mm sodium phosphate pH 6.8 with 300 mm sodium chloride running at a flow rate of 0.35 mL/min.

Results and Conclusion

Since EPO is approximately 30 kDa molecular weight in its glycosylated form (approximately 18 kDa for protein), a BioSep 2000 series column was used for all separations as it provides the largest separation window for proteins below 100 kDa molecular weight. Figures 1 and 2 show GFC chromatography on the BioSep 2000 column for two different samples of EPO. Figure 1 is a chromatogram of a freshly-frozen EPO sample and Figure 2 is a chromatogram of an EPO sample that had been frozen for over a year. While one can see peaks for aggregate, monomer EPO, and buffer salts for both samples, note the increase in a dimer peak for EPO for the sample frozen for more than a year. This increase in EPO dimer suggests that the formulation used was less than ideal for this sample.

Also note that the chromatography for the EPO sample run on the BioSep 2000 shows good resolution between the monomer and dimer peaks of the EPO sample (in Figure 2) despite the sample being heavily overloaded to visualize the dimer peak. The assigned aggregate peak in both chromatograms is also very low level, but is well recovered and somewhat included into the pores of the BioSep 2000 suggesting that aggregate has a finite size or that the early eluting peak is a different high molecular weight protein impurity in the sample. Regardless, the good peak separation, wide resolution window for low molecular weight proteins, and inertness makes the BioSep 2000 column an excellent GFC solution for aggregate analysis of EPO and other low molecular weight biosimilar proteins.

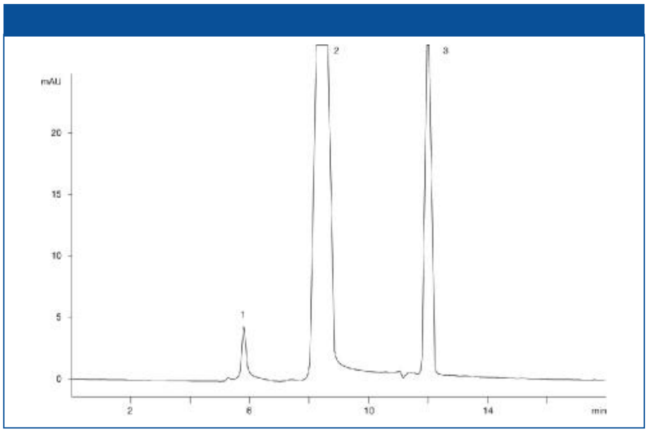

Figure 1: A freshly-frozen EPO sample run on a BioSep-SEC-S 2000 column. Note the early eluting high molecular weight protein (assumed to be EPO aggregate), the monomer EPO peak at 9 min RT, and the low molecular weight peak at the void of the column (assumed to be buffer salts in the diluent). Little or no dimer appears to be present.

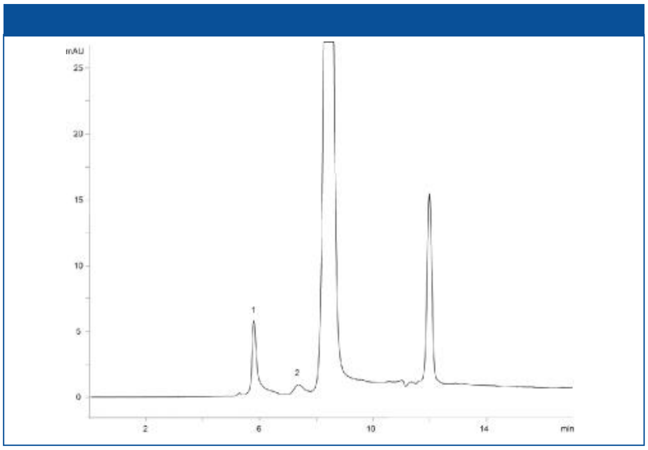

Figure 2: An EPO sample stored at -20 °C for more than a year run on a BioSep-SEC-S 2000 column. The good resolution between peaks makes the BioSep 2000 column an excellent choice for aggregate analysis of EPO and other biosimilar proteins.

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TOSOH BIOSCIENCE

Characterization Studies of PEGylated Lysozyme Using TSK-GEL® HPLC Columns

Jessica Christel, Werner Conze, Achim Sprauer, Volker Noedinger, and Egbert Mueller, Tosoh Bioscience GmbH

Chemical modification of therapeutic proteins in order to enhance their biological activity is of increasing interest. One of the most frequently used protein modification methods is the covalent attachment of poly (ethylene glycol), which is referred to as PEGylation. This polymeric modification changes the biochemical and physicochemical properties of the protein, which can result in several important benefits, among them more effective target delivery, slower in-vivo clearance, and reduced toxicity and immunogenicity of therapeutic proteins. After PEGylation reaction, the mixture has to be purified in order to remove nonreacted protein and undesired reaction products. Liquid chromatography is the most common purification method of PEGylation reaction products. Since the extent of PEGylation influences masking and shielding effects of the covalently linked PEG molecule, there is increasing demand for chromatographic methods to separate the modified isoforms from the native protein. This application note describes the use of size exclusion and ion exchange chromatography for the characterization of PEGylated lysozyme.

Lysozyme is a well known standard protein and is often used to determine the dynamic binding capacity of ion exchange chromatography (IEC) resins; therefore we decided to use PEG-lysozyme as a model protein in our study. PEGylated lysozyme was synthesized from methoxy PEG aldehyde (with a MW of 5 kDa, 10 kDa, and 30 kDa) and chicken egg white lysozyme in phosphate buffer in the presence of sodium cyanoborohydride ($NaCNBH_3$) as a reducing agent. The PEGylation reaction takes place between the aldehyde group of methoxy PEG aldehyde and the free amino acid group (NH_2-group) of lysine residues within the lysozyme molecule (see Figure 1).

The product mixture was analyzed with a TSKgel® G3000SW$_{XL}$ size exclusion column, SDS-PAGE (not shown), a TSKgel SP-5PW strong cation exchange column, a TSKgel SP-NPR strong cation exchange column and subsequent MALDI-TOF MS analysis (not shown).

Materials & Methods

PEGylation of egg white lysozyme:

5, 10, 30 kDa methoxy PEG aldehyde; 100 mmol/L phosphate buffer (Na_2HPO_4, NaH_2PO_4) pH 6.0; PEGylation by reductive alkylation; 20 mmol/L $NaCNBH_3$ to reduce a Schiff base; 100 mmol/L HCl to stop PEGylation reaction.

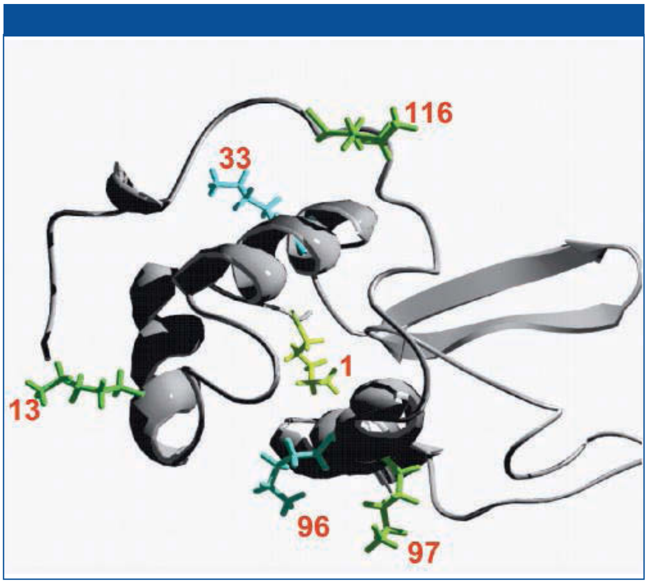

Figure 1: Lysozyme has six lysine residues as possible PEGylation reaction sites.

Size Exclusion:
Column: TSKgel G3000SW$_{XL}$, 5 µm, 7.8 mm ID × 30 cm
Mobile phase: 0.1 mol/L phosphate buffer,
0.1 mol/L Na_2SO_4, pH 6.7
Flow rate: 1.0 mL/min
Detection: UV@ 280 nm
Injection vol.: 20 µL

Ion Exchange (low pressure):
Column: TSKgel SP-5PW, 20 µm, 6.6 mm ID × 22 cm
Buffer A: 25 mmol/L phosphate buffer,
0.1 mol/L Na_2SO_4, pH 6.0
Buffer B: A + 0.5 mol/L NaCl
Flow rate: 0.85 mL/min
Detection: UV@ 280 nm
Injection vol.: 100 µL

Ion Exchange :
Column: TSKgel SP-NPR, 2.5 µm, 4.6 mm ID × 3.5 cm
Buffer A: 25 mmol/L phosphate buffer,
0.1 mol/L Na_2SO_4, pH 6.0
Buffer B: A + 0.5 mol/L NaCl
Flow rate: 1.0 mL/min
Detection: UV@ 280 nm
Injection vol.: 5 µL

Results

PEGylation of lysozyme

Figure 2 shows a typical chromatogram of a reaction mixture of PEGylated lysozyme separated on a TSKgel SP-5PW column. PEG chain lengths of 5 kDa and 30 kDa are shown. The profiles indicate a similar reaction characteristic. Nonreacted lysozyme remained in the reaction mixture; mono-PEGylated lysozyme as well as poly-PEGylated lysozyme was formed during the reaction.

Size exclusion chromatography was performed as shown in Figure 3. The retention volumes of PEGylated lysozymes were used to assign the peaks in Figure 3 based on a standard calibration curve.

Selectivity

As expected, particle size greatly influenced resolution, while differences in particle and ligand chemistry contributed to selectivity differences. The nonporous particle resin of the prepacked TSKgel SP-NPR column in particular showed very high resolution; with a number of mono-PEGylated isoforms resolved while two isoforms were visible for di-PEGylated lysozyme. TSK-GEL SP-5PW (20 µm) is a polishing resin with a particle size that is almost ten times larger than the TSK-GEL SP-NPR matrix and also a much higher binding capacity as the TSK-GEL SP-5PW particles are fully porous. As shown in Figure 4, although the resolution on the TSKgel SP-5PW column is lower, two mono-PEGylated isoforms remained visible.

Discussion

Lysozyme, as the model protein, was PEGylated to examine the behavior of PEGylated proteins in cation exchange chromatography. A random PEGylation of lysozyme using methoxy PEG aldehyde of sizes 5 kDa, 10 kDa, and 30 kDa was performed.

As a result of PEGylation, we observed a large increase in the size of lysozyme by size exclusion chromatography. The SEC elution position of lysozyme modified with a 30 kDa PEG was equivalent to that of a 450 kDa globular protein. There was a linear correlation between the theoretical MW of PEGylated protein and the MW calculated from SEC. This result illustrates the strong effect that PEG has on the hydrodynamic radius of the resulting PEGylated protein.

Selectivity comparison

Cation exchange chromatography was capable of resolving the PEGylated isomers, which are products of the random PEGylation. Best resolution was obtained with a nonporous TSKgel SP-NPR column. This is due to the faster mass transfer kinetics for large molecules on small, nonporous particles. Despite lower resolution, a porous resin with larger particle size for the first chromatographic step was useful because of higher capacity and better pressure-flow characteristics.

Conclusion

The selectivity of various cation exchange resins were evaluated with random PEGylated lysozyme (chicken egg white). It is

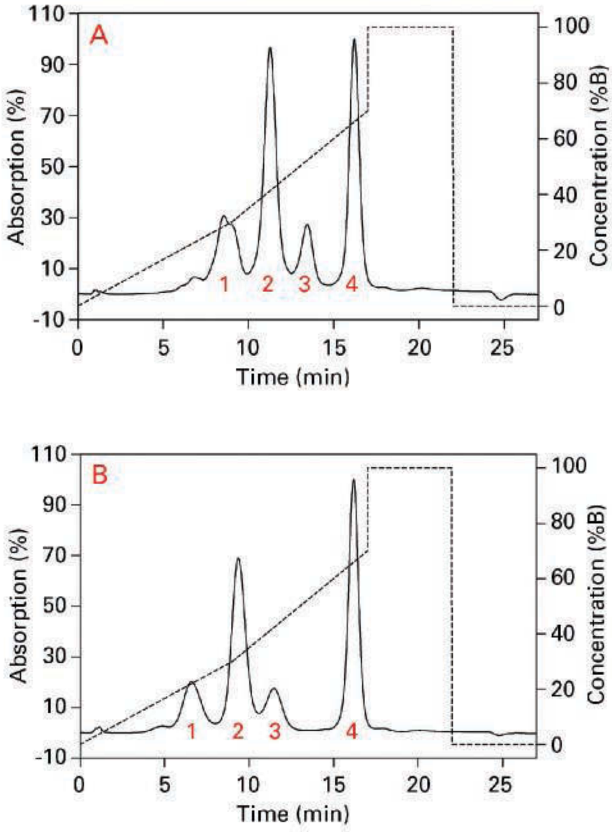

Figure 2: Separation of PEGylated lysozymes on an analytical TSKgel SP-5PW column: mPEG5-aldehydes (A) and mPEG30-aldehydes (B). Peaks were identified by MALDI-TOF analysis. Identical sizes were numbered consecutively.

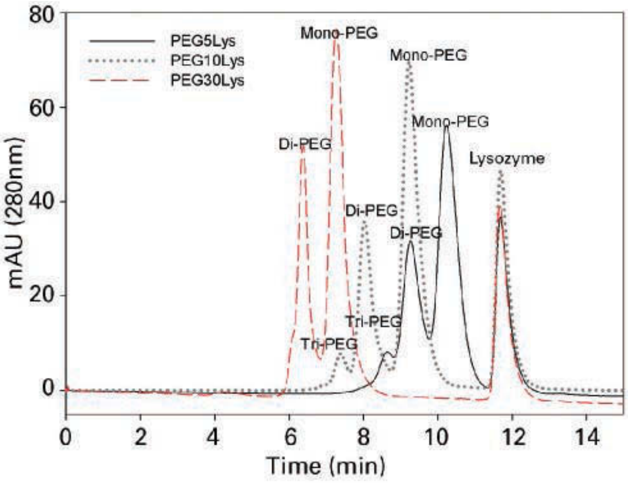

Figure 3: SEC analysis of reaction mixtures performed with a TSKgel G3000SW$_{XL}$ column. Lysozyme and PEGylated lysozyme derivates are shown.

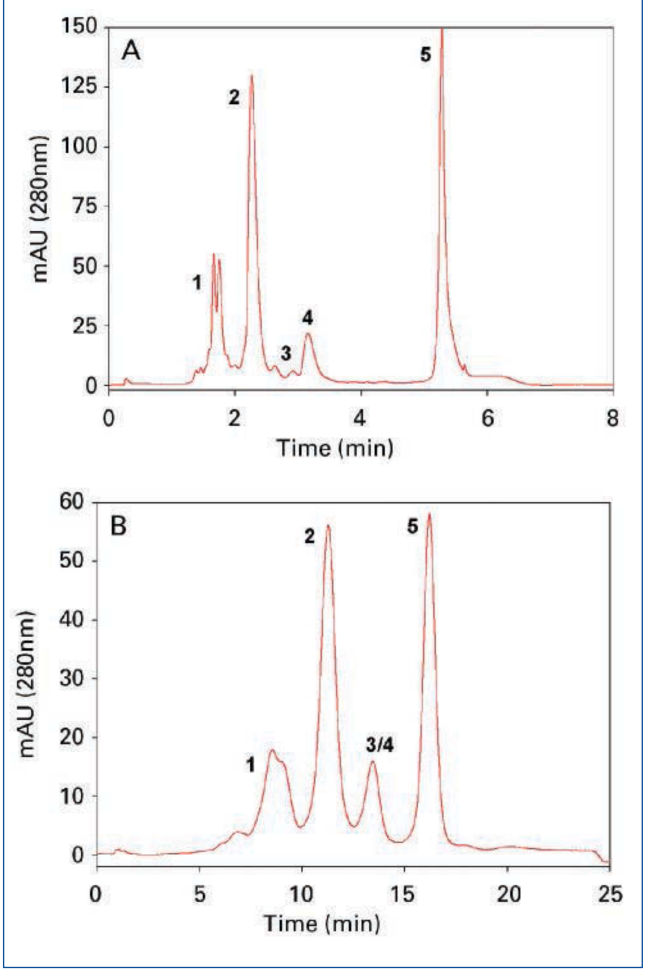

Figure 4: Resolution dependency on particle size shown with 5 kDa PEGylated lysozyme reaction mixture. (A) TSKgel SP-NPR, (B) TSKgel SP-5PW; (1) poly-PEG5Lys, (2) 1-mono-PEG5Lys, (3) 2-mono-PEG5Lys, (4) 3-mono-PEG5Lys and (5) lysozyme.

shown that the selectivity for PEG-modified proteins depends on particle size of the resin. All PEGylated lysozyme species could be resolved on a TSKgel SP-NPR column with a particle size of 2.5 µm and on a TSKgel SP-5PW column packed with 20 µm particles.

Acknowledgement

We thank our colleagues from Institute of Bioprocess Engineering, Friedrich-Alexander University Erlangen-Nuernberg, for carrying out the MALDI-TOF analysis. This work was supported by the Federal Ministry of Education and Research (BMBF), Germany.

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Thermo
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Increasing Proteome Coverage with ETD and CID in One Run Using Intelligent Data-Dependent Decision Tree Logic

Martin Zeller, Torsten Ueckert, Bernard Delanghe, and Thomas Moehring, Thermo Fisher Scientific

Since the addition of electron transfer dissociation (ETD) to the Thermo Scientific LTQ Orbitrap hybrid mass spectrometers, ETD has become a standard tool for proteomics research together with the traditional collision-induced dissociation (CID) (1). Whereas CID produces y- and b-fragment ions, ETD predominantly produces c- and z-type fragment ions.

ETD has shown to be a complementary fragmentation technique to CID and Josh Coon and coworkers have found rules for on-the-fly decisions whether to use CID or ETD, depending on the peptide charge state z and mass-to-charge ratio (*m/z*) which more likely results in a confident peptide identification upon database search (2). These rules have been implemented in a data-dependent decision tree (DDDT) logic in the instrument method setup.

A raw file acquired with the DDDT method, therefore contains both CID and ETD MS-MS spectra which require different search parameters for a database search, and, in addition, ETD spectra require preprocessing before submission to the search engine.

The setup of the DDDT method within the Thermo Scientific Xcalibur method editor is straightforward. In the standard DDDT method, all doubly charged are fragmented with CID, for higher charged peptides CID or ETD is used depending on the peptide's *m/z* (see Table I). For peptides with charge states higher than five ETD is used. In this work, we compare the results of the DDDT method with the results of CID and ETD only methods.

Table I: Settings for the DDDT for fragmentation mode real-time decisions

Charge state	*m/z*	Dissociation technique
2	all	CID
3	< *m/z* 650	ETD
4	< *m/z* 900	ETD
5	< *m/z* 900	ETD
> 5	all	ETD

Experimental

All spectra were acquired on the Thermo Scientific LTQ Orbitrap XL ETD. 500 ng of the complex *Saccharomyces cerevisiae* samples were separated by nano-LC in a 130 mingradient. The LTQ Orbitrap XL ETD™ performed a high-resolution, accurate mass survey scan followed by eight data-dependent MS-MS scans with detection of the fragment ions in the linear ion trap.

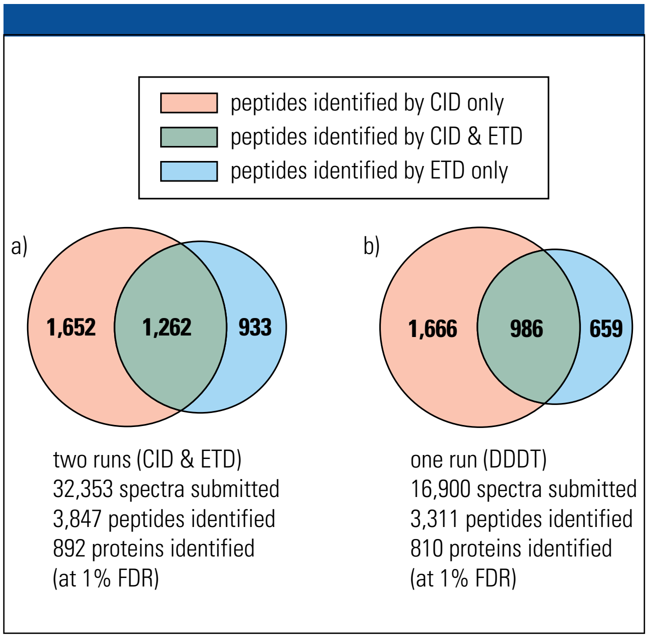

Figure 1: Venn diagrams of the identified peptides using CID and ETD method (a) and DDDT (b).

Data analysis was done using Thermo Scientific Proteome Discoverer 1.1 software suite using Sequest™, Mascot™, and ZCore. Peptide precursor mass tolerance was set to 10 ppm and fragment ion mass tolerance to 0.6 Da for CID and 1.1 Da for ETD (taking hydrogen re-arrangement for ETD fragment ions into account). Carbamidomethylation on cysteine residues was used as fixed modification and oxidation of methionine as variable modifications. ETD spectra were pre-processed using the "Non-Fragment Filter" (3, 4). For the DDDT data, the MS-MS spectra were filtered for activation type and the appropriate search parameters for CID and ETD were used.

All spectra were searched against NCBI database filtered for yeast proteins containing 26,918 entries using a target false discovery rate (FDR) of 1%.

Results

To make use of the complementary character of CID and ETD without the DDDT logic, samples would have to be injected twice (once for the CID run and once for the ETD run). This approach doubles instrument time as well as sample consumption.

Figure 1 illustrates the results of the two approaches using Venn diagrams for the distribution of identified peptides for the two runs in Figure 1a and the one DDDT run in Figure 1b. For the two runs with the CID and ETD method almost twice (91%) as many MS-MS spectra have been triggered but only 16% more peptides and 10% more proteins are identified compared to the single DDDT run. The number of identified peptides using CID is almost the same for both approaches whereas the number of peptides identified by CID and ETD or by ETD only is less than for the DDDT run. The reason for this is twofold: 1) The sample was enzymatically degraded to rather short peptide stretches which predominately ionized doubly charged peptides; 2) CID is superior for doubly charged peptides compared to ETD whereas ETD is more efficient for higher charged peptides. This behavior underscores the power of the DDDT approach to choose the appropriate dissociation method that most likely result in confident peptide identification.

Conclusion

We have shown that the data-dependent decision tree method improves peptide and protein identifications compared to separate runs using CID and ETD. This is especially useful when low sample amounts and/or limited instrument time are available. Furthermore, we show that the Proteome Discoverer™ software has all the tools that are necessary for data mining of mixed raw files containing CID and ETD spectra.

Acknowledgements

We would like to thank Dr. David M. Good (Karolinska Institute Stockholm, Sweden) for the kind gift of the yeast digests.

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A Flexible SPE-LC–MS–MS Platform for the Simultaneous Quantification of Multiple Amyloid β Peptides in Cerebrospinal Fluid

Erin E. Chambers[1], Mary E. Lame[2], and Diane M. Diehl[1], [1]Waters Corporation and [2]Pfizer, Neuroscience Research Unit

Fast, flexible platforms for peptide quantification are needed, particularly for a discovery setting. This type of methodology would be especially advantageous in the case of amyloid beta (aβ) peptides. The deposition/formation of insoluble aggregates, or plaques, of aβ peptides in the brain is considered to be a critical event in the progression of Alzheimer's Disease (AD) and thus has the attention of many researchers. Historically, quantification of aβ peptides in biological fluids has relied mainly on the use of immunoassays, such as ELISA. These assays are time consuming, expensive to develop and labour intensive. They are subject to cross reactivity and an individual assay is required for each peptide. To meet the throughput requirements and constant flow of demands for new peptide methods in a discovery setting, there is a need for a highly specific yet flexible methodology based on an LC–MS-MS platform. In this work, this platform is coupled with selective sample preparation for the simultaneous quantification of multiple aβ peptides. This work focuses on methods for the 1-38, 1-40, and 1-42 aβ peptides, in support of preclinical studies.

Experimental

SPE-LC–MS-MS conditions

LC system:	Waters ACQUITY UPLC system
Column:	ACQUITY UPLC BEH C18, 300 Å, 2.1 × 150 mm, 1.7 μm, Peptide Separation Technology
SPE device:	Oasis MCX μElution 96-well plate
MS system:	Waters Xevo TQ MS, ESI$^+$

Mass spectrometry

MS was performed in positive ion mode because CID of the 4+ precursor ion yielded several distinct product ions corresponding to inherently specific b sequence ions (representative spectrum shown in Figure 2).

UPLC separation

Separation of the three amyloid β peptides is shown in Figure 3.

Sample preparation: SPE

SPE was performed using Oasis MCX, a mixed-mode sorbent, to enhance selectivity of the extraction. The sorbent relies on both reversed-phase and ion exchange retention mechanisms to selectively separate the aβ fraction from other high abundance polypeptides in complex CSF samples. The Oasis μElution plate (96-well format) provided significant concentration while eliminating evaporation and reconstitution, minimizing peptide losses.

Amyloid β 1–38
DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGG
MW4132pI 5.2

Amyloid β 1–40
DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV
MW4330pI 5.2

Amyloid β 1–42
DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
MW4516pI 5.2

Figure 1: Sequence, MW, and pI information for amyloid β peptides.

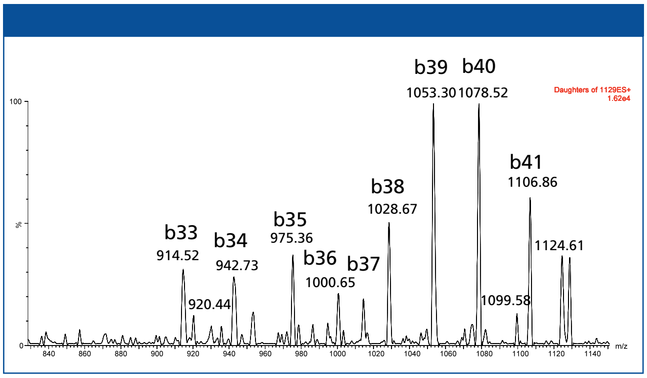

Figure 2: Representative ESI+ MS-MS spectrum for amyloid β 1-42 with fragment sequence ions labelled.

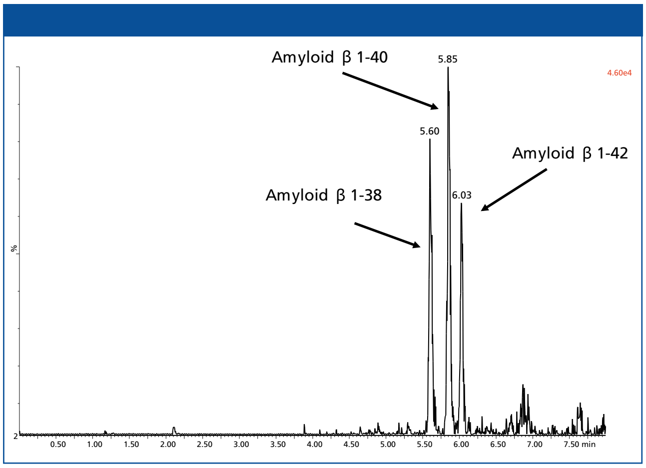

Figure 3: UPLC–MS-MS analysis of amyloid β 1-38, 1-40 and 1-42 peptides extracted from artificial CSF + 5% rat plasma.

Table I: Representative results from analysis of QC samples prepared in pooled human CSF, source 3

Amyloid β 1-38

Over-spike conc. ng/mL	QC conc.	Mean calculated conc.	Std deviation	% CV	Number of replicates passed	Mean accuracy
0.2	1.490	1.355	0.071	5	3/3	90.9
0.8	2.090	1.843	0.118	6	3/3	88.2
2	3.290	3.287	0.319	10	3/3	99.9
6	7.290	7.701	0.478	6	3/3	105.6

Amyloid β 1-40

Over-spike conc. ng/mL	QC conc.	Mean calculated conc.	Std deviation	% CV	Number of replicates passed	Mean accuracy
0.2	2.750	2.359	0.015	1	2/3	85.8
0.8	3.350	3.054	0.016	1	2/3	91.2
2	4.550	3.929	0.011	0	3/3	86.4
6	8.550	8.209	0.500	6	3/3	96.0

Amyloid β 1-42

Over-spike conc. ng/mL	QC conc.	Mean calculated conc.	Std deviation	% CV	Number of replicates passed	Mean accuracy
0.2	0.663	0.665	0.070	11	3/3	98.8
0.8	1.263	1.145	0.058	5	3/3	90.7
2	2.463	2.403	0.121	5	3/3	97.5
6	6.463	5.986	0.701	12	3/3	92.6

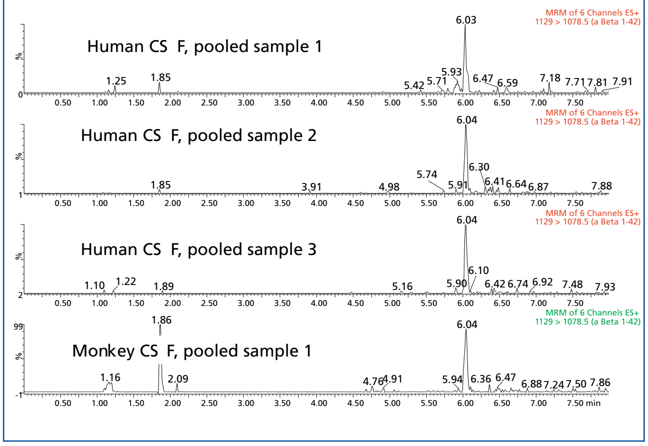

Figure 4: Representative chromatogram showing basal levels of amyloid β 1-42 extracted from 3 sources of human and 1 source of monkey CSF.

Linearity, Accuracy, and Precision

N15-labelled internal standards were used for each peptide. Standard curves for all 3 aβ peptides were linear (1/× weighting) from 0.1 to 10 ng/mL in artificial CSF + 5% rat plasma. Basal levels of amyloid β 1-42 extracted from 3 sources of human and 1 source of monkey CSF are shown in Figure 4. Overspike QC samples were prepared in 3 sources of pooled human CSF and 1 source of pooled monkey CSF at 0.2, 0.8, 2, and 6 ng/mL. Accuracy and precision values for all 3 peptides met the regulatory criteria for LC–MS-MS assays. Representative results from QC sample analysis are shown in Table I.

Results and Conclusions

- An SPE-UPLC–MS-MS bioanalytical method was developed and validated for the simultaneous quantification of multiple amyloid β peptides in human and monkey CSF.
- 96 samples can be extracted and ready for injection in < 30 min, providing the sample prep throughput required for pre-clinical and clinical studies.
- The method described herein eliminates time-consuming immunoassays or immunoprecipitation steps for pre-clinical work.
- This approach also allows one assay for the simultaneous measurement of several different amyloid β peptides from a single sample. This single assay provides a high degree of selectivity and specificity in a high-thoughput format while still achieving the high sensitivity required for low level endogeneous amyloid β peptides.
- The use of a single UPLC–MS-MS assay represents a significant advantage over an ELISA assay, which would require multiple assays with multiple antibodies to quantify each of the relevant peptides.

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Virus Particle Characterization

Wyatt Technology Corporation

Viruses are packets of infectious nucleic acid (either DNA or RNA) surrounded by a protective coat consisting of a large number of protein subunits. Since viruses can cause various diseases — some life-threatening — characterizing virus particles thoroughly in terms of their size distribution, aggregation, and absolute counts-per-unit volume is of extreme importance.

Though size exclusion chromatography (SEC) has been the primary tool for separating aggregates of molecules, it is generally *not* appropriate for fractionating viruses because viruses and their aggregates are subject to shearing degradation by the stationary phase. Moreover, they can also be caught by the columns due to their large sizes.

The Eclipse, a particle separation system based upon field flow fractionation (FFF), effectively replaces SEC as a perfect alternative for virus characterization. Since it is analogous to an HPLC separation, the Eclipse — combined with MALS — makes a physical separation of the particles and then sizes them directly as they elute.

This application note reports the results obtained from a set of virus particles fractionated by Eclipse and sized by the 18-angle DAWN EOS on-line multi-angle light scattering instrument.

Figure 1 shows the radius measured by the DAWN EOS detector (from initial slope of angular dependence) *versus* elution time for two different virus strains. The plots show clearly that aggregates were found in *both* viruses and much more in Virus 2. Results from duplicate injections of Virus 1 show the excellent reproducibility of the Eclipse-MALS system.

The results are also shown by the cumulative number distribution plots in Figure 2. The cumulative number distribution yields quantitative information on the *percentage* of aggregates present in each virus strain: 5% for Virus 1 and 95% for Virus 2.

The success of the virus characterization demonstrated above confirms that the Eclipse-MALS approach is an indispensable tool for charactering virus particles — and other colloidal particles — in solution. This approach is especially important when the *absolute* particle distributions are vital.

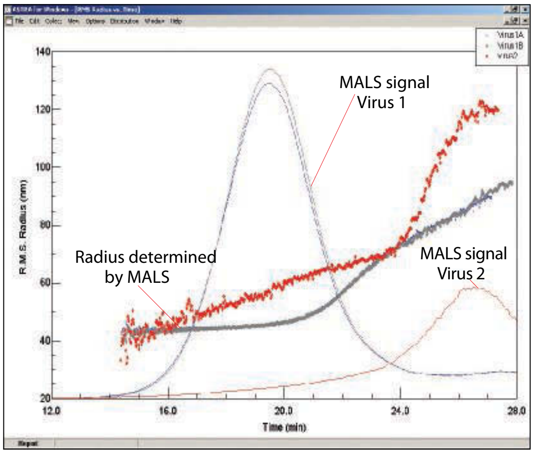

Figure 1: Radius of the virus vs. elution time obtained from the Eclipse-DAWN analysis. The results from two injections of Virus 1 are shown.

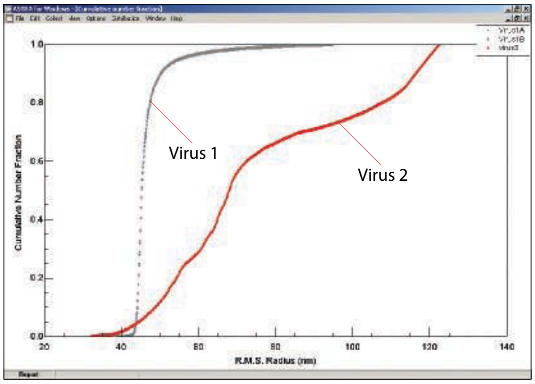

Figure 2: Cumulative number fraction plots of the two virus strains. As shown in the plots, only 5% of Virus 1 (by number) is aggregated; whereas, over 95% of Virus 2 forms aggregates.

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Hydroxyethylstarches (HES)

Wyatt Technology Corporation

Hydroxyethylstarches (HES) are used increasingly as plasma expanders in medical applications. The HES' circulation time in the blood depends strongly on its molar mass distributions. Historically, polysaccharide characterization by gel permeation chromatography (GPC) has been problematic, especially if high molar mass components are present. Due to its superior separation capability, especially on molecules exceeding 50 kDa, flow field-flow fractionation (Flow-FFF) is an excellent separation alternative. By coupling this technology to a multi angle light scattering (MALS) detector, such as a DAWN or miniDAWN, absolute values can be determined without making any assumptions.

We characterized 0.2% (w/v) HES solutions in doubly-distilled water. The HES types were 200/0.5, 130/0.42, and 70/0.5 from Serumwerk Bernburg, Germany. The FFF system was an Eclipse connected to a DAWN EOS and an RI detector (Shodex 101). Volumes of 100 µL were injected into the 350 µm spacer channel containing a 10 kDa regenerated cellulose membrane. A channel flow of 1 mL/min was kept constant while the cross flow decreased linearly from 2 mL/min to 0 mL/min within 20 min. Data were evaluated using Wyatt's ASTRA software package.

The aF-FFF/MALS fractograms of the HES types are compared in Figure 1. Corresponding to normal mode Flow-FFF theory, samples with smaller average molar mass elute faster. In Figure 2, the molar mass distributions are given as cumulative weight fraction plots. As is evident in the plots, monomodal distributions were successfully achieved. The values covered a range from approximately 20 kDa to approximately 600 kDa and up to 2 GDa in size, depending on the characterized HES type. Thus, the average molar mass value was mainly influenced by high molar mass fractions. This was also indicated by higher polydispersity values for HES types with higher molar mass.

Asymmetrical FFF/MALS is, therefore, an excellent method for characterizing medical polysaccharides such as HES. The main advantage of this technique is that molar mass distributions can be determined from absolute values over an extremely wide range of masses.

This note graciously submitted by C. Augsten, K. Mäder, Institute of Pharmaceutics and Biopharmaceutics of the University Halle, W.-Langenbeck-Str. 4, 06120 Halle/Saale, Germany.

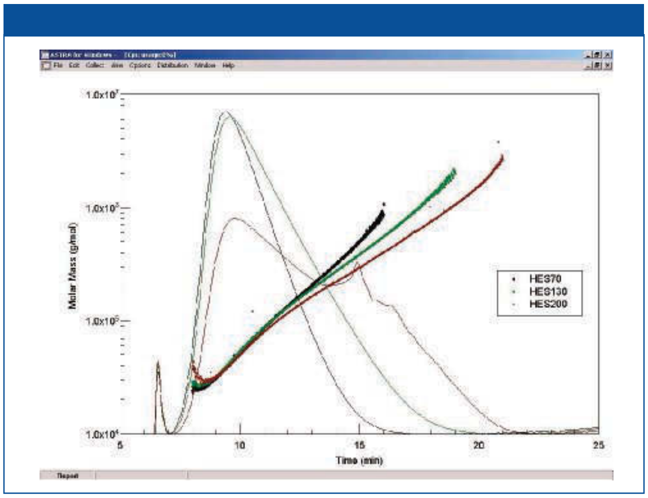

Figure 1: The fractograms of the three samples with their molar mass values overlaid.

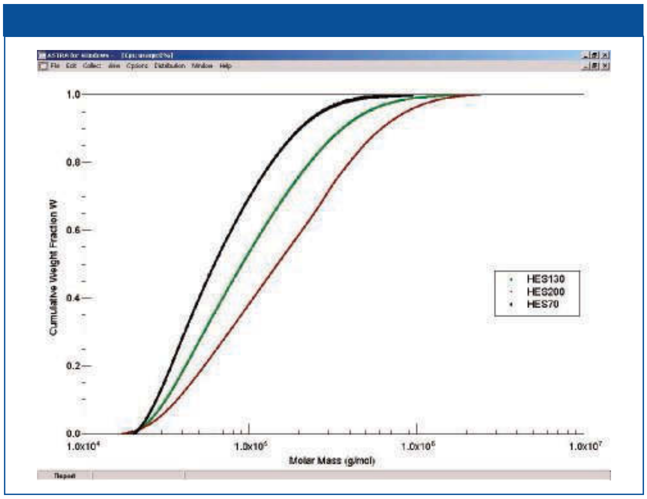

Figure 2: Three different HES samples shown on the cumulative weight fraction plot of ASTRA, indicating the large differences among them.

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The Separation of Racemic Flavanone on Epitomize™ CSP-1 Chiral Stationary Phases

David W. House and Asha Oroskar, Orochem Technologies, Inc.

The optical isomers of flavanone have been separated using the Epitomize™ CSP-1 series of chiral stationary phases. A separation value of 2.52 was obtained using CSP-1A with methanol as the mobile phase. A value of 1.70 was obtained using CSP-1K with a 95/5 blend of heptane/2-propanol as the mobile phase.

The Epitomize CSP-1 series of CSPs includes four derivatized polysaccharides that are coated onto modified silica gel. These CSPs, which are described in Table I, have been used to separate racemic flavanone using both normal and polar mobile phases.

Table I: The Epitomize™ CSP-1 Series of chiral stationary phases

CSP	Chiral Phase
CSP-1A	Amylose tris-(3,5-dimethylphenylcarbamate)
CSP-1C	Cellulose tris-(3,5-dimethylphenylcarbamate)
CSP-1K	Amylose tris-(3-chloro-4-methylphenylcarbamate)
CSP-1Z	Cellulose tris-(3-chloro-4-methylphenylcarbamate)

Experimental

All columns were 4.6 mm I.D. by 250 mm long and the CSP particle size was 5 microns. The normal phase mobile phases were blends of 2-propanol (IPA) and n-heptane and the polar phases were methanol or acetonitrile. The flow rate was 1 mL/min in all cases. The column temperature was 25 °C and detection was at 254 nm.

Results

The best separation value observed using normal phase conditions was 1.70 and was obtained using CSP-1K with 95/5 heptane/IPA as the mobile phase. Table II summarizes the results for flavanone on all four CSPs with normal phase mobile phases.

Table II: Normal phase separation of flavanone on CSP-1A, 1C, 1K, and 1Z

CSP	Hep/IPA	Sep Val.
1A	90/10	1.0
1C	90/10	1.35
1K	95/5	1.70
1Z	90/10	1.07

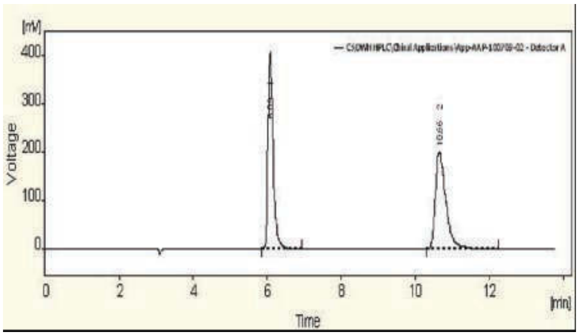

Figure 1: Separation of flavanone on CSP-1A, 5 µm, using methanol at 1 mL/min. Separation value of 2.52.

Flavanone was rerun on CSP-1A and CSP-1C with methanol as the mobile phase at a flow rate of 1 mL/min. The results obtained for CSP-1A are particularly interesting. Although CSP-1A showed essentially no separation of racemic flavanone using 90/10 heptane/IPA as the mobile phase, when pure methanol was used the separation value was 2.52. This separation is shown in Figure 1. Using acetonitrile as the mobile phase led to a separation value of 1.98. The separation of racemic flavanone on CSP-1C with methanol as the mobile phase showed a decrease compared to heptane/IPA. These results are listed in Table III.

Table III: Polar phase separation of flavanone on CSP-1A and 1C

CSP	Eluent	Sep Val.
1A	Methanol	2.52
1A	Acetonitrile	1.98
1C	Methanol	1.26

All of these separations can be scaled up to preparative and simulated moving bed (SMB) chromatography.

Conclusion

Excellent separation of the optical isomers of flavanone can be obtained using Epitomize CSP-1K with normal phase, mobile phases, and CSP-1A with polar mobile phases.

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Effects of Various Acids and Acid Concentration on a ChiroSil® Crown-Ether Chiral Stationary Phase

Ted Szczerba, Regis Technologies, Inc.

The Chirosil® RCA(+) and SCA(−) chiral stationary phases (CSPs) are specialized chiral phases very effective in separating various natural and unnatural amino acids as well as compounds containing primary amines. They are also successfully used for chiral resolution of chiral amino alcohols including therapeutically active compounds such as amphetamine, phenylethanolamine, octopamine, and norepinephrine. The Chirosil® RCA(+) and SCA(−) are covalently bonded, show high performance and durability, and have the ability to invert elution order.

The experimental analysis was performed on the (D,L)-DOPA in order to study the effects of various acids and acid concentration in the method development process.

An Agilent 1200 HPLC system was used for the study. Chromatography was performed on a Chirosil® SCA(−) chiral column, 15 cm × 4.6 mm. The effect of acidic modifiers in the mobile phase was studied using four acids: trifluoroacetic acid (TFA), perchloric acid, phosphoric acid, and acetic acid. The aim of the study is to demonstrate the scope of method development options and propose a simple way to optimize separation methods.

Table I summarizes the effect of different acids. Chromatogram Set #1 (Figure 1) illustrates the chromatograms.

Table I: Summary of the effect of different acids

Acid	k'_1/k'_2	Alpha	Rs
TFA	0.35/0.50	1.41	1.02
Perchloric	0.61/0.80	1.32	1.12
Phosphoric	0.92/1.81	1.97	2.64
Acetic	2.14/4.27	1.99	2.77

Table II summarizes the effect of acid concentration. Chromatogram Set #2 (Figure 2) illustrates the chromatograms.

Table II: Summary of the effect of acid concentration

Acid Concentration	k'_1/k'_2	Alpha	Rs
0.1% TFA	0.35/0.50	1.41	1.02
0.075% TFA	0.38/0.58	1.51	1.25
0.05% TFA	0.42/0.69	1.65	1.50
0.025% TFA	0.58/1.07	1.86	2.07
0.01% TFA	0.83/1.68	2.01	2.48

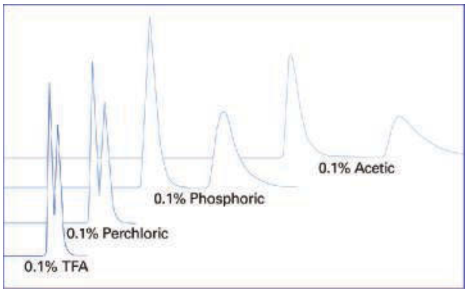

Figure 1: Chromatogram Set #1: Column: 15 cm × 4.6 mm Chirosil® SCA(−). Mobile Phase: Methanol/Water (70/30) + acid. Flow Rate: 1.0 mL/min.

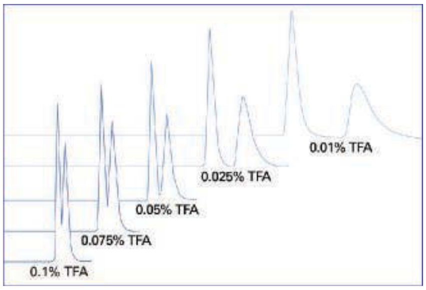

Figure 2: Chromatogram Set #2: Column: 15 cm × 4.6 mm Chirosil® SCA(−). Mobile Phase: Methanol/Water (70/30) + acid. Flow Rate: 1.0 mL/min.

Conclusions

Different acids and acid concentrations play a key role in the method development process on a Chirosil® Crown Ether column. The behavior of each acid is sample dependant. All four acids should be tried to achieve the best separation and resolution of your test analyte. The separation can be further optimized by adjusting the acid concentration. Other parameters that should be explored are organic modifier choices (ethanol, methanol, acetonitrile, etc.) in the mobile phase and column temperature.

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Biotage

Extraction of 14 Benzodiazepines by a Supported Liquid Extraction Plate, ISOLUTE® SLE+

Lee Williams, Rhys Jones, Steve Jordan, Richard Calverley, Claire Desbrow, Gary Dowthwaite, and Elena Gairloch,
Biotage GB Limited

Benzodiazepines are commonly assayed to determine therapeutic dose levels and for forensic investigation. These drugs are commonly prescribed to alleviate pain and therapeutic dosing needs to be monitored. Additionally, the use of this class of drugs in "date rape" cases has lead to a need for forensic monitoring. Biotage have successfully applied supported liquid extraction, utilizing ISOLUTE® SLE+, to analyze 14 benzodiazepines by LC–MS from whole blood, plasma, and urine. These procedures are fully automation compatible, dramatically reducing cost and increasing throughput.

Method

ISOLUTE SLE+ Supported Liquid Extraction Plate, 200 µL sample size, part number 820-0200-P01, was used. Blank matrix (100 µL) was spiked with the benzodiazepines at 50 ng/mL. The matrix was then diluted 1:1 v/v with H_2O prior to loading. This sample dilution results in an approximate loading pH of 8.0. For the whole blood cell lysis, samples were sonicated for 10 min in buffer, followed by centrifugation at 11,000 rpm for 10 min and the cellular debris discarded. 200 µL of pretreated plasma was applied to the plate and a pulse of vacuum applied to initiate flow. The samples were then left to absorb for 5 min. Analytes were eluted by the addition of 1 mL of various water immiscible extraction solvents. The extraction solvents evaluated were DCM, 95:5 (v/v) DCM/IPA, MTBE, and EtOAc. The eluate was then evaporated to dryness and the analytes reconstituted in 500 µL of 80:20 (v/v) H_2O/MeOH prior to analysis.

Results

Recoveries of greater than 80% were observed for the majority of analytes from all three matrices using various extraction solvents. RSDs were below 10%, demonstrating reproducible extraction and recoveries. Pretreatment with 1% formic acid, 0.1% formic acid, H_2O and 0.5M NH_4OH for plasma and urine and 0.1% formic acid and H_2O for whole blood were also investigated (data not shown, available upon request). These pretreatment conditions yielded a range from pH 3.2–10.4. Consistently high recoveries were observed using H_2O pretreatment of plasma, urine, and blood for all extraction solvents (Figures 1–3, respectively). Similar, though slightly attenuated, recoveries were obtained from all analytes across the full pretreatment pH range (pH 3.2–10.4). These results allow for the use of a broad range of buffer conditions to be used at a pH compatible with other analytes of interest. This easily automatable methodology allows for high recoveries of a broad range of analytes using various buffers and extraction solvents.

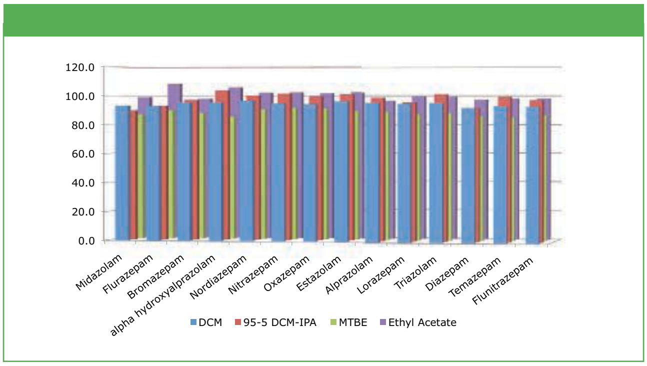

Figure 1: Absolute recoveries of the aromatic and aliphatic fractions.

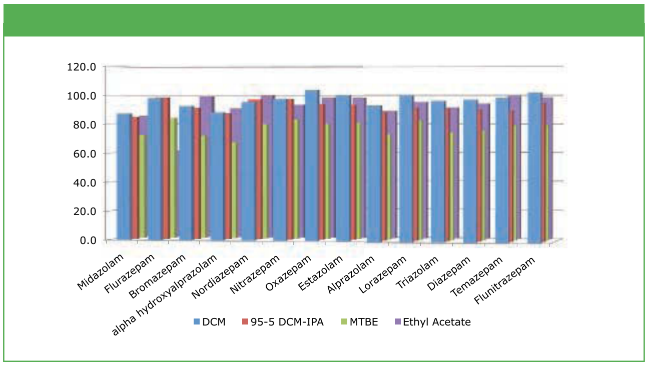

Figure 2: Post-fractionation of PAH-TPH mix– aliphatic fraction.

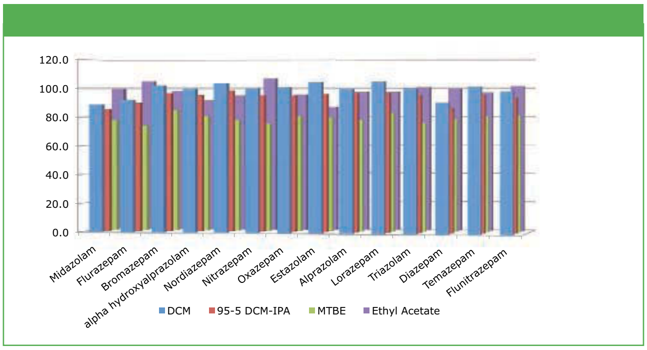

Figure 3: Benzodiazepine recovery profile from whole blood using H_2O pretreatment.

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Nitrosamines in Drinking Water

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N-Nitrosodimethylamine and other structurally similar nitrosamines are classified as probable carcinogens by the U.S. EPA, and their presence has been reported in California drinking water since 1998. Established notification levels for these nitrosamines have been set to 10 ng/L (parts per trillion).

Analysis of trace levels of nitrosamines in water samples involves solid phase extraction (SPE) and concentration, followed by liquid or gas chromatographic (LC or GC) separation with a sensitive and selective detector, preferably mass spectrometry (MS or MS-MS).

The following describes an LC–MS method for improved chromatographic separation and detection using an MSQ™ Plus single quadrupole mass spectrometer.

Experimental

A Dionex UltiMate® 3000 HPLC system was coupled to an MSQ Plus MS by an electrospray ionization (ESI) interface. Chromatographic separation was achieved on an Acclaim® PA2 column with gradient elution at 0.25 mL/min: 0.5% to 80% acetonitrile from 5 to 21 min, and held constant at 80% for 2 min, 0.005% formic acid was held constant throughout the run. The column temperature was set at 10 °C and the injection volume was 20 μL. The MSQ Plus MS was operated in selected ion monitoring (SIM) mode and the protonated molecular ion $[M+H]^+$ was used as the quantification ion for each of the analytes as shown in Table I.

MS parameters, such as probe temperature, needle voltage, and cone voltages, were optimized to provide the best sensitivity.

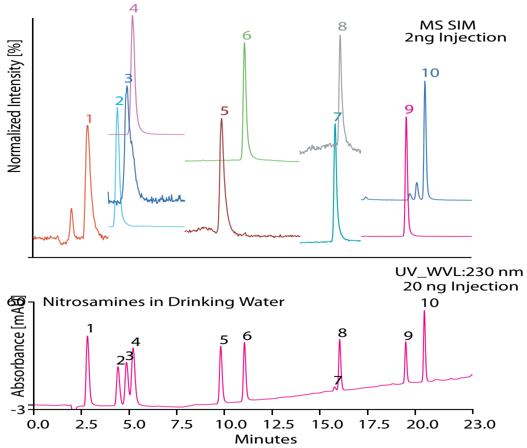

Figure 1: N-Nitrosodimethylamine and other nitrosamines in drinking water by LC–MS.

Table I: SIM scan parameters for studied compounds.

Peak No.	Analyte	Abbreviation	Retention Time	SIM	Cone Voltage
1	N-Nitrosodimethylamine	NDMA	2.9	75.1	60
2	N-Nitrosomorpholine	NMOR	4.4	117.1	45
3	N-Nitrosomethylethylamine	NMEA	4.9	89.1	35
4	N-Nitrosopyrollidine	NPYR	5.3	101.1	45
5	N-Nitrosodiethylamine	NDEA	9.9	103.1	40
6	N-Nitrosopiperidine	NPIP	11.1	115.1	50
7	N-Nitroso-n-propylamine	NDPA	16.1	131.1	30
8	N-Nitroso-n-butylamine	NDBA	19.6	159.2	50
9	N-Nitrosodiphenylamine	NDPHA	20.5	199.0	40

Results

In Figure 1, the UV chromatogram shows well resolved chromatographic separation (20 ng of each analyte in a 20 μL injection), and the MS SIM chromatograms demonstrate the advantage of MS for selective and sensitive detection (2 ng of each analyte in a 20 μL injection). For N-Nitrosodiphenylamine (peak 10 in Figure 1), interferences were chromatographically separated from the target analyte, showing the combined benefits of chromatographic separation and MS detection — in achieving a high level of confidence in data acquisition. With the addition of proper SPE sample enrichment, this method provides sufficient sensitivity to enable detection of nitrosamines in water samples at the low parts per trillion levels.

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Making Non-Electrochemically Active Compounds Electrochemically Active

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Electrochemical detection (ECD) is both selective and extremely sensitive. However, relatively few compounds are electrochemically active, therefore limiting its application. To extend the usefulness of ECD, the authors investigated a process termed electrotagging that renders inert compounds electrochemically active. A boron-doped diamond (BDD) electrode was used at high cathodic potentials under aqueous conditions to generate hydroxyl free radicals (1, 2). These radicals reacted with inert aromatic rings forming an electrochemically active adduct that may then be measured on the same BDD electrode. The authors have previously shown the application of this approach for the measurement of aromatic genotoxins, including aminopyridines, besylates, and tosylates (3, 4, 5). Here, they explore the use of electrotagging and gradient elution for the measurement of more complex aromatic systems–polyaromatic hydrocarbons (PAHs).

Sixteen PAHs were selected for evaluation using this method, as listed in Figure 1.

Materials and Methods

A high-pressure gradient HPLC system was used consisting of two pumps, a refrigerated autosampler, an on-line degasser, a thermal organizer, and a Coulochem® III Electrochemical Detector equipped with a Model 5020 Guard Cell and a Model 5040 amperometric cell with a BDD working electrode.

Results

All sixteen PAHs were detected by ECD and were well resolved in under 30 min. Overlaid chromatograms (313–5000 pg on column) are presented in Figure 1. Linear relationships were found for all 16 PAHs from amounts of 39 to 5000 pg on column for the first 13 PAHs and from 313 to 5000 pg on column for the last 3. Correlation coefficients were all > 0.994. LOD values of 20 pg on column for analytes 1–10, 80 pg on column for analytes 11–13, and 300 pg on column for analytes 14–16 were determined using a signal-to-noise ratio of 3:1. Precision was acceptable at < 5% RSD for the majority of analyte concentrations.

Conclusion

The BDD working electrode, with its ability to be used with gradient elution and at high potentials, extends the range of compounds that can be measured electrochemically. By generating free radicals, aromatic compounds can both be hydroxylated *in situ* and detected with the BDD electrode. This approach may be used to measure, at ppb sensitivity, numerous aromatic compounds, such as drugs and environmental pollutants, that cannot be analyzed by traditional ECD approaches.

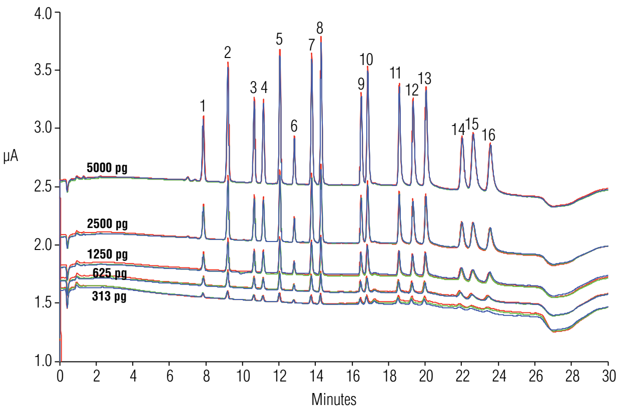

Figure 1: Triplicate injections of 16 PAH analytes (313–5000 pg on column). Column: Pinnacle® II, 2.1 × 100 mm, 4 µm; Column Temp.: 35 °C; Sample Temp.: 10 °C; Mobile Phase A: Buffer/water/acetonitrile (225:675:100), 25 µL/L H_2O_2; Mobile Phase B: Buffer/acetonitrile (200:800), 25 µL/L H_2O_2; Buffer: 200 mM sodium perchlorate, 100 mM perchloric acid in polished, deionized water; Flow Rate: 0.60 mL/min; Injection Vol.: 10 µL; Guard Cell: +500 mV (vs Pd reference); BDD Cell: +1750 mV (vs Pd reference); Filter: 5 s; Gradient Time (min), %B: 0.0, 30.0; 7.0, 58.0; 16.0, 100.0; 25.0, 100.0; 25.1, 30.0; 30.0, 30.0. Peaks: 1. naphthalene; 2. acenaphthylene; 3. acenaphthene; 4. fluorine; 5. phenanthrene; 6. anthracene; 7. fluoranthene; 8. pyrene; 9. benzo[a]anthracene; 10. chrysene; 11. benzo[b]fluoranthene; 12. benzo[k]fluoranthene; 13. benzo[a]pyrene; 14. dibenzo[a,h]anthracene; 15. benzo[g,h,i]perylene; 16. indeno[1,2,3-c,d]pyrene.

References

(1) Luong, et al. *Analyst*, **134**, 1965–1979 (2009).
(2) Oliveira, et al. *Chemosphere,* **66**, 2152–2158, (2007).
(3) ESA Application Note 70-8311, Routine HPLC Determination of Aminopyridine Compounds with a Boron-Doped Diamond Electrode.
(4) ESA Application Note 70-9063, Genotoxic Alkyl Besylates by BDD.
(5) ESA Application Note 70-8966, Genotoxic Alkyl Tosylates by BDD.

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Determination of Trace-Level Endocrine Disruptors in Seawater by Online SPE-HPLC Using the SolEx HRP Cartridge

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In this application note we present an online SPE-LC method for the determination of trace-level endocrine disruptive compounds (EDCs) in seawater using the new SolEx® HRP cartridge as the solid-phase extraction (SPE) phase. This method shows good recovery of EDCs in 2% sodium chloride solution and seawater.

EDCs in seawater are an environmental concern. It has been reported that there is global contamination of sea sand and seawater with the endocrine disruptor bisphenol A (BPA) (1). Scientists found BPA in sand and seawater from Southeast Asia and North America ranging from 0.01 parts per million (ppm) to 50 ppm (1). In this proposed method, EDCs in seawater are preconcentrated by online SPE using the SolEx HRP cartridge followed by separation on the Acclaim® PolarAdvantage reversed-phase column with UV detection.

Experimental

A Dionex UltiMate® 3000 system with a DGP-3600M dual gradient pump, a SRD 3600 solvent rack with integrated vacuum degasser, a TCC-3200 thermostatted column compartment with two two-position, six-port valves, an AS-HV high volume autosampler, and a PDA-3000 UV detector were used. A SolEx HRP cartridge, 12 µm (2.1 × 20 mm) was used for solid phase extraction; an Acclaim PolarAdvantage reversed-phase column, C16, 3 µm (2.1 × 150 mm) was used for separations.

Results and Discussion

Figure 1 shows online SPE-LC of bisphenol A, β-estradiol, and estrone. Seawater was prefiltered through a 0.45 µm membrane. After preconcentration on the SPE phase, 6 mL DI water followed by 4 mL 5% acetonitrile (CH_3CN) were used to wash the SPE column in order to remove the salty matrix. EDCs trapped in the SPE phase were transferred to the Acclaim PA column using 35% CH_3CN for separation. Peak area RSDs ranged from 0.4% to 0.7% for BPA; 1.4% to 3.7% for β-estradiol; and 0.3% to 1.1% for estrone. Retention time precision was excellent, with RSDs ranging from 0.04% to 0.09%.

Recovery of these three EDCs in 2% sodium chloride solution and seawater collected at Half Moon Bay are calculated by comparing the area responses of EDCs to the same sample spiked in deionized water. All the three EDCs studied show good recovery.

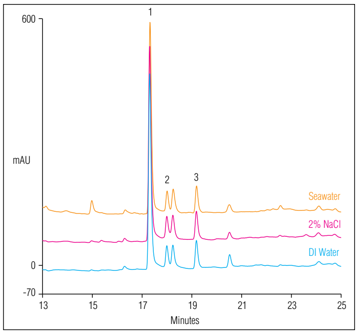

Figure 1: Online SPE-LC of EDCs spiked into deionized water, 2% sodium chloride, and Half Moon Bay, CA seawater. Flow Rate: 300 µL/min; Injection Volume: 10 mL; Temperature: 25 °C; Detection: UV at 210 nm; Mobile Phases: A) water, B) Acetonitrile, C) 100mM pH 5.4 ammonium acetate; Gradient: to 10.0 min: 55% A, 35% B, 10% C; 20.0 min: 30% A, 60% B, 10% C; 25.0 min: 30 % A, 60% B, 10% C; 25.1 min: 55% A, 35% B, 10% C; Peaks (5 µg/L each): 1). bisphenol A; 2) β-estradiol; 3) estrone.

Table I: Recovery of endocrine disruptive compounds in 2% sodium chloride and Half Moon Bay seawater

Analyte, 5 ppb	2% NaCl	Seawater
Bisphenol A	98.01%	97.18%
β-Estradiol	93.81%	93.39%
Estrone	103.28%	97.81%

Conclusion

Using online SPE and HPLC-UV, EDCs were analyzed at low ppb levels on the new Dionex SPE phase, SolEx HRP cartridge. The method was applied to 2% sodium chloride solution and Half Moon Bay seawater sample extracts, showing good recovery at 50 ng. Lower detection limits can be obtained using large injection volume. Replicate injections of standard with very low RSDs of retention times and peak areas confirm the reproducibility of this method.

References

(1) American Chemical Society 239th National Meeting news releases, San Francisco, Mar. 23, 2010.

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Determining the Health Impact of Burning Mosquito Coils Using Versatile Micro-Chamber Sampling and TD-GC–MS

Lara Kelly, Markes International, Ltd.

Burning mosquito coils to repel these pests is common in many countries; however the emission of various, potentially toxic volatile organic compounds (VOCs) is cause for concern. Novel sampling equipment with TD-GC–MS is used to extract, concentrate, and analyze the emissions of two burning coils and determine any potential impact on consumer health.

Mosquito coils are a form of incense whose active ingredients are usually derived from pyrethin, a natural insecticide. When burnt, the smoke repels, paralyses, or kills mosquitoes. These coils are very popular in Asia, Africa, and South America; however, there has been concern about levels and potential harm of chemicals emitted, e.g., polycyclic aromatic hydrocarbons (PAHs) or aldehydes (types of volatile organic compounds [VOCs] with known health effects), particularly when burned for long periods in confined or poorly ventilated areas (1). Two types of coil (one red, one black) were obtained and burnt, and their VOC emissions compared to estimate their safety.

To analyze the emissions of a particular substance, novel micro-scale chamber equipment provides a compact, portable unit that allows fast screening of several samples, simultaneously (Figure 1) (2). For bulk emissions testing, a small amount of a sample is placed in the bottom of an individual chamber pot. Several pots can then be inserted into the micro-chamber unit set to a prescribed temperature. Emitted vapor is extracted and retained using sorbent tubes, and its VOC composition determined by GC(MS).

To obtain a comprehensive representation of the VOC emissions, thermal desorption of the extracted vapors was performed prior to GC–MS analysis. This well-established technique concentrates the VOCs from a large sample into a narrow band of vapor and efficiently injects this directly into the GC column, giving results with far higher sensitivity, therefore allowing more reliable compound identification.

Figure 1: A micro-chamber sampling unit with six individual chambers.

Experimental Conditions

Sections (~40 mm) of both red and black mosquito coils were cut and lit. These were then placed inside individual micro-chamber pots (constructed from inert-coated stainless steel), which were inserted into the micro-chamber sampling unit and sealed. The samples were left to equilibrate for 10 min under a constant

Table I: Identification of VOCs found in mosquito coil emissions (* only found in red coil)

Peak #	Compound	Peak #	Compound	Peak #	Compound
1	Propene	18	2-Pentanone	35	Phenyl ester acetic acid
2	Methanol	19	Pentadione	36	Butyl benzene*
3	Chloromethane	20	2,4-Dimethylfuran*	37	2-Methoxyphenol
4	Acetonitrile	21	1-Methyl pyrrole*	38	Benzoic acid methyl ester
5	Acetone	22	Toluene	39	2-Methyl benzofuran*
6	Propanol	23	Hexanal	40	Tetramethylbenzene
7	Furan	24	Furfural	41	Phenylmethyl ester acetic acid
8	Acetic acid methyl ester	25	Ethylbenzene	42	2-Methoxy-4-methyl phenol*
9	2,3- Butanedione	26	Xylene	43	Naphthalene
10	2-Butanone	27	Styrene*	44	4-Ethyl-2-methoxy-phenol*
11	Hexene*	28	Methoxy benzene*	45	Tridecane
12	3-Methyl furan	29	Benzaldehyde	46	Dimethoxy phenol
13	Hexane	30	Benzonitrile	47	Biphenyl
14	2-Methyl furan	31	Phenol	48	Tetradecane
15	Methyl propionate*	32	Benzofuran	49	Pentadecene
16	Benzene	33	Decene*	50	Pentadecane
17	1-Hydroxy-2-propanone	34	Limonene*	51	Allethrin*

flow of dry air (to ensure lit samples were not extinguished). A conditioned TD tube containing a combination of sorbents (allowing the retention of a broad range of analytes) was placed on the outlet port of each of the chambers, with a diffusion locking cap (Markes International Ltd., UK) on the nonsampling end to allow air flow and prevent contaminant ingress. The tubes were removed after 30 min.

Following sampling, the tubes were placed onto the TD system, desorbed, and then analyzed by GC–MS. Full conditions are listed below.

Instrument conditions

Micro-chamber sampling:

Instrument: Micro-Chamber/Thermal Extractor™ (Markes International Ltd.)
TD tube sorbents: Tenax® TA (Buchem B.V., The Netherlands) and UniCarb™ (Markes International)
Carrier gas: Dry air
Flow rate: 50 mL/min
Temperature: 40 °C

Thermal desorption:

Instrument: UNITY 2™ (Markes International)
Flow path: 200° C
Trap purge time: 1 min
Primary desorption: 10 min, 300 °C
Trap conditions: −10 °C, 300 °C, hold 5 min
Trap type: T6-SUL-2S (sulphur trap) (Markes International)

GC–MS:

Instrument: Agilent 7890/5975
Column: Ultra 2.5% PMS, 50 m, 0.32 μm, 0.52 μm
Column flow: 1.8 mL/min, constant flow
Temperature program: 50 °C (2 min), ramp at 25 °C/min to 160 °C, then ramp at 10 °C/min to 280 °C, hold for 30 min
Carrier gas: He
Mass scan range: 35–300
MS source temperature: 230 °C
MS quad temperature: 150 °C

The GC–MS data file produced was imported into TargetView™ (ALMSCO International, UK) software to deconvolute the data and identify compounds automatically (3).

Results and Discussion

Figure 2 displays the VOC profiles of both the red and the black mosquito coils when burning. The complexity of the chromatograms gives an indication to the high chemical emissions and the efficiency of the extraction/concentration technique used. The differences between the two mosquito coils are visible; although there are many common compounds, these are often present in different abundances. TargetView, a deconvolution and identification software package, was used to automatically and quickly determine the identity of peaks in the complex data with high confidence (a match co-efficient exclusion value of >0.7 was used); these are listed in Table I. Compounds that are present in the red mosquito coil only are marked with an asterisk.

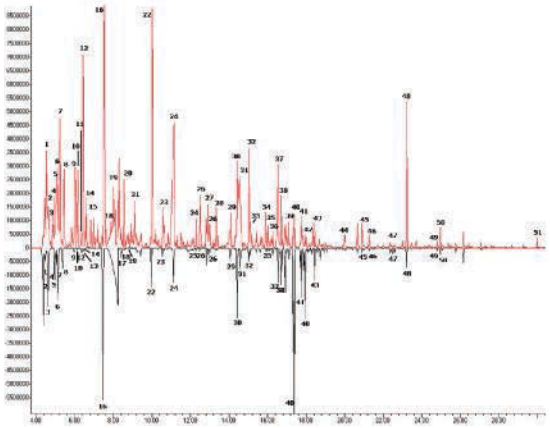

Figure 2: Chromatograms of VOCs from red mosquito coil (upper) and black mosquito coil (lower; inverted).

Some of the compounds found were nontoxic fragrance compounds. However, of concern, the most abundant compound emitted from the red coil, and a significant contributor to emissions of the black coil, is benzene, a known carcinogen and one linked to many serious health problems. The chromatograms also show that many other suspected carcinogens, e.g., furan, ethylbenzene, and naphthalene (which are categorized as IARC Group 2B carcinogens), or respiratory tract irritants, e.g., benzaldehyde, tridecane, and phenol, are observed in both but are more abundant in the red coil emissions. It is also interesting to note that allethrin, a synthetic pyrethroid (natural insecticide), is found only in the red coil emissions. In addition, the compound 2,3-butanedione, which is another mosquito repellent, is found at a significantly higher concentration in the red coil than the black.

Conclusion

The introduction of micro-chamber sampling technology has made in-house screening of product emissions far more accessible to manufacturers due to its simplicity, size, and speed.

Via this simple, yet efficient, method of chemical extraction and sample concentration for GC–MS analysis, comprehensive VOC profiles of both mosquito coils were obtained. The results suggest that though the red coil has significantly higher VOC emission levels, either coil would give cause for concern if burned for a prolonged duration in a poorly ventilated area.

References

(1) W. Liu, J. Zhang, J.H. Hashim, J. Jalaludin, Z. Hashim, and B.D. Goldstein. *Environmental Health Perspectives 111* **(12)**: 1454–1460 (2003).

(2) Markes International TDTS 67: Introducing the Micro-Chamber/Thermal Extractor™ (μ-CTE™) for rapid screening of chemicals released (emitted) by products and materials.

(3) Markes International TDTS 90: Automatic detection of trace target compounds in complex chemical emission profiles.

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Analysis of Aircraft Cabin Air by TD-GC×GC-TOFMS

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A sample of ambient cabin air from a commercial airliner was collected by stir bar sorption extraction (SBSE) and analyzed by thermal desorption GC×GC-TOFMS. The cabin air is a mixture of recirculated cabin air and bleed air from the compressor stages of the aircraft's turbine engines. Volatile organic compounds in cabin air, tricresyl phosphate in particular, have been the subject of recent discussion.

Experimental Conditions

In this study, analyses were performed with a LECO Pegasus® 4D GC×GC-TOFMS system.

Primary Column: 10.0 m × 0.18 mm ID × 0.18 µm d_f DB-5
Secondary Column: 1.00 m × 0.10 mm ID × 0.10 µm d_f
Rxi-17
Carrier Gas Flow: 1.2 mL/min Corrected Constant Flow

Oven Program: 30 °C 3 min 5 °C/min 295 °C 3 min
Column Offset: +10 °C
Modulator Offset: +15 °C
Modulation Period: 5 s
Hot Pulse Duration: 0.800 s

Source Temperature:200 °C
Acquisition Rate: 200 spectra/s
Acquisition Range: *m/z* 40 to 400

A GERSTEL Twister Desorption Unit (TDU) connected in-series with a GERSTEL CIS4 inlet was operated in Solvent Vent mode at 300 °C for 2 min and 50 mL/min flow. The CIS4 was held at −120 °C. The injection from the CIS4 to the column was Splitless at 300 °C and at column flow.

The sample was collected on a pair of 10 mm × 1.0 mm d_f PDMS GERSTEL Twister stir bars suspended from a wire scaffold in a 20 mL scintillation vial. The sample was collected by placing the apparatus ~8 cm from the overhead air vent operating at maximum flow for a period of 30 s.

Results

The sample was data processed and 2147 peaks were found with a s/n ratio threshold ⩾100:1. Tricresyl phosphate is an organophosphate that is a known neurotoxin that is present in the oil used in aviation turbine engines. Tricresyl phosphate was not found in the sample. Propanoic acid esters were a dominant feature.

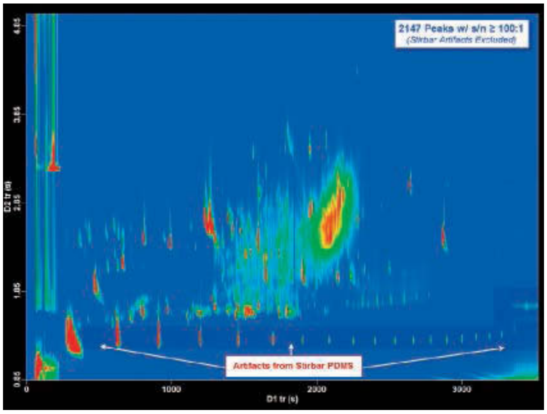

Figure 1: A contour plot of the sample.

Conclusions

The recent global outbreaks of SARS, influenza, and other highly contagious conditions have led commercial aircraft manufacturers and operators to equip aircraft ventilation systems with HEPA particle filtration. While HEPA filtration does provide a level of protection against particulate pathogens, it does not provide protection from chemical vapors present in the aircraft cabin and ventilation systems. The work presented here provides an example of a simple sampling technique for VOCs and SVOCs in a commercial aircraft cabin. This sample is complex and contains components over a wide range of volatilities and functionalities. When analyzed by GC×GC-TOFMS, the large number of individual components can be resolved and identified in a single analysis, providing an efficient means for the analyst to obtain a large amount of information about the sample with a minimum amount of effort. LECO's Pegasus 4D GC×GC-TOFMS system and ChromaTOF software are an excellent choice for the analysis of highly complex samples such as that demonstrated in this work.

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GPC Clean-Up of Black Pepper Prior to Analysis for Organochlorine (OC) Pesticides by GC/XSD

Laura Chambers and Elizabeth Badgett, OI Analytical

Gel-permeation chromatography (GPC) is a highly effective postextraction cleanup method for removing high molecular-weight interferences such as lipids, proteins, and pigments from sample extracts prior to analysis. Postextraction GPC cleanup is used extensively for preparing food and environmental samples prior to analysis for pesticides, fungicides, semivolatiles, and other environmental contaminants. The FDA Pesticide Analytical Manual and the European Committee for Standardization (EN 1528) both recommend using GPC for separating fats and oils from food matrices prior to pesticide analysis by gas chromatography (GC). If sample extracts with high-lipid content are injected onto a GC column, the injection port and column can easily become contaminated, resulting in recovery losses and poor chromatography.

The OI Analytical GPC AutoPrep 2000 fully automates the GPC cleanup process. This system uses an autosampler for injecting sample extracts and collecting cleaned fractions into a variety of collection vessels, and features a modular design with electronic valve actuation. WinSEP™, a Windows®-based program, controls the GPC AutoPrep 2000 and includes extensive quality monitoring features.

This study used the GPC AutoPrep 2000 for cleanup of black pepper samples prior to analyzing for OC pesticides by GC with the OI Analytical 5360A Halogen Specific Detector (XSD™). The XSD is the preferred detector for analyzing OC pesticides because of its extreme selectivity, ease of use, and low maintenance requirements.

Experimental Conditions

Pesticide-fortified and blank black pepper were extracted by mixing with 1:1 acetonitrile:water, filtering, and then extracting with petroleum ether by liquid-liquid partitioning. The petroleum ether extract was evaporated to dryness using a Labconco RapidVap® N2 System and reconstituted in 10 mL of GPC mobile phase for cleanup. Cleanup used the GPC AutoPrep 2000 equipped with a 700 mm × 25 mm glass column containing 60 g of Envirobeads® S-X3 resin. The system used a 5-mL sample loop with a 50:50 mixture of ethyl acetate:cyclohexane as the mobile phase (5 mL/min flow rate). The GPC column was calibrated with a calibration standard and UV detector as described in USEPA Method 3640A. The collected fractions were evaporated using the RapidVap N2 system and reconstituted in appropriate solvent for GC analysis. The reconstituted extracts were analyzed for OC pesticides using an XSD and a 2-mL splitless injection. The GC was configured with an HP5 column and ramped oven program.

Results and Conclusion

The GPC AutoPrep 2000 was highly effective at removing the high molecular-weight interferences from black pepper prior to GC/XSD analysis. The XSD yielded excellent detection of 18 OC pesticides with no residual chromatographic interferences from sample lipids (Figure 1). Capsaicin, a component of black pepper, was detected in the GPC extracts but did not interfere with pesticide detection using this detector.

References

(1) FDA. Pesticide Analytical Manual, 1 Sec. 302, 303, (2002).
(2) EPA. Test Methods for Evaluating Solid Waste, Physical/Chemical Methods; Method 3640A (1994).

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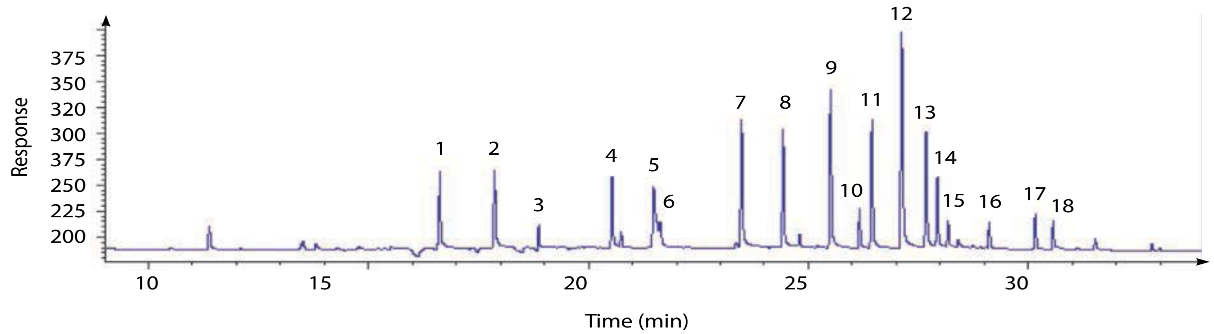

Figure 1: GC/XSD chromatogram of black pepper extracts following GPC clean-up. The sample was fortified with 1 ppm of 18 OC pesticides. 1 = α-BHC; 2 = β-BHC; 3 = γ-BHC; 4 = δ-BHC; 5 = Aldrin; 6 = Heptachlor; 7 = Heptachlor epoxide; 8 = Endosulfan I; 9 =4,4′-DDE; 10 = Dieldrin; 11 = Endrin; 12 = Endosulfan II; 13 = 4,4′-DDD; 14 = Endrin aldehyde; 15 = Endosulfan sulfate; 16 = 4,4′-DDT; 17 = Endrin ketone; 18 = Methoxychlor.

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Calibrating Gasoline Range Organics (GROs) Using an Automated Dilution Scheme with the Atomx Concentrator/ Multi-Matrix Autosampler

Tyler Trent and Nathan Valentine, Teledyne Tekmar

In developing the Atomx automated sample prep system, Teledyne Tekmar integrated both a P&T concentrator and a multi-matrix autosampler into a single platform system that can be used in the analysis of soil, water, and soils requiring methanol extraction. This "all-in-one" setup has allowed for greatly increased throughput and efficiency through the built in features it provides.

In this study, the sample syringe dilution feature was used to generate a calibration curve from a single stock solution for a subset of EPA method 8260B compounds (1). This study not only validates the capability to perform accurate and precise dilutions, but also removes the risk of errors that can occur from manually preparing multiple standards. Lastly, it shows that a considerable amount of labor time can be saved when using this feature.

Table I: MDL and calibration %RSD for target and surrogate compounds

Compound	MDL	Calibration % RSD
MTBE	0.73	4.76
Dibromofluoromethane (surr)	0.72	4.47
Benzene	0.61	4.59
Toluene-d8 (Surr)	0.66	8.02
Toluene	0.60	5.4
Ethylbenzene	0.55	7.58
M&P Xylene	1.11	7.44
Ortho Xylene	0.58	6.46
BFB (Surr)	0.52	11.69
1,3,5-Trimethylbenzene	0.57	10.08
1,2,4-Trimethlybenzene	0.53	8.22
Naphthalene	0.53	6.99

Experimental/ Instrument Conditions

The Atomx, equipped with a #9 adsorbent trap, an Agilent 7890A GC, and 5975C inert XL MSD were used for this analysis. Column: Restek Rtx-624 20 m × 18 mm ID × 1 µm, Constant flow 1.2 mL/min; Oven: 35 °C for 4 min, 16 °C to 85 °C for 0 min, 30 °C/min to 210 °C for 3 min, 14.29 min runtime. MS: 35–300 *m/z*.

Calibration and Minimum Detection Limits

A 100 ppb working calibration standard containing the target and surrogate compounds (PVOC/GRO mix) was prepared in 500 mL of deionized water. After transferring the standard to six VOA vials, a calibration range of 2–100 ppb was achieved using the following dilution volumes: 1:1= 5 mL, 1:2= 2.5 mL, 1:5= 1 mL, 1:10= 500 µL, 1:25= 200 µL, and 1:50= 100 µL.

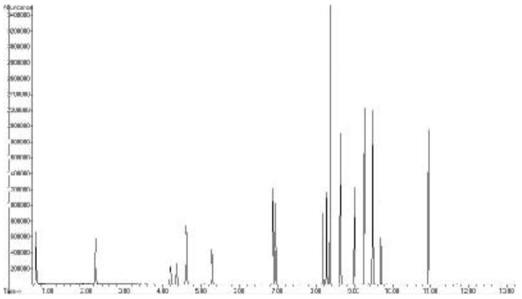

Figure 1: Static experimental results and TIC of 50 ppb GRO calibration standard.

All dilutions were automatically brought to a 5 mL final volume with deioninzed water using the instrument supplied water reservoir. MDLs were generated at 1 ppb using the 1:100 dilution. A 125 ppm internal standard (IS) was prepared in methanol and injected in 1 µL volumes using the internal standard addition feature, providing a constant 25 ppb final concentration.

Agilent Chemstation software was used to process the calibration data. The relative response factors (RRF) of all the analytes were evaluated for %RSD. %RSD met EPA method 8260B performance criteria.

Conclusions

Teledyne Tekmar's Atomx continues to improve efficiency and throughput. By automating the calibration process using the sample dilution feature in conjunction with the automated internal standard addition feature, the calibration curve met the performance criteria. Using the Atomx, only one stock standard solution needs to be made, rather than an entire series. Switching to an automated calibration allows for the same performance, but with the benefit of less prep time. This automated calibration, as well as the other enhancement tools, aid the user in both productivity and cost savings.

Reference

(1) USEPA Method 8260B Volatile Organic Compounds by Gas Chromatography/Mass Spectrometry (GC/MS) Revision 2, December 1996.

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Agilent Technologies

Fast, Low Pressure Analysis of Food and Beverage Additives Using Agilent Poroshell 120

Anne E. Mack and William J. Long, Agilent Technologies, Inc.

Agilent Poroshell 120, a superficially porous particle column, offers resolution and speed similar to columns packed with 1.8-µm totally porous particles, without generating high back pressure. The high efficiency of Poroshell 120 particles is due to short mass transfer distance and substantially narrower particle size distribution. The larger 2.7-µm Poroshell 120 particles generate very low back pressure, about 40–60% of sub-2-µm totally porous particles. This allows the columns to run at faster flow rates without exceeding HPLC pressures. Poroshell 120 columns with 2-µm frits are more forgiving with dirty samples than 1.8-µm columns, providing a more seamless method transfer from traditional 5-µm columns.

A group of 11 non-nutritive food and beverage additives are used to demonstrate this method translation. These compounds include preservatives, artificial sweeteners, an energy supplement, and a flavoring agent.

Results and Discussion

Figure 1 shows an overlay of the original 250-mm, 5-µm method compared to the new 100-mm Poroshell 120 method. Flow rate for the Poroshell 120 column is adjusted to compensate for the smaller column ID and particle size, while gradient time is scaled proportionally. Analysis time is reduced by 80% — from 13.1 to 2.6 min — with the post run time reduced from 7 to 1.8 min. Solvent and mobile phase consumption are also reduced by more than 80%. Resolution of critical pair (dehydroacetic acid and methylparaben) improved from 1.79 to 3.01 on Poroshell 120, compared to the longer 5-µm Eclipse Plus method. Note in Figure 1, that the last peak on Poroshell 120 elutes at approximately the same time as the first peak on 5-µm Eclipse Plus.

When high throughput is most important, and HPLC system limits allow, flow rate can be increased with little loss in chromatographic quality, as shown in Figure 2. Flow rate on the 3.0 × 100-mm Poroshell 120 column can be increased from 0.851 to 1.489 mL/min to further reduce run time by 40% in under 600 bar. This achieves little loss in resolution of the critical pair and has minimal effect on conditional peak capacity (n_c). The result is a baseline separation of 11 compounds in 1.5 min, in under 600 bar.

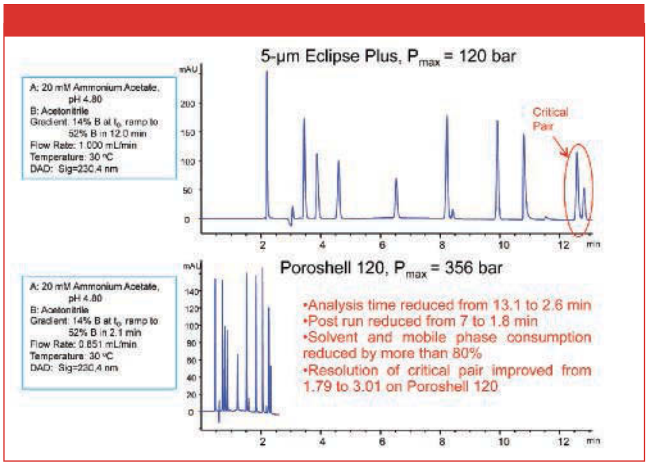

Figure 1: Fast, low pressure analysis; all 11 peaks on Poroshell 120 are resolved by the time the first peak elutes on the original 5-µm Eclipse plus method.

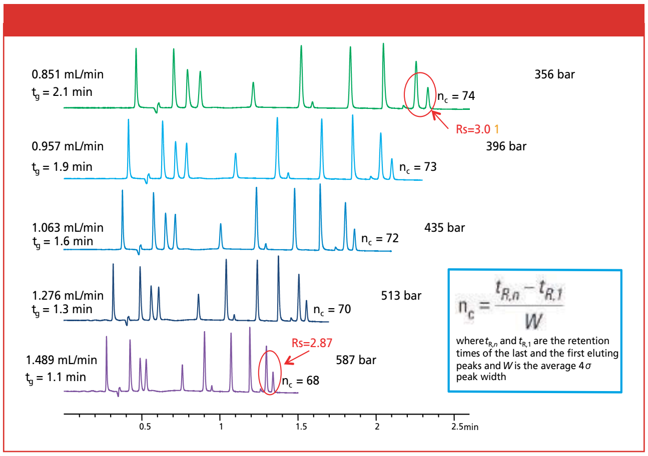

Figure 2: Flow rate on Poroshell 120 can be increased to further enhance productivity with minimal impact on resolution and conditional peak capacity (n_c).

Conclusion

Due to the similar selectivity between Poroshell 120 EC-C18 and Eclipse Plus C18, methods can easily be transferred from older Eclipse Plus C18 columns to new Poroshell 120 EC-C18 columns. Methods developed on Poroshell 120 can be transferred to 600 bar instruments for even greater productivity gains. More details of this analysis are found on the Agilent website; search for the full-length version of this application note using publication #5990-6082EN.

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Pesticide Residues Screening in Food Samples Using High Resolution and Accurate Mass LC–MS-MS

André Schreiber, AB SCIEX

This paper describes the use of the AB SCIEX TripleTOF™ 5600 LC–MS-MS system for targeted and nontargeted screening for pesticide residue in food samples. High resolution and accurate mass MS information was used to quantify compounds and MS-MS spectra were used to identify compounds based on mass spectral library searching.

Experimental Conditions

Fruit and vegetable samples were extracted using a QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) procedure (1, 2). Extracts were diluted 10× to minimize possible matrix effects. UHPLC separation was achieved using a Shimadzu UFLCXR system with a Restek Ultra II Aqueous C18 2.2 µm (100 × 2.1 mm) column and a gradient of water and methanol with ammonium formate buffer. The AB SCIEX TripleTOF™ 5600 LC–MS-MS equipped with Turbo V™ source and electrospray ionization (ESI) probe was used. Full scan MS spectra were acquired over a mass range of 100–1000 Da with an accumulation time of 100 ms. Information dependent acquisition (IDA) was used to collect up to 30 MS-MS spectra with an accumulation time of 25 ms for compound identification based on mass spectral library search.

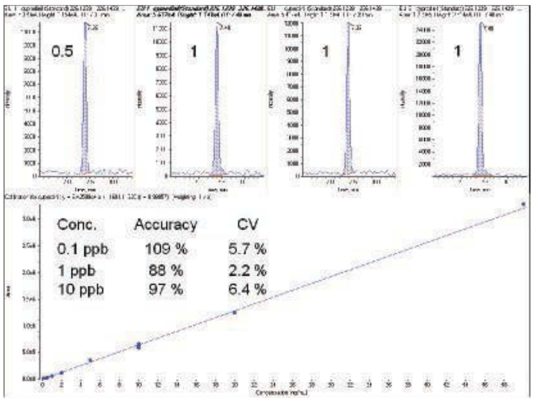
Results and Discussion

Targeted pesticides were quantified with high selectivity in fruit extracts from extracted ion chromatograms using an extraction window of ±10 mDa. The sensitivity of the developed method was sufficient to allow 10× extract dilution to minimize possible matrix effects, such as ion suppression, and still reach the target limit of quantitation for all analytes of less than 10 ppb (µg/kg).

An example of positive findings of pesticide in a red bell pepper sample is shown in Figure 2. The accurate mass MS and MS-MS information was used to identify Acephate, Carbaryl, Clothianidin, Dimethoate, Imidacloprid, Methamidophos, Metalaxyl, Myclobutanil, Omethoate, Propamocarb, Spinosyn A, Spinosyn D, and Thiamethoxam with high confidence. MS-MS library searching resulted in FIT values greater than 80%.

Summary

The new AB SCIEX TripleTOF™ 5600 LC–MS-MS system was used to screen for, quantify, and identify pesticide residues in food extracts. High sensitivity of detection allowed dilution of QuEChERS extracts to minimize possible matrix effects. Accurate mass MS-MS spectra were searched against an online LC–MS-MS library of pesticides to confirm the identity of quantified analytes (3).



Conc.	Accuracy	CV
0.1 ppb	109 %	5.7 %
1 ppb	88 %	2.2 %
10 ppb	97 %	6.4 %

Figure 1: Extracted ion chromatograms, reproducibility, and calibration curve (0.05 to 50 ppb of Cyprodinil).

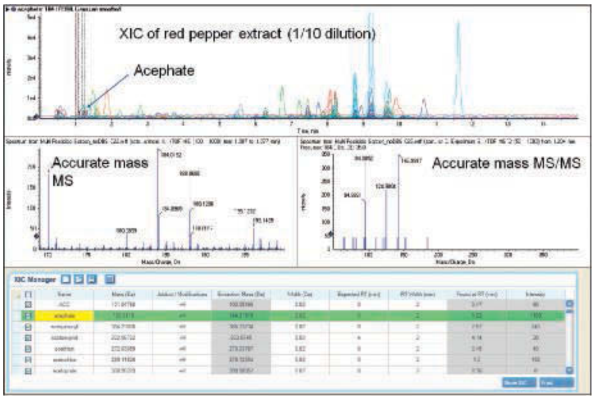


Figure 2: Identification of Acephate in a red bell pepper sample using accurate mass and high resolution MS and MS-MS data.

References

(1) M. Anastassiades, et al., *J. AOAC Int.* **86**, 412–431 (2003).
(2) EN 15662:2007: (2007).
(3) www.alanwood.net/pesticides.

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Determination of Furan in Food by Gas Chromatography–Mass Spectrometry and Headspace Sampling

Padmaja Prabhu, PerkinElmer, Inc.

Furan is naturally occurring at low levels in many foods and drinks (1). Based on studies in laboratory animals, furan consumption has been classified by IARC as possibly carcinogenic to humans. The primary source of furan in food is considered to be thermal degradation of carbohydrates, such as glucose, lactose, and fructose.

This application note demonstrates a rapid method for the identification and quantification of furan in food samples using gas chromatography (GC), headspace sampling (HS), and mass spectrometry (MS). In addition to method optimization and standard analysis we have analyzed a number of food samples for furan.

The PerkinElmer® Clarus® 680 Gas Chromatograph, Clarus 600 C Mass Spectrometer, and a TurboMatrix™ HS-40 system was used for this application. The separation was achieved on an Elite™-624 column ramped from 40 °C to 250 °C over 20 min. The mass spectrometer was scanned from *m/z* 35–150 with 5 scans per s.

Headspace was chosen as the sample introduction technique because furan can easily be partitioned from liquid matrices, and headspace facilitates automated extraction of furan without the instrument contacting the sample matrix. Caution must be taken when setting the vial oven temperature; a high temperature can result in furan formation in the sample during analysis. To reduce this risk, the method presented here uses a temperature of 60 °C.

The GC–MS was calibrated across the range of 1.0 to 40 ng/mL, each calibration point was run in triplicate to demonstrate the precision of the system, the average %RSD across the range was 4.3%. The coefficient of determination for a line of linear regression across the range of 1.0 to 40 ng/mL was 0.9997.

Sample Preparation

Samples were collected from the local food market. These samples included coffee, milk, canned foods, sauces, peanut butter, and apple juice. 10 mL of sample was transferred into a headspace vial; 4 g of NaCl was added to it. Milk and other viscous samples were diluted with water (1:2 or 1:4). The semi-solid samples were ground and 5 g of sample was added to the headspace vials with 5 mL of saturated salt (NaCl) solution. Coffee powder was dissolved following directions on the package, and then treated like a nonviscous liquid sample.

The recovery of the method was tested with the analysis of the brewed coffee sample spiked at three different levels: 2, 5, and 10 µg/L. The measured amount was 2.03, 5.44, and 9.54 µg/L demonstrating the technique to be quantitative.

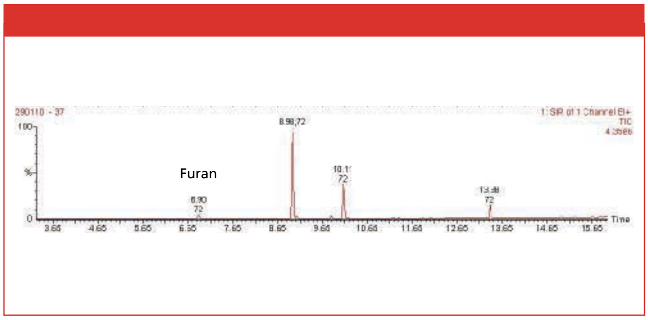

Figure 1: Chromatogram furan peak in coffee sample.

Results

Eight samples of common beverages were analyzed using the HS-GC–MS method developed here. Brewed coffee was demonstrated to have the highest levels of furan at 250 µg/L.

Conclusion

This application provides a method for the determination of furan in beverages using headspace sample introduction. Headspace-GC is fast, reliable, and can be used for the quantification of furans in common beverages. The internal standard calibration of furan across 1–40 µg/L fit responded linearly. Beverages were analyzed and the level of furan was determined. Furan was identified by both the retention time and the MS fragmentation pattern. This method was validated at several levels on coffee matrix recovery, with values between 95–101%.

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Analysis of Aflatoxins in Ground Peanuts and Peanut Butter by HPLC with Photochemical Derivatization

Maria Ofitserova, Pickering Laboratories, Inc.

Aflatoxins occur naturally in peanuts, cottonseed, corn, and dried chili pepper, as well as many mixed or processed foods and feeds. A simple, sensitive, and robust HPLC method with post-column photochemical derivatization and fluorescence detection was used to analyze Aflatoxins B1, B2, G1, and G2 in peanut butter and ground peanuts. The UVE™ (LCTech, Germany) photochemical reactor requires no additional reagents and is easy to install between the HPLC column and FLD detector.

The extracts were cleaned by using AflaCLEAN™ (LCTech, Germany) Immunoaffinity columns that offer high recoveries for Aflatoxins even with the most challenging matrices. No matrix interferences were present after the sample clean up. AcceCLEAN™ (LCTech, Germany), an automated system that processes three samples at a time, was used for handling the Immunoaffinity columns. Instrumentation allows for quick and efficient detection of Aflatoxins at low ppb level.

Sample Preparation

Blend 20 g of sample at high speed with extraction solution (100 mL of methanol/water 80/20, 50 mL of Hexane, 2 g NaCl) and filter through fluted paper. Dilute 14 mL of aqueous layer with 86 mL of PBS buffer (pH 7.2), filter and apply 11 mL of solution on AflaCLEAN™ Immunoafinity column. The toxins are eluted with 2 mL of methanol and analyzed using HPLC with photochemical derivatization.

Table I: Peanut butter (NIST SRM2387) – control sample

	Aflatoxin B1	Aflatoxin B2	Total Aflatoxins
Target value, ng/g	4.2 ± 0.9	0.7 ± 0.3	5.0 ± 0.5
Found value, ng/g	4.51	0.86	5.37
RSD_r, %, n=6	6.9	7.2	5.9

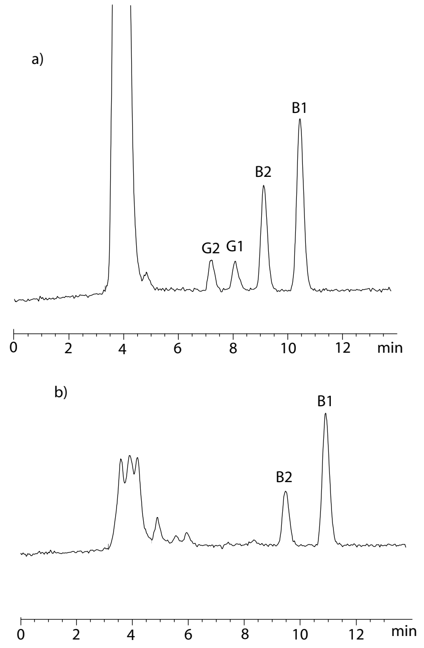

Figure 1: Chromatograms of a) naturally contaminated peanuts; b) NIST SRM2387 peanut butter sample. All samples are part of NIST Exercise E (April 2010).

Injection volume: 30 µL
FLD Excitation 365 nm, Emission 430 nm

Results and Discussion

The 6-point calibration curves were build in a range of 11.49–0.24 ppb for B1, 3.29–0.07 ppb for B2 and G2, 9.85–0.21 ppb for G1 with R2 exceeding 0.999.

Table II: Naturally contaminated ground peanuts

	Aflatoxin B1	Aflatoxin B2	Aflatoxin G1	Aflatoxin G2	Total Aflatoxins
Contamination Level, ng/g	5.93	1.64	2.11	1.41	11.09
RSD_r, %, n=6	7.1	10.3	18.4	16.6	3.1

Analytical Method

Analytical column: Mycotox™ (Pickering Laboratories, Inc), C18, 4.6 × 250 mm
HPLC eluent: Sodium Phosphate buffer (Cat #1700-1108)/methanol/acetonitrile (57/28/15)
Flow rate: 1 mL/min

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Determination of Furan in Coffee by Static and Dynamic Headspace-GC–MS

Roger Bardsley, Teledyne Tekmar

Coffees have aroma and taste based on where the beans are grown, roasting techniques, and flavor additives. One component of the bean responsible for the aroma and some of the taste are volatile organic compounds (VOCs). Furan is one VOC that has recently been investigated in food (1).

An FDA method for furan in food uses static headspace and requires 30 min of incubation time at 60 °C (2). This paper presents data comparing the determination of furan in dry coffee by both static and dynamic headspace techniques. In the dynamic headspace method analytes are swept and concentrated on a sorbent trap that greatly increases compound sensitivity and decreases sample preparation time.

Experimental- Instrument Conditions

Coffee samples of 5 grams were weighed into vials and crimp capped. Both the static and dynamic methods maintained the sample at 60 °C with the valve oven and transfer line held at 100 °C. The static method followed the FDA's 30 min sample heating time (2). The vial was then pressurized to 10 psi and the loop filled to 5 psi. The loop contents were transfered to the Thermo Focus DSQ™II GC–MS for detection and quantitation with EnviroLab Forms™ 3.0 mass spectrometry software.

The dynamic method trapped the VOCs on a proprietary #9 trap by sweeping the sample for 15 min at 50 mL/min. The VOCs were rapidly desorbed from the trap at 250 °C and detected with the Thermo Focus DSQ™II GC–MS.

A Restek Rtx®-VMS, 20 m by 0.18 mm internal diameter column with a 1.0 m film thickness was used for both static and dynamic headspace samples. The column had a constant flow of 1.0 mL/min helium and an oven program of 35 °C for 4 min, then 16 °C/min to 80 °C, then 30 °C/min to 230 °C with a final hold of 3 min. The inlet was held at 220 °C with a split ratio of 80:1. The mass spectrometer was scanned from 35 *m/z* to 300 *m/z* with the source at 230 °C and a scan rate of 1492.11.

Results

The peak area of the 68 *m/z* ion specific for furan with both the static and the dynamic methods were compared. Table I is the furan peak area data from the mass 68 *m/z* chromatogram in Figure 1.

Table I: Furan peak area data from the mass 68 *m/z* chromatogram in Figure 1

Sample Temp	Static	Dynamic
60 °C	14664294	495186280

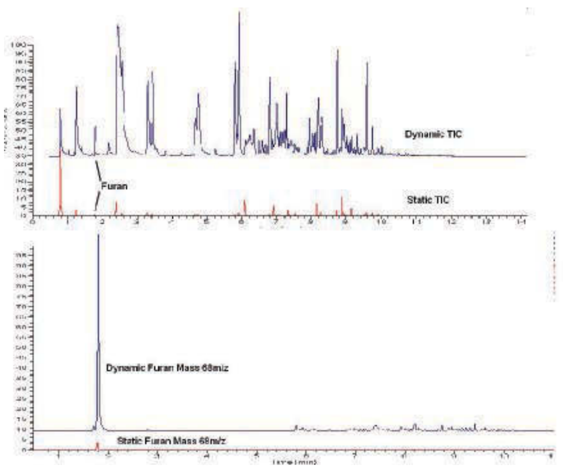

Figure 1: Comparison of the Dynamic (Blue) and Static (Red) total ion chromatogram (TIC) (top) and the specific mass Ion 68 *m/z* for Furan (bottom).

Conclusions

The dynamic technique detects approximately 30 times more furan from the same size sample when compared to a static method. In addition the dynamic option shortens the analysis time to 15 min.

The HT3 system offers food scientists a powerful analysis tool that can perform both static and dynamic techniques in a single package thus optimizing performance as well as efficiency.

References

(1) Report of the Scientific Panel on Contaminants in the Food Chain on Provisional Findings on Furan in Food, Adopted on 7 December 2004, *The EFSA Journal,* **137**, 1–20, http://www.efsa.eu.int (2004).

(2) Determination of Furan in Foods, US Food and Drug Administration, October 27, 2006.

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GC Column Selection for the Analysis of Volatiles and Higher Alcohols in Whisky

Luisa Pereira, Thermo Fisher Scientific

Whisky is a complex and variable material, the quality of which is affected by the raw materials and processing. The overall flavor and quality results from the combination of levels of higher alcohols and other flavor components generated as a result of the fermentation process.

There are two basic types of whisky, pure malt and grain. Single malt is considered the best quality with premium brands claiming individual, unique flavors. Grain whisky is not commonly available and many commercial whisky brands are a blend of malt and grain being labelled "blended whisky."

The compounds of interest in the analysis of whisky quality and for whisky differentiation are:

Acetaldehyde, Methanol, n-Propanol, isobutanol (used for brand differentiation), Ethyl acetate, and 3-Methylbutanol and 2-Methylbutanol (not present in grain whiskies, indicate quality of blend).

Whisky analysis has traditionally been carried out using a polar, wax type column, however this has some limitations. Firstly, the very volatile components, especially those eluting prior to the ethanol peak are difficult to separate on a wax column due to their short residence time on the stationary phase. Secondly, the wax phase in not able to resolve 2- and 3-methylbutanol (amyl alcohols). The use of a nonpolar phase enables separation of the 2 critical amyl alcohols as well as shifting the elution of some volatiles to positions where they are easily measured.

Materials & Methods

Equipment

A Thermo Scientific TRACE™ GC ULTRA, fitted with a FID detector, split/splitless injector and Helium carrier gas was used. All injections were 1 µL dispensed by a Thermo Scientific TriPlus™ autosampler.

Columns

Thermo Scientific TRACE TR-WaxMS 30 m × 0.25 mm × 0.25 µm; Thermo Scientific TRACE TR-V1 30 m × 0.25 mm × 1.4 µm.

The method was optimised for each column (1).

Samples

A set of standards was run on each column to establish the position of each alcohol peak. Whisky samples (commercially available whiskies) were injected neat.

Results

Figure 1 shows the analysis of The Balvenie® 10-year-old Scottish whisky, a Speyside single malt brand on the two columns.

The profile for the polar TR-WaxMS column shows the close elution of ethyl acetate and acetaldehyde diethyl acetal (peaks 5 and 8) and the two amyl alcohols (peaks 9 and 10) are unresolved.

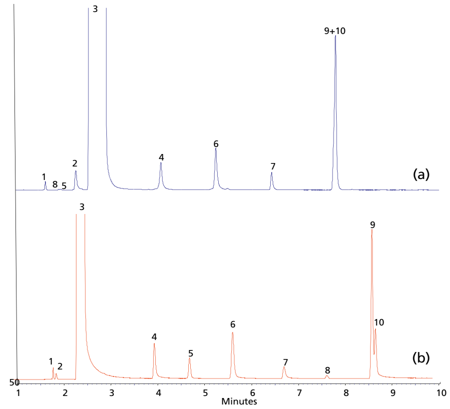

Figure 1: Speyside whisky analysis on (a) TR WaxMS and (b) TR V1. Compounds: 1. Acetaldehyde, 2. Methanol, 3. Ethanol, 4. n-Propanol, 5. Ethyl acetate, 6. Isobutanol, 7. n-Butanol, 8. Acetal, 9. 3-Methylbutanol, 10. 2-Methylbutanol. Experimental conditions in reference 2.

The polar TR-V1 column gives a better separation of all the required compounds. The 3-methyl butanol and 2-methyl butanol are separated, making it possible to better assess the quality of the whisky. Some tailing occurs but statistical analysis of 10 injections confirmed good reproducibility for measured amyl alcohols.

Conclusions

The advantage of the wax type column is better peak shape and being well establish in this type of analysis. The disadvantage is the co-elution of the low polarity compounds prior to the ethanol peak which may be required as a measurement of the wash quality. The TR-V1 column (cyanopropylphenyl) gave better resolution for all compounds. This column is also suitable for detailed analysis including characteristic flavor and phenolic compounds.

References

(1) Whisky, Technology, Production and Marketing, Pub. Academic Press. ISBN 0-12-669202-5.
(2) J. Warden, L. Pereira, Thermo Scientific Poster PO20373_E02/07.

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Extraction of Polycyclic Aromatic Hydrocarbons (PAH) from Fish Using the QuEChERS Approach*

Don Shelly and Craig A. Perman, UCT, LLC

The QuEChERS approach is used for the extraction of 16 PAH compounds from fish with analysis by LC using fluorescence detection.

Instruments

Extracts were analyzed using a Shimadzu LC system (Shimadzu Corporation, Kyoto, Japan) equipped with a LC-20AB pump, DGU-20AS degasser, and a fluorescence RF-10AXL (FLD) detector.

1) Sample Preparation

a) Transfer 5 g homogenized fish to a 50 mL centrifuge tube
b) Add 10 mL acetonitrile
c) Shake vigorously
d) Add the contents of UCT ECMSSC50CT-MP pouch containing 4 g $MgSO_4$ & 1 g NaCl to the tube
e) Immediately vortex the mixture for 3 min
f) Centrifuge the tube for 3 min at 3400 rpm
g) The supernatant is ready for clean-up

2) Sample Cleanup

a) Add 3 mL of the supernatant to centrifuge tube UCT ECMPSC1815CT (900 mg $MgSO_4$, 300 mg PSA, and 150 mg endcapped C18)
b) Shake for 1 min
c) Centrifuge for 1 min at 3400 rpm
d) Filter supernatant using 0.20 µm PTFE membrane filter
e) Sample is ready for analysis

3) Analysis Conditions

a) Separation is performed using a C18 column (CC 150/4 Nucleosil 100-5 C18 PAH, 150 × 4.0 mm; 5 µm particle size; Macherey-Nagel, Duren, Germany) or equivalent. Maintained at room temperature.
b) Inject 15 µL
c) Mobile phase: Initial 50% ACN and 50% water
d) Use a linear gradient to 100% ACN over 15 min
e) Flow rate is 0.8 mL/min
f) Hold for 13 min
g) Initial conditions are achieved in 1 min and maintained for 6 min before next run
h) Total run time is 40 min
i) Fluorescence Wavelength Program: Each compound is detected at its optimum excitation/emission pair.
315/260 nm naphthalene, acenaphthene, fluorene
366/260 nm phenanthrene
430/260 nm anthracene, fluoranthene, pyrene, benz(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, dibenz(a,h)anthracene, benzo(g,h,i)perylene and dibenzo(a,l)pyrene
505/290 nm (indeno(1,2,3-cd)pyrene

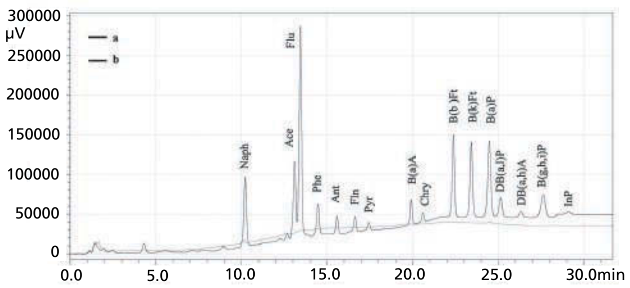

Figure 1: Overlay LC-FLD chromatograms of a matrix-matched standard PAHs mixture (a) containing 16 PAHs (Naph-100.3 µg/L, Ace-100.3 µg/L, Flu-19.8 µg/L, Phe-10.0 µg/L, Ant-10.0 µg/L, Fln-19.7 µg/L, Pyr-9.69 µg/L, B(a)A-9.84 µg/L, Chry-9.91 µg/L, B(b)Ft-20.1 µg/L, B(k)Ft-9.98 µg/L, B(a)P-10.0 µg/L, DB(a,l)P-20.0 µg/L, DB(a,h)A-20.0 µg/L, B(g,h,i)P-19.9 µg/L and InP-10.0 µg/L) and (b) a blank horse mackerel extract.

Conclusion

This QuEChERS approach allows efficient extraction of PAHs from multiple homogenized fish samples in 20 min or less while providing a clean extract. Sample clean-up prevents instrument downtime.

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*Adapted from: Ramalhosa, Maria Joao, et. al., "Analysis of polycyclic aromatic hydrocarbons in fish: evaluation of a quick, easy, cheap, effective, rugged, and safe extraction method," *J. Sep. Sci.*, **32**, 3529–3538 (2009).

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Extending the Range of High Temperature Simulated Distillation (ASTM D7169) to C120 Using New Zebron ZB-1XT SimDist GC Columns

Kory Kelly, Chris Fernandez, and Ngoc Nguyen, Phenomenex, Inc.

An extension of ASTM D7169 is shown using new Zebron ZB-1XT SimDist metal GC columns. This method extends the analysis to C120 while easily surpassing method requirements including peak shape and efficiency.

When refining petroleum, it is necessary to determine the composition of raw materials as well as finished products to ensure that reactants are being processed effectively and that the highest possible efficiency is maintained. A sample's composition is determined by creating a boiling point distribution profile that correlates to the hydrocarbon distribution within that sample. Refineries continually try to extend the range of analysis to include wider boiling points because that gives more information about their materials and allows them to better plan processing.

Typically, crude oil and heavier range products contain a lot of high molecular weight compounds that require high boiling point simulated distillations. ASTM D7169 is such a method. It extends the range of the previous method ASTM D6352 from C90 to C100.

The method presented here extends that range even further by going to C120, while still surpassing the performance requirements of the current ASTM D7169 method.

Experimental Conditions

A Zebron ZB-1 SimDist 5 m × 0.53 mm × 0.09 µm column was used with an oven program of 35 °C to 430 °C at 9 °C/min for 10 min. A 1.0 µL injection of hydrocarbon standards with POLYWAX 1000 in carbon disulfide was made on-column with FID detection at 450 °C. Helium carrier gas was used at 34.0 mL/min.

Results

The ASTM method D7169 has system performance requirements for resolution, peak skew, and temperature program. Resolution between C50 and C52 must be between 1.8 and 4.0. The skew of any peak between C12 to C24 needs to be between 0.8 and 1.2. The last compound that can be included in the analysis needs to elute on the temperature program.

Using a Zebron ZB-1XT SimDist GC column, all method requirements were met. The minimum resolution requirement was surpassed with a value of 2.55. The average peak skew of all peaks from C12–C24 had near perfect symmetry of 0.99 and covered a narrow range from 0.94 to 1.06. The last peak that elutes on the temperature program has been extended to include the very high boiling C120 (Figure 1).

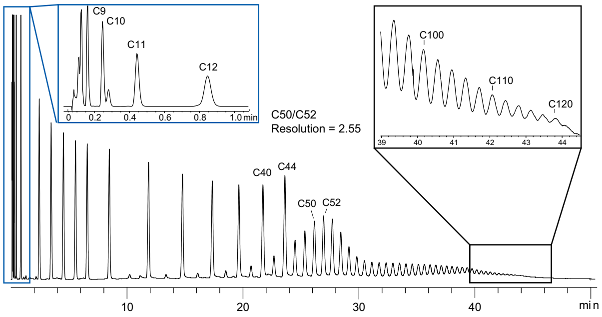

Figure 1: ASTM D7169 standard using a Zebron ZB-1XT SimDist column of 5 m × 0.53 mm × 0.09 µm dimensions with elution of C120 on the temperature ramp.

Conclusions

The new Zebron ZB-1XT SimDist metal columns show excellent performance for high temperature simulated distillation methods like ASTM D7169. The requirements of the method were easily met while still allowing for an extended range of hydrocarbons up to C120 program without going above 430 °C. The ease of meeting system requirements means less downtime for maintenance. The extended range of compounds from this analysis gives a refinery a greater understanding of its samples. This knowledge can aid in process planning and increase product yields which directly affect the bottom line.

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Achieving Enantiomeric Separation of Anti-Ulcer Drugs Using Lux Cellulose-4

Scott Krepich and Christopher Fernandez, Phenomenex Inc.

Polysaccharide-based chiral stationary phases (CSPs) are often utilized for a broad range of enantiomeric chromatographic separations due to their wide chiral recognition ability. A new chiral column with unique selectivity, Lux Cellulose-4, was used to achieve good separation of anti-ulcer drugs under reversed phase conditions.

In many cases in pharmaceutical drug development, one of the two enantiomers of a specific compound is the active pharmaceutical ingredient (API) and the other enantomer is either inert or toxic. Researchers look to chiral chromatography to purify these enantiomers and often a unique chiral selectivity is required to separate specific classes of compounds. Lux Cellulose-4 is a new unique chiral stationary phase using a tris (4-chloro-3-methylphenylcarbamate) derivative of cellulose (Figure 1). Here, its chiral recognition abilities under reversed phase conditions were demonstrated for the anti-ulcer drugs; lansoprazole and omeprazole.

Materials and Methods

All chemicals were obtained from Sigma Chemicals (St. Louis, MO) and solvents were purchased from EMD (San Diego, CA). All compounds were analyzed on an Agilent 1100 HPLC with an autosampler, column oven, variable wavelength detector, and data was collected using Chemstation software. The column used for all separations was a Lux 5 µm Cellulose-4 250 × 4.6 mm (Phenomenex, Torrance, CA) and flow rate was 1 mL/min. Separations were monitored at 220 nm. Mobile phase composition was 20 mM ammonium bicarbonate/0.1% diethylamine with varying amounts of acetonitrile (70:30 for lansoprazole and 80:20 for omeprazole).

Results and Conclusions

While some trends are often seen between classes of compounds, chiral separations are usually very compound and phase specific with no set chromatographic rules for selectivity. Polysaccharide-based chiral stationary phases tend to have the most widespread applicability for a variety of compounds, but still different types of polysaccharide columns and mobile phase conditions must be screened to achieve maximal separation of enantiomers. For the anti-ulcer compounds, lansoprazole and omeprazole, the maximum resolution and best overall chromatography was achieved under reversed phase conditions on Lux Cellulose-4 (data not shown). After the initial screen of chiral stationary phases using generic conditions, mobile phase was optimized to increase resolution of the enantiomers. Chromatograms of the optimized conditions for the enantiomeric separation of each compound are show in Figure 2. In the case of lansoprazole, the optimal mobile phase condition was 70:30 acetonitrile: buffer, and for omeprazole 80:20 acetonitrile: buffer on the Lux Cellulose-4 column.

These results show an example of the utility of the Lux Cellulose-4 media as a unique chiral selective phase for the separation of these anti-ulcer enantiomers.

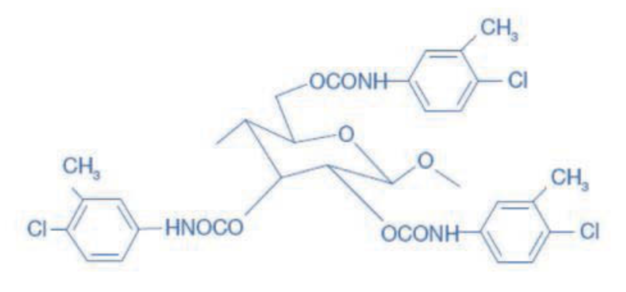

Figure 1: Chemical structure of the Lux Cellulose-4 polysaccharide-based chiral phase.

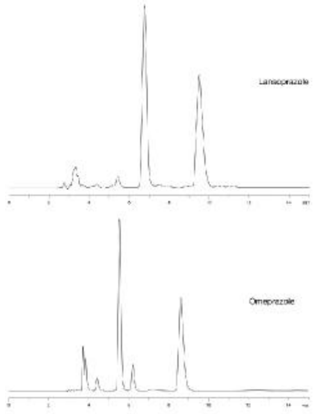

Figure 2: Separation of anti-ulcer enantiomers lansoprazole and omeprazole on the Lux 5µ Cellulose-4 chiral column. Note the good separation of the different enantiomers of each compound. Lux Cellulose-4 provides a unique selectivity for different chiral compounds.

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Polymer Char

Microstructure Characterization of Polyolefins by Automated Cross-Fractionation Chromatography (CFC)

Alberto Ortín, Benjamín Monrabal, and Pilar del Hierro, Polymer Char

Complete high resolution cross-fractionation of Polyolefins in the molar mass and composition dimensions is achieved with a new benchtop CFC analytical instrument. This method has no information loss on the polymer microstructure heterogeneity. Operational details and examples of real data are presented, highlighting the results as contour and surface plots.

Polyolefin technology has made remarkable progress in the last decades through the development of new technologies which allow more control of the polymer structures by the product designers, so high value products are produced with varying and increasing microstructure complexity. Polymer characterization provides information about the molecular structure, which builds a bridge between polymer properties and polymerization conditions (1).

In most industrial polyolefins there are essentially two molecular parameters of interest: the MMD (molar mass distribution) and the CCD (chemical composition distribution), which are manipulated to impart desired properties to the final product. The MMD tells how much material of a certain molar mass is present. The CCD tells how much material of a certain composition, or comonomer content, is present.

Separate information on MMD and CCD, which are routinely determined by SEC/GPC (size exclusion chromatography, gel permeation chromatography) and TREF (temperature rising elution fractionation) or CRYSTAF (crystallization analysis fractionation) respectively, although important and in many cases sufficient, are not enough to fully characterize a Polyolefin resin and the full bivariate distribution is required (2, 3).

Hyphenated techniques, such as SEC-FTIR and TREF-LS that carry out the fractionation only along one molecular axis and determine some average of the other, have become popular in the last years, although they result in some information loss, the extent of which depends on the method employed and on the shape of the CCD × MMD surfaces of the materials (Figure 1) (1).

Thus, it is generally preferable to access the full CCD × MMD, which has been traditionally a challenging and time consuming task (4).

With the introduction by Polymer Char of the CFC automated analytical-scale instrument for polyolefin cross-fractionation, it is now possible to fully characterize complex comonomer/molar mass distributions on the order of hours instead of days and without the need of intermediate manual steps in a bench top instrument (5). Very high resolution can be achieved in both dimensions by using optimized analytical columns, analysis conditions, and sample sizes.

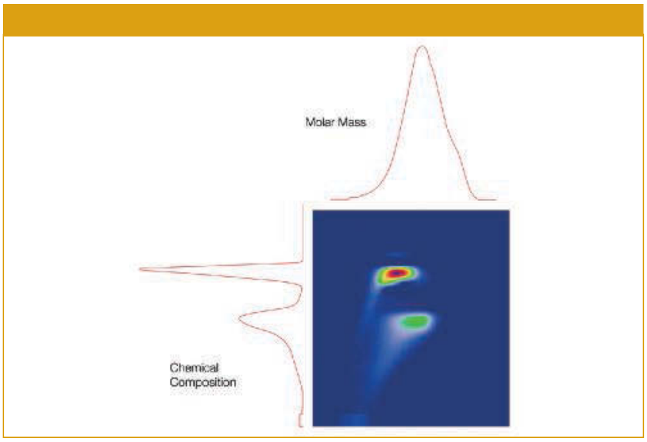

Figure 1: Contour plot of the CCD × MMD and individual distributions as measured by TREF and GPC/SEC.

Experimental

The instrument is based on a high resolution analytical TREF apparatus combined with a dedicated GPC/SEC columns oven, and equipped with five vessels for sample preparation so up to 5 samples can be analyzed sequentially. Polymer detection is made through an IR4 infrared detector for maximum sensitivity in polyolefin applications, which also provides excellent long term baseline stability.

The polymer sample is placed in solid form (40–200 mg) into a stainless steel vessel, where it is dissolved in a proper solvent, o-DCB or TCB, at temperatures of 140–160 °C for 60–90 min under stirring. The instrument works by loading the polymer solution into the TREF column where it is crystallized by cooling down the TREF oven and then eluting the fractions in step-wise temperature increments towards the GPC columns.

A series of chromatograms are collected at the different fractionation temperatures (Figure 2). Those chromatograms describe the molar mass distribution of every compositional fraction. The relative area of the individual chromatograms reflects the compositional heterogeneity, while their retention time and shape relates to the molar mass dimension.

Experimental conditions may be varied to optimize resolution and signal quality depending on the chemical composition distribution, by setting any number and arrangement of temperature fractionation points as required: evenly distributed in all the range every 2 °C or 3 °C for a typical Z-N LLDPE sample having broad CCD, or concentrating more fractionation points every 1 °C in a narrow range around 85–95 °C for HDPE which tends to have quite narrow composition distribution. Sample size is also optimized according to the fractionation conditions in order to prevent overloading of the SEC/GPC columns while keeping good enough signal from the IR detector.

An SEC/GPC columns calibration curve is established so that MMD and molar mass moments are calculated at every elution temperature. The relative weight fraction is obtained from the area under each chromatogram and it is combined with the individual fractions MMD to obtain the full CCD × MMD distribution for the sample. All the data processing and calibration is performed within a dedicated and comprehensive software package, which additionally provides the TREF profile and MMD for the whole sample.

Results and Discussion

A synthetic blend of two single site catalyst polyethylene-co-octene copolymers having different densities and molar masses was analyzed automatically using the CFC instrument. The full CCD × MMD is directly measured with high resolution in 12 h by elution of 24 TREF fractions into the GPC columns.

The bivariate distribution is presented in a tridimensional surface plot (Figure 3), which can be processed in several ways within the software. The two components are observed in the graph, which also reveals clearly their distinct position in the molar mass/chemical composition plane. In this case, more comonomer was added to the higher molar mass component of the blend, as highlighted by the lower temperature peak which appears at a higher value in the log M scale.

The TREF profile is recovered from the fractions relative weights using a smoothing fitting routine to obtain the differential weight fraction versus elution temperature.

The two dimensional data is also processed to obtain the molar mass distribution of the whole sample by integration over the temperature dimension providing all the distribution averages as well as overall polydispersity. Additionally, the software allows the integration of data across specific temperature ranges in order to study the MMD at different comonomer levels. The microstructural characterization of different types of polyolefin products can benefit from the automation of this powerful technique: ZN-LLDPE, polypropylene impact copolymers, dual reactor products, or polymer blends and also high density polyethylene with low comonomer levels.

The result of this cross-fractionation analysis can be presented as contour plots, which map the molecular structure of polyolefins (in terms of comonomer and molar mass). Families of related or competitive products are easily compared in this way (Figure 4), helping the product designer identify the relevant structural features and assisting to establish basic property-structure relationships.

Conclusions

A new integrated benchtop instrument is introduced, which enables the direct measurement of the CCD × MMD surface plot in polyolefins on the order of hours, and in a fully automated way. This method is capable of describing the resin microstructure without any information loss. Applications are found in all types of polyolefins when accurate knowledge of their microstructure is required.

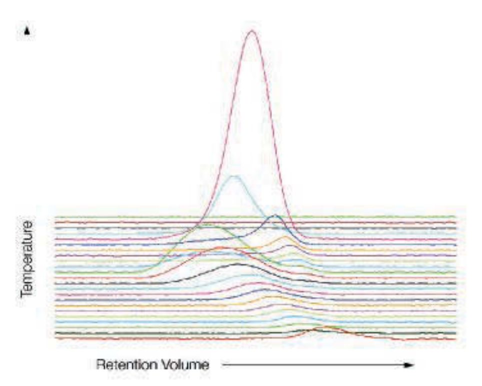

Figure 2: GPC/SEC chromatograms collected at 23 different fractionation temperatures, showing both the molar mass and the compositional heterogeneity of the analyzed PE blend.

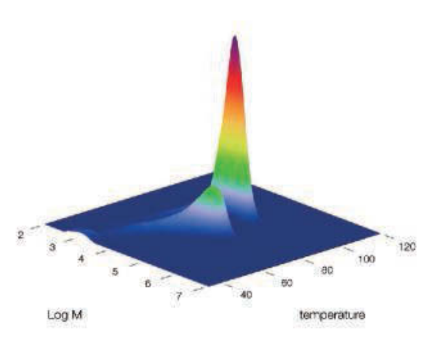

Figure 3: MMD × CCD surface plot of a two-component PE blend.

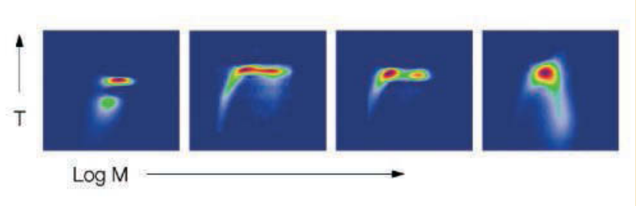

Figure 4: Contour plots of different PE products showing distinct microstructural features.

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Use of Alternative Solvents for Liquid Chromatographic Analysis of Cationic Surfactants

Mark Tracy and Xiaodong Liu, Dionex Corporation

Cationic surfactants are used as disinfectants, corrosion inhibitors, phase-transfer catalysts, wetting agents, dispersants, and even mobile phase modifiers in liquid chromatography. They face two difficulties for successful analysis by LC. First, they tend to have very poor peak shape on conventional reversed-phase columns due to strong ionic interactions with residual silanol groups. Second, many cationic surfactants have no useful UV chromophore. The first problem may be solved by the use of specialized columns. The second problem may be solved by the use of mass spectrometry, charged aerosol detection (CAD), or evaporative light scattering detection (ELSD) with a volatile mobile phase.

The Acclaim® Surfactant is a high-efficiency, silica-based, specialty reversed-phase column designed for the analysis of anionic, cationic, nonionic, and zwitterionic surfactants. Here, the authors compare acetonitrile and four less expensive and less toxic solvents — methanol, ethanol, isopropanol, and acetone for the analysis of cationic surfactants using ELSD.

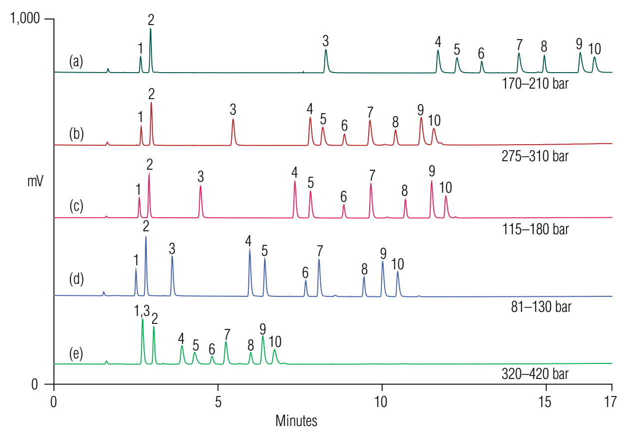

Figure 1: Alternative solvents for the analysis of cationic surfactants. Column: Acclaim Surfactant 3 µm, 3 × 150 mm; column temperature: 25 °C; flow rate: 0.425 mL/min; ELSD spray temperature: 55 °C; drift tube: 65 °C. Gradient program: initially at 35% organic solvent until 1.0 min; linear gradient to 75% organic until 13.5 min; maintained at 75% organic until 17.0 min. Mobile phases: buffer, 100 mM acetic acid in water, adjusted to pH 5.0 with ammonium hydroxide; organic solvent (a) methanol; (b) ethanol; (c) acetone; (d) acetonitrile; (e) isopropanol. Components approx. 75 µg/mL: 1. chloride; 2. bromide; 3. decyltrimethylammonium; 4. dodecyltrimethylammonium; 5. dodecylpyridinium; 6. dodecylbenzyldimethylammonium; 7. tetradecyltrimethylammonium; 8. tetradecylbenzyldimethylammonium; 9. hexadecyltrimethylammonium; 10. hexadecylpyridinium.

Experimental Conditions

UltiMate® HPG 3400 RS pump, WPS 3000 TRS sampler, TCC 3000 column oven (Dionex Corporation, Sunnyvale, CA) and SofTA model 1400 evaporative light scattering detector (SofTA Corporation, Westminster, CO). Acetonitrile and methanol UV grade from B&J; ethanol, denatured 3A from BDH; isopropanol and acetone, semiconductor grade from General Chemical.

Results and Discussion

Figure 1 shows a comparison of the chromatograms of the five solvents under the same gradient program. Compared to acetonitrile (trace d), isopropanol (e) was a stronger solvent, while acetone (c), ethanol (b), and methanol (a) were the weaker solvents. The selectivity, efficiency, and resolution were quite similar for acetonitrile and acetone. Alcohols have higher viscosity than acetonitrile or acetone, which results in high operating pressures (as noted in Figure 1) and lower peak efficiency.

Conclusion

The Acclaim Surfactant column provides excellent separation capability for cationic surfactants. Several less-toxic, lower-cost, and more readily available solvents can replace acetonitrile. When using ELSD or CAD detectors, acetone can substitute for acetonitrile. When UV detection is required, methanol and ethanol are useful alternatives.

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Rapid UHPLC Determination of Common Preservatives in Cosmetic Products

Njies Pedjie, PerkinElmer Inc.

P-hydroxybenzoic acid esters, also called parabens, are widely used as antimicrobial preservatives in cosmetic products. Parabens have a potential of endocrine effect — as a result, their use is limited in EU countries to a maximum concentration of 0.4% when used individually and 0.8% as a mixture. In the US, it is against the law to market products with ingredients that may cause injuries under label conditions, thus parabens are typically used at levels between 0.01 to 0.3%. This application note will present a fast, sensitive, and reliable UHPLC determination of six common parabens.

This application will discuss throughput improvements and reductions in solvent consumption compared to a conventional HPLC analysis of parabens. In addition, method conditions and performance data will be presented.

Experimental

The PerkinElmer Flexar FX-15 with Flexar UV-vis detector provided the UHPLC platform for this application. The separation was completed on a PerkinElmer Brownlee Analytical C18, 1.9 µm 50 mm × 2.1 mm column. The run time was approximately 3 min with a back pressure of 8100 psi (559 bar).

The separation was characterized and system calibrated with a mixture of parabens diluted from neat material. Repeatability was studied with six injections of the working standard (1200 ng/mL). Linearity was determined across the range of 30–1200 ng/mL. Samples of face hydrating lotion and cocoa butter body lotion were tested.

Results and Discussion

A conventional HPLC method would take approximately 16 min to resolve the parabens discussed here. This method would use approximately 24 mL of solvent.

Compared with conventional HPLC methodology, the UHPLC run time was dramatically shortened to about 3 min, the resolution of analyte peaks and sensitivity of the determination were improved and the total solvent usage was reduced to 2.1 mL per injection.

A representative chromatogram from standard solution analysis under UHPLC conditions is presented in Figure 1.

The method performance was outstanding. The linearity of the analysis achieved an average r^2 value of 0.998. The average precision was less than 1% RSD, ranging from 0.9 to 0.2%.

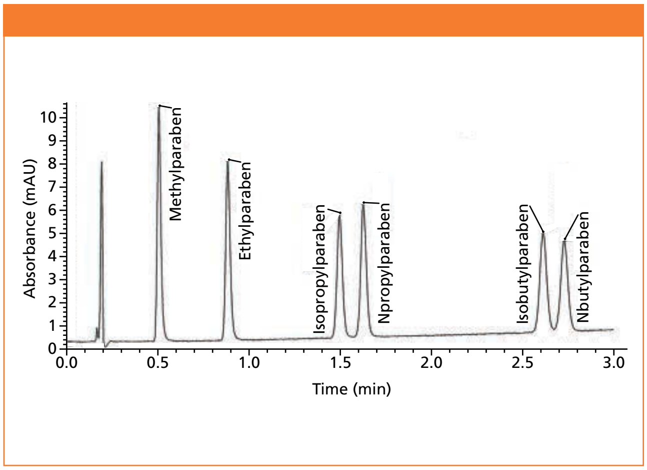

Figure 1: Example chromatogram from the analysis of a standard solution of common parabens with UHPLC and a 1.9 µm particle size column.

Conclusion

The application of UHPLC to the analysis of common antimicrobials in cosmetics has resulted in a 13 min or 80% reduction in run time as well as a reduction of solvent usage of 22 mL, or about 90%. The PerkinElmer Flexar FX-15 UHPLC system and Brownlee Analytical C18, 1.9 µm 50 × 2.1 mm column resolved all the six parabens in about 3 min. The method was shown to be linear and precise. When applied to hand and body lotions, parabens were detected and their amounts complied with the EU and US Cosmetic recommendations.

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Extend UHPLC Column Lifetime with KrudKatcher™ Ultra In-Line Filters

Philip J. Koerner, J.T. Presley, Tom Cleveland, and Jason A. Anspach, Phenomenex, Inc.

For many years, the use of guard columns has been advocated by column manufacturers and other experts to protect and extend column lifetime and performance of analytical columns from potential damage caused by the presence of chemical contaminants and microparticulates in the sample and mobile phase. The introduction and adoption of smaller particle size (< 3 µm) and narrow ID columns (≤ 2 mm) columns for ultra-high performance liquid chromatography (UHPLC) presents additional challenges. Specifically, these ultra-high performance columns are very sensitive even to small increases in dead volume and the use of traditional guard columns or guard cartridge systems will show a marked decrease in column performance (efficiency and resolution). However, small particle columns are even more susceptible to contamination and blockage from microparticulates that may be present in both the sample and mobile phases as they can occlude the smaller porosity column frits and dramatically shorten column lifetime.

Results and Discussion

KrudKatcher Ultra in-line filters are specifically designed for use with ultra-high performance columns from 1.0 to 4.6 mm ID and are pressure rated to 20,000 psi (1,375 bar). The filter body houses an integrated 0.5 µm 316 stainless steel depth filter that efficiently removes microparticulates from the flow stream without contributing to system backpressure or dead volume (<0.2 µL).

To illustrate the benefit of using the KrudKatcher Ultra in-line filter for extending column lifetime, a chromatographic separation of a ketone mixture on a Kinetex® 2.6 µm PFP 50 × 2.1 mm column was set up on a UHPLC system (Figure 1). The initial injection shows excellent peak shape and was maintained for over 1,100 injections (1a). However, after 1,175 injections the chromatographic performance has noticeably degraded resulting in split peaks with some peak broadening and tailing evident (1b). Simply replacing the KrudKatcher Ultra and re-injecting the sample returns the original chromatographic performance (1c). The KrudKatcher Ultra in-line filter successfully protected the ultra-high performance Kinetex column from damaging microparticulates in the mobile phase flow path and sample that would have dramatically shortened the column lifetime.

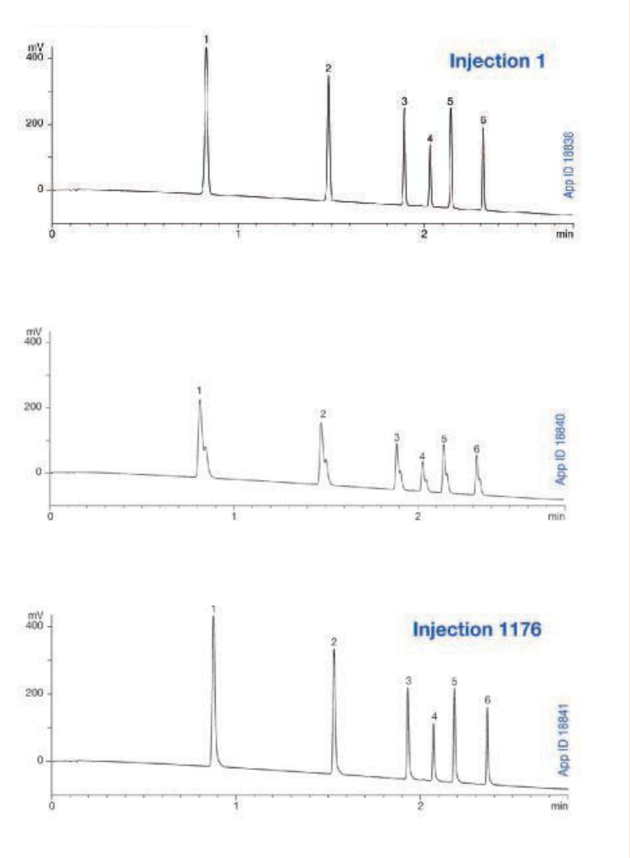

Figure 1a: Initially, both the KrudKatcher Ultra in-line filter and UHPLC column show excellent performance.
Figure 1b: After extended use the chromatography starts to degrade producing split peaks.
Figure 1c: Replacing the KrudKatcher Ultra in-line filter extends the lifetime of the column.

Conclusion

The regular use of KrudKatcher™ Ultra in-line filters with ultra-high performance UHPLC columns protects and extends the lifetime by protecting them from damaging microparticulates that may be present in the sample and mobile phase. Additionally, the KrudKatcher Ultra does not have a detrimental effect on chromatographic performance (retention time, peak width, resolution, or efficiency). For additional information, please request technote TN-1063.

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Analyzing The SAMHSA Drug Panel on Kinetex Core-Shell Media

Jeremy Bierman and Carl Sanchez, Phenomenex, Inc.

Urine is often used to analyze drugs of abuse because it is easy to collect and is available in copious amounts. However, it can be challenging to analyze urine samples due to the variances in the endogenous nature of the matrix, which can impact the chromatographic separation and quantitation. A high-throughput SPE plus LC–MS method using core-shell UHPLC columns to screen for a panel of 11 drugs of abuse (Substance Abuse and Mental Health Service Administration, SAMHSA) was developed using Kinetex core-shell media. This new high-throughput method demonstrates the power of new-generation HPLC media, as well as considerations when developing methods for LC–MS analysis.

Materials and Methods

Urine samples from five different lots were hydrolyzed to convert the glucuronide metabolites to native form using β-glucuronidase. Hydrolyzed samples were then adjusted to a pH of 5.95 with 100 mM ammonium acetate buffer. SPE clean-up was performed using Strata™-X-C, a high cation exchange capacity polymeric material, in a 30 mg/3 mL tube. Analysis of the 11 compounds was done using an Agilent® 1200SL HPLC coupled with an Applied Biosystems® 4000 QTRAP® (with Turbo V™ ion source) at different concentrations on a Kinetex® 2.6 µm C18 50 × 2.1 mm core-shell column. A gradient separation method was used with an aqueous mobile phase of 0.1% formic acid in water (A) and organic mobile phase of acetonitrile (B). The gradient used was from 5% to 95% B in 1.7 min followed with a 0.2 min hold at 95% B and re-equilibration at initial conditions for a total cycle time of 2.6 min. The flow rate was 1.2 mL/ min and the column oven was set at 25 °C.

Results and Conclusion

The analysis of 11 drugs of abuse at cut-off concentrations was completed in less than 2 min (Figure 1). The use of the new Kinetex core-shell HPLC column technology provided extremely sharp chromatographic peaks with average peak width of 1–2 s at the base. Because of the narrow peak width for each analyte, a timed acquisition program for the MS was used to achieve accurate peak quantitation. The analytical method resulted in accuracies between 85–99% for all analytes at the LLOQ. At LLOQ and 3 × LLOQ all RSDs were below 15%, indicating a wide linear working range for all compounds. The Strata-X-C SPE protocol has been optimized for the 11 analytes and the internal standards. Endogenous contaminants with low pKa values were not retained which allowed for a 100% methanol wash to more effectively remove contaminants, such as uronilin which can interfere with LC–MS analysis.

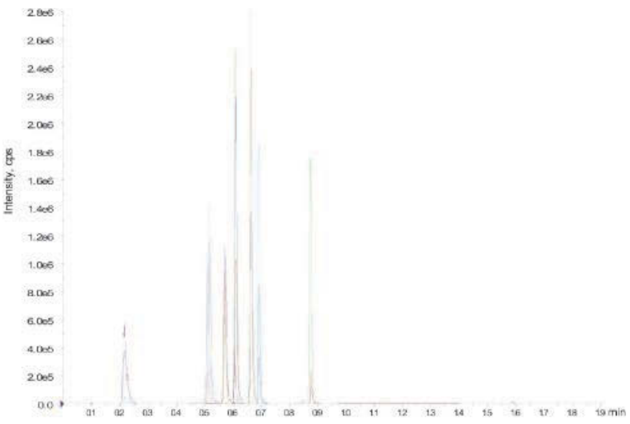

Figure 1: LC–MS-MS analysis of 11 drugs of abuse compounds ran on core-shell Kinetex C18 columns. Note the good resolution and narrow peaks for most compounds. The high performance column allowed for analysis of the SAMHSA panel in under 2 min.

The combination of an extremely targeted and consistent sample preparation method and the highly efficient Kinetex column yielded a significant increase in productivity and throughput while solvent usage and analysis time were minimized. Core-shell media utilizes optimized particle geometry to generate high efficiency separations on par with sub-2 µm UHPLC columns except with much lower back pressures. Key to maximizing performance on older HPLC's is to match detector acquisition rate with core-shell Kinetex column performance. High efficiencies close to 300,000 plates per meter for Kinetex core-shell HPLC columns can result in peak widths as narrow as 1 s in width. The method for analyzing the SAMHSA panel on Kinetex 2.6µ core shell media is sensitive and accurate and a significant improvement over existing GC–MS or LC–MS methods using standard HPLC columns.

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SHISEIDO

Next-Generation Autosampler for LC–MS Equipped with Straight Injection Technology™

Yoshikazu Sugito, Tsuneaki Kaneko, and Osamu Shirota, SHISEIDO Co., Ltd.

This article reports a new sample injection mechanism for an autosampler that directly transfers a sample to column without passing it through any flow-changing device, and hence minimizes a delay volume and reduces a carryover signal to the extreme level.

Carryover is one of the most common problems one often encounters in wide dynamic-range measurements with a high-end mass spectrometer. There are various sources leading to the carryover, such as adsorption of low-solubility compounds on column and instrumental surfaces, especially in gradient elution. In many cases, an autosampler seems to be the most responsible factor to the problem. Although it has been largely improved, anti-carryover performance in current HPLCs may not be adequate to fully exploit the sensitivity of high-end mass spectrometers. This article describes a new autosampler having straight injection technology (SIT), which can drastically reduce carryover in LC–MS.

Experimental

Two autosamplers, with and without SIT, were used for the comparative study. Carryover values of reserpine were measured under isocratic conditions, and those of chlorhexidine were measured under gradient conditions. An API5000™ mass spectrometer (Applied Biosystems) and a UV detector were used as detectors.

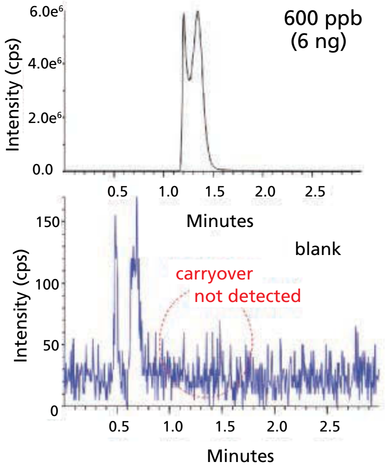

Figure 1: Carryover of reserpine under isocratic conditions. Column: CAPCELL PAK C18 MGII S3 2.0 mm i.d. × 50 mm, Mobile phase: A) 0.1 vol % HCOOH, B) CH_3CN, A/B = 60/40, Flow rate: 200 mL/min, Temperature: 40 °C, Detection: ESI Positive (API5000™).

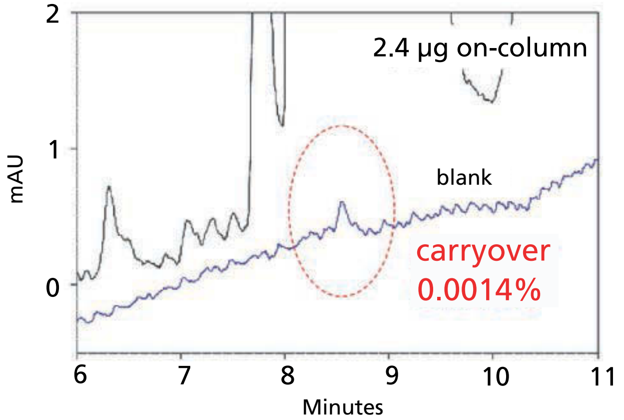

Figure 2: Carryover test of chlorhexidine under gradient conditions. Column: CAPCELL PAK C18 MGIII S5 2.0 mm i.d. × 150 mm, Mobile phase: A) 10 mmol/L KH_2PO_4, 100 mmol/L $NaClO_4$ (pH = 2.6), B) CH_3CN, B: 30% (0.0min) – 70% (10.0 min) gradient, Flow rate: 200 mL/min, Temperature: 40 °C, Detection: UV 260 nm.

Results

The newly-designed autosampler did not show any carryover of reserpine (Figure 1) in an isocratic elution even after introduction of overloading quantity. For chlorhexidin, a carryover peak of magnitude one tenth that of the conventional model (Figure 2) was slightly observed under gradient conditions. These results indicated that the direct transfer of samples to a column greatly contributed to the reduction of carryover.

The simplified sample transfer pathway from a sample-collection needle to a separation column also contributed to reproducibility of peak area and retention time, number of theoretical plates, and resolution between peaks. The ultimately reduced delay volume and shortened pathway seem to have reduced sample diffusion to a large extent. Using stainless steel-reinforced polyetheretherketone (PEEK) tubing, the autosampler could tolerate 70 MPa and must be the most pressure-resistant among "inert" HPLCs.

Conclusion

The newly developed injection mechanism improved separation efficiency and precision in HPLC and LC–MS, including those obtained under gradient conditions.

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Confirmation and Quantitation of Cocaine and Major Metabolites in Urine Using the ISQ Single Quadrupole GC–MS

Matthew Lambing, Eric Phillips, and Trisa Robarge, Thermo Fisher Scientific

Cocaine is a central nervous system stimulant derived from *Erythroxylon coca*. It is metabolized in-vivo resulting in the formation of ecgonine methyl ester, norcocaine, and benzoylecgonine. Cocaethylene is a substance formed when cocaine and ethanol are coadministered (1). A forensic toxicology method for the confirmation and quantitation of ecgonine methyl ester (EME), benzoylecgonine (BE), cocaine (COC), and cocaethylene (CE) in human urine was developed using the Thermo Scientific ISQ single quadrupole GC–MS system.

Methods

Each sample batch contained a matrix-matched single point calibrator (at 150 ng/mL), quality control samples set to contain each target compound at 40% and 125% of the calibrator (60 ng/mL and 187.5 ng/mL respectively), and a negative control (blank urine with internal standard only). Thermo Scientific HyperSep Verify-CX solid phase extraction columns were used for sample extraction. Samples were derivatized with hexafluoroisopropanol (HFIP) and pentafluoropropionic acid (PFPA or PFAA).

The ISQ™ mass spectrometer system was operated in selected ion monitoring mode, collecting 3 ions for each target compound, and 2 ions for each deuterated internal standard (Table I). A Thermo Scientific AS 3000 II autosampler and a Thermo Scientific TRACE GC Ultra gas chromatograph, equipped with a split/splitless injection port, provided sample introduction and separation. A 15 m × 0.25 mm i.d. × 0.25 µm film thickness Thermo Scientific TraceGOLD TG-5MS analytical column was used to enhance separation of the target cocaine class compounds from each other and from matrix components. Thermo Scientific ToxLab Forms software automated the acquisition and processing of all data, including quantitation and ion ratio confirmation calculations. For precision analyses, a coefficient of variation of <10% of the average calculated quality control amounts were required for each analyte, and inter-day percent differences of calculated amounts also had to be less than 10%.

Results

- Assay linearity ranged from 15 ng/mL to 12,500 ng/mL for BE, EME, and CE, and 15 ng/mL to 5,000 ng/mL for cocaine.
- Limits of detection and quantitation of 15 ng/mL using a 2 mL sample size.
- Intra- and inter- day precision of <10% CV at the quality control levels of 60 ng/mL and 187.5 ng/mL.
- Correlation coefficient (R^2) better than 0.9990 for cocaine, benzoylecgonine, ecgonine methyl ester, and cocaethylene based on a one point calibration.

Table I: Retention times and ions monitored for cocaine and metabolite analytes and their deuterated internal standards

Analyte	Retention Time (min.)	Quan Ion *(m/z)*	Qual Ion(s) *(m/z)*	Dwell Time (ms)
EME-d3	1.34	348	317	15
EME	1.35	182	345, 314	15
Benzoylecgonine-d3	2.91	321	442	15
Benzoylecgonine	2.91	318	334, 272	15
Cocaine-d3	3.51	185	306	15
Cocaine	3.52	82	182, 303	15
Cocaethylene-d3	3.65	199	275	15
Cocaethylene	3.66	196	317, 212	15

- Pseudoephedrine at a concentration of 20,000 ng/mL showed interference with EME at the 40% and 125% QC levels.
- Norcocaine at 10,000 ng/mL demonstrated no interference with any analyte tested, but limited co-elution was observed with cocaethylene. Relative retention time to CE = 1.005.

Conclusion

A method was developed to demonstrate the performance of the ISQ GC–MS system for the confirmation and quantification of cocaine and its major metabolites in a urine matrix. The assay described offers broad linearity to cover a wide range of analyte concentrations, thus, reducing the need for dilutions or repeat extractions. Excellent precision was also demonstrated around the 150 ng/mL cutoff, with CV measurements of 10% or less over the study. Limits of detection and quantification at 15 ng/mL ensure sensitive performance for retest and directed assay samples. The methodology described offers a means for a forensic toxicology laboratory to confirm and quantify cocaine, benzoylecgonine, ecgonine methyl ester, and cocaethylene in human urine.

References

(1) Disposition of Toxic Drugs and Chemicals in Man, Eighth Edition. Randall C. Baselt, Biomedical Publications, 2008.

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An Accurate and Robust LC–MS Method for the Identification of Illicit Drug Salt Forms

Guifeng Jiang, Terry Zhang, and Kathryn Preston, Thermo Fisher Scientific

Illicit cocaine, amphetamine, methamphetamine, and ecstasy (MDMA) are often encountered in the form of salts and identification of the specific counter ion present may provide valuable information about the source of production and the manufacturing pathway. Screening and identification of these stimulants in seized evidence requires an analytical method that is capable of detecting the salt forms.

LC–MS Analysis

The Thermo Scientific Accela LC system offers the flexibility of performing both conventional and high-speed LC separations on a single platform, and seamlessly integrates with the Thermo Scientific MSQ Plus Mass Detector, a sensitive and fast scanning single quadrupole mass spectrometer. The rapid polarity switching capability of the MSQ™ Plus Mass Detector enables simultaneous detection of cations and anions in a single analysis.

Results and Discussion

1. Separation and MS detection of drug and inorganic anion standards

Common salt forms of cocaine are chloride and bromide. Amphetamine is usually found in the chloride and sulfate forms. Bromide and chloride forms of methamphetamine are common, but iodide, phosphate, and nitrate forms may also be encountered. For 3,4-MDMA, bromide and chloride are the most common salt forms, although a phosphate form may occasionally be detected.

Separation of a mixture of cocaine, amphetamine, methamphetamine, and 3,4-MDMA standards as well as a mixture of six inorganic anions was achieved using a Hypercarb column and a single LC–MS method (Figure 1). Gradient elution was used to control selectivity and retention of the inorganic anions and the organic cations. The inorganic anions were separated within 3 min with an elution order of phosphate, chloride, bromide, nitrate, iodide, and sulfate. The drug standards were baseline-resolved in under 7 min and eluted in order of increasing hydrophobicity: amphetamine, methamphetamine, 3,4-MDMA, and cocaine.

The most abundant ions for the drug standards are the $[M+H]^+$ ions, at *m/z* 136.12, 150.10,194.15, and 303.95 for amphetamine, methamphetamine, 3.4-MDMA, and cocaine, respectively.

2. Linearity and sensitivity

Excellent linearity in detector response was observed over the range of 0.125–2000 ng/mL for the drug standards and over the range of 1.14–29400 ng/mL for the inorganic ions, with correlation coefficients greater than 0.996 for all analytes.

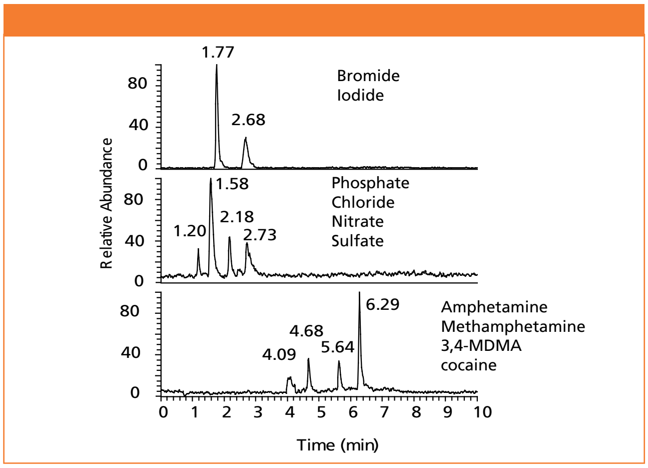

Figure 1: Separation and detection of four drug standards and six inorganic anion standards.

Limits of detection (LODs) and limits of quantitation (LODs), are defined as S/N ratio of 3 and 10, respectively. For drug standards, LODs ranged from 0.3 to 0.5 ng/mL, and LOQs ranged from 1 to 2 ng/mL. For the inorganic ions, LODs were between 1.1 and 30 ng/mL, and LOQs ranged from 3.3 to 100 ng/mL.

3. Reproducibility

Reproducibility was investigated by analyzing three replicate injections of each analyte. Retention time RSDs ranged from 0.35–0.92%, indicating excellent method reproducibility, particularly of the Accela UHPLC pump.

Conclusion

An accurate and robust LC–MS method for the identification of illicit drug salt forms was developed. Simultaneous separation and detection of cations and anions was achieved using a Hypercarb LC column and the polarity switching mode of the MSQ Plus MS. Furthermore, this method utilizes two uncorrelated parameters — retention time and mass spectral signature — to establish the identity of drug salt forms.

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How Clean Are Your Vials and Closures?

L. Pereira, T. Edge, L. Shick, and M. Slade, National Scientific, Part of Thermo Fisher Scientific

Purpose: The evaluation of a new vial and closure for reducing interferences.

Methods: Vials and closures were exposed to acetonitrile for 2 h and subsequently analysed by LC-UV, LC–MS, and GC–MS to characterize the interferences. Comparisons were made between precleaned MS certified vials and closures, a control, and a competitor vial and closure set.

Improvements in chromatographic techniques, instrumentation, and sample handling continue to push the limits of detection in trace analysis. As such, the cleanliness of the total workflow process becomes even more important to reduce the potential for interferences and contamination that can ultimately reduce the sensitivity of the assay. The selection of the correct autosampler vial and closure is thus an important consideration.

Vials that are not effectively cleaned can introduce particulate matter that can cause blockages and accumulation of foreign material at the head of the separation column affecting chromatographic performance. Additionally, residual organic compounds that might survive the glass forming process or that leach from the closure when exposed to the sample solvent can reduce the analysis sensitivity.

The work presented in this poster evaluates the performance of the new MS certified vial and an ultra high pure bonded PTFE/silicone closure compared with a control and a competitor vial and closure set.

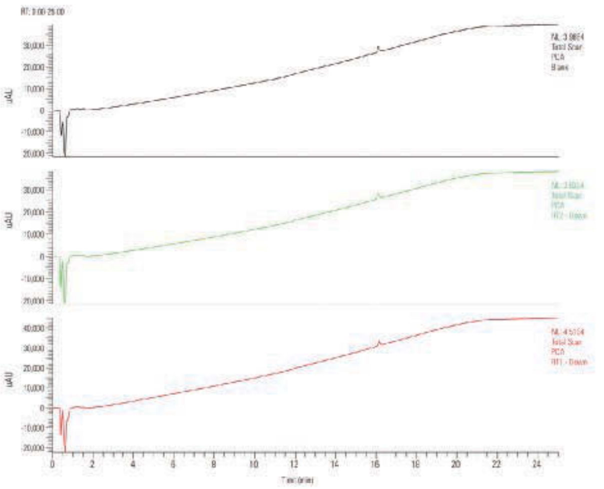

Figure 1: LC-UV chromatograms of a blank, a vial with PTFE/silicone closure, and a vial with pre-slit closure, incubated at room temperature in the inverted position.

Materials & Methods

LC–MS and LC-UV

Instrument:	Surveyor with LCQ Deca XP	
Column:	Hypersil GOLD 3 µm, 50 × 2.1 mm	
Mobile phase:	A — H_2O + 0.1% formic acid;	
	B — MeOH + 0.1% formic acid	
Gradient:	Time (min)	%B
	0	10
	10	50
	20	100
	25	100
	25.01	10
	30	10
Flow rate:	0.3 mL/min	
Temperature:	40 °C	
Injection vol.:	10 µL (2 µL for system suitability test)	
UV:	190 to 500 nm	
MS:	Full scan 50 to 1500 amu	

GC–MS

Instrument:	DSQII DurabriteXL GC–MS with Triplus autosampler
Column:	TRACE TR-5MS, 30 m × 0.25 mm × 0.25 µm
Carrier gas:	Helium
Flow rate:	1.2 mL/min
Oven program:	40 °C, hold for 0.5 min; 15 °C to 150 °C, hold for 1 min; 10 °C to 290 °C, hold for 5 min
Inlet temperature:	250 °C; Split flow: 50 mL/min
Injection vol.:	1 µL splitless
MS transfer line:	290 °C
MS ion source:	230 °C
MS detection:	EI; Full scan 50 to 650 amu

Sample Preparation

- Vials were filled with 1.0 mL of acetonitrile (LC–MS grade).
- The filled vials were capped and the vial + closure incubated at room temperature in the upright position and in the inverted position (in duplicate) for 2 h.

System Suitability Tests (SST)

- LC–MS — 5 repeated injections of reserpine solution at 10 ng/μL in water.
- GC–MS — 5 repeated injections of phenanthrene at 1 ng/μL in acetonitrile. SSTs were injected before each set of injections for each vial + closure type.

Control

The control (or blank) was generated by rinsing a vial (with no cap) twice with injection solvent (acetonitrile). 1 mL of acetonitrile was measured into the vial and vial with no closure was placed in the autosampler tray for analysis.

Figure 1 illustrates the UV traces for a control, a vial with PTFE/silicone closure and a vial + closure with pre-slit, incubated in the inverted position at room temperature. The three traces are identical, demonstrating that in LC-UV it is not feasible to see the interferences using this detector technology.

Figure 2 shows the LC–MS analysis of a control, a competitor vial + closure, and an MS certified vial + closure. The LC–MS chromatograms for the control and the MS certified are very similar, suggesting that there is minimal interference from the vial + closure. However, the total ion chromatogram (TIC) obtained for the competitor vial shows a substantial amount of contamination. Combining the MS spectra of the latter part of the chromatogram reveals that the majority of the peaks in the positive ESI spectra are 74 units apart, suggesting the presence of polydimethylsiloxane $(C_2H_6OSi)_n$.

Figures 3 show the results of the GC–MS analysis of the MS certified vials compared to the control. Both sets of chromatograms and spectra clearly demonstrate that there is minimal difference between the MS certified vials and the control.

Conclusions

- UV detection is generally not sensitive enough to detect trace amounts of nonvolatile organic contaminants present in autosampler vials.
- Some commercially available autosampler vials can leach organic compounds into the sample, particularly if the sample solvent comes into contact with the closure.
- The National Scientific mass spec certified vials showed no evidence of sample contamination in LC–MS with ESI or GC–MS with EI ionization, even when the sample solvent came into contact with the closure.

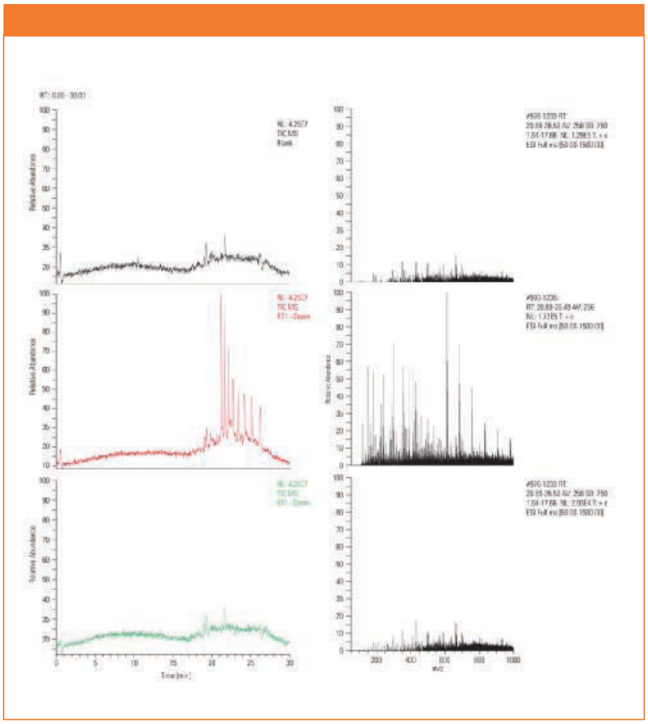

Figure 2: LC–MS +ve ESI chromatograms obtained for different sample types. The MS spectra are obtained by summing the spectra over the last portion of the chromatogram.

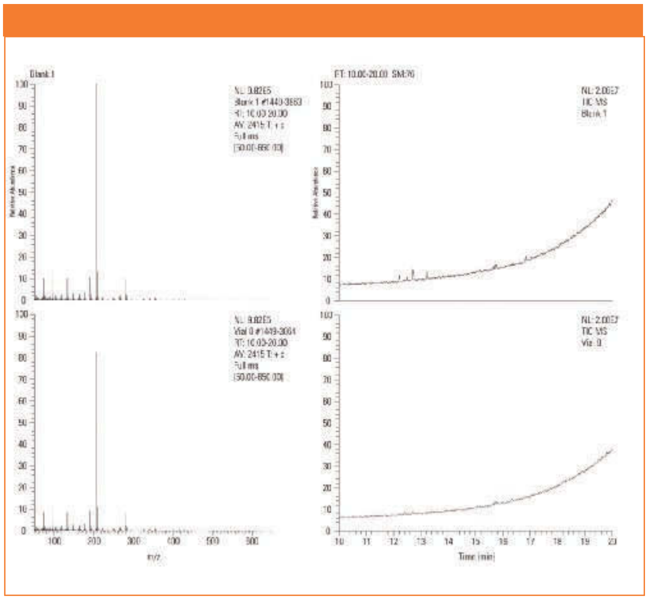

Figure 3: GC–MS data for the MS certified vials. Comparison to control TIC and spectrum across the full time span, demonstrate that there is minimal difference between the two.

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VARIAN

Is There Really a Difference Between Flash and HPLC for LC Purification?

L. Lloyd, S. Ball, and K. Mapp, Varian, Inc. — now a part of Agilent Technologies

This note examines the benefits of using flash or HPLC for compound purification. Results show that flash is a viable alternative to HPLC.

With the trend towards the use of smaller sub-2 µm particle materials for analytical HPLC to run the analysis faster, one would also expect a move to these materials for purification. While high efficiency separations performed at speed are utilized for purification, the capital cost of high-pressure equipment and columns makes the technique unsuitable for routine purification or when larger amounts of compound are required.

There are a number of factors that need to be considered before starting an LC purification of a target compound.

- Difficulty of the separation
- Required purity
- Required yield
- Amount of compound needed
- Purification throughput
- Available instrumentation

In all cases, the driver for the purification will be the economics of achieving the final compound or compounds at the required purity and with acceptable recovery.

A wide range of instruments, columns, and media are available for LC purification, ranging from flash to HPLC, and so we examined the advantages and disadvantages of using these techniques for compound purification.

Resolution Equation

Flash and HPLC are subsets of liquid chromatography and as such the same chromatographic theory will apply. The resolution equation (Equation 1) is applicable to both techniques. It contains three terms: efficiency, influenced by the particle size and shape of the media; selectivity, influenced by the total functionality of the media and eluents used for separation; and retention capacity, relating to the residence time of the solute in the column and influenced by the eluent composition.

$$R = \sqrt{N}/4 \times [(\alpha-1)/\alpha] \times [K'/(1+K)]$$

where N = efficiency, α = selectivity, and K' = retention capacity

Equation 1: Resolution equation for liquid chromatography.

Table I: Comparison of flash LC and HPLC parameters that influence the resolution equation

	Flash	HPLC
Particle shape	Irregular	Spherical
Particle size distribution	Wide	Narrow
Particle size	35 µm to 50 µm	< 1.9 µm to 50 µm
Functionalities	Normal and reversed phase	
Solvent compatibility	Determined by silica and bonding chemistry	

Experimental Conditions

HPLC and flash columns are available with a wide range of media types. Two silica-based C18 materials were chosen in this study. The HPLC media was SepTech™ ST60 10-C18, a 60Å, 10-µm spherical silica particle, and the flash media was SuperFlash™ C18, a 60Å, 50-µm irregular silica particle. Separation of a five-component test mix was used to assess the performance of the two materials (Figures 1A and 1B).

Column A: SuperFlash SF10-5.5 g C18
Column B: SepTech ST60 10-C18
Eluent: 60:40 Acetonitrile:water

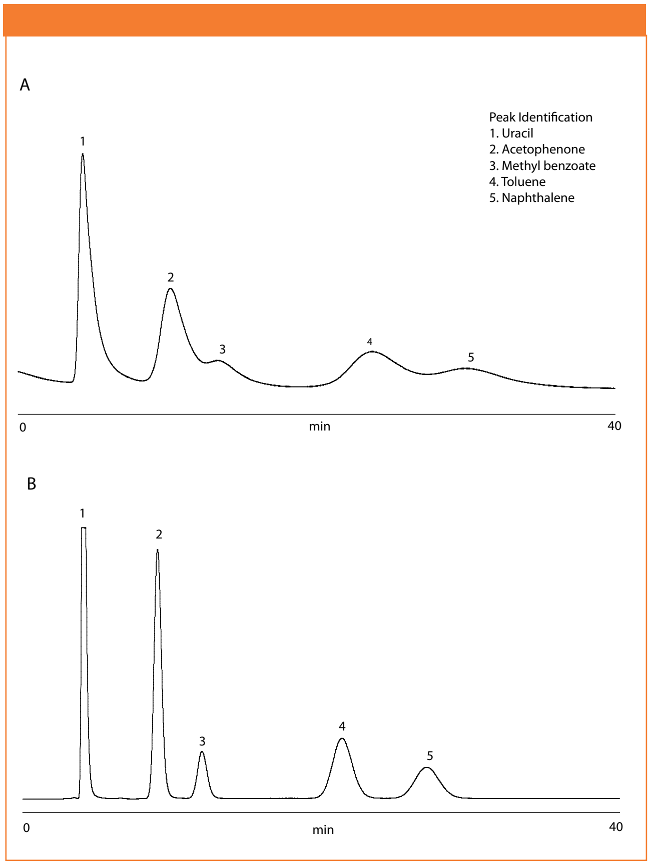

Figure 1: Selectivity comparison of SF10-5.5g C18 (A) and SepTech ST60 10-C18 (B), with 60:40 MeCN/H$_2$O.

Results

As would be expected, the peak widths were much broader with the SuperFlash column but the separations appeared comparable.

To determine differences in resolution between the SepTech and SuperFlash materials due to contributions from selectivity and retention capacity, these values were calculated for adjacent pairs of peaks. From the comparisons shown in Figures 2 and 3, it is clear that the two materials were very similar in their chromatographic performance. The difference in resolution of the sample components was due to the difference in efficiency, because of particle size and shape, and not to compound selectivity or retention capacity.

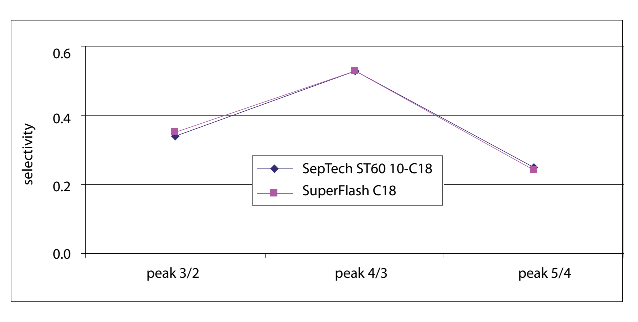

Figure 2: Comparison of SepTech ST60 10-C18 and SuperFlash C18 selectivity.

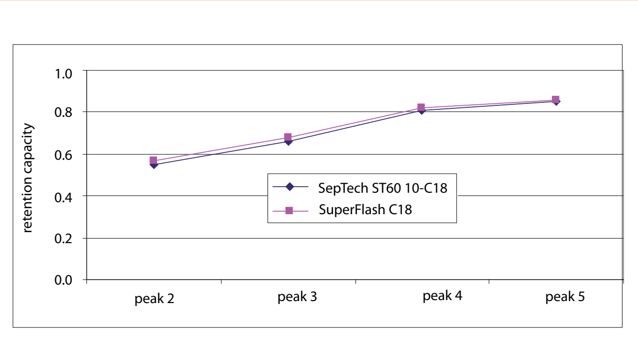

Figure 3: Comparison of SepTech ST60 10-C18 and SuperFlash C18 retention capacity.

Conclusions

Flash and HPLC are sub-sets of liquid chromatography and the resolution equation of LC is applicable to both. Efficiency, selectivity, and retention capacity components were evaluated for flash and HPLC media using SuperFlash C18 and SepTech ST60 10-C18 media, respectively. As expected from differences in particle shape, size, and size distribution, the biggest performance variations were seen in efficiency, otherwise the media were very similar in retention and selectivity. Therefore, where purifications do not require high efficiency, flash is a viable alternative to HPLC for compound purification.

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Orthogonal Selectivity of ZirChrom®-CARB

Dr. Bingwen Yan and Dr. Clayton McNeff, ZirChrom Separations, Inc.

Method development in reversed-phase liquid chromatography (RPLC) can be challenging for structurally similar compounds. Validation methods often require a column of orthogonal selectivity be used to ensure no impurities are missed, carbon-clad zirconia phase offers dramatically different chromatographic selectivity when compared to traditional silica-based bonded phases. This note shows selectivity comparison of the zirconia based, carbon-clad ZirChrom®-CARB HPLC column versus a typical silica bonded phase for 22 nonelectrolyte solutes with varying chemical properties.

Method development in RPLC has traditionally been difficult for molecules which are geometric isomers or structurally very similar. In bonded-phase silicas, the partition mechanism responsible for retention in RPLC often does not offer adequate chemical selectivity for such compounds. On the other hand, carbon-based phases provide retention in RPLC through an adsorption mechanism which often increases the chromatographic selectivity for these types of compounds and dramatically increases the chances of resolving pairs of these analytes.

In addition to this enhanced and often orthogonal chromatographic selectivity, carbon-based phases also offer increased chemical and thermal stability of the stationary phase. Zichrom Separations, Inc. has developed ZirChrom®-CARB using zirconia as a stationary phase support, and patented chemical vapor deposition technology to produce a carbon-clad stationary phase suitable for use in reversed-phase liquid chromatography. The graphitized carbon surface is extremely resistant to chemical and thermal attack. The authors have run mobile phases at very high pH (1 M NaOH), very low pH (0.5 M HNO_3), and at elevated temperature (200 °C) and have not observed loss of stationary phase. This unique chemistry results in a very selective phase with no detectable bleed by LC–MS.

Experimental

22 non-electrolyte solutes were injected onto two HPLC columns of equivalent configuration; 1) ZirChrom®-CARB, 3 µm particle size and 2) a leading traditional silica based bonded phase (ODS), 3 µm particle size. For both HPLC columns the k' for each solute was calculated from the resulting data. Each k' was then converted to log k' and graphed, see Figure 1.

Column: ZirChrom®-CARB, 50 mm × 4.6 mm i.d.
(Part Number: ZR01-0546)
Mobile Phase: 40/60 acetonitrile/water

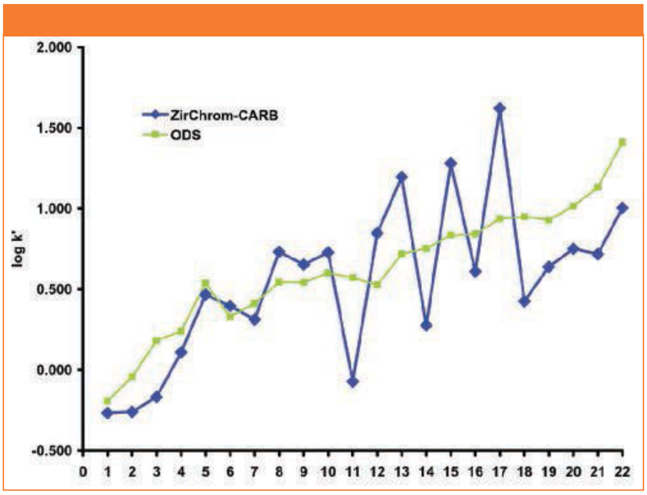

Figure 1: Selectivity comparison for 22 nonelectrolytes. Solutes:
1. n-Benzyl formamide
2. Benzylalcohol
3. Phenol
4. 3-Phenyl propanol
5. p-Chlorophenol
6. Acetophenone
7. Benzonitrile
8. Nitrobenzene
9. Methyl benzoate
10. Anisole
11. Benzene
12. p-Chlorotoluene
13. p-Nitrobenzyl chloride
14. Toluene
15. Benzophenone
16. Bromobenzene
17. Naphthalene
18. Ethylbenzene
19. p-Xylene
20. p-Dichlorobenzene
21. Propylbenzene
22. n-Butylbenzene

Temperature: 30 °C with Metalox™ 200-C column heater
Flow Rate: 1 mL/min
Injection Vol.: 5 µL
Pressure Drop: 50 bar
Detection: UV at 254 nm

This method can be tailored to your specific application needs. ZirChrom technical support can help to optimize and transfer this method to your site. Please contact ZirChrom technical support at 1-866-STABLE-1 or support@zirchrom.com for details.

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Real-Time Profiling of Volatile Malt Aldehydes Using Selected Ion Flow Tube Mass Spectrometry

The potential of selected ion flow tube mass spectrometry (SIFT-MS) to differentiate malted barley cultivars on the basis of their headspace profiles has been investigated. From a broad range of volatiles, marker aldehydes were selected because they are associated with malt quality and beer flavor stability. The authors used dynamic headspace SIFT-MS to identify the target volatiles in the different malt headspaces. The technique exhibited an increase in specificity and speed compared with the headspace solid-phase microextraction (SPME) gas chromatography–MS method currently used. The unique feature of SIFT-MS to analyze sample headspaces rapidly and directly without the need for sample preparation, derivatization, or chromatographic preseparation is demonstrated.

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Malt, the main raw material for beer production, is made from selected cereal grain, usually barley (*Hordeum vulgare L.*), by steeping, germination, and drying (kilning). By varying the processing parameters during germination and drying, various types of malt are obtained (1). The strong influence of malt composition on final beer quality and beer flavor stability is generally acknowledged (2–4). In particular, flavor stability remains one of the main quality criteria for beer, and the urgency to control it is endorsed by the global beer market and its allied need for longer storage times for exported beer.

Formation and release of volatile aldehydes is recognised as one of the main causes of beer flavor deterioration upon storage (5–7). Most of these compounds pre-exist abundantly in malt, and can vary significantly between different malt types. As such, the staling potential of finished beer is determined largely by the type of malt used in the brewing process (8–12). Therefore, each modern brewery that aims at a pleasant and consistent beer flavor has to take appropriate measures from the onset of the brewing process by selecting high-quality malt. Consequently, knowledge of malt aldehyde content is indispensable for brewers in view of quality control, selection of the appropriate malt variety, and objective assessment of flavor stability of the processed beer.

Different procedures have been applied to isolate carbonyl compounds from malt (13,14). Their isolation from the complex malt matrix is far from easy and requires an appropriate sample preparation procedure. Traditional extraction techniques such as vacuum distillation or continuous steam distillation–solvent extraction (that is, Likens–Nickerson extraction) with subsequent Kuderna–Danish evaporation before gas chromatography (GC) analysis, are very cumbersome and time-consuming (15,16). To increase throughput and selectivity and maintain sensitivity, headspace analysis, preferably in combination with solid-phase microextraction (SPME), is the method of choice (16). SPME is a well-known sample preparation technique that is solvent-free, fast, inexpensive, easily amenable to GC, and has proven to be extremely powerful for analyzing volatile as well as semivolatile compounds at trace and ultratrace levels (17).

Table I: Overview of SIFT-MS parameters. The branching ratio is the fractional probability (represented as a percentage) of a product ion being formed from a single reaction between an ion and an analyte.

Component	Reagent	Branching ratio (%)	Mass	Product
2,3-Butanedione	NO^+	65	86	$C_4H_6O_2^+$
Formaldehyde	H_3O^+	100	31	CH_3O^+
	O_2^+	40	29	HCO^+
	O_2^+	60	30	H_2CO^+
Acetaldehyde	H_3O^+	100	45	$C_2H_5O^+$
Hexanal	NO^+	100	99	$C_6H_{11}O^+$
trans-2-Pentenal	NO^+	95	83	$C_5H_7O^+$
	NO^+	5	114	$C_5H_8O.NO^+$
trans-2-Hexenal	NO^+	85	97	$C_6H_9O^+$
	O_2^+	30	69	$C_5H_9^+$
trans-2-Heptenal	NO^+	85	111	$C_7H_{11}O^+$
	NO^+	15	142	$C_7H_{12}O.NO^+$
trans-2-Octenal	NO^+	80	125	$C_8H_{13}O^+$
	NO^+	20	156	$C_8H_{14}O.NO^+$
trans-2-Nonenal	NO^+	80	139	$C_9H_{15}O^+$
	NO^+	20	170	$C_9H_{16}O.NO^+$
trans,trans-2,4-Decadienal	H_3O^+	100	153	$C_{10}H_{17}O^+$
2-Methylpropanal	NO^+	100	71	$C_4H_7O^+$
2-Methylbutanal	NO^+	98	85	$C_5H_9O^+$
3-Methylbutanal	NO^+	100	85	$C_5H_9O^+$
Methional	NO^+	95	104	$C_4H_8OS^+$
Phenylacetaldehyde	H_3O^+	100	121	$C_8H_8O.H^+$
Benzaldehyde	H_3O^+	100	107	$C_7H_7O^+$
Furfural	H_3O^+	100	97	$C_5H_5O_2^+$

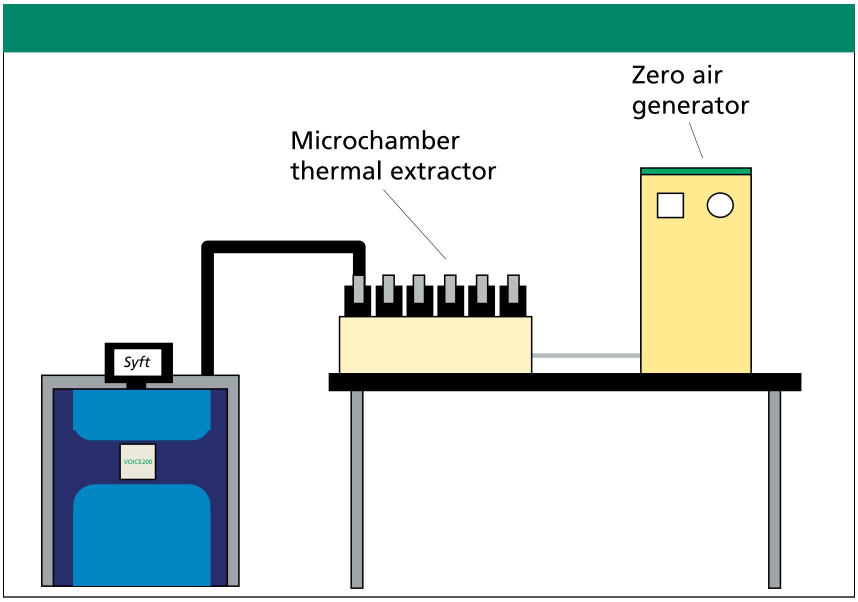

Figure 1: Schematic representation of the analytical set-up for volatile malt composition analysis by dynamic headspace SIFT-MS.

Unfortunately, headspace SPME of malt and beer samples suffers from the interference of other compounds that are abundant in the matrix. To eliminate these interferences, SPME of aldehydes generally is carried out in combination with a selective extraction, on-fiber derivatization, for example, with *o*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine (PFBHA) (18–20). Converting the aldehydes to their pentafluorobenzylhydroxylamine derivatives is not only beneficial with respect to extraction selectivity but also has a positive effect on GC performance. Additional effects, which are due predominately to the nature of the SPME procedure — that is, equilibrium extraction — and which hamper accurate quantification, require the use of internal standards and standard addition, which complicates the overall procedure even further. Moreover, milling and exposure to ambient air during sample preparation and extraction induces the pro-oxidative enzyme potential of malt, which leads to the formation of unwanted artifacts and a severe risk of biased results.

Selected ion flow tube mass spectrometry (SIFT-MS) is an analytical technique that is based upon soft chemical ionization taking place in a flow tube reactor. First introduced by Smith and Spanel, SIFT-MS is now an established technique for volatile organic compound (VOC) analysis that has advantages over many other analytical approaches (21). SIFT-MS provides a quantitative measure of analytes in air mixtures in real time at sensitivities in the low parts-per-billion (ppb) level, and more recently, the parts-per-trillion (ppt) level without the need for external calibration (absolute quantification) (22,23). These very low quantification limits are enabled by a thorough understanding of the chemical kinetics of an analyte with each of the SIFT-MS reagent ions H_3O^+, NO^+, and O_2^+ (21,24).

Initially, the instrumentation used to exploit the SIFT-MS technique was large and cumbersome and only able to be operated by highly skilled laboratory scientists. Furthermore, early SIFT-MS instruments had very poor limits of detection due to low ion currents. New instrumentation specifically designed for quantitative measurements often will generate total ion signals greater than 2×10^7 cps, which leads to routine measurements in the parts-per-trillion range being possible (23,25,26).

To properly utilize SIFT-MS as an analytical technique, at a level that will provide analyte quantification, requires knowledge of rate coefficients, product ion channels and their respective branching ratios, reactions of the water cluster ions with the analyte, and secondary reactions of major product ions with H_2O. Currently, the database of this knowledge contains over 400 compounds that can be quantified by SIFT-MS without

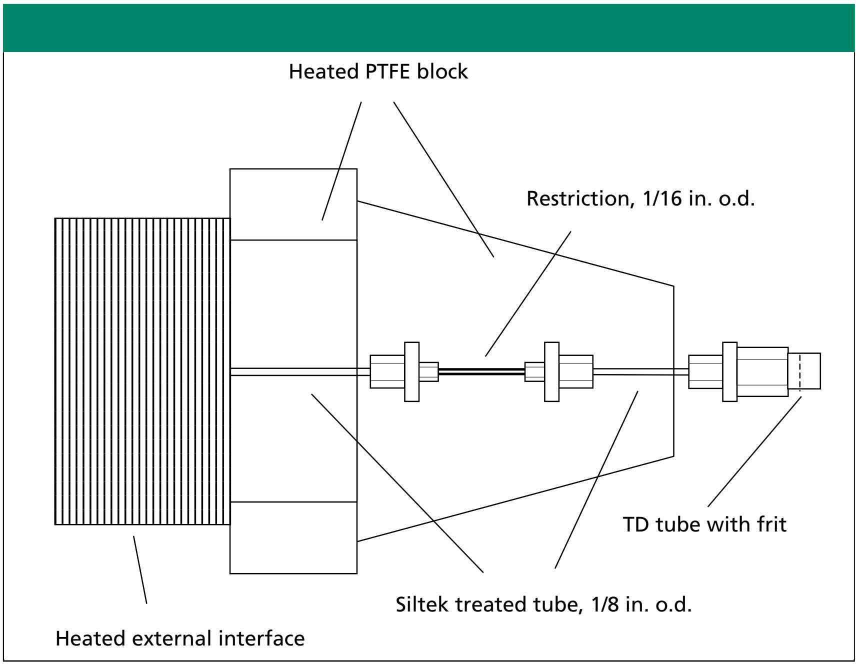

Figure 2: Schematic drawing of the SIFT-MS system's heated external interface with module for direct coupling to a microchamber–thermal extractor.

Table II: Overview of volatile aldehydes in the headspace of different malts after headspace SPME GC–MS expressed in µg/kg malt. All compounds were PFBHA derivatized and detected by monitoring *m*/*z* = 181 in SIM mode.

Component	Malt sample				
	A	B_1	B_2	B_3	C
Hexanal	173 ± 10	705 ± 37	834 ± 27	1010 ± 41	736 ± 5
trans-2-Nonenal	29 ± 2	41 ± 1	58 ± 2	74 ± 3	66 ± 3
2-Methylpropanal	612 ± 17	1668 ± 41	1885 ± 5	2311 ± 39	1475 ± 99
2-Methylbutanal	467 ± 21	829 ± 19	952 ± 19	1119 ± 111	749 ± 82
3-Methylbutanal	1213±137	3270±308	3674±120	4197±192	4271±341
Methional	281 ± 29	366 ± 11	224 ± 7	377 ± 5	566 ± 5
Phenylacetaldehyde	400 ± 19	725 ± 46	736 ± 31	853 ± 35	617 ± 70
Benzaldehyde	64 ± 4	81 ± 3	86 ± 23	93 ±10	99 ± 16
Furfural	285 ± 6	291 ± 46	404 ± 63	416 ± 1	412 ± 30

the need for an external calibrant.

A unique feature of SIFT-MS is its ability to quickly and directly analyze the headspace samples mentioned earlier, without requiring specific sample preparation or derivatization techniques. Moreover, the use of three reagent ions, generally, creates sufficient selectivity for real-time analysis without the need for (time-consuming) chromatographic separations. The SIFT-MS application range is already very broad and is still broadening as this article goes to press. Applications such as breath analysis (21,27), environmental monitoring (28), oil exploration (29), ambient air monitoring for occupational safety and health (30), and the detection of chemical warfare agents (26) and peroxide-based explosives (31) have been published recently.

In this article, we present our results on the evaluation of dynamic headspace SIFT-MS to discern volatile profiles and composition of various malted barley cultivars. Prime focus is on the analysis of aldehydes and particularly as compared to the headspace SPME procedure currently used.

Experimental

Reagents: All chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri) at the highest purity available.

Malt Samples: Five different malt samples were used during this study. All were produced from barley on industrial scale, and referred to as A, B_1, B_2, B_3, and C. Three different barley cultivars were distinguished: A, B and C. From the single-variety industrial malt B, three different harvest years were studied — B_1, B_2, and B_3. Samples A, B_1, B_2, and B_3 were supplied by the same malting plant.

Headspace SPME GC–MS Analysis: Volatile aldehydes in malt were quantitatively determined according to Vesely and colleagues (19). Extraction of marker aldehydes from CO_2-milled malt samples (0.25 g in 10 mL water) was performed by headspace SPME with on-fiber PFBHA derivatization using a 65-µm PDMS-DVB coated fiber (Supelco, Bellefonte, Pennsylvania). The PFBHA (1 g/L) was loaded during 10 min at 50 °C, after which extraction and derivatisation was carried out for 30 min. The carbonyl derivatives were analyzed using a TraceGC/DSQ II GC–MS system (Thermo Fisher Scientific, Madison, Wisconsin) purchased at Interscience (Louvain-la-Neuve, Belgium). The system was equipped with a CTC CombiPAL autosampler, a split–splitless injector with narrow-bore glass inlet liner, and an RTX-1 fused-silica capillary column (40 m × 0.18 mm, 0.2-µm film thickness, Restek). Helium was used as carrier gas at 0.8 mL/min. The inlet temperature was set at 250 °C, and injection was carried out in split mode (split ratio 50:1). The oven temperature was kept at 50 °C for 2 min, then raised to 210 °C at 6 °C/min, followed by an increase to 250 °C at 15 °C/min, and finally held at 250 °C for 5 min. The MS transfer line was set at 260 °C. Ionization of the carbonyl derivatives was obtained by electron ionization and the main fragment ion (*m*/*z* = 181) was detected in the single ion monitoring scan mode (19). Data were processed with XCalibur software (Thermo Fisher Scientific).

SIFT-MS Analysis: A commercial SIFT-MS instrument (Voice200, Syft Technologies, Christchurch, New Zealand), was used for this work. The system was equipped with a direct inlet and a heated external interface, which provided

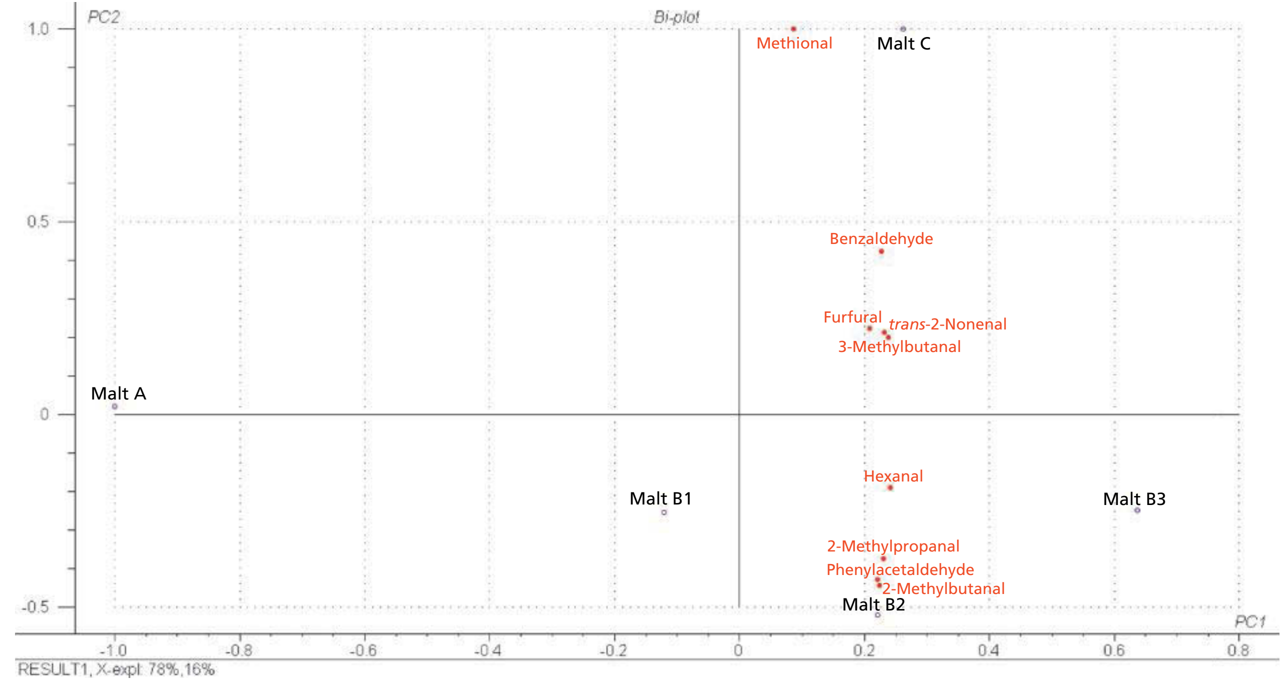

Figure 3: Combined score/loading plot as the result of principal component analysis (PC1 versus PC2) displaying the differentiation of malt varieties (objects) on the basis of their volatile pattern targeted by headspace SPME GC–MS (variables).

direct entry to the flow tube. The external interface was Siltek treated (Restek) to minimize activity.

Target compounds were analyzed using selected ion mode (SIM). Here, abundance is derived from the measured signal intensity at the specific product ion masses. The following compounds were targeted: 2,3-butanedione, formaldehyde, acetaldehyde, hexanal, *trans*-2-pentenal, *trans*-2-hexenal, *trans*-2-heptenal, *trans*-2-octenal, *trans*-2-nonenal, *trans,trans*-2,4-decadienal, 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, methional, phenylacetaldehyde, benzaldehyde, and furfural. All relevant instrumental settings, target and product ions, monitored reactions, and branching ratios are summarized in Table I. The majority of the data was obtained from a proprietary compound library database included with the instrument (25). Care was taken not to induce any conflicts in selecting target product ion masses. Soft ionization with minimal fragmentation in combination with three readily available reagent ions offers sufficient selectivity to discern between most isobaric compounds, for example trans-2-hexenal and furfural. Of course, this selectivity is not ultimate, such as with 2-methylbutanal and 3-methylbutanal, two isomers that could not be discerned from each other.

Static Headspace SIFT-MS Analysis: Static headspace analyses were carried out with a CTC CombiPAL autosampler (Thermo Fisher). The system was installed on a TraceGC system (Thermo Fisher), which was equipped with a standard split–splitless injector with a laminar cup liner. Due to the size and weight of the SIFT-MS instrument (900 × 725 × 875 mm, 212 kg), the SIFT-MS system had to be placed on the laboratory floor, close to the GC system. Hyphenation was achieved by means of the instrument's heated external interface, which entered the GC oven at the left-hand side, similar to a regular benchtop MS. The SIFT-MS and GC–MS instruments were connected directly to each other without any restriction installed in the heated external interface, by means of a piece of 5-m of deactivated fused-silica capillary tubing (Siltek 0.25 mm i.d., Restek). During analysis, the split–splitless injector, GC oven, and heated external interface were kept at 200 °C. Carrier gas was helium at 70 kPa, which corresponded with a flow rate of 10 mL/min. Injections were made in splitless mode.

Ungrounded malt grains (±5 g) were placed in a 20-mL headspace vial, capped, and subsequently placed on the autosampler tray. A blank sample was prepared by analyzing an empty vial (laboratory air). All samples were equilibrated at 50 °C or 75 °C for 10 min. Afterwards, part of the headspace was sampled by means of a gas-tight syringe (2.5 mL, Hamilton, Reno, Nevada) and transferred to the split–splitless injector. Other headspace conditions were set as follows: syringe temperature, 150 °C; agitation speed, 500 rpm; syringe fill speed, 100 µL/s; injection volume, 2.5 mL; and injection speed, 10 µL/s.

Dynamic Headspace SIFT-MS Analysis: Volatile emissions from ungrounded malt grains were sampled with a micro-chamber–thermal extractor (µ-CTE, Markes International, Llantrisant, UK). The system consisted of six Siltek-treated stainless steel sample containers (44 mL capacity each), which could be sealed from exterior air to prevent contamination from occurring. Usually, the micro-chamber–thermal extractor is used as a miniaturized alternative to carry out material emission testing. Therefore, the entire assembly is fed with a constant flow of (inert) gas and brought to high temperature (120 °C max). At the same time, each active sample container is fitted with a thermal desorption tube, which is filled with an appropriate packing material to enrich the released volatiles. Afterwards, compounds are removed from the tube by means of thermal desorption or another appropriate technique.

A schematic representation of the analytical set-up for volatile malt composition analysis with the SIFT-MS instrument and the microchamber–thermal extractor for dynamic headspace sampling is depicted in Figure 1. Practical hyphenation between both instruments was achieved by connecting the SIFT-MS instrument heated external interface with the outlet of the microchamber–thermal extractor sample container. To achieve this, a small piece of an empty thermal desorption tube was connected to the external interface by means of a 1/4-in. nut and ferrule. The metal frit, which normally is used to secure the packing material, was left in place to serve as a filter to prevent small dust particles from entering the flow tube. Contrary to the static headspace set-up, the external interface was furnished with an internal restriction to control and reduce the flow towards the flow tube. The restriction reduced the flow to 10 mL/min and simplified handling, which permitted easy changeover from container to container without vacuum disturbance. A schematic drawing of the interface and a representation when in use are given in Figure 2. Approximately 25 g of malt was used for analysis. During sampling, the microchamber–thermal extractor was kept at 50 °C, while the flow rate was set at 10 mL/min (nitrogen). The total test time was 20 min — 10 min equilibration and 10 min actual data acquisition.

Principal Component Analysis: Principal component analysis (PCA) was performed for interpretation of the results in a statistical way. PCA is a projection method (bilinear modeling method) that offers an interpretable overview of the main information in a multidimensional data table. The information carried by the original variables is projected onto a smaller number of underlying variables (principal components). The first principal component (PC1) covers as much of the variation in the data as possible. The second principal component (PC2) is orthogonal to the first and covers as much of the remaining variation as possible, and so on. The result of PCA is displayed graphically to facilitate the identification of patterns in data and to detect interrelationships between different variables.

In this study, PCA was used for differentiation of the different malt samples on the basis of particular compounds in their volatile analytical pattern, which was obtained by headspace SPME GC–MS and by dynamic headspace SIFT-MS, respectively. PCA was done by means of multivariate data analysis software (The Unscrambler v9.2, CAMO, Oslo, Norway).

Results and Discussion

Headspace SPME GC–MS Analysis: Quantitative profiling of aldehyde markers was performed on all malt samples A, B_1, B_2, B_3, and C according to Vesely and colleagues (19) by headspace SPME with on-fiber PFBHA derivatization and capillary GC in combination with a quadrupole mass spectrometer operating in the single ion monitoring mode (*m/z* = 181; see Experimental section). The investigated aldehyde markers can be classified into Strecker degradation aldehydes (2-methylpropanal, 2- and 3-methylbutanal, methional, benzaldehyde, and phenylacetaldehyde), aldehydes formed during Maillard reactions (furfural) and lipid oxidation aldehydes (hexanal and *trans*-2-nonenal). These compounds are to be regarded as true markers for flavor instability of beer and are determined on a routine basis (32).

The quantitative results summarized in Table II are the mean values of two measurements with coefficients of variation situated between 0.2% and 25%. The malted barley cultivar A has a relatively low content of aldehyde markers compared with the cultivars B and C. Different crops from the same barley cultivar B, referred to as B_1, B_2, and B_3, vary considerably in their aldehyde concentrations. The aldehyde profile of variety C is characterized by a high concentration of 3-methylbutanal and methional.

In order to visualize the differentiation of the malt samples, the quantitative headspace SPME GC–MS data were processed with a multivariate data analysis software package described earlier (CAMO). The biplot as depicted in Figure 3 is the result of PCA on the data matrix composed of the different malt samples (objects) and the measured volatiles (variables) in each sample. The two first principal components explain 94% (PC1 78%, PC2 16%) of the total variance. Based upon their volatile composition, the various malt varieties A, B, and C are differentiated clearly by means of PCA. Malt A was characterized by the substantially lower concentrations of the selected aldehyde markers than were found for malt samples B and C. Malt C is differentiated from the other malt varieties by its higher amount of methional present. From malt variety B, harvest year B_1 is distinguished from B_2 and B_3 because lower concentrations were measured for this crop.

On the basis of quantitative GC–MS profiling of the selected aldehyde markers, clear classification of the malt samples was obtained by visualization of the data matrix by PCA. To evaluate the true potential of headspace SIFT-MS, the headspace SPME GC–MS analyses on the various malt varieties as described earlier were repeated using this innovative technique of real-time measurement. It was verified by multivariate data analysis that an equivalent differentiation of the various malt varieties can be obtained on the basis of their headspace profiles acquired by SIFT-MS.

Static Headspace SIFT-MS Analysis: Static headspace injection is by far the easiest way for automated analysis of volatile components by means of SIFT-MS. In the first test to determine overall method performance and sensitivity, malt type A was analyzed. Therefore, ungrounded malt grains were weighed into a headspace vial, equilibrated and analyzed. In total, 17 carbonyl compounds were measured, including the marker aldehydes determined by headspace SPME GC–MS. As an illustration, the resulting SIFT-MS trace for benzaldehyde after 10 min equilibration at 50 °C is depicted in Figure 4. The results of the other compounds are summarized in the bar graph in Figure 5. Compared to the blank analysis, of which the results also are depicted in Figures 4 and 5, only minor differences can be discerned. To increase the response of the target compounds, the extraction temperature was increased to 75 °C (see Figure 5). As expected, this led to a proportional increase in signal intensity for some of the volatile components, such as acetaldehyde, hexanal, furfural, 2,3-butanedione, 2-methylpropanal, 2-methylbutanal, and 3-methylbutanal. The perceived response increase, however, is not necessarily due to the elevated extraction temperature alone, but also is affected by

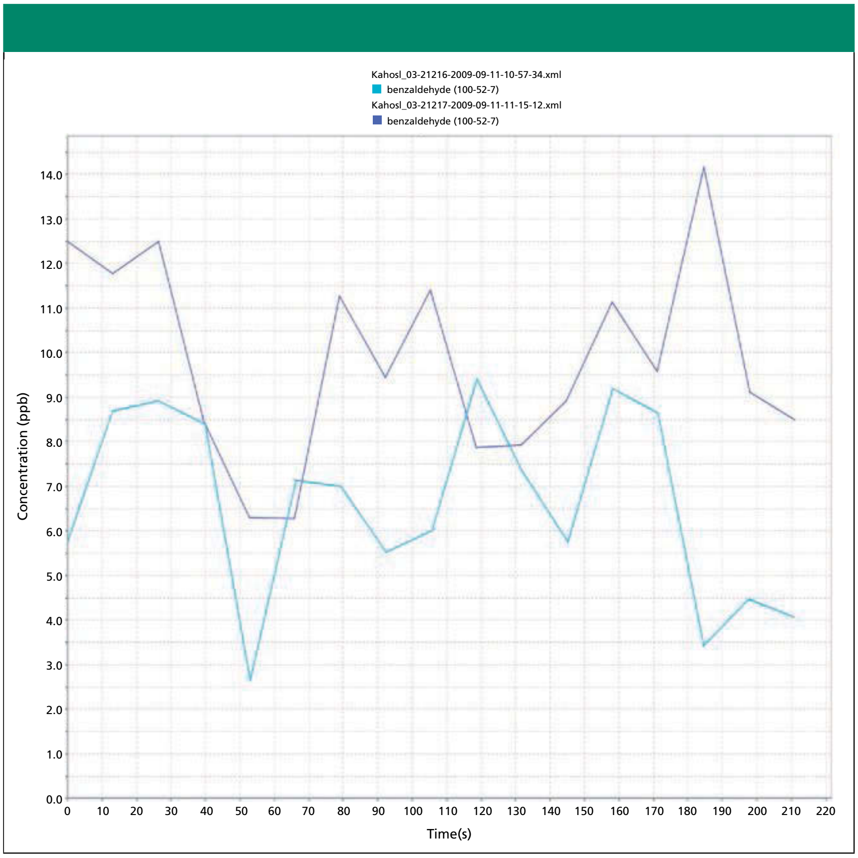

Figure 4: SIFT-MS trace of benzaldehyde after static headspace injection. Light blue trace shows the analysis of a blank sample (laboratory air); dark blue trace shows the analysis of malt A.

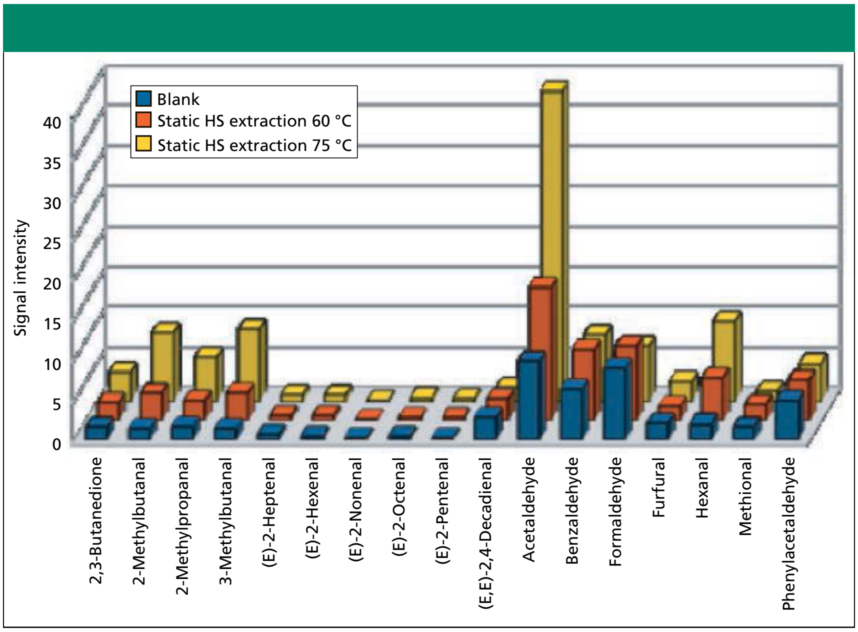

Figure 5: Volatile compounds targeted by static headspace SIFT-MS scans for malt type A.

the induction of oxidative processes and heat load, which creates artifacts. To avoid these oxidative reactions during analysis, sampling temperature is, preferably, kept as low as possible. As a result, static headspace injection was not considered a valuable injection technique for the analysis of aldehydes in malt.

Dynamic Headspace SIFT-MS Analysis: When directly analyzing the headspace of intricate samples such as malt, the sampling technique used in combination with SIFT-MS is of vital importance. Because the static headspace analyses did not produce significant levels of target volatiles, the experiment was repeated using dynamic headspace SIFT-MS. Compared to the static headspace procedure discussed earlier, dynamic headspace extraction with the microchamber–thermal extractor uses a substantially higher amount of material (25 g versus 5 g). As a result, higher absolute responses are obtained easily without the need to increase extraction temperature and without inducing stress-related emissions.

Dynamic headspace SIFT-MS was applied to the analysis of the target volatiles in all malted barley samples. Figures 6 and 7 show qualitative data for the less and more abundant volatiles, respectively. Each result is the mean of two replicate measurements with coefficients of variation situated between 0.5% and 15%. As can be deduced from both figures, headspace profiles clearly differ between malt variety as well as harvest year. For example, malt sample A is characterized by low levels of aldehydes and other target volatiles, and was received from the same malting plant as malt samples B_1, B_2, and B_3, but produced from a different barley cultivar. Depending on harvest year, the signal intensity of the target compounds varied substantially, as illustrated by malt samples B_1, B_2, and B_3. All originated from the same barley cultivar, but from different crops. Malt sample C was produced from a different barley variety than A and B, and malted by another malting plant, which expressed itself in a dissimilar headspace profile. Phenylacetaldehyde and benzaldehyde showed the highest signal intensities in malt samples B_1, B_2, B_3, and C. Obviously, malt sample A, which was characterized by only a limited number of abundant compounds, shows the lowest signal intensities for all detected volatiles. Knowledge of these variations in headspace profiles among various malted barley types, varieties, and harvest years is of great interest to many commercial users. Indeed, many of these compounds are described in literature as key odorants in barley and malted barley, and both the organoleptic quality of beers and their flavor stability during aging are affected by them (33–35).

The SIFT-MS results for the aldehyde markers are in accordance with the headspace SPME results earlier described. To verify the grouping of different malt

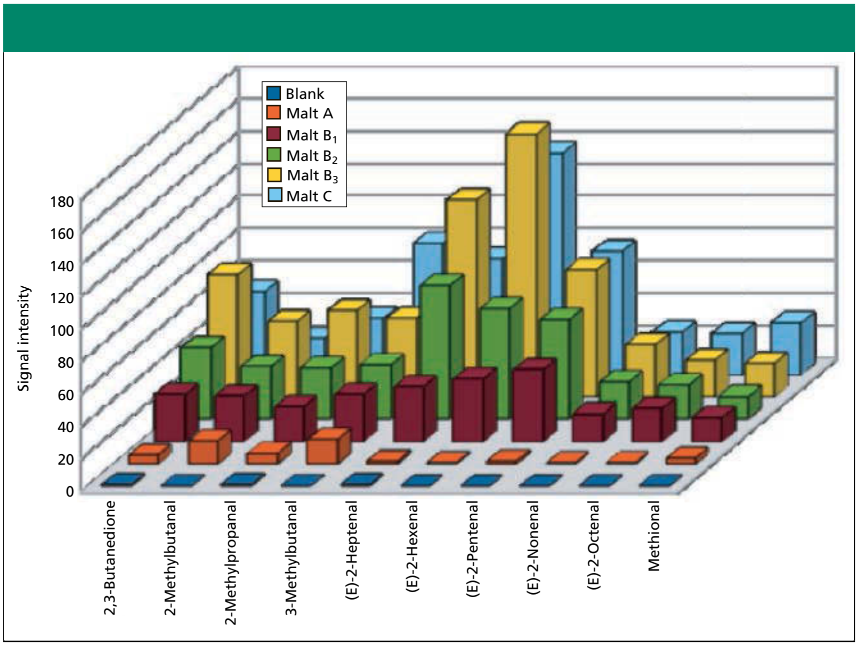

Figure 6: Minor volatile compounds targeted by dynamic headspace SIFT-MS scans for different malt types.

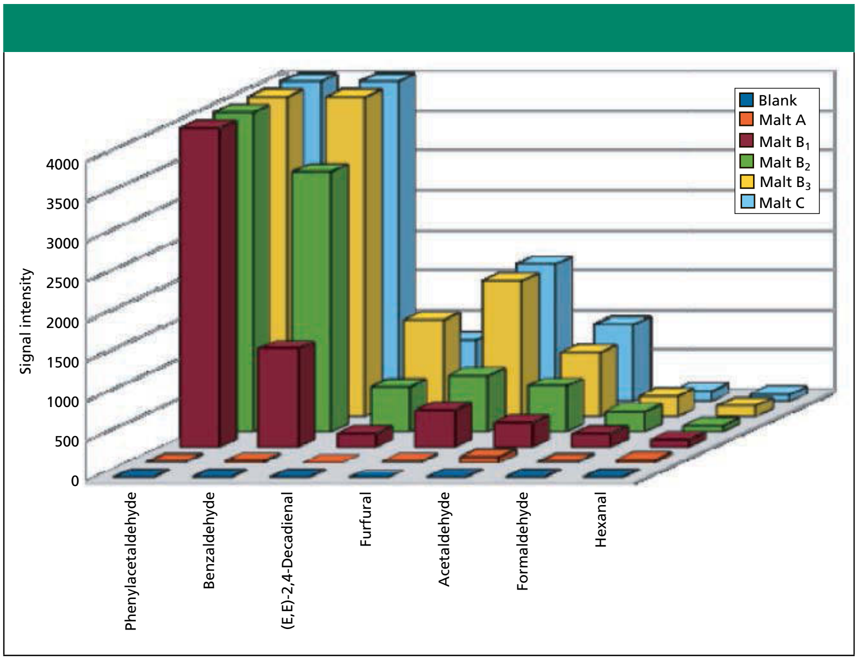

Figure 7: Major volatile compounds targeted by dynamic headspace SIFT-MS scans for different malt types.

varieties by PCA as was obtained with headspace SPME GC–MS, the dynamic headspace SIFT-MS data were processed with the multivariate data analysis software. To compare the results, the data matrix for PCA was composed of only the measured volatiles 2-methylpropanal, 2- and 3-methylbutanal, methional, benzaldehyde, phenylacetaldehyde, furfural, hexanal, and *trans*-2-nonenal (variables) in each of the malt samples (objects). Figure 8 represents the combined score–loading plot (biplot). Based upon the headspace profiles as acquired by SIFT-MS, this biplot bears comparison with the PCA-plot as was obtained previously from the headspace SPME GC–MS results depicted in Figure 3. Next to a similar, significant classification of the malt samples, Figure 8 also displays good reproducibility of the SIFT-MS results and exhibits that 95% of the total variance is explained by PC1 (79%) and PC2 (16%). The biplot in Figure 8 clearly visualizes the high potential of the SIFT analysis for a fast classification of the different malt samples on the basis of the selected measured volatiles. Moreover, this technique allows real-time measurement of substantially more volatiles than is done with headspace SPME GC–MS.

Conclusion

The objective of this study was to distinguish malt varieties on the basis of their headspace profiles by means of headspace SIFT-MS. A broad range of volatiles were identified readily by direct analysis of malt headspaces without sample preparation, derivatization, or chromatographic preseparation. Cycle time per individual sample could, therefore, be reduced to 10 min, which is substantially lower than the more commonly applied technologies.

The results demonstrate the potential of SIFT-MS for profiling different malts on the basis of their volatile composition (profiling) and for detecting the volatiles associated with malt quality (quality control, cultivar selection).

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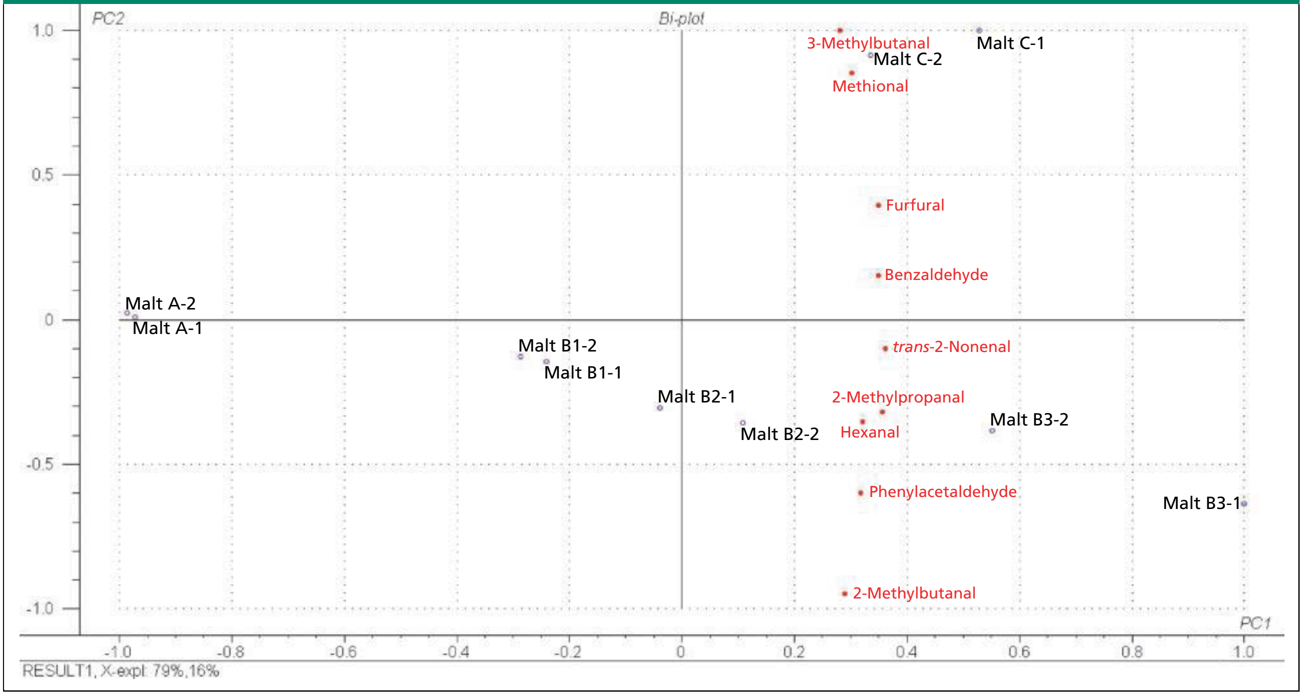

Figure 8: Combined score/loading plot as the result of principal component analysis (PC1 versus PC2) displaying the differentiation of malt varieties (objects) on the basis of their volatile pattern targeted by dynamic headspace SIFT-MS scans (variables).

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For more information on this topic, please visit www.chromatographyonline.com

THE APPLICATION NOTEBOOK

Call for Application Notes

LCGC is planning to publish the next issue of *The Application Notebook* special supplement in December. The publication will include vendor application notes that describe techniques and applications of all forms of chromatography and capillary electrophoresis that are of immediate interest to users in industry, academia, and government. If your company is interested in participating in these special supplements, contact:

Michael J. Tessalone, Group Publisher,
(732) 346-3016
Edward Fantuzzi, Associate Publisher,
(732) 346-3015
Stephanie Shaffer, East Coast Sales Manager,
(508) 481-5885

Application Note Preparation

It is important that each company's material fits within the allotted space. The editors cannot be responsible for substantial editing or handling of application notes that deviate from the following guidelines: Each application note page should be no more than 500 words in length and should follow the following format.

Format

- **Title:** short, specific, and clear
- **Abstract:** brief, one- or two-sentence abstract
- **Introduction**
- **Experimental Conditions**
- **Results**
- **Conclusions**
- **References**
- **Two graphic elements:** one is the company logo; the other may be a sample chromatogram, figure, or table
- **The company's full mailing address, telephone number, fax number, and Internet address**

All text will be published in accordance with *LCGC*'s style to maintain uniformity throughout the book. It also will be checked for grammatical accuracy, although the content will not be edited. Text should be sent in electronic format, preferably using Microsoft Word.

Figures

Refer to photographs, line drawings, and graphs in the text using arabic numerals in consecutive order (Figure 1, etc.). Company logos, line drawings, graphs, and charts must be professionally rendered and submitted as .TIF or .EPS files with a minimum resolution of 300 dpi. Lines of chromatograms must be heavy enough to remain legible after reduction. Provide peak labels and identification. Provide figure captions as part of the text, each identified by its proper number and title. If you wish to submit a figure or chromatogram, please follow the format of the sample provided below.

Tables

Each table should be typed as part of the main text document. Refer to tables in the text by roman numerals in consecutive order (Table I, etc.). Every table and each column within the table must have an appropriate heading. Table number and title must be placed in a continuous heading above the data presented. If you wish to submit a table, please follow the format of the sample provided below.

References

Literature citations must be indicated by arabic numerals in parentheses. List cited references at the end in the order of their appearance. Use the following format for references:

(1) T.L. Einmann and C. Champaign, *Science* **387**, 922–930 (1981).

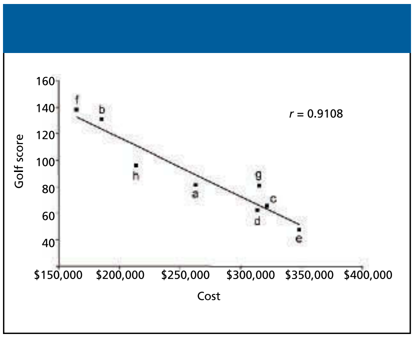

Figure 1: Chromatograms obtained using the conditions under which the ion suppression problem was originally discovered. The ion suppression trace is shown on the bottom. Column: 75 mm × 4.6 mm ODS-3; mobile-phase A: 0.05% heptafluorobutyric acid in water; mobile-phase B: 0.05% heptafluorobutyric acid in acetonitrile; gradient: 5–30% B in 4 min. Peaks: 1 = metabolite, 2 = internal standard, 3 = parent drug.

Table I: Factor levels used in the designs

Factor	Nominal value	Lower level (−1)	Upper level (+1)
Gradient profile	1	0	2
Column temperature (°C)	40	38	42
Buffer concentration	40	36	44
Mobile-phase buffer pH	5	4.8	5.2
Detection wavelength (nm)	446	441	451
Triethylamine (%)	0.23	0.21	0.25
Dimethylformamide	10	9.5	10.5

The deadline for submitting application notes for the December issue of **The Application Notebook** is:

October 22, 2010

This opportunity is limited to advertisers in ***LCGC North America***. For more information, contact:
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