THE APPLICATION NOTEBOOK

SUPPLEMENT TO

LC GC north america

solutions for separation scientists

How can we help your *sample preparation* today?

Improve *cost efficiency, productivity and accuracy* in your analysis of organic compounds.

A SUPPLEMENT TO LCGC NORTH AMERICA

September 2013

Horizon Technology can provide *automation* to help with:

- Reduced manual labor to allow technicians to focus on other tasks
- Minimize technique variability for improved reproducibility
- Decrease solvent usage by up to 90%
- Meet the most stringent regulatory requirements
- Improve technician safety and reduce exposure to solvents

From ***sample extraction*** using solid phase extraction with the **SmartPrep® cartridge extractor...**

to ***concentration*** with the **XcelVap™ Evaporation/ Concentration System.**

We can help make your laboratory *more efficient!*

Horizon technology

PREPARE FOR BETTER RESULTS

Call or check out our website: 1.800.997.2997 (US toll-free) • 1.603.893.3663 • www.horizontechinc.com

SUPPLEMENT TO
LC GC
north america
solutions for separation scientists

THE APPLICATION NOTEBOOK

September 2013
www.chromatographyonline.com





THE APPLICATION NOTEBOOK
A SUPPLEMENT TO LCGC NORTH AMERICA
ADVANSTAR SCIENCE
September 2013

If the first RI Detector for UHPLC were any more sensitive, it would need a special lab bench.

The Optilab® UT-rEX™ from Wyatt Technology provides 256 times the detection power of any refractive index detector. And up to 50 times the dynamic range. In fact, the Optilab delivers its extreme sensitivity over its entire range, picking up large *and* small signals within the same data run. Which is what you would expect from the first new RI technology in over 40 years.

The UT-rEX can be connected to *any* UHPLC system, so you can finally get information that was undetectable with UV and unavailable from mass spec or ELSD. No wonder other RI's are so depressed.

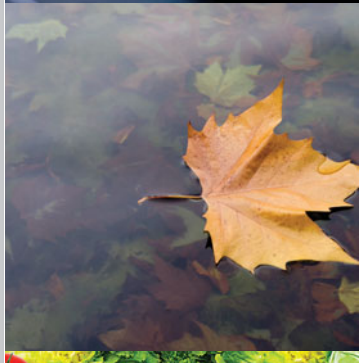
Since the UT-rEX sensitivity doesn't depend on chromophores, you can examine samples that were impossible to analyze using UHPLC.

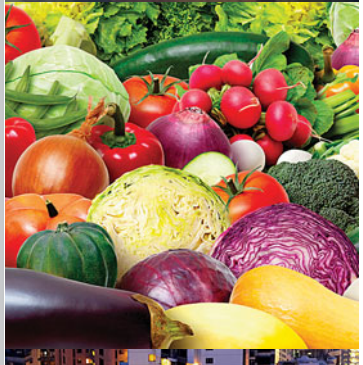
And how does it do it? Well, its small flow cell minimizes sample broadening issues and ensures temperature stability. And the built-in Peltier lets you control temperatures anywhere from 4°C to 50°C.

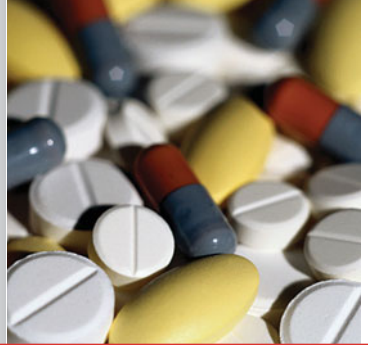
For more information on the Optilab UT-rEX, please call 805-681-9009 or visit us at **wyatt.com**. We'll provide all the analysis you need without driving you crazy.

WYATT TECHNOLOGY
www.wyatt.com

THE APPLICATION NOTEBOOK



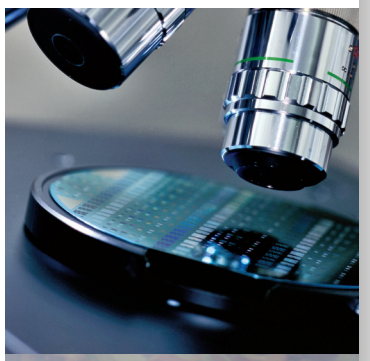


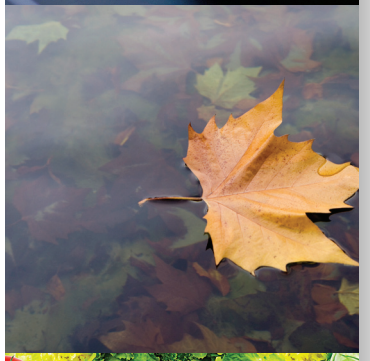


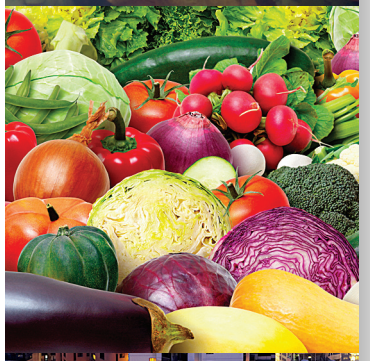
Medical/Biological

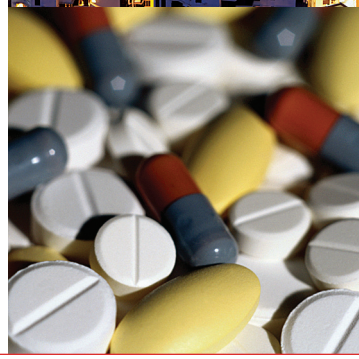
Chiral

Environmental







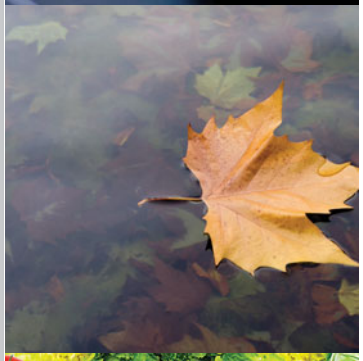
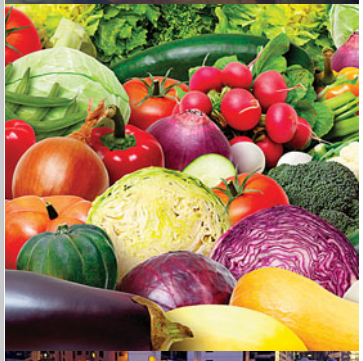


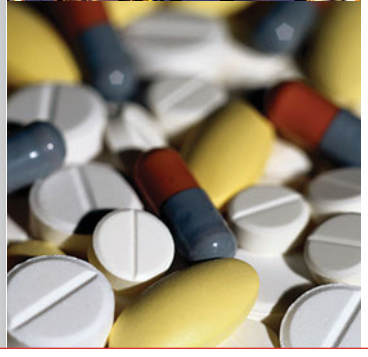
Food and Beverage

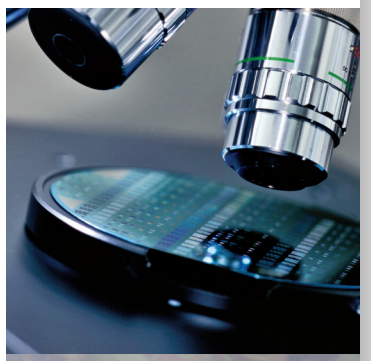
Industrial

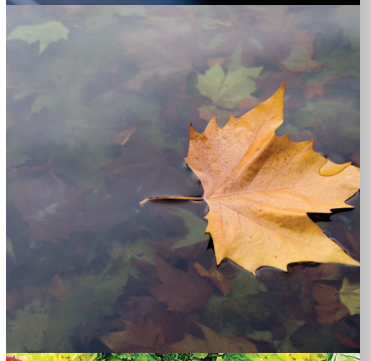
Pharmaceutical/Drug Discovery

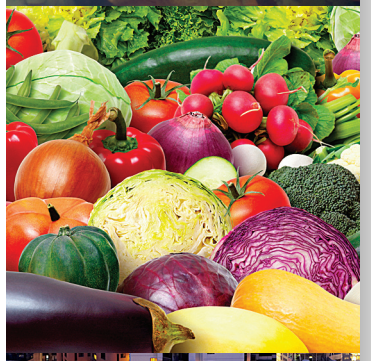
Polymer

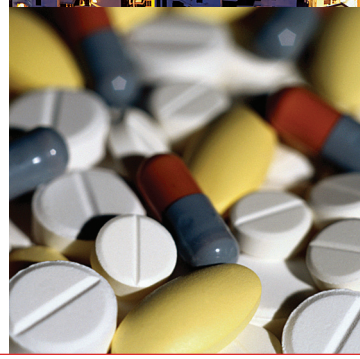














Articles

Departments

Cover Photography: Getty Images

LCGC® north america

MANUSCRIPTS: For manuscript preparation guidelines, see *LCGC* **30**(12), 1063 (2012), or call The Editor, (732) 596-0276. *LCGC* welcomes unsolicited articles, manuscripts, photographs, illustrations, and other materials but cannot be held responsible for their safekeeping or return. Every precaution is taken to ensure accuracy, but *LCGC* cannot accept responsibility for the accuracy of information supplied herein or for any opinion expressed.

SUBSCRIPTIONS: For subscription and circulation information: *LCGC*, P.O. Box 6168, Duluth, MN 55806-6168, or call (888) 527-7008 (7:00 a.m.–6:00 p.m. central time). International customers should call +1-218-740-6477. Delivery of *LCGC* outside the United States is 14 days after printing. For single and back issues, call (800) 598-6008 or (218) 740-6480. (*LCGC Europe* and *LCGC Asia Pacific* are available free of charge to users and specifiers of chromatographic equipment in Western Europe and Asia and Australia, respectively.)

CHANGE OF ADDRESS: Send change of address to *LCGC*, P.O. Box 6168, Duluth, MN 55806-6168; alternately, send change via e-mail to magazines@superfill.com or go to the following URLs:
• Print: https://advanstar.replycentral.com/Default.aspx?PID=469
• Digital: https://advanstar.replycentral.com/?PID=469&V=DIGI
Allow four to six weeks for change. PUBLICATIONS MAIL AGREEMENT No. 40612608. Return all undeliverable Canadian addresses to: IMEX Global Solutions, P.O. Box 25542, London, ON, N6C 6B2, CANADA. Canadian GST number: R-124213133RT001.

DIRECT MAIL LIST RENTAL: Contact Tamara Phillips, tel. (440) 891-2773, e-mail TPhillips@advanstar.com.

REPRINTS: Reprints of all articles in this issue and past issues of this publication are available (500 minimum). Call 877-652-5295 ext. 121, or email bkolb@wrightsmedia.com. Outside US, UK, direct dial: 281-419-5725. Ext. 121.

MARKETING DEVELOPMENT/CLASSIFIED: Contact Tod McCloskey, tel. (440) 891-2739, fax (440) 826-2865.

RECRUITMENT: Contact Tod McCloskey, tel. (440) 891-2739, fax (440) 826-2865.

INTERNATIONAL LICENSING: Contact Maureen Cannon, tel. (440) 891-2742, fax (440) 891-2650, or e-mail mcannon@advanstar.com.

Advanstar Communications Inc. (www.advanstar.com) is a leading worldwide media company providing integrated marketing solutions for the Fashion, Life Sciences and Powersports industries. Advanstar serves business professionals and consumers in these industries with its portfolio of 91 events, 67 publications and directories, 150 electronic publications and Web sites, as well as educational and direct marketing products and services. Market leading brands and a commitment to delivering innovative, quality products and services enables Advanstar to "Connect Our Customers With Theirs." Advanstar has approximately 1000 employees and currently operates from multiple offices in North America and Europe.

Peptide Mapping of Glycoprotein Erythropoietin by HILIC LC–MS and RPLC–MS

Alex Zhu, James Martosella, and Phu T. Duong, Agilent Technologies Inc.

Peptide mapping is an important technique for the comprehensive characterization of protein biotherapeutics. Reversed-phase ultrahigh-pressure liquid chromatography/high performance liquid chromatography (UHPLC/HPLC) is routinely used, but if the digest contains hydrophilic peptides, valuable information can be missed. In this work, we demonstrate peptide mapping of digested glycoprotein erythropoietin (EPO) using hydrophilic interaction chromatography (HILIC) as a complementary approach to reversed-phase chromatography peptide analysis. An Agilent ZORBAX Rapid Resolution High Definition (RRHD) 300-HILIC 1.8 µm LC column and AdvanceBio Peptide Mapping RP column, in combination with time-of-flight (TOF) mass spectrometry (MS), were used for mapping EPO protein. Taking advantage of the high organic solvent system of the mobile phase for HILIC, the digested peptides from these analyses were evaluated and compared for sequence coverage and peptide identification. This work demonstrates the utility of HILIC as an orthogonal and complementary approach to reversed-phase LC–MS for peptide analysis.

Table I: Peptides common to both columns

Peptide	Sequence	Hydrophobicity	RP Retention Time (min)	HILIC Retention Time (min)
P1	APPR	1.83	2.103	10.259
P2	GKLK	3.84	2.118	8.437
P3	ALGAQK	4.57	2.119	9.181
P4	AVSGLR	9.15	2.13	8.316
P5	YLLEAK	19.64	15.698	8.014
P6	VYSNFLRGK	23.14	18.742	6.583
P7	SLTTLLR	24.79	20.109	7.492
P8	VNFYAWKR	27.64	22.87	0.587

Table II: Peptides identified only from the HILIC column

No	Sequence	Hydrophobicity	Retention Time (min)	Height
1	VLER	6.24	6.747	1396603
2	LKLYTGEACRTGDR	18.13	9.175	2192
3	ALGAQKEAISPPDAAS-AAPLRTITADTFR	37.09	11.059	5263
4	APPRLICDSRVLER	27.7	6.493	1485
5	GQALLVNSSQPWE-PLQLHVDK	40.19	9.103	1995
6	KLFRVYSNFLR	36.51	4.629	3745
7	LFRVYSNFLR	35.24	4.945	1972

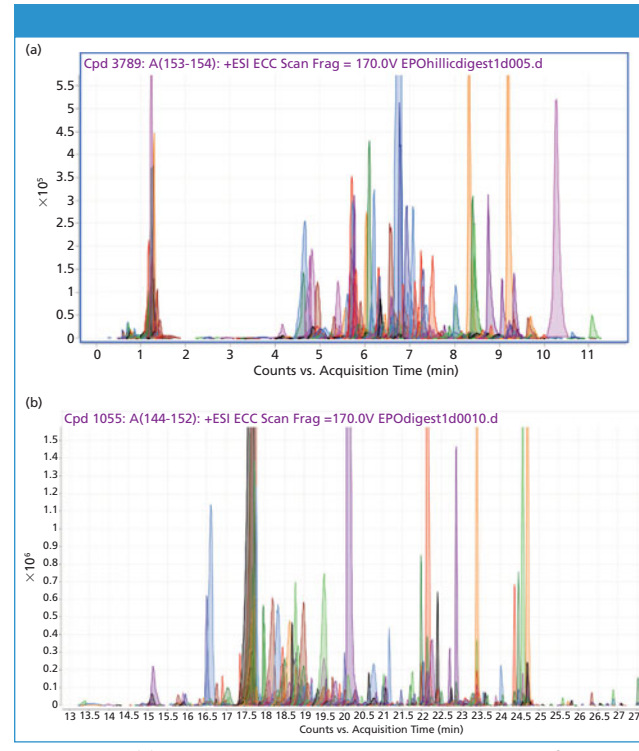


Figure 1: (a) Extracted compound chromatograms of matched erythropoietin (EPO) digested peptides from an Agilent ZORBAX RRHD 300-HILIC column and (b) an Agilent AdvanceBio Peptide Mapping RP column, both using the Agilent MassHunter molecular feature extractor.

Results and Discussion

The elution order in reversed-phase LC and HILIC is orthogonal. In reversed-phase separation, the digested peptides from EPO protein are eluted in order of increasing hydrophobicity, but with HILIC the least hydrophobic peptides (hydrophilic) will be retained most strongly by the column. Subsequently, the elution order is reversed. The use of HILIC columns for the analysis of the peptides obtained from an enzymatic digest of a protein would therefore be expected to provide increased retention and resolution of the hydrophilic peptides, including glycopeptides, compared with reversed-phase columns. Hence, digested peptides can be identified by HILIC that may not have been retained and resolved by reversed-phase chromatography.

The biotherapeutic glycoprotein, EPO, is a small protein and has a molecular weight of approximately 34,000 Da. It is known to be heavily glycosylated and therefore a tryptic digest would be expected to contain a range of peptides, including hydrophilic peptides and glycopeptides.

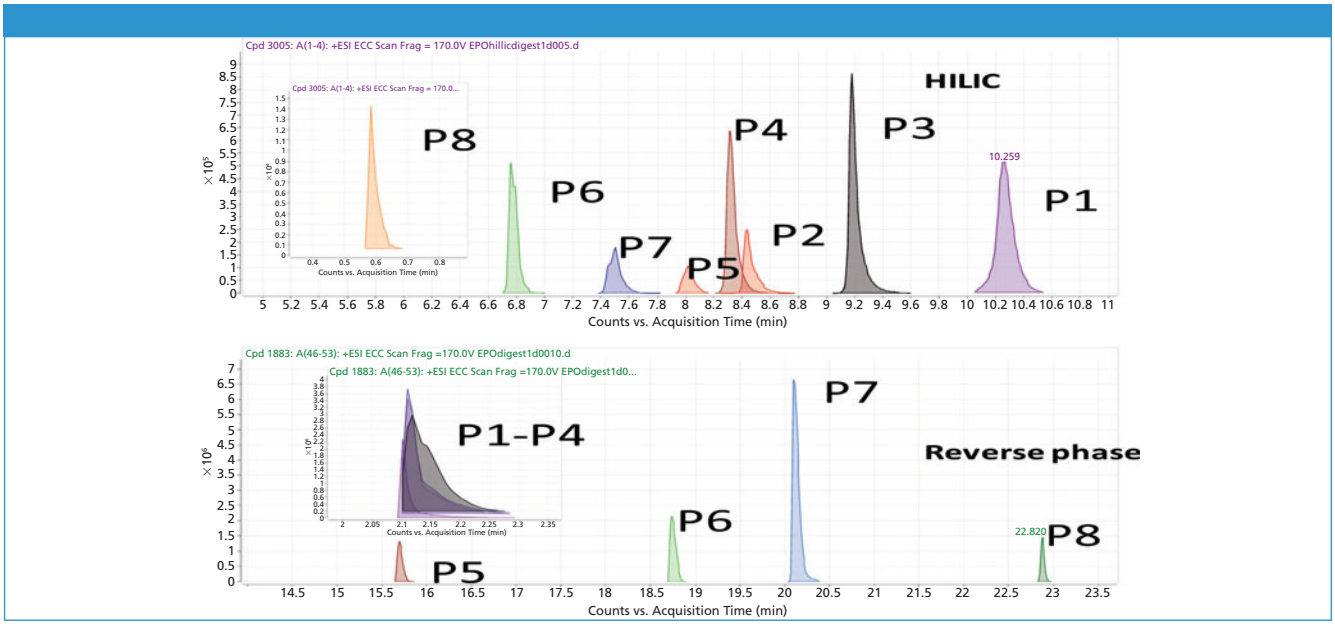


Figure 2: Comparing eight peptides from both columns for their retention times and resolutions.

Table III: Glycopeptides from trypsin-digested erythropoietin (EPO) glycoprotein found using the ZORBAX RRHD 300-HILIC column

	Sequence	Seq. Location	RT	Glycosylation
1	EAENITTGCAEHCSLNENITVPDTK	21-45	9.425	1111 0A 1G (+1710.5977)
2	EAENITTGCAEHCSLNENITVPDTK	21-45	9.392	3022 2A 0G (+2407.8518)
3	EAENITTGCAEHCSLNENITVPDTK	21-45	9.425	3021 1A 0G (+2407.8518)
4	EAENITTGCAEHCSLNENITVPDTK	21-45	9.500	3020 0A 0G (+1825.6610)
5	GQALLVNSSQPWEPLQLHVDK	77-97	9.103	0100 0A 0G (+1038.3751)

A comparison of mass spectrometry analysis of digested peptides from EPO glycoprotein using ZORBAX RRHD 300-HILIC and AdvanceBio Pepping Mapping RP column is shown in Figure 1a and 1b.

The HILIC LC–MS results were extracted using the Agilent MassHunter molecular feature extractor and then matched to the digested EPO protein sequence, showing that the sequence coverage was 100% (Figure 1a). It is noteworthy that the separation took less than 15 min.

The same sample was then analyzed using the AdvanceBio Peptide Mapping RP column. Extracted compounds of matching EPO-digested peptides again showed 100% sequence coverage (Figure 1b).

Peptides Common to HILIC and Reversed-Phase

Eight peptides were present from both columns when the data were compared. This indicated that these peptides had affinity for both modes of chromatography (see Table I). Generally, the elution order of the HILIC profile will be opposite to that of the reversed-phase profile, but not always. Elution orders are dictated by hydrophobicity and charge (on the peptides). Therefore, the HILIC order does not necessarily go from 8 to 1 as shown in Figure 2.

Peptides P1 to P4 were resolved better with the ZORBAX RRHD 300-HILIC column, as shown in the top and bottom panels of Figure 2. They eluted together on the reversed-phase column.

Peptides Found Only from HILIC

Generally, under reversed-phase conditions, the least hydrophobic peptides (hydrophilic) will elute early, making their quantitation by MS analysis more difficult. Moreover, some very hydrophobic peptides are difficult to dissolve in aqueous conditions, which are usually used as solvents for reversed-phase LC–MS analysis. This also leads to lower sensitivity. Therefore, with high organic solvent mobile phase, and the sample mixed with a high percentage organic solvent, some highly hydrophobic peptides will be dissolved and separated better with the HILIC column. Data from Table II provides an example showing hydrophilic peptides that are only be identified by HILIC LC–MS.

Glycopeptides from Trypsin-Digested EPO Protein Found from HILIC Column

From the data in Table II, peptide number 5 is the glycopeptide found only from the HILIC column. Its sequence location, retention, and glycosylation identification are indicated in Table III. Moreover, one additional glycopeptide with the sequence EAENITTGCAEHCSLNENITVPDTK was identified using ZORBAX RRHD 300-HILIC column and has four different glycoforms (Table III).

EPO glycoprotein has three N-glycosylation sites at sequence locations 24, 38, and 83. The glycopeptides identified by the HILIC column are listed in Table III with the glycosylation sites presented in red color. Based on their retention times of the MS chromatogram (Figure 1a), these glycopeptides are very hydrophilic peptides. Similarly, four glycoforms of glycopeptide EAENITTGCAEHCSLNENITVPDTK were identified by the reversed-phase column. However, these glycoforms were eluted in void volume of the reversed-phase column without being resolved.

Experimental

Sample Preparation

A sample of trypsin-digested EPO glycoprotein was purchased from Bio Creative, Shirley, New York; 100 µL of the sample (2 mg/mL) was mixed with 100 µL of HILIC or reversed-phase eluent A solvent, as appropriate.

Operating Conditions

Experiments were performed on an UHPLC–TOF system, comprising an Agilent 1290 Infinity LC, accurate-mass 6224 TOF-LC–MS, with dual electrospray ionization (ESI) source in positive mode. Peptides from trypsin-digested EPO protein were separated using different HILIC and reversed-phase conditions.

Conditions, HILIC

Columns: Agilent ZORBAX Rapid Resolution High Definition 300-HILIC, 2.1 × 100 mm, 1.8 µm (p/n 858750-901)
Eluent: A = 95% acetonitrile + 5% water; B = 50 mM ammonium formate, pH 4.0
Flow rate: 0.4 mL/min

Gradient: Time (min)	% B
0	0
15	100
15.1	0
20	0

Temperature: 55 °C

Conditions, Reversed-phase

Column: Agilent AdvanceBio Peptide Mapping, 2.1 × 250 mm, 2.7 µm (p/n 653750-902)
Eluent: A = 100% water, 0.1% formic acid; B = 100% acetonitrile, 0.1% formic acid
Flow rate: 0.4 mL/min

Gradient: Time (min)	% B
0	3
3	3
33	45
38	60

Temperature: 55 °C

MS Conditions

Gas temperature: 350 °C
Gas flow: 10 L/min
Nebulizer: 45 psi
Capillary voltage: 3500 V
Fragmentor: 170 V
Scan rate: 2 spec/s
Mass range: 400–3200 *m/z*

Conclusions

The use of ZORBAX Rapid Resolution High Definition 300-HILIC could aid the mapping and identification of hydrophilic peptides that were not resolved by reversed-phase chromatography. Therefore, coupling this column with MS could be an orthogonal and complementary approach to reversed-phase LC–MS, to provide better retention for hydrophilic peptides including glycopeptides, therefore, potentially providing better sequence coverage and protein characterization.

Agilent Technologies

Agilent Technologies Inc.
2850 Centerville Road
Wilmington, DE 19808
www.agilent.com/chem/advancebio

Molecular Weight Determination of Low-Molecular-Weight Heparins: SEC-MALS vs. SEC-UV-RI

Wyatt Technology Corporation

Low-molecular-weight heparins (LMWHs) are obtained by fractionation or depolymerization of natural heparins. They are defined as having a mass-average molecular weight of less than 8000 and for which at least 60% of the total weight has a molecular mass less than 8000.

Size-exclusion chromatography (SEC) has been the most common way of measuring the molecular weight and molecular weight distributions of LMWHs by using the two most common detection technologies: ultraviolet (UV) coupled with refractive index (RI) detection. However, these detectors embody a relative method in order to determine molecular weights, requiring calibration standards. A newer, absolute method involves the use of multi-angle light scattering (MALS), which does not require any standards. The European Pharmacopeia (EP) monograph for LMWH specifies the use of the UV–RI detection method and provides a known calibration standard. Many laboratories around the world have adopted this method.

We previously developed an SEC–MALS method and found it to be very suitable for the analysis of LMWHs. We have recently adopted the UV-RI method described in the EP monograph and compared the molecular weight results generated for LMWH using each detection type. The adopted method uses an Agilent LC-1200 series HPLC, 0.2 M sodium sulfate pH 5.0 mobile phase, Tosoh TSK-gel G2000 SWxl column with Tosoh TSK-gel Guard SWxl, Waters 2487 dual wavelength UV detector, and Wyatt Optilab rEX refractive index detector. For MALS analysis, the UV detector was replaced with a Wyatt miniDAWN TREOS detector; all other methods aspects remained the same.

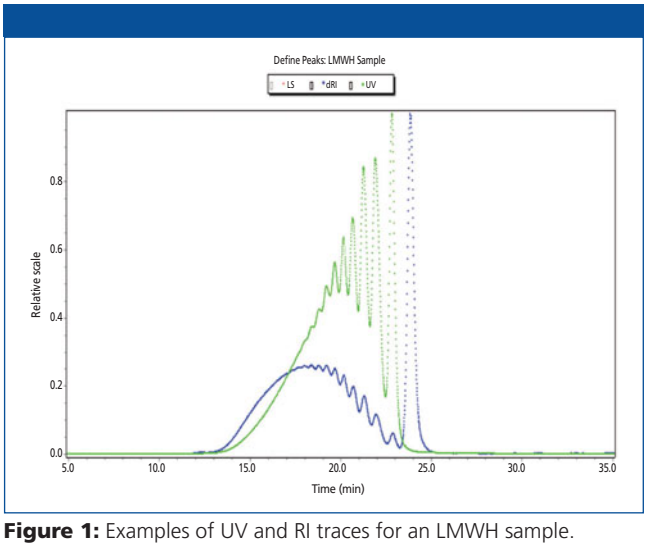


Figure 1: Examples of UV and RI traces for an LMWH sample.

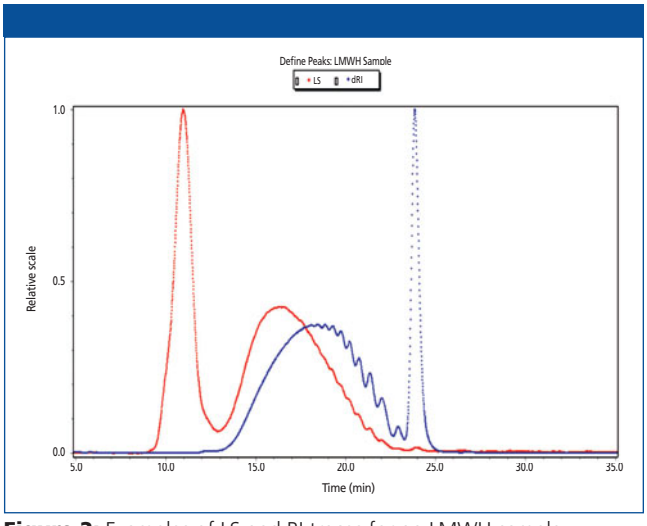


Figure 2: Examples of LS and RI traces for an LMWH sample.

The results indicated that both detection types are suitable and acceptable for the analysis of LMWHs. The molecular weight and distribution results generated using each detection type are comparable. This indicates that a SEC–MALS method could be adopted in place of the SEC–UV-RI method currently required by the EP monograph, and that it would result in less time because it obviates the need for calibration standards.

This note was graciously submitted by Lin Rao and John Beirne of Scientific Protein Laboratories LLC.

Wyatt Technology Corporation
6300 Hollister Avenue, Santa Barbara, CA 93117
tel. (805) 681-9009, fax (805) 681-0123
Website: www.wyatt.com

Separation of a Monoclonal Antibody Monomer from Its Impurities Using New TSKgel® SW mAb Columns

Atis Chakrabarti, PhD and Justin Steve, Tosoh Bioscience LLC

The analysis of monoclonal antibodies (mAb) is growing in importance in the field of biotherapeutics for the treatment of a variety of diseases. Quality control of therapeutic mAb is essential, as the introduction of species to the body other than the monomer may induce toxic side effects. Therefore, the pure antibody monomer must be very well resolved from its dimer and higher molar mass aggregates, as well as the antibody fragments. Size exclusion chromatography (SEC) is the best choice for determining mAb monomers and their impurities, including aggregates, oligomers, and mAb fragments.

Tosoh Bioscience has answered the call for dedicated SEC columns for the high resolution separation of mAb with the new silica-based 4 µm TSKgel SuperSW mAb HR column, for high resolution separation of the monomer and dimer, and the 3 µm TSKgel UltraSW Aggregate column for the separation and quantification of mAb aggregates and oligomers. This application note demonstrates the superb performance of these new columns for the analysis of monoclonal antibodies.

Experimental Conditions

Column:	TSKgel SuperSW mAb HR, 4 µm, 7.8 mm i.d. × 30 cm TSKgel G3000SWXL, 5 µm, 7.8 mm i.d. × 30 cm
Mobile phase:	200 mmol/L potassium phosphate buffer + 0.05% NaN_3, pH 6.7
Flow rate:	1.0 mL/min
Detection:	UV @ 280 nm
Temperature:	25 °C
Injection vol.:	10 µL
Sample:	10 g/L IgG digested with papain for 0–24 h
Column:	TSKgel UltraSW Aggregate, 3 µm, 7.8 mm i.d. × 30 cm
Mobile phase:	100 mmol/L potassium phosphate buffer, 100 mmol/L sodium sulfate, pH 6.7 + 0.05% NaN_3
Flow rate:	1.0 mL/min
Detection:	UV @ 280 nm
Temperature:	60 °C
Injection vol.:	20 µL
Sample:	BI-mAb-02 (4.6 mg/mL)

Results and Discussion

IgG monomer, dimer, and fragments digested by papain over a 24 h period were analyzed using the TSKgel SuperSW mAb HR column (Figure 1). The results exhibit the superior resolving power of this column for monomer/fragment and monomer/dimer separation (Rs = 2.87 and 2.02 respectively).

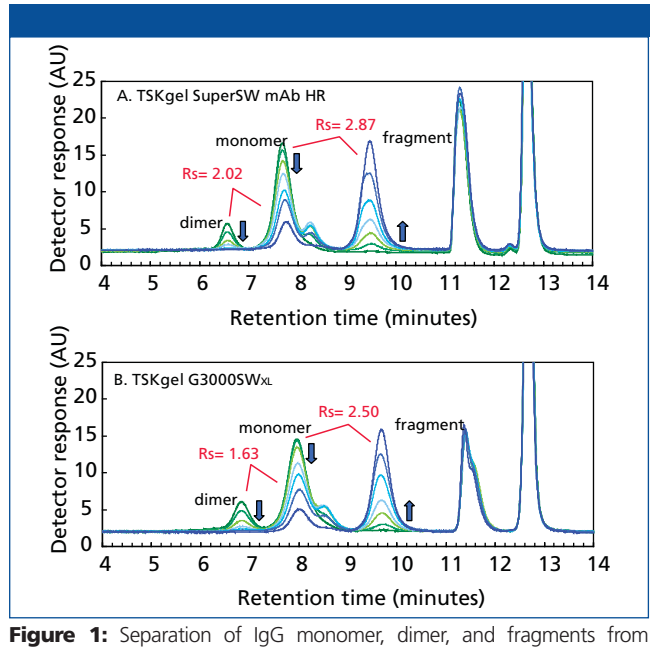


Figure 1: Separation of IgG monomer, dimer, and fragments from papain digested IgG by TSKgel SuperSW mAb HR and TSKgel G3000SWXL columns.

The results also show that the TSKgel SuperSW mAb HR column has superior performance of mAb separation in comparison to the TSKgel G3000SWXL column. While TSKgel G3000SWXL has set the standard for the separation of general proteins for more than 25 years, the new TSKgel SuperSW mAb HR column is more specifically suited for the analysis of mAb, as seen in the results of the analysis of IgG.

A heat denaturation study of a monoclonal antibody was conducted using a TSKgel UltraSW Aggregate column. The column was used to monitor the denaturation of the antibody as a function of time at pH 5.5 and 60 °C. Heating for 1 h at 60 °C results in almost complete breakdown of the monoclonal antibody and the formation of very large aggregates that extend to the exclusion volume of the column. As seen in Figure 2, the efficient separation of aggregates from the monomer, induced by heat denaturation, could be achieved using the TSKgel UltraSW Aggregate column. Also shown in Figure 2, an "unknown" aggregate peak of intermediate molar mass between the monomer and dimer and several higher order aggregate peaks, in addition to the presumed dimer peak at 8.5 min, was seen.

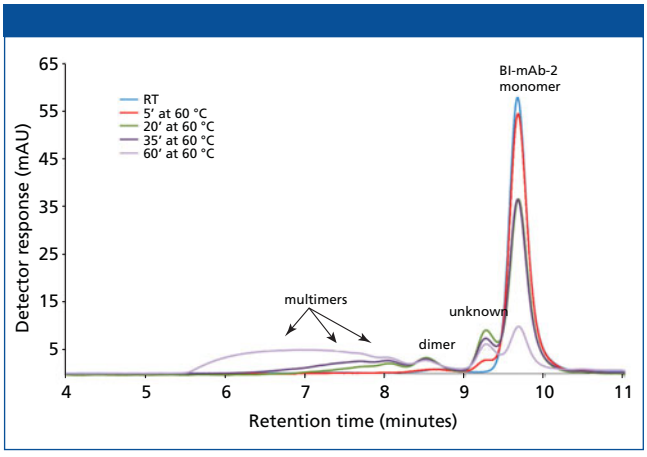


Figure 2. Heat denaturation study of monoclonal antibody (BI-mAb-02) using a TSKgel UltraSW Aggregate column.

Conclusions

The results of both analyses show the superb performance of the new TSKgel SuperSW mAb HR and UltraSW Aggregate columns for the analysis of monoclonal antibodies. The TSKgel SuperSW mAb HR column exhibited superior resolving power for IgG monomer, dimer, and fragments, while the TSKgel UltraSW Aggregate column demonstrated efficient separation of aggregates from the monomer peak. These new additions to the TSKgel SW-type column line are an excellent choice for your mAb analysis: TSKgel SuperSW mAb HR for high resolution monomer, dimer, and fragment analysis, TSKgel UltraSW Aggregate for superior resolution of aggregates.

TOSOH BIOSCIENCE

TOSOH

Tosoh Bioscience LLC
3604 Horizon Drive, Suite 100, King of Prussia, PA 19406
tel. (484) 805-1219, fax (610) 272-3028
Website: www.tosohbioscience.com

HPLC Separation of Anabolic Steroids on ZirChrom®-PBD

Dr. Dwight Stoll and Dr. Clayton V. McNeff, ZirChrom Separations, Inc.

This note shows the separation of three closely related anabolic steroids, boldenone, nandrolone, and testosterone, using a ZirChrom®-PBD column. A typical analysis of these compounds involves derivatization and subsequent quantitation by GC–FID or GC–MS, however these methods tend to be labor intensive, and analytically unreliable. Baseline resolution of all three compounds was obtained on ZirChrom®-PBD at slightly elevated temperature in under 10 min using isocratic conditions.

Pressure Drop: 160 bar
Temperature: 60 °C with Metalox™ 200-C Column Heater
Detection: UV at 215 nm

This separation allows for clear identification and quantification of these compounds without the use of expensive MS detection. The separation also requires no complex buffers and uses a minimum of organic modifier.

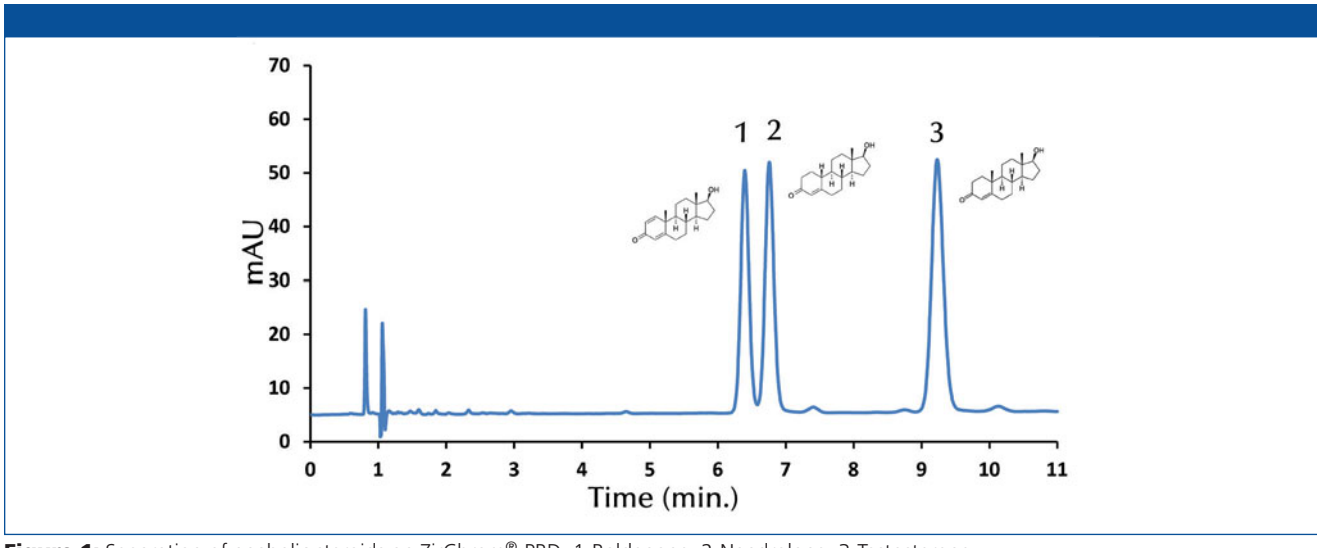


Figure 1: Separation of anabolic steroids on ZirChrom®-PBD, 1-Boldenone, 2-Nandrolone, 3-Testosterone.

Introduction

The rapid and accurate detection of anabolic steroids is crucial in today's sporting world. Historically the structural similarity of these compounds has made quantitative analysis by reversed-phase HPLC difficult at best. These steroids are very difficult to separate on silica ODS phases and provide nearly identical mass spectra. This method capitalizes on the unique temperature stability and surface chemistry of a zirconia-based stationary phase to achieve baseline resolution of these compounds in less than 10 min.

Experimental

A mixture of anabolic steroids was separated at 60 °C using a ZirChrom®-PBD column (see Figure 1) using the following conditions.

Column: 150 mm × 4.6 mm i.d. ZirChrom®-PBD
Mobile Phase: 15/85 ACN/Water
Flow Rate: 1.5 mL/min
Injection Vol.: 5 µL

Acknowledgments

(1) Walter Hyde, Iowa State University.

Visit www.zirchrom.com for more application notes using ultra-stable, high efficiency ZirChrom columns.

ZirChrom Separations, Inc.
617 Pierce Street, Anoka, MN 55303
tel. 1-866-STABLE-1
Website: support@zirchrom.com

Summarization of Screening Hits on the Whelk-O®1, RegisPack®, and RegisCell® Chiral Stationary Phases (CSPs)

Ted Szczerba, Regis Technologies, Inc.

The determination of enantiomeric purity is crucial in new drug development. The number of diverse chiral compounds is increasing and sensitive chiral methods are often needed quickly. With many new CSPs on the market, it is challenging to select the most important ones for the initial screening stages and to expedite method development. The focus of this study is to evaluate high selectivity Regis CSPs and to suggest the best screening method with a limited number of high success rate chiral columns.

Five-hundred and nineteen test compounds were screened on a number of different CSPs. These are client-submitted samples for Regis' free chiral screening service. The test library consists of active pharmaceutical ingredients (APIs), potential drug candidates, proprietary research compounds, or simple commercially available compounds.

While we decided to summarize results for all the samples screened, a number of these compounds are unique and there is no guarantee that they are truly chiral. It has been proven on numerous occasions that some of the samples submitted are achiral and cannot be separated on chiral columns.

The focus was on three of the most versatile columns on the market. They consist of the Whelk-O®1, RegisPack®, and RegisCell® CSPs. These three phases show a high success rate and broad versatility. In addition, these three CSPs are complementary in selectivity, as demonstrated by the number of unique hits for each CSP. All the columns were standard dimensions, 25 cm × 4.6 mm i.d. packed with a 5 µm particle size. Each compound was screened individually and a number of different mobile phases were applied during the screening process to achieve optimal separation.

Table I: Screening summary results of 519 test compounds

CSP	# of Hits	%
RegisPack®	238	46%
Whelk-O®1	174	34%
RegisCell®	174	10%
All Other CSPs	19	4%
# Not separated	140	—

# of hits on multiple CSPs
- Whelk-O®1 and RegisPack® – 63
- Whelk-O®1 and RegisCell® – 9
- RegisPack® and RegisCell® – 12
- Whelk-O®1, RegisPack®, and RegisCell® – 13

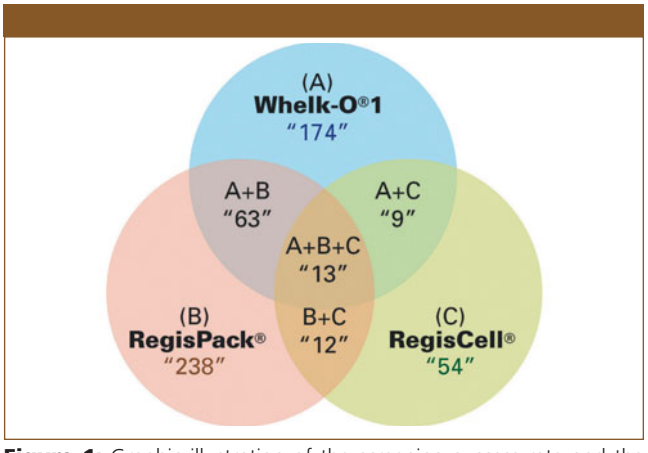


Figure 1: Graphic illustration of the screening success rate and the summary of results.

# of unique hits
- Whelk-O®1 – 89
- RegisPack® – 150
- RegisCell® – 20

Conclusion

Out of a total of 519 compounds screened, 379 compounds were separated on numerous CSPs. Out of the 379 compounds separated, 360 compounds were separated on either the Whelk-O®1, RegisPack®, or RegisCell® CSPs for a hit ratio of 95%. Some compounds were separated on more than one column (see Figure 1). These three CSPs account for a 70% success rate out of the total of 519 compounds screened.

With the Whelk-O®1, RegisPack®, and RegisCell® columns installed on a screening station, the chromatographer can expect greater than a 70% success rate in the screening process.

Note

If we were guaranteed that all of the compounds were truly racemic, the success rate would most likely be higher.

Regis Technologies, Inc.
8210 Austin Ave., Morton Grove, IL 60053
tel. (847) 583-7661, fax (847) 967-1214
Email: teds@registech.com, Website: www.registech.com/chiral

Polynuclear Aromatic Hydrocarbons in Water by Automated Solid Phase Extraction

FMS, Inc.

Polynuclear aromatic hydrocarbons (PAHs) consist of fused aromatic rings and are produced as byproducts of fuel burning. As pollutants, they are of concern because some compounds have been identified as carcinogenic, mutagenic, and teratogenic. PAHs are lipophilic and therefore found in the environment primarily in soil, sediment, and oily substances. However, they also appear in surface and ground water indicating a source of pollution.

Instrumentation and Consumables

- FMS, Inc. TurboTrace SPE system (solid phase extraction)
- FMS, Inc. SuperVap Concentrator
- FMS, Inc. 1 g PAH C18 cartridge
- Waters Alliance 2695 HPLC, UV254

Method Summary

TurboTrace SPE

1. Condition cartridge with 15 mL dichloromethane
2. Condition cartridge with 15 mL methanol
3. Condition cartridge with 35 mL water
4. Load 1 L of water sample
5. Dry cartridge for 20 min
6. Rinse sample bottle with 35 mL dichloromethane
7. Elute sample from cartridge with bottle rinse
8. Elute sample with additional 25 mL dichloromethane

SuperVap Concentrator

1. Pre-heat temp: 60 ºC
2. Pre-heat time: 20 min
3. Heat in Sensor mode: 60 ºC
4. Nitrogen pressure: 9 PSI

Table I: Results for three 1-L samples

Compound	Amount Spiked µg/L	Mean Recovery µg/L	SD	Percent Recovery
Naphthalene	5.00	3.57	0.67	71%
2-Methylnaphthalene	5.00	3.95	0.70	79%
Acenaphthylene	5.00	3.71	0.78	74%
Acenaphthene	5.00	4.08	0.71	82%
Fluorene	5.00	4.19	0.59	84%
Phenanthrene	5.00	4.71	0.44	94%
Anthracene	5.00	4.79	0.41	96%
Fluoranthene	5.00	5.22	0.33	104%
Pyrene	5.00	5.30	0.31	106%
Benzo(a)anthracene	5.00	5.34	0.35	107%
Chrysene	5.00	5.36	0.35	107%
Benzo(b)fluoranthene	5.00	5.35	0.38	107%
Benzo(k)fluoranthene	5.00	5.36	0.29	107%
Benzo(a)pyrene	5.00	5.35	0.37	107%
Indeno(1,2,3-cd)pyrene	5.00	5.22	0.41	104%
Dibenzo(a,h)anthracene	5.00	5.31	0.42	106%
Benzo(g,h,i)perylene	5.00	5.26	0.40	105%

Procedure

Three, 1 L samples were acidified to pH 2 with 6 N hydrochloric acid and spiked with 5 µL of Restek 31458 MA EPH aromatic hydrocarbon solution. Sample were then loaded onto the FMS TurboTrace SPE system using vacuum and passed across an FMS PAH C_{18} cartridge. After sample loading, the cartridges were dried automatically using a stream of nitrogen until no residual water was present, and the cartridges were subsequently eluted using dichloromethane from both the automatic sample bottle rinse and from the elution solvent reservoir. The extracts were concentrated to 1.0 mL and 5 mL acetonitrile was added for solvent exchange. The sample was concentrated to a final volume of 1 mL for HPLC analysis.

Conclusion

The FMS TurboTrace SPE system produces consistent reliable, reproducible results for polynuclear aromatic hydrocarbons in water. The FMS PAH SPE cartridge achieves high recovery extraction in water. Results reported from the TurboTrace™ SPE and SuperVap™ Direct-to-Vial Concentration system (Table I) show high levels of precision and accuracy for the parallel extraction and concentration of PAHs using the FMS integrated Sample-to-Vial systems. With expanded modules, the TurboTraceTM SPE system enables the lab to simultaneously extract eight samples hands free. Compared to manual extraction processes, this automated technique frees up chemists to focus on increasing sample throughput. The addition of direct to GC vial concentration vessels eliminates the need to manually transfer extract from the concentration tubes to the vials and reduces operator error.

FMS Inc.
580 Pleasant Street, Watertown, MA 02472
tel. (617) 393-2396, fax (617) 393-0194
Website: www.fmsenvironmental.com

Analysis of Perchlorate and Oxyhalides by HPLC–ESI–MS

Kanna Ito, Shodex/Showa Denko America Inc.

Perchlorate is reported to interfere with iodide uptake by the thyroid gland and may influence thyroid hormone production. In 2011, the US Environmental Protection Agency (EPA) issued a "regulatory determination" of perchlorate to meet the Safe Drinking Water Act criteria for regulation as a contaminant. EPA method 6850 "Perchlorate in water soils and solid wastes using high performance liquid chromatography/electrospray ionization/mass spectrometry (HPLC/ESI–MS or HPLC–ESI–MS-MS)" is designed for low level detection of perchlorate. The method calls for an analytical column that is capable of separating perchlorate from the sample matrix.

Shodex RSpak JJ-50 2D is packed with polyvinyl alcohol base gel modified with quaternary ammonium group. The separation can be achieved by a combination of partition/adsorption and anion exchange modes. Using JJ-50 2D, the mobile phase composition and flow rate were optimized for the separation of perchlorate with MS detection.

Experimental Conditions

(1) Column: Shodex RSpak JJ-50 2D, 2.0 mm i.d. × 150 mm length
(2) Detector: ESI-MS (SIM)
(3) Sample: Sodium salt of perchlorate (ClO_4^-), chlorate (ClO_3^-), bromate (BrO_3^-), chlorite (ClO_2^-), and chloride (Cl^-) were prepared in water. Tap water was directly injected.
(4) Injection volume: 5 µL

Results

The mobile phase and flow rate were optimized for the best separation of perchlorate and other anions. Also, they were optimized to achieve high sensitivity detection by the MS. Figure 1 shows sample chromatograms. The optimized eluent condition obtained was 50 mM ammonium formate (aq) / acetonitrile = 25/75 and the flow rate was 0.2 mL/min. The column dimension of 2.0 mm i.d. × 150 mm length is suitable for the flow rate selection for the MS. Using this method, quantification limit of ~0.1 µg/L was possible.

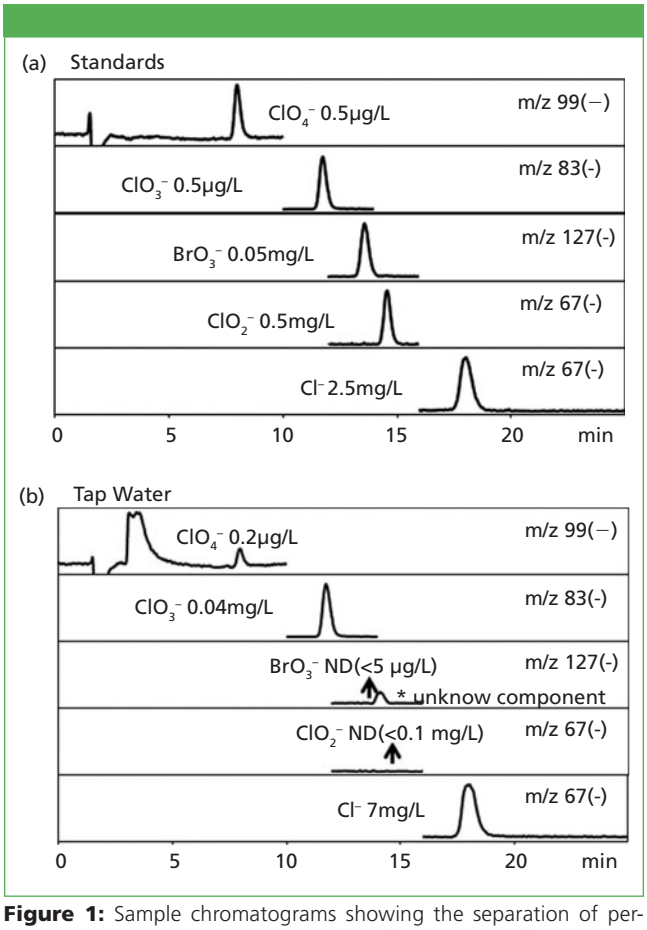


Figure 1: Sample chromatograms showing the separation of perchlorate and other oxyhalides. Sample: (a) standards, (b) tap water, eluent: 50 mM ammonium formate (aq) / acetonitrile = 25/75; flow rate: 0.2 mL/min; column temp.: 30 ºC; detector: ESI-MS (SIM); injection volume: 5 µL.

Conclusions

Shodex multi-mode column, RSpak JJ-50 2D, is suitable for the low detection limit analysis of perchlorate by HPLC–ESI–MS method which meets the requirement of EPA method 6850. A simple mobile phase used here was able to separate perchlorate from other components as well as from other oxyhalides.

Shodex/Showa Denko America Inc.
420 Lexington Avenue Suite 2335A, New York, NY, 10170
tel. (212) 370-0033 x109, fax (212) 370-4566
Website: www.shodex.net

GC×GC Forensic Analysis of Oil Sheens at the Deepwater Horizon Disaster Site Helps Pinpoint the Source of Oil Leakage

Robert K. Nelson[1], Christoph Aeppli[2], Catherine A. Carmichael[1], Matthias Y. Kellerman[3], David L. Valentine[3], and Christopher M. Reddy[1], [1]Dept. Marine Chemistry & Geochemistry, Woods Hole Oceanographic Institution, [2] Bigelow Laboratory for Ocean Sciences, [3]Dept. of Earth and Marine Science Institute, University of California, Santa Barbara

Background Information

On April 20, 2010 an explosion and fire aboard the drilling rig *Deepwater Horizon* killed 11 crew members and marked the beginning of the largest offshore oil spill ever to occur in US territorial waters. The *Deepwater Horizon* capsized and sank on April 21, 2010 coming to rest 400 m northwest of the well head at a depth of approximately 1500 m. Crude oil from the Macondo well flowed into the northern Gulf of Mexico between April 20th and August 4th 2010 before the well was finally sealed with drilling mud and cement. Over the course of the spill an estimated 4.9 million barrels of oil flowed into the Gulf of Mexico according to a US Federal On-Scene Coordinators (FOSC) report. Crude oil from this spill impacted beaches and marshes in Texas, Louisiana, Mississippi, Alabama, and Florida.

Scope of this Study

Alkenes commonly found in synthetic drilling-fluids were used to identify sources of oil sheens that were first observed in September 2012 close to the *Deepwater Horizon* (DWH) disaster site, more than two years after the Macondo well (MW) was sealed (Figure 1). Exploration of the sea floor by BP confirmed that the well was capped and sound. BP scientists and engineers identified the likely petroleum source as leakage from an 80-ton cofferdam abandoned during the operation to control the MW in May 2010. We acquired and analyzed sheen samples at the sea-surface above the DWH wreckage as well as oil samples collected directly from the cofferdam, the MW, and natural seeps in the area. GC×GC allowed the identification of drilling fluid C_{16}- to C_{18}-alkenes in sheen samples. Drilling fluid alkenes were absent in the cofferdam oil (Figure 2a), reservoir, and natural seeps. Furthermore, the spatial pattern of evaporative losses of sheen oil alkanes indicated that oil surfaced closer to the DWH wreckage than the cofferdam site. Lastly, ratios of alkenes and oil hydrocarbons pointed to a common source of oil found in sheen samples and recovered from oil-covered DWH debris collected shortly after the explosion. These lines of evidence suggest that the observed sheens do not originate from the MW, cofferdam, or from natural seeps. Rather, the likely source is oil in tanks and pits on the DWH wreckage, representing a finite oil volume for leakage.

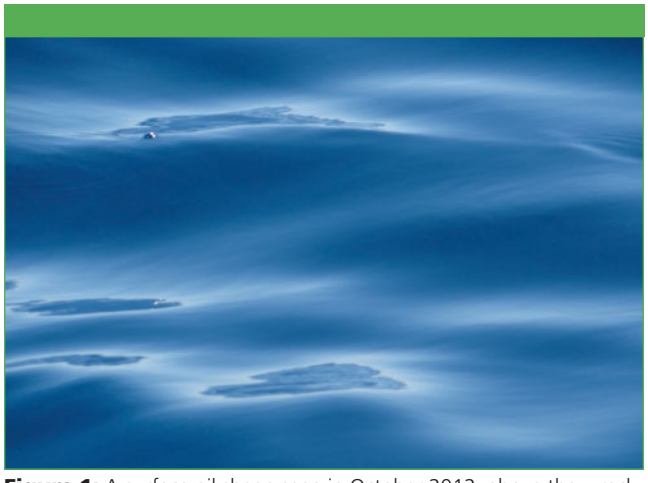


Figure 1: A surface oil sheen seen in October 2012, above the wreckage of the Deepwater Horizon drilling rig. (Courtesy of Chris Reddy.)

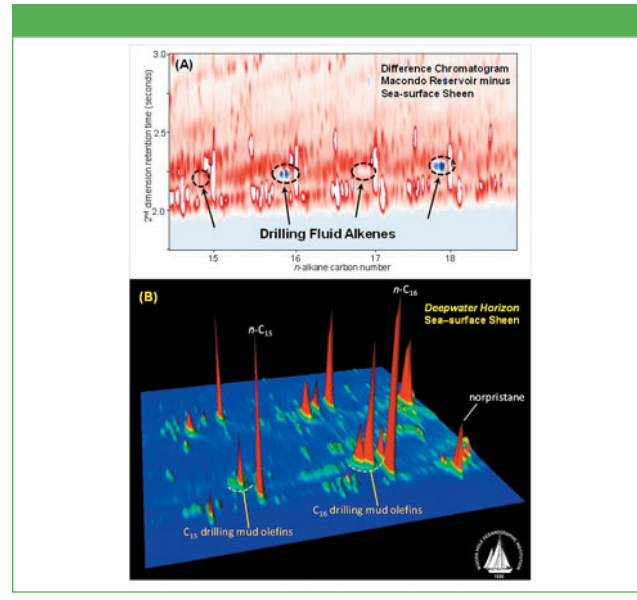


Figure 2: (a) Difference chromatogram made by normalizing Macondo well petroleum and a surface sheen sample collected in October 2012. The red peaks represent components that are more abundant in the Macondo reservoir sample while blue peaks represent components that are more abundant on the sheen sample. (b) Zoomed in view of a GC×GC analysis of a sea-surface sheen sample containing drilling fluid alkenes collected in October 2012.

Experimental

Instrument:	LECO Pegasus IV GC × GC-TOF
Columns:	1st Dimension, Restek Rtx-1ms, 60 m × 0.25 mm × 0.25 µm 2nd Dimension, SGE BPX-50 1.5 m × 0.10 mm × 0.10 µm
Injection:	Splitless at 300 °C
Carrier Gas:	Helium at 1mL/min, constant flow

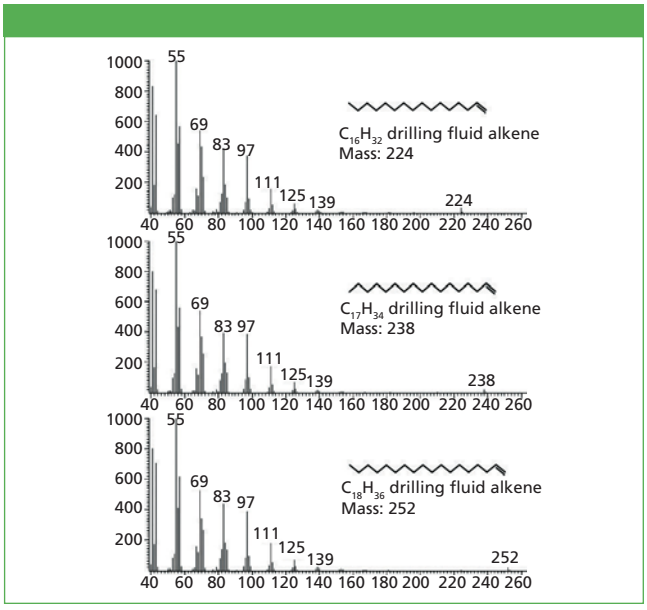


Figure 3: Mass spectra obtained from drilling fluid alkene peaks first identified in the difference chromatograms from Figure 2a.

Temperature Ramps	
Oven 1:	60 °C (10 min), 1.25 °C/min to 325 °C (5 min)
Oven 2:	65 °C (10 min), 1.25 °C/min to 330 °C (5 min)
Modulation Period:	10 s
Mass Range:	35–650 m/z
Acquisition Rate:	50 spectra/s
Source Temp:	225 °C
Transfer Line:	300 °C

Results and Discussion

Identification of similarities and differences between complex mixtures such as petroleum is extremely difficult, tedious, and time consuming. GC–MS experiments in our lab and in other labs failed to pick up the drilling fluids in the sea-surface sheens therefore, due the large number co-eluting components in the saturate/olefin region and the very low concentrations of drilling fluid alkenes, GC×GC was the only option for this work. We have developed a methodology which uses difference chromatograms to rapidly distinguish between two complex mixtures (1). Briefly, chromatograms are normalized with a component that is common to both samples then one chromatogram is subtracted from the other. Positive values are assigned one color and negative values another color. In this case (Figure 2a), components that were unique to the oil surfacing and forming sheens in the Gulf of Mexico are shown in blue and components that are present in higher abundance in the MW reservoir are shown as red. Analyzing GC×GC chromatograms in this manner allowed us to quickly focus on the drilling fluid components present in surface sheens. Mass spectral examination of the peaks identified in the difference chromatogram confirmed that these compounds were alkenes derived from the drilling fluids used during drilling operations on the *Deepwater Horizon* rig (Figure 3).

Conclusions

GC×GC forensic analysis of surface sheens collected above the *Deepwater Horizon* enabled us to eliminate a number of possible leakage sources based upon direct comparison with each source sample. We were able to confidently rule out the Macondo reservoir, the cofferdam, and natural oil seeps because none of these possible leakage sources contained drilling fluid alkenes. Comparison of the sheen samples with oil covered floating riser pipe buoyance compensator debris collected shortly after the explosion showed very high similarity (both contained drilling fluids) and lead to the conclusion that the source of oil surfacing 2 years after the explosion must be the wreckage of the *Deepwater Horizon (*2-5).

References

(1) R.K. Nelson, B.S. Kile, D.L. Plata, S.P. Sylva, L. Xu, C.M. Reddy, R.B. Gaines, G.S. Frysinger, and S.E. Reichenbach, *Environ. Forensics* **7**, 33–44 (2006).

(2) C.M. Reddy, J. Arey, J.S. Seewald, S.P. Sylva, K.L. Lemkau, R.K. Nelson, C.A. Carmichael, C.P. McIntyre, J. Fenwick, G.T. Ventura, B.A.S. Van Mooy, and R. Camilli, *Proceedings of the National Academy of Sciences of the United States of America* **109,** 20229–20234 (2011).

(3) C.A. Carmichael, J.S. Arey, W.M. Graham, L.J. Linn, K.L. Lemkau, R.K. Nelson, and C.M. Reddy, *Environ. Res. Lett.* **7**, 015301 (2012).

(4) H.K. White, P.Y. Hsing, W. Cho, T.M. Shank, E.E. Cordes, A.M. Quattrini, R.K. Nelson, R. Camilli, A.W.J. Demopoulos, C.R. German, J.M. Brooks, H.H. Roberts, W. Shedd, C.M. Reddy, and C.R. Fisher, *Proceedings of the National Academy of Sciences of the United States of America* **109**(50),, 20303–20308 (2012).

(5) C. Aeppli, C.M. Reddy, R.K. Nelson, M.Y. Kellerman, and D.L. Valentine,, *Environ. Sci. Technol.,* **47**(15), 8211–8219 (2013).

Determination of PAH Compounds from Aqueous Samples Using a Non-Halogenated Extraction Solvent and Atlantic C18 Disks

Jim Fenster, Kevin Dinnean, David Gallagher, and Michael Ebitson, Horizon Technology, Inc.

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants, yet can be a health hazard and are therefore measured and regulated. The traditional extraction solvents used for solid phase extraction (SPE) methods involving PAH compounds are dichloromethane (DCM) and acetone. DCM has been used in the past because of its excellent solvating properties and its low boiling point which results in higher yields after extraction, drying, and concentration. DCM however is dangerous to work with as it has been proven to be a carcinogen at very low exposure levels. As such, many laboratories have now mandated that solvent extractions in environmental methods not use any halogenated solvents, in particular DCM. Previous work, done by Frederick Werres (1), has demonstrated good extraction efficiencies using acetone as the eluting solvent. However; acetone creates a problem with residual water in the final extracts due to its miscibility with water and a more optimal non-halogenated, non-polar solvent would improve performance. This application note demonstrates an improved extraction of 16 PAH compounds listed by the US Environmental Protection Agency (EPA) as priority pollutants, including all PAHs listed in the content of the EU Water Framework Directive, using the Horizon Technology SPE-DEX® 4790 automated SPE extraction system with Atlantic® C18 disks and Fast Flow Sediment Disk Holder (FFSDH) setup which is optimized for use with highly particulated samples.

The data in Table I show very good recoveries for the PAH analytes from 79% to 92%. The light end PAH analytes are affected more than the heavy end components when air dry times are reduced from 2 min to 1 min. The 1-min air dry times gave a result with a low of 79% for naphthalene and a high of 92% for both dibenz(ah)anthracene and benzo(ghi)perylene (avg. RSD 4.69%). The 2-min air dry times gave a recovery range of 73% to 90% (avg. RSD 10.26%). For more information on method development please see reference 2.

Conclusions

This application note demonstrates an efficient SPE disk extraction scheme for PAH compounds in aqueous samples. The method demonstrated excellent recoveries for an extraction scheme which uses acetone and hexane in place of chlorinated solvents for clean or dirty samples.

References

(1) F. Werres, P. Balsaa, and T.C. Schmidt, *J Chromatogr. A* **1216,** 2235–2240 (2009).

(2) J. Fenster, K. Dinnean, D. Gallagher, and M. Ebitson, "Determination of PAH Compounds from Aqueous Samples Using a Non-Halogenated Extraction Solvent and Atlantic C18 Disks," Horizon Technology, Inc., Salem, New Hampshire, Application Note AN072 (2012).

Horizon Technology, Inc.
16 Northwestern Drive, Salem, NH 03079
tel. (800) 997-2997 (US Toll-free)
Website: www.horizontechinc.com

Table I: Recovery data of 20 µg PAH spike from clean aqueous extractions using FFSDH and C18 disks 2 and 1 min air dry times

Air Dry Time Collection Vessel	2 min 125 mL Erlenmeyer					1 min 125 mL Separatory Funnel				
Compound	% Rec	% Rec	% Rec	Average % Rec	% RSD	% Rec	% Rec	% Rec	Average % Rec	% RSD
Naphthalene	82	68	70	73	10.51	77	78	84	79	4.78
Acenaphthylene	83	67	71	74	11.4	77	85	86	83	5.84
Acenaphthene	83	70	73	75	9.45	82	87	88	85	3.77
Fluorene	87	71	72	77	11.69	79	87	88	85	5.71
Phenanthrene	87	72	73	77	10.84	80	85	87	84	4.32
Anthracene	82	70	69	74	9.82	80	88	87	85	5.13
Fluoranthene	88	72	76	78	10.53	84	88	91	88	4.31
Pyrene	88	72	76	78	10.65	84	89	92	88	4.55
Benz(a)anthracene	96	81	81	86	10.45	85	92	91	89	4.26
Chrysene	90	76	78	81	9.70	83	87	90	86	4.08
Benzo(b)fluoranthene	100	84	84	89	10.53	88	90	90	89	1.49
Benzo(k)fluoranthene	96	79	82	85	10.97	84	90	93	89	5.24
Benzo(a)pyrene	98	82	82	87	10.95	85	91	91	89	3.75
Indeno(1,2,3-cd)pyrene	97	87	86	90	6.96	83	92	93	89	6.17
Dibenz(ah)anthracene	99	84	83	89	10.28	86	94	98	92	6.68
Benzo(ghi)perylene	95	85	79	86	9.36	87	93	96	92	5.01
			Averages	81	10.26			Averages	87	4.69

Determination of Geosmin in Water Samples Using Person-Portable GC–MS and Sample Preparation Instruments

Bruce E. Richter, Tai V. Truong, Tiffany C. Brande, Charles S. Sadowski, and Douglas W. Later, Torion Technologies Inc.

Geosmin is a naturally occurring compound released when soil-present microbes die. Communities can periodically experience episodes of unpleasant-tasting water when a sharp drop in this microbe population releases geosmin into ground water. Geosmin can be difficult to remove from water with standard water treatment processes, and knowing in advance that an episode has occurred will save time and money. A field method has been developed to detect geosmin in water at ppt levels.

Experimental Conditions

Field samples were collected from a river, creek, and lake located near the laboratory. Geosmin was extracted from each 500 mL sample by passing the water through a CUSTODION™-CT (conventional trap) at ambient temperature using a CLAIRION™ pump.

The extracted geosmin was then transferred to a CUSTODION-NT (needle trap) which introduces samples into the person portable TRIDION®-9 GC–MS, for compound analysis in the field. The geosmin was thermally desorbed from the CUSTODION-CT to NT using the FUZION™-3 SD (sample desorption) module.

Samples were injected into the TRIDION-9 GC–MS, which has a low thermal mass, capillary GC column (MXT-5, 5 m × 0.1 mm, 0.4 µm d_f), coupled to a toroidal ion trap mass spectrometer (TMS) detector having a mass range of 45–500 m/z.

Geosmin spiked in water at concentrations of 1, 10, 20, and 70 ppt (ng/L) was used to develop a linear calibration. Three replicates at each concentration were analyzed and included, which generated a standard curve with $R^2 = 0.9986$.

Results/Conclusions

Geosmin was not present at levels above 1 ppt in the creek or river water samples. However, geosmin was detected at 2.5 ppt in the lake water sample, shown in Figure 1, the reconstructed m/z 112 ion chromatogram below (red). This is understandable since the sample was collected in July during the hot summer months in Utah. Geosmin can be detected at low ppt levels in water samples using the CUSTODION sample collection and extraction devices along with the TRIDION-9 GC–MS. These field portable devices represent a powerful combination for on-site sample preparation and detection of target compounds, saving time and money.

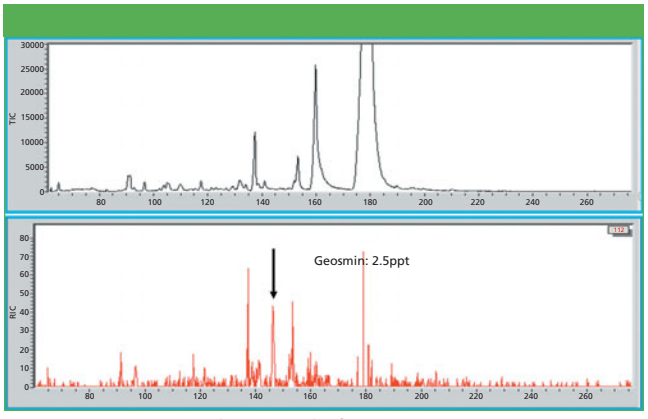


Figure 1: TIC and RIC (m/z = 112) of lake water sample processed and analyzed for geosmin. 2.5 ppt were detected in the sample as received.

Acknowledgments

TRIDION™ and CUSTODION® are trademarks of Torion Technologies Inc. The TRIDION-9, CUSTODION SPME syringe, and CHROMION® operating software are manufactured and sold under U.S. Patents and Applications 7,075,070, 7,075,064, 11/639,373, 11/379,716 and/or any division, continuations, revisions or foreign filings thereof.

These data represent typical results. For further technical assistance or purchasing information, contact:

Torion Technologies Inc.
796 East Utah Valley Dr., Suite 200, American Fork, UT 84003
tel. (801) 705-6600, fax (801) 705-6649
Website: www.torion.com

Thermal Treatment Analysis — Determination of 3,5-stigmastadienes in Olive Oil Using the Agilent Infinity 1220 LC System with Diode Array Detector

Sonja Schneider, Agilent Technologies Inc.

3,5-stigmastadienes were analyzed in seven olive oil samples using the Agilent 1220 Infinity Mobile LC Solution to differentiate virgin from refined or other thermally-treated olive oil. Because of the robust and rugged 1220 Infinity Mobile LC Solution, it is possible to perform olive oil analysis on-site as a starting point for quality analysis of virgin olive oils.

Introduction

Virgin olive oil can be created only by mild, cold pressing of the olives (*Olea europea L.*). Thermal or chemical treatment is not allowed in the procedure. There are different analytical methods to differentiate virgin from refined or thermally-treated olive oils. In addition to the determination of stigmastadienes and chlorophyll degradation products, the analysis of the concentration of polymerized triacylglycerides in olive oil is another important factor. The amount of stigmastadienes in commercially refined vegetable oils is dependent on the conditions applied during the refining process. The determination of stigmastadienes in olive oils also detects minor amounts of refined oils in virgin olive oils and is, therefore, an important quality characteristic for virgin olive oils. Because of the ultraviolet (UV) detection of the stigmastadienes analysis method, the 1220 Infinity Mobile LC Solution can be used in a mobile laboratory as a starting point for olive oil quality analysis before further quality analyses are applied in a stationary laboratory.

Experimental Conditions

Column: Agilent LiChrospher C18, 4 × 250 mm, 5 µm (p/n 79925OD-584), Agilent ZORBAX Extend-C18 RRHT, 4.6 × 50 mm 1.8 µm (p/n 727975-902)
Mobile phase: Acetonitrile/methyl tert-butyl ether (70:30)
Flow: 1 mL/min
Stop time: 30 min or 5 min
Injection volume: 10–50 µL, 20 µL
Column temperature: 25 °C
UV: 235 nm/4 nm Ref.: off
Peak width: >0.05 min (1.0 s response time) (5 Hz)
Sample preparation was carried out according to EN ISO 15788-3:2004 (D) using the internal standard method.

Results

In contrast to virgin olive oils, 3,5-stigmastadienes were detected in partly refined olive oil, see Figure 1. To accelerate analysis time, the run was shortened to 5 min using a 50-mm, sub-2 µm column (Agilent ZORBAX Extend-C18 RRHT, 4.6 × 50 mm 1.8 µm), still obtaining good resolution of the analytes in partly refined olive oil.

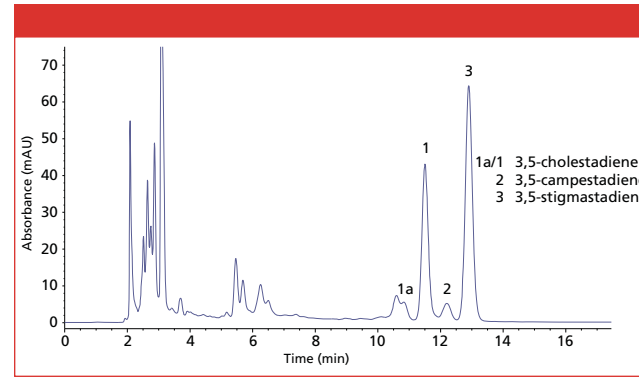


Figure 1: Detection of 3,5-stigmastadienes in partly refined olive oil.

Conclusion

Seven olive oils were analyzed for 3,5-stigmastadiene to determine refining processes or other thermal treatments according to EN ISO 15788-3:2004 (D). As expected, no 3,5-stigmastadienes were detected in any of the tested virgin oils. In contrast, in a sample containing refined and virgin oils, the amount of 3,5-stigmastadienes found was 0.63 mg per kg sample. The analysis time could be shortened to 5 min using a 50-mm, sub-2 µm column.

References

(1) Quality Analysis of Virgin Olive Oils — Part 3, Agilent Application Note, Publication Number 5991-1896EN, (2013).
(2) Quality Analysis of Virgin Olive Oils — Part 2, Agilent Application Note, Publication Number 5991-1895EN, (2013).
(3) Dobarganes et al., *Pure & Appl. Chem.* **71**(2), 349–359 (1999).
(4) L. Brühl and H.J. Fiebig, *Fat. Sci. Technol.* **97**(6), 203–208 (1995).
(5) H.J. Fiebig, *Fett/Lipid* **101**, 442–445 (1999).

Agilent Technologies

Agilent Technologies Inc.
5301 Stevens Creek Blvd., Santa Clara, CA 95051
tel. (800) 227 9770
Website: www.agilent.com/chem/infinity-mobile-lc

Analysis of Maleic Acid in Starch-Based Products Using a New Bonded Zwitterionic HILIC Column and Low UV Wavelength Detection

Jeffrey Lei and Patrik Appelblad, EMD Millipore

Organic acids are hydrophilic compounds with acidic properties where the carboxylic acids are predominant. Organic acids are generally weak acids that do not dissociate completely in water and they are present in every meal we eat. Organic acids are also used in food preservation because they can penetrate bacteria's cell wall and disrupt their normal physiology. Ion chromatography is the favored analytical technique for quantitative and qualitative purposes, but reversed-phase (RP) chromatography coupled to various detection techniques such as electrochemical, UV, RI, or MS is also common. To retain organic acids in reversed phase mode a requirement is to add ion-pairing reagents, work at low pH, and/or use completely aqueous mobile phases. Citric and tartaric acid are difficult to retain and resolve sufficiently in RP mode, and often there is co-elution of malic acid and succinic acid when using ion chromatography.

Hydrophilic interaction liquid chromatography (HILIC) has appeared and proven as an attractive technique for separation of small polar molecules such as organic acids. HILIC is considered as a MS friendly technique using volatile acetate or formate buffers in the mobile phase, conditions preventing analysis at low UV wavelength. However, bonded zwitterionic HILIC columns can be used with inorganic buffers like phosphate despite the limited solubility of potassium phosphate in high acetonitrile eluents. There are though some useful guidelines when using inorganic buffers (i.e. phosphate) in HILIC but the same also apply to RP when using a high proportion of acetonitrile in the eluent. Use premixed mobile phase and avoid pure acetonitrile as one mobile phase constituent. Precipitation of salt generally occur when using over 80 volume-% acetonitrile in the mobile phase, though at low buffer strengths 85% is the absolute maximum. In gradient mode, the difference between mobile phase A and B should be as small as possible and HILIC gradients should be shallower than in RP since changes in mobile phase composition has a larger effect in HILIC than in RP and thus require longer column equilibration.

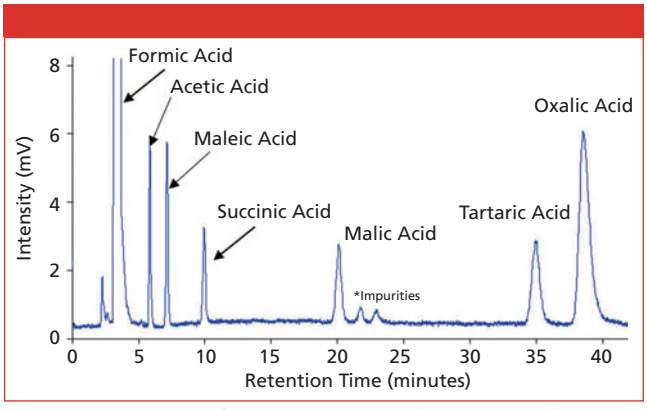


Figure 1: Separation of seven organic acids (20 µL injection) on a SeQuant® ZIC®-cHILIC (3 µm/100 Å), 250 × 4.6 mm column using a mobile phase consisting of 80:20 (v/v) acetonitrile and potassium phosphate 25 mM pH 6.0 (5 mM total ionic strength). The flow rate was 1.0 mL/min and the UV detector was set at 205 nm.

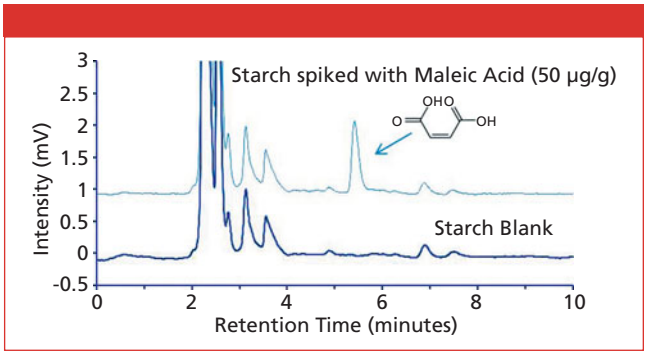


Figure 2: Chromatogram showing the analysis of a) starch sample and b) maleic acid spiked starch sample on a SeQuant® ZIC®-cHILIC (3 µm/100 Å), 250 × 4.6 mm column using a mobile phase consisting of 77:23 (v/v) acetonitrile and di-potassium hydrogen phosphate 20 mM pH 7.0 (4.6 mM total ionic strength). 20 µL samples (dissolved in mobile phase) were injected and analyzed at a flow rate of 1.0 mL/min using a UV detector set at 214 nm.

This application note shows that organic acids can be successfully analyzed with high sensitivity, low UV wavelength detection, using phosphate-based buffer systems and zwitterionic SeQuant® ZIC®-cHILIC columns, see Figure 1. A key characteristic of the ZIC®-cHILIC column in this separation is the controlled ionic interactions offered by its zwitterionic phosphorylcholine group orientation, which results in higher retention and thus allow the use of lower amounts of acetonitrile, fully compatible with the solubility levels of phosphate buffer. At the same time the ionic interactions between the organic acids and the ZIC®-cHILIC column are weak enough to not give excessive retention or poor selectivity dominated by extremely strong ionic interaction. Recently this column has proven useful in Taiwan for detecting maleic acid in tainted starch based products, see Figure 2.

EMD Millipore.
290 Concord Rd., Billerica, MA 01821
tel. (800) 645-5476
Website: www.emdmillipore.com

Structural Differences in Modified Starches

Malvern Instruments Ltd.

Modified starches are important materials used in many applications including foodstuffs. The starches are modified by a number of methods — both physical and chemical — to tailor the properties to the required application. Most commonly the starches are modified to give a particular texture to a finished foodstuff; for example, to give extra thickening in puddings.

In this application note we show how two modified starch samples with essentially the same molecular size in solution can be easily differentiated and characterized by triple detection size-exclusion chromatography (TD-SEC).

Triple Detection SEC

In the advanced technique of TD-SEC, the sample, after separation on the chromatography column, is passed though a series of detectors to provide a complete analysis of the molecules: The low angle light scattering detector (LALS) provides a direct measure of the molecular weight; the refractive index (RI) detector measures the concentration; and the differential viscometer measures the intrinsic viscosity (IV). From the measured IV and molecular weight (MW) values a Mark-Houwink (M-H) plot showing structural changes can be made.

Instrumentation and Conditions

SEC system comprising the Viscotek GPCmax (degasser, pump, autosampler) with the Viscotek TDA detector equipped with the following detectors: Low angle light scattering; differential viscometer; RI. The data were all calculated using OmniSEC software.

Discussion

The triple chromatogram of one of the modified starch samples is shown in Figure 1. The signal-to-noise on all three detectors is excellent, which ensures the quality of the calculated data. The data are calculated directly from the chromatograms by the OmniSEC software and the results for both samples are shown in Table I. Note that the hydrodynamic radius (R_H) of both samples is within 0.2 nm. This means that by traditional GPC/SEC techniques the molecular weights based on retention volumes would be the same. However, TD-SEC clearly shows the weight average molecular weight of sample

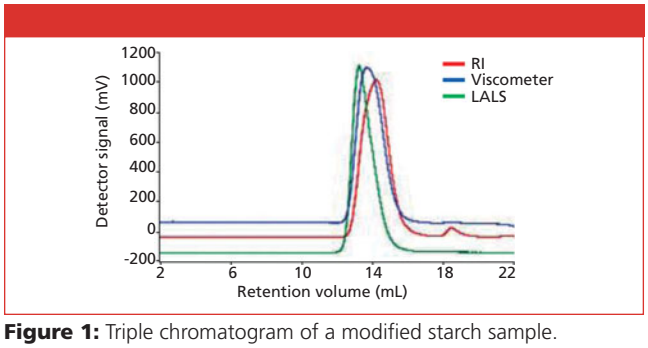


Figure 1: Triple chromatogram of a modified starch sample.

Table I: Weight average molecular weight, number average molecular weight, intrinsic viscosity, and hydrodynamic radius data

Sample	Mw(D)	Mn(D)	IV(dL/g)	R_H(nm)
Modified starch A	241.780	123.780	0.117	7.2
Modified starch B	399.020	169.620	0.081	7.4

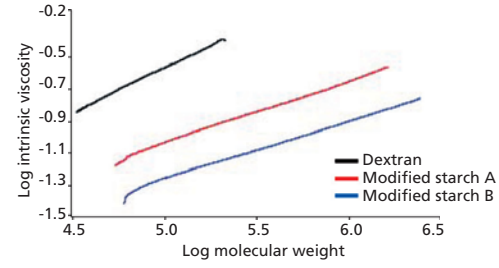


Figure 2: Mark-Houwink (Structure) plot.

A is only 60% of sample B. We can also see that the viscosity of A, despite the lower molecular weight, is higher than B.

By looking at the structure plot (M-H plot, Figure 2) of both modified samples (with a dextran T70 sample as reference), it is clear that the two modified starches have very different molecular structures. Sample B has a much more compact structure than sample A; shown by the fact it appears lower on the M-H plot. This means that despite higher molecular weight the molecules in sample B are denser — because of the different modification — resulting in a lower intrinsic viscosity. The dextran T70 material is shown for reference. It indicates, as expected, that modified starches have a much more compact structure than dextran.

Conclusions

The Viscotek triple detection system provides a convenient and rapid way to characterize starches and modified starches. The instrument allows determination of molecular weight and molecular size in a single run using normal conditions and sample concentrations. The IV and size data allow differentiation between molecules of differing structures. The technique is equally applicable to other polysaccharides and all other synthetic or natural polymers such as proteins and DNA.

Malvern Instruments Ltd.
Enigma Business Park, Grovewood Road, Malvern, UK
tel. +44 (0) 1684 892456
E-mail: salesinfo@malvern.com
Website: www.malvern.com

Determination of Pesticide Residue in Apple Juice Using the AutoMate-Q40

Tyler Trent, Teledyne Tekmar

QuEChERS is a Quick-Easy-Cheap-Effective-Rugged-Safe extraction method that has been developed for the determination of pesticide residues in agricultural commodities (1). The rise in popularity of the QuEChERS extraction has increased the demand for an automated workflow solution to this labor intensive technique. The AutoMate-Q40 fulfills this need by automating the process from the homogenate to the final extract, with or without sample clean-up.

The goal of this work is to utilize the AutoMate-Q40 for automating the QuEChERS extraction in a multi-lab validation study (Teledyne Tekmar and Pacific Agricultural Lab) for the determination of pesticides in apple juice. Pesticide residues were extracted from the apple juice by using the AutoMate-Q40 workflow solution. Quantification was based on matrix-matched calibration curves with the use of internal standard to ensure method accuracy. Quality control samples were evaluated at levels of 10, 50, and 100 ng/g to ensure the precision and accuracy of the AutoMate-Q40.

Extraction/Cleanup

Figure 1 shows the flow chart for the AOAC QuEChERS extraction procedure for both analytical labs for the AutoMate-Q40.

Results and Discussion

A precision and accuracy study was performed in both labs using the AutoMate-Q40. A 6 µg/mL stock pesticide solution was used to fortify the apple juice samples. Using the AutoMate-Q40, the system is able to spike the following samples with 25.0, 125.0, and 250.0 µL of the pesticide standard that yielded a 10.0, 50.0, and 100 µg/L check samples. Also, the AutoMate-Q40 spiked in 75.0 µL of the internal standard TPP in each sample that yielded a 100.0 µg/L of TPP. These QC samples were quantitated against their corresponding matrix matched calibration curve. Table I demonstrates the excellent recoveries achieved when using the AutoMate-Q40 and exceptional precision for the automated QuEChERS extraction.

Table I: Average LC–MS-MS recovery and %RSD for the AutoMate-Q40 (n = 7)

		AutoMate-Q40 Comparison			
		Un-Clean Samples		Cleaned Samples	
	Concentration	Avg. Recovery	Avg. %RSD	Avg. Recovery	Avg. %RSD
Teledyne Tekmar	10.0 ng/g	103.68	7.5	100.32	8.4
	50.0 ng/g	100.47	3.2	103.21	3.7
	100.0 ng/g	100.73	4.0	100.46	4.2
Pacific Agricultural Lab	50.0 ng/g	91.04	12.4	94.43	10.9
	100.0 ng/g	98.28	6.5	100.78	6.7

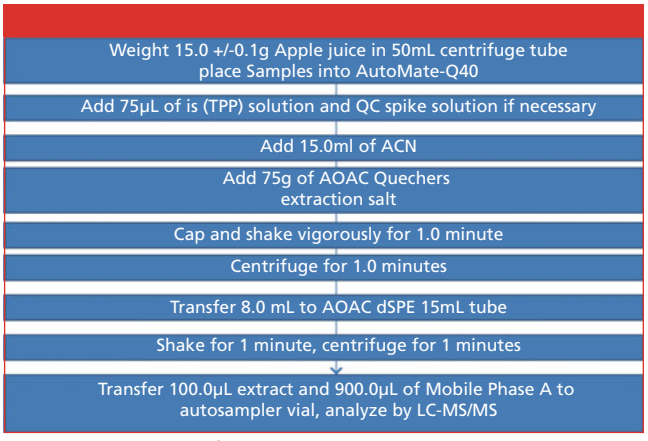


Figure 1: Flow chart for the AOAC QuEChERS extraction procedure for both analytical labs for the AutoMate-Q40.

Conclusion

This study demonstrates the feasibility of automating the QuEChERS extraction method using the AutoMate-Q40. By automating the liquid handing, addition of salt/buffers, sample mixing, pipetting, and liquid level sensing using the patent pending VialVision™. The extraction process is more reliable, and easier. This enables time and labor savings, while improving consistency and repeatability of the extraction. As shown above in Table I the combined average pesticide spikes recovered at 100.17%, with an average RSD of 6.76%. These numbers indicate superb precision and accuracy from two independent testing sites validating the performance of the AutoMate-Q40 as an excellent analytical tool.

References

(1) AOAC Official Method 2007.07 Pesticide Residues in Food by Acetonitrile Extraction and Partitioning with Magnesium Sulfate. Gas Chromatography/Mass Spectrometry and Liquid Chromatography/Tandem Mass Spectrometry, First Action 2007

To view the full application note please visit
http://info.teledynetekmar.com/pesticide_residue_apple_juice_lcgc

TELEDYNE TEKMAR
Everywhereyoulook™

Teledyne Tekmar
4736 Socialville-Foster Rd., Mason, OH 45040
tel. (513) 229-7000
Website: www.tekmar.com

Analysis of Polyether Antibiotics in Animal Feeds by HPLC with Post-Column Derivatization

Maria Ofitserova and Sareeta Nerkar, Pickering Laboratories, Inc.

Polyether antibiotics are commonly used for preventing coccidiosis and other infections in poultry and for improving feed efficiency for beef cattle and swine. The use of polyether antibiotics is strictly regulated, with only specific ionophores approved for use in feeds intended for different animals.

Analysis of polyether antibiotics by HPLC with post-column derivatization and UV–vis detection has been proven to successfully identify and quantify monensin, narasin, and salinomycin in medicated feeds, supplements, and premixes as well as to determine trace contamination levels in non-medicated feeds (1,2).

Post-column derivatization of polyether antibiotics is done using highly acidic vanillin or DMAB reagents. Pinnacle PCX derivatization system (Pickering Laboratories, Inc.) has an inert flow path and automated system wash capabilities that make it uniquely suitable for handling corrosive reagents. The two-pump system is recommended to extend reagent stability, but the single-pump system for this application is also available.

Adding a fluorescence detector to the instrumentation allows for using the same extraction procedure and HPLC conditions to also determine lasalocid which doesn't require post-column derivatization.

Method

Sample Preparation

To 25 g of finely ground feed sample, add 100 mL of extraction solution (90% methanol—10% water). Shake for 1 h at high speed using mechanical shaker. Let the solids settle and filter an aliquot of the extract for injection. Dilute with extraction solution if needed to fit the calibration curve. Use 2.5 g portion when testing premixes.

Table I: Polyether antibiotics in certified medicated feeds

	Feed Type	Certified Amount	Found in Sample	Recoveries	RSD, $N = 4$
Monensin	Beef feed	267 g/ton	275 g/ton	103%	0.7%
Lasalocid	Milk replacer	72 g/ton	69 g/ton	96%	3.3%

Table II: Spike recoveries for monensin

	Non-Medicated Bird Feed		Non-Medicated Rabbit Feed	
	Monensin A	Monensin B	Monensin A	Monensin B
Spike Level	172 g/ton	8 g/ton	86 g/ton	4 g/ton
Recoveries	100%	100%	101%	102%
RSD, $N = 3$	1.9%	2.1%	1.1%	0.6%
Spike Level	3.44 g/ton	0.16 g/ton	3.44 g/ton	0.16 g/ton
Recoveries	96%	95%	94%	88%
RSD, $N = 3$	0.7%	3.1%	0.9%	1.6%

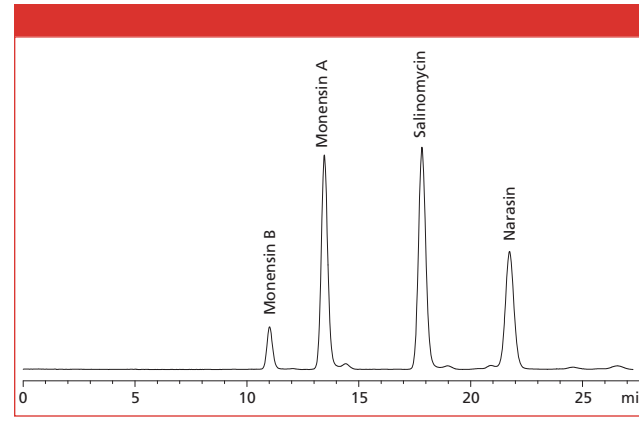


Figure 1: Standard mixture of monensin, salinomycin, and narasin.

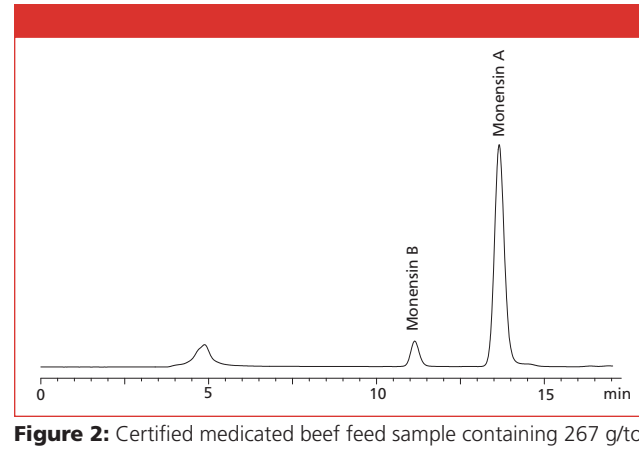


Figure 2: Certified medicated beef feed sample containing 267 g/ton of monensin.

Analytical conditions

Analytical Column: Polyether Column, C18, 4.6 × 250 mm, Catalog No. 2381750
Temperature: 40 °C
Flow rate: 0.7 mL/min
Mobile Phase: 90% methanol, 10% of 5% acetic acid solution in water, isocratic
Injection volume: 20 µL

Post-Column Conditions

Post-column System: Pinnacle PCX
Reactor Volume: 1.4 mL

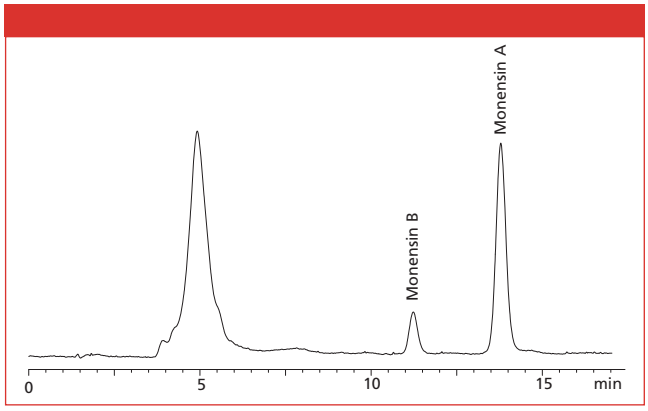


Figure 3: Non-medicated bird feed sample spiked with monensin A (3.44 µg/g) and monensin B (0.16 µg/g).

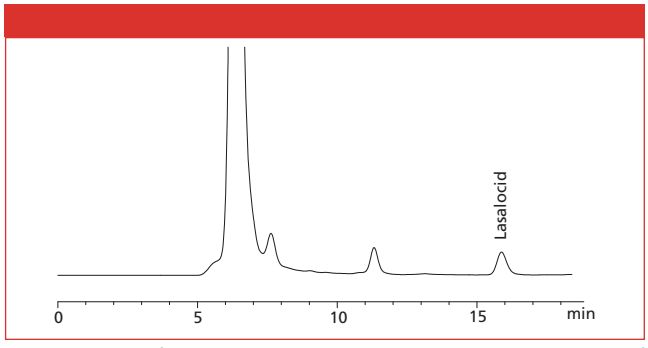


Figure 4: Certified medicated milk replacer containing 72 g/ton of lasalocid.

Reactor Temperature: 90 °C
Reagent 1: Concentrated sulfuric acid/methanol (4:96 v/v)
Reagent 2: 60 g of vanillin in 950 mL of methanol
Reagents Flow Rate: 0.3 mL/min
Detection: UV-vis 520 nm (for Lasalocid – FLD, Ex. 322 nm, Em. 370 nm)

Calibration

Monensin A: 0.1 ppm–50 ppm, $R^2 = 0.999$
Monensin B: 0.0035 ppm–0.7 ppm, $R^2 = 0.999$
Lasalocid acid: 0.25 ppm–50 ppm, $R^2 = 0.999$

Conclusion

Analysis of polyether antibiotics by HPLC with post-column derivatization is a robust and sensitive method that utilizes standard equipment and could easily be adopted by testing laboratories. It allows for testing of different ionophores at wide range of concentrations, including at trace levels. Using Pinnacle PCX post-column derivatization system, factory configured for the analysis, guarantees stable and reproducible results.

References

(1) H. Campbell and G. Nayeri, *J. AOAC Int.* **89**, 1229–1242 (2006).
(2) AOAC Official Method 997.04. Monensin in Premix and Animal Feeds.

PICKERING LABORATORIES

Pickering Laboratories, Inc.
1280 Space Park Way, Mountain View, CA 94043
tel. (800) 654-3330, (650) 694-6700
Website: www.pickeringlabs.com

Determination of Pesticides in Banana by AOAC QuEChERS and LC–MS-MS Detection

Xiaoyan Wang, UCT

This application note presents a simple and cost-effective method for the fast determination of pesticides in bananas. The method employs the AOAC QuEChERS approach, which yields higher recovery for several sensitive pesticides, such as pymetrozine and Velpar. A 15 g sample of homogenized banana is hydrated with 5 mL of reagent water to give a sample with >80% water. The hydrated sample is extracted using 15 mL acetonitrile with 1% acetic acid, this is followed by the addition of magnesium sulfate and sodium acetate. After shaking and centrifugation, 1 mL supernatant is cleaned in a 2-mL dSPE tube containing 150 mg $MgSO_4$, 50 mg primary secondary amine (PSA), and 50 mg C18. $MgSO_4$ absorbs residual water in the extracts; PSA removes organic acids and carbohydrates; while C18 retains fatty acids and other non-polar interferences. The result is a clean extract for LC–MS-MS analysis.

Extraction and cleanup products	
RFV0050CT	50 mL polypropylene centrifuge tube
ECMSSA50CT-MP	6 g $MgSO_4$ and 1.5 g NaOAc in Mylar pouch
CUMPSC18CT	150 mg $MgSO_4$, 50 mg PSA, and 50 mg C18 in 2 mL centrifuge tube

QuEChERS Extraction

1. Weigh 15 ± 0.15 g of peeled and homogenized banana sample into a 50-mL centrifuge tube **(RFV0050CT)**.
2. Add 5 mL of reagent water to increase the water content in banana from 74% to >80%.
3. Add an internal standard to all samples, and appropriate amounts of pesticide spiking solution to fortified samples.
4. Add 15 mL of acetonitrile with 1% acetic acid.
5. Cap and shake for 1 min at 1000 strokes/min using a Spex 2010 Geno/Grinder.
6. Add salts (6 g $MgSO_4$ and 1.5 g NaOAc) in Mylar pouch **(ECMSSA50CT-MP)** to each tube, and vortex for 10 s to break up salt agglomerates.
7. Shake for 1 min at 1000 strokes/min using Spex Geno/Grinder.
8. Centrifuge the samples at 3830 rcf for 5 min.

dSPE Cleanup

1. Transfer 1 mL supernatant into 2-mL dSPE tube **(CUMPSC18CT)**.
2. Shake for 2 min at 1000 strokes/min using Spex Geno/Grinder.
3. Centrifuge at 15300 rcf for 5 min.
4. Transfer 0.3 mL of the cleaned extract into a 2-mL autosampler vial.
5. Add 0.3 mL of reagent water, and vortex for 30 s.
6. The samples are ready for LC–MS-MS analysis.

LC–MS-MS Method

System: Thermo UltiMate 3000 LC with Vantage MS-MS, ESI+

Table I: Accuracy and precision data (n = 5)

Analyte	Spiked at 10 ng/g		Spiked at 50 ng/g	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Methamidophos	97.3	5.9	100.2	4.6
Pymetrozine	96.5	4.7	99.3	3.8
Carbendazim	103.5	3.3	107.3	5.3
Dicrotophos	101.8	4.1	104.8	4.8
Acetachlor	121.0	2.8	126.2	4.5
Thiabendazole	133.8	5.8	111.0	4.9
DIMP	89.2	6.0	92.1	7.7
Tebuthiuron	105.2	7.9	112.2	5.1
Simazine	96.3	4.6	101.2	4.8
Carbaryl	93.3	10.8	96.4	7.1
Atrazine	97.6	12.8	101.5	7.1
DEET	86.9	12.8	93.6	7.3
Pyrimethanil	100.6	8.0	97.0	5.7
Malathion	103.9	2.6	100.2	4.8
Bifenazate	84.4	13.7	85.4	3.2
Tebuconazole	90.0	1.2	88.2	1.5
Cyprodinil	97.3	3.1	96.0	1.8
Diazinon	104.1	1.7	99.8	2.9
Zoxamide	104.3	2.7	98.9	4.4
Pyrazophos	105.4	3.3	106.1	5.2
Profenofos	95.8	8.8	96.4	8.7
Chlorpyrifos	86.8	14.3	90.7	12.3
Abamectin	81.7	7.8	80.6	16.3
Bifenthrin	90.9	2.6	88.4	7.8
Overall mean	**98.7**	**6.3**	**98.9**	**5.9**

Injection: 10 µL at 10 °C

LC column: Thermo Accucore aQ, 100 × 2.1 mm, 2.6 µm, at 40 °C

Mobile phase: (A) 0.3% formic acid and 0.1% ammonia formate in water; (B) 0.1% formic acid in methanol

Gradient program and SRM transitions are available upon request.

Conclusion

A simple, fast, and cost-effective method has been developed for the determination of pesticides in banana samples. Pesticide residues in bananas were extracted using the AOAC version of the QuEChERS approach, followed by dSPE cleanup using $MgSO_4$, PSA, and C18. Excellent accuracy and precision were obtained, even for pymetrozine (recovery > 95%), a sensitive pesticide with limited recovery when the original or EN versions of QuEChERS approach is employed. The analytical run time was 20 min and the overall mean recovery for the 24 pesticides tested were 98.7% and 98.9% for the fortified banana samples at 10 ng/g and 50 ng/g, respectively.

UCT, LLC
2731 Bartram Road, Bristol, PA19007
tel. (800) 385-3153
Email: methods@unitedchem.com
Website: www.unitedchem.com

Determination of Sulfur in Natural Gas by ASTM Method D 6228-11

Laura Chambers and Gary Engelhart, OI Analytical

Natural gas and other gaseous fuels contain varying amounts and types of sulfur compounds which can be corrosive to equipment and can inhibit or destroy gas processing catalysts. Small amounts of sulfur odorants are added to natural gas and liquefied petroleum gases (LPGs) for safety purposes. Accurate measurement of sulfur species ensures proper process operation and odorant levels for public safety.

This application note describes the use of a pulsed flame photometric detector (PFPD) for determination of sulfur species in natural gas and LPGs by ASTM Method D 6228-11: Standard Test Method for Determination of Sulfur Compounds in Natural Gas and Gaseous Fuels by Gas Chromatography and Flame Photometric Detection.

Experimental Conditions

Instrumentation used for this study was an OI Analytical SPRO-Select GC system equipped with a 5380 Pulsed Flame Photometric Detector. Two capillary PLOT columns were evaluated: the Agilent J&W Select Low Sulfur column and Agilent GS-GasPro column.

Results

Two natural gas samples were blended for this project. Both contained five sulfur compounds at concentrations ranging from 3 to 6 ppmv, and one or more representative hydrocarbons found in different grades of natural gas. The composition of Sample #1 and its repeatability results on two different columns are shown in Table I.

Any capillary column that can provide adequate separation of the target sulfur compounds can be used with the PFPD for ASTM Method D 6228-11. The columns evaluated in this study were chosen because of their superior peak shape, excellent sensitivity for sulfur compounds, and retention time repeatability. Figure 1 illustrates the simultaneous sulfur and hydrocarbon chromatograms obtained from Sample #2 using an OI Analytical SPRO-Select GC system and Agilent Select Low Sulfur column. For complete results of this study, refer to OI Analytical Application Note #3671 (1).

Table I: Blended natural gas sample #1 and repeatability results on two GC columns

Compound	Concentration	Repeatability %RSD (n = 20)	
		GS-GasPro Column	Select Low Sulfur Column
COS	3.45 ppmv	1.2	2.1
H_2S	4.83 ppmv	2.9	2.5
DMS	4.11 ppmv	2.5	2.0
1-Propanethiol	5.92 ppmv	1.5	1.9
THT	4.46 ppmv	3.3	1.5
Methane UHP	Balance	-	-

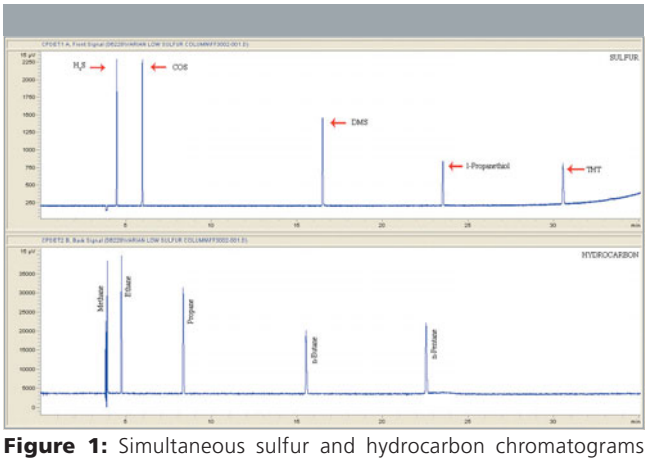


Figure 1: Simultaneous sulfur and hydrocarbon chromatograms obtained from a blended natural gas sample using the SPRO-Select GC system and Agilent J&W Select Low Sulfur column.

Conclusions

The SPRO-Select GC system equipped with a PFPD detects and measures sulfur species in natural gas by ASTM Method D 6228-11 with a high level of precision and accuracy, meeting all method requirements. Both capillary PLOT columns evaluated in this study yielded reproducible chromatograms with symmetric peak shape and chromatographic resolution of the sulfur and hydrocarbon peaks of interest.

References

(1) OI Analytical Application Note #3671, "Determination of Sulfur in Natural Gas by ASTM Method D 6228-11 Using a Pulsed Flame Photometric Detector (PFPD)."

OI Analytical
P.O. Box 9010, College Station, TX 77842
tel. (800) 653-1711, or (979) 690-1711, fax (979) 690-0440
Email oimail@oico.com, Website: www.oico.com

Simultaneous Quantitative and Qualitative Measurements in a Single Workflow to Increase Productivity in Primary Drug Metabolism Investigations

Bruker Daltonics

The ability to simultaneously collect quantitative and qualitative information from a DMPK analysis has the potential to significantly increase productivity in pharmaceutical drug discovery and development. We present a single workflow allowing P450 drug clearance values to be determined as well as metabolites identified, profiled, and their structures elucidated. To be able to do all of this on a high throughput UHPLC chromatographic timescale is essential for the high levels of productivity required for today's DMPK screening laboratories. Haloperidol provides a good example of what can be achieved.

Haloperidol

$C_{21}H_{23}NO_2FCl$ $M+H^+ = 376.1474$

Workflow and Protocol

Microsomal incubations were carried out by Unilabs Bioanalytical Solutions at 1 µM drug concentration and a protein concentration of 0.5 mg/mL. Aliquots were taken and quenched with acetonitrile containing propranolol as an internal standard at eight time points over a period of 60 min.

Chromatography

Column: Fortis, 1.7 µm, H_2O, 2.10 mm × 30 mm
Column temperature: 30 °C
MPA: 0.1% formic acid in 95% H_2O/CH_3CN
MPB: 100% CH_3CN

Gradient:	0.0	0.3	2.0	2.5	2.6	3.0	min
MP %:	95	95	5	5	95	95	%

Flow rate: 300 µL/min
Injection volume: 5 µL

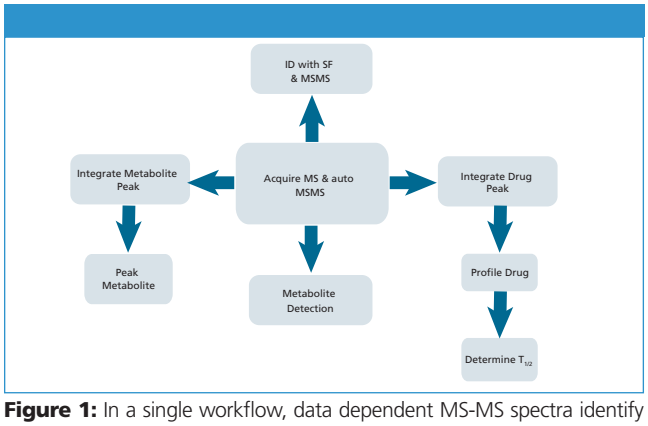


Figure 1: In a single workflow, data dependent MS-MS spectra identify and elucidate metabolite structures and drug clearance is measured.

The high surface area and lipophilic ligand combined with a hydrophilic end cap give this stationary phase a broad selectivity and resolving power for the target drug and the metabolites. The use of small particles allows UHPLC to compress the peak into a tighter and taller peak, therefore enhancing detection of very low level analytes.

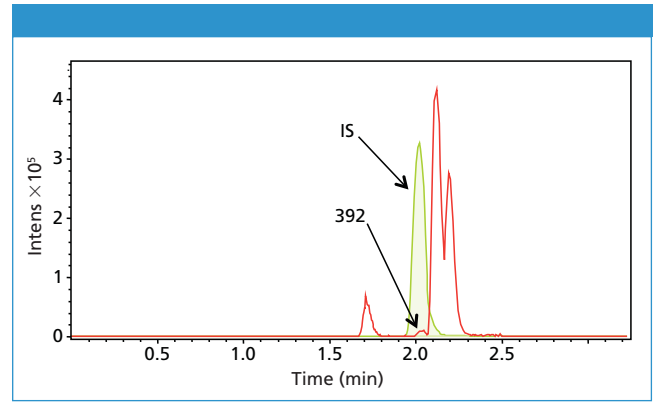


Figure 2: Metabolite detection software compares the data file for the drug (in this case t_{60}) with the corresponding control sample. A base peak chromatogram of the difference is created allowing the metabolites to be easily observed and their mass determined to 4 decimal places.

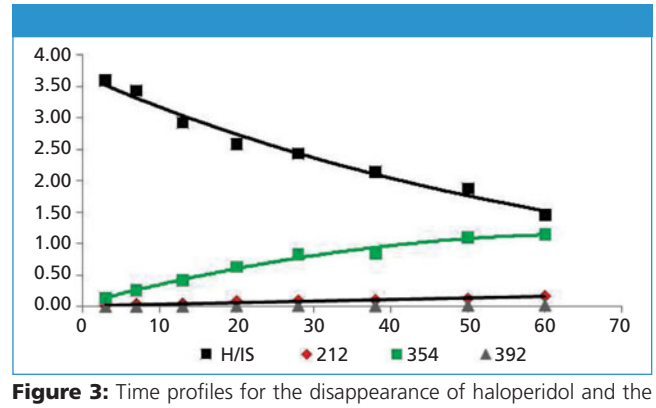


Figure 3: Time profiles for the disappearance of haloperidol and the appearance of three metabolites.

Metabolite Detection

Metabolite detect software compares the data file for the drug (in this case t_{60}) with the corresponding control sample. A base peak chromatogram of the difference is created allowing metabolites *m/z* 354, 212, and even 392 to be easily observed.

Metabolite detection software is able to detect the *m/z* = 392 metabolite even though it co-elutes with the internal standard.

Drug and Metabolite Profiles

Integration is carried out on the XIC for the measured *m/z* of each metabolite +/– 0.005 Da. Plotting the ratio of metabolite to internal standard (M/IS) versus time produces the metabolite profiles. Half-life and clearance values are determined from the natural log (ln) of the drug profile versus time plot.

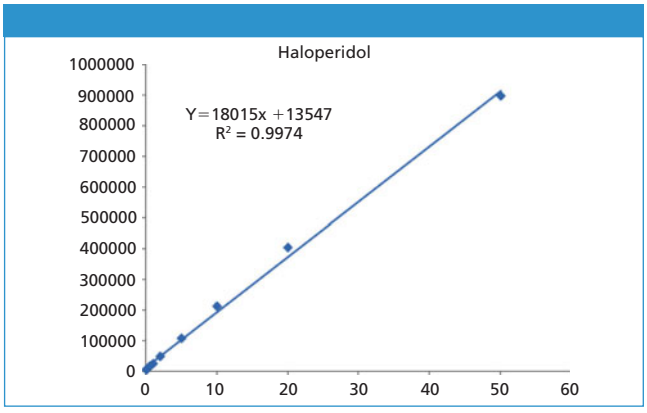


Figure 4: Linear calibration of 50 pg/mL to 50 ng/mL (3 decades) was achieved using the XIC for the measured *m/z* of each metabolite +/- 0.005 Da. R^2= 0.9974.

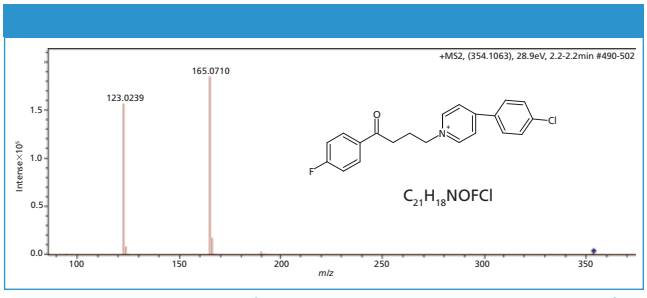


Figure 5: The structure of metabolite *m/z* = 354 is easily identified using Smartformula3D to understand the fragmentation pattern.

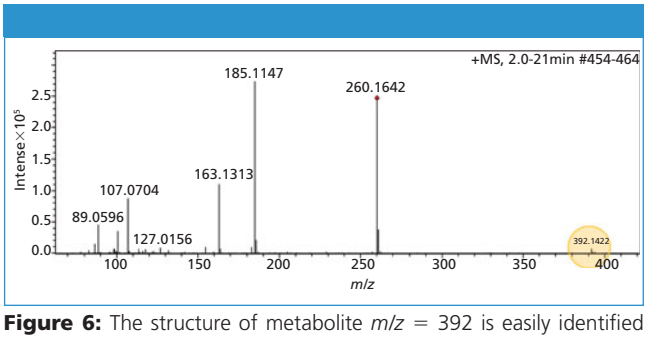


Figure 6: The structure of metabolite *m/z* = 392 is easily identified using Smartformula3D to understand the fragmentation pattern.

Linearity

MS–MS data was not available for *m/z* = 392 because of co-elution with the internal standard. The high quality data available, even for such a small peak, means SmartFormula is still able to predict the formula and deduce that it is a mono-oxidative metabolite.

m/z = 392.2422 Δm = 0.1 mDa (0.3 ppm)

$C_{21}H_{23}NO_3FCl$ Isotope fit = 23 ms

Comparison with 3Q

Both the AB Sciex API 5000 and Bruker **impact** QTOF yield equivalent results for the clearance values. This can be clearly seen by comparing the ln [Drug]/[IS] versus time plots.

The linearity and gradients of these plots are nearly identical and result in values for $t_{1/2}$ of 45 and 47 min, respectively.

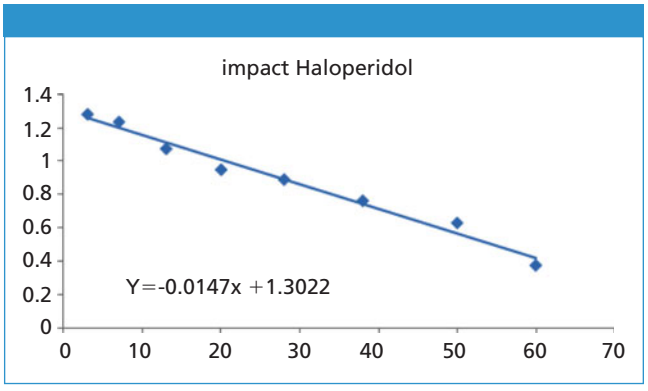


Figure 7: Clearance data from impact.

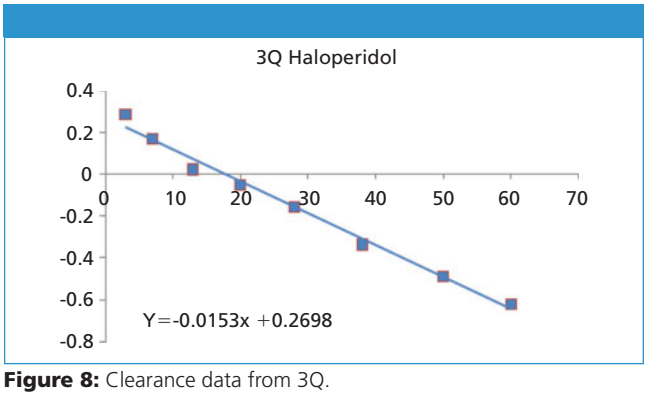


Figure 8: Clearance data from 3Q.

The difference in y intercept is a result of a difference in relative response of the internal standard and has no influence on the clearance results.

Conclusions

The quan–qual workflow is effective and robust using a rapid analytical method suitable for high throughput screening at 1 µM drug concentrations.

Metabolite detection software allows metabolites to be rapidly identified and profiled even when compounds co-elute.

Bruker Daltonics Inc.
40 Manning Road, Billerica, MA 01821
tel. (978) 663-3660, fax (978) 667-5993
Website: www.bruker.com

Measuring Antibody Molecular Weight by SEC-MALS

Malvern Instruments Ltd.

A purified polyclonal antibody (IgG) is separated and fully characterized using the Viscotek SEC-MALS 20, allowing calculation of molecular weight and radius of gyration (Rg).

Therapeutic recombinant antibodies represent a growing proportion of biopharmaceuticals and are primarily classed as Immunoglobulin G (IgG). However, proteins have a tendency to aggregate over time and one challenge for biologic drugs is that the presence of aggregates will stimulate an immune response. Size-exclusion chromatography (SEC) is a powerful tool that is commonly used to look at the aggregation of proteins. While most SEC systems use a single concentration detector such as ultraviolet (UV), the addition of light scattering allows the molecular weight of the protein to be measured independent of its retention volume. The new SEC-MALS 20 detector, which uses multi-angle light scattering (MALS), is ideal for this application. In addition, the MALS detector makes it possible to measure the radius of gyration (Rg) of molecules that scatter light anisotropically.

In this application note, a purified polyclonal antibody (IgG) is separated using SEC and characterized using the Viscotek SEC-MALS 20.

Experimental Conditions

Samples were analyzed using a Viscotek TDAmax system connected to Viscotek SEC-MALS 20. The mobile phase was phosphate buffered saline, which was also used to prepare the IgG for analysis.

Results

The SEC-MALS results are presented in Table I. The monomer (15.80 mL) and dimer (14.00 mL) peaks are clearly identified by the measured molecular weights and low polydispersity (Mw/Mn). No size (Rg) can be measured for these peaks as they are below the isotropic scattering threshold of 10–15 nm. Studying Figure 1, it is just possible to see that the SEC-MALS 20 show the same response for the monomer peaks at all angles. The aggregate peak (13.23 mL) is clearly different. The molecular weight is higher and more polydisperse, which shows that there is a variable composition of molecules within the aggregate peak. Because it is large, the light scattering response varies with angle and can be used to measure the Rg.

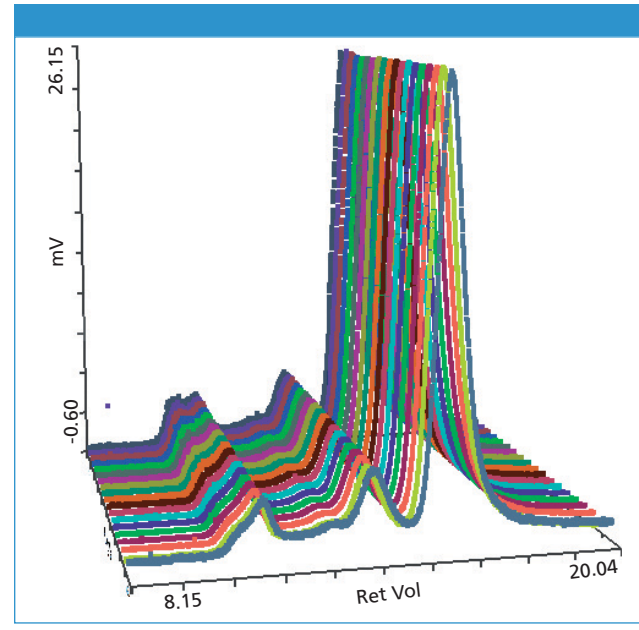


Figure 1: Overlay of MALS detector responses for IgG.

Table I: Measured molecular weights of the different peaks of the IgG sample

	Aggregates	Dimer	Monomer
Peak RV - (mL)	13.23	14.00	15.80
Mn - (kDa)	674.12	308.6	147.2
Mw - (kDa)	7661.00	309.2	147.4
Mw / Mn	11.364	1.002	1.001
Rg (w) - (nm)	26.6	N/C	N/C
Wt. Fr. (Peak)	0.014	0.065	0.921
Weight Fraction % (Peak)	1.4	6.5	92

Malvern Instruments Ltd.
Enigma Business Park, Grovewood Road, Malvern, UK
tel. +44 (0) 1684 892456
E-mail: salesinfo@malvern.com
Website: www.malvern.com

Introducing Titan UHPLC Columns Featuring Monodisperse Porous Particles

Wayne Way, Supelco

A careful investigation of columns prepared with monodisperse, solid-core silica particles has shown that an important contribution to their surprising efficiency arises from a very low eddy diffusion (multipath) term in van Deemter plots. The very narrow particle size distribution of 6% RSD for 2.7 µm Fused-Core® silica particles may create more uniform column beds and flow paths than particles with broader distribution.

Uniform column beds may also preserve initial high efficiency by providing more resistance to voiding and channeling under variable flow and pressure conditions. If small porous silica particles can be made economically with a similar narrow size distribution, they may also allow preparation of columns with higher efficiency and greater bed stability. Because porous silica particles made by usual processes have broader distribution and can be costly to size, a new process was developed to create silica particles that are already monodisperse and ready-to-use for UHPLC. The unique process allows monodisperse silica to be prepared in a range of porosities and sizes with <6% RSD.

Previously, a size range of 15–25% RSD has been more typical for porous silica particles, even after special sizing procedures have been applied. Titan™ columns prepared with monodisperse 1.9 µm C18 silica have exhibited more than 300,000 plates per meter for small molecules and can greatly enhance the performance of UHPLC instruments. Titan UHPLC columns are the outcome of the patent pending Ecoporous™ process, a process that provides an economical route to UHPLC grade silica.

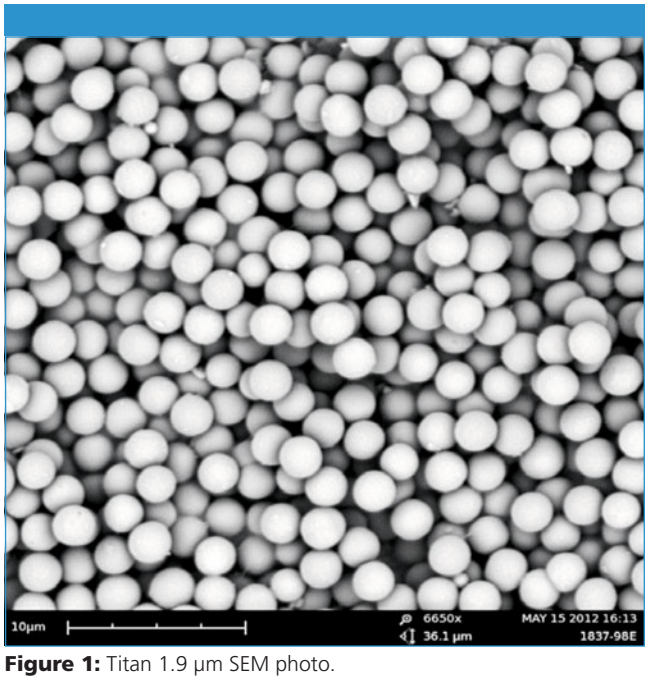


Figure 1: Titan 1.9 µm SEM photo.

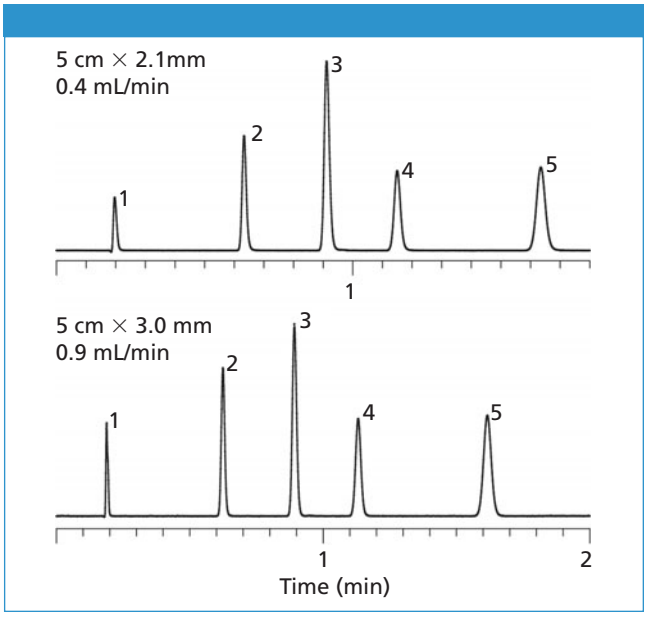


Figure 2: Titan C18 performance at different column i.d.s, columns: Titan C18, 1.9 µm, mobile phase: 50% acetonitrile, temp.: 35 °C, det.: 254 nm. Peaks: 1. Uracil, 2. Diazepam, 3. Toluene, 4. Naphthalene, 5. Biphenyl.

Titan exhibits the narrowest particle size distribution for any known UHPLC grade porous silica. Figure 1 shows an SEM image of the Titan silica. D90/10 values have been consistently measured less than 1.2 for this silica. Chromatographic data for two column internal diameters is shown in Figure 2 for a set of probes including a pharmaceutical base. Excellent efficiency and peak shape are demonstrated in this data set.

In conclusion, our results indicate a higher performance to pressure ratio for Titan columns compared to many commercial UHPLC columns. Very narrow PSD porous silica promises to become another important development in this era of rapid advancements in UHPLC technology.

Size Exclusion Analysis of Tween-Containing IgG Formulations

Supelco

During purification and in final formulation, proteins, including monoclonal antibodies and other biotherapeutic agents, are often in the presence of a low concentration of surfactant (<0.01%) to inhibit protein aggregation or adsorption (1–4). High performance silica-based gel filtration columns, such as 5 µm packed SRT® SEC-300 columns, allow for an accurate assessment of the influence of Tween® 20 or Tween 80 surfactants on the level of aggregation in protein drug formulations.

Polysorbates 20 and 80, commercially available as Tween 20 and Tween 80, are among the most popular non-ionic surfactants used to limit the formation of aggregates in biotherapeutic solutions. Since the presence of aggregates in protein drug formulations may result in an immunological response, manufacturers are required to determine the level of aggregation in such formulations. Size exclusion chromatography is an accepted method to determine the level of aggregation in formulations of biotherapeutic products.

Since the composition of an SEC mobile phase differs from the composition of the formulation, it is important to determine that the mobile phase conditions under which the biotherapeutic elutes from the column, does not influence the level of aggregation that is present in the formulation. Protein aggregates often form irreversible soluble aggregates which are not influenced by the mobile phase composition, although many in-process aggregates do not form irreversibly (4). As part of such a study it is also important to determine the effect of adding surfactant to the sample to ascertain that the surfactant concentration at the time of protein and aggregate elution is similar to the surfactant concentration in the formulation. In this study we investigated the effect of adding Tween 20 and Tween 80 to the elution positions of a proprietary monoclonal antibody (mAb 221) under SEC conditions.

Discussion

We used 25 cm × 4.6 mm i.d. columns packed with 300 Å pore size silica particles of 5 µm size, which were bonded with a hydrophilic functional group. Figure 1 shows the UV traces of successive injections of water, 0.1% Tween 20, mAb 221, and 0.1% Tween 20 added to mAb 221. The chromatogram of a high concentration sample of monoclonal antibody 221 was expanded to clearly show the small aggregate peak in front of the main component. The effect of adding Tween 20 to the sample did not have a measurable effect on the level of aggregate. Note that the nature of the interfering fragment on the trailing side of the main component was not established.

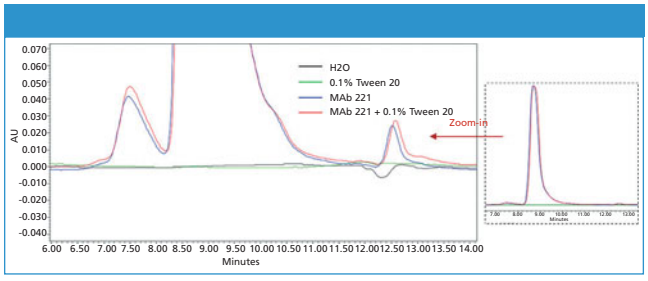


Figure 1: No interference from Tween 20 in SEC of mAb221. Column: SRT SEC-300 (5 µm, 300 Å, 4.6 × 30 cm), mobile phase: 150 mM sodium phosphate buffer, pH 7.0; flow rate: 0.35 mL/min, temp.: ambient, pressure: 800 psi, detection: UV@214 nm, sample: mAb 221 #1, inj. volume: 3 µL.

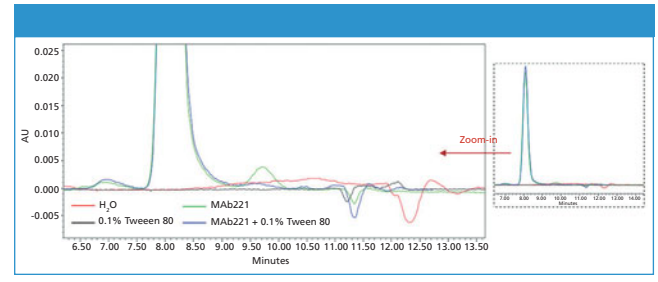


Figure 2: Effect of Tween 80 on the SEC analysis of mAb 221 by size exclusion chromatography. Conditions as in Figure 1, except sample: mAb 221 #2.

Using the same SRT SEC-300 column, we also looked at the effect of Tween 80 on a lower concentration lot of mAb 221; see Figure 2. This sample showed a smaller aggregate peak, and an additional peak after the main component, while there is at best only a slight indication of a fragment peak interfering with the monoclonal antibody. In this case, we found that adding Tween 80 to the antibody sample changed the aggregate profile, while the large unknown peak eluting at 9.72 min greatly diminished in size.

References

(1) S. Bondes and A. Bicknell, *Anal. Biochem.* **316,** 223–231 (2003).

(2) B. Kerwin, *J Pharm Sci.* **97**(8), 2924–2935 (2008).

(3) *Aggregation of Therapeutic Proteins,* W. Wang and C.J. Roberts, Eds. (John Wiley & Sons, Inc., Hoboken, New Jersey, 2010).

(4) L. Narhi, Y. Jiang , R. Deshpande, S. Kang, and J. Shultz, in *Aggregation of Therapeutic Proteins,* W. Wang and C.J. Roberts, Eds., (John Wiley & Sons, Inc., Hoboken, New Jersey, 2010), Chapter 6.

SEC-MALS of Silicones

Wyatt Technology

Polydimethylsiloxane is the world's most common silicone. Its applications range from contact lenses and medical devices to elastomers, caulking, lubricating oils, and heat resistant tiles. For all of its applications, the weight-average molar mass (and its distribution) is directly associated with the performance of the product. A DAWN DSP multi-angle light scattering (MALS) detector coupled with a size-exclusion chromatograph (SEC) provides the perfect tool for making molecular weight determinations without reference to standards or column calibration.

For this note, a polydimethylsiloxane sample was analyzed by SEC in toluene, using Wyatt Technology's DAWN and an Optilab refractometer as the respective MALS and concentration detectors.

Figure 1 shows the chromatograms of polydimethylsiloxane with signals from the light scattering at 90° (top) and the RI (bottom) detectors. The RI signal is negative because the refractive index increment (dn/dc) of polydimethylsiloxane in toluene is negative. A positive signal can be obtained if the polarity of the signal output is reversed. Because the light scattering signal is proportional to dn/dc squared, its signal is positive.

By combining the DAWN and Optilab data, the absolute molar masses of this siloxane were calculated without making any assumptions about the polymer's conformation or elution time.

A polystyrene standard with a molar mass of 200 kD was analyzed under the same conditions, as it is frequently used to calibrate columns for conventional chromatography. Both results are plotted in Figure 2. Even though polydimethylsiloxane is a linear polymer, just as is this polystyrene standard, the molar masses at the same elution time are not identical for the two polymers.

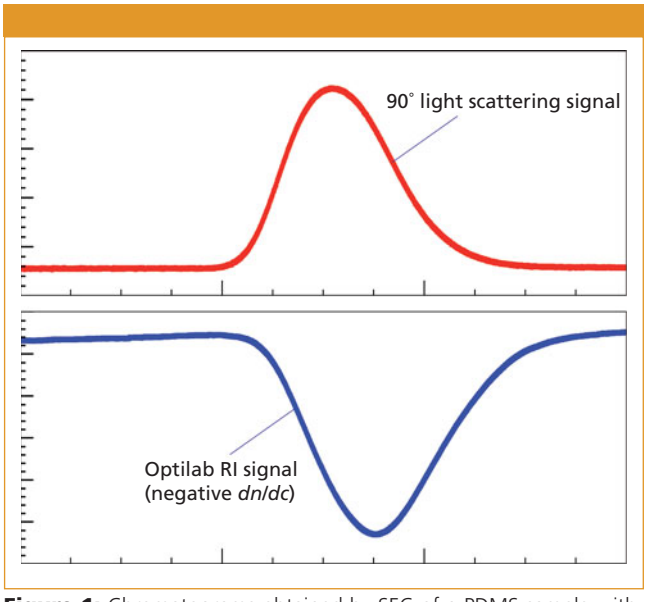


Figure 1: Chromatograms obtained by SEC of a PDMS sample with-signals from the DAWN DSP (top) and the Optilab RI (bottom).

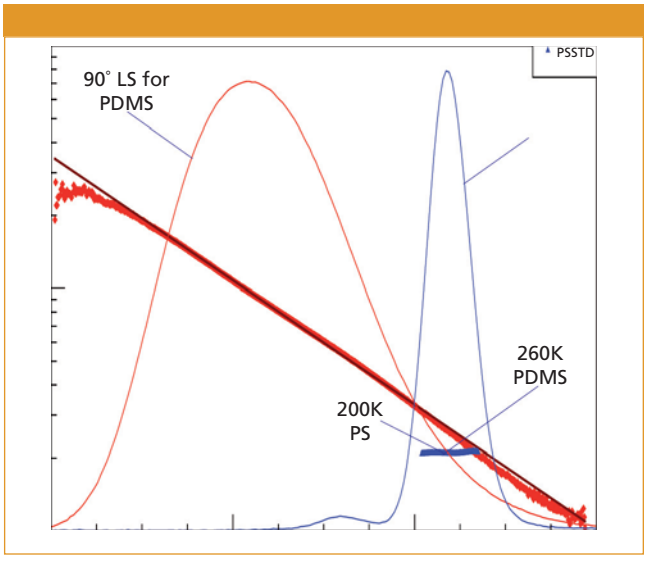


Figure 2: Plots of the molar mass versus elution time superimposed over the signals from the DAWN, for the PDMS sample and the polystyrene "standard" showing the large errors associated with conventional column calibration.

If polystyrenes had been used as calibration standards, the molar mass for polydimethylsiloxane would have been erroneous. The results once again demonstrate the power of MALS in determining absolute molar masses of polymers without any reference to calibration routines or polymer standards — even when those polymers appear to share the same conformation as the standards.

Wyatt Technology
6300 Hollister Avenue, Santa Barbara, CA 93117
tel. (805) 681-9009, fax (805) 681-0123
Website: www.wyatt.com

Troubleshooting GC Selectivity, Resolution, and Baseline Issues

Understanding the chemistry behind gas chromatography (GC) separations can lead to faster problem identification and improved troubleshooting.

Fortunately, sudden selectivity changes in GC are fewer than in other forms of chromatography — it's not as if we can make up the eluent with the incorrect amount of organic or at the wrong pH after all! However, changes in selectivity (the distance between the apices of two peaks, measured as a ratio of their retention factors) do occur and the causes can usually be traced to one of few usual suspects. The selectivity of a separation is affected predominantly by the strength of the interaction of each analyte with the two phases between which it is portioning, and in GC this interaction is affected most by the stationary-phase chemistry and the temperature of the mobile phase. If the relative peak spacing suddenly changes, one should check that the correct stationary-phase chemistry is being used and that the temperature (or temperature program) is as it should be. If the temperature program has been entered correctly into the instrument, one might check the oven temperature over the course of the analysis using a resistive thermocouple and digital thermometer. If the oven temperature is not following the program to within a few degrees, one might suspect a problem with the heater and a visit by your service provider may be required. If all is well, ensure that the column equilibration time (the wait time after the oven has reached its initial temperature set point before sample injection) is sufficient to establish the correct (homogeneous) temperature throughout the length and across the diameter of the whole column. Even though capillary GC columns have relatively low thermal mass, we need to ensure that the column and carrier gas passing through it, are at the correct initial temperature to begin the analysis, and this can sometimes take a surprisingly long time (>1 min for columns with thicker films). I have often seen variations in column equilibration time give rise to insidious issues during method transfer exercises and, although this parameter may seem minor, bear in mind the oft quoted fact that a difference in temperature of 23 °C can half the retention time of an analyte. This is particularly important during splitless injection where the initial column temperature plays a vital role in determining peak shape and may influence relative analyte retention. One final check into selectivity changes would involve trimming the first 5% of the column length to remove any stationary phase whose chemistry has been modified by the adsorption of sample components or whose phase has been stripped to reveal the underlying silica, both of which may have subtle influences over the relative band spacing of analytes; this initial column section has a large influence over band spacing in GC.

As we know, resolution in chromatographic separations is influenced by efficiency (N), selectivity (α), and retention factor (k) as defined by the fundamental resolution equation. Having taken care of any issues with selectivity, we must ensure that the efficiency of the system is as it should be, because reduction in efficiency is perhaps the most common cause of loss of resolution in GC. The efficiency of the GC column will decrease gradually over time, primarily because of the loss of bonded phase through chemical and thermal degradation; this should be noted, with a minimum acceptable efficiency established for system suitability testing, especially where resolution between critical peak pairs is reliant on achieving a certain peak width or plate count. Trimming the first 1–5% of the column length can help to temporarily restore column efficiency, but note that retention times may decrease as a consequence. The other main cause of efficiency loss is poor installation of the column into the inlet and detector. Ensure that you follow your manufacturer's instructions carefully to avoid issues with sample introduction into the column or dead volumes at the detector connection.

Baseline position in GC will tend to shift with temperature but also with flow rate for certain detectors which are known to be "mass/flow" sensitive — that is, response depends not only on the amount of substance entering, but also the rate at which it enters. We typically see a shift in baseline position when thermally equilibrating the column, but a rising baseline may also be seen when using a temperature program. This may be normal at high temperatures (due to increased phase bleed for example), but rising baselines at lower temperatures can be mitigated by using a constant-flow mode of operation as opposed to constant pressure where the carrier flow will reduce with increased oven temperature.

Noisy baselines in GC can arise from a poorly equilibrated detector system (especially with nitrogen–phosphorus detection and electron-capture detection systems) as well as from column and septum bleed. Ensure that your column is fully thermally equilibrated by purging with carrier gas at room temperature for 10 min followed by a ramp to 10 °C above your upper method temperature, or to the isothermal temperature maximum of the column, whichever is lower. Purging with carrier gas at room temperature for a short while will initially reduce the amount of column bleed substantially that occurs during thermal equilibration. Ensure that you use low-bleed septa and that your septum purge flow is working to reduce the amount of bleed product emanating from the septum. Replace septa regularly before they core or become worn and release shards of material into the inlet liner.

The issues above are among those considered within the CHROMacademy tutorial on GC troubleshooting — follow the link shown in the lower left corner of this page to discover other causes and remedies for issues relating to selectivity, resolution, and baseline issues.

More Online:

Get the full tutorial at
www.CHROMacademy.com/Essentials
(free until September 20).

Material Identification by HPLC with Charged Aerosol Detection

Material identification is a common need in many industries, most notably for pharmaceutical manufacturing where the United States Pharmacopeial Convention (USP) defines many identification tests. A new platform approach to material identification was developed using high performance liquid chromatography (HPLC) with charged aerosol detection (CAD). The method described in this article incorporates a mobile-phase gradient consistent with a hydrophilic interaction liquid chromatography (HILIC) separation mechanism and uses a mixed-mode column, which provides reversed-phase and cation- and anion-exchange properties. This technique provides a rapid and flexible alternative to *USP* <191>, and other analytical identification techniques. The chromatographic separation of 13 substances included in *USP* <191> has been demonstrated. Furthermore, simultaneous quantitation and impurity detection is achievable within the same analytical run.

Brandon Scott, Kelly Zhang, and Larry Wigman

Material identification is an integral part of the quality control process for chemical manufacturing and research, both of which need a generic, fast, and specific test method for a wide range of compounds. The typical practice in the pharmaceutical industry for raw material acceptance in manufacturing is minimally based on a vendor's "Certificate of Analysis," appearance and identification testing.

Identification testing of commonly used raw materials is often included in the United States Pharmacopeial Convention (USP). The USP sets quality and purity standards for products ranging from medicines to food ingredients that are enforceable in the United States by the Food and Drug Administration (FDA). *USP* chapter <191> (1) defines general identification tests. The materials described are identified by a distinctive set of tests based on their unique physiochemical properties. Identification of a single substance can range from the formation of a single precipitate to performing a flame test followed by a multistep precipitation and dissolving matrix. While these are acceptable methods for material identification in the pharmaceutical industry, many other technologies provide more rapid and generic capabilities for material identification.

Depending on the industry and application, material identification may be required for compounds from inorganic salts to heavy metals to organic solvents. Raw materials for pharmaceutical manufacturing do not contribute to the structure of the final active pharmaceutical ingredient (API) molecule; however, positive identification is generally required before use in the production process. Suitable identification techniques are selected based on unique material properties of the raw material of interest. Furthermore, confirmation of identification will be shown by comparison to a reference standard, which contains closely related substances (2). Not only will the reference standard confirm a positive identification, but also show that a positive response is not obtained from the closely related substances or possible interfering materials.

Many technologies are currently used for identification. These include, but are not limited to, Fourier transform infrared spectroscopy (FT-IR), ion chromatography (IC), pK_a determination, and Raman spectroscopy. Technologies such as these are often limited by their inability to analyze multiple substances at the same time or within the same analytical run, and each technique is limited in the set of substances that it can detect. FT-IR lacks the ability to detect substances like inorganic salts or solutions in water, IC cannot detect neutral compounds, pK_a determination cannot distinguish between acids with similar pK_a values, and identification by Raman spectroscopy fails for compounds that fluoresce.

Charged aerosol detection (CAD) is a universal detection method for nonvolatile compounds or substances (3). CAD nebulizes eluent from the high performance liquid chromatography (HPLC) analysis to form particles. Ionized nitrogen gas then charges the particles and those charges are detected by an electrometer. Using CAD as a tool for identification allows us to take advantage of its inherent property as a universal detection method and detect significantly more substances than similar identification technologies or techniques.

The purpose of this work was to show that HPLC with CAD can be used for material identification. The identification includes, but is not limited to, many materials listed

Table I: Chromatographic conditions for the HPLC–CAD identification method				
Flow rate	1.0 mL/min			
Column temperature	35 °C			
Injection volume	5 µL			
Mobile phase A	Water			
Mobile phase B	Acetonitrile			
Mobile phase C	200 mM ammonium formate (pH 4.0)			
Gradient	Time (min)	% Mobile-Phase A	% Mobile-Phase B	% Mobile-Phase C
	0	48	50	2
	1	45	50	5
	5	45	25	30
	7.5	5	5	90
	12	5	5	90

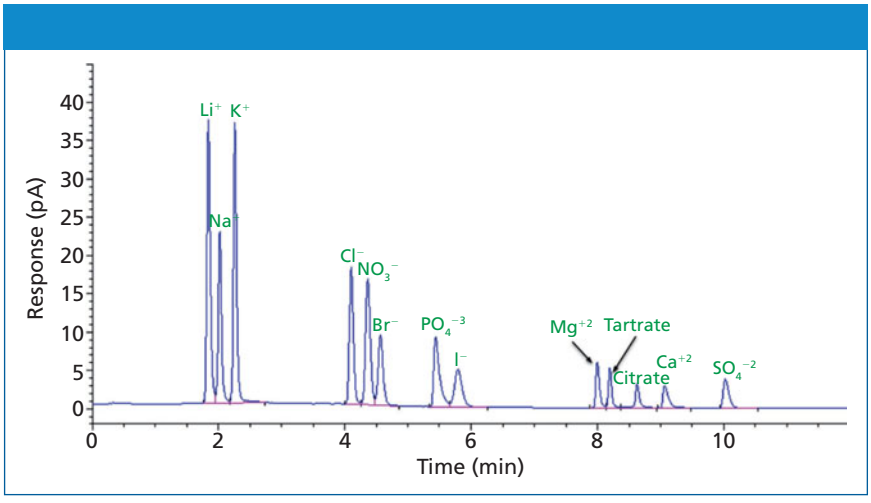


Figure 1: Chromatogram showing 13 substances in *USP* <191> separated using the conditions in Table I.

in *USP* <191>, which describes material identification for many alkali metals, halides, heavy metals, and anionic polyatomic species like sulfate.

Experimental

Reagents and Materials

The materials used for this work were all obtained from Sigma-Aldrich and were ACS grade or better. The materials used include sodium sulfate, sodium chloride, sodium phosphate, sodium nitrate, lithium hydroxide, magnesium lactate, sodium citrate, potassium phosphate, and ammonium formate for HPLC mobile phase.

Deionized water (>18.2 MΩ) was purified from a Milli-Q water purification system (Millipore). HPLC-grade acetonitrile was purchased from Burdick and Jackson.

Instrumentation

The chromatographic system was an Agilent 1260 HPLC system (Agilent) equipped with an on-line degasser, a quaternary pump, an autosampler, a column thermostat, a diode-array UV detector, and a Corona Plus CAD detector (ESA Inc., A Dionex Company). The column was a 50 mm × 3.0 mm, 3-µm d_p Thermo Scientific Acclaim Trinity P1.

Results and Discussion

HPLC–CAD Method Development

Method specificity is a critical parameter for any identification method or technique (2). Separation-based method identification typically uses retention time of the sample compared to a standard; for instance, identification by HPLC–UV is commonly used. Our aim is to have a generic identification method, thus 13 common substances used in pharmaceutical manufacturing that are typically identification tested per *USP* <191> were selected: sodium, potassium, lithium, chloride, bromide, iodide, nitrate, sulfate, tartrate, calcium, phosphate, citrate, and magnesium. The method development was based on a method we published previously for ion analysis, in which 11 of these substances were separated using a mixed-mode column and hydrophilic interaction liquid chromatography (HILIC) conditions (4). The addition of bromide and lithium was necessary to provide additional coverage for the most common raw materials used in pharmaceutical manufacturing. Thus, there were three areas of focus for method development. These include adding lithium and bromide to the analysis, decreasing overall analysis time, and maintaining specificity by making gradient modifications.

The most desirable conditions satisfying all method development goals were achieved by increasing the flow rate to 1 mL/min, decreasing the starting acetonitrile to 50% while maintaining 2% 200 mM ammonium formate at the starting conditions for 1 min, and using a 7.5 min gradient. Table I shows the final method chromatographic conditions. Specificity was maintained as shown in Figure 1 despite gradient modifications. Furthermore, a reduction in analysis time was achieved, from 20 min to 12 min.

Identification by HPLC–CAD

Identification by HPLC–CAD has many advantages compared to other techniques. One primary advantage of this technique is the rapid identification of a substance, such as potassium phosphate shown in Figure 2. For example, a previously prepared standard is run confirming the retention times of 13 substances, as shown in Figure 1. A solution of the sample (potassium phosphate), prepared at about 0.1 mg/mL, is injected after the standard. By comparison of retention times, potassium phosphate

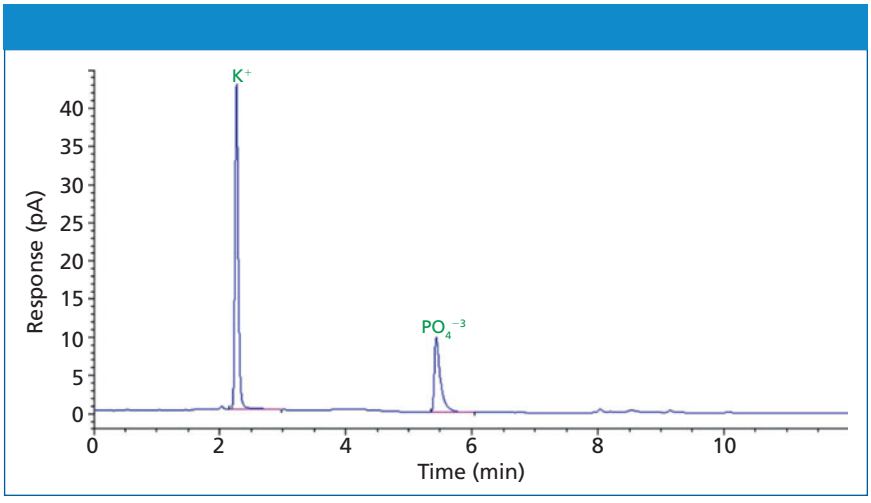


Figure 2: Chromatogram of potassium phosphate using the conditions in Table I.

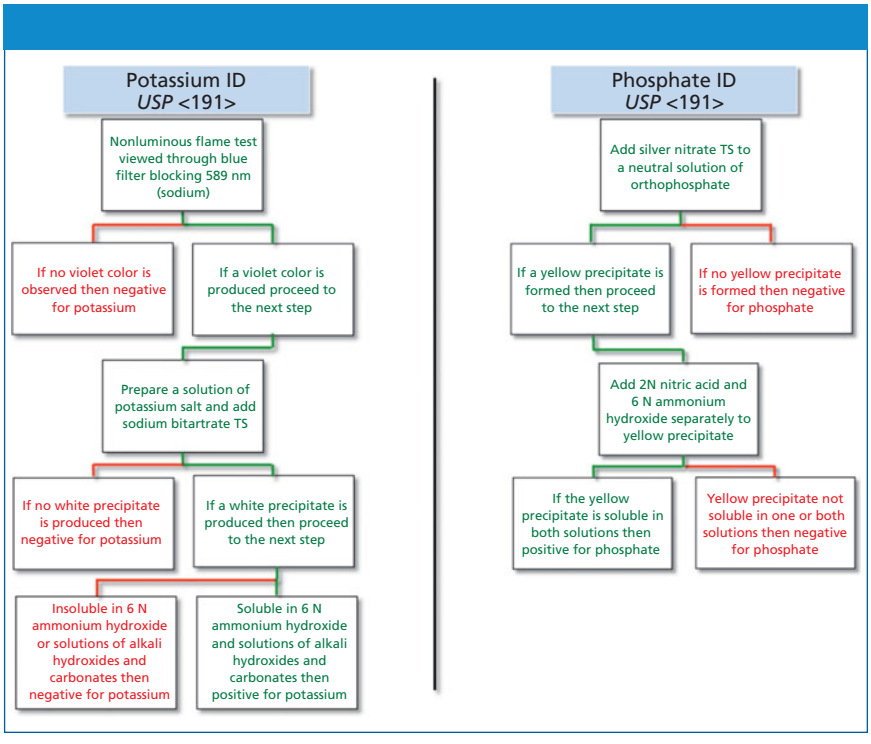


Figure 3: Flow chart depicting steps necessary to identify potassium and phosphate per *USP* <191>.

is easily identified. With each standard and sample injection taking less than 20 min, depending on equilibration time, the identification takes less than 40 min. Where this application excels is its ability to identify multiple substances within the same analytical run. For example, the analysis time for four different compounds, or seven substances (sodium chloride, potassium nitrate, lithium bromide, citric acid) would take less than 100 min, illustrating not only the application's diversity but also its efficiency.

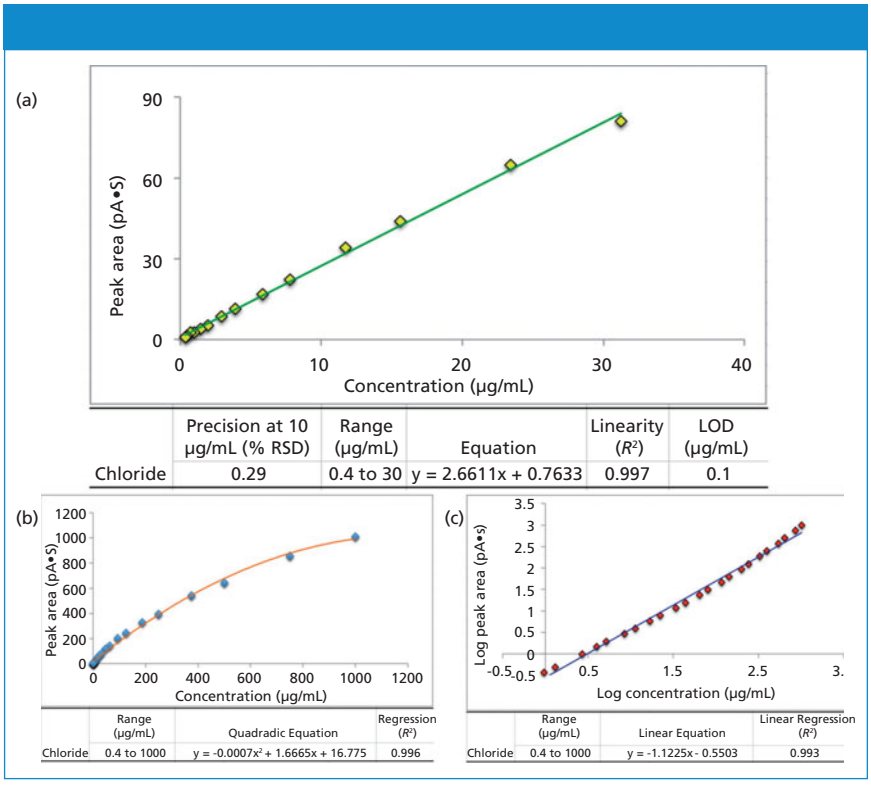
Adding to the efficiency of the HPLC–CAD analysis is the ease of system setup and sample or standard preparation. For this specific application, all samples were prepared at 0.1 mg/mL in 80:20 (v/v) water–acetonitrile. Two approaches can be taken for standard preparation. Approach one is to prepare a standard, which represents only the substances to be identified. A second approach would be to prepare a stock solution of multiple substances as used in the potassium phosphate example. The stock standard would then be stored for future use, thus additional time can be saved. For both approaches, the standards are prepared quickly from commercially purchased standards such as 1000 ppm chloride standard for IC.

Identification by HPLC–CAD Versus *USP* <191>

Identification by HPLC–CAD has many advantages when compared to *USP* <191>. As shown in Figure 3, the identification of potassium and phosphate using *USP* <191> uses a large number of reagents. Some reagents can be purchased commercially whereas others may need to be freshly prepared. For example, to identify potassium and phosphate, eight reagents are required. In addition, equipment for a flame test is required for potassium identification. After an analyst has acquired all reagents, equipment, and glassware, the identification can be started. In total, nine steps are required to identify both substances and this would take significantly longer than the same identification by HPLC–CAD, which requires approximately 2 h of analyst time. A comparison of the required analyst time for identifying four compounds by HPLC–CAD as described above exhibits a larger time advantage. Using the *USP* <191> method for each substance, an analyst would spend many hours identifying each, whereas the analyst time commitment using HPLC–CAD would be no more than 1 h.

Furthermore, if multiple lots of the same substance need to be identified per *USP* <191>, then each would require the same amount of steps and nearly double the analyst time commitment. In comparison, if HPLC–CAD was used, samples from each additional lot would be prepared in about 5 min and added to the same analysis.

One advantage that *USP* <191> possesses is the ability to identify substances such as ammonium, that are too volatile for detection using CAD. CAD cannot detect highly volatile



	Precision at 10 µg/mL (% RSD)	Range (µg/mL)	Equation	Linearity (R^2)	LOD (µg/mL)
Chloride	0.29	0.4 to 30	y = 2.6611x + 0.7633	0.997	0.1

	Range (µg/mL)	Quadradic Equation	Regression (R^2)
Chloride	0.4 to 1000	$y = -0.0007x^2 + 1.6665x + 16.775$	0.996

	Range (µg/mL)	Linear Equation	Linear Regression (R^2)
Chloride	0.4 to 1000	y = -1.1225x - 0.5503	0.993

Figure 4: Method qualification results for the determination of chloride concentration with (a) simple linear regression; (b) quadratic regression; (c) log–log plot linear regression.

substances because particles cannot be formed when leaving the nebulizer.

Identification by HPLC–CAD Versus Other Technologies

Other technologies are also available for identification of substances. Identification by IC has few, if any, advantages over HPLC–CAD. Performing an identification on an IC system will require only highly purified water, a column for retaining anions or cations, and an eluent generator, such as hydroxide. For a single substance like phosphate, this technique is quick and requires little analyst time. However, to identify phosphate, three instrument modifications are needed, including switching the eluent generator to methanesulfonic acid, changing to a cation retaining column, and switching the capillary connections between the detector and the negative ion suppressor to the detector and the positive ion suppressor. Compared to an HPLC–CAD technique, traditional IC techniques have no advantage because they cannot detect anions and cations simultaneously without instrument modification and a second analysis. IC also cannot detect uncharged substances, and although the method presented in this paper only describes charged substances, the CAD can also detect uncharged substances when combined with the right chromatographic conditions.

Simultaneous detection of anions and cations is possible by chelating the positively charged ions with the ligand EDTA, then an IC setup in anion mode can detect the anion and precomplexed cation EDTA ligand (5). Although this technique achieves some similarities to the HPLC–CAD technique, using it for multiple substances would require extensive method development. Other IC techniques are available including multieluent systems, in-series columns, zwitteric ion-exchange columns, and multidetector systems, making multi-substance detection possible (6). Unfortunately, these options include columns that are not commercially available and have complex instrumentation configurations, and uncommon mobile phases.

Infrared spectroscopy is a common technique for the identification of substances, including those in the pharmaceutical industry. Although FT-IR can be used for identification of many substances by comparing them to a reference standard spectrum, inorganic salts like sodium chloride and potassium bromide are infrared-transmitting substances and are therefore not susceptible to identification. Substances in water, such as 1 M citric acid, would be difficult to identify due to large interference from water.

Potentiometric titration could be used to identify acids such as fumaric acid and tartaric acid by their pK_a values. However, fumaric acid and tartaric acid have nearly identical pK_{a1} and pK_{a2} values (3.03 and 4.38 for fumaric acid, 3.02 and 4.36 for tartaric acid), so identification cannot be confirmed.

Raman spectroscopy can be used to identify substances such as manganese sulfate. A noninvasive technique using spatially offset Raman spectroscopy, offers quick scanning for identification (7). However, substance identification is limited due to fluorescence, which obscures the Raman spectra. Although multiple technologies are available to identify substances, all of them can only be used to identify a narrow set of compounds compared to HPLC–CAD.

Additional Advantages of HPLC–CAD

Although the goal of this article is to show how HPLC–CAD can be used for substance identification, the HPLC–CAD combination has additional applications (4) that can be performed simultaneously. One application is the ability to perform substance quantification. For example, using current chromatographic conditions, chloride has been shown to be linear from 0.4 µg/mL to 30 µg/mL and all 13 substances have similar linear ranges. Figure 4a shows the linearity, injection precision, and limit of detection (LOD) method qualification results that would satisfy regulatory requirements for assay methods.

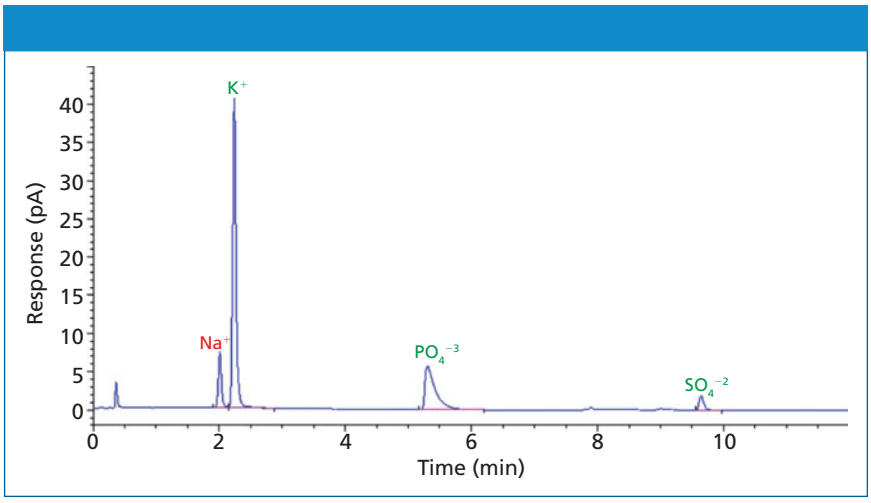


Figure 5: Identification of a potassium phosphate sample showing sodium and sulfate contamination.

Method sensitivity is typically not required for a material identification method, but generally, CAD can achieve detection to the single-digit nanogram range. Detailed information regarding CAD sensitivity can be found in the literature (4). A simple linear fit with a dynamic range of two orders of magnitude was used here in Figure 4a, and it is adequate for assay method requirements. If a wider dynamic range is desired, a quadratic plot or linear log–log plot (8) can be used, as shown in Figures 4b and 4c, respectively, where the dynamic range of up to four orders of magnitude can be achieved.

Another advantage is the ability to collect impurity data, which again can be collected simultaneously. Because CAD is a universal detection method, impurities present in a sample are most likely to be observed. A major impurity in potassium phosphate is sulfate, which, as shown in Figure 5, is observed along with sodium. If a standard, as shown in Figure 2, is prepared quantitatively, then the amount of impurities can be determined. Thus, in one sample and analysis, the identity of potassium phosphate was confirmed and the purity of the sample was ascertained.

The optimized method is not limited to only 13 substances. The method can be modified to include additional substances, either from *USP* <191> or not, depending on future identification needs.

Limitations of HPLC–CAD for Material Identification

As demonstrated above, HPLC–CAD for material identification based on the retention time of samples and standards provides significant practical advantages, especially for raw material identification in which the sample matrix is typically simple. However, CAD response is generally mass dependent and not spectral or physicochemical property dependent. Although this is an advantage for universal detection, it lacks specific structural information. For samples with a complex matrix or an unknown mixture in which definitive identification of impurities is needed, orthogonal detection methods or specific techniques such as mass spectrometry should be used.

Another limitation as mentioned earlier is that CAD cannot detect volatile compounds, such as ammonium acetate and formate. On the other hand, this is why ammonium acetate and formate are good mobile-phase modifiers for HPLC–CAD.

Conclusions

A new generic approach for material identification has been shown using a combination of an HPLC, CAD, and a mixed-mode column. Current industry standards in the pharmaceutical industry rely on *USP* <191> for material identification. However, our approach is a rapid and concise way for replacing many sections of *USP* <191> for material identification. Furthermore, this approach is a practical alternative for material identification in other industries or applications. Together with identification, the technique can simultaneously perform quantitation and detect impurities. However, for structural information, specific detectors, such as mass spectrometers, should be used.

Acknowledgments

We thank Sigrid Hubbell (Genentech) for manuscript review and helpful suggestions.

References

(1) General Chapter <191> "Chemical Tests and Assays, Identification Tests — General" in *United States Pharmacopeia 35–National Formulary 30* (United States Pharmacopeial Convention, Rockville, Maryland, 2012).

(2) International Conference on Harmonizaiton (ICH) Guidelines. Validation of Analytical Procedures Q2 (R1), Nov. 2005.

(3) S. Almeling, D. Ilko, and U. Holzgrabe, *J. Pharm. Biomed. Anal.* **69,** 50–63 (2012).

(4) K. Zhang , L. Dai, and N.P. Chetwyn, *J. Chromatogr., A* **1217**(37), 5776–5784 (2010).

(5) M. Bruzzoniti, E. Mentasti, and C. Sarzanini, *Anal. Chim. Acta* **382,** 291–299 (1999).

(6) P. Nesterenko, *TrAC, Trends Anal. Chem.* **20,** 311–319 (2001).

(7) M. Bloomfield, D. Andrews, P. Loeffen, C. Tombling, T. York, and P. Matousek, *J. Pharm. Biomed. Anal.* **76,** 65–69 (2013).

(8) T. Vehovec and A. Obreza, *J. Chromatogr., A* **1217**(10), 1549–1556 (2010).

Brandon Scott, Kelly Zhang, and Larry Wigman are with Small Molecule Pharmaceutical Science, at Genentech Inc., in South San Francisco, California. Direct correspondence to: zhang.kelly@gene.com ■

Applying Comprehensive Analysis to EPA Method 1613B Samples — Discover Those Compounds Usually Discounted in Environmental Samples

Polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are ubiquitous environmental pollutants that are persistent and toxic. The historical quantitative analysis of these compounds has been achieved with high-resolution targeted analysis using magnetic sector instruments, with subsequent lower resolution analysis to identify other contaminants. Advances in technology have led to comprehensive time-of-flight (TOF) mass spectrometers that can quantify PCDDs or PCDFs while simultaneously acquiring data on other contaminants in the samples. Samples that had been analyzed using EPA Method 1613B conditions on a sector instrument were analyzed with a high performance TOF mass spectrometer and a low-resolution comprehensive two-dimensional gas chromatography (GC×GC) TOF instrument. The quantitative results from the sector, the high-resolution TOF, and the GC×GC-TOF systems are compared in this article.

Jayne de Vos, Jack Cochran, Eric J. Reiner, and Peter Gorst-Allman

Polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are ubiquitous environmental pollutants formed as by-products of industrial and thermal processes. They form part of a class of compounds known as *persistent organic pollutants* (POPs), as defined in the Stockholm Convention (SC), that are persistent, geographically widely distributed, bioaccumulative, and have the potential to cause adverse health and environmental effects (1). The historical analysis of these compounds has required the utilization of two technologies; magnetic sector instruments and lower resolution mass spectrometry (MS) (that is, quadrupole or ion trap) with multiple stages of analysis. High-resolution targeted analysis on a magnetic sector instrument is applied for quantitation of dioxins followed by lower resolution analysis to characterize other possible contaminants (2). The quantitative standard by which all other analyses for these compounds are compared is EPA Method 1613B (3) and its derivatives. Recent advances in MS technology have led to comprehensive time-of-flight (TOF) mass spectrometers that can provide quantitation of dioxins through much of the mandated concentration range while simultaneously acquiring data on other contaminants in the samples.

This article compares results from samples prepared and analyzed according to EPA Method 1613B with those from a high-resolution, accurate mass TOF mass spectrometer and a low-resolution TOF mass spectrometer interfaced to a comprehensive two-dimensional gas chromatography (GC) system (GC×GC–TOF-MS). The TOF systems were able to detect dioxins across a broad concentration range covering more than 75% of the reported results obtained under EPA Method 1613B conditions. Quantitation was established in all instances using commercial standards and confirmed that the TOF systems could successfully achieve or surpass (4) detection at the mandatory lower limit required by EPA Method 1613 (TCDD at 500 fg) (3). (Note: Throughout this article the relevant PCDD and PCDF will be described using the following abbreviations: XCDD and XCDF, where X = T [tetra], P [penta], Hx [hexa], Hp [hepta], and O [octa]). The quantitative results obtained from the GC–high-resolution TOF (GC–HRTOF-MS) system and the GC×GC–TOF-MS systems were correlated with those obtained under EPA Method 1613B conditions.

More significantly, while providing comparable quantitative results, the TOF systems simultaneously allowed for the identification of a diverse set of other POPs not detected under the highly selective, selected ion monitoring (SIM) conditions defined in EPA Method 1613B. SIM analyses require prior knowledge of the compounds to be analyzed. The retention times and windows for mass-to-charge are

Table I: GC–MS conditions for PCDD and PCDF analyses			
Parameter	GC–HRMS	GC–HRTOF-MS	GC×GC–TOF-MS
Amount injected (µL)	1	2	2
Inlet temperature (°C)	280	280	250
Helium flow (mL/min)	0.8	1	1.4
Column 1	DB-5 (40 m × 0.18 mm, 0.18 µm d_f)	Rtx-Dioxin 2 (40 m × 0.18 mm, 0.18 µm d_f)	Rtx-Dioxin 2 (40 m × 0.18 mm, 0.18 µm d_f)
Column 2	—	—	Rxi-17Sil MS (1 m × 0.15 mm, 0.15 µm d_f)
Oven 1 (°C)	140 (hold 1 min) to 200 (52/min) to 235 (2.9/min hold 3 min) to 267 (3/min) to 310 (7/min) (hold until OCDD is eluted)	140 (hold 1 min) to 200 (50/min) to 260 (3/min) to 280 (1/min) to 310 (6/min) (hold 5 min)	120 (hold 2 min) to 200 (20/min) to 320 (5/min) (hold 3 min)
Oven 2 (°C)	—	—	125 (hold 2 min) to 205 (20/min) to 325 (5/min) (hold 3 min)
Transfer line (°C)	280	300	320
Modulation period (s)	—	—	2
Ion source (°C)	280	280	250
Start mass (amu)	SIM	140	45
End mass (amu)	SIM	520	750
Acquisition rate (spec/s)	—	3	100
Electron energy (eV)	35	50	70
Calibration	PFK	PFTBA	—
SIM = single ion monitoring. See method for details.			

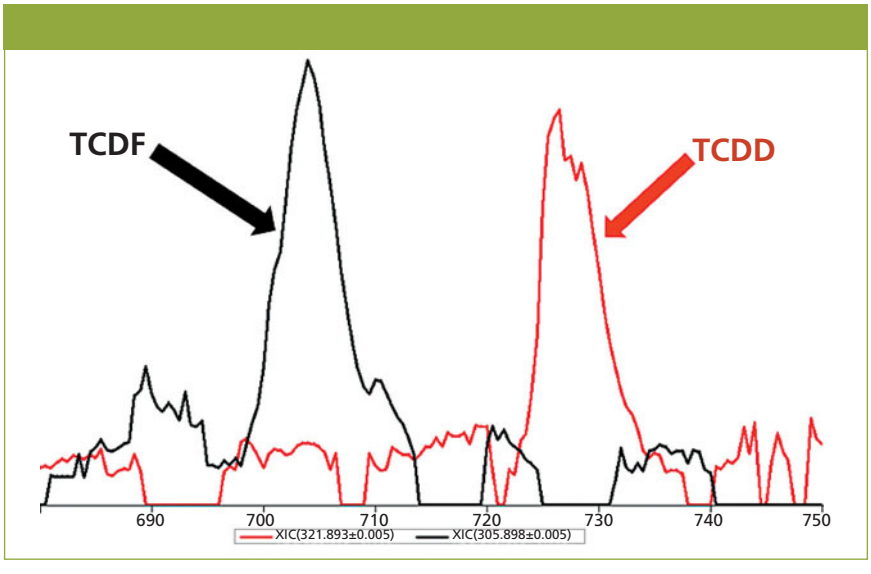


Figure 1: Selected ion chromatogram (*m/z* 321.893) for TCDD at 500 fg/µL (CS1 standard) on the HRT system.

highly constrained to limit interference and provide selective detection with correlation to labeled standards. Because of the constrained conditions for mass analysis under Method 1613B, other analytes would not be detected, while the TOF systems, not suffering the same constraints, always yield full range mass spectra and provide considerable additional sample information. The absence of constraints (that is, comprehensive analysis) enables a single experiment to obtain good quantitative correlation with regulated methods while data for prospective or retrospective analysis of unknowns is also acquired. The isotopic abundance of the analyte ions, mass accuracy, and resolving power achieved during the comprehensive experiment on the GC–HRTOF-MS system provide for both robust quantitative information and confident analyte identification. In the case of the GC×GC–TOF-MS system, the added chromatographic resolving power and cryofocusing effect of thermal modulation provides added sensitivity and selectivity.

Legislative Considerations

The analysis of PCDDs and PCDFs is regulated globally and concentrations are determined using a variety of EPA methods (such as 1613B and 8290A) (3,5), and by Council Directives in the European Union (6). In most other countries requiring dioxin analysis, modified forms of these methods govern testing protocols. These methods prescribe the use of GC coupled with high-resolution mass spectrometry (HRMS) and stipulate minimum quantifiable levels of the analyte (TCDD at 500 fg) for an analytical system and the testing to be deemed acceptable. Any alternative technology

Table II: Quantitative values (pg/g) for PCDDs and PCDFs (T = tetra; P = penta; Hx =Hexa; Hp = hepta; O = octa)									
Sample	Instrument	2378-TCDF	2378-TCDD	12378-PCDF	23478-PCDF	12378-PCDD	123678-HxCDF	123478-HxCDF	
1	GC–HRTOF-MS	3.5	3.0	2.7	4.5	1.4	2.2	1.7	
	GC–HRMS	3.7	2.6	1.7	5.6	1.1	5.4	1.7	
	GC×GC–TOF-MS	ND	ND	ND	ND	ND	11	ND	
	GC×GC–TOF-MS (×5)	3.5	5.6	6.6	12	ND	9.8	5.7	
2	GC–HRTOF-MS	20	28	21	21	2.8	76	32	
	GC–HRMS	26	32	18	19	2.8	85	35	
	GC×GC–TOF-MS	22	48	18	20	ND	90	40	
3	GC–HRTOF-MS	48	55	22	140	10	215	37	
	GC–HRMS	46	69	14	180	3.0	240	21	
	GC×GC–TOF-MS	40	73	32	150	21	210	46	
4	GC–HRTOF-MS	52	3.2	120	39	5.1	280	190	
	GC–HRMS	59	3.9	140	55	4.7	330	210	
	GC×GC–TOF-MS	48	5.8	130	36	14	210	210	
5	GC–HRTOF-MS	40	ND	35	24	ND	97	35	
	GC–HRMS	50	0.7	40	31	1.9	120	48	
	GC×GC–TOF-MS	43	ND	43	30	ND	120	47	
6	GC–HRTOF-MS	12	15	16	17	5.2	71	30	
	GC–HRMS	18	20	19	17	2.5	73	28	
	GC×GC–TOF-MS	15	21	21	18	ND	59	28	

GC–HRTOF-MS = Pegasus HRT; GC–HRMS = AutoSpec (Waters); GC×GC–TOFMS = Pegasus 4D;
GC×GC–TOFMS (×5) = Pegasus 4D with 5-µL injection

should be able to achieve the levels of detection required by these methods and the capabilities must be verified through testing.

Analytical Considerations

The limitation of the sector HRMS approach lies in its targeted approach that provides accurate information on quantitative levels of PCDDs and PCDFs, but gives no information on the presence of other POPs in the sample. This has to be determined in separate runs, and is generally done using low-resolution mass spectrometry. To be able to do both quantification and sample component investigation in a single analysis holds obvious advantages.

Samples

Six samples were investigated in this study. These were sourced from the Ministry of the Environment Laboratory Services Branch in Toronto, Canada. All samples were prepared according to standard methods used for the preparation of samples for PCDD and PCDF analysis by HRMS (7).

Instrumentation

Conditions for EPA Method 1613B acquisition were achieved using a Waters Autospec magnetic sector mass spectrometer at a resolving power of 10,000 interfaced to an HP6890 gas chromatograph (Agilent Technologies) using a 40 m × 0.18 mm, 0.18-µm d_f DB-5 column (J&W Scientific).

The GC×GC–TOF-MS system consisted of a Pegasus 4D time-of-flight mass spectrometer (LECO Corporation) coupled to an Agilent 7890 GC system equipped with an Agilent 7683B autosampler, a secondary oven and a dual-stage, quad-jet thermal modulator. Liquid nitrogen was used for the cold jets and synthetic air for the hot jets. The liquid nitrogen levels were maintained using an AMI model 186 liquid level controller.

The HRTOF-MS system was a Pegasus HRT (LECO Corporation) using Folded Flight Path technology (8,9). The system included an Agilent 7890 GC system equipped with an Agilent 4513A autosampler.

Experimental

EPA Method 1613B calibration and verification solutions (EPA-1613CVS), labeled calibration solutions (EPA-1613LCS), internal standard spiking solution (EPA-1613ISS), and cleanup standard stock solution (EPA-1613CSS) were selected for spiking and calibration purposes. These solutions were purchased from Wellington Laboratories and contained the 17 native and corresponding mass-labeled PCDD and PCDF congeners in nonane.

	123478-HxCDD	123678-HxCDD	123789-HxCDD	234678-HxCDF	1234678-HpCDF	1234678-HpCDD	1234789-HpCDF	OCDF	OCDD
	ND	3.1	ND	ND	12	33	ND	19	147
	0.8	1.7	1.6	0.6	15	24	1.5	24	170
	ND	ND	ND	ND	ND	31	16	ND	160
	ND	6.3	ND	ND	ND	31	16	31	170
	10	13	14	8	290	170	55	2400	1200
	3.7	9.3	6.0	2.3	300	160	47	2600	1900
	ND	ND	ND	ND	410	230	120	2400	1500
	17	18	28	14	620	240	51	980	790
	5.2	14	8.6	1.5	990	230	17	1100	1300
	18	41	32	36	950	210	58	980	990
	5.1	13	20	86	710	65	380	6500	170
	4.1	9.9	8.1	50	1000	70	470	5200	220
	23	14	18	100	1000	66	460	5100	190
	ND	ND	14	17	290	71	72	1100	370
	2.9	5.8	3.9	4.7	420	75	79	1200	550
	ND	10	15	38	400	78	90	1200	480
	5.8	10	12	6.7	180	120	30	1800	1100
	2.9	8.3	5.2	2.7	250	130	38	1800	1500
	7.8	10	ND	15	220	110	31	1900	1400

All instrument functions and data processing for the GC×GC–TOF-MS were managed with the LECO ChromaTOF software (version 4.44). All instrument functions and data processing for the Pegasus HRT system were managed using LECO ChromaTOF software (version 1.61). Manual review of all peak identifications and integrations was performed using this software. Library searching was performed using a PCDD and PCDF user library compiled from the PCDD and PCDF standards. GC–HRMS data were obtained using conditions and processing as specified in EPA Method 1613B.

Experimental conditions used for the analysis of the samples are shown in Table I.

Results

Limits of Detection

The results obtained from the EPA Method 1613B (3) standard set provides an estimate of the limits of detection (LOD) possible using the GC×GC–TOF-MS system and GC with HRTOF-MS detection, with the understanding that matrix interference and sample preparation effectiveness will contribute significantly. The low-level standard (CS1), which contains 2,3,7,8-TCDD at 0.5 pg/µL, was used to provide an estimated LOD using GC×GC–TOF-MS. Using the methodology described in EPA Method 1613B, the signal-to-noise ratio (S/N) for the ion of *m/z* 322 for 2,3,7,8-TCDD was calculated as 20 (EPA Method 1613 requires this ratio to be >10 [3]).

However, for GC–HRTOF-MS the prescribed approach is not applicable. Modern high-resolution mass spectrometers show little chemical noise on the plot of an exact mass ion owing to the elimination of noise during processing and acquisition. This is demonstrated in Figure 1, which shows a plot of the ion at *m/z* 321.893 for TCDD at 500 fg/µL (CS1 standard). As can be seen, the compound is readily detected at the lowest level (500 fg/µL) required by EPA Method 1613B indicating a capability of achieving the minimum levels. It is, however, often desirable to detect lower in routine analyses to ensure compliance and analytical capability.

Quantitative

The quantitative values for the 17 PCDDs and PCDFs whose levels are regulated using Method 1613B are shown in Table II. Values obtained using GC×GC–TOF-MS and GC–HRTOF-MS are compared to the levels acquired using 1613B conditions. At the lowest levels, the GC×GC–TOF-MS system is not capable of detecting analytes, but this may be partially offset using the concurrent

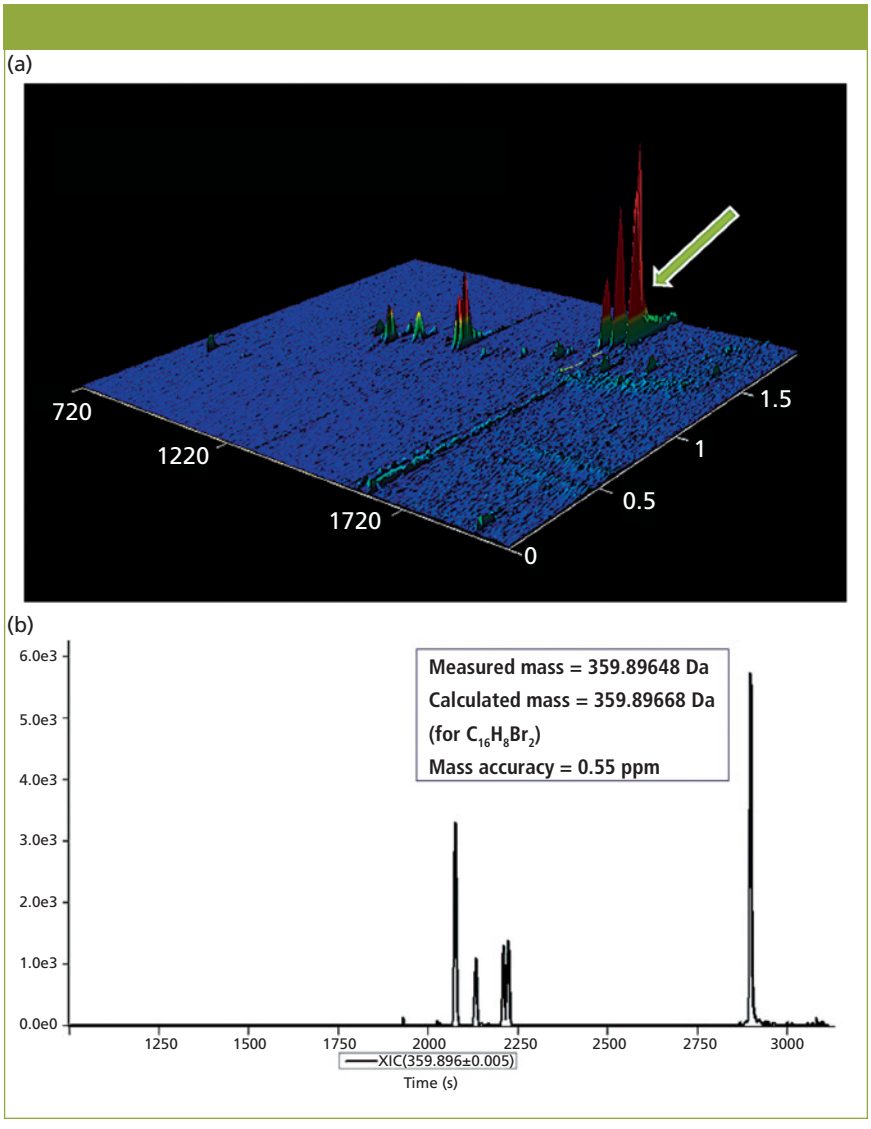


Figure 2: (a) GC×GC–TOF-MS surface plot showing the *m/z* 360 extracted ion. A dibromopyrene congener is one of a cluster of peaks in the top right corner. (b) GC–HRTOF chromatogram showing the *m/z* 359.896 extracted ion and the 2,7-dibromopyrene peak on the right side.

solvent recondensation technique of large-volume splitless injection (10). This technique, originally described by Magni and Porzano (10), can be done in an unmodified split–splitless injection port, provided that a guard column is used to protect the analytical column and that the matrix is clean to minimize fouling of the inlet and columns. For dioxin analysis in which the sample has been subjected to considerable cleanup, this latter condition is met, and the technique is advantageous when trying to analyze the trace-level PCDDs and PCDFs in the sample on the low-resolution system.

The data show strong correlation between Method 1613B results and those from GC×GC–TOF-MS analysis, suggesting its viability as a screening tool for dioxin levels in complex samples prepared using Method 1613B protocols.

Similarly, when compared to one another, the results from the two high-resolution systems are generally in excellent agreement, showing the high-resolution TOF system to provide quantitative results comparable to those obtained under Method 1613B criterion when concentrations are above a threshold near 500 fg/µL.

Comprehensive

Targeted dioxin analysis, as obtained with Method 1613B, fails to provide a comprehensive picture of the samples. Only the targeted PCDDs and PCDFs are detected, and additional POPs and analytes with dioxin-like properties present in the samples are transparent to detection. The capability of TOF instrumentation to generally reach or approach the required low levels, while still acquiring full-range mass spectra, means that these systems can be used not only to quantify the PCDD and PCDF components, but also to detect and identify additional components in the sample in the same run. The ability to detect and identify analytes not specifically monitored in Method 1613B is a feature demonstrated by both the GC×GC–TOF-MS and GC–HRTOF-MS systems.

For example, the GC×GC–TOF-MS chromatogram for sample 2 (Figure 2a) shows a high-boiling compound containing a prominent molecular ion cluster at *m/z* 360, not specified in the ions monitored in Method 1613B. The deconvoluted mass spectrum obtained for this compound can be library-searched to obtain the result shown in Figure 3. The compound is identified as 2,7-dibromopyrene, that can be confirmed against a standard to verify the congener, with a spectral match of 84% and would have gone undetected under Method 1613B conditions. Bromopyrenes have been shown to be irritating to the skin, eye, and respiratory systems (11) and while not the health concern of a dioxin, this compound represents what could be missed and may provide valuable insight should a contamination with dioxins occur. Although the toxicity of this specific dibromopyrene is not known, it may well contribute to the overall sample toxicity and so its presence in the sample may be of importance.

This same compound can be located in the chromatogram of sample 2 obtained when using the GC–HRTOF-MS system (Figure 2b). In this case, the relevant mass is measured as 359.89648, which can be used

Table III: A selection of other potentially harmful compounds contained in the samples analyzed, and detected by a comprehensive analysis

Sample	Compound	Similarity	Mass Accuracy (ppm)
1	Tetrachlorobiphenyl	516	2.20
1	7H-Benz[*de*]anthracen-7-one	816	-0.66
2	Perylene	832	-0.71
2	Trichloropyrene	702	0.29
2	Trichloroterphenyl	608	-1.74
2	Benz[*a*]anthracene	942	-0.22
2	Dichloroanthracene	958	-0.11
3	Tetrachlorobiphenyl	854	-0.15
4	Benzo[*e*]pyrene	824	0.23
5	Coronene	860	-0.16
5	Tetrachlorobiphenyl	750	1.57
5	Pentachlorobiphenyl	769	0.51
6	Pentachlorobiphenyl	664	0.84
6	Hexachlorobiphenyl	853	1.82
6	Benzyl butyl phthalate	927	-0.41
6	Tetrabromodiphenylether	653	0.62

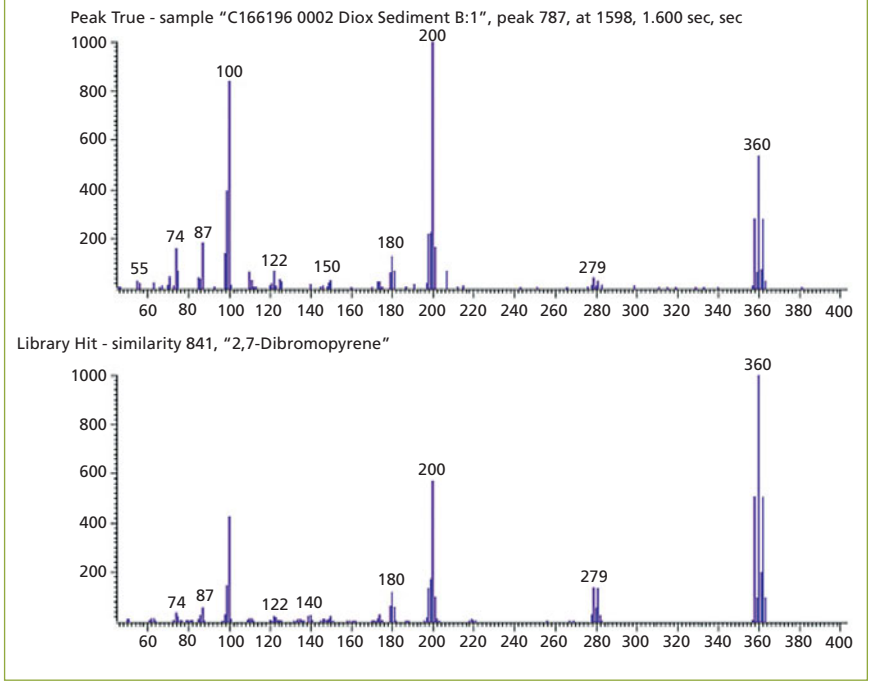


Figure 3: Mass spectrum of high boiling compound from sample 2, which gives an 84.1% match with dibromopyrene.

to determine the formula $C_{16}H_8Br_2$ with a mass accuracy of -0.55 ppm. In the case of the GC×GC experiment, it is library match alone that provides identification. In the case of the GC–HRTOF-MS analyses, accurate mass provides a clear indication of the likely formula and confirms the library match.

Numerous other POPs, which would not have been detected in the sector data, have been found in the samples. Examples of these are shown in Table III. It should be noted that the compounds shown are a random selection among many. It is only possible to obtain a full picture of the sample when a comprehensive analysis is performed. By focusing just on the PCDDs and PCDFs, only a partial understanding of the total toxicity factor is obtained. It should also be kept in mind that the sample has undergone rigorous cleanup before analysis. It is likely that this cleanup would remove other potentially harmful components (for example, pesticides).

Mass Accuracy and Isotope Abundance

An important part of the identification of unknown compounds is the measurement of accurate mass (see above) and the robust determination of isotope abundance. For example, one of the important POPs identified in sample 6 is tetrabromodiphenylether (TBDE), a member of a class of compounds known as brominated flame retardants (BFRs), with a chemical formula $C_{12}H_6Br_4O$, and a molecular mass of 482. The measured molecular ion was 481.71496 Da and the calculated value is 481.71455 Da, with a Δm of 0.00041 Da (or 0.85 ppm) leading to confident confirmation of its identity.

In the case of compounds with pronounced molecular ion clusters (arising from the presence of chlorine or bromine in the molecule), measurement of the relative isotopic abundance in the molecular ion cluster is also an important confirmation of molecular formula. In the case of the tetrabromodiphenylether, a high-resolution mass spectrum is shown in Figure 4 and the calculated values comparing measured with theoretical are provided in Table IV.

In general, differences of up to 30% are considered acceptable (Method 1613B) when working with PCDDs

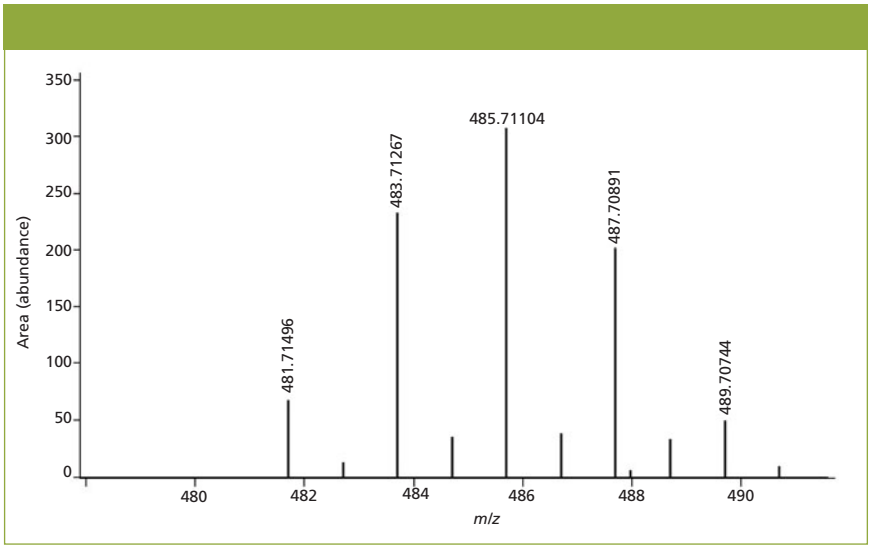


Figure 4: Measured molecular ion cluster for tetrabromodiphenylether.

Table IV: Ion abundance differences between measured and calculated relative abundances for tetrabromodiphenylether

Ion (Da)	Height	Measured Relative Abundance	Calculated Relative Abundance	Difference (%)
481.71	48	26.2	17.3	51.62
483.71	152	83.1	67.8	22.51
485.71	183	100.0	100	–
487.71	119	65.0	65.8	-1.17
489.71	38	20.8	16.5	25.85

and PCDFs at low levels. If that criterion is applied to the spectrum of the low-level tetrabromodiphenylether then, as can be seen from Table IV, all of the ions fall within the acceptable level except for the low-level ion at 481.71 Da. These values, coupled with the excellent mass accuracy obtained on the molecular ion provide strong confirmatory evidence of the proposed identification.

Conclusion

Analysis of samples suspected to contain PCDDs and PCDFs using GC–MS with appropriate selectivity and sensitivity requires a targeted approach. EPA Method 1613B uses GC–HRMS using SIM analysis. This requirement compromises the ability to identify other priority POPs that may be present in the samples in the same run using a single analytical technique. Time-of-flight mass spectrometry, implemented either as a high-resolution, accurate mass instrument, or as a low-resolution instrument coupled with comprehensive two-dimensional GC, provides an alternative to the traditional, regulated approach. TOF systems have the sensitivity to achieve the low detection levels mandated by regulated methods while showing a strong correlation to results obtained using the regulated methods, and in addition provide the flexibility to detect and identify other priority pollutants in the same analytical run. Although not able to achieve detection in 100% of the instances, the benefit of providing a comprehensive result and detection of EPA Method 1613B analytes at or above the limits provides advantages. These capabilities lead to the opportunity for considerable savings of time and money. As such, these comprehensive technologies form a potent weapon in the hands of the environmental analyst.

References

(1) Stockholm convention on persistent organic pollutants. Website: http://chm.pops.int (2011).

(2) E.J. Reiner, *Mass Spectrom. Rev.* **26,** 526–259 (2010).

(3) EPA Method 1613: Tetra through octa-chlorinated dioxins and furans by isotopic dilution HRGC/HRMS. Website: http://www.epa.gov/region3/1613.pdf (1994).

(4) J. de Vos, P. Gorst-Allman, and E. Rohwer, *J. Chromatogr., A* **1218,** 3282 (2011).

(5) EPA Method 8290A: Polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) by high-resolution gas chromatography/high-resolution mass spectrometry (HRGC/HRMS).

(6) Official Journal of the European Communities, L221/8 (2002).

(7) Ontario Ministry of the Environment. The determination of polychlorinated dibenzo-*p*-dioxins, polychlorinated furans and dioxin-like PCBs in environmental matrices by GC-HRMS. Report number DFPCB-E3418 (2010).

(8) A.N. Verentchikov, M.I. Yavor, Y.I. Hasin, and M.A. Gavrik, *Tech. Phys.* **50**, 73–81 (2005).

(9) A.N. Verentchikov, M.I. Yavor, Y.I. Hasin, and M.A. Gavrik, *Tech. Phys.* **50**, 82–86 (2005).

(10) P Magni and T Porzano, *J. Sep. Sci.* **26**, 1491 (2003).

(11) www.lookchem.com, Safety Data Sheet.

Jayne de Vos is with the National Metrolgy Institute of South Africa in Pretoria, South Africa. **Jack Cochran** is with Restek Corporation in State College, Pennsylvania. **Eric J. Reiner** is with Ontario Ministry of the Environment in Ontario, Canada. **Peter Gorst-Allman** is with LECO Africa in Pretoria, South Africa. Direct correspondence to: peter@lecoafrica.co.za ■

Green Foodomics

Is "green foodomics" another buzzword or a new direction in food analysis? To find out more, *LCGC* spoke to Professor Elena Ibañez of the Institute of Food Science Research (CIAL) in Madrid, Spain.

What is foodomics?

Ibañez: Our research group defined foodomics for the first time in 2009 as "a new discipline that studies the food and nutrition domains through the application and integration of advanced –'omics' technologies to improve (consumer) well-being, health, and knowledge." Basically, we believe that foodomics can help provide new answers to some of the important challenges (such as food safety and quality, traceability, new foods for health improvement, and disease prevention) that society is facing in the 21st century.

What chromatographic techniques are commonly used in foodomics?

Ibañez: The techniques typically used in foodomics are those typically used in proteomics and metabolomics, such as liquid chromatography (LC), ultra-high-pressure liquid chromatography (UHPLC), nano-LC, gas chromatography (GC), and capillary electrophoresis (CE) hyphenated to high-resolution mass spectrometry (MS). These techniques are able to provide us with a great deal of information at different expression levels, including proteins and metabolites. Logically, an important additional step here is the use of adequate sample preparation techniques.

When was the term "green foodomics" coined and what does it involve?

Ibañez: Foodomics can be understood as a global framework that gathers all the new challenges that the food science domain will be facing in the current postgenomic era (some of which were unthinkable a few years ago) and providing new answers through the development and application of new strategies, mainly based on "omics" approaches for large-scale analysis. In this regard, one of the challenges that can impact future generations is sustainability, which is understood as a rational way of improving processes to maximize production while minimizing the environmental impact or, in the words of the Environmental Protection Agency (EPA), "sustainability creates and maintains the conditions under which humans and nature can exist in productive harmony, that permit fulfilling the social, economic and other requirements of present and future generations." Thus, the term "green foodomics" was coined as a way to highlight foodomics goals with regards to green chemistry principles; bearing in mind that sustainability can be not only a word but also a way of doing things.

How easy is it to translate regular chromatography techniques to the green foodomics approach (and can it be cost-effective)?

Ibañez: The application of green chemistry principles to analytical chemistry is not new, although it is true that not much attention has been given to this approach until recently. Although the analytical community has always been environmentally sensitive and the idea of improving analytical methods by reducing the consumption of solvents and reagents has always been at the forefront of the analytical chemists' mind, the first descriptions of "green analytical chemistry" (or clean analytical methods) appeared in the mid-1990s (1). The concept and use of such an approach has evolved over the years reaching approximately 100 publications by 2011. This evolution positively affects foodomics (and green foodomics) since some of the mentioned applications deal with advanced analytical methodologies applied to food science.

The key aspects that should be considered when regarding the adverse environmental impact of analytical methods deal with reducing the amount and toxicity of solvents during sample pretreatment, minimizing solvents and reagents during the separation and measurement steps, and developing alternative direct analytical methods that do not require solvents or reagents. Moreover, they should also consider developing methods that are able to consume fewer resources. All of this has to be done whilst maintaining or improving the analytical performance of the method. This is probably the most difficult task and is responsible for a limited translation of conventional methods to greener ones.

Undoubtedly, laboratories that follow the green analytical chemistry principles, applied or not to foodomics, can have many benefits, which include the cost in terms of waste generation and management, health risks, and resources preservation.

Does green foodomics benefit the consumer?

Ibañez: Green foodomics can highly benefit the consumer since it attempts to improve consumer well being and confidence while, at the same time, decreasing contamination and health risks and preserving sustainability.

Reference

(1) S. Armenta, S. Garrigues, and M. de la Guardia, *TrAC - Trends in Analytical Chemistry* **27**(6), 497–511 (2008).

This interview was edited for length and clarity.

Read the rest of this interview at:
chromatographyonline.com/Ibanez

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 fit 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 issue. 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).

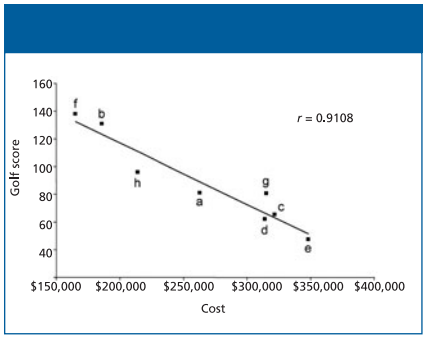


Figure 1: Chromatograms obtained using the conditions under which the ion suppression problem was originally discovered. The ion suppression trace is shown on the bottom. Column: 75 mm × 4.6 mm ODS-3; mobile-phase A: 0.05% heptafluorobutyric acid in water; mobile-phase B: 0.05% heptafluorobutyric acid 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 21, 2013

This opportunity is limited to advertisers in *LCGC North America*.
For more information, contact:
Mike Tessalone at (732) 346-3016, Ed Fantuzzi at (732) 346-3015, or Stephanie Shaffer at (508) 481-5885.

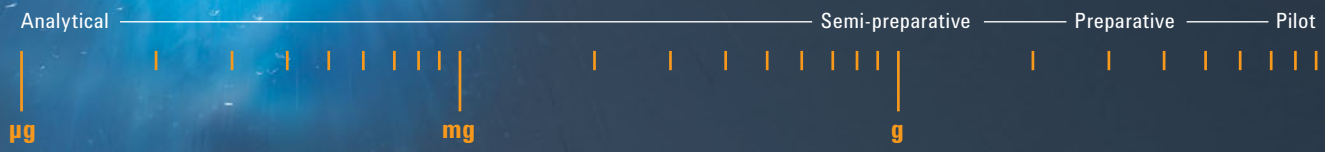
PURIFY YOUR WAY

What if you could purify exactly how you want?
What if you could trust your system 100 percent?
What if you could meet all your purification challenges?

Micrograms to multiple grams.
Highest purity with maximum recovery.
Save time and cut costs.

Agilent solutions can help you do just that.
Instruments. Columns. Software. Services.

Everything you need to purify your way:
www.agilent.com/chem/purifyyourway



The Measure of Confidence

Agilent Technologies

BRUKER

Trade In Your Current Benchtop Mass Spec and Upgrade To The Power and Reliabilty of Bruker

Bruker has always been driven by one idea: to provide the best technological solution for your analytical task. We understand the challenges you face, and the importance of getting the right answer, the first time. Our turnkey, easy to use, sytems offer superior performance and value.

We're so confident of our instruments that, for a limited time*, we're offering a second-year full coverage warranty when you upgrade your current benchtop instrument to a Bruker MALDI-TOF or LC-MS solution.

Experience for yourself why Bruker is number one in MALDI and the technology leader in LC-MS.
Contact us at ms-sales@bdal.com or visit www.bruker.com.

**Offer good until December 31, 2013, valid in North Ameria only.*

MASS SPECTROMETRY

Innovation with Integrity