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A Fusion of Triple Quad LC/MS/MS Speed & Sensitivity

UFMS
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High-throughput LC/MS/MS Analysis at Lower Levels of Detection

Combine Shimadzu's ultrafast LCMS-8040 with Nexera UHPLC rapid cycle times for superior laboratory throughput

Utilizing proprietary high-speed technologies, with enhanced sensitivity even at faster scan speeds, Shimadzu's new triple quad LC/MS/MS maximizes throughput in the laboratory. Plus, newly improved ion optics, integrating two multi-pole RF ion guides, maintain signal intensity and suppress crosstalk, even for high-speed or simultaneous multi-component analysis. This increases sensitivity for MRM and scan mode measurements, significantly expanding the application range and ensuring high-throughput analysis at lower levels of detection.

Shimadzu's LCMS-8040 Triple Quad features:

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- Ultrafast MRM transition speeds, up to 555 MRMs per second
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- Easy-to-use software with powerful quantitation and data browsers
- Nexera UHPLC features the world's fastest cycle time (14 seconds)

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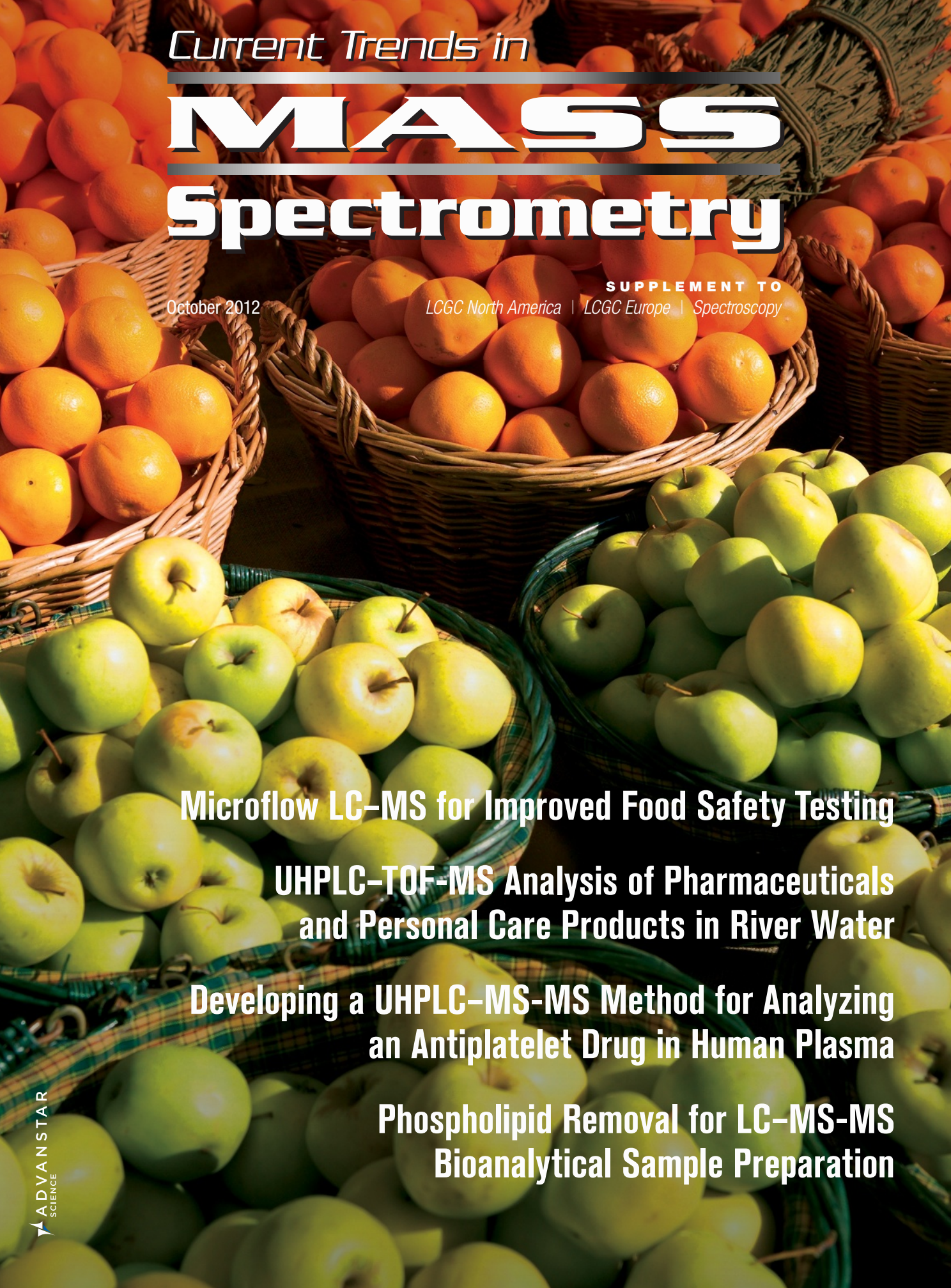

Current Trends in MASS Spectrometry

October 2012

SUPPLEMENT TO
LCGC North America | *LCGC Europe* | *Spectroscopy*

Microflow LC–MS for Improved Food Safety Testing

UHPLC–TOF-MS Analysis of Pharmaceuticals and Personal Care Products in River Water

Developing a UHPLC–MS-MS Method for Analyzing an Antiplatelet Drug in Human Plasma

Phospholipid Removal for LC–MS-MS Bioanalytical Sample Preparation

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Current Trends in MASS Spectrometry

October 2012

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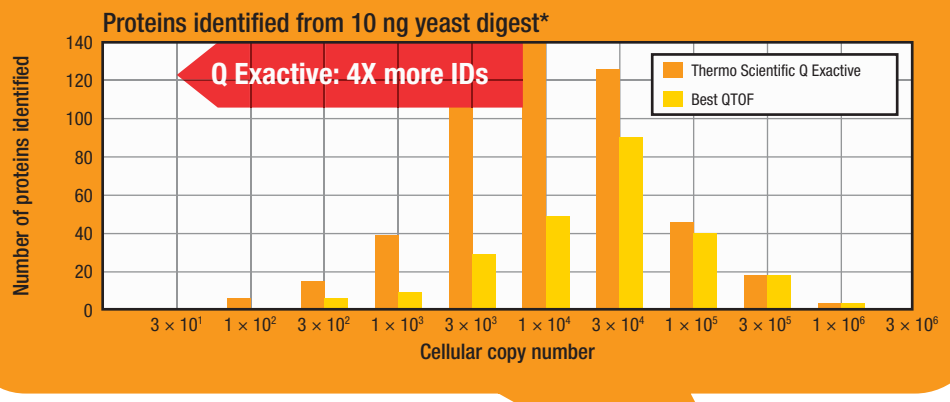
When it comes to scan rates, the faster the better.

Think again!

Thermo Scientific Q Exactive identified more low abundance proteins than QTOF.

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* Hao Z, Zhang Y, Eliuk S, Blethrow J, Horn D, Zabrouskov V, Kellmann M, and Huhmer A. A Quadrupole-Orbitrap Hybrid Mass Spectrometer Offers Highest Benchtop Performance for In-Depth Analysis of Complex Proteomes; Thermo Scientific Application Note 552; April 2012.

Thermo SCIENTIFIC

Analysis of Pharmaceuticals and Personal Care Products in River Water Samples by UHPLC–TOF-MS

In this study, ultrahigh-pressure liquid chromatography–time-of-flight mass spectrometry (UHPLC–TOF-MS) was used to analyze nine targeted pharmaceuticals and personal care products and several unknown compounds in North American river water samples.

Sharanya Reddy, Sergey V. Rakov, and Blas Cerda

Several pharmaceuticals and personal care products (PPCPs) have been detected in surface river waters, raising concerns about water pollution (1). Many of these PPCPs are released into rivers and streams anthropogenically from municipal sewage treatment plants. There is inefficient breakdown of the chemically synthesized PPCPs in the sewage treatment plants and the resulting effluent released from these plants carries the products into rivers. There is a growing concern for detecting PPCPs in aquatic systems because of the potential risk they pose to aquatic and human life. Reliable analytical techniques to identify and quantify PPCPs are important to develop. The diversity in the chemical nature of PPCPs, along with low concentrations of these analytes in complex matrices, makes these methods challenging to develop.

PPCPs have been quantified using gas chromatography–mass spectrometry (GC–MS) methods, which require derivatization to make the analytes more volatile (2). Besides GC–MS, liquid chromatography (LC)–triple-quadrupole MS methods have been developed, which do not require derivatization and are less tedious (3). However, triple-quadrupole mass spectrometers are often set to acquire in multiple reaction monitoring (MRM) mode, which does not allow for the simultaneous acquisition of full spectral information. Besides, quadrupole MS systems exhibit very poor sensitivity in scan mode, which limits their use to identify unknown analytes such as the wide variety of PPCPs. Unlike quadrupole instruments, time-of-flight (TOF) mass spectrometers show high sensitivity while acquiring full spectrum information, making them ideal for analyzing and identifying an unlimited number of compounds without prior knowledge of target analytes or when reference standards are not available. The parts-per-million mass accuracy provided by TOF mass spectrometers helps to determine elemental composition, thereby aiding identification of unknowns. The TOF systems fitted with newer analog-to-digital conversion technologies offer a wide dynamic range equivalent to triple-quadrupole systems. Similar to quadrupole instruments, TOF mass spectrometers can be used for quantifying known targets yet have the added advantage of identifying unknown compounds.

Here, we present a study of PPCPs in river water samples from northeastern United States using ultrahigh-pressure liquid chromatography (UHPLC) coupled with TOF-MS for both targeted and nontargeted analytes. In this study, we show how high mass accuracy information along with compound identification software can be used to identify unknown analytes in surface river waters.

Experimental Conditions

Sample Collection and Preparation

River samples (400 mL) were collected in 1-L amber bottles about 1 mile downstream of a sewage treatment plant. Samples were filtered through Whatman glass fiber filters (GF/C, 1.2 µm) and stored at 4 °C until analysis. Before analysis, the samples were extracted through C18 solid-phase extraction (SPE) cartridges.

SPE

Phenomenex Strata-X SPE cartridges (500 mg/6 mL) were used for extraction. The cartridges were initially conditioned with methanol (5 mL) followed by water

(5 mL). The filtered river sample was loaded on the cartridges with or without a spike of standard PPCPs (100 ng each) and extracted through the cartridge at 5–6 mL/min. The cartridge was dried under vacuum for ~20 min before analytes were eluted with methanol (4 mL) and acidified methanol (containing 2% formic acid, 4 mL). The eluate was dried under nitrogen to ~0.3 mL and diluted to 0.5 mL with water.

LC Conditions

A PerkinElmer Flexar FX-10 pump was used for LC analysis. Mobile-phase A was water containing 0.1% formic acid, and mobile-phase B was acetonitrile containing 0.1% formic acid. The gradient used was 10–90% B in 5 min. An injection volume of 3 µL was used in partial fill mode. A 50 mm × 2 mm, 2.7-µm d_p, PerkinElmer Brownlee SPP C18 column was used for the separation.

MS Conditions

A PerkinElmer AxION 2 TOF MS system was used with an Ultraspray 2 dual electrospray ionization source. Other conditions were as follows: ionization mode: positive; pulse mode: 100–800 *m/z*; capillary exit voltage: 100 V; TrapPulse mode: 100–800 *m/z* (D7:42, D8:63).

Internal calibration was done using two ions, *m/z* 118.08625 and 622.02896, as lock mass ions.

AxION EC ID software (PerkinElmer) was used for compound identification.

Results

In this study, a TOF-MS system was used to analyze nine targeted PPCPs and several unknown compounds present in the river water samples. The separation of the nine target analytes was achieved in less than 3 min on column (Figure 1). The low concentration of the analytes in surface waters made it necessary to preconcentrate using SPE cartridges. The SPE extraction procedure applied for a wide range of analytes resulted in an estimated 75% or greater recovery of the majority of the analytes (based on 100 ppb standard spiked in MilliQ water [Millipore]) except for the

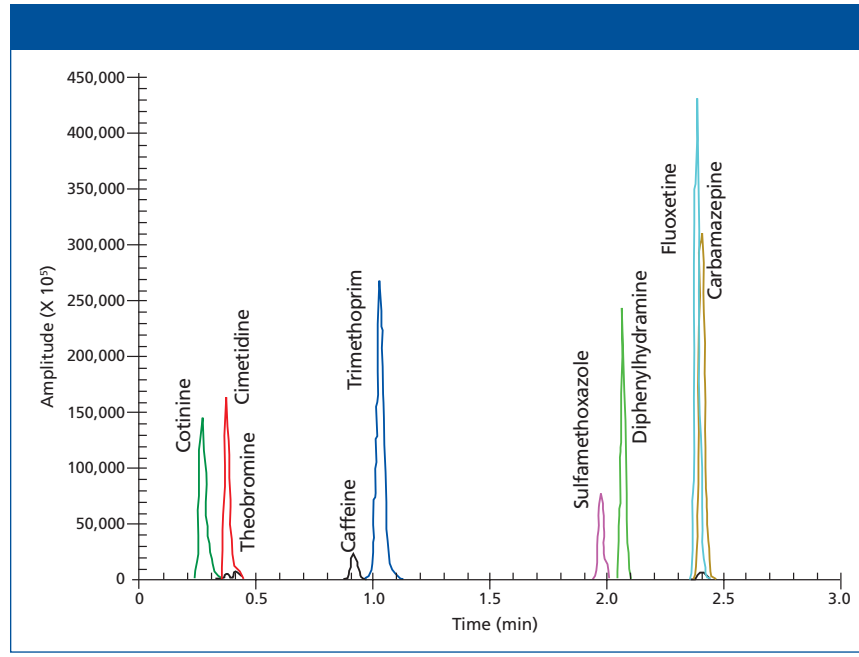

Figure 1: The separation of PPCPs in under 2.5 min.

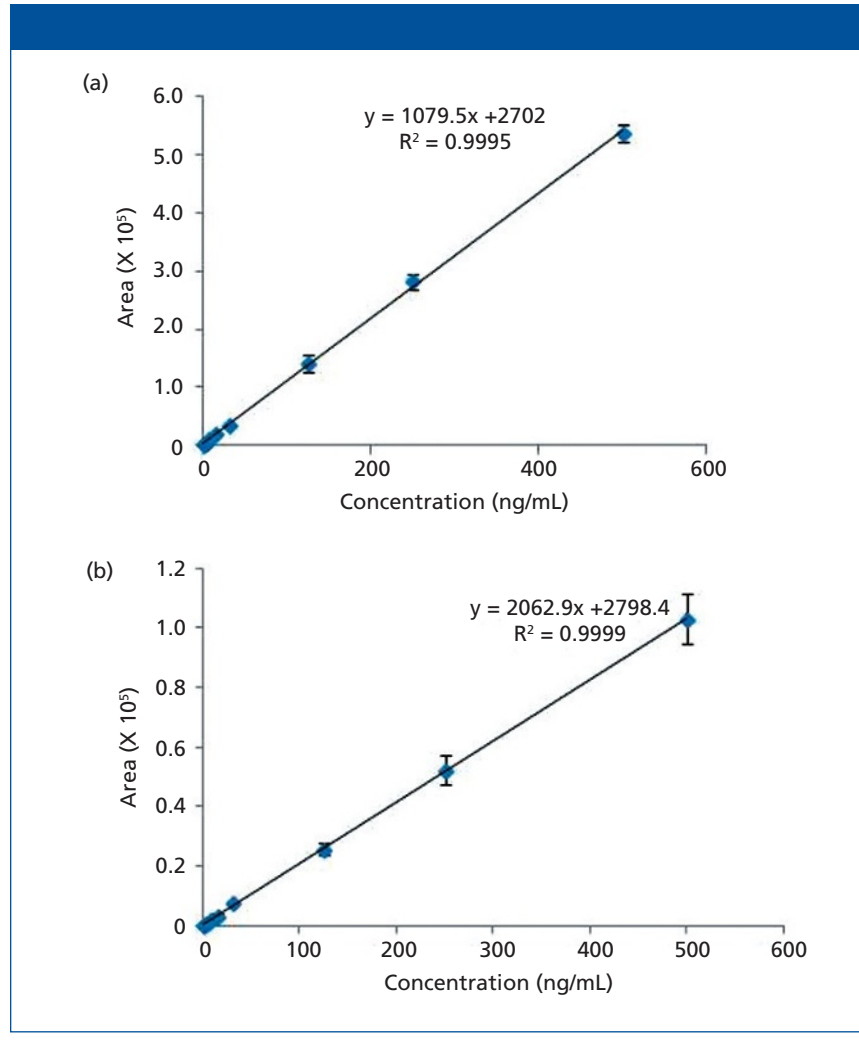

Figure 2: The calibration curves for (a) sulfamethoxazole and (b) diphenylhydramine.

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The Measure of Confidence

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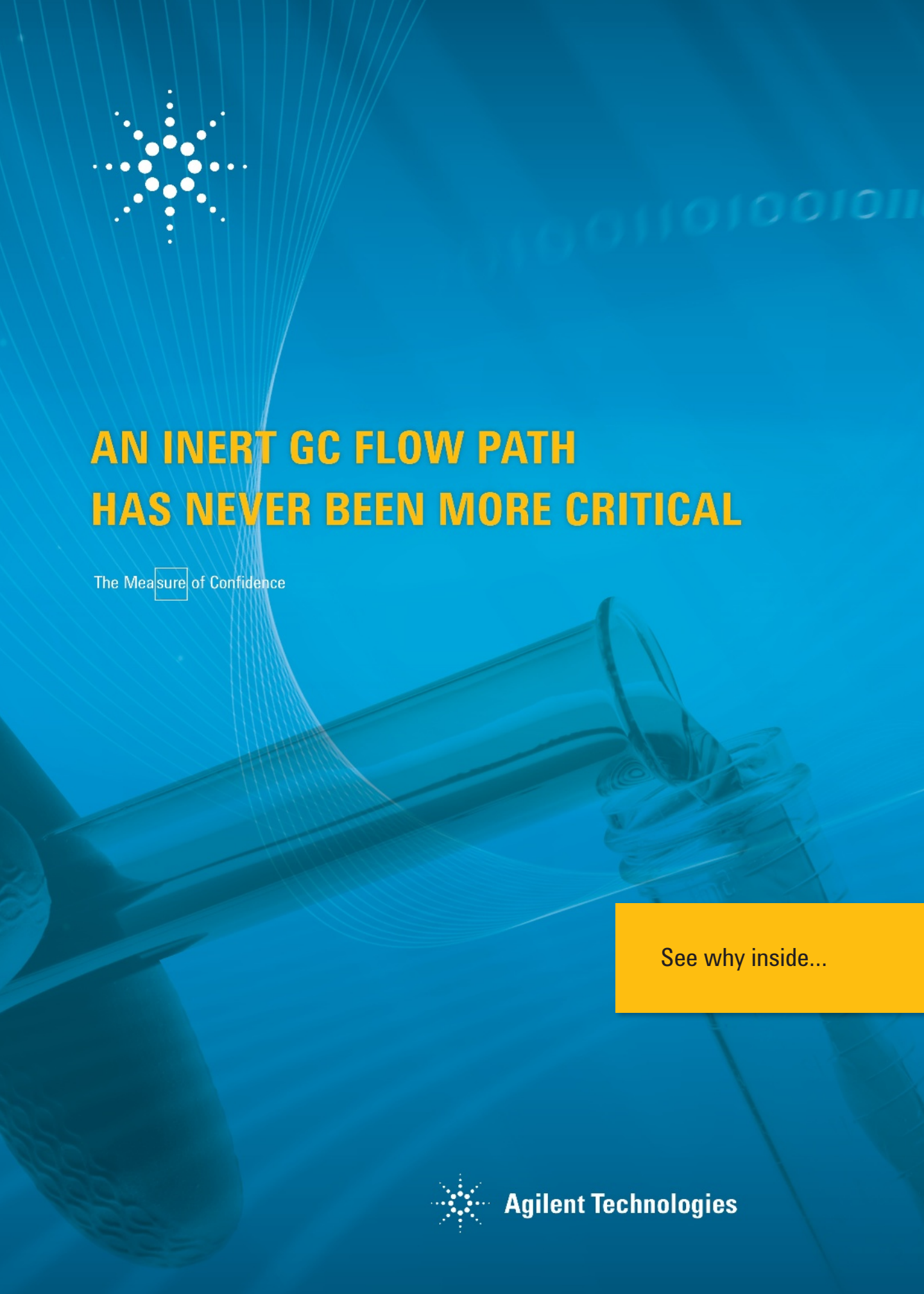


AN INERT GC FLOW PATH HAS NEVER BEEN MORE CRITICAL

The Measure of Confidence

See why inside...

Agilent Technologies

As samples become increasingly active and more complex, you cannot afford interferences introduced by the flow path. A non-inert flow path can cause peak tailing and signal loss, it can also take or hide components in your sample, so you would never know what was missing. Repeating or verifying suspect analyses wastes resources, hinders productivity, and costs you money. And, unreliable results can have catastrophic implications in terms of environmental safety, food quality, and inaccurate accusations of drug abuse.

To achieve the lower detection limits demanded by increasingly tough regulatory obligations, and quantify active analytes with confidence, you need the most inert GC flow path you can get.

Where's the problem?

Every stage of the flow path can degrade your results, from the inlet liner to the ion source. *Figure 1* shows the different components of the flow path where a lack of inertness can impact your results.

What's the solution?

Here are **Agilent's top five tips for GC flow path inertness** to give you confidence that nothing has been lost from your sample, even at trace levels, and that optimum productivity is achieved.

1. Maintain the inlet to maintain results

Inlet cleanliness is critical to reliable, repeatable GC results. The choice of consumables, including septa and liner O-rings, affects the speed and quality of routine inlet maintenance. This is particularly important if you're working with trace, ECD and MSD analyses, when out-gassing or silicone residue can be a problem. Inlet

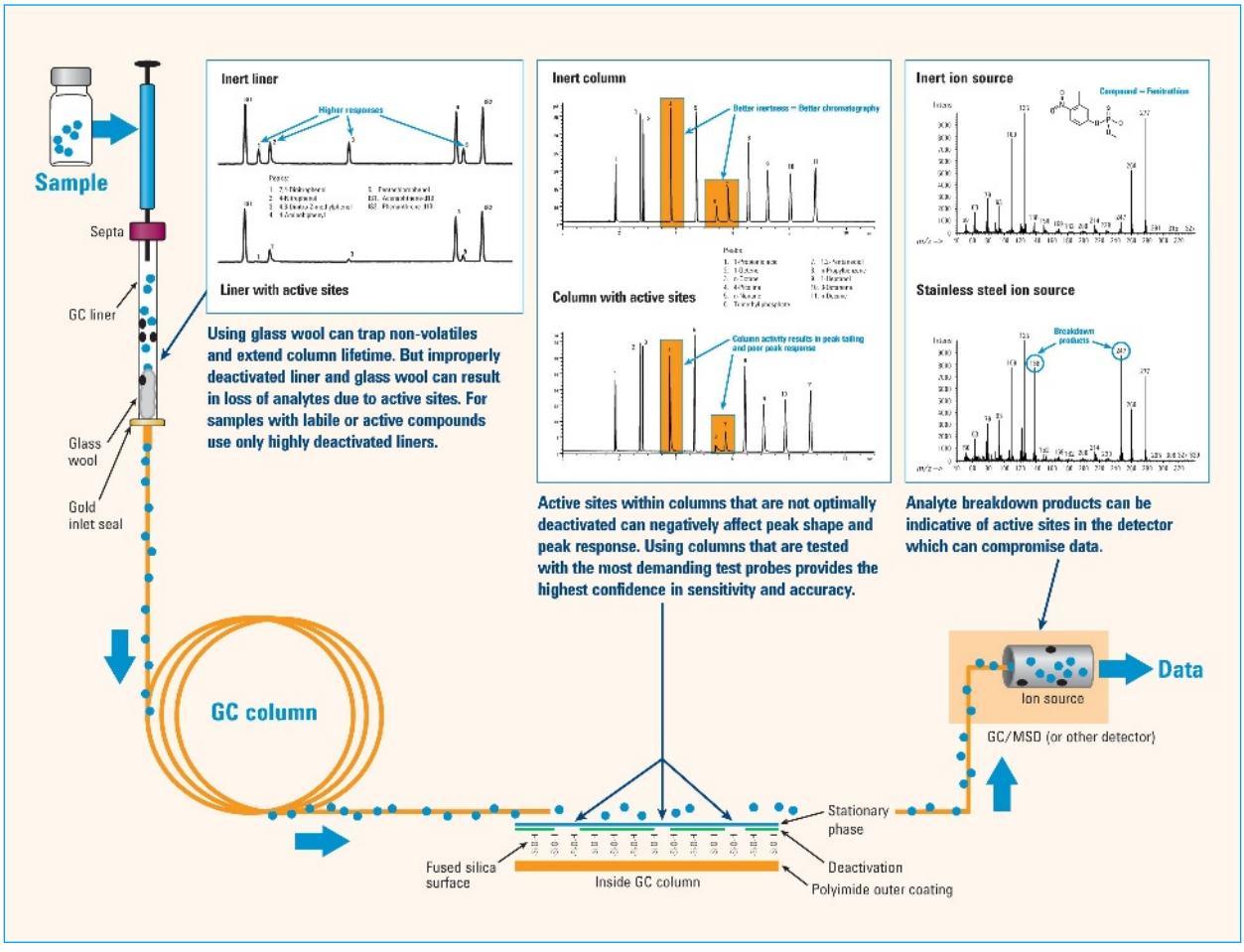


Figure 1. *Are you building the most inert flow path?*

cleanliness is thus a major concern in GC. The best option is to use Agilent septa and O-rings made from the purest materials, manufactured in clean facilities, and purposefully packaged to maintain cleanliness and prevent contamination during shipping and handling.

Silicone components in the heated inlet are known to stick to hot metal surfaces. Adherent residues force unscheduled inlet cleaning, reducing productivity. You can avoid this problem by selecting treated O-rings and septa that stops them from sticking to the metal surface of the inlet. The contaminant-free material prevents adhesion and unnecessary inlet cleaning, saving downtime and expense.

Preventive maintenance helps ensure peak instrument performance and productivity. Inspect and replace worn or dirty flow path supplies, such as syringe needles, septa, ferrules, and inlet seals on a regular basis, to eliminate leaks and minimize downtime. Record any changes in your lab book. Using Agilent certified vials, caps, septa, ferrules, and gold inlet seals can also extend the inert GC flow path. Gold seals are made from stainless steel, electro-polished and gold plated. The smooth exterior provides an inert surface that reduces breakdown of active compounds, as well as reducing the risk of leaks.

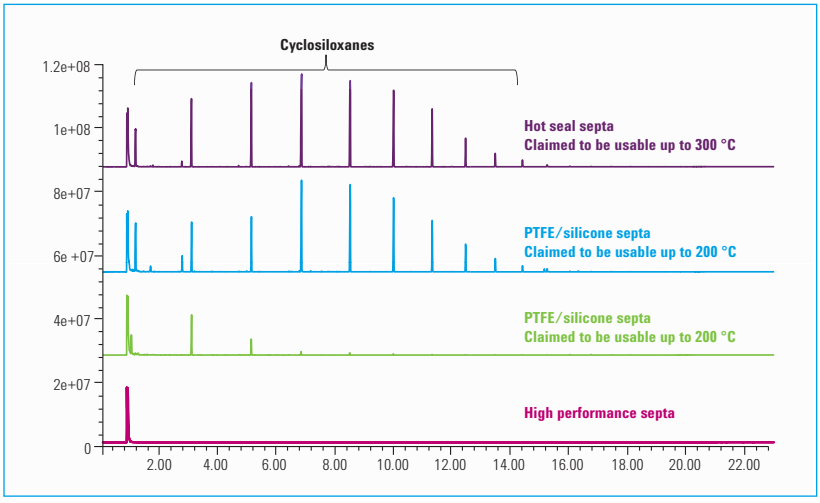


Figure 2. *GC/MS chromatogram comparison of vial blank with different PTFE/silicone headspace septa and Agilent high performance septum. Vials were equilibrated at 300 °C for 30 minutes. Using a high performance septum delivers a chromatogram free of contamination.*

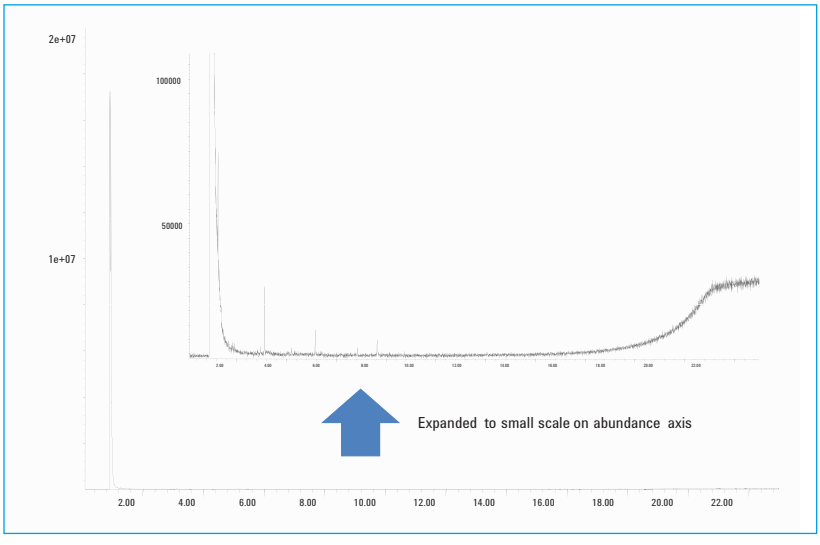


Figure 3. *The Agilent high performance septum provides significantly cleaner blank background at high temperature headspace testing. Even with an expanded abundance scale, the 300 °C vial blank chromatogram with an HP septum shows few siloxane peaks, with very low abundance.*

You can also use Agilent high performance (HP) septa that are manufactured from a material lined with a very robust silicone/rubber. These heat treated septa significantly reduce the amount of siloxanes that leach out of the material, and offer favorable chemical compatibility. Because they provide dramatically cleaner backgrounds under very stringent operating conditions. HP septa are your best option for reliable and efficient headspace analysis-high-temperature GC.

Figure 2 shows a comparison of high performance and

(Continued)

"In my 30 years of experience in running virtually every US EPA semi-volatile method... like Methods 608, 625, 508, 525.2 and of course 8270... the performance of the ***Agilent J&W DB-UI8270D GC column*** *has raised the bar for semi-volatile analysis. With both conventional inlet technologies (Split/Splitless and LVI inlets) and on-column introduction, the Agilent J&W DB-UI8270D has demonstrated:*

- *Superior chromatographic resolution (PAHs specifically are phenomenal!)*
- *Superior inertness (reactivity of acid-fraction components like 4-Nitrophenol and 2,4-Dinitrophenol which have low pKa values is minimal)*
- *Superior breakdown characteristics (4,4'-DDT and Endrin breakdown is well below all method requirements and in many examples <5%!)*
- *Superior peak tailing performance of Pentachlorophenol and Benzidine (Benzidine, for example is Gaussian in peak shape)*

As a result of my continued research and work with clients, the Agilent J&W DB-UI8270D GC column will be the column of choice for my continued work and the recommended GC column to my clients analyzing under the core set of US EPA methods for semi-volatile analysis."

Jeffery S. Hollis
Owner/Consultant
AnalySense - Sacramento, CA

non-high performance septa. The contaminant-free cleanliness of the chromatogram produced using HP septa is clearly evident. *Figure 3* is an expanded view, showing how high performance septa provide industry-leading chromatographic purity at 300 °C.

2. **Don't lose sample at injection**
Inlet liners are critical links in the sample flow path, and can be a source of activity and analyte loss. Liner design and chemistry impact the transfer of compounds into the column because active sites in the liner and the glass wool can cause loss of analyte; therefore, you should always use a reliably deactivated liner suited to your injection technique, and change the liner as needed. This will maximize sample transfer and minimize sample loss.

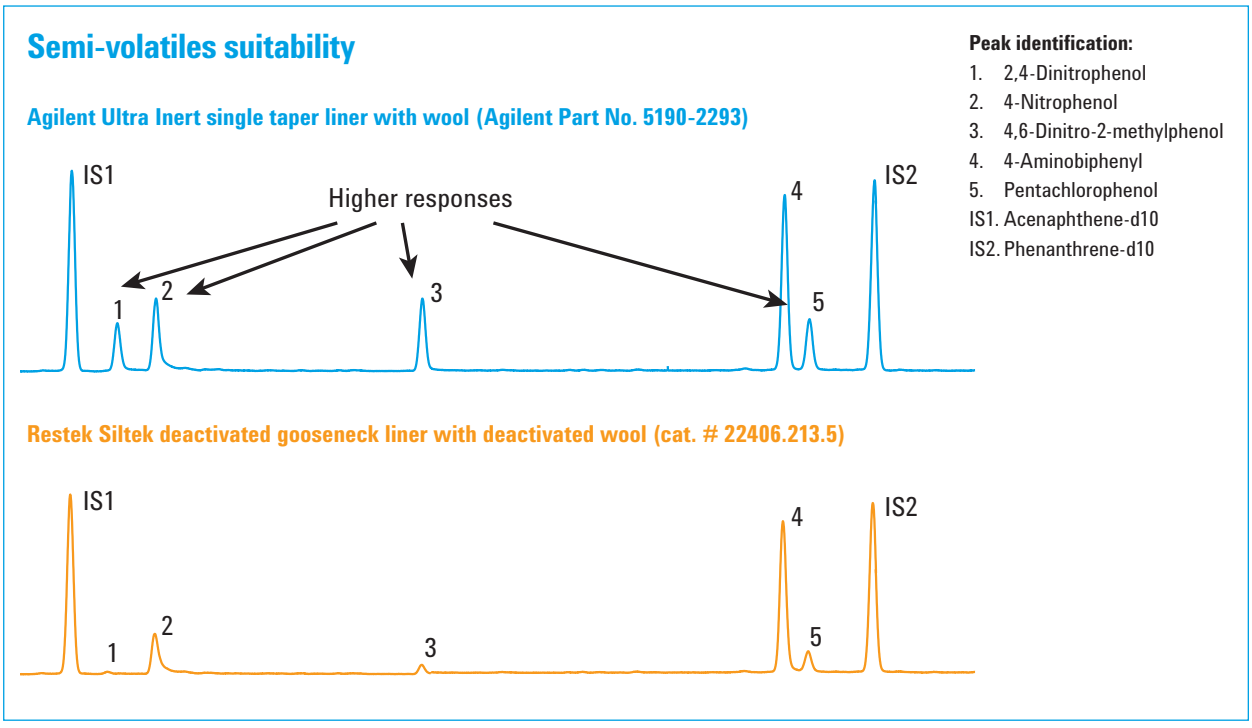


Figure 4. *Using an Agilent Ultra Inert Inlet Liner preserves analyte integrity (above) compared to a liner without this capability (below)*

Inlet liners with wool are widely used because the wool promotes homogenous sample mixing and better quantitation. Using a liner with wool seems like the obvious solution to trap high-boiling point matrix interference and prevent 'junk' from contaminating GC or GC/MS systems. However, glass wool liners can have drawbacks. The active sites on the wool surface can trap sensitive analytes, preventing these compounds from being delivered to the column for separation and analysis, and therefore causing significant loss of system sensitivity.

Now, you can inject heavy matrix samples and retain sensitivity by using Agilent Ultra Inert Inlet Liners with wool, for trace level analyses such as semi-volatiles, pesticides, and even drugs of abuse. As well as protecting the inlet and column, and ultimately the MS source, the highly deactivated surfaces of these liners and wool are so inert that the negative impact of surface activity is significantly reduced, as shown in *Figure 4*.

If you test environmental samples, or samples extracted from plasma or urine, you will be dealing with heavy matrix samples that can have a marked effect on instrument condition every day. This is not an issue for Agilent Ultra Inert Inlet Liners, which provide excellent consistency even with heavy matrix samples.

The high level of inertness permits use of glass wool to trap the non-volatiles in the matrix, extending lifetime and protecting the column and the detector.

3. Use an Ultra Inert column with low activity

Peak shape and response can also be adversely affected by what happens to analytes in the column, so inertness is very important. High column inertness minimizes compound loss and degradation for more accurate quantitation of active analytes, especially at trace levels of acids, bases, and other active compounds.

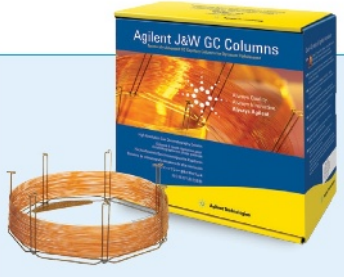
To ensure consistent column inertness, it is vital to choose a column that has been tested to

(Continued)

ensure optimal inertness and low bleed.

For example, the unique ultra inert testing that only Agilent J&W offers ensures the most reliable qualitative and quantitative results, and unmatched column-to-column reproducibility, for your most challenging compounds. With the industry's only Ultra Inert testing, every column is tested for peak height ratios and tailing for acids, bases, and other chromatographically demanding compounds so you can have utmost confidence in your challenging trace-level results. And you can see ultra inert performance yourself as an individual performance summary sheet is shipped with every Agilent J&W Ultra Inert GC column.

When installing the column, start with high-quality ferrules and examine column ends for chips and burrs under magnification. Make sure the column is positioned at the recommended depth into the inlet and detector.



Industry leading Agilent J&W Ultra Inert GC columns

Agilent J&W Ultra Inert GC columns push industry standards for consistent column inertness and exceptionally low column bleed, resulting in lower detection limits and more accurate data for difficult analytes. Learn about the new DB-624UI GC column for environmental, food and pharma at **www.agilent.com/chem/624UI** Learn how to get optimal inertness for environmental semi-volatile analysis with the new DB-UI8270D at **www.agilent.com/chem/UI8270D**

4. **Don't forget the detector**
To ensure accurate quantification and high sensitivity, the entire flow path must be highly inert, including detector surfaces. This is especially true of mass spectrometers, where an inert ion source is necessary to prevent active compounds from attaching to metal surfaces. Analyte breakdown products can indicate active sites in the detector that can compromise your data. The best inert sources are constructed of a solid inert material, as opposed to an inert coating that can wear away over time.

5. **Profit by purifying your gases**
Ensuring gas hygiene is one of the most important steps you can take to optimize GC system performance. Impure gases can introduce contaminants, or cause installation delays, premature instrument failure, and flawed results. Plus, the inefficient use of increasingly expensive and rare gas can go right to your bottom line.

Cleanest gas delivery for your most reliable analysis

Clean gases reduce the risk of column damage, sensitivity loss, and instrument downtime, while contaminants in gases can significantly affect your analysis. Learn about Agilent gas clean products at **www.agilent.com/chem/gasclean**

Impurities in gases can activate glass wool in liners and accelerate septum degradation, causing high background signals and ghost peaks. This leads to time-consuming troubleshooting. Inserting gas filters in the gas line immediately before your GC inlet greatly reduces the level of impurities, thus improving trace analysis. Contaminants entering the GC column will also be reduced, which is critical for high temperature analysis and is essential for longer column lifetime. Gas filters also ensure clean gas delivery, provide fast stabilization and reduce helium gas consumption.

Total costs *vs.* helium consumption

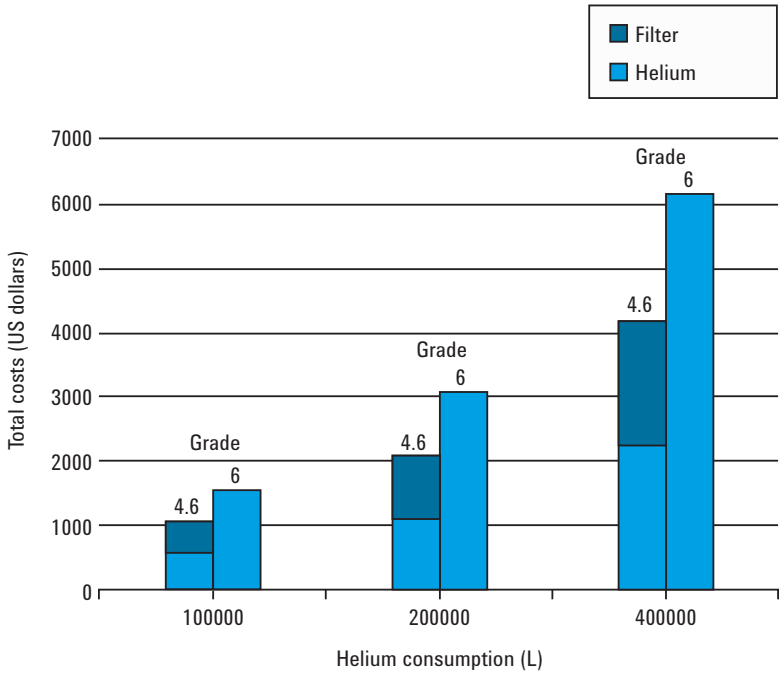


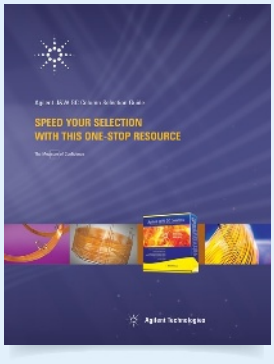
Figure 5. *Potential cost savings when using the Agilent Gas Clean Filter System and 4.6 grade helium rather than 6.0 grade helium.*

A good gas clean filter system lets you use 99.996% (4.6) pure helium and get high quality analytical results, instead of the more expensive 99.999% (5.0) or 99.9999% (6.0) grade. *Figure 5* compares the costs of filtered and non-filtered carrier gas using helium grade 4.6 and 5.0. The expected cost saving is around 30%.

Use the Agilent Gas Clean Filter System for oxygen, hydrocarbons and moisture to avoid loss of sensitivity and accuracy of the GC, and damage to your system.

Keep your GC clean – and working

With a busy schedule and countless demands on your time, it can be easy to lose sight of the need to optimize for an inert flow path. However, if you don't manage the inertness of your system, you risk jeopardizing your instrument, your column, and your results, with a potentially serious impact on economy and productivity. So ensuring an inert GC flow path has never been more critical. And Agilent continues to lead the way with inert flow path solutions. ♦



Speed up GC column selection with the Agilent J&W GC Column Selection Guide

The *Agilent J&W GC Column Selection Guide* makes it easier to choose the right GC column for all your applications. The step-by-step guide helps you choose a stationary phase based on factors such as selectivity, polarity, and phenyl content and understand how column diameter influences factors such as efficiency, solute retention, head pressure, and carrier gas flow rates. Order your copy at **www.agilent.com/chem/getguides**

Quantify active analytes with confidence

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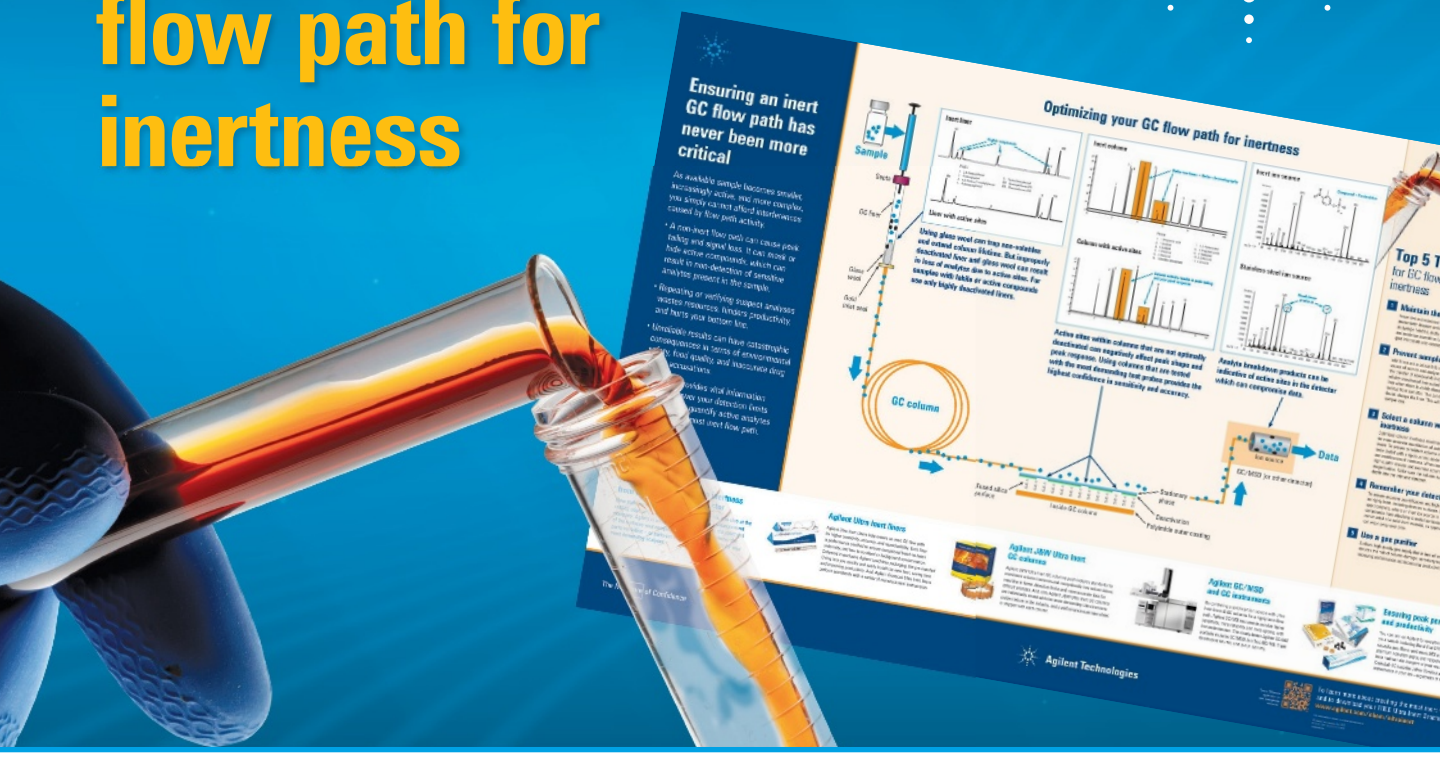

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Table I: Instrument detection limits of PPCPs

Analyte	Instrument Detection Limits (ng/mL or ppb)
Cimetidine	0.5
Carbamazepine	0.2
Cotinine	0.5
Sulfamethoxazole	0.5
Diphenylhydramine	0.5
Fluoxetine	0.2
Caffeine	4.0
Theobromine	8.0
Trimethoprim	0.5

very hydrophilic analyte, cotinine, which resulted in only 20% recovery (data not shown). The operation of the TOF-MS system was in TrapPulse mode, which increases duty cycle and results in improved sensitivity (a signal increase of up to 10-fold can be observed). The instrument detection limits (Table I) for the majority of the PPCPs was less than 1 ppb except for caffeine and theobromine (4 and 8 ppb, respectively). The linearity of the PPCPs tested over 0.5–500 ppb showed excellent r^2 values (Figures 2a and 2b).

Analysis of Target Analytes in River Water Samples

The exact mass capabilities of TOF mass spectrometers along with reproducibility in LC retention time can be used in combination to identify the presence or absence of target analytes in samples. Table II shows the <2 ppm mass accuracy for all the PPCPs. The surface river water samples were collected about 1 mile downstream from a sewage treatment plant and were processed and analyzed as described in the methods section. The analysis revealed the presence of carbamazepine, diphenylhydramine, sulfamethoxazole, and fluoxetine in the river water samples (Figure 3). The presence of these compounds was confirmed by accurate mass and retention time matching with standards. The concentration of these analytes in river water was estimated at 0.5–2 ppb.

Analysis of Nontarget Analytes in River Water Samples

The river sample data were further examined for unknown nontarget analytes. A major chromatographic peak eluted at ~2.7 min (Figure 4) was analyzed using the compound identification software. Accurate monoisotopic mass and isotope ratio information is used by the software to search the PubChem database for potential molecular formula matches. It provides a ranked summary of the potential matches as well as suggestions for possible compound structures for a given elemental composition. In this example, the elemental composition $C_{12}H_{17}NO$ was listed with highest score and the top candidate was identified as *N,N*-diethyl-*m*-toluamide (DEET) based on the number of active assay identifications reported. DEET is a common ingredient in insect repellent and the accurate mass of the unknown compound was verified to be within 2 ppm mass error of the expected mass of DEET (Figure 5). The presence of DEET in the river sample was further confirmed by retention time matching with the standard. Using a similar approach, acetaminophen, a commonly used analgesic drug, was also identified in the river water (data not shown).

Conclusions

PPCPs in the environment include a diverse variety of chemicals including prescription drugs, over-the-counter drugs, chemicals used in agribusiness such as veterinary drugs, cosmetics, and fragrances. It is becoming increasingly important to understand the fate and occurrence of these chemicals in the environment since many of the

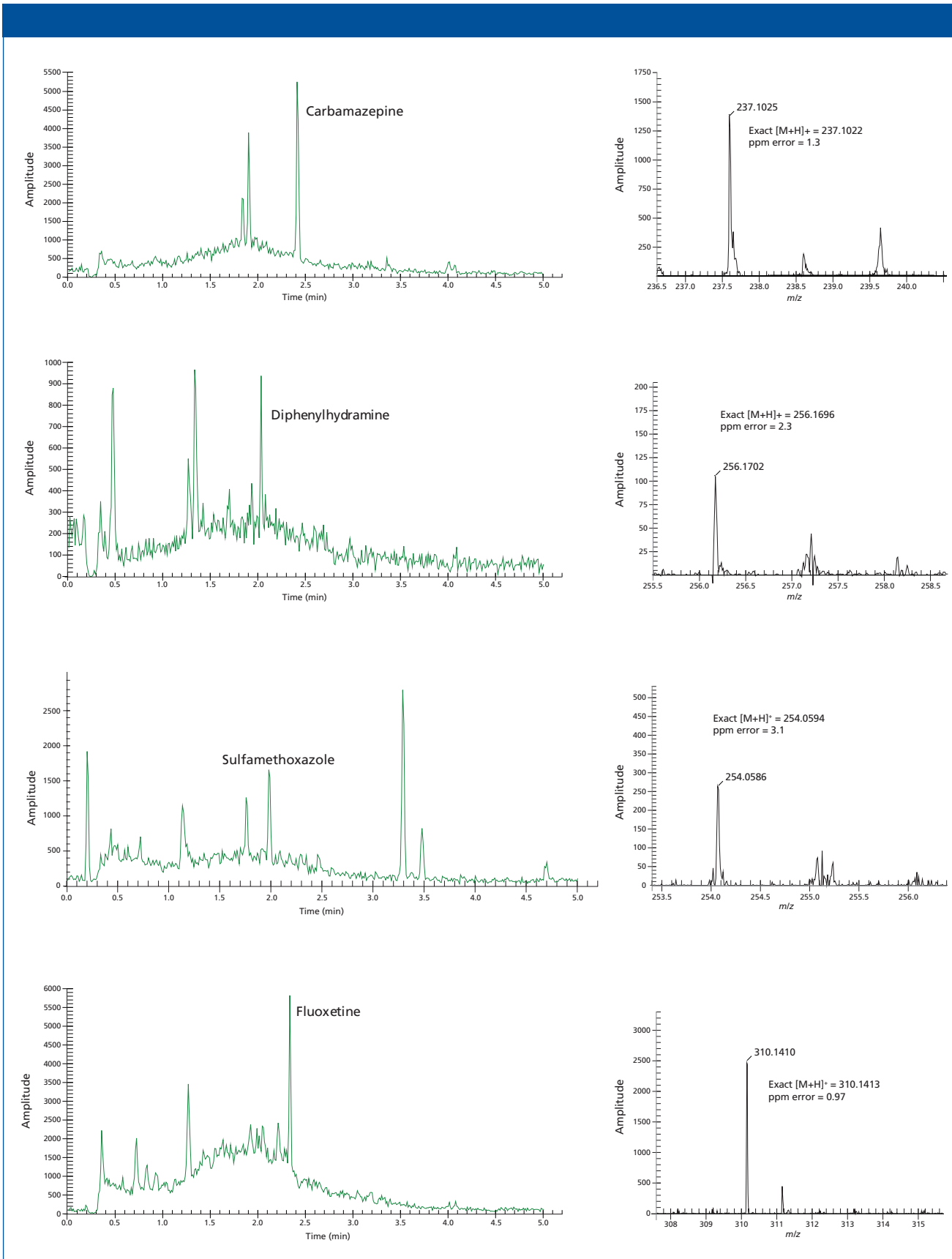

Figure 3: Identification of carbamazepine, diphenylhydramine, sulfamethoxazole, and fluoxetine in river water using accurate mass and retention time matching with standards.

Table II: Theoretical mass, observed mass, and mass error of PPCPs

Analyte	Observed Mass	Accurate Mass	Error (ppm)
Cotinine	177.1029	177.1032	-1.69
Cimetidine	253.12299	253.1229	0.36
Theobromine	181.072	181.0718	1.10
Caffeine	195.0877	195.0878	-0.51
Trimethoprim	291.1452	291.145	0.69
Sulfamethoxazole	254.0594	254.0594	0.00
Diphenylhydramine	256.1696	256.1693	1.17
Fluoxetine	310.1413	310.1411	0.64
Carbamazepine	237.1022	237.1027	-2.11

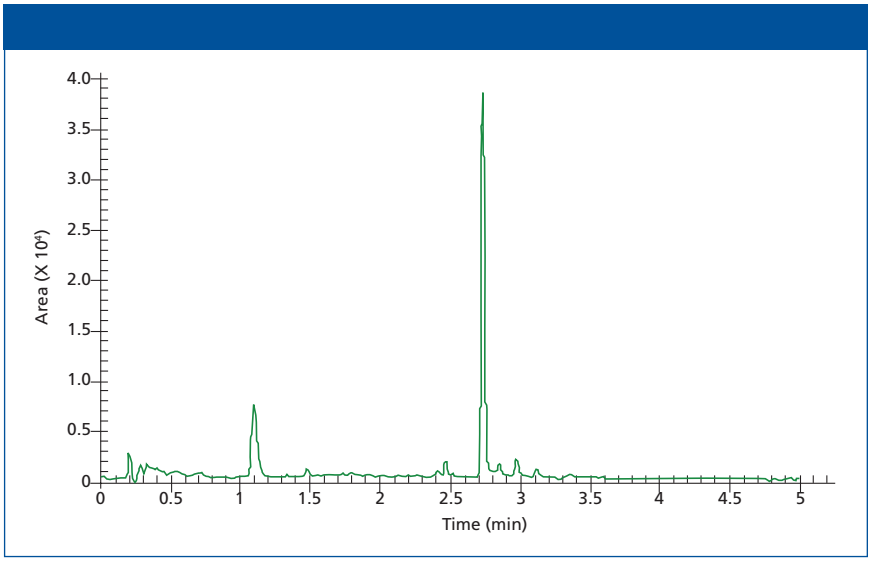

Figure 4: A major unknown target analyte eluted at ~2.7 min.

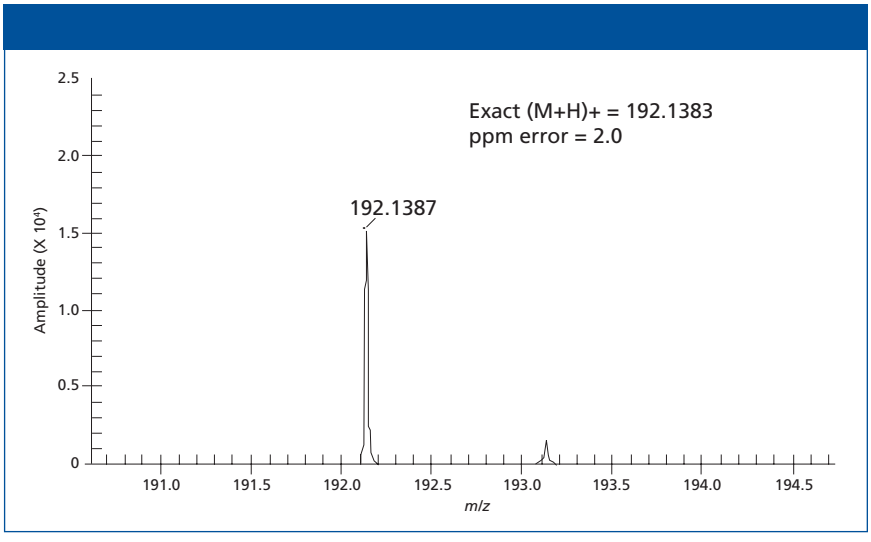

Figure 5: The presence of DEET was confirmed by accurate mass data.

chemicals are considered to cause ecological harm to animals, including possible endocrine disruption (4). Using the accurate mass capabilities of the TOF mass spectrometer, we have shown the high specificity in analysis of many of the target analytes in river water including antidepressants, antihistamines, antiseizure compounds, and antibiotics. Because of the diversity of chemicals found in the environment, there is an increasing trend to re-examine already acquired data for unknown, unexpected, or nontargeted analytes. This study shows examples of nontarget compounds that can be identified in river samples using the accurate mass and isotope ratio provided by the TOF mass spectrometer along with powerful database search tools such as the compound identification software.

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Development of a High Sensitivity Method for the Analysis of Clopidogrel and Clopidogrel Carboxylic Acid Metabolite in Human K_2EDTA Plasma Using UHPLC–MS-MS

Bioanalytical methods often involve the quantification of a parent compound to determine the pharmacokinetic properties of a potential drug. In some cases, it may also be necessary to quantify a metabolism product in addition to the parent molecule as these may be either active or toxic. Simultaneous determination can be challenging because of differences in chemical properties, such as acidity, basicity, and polarity, which can significantly increase the difficulty in developing both the extraction method as well as suitable chromatography. Developing chromatographic conditions involves not only separating the parent from the metabolite, but also effectively separating both compounds from potential coeluted contaminants such as phospholipids that may affect ionization efficiency and cause suppression or enhancement. The stabilities of the parent and metabolite also must be considered, as any interconversion between the two during the bioanalytical procedure may lead to variability within the results. In this article, we discuss the method development process required for the accurate quantification of both clopidogrel and its acid metabolite with a lower limit of quantification (LLOQ) of 1 pg/mL in human plasma.

Jennifer L. Simeone, Paul D. Rainville, and Robert S. Plumb

It is of great importance to integrate pharmacokinetic, pharmacodynamic, and toxicokinetic information in the process of drug development. Serious decisions are often made based on bioanalytical data, so it is vitally important that these data are precise and accurate. Before any bioanalytical method can be used for the analysis of samples acquired from dosed subjects, it is necessary to validate the method according to the appropriate guidelines, set forth by the US Food and Drug Administration (FDA), the European Medicines Agency (EMA), and others. Because of the strict requirements for method validation, it is essential to be thorough during the method development process and to identify any potential issues before the validation process. The best strategy for method development is to take a systematic approach with the three main aspects as follows: the extraction method, the liquid chromatography (LC) method, and the detection method. For the develop-

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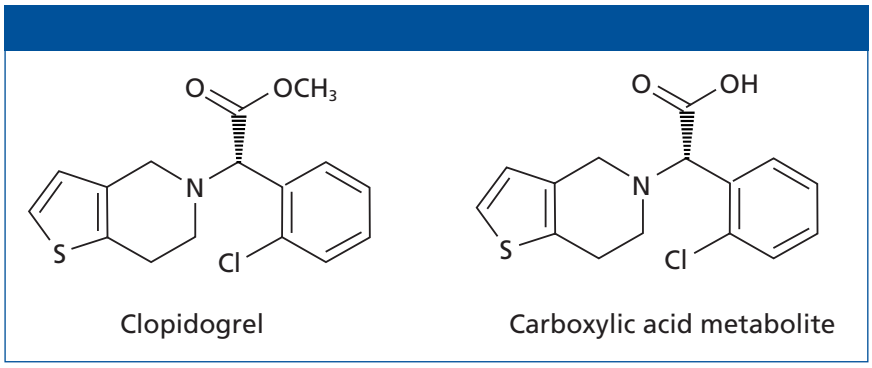

Figure 1: Chemical structures of clopidogrel and clopidogrel carboxylic acid metabolite.

ment of a sensitive, robust, and accurate method, all three portions are equally important.

As stated above, many bioanalytical methods require the quantification of more than one component. In many cases, the quantification of one or more metabolism products is essential for obtaining accurate data regarding the drug's pharmacokinetic profile. When an assay requires the quantification of multiple components, the development process becomes more difficult than it would be for a single compound. For some assays, the relative concentration requirements of the parent and metabolite may be vastly different because of the expected levels found in vivo. In this case, it may be best to optimize the method for the compound requiring the lower limit of quantification (LLOQ), and then make any necessary modifications to accommodate the other components.

Clopidogrel (trade name Plavix, Figure 1), is a thienopyridine derivative antiplatelet prodrug used in the prevention of artherosclerotic events. It is dosed in an inactive form and requires a hepatic biotransformation to yield the active thiol-metabolite, which binds to cell receptor P2Y12, irreversibly inhibiting the platelet activation process (1). In addition to the active metabolite, an inactive carboxylic acid metabolite is also formed. This acid metabolite accounts for the majority of circulating clopidogrel-related material with very low levels of the active metabolite and unchanged prodrug present (2,3). Because of the reactivity of the thiol metabolite, coupled with the low levels of the unchanged prodrug, most quantitative studies are based on the circulating levels of the inactive metabolite. The ability to detect the low levels of unchanged prodrug will provide more accurate data on the pharmacokinetics of clopidogrel, allowing improved evaluation of the bioavailability of new formulations.

Experimental

The finalized experimental conditions for the extraction procedure and ultra-high-pressure liquid chromatography coupled with tandem mass spectrometry (UHPLC–MS-MS) are described below.

Solid-Phase Extraction

Plasma samples were extracted by diluting 350 µL of plasma sample, containing 10 µL of internal standard solution at a concentration of 250 pg/mL, with 350 µL of Milli-Q (Millipore Corporation) water. Samples were added to an Oasis hydrophilic-lipophilic balanced (HLB) µElution plate (Waters Corporation) after preconditioning the plate with 200 µL of methanol followed by 200 µL of water. Samples were drawn through under vacuum, and then washed with 200 µL of water and 200 µL of 5% methanol–water. The sample was eluted with 2 × 25 µL of methanol and then diluted with an equal volume of water before injection. Samples were prepared at the following concentrations: 1.00, 2.50, 5.00, 10.0, 25.0, 50.0, 100, 250, and 500 pg/mL.

UHPLC–MS Conditions

The UHPLC–MS system consisted of an Acquity UPLC system (Waters Corporation) coupled to a tandem quadrupole Xevo TQ-S mass spectrometer (Waters Corporation) equipped with a prototype low-flow capillary for use with 1.0-mm i.d. columns. Chromatographic separations were performed on a 50 mm × 1.00 mm, 1.7-µm d_p Waters Acquity UPLC BEH C18 column (Waters Corporation) maintained at 45 °C. Mobile-phase A consisted of 0.1% formic acid and mobile-phase B was 100% acetonitrile. The flow rate was 0.140 mL/min. Starting conditions of 10% B were held for 0.5 min, then components were eluted via a linear gradient of 10–90% B over 2.5 min. The MS system was operated in positive electrospray ionization (ESI^+) mode, and the following multiple reaction monitoring (MRM) transitions were used: 322.1→212.0 for clopidogrel, 326.1→216.1 for d_4-clopidogrel, 308.1→198.1 for clopidogrel acid metabolite, and 312.1→202.1 for the d_4-clopidogrel acid metabolite. The capillary and cone voltages were set to 0.5 kV and 35 V, respectively. The optimized collision energies for all four components were determined to be 16 V. All data integration and calculations were done using Waters UNIFI Scientific Information System software (Waters Corporation).

Results and Discussion

Most published methods for the analysis of clopidogrel or its carboxylic acid metabolite use a liquid–liquid extraction (LLE) technique, often requiring a double LLE before UHPLC–MS (3,4). These methods are time consuming and tedious, and require large volumes of harmful chemicals such as hexane and diethyl ether. For these reasons, the use of alternative solid-phase microelution technology was investigated. This methodology provides an increase in throughput while decreasing solvent consumption. A typical LLE will consume 2–8 mL of organic solvent per sample, in contrast to microelution solid-phase extraction (SPE) methods, which require less than 0.5 mL of organic solvent per sample. Because of the fact that the carboxylic acid metabolite is present at much higher concentrations in circulating blood, development of an extraction procedure was optimized for the prodrug, clopidogrel. Results from initial method development experiments showed that mixed-mode cation exchange and HLB were the two

Table I: QC statistical data for QC levels at LLOQ, low, mid, and high values

	Clopidogrel QC Statistics					Metabolite QC Statistics			
	QC LLOQ	QC Low	QC Mid	QC High		QC LLOQ	QC Low	QC Mid	QC High
	1.00 pg/mL	3.00 pg/mL	30.0 pg/mL	200 pg/mL		1.00 pg/mL	3.00 pg/mL	30.0 pg/mL	200 pg/mL
	1.154	3.11	29.0	195		0.937	3.05	29.5	193
	1.29	3.09	30.9	203		0.937	3.48	29.9	199
	0.99	3.07	29.8	199		0.977	3.23	30.9	190
	1.036	3.04	30.0	204		0.907	2.94	30.6	198
	1.17	3.18	29.3	200		1.04	2.88	31.3	201
Mean	1.13	3.10	29.8	200	Mean	0.966	3.11	30.4	196
Std. Dev.	0.118	0.0516	0.725	3.36	Std. Dev.	0.0488	0.244	0.720	4.57
% CV	10.5	1.7	2.4	1.7	% CV	5.1	7.8	2.4	2.3
% Bias	12.8	3.2	-0.7	0.0	% Bias	-3.4	3.8	1.4	-1.9

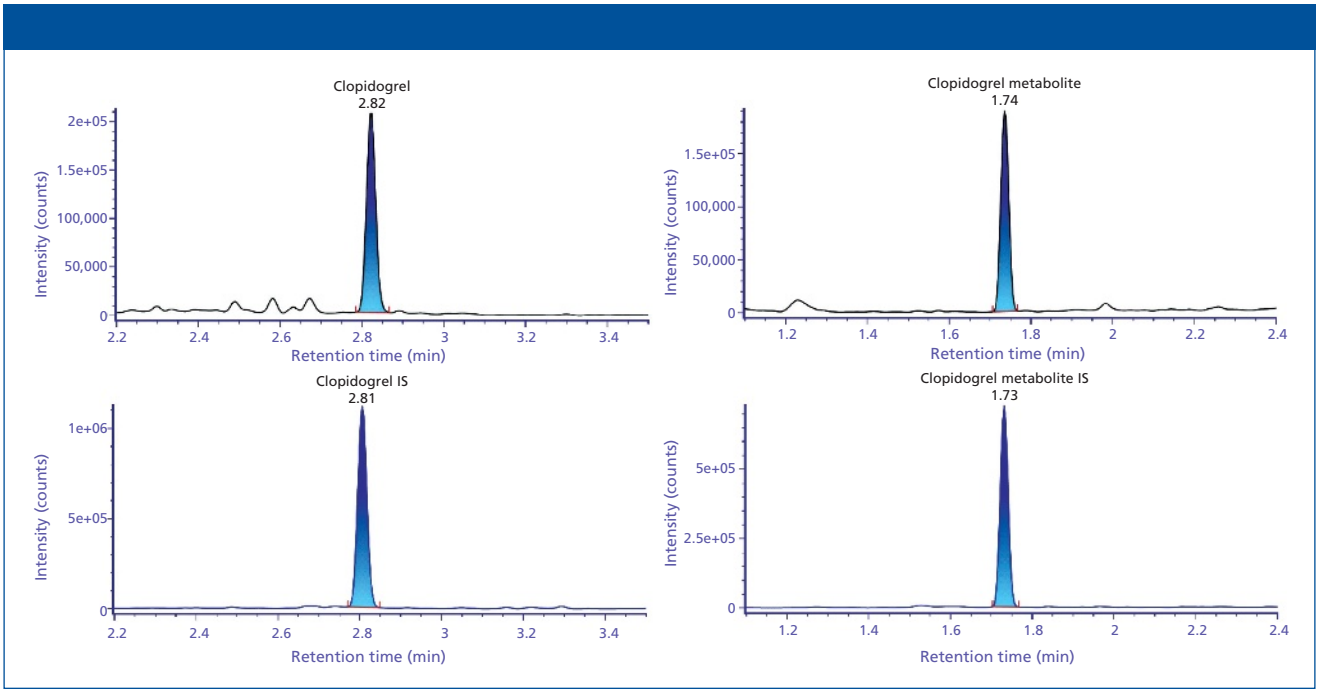

Figure 2: Example chromatograms of clopidogrel (top left), deuterated clopidogrel (bottom left), carboxylic acid metabolite (top right), and deuterated carboxylic acid metabolite (bottom right).

most promising SPE sorbents to further optimize. Both the analyte and metabolite were shown to extract well using these methods, and there was no loss shown of either component during the wash steps. Use of the microelution plate made it possible to load 350 µL of plasma sample (diluted 1:1 with aqueous solution before loading), and elute the analytes with only 50 µL of solvent. This gives a sevenfold increase in concentration and also eliminates the need for a time-consuming dry down step that is found in most bioanalytical methods, especially LLEs. Because of the chromatographic differences in the two compounds (discussed later in this article), a 1:1 dilution of the SPE eluent with water was required before injection.

The next step in the method development process was to conduct a mobile-phase screen to determine the mobile-phase composition that would provide both the best MS ionization characteristics and separation of clopidogrel from any coeluted interferences. Detection was carried out on a triple-quadrupole mass spectrometer operating in ESI^+ conditions. The initial screening was conducted on a 50 mm × 2.1 mm, 1.7-µm d_p UHPLC C18 column. The results for all combinations of either acidic or basic aqueous mobile phase modifier when used in combination with acetonitrile or methanol as the organic eluent indicate that 0.1% ammonium hydroxide with acetonitrile gave the greatest response as well as the greatest signal-to-noise value. Although a majority of mobile phases used in LC–MS are traditionally acidic, such as 0.1% formic acid, many recent studies show that for a wide range of pharmaceutical compounds, high pH often provides the best conditions for ionization (5).

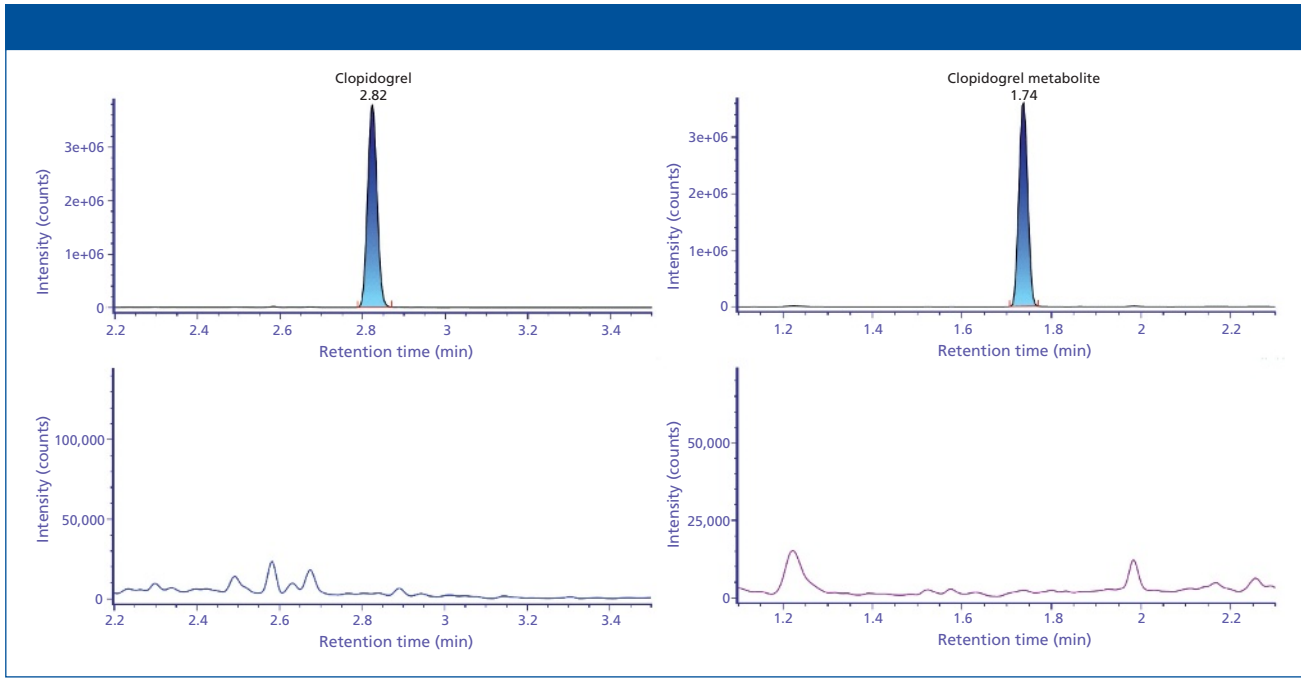

Figure 3: No detectable carryover was observed for both clopidogrel (upper limit of quantification [ULOQ] top left, blank bottom left) and the carboxylic acid metabolite (ULOQ top right, blank bottom right).

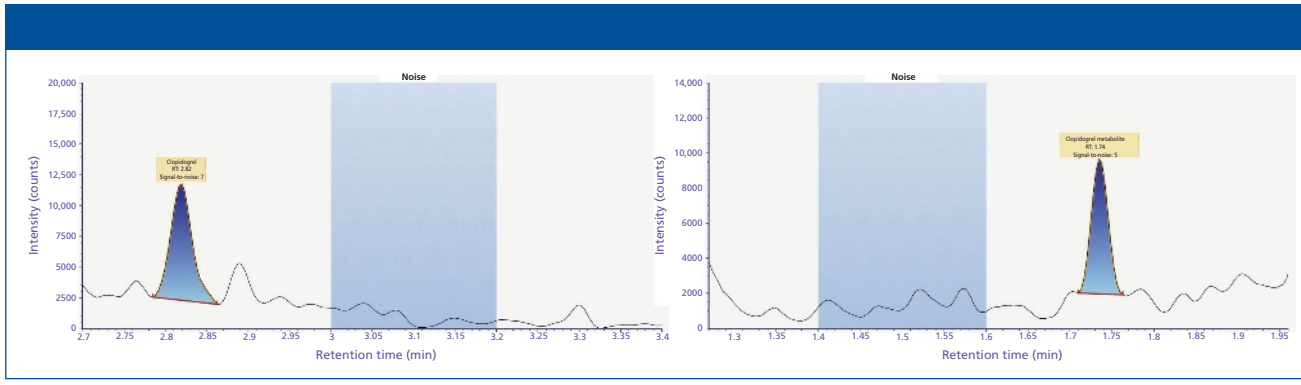

Figure 4: Signal-to-noise values for clopidogrel (left) and carboxylic acid metabolite (right).

Typically, a method developer seeks to find chromatographic conditions that yield the highest signal-to-noise ratios. This provides a lower detection limit, which is often the limiting factor in a bioanalytical method. However, one must also take into account any coeluted interferences that may potentially impact the assay via suppression, by enhancement, or by producing variability within the assay. Some of the most common interferences within a bioanalytical assay arise from the choline-containing lipids found in the matrix, which share a common fragmentation of 184 *m/z*. Using this piece of information, it is possible to monitor a majority of these compounds either by using a generic 184→184 *m/z* MRM transition or by collecting precursor data. For clopidogrel, acquisition of both clopidogrel MRM 322→212 *m/z* along with a precursor scan (precursors of *m/z* 184, from 450 *m/z* to 800 *m/z*) were collected simultaneously. Evaluation of the acquired data for both the acidic and basic aqueous mobile phase combined with acetonitrile showed that clopidogrel is eluted in the middle of the phospholipid region when basic solution is used. In contrast, for 0.1% formic acid with acetonitrile, clopidogrel is on the leading edge of the phospholipid region. In this case, it is much easier to chromatographically separate the clopidogrel from the phospholipid fraction. Although the response of the clopidogrel was not obviously suppressed by the coelution of lipids, it can still lead to inconsistent data because of varying amounts of lipids within the sample, or varying interactions with the lipids that may affect ionization. Both of these scenarios have the potential to change on a sample-by-sample basis, which may lead to assay irreproducibility. For these reasons, the use of ammonium hydroxide with acetonitrile was not used for further method optimization.

Because of the fact that phospholipids are not the only potential interferences, the full scan data as well as the 184 *m/z* precursor scan data were used to determine the best column choice. The UHPLC 1.7-µm d_p C18 column

showed the lowest response for both the precursors of 184 *m/z* and full-scan data in the region of the clopidogrel peak elution. The next step was the development of the LC conditions to accommodate both the clopidogrel and its acid metabolite. In light of the differences in structure and hydrophobicity, the elution composition for each component was drastically different. Because of the hydrophobic property of clopidogrel, it is advantageous to use LC gradient starting conditions with a higher percentage of organic solvent to expedite the elution and thus shorten the run time. In contrast, the acid metabolite is eluted much earlier in the gradient profile, thus if starting conditions contain too much organic, the *k* (retention factor) value will not be optimal and peak shape may suffer. These factors also had to be taken into account before introduction of the sample into the UHPLC system. If the injection solution percent organic is too high, the peak shape of the acid metabolite may potentially suffer via early elution. For these reasons, the development of the optimal method contained a 90:10 aqueous–organic hold for 0.5 min before the gradient start. This provided a *k* value of 1.6 for the metabolite and resulted in no discernible band spreading for the 50:50 aqueous–organic injection.

By using a microelution SPE plate for sample preparation, the resulting bioanalytical method yielded an LLOQ of 1 pg/mL for both the parent and acid metabolite. The final method used d_4-labeled internal standards for both clopidogrel and its carboxylic acid metabolite to account for any variability within the extraction and detection. The next step in creating a bioanalytical method that is appropriate for use is to test for robustness and reproducibility. In a regulated setting where the method would be used to analyze study samples, a formal method validation would be required before use. After several accuracy and precision batches were extracted and injected, it was noticed that the results for both the parent and metabolite were very erratic, especially near the lower end of the calibration range. To determine whether it was an effect of some components in the plasma, the extraction was performed using calibration standards and quality controls (QCs) prepared both in human plasma and in aqueous solution. The results showed the same nonreproducibility for both matrices, indicating that the unreliable results were not because of any of the matrix components. Replicate injections of the same sample, whether in plasma or in solution, produced MS responses with very low variability. This indicated that the erratic results were not because of the UHPLC–MS portion of the analysis. For example, any problem with column re-equilibration, insufficient ionization, or hardware (such as leaks) would have been noticed when injecting replicates of the same sample. The problem, therefore, seemed to lie somewhere within the extraction method; thus an alternative HLB reversed-phase extraction method was investigated.

From initial method development, the use of the HLB sorbent gave the second best recovery and signal to noise values. In addition, the use of HLB sorbent did not produce acidic or basic extracts, which may have been the cause of the nonreproducibility when using the mixed mode cation exchange sorbent plate. Because it is the methyl ester of clopidogrel that undergoes a metabolism to yield the carboxylic acid metabolite, it is reasonable to assume that there may be conversion or back-conversion within the extraction procedure. Even if the reaction occurred to a very minor extent, there is still potential to impact assay results obtained given the extremely low detection levels. As such, the HLB plate was investigated to see if it generated more-reproducible results. As part of the development of the HLB method, wash solutions containing 5%, 10%, and 15% methanol were tested. The use of 10% and 15% methanol resulted in loss of the metabolite during the wash steps, so only one organic wash step using 5% methanol was used.

Because of the lower sensitivity of the HLB method compared to the mixed-mode cation exchange method, it was not possible to obtain the 1 pg/mL LLOQ using the previously established UHPLC–MS-MS conditions. The use of 1.0-mm i.d. (microbore) chromatography is known to give increases in sensitivity of up to fourfold over that

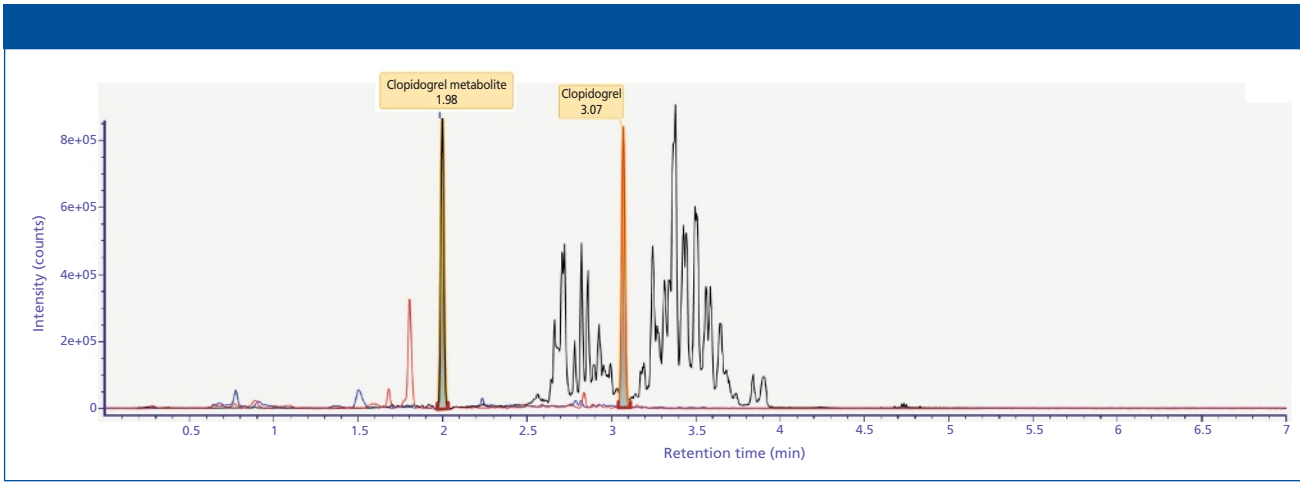

Figure 5: Chromatographic resolution of clopidogrel and carboxylic acid metabolite from choline-containing phospholipids.

obtained with a standard 2.1-mm column, thus a smaller diameter column was used. The UHPLC method also needed to be updated to accommodate the lower flow rate and the longer column re-equilibration time required for the 1-mm column. The resulting method showed reproducible results for the entire calibration range of 1–500 pg/mL.

Assay Results

The resulting chromatograms obtained for standards clopidogrel and clopidogrel carboxylic acid, as well as the internal standards d_4-clopidgrel and d_4-clopidogrel carboxylic acid are shown in Figure 2. The metabolite and deuterated internal standard are eluted at 1.7 min, while clopidogrel and its internal standard are eluted at 2.8 min. The calibration curve was linear over the range of 1.00–500 pg/mL, with no carryover detected for either component (see Figure 3). The use of microelution SPE coupled with a 1.0-mm i.d. column gave rise to an LLOQ of 1 pg/mL for both clopidogrel and its metabolite. Clopidogrel showed a signal-to-noise ratio of 7:1 while the metabolite showed a signal-to-noise ratio of 5:1 (Figure 4). QC samples were injected in replicates of five at four different levels spanning the range of the calibration curve. All QCs met acceptance criteria of accuracy and precision ±20% for the LLOQ and accuracy and precision ±15% for all other QC levels (Table I).

As previously stated in the method development discussion, another essential part of method reliability and robustness is the ability to separate the analytes of interest from any background interferences, such as choline-containing lipids. Comparison of the two MRM channels for clopidogrel and the acid metabolite with the precursor scan of 184 *m/z* over the mass range of 490–760 *m/z* is shown in Figure 5. As can be seen from the chromatogram, clopidogrel is eluted between two major regions of interference while the metabolite is eluted over 1 min before elution of the choline-containing lipids. Thus, successful separation of the components of interest from any potential impact of coeluted phospholipids was achieved.

Conclusions

A systematic approach to the method development process was successfully carried out for clopidogrel and its inactive carboxylic acid metabolite. The optimal extraction method was determined to be by a solid-phase microelution extraction. The chromatographic conditions were then examined, leading to successful separation of clopidogrel and the carboxylic acid metabolite from coeluted phospholipids. This successful separation allowed for the development of a high sensitivity method for both components with an LLOQ of 1 pg/mL. A mock-validation accuracy and precision batch was run, and all standards and quality control samples met the required acceptance criteria put forth by the FDA. For each QC level, multiple replicates injected showed low variability between extracted samples. In addition, there was no detectable carryover for either compound.

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Lean, Green Food-Testing Machines: How Innovations in Microflow LC–MS Can Improve Food Safety Test Methods

Food testing labs work endlessly to test our food supply for hazardous chemicals and contaminants to ensure human and animal safety. For a routine food testing laboratory, this typically means they must prepare hundreds of food samples daily for analysis, analyze those samples for hundreds of contaminants, and do it all with a fast turnaround of results. In recent years, innovations in microflow liquid chromatography (LC) coupled to mass spectrometry has created opportunities for laboratories to do more with less — more sensitivity, higher throughput, and more robustness in their analyses but in less time, with lower cost, and with less hazardous solvent consumption. For a high-throughput food testing laboratory, microflow LC coupled to mass spectrometry is an innovation that can push routine food testing analysis to the next level. Here, we describe how a food testing laboratory can transition routine high performance liquid chromatography (HPLC) methods to microflow LC and show performance results that showcase improved sensitivity, throughput, and robustness of analysis, with the added benefits of reduced solvent waste and overall analysis costs.

Stephen Lock and Lauryn Bailey

Food is a vital part of life, and many food scientists and chemists work hard each day to test food products and ingredients for safety and quality. From pesticides to antibiotics, allergens to natural toxins, and so many others, the number of contaminants that can often be found in foods, ingredients, and assorted consumer products continues to increase.

As the demand to test more products for more types of contaminants increases, the workload on food testing laboratories increases as a direct result. With this increase, food testing scientists are continuously looking for new methods and technologies to improve their workflows. From more efficient and effective ways to extract contaminants and remove matrix components in sample preparation, to faster and higher quality chromatography as well as more sensitive and selective detection approaches, food testing scientists are in constant pursuit to test more samples with faster turnaround of results with the highest level of accuracy and reliability.

As an added pressure, modern analytical equipment can sometimes require an abundance of resources, including consumable resources such as solvents, chemicals, and disposable equipment as well as personnel resources such as instrument run management and maintenance. These resource requirements can also put a strain on food testing laboratories, which endlessly strive

to keep their cost per analysis low and their efficiency to produce results high.

Fortunately, advances in modern analytical instrumentation can relieve some of these strains on routine food testing laboratories. From better sample preparation approaches to new liquid chromatography coupled to tandem mass spectrometry (LC–MS-MS) technology, food testers now have better accessibility to techniques that will save them time, save them money, and give them better results than ever before.

LC has been a preferred methodology for food testing laboratories for many years, allowing ideal separation, identification, and detection of contaminants in complex food samples. Routine food testing laboratories run samples around the clock, which results in considerable resource consumption, excessive expenses in consumable costs, and considerable production of organic solvent waste.

New innovations in LC technology, such as microflow LC, present laboratories with a more efficient alternative to higher-flow LC methods. Microflow LC requires significantly less solvent usage and less analytical sample consumption, without sacrificing any of the performance features of normal-flow LC, making it substantially more cost and time efficient for a high-throughput testing laboratory while still providing the best analytical results. Furthermore, the reduced solvent and chemical consumption has a reduced environmental impact, making microflow LC a leaner, "greener" choice for environmentally conscious laboratories.

In this article, we present a research study that shows how routine food testing methods, such as pesticide analysis in spices, can be transferred from traditional LC–MS-MS to microflow LC–MS-MS to save laboratories time, money, and resources without sacrificing analytical performance and quality of results.

Material and Methods

Two food samples were prepared and analyzed on both a conventional LC–MS-MS system and on a microflow LC–MS-MS system. The food samples (chili powder and fresh basil, two notoriously difficult matrices for food testing laboratories) were prepared using a standard QuEChERS (quick, easy, cheap, effective, rugged, and safe) approach (1). Briefly, 5 g of herb or spice was homogenized, hydrated with water (10 mL), and extracted with acidified acetonitrile (0.05% acetic acid in acetonitrile, 10 mL). QuEChERS salts (magnesium sulfate) were added to the sample, and the sample tube was rigorously shaken and centrifuged. An aliquot of the supernatant (6 mL) was mixed with dispersive solid-phase extraction (SPE) cleanup solids (PSA and C18), shaken vigorously, and centrifuged. The resulting supernatant was diluted 1:10 with water before analysis. The Sciex iDQuant standards kit (AB Sciex) for pesticide analysis (containing approximately 200 pesticide compounds) was used to prepare the calibration solvent standards as well as

Table I: Comparison of LC–MS sensitivities of nine pesticides spanning the analytical run for the conventional LC vs. the microflow LC system

Compound	Retention Time (min)	LC–MS-MS Signal-to-Noise Ratio	Microflow LC–MS-MS Signal-to-Noise Ratio	Signal-to-Noise Improvement Factor
Monocrotophos	4.05	229	1083.5	4.7
Tricyclazole	5.62	56.8	758.4	13.4
Simetryn	6.18	126.3	414.8	3.3
Monolinuron	6.89	40.2	432.6	10.8
Isoproturon	7.57	65.7	613.5	9.3
Terbutryn	8.03	92.5	883.7	9.6
Flutolanil	8.77	80.7	416.9	5.2
Fenoxycarb	9.44	16.7	99.8	6.0
Pyridaben	10.62	22.9	903.7	39.5

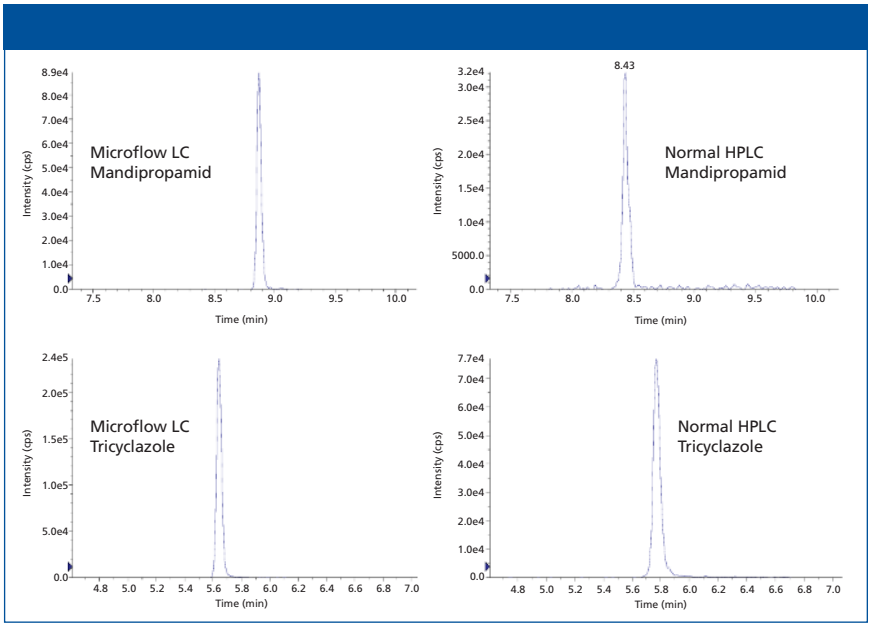

Figure 1: Comparison of the peak shapes of two pesticides using microflow LC (left-hand panel) and conventional LC (right-hand panel).

to prepare pesticide-spiked matrix samples.

Two LC–MS-MS systems were used for the comparative evaluation, one with the MS system coupled to a conventional high performance liquid chromatography (HPLC) system and the other to a microflow LC system. For the conventional HPLC system, a 5.0 mm × 4.6 mm, 2.6-µm Kinetex core–shell column (Phenomenex) was used with temperature set at 50 °C. For the MS-MS set up (QTRAP 4500, AB Sciex), a standard 100-µm i.d. Turbo V electrospray ionization (ESI) probe (AB Sciex) was used, with a source temperature of 550 °C, nebulizer gas (GS1) setting of 50 psi, heater gas (GS2) setting of 60 psi, and source voltage of 5500 V. For the microflow LC system, a 5.0 mm × 4.6 mm Halo C18 column (Advanced Materials Technology) was used with the temperature set at 50 °C. For the MS-MS setup, a microflow LC hybrid electrode (50 µm i.d.) (2) probe was used, with a source temperature of 450 °C, nebulizer gas (GS1) setting of 25 psi, heater gas (GS2) setting of 25 psi, and source voltage of 5500 V.

Identical mobile phases and gradients were used for the preliminary evaluation to compare conventional LC and microflow LC performance. The mobile-phase gradient of 98% water to 95% methanol (containing 2 mM ammonium acetate and 0.1% formic acid modifiers) had a 15-min total run time. The injection volume on both systems was 2 µL, and the flow rates were 400 µL/min and 40 µL/min for the conventional HPLC and microflow LC systems, respectively.

The Scheduled MRM algorithm was used for data acquisition to maximize the number of data points across each peak for the 125 pesticides assessed in the study (two MRM transitions per pesticide were used for a total of 250 MRM transitions).

Data were processed and analyzed using Analyst software version 1.6 and MultiQuant software version 2.1 (AB Sciex).

The following performance criteria were used to evaluate and compare the two instruments: peak shape and sensitivity, linearity, injection carryover, robustness, and solvent consumption.

Results and Discussion

The aim of this study was to assess the applicability of microflow LC for routine food testing applications and compare the sensitivity and performance with a traditional, higher-flow method already established for pesticide analysis in food samples. In this study, the chromatography was not optimized for speed, although it should be noted that the methods, particularly the microflow LC method, could be optimized for faster chromatography and reduced run times.

Peak Shapes and Sensitivity

To evaluate the peak shape and sensitivity of the two systems, a pesticide

standard containing 2 ppb each of more than 200 common pesticides was injected in each system. The extracted ion chromatograms of one MRM transition for two different pesticides are shown in Figure 1. The results show excellent peak shapes on both systems.

Signal-to-noise ratios (taken directly from MultiQuant software) were used to assess the relative sensitivity of the two systems, using selected pesticides that were eluted at various times throughout the run. A sensitivity comparison of nine pesticides is shown in Table I. The results show an increase in the MS instrument response when the microflow LC is used, with little increase in noise, resulting in signal-to-noise increases ranging from threefold to 10-fold relative to conventional flow LC.

A benefit of the added sensitivity of microflow LC is the ability to dilute samples further to reduce matrix effects on the results and also to potentially reduce injection volumes, lowering the amount of sample loaded onto the chromatography column, which could improve the robustness of the analysis, extend the life of the column, and reduce the maintenance required for the LC–MS-MS system.

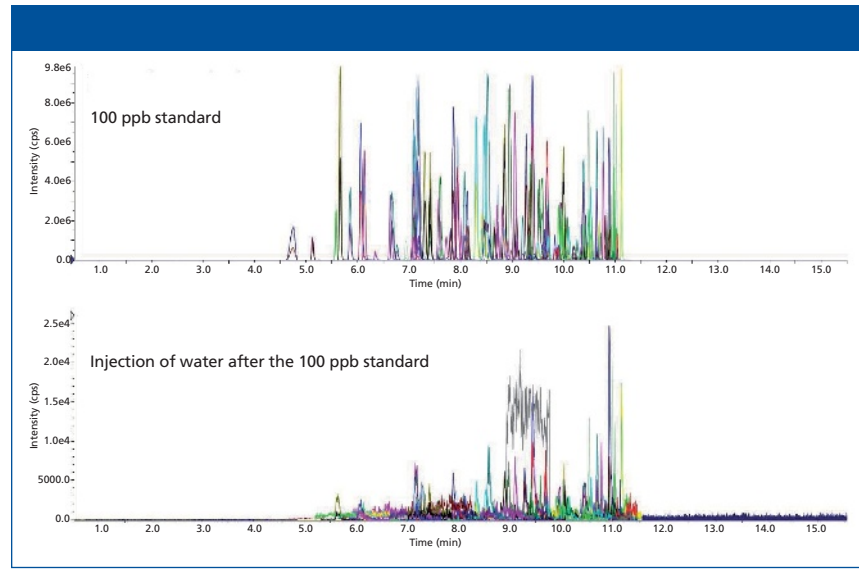

Figure 2: Carryover study showing a 100 ppb pesticide mix standard injection (top panel) and a water blank injection immediately after (bottom panel).

Linearity

Linear response of analytical instrumentation is highly important for

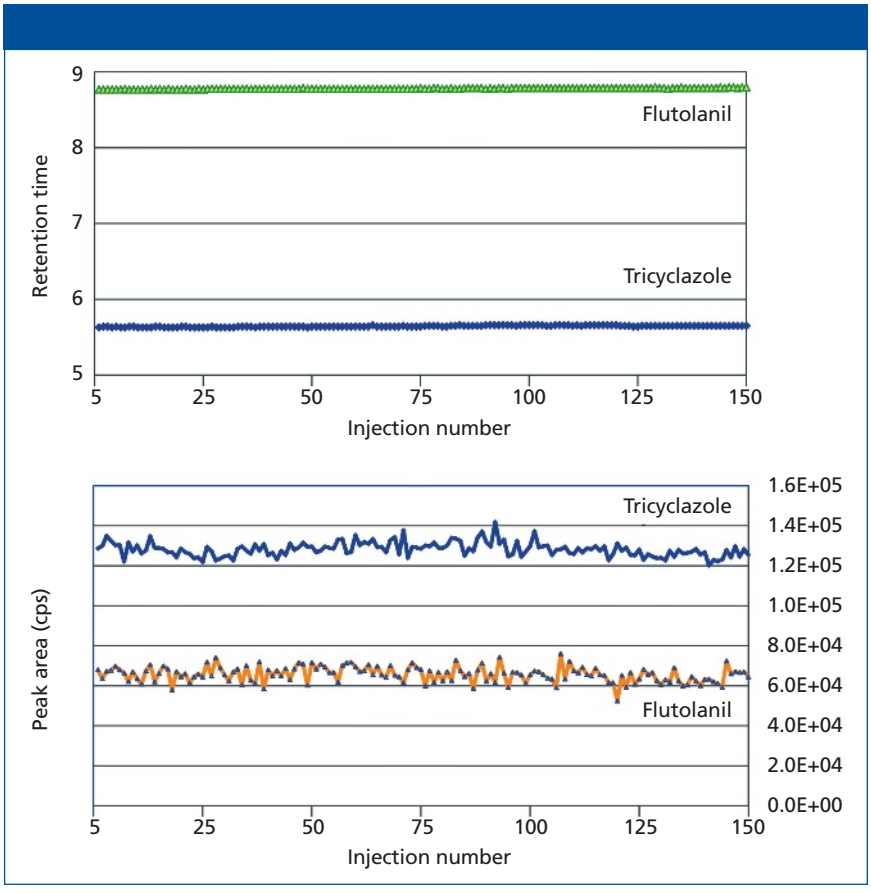

Figure 3: Results showing the retention times and peak areas observed for two pesticides (flutolanil: yellow trace and tricyclazole: blue trace) after repeated injections of a crude spice sample using the microflow LC–MS-MS system. These two pesticides were selected to assess robustness early in the analytical run as well as later in the analytical run.

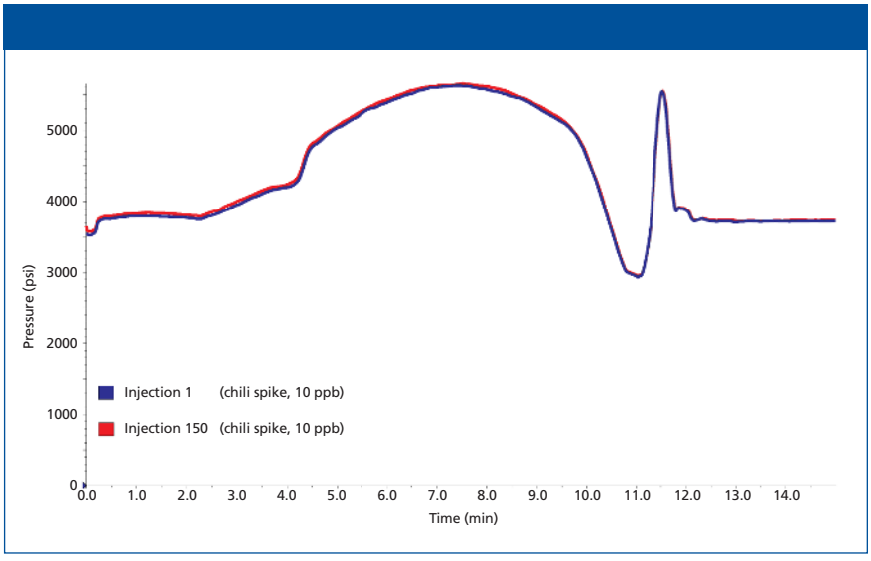

Figure 4: Pressure profile obtained from the microflow LC for the first injection of the spice sequence and the 150th injection of the spice sequence. Results show that the system is highly robust for long runs with "dirty" samples.

food testing applications because of the need for accurate quantitation over a wide range of concentrations. Conventional LC methods typically show excellent linear range; therefore, we tested the microflow LC system to confirm that the linear response is preserved using this front-end chromatography system. The linearity was assessed for a pesticide concentration range of 0.2–100 ppb with a linear regression fit, and the *r* value obtained for the majority of the pesticides was greater than 0.999. The results confirmed that linearity for quantitative LC–MS is preserved with the microflow LC chromatography.

Injection Carryover

A big concern in LC analysis, and an important feature of suitable LC systems and conditions, is that carryover between injections must be minimal to nonexistent. We wanted to verify that the carryover using the microflow LC front end was suitable and comparable to conventional LC systems, which typically are listed as having carryover values of 0.05% or less. To assess the carryover, a 100 ppb pesticide mix standard (which produces a saturated response in the mass spectrometer for most pesticides) was injected, and the carryover from the injection was assessed by evaluating the pesticide responses in a water blank injected immediately following the 100 ppb standard injection. The results of this assessment are shown in Figure 2. It was noted that for the majority of pesticides, no carryover was observed in the water blank, and the overall carryover was estimated at <0.1%.

Robustness

Food testing laboratories often put excessive stress on their analytical instruments, running many samples (often samples containing high levels of "dirty" matrix components) around the clock. As a result, instrument robustness is essential as the results obtained in the final run of the batch must be just as reliable as the results obtained in the first run of the batch. To test the robustness of the microflow LC system, repeated injections of unfiltered chili powder extract, a notoriously difficult matrix for food laboratories, were made, totaling more than 150 subsequent

injections without changing the column, cleaning the system, or performing any maintenance.

The retention times and peak areas of the pesticides were assessed for the duration of the 150 injections (Figure 3), and the pressure profile of the microflow LC system was plotted at the first injection and again at the 150th injection (Figure 4).

The results show that the LC–MS retention times are rock solid for the duration of the 150 injections, with little to no variability observed. These results confirm that fast equilibration times are possible with the microflow LC system, and also verify the benefits of the reduced dead volume of the system. Additionally, peak area responses measured throughout the injection sequence revealed no deterioration in total signal. This result was surprising because with repeated injections of dirty matrix samples, some peak response loss is typically observed. However, it was shown that the robustness of the microflow LC–MS-MS withstands these fluctuations, potentially in part due to the low sample injection volume required for analysis using micro-flow LC.

Solvent Consumption

Results showed that the performance of microflow LC was as good as that of conventional LC, and even better in some cases. Given the reduction in solvent consumption of the microflow LC system (40 µL/min flow versus 400 µL/min flow), we did one final evaluation calculating the relative solvent consumption of the microflow LC system versus the conventional LC system.

With the following assumptions:

- 15-min run time per injection
- Gradient consumes approximately 50% organic phase
- Methanol cost estimate of $1000 per 4 × 4 L case
- Approximately 200 samples injected per day
- Samples run five days per week, 52 weeks per year.

A laboratory could see cost savings of nearly $9000 just in solvent consumption per year (which does not include any additional costs incurred in waste disposal of solvents) by transitioning methods from conventional LC to microflow LC.

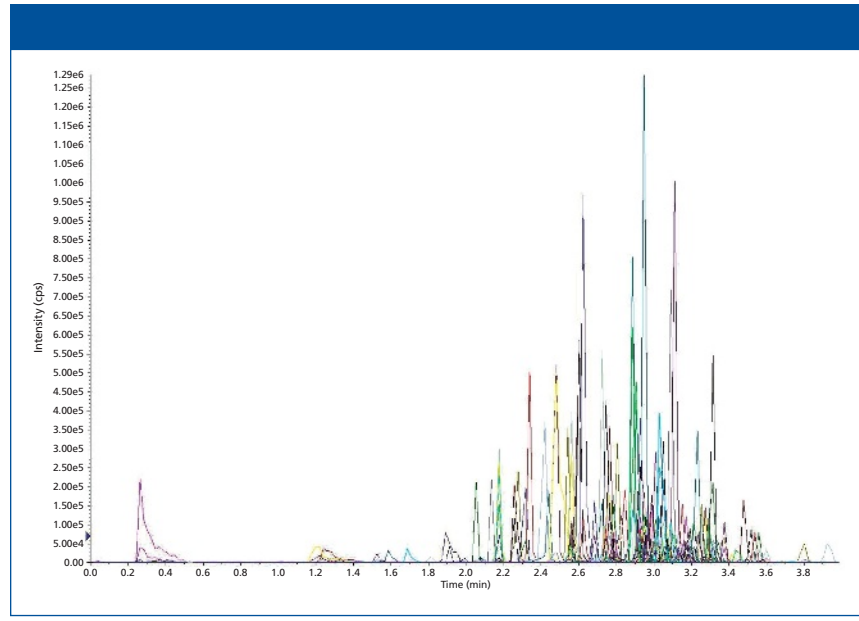

Figure 5: Injection of a 1 ppb pesticide standard with a rapid microflow LC gradient.

Microflow LC also can provide improved separation over normal LC, allowing the potential to reduce run times and greatly improve not only sample throughput, but also provide cost savings in solvent consumption. Figure 5 shows an example of a rapid gradient for the analysis of pesticides in food, where run time is reduced from 15 min per injection to 4 min per injection.

Conclusions

This study has demonstrated the capabilities of microflow LC–MS–MS for routine testing of contaminants in food samples. With the increasing demand on food testing laboratories to test more samples (and a larger variety of matrices) for more compounds with better performance, while also improving throughput and efficiency, it is clear that new techniques such as microflow LC can have a strong impact in routine food-testing laboratories. With improved sensitivity and throughput, without any sacrifice in analytical performance and reliability in results, microflow LC is a viable alternative to conventional LC for food laboratories striving to improve their methods while also improving their bottom line.

Also, with the reduced solvent consumption associated with microflow LC, environmentally conscious scientists can feel better about using more sustainable technology with reduced environmental impact. As these results show, becoming a lean, "green" food-testing machine can now be as easy as a simple swap from LC to microflow LC, and the graduation to the next generation of sustainable LC–MS-MS technology.

References

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(2) K. Mriziq et al., Technical Note Eksigent (2011) # 4590211-01.

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An Improved Method for Eliminating Ion Suppression and Other Phospholipid-Induced Interferences in Bioanalytical Samples

Phospholipids are perhaps one of the most troublesome components in bioanalytical samples when performing liquid chromatography coupled to tandem mass spectrometry (LC–MS-MS) analysis. Shortened column life, ion suppression, and an increase in MS system maintenance are just a few of the downfalls that can occur if phospholipids are not sufficiently removed from bioanalytical samples before analysis. Rapid crude sample preparation methods such as protein precipitation are sometimes preferred over more focused sample cleanup techniques like solid-phase extraction (SPE); however, such crude sample preparation techniques primarily reduce protein content and only slightly reduce the phospholipids present in the sample. Method developers are faced with a dilemma: Choose a sample preparation method that is quick but prone to method interferences because of the presence of phospholipids, or develop an optimized SPE method to achieve a cleaner, phospholipid-free sample. This article examines the various effects that phospholipids play in LC–MS-MS analysis and demonstrates a new phospholipid-removal approach that is simple and rapid like protein precipitation, yet removes phospholipids similar to a SPE procedure, but without the required method development.

Stuart Kushon and Erica Pike

In the past, the analysis of drugs, metabolites, and toxins in biological fluid was performed using a developed sample preparation technique that included either solid-phase extraction (SPE) or liquid–liquid extraction (LLE) before the sample was analyzed by liquid chromatography–mass spectrometry (LC–MS). As the size and complexity of studies increased, many groups undertook efforts to streamline methods and increase throughput by reducing LC run times and moving away from complex sample preparation methods. Indeed, many groups have simplified sample preparation methods such that extraction methods have been completely abandoned and a simple protein-precipitation method is used before LC–MS analysis. In addition, LC–MS run times have been reduced so that the chromatography is little more than a desalting step. Unfortunately, such

Table I: Sample preparation protocols

Protein-Precipitation Method	Phospholipid-Removal Method (Phree)
1. Add 300 µL acetonitrile to the wells of a collection plate	Add 300 µL acetonitrile with 1% formic acid to the wells of the phospholipid-removal plate
2. Add 100 µL of plasma directly into the acetonitrile	Add 100 µL of plasma directly into the acetonitrile
3. Vortex for 2 min at maximum possible speed	Vortex for 2 min at maximum possible speed
4. Pellet precipitated proteins by centrifuge	Filter using vacuum at 2–7 in. Hg for up to 5 min*
5. Collect supernatant, taking care not to disrupt pellet	
*Centrifugation and positive pressure may also be used.	

reductions in run time and sample preparation have not come without encountering issues with sample matrices.

Interference caused by phospholipids in biological fluid samples has been a long-standing topic of debate because numerous groups have repeatedly demonstrated that most ion suppression in LC–MS-MS analysis of protein precipitated and direct inject sample is because of the presence of phospholipids. It has also been observed that phospholipids can cause several other negative effects in LC–MS-MS analysis such as a decrease in high performance liquid chromatography (HPLC) or ultrahigh-pressure liquid chromatography (UHPLC) column lifetimes, a decrease in MS sensitivity, and a subsequent increase in MS maintenance because of build up of nonionized lipids on the MS ion source.

These many adverse effects of phospholipids on LC–MS-MS analysis have spurred an increase in targeted sample preparation to selectively remove phospholipids from bioanalytical samples. In the past, it was difficult to selectively remove phospholipids because they exist in several compound classes with unique chemical and retentive properties. Of particular concern and interest in rapid LC–MS analyses are the phosphatidyl cholines and lysophosphatidyl cholines. Both classes of phospholipids can be visualized by LC–MS-MS by monitoring the 184→184 mass transition. Using this approach, this study looks at the effects of phospholipids in plasma samples following the use of two different sample preparation techniques: a standard protein-precipitation method and a simultaneous protein-precipitation and phospholipid-removal method

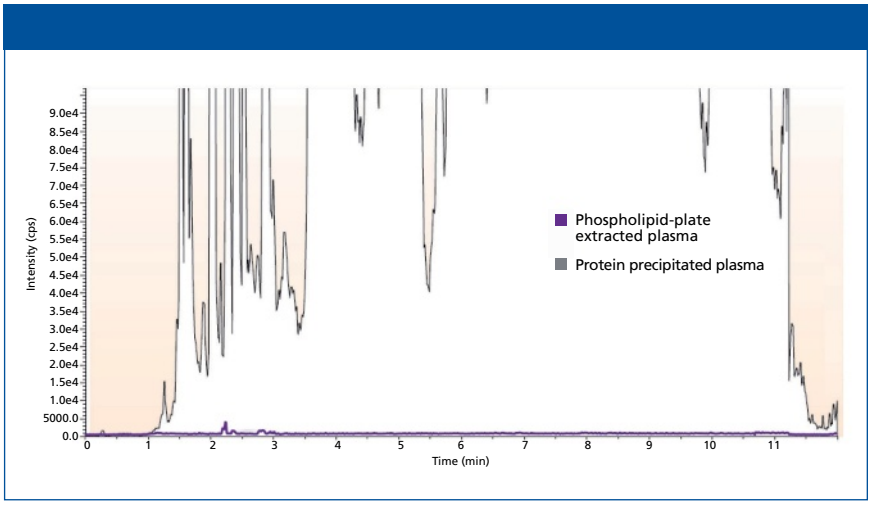

Figure 1: Comparison between protein-precipitated plasma and phospholipid-removal plate (Phree) extracted plasma samples. The MS-MS MRM transition for phospholipids is monitored across a gradient on a Kinetex C18 column. Note that phospholipid-removal plate samples exhibit a dramatic reduction in MS signal because of the selective removal of phospholipids.

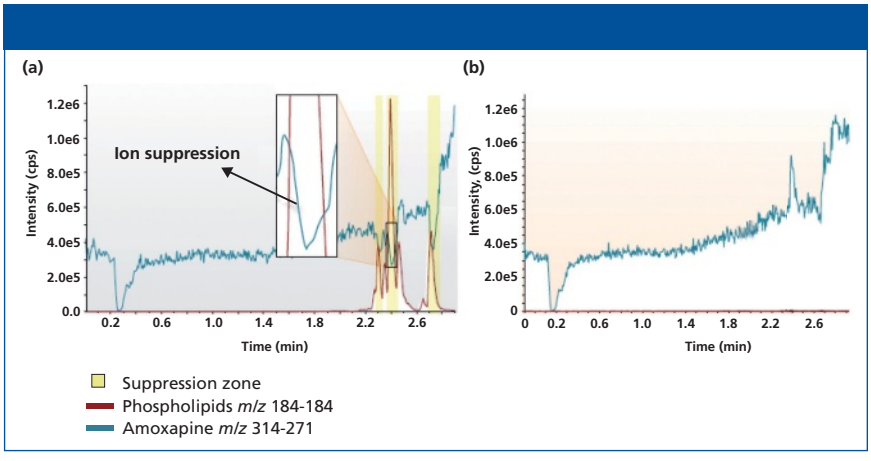

Figure 2: Ion suppression effect across an LC gradient for (a) protein-precipitated plasma and (b) plasma treated using a phospholipid-removal plate. Amoxapine post-column infusion maintains a 314–271 MS-MS signal across the gradient. Any rapid deviations (peaks or valleys) from baseline are indicative of ion suppression because of phospholipids. Note that with the protein-precipitated sample, a major depression in amoxapine signal at 2.4 min retention time corresponds to the phospholipids peak at the same time. The same sample run on the phospholipid-removal plate shows minimal signal suppression across the entire gradient.

using a 96-well phospholipid-removal plate.

Experimental Conditions

All solvents and buffer reagents were purchased from EMD. Laboratory chemicals were obtained from Sigma Chemicals and plasma samples were purchased from Bioreclamation.

Plasma samples were prepared using the two sample preparation techniques described in Table I. A standard protein-precipitation method was compared to a slightly modified procedure using a 96-well phospholipid-removal plate, Phree (Phenomenex). Each sample preparation technique called for up to five simple steps and required no sample specific method development outside the listed protocol. In an effort to maintain equivalence between methods, plasma samples from the same lot were used for each cleanup method, which ensured that the starting level of phospholipids was equivalent in each prepared sample.

After cleanup, samples were injected onto an Agilent 1200 HPLC system, which included an autosampler, solvent degasser, and column oven. The equipment was operated using Chemstation software (Agilent). The UHPLC column used was a 50 mm × 2.1 mm, 2.6-µm Kinetex C18 core–shell column (Phenomenex) that was coupled to an API 3000 mass spectrometer (AB Sciex). Phospholipids in each sample were then monitored using *m/z* 184–184. Mobile-phase A was 0.1% formic acid in water and mobile-phase B was 0.1% formic acid in acetonitrile. A gradient consisting of 5–95% B in 5 min or 15 min (Figures 1 and 2, respectively) was used, and the flow rate was 0.35 mL/min.

Results

An overlay between the phospholipid-focused LC–MS analysis of protein-precipitated plasma sample and the same sample using a phospholipid-removal plate is shown in Figure 1. Contrary to some that report phospholipids only being at the beginning and end of a gradient, when a focused analysis is performed one can see phospholipids eluted across the entire UHPLC gradient in the protein-precipitation sample.

Even though phospholipids can be seen across the whole gradient when analyzing the low-level MS signal on the C18 column, their presence does not necessarily impact quantitation of all compounds in plasma samples. To better study the influence of ion suppression on the quantitation of an analyte, amoxapine was infused post-column to establish an ion suppression and enhancement profile in each of the prepared samples. Two mass transitions were then analyzed, *m/z* 184–184, which showed the total phospholipid profile, and *m/z* 314–271, which monitored for the amoxapine that was introduced via post-column infusion (Figure 2). Dramatic changes in the MS base-

line (up or down) are indicative of suppression or enhancement effects of phospholipids in a plasma sample.

In addition to the deleterious influences on analyte quantitation, a common finding from groups using protein precipitation has been that HPLC column lifetimes have been greatly reduced compared to columns used with other sample preparation techniques. A lifetime study was performed to determine the effects each sample preparation technique (protein precipitation vs. phospholipid-removal plate) had when multiple samples were repeatedly injected onto the LC–MS system. Repetitive 20-µL injections of diclofenac in protein-precipitated plasma versus diclofenac in phospholipid-removal plate extracted plasma (as prepared in Table I) were made onto the LC–MS system (Figure 3).

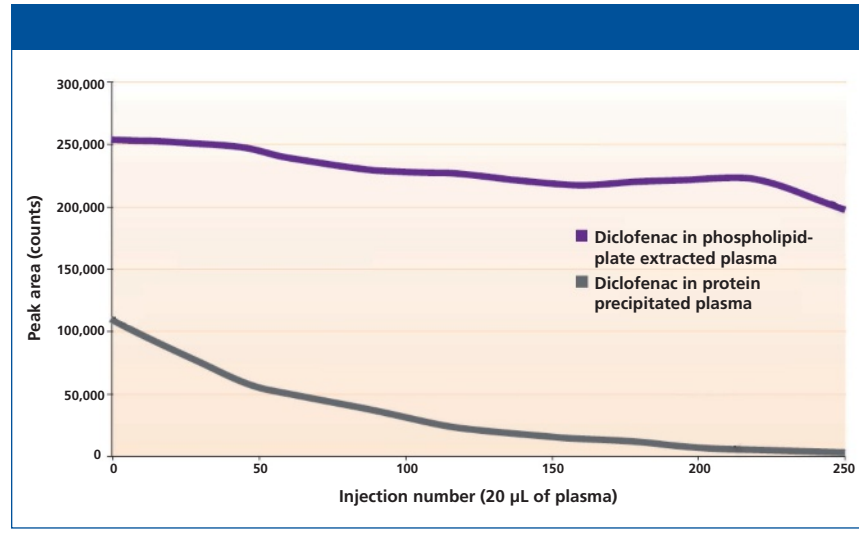

Figure 3: Column sensitivity after 250 injections.

Discussion

Our study revealed that the protein-precipitated plasma sample contained a significant amount of phospholipids (phosphatidyl cholines and lysophosphatidyl cholines) when monitoring for *m/z* 184–184. When monitoring the same mass transition, the phospholipid-removal plate extracted plasma samples showed a significant decrease in the presence of phospholipids to the point where they were virtually eliminated.

After confirming the presence and absence of phospholipids between protein-precipitation and phospholipid-plate extracted plasma, respectively, ion suppression in each prepared sample was studied. It was shown that a large region of suppression occurred in the protein-precipitated plasma when monitoring *m/z* 314–271 (amoxapine) (Figure 2). This region of suppression directly corresponded to a spike in phospholipid signal that was monitored using *m/z* 184–184. To confirm that phospholipids were the cause of the observed ion suppression, the same post-column infusion study was performed on the phospholipid-removal plate extracted plasma that we previously determined had been stripped of virtually all phospholipids. This study did not show a region of ion suppression and instead displayed a steady signal for the amoxapine infusion. It was therefore determined that the presence of phospholipids in the protein-precipitated sample caused a region of ion suppression in the amoxapine infused sample that did not occur in the sample that had been stripped of phospholipids.

Ion suppression is not the only negative effect that phospholipids can cause in LC–MS-MS analysis. Column lifetime and MS sensitivity are also at risk, so we studied the effect of phospholipids on each. It was shown that the phospholipid-removal plate extracted sample immediately yielded a signal that was 2.5 times stronger than the protein precipitated sample when the first injections were made. After repetitive injections, the signal from the protein-precipitated samples quickly decreased by more than 100,000 peak area counts to virtually zero after 250 injections. On the other hand, the signal from the phospholipid-removal plate extracted samples decreased only slightly, by 50,000 peak area counts after 250 injections (Figure 3).

Conclusion

Phospholipids are thought to play many negative roles in LC–MS analysis. Our study found that by using the correct sample preparation technique, phospholipids can be removed from samples before being injected onto an HPLC or UHPLC column. Protein precipitation, while a quick and easy method, was not able to remove phospholipids and resulted in several downfalls including ion suppression and an immediate decrease in sensitivity and column lifetime. Using a phospholipid-removal device, samples could be prepared using the same steps required in a traditional protein precipitation; however, the approach provided the added benefit of removing phospholipids. Extracted samples showed virtually no sign of phospholipids and immediately provided a 2.5 increase in sensitivity as well as an increased column lifetime compared to protein precipitated samples.

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Application of IC–MS and IC–ICP-MS in Environmental Research

Since its introduction in 1975, ion chromatography (IC) has been used in most areas of analytical chemistry and has become a versatile and powerful technique for the analysis of a vast number of ions present in the environment. Although conductivity detection is still the most popular detection method, other types of detection can be applied for different analytes. These include the following methods: electrochemical (for example, amperometric and potentiometric), photometric (UV–vis and chemiluminescence), and spectrometric (used mainly in hyphenated techniques). The most versatile and powerful detection method is mass spectrometry (MS). The main advantages of IC–MS are extremely low detection and quantification limits, insignificant interference influence, and high precision and repeatability of the determinations. This article is a review of possible uses of IC in combination with MS detection for environmental research.

Rajmund Michalski

Speciation, as a word borrowed from biology, is a term describing the existence of various chemical and physical forms of a particular element, and speciation analytics denotes the determination of those forms (1). The notion of speciation is used in chemistry to determine the occurrence of diverse forms of a given element (for example, elements at various oxidation states or bound with different ligands) in the analyzed sample. The forms might differ in physical and chemical characteristics as well as in the influence they exert on living organisms. In the last several decades, speciation analytics has become one of the most central issues in analytical chemistry. Even though its cost is significant, speciation analytics has become more important when it comes to solving problems that concern not only the determination of total element contents, but also taking into account various forms of occurrence. It plays an exceptional role in the examination of biochemical cycles of selected chemical compounds, determination of toxicity and ecotoxicity of selected elements, food and pharmaceutical product quality control, and technological process control as well as health risk assessment and clinical analytics (2).

It is reasonable to differentiate between chemical and physical speciation. In chemical speciation, it is possible to distinguish between *screening speciation*, which searches for and determines selected chemical forms, and *distribution speciation*, which searches for and determines selected chemical individuals in specific elements of the examined sample. Another division within chemical speciation concerns *group speciation*, which is defined as searching for and determining specific groups or classes of chemical forms, and *individual speciation*, which is searching for and determining all chemical individuals present in the sample. When it comes to liquid sample analyses, the most common technique is the one developed by Florence and Batley (3). According to this method, a water or wastewater sample filtered through a 0.45-µm filter is divided into a solid phase and a mobile phase, in which the determinations of total metal contents and the metal labile and bound forms are carried out. The division suggested by Tessier and colleagues (4) is recommended in the research concerning the speciation of heavy metals in bottom sediments. They distinguished and defined five fractions: exchangeable metals,

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carbonate-bound metals, iron and manganese oxides-bound metals, organic matter-bound metals, and other mineral-bound metals. Nonetheless, this method of speciation does not differentiate between oxidation states of elements, which may be of great importance when considering their toxicity.

Lowering the detection limits of analytes to extremely low concentration levels resulted in methods that did not always meet the necessary requirements. For that reason, there has been a tendency to combine various methods and techniques. These combinations are known as hyphenated techniques. A suitable hyphenated technique should be selective toward determined analytes, should be sensitive within a wide range of concentrations, and should enable the best possible identification of the determined substances.

In speciation analytics, chromatographic methods are largely used for separation whereas spectroscopic ones are used for detection (5).

The application of hyphenated techniques entails a perfect understanding of analytical methodologies and detailed knowledge of instrumentation. These are expensive systems used in scientific research rather than in routine analyses. The earliest hyphenated techniques were developed by coupling gas chromatography (GC) with various detectors. The following systems were then developed: gas chromatography–atomic absorption spectrometry (GC–AAS), gas chromatography–atomic emission spectrometry (GC–AES), gas chromatography–mass spectrometry (GC–MS), and gas chromatography–inductively coupled plasma–time-of-flight mass spectrometry (GC–ICP-MS-TOF). Because of technological reasons, systems using liquid chromatography (LC) methods for the separation of analyzed substances, such as high performance liquid chromatography (HPLC)–ICP-MS, appeared in the market slightly later.

There are couplings of various LC types among the most popular hyphenated techniques used to determine different ionic forms of metals and metalloids. These include HPLC, ion chromatography (IC), ion-exclusion chromatography, and size-exclusion chromatography (SEC) with ICP-MS or electrospray ionization (ESI) MS. The most popular hyphenated techniques using ion chromatography are IC–ICP-MS and IC-MS (6).

IC–ICP-MS and IC–MS

Ion chromatography is the most popular method used to separate and determine organic and inorganic ionic substances (7). Ion chromatography as a kind of liquid chromatography is primarily applicable to (in terms of the hyphenated techniques) the determination of inorganic ions. Determination and separation of ions (which until recently have been thought of as difficult or even impossible to separate especially in the case of complex matrix samples) has become more effective with the implementation of new highly selective stationary phases in ion-exchange columns and new modes of detection.

Using an IC–MS system allows users to not only obtain information about the quality and quantity composition of the sample, but also to define the structure of analytes and their molar weights. It is necessary to maintain extremely low pressure in the spectrometer, and the separated ions of the analyte leave the chromatographic column under relatively high pressure. Research into such applications started in the 1980s, but it was only recently introduced on a commercial scale.

The ion source is used for transferring the eluent into the spectrometric detector. It consists of the conversion of the liquid eluent containing the analyte into its gas phase under atmospheric pressure. The analyte must be thermally stable and the eluent should vaporize without leaving salts behind. Organic solvents such as methanol and acetonitrile are sometimes added to the eluent to assist its vaporization.

The application of MS detection allows users to obtain information about the qualitative and quantitative content of the sample and also to determine the structure and molar masses of the analytes. The main difficulties in using an MS detector coupled with chromatographic methods result from the fact that it is necessary to maintain very low pressure in the spectrometer while separated analyte ions leave the chromatographic column under comparatively high pressure. Another combination is ion chromatography coupled with negative thermal ionization isotope dilution mass spectrometry (IC–NTI-IDMS) analysis (9) and ion chromatography coupled with electrospray ion tandem mass spectrometry (IC–MS-MS) (10). The application of atmospheric pressure ionization-mass spectrometry (API-MS) coupled with ion chromatography demonstrated performance comparable to that of IC–MS-MS and IC–ICP-MS (11). Compared with IC–API-MS, IC–ICP-MS can tolerate a higher salt concentration in the eluent, which allows for the use of high-capacity columns and larger sample volumes. These capabilities lower the detection limits by one order of magnitude for ICP-MS detection compared to API-MS.

Various sources of ionization may be used in HPLC-MS systems (12), including ESI, atmospheric pressure chemical ionization (APCI), and atmospheric pressure photochemical ionization (APPI). The scope of these applications depends on the polarity and molar mass of analytes, as well as the eluent flow rate. Only the first variant of the above-mentioned ionization types is used in IC–MS systems. ESI is the so-called soft ionization method. In contrast to other ionization methods, it is able to convert multivalent ions into the gas phase. The MS detection can be conducted in two different modes: selected ion monitoring (SIM) or scan mode. In SIM mode, the information on the analyte molar mass is obtained and the method is usually applied for quantitative analyses. In scan mode, the retention time, mass spectra, and mass distribution in-

formation is obtained. This mode is primarily used in qualitative analyses. Identification is relatively simple when it pertains to analytes with low molar mass. The identification problems concerning large molecules are primarily related to the higher number of possibilities in terms of obtaining spectra with the same molar mass-to-charge ratios.

Coupling ion chromatography with ICP-MS is a powerful tool to determine unambiguously different organic and inorganic compounds in a single run. IC–ICP-MS is a suitable technique for complex speciation because the conditions of the mobile phase can be manipulated accordingly to provide optimal separation. Liquid sample introduction is a standard in ICP-MS. Therefore, the simplest form of LC and ICP-MS coupling is the connection of the column outlet with the nebulizer of the sample introduction system via transfer tubing. It should come as no surprise that the hyphenated system that results from the coupling of LC and ICP-MS is the system most often used for speciation analysis related to ICP-MS detection.

Despite its advantages, this hyphenated technique, like other methods, has some drawbacks. For example, one of the major limitations of As or Cr speciation with ICP-MS is the formation of ^{75}As or ^{52}Cr isotopically equivalent species such as $^{40}Ar^{35}Cl^+$ and $^{40}Ar^{12}C^+$ in the plasma, because of the presence of chlorides or carbon in the matrix that interfere with the accurate determination of As at *m/z* 75 or Cr at *m/z* 52. There are two main approaches to address this problem. The first approach is to use ion chromatography to separate interferences, such as chloride, from arsenic before the introduction of the sample into the plasma. It usually can be accomplished during IC by a simple manipulation of the mobile phase. The other approach developed in parallel to the ion chromatography solution is the reduction or elimination of the $^{40}Ar^{35}Cl^+$ and $^{40}Ar^{12}C^+$ interferences after the sample introduction into the plasma by using collision–reaction cell techniques (13). Some instruments use a collision mechanism to dissociate polyatomic interferences, whereas others use gas-phase reaction chemistry to specifically induce dissociation or formation of secondary species that can be rejected by the mass analyzer. The isobaric overlaps are generally not an issue when using a double focusing sector field instrument that offers the higher resolution that may be required for the interference-free determination of sulfur, arsenic, or chromium. However, an increase in the resolution inevitably leads to a dramatic decrease in sensitivity. It should also be noted that the sensitivity of the latest generation of quadrupole instruments is only a factor of 2–3 lower than that of high-resolution ICP-MS operated in low-resolution mode. A good tradeoff between sensitivity, freedom from isobaric interferences, and price is offered by ICP-MS instruments equipped with a collision cell.

Applications of IC–MS and IC–ICP-MS systems include, among other things, the determination of fluoroacetate (14), endothal (14), ascorbic acid (15), perchlorate (16,17), chlorophenols (18), phosphorous oxyanions (19), chelating agents (20), amines (21), polychlorinated biphenyls (22), epichlorohydrin (23), and metal–EDTA complexes (24).

The main applications of ion chromatography in speciation analytics can be divided into three areas:

- Determination of nitrogen (for example, NO_2^-, NO_3^-, and NH_4^+) (25) and sulfur (such as S^{2-}, SO_3^{2-}, SO_4^{2-} $S_2O_3^{2-}$, and SCN^-) (26) ions.
- Determination of inorganic water disinfection by-products (for example, BrO_3^-, ClO_2^-, and ClO_3^-) (27) and other halide ions (such as ClO_4^- and IO_3^-) (28).
- Determination of metals (such as Cr[III] or Cr[VI] and Fe[II] or Fe[III]) and metalloids (for example, As[III] or As[V] and Se[IV] or Se[VI]) (29) ions.

Determination of nitrogen and sulfur ion forms has been performed since the beginning of ion chromatography and is usually carried out with the classic ion chromatograph equipped with an appropriate anion- or cation-exchange column and suppressed conductivity detection. From an environmental and toxicological point of view, the most important ion determinations are halides and metals or metalloids.

Protecting people against health-hazardous microorganisms present in drinking water requires disinfecting the water by various methods. Water chlorination is a well-known and effective technology that has been used for many years; however, it can cause the formation of dangerous by-products such as trihalomethanes. Because of that drawback, there has been a search for other water disinfection methods, among which ozonation has become the most popular one. Even though modern water disinfection methods have their undeniable advantages, they also have certain negative aspects and limitations. These limitations mainly involve the formation of inorganic oxyhalide by-products such as bromate, chlorite, and chlorate.

Bromate can form in raw water containing bromides that are subjected to the ozonation process. The International Agency for Research Cancer (IARC) classified bromate as a potential carcinogen (B2 group), whereas the World Health Organization (WHO) and the United States Environmental Protection Agency (US EPA) initially established 0.8 μg/dm^3 as the bromate level that is safe for human consumption. Because there is no simple analytical method to make the determination of such a low concentration of bromate, the provisional permissible content in drinking water was increased to a level of 25 μg/dm^3. Nowadays, in most highly industrialized countries the permissible bromate content in drinking water is 10 μg/dm^3. The methods to determine bromate, chlorite, and chlorate in water with ion chromatography can be categorized into three groups depending on the detection mode (30):

- Direct methods (conductivity detection);

- Indirect methods (UV–vis detection);
- Hyphenated techniques (MS and ICP-MS detection).

The direct methods rely on the selective BrO_3^- ion separation in the presence of other anions in the sample and its detection with suppressed conductivity detection. These methods are relatively simple and inexpensive but their main flaw is difficulty with the appropriate separation of BrO_3^- and Cl^- ions, whose concentrations in real samples differ significantly. The derivatization methods belong to the indirect methods category and involve converting the determined substance (after its separation in the analytical column) into its derivatives that can be then detected with the UV–vis detector. The third category encompasses the hyphenated techniques such as IC–ICP-MS and IC–MS.

The simultaneous separation and determination of metals and metalloid ions at different oxidation states belongs to another important area of ion chromatography: applications in speciation analytics (31).

Summary

Because of a strong environmental impact, metals or metalloids and selected halide ions determination and speciation have received significant attention in the past few years. Ion chromatography has become one of the main powerful analytical tools for the analysis of complex matrices and speciation studies in that field of analysis.

The hyphenated techniques IC–ICP-MS and IC–MS create completely new and immense possibilities in speciation analysis. The main advantages of those techniques are extremely low detection and quantification limits, insignificant interference influence, and high precision and repeatability of the determinations.

Like all other methods, the hyphenated techniques have their shortcomings. The limitations include issues such as the high price of the apparatus and their complexity, which causes their practical limited availability and usage in laboratories. Using hyphenated techniques requires an in-depth understanding of the analytical methodologies and instrumentation. The systems discussed are expensive, which has limited their use to scientific research rather than routine analyses. Nevertheless, the development of hyphenated techniques is becoming more and more important and the growing number of works concerning this subject seems to corroborate that (32).

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PRODUCT RESOURCES

TOF mass spectrometers

The Xevo G2-S QTof and Xevo G2-S Tof benchtop time-of-flight mass spectrometers from Waters are designed with the company's StepWave ion optics and off-axis ion source technology. According to the company, the systems are as much as 20× more sensitive than earlier generation instruments and critical components stay clean longer.
Waters Corporation,
Milford, MA; www.waters.com

Card extraction LC–MS system

Agilent's automated card extraction LC–MS system is designed as an integrated instrument for the analysis of dried blood spots and other dried media. The system reportedly incorporates the company's 6400 Series triple-quadrupole LC–MS system and is suitable for pharmaceutical and clinical research laboratories. **Agilent Technologies,**
Santa Clara, CA; www.agilent.com

MS instrument control software

Thermo Fisher Scientific's instrument control software for its Q Exactive high performance quadrupole-Orbitrap LC–MS-MS system is designed to allow the instrument to collect MS-MS spectra on ions in a sample by selecting broad mass-to-charge ratio windows and fragmenting all precursors in the window. According to the company, the instrument control software allows users to perform data-independent acquisition and targeted quantification experiments on the same instrument.
Thermo Fisher Scientific, San Jose, CA; www.thermoscientific.com

Mass spectrometers

The EVOQ Qube and EVOQ Elite LC–triple-quadrupole mass spectrometers from Bruker incorporate the company's Advance UHPLC systems. According to the company, both systems have a vacuum-insulated probe heated electrospray ionization source, an atmospheric pressure ionization source, a flat-tuning interlaced quadrupole dual funnel, and software that enables "exception-based data review," which reportedly reduces the error rate for quantitative analysis. **Bruker Daltonics,** Billerica, MA; www.bdal.com

Mass spectrometer

The LCMS-8040 triple-quadrupole mass spectrometer from Shimadzu is designed to provide polarity switching at 15 ms and a high speed-scanning rate of 15,000 u/s. According to the company, the instrument's UFsweeper II collision cell design enables MRM transition speeds of up to 5555 MRM/s.
Shimadzu Scientific Instruments, Inc.,
Columbia, MD; www.ssi.shimadzu.com

High-throughput screening software

LibraryView software from AB Sciex is designed for use in identifying contaminants in food, forensic toxicology samples, and the environment, and can be used for clinical research. According to the company, coupled with its QTRAP and TripleTOF technologies, the software enables acquisition of full-scan MS-MS spectra of compounds or contaminants present in a sample. **AB Sciex,** Framingham, MA; www.absciex.com

LC–MS system

The 6550 iFunnel quadrupole time-of-flight liquid chromatography–mass spectrometry system from Agilent is designed to increase TOF sensitivity to low femtogram levels. According to the company, the system delivers acquisition to 50 spectra/s and up to five orders of intra-scan dynamic range.
Agilent Technologies,
Santa Clara, CA. www.agilent.com

Portable GC–MS system

The Tridion-9 portable GC–toroidal mass spectrometry system from Torion is designed for field GC–MS applications. The system reportedly combines an electronic pressure controlled gas chromatograph with an amplitude scanning toroidal ion trap mass spectrometer. According to the company, the system can be used with SPME syringes for sample introduction. **Torion Technologies Inc.,**
American Fork, UT; www.torion.com

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AD INDEX

The Benefits of Switching from Helium to Hydrogen as a Carrier Gas

John Speranza, Proton OnSite

The most common carrier gas used for gas chromatography (GC) in the US is helium. But due to dwindling supplies, costs are rising. In this note we discuss why chromatographers may choose to switch to hydrogen, a gas easily and inexpensively produced safely through an on-site generator via electrolysis.

Helium has always been a prized resource, sealed in caverns filled with the gas to create the Helium National Reserves. But in 1996, legislation (1) allowed the sale of 0.6 billion ft^3 of gas between 2005 and 2015. The law also set the price of crude helium to approximately $43/1000 scf. In 2010 the Federal Bureau of Land Management reset prices to $64.75/1000 scf, based on the Consumer Price Index (2). This increased to $75.75/1000 scf for FY 2012. Critics argue this price is still too low when assessing remaining reserves of helium (3).

In addition to the economic issues around using helium in GC, there is a question of efficiency. Both helium and hydrogen are very efficient gases to use for GC. But the differences in the properties of the gases will lead to a difference in the efficiency of the separation. Using the van Deemter equation, which predicts an optimum velocity at which there will be the minimum variance per unit column length and, hence, a maximum efficiency (4), one can deduce that linear flow rate of hydrogen can be greater than that of helium and offer equal efficiency in the gas's ability to separate peaks.

Experiment

This application note is a comparison of similar-sized columns; one of helium and one of hydrogen is presented (5). Spearmint oil is separated using both a helium carrier and a hydrogen carrier (Table I).

Results

Using hydrogen as a carrier gas in conjunction with the high efficiency column resulted in an overall speed gain of 61% compared to the original method (Figure 1).

Conclusions

Hydrogen can be produced safely on-site compared to helium, which occurs rarely and is a finite resource. It should be noted that the use of stored, cooled, or pressurized hydrogen can bring with it fire and explosion (6).

Also, using the van Deemter curve, it is apparent that using hydrogen as a carrier gas instead of helium will allow an increased flow rate without effecting the efficiency of the separation, thus increasing the throughput of a laboratory.

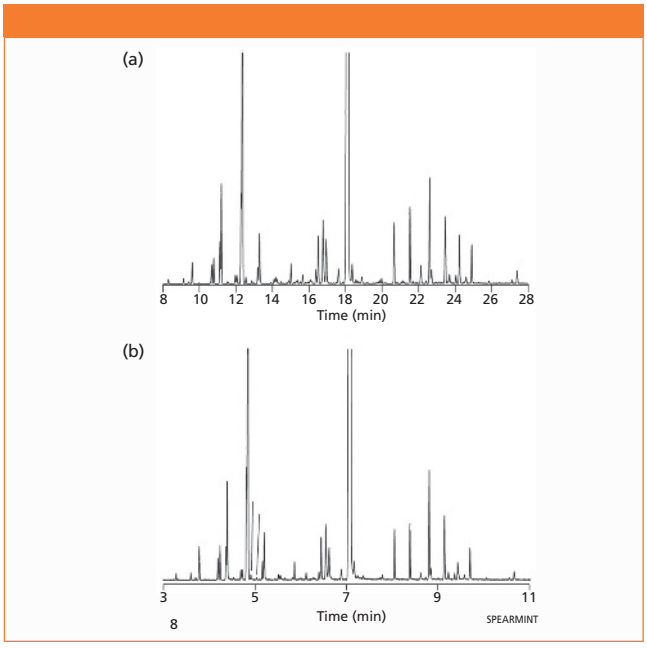

Figure 1: The separation of spearmint oil, obtained using the conditions outlined in Table I. The first separation, (a), used a helium carrier gas and obtained results between 8 and 28 min to separate. The second separation, (b), used hydrogen carrier gas and obtained comparable resolutions between 3 and 11 min.

Table I: GC column and flow details

	Column A - helium	Column B - hydrogen
Column	Agilent DB-1,122-1032	Agilent DB-1, 121-1022
Column dimensions	30 m × 0.25 mm, 0.25 µm	20 m × 0.18 mm, 0.18 µm
Flow rate	25 cm/s measured at 40 °C	47 cm/s measured at 40 °C
Oven	40 °C hold 1 min, 5 °C/min to 290 °C	40 °C hold 0.38 min, 13 °C/min to 290 °C hold 3.09 min
Injection	250 °C, Split 40:1, 1 µL injection	250 °C, Split 40:1, 1 µL injection

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Comparing Single Quadrupole with Triple Quadrupole GC–MS-Based Metabolomics

Rob Trengove, Separation Science & Metabolomics Laboratory, Metabolomics Australia, Murdoch University, Murdoch, Western Australia

The emerging field of metabolomics is defined as the nontargeted detection and quantification of small molecule metabolites that are found in biological materials. With the advantage of noninvasive techniques, metabolomics is finding a growing number of applications in: chronic disease, environmental exposure, functional genomics, neonatal screening, nutrition, sports science, toxicology, and transplantation to monitor the health of the transplant organ.

To identify and quantify the vast range of chemically diverse primary and secondary metabolites, any analytical approach must encompass reliable sampling, precise detection methods, and advanced data handling and interpretation capabilities. With two recognized approaches to metabolomics, the first is referred to as "footprinting" (1) and is used when profiling metabolites within the environmental medium into which they were secreted by cells. Alternatively, a more untargeted method to metabolomics would be to analyze all metabolites present in a more blanketed approach much simpler and required less frequently, resulting in a more robust system for long operation.

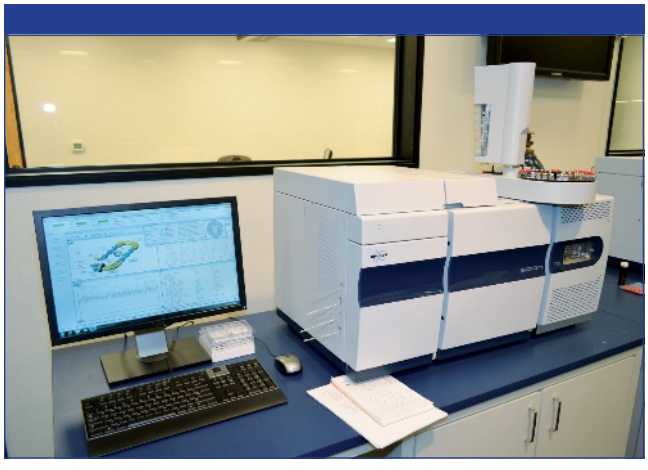

Figure 1: Bruker's SCION GC–MS TQ platform with PC illustrating the compound-based scanning (CBS) software.

Transitioning Single Quadrupole (SQ) to Triple Quadrupole (TQ) GC–MS

While metabolomics studies can be carried out using single quadrupole (SQ) GC–MS instruments, these rely heavily on mass spectra deconvolution and some derivatization for polar compounds such as amino acids, organic acids, and simple sugars (2). The application of single quadrupole GC–MS requires chromatographic temperature ramps and spectral scan rate to be matched in order to maximize the number of compounds that can be routinely identified for metabolite profiling. The scan rate becomes a limiting factor in metabolite profiling and the ability to effectively deconvolute data sets is critical.

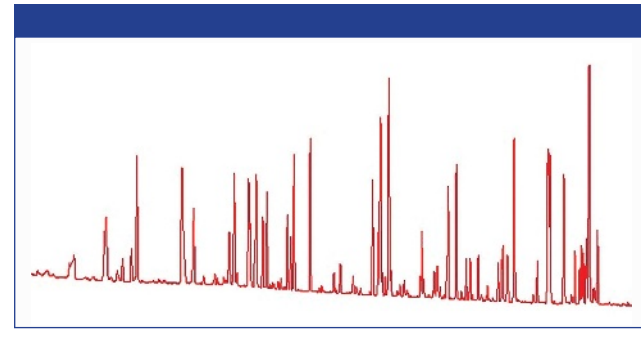

Figure 2: Full scan mass spectrometry of 43 components QA/QC mix (TMS).

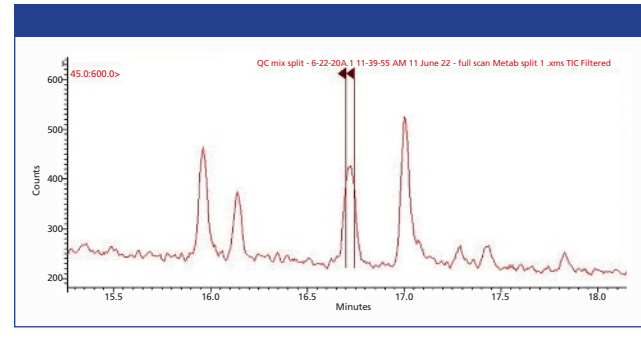

Figure 3: Upper: Full scan mass spectrometry of 43 components QC mix split (TIC filtered) with coelution at one peak.

To lessen the load of quantitative metabolomics, triple quadrupole (TQ) GC–MS has been utilized to excellent effect. The SCION™ TQ hardware (Figure 1) provides higher throughput and faster scan speeds for more efficient sampling runs, greater sensitivity, and zero cross talk and improved signal-to-noise ratio (S/N). Final outcomes include the ability to process more samples, resolve more compounds, and improve identification of compounds.

Experimental

The transition from single quad to triple quad can be illustrated with the use of GC–MS-MS with a standard 43 component mixture used for a QA/QC mix with derivatization (2).

Figure 2 illustrates a typical full scan with a significant peak (3). Following close examination of the peak, total ion chromatogram coelution of proline and leucine was identified. The need for deconvolution to resolve these compounds was necessary (see Figure 3).

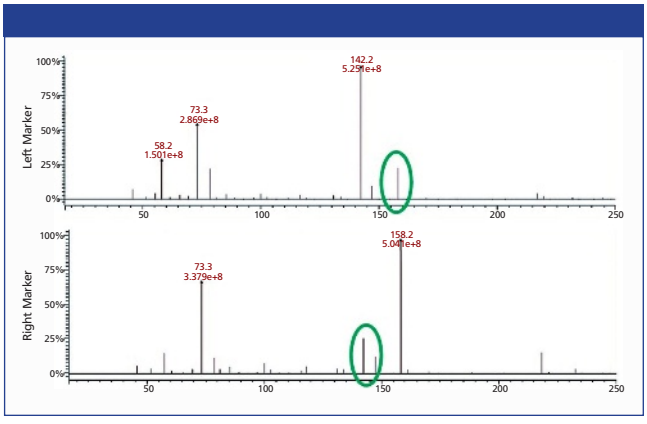

Figure 4: Deconvolution of proline and leucine by compound-based scanning (CBS) software.

Comparative Results: SQ vs TQ

When investigating metabolomic samples there are several hundreds of compounds and several may coelute. These compounds can be very similar in structure, such as sugars and deconvolution may not resolve them. Expanding the capabilities of the GC–MS-MS system is ideal with an instrument that undertakes data dependent MS and combines it with a series of scheduled multiple reaction monitoring (MRM) transitions. This extended system can be set-up to reference known coelution and accurate retention times of compounds.

In Figure 4 the mass spectra of the left and right markers are quite similar and dependent on the speed at which data can be collected. Deconvolution may not resolve the two compounds in the peak, but if analysis by MS-MS is undertaken (Figure 5), the baseline is cleaned up significantly improving S/N, and the limits of detection (LOD) and quantification are greatly improved. It then becomes obvious that two peaks exist at this retention time (RT), whereas in full scan it was only a single peak with coelution. Using the triple quad system, it only takes one transition to resolve the compounds in this instance.

Conclusion

A triple quadrupole operating in MRM mode with compound-based scanning (CBS) software provides enhanced duty-cycle coverage so that resolution of co-eluted compounds is possible in the SCION GC–MS TQ. It also provides a faster scan rate with lower LOD and this will lead to improved metabolome coverage with routine metabolite profiling using built-in libraries.

The TQ can also deliver precursor ion scans, product ion scans, neutral loss scans, and MRM to assist in the identification of unknowns as well as quantitatively detect metabolites in really complex mixtures at very low levels.

Chemical ionization (CI) in combination with MRM mode provides high throughput metabolomics capability, where as full scan struggles to resolve compounds. In addition, when using CI, an improved transmission is possible. With the right combination of reagent gases, compound identification is further improved, more compounds are resolved, and S/N is substantially better. CI

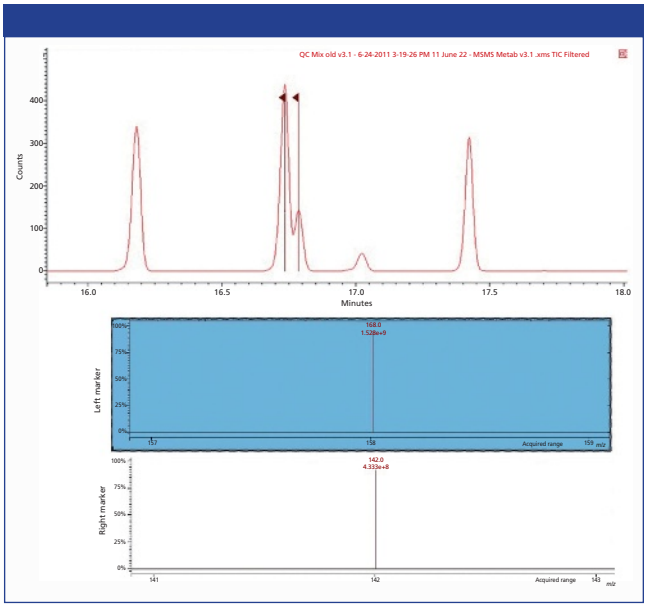

Figure 5: GC–MS-MS TQ of 43 components QA/QC mix illustrating two peaks for coeluted compounds proline and leucine.

also provides higher mass to charge (*m/z*) species of precursor ions for MS-MS with far less interference from the matrix. TQ MS that combines CI and MRM offers the potential to increase metabolome coverage and identification and provides both the LODs and improved precision required to do so.

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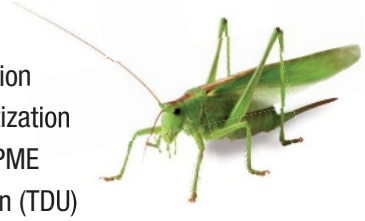

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