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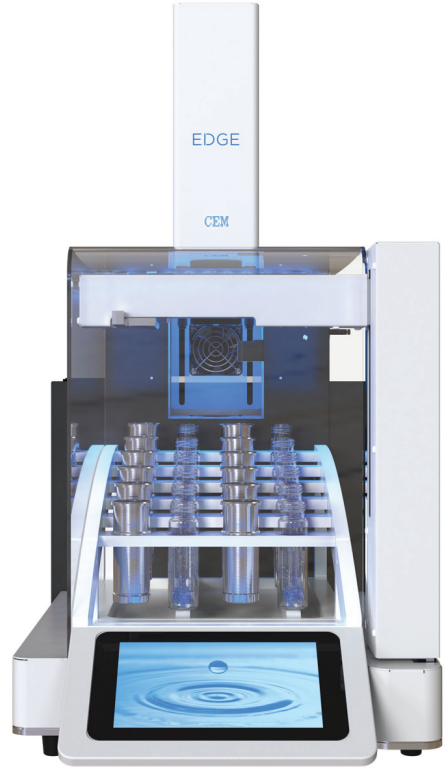

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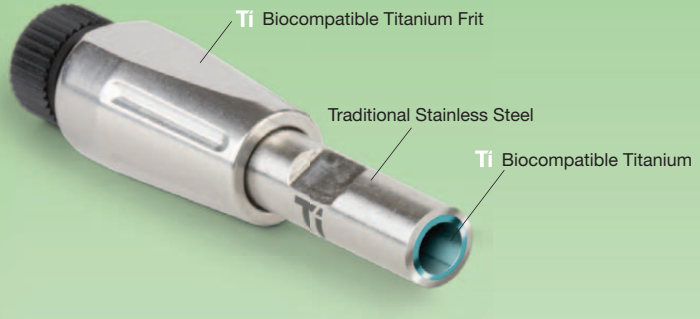

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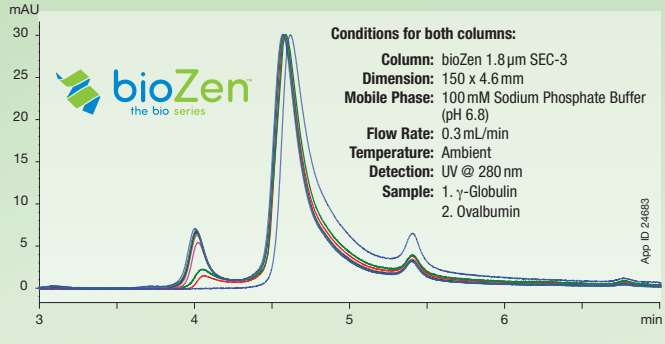

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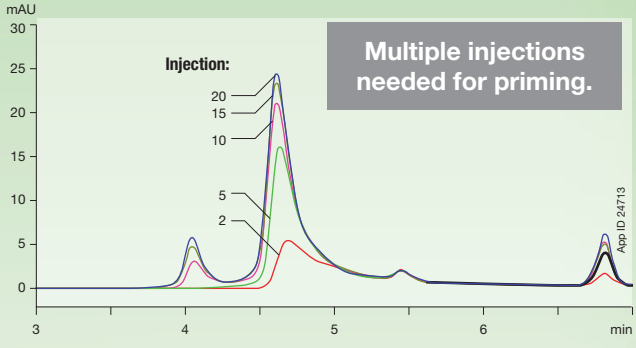

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LCGC North America (ISSN 1527-5949 print) (ISSN 1939-1889 digital) is published monthly by UBM LLC, 131 West First Street, Duluth, MN 55802-2065, and is distributed free of charge to users and specifiers of chromatographic equipment in the United States and Canada. Single copies (prepaid only, including postage and handling): $15.50 in the United States, $17.50 in all other countries; back issues: $23 in the United States, $27 in all other countries. LCGC is available on a paid subscription basis to nonqualified readers in the United States and its possessions at the rate of: 1 year (13 issues), $74.95; 2 years (26 issues), $134.50; in Canada and Mexico: 1 year (13 issues), $95; 2 years (26 issues), $150; in all other countries: 1 year (13 issues), $140; 2 years (26 issues), $250. Periodicals postage paid at Duluth, MN 55806 and at additional mailing offices. POSTMASTER: Please send address changes to LCGC, P.O. Box 6168, Duluth, MN 55806-6168. PUBLICATIONS MAIL AGREEMENT NO. 40612608, Return Undeliverable Canadian Addresses to: IMEX Global Solutions, P. O. Box 25542, London, ON N6C 6B2, CANADA Canadian GST number: R-124213133RT001. Printed in the USA.

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PEAKS of Interest

New Teaching and Research Center for Separation Science is Launched

Agilent Technologies (Santa Clara, California) and the University of Duisburg-Essen (Germany) will collaborate to pair the company's analytical technologies with the university's researchers. As part of the collaboration, the company will support the university with a broad range of instruments to equip the new Teaching and Research Center for Separation (TRC).

The focus of the TRC will be teaching students, industry employees, technicians, managers, graduates, and postdoctoral researchers about separation science, and training them in the use of modern analytical equipment. The TRC will support an extensive cross section of research activities in biomedicine, nanotechnology, and other life science specialties.

The company has developed a global network of world-class Centers of Excellence; the University of Duisburg-Essen is the fifth university to join this network.

For more information about the TRC, please visit: www.trc-separation.com/home-en.

Calls for Nominations for 2019 CACA Young Investigator and Student Excellence Awards

The Chinese American Chromatography Association (CACA) invites nominations for the 2019 CACA Young Investigator Award and the 2019 Student Excellence Awards, to be presented at Pittcon 2019.

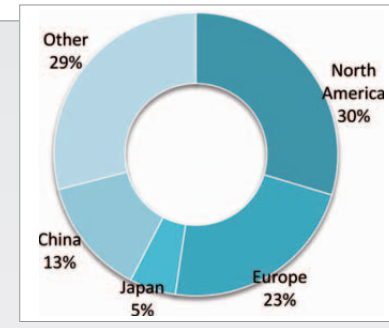
Both awards recognize outstanding contributions to the development of separation science and its applications, especially in the field of chromatography. The 2019 CACA Young Investigator Award, sponsored by Advanced Materials Technology, is open to all CACA members who are within 10 years of receiving their highest degree at the time of the award session. The CACA Student Excellent Awards, sponsored by Mac-Mod Analytical, are open to all CACA members who are graduate students at the time of the award session. Two student awards are planned at this time. Candidates may join CACA for free before applying for the awards.

Applicants are judged on the originality and overall quality of research, significance to the advancement of separation science, and other supporting factors.

For more information about the awards, please visit the CACA website at http://www.ca-ca.org. ■

CHROMATOGRAPHY MARKET PROFILE

Process chromatography



Demand by region for process chromatography in 2017.

Process chromatography, in particular process gas chromatography (GC), is a well-established technique within the oil and gas, refining, and petrochemical industry. The technique is generally used in process plants to determine product quality and yield. Like most process analytical instruments, its primary role is to monitor conditions at critical points in the overall process. The investment for process GC instruments is typically justified by inherently providing data that would lead to an increase in a product's yield.

There are three distinct categories of process GC systems. Process GC instruments are traditional GC instruments for rigorous process applications. Process GC instruments also include special-purpose (often rack-mounted) GC instruments used for selected applications such as monitoring volatile organics, or VOCs, in environmental monitoring and occupational safety and health. British thermal unit (BTU) systems are dedicated process GC instruments used to measure BTU or calorific value of natural gas. The final category is process liquid chromatography (LC), which includes high-pressure liquid chromatography (HPLC) systems and ion chromatography (IC) instruments used for process analytics.

With the trend toward digital solutions and the Internet of Things (IoT), plants are learning more about their processes and instruments that are increasing overall performance and reducing operating costs. New plants are installing modern process GC instruments that do more than just increase product's yield; these instruments also can play a role in improving the overall plant operation by helping to preventing unscheduled downtimes, enabling remote monitoring, and providing system diagnostics.

The total demand for process chromatographs was measured at more than $200 million in 2017, including instruments, parts and consumables, and service. Service continues to play a significant role in the market and is expected to outpace instrument sales. North America and Europe account for about half of the market, fueled by robust demand for BTU analyzers. North American and (Western) European markets will be characterized by ongoing replacement and upgrade sales to change outdated units in the installed base that do not include the most current communication and diagnostic functions, or that are obsolete and no longer supported by the supplier. Leading suppliers of process chromatographs include ABB, Emerson, Siemens, and Yokogawa.

Market size and growth estimates were adopted from *TDA's Industry Data,* a database of technology market profiles and benchmarks, as well as the *2018 Instrument Industry Outlook* report from independent market research firm Top-Down Analytics. For more information, contact Glenn Cudiamat, general manager, at (888) 953-5655 or glenn.cudiamat@tdaresearch.com. Glenn is a market research expert who has been covering the analytical instrumentation industry for more than two decades.

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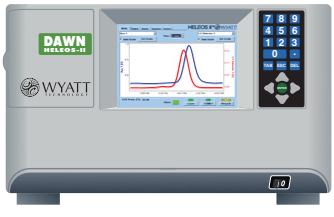

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COLUMN WATCH

LC–MS Sensitivity: Practical Strategies to Boost Your Signal and Lower Your Noise

Liquid chromatography–mass spectrometry (LC–MS) has become the preferred analytical technique for many challenging assays based on its selectivity, sensitivity, and broad applicability to compounds of varying polarity. Despite the advantages of the technique, the complexity of LC–MS systems often leaves analysts struggling to meet method detection limits. In this installment of "Column Watch," several strategies are discussed to improve method sensitivity through the reduction of contaminants, the careful selection of LC method conditions, and the optimization of MS interface settings. By understanding the relationship between these parameters and ionization efficiency, analysts can enhance their signal-to-noise ratio and realize the hidden potential of the LC–MS technique.

Sharon Lupo

In mass spectrometry (MS), the term *sensitivity* can have several meanings that are often used interchangeably. *Sensitivity* may be defined as the change in signal per unit change in concentration of an analyte (such as the slope of the calibration curve) (1). More commonly, it is used to reference the magnitude of the signal produced by the analyte in the MS detector. In this latter usage, MS sensitivity is often used to compare detectors.

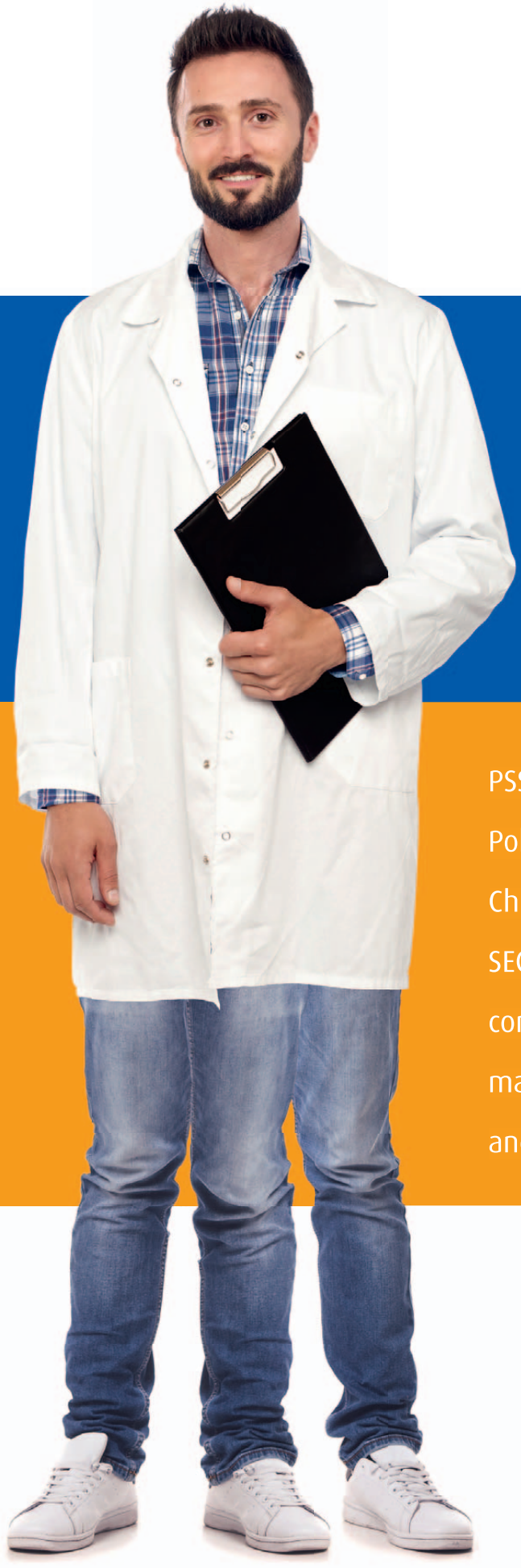
Fundamentally, the ability of a detector to provide quantitative data is a function of the signal-to-noise ratio (S/N) for an analyte. The limit of detection (LOD) is determined from the analyte S/N and is the lowest concentration of a substance where its signal can be distinguished from system noise (2). As shown in Figure 1, the higher the sensitivity of the MS, the greater the value of S/N for a given method LOD if background noise remains constant. Therefore, improvements in sensitivity can occur through manipulation of S/N. MS optimization, sample pretreatment strategies, mobile-phase composition, and LC column characteristics are all integral to ionization efficiency and will improve analyte signal when optimized. Likewise, limiting contaminants that contribute to signal suppression or adduct formation may also enhance response.

MS Optimization

In liquid chromatography–mass spectrometry (LC–MS), sensitivity directly relates to the effectiveness of producing gas-phase ions from analytes in solution (ionization efficiency) and the ability to transfer them from atmospheric pressure to the low pressure zone of the MS system (transmission efficiency) (3). The optimization of ionization and transmission efficiency is dependent on the LC method parameters and the target analyte or analytes. To make the appropriate adjustments, it is necessary to have a basic understanding of the mechanisms taking place within the MS source.

Electrospray ionization (ESI) is one of the most popular ionization techniques; therefore, it will be the focus of this column installment. It is important to note, however, that optimization of the source parameters is necessary regardless of the ionization mode selected. As the LC mobile phase flows into the sample capillary, positive and negative ions are separated based on the polarity chosen. In positive ESI, the negative ions are neutralized on the capillary wall, and the positive ions continue with the mobile phase to the capillary tip where the charged analytes accumulate into a droplet. Under the influence of an applied voltage, a Taylor cone is formed (4). Electrostatic repulsion causes the cone to break up into small, electrically charged droplets, which then travel toward the sampling orifice under the guidance of the applied potential difference between the capillary tip and the sampling plate. As the tiny droplets progress toward the orifice, the solvent evaporates with the aid of drying gas and heat, causing the droplet surface area to decrease and an increase in charge density. Ultimately, repulsive forces overcome the droplet surface tension and the droplet explodes into even smaller droplets. The process repeats itself until the droplets are so small that gas-phase ions are emitted (5). The cloud of ions formed is known as the *ion plume*.

Choosing the appropriate polarity is the first step in developing a sensitive LC–MS method. The capillary polarity is selected to match the charge of the ana-



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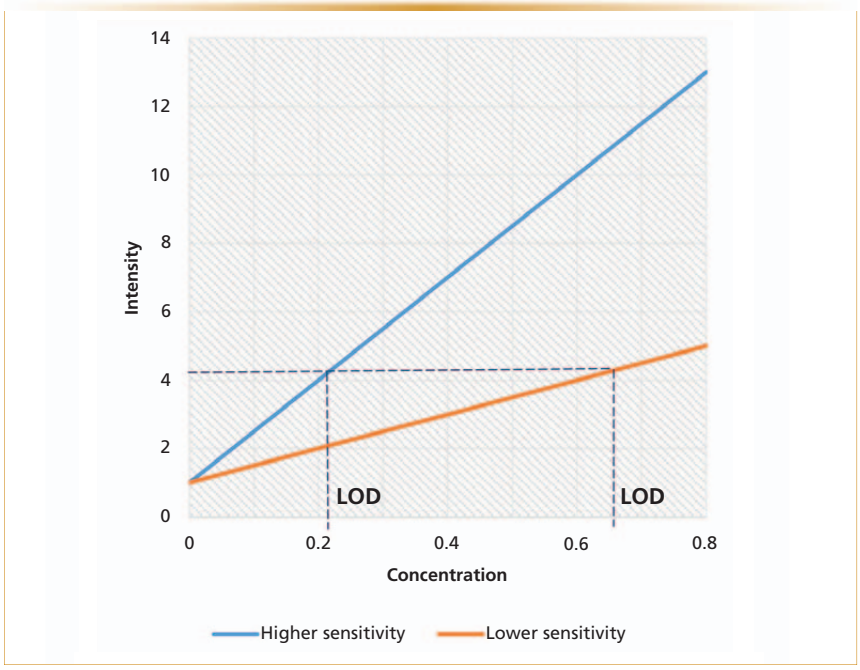

FIGURE 1: A hypothetical demonstration of the effect of increased sensitivity on limits of detection (LOD) assuming linear calibration and fixed background noise.

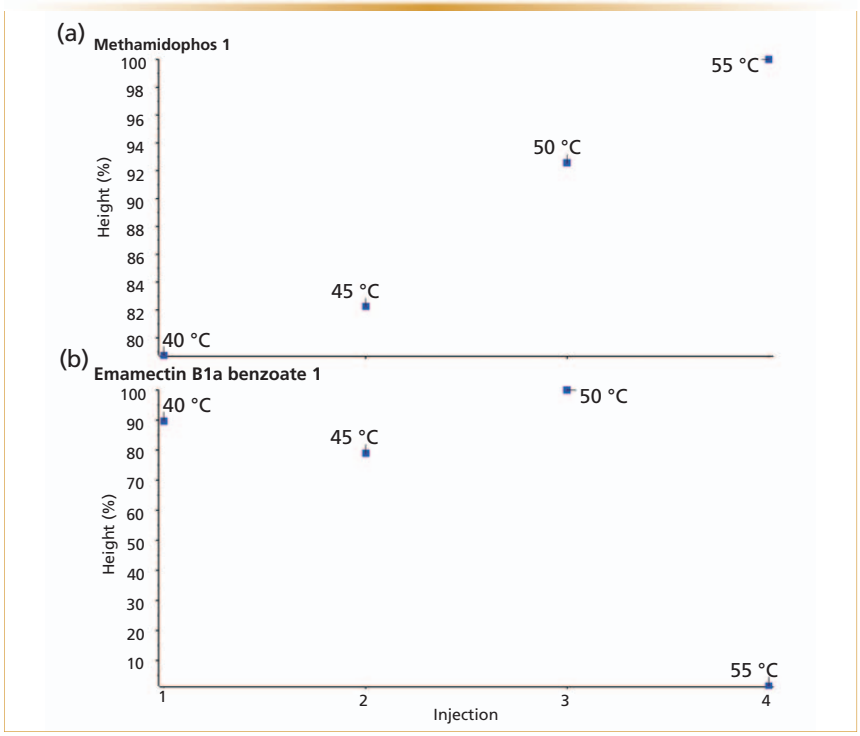

FIGURE 2: LC–MS/MS optimization of desolvation temperature for (a) methamidophos and (b) emamectin B1a benzoate over four successive injections. Column: 100 mm x 2.1 mm, 3-µm fully porous C18; mobile-phase A: water + 2 mM ammonium acetate + 0.1% formic acid; mobile-phase B: methanol + 2 mM ammonium acetate + 0.1% formic acid; gradient %B (time): 5% (0 min), 5% (1.5 min), 70% (6 min), 70% (9 min), 100% (10 min), 100% (12 min), equilibrate; flow rate: 0.5 mL/min; polarity: ESI+; curtain gas: 30 psi; nebulizer gas: 45 psi; drying gas: 55 psi; capillary voltage: 5.5 kV; collision gas: 10 psi.

lytes of interest. Typically, basic analytes will ionize most efficiently in positive ion mode by accepting a proton $(M+H)^+$, while acidic analytes will produce the strongest signal in negative ion mode by donating a proton $(M-H)^-$. However, it can be difficult to predict the best polarity mode for more-complex molecules. In addition, analyte behavior and response varies by instrument platform. Therefore, it is beneficial to screen analytes using both polarity modes during initial method development or when transferring an existing method to a new instrument (6).

Ionization efficiency is strongly influenced by flow rate, mobile-phase composition, and the physicochemical properties of the target analytes. The capillary voltage setting is dependent on the analytes, eluent, and flow rate and can have a significant impact on method reproducibility. The applied potential difference between the capillary tip and sampling plate is responsible for maintaining a stable and reproducible spray (7). Problems with variable ionization and precision can arise if the capillary voltage is set incorrectly. Optimal nebulizing gas flow and temperature are also eluent dependent. The nebulizing gas constrains the growth of the droplet, while charge accumulates and also affects the size of the droplets emitted from the capillary. The nebulizing gas flow and temperature should be increased for faster LC flow rates or when using highly aqueous mobile phases. Similarly, drying gas flow and temperature can be critical for effective desolvation of the LC eluent and the successful production of gas-phase ions. As a caution, when analyzing thermally labile analytes, care must be exercised to prevent their degradation in the source.

The location at which gas-phase ions are produced within the ionization source is important for optimal transmission into the MS system. The size of the ion plume is dependent on the number of fission events required to emit gas-phase ions and its distance from the sampling orifice. Sampling of the ion plume can be optimized by adjusting the position of the capillary tip in relation to the orifice based

on the LC flow rate. At faster flow rates, the capillary tip should be placed further from the sampling orifice to allow for adequate desolvation and an increased number of fission events. Although extending the distance will allow for an increased number of gas-phase ions to be produced, repulsive forces will also increase proportionally, causing the size of the ion plume to expand and the density of gas-phase ions to be reduced. As a result, the number of ions entering the sampling orifice could decrease, causing a drop in signal intensity (3). At slower flow rates, smaller droplets are formed, allowing the capillary tip to be placed closer to the sampling orifice. Smaller droplets desolvate more easily and require fewer fission events, reducing the impact of repulsive forces and inhibiting the size of the ion plume. The decreased distance between the capillary tip and sampling orifice increases ion plume density and improves analyte ionization efficiency and transmission (3).

Optimization of the ionization source parameters described above could potentially bring sensitivity gains of two- to threefold, as demonstrated by Szerkus and colleagues for the analysis of 7-methyl guanine and glucuronic acid in urine (8). When optimizing the source conditions, it is important to use the intended LC mobile phase and flow rate. One method of optimization is to inject a standard solution several times, and alter a specific source parameter stepwise with each injection. Figure 2 demonstrates this process for the evaluation of optimal desolvation temperature for two pesticides: methamidophos and emamectin B1a benzoate. A 20% increase in response for methamidophos was achieved by increasing the desolvation temperature from 400 °C to 550 °C. In contrast, emamectin benzoate B1a experiences complete signal loss if the desolvation temperature is increased beyond 500 °C because of the thermal lability of that compound. Alternatively, source conditions may be optimized by teeing a constant flow of analyte into the LC eluent and monitoring the analyte TIC. This technique allows for adjustments to be made on the fly. Methods using gradient elution of multiple compounds should be optimized by estimating the organic concentration at the time of elution. Although this step can be overwhelming, the process can be simplified by concentrating efforts on only critical or low intensity analytes.

Sample Pretreatment

Sample pretreatment is an essential part of the LC–MS analytical workflow, particularly when analyzing complex samples containing target analytes at low concentrations. Removal of non-target sample components can minimize matrix interferences and improve the S/N ratio for the analytes of interest. Matrix compounds coeluted with a target analyte may cause suppression or enhancement of the ana-

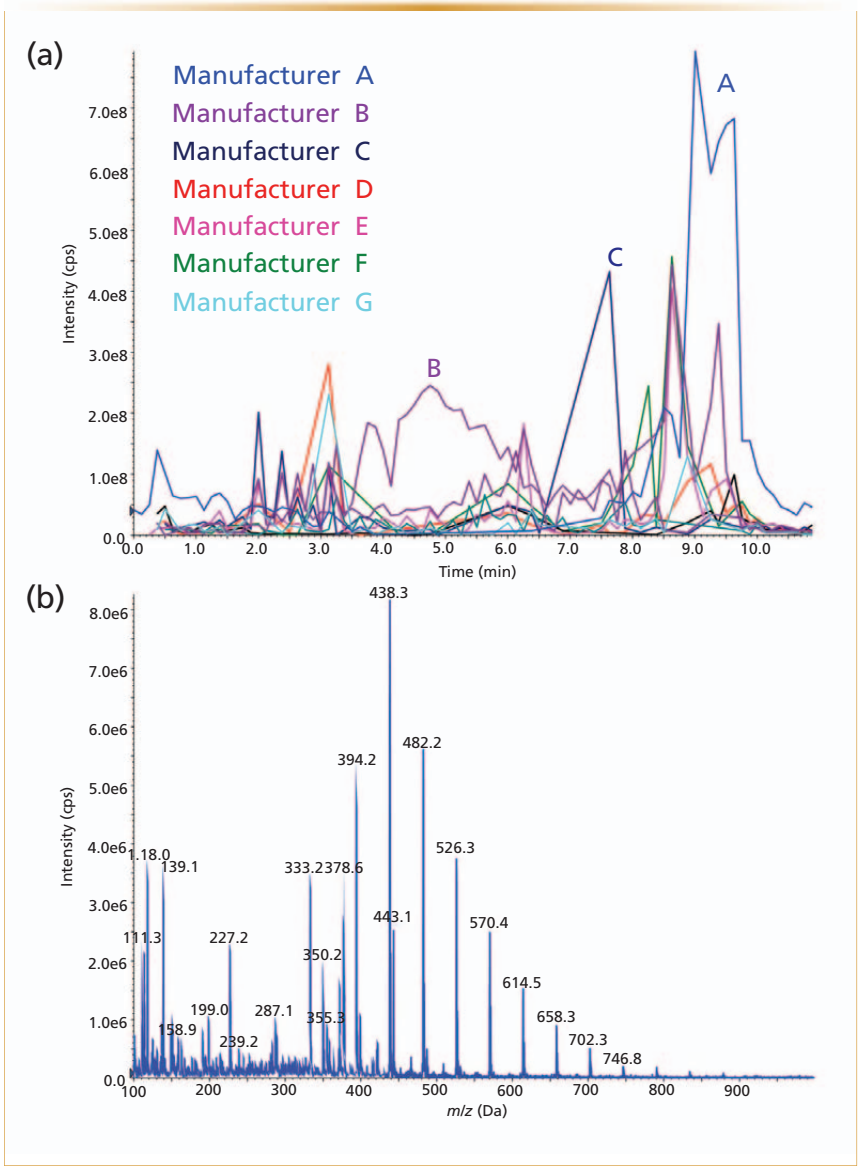

FIGURE 3: Contaminants extracted with acetonitrile from polymeric solid-phase extraction reversed-phase 96-well plates and analyzed by LC–MS/MS: (a) Overlay of background subtracted TIC from seven manufacturers. (b) Averaged spectra collected from peak C located at 6.5–8 min. Column: 100 mm x 2.1 mm, 2.7-µm superficially porous C18; mobile-phase A: water + 1 mM ammonium acetate + 1% acetic acid; mobile-phase B: methanol; gradient %B (time): 5% (0 min), 100% (8 min), 100% (9 min), equilibrate; flow rate: 0.5 mL/min. (Methodology developed by Hua and Jenke, reference 10.).

lyte signal; these interferences are known as *matrix effects*. Matrix effects often manifest as a loss in MS sensitivity or specificity, and are prevalent in ESI because of the potential for charge competition on the droplet surface prior to emitting gas-phase ions. As an alternative, atmospheric pressure chemical ionization (APCI) may be employed if the analytes of interest are thermally stable and of moderate polarity (1). In APCI, the LC eluent is completely evaporated into a gas before ionization by the applied voltage of the corona needle. The ionized mobile-phase vapor then reacts with the analyte molecules to produce charged ions. Matrix effects tend to be less extensive in APCI, since ions are produced through gas-phase reactions instead of liquid-phase reactions (9).

Various sample preparation strategies are available to extract target analytes from potential interfering matrix components. The appropriate technique is dependent on the sample matrix, sample volume, target analyte concentration, and analyte physicochemical properties. If the sample is clean and known to contain high concentrations of the target analyte, simple filtration and dilution is a quick and convenient way to reduce the concentration of potential interferences. On the other hand, complex samples known to contain low target analyte concentrations will require a more rigorous extraction procedure to improve signal intensity. Although more stringent sample preparation procedures may not be desirable because of the cost and time investment required, injecting cleaner samples will decrease the likelihood of matrix effects from endogenous interferences while simultaneously increasing analyte response and instrument reproducibility.

Regardless of the sample preparation technique chosen, it is important to consider that matrix effects can result from the presence of endogenous or exogenous substances. Whereas endogenous constituents are already present in the sample (proteins, lipids, pigments, and so forth), exogenous compounds are introduced into the sample during the sample pretreatment process. These compounds can leach from plastics used in centrifuge tubes, well plates, and pipette tips, and may include by-products and residues from the manufacturing processes (for example, molding agents, plasticizers, stabilizers, and releasing agents). The amount and type of contaminants varies from manufacturer to manufacturer, as shown in Figure 3a. In this experiment, contaminants extracted from polymeric solid-phase extraction (SPE) reversed-phase 96-well plates were compared for seven manufacturers. The extracts were analyzed by LC–MS, and the resulting data were background subtracted to remove contributions from the solvent and the analytical column. An overlay of the resulting chromatograms shows the presence of multiple chemical contaminants between the various manufacturers. The spectra for polyethylene glycol (PEG)

was clearly identified in manufacturer C based on the series of repeating ions separated by 44 Da (Figure 3b).

Other sources of exogenous chemicals include glassware (especially when cleaned with detergents), the use of non-MS-grade solvents and additives, or careless work practices that can introduce chemicals from the skin or surrounding environment. Without appropriate laboratory procedures and careful screening of sample pretreatment products, it is possible to inadvertently introduce contaminants into a sample. If samples are subjected to a concentration step, a decrease in S/N ratio may be observed, because both analytes and contaminants will be concentrated (9).

Mobile-Phase Composition

The mobile phase plays a key role in LC–MS sensitivity by influencing the retention and ionization of target analytes. The use of high-purity solvents and additives is of utmost importance to prevent unwanted adduct formation and increased MS background. Similarly, only ultrapure water from a water purification system or bottled water suitable for LC–MS should be used for mobile-phase preparation. LC–MS spectra collected for MS-grade and HPLC-grade methanol showed significantly increased impurities in the HPLC-grade methanol, particularly in the low-molecular-weight ranges common for small-molecule analysis (Figure 4). It is apparent from this data how the use of lower grade solvents could contribute to reduced sensitivity and convoluted spectra, making accurate quantitation or spectra interpretation difficult. Mobile phases should be stored in borosilicate glass containers and "topping off" solvents should be avoided to prevent the accumulation of contaminants.

The incorporation of volatile buffers and acids into the mobile phase enables control over the ionization state of the target analytes so that retention can be manipulated. Analyte retention affords the LC–MS analyst several advantages. First, increased retention of analytes means a higher organic solvent concentration is required to elute the analyte from the column during gradient LC. It has been shown that droplets with a higher organic concentration are desolvated more efficiently in the MS source, leading to improved MS sensitivity (11). Second, greater chromatographic selectivity makes it possible to avoid coeluted matrix effects that can be detrimental to analyte response. Areas of retention and matrix suppression can be monitored chromatographically by simultaneously infusing the analyte post column while performing an LC injection of an extracted blank matrix sample through the analytical column (12). Areas of matrix suppression are characterized by a decrease in analyte signal. In this way, analyte retention can be adjusted to

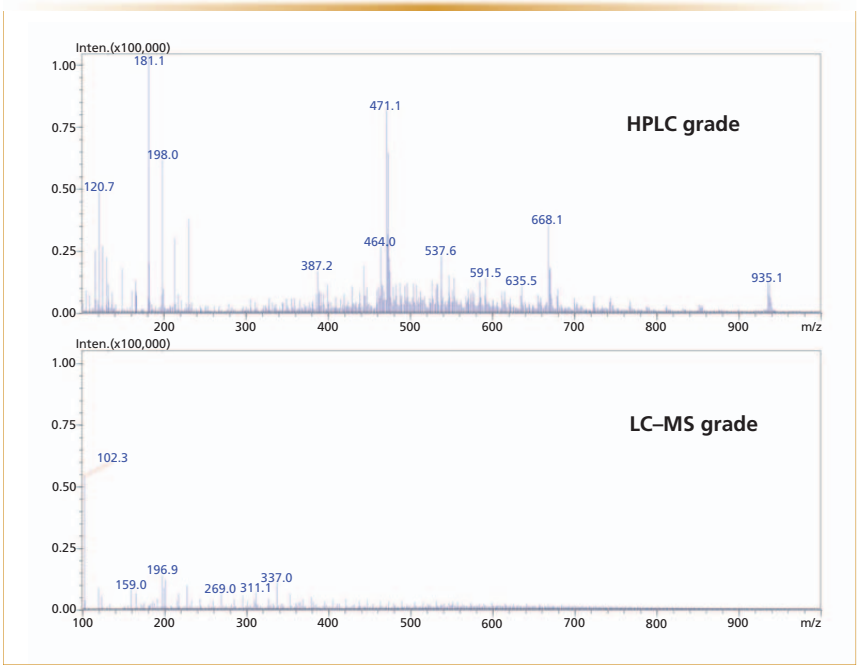

FIGURE 4: Comparison of averaged spectra for HPLC-grade methanol and LC–MS-grade methanol. Mobile phase: unmodified methanol as indicated; flow rate: 0.5 mL/min; system: LC–MS with ESI+ ionization; scan range: 100–2000 *m/z*.

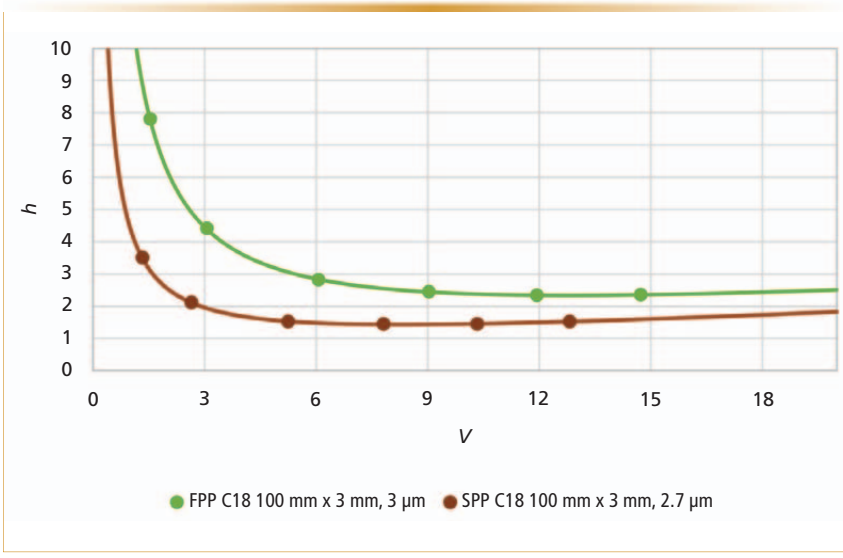

FIGURE 5: Van Deemter plot comparing efficiency between a 3-µm fully porous C18 column and a 2.7-µm superficially porous C18 column. Mobile-phase A: 45% water; mobile-phase B: 55% acetonitrile; detection: photodiode array, 254 nm; injection volume: 1 µL; sample: 0.03 mg/mL biphenyl prepared in 25:75 acetonitrile–water.

avoid zones of significant suppression in the chromatogram.

Mobile-phase buffers and acids also affect ionization efficiency. This statement is especially true for ESI because it is susceptible to the reduction of detector response because of competition for ionization. To reduce the likelihood of buffer-induced suppression, concentrations should generally be kept to a minimum. Alternatively, mobile phases containing formic acid can minimize unwanted metal adducts. The excess in protons provided by the acid drives the majority of ion formation to the protonated molecule $[M+H]^+$, resulting in an overall improvement in response since it would no longer be distributed across multiple charged species (13).

Enhancements in ionization efficiency have been observed by donating protons in the case of an acid modifier in positive-ion mode or by accepting protons in the case of a basic modifier in negative-ion mode. The latter was demonstrated for the negative ionization of two neutral estrogens, estrone and estriol, where their response triples when they are prepared in diluent containing 0.2% ammonium hydroxide compared to one that contains 0.2% acetic acid (14). Buffer salts containing ammonia (for example, ammonium formate or ammonium acetate) can increase the ionization efficiency of polar neutral compounds that cannot be ionized on their own by forming ammonium adducts. Ammonium salts can be used to prevent the formation of unwanted adducts by providing a constant supply of ammonium. For example, the LC–MS analysis of two cardiac glycosides, digoxin and digitoxin, is performed almost exclusively with ammonium formate modified mobile phases. Without ammonium formate, these compounds tend to form sodium adducts, which are difficult to fragment when analyzed by tandem MS (15).

LC Column Characteristics

The desire for increased LC–MS sensitivity has trended towards the implementation of highly efficient LC columns using smaller particles (sub-2 µm) in combination with reduced column diameters (≤ 2.1 mm). The introduction of superficially porous particles (SPPs) has allowed for increased efficiency while reducing system pressure when compared to fully porous particles (FPPs). High efficiency columns theoretically translate to improved sensitivity; however, LC–MS system extracolumn volume, ionization efficiency, and data sampling rates must be considered to fully realize the benefits.

The ability of a column to provide narrow chromatographic peaks is characterized as its efficiency (*N*), and is defined

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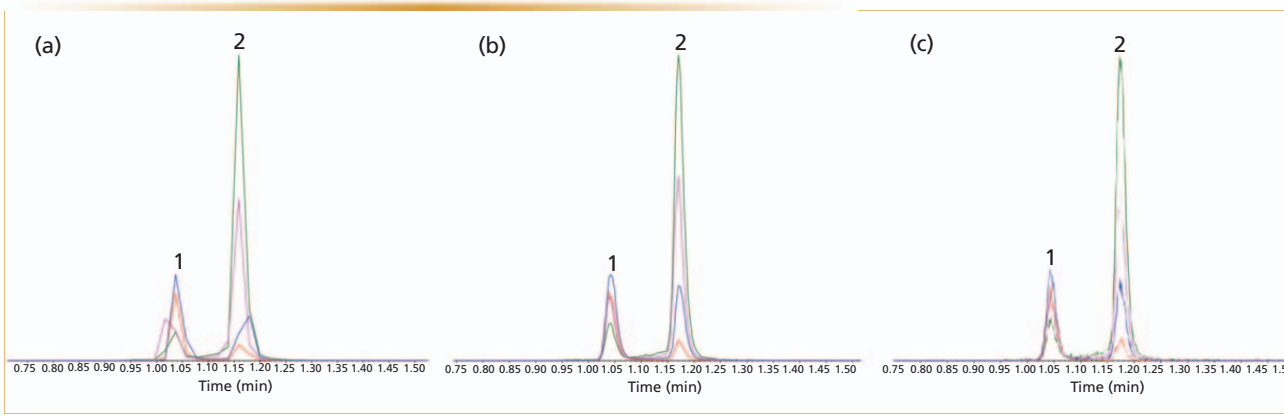

FIGURE 6: Comparison of data collected with dwell times of (a) 300 ms, (b) 50 ms, and (c) 5 ms, and their contribution to time-related band broadening. System: LC–MS/MS; polarity: ESI+. Peaks: 1 = morphine, 2 = hydromorphone.

by its plate height (*H*). The efficiency of a peak is a function of its width and retention time. There are several processes that contribute to peak broadening inside and outside of the column. The injector, connecting tubing, and detector are all sources of extracolumn peak broadening.

Inside the column, eddy diffusion (*A*), longitudinal mass transfer (*B*), and mobile-phase and stationary-phase mass transfer (*C*) all contribute to peak dispersion. Collectively, these terms make up the van Deemter equation:

$$h = A + B/v + Cu \quad [1]$$

where *h* is reduce plate height and *v* is the mobile-phase linear velocity (2). The van Deemter equation serves as a basis from which column performance is compared.

One way to increase column efficiency is by decreasing the particle size. Decreasing the overall peak width will cause an overall increase in peak height. Assuming that detector noise remains constant, taller peaks result in improvements in S/N and a boost in sensitivity. Additionally, highly efficient peaks are likely to be more resolved, reducing the likelihood that matrix interferences will impact ionization efficiency.

Smaller-particle columns also allow for the use of faster optimal linear velocities, and by extension, faster flow rates—without experiencing significant losses in efficiency. Unfortunately, because of the mechanisms that govern ESI, faster flow rates are generally a detriment to sensitivity since all eluent must be removed for successful formation of gas-phase ions. Although some manufacturers claim instrument compatibility with eluent flow rates up to 1 mL/min, the best performance for standard flow LC–ESI–MS systems has been reported to occur in the range of 10–300 µL/min (16). To accommodate small particles and their associated high linear velocities, 2.1-mm i.d. columns have become the preferred size for standard-flow LC–ESI–MS systems with optimal flow rates of 200–300 µL/min.

Changing the particle morphology is yet another way to improve column efficiency. Superficially porous particles are different from fully porous particles in that they have a thin porous shell surrounding a solid core. They are able to provide a significant increase in efficiency because of decreases in longitudinal diffusion (*B*) and eddy diffusion (*A*) that result from their narrow particle size distribution, reduced permeability, and rough surface exterior (17). Figure 5 compares the kinetic performance of a fully porous 3-µm C18 column to a superficially porous 2.7-µm C18 column of the same dimension using a van Deemter plot. The superficially porous column displays up to a 60% increase in efficiency over the fully porous particle.

Utilizing columns with narrow inner diameters minimizes analyte dilution, which takes place during the chromatographic separation. Because of on-column dilution, analyte sensitivity is inversely proportional to the square of the column inner diameter for concentration dependent detectors (18). Therefore, switching from a 2.1-mm i.d. column to a 0.3-mm i.d. column would theoretically increase sensitivity by a factor of 50, assuming the same volume of sample can be injected in both cases (19). Likewise, smaller inner diameter columns maintain the same linear velocity with reduced flow rates, which is beneficial in terms of ionization efficiency. The use of very slow flow rates (nanoliters per min) has gained popularity for applications that require high sensitivity with limited sample amounts. At these flow rates, desolvation becomes so efficient that matrix effects actually cease to be a concern (1).

There are several implications when coupling high-efficiency, narrow-bore columns with mass spectrometry. The decrease in column inner diameter, in conjunction with increased efficiency, leads to substantial decreases in peak volume. Without minimizing extracolumn volume in the instrument, column performance will be compromised, making it difficult to realize any significant gains in sensitivity. Most extracolumn-volume contributions can be attributed to the LC system with MS contributions being negligible. However, the tubing used to interface the LC column to the MS system was found to be critical since this tubing is located post-column, where the focusing effects that compensate for band broadening do not occur (20). As a rule of thumb, the extracolumn volume should not exceed one-third of the peak volume of the narrowest peak in the chro-

matogram (21). For example, a 1.8-µm, 100 mm x 2.1 mm column produces a peak volume of approximately 8 µL (16). Therefore, the maximum extracolumn volume should be <3 µL to negate system related losses to efficiency.

Smaller peak volumes also imply that a fast acquisition rate is required to collect the minimum 15–20 data points across a peak needed for quantitative data. Time-related band broadening effects can result from insufficient dwell times and excessive data smoothing. In Figure 6, morphine and hydromorphone were analyzed using three scan rates (300 ms, 50 ms, and 5 ms). Artificial broadening is apparent for the 300-ms data whereas the 5-ms data show excessive noise that is characteristic of over sampling. Improper dwell time settings can have a profound impact on data quality and S/N ratios. When analyzing a large number of compounds, increased cycle times can be achieved by collecting data in selected-ion monitoring (SIM) or multiple reaction monitoring (MRM) mode to reduce the occurrence of time related band broadening effects. In addition, most software allows for timed data collection, enabling data to be collected for a particular compound over a predefined window of time, extending the cycle time of a system.

Conclusions

Developing a sensitive and robust LC–MS method is a difficult task. Equipped with an understanding of the physicochemical properties of their target analytes, as well as the mechanisms and limitations of MS ionization and transmission efficiency, analysts can begin to make educated decisions to optimize overall response. The easiest and most effective way to improve sensitivity is through optimization of the ionization source conditions to ensure maximum production and transfer of gas-phase ions into the MS system. Careful selection of sample pretreatment procedures can reduce limits of detection by improving response and reducing interferences that could contribute to matrix effects and baseline noise. The use of efficient, narrow-bore LC columns, slower LC flow rates, and logical mobile phases can facilitate gains in signal intensity assuming extracolumn volumes are minimized and the data acquisition rates are appropriately set.

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LC TROUBLESHOOTING

Tips, Tricks, and Troubleshooting for Separations of Biomolecules, Part II: Contemporary Separations of Proteins by Size-Exclusion Chromatography

Several new materials and columns have been introduced in recent years for size-exclusion separations of proteins. How do I know which one to choose, and which separation conditions will be the best for my protein separation?

Szabolcs Fekete, Davy Guillarme, and Dwight R. Stoll

In Part I of this series (1), we focused on reversed-phase separations of proteins. In recent years, many new materials and columns have been introduced that provide potential for substantially better separations compared with those from one or two decades ago. Although some things have stayed the same, much of the old conventional wisdom has been overturned with the development of better stationary-phase chemistries and new research that has provided deeper insights into why we observe some phenomena (for example, low recovery of proteins from reversed-phase materials under some conditions). This research has also led to new guidance for operating conditions that improve the likelihood of obtaining acceptable chromatographic results.

Over the past few years, we have seen tremendous expansion in commercially available offerings for size-based separations of proteins as well. These separations are most commonly referred to as size-exclusion chromatography *(SEC), and we will use that term here. As with reversed-phase separations of proteins, the upside to having more commercially available columns to choose from is that we can more precisely tailor our column choices to the needs of our applications. However, the downside to more options is that we have to choose which one is the most suitable, and in some cases, this can be a challenging task in itself. On the other hand, recent research studies have added considerable insights to the existing knowledge base to support this decision-making process. Even if we don't fully understand why SEC materials behave the way they do in every situation (for example, see reference 2), we are in a much better position today to make good choices about columns and operating conditions than we were five years ago.*

For this installment of "LC Troubleshooting," I have asked two of my collaborators in the biomolecule application space, and genuine experts in SEC separations of proteins, to join me in sharing some of the details that we have found to be particularly important to successful SEC separations.

Dwight Stoll

Basics of SEC Separations

From a theoretical point of view, SEC is arguably the simplest of all chromatographic separation modes. In reversed-phase mode and other separation modes, we spend a lot of time thinking and talking about retention (that is, retention factors greater than zero are very important!), which is a function of differences between the strength of intermolecular interactions between analytes, mobile phase, and stationary phase. It is differences between the way one analyte interacts with the mobile and stationary phases compared to another analyte that give rise to differences in retention (that is, selectivity) and ultimately resolution of two analytes. In this way, resolution in reversed-phase and similar separation modes (sorptive modes) is inherently chemically driven. SEC, on the other hand, is completely different, at least in the ideal case. Here, resolution has a physical basis, rather than a chemical one, and in the ideal case, there is no retention of the analyte by the stationary phase (that is, retention factors are zero or apparently negative). Instead, separation arises from differences in the physical limitations that analytes of certain sizes experience, preventing them from exploring the entire pore network of porous particles used in SEC columns. Very small analytes in a sample will be able to explore most of the pore network. On the other hand, larger analytes that are too big to explore all of the pores will travel through the column with a higher velocity, and be observed flowing from the column earlier than the small analytes. From the point of view of the large analytes, the mobile phase volume inside

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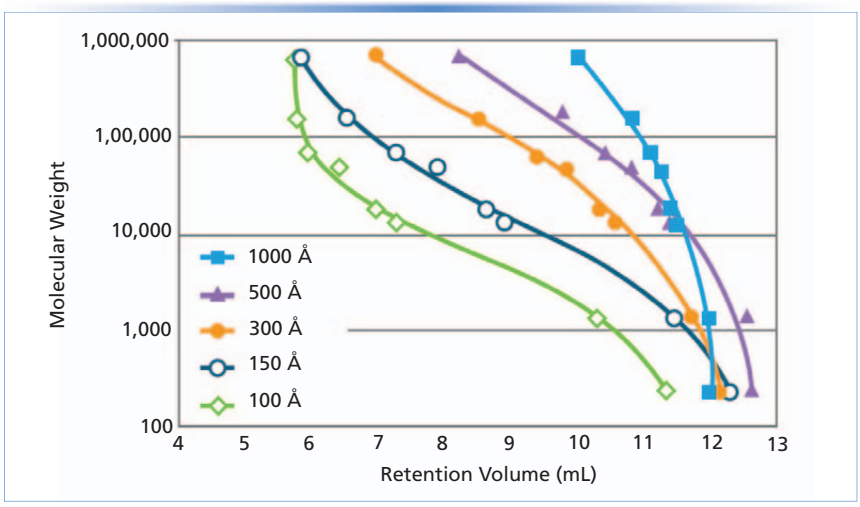

FIGURE 1: Molecular weight vs. retention volume plots for SEC columns having particles with different average pore sizes. The smallest two molecules in the dataset are uracil (112 Da) and vitamin B12 (1350 Da), and the largest two molecules are gamma-globulin (158 kDa) and thyroglobulin (670 kDa). Adapted with permission from reference 6.

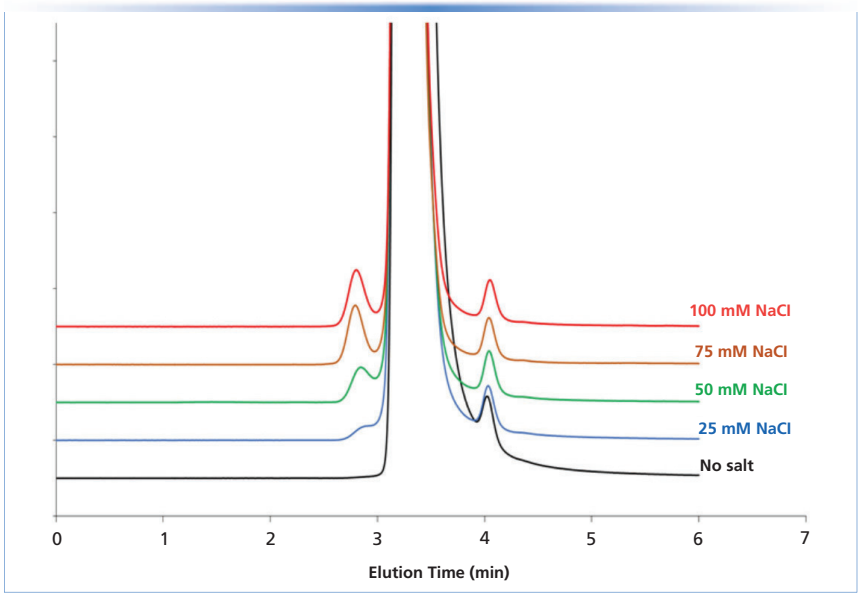

FIGURE 2: Impact of mobile phase salt concentration on detected concentration of aggregates in a sample of the therapeutic protein adalimumab. Conditions: Column: 150 mm x 4.6 mm, 2.7-µm Agilent AdvanceBioSEC; mobile phase: 100 mM phosphate buffer, pH 6.8, + indicated concentrations of sodium chloride; flow rate: 350 µL/min. Peaks in order of elution: Dimer, monomer, and fragment (3). Unpublished data from the laboratory of D. Guillarme.

the column is effectively smaller. Under ideal circumstances (that is, no retention due to intermolecular interactions), very small analytes will be eluted at what we would normally refer to as the dead time (t_m) in reversed-phase separations. The mobile-phase volume associated with this time (that is, t_m x F) is referred to as the *inclusion volume* (corresponding to the total porosity of the column). Larger analytes will elute at earlier times, before the inclusion volume.

Decision 1—Choosing the Column

Before we dive into the details here, we want to be clear about our intent for this installment. A tremendous amount of very good information on the following topics has been published in recent years. Our discussion here is limited to a survey of highlights of that work. Readers interested in the details behind our discussion are strongly encouraged to engage the literature cited here to learn more.

Particle Size and Column Length

Before the advances in column technology for SEC in recent years, most SEC columns in use were relatively large—typically 7.8 mm in diameter, and 150 to 300 mm in length. The long column lengths were required because of the large particles that were used, most of which did not have high mechanical strength and had to be used at relatively low pressures. The recent trend in column technology for SEC has been focused on the development of columns with smaller particles (<3 µm), in shorter columns (the standard now is 15 cm), and in smaller diameters (typically 4.6 mm). This trend has been supported by the development of particle chemistries that are both sufficiently mechanically stable to be used at the higher pressures that accompany the smaller particle sizes, and sufficiently inert toward biomolecules, to produce separations based mostly on molecular size. The move to smaller particle diameters also provides opportunities to improve separation speed by using higher flow rates through these columns. With larger particles, using high flow rates tends to result in decreases in efficiency (that is, plate number) and resolution, but the price paid for doing so with smaller particles is not as severe.

Although we must be careful with generalizations, it is useful to think a bit about what the trend toward the use of small particles can do for us, in a practical sense. In rough terms the plate height scales with the particle diameter. So, upon moving from a 5-µm particle to a 2-µm particle, the plate height should decrease by about a factor of two (3). There are two main ways we can capitalize on this improvement in plate height—we

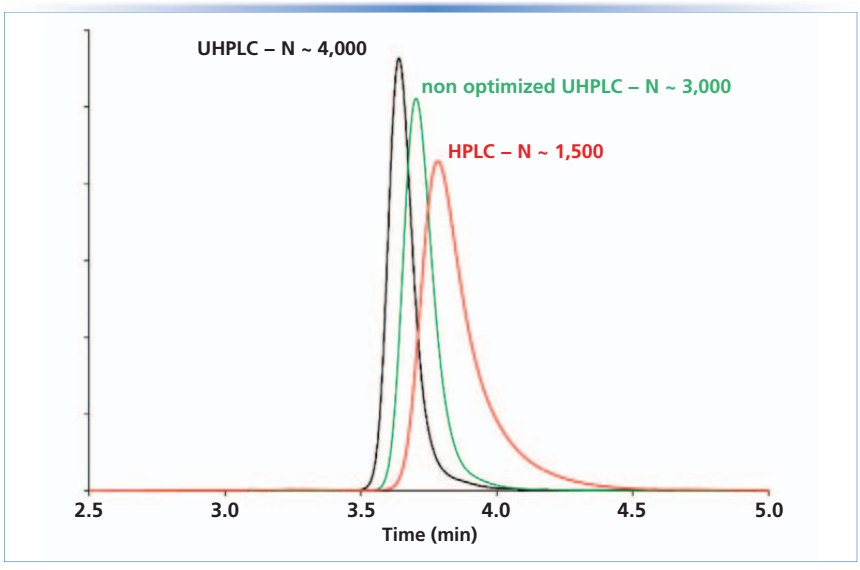

FIGURE 3: Observed chromatograms for a mAb monomer on three different LC systems. Column: 150 mm x 4.6 mm, 1.8 µm; flow rate: 0.3 mL/min; temperature: ambient. Adapted with permission from reference 11.

can either improve resolution while using a column of the same length, or we can decrease analysis time while maintaining resolution. In the first case, if we use two columns of the same length—one with 5-µm particles and one with 2-µm particles—the plate number for the 2-µm particle should be approximately double that of the column with 5-µm particles. Since resolution scales with the square root of plate number, we should expect the resolution to improve by about 40%. In the second case, the plate number is directly proportional to column length, and inversely proportional to plate height. If the plate height decreases by a factor of two with the smaller particles, then we can decrease the column length by a factor of two, while maintaining the same plate number and resolution. If the same flow rate is used in both cases, we should expect this to immediately result in a 50% decrease in analysis time. This is a simple but useful view of these scenarios. There are number of other factors to think about when considering the move to smaller particles, including the pressure limitations of the column and parti-

cles, and specifications of the instrument. More-detailed discussions of the theory relevant to these considerations can be found elsewhere (4,5).

Average Pore Size and Distribution

As described above, the velocity of a particular molecule through a SEC column depends on the extent to which it can explore the pores of the particles. For particles with a well-defined pore size distribution, there is a range of molecular sizes for which a particular particle will be effective for size-based separations. The calibration curve shown in Figure 1 shows the selectivity (that is, difference in elution volume for a given change in molecular weight) for particles with different average pore diameters. We see that with small-pore columns there is good selectivity for small molecules, but the largest molecules will effectively be coeluted. On the other hand, the very large pore materials effectively separate the largest molecules, but the smallest molecules are coeluted. This type of plot can be used to decide which pore size will be most effective for the application at hand. For protein characterization, typical pore sizes between 150 and 500 Å are used. For common therapeutic proteins (MW ≈15–80 kDa), a pore size of 150–200 Å works well, while a 200–300 Å pore size is usually used for monoclonal antibodies (mAbs, MW ≈ 150 kDa). For very large proteins (MW > 200 kDa, for example, pegloticase or PEGylated proteins), typically the 500–1000 Å materials offer the most appropriate selectivity.

The pore size distribution has an impact on the slope of the calibration curves. The wider the pore size distribution, the steeper the curve is. Therefore, with a wide pore size distribution, the selectivity will be lower but the range of the analytes that can be separated will be broader. A narrow pore size distribution provides higher selectivity between species with slight differences in size, but only a limited size range of analytes can be separated.

The challenge in practice is that the only information that is readily available from column manufacturers is the nominal pore size. Unfortunately there is not broad agreement about how exactly to report pore size, and most of these measurements are based on gas adsorption/desorption measurements and may not be very meaningful for protein analyses. Thus, from the point of view of users of these columns, it is practically useful to experimentally determine the calibration curve by injecting a mixture of standard proteins in order to get a good sense for the selectivity that can be expected for a given protein sample.

Decision 2—Choosing the Mobile Phase

After choosing the column, the next most important decision involves choosing exactly what will go into the mobile phase. As described above, one of the basic tenets of SEC separations is that conditions should be chosen so that retention (in a chemical sense) is minimized. If achieved, this approach ensures that the elution volume is an indicator of molecular size (as in a calibration curve of the type shown in Figure 1) and nothing else. At first glance, this seems like it should be straightforward—we should just choose a stationary phase that does not interact strongly through specific types of interactions with the analyte, and choose a mobile phase in which the analyte has a high solubility and that is able to minimize analyte–stationary phase interactions. But, if we've learned anything from 50 years of liquid chromatography, one of the big lessons has been that apparently tiny changes in the chemistry or structure of a stationary phase or analyte can lead to big changes in retention. Indeed, we often exploit these interactions to great effect in reversed-phase separations when developing a new method. However, implementing this approach also means that achieving the "no retention" condition in SEC separations of proteins can be quite difficult in practice. There is a rich literature describing studies that have explored the use of different mobile phase modifiers and conditions to minimize stationary phase–analyte interactions.

It has been our experience that many of the specific effects of different mobile-phase conditions are protein- or stationary-phase specific (or both), and thus some amount of exploration of variables is a necessary part of method development when starting work with a new molecule. However, based on our experience and the literature available to date, we can provide some suggestions for starting conditions:

- **pH:** When the isoelectric point (pI) of the protein is known, the mobile-phase pH should be adjusted to approximately match the pI of the protein. If the pI is not known, pH 6.5 is a good starting point. One should ensure, either based on existing literature or by experiment, that the protein is both highly soluble and chemically stable at the pH that is planned for.
- **Salts:** Various additives have been tested as a means to reduce non-specific interactions and retention of proteins under SEC conditions. For example, high concentrations (~0.2 M) of arginine have been used in the past (7). Arginine and other amino acids can interact with the protein and therefore decrease the accessible charges and possible electrostatic (ion-exchange) interactions. More commonly though, significant concentrations of sodium and potassium salts are used to suppress electrostatic interactions between the stationary phase and protein (8,9). An example of the effect of adding increasing levels of sodium chloride to a phosphate buffered mobile phase at pH 6.8 is shown in Figure 2 for the therapeutic protein adalimumab. Here, we see two major effects, both of which evidently result from decreased interactions between the protein and the stationary phase. First, the detected concentration of the mAb dimer (peak eluted before the monomer) increases dramatically (higher recovery) from barely detectable with no salt added, to easily detected at 100 mM sodium chloride added. Second, the elution

volume of the dimer also decreases, again because interactions with the stationary phase are decreased, such that the resolution of the dimer and monomer increases.

- **Organic solvents:** Although most proteins are sufficiently hydrophilic that completely aqueous mobile phases will yield acceptable SEC results, hydrophobic proteins may require small additions of solvent to improve recovery and peak shape. In particular, antibody–drug conjugates (ADCs) are a class of molecules of current interest that may benefit from addition of organic solvent (10). In these cases, addition of 10–15% of isopropanol to the mobile phase is a good starting point.

And What About the Instrument?

There are at least two major issues we could discuss here—the impact of system dispersion on the performance of high-quality SEC separations, and the impact of instrument construction and the use of bioinert, biocompatible materials. The latter topic is complex and we will reserve that discussion for a later date. On the topic of system dispersion, we have to recognize that SEC separations are particularly prone to the negative effects of peak dispersion outside of the column (that is, extracolumn dispersion) because, again, the peaks are eluted with no retention or even before the inclusion volume. In separation modes where retention is desirable, the effects of extracolumn dispersion are less severe for peaks that are more retained, and in the case of gradient elution in many cases nearly all precolumn dispersion can be eliminated. Not so in SEC, because no peaks are retained, and all separations are isocratic.

As discussed above, until relatively recently most SEC columns in use were large in diameter (~7.8 mm) and long (300 mm). This resulted in separations where the peak volumes (that is, the peak width in time units, times the flow rate) were large enough in comparison to the injector-to-detector volumes of LC systems they were connected to. However, with the improved plate heights and smaller volumes of state-of-the-art columns, the peak volumes are small enough that extracolumn dispersion has become a very important issue again (11). Figure 3 shows a comparison of the detected peak for a monoclonal antibody monomer obtained on three different LC systems with different levels of extracolumn peak dispersion. Given that resolution is often very valuable in SEC separations, this comparison makes it clear that one should seriously consider the effect of extracolumn dispersion on the observed chromatography, particularly when using modern SEC columns with small volumes and small particles.

When working with a state-of-the-art 150 mm x 4.6 mm SEC column, for a small analyte that is eluted near the inclusion volume, only 25–60% of the intrinsic column efficiency can be attained on conventional high performance liquid chromatography (HPLC) systems. The situation is even worse with a partially excluded analyte. Optimized ultrahigh-pressure liquid chromatography (UHPLC) systems having very low extracolumn volumes (typically V_{ec} < 10 µL) have to be used to properly operate these columns. Therefore connector tubing volume and detector cell volume must be as low as possible. As most SEC separations are performed at ambient temperature, the mobile-phase preheater unit can also be removed to further gain in apparent efficiency. Another interesting finding is that conventional HPLC systems also have a big impact on the apparent elution time of proteins—and therefore on mass-calibration curve—when working with 150 mm × 4.6 mm columns. Under these conditions the resulting calibration data will not be reliable, except if corrected for extracolumn residence time.

Summary

Developing effective and high performing SEC separations for proteins requires attention to all facets of the method, including choices around stationary phase, particle size, and column dimensions, mobile-phase conditions, and instrument effects on chromatographic efficiency and resolution. Several research groups are continually contributing to our understanding of the effects of all of these decisions on separation performance. Although we certainly are very far from a complete understanding, we are in a better position than ever before to leverage the information we do have to develop the best methods possible today.

A Note to Readers

With this installment of "LC Troubleshooting," I am approaching my first full year of writing monthly columns that address some of the pain points we experience as practitioners of liquid chromatography. As I have said many times already here, some new problems emerge as technology changes and we adapt to the new behaviors of instruments and columns, but there are also many problems that nominally remain the same over time. I will continue working to bring a mix of discussions of old and new topics to the column, but I am also particularly interested to hear what you, as a regular consumer of the column, have to say about topics you would like to see addressed here. Are there topics that are emerging challenges that you have not seen addressed in the past? Are there "old" topics that you would like to see addressed in more depth? I'd love to hear your topic suggestions! Please send them along to LCGCedit@ubm.com.

Dwight Stoll

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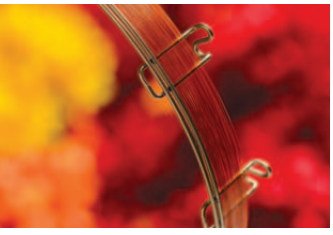

GC CONNECTIONS

Safely Delivering the Best Possible Carrier and Detector Gases to Your GC System

The quality of a gas chromatogram depends heavily on the quality of the separation and detection gases, among many other factors. In this month's installment, "GC Connections" discusses ways in which chromatographers can ensure a safe working environment while delivering gases that are up to the requirements of the separations at hand, in the context of moving a laboratory to a new location.

John V. Hinshaw

Earlier this year, the laboratory I use was moved to a new location several miles away. In the course of setting up the new laboratory, the gas chromatography (GC) carrier and detector gas supplies had to be torn down and rebuilt. This laboratory is similar to many industrial GC laboratories—it contains a number of GC systems, plus a variety of specialized test equipment. The laboratory has two double-wide gas cylinder corrals that hold helium, air, nitrogen, hydrogen, and an assortment of gas standards. Cylinders not in use are stored in gas safety storage cages in another room. Occasionally a cylinder is secured next to an instrument, but lengths of manifolded tubing anchored to the walls deliver the gases for permanent use from the corrals to the instruments.

The new laboratory is somewhat larger and requires longer tubing runs between the cylinder corrals and the instruments. The challenge was to reuse as much of the existing hardware—regulators, tubing, fittings and valves—as possible, to save costs, while maintaining the integrity of the connections and purity of the delivered gases. The leak-tight aspect is absolutely crucial for laboratory safety, because some of the gas standards contain high levels of toxic substances, and, of course, hydrogen is quite flammable.

How did it go, and what lessons were learned? Let's take a look.

Safety First

Gas cylinder safety has been addressed many times in this column as well as in multiple safety guides and government regulations. The topic was covered recently in two "GC Connections" installments from 2016 (1,2). Good safety practice centers around proper training and equipment. Gas safety training should include both general procedures and practices as well as topics specific to the gases in use, emergency procedures, and appropriate training on how to make and break the various gas-tight fittings found in the work environment. Beyond cylinder restraints and carts, safety equipment also includes goggles, gloves, and safety shoes, plus correctly sized and rated regulators, tubing, valves, gas filters, and fittings. Please see the two installments (1,2), as well as the references inside, for additional gas safety information.

In this laboratory, tanks of air, hydrogen, nitrogen, and helium are secured alongside gas standard cylinders. Liquid nitrogen tanks are used occasionally. Beyond dangers directly attributable to the gas cylinders, such as high internal pressures and risks from handling heavy objects improperly, the cylinder contents create hazards of toxicity, flammability, oxygen displacement, and cryohazards. See Table 1 in reference 1 for more details about the various commonly used gases and associated hazards.

The primary lines of defense against these gas hazards are proper cylinder handling and regular verification of the leak-free state of all gas feeds. These hazards are mitigated further by providing the laboratory with high-volume heating, ventilation, and air conditioning (HVAC) air flow and suitable ventilation of gas streams, along with toxic, flammable, and oxygen-depletion gas sensors wired to the building alarm system.

When the new laboratory was configured, the gas sensors were brought over intact, and a larger HVAC system was installed to create an improved gas-safety work environment. During the interim period between moving the gas sensors to the new building and leaving the old laboratory, sets of similar portable gas sensors were leased and placed in key spots in the old location, where some work continued right up to the move date.

Immediately after the move, some of the built-in sensors were found to be near their rated service period and were replaced. It is difficult to perform regular checks for this type of safety failure. Fire extinguishers, for example, are checked periodically for expiration as specified in fire safety regulations. Equipment like the gas sensors sits quietly for extended periods of time without alarming and so can fade into the background and not receive sufficient attention. Another example like this that I have encountered is eye wash equipment in a very expired condition, the type that

has a fluid reservoir instead of a plumbed water connection. Thus gas safety sensors, as well as all of the other safety equipment related to gases or for other purposes, should be checked regularly for function and expiration date where appropriate.

Making the Move

The move was a multistep process. One of the first steps was to disconnect the GC gas filters. The incoming gas pressures on the carrier and detector lines were reduced, the gas lines to the instrument were disconnected, and the filter fittings were quickly capped under gas flow. After disconnecting and capping the bulkhead fittings at the back of the instruments, the gas line pressures were shut off at the regulator outlet valves, the tubing was disconnected from the filters one by one, and the filter inlet fittings were capped. This approach allowed as much pure gas as possible to be retained inside the filters. The intent was to reuse each filter at the new location.

The tubing, fittings, and valves were disconnected from the regulators, and the longer tubing runs with intermediate unions were disconnected as well. The first tubing sections, starting at the tank regulators, are six-foot lengths of flexible hose, which makes connecting the cylinders much easier and adaptable. The same is true for the gas connections at the outlet ends of the carrier gas lines. Where possible, fittings were left intact, because they were likely to remain leak-tight through the move. The 0.125-in. diameter tubing was coiled as smoothly as possible, while the 0.25-in. pieces had to be moved with straight sections and bends left intact. It was not practical to cover all of the exposed fitting ends, so they were taped over with low-residue blue painter's tape. Of course, the new laboratory configuration is not the same, and so a significant number of pieces of bent tubing would not fit anywhere at the destination.

The tank regulators were vented and then packed a few to a box with bubble wrap or foam surrounding them. Although cylinder regulators don't look very fragile, their gauges and valve stems are prone to impact damage during transport. It's a good idea to cover the regulator gas inlets with low-residue blue painter's tape as well, to prevent ingress of particles. Regulator outlets are best sealed with matching caps.

Before the move, the gas cylinders were inventoried, and any that were no longer needed were returned to the supplier. When the time came to break down and pack the laboratory gas systems, rather than attempting to put the cylinders on a truck and run afoul of state or federal Department of Transportation, Occupational Safety and Health Administration (OSHA), and who knows what other regulations while creating a true public safety hazard, the commercial gas suppliers were engaged instead to move their cylinders themselves. The cylinders were disconnected from their regulators, capped, and then packed by the gas suppliers onto suitable pallets for the short

journey to the new location. Once on site they were unloaded from the pallets and placed back into the in-lab corrals or into one of the gas storage cages.

Reassembly

At the destination, the lengths of tubing were assembled as best they could be positioned to bring the carrier and other gases to the instrumentation. Nearly all the tubing is stainless steel, so there was little concern that the tubing would fail as a result of stress fracturing across multiple bends. A few of the lines are the heat-treated copper 0.125-in. outer diameter type provided by some instrument manufacturers. These lines were left intact and were disconnected only at the instrument bulkhead and the corresponding gas filter, then reassembled to exactly the same fittings with as little rebending as possible. No fractures occurred.

New tubing was used in some places where nothing fit the required lengths. When all was complete, about 75% of the tubing in the new laboratory was recovered from the old lab. Much of the new tubing used was needed to extend the tubing runs for the increased distance between the tanks and the instruments.

Special attention was paid to any reused swaged fittings. Each fitting was inspected first for over-tightening symptoms of a bulging tubing end, or distorted ferrules. Another problem can arise from mis-matched fittings from two different manufacturers. A recent installment of "GC Connections" (3) has some good photos of what to look for in this regard as well as an informative discussion on how to make the connections.

A trial attempt at making each connection was performed. If the fitting nut did not engage the threaded union or valve thread smoothly and without requiring the force of a wrench, then the nut and ferrule portion on the tubing were discarded. If the union or valve was not new, it was also discarded since it was likely that the damage extended to both sides of the connection.

After making a new clean cut on the tubing, and using a new union or valve, a new connection was made following the fitting manufacturer's procedures. Overall a good recovery of used fittings was achieved, around 80%. This good recovery was due to having paid attention to the quality of the original installation of the fittings in the old laboratory, which paid off handsomely for the move to the new lab.

After a tubing run was complete, the exit was sealed temporarily with a plug and the line was pressurized with helium. The first leak check consisted of turning off the tank valve after pressurization, leaving the regulator's outlet valve open, and observing the high-pressure tank gauge for up to 30 min. If any observable pressure drop was seen, a quick check of the fittings with a helium leak detector usually revealed one or more leaking fittings, which were duly repaired or replaced. Sometimes all that was needed was the audible hiss of an untightened fitting! If no pressure drop was observed, then the fittings were checked more carefully to be sure there were no microleaks. Liquid leak checking solutions were not used.

Finally the intended gas, if other than helium, was connected and another pressure drop check was made for air or nitrogen, or a leak tester check for hydrogen, after which the line was deemed ready for service. Note that flushing a hydrogen line with helium for the initial leak check is a good idea, as it avoids potentially venting a lot of hydrogen into the air uncontrollably in the event of a large unintentional leak.

Leak checking was performed before connecting tubing to the inlets of any gas-scrubbing filters, to avoid forcing any more air than necessary into them. After a line was leak tight, it was purged with the appropriate gas before connecting to the filter, and then the filter was purged before connecting to the instrument. Fortuitously, none of the water or oxygen indicating filters exhibited significant degradation from before to after the move after following the above procedures.

Caution is advised when venting hydrogen lines. Hydrogen diffuses away into the room air quite rapidly, because of its buoyancy and high diffusivity. Using a low pressure in the line during purging helps limit the amount of hydrogen that is released. I can say, from the experience of unintentionally testing a combustible gas detector, that it takes about two seconds for hydrogen to make its way up to a combustible gas detector near a 10-ft ceiling and halfway across the lab!

After the gas lines were set, one of the GC systems was powered on and a series of baseline runs were made. They followed a normal sequence with some ghost peaks and baseline instability in the first couple of runs, and then settled down nicely. The move was deemed successful, and we resumed our normal work.

Conclusions

One of the lessons learned in the move was that it is absolutely necessary to maintain accurate records of the age of consumable components in the laboratory. In this case, expired gas detectors were discovered and replaced. The GC gas filters, at least the indicating ones, appeared to survive the move well, but the non-indicating filters may or may not be in good shape today. It is difficult to track how well they perform, unless a small indicating filter is inserted downline. These will be replaced as necessary and feasible.

Another observation: Treat your fittings well and they will repay you with multiple make–break cycles. You will avoid having to replace them often to keep the gas system leak tight, and reduce correspondingly the expense of new fittings.

Although I would not choose to move a workplace very often, with proper planning, organization, and attention to the technical requirements for both safety and gas handling, a move can be made without major unplanned interruption or equipment losses.

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Chemical Fingerprinting of Mobile Volatile Organic Compounds in Soil by Dynamic Headspace–Thermal Desorption–Gas Chromatography–Mass Spectrometry

The chemical analysis of organic compounds in environmental samples is often targeted on predetermined analytes. A major shortcoming of this approach is that it invariably excludes a vast number of compounds of unknown relevance. Nontargeted chemical fingerprinting analysis addresses this problem by including all compounds that generate a relevant signal from a specific analytical platform, and so more information about the samples can be obtained. A dynamic headspace–thermal desorption–gas chromatography–mass spectrometry (DHS–TD–GC–MS) method for the fingerprinting analysis of mobile volatile organic compounds (VOCs) in soil is described and tested in this article. The analysis parameters, sorbent tube, purge volume, trapping temperature, drying of sorbent tube, and oven temperature were optimized through qualitative and semiquantitative analysis. The DHS–TD–GC–MS fingerprints of soil samples from three sites with spruce, oak, or beech were investigated by pixel-based analysis, a nontargeted data analysis method.

Peter Christensen, Majbrit Dela Cruz, Giorgio Tomasi, Nikoline J. Nielsen, Ole K. Borggaard, and Jan H. Christensen

Environmental samples contain thousands of organic compounds in complex mixtures (1), but the chemical analysis of organic compounds in environmental samples is typically targeted at a few chemical constituents that are already known and are expected to be present (2–4). In contrast, chemical fingerprinting aims to analyze all compounds from a complex mixture, which can be monitored with the selected analytical platform. The concept of chemical fingerprinting was first used in the 1970s for oil hydrocarbon fingerprinting to determine the source and weathering of crude oil and refined petroleum products (5). Since then, oil hydrocarbon fingerprinting has developed extensively and modern methods can now be used to monitor more than 1000 compounds in one single analysis (6). In the 1990s, fingerprinting methods were used for metabolomics and proteomics studies (7,8), and are now also used for plant and air matrices (9–11). Although the overall aim of chemical fingerprinting is to obtain a complete representation of a sample (for example, the whole metabolome of a cell), no single analytical technique exists that can fulfill this aim. Analytical techniques such as gas chromatography (GC) with mass spectrometry (MS) detection and liquid chromatography (LC) with MS detection are complementary methods that can be used with varying sensitivity to monitor compounds with different physical and chemical properties (for example, volatility and polarity). Each of these methods can be tuned to address different chemical windows by the choice of chromatographic mode or ionization source. Within soil science, substances in soil that can evaporate into the atmosphere, leach to surface and subsurface water, or can be taken up by living organisms are of great interest for environmental, human health, and food perspectives (12). Several extraction techniques have been developed to transfer VOCs from various matrices to a GC system (13,14). Most of these techniques can be grouped into solvent extraction, solid-phase extraction (SPE), gas extraction, and passive extraction (14). The U.S. Environmental Protection Agency Method 5035 for soil and waste samples recommends solvent extraction with methanol or polyethylene glycol for samples with high VOC concentration and gas extraction by purge-and-trap for VOC concentrations of less than 200 µg/kg (15). Purge-and-trap is able to automatically extract, concentrate, and transfer analytes to a GC system with little loss to the surroundings, and this is especially useful when working with trace amounts of VOCs (16,17). Dynamic headspace (DHS) is an alternative to purge-and-trap. In DHS the headspace above the sample, such as a soil slurry, is purged with inert gas during shaking or stirring and the VOCs are trapped on a sorbent tube. The sorbent tube is transferred to a thermal desorption unit (TDU), which is then heated for desorption (thermal desorption [TD]) of the VOCs and an inert gas carries the VOCs to the GC inlet. In this step, the direction of the gas flow thorough the desorption tube is reversed compared to the gas flow in the trapping phase. At the GC inlet the VOCs are focused, either cryogenically or by a sorbent before transfer to the GC column. By using DHS, the VOCs are dynamically removed from the sample, which mimics natural conditions better than batch

extraction (18). The aim of this study was to develop and test a method for chemical fingerprinting of the mobile fraction of VOCs in soil using DHS–TD–GC–MS. Several parameters were optimized with a focus on optimal transfer of VOCs, while also reducing transfer of water. Following method optimization, soil samples representing three vegetation types were analyzed and a pixel-based chemometric approach was used to compare them to search for specific markers for land use.

Materials and Methods

Standards and Chemicals

EPA VOC Mix 6, EPA Appendix IX Volatiles Calibration Mix, and calcium chloride hexahydrate were supplied by Sigma Aldrich Denmark A/S. D8-Naphthalene (Cambridge Isotope Labs., Inc.) was obtained from VWR International A/S. Stock solutions and dilutions of mixtures were prepared in methanol (HPLC-grade, Rathburn Chemicals Ltd.) supplied by Mikrolab Aarhus A/S. Purified water was produced by a Millipore Milli Q Plus system.

Artificial Sample for Method Optimization

A test mix of EPA VOC Mix 6 and EPA Appendix IX Volatiles Calibration Mix was prepared by adding 10 µL of each mix to 180 µL of methanol to reach a final concentration of 100 ppm for each VOC. An artificial sample was then prepared in headspace vials (20 mL) containing 5 g of Ottawa sand and 10 mL of milli-Q water spiked with 1.0 µL of the test mix. Compounds, retention times, target and qualifier ions, and VOC group for the test mix are listed in Table 1.

Soil Samples

Soil samples were collected from three closely spaced forest sites in Vestskoven in Denmark during March 2017. According to the American Soil Taxonomy system, the soils at the three sites were classified as Typic Hapludalfs, which are important, productive, mainly temperate area soils (19). Each site represents a different vegetation type: beech (*Fagus sylvatica*), Norway spruce (*Picea abies*), and oak (*Quercus robur*), which were planted on former farmland in the early 1960s. At each site, the top 30 cm was removed from an area of 0.5 × 0.5 m and approximately 500 g of soil from the sides of the hole at a depth of 10–20 cm were transferred to 1-L blue-cap bottles. Six samples were collected from each site and transferred to the laboratory. Each bottle was filled to the neck with 0.01 M calcium chloride and shaken for 1 h in a bottom-over-end rotator at 10 rpm. From each sample, 10 mL of slurry was transferred to a 20-mL amber headspace vial, avoiding plant debris floating on the top. Quality control (QC) samples were prepared by mixing 350 mL from one beech sample, 350 mL from one oak sample, and 450 mL from one spruce sam-

TABLE I: Monitored compounds used for the method optimization together with retention times, target and qualifier ion(s), and grouping of VOCs based on boiling points (bp). VOC group 1, bp < 35 °C; VOC group 2, 35 °C ≤ bp < 100 °C, and VOC group 3, 100 °C ≤ bp ≤ 218 °C (bp of naphthalene).

Compound	Retention Time (min)	Target Ion	Qualifier Ion(s)	VOC Group
Dichlorodifluoromethane	4.75	85	87/101	1
Chloromethane	5.98	50	52/15	1
Chloroethene	6.51	62	27/64	1
Bromomethane	7.33	94	96/79	1
Methanol	7.48	30	15/28	
Vinyl chloride	7.59	64	29/66	1
Trichloromonofluoromethane	8.00	101	103/105	1
1,1-Dichloroethene	8.61	61	96/98	1
Carbon disulfide	8.76	76	44/32	2
Acetonitrile	8.93	41	40/39	2
Allyl chloride	8.98	41	39/76	2
Dichloromethane	9.10	84	49/86	2
Water	9.26	16	19/20	
Acrylonitrile	9.29	53	52/26	2
(E)-1,2-Dichloroethene	9.32	61	96/98	2
1,1-Dichloroethane	9.66	63	65/27	2
Chloroform	9.68	83	85/47	2
Propionitrile	10.09	54	28/26	2
Methacrylonitrile	10.19	41	67/39	2
1,1,1-Trichloroethane	10.40	97	99/61	2
Carbon tetrachloride	10.51	117	119/82	2
2-Methyl-1-propanol	10.55	31	41/42	2
Benzene	10.63	78	77/52	2
1,2-Dichloroethane	10.67	62	27/49	2
Trichloroethylene	11.06	130	95/132	2
Methyl methacrylate	11.22	41	69/39	3
1,2-Dichloropropane	11.23	63	62/41	2
1,4-Dioxane	11.27	88	28/29	3
Dibromomethane	11.28	174	93/95	3
Bromodichloromethane	11.38	83	85/129	3
(Z)-1,3-Dichloro-1-propene	11.67	75	39/110	3
(E)-1,3-Dichloro-1-propene	11.75	75	39/49	3
Pyridine (from pyridine trifluoroacetate)	11.82	79	52/51	3
Toluene	11.90	91	92/65	3
Ethyl methacrylate	12.05	69	41/39	3
1,1,2-Trichloroethane,	12.17	97	83/61	3
Tetrachloroethylene	12.24	166	164/131	3
Dibromochloromethane	12.44	129	127/131	3
1,2-Dibromoethane	12.53	107	109/27	3
Chlorobenzene	12.86	112	77/114	3
1,1,1,2-Tetrachloroethane	12.92	131	133/117	3
Ethylbenzene	12.92	91	106/77	3

(Table continues on page 676)

ple. The QC mix was shaken and 10 mL was transferred to each of six amber headspace vials. Six controls were also prepared in the same way as the soil samples but without adding soil.

Apparatus

The sample handling was performed by a MultiPurpose MPS2 autosampler equipped with a DHS station and agitator (Gerstel GmbH & Co. KG). The GC system was a 7890A with a 5973N MS (Agilent Technologies).

Analytical Method

A 1-µL volume of deuterated internal standard solution (68 µg/mL d8-naphthalene in methanol) was added to each sample and was then shaken at 1500 rpm for 3 min in the DHS station. The DHS extraction was performed with a nitrogen purge flow of 50 mL/min for 10 min at 20 °C, and analytes were trapped on sorbent tubes packed with Carbopack B + C and Carbosieve SIII (Gerstel GmbH & Co. KG) at 70 °C.

For transfer of analytes to the GC system, the sorbent tube was moved to the TDU, which was in solvent vent mode. Initially the total He flow rate was 53.5 mL/min, the septum purge flow rate was 0 mL/min (fixed), and the desorption flow rate was hence 53.5 mL/min. The TDU purge flow rate was 3 mL/min (fixed), the TDU split flow rate was 50 mL/min, and the column flow rate was 0.5 mL/min.

At 0.50 min (after the sorbent tube was moved to the TDU) the TDU split flow was changed to the PTV (programmable temperature vaporizing) inlet split flow and kept at 50 mL/min (Figure 1). The pressure in the PTV inlet was 0.772 psi. The temperature of the TDU was held at 50 °C for 0.50 min, ramped to 330 °C at 720 °C/min, and held for 3 min (Figure 1). The analytes were cryo-focused in the liner in the PTV inlet at -150 °C during the thermal desorption step. To avoid excessive use of liquid nitrogen, oven cooling was initiated after the thermal desorption step. The oven program was hence started at 35 °C, decreased to -40 °C at 120 °C/min, held for 2.875 min, increased to 200 °C at 20 °C/min, held for 5 min, and decreased to 35 °C at 25 °C/min (Figure 1). The oven reached -40 °C but it was not possible to keep a rate of -120 °C/min. The hold time of 2.875 min was set to ensure that the -40 °C was reached. Transfer of analytes to the GC system can be improved by increasing the column flow rate before the PTV is heated. This was achieved with a column flow program starting at 0.5 mL/min, ramped to 5 mL/min at 1.95 mL/min per min, held for 1 min, and decreased to 1.1 mL/min at 5 mL/min per min (Figure 1). At the end of the flow program, the temperature program of the PTV was initiated. Here the temperature was increased by 12 °C/s to 250 °C, held for 5 min, increased by 10 °C/s to 300 °C, and held for 5 min.

The MS transfer line, ion source, and quadrupole temperatures were 230 °C, 230 °C, and 150 °C, respectively. Samples were analyzed in scan mode with a scan range of 10–300 mass-to-charge ratio (m/z). A 30 m × 0.25 mm, 1.4-µm VF-624ms column (Agilent J&W) was used.

TABLE I (CONTINUED): Monitored compounds used for the method optimization together with retention times, target and qualifier ion(s), and grouping of VOCs based on boiling points (bp). VOC group 1, bp < 35 °C; VOC group 2, 35 °C ≤ bp < 100 °C, and VOC group 3, 100 °C ≤ bp ≤ 218 °C (bp of naphthalene).

Compound	Retention Time (min)	Target Ion	Qualifier Ion(s)	VOC Group
o-Xylene	13.01	91	106/77	3
p-Xylene	13.01	91	106/77	3
m-Xylene	13.29	91	106/77	3
Styrene	13.29	104	103/78	3
Bromoform	13.44	173	171/175	3
1,1,2,2-Tetrachloroethane	13.74	83	85/95	3
(E)-1,4-Dichloro-2-butene	13.78	53	75/89	3
1,2,3-Trichloro-propane	13.79	110	75/77	3
Pentachloroethane	14.24	167	117/165	3
1,3-Dichlorobenzene	14.48	146	148/111	3
1,4-Dichlorobenzene	14.55	146	148/111	3
1,2-Dichlorobenzene	14.82	146	148/111	3
Hexachloroethane	15.04	201	117/119	3
1,2-Dibromo-3-chloropropane	15.40	157	75/155	3
Naphthalene	16.30	128	127/102	3

Optimization Steps

Several parameters were optimized for the final method: type of sorbent tube, purge volumes, trapping temperature, drying of the sorbent tube, and initial oven temperature (Table 2).

The optimization steps for sorbent tube, trapping temperature, drying of the sorbent tube, and oven temperature were performed with a 30 m × 0.15 mm, 0.85-µm VF-624ms column (Varian) and modified methods compared to the final method described above were used.

For the optimization of the purge volume, the flow was kept constant at 50 mL/min and time was set to reach the designated purge volumes. To evaluate the sorbent tubes, the DHS extractions were performed with a purge flow of 25 mL/min for 8 min. The trapping temperature was 40 °C for the Tenax-based tubes (Table II, tubes 2 and 3) and 50 °C for the Carbopack tubes (Table II, tubes 1, 4, and 5).

Data Analysis

For each optimization step, peaks were integrated and divided into their respective VOC group (Table I). Evaluation of the parameters was based on the area of the VOCs and the area of the water peak (*m/z* 16). Overloading of the MS system occurred for *m/z* 17 and *m/z* 18 and therefore *m/z* 16 was the preferred choice for determination of the area of the water peak.

The total ion chromatograms (TICs) obtained from DHS–TD–GC–MS analysis of the soil extracts were investigated using a pixel-based chemometric approach where entire sections of chromatograms are analyzed without peak extraction (20). Mass-to-charge ratios below 35 as well as *m/z* 44 were removed from

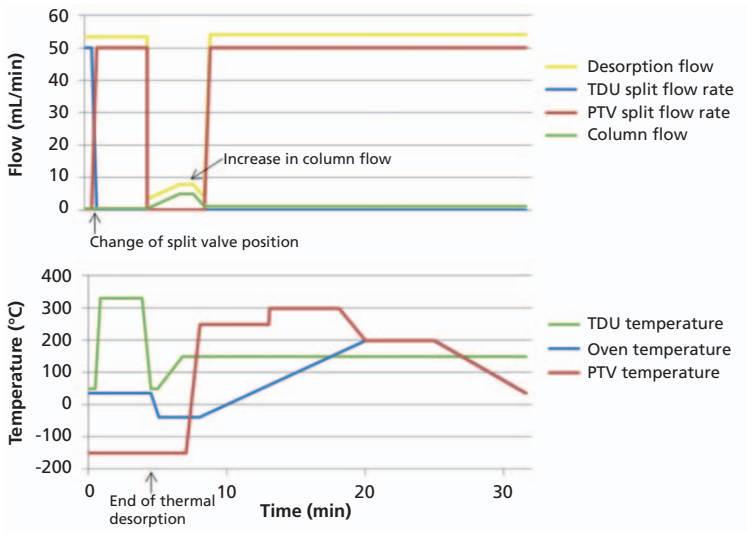

FIGURE 1: Flow and temperature scheme for the analytical method.

TABLE II: Optimization parameters and chosen settings for method optimization. Bold indicates setting chosen for the final method.

Sorbent Tube	Setting Evaluated				
	Carbopack C, Carbopack B, Carbosieve S-III	Tenax GR	Tenax TA	Carbopack B, Carbopack X	Carbopack B, Carbopack X, Carboxen-1000
Purge volume (mL)	100	200	300	400	**500**
Trapping temperature (°C)	30	50	**70**		
Drying of sorbent tube in DHS station (mL)	**0**	75	50		
Drying of sorbent tube in the TDU (mL)	**0**	75	150	225	
Oven temperature (°C)	**-40**	-20	0	35	

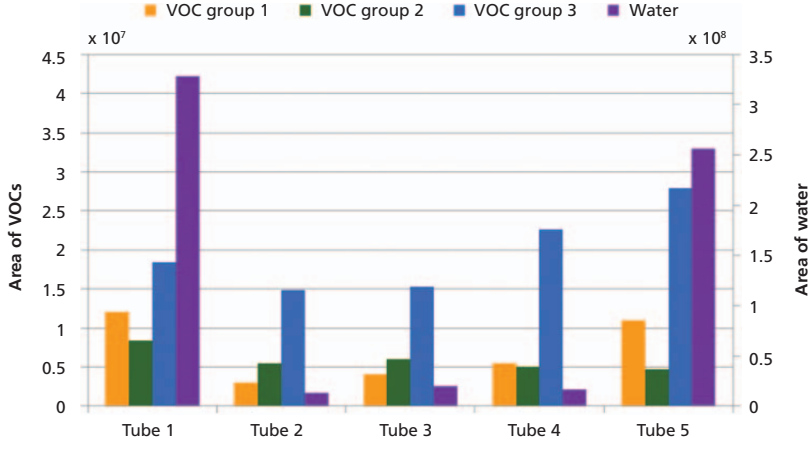

FIGURE 2: Area of VOCs and water for the five sorbent tubes (see Table II for further information, $n = 1$).

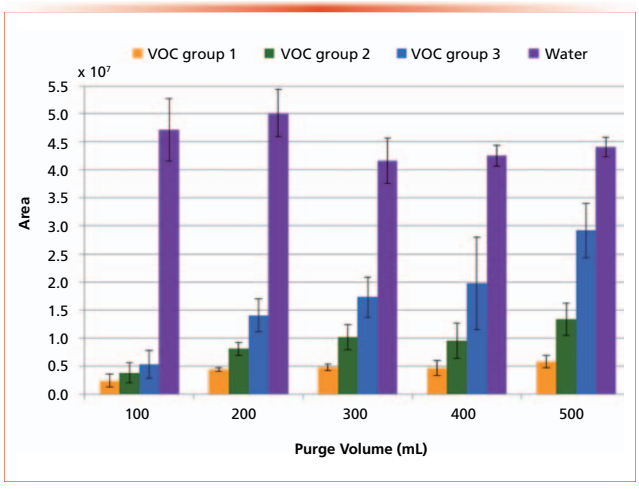

FIGURE 3: Transfer of VOCs and water for five purge volumes during the DHS extraction (n = 3). Error bars are ± 1 standard deviation.

the TIC to exclude water, oxygen, nitrogen, and carbon dioxide. Baselines were removed by piecewise linear subtraction of the lower part of a convex hull of each chromatogram (21) and samples were aligned using correlation optimized warping (COW) (22); the optimCOW procedure devised by Skov and colleagues (23) was used to find the optimal warping parameters. The scans before 9.25 min were excluded before alignment because the large irregular shifts in the early part of the chromatogram could not be satisfactorily aligned. The TICs were subsequently normalized to Euclidean norm, thus removing information on analytical changes in signal intensity and concentration (21,24). The data were analyzed by principal component analysis (PCA), which was fitted according to a weighted least squares criterion using the inverse of the relative standard deviation of the QC samples as weights (25,26).

Results and Discussion

Optimization

One of the major challenges when analyzing VOCs in water samples and water suspensions on DHS–TD–GC–MS is to trap and isolate a large fraction of the VOCs and still eliminate water. Water can lead to chromatographic problems, such as poor peak shapes and split peaks, as well as retention time shifts as a result of solvent flooding (27). High amounts of water can also lead to carryover, higher detection limits, and poor reproducibility during the rapid heating of the inlet because of sample expansion beyond the capacity of the liner volume. Type of sorbent tube, purge volume, temperature during trapping, drying of the sorbent tube, and initial oven temperature were optimized to reduce the amount of water transferred from the sample while still obtaining high extraction efficiency and transfer of the VOCs from the sorbent tube to the GC column. The method targeted compounds with boiling points up to 218 °C. However, compounds with different boiling points were not necessarily affected the same way during extraction, trapping, transfer, and analysis. Therefore, the optimization parameters were evaluated based on a division of the VOCs into three groups. VOC group 1 included compounds with boiling points below 35 °C. These can easily volatilize at the sampling site and can be difficult to sample. VOC group 2 included compounds with boiling points between 35 °C and 100 °C. These are still very volatile, but are easier to sample compared to VOC group 1. VOC group 3 included compounds with boiling points between 100 °C and 218 °C. These are less likely to volatilize during sampling, but are also harder to extract with DHS than VOC groups 1 and 2 because they have a lower vapor pressure.

The most suitable sorbent tube traps all VOCs and is able to release them again during thermal desorption in the TDU, but does not trap any water and does not affect the VOC composition. Five sorbent tubes were tested for the trapping of VOCs. VOCs with boiling points below 100 °C (VOC groups 1 and 2) are likely be found at lower concentrations in soil samples than VOCs with boiling points above 100 °C as a result of volatilization in the field. Tube 1 was selected for the final analytical method because it provided the most efficient trapping of these low-boiling point VOCs and was the only sorbent tube that was able to trap the most volatile compound, dichlorodifluoromethane (Figure 2).

The purge volume for extraction should ensure highest possible transfer of VOCs, but not at the expense of also transferring

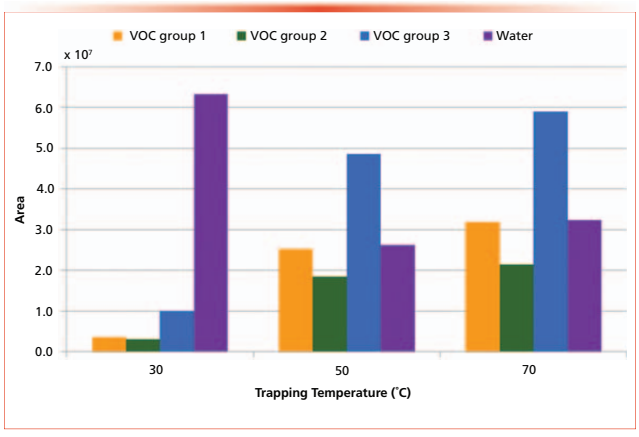

FIGURE 4: Area of VOCs and water for three trapping temperatures (n = 1).

a lot of water. Initial screening indicated that purge volumes of 30–400 mL during the DHS extraction were optimal and therefore purge volumes between 100–500 mL were tested in triplicates. The amount of water transferred to the sorption tube was relatively stable for the evaluated purge volumes (Figure 3). Transfer of VOCs largely increased with increasing purge volume, with VOC group 3 more affected than VOC groups 1 and 2. The optimal purge volume for all VOC groups was at 500 mL (Figure 3) and not at 300–400 mL as was found in the initial screening tests.

By increasing the trapping temperature, trapping of water can be limited. Trapping temperatures of 30 °C, 50 °C, and 70 °C were tested once. At trapping temperatures of 50 °C and 70 °C, trapping of water was reduced by approximately 50% compared to a trapping temperature of 30 °C (Figure 4). VOCs were trapped the least at 30 °C and slightly better at 70 °C than at 50 °C (Figure 4). The trapping temperature of 70 °C was therefore chosen.

Another way to remove water is by drying the sorption tubes in either the DHS station or in the TDU. Drying in the DHS station was performed with a nitrogen flow through the tube (from the bottom and up), in the same way as the headspace was purged during the trapping. In the TDU, the drying was performed with a helium flow from the top of the sorption tube to the bottom. The removal of water and VOCs was tested with a drying temperature of 70 °C, a flow of 35 mL/min in the TDU and DHS station, and with flow volumes in the range of 0–225 mL. Drying did not improve the VOC–water ratio and was therefore not implemented in the analytical method.

For the successful transfer of VOCs to the GC system, initial oven temperatures were also evaluated. The oven was cooled to initial temperatures of -40 °C, -20 °C, 0 °C, and 35 °C by the use of liquid nitrogen (except for 35 °C). The initial temperature of -40 °C gave the highest and narrowest peaks (Figure 5); this was further improved for the final method using the same column as before with a larger inner diameter (0.25 mm instead of 0.15 mm) and film thickness (1.4 µm instead of 0.85 µm) leading to improved focusing on the column. The effect of the initial oven temperature was not seen for the very late-eluted compounds (Figure 5).

Soil Samples

The PCA of the preprocessed TICs showed a clear separation of spruce samples from the remaining samples along principal component (PC) 2. PC1 described variations in hexamethylcyclotrisiloxane, octamethylcyclotrisiloxane, and diethyl phthalate. Spruce samples have positive PC2 score values while beech and oak samples have large negative PC2 scores (Figure 6). The separation in the PCA score plot can be explained from the corresponding loading plot (Figure 7). The positive scores indicate that the spruce samples contain relatively more (with respect to the average sample, which has score 0 by definition) of the compounds whose peaks have positive PC2 loading coefficients and relatively less of those with negative coefficients. For beech and oak samples the opposite is the case. Representative TICs of soil extracts from spruce, beech, and oak forest show that the TICs of soil extracts from spruce forest contain a number of peaks with positive PC2 loading coefficients that are not present in soil extracts from the beech and oak forests (Figure 8). The peaks with the largest PC2 loading coefficients were tentatively identified via a search in the NIST14 database. The majority of peaks with positive PC2 loading coefficients were terpenes,

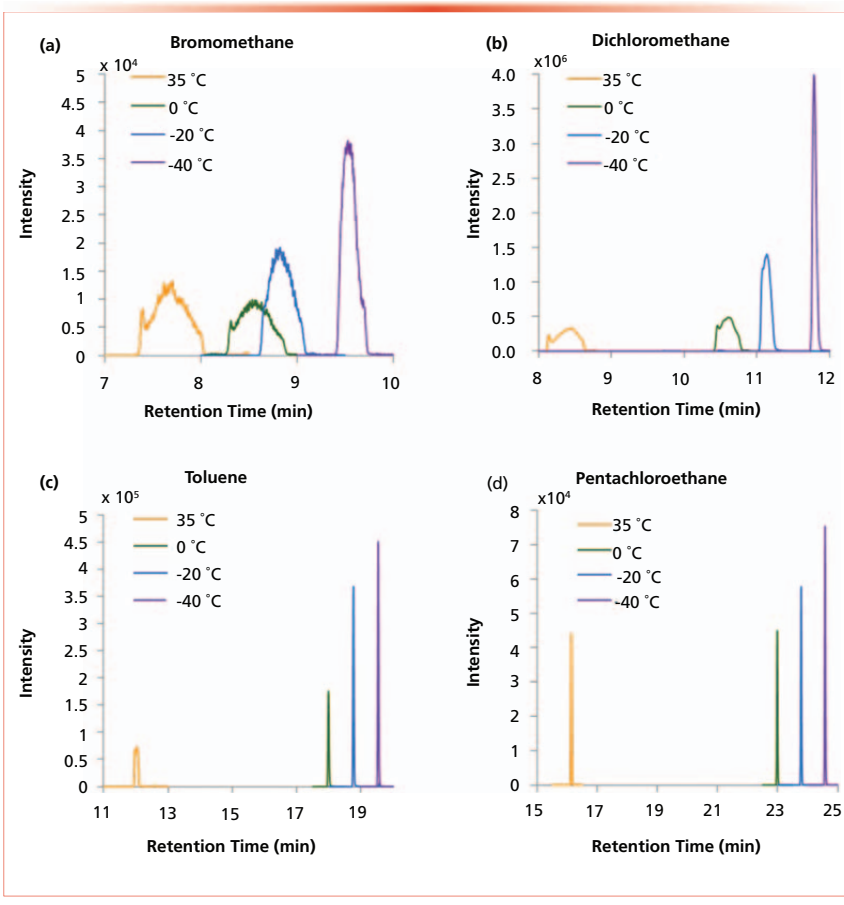

FIGURE 5: Extracted ion chromatogram of (a) bromomethane (*m/z* 94, VOC group 1), (b) dichloromethane (*m/z* 84, VOC group 2), (c) toluene (*m/z* 91, VOC group 3), and (d) pentachloroethane (*m/z* 167, VOC group 3) at initial oven temperatures of 35, 0, -20, and -40 °C.

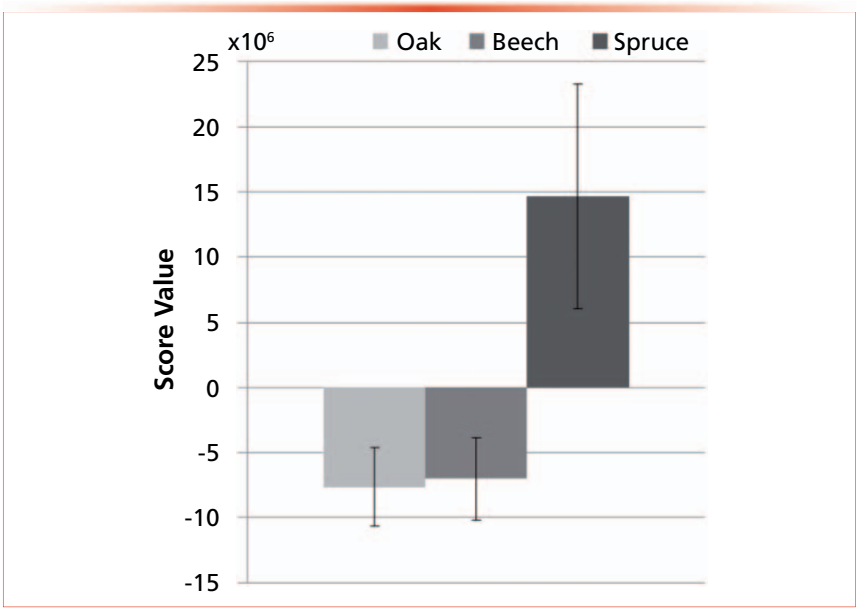

FIGURE 6: Average PC2 score values and standard deviations for samples representing oak, beech, and spruce (n = 6). Error bars are ± 1 standard deviation.

while peaks with negative PC2 loading coefficients were peaks that could also be found in the blank samples, such as *d*8-naphthalene and hexamethylcyclotrisiloxane (Figure 7). The terpenes tentatively identified were α-pinene, β-pinene, camphene, 3-carene, D-limonene, *o*-cymene, and β-phellandrene.

In Figure 9 the precision of the terpenes is given based on the relative peak areas of the terpenes with respect to *d*8-naphthalene for the quality control (QC) samples and the samples representing spruce. The samples representing beech and oak did not contain any of the terpenes. The precision of samples representing spruce was influenced by sample heterogeneity, as well as sampling and analytical variations. The QC samples were used to determine the analytical precision (repeatability) of the analytical method because these samples are analytical replicates. The repeatability calculated as relative standard deviations of the *d*8-naphthalene standardized peak areas of terpenes in the QC samples was on average 27.5% (range 22.2–32.4%) and the sampling and analytical variation was on average 59.4% (range 46.1–68.1%) when calculated based on soil samples representing spruce. This means that the sampling variation can be estimated to an average value of 52.7%. These results demonstrate that the analytical uncertainty is acceptable and only contributes a little to the total uncertainty (59.4%).

With an unknown chemical profile of soil samples the benefit of calculating recoveries for the compounds in the test mixture is limited because these are not necessarily the compounds that are detected in the soil samples. All compounds in the test mix were detected at a level of 10 ng/mL in the artificial samples. The signal-to-noise ratio (S/N) was calculated for bromomethane, dichloromethane, toluene, pentachloroethane, and naphthalene as representatives of the three VOC groups. The S/N was in the range of 1300–6000 for the selected compounds in the test mix, which indicates that detection limits for these compounds are in the range of 5–23 ng/L.

The method was optimized to allow

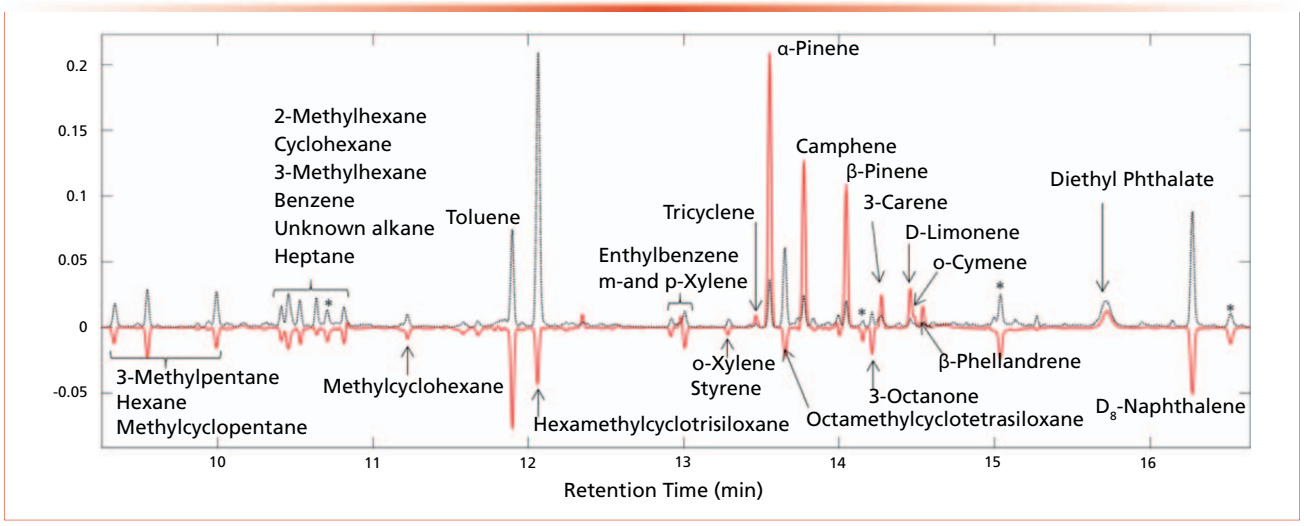

FIGURE 7: PC2 loading plot. Red line indicates PC2 loading coefficients and dotted line indicates the average TIC. Terpenes have positive loading coefficients while most remaining peaks have negative coefficients. Compounds have been tentatively identified through a search in the NIST14 database. Asterisks indicate unknown compounds.

for nontargeted fingerprinting of soil samples. The method optimization was therefore based on peak areas and the chemometric analysis was performed on TICs. Thus only qualitative and semi-quantitative data were presented. The nontargeted approach included all compounds that were detected compared to a targeted approach where only known constituents are analyzed. This provides improved information about the samples, and in this case, explains why soil

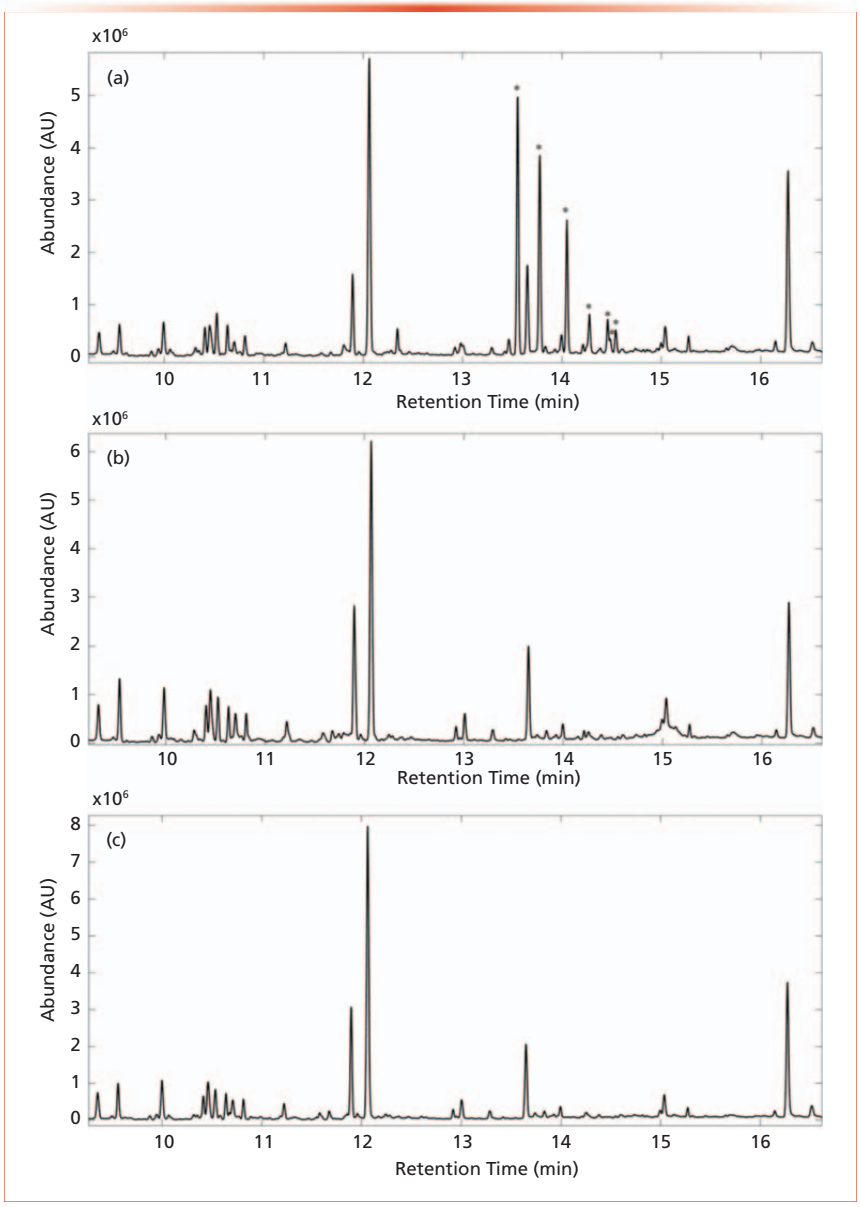

FIGURE 8: Representative TICs of (a) spruce, (b) beech, and (c) oak where *m/z* 1–34 and 44 have been removed. Tentatively identified terpenes are marked with an asterisk (see Figure 9 for names).

samples from a spruce forest are different from soil samples from beech and oak forests. This could potentially lead to identification of new biomarkers for land use. For full quantitative analysis, it would be necessary to run standards and obtain better estimates of detection limits and limit of quantifications and recoveries specifically for the terpenes detected in the nontargeted fingerprinting to improve their applicability as a biomarker for land use.

Conclusion

A DHS–TD–GC–MS method was successfully optimized through qualitative and semiquantitative analysis and applied to soil samples representing spruce, oak, and beech. Nontargeted chemical fingerprinting analysis of the TICs of soil sample extracts showed that soil samples representing spruce differed from soil samples representing beech and oak because of the presence of terpenes. The optimized method was successfully used for the comparison of VOCs in soil samples from the three forest areas and for detection of terpenes as potential biomarkers for land use. The fingerprinting approach could be useful in other areas of research, such as metabolomics and petroleomics, and is not limited to environmental samples.

Acknowledgment

The authors acknowledge the Water Research Initiative at the University of Copenhagen for funding the PhD project of Peter Christensen.

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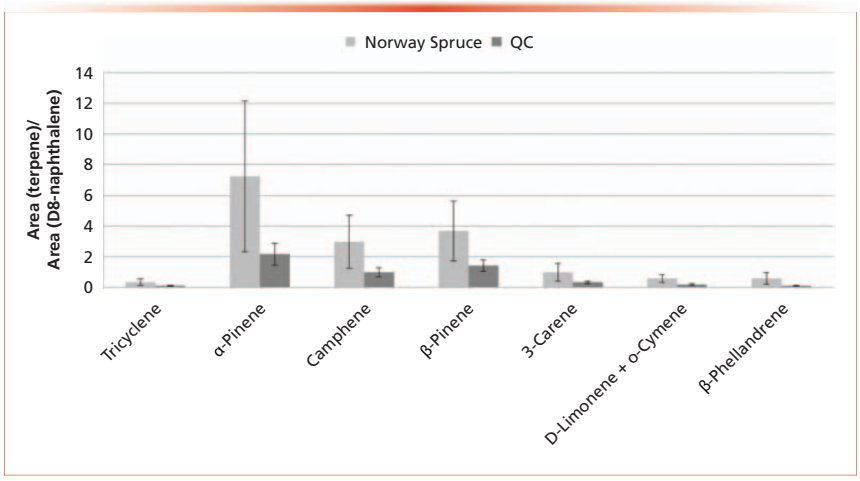

FIGURE 9: Precision of selected terpenes based on the area of the terpene divided by the area of d8-naphthalene for QC samples (analytical precision) and samples representing spruce (combined sampling and analytical variation, $n = 6$). Error bars are ± 1 standard deviation.

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A Q&A

Innovative and Robust Analytical Technologies to Address Key Challenges of Critical Quality Attributes Monitoring in Biopharmaceutical Development

Padraig McDonnell
Executive Vice President and General Manager
Chemistries and Supplies Division
Agilent Technologies

Biotherapeutics development requires accurate and robust analytical testing methodologies with dependable separations. Agilent is committed to the biopharmaceutical market, and has a company-wide initiative to leverage the entire product portfolio, application-specific total workflow solutions, and global presence to deliver the support customers rely on to make trusted decisions. In this interview with *BioPharm International* and *LCGC*, Padraig McDonnell, executive vice president and general manager for the Chemistries and Supplies Division at Agilent Technologies, explains how Agilent's products address customer pain points including poor reproducibility and difficulties with their instrumentation and methods.

BioPharm International and LCGC: Can you tell us a bit about your customers in the biopharmaceutical market and some of their specific challenges?

McDonnell: The future of biopharmaceuticals looks promising with life-changing treatments, and the field keeps growing, powered by innovative groundbreaking therapies to treat cancer and autoimmune diseases. Advancing these novel biotherapeutics safely in the clinic requires reliable manufacturing and quality control processes.

The complex heterogeneous nature of biotherapeutics requires accurate and robust analytical testing methodologies with dependable chromatographic separations. Identifying critical quality attributes (i.e., impurities that could impact the product safety and efficacy) is the most difficult step in the implementation of a Quality by Design approach for biopharmaceutical development and production.

Defining each product attribute is extremely challenging. Therefore, consistency of product quality becomes even more important. Some of the key challenges are accuracy, robustness, and reproducibility of the data. It all comes down to speed and efficiency of the workflow.

BioPharm International and LCGC: You mentioned speed and efficiency workflow as being of major importance. What can Agilent bring to those customers to help them solve some of those challenges and meet those demands?

McDonnell: We, at Agilent, design and manufacture our own AdvanceBio LC columns and consumables to match our customers' needs. It is important to recognize that several analytical techniques are used as part of our workflow solution. This includes sample preparation, separation detection, and data analysis. Each part of the workflow must work seamlessly with the other components to ensure trusted answers.

At the 2017 American Society of Mass Spectrometry (ASMS) conference in Indianapolis, we launched an end-to-end AdvanceBio solution for biologics characterization, focused on intact protein and peptide mapping, which are critical quality attributes. Reproducible chromatographic separation is key to these workflows.

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Our AdvanceBio Peptide Plus columns and PLRP-S columns are designed for reproducible performance. There is quality at every step of the columns manufacturing from the receipt of raw materials to the finished column. We know that quality is an important step to be productive and gain efficiencies in the overall process.

BioPharm International and LCGC: Can you provide examples of innovative solutions that your organization has brought to the industry in the context of biologics characterization workflows?

McDonnell: Let me pick one example among many, where Agilent has redesigned a product to address our customers' needs. During the biotherapeutic manufacturing process, there are many things that can cause a protein to aggregate into dimers, trimers, and higher order aggregates. This can be caused by many things: changes in temperature, concentration, pH, and so on.

Size-exclusion chromatography (SEC) is the preferred technique for quantifying these aggregates. Agilent developed an entirely new LC column for this purpose, and then demonstrated superior performance in terms of data reliability and quality. Not only did we address the issue of the column lifetime that presented challenges to customers analyzing aggregation, we also ensured the AdvanceBio SEC product would work with more complex molecules such as antibody–drug conjugates or ADCs.

BioPharm International and LCGC: Many companies are focused on biopharma, but what puts Agilent in a strong position to serve these customers also in the mid-to-long term?

McDonnell: We differentiate our commitment to biopharma in three major ways. First, about three and a half years ago, Agilent launched a strategic initiative to help solve our biopharma customers' challenges by offering complete end-to-end solutions. Since then, we've been bringing several innovative and easy-to-use solutions to the market. I talked about a recent solution launched at the ASMS 2017 that shows our continued commitment.

Second, Agilent is in a unique position, compared to any other vendor in the market, to leverage the entire product portfolio. Automated sample prep using the AssayMAP Bravo Platform, InfinityLab Bio-inert HPLC systems, AdvanceBio columns, AdvanceBio Standards, application-specific total workforce solutions, a global presence through Agilent CrossLab services and throughout Agilent, collaborating to deliver support to our customers that rely on making trusted decisions.

Regular customer contact and integrating the voice of customer is really critical to us. It gives us exceptional insight into the scientific challenges our customers face. These insights enable Agilent to put an intentional focus on biopharma, as we continue to develop new products and services that help our customers. Collaboration and cross-functional teamwork have enabled us to deliver new products and workflow solutions that better address customer analytical needs.

Third, we continue to invest in the biopharma business. On June 28, 2018, we announced that we entered into a definitive agreement to acquire privately held ProZyme, Incorporated, a leading provider of glycan analysis kits and standards. The acquisition will expand Agilent's portfolio of biopharma consumables in the fast-growing glycans space. We have a strong base of customers and expanding our consumables portfolio is key to our strategy. This fits our strategy to provide a complete workflow solution that will help biopharmaceutical companies reduce the cost and time required to bring new therapeutics to market. We stand by our customers on this journey, to help them develop safer and more effective biotherapeutics more quickly.

LCGC: Anything new on the horizon that we should look out for?

McDonnell: I would like to also tell you about the latest product that Agilent is launching, the AdvanceBio HIC column. Hydrophobic Interaction Chromatography is a powerful tool that can be used to separate out impurities that can prove too difficult to analyze by any other analytical approach.

The biotherapeutic industry has struggled with the products that were already on the market. Our customers explained the challenges they faced and we listened. Things like poor reproducibility, as well as difficulties with their instrumentation and methods, due to mobile phase conditions that are used (HIC often requires a high concentration of salt) were recurring themes that we heard from our customers.

Agilent has once again listened closely and used our R&D resources to address the pain points customers experience right now. As a result, we have developed a new product, the AdvanceBio HIC column, that has designed-in features to overcome many of these problems.

Data Integrity in the Chromatography Laboratory, Part VI: Open Culture, Training, and Monitoring Metrics

In the first five parts of this series, we have discussed data integrity throughout the analytical process. In the final installment, we look at three requirements for establishing and supporting data integrity in a regulated laboratory. These are an open culture, data integrity training, and quality and data integrity metrics.

Mark E. Newton and R.D. McDowall

This is the last of six articles on data integrity in a regulated chromatography laboratory. The first article introduced a four-layer data integrity model and then discussed sampling and sample preparation (1), the second focused on preparing the instrument for analysis and acquiring data (2) and the third discussed integration of acquired chromatograms (3). Article four discussed calculation of the reportable result (4) and the fifth one presented second-person review (5).

The Foundation of Data Integrity

A data integrity model was presented in that first article that consisted of four layers: a foundation layer and three levels above it (6,7). The model works like building a house: A firm foundation allows the three levels above it to function correctly. Therefore, for the final part of this series, we look at three topics within the foundation layer that are essential for supporting data integrity throughout the analytical process:

- An open culture
- Training for data integrity
- Metrics to monitor the analytical process and data integrity.

Establishing and Maintaining an Open Culture

Establishing and maintaining an open culture is the hardest part of a data integrity program. You can have all the procedural and technical controls plus training, but if you don't have the open culture and ethos, it will be wasted because management can put pressure on staff to cut corners.

The following sections discuss some of the key elements of an open culture.

Leading from the Top

Data integrity comes from the top of the organization. Senior management must ensure that they communicate their requirements for data integrity, and obtain feedback to ensure that their requirements are met. Communication is not a single e-mail to all staff, but is reinforced by including data integrity requirements in everybody's job description and objectives; also, an individual's performance for data integrity, in part, should be linked to pay.

In parts I–V of this series (1–5), we have had a running section "Is Management the Problem?" to discuss the impact management can have on a laboratory's approaches to data integrity. These are additional areas where management must be aware, to ensure that the laboratory staff protect data integrity and don't just pay lip service.

Changing the Mindset

A laboratory must move from a blame culture to a learning organization. This approach is illustrated by a quote from Deming (8):

> *"Fear invites wrong figures. Bearers of bad news fare badly. To keep his job, anyone may present to his boss only good news."*

There must be the ability to allow staff members to own up if they have made a mistake without the fear of being ridiculed or pointed out as inept. At this point, it is worth quoting from the U.S. Food and Drug Administration's (FDA's) Out of Specification (OOS) guidance on analyst responsibilities (9):

> *"If errors are obvious, such as the spilling of a sample solution or the incomplete transfer of a sample composite, the analyst should immediately document what happened. Analysts should not knowingly continue an analysis they expect to invalidate at a later time for an assignable cause (that is, analyses should not be completed for the sole purpose of seeing what results can be obtained when obvious errors are known)."*

Here is a requirement from the FDA for openness and honesty. The move to a learning organization now allows you to ask why a mistake was made. Can we learn from this and improve and prevent the situation from re-occurring? Following are a few examples of reasons for a mistake:

- A procedure is too complex to follow consistently.
- There is too much pressure to release a batch as production is waiting to ship.

TABLE I: Corporate procedures for data integrity

Document Title	Contents of the Document
Data Integrity Policy	• Corporate expectations for data integrity and ethics • Roles and responsibilities of all staff for data integrity • Open culture, expected behavior, and ability to admit mistakes • Raising data integrity issues in confidence • Investigation of data integrity violations
Good Documentation Practices	• Principles and requirements of good documentation practices • Defining raw data and complete data • Documenting paper processes • Documenting hybrid processes: paper and electronic records • Documenting electronic processes: electronic records
Evaluating and Selecting Analytical Instruments	• Process for evaluating, selecting and purchasing new analytical instruments and systems • Laboratory user requirements specification • Scientific evaluation of the instrument • Compliance evaluation of the associated software for regulatory compliance and data integrity gaps

- Missing a turnaround target time has too much influence on data integrity and data quality.

The GAMP Guide on Records and Data Integrity details the types of mistakes and their impact (10).

Observing Actual Practices

Closely linked to management leadership is a *gemba walk*, where managers get out of their offices and see what is happening first hand, rather than filtered through organizational layers. This practice is an opportunity for management to encourage data integrity, and for staff to inform management of problems with processes and systems. In part V (5), we mentioned that, without investment in laboratory automation and systems, the second-person review now can take longer than the actual analysis, slowing release of product to the market. Management must be made aware of such issues.

Equally so, a gemba walk can be an opportunity or staff to show management where data integrity successes have occurred, say by the elimination of a hybrid system as a result of auto-

TABLE II: Procedures for data Integrity in a chromatography laboratory

Document Title	Contents of the Document
Chromatographic Integration	• How to integrate chromatography peaks • Order of injection integration: SSTs, standards, and samples • Analytical procedures when you can and cannot integrate peaks • See part III for the content of this procedure (3)
Calculation and Rounding of Data	• How to round numbers • When to round numbers • See Part IV for the content of this procedure (4)
Second Person Review	• Who can be a second person reviewer • Procedure covers manual, hybrid, and electronic processes • See Part V for the content of this procedure (5)
Out of Specification Investigations	• Trigger for invoking the procedure • Laboratory investigation options • Scientific basic for OOS invalidation • See Part V and the FDA OOS Guidance for the content of this procedure (5,9)

mation. For more information on an open culture, see the ISPE Cultural Excellence Report (11).

Training for Data Integrity

One of the keys to success, ensuring both data integrity and regulatory compliance, is adequately trained and competent analysts. There are several policies and procedures that we first need to introduce, and then we can discuss how training needs to take place. First, we will consider procedures at a corporate level and second, discuss chromatography laboratory standard operating procedures (SOPs).

There are three high-level policies or procedures shown in Table I that we will discuss first along with the approaches for training.

A Data Integrity Policy lays out the principles for data integrity and ethos within the organization along with the expected behavior of all staff (6,7,10). This document is too important for a read-and-understand approach when training the staff; additionally, such an approach will not lead to consistency of action. A much better approach is offered by the National Environmental Laboratory Accreditation Conference (NELAC) (12), and outlined in more detail (6,7). There needs to be an introduction to the session by management in which the policy is viewed and explained with examples of both required and prohibited actions. To reinforce the training, copies of the policy and all training materials should be given to each attendee to make their own notes. Because of the importance of this subject, we recommend an assessment at the end with a high pass mark. After the training has been passed, each employee should sign a form declaring that he or she understands the training and the consequences of failing to follow the policy. Staff that fail the assessment should retake the whole of the training and assessment.

Good Documentation Practices (GDocP) training needs to be undertaken in a similar way to the data integrity policy with a copy of the procedure and the training materials followed by an assessment (6,7). Although most laboratories have a procedure for GDocP, those procedures focus mainly on paper records. This policy needs to be extended to include hybrid systems (including record–signature linking) and electronic systems. The procedure needs to cover what is meant by *complete data* and *raw data* (13) in a laboratory.

Evaluation and Selection of Analytical Instruments and Systems. With the issue of the new version of *USP* <1058> on Analytical Instrument Qualification (14), there is an opportunity to update laboratory procedures to ensure correct specification, evaluation, and selection of new instruments and systems (15). There is little point in assessing and remediating current processes and systems if the laboratory continues to purchase inadequate systems that also require remediation before they are operational. Accepting these inadequate systems increases the use of logbooks, which slows the second-person review, as discussed in the fifth article of this series (5).

Focusing on the chromatography laboratory, there are four main SOPs that impact data integrity, as shown in Table II:

- Chromatographic integration
- Calculation and rounding
- Second-person review
- OOS investigations.

Because these SOPs have been covered earlier in this series, we do not propose to discuss them further and readers are referred to the applicable part of this series in Table II.

Data Integrity Metrics

As background for data integrity metrics, Newton and McDowall published an overview on the subject in *LCGC Europe* (16). This article contains the requirements from the various data integrity guidance documents on quality metrics (17,18). It is worth quoting the following note of caution before any metrics are considered (18):

> *"Caution should be taken when key performance indicators are selected, so as not to inadvertently result in a culture in which data integrity is lower in priority."*

Metrics should be collected automatically to prevent bias. When starting to use metrics, keep it simple at first (16). Some key metrics can be used to monitor the calculation process, as described below.

Runs Aborted

Reporting runs that were started, but not concluded, can point toward analysts

TABLE III: Key learning points from the six-part data integrity series

Topic of the Part	Key Data Integrity Issues
Sampling and sample preparation	• Ensure each sampling plan is scientifically sound and that samples are taken and labeled correctly. • Automate the collection of sampling information. • Automate sampling and sample preparation to reduce dilution and extraction errors.
Preparing a chromatograph and acquiring data	• Consider risks for entry or transfer of factors, weights, or other assay values for calculations. • Limit storage locations of injection results to avoid diversion of data and potential of creating orphan data. • System suitability failures must be scrutinized to rule out the possibility of using suitability failure to eliminate undesirable sample results.
Integrating and interpreting data	• Do not use integration techniques to mask a poor method; instead, fix the method. • All actions for integration, calculation, and reporting of data should be directed from a chromatography SOP. • Sample results should not be integrated until after a run's acceptability is established, to avoid potential issues of testing into compliance.
Calculation of the reportable result	• The order of calculations is important to avoid testing into compliance. • Use CDS or LIMS/ELN for method calculations whenever possible. • Avoid the use of spreadsheets due to their data integrity risks. • Interfaces (human and machine) are potential data integrity issues and their risks must be managed.
Second-person review	• The review must include all raw data necessary to conform to regulations. • Testing data excluded from final result calculations must be included for review. • Paper records—especially logbooks—can make second-person review longer to complete than performing the analytical method. This timing is improved with electronic records.
Training, culture, and metrics	• An open culture is a critical factor to maintain data integrity. • Training must include every person's responsibility to ensure data integrity. • Automated metrics can help identify potential signals or trends that merit additional scrutiny.

looking at data during the run, then making the decision to terminate the run to avoid accepting data they believe may be OOS, out of trend (OOT) or out of expectation (OOE). Aborted runs, in a well-controlled GMP environment, should always be viewed with a suspicious eye.

Short Runs

Reporting runs that have fewer than an accepted number of injections (for example, three injections) is a means of detecting analysts who re-inject a sample to obtain a new result that can replace one from a previous injection.

Run Evaluation Sequence

As mentioned in part III of this series (3), there should be a procedural order for processing a chromatography run:

1. evaluation of system suitability
2. evaluation of reference standard acceptability
3. evaluation of method acceptance criteria
4. evaluation of sample results.

It is possible to create reports that ensure this sequence of events is happening, based on time stamps of events. This report can point toward analysts evaluating sample results before other acceptance criteria, then finding means to reject the run, such as manipulating standards or suitability to ensure failure of the run—a type of "testing into compliance."

Recalculated Dataset

Monitoring runs that are calculated more than once has two benefits: It is one means of looking across runs for potential improper activities, but it also can point out methods that are not well configured, and therefore require additional manual intervention. Recalculations and manual integrations not only have data integrity impact, but lab efficiency as well.

Manual Integration

For each analytical method at each site, report the number of peaks automatically integrated and manually integrated. This metric provides insights that lead to more automated integration. For example, Site A automatically integrates 80% of all peaks for method Y, whereas all other sites using the same method automatically integrate only 30% of their peaks. What do analysts at Site A know about this method that permits such a high level of automated integration?

Benchmarking

For each report type, generate a summary report that compares the number of records found by site. This summary report permits comparisons, and reveals sites that have unusually high (or low) activity compared to other sites. For example, a site with twice the number of aborted runs as other sites might lead to a quality assurance inquiry to understand the high number of aborts. Perhaps equipment issues, a fragile method, or poor behaviors are the root of the issue, but the report creates the signal that starts the investigation.

Metrics Governance

For companies with multiple sites of operation, a supervisory layer of metrics should be created to provide a view of metrics reports. At a minimum, this supervisory layer should provide counts for the type and number of reports generated (either visually or on paper) for each site. This provides insight to

A Q&A

Improving Sample Preparation for LC and GC Analysis of Plastics, Foods, and More

Alicia D. Stell, PhD
Product Manager
CEM Corporation

New technology helps labs streamline sample setup and extraction for faster progression to analysis.

Time is money for analytical laboratories, and a new technology for liquid chromatography (LC), gas chromatography (GC), and mass spectrometry (MS) sample preparation is a breakthrough for such labs—yielding comparable recoveries with traditional techniques in a fraction of the time. EDGE, an automated system produced by CEM Corporation, combines pressurized fluid extraction with dispersive solid-phase extraction to prepare samples in as little as five minutes. *LCGC* recently spoke with Alicia D. Stell, PhD, product manager at CEM Corporation, about how the EDGE accelerates steps from extraction to analysis.

LCGC: To start, what is the EDGE?

Stell: That's a great question. The EDGE is an acronym: energized dispersive guided extraction. It's a new technology that combines the processes of pressurized fluid extraction and dispersive solid-phase extraction in one instrument. We can accomplish this because of our innovative Q-Cup technology, which enables us to have a dispersive open environment within a pressurized cell.

With this technology, solid or semisolid samples can be extracted in five minutes using about 30 mL of solvent. So, the entire process—extraction, filtration, cooling, and washing—is complete in five minutes, and then the sample is ready for analysis.

LCGC: Who benefits from using the EDGE?

Stell: It's very widely applicable; anybody doing organic sample preparation would benefit from the EDGE. So, if you're doing an extraction of a solid or semisolid sample and then conducting LC or GC–MS analysis, you should consider using the EDGE.

Some existing techniques you might be using include pressurized fluid extraction, Soxhlet, or QuEChERS. Typically, you wouldn't hear those three techniques and think, "Hey, if I'm using any of these, I can think about using the EDGE." But because the EDGE combines both pressurized fluid extraction and dispersive solid-phase extraction, it really opens up many different techniques you might be using on just one instrument: the EDGE.

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LCGC: How does the EDGE extract fats from foods?

Stell: That's a good question. With the extraction of fats from food, you have this important consideration: Are you looking at just bound fat, or at both bound *and* unbound fat?

If you only have unbound fat—samples such as meats, nuts, or grains—you can directly extract on the EDGE. You'd just run your sample in a typical solvent, such as petroleum ether, and in five minutes, the total fat is extracted and you're ready to go to your analysis.

But if you had a sample with both bound and unbound fat, then you'd need to do a pre-hydrolysis, followed by your extraction on the EDGE.

Pay attention to CEM's website because within the year, we'll be releasing a hydrolysis unit in which you'll be able to do both those steps. Right now, the EDGE can accommodate any sample that contains unbound fat, the soon-to-be released hydrolysis unit will be able to accommodate any sample that would need hydrolysis and extraction such as dairy and seafood.

LCGC: Can the EDGE perform QuEChERS?

Stell: EDGE is an alternative to QuEChERS. QuEChERS, by definition, is a two-step process that does salt-partitioning extraction and then a dispersive solid-phase extraction cleanup. The EDGE does pressurized fluid extraction, so the actual extraction process is different. It can also do in-cell cleanup. So, that dispersive solid-phase extraction is essentially the same, but it happens in just one step on the EDGE.

For any sample that you're currently using with QuEChERS for dispersive solid-phase extraction, whatever sorbents you're using will be compatible with the EDGE. If you're preparing your sample for the EDGE, you'd simply pour in your sorbents for the dispersive solid-phase extraction cleanup step, add your sample, and then load that into the EDGE. And in five minutes, you've got your extracted, cleaned-up sample.

LCGC: Are there any applications for consumer products?

Stell: With now being summer, I was thinking about sunscreen. On the EDGE, not only can you extract the sunscreen to look for a particular allergen to make sure that the product's appropriate for a wide range of end users, but you could also think about the sunscreen bottle itself and see if there's anything leaching out that could potentially be harmful. You could perform both of those cases—extracting the sunscreen or extracting the components of the bottle—on the EDGE.

Consumer products hit us all personally—we can all relate to them—and they bring home how applicable the EDGE can be to helping us figure out what contaminants could be in the materials we're using on an everyday basis.

LCGC: Are there carryover concerns with EDGE?

Stell: No. In any situation where you're doing a serial extraction, carryover will be a concern because that same set of lines that your sample saw is going to see your next sample. So, you want to make sure that you do a really good wash.

On the EDGE, we have great control over our washing parameters. You can do up to five different washes using a different solvent for each one, and each wash could be from 5 mL to 30 mL.

The EDGE gives you so much control. Let's say you're extracting fat—pepperoni could be up to 40% fat, for instance, so there's a lot of fat that could potentially carryover. But we make sure that won't be a problem on the EDGE because we have such fine control over washing. And that gives us such versatility there.

LCGC: Ultimately, how does EDGE compare to other techniques?

Stell: If we compare EDGE to general pressurized fluid extraction, I think about simplicity. The cell design has two simple pieces and the Q-Disc for filtration. So, in a matter of seconds, you can assemble your cell and begin extraction.

That time saved—literally preparing your sample in seconds, and then analyzing it five minutes later—is a big advantage. If you were doing an ultrahigh-pressure liquid chromatography (UHPLC) analysis and a standard 15-minute run, you'd literally be looking at your data in less than 30 minutes and figuring out how to fine-tune the method.

But on an even grander scale, I'd look at Soxhlet. Soxhlet is a gold-standard method: it's tried and true, and it works. But it takes a long time—hours—and hundreds of milliliters of solvents. Meanwhile, the EDGE yields the same efficiency in terms of extraction, but in a matter of minutes, with only 30 mL of solvent.

the question, "Are people using the reports in our operations?" Failure to use reports indicates either a lack of understanding about the reports, or a lack of report effectiveness. In addition to use frequency, the number of investigations and number of issues uncovered should be monitored to assess the effectiveness of metrics. Reports that seldom lead to discovering real issues should be modified or replaced with more effective reports.

Ideas for Metrics

The best ideas for monitoring metrics often come from regulatory enforcement actions (for example, Warning Letter, Notice of Concern, and so forth). The only twist is to read the cited deficiency and ask yourself, "How would we detect this situation in our own operation?" This question will cause you to think about the data patterns that accompany the behavior and then to formulate a query that could detect the data pattern. For example, a firm is cited for manipulating the system clock to falsify timestamps in audit trail records. If this falsification happens, there could be a series of system audit trail entries, one for each clock adjustment. In addition, there will be some frequently written audit trails (such as intersystem messages) where the clock will appear to go backward because of the clock manipulation. So, a query that checks for clock entries that do not continue to increase could flag clock manipulation behavior.

Limitations of Metrics

It is important to remember that all metrics are not created equal; some will prove more effective than others in your operation. In addition, metrics seldom identify a true issue with every reported record in a report. Rather, they highlight suspicious records that require a human to investigate. This investigation requires a time investment, and therefore becomes a limitation on reporting effectiveness. Finally, some real issues will not be detected in a report, such as reanalyzing a sample on a simple instrument (for example, a pH meter), picking the desired outcome and forwarding it to laboratory information management system (LIMS). This data integrity issue will not be detected on any report.

Summary

Over the six parts of this series, we have covered the whole of the analytical process for chromatography. To conclude, we would like to summarize the key points from each article (see Table III).

Data integrity in the chromatographic process requires a holistic look at the end-to-end process, identifying places in the process where actions can impact the integrity of the reportable results, then putting controls in place to mitigate the risks. In addition, metric reports must be identified from known issues, to observe the process at a more abstract level, looking for potential signals or trends that deserve closer investigation by qualified personnel.

These actions require the support of senior management, who provide the needed resources for governance and training, and more importantly, who lead by example and regularly inspect the operation to ensure that controls are both used and effective for their purpose.

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Instrument Considerations in the Transfer of Chromatographic Methods, Part I: Method Considerations

Successful transfer of chromatographic methods often proves more challenging than expected. This three-part article series will summarize the details of this process with particular attention to the sources of confusion and to some suggested solutions for the observed difficulties. In part I, the focus will be on the method as a whole and considerations for avoiding and eliminating ambiguities and inconsistencies between laboratories. In part II, the characterization of the system will be emphasized. In part III, emphasis will be placed the techniques for bringing the modules of the two systems into alignment.

Thomas E. Wheat

Liquid chromatography (LC), in its instrumental form, is among the most common tools used for chemical analysis. The technique is fundamental in research, product development, quality control, natural product analysis and purification, and clinical testing across a wide range of application areas. Much effort and time is devoted to developing chromatographic methods for routine use. These methods must reliably separate each potential sample component in a way that is suitable for unequivocal identification and for quantitative analysis. These well-developed methods become part of standard operating procedures and, ultimately, regulatory documents, as well as being very desirable for use in related laboratories. This transfer of methods has proven generally challenging in many cases. This is a very large topic, and we will focus this discussion on some subsets of the general problem.

Method transfer can be attempted for several reasons. A laboratory may try to implement an established method when working on the same or a similar analytical problem. This process tends to be fairly simple since the original method is only a guidance to a starting point. At the other extreme, a laboratory may be implementing a method to obtain the exact same result as the originating laboratory. This approach can occur within an organization or company because of a need for expanded capacity, or to analyze the same sample types at a new location. In the latter case, it is planned to duplicate exactly the chromatogram obtained in the originating laboratory as documented in a standard operating procedure or regulatory document. In this case, the same qualitative and quantitative analytical results are required. Between these extremes, the range of possible objectives includes a desire to improve a method in any of several ways, including modernizing the materials and instrumentation, reducing run time or operating cost, improving sensitivity or accuracy, or adding validation for additional analytes like newly recognized impurities. For this discussion, we will focus on the most rigorous method transfer leading to the duplication of results of the established methods without improvements to the method. The considerations in this approach also apply to the other forms of transfer, with some additional factors to be considered elsewhere. We will assume that the methods under consideration include detailed materials and methods, system suitability criteria, and expected results with acceptable limits. We will also include consideration of the many things that can go wrong in such an exercise.

This discussion will be divided into three parts. In this first part, we will consider those aspects of the method itself that affect the transfer of the method. In the future, part II will address the chromatographic systems, and part III will consider the details of aligning individual instrument modules.

General Considerations for Effective Method Transfer

A consistent principle in the discussion to follow is the elimination of all sources of variability, particularly in the early stages of the method transfer. The use of identical materials and methods creates a stable point for any further use. After the method is operating as specified, modifications and substitutions can be made in a controlled way. Although this principle seems obvious, it is often not observed in practice. At the same time, as we will emphasize below, it is assumed that the originator of the method followed all the same principles and controls in developing the method. Uncontrolled and undocumented elements contribute to a method that is difficult or impossible to transfer.

The elements that must be considered in a successful method transfer

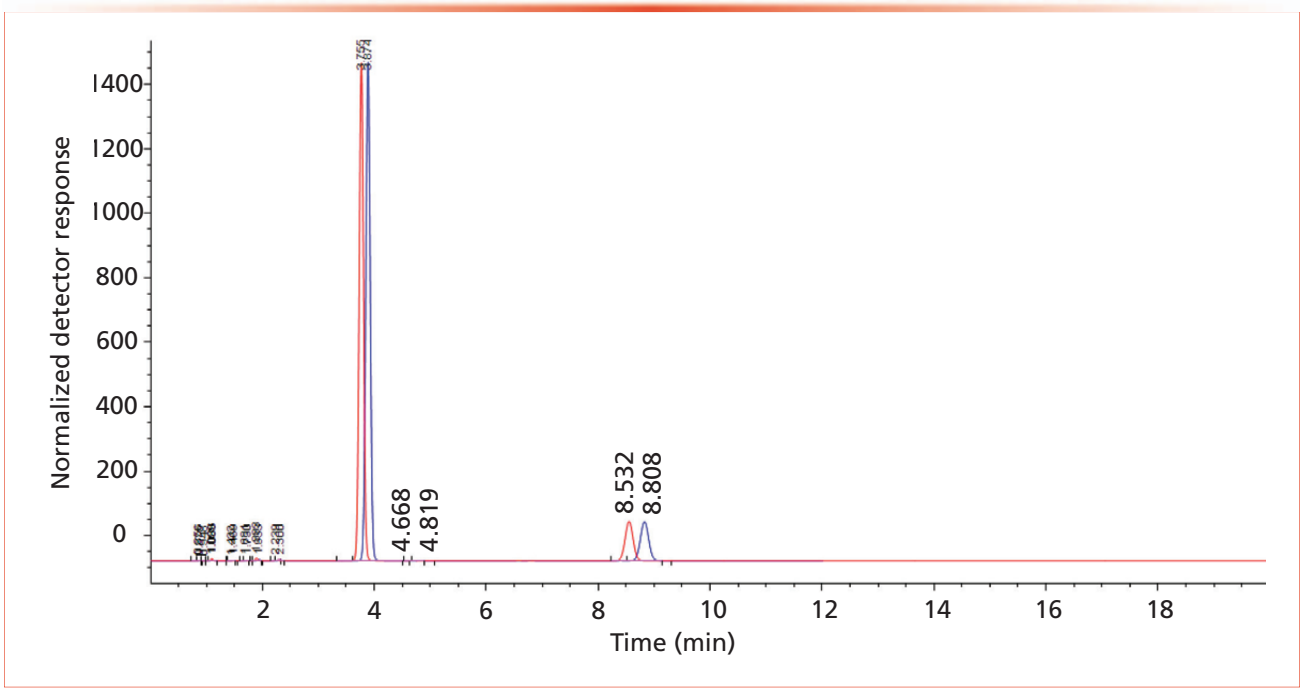

FIGURE 1: Effect of Solvent Preparation Protocol: Red trace – Mobile phase prepared on demand with instrument blending; Blue trace – Mobile phase prepared volumetrically by laboratory scientist.

include the procedures, the sample and its preparation, the available chemicals, the ancillary laboratory instruments and equipment, the chromatographic column, the mobile phase, and the chromatographic instrument itself. In addition, the skills and experience of the laboratory scientist or operator are often overlooked variables in the process.

Even highly skilled laboratory personnel differ in their knowledge and in the exact way that they do particular tasks. If at all possible, it is desirable to establish communication between the new operator and either the developer of the method or a person who has routinely and successfully executed the method. The consultation should begin at the start of the process, and be available until successful transfer is accepted. If such collaboration is not possible, it would be useful for two people to share the transfer exercise to allow for different experiences and consideration of different details in execution of procedures.

The written procedures for the established method are expected to provide sufficient information and detail for reasonably trained and skilled scientist. Every individual who prepares such a document tries to meet these criteria. We must, however, recognize that each of us who writes such procedures is affected by the things that they know and by the details that they recognize without additional thought. We all omit writing down the things that "everybody knows." Our intended audiences do, however, know somewhat different things. We should remember for example that there are at least four reasonable ways to prepare 50:50 methanol–water, as discussed below. The same is true of a buffer, such as 25 mM sodium phosphate, pH 7, also with four different recipes. An example of the differences in the separation resulting from different preparation protocols is shown in Figure 1. The differences in retention are small, but they exceed the width of the peak. Each detail must be described as explicitly as possible to ensure that it will be faithfully replicated in the new adopter laboratory. This guideline becomes more important each year with increasing globalization. The receiving laboratory may not share the primary language of the originator. Incorporation of more detail, rather than less, is the best practice.

The sample and its preparation can often prove the most challenging aspect of a method transfer. Because this is such a large and diverse topic, we will not consider it in this discussion. It is worth noting that the steps in sample preparation, including preparation of the standard, require the same level of detail and specificity as the other parts of the method.

The chemicals chosen for the transferred method will often be chosen based on those readily available in the laboratory or in a local stock room. This approach often proves unwise. The reagents used for standard and sample preparation may contribute chromatographic peaks or compromise the recovery of important analytes. The best practice is to duplicate the reagent set, not just grades but even the same chemical vendor where possible. Although it may seem unnecessary to purchase new supplies of common reagents, it is desirable for the first trials in a method transfer to eliminate as many variables as possi-

ble. After successful chromatographic analyses are obtained, it is possible to change reagents in a controlled way, confident that any consequences of reagent composition can be quickly recognized. This statement assumes that the originating laboratory used reagents that were relatively fresh and uncontaminated.

A variety of laboratory equipment, in addition to the liquid chromatograph, will be used in executing the method. This equipment includes instruments such as pH meters, balances, digital pipets. All these devices must be maintained in a reliable, calibrated state to be used in a successful method transfer. It should be obvious that this calibration and maintenance must be rigorous on both sides of the transfer. For example, incorporating an erroneously measured pH into a procedure can preclude successful use of the method by anyone else. In addition, it is commonly found that accessory instruments are not identical. Particularly with respect to sample preparation, the results can be different with some types of tools. This advice applies not so much to balances and pH meters as it does to homogenizers or centrifuges.

The Chromatographic Column

The chromatographic column is a frequent source of discrepancies between laboratories. Such difficulties should be easily avoided by restricting initial trials to the use of only the exact column specified in the method. The major column manufacturers have become quite rigorous in maintaining consistent properties within a particular brand of column of a given particle size and dimensions. Batch controls are good, and it is possible to obtain multiple columns from a single batch, as well as columns from a representative set of batches. Problems can arise when a different column is substituted for that specified in the method. In addition, difficulties have often arisen when a previously used column is chosen for a transferred method. This approach is extremely unwise because the history of a column is usually unknown and impossible to duplicate. Such prior use may leave some trace residue on the column surface, or otherwise modify the chemistry. As always, this principle can only be successful when the originator of the method established operating limits with a column with no history, and screened multiple columns from multiple batches.

Mobile-Phase Preparation

Preparation of the mobile phase has proven to generate more difficulties than one would anticipate. Such problems generally arise from either the quality of the mobile-phase components or from the preparation. The components of the mobile phase, both aqueous and organic solvents, can distort the baseline, contribute additional chromatographic peaks, or alter the selectivity of the separation. Water is an especially variable solvent, so either an on-demand laboratory water purification system or high-purity bottled water is safest. Organic solvents should be the highest possible quality, especially for the first test, and they must be at least the specification used in the originator trials. All solvent components, including any additives, should be freshly opened and prepared.

It is possible to prepare the mobile phase differently from the originator laboratory. As noted above, there are at least four reasonable ways to make 50:50 methanol–water: place 500 mL of water in a 1000-mL volumetric flask and bring to volume with methanol; place 500 mL of methanol in a 1000-mL volumetric flask and bring to volume with water; measure 500 mL of methanol in a graduated cylinder and 500 mL of water in a second graduated cylinder; and weigh 500 g of methanol and 500 g of water. These four formulations will give measurably different retention and chromatographic selectivity. There are even more ways to prepare 25 mM sodium phosphate, pH 7.00: titrate 25 mM phos-

phoric acid with concentrated sodium hydroxide; titrate 25 mM monobasic sodium phosphate with concentrated sodium hydroxide; titrate 25 mM dibasic sodium phosphate with phosphoric acid; blend solutions of 25 mM monobasic sodium phosphate and 25 mM dibasic sodium phosphate; mix specified volumes of 25 mM monobasic and dibasic sodium phosphate as calculated from published values for pK_a or from commonly available tables in reference books; or weigh the amounts of solid monobasic and dibasic phosphate salts, again as calculated. These different formulations may give altered chromatography in reversed phase, especially for ionic analytes, and will certainly affect ion exchange chromatography. In addition, problems commonly arise from failure to observe the different formula weights associated with different hydration states of salts and from not correcting for the effects of temperature on pH measurement.

It would be best practice in developing and documenting a standard method to choose one of the alternatives described above and to write a detailed description of what was actually done. Gravimetric preparation of aqueous–organic mixtures is probably most exactly communicated, but allowance must be made for not every laboratory having a balance of sufficient capacity for accurately weighing the required amount of solvent, typically more than a kilogram.

An alternative approach to mobile-phase preparation has been suggested and is somewhat frequently used. It is possible to use a multisolvent chromatographic pumping system to blend pure solvents on demand. The desired percentages of each solvent are programmed into the gradient table or the isocratic pump control. This approach removes the manual preparation steps to reduce labor. It tends to be more reproducible than manual blending since fewer measurements are made. Although the technique generally gives accurate results, there are sources of imperfect results that are discussed in some depth below in the context of instrumental characteristics. Purely from the perspective of solvent blending, it would be good practice to compare a batch of preblended mobile phase with the results from an instrument-blend. If the results are both within the specified limits, the labor-saving technique can be implemented. Typical results are shown in Figure 1.

Instrumentation—General Considerations

The last topic to be considered, the chromatographic instrument itself, is also the largest. The common principle applied for all other considerations—use exactly what was used in the originator's laboratory—is desirable here. It is, however, very often impossible to maintain consistency. The instruments selected in various laboratories are often different models or brands, and it is not usually financially sensible to purchase chromatography instruments for each specific, new method to be implemented. Furthermore, the usable lifetime of a method is often much longer than that of an instrument. Matching an instrument, therefore, may not be possible to begin and execute a method transfer. We must, therefore, consider the differences among instruments that can affect method transfer. The transfer of a method from one instrument to another may require some adjustment of the method. Many laboratories adhere to the guidelines found in Chapter 621 of the current *United States Pharmacopeia* (*USP*) (1). The currently applicable chapter specifically states

> *"Adjustments to the specified chromatographic system may be necessary in order to meet system suitability requirements. Adjustments to chromatographic systems performed in order to comply with system suitability requirements are not to be made in order to compensate for column failure or system malfunctions. Adjustments are permitted only when . . . adjustments or column change yields a chromatogram that meets all the system suitability requirements specified in the official procedure."(1)*

These guidelines, often mentioned as "<621>," specify ranges of changes to the method that may be implemented without revalidating the method. The chapter has been summarized in many places, but the original document should always be consulted. We will allude to specific items in the context of specific challenges in method transfer. It should be emphasized that many laboratories follow these limits and practices, but they are not universal regulations. They are absolute requirements only for *USP* compendial methods.

Conclusions

We have considered, to this point, the characteristics of a method that can affect the transfer of a chromatographic method from one user or laboratory to another. Some suggestions have been included for ways to avoid difficulties. It has been noted that many of these difficulties are rooted in method descriptions that can be interpreted differently by well-trained scientists or executed in alternative ways by skilled laboratory workers. There has been an emphasis on providing detailed descriptions that can minimize such differences. In the next installment, the focus will shift to the instruments used for the origination and the execution of the chromatographic method.

References

(1) General Chapter <621> "Chromatography" in *United States Pharmacopeia 40 National Formulary 35* (USP 40-NF 35, United States Pharmacopeial Convention, Rockville, Maryland, 2017), pp. 508–520.

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THE ESSENTIALS

Excerpts from *LCGC's* professional development platform, CHROMacademy.com

LC–MS Peptide Mapping: Where to Start, and What It Can Tell Us

To identify or fully characterize a protein biopharmaceutical, it must be broken down into smaller segments or peptides. This process requires proteolytic enzymes to digest the protein into peptides, and is referred to as *bottom-up proteomics*. A large amount of information can be acquired from biopharmaceutical analysis, including specific post-translational modifications (PTMs) and the protein glycoprofile (the degree and type of glycosylation). However, PTMs can only be isolated to specific amino acid residues when assessed at the peptide level. Great care and consideration is therefore required during the digestion process, because the proteolytic enzymes used and the conditions employed (pH, temperature, and storage time) not only affect the overall number of peptides liberated, but also the stability of associated PTMs, and can even introduce protein modifications of their own.

Broadly speaking, the digestion process can be broken down into three discrete and separate steps: reduction, alkylation, and digestion.

Reduction is commonly accomplished with an acid-labile surfactant that acts to remove the higher order structure of the protein, and exposes internal disulfide bonds ready for reduction by dithiothreitol (DTT), a small-molecule redox reagent. The pH is maintained at physiological levels throughout the process and buffers are used to ensure that the pH levels are appropriate. To prevent reformation of disulfide bridges across the thiol groups of the cysteine (C) residues, the protein is then incubated with an alkylating agent such as 2-iodoacetamide (IAA), once again at physiological pH. The final stage is the addition of a proteolytic agent (trypsin, for example), which is capable of site-specific protein digestion. Trypsin cleaves proteins at the C-terminal side of both lysine (Lys/K) and arginine (Arg/R) residues, unless either is proceeded by a proline (such as KP or RP). For this reason, all resultant peptides, apart from the C-terminal peptide, terminate in either a lysine or arginine residue. Additional and alternative proteolytic enzymes that produce other specific cleavage sites are available and routinely used.

Typical ultrahigh-pressure liquid chromatography (UHPLC)–UV conditions for the separation of the peptides created by digestion follow:

Instrument:	UHPLC
Column:	250 mm x 2.1 mm, <2-µm d_p fully porous particles (FPPs) or <3-µm d_p superficially porous particles (SPPs), C18
Mobile-phase A:	0.05% trifluoroacetic acid
Mobile-phase B:	0.05% trifluoroacetic acid in acetonitrile
Flow rate:	300 µL/min
Gradient:	0–2 min: 1% B, 2–35 min: 1–45% B
Column temp.:	60 °C
UV:	214 and 280 nm

Long (250 mm) but narrow (2.1 mm) high performance liquid chromatography (HPLC) columns, packed with either sub-2-µm FPPs or modern sub-3-µm SPPs bonded with C18 alkyl chain ligands as the stationary phase, are used to generate sufficient peak capacity to separate the large number of peptides created. Typically larger pore sizes (100–300 Å) are used to avoid peak broadening.

Gradients start with very high aqueous, sometimes as high as 100%, and the organic is then ramped up to mid concentrations, typically around 50%, over approximately 30 min. Care is needed to optimize the gradient slope (ramp time) to optimize the separation, and one should note that elution order (selectivity) can be greatly affected by small changes in the gradient ramp rate.

Standard mobile phases are 0.05– 0.1% trifluoroacetic acid in aqueous solution as the polar (A) solvent, and 0.05–0.1% trifluoroacetic acid in acetonitrile as the organic (B) solvent, with trifluoroacetic acid acting to reduce pH to afford good ionization of the peptide, suppress stationary-phase silanol ionization to give good peak shape, and afford retention of the peptides via ion pairing. One should note that lower concentrations or alternative reagents may be required if mass spectrometric detection is required.

The flow rate very much depends on the internal diameter of the column, but volumetric flow rates of 200–300 µL/min for 2.1-mm i.d. columns are the norm. Wider columns will require higher volumetric flow rates to maintain a suitable linear velocity.

Elevated temperatures are common, with 60 °C often favored to improve mass transfer kinetics and maintain good peak efficiency (sharper peaks). In routine production and QC environments, two detection wavelengths (214 and 280 nm) are typically monitored to give good sensitivity for the various peptide subunits.

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A Q&A

GPC/SEC Adventures in (Bio)Polymer Analysis: *"Perfect Separation Solutions"* from Our Contract Analysis Lab

Claudia Lohmann, PhD
Independent Polymer Consultant
PSS-Polymer Standards Service – USA Inc.

More sophisticated chromatographic techniques are needed to characterize increasingly complex macromolecular materials.

Modern macromolecular materials can be tailored to fit virtually any application, but their characterization has become more challenging as a result. More sophisticated chromatographic techniques are required to reveal important architectural details in the molecular structures. The separation techniques applied can be as diverse as the macromolecules themselves. *LCGC* recently sat down with Claudia Lohmann, PhD, independent polymer consultant to PSS-Polymer Standards Service – USA Inc., to discuss how PSS can help solve these analytical problems, who its customers are, and samples that are particularly challenging to analyze.

LCGC: Why is it an adventure when you work with macromolecules?

Lohmann: Macromolecules, especially modern purpose-design materials, have so many interesting features and applications. Macromolecules can be tailored to fit virtually any requirement and are used in all industries. For instance, without plastic, there would be no civilization. At PSS, we are constantly surprised and amazed to learn about the different applications of macromolecules.

Because of the complex and sophisticated nature of these macromolecular materials, conventional calibration techniques based on narrow standards no longer provide a complete answer with respect to molar mass and chemical composition distribution. It is very challenging. The analysis of modern polymers can be compared with trying to find the best way out of a maze or a jungle.

At PSS, we appreciate and understand the complexities of these materials. Therefore, we can apply our expertise to the characterization of large molecules with respect to correct sample preparation and choice of separation technique/s to produce accurate, reliable, and useful data.

For example, PSS was able to identify the components in a polymer protection film made the late 1950s that nearly destroyed a historic document from the 12th century. In other examples, we discovered why paint wouldn't stick on the heels of shoes and how milling degrades different types of starches. In a more modern instance, PSS quantified the copolymer in a tablet coating, and we developed and implemented a method for European gelatin manufacturers.

LCGC: How can PSS Labs help with solving problems?

Lohmann: The scope is broad, ranging from simple batch-to-batch comparisons to complex detective work. PSS can establish and perform quality-control procedures to run samples under standardized conditions with dedicated instrumentation or column sets.

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PSS can also deformulate products to investigate possible patent or intellectual property infringement and provide a project plan for how to identify specific sample components, such as polymers and additives etc. PSS can help develop gel permeation chromatography (GPC) and interaction polymer chromatography (IPC) methods that can be easily transferred to a customer's lab.

Importantly, PSS tailors conditions to accommodate the customer's sample requirements instead of trying to make things fit that just do not fit. The bottom line is, PSS does not just give numbers to its customers. We supply answers that are both needed and useful.

LCGC: How is PSS of service and whom does PSS serve?

Lohmann: PSS works with customers in all aspects of academia and industry. Our industrial clients dealing with polymers are in the automotive, food, wood, biotech, and pharmaceutical industries and also work with medical devices, and specialty chemicals. Our customers in academia, at universities, and in renowned research facilities all value PSS's scientific input.

Customers contact PSS with either a specific application problem or a request for routine analysis. We then have an interactive discussion with the customer to confirm the initial analysis request. In some cases, we can provide extra information that the customer was not aware would be possible.

For example, in addition to molar mass PSS can supply composition information. If the request is straightforward (e.g., determination of a mass distribution by a conventional calibration or light-scattering detection or a simple batch-to-batch comparison), PSS provides the customer with a quote and a sample information sheet. The turnaround time is rapid.

LCGC: In your experience, what has been the most challenging sample to analyze, and why?

Lohmann: A very challenging (and smelly!) project was on sewage sludge. The objective was to find out if the flocculants would degrade over time, when the sludge is spread on fields. The degradation was monitored over time under close-to-natural conditions after extracting the polymer from the soil. The biggest challenge was the sample matrix because it contained humic acids, which made detection nearly impossible. With a lot of effort, PSS determined that the polymers in the soil do degrade over time, thus allowing the sewage sludge to be spread on fields that are cultivated.

Another interesting example, from a chromatographic point of view, was a comparison of two polyvinylchloride samples. The molar masses of the polymers were almost identical. There seemed to be only one small peak, coming from an additive, on the low molecular end of the chromatogram.

However, these materials behaved differently. With the help of FT-IR and ESI-MS hyphenation, PSS was able to finally identify that the small additive peak of one sample consisted of three co-eluting species, whereas the one of the other sample contained only one specie. In this case, there was much more than met the eye.

LCGC: Are there any samples that cannot be analyzed directly by GPC/SEC?

Lohmann: The first things that come to mind are usually gels, cross-linked samples, or ultra-high molar mass samples. Generally speaking, if the sample is not soluble, then gel permeation chromatography (GPC) or interaction polymer chromatography (IPC) cannot be used. However, other options and alternative techniques are available.

For example, inverse GPC allows PSS to measure the pore size distribution. Or, PSS could determine the soluble portion of a sample or its gel content, if the sample contains a gel. High molar mass samples are definitely a challenge. However, PSS has experience with those fragile samples. PSS scientists know how to prepare them with care and how to make the chromatography work.

Another example is reactive samples. These must be analyzed in close cooperation with the customer to prevent any damage to the equipment.

LCGC: To summarize, why is PSS the perfect partner for liquid chromatography (LC) of polymers and biopolymers?

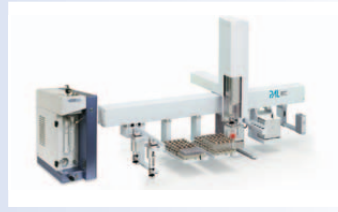
Lohmann: PSS, which has been in business for more than 30 years, has a creative team of scientists who have extensive experience with macromolecules. We have state-of-the-art equipment for ambient-temperature liquid chromatography and high-temperature GPC. Multiple LC systems are set up, featuring specialty detectors necessary for sophisticated characterization.

Solvent-wise, we can cover the entire polarity chart. Analyses are performed in common (e.g., tetrahydrofuran) or uncommon (e.g., hexafluoroisopropanol or N-methyl-2-pyrrolidone) GPC solvents. PSS produces GPC columns, has its own software, and makes polymeric reference materials.

PSS's close interdepartmental cooperation allows the development of long-term stable methods, grants unparalleled in-house support, and provides access to advanced products and the latest developments. In addition, PSS is certified according to ISO 9001:2015.

PRODUCTS & RESOURCES

Purge-and-trap concentrator

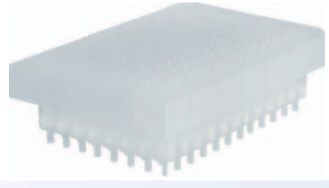


CDS Analytical's 7000C purge-and-trap concentrator, designed for use with PAL RTC systems, automates purge-and-trap sampling for the trace analysis and measurement of purgeable organic compounds in water. According to the company, the system is compliant with the official international standards methods DIN-EN ISO 15009, and U.S. EPA series 500 and 8000 for volatiles in water.
CDS Analytical LLC, Oxford, PA. www.cdsanalytical.com

Reference materials

Reference materials from LGC Standards are available for allergens, veterinary drugs, pesticides, dyes, toxins, and other organic materials. According to the company, its Dr. Ehrenstorfer brand of standards has the world's largest portfolios of organic contaminant standards.
LGC Standards,
Manchester, NJ.
www.lgcstandards.com

Polymeric SPE adsorbent



Machery-Nagel's hydrophilic-lipophilic balanced (HLB) polymeric solid-phase extraction (SPE) adsorbent Chromabond HLB is designed for the enrichment of hydrophilic analytes such as pharmaceuticals and pesticides from polar matrices like blood, water, and food. The company also offers Chromabond MULTI 96 HLP plates for high-throughput SPE in a 96-well format.
Macherey-Nagel Inc., Bethlehem, PA.
www.mn-net.com

Fixed-ratio flow splitters

Mott's PerfecPeak fixed flow splitters are designed to provide improved peak resolution and accurate splitting with a finger-tight design. According to the company, the design allows for low internal volume, and the splitters are equipped with interchangeable splits and a 0.1-µm replaceable prefilter.
Mott Corporation,
Farmington, CT.
www.mottcorp.com

Mass flow controller

Parker Hannifin's X-Flow mass flow controller is designed for instruments, laboratories, and process needs. According to the company, the controller is calibrated to the user's specific conditions and includes the Parker Tracking System feature to assist with annual asset calibration.
Parker Hannifin,
Cleveland, OH.
www.parker.com

LC columns

The Luna Omega SUGAR liquid chromatography columns from Phenomenex are designed for carbohydrate separation and analysis from food, beverage, and pharmaceutical matrices, such as milk, animal feed, wine, soda, fruit, and tablets. According to the company, the HILIC stationary phase incorporates an amide polyol, an amino group with linker, and polar endcapping.
Phenomenex,
Torrance, CA.
www.phenomenex.com

Syringes

VICI Precision Sampling Pressure-Lok analytical syringes are made with polytetrafluoroethylene (PTFE) plunger tips. According to the company, the tips are designed to remain smooth, without the seizing or residue of conventional metal plunges, and have leak-proof seals.
Valco Instruments Co., Inc.,
Houston, TX.
www.vici.com

Separation system

The Eclipse DualTech separation system from Wyatt Technology is designed for both hollow-fiber flow field-flow fractionation (HF5) and asymmetric-flow field-flow fractionation (AF4) techniques. According to the company, both techniques may be integrated into one instrument, and coupled to the company's DAWN HELEOS II detector.
Wyatt Technology Corp.,
Santa Barbara, CA. www.wyatt.com

Automated sample preparation system

Gerstel's MultiPurpose Sampler automated sample preparation system is designed with automation and quality control options included. According to the company, the autosampler features a barcode sampler that scans samples either one by one before analysis, or as a batch up front.
Gerstel,
Linthicum, MD.
www.gerstel.com

Headspace syringe

Hamilton Company's HDHT headspace syringe is designed for high-temperature applications up to 200 °C. According to the company, the syringe's high-dynamic HD plunger uses a spring in the plunger tip that compensates for the materials' different expansion coefficients.
Hamilton Company,
Reno, NV.
www.hamiltoncompany.com

GCxGC TOF-MS system

The Pegasus BT 4D GCxGC TOF-MS system from LECO is designed to interrogate challenging samples. According to the company, software and hardware features simplify quantitation, and the system's StayClean ion source eliminates the need for source cleaning.
LECO Corporation,
St. Joseph, MI.
www.leco.com

Sampling tubes for thermal desorption

Markes' sorbent tubes are designed for sampling and analytical performance for volatile and semivolatile organic compounds. According to the company, the tubes are available in metal, inert-coated, or glass versions, with a range of packing materials suitable for various applications.
Markes International Ltd.,
Llantrisant, UK.
www.markes.com

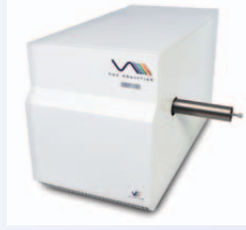
Artificial body fluids

Pickering's artificial body fluids are designed to meet official product testing specifications from AATCC, ISO, DIN, BS, EN, and other worldwide standards organizations. According to the company, the artificial body fluids are suitable for product development, quality testing, and research applications.
Pickering Laboratories,
Mountain View, CA.
www.pickeringlabs.com

LC–MS/MS system

The Nexera Mikros Microflow liquid chromatography–mass spectrometry system from Shimadzu Scientific is designed with a direct injection system for sample volume-limited analyses for highly sensitive micro LC–MS analysis without sample loss. According to the company, a trap-and-elute system is available for analyses with larger injection volumes or when some degree of sample cleanup is desirable. **Shimadzu Scientific Instruments,** Columbia, MD. www.ssi.shimadzu.com

GC–VUV detector



VUV Analytics' VGA-101 gas chromatography–vacuum ultraviolet (GC–VUV) detector is designed to meet the requirements of customers with advanced GC application needs. According to the company, the detector features an expanded wavelength spectrum and a higher allowable maximum operating temperature. The detector's unique capabilities reportedly include isomer differentiation, resolution of coeluted peaks, shorter GC run times, and automated data analysis.
VUV Analytics, Inc., Cedar Park, TX. www.vuvanalytics.com

Septa

Merlin Microseal septa from Restek are designed for traditional and solid-phase microextraction (SPME) fiber injections. Users reportedly can use the septa for SPME Arrow applications. According to the company, advantages of the septum include elimination of septum coring, long life, and low insertion force for injections.
Restek Corporation,
Bellefonte, PA.
www.restek.com

(Continued from page 667)

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Szabolcs Fekete holds a PhD degree in analytical chemistry from the Technical University of Budapest, Hungary. He worked in the analytical R&D department of the Chemical Works of Gedeon Richter Plc for 10 years. Since 2011, he has been working at the University of Geneva in Switzerland. He contributed to more than 100 journal articles and authored book chapters and edited handbooks. His main interests include liquid chromatography (reversed-phase, IEX, SEC, HIC, SFC, HILIC), column technology, method development, pharmaceutical, and protein analysis.

Davy Guillarme holds a PhD degree in analytical chemistry from the University of Lyon, France. He is now senior lecturer at the University of Geneva in Switzerland. He has authored more than 200 journal articles related to pharmaceutical analysis. His expertise includes HPLC, UHPLC, HILIC, LC–MS, SFC, analysis of proteins and mAbs. He is an associate editor of *Journal of Chromatography B* and editorial advisory board member of several journals including *Journal of Chromatography A*, *Journal of Separation Science*, *LCGC North America*, and others.

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Dwight R. Stoll is the editor of "LC Troubleshooting." Stoll is a professor and co-chair of chemistry at Gustavus Adolphus College in St. Peter, Minnesota. His primary research focus is on the development of 2D-LC for both targeted and untargeted analyses. He has authored or coauthored more than 50 peer-reviewed publications and three book chapters in separation science and more than 100 conference presentations. He is also a member of *LCGC*'s editorial advisory board. Direct correspondence to: LCGCedit@ubm.com

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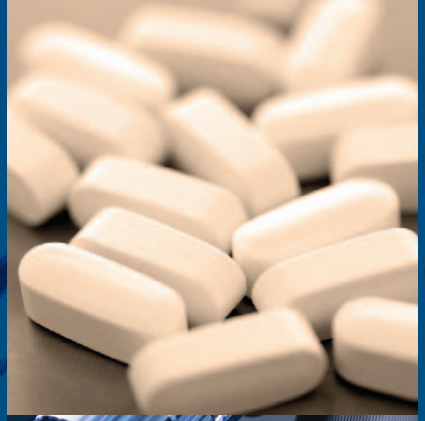

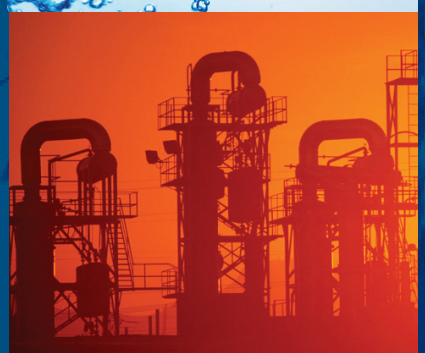

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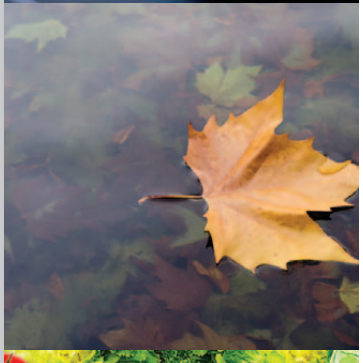
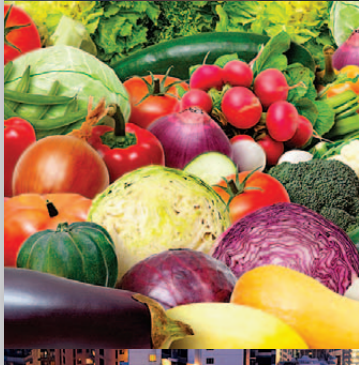
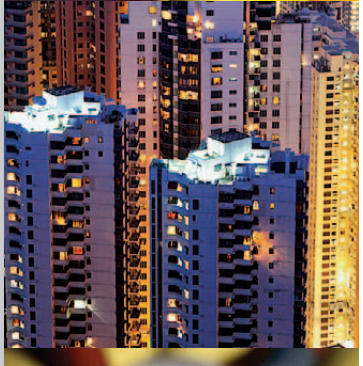


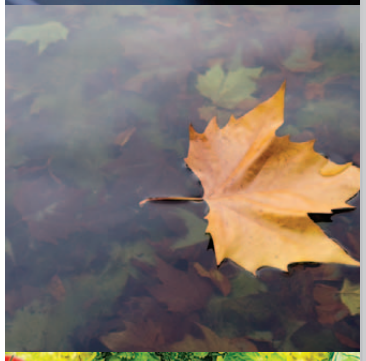
Environmental

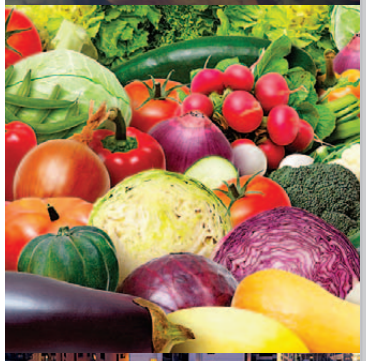


Food and Beverage



Medical/Biological





Medical/Biological (continued)

Pharmaceutical/Drug Discovery

Polymer

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Improving the Quality and Productivity of Environmental Extraction

Milestone Inc.

Microwave-assisted extraction technology offers multiple benefits over traditional Soxhlet extraction and other non-conventional systems. ETHOS X, with its new FastEX-24 rotor and disposable glass vials, offers reliable extraction of contaminants from soil in compliance with EPA 3546, along with easy handling and high extraction efficiency.

The extraction of contaminants, such as PCBs, semivolatile organic compounds, and PAHs, from soils requires solvent extraction. Microwave-assisted extraction (MAE) overcomes the limitations of Soxhlet extraction, resulting in rapid sample preparation with reduced amounts of solvents while working at higher temperatures and pressures. The process is a partitioning of the compounds of interest from the sample matrix into the solvent within a closed vessel. EPA 3546 method provides guidelines to work with the MAE technology, thus improving the quality and productivity of environmental laboratories.

Instrumentation

Milestone's new ETHOS X microwave extraction system can extract organic target compounds from soils, in full compliance with EPA 3546 (100–115 °C and 50–150 psi). Disposable glass vials and contactless temperature control in all positions makes the Milestone ETHOS X with the FastEX-24 rotor a unique and innovative solution for the extraction of contaminants from soils, providing unmatched ease of use and low running costs. The ETHOS X is capable of processing up to 30 g of sample per vessel (up to 24 samples simultaneously), thereby improving the limit of quantitation (LOQ) for analysis. The handling is very easy: as the sample is weighed directly into the disposable glass vial, hexane/acetone or CH_2Cl_2/acetone (1:1) is added, and the vessel is loaded into the FastEX rotor. After 10–20 min of microwave heating, the sample is ready to be filtered and analyzed by gas chromatography.

Table I: Efficiency of PCB extraction evaluated on multiple LCS samples spiked at 20 mg/L; analysis by GC–MS.

Target compounds	Recovery (%)	RSD % (*n*=4)
Tetrachloro-m-xylene	88.5	3.6
Decachlorobiphenyl (DCB)	93.4	4.5

Table II: Efficiency of semivolatile organic compounds extraction evaluated on multiple LCS samples spiked at 50 mg/L; analysis by GC–MS.

Target compounds	Recovery (%)	RSD % (*n*=4)
2-Fluorophenol	89.3	2.3
Phenol-d5	90.1	4.8
Nitrobenzene-d5	81.3	3.5
2-Fluorobiphenyl	87.3	4.2
2,4,6-Tribromophenol	96.4	1.8
p-terphenyl-d14	98.2	3.4

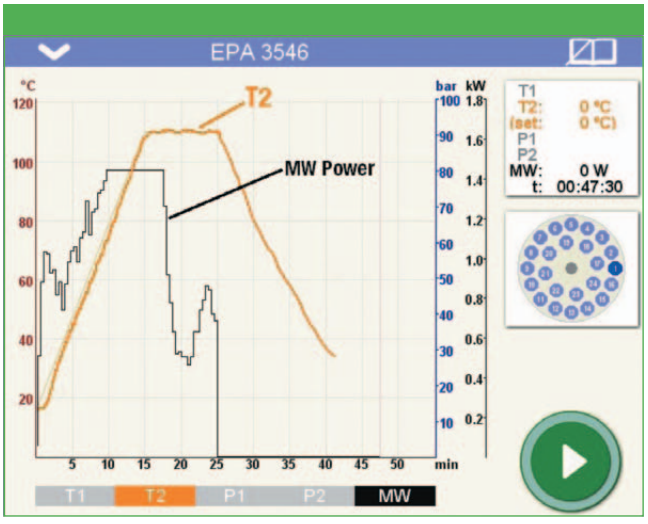

Figure 1: ETHOS X extraction program with 24 × 15 g dried soil samples. The line T2 shows the actual extraction temperature achieved.

Figure 2: ETHOS X system equipped with the FastEX-24 rotor incorporates Weflon™ sleeves and disposable glass vials to ensure maximum productivity.

Conclusion

The ETHOS X enables simultaneous solvent extraction of up to 24 samples (from weighing to filtration) in only 40 min. This results in the capacity to extract over 200 samples in an 8-hour workday. Contamination, memory effects, and cleaning are eliminated due to the use of disposable glass vials. The use of contactless temperature control ensures high reproducibility and full recovery of the target analytes for full compliance with EPA 3546. The ETHOS X, with all its features, fully addresses the needs of environmental laboratories in terms of productivity, ease of use, running costs, and extraction quality. The ETHOS X equipped with the FastEX-24 rotor provides superior extraction capability for easier analysis.

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Shodex HILICpak VT-50 2D: A HILIC + Anion Exchange HPLC Column for Polar Pesticide Analysis Including Glyphosate

Leah Block Sullivan, Showa Denko America, Inc.

There has recently been a peak in research involving herbicides, food additives, and GMO-related topics. Herbicides are generally referred to as a poison that can kill weeds or specific plants. Over the past century, there has been a steady rise in the use of herbicides in the agricultural sector. The main benefit is that herbicides allow the farmer to select which plants will flourish and what size the produce will reach, as well as other factors. However, not all herbicides have been deemed "safe" or approved for agricultural usage.

Glyphosate is a non-selective herbicide specifically used in agriculture for the control of weeds and shrubs. The most common brand name using glyphosate as an active ingredient is Monsanto's Roundup (1). Glyphosate has also been used on genetically modified produce. Europe has restricted glyphosate, as well as some other pesticides, due to adverse effects on the soil and surrounding areas. With new restrictions, methods were developed to detect targeted compounds including aminomethylphosphonic acid, chlorate, ethephon, fosetyl aluminum, glufosinate, glyphosate, maleic hydrazide, and phosphonic acid.

Companies including Waters (2) have developed a method for the best way to analyze herbicides in accordance with the Quick Polar Pesticides (QuPPe) extraction method (3). They have selected the Shodex VT-50 2D column to successfully complete this task.

The organophosphate herbicides easily form metal complexes with SUS housing, so PEEK housing has been used for the VT-50 to avoid tailing. The developed method shows a fast and stable analysis of organophosphate herbicides and related compounds without the use of pre-column derivatization, ion-pair reagents, nor gradient elution.

Shodex analyzed four variations of herbicides using a Shodex HILICpak VT-50 2D column under LC–MS conditions. The sample contained different active ingredients in common pesticides, providing different functions. This analytical condition can also be used with other detectors including RI, ELSD, and corona CAD.

Experimental Conditions

The analysis of samples containing aminomethylphosphonic acid, glufosinate, glyphosate, and 3-methylphosphinicopropionic acid was accomplished using the Shodex HILICpak VT-50 2D (2.0 mm ID × 150 mm ID, 5 µm) a HILIC column suitable for LC–ESI–MS. The column temperature was 40 °C and flow rate was 0.3 mL/min. The eluent conditions were H_2O/ 1% HCOOH aq./CH_3CN: 70/20/10. An injection volume of 5 µL of 1µg/mL of each sample was used for the experiment. The HPLC system was coupled with an ESI-MS (SIM) detector.

Results

The aqueous sample containing aminomethylphosphonic acid, glufosinate, glyphosate, and 3-methylphosphinicopropionic acid was analyzed successfully using HILIC and ESI-MS detection with Shodex HILICpak VT-50 2D (Figure 1). Each herbicide or related metabolite was prominently detected.

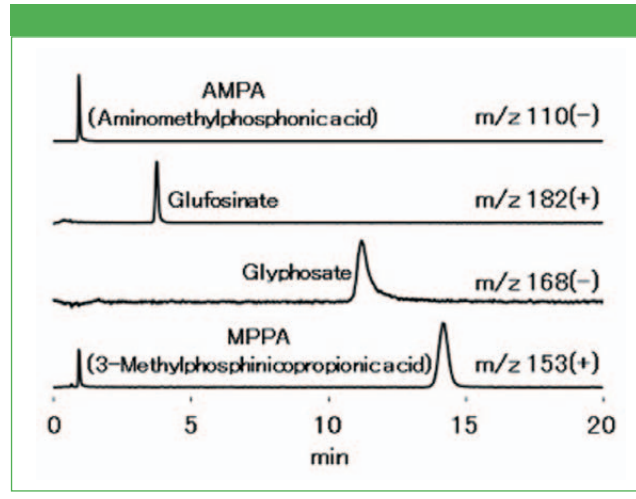

Figure 1: The analysis of phosphorylated saccharides using Shodex VT-50 2D.

Conclusions

Shodex HILICpak VT-50 2D, a hydrophilic interaction chromatography (HILIC) column is suitable for the analysis of phosphorous-containing amino acids herbicides including glyphosate, glufosinate and their metabolites using ESI-MS detection. The polymer-based packing material provides excellent chemical stability and minimum deterioration over extended periods of time.

References

(1) RoundUp is a registered trademark of Monsanto.

(2) www.waters.com/posters

(3) www.eurl-pesticides.eu/docs/public/tmplt_article.asp?CntID=88&LabID=200&Lang=EN

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Analysis of Acrylamide in Potato Chips by UHPLC–MS/MS

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Acrylamide is viewed as a human health concern and found in certain foods after preparation or processing at high temperatures. World experts recommend reducing acrylamide in our diet and suggest long-term research studies to determine its potential risk. In this application we present an HPLC–MS/MS method for analysis of acrylamide in potato chips, using a simple sample preparation procedure.

Experimental Conditions

Stock solution of acrylamide (0.20 mg/mL) was prepared by dissolving 20 mg of the compounds in 100 mL of methanol. The standard working solutions were prepared by serial dilution sample; calibration levels were: 0.5–1.0–5.0–10–50–100 µg/L in methanol/water 50/50 and containing 100 µg/L of isotopic ^{13}C-acrylamide. Samples were previously homogenized. Acrylamide extraction was as follows:

(1) Analytically weigh 1.0 ± 0.1 g crushed potato chips.
(2) Add 100 µL of 10 mg/L standard solution of ^{13}C-acrylamide.
(3) Add 25 mL of 0.1% formic acid in methanol solution and mix in a shaker for 15 min.
(4) Centrifugation for 5 min (3000 rpm).
(5) Add 1 mL of extract to 1 mL of water.
(6) Condition C18 SPE tube (6 mL, 500 mg) with 2 mL methanol and 2 mL water/methanol 50/50: then, dry under vacuum.
(7) Apply 2 mL of extracted potato chip solution to SPE tube.

Acrylamide was separated on a PerkinElmer QSight® LX50 HPLC system using a UHPLC BEH C18 column (2.1 × 50 mm, 2.7 µm). Column temperature was set at 40 °C.

Detection of acrylamide was carried out on a QSight triple quadrupole mass spectrometer equipped with an electrospray ionization source operating in positive ion mode and multiple reaction monitoring mode (MRM). Mass spectrometer conditions were as follows: ElectroSpray, 5000 V; Source Temp, 300 °C; HSID Temp, 275 °C; Drying Gas, 100 mL/min; Nebulizer Gas, 300 mL/min. Data acquisition and processing was performed using the Simplicity™ 3Q software.

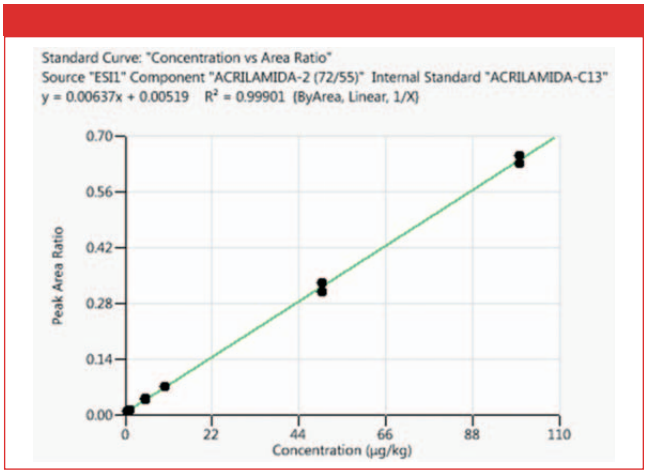

Figure 1: Linearity plot for standard solution over a range of 0.5–100 µg/L (internal standard).

Results

The selectivity of the method was evaluated by adding a known concentration of acrylamide equivalent to the limit of quantification to a matrix target (25 µg/kg). No matrix effect was observed by comparing solvent solution and extract solutions (the variation of the mean of the responses of 10 trials between both samples was less than 10%). Matrix concentration in sample extracts is very low (20 mg/mL).

Based on the calculated LOQ of 25 µg/kg, this method was in compliance with the technical requirements set by Regulation (EU) 2017/2158 (recovery 75-110%, RSD < Horwitz modified, LOQ ≤ 50 µg/kg). Very good recoveries were obtained (from 85 to 109%).

Conclusions

The QSight triple quad provides a robust platform for the analysis of trace acrylamide levels in compliance with European Regulations. This rapid, sensitive, and reproducible method can also be applied to other types of regulated matrices such as bread, biscuits, breakfast cereals, and more.

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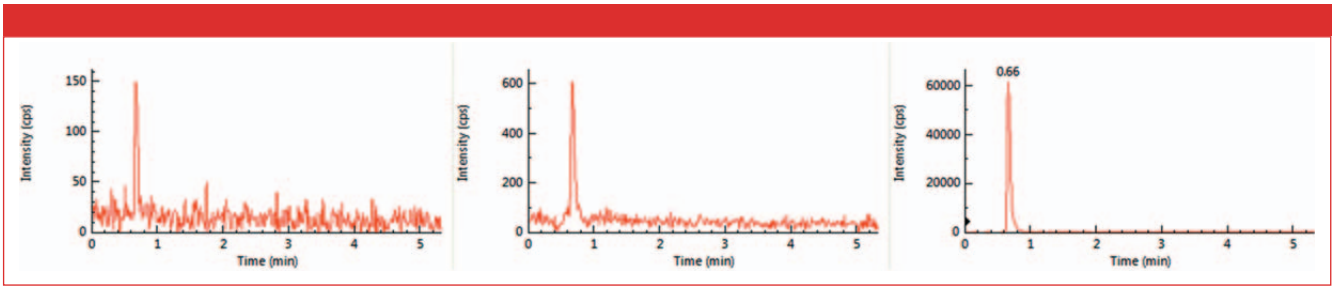

Figure 2: MRM chromatogram of 0.5 and 5 µg/L acrylamide calibration solutions.

Analysis of Fentanyl and Its Analogues in Human Urine by LC–MS/MS

Shun-Hsin Liang and Frances Carroll, Restek Corporation

Abuse of synthetic opioid prescription painkillers such as fentanyl, along with a rapidly growing list of illicit analogues, is a significant public health problem. In this study, we developed a simple dilute-and-shoot method that provides a fast 3.5-min analysis of fentanyl and related compounds (norfentanyl, acetyl fentanyl, alfentanil, butyryl fentanyl, carfentanil, remifentanil, and sufentanil) in human urine by LC–MS/MS using a Raptor Biphenyl column.

In recent years, the illicit use of synthetic opioids has skyrocketed, and communities worldwide are now dealing with an ongoing epidemic. Of the thousands of synthetic opioid overdose deaths per year, most are related to fentanyl and its analogues. With their very high analgesic properties, synthetic opioid drugs such as fentanyl, alfentanil, remifentanil, and sufentanil are potent painkillers that have valid medical applications; however, they are also extremely addictive and are targets for abuse. In addition to abuse of these prescription drugs, the current opioid crisis is fueled by a growing number of illicit analogues, such as acetyl fentanyl and butyryl fentanyl, which have been designed specifically to evade prosecution by drug enforcement agencies.

As the number of opioid drugs and deaths increases, so does the need for a fast, accurate method for the simultaneous analysis of fentanyl and its analogues. Therefore, we developed this LC–MS/MS method for measuring fentanyl, six analogues, and one metabolite (norfentanyl) in human urine. A simple dilute-and-shoot sample preparation procedure was coupled with a fast (3.5 min) chromatographic analysis using a Raptor Biphenyl column. This method provides accurate, precise identification and quantitation of fentanyl and related compounds, making it suitable for a variety of testing applications, including clinical toxicology, forensic analysis, workplace drug testing, and pharmaceutical research.

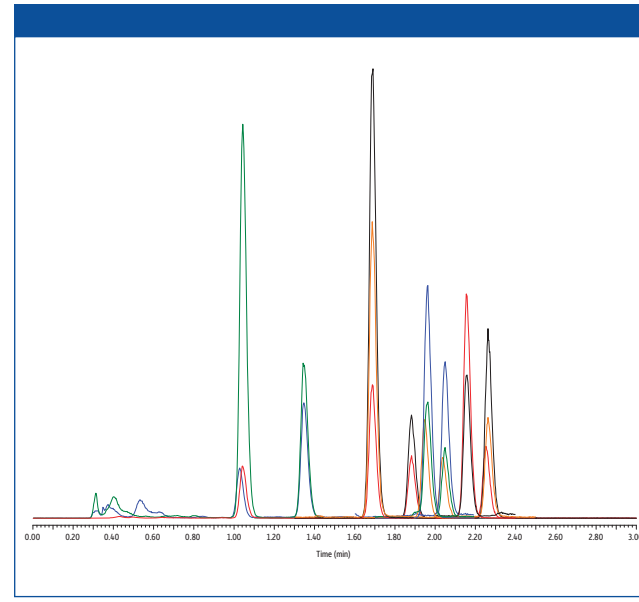

Figure 1: The Raptor Biphenyl column effectively separated all target compounds in urine with no observed matrix interferences. Peak elution order: norfentanyl-D_5, norfentanyl, remifentanil, acetyl fentanyl-$^{13}C_6$, acetyl fentanyl, alfentanil, fentanyl-D_5, fentanyl, carfentanil-D_5, carfentanil, butyryl fentanyl, sufentanil-D_5, sufentanil.

Table I: Analyte transitions

Analyte	Precursor Ion	Product Ion Quantifier	Product Ion Qualifier	Internal Standard
Norfentanyl	233.27	84.15	56.06	Norfentanyl-D_5
Acetyl fentanyl	323.37	188.25	105.15	Acetyl fentanyl-$^{13}C_6$
Fentanyl	337.37	188.26	105.08	Fentanyl-D_5
Butyryl fentanyl	351.43	188.20	105.15	Carfentanil-D_5
Remifentanil	377.37	113.15	317.30	Norfentanyl-D_5
Sufentanil	387.40	238.19	111.06	Sufentanil-D_5
Carfentanil	395.40	113.14	335.35	Carfentanil-D_5
Alfentanil	417.47	268.31	197.23	Acetyl fentanyl-$^{13}C_6$
Norfentanyl-D_5	238.30	84.15	—	—
Acetyl fentanyl-$^{13}C_6$	329.37	188.25	—	—
Fentanyl-D_5	342.47	188.27	—	—
Sufentanil-D_5	392.40	238.25	—	—
Carfentanil-D_5	400.40	340.41	—	—

Experimental Conditions

Sample Preparation

The analytes were fortified into pooled human urine. An 80 µL urine aliquot was mixed with 320 µL of 70:30 water–methanol solution (fivefold dilution) and 10 µL of internal standard (40 ng/mL in methanol) in a Thomson SINGLE StEP filter vial (Restek cat. #25895). After filtering through the 0.2 µm PVDF membrane, 5 µL was injected into the LC–MS/MS.

Calibration Standards and Quality Control Samples

The calibration standards were prepared in pooled human urine at 0.05, 0.10, 0.25, 0.50, 1.00, 2.50, 5.00, 10.0, 25.0, and 50.0 ng/mL. Three levels of QC samples (0.75, 4.0, and 20 ng/mL) were prepared in urine for testing accuracy and precision with established calibration standard curves. Recovery analyses were performed on three different days. All standards and QC samples were subjected to the sample preparation procedure described.

LC–MS/MS analysis of fentanyl and its analogues was

performed on an ACQUITY UPLC instrument coupled with a Waters Xevo TQ-S mass spectrometer. Instrument conditions were as follows, and analyte transitions are provided in Table I.

Analytical column:	Raptor Biphenyl (5 µm, 50 mm × 2.1 mm; cat. #9309552)	
Guard column:	Raptor Biphenyl EXP guard column cartridge, (5 µm, 5 mm × 2.1 mm; cat. #930950252)	
Mobile phase A:	0.1% Formic acid in water	
Mobile phase B:	0.1% Formic acid in methanol	
Gradient	Time (min)	%B
	0.00	30
	2.50	70
	2.51	30
	3.50	30
Flow rate:	0.4 mL/min	
Injection volume:	5 µL	
Column temp.:	40 °C	
Ion mode:	Positive ESI	

Results

Chromatographic Performance

All eight analytes were well separated within a 2.5-min gradient elution (3.5-min total analysis time) on a Raptor Biphenyl column (Figure 1). No significant matrix interference was observed to negatively affect quantification of the fivefold diluted urine samples. The 5-µm particle Raptor Biphenyl column used here is a superficially porous particle (SPP) column. It was selected for this method in part because it provides similar performance to a smaller particle size fully porous particle (FPP) column, but it generates less system back pressure.

Linearity

Linear responses were obtained for all compounds and the calibration ranges encompassed typical concentration levels monitored for both research and abuse. Using 1/x weighted linear regression (1/x^2 for butyryl fentanyl), calibration linearity ranged from 0.05 to 50 ng/mL for fentanyl, alfentanil, acetyl fentanyl, butyryl fentanyl, and sufentanil; from 0.10 to 50 ng/mL for remifentanil; and from 0.25 to 50 ng/mL for norfentanyl and carfentanil. All analytes showed acceptable linearity with r2 values of 0.996 or greater and deviations of <12% (<20% for the lowest concentrated standard).

Accuracy and Precision

Based on three independent experiments conducted on multiple days, method accuracy for the analysis of fentanyl and its analogues was demonstrated by the %recovery values, which were within 10% of the nominal concentration for all compounds at all QC levels. The %RSD range was 0.5–8.3% and 3.4–8.4% for intraday and interday comparisons, respectively, indicating acceptable method precision (Table II).

Conclusions

A simple dilute-and-shoot method was developed for the quantitative analysis of fentanyl and its analogues in human urine. The analytical method was demonstrated to be fast, rugged, and sensitive with acceptable accuracy and precision for urine sample analysis. The Raptor Biphenyl column is well suited for the analysis of these synthetic opioid compounds and this method can be applied to clinical toxicology, forensic analysis, workplace drug testing, and pharmaceutical research.

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Table II: Accuracy and precision results for fentanyl and related compounds in urine QC samples

	QC Level 1 (0.750 ng/mL)			QC Level 2 (4.00 ng/mL)			QC Level 3 (20.0 ng/mL)		
Analyte	**Average Conc. (ng/mL)**	**Average % Accuracy**	**%RSD**	**Average Conc. (ng/mL)**	**Average % Accuracy**	**%RSD**	**Average Conc. (ng/mL)**	**Average % Accuracy**	**%RSD**
Acetyl fentanyl	0.761	102	1.54	3.99	99.7	2.08	19.9	99.3	0.856
Alfentanil	0.733	97.6	3.34	3.96	98.9	8.38	20.9	104	6.73
Butyryl fentanyl	0.741	98.9	6.29	3.77	94.3	6.01	20.8	104	4.95
Carfentanil	0.757	101	7.34	3.76	94.0	4.64	20.6	103	4.24
Fentanyl	0.761	102	1.98	3.96	99.1	2.31	19.9	99.6	1.04
Norfentanyl	0.768	103	6.50	4.04	101	1.84	20.1	101	2.55
Remifentanil	0.765	102	3.42	3.97	99.2	3.68	20.8	104	4.14
Sufentanil	0.752	100	1.67	3.93	98.3	1.28	20.1	100	0.943

Novel Stationary Phase Aids in the Fight Against Cardiovascular Disease

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Elevated ADMA, SDMA and NMMA are early biomarkers for cardiovascular disease. LC–MS methods are challenging due to the high similarity of these compounds, often requiring derivatization. Here we show successful non-derivatized LC–MS separation of these and other related compounds, on a novel stationary phase.

Factors leading to the reduction of nitric oxide (NO) production, such as an increase in asymmetric and symmetric dimethyl-arginine (ADMA/SDMA) (1,2) and NG-monomethyl-arginine (NMMA) 3,4, as well as a decrease in homoarginine (hArg) 5,6,7, negatively impact endothelial function and promote the development of atherosclerosis. Therefore, early detection of these biomarkers is critical in the fight against cardiovascular disease (CVD).

There are several challenges in developing an LCMS method which can reliably separate these compounds, due to their similarities. For example, ADMA and SDMA are isomers, differing only in the position of two methyl groups, making them difficult to resolve chromatographically using traditional columns and methods. They also share the same mass/charge ratio of 203.1 m/z, meaning that they cannot be distinguished by mass spectrometry alone. NMMA and hArg also share the same m/z of 189.1, adding to the complexity of this analysis.

Current methods rely on complicated derivatizations, making them cumbersome and unreliable. Here, we show an LC–MS method using our Intrada Amino Acid column, which is designed specifically to separate amino acids and similar compounds, without the need for analyte modification.

Experimental Conditions

See Figure 1. Standards were purchased from Sigma Aldrich and tested by LC–MS without derivatization, using the following column and conditions:

Intrada Amino Acid (Imtakt Corp., Kyoto, Japan)
100 × 3 mm (WAA34)
A: 50 mM ammonium formate
B: 100 mM ammonium formate/methanol = 70/30
20–50%B (0–15min), 100%B (15–20 min)
0.5 mL/min (8MPa), 37 °C, 10 µL (0.66–2.4 nmol/mL, 1% formic acid)
Single Quad. MS (ESI, positive)

Result and Discussion

ADMA/SDMA were baseline resolved, in just over 10 min, despite being isomers. NMMA and hArg are also well resolved on the Intrada Amino Acid column. Arginine, the common precursor molecule to the other compounds shown, has excellent peak shape in this method, and should be considered in the total characterization of a patient's risk for CVD.

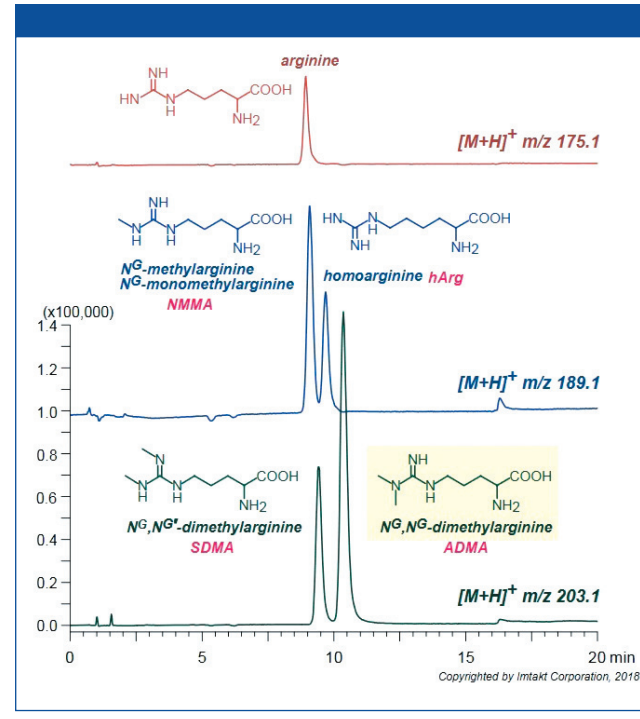

Figure 1: LC–MS analysis of asymmetric/symmetric dimethylarginines (ADMA/SDMA) and related compounds.

Conclusion

In the laboratory, accurate LCMS testing of these compounds is challenging due to their similarities, and rarely accomplished without derivatization. Here, we show that the novel stationary phase of our Intrada Amino Acid column is able to resolve all of these compounds without any analyte modification, which will likely improve the accuracy and ease for clinical reporting. The simplicity of this analysis is sure to make this method an attractive alternative to current strategies, as a vast improvement to aid in the fight against CVD.

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Quantitative Analysis of Benzodiazepines in Whole Blood by QuEChERS and LC–MS/MS

Tina Fanning, UCT, LLC

Common sample preparation methods for biological samples include a protein precipitation step followed by liquid–liquid extraction or solid phase extraction. This application describes an easy, fast, and effective method using QuEChERS for the quantitative analysis of benzodiazepines in whole blood. Benzodiazepines are psychoactive drugs widely prescribed for treating anxiety, insomnia, agitation, seizures, muscle spasms, and alcohol withdrawal.

Extraction/Analytical Materials	
ECQUUS15CT	15 mL centrifuge tube with 400 mg $MgSO_4$ and 100 mg NaOAc
CUMPSC18CT	2 mL centrifuge tube with 150 mg $MgSO_4$, 50 mg PSA and 50 mg C18
SLDA100ID21-3UM	Selectra® DA HPLC column 100 × 2.1 mm, 3 µm
SLDAGDC20-3UM	Selectra® DA guard column 10 × 2.1 mm, 3 µm

Procedure

a) Add 2 mL of MeCN with 0.4% Formic Acid to ECQUUS15CT
b) Add internal standards and 1 mL whole blood
c) Cap and shake for 1 min at 1000 strokes/min using a Spex Geno-Grinder
d) Centrifuge for 5 min at 3000 g
e) Transfer 1 mL of supernatant to CUMPSC18CT
f) Cap and shake for 1 min at 1000 strokes/min using a Spex Geno-Grinder
g) Centrifuge for 5 min at 3000 g
h) Transfer 0.4 mL of the cleaned extract into an autosampler vial. Add 0.4 mL D.I. H_2O and vortex for 30 s

Instrumental

LC–MS/MS: Agilent™ 1200 HPLC and AB Sciex™ 4000 Q Trap (MS/MS)
Column: UCT Selectra® DA HPLC Column 10 × 2.1 mm, 3 µm
Guard Column: UCT Selectra® DA Guard Column 10 × 2.1 mm, 3 µm
Injection Volume: 10 µL
Mobile Phase A: D.I. H_2O + 0.1% Formic Acid
Mobile Phase B: MeOH + 0.1% Formic Acid
Column Flow rate: 0.30 mL/min

Table I: Linearity and Matrix Effect

Compound	Solvent standard		Matrix-matched standard		Matrix Effect (%)
	Slope	Linearity (R^2)	Slope	Linearity (R^2)	
7-aminoclonazepam	0.00823	0.9993	0.00646	0.9998	-22
α-Hydroxy-Alprazolam	0.00646	0.9990	0.00764	0.9996	18
Alprazolam	0.00041	0.9990	0.00048	0.9989	18
Clonazepam	0.00443	0.9995	0.00497	0.9999	12
Diazepam	0.01330	0.9997	0.01460	0.9996	10
Lorazepam	0.00306	0.9999	0.00340	0.9997	11
Midazolam	0.00656	0.9989	0.00675	0.9963	3
Nordiazepam	0.00703	0.9999	0.00754	0.9998	7
Oxazepam	0.00987	1.0000	0.01070	1.0000	8
Temazepam	0.00641	0.9998	0.00709	0.9999	11

Table II: Recovery and RSD% from Whole Blood Spiked at 2 Levels (n=6)

Compound	10 ng/mL		50 ng/mL	
	Recovery%	RSD%	Recovery%	RSD%
7-aminoclonazepam	88.6	7.5	96.9	2.1
α-Hydroxy Alprazolam	101.2	3.4	91.0	2.0
Alprazolam	92.3	10.7	90.2	4.0
Clonazepam	96.4	3.6	105.0	3.2
Diazepam	85.5	3.3	103.0	2.7
Lorazepam	96.9	5.1	93.7	4.1
Midazolam	96.7	2.7	101.6	2.7
Nordiazepam	88.4	3.9	99.7	2.5
Oxazepam	86.5	1.9	93.8	2.4
Temazepam	96.7	2.7	101.6	2.7

Conclusion

The matrix effects were found to be minor, ranging from -22 to 18%. This indicated that the QuEChERS method with dSPE cleanup sufficiently removed matrix interferences that may cause significant ion suppression or enhancement. Excellent recoveries (85.5–105%) and relative standard deviations (RSD% ≤ 10.7%) were obtained.

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RPC-MALS Analysis of Protein Oligomers

Wyatt Technology

Reverse-phase chromatography can be combined with multi-angle light scattering to separate and characterize protein oligomers and isoforms not resolvable by size-exclusion chromatography.

Reversed-phase chromatography (RPC) represents one of the most popular applications of HPLC, with particular importance for protein characterization. Because elution depends on the hydrophobicity of the sample, it is generally impossible to identify the separated products on the basis of their elution time (volume). Frequently, each eluted fraction must be isolated further and analyzed with other techniques in order to gain some understanding of the molecular behavior of the protein.

Adding a miniDAWN® or DAWN® multi-angle light scattering (MALS) detector to one's HPLC system, however, simplifies protein identification significantly. It allows one to measure absolute molar masses directly, irrespective of retention time, and to observe the properties of the protein in solution.

Experimental

The experimental system used to collect the data shown here consisted of a miniDAWN connected downstream of an HPLC quaternary pump, degasser, autoinjector, and UV diode-array detector. A Vydac Protein C-4 column (150 mm × 4.6 mm, 300 Å) was used with a flow rate of 0.7 mL/min. ASTRA® software collected and analyzed the data to determine absolute molar mass and size.

Results

The inset of Figure 1 shows a chromatogram of basic fibroblast growth factor (bFGF) with a particular degree of oxidation produced by 0.2 equivalents of DTNB (1). The light scattering signal for each of the first two peaks is approximately twice the UV signal, while for the third peak the two signals are equal. This shows at a glance that the three peaks correspond, from left to right, to two types of dimers and a single monomer of bFGF; calculations show the molar masses to be 33 kDa, 34 kDa, and 17 kDa, respectively.

The main part of Figure 1 presents a superposition of molar mass versus elution volume from four different separations, each based on a different degree of oxidation. The presence of dimers, trimers, tetramers, etc. is clearly evident, yet there is no consistency in the order of the elution volume.

MALS measurements made with the miniDAWN permit the absolute identification of each eluting peak. In addition, the root-mean-square radius of an eluting molecule may often be determined. For example, the rms radius of the bFGF pentamer was shown to be about 13 nm, indicative of a rod-like structure for this 85 kDa multimer.

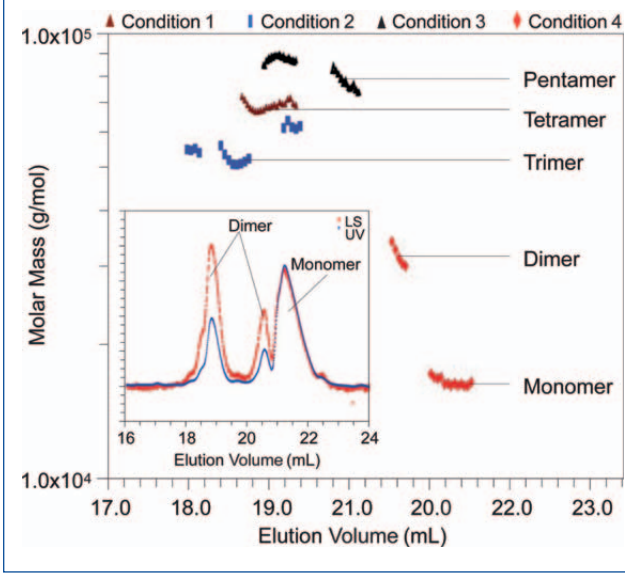

Figure 1. Inset: UV signal of basic Fibroblast Growth Factor (bFGF) with 90° light scattering (LS) signal corrected for gradient baseline shift. Main: Absolute molar masses at the peak regions for bFGF prepared under four different oxidation conditions.

Conclusions

Simply adding a miniDAWN to RPC permits the absolute determination of the molar mass of each eluting fraction, the detection and identification of different multimeric forms, and the size and conformation of the separated molecules in solution.

References

(1) I. V. Astafieva, G.A. Eberlein, and Y.J. Wang, *J. Chromat. A* **740**(2), 215–229 (1996).

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Fast Separation of Triptans in Rat Plasma on ZirChrom®-PBD

Sameh Ahmed*, and Noha Atia†, and ZirChrom Separations, Inc., *Taibah University, †Assiut University

The following reviews a published comparison of the ZirChrom®-PBD column to the Hypersil™ BDS C18 column for the analysis of triptans in rat plasma. This work concluded that the ZirChrom®-PBD phase had a superior selectivity for these analytes; allowing for an isocratic method with comparatively enhanced selectivity, peak shape and efficiency with an analysis time of less than six minutes.

Triptans are most often prescribed for the acute treatment of migraine headaches. By stimulating the brain's seratonin receptors, triptans allow the constriction of dilated blood vessels and thus alleviate pain and pressure associated with a migraine (1,2).

Traditional HPLC analysis of triptans has been complicated by the fact that they are very basic drugs. The amine moieties have a strong affinity for the silanol groups present on silica based HPLC columns causing poor peak shape, short lifetime and irreproducibility (2).

The following rapid analysis, developed and validated by Ahmed and Atia, at Taibah University (Saudi Arabia) and Assiut University (Egypt) respectively, strove to improve upon currently available methods (2). The zirconia-based ZirChrom®-PBD was chosen by the authors for its lack of silanol groups, different selectivity, and unparalleled thermal and chemical stability.

Experimental

Four triptans were analyzed: Sumatritan succinate (SMT), Zolmitriptan (ZLT), Eletriptan hydrobromide (ELT) and Rizatriptan benzoate (RZT). Standard stock solutions were prepared by dissolving the samples in pure acetonitrile to a concentration of 1 mg/mL. The samples were then diluted using the appropriate mobile phase and used to spike a sample of processed rat plasma to a final concentration of 1000 ng/mL. The following chromatographic conditions were used:

Column:	A:	ZirChrom®-PBD, 150 mm × 4.6 mm, 3 um (part # ZR03-1546)
	B:	Hypersil™ BDS C18 150 mm × 4.6 mm, 3 um
Mobile Phase:		A: 20/80 acetonitrile/10 mM sodium dihydrogen phosphate buffer pH 3.0
		B: 40/60 acetonitrile/10 mM sodium dihydrogen phosphate buffer pH 3.0
Temperature:		50 ºC
Flow Rate:		1 ml/min.
Detection:		UV at 225 nm

In Figure 1, the ZirChrom®-PBD clearly provided superior selectivity and was able to do so faster, more efficiently and with less organic solvent used than the silica column (2). The unique selectivity and thermal stability of the ZirChrom®-PBD phase allows for baseline resolution of the four triptans in under six min. The longer elution time and poor peak shape of the compounds on the Hypersil™BDS C18 column was attributed to residual silanol interactions.

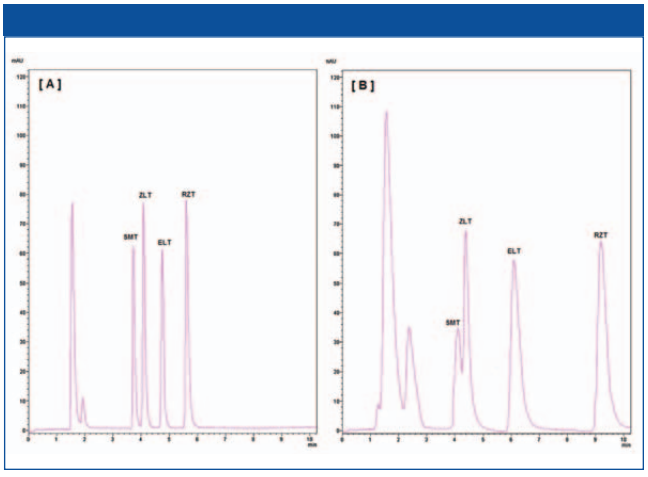

Figure 1: Comparison of (A) ZirChrom®-PBD and (B) Hypersil™ BDS C18 for the analysis of four triptans. Used with permission (2).

The efficiency (N - theoretical plates) on ZirChrom®-PBD improved for all and for three of the compounds the improvement was ten-fold (2). When validating this method on ZirChrom®-PBD the authors found that the ZirChrom®-PBD column had a wider calibration range and improved sensitivity when compared to other HPLC and UV methods (2).

References

(1) H.C. Diener, H. Kaube, *J. Neurol.,* **246,** 515–519 (1999).

(2) S. Ahmed, N.N. Atia, *J. Pharm. Biomed. Anal.,* **143,** 241–251 (2017).

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Determination of Pharmaceuticals from Serum

Hans Rainer Wollseifen, Johannes Brand, and Detlef Lambrecht, Macherey-Nagel GmbH & Co. KG

This application note describes the determination of pharmaceuticals from serum using solid-phase extraction with the hydrophilic-lipophilic balanced SPE phase CHROMABOND® HLB for analyte enrichment and for sample clean-up. The eluates from SPE are finally analyzed by HPLC–MS/MS on a NUCLEOSHELL® PFP core-shell phase.

Nowadays, people are suffering from various diseases. Therefore, they are prescribed many types of pharmaceuticals as part of their treatment, for instance anesthetics, antibiotics, anticholinergics, anticonvulsants, etc. In order for the treatment to be successful, it is necessary to keep controlling the levels of the pharmaceuticals to provide an accurate dosage. This leads to an increasing demand for the development of accurate and sensitive analytical methods for the pharmaceuticals from serum to protect human health.

Table I: SRM transitions for the investigated pharmaceuticals.

Analyte	Retention time [min]	[M-H]-	Q1 (Quantifier)	Q2 (Qualifier)
Atenolol	1.12	267.2	145.2	74.1
Sulfapyridine	1.72	242.9	130.9	96.9
Atropine	1.81	290.2	124.2	93.0
Sulfamerazine	1.82	265.1	156.0	91.9
Ketamine	1.87	238.2	125.1	179.1
Chlorpheniramine	2.27	275.1	230.0	167.0
Sulfachloropyridazine	2.47	285.1	156.0	91.9
Sulfadoxine	2.63	311.1	156.0	92.1
Sulfamethoxazole	2.70	254.1	155.8	91.8
Propanolol	2.74	260.2	116.2	182.9
Diphenhydramine	2.94	256.1	166.9	152.1
Amitriptyline	3.04	278.2	223.0	91.0
Sulfaquinozaline	3.14	301.1	156.1	92.1
Nortriptyline	3.32	264.2	232.9	91.1
Verapamil	3.36	455.2	165.0	150.1
Trimipramine	3.41	295.2	100.1	58.0
Carbamazepine	3.50	237.1	194.1	193.0
Clomipramine	3.67	315.1	86.1	58.0
Indapamide	3.77	366.1	132.1	91.1
Ketoprofen	4.28	255.1	77.0	105.0

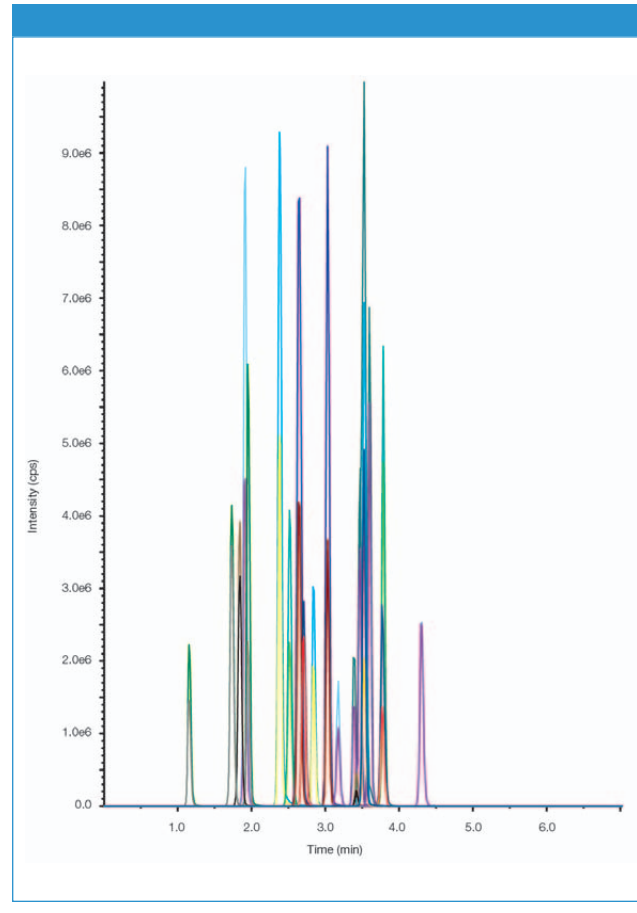

Figure 1: Chromatogram of serum sample spiked with 10 ng/mL for each pharmaceutical.

Solid-Phase Extraction (1)

SPE column: CHROMABOND HLB, 1 mL, 30 mg, Macherey-Nagel REF 730921
Column conditioning: 1 mL methanol, then 1 mL water
Sample application: 1 mL spiked serum sample is passed through the column by vacuum.
Washing: 1 mL water
Drying: 10 min with vacuum
Elution: 2 mL methanol
Eluent exchange: Eluate is evaporated to dryness at 40 °C under a stream of nitrogen and reconstituted in 1 mL water–acetonitrile (95:5, v/v).

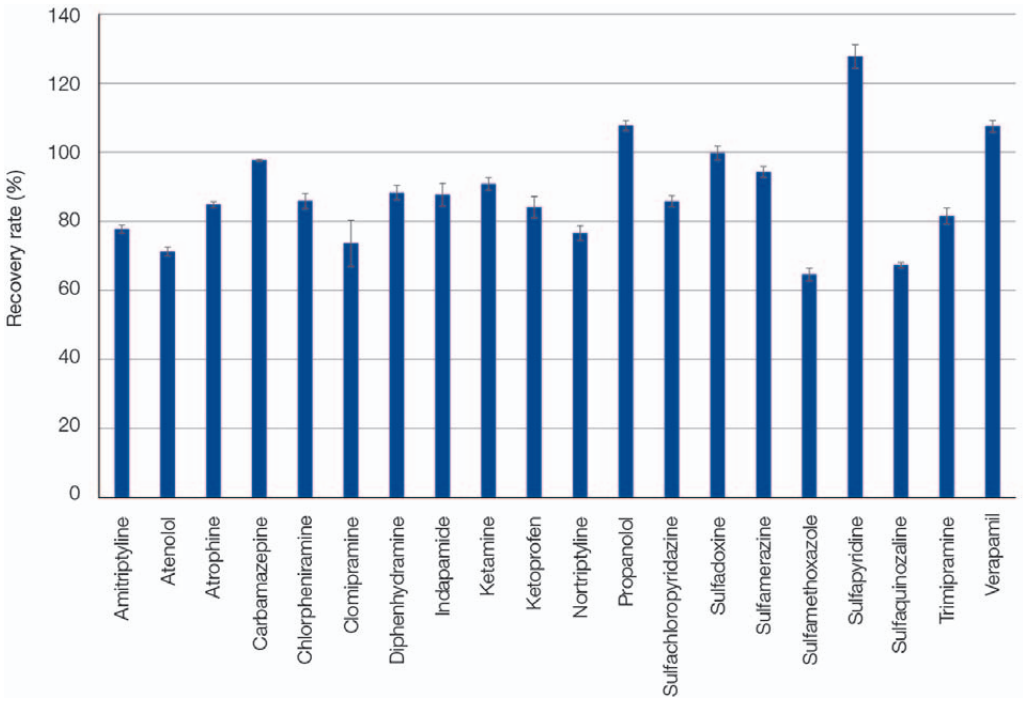

Figure 2: Recovery rates for solid-phase extraction method of pharmaceuticals from serum.

Subsequent Analysis: HPLC–MS/MS (2)

HPLC column: EC 50/2 NUCLEOSHELL PFP, 2.7 µm, Macherey-Nagel REF 763532.20
Eluent A: 0.1% formic acid in water
Eluent B: 0.1% formic acid in acetonitrile
Gradient: 5–95% B in 7.5 min, 95% B for 1 min, 95–5% B in 0.5 min, 5% B for 5 min
Flow rate: 0.3 mL/min
Temperature: 30 °C
Injection volume: 5 µL
MS/MS detection: API 5500 (AB Sciex GmbH, Germany), ion source ESI, positive ionization mode, scan type Selected Reaction Monitoring (SRM, for transitions see Table I), detection window 90 s, curtain gas 40 psig, ion spray voltage 5500 V, temperature 500 °C, nebulizer gas 45 psig, turbo gas 45 psig, CAD medium

Results

The recovery rates show that the determination of pharmaceuticals from serum could be carried out successfully (Figure 2). By using SPE with CHROMABOND HLB it was possible to recover nearly all pharmaceuticals from serum on average with good reproducibility. Regarding the different types pharmaceuticals of the average recovery rates were: for anesthetics 90.8%, for antibiotics 94.4%, for anticholinergics 84.8%, for anticonvulsants 97.7%, for antidepressants 77.4%, for antihistamines 87.1%, for anti-inflammatory drugs 84.1%, for beta blockers 89.5%, for calcium channel blockers 107.5%, and for diuretics 87.7%

The identification and quantification of pharmaceuticals in the solid-phase extracts were carried out by ESI mass spectrometry on an EC 50/2 NUCLEOSHELL PFP column. The chromatogram in Figure 1 shows the results of solid-phase eluate spiked with 10 ng/mL serum for each pharmaceutical.

Conclusion

The presented application describes a quick and convenient method for the determination of pharmaceuticals from serum by SPE with a hydrophilic-lipophilic balanced phase, followed by HPLC–MS/MS analysis.

References

(1) Application No. 306510, MACHEREY-NAGEL, available from www.mn-net.com/apps
(2) Application No. 128200, MACHEREY-NAGEL, available from www.mn-net.com/apps

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Analysis of Mycotoxins in Cannabis Plant and Cannabis-Containing Products

Maria Ofitserova, Sareeta Nerkar, Pickering Laboratories, Inc.

As medical and recreational cannabis use gains broader acceptance, regulations are being put in place to mandate the testing of consumer products containing cannabis. Legally available cannabis plant and cannabis-containing edible products are tested for the presence of pesticides, heavy metals, residual solvents, and other harmful substances. Mycotoxins is another group of contaminants that state regulations have established maximum allowed levels for. In cannabis products sold to consumers the maximum allowed levels for total aflatoxins G1, G2, B1, and B2 are set at <20 ppb and for ochratoxin A at <20 ppb.

Pickering developed an easy and sensitive method to analyze aflatoxins B1, B2, G1, G2 and ochratoxin A in cannabis plant and edible products. Mycotoxins are isolated using immunoaffinity clean-up columns and analyzed with fluorescence detection. To increase sensitivity of aflatoxins B1 and G1, an in-line photochemical reactor is installed before the detector. This method utilizes standard HPLC equipment and allows laboratories to easily determine mycotoxins at levels below the limits established by state regulations.

Method

Isolation of Aflatoxins B1, B2, G1, G2 and Ochratoxin A

Blend 1 g of finely ground sample with extraction solution (10 mL of methanol/water 80:20, 5 mL of hexane, 0.1 g of NaCl) using a handheld homogenizer. Centrifuge for 10 min. Mix 2 mL of the aqueous layer with 12 mL of PBS buffer (pH 7.2) containing 4% of Tween 20. Apply the solution to AflaOTAClean™ Immunoaffinity column at a flow rate of 1–2 drops/sec.

Wash the column with 10 mL of water at a flow rate of 1–2 drops/s.

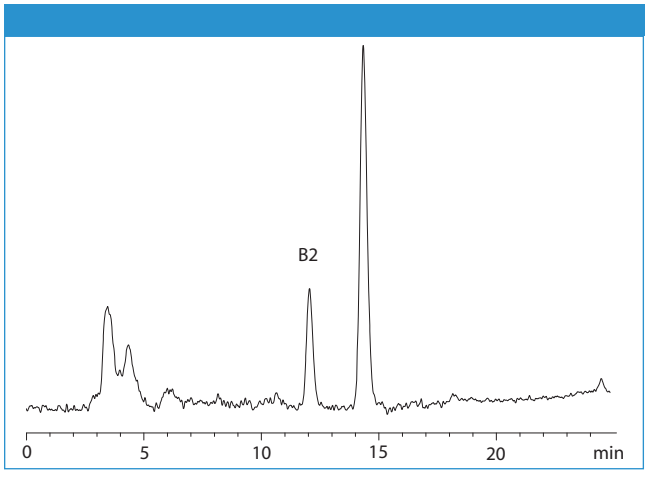


Figure 1: Chromatogram of cannabis-containing peanut butter cookie sample naturally contaminated with 1.58 ng/g of aflatoxins B1 and 0.26 ng/g of B2.

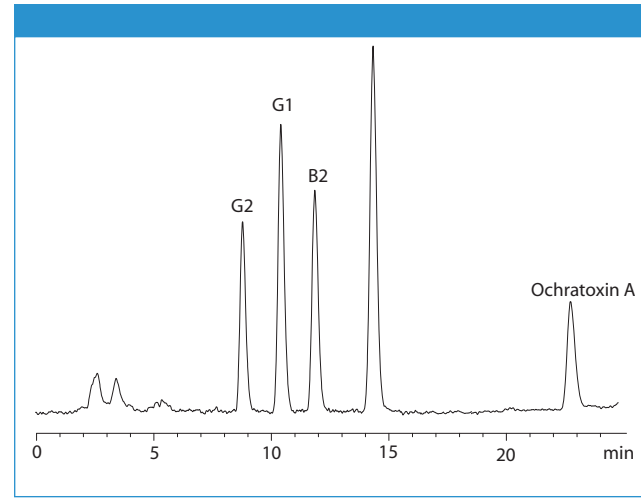


Figure 2: Chromatogram of cannabis pre-roll sample spiked with 6 ng/g of aflatoxin B1; 1.8 ng/g of aflatoxin B2; 5.94 ng/g of aflatoxin G1; 1.8 ng/g of aflatoxins G2 and 20 ng/g of ochratoxin A.

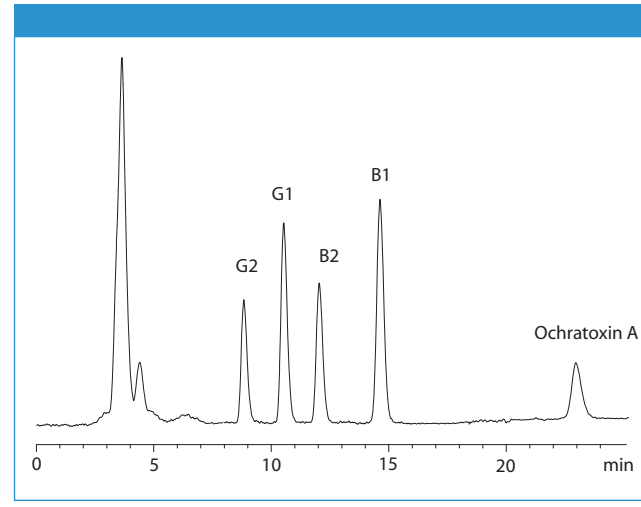


Figure 3: Chromatogram of cannabis inflorescence sample spiked with 6 ng/g of aflatoxin B1; 1.8 ng/g of aflatoxin B2; 5.94 ng/g of aflatoxin G1; 1.8 ng/g of aflatoxins G2, and 20 ng/g of ochratoxin A.

Elute the toxins with two 1-mL portions of methanol at a flow rate of 1 drop/s. Allow 5 min before applying the second portion of the methanol to ensure complete breaking of the antibody-toxin bond.

Evaporate to dryness at 55 °C. Reconstitute in 1 mL of methanol/water 50:50. Other immunoaffinity columns, such as Vicam's Afla-Ochra HPLC, could be used for sample clean up as well.

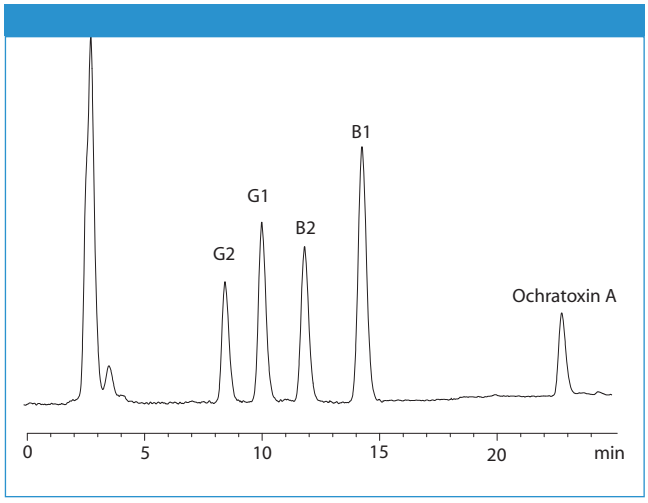


Figure 4: Chromatogram of cannabis-containing chocolate chip cookie sample spiked with 6 ng/g of aflatoxin B1; 1.8 ng/g of aflatoxin B2; 5.94 ng/g of aflatoxin G1; 1.8 ng/g of aflatoxins G2 and 20 ng/g of ochratoxin A.

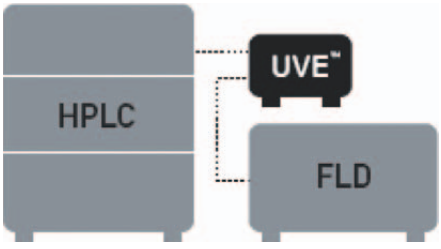
Analytical Conditions

Analytical Column:	Mycotox™ (Pickering Laboratories, Inc), C18, 4.6 ×2 50 mm
HPLC Eluent:	Sodium phosphate buffer (Cat #1700-1108), methanol, acetonitrile (57:28:15)
Flow Rate:	1 mL/min
Injection Volume:	100 uL
FLD:	Excitation 365 nm, Emission 430 nm for aflatoxins Excitation 333 nm, Emission 477 nm for ochratoxin A

Calibration

The 5-point calibration curves were built in the ranges of 0.25–5 ppb for B1, 0.075–1.5 ppb for B2, 0.248–4.95 ppb for G1, 0.075–1.5 ppb for G2, and 1–10 ppb for ochratoxin A. Correlation coefficient $R^2 > 0.999$ for all toxins. All calibration standards were prepared in methanol/water 50:50

Flow diagram for UVE™ photochemical reactor



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Rapid Separation of Basic Drug Compounds on pH-Stable Hamilton PRP™-C18

Derek Jensen and Mark Carrier, Hamilton Company

Mobile-phase pH is a powerful tool in method development, particularly for separation of neutral forms of amines or other organic bases under alkaline conditions. In this study a generic, 5-min linear gradient was used to separate six basic drug compounds on a short (50 mm) PRP-C18 column.

More than 70% of all pharmaceutical drug compounds are cationic solutes that carry a formal positive charge below pH 7. Separation of these and other organic bases has historically been problematic. Ionization has a dominating effect in reversed-phase chromatography that tends to dictate retention. Consequently, the elution window for a sample of ionized amines is narrow. The task is further complicated by secondary interactions that occur between positively charged solutes and residual silanols on the column stationary phase. These secondary mechanisms of retention are the principle source for anomalous chromatographic activity, such as poor peak shape, shifts in retention times and loss of efficiency that progressively worsen over the life of the column.

The PRP-C18 is a new column designed for high-efficiency reversed phase separations under any mobile phase conditions. The stationary phase for the PRP-C18 is devoid of free silanols, does not strip, bleed, or dissolve at any pH, and therefore can be expected to perform reliably and reproducibly throughout the extended life of the column, regardless of mobile-phase conditions. Use of alkaline mobile phase (pH > 11) permits separation of basic solutes in their neutral forms. This broadens the window for elution, whereby subtle structural nuances among chemically similar compounds can be exploited to effect resolution.

Although some recent C18 columns boast stability in alkaline pH, all silica-based supports experience measurable degradation at pH > 6, where column life is still considerably shorter than if used under more favorable conditions. On the other hand, the PRP-C18 stands up to prolonged exposure to concentrations as high as 1 M NaOH and H_2SO_4, with no measurable decrease in performance.

Experimental Conditions

Column:	PRP-C18, 4.1 × 50 mm, 5 µm
Instrumentation:	Agilent 1100 quaternary pump with UV detector
Standards:	nicotine, metropolol, quinine, doxylamine, dexmethorphan, amitriptyline
Mobile phase A :	30 mM Diethylamine
Mobile phase B:	A + 95% ACN, 5% H_2O
Gradient:	10 to 100% B in 5 min
Flow rate:	2 mL/min
Temperature:	Ambient
Injection volume:	10 µL
Detection:	UV at 265 nm

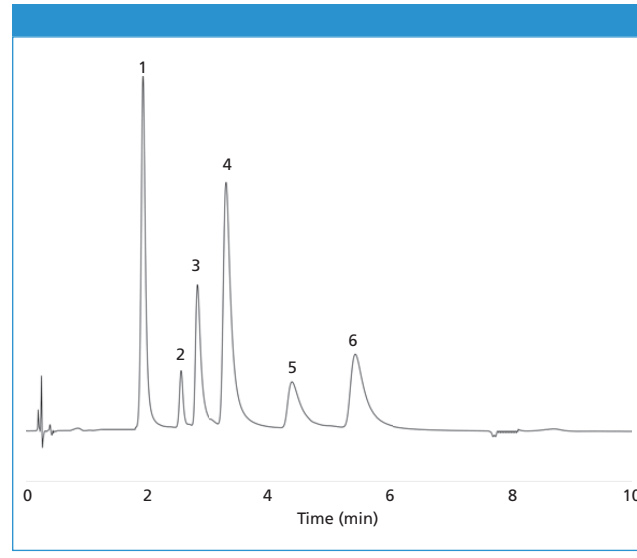

Figure 1: Rapid separation of six basic drug compounds on a 50 mm PRP-C18.

Results and Conclusion

In modern drug discovery science where analytical HPLC can be a bottleneck, the trend is to streamline production through the use of shorter columns with smaller particles operated at elevated flow rates. The flexibility to employ a high pH mobile phase is another valuable tool that permits separation of basic solutes in their neutral forms. Oftentimes, this greatly simplifies the process of methods development. In this study, separation of a set of structurally diverse pharmaceutical compounds is achieved on a short (50 mm) PRP-C18 column using a generic 5 min linear gradient.

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Pyrolysis GC–MS Reveals Different Phthalate Isomers in Vinyl Polymers

Karen Sam, CDS Analytical

CDS 6000 Series Pyroprobe coupled to a GC–MS is beneficial in phthalate analysis of plastics. This application uncovers intriguing information about phthalates used in vinyl products.

Because pure vinyl is a rigid material, flexible finished vinyl products contain a high amount of phthalate plasticizer. During analytical pyrolysis, these plasticizers are easily thermally desorbed, producing a large peak at the end of the pyrogram, which can be identified using a library search and their unique retention times.

These phthalate plasticizers may also produce unique decomposition products. Electrical tape has a peak for dioctyl phthalate (DOP), and among other things, a decomposition product of DOP, phthalic anhydride (Figure 1).

Unique decomposition products can help with phthalate identification. In the next example (Figure 2), a clear vinyl and a green vinyl each contain typical pyrolysis products of PVC, like the aromatics benzene, toluene, and indene. Each vinyl also has large plasticizer peak. Each peak has a similar retention time and similar mass spectra. With such similar mass spectra, a library search could easily mischaracterize them.

However, each plasticizer has a unique thermal decomposition product, circled in the figure. An ortho-substituted phthalate in the clear vinyl generates phthalic anhydride, but the para-substituted phthalate in the green vinyl generates 2-ethyl hexyl benzoate, helping to simply distinguish between the two.

It is interesting to note that the green vinyl was taken from a child's toy, in which certain phthalate plasticizers are regulated, and there are no regulation requirements for para-substituted phthalates.

The data presented here show clear advantages to identifying phthalates in vinyl materials using pyrolysis GC–MS.

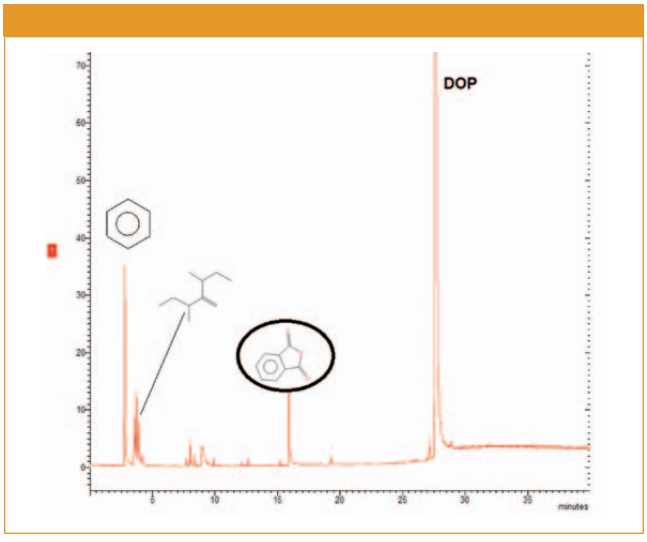

Figure 1: Electrical tape at 300 °C.

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Website: www.cdsanalytical.com/pyrolyzer

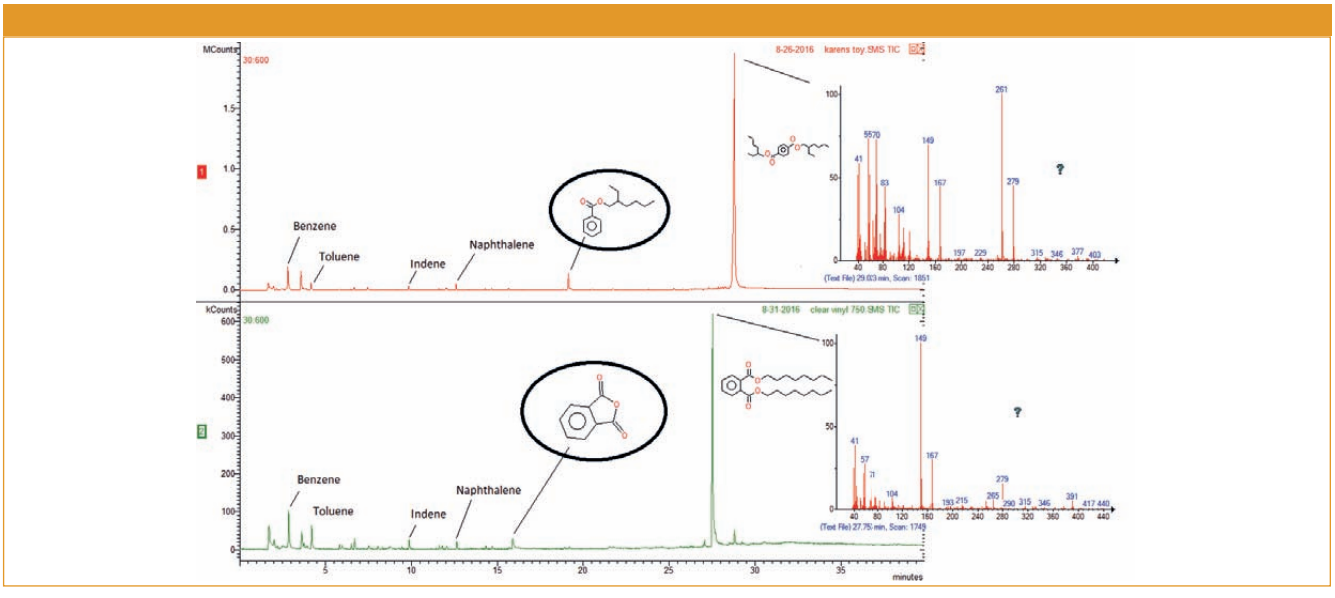

Figure 2: Green Vinyl (top), Clear Vinyl (bottom), 700 °C. Phthalate decomposition products circled.

Analysis of Cellulose Molecular Weight Distributions in DMAC

Wyatt Technology

SEC-MALS analysis of cellulose provides absolute molar mass distributions to understand the impact of different extraction processes. The biopolymer is solubilized in DMAC, enabling liquid chromatography without degradation.

Cellulose, a biopolymer of great importance to the fiber and paper industries, is difficult to characterize because of its high molar mass. Its intractable nature means it cannot be dissolved in conventional solvents without chemical modification. With tedious effort, it can be modified so that it can be dissolved in an easy-to-use solvent like THF, but when the cellulose is so modified it is degraded and the analysis does not represent the source material.

Unmodified cellulose can be dissolved in dimethyl acetamide (DMAC) with LiCl added. The problem remains, how to characterize it without reference to column calibration standards that typically do not have the same conformation as cellulose. Absolute characterization is performed by combining multi-angle light scattering with size exclusion chromatography (SEC-MALS) to determine molar mass, independently of elution standards.

Experimental Conditions

Separations were performed on a set of SDV-GPC columns in DMAC/LiCl. The separation columns were followed by the HPLC's UV detector, a DAWN® MALS detector (Wyatt Technology, Santa Barbara) and an Optilab® differential refractive index (dRI) detector (Wyatt Technology).

Data collection and analysis were performed in the ASTRA® software (Wyatt Technology) using empirically determined differential refractive index increments (dn/dc). Polymer molar mass M was calculated at each elution volume using signals from the two detectors.

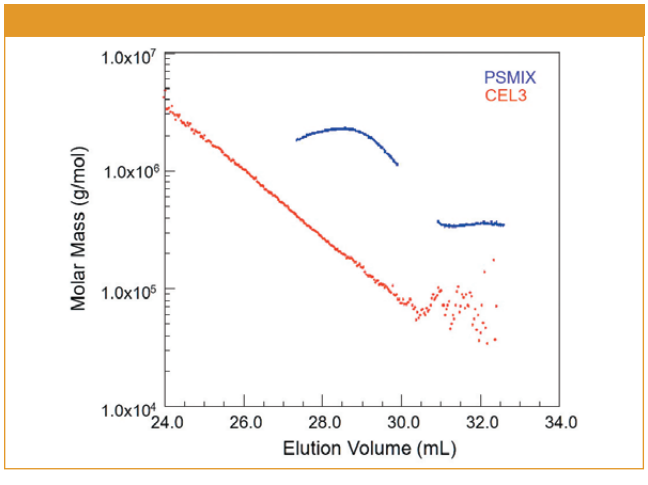

Figure 1: Two narrow polystyrene standards and a cellulose. Note that at the same elution volume, the "standard" gives a molar mass 10 times larger than the cellulose value.

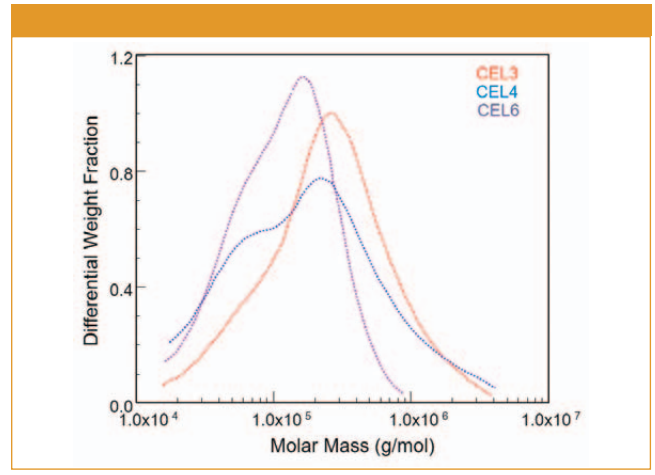

Figure 2: ASTRA's Differential Weight Distribution plot shows how different extraction processes create large variations in cellulose molar mass distributions.

Results

Molar masses determined by MALS in Figure 1 follow the usual logarithmic variation with elution volume. For the sake of comparison, a run of two mixed polystyrene standards is overlaid in a plot of molar mass versus elution volume. As can be clearly seen, a calibration based on polystyrene standards would overestimate the molar mass by more than a factor of five. This discrepancy is usually a result of branching, typical for cellulose in the MW range of 10^5–10^6 and above.

The technical process of extracting the cellulose from the wood pulp can have a profound effect on the molar mass distributions. Figure 2 shows the differences in molar mass distributions arising from different extraction processes. Only a MALS detector can reveal and quantify those differences and thereby MALS has become an important tool in optimizing the production processes for cellulose.

Conclusions

The SEC-MALS results prove that the lengthy process of solubilizing the cellulose has been mastered, enabling the manufacturer to optimize the cellulose extraction process.

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