ENVIRO-CLEAN® WAX

Your Go-To PFAS Clean-up solution for EPA Method 533.
Featuring a weak anion exchange polymeric sorbent bed with maximum exchange capacity, our cartridges provide robust recovery and reproducibility for diverse PFAS monitoring lists.

Available configurations:

- **ECWAX126-P**
  200mg bed volume / 6mL SPE cartridge

- **ECWAX156-P**
  500mg bed volume / 6mL SPE cartridge

Order samples today

Visit us online [unitedchem.com](http://unitedchem.com)
Shimadzu’s new **Nexera QX Multiplex LC-MS/MS System** incorporates innovative LC-MS multiplexing technology using dedicated sample introduction streams for continuous operation of the mass spectrometer. Combined with newly designed software control, Nexera QX significantly increases laboratory throughput and profitability.

> **Maximize Mass Spectrometer Productivity**
> Multiple Stream technology eliminates waiting for column equilibration or system flushing

> **Hardware Engineered for Multiplexing**
> Nexera SIL-40 UHPLC autosamplers, stream-dedicated injection valves and washing pumps deliver ultra-fast performance with ultra-low carryover

> **Software Designed for Multiplexing**
> Offers an intelligent, automated single point of control for multiple UHPLC streams and the LC-MS/MS

[www.ssi.shimadzu.com](http://www.ssi.shimadzu.com)

Shimadzu Scientific Instruments, 7102 Riverwood Drive, Columbia, MD 21046, 800-477-1227
THEIRS is one-size-fits-all.
OURS has room to grow.

Say goodbye to your comfort zone.
Learn more at ic.metrohmusa.com
CONTENTS

LC TROUBLESHOOTING
Surfing on Mobile Phase, Part I: Origins of Mobile-Phase Composition Waves and Their Effects on Detector Baselines
Dwight R. Stoll

Liquid chromatography (LC) pumps produce mobile-phase streams with small short-term variations in mobile phase composition. We explain the origin of these variations and their effects on chromatographic performance.

COLUMN WATCH
Modern Trends in Mixed-Mode Liquid Chromatography (LC) Columns
David S. Bell

Mixed-mode chromatography columns are on the rise. We review recent fundamental research, stationary phase development and design, and areas of application.

GC CONNECTIONS
Flying High with Sensitivity and Selectivity: GC–MS to GC–MS/MS
Nicholas H. Snow

By moving from GC–MS to GC–MS/MS, you can have both universal and selective detection along with low detection limits. Here’s how it works.

VIEWPOINTS
How Pharmaceutical Innovation Is Saving the World
Michael J. Hennessy

The COVID-19 vaccine, and the speed at which it was developed, is the medical breakthrough of our lifetimes.

PEER-REVIEWED ARTICLES

Online Hydrophobic Interaction Chromatography–Mass Spectrometry (HIC–MS) Analysis of Proteins
Andrew J. Alpert

Recent advances in stationary phases for hydrophobic interaction chromatography (HIC) permit HIC–MS analysis of intact antibodies and other proteins using direct flow to the mass spectrometer.

Ultra-Sensitive and Rapid Analysis of Per- and Polyfluoroalkyl Substances (PFAS) in Water
Emily Parry and Tarun Anumol

A method is described using a triple quadrupole LC–MS instrument with isotopic dilution to obtain the highest accuracy and confidence for analysis of per- and polyfluoroalkyl substances (PFAS) in water. Excellent method spike recoveries and robustness were found in wastewater.

FEATURE ARTICLE

The 2021 Winners of the Lifetime Achievement and Emerging Leader in Chromatography Awards
Jerome Workman, Jr.

Paul Haddad and Erik L. Regalado are the winners of the 14th annual LCGC Lifetime Achievement and Emerging Leader in Chromatography Awards, respectively. We review their achievements.
INTRODUCING THE NEW VICI®
D-3 PULSED DISHCARGE HELIUM IONIZATION DETECTOR

DESIGNED FOR THE AGILENT 8890 GC

VICI model D-3-I-8890 for the Agilent 8890 joins the lineup of Pulse Discharge Detectors already available for plug-and-play installation on the Agilent 6890 and 7890, and is optimized for trace level work in the helium ionization mode. Each kit includes all components required for installation:

- Detector
- Helium purifiers
- Pulser module
- Power supply
- Tubing
- Fittings

FEATURES

- Universal detector
- Wide linear dynamic range (>5 orders LDR, from low ppb to high ppm)
- Concentration Sensitive
- Easily added and configured on new or existing 8890 GC, using Agilent electrometer and interface boards

www.vici.com  800-367-8424  sales_usa@vici.com
Surfing on Mobile Phase, Part I: Origins of Mobile-Phase Composition Waves and Their Effects on Detector Baselines

The most commonly used designs for modern liquid chromatography (LC) pumps produce mobile-phase streams with small short-term variations in mobile phase composition. Understanding the origin of these variations and their effects on chromatographic performance can help us develop high-performing methods. In this installment, we focus on the effect of these mobile-phase composition “waves” on detector baselines.

Dwight R. Stoll

In this month’s installment of “LC Troubleshooting” I review the operating principles of LC pumps that rely on low- or high-pressure mixing approaches, describe how waves of solvent composition can develop in the mobile phase, and evaluate the potential impacts of these waves on detector baselines. In next month’s installment, I will discuss the results of simulations that show how these waves can impact variability in retention time along with some solutions to these problems. One could devote an entire book chapter to these topics, so I discuss the highlights here, providing a concise overview of LC pump technology.

Review of LC Pumping Principles—Low- and High-Pressure Mixing Designs

Figure 1 illustrates the basic principles of the two of the most common designs for pumping systems used in LC. In the case of the high-pressure mixing approach, two independent pump heads, each capable of producing a high-pressure stream of a mobile-phase component, draw in and discharge solvent at a consistent flow rate (for isocratic operation). For example, if the total flow rate through the column is 1.0 mL/min, and the desired mobile-phase composition is 40:60 acetonitrile:water, then one pump head discharges acetonitrile continuously at 0.4 mL/min, and the other pump head discharges water continuously at 0.6 mL/min. The two streams discharged from the pump heads then converge and pass through a mixer, and the mixed mobile phase proceeds to the sampler, and eventually the column. In the case of the low-pressure mixing approach, there is only one pump head that pressurizes the mobile phase to drive it through the column. The mobile-phase composition is determined by assembling small “packets” of individual solvents in a serial fashion into a mobile-phase stream that is drawn into the high-pressure pump. In most modern pumps of this design, the volume of each solvent packet is determined by the length of time a solenoid-type valve is open between the solvent bottle and the proportioning valve unit. Furthermore, these times are also related to the volume of each stroke of the high-pres-
Cells beyond their sell-by date exhibit a higher probability to propagate damaged cellular components like DNA, which leads to disease. It is therefore necessary for the body to remove these old malfunctioning cells. One method the body uses to remove these cells is programmed cell death, or apoptosis. Garlic has shown a remarkable ability to induce apoptosis of damaged or malfunctioning cells in multiple studies. Induction of senescent cell death is not the only role garlic plays in the body. It has also been documented as providing proliferative benefits in the digestive, cardiac, and neural areas of the body. These wonder chemicals have shown positive affects in the fight against the number one killer of people in the United States: cardiac complications. The compounds in garlic inhibit the aggregation of platelets in the blood stream and reduce the likelihood of thrombotic events.

With a multitude of health applications, garlic separations appear to be a necessary quest. All of the active components of garlic are easily isolated using the 5 µm PRP-C18 HPLC column from Hamilton Company. Good peak shape is observed on both the hydrophilic and hydrophobic regions of the chromatogram. The beauty of garlic is the multitude of active components containing both lipophilic and lipophobic properties all combined into one clove. Isolation of any of the active components is easily achieved due to the high analyte loading capacity associated with this column, making it a great choice when scaling up from analytical to preparatory isolation is desired. An added benefit when using the PRP-C18 column is the ability to easily regenerate the column if previous separations had fouled the surface chemistry. The regeneration can restore the media back to the original peak shape and reproducibility, thereby enhancing the longevity and value of the column.

Column Information

<table>
<thead>
<tr>
<th>Packing Material</th>
<th>PRP-C18, 5 µm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimensions</td>
<td>150 x 4.6 mm</td>
</tr>
<tr>
<td>P/N</td>
<td>79676</td>
</tr>
</tbody>
</table>

Chromatographic Conditions

<table>
<thead>
<tr>
<th>Gradient</th>
<th>Intensity (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00 – 12.5 min.</td>
<td>2–30% B</td>
</tr>
<tr>
<td>12.51 – 17.0 min.</td>
<td>30% B</td>
</tr>
<tr>
<td>17.01 – 19.0 min.</td>
<td>30–99% B</td>
</tr>
<tr>
<td>19.01 – 22.0 min.</td>
<td>99% B</td>
</tr>
<tr>
<td>22.01 – 25.0 min.</td>
<td>2% B</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Temperature</th>
<th>35°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detection</td>
<td>UV at 215 nm</td>
</tr>
<tr>
<td>Eluent A</td>
<td>Water</td>
</tr>
<tr>
<td>Eluent B</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>Flow Rate</td>
<td>1.0 mL/min</td>
</tr>
</tbody>
</table>

Compounds:
1. Cysteine
2. Alliin
3. Allyl Cysteine
4. Diallyl Trisulfide
5. Allyl Methyl Sulfide
6. Allyl Sulfide
7. Dipropyl Disulfide

For more information on Hamilton HPLC columns and accessories or to order a product, please visit www.hamiltoncompany.com or call (800) 648-5950 in the US or +40-356-635-055 in Europe.
Sure pump and the mobile-phase flow rate. For example, suppose the stroke volume is 100 µL, the desired mobile-phase composition is 40:60 acetonitrile:water, and the flow rate is 1 mL/min. The period of each pump stroke will be 6 s; the solenoid for the acetonitrile line will be open for 2.4 s, drawing pure acetonitrile into the tubing leading from the proportioning valve to the pump head. Then, this valve closes, and the solenoid for the water line opens for 3.6 s and pure water is drawn into the tubing. This completes one cycle of mobile-phase composition proportioning. The solvent composed in this way is mixed extensively as it travels through the high-pressure pump head itself, and an additional mixer is positioned between the pump and the sampler.

Origins of Solvent Waves and Their Impacts on Detector Baselines

Each of the pump designs discussed above has several strengths and weaknesses. In both cases, however, the mobile-phase composition at the pump outlet will not be perfectly smooth (that is, no variation in composition during isocratic operation; in gradient elution, the change in composition over time would ideally be smooth without any short-term noise). The primary causes of the deviations of the actual mobile-phase composition from what is programmed are different for the two designs. In the case of the low-pressure mixing approach, it is intuitive that there would be short-term variations on the time scale of one pump stroke. During the pump stroke, there are times when the fluid entering the high-pressure pump is literally all A or all B. This is illustrated in Figure 2b. The resulting variation in mobile-phase composition can be smoothed to a large degree with effective mixing downstream from the high-pressure pump, but completely eliminating the variation would require a large volume mixer. If the detector is capable of detecting small variations in composition (for example, refractive index detection, or ultraviolet [UV] detection in the case where mobile-phase additives absorb UV light, such as TFA), then they will be observable in the detector signal as “waves,” or short-term noise. Adding a large mixer introduces other problems, such as a large delay between the time of a programmed change in composition and when that change arrives at the column (in gradient elution this appears as the “gradient delay” or “dwell” time). Thus, the configurations of these pumps as received from manufacturers reflect a compromise between

---

**FIGURE 1:** Block diagrams for the two most commonly used designs of high performance liquid chromatography (HPLC) pumps in use today: (a) binary pump with high-pressure mixing; and (b) quaternary pump with low-pressure mixing.

**FIGURE 2:** Conceptual illustration of the origin of mobile phase composition waves in the case of (a) high-pressure mixing and (b) low-pressure mixing designs used in modern pumps.

**FIGURE 3:** Comparison of ultraviolet (UV) absorbance signals (214 nm) obtained with different mixers in use. The pump was a high-pressure binary mixing system (Agilent 1290, Infinity II). The column was a 30 mm x 2.1 mm i.d. Agilent SB-C18, and gradient elution was used. Solvent A was 0.1% trifluoroacetic acid (TFA) in water, solvent B was 0.1% TFA in acetonitrile (ACN), and the gradient ran from 2–40% B in 4 min.
doing enough mixing to smooth out these waves to a large extent and not adding a mixer that is so large that it causes other problems. Indeed, pump manufacturers offer mixers of different volumes that allow the user to choose a larger volume mixer for applications that are expected to be especially sensitive to short-term variations in mobile-phase composition (for example, applications involving TFA).

The primary origin of solvent waves in the case of high-pressure mixing is fundamentally different. In this case, if the flow rate from each pump head were perfectly consistent over time, the composition of the mixed mobile phase would be perfectly consistent over time. But, in a reciprocating piston design (which is the dominant design in use today), there are small changes in the flow from each pump head at the end of a piston stroke due to the imperfect operation of check valves. These small changes in flow are illustrated in Figure 2a. If these flow rate changes are different for channels A and B, and they are not perfectly synchronized in time, then there will be a small, short-term variation in the composition of the mixed mobile phase. The lengths of the resulting waves in this case tend to be shorter in comparison to the low-pressure mixing case, as illustrated in Figure 2. These waves can also be greatly minimized by introducing a mixer between the solvent convergence point in the pump and the LC column. However, the same challenge exists here as with the low-pressure mixing design: Completely eliminating the waves requires a large mixer, so what we use in practice represents a compromise between smoothing the mobile-phase composition and having a low gradient delay volume (which is essential for fast gradient elution separations, for example).

As mentioned above, the mobile-phase composition waves that flow from the pump can impact the quality of detector baselines (as measured by noise and drift) if the detector signal is dependent on the composition of the mobile phase itself. Most of the time, this can be avoided or minimized through judiciously choosing the conditions and instrument parameters. For example, acetonitrile is attractive as a mobile-phase organic solvent modifier for reversed-phase liquid chromatography (RPLC) when using UV detection at low wavelengths (< 230 nm). If the mobile-phase components are effectively transparent to the detector, then small variations like waves in the composition will not impact the quality of the detector baseline signal. Sometimes, though, this is impossible, or at least very difficult to avoid. One well known and studied example is the case where TFA is used as a mobile-phase modifier for RPLC separations of peptides involving UV detection. TFA is attractive because it tends to improve peak shapes for peptides and increase retention for hydrophilic peptides. However, a disadvantage of TFA in this context is that it absorbs a significant amount of UV light at 214 nm, which is the wavelength typically used for peptide mapping applications. Furthermore, the TFA itself is somewhat retained by RPLC stationary phases. When a mobile-phase composition wave travels through the column, the acetonitrile-rich part of the wave will decrease the local retention of TFA, dumping more of it into the mobile phase where it will absorb more UV light. This is a very complex situation that cannot be thoroughly discussed here, but there are at least two excellent papers (5, 6) that describe all of the factors involved and demonstrate the impact of different chromatographic variables on baseline quality when using TFA in the mobile phase with UV detection. I strongly encourage readers interested in learning about this situation in more detail to consult these papers.

In a previous installment of “LC Troubleshooting”, I discussed why mobile-phase mixers are needed following LC pumps, and the types of situations when a change in the type of mixer might be needed (7). Figure 3 shows the effect of increased mixer volume on the noise level in UV detector baselines when using TFA in the mobile phase delivered by a binary pump. Although the 35 µL mixer might be adequate for other applications, a larger volume mixer is helpful for reducing baseline noise in this case because of the increased sensitivity of the baseline noise to mobile-phase composition waves when using TFA as an additive. In this column, I have reviewed the operating principles of modern LC pumps based on low- and high-pressure mixing designs and explained how these pumps produce mobile-phase streams with small short-term variations in mobile-phase composition. These composition “waves” can negatively affect detector baseline quality and also retention time variability. In next month’s installment of “LC Troubleshooting”, I will continue exploring this topic by discussing the results of simulations that illustrate the effect of method parameters including the amplitude of the composition waves, flow rate, and pump stroke volume on retention time precision.

References
Modern Trends in Mixed-Mode Liquid Chromatography (LC) Columns

Commercialization of columns that provide multiple modes of chromatographic separations have recently been on the rise. For example, combinations of retention modes, such as ion-exchange and reversed-phase, often enable the separation of complex mixtures of analytes not possible using single-mode columns. In this work, recent trends in what is often referred to as “mixed-mode” phase are investigated. Particular attention is paid to recent fundamental research, stationary phase development and design, and areas of application.

David S. Bell

In recent LCGC reviews of newly launched high-performance liquid chromatography (HPLC) columns, it was observed that several stationary phase developments fell into a category that can be characterized as “mixed-mode” (1,2). The idea of mixed-mode chromatography is not new. However, the observation prompted the question, “What is the current status of mixed-mode chromatography?”

An initial search through the literature and various websites revealed that there have been a multitude of publications, including reviews, that have written about mixed-mode chromatography in recent years. For example, West and others published a review of mixed-mode chromatography that covered many important developments, including combinations of reversed-phase liquid chromatography (RPLC) and ion-exchange chromatography (IEC), as well as hydrophilic interaction liquid chromatography (HILIC) and ion-exchange (3). Zhang and Liu published another notable review that focused on mixed-mode chromatography in which they described the potential to address a number of analytical challenges. Mixed-mode chromatography has played a significant role in modern chromatographic practices and has been applied to areas such as oligonucleotides, peptides, proteins, metabolomics, pharmaceutical analysis, and natural product studies, among many others (4).

In an attempt to take an even more recent pulse on the level of interest and utilization of mixed-mode chromatography, a non-exhaustive search of publications in the past year along with a perusal of well-known column vendor websites was conducted. The limited search revealed a significant number of papers and technical notes in this realm, indicating continued interest. What follows is a brief synopsis of what was found, broken down into fundamental studies, new stationary phase designs, and applications of the technology.
**Fundamental Research**

The application of mixed-mode chromatography is often limited, because of the complexity of the resulting chromatography. With more mechanisms playing a significant role, additional parameters that control these mechanisms need to be taken into account during method development and practice of the resulting procedures. In addition, it is often difficult to find a full description of surface chemistry from vendors or detailed characterization results (8). Fundamental studies that provide the user with information to help guide method development and understand both the potential and limitations of stationary phases is of utmost importance.

Lämmerhofer and associates used chromatographic, molecular modeling and electrochemical techniques to characterize chiral zwitterionic materials (7). Although the target phases were commercialized for use in chiral separations, the group notes significant achiral utility. The columns studied were described as cinchona carbamate stationary phases decorated with cyclohexylsulfonic acid and carbamate residues and are thus likely to provide multiple dominant retention mechanisms. Indeed, the group found the phases to exhibit moderate hydrophobicity, the potential for HILIC operation, and ion-exchange character. A unique aspect of this research was that investigators used a sequential building and analysis of intermediary versions of the stationary phases to better understand the individual structural contributors to retention mechanisms.

Gilar and co-workers investigated the chromatographic attributes of several commercially available columns, including single-mode as well as a recently launched mixed-mode column combining C18 and anion-exchange chemistry (5). The group focused their efforts on understanding the ion-exchange interaction potential of the columns across a wide pH range. Fundamental information regarding the interactions available across a number of different conditions is important to method developers for both selecting

**FIGURE 1:** Optimized separation of seven artificial sweeteners on a mixed-mode stationary phase. Reproduced with permission from reference (17). ACN is acetonitrile.
and utilizing various stationary phases. For instance, the researchers were surprised to find that the ion-exchange interactions for the mixed-mode phase dropped off at a pH value much lower than the ligand pKₐ value. The observation was attributed to the impact of ionized surface silanols on the overall surface charge and demonstrates both the complexity and the need to carefully study mixed-mode systems.

New Stationary Phase Research
The majority of the published research over the past year has focused on the development of new stationary phases and their characterization. What follows is not intended to be an exhaustive list, but it should provide an overview of the types and breadth of stationary phases being investigated across the globe in this field. Shields and Webber reported on the development of a mixed-mode, reversed-phase cation-exchange system based on a thiol-yne reaction. The intent was to develop a mixed-mode column with low pH stability. The authors used the separation of monoamine neurotransmitters to demonstrate both the ion-exchange and partition properties of the phase (9). Guo and others published a paper describing the interesting combination of MOF-235, polyethylene glycol, and silica, in a core–shell-based format. The authors indicate a combination of HILIC and ion-exchange is possible with the phase composition (10).

Li and associates described the use of modified dialdehyde cellulose as a substrate for developing stationary phases that exhibit both HILIC and ion-exchange properties. The authors note facile functionalization and suggest this approach as the basis for further stationary phase development (11).

Several stationary phase developments have been recently published by Wang and co-workers. The authors describe a poly(ethyleneimine) embedded N-acetyl-L-phenylalanine stationary phase that is shown to exhibit RPLC, HILIC, and ion-exchange characteristics (12). In a second paper from the group, a stationary phase co-modified with N-isopropylacrylamide and aminophenylboronic acid is described. The resulting phase was also characterized as providing both hydrophobic and hydrophilic retention capabilities along with anion-exchange properties (13). Wang and associates also reported on the incorporation of ionic liquids with C18 and cyclodextrin moieties bonded to silica supports as potential stationary phases for liquid chromatography (LC) (14). The group characterized the phases as exhibiting hydrophobic and ion-exchange character as well as the potential for use in the HILIC domain. A positive comparison with commercially available, single-mode columns was provided.

Heydar and Hosseini published a paper describing the preparation of a novel, silica-based stationary phase using 9-methyllacridine and 9-undecylacridine (15). The phases were shown to exhibit hydrophobic and hydrophilic partitioning as well as anion-exchange characteristics. Finally, Wolrab and associates investigated a series of zwitterionic and strong cation-exchange based mixed-mode phases under RPLC, HILIC, and supercritical fluid chromatography (SFC) conditions (16). The authors noted that ion-exchange interactions appear to prevail over others for the phases studied. However, other interactions, such as partitioning, can be enhanced or attenuated using various mobile phase conditions.

Applications
Mixed-mode chromatography is powerful, but complex. As noted above, when multiple dominant retention mechanisms are invoked, many variables must be controlled to produce a robust and repeatable method. Mixed-mode chromatography is therefore most often applied only to complex systems. The following are examples of mixed-mode applications found in recent literature.

Artificial Sweeteners in Surface Waters
The combination of anion-exchange and RPLC was used to analyze artificial sweeteners in surface waters. Anionic sulfamates (acesulfame, cyclamate, saccharin), zwitterionic dipeptides (asparagine, neotame), and polar derivatives of natural products (sucralose, neohesperidin dihydrochalcone [NHDC]) were all efficiently separated on an octadecylsilane (C18)-strong anion exchanger (SAX) combination phase, as shown in Figure 1. One of the major advantages of the mixed-mode approach noted by the authors was the ability to inject large volumes of sample, presumably because of the accumulation effect of the ion-exchange character (17).
Separation of Oligonucleotides
Zhang and associates recently reported on the use of mixed-mode chromatography for the analysis of oligonucleotides. The use of ion-pair reversed-phase chromatography (IP-RPLC) appears to be the most heavily employed mode of separation for oligonucleotides to date. However, anion-exchange, HILIC, and mixed-mode methods have also been successful. According to the authors, the use of mixed-mode separations for oligonucleotides dates back to the early 1980s. The authors also note that RPLC and ion-exchange mixed-mode approaches have displayed improved separations over either mode alone. In addition, it is speculated that multidimensional liquid chromatography (mLC) is poised to deliver much of what mixed-mode chromatography can do and is expected to continue to grow in this space (18). Lämmerhofer and co-workers also proposed the use of a chiral zwitterionic phase, which exhibits both partition and anion-exchange properties, for the first dimension separation of oligonucleotides using RPLC as the second dimension (19).

Underivatized Amino Acids
The application of mixed-mode chromatography for the separation of underivatized amino acids was demonstrated by Moussa and associates (20). The traditional amino acid analysis routine consists of an ion-exchange based separation followed by reaction with ninhydrin or similar reagent. The traditional approach is noted as being time-consuming, nonspecific, and requires a dedicated analyzer. The authors used a “trimodal” column consisting of anion-exchange, cation-exchange, and hydrophobic properties to separate 52 amino acid-related compounds in 18 min with minimal sample preparation. The authors noted that the bimodal systems investigated did not provide the necessary separation of critical pairs.

Deamidation of Proteins
Sze and co-workers reported on the utility of employing ion-exchange in combination with reversed-phase and HILIC separations for the improved analysis of deamidation in proteins (21). The authors note that deamidation of proteins results in several species of very similar hydrophobicities that are thus difficult to separate using partition chromatography alone. The authors suggest that the use of electrostatic interactions—coupled with HILIC chromatography, along with improved sample preparation and advanced mass spectrometric techniques—will help serve to fill deficiencies in this important area of research.

Pentacyclic Triterpenoids
Another application of mixed-mode chromatography published in 2020 tackled the separation of pentacyclic triterpenoids using a mixed-mode, weak-anion-exchange stationary phase (22). The critical pairs of erythrodiol and uvaol, as well as oleanolic acid and ursolic acid, were only resolved with a combination of reversed-phase or HILIC with ion-exchange mechanisms, whereas both RPLC and HILIC alone...
were found to be insufficient. As shown in Figure 2, the mixed-mode column utilized provided the separation of 10 analytes in approximately a 7-min run time.

It should also be noted that many vendors provide application data in support of the use of mixed-mode phases. A cursory search through websites of many prominent vendors showed recent activity in this realm. The reader is encouraged to visit column vendor sites for more information.

Conclusions
A non-exhaustive literature search and perusal of web-based information revealed that interest in mixed-mode chromatography is alive and well. Recent applications in environmental, pharmaceutical, and biopharmaceutical areas demonstrate that the use of multiple dominant retention mechanisms continues to assist researchers in meeting the need for complex separations. The breadth of research toward the development of new mixed-mode stationary phases and their subsequent characterization over the past year indicates there is significant space for, and interest in, new discoveries. Finally, fundamental research and characterization of commercially available columns is paramount and is expected to greatly facilitate the intelligent use of these powerful stationary phases.

References

ABOUT THE AUTHOR
David S. Bell is a director of Research and Development at Restek. He also serves on the Editorial Advisory Board for LCGC and is the Editor for “Column Watch.” Over the past 20 years, he has worked directly in the chromatography industry, focusing his efforts on the design, development, and application of chromatographic stationary phases to advance gas chromatography, liquid chromatography, and related hyphenated techniques. His main objectives have been to create and promote novel separation technologies and to conduct research on molecular interactions that contribute to retention and selectivity in an array of chromatographic processes. His research results have been presented in symposia worldwide, and have resulted in numerous peer-reviewed journal and trade magazine articles. Direct correspondence to: LCGCedit@mmhgroup.com
Flying High with Sensitivity and Selectivity: GC–MS to GC–MS/MS

Mass spectrometry (MS), often termed mass selective detection, is the most powerful detector available for gas chromatography (GC). Multidimensional mass spectrometry (MS/MS) takes mass selective detection to another level on benchtop systems, offering both universal and selective detection along with low detection limits. In this installment of “GC Connections,” we review the fundamentals of MS/MS and how they relate to MS as a detector for GC. We see how using full-scan analyses can make the detector universal, and how, by using selected ion monitoring and multiple reaction monitoring, the detector can be so selective and noise-free that femtogram quantitative analysis is commonplace. We then examine some scenarios that should lead analysts to consider using GC–MS/MS to solve complex problems.

Nicholas H. Snow

High sensitivity and selectivity are among the most important goals of any chromatographic method development or optimization process. Instruments, stationary phases, and detectors are usually chosen with one or both of these goals in mind. In gas chromatography, mass selective detectors (MSDs or mass spectrometers) have been used for decades to provide both high selectivity and high sensitivity. Capillary gas chromatography coupled to mass spectrometry (GC–MS) is a straightforward, yet powerful, coupling of the selectivity of GC with the high sensitivity and option of universal or selective detection of MS. Traditional GC–MS provides multiple dimensions of separations and low detection limits in benchtop or smaller instruments.

Before flying into the details of MS/MS, we should briefly review some of the terminology specific to MS as a detector for GC. Mass selective detectors operate in two modes. The first mode is full-scan, in which spectra are continuously collected in quadrupole systems at rates usually up to 10–20 spectra per second, depending on the mass range selected. The second mode is selected ion monitoring (SIM), in which one or more individual ions are monitored. The data can be obtained in three forms:

- A total ion chromatogram (TIC) is the sum of all signals that reach the detector and is a demonstration of nearly universal detection. The full mass spectrum can be obtained at any point on the chromatogram.
- An extracted ion chromatogram (EIC) is obtained from the TIC by choosing one or more individual masses and extracting these from the full data set. This allows both universal and selective detection in a single experiment, since the ion chosen for analysis can be characteristic of a single compound or compound class.
- Selected ion monitoring (SIM) is obtaining a TIC in which the detector is set to monitor only one or a few ions. If a spectrum is selected from the TIC, it will only show the few ions that were selected when the experiment was set up.

There are several common GC–MS and GC–MS/MS instruments. Single dimension, classical GC–MS is mainly performed using quadrupole mass analyzers. Ion trap, a derivative of quadrupole instruments, and time-of-flight (TOF) are also used for specific analyses. Quadrupole-based systems are simpler and less expensive; GC–TOF-based systems offer the highest sensitivity and much greater mass precision and accuracy.

GC–MS/MS can be achieved through several configurations, with a wide range of capability and complexity. The most common of these is GC–triple quadrupole-MS (GC–TQMS), while GC–ion trap-MS (GC–ITMS) and GC–quadrupole time-of-flight MS (GC–QTOF-MS) are also available. A brief discussion of the evolution of ion trap and triple quadrupole mass analyzers over the years is provided in the brochure by Huebschmann (1). Professors Chris Enke and Rick Yost, inventors of the TQMS, have provided two excellent video interviews discussing the development of the technique in detail (2,3).

Figure 1 shows a block diagram of the detector on a GC–TQMS system compared to a traditional GC–MS system. Both are available in benchtop configurations. The main difference between the two systems is the presence of three quadrupole mass filters on the GC–TQMS system and one on the GC–MS system. Both systems use a transfer line with a capillary direct interface into the ion source and...
In the second part of this three-part series, LCGC continues its conversation with Christoph Johann, global product manager at Wyatt Technology, as he discusses the benefits of coupling light scattering online, field-flow fractionation’s (FFF) role in the pharmaceutical industry, its acceptance by regulatory agencies, and more.

**LCGC: Can you explain the benefit of coupling light scattering online with FFF?**

**Christoph:** While FFF generally separates particles according to hydrodynamic size, following strict fluid dynamic equations, there are sufficient uncertainties in channel and membrane properties that you cannot rely on retention time only to determine size accurately. In this sense, it is very similar to size-exclusion chromatography (SEC): online multi-angle light scattering (MALS) and dynamic light scattering (DLS) detectors are crucial for reliable and accurate characterization of the particle size, concentration, conformation, and the molar mass.

As in chromatographic methods, additional detectors can be added for deeper characterization. Typically, refractive index, UV/Vis, and fluorescence HPLC detectors may be included, enabling quantification of drug or nucleic acid loading, encapsulation, and similar properties that relate to particle composition and conjugation.

So, it is the complete system of robust FFF separation, with powerful light-scattering and spectroscopic detection, that provides comprehensive characterization capabilities.

**LCGC: Where do you see the increasing need for FFF in the pharmaceutical industry?**

**Christoph:** There are two parallel and similar paradigm shifts going on at full speed. In traditional small-molecule pharmaceuticals, more drugs are formulated as nanoparticles, whether as emulsions, nanosols, or encapsulated in liposomes or other nanocarriers, including lipid nanoparticles, polymer micelles, polymersomes, albumin particles, polyplexes, etc.

In biopharmaceuticals, commercialization of gene therapies—the delivery of DNA or RNA by viral or non-viral gene vectors—is in high gear. SEC-MALS is suitable for small vectors like adeno-associated virus, but larger vectors such as lentivirus or adenovirus require separation by FFF. Non-viral vectors are very similar to small-molecule nanodrug-delivery systems, e.g., lipid nanoparticles or polymersomes. In both cases, the trend is to deliver therapeutic payloads in delivery vehicles that are in the size range of 30 to 300 nanometers, which is very different from the size of current drugs based on small molecules, peptides, proteins, or microparticles.

The standard tools in place for characterizing these new modalities—whether batch DLS or nanoparticle tracking analysis for nano drug delivery systems (DDS) or qPCR and ELISA for gene vectors—are simply insufficient to meet the challenges and analytical needs presented by these complex therapeutics. FFF with MALS, DLS, and spectroscopic detectors provide a powerful and versatile characterization platform that is perfectly matched to these products.
insufficient to meet the challenges and analytical needs presented by these complex therapeutics. FFF with MALS, DLS, and spectroscopic detectors provide a powerful and versatile characterization platform that is perfectly matched to these products.

"The need for FFF-MALS-DLS in characterization for regulatory fillings of drugs, and eventually quality control of nanomedicines, is fully recognized by regulatory agencies and the institutions and organizations developing standards for the pharmaceutical industry.

LCGC: How complicated are these analyses?

Christoph: Setting up the separation method is pretty straightforward, especially with our SCOUT method simulation software. The actual operation is very similar to HPLC—the samples are loaded into vials, placed in the autosampler, and run through fully automated sequences. Basic analyses like average size, size distribution, shape, and particle concentration for each fraction are also straightforward and can be fully automated. Analyses of composition and encapsulation require a combination of multiple detector signals, which may require method development and calibration steps, but once those are done, the rest is automated and easy. Best of all, all of these characterizations are completed in a single injection. Replicates, of course, are elementary.

Wyatt Technology is the recognized leader in light-scattering instrumentation for characterizing macromolecules and nanoparticles in solution. Wyatt’s products determine absolute molar mass, size, charge, interaction properties, conformation, and conjugation. The company offers a complete suite of multi-angle light scattering (MALS) instruments, field-flow fractionation (FFF) systems, dynamic light scattering (DLS), and zeta potential instruments and detectors to measure refractive index and intrinsic viscosity.
Both operate with the ion source and mass analyzer at high vacuum, and use a classical electron multiplier to detect ions that pass through the ion source. As described in more detail below, the first quadrupole (Q1) performs in the same manner as the single quadrupole in traditional GC–MS, selecting the ions that are ultimately passed to the electron multiplier detector. It can operate in either full-scan or selected ion monitoring modes. The second quadrupole (Q2) is used as a medium for collision induced fragmentation of ions passed through the first quadrupole to produce new fragments, and the third quadrupole (Q3) is used to select and analyze these new fragments.

MS/MS is among the most flexible of all detectors, as it operates in several modes. In traditional GC–MS, full-scan MS provides a nearly universal detector; any analyte that can be ionized within the ion source can be detected. SIM-MS is a highly selective detector; the signals for the chosen ions are the only ones recorded. SIM is used for quantitation as the reduction in the signals being monitored versus full scan also reduces the noise, increasing the signal-to-noise (S/N) ratio, and therefore lowering the detection limit.

Figure 2 shows how the most common modes of quadrupole MS and MS/MS detection work. Full-scan and SIM single quadrupole detection are seen in the left side of the figure, in the ion source and Q1 images. The ion source generates ions including many masses; the quadrupole can either pass all of them (full scan) or selected ions (SIM). Triple quadrupole MS offers even more flexibility, since two additional quadrupoles are employed, as seen in Figure 2. Note that in both GC–MS and GC–MS/MS, classical electron ionization (EI) is by far the most commonly used ion source mechanism, so this is assumed in the following discussion.

A GC–MS/MS system can be operated exactly as a single quadrupole system. The second and third quadrupoles can be set to pass ions through without any further separation or reaction. This is often the first step in developing a method or transferring one to GC–MS/MS as it provides a traditional total ion chromatogram and traditional mass spectra of the analytes as a starting point.

In a product ion scan, the first quadrupole (Q1) can be set for selected ion monitoring, as in traditional GC–MS. Collision-induced ionization then occurs in the second quadrupole (Q2) to generate further fragmentation of the chosen ion, providing additional fragmentation that is analyzed by scanning the third quadrupole (Q3). This is a powerful tool for qualitative analysis since larger fragments from a traditional single dimension mass spectrum can be further fragmented to...
True Food Safety Starts with LECO

Are you identifying both known and unknown pesticide residues? LECO instruments, featuring our exclusive ChromaTOF software, perform a rapid full-scan screening for all pesticide residues that may be present for a more accurate snapshot of what’s hiding in your fruits and vegetables.

True food safety should not only be about performing a risk analysis based on a fixed list. Identify unknown residues and create greater confidence in your results, in your food safety monitoring program, and in your products with instruments from LECO.

Phone: 1-800-292-6141 | info@leco.com
www.leco.com | © 2021 LECO Corporation

PEGASUS® HRT® GCMS

PEGASUS® BT GCMS
aid in confirming the correct structure. A product scan is also used for choosing transitions in initial method development for multiple reaction monitoring.

In a precursor ion scan, Q1 can be operated full scan, which passes all of the fragments generated in the ion source; think of traditional MS without the detector, with all of the resulting fragments reionized in Q2, and a single fragment chosen for monitoring in Q3. This is very similar to SIM in traditional GC–MS and is useful for quick quantitative analysis method development.

In multiple reaction monitoring (MRM), the most sensitive mode for quantitative TQMS, both Q1 and Q3, are set for single ion analysis. Based on the full-scan mass spectrum or a product ion scan, a fragment from Q1 is chosen and then, based on the further fragmentation in Q2 as seen by scanning Q3, a single fragment from Q3 is chosen. This provides possibly the ultimate selectivity as the possibility of two compounds, even closely related isomers, having the same transitions from precursor ion fragment to product ion fragment and the same (or close) retention time in the column decreases greatly. MRM also provides very low detection limits by significantly reducing noise in both dimensions.

Figure 3 shows the well-known mass spectrum for caffeine, which provides an example for the utility and power of MRM. The mass transitions that generate the signals seen in the mass spectrum are also provided. In MRM, this full-scan spectrum is the starting point for method development, either determined experimentally or obtained from the literature. As this is an election ionization spectrum, it is good practice to interpret the spectrum, using traditional spectral interpretation rules at this point for a full understanding of the structural elements and decomposition reactions that generate each of the fragments. There are multiple excellent tutorial books relating to election ionization mass spectral interpretation available (4,5). Should there be additional spurious peaks, these can be identified at this point so they do not cause confusion later.

In traditional single dimension GC–MS, the full-mass spectrum is used to identify the analyte. Quantitation is performed using either extracted ion chromatograms obtained by selecting one or more individual masses from the total ion chromatogram or by selected ion monitoring. SIM offers lower detection limits by reducing noise as most of the mass signals seen in the full-scan spectrum and their accompanying noise are eliminated.

In MRM, further advantage is taken of this noise reduction. One or more peaks from this initial mass spectrum is then chosen for further fragmentation. Usually this is the largest (base) peak, or it can be another strong signal that may be more characteristic of that compound. With this mass chosen, a second experiment is performed with Q1 operating in SIM mode to pass the one ion, termed a Precursor ion, with that ion being reionized in Q2, with the resulting new fragments, termed product ions, passed to Q3 operating in scan mode as seen in the Figure 2 product scan, to generate a mass spectrum of the fragment. One or more ions from this product spectrum can then be chosen for the final quantitative method. Any of the transitions seen in Figure 3, such as those from mass 194 to mass 109 are termed MRM transitions, and are often reported in the literature for completed methods. Any of the transitions can be used with one for quantitation and additional transitions for confirmation. Selectivity is generated because, instead of looking at individual masses that may not be unique to a compound, this is looking at transitions, which are very unique, especially when multiple transitions are used.

The ability to obtain spectra from precursor ions and to perform MRM leads to three situations in which GC–MS/MS is especially useful:

1. Targeted analysis of a few analytes, in which you know the identity of the analyte or analytes, for which extreme sensitivity or low detection limits are required and/or the sample matrix is highly complex.
2. Simultaneous targeted analysis of many analytes whose chromatographic peaks are not fully resolved.
3. Untargeted analysis in which the matrix is complex, analyte chromatographic peaks are overlapped and the additional qualitative information about fragmentation is needed.

Analysis of extremely low levels of emerging contaminants in environmental water samples is an example of the first case. These appear at very low levels, and are the result of human activity. One application, freely available online shows analysis of several steroids in water at low parts per billion (ppb) and parts per trillion (ppt) levels using solid-phase micro-extraction coupled to
GC–MS/MS (6). In this work, the sample preparation and detection were optimized but the chromatography was not, so it also illustrates the second problem: the analyte peaks are not resolved by the chromatography but are easily resolved using their differing MRM transitions. In a more extreme example, over 300 pesticides were extracted from apples and determined simultaneously in a single run using GC–MS/MS (7). This work illustrates the second and third cases: there are many analytes, the analysis can be either targeted or untargeted and the sample matrix is a complex food sample.

MS/MS provides the ultimate in detection for capillary gas chromatography. It can be both universal (full scan) or selective (SIM or MRM) and it is highly sensitive with detection limits of femtograms readily available. MS/MS is especially suited to the most difficult separation and detection problems. The trade off of this capability is capital and ongoing expense. MS/MS detectors are expensive, with fully loaded systems costing hundreds of thousands of U.S. dollars and have higher ongoing costs than traditional GC–MS systems, requiring special training to operate and needing additional maintenance compared to GC and GC–MS systems. The high sensitivity and low detection limit of MS/MS makes it especially sensitive to laboratory conditions such as clean carrier gases and careful sample preparation. Errors in sample preparation and contamination issues are often amplified when instrumental noise from the detector is lowered, so special care in sample preparation and laboratory management of GC–MS/MS is required. The unmatched combination of sensitivity with the ability to be both selective and universal makes MS/MS the most powerful detection tool available for capillary gas chromatography.

References
(2) A Look Back at the Birth of the Triple Quadrupole Mass Spectrometer (video), https://www.youtube.com/watch?v=whEO8kspM_g&vl=en Agilent Technologies (Santa Clara, California, 2017).

Nicholas H. Snow is the Founding Endowed Professor in the Department of Chemistry and Biochemistry at Seton Hall University, and an Adjunct Professor of Medical Science. During his 30 years as a chromatographer, he has published more than 70 refereed articles and book chapters and has given more than 200 presentations and short courses. He is interested in the fundamentals and applications of separation science, especially gas chromatography, sampling, and sample preparation for chemical analysis. His research group is very active, with ongoing projects using GC, GC–MS, two-dimensional GC, and extraction methods including headspace, liquid–liquid extraction, and solid-phase microextraction. Direct correspondence to: LCGCedit@mmhgroup.com

ABOUT THE AUTHOR
Hydrophobic interaction chromatography (HIC) is the chief alternative to ion-exchange chromatography (IEC) for the non-denaturing analysis of proteins. Separation by ion-exchange is based on differences in electrostatic charge, while separation by HIC is based on polarity differences, generally involving the hydrophobic residues. Its selectivity is then complementary to that of ion-exchange. Polarity differences are also the basis of separation in reversed-phase liquid chromatography (RPLC), but because the conditions of RPLC denature proteins, then the two modes, RPLC and HIC, have access to different sets of hydrophobic residues. As a result, their selectivity differs as well. HIC has emerged as the chief mode used for analysis of antibody-drug conjugates (ADCs) in which the ligands are linked through cysteine residues (1,2); an example is presented in Figure 1 (3).

Recent advances in technology have made it feasible to analyze large intact proteins by mass spectrometry. These advances have created a demand in top-down proteomics for separation methods that do not denature proteins. The reason is that the mass spectra of proteins with native structure have lower charge states and are appreciably less complex than those of denatured proteins, and therefore, they are easier to deconvolute. Conventional HIC is eliminated from consideration. While it preserves structure, it also involves gradients beginning with high concentrations of nonvolatile salts such as ammonium sulfate. Ammonium acetate is a volatile salt, but retention in HIC comparable to the level obtained with ammonium sulfate would require 3–4 M of ammonium acetate, a level that would clog the orifice of current mass spectrometers (4). A recent study (5) took note of the fact that the more hydrophobic a HIC material was, the less salt was required for retention. Accordingly, the ligand in the HIC coating was expanded beyond the usual propyl- or butyl- to encompass everything from pentyl- to decyl-ligands. The pentyl- and hexyl-ligands proved to confer adequate retention for small proteins starting with 1 M ammonium acetate, a concentration that a mass spectrometer can handle (Figure 2). This approach also works well for proteins as large as monoclonal antibodies (mAbs) (Figure 3) (6).

The selectivity and resolution obtained with the new columns and mobile phases compare favorably with those obtained with columns and running conditions used for conventional HIC, as seen in Figure 4.

For most proteins, an appropriate gradient runs from a high level of ammonium acetate, typically in the range 0.7–1.0 M, to a low concentration plus 50% organic solvent (either acetonitrile or propanol). One recurring question about this method is why the organic solvent doesn’t denature the proteins. The answer presumably lies in the kinetics involved. When left in the final mobile phase for hours, most susceptible proteins probably would denature. For that matter, leaving a protein sitting in a conventional HIC column for hours also leads to its denaturation (8). Under the conditions used here, the kinetics of chromatography are faster than the kinetics of denaturation, and proteins enter the orifice of the mass spectrometer with their structure intact. One reason for the durability of the protein structure may lie in the salt used. High concentrations of conventional HIC salts, such as ammonium sulfate, strip away a protein’s sphere of hydration. No longer solvated by the medium, the protein tends to partition into another phase, either via self-association as a solid (“salting out”) or via adsorption onto a HIC stationary phase surface. Acetate salts tend to have a protective effect on the sphere of hydration (9), and may play a role in the ruggedness of the protein’s retention of native structure. Such speculation can be assessed through the effects of using lower acetate concentrations. Figure 5 examines the consequences with lysozyme.

The top and middle panels involved conditions where lysozyme was in 20 mM ammonium acetate prior to introducing acetonitrile. The low concentration of acetate presumably left hydrophobic patches on the protein’s surface less than fully hydrated, making them more accessible to the stationary phase. A subsequent gradient to 50% acetonitrile failed to elute the protein in a reasonable time frame. By contrast, when the protein was exposed to acetonitrile while there was still a high concentration of acetate present, access to the hydrophobic patches was limited and the protein was eluted readily with good peak shape. This subject has been further explored in two subsequent papers. In the first, a group at Genentech took advantage of the greater retention at low concentrations of acetate to optimize conditions for analysis of variants of mAbs (10). A combination of a less-hydrophobic stationary
phase (PolyPROPYL A) and 150 mM ammonium acetate permitted the isocratic separation of a mAb from a number of its variants, as seen in Figure 6.

In the second study, Chen and others (11), offer additional insight into the impact of these running conditions on the stability of protein structure. Silica particles were given a coating of methyl methacrylate and then used for analysis of two ADCs under conditions similar to those described above for HIC–MS. The authors used a gradient to 50% 2-propanol with a constant concentration of 50 mM ammonium acetate; these conditions were termed native reversed-phase liquid chromatography (nRPLC). This term is as acceptable as HIC–MS; the HIC conditions described above do seem to obliterate the boundaries between HIC and RPLC. The ADCs were eluted in a profile typical of cysteine-linked ADCs in conventional HIC, judging from the ultraviolet (UV) absorbance traces (Figure 6b). However, the mass spectra of the variants of the ADC (Figure 7a) exhibit considerable dissociation of noncovalently attached light chains from the antibody complex. Dissociation to this extent is not evident in the UV trace. Evidently, these conditions neither denatured the original antibody (the DAR-0 form) nor was the original antibody dissociated from the noncovalent ADC complex evident in the UV trace. However, in the presence of a low concentration of ammonium acetate and a high concentration of organic solvent, the complex was sufficiently fragile that dissociation occurred from the greater stress encountered at the orifice of the mass spectrometer,
Forever Chemicals: Understanding Their Origins, Evolution, and Overcoming the Analytical Separation Challenges They Present

What are these pervasive forever chemicals? They have been around for a long time, and they are everywhere, but why don’t we know more about them, and how can industry improve detection and measurement capabilities?

**LCGC:** What are per- and polyfluorinated alkyl substances (PFAS) compounds, and why are we hearing so much about them lately?

**POWLEY:** In general, most people have heard about only one of the many PFAS chemicals, perfluorooctanoic acid, commonly known as PFOA or C8. It’s the subject of a documentary on Netflix called The Devil We Know and the 2019 film Dark Waters. They are a class of forever chemicals that are everywhere, and since 2000, there has been concern about them. They do not break down and accumulate, hence forever, and they have been linked to different types of cancers and other health effects. There are concerns about PFOA even at part per trillion levels in drinking water.

**LCGC:** Where did these compounds originate, and where are they now found?

**POWLEY:** All these chemicals are manmade fluorochemicals, so they don’t naturally occur—they have to be manufactured in a chemical manufacturing plant. These chemicals are used in a variety of products such as non-stick cookware, fabric coatings to protect carpets, firefighting foams, and other similar products.

As a result of their use over the past 50 years, they are now found everywhere because they don’t degrade, and they get around and move through the environment very easily. Just about any water supply that is near a relevant industry can be impacted, be it a manufacturing plant, a brownfield or superfund site, airport, or military base. They’re also highly mobile in aquifers and can even show up in arctic animals at the North and South Pole. All humans have some levels of these compounds in their blood serum, typically low part per billion.

**LCGC:** Why do we not know more about them?

**POWLEY:** Prior to 2000, the analytical tools we had available such as GC-MS and early versions of LC-MS were not sensitive or selective enough. In the case of LC-MS, it wasn’t quantitative. The big game changer was the commercialization of the electrospray interface for LC-MS/MS around 1998.
Once that came out, we could detect even part per trillion levels in water and single-digit part per billion levels in blood serum. Water and blood serum are the two major areas of concern. Now that we could measure them accurately and at these low levels, we saw them everywhere.

Beyond instrumentation, there was a lack of standards and issues with contamination in labs and samples. Because these compounds are everywhere, they are used in a lot of laboratory equipment, so it is hard to compensate for or get rid of the contamination. There are fewer than 100 of these compounds that can be measured. More are known, but authentic standards are not yet available and several thousand still remain.

A big opportunity of interest is high-performance liquid chromatography (HPLC) column technology.

**LCGC**: Where is the most opportunity for future advances in measuring PFAS compounds?

**POWLEY**: A big opportunity of interest is high-performance liquid chromatography (HPLC) column technology. We need columns that can chromatograph both the long- and the short-chain versions of these—fewer than four, maybe down to two perfluorinated carbon units and all the way up to 20 units.

The challenges with the column technology for the short-chain compounds is getting decent peak shapes and for long-chain compounds is eluting them and getting them off the column in one run. We will have to continue to fight with contamination and background reduction, and we need better strategies for that because there are so many of these compounds in regular use everywhere. The more we add, the more we'll find in our background due to their ubiquitous nature.

In terms of mass spectrometry, the current offerings are adequate, but the data-processing software could be improved to match the methods. Right now, most of the data-processing software for LC-MS/MS is geared toward pharma because they buy many more instruments than the environmental field. Better data-processing techniques for the environmental workflow could also speed things up quite a bit in terms of timely reporting.

**POWLEY**: There are several. As I mentioned, there are thousands of these compounds in the environment, and we don't know much about them. So, the first thing that needs to be done is to improve non-targeted analytical methods to identify more of them. Then regulatory agencies need to decide which of these thousands we need to measure. This would most likely involve designating certain ones as representative or marker compounds. The next step would be to develop quantitative targeted methods with available analytical standards for all of these.
These new combinations of stationary and mobile phases combine the protein compatibility and high resolution of HIC with the ever-increasing capabilities of mass spectrometry. HIC–MS promises the analysis of intact proteins online, without the delay, inconvenience, and potentially incomplete sequence coverage of middle-down or bottom-up approaches to proteomics.

which indicates that the ruggedness of structure of proteins, such as a mAb or a noncovalent complex, involves a balance of factors. Successful MS analysis of noncovalent complexes will presumably require further optimization of the chromatography conditions.

Summary

These new combinations of stationary and mobile phases combine the protein compatibility and high resolution of HIC with the ever-increasing capabilities of mass spectrometry. HIC–MS promises the analysis of intact proteins online, without the delay, inconvenience, and potentially incomplete sequence coverage of middle-down or bottom-up approaches to proteomics.

Acknowledgments

Adcetris is a trademark of Seattle Genetics. TSKgel Butyl-NPR is a trademark of Tosoh Corporation. PolyPROPYL A, PolyPENTYL A, and PolyHEPTYL A are trademarks of PolyLC Inc.

References


Andrew J. Alpert is the president of PolyLC Inc., in Columbia, Maryland. Direct correspondence to: aalpert@polylc.com.
HILICpak VN-50
- Unpurified oligo DNA analyzed with LC/UV/MS detection
- No ion pair reagent

Column Specs
- Modified diol groups
- Housed in PEEK
Ultra-Sensitive and Rapid Analysis of Per- and Polyfluoroalkyl Substances (PFAS) in Water

Per- and polyfluoroalkyl substances (PFAS) are chemicals widely used in consumer products because of their unique and desirable chemical properties. Because of widespread usage and environmental persistence, legacy PFAS are ubiquitous in the environment, and new fluorochemicals are being found in the environment frequently. Public interest and concern has increased pressure to develop comprehensive methods for sensitive analysis in different types of water. We discuss a method for 35 PFAS compounds analyzed in nonpotable waters that is sensitive, robust, and precise, and has high-throughput capabilities because of the fast preparation that makes it ideal for laboratories looking to run at high capacity. The analyte lists overlap with American Society for Testing and Materials (ASTM) 7979 and Draft Environmental Protection Agency (EPA) 8327, with some additions of emerging PFAS, such as Adona and the components of F53B because of their increasing presence in the environment. Sample preparation was minimal with 1:1 dilution with methanol followed by filtration. Compound separation was achieved on a 1260 liquid chromatograph (LC) and analyzed with an Agilent 6495 triple quadrupole LC–MS instrument. Compounds were quantitated with isotopic dilution to obtain the highest accuracy and confidence. Excellent method spike recoveries and robustness when running hundreds of wastewater samples were seen, proving this methodology is suitable for use in a high-throughput laboratory with high data accuracy and good sensitivity.

Emily Parry and Tarun Anumol

Per- and polyfluoroalkyl substances (PFAS) are a group of >4000 man-made substances that have been in use since the 1940s (1). These substances have unique chemical properties because of the carbon–fluorine bond that make their use desirable in a number of industrial and household products. Common applications of PFAS are in products used as fire-fighting foams, nonstick cookware, water repellent clothing and furniture, food-packing materials, and many others. The same properties that make them tough, durable, and resistant to temperature and degradation also mean they can be persistent in the environment (2,3). These substances, particularly those with less than seven carbon-chain lengths, are bioaccumulative and have various adverse health effects in humans and wildlife (4,5).

There are several hundred PFAS that have been detected in the environment and studies have shown their presence in many major water bodies, soil, air, food, and even in polar bears in the arctic. Several studies have also detected PFAS in human blood across the globe (6–9). Although there are several thousand PFAS known to have been created, they can largely be divided in 10 sub-classes. Of these, the most commonly studied classes are perfluorocarboxylic acids (PFCAs) and perfluorosulfonic acids (PFSA). Two PFAS in particular, perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA), have been widely used in the past and are being considered for regulatory determination globally. These two compounds have been detected frequently in the environment, and there is mounting toxicological data that points to adverse effects in animals and humans (10). As a result, these compounds are designated as persistent organic pollutants (POPs) under the United Nations Stockholm Convention, which is an international treaty aimed at eliminating the use of POPs globally. In 2015, several major manufacturers of these chemicals decided to voluntarily phase out the manufacture and use of PFOA and PFOS in the United States as part of a stewardship program with the U.S. Environmental Protection Agency (USEPA) (11). This decision has led to a change in manufacturing and production of shorter-chain PFAS compounds. Subsequently, we have started to detect these compounds in the environment with hexafluoropropylene oxide dimer acid (HFPO-DA), also commonly referred to by the brand name GenX, a particular PFAS of interest in the research and regulatory community.

Because of their potential adverse effects, frequent detections in the environment, and the resulting public outcry, PFAS compounds, especially PFOA and PFOS, are under review for regulation in water globally. In the United States, the EPA has established a drinking water health advisory level of 70 ng/L for PFOA and PFOS combined and is evaluating setting a drinking water maximum contaminant level (MCL) for these compounds soon. More than 25 states in the United States have implemented their own regulatory limits for PFAS in drinking water, with many being much more stringent than the USEPA’s value and around the single-digit nanogram per liter level. The European Union has listed PFOA and PFOS as priority hazardous substances under its Water Framework Directive (WFD) and several other PFAS are listed under the Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH) regulation to restrict their use and manufacture in commercial and household products. Several European states, such as Germany, Sweden, and Denmark also
FIGURE 1: Quantification transition chromatogram for each analyte except fluorotelomer acids (FTAs), which had higher method detection limits (MDLs). Axes labels are Counts (%) for ordinate and Acquisition time (min) for abscissa.
have individual limits for specific PFAS in water and soil. Many other countries like Japan, Korea, Australia, and others have also set preliminary or final regulatory limits for certain PFAS compounds in water. It is expected, with the increased toxicological information and identification of newer PFAS, that these regulatory lists will only increase in scope and lead to lower regulatory limits globally. It is thus prudent to have sensitive analytical techniques to measure a vast range of legacy and emerging PFAS in water to have adequate baseline monitoring data to assist regulators in setting regulatory guidance and ensuring safe water.

Analysis of PFAS in the environment can be divided into quantification and identification of new PFAS. Identifying new PFAS is best performed using a high-resolution accurate mass spectrometer (MS), such as quadrupole time of flight (QTOF) instruments, that measure molecular masses accurately and then, with the help of sophisticated software platforms, identify chemical structures. One example is the identification of perfluoroether carboxylic acids (PFECAs) like GenX using LC–QTOF by Strynar and others (12). The gold standard for quantifying PFAS at low nanogram per liter levels with robustness and reliability though is with tandem quadrupole LC–MS/MS instruments (13). Several standard methods for quantification of sub-sets of PFAS in water exist now. The USEPA has released two methods for analysis of PFAS in drinking water. In 2018, EPA method 537.1 was released to analyze 18 PFAS using solid phase extraction (SPE) and LC–MS/MS while EPA method 533, released in December 2019, analyzes 25 PFAS, including short-chain ones using SPE and LC–MS/MS (14,15). Both of these methods were developed for analysis in drinking water only. In 2019, the EPA released a draft of EPA 8327 that analyzes 28 PFAS in non-potable waters (surface water, ground water, and wastewater) using a dilute-and-shoot technique with minimal sample preparation (16). This method allows much faster analysis and turnaround of samples because it does not require SPE, but as a result needs a more sensitive MS/MS instrument. EPA 8327 is similar to American Society for Testing and Materials (ASTM) 7979 that can be used for analysis of PFAS in nonpotable waters as well. The ISO/DIS 21675 is a method developed for analysis of PFAS in drinking water, natural water, and wastewater using SPE and LC–MS/MS as well.
Adeno-associated viruses (AAVs) are increasingly used for gene therapy due to their versatility and safety. One of the biggest concerns for manufacturing a uniform AAV suspension is the presence of viral aggregates, which can create problems with transduction efficiency, biodistribution, and immunogenicity. These large AAV aggregates are challenging to separate and characterize by traditional column-based chromatography techniques such as size exclusion chromatography (SEC).

Asymmetrical Flow Field-Flow Fractionation with Multi Angle Light Scattering (AF4-MALS) can separate and size large AAV aggregates, and discern a difference in aggregate concentration due to the stressing protocol. Some or all of the large aggregates would be filtered out by SEC, resulting in incorrect determination of the aggregate content or the false conclusion that no aggregates are present.

For more details visit www.postnova.com and search for ‘virus’.

Contact us for more information: www.postnova.com
# TABLE I: Analyte list including MRM data

<table>
<thead>
<tr>
<th>Methods</th>
<th>Analyte Name</th>
<th>Abbreviation</th>
<th>$t_R$</th>
<th>Transition</th>
<th>CE</th>
</tr>
</thead>
<tbody>
<tr>
<td>537.1, 533, ISO</td>
<td>Potassium 11-chloroeroicosfluoro-3-oxaundecane-1-sulfonate</td>
<td>11CL-PF3OUDS</td>
<td>12</td>
<td>630.9&gt;450.9</td>
<td>36</td>
</tr>
<tr>
<td>533, 8327, ISO, ASTM</td>
<td>Fluorotelomer sulfonate 4:2</td>
<td>4-2 FTS</td>
<td>6.3</td>
<td>327&gt;306.9</td>
<td>16</td>
</tr>
<tr>
<td>533, 8327, ISO, ASTM</td>
<td>Fluorotelomer sulfonate 6:2</td>
<td>6-2 FTS</td>
<td>8.2</td>
<td>427.0&gt;406.8</td>
<td>28</td>
</tr>
<tr>
<td>533, 8327, ISO, ASTM</td>
<td>Fluorotelomer sulfonate 8:2</td>
<td>8-2 FTS</td>
<td>10.5</td>
<td>527.0&gt;506.8</td>
<td>32</td>
</tr>
<tr>
<td>ASTM</td>
<td>2-Perfluorohexyl ethanoic acid</td>
<td>FHEA</td>
<td>7.5</td>
<td>377.0&gt;312.8</td>
<td>4</td>
</tr>
<tr>
<td>ASTM</td>
<td>2H-Perfluoro-2-octenoic acid</td>
<td>FHUEA</td>
<td>7.4</td>
<td>357.0&gt;293.0</td>
<td>4</td>
</tr>
<tr>
<td>ASTM</td>
<td>2-Perfluoroocetyl ethanoic acid</td>
<td>FOEA</td>
<td>9.8</td>
<td>477.0&gt;393.0</td>
<td>8</td>
</tr>
<tr>
<td>ASTM</td>
<td>2H-Perfluoro-2-decenoic acid</td>
<td>FOUEA</td>
<td>9.7</td>
<td>457.0&gt;393.0</td>
<td>4</td>
</tr>
<tr>
<td>ASTM</td>
<td>2-Perfluorodecyl ethanoic acid</td>
<td>FDEA</td>
<td>12.1</td>
<td>577.0&gt;493.1</td>
<td>4</td>
</tr>
<tr>
<td>ASTM</td>
<td>2H-Perfluoro-2-dodecenoic acid</td>
<td>FDUEA</td>
<td>11.9</td>
<td>557.0&gt;493.1</td>
<td>4</td>
</tr>
<tr>
<td>537.1, 533, ISO</td>
<td>Potassium 9-chlorohexadecafluoro-3-oxanone-1-sulfonate</td>
<td>9CL-PF3ONS</td>
<td>10.0</td>
<td>530.9&gt;350.9</td>
<td>28</td>
</tr>
<tr>
<td>537.1, 533, ISO</td>
<td>Sodium Dodecafluoro-3H-4, 8-dioxanone</td>
<td>ADONA</td>
<td>7.3</td>
<td>377.0&gt;250.9</td>
<td>8</td>
</tr>
<tr>
<td>Labeled compound</td>
<td>N-methyl-d3-perfluoro-1-octanesulfonamidoacetic acid</td>
<td>D3 N-MEFOSAA</td>
<td>11.0</td>
<td>573.0&gt;418.9</td>
<td>20</td>
</tr>
<tr>
<td>Labeled compound</td>
<td>N-ethyl-d5-perfluoro-1-octanesulfonamidoacetic acid</td>
<td>DS-N-ETFOSAA</td>
<td>11.59</td>
<td>589.0&gt;418.9</td>
<td>16</td>
</tr>
<tr>
<td>Labeled compound</td>
<td>2-Perfluorohexyl-[1,2-13C2]-ethanoic acid</td>
<td>MFHEA</td>
<td>7.5</td>
<td>379.0&gt;294.0</td>
<td>4</td>
</tr>
<tr>
<td>Labeled compound</td>
<td>2H-Perfluoro-[1,2,13C2]-2-octenoic acid</td>
<td>MFHUEA</td>
<td>7.4</td>
<td>359.1&gt;294.0</td>
<td>4</td>
</tr>
<tr>
<td>Labeled compound</td>
<td>2-Perfluoroocetyl-[1,2-13C2]-ethanoic acid</td>
<td>MFOEA</td>
<td>9.8</td>
<td>479.0&gt;394.0</td>
<td>4</td>
</tr>
<tr>
<td>Labeled compound</td>
<td>2H-Perfluoro-[1,2,13C2]-2-decenoic acid</td>
<td>MFOUEA</td>
<td>9.7</td>
<td>459.1&gt;394.1</td>
<td>4</td>
</tr>
<tr>
<td>Labeled compound</td>
<td>2-Perfluorodecyl-[1,2-13C2]-ethanoic acid</td>
<td>MFDEA</td>
<td>12.1</td>
<td>579.0&gt;494.1</td>
<td>4</td>
</tr>
<tr>
<td>Labeled compound</td>
<td>2H-Perfluoro-[1,2,13C2]-2-dodecenoic acid</td>
<td>MFDUEA</td>
<td>11.9</td>
<td>559.1&gt;494.0</td>
<td>4</td>
</tr>
<tr>
<td>Labeled compound</td>
<td>Sodium 1H,1H,2H,2H-perfluoro-1-[1,2-13C2] hexane sulfonate (4:2)</td>
<td>M2 4-2 FTS</td>
<td>6.3</td>
<td>329.0&gt;309.0</td>
<td>12</td>
</tr>
</tbody>
</table>

Continued on page 80
Experimental Standards and Water Samples

Native and labeled PFAS compounds (Table I) were acquired from Wellington Laboratories (Guelph, Ontario, Canada). Calibration and fortification experiments were prepared with 18 MΩ water. Surface water, drinking water, and wastewater effluent samples were all collected in Wilmington, Delaware.

Sample Preparation

First, 5 mL samples were collected in polypropylene centrifuge tubes. Labeled surrogates were added and the samples were diluted with 5 mL volume of methanol and inverted for 2 min. The entire sample was then filtered through a 0.2 µm syringe filter followed by acidification with 10 µL of acetic acid. 1 mL of the sample was then placed into a polypropylene vial and closed with polypropylene caps, now ready for LC–MS analysis. Both polypropylene and deactivated glass vials were tested, but longer chain compounds showed significant sorption to glass vials with time in the autosampler.

Instrumental Analysis

The samples were analyzed on an Agilent 1260 Infinity II liquid chromatograph (LC) equipped with a high-speed binary pump and multicolumn compartment coupled to an Agilent 6495C Tandem Quadrupole LC–MS. For this experiment, 57 PFAS (35 native and 25 internal standards) were analyzed simultaneously using dynamic multiple reaction monitoring (DMRM) with data acquisition and processing done using MassHunter software. All compound parameters and MS source parameters were optimized automatically using the Agilent Compound Optimizer and its source optimizer features. LC and MS parameters are provided in Tables II and III, respectively.

Quantification

Isotopic analogues were associated with native compounds. If a labeled compound was not available, compounds were corrected by either PFOA or PFOS, depending on whether the target was a sulfonate or an acid (that is, PFNS was corrected M8PFOS).

All calibration curves were linear with the origin ignored and 1/x weighting used with a minimum of seven points. Ranges are shown in the Table IV and for most PFAS the lower limit of quantification of 2 ng/L was chosen. The fluorotelomers had a higher calibration range of 200–8000 ng/L because of lower sensitivity, but this is consistent with the EPA 8327 and ASTM 7979 methods.

Method Performance Evaluation

Method Detection Limits (MDL)

Over the course of three different days, a total of nine reagent water samples were spiked with native PFAS (see Table IV) concurrently with nine method blanks. Samples were prepared as described in the sample preparation section. MDLs were calculated as described in equation 1:
MDL = Standard deviation of replicates
\times \text{ Students } t\text{-value}_n \text{,}_1 \quad [1]

with \( n \) being the number of replicates.

Matrix Recovery

To investigate the effect of matrix on method performance and viability of the method to different waters, three different water types were tested. Within each group, blanks and spikes were processed. Surface water (\( n = 3 \)), wastewater effluent (\( n = 4 \)), and reagent water (\( n = 3 \)) were prepared as described in the sample preparation section and analyzed with the method. For compounds with labeled surrogates, method performance was evaluated by percent recovery of isotopic analogue. Compounds that did not have a labeled analogue percent recovery were calculated from spike recovery with consideration of whether the compound was present in the blank of that water type.

Instrument Robustness

Wastewater effluent is the dirtiest matrix in the study and was used to determine

<table>
<thead>
<tr>
<th>Methods</th>
<th>Analyte Name</th>
<th>Abbreviation</th>
<th>( t_R )</th>
<th>Transition</th>
<th>CE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Labeled compound</td>
<td>Sodium 1H,1H,2H,2H-perfluoro-1-[1,2,3C2] octane sulfonate (6:2)</td>
<td>M2 6-2 FTS</td>
<td>8.2</td>
<td>429.0&gt;80.9</td>
<td>48</td>
</tr>
<tr>
<td>Labeled compound</td>
<td>Sodium 1H,1H,2H,2H-perfluoro-1-[1,2,3C2] decane sulfonate (8:2)</td>
<td>M2 8-2 FTS</td>
<td>10.5</td>
<td>529.0&gt;509.0</td>
<td>28</td>
</tr>
<tr>
<td>Labeled compound</td>
<td>Perfluoro-n-[1,2-13C2] dodecanolic acid</td>
<td>M2PFDOA</td>
<td>12.6</td>
<td>614.9&gt;570.0</td>
<td>8</td>
</tr>
<tr>
<td>Labeled compound</td>
<td>Perfluoro-n-[1,2,13C2] tetradecanolic acid</td>
<td>M2PFTRA</td>
<td>14.4</td>
<td>715.0&gt;670.0</td>
<td>13</td>
</tr>
<tr>
<td>Labeled compound</td>
<td>Sodium perfluoro-1-[2,3,4-13C3] butane sulfonate</td>
<td>M3PFBS</td>
<td>5.8</td>
<td>302.0&gt;80.0</td>
<td>41</td>
</tr>
<tr>
<td>Labeled compound</td>
<td>Sodium perfluoro-1-[2,3,4-13C3] hexane sulfonate</td>
<td>M3PFHXS</td>
<td>7.3</td>
<td>402.0&gt;80.0</td>
<td>53</td>
</tr>
<tr>
<td>Labeled compound</td>
<td>Perfluoro-n-[1,2,3,4-13C4] butanoic acid</td>
<td>M4PFBA</td>
<td>3.94</td>
<td>217.0&gt;172.0</td>
<td>4</td>
</tr>
<tr>
<td>Labeled compound</td>
<td>Perfluoro-n-[1,2,3,4-13C4] heptanoic acid</td>
<td>M4PFHPA</td>
<td>7.2</td>
<td>367.0&gt;322</td>
<td>8</td>
</tr>
<tr>
<td>Labeled compound</td>
<td>Perfluoro-n-[1,2,3,4,6-13C5] hexanoic acid</td>
<td>M5PFHXA</td>
<td>6.4</td>
<td>318.0&gt;273.0</td>
<td>4</td>
</tr>
<tr>
<td>Labeled compound</td>
<td>Perfluoro-n-[1,2,3,4,5-13C5] pentanoic acid</td>
<td>M5PFPEA</td>
<td>5.6</td>
<td>268.0&gt;223.0</td>
<td>4</td>
</tr>
<tr>
<td>Labeled compound</td>
<td>Perfluoro-n-[1,2,3,4,5,6-13C6] decanoic acid</td>
<td>M6PFDA</td>
<td>10.5</td>
<td>519.0&gt;474.0</td>
<td>8</td>
</tr>
<tr>
<td>Labeled compound</td>
<td>Perfluoro-n-[1,2,3,4,5,6,7-13C7] undecanolic acid</td>
<td>M7PFUNA</td>
<td>11.6</td>
<td>570.0&gt;525.0</td>
<td>12</td>
</tr>
<tr>
<td>Labeled compound</td>
<td>Perfluoro-1-[13C8] octane sulfonamide</td>
<td>M8 FOSA</td>
<td>11.0</td>
<td>506.0&gt;779.0</td>
<td>44</td>
</tr>
<tr>
<td>Labeled compound</td>
<td>Perfluoro-n-[13C8] octanoic acid</td>
<td>M8PFQA</td>
<td>8.23</td>
<td>421.0&gt;376.0</td>
<td>4</td>
</tr>
<tr>
<td>Labeled compound</td>
<td>Sodium perfluoro-1-[13C8] octane sulfonate</td>
<td>M8PFOS</td>
<td>9.4</td>
<td>507.0&gt;80.0</td>
<td>58</td>
</tr>
<tr>
<td>Labeled compound</td>
<td>Perfluoro-n-[13C9] nonanoic acid</td>
<td>M9PFNA</td>
<td>9.4</td>
<td>472.0&gt;427.0</td>
<td>8</td>
</tr>
<tr>
<td>537.1, 8327</td>
<td>N-ethyl-N-((heptadecafluorooctyl) sulfonyl) glycine</td>
<td>N-ETFOSAA</td>
<td>11.6</td>
<td>584.0&gt;526.0</td>
<td>20</td>
</tr>
<tr>
<td>537.1, 8327</td>
<td>N-(Heptadecafluorooctylsulfonyl)-N-methylglycine</td>
<td>N-MEFOSAA</td>
<td>11.0</td>
<td>570.0&gt;419.0</td>
<td>20</td>
</tr>
<tr>
<td>533, 8327, ISO, ASTM</td>
<td>Perfluorobutanoic acid</td>
<td>PFBA</td>
<td>3.9</td>
<td>213&gt;169</td>
<td>4</td>
</tr>
<tr>
<td>537.1, 533, 8327, ASTM, ISO</td>
<td>Perfluorobutane sulfonate</td>
<td>PFBS</td>
<td>5.8</td>
<td>298.9&gt;80</td>
<td>41</td>
</tr>
<tr>
<td>537.1, 533, 8327, ASTM, ISO</td>
<td>Perfluorododecanoic acid</td>
<td>PFDOA</td>
<td>12.7</td>
<td>613.0&gt;569.0</td>
<td>10</td>
</tr>
</tbody>
</table>

Continued on page 81
instrument robustness. The wastewater effluent samples were injected continually for 36 h, bracketed every 10 samples by a continuing calibration check (CC) at 80 ng/L. The raw and uncorrected response of the CC was compared across the course of the worklist to evaluated how continual introduction of matrix affected instrument response and performance of the MS. Method performance specification require deviations to remain less than 30%.

**Results**

**Background and Compound Separation**

Instrument background was not noticed for all the PFAS after the installation of the PFAS elimination kit and delay column. The 35 native PFAS analyzed in this method were separated using an Agilent Poroshell 120 EC-C18 column (with column dimensions of 2.1 mm x 100 mm, 2.7-µm). Figure 1 shows the individual MMRs of the PFAS analyzed at a 2 ng/L concentration standard. It can be noticed that for most compounds lower concentrations can be analyzed. PFBA does show peak fronting, likely because of a large volume injection of a solvent other than the initial gradient conditions (nominally 5% methanol). Different mobile phase combinations and columns were tried; however, 5 mM ammonium acetate provided the best sensitivity for the widest range of compounds. One study showed good PFBA peak shape with a Zorbax SB-C18
column; however, the mobile phase pH was lowered with acetic acid to maintain the improved peak shape (18). With the additional analytes added and the goal of ultimate sensitivity, it was decided to compromise PFBA peak shape for enhanced sensitivity.

**Method Detection Limits**

In the ASTM 7979 and EPA draft 8327 method, the lowest reporting limit is 10 ng/L for some PFAS, while many are higher (17). The fluorotelomer acids (FTAs) have a lowest limit of quantification in the method at 200 ng/L. In this method, employing the 6495C tandem quadrupole LC–MS, calculated method detection levels were below 10 ng/L for all PFAS tested except the three fluorotelomer acids. In fact, greater than 80% of the PFAS tested had method detection levels less than 2.5 ng/L, which is four times lower than the 10 ng/L level suggested for any PFAS in the ASTM or EPA methods. This shows the enhanced sensitivity of the instrument. The benefits of sensitivity do not extend only to lower reporting levels which may be critical in future proofing a laboratory against expected reduction in regulatory PFAS limits. Additionally, the enhanced sensitivity allows a laboratory to reduce injection volumes or extract less sample that can lead to several benefits, like less contamination going into the MS instrument and lower frequency of maintenance along with ancillary benefits of lower sampling and shipment costs required because of the smaller sample usage.

**Matrix Spike Recovery**

Figure 2a shows the surrogate recovery of the 25 isotopically labeled PFAS spiked at 80 ng/L in the three different water matrices tested. The average recovery and relative standard deviation (n = 4) of the samples is plotted in the figure. All water types, namely reagent water, surface water, and wastewater, were within the ASTM and USEPA method guidelines of 100% ± 30% surrogate recovery. Average recovery of the surrogates was much closer to a 100% for most analytes with ranges of 87–104% in reagent water, 83–107% in surface water, and 83–107% in wastewater.

Table 2b shows the average recovery of nine additional PFAS tested that did not have a matching isotopically labeled standard in the three water matrices. Perfluoro-4-methoxybutanoic acid (PFMBA) was present at low levels in the unspiked wastewater replicates so recoveries were corrected to take into account the background in the wastewater sample. All other compounds had clean matrix blanks with concentrations lower than limits of detection and recoveries were within method guidelines. All nine had recoveries without any correction between 70–130% in all three water matrices, indicating good recovery and very accurate detection.

**Instrument Robustness**

Because this method uses minimal sample preparation and cleanup, it is critical that the instrument be robust and stable across long periods so there is no drift

### Table II: LC separation conditions

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC</td>
<td>Agilent 1260 Infinity II LC</td>
</tr>
<tr>
<td>Autosampler</td>
<td>Agilent 1260 Multisampler</td>
</tr>
<tr>
<td>Delay column</td>
<td>Zorbax Eclipse Plus C18, 4.6 mm x 50 mm, 1.8-um</td>
</tr>
<tr>
<td>Analytical Column</td>
<td>Poroshell 120 EC-C18, 2.1 mm x 100 mm, 2.7-um</td>
</tr>
<tr>
<td>Column Temperature</td>
<td>50 ºC</td>
</tr>
<tr>
<td>Mobile Phase</td>
<td>A: 5 mM Ammonium Acetate B: 95:5 Methanol : Water, 5 mM Ammonium Acetate</td>
</tr>
<tr>
<td>Flow Rate</td>
<td>0.4 mL/min</td>
</tr>
<tr>
<td>Injection Volume</td>
<td>30 uL % B</td>
</tr>
<tr>
<td>Gradient</td>
<td>10</td>
</tr>
<tr>
<td>0.5</td>
<td>60</td>
</tr>
<tr>
<td>3.0</td>
<td>95</td>
</tr>
<tr>
<td>15.0</td>
<td>100</td>
</tr>
<tr>
<td>17.0</td>
<td>10</td>
</tr>
<tr>
<td>17.1</td>
<td>5.5</td>
</tr>
</tbody>
</table>

### Table III: Mass spectrometer conditions

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS</td>
<td>Agilent 6495C</td>
</tr>
<tr>
<td>Ionization Mode</td>
<td>Negative</td>
</tr>
<tr>
<td>Drying Gas</td>
<td>250 ºC</td>
</tr>
<tr>
<td>Drying Gas Flow</td>
<td>11 L/min</td>
</tr>
<tr>
<td>Nebulizer</td>
<td>25 psi</td>
</tr>
<tr>
<td>Sheath Gas Temperature</td>
<td>375 ºC</td>
</tr>
<tr>
<td>Sheath Gas Flow</td>
<td>11 L/min</td>
</tr>
<tr>
<td>Capillary</td>
<td>2500</td>
</tr>
<tr>
<td>Nozzle</td>
<td>0</td>
</tr>
<tr>
<td>High Pressure ifunnel RF</td>
<td>70 V</td>
</tr>
<tr>
<td>Low Pressure ifunnel RF</td>
<td>60 V</td>
</tr>
</tbody>
</table>
or drop in sensitivity. Additionally, it is critical for the instrument to keep a stable response and not let matrix effect the MS instrument, which will lead to frequent cleaning, maintenance, and downtime. The ASTM 7979 method also uses a similar sample preparation but does not require use of internal standards so true and raw recovery is critical for the MS instrument to maintain across a large run. In this study, a worklist of wastewater effluent samples were run across 36 h and after every 10 samples a continuing calibration standard of all 35 PFAS was run at 80 ng/L. The raw and uncorrected responses of each analyte are normalized and plotted across the 36 h in Figure 3. The system showed excellent robustness across repeated injections of wastewater. Of the 35 analytes, 61% had RSDs that remained below 6% for the duration of the 36 h test and 85% of the analytes had RSDs that remained below 10% without any correction of response with internal standards (ISTDs). Only two compounds exceeded the method guidelines of 30%, 2H-Perfluoro-2-dodecenoic acid (FDUEA) and 2-perfluorodecylethanoic acid (FDEA). Those two compounds, as well as some of the longer chain PFCAs (PFTreA, for example), showed a downward trend after 24 h had passed. Rather than instrument performance, an alternative explanation for this trend is that these compounds are less soluble and stable in the 50:50 methanol:water solution and could be falling out of solution. Re-vortexing the autosampler vials might resolubilize them and could be tested as one potential solution. The multisampler was kept at room temperature during analysis in an attempt to maintain solubility. The results, however, indicate excellent robustness of the system under long-term stress of heavy matrix wastewater samples.

Conclusions

With the regulatory landscape around PFAS changing globally and driving expected measurement levels down, in addition to a significant increase in sites with potential PFAS contamination coming to light, it is critical to have analytical methods that offer ultimate sensitivity, while maintaining robust and reliable quantification data even when challenging matrices are tested and minimal sample preparation is employed to have high-throughput. The method demonstrated here analyzes more than 50 PFAS in different and challenging water matrices, achieving low detection levels with high data quality and robustness, meeting and exceeding established guidelines by standard methods.

References

The 2021 Winners of the Lifetime Achievement and Emerging Leader in Chromatography Awards

Paul Haddad and Erik L. Regalado are the winners of the 14th annual LCGC Lifetime Achievement and Emerging Leader in Chromatography Awards, respectively. Here, we review their achievements.

Jerome Workman, Jr.

Paul Haddad and Erik L. Regalado are the winners of the 14th annual LCGC Lifetime Achievement and Emerging Leader in Chromatography Awards, respectively. The LCGC Awards honor the work of leading separation scientists for lifetime achievement and emerging potential (Table I). The award winners will be honored at an oral symposium as part of the PittCon 2021 conference, which will be held virtually this year from March 8–12, 2021. The presentations will feature talks by each award winner as well as other leaders in the field of separation science.

The Lifetime Achievement in Chromatography Award

Paul Haddad, the 2021 winner, is an Emeritus Distinguished professor at the University of Tasmania in Australia and was the foundation Director of the Australian Centre for Research on Separation Science (ACROSS). He is best known for his research on the theory, mechanisms, and applications of analytical separation science in liquid phases, with particular emphasis on the separation and quantification of ionic species.

He received his PhD in analytical chemistry from the University of New South Wales, Australia, in 1975, and his BS degree in 1971 from the same institution. He also holds a diploma of Military Studies (Science) awarded in 1969 by the Royal Military College in Duntroon, Australia. In 1996, he was awarded a Doctor of Science for a career of scientific contributions by the University of New South Wales in Australia.

Haddad has had a broad impact in the field of separation science, across many techniques, including high performance liquid chromatography (HPLC), ion chromatography (IC), capillary electrophoresis (CE), and capillary electrochromatography (CEC). The focus of his research has been to study separation mechanisms and methods of detection, the goal being to develop mathematical models that improve fundamental understanding of separation mechanisms and apply this understanding to the development of new methods of analysis. Another important area of his research work has been studying detection methods, with an emphasis on potentiometric detection using reactive metallic electrodes and the theory and application of indirect methods of spectrophotometric detection. The separation of complex metal ions and sample handling methods have comprised further major research themes of Haddad and coworkers.

Among Haddad’s greatest contributions is his establishment of ACROSS, where he served as the foundation director from 2001 to 2014, and of the Pfizer Analytical Research Centre (PARC), which he led from 2006 to 2012. ACROSS is recognized worldwide as a leading center of research excellence in separation science. Over the past 16 years, the center has published more than 1000 peer reviewed papers, has graduated 110 PhD students (with 45 currently active PhD students), and presented more than 1500 oral papers or posters at international scientific meetings. PARC was a multimillion dollar research center funded by Pfizer.

Research in Modeling and Optimization of Separation Mechanisms

Haddad’s modeling research has led directly to 115 publications on the fundamentals of separation mechanisms in various chromatographic and electrophoretic systems and the use of mechanistic models for computer-assisted selection of optimal separation conditions. Haddad’s major achievements in this area include the derivation and experimental validation of retention models for cations in the presence of complexing eluents, ion-exclusion chromatography, migration processes in micellar electro-
kinetic capillary chromatography, and anion separations methods using IC.

The extensive studies carried out by Haddad and his team of computer-based optimization in IC and CE, led to revolutionary new software enabling rapid and accurate simulation and optimization of IC separations in isocratic and gradient elution conditions. This software has been commercialized by Thermo Fisher Scientific as a product called “Virtual Column,” introduced in 2004, and now is reportedly in use in over 2000 laboratories across 30 countries.

Haddad also has explored the use of quantitative structure–retention relationships to predict chromatographic retention in reversed-phase LC (RPLC), hydrophilic interaction chromatography (HILIC) and IC, based only on the chemical structures of analytes. This work sits at the very forefront of research in separation science and the predictions of retention have the best accuracy reported to date.

**TABLE I: History of winners of the LCGC Awards**

<table>
<thead>
<tr>
<th>Year</th>
<th>Lifetime Achievement</th>
<th>Emerging Leader</th>
</tr>
</thead>
<tbody>
<tr>
<td>2008</td>
<td>Walt Jennings</td>
<td>Gert Desmet</td>
</tr>
<tr>
<td>2009</td>
<td>Harold McNair</td>
<td>Kevin Schug</td>
</tr>
<tr>
<td>2010</td>
<td>Georges Guiochon</td>
<td>Jared Anderson</td>
</tr>
<tr>
<td>2011</td>
<td>James W. Jorgenson</td>
<td>Dwight Stoll</td>
</tr>
<tr>
<td>2012</td>
<td>Lloyd Snyder</td>
<td>Emily Hilder</td>
</tr>
<tr>
<td>2013</td>
<td>Peter W. Carr</td>
<td>Davy Guillaume</td>
</tr>
<tr>
<td>2014</td>
<td>Fred E. Regnier</td>
<td>Andre’ De Villiers</td>
</tr>
<tr>
<td>2015</td>
<td>Joseph Jack Kirkland</td>
<td>Caroline West</td>
</tr>
<tr>
<td>2016</td>
<td>Milton L. Lee</td>
<td>Debby Mangelings</td>
</tr>
<tr>
<td>2017</td>
<td>Pat Sandra</td>
<td>Deirdre Cabooter</td>
</tr>
<tr>
<td>2018</td>
<td>Ronald E. Majors</td>
<td>Zachary S. Breitbart</td>
</tr>
<tr>
<td>2019</td>
<td>Milos V. Novotny</td>
<td>Ken Broeckhoven</td>
</tr>
<tr>
<td>2020</td>
<td>Daniel W. Armstrong</td>
<td>Szabolcs Fekete</td>
</tr>
<tr>
<td>2021</td>
<td>Paul Haddad</td>
<td>Erik L. Regalado</td>
</tr>
</tbody>
</table>

Studies on the Control of Separation Selectivity in Chromatographic and Electrophoretic Systems

Haddad has conducted systematic studies leading to 182 publications on parameters that influence separation selectivity in chromatographic and electrophoretic systems. One of his major achievements...
in this area includes the use of function-
alized latex nanoparticles for selective ion-exchange separations for microfluid-
ics, CE, and micro-chromatography. He has explored the theory and applications of pseudo-stationary phase CE and CEC systems having variable and predictable selectivity. Haddad has led the study of the fundamentals and applications of electrostatic ion chromatography (EIC) for the determination of inorganic and organic ions in sample matrices of high ionic strength. Haddad first reported separation of all stable metallocyanide complexes, thiocyanate, and free cyanide. He has also designed IC and CE systems for the rapid separation of ions in pre-blast and post-blast samples of improvised explosives leading to the development of instrumentation and methodologies in active use by law enforcement agencies.

Exploring Detection and Sample Preconcentration Methods in IC and CE
In another major area of focus, Haddad has produced a large body of work, consisting of 87 publications, on the theory and application of detection methods for a range of chromatographic techniques. His significant contributions in this area include the design and elucidation of electrode response theory for a metallic copper potentiometric detector for IC and CE, the elucidation of the source of “system peaks” in indirect detection in IC and CE, and the comprehensive studies of the theory and fundamentals of online preconcentration using ion-exchange, covering flow-path design, eluent selection, sample loading parameters, and ion-exchange capacity of the concentrator column. Haddad has completed derivation of the fundamental theory of response for indirect detection in CE, and recognition of the key role of the transfer ratio concept. He also has developed an online sample preconcentration methodology for CEC using alatex-coated precapillary and a transient isotachophoretic gradient. Haddad and coworkers have performed extensive studies on the theory and application of contactless conductivity detection for IC and CE, including microfluidics. Haddad has designed IC suppressors suitable for the detection of organic ions; developed universal response models for aerosol-based detectors in HPLC; and co-invented an on-capillary sample enrichment method for CE based on analyte focusing after micelle collapse.

Key Recent Publications
Haddad's lifetime of contributions to the field of separation science has continued with significant developments in recent years. Recent research by Haddad and colleagues, for example, has led to the development of a miniaturized deep-ultraviolet light emitting diode (LED)-based detector for use in a portable capillary-scale liquid chromatograph (LC) system (1). The detector emits at 235 nm with a full width at half maximum bandwidth of 12 nm. The design includes a high-sensitivity Z-shaped flow cell. This system was reported to yield a 20-fold higher optical output and approximately 35 times increase in external quantum efficiency (EQE). The detector was fitted with an all-aluminum housing and cooled using a fan. The detector demonstrated linearity with low stray light (0.06%), using an effective pathlength of 1.1 mm.
This detector was incorporated into a briefcase-sized portable capillary HPLC system and was tested using a mixture of 13 test compounds at the sub-mg/L concentrations with analysis times less than 5 min, using gradient elution.

Haddad also has continued his research and development of a miniature ultraviolet absorbance detector for capillary LC that is based on LED technology and is capable of scanning detection from 230 to 300 nm as well as individual wavelength detection (240, 255, and 275 nm) (2). The detector has been applied and tested for a miniaturized LC system with reported initial stray light of less than 0.4%, low noise at a 300 μAU level, as well as a wide dynamic measurement range of 0.5 to 200 μg/mL for compounds tested, such as sulfamethazine, carbamazepine, and flavone.

Initial research into the development of a portable, modular capillary LC system has been reported recently by Haddad and associates (3). The system has demonstrated high performance and includes custom syringe pumps with a wide range of capillary flow rates. This system has been reported to achieve high repeatability (precision) of less than 1.5% relative standard deviation (RSD) for peak area and less than 0.4% RSD for retention time (n = 6). This performance was demonstrated for the separation of a five-component mixture using a 50 mm x 530 μm ID C18, 3-µm particle capillary column.

Haddad also has explored modifying the surface of silica nanoparticles using octadecyl silica (ODS) chains for use as a sorbent for in-tube solid-phase microextraction (IT-SPME) sampling (4). For this work, chemical coating using nanostructured octadecyl silica particles and a nucleosynthesis procedure was applied to the internal surface of a fused-silica capillary. The result is the internal surface of the fused-silica capillary is more porous, more adsorptive, and more resistant to chemical and mechanical stresses. The modified capillary was developed as an IT-SPME device for the extraction and preconcentration of ultra-trace levels of polycyclic aromatic hydrocarbons (PAHs) in aqueous samples, followed by analyte determination using a gas chromatography–flame ionization detection (GC-FID) system.

Given Haddad’s research focus of formulating models to predict retention times in LC, he has also recently published an excellent review on this topic, covering 2015–2020 (5). The review article focused on RPLC, hydrophilic-interaction chromatography (HILIC), and IC, and covered multiple analytes.

**Key Career Publications: Most Cited**

Haddad’s key publications include two reference textbooks. The first is Ion Chromatography: Principles and Applications (Elsevier, 1990) (6), The first book currently has 731 Google Scholar (G.S.) citations and is widely used as a comprehensive reference for ion chromatography (IC). With 22 chapters and more than 750 pages of detail, the book is a wealth of information, providing IC researchers and professional analytical scientists the type of information they would find most valuable and helpful. The book contains many equations, figures, and data tables and is prolifically referenced. The second book is Principles and Practice of Modern Chromatographic Methods (Academic Press, 1994) (7). With nine chapters and nearly 500 total pages, it covers the theory and practice of chromatographic methods and topics, such as GC, planar chromatography, HPLC instrumentation and techniques,
HPLC separations, supercritical fluid chromatography (SFC), sample handling, and qualitative and quantitative analysis. This text has 283 G.S. citations.

Haddad’s paper with the third highest citations (272 G.S.) reviews the subject of leaching and recovery of gold using ammoniacal thiosulfate liquors (8). The review describes the current application of ion exchange resins for recovery of gold and silver from such leach liquors. Thiosulfate leaching chemistry is compared with cyanide leaching. Comparisons are also made with other leach liquor recovery processes, such as carbon adsorption, solvent extraction, electrowinning and precipitation.

Another review by Haddad received the fourth highest level of citations (187 G.S.) (9). The review describes the details of recent developments (circa 2008) in the field of ion chromatography (IC). The topics covered in the review are new stationary (monolithic) phases, miniaturized IC systems, enhanced peak capacity through computer tools for simulation and prediction of retention, hyphenated IC systems and their use for speciation and metallomics studies, and IC in bioanalysis.

The fifth highest cited work from Haddad (159 G.S.) is a review paper describing the integration of conductivity detection for conventional and miniaturized capillary electrophoresis (CE) systems (10). As the paper points out, the application of microfabrication technology enables precise alignment of electrodes, resulting in the widespread use of conductivity detection for microfluidic devices. For contactless conductivity detection (CCD) used in CE, the precise alignment of the electrodes with respect to the capillary element is not critical. In addition, a description of contactless conductivity detection (CCD) as applied for CE systems is included within this review paper.

Collaborative Research
Numerous scientists have enjoyed collaborating with and being supported by Haddad over the years. One of those is Brett Paull, the Associate Dean of Research of the College of Science and Engineering at the University of Tasmania. When Paull joined the chemistry department in 1995 at the university as a junior lecturer, he immediately began working with the rapidly growing Separation Science Research Group (SSRG).

“Paul was head of the group and I spent the next two and a half years working with Paul on various projects,” Paull said. “Paul provided great mentorship and support during that time, and ever since them we have remained close friends and research collaborators.”

Today, Paull and Haddad collaborate within ACROSS, the living descendant of that early SSRG.

Professor Emily Hilder, who is with the Defense Science and Technology Group at the Department of Defense in Australia, remembers being inspired by Haddad when he was her professor of chemistry.

“It was Paul who first introduced me to chromatography and I credit him with inspiring my love of chromatography and shaping my future career direction,” she said.

Michael Breadmore, a Professor of Chemistry and the current director of ACROSS has known Haddad since 1995.

“Paul was my first research project supervisor and the one that accepted me into his research group to do a PhD—Paul is generous, a man of integrity, and trustworthy,” he says.

Purnendu “Sandy” K. Dasgupta, the Hamish Small Chair in Ion Analysis in the Department of Chemistry and Biochemistry at the University of Texas at Arlington, remembers when he first met Haddad at the inaugural International Ion Chromatography Symposium in 1989. Dasgupta was immediately impressed by Haddad’s early work, and still is to this day.
“As the saying goes, imitation is the highest form of flattery—some four decades later I am presently admiring the beauty of doing electrochemistry at copper electrodes,” he said. “So it is fair to say I have been paying my tributes to Paul through my actions throughout the years up till now.”

Wolfgang Buchberger is a Full University Professor in Analytical Chemistry at the Johannes-Kepler-University Linz in Austria and has known Haddad since the late 1980s when Buchberger joined him as a post-doc at the University of New South Wales in Sydney.

“In the early 1990s Paul moved to Hobart (Tasmania). During the process of establishing a research group in separation science he offered me a position to be part of his team—I considered this a privilege,” he said.

Buchberger joined Haddad’s team for 1993 and 1994 and has continued to stay connected and collaborate since that time.

Greatest Contributions

When we asked others in the field to assess Haddad’s most important contributions to separation science, a common theme was that he made an impact in many ways.

Paul focused on Haddad’s development of many aspects of ion chromatography, and other modes of chromatography and electro-separation methods, targeted to small inorganic and organic ions. “He has gained a considerable reputation in the area of chromatographic modelling and retention time prediction algorithms, including work which has led to commercial products,” Paul says. “He has been involved in numerous applied projects across a whole range of industries, from mining to pharmaceuticals, and has led as director of multiple industry supported large-scale research initiatives and centres.”

“Paul has made outstanding contributions to the field of ion chromatography and is one of the pioneers and gurus in this field,” Hilder said.

Breadmore sees Haddad’s major influence in separation science in three main areas. First is the field of small-molecule analysis by ion chromatography, capillary electrophoresis, and more recently, by HILIC.

“He was, and still is, one of the most published authors in ion analysis,” he said. Second is the field of computer aided optimization and method development. “The development of software to help optimize conditions has been a significant aid to many users of ion chromatography,” he said. “Haddad’s recent work with other forms of chromatography for pharmaceuticals will hopefully have similar impact.”

Breadmore said that Haddad’s third main contribution is in training outstanding researchers that have gone on to make significant contributions to academia and industry.

Buchberger also named multiple areas, including Haddad’s work in modeling of chromatographic separations, prediction of retention times, systematic optimization of chromatographic resolution, and the foundation of ACROSS.

Dasgupta says that choosing Haddad’s most important contribution is difficult, because he has contributed to so many areas.

“I think Paul’s most lasting contribution is ACROSS, and specifically all the students and colleagues who flourished under his leadership,” he said. “It is hard to think about analytical chemistry in Australia if we take out all those researchers that ACROSS has touched in some way.”

Teacher and Mentor

Many expressed that Haddad is not just an outstanding scientist, but an outstanding teacher and mentor.

“I have learnt much more than chemistry and chromatography from Paul—about how to lead and manage people effectively, working effectively across academia and industry, and many other things without which I would not have been able to achieve so much in my own career to date,” says Hilder. “Paul’s legacy is not just his contributions to chromatography, which alone are very significant, but what he has done to build and support the careers of others, especially through his establishment and leadership of ACROSS.”

Brett Paull agrees. “He is well known for his unending support and loyalty to his past students and post-docs, right throughout their careers, rarely having a negative word to say,” Paull said. “In fact, that is a trait of Paul’s which is truly admirable, in that he has always been a leader who lets people make their own mistakes, without rushing to criticize, only provide support and advice.” Paull adds that Haddad is an extremely loyal and
reliable friend. “He offers up his expert help for almost anything needed, from editing a first draft manuscript, all the way to house painting.”

Closing Remarks

Although Haddad has officially retired from the university, he has not stopped working—as evidenced from his 2020 publications. “He still co-supervises multiple PhD students, several with me, and is also still a named investigator on active research grants,” Paull said.

Dasgupta said, “Paul has not stopped, may this award be additional impetus for him to keep keeping on.”

“He contributions to date have been phenomenal and I think he deserves a rest if he wants one,” says Breadmore, “If he doesn’t, I think he will continue to explore the idea of predicting chromatographic behavior a priori using computer aided method development.”

References

(7) K. Robards, P.R. Haddad, and P.E. Jackson, Principles and Practice of Modern Chromatographic Methods (Academic Press, Boston, Massachusetts, 1994).

The Emerging Leader Award

The Emerging Leader in Chromatography Award recognizes the achievements and aspirations of a talented young separation scientist who has made strides early in his or her career toward the advancement of chromatographic techniques and applications.

Erik L. Regalado, the 2021 winner, is a Principal Scientist in the analytical research and development (AR&D) department at Merck Research Laboratories, where he leads the Method Screening and Purifications group. His work focuses on analytical and preparative enabling technologies that accelerate the development of new pharmaceuticals, including automated method screening, multidimensional chromatography, high-throughput analysis, and ultrafast and computer-assisted separations.

Regalado received a PhD in chemistry from the University of Havana, in Cuba, in collaboration with the University of Nice Sophia Antipolis in France in 2011. He received a MS in organic chemistry in 2007, and a BS in chemistry in 2003, both from the University of Havana in Cuba.

Key Research Publications

In his efforts to improve the performance of analytical and purification separations of pharmaceutical compounds, Regalado has employed a range of separation techniques and modeling approaches. Here are his latest publications as examples.

In his most recent paper, an extract of a Dactylia sp. nov. marine sponge, Regalado and colleagues have identified seven new arylpyrrole alkaloids and four known compounds by interpretation of both nuclear magnetic resonance (NMR) and mass spectrometry (MS) data (11). The molecular structures of denigrins D–G, dactylypyrrols A–C, denigrin D, and dictyodendrin F were described in detail using these analytical techniques.

In another recent study, Regalado and colleagues introduced multifactorial peak crossover (MPC) via computer-assisted chromatographic modeling to address challenging coelutions of critical pairs and poor chromatographic productivity of purification methods (12). This MPC approach allows mapping of separation parameters in pharmaceutical mixtures to enable switching the elution order of target analytes. The MPC technique is reported to dramatically reduce development time for analytical and preparative-scale separations. In addition, MPC chromatography is reported to improve signal-to-noise ratio (S/N) for parts per billion (ppb) detection of metabolites and genotoxic impurities.

Regalado has reported the development and implementation of a customized online multicomponent two-dimensional liquid chromatography–diode array detection–mass spectrometry (2D-LC-DAD-MS) workflow system (13). The system is designed for method development and screening specifically to analyze multicomponent biopharmaceutical mixtures. Excellent chromatographic performance was demonstrated for peak shape, selectivity, and peak reproducibility for reversed-phase liquid chromatography (RP-LC), strong cation exchange (SCX), strong anion exchange (SAX), and size exclusion chromatography (SEC). A sub-2-μm column was used for the first dimension combined with multiple column in the second dimension. This technique may be used for semi-automated development of 2D-LC methods, especially for protein-based drug analysis.

Another important publication of Regalado’s introduces a new concept for multidimensional chromatography: the first report of automated multicomponent 2D-LC screening for facile selection of stationary- and mobile-phase conditions in both dimensions (14).

In another recent paper, Regalado and associate researchers demonstrated a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)-MS method for determining peptide and protein permeability through an artificial liposome membrane using a hydrogen-
deuterium exchange (HDX) technique (15). The technique is referred to as the liposome artificial membrane permeability assay (LAMPA) workflow and was used to study three previously characterized membrane-active model peptides—melittin, alamethicin, and gramicidin. In this work a non-aqueous (aprotic) matrix solution was designed to quench unwanted HDX, and peak area ratio was calculated for labeled vs. unlabeled peptides using MALDI to indicate permeability. The method requires labeling peptides on the outside of the liposomes via exposure to deuterium oxide, while the peptides inside the liposomes are not labeled. The proposed LAMPA-MALDI-HDX-MS technique can be used for ultra-high-throughput analysis to rank-order peptides and small proteins for their membrane permeability properties.

A recent review paper published by Regalado includes academic and industry reports on the emergence of universal chromatographic methods in the research and development of new drug substances (16). Regalado also has become one of the leaders of this field that is transforming analysis in pharmaceutical R&D laboratories. Other work focuses on improving supercritical fluid chromatography for preparative-scale purifications. By introducing a simple and highly efficient enhanced SFC (eSFC) approach, Regalado and coworkers reported analysis and purification of over 40 α,α-diaryl primary amine mixtures, overcoming limitations of traditional chromatographic methods

Regalado and Makarov at the HPLC 2019 conference in Milan.
The authors report that more than 1500 methods (17). The eSFC approach is reported to deliver significant method improvements for simplicity and greenness, such as improved chromatographic performance; augmented eSFC–MS and preparative MS purifications; and direct solvent removal with minimal solvent use. He also recently published work that demonstrates a revolutionary concept that introduces chaotropic effects in SFC (18). This new strategy has enabled Regalado’s group at Merck to deliver highly pure cyclic peptides at kilogram scale, something unprecedented in purification laboratories. In addition, the concept helped extending the reach of SFC to molecules that were previously considered out of the scope of the technique.

**Key Career Publications: Most Cited**

Regalado’s most cited paper (264 citations in Google Scholar (G.S.)), demonstrates a method for direct photocatalytic C–H fluorination used in the preparative synthesis of odanacatib (20). This reaction advanced the synthetic pathway for odanacatib—an investigational drug treatment for osteoporosis and bone metastasis. In a paper receiving 86 citations, Regalado and coworkers described a methodology for ultrafast chiral separations useful for high throughput analysis of drug enantiopurity (21). The authors reported that enantioseparations, using fast chromatographic techniques, allow high throughput analysis of enantiopurity within a few seconds for pharmaceutical drugs and intermediates. This research demonstrated that ultrafast resolutions (≤1 min) are possible using both SFC and RPLC.

Regalado published a paper receiving 73 citations that proposed and demonstrated ultrafast two-dimensional liquid chromatography (2D-LC) for chiral separations in pharmaceutical products (22). The incorporation of a 2D-LC technique improves peak capacity and selectivity for chromatographic separations. Special combinations of achiral and chiral narrow-bore columns in the first dimension with highly efficient chiral selectors in the second dimension were successfully applied to the separation and analysis of complex mixtures for pharmaceuticals and synthetic drug intermediates.

In work during his graduate studies, five new hydantoin alkaloids, named parazoanthines A–E, were isolated by Regalado and colleagues as the major constituents of the Mediterranean sea anemone Parazoanthus axinellae using NMR spectroscopic and mass spectrometric analyses (23). The absolute configuration of two of the chiral compounds 1 and 4 was determined by comparison between experimental and time-dependent density functional (TDDFT) calculated circular dichroism (CD) spectra. This paper has received 71 citations to date.

**An Innovator and Emerging Leader**

Numerous collaborators and mentors have praised Regalado’s work and innovation. Abilio Laguna Granja is currently a production researcher at Synergy Semichemicals Ltd., in Surrey, British Columbia, Canada. He was Regalado’s mentor for his Master’s and PhD theses, together with Professor Clara Nogueiras from Havana University, and worked with Regalado from 2004 to 2012 in the Department of Chemistry at the Center for Marine Biology (CEBiMar).

“Since I have known Erik I have considered him the best young scientist I ever met,” he said.

“Erik has the ability to synthesize empirical facts into a clear picture,” said Alexey A. Makarov, a principal scientist in the AR&D group. "I believe one of his most significant accomplishments in the field of chromatography is revealing chaotropic effects on peptides during SFC separation.”

This approach has since been extensively applied to the separation and purification of reaction mixtures of many polar pharmaceuticals, such as organohalogenated species, nucleobases, nucleosides, nucleotides, sulfonamides, and cyclic peptides, and resulted in a publication in *Analytical Chemistry*.

Robert Hartman is currently the Director of Integrated Development and Supply Team, Center of Excellence (IDST CoE) for Quality at Merck Research Laboratories in Rahway, New Jersey. Regalado worked with Hartman for several years when Hartman led the AR&D group.
Follow us on social media for more updates on the field of chromatography industry.

Join your colleagues in conversation and stay up-to-date on breaking news, research, and trends associated with the industry.

- [LinkedIn](linkedin.com/company/lcgc)
- [@lcgcmagazine](twitter.com/lcgcmagazine)
- [@LC_GC](twitter.com/LC_GC)
“Erik is one of the most enthusiastic and hardworking analytical chemists that I’ve had the pleasure of knowing,” Hartman said, adding that Regalado would often call or text him late at night or during the weekend to share a new idea he had or a recent breakthrough in an analytical challenge. “Erik truly loves being an analytical chemist, solving challenging problems and sharing these learnings through presentations, conferences, publications and late-night calls to his friends.”

Christopher Welch is the Executive Director of the Indiana Consortium for Analytical Science & Engineering (ICASE) in Fishers, Indiana, and the former Distinguished Scientist for Process & Analytical Chemistry at Merck, where he hired and supervised Regalado’s post-doctoral research.

“When I met Erik in the interview stage, it was clear that we would work well together and that he wanted the same thing that I wanted . . . an opportunity to work hard, carry out interesting research, and publish some great science,” Welch said.

Daniel W. Armstrong, the R.A. Welch Distinguished Professor at the University of Texas at Arlington, first got to know Regalado in 2010 after the two met at various conferences. They discovered their mutual interests in chiral separations, fast separations, and super critical fluid chromatography—among others, and ended up having several joint publications.

“Erik has made contributions in many different areas, but his work in high throughput analyses, particularly of pharmaceutically important compounds, certainly stands out,” Armstrong said.

Davy Guillarme is a senior lecturer and research associate at the University of Geneva in Switzerland and has known Regalado since 2013, when Erik was doing his post-doctoral work at Merck. The two began collaborating in 2018.

“Regalado has developed a significant number of platform methods for a wide range of molecules having very diverse physico-chemical properties,” Guillarme says. “In particular, he has developed some analytical strategies making it possible to obtain very fast chiral separations and solutions to obtain both chemical and enantioselectivity within the same run, by combining achiral and chiral LC in a 2D-LC setup.”

Ian Mangion is the Executive Director of Analytical Enabling Technologies at Merck in Cranford, New Jersey. “I rely on Erik to drive innovation and advances in automation and modeling in method development and separations of a wide diversity of chemical matter across modalities.” Mangion said. “Erik is reliably one of the most productive innovators in the field of separation sciences, but perhaps his greatest contribution is the way he has collaborated with leading research labs and his pharmaceutical colleagues to facilitate substantive advances in the speed and productivity of our work.”

The Future

Given Regalado’s impressive work so far—exemplified by his 18 impactful publications during the first year of his postdoctoral fellowship, including contributing to a publication in the journal Science—the community expects his impact to continue.
“I know that he will continue working hard while obtaining many great results that will be published in top level journals,” said Laguna Granja. “I hope that Dr. Regalado will continue to increase his knowledge in the field of LC and GC chromatography and will become one of the top leaders in the field.”

Makarov says that Regalado is growing to become a great scientific and group leader at Merck.

“He is now leading the Purification and High-Throughput Screening Lab,” Makarov said. “This brings him to a new level of influence and innovation leadership.”

Hartman said that he sees Regalado continuing to advance his career as a leader in Merck’s analytical mission.

“Erik is one of the most enthusiastic and hardworking analytical chemists that I’ve had the pleasure of knowing,” Hartman said. “He is doing well at Merck and leading a strong group. I expect great things from him.”

“Based on his qualities, I am sure Erik will become one of the most impactful people in pharmaceutical analysis in the future,” commented Guillarme “Indeed Erik’s inventiveness and practical problem-solving in chromatography is largely renowned, bringing great value to both pharmaceutical industry and academia.”

“What he has done as an industrial chemist is almost unbelievable; he has more and better research publications than most academic chemists in the field,” says Armstrong. “I can only see him continuing to go onwards and upwards in his research reputation and contributions.”

References

Jerome Workman, Jr. is Senior Technical Editor for LCGC North America and Spectroscopy. Direct correspondence about this article to jworkman@mmhgroup.com
Follow us on social media for more updates on the cannabis industry

Join your colleagues in conversation and stay up-to-date on breaking news, research, and trends associated with the industry.

linkedin.com/company/cannabis-science-and-technology/
@CannSciTech
@CannabisSciTech
@CannabisScienceTechnology
THE APPLICATION NOTEBOOK
Environmental

99 Using the Empore™ EZ-Trace SPE Workstation to Perform Efficient and Reproducible Extractions of SVOCs
Xiaohui Zhang, Michael Apsokardu, and Guotao Lu, CDS Analytical

Food & Beverage

100 Separation of Aflatoxins on HxSil C18
Adam L. Moore, PhD, Hamilton Company Inc.

101 Summary of FDA Method C-010.01: Determination of 16 Perfluoroalkyl and Polyfluoroalkyl Substances (PFAS) in Food Using Liquid Chromatography–Tandem Mass Spectrometry (LC–MS/MS)
Susan Genualdi and Lowri deJager, FDA Foods Program
Compendium of Analytical Laboratory Methods

102 Starch Amylose and Amylopectin Molar Mass and Size Distributions by FFF-MALS
Rick White and Eija Chiaramonte, The Procter & Gamble Company

Pharmaceutical/Drug Discovery

103 Shodex HILICpak VC-50 2D for All Essential Amino Acids Detection
Leah Sullivan, Showa Denko America, Inc.

104 Characterization of Recombinant Protein Biotherapeutic Using TSKgel® UP-SW2000 Size Exclusion Chromatography Column In-Line with LenS3™ MALS Detector
Tosoh Bioscience LLC

Cover Photography: Getty Images
Using the Empore™ EZ-Trace SPE Workstation to Perform Efficient and Reproducible Extractions of SVOCs

Xiaohui Zhang, Michael Apsokardu, and Guotao Lu, CDS Analytical

This application note demonstrates how the Empore EZ-Trace SPE system is used to perform multiple extractions simultaneously while achieving clean and reproducible results using EPA Method 525.3 as an example application.

The Empore EZ-Trace (Figure 1) is a manual, vacuum-controlled extraction workstation designed to perform up to four extractions simultaneously to improve efficiency in a clean and reproducible manner. The Empore EZ-Trace workstation is universally compatible with all Empore 47/90 mm disks and cartridges. The unique independent channel design ensures that each extraction is precisely controlled, preventing cross-contamination with the other channels. High-throughput flow path switches provide a unique, environmentally friendly extraction by collecting organic and aqueous wastes in separate reservoirs. This is demonstrated using an example application of EPA Method 525.3, which is designed for identification and quantification of semivolatile organic compounds (SVOCs) such as PCBs, PAHs, and pesticides. Under this method, SVOCs are extracted from three reagent water samples having different concentrations and one sample of ground water, with four replicate measurements required for each sample. Methods such as EPA 525.3 require ways to improve efficiency when numerous extractions are needed.

Four extractions were done simultaneously using the Empore EZ-Trace system (catalog #: 8000) by following EPA 525.3 extraction procedures to extract SVOCs onto an Empore SDB-XC 47mm disk (catalog #: 2240). The extract was then evaporated, diluted, and then analyzed with a Shimadzu GC-2010 instrument using a Restek Rxi-5Sil-MS system (30 m x 0.25 mm x 0.25 µm) column for separation and MS QP2010 for detection. In this method, 125 analytical calibration standards, three surrogates, and four internal standards compounds were efficiently separated (Figure 2). The recoveries and concentrations of 125 analytes were determined from four replicate measurements using a five-point calibration curve. For all compounds except for two, the acceptable range of recoveries is 70–130%. The accepted range for the other two compounds is 60–130%. The combined recovery results are summarized in Table I. For each sample, the average recovery is >89.9% with <7.4% RSD. These results demonstrate how the Empore EZ-Trace provides a clean, efficient, and reproducible extraction.

![Figure 1: Empore EZ-Trace extraction workstation for multiple extractions using Empore disks and cartridges.](image)

![Figure 2: Chromatogram showing the separation of 125 analytical, three surrogates, and four internal standards compounds extracted from a reagent-grade water sample under EPA Method 525.3.](image)

<table>
<thead>
<tr>
<th>Water</th>
<th>Reagent Grade</th>
<th>µg L⁻¹</th>
<th>Ground</th>
<th>µg L⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>2.0</td>
<td>5.0</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>≥70%</td>
<td>118</td>
<td>123</td>
<td>123</td>
<td>123</td>
</tr>
<tr>
<td>avg Rec. (%RSD)</td>
<td>89.9 (7.4)</td>
<td>90.9 (5.8)</td>
<td>94.4 (3.4)</td>
<td>93.4 (4.5)</td>
</tr>
</tbody>
</table>
Since the discovery of aflatoxins in 1960, many areas of human food sources have shown considerable propagation of the fungal infection. Although the majority of food samples are cereals, the toxins can be found in a variety of medias, including corn, peanuts, legumes, livestock, milk, and even cannabis, to name a few. The aflatoxins mentioned above are part of approximately 40 secondary metabolites derived from the fungus, *Aspergillus flavus*. An *A. flavus* infection can be found in both the pre-harvest and the post-harvest analysis of crops. Analogously, the effects from these metabolites can be either acute or chronic, and have been found to be teratogenic, mutagenic, carcinogenic, immunotoxic, or hepatotoxic in nature. Due to the nature of these attributes, the toxins have been implicated in liver cancer, Reyes syndrome, cirrhosis, and chronic gastritis. As such, the FDA has imposed strict guidelines on the amounts of acceptable aflatoxins present in food, <20 ppb, with even stricter guidelines for milk, at <0.5 ppb.

In this application note, we have developed an HPLC method that isolates five different aflatoxins with the aid of Hamilton’s HxSil-C18 (3 μm) column. The fast isocratic method is completed in under 6 min, and provides excellent resolution between the various aflatoxins. The excellent sensitivity shown in the method affords the analyst a robust, accurate, and affordable product.

**Table 1: Column Information and Chromatographic Conditions**

<table>
<thead>
<tr>
<th>Column Information</th>
<th>HxSil, 3 μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Part Number</td>
<td>79641</td>
</tr>
<tr>
<td>Dimensions</td>
<td>150 × 4.6 mm</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chromatographic Conditions</th>
<th>Isocratic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>40 ºC</td>
</tr>
<tr>
<td>Injection Volume</td>
<td>2 μL</td>
</tr>
<tr>
<td>Detection</td>
<td>UV at 365 nm</td>
</tr>
<tr>
<td>Eluent</td>
<td>Water:Acetonitrile:Methanol (50:25:25)</td>
</tr>
<tr>
<td>Flow Rate</td>
<td>1.0 mL/min</td>
</tr>
</tbody>
</table>

**Figure 1: Separation of aflatoxins on HxSil C18**

Compounds:
1. Aflatoxin M1 (2.5 ng)
2. Aflatoxin G2 (20 ng)
3. Aflatoxin G1 (10 ng)
4. Aflatoxin B2 (10 ng)
5. Aflatoxin B1 (10 ng)
Summary of FDA Method C-010.01: Determination of 16 Perfluoroalkyl and Polyfluoroalkyl Substances (PFAS) in Food Using Liquid Chromatography–Tandem Mass Spectrometry (LC–MS/MS)

Susan Genualdi and Lowri deJager, FDA Foods Program Compendium of Analytical Laboratory Methods

Summary
Per- and polyfluoroalkyl substances (PFAS) are a diverse group of synthetic organofluorine compounds that have been widely used in industrial applications and consumer products, such as nonstick cookware, food packaging, fire-fighting foams, carpeting, apparel, and metal plating. PFAS are persistent in the environment and bio accumulative in humans and wildlife, and said to cause reproductive and developmental toxicity. This application note summarizes a validated procedure developed by the US Food and Drug Administration (FDA) for the measurement of 16 PFAS in food matrices using a QuEChERS sample preparation approach and LC–MS/MS analysis.

Sample Extraction
The edible portion of the food sample is collected and homogenized using an IKA tube mill with a disposable 100 mL polypropylene grinding chamber (an alternative homogenizer may also be used for this step). Samples are ground at 5000 rpm for approximately 2 min. The minimum sample size for analysis is 5 g.

1. Add amount of sample and LC–MS grade water based on Table II and commodity type to a 50 mL polypropylene (PP) centrifuge tube.
2. Add 10 µL of 1 µg/mL isotopically labeled surrogate standard solution to the sample.
3. Add 10 mL acetonitrile and 150 µL formic acid to the 50 mL PP conical centrifuge tube.
4. Shake vigorously for 1 min.
5. Add QuEChERS salt packet and shake for 5 min.
   - Glas-Col® digital pulse vortexer at 1500 rpm with pulse set to 70
   - Geno/Grinder® 2010 at 1500 rpm
6. Centrifuge the samples at 10000 rcf for 5 min.

Sample Clean-up
1. Transfer supernatant to the 15 mL dSPE tube
2. Vortex/shake for 2 min.
3. Centrifuge the samples at 10000 rcf for 5 min.
4. Filter 5 mL of the extract with a 0.2 µm nylon syringe filter and transfer to a 15 mL conical centrifuge tube.
5. Add internal standard:
   - For samples that do not require nitrogen concentration:
     • Add 5 µL of 1 µg/mL d5-N-EtFOSAA internal standard solution to the 5 mL extract to give a final concentration of 1 ng/mL. Surrogates will also have a final concentration of 1 ng/mL in the final extract.
   - For samples that require nitrogen concentration:
     • Concentrate to near dryness with nitrogen and reconstitute to 0.5 mL with methanol.
     • Add 5 µL of the 1 µg/mL d5-N-EtFOSAA internal standard solution to give a final concentration of 10 ng/mL in solution.
   Surrogates will also have a final concentration of 10 ng/mL in the final extract.
6. Briefly vortex and shake
7. Transfer sample to a polypropylene autosampler vial for analysis by LC–MS/MS.

Results
A level 2 single laboratory validation was conducted under the Guidelines for the Validation of Chemical Methods for the FDA FVM Program 2nd Ed. A total of four different types of foods and beverages were evaluated. These include produce, milk, fish, and bread. The method was validated at six concentrations (0.05, 0.15, 0.5, 1.5, 2, 5 ng/mL) in four food matrices. Acceptable recovery ranges for these compounds based on the FDA guidelines for the validation of chemical methods is 40–120% for concentrations spiked at 1 ng/mL. All compounds were within the acceptable range, except for 11Cl-PF3OUdS in bread samples which were on the lower side at 26–42% recovery.

Table I: Extraction/Analytical Materials Ratio

<table>
<thead>
<tr>
<th>Material</th>
<th>Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECMSSCS-MP</td>
<td>Mylar pouch containing 6 g MgSO₄ and 1.5 g NaCl</td>
</tr>
<tr>
<td>ECMPSB15CT</td>
<td>15 mL dSPE tube with 900 mg MgSO₄, 300 mg PSA and 150 mg GCB</td>
</tr>
</tbody>
</table>

Table II: Sample Preparation Conditions Based on Food Commodity Type

<table>
<thead>
<tr>
<th>Commodity Type</th>
<th>Sample Amount</th>
<th>Water Added (mL)</th>
<th>ACN Added (mL)</th>
<th>Concentrate to Dryness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fruits &amp; Vegetables</td>
<td>5 g</td>
<td>5</td>
<td>10</td>
<td>No</td>
</tr>
<tr>
<td>Bread</td>
<td>5 g</td>
<td>15</td>
<td>10</td>
<td>No</td>
</tr>
<tr>
<td>Milk</td>
<td>5 mL</td>
<td>5</td>
<td>10</td>
<td>No</td>
</tr>
<tr>
<td>Cheese</td>
<td>1 g</td>
<td>5</td>
<td>10</td>
<td>No</td>
</tr>
<tr>
<td>Other Dairy</td>
<td>5 g</td>
<td>5</td>
<td>10</td>
<td>No</td>
</tr>
<tr>
<td>Meat</td>
<td>5 g</td>
<td>5</td>
<td>10</td>
<td>No</td>
</tr>
</tbody>
</table>

For samples that require nitrogen concentration:
• Concentrate to near dryness with nitrogen and reconstitute to 0.5 mL with methanol.
• Add 5 µL of the 1 µg/mL d5-N-EtFOSAA internal standard solution to give a final concentration of 10 ng/mL in solution.

Surrogates will also have a final concentration of 10 ng/mL in the final extract.

UCT, Inc.
2731 Bartram Road, Bristol, PA 19007
Tel. (215) 781-9255, Fax (215) 785-1226
Website: www.unitedchem.com
Starch contains large homopolymers of linear amylose (AMY) and branched amylopectin (AMP). Its functional properties are influenced by the ratio and molar masses of its macromolecular constituents, which vary with source, crop year, and climate.

With average radii in the hundreds of nanometers, and molecular weights ranging into the hundreds of millions, starch polymeric components cannot be separated by GPC. However, field-flow fractionation coupled to multi-angle light scattering (FFF-MALS) is suitable for separation and characterization of polymers and nanoparticles from 1 to 1000 nm, and hence is applicable to starch analysis. FFF performs non-shearing separation by hydrodynamic size, and MALS analyzes absolute molar mass and size regardless of conformation or retention properties.

This note demonstrates the application of FFF-MALS to separating AMY and AMP in order to calculate their mass ratio, determine molar mass distributions, the average molecular weights of AMY and AMP, and the z-average root-mean-square radius $R_{g,z}$ and polydispersity $M_w/M_n$ of the AMP component.

Materials and Methods
Separation was performed by an Eclipse® FFF system with short channel, a 350 µm spacer, and a 10 kDa cutoff regenerated cellulose membrane. Detection was accomplished with DAWN® 18-angle MALS and Optilab® refractive index (RI) detectors. The channel flow was maintained at 1.0 mL/min and the cross-flow was varied linearly from 1.0 to 0.1 mL/min for 10 min, then switched to 0.0 mL/min.

Results and Discussion
Figure 1 shows that FFF-RI fractograms separate into distinct peaks for AMY and AMP. The molar mass distributions determined for these samples ranged from 10 kDa to 1 GDa, though there is evidence for smaller starch components as well.

Table I summarizes the results of the analyses. Integration of the respective peak areas enabled calculation of the AMY:AMP ratios, in excellent agreement with the nominal values. The values for $M_w$ and $R_{g,z}$ fall within the generally accepted limits found in the literature. Polymer conformation is assessed by comparing $R_g$ with molar mass. Conformational plots for the AMP component (not shown) indicate a log-log slope of 0.39–0.41 for all starches measured, verifying their branched nature. Species-specific differences in the overall density of the amylopectins are apparent in the vertical offsets of the conformation plots.

Conclusions
Starch characterization by size-exclusion chromatography can be limited due to shear degradation or column adsorption of the higher mass fractions of AMP, and SEC columns cannot cover the entire size range. Due to branching, the usual linear calibration standards would not have been applicable. The open-channel separation of FFF eliminates these limitations and enables recovery of intact AMP. The molar masses and mass ratios of AMY:AMP were accurately determined over five orders of magnitude. Only FFF-MALS fully addresses the multiple analytical challenges presented by starch.
Amino acids have significant biological importance, as they are the building blocks of life, and they are also used in nutritional supplements, fertilizers, and food technology. Industrial amino acid use includes the production of drugs, biodegradable plastics, and even chiral catalysts. With the wide variety of uses, there are many ways to classify amino acids, including polarity, pH, size, hydrophobicity, side chain group type, and so on. However, the ideal quantitative analysis of amino acids combines speed and sensitivity with reliability of the analytical technique.

Many columns cannot offer a single method to analyze the 20 building block amino acids. Shodex introduces the new HILICpak VC-50 2D column, which can offer speed, sensitivity, and reproducibility. This column is packed with polyvinyl alcohol material with carboxyl functional groups. When used according to the protocol described below, the column enables the user to analyze all 20 amino acids using a single method. A single run can be completed in 25 min with good resolution. In addition to analyzing the essential amino acids, the VC-50 2D has been shown to separate amino sugars.

**Experimental Conditions**
Each amino acid was dissolved in water and diluted to 0.1 μg/mL. The analysis of all 20 amino acids was successfully accomplished with the Shodex HILICpak VC-50 2D (2.0 mm ID x 150 mm), a polymer-based column for HILIC analysis. A 5 μL measure of each sample was injected into the system. The column temperature was kept at 30 °C and a flow rate of 0.2 mL/min. Eluent conditions were H₂O/100 mM HCOOH aq./CH₃CN = 30/20/50 to 0/90/10 (0 to 5 min), 0/90/10 (5 to 30 min) in a lower pressure linear gradient. The HPLC system was coupled with an ESI-MS SIM detector.

**Results**
The Shodex team has successfully developed a method for detecting 20 amino acids using a single method on the HILICpak VC-50 2D under positive MS mode. From the data shown above, isoleucine and leucine have some overlap. Through some modifications to the method, this analysis can result in a better separation. The Shodex HILICpak VC-50 2D is an ideal column for amino acid analysis.
Characterization of Recombinant Protein Biotherapeutic Using TSKgel® UP-SW2000 Size Exclusion Chromatography Column In-Line with LenS₃™ MALS Detector

Tosoh Bioscience LLC

Biotherapeutics are generally larger molecules such as peptides, proteins and monoclonal antibodies with monomer molecular weight ranging from 3,000 to 150,000 Da. The drug must remain free from impurities such as fragment, dimer, and other higher order aggregates because they may cause severe immunogenic response. This becomes even more important particularly if the biotherapeutic protein is thermally susceptible. Historically, size exclusion is the preferred mode of chromatography for separation and characterization of such applications. Here we report the online detection of absolute molecular weight of two recombinant protein samples using a 2 μm size exclusion chromatography (SEC) column directly connected to the LenS₃ Multi-Angle Light Scattering (MALS) detector.

Materials and Methods

Samples: BSA (Calibration standard)
- Sample 1 – Recombinant proteins (~90 kDa)
  at 1.72 mg/mL in mobile phase
- Sample 2 – Recombinant proteins (~90 kDa)
  at 3.64 mg/mL in mobile phase

The samples were stored at -20 °C and thawed to 8 °C just before analysis. The concentration was adjusted by diluting the sample in mobile phase pre-chilled at 8 °C.

Chromatographic Conditions

Instrument: ThermoFisher Ultimate® 3000 UHPLC and Chromeleon® software
Column: TSKgel UP-SW2000, 2 μm, 4.6 mm ID x 30 cm
Mobile phase: BupH modified Dulbecco's phosphate buffer prepared in light scattering grade water and filtered through a 0.1 μm PES membrane (The buffer was prepared from saline packs as per directions contained in Thermo Scientific–catalog # 28374, lot TL275790)
Flow rate: 0.20 mL/min
Detectors: UltiMate 3000 WWD variable wavelength detectors @ 280 nm wavelength and Tosoh LenS₃ MALS detector (positioned in series: Column → UV → MALS)

Results and Discussion

The multi-detector setup was calibrated using a freshly prepared bovine serum albumin (BSA) solution. Figure 1 shows the overlay trace of UV and MALS detectors. The one-step calibration procedure in the SECview™ software adjusted the dead volume between the detectors and corrected for the band-broadening effect caused by the in-series detector configuration while determining the detectors’ calibration constants and offsets.

Figures 2 and 3 illustrate the UV detector overlays for two consecutive injections of Sample 1 and Sample 2, respectively. Zoomed-in figures (inset) show the excellent separation resolution between the monomer and the aggregates obtained from TSKgel UP-SW2000 column.
Figures 4 and 5 demonstrate the molecular weight (green) profiles for Sample 1 and Sample 2, respectively. The concentrations of the aggregate contents differ in the two samples.

Looking closer at the UV trace, it appears that the monomer peaks in both samples illustrate a slight shoulder on the higher retention region, suggesting a bimodal shape. Further analysis using the molecular weight trace by the MALS detector reveals two separate populations of molecular weights. Figures 6 and 7 zoom in on the monomer peaks and demonstrate the two molecular weight plateaus, 1a and 1b, in both samples. Considering the sensitive nature of these recombinant proteins to the ambient conditions, the shoulder peak (1b) suggests the beginning of temperature-induced modifications, which varies in extent in both samples.

Tables I and II list the results of the chromatography analysis, including molecular weight and percent content, for the identified aggregate peaks.

**Conclusions**

This study shows that the molecular weight species including monomer and multiple aggregate levels present in the recombinant protein therapeutics could be determined and quantified using a SEC-MALS configuration. The TSKgel UP-SW2000 demonstrates excellent separation of the higher order aggregates from the monomer in both Sample 1 and Sample 2, as well as the slightly higher molecular weight temperature-induced variants that are almost co-eluted with the monomer. The column yields reproducible results, allowing accurate and precise chromatograms to be analyzed using the LenS3 MALS detector. The LenS3 accompanied by the SECview software also produces reproducible, accurate results in terms of MW determination for all peaks, in addition to area calculations, even at extremely low concentrations or in the presence of the aggregates.

---

**Table I: Results table for Sample 1**

<table>
<thead>
<tr>
<th>Peak</th>
<th>Retention Time</th>
<th>Peak MW</th>
<th>% UV Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>7.220</td>
<td>93,564 Da</td>
<td>98.06%</td>
</tr>
<tr>
<td>1b</td>
<td>-</td>
<td>95,811 Da</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>6.593</td>
<td>229,024 Da</td>
<td>0.69%</td>
</tr>
<tr>
<td>3</td>
<td>6.355</td>
<td>276,217 Da</td>
<td>0.73%</td>
</tr>
<tr>
<td>4</td>
<td>5.533</td>
<td>1,285,996 Da</td>
<td>0.51%</td>
</tr>
</tbody>
</table>

* Retention times are reported in minutes.

**Table II: Results table for Sample 2**

<table>
<thead>
<tr>
<th>Peak</th>
<th>Retention Time</th>
<th>Peak MW</th>
<th>% UV Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>7.242</td>
<td>93,106 Da</td>
<td>98.73%</td>
</tr>
<tr>
<td>1b</td>
<td>-</td>
<td>93,602 Da</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>6.282</td>
<td>251,078 Da</td>
<td>1.04%</td>
</tr>
<tr>
<td>3</td>
<td>5.540</td>
<td>1,631,227 Da</td>
<td>0.23%</td>
</tr>
</tbody>
</table>

---

TSKgel and Tosoh Bioscience are registered trademarks of Tosoh Corporation.
LenS is a trademark of Tosoh Bioscience LLC.
SECview is a registered trademark of Tosoh Bioscience LLC in the USA and EU and of Tosoh Corporation in Japan. UNIMatch and Chromeleon are registered trademarks of the Dionex Corporation.
Nine months. In March 2020, the United States was in the early stages of the COVID-19 pandemic. We shut the entire country down, and ground the economy to a halt, in an effort to slow the spread of the virus. Think back to March, and how much uncertainty we were living under.

Nine months later, the FDA approved two COVID-19 vaccines under emergency authorization. Before New Year’s Day, millions of Americans had received the vaccine, including front-line physicians, health care providers, and nursing home patients, our most vulnerable citizens.

Nine months. Take a moment to let that sink in.

The mainstream media has crafted a narrative around the COVID-19 pandemic that is almost entirely negative. For the purpose of ratings, they have described the U.S. response to the pandemic as blundering from one mistake to the next. This narrative is false.

There is another way—a more accurate and underappreciated way—to tell the story of the last nine months. It is a story of heroism, innovation, and precise science, performed under unbelievable pressure.

Let’s not mince words: The United States and the world needs to appreciate the role of the pharmaceutical industry—the researchers, physicians, and business leaders—who are rescuing the world from COVID-19. It’s the medical breakthrough of our lifetime.

Instead of dwelling on why many in the media are ignoring this, let’s review some facts:

- Since the discovery of COVID-19, here is what scientists have accomplished: They identified a novel virus, unlocked and sequenced its genetic code, created new therapies to save lives, and developed multiple safe and effective vaccines using messenger RNA technology, a technology hopefully applicable to future vaccine development. Margaret Liu, MD, a member of the MJH Life Sciences COVID Coalition, called it a breakthrough for mRNA vaccines.
- The United States has two vaccines approved for emergency use, one from Pfizer/BioNTech and another from Moderna, and the AstraZeneca/Oxford vaccine has been approved for emergency use in the United Kingdom. In addition, there are 64 vaccines undergoing clinical trials at the moment, including 20 in phase 3 trials. In the United States and around the world, the pharmaceutical industry has answered the call and invested heavily in this effort.
- This was the fastest vaccine development program in history, and it’s not even close. David Pride, MD, a microbiologist at the University of California San Diego, estimates that vaccines typically take 10–15 years to develop. Until the COVID-19 pandemic, the fastest development timeline was four years, for the mumps vaccine.
- Many government systems moved quickly to lessen the burden of onerous regulations, and provide funding so that vaccines could be developed quickly, but with still rigorous standards. Perhaps it should be a lesson to all of us that regulation and innovation can be calibrated more effectively during “normal” times as industry races to develop new therapies for our world’s other pandemics—cancer, diabetes, heart diseases, and more.

The next step of the process—distribution of the vaccine—will be as challenging as the development phase, if not more so. But again, the pharmaceutical industry is rising to the occasion. Factories around the world are working in overdrive to produce hundreds of millions of vaccine doses.

Already, more than 15.4 million doses of vaccine have been distributed across the country, and more than 4.6 million people have received their first dose, according to CDC data. Many patients are already receiving their second dose.

While 15.4 million doses are impressive, some expected 20 million doses. But even that is moving the goal line a bit, as six months ago many observers didn’t think we’d get a vaccine until 2021.

Members of our COVID Coalition told us that the holidays slowed the rollout considerably. Nancy Messonnier, MD, a physician with the National Center for Immunization and Respiratory Diseases at the CDC, expects a rapid jump in administered vaccines during these first few days of 2021.

Every day, more people will be vaccinated. After health care workers and our most vulnerable citizens, other frontline workers will be next. Teachers will be vaccinated so our children can return to school. And soon, all Americans will be able to go to their doctor, or walk into a CVS or Walgreens, and receive the vaccine.

Remember, we did all this in nine months, with the help, dedication, and expertise of our pharmaceutical industry heroes. Next time you turn on the television and see negativity, turn it off and imagine instead where we will be nine months from now.

Michael J. Hennessy is the founder and Chairman of MJH Life Sciences.
With so many laboratory partners focused on the health and safety of their employees, our return to normal work schedules will no doubt include many new behaviors and norms. For over 35 years, Parker Gas Generators have saved millions of dollars by producing UHP quality gases in-house, all while eliminating potential points of viral introduction by reducing the need for regular outside deliveries.
Sign up today to access Restek’s years of chromatography knowledge at
www.restek.com/advantage

Peak GC Performance Ensured by the Proper Inlet Supplies, Maintained Properly

With Restek’s guidance and our comprehensive list of high-quality GC inlet supplies, performing proper GC inlet maintenance—to help maximize your uptime and ensure accurate, reproducible data—is easy as replacing a few key items. Download our guide or other chromatography tips at www.restek.com/advantage.