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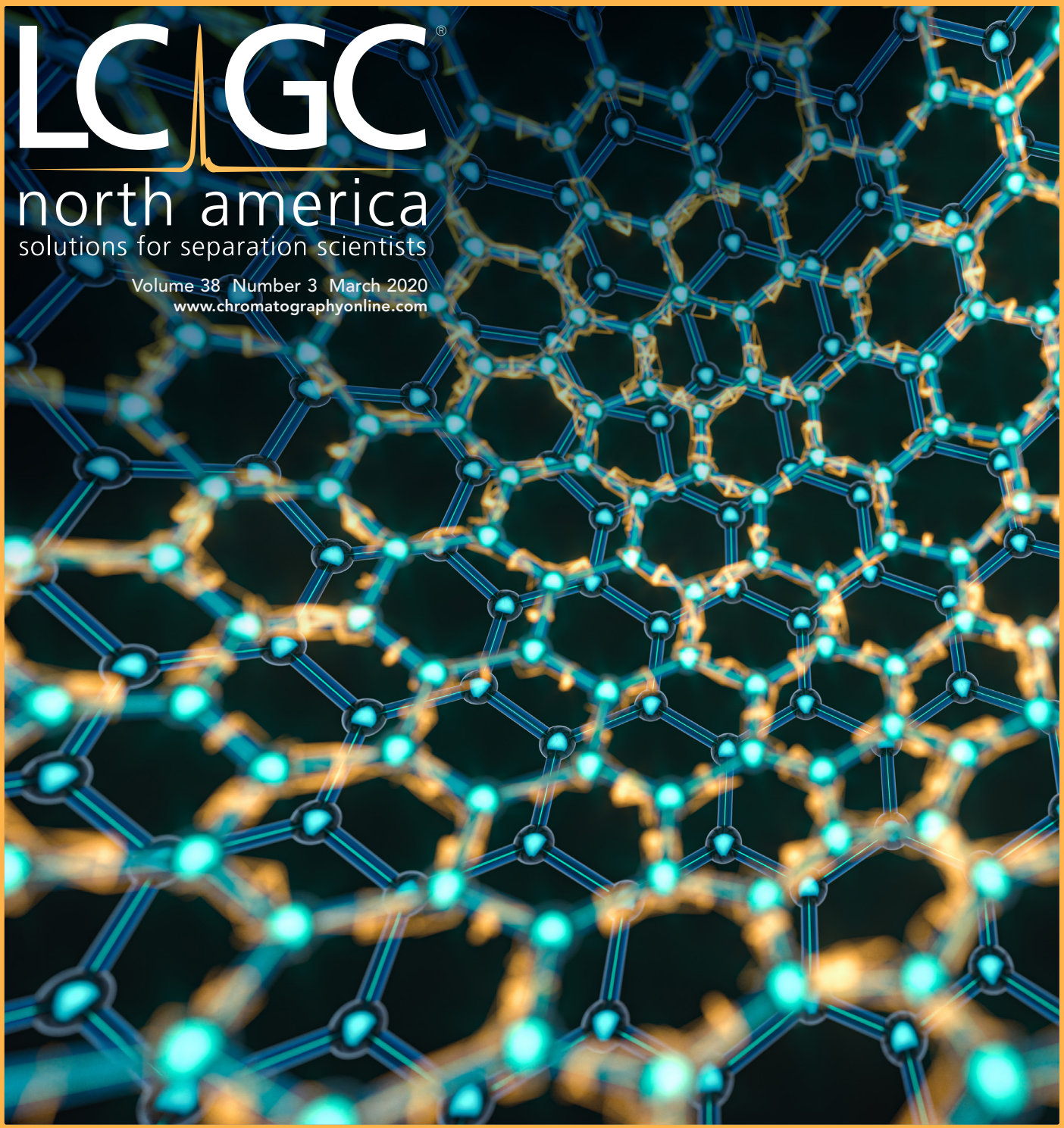

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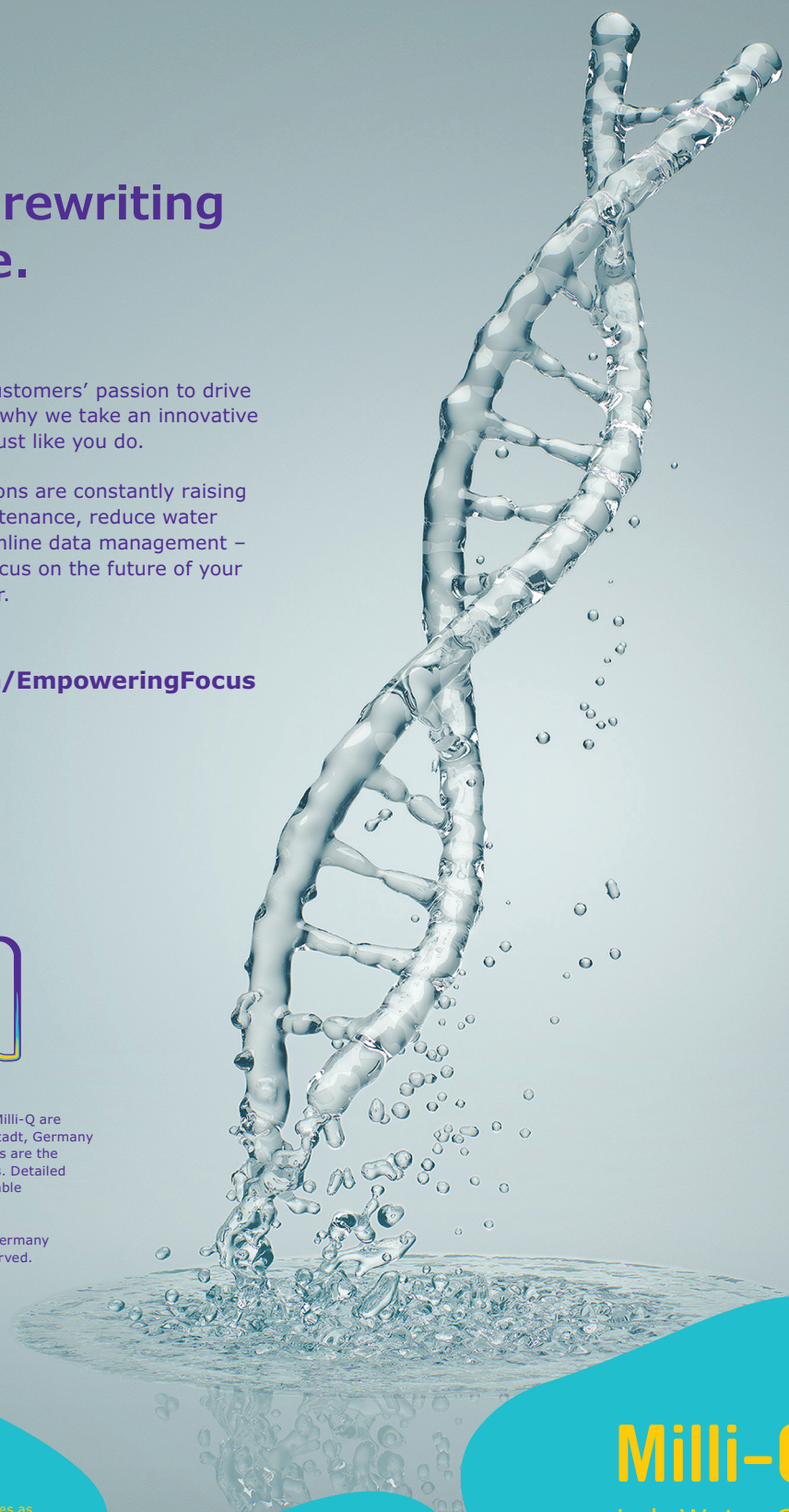

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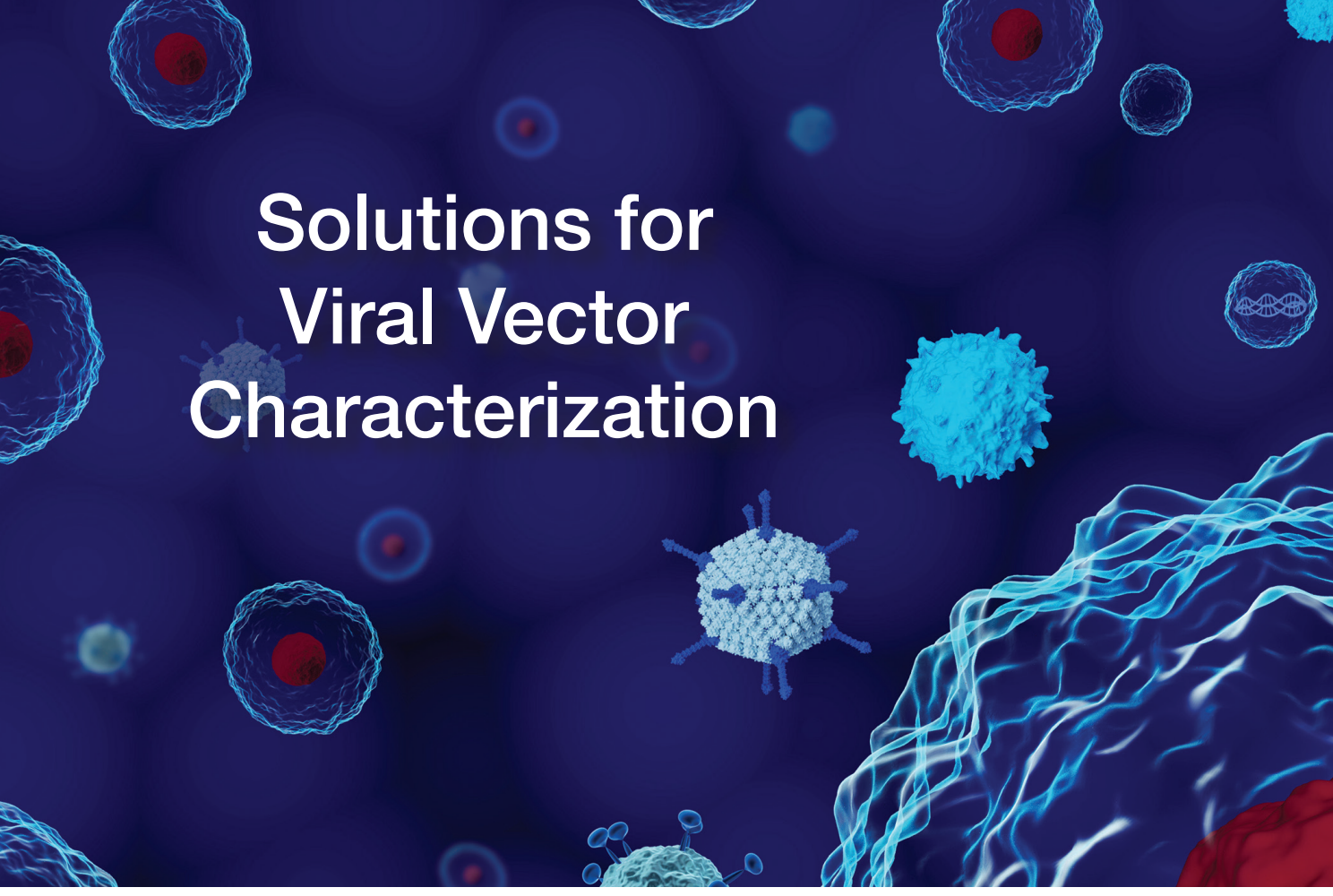

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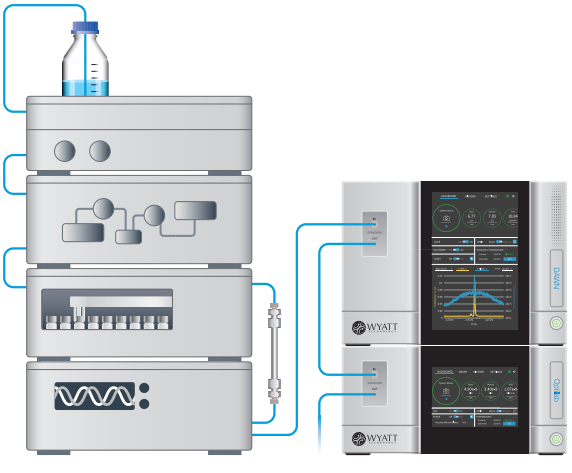

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PEAKS of Interest

Ron Majors Wins The Chromatography Forum of Delaware Valley Dal Nogare Award

Ron Majors is the 2020 winner of the Dal Nogare Award, which was presented this March at Pittcon, in Chicago. This award is given to an outstanding scientist in the field of chromatography. Awardees are selected on the basis of his or her contributions to the fundamental understanding of the chromatographic process.

Majors retired from Agilent Technologies, where he worked in sample preparation and column technology. He is a former *LCGC* columnist, writing both the "Column Watch" and "Sample Prep Perspectives" columns for *LCGC North America*. Currently a member of *LCGC's* editorial board, Majors has authored more than 150 publications in HPLC, GC, sample preparation, and surface chemistry.

He received his B.S. from California State University, Fresno, and his PhD from Purdue University. His PhD thesis was on molecular-imprinted phases for chromatography and sample preparation. Majors has served as the Chairman of HPLC '86 and Anabiotec '90, and on the Instrumentation Advisory Board of *Analytical Chemistry*.

Katelynn Perrault Wins Satinder Ahuja Award for Young Investigators in Separation Science

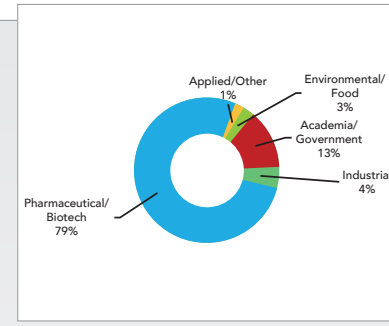
Katelynn A. Perrault, an assistant professor of forensic sciences and chemistry at Chaminade University of Honolulu in Hawaii, is the winner of the 2019 Satinder Ahuja Award for Young Investigators. The award recognizes and encourages outstanding contributions to the field of separation science by a young chemist or chemical engineer. It was presented to Perrault at Pittcon 2020 in Chicago this March. Perrault's work focuses on the use of multidimensional chromatography for odor analysis applications.

Perrault earned her PhD from the University of Technology Sydney in 2015 with a focus on forensic chemistry. Her doctoral studies took place at the University of Liège, Belgium, where she applied novel analytical approaches to challenging matrices in fields such as food science, archaeology, and forensic science. This research has allowed her to cultivate an international network of collaborators in academia, police, and government agencies.

Perrault is developing a career with synergistic approaches for novel research undergraduate education and science outreach. ■

CHROMATOGRAPHY MARKET PROFILE

Flash Chromatography



Flash chromatography market demand by industry for 2019.

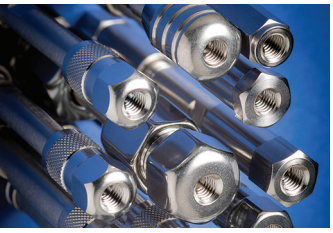
Flash chromatography is a purification technique that is designed for rapid separation by using air pressure as opposed to slow and inefficient gravity-fed chromatography. It differs from the conventional column technique by using slightly smaller silica gel particles and pressurized gas at 50–200 psi. Flash chromatography columns are typically prepacked plastic cartridges with silica gel particle sizes between 40–60 mm. Automated flash chromatography systems are composed of parts normally found on HPLC systems such as a gradient pump, injection ports, a UV detector, and a fraction collector to gather the eluent.

The earliest report of flash chromatography was by Clark Still over 40 years ago, but development was still in its infancy, as the newfound method was laborious and held the risk of the glass column shattering. However, by 1994, disposable plastic cartridges reduced preparation, improved reproducibility, and decreased separation time.

Flash chromatography is widely useful in the separation of closely related organic compounds. In the pharma industry, it can be used to purify various peptides, antibiotics, and related drug intermediates for drug discovery and development. It can also be used to fractionate natural products, such as tocopherols, alkaloids, xanthones, flavonoids, and cannabinoids.

The total market for flash chromatography was measured at around $150 million in 2019. In the past decade, the biopharma market has progressively expanded its use, accounting for more than three quarters of the demand. While solid growth is expected from flash columns and cartridges, demand from flash instrument systems are expected to be robust. Users continue to favor flash instruments coupled with advanced detectors such as evaporative light scattering detector (ELSD) and single quad mass spectrometers (SQMS). TDA estimates flash-ELSD systems captured the highest share of the market, but flash-SQMS systems will likely take over the top spot within the next few years.

Market size and growth estimates were adopted from TDA's *Industry Data*, a database of technology market profiles and benchmarks covering laboratory and process analytical instrumentation that are updated quarterly. It also includes data from the *2020 Instrument Industry Outlook* report from independent market research firm TDA. For more information, contact Glenn Cudiamat, general manager, at +1 (310) 871-3768 or glenn.cudiamat@tdaresearch.com.



LC TROUBLESHOOTING

Troubleshooting LC Separations of Biomolecules, Part I: Background, and the Meaning of *Inertness*

How do bioinert and biocompatible LC systems and columns improve separations of biomolecules? How do I know when these systems are required for my separation?

Jordy J. Hsiao, Gregory O. Staples, and Dwight R. Stoll

We are witnessing tremendous growth in the life science and biopharmaceutical research areas, and separation scientists have risen to the myriad challenges that have presented themselves in these industries. Increasingly, analytical workflows are being designed with special measures taken to improve separation performance for biomolecules. Thus, terms like **bioinert**, **biocompatible**, **biocolumn**, *and* **large molecule liquid chromatography LC (bio-LC)** *have arrived on the liquid chromatography scene. For this month's installment of "LC Troubleshooting," I've asked two my research collaborators, Jordy Hsiao and Greg Staples, to join me to address these topics from the point of view of troubleshooting. In this first installment, we'll dive into some of the background behind these terms and their relevance to bioseparations, and how examples of paying attention to specific characteristics of LC systems and separation conditions can have dramatic effects on the separation of biomolecules.*

Dwight Stoll

Background

Several years ago, some of our own work focused on using hydrophilic interaction chromatography (HILIC) to separate biologically relevant small molecules. The preliminary results were quite exciting, as we were able to achieve good separations of underivatized amino acids. However, we had difficulty detecting and obtaining good peak shapes for acidic metabolites containing phosphate groups or multiple carboxylate groups. These compounds are important because they are involved in many crucial cellular pathways (for example, the tricarboxylic acid cycle). At that time, we embarked on a journey to systematically study these effects, with the hope that a more detailed understanding of the observations would lead to better separations in the long run. It turned out to be a long trip, and the path is one that many analytical scientists have travelled. We began assembling other reports of deleterious interactions between biomolecules and high performance liquid chromatography (HPLC) systems, cataloging problems with phosphopeptides (1,2), phosphorylated glycans (3), monoclonal antibodies (4), and pharmaceutical compounds (5). From this survey, we observed that these problems were reported to occur on a variety of stationary phases in addition to HILIC, including reversed-phase, ion exchange, size-exclusion chromatography (SEC), and others. We were eventually able to develop robust, high performing separations of acidic metabolites. We hope some of the general lessons we've learned through these experiences can be leveraged by others to save significant time in tackling tricky biomolecule separations.

How to Spot Problems in Bioseparations

Poor performance for separations of biomolecules can manifest in a number of ways. Biomolecules as a class of analytes comprise a vast array of molecules that vary in terms of both physico-chemical properties and size, and it's important to note that chromatography problems can arise for species that are either small (such as metabolites, glycans, peptides), or large (such as proteins).

1. Peak tailing: Often, analytes are eluted with undesirable peak tailing, which can make quantitation difficult. Sometimes these peak shapes gradually improve over the course of days or weeks, while other times their poor shapes can remain the same or continue to worsen. A good example of such peak tailing for a biomolecule is the separation of the protein cytochrome C using SEC. Figures 1a and 1b show such a separation on a column housed in stainless steel (SS) versus a column housed in polyether ether ketone (PEEK). The tailing factor of the protein on the SS column is significantly worse than on the PEEK column.

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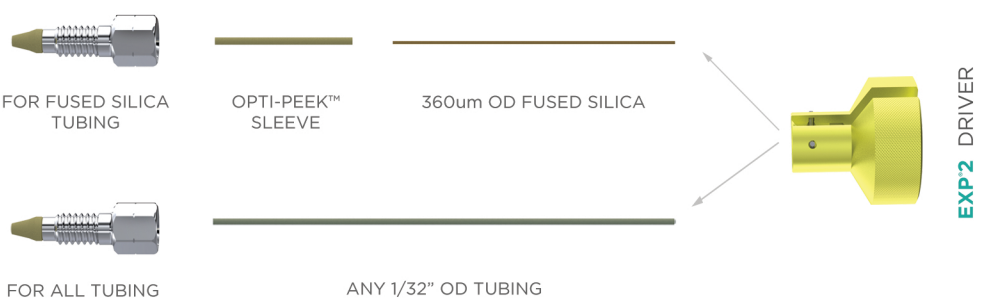

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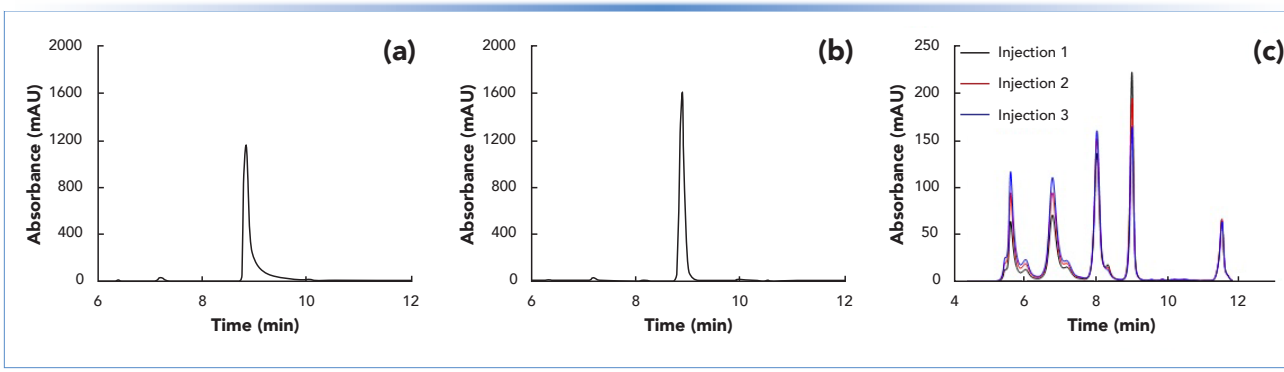

FIGURE 1: Separation of cytochrome C protein on SEC columns in (a) SS or (b) PEEK column hardware. Panel (c) shows how the area of some peaks can increase when a protein sample is injected repeatedly on a SEC column with metal hardware.

2. Drifting peak area: Another situation that is commonly observed is an increase in peak area upon repeated injections of a sample, which can apply to individual or groups of analytes in the sample. An example of this is shown in Figure 1c. The peak areas can eventually stabilize, but, in some cases, the stabilization can be very slow and require many injections. Sometimes, it's impossible to be sure that the separation has stabilized at all.
3. No observable elution: Perhaps most concerning are situations where an analyte is not detected at all because it is stuck somewhere inside the instrument between the sample vial and the detector.

We'll address some of the countermeasures for the situations listed above in future installments of "LC Troubleshooting." In the meantime, you might be wondering about the root cause of these problems. There are unfortunately many culprits, but a common issue involves the interaction between biomolecules and metals in HPLC systems, the most common of which is the iron in stainless steel. There are several ways that biomolecules can interact with iron, in a physico-chemical sense. For example, the phosphate groups in metabolites, glycans, and phosphopeptides can act as Lewis bases that tend to interact strongly with Lewis acids, such as iron. Moreover, molecules that contain multiple acidic functional groups (for example, malate, adenosine diphosphate) can bind metals with extraordinary affinities as a result of interaction geometries that are particularly favorable (for example, this is what makes the interaction between ethylenediaminetetraacetic acid [EDTA] and metals so strong). With proteins in particular, it is difficult to develop clear and robust rules about what will or will not interact strongly with metals, because there is the potential for so many amino acid sequence-specific effects, as well as a strong dependence of the interaction on secondary and tertiary structure of the protein.

Bioinert to the Rescue!

Let's return to the terms we called out earlier, *bioinert* and *biocompatible*. These terms are used interchangeably in the literature, and in the context of biomolecule separations describe products that are designed to reduce problematic interactions with biomolecules. This is primarily accomplished by using alternatives to SS in the flow

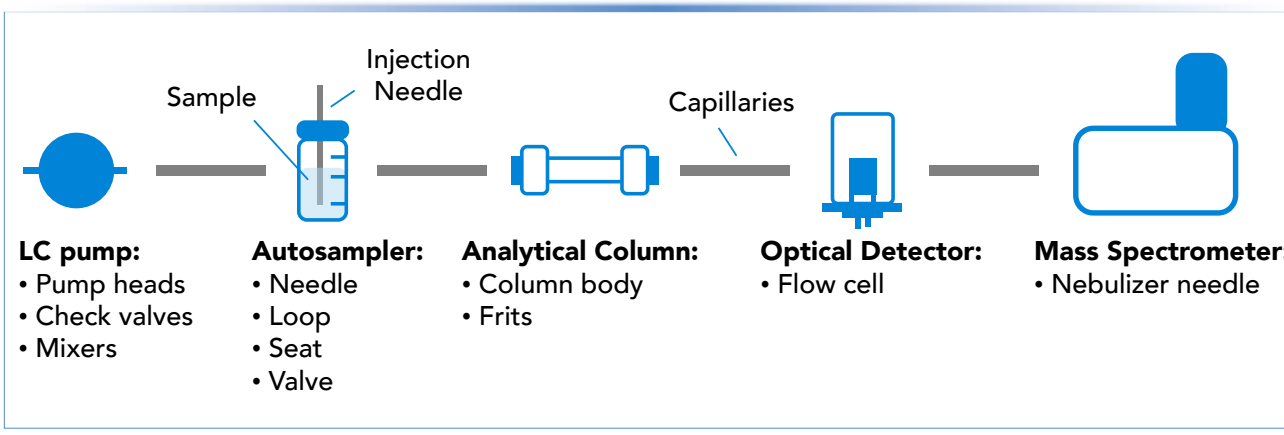

FIGURE 2: Typical LC–MS instrument setup. This simplified illustration includes the HPLC pump, autosampler, analytical column, detectors (with both an optical detector and a mass spectrometer illustrated here), and the connection capillaries.

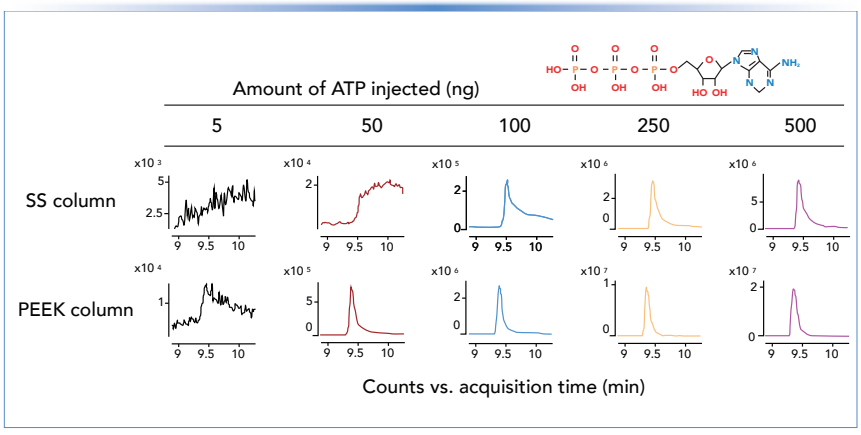

FIGURE 3: Column hardware comparison experiment for the analysis of phosphorylated metabolites using LC–MS. Chromatographic performance of ATP with HILIC media packed in either SS or PEEK column hardware.

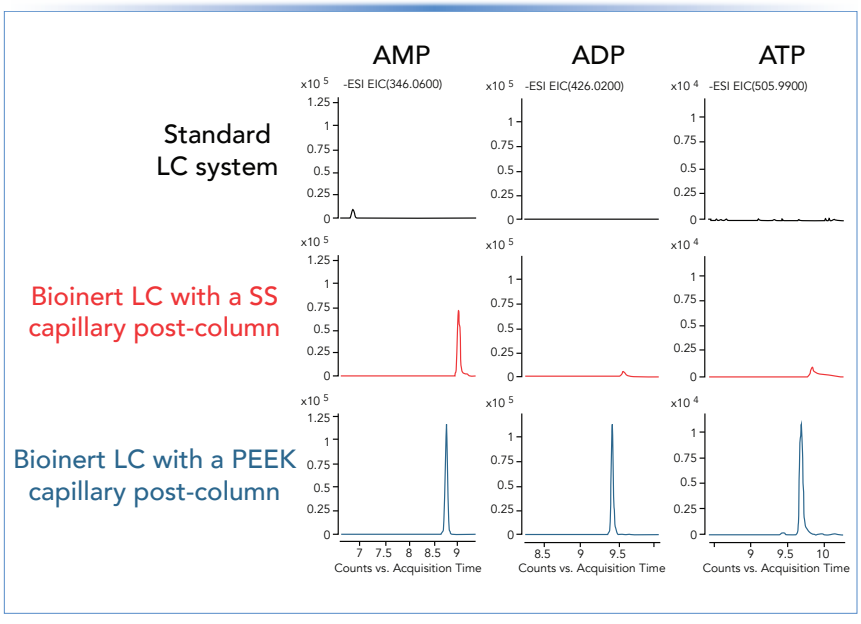

FIGURE 4: Sensitivity for HILIC-LC–MS improves as individual metal components are removed from the analyte flow path.

path of the separation. Some examples of materials that can be found in bioinert products are PEEK, alloys of titanium, ceramic, and MP35N (an alloy whose main components are Ni, Co, Cr, and Mo). Now that we are thinking of each of these different materials, it's a good time to reflect on the many components of a UHPLC–mass spectrometry (MS) system, and consider how they relate to the interaction of biomolecules with metals. Figure 2 shows a block diagram of a typical LC–MS system. Metal containing components can generally be divided into two categories: those that physically contact the sample, and those that do not. The former includes things like the sample vial, the autosampler needle and loop, the HPLC column, any optical flow cells, transfer capillaries, and, in the case of MS, the nebulizer needle. The latter includes solvent bottles, pump heads, and mixers. It's also worth noting that samples of biological origin may themselves contain metal ions, either inherently or by design.

Column Technologies for Biomolecules

Analyte interaction with HPLC columns has been the subject of much attention from column manufacturers. Analytes can potentially interact with the walls of the column tubes, as well as the inlet and outlet frits that hold the stationary phase in place. Despite their small size, column frits can actually have surface areas in the same order of magnitude as the column walls. In some of our own work, we compared SS and PEEK HILIC columns for adenosine triphosphate (ATP) analysis. In the experiment shown in Figure 3, low amounts of ATP injected onto SS columns were not detectable. Only with larger injection amounts (above 250 ng) does the peak shape and intensity for ATP improve. This effect is mitigated using PEEK-lined columns, and more importantly, the sensitivity and peak shape improved significantly using PEEK hardware. Another similar comparison study has demonstrated better recovery and peak shape for phosphorylated N-glycans when using PEEK-lined column hardware (3). Alternatives to SS column hardware are available from column vendors, and comprise a variety of different bioinert materials in addition to PEEK (for example, titanium, glass).

Biocompatible HPLC Instrument Components

Let's move on to the components of the HPLC–MS system that are relevant to metal–analyte interaction, keeping in mind the components in the flow path in Figure 2. It's intuitive that there can be problems when the sample is in contact with a metal surface, but what about metal surfaces which the sample does not contact, like the pump heads? Indeed, these surfaces can be problematic when they leach (often variable) levels of metal ions into the mobile phase (6). This phenomenon can cause some serious complications, especially if leached metal ions accumulate on the column stationary phase, rendering the use of bioinert column hardware ineffective. Given that an HPLC–MS system has many potential sites of metal introduction or interaction, the effect on the performance of a biomolecule separation is cumulative. To illustrate this, consider the data in Figure 4, which examines separations of the metal sensitive analytes AMP, ADP, and ATP as markers for HILIC-LC–MS performance. We initially started with an LC system composed of a SS pump head, an autosampler with a SS injection needle, and SS capillaries connected to a quadrupole time-of-flight (QTOF) mass spectrometer (top row). Next, we changed the HPLC system to one with a titanium pump head, ceramic injection needle, and PEEK-lined connection capillaries, but with a single SS capillary post-column (middle row). In the last step, the post-column capillary was changed from SS to PEEK (bottom row). These results demonstrated that the signal intensities significantly increased as individual SS components were removed from the sample flow path. More notably, even a single SS capillary can negatively impact the detection limits for phosphorylated metabolites (compare middle and bottom rows).

Summary

In this first installment of "LC Troubleshooting," we've worked to highlight some of the problems you may encounter when developing a method for the analysis of biomolecules that are related to deleterious interactions between the analytes and the LC system. Such problems often show up in the form of lower than expected sensitivity, poor peak shape, and poor reproducibility. When working with analytes that have the potential to interact strongly with metals, it's useful to know what to look for and how you can assess the overall performance of your system. In the best case, such separations should be developed using systems that limit or eliminate SS components. When this is not possible, there are other options available, including passivation and mobile phase additives, and we'll discuss details of these approaches in a future installment of "LC Troubleshooting." Arming yourself with knowledge about the sources of metals in LC systems and the mechanism of interaction of biomolecules with these metals can be helpful in any troubleshooting you do, and facilitate the development of robust methods for accurate biomolecule determinations.

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ABOUT THE COLUMN EDITOR

Dwight R. Stoll is the editor of "LC Troubleshooting." Stoll is a professor and co-chair of chemistry at Gustavus Adolphus College in St. Peter, Minnesota. His primary research focus is on the development of 2D-LC for both targeted and untargeted analyses. He has authored or coauthored more than 60 peer-reviewed publications and four book chapters in separation science and more than 100 conference presentations. He is also a member of *LCGC*'s editorial advisory board. Direct correspondence to: LCGCedit@mmhgroup.com

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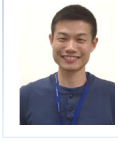
Gregory Staples leads an R&D team focused on creating and developing separation, reagents, sample preparation, and analysis solutions for biomolecules at Agilent Technologies, in Santa Clara, California.



Jordy Hsiao is an R&D scientist at Agilent Technologies, in Santa Clara, California.

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GC CONNECTIONS

Go With the Flow: Thinking About Carrier Gas Flow in GC

Mobile phase flow is critically important, and must be carefully controlled. In this installment of "GC Connections," we discuss carrier gas flow and its importance in successful gas chromatographic analysis. We will begin with a short review of fundamental theories showing why flow is important, and move into a discussion of how flow rate is measured and controlled on modern instruments. Finally, we will discuss the effect of flow-related parameters, including carrier gas choice, the difference between constant pressure and constant flow modes, and some new thinking about flow rate optimization. We conclude with some takeaways that should assist gas chromatographers with effective carrier gas management.

Sean P. McCann, Hetal Rana, Brittany A. Handzo, and Nicholas H. Snow

When a sample is injected into a column in any mode of chromatography, its molecules spend time in one of two phases, the stationary phase or the mobile phase, as illustrated in the very familiar equation 1.

$$t_R = t'_R + t_M \quad [1]$$

As we all know, t_R represents the total retention time, t'_R represents the adjusted retention time, or the time spent sorbed (not moving) in the stationary phase, and t_M represents the holdup time, the time spent moving in the mobile phase, also defined as the time required for an unretained substance to traverse the column. The carrier gas flow rate obviously has a very strong impact on t_M. We are also very familiar with equation 2, which represents the retention factor.

$$k = \frac{t'_R}{t_M} \quad [2]$$

Most experts in method development will suggest that the best separations occur when k is between about 2 and about 10. The lower limit allows enough total time in the column for effective separation. The upper limit comes from a diminishing return for better resolution as retention times get very long. As k gets smaller, the importance of t_M (flow) becomes larger. At k = 2, the analyte spends one third of the retention time in the mobile phase. At k = 10, it spends about 9% (1/11) of the retention time in the mobile phase. In any case, precise flow control is important for retention times, retention factors, and all of the calculations that we base on them to be reproducible.

Equation 3 relates the gas holdup time to the average column volumetric flow rate.

$$\overline{F_c} = \frac{\pi r^2 L}{t_M} \quad [3]$$

The numerator of equation 3 represents the total volume of the column (think of it as a long skinny cylinder). Note that the stationary phase film thickness is not considered; it usually does not contribute much to the total volume inside the fused-silica tube. Also, note that this is an average flow rate. In contrast to high performance liquid chromatography (HPLC), in which the liquid mobile phase is essentially non-compressible, the gas mobile phase in gas chromatography (GC) is compressible. According to Boyle's Law, pressure and volume are inversely proportional. As the gas moves from the higher- pressure column head to the lower pressure outlet, it expands, increasing in volume. Thus, the volumetric flow rate calculated by equation 3 is the average volumetric flow rate and it is usually expressed in units of cm^3/min or mL/min.

A related term, average linear gas velocity, which is simply the average speed (usually expressed in cm/s) of the gas flowing through the column, is given in equation 4.

$$\bar{u} = \frac{L}{t_M} \quad [4]$$

As we saw above, the gas is expanding as it moves along the column. Since the column constricts the gas flow, the velocity of the gas must also increase as it moves down the column. The average linear gas velocity is most commonly used to examine the effect of flow on peak width and the construction of "van Deemter" plots, the classical method for optimizing the flow rate, discussed below. In a previous "GC Connections" column, Hinshaw provided a detailed description of the relationships between carrier gas flow and velocity (1).

There are several variables that determine the gas holdup time and some complexity in how gas molecules move through the column. Blumberg has provided a detailed description and equations describing the fundamental basis of the gas holdup time (2). The relationships involved are surprisingly complex, but can be summarized by stating that the gas holdup time is a function of column dimensions (length and inside diameter), the inlet and outlet pressures and the viscosity (resistance to flow) of the carrier gas. The viscosity of a gas is a function of temperature, and increases as temperature increases. This is the opposite behavior seen with liquids, where the viscosity decreases with increasing temperature. Some instruments, data systems, and computer simulation programs use equations based on the these relationships to determine gas holdup times from the known variables, lessening the need to physically measure t_M. Finally, remember that the calculated flow rate and linear gas velocity are average values, and they assume isothermal operation throughout the run.

Measuring the Gas Holdup Time

In a recent "GC Connections" column, Hinshaw suggested keeping a manual syringe and a butane lighter handy as part of the supplies kit for any gas chromatograph (3). Injecting butane from a lighter serves two very important purposes. First, butane is not retained on most capillary columns at most temperatures used in GC, so it can be used as the analyte to directly measure the gas holdup time, t_M. Second, the shape of the butane peak, injected following a column change or other maintenance, provides a confirmation that the column is connected properly and the connections are leak-free. If the butane peak exhibits tailing or a poor peak shape, then there is likely a leak or a problem with the connections. Beware that butane may be retained on some thick film columns, so methane is preferred when using them.

The technique of injecting butane was developed in times when most GC was performed on packed column instruments, with manual injection and a manually operated chart recorder as the data system. Classically, injecting butane or methane (we used Bunsen burner gas collected from the laboratory gas jets or in a small lecture bottle) requires training that may not be commonly known today. Also, if using an autosampler, the autosampler may need to be dismounted from the instrument and a manual method set up in the data system. This makes injecting butane, while it may be a necessity, potentially problematic. Interestingly, this problem was addressed as early as 1959 by Peterson and Hirsch, who developed an equation for determining the holdup time from the retention times of three homologous fatty acid methyl esters (4). As recently as 2013, Wu and associates provided an excellent review and comparison of many techniques that have been used to determine gas holdup times. They correctly pointed out that all substances are retained to some degree, and that variations in gas holdup time measurements

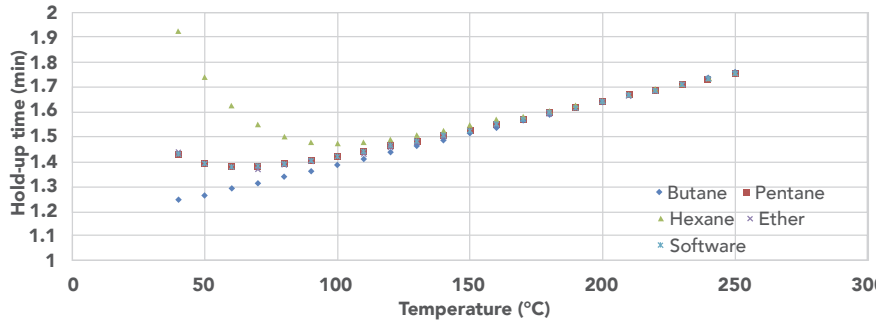

FIGURE 1: Gas hold-up time versus temperature for several analytes. The figure shows data plotted for butane, pentane, hexane, ether, and software.

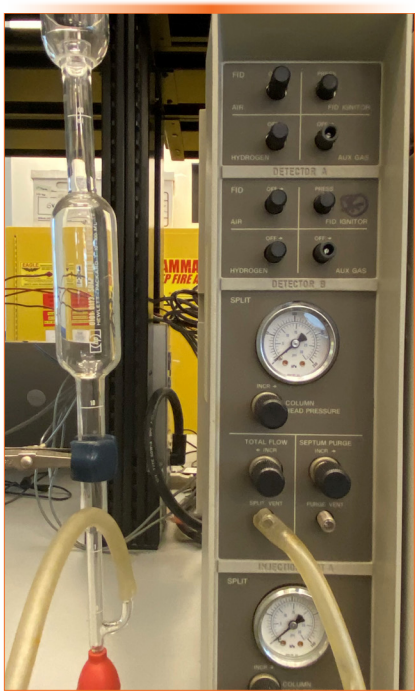

FIGURE 2: Soap film flowmeter connected to the split vent of a classical pneumatic gas chromatograph.

may lead to variations in calculations that are based on them, including retention factors, partition coefficients, and retention indexes (5).

Figure 1 shows the retention time for several analytes injected neat, measured on a common capillary column under isothermal conditions at temperatures ranging from 40 to 250 °C. Butane was injected by drawing about 5 µL of vapor from a lighter (just hold down the handle on the lighter while inserting the syringe needle and moving the plunger). The liquids (pentane, hexane, and ether) were injected by placing about 100 µL of each into a standard 2 mL vial, sealing the vial and injecting 5 µL of the headspace vapor, using a standard syringe and the autosampler. The t_M values were also calculated using equation 5 and retention factors for tetradecane provided by the data system.

$$t_M = \frac{t_R}{1+k} \quad [5]$$

With all variables other than temperature held constant, the retention time of an unretained substance, the gas holdup time, should increase with temperature, related to the carrier gas viscosity. Viscosity of a gas is related to temperature by an exponential relationship that can approximate to a linear relationship in the temperature ranges used in GC (6). In short, if a substance is unretained, the relationship between retention time and temperature should be linear with a positive slope. In Figure 1, this is seen at all temperatures for butane, and at temperatures above about 150 °C for pentane and diethyl ether. The calculated value also almost exactly overlapped with the butane results over the entire range, indicating agreement between the measured and calculated values. As the temperature decreases, however, the pentane, hexane, and diethyl ether become clearly retained, eventually exhibiting the expected behavior with the retention time becoming longer as the temperature is lowered. When measuring holdup time, take care to ensure a symmetrical peak; if the peak is asymmetrical, the retention time will not be accurate.

Measuring Flow Rates

As we have seen above, the average carrier gas flow rate can be calculated by measuring the gas holdup time, t_M. However, the average flow rate is not, by itself, a very useful measure for most practical situations. For example, the split ratio, calculated as the split vent flow divided by the column flow, requires the column flow to be measured at the inlet to be accurate. In packed-column GC, where column flow rates were much larger than with today's capillary columns, the average and inlet flow rates are calculated from the measured outlet flow rate by applying appropriate correction factors (7).

Also in packed column GC, the column flow rate is most easily measured by attaching a soap-film flowmeter, as seen in Figure 2, to the column outlet. These are inexpensive, and are operated by generating a soap bubble from the bulb at the bottom, and measuring the time required for 1, 10, or 100 mL of gas to flow from the column. This time is converted to a flow rate in mL/min. Soap film flow meters can also to measure split vent flow, as seen in Figure 2, and detector gas flow rates in capillary column systems. Today, fully electronic flow meters are available from a number of vendors. An electronic flow meter and an electronic leak detector are both must-haves in a modern GC laboratory.

Capillary column flow rates are more difficult to measure than packed column rates, as they are generally much lower, typically on the order of 1–2 mL/min and the measurement is often needed when the system is running. It is not wise to place the inlet of a flowmeter into a heated and running flame ionization detector (FID)! It is impos-

sible to directly measure the outlet flow rate on an mass selective detector (MSD). Today's electronically controlled systems calculate column flow rates automatically, using the known column dimensions, choice of carrier gas, inlet and outlet pressure, and temperature and relationships, such as those discussed in this article. Users should be certain that the column dimensions are accurately entered into the data system, and should double check that they are correct if the system enters them automatically.

Constant Pressure vs. Constant Flow

Prior to the advent of the solid-state electronic pneumatic controls, used in most new GC instruments today, nearly all commercially available GC systems for both packed and capillary columns operated at constant column head pressure. For packed column systems, this allowed very simple pneumatics, essentially just a flow controller and a pressure regulator. Today's capillary column systems with electronically controlled pneumatics can operate in two modes: constant pressure and constant flow. Shortly after the advent of electronic control of the pneumatic systems in gas chromatographs, Blumberg, Wilson, and Klee compared column performance characteristics in constant pressure and constant flow with temperature programming (8). They noted that there is little difference in overall column performance between the two options, so other considerations determine the choice of mode.

In constant pressure operation, the column head pressure is constant throughout the run. This is the more common operating condition and is seen is most of the GC literature, especially papers and methods more than 10 years old and in many compendial methods. In a temperature-programmed run the carrier gas viscosity will increase with the temperature, causing a decrease in the flow rate as the run proceeds. This decrease is partly offset by an increase in volume (Charles' Law) as the temperature increases. Overall, however, in most cases, the volumetric flow rate will decrease as temperature increases in a temperature-programmed run.

In constant flow operation, the electronic flow controller increases the head pressure as the temperature is increased, to maintain a constant flow rate. Figure 3 shows chromatograms of the same sample, run under the same temperature program and same initial column head pressure. The chromatogram on top was run in constant pressure mode and the chromatogram on the bottom was run under constant flow mode. Note that the retention times are slightly shorter in the constant flow chromatogram, however, in agreement with Blumberg and colleagues, the peak widths and spacing are about the same. Not surprisingly, the effect on retention time is greater for the later eluting peaks.

An article on ChromAcademy, *LCGC*'s learning platform, describes fundamentals of setting up gas flows in GC and discusses constant pressure and constant flow (9). Some ideas to consider when choosing which mode to use include:

- Detector performance. Mass-sensitive detectors such as FID and MSD may perform better with constant flow.
- Translating methods. Constant pressure may be preferred when a method is translated between differing column dimensions or stationary phases.

TABLE I: Characteristics of common carrier gases

Characteristic	Hydrogen	Helium	Nitrogen
Cost	$$-$$$	$$	$
Safety	+	+++	+++
Inertness	++	+++	+++
Speed	+++	++	+
Resolution	+++	++	++
Use with FID	+++	+++	+++
Use with MSD	+++	+++	No
Molar Mass (da)	2	4	28
Viscosity at 60 °C (µP)	95	211	190

Key: (+ = good; ++ = better; +++ = best)

- Speed. Constant flow methods will often be slightly faster than constant pressure.
- Lower temperature. Later eluted peaks will be eluted at lower temperature in constant flow mode.
- Electronic pneumatic control-based techniques for ensuring system-to-system and laboratory-to-laboratory reproducibility, such as retention time locking and method translation often require constant flow mode.

When optimizing, adapting, or attempting to repeat a method from the literature, it is important to note which mode was used for the original method, and either ensure that you are using the same mode, or be prepared to perform additional optimizing.

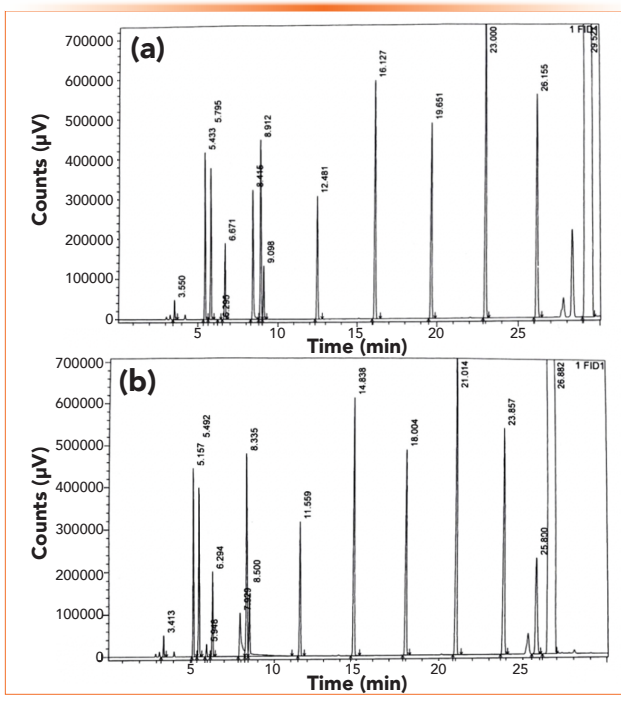

FIGURE 3: Constant pressure and constant flow chromatograms of a column test mixture. (a) constant Pressure, 10 psi initial pressure. (b) constant flow, 2.35 mL/min. Column: DB-624, 30 m x 0.32 mm x 1.8-µm. Temperature program: 40 °C with no initial hold time, 5 °C/min to 250 °C, final hold time for 2 min. Injection: split 50:1. Detector FID, 250 °C. Sample: mixture of alkanes and McReynolds constant test probes.

Choosing a Carrier Gas

Carrier gases for GC must meet several requirements to be useful. The carrier gas must be inert, safe, dry, highly pure, suitable for the detector, and inexpensive. Helium, which readily meets all of these characteristics, has been the carrier gas of choice for most capillary GC work, especially gas chromatography–mass spectrometry (GC–MS), for decades, used in over 90% of recent research articles describing GC methods. Recently, however, several incidents of regional and global shortages have caused helium to be expensive or unavailable at times. As helium becomes less available, hydrogen and nitrogen emerge as leading alternatives, because they meet all of the necessary characteristics. Table I provides a brief comparison of the three gases.

As seen in Table I, each gas has benefits and difficulties. Helium has been the gas of choice, because with moderate cost, it is highly pure, inert, and fast, gives high resolution, and is compatible with nearly all detectors, including mass spectrometers. With the rising cost of helium, many experts are recommending changing to hydrogen, which, given its lower molecular weight and viscosity than helium, can generally provide faster separations with similar or improved resolution. If obtained from cylinders, hydrogen is

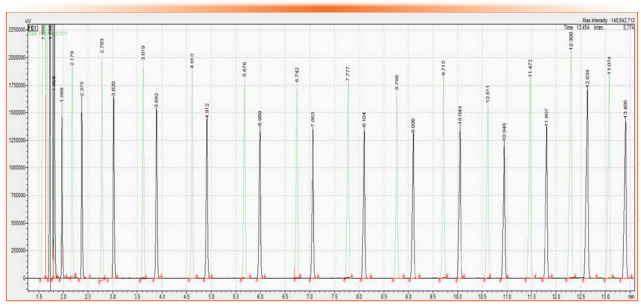

FIGURE 4: Overlaid chromatograms of an n-alkane mixture (C_6-C_{20}) using nitrogen (green plot) and helium (black plot). Column: 30 m x 0.25 mm ID x 0.25-µm, 5% phenyl polydimethylsiloxane. Temperature program: 70–250 °C at 13 °C/min. Inlet: split 50:1, 15 psi head pressure. Detector FID, 250 °C.

also moderately expensive, offers better resolution, and is often the carrier gas of choice when extremely narrow bore columns (0.1 mm inside diameter) are used, and for fast GC. Many laboratories are now using hydrogen generators, which offer high purity hydrogen, but with high up-front capital cost, ongoing maintenance, and potential downtime if there is a failure of the generator. If you consider using hydrogen, be sure to consult with your instrument manufacturer, because modifications or adjustments may be needed in order to prevent hydrogen form leaking into the laboratory.

Most experts do not recommend nitrogen for capillary column work. Although the cost is low, it is compatible with most detectors, and the viscosity is similar to helium, the high molecular weight leads to lower resolution through increased diffusion rates for analyte molecules in the gas phase. Furthermore, nitrogen is detected within the mass range for most benchtop mass spectrometers, so it is not generally compatible with MS detection. In December 2018, a helium shortage struck our facility, and for several months we were not able to purchase helium cylinders at any price. Lacking enough capacity in our hydrogen generators to run carrier gas as well as FID gas, we decided to switch to nitrogen and take our chances. Figure 4 shows overlaid FID chromatograms of a simple (C_6-C_{20} n-alkanes) hydrocarbon mixture, using helium (black) and nitrogen (green) with a simple linear temperature program, a split injection, and a common capillary column operated in constant pressure mode.

As seen in Figure 4, the two chromatograms are remarkably similar, with the main difference being slightly shorter retention times for nitrogen, due to the lower viscosity of nitrogen versus helium, as seen in Table I. The peak widths and separation numbers (a measure of the spacing between the peaks) are nearly identical. This is not the result that most chromatographers would expect; we would have expected the nitrogen separation to exhibit significantly poorer performance. Remember that most of our common understanding about carrier gas flow and characteristics is based on isothermal analysis. Changing the temperature as the run proceeds impacts nearly every physical parameter that affects transport of molecules along the column. We addressed the overall peak widths seen in both chromatograms in a previous "GC Connections" column (10). In short, Figure 4 leads to the recommendation that, if using helium is becoming problematic, nitrogen should be considered, as it is very abundant, inexpensive to obtain, may give adequate separation performance, and will not damage the instrumentation if it does not work. With more helium shortages looming, there is already a lot of discussion and advertising around alternatives. When choosing an alternative to helium, chromatographers should carefully evaluate both hydrogen and nitrogen, and make the best choice for their own laboratory.

New Directions

Classically, most gas chromatographers optimize the flow rate for a separation by trial and error, by making a Van Deemter or Golay plot of the height equivalent to a theoretical plate (*H*) vs. the average mobile phase velocity or the average flow rate, or by doing nothing and simply setting a column head pressure they have "always used." Trial and error and doing nothing are obvious approaches and numerous references that discuss making van Deemter plots are available elsewhere (11). All three of these approaches have limitations; for trial and error and doing nothing, the limitations are obvious. Van Deemter and Golay plots have been heavily used for flow optimization since the early days of GC, and therein lies the limitation. The theory, while illustrative, was developed for the limited conditions of the inlet and outlet pressure ratio near unity and constant temperature (12,13). Quoting Golay, the equations "are applicable to columns of uniform cross-sections in which the

input to exit pressure ratio is near unity." This means that the commonly used Golay equation is only applicable in cases where the pressure drop between the column inlet and outlet is very small. In today's capillary GC, this places a potentially severe limitation on the actual utility of the plots, given the common use of small-diameter columns and large pressure drops. It is generally not applicable to larger pressure drops and vacuum outlet detectors, such as MSD, common in today's capillary GC. The Golay equation is also not applicable at all to temperature programming. In a book and a book chapter, Blumberg addresses this problem in detail, but a simplified optimization process to replace van Deemter and Golay plots is still eluding most chromatographers (14,15). Be careful when using classical van Deemter or Golay equation plots to select an optimum flow rate; they may not be applicable to your situation.

Conclusions

Although measuring the carrier gas flow rate in GC is very straightforward, and optimizing it may be as simple as a trial-and-error approach, the underlying principles behind how the carrier gas flows through the column, and how this affects retention time and peak broadening, are not so simple and have been under discussion since the inception of GC. In thinking about carrier gas flow and gas holdup time, some lessons and takeaways become apparent:

- Given that the gas holdup time may be up to half of the time an analyte spends in the column, precise flow control is critical in capillary GC.
- Calculated flow rate and linear gas velocity are average values, and they assume isothermal operation throughout the run.
- When measuring gas holdup time (t_M), take care to ensure a symmetrical peak; if the peak is asymmetrical, the retention time will not be accurate, and the asymmetry indicates a problem with the inlet or gas flows.
- An electronic flow meter and an electronic leak detector are both must-haves in a modern GC laboratory.
- Be certain that the column dimensions are accurately entered into the data system, or double check that they are correct if the system enters them automatically.
- When optimizing, adapting, or attempting to repeat a method from the literature, note which mode (constant pressure or constant flow) was used for the original method, and either ensure that you are using the same mode, or be prepared to perform additional optimizing.
- When choosing an alternative to helium, chromatographers should carefully evaluate both hydrogen and nitrogen, and make the best choice for their own laboratories.
- If you consider using hydrogen, be sure to consult with your instrument manufacturer, as modifications or adjustments may be needed in order to prevent hydrogen form leaking into the laboratory.
- Be careful in using classical van Deemter or Golay equation plots to select an optimum flow rate; they may not be applicable to your situation.

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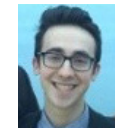

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SAMPLE PREP PERSPECTIVES

Sample Preparation in the Rearview Mirror: Looking Back on 2019

This month's installment of "Sample Prep Perspectives" is the first of two looking the state of sample preparation in 2019; in May, we present our annual review of new product introductions in the past year. For this month's column, we asked several thought leaders in the field their opinion on the most influential journal articles on chromatographic sample preparation. The results present a forecast of emerging technologies that may impact the future of the field.

Douglas E. Raynie

During the week before the new year, electronic and print media are inundated with lists of the top happenings in the previous twelve months. In that spirit, I reached out to several thought leaders in the field (see Table I) to discuss what they believed to be the most influential paper in the area of chromatographic sample preparation published in 2019, with a brief description of why, in their opinion, the article was impactful. The results cover a variety of applications and reviews of emerging techniques that may soon become more common in the sample preparation arsenal. Other than the common theme of chromatographic sample preparation, the only unifying theme was the comments on the difficulty of this task.

Electromembrane Extraction

Norwegian researcher Stig Pedersen-Bjergaard is arguably the leading proponent of electromembrane extraction (EME), and selected an article in this area (1). He notes that EME, or electrophoresis across an oil membrane, is becoming of increasing importance. Publication of new manuscripts is vital to understand where the EME principle fits relative to existing approaches. The unique feature presented in this article is the use of three-dimensional (3D) printing for fabrication of the EME device. This device included a conductive polylactic acid filament, and accommodated a 1 mL sample with anion migration through the 3D printed porous material to the acceptor solution (see Figure 1). The inner, concentric vial contains the acceptor solution. Combined with capillary electrophoresis, submillimolar detection limits were obtained from the anions of strong acids. More importantly, a large step forward in the fabrication of EME devices with unique geometries and novel materials was demonstrated.

Vortex-Assisted Liquid–Liquid Microextraction

Another newer approach for the micro-scale isolation of solutes from aqueous solution is vortex-assisted liquid–liquid microextraction (VALLME). Elia Psillakis of the Technical University of Crete proposed, and has spent the last decade developing, the technique. Naturally, the most influential article in this area is one of her reviews, which serves as both a presentation of the fundamental understanding of VALLME and a tutorial on its use (2). Hydrodynamic, interfacial, and mass transfer concepts provide a deeper treatment. VALLME uses vortex agitation to disperse microliters of water-immiscible solvent into an aqueous sample. Following extraction, centrifugation promotes phase separation, and the isolated extracting solvent is ready for analysis. One particular advantage of the use of vortex agitation is that dispersive liquid–liquid microextraction (DLLME) can occur without the use of a disperser solvent. In this review, essential considerations are given thorough treatment, including drop breakup and emulsion formation, mass transfer rates in disperse systems, and emulsion destabilization and mechanisms of phase separation. Psillakis discusses the effects of extracting solvent type, solvent and sample volumes, surfactant type and concentration, pH and ionic strength, and vortex speed and time. Intensification of the phase separation via centrifugation, magnetic stirring, ionic strength adjustment (such as through salting out), and other means to increase drop coalescence are discussed. Though understanding of the principles of emerging analytical techniques, such as presented here for VALLME, is necessary for the transfer of knowledge required for technique development, instrumentation considerations, method development and transfer, and ultimately widespread acceptance.

Metal-Organic Frameworks

Crystalline porous networks made from metal ions or clusters coordinated with organic linkages, known as metal–organic frameworks (MOFs) is the topic of the

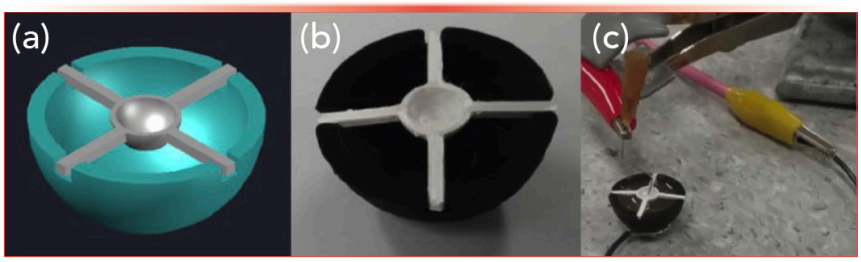

FIGURE 1: (a) Computer-aided design schematic of EME device, (b) the 3D printed device, and (c) experimental set-up, reproduced from reference (1).

TABLE I: Researchers who named the most influential sample preparation articles of 2019

Researcher Name	Institution Name
Mihkel Koel	Institute of Chemistry, Tallinn University of Technology (Estonia)
Hian Kee Lee	Department of Chemistry, National University of Singapore
Stig Pedersen-Bjergaard	School of Pharmacy, University of Oslo (Norway)
Elia Psillakis	School of Environmental Engineering, Technical University of Crete (Greece)
Kevin Schug	Department of Chemistry and Biochemistry, University of Texas at Arlington (USA)

influential paper recommended by Mihkel Koel of the Tallinn University of Technology (3). MOFs are used in a variety of forms of solid-phase extraction (SPE), including the conventional cartridge and pipet-tip formats, stir-bar sorptive extraction (SBSE) and in dispersive- and magnetic-SPE. Each of these forms of SPE approaches is discussed relative to MOFs, and the green emphasis of MOFs particularly impressed Koel. The thoroughly referenced (105 references) review presents the stability of MOFs in aqueous systems, such as collapse of the MOF due to competitive coordination with water, metal–ligand coordination geometry, surface hydrophobicity, crystallinity, and presence of defects. The hard/soft acid/base (HSAB) principle is used to describe the coordination between organic moieties and metal ligands. The manuscript also discusses the mechanisms of metal ion extractions with MOFs, including ion exchange and Van der Waals, hydrogen bonding, Lewis acid-base, chelation, and coordination electrostatic forces, with Lewis acid-base interactions being the most common. Applications in the extraction of palladium, lead, mercury, copper, cadmium, thorium, uranium, selenium, and multiple elements from aqueous samples with MOFs are presented. Two specific subclasses of MOFs are highlighted. Zinc (II) or cobalt (II) ions with imidazoles, or ZIFs, combine the benefits of zeolites and MOFs, especially for magnetic-SPE. MOF analogs with covalent organic frameworks (COFs) (consisting of H, O, C, N, B, and Si) connected via strong covalent bonds to organic monomers are a novel type of ordered crystalline porous polymers. COFs show low crystal density, high specific surface area, tunable pore size, and good thermal stability for adsorption of trace elements like Cr^{3+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+}, V^{5+}, Cu^{2+}, As^{3+}, Se^{4+}, and Mo^{6+}.

Analytical Reliability

From a more holistic perspective, Koel also suggested a recent paper that developed the concept of "analytical reliability" as important (4). In this manuscript, the need for quality assurance provided by rapid, simple, and direct analytical processes is claimed. Ten limitations to chemical analysis are identified: instrumentation and sensing systems, lack of involvement of analytical chemists in the knowledge generation from their data, publication vs. usefulness in solving analytical problems, lack of true innovation, lack of harmonization between basic and applied studies, importance of the transfer of knowledge and technology, lack of an interdisciplinary context, lack of interest in the social consequence of analytical activities, foundational education in analytical chemistry vs. analytical knowledge building, and a misunderstanding between method validation and performance characterization and fitness for purpose. Using these constraints, an approach to describing reliability (qualitative analysis) and uncertainty (quantitative analysis) is developed, with reliability expressed as a percentage based on bias and uncertainty.

Other Influential Trends

Rather than singling out a specific article, Hian Kee Lee from the National University of Singapore instead mentioned the evolving trends between new and emerging materials and both solid- and liquid-based sample preparation. Specifically, two-dimensional materials and liquids made by mixing and heating solids are of interest; both MOFs and deep eutectic solvents (DES) would be included in this emerging trend. Meanwhile, Kevin Schug from the University of Texas, Arlington presented the scenario related to the desire to track protein biomarker targets for disease diagnosis, prognosis, and treatment effectiveness (5). Current work in our laboratory extends this concept from the human biopharmaceutical industry to livestock reproduction. Borrowing from the approach for small-molecule analysis, Schug calls for commercial development of large-pore-size SPE materials for selective isolation of intact proteins of varying isoelectric points and molecular weights. Immunoaffinity approaches should be avoided because multiple, diverse proteins may be of interest. Such an approach will provide for the top-down (absolute) quantification of multiple intact proteins from biological samples using liquid chromatography with triple quadrupole mass spectrometry (LC–MS/MS).

Separate from journal articles or specific sample preparation methodologies, perhaps as noteworthy is the creation in 2019 of the Task Force on Sample Preparation by the European Chemical Society's Division of

Separation of Fat Soluble Vitamin K Congeners in under 6 minutes

The coagulation benefits of vitamin K1 (phylloquinone) are well known, however, the other congeners of the vitamin series are not as common (K2, K3, and K4), but equally contribute to a healthy disposition. K1 is abundantly found in leafy greens due to its role in photosynthesis. The absorption of K1 can be enhanced if a fat source like oil or butter is consumed with the greens, owing to the vitamin's inherent lipophilicity.[1] Vitamin K2, or menaquinone's (MK's), are identified by their various isoprenoid sidechain lengths (4–13). The MK4 subtype, that is 4 isoprenoid repeating units, can be synthesized through normal gut bacteria, but the other subtypes are primarily produced through non-human gut bacterial modalities. Recently MK4 and MK7 have gained interest due to reports indicating an increase in bone health, dental health, and arterial plasticity as well as a decrease in cardiovascular disease and have been linked to an increase in adipose metabolism.[2] Reports have indicated that an increase in MK7 can lead to better glucose homeostasis and offers a protection from the detrimental turnover of lipid and protein structures of the body.[2] Unlike K1 and K2, which are naturally abundant, K3 and K4 are synthetically produced and have been successfully utilized in the inhibition of tumor growth while increasing the occurrence of apoptosis of such cells in multiple types of cancers including: leukemia, hepatocellular carcinoma, lung, breast, oral, bladder, bone, and prostate cancers.[3] The addition of vitamin C to K3 (menadione) was shown to increase the efficacy of the anti-tumor properties through an increase in oxidative stress in tumor cells leading to cell apoptosis and an arrest of S phase cell division.[4] K4 was similarly shown to have an analogous apoptotic pathway as K3 in its anti-cancer activity in prostate cancer cells. In addition to its use in cancer treatments, K4 has been used in the treatment of vitamin K deficiency bleeding (VKDB) in newborns.[5]

Column Information

Packing Material	P/N
PRP-C18 (5 µm)	79676

Chromatographic Conditions

Gradient	0.00–1.00 min, 60% B 1.00–6 min, 60–100% B 6.01–8.00 min, 100% B
Temperature	40°C
Injection Volume	5 µL
Detection	UV at 254 nm
Dimensions	150 x 4.6 mm
Eluent A	25 mM HAA pH 9.2
Eluent B	THF
Flow Rate	1.0 mL/min

With the growing utility of the K vitamin series of molecules, a need appears relevant for both food labeling of constituents and for research based separation of the natural and synthetic compounds of vitamin K. The fast simple method developed by Hamilton Company using the PRP-C18 5 µm 150 x 4.6 mm column allows for simple efficient identification of the whole vitamin series. The use of the ion-pairing agent, hexylamine acetate (HAA) provided the best separation with the most efficient peak height. HAA offered a good interaction between both the analytes of interest and the lipophilic PRP-C18 resin. To elute the analytes, the stronger chromatographic reversed-phase eluent, tetrahydrofuran (THF) was used. The devised method, though developed with UV detection, is compatible with the standard post column conditions that are typically used with the detection of vitamin K via fluorescence detection.

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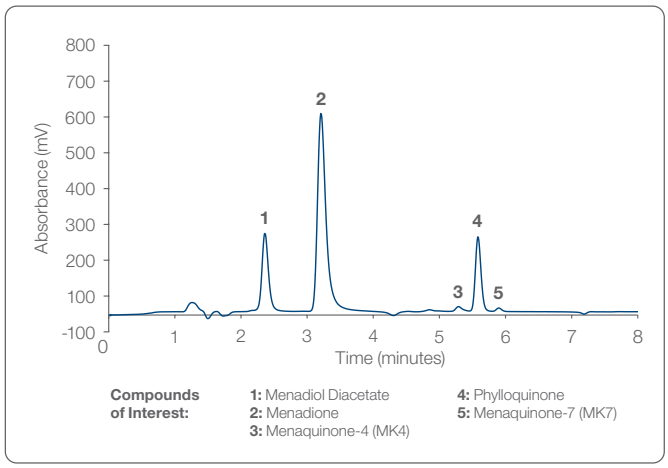

Compounds of Interest:
1: Menadiol Diacetate
2: Menadione
3: Menaquinone-4 (MK4)
4: Phylloquinone
5: Menaquinone-7 (MK7)

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Analytical Chemistry. The Sample Preparation Task Force aims to promote sample preparation through creating networks and clusters, organizing events, engaging technical communication and information exchange, and promoting fundamental studies and cross-disciplinary collaboration. Innovation and entrepreneurship, as well as involvement of early stage researchers, is encouraged. The task force is led by Elia Psillakis (Professor, Technical University of Crete), and includes Manuel Miró (Professor, University of Balearic Islands), Stig Pedersen-Bjergaard (Professor, University of Oslo), Marcela Segundo (Research Leader, REQUIMTE, Portuguese Government Associate Laboratory for Green Chemistry), Charlotta Turner (Professor, University of Lund), Barbara Bojko (Associate Professor, Nicolaus Copernicus University), Ezel Boyaci (Associate Professor, Middle East Technical University of Turkey), with Janusz Pawliszyn (Professor, University of Waterloo) and Gangfeng Ouyang (Professor, Sun Yat-sen University) as International Guest Members. The network consists of three working groups: science and fundamentals; automation, innovation and entrepreneurship; and information exchange and networking. An open invitation for membership in these networks is extended to all researchers, European and non-European. More details on the Sample Preparation Task Force and Network are found at their website (see reference [6]). If fully successful, this initiative should drive educational and research efforts in sample preparation for some time to come.

Final Assessments of the State of Sample Preparation in 2019

In addition to the list of the "most influential" articles chosen by thought leaders for this column, the first issue of *Analytical Chemistry* in 2020 presented a series of three critical reviews (7–9) that also assess the current state of sample preparation in the past year. To focus an annual review on emerging analytical extraction strategies, Hanson and Pedersen-Bjergaard centered their article on new liquid and solid extraction phases and on microextraction systems. Specifically, they address liquid extraction phases (including ionic liquids, DES, and nanostructured supramolecular solvents), solid extraction phases (including molecularly imprinted polymers [MIPs] and MOFs), liquid-phase microextraction, solid-phase microextraction, SBSE, micro-solid-phase extraction and microextraction by packed sorbent, and dispersive SPE. Regarding liquid phases, important features included improved environmental sustainability, reduced toxicity, enhanced extraction of polar analytes from aqueous samples, high selectivity, and improved compatibility with analytical instrumentation. Research efforts toward solid extraction phases include increased mass transfer and extraction capacity, increased selectivity or specificity, improved chemical and thermal stability, compatibility with complex samples, improved environmental sustainability, and lower cost. An annual review (8) of sample preparation using MIPs presented choice of reagents including from a green approach; MIPs synthesis for proteins; use of MIPS in SPE (33% of extraction methods published in 2018–2019), dispersive SPE (51%), SPME (5%), SBSE (3%), and with membranes or plates (8%). Applications of extractions with MIPs were categorized in compound classes of natural products (30% of 2018–2019 publications), drugs (27%), emerging contaminants (21%), proteins (12%), and pesticides (10%). The authors suggest that recent results with protein isolation will lead in the near future to an alternative to the use of antibodies in clinical analysis. Might MIPs address the top-down protein biomarker quantification concerns in the previously discussed blog (5) by Schug?

The final of the three critical reviews (9) discusses progress in fast sample preparation techniques, including extraction acceleration by energy exchange (including acoustic waves, microwave, electric field, and multiple fields), materials (such as phase adsorption and partitioning, chemical transformation, size recognition, and mass transfer acceleration), size reduction, and increased throughput.

Conclusions

Although the identification of influential sample preparation articles in 2019 was performed by a select few, the ideas presented may present techniques and approaches that will drive the future of the field. It is interesting that, with the exception of the critical review on fast sample preparation (9), techniques applied to the extraction of environmental solids, foods, and related solid or semi-solid matrices were not discussed. Perhaps this was an artifact of the selection of thought leaders who contributed their ideas, or perhaps research and technology pertaining to these application areas has stagnated. EME, VALLME, MOFs, and other subject matter presented, including expected outcomes from the European Task Force on Sample Preparation, are worth keeping an eye on and represent potential topic areas for upcoming installments of "Sample Prep Perspectives."

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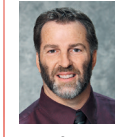
ABOUT THE COLUMN EDITOR



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The Why, What, and How of CDS Audit Trail Review

Audit trail review is a key component of the second person review of chromatographic analysis for compliance with regulations, procedures, and analytical science. We focus on what the review of audit trail entries means, and how to review by exception if the CDS has appropriate technical controls.

R.D. McDowall

Examination of audit trail entries for an analysis is a key part of the second person review. It is over 20 years since EU GMP Annex 11 and 21 *CFR* 11 required audit trails in regulated applications, including the chromatography data system (CDS). What do the regulations say about review of audit trails? How can we speed up the review process? What is review by exception? How can we use this to save review time?

Where It All Began

All those lucky chromatographers who must review CDS audit trails have Able Laboratories to thank for the drudge of ensuring that the analysis has been performed correctly. As citation 1 of the Able Laboratories 483 Observation states:

> *...The Quality Unit failed to: review electronic data as part of batch release, review computer audit trails in the <Redacted> Data Acquisition System, and provide adequate training to analytical chemists (1).*

Here we have the first regulatory citation for failure to review audit trails. However, audit trails, however rudimentary, have been included in major laboratory informatics applications such as laboratory information management system (LIMS) since the 1980s. The problem was that there was little agreement on what was required from a regulatory perspective.

Four Eyes Principle and Second Person Review

The generation of sound analytical results is based on the established "four eyes" principle; one person to perform the chromatographic analysis, and a second person to review the data to show that the work has been performed correctly and that no mistakes have been made. The involvement of a second person is to look with a fresh pair of eyes for anything that the analyst may be overlooked. Now, with the emphasis on data integrity, the second person review has been expanded to check that work has not been falsified, and must include review of the CDS audit trail entries.

The scope of the review must cover the whole of the analytical process: from sampling to the calculation of the reportable result, however we will only consider the audit trail review here. The terms performer and reviewer are stated in 21 *CFR* 211.194(a) (2). I will use the term "reviewer" or "second person reviewer" to indicate the one individual who conducts the checks to ensure that work has been performed correctly, and all data and records have been collected. The reviewer will use a general second person review standard operating procedure (SOP) to control their work, and will, in all probability, have a linked work instruction for each different CDS audit trail to be reviewed (unless you have standardized on a single CDS). Ideally, the CDS can support the audit trail review process with software functions (technical controls) to make it quick and efficient, as we shall discuss later.

What is an Audit Trail?

Before we can review audit trail entries, we need to define what and audit trail is, and then understand the regulations surrounding it and the review process. The simplest definition is found in the 2018 FDA guidance on Data Integrity and cGMP, where question 1c asks, "What is an audit trail?"

> *...audit trail means a secure, computer-generated, time-stamped electronic record that allows for reconstruction of the course of events relating to the creation, modification, or deletion of an electronic record. For example, the audit trail for an HPLC run should include the user name, date/time of the run, the integration parameters used, and details of a reprocessing, if any. Documentation should include change justification for the reprocessing. Audit trails include those that track creation, modification, or deletion of data (such as processing parameters and results) and those that track actions at the record or system level (such as attempts to access the system or rename or delete a file)...(3).*

How did we get here?

Audit Trail Regulations

21 *CFR* 11 (Electronic Records and Electronic Signatures) regulations has clause 11.10(e) that requires:

> *Use of secure, computer-generated, time-stamped audit trails to independently record the date and time of operator entries and actions that create, modify, or delete electronic records.*
>
> *Record changes shall not obscure previously recorded information.*

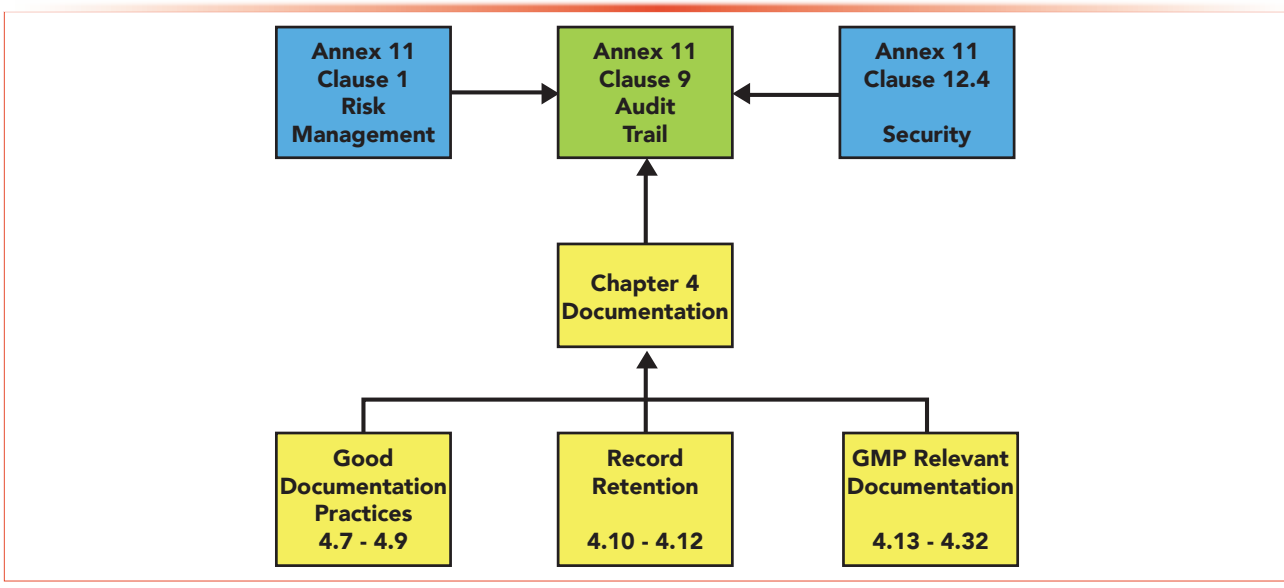

FIGURE 1: Key clauses of EU GMP Annex 11 and interaction with EU GMP Chapter 4 on documentation.

> *Such audit trail documentation shall be retained for a period at least as long as that required for the subject electronic records and shall be available for agency review and copying (4)*

See where the definition of audit trail comes from in the FDA data integrity guidance? Straight out of the Part 11 regulation. However, life is not always simple, and Part 11 is no exception.

Interpretation of Part 11 by the Predicate Rule

Part 11 only defines the requirements for electronic records and electronic signatures, as that is the role of the predicate file. In our case, the applicable FDA predicate rule is either Good Laboratory Practice (GLP or 21 *CFR* 58) or Good Manufacturing Practice (GMP or 21 *CFR* 211), and you have to interpret these regulations for a complete understanding of FDA regulations for audit trails. For example, there are differences between the two predicate rules. GLP requires a reason for data change in 21 *CFR* 58.130(e) (5), but GMP does not (2). However, it would be a foolish quality control (QC) laboratory that did not implement a reason for change in today's data integrity environment.

This now brings up to 2005, and the Able Laboratories case. How did the FDA cite Able for failure to review audit trail entries? Enter the GMP predicate rule, and specifically 21 *CFR* 211.194(a) for laboratory records. There are two specific requirements:

> *Laboratory records shall include complete data derived from all tests necessary to assure compliance with established specifications and standards, including examinations and assays, as follows:*
> *(1–7) ...*
> *8) The initials or signature of a second person showing that the original records have been reviewed for accuracy, completeness, and compliance with established standards (2).*

Although this regulation has been effective since 1978, only since 2005 has the FDA interpreted it to include review of audit trails since the Able Laboratories fraud case. Now we see that when a CDS is involved, a key component of the second person review is to review audit trail entries.

Woah!

For a networked CDS there will be thousands of audit trail entries – must I review all of them? To give a rational answer to this, we have to move to Europe, and the update of EU GMP Annex 11 in 2011, to see how regulators have coped post Able with data integrity and any possible data falsification.

Update of EU GMP Annex 11

In 2011, the update of Annex 11 was issued (6). Before we discuss the specific requirements for audit trail in the new version, it is important to understand that the full interpretation of Annex 11 requires an understanding of EU GMP Chapter 4 on Documentation (7). The updates of both these regulations were issued at the same time. Figure 1 shows the key sections of Annex 11 and Chapter 4 that are pertinent to our audit trail discussion.

Let us work through Figure 1 to understand the most recent regulations for audit trails and their review. First, there is the Chapter 4 requirement for Good Documentation Practices (GDocP) in section 4.7–4.9 (8), this was discussed in one of last year's Data Integrity Focus articles (9), and will not be repeated here. However, the inter-

pretation of these GDocP requirements on audit trail entries is very relevant, therefore all audit trail entries must be:

- Legible and understandable
- The old and new value for a change must be recorded, along with who made the change
- A reason for changing data is required for all modifications and deletions
- Entries must be date and time stamped. The format of this must be unambiguous, and may also require the time zone, especially for multinational companies.
- Audit trails need to be associated with the activities supported, and it must be possible to search the entries for specific events
- Audit trails must be secure from change
- Audit trails must be retained and be readable for the record retention period (defined in sections 4.10 to 4.12), and this is at least five years after release of the batch by a qualified person.

This interpretation comes only from the requirements of GDocP and records retention sections in Chapter 4 (7). We now turn to see what the audit trail requirements are in Annex 11.

Annex 11 Requirements for Audit Trail

The updated version of Annex 11 has the following requirements for audit trail documented in clause 9:

> *Consideration should be given, based on a risk assessment, to building into the system the creation of a record of all GMP-relevant changes and deletions (a system generated "audit trail").*
> *For change or deletion of GMP-relevant data the reason should be documented.*
>
> *Audit trails need to be available and convertible to a generally intelligible form and regularly reviewed (6).*

Normally, the requirements are presented as a single paragraph, but, for the purposes of this discussion, I have broken these requirements into sentences and clauses. First and foremost, an audit trail is not mandatory, as the phrasing is "consideration … based on a risk assessment…". Before you all rush to turn your CDS audit trails off, please consider the following issues:

- There are many regulatory citations for CDS with audit trails either not turned on, or turned off and then on, to hide falsification activities
- In the absence of an audit trail, Clause 12.4 requires that management systems for data and for documents should be designed to record the identity of operators entering, changing, confirming, or deleting data, including date and time (6).

Therefore, we will not be conducting a risk assessment to justify not having an audit trail, as you will have to com-

TABLE I: What an audit trail is and is not

An Audit Trail Is	An Audit Trail Is Not
• Generated automatically • Secure and linked to a trusted time source • Built within a GMP application (ideally within a database) • Focused on GMP data generation, modification, and deletion • Can allow an authorised user to enter a reason for change (free text or default entry)	• A text file that is unsecured • Built into a data file as the file cannot monitor its own deletion • A repository for anything to do with the system • A system log • An operating system event log

TABLE II: Identifying some GMP relevant data changes

GMP Relevant Changes in an Audit Trail	General Audit Trail Entries
• Change batch number of sample • Move a sample in an injection sequence • Modify a sample weight • Abort a sequence • User manually integrates a peak • User electronically signs an analysis report	• Logon or logoff by a user • User locks their account • Administrator unlocks user account • Create a new user • Administrator archives an analysis project • Change application configuration setting

ply with clause 12.4. The next is that the audit trail focuses on GMP-relevant changes and deletions, unlike a Part 11 audit trail that also includes creation of the records in 11.10(e) (4). You have to interpret what are GMP-relevant changes and deletions.

Any change to data requires a reason for change, as this is consistent with the GDocP requirements of Chapter 4 (7). This can be implemented either by a drop-down list of context sensitive options to save typing, or by free text. Personally, I prefer the default reasons for change, as this ensures consistency. This option can take time to implement, but the basic functionality must be validated. However, when the CDS is operational, it is best controlled by procedure for adding reasons for change (10).

There is the requirement for audit trails to be available in a generally intelligible form, this refers back to the requirements for GDocP discussed above, but also the need for audit trail entries to be easy to understand and follow.

Finally, the three words that come directly from Able Laboratories: "And regularly reviewed." This is the first, and currently only, explicit regulatory requirement to review audit trail entries, but interpretation always causes much debate and discussion. We shall discuss frequency of review later in this article.

What is An Audit Trail?

Now we have presented and discussed the three main regulations issued over the past 20–30 years for computerized system audit trails, which should be straightforward to interpret in practice.

But, no. We still lack adequate audit trails, or even the existence of an audit trail in many laboratory informatics systems as evidenced in a review of infrared spectroscopy software citations by Smith and McDowall in *Spectroscopy* (11). Therefore, we need to briefly discuss what is an audit trail, and this is presented in Table I.

From all of the regulations, we can show what an audit trail is and is not in Table I:

The key requirement is that an audit trail is an integral function in any CDS or any laboratory informatics application. As such, it cannot be bolted on as an afterthought of system design. To be encompassing and effective, the foundation of any audit trail in any application must be a database. The debate between a single audit trail containing all entries versus one for system related entries and one associated with data is outside the scope of this article.

What Are GMP-Relevant Changes?

For an effective and efficient review of audit trail entries, it is essential to understand what the phrase "GMP relevant changes and deletions" in Annex 11 means in practice. As the laboratory has configured and validated all laboratory user roles without deletion privileges, a reviewer will not be looking for any deletion entries, will they? That leaves us with just GMP relevant changes, Table II lists some audit trail entries that could be found within the audit trails of most, if not all, CDS applications. They are divided into general entries in the right-hand column and GMP relevant changes in the left one.

Let us take the entries in the right-hand column and discuss them first. Here we have logon, logoff, and failed logons, as well as creating an account and unlocking an account by an administrator. Are these GMP relevant changes? Your answer should be an unequivocal "no," as no data have been changed. Equally so, are the archiving of an analyst's project and changing a configuration setting of the application? At this point, there are probably wails of anguish coming from the direction of the quality assurance (QA) department. Let me be very clear here: These last entries are not part of a second person review process. But they will be covered by a QA led data integrity audit to ensure that these

actions have the correct authorization, and have followed the appropriate procedure. The entries in the left-hand column of Table II are GMP relevant, and must be reviewed during a second person review of any analysis.

However, if you only have a single audit trail covering the whole system, this can present problems, as entries for all analyses, all user logon and logoffs,or any configuration changes can be found in one huge dustbin. To identify GMP relevant changes for the specific analysis that you are going to review needs good search routines.

Risk Management of Audit Trail Review

Next to discuss is a very important clause in Annex 11 that has a major impact on our discussion. Clause 1 states that risk management should be applied throughout the lifecycle… taking into account patient safety, data integrity and product quality (6). Risk management applies not just in the validation of the system, but also during operation of the CDS. Unsurprisingly, this should include audit trail review, but it often does not. Therefore, we need to consider how we can use risk management to reduce our work reviewing audit trail entries. Of necessity, this approach includes utilizing any technical controls that can be implemented in the CDS application to reduce the amount and number of entries to review.

- Can a user delete data? If all user roles can be configured so that no user has delete privileges, then why should a reviewer look for deletion? To achieve this, there must be a record of how each user role is configured and this must be tested in the system validation. Checks will be performed during data integrity audits that these controls remain in place but, reiterating points made above, do not have to be performed during second person review.
- Can locations where chromatographic data are stored be changed by an analyst? If locations for data storage are controlled by the administrator, and these cannot be changed by a user, then the reviewer need not look at locations for unofficial testing. The procedure and specifications must also be included in the CDS validation.
- Activate the CDS technical controls for audit trail review. Understand and implement any technical controls in your CDS software, such as how does the application highlight SST results not meeting acceptance criteria, files that have been manually integrated, changes to sample weights, purity factors, calculations, etc.?
- Is there an effective audit trail search function? This is to look for activities such as short injection sequences, repeated sequences, or aborted runs as possible poor data management practices.

- Does the system have a function to document audit trail review, or must this be done by procedure? If there is an audit trail review button, then this must be specified in the system User Requirements Specification and be validated. Otherwise, control of audit trail review will involve an SOP.
- Evaluate review by exception. If there are adequate technical controls that identify changes to data (you will not have enabled delete options), and these have been validated, then you can consider audit trail review by exception. This works when there are no data modifications identified by the technical controls monitoring the audit trail. We will discuss this later.

Who Should Review Audit Trail Entries?

One of the main discussion points in training courses that I have participated in is who should be responsible for reviewing audit trail entries. Answers have varied from analytical development/QC, QA, or even (horror of horrors) IT! How can people and organizations get this so wrong? Audit trail review as part of second person review is a laboratory function. The rationale for my view? Its in the regulations, specifically 21 *CFR* 211.194(a) (2), and EU GMP Chapter 6.17 vii (12).

Recent guidance documents have reinforced this. The FDA's approach in Question 7 of their data integrity guidance (3) is that the people responsible for record review under CGMP should review the audit trails that capture changes to data associated with the record as they review the rest of the record (for example, 211.194(a)(8) [2]). Similarly, PIC/S PI-041 guidance in section 9.5 states that audit trails for each batch should be independently reviewed with all other records related to the batch and prior to the batch's release, so as to ensure that critical data and changes to it are acceptable .. and performed by the originating department, which is the laboratory (13). QA, please note! Your department can verify the effectiveness of the review during data integrity audits or investigations (13–15).

How Regular is a Regular Review?

This is another question with a multitude of wrong answers! Let's see what is discussed in the data integrity guidance documents. FDA's view, stated in question 8 of the 2018 guidance, (3) is that if audit trail review is mandated in 21 *CFR* 211, then this is the review frequency. If the interval is not specified, then determine this according to a risk assessment (a lovely get out of jail excuse!) based on knowledge of the process and the functions of the CDS application, and also include evaluation of data criticality, control mechanisms, and impact on product quality, to ensure that CGMP requirements are met, appropriate controls are implemented, and the reliability of the review is proven (3).

To help you let you understand this, let us move from the vague to the specific, and give laboratory examples. What audit trail review would you conduct for:

- Method development: As method development is seen as outside of GMP, there is no need for a review of audit trail entries. This may be so as there is no mention of method development in ICH Q2(R1) (16). However, this is the critical foundation of a robust analytical procedure, and times are changing, as the analytical world is going to a lifecycle methodology with the publication of a draft USP <1220>, a revision of ICH Q2(R1) in the works. This will be the subject of the next "Data Integrity Focus" article.
- Method validation: Audit trail review of the work must be performed before release of the report, but my preference would be at the completion of each experiment to ensure that integrity and quality of the data before the whole validation data become too large.
- Batch release: As discussed earlier, this is mandated by GMP regulations, and therefore must be done before signing of each chromatographic test by the reviewer.
- Stability testing: Similar to batch release, it is important to review audit trail entries after each pull of samples rather than wait for the whole stability study to finish. This is because there are requirements to inform regulatory authorities if an out of specification (OOS) result is obtained.

Performing the Audit Trail Review

Now we can get down to describing how to perform an actual review of audit trail entries. Remember that an audit trail review is only a portion of the overall second person review that starts with sampling and finishes with the calculation of the reportable result. In this section, we will focus on the CDS audit trail review for one analytical run. In this discussion, the technical controls presented earlier are in place and validated to make the review process easier, and there is a procedure in place for review by exception.

First up, do we review audit trail entries on screen, or do we print them out? For those readers that selected the latter option, please write a letter of resignation immediately and reserve a bed at the local lunatic asylum, as you don't understand how to make a process easier. We will be reviewing on screen. The reason for this is that an audit trail can contain much more information that fits on a screen, and printing can generate much, much more paper than anticipated. When it comes to on screen review, it is important that the reviewer has one and preferably two large high-resolution screens. Perhaps it is time to ask your boss for a 55 inch 8K TV, I mean monitor, for the review?

Seriously though, this is an important task and the reviewer needs

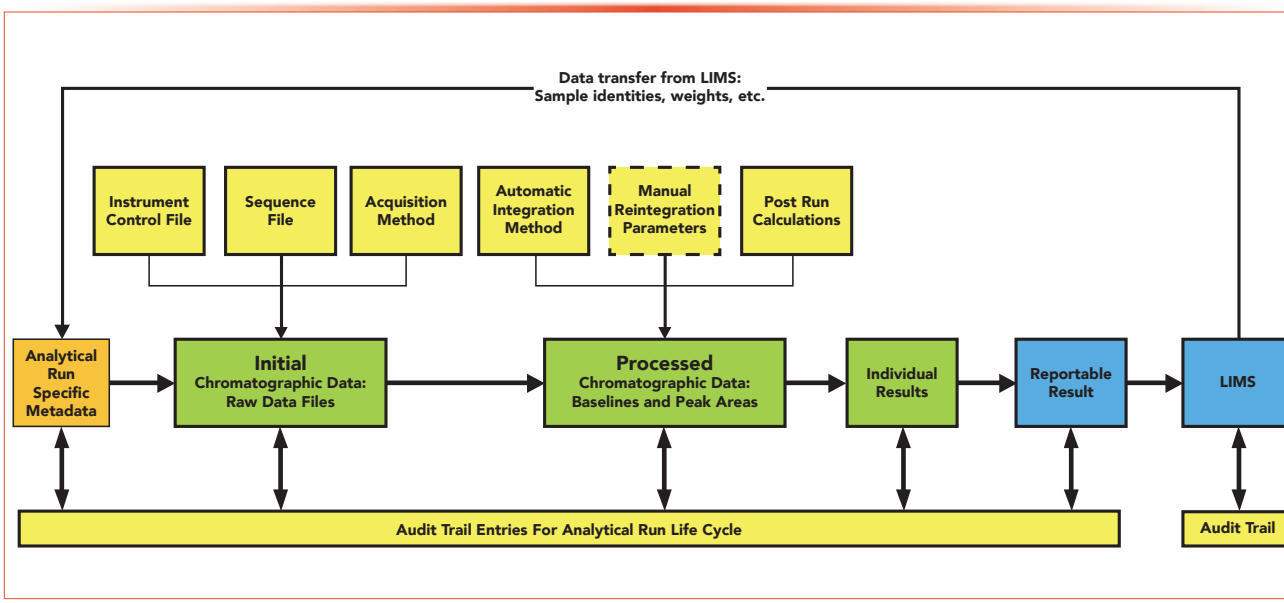

FIGURE 2: Audit trail review when a LIMS is interfaced to a CDS.

the right tools. Having an expanded chromatogram on one screen and the pertinent audit trail events on another can help understand and recreate activities more easily than on one small monitor and save switching between views of the data. Comparison and correlation are much easier on two screens, and faster.

Next, let us look at where in the CDS application that there could be GMP-relevant modifications (remember, deletions are not configured for the users). Here are some changes that should trigger audit trail entries:

- Data entered manually and then corrected. Typographical errors will inevitably occur when data are entered manually into the sequence file. Ideally, they should be found by the performer of the test or the reviewer for the performer to correct. There will be corresponding audit trail entries with reasons for change.
- Failures of SST injections to meet acceptance criteria. There should be entries in the audit trail, but they also need to be cross referenced with entries in the instrument log together with any corrective action and any requalification work, such as replacement of pump seals.
- Changes to instrument and processing parameters, if allowed by the CDS
- Manual integration of peaks (if allowed) which will be the subject of a later Data Integrity Focus article.
- Changes to calculation formulae

These are some of the areas where there might be audit trail entries containing GMP-relevant changes.

Review by Exception

Review by exception is a term used to review only the exceptions in any analytical run, rather than each and every audit trail entry. In the discussion above Annex 11 required an audit trail for GMP-relevant changes and deletions (6). If no deletions are allowed in the CDS, then all you need to look for are the modifications or the exceptions to normal working of the system.

Consider an analysis: if all peaks are integrated automatically, do you need to look at the audit trail entries for manual integration? No. Each peak integration shows if a baseline has been placed automatically or manually with the integration codes BB or Bb for example. In the former, both the start and end baselines have been determined by the system; in the latter case, the trailing baseline has been positioned by a chromatographer. This is a slow process of looking at each chromatogram individually, and is only marginally more interesting than watching paint dry.

Does the CDS Aid Review by Exception?

A much better approach is if the CDS can highlight that there is no manual integration at the injection level AND this function has been validated, if there are no exceptions (manual integration) for the run, and you don't need to review the pertinent audit trail entries.

The exceptions the reviewers should be identifying are the GMP relevant changes such as those listed in Table II. This is where the supplier of your CDS can be a great help or a hinderance to the second person review process. The technical controls built into the application are enabled and validated to highlight changes to data so that a reviewer can focus their attention on the key items. This is risk assessment in practice. The ways that an application can identify changes to data are color coding (for example, a traffic light approach with

A Q&A

GC×GC Analysis of Environmental Samples

Christopher Reddy
Senior Scientist
Department of Marine Chemistry and Geochemistry
Woods Hole Oceanographic Institution

GC×GC tells a rich and exciting story about what happens to pollutants in the environment.

Comprehensive two-dimensional gas chromatography (GC×GC) offers dramatic improvements over traditional gas chromatography for the analysis of complex mixtures in numerous applications. This is due to the increased chromatographic resolution, and other benefits, gained by adding an orthogonal second dimension of a chromatographic separation. Christopher Reddy, senior scientist in the Department of Marine Chemistry and Geochemistry at the Woods Hole Oceanographic Institution in Cape Cod, Mass., recently sat down with *LCGC* to discuss his past, present, and future with GC×GC analysis. From his start with a 50-year-old oil spill in Buzzards Bay, Mass., to his thoughts on the current developments of GC×GC technology, Chris has 20 years of experience in the world of comprehensive two-dimensional gas chromatography.

LCGC: How did you first get involved in comprehensive GC×GC of complex environmental samples?

Reddy: I became interested in GC×GC after reading a few papers written in the late 1990s—I thought it was a powerful platform for expanding the field of environmental chemistry. I was studying an oil spill that occurred in 1969 in my backyard in Massachusetts and contacted Captain Rick Gaines and Professor Glenn Frysinger, GC×GC trailblazers at the United States Coast Guard Academy in Connecticut. They ran some of my first samples and helped me interpret the results. I was hooked and had my first instrument in 2002. I have not looked back.

LCGC: Can you cite any examples of the types of chromatographic challenges that illustrate the power and utility of GC×GC technology?

Reddy: Yes, absolutely. GC×GC is capable of answering questions that were previously unattainable. The power of GC×GC isn't that you're seeing more compounds or making pretty pictures alone; it's that it delivers content that you couldn't get any other way and allows you to visualize and quantify what you know, what you don't know, and tell a better story about what happens to pollutants in the environment. With that said, as much as I've really enjoyed studying pollution, it's inherently contagious among our colleagues who have asked us to look at other samples. We have gone from providing molecular evidence for the existence of Archaea in 2.7-billion-year-old rocks from Canada to other legacy pollutants like DDT that was dumped off the coast of California. We've looked at biofuels and studied reaction mechanisms in synthetic organic chemistry. Our work has found its way into some really topical areas. I'm particularly proud of the science we published on the Deepwater Horizon disaster, which occurred in 2010 in the Gulf of Mexico spilling approximately 160 million gallons of crude oil.

LCGC: What innovations would you like to see in the near future to help GC×GC grow?

Reddy: I have the luxury of having a colleague, Bob Nelson, who has been

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with me since day one. He is a fantastic, knowledgeable operator of GC×GC platforms and fine geochemist. Also, manufacturers like LECO have made it a lot more user-friendly. But we wish, almost daily, for more advanced and novel stationary phases for gas-chromatographic columns, in particular chiral, or columns that allow us to separate similar but different compounds based on their size or shape. Right now, they are not thermally stable for separating many of the larger, less volatile compounds we study, so we can only study certain types of compounds with these columns—this really limits our capacity to study a broader range. This is a plea to all of the manufacturers of gas chromatographic columns—we'd love to see more non-traditional columns that can operate at higher temperatures.

> "It's been a delight to see these colleagues use the preliminary data generated in our laboratory to acquire their own instruments."

While not necessarily an innovation, people are paramount to the continuing excellence of GC×GC. I am a firm believer that collaborating and working with talented and generous people is the key to success, and GC×GC is one field where the human dimension is critical. I can point to the generosity of Rick and Glenn, where they not only ran samples but answered many questions that Bob and I had after we acquired our first GC×GC. They hosted us in their laboratory many times and helped with the maintenance of our instrument. As a result, Bob and I have also hosted many visitors in our laboratory and run countless samples for scientists. And I encourage others to follow the example set by Rick and Glenn.

It's been a delight to see these colleagues use the preliminary data generated in our laboratory to acquire their own instruments. And most importantly, mastering GC×GC does not happen in a day or a month. It takes time, but it's worth it. Hence, I ask laboratory managers and academic advisors to be patient and support their staff and students as they learn GC×GC. Find ways to provide opportunities for them to attend workshops or training sessions.

LCGC: Are there some current developments that you find exciting?

Reddy: The most exciting development in our laboratory is having a GC×GC with a mass spectrometer that allows us to "confirm" or "characterize" unknown compounds in our samples. This is not just a case of bookkeeping; every compound in a complex mixture provides a clue to studying the problem at hand. Mass spectrometers come in all sizes and shapes, and the newest one in our laboratory is a high-resolution mass spectrometer made by LECO that gives us a more powerful means to interrogate what we see and what it is.

LCGC: How are you using GC×GC in your lab right now?

Reddy: We have several GC×GC platforms that have different attributes for studying challenging research questions. One platform for most laboratories would be more than enough, but in our case, we take the information that we get from these different instruments and create a comprehensive understanding of the samples we're analyzing.

LCGC: How has GC×GC impacted or changed your job?

Reddy: Fantastically. I want to know how Mother Nature responds to an uninvited guest like an oil spill. When a spill occurs, it's like putting nature on a treadmill. In terms of traditional GC, you can monitor the pulse and blood pressure of nature, but when you have a GC×GC, you can take an MRI of nature. That has really helped propel my career, allowing me to tell a story about what happens when oil gets released, how it behaves, how long it will last, and how long it might be potentially toxic. GC×GC takes us further than traditional platforms.

At this point in my career, I am happiest when my colleagues excel. I love to hear when they get funded, finish their Ph.D., get their first job, awarded tenured, or win an award. And many of these colleagues have benefited from GCxGC.

green indicating no changes, yellow to highlight any data modifications, and red for deletions), or by flagging data changes. This functionality is important to avoid the reviewer from drowning in data.

One area that will NOT be subject to review by exception and MUST be performed in all the second person reviews, even if there is no indication manual integration, is viewing all chromatograms on screen. This can be either singly or overlaid to ensure peak shape and resolution are as expected and consistent throughout the run. This is good analytical science, and must be performed for all analyses to ensure the integrity and quality of the results. There are no exceptions!

Documenting the Review

Few laboratory informatics applications have the ability to document if an audit trail has been reviewed, by whom, and when. Ideally, this is the best approach. However, in many cases, the review must be done procedurally, and the SOP must state that the meaning of the signature of the reviewer includes review of applicable audit trail entries. Sometimes, auditors insist that laboratories print out audit trail entries and sign them to provide documented evidence of the review. This approach should be resisted, as it is unrealistic and untenable, and laboratories should ask where is the specific regulation for this approach. This is an area where users require an electronic audit trail review function. In the absence of this, data integrity audits should focus on the effectiveness of a procedural audit trail review, and be conducted more frequently.

AT Review: System vs. Process

Up to now, we have just considered audit trail review in a CDS that is not interfaced to any other application. However, there are situations where a CDS is interfaced to another informatics application, such as a laboratory information management system (LIMS); how should an audit trail review be conducted in this situation? We must consider process taking precedence over system when considering audit trail review.

Figure 2 depicts a LIMS interfaced with a networked CDS. As I mentioned above, we have to consider process, not system, otherwise potential issues will fall into the interfaces and not be identified in any review.

- The process starts in the LIMS, where sample weights and sample identities are downloaded to the CDS
- Any run specific metadata (such as, for example, dilutions) are manually entered into the sequence file of the CDS by the analyst
- The analysis takes place in the CDS, with the performer calculating the reportable result
- At the end of the analysis, the result is transferred automatically from the CDS to the LIMS.

Now, we have to consider how we need to conduct the audit trail reviews with the two systems. We will only focus on the transfers between the two at this point.

- The export of the sample identities and weights will be recorded in the LIMS audit trail, and there should be a corresponding import in the CDS audit trail
- Time (and date) synchronization is very important here. There should be a delay between the LIMS data export and the import into the CDS; how much would be determinized in the validation of the interface (from microseconds to minutes, depending on the transfer mechanism). An important issue could be if the two systems were in different timezones; however, this should be already resolved in the system validation.
- At the completion of the analysis, there must be an export of the data from the CDS recorded in the CDFS audit trail, and, after a delay, an import into the LIMS, with a corresponding record in the audit trail.

Compliance Features to Consider When Purchasing a CDS

Apart from the chromatographic and instrument control functionality, one of the key requirements when selecting a new CDS are the technical control available to help protect electronic records, implement electronic signatures, and audit trail functionality. Ensuring regulatory compliance and data integrity are essential criteria for system selection now. These functions are often overlooked in selection of a CDS. Here are some of my compliance criteria that you should include when selecting a new system:

- Database as the foundation for managing data and building an effective audit trail.
- Flexible data storage to separate active data projects from inactive or archived ones.
- Configuration at the application level to protect electronic records.
- Configurable user roles or types to avoid conflicts of interest, such as, for example, no user should have administration privileges. Note that there will also be laboratory administrators for building custom calculations and reports.
- Audit trail functionality covering the whole system. Within this umbrella, there are two options: either a single audit trail for the application coupled with effective search routines to find all entries associated with a specific analysis; or two separate audit trails, one at the system level and one at the data level. When a project is created, it will have a data level audit trail within it, making it easier to search events within the analysis.
- Technical controls within the audit trail to highlight data changes and deletions to facilitate the review process, as well as enable review by exception, plus the ability to create efficient search routines within an individual project or the

Continued on Page 193

Hydrophilic-Interaction Chromatography: An Update

This article is an update on the technique of hydrophilic-interaction chromatography (HILIC) and covers recent ideas on the mechanism of separation, and how it may be manipulated to suit the separation of particular sample types. The advantages of HILIC are discussed, and also the actual and perceived disadvantages of the technique and how the latter can be overcome. Some new applications of HILIC for characterization of biopharmaceuticals, where it can even be applied to the separation of intact proteins, and to applications in metabolomics, will be discussed.

David V. McCalley

It is now 30 years since the publication of Alpert's landmark paper that named the technique of hydrophilic-interaction chromatography (HILIC) and discussed its mechanism and applications (1). Alpert clearly recognized that separations were influenced by the partition of solutes between a water layer held on the surface of a polar column and the bulk mobile phase rich in an organic solvent such as acetonitrile. Additional mechanisms, such as ionic retention and adsorption, can be superimposed on this process. Retention increases with increasing polarity of the solute, broadly opposite to that in reversed phase. Nevertheless, there are considerable differences in the selectivity of the techniques, indicating their complementary nature and orthogonality, which is also an advantage for two-dimensional (2D) separations. Polar solutes are retained in HILIC that have little or no retention in reversed phase; for example, uracil can show good retention in HILIC, whereas it is used as a void volume marker in reversed-phase chromatography.

There are many advantages of HILIC over reversed-phase chromatography; indeed, for some polar neutral solutes, there is hardly an alternative to HILIC to achieve sufficient retention for separation. However, there are real and perceived disadvantages of HILIC that can provide a barrier to more widespread uptake of the technique. The aim of this paper is to provide an update on the mechanism of separation of HILIC, to discuss the manipulation of its selectivity, its advantages, and its limitations (and how they may be overcome), together with some new applications of the technique.

Which Solutes are Suitable for HILIC?

Polar and ionized solutes that are hydrophilic are likely to be retained in HILIC; as a guide, the log of the solute distribution ratio between octanol and water can be considered where:

$$\text{Log } D_{ow} = \log \{[\text{neutral + ionized solute}]_{\text{octanol}}] / [\text{neutral + ionized solute}]_{\text{water}}\} \quad [1]$$

Log D_{ow} values can be measured experimentally, or obtained from a number of commercial simulation software packages. Hydrophobic solutes have a high positive log D value; that is, they prefer to partition into the relatively nonpolar (octanol) in a shake flask experiment (this simulates the bulk mobile phase in HILIC), and thus they are less retained. Conversely, very hydrophilic solutes have a large negative value (prefer to partition into water), and thus are more retained. Typically, solutes with a value of log D > ~+1 will show low retention in HILIC. However, ionic effects can contribute to retention; for example, the protonated base nortriptyline (log D ~+1) gives high retention on a silica column using aqueous acetonitrile buffered at $_{w}^{w}$ pH 3 because of interaction with negatively charged silanols (2). Figure 1 indicates a rough correlation on three HILIC stationary phases between log D_{ow} and retention for some acidic, basic, and neutral solutes. The correlation is best for TSKgel amide (r = 0.83), which has a thick neutral polymer layer that shields stationary phase silanol groups (negatively charged) from ionic retention of protonated bases or repulsion of charged acids. The bare silica column shows no such shielding from ionic effects, resulting in a much poorer correlation (r = 0.42). Clearly, the retention of neutrals by partition should depend on the extent of the water-rich layer on the surface, while ionic retention–repulsion and adsorption effects will also depend on the particular stationary phase used. These factors contribute to the substantial selectivity obtainable by changing the stationary phase in HILIC (see below).

Whereas HILIC has classically been applied to small molecules, it has recently been successfully applied to the separation of peptides and even intact proteins (see below).

The Mobile Phase in HILIC

Organic Solvent Concentration

The ratio of organic solvent to water in the mobile phase is a crucial factor in controlling overall retention in HILIC. Reducing the water (the strong solvent) concentration increases retention, which is the opposite effect to that in reversed phase. Retention can increase exponentially at high levels of acetonitrile (80–95%) (3). Although other water-miscible polar organic solvents have also been used (such as methanol), acetonitrile is by far the most commonly employed, and usually in the concentration range ~ 60–95%. As the water concentration increases in the mobile phase, polar solutes increasingly partition into it, reducing retention.

Buffers and Mobile Phase pH

Approaches to pH Measurement

Buffers are necessary to stabilize the charge both on the solute and the stationary phase. The choice of buffer is rel-

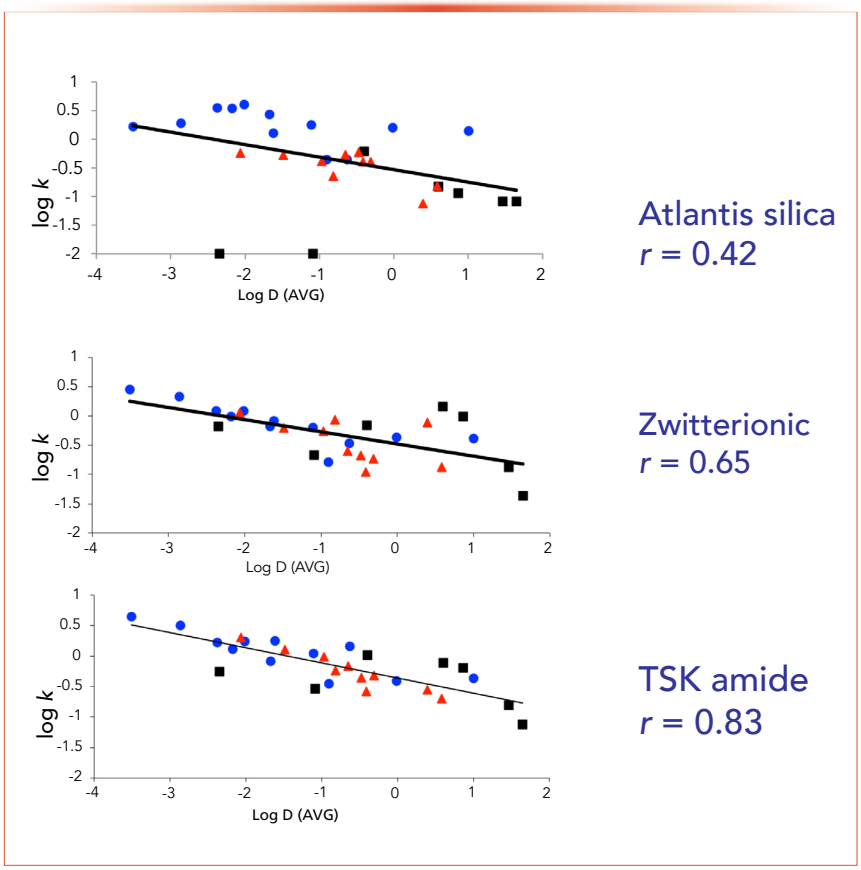

FIGURE 1: Plots of log k versus average log D (from three calculation programs) on three stationary phases. Mobile phase 5 mM ammonium formate pH 3.0 in 85% acetonitrile. Blue circles = basic solutes; black squares = acidic solutes; red triangles = neutrals. Adapted with permission from reference 2.

atively restricted as a result of solubility problems in high concentrations of acetonitrile; ammonium formate (AF, ${}_w^w pK_a$ = 3.75) and ammonium acetate (AA, ${}_w^w pK_a$ = 4.75) are commonly used. In addition to favorable solubility, these buffer salts are volatile and thus useful for mass spectrometry (MS) detection. High concentrations of acetonitrile do, unfortunately, influence pH measurement. Typically, pH is measured in the aqueous component of the mobile phase (${}_w^w$ pH), but is more informatively measured after addition of the organic solvent, directly in the final mixture (${}_w^s$ pH). This measurement (by use of published delta "correction" factors) can be related to the true thermodynamic pH of the solution (${}_s^s$ pH):

$$ {}_s^s pH = {}_w^s pH - \delta \qquad [2] $$

The ${}_s^s$ pH ultimately determines the retention properties of the solute in the system. Correspondingly, the ionization of buffers is also governed by their ${}_s^s pK_a$ values. Unfortunately, these values are rarely available, leading to the widespread adherence to the (less correct and less informative) ${}_w^w$ pH and ${}_w^w pK_a$ values. This difficulty can be considered a disadvantage of the HILIC technique, because it complicates interpretation and prediction of retention.

Effect of Buffer pH

McCalley studied the use of volatile AF, AA, and ammonium bicarbonate buffers at ${}_w^w$ pH 3.0, 4.4, 6.0, and 9.0 for the analysis of acidic, neutral, and basic solutes on a relatively inert amide column, containing a low concentration of acidic silanols and a neutral bonded ligand (see Figure 2). The neutral solutes, thiourea (peak 3) and uracil (peak 4), showed little variation in *k* over the entire pH range. The bases, procainamide and nortriptyline (peaks 5 and 6), gave increased retention as the pH was increased from 3 to 6. It is possible that increased silanol ionization over this range produces increased ionic retention of these basic solutes. At pH 9, however, their retention dropped, probably as a result of suppression of solute ionization and thus diminution of ionic attraction of ionic attraction in addition to reduced solubility in the aqueous stationary phase layer. The strong sulfonic acids (peaks 1 and 2) showed little retention, which further decreased as the pH was raised from 3 to 9, attributable to increased solute repulsion of these fully ionized acids from increasingly ionized silanols as the pH was raised. The weak acid 4-hydroxybenzoic acid (peak 7) showed increased retention as the pH was raised from 3 to 6. The increase in pH causes increased solute hydrophilicity, reflected in a decreased log *D* value as the solute was increasingly ionized. It may also encourage hydrogen bonding between ionized acidic solute and neutral polar column groups. However, at pH 6, the acid was mostly ionized and repulsion effects from increasingly ionized silanols dominated, causing reduced solute retention at pH 9. The retention of many weak acids on silica-based columns can be explained by a balance of the opposing effects of increased ionization and hydrophilicity against increased repulsion as the pH is raised (4).

Changing the mobile phase pH is a powerful means of adjusting the selectivity in a HILIC separation, even when restricting the choice to volatile "mass spectrometer-friendly" buffer systems.

Effect of Buffer Concentration

An increase in volatile buffer concentration of AA or AF tends to increase the retention of neutral compounds. This result may be a result of the salt increasing the volume of water in the stationary phase layer, a selective process that may depend on the nature of the salt. For basic compounds, retention decreases with increasing buffer concentration on silica-based columns, as a result of the competitive interaction of the buffer cation with ionized stationary phase silanols.

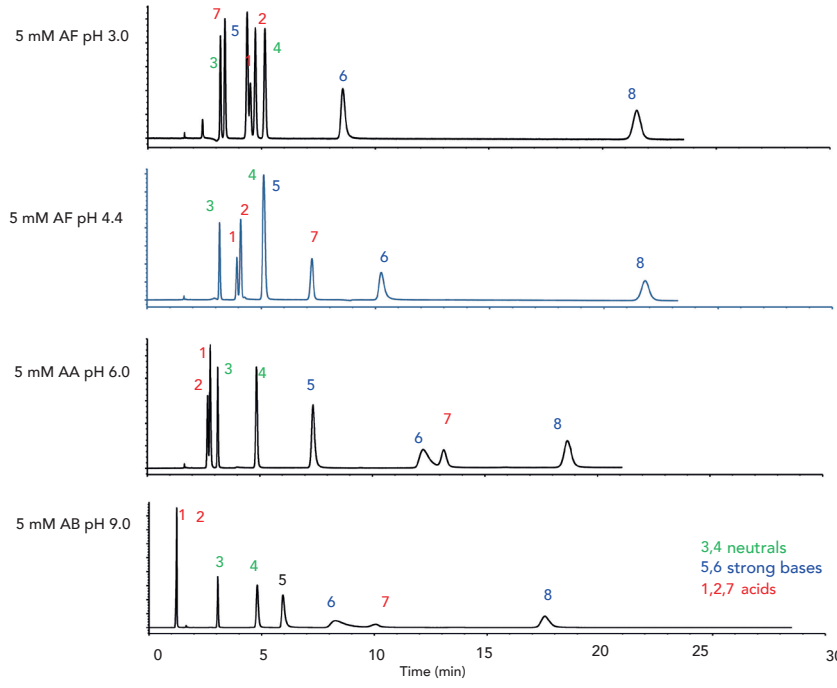

FIGURE 2: Chromatogram of a mixture of acidic, basic, and neutral test compounds on a BEH amide column using 95% acetonitrile containing 5 mM ammonium formate buffer $_{w}^{w}$pH 3 and 4.4, ammonium acetate buffer pH 6.0, and ammonium bicarbonate buffer pH 9.0. Peak identities as Figure 3. Adapted with permission from reference 4.

Conversely, the insulating effect of buffer ions on charged silanols results in reduced repulsion from the column and a higher retention of acidic solutes. Clearly, ionic interaction effects (both attractive and repulsive) could be minimized, or in some cases even eliminated, by the use of high buffer concentrations, although their deliberate suppression is questionable because they can give rise to useful selectivity effects. In addition, high buffer concentrations are undesirable for use in liquid chromatography (LC)–MS because they can suppress solute ionization, and thus reduce detection sensitivity (3).

Alternative Buffers to AA and AF

Simple organic acid additives, such as 0.1% formic and acetic acid, have been employed instead of salt buffers because they give reduced ion suppression effects in MS; unfortunately, they can give poor peak shapes for some acidic and basic solutes. The inferior peak shapes may be a result of the very low ionic strength of simple acids, especially in high concentrations of acetonitrile, causing overloading effects even at relatively low solute concentrations (5). These effects may be in addition to the lack of deactivating effect of the ammonium ion (present in the usual salt buffers) on ionized silanols. Stronger acids (for example, 0.1% trifluoroacetic acid [TFA]) give access to lower $_{w}^{s}$ pH values while retaining sufficient ionic strength in high concentrations of acetonitrile to yield satisfactory peak shapes (6). TFA may also improve peak shape through ion pairing effects. TFA gives unusually increased retention of strongly

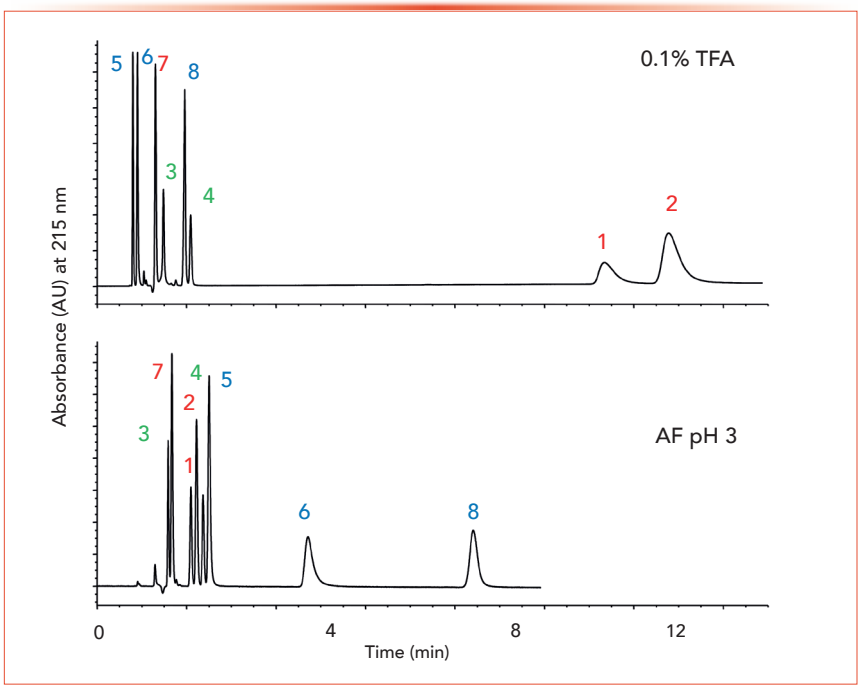

FIGURE 3: Chromatograms of acidic, basic, and neutral solutes on a glycan (amide shell) columns (Agilent) using 95% acetonitrile containing 0.1% trifluoroacetic acid or 5 mM ammonium formate pH 3.0. Peak identities: 1 = p-xylenesulfonic acid; 2= naphthalene-2-sulfonic acid; 3 = thiourea; 4 = uracil; 5 = nortriptyline; 6 = procainamide; 7 = 4-hydroxybenzoic acid; 8 = cytosine. Adapted with permission from reference (6).

acidic solutes on silica-based columns, partially as a result of reduction of silanol repulsion. In addition, the column surface may become positively charged, attracting anionic solutes (see Figure 3).

Alpert explored a much more diverse series of buffer compounds, including nonvolatile salts that are not suitable for mass spectrometry (7). In particular, he aimed to study the effects on retention of kosmotropic ions, such as sulfate, which are well hydrated, and chaotropic ions, such as perchlorate, which are poorly hydrated. The former was found to promote partitioning of charged solutes into the immobilized aqueous layer. The effects on neutral solutes were more modest; retention times were unchanged or increased slightly with an increase in concentration of any salt. Concentrations of salt ranged from 5 to 120 mM at $_{w}^{w}$ pH values of both 3 and 6.

The Stationary Phase

Stationary phases can be divided broadly into those that have an essentially neutral surface, for example, amide or diol; those that have an acidic surface, for example, poly(2-sulfoethyl), which possesses sulfonic acid groups; and those that are basic, for example, those with alkyl amino groups. There are also other categories, such as zwitterionic columns, that can behave in a somewhat similar way to neutral columns, because of the proximity of negatively and positively charged ligands (but with some superimposed ionic retention behavior). Bare silica columns are another distinct group that show acidic properties, because of the presence of negatively charged silanol groups, particularly at higher pH. Principal component analysis (8) or correlation analysis considering retention data for different probe solutes has demonstrated that columns in these various categories show distinct retention behavior.

The retention of neutral solutes is influenced by partitioning into the water layer on the column surface and it is therefore hardly surprising that their retention is affected by the thickness of this layer, which varies on different columns. Commercially available polymeric zwitterionic phases have an extensive water layer, which may result from their thick layers of bonded polymeric stationary phase ligands. In comparison, diol phases and silica hydride have thinner water layers. Adsorption may be a more important mechanism here, especially on the latter phase. Neutral solute retention is affected by water layer thickness and can be assessed by measuring the hydrophilic selectivity of the phase αOH (9):

$$\alpha_{OH} = k_{uridine}/k_{deoxyuridine} \quad [3]$$

Dexyuridine has one less –OH group than uridine, resulting in less hydrophilicity and reduced retention compared with uridine. Measurements of this parameter show a broad correlation with estimates of water layer thickness (2). For example, using 5 mM AF pH 3.0 in 95% acetonitrile gave αOH as 1.6, 2.3, and 3.0 for a bare silica, amide, and zwitterionic phase, respectively. Clearly, the zwitterionic phase should give the greatest retention of neutral solutes.

The propensity of a phase to retain cationic solutes over neutral solutes can be assessed by measuring its cationic selectivity αCXT:

$$\alpha_{CXT} = k_{TMPAC}/k_{uridine} \quad [4]$$

where TMPAC is trimethylphenylammonium chloride, a quaternary salt that is ionized under all conditions. Using 5 mM AF in 85% acetonitrile, silica, zwitterionic, diol, and amide columns gave values of 7.1, 1.0, 0.6, and 0.5, respectively, for this parameter, demonstrating considerable preferential retention of cationic solutes on bare silica. Similarly, the anionic selectivity of columns can be measured from:

$$\alpha_{AS} = k_{BSA}/k_{uridine} \quad [5]$$

where BSA is the strong acid benzenesulfonic acid. Once again when using 5 mM AF in 85% acetonitrile, amino and amide columns gave values of 6.6 and 0.33, respectively, demonstrating the considerably increased selectivity for acids of the amino phase (4).

Changing the stationary phase is probably the most effective way to change the selectivity of the separation (2), as shown in Figure 4 for neutral uridine, the acid 4 –hydroxybenzoic acid, and the base nortriptyline (10). Both bare silicas show preferential retention of nortriptyline because of attractive interactions with negatively charged silanols. The low pore occupancy of water in silica columns (7–9% in 89% acetonitrile [11]) and their poor hydrophilic selectivity explains their low retention of uridine. Bonded phases have fewer silanols, which may in addition be partially shielded, resulting in low retention of nortriptyline on the zwitterionic and amide phases. Reduced silanol interactions and good hydrophilic selectivity on these phases may also explain their improved retention of neutral and acidic probes. A "toolbox" of phases of different selectivity might first contain the neutral and quasi-neutral amide and zwitterionic columns, which are good "general-purpose" phases. A bare silica column or phase containing bonded acidic groups (for example, sulfonic acid) should be included to give good retention or separation of bases and an amino column could be used for the separation of acids. The substantial variation in selectivity between different columns in HILIC is an advantage over reversed-phase methods, where interchange of stationary phases has a relatively smaller effect on selectivity.

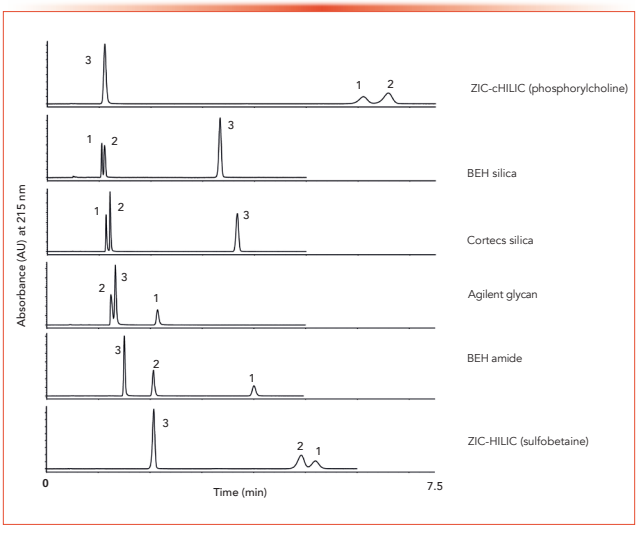

FIGURE 4: Selectivity of different columns using mobile phase 5 mM ammonium formate pH 4.4 in 95% acetonitrile. Peak identities: 1 = uridine; 2 = 4-OH benzoic acid; 3 = nortriptyline. Adapted with permission from reference (10).

Advantages of HILIC

As well as the high retention of polar and ionized compounds, the complementary nature to reversed-phase chromatography, and the beneficial changes in selectivity of different stationary phases, HILIC demonstrates a number of other advantages over reversed-phase chromatography.

Low Viscosity of the Mobile Phase

Many of the advantages of HILIC stem from its use of high concentrations of acetonitrile (typically 60–95%) in the mobile phase, giving low viscosity, and allowing the use of long columns, or fast flow with conventional columns. A 45 × 0.46 cm column packed with 2.7-µm shell particles operated at a flow of 2.0 mL/min generated over 100,000 theoretical plates for four basic drugs in a reasonable analysis time (<15.0 min) (12). For solutes amenable to either HILIC or reversed phase (such as moderately hydrophobic base nortriptyline), the organic-rich mobile phase used in the former technique provides increased solute diffusion and thus flatter van Deemter curves in the *C*-term region (mass transfer). However, if the increase in solute diffusion is factored out using reduced plots, reversed phase shows a slight advantage in the *C*-term region at fast flow rates. This means that a somewhat improved performance at high flow rates is obtained for hydrophobic solutes using reversed phase rather than hydrophilic solutes using HILIC (13). However, HILIC is clearly favoured in many cases by the possibility of longer columns (at normal or reduced flow) to generate high efficiency.

Peak Shape of Ionogenic Compounds

Peak shapes of basic compounds can be surprisingly good in HILIC. The basic drugs diphenhydramine, procainamide,

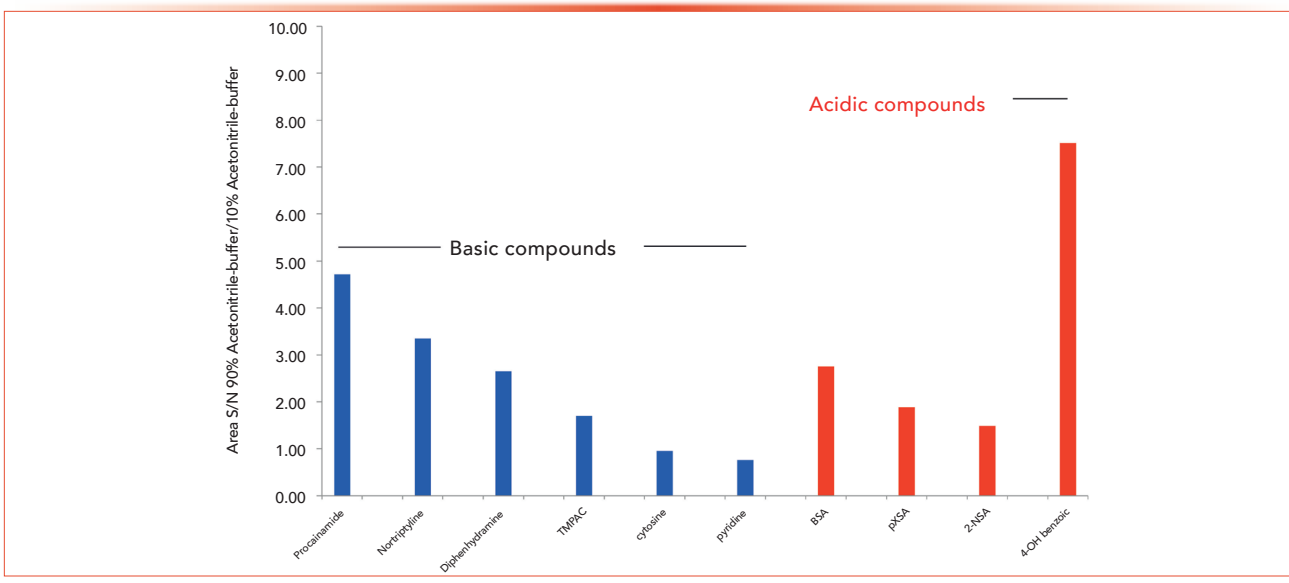

FIGURE 5: Signal-to-noise ratios using flow injection analysis coupled with optimized MRM triple quadrupole mass spectrometry for ten solutes with a HILIC mobile phase (5 mM ammonium formate pH 3.0 in 90% acetonitrile) and a reversed phase (5 mM AF pH 3.0 in 10% acetonitrile).

and nortriptyline gave excellent peak shapes using a bare silica column with a simple AF buffer pH 3 in acetonitrile. This column has a high cationic selectivity; in reversed-phase chromatography, strong ionic interactions can be associated with poor peak shape, which is not always true in HILIC. Good peak shapes may be associated with higher sample capacity in HILIC, with column efficiency being maintained at much higher solute mass than in reversed phase (14).

Compatibility with Electrospray Ionization Mass Spectroscopy (ESI-MS) and Other Evaporative Detectors

The low viscosity, high volatility, and low surface tension of high concentrations of acetonitrile are conducive to higher sensitivity in electrospray ionization mass spectroscopy (ESI-MS) (15).

Figure 5 compares the relative signal-to-noise (S/N) ratio for six basic and four acidic solutes by flow injection analysis (FIA) ESI-MS using a triple quadrupole MS and multiple reaction monitoring (MRM) conditions optimized for each solute. On average, a sensitivity increase of ~3× for the HILIC conditions (90% acetonitrile–10% 5 mM AF pH 3.0) compared with reversed-phase conditions (10% acetonitrile–90% 5 mM AF pH 3.0) was obtained. The use of FIA avoids introduction of the samples into a chromatographic surface, which potentially might confound the results with adsorption effects. Gradient elution of mixtures of solutes using an appropriate column can also be used to assess difference in detection sensitivity. While possible column adsorption effects may indeed confound the results, as well as possible elution in different mobile phase composition (dependent on the column), this method can be used to evaluate the response of solutes in mixtures simultaneously, as they can be separated on the column. As FIA has no separation stage, solutes are best evaluated individually, making the procedure more laborious but not susceptible to variable chromatographic effects. The average gain in sensitivity was reported as 7–10 times (16), but improved modern interface designs showed more modest gains (17). Similar beneficial increases in sensitivity can be obtained with other mobile phase evaporation detectors, such as the charged aerosol detector (18).

Disadvantages of HILIC and How They May be Overcome

Sample Injection

Using injection solvents of higher eluotropic strength than the mobile phase (that is, increased water concentration) gives increasing deterioration in peak shape (19). This effect can be problematic if the sample is not soluble in high concentrations of acetonitrile (that is, in appropriately "weak" mobile phases). The effect can be moderated by injecting small volumes. Alternatively, for small-molecular-weight (MW) compounds, isopropyl alcohol (IPA) or a mixture of 50:50 acetonitrile–IPA has been recommended. For drug discovery applications, dimethylsulfoxide in at least 80% acetonitrile can be used, whereas for peptide analysis, pure ethanol or IPA is possible (20).

Use of the mobile phase or a weaker solvent as an injection solvent to avoid peak distortion is not normally possible with biopharmaceuticals because of limited solubility in high concentrations of acetonitrile and possible protein denaturation or precipitation. Therefore, in one study, an aqueous sample was injected followed by a fast gradient ramp incorporating a high percentage of acetonitrile at the beginning of the method,

in addition to a small injection volume (21), which produced good results.

Long Equilibration Times

Full isocratic equilibrium of the column (where retention times stabilized to 99–101% of the value at "infinite" equilibration time), with buffered acetonitrile mobile phase, can require more than 20 min (> 40 column volumes) when purging the 10× 0.21-cm columns at 0.5 mL/min (10). In isocratic analysis, full equilibration is necessary because the selectivity of the separation can change with equilibration time. Full equilibration was found to depend on the nature of the HILIC stationary phase, the purging flow rate, and the original or "storage" solvent. These long equilibration times are not, however, a barrier to the use of gradient elution in HILIC. In gradient elution, a repeatable partial equilibrium was demonstrated (10) in an equilibration time of as little as 5 min, implying that HILIC can be reliably used under these conditions. Selectivity changes can occur in the separation dependent on the particular equilibration time between gradient runs. Therefore, this parameter must be kept constant in a series of analyses; however, this does not appear to be a problem when using modern HPLC instruments.

Retention Time Instability and Drift

HILIC has sometimes been found to suffer from irreproducible or drifting retention times. This problem is often associated with insufficient equilibration of the column (see above), especially in isocratic applications. However, one study found retention time irreproducibility was associated with storage of the (organic-rich) mobile phase while connected to the instrument, rather than in tightly sealed bottles, which gave excellent day-to-day repeatability of retention (22).

Extra care may be necessary in mobile phase preparation. The commonly used buffer salts AF and AA are hygroscopic and should be stored in a desiccator at room temperature to improve reproducibility of buffer preparation. For isocratic analysis using high concentrations of acetonitrile (low concentrations of water), some consideration should be given to preparing the mobile phase by premixing aqueous and organic liquids by weight, taking into consideration the density of the liquids. Errors might otherwise result if metering relatively small volumes of aqueous phase using the HPLC instrument.

In gradient analysis, particularly with high-pressure mixing systems that use a separate pump for each solvent flow, the use of one channel delivering small volumes (difficult to achieve reproducibly and accurately) throughout the gradient run should be avoided. This is especially true when the total flow is relatively low, which is necessary with a small-diameter column, and when a relatively shallow gradient is employed, as is often the case with HILIC. Therefore, a gradient from 90% to 80% acetonitrile is best not devised with bottle A containing 90% acetonitrile and bottle B 0% acetonitrile at a total flow of 0.4 mL/min. This would result in pump B delivering only ~0.045 mL/min, even at its maximum flow at the end of the gradient.

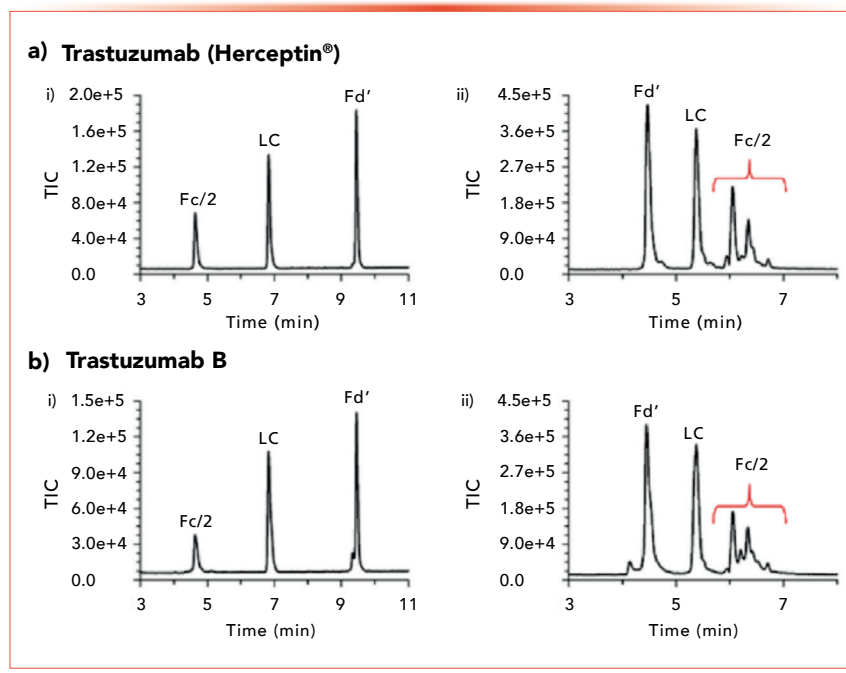

FIGURE 6: Middle-up analysis of (a) Trastuzumab and (b) Trastuzumab B (biosimilar). Total ion chromatograms using i) reversed-phase LC–MS and ii) HILIC–MS. Adapted with permission from reference (21), copyright 2016, American Chemical Society.

Some New Applications of HILIC

Analysis of Glycans "Bottom-Up" Methods

HILIC has established itself as an essential technique for monitoring glycans in monoclonal antibody (mAb) drugs designed to target specific antigens (mAbs have MW ~150,000, of which about 5% by weight can be glycans). Much analytical work needs to be performed in the characterization of mAbs or their biosimilars that have similar efficacy and safety to the original drug, or biobetters, which are improved products. Many of the original biopharmaceuticals are coming off patent, giving scope for the development of these substitute drugs. Glycosylation is one of the important causes of microheterogeneity caused by post-translational modification (PTM) that can occur, for example, during production and storage; thus, its characterization is of great importance to enable potential differences in the products to be assessed.

Glycans may be attached to the Fc fragment of the mAb. A glycan is a mono-, poly-, or oligosaccharide, but typically contains ~10 monosaccharides. *N*-gly-

cans can be cleaved from the mAb by an enzyme, such as PNGase-F. The resulting free *N*-glycans can be analyzed in their native state or reacted with 2-amino benzamide (2-AB) or procainamide to give sensitive fluorescent derivatives. However, 2-AB derivatives are difficult to detect by ESI-MS because of poor ionization efficiency. An alternative derivative after PNGase-F deglycosylation, which apparently gives both good fluorescent and ESI-MS sensitivity, has recently been proposed (23). In addition to releasing *N*-glycans from IGg Fc domains, the proposed approach also produced complete release of Fab domain *N*-glycans. Gradient elution analysis on a wide-pore (300 Å) amide column was used in their analysis. Complete release of *N*-glycans from Fab domains was obtained using this procedure with HILIC–MS, showing a peak of mass 148.4 kDa before deglycosylation and 145.3 kDa after, implying the loss of two N-glycans.

Characterization of Intact or Large Fragment Protein Biopharmaceuticals and mAbs

Guillarme and co-workers (24) employed a wide-pore sub-2-µm amide HILIC column to successfully characterize intact and digested (25–100 kDa fragment) protein biopharmaceuticals using gradients of 65–80% acetonitrile and 0.1% TFA. The 300-Å pore size packing allowed the accommodation of large biomolecules and fragments without resulting in restricted diffusion. The separations were reported to be highly orthogonal to reversed-phase LC, while the kinetic performance remained comparable. The authors stressed the following advantages of HILIC: i) compatibility with MS, ii) reduced requirement of high temperatures that are necessary in reversed-phase LC to limit undesirably strong adsorption, and iii) the possibility of coupling columns in series to gain extra resolving power. Applications were shown to the analysis of six different insulins (one of the oldest biopharmaceuticals, RMM~6000); reversed-phase LC and HILIC were shown to be complementary, with better separation of insulin and insulin glulisine by HILIC, but superior separation of insulin and insulin lispro by reversed-phase chromatography. High efficiencies were obtained in both (isocratic) analyses. The authors compared characterization of trastuzumab by HILIC, reversed phase, and ion exchange, even gaining some results with intact proteins, although reduction of disulphide bonds prior to chromatography or partial digestion of the mAb typically yields better results in terms of both chromatographic and mass spectrometry characterization. Trastuzumab (Herceptin) is a mAb widely used for the treatment of some types of breast cancer. Whether these large molecules are denatured during separation (which should still allow for their analytical—if not their preparative—separation, retaining their biological activity) remains to be confirmed.

HILIC–MS was used to compare originator and biosimilar therapeutic mAbs at the intact and the so-called "middle-up" level of analysis, again using a wide-pore 300-Å amide column (21). While "bottom-up" analysis (where the sample is processed to give the simplest building blocks, for example, liberated peptides or glycopeptides) is most often used as the subsequent analysis of these smaller molecules is easier, it loses structural information on these complex molecules. In the HILIC analysis, 0.1% TFA was again used as mobile phase additive, even though it can cause some ion suppression in MS detection. It was preferred due to its solubilizing effect on proteins and its low pH suppression of silanol ionization. The intact mAb was digested, then reduced to give Fd', light chain (LC), and Fc/2 subunits (two of each) of about 25 kDa, containing attached intact glycans, and then subjected to reversed-phase and HILIC analysis. The two techniques were complementary, as can be seen in Figure 6, which shows middle-up separations of Trastuzumab (Herceptin) and its biosimilar Trastuzumab B. Reversed-phase analysis gave a separation of the three main fragments, but offered information about the glycosylation pattern only after examination of the MS data. In contrast, HILIC–MS on the same sample allowed for a direct and immediate comparison of the glycosylation profiles. Furthermore, Guillarme (25) demonstrated the use of HILIC coupled with MS to characterize the antibody-drug conjugate (ADC) Brentuximab vedotin, which is used in the treatment of Hodgkin's lymphoma. ADCs enable the delivery of cytotoxic drugs (present in this case with an average drug-to-antibody ratio of four) to therapeutic targets with an antibody-directed mechanism. As with mAbs, these materials need to be characterized because of the structural complexity and heterogeneity. A middle-up approach with fragment (~25 kDa) analysis using a 300-Å amide column and a gradient of 85–73% acetonitrile with 0.08% TFA and 0.02% formic acid was used. It was found that HILIC analysis offered a completely complementary and orthogonal set of information to reversed-phase analysis; elution order was essentially the opposite to that observed in reversed phase. Only one HILIC–MS run was necessary to obtain highly important information on structural microheterogeneity.

HILIC in Metabolomics

The human metabolome consists of small molecules—generally accepted as having a MW <1500 Da—dictated by the genes of the individual and also the individual's environment, and therefore it consists of a mixture of endogenous and exogenous compounds. Whereas nuclear magnetic resonance (NMR) spectroscopy provides standard analytical methodology common to most samples and does not require analytical development, it suffers from spectral overlap and low sensitivity. Thus, MS, which is considerably more sensitive, coupled with separation techniques such as gas chromatography (GC) and LC provides a valuable alternative to NMR. LC clearly has broader application possibilities than GC because it is amenable to nonvolatile, highly polar, and thermally labile samples without derivatization. The complementary use of HILIC and reversed-phase chromatography coupled with MS expands the number of detected analytes and provides considerably more comprehensive analyte coverage (26). Important classes of compound that

have been analyzed by HILIC include phospholipids, which are the main constituents of biological membranes. They are important signalling molecules and potential biomarkers for ovarian cancer, diabetes mellitus, and other conditions. They consist of a polar phosphate head group, two fatty acid chains, and a glycerol group. HILIC eluents are more compatible with ESI-MS than normal-phase chromatography eluents, which are traditionally used for these solutes. Typical mobile phases are acetonitrile containing AA or AF buffers, in conjunction with bare silica or diol columns. Organic acids, sugars, amino acids, nucleosides, and nucleotides, which can be biomarkers of inherited metabolic disease, are other examples of metabolites amenable to HILIC. A comparative study of the analysis of 764 metabolites using either HILIC or reversed-phase analysis showed that HILIC methods markedly improved the coverage of polar metabolite groups, such as phosphates or carbohydrates, and therefore represented a worthy alternative to reversed-phase separations (27). Zwitterionic sorbents, such as those containing sulfobetaine groups, had a particular broad application range. In addition, selectivity was highly diverse among the HILIC methods investigated (which used different stationary phases). For example, an amide column gave good retention of nucleosides, whereas a phophorylcholine sorbent was most appropriate for the separation of carbohydrates.

A further advantage of HILIC over reversed phase in metabolic studies appears to be that glycerophospholipids, generally observed in cell and plasma samples, tend to appear in narrow retention time ranges instead of covering major parts of the retention window, which is often found for reversed-phase separations (28). These compounds give extensive ion suppression in ESI-MS. For analysis of a broad range of metabolites, the risk of suppression increases with increased spreading of the retention window of the interferents. In the same article, considerable differences in selectivity for metabolites and matrix interferents were shown between bare silica, zwitterionic, and amide HILIC stationary phases. Thus, a particular HILIC column may be the optimum for each individual application.

Conclusions

HILIC has become an indispensable technique for the analysis of polar and ionized solutes poorly retained by traditional reversed-phase methods. For samples amenable to both HILIC and reversed-phase analysis, the techniques show a complementary nature. In fact, for such samples, the use of HILIC may be advantageous because of the favorable coupling with MS and other evaporative detectors, the low viscosity of the mobile phase (allowing the use of long columns), and good peak shapes for some basic pharmaceuticals. The mechanism of HILIC separation, however, appears complex, which can pose a barrier to the more widespread adoption of the technique. Nevertheless, a greater understanding of the effect of some simple parameters could lead to more straightforward method development. Problems such as longer equilibration times are not a barrier to the use of gradient methods. Some new applications, such as the characterization of biopharmaceuticals, and its use in metabolomics, indicate good potential for the use of the technique in these areas, where it is complementary to reversed-phase methodology.

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Advancing Separation Science Throughout a Lifetime of Achievements: Daniel W. Armstrong, the Winner of the 2020 Lifetime Achievement in Chromatography Award

Daniel W. Armstrong, of the University of Texas at Arlington, has a lifetime of achievements in separation science, including his most widely recognized contributions in the field of enantiomeric separations. His contributions are known in the fields of molecular recognition (chiral and isomeric), ionic liquids (synthesis, characterization and use), ordered media (including micelles and macrocyclic compounds), enantiomeric separations, mass spectrometry (MS), and applied work in drug development, environmental, and food analysis. He is the winner of the 2020 *LCGC* Lifetime Achievement in Chromatography Award, which honors an outstanding and seasoned professional for a lifetime of contributions to the advancement of chromatographic techniques and applications. He recently spoke to *LCGC* about his work and his career.

Jerome Workman, Jr.

Many consider you to be the "father" of micelle and cyclodextrin-based separations, because your work elucidated the first chiral recognition mechanism by cyclodextrins (1–4). You have many publications related to this subject. What papers represent the most seminal work in this area? What prompted you to investigate this separations approach? What was your most surprising discovery from this work?

While it was not our first publication on cyclodextrin isomeric liquid phase separations and chiral recognition, our 1986 *Science* paper (5) probably received the most attention for a number of reasons. We easily separated underivatized drug enantiomers. It was the first paper of its type on associative small-molecule molecular modeling with energy minimization calculations. At that time there were only two computer systems in the world that could do this. We compared two cases where there was chromatographic retention, but only one was enantioselective. We then definitively showed why this occurred. The knowledge gained from this study led to the successful development of a number of new chiral selectors. We were told that this paper, in part, provided impetus for the FDA to pass their 1992 guidelines for stereoisomeric drugs.

The 1992 *Analytical Chemistry* paper (6) was a mechanistic study involving gas chromatography (GC). We showed that there were multiple enantioselective retention mechanisms and that inclusion complexation was not necessary in many cases. Subsequently, this was shown to be true for liquid chromatographic (LC) separations as well. This work was preceded by an important theoretical study (7) that doesn't often get its due credit because it was well beyond the simple practical applications focus of the time. In late 1983, cyclodextrin-based LC stationary phases became the first reversed phase "chiral columns" to be commercialized (by Advanced Separation Technologies).

You and your research group were the first to develop macrocyclic antibiotics as chiral selectors, and you are recognized as one of the world's leading authorities on the theory, mechanism, and use of enantioselective molecular interactions (8). This work has been cited numerous times by other researchers. How was your specific research approach different from your contemporaries at that time?

Early on, it was never our goal to develop columns for practical use or for commercial purposes. We were interested in understanding molecular recognition. That remains a prime focus of much of our work. Of course, there is a correlation between enhanced molecular recognition and chromatographic selectivity. Also, chromatography provides a simple way to study the effects of different solvents, salts, temperatures, and so forth on chiral recognition. It also provides a means to obtain relevant physicochemical information. The macrocyclic glycopeptides (antibiotics) are a case in point. They probably provide a greater variety of functionality than any other chiral selector and in a relatively compact "package". Consequently, they may be the most broadly selective class of chiral selectors, however, their chiral selectivity differs considerably in different chromatographic modes and conditions. How and why this occurs can provide numerous avenues for research. Such studies have led us to synthetically modify essentially all chiral selectors that we have worked with. Indeed, modified chiral selectors often provide the more interesting results. Proper synthetic changes can enhance beneficial properties and often, just as importantly, dimin-

captioncredit_photo

ish unwanted properties of a chiral selector. This entire process leads to a better understanding of chiral recognition and enantiomeric separations. Of course, it would be short-sighted to ignore the obvious practical consequences of this work.

What do you consider the most important research publications you have produced relative to chiral separations over the last two- and one-half decades of your research in this field?

So, this is research that was published after the initial exciting and transformative decade of "chiral separations." Certainly, the first paper on macrocyclic antibiotics as chiral selectors appeared just after that time period (9). The first cyclofructan based stationary phase represents the most recent major class of chiral selectors published (10). Perhaps the most important, relatively recent, advance in chiral separations has not been in new chiral selectors or chiral recognition theory, but rather the advent of new "high efficiency" supports, such as superficially porous particles. This moved enantiomeric separations into the realm of ultrafast and even sub-second separations as we demonstrated in 2015 (11) and 2018 (12).

There is one area which much of this research impacts that I have been convinced for years is important but has been relatively ignored. It is D-amino acids in biological systems. The importance of D-amino acids is slowly being recognized in many different fields. Our 2017 paper on this topic (13) is a case in point. Such biological studies are becoming more prevalent over the last several years and will continue to escalate in number and importance.

Your work to characterize the solvent properties of room-temperature ionic liquids (ILs) has become essential to multiple fields in chemistry (14–16). What do you feel are the most important applications of room-temperature ILs for the analytical sciences?

Research involving ILs is pervasive and has impacted most areas of science and technology. Certainly, ILs in synthesis and process chemistry have received a lot of attention, but important advances in the analytical sciences also have been reported over the last two decades. There are several reviews on the topic. The intrinsic properties of ILs have made them useful in electrochemistry and battery research. All types of sensors have used ILs to broaden and improve their utility. Applications in areas of spectroscopy have been reported. Of course, ILs have become very important in separation science, no more so than in extractions, headspace solvents and as stationary phases for GC. We have been involved in the later areas of this research. We have probably synthesized and tested a greater variety of ILs than any other laboratory. Our goals were to knowledgeably enhance certain desired properties of ILs. These properties included thermal stability, fluidity, viscosity, site specific interactions, and so forth. As a practical consequence of our work, the first new class of commercial GC stationary phases in several decades was produced. This includes the most polar stationary phases known. IL stationary phases are often preferred in comprehensive GC×GC. Their nonvolatile nature makes them exceptional headspace solvents. Also, they are preferred for the GC analysis of water.

You and your research team have developed the new enhanced mass spectrometry (MS) technique of (paired ion electrospray ionization (PIESI) as one of the most sensitive methods for ultra-trace anion analyses and speciation (17,18). What are some of the greatest insights or advances that the development and application of the PIESI technique has allowed you to discover?

Our PIESI work evolved from our synthetic work on dicationic and polycationic ionic liquids. My good friend and colleague Sandy Dasgupta thought that very dilute solutions of such molecules could be useful for electrospray ionization (ESI)-MS detection of perchlorate in the positive mode. Indeed, it worked beautifully and easily provided sub parts per trillion detection limits. We went on to test our di- and trications (as fluoride ion salts) on a large variety of anions and their utility was quite broad. However, the structure of the PIESI reagents made a significant difference as to their efficacy. This led to our studies on the "PIESI mechanism." By understanding the mechanism, we were able to synthesize the next generation of PIESI reagents, which became available commercially. This technique has been applied to all manner of anionic species from simple inorganic ions to pesticides (and their degradants), phospholipids, nucleotides, anionic drugs, and chelated metal ions.

You have developed the first high efficiency capillary electrophoresis (CE) separation approach for microorganisms, such as bacteria, viruses, and fungi (19,20). How has this breakthrough extended the use of separation science into the mainstream of biology and colloid science?

Doing CE on intact microorganisms was very different than analyzing molecules in that molecules tended to behave much more predictably and reproducibly. Such is the difference between chemistry and biology. However, the results were so interesting and potentially important that we had to push on and see where it would lead. In this technique the term high efficiency must be qualified as under many conditions, focusing of the microorganism occurs and so a true capillary column efficiency cannot be obtained. Regardless, this work provided a new, nontraditional (for biology) approach for the analysis and quantification of microbes. It also can be used to determine whether the analyzed microorganisms are alive or dead and can give the percentages of each. Since laser-induced fluorescence (LIF) detectors can detect a single cell, this approach can be used as a test for sterility of samples. It has even been used on sperm cells as a fertility test. Today, we more often see such microbial studies done in a microfluidic or chip-based format.

What do you consider to be the most important new areas of research in the chromatography field? What do you see as your greatest contribution to the field?

I sincerely hope one area will be molecular rotational resonance spectroscopy-based detectors for GC and LC, since we just did the first paper on it (21). This technique can have greater specificity than high-resolution MS and nuclear magnetic resonance (NMR). High-speed separations and multidimensional separations will continue to grow. Separations-based sensors could be very important. Separations-based diagnostic devices may be the wave of the future if they are simple, sensitive, selective, and effective.

I've worked in many areas and will continue to. It is for posterity to decide the value of any research contributions I've made. That said, perhaps my greatest contribution is the over 100 analytical PhDs I've mentored and nearly an equal number of postdocs and visiting scholars and collaborators. The majority of my graduate students were the first person in their family to go to college at any level. They have all done well and that makes me quite happy.

What words of advice do you have for young researchers just getting started, or even undergraduates considering a future career in science?

I love doing research and working with graduate and undergraduate students. It has been fun and very rewarding. I think it is a great career choice. However, you should know that this is not the only rewarding career for chemistry or other science majors. Approximately 60% of my graduate students work in the pharmaceutical industry and many of those are doing fundamental work in drug discovery and development. Some work for instrument manufacturers, column companies, or consumer product companies. Often, they are "troubleshooters" or problem solvers, a role that involves scientific detective work. I have done a lot of work with brilliant scientists that also have law degrees. They work in patent law and are at the forefront of commercially important science and technology—and they are well rewarded. In all cases, the common factors for success or advancement are working hard, working smart, and constructive imagination.

How do you organize your work schedule to enable you to teach, mentor, guide research, invent, and start new companies? What skill set is needed to accomplish all this?

Basically, you have to be willing to work many weekends, holidays, and nights. Also, it is beneficial to have good people working with you. You have to be able to minimize nonproductive, time-wasting activities such as meetings, most non-research related paperwork, social media, most administrative activities, administrators, and so forth.

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ABOUT THE AUTHOR

Daniel W. Armstrong, the 2020 winner Lifetime Achievement in Chromatography Award winner, received his B.A. degree from Washington & Lee University, Lexington city, Virginia; and his M.S. degree in Oceanography, and a Ph.D. in Chemistry from Texas A&M University. He is the R.A. Welch Distinguished Professor of Chemistry and Biochemistry at the University of Texas at Arlington. He has worked on an extremely broad range of separation techniques including high performance liquid chromatography (HPLC), gas chromatography (GC), supercritical fluid chromatography (SFC); micellar liquid chromatography, thin-layer chromatography; countercurrent chromatography; capillary electrophoresis (CE); and field flow fractionation, among others. He developed the theory and mechanistic background behind many of the practical advances in these techniques. Further, he advanced the use of separations techniques as a means to obtain important physico-chemical data. His most recent work in ultrafast separations and signal processing is driving fundamental changes in the field. Armstrong has over 700 publications, including 33 book chapters, and 35 patents. He has been named by the Scientific Citation Index as one of the world's most highly cited scientists; his work has been cited over 43,000 times, having a Hirsch (h-) index of 105 (G.S.).

Research in Protein Chromatography, Pharmaceutical Analysis, and Fundamental Studies in LC and Method Optimization: Szabolcs Fekete, the 2020 Winner of the Emerging Leader in Chromatography Award

Szabolcs Fekete is currently a scientific collaborator at the University of Geneva. His work focuses on finding new possibilities in protein chromatography; on characterizing therapeutic proteins, on studies of liquid chromatography (LC) column technology, and on method optimization. He is actively investigating aspects of retention modeling and fundamental attributes of LC. He is the 2020 winner of the *LCGC* Emerging Leader in Chromatography Award which 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. He recently spoke to *LCGC* about his current research work and his career aspirations.

Jerome Workman, Jr.

In one of your studies, you experimentally determined the impact of operating pressure on the retention of large solutes (proteins) and selectivity in reversed-phase LC (1). A huge impact was observed even in gradient elution mode, which was not expected. What prompted you to specifically investigate operating pressure as a separation variable? How is what you have discovered different from what others have thought previously?

It is well-known that working at very high pressures affects various chromatographic parameters. There is a possible complication with ultra-high-pressure liquid chromatography (UHPLC) regarding the effect of pressure and mobile phase velocity (friction) on both retention and band-broadening.

First, Giddings showed that increased pressure could induce significant changes in molecular volume and the ability of molecules to crowd together to reduce molecular volume upon adsorption. Later on, Guiochon and McCalley reported interesting results observed in isocratic mode and mostly for small-molecule separations. However, the change in retention and selectivity caused by pressure was relatively minor when eluting small solutes (in the pressure range up to 1000 bar). Our hypothesis was that, in the case of large biomolecules, the changes of molecular conformation caused by the pressure or high flow rates (thermal effects) will play a much more important role in changing retention and selectivity. Especially when considering their "bind and elute" type retention behavior. In our work, this huge conformational change was experimentally verified both by fluorescence emission measurements and by the analysis of native and reduced forms of various proteins which possess the same molecular weight but a different conformation.

Finally, a substantial effect was observed and demonstrated that the operating pressure is a useful method variable to adjust selectivity of large molecule separations. On the other hand, this extreme effect could cause serious issues when transferring previously developed conventional methods to UHPLC method.

You have also published valuable work on the retention modeling of large proteins (monoclonal antibodies and related products such as antibody-drug conjugates [ADCs]) using computer simulation (2), and suggest a generic method development approach, along with platform methods, which could be very useful for industrial pharmaceutical laboratories. How will this new information assist a broader biomedical and pharmaceutical analytical community?

We hope that this information will make the life of practicing chromatographers easier! In the common practice of analytical R&D laboratories located in most biopharmaceutical companies, when developing a new method, several method variables are typically screened over a wide range. This is time consuming and above all unnecessary in many cases. As an example, we often see that people optimize their mobile phase temperature for antibody separations, in reversed-phase mode, over a low temperature range such as 30 to 60 °C. When they do not see peaks in their

chromatogram, they ask us what is wrong with their separation. We only need to suggest that they perform one experiment at 80 or 90 °C, after which, miraculously their protein elutes in a sharp peak.

Therefore, we promote a generic method development approach which is based on the fact that proteins of the same, or similar, family (for example, mAbs or cys-linked ADCs) show very similar retention behavior. By knowing this, once you have developed a method for one mAb then it is possible to use these insights for another protein as it will–elute with similar mobile phase strength, show good recovery only at high temperature (75 to 90 °C), and only elute in a sharp peak when using an ion-pairing agent and low pH. Considering these facts, at the end you only need to perform very few experiments (in a more limited range of two or three method variables) as input for further method optimization. The same input experiments can be performed for any mAb – no need to study again the impact of method variables. Therefore, to optimize the method, retention modeling (computer simulation) can indeed be helpful. It is true that in a wide range of method variables, the retention behavior of large proteins is complicated due to possible (either reversible or irreversible) conformational changes (caused by pressure, temperature, organic solvents, pH, and so forth) which impacts the molar volume and thus the retention. Consequently, accurate modeling of retention in the full range of method variables is most likely impossible. Fortunately, as explained previously, there is no need to model retention in a large design space, as only a limited range needs to be modeled. In that limited range, the most common models (the stoichiometric displacement model, linear solvent-strength model, adsorption model, and so forth) describe the retention of proteins very precisely. For such optimizations, any chromatographic modeling software (such as DryLab) or even some less specific software can be applied (for example, Statistica or even MS Excel).

It is worth mentioning that the effect of operating pressure and the heat developed by friction can also be modeled for protein separations, however this requires more experiments and more sophisticated models.

In other work you have carried out fundamental studies in which the effect of longitudinal temperature gradient on retention, caused by frictional heating, was experimentally dissociated from the combined effect of pressure and frictional heating (3). Through this work, the specific contributions of these effects to the overall retention were determined for both small and large solutes. Would you explain the significance and meaning of this work for the readers of *LCGC North America*?

The interesting thing here is that operating pressure and longitudinal temperature gradients caused by friction have contrasting effects on solute retention and it is not obvious how to distinguish these two effects. Frictional heat effects tend to decrease retention (in still-air ovens) while pressure inherently increases solute retention. In the past, pressure effects were mostly studied by varying the flow rate. However, within this we need to be careful as both the pressure and temperature gradients are strongly affected by changing the flow rate. Therefore incorrect (not accurate) conclusions have been drawn in several published studies. Our purpose was to dissociate these two contrasting effects and determine their individual contributions to the retention of solutes of various sizes. To realize that, we suggested two sets of experiments: one performed at constant inlet pressure and at varied flow rates, and the other at varied inlet pressure.

We saw that friction related effects were more important for small molecule separations whilst pressure effects were much more significant for protein separations. Insulin was an attractive example as we could clearly see the decrease of retention in constant inlet pressure mode and the increase of retention in variable inlet pressure mode when increasing the flow rate. With this information, the developed heat power and outlet column temperature could also be estimated.

What issues and problems would you define as previously ignored or neglected specifically in the field of UHPLC, and more generally in the separation sciences? What is your vision for improvements that could be made in separation instrumentation, columns, data processing algorithms, or high-speed computing power?

One of the main limitations in liquid chromatography today is still the significant extra-column volume contribution of commercial chromatographic systems to band broadening. I believe that current instruments have reached their limits and therefore one might expect some completely new integrated system designs (for example, a slot for the column, on-column injection and on-column detection) alongside developments of column technology. The adiabatic isolation of the stationary phase would probably be a huge step forward in UHPLC and supercritical fluid chromatography (SFC) and the idea and design suggested by Fabrice Gritti–called a vacuum jacketed column–is obviously marvelous. Through this the detrimental band broadening caused by frictional thermal effects could be eliminated and I would expect the commercialization of such columns to be seen in the near future.

Now it seems that column wall coatings and using frits made of inert materials are also interesting, especially for protein separations, to eliminate non-desired secondary interactions. For protein separations, it is also a hot topic today to couple non-denaturing

modes, such as size-exclusion chromatography (SEC), ion-exchange chromatography (IEC or IEX), or hydrophobic interaction chromatography (HIC) to mass spectrometry (MS) detection. However, those chromatographic modes are inherently not compatible with MS and therefore there is a need for finding novel volatile mobile phase buffer systems. Very interesting recent work by Mary Wirth should also be mentioned here, namely an MS-compatible alternative to HIC called native reversed-phase liquid chromatography (nRPLC).

Improving data processing algorithms and computing power today, in my opinion, is not required. However, it would probably be interesting for very fast separations using very short columns and improving online data processing (for deconvolution, Fourier analysis, or peak fitting). Daniel Armstrong has very recently illustrated the possibility to perform sub-second separations using 5-mm long columns on current instrumentation. For such applications it could make sense to further improve computational power.

For modeling, there is still a lack of available model parameters (or variables) that can put into model equations because they are very difficult to measure, such as the tortuosity factor, obstruction factor, diffusion coefficients, and so on. There is therefore still a need to develop experimental methodologies to obtain or derive accurate model parameters.

Based on your work in analysis of pharmaceuticals (4), where do you see the need for the most future research? Would it be in the area of component separation, speed of analysis, detection limits, automation, or other areas? What major breakthrough would you like to see for faster liquid chromatography analysis?

Honestly, I do not think there is a need for faster separations or lower detection limits than are available today, however, I believe the problem is that pharmacopeia methods are still old-fashioned and do not take into consideration the benefits of current possibilities. UHPLC was commercially introduced in 2004 (15 years ago) however in many quality control (QC) labs, old conventional high performance liquid chromatography (HPLC) methods are still used and 40 to 90-minute-long separations are routinely performed to determine 5-6 impurities of an active pharmaceutical ingredient (API), simply because those labs need to follow pharmacopeia instructions. To me, that is nonsense.

On the other hand, I think that it is not the technical possibilities but the new samples to be analyzed (the increase in complex samples such as therapeutic proteins, oligonucleotides, single cell analysis, and so forth) will shape the chromatographic needs, which is now difficult to predict.

In one of your more recent papers, you describe the potential benefits and theory of using columns packed with particles of decreasing size (particle size gradient) in liquid chromatography (5). What can you tell us about how particle size gradient affects the separation and what improvements can be made by understanding the particle size gradient effect?

That was a funny project. Some friends and colleagues (Balázs Bobály, Róbert Kormány, Krisztián Horváth) and I were together during a conference and discussing chromatographic questions. One of those was: What happens when coupling two or more columns in series, which have a different number of theoretical plates? We were aware of the earlier amazing work of Calvin Giddings on plate heights of non-uniform columns, of Leonid Blumberg's variance of zone migration in non-uniform medium, and also of Deirdre Cabooter's works on multi-column systems. However, we were interested in the gradient elution mode (since in practice most separations are performed in gradient mode) and in peak widths: whether the peak width changes if one of the columns that is used for the coupled systems loses a bit of its initial efficiency? If yes, then does the order of the columns impact the apparent efficiency of the system or not? These questions seem to be simple and easy at first sight but the more you think on them the more difficult they become. From here the project went on and has almost developed by itself as questions have

arisen. We asked for the help of other prominent colleagues (Davy Guillarme, Santiago Codesido, Gert Desmet) and in the end, some very interesting conclusions were drawn. We found that, in the case where the later columns in the row have high enough efficiency, then the gradient band compression effect outperforms the band broadening effect and finally a "peak sharpening" will be observed during the travel of the solute along the column. That was really exciting, and it motivated us to further explore the potential gain in efficiency when sequentially placing columns according to their increasing efficiency. The next rational question was: why not apply a particle size gradient (as a limiting case of a large number of serially coupled columns). And indeed, in the best case, about 15–20% gain in efficiency could be expected at a given retention when utilizing a particle size gradient, compared to constant particle size. Conversely, when fixing efficiency, the analysis time could be decreased by about 15% with an optimal particle size gradient. In theory, applying a particle size gradient can be a good possibility to improve the quality of separations but in practice it is not easy to implement since packing columns with different particle size gradients might be challenging to achieve.

What can you share with our readers regarding your next area of interest for your research?

We are planning to work a lot with new biopharmaceutical products (mAbs, ADCs, bispecific monoclonal antibodies (bsAbs), and fusion proteins) since there are still many things to understand and develop. We are also involved in the development of new column designs and stationary phases which we believe will change the current practice of protein chromatography.

Would you like to acknowledge any coworkers, professors, or mentors that have been a great help to you early in your career?

During my second year at the Technical University of Budapest I met Prof Jenő Fekete for the first time and was fascinated by his skills, knowledge and experiences. Before this encounter, I spent most of the semester on basketball courts and athletics tracks. Jenő completely shifted my interest, refocused my attention to attending lectures and I found I enthusiastically participated in his research projects. Later on, Jenő was my supervisor during my PhD studies and we became very good friends.

Another person who had a significant influence on my carrier was Katalin Ganzler at Gedeon Richter Plc (GR, Hungarian pharmaceutical company). I was very impressed by her talent and the way she thinks. She has a thorough global view on any topic whilst at the same time she can quickly identify all the tiny details in a way I am not able to do myself. Moreover, she always supported and encouraged me and helped me considerably in private life.

While working at GR, I soon met Imre Molnár (Molnar-Institute). He was giving courses on LC method development. Thanks to him, I learnt a considerable amount about the pioneers of chromatography (Csaba Horváth, István Halász, Lloyd Snyder, and John Dolan). He often told personal and funny stories about those legends which made his courses unique and entertaining. Since then we have had a long and fruitful collaboration together.

I would also like to thank Professor Sándor Görög; who was the former director of analytical research at GR and editor of *Journal of Pharmaceutical and Biomedical Analysis*. We worked in different departments, however I often went to him to discuss ideas and his help was extremely valuable when writing my first journal articles. Moreover, he was the person who first contacted Professor Jean-Luc Veuthey (University of Geneva) and recommended me for a post-doc position–thanks to him I moved to my current position at the University of Geneva.

Finally, the two people I would most like to thank are Professor Jean-Luc Veuthey and Davy Guillarme at the University of Geneva. They are both well-recognized and outstanding scientists, while more importantly, these two guys are exceptional people too. They are very friendly, helpful, calm and fair. These are two persons in whom you can fully trust, who always do their best to support the colleagues, students and the group. I count myself extra-lucky and I am proud to be a member of their group.

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Szabolcs Fekete the 2020 winner of the Emerging Leader in Chromatography Award, earned his PhD degree in 2011 from Technical University of Budapest, Hungary, and is currently a scientific collaborator at the University of Geneva. His work focuses on finding new possibilities in protein chromatography; characterizing therapeutic proteins, LC column technology; LC method development, optimization, retention modeling and other fundamental studies. Fekete has published about 130 papers, with more than 4000 citations. He has also given more than 60 oral and 30 poster presentations at scientific conferences, and has won five "best poster" awards at international conferences. He also received the György Oláh award from the University of Technology of Budapest in 2011.

44th International Symposium on Capillary Chromatography and 17th GC×GC Symposium

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The ISCC/GC×GC scientific program will be a rich one, it being characterized by:

- invited contributions from leading scientists reporting the latest most exciting developments
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Researchers in all areas relevant to the subjects of the symposia are invited to submit abstracts. As is traditional for the Riva meetings, the majority of presentations will be in a poster format and the Scientific Committee will select contributions for oral presentations. As always, many awards will be assigned in both the ISCC and GC×GC events, recognizing excellence in both established and young scientists, in oral and poster presentations. Exhibitors and sponsors are a fundamental part of the meeting (without them...Riva wouldn't be Riva!) and are encouraged to participate by reserving booth space, becoming a sponsor and to promote the ISCC and GC×GC events.

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PRODUCTS & RESOURCES

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Macherey-Nagel Inc.,
Bethlehem, PA.
www.mn-net.com

Phospholipid removal microplate

Microlut PLR, a 09-well microplate from Porvair Sciences, is designed to provide effective removal (>99%) of phospholipids and proteins. According to the company, the microplate delivers unmatched levels of reproducibility from plasma and serum samples, while maintaining maximum recovery of target analytes.
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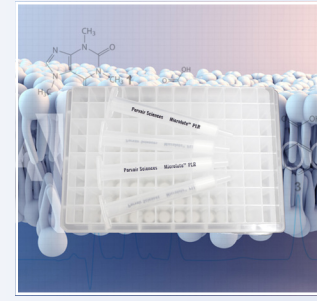
Wales , UK.
www.microplates.com/microlute-plr/



Method translation software

The Pro EZLC method translation software from Restek is designed to simplify and streamline the process of properly adjusting liquid chromatography (LC) conditions. According to the company, the translator allows users to input their current column dimensions and method conditions, and specify the dimensions of the new column that the user wants to try, automatically generating new injection volumes and isocratic or gradient program conditions.
Restek Corporation,
Bellefonte, PA.
www.restek.com/ezlc

MALS scattering instrument

Wyatt's NEON line of multi-angle light scattering (MALS) instruments are designed with an advanced platform based around a modern user interface that guides the user toward making optimal measurements. According to the company, a large display incorporates multi-touch interactivity with swipe and zoom capabilities, giving it the feel of a modern tablet or smartphone application.
Wyatt Technology Corporation,
Santa Barbara, CA.
www.wyatt.com

Headspace syringe

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

HPLC columns catalog

A catalog of reversed-phase, Cogent high performance liquid chromatography (HPLC) columns is available from Cornerstone Scientific. According to the company, the columns are suitable for use in old and new methods, especially for USP legacy methods, and lifetime technical support is provided free with every column from the manufacturer.
Cornerstone Scientific,
Greater Wilmington, NC.
www.cornersci.com

Derivatization instrument

The Onyx PCX post-column derivatization instrument from Pickering is designed with reactor temperature and reagent flow control, an inert flow path, and a column oven with gradient capabilities. According to the company, the instrument is suitable for laboratories involved in testing amino acids, glyphosate, antibiotics, mycotoxins, and other ingredients and contaminants.
Pickering Laboratories, Inc.,
Mountain View, CA.
www.pickeringlabs.com

HPLC and SFC phases

Princeton Chromatography offers reproducible high performance liquid chromatography and supercritical fluid chromatography columns in a variety of phases and dimensions to support analytical, prep, and ultrahigh-pressure liquid chromatography workflows. According to the company, popular phases include C18, 2-Ethylpyridine, Diol, and Cyano, and custom column packing services, including bulk chromatographic material, are provided.
Princeton Chromatography Inc.,
Cranbury, NJ. www.pci-hplc.com

PRODUCTS & RESOURCES

Sulfur chemiluminescence detection system

The Nexis SCD-2030 chemiluminescence (CL) detection system from Shimadzu is designed with a horizontally positioned redox cell. According to the company, the redox cell can ensure an ample reaction time and reaction zone within the cell, which promotes the sample's redox reaction and achieves long-term stability.

Shimadzu Scientific Instruments, Columbia, MD.
www.ssi.shimadzu.com

Bioinert coating for metal-free HPLC

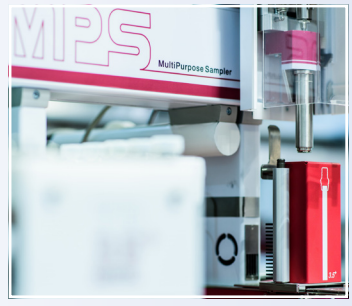
SilcoTek's Dursan bioinert coating is designed to improve the reliability, sensitivity, and speed of HPLC instruments by eliminating the effects of metals in the flow path. According to the company, the coating can be applied to pumps, columns, frits, and other sample transfer components.

SilcoTek Corporation, Bellefonte, PA .
www.silcotek.com/lc

GC–MS thermal desorption system

Gerstel's MPS TD system is designed as a dedicated sampler for automated thermal desorption, thermal extraction, and dynamic headspace analysis. According to the company, the system can process up to 240 samples, and is operated with one integrated method and one sequence table.

Gerstel, Inc.
Linthicum, MD.
www.gerstel.com



Hydrogen gas generators

VICI DBS has recently expanded its core range of NM hydrogen gas generators for gas chromatography. According to the company, gas purity is to +99.99996%, pressure of 160 psig, and flow to 1,350 mL/min, and the internal software system continuously monitors key parameters for reliable safe operation.

VICI DBS USA,
Houston, TX.
www.vici-dbs.com

CALENDAR

29 March–2 April 2020
MSACL 2020 US: 12th Annual Conference & Exhibits
Palm Springs, California
https://www.msacl.org

31 March–3 April 2020
analytica 2020
Munich, Germany
https://www.analytica-world.com

6–8 April 2020
Cannabis Science Conference East
Baltimore, Maryland
https://www.cannabisscienceconference.com

28–30 April 2020
Interphex 2020
New York, New York
https://www.interphex.com

31 May–3 June 2020
PREP 2020: 33rd International Symposium on Preparative and Process Chromatography
Baltimore, Maryland
https://prepsymposium.org

31 May–4 June 2020
68th ASMS Conference on Mass Spectrometry and Allied Topics
Houston, Texas
https://asms.org/conferences/annual-conference

4–5 June 2020
ICABC 2020: Analytical and Bioanalytical Chemistry Conference
New York, New York
https://waset.org/analytical-and-bioanalytical-chemistry-conference-in-june-2020-in-new-york

9–10 June 2020
11th World Congress on Green Chemistry and Technology
Geneva, Switzerland
http://htc2020.hu

20-25 June 2020
HPLC 2020: 50th International Symposium on High Performance Liquid Phase Separations and Related Techniques
San Diego, California
http://www.hplc2020-usa.org

22–25 June 2020
9th International Conference on High Temperature Capillarity (HPC)
Visegrád, Hungary
http://htc2020.hu

26–29 July 2020
57th Annual North American Chemical Residue Workshop (NACRW)
Fort Lauderdale, Florida
https://nacrw.org

3–7 August 2020
National Environmental Monitoring Conference (NEMC)
Minneapolis, Minnesota
https://www.nemc.us

31 August–2 September 2020
Cannabis Science Conference West
Portland, Oregon
https://www.cannabisscienceconference.com

21–25 September 2020
40th Society of Forensic Toxicologists
San Diego, California
http://www.soft-tox.org

24–25 September 2020
ICACEA 2020: International Conference on Analytical Chemistry and Engineering Applications
San Francisco, California
https://waset.org/analytical-chemistry-and-engineering-applications-conference-in-september-2020-in-san-francisco

12–15 October 2020
American Council of Independent Laboratories (ACIL) Annual Meeting
Long Beach, California
https://www.acil.org

13–14 October 2020
Gulf Coast Conference
Galveston, Texas
http://www.soft-tox.org

25–29 October 2020
American Association of Pharmaceutical Scientists (AAPS) Annual Meeting
New Orleans, Louisiana
https://www.aaps.org

8–11 November 2020
40th International Symposium on the Separation of Proteins, Peptides, & Polynucleotides (ISPPP)
Porto, Portugal
https://www.isppp.net

6–10 March 2021
Pittcon 2021
New Orleans, Louisiana
https://pittcon.org

Ad Index

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whole database to identify data trends and inconsistencies.

- Functionality within the CDS application to document that audit trail entries have been reviewed.

Summary

We have looked at the regulations and regulatory guidance for audit trail review, which is a key component for second person review of chromatographic analysis. To facilitate an effective review by exception, technical controls need to be included in the CDS application to identify data changed during an analytical run, including a function to document the review itself.

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(3) *FDA Guidance for Industry Data Integrity and Compliance With Drug CGMP Questions and Answers* (Food and Drug Administration, Silver Spring, Maryland, 2018).

(4) *21 CFR 11 Electronic records; electronic signatures, final rule, in Title 21* (Food and Drug Administration: Washington, DC, 1997).

(5) *21 CFR 58 Good Laboratory Practice for Non-Clinical Laboratory Studies* (Food and Drug Administration: Washington, DC 1978).

(6) EudraLex - Volume 4 *Good Manufacturing Practice (GMP) Guidelines, Annex 11 Computerised Systems*. (European Commission, Brussels, Belgium, 2011).

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FUNDAMENTALS

HPLC Diagnostic Skills—Noisy Baselines

Tony Taylor

Just as medical practitioners are able to discern worrying features from a variety of medical physics devices (electrocardiogram, electroencephalogram, and ultrasound, for example), we need to develop the skill to identify worrying symptoms from our high performance liquid chromatography (HPLC) instrument output. Medical professionals learn an innate ability to identify critical symptoms (signals) from the noise or random variation in the instrument output, and we need to develop these same skills to avoid production of data not fit for purpose, or instrument failure.

One of the most useful diagnostics in HPLC is the nature of the baseline produced by the detector while the eluent is flowing. While there can be many baseline characteristics, such as drift, irregular, or more regulation cycling (pulsations), baseline noise is perhaps the most commonly encountered, and can arise from a variety of different sources. One needs to be aware of what constitutes "normal" baseline, as opposed to "unusual" levels of baseline, depending upon the instrument configuration. Of course, the business imperative is not only to spot problems, but also to quickly and efficiently deal with them, and that is the subject of this article.

The signal to noise (S/N) of the HPLC output is usually measured as the ratio of the detector signal to the inherent background signal variation, and is a useful measure of the "normal" noise within the system (Figure 1). The inherent or background noise is typically measured over a predefined portion of the baseline, and most data systems will be capable of making this measurement and reporting the result.

When inherent or background noise within the system is unusually high, this can affect system performance, and will usually result in an increase in the limit of quantitation and issues with reproducible integration. This is why, as chromatographers, we get so worked up about noise levels that are higher than expected.

The smallest detectable signal is usually estimated to be equivalent to three times the height of the average baseline noise. This would give a S/N ratio of 3:1 for the limit of detection (LOD) of the detector. If the amount of analyte injected is less than this, then the signal ceases to be distinguishable from noise. For quantitative analysis a S/N ratio of 10:1 is recommended for the limit of quantitation (LOQ).

The magnitude of the analyte signal cannot be used in isolation when calculating detector sensitivity; the sensitivity of detection is usually defined in terms of S/N ratio, a measurement of the ratio of the analyte signal to the variation in baseline. S/N measurements are usually performed by the data system.

One needs to begin by establishing, preferably for each method and set of instrument conditions, the S/N when the method (or instrument) is performing well, and perhaps even set a system suitability performance criterion (usually a range or lower acceptable limit) for the determination. Of course, the seasoned chromatographer will typically know by glancing at the baseline whether the inherent noise is "usual," and this comes only through experience. One should also take care to assess the noise at a reasonable screen magnification or signal attenuation, as any baseline can be made to appear noisy with the correct level of magnification!

However, once again the data system may be able to help us out by reporting what is known as the peak-to-peak noise, which may be expressed as absorbance units. This measurement is of the variation in the normal baseline portion, rather than a ratio to the height of a signal, and can be very useful at establishing acceptable limits for the background noise. Most HPLC detectors will run a noise test evaluation as part of their initialization routine, or can perform a longer test using ASTM criteria with HPLC-grade water flowing through the flow cell. Specifications for acceptable noise levels will be given in the manufacturer's literature.

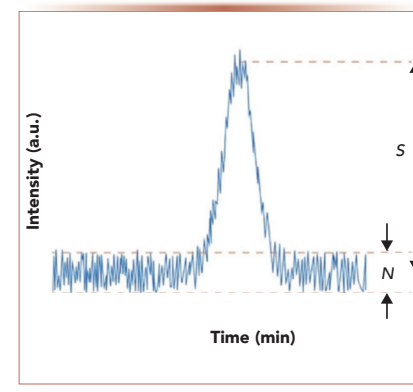

FIGURE 1: Signal (S) to noise (N) measurement of 5:1.

Although typically associated with detector phenomenon, there are many contributors to the noise within an HPLC system. Noise can be both random and periodic, depending upon the nature of the underlying cause of the problem, and this difference can, in itself, give us some clues to the nature of the issue.

In future installments, we will examine a few of the main culprits of baseline noise.

This article is an excerpt of an installment of the LCGC blog .

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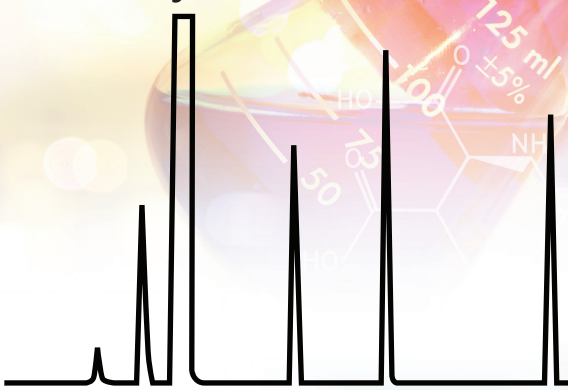
The Minnesota Chromatography Forum (MCF) invites you to our 41st Annual Spring Symposium.



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- April 1st, 2020

Symposium and Short Course Early Registration Deadline
- April 14th, 2020

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- May 1st, 2020

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- May 1st, 2020

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