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Physical Causes of Peak Asymmetry in Liquid Chromatography

What Harold McNair Taught Us About GC
Concern over detrimental greenhouse gases produced from the combustion of fossil fuels has been growing for the past few decades. Identification of new and more diverse methods of energy use has been investigated around the world. One such avenue is combustible alcohols produced from biomasses like sugar beet or sugar cane. To reduce dependence on fossil fuels, a concerted effort has been made to develop processes where biomass can be fermented biologically by bacteria or yeast to produce combustible alcohols. Combustible alcohols are desired over other technologies due to the convenience of switching from petrol based fuels. Ethanol is already added to most gasolines to increase octane efficiency. With anywhere from 5–25% ethanol being added to petrol products in most of the world and up to 100% ethanol fuel in Brazil. 

Production of ethanol from biological sources produce side products, specifically propanol and methanol amongst other ketones, fatty acids, and esters. Due to azeotropic formation of ethanol with water it is difficult to distill pure 200 proof ethanol products. However, HPLC analysis provides a good platform for ethanol determination after biomass fermentation. To aid the detection of ethanol purity, Hamilton has developed a method utilizing the HC-75 (H⁺ Form) HPLC column, which provides good separation of the desired analytes. With ~8% crosslinking of the PS-DVB backbone the resulting porous gel stationary phase matrix allows for enhanced interactions between stationary phase and analyte. Detection was accomplished with refractive index, but other methods of detection can be applied (i.e. UV, or mass spectrometry).
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From the Chairman

Mike Hennessy, Sr.
Chairman & Founder, MJH Life Sciences

Research on the toxicity of per- and polyfluoroalkyl substances (PFAS) is raising increasing concern. PFAS are often referred to as forever chemicals because they do not easily break down in the environment. Recent years have seen the publication of EPA methods 533 and 537.1 for PFAS analysis in drinking water using solid-phase extraction (SPE). In this month’s “Focus on Environmental Analysis” column—our LCGC North America cover story for September—Rudd Addink and Tom Hall at Toxic Report Laboratories report on the use of semi-automated SPE to prepare drinking water samples for PFAS analysis with these EPA methods. This a semi-automated approach combines the best features of both manual and automated methods, leading to an approach to that is reliable and relatively inexpensive, and shows good reproducibility.

As both natural medicines continuously evolve, so do the methods used to analyze them. Researchers at Kunming Medical University in China and co-authors have synthesized two dioscin-based liquid chromatography stationary phases for high performance liquid chromatography (HPLC) quality control analysis of natural products. This month’s peer-reviewed research evaluates the use of those stationary phases for both chiral and achiral analysis of gongxuening, a traditional Chinese medicine.

A different—and exciting—approach to HPLC column synthesis involves the use of additive manufacturing, better known as three-dimensional (3D) printing. In “Column Watch,” Suhas Nawada of the University of Amsterdam and Tristrum Budel of Autum3D present a 3D printing method that overcomes the limitations of other approaches to produce both analytical- and preparative-scale columns with a feature resolution of 10 μm.

But let us not focus only on HPLC. “GC Connections” examines the career of the late Harold McNair and his impact on the field of gas chromatography, focusing on three key lessons he taught us. “Sample Prep Perspectives” in turn, discusses fundamentals of five important sample preparation methods, explaining their initial conception and development and what those mean for understanding the role of these approaches in the current analytical toolbox.

We close the issue with a provocative opinion piece on the future of separation science. As his title indicates, Mark Hayes anticipates that his provocative piece might rile up the community. What is your take?
But My Peaks Are Not Gaussian! Part II: Physical Causes of Peak Asymmetry

Although symmetric peaks with Gaussian shapes are predicted by models of the chromatographic process, “perfect peaks” are not observed very often outside of textbooks. Several physical phenomena can lead to asymmetric peak shapes, including heterogeneity of the particle density inside the column, rearrangement of the particles over time, and accumulation of debris at the column inlet frit. Understanding these phenomena can help identify whether the cause of asymmetry is most likely to have a physical or chemical origin, which, in turn, dictates which troubleshooting steps to start with when dealing with poor peak shapes.

Dwight R. Stoll

Last month, in the first installment of this series of LC “Troubleshooting” articles on peak asymmetry, I discussed some basic concepts in peak asymmetry, including commonly used models of chromatographic peak shapes, how to quantify peak tailing, and the impact of peak tailing on separation performance. I then went on to discuss in some detail how poorly-made connections between the column and the rest of the LC system can lead to serious peak tailing. The good news is that this particular cause of peak tailing can usually be fixed rather easily by carefully considering the parts (such as capillaries and unions, to name two) used in the flow path between the injector and detector, and replacing those that are inappropriately sized (for example, a union with a very large through-hole) or somehow improperly connected (for example, a ferrule set too shallow). In addition to these problems, there are many other potential causes of peak asymmetry—too many to cover in a single one of these articles—some having primarily physical origins, and some having primarily chemical origins. In this month’s installment, I will focus on several other potential physical causes, including problems with column packing, changes in the packed particle bed over time, and accumulation of debris in the column. A common symptom of all of the physical causes of peak asymmetry discussed here is that all peaks in a chromatogram will be affected similarly. This can be an important clue to help determine if the source of the asymmetry is more likely to have chemical or physical origins. If all of the peaks in a chromatogram are either fronting, or tailing (or both [1]), the cause is most likely physical in origin. If only some of the peaks are fronting or tailing, but the other peaks look good, then it is most likely that the cause of the poor peak shape is chemical in nature. This distinction is helpful when deciding which potential solutions to improve the peak shape to try first.

Brief Review of Flow Through a Packed Bed of Particles

To understand the different peak shapes that can be observed as a result of different physical problems within a LC column, it is helpful to first review some foundational concepts related to mobile phase flow through a column packed with small particles like those used in HPLC. Figure 1a shows a highly idealized illustration of the organization of particles (depicted as porous spheres) in a perfectly packed bed. Such perfect arrangements of particles are not achievable in practice, though some come close, in the case of small very small (<1 µm) particle columns (2). The main point of this illustration is that a highly ordered, consistent arrangement of the particles in the column leads to highly consistent mobile phase velocities across the column radius. Since the rate of migration of analyte molecules (that is, analyte velocity) from the inlet to the outlet of the column is proportional to the mobile phase velocity, the consistency in mobile phase velocity directly translates into consistent analyte velocities, and thus a symmetric analyte peak observed at the detector. In the case of perfectly consistent mobile phase velocity across the column diameter, there would be no “A-term broadening” of peaks, and their widths would be dictated primarily by diffusion of analytes along the long axis of the column, and into and out of pores in the particles. The consistency of the mobile phase velocity in this case is communicated in Figure 1a with yellow arrows of the same size.
Variations in Particle Packing Tightness Throughout the Column Can Lead to Asymmetric Peaks

In contrast to the highly ordered bed structure illustrated in Figure 1a, Figure 1b shows that, in real columns, the structure of the particle bed is rather disordered, leading to regions where the particle density (that is, the number of particles per volume of column, not the density of the particles themselves) is much higher than others. In recent years, several studies discussed in the literature have made clear that much of this heterogeneity in packing density throughout the column arises as a result of friction between the column wall and the particles as they are pushed into the tube. This results in large variations in density near the wall, moving from the wall toward the center of the tube. This is sometimes referred to as a wall effect on the packing density. The net effect of this variation, though, is that the mobile phase velocity will be lower in regions of high density, and higher in regions of low density, because there is less friction opposing the flow of mobile phase as the spaces between adjacent particles increase. This variation in mobile phase velocity then manifests as an “A-term type of peak broadening,” and results in peaks that are broader compared to the situation where there is no variation in mobile phase velocity across the radius of the column. In the particular situation shown in Figure 1b, the packing density near the wall is significantly lower compared to the center of the column. If the difference in densities is large enough, this can actually result in peak asymmetry like that shown in this case, which we refer to as peak fronting. We typically do not observe this behavior much in commercial LC columns, because the manufacturers do a good job of mitigating this particular problem, but I’ve packed enough columns during my days working in the column business to know that this effect can be very serious, and must be solved through optimization of column packing procedures. During the development of new stationary phases and column technologies, column manufacturers study the impact of a large number of variables on the packing quality, including things like the slurry solvent (that is, the solvent used to suspend the particles to flow them into the column), slurry concentration (particles per volume of solvent), packing flow rate, and packing pressure. Readers interested in learning more about the column packing process and the topic of heterogeneity of the particle bed are referred to recent articles on the topic (3).
modern materials. In principle, these high pressures force the particles into an arrangement that is unlikely to change significantly when used in an LC instrument at lower pressures. In the early days of HPLC it was not uncommon for column performance to deteriorate upon settling or rearrangement of the particle bed. Fortunately, modern manufacturing procedures have generally improved the robustness of LC columns significantly, and I have to say I am often impressed at just how resilient modern columns are, surviving instrument failures (for example, pressure spiking due to sticky check valves) or user error (for example, allowing columns to dry out without flushing first with an organic/water mixture to remove buffer salts). However, rearrangement of the particle bed can still happen—for example, in response to physical stress on the column (such as repeated pressure fluctuations [4]). Although there are many ways that the particle bed could conceivably rearrange, the classical observation is that the particle bed consolidates in the direction of the outlet (think of sand settling in a bucket when vibrated), leaving a significant “void” at the column inlet where there are no particles and only mobile phase. These void spaces can then act like small mixing chambers and lead to significant peak tailing like that shown in Figure 1c. Sometimes reversing the flow direction can restore some of the column performance, but, in my experience, this performance is usually very short-lived, and when a void develops in a column it is best to just replace the column. Although many modern LC columns are highly resilient to various physical stressors, it is still helpful in the long run to avoid causes of such stress in order increase column lifetime. This includes avoiding major pressure fluctuations (for example, due to air bubbles in pumps), and properly flushing columns according to manufacturer recommendations before storing the column for more than a few days.

**Accumulation of Debris on Column Frits Can Lead to Asymmetric Peaks**

As I’ve written about in the past, one of my favorite troubleshooting tips is to consistently use inline filters in LC systems, especially immediately upstream from the column (5). There are many ways that insoluble debris can make its way to the column inlet, including particulate matter in the sample that is injected, particulate matter in the mobile phase that comes from unfiltered solvent feeding the pump, and polymeric material that is shed by valves (for example, from a rotor seal) like those found in autosamplers. If an inline filter is not used between the sample injector and the column, then much of this insoluble debris will accumulate on the inlet frit of the LC column.

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I’ve looked at many inline filters when replacing them after they’ve become blocked, and, without exception, I see that debris never accumulates across the frit in a uniform way. Sometimes it is concentrated at the edges, sometimes in the middle, and other times there is no obvious pattern. However, the non-uniform distribution of the accumulated material again means that flow through the column inlet frit will also be non-uniform, leading to different mobile phase velocity streams at the column inlet, peak dispersion, and asymmetric peak shapes. In the illustration in Figure 1d, I’ve attempted to show accumulation of debris at the center of the inlet frit, and that in some cases this can lead to severely distorted split peaks as shown at right. The good news is that this particular cause of peak asymmetry can largely be avoided through consistent use of inline mobile phase filters directly upstream from the analytical column. It is also possible in many cases to physically replace the inlet frit on the LC column, but this requires extraordinary care to avoid disrupting the particle bed, and I would not recommend this remedy as a routine practice.

**Summary**

In this installment of “LC Troubleshooting,” I’ve discussed several of the common physical causes of peak asymmetry in LC, and remedies for some of them. In some cases there is not a whole lot the user can do to address the root cause of peak asymmetry, as some problems occur at the point of manufacture of the column, but in the spirit of increasing our troubleshooting knowledge, it is still helpful to know what all can go wrong, and this knowledge can also help determine if the problem is likely to be solvable, or if the column must simply be replaced.

In the next installment in this series, I will discuss several chemical causes of peak asymmetry, where we as users typically have more opportunities to improve peak shape through changes in operating conditions.

**References**


**ABOUT THE COLUMN EDITOR**

Dwight R. Stoll is the editor of “LC Troubleshooting.” Stoll is a professor 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 co-authored more than 75 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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A Novel 3D-Printing Method to Create Liquid Chromatography Columns

For approximately a decade, three-dimensional (3D)–printed columns have been hailed as the future of liquid chromatography (LC). However, the resolution of typical printing methods has fallen short of the requirements to make an effective analytical- or preparative-scale column. We describe a new 3D-printing method that can create large-volume columns with a feature resolution of 10 μm.

Suhas Nawada and Tristram Budel

In the ongoing quest for fundamental advances in high performance liquid chromatography (HPLC) column performance in the past two decades, several methods of producing ordered stationary phases have been explored. To quote John Knox, “The overall effect of a very homogenous bed is to greatly enhance chromatographic efficiency” (1). Over the past decade, the emergence of additive manufacturing, or three-dimensional (3D) printing, has led many in the chromatography field to consider it a promising technique for creating ordered stationary phases with tailored geometries (2).

Studies have been performed on orientations (3), element shapes (4), and novel porous geometries that are best suited for 3D-printed columns. The efficiency of such structures has been demonstrated in both computational simulations and experimental tests, with reduced chromatographic plate heights as low as 1.12. Furthermore, a bifunctional resin has been developed that allows for direct printing of anion exchange columns (5,6).

However, a limitation in such studies has been the feature size of the porous beds that have been created. This limitation results from the limited resolution of most 3D-printing methods. Common printing methods, such as fused deposition modeling, stereolithography, and selective laser sintering, typically operate with nominal resolutions in the order of 20–100 μm. By operating with nominal resolutions, common printing methods generate feature sizes of porous beds typically greater than 300 μm, which is two orders of magnitude short of the feature sizes that are necessary for use in HPLC columns.

In contrast to conventional methods, the high-resolution 3D-printing method called two-photon polymerization is capable of creating pore sizes of under 1 μm with high fidelity. However, as detailed in a previous article by De Malsche and coworkers (7), the printing method requires impractically long production times for analytical-scale columns typically used in HPLC. Therefore, the current capabilities of 3D-printing technologies are inadequate to create analytical- or preparative-scale columns because of limitations in both resolution and printing speeds.

To address these limitations, we have developed a 3D-printing technique called hybrid stereolithography (HSLA) (8), which combines traditional stereolithography and photolithography, and is capable of high-resolution and fast printing of high-volume structures.

Figure 1a shows a prototype HSLA setup during the printing process. A lower resolution pattern is illuminated, using an ultraviolet (UV) light source (shown in blue in Figure 1a) and a digital mirror device. To achieve higher resolution than are achieved with traditional stereolithographic methods, a high-resolution photomask with predefined patterns is used between the resin tray and the digital pattern. After a layer is cured, the build platform lifts to peel the printed piece from the resin tray.

The primary advantage of such a system is that it prints high-resolution repetitive features such as ordered LC column microstructures. The photomask patterns can be used to print grids with feature sizes as low as 10 μm without compromising on printing speeds. Because all regions of a pattern are cured simultaneously, the column i.d. is entirely independent of the print speed. For example, the 40-mm i.d. column seen in Figure 1a was printed at a speed of 5 mm of column length per hour, translating to 6.5 mL/hr. The 40-mm i.d. column represents an improvement of greater than three orders of magnitude compared to the fastest two-photon polymerization systems for a similar resolution (9). The high printing speed can be used to create large preparative-scale columns, or several analytical-scale columns in parallel—up to 32 columns with a 4.6-mm i.d., like the one shown in Figure 1a.

Figures 1b and 1c show axial and radial cutaways of two different lattices printed using HSLA. Figure 1b shows a 50-μm simple cubic grid printed in the setup shown in Figure 1a, with a designed porosity of 50% and a layer thickness of 25 μm. Figure 1c shows a lattice with a feature size of 20 μm with a
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layer thickness of 10 µm. In both cases, the ordered nature of the printed structures is evident, with the photomask features being imprinted on the structure with good fidelity.

To create a 3D structure, several patterns were defined on the photomask, with a linear stage moving the photomask after a layer is cured. A photomask-switching system with two patterns, shown in Figure 2, was used to create the lattice seen in Figure 1b. More-complex 3D structures can also be created using more photomask patterns. Because LC column microstructures typically consist of simple, repeatable geometries, a photomask-switching system is well-suited for column production.

A commercial nonporous high-resolution photopolymer resin called “Nanoclear” (FTD Resins) was used for all tests. For this study, 1-, 2.1-, 4.6-, 10-, and 20-mm i.d. columns were produced and thoroughly flushed using isopropanol to clear the porous structure of uncured resin. An external casing with fittings and flow distributors was used to connect the column to an HPLC system.

Uracil injections were performed in triplicate on the 4.6-mm i.d. columns from two directions. The resulting plate heights show a classical van Deemter curve, with a clear column efficiency minimum ($Q_{\text{min}}$) of 0.25 mL/min. As seen in Figure 3b, aligning the flow direction with the print direction (that is, top to bottom, as seen in Figure 3a) is clearly advantageous compared to orienting the column against the direction of the print. A minimum absolute plate height of $H = 112.4$ µm (a reduced plate height of $h = 2.25$) was demonstrated with the column oriented in the direction of the print. To the best of our knowledge, these represent the lowest absolute plate heights for 3D-printed columns, and a five-fold improvement compared to previous tests using conventional printing methods (3). In the region of the optimum flow rate, the column also exhibited low relative standard deviations of 4 and 7%, for the first moment and plate height, respectively.

Switching the flow direction of the column resulted in a near-doubling of plate heights. This difference can be explained by the minor printing artifacts that occur because of a phenomenon called back-curing, in which a small potion the bottom of a pore in a lattice layer cures and solidifies (10).

In addition to determining the plate heights, the scalability of the printing method was tested by measuring the void volumes of columns of different internal diameters. Columns with internal diameters of 1–20 mm × 2.5 cm L were printed in triplicate and measured. As seen in Figure 4, the volume of the printed columns was consistently between 90 and 100% of the designed volume, indicating good fidelity between the designed photomasks and the printed structures. However, larger columns (10- and 20-mm i.d.) showed greater variation in measured void volumes. A possible cause for larger columns showing a greater variation in void volumes is the post-processing method that was used to remove the uncured resin from the printed lattices. Isopropanol was flushed through the columns using an analytical-scale HPLC system for a fixed column volume. However, to achieve constant flushing velocity, the optimum flow rates for the larger i.d. columns were sig-
nificantly higher than the maximum flow rate limits that HPLC systems will allow. As a result, the larger printed columns delivered incomplete removal of the uncured resin. Tests with preparative-scale systems are necessary, not just to perform separations on such larger i.d. columns, but to achieve the desired porosities and void volumes.

Conclusion
This article outlines the development and operation of a novel 3D-printed method, HSLA, geared toward the production of liquid chromatography columns. The method is capable of operating on length scales of micrometers to >40 cm to produce analytical- and preparative-scale column structures in reasonable timeframes, representing a significant improvement compared to existing 3D-printing methods. The characterization of the printed structures shows good agreement between the designed and measured void volumes and reduced plate heights of $h = 2.25$. The results here show that full-scale analytical HPLC and even industrial preparative-scale 3D printed columns with an organized internal structure that is consciously designed to fit the application are soon to be a widespread reality.

References

ABOUT THE AUTHORS
Suhas Nawada is with the Van’t Hoff Institute for Molecular Sciences at the University of Amsterdam, in Amsterdam, The Netherlands.

Tristram Budel is with Atum3D, in Gouda, The Netherlands.

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

Size-exclusion chromatography combined with multi-angle light scattering (SEC-MALS) is a well-established biophysical characterization tool used to quantify critical quality attributes (CQAs) of adeno-associated virus (AAV).

**LCGC:** How did Wyatt get involved in multi-CQA quantification of viral vectors?  
**CHEN:** The short answer is because our customers wanted it. Many of them are working in the gene-therapy field using AAV. To ensure the safety and efficacy of AAV-based products, it is essential to understand and measure the CQAs throughout the development cycle as well as manufacturing.

I met a few customers working on AAV assay development, and they challenged us to develop a SEC-MALS method to quantify quality attributes because the traditional assays they used at that time lacked robustness, speed, and automation. After studying and learning about their needs, we developed such a method using two options: MALS with two UV wavelengths, and MALS with one UV wavelength and refractive index (RI) detection.

**LCGC:** Tell us more about SEC-MALS.  
**CHEN:** SEC-MALS is a well-established technique for characterizing proteins, polysaccharides, and synthetic polymers. It measures the absolute molecular weight, quantifies the composition of protein conjugates or copolymers, detects aggregation with much higher sensitivity than SEC-UV, and analyzes many other attributes depending on the particular analyte.

It has been adopted as a release assay in QC for polysaccharides and certain protein conjugates in cases where standard analytical SEC, which is used with just a concentration detector, is inadequate. There’s a specific protein conjugate analysis module in ASTRA, which is our data collection and analysis software. In the SEC-MALS paradigm, AAV is also a type of protein conjugate, a conjugate of protein and DNA. By using the protein conjugate calculation, we were able to determine the molecular weights of the capsids and encapsulated DNA, and then further calculate AAV-specific attributes.

**LCGC:** What can SEC-MALS accomplish for AAVs?  
**CHEN:** This method can measure three key AAV quality attributes: total AAV particle concentration, empty-to-full or Vg/Cp ratio, and aggregate content. It can measure molecular weights of the capsid
and the DNA, as well as provide an impurity profile. Extracting all that information from the standard Protein Conjugate Analysis module takes a fair amount of effort. To help our customers with the analysis, we added a new Viral Vector Analysis module in ASTRA earlier last year, which takes care of all the calculations, graphing, and reporting.

The SEC-MALS AAV method has been tested by quite a few customers. After successfully validating it, many told us they hope to use it as a platform assay that will support multiple AAV programs throughout the R&D cycle and as a release assay for quality control in production. We developed a standard operating procedure (SOP) guidance manual for AAV analysis of SEC-MALS, released in January 2021, to provide further support in implementing SEC-MALS as a platform R&D and QC assay.

**LCGC:** What about in-process samples?
**CHEN:** SEC-MALS is the perfect match for in-process AAV samples, which may not be pure enough for other techniques. Those samples often have concentrations between $1 \times 10^{11}$ to $1 \times 10^{16}$ particles per mL, which is well within the range for SEC-MALS $5 \times 10^{10}$ AAV particles per mL up to $10^{15}$ AAVs per mL. Because SEC fractionates AAV monomers and oligomers, as well as other impurities with different sizes, SEC-MALS can tackle samples throughout the AAV downstream process and even some steps in the upstream process. Our application scientists can review the specifics of any application with customers interested in exploring the suitability of the SEC-MALS for their AAV needs.

**LCGC:** Has any work using the SEC-MALS AAV method been published?
**CHEN:** Yes, two groups have recently published their work. Scientists at BioMarin published “Comprehensive Characterization and Quantification of Adeno-associated Vectors by Size Exclusion Chromatography and Multi-Angle Light Scattering” in the open-access journal *Nature Scientific Reports*, February 2021. Another recent paper, “Detailed Protocol for the Novel and Scalable Viral Vector Upstream Process for AAV Gene Therapy Manufacturing,” published online in *Human Gene Therapy* in March 2021, was authored by scientists at Frederick National Laboratory for Cancer Research. Many of our customers have presented results from the SEC-MALS method at various conferences as well.

**LCGC:** Can you summarize the limitations of the SEC-MALS method?
**CHEN:** Since empty, full, and partials are not physically separated by SEC, SEC-MALS only provides an average level of Vg/Cp at each eluting slice, which can be divided across two states such as empty and full or half-full and full, but it is not able to tease out the ratio of three AAV fill states such as empty, full, and half-full. But again, this method is very sensitive in detecting a small change in Vg/Cp.

The second limitation is the need to assume 100% mass recovery from the column when calculating the total AAV concentration. Under our SEC conditions, this assumption has been demonstrated to be valid. Nevertheless, we want our customers to be aware of this assumption and verify during method validation. The last limitation is that SEC cannot quantify large aggregates properly because the large aggregates might be removed or altered by the column. For separation and analysis of large aggregates, we offer field-flow fractionation coupled to MALS (FFF-MALS).

**LCGC:** What about the advantages?
**CHEN:** This method has many advantages. It is robust with high accuracy and precision, automated with little-to-no hands-on time, fast (typically 15 - 30 minutes per sample depending on the flow rate used), measures multiple quality attributes within one run, no need for reagents, calibration, or labeling, and it can measure in-process samples from downstream or even upstream process steps.
The Origins of Sample Preparation Technologies

In this column, and elsewhere throughout the research community devoted to chromatographic sample preparation, the importance of the knowledge of the fundamental principles underlying sample preparation is emphasized. What is the role of solubility, diffusion, surface tension, and other parameters on the efficacy of extraction, selectivity, and so on? Of course, the answer is partly based on insight into the physicochemical principles upon which they are based, and partly based on serendipity. This month, we take a look at some of the more popular sample preparation methods, and present a discussion of their initial conception and development, to shed light on their current roles in our analytical "toolbox."

Douglas E. Raynie

Two years ago, we presented a retrospective look at the classical Soxhlet extraction, including the origins, operation, standard methods, and derivations (1). One can learn about the principles of modern techniques for extracting solid samples, such as via supercritical fluid extraction (SFE), pressurized liquid extraction (PLE), and microwave-assisted extraction (MAE), from a working knowledge of Soxhlet extraction, according to our premise. Now, let's take it to the next step and investigate the origins of more modern treatments of extraction. To do this, we look to the chemical literature, but more importantly, some of the folklore is presented based on my conversations or subsequent communications with key players in the field of sample preparation. Last year, my colleague Brian Logue described his thought process in combining freeze concentration with stir-bar sorptive extraction (SBSE) to form a novel approach called ice concentration linked with extractive stirrer (ICECLES) (2). This is a great example of combining natural curiosity and an understanding of fundamental principles to develop modern approaches to analytical problem solving. Stories about the development of modern analytical extractions follow.

Solid-Phase Extraction
Perhaps the place to start is the beginning...of chromatography. Of course, A.J.P. Martin and R.L.M. Synge are foremost among the pioneers of analytical separations. In 1941, they separated amino acids via partition between aqueous and organic phases by designing a "mixer-settler" extractor (3). They abandoned this approach when they encountered mechanical difficulties with their apparatus, and turned their attention toward the development of liquid–liquid (partition) chromatography (LC). However, L.C. Craig picked up on this work in refining his countercurrent extractor.

As a side note, when I was in graduate school, Milton Lee always showed a Craig countercurrent apparatus during his separations course. Each time, one or more of the glass tubes broke off. I feel sorry for the more recent students that probably never observed a somewhat intact device.

QuEChERS
One of the most recent, and more intriguing, techniques in the sample preparation arsenal is that known as QuEChERS or quick, easy, cheap, effective, rugged, and safe extraction, developed in the 2000s. This approach...
has been adapted for use with the extraction of almost any analyte from almost any matrix, or so it seems. Steve Lehotay in the U.S. Department of Agriculture’s Agricultural Research Service laboratory in suburban Philadelphia and his colleague Michelangelo Asastassiades at the EU Reference Laboratory for Pesticides set out to develop what Lehotay described in an email message as, “the most efficient method for multiclass, multiresidue analysis of pesticides in foods.” (5)

Previously, both researchers had explored SFE and taxed it to its limits. Steve describes their collaboration as something like, “the Holy Grail of regulatory and industry laboratories for decades.” He explained further:

Increasing concerns about excessive glassware, use of chlorinated solvents, high labor needs, and low sample throughput were putting more pressure on laboratories to adopt more efficient practices. More sensitive, smaller, and less expensive gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–tandem mass spectrometry (LC–MS/MS) instruments were also being introduced at about that time, which permitted an even broader scope of analysis while still maintaining high selectivity in detection. This meant that sample preparation needed to recover a wide range of polar and nonpolar pesticides, but cleanup was still needed to avoid instrument contamination. (5)

Ultimately, it became a brute force effort. They were aware of magnesium sulfate as both a drying agent and an extraction aid, so that was a starting point. They isolated and studied solvent type, ratio of sample size to solvent volume, sample and buffer pH, salting-out salts and amounts, and extraction time and temperature. They determined recoveries depending on analyte polarity relative to the amounts of water and solvent used, along with measuring co-extractives. After tailoring the extraction for a broad range of pesticides and food samples, their attention turned to the dispersive-SPE step, addressing the perceived disadvantages of matrix-solid phase dispersion, the cost of cartridges, and related factors. Since that time, several vendors have modified the salts or parameters to expand the scope of the technique, and Lehotay has refined the approach to a new QuEChERSER which will be the subject of an upcoming “Sample Prep Perspectives” column.

In the food industry, it is customary to name extraction techniques after those who developed them.
such as the Luke or Randall methods. However, the inventors apparently felt that the “Lehotay and Asastassiades Method” didn’t roll off the tongue with the same impact as listing the key attributes of the technique!

**Solid-Phase Microextraction**

Continuing along the theme of extraction techniques based on chromatographic sorbents is solid-phase microextraction (SPME). Despite its name, SPME is not a miniaturized version of SPE. It is described as an open bed, diffusion rate-controlled technique (6). While numerous versions of the approach continue to appear, our discussion will focus on the original, traditional variant of SPME, and this is perhaps a case where sometimes a researcher can be too advanced.

The story of SPME begins in 1987, when Pawliszyn and Liu described sample introduction in capillary gas chromatography (GC) using laser desorption of the sample from the end of an optical fiber, as seen in Figure 2 (7). This provided inspiration to Pawliszyn, who realized that the fiber, clad with a stationary phase like polydimethylsiloxane (PDMS), could also extract organic compounds. The fiber optic replaced the wire used in microsyringes.

Stir-Bar Sorptive Extraction

SBSE represents an interesting example of applying your expertise to a seemingly unrelated situation, at least partially according to the folklore. We remember overhearing a conversation involving Pat Sandra from Belgium’s Research Institute of Chromatography, where he claimed that his inspiration was reading one of his colleague’s papers where, in performing a mass balance for an extraction, the author looked at sample losses due to volatilization, adsorption to the walls of the glassware and stir bar, and other reasons. Sandra thought he could apply a stationary phase to the stir bar and intentionally adsorb the analyte onto the device for subsequent desorption.

David, Ochiai, and Sandra note that, in the early days of SPME, a controversy existed regarding the nature of the sorption phenomenon observed (8). Apolar solutes could potentially adsorb onto the Teflon-coated stir bar used for sample agitation, as well as onto the PDMS SPME coating. This led to the development, and subsequent commercialization, of PDMS-coated stir-bars for extraction, the advent of
SBSE. In the two decades since Sandra’s keen insight, approximately 1000 reports of SBSE are in the literature.

**Pressurized Liquid Extraction**
The 1990s are considered the heyday of the development of modern instrumental approaches to sample preparation, including SFE and MAE. In the early part of that decade, Dionex/Lee Scientific introduced the Model 703 supercritical fluid extractor. Within a very short period, their competitors responded with their SFE offerings and with the more advanced developments, shortcomings in the earlier equipment rose to the forefront. In response, the Dionex Salt Lake City Technical Center, home to the SFE development, convened a customer focus group to “fix the SFE.” Led by Bruce Richter and Brian Jones, the conversation led to questions like “If nearly all SFE requires the use of an organic co-solvent, what is the role of the carbon dioxide?” From here, the concept evolved into the concept of an “analytical pressure cooker”—that is, the application of pressure to an extracting solvent with the intent of increasing its boiling point to create enhanced extracting conditions. Both kinetic and thermodynamic factors become favored at temperatures of 100–150 °C. The resulting technique became Accelerated Solvent Extraction (ASE), a trade name more generally referred to as ASE. During the development of SFE instrumentation in the 1990s, the U.S. Environmental Protection Agency’s Office of Solid Waste issued a memo to equipment manufacturers delineating the requirements to gain approval for an official EPA method utilizing SFE. Dionex followed this guidance during their development of the ASE technique, and they received early market acceptance. As a result of the introduction of ASE, not only did other vendors follow suit in creating competitive instrumentation, but the overhype of SFE declined. Applications of SFE became less risky and increasingly oriented toward those applications, like foods, flavors, or polymers, where supercritical fluids truly had advantages.

**Vaping as Sample Preparation**
Finally, we introduce a novel sample preparation approach based on knowledge of extraction fundamentals and creative reapplication of casual observations. While I know little about the phenomenon of vaping, I realized that this approach is fundamentally sample preparation. The vaping device, via application of elevated temperature, vaporized sample components for delivery by inhalation. We obtained a dry herb vaporizer and, as the final part of his dissertation (8), one of our graduate students, Ahsan Ahmed, evaluated its use in analytical extractions. The particular device we used allowed controllable temperatures up to 240 °C. What was intriguing was that, not only could we employ temperatures similar to conventional headspace techniques and thermal desorption, but also at often unexplored temperatures intermediate between these techniques and pyrolysis. Ahsan used the vaping pen with little modification and SPME to collect the emitted vapors. Due to the volume of the vaping chamber, improved sensitivity resulted. Figure 3 shows the GC–MS results for the extraction, or vaping, of horseradish root as a function of temperatures from 150 to 240 °C. While further investigation is warranted and improvements to the device may be necessary, it shows promise for use in analytical laboratories, perhaps even for applications done “in the field.”

Our initial paper describing this approach, our application to food and environmental samples, and comparison to headspace sampling is in preparation, with the intent of journal submission this fall.

**Conclusions**
We’ve presented the told and untold stories surrounding the development of several modern extraction techniques. These examples confirm that the invention process requires understanding of the fundamental principles in a given field, often reapplied in a unique setting. While some of these reapplications may seem intuitive in retrospect, what is obvious to one investigator may not be so to others. Of course, serendipity also plays a role in the creative process. Perhaps, sometime down the road, readers of this column may be inspired to develop new approaches by employing their knowledge and experience.

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**ABOUT THE COLUMN EDITOR**

Douglas E. Raynie

“Sample Prep Perspectives” editor Douglas E. Raynie is a Department Head and Associate Professor at South Dakota State University. His research interests include green chemistry, alternative solvents, sample preparation, high-resolution chromatography, and bioprocessing in supercritical fluids. He earned his PhD in 1990 at Brigham Young University under the direction of Milton L. Lee. Raynie is a member of LCGC’s editorial advisory board. Direct correspondence about this column via e-mail to LCGCedit@mjhlifesciences.com
Nicotine: Smoking Out the Truth, Part 1

Part 3 of the Pharmaceutical Roots content series from LGC Mikromol covers the history of nicotine use.

Humanity has had a tumultuous history with nicotine, with use of nicotine-rich tobacco for ceremonial and social functions starting as early as 1400 BC, before being introduced to Europe as a medicinal item in the 16th century. In the last 100 years, nicotine reached peak popularity through cigarettes, and then fell from favor, as their addictive nature and danger to health became apparent.

But has nicotine been given an unfairly bad rap? More recent research has shown that, outside its association with smoking, nicotine is actually a promising treatment for psychiatric and cognitive disorders, including Alzheimer’s disease, Parkinson’s disease, and dementia.

THE HISTORY

Nicotine is an organic compound present in the leaves of plants of the genus *Nicotiana*, which numbers more than 60 species, generally characterized by tubular flowers and large leaves. *Nicotiana* species are native to North and South America, Australia, Africa, and the South Pacific, and are widely grown in China, India, and elsewhere.

While all are commonly called tobacco plants, it is *Nicotiana tabacum* that is cultivated worldwide for tobacco and is the principal source of nicotine, with many other species ornamental. Other plants in the genus containing nicotine at a significant level include:

- *N. glauca* (Brazilian tree tobacco)
- *N. alata* (Jasmine tobacco)
- *N. rustica*

Nicotiana is part of the nightshade family (*Solanaceae*), which may also contain nicotine. *Duboisia hopwoodii* (pituri) from Australia is one example, from which the leaves have been chewed as a stimulant by indigenous communities for centuries. Even potatoes, tomatoes, aubergines, and peppers contain very small amounts of nicotine!

As an addictive stimulant, nicotine is the ingredient in tobacco that makes its use pleasurable, but it wasn’t until 1828 that it was isolated from the tobacco plant and recognized as a chemical. Use of nicotine in tobacco, however, began long before that, potentially as early as 1400 BC, with evidence of tobacco cultivation by Mexicans and Native Americans. Tobacco was smoked socially, medicinally, and ceremonially, but also used for trade and currency: in colonial Virginia...
promissory notes payable in tobacco were accepted, and the cost of commodities from goods to wives was given in pounds of tobacco.

When tobacco was introduced to Europe in 1559, it was promoted as a medicinal treatment; the French ambassador to Portugal, Jean Nicot de Villemain—from whose name we get Nicotiana and nicotine—helped popularize its use in society, sending tobacco and its seeds to the French king in 1560.

Smoking was believed to help prevent people from contracting diseases, in particular the plague—this is why those involved with burying the dead during outbreaks smoked clay pipes of tobacco.

This belief persisted for centuries, until concerns about smoking began to grow in the early 20th century, leading to increasing government campaigns encouraging people to quit, and eventually bans on smoking in enclosed places in many countries in the 2000s.

As tobacco’s star waned, nicotine replacement treatments such as patches and gum started to be used as an aid to help people overcome nicotine withdrawal, and e-cigarettes (vapes) were developed, which replace tobacco leaves with nicotine and water vapor. A 2019 clinical trial found that e-cigarettes were almost twice as effective as traditional nicotine replacement treatments in helping smokers to quit, though the Royal College of Physicians has cautioned that “there is a need for regulation to reduce direct and indirect adverse effects” and ensure quality standards for e-cigarettes are in place.

CHEMISTRY AND PHARMACOLOGY
Nicotine is a chiral alkaloid that makes up over 90% of tobacco alkaloid content, constituting 2-8% of the dry mass of tobacco leaves. The nicotine used in medication and e-cigarettes is also sourced from tobacco plants, and the extraction process can produce many potential impurities.

Most alkaloids contain oxygen in their molecular structure, and exist as colorless crystals. Nicotine contains no oxygen atoms, and is one of the few alkaloids that exist in liquid form when pure. It turns brown after exposure to light or air.

Other nicotine-related alkaloids include cotinine (a metabolite of nicotine in humans), nicotine N-oxides, myosmine, beta-nicotyrine, and beta-nornicotyrine.

Nicotine is a powerful psychoactive neurotoxin that acts both as a stimulant and a sedative and is absorbed and metabolized by the liver. It binds to receptors in the adrenal glands, stimulating an adrenaline release that causes an increase in heart rate, blood pressure and respiration. At the same time, it also activates nicotinic acetylcholine receptors (nAChR) in the brain and appears to cause a release of both dopamine and endogenous opioids, which activate opioid pathways in the neural reward system, producing feelings of pleasure and contributing to its reinforcing effects.

Its pharmacologic and psychodynamic effects make stopping nicotine consumption very difficult, with potential withdrawal symptoms including cravings, anxiety, difficulty focusing, irritability and depression. A 2011 National Institute on Drug Abuse study found evidence that nicotine consumption made cocaine more addictive in mice, and the American Heart Association has said that nicotine consumed through tobacco is one of the hardest substances to quit—on a par with heroin.

The next segment of this series will cover the most recent research behind the potential health benefits of nicotine.
Remembering Harold McNair: Three Fundamental Areas of Understanding in Gas Chromatography

On June 27, 2021, Harold Monroe McNair passed away peacefully in his home, surrounded by his family. Harold’s career as a chromatographer spanned over 60 years, from his 1959 authoring of the first gas chromatography (GC) doctoral thesis in the United States on stationary phase chemistry to the first edition of his classic book, Basic Gas Chromatography, in 1964, to a 2019 journal article on the diet of post-weaning heifers (cows). McNair’s remarkable career and writings spanned the entire breadth of chromatography and separation science. Using McNair’s early writings and works as a guide, I explore three fundamental areas of understanding in GC, seeing ideas about problems that still challenge gas chromatographers today. With McNair, we explore stationary phase chemistry, computers in chromatography, and liquid chromatography (LC) compared to GC.

Nicholas H. Snow

H arold McNair had a remarkable 60-year career as a chromatographer, and his writings spanned the entire breadth of chromatography and separation science. By looking back at some of his key early writings, we have an opportunity to explore three key areas in gas chromatography (GC), which are still relevant today: stationary-phase chemistry, chromatography and computers, and liquid chromatography (LC) versus GC.

Understanding Stationary-Phase Chemistry
Harold McNair’s doctoral dissertation, presented to the faculty of Purdue University in 1959, entitled “Efficiency of Solvents in Gas Chromatography,” was the first doctoral thesis on GC in the United States (1). The original thesis is freely available by open access through the university and ProQuest. The thesis title alone offers interesting historical insight because the thesis was written prior to the definition of many of the currently used terms in chromatography (2). When we think of efficiency today, we mostly discuss peak widths and theoretical plates. In 1959, McNair was discussing retentiveness and selectivity. When we think of solvents today, we would likely think of sample preparation or dilution solvents; McNair was discussing the stationary phase. Translated to the terminology of today, this title might read something like: “Retentiveness of stationary phases in gas chromatography.” McNair performed the first systematic study of retention and polarity of stationary phases in GC, which is a topic that we are still debating and discussing today.

McNair’s thesis also provides insight into how gas chromatographs were constructed back in the 1950s before commercial instruments were available. Figure 1 shows a schematic of McNair’s home-built GC instrument. A complete description of the construction can be found in the thesis. A few construction details about this instrument provide lessons about gas chromatographs and columns that are still relevant today. McNair describes making and packing the columns using 6–10 ft lengths of copper tubing, which can easily be coiled, handled, and fitted into the instrument. Although stainless steel and glass columns would be more inert, McNair was thinking practically about ease-of-use. Today’s fused-silica capillary columns are designed with ease-of-use in mind as well. Other materials might be more inert and more temperature stable, but fused silica columns are easy to use.

McNair’s GC instrument did not have a column oven as we have come to know them. Traditional ovens of the day at a reasonable cost would not likely have provided precise enough temperature control for systematic and reproducible studies of retention. In McNair’s gas chromatograph, the column and detector were placed in a vapor jacket that contained the vapor of a refluxing solvent at constant temperature. Using acetone, water, and anisole provided stable constant temperatures at 55 °C, 100 °C, and 153 °C, respectively. Although this arrangement provided stable temperatures, it was obviously difficult to change temperature. Using acetone, water, and anisole provided stable constant temperatures at 55 °C, 100 °C, and 153 °C, respectively. Although this arrangement provided stable temperatures, it was obviously difficult to change temperature. Temperature programming, a new concept at the time, was out of the question.

McNair recognized the need for strong analyte retention in studies of stationary phase properties, so he prepared packed columns using 30% by weight of each stationary phase with the other 70% being the solid support particles. He commented that this was a higher than usual loading of the stationary liquid phase onto the solid par-
articles, to ensure greater retention of the analytes through having a higher mass of stationary phase in the column. Today, the equivalent thinking is to use a thicker film capillary column to increase retention.

Finally, McNair provides a summary of classical early works on chromatography, some from before the beginnings we think of today. He notes an original work describing gas–solid chromatography but not described as chromatography, as it predates Tswett’s coining of the term chromatography by several years (3,4). In 2010, McNair provided an excellent summary of the history of GC for LCGC North America (5).

McNair’s classic book, Basic Gas Chromatography, was first published in 1964 and became the standard by which all other books intended for new users to learn analytical instruments is measured (6). Figure 2 is a photograph of my own well-worn copy that I purchased upon joining his research group in the 1980s. Of course, this book was required reading in his courses and at the time it still contained much timely information. Today, the original versions with the instantly recognized classical green cover seem dated but the concise descriptions of stationary phases, detectors, and troubleshooting are still relevant. Over the decades, the original Basic Gas Chromatography sold more than 130,000 copies and was published in eight languages. The original paperback is out of print, but second-hand copies are often available through online booksellers. The most recent edition, published by John Wiley and Sons, with me and Professor James Miller as co-authors, was published in 2019.
The more modern discussion of stationary phase chemistry and polarity presented in the later editions Basic Gas Chromatography demonstrate the continuing timeliness and relevance of this discussion, which began with McNair's thesis and was re-energized with the development of ionic liquid-based stationary phases in the 2000s (7). When these highly polar stationary phases were being developed, it was quickly discovered that the chemical names of the compounds used to make them were far too complex for marketing purposes, or even for most chemists to remember. In 2011, to simplify the discussion of the polarity of these new stationary phases, Mondello seized on an idea presented in Basic Gas Chromatography that an overall stationary phase polarity value could be generated using the sum of the McReynolds constants for that phase (8). Mondello's polarity numbers, calculated from the sum of McReynolds constants, are now widely used to express the overall polarity of stationary phases (9).

Since its start in 1965 and written in a style that does not require the reader to be a highly experienced chemist, Basic Gas Chromatography has been the "go-to" first book on GC for analysts all over the world.

McNair's publications also provide insight into major developments in chromatography over the years. Although he is best known as a pioneer in GC, McNair's publication list includes many important works in high performance liquid chromatography (HPLC), supercritical fluid extraction (SFE), supercritical fluid chromatography (SFC), capillary electrophoresis (CE), mass spectrometry (MS), and many more techniques. Perhaps the most important lesson from McNair's publication list is to not just focus on a single technique, but think about solving problems. Thinking about or learning one technique is too limiting for the complex scientific problems of today.

Chromatography and Computers

In 1972, McNair hosted the first International Symposium on Computer Chromatography and Associated Techniques in Mainz, Germany. His editorial introducing the symposium, which was captured in a special issue of Chromatographia, included several comments about data systems and chromatographic data analysis that remain relevant today (10). McNair asked the following two questions: "What are we doing with computers?" and "What should we be doing with computers?" The symposium volume provides an excellent set of answers to the first question, which included developing interfaces for single and multiple instruments, acquiring the data, and processing the data. McNair commented that the introduction of computers to chromatography caused chemists to complain about the large amounts of data generated each morning. This complaint rings true today in the fast GC and comprehensive two-dimensional gas chromatography (GCxGC) communities, which often deal with large data sets. A few of the article titles from this 1972 symposium, including Kaiser's "PPB-Analysis Computerized?" and Battista's "Computerized Blood Alcohol Analysis" demonstrate the intense interest in quantitative analysis. Several other articles on data analysis and evaluation include diagrams showing integration schemes for overlapping and asymmetrical peaks that look very similar to those used to describe these processes in today's modern data systems.

McNair emphasized the need to use computers for qualitative analysis, mentioning the early work of Schomburg and Ziegler on the use of Kovats retention indexes to identify analytes. These early ideas led to the development of retention index libraries that are still in common use today. I was fortunate to work with McNair and Sadtler Research Laboratories in the 1980s on the development of their retention index library into a "poor man's gas chromatography–mass spectrometry (GC–MS)" concept, whereby retention indexes measured on two significantly different stationary phases could be compared to libraries to generate an accurate qualitative analysis. With the rise of benchtop GC–MS and GCxGC–MS instruments, tools for qualitative analysis have been included in many of today's data systems. McNair's comments about qualitative analysis and large data sets still resonate in the GCxGC community, where both are the norm, and the chromatograms themselves are increasingly complex.

LC versus GC

In 1974, McNair posed a question that is still commonly asked today in some form: "How soon will liquid chromatography replace gas chromatography?" (11). He then comments that asking the question at all may demonstrate a general lack of understanding about the two techniques. Difficulties in fundamental understanding exist today. In a comprehensive edited volume on GC published this year, Poole devotes a short section and commentary to this challenge (12). Interestingly, having published exclusively on GC until then, McNair built an extensive publication record on HPLC and other techniques, including GC. When I was in his group, there were two active laboratories in the group: the "LC group" and the "GC group." The LC and GC laboratories were even in separate buildings for a time. McNair kept us all working together: LC group members worked with and challenged GC group members and vice versa. He made sure we were all cross-trained and that we all fundamentally understood separation science, not just individual techniques, and problem solving. The answer to the question of LC replacing GC was never fully decided, and McNair's response is as true today as it was back in 1974. McNair's concise two-page comparison of LC and GC, including thoughts on the separation principle, sample types, minimum detectable quantities, analysis time, theoretical plates, preparative capability, and price range, is as useful today as it was back then. Viewing LC and GC as complementary highlights an important principle of analytical chemistry—that the problem should be considered before the technique, and the technique should be designed to solve the problem, not the other way around. Viewing LC, GC, and other analytical methods as competitive to one another only limits the work and experience to a single technique, which is an elementary mistake new analytical scientists can make.

In 1984, Bowermaster and McNair demonstrated the fundamental complementary nature of LC and GC, describing temperature programming in HPLC (13). In both GC and LC, partitioning between the mobile and stationary phases is an equilibrium-controlled process so both techniques are subject to similar phase thermodynamic
principles that govern retention. As a simple analogy, mobile-phase strength in LC is like temperature in GC. However, in LC, temperature can also be used as an additional variable to control retention. Temperature-programmed versus isothermal LC runs showed very similar behavior to temperature-programmed runs in GC, with the temperature-programmed runs showing roughly equal spacing of the peaks versus the exponential spacing seen in isothermal runs. Although temperature-programmed HPLC has not caught on very much, precise temperature control, obviously necessary in GC, which Bowermaster and McNair also discussed in detail, has been incorporated into nearly all of today’s commercial HPLC systems.

These brief vignettes provide a small taste of Professor Harold McNair’s massive contributions to separation science. His publication record alone does not capture the whole story of a career and life devoted not only to the science but to people all over the world. There are few separation scientists of my own and previous generations that did not meet or interact with McNair at a conference, a short course, or in the laboratory. He was quick to invite visitors from all over the world to visit his laboratory in Blacksburg, Virginia to share knowledge, experience, and good times. He was a world traveler who routinely visited the laboratories of colleagues all over the globe.

We had a globe of the earth in our laboratory with a small arrow attached, pointing to wherever in the world “Doc” was, often not Blacksburg. Whether through publication, working in the laboratory together with other scientists, or from conversations, Harold McNair freely shared his knowledge and experience. His work and spirit influenced separation science far beyond GC and he is easily considered among the most influential analytical scientists of the past 60 years.

Thank you, Harold.

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ABOUT THE AUTHOR
Nicholas H. Snow is the Founding Endowed Professor in the Department of Chemistry and Biochemistry at Seton Hall University, and an Adjunct Professor of Medical Science. During his 30 years as a chromatographer, he has published more than 70 refereed articles and book chapters and has given more than 200 presentations and short courses. He is interested in the fundamentals and applications of separation science, especially gas chromatography, sampling, and sample preparation for chemical analysis. His research group is very active, with ongoing projects using GC, GC–MS, two-dimensional GC, and extraction methods including headspace, liquid–liquid extraction, and solid-phase microextraction. Direct correspondence to: LCGCedit@mmhgroup.com
Analyzing Per- and Polyfluoroalkyl Substances in Drinking Water Using EPA Methods 533 and 537.1 with Semi-Automated Solid-Phase Extraction (SPE)

Per- and polyfluoroalkyl substances (PFAS) are compounds that contain perfluorinated or polyfluorinated carbon chain moieties such as F(CF$_2$)$_n$- or F(CF$_2$)$_n$-(CH$_2$)$_m$. PFAS are widely used in consumer and industrial applications. There has been increased concern over the levels of PFAS, such as perfluorosulfonate (PFOS) and perfluoro-octanoic acid (PFOA), in the global environment and their potential adverse effects on the environment. In the United States, recent EPA methods 533 and 537.1 were released for analyzing PFAS in drinking water samples. A semi-automated system that combines elements of fully manual and automated procedures was developed and tested. Both methods use 250 mL water samples that were spiked with 2–32 ng/L PFAS standards. Styrenedivinylbenzene (SDVB) cartridges were installed on the system and the vacuum was turned on. The cartridges were conditioned, and then the samples were loaded across the cartridges (25-50 min). The sample bottles were automatically rinsed with water and the rinses were also loaded. Cartridges were dried and eluted for collection. Samples were reduced in volume using an automated concentrator. A total of ~30 PFAS were analyzed with recoveries well within the method acceptance windows (70–130%). Data showed good reproducibility with native background contributions from the system (<0.20 ng/L). Total processing time was between 50 min (537.1 method) and 75 min (533 method).

Ruud Addink and Tom Hall

Per- and polyfluoroalkyl substances (PFAS) are compounds that contain perfluorinated or polyfluorinated carbon chain moieties, such as F(CF$_2$)$_n$- or F(CF$_2$)$_n$-(CH$_2$)$_m$. Recent years have seen a great increase in the need for fast and reliable PFAS analyses in various types of environmental samples, especially in drinking water.

PFAS have been widely used in various consumer and industrial applications. These applications include firefighting foams, floor polishes, grease-proof coatings for paper products approved for food contact, insecticide formulations, mining and oil well surfactants, stain-resistant coatings for textiles, leather, and carpets. Some of the PFAS, such as perfluorosulfonate (PFOS) and perfluoro-octanoic acid (PFOA), have been studied extensively for their increasing levels in the environment. In animal studies, adverse effects on multiple organs (liver and pancreas), developmental problems in rodent offspring exposed in the womb, endocrine disruption, and reduced immune function, have been found (1). Possible effects on human health are still being investigated (2).

PFAS have been added in 2009 and 2019 to the 2004 Stockholm Convention to which the majority of countries in the world have acceded (3). The U.S. Environmental Protection Agency (EPA) has established an advisory level of drinking water not to exceed 70 ng/L of PFAS (4). Recent years have seen the releases of EPA methods 533 and 537.1 for PFAS analyses in drinking water using solid-phase extraction (SPE), which are being followed by commercial and governmental environmental laboratories in North America (5,6).

Manual methods for PFAS extraction generally involve using a vacuum manifold with cartridges placed on top to load the samples and then elute them into collection vessels. Because this system is open to the laboratory background, it is prone to lead to sample contamination. Water present in the extracts needs to be removed after elution of the cartridges in a separate step. The extractions typically require the attention of a laboratory technician throughout the entire procedure. Fully automated methods have the advantage of a closed system, which reduces the chances of background contamination. Solvent use for cleaning the system is likely to be less than with the manual method since the...
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Automated systems run cleaning solvents through the sample lines and connections only. Water removal from the extracts can be done with inline drying cartridges (sodium sulfate). Automated systems are typically run with a built-in computer which allows for unattended extractions freeing up workers for other tasks (7).

Combining the best features of both the manual and automated methods can lead to a semi-automated system that is relatively inexpensive and able to extract drinking water samples for PFAS reliably and with good reproducibility. Many laboratories do not have sufficient funds available to purchase fully automated systems. However, the semi-automated method described here is a good solution for laboratories that do not have as much financial capital.

Materials and Methods

Semi-Automated System

The system can extract six or 12 samples of 250 mL drinking water in polypropylene bottles with Delrin fittings (Fluid Management Systems). The tubing is made of polyethylene so that background concentration of PFAS (as present in Teflon) is greatly reduced. The system operates under the vacuum, which stays on during the entire procedure. Various features of a semi-automated SPE system are shown in Figure 1. The system has a stage 1 manifold with stopcocks for cartridge conditioning, sample loading, and automated bottle rinsing. Stage 2 is the manifold used for elution, and it contains a glass tank with six or 12 positions for collection vessels. Each of the manifolds are comprised of a vacuum regulator and a vacuum gauge. On the front of the base of the system, there is a nitrogen pressure gauge, a nitrogen gas regulator and valve (for drying and automated bottle rinsing), a valve that switches the vacuum suction between stages 1 and 2, and a valve that sends the waste to either aqueous waste or organic waste.

Method

Samples were prepared in deionized water (250 mL) containing 1 g/L ammonium acetate. Acetic acid was used to adjust the pH from 6 to 8. The sample bottles were then spiked with relevant native 533 PFAS standards. Next, 500 mg (6 mL volume) styrene-divinylbenzene (SDVB) cartridges (Fluid Management Systems) were put in each of the manifold one positions. The vacuum was turned on, and the cartridges were conditioned with 10 mL methanol, 10 mL 0.1 M phosphate buffer, and 3 mL phosphate buffer with 2 mL of water. The cartridges were kept wet during the entire conditioning process. The samples were then loaded across the cartridges at 5 mL/min. Sample bottles were then rinsed automatically with 10 mL of 1 g/L ammonium acetate.

Recoveries of native PFAS compounds in EPA method 533 extracts at 25 ng/L. The abscissa label is native PFAS compounds.

<table>
<thead>
<tr>
<th>Native PFAS Compounds</th>
<th>Percentage Recovery</th>
<th>0.0</th>
<th>20.0</th>
<th>40.0</th>
<th>60.0</th>
<th>80.0</th>
<th>100.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**TABLE I:** Gradient solvent program for chromatography method

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow (mL/min)</th>
<th>%A</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>0.4</td>
<td>75</td>
<td>25</td>
</tr>
<tr>
<td>1.00</td>
<td>0.4</td>
<td>75</td>
<td>25</td>
</tr>
<tr>
<td>1.50</td>
<td>0.4</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>9.60</td>
<td>0.4</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>13.50</td>
<td>0.4</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>14.50</td>
<td>0.4</td>
<td>75</td>
<td>25</td>
</tr>
</tbody>
</table>
acetate in water, which was loaded across the cartridges, followed by rinsing bottles with 1 mL methanol that was then also loaded. Cartridges were then dried for 5 min under nitrogen. The cartridges were then transferred to the stage 2 side for elution. The rinse bottles were filled with 5 mL 2% ammonium hydroxide in methanol, and this solution was sprayed across the walls of the samples bottles automatically using nitrogen gas. The rinses were then pulled across the cartridges under vacuum. The procedure was then repeated resulting in a total of 2 x 5 mL of elution across the cartridges. The eluents were collected for further concentration and analysis.

537.1 Method
For the analysis, 250 mL water samples were prepared and spiked with 537.1 PFAS standards. Sample bottles were loaded onto the system and rinse bottles were filled with 7.5 mL water. Next, 6 mL 500 mg SDVB cartridges were put in each of the manifold one positions, and the vacuum was turned on. The cartridges were conditioned with 15 mL methanol (soak 1 min) and then with 18 mL water. Samples were loaded across the cartridges under vacuum (20–25 min, ~8 in. mercury). The sample bottles were then rinsed automatically with nitrogen with 7.5 mL water twice (total 15 mL water) and loaded across cartridges (dry for 5 min under vacuum). The cartridges were then transferred to the stage 2 side for elution. Rinse bottles with 4 mL methanol were used to rinse sample bottles automatically and rinses were loaded across the cartridges (twice, 1 min soak, 8 mL methanol total). Eluent was collected for analysis into collection vessels.

Sample Concentration
Extracts were concentrated to dryness in an automated concentrator (Fluid Management Systems). The concentrator has 24 positions for concentration at 65 °C under a nitrogen flow of 9 psi. The polypropylene collections vials are graduated for 15 mL and are conical in shape. The system uses a dry bath heating element with a time-based endpoint, and the temperature and time log were saved.

Analysis
A Waters Acuity ultrahigh-pressure liquid chromatography (UHPLC) system coupled to a quadrupole time-of-flight (QTOF) Xevo G2-XS mass spectrometer and high resolution mass spectrometry (HRMS) was used for analyzing PFAS. Separation was performed with a Waters Acquity HSS T3 column (2.1 mm x 100 mm, 1.8 µm) and samples were analyzed by electrospray in negative ionization mode. Samples were reconstituted in 1 mL methanol (1:3 ratio) and then sonicated prior to injection (20 µL).

A gradient solvent program was used with 0.1% formic acid in liquid chromatography–mass spectrometry (LC–MS) grade methanol (solution A) and 0.1% formic acid in LC–MS grade water (solution B). The details of the gradient method are seen in Table I.
Samples were analyzed in negative electrospray ionization (ESI) mode. Desolvation gas (nitrogen) flow rate was kept at 750 L/h, capillary voltage at 0.5 kV, and the source and desolvation temperatures at 120 ºC and 250 ºC, respectively. Mass range was set from 50 to 950 Da with a scan rate of 0.055/s.

The concentration and recovery of each PFAS was determined by comparing the spiked sample response to that of a reference standard at the same concentration (0.5 ng/mL). A labeled PFAS (d$_3$-N-MeFOSA-M) was spiked to each sample and reference standard in order to obtain a normalized response for accuracy (8).

### Discussion and Results

Figure 2 shows recoveries for PFAS analyzed in accordance with EPA method 533. This method is comprised of perfluorocarboxylic acids that have a linear chain of carbon atoms all substituted with fluor and a terminal carboxylic acid group, perfluorosulfonic acids that are similar in structure but have a terminal sulfonic acid functional group, and three other compounds (4:2 FTS, 6:2 FTS, and 8:2 FTS) that are sulfonic acids in which the two carbon atoms nearest to the sulfonic functionality have no fluor atoms substituted. Average recoveries were between 80–105% for a set of samples with relative standard deviations (RSDs) shown as error bars. The recoveries were all well within the acceptance window of the method, which is 70–130% in this instance.

Table II shows recoveries for method 537. PFAS run on the system. Samples were run at 2, 4, 24, and 32 ng/L. Data are between 80–120% and again all within the 70–130% acceptance window of the method. The data also shows that good recoveries can be obtained for both lower and higher PFAS concentrations.

Figure 3 has background concentrations for method blank runs done on the system. The contributions from the system are low: the highest blank concentration found is that of PFOA at 0.2 ng/L. The other PFAS shown are present at lower concentrations, mostly <0.05 ng/L. The system is clearly suitable for trace level determination of PFAS in water. The use of polyethylene and polypropylene materials is clearly very effective in keeping native background of PFAS to a minimum.

### Conclusions

Sample data shows high recoveries for all spiked native PFAS for both 533 and 537.1 methods. All native recoveries are well within the 70–130% acceptance windows for both methods. RSDs are mostly low.

### TABLE II: Recoveries of native PFAS compounds in method 537.1 extracts at 2–32 ng/L.

<table>
<thead>
<tr>
<th>PFAS Compound</th>
<th>2 ppt</th>
<th>4 ppt</th>
<th>24 ppt</th>
<th>32 ppt</th>
<th>EPA window</th>
</tr>
</thead>
<tbody>
<tr>
<td>PFBS</td>
<td>94%</td>
<td>93%</td>
<td>98%</td>
<td>99%</td>
<td>70%-130%</td>
</tr>
<tr>
<td>PFHxS</td>
<td>99%</td>
<td>104%</td>
<td>101%</td>
<td>109%</td>
<td>70%-130%</td>
</tr>
<tr>
<td>GenX</td>
<td>102%</td>
<td>98%</td>
<td>106%</td>
<td>123%</td>
<td>70%</td>
</tr>
<tr>
<td>PFHpA</td>
<td>99%</td>
<td>103%</td>
<td>102%</td>
<td>103%</td>
<td>70%-130%</td>
</tr>
<tr>
<td>PFHxS</td>
<td>95%</td>
<td>97%</td>
<td>97%</td>
<td>102%</td>
<td>70%-130%</td>
</tr>
<tr>
<td>ADONA</td>
<td>90%</td>
<td>97%</td>
<td>99%</td>
<td>104%</td>
<td>70%-130%</td>
</tr>
<tr>
<td>PFOA</td>
<td>116%</td>
<td>109%</td>
<td>105%</td>
<td>103%</td>
<td>70%-130%</td>
</tr>
<tr>
<td>PFNA</td>
<td>95%</td>
<td>107%</td>
<td>111%</td>
<td>110%</td>
<td>70%-130%</td>
</tr>
<tr>
<td>PFOS</td>
<td>93%</td>
<td>96%</td>
<td>95%</td>
<td>101%</td>
<td>70%-130%</td>
</tr>
<tr>
<td>9Cl-PF3ONS</td>
<td>88%</td>
<td>88%</td>
<td>95%</td>
<td>100%</td>
<td>70%-130%</td>
</tr>
<tr>
<td>PFDA</td>
<td>91%</td>
<td>99%</td>
<td>105%</td>
<td>111%</td>
<td>70%-130%</td>
</tr>
<tr>
<td>N-MeFOSAA</td>
<td>93%</td>
<td>97%</td>
<td>92%</td>
<td>92%</td>
<td>70%-130%</td>
</tr>
<tr>
<td>PFudA</td>
<td>93%</td>
<td>101%</td>
<td>104%</td>
<td>108%</td>
<td>70%-130%</td>
</tr>
<tr>
<td>N-EtFOSAA</td>
<td>95%</td>
<td>110%</td>
<td>98%</td>
<td>98%</td>
<td>70%-130%</td>
</tr>
<tr>
<td>11Cl-PF3OUDS</td>
<td>86%</td>
<td>88%</td>
<td>86%</td>
<td>91%</td>
<td>70%-130%</td>
</tr>
<tr>
<td>PFDoA</td>
<td>90%</td>
<td>92%</td>
<td>99%</td>
<td>101%</td>
<td>70%-130%</td>
</tr>
<tr>
<td>PFTrDA</td>
<td>86%</td>
<td>89%</td>
<td>97%</td>
<td>93%</td>
<td>70%-130%</td>
</tr>
<tr>
<td>PFTeDA (PFTA)</td>
<td>84%</td>
<td>82%</td>
<td>91%</td>
<td>93%</td>
<td>70%-130%</td>
</tr>
</tbody>
</table>

FIGURE 3: Native background of 537.1 PFAS compounds using the semi-automated system. The abscissa label is native PFAS compounds.
showing that the semi-automated system gives very good reproducibility. Native background contributions from the system are very low. The automated sample concentration step (to dryness) accounts for little loss of these compounds. The semi-automated system provides an inexpensive alternative to both manual and automated methods, with fast processing times between 50 and 75 min (depending on the different loading times described in the methods). Samples can be taken from collection bottle to a LC vial in one quick, consistent, reproducible process.

Wastewater samples can be run on the semi-automated system too, using larger 25 mL volume cartridges—still with 500 mg SDVB—that make it possible to fill up the cartridge barrel with Delrin plastic filtration wool. Using a semi-automated system to run wastewater samples will slow down movement of the particulate matter in the waste water and prevents plugging of the cartridge.

References

ABOUT THE AUTHORS
Ruud Addink is Technical Director of Toxic Report Laboratories in Billerica, Massachusetts. Direct correspondence to: ruudaddink@toxicreports.com
Tom Hall is Manager of Toxic Report Laboratories and Vice-President of Sales at Fluid Management Systems in Billerica, Massachusetts.
Evaluating Two Dioscin-Based Silica Stationary Phases and their Application to Achiral and Chiral Separations

In a previous study, polyphyllin III, an important natural dioscin ingredient, was bonded to silica particles using the “one-pot method” for preparing a stationary phase, called SP-D. SP-D was further modified to produce two stationary phases, namely the phenyl isocyanate-dioscin (Phe-D) and 3,5-dimethyl phenyl isocyanate-dioscin bonded silica stationary phase (DMP-D), respectively. The Phe-D and DMP-D were evaluated by achiral and chiral analytes. The high performance liquid chromatography (HPLC) method was optimized and applied to the analysis of the main active ingredient, polyphyllins, that were contained in gongxuening capsules, a traditional Chinese medicine (TCM) widely used to treat gynecological diseases. The two synthesized stationary phases were also applied to the separation of the amino acid (AA) enantiomers. For this study, 10 α-AAs (lysine, leucine, cysteine, arginine, isoleucine, threonine, serine, valine, alanine, and histidine) were selected and studied for chiral separation using Phe-D and DMP-D stationary phases. In this study, 10 observed AA enantiomers were separated to different degrees on at least one stationary phase whereas four AAs were enantioseparated on both Phe-D and DMP-D. These results indicated that the two synthesized stationary phases have potential applicability in quality control (QC) of TCM and chiral separation as well as offering a new application choice for analyses of natural products.

Xiaojuan Gu, Kongchun Sun, Yaqiong Han, Bingquan Chang, Wen Shu, Liangyu Chen, Canyu Yang, and Baochun Shen

As the pharmaceutical and natural medicine industries continue to evolve, new separation issues posed by various samples need to be resolved. As a result, the demand for more reversed-phase high performance liquid chromatography (HPLC) stationary phases is becoming significant. The ongoing research in designing, synthesizing, and testing the performance of stationary phases, is therefore crucial (1,2).

It is possible to prepare a stationary phase that possesses both support and active functions by bonding the active ingredients to the surface of the silica gel base material. Some stationary phases with ligands extracted from medicinal plants such as emodin, curcumin, saponin, magnolol, allicin, quercetin, and isatin have been prepared (3–6). This type of stationary phase can provide not only the functions of hydrophobic interaction, but also hydrogen-bonding interaction, π–π, and n–π conjugation. They are quick and highly selective for structurally similar compounds (7–9). Modified or derivative natural products can be bonded to the surface of the silica gel to prepare HPLC chiral stationary phases (CSP). These CSPs, based on products such as cyclodextrins, polysaccharides, and macrocyclic glycopeptide antibiotics and proteins, were proven to be highly efficient (10–15). The interactions, hydrogen-bonding, π–π stacking, inclusion interaction, and ion exchange, between the solute and CSP played a key role in chiral resolution (16,17).

Dioscin, one active compound in Dioscorea rhizoma, and several Discordance plants, including Dioscorea zingiberensis C.H. Wright (D. zingiberensis) and Dioscorea nipponica Makino (DNM), possess anti-inflammatory, immunoregulation, hypolipidemic, antiviral, antifungal, and antiallergic effects (18). Dioscin is also used as an important ingredient for the synthesis of various steroid hormone drugs.

Figure 1 shows the structural formula of dioscin. As the figure shows, dioscin contains active hydroxyls that provide favorable conditions for binding events. The multiple hydroxyls and double bonds can participate in hydrogen-bonding interaction, dipole–dipole interaction, and π–π interactions, and the structure is large enough to shield the influence of the acidic Si-OH residue on the stationary phase and improve the separation process. The plane and rigid structure of dioscin can also improve the stereoselectivity by providing a larger contact surface for the solute molecules. In this case, we bonded dioscin to silica gel to obtain a dioscin-bonded stationary phase, called SP-D. Then, two derived stationary phases were produced, namely phenyl isocyanate-dioscin bonded silica stationary phase (Phe-D) and 3,5-dimethyl phenyl isocyanate-dioscin bonded silica stationary phase (DMP-D), respectively. In a previous study, these two derived stationary phases were evaluated, and it was determined that they had typical reversed-phase (RP) chromatographic performance, which makes both similar to the performance of octadecylsilyl (ODS) columns (19).

Quality control (QC), including qualitative and quantitative analysis, is a major bottleneck for traditional Chinese medicines (TCM) with their increased use worldwide (20). There are many ingredients, including active ingredients, auxiliary components, and inactive ingredients in TCM. Because of the sheer quantity of ingredients in TCM, it is too difficult to identify and characterize all of them. Therefore, a characteristic HPLC chromatogram is always chosen to compare and evaluate TCM (21,22). For quantitative analysis, a single component cannot represent the full
efficacy of any TCM. Analysis of multiple active components, through quality markers (Q-markers) detection is therefore necessary (23,24). As a result, the combination of a characteristic chromatogram and quality markers detection is a powerful way to ensure quality of TCMs.

Chirality caused by molecular asymmetry has special effects on physical, chemical, biological, and pharmacological properties appearing at a molecular level (25). Amino acids (AA), the simplest chiral molecule, have at least one stereogenic center (with the exception of glycine). The important analytical task of the chiral separation of an amino acid is achieved mainly by chromatographic methods, especially HPLC, including direct and indirect separation (26).

In a previous study, we prepared two natural active ingredient dioscin-based stationary phases, Phe-D, and DMP-D, respectively. The bonding method, the “one-pot method,” used to produce the stationary phases directly without tedious intermediate steps, was proven to be simple and convenient. The Phe-D and DMP-D stationary phases were characterized by elemental analysis, scanning electron microscopy, thermogravimetric analysis, and infrared (IR) spectroscopy. These new stationary phases were evaluated by benzene and its homologs, polycyclic aromatic hydrocarbons, and other solutes as probes. Through these evaluations, Phe-D and DMP-D were verified to have typical RP chromatographic performance, similar to that of ODS columns, and the separation principle was shown to be related to the hydrophobic effect. In addition, these stationary phases were confirmed to have the ability to provide hydrogen bonding and π-π conjugation sites for solutes (19).

To evaluate and compare these two stationary phases, we investigated their application to achiral and chiral separation. In this study, we used HPLC characteristic chromatograms of 10 batches of gongxuening capsules and determined the content of six main polyphyllins in 10 batches of gongxuening capsules. Then, the application of Phe-D and DMP-D to chiral separation of 10 amino acids was studied and evaluated.

Experimental
Reagents and Chemicals
Polyphyllin I and II (Guizhou Dida Biochemistry Co.), polyphyllin III (Nanjin Biochemistry Co.), polyphyllin V (Chengdu Herbpurify Co., Ltd.), polyphyllin VI and VII (Sichuan Weikeji Biotech Co., Ltd.), and gongxuening capsules (Yunnan Baiyao Group Co., Ltd.) were used. The AA enantiomers lysine, leucine, cysteine, and arginine came from Shanghai Lanji Technologies Co., Ltd. The AA enantiomers isoleucine and threonine came from the Chinese Academy of Sciences’ Institute of Biochemistry. For the remaining AA enantiomers, serine came from the Shanghai Chemical Reagents Procurement Station, valine came from Feinbiochemical Heidelberg, alanine from the Shanghai Third Reagent Factory, and histidine from the Shanghai Renfa Comprehensive Utilization Factory.
All the other reagents were analytically pure and procured through commercial channels.

**Experimental Instrument and Chromatographic Conditions**

The Agilent 1260 HPLC system, which included the G1322A Vento, a G1312C delivery pump, a G1329B standard automatic sampler, a G1316A column oven, a G1315D detector, and the Agilent 1200 LC chromatographic work station, was used as the instrumentation for this study. The separation was performed on two columns (Phe-D: phenyl isocyanate-dioscin bonded silica gel stationary phase, 4.6 × 250 mm; and DMP-D: 3,5-dimethyl phenyl isocyanate-dioscin bonded silica gel stationary phase, 4.6 × 250 mm). The mobile phase consisted of acetonitrile and 0.01% phosphoric acid for QC, and for the chiral separation, methanol and a 0.04 mol/L buffer solution were used. The flow rate was 0.4 mL/min. The injection volume was 10 µL. The column oven was maintained at 35 °C, and the UV detector was set at 203 nm for quality control. The column oven was maintained at 25 °C, and the UV detector was set at 210 nm for chiral separation. Column efficiency was determined with biphenyl, the theoretical plate number of Phe-D is 4776 block/m, and the theoretical plate number of DMP-D is 5028 block/m.

**Preparation of Reference and Sample Solutions**

**Preparation of Reference Solutions**
The appropriate amount of Polyphyllin I, II, III, V, VI, and VII were dissolved in methanol to prepare the reference solutions of various concentrations (0.375 mg/mL, 0.375 mg/mL, 0.400 mg/mL, 0.300 mg/mL, 0.450 mg/mL, and 0.450 mg/mL, respectively).

**Preparation of Sample Solutions**
The content of 20 capsules from 10 different batches of gongxuening capsule was weighed and porphyrized. Next, 1 g of the substance was added into a conical flask with cover along with 25 mL of methanol. The flask was then sealed and weighed. This solution in the flask was treated ultrasonication for 40 min and then cooled to room temperature. Afterward, methanol was added to the solution to compensate any weight loss. The solution was shaken well and filtered with a 0.22-µm microfiltration membrane to remove particulates and the subsequent filtrate was kept as the sample solution.

**Preparation of Sample and Buffer Solution for Chiral Separation**

Racemic lysine, leucine, cysteine, arginine, threonine, serine, valine, alanine, isoleucine, and histidine were separately dissolved in water, and the solutions were adjusted to the appropriate concentration and then filtered with a 0.22-µm microfiltration membrane to remove particulates and the subsequent filtrate was kept as the sample solution.

**Determining Linearity, Precision, Recovery, and Stability**

A series of mixed reference solutions with polyphyllin I, II, III, V, VI, and VII at

---

**TABLE I: The results of similarity calculation**

<table>
<thead>
<tr>
<th>Batch</th>
<th>Similarity*</th>
<th>Similarity**</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>0.991</td>
<td>0.991</td>
</tr>
<tr>
<td>S2</td>
<td>0.982</td>
<td>0.982</td>
</tr>
<tr>
<td>S3</td>
<td>0.942</td>
<td>0.942</td>
</tr>
<tr>
<td>S4</td>
<td>0.913</td>
<td>0.913</td>
</tr>
<tr>
<td>S5</td>
<td>0.999</td>
<td>0.999</td>
</tr>
<tr>
<td>S6</td>
<td>0.974</td>
<td>0.974</td>
</tr>
<tr>
<td>S7</td>
<td>0.951</td>
<td>0.951</td>
</tr>
<tr>
<td>S8</td>
<td>0.979</td>
<td>0.979</td>
</tr>
<tr>
<td>S9</td>
<td>0.917</td>
<td>0.917</td>
</tr>
<tr>
<td>S10</td>
<td>0.996</td>
<td>0.996</td>
</tr>
</tbody>
</table>

Stationary phase: *Phe-D; **DMP-D
five levels (3, 6, 9, 12, and 15 μL) were analyzed. The standard curve was drawn with the concentration (x) of reference solution as the abscissa and the area of chromatographic peak (y) as the ordinate. Linearity was evaluated using a linear calibration curve and by calculating the coefficient of determination. A mixed reference solution with polyphyllin I, II, III, V, VI, and VII with the volume of 10 μL was analyzed six times. Each peak area was recorded for the real standard deviation (RSD) calculation.

To determine the recovery rate, the reference substances (polyphyllin I, II, III, V, VI, and VII) were added to the same sample of gongxuening capsule one by one. Then, a 1.0 g spiked sample of gongxuening was used in conjunction with methanol as previously described in the "Experimental Instrument and Chromatographic Conditions" section. The amount of each reference substance in the sample was calculated by the linearity equation.

Six gongxuening capsule samples from the same batch were prepared with methanol. These solutions were injected into the HPLC column and analyzed. Each common peak area was recorded to analyze the consistency of the relative retention time and relative peak area. A sample solution was placed at room temperature and the sample solution was injected into the HPLC system after 0 h, 4 h, 8 h, 12 h, and 16 h, respectively.

Results and Discussion

The Application of Phe-D and DMP-D to Quality Control of the Gongxuening Capsules

A gongxuening capsule is comprised of the ethanol extract of the traditional Chinese herbal medicine *Paris polyphylla var. yunnanensis* (Franch.) Hand.-Mazz (27). It is widely used to treat diseases such as menometrorrhagia, metrorrhagia, uterine hemorrhages caused by insufficient contraction after delivery or abortion, pain in lateral lower abdomen or lower back, and morbid leukorrhea caused by chronic pelvic inflammatory diseases. It is confirmed that the main active ingredients of the gongxuening capsules are polyphyllins, such as polyphyllin I, II, and VI. The QC procedure for gongxuening capsules in the *Chinese Pharmacopoeia* is simple, which selects the polyphyllins V and VI as the identification references and the polyphyllin VI as the assay marker.

For HPLC method optimization, different mobile phases, including methanol–water, ethanol–water, acetonitrile–water, methanol–acetonitrile–water, ethanol–acetonitrile–water, acetonitrile–aqueous phosphoric acid, and acetonitrile–aqueous acetic acid were studied. The terminal absorption of methanol caused the baseline to drift, and excessive viscosity of ethanol increased the back-pressure of the columns, so acetonitrile was selected as the organic solvent. The combination of acetonitrile and 0.01% aqueous phosphoric acid (v/v) proved to be an optimal mobile phase with the gradient elution conditions. An HPLC method was applied by gradient elution using a) acetonitrile and b) 0.01% phosphoric acid as the mobile phase for QC. For Phe-D, results were taken at the following parameters: 0 min (40% A), 15 min (40% A), 40 min (55% A), 60 min (90% A), and at 75 min (90% A). For DMP-D, results were recorded at the following parameters: 0 min (40% A), 10 min (40% A), 35 min (60% A), 60 min (100% A), and at 75 min (100% A).

The proposed method for chromatographic analysis was validated in terms of precision, repeatability, and stability. Polyphyllin I was chosen as the internal reference peak. On both stationary phases, the precision was assessed by analyzing six replicate samples and the RSD of relative retention times (RRT) and relative peak areas (RPAs), were below 0.75 and 2.34%, respectively. The repeatability test results showed that the RSD of RRTs was below 0.75%, and the RSD of RPAs was below 2.83%. Within 24 h, the RSD of RRTs were below 0.82%, and the RSD of RPAs was below 2.69%. These results showed that the proposed methods for chromatographic analysis were reliable, consistent, and stable.

Similarity is one of the most commonly used parameters for evaluating what is termed characteristic chromatograms for TCM. Usually, similarity data for characteristic chromatograms is used to evaluate whether the quality of TCM is controllable and stable (17,18). Characteristic chromatograms obtained from 10 batches of gongxuening capsules with Phe-D are shown in Figure 2 and the similarity values, calculated by “Similarity Evaluation System for characteristic chromatograms of Traditional Chinese Medicine (version 2004A),” are reported in Table I. Each
peak relative to all sample chromatograms was called the “common peak,” and 14 common peaks were observed in all 10 batches among the total 36 peaks. The peak with the retention time of 31.592 min, which was identified as polyphyllin I, was one of the most important active constituents of the gongxuening capsules and was chosen as the internal reference peak to calculate the RRT and the RPA of the other peaks. The RSDs of RRT and RPA of other common peaks with respect to polyphyllin I from all samples were in the ranges of 0.14–0.77% and 1.35–59.95%, respectively.

From Figures 2 and 3, it can be seen that six polyphyllins had the same elution order on Phe-D and DMP-D, but had a longer retention time on Phe-D. The unique difference of the two stationary phases was two methyl groups connecting to the phenyl ring on DMP-D; the results showed that the electron density of the phenyl ring affected the retention of analytes.

For the determination of the main polyphyllins in gongxuening capsules, the appropriate amount of six standard substances, polyphyllin I, II, III, V, VI, and VII, was dissolved in methanol to prepare the reference solution at various concentrations. Under the analytical methods, polyphyllin II and III were not separated, thus the content value was determined based on the sum of the two compounds.

The linear equation results of each polyphyllin are as follows: For Phe-D, the linear equations were as follows: $y = 1480x - 1088.4$ ($R^2 = 0.9777$) for polyphyllin I; $y = 724.26x - 566.17$ ($R^2 = 0.9764$) for polyphyllin II and III; $y = 1014.9x - 930.39$ ($R^2 = 0.9566$) for polyphyllin V; $y = 908.61x - 33.987$ ($R^2 = 0.9998$) for polyphyllin VI; and $y = 620.13x - 29.652$ ($R^2 = 0.9998$) for polyphyllin VII. For DMP-D, the linear equations were as follows: $y = 1438.5x - 195.19$ ($R^2 = 0.9989$) for polyphyllin I; $y = 755.28x - 217.99$ ($R^2 = 0.9995$) for polyphyllin II and III; $y = 658.75x - 18.738$ ($R^2 = 0.9999$) for polyphyllin V; $y = 984.12x - 59.914$ ($R^2 = 0.9999$) for polyphyllin VI; and $y = 656.61x + 0.0457$ ($R^2 = 0.9999$) for polyphyllin VII. Table II shows the recovery rate of six polyphyllins on Phe-D and DMP-D. The data were determined six times. The precision results for each polyphyllin showed that the RSD% were less than 1.60% and 1.13% for Phe-D and DMP-D, respectively, and the RSD% for each polyphyllin in 16 h were less than 1.60% and 1.13% for Phe-D and DMP-D, respectively.

The samples were chosen from 10 batches of gongxuening capsules and tested. The peak area of all polyphyllins was measured five times with each sample and the average value was recorded. The content of each polyphyllin was calculated by the linear equation and the results are listed in Table III. As can be observed, the content of polyphyllin varied among different batches of capsules. For example, the content of polyphyllin VI was 0.0724 mg/g in batch 1 while 0.0336 mg/g in batch 6, half of that in batch 4.

Above all, either the relative content of common peaks, or the content of main polyphyllin, varied between batches. As mentioned above, the quality standard for gongxuening capsules specified in the Chinese Pharmacopoeia is merely based on the content of polyphyllin VI. This quality control method listed in the Chinese Pharmacopoeia.

**FIGURE 3**: (a) Characteristic chromatograms obtained from 10 batches of gongxuening capsules on DMP-D, and (b) chromatogram of mixed standard compounds on DMP-D.
seemed insufficient because the content of polyphyllin VI is not sufficient to evaluate the quality of gongxuening capsules. Therefore, a useful approach could be the combination of the typical chromatograms recorded and the determination of multiple evaluation markers. The QC method of the raw material, Pari-dis rhizoma, is meant to determine the sum of polyphyllins I, II, VI, and VII, which can be the reference for multiple evaluation markers for gongxuening capsules.

Application of Phe-D and DMP-D to the Chiral Separation of Amino Acids
Polyphyllin III contains, in its chemical structure, several asymmetric centers. Therefore, it can be supposed that the two stationary phases have the potential capability of enantioseparation. To test and evaluate the chiral separation capability of the two dioscin stationary phases, 10 native amino acids, namely lysine, leucine, cystine, threonine, serine, valine, alanine, arginine, isoleucine, and histidine, were chosen as chiral analytes. Because of the good water solubility of amino acids, a water-based mobile phase was selected. The mobile phases were investigated considering the type and concentration of the organic additive, and the concentration and the pH value of the buffer to get the best separation results. The

<table>
<thead>
<tr>
<th>Polyphyllin</th>
<th>Sample (mg/g)</th>
<th>Spiked (mg/g)</th>
<th>Spiked Sample (mg/g)</th>
<th>Recovery (%)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phe-D</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>0.7142</td>
<td>0.6801</td>
<td>1.3983</td>
<td>100.28</td>
<td>0.69</td>
</tr>
<tr>
<td>II,III</td>
<td>2.6691</td>
<td>2.0922</td>
<td>4.7570</td>
<td>99.92</td>
<td>1.12</td>
</tr>
<tr>
<td>V</td>
<td>0.8801</td>
<td>0.6271</td>
<td>1.5374</td>
<td>101.99</td>
<td>0.81</td>
</tr>
<tr>
<td>VI</td>
<td>0.4231</td>
<td>0.6190</td>
<td>1.0463</td>
<td>100.35</td>
<td>0.83</td>
</tr>
<tr>
<td>VII</td>
<td>0.3315</td>
<td>0.2611</td>
<td>0.5962</td>
<td>100.60</td>
<td>0.78</td>
</tr>
<tr>
<td>DMP-D</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>0.7141</td>
<td>0.6803</td>
<td>1.4080</td>
<td>101.04</td>
<td>0.52</td>
</tr>
<tr>
<td>II,III</td>
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<td>2.0924</td>
<td>4.8951</td>
<td>102.81</td>
<td>0.99</td>
</tr>
<tr>
<td>V</td>
<td>0.8803</td>
<td>0.6271</td>
<td>1.5191</td>
<td>100.82</td>
<td>0.61</td>
</tr>
<tr>
<td>VI</td>
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<td>0.6190</td>
<td>1.0291</td>
<td>98.73</td>
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<tr>
<td>VII</td>
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<td>0.2614</td>
<td>0.5852</td>
<td>98.80</td>
<td>0.59</td>
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</table>
enantioseparation results are reported in Table IV and the representative chromatograms are illustrated in Figure 4.

As shown in Table IV, 10 observed amino acid enantiomers saw different degrees of separation, indicating that hydrogen-bonding, and dipole–dipole interactions between the carboxyl (-COOH), amino (-NH₂) group and the SP-D benefitted the enantioseparation. Leucine, lysine, serine, and isoleucine enantiomers were resolved using both Phe-D and DMP-D and the chiral separation trend of these three amino acids were similar. Cystine, arginine, and histidine were resolved in their enantiomers only on Phe-D, while threonine, valine, and alanine could only be enantioseparated on DMP-D.

Furthermore, from the results listed in Table IV, histidine had a longer retention time on DMP-D \((k_r = 1.34)\) and a shorter retention time on Phe-D \((k_r = 1.04)\). The unique difference of the two stationary phases was two methyl groups connected to the phenyl ring on DMP-D such that the electron density of the phenyl ring of DMP-D is larger. The result showed that π-π stacking between the aromatic ring of histidine and DMP-D affected the retention time. Lysine had the best enantioseparation on both Phe-D and DMP-D \((\alpha = 1.28, 1.13)\), indicating that the hydrogen bonding between the free -NH₂ of lysine and SP-D contributed to the resolution, because the free -NH₂ can be hydrogen-bonding donor as well as receptor. Lysine had a greater selectivity factor on Phe-D, which may be attributed to the steric structure of the SP-D.

**Conclusions**

The two dioscin stationary phases can be applied to QC of gongxuening capsules, including qualitative and quantitative analysis, indicating that they have potential applicability for TCM QC. The ingredients of gongxuening capsules had the same elution order on Phe-D and DMP-D, but had a longer retention time on Phe-D. The content of many ingredients varied from batch to batch, so for the QC of the gongxuening capsules, the combination of the typical chromatograms and the multiple evaluation marker determinations, is required. In addition, because of the chirality of the two dioscin stationary phases, they showed potential chiral separation ability for amino acids. The 10 observed amino acid enantiomers showed different degrees of separation. The hydrogen-bonding, dipole–dipole interactions, and the π-π stacking and steric effect between the amino acids and the SP-D contributed to the enantioseparation. These results show that two synthesized dioscin stationary phases, Phe-D and DMP-D, exhibited potential applicability for TCM quality control and chiral separation.

**Funding**

This work was supported by the National Natural Science Foundation of China [number 81102408 and 81860632], the Yunnan Provincial Science and Technology Department with the Kunming Medical University Applied Basic Research Joint Special Fund Project [number 2017FE468(-172)]; the Science Research Foundation of Yunnan Education Bureau [number 2019J1190]; and the Yunnan Characteristic Plant Polysaccharide Engineering Research Center of Yunnan Province Colleges and Universities (2019).

**Compliance with Ethical Standards**

Conflict of Interest

The authors declare that they have no conflict of interest.

**TABLE III: Contents of 6 polyphyllins in 10 batches of gongxuening capsules**

<table>
<thead>
<tr>
<th>Batch</th>
<th>Content (mg/mL)*</th>
<th>Content (mg/mL)**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>II, III</td>
</tr>
<tr>
<td>1</td>
<td>2.51</td>
<td>9.34</td>
</tr>
<tr>
<td>2</td>
<td>2.42</td>
<td>9.25</td>
</tr>
<tr>
<td>3</td>
<td>2.15</td>
<td>8.63</td>
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<tr>
<td>4</td>
<td>2.13</td>
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<tr>
<td>5</td>
<td>2.65</td>
<td>10.53</td>
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<tr>
<td>6</td>
<td>2.32</td>
<td>9.56</td>
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<tr>
<td>7</td>
<td>2.15</td>
<td>8.59</td>
</tr>
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<td>8</td>
<td>2.10</td>
<td>8.58</td>
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<tr>
<td>9</td>
<td>2.22</td>
<td>9.44</td>
</tr>
<tr>
<td>10</td>
<td>2.44</td>
<td>9.13</td>
</tr>
</tbody>
</table>

*Stationary phase: Phe-D; **Stationary phase: DMP-D
TABLE IV: Results of the chiral separation of amino acids

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Phe-D</th>
<th></th>
<th></th>
<th>DMP-D</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k'$</td>
<td>$\alpha$</td>
<td>$R$</td>
<td>$k'$</td>
<td>$\alpha$</td>
<td>$R$</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.92</td>
<td>1.28</td>
<td>1.39</td>
<td>0.90</td>
<td>1.13</td>
<td>1.35</td>
</tr>
<tr>
<td>Leucine</td>
<td>1.45</td>
<td>1.02</td>
<td>0.38</td>
<td>1.18</td>
<td>1.08</td>
<td>0.55</td>
</tr>
<tr>
<td>Cystine</td>
<td>1.18</td>
<td>1.17</td>
<td>1.44</td>
<td>1.38</td>
<td>1.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Threonine</td>
<td>1.16</td>
<td>1.00</td>
<td>0.00</td>
<td>1.18</td>
<td>1.08</td>
<td>0.67</td>
</tr>
<tr>
<td>Serine</td>
<td>1.17</td>
<td>1.04</td>
<td>0.20</td>
<td>1.18</td>
<td>1.06</td>
<td>0.59</td>
</tr>
<tr>
<td>Valine</td>
<td>1.20</td>
<td>1.00</td>
<td>0.00</td>
<td>1.18</td>
<td>1.08</td>
<td>0.27</td>
</tr>
<tr>
<td>Alanine</td>
<td>1.20</td>
<td>1.00</td>
<td>0.00</td>
<td>1.18</td>
<td>1.06</td>
<td>0.57</td>
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<tr>
<td>Arginine</td>
<td>0.92</td>
<td>1.26</td>
<td>0.78</td>
<td>1.02</td>
<td>1.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>1.15</td>
<td>1.09</td>
<td>0.30</td>
<td>1.18</td>
<td>1.06</td>
<td>0.55</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.06</td>
<td>1.11</td>
<td>0.68</td>
<td>1.34</td>
<td>1.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

$k'$, retention factor of the first elution compound; $\alpha$, separation factor; $R$, resolution

Chromatography conditions:
Stationary phases: Phe-D: phenyl isocyanate-dioscin bonded silica gel stationary phase; DMP-D: 3, 5-dimethyl phenyl isocyanate-dioscin bonded silica gel stationary phase
Mobile phase: methanol: 0.04 mol/L buffer solution (0.04 mol/L ammonium acetate pH 4.7) = 85:15 (v:v), flow rate: 0.4 mol/L, ultraviolet (UV): 210 nm, column temperature: 25 °C.

Ethical Approval
This article does not contain any studies with human participants or animals performed by any of the authors.

References
(3) L.L. Li, L.S. Li, and H.R. Yang, Chin. J. Chromatogr. 35(9), 44–49 (2007).
The Future of Separation Science: Goodbye Old Friends

Mark A. Hayes

I grew up professionally in a laboratory with two HPLC systems, along with capillary and packed-column gas chromatography (GC) instruments. I moved on to be a technical support chemist for J&W Scientific, a pioneering company in capillary GC columns (now part of the Agilent family).

My graduate studies were centered in capillary zone electrophoresis, and I have since contributed to microfluidic separations. I have a deep history and real appreciation for separations science—I have taught it at the graduate level for twenty-five years. I am currently the Chair of the ACS Sub-committee on Chromatography and Separations Chemistry (SCSC).

That being said, I have struggled to write this article. Why? I wasn’t sure. I basically write for a living, so 900 words should not be that hard. But I think I have figured it out, and this isn’t going to be a popular opinion: I do not believe in classic linear separations anymore; further, I think separations science is wildly underdeveloped and under-appreciated.

I do not think it will be a popular opinion because many people have spent their lives learning and developing these techniques, and many people (myself included) have made a good living working within these industries. Suggesting they are not as useful as they could be and may need to be replaced is discomforting.

Okay, Mark, now explain yourself.

So where is this coming from? Well, I guess it gets back to seeing and thinking about the stunningly small injection volumes and fast total analysis times from microfluidics and high-speed GC. Couple that with multidimensional separations in general, then add in hybrid 3D fabrication (fab) techniques for microscale systems and gradient style techniques (increasing concentration while separating), and one can quickly surmise the days of long linear runs of univariate data are numbered.

Yeah, well, not so quick.

I was around for the introduction of capillary GC and capillary electrophoresis, which were to replace the packed column GC immediately (it took twenty years or more) and HPLC (it never happened). Things change slowly. Acceptance of new techniques is limited. If engineers and scientists have existing working solutions, they do not really need to change.

That being said, where do I think separations science will be in 10 years? 50 years?

Extending observations about injection bandwidth, separation speed, available resolution, developing gradient techniques, orthogonality of techniques, and hybrid 3D fab capabilities, well…at a pretty amazing place.

There will be “on demand” capability, where the desired target from a complex sample is isolated, concentrated, purified to homogeneity, and delivered to a detector or to a next step. The internal workings will be a highly efficient, multidimensional programmable separations scheme set on foundational physical interaction and hard-won empirical results. All this will be held in a volumetrically small system engineered and created using hybrid 3D fab based on the fundamentals of the separations sciences we all know and love. It will probably look like the Borg ship from “Star Trek,” but our beloved linear separations schemes will be gone. We have witnessed similar evolutions and revolutions in various spectroscopies (nuclear magnetic resonance [NMR] is an exemplar) and especially mass spectrometry (MS). Although, admittedly, they do not resemble the Borg ship. We can get there, but it will take several axes of separation and uber-efficient sample transfer.

If we can do separations on demand, we can do what I call “whole sample information extraction”—that is, analyze everything (total information theory) in a sample with a gigantic and complex scanning mode. Imagine starting with a blood sample, separate based on size, various similar cells gathered together, then separate on some physical principle such that all cells in a fraction are identical. Then, with each of these fractions, disrupt the cells and separate all the organelles and biochemicals to pure complex structures, disrupt these and separate the resulting complexes or pure materials and delivery to the detector (or multiple detectors). The now-pure homogeneous fractions can be fed into information-rich detection systems (MS, cryoEM, NMR, whole genome sequencing, lipidomics, and so on).

Since this system can be operated in a dynamic mode, the various fractions can be queried against a variable input (diseased or healthy, polluted or pure, immune response or non) and AI or machine learning strategies can be employed to determine the most important elements.

Well, I suspect I have made myself a pariah in my own scientific field—a lot of my friends are making a very good living using and improving our current techniques. However, if my students and younger colleagues’ enthusiasm is any indicator, we will be 3D printing our way into a very interesting future based on our beloved and respected principles of separations. I look forward to a much greater impact on our society as these capabilities are realized.
THE APPLICATION NOTEBOOK

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Restek Corporation

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*Showa Denko America, Inc., †Showa Denko K.K., Japan

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Wyatt Technology

Cover Photography: Getty Images
Comprehensive Analysis of C2–C8 PFAS Using a Novel LC Column

Restek Corporation

While not currently regulated, ultrashort-chain (C2–C3) per- and polyfluoroalkyl substances (PFAS) are of great interest. Current testing methodologies using reversed-phase liquid chromatography (LC) columns cannot be used because of a lack of retention, so either a separate method or a different column is required.

A unique, hybrid ion-exchange/HILIC column (Raptor Polar X) was used to develop a comprehensive LC–MS/MS method for the analysis of ultrashort-chain through long-chain, and alternative PFAS in water sources (tap, river, groundwater, and sewage effluent). The Raptor Polar X’s multimode retention mechanisms allow for retention with a single isocratic run.

**Experimental**

Chromatographic conditions are reported in Figure 1.

To avoid introducing background contamination, polypropylene vials and caps were used during sample preparation.

Each water sample of 250 μL was mixed with 250 μL of methanol and 5 μL of internal standard solution (10 ng/mL of 13C2-PFHxA, 13C2-PFOA, 13C3-PFBS, 13C4-PFOS in methanol).

Calibration standards were prepared by using deionized water and fortified with 14 analytes (see Figure 1) at a range of 10–800 ng/L. The calibration standard solutions were diluted 1:1 as above.

A Restek tap water sample, along with three water samples (river, ground, and sewage effluent) supplied by the United States Environmental Protection Agency were fortified at 40 and 160 ppt. Blank and fortified water samples were diluted 1:1 in methanol as above for chromatographic analysis. For TFA measurement in groundwater, the sample was diluted fivefold with deionized water before fortification due to its high TFA concentration.

**Results and Discussion**

All analytes were eluted in 4 min with good peak shapes (Figure 1). The overall analytical cycle time was 8 min to ensure no matrix-related interferences.

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<th>Detector</th>
<th>MS/MS</th>
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<td>Ion Mode</td>
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<td>BRM</td>
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<td>Temp.</td>
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<td>ESI-</td>
<td>BHM</td>
</tr>
<tr>
<td>Sample</td>
<td>Deionized Water + methanol</td>
<td>Methanol</td>
<td>BHM</td>
</tr>
<tr>
<td>Mobile Phase</td>
<td>Water: 10 mM ammonium formate, 0.05% formic acid</td>
<td>0.05% formic acid</td>
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</tr>
<tr>
<td>Time (min)</td>
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<td>0.5</td>
<td>45</td>
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</tbody>
</table>

**Figure 1:** Chromatogram of a 400 ng/L standard
Method linearity from 20–800 ppt for trifluoroacetic acid (TFA) and 10–800 ppt for all other analytes provided $r^2$ values $>0.996$ and deviations $<20\%$ using a $1/x$ weighted quadratic regression.

Samples were fortified at the low and high concentrations of their calibration ranges and run in duplicate for each analytical batch. A total of three batches were measured on different days. Concentrations of fortified samples were adjusted to account for any observed background contamination in sample blanks. Results are presented in Table I.

### Conclusions
These results demonstrate that switching to a mixed-mode LC column provides the capability to analyze currently monitored and emerging PFAS contaminants in a single, short, isocratic run, preparing laboratories for the future of PFAS testing.
Mono and disaccharides are of increasing importance in the growing industrialized food industry. With the rise of diabetes and other metabolic diseases, nutritional content has found itself at the forefront of consumer concern. Investigation of the constituents found on the packaging is now commonplace. Consumer concern, coupled with the increase in FDA regulation on consumer products, has brought about a change in individual ingredients reported on labels. Identification of the five most common mono and disaccharides found in packaged foods, lactose, maltose, sucrose, fructose, and glucose, is easily resolved on any of the RCX-30 family of columns offered by Hamilton Company.

Ultraviolet absorption, normally the detection method of choice for most chromatography separations, is unsuitable for carbohydrates. This means other analyte detection methods must be used, such as refractive index (RI), conductivity, and pulsed amperometric detection (PAD). Both RI and conductivity are excellent choices for detection if only isocratic methods are needed. PAD, however, offers the advantage of detection from either gradient or isocratic elution. An additional consideration when choosing a detector is sensitivity. RI and conductivity provide nanomolar detection levels while the detection limit for PAD is an order of magnitude lower.

The RCX-30 columns, powered by a propyl trimethylamine functionality, use a strong anion exchange mechanism to aid carbohydrate separation. Increasing saccharide retention is easily achieved by increasing pH or decreasing hydroxide concentration.

Similarly, initiating the opposite operations facilitate a decrease in retention and allow a fine-tuning of the individual analytes. The PS-DVB particles featured in all Hamilton polymeric columns provide exceptional column lifetime and reduced backpressure. Polymeric columns always provide excellent pH stability in alkaline mobile-phase conditions because there are no silanols to degrade, offering consistent performance, injection after injection.
The timsTOF Pro platform, introduced in 2017, already featured a sensitivity boost from the Parallel Accumulation Serial Fragmentation (PASEF®) technology, which provides time-and-space focusing of the ions in the Trapped Ion Mobility Spectrometry (TIMS) tunnel. The timsTOF SCP platform further enhances the sensitivity with robustly modified ion optic design. This boost in sensitivity enables measurement of low nanogram and sub-nanogram peptide loads, resulting in quantification of a few thousand protein groups per injection. Our data from as low as 200 pg of peptide loads demonstrates the applicability to unbiased true single cell proteomics in a routine fashion.

Single-cell 'omics in recent years has highlighted the microheterogeneity in clonal populations. Unlike other 'omics technologies, single-cell proteomics is hindered by lack of amplification techniques for protein molecules. Single-cell proteomics is now beginning to get attention, and one common strategy is to multiplex labeled single cells together with carrier samples to boost the sensitivity in detection. Further improvements in sophisticated sample preparation techniques have resulted in increased peptide yield that is delivered to the liquid chromatography–mass spectrometry (LC–MS) instrumentation (1). While these improvements upstream of sample measurement have improved the analysis depth, the raw sensitivity requires improvement as well without compromising robustness. Parallel Accumulation and Serial Fragmentation (PASEF) (2) on the timsTOF Pro platform makes efficient usage of the ion beam and with intelligent precursor placement within a TIMS cycle achieves rapid-sequencing speed. In addition, the ions get focused in space and time within the TIMS cell, resulting in a significant boost in sensitivity. This enables the analysis of low sample amounts, in the range of low-nanogram peptide loads. The newly designed timsTOF SCP’s ion optics allow a 4–5× improvement in ion current by increasing the ion brightness while maintaining the robustness of the timsTOF Pro. As the yield of the electrospray ionization increases with lower flow rates, we further enhanced the experiment’s overall sensitivity by coupling the timsTOF SCP to an Evosep One (Evosep Biosystems) instrument operated with the new low-flow Whisper methods. We have characterized the resulting system’s performance by injecting and measuring peptide loads mimicking the amount resulting from a single-cell preparation.

Methods
The new ion optic design of the timsTOF SCP system includes switching the orientation of the ion optics with inclusion of an additional ion funnel and additional orthogonal turns of the ion beam to preserve the robustness of the instrument. The source contained a wider glass capillary orifice that draws more ions into an additional funnel housed in a multi-stage differentially pumped region. Our initial experiments
demonstrated that in addition to the brighter ion beam these dedicated modifications were crucial to gain a factor five boost in ion current. For ultrahigh sensitivity measurements from 200 pg to a few nanograms of peptide, we coupled an Evosep One system (Evosep Biosciences) to the timsTOF SCP instrument and used a ~28 min gradient Whisper 40SPD method that offers a constant flow of 100 nL/min. Evotips were loaded with K562 (Promega) peptides according to the vendor instructions. Data were acquired in a DIA mode with window placements as shown (Figure-1B). All data were processed using Spectronaut software version 14 with default settings applying a hybrid library.

**Results and Discussion**

A dilution series of peptide load was performed starting from 200 pg to 25.6 ng in replicates using the ultralow flow method—Whisper 40SPD—from Evosep Biosystems. This method delivers gradient at a flow rate of 100 nL/min further boosting the sensitivity of the platform. About 1200 protein groups could be quantified from the 200 pg loads, and that number increased to an excess of 4000 protein groups for 6.4 ng loads. Then 250 and 500 pg loads, mimicking the amount of peptides resulting from the digestion of one or two isolated cells, were used to test the accessible proteome depth. These samples were analyzed using the Whisper 40 samples per day (SPD) method applying dia-PASEF® methods with a 0.7 cycle time method that covers between 400 and 1000 m/z. The data were processed with a library consisting of 5200 protein groups and about 54,000 peptides. From 250 and 500 pg loads on average 1542 and 2146 protein groups were quantified, respectively.

Work done in the laboratories of Prof. Matthias Mann with the timsTOF SCP combined with robust low flow Evosep One when applied with efficient sample preparation yields exciting results on the biology of the cell cycle (3,4).

**Conclusions**

- timsTOF SCP provides robust proteome coverage with peptide loads in the range of 250 pg.
- Combination of timsTOF SCP with Whisper methods on the Evosep provide a robust and sensitive platform to perform single-cell proteomics.

**References**

4. A. Mund et al., https://doi.org/10.1101/2021.01.25.427969

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**Figure 1:**
(a) Dilution series of peptides with Whisper 40SPD. (b) Multiple injections of 250 and 500 pg runs using Whisper 40SPD. (c) dia-PASEF® window scheme used for low sample amount.
Mass spectrometry

Therapeutic antibody technology currently dominates the biologics market, and the last decade has seen drugs based on monoclonal antibodies (mAbs) progressively overtake their small-molecule alternatives. mAbs have shown remarkable efficacy to treat a plethora of indications, including cancers, infections, autoimmune disorders, and cardiovascular and neurological diseases. Because the whole antibody therapeutics platform is regarded as one of the most promising classes of pharmaceutical technology to date, there is growing interest in developing integrated analytical methods to provide layered information on the structure, purity, and stability of mAbs.

The rise of proteomics has generated remarkable technological advances in mass spectrometry (MS), especially in the field of high-resolution MS (HRMS), leading to an increased impact of MS on the field of structural biology. Electrospray ionization (ESI) has proven to be a very useful technique for obtaining multiply charged ions of intact proteins, providing the biopharmaceutical industry with a unique opportunity to characterize and control mAb quality during clinical development and commercial production. U/HPLC methods that allow hyphenation with MS are of particular interest for this purpose, providing users with an additional dimension in their mAb characterization (molecular weight determination and structural information). Although direct infusion in the mass spectrometer remains the most straightforward approach to conduct intact mAb characterization, it is typically hampered by the various contaminants (such as salts, stabilizers or detergents) present in the matrix of recovered and purified mAbs. Sample preparation before MS analysis is therefore essential to ensure accurate mass and structural characterization.

With the use of MS-compatible eluents, reversed-phase (RP) chromatography is particularly well suited for protein desalting prior to MS analysis. Using a gradient from low to high organic content, the protein binds to the column while MS-interfering contaminants are eluted. Additional benefits to RP desalting include the increased mobile-phase volatility at the source for an improved ionization process. Nevertheless, the use of organic solvent in the mobile phase causes denaturation of the protein, which results in increased charge (z) values and often complicates MS data interpretation. In addition, mAb analysis by RP requires high temperatures (usually >50 °C) to reduce the strength of secondary interactions between mAb and the stationary phase, which can result in unwanted on-column fragments generation or loss of specific sugars from the glycan moieties.

Size-exclusion chromatography (SEC) remains the gold standard for determining the molecular weight (MW) distribution of mAbs expressed in mammalian cell culture. Obtaining structural information beyond the physical size (hydrodynamic volume) typically requires the combination of SEC with MS. Native ESI has proven particularly useful for generating multiply charged ions of intact proteins with lowered charge states, providing increased spectral resolution at higher m/z values. Still, the analysis remains challenging and involves biomolecule-specific optimization on both the chromatography and mass spectrometry side.

This application note describes the effective use of MS-compatible mobile-phase compositions in the analysis of mAbs using both RP (denaturing ESI) and SEC (native ESI) modes with in-line native ESI-MS detection on a hybrid Q-TOF instrument.

Experimental Conditions

Reversed Phase Analysis (Denaturing)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>TSKgel® Protein C18-300, 3 μm, 2.0 mm ID × 5 cm</td>
</tr>
<tr>
<td>HPLC Instrument</td>
<td>Nexera® XR UHPLC system</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>A: H2O containing 0.1% formic acid (FA)</td>
</tr>
<tr>
<td></td>
<td>B: acetonitrile (CH3CN) containing 0.1% FA</td>
</tr>
<tr>
<td>Gradient</td>
<td>0 to 1 min at 5% B from 1 (5% B) to 2.5 min (90% B)</td>
</tr>
<tr>
<td></td>
<td>2.5 to 3.5 min at 90% B back to 5% B at 3.7 min</td>
</tr>
<tr>
<td></td>
<td>3.7 to 8 min at 5% B</td>
</tr>
<tr>
<td>Flow rate</td>
<td>0.2 mL/min</td>
</tr>
<tr>
<td>Detection</td>
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<td>Temperature</td>
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<tr>
<td>Injection vol.</td>
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</tr>
<tr>
<td>Sample</td>
<td>NIST mAb @ 0.5 mg/mL</td>
</tr>
<tr>
<td>MS Instrument</td>
<td>SCIEX X500B QTOF</td>
</tr>
<tr>
<td>Ionization mode</td>
<td>Electrospray ionization, positive mode</td>
</tr>
<tr>
<td>MS mode</td>
<td>Scanning, TOF MS m/z 900-4000</td>
</tr>
<tr>
<td>Ion source gas 1</td>
<td>45 psi</td>
</tr>
<tr>
<td>Ion source gas 2</td>
<td>45 psi</td>
</tr>
<tr>
<td>Curtain gas</td>
<td>30 psi</td>
</tr>
<tr>
<td>CAD gas</td>
<td>7 psi</td>
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<tr>
<td>Spray voltage</td>
<td>5000 V</td>
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<tr>
<td>Source temperature</td>
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<tr>
<td>Declustering potential</td>
<td>275 V</td>
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<tr>
<td>Collision energy</td>
<td>20 V</td>
</tr>
</tbody>
</table>
Accumulation time: 0.5 s
Time bins to sum: 120
Script of Intact Protein Mode: ON
Q1 transmission window: 100% at 2250 Da

**SEC Analysis (Native)**
Column: TSKgel UP-SW3000, 2 μm, 4.6 mm ID × 15 cm
HPLC Instrument: Nexera XR UHPLC system
Mobile phase: 50 mmol/L ammonium acetate, pH 6.8
Gradient: isocratic
Flow rate: 0.2 mL/min
Temperature: 25 °C
Injection vol.: 10 μL
Sample: NIST mAb @ 0.2 mg/mL

MS Instrument: SCIEX X500B QTOF
Ionization mode: Electrospray ionization, positive mode
MS mode: Scanning, TOF MS
Ion source gas 1: 50 psi
Ion source gas 2: 50 psi
Curtain gas: 30 psi
CAD gas: 7 psi
Spray voltage: 5000 V
Source temperature: 250 °C
Declustering potential: 275 V
Collision energy: 5 V
Accumulation time: 0.5 s
Time bins to sum: 80
Script of Intact Protein Mode: ON
Q1 transmission window: 100% at 2250 Da

**Results and Discussion**

**Denaturing vs. Native ESI-MS Analysis**
The main difference between traditional denaturing and native ESI–MS is the composition and pH of the employed mobile phases (see Experimental Conditions). Denaturing solutions, containing organic solvents and non-neutral pH conditions, produce unfolded proteins by disrupting noncovalent interactions, causing molecules to unfold and expose sites of protonation. Conversely, native solutions at neutral pH (~7) preserve noncovalent interactions, as these molecules transition to the gas phase. Charge state and intensity during electrospray ionization are mainly determined by the number of ionizable sites. ESI–MS of denatured species typically produces a Gaussian distribution of highly charged ions in a wider charge state distribution, while native ESI produces ions with lower charge states and a narrower charge state distribution. This is largely due to the globular nature of folded proteins, limiting solvent accessible residues to only those on the surface of the protein. This is illustrated in Figure 1, which depicts the charge state envelopes obtained for the NIST mAb in both denaturing (RP-MS) (Figure 1B) and native conditions (SEC-MS) (Figure 1E).

Both RP (on a TSKgel Protein C4-300 column) and SEC (on a TSKgel UP-SW3000 column) analyses of the ~150 kDa NIST mAb coupled to a HRMS QTOF mass spectrometer led to robust separation and detection of the mAb. The main mAb RP peak eluting at 6 min (Figure 1A) exhibits a mass spectrum made of a charge distribution ranging from ~40+ to ~60+, with the Gaussian distribution representative of its unfolded form centered around m/z ~2900. In contrast, the main mAb SEC peak eluting at 10.5 min (Figure 1D) exhibits a less complex mass spectrum made of a charge distribution ranging from 24+ to 30+, with the Gaussian distribution representative of its native, folded form centered around m/z ~5300 (Figure 1E). Intact protein mass values—also called the zero-charge state—from these pseudo-molecular ions can be determined using deconvolution of protein charge states. MS deconvolution results for the denatured and native NIST mAb are presented in Figures 1C and F, respectively, exhibiting low mass errors (average: <10 Da) and achievement of MS resolution for the major glycoforms of the NIST mAb.
Buffer Consideration for Intact Protein Analysis by LC–MS
Making your liquid chromatography (LC) method compatible with MS is a crucial first step in your method development or transfer. When using MS detection, your analytes must be “transferred” from the liquid phase into the gas phase, which is quite different from most optical detectors classically hyphenated with LC (for example, UV and fluorescence detectors) where your sample is maintained in the liquid phase. Hyphenation with MS involves the process of ionization, which occurs in the ion source of your MS equipment, making your mobile phase (and all its additives) a crucial part of a successful method development and/or transfer.

RP is easily amenable to MS compatibility, with the focus being on the acidifier additive. Traditional RP-UV methods have used trifluoroacetic acid (TFA), which needs to be replaced by a volatile acidifier such as formic acid (FA) or difluoroacetic acid (DFA). In the case of SEC, although they are best suited to maintain the native folded structure of your mAb of interest, phosphate-based buffers cannot be used as they will contaminate your ion source. Switching to volatile buffers (such as ammonium acetate, ammonium formate or ammonium bicarbonate) is required. When doing so, considering your mobile phase from both the SEC and the MS point of view is crucial, as both LC performance (retention times, peak shape, resolution) and MS performance (ionization efficiency, maintenance of proteins’ native structures) can be affected by buffer type and buffer concentration.

The effects of MS-compatible volatile SEC buffers on mAb elution profiles are illustrated in Figure 2, where the separation of Humira® biosimilar on the TSKgel UP-SW3000 column was conducted using four different buffers at 50 mmol/L concentration. A sharp monomer peak at 5.7 min was observed when using a non-volatile buffer (MES) containing 150 mmol/L NaCl. When testing three volatile, MS compatible buffers at 50 mmol/L, three different peak shapes and retention times were observed, with 50 mmol/L ammonium formate offering the best chromatographic performance.
Experimental Considerations for a Successful Intact mAb Characterization by LC–ESI–MS

Below are the three main steps of how to optimize a successful intact mAb analysis using LC–MS:

1. Consider the various aspects of chromatographic separation and how to make them compatible with MS analysis.
2. Electrospray ionization: Identify the various parameters that can influence ionization efficiency and desolvation when you hyphenate LC with MS.
3. The MS detection and analysis: What parameters to consider on your MS instrument.

Table I presents a non-exhaustive summary of the various parameters and variables to consider when developing intact mAb ESI–MS experimental workflow, including MS platform-specific factors.

Conclusions

Monoclonal antibodies are highly complex biomolecules, requiring high resolution, precision, and dynamic range to fully characterize them with confidence. This application note illustrates an intact mAb analysis workflow solution integrating U/HPLC technologies, high-resolution mass spectrometry on a QTOF instrument and software for automatic data processing.

The workflow permits rapid and accurate mass characterization of mAbs, using either denaturing ESI using reversed-phase chromatography or native ESI using size-exclusion chromatography, leading to excellent mass accuracy for glycoform distribution. Detailed information was obtained about the heterogeneous composition of mAb proteins, with minimal sample preparation involved.

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Orbitrap is a registered trademark of Thermo Fisher, LLC

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Analytical toxicology involves methods for comprehensive screening of biological matrices for the presence of abused drugs. Routine analysis of samples in clinical and forensic settings demands quick and efficient extraction procedures. Smaller sorbent amounts utilized by solid-phase extraction (SPE) products allow scaling-down of starting sample size and minimize the total solvent volumes required to wash matrix components and elute the target analytes. A 2 mg or less measure of sorbent particles embedded in a disc membrane allows for sample enrichment and high throughput processing. As compared to loose sorbent, disk format eliminates channeling effects and reduces dead volume. Removal of the evaporation step from the procedure also decreases overall turn-around time.

In this application note, methods for extracting a large drugs of abuse panel from urine and plasma using UCT’s Micro-Prep™ HLB (W96-XTMC-HLB) and MMCX (W96-XTMC-MMCX) microelution plates have been described. HLB consists of a highly retentive uncharged hydrophilic and lipophilic sorbent which can effectively retain a range of acids, neutrals and bases via reverse-phase. The mixed-mode cation exchange chemistry of MMCX allows extraction of polar and non-polar analytes from aqueous samples. HPLC separation was carried out using UCT’s Selectra® PFPP column prior to detection by LC–MS/MS. The pentafluorophenylpropyl phase can undergo dipole-dipole, and pi-pi interactions, imparting unique selectivity and retention mechanisms to the column that distinguish it from a traditional biphenyl phase. Water and methanol consisting of 5 mM ammonium formate and 0.1 % formic acid were used as mobile phase. The total run time was 13 min at a 0.4 mL/min flow rate.

Results
HLB microelution plate utilized to extract urine and plasma quality control samples yielded excellent recoveries for a majority of the analytes in the panel. From a total of 47 drugs, >80% recoveries were achieved for 37 drugs fortified at 5 ng/mL and for 43 drugs spiked at 50 ng/mL. Corresponding RSD values were <10% at both concentration levels. From a total of 50 drugs extracted on the MMCX microelution plate, 45 and 48 drugs showed >80% recoveries at 5 ng/mL and 50 ng/mL respectively. The RSD values for both concentrations were <20%.

Conclusion
The use of UCT Selectra PFPP UH PLC column resulted in excellent peak shape and good linear calibration curves for all the analytes. Excellent recoveries and relative standard deviation (RSD) values confirm both the microelution extraction methods to be efficient. In addition to using minimal wash and elution solvent volumes, the elimination of the drying step reduced the overall processing time to approximately less than 30–40 min. The potential for automation and the option to load the collection plate directly on to the autosampler make this extraction technique very convenient for high throughput forensic and clinical laboratories.

<table>
<thead>
<tr>
<th>HLB Sample Extraction Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Sample Preparation</td>
</tr>
<tr>
<td>• 300 µL sample + ISTD</td>
</tr>
<tr>
<td>• 300 µL 100 mM pH 10.0 Sodium car/bicarbonate buffer</td>
</tr>
<tr>
<td>2) Condition (Optional)</td>
</tr>
<tr>
<td>• 100 µL MeOH</td>
</tr>
<tr>
<td>• 100 µL 100 mM pH 10.0 Sodium car/bicarbonate buffer</td>
</tr>
<tr>
<td>3) Apply sample</td>
</tr>
<tr>
<td>• Load 400 µL sample onto the microelution plate</td>
</tr>
<tr>
<td>4) Wash column</td>
</tr>
<tr>
<td>• 100 µL 5% MeOH in DI H₂O</td>
</tr>
<tr>
<td>5) Elute</td>
</tr>
<tr>
<td>• 50 µL 2% Formic acid in MeOH</td>
</tr>
<tr>
<td>6) Post Elution (Optional)</td>
</tr>
<tr>
<td>• Evaporate &amp; Reconstitute in mobile phase or</td>
</tr>
<tr>
<td>• Add 50 µL DI H₂O</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>MMCX Sample Extraction Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Sample Preparation</td>
</tr>
<tr>
<td>• 300 µL sample + ISTD</td>
</tr>
<tr>
<td>• 300 µL 100 mM pH 6.0 Phosphate buffer</td>
</tr>
<tr>
<td>2) Condition (Optional)</td>
</tr>
<tr>
<td>• 100 µL MeOH</td>
</tr>
<tr>
<td>• 100 µL 100 mM pH 6.0 Phosphate buffer</td>
</tr>
<tr>
<td>3) Apply sample</td>
</tr>
<tr>
<td>• Load 400 µL sample onto the microelution plate</td>
</tr>
<tr>
<td>4) Wash column</td>
</tr>
<tr>
<td>• 100 µL 100 mM Glacial acetic acid in DI H₂O</td>
</tr>
<tr>
<td>• 100 µL 40% MeOH</td>
</tr>
<tr>
<td>5) Elute</td>
</tr>
<tr>
<td>• 50 µL 2% NH₄OH in MeOH</td>
</tr>
<tr>
<td>6) Post Elution (Optional)</td>
</tr>
<tr>
<td>• Evaporate &amp; Reconstitute in mobile phase or</td>
</tr>
<tr>
<td>• Add 50 µL 2% Formic acid in DI H₂O</td>
</tr>
</tbody>
</table>
Once thought of as garbage bins, extracellular membrane vesicles full of cellular remnants responsible for the possible spread of many diseases, exosomes are viewed now as potential vehicles for regenerative medicine and targeted therapies for chronic and degenerative diseases, certain genetic disorders, musculoskeletal pain, and even Alzheimer’s disease. Targeted therapies may use a variety of targeting or signaling molecules such as RNA (messenger RNA and small interfering RNA, for example), DNA fragments, peptides, proteins, and lipids (1).

However, it is important to separate and purify the desired exosome from impurities during production. In this example of exosome (EV) analysis, the EV preparation process from cell culture supernatant was followed by a combination of polymer-based aqueous SEC (GFC) column OHpak SB-806 HQ and various detectors. Ultraviolet (UV) at 280 nm covers general culture-derived impurities, and fluorescence (Ex at 280 nm and Em at 348 nm) responds mainly to proteins via tryptophan residue fluorescence. In addition, MALS scattered light (LS) provides a highly sensitive response especially for large objects like nanoparticles. Moreover, MALS gives an estimate of the target RMS (root mean square) radius. The fraction consisted mainly EV was separated from many culture-derived impurities and was found around 8 min. While UV and fluorescence provide important insights into the progress and efficacy of the purification process and profiling of purified products, they are less sensitive to EVs mostly composed of lipid membranes and containing trace amounts of protein/nucleic acid cargo. Light scattering (LS) is an effective EV tracking method, especially in the early stages of purification. SB-806 HQ is a high-performance aqueous SEC (GFC) column suitable for bioproducts with a sufficient pore size to hold and separate EV-class nanoscale objects. Combined with a variety of detectors, a comprehensive analysis of the complex bio-nano target preparation process is achieved.

<table>
<thead>
<tr>
<th>Table I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
</tr>
<tr>
<td>Eluent</td>
</tr>
<tr>
<td>Flow rate</td>
</tr>
<tr>
<td>Detector</td>
</tr>
<tr>
<td>Column temperature</td>
</tr>
</tbody>
</table>

Figure 1: Sample 1: Cell culture supernatant 50 μL inj. 2: Concentrate 50 μL inj. 3: Crude product 15 μL inj. Process: supernatant concentrated by ultrafiltration; crude product obtained by affinity chromatography.
Liposome Size, Concentration, and Structural Characterization by FFF-MALS-DLS

Wyatt Technology

Liposomes and lipid nanoparticles are often used as nanocarriers to encapsulate fragile nucleic acids and hydrophobic or highly toxic drugs, and to safely deliver them to target tissue. During drug nanocarrier product and process development, as well as quality control, it is of great importance to monitor liposome size distributions accurately while also verifying drug encapsulation. FFF-MALS-DLS, consisting of field-flow fractionation (FFF) combined with multi-angle light scattering (MALS) and dynamic light scattering (DLS), is a powerful tool for characterizing the size, concentration, and structure of large nanoparticle ensembles.

Method

Encapsulation might cause changes in liposomal dimensions, but that is not always the case and other effects could cause such changes. Therefore, a more sophisticated analysis is warranted than mere size. Here, we report the analyses of two liposome samples, one empty and one filled with drug, by means of an Eclipse™ FFF system followed by a DAWN® MALS detector with embedded WyattQELS™ DLS module. The FFF separation method was optimized with the aid of Wyatt’s proprietary FFF simulation software. ASTRA software was used to collect and analyze the light scattering data to determine size and concentration (number density).

Results and Discussion

FFF separates particles according to hydrodynamic radius. Thanks to upstream separation, quantitative size distributions by FFF-MALS-DLS provide far more resolution and quantification than batch (unfractionated) DLS. Online DLS directly measures the hydrodynamic radius, $R_h$, sequentially for each eluting size fraction, while MALS simultaneously measures the root-mean-square radius, $R_g$. The shape factor $\rho$, which is defined as the ratio $R_g/R_h$, provides important structural information: it can discriminate between empty and filled shells or quantify the axial ratio of a uniform ellipsoid.

Both $R_h$ and $R_g$ are plotted against elution time in Figure 1. The results from duplicate runs demonstrate clean separation and excellent reproducibility of the FFF-MALS-DLS method. Figure 1(a) shows that the $R_h$ values for both empty and filled liposomes are well-overlaid, which is expected since FFF separates according to hydrodynamic size. However, as shown in Figure 1(b), $R_g$ values for these two liposomes do not overlay, which indicates different internal structures.

Figure 2(a) plots $R_g$ against $R_h$; $\rho$ is the slope of the linear fit. Interpretation of $\rho$ requires an assumption about the shape of the particles; here we make use of a priori knowledge that they are spherical. The values of $\rho$ for these two populations then correlate precisely to empty and filled liposomal structures.

In addition to size and structure, FFF-MALS can determine quantitative size distributions (number density vs. size) of size-fractionated nanoparticles if the refractive index of the constituent material is known. For lipids this is quite straightforward and Figure 2(b) provides the quantitative nanoparticle concentration analysis.

Conclusion

For liposomes or other nanoparticles, FFF-MALS-QELS provides an easily adaptable yet powerful characterization tool to obtain information on particle size, size distribution, particle count, as well as structure—all without making assumptions about the particles or their composition. FFF-MALS-DLS instrumentation is essential for robust drug nanocarrier development and quality control.
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