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485F US Highway One South, Suite 210
Iselin, NJ 08830
(732) 596-0276 • Fax: (732) 647-1235

Michael J. Tessalone
Vice President/
Group Publisher
Michael.Tessalone@ubm.com

Edward Fantuzzi
Publisher
Edward.Fantuzzi@ubm.com

Stephanie Shaffer
Sales Manager
Stephanie.Shaffer@ubm.com

Brianne Molnar
Sales Manager
Brianne.Molnar@ubm.com

Michael Kushner
Senior Director,
Digital Media
Michael.Kushner@ubm.com

Kristen Moore
Webcast Operations
Manager
Kristen.Moore@ubm.com

Vania Oliveira
Project Manager
Vania.Oliveira@ubm.com

Sabina Advani
Digital Production Manager
Sabina.Advani@ubm.com

Kaylynn Chiarello-Ebner
Managing Editor,
Special Projects
Kaylynn.Chiarello.
Ebner@ubm.com

Anne Lavigne
Marketing Manager
Anne.Lavigne@ubm.com

Melissa Stillwell
C.A.S.T. Data and
List Information
Melissa.Stillwell@ubm.com

Laura Bush
Editorial Director
Laura.Bush@ubm.com

Megan L'Heureux
Managing Editor
Meg.L'Heureux@ubm.com

Stephen A. Brown
Group Technical Editor
Stephen.Brown@ubm.com

Cindy Delonas
Associate Editor
Cindy.Delonas@ubm.com

Dan Ward
Art Director
dward@hcl.com

Rajesh Thangappan
Graphic Designer
Rajesh.Thangappan@hcl.com

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Jesse Singer
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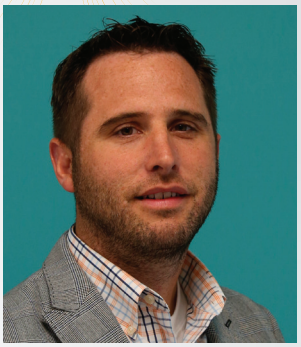

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COLUMN WATCH

Peak Shapes and Their Measurements: The Need and the Concept Behind Total Peak Shape Analysis

Gaussian peak shapes in chromatography are indicative of a well-behaved system. Such peak shapes are highly desirable from the perspective of column packing technology. From an analyst's point of view, Gaussian peaks provide improved sensitivity (lower detection limits) and allow ease of quantitation. In practice, one can obtain peaks that tail, front, or concurrently front and tail for reasons such as column packing issues, chemical and kinetic effects, and suboptimal high performance liquid chromatography (HPLC) system plumbing and detector settings. Here, we discuss a number of approaches for peak shape measurement that are available in modern chromatography software, along with their advantages and drawbacks. A new "total peak shape analysis" approach is suggested that facilitates detection and quantification of concurrent fronting and tailing in peaks. Several remediation approaches are proposed that can help chromatographers analyze and improve peak shapes.

M. Farooq Wahab, Darshan C. Patel, and Daniel W. Armstrong

Why do separation scientists care about peak shapes so much? The analysis of peak shape is critical during the development and optimization of synthetic approaches of new stationary phases as well as the quality of packing for all stationary phases (1). To the end user, Gaussian peaks are more amenable to integration than non-Gaussian shapes, provide higher detection sensitivity, and allow a higher number of peaks within a given run time—that is, increased peak capacity. Peak distortion may also indicate a closely eluted component. From an instrumentation designer's perspective, obtaining a narrow dispersion of analyte peaks is an indication of a well-behaving instrument. Unfortunately, peak shapes encountered in practice are rarely ideal in the majority of the separation modes. The shape of the peak can be affected by factors such as the column packing, secondary interactions of the analyte with the stationary phase, the connection tubing from the injector to the detector inlet, the detector sampling rate, and the nature of the digital filter (mathematical elimination of noise) in the chromatographic software (2). These factors result in a different efficiency, retention time, and selectivity or lower overall resolution than expected for the particle size of a given support. Table I summarizes the origins of undesired peak shapes with their underlying causes.

How to Make an Initial Assessment of an Experimental Peak Shape

The simplest way to assess the quality of the chromatographic signal is to visually inspect the peak for mirror image symmetry and a measurement of its width *W*, which can be measured at a given height. These measurements allow the calculation of two chromatographic figures of merit: the theoretical plates (*N*) and the peak asymmetry. Like an engine's horsepower, *N* indicates the "horsepower" of a chromatography column. A larger value of *N* indicates that the column can produce narrower peak widths and can separate more peaks in a given time window. The features of a typical peak obtained in chromatography are labeled in Figure 1 (3). Figure 1a shows how *N* can be calculated. Similarly, Figure 1b shows how peak shapes are measured at various heights using two popular quantities: the United States Pharmacopeia (USP) tailing factor and the asymmetry factor. If the users assume that peaks are visually Gaussian (the judgement depends on the user), the equation to calculate *N* is given by equation 1:

$$N = a\left(\frac{t_R}{W}\right)^2 \quad [1]$$

where t_R is the retention time, and *W* is the width of a peak at a given height. Because it is possible to measure *W* at various heights, the factor *a* is adjusted accordingly. Unfortunately, even for extremely high efficiency columns, perfect Gaussian peaks are rarely observed. Because we are interested in measuring the actual peak shapes, the method of moments is the most accurate measure of peak properties. It involves slightly tedious calculations, but several computer data systems (CDS) readily allow

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TABLE I: Origin of peak distortions in chromatography (thermodynamic, kinetic, peak processing, and fluid dynamical reasons)

Origin of Peak Distortion	Peak Shape	Phenomenological Cause	Correctable by User?
Thermodynamic and Kinetic Origins			
Stationary-phase characteristics: carbon load, particle or pore size distribution	Variable	Thermodynamics–kinetics; higher bonding coverage may slow kinetics; wide particle size distribution results in poor bed structure	No: Stationary phase synthesis issues
Sample concentration effects (analyte and other components)	Tailing, fronting, split tailing and split fronting, retention time shift	When analyte concentration exceeds the adsorption capacity of the stationary phase/mobile phase	Yes: Lower analyte concentration
Solvent mismatch between diluent and the mobile phase	Possibly distorted or split peaks, viscous fingering	The elution strength of the diluent is significantly stronger than the mobile phase or possible immiscibility issues	Yes: Match the diluent composition with the mobile phase
Frictional effects of the mobile phase	Peak distortion, shoulders in worst case	Radial frictional heating of the column at high flow rates	Yes: Use narrow inner diameter columns or decrease the flow rate; use still-air–based temperature control
Fluid Mechanics			
Slurry packing process of columns	All shapes possible because of axially and radially heterogeneous bed	Suspension rheology during column packing under pressure	No: Can only be changed by the column packer
Injector–connection tubing, frits, detector design	Tailing, peak broadening, and shoulders (in case there are dead volumes or clogged frits)	Fluid dynamics	Partly yes: Use the shortest possible and narrower inner diameter tubing, low-volume flow cell, zero-dead-volume fittings
Mathematical Processing of the Chromatographic Data			
Digital filters in the instrument's software	Symmetric widening or tailing, never fronting; can produce dips on chromatogram; can deceptively make peaks look more symmetric (with a Gaussian filter)	Mathematical operations on the raw chromatogram to decrease noise level	No: The filters are mathematics embedded in the software; can be circumvented by collecting analog output and users can apply smoothing filter of their choice to improve S/N

users to calculate moments as shown in Figure 2.

$$N = \left(\frac{m_1^2}{m_2}\right) \quad [2]$$

In equation 2, m_1 is the centroid or center of gravity of the peak, and m_2 is the second moment or variance of distribution of the analyte in time. The definitions are provided in Figure 2. The definition of *N* described in equation 2 does not assume any peak shape, because the centroid and variance can be determined for any peak shape encountered in chromatography (that is, fronting, tailing, split, shouldering, horned, and so forth). The main drawback of moments is that they are very sensitive to peak start (t_1) and peak end (t_2), and noise in the signal $S(t)$ (Figure 2). Secondly, moments also depend on the data sampling rate. Typically, the signal-to-noise ratio (S/N) should be 200 or above for obtaining reliable moment values (4). All the first three moments can be calculated in Microsoft Excel by estimation of the equations shown in Figure 2 (4). Slightly incorrect peak integration could lead to poor precision; therefore, the moment analysis for total shape analysis is too extensive and sensitive for routine work, despite their easy availability in modern data acquisition and analysis software.

Let us compare the width-based measurements of the two peaks obtained on a 15 cm x 0.46 cm, 2.7-µm d_p C18 core–shell column. The manufacturer's quality control test reports an exceptionally high plate number (39,000 per column). When tested, indeed the Gaussian efficiencies are 38,000 and 39,700 for uracil and phenol, respectively (equation 1). However, *N* values obtained from moment analysis are 24,000 and 26,000, respectively, as calculated by Agilent's ChemStation software (equation 2). There is a surprising difference of more than 10,000 plates, proving the point that even the most efficient columns today do not produce pure Gaussian peaks. If the peaks were perfectly Gaussian, the plate numbers from equations 1 and 2 would match. Chromatographers are used to looking at large numbers for efficiency; most column manufacturers use the simplified calculation of efficiency since the method of moments often results in very low values that do not reflect favorably on a column's performance. The efficiency only carries the information about the width of a peak, and nothing about the nature of its entire shape.

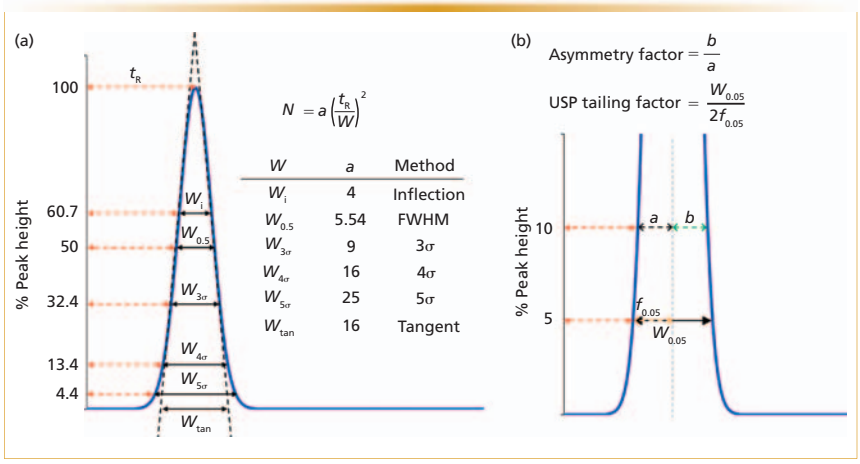

FIGURE 1: (a) Illustration of the features of a Gaussian peak profile. The theoretical plates can be calculated for various heights with an appropriate factor *a*. (b) Definitions of the commonly used measures of peak shape.

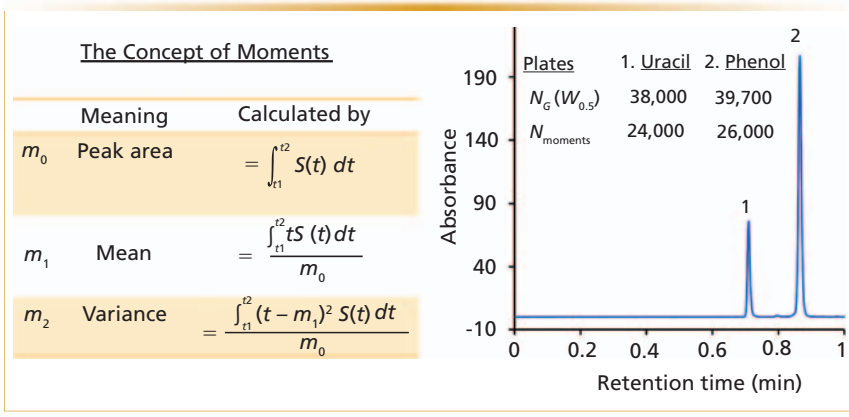

FIGURE 2: A comparison of different indicators of peak width. The calculation of column efficiency using two different approaches for calculating theoretical plates; N_G assumes that the experimental peaks are perfect Gaussian peaks and efficiency is measured using peak width at half height ($W_{0.5}$). Moment analysis considers the exact peak shape. The discrepancy in plates $N_{moments}$ shows that even high-efficiency columns may not produce ideal Gaussian peaks. See moment equations on the left. *S* refers to the chromatographic signal. The peak start and the end times are indicated by t_1 and t_2.

With chiral separations, even when the peaks may have very high efficiency, the peaks are often asymmetric. In the unusual case of β-blockers or compounds such as hydantoins on chiral columns, the peaks have a slight ascending fronting as well as a tailing. Both of these shapes can originate because of column packing or kinetic band broadening effects, or both. We have aptly named such peaks *Eiffel Tower peaks* because the top is mostly very narrow, yet the ascent and the descent are rather bent like the Eiffel Tower (Figure 3) (5). The Gaussian efficiencies of peaks 1 and 2 in Figure 3 are 10,000 and 6400, respectively; however, keep in mind that these peaks are not even visually Gaussian. The plates are vastly overestimated and the efficiency by moments tells us the actual efficiencies are 3300 and 1800 for peaks 1 and 2, respectively. These points again show that we need to come up with an improved measure to assess peak shapes. There are several ways to measure peak asymmetry, many of which are included in chromatography data acquisition software for reporting. The definitions of various peak shape measurements are shown in Table II. Figure 3 shows that USP tailing factor of peak 1 is 1.22, the asymmetry factor is 1.33, the symmetry is 1.72, and the moment-based measure called *skew* is 1.64. All of these numbers tell us that the peaks are tailing in a net fashion. These methods assign a unique number to a given peak, an approach that may not present the full picture. Indeed, they only indicate the contributions to the asymmetry that is in excess. A careful examination of the chromatographic peaks in Figure 3 reveals that tailing is coupled with fronting, which is rarely detected and never quantified. The USP tailing (*T*) is the most common measurement and is required by the U.S. Food and Drug Administration (FDA). The FDA recommends a tailing factor of ≤2. A peak shape *T* of ~2 is visually very asymmetric and deformed.

The Concept of Total Peak Shape Analysis

How can we detect peak deformations throughout the entire peak rather than rely on a single value? Such an analysis for the whole of the peak is beneficial in troubleshooting the peak shape problems highlighted in Table I. We developed two simple tests to study complete peak shapes graphically (5). Both methods are intuitive and can be used with Microsoft Excel. The first one is the derivative test and the second one is the Gaussian test.

The Derivative Test

Taking the derivative with respect to time of a given peak is the most straightforward approach to assess total symmetry and peak shapes. If *S* is the chromatographic signal, then the derivative is

$$\frac{dS}{dt} = \frac{S_2 - S_1}{t_2 - t_1} \qquad [3]$$

Equation 3 merely states that we find the difference between two consecutive signal values (S_2 and S_1) and divide it by the sampling interval. Like moment analysis, this peak shape test does not preassume any peak model. Thus any peak shape can be analyzed. The sampling interval can be determined as fol-

TABLE II: Peak shape measurements and their availability in chromatography data acquisition software

Names	Definition	Data Acquisition Software	Details
USP tailing factor	$T = W_{0.05}/2f_{0.05}$ where $W_{0.05}$ = peak width at 5% peak height, and $f_{0.05}$ = distance from the leading edge of the peak to the peak maxima at 5% peak height.	Universally present in all major software. Symmetry factor (JP) or (EP) is identical with the tailing factor (USP). Chromeleon also calls it "skewness."	1, perfect symmetry; <1, net fronting >1, net tailing Measured at 5% height
Asymmetry	$T = b_{0.1}/a_{0.1}$ where $a_{0.1}$ = distance from leading edge of the peak to the peak maxima at 10% peak height, and $b_{0.1}$ = distance from peak maxima to the trailing edge at 10% peak height	OpenLAB, Empower, Atlas, Chromeleon, ChromNAV, ChemStation	1, perfect symmetry; <1, net fronting >1, net tailing Measured at 10% height
Symmetry	$\sqrt{\frac{m_1+m_2}{m_3+m_4}}$ m_i is the ith moment, where $i = 1$, represents mean; $i = 2$ represents the variance; $i = 3$, vertical symmetry; and $i = 4$ is a measure of the compression or stretching of the peak along a vertical axis	ChemStation	Proprietary formula based on moments (6)
Skew (7)	$\frac{m_3}{m_2^{3/2}}$	ChemStation, Empower, many software programs use their own equations.	0, perfect symmetry <0, net fronting >0, net tailing with respect to the centroid (mean retention time)
Derivative test	$\frac{dS}{dt} = \frac{S_2-S_1}{t_2-t_1}$	Chromeleon Test of peak symmetry, no shape assumed	Same absolute value of the maximum and minimum indicates the peak is perfectly symmetric
Gaussian test	Graphical representation, divides the peak in half and assumes the top 15–20% of the peak is a perfect Gaussian; measures residuals	New approach suggested for graphical peak shape analysis	Minimum fronting and tailing residuals

lows: If the data are sampled at 160 Hz (160 data points per second), the sampling interval is 1/160 s or 1/(60x160) min. The only requirements of the derivative test is to ensure a high sampling rate (80 Hz and above), to have low response time settings (< 0.1 s), and a high signal-to-noise ratio. When time and the derivative are plotted on the same axis as the original chromatogram, the derivative intersects the *x*-axis at the same position as the maximum of the original peak (Figure 4). Figure 4a shows how the derivative of a pure Gaussian peak (ideal chromatography) would look. The maximum and minimum values are identical, which would be true for any symmetric peak. If a peak has a very slight tail as in Figure 4b, then the left maximum has a larger absolute value than the right minimum. This tailing is coming from a very short column (0.5 cm x 0.46 cm). As indicated in Table I, the tailing is originating from the column packing as well as from extracolumn effects. If there is concurrent fronting or tailing, then the absolute values will depend on which half of the peak is dominating, as we will show later. The gist of the derivative test is that if the absolute values of the maxima and minima of the first derivative of a peak do not match, the slope of the leading edge of the peak differs from the slope of the trailing edge and reveals the presence of asymmetry. For the derivative test, the excess magnitude of the positive end indicates tailing and the excess magnitude of the negative end suggests a fronting element to the peak. The derivative test is a very sensitive test to detect the peak asymmetry, and it does not rely on the user to choose peak start and end times, which makes it independent of integration errors.

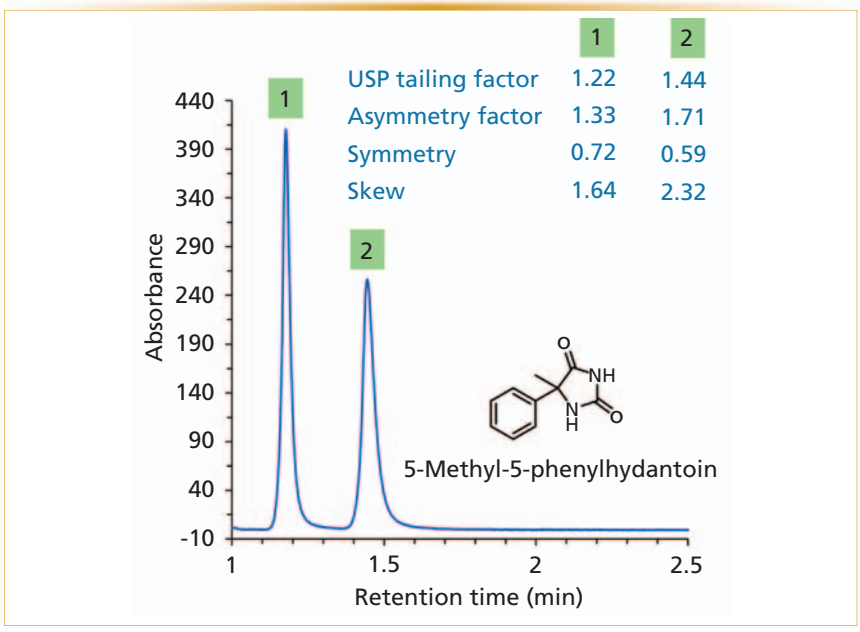

FIGURE 3: Comparison of single valued measures of peak shapes of the enantiomers of 5-methyl-5-phenyl hydantoin on a chiral column. The USP tailing factor, symmetry, and skew were obtained using Agilent Chemstation software. The asymmetry factor was obtained using Microsoft Excel. Manually calculated values may slightly differ, however the trend is the same. Eiffel Tower–shaped peaks were obtained in this case. A visual examination shows that the peaks have fronting and tailing attributes yet the single value descriptors only indicate tailing.

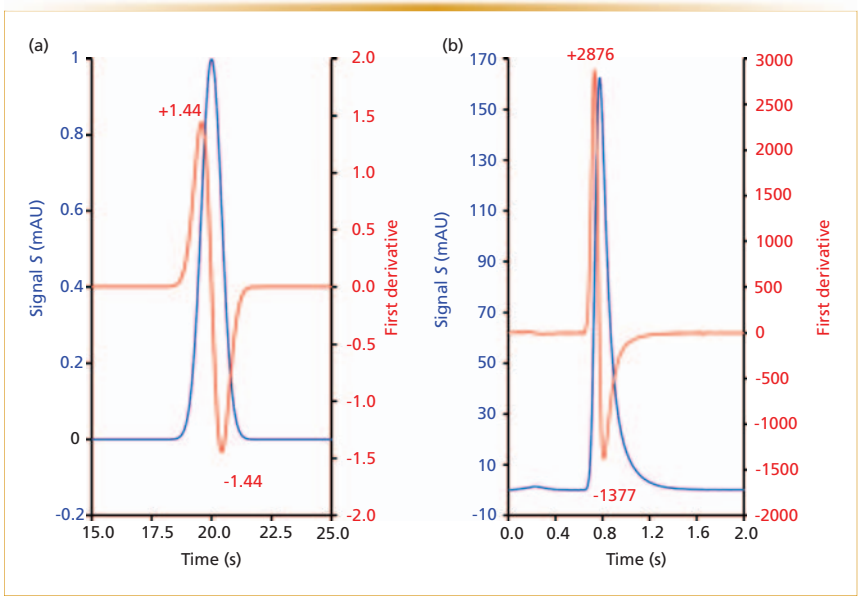

FIGURE 4: Illustration of a derivative test for testing peak symmetry (a) for a perfect Gaussian peak and (b) for a peak obtained on a 0.5-cm column at 5 mL/min on a custom-built chromatographic system. Note how the absolute values of the maximum and minimum match for a symmetric peak as in (a). Any symmetric peak shape will show the same values of maximum and minimum.

The Gaussian Test

The Gaussian test is a graphical and quantitative approach for analyzing the total peak shape and its departure from an ideal Gaussian shape. It concurrently determines the extent of a peak's deviation from a perfect Gaussian form on the leading and the trailing edge and allows detection and quantitation of fronting and tailing peaks. As discussed earlier, Table II compares what measurement methods are available in chromatographic software for analyzing experimental peaks. Ideal chromatography peaks typically follow the Gaussian peak profile $G(t)$ and amplitude A can be modeled using equation 4:

$$G(t) = A\ exp\left(-\frac{(t-t_R)^2}{2\sigma^2}\right) \quad [4]$$

The standard deviation or σ of a peak can be obtained at any peak height using equation 5:

$$\sigma = \frac{w_H}{2\sqrt{2\ln(\frac{1}{H})}} \quad [5]$$

If we know the experimental standard deviation and retention time, we can construct a complete Gaussian peak using equation 4. This method makes the critical assumption that even in the most distorted peaks, the upper regions of a peak (80% peak height or above) follow the Gaussian peak shape; the peak shape analysis is approached from the top in this method. The idea behind the Gaussian test is as follows:

1. Normalize the experimental peak height to unity. This process simplifies the visualization and calculations.
2. The top section of most chromatographic peaks is almost an ideal Gaussian shape—for example, the >80% height (Figure 1). To confirm this hypothesis, the standard deviation should be extracted at other heights (for example, 85%, 90%). The σ values should closely match.
3. Extract the standard deviation of the experimental peak at a given height (>80% peak height) from equation 5, and determine the peak maximum (that is, the retention time).
4. Plot a pure Gaussian peak, using equation 4 with the retention time and standard deviation extracted from the experimental peak. Graphically, superimpose the ideal peak on a real peak.

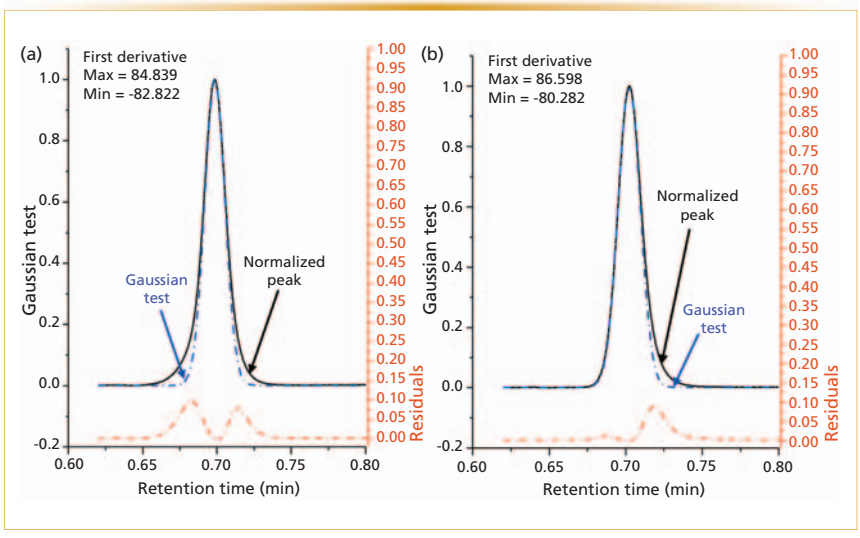

FIGURE 5: The Gaussian test applied on examples of (a) asymmetric peak (which deceptively appears symmetric) obtained from the use of a nonoptimal (2.3% w/v) slurry concentration and (b) improvement of peak shape by using an optimal (16% w/v) slurry concentration during column packing. The experimental peaks were obtained using 5 cm × 0.3 cm columns packed with native 2.7-µm superficially porous particles at different slurry concentrations. The black curve shows the raw data, and the superimposed blue dashed lines show the ideal peak shape. The residuals in red show the regions where the experimental peak does not match the ideal peak.

5. Find the differences at each point of the pure Gaussian peak and the experimental peak. These values are called *residuals*. Plot the residuals on the same graph against retention time. The residuals show both if the peak fronts or if there is a shoulder, or if the peaks tail and how much they tail.
6. Express the percentage of fronting residual and tailing residual as the fraction of the sum of all residuals.

Herein we assume that S/N is high, and the baseline is not drifting. We wish to emphasize here that this test is not simply a curve-fitting procedure, where the only goal is to minimize the residuals by the method of least squares. Although we fit the curve on the experimental peak, mathematical constraints are placed in this process (see steps 2 and 3). To make the technique easily accessible, a useful Excel template with prefilled formulas is available (5) that ultimately automates the derivative and the Gaussian test. In the Excel file, the user simply pastes the retention time as well as a chromatographic signal (absorbance, fluorescence, refractive index, and so forth) and allows Excel to perform the rest of the calculations.

Figure 5 provides examples where we show the utility of this Gaussian test. During initial column packing experiments, a core–shell material was producing peak shapes that were seemingly Gaussian, but only deceptively symmetric. The column efficiency was acceptable (for a 5-cm, 2.7-µm column, ~8000–9000 plates). The USP tailing factor was 0.93 (Figure 5a), which can indicate a relatively symmetric peak shape with a net fronting; however, a visual examination showed that there is coupled fronting and tailing, which had to be eliminated experimentally by optimizing the packing conditions. Modern columns are packed as a slurry of particulates suspended in an appropriate solvent. The concentration of the slurry and the nature of the solvent affects the peak shape of the packed column. In Figure 5a, the Gaussian test (steps 1 to 5) was performed on this initial packing condition with a low slurry concentration; the derivative test confirms the presence of peak distortion (maximum = 84.839 and minimum = −82.822). Only the full peak shape analysis identifies the problematic regions of the entire peak. The presence of residuals shows significant areas of fronting plus tailing elements despite an indication from the USP tailing factor that the peak only fronts. The contribution to peak distortion is 58% from fronting and 42% from tailing. In another set of conditions, the slurry concentration was increased for optimization. As shown in Figure 5b, the fronting element has become negligible and the left side of the peak has nearly a perfect Gaussian character. The value of the derivative still detects asymmetry (maximum = 86.598, minimum = –80.282). The peak shape distortion from the Gaussian tests shows 10% contribution from fronting and 90% from tailing. Although a visual inspection may lead users to assume that the original peak shown in Figure 5a is desirable over the optimized condition shown in Figure 5b based on the USP tailing factor, the Gaussian test disproves this assumption by showing the problematic regions of the peak with concurrent fronting and tailing.

Indeed, where resolution from a closely eluted impurity or a low-level analyte eluted before the main peak is of concern, the peak shape shown in Figure 5a may conceal the smaller impurity despite its seemingly more symmetric peak shape. In such a case, despite a larger USP tailing factor, the peak shape shown in Figure 5b would be desired because it may facilitate improved resolution of the impurity; the main peak has practically no fronting. Note that since uracil is an early eluted analyte on a narrow-bore column, the persistent presence of tailing has its origins in extracolumn connections plus a nonoptimized slurry solvent.

Conclusions

An ideal Gaussian peak shows that the system (column and the instrument) are well-behaved. When a peak is asymmetric, single-valued descriptors of peak shapes such as USP tailing, asymmetry factor, symmetry, and skew can be inadequate because they do not

give a complete picture of the overall peak shape. Researchers engaged in instrument design and stationary-phase development need to analyze peak shapes during synthesis and column packing. The derivative test is based on the concept that if a peak is symmetric, their inflection points will be mirror images. The Gaussian test superimposes a Gaussian model on a normalized peak with its set of constraints and shows the problematic regions of the peak. The standard deviation is extracted from the upper section (>80% height) of the peak rather than the conventional half-height approach. This total peak shape analysis approach can uncover the concurrent fronting and tailing in peaks. The origin, underlying phenomenological cause, and possible remedies are highlighted.

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ABOUT THE AUTHORS

M. Farooq Wahab is currently a research engineering scientist at the University of Texas at Arlington. He received a postdoctoral fellowship with Professor Armstrong after completing his PhD at the University of Alberta. His research interests include fundamentals of separation science, ultrahigh-efficiency phases, hydrophilic interaction chromatography, supercritical fluid chromatography, data processing, and subsecond separations in LC. For the Excel template and correspondence contact: mwahab@ualberta.ca

Darshan C. Patel received his PhD from the University of Texas at Arlington, where he developed new technologies for ultrahigh-throughput LC under the direction of Dr. Armstrong. In his current role, as a senior scientist at AbbVie Inc., in Process Research and Development, he focuses on the development of process analytical technologies for in-process sampling and automation.

Daniel W. Armstrong is the Welch Distinguished Professor of Chemistry at the University of Texas at Arlington. Professor Armstrong has received more than 30 national and international research and teaching awards. His current interest involves chiral recognition, macrocycle chemistry, synthesis and use of ionic liquids, separation science, and mass spectrometry. He has more than 600 publications, one book, and 30 patents. For correspondence and a copy of the Excel template contact: sec4dwa@uta.edu

ABOUT THE COLUMN EDITOR

David S. Bell is a manager in Separations Technology and Workflow Development at MilliporeSigma (formerly Sigma-Aldrich/Supelco). With a B.S. degree from SUNY Plattsburgh and a PhD in Analytical Chemistry from The Pennsylvania State University, Dave spent the first decade of his career within the pharmaceutical industry performing analytical method development using various forms of chromatography and electrophoresis. During the past 20 years, working directly in the chromatography industry, Dave has focused his efforts on the design, development, and application of stationary phases for use in HPLC and hyphenated techniques. In his current role at MilliporeSigma, Dr. Bell's main focus has been to research, publish, and present on the topic of molecular interactions that contribute to retention and selectivity in an array of chromatographic processes. Direct correspondence to: LCGCedit@ubm.com

LC TROUBLESHOOTING

Resources for LC Practitioners in 2017: What's on Your Bookshelf and in Your Web Browser?

What are the most useful chromatography books on your bookshelf? What are the most useful web-based resources (such as websites, downloadable documents, videos) about separation science? What are the most useful tools supporting your work (such as calculators and simulators)? This installment compiles input from the separation science community (both individuals and vendors) to guide you to the resources that people find most useful.

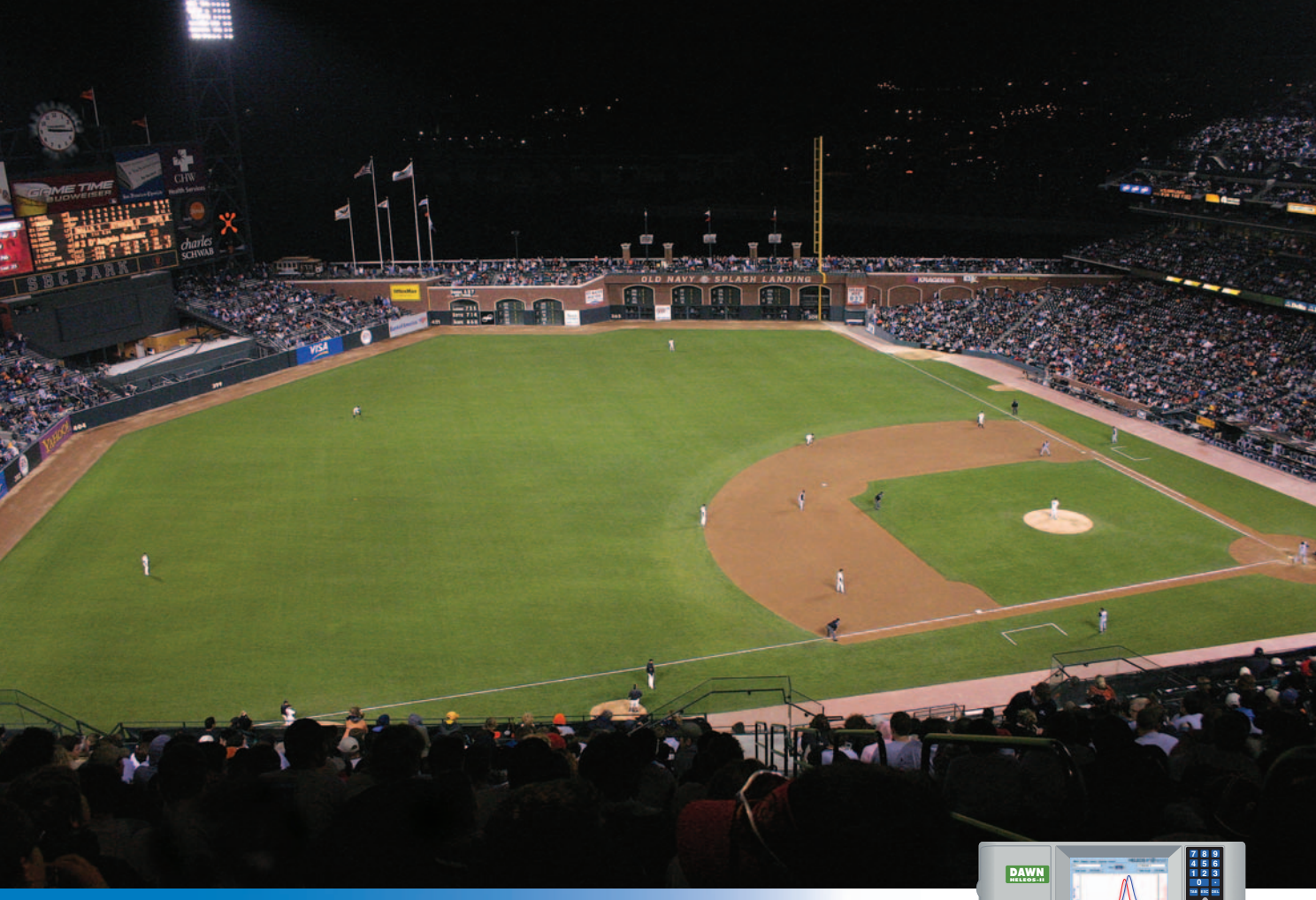
Dwight R. Stoll

Since I started thinking several months ago about putting this installment of "LC Troubleshooting" together, my thoughts have been focused on two themes. First, the information landscape is changing very rapidly in most fields, and separation science is not immune to this trend. There are so many different vehicles for sharing information available now, including webinars, blogs, and websites, in addition to the more traditional resources of journals and books. As a result, it really is difficult—if not impossible—for any one of us to keep up with changes in the field that are important to us. I don't think any of my chromatographer friends would say they feel confident that they have a good handle on the literature and other information at any moment in time. Second, I think that a point John Dolan made in his final "LC Troubleshooting" column installment in October (1) is astute and valuable: Toward the end of that installment he noted that over the years he aimed to help readers of his column be better troubleshooters by supporting their learning about chromatography concepts and how chromatography instruments work. In other words, the more deeply we understand how the instrument works and what is going on inside the box chemically and physically, the more likely it is that we as practitioners can solve problems we encounter and make our work more efficient and effective (and enjoyable!). The more I have thought about John's point, the more I agree with it. To that end, I am hopeful that the resources that I've pulled together in this installment will yield at least one gem for each reader. I hope you find one new book, website, or tool that you had not considered before, and that it helps you in your work.

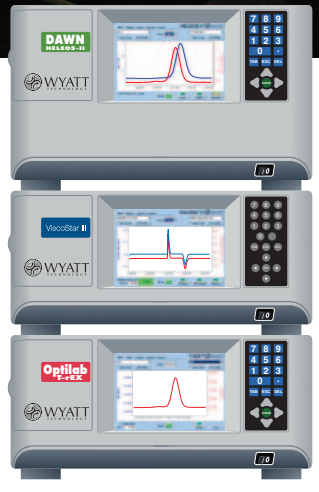
Now, the problem of course is that I don't think there is any single person in the chromatography community that has the "right" answer to the following question: Which resources are the best ones? So, the approach I have taken here is to crowdsource that information, with the hope that the cream has floated to the top. I've asked instrument and consumables companies that regularly contribute to *LCGC* to send me a list of resources highlighting those they think are most useful to the community. I've also reached out to more than 60 individuals in my professional network who I view as experts in their respective research areas, or at least as highly experienced chromatographers. This group is roughly composed of 60% from industry, 40% from academia, and a few folks from government laboratories. They represent six continents and eight countries. A large majority of respondents were in the age range of 31–50 years old; however, the range spanned from one person in his 20s to several over 70. I asked them to respond to the following questions, with a focus on those resources they find most helpful in whatever work they do that involves liquid chromatography (LC):

- What are the five most useful books on your bookshelf?
- What are the five most useful web-based resources (such as websites, downloadable documents, or videos)?
- What are the five most useful tools supporting your work (such as calculators or simulators)?

In my presentation, discussion, and summary of the input received from individuals and suppliers I have focused the attention on those resources that people find most useful. Of course, it is impossible to summarize all of the resources available to us in a few pages, so the perspective here is not intended to be comprehensive. However, I'd love to hear from you if you think there is a gem that has been overlooked here, and I'll be sure to call attention to it in a future article (please see contact information at the end of this article). You can also share suggestions through the commenting function on the online version of this arti-



Our Triple is a Home Run



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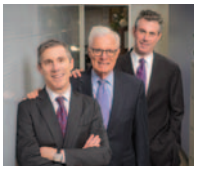

TABLE I: Most useful books supporting work in liquid chromatography

Title	Authors/Editors	Year	ISBN	Votes
Introduction to Modern Liquid Chromatography	Snyder, Kirkland, and Dolan	2009	978-0470167540	20
Practical HPLC Method Development	Snyder, Kirkland, and Glajch	2011	978-8126528530	13
HPLC Columns: Theory, Technology, and Practice	Neue	1997	978-0471190370	12
High-Performance Gradient Elution: The Practical Application of the Linear-Solvent-Strength Model	Snyder and Dolan	2006	978-0471706465	11
Unified Separation Science	Giddings	1991	978-0471520894	10
Practical High-Performance Liquid Chromatography	Meyer	2010	978-0470682173	7
Dynamics of Chromatography	Giddings	2002	978-0824712259	6
Essence of Chromatography	Poole	2002	978-0444501998	5
Introduction to Separation Science	Karger, Snyder, and Horvath	1973	978-0471458609	4
Quantitative Chemical Analysis	Harris	2015	978-1464135385	4
Troubleshooting LC Systems	Dolan and Snyder	1989	978-0896031517	3
Modern Size-Exclusion Liquid Chromatography	Striegel, Yau, Kirkland, and Bly	2009	978-0471201724	3
Modern HPLC for Practicing Scientists	Dong	2006	978-0471727897	3
HPLC for Pharmaceutical Scientists	Kazakevich and LoBrutto	2007	978-0471681625	3
Chiral Separation Techniques: A Practical Approach	Subramanian	2006	978-3527315093	2
Multidimensional Liquid Chromatography	Cohen and Schure	2008	978-0471738473	2
Comprehensive Chromatography in Combination with Mass Spectrometry	Mondello	2011	978-0470434079	2
Chromatography: Concepts and Contrasts	Miller	2009	978-0470530252	2
The HPLC-Expert II: Optimizing the Benefits of HPLC/UHPLC	Kromidas	2017	978-3527339723	1
The HPLC–MS Handbook for Practitioners	Kromidas	2017	978-3527343072	1
Chromatography: Principles and Instrumentation	Vitha	2016	978-1119270881	1
Cromatografia Líquida - Novas Tendências e Aplicações	Cass and Cassiano	2015	978-8535275971	1
UHPLC in Life Sciences	Guillarme and Veuthey	2015	1849735492	1
Chromatography Today	Poole and Poole	2014	978-0444596192	1
Beginners Guide to UPLC: Ultra-Performance Liquid Chromatography	Waters Corporation	2014	978-1879732070	1
Pitfalls and Errors of HPLC in Pictures	Meyer	2013	978-3527332939	1
Hydrophilic Interaction Chromatography: A Guide for Practitioners	Olson and Pack	2013	978-1118054178	1
HPLC of Polymers	Pasch and Trathnigg	2013	978-3540655510	1
Multidimensional HPLC of Polymers	Pasch and Trathnigg	2013	978-3642360794	1
Hydrophilic Interaction Liquid Chromatography (HILIC) and Advanced Applications	Wang and He	2011	978-1439807538	1
Solvents and Solvent Effects in Organic Chemistry	Reichadt and Welton	2011	978-3527324736	1
Handbook of Modern Pharmaceutical Analysis	Ahuja and Scypinski	2010	978-0123756800	1
Chiral Recognition in Separation Methods: Mechanisms and Applications	Berthod	2010	978-3642124440	1
Lipid Analysis, Fourth Edition: Isolation, Separation, Identification and Lipidomic Analysis	Han and Christie	2010	978-0955251245	1

Table continued on next page

TABLE I: (continued) Most useful books supporting work in liquid chromatography

Title	Authors/Editors	Year	ISBN	Votes
Basic Gas Chromatography	McNair and Miller	2009	978-0470439548	1
Fundamentals of Preparative and Nonlinear Chromatography	Guiochon, Felinger, Shirazi, and Katti	2006	978-0123705372	1
Coulson and Richardson'S Chemical Engineering, Volume 2, 5th Edition: Particle Technology and Separation Processes	Richardson	2006	978-8181471444	1
Principles of Instrumental Analysis	Skoog and Holler	2006	978-8131525579	1
Chirality in Natural and Applied Science	Lough and Wainer	2002	978-0849324345	1
Solvent Mixtures: Properties and Selective Solvation	Marcus	2002	978-0824708375	1
The Properties of Gases and Liquids	Poling and Prausnitz	2000	978-0070116825	1
Contemporary Instrumental Analysis	Rubinson and Rubinson	1999	978-0137907267	1
Basic HPLC and CE of Biomolecules	Cunico, Gooding, and Wehr	1998	978-0966322903	1
Handbook of HPLC	Katz, Eksteen, Schoenmakers, and Miller	1998	978-0824794446	1
Manuel pratique de chromatographie en phase liquide	Rosset, Carde, and Jardy	1997	978-2225851261	1
Chromatographic Detectors: Design: Function, and Operation	Scott	1996	978-0824797799	1
High Performance Liquid Chromatography, Fundamental Principles and Practice	Lough and Wainer	1995	978-0751400762	1
Packed Column SFC	Berger	1995	978-0854045006	1
HPLC: A Practical User's Guide	McMaster	1994	978-0471185864	1
High Performance Liquid Chromatography	Brown and Hartwick	1989	978-0471845065	1
Multidimensional Chromatography: Techniques and Applications	Cortes	1989	978-0824781361	1
Quantitative Gas Chromatography	Guiochon and Guillemin	1988	Journal of Chromatography Library	1
Gradient Elution in Column Liquid Chromatography, Volume 31: Theory and Practice	Jandera and Churacek	1985	978-0080858340	1
Contemporary Practice of Chromatography	Poole	1985	978-0444425065	1
Instrumental Methods of Chemical Analysis	Ewing	1985	978-0070198579	1
Practical Liquid Chromatography: An Introduction	Ettre and Yost	1980	B000JJLTAM (ASIN)	1
Modern Practice of Liquid Chromatography	Kirkland	1971	978-0471488781	1
Principles of Adsorption Chromatography	Snyder	1968	978-0824716394	1
Advances in Chromatography series (CRC Press)	Multiple authors			1

cle at www.chromatographyonline.com/resources-lc-practitioners-2017-what-s-your-bookshelf-and-your-web-browser.

Books

In total, 58 different books were cited by respondents as being on their list of five most useful for their work in LC. Many of these were only cited once by the group, but I've chosen to share them all because there is quite a lot of diversity of material represented in the list. Table I shows the list, sorted by descending frequency of mention by the group, where the "votes" column indicates the frequency of mention. Those titles receiving the fewest mentions are sorted by year of publication. A few comments come to mind after looking at this list. First, I think it makes a great shopping list for

young scientists. Second, several respondents pointed out that the most useful books on their shelves are not about chromatography per se, and this observation is evident from the table. For example, the books by Reichadt and Welton (*Solvents and Solvent Effects in Organic Chemistry*) and Poling and Prausnitz (*The Properties of Gases and Liquids*) are not focused on chromatography, but contain information about solvent properties that help us understand how chromatography works. Similarly, some respondents referred to books that focus on a different mode of chromatography (for example, Berger's *Packed Column SFC*), because they contain good ideas and explanations of chromatography that are generally applicable. Again, I've intentionally included the less frequently mentioned books. Some of the newer ones will benefit from more consideration by a wide audience (for example, Vitha's *Chromatography: Principles and Instrumentation*), while some of the older ones are classics that younger scientists simply may not be aware of (for example, Snyder's *Principles of Adsorption Chromatography*).

User-Identified Web-Based Resources

The books listed in Table I provide an incredible amount of information to support anyone working in LC. Increasingly, though, web-based resources provide information and tools that are difficult, if not impossible, to deliver in the form of a traditional printed book. Table II contains a summary of the web-based resources mentioned by respondents to my user survey. Again, this collection is not intended to be comprehensive. Rather, it represents a first pass at identifying those resources users find most useful in their day-to-day work with LC. The printed version of Table II only contains the resource name and a short description, but the online version of this article contains hyperlinks. And in principle, these are discoverable with a simple web search. Many of these resources are self-explanatory, but I'd like to highlight a few things in each category.

Resources, Broadly Defined

Many users commented on the usefulness of John Dolan's past "LC Troubleshooting" articles. These are all accessible through the *LCGC* website, but they have also been organized into John's "LC Troubleshooting Bible." The web version of this resource is searchable by keyword and enables rapid discovery and access to the troubleshooting ideas you need most.

Tutorials, Primers, and Guides

Much of the information cited in this box did not exist five years ago, and certainly not 10 years ago. The resources listed here are incredibly rich, with accurate information that is relevant to modern high performance liquid chromatography (HPLC). ChromAcademy was mentioned by users most frequently, and it is an excellent tool for learning about HPLC and related technologies. I personally use it extensively in my own teaching—both for classroom teaching and with my research students—in addition to the excellent primers on various topics offered by Waters and Agilent.

Tools for LC Simulation and Calculation

When we get to the tools in this column, the value of web-based resources really becomes apparent. It is said that a picture is worth 1000 words. A video or animation, then, must be worth at least 20,000 words. The tools listed here, like animations in ChromAcademy, enable users to explore and study the complex physical relationships that are central to the way chromatography works. The HPLC simulators listed in this box offer users a number of different options for simulating HPLC separations with varying degrees of sophistication. There are also dedicated tools to assist users with transferring methods from one instrument to another (2), or to improve analysis time by taking advantage of recent improvements in particle and instrument technology. The commercial product DryLab was mentioned frequently as an aid to method development. This sophisticated tool was originally developed by LC Resources, and is currently available from the Molnar Institute.

TABLE II: Most useful web-based resources for liquid chromatography mentioned by users

Resources, Broadly Defined	Tutorials, Primers, and Guides	Tools for LC Simulation and Calculation
• ***LCGC* website** – Great free resource for all aspects of separation science • **John Dolan's LC Troubleshooting Bible** – All of Dolan's "LC Troubleshooting" articles, searchable by keyword • Miscellaneous resources curated by **LC Resources** • **Analytical Sciences Digital Library** – Collection of resources developed with funding from the U.S. National Science Foundation • **Agilent Teaching Resources for Academia** – Collection of videos and animations demonstrating the principles of various LC and mass spectrometry (MS) approaches	• **ChromAcademy** – Full learning management system of >1000 animated and video training courses, with customizable learning paths, and online troubleshooting tools, in chromatography, sample preparation, mass spectrometry, spectroscopy, and basic laboratory skills. Constantly updated. • **Chromedia** – Multimedia learning resources for sample preparation, chromatography, and mass spectrometry. • **Wall Charts and Quick-Start Guides –** – *LCGC*'s Sample Preparation Guide – *LCGC*'s LC Troubleshooting Guide • **Primers by Agilent** – Introduction to Liquid Chromatography – Two-Dimensional Liquid Chromatography • **Primers by Waters** – Beginners Guide to HPLC • **Introductory video about HPLC** – Produced by the Royal Society of Chemistry, captures in 5 minutes the essence of how HPLC works and why it is so useful. • **Supelco Resources for Chiral Chromatography** – Multimedia learning resources for chiral separations	• **HPLC Simulator** (www.hplcsimulator.org) – Dynamic simulator for exploring the effects of operating variables on reversed-phase separations; developed at the University of Minnesota; Java application (free) • **HPLC Simulator** – More-detailed simulator compared to hplcsimulator.org; developed at the University of Geneva; Excel spreadsheet (free) • **HPLC Teaching Assistant** – Calculators to facilitate teaching and learning about HPLC concepts; developed at the University of Geneva; Excel spreadsheet (free) • **Calculator Apps for Parameter Estimation** – Apps for estimating parameters such as pressure versus flow rate; Agilent (free) (web, iOS) • **Method Transfer Tools** - Calculators for scaling to different particle sizes, movement between instruments, and so on; Thermo Excel spreadsheet (free; Waters Desktop application (free) • **Buffer Wizard** – Web-based application designed to assist with calculations needed for preparing buffers (free and paid versions available)
Tools for Column Selection and Characterization	**Tools for Molecular Properties**	
• **PQRI Website** – Database of column parameters based on the hydrophobic subtraction (HS) model developed by Snyder, Dolan, and coworkers; maintained by United States Pharmacopeia • **hplccolumns.org** – Database of column parameters based on HS model, presented in a different way compared to USP PQRI site, and with unique tools for visualization; maintained by Stoll Laboratory • **Waters RP Column Selectivity Chart** – Tool for comparing selectivities of different reversed-phase chemistries, and columns from different vendors • **ICOA Website** – Classification tool for reversed phases developed at the Institut de Chimie Organique et Analytique	• **ChemSpider** – Free chemical structure database providing access to millions of structures from hundreds of data sources. Enables predication of properties including pK_a and log *P* using a number of different algorithms • **NIST WebBook** – Free access to physical and chemical property data compiled by U.S. National Institute of Standards and Technology (NIST)	

Please see the online version of this article (www.chromatographyonline.com/resources-lc-practitioners-2017-what-s-your-bookshelf-and-your-web-browser) for the hyperlinks to all of these resources.

Tools for Molecular Properties

Effective and efficient method development in HPLC usually benefits from a detailed understanding of the physical and chemical properties of the compounds we are trying to separate. Users pointed to a number of tools that are useful for this purpose. I've only listed two in this box, because the ChemSpider website actually leverages several individual tools for molecular property prediction. For example, ChemSpider allows the user to predict (for free) properties such as dissociation constants (pK_a) and partition coefficients (log *P*) using the EpiSuite algorithms from the U.S. Environmental Protection Agency (EPA), tools from ACD Labs, Inc., and ChemAxxon (for example, www.chemicalize.com). As someone who also spends quite a lot of time teaching acid–base chemistry in the context of quantitative chemical analysis, I find these tools invaluable.

TABLE III: Most useful web-based resources for liquid chromatography identified by vendors

Vendor	Handbooks and Guides	Apps, Calculators, and Selection Tools	Applications Database	Other
Agilent Technologies	• LC Handbook (www.agilent.com/cs/library/primers/Public/LC-Handbook-Complete-2.pdf) • 2D-LC Primer (www.reolgrade.ru/docs/documents/news/2017-04-21/5991-2359EN.pdf)	LC Columns Navigator (navigator.chem.agilent.com/)	Application Finder (www.agilent.com/chem/application-finder)	
Chiral Technologies	Chiral Separation Method Development Guide (chiraltech.com/method-development-strategies/)		Generic Drug Application Index (chiraltech.com/global-application-center/)	
Phenomenex	• What is USP Chapter 621? (phenomenex.blog/2017/06/28/what-is-usp-chapter-621/) • Resources for Scaling up to Prep (phenomenex.blog/2017/03/14/preparative_separation/)			• Stages of Column Death (https://phenomenex.blog/2017/07/11/stages-of-column-death/) • Method Development for Size Exclusion Separations of Antibodies (https://phenomenex.blog/2017/08/17/hplc-method-development-monoclonal-antibody-aggregates-sec/)
Restek Corporation	Troubleshooting and FAQs (www.restek.com/Technical-Resources/Technical-Library)		Application Notes (www.restek.com/Technical-Resources/Technical-Library)	Physical Characteristics of HPLC Columns (www.restek.com/Chromatography-Columns/HPLC-UHPLC-Columns/LC-Columns-Physical-Characteristics-Chart)
Thermo Fisher Scientific	Charged Aerosol Detection for Liquid Chromatography and Related Separation Techniques (www.wiley.com/WileyCDA/WileyTitle/productCd-0470937785,subjectCd-LS35.html)	• LC Method Transfer Calculator (www.separatedbyexperience.com/uhplc/calculator.html) • HPLC Troubleshooting Guide App (www.thermofisher.com/us/en/home/life-science/lab-data-management-analysis-software/lab-apps.html)		
Tosoh Bioscience	Principles of Chromatography (www.separations.us.tosohbioscience.com/service--support/technical-support/resource-center/principles-of-chromagraphy)	Chromatography Calculator App (Android, iOS) (play.google.com/store/apps/details?id=com.app_tosohchrom.layout&hl=en)	Applications Database (https://www.separations.us.tosohbioscience.com/applications-database)	Gel Permeation Chromatography Glossary (Android, iOS) (play.google.com/store/apps/details?id=com.app_tosoh.layout&hl=en)
Waters Corporation	• Beginners Guide to HPLC (www.waters.com/waters/en_US/HPLC---High-Performance-Liquid-Chromatography-Explained/nav.htm?cid=10048919) • Beginners Guide to UPLC (www.waters.com/waters/en_US/UPLC---Ultra-Performance-Liquid-Chromatography-Beginner%27s-Guide/nav.htm?cid=134803622)	• Interactive Column Selectivity Chart (www.waters.com/waters/promotionDetail.htm?id=10048475&alias=Alias_selectivitychart__CHEMISTRY) • Filter Selector (www.waters.com/app/selector/en/filters.html)		

Tools for HPLC Column Comparison and Selection

Finally, several web-based resources were mentioned by users as being useful for the purpose of understanding the relationships between different column chemistries and columns from different vendors. To some extent these tools also support column selection, especially in cases where a column is very similar to, or very different from, a column already in hand.

Vendor-Identified Web-Based Resources

In addition to my survey of individual experienced chromatographers, I also asked several vendors of chromatography instrumentation, consumables, and software to send me references for up to five web-based resources that they believe are particularly useful to the community. Of these, I selected up to four in each case, and organized those into the categories presented in Table III.

Surprisingly, there is not too much overlap between Tables II and III. Perhaps some of the resources identified by vendors have been a bit hidden, and users will find them useful now that they have been brought to light. I think most of the items in Table III are self-explanatory, especially given the prior discussion of Table II, and I will not comment further on them here. However, there is one item that I found to be unique—the Phenomenex blog post "What is USP Chapter 621?"—which may be of particular value to many readers. In conversation with many users and vendors I find there is a lot of confusion around *United States Pharmacopeia* (*USP*) Chapter 621 (3), part of which is concerned with allowable changes to a USP method. I hope that resource will bring some clarity to this topic.

Closing Thoughts

I hope this installment achieves two things. First, I hope that all readers find a resource identified here that they were not previously familiar with, and helps them in their work on LC. Second, I expect that identifying these resources will lead to conversation that uncovers other gems, and perhaps even inspires individuals or groups to develop new resources that address existing gaps in these materials. If you know of a useful resource that you would like to see discussed, please let me know and I will draw attention to it in the future.

Two final points are worth mentioning here. First, a few respondents pointed to online discussion groups such as www.chromforum.org as resources for questions that the chromatography community might have answers to. Second, some respondents pointed out the value of hands-on training in learning about chromatography. Although the paper and web-based resources discussed here are very useful, I would certainly agree with the idea that there is no more effective way to learn about the practice of LC than through hands-on experience, recognizing that this can also involve significant upfront cost.

Acknowledgments

I want to extend a sincere and hearty thank you to all of the individuals and vendors who took the time to respond to my questions.

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ABOUT THE COLUMN EDITOR

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

FOCUS ON ENVIRONMENTAL ANALYSIS

A Look at Matrix Effects

All quantitative environmental analytical chemistry methods probably have matrix effects to some extent. However, the natural tendency for a high-volume testing laboratory is to blame the sample matrix when matrix spike recoveries are outside of limits and move on to the next sample. Increasingly, approved regulatory methods require that results for samples with out-of-limits matrix spike recoveries not be used for regulatory compliance reporting. The environmental analytical chemistry literature on matrix interferences and matrix effects is small, and the topic doesn't appear to have been addressed in a fundamental way. This installment of "Focus on Environmental Analysis" examines the possible types of matrix interferences or matrix effects. It examines method performance using routine quality control indicators to gauge which methods appear to have a tendency for matrix interference problems and suggests ways to reduce matrix effects. It ends with a look at cyanide as the "baddest" bad actor regarding matrix effects.

Michael F. Delaney

Environmental analytical chemistry testing performed for regulatory purposes generally uses standardized quality control (QC) indicators to demonstrate that the testing was performed properly and usable results were obtained. This includes the following "batch QC" samples:

- Laboratory reagent blank (LRB) or method blank (MB) is a clean matrix, like deionized water, carried through the test procedure.
- Laboratory control sample (LCS) or laboratory fortified blank (LFB) is a known amount of target analyte in a clean matrix carried through the test procedure.
- Matrix spike (MS)–matrix spike duplicate (MSD) or laboratory fortified matrix–laboratory fortified matrix duplicate is a known amount of target analyte added to a sample in the batch, carried through the test procedure.

If you look closely enough, all quantitative analytical chemistry methods probably have matrix effects to some extent. However, the natural tendency for a high-volume environmental testing laboratory is to blame the sample when MS–MSD recoveries are outside of laboratory or method required control limits and move on to the next sample. ("It's a matrix effect!") Environmental Protection Agency (EPA)-approved methods vary in how out-of-limits MS–MSD recoveries are addressed, but often state that the associated sample results are "suspect" and "may not be reported for regulatory compliance purposes" (1). These methods often do not acknowledge that for multianalyte methods, QC indicators will occasionally exceed statistically derived control limits, just by random variation.

Blame the Sample

To stay in business at high-volume commercial and government environmental labs you need to have robust, consistent procedures to get the work done efficiently. Samples are processed in batches and need to have good batch QC to show that things are working and you can move on to the next batch. If the LCS worked and the MS didn't (and the MSD agrees with the MS), then it's the sample's fault. It's a matrix effect. Move on!

A high-volume laboratory doesn't have control over the clients' sample matrices. You can't shut down the operation to investigate one particular sample. And for organic analyses, such as pesticides, volatiles, and semivolatiles, there are so many target analytes, that something is always out of statistically set recovery control limits just because of random variation. However, the spike recovery is telling you something about that analyte in that sample analyzed by that particular analysis method. But who has time to investigate?

A Little History on Matrix Effects

An online search of the American Chemical Society (ACS) journals for the first use of the term "matrix effect" found a 1951 paper on the spectroscopic determination of vanadium in fuel oil (2), in which it was stated that "it was assumed that the use of silica and graphite eliminated any matrix effect that might otherwise have been caused by the chemical character of the fuel oil." The first use of the term "matrix interference" appears in a 1962 paper on neutron activation analysis (3): "The method is rapid, sensitive, and selective, and is free from most matrix interferences." The term "matrix effects" was also used in a 1980 instrumental analysis text for undergraduates (4).

According to the ACS search performed in 2016, the term "matrix effects" was used in 792 out of 78,769 articles (1.0%) and "matrix interferences" was used in 3189 out of 78,769 articles in the journals *Analytical Chemistry* and *Environmental Science and Technology*

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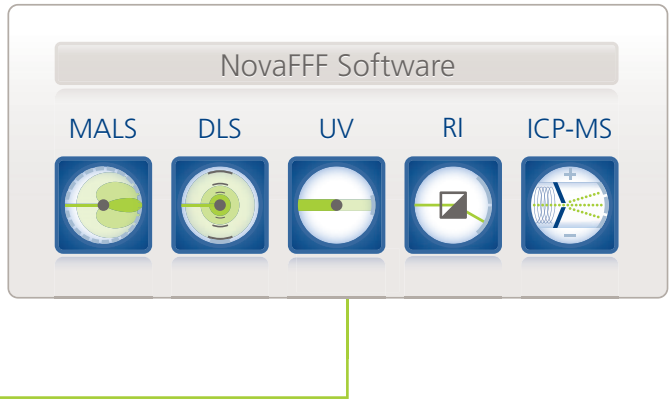

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TABLE I: Statistical analysis of LCS and MS–MSD recoveries for selected target analytes. When F_{calc} is greater than $F_{critical}$ there is a statistical indication of a significant matrix effect at the 95% confidence level.

Analyte	Method	*N* (LCS)	*N* (MS–MSD)	*s* (LCS)	*s* (MS–MSD)	F_{calc}	$F_{critical}$	Significant at 95%?
Benzene	624	1141	584	7.66	7.81	1.040	1.124	N.S.
Benzo[a]pyrene	625	652	569	13.26	16.62	1.571	1.143	S
Benzoic acid	625	652	567	9.93	147.77	221.672	1.143	Highly S
Acrylonitrile	624	1725	1141	14.48	14.67	1.025	1.093	N.S.
Acrolein	624	584	1141	28.54	43.40	2.312	1.124	S
Acrolein	603	25	50	12.08	27.02	5.001	1.727	S
NH_3	AAN	150	232	3.83	5.90	2.377	1.274	S
PO_4	AAN	107	118	3.98	6.53	2.690	1.368	S
NO_2 (by diff)	AAN	180	212	2.60	11.29	18.842	1.266	S
NO_3/NO_2	AAN	178	211	3.57	5.39	2.276	1.268	S
S--	Titration	308	584	8.18	7.90	0.932	1.176	N.S.
S--	UV–vis	325	800	6.60	7.58	1.317	1.163	S
NH_3	ISE	320	277	7.21	10.90	2.286	1.212	S
CN Total	AAN	267	701	5.87	20.89	12.665	1.179	S
CN Total	FIA	79	219	3.86	10.00	6.719	1.346	S

Note: *N* = number of samples. *s* = standard deviation. F_{calc} = the calculated *F*-statistic. $F_{critical}$ = the critical *F*-value for the indicated degrees of freedom. S = statistical significant. N.S. = not statistically significant. AAN = autoanalyzer. UV–vis = ultraviolet–visible spectrophotometry. ISE = ion selective electrode. FIA = flow injection analysis.

(4.0%). Or, in other words, 95% of the articles in these journals used neither term.

What Is a Matrix Effect?

Environmental analytical chemists blithely toss around the term "matrix effect," but are rarely asked to define it. We can start with an EPA definition (5):

> *Manifestations of non-target analytes or physical/chemical characteristics of a sample that prevents the quantification of the target analyte (i.e., the compound or element of interest being effectively quantified by the test method) as it is routinely performed, typically adversely impacting the reliability of the determination. For example, a matrix effect can give rise to a high or low bias.*

There doesn't seem to be an EPA definition of matrix interference. There is an International Union of Pure and Applied Chemistry (IUPAC) definition (6) of the term matrix effect: "The combined effect of all components of the sample other than the analyte on the measurement of the quantity." And also interference, "If the specific component can be identified as causing an effect then this is referred to as an interference."

Interestingly, the 2016 NELAC Institute (TNI) standard (7) only mentions "the effect of the matrix" in its definition of matrix spike:

> *A sample prepared, taken through all sample preparation and analytical steps of the procedure unless otherwise noted in a referenced method, by adding a known amount of target analyte to a specified amount of sample for which an independent test result of target analyte concentration is available. Matrix spikes are used, for example, to determine the effect of the matrix on a method's recovery efficiency.*

The key aspect of a matrix effect or matrix interference is usually bias, and can be summarized as follows:

- matrix interference if you know what is causing the bias.
- matrix effect if you don't know what is causing the bias.

Or it could be that there is something fundamentally wrong with the method itself and it is affecting the target analyte.

What Do EPA Methods Say About Poor Matrix Spike Recoveries and Matrix Effects?

I always believed that the 600-series Clean Water Act, the National Pollutant Discharge Elimination System (NPDES) wastewater methods (for example, 608, 624, and 625), were relatively forgiving with regard to poor matrix spike recoveries, but that belief is actually erroneous. For example, wastewater semivolatile Method 625 (8) says this in section 8.4.3:

> *If the recovery of any such parameter falls outside the designated range, the laboratory performance for that parameter is judged to be out of control, and the problem must be immediately identified and corrected. The analytical result for that parameter in the unspiked sample is suspect and may not be reported for regulatory compliance purposes.*

Not being able to use the results for regulatory reporting is especially problematic for a treatment plant laboratory that must meet monthly reporting deadlines. After the month is over you can't go back and

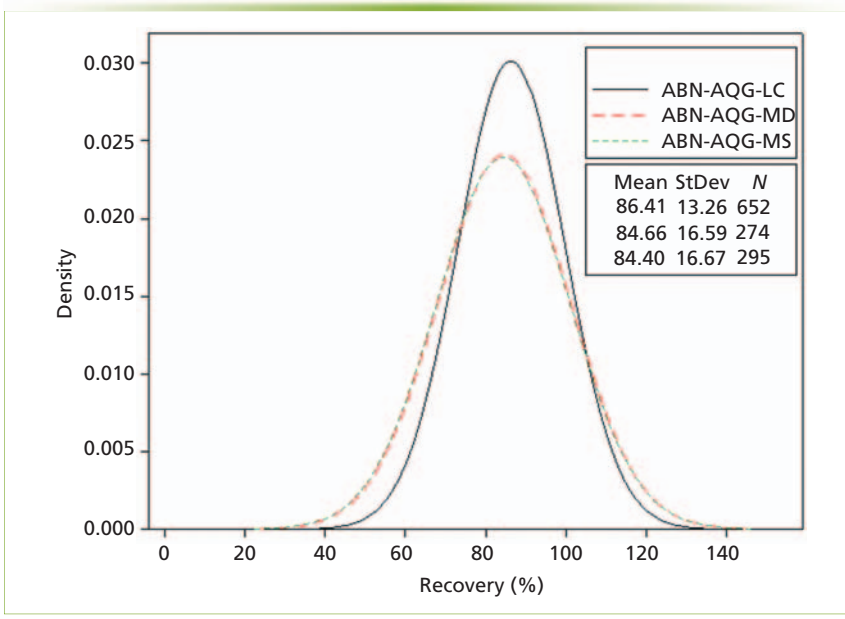

FIGURE 1: Distribution of LCS and MS–MSD recoveries for benzo[a]pyrene by EPA Method 625.

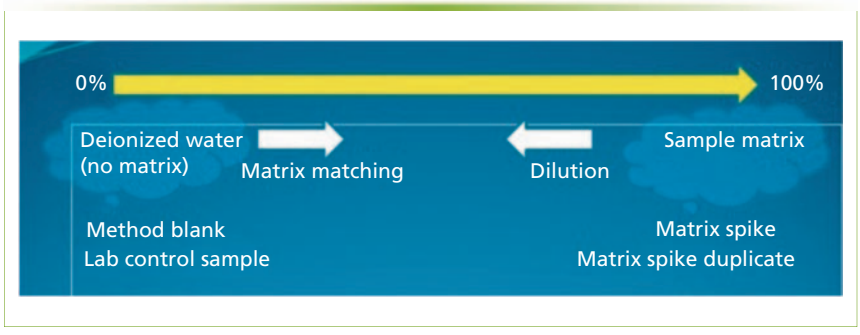

FIGURE 2: The matrix spectrum.

resample, so the testing laboratory needs to do the best job possible with each regulatory sample. The EPA has provided guidance on how to document and resolve matrix effects for wastewater samples (9).

For solid waste and hazardous waste, the corresponding language in EPA SW-846 methods compendium is more forgiving (10): "If the results are to be used for regulatory compliance monitoring, then the analyst must take steps to demonstrate that the analytes of concern can be determined in the sample matrix at the levels of interest." Even the language in the corresponding drinking water method, 525.2, is tolerant: "If the recovery data for a LFM [MS] does not meet the criteria . . . and LFBs [LCSs] show the laboratory to be in control, then the samples from that matrix (sample location) are documented as suspect due to matrix effects" (1). Suspect means flag or qualify the data and move on.

How Prevalent Are Matrix Effects?

Matuszewski and colleagues (11) presented an interesting way of examining matrix effects, noting that a matrix effect can be quantified by examining target analyte recovery with and without sample matrix. For environmental testing, these recoveries are the LCS and MS–MSD. The magnitude of the matrix effect (ME) is calculated as follows:

ME (%) = MS Recovery/LCS Recovery x 100

If the MS and LCS give the same recovery, then ME = 100%, meaning no matrix effect is evident. If ME > 100% there is signal enhancement and if ME < 100% there is signal suppression. Using about six years of QC results in our laboratory information management system (LIMS), we can use the variability of MS–MSD and LCS recovery data in bulk to go looking for significant matrix effects using a simple *F*-test for:

$$F_{calc} = s^2_{MS/MSD}/s^2_{LCS} \text{ compared to } F_{critical}$$

where s is standard deviation. If the calculated F_{calc} is larger than the $F_{critical}$, there is a significant matrix effect.

Table I shows some representative results of this calculation using approximately six years of data from our LIMS. An example for benzo[a]pyrene by EPA Method 625 for semivolatiles is shown in Figure 1, demonstrating a small, but statistically significant, matrix effect. Note that nearly all the analytes presented in Table I showed a statistically significant matrix effect.

Types of Matrix Effects

There isn't a generally embraced systematic way of characterizing or organizing the various types of matrix effects. Perhaps it's helpful to think about the impact of the matrix effect on the calibration curve relationship between target analytes and the observed signal. Additive effects move the calibration curve up or down and multiplicative effects change the slope of the calibration curve.

It might be helpful to characterize matrix effects as simple or subtle. Simple matrix effects include matrix interferences that can be addressed by improving the method. For example, in a chromatographic analysis, if a nontarget interfering compound is coeluted with the target analyte, the matrix interference can be decreased by

- Better cleanup. Remove the interference.
- Better chromatography. Separate the interference from the target analyte.
- Better detector—more selective. Detect the target analyte but not the interference.

Subtle matrix effects are, by definition, harder to characterize. Perhaps it's helpful to think about ways to address subtle matrix effects using the "matrix spectrum," shown in Figure 2, which runs from 0% matrix to 100% matrix. Real samples, as well as MS–MSD, contain 100% matrix while calibration

A Q&A

How I Optimized My Solid Waste SVOC Analysis with a GC-MS Workflow

Don Venturini
Laboratory Manager
Heritage Thermal Services

Environmental labs are often looking for new ways to streamline laboratory workflows through better data processing, automation, and improved instrument control. To talk about these issues, *LCGC* recently sat down with Don Venturini, a laboratory manager at Heritage Thermal Services, to discuss applications of the Thermo Scientific™ ISQ™ Series Quadrupole GC-MS System and Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software for environmental monitoring.

LCGC: Can you tell us about your company?

Venturini: Heritage Thermal Services is a hazardous waste management company that specializes in the incineration of hazardous and non-hazardous waste. Some of the waste we receive includes corrosive liquids, reactives, flammable liquids, organic peroxides, oxidizers, pesticides, aerosols, lab packs, consumer commodities, product recalls, and miscellaneous hazardous waste. We are also the first incinerator in the industry to be ISO-14001, ISO-9000, and OSAF-18001 certified.

LCGC: Can you tell us what samples you analyze with the ISQ GC-MS system?

Venturini: We analyze the ash and slag that are produced from the incineration process. Based on the different waste products that are processed, the components in ash and slag are different. We analyze for 102 compounds that include ketones, chloro-anilines, phthalates, aromatics, methyl-phenols, and PAHs ranging from pyridine to benzo (G, H, I) perylene. Because our analyte list is so diverse, the GC-MS system is the most appropriate tool for our needs.

LCGC: Why is your analysis important? How are these analytes regulated?

Venturini: This analysis is important because we need to verify that all the organic compounds that have been processed through the kiln are below the land disposal limits before we can send the slag to a hazardous waste landfill. The analytes that we look for are all regulated by the US Environmental Protection Agency (EPA) under the Resource Conservation and Recovery Act (RCRA).

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Our facility specializes in the incineration of RCRA hazardous and nonhazardous wastes. All waste types undergo a rigorous analysis before the waste is accepted. We ask customers to provide a profile (a description) of the material along with a physical sample (pre-approval). We analyze the sample thoroughly to verify it conforms with the profile.

When the results match the profile and meet the regulatory requirements, we schedule the waste for shipment. When the shipment arrives at our facility, we pull a sample for additional analysis. Those results are compared to the preapproval sample's results. If both sets of results match the delivery, the waste is accepted and processed.

LCGC: What regulatory methods do you use? How are your GC-MS instruments and software adapted specifically for this method?

Venturini: We use EPA SW-846 Method 8270D for semivolatile organic compounds. The GC-MS system is easily operated using the Chromeleon CDS software with the environmental package. It has the EPA 8270D method requirements built into the software as part of an e-workflow. Using the e-workflow we can create a complete sequence with a few clicks before run. The Decafluorotriphenylphosphine (DFTPP) tune parameters and initial calculations are also built into the e-workflow. At the end of the run, all the required reports are printed without the need of data transfer and calculations.

LCGC: What are some challenges that you face? What are the requirements for the instrument to deliver accurate results?

We also need an instrument that is robust and can run samples day after day with very little change in the chromatographic conditions so that we can continue to get reproducible results.

Venturini: Our sample matrix can be dirty at times and contain high levels of interferences. We need an instrument that can handle this complicated matrix without compromising sensitivity or lower detection limits that are required to meet regulatory compliance. We also need an instrument that is robust and can run samples day after day with very little change in the chromatographic conditions so that we can continue to get reproducible results.

LCGC: What specifically do you like about the ISQ GC-MS system and how the system and software help you simplify your workflow?

Venturini: I like how easy it is to do maintenance on the ion source. We can change a dirty source with a clean one within a few minutes while the MS is still under vacuum. I also like the modularity of the injection port and that the ISQ GC-MS system uses the same consumables as other manufacturers. Finally, the customization of the Chromeleon software for integrating EPA Method 8270D is a great benefit. It helps us save time and minimize errors.

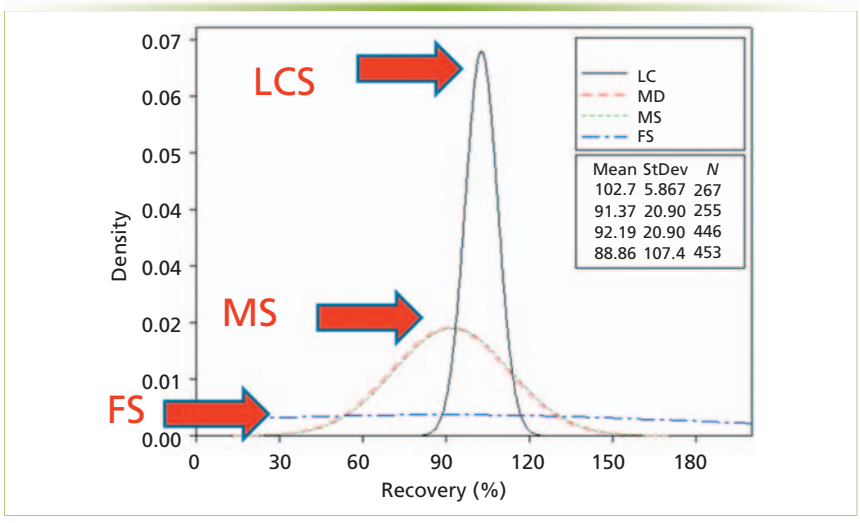

FIGURE 3: Distribution of LCS, MS–MSD, and field spike (FS) recoveries for total cyanide by autoanalyzer (AAN).

standards, method blanks, and LCS are typically formulated with deionized water, therefore consisting of 0% matrix. Techniques for decreasing matrix effects can be envisioned as moving along the matrix spectrum. For example, in metals analysis, matrix matching is when the calibration standards are prepared in a matrix more similar to the prepared samples (such as digestates). This approach changes the matrix of the standards from 0% matrix toward 100% matrix. In organics analysis, internal standards may be added to the prepared standards and samples (for example, extracts) to compensate for various effects of the sample matrix on the analysis, corresponding to 100% matrix. The standard additions approach (12) is perhaps the ultimate in compensating for matrix effects because known amounts of the target analyte are added directly to the samples, thus forming the calibration curve under conditions of 100% matrix. However, this technique is not commonly used in environmental analysis because each sample must be spiked and analyzed one or more times—decreasing throughput.

Perhaps the most common way of addressing a matrix effect is by what might be called *matrix minimization*, but is more commonly known as *dilution*. Minimizing the sample matrix by dilution is used in a variety of ways, such as diluting extracts or digestates, or taking smaller volumes of the native sample for sample preparation. Dilution amounts to moving along the matrix spectrum (Figure 2) from 100% matrix toward 0% matrix.

Dilution is especially useful when the analytical technique has sensitivity to spare. A good example of this is high performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS). This is a highly sensitive and selective technique that is also prone to matrix effects (13,14), but dilution has been shown to be effective in reducing electrospray ion suppression and other matrix effects (15–18).

An interesting approach to systematically using dilution to improve quantitation, called *standard dilution analysis* (19), combines the method of standard additions and internal standards to simultaneously correct for matrix effects and other variations and instrumental parameters. However, this approach probably won't have a major impact on high-volume laboratory operations.

It would be remiss not to mention the work on matrix effects by Kalivas and Kowalski (20), but here we are about 35 years later and the chemometric approach really hasn't had an impact except in cases where the math might be imbedded in commercial instrument software.

Cyanide Is the "Baddest" Bad Actor

Of all the tests routinely performed in environmental laboratories, it can be argued that cyanide is the worst in terms of method performance. There is a fair bit of literature on the "bad behavior" of cyanide in wastewater and drinking water testing (21–25). Cyanide is notable because it can be formed or destroyed, and this can happen during sampling, preservation, storage, and testing. Not only that, the methods are prone to interferences such as aldehydes, color, dissolved solids, fatty acids, mercury, metal anions, metal cations, nitrate, nitrite, oxidants, photodecomposition, sugars, sulfides, turbidity, sulfur compounds, thiocyanate, and "unknowns that cause negative results" (26).

Because of all the potential matrix interferences and matrix effects, the performance of cyanide testing matrix QC indicators tends to be very variable. Figure 3 shows the distribution of total cyanide recoveries for LCS and MS–MSD over four years using EPA Method 335.4 (27), an automated colorimetric method (autoanalyzer). These are primarily wastewater samples from industrial dischargers into the sewer system. Also shown on this graph are field spike (FS) recoveries, obtained using a known amount of a complex cyanide added to a portion of wastewater samples at the time of sample collection. We have demonstrated the utility of field spikes for this analysis (22). Note the wide distribution of MS recoveries and the very wide distribution of FS recoveries.

Field Dilution Is a Solution

The FS recoveries for total cyanide have allowed us to identify industrial sample matrices that have a strong matrix effect on the total cyanide analysis. This knowledge allows us to go back to the site and use alternative dechlorination agents, interference treatments, and even field dilution, to obtain reasonable FS recoveries, demonstrating that we have maintained sample integrity for cyanide and have met the requirements of the cyanide footnote in 40 *Code of Federal Regulations* (*CFR*) 136 Table II, footnote 6, which states (27)

> *Any technique for removal or suppression of interference may be employed, provided the laboratory demonstrates that it more accurately measures cyanide through quality control measures described in the analytical test method.*

Especially for industrial wastewater samples, we have found the combination

of field dilutions, field spikes, and careful field preservation to be vital in demonstrating accurate total cyanide analyses. However, keep in mind that when you dilute the sample you raise the reporting limit by a comparable factor, which could be above the regulatory limit.

Use a Better Method

Finally, there's no better substitute to minimizing matrix effects than using a better method, although regulatory environmental analyses are often limited to using an EPA-approved method. For both total and free cyanide analyses we have found the flow injection analysis (FIA) methods that use online ultraviolet (UV) digestion (for total cyanide) membrane diffusion, and amperometric detection to perform better than distillation–spectrophotometric methods. This includes Methods OIA-1677-DW (28) for free cyanide in drinking water and ASTM D7511-09 (29) for total cyanide in wastewater. For wastewater, we have found that the distribution of FS recoveries using FIA the method is significantly tighter than for the distillation–autosampler method. The FIA methods have the added benefit that they are highly automated, which allows more analyst time to investigate problematic samples.

Unfortunately, method modifications are generally not allowed by the EPA for drinking water analyses. National modifications can be obtained through the EPA's national Alternative Test Procedure (ATP) program (30). For wastewater testing under the Clean Water Act (CWA), certain method flexibility is afforded in 40 *CFR* 136.6 (27). For hazardous and solid waste testing, methods in the EPA's compendium SW-846 are often regarded as advisory and can be modified for a particular project. Check with your local environmental permitting authority for regulatory requirements in their jurisdiction.

Conclusion

Matrix effects are everywhere in environmental testing and, unfortunately, the regulatory requirements often make it difficult for analytical chemists to improve method performance. Also, high-volume laboratories don't have the luxury to treat each sample as a special project. But if environmental laboratories keep running routine samples efficiently, perhaps that would allow some time for dealing with problematic samples or method improvements.

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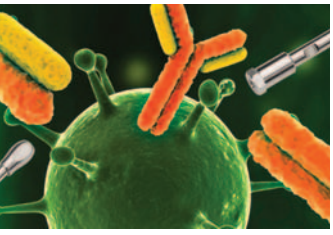
ABOUT THE AUTHOR

Michael F. Delaney, PhD, is the Director of Laboratory Services at the Massachusetts Water Resources Authority in Winthrop, Massachusetts. Direct correspondence about this column to LCGCedit@ubm.com



FOCUS ON BIOPHARMACEUTICAL ANALYSIS

Biolayer Interferometry as an Alternative to HPLC for Measuring Product Concentration in Fermentation Broth

In this installment, we showcase the use of biolayer interferometry (BLI) for measuring the titer of a product: a fragment antigen-binding (Fab) fragment that has been expressed in the periplasm of *E. coli*. High performance liquid chromatography (HPLC)-based quantification is also performed to showcase the high-throughput characteristics of BLI versus HPLC.

Anurag S. Rathore, Deepak Kumar, Jyoti Batra, and Ira Krull

The rising number of life-threatening diseases has translated into a steady increase in demand for biotherapeutics. However, biotherapeutics continue to suffer from poor affordability when compared to other pharmaceutical products. This anomaly has created a significant pressure toward increasing the productivity of biotech processes. *Escherichia coli* (*E. coli*) is the most widely used expression system (~30%) because it offers high density fermentations and scale-up production at a minimum cost (1). In terms of scale, productivity and optimal protein folding, and downstream processing, cytoplasmic and periplasmic expression are the sought after means of production (2–4). However, it should be noted that an unbalanced equilibrium between protein aggregation and solubilization results in inclusion body (IB) formation, protein toxicity and inactivity, and low titers of target proteins (5,6).

The product in *E. coli* is expressed either in cytoplasm or periplasm, or is directly secreted in culture medium (5). The choice of the site of expression depends on a number of factors that include ease of refolding, the role of disulfide bonds on the biological activity of the target proteins, and whether the target protein performs any detrimental and undesirable function in the host cell that interferes with the normal proliferation and homeostasis of the microorganism (7–9). Note that in *E. coli*, the cytoplasm has a more negative redox potential and this reducing environment is maintained by the thioredoxin–thioredoxin reductase (trxB) and the glutaredoxin–glutaredoxin reductase (gor) system (10). Any disulfide bond formation in the cytoplasm often leads to protein inactivation, misfolding, and aggregation. In contrast, myriad enzymes particularly from the Dsb family catalyze the cysteine oxidation via disulfide exchange reactions in the periplasm where a naturally oxidizing environment is present (11). The advantages and disadvantages of the different sites of protein localization are summarized in Table I. The site of protein localization significantly impacts the consideration of choosing the site of expression of recombinant proteins. Often the decision is guided by several factors including the importance of disulfide bonds for the activity of the protein, if the protein can easily be refolded into its native three-dimensional (3D) form in vitro where the protein expression is in the form of IBs, and if any instances of protein toxicity to the host organism are observed.

After the subcellular location for protein expression has been decided, the next challenge is the availability of an appropriate analytical method that can measure the concentration of the target analyte in complex matrices that fermentation broth usually offers (12). In addition, since bioprocessing of protein therapeutics involves multivariate interactions among feed materials, process variables, and product attributes, it is important to screen as many product attributes as possible with regard to the input variables for obtaining an optimal production strain (13). Specifically, in the case of antibody fragments, the product concentration in the fermentation broth is quite low and in addition there is a heterogeneous population of the product in the broth. When identifying the analytical method of choice, desirable attributes of the method include accuracy, sensitivity, dynamic range, reproducibility, time-to-results, cost, and throughput (14).

Commonly used analytical methods that are employed for antibody fragment quantification include enzyme-linked immunosorbent assay (ELISA), electrophoretic techniques such as sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), immunospecific methods such as western blotting, and chromatographic techniques such as reversed-phase high performance liquid chromatography (HPLC). More recently, the use of optical techniques measuring biomolecular interactions (biolayer interferometry [BLI] and surface plasmon resonance [SPR]) have been

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TABLE I: The subcellular location of protein expression and the associated pros and cons for different protein classes

Protein Type	Location	Degree of Solubility	Advantages	Disadvantages
Native protein	Cytoplasm	High	Direct purification with high yield, recovery, and level of expression	Susceptible to proteolysis. High cellular native protein content.
	Cytoplasm	Low	High level of expression. Toxicity effects of protein to cell may be avoided. Easy partial purification.	Protein folding must be carried out. Recovery of the purified native protein can be low or even zero.
Fusion protein	Cytoplasm	High	High level of expression. Purification may be aided with affinity-tagged protein. Solubility and stability may be enhanced by fusion partners.	Site-specific cleavage of fusion peptide required. Overall yield may be low.
Fusion protein directed to secretion	Cytoplasm	Low	High level of expression, may prevent proteolysis.	Signal peptide unprocessed, purification usually not attempted.
	Periplasm space/ medium	High	Ease of purification	Expression level and recovery may be low. Diluted product.

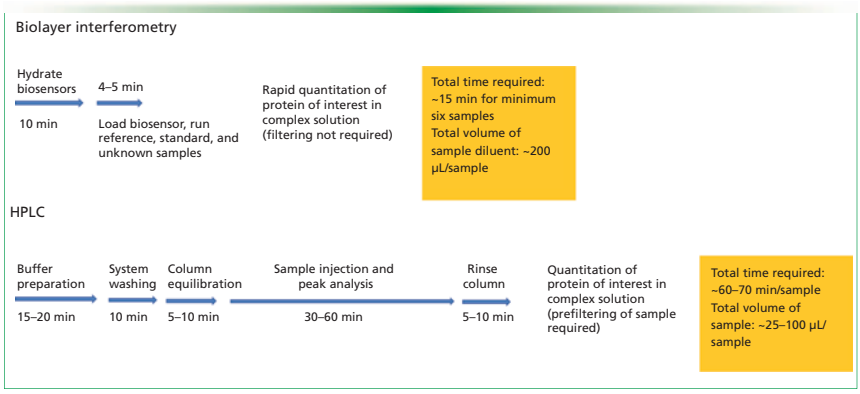

FIGURE 1: Illustration depicting the screening workflow in the BLI system and the HPLC system.

explored by researchers (15). Among these, ELISA is the most widely used technique for quantification, but these techniques have limited accuracy especially in the case of complex mixtures like culture broth and crude extracts (16). Also, these techniques cannot discriminate amongst the molecular variants of the product (for example, mass and charge isoforms) versus its native form. Chromatographic quantification methods are well proven and widely accepted, but require laborious and time intensive sample pretreatment and analysis, in addition to requiring significant capital investment for equipment.

Biosensor-based fragment screening is gradually becoming an established practice in drug discovery (17). BLI and SPR spectroscopy are being increasingly used for this purpose (18). Unlike SPR, BLI does not rely on samples flowing through microfluidics, and up to 8–16 protein-labeled sensors can be simultaneously dipped directly into different solutions of small molecules arrayed in a 96- or 384-well plate.

In this installment, we showcase the use of BLI for measuring titer of a product: a fragment antigen-binding (Fab) fragment that has been expressed in periplasm of the *E. coli*. HPLC based quantification is also performed to showcase the high throughput characteristics of BLI versus HPLC. The screening workflow of sample analysis by BLI and HPLC is illustrated in Figure 1.

Theory

Biolayer Interferometry

BLI is a label-free optical analytical technique for measuring molecular interactions between proteins, peptides, and other molecules in a high-throughput manner using nanomole amounts of samples. The method relies on developing an interference pattern from constructive and destructive waves of a reference layer compared with the reflection at the biolayer (Figure 2). The interference pattern is in terms of the wavelength shift proportionate to the changes in thickness as well as local refractive index changes located at the end of the biolayer interferometry biosensor tip as a result of the molecule binding to the biolayer. The shift in the wavelength is in turn proportional to the number and mass of molecules binding on the biosensor tip. This value is recorded as Δnm or $\Delta\lambda$. The small sample requirements make this technique an excellent choice for the analysis of proteins that are otherwise challenging to isolate. In addition, it is also possible to carry out experiments in parallel making this technique suitable for high-throughput analysis of a large number of samples.

Another feature of BLI that is relevant to the analysis of biopharmaceuticals is that the refractive index changes in the sample do not affect shifts in the interference pattern (19). This lack of effect is due to the property of the BLI signal detection, which occurs in response to interactions at the tip of the biosensor, and any changes to the matrix and unbound proteins in solution (typically found in crude lysates) have a minimal effect on the signal. This property enables BLI to quantify protein concentrations from heterogeneous crude lysates, thereby circumventing the need for extensive sample preparation steps involving purification and dilution. This advantage allows for significant reduction in the analysis time with significantly high accuracy and precision when compared to traditionally used alternatives. Also, appropriate subtraction methods during data analysis can be effectively used to alleviate nonspecific interactions.

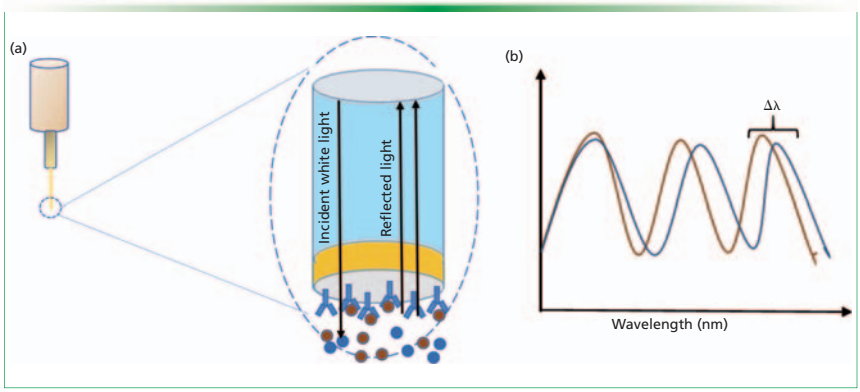

FIGURE 2: (a) Schematic of BLI biosensor tip, and (b) the wavelength shift.

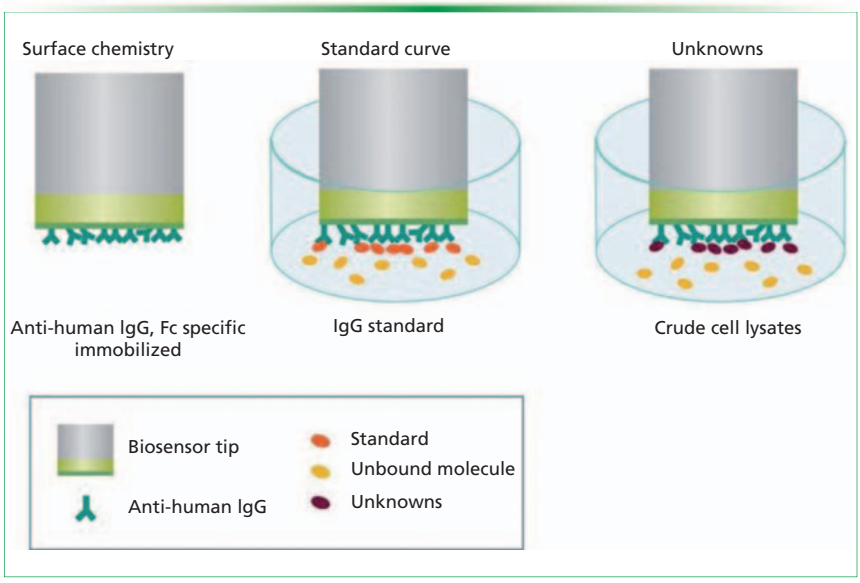

FIGURE 3: Depiction of the principle of how BLI is used for quantitation of target proteins from a complex matrix.

Various biosensor choices based on different chemistries are available that enable quantitation of the proteins. These include anti-human IgG Fc, anti-murine IgG Fv, protein A, protein G, protein L, anti-penta-HIS, streptavidin, anti-human Fab-CH1, anti-GST, and Ni-NTA based biosensors (20). All of these chemistries have very high specificity to the target analyte and can measure in the presence of complex host cell proteins and other components, thereby simplifying analysis at all stages of bioprocess development. In contrast, the traditional techniques for quantitation such as ELISA and HPLC require extensive labor input, sample preparation, and longer time to results. A comparison of these attributes of BLI, ELISA, and HPLC is shown in Table II.

Several key applications of BLI in pharmaceutical development have emerged recently. These include identification of antibody candidates with promising affinities and dissociation kinetics, screening of crude hybridoma supernatants, libraries to identify clones with high affinities and low off-rates, and secondary screening for scFv, Fab, and other biologics from phage or yeast display libraries. Additionally, BLI has been used to characterize antibodies that have undergone affinity maturation, thereby guiding the iterative evolution of antibody hits into lead candidates.

Principle of Fab Quantification by BLI

Protein L possesses a high affinity for the kappa light chain of antibodies and antibody fragments (21). The assay involves coating the tip of the biosensor with a special optical layer followed by capturing molecules (protein L) on this tip (Figure 3). The tip is then dipped into the sample containing the target molecule. The target molecules, upon binding to the captured molecule, form a molecular layer. A white light directed to this assembly gets reflected into two beams. The first beam comes from the tip as a reference while the second light comes from the molecular layer. The difference of the two beams results in the formation of a spectrum color pattern, as depicted in Figure 2b. The phase is a function of the molecular layer thickness and corresponds to the number of molecules on the tip surface.

Materials and Methods

Cultivation

The therapeutic antibody fragment used in this study was expressed in *E. coli* BL21 (DE3) using rhamnose promoter (pRha). Bacterial cells were cultivated in chemically defined R9 medium containing kanamycin (30 µg/mL) at 30 °C and 200 rpm (22). Protein expression was induced by the addition of 50 mM rhamnose at an OD600 of ~6 and the protein was extracted from periplasm by osmotic shock treatment.

Fab Quantification by Reversed Phase-HPLC

Samples were analyzed by reversed-phase HPLC on an Ultimate 3000 HPLC system (Dionex, Thermo Scientific) using a 150 mm x 4.6 mm Zodiacsil column operated at 70 °C with a flow rate of 0.5 mL/min. Mobile-phase A was composed of 0.1% trifluoroacetic acid in purified water (MilliQ, EMD Millipore) and mobile-phase B was composed of 0.1% trifluoroacetic acid in acetonitrile. The equilibration of the column was achieved using 45% B for 5 min. The elution was performed using a linear gradient of 45–100% B in 25 min. The column was regenerated by using 45% B for 15 min. Protein detection was performed by ultraviolet (UV) absorption at 214 nm.

Fab Quantification by BLI

An Octet RED96 BLI system (Fortebio, Pall) was used for quantification of the anti-

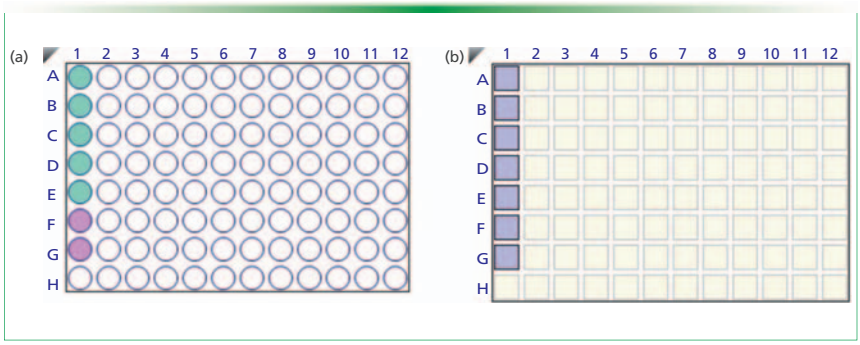

FIGURE 4: (a) Layout of standard solution (green) and unknown samples (purple) in the sample plate. (b) Layout depicting the position of protein L sensors in the sensor plate.

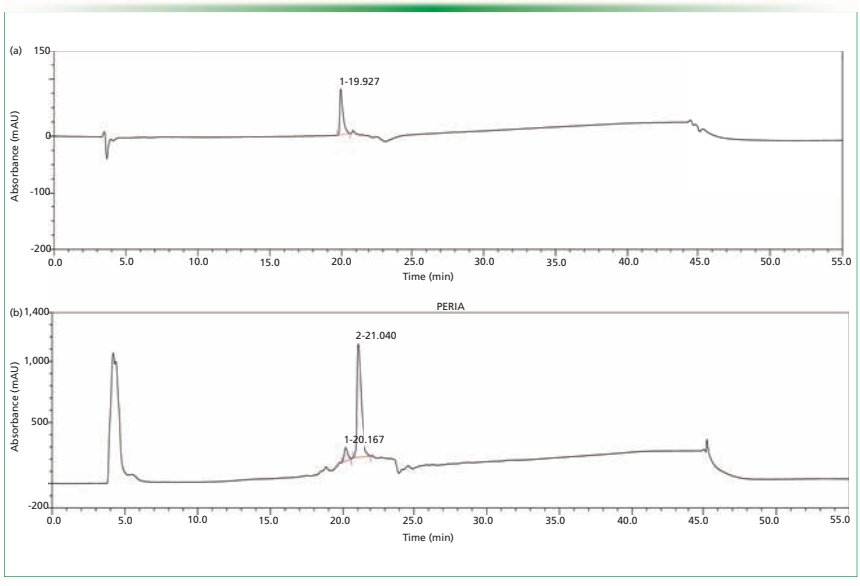

FIGURE 5: (a) Reversed-phase-HPLC chromatogram of standard molecule. (b) Reversed-phase-HPLC chromatogram of Fab molecule expressed in the periplasmic compartment of *E. coli*.

body fragment using protein L biosensors (ForteBio). Black 96-well microplates were obtained from Greiner Bio-One. A commercial standard of the therapeutic antibody was used as a calibration standard. All the samples analyzed were diluted in sample diluent (ForteBio), consisting of 10 mM PBS along with 0.1% BSA and 0.02% Tween 20, pH 7.4.

Assay Protocol

Preparation of Samples and Calibration Standards

The samples, calibration standards, and hydration solution in the sensor plate (200 µL/well) were prepared as per the procedure illustrated in Figures 4a and 4b. Hydration of the protein L sensor was performed in phosphate-buffered saline (PBS), pH 7.4. The regeneration was performed in 10 mM glycine, pH 1.5, and the neutralization buffer was kept identical to the hydration buffer. Since exposure of hydrated biosensors to air may affect performance, the hydrated biosensors were kept ready for the binding experiments with the analyte. Biosensors hydrated in the BLI kinetics buffer retain activity when stored overnight in the BLI kinetics buffer at 4 °C.

Preparation of Assay Plate and Biosensors

The control and assay samples were pipetted (200 µL/well) and the hydration solution was placed in the wells corresponding to the position of the biosensor to be used in the analysis. Next, they were hydrated for 10 min before the experiment followed by sample measurement at 1000 rpm for 300 s. Alternatively, the delay timer can be used to automatically start the assay after 10 min. All experiments were performed at 30 °C. The assay was performed in the basic quantitation with regeneration assay format of the Octet data acquisition software.

Data Analysis

Data analysis was performed using Octet data analysis software version 7.1. The acquired data were corrected by subtracting the black matrix from the sample matrix. The reference subtracted data was analyzed using the initial slope binding rate equation.

TABLE II: Comparison of analysis attributes of ELISA, HPLC, and BLI

Method	ELISA	HPLC	BLI
Number of assay steps	7	4	2
Labor time (h)	3	0.5	<0.2
Total time to results (h)	>6	10	0.5
Precision (%CV)	>10	<5	<10

TABLE III: Calculated concentrations of Fab using the BLI system and reversed-phase HPLC

Sample	BLI	HPLC	% Difference
A1	0	0	0
C1	6.58	5.9	10
D1	6.44	6.5	-1
E1	5.34	5.5	-3
F1	6.94	7.09	-2
G1	8.59	8.05	6
H1	8.37	7.25	13

Results and Discussion

Fab Quantification Using HPLC

Reversed-phase HPLC plays a critical role with respect to characterization of monoclonal antibodies (mAbs). These products include full-sized intact mAb, Fab, and Fc fragment molecules, reduced heavy- and

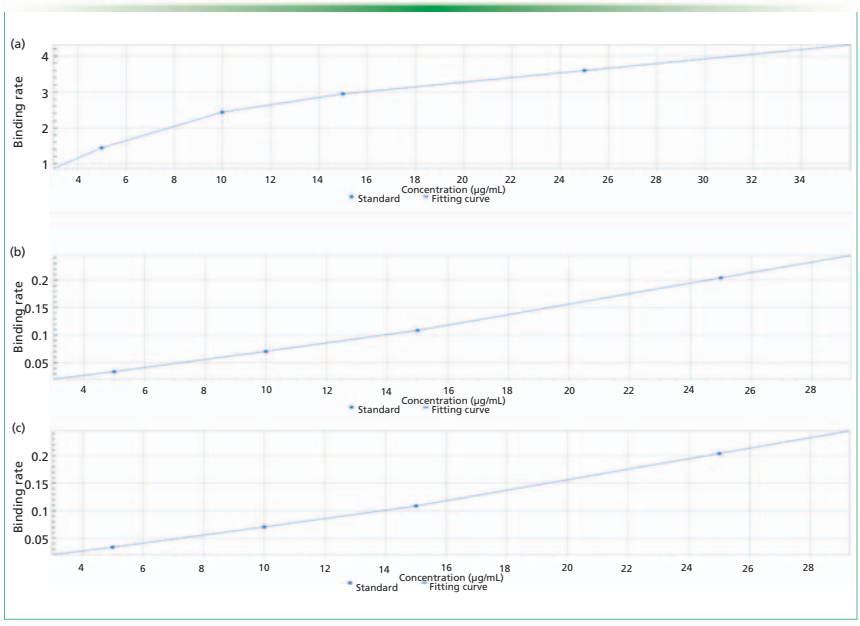

FIGURE 6: Standard curve generated using various dilutions of culture media: (a) neat; (b), twofold, 50% culture medium; (c) fivefold, 20% culture medium.

TABLE IV: Key features of BLI and HPLC; a comparative analysis

Sample	BLI	% Difference
Rapid high throughput screening using biosensors (96- or 384-well screening)	Yes (~5–10 min)	No
Crude sample screening	Yes	No (prefiltration requirement)
Kinetics determination	Yes	No
Quantitation of functional activity of antibody molecules or therapeutic proteins	Yes	No
Sample recovery possible	Yes	No
Application as PAT tool	Yes	Yes
Dynamic range	Wide	Limited
Sample concentration requirement	Low	High
Instrumentation cost involved	High	Low
User expertise required	Low	High
Detection limit	ng	µg

light-chain species, and peptide maps generated by proteolytic digestion. There are many reasons that make reversed-phase HPLC a popular tool, including the well-studied hydrophobic separation mechanism, the availability of efficient reversed-phase-HPLC columns packed with small-particle, nonporous, fully porous, or superficially porous (SPP) materials, and the use of mobile phases compatible with mass spectrometry (MS) for peak identification and structure elucidation.

Figures 5a and 5b illustrate the chromatograms of the standard molecule and the target protein (Fab protein) obtained using reversed-phase-HPLC. The quantification was done by comparing the peak area of the standard protein (known concentration) to the peak area of the target protein in the periplasm extract (prefiltered) based on identical retention times. Protein samples were concentrated by using 10-kDa cut-off centrifugal filtration and retentate. In addition, filtrate proteins were analyzed for estimation of the Fab molecule.

As indicated in Table II, the advantage that reversed-phase HPLC offers is the high resolution for separation of antibody fragments. The analysis time is about 45 min per sample. In addition, every consecutive sample injection requires intermittent blank runs to prevent carryover. Thus, in cases for which a large number of samples are to be analyzed, such as drug discovery or during process development, HPLC analysis is often the bottleneck.

Fab Quantification Using BLI

First, standard curves were constructed using a purified Fab molecule at concentrations of 5, 10, 15, and 25 µg/mL. Standard curves were assayed in different well locations in three independent experiments to account for any well-to-well variability. A consistent assay performance across all the wells was observed with average coefficient of variation (CV) for the entire standard curve range of less than 11.5%.

Next, possible interference of the media components with the analysis was assessed by constructing standard curves using various dilutions of the culture media and then overlaying and assessing the degree of variability at each of the concentrations. The assay was found to tolerate up to 100% culture media, with comparable recoveries of spiked standards in all dilutions and an overall CV of <10% (Figure 6). These results indicate that BLI offers a robust method for analysis of upstream process samples with minimal preprocessing.

Figures 7a–7c represent the binding curve generated for the standard Fab molecule. The binding rates of the test sample were measured and interpolated from the standard curve to determine the concentration. Data for eight wells were acquired every 2 min, thereby enabling analysis of 96 samples in ~30 min. Significantly, there are no wash steps required during analysis from one well to the other. It should be noted that the use of a standard curve for determining the concentration of the test sample is optional; however, it is recommended that separate standard curves be constructed for different culture broths. The reported values of the assay sensitivity for Fab fragment quantification were 0.05–300 µg/mL. For the present study, the dynamic range was found to be 100–300 µg/mL.

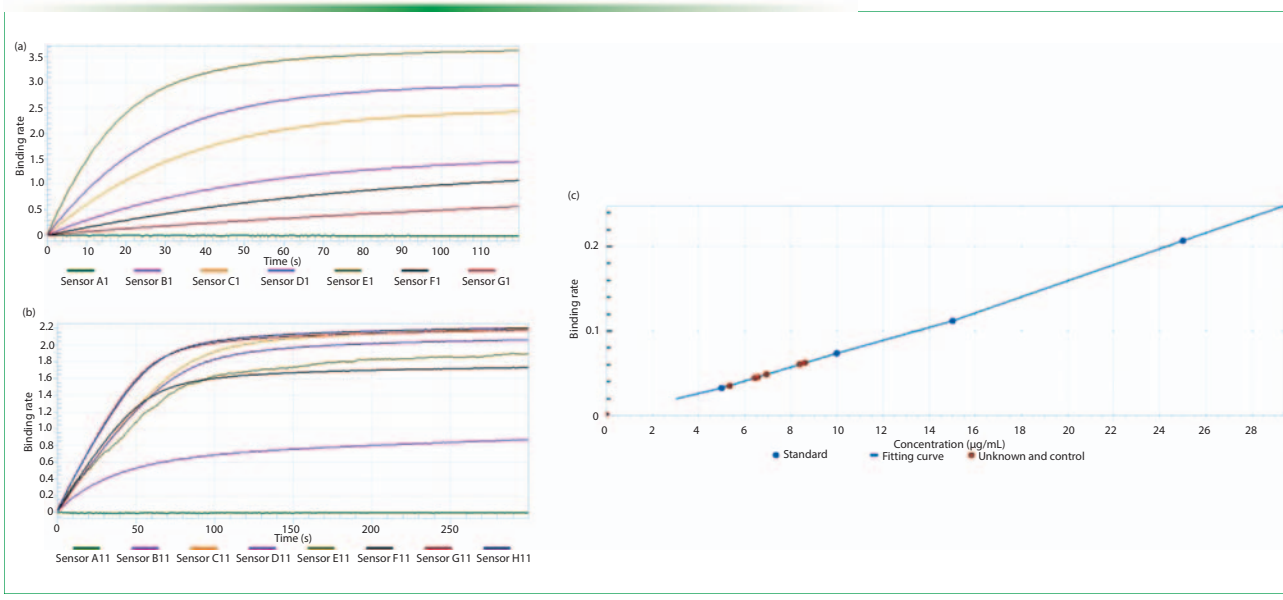

FIGURE 7: Real-time binding data of (a) standard Fab molecule and (b) expressed Fab molecule, and (c) a graph depicting the binding rate of standard as well as target Fab protein.

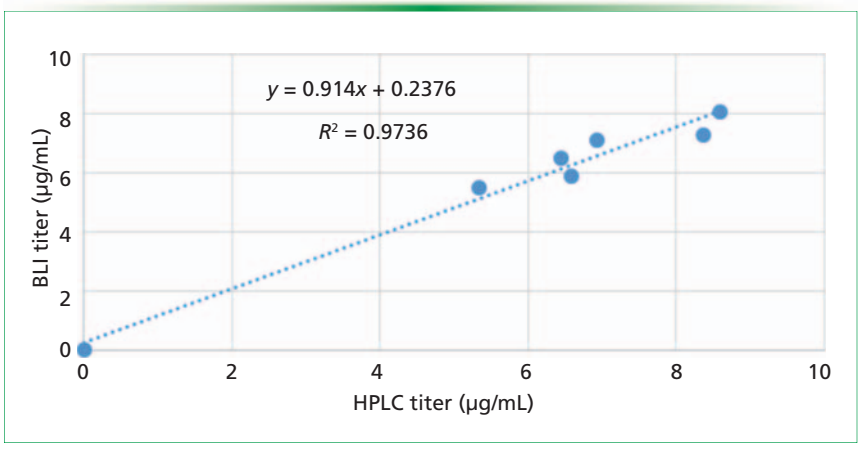

FIGURE 8: Comparison of Fab measured by BLI and reversed-phase HPLC methods.

Comparison of Titer Measurement by HPLC and BLI

A comparison of the performance of HPLC and BLI to quantify the Fab fragment was performed using seven periplasmic-expressed Fab proteins. For BLI, the samples were diluted 40-fold so that the concentration was brought within the dynamic range of the instrument. For HPLC, the analysis was performed by injecting 100 µL of the undiluted samples. There was no other difference in the sample preparation strategy for the analysis using these two platforms; the goal was to obtain an unbiased comparison of the two methods.

Titer values measured with both techniques showed reasonable comparability with a deviation of ~13% between the two techniques (Table III). However, for most of the samples, the differences between the values obtained using the two techniques were well within 10%.

With regards to the total analysis time, it is clear that BLI offers a significantly faster analysis time (0.5 h versus 90 h) for 96 samples. The time includes any intermediate processing steps that may be required during analysis using both techniques. Note that the injection of crude sample will reduce the column life; 200 injections of crude samples versus 1000 injections of clean samples. In contrast, BLI does not suffer from these shortcomings.

Reversed-phase HPLC, however, can provide information about the presence of various molecular variants and other product heterogeneities of the sample. BLI is unable to provide this information. Thus, the two techniques complement each other and together they can be used for a comprehensive quantitation and analysis of Fab fragments in complex culture media. Table IV compares the various characteristics of sample analysis by HPLC and BLI.

Limitations of Affinity Recognition–Based Approaches

There are, however, a few generic deficiencies in any analytical assay that utilizes affinity recognition principles to detect or identify the target protein or antibody. These deficiencies have been known for decades, ever since ELISA was discovered and promulgated around the world, to the present day. First off, molecular recognition entities, especially antibodies (mAbs), proteins, or peptides, are not always easy to manufacture in a totally reproducible manner, batch to batch, even for analytical reagent purposes. The current dilemma in the scientific literature related to a general inability to reproduce certain publi-

cations using antibodies, such as ELISA, has been attributed to the difficulties in obtaining identical mAbs or proteins from the same vendor, year to year, or from different vendors at any given time. This inconsistency then leads to a general, at times, lack of reproducibility of the original studies, unless the authors had done a thorough analytical method validation demonstration in their original publication (not usually the case today).

A second, generic problem in using affinity recognition is that very few such reagents recognize only the analyte molecule of interest in developing the original assay, whatever that might have been. Biological routes to recognition molecules rarely evolve any protein that will recognize only a single, molecular entity. This drawback has been known from the very beginning of ELISA, which also should not be used alone or entirely for identifying any specific antigenic species until it is shown to be 100% specific. That is almost never the case, nor will it be for the recognition entities in BLI.

There is a third possible problem with using BLI alone to identify the precise structure of any protein: not knowing if that protein is alone at the biorecognition step. BLI does not resolve all the entities that might be present in the final sample under analysis—it is not chromatography or electrophoresis and has no separation powers beyond affinity recognition, by and large.

Finally, BLI does not provide any indication of the molecular weight or structure of the antigenic species under analysis. Though it may be used for approximate quantitation, it does not truly identify the analyte species, other than that it recognizes it by some form of molecular interaction processes. It provides no structural or molecular weight information, no fragmentation data, and no database that could be approached to confirm its recognition by molecular interactions or fragmentation patterns, which are so common in MS methods of analysis and quantitation today.

Conclusions

Despite the caveats discussed above, it should be noted that affinity recognition is playing an increasingly significant role in many protein analysis methods now being developed, especially in bioanalysis. BLI can be considered a rapid, inexpensive, and fully automatable approach for affinity recognition, as in ELISA, and partial qualitative identification of a protein in a complex sample or biofluid, along with suitable quantitation having authentic reference standards run alongside the actual samples of interest. This study amply demonstrates the utility of BLI as an alternative to HPLC for quantitation of protein samples in complex media matrices. The utility is particularly more pronounced where there is a demand for high-throughput analysis without affecting sensitivity and robustness of analysis. The ability of protein L to retain its binding activity ensures that the biosensors can be regenerated and the necessary optimization of conditions would maximize the recovery.

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ABOUT THE AUTHORS

Deepak Kumar is a post-doctoral fellow in the Department of Chemical Engineering at the Indian Institute of Technology in Delhi, India.

Jyoti Batra is a research scientist at Vallabhbhai Patel Chest Institute, University of Delhi, India.

Anurag S. Rathore is a professor in the Department of Chemical Engineering at the Indian Institute of Technology in Delhi, India.

Ira S. Krull is a Professor Emeritus with the Department of Chemistry and Chemical Biology at Northeastern University in Boston, Massachusetts, and a member of *LCGC*'s editorial advisory board.

Recent Advances in Comprehensive Chromatographic Analysis of Emerging Drugs

The analysis of seized drugs containing emerging drugs, which are synthesized to skirt the controlled substances laws, is complicated by a large array of similar drugs including analogues, homologues, positional isomers, and diastereomers. Chromatographic techniques such as gas chromatography (GC) and liquid chromatography (LC), which are commonly used with mass spectrometry (MS) detection, have certain limitations—especially for positional isomers and diastereomers. This article discusses the use of emerging technologies that are complementary to established techniques, to significantly reduce these shortcomings for both synthetic cannabinoids and synthetic cathinones. In this vein, the utility of recently reported approaches including ultrahigh performance supercritical fluid chromatography (UHPSFC)–photodiode array (PDA) ultraviolet (UV)–MS, and GC–vacuum UV is discussed. To increase the specificity of analysis, multiple chromatographic techniques are commonly used. For the analysis of emerging drugs, a combination of GC and UHPSFC is recommended. The utility of a previously unreported coupled-columns approach for UHPSFC to significantly enhance resolution of synthetic cathinones is presented.

Ira S. Lurie, Lauriane Tremeau-Cayel, and Walter F. Rowe

Emerging drugs are synthetically produced modifications of controlled drugs designed to skirt laws banning their use as recreational drugs. These changes often result in structural analogues, structural homologues, as well as positional isomers and stereoisomers. The identification of existing and new emerging drugs is complicated by the similarity in structure, lack of reference materials, insufficient libraries, lack of molecular ions for certain solutes (when electron ionization [EI] mass spectrometry [MS] is used), difficulty in deconvoluting spectra for coeluted solutes, and the similarity in MS spectra for diastereomers and positional isomers. Therefore, as a complement to MS detection, chromatographic resolution is particularly important. In this vein, gas chromatography (GC) is commonly used for the analysis of seized drugs, followed by liquid chromatography (LC) (specifically, high performance liquid chromatography [HPLC] and ultrahigh-pressure liquid chromatography [UHPLC]). Although GC has inherently higher resolving power (peak capacity) than HPLC, the former technique is disadvantageous for solutes (including many emerging drugs) that are thermally labile, polar, and nonvolatile.

For certain emerging drugs such as synthetic cathinones, GC–EI-MS suffers from extensive fragmentation in the source, which results in a lack of molecular ions, and a lack of diagnostic fragment ions (1). Differentiating between positional isomers for these solutes is particularly difficult using EI spectra, especially when substitution occurs on the benzene ring (2,3). Although LC–electrospray ionization (ESI)–MS/MS and LC–ESI-quadrupole time-of-flight (QTOF)-MS will provide molecular ions for synthetic cathinones, there is a scarcity of diagnostic fragment ions and difficulty in distinguishing between positional isomers (1). For other emerging drugs, such as synthetic cannabinoids, difficulty arises in distinguishing positional isomers using UHPLC–ESI-TOF-MS (4). Both GC–EI-MS (5) and LC–MS/MS (6) cannot distinguish between diastereomers. GC (4,7–9), HPLC (10,11), and UHPLC (4,12–14) have been used for the separation of emerging drugs. For mixtures of both controlled synthetic cannabinoids and controlled synthetic cathinones, neither GC nor reversed-phase UHPLC will resolve all these solutes, with GC slightly outperforming UHPLC (4,14). The controlled synthetic cathinones were poorly resolved using UHPLC in the hydrophilic interaction chromatographic (HILIC) mode (14). Both GC and reversed-phase UHPLC exhibit poor resolution for positional isomers of synthetic cannabinoids and synthetic cathinones (4,14), with better (but still incomplete) resolution of positional isomers of synthetic cathinones obtained using HILIC (14). GC (10) and HPLC (11) can exhibit poor resolution for diastereomers of synthetic cannabinoids.

Ultrahigh performance supercritical fluid chromatography (UHPSFC) uses SFC with packed columns that contain sub-3-µm particles, or columns that give equivalent performance such as core–shell, to produce highly efficient and rapid separations (15–18). Similar to LC techniques, SFC can analyze compounds that are thermally labile, polar, or nonvolatile without pretreatment or derivatization. Compared to UHPLC mobile phases, those used in UHPSFC are more diffusive with lower viscosities, which can result in separations that are up to four times faster.

The utility of ultraviolet (UV) detec-

TABLE I: Comparison of the various separation methods for the synthetic cannabinoids

Chromatographic Mode (Column)	Resolution ≥1 22 Controlled*	Resolution ≥1 10 JWH-018 Positional Isomers	Time of Analysis (min)†
UHPSFC (CEL1)	11	10	11.3‡
UHPLC (SPP C18)	15	3	16§
GC (Elite 5-MS)	17	4	25.5‖

*23 controlled synthetic cannabinoids separated by UHPLC and GC; †mixture of controlled synthetic cannabinoids; ‡includes 1 min gradient re-equilibration; §includes 5 min gradient re-equilbration; ‖includes 0.5-min temperature re-equilibration

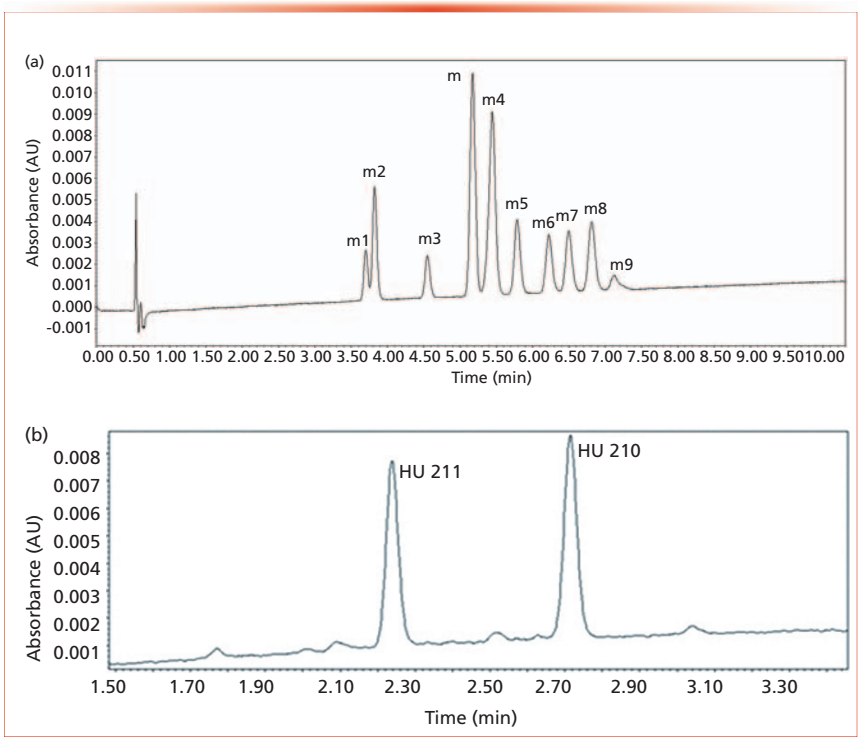

FIGURE 1: (a) UHPSFC separation of JWH 018 and nine of its positional isomers. Column: 15 cm x 3.0 mm, 2.5-µm d_p Acquity UPC^2 Trefoil CEL1; initial mobile-phase conditions: 20% isopropanol, 80% carbon dioxide; final mobile-phase conditions: 31% isopropanol, 69% carbon dioxide; gradient: 10.3 min, linear, with a 1.0-min gradient reequilibration; flow rate: 1.25 mL/min; temperature: 55 °C; automated back-pressure regulator setting: 2200 psi; detection: UV absorbance at 273 nm; injection volume: 1 µL. Peaks (5 µg/mL): m = JWH-018, m1 = JWH 018 2′-naphthyl-*N*-(1, 2-dimethylpropyl) isomer, m2 = JWH 018 2′-naphthyl-*N*-(1 ethylpropyl) isomer, m3 = JWH 018 2′-naphthyl-*N*-(1 methylbutyl) isomer, m4 = JWH-016, (m5) JWH 018 2′-naphthyl-*N*-(1,1-dimethylpropyl) isomer, m5 = JWH 018 2′-naphthyl-*N*-(1,1-dimethylpropyl) isomer, m6 = JWH 018 2′-naphthyl-*N*-(2 methylbutyl) isomer, m7 = JWH 018 2′-naphthyl-*N*-(2, 2-dimethylpropyl) isomer, m8 = JWH 018 2′-naphthyl-*N*-(3 methylbutyl) isomer, m9 = JWH 018 2′-naphthyl isomer. (b) UHPSFC separation of HU-210 and HU-211. Column 15 cm x 3.0 mm, 2.5-µm d_p Acquity UPC^2 Trefoil AMY1; initial mobile-phase conditions: 18% isopropanol, 80% carbon dioxide; final mobile-phase conditions: 53% isopropanol, 82% carbon dioxide; gradient: 5 min, linear, hold for 1 min, 1.0 min gradient reequilibration; flow rate: 1.25 mL/min; temperature: 45 °C; automated back-pressure regulator setting: 2200 psi; detection: UV absorbance at 273 nm; injection volume: 1 µL. Adapted with permission from reference 26.

tion subsequent to HPLC and UHPLC separation to aid in the identification of emerging drugs, including positional isomers on the benzene ring, has been described (2,12,19). Vacuum UV (VUV) detection, which is easily interfaced to GC, can measure solute absorbance between 120 and 430 nm, and is capable (in contrast to conventional UV detection) of probing $\sigma \rightarrow \sigma^*$, and short wavelength $\pi \rightarrow \pi^*$ transitions (20). VUV detection can not only demonstrate class similarities, but also allows for differentiation within a class, including most isomers (21). In addition, measured spectra can be matched against an existing library to identify the compounds (21). Furthermore, since UV spectra are additive, spectra can also be easily deconvoluted if coelution exists (22). The identification of coeluted compounds by GC–EI-MS would require altering chromatographic conditions, including changing columns, or using multidimensional GC, which may not be readily available (21,22).

Coupled columns (two or more columns, with or without the same stationary phase combined in series) can be used to improve separations by a linear increase in peak capacity. The same mobile phase can be used throughout both columns unlike in multidimensional LC where different mobile phases are usually required (23,24). However, for multidimensional chromatography, unlike for coupled columns, peak capacity is the product of the peak capacity of the individual columns. Because SFC uses mobile phases of significantly lower viscosity, there is less concern for back-pressure drops, which facilitates the use of coupled columns (24).

In this article various approaches are discussed to provide improved chromatographic resolution for emerging drugs, particularly for positional isomers and diastereomers, including the use of UHPSFC both in the single and tandem column mode. In addition, for synthetic cathinones, the use of UV detection for UHPSFC in tandem with MS detection, and VUV detection for GC to complement MS detection, which is particularly useful for positional isomers, is discussed.

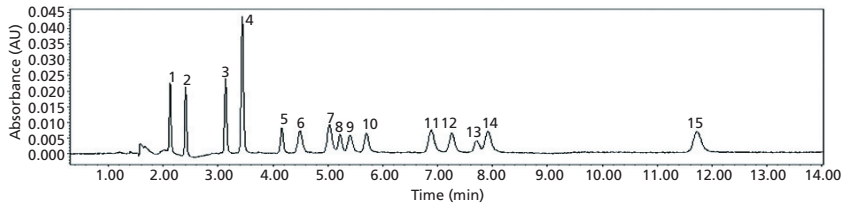

FIGURE 2: Coupled-column chromatogram of a standard mixture of synthetic cathinone controlled substances. Columns: 10 cm x 3.0 mm, 1.7-µm d_p Acquity UPC^2 Torus Diol connected in series with a 10 cm x 3.0 mm, 1.7-µm d_p Acquity UPC^2 Torus 2-PIC column; mobile phase: 3% methanol (10 mM ammonium formate in methanol), 97% carbon dioxide; flow rate: 1.00 mL/min; temperature: 40 °C; automated back-pressure regulator setting: 1800 psi; detection: UV absorbance at 230 nm; injection volume: 0.5 µL. Peaks (50 µg/mL concentration): 1 = α-PVP, 2 = α-PBP, 3 = MDPV, 4 = naphyrone, 5 = 4-MePPP, 6 = pentedrone, 7 = buphedrone, 8 = 3-fluoromethcathinone, 9 = 4-methylethcathinone, 10 = 4-fluoromethcathinone, 11 = pentylone, 12 = methcathinone, 13 = butylone, 14 = mephedrone, 15 = methylone .

TABLE II: Comparison of the various separation methods for the synthetic cathinones

Chromatographic Mode (Column)	Resolution ≥1 15 Controlled*	Resolution ≥1 34 Positional Isomers†	Time of Analysis (min)‡
UHPSFC (Diol)	11	28	8§
UHPLC (SPP C18)	12	7	16‖
UHPLC (SPP HILIC)	5	27	12§
GC (Elite 5-MS)	13	22	13.5#

*The number of controlled synthetic cathinones separated in mixture 1; †the sum of the number of positional isomers separated in mixtures 1–9; ‡mixture of controlled synthetic cathinones; §isocratic analysis; ‖includes 5-min gradient reequilibration; #includes 0.5-min temperature reequilibration. Adapted with permission from reference 14 and John Wiley and Sons

The Utility of UHPSFC for the Analysis of Emerging Drugs

UHPSFC has been used for the separation of synthetic cannabinioids (25,26) and synthetic cathinones (14,27). For a large array of these solutes, including sets containing up to 10 positional isomers and difficult-to-separate diastereomeric compounds, UHPSFC has been compared to both GC and UHPLC (4,10,11,14,26). For these latter studies, various columns and mobile phase conditions were evaluated. The columns included four achiral stationary phases including Acquity UPC^2 Torus 2-PIC, Acquity UPC^2 Torus Diol, Acquity UPC^2 Torus DEA, and Acquity UPC^2 Torus 1-AA (100 mm × 3.0 mm, 1.7 µm), and three chiral columns, including Acquity UPC^2 Trefoil AMY1, Acquity UPC^2 Trefoil CEL1, and Acquity UPC^2 Trefoil CEL2 (150 mm × 3.0 mm, 2.5 µm) (all from Waters). For the mostly neutral synthetic cannabinoids (JSW-200 basic) a 5-min gradient was conducted (usually with a 1-min hold) using carbon dioxide with methanol, ethanol, isopropanol, or acetonitrile as a modifier. A fine-tuned separation was obtained by varying gradient steepness (time of gradient), and temperature. For the basic synthetic cathinones, 5-min gradients (1-min hold) were conducted or isocratic analysis (usually under 6 min) using carbon dioxide with methanol, ethanol, or isopropanol as the modifier or ammonium formate or ammonium hydroxide as the additive. A fined-tuned separation was obtained by varying the temperature. For the gradient separations, conditions were adjusted so that the chromatographic peaks occupied approximately the entire gradient time.

It is of interest to compare the separation of the synthetic cannabinoids by UHPSFC, GC, and UHPLC (see Table I). Of particular interest for the synthetic cannabinoids were the separations of JWH-018 and nine positional isomers, and the diastereomers such as HU210 and HU211. For this purpose, the polysaccharide-based chiral columns performed well, allegedly because of their selectivity arising from dipole–dipole and steric interactions (26). The near-baseline separation of JWH-018 and nine positional isomers, as well as the well-resolved separation of HU-210 and HU-211 are shown in Figures 1a and 1b, respectively. In comparison for JWH-018 and it positional isomers, GC (5% phenyl column) resolved four out of 10 solutes, while at best UHPLC (reversed-phase mode with a C18 column) resolved three out of 10 (4). HU-210 and HU-211 were not resolved using GC (10) or HPLC (11). In regards to the separation of a diverse mixture of controlled synthetic cannabinoids, 11 out of 22 were separated (resolution ≥ 1) using UHPSFC (10-min gradient plus 1-min gradient reequilibration) using the same system employed for JWH-018 and it positional isomers (26). In contrast, 17 out of 23 were separated (resolution ≥ 1) using GC for the same system used for the positional isomers (24-min temperature-programmed run plus 0.5-min temperature reequilbration), while at best 15 out of 23 were separated (resolution ≥ 1) using UHPLC (11-min gradient plus 5-min gradient equilibration) (4).

Similarly, it is of interest to compare the separation of the synthetic cathinones by UHPSFC, GC, and UHPLC (see Table II). To evaluate the utility of UHPSFC for the separation of positional isomers of synthetic cathinones, nine mixtures containing different sets of masses for a total of 34 positional isomers were investigated

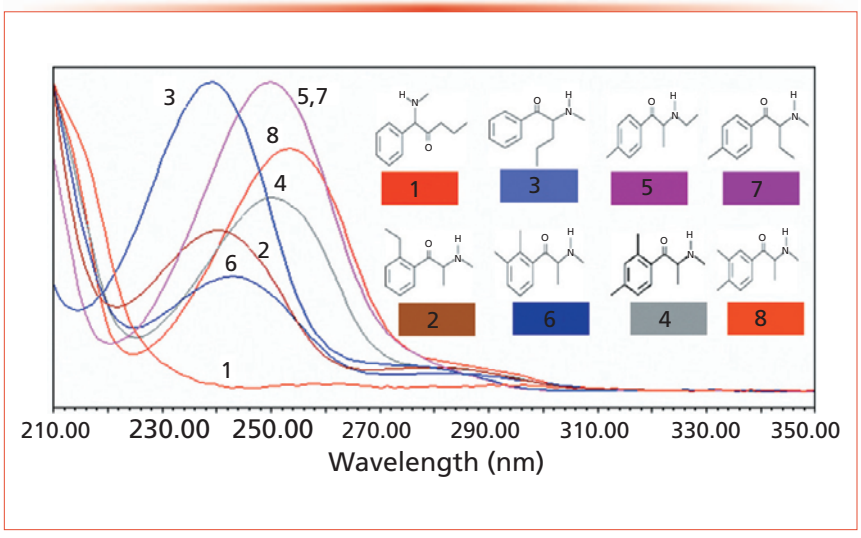

FIGURE 3: Normalized UV spectra of synthetic cathinone positional isomers with molecular mass 191 acquired by PDA-UV detection subsequent to UHPSFC separation. Column: 10 cm x 3.0 mm, 1.7-µm d_p Acquity UPC^2 Torus Diol; mobile phase: 3% methanol (10 mM ammonium formate in methanol), 97% carbon dioxide; flow rate: 1.25 mL/min; temperature: 40 °C; automated back-pressure regulator setting: 2200 psi; injection volume: 0.5 µL. Spectra: 1 = isopentedrone, 2 = 2-ethylmethcathinone, 3 = pentedrone, 4 = 2,4-dimethylmethcathinone, 5 = 4-methylethcathinone, 6 = 2,3-dimethylmethcathinone, 7 = 4-methylbuphedrone, 8 = 3,4-dimethylmethcathinone. Adapted with permission from reference 3 and John Wiley and Sons.

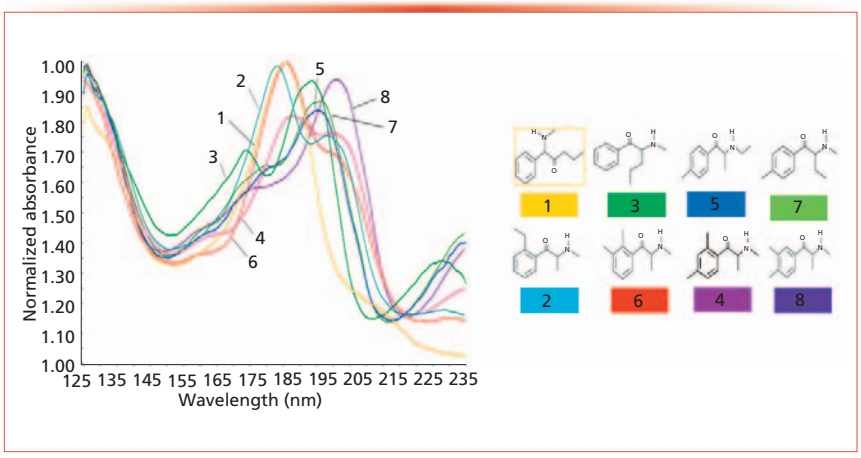

FIGURE 4: Normalized VUV spectra of synthetic cathinone positional isomers with molecular mass 191 acquired by VUV detection subsequent to GC separation. Column: 30 m x 0.25 mm, 0.25-µm d_f PerkinElmer Elite-5MS; inlet temperature: 230 °C; injection volume: 2 µL (1:10 split); oven program: 80 °C initial temperature for 1.0 min, ramp to 320 °C at a rate of 20 °C/min, and a temperature hold for 1.5 min; VUV system temperature :300 °C; VUV makeup gas pressure: 0.5 psi. Adapted with permission from reference 31.

(14). The criterion used to evaluate a chromatographic system was to sum the total number of isomers resolved with a resolution ≥ 1 for each mixture. Using a diol column operating under isocratic conditions, with the mobile phase containing a methanol modifier and an ammonium formate additive, 28 out of 34 positional isomers were separated. Since the synthetic cathinones are chiral, an achiral column was preferred to reduce the complexity of the separation. In practice, since all optical isomers of a given synthetic cathinone are controlled, enantiomeric resolution is of limited forensic value. In comparison, GC with a 5% phenyl column resolved 22 out of 34 positional isomers, while at best UHPLC (HILIC mode with a HILIC column) resolved 27 out of 34 (14). Note that UHPLC using the commonly employed reversed-phase mode with a C18 column only resolved 7 out of 34. For the separation of a diverse mixture of controlled synthetic cathinones, 11 of 15 were separated (resolution ≥ 1) using UHPSFC (8-min isocratic run using the same system employed for the positional isomers) (14). For the same systems used for the positional isomers, 13 out of 15 were separated by GC (13-min temperature program plus a 0.5-min temperature reequilbration), while 12 out of 15 and five out of 15 were obtained using UHPLC in the reversed-phase mode (11-min gradient plus a 5-min gradient reequilibration) and HILIC modes (12-min isocratic), respectively (14).

For the analysis of seized drugs, it is important to use more than one non-correlated chromatographic system to decrease the likelihood of the misidentification of a solute of interest. In this vein, the orthogonality of UHPSFC to both GC and UHPLC for both synthetic cannabinoids and synthetic cathinones was examined. For this purpose, principal component score plots were generated for mixtures of the controlled substances of both the synthetic cannabinoids (26) and synthetic cathinones (14). For both classes of drugs, the UHPSFC systems stand by themselves, highly orthogonal to both GC and UHPLC using a C18 column operating in the reversed-phase mode. For synthetic cathinones, UHPSFC is also highly orthogonal to UHPLC using a HILIC column operating in the HILIC mode. Given that UHPLC in the reversed-phase mode could provide poor overall resolution of positional isomers and that UHPLC in the HILIC mode could provide poor resolution of a diverse mixture of controlled

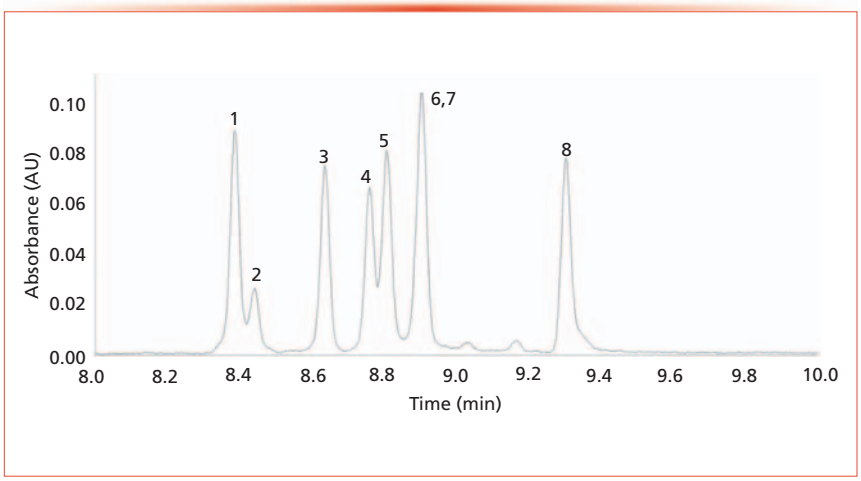

FIGURE 5: GC–VUV separation of synthetic cathinone positional isomers with molecular mass 191. See Figure 4 for GC conditions and peak identity. Adapted with permission from reference 31.

substances, a viable combination of chromatographic techniques for the analysis of emerging drugs would be GC and UHPSFC.

The use of serial coupled columns with different stationary phases (Diol + 2PIC) for UHPSFC significantly improved the overall resolution of synthetic cathinones. Using a previously reported experimental setup for single columns (14), a mixture of 15 controlled synthetic cathinones was resolved (Figure 2). In addition, under the same experimental isocratic conditions, 32 out of 34 positional isomers were also resolved.

The Use of Complementary Detection Schemes for Both UHPSFC and GC Analysis of Synthetic Cathinones

Not only are GC and UHPSFC highly complementary separation techniques for emerging drugs such as synthetic cathinones, but the choice of detection techniques can also provide essential complementary information. Along these lines, for UHPSFC the use of photodiode-array (PDA) UV detection in tandem with ESI-MS detection provides complementary data to GC with EI-MS detection. In contrast to GC–EI-MS detection for synthetic cathinones, UHPSFC with tandem UV and ESI-MS detection will provide molecular mass information as well as distinguish between positional isomers where substitution occurs on the benzene ring. The great utility of UV detection for positional isomers (for example, molecular mass 191) is shown in Figure 3. For compounds that differ in substitution on the benzene ring, such as 2-ethylmethcathinone, 2,4-dimethylmethcathinone, 2,3-dimethylmethcathinone, and 3,4-dimethylmethcathinone, unique UV spectra are obtained. It should be noted that these solutes are resolved by both UHPSFC and GC, further adding to the specificity of their determination (14). 4-methylethcathinone and 4-methylbuphedrone, which differ in substitution on the aliphatic portion of the molecule, give identical UV spectra. UV spectra can also help identify which class or subclass of an emerging drug is present (3,22), which is an especially useful aid for the identification of unknown compounds.

With the mobile-phase conditions used with UHPSFC to acquire UV spectra, there was at least a 10-nm blue shift (shift to shorter wavelengths) compared to UHPLC mobile-phase conditions (3). This effect was largely caused by the presence of carbon dioxide as a predominant component in the mobile phase, as demonstrated by the much smaller blue shift observed when carbon dioxide was replaced with methanol under otherwise identical mobile-phase conditions. The trends in the data can be explained as the result of the dielectric constants of the media surrounding the analyte molecules. Methanol is a highly polar solvent with a high dielectric constant of 32.6 at 20 °C (28). In contrast, supercritical carbon dioxide with a small dipole moment has a small dielectric constant of approximately 1.45 at 40 °C (29). Kadum and colleagues (30), who explored the effects of nonpolar and polar solvent on the $\pi \rightarrow \pi^*$ transitions of benzaldehyde and acetophenone (solutes with a similar chromaphore to the synthetic cathinones), reported that the $\pi \rightarrow \pi^*$ transitions shifted to longer wavelengths with increasing solvent dielectric constant.

In contrast to conventional UV detection for supercritical fluid or liquid-phase separation techniques, VUV detection examines enhanced $\pi \rightarrow \pi^*$ as well as $\sigma \rightarrow \sigma^*$ transitions for gas-phase separations. Therefore, the latter technique is not only capable of distinguishing between positional isomers where substitution occurs on the benzene ring, but also of distinguishing between positional isomers of synthetic cathinones where substitution also occurs on the aliphatic portion of the molecule (31). As shown in Figure 4, all of the eight *m/z* 191 positional isomers, in contrast to UV detection, yield unique VUV spectra, including 4-methylethcathinone and 4-methylbuphedrone, which differ in substitution on the aliphatic portion of the molecule. It should be noted (see Figure 5) that two of the solutes, 4-methylbuphedrone and 2,3-dimethylmethcathinone, are coeluted in the GC separation. A nice feature of VUV detection is the ability to deconvolute coeluted peaks and obtain the subsequent individual VUV spectra (32). Therefore, another viable approach for the analysis of emerging drugs would be to use UHPSFC

with MS/MS or QTOF detection and GC–VUV detection. This complementary detection scheme would provide molecular mass information, diagnostic fragmentation, information about the class or subclass of emerging drugs, and the ability to distinguish between positional isomers. In addition, similar to UV detection, VUV detection is excellent for quantitative analysis (31). Unlike flame ionization detection for GC, which is also excellent for quantitative analysis, VUV detection provides qualitative information besides retention time, including possible coelution (31).

Conclusion

The ability to distinguish between emerging drugs using chromatographic techniques with various detection schemes has been recently enhanced by emerging techniques such as UHPSFC and VUV detection. These approaches are particularly useful for distinguishing between positional isomers. For the identification of regioisomers, the coupling of GC with VUV and MS would be useful.

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Ira S. Lurie and Walter F. Rowe are with the Department of Forensic Sciences at George Washington University in Washington, D.C. Lauriane Tremeau-Cayel is an Associate Scientist 11 with Cayman Chemical in Ann Arbor, Michigan. Direct correspondence about this article to: islurie@email.gwu.edu ■

PRODUCTS & RESOURCES

Dynamic headspace analyzer

The Micro-Chamber dynamic headspace analyzer from Markes International is designed for the rapid, method-compliant screening of chemicals emitted from a wide variety of products, materials, and foodstuffs. The compact, stand-alone unit reportedly can be used for applications in the automotive, building products, consumer goods, and food and beverage industries.
Markes International, Llantrisant, UK.
http://chem.markes.com/UCTE

Preparative system

The Quattro countercurrent and centrifugal partition chromatograph and extractor from AECS-QuikPrep is designed to work with and complement standard flash and HPLC laboratory and process instrumentation. According to the company, standard biphasic solvents, ionic liquids, liquid chiral selectors, and ion exchangers may all be used. **AECS-QuikPrep Ltd.,** London, UK.
www.quattroprep.com

GC–MS system

Shimadzu's GCMS-QP2020 high-sensitivity GC–MS system is designed with a multifunction ion source, high-speed scan control, and an ultrafast turbomolecular pump. According to the company, comprehensive databases and multiple sample introduction devices enable custom configurations for use in environmental, food, and forensics laboratories.
Shimadzu Scientific Instruments, Columbia, MD.
www.ssi.shimadzu.com

SEC–MALS detector for UHPLC

The µDAWN multiangle light-scattering detector from Wyatt Technology is designed to be coupled to any UHPLC system to determine absolute molecular weights and sizes of polymers, peptides, and proteins or other biopolymers directly, without column calibration or reference standards. The detector reportedly connects to the company's Optilab UT-rEX differential refractive index detector.
Wyatt Technology Corp., Santa Barbara, CA.
www.wyatt.com

LC purification systems

Agilent's InfinityLab LC purification systems consist of a range of analytical-scale and preparative-scale purification systems. According to the company, the systems comprise 11 modules plus accessories and software, allowing laboratories to tailor purification systems to meet their needs.
Agilent Technologies, Santa Clara, CA.
www.agilent.com/chem/livepreplc

Autosampler syringes

Two brand-specific syringes from Hamilton are designed for use with CTC PAL liquid chromatography autosampler systems. According to the company, the direct attachment design of the C-Line syringe needle to the barrel eliminates contact between the sample solvent and adhesive. The X-Type syringes reportedly feature near-zero carryover.
Hamilton Company, Reno, NV.
www.hamiltoncompany.com

Biopharmaceutical application note

An application note from Tosoh Bioscience titled "DAR Analysis of Antibody Drug Conjugates using a TSKgel HIC Column" describes the separation of unconjugated and drug conjugated trastuzumab samples with baseline resolution using a TSKgel Butyl-NPR column. According to the company, the baseline resolution enabled an easy integration and quantification of different drug payloads in ADC characterization.
Tosoh Bioscience, LLC, King of Prussia, PA. www.separations.us.tosohbioscience.com/HPLC_Columns/id-7010/TSKgel_Butyl-NPR

HPLC tubing tool

The Beta Tool-2 tubing tool from MicroSolv is designed for use in HPLC laboratories to straighten or bend stainless steel tubing for a tight fit while keeping the integrity of the tubing's internal diameter, which minimizes band broadening that occurs from kinked tubing. According to the company, the tool's snub nose allows for greater access to tubing that is located in tight spaces, such as inside instruments.
MicroSolv Technology Corp., Eatontown, NJ. www.mtc-usa.com

THE ESSENTIALS

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

Tips for Troubleshooting Liquid–Liquid Extractions

Liquid–liquid extraction (LLE) is the most widely used extraction technique for liquid samples. The information provided in this article focuses on LLE using a separatory funnel. Some of the potential issues that may require troubleshooting when using LLE include (1):

- Emulsion formation
- Analytes strongly adsorbing to particulates
- Analytes binding to high-molecular-weight compounds (protein–drug interactions)
- Mutual solubility of the two phases
- Potential for solvent mediated decomposition
- Method robustness and transfer issues because of manual processing

The formation of emulsions is at the top of the list because it is a very common problem. Let's focus on how to troubleshoot and avoid this issue, as well as offer an alternative extraction technique that precludes emulsion formation.

Emulsions commonly occur when a sample contains a high amount of surfactant-like compounds (that is, phospholipids, free fatty acids, triglycerides, proteins, and so on). These surfactant-like molecules are large and will have mutual solubility in the aqueous and organic solvents which results in the formation of an emulsion in a mid-zone between the two phases. This intermediate solubility in each of the two phases makes it difficult to quantitatively collect one phase or another. Furthermore, the emulsion can also trap some of the analyte of interest, leading to quantitative problems. Emulsions often occur with samples where the animal (or human) diet is high in fats. Thus, emulsions sometimes appear when passing from preclinical trials with animals on low-fat controlled diets to clinical trials with humans who may be on high-fat diets. This characteristic problem makes LLE a less dependable procedure if it is expected that the same extraction protocol will be used for both preclinical and clinical samples. If this problem is anticipated, it is worth trying high-fat samples during method development in addition to the standard test matrices.

There are a few tricks of the trade to either stop emulsions from forming in the first place or to disrupt them if they do form. As a general rule, it is easier to prevent emulsion formation than to break it after one has formed. The simplest way to prevent the formation of an emulsion is to gently swirl instead of shake the separatory funnel. By swirling the separatory funnel the agitation that can cause the emulsion to form is reduced, but the surface area of contact between the two phases is maintained to allow for extraction to occur.

Emulsions can be disrupted by the addition of brine or salt water, which increases the ionic strength of the aqueous layer and facilitates separation of the two phases by forcing the surfactant-like molecule to separate into one phase or the other—this technique is known as *salting out*.

The individual layers or emulsion can often be separated via filtration through a glass wool plug (to remove the emulsion) or a phase separation filter paper (to isolate a specific layer). Phase separation filter papers are highly silanized and, depending on the type of paper, will allow either the aqueous or organic phase to pass through and be isolated. Centrifugation of the separation can also be used to isolate the emulsion material in the residue of the centrifugation.

Addition of a small amount of a different organic solvent will adjust the solvent properties of the separation and can result in the surfactant-like molecule being solubilized in either the organic or aqueous layer to a greater extent, which breaks the emulsion.

Supported liquid extraction (SLE) is a technique that can be used for samples that are prone to emulsion formation (2). Analytes are separated based on differential solubility. The aqueous sample may be pretreated—for example, the pH can be adjusted so that the analytes are in a suitable form to be extracted into an organic solvent. Following this adjustment, the sample is applied to a solid support (often diatomaceous earth), which creates an interface for extraction. A small volume of water-immiscible organic solvent is subsequently passed over the matrix holding the aqueous layer and the analytes partition into the organic phase. The extraction solvent is allowed to percolate by gravity; sometimes a gentle vacuum or pressure is applied. Organic solvents that are commonly used include ethyl acetate, methyl *tert*-butyl ether (MTBE), dichloromethane, hexane, and mixtures thereof.

MORE ONLINE:

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