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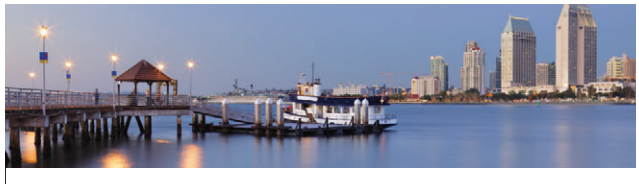

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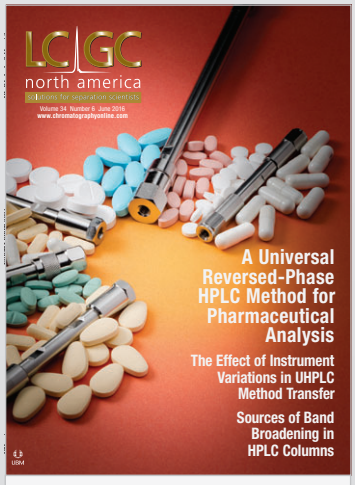

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The Main Causes of Laboratory Water Contamination and Strategies to Avoid Them
Anastasia Domanov, Millipore Sigma

GC–MS-MS-Based Multi-Residue Determination of Pesticides in Difficult Matrices
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JUNE 2016

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PEER-REVIEWED ARTICLE

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

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PEAKS of Interest

Deirdre Cabooter Joins LCGC's Editorial Advisory Boards

LCGC magazine is pleased to announce the addition of Deirdre Cabooter to the editorial advisory boards of *LCGC North America* and *LCGC Europe*.

Cabooter is an assistant professor in the Department of Pharmaceutical and Pharmacological Sciences at the University of Leuven in Belgium. Her work focuses on a broad range of topics in separation science, including the fundamental investigation of novel chromatographic supports, the comparison and evaluation of liquid chromatography techniques using the kinetic plot method, the development of generic automatable method development strategies for the analysis of complex samples, and the evaluation and application of new microfluidic devices for chromatographic separations.

In 2015, Cabooter became a member of the editorial board of *Journal of Chromatography A*. She has been nominated for the Csaba Horváth Young Scientist Award at the international Symposium on High Performance Liquid Phase Separations and Related Techniques (HPLC conference) in 2010, 2011, and 2014. She received her PhD in chemical engineering in 2009 from the Vrije Universiteit Brussel, in Brussels, Belgium.

Shimadzu Lab4You Accepting Applications

Following a successful launch last year, Shimadzu's Lab4You program is accepting applications from young and enthusiastic researchers hoping to advance their research.

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The Lab4You program emerged from Shimadzu's 2013 Laboratory World opening in Duisburg, Germany. The 1500 m^2 testing facility contains Shimadzu's entire product range and was used for customer demonstrations and application training.

Minimum requirements for candidates include an undergraduate degree with a science background, an approved topic of research (MSc or PhD thesis or postdoc research), and some practical experience in analytical chemistry.

Interested students can apply by submitting a short abstract of their research at www.shimadzu.eu/lab4you. The deadline for submission is October 31, 2016. ■

SURVEY SAYS...

LCGC DATA INTEGRITY POLL

Data integrity is currently a hot issue in regulated laboratories. *LCGC* ran a poll to ask readers who work in regulated laboratories what their firms are currently doing about it. As Figure 1 shows, more than two-thirds are taking significant steps. About 12% are doing nothing; when asked why, however, most said that their computerized systems are validated, their analytical instruments are qualified, and their staff are already trained. A few said they do not work in regulatory laboratories, so the issue does not pertain to them. Some readers—about 18%—indicated that they are just getting started looking into the issue.

Figure 1: What is your company or laboratory doing about data integrity?

Figure 2: If your company or laboratory is working on data integrity, where are you in the process?

We also asked those who are taking action where they are in the process. As shown in Figure 2, about 21% said that their management is leading the project. Another 40% are in the very early stages, assessing their systems and processes. The rest are split among steps from remediating systems, to having a policy in place with staff having received training. About a fifth of respondents said their firms already have a procedure in place.

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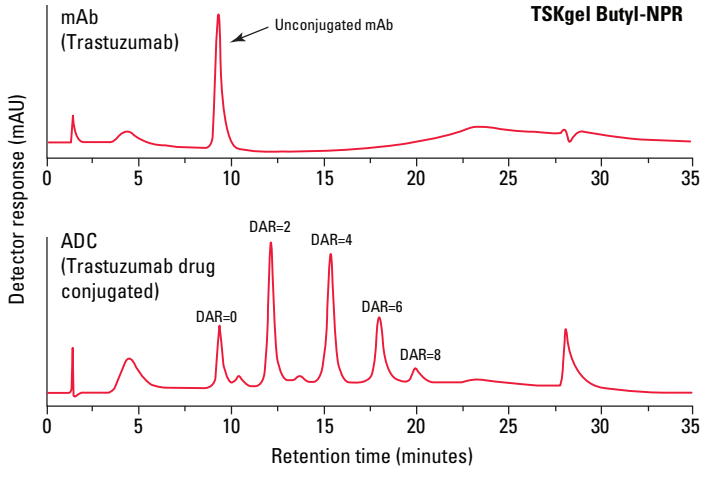

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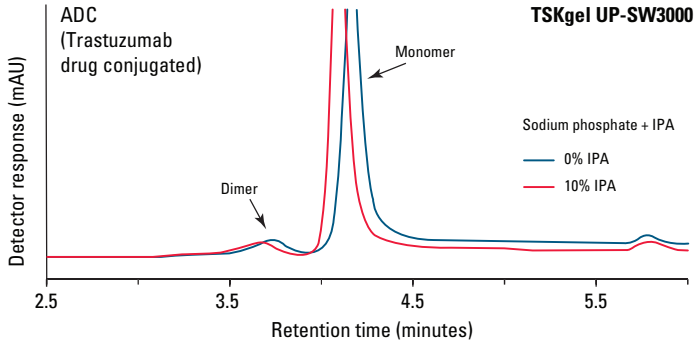

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A Q&A

Challenges & Benefits of Using SFC

A Q&A with Ted Szczerba, Technical Director of Chromatography and SFC Separations at Regis Technologies, Inc.

To learn more about the challenges and benefits of using supercritical fluid chromatography (SFC), *LC/GC* Magazine talked with Ted Szczerba, Technical Director of Chromatography and SFC Separations at Regis Technologies, Inc. With over 30 years' experience working with chiral compounds and over 10 years running SFC, Ted assessed the value of SFC versus the drawbacks of other techniques available in the field today.

LC/GC Magazine: How long have you been performing SFC analysis?

Ted Szczerba: I've been involved in LC for over 35 years and proud to say that all of those years have been spent with Regis Technologies. During that period, I've been exposed to a wide variety of LC methods and some very unique stationary phases, like the restricted access media (RAM) materials and immobilized artificial membranes (IAM). During the last 30 years, my main focus has primarily been chiral chromatography. I've been involved in SFC for roughly the past 10 years.

LC/GC Magazine: Why is SFC valuable?

Ted Szczerba: SFC is a mainly normal phase LC with hexane replaced by CO_2. The number one advantage is the speed of the analysis; the separations by SFC are typically four to five times faster than LC. Another advantage is solvent consumption. SFC uses very little solvent during the separation process due to the fact that CO_2 is one of the primary co-solvents in the mobile phase.

LC/GC Magazine: Can you describe a typical screening day?

Ted Szczerba: As everyone knows, chiral stationary phase columns are very expensive. Several years ago Regis started offering a free chiral screening service, probably one of the greatest sales tools that we came up with. Typically when a compound comes to Regis, we look at it both by LC as well as SFC. If someone is looking for a column or method, we typically perform just an LC screen. QC testing is mostly performed by LC, as LC methods are a little bit more stringent and accurate. If a compound comes in that is a candidate for a preparative separation, we use SFC. At Regis, we have four analytical LC systems and two analytical SFC systems. We usually require a minimum of 10 mg and a chiral screening document and as much information as we can get regarding safety, solubility, structures, etc. We look at a number of columns, typically around 12. The majority of those 12 columns are all manufactured and/or packed at Regis Technologies. If we happen to fail with those 12 columns, we do have a separate library of competitor columns that we also employ. I have the advantage that I can do what I want with these columns and I don't worry about destroying them. In a sense, however,

having this number of columns available is actually a drawback because the screening approach with 12-plus columns can be time consuming. From our experience at Regis, at the end of the day, 75 to 80 percent of all chiral separations can be performed on three of the most commonly used column phases (Whelk-O® 1, RegisPack™, and RegisCell™). But, you can't eliminate all these other phases because one of them, even if it has one chance out of 100, one of the other less commonly used column stationary phases might be the only one that can provide the required separation. So, in a way, at Regis having all these columns at our disposal is a luxury, but since multiple-column screens are time consuming, sometimes it's more of a hindrance than a help.

LC/GC Magazine: What is the ideal candidate for screening or prep separation analysis?

Ted Szczerba: The ideal candidate is highly soluble in a common solvent such as ethanol, IPA, methanol or acetonitrile. A separation can have all of the resolution in the world but if the compound is not soluble, chromatography will be a challenge. A chromophore for detectability is also required and a robust method.

LC/GC Magazine: What challenges do you have with SFC?

Ted Szczerba: With SFC there really aren't too many challenges aside from solubility. However, SFC is a closed system, so once you inject the sample, you really don't always know what's going on in that system. For example, if a compound has limited solubility in ethanol, it is difficult to determine if CO_2 helps or hinders the solubility. The only way you will know if solubility is an issue is if, after a number of injections, a high back pressure is observed indicating that the compound is precipitating out on the surface of the column.

LC/GC Magazine: What improvements are needed to improve the usefulness of SFC?

Ted Szczerba: First and foremost are improvements in the hardware to enable work at high pressures. Currently, high pressures are tough on both equipment and columns, and large columns are expensive. Some equipment has limited flow rate and pressure ranges, an issue using larger preparative columns. Consequently, improvements in both column hardware and equipment capable of handling higher flow rates and pressures are needed to make full use of SFC.

Regis Technologies, Inc., has been a trusted partner to expedite drugs to market for 60 years. They offer cGMP and non GMP synthesis and separations services to the pharmaceutical, biotechnology, and other industries worldwide. With over 30 years of manufacturing unique chiral stationary phases and nearly ten years of offering SFC separations services, they are an expert resource for chiral separations.

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COLUMN WATCH

Effects of Column Inner Diameter and Packed Bed Heterogeneities on Chromatographic Performance

In recent years industry has been moving to columns with smaller and smaller inner diameters—moving from 4.6 and 3.0 mm i.d. columns to 2.1 mm, 1.0 mm, and even smaller. While small inner diameter columns have some clear advantages, they also bring challenges. Reduction of extracolumn volumes must be given greater consideration by both customers and manufacturers. This installment of "Column Watch" focuses on the sources of band broadening within high performance liquid chromatography (HPLC) columns with an emphasis on eddy dispersion. The physical mechanisms of dispersion are discussed and a review of the current literature as it pertains to small inner diameter columns is presented.

Edward G. Franklin and Ty W. Kahler *are the guest authors of this installment.*
David S. Bell *is the editor of Column Watch.*

The observed efficiency of an isocratic chromatographic separation is a function of the column itself (that is, its intrinsic efficiency) and the band broadening contributions of the instrument (for example, the injection process, the connecting tubing, the detector volume, and so forth) (1). The volume variances of these elements are additive, as shown in equation 1:

$$\sigma^2_{vol,obs} = \sigma^2_{vol,col} + \sigma^2_{vol,ext} \quad [1]$$

where $\sigma^2_{vol,obs}$ is the volume variance of the observed peak, and $\sigma^2_{vol,col}$ and $\sigma^2_{vol,ext}$ are the variance contributions of the column and the extracolumn or instrument volumes, respectively.

Before the introduction of ultra-high-pressure liquid chromatography (UHPLC) and columns packed with sub-2-µm particles, extracolumn volume contributions to the measured widths of chromatographic peaks were rarely seen as problematic (2). For example, a 200 mm × 4.6 mm column packed with 5-µm fully porous particles might have an intrinsic efficiency of 20,000 plates under typical isocratic elution conditions. The peak volume of a lightly retained analyte (k' = 1) is approximately 132 µL. Using an instrument with even a relatively large variance contribution (50 μL^2) results in an observed efficiency loss of less than 5%. A 100 mm × 2.1 mm column packed with 2.5-µm fully porous particles would exhibit a similar intrinsic efficiency (N = 20,000), but the peak volume would be much smaller (<14 µL), and the variance contribution of the instrument would be detrimental; approximately 3800 plates would be observed. Fortunately, a generation of UHPLC instruments have been designed that address extracolumn volumes and limits system volume variances to 1–10 μL^2 (3). In a best case scenario, the previously described 100 mm × 2.1 mm column would exhibit approximately 92% of its intrinsic efficiency for a lightly retained analyte (k' = 1) and 98% for an analyte with a retention factor of 3. Figure 1 shows the extracolumn volume effects on the observed efficiencies of columns capable of generating 20,000 theoretical plates.

Instrument design continues to be pushed by advancements in column technologies. Ever smaller particles are consistently attractive because plate height (H) scales with particle diameter (d_p). However, the use of higher operating pressures, which are needed to realize those theoretical efficiency gains, results in frictional heating. Frictional heating can give rise to radial temperature gradients within the column that lead to decreased efficiency (4). Reducing the inner diameter of the column improves heat dissipation and also allows for the use of lower flow rates. This reduction of column diameter and flow rate is attractive from an economic perspective (that is, in the reduction of mobile phase solvent costs) as well as in clinical, forensic, environmental, and pharmaceutical settings where samples may be especially precious or limited. Reduced flow rates are also advantageous when attempting to improve the limits of detection (LOD) of methods that couple

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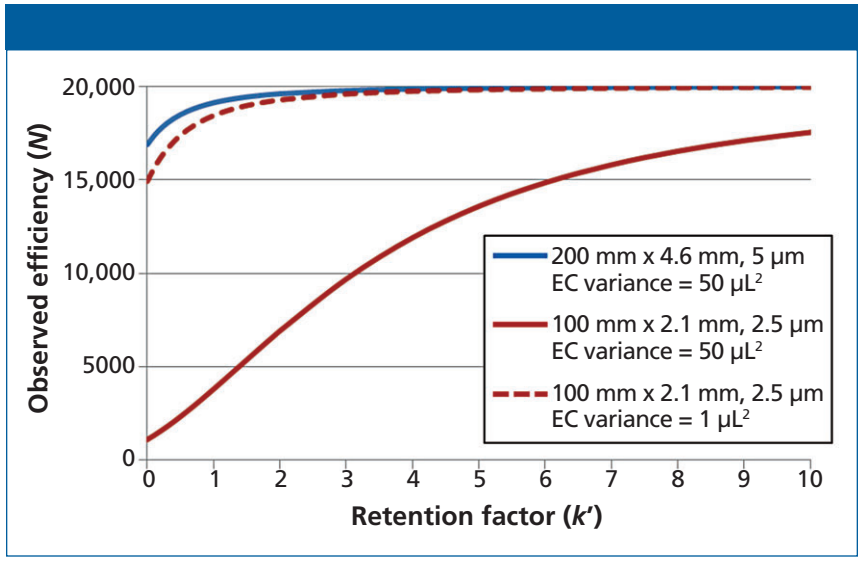

Figure 1: Observed column efficiency (*N*) as a function of retention factor (*k'*) for instruments with volume variance contributions of 1 and 50 µL^2.

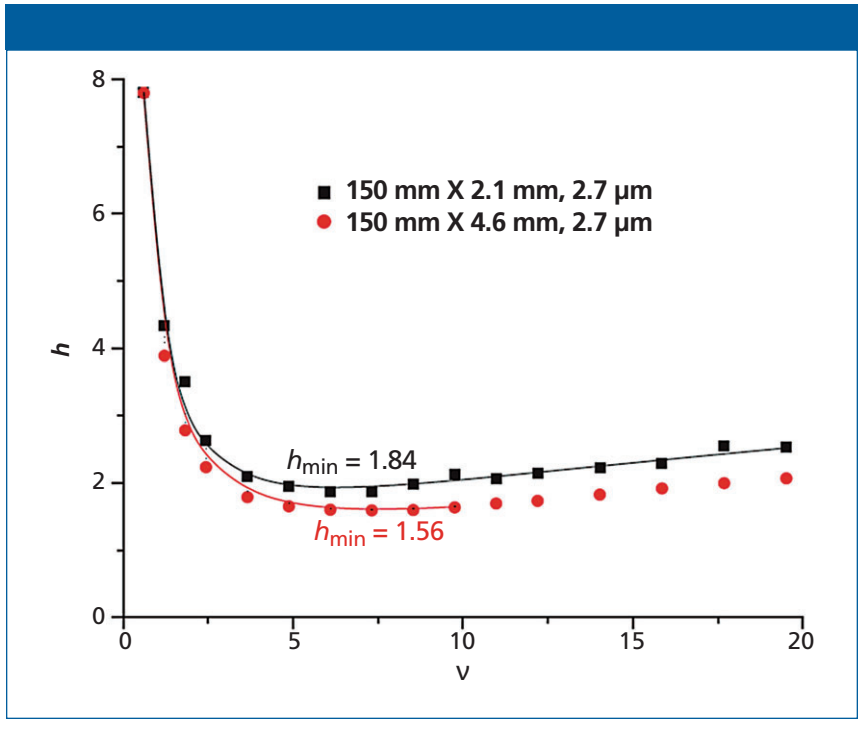

Figure 2: *h–v* curves of columns packed with 2.7-µm core–shell particles. The 4.6-mm i.d. column has a smaller minimum intrinsic plate height than the 2.1-mm i.d. column. (Adapted with permission from reference 10.)

high performance liquid chromatography (HPLC) to electrospray ionization mass spectrometry (ESI-MS) (5).

Chromatographic Band Broadening: The Column

Given what has been said about observable column efficiencies as a function of instrument dispersion, it can be seen how highly efficient, narrow-bore columns packed with small particles challenge the present state of instrument design. But now we turn our attention to the band broadening contributions of the column itself. The van Deemter equation is a classical expression of band broadening that groups the various contributions according to their dependencies on mobile-phase linear velocity, u_{avg}. It is commonly expressed in reduced terms so that meaningful comparisons between columns can be made, regardless of particle size:

$$h = a + \frac{b}{v} + cv \quad [2]$$

where h is the reduced plate height (H/d_p), v is the reduced velocity ($u_{avg} d_p/D_m$), and D_m is the bulk molecular diffusion coefficient. The a, b, and c terms are coefficients that describe the band broadening contributions from eddy dispersion, longitudinal diffusion, and resistance to mass transfer, respectively. By a first approximation, we might expect to achieve the same intrinsic efficiency and reduced plate height (for example, h = 1.8) with a 50 mm × 1.0 mm column packed with 1.25 µm particles (H = 2.25 µm; N = 22,000) as we would with a 200 mm × 4.6 mm column packed with 5-µm particles (H = 9 µm; N = 22,000) in 1/16 the time and with a fraction of the flow rate. However, this would be true only if the structures of the packed beds were conserved independently of particle size and column diameter such that each of the terms in equation 2 remained constant (6). A comparison of experimental *h–v* data collected using columns packed with several types of core–shell particles reveals that these values are often affected by column inner diameter (7–10). Without exception, the minimum intrinsic plate heights obtained when using 4.6-mm i.d. columns are smaller than those obtained when using 2.1-mm i.d. columns packed with identical particles, as exemplified by the data presented in Figure 2. Likewise, the slopes of the *h–v* curves in the mass transfer (*c* term) dominated regions are slightly larger for the 2.1-mm i.d. columns than for the 4.6-mm i.d. columns. The mechanisms of mass transfer alone cannot explain these observations (6). Let us therefore turn our attention to a more rigorous model of band broadening capable of providing additional insight.

The Giddings model describes plate height as a function of average mobile-phase linear velocity, and it couples transverse (that is, radial) diffusion of the analytes and fluctuations in linear velocity, which occur in different regions of the packed bed (11):

$$h = \frac{b}{v} + \sum_{i=1}^{3} \frac{2\lambda_i}{1 + \left(\frac{2\lambda_i}{\omega_i}\right) v^{-1}} + cv \quad [3]$$

The first and last terms describe longitudinal diffusion and mass transfer, respec-

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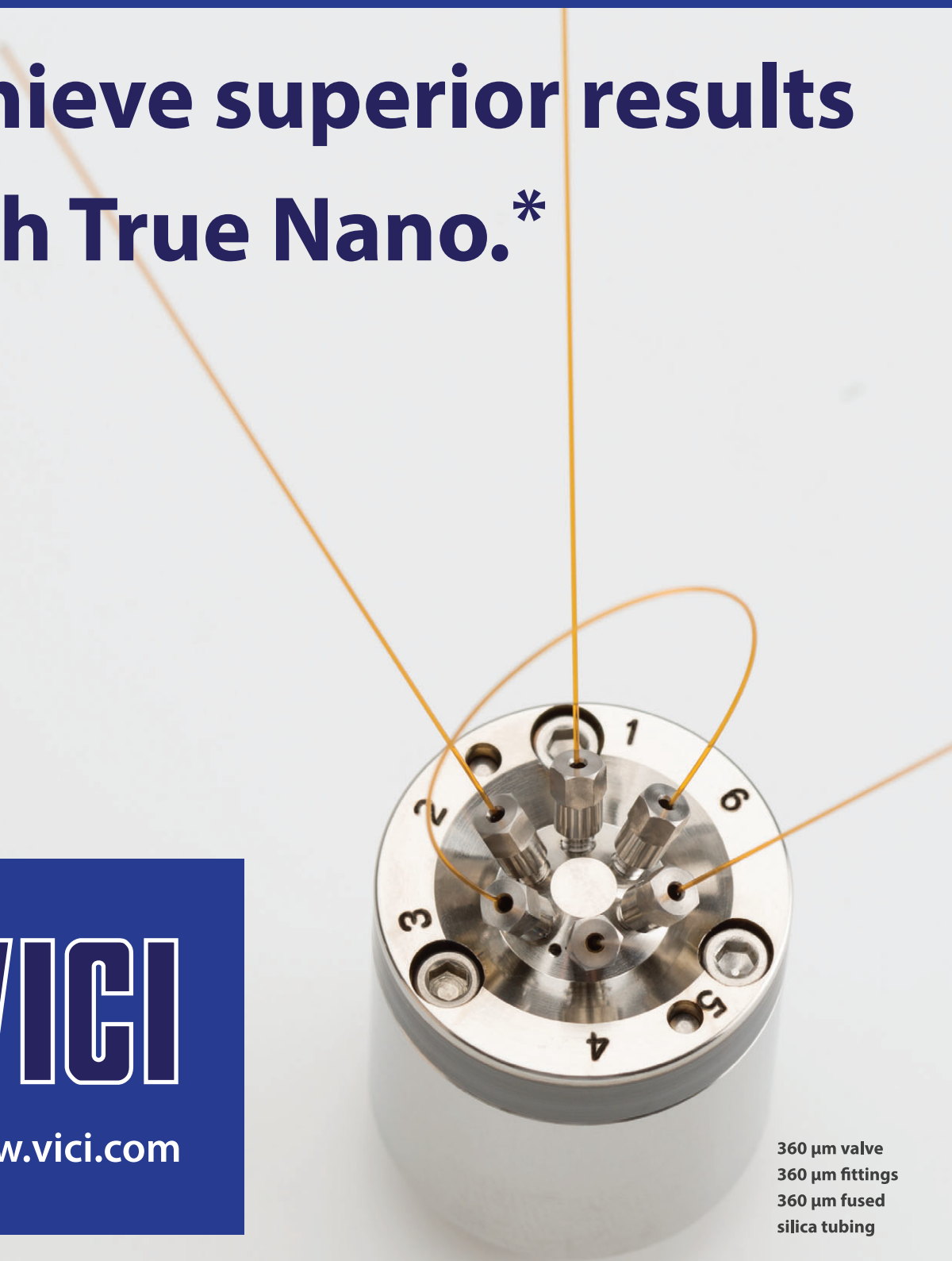

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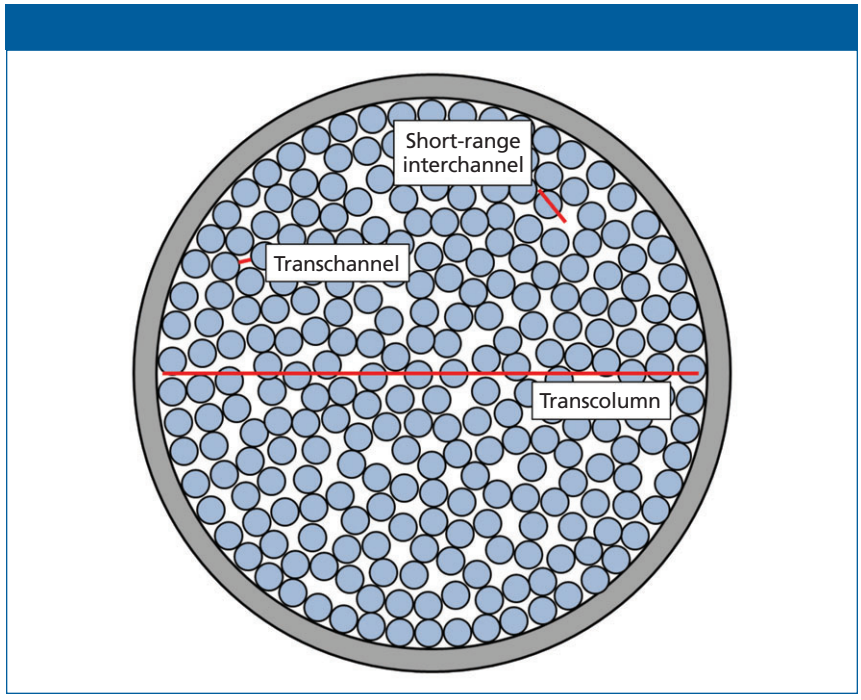

Figure 3: Cross-section of a particle-packed column showing the three categorical distances over which mobile phase velocity fluctuations occur. Each distance is related to an eddy dispersion term in the Giddings model to describe plate height: transchannel (i = 1), short-range interchannel (i = 2), and transcolumn (i = 3).

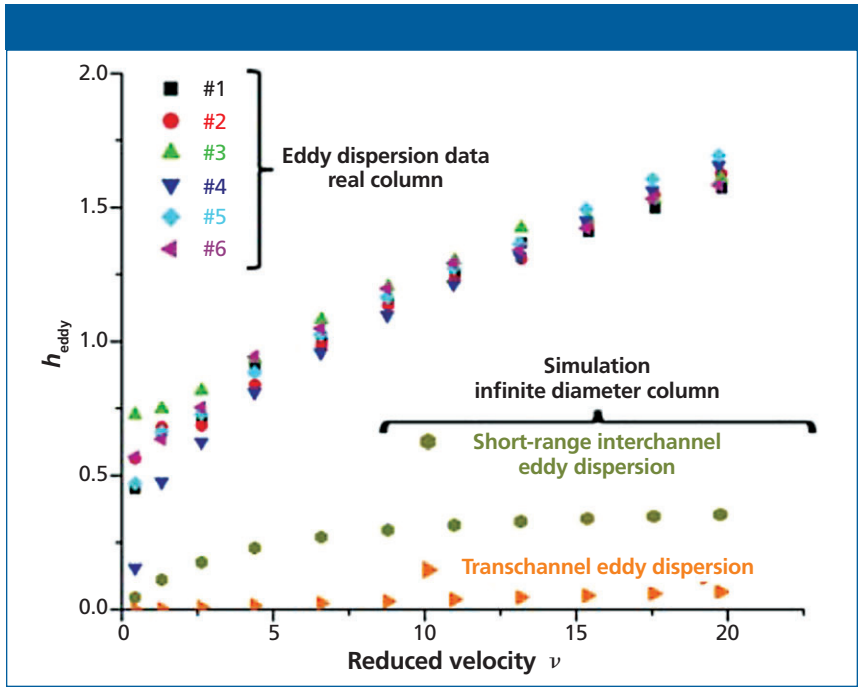

Figure 4: Real eddy dispersion data collected using 100 mm × 4.6 mm columns packed with 2.7-µm core–shell particles compared to dispersion data calculated for a simulated "infinite bed." The differences between the total eddy dispersion of the real data and the sums of the short-range interchannel and transchannel eddy dispersion contributions from the simulated infinite bed data are largely attributable to transcolumn dispersion, induced by wall effects. (Adapted with permission from reference 13.)

tively. The remaining terms characterize eddy dispersion on time scales consistent with three categorical distances within the column, as shown in Figure 3.

Unlike the van Deemter equation, eddy dispersion is presented here as velocity-dependent. λ_i and ω_i represent structural parameters of the packed bed characteristic of each eddy dispersion term. As such, this coupling model provides physical correlatives to the parameters involved in describing eddy dispersion. The transchannel contribution (i = 1) accounts for mobile phase velocity differences within the small, interstitial channels between neighboring particles. Although the channels in packed beds have very complicated geometries, a parabolic flow profile with the highest velocities occurring at the channel centers and the lowest velocities occurring at the particle interfaces can be envisioned. Short-range interchannel contributions (i = 2) describe the effects of velocity differences between channels separated by only a few particle diameters. These differences can arise because of small regions within the packed bed where particles group and pack more tightly than other particles in the immediate surrounding region. Finally, the transcolumn contribution (i = 3) characterizes the analyte band broadening that results from velocity differences existing between different regions of the column, such as between the center of the column and the column walls. These velocity differences are the result of radial heterogeneities in the packed bed which are largely attributable to the effects of the column walls (12).

The Column: Eddy Dispersion

The extent of analyte band broadening attributable to each of the eddy dispersion terms mentioned above (transchannel, short-range interchannel, and transcolumn) are characterized by their respective quantities λ_i and ω_i. Looking at equation 3, we can identify reduced velocities at which the plate height contributions of each term reach half their limiting values, after which the individual $h_{eddy,i}$–ν curves notably flatten. These are known as the transition velocities, $\nu_{1/2}$ (12):

$$\nu_{1/2} = \frac{2\lambda_i}{\omega_i} \quad [4]$$

Before the transition velocity, the magnitude of each term increases with linear velocity such that the contribution appears indistinguishable from the mass transfer term ($c\nu$). The transchannel and transcolumn contributions are characterized by very high transition velocities. Only the values of λ_2 and ω_2 are such that the coupling

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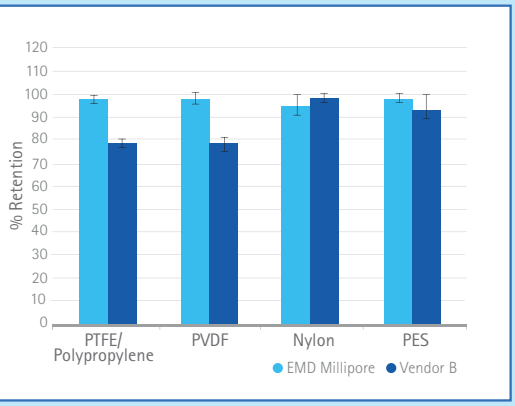

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characteristic of the short-range interchannel term remains relevant to the practical range of HPLC operation ($1 \leq v \leq 20$). This is why the van Deemter equation remains an accurate quantitative expression of plate height data even as it assigns incorrect relative plate height contributions to each of the *a*, *b*, and *c* terms. The physical and morphological characteristics of the packed bed that contribute to eddy dispersion are manifested in the *c* term (12,13).

Simulations of band broadening in computer-generated sphere packings allow for comparison of the relative contributions of transchannel, short-range interchannel, and transcolumn eddy dispersion to total eddy dispersion (12). By simulating an "infinite bed," total eddy dispersion is reduced to the sum of the transchannel and short-range interchannel terms. An experiment comparing real eddy dispersion data collected using 100 mm × 4.6 mm columns packed with 2.7-µm core–shell particles to simulated data generated from a corresponding "infinite bed" reveals the relative importance of the transcolumn contribution (13). As shown in Figure 4, the total eddy dispersion observed with the real columns is nearly three times higher than that of the simulated infinite bed, with the difference being mainly attributable to transcolumn dispersion. It is clear, then, that the efficiency of HPLC columns is largely affected by the confinement of the pack bed (that is, wall effects), which results in radial heterogeneity and a significantly large transcolumn dispersion term.

The Column: Wall Effects and Radial Heterogeneity

There are two types of wall effects that result in the radial heterogeneity of packed beds (14). The first is a geometrical effect occurring at the column wall. Particles abut the wall but cannot penetrate it. This leads to a highly ordered monolayer of particles along the wall. Subsequent layers are increasingly disordered to a distance of approximately 5 d_p from the column wall, after which a random packing arrangement is achieved (12). These differences in packing arrangements and densities along the column radius mean that there are different interstitial porosities at different points in the column. This leads to higher-than-average mobile-phase velocities at the wall regions compared to the center of the column. A chromatographic band broadens as analyte molecules sample these different velocity flow paths, and the extent of this broadening is characterized in the transcolumn eddy dispersion term. The second wall effect is manifested as a region of higher-than-average packing density occurring between 5 and 50 d_p from the column wall. The extent of this effect is dependent upon the conditions used to pack the column and is due to the combination of radial and axial stresses present during bed consolidation (14,15).

Column Inner Diameter: 4.6 and 2.1 mm

Keeping in mind the contributions to total eddy dispersion, let us return our attention to the relative performances of 4.6-mm i.d. and 2.1-mm i.d. columns packed with identical particles. As mentioned, 4.6-mm i.d. columns consistently exhibit lower intrinsic minimum plate heights and better performance at high mobile-phase linear velocities than their 2.1-mm i.d. counterparts (10). Gritti and Guiochon (13) attribute this to the respective heterogeneities of the packed beds (that is, differences between the respective transcolumn eddy dispersion terms). In other words, the empirically determined values λ_3 and ω_3 increase with decreasing column radius.

So far we have been concerned with the radial heterogeneity of packed beds and its effect on plate height. At this point, we should also mention a specific matter with regard to these column dimensions. When sample is introduced onto a column, it occupies a relatively narrow region at the center of the packed bed, characterized by the average flow velocity. As the sample migrates axially down the column, it also disperses radially outward toward the column walls (16). For columns with inner diameters ranging from 2.1 to 4.6 mm and lengths between 5 and 25 cm, lightly retained analytes often are eluted from the column in less time than it takes for analyte molecules to reach the column walls where mobile-phase flow velocity can be much different than it is at the center of the column. In such cases, there is an increasing total eddy dispersion contribution to plate height with decreasing column diameter. In addition to wall effects, border effects at the outlet of the column become increasingly important with decreasing inner diameter. With narrower inner diameter columns, analytes have a greater chance to reach the radial edge of the outlet frit where stagnant regions of mobile-phase eluent may be found. These outer flow streams must then distort and converge upon the narrow outlet aperture to exit the column, thus leading to increased band broadening and peak tailing (13). Axial and transverse dispersion in relatively short, wide columns is illustrated in Figure 5a.

Parallel segmented flow chromatography (PSFC) has been suggested as a means to improve the performance of columns that are relatively wide and short (17–19). With this technology, a column is equipped with an outlet fitting that features a num-

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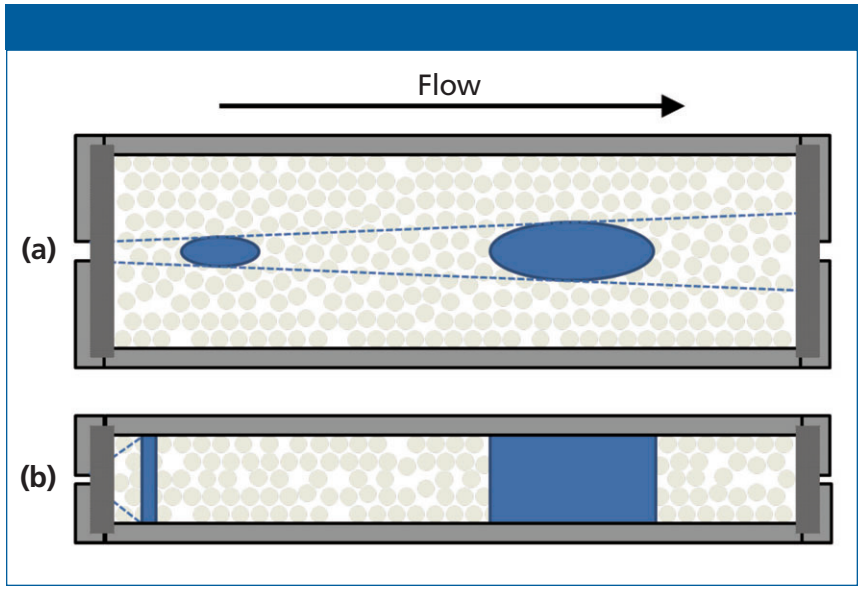

Figure 5: Representations of axial and transverse dispersion of an analyte zone within a column. (a) In short and wide columns, lightly retained analytes often are eluted from the column in less time than it takes for analyte molecules to reach the column walls. As the column inner diameter decreases, wall and border effects become increasingly important. (b) In capillary columns, the retention times of analytes are much longer than the time it takes for analyte molecules to sample the column diameter many times. Radial heterogeneities induced by wall effects are a dominating factor in column efficiency (13).

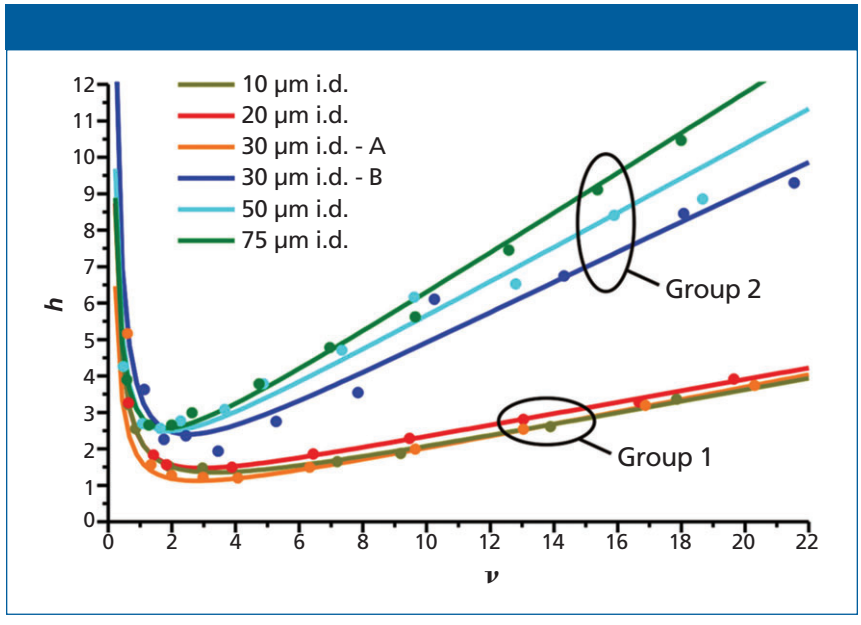

Figure 6: *h*–*v* curves of different inner diameter capillary columns packed with 1.7-µm fully porous particles. Confocal scanning laser microscopy (CLSM) data reveals the extent of radial heterogeneity for the poorer performing columns in Group 1 to be greater than the heterogeneities of columns in Group 2. (Adapted with permission from reference 23.)

ber of peripheral exit ports and a single central exit port. Mobile phase and analytes exiting through the central port are sent on for detection, while flow streams from along the walls of the column are collected and discarded through the peripheral ports. Improvements are realized if, for instance, the average mobile phase velocity at the center of the column is higher than that at the wall regions (see the second wall effect described above). In such a case, elimination of the peripheral flow streams can increase the observed efficiencies and eliminate tailing. PSFC has been shown to dramatically improve the performance of 30 mm × 4.6 mm columns by reducing the combined influence of wall and border effects. In longer, narrower columns, analyte molecules have enough time before elution to reach the column wall and to statistically sample all of the available flow path velocities. There are no improvements to observed efficiencies when using PSFC in such cases.

Column Inner Diameter: Capillary Columns

As the diameter of the column narrows into the capillary range, the elution times of analytes become much longer relative to the times needed for analyte molecules to radially disperse to the column walls (13). The analyte molecules are thus able to sample the different velocity flow paths (induced by heterogeneities in the packed beds) many times. In such cases, wall effects are a dominating factor in column efficiency. This situation is illustrated in Figure 5b. Bruns and colleagues (20) describe an experiment wherein 20-cm capillaries with inner diameters of 10, 20, 30, 50, and 75 µm were packed with 1.7-µm fully porous particles, and reduced plate heights as a function of linear velocity were observed. Representative segments of each packed bed were reconstructed through confocal scanning laser microscopy (CLSM), and radial interparticle porosity profiles were determined to quantify the extent of transcolumn heterogeneity. Capillary columns with larger inner diameters (that is, 30, 50, and 75 µm) were found to exhibit larger porosity fluctuations at the column walls before converging to the mean porosity values characterized by the random packing arrangements at the column centers. Analyte molecules experience more mobile-phase flow velocity differences across the diameter of the column, thus leading to high transcolumn dispersion and poorer chromatographic performance, as shown in Figure 6. Capillary columns with smaller inner diameters (that is, 10, 20, and 30 µm) were found to exhibit smaller radial porosity fluctuations, and thus performed better chromatographically. This latter result is partially explained through envisioning the lower aspect ratios of the column to particle diameters as substantially eliminating the bulk regions of the packings. In other words, packed-bed morphologies in very

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narrow capillaries are dictated almost entirely by wall effects and are therefore much more radially homogeneous (21,22).

At this point, it may be clear that reducing the radial heterogeneity of packed beds to reduce transcolumn dispersion is of special importance. Reising and colleagues (6) describe an experiment seeking to correlate column packing procedures, chromatographic performance, and the physical morphologies of packed beds as determined by CLSM. Nine 34 cm × 75 µm columns packed with the same batch of 1.3-µm fully porous particles using slurry concentrations ranging from 5 to 50 mg/mL were prepared. Examination of the physical morphologies revealed two competing effects of using increasingly concentrated particle slurries. Firstly, higher slurry concentrations effectively reduced the heterogeneities induced by the wall regions (6,23). Secondly, however, higher slurry concentrations resulted in a greater number of void regions within the packed bed. The effects of such voids can be manifold. Additional transchannel and short-range interparticle contributions to eddy dispersion are likely, and additional transcolumn contributions are also possible if the voids are preferentially located in the wall regions. Chromatographic testing showed the column prepared with the 20 mg/mL slurry to perform best while those prepared with lower and higher slurry concentrations exhibited higher minimum plate heights and larger slopes in the *h–v* curves. There exists, then, an optimum intermediate slurry concentration where all other column packing conditions are more or less constant. This work suggests the possibility of improved chromatographic packings through the use of packing conditions that minimize wall effects and reduce radial heterogeneity while also avoiding the formation of larger voids (24).

Summary

Intrinsic column performance as a function of column inner diameter has been observed and investigated by a number of practitioners and researchers, and the morphological structures of packed beds have received particular attention. The radial heterogeneity of a packed bed, specifically the differences between the packing structures at the column walls and the center of the column, is a key contributor to chromatographic band broadening. The arrangement of particles at the column walls are often such that the interstitial porosities in those regions are higher than the porosities of the packing arrangements at the center of the column. This leads to mobile-phase flow velocity differences across the column diameter, which result in transcolumn eddy dispersion. It has been estimated that up to 70% of the total dispersion of a chromatographic peak is attributable to transcolumn eddy dispersion (13).

The effects of column radial heterogeneity (and border effects) become increasingly pronounced as column inner diameter decreases from 4.6 mm to 2.1 mm, as reflected in the diminished chromatographic performance of 2.1-mm i.d. columns (10,13). As column diameter narrows further into the capillary regime, wall-induced radial heterogeneities become more and more important because analyte molecules have sufficient opportunity to sample the column diameter (and the different velocity flow paths) many times before elution. Very narrow microcapillary columns have displayed excellent intrinsic efficiencies because, as the aspect ratio of the column to particle diameter decreases, packed-bed morphologies are almost entirely dictated by the column walls and are therefore more radially homogeneous (20–22).

Given the attractiveness of reduced solvent consumption and increased sensitivity that is associated with the use of smaller inner diameter columns, instrument manufacturers, column manufacturers, and users continue striving to improve the observed performances of such columns. The reduction of extracolumn volumes and the design of less dispersive sample injection and detection schemes remain relevant along with methodological approaches involving sample trapping and gradient elution. Parallel segmented flow has been used to operate 4.6-mm i.d. columns as virtual 2.1-mm i.d. columns by tuning the ratio of peripheral flow stream mobile phase that is discarded before detection. This approach has been shown to significantly improve observed efficiencies (25). In capillaries, the correlation of column packing parameters and chromatographic performance to the morphological structures of packed beds suggests possible means to improve the intrinsic efficiencies of columns by improving radial homogeneity and reducing transcolumn eddy dispersion (6). Such approaches may be applicable across a range of column inner diameters.

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Erratum

In the April 2016 installment of "Column Watch," there was an error in Table VI (SEC columns). The particle type in the Agilent AdvanceBio column is fully porous, not superficially porous.

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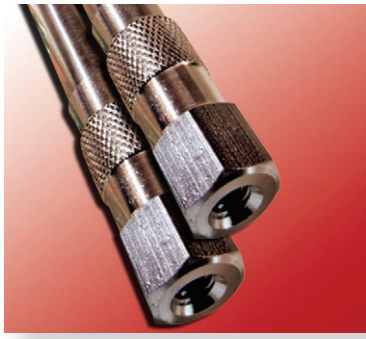

LC TROUBLESHOOTING

How Does It Work? Part II: Mixing and Degassing

Different techniques of liquid chromatography (LC) mobile-phase mixing can give different results and have different problems.

John W. Dolan
LC Troubleshooting Editor

In last month's "LC Troubleshooting" we reviewed the mechanics and operation of the most popular liquid chromatography (LC) pump designs (1). This month, we look at how the mobile phase is mixed and some potential problems that might be encountered. Most of us opt to have the LC instrument do the mobile-phase mixing for us, but we'll look briefly at manual mobile-phase preparation first.

The Human Touch

When isocratic separations are used, in which the mobile-phase concentration is constant, hand-mixed mobile phase is an option. In addition, many gradient methods require some hand-mixing of the mobile phase, such as when a buffer is used. The simplest and most accurate method of preparing a buffer is to weigh the appropriate amounts of the acidic and basic components, dilute them in high performance liquid chromatography (HPLC)-grade water, mix, and check the pH of an aliquot of this solution with a pH meter. Other techniques include preparing equimolar solutions of the acid and base and blending them until the desired pH is obtained, or preparing the base (or acid) at the desired concentration and titrating with a concentrated acid (or base) until the desired pH is obtained. The latter techniques are more error prone and require more steps than the gravimetric method.

Once the aqueous component of the mobile phase has been prepared, it is blended with the organic component (usually acetonitrile or methanol) to reach the desired aqueous–organic ratio. It should be noted that LC mobile phases are prepared on a volume-to-volume basis, not by the *quantum sufficit* (q.s.) method, where one component is added to the other to reach a specific volume. For example, when preparing 1 L of 40:60 water–methanol, 400 mL of water and 600 mL of methanol are combined to yield approximately 1 L of mobile phase. The alternative of adding a sufficient volume of methanol to 400 mL of water to yield 1.0 L would give a mixture of approximately 3.5% more methanol. This is illustrated in Figure 1, where the volume change on mixing is illustrated for mixtures of water with acetonitrile, tetrahydrofuran, or methanol (2). It can be seen in Figure 1 that at 60% methanol–water, the volume of the solution is ~3.5% less than would be expected with no volume change on mixing. Acetonitrile–water solutions lose only about half the volume of methanol–water.

On-Line Mixing Options

As mentioned above, most of us rely on on-line mixing for both isocratic and gradient LC methods. There are two on-line mixing options that are popular with today's LC systems, as illustrated in Figure 2.

High-pressure mixing, as the name implies, relies on mixing the mobile-phase components on the high-pressure side of the pump. Figure 2a shows that this requires a separate pump for each solvent. In this case, one pump delivers the A-solvent (usually the aqueous component in reversed-phase separations), and the other pump delivers the

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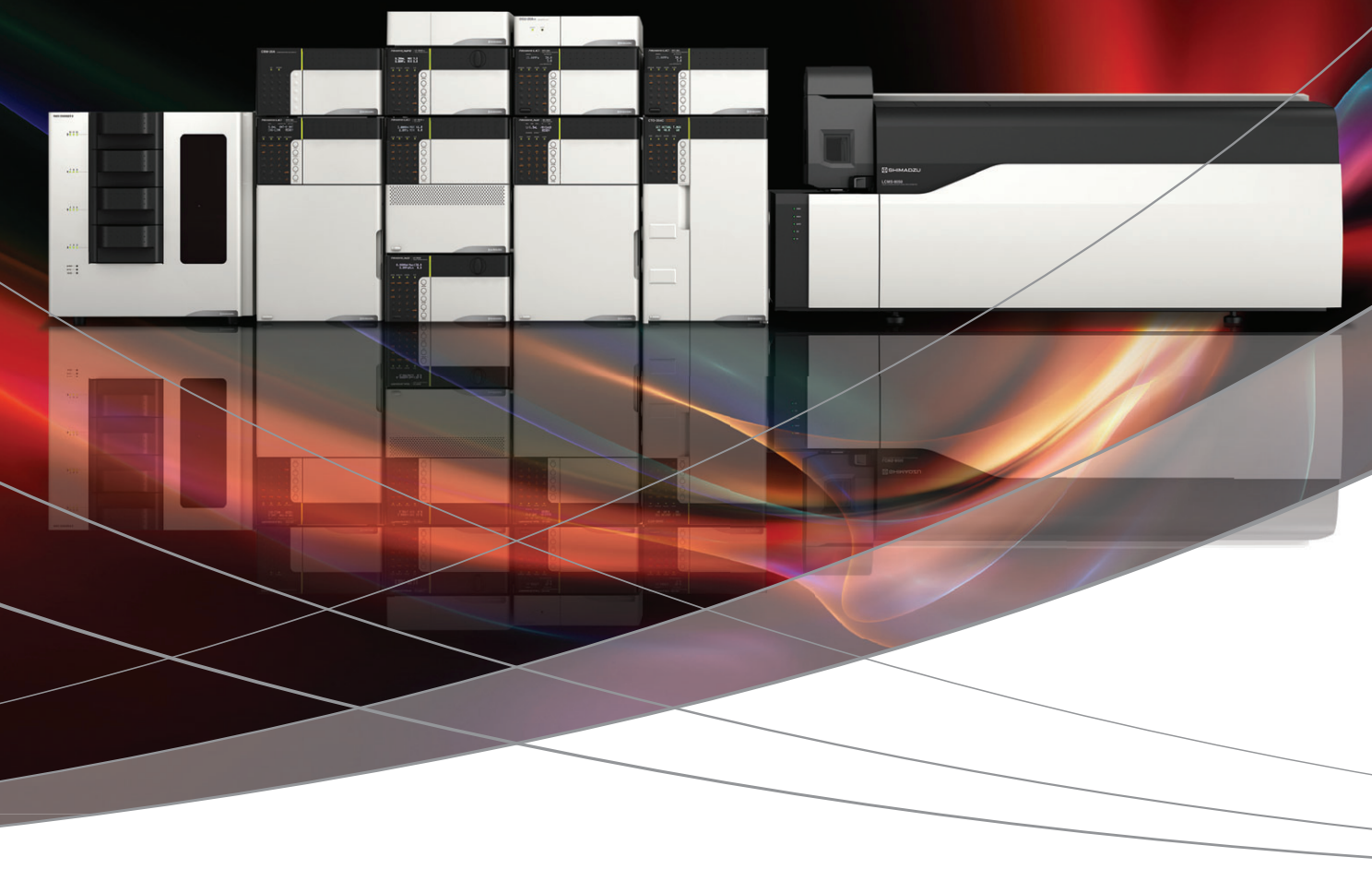

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B-solvent (organic—most commonly acetonitrile or methanol). The actual mobile-phase mixture is determined by the relative flow rates of the two pumps. For the above example of 60:40 methanol–aqueous example and a flow rate of 1 mL/min, the A-pump would pump at 0.4 mL/min and the B-pump at 0.6 mL/min. The solvents would be delivered to a mixer for blending and then to the injector and column. Note that in this case, the volume reduction mentioned above would also occur. So the flow rate would actually be ~3.5% lower, or ~0.965 mL/min. This, in turn, would increase retention times by 3.5%, so a nominally 5.00 min retention time would increase to 5.00/0.965 = 5.18 min. This change in retention would probably be of little concern, because changes in retention of ±0.1–0.2 min are not unreasonable when changing from one LC system to another or when replacing an old column with a new one, and retention would be constant for one system configuration. For gradient operation, the flow rates of the two pumps would change during the gradient to provide the desired gradient profile. For example, if a 5–95% gradient at 1 mL/min was required, the gradient would start with the A-pump running at 0.05 mL/min and the B-pump at 0.95 mL/min. As the gradient ran, the flow rate for the A-pump would increase and the B-pump would decrease. There would be a simultaneous change in mobile-phase volume (and thus flow rate) that would vary during the gradient according to Figure 1. It is possible to compensate for flow changes due to changes in mobile phase volume (or mobile phase compressibility) through special pump-control software. Such adjustments are likely of little practical consequence because any observed retention changes will be consistent for both reference standards and samples, so the changes would cancel each other.

The alternative form of mixing is low-pressure mixing. In this case, the mobile-phase components are blended prior to reaching the pump, on the low-pressure side of the pump, as illustrated in Figure 2b. Although Figure 2b shows only two solvents, typically low-pressure mixing systems are configured for four solvents (A, B, C, and D). This configuration offers more flexibility and less cost than a comparable high-pressure mixing system that would require four LC pumps for the same capability.

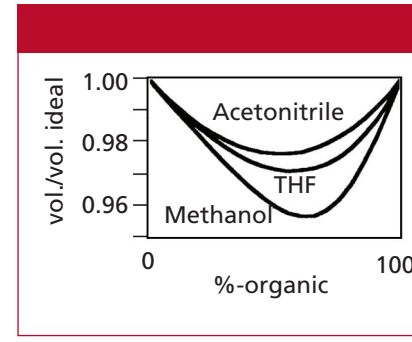

Figure 1: Volume change of solutions when water is mixed with acetonitrile, tetrahydrofuran (THF), or methanol. Volume relative to the ideal volume with no change on mixing. Adapted with permission from reference 2.

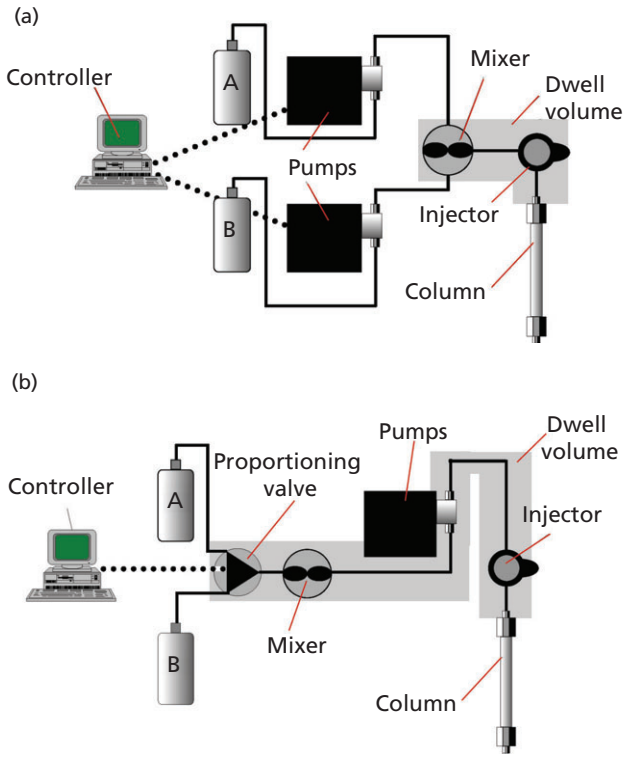

Figure 2: (a) LC system configuration for high-pressure mixing (detector not shown); (b) low-pressure mixing (proportioning valve and mixer are usually combined).

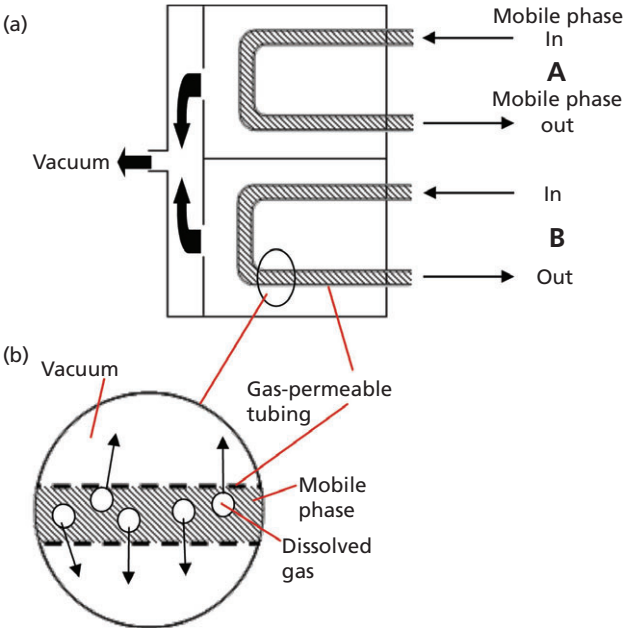

Figure 3: In-line membrane degasser. (a) Schematic for two-solvent degasser; (b) expansion showing gas-permeable membrane.

To prepare a 60:40 methanol–aqueous mobile phase delivered at 1 mL/min, the low-pressure mixing system would open the A-solvent proportioning valve for 40% of the time and the B-valve for 60% of the time (usually in short pulses), with the pump running at a constant 1 mL/min. Any time two solvents are mixed, the same volume change on mixing occurs, so the same 3.5% reduction in volume would occur here in the same way as was seen in the earlier examples. However, because the pump is pumping at 1.0 mL/min, the flow to the column would be 1.0 mL/min. You can see that under the same systems settings (same column, temperature, set flow rate, and so forth), the low-pressure mixing system would give slightly shorter retention times than the corresponding high-pressure mixer. However, as mentioned above, this small change is likely to go unnoticed because it is in the same scale as normal system-to-system or column-to-column variability. When a gradient is desired, the pump runs at a constant flow rate and the ratio of the cycle times of the proportioning valves gradually changes to deliver the proportion of solvents dictated by the gradient program.

If the mobile phase is supersaturated with air, any nucleation site in the system can cause outgassing.

Potential Problems

Let's look next at two potential problems that can occur when on-line mixing is used. These are mobile-phase outgassing and solvent proportioning errors.

Outgassing

When water is blended with acetonitrile or methanol, the resulting mixture has a lower capacity for dissolved air than the pure solvents. If the starting solvents are saturated with air, as is the case when they are equilibrated with the atmosphere, the mobile-phase mixture will become supersaturated with air and often outgassing will occur. If the mixture is supersaturated, any nucleation site in the system, such as a frit or the sharp edge of a tube end, can cause outgassing. Under high-pressure mixing conditions, the solvents are mixed at pressures well above atmospheric pressure, so the excess gas tends to stay in solution. As a result, high-pressure mixing systems are less susceptible to pumping problems caused by outgassing than are low-pressure mixing systems. However, when the mobile phase exits the column, it will

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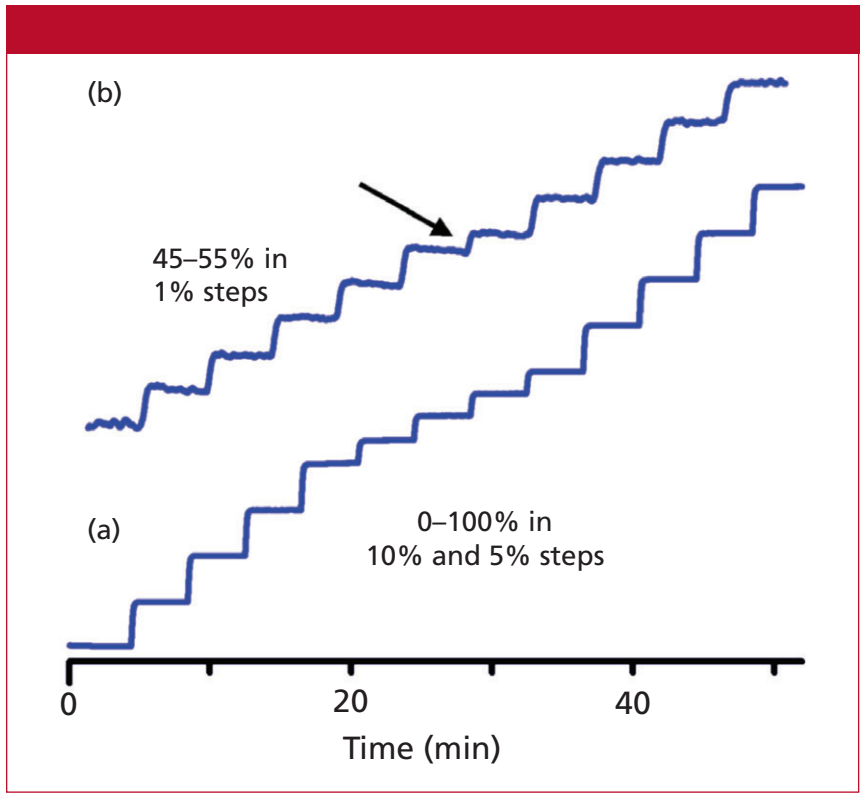

Figure 4: Results of a mobile-phase proportioning step-test. (a) Steps of 0%, 10%, 20%, 30%, 40%, 45%, 50%, 55%, 60%, 70%, 80%, 90%, and 100% B. (b) Expanded view of step-test with steps of 45–55% B in 1% increments; arrow shows deviation in 50–51% B transition. See text for details.

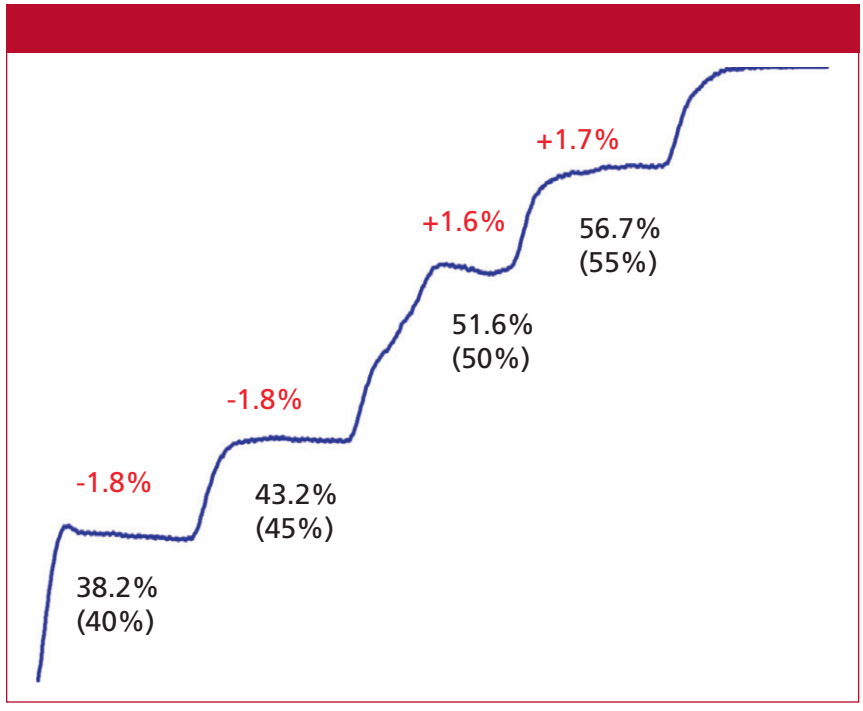

Figure 5: Failed step-test. Actual step values shown below steps with nominal (ideal) values in parentheses. Red values above steps show deviation from nominal.

return to (near) atmospheric pressure and often outgassing will occur, causing problems with air bubbles in the detector.

Low-pressure mixing systems blend the solvents before they reach the pump, and at or slightly below atmospheric pressure. Such conditions encourage solvent outgassing, and the resulting bubbles are likely to interfere with pump performance exhibited as pressure or flow pulsations. It is rare for low-pressure mixing systems to run reliably unless degassing is used.

Outgassing problems can be minimized by degassing the mobile-phase components. Historically, this was accomplished by helium-sparging the solvents in the mobile-phase reservoirs. Although helium-sparging is still the most effective degassing technique, helium is expensive and the process is inconvenient, so it has gone out of favor. Today most LC systems incorporate in-line degassers, which provide an adequate level of degassing for most applications. The operation of the in-line degasser is illustrated in Figure 3. The degasser relies on a gas-permeable membrane over which the solvent passes, and a partial vacuum on the other side of the membrane. Figure 3a shows a possible configuration for a two-solvent degasser (most accommodate four separate solvents). In this case, solvents flow through gas-permeable tubing that passes through a vacuum chamber. The inset (Figure 3b) illustrates that the gas in solution passes through the membrane because of the difference in pressure, but solvent is unable to penetrate the pores of the membrane. This is much like the function of a GoreTex rain jacket, where water runs off the surface, but the fabric is "breathable." The in-line, or membrane, degasser does not remove 100% of the dissolved gas, but it reduces the gas load of the solvents such that the mobile-phase mixtures are no longer saturated with dissolved air. In practice, the in-line degasser is placed so that the solvents pass through it before reaching the pumps in high-pressure mixing systems or before reaching the proportioning valves in low-pressure mixing systems.

It is the best practice to always use an in-line degasser (or other degassing technique) for reversed-phase LC operation. To give an idea of the effectiveness of these degassers at preventing problems, consider this

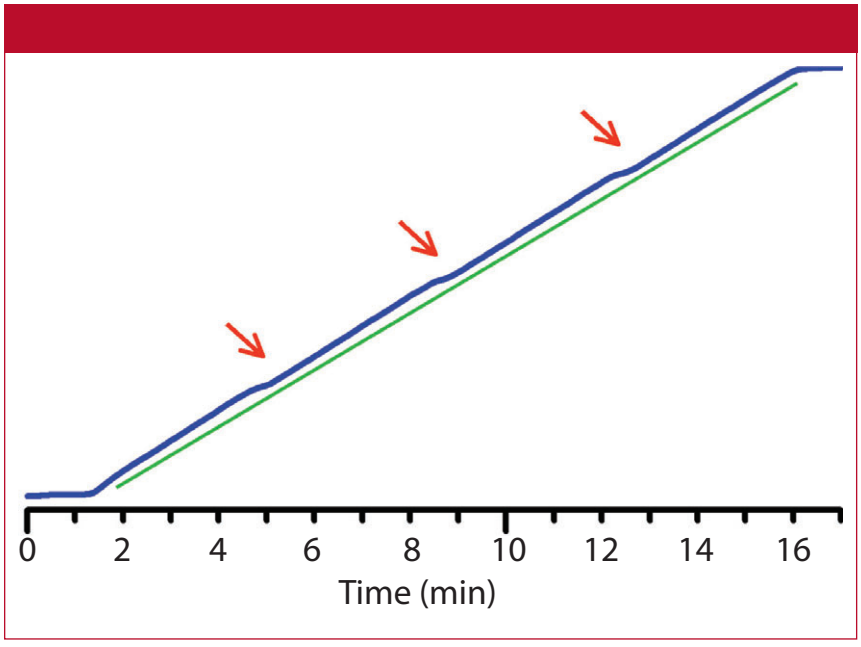

Figure 6: Errors in gradient linearity test at ~25%, ~50%, and ~75% B (arrows). Experimental gradient trace in blue; linear reference line in green. Corresponds to data of Figure 4. See text for details.

observation based on the inquiries I receive from readers of this column: Before in-line degassers were widely used, problems related to air bubbles in the system were easily the most common problems reported. Since the wide acceptance of these devices, I almost never get a question about a problem resulting from air bubbles in the LC system.

Solvent Proportioning Problems

On-line mixing is convenient, and when it works properly is very reliable. However, it is a good idea to check the mixing performance periodically. Often this is done as part of an annual preventive maintenance procedure for the instrument. The simplest way to check for proportioning errors is to use a step test. In this test, water is placed in the A-reservoir and water spiked with 0.1% acetone is placed in the B-reservoir. The column is replaced with a short length of capillary tubing, and an ultraviolet (UV) detector is set to 265 nm for strong response to the acetone in the B-solvent. A stair-step program is entered into the system controller. Typically, the program is 0–100% B in 10% steps; I like to add an extra step at 45% and 55% B to generate a little more information near the midpoint of the test. The flow rate is set to a typical operating condition, such as 1 mL/min. The time for each step is set to allow each step to stabilize before moving to the next step, for example, 1–2 min/step. I usually set a specification that each step should be within ±1% of the programmed value. An example of a satisfactory step test is shown in Figure 4a. The steps are even and within 1% of the setpoint in each case. Figure 5 shows an example of part of a failed step test. In this case, the steps at 40% and 45% are 1.8% low and the 50% and 55% steps are 1.6% and 1.7% high, respectively. In addition, the transition at the beginning of the 40% step has a little bump on it and the step from 45% to 50% is actually 8.4% and is distorted. The problems with step size and accuracy were most likely caused by errors in the proportioning-valve control program, whereas the distortions in step shape were probably due to bubbles or a malfunctioning check valve.

The same solvents can be used to test gradient linearity, as shown in Figure 6. Here, the overall gradient was linear (compare the blue experimental gradient line to the green reference line), but there was a small nonlinear portion at ~25%, 50%, and 75% B. Although this system passed the initial step test (shown in Figure 4a), a modified step test exposed the problem. When the step test was modified to change steps in 1% increments in the 20–30%, 45–55%, and 70–80% B regions, a discontinuity was seen, as illustrated in Figure 4b. Each step is the desired 1% in magnitude, except the transition from 50% to 51% B (arrow); similar results were seen for the 25–26% and 75–76% steps. Further troubleshooting isolated the problem to a single proportioning valve; see reference 3 for further discussion of this problem.

Summary

We have looked at the three common mobile-phase preparation techniques: hand mixing, high-pressure mixing, and low-pressure mixing. Each of these has advantages and disadvantages. We also considered potential problems related to solvent volume changes on mixing, mobile-phase outgassing, and solvent proportioning errors. Successful LC operation can be obtained with both low- and high-pressure mixing systems, but it is wise to always use degassed solvents and to periodically verify that the solvent proportioning system is working properly.

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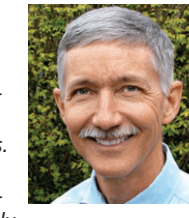

John W. Dolan

"LC Troubleshooting" Editor John Dolan has been writing "LC Troubleshooting" for LCGC *for more than 30 years. One of the industry's most respected professionals, John is currently the Vice President of and a principal instructor for LC Resources in Walnut Creek, California. He is also a member of* LCGC*'s editorial advisory board. Direct correspondence about this column via e-mail to LCGCedit@ubm.com*

PERSPECTIVES IN MODERN HPLC

A Universal Reversed-Phase HPLC Method for Pharmaceutical Analysis

A universal generic high performance liquid chromatography (HPLC) or ultrahigh-pressure liquid chromatography (UHPLC) method with a primary modern column that works well for most drug analyses in a few minutes would be an attractive idea for many laboratories. With advances in column technologies, this ideal scenario is becoming more realistic, as demonstrated in the proposed 2-min generic method shown here. In addition, rationales for the selection of column and operating conditions are discussed, together with ways to extend this generic method as a starting point for stability-indicating applications by simple adjustments of gradient time and range.

Michael W. Dong
Perspectives in Modern HPLC Editor

A generic high performance liquid chromatography (HPLC) method that works well for most small organic molecules is not a new idea. Most pharmaceutical laboratories use ballistic-gradient HPLC–ultraviolet (UV)–mass spectrometry (MS) methods for high-throughput screening (HTS), in-process control (IPC), and drug metabolism pharmacokinetics (DMPK) applications with gradient times (t_G) ranging from 1 to 10 min depending on resolution (R_s) requirements (1,2). For instance, a 2-min reversed-phase ultrahigh-pressure liquid chromatography (UHPLC) screening method using both acidic and basic mobile phases was routinely used for many years to support high-throughput purification in drug discovery (3). But how about extending this generic HTS–IPC approach further for multicomponent analysis or even purity assays? In 2012, a 10-min HPLC–UV cleaning verification method developed for a specific new chemical entity (NCE) was reported to work well for many drug candidates in a small-molecule portfolio with limits of quantitation down to 0.03–0.05 µg/mL (4). In 2013, the use of generic broad-gradient methods for purity analysis of raw materials, starting materials, and some active pharmaceutical ingredients (APIs) was proposed as part of a three-pronged template approach strategy for rapid HPLC method development in pharmaceutical development (5). In this installment, further refinements of this generic gradient method approach using the latest column technologies are proposed and exemplified in a 2-min HPLC assay for multiple NCEs. With the same column and mobile phase, higher peak capacities ($P_c \approx 200$) and resolution can be obtained by increasing t_G and gradient range–segments. Explanations for the selection of appropriate columns and operating conditions to maximize flexibility and compatibility with quality control (QC) applications are discussed together with ways this generic methodology can be extended for stability-indicating applications of more-complex drug molecules with simple adjustments of gradient conditions.

A Proposed Universal HPLC Method for Multiple NCEs

A 2-min generic gradient method amenable to the assays of multiple NCEs was proposed (6) and an example chromatogram on the separation of a test mixture of 12 NCEs is shown in Figure 1a. These NCEs were randomly selected and represented drug candidates with optimized drug-like properties and binding constants to different disease targets with diversified pK_a and log P values. The selection of the appropriate column, mobile phase, and operating conditions is discussed below. Note that the selection process and the final method parameters in this case study represented the end result, which balanced many analytical trade-offs (for example, analysis time, operating pressure, peak capacity, ease of use and ease of method transfers, and so forth) using available columns during laboratory evaluations. This example is used to illustrate the performance and usefulness of this generic gradient approach with modern columns under optimized operating conditions. It is expected that other similar choices on col-

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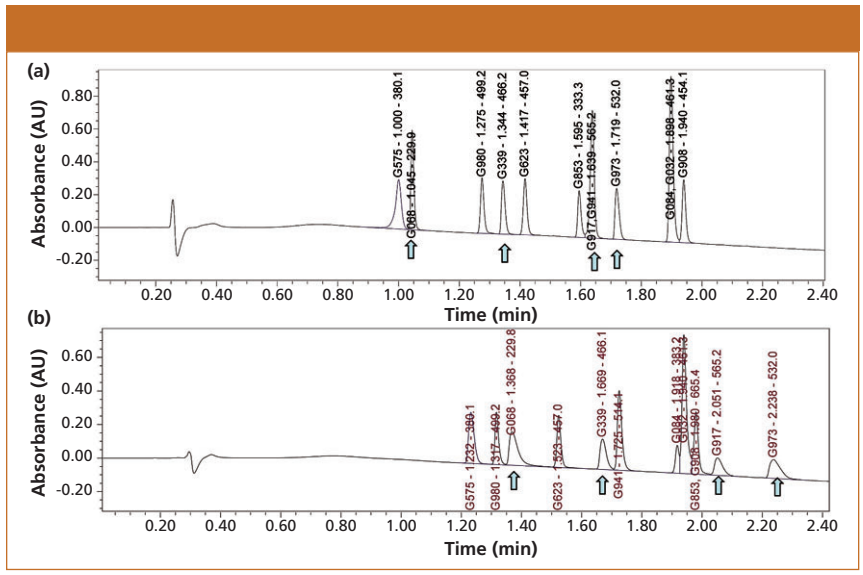

Figure 1: Chromatograms of multiple NCEs obtained using the proposed universal HPLC method (a) with a 2.7-µm SPP C18 column and (b) with a 1.7-µm C18 column. Column (a): 50 mm × 3.0 mm, 2.7-µm Waters Cortecs C18+; column (b): 50 mm × 3.0 mm, 1.7-µm Waters BEH C18; mobile-phase A: 0.05% formic acid; mobile-phase B: acetonitrile; gradient: 5–60% B in 2 min, 60–95% B in 0.5 min, 95–5% B in 0.1 min; flow rate: 1.0 mL/min; temperature: 40 °C; pressure: 3300 psi; detection: UV absorbance at 220 nm and MS (ESI+); system: Waters Acquity UHPLC equipped with a 425-µL peptide mapping mixer with diode-array and MS detectors; dwell volume: ~0.5 mL; sample: 1 µL of test mix of 12 NCEs at 50–100 ng/mL. Peaks are designated by code name, retention time (min) and M+1 parent ion. (Figure 1a is adapted with permission from reference 6.)

umn and operating conditions may yield comparable and equally acceptable results.

The Column

In this case study, the column selected (support type, bonded phase, particle size, column length, and inner diameter) is based on chromatography principles (1,2), scientific literature, laboratory evaluations, considerations for QC applications, and compatibility with both HPLC and UHPLC equipment.

The selection of a superficially porous particles (SPP) column such as the Waters Cortecs column was well justified because of the significantly higher efficiency performance (20–40%) versus those of their totally porous counterparts of the same particle diameter (7,8). The Cortecs C18+ bonded phase material was selected based on the better tailing factors produced for basic analytes (9,10). This C18+ phase has a positively charged surface, which yields superior peak shape performance (less tailing factors) for highly basic NCEs (with multiple positive charges under acidic

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pH, which are labeled with arrows in the chromatograms) when used with a low ionic strength mobile phase (for example, 0.05% formic acid) as shown in Figures 1a and 1b (11). Other column choices are discussed next.

A Cortecs C18+ column packed with sub-3-µm particles (d_p = 2.7 µm) was selected as the primary column to provide better compatibility with both HPLC and UHPLC equipment for easier method transfers in global manufacturing situations (2). The Cortecs 1.6-µm SPP column was not chosen because the pressure drop is threefold higher (1), even though it can deliver higher efficiency and resolution. The 1.6-µm column in a narrow-bore format (2.1-mm i.d) would be the better choice for HTS applications using exclusively UHPLC equipment at high linear velocities (3).

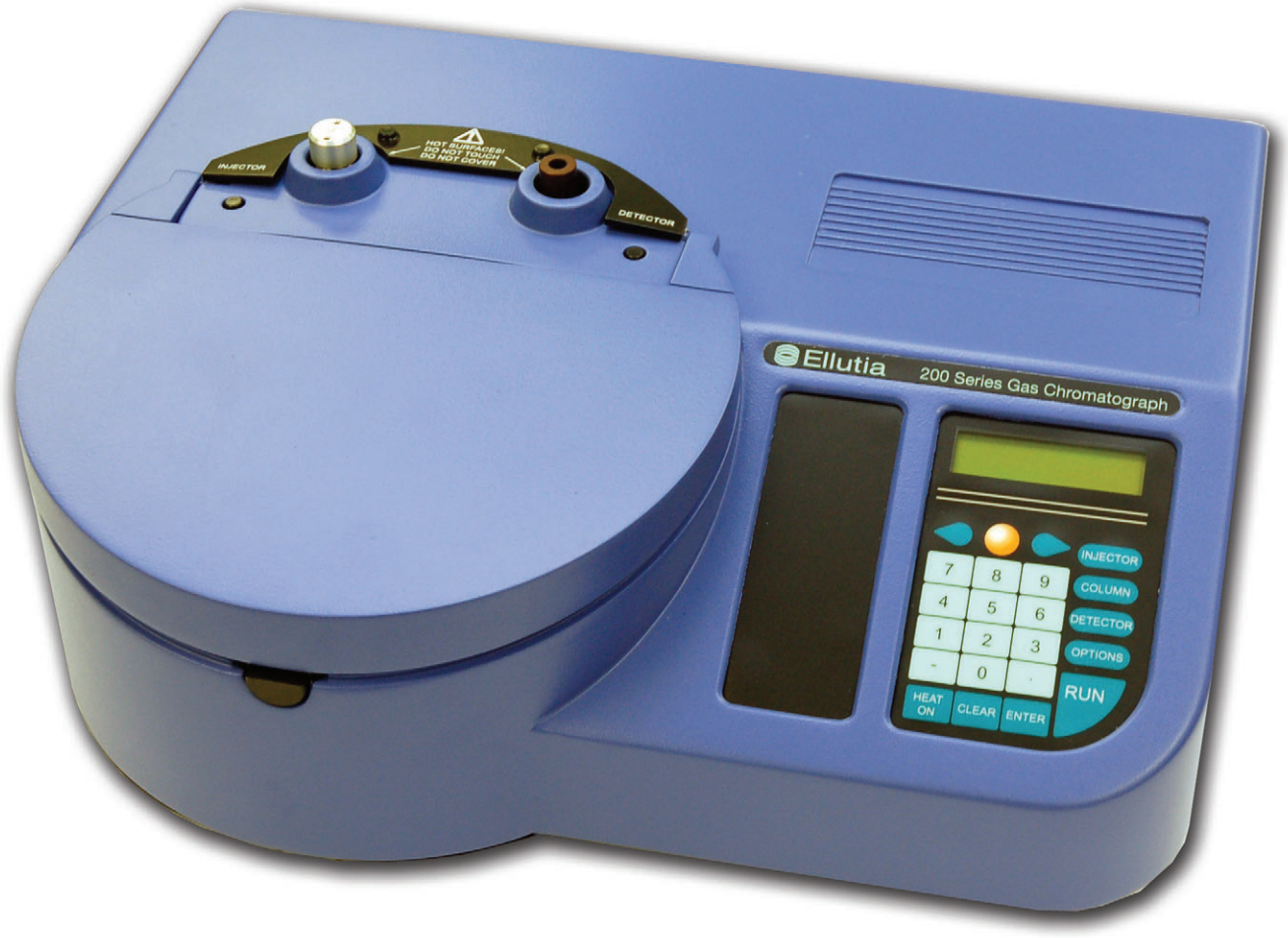
A short column length of 50 mm was selected for faster analysis. A short column allows excellent method flexibility for fast analysis and the ability of improving P_c by increasing t_G (12,13).

Figure 2 shows comparative chromatograms illustrating the increase of P_c from 100 to 300 by increasing t_G from 1 to 10 min. This simple approach of increasing t_G in conjunction with a narrower gradient range is an effective means to customize a generic method for stability-indicating assays, as demonstrated in the next section. The 3.0-mm i.d. format (rather than the more common UHPLC column format of 2.1 mm i.d.) was selected to yield better compatibility with HPLC equipment having higher system dispersion (1). The normal flow rates and injection volumes used for 3.0-mm i.d. columns are closer to those for standard 4.6-mm i.d. columns compared to those for 2.1-mm i.d. columns.

The Mobile Phase

A simple mobile-phase A of 0.05% formic acid was selected initially to permit easy mobile phase preparation in addition to its ability to support excellent mass spectrometry (MS) ionization efficiency (14). An alternate mobile-phase A of 20 mM ammonium formate with a higher ionic strength buffered at pH 3.7 would likely yield better peak shapes for most NCEs on a wider selection of bonded-phase columns (10,11). A buffered mobile phase may also be beneficial for critical separations that require tighter control of the mobile phase pH (14). Nevertheless, the simple formic acid-only mobile phase worked well, yielding good peak shapes for all NCEs tested with the recommended columns in this case study. An example chromatogram on the separation of the same 12-NCE test mixture by the primary column using this formate buffer is shown in Figure 3a. Example chromatograms showing results for the 12-NCE test mixture using the primary Cortecs C18+ column for mobile-phase A with formic acid and ammonium formate are shown in Figures 1a and 3a, respectively. Note that the chromatogram in Figure 3a shows analytes that have higher retention and different selectivity in comparison to those in Figure 1a because of higher mobile phase pH (pH 3.7 versus ~3.0) and ionic strength.

Acetonitrile, used as mobile-phase B, provides higher peak capacities and lower pressure drop than methanol because of acetonitrile's lower viscosity (1). Acetonitrile with 0.03% formic acid (v/v) should be used with 0.05% formic acid (v/v)



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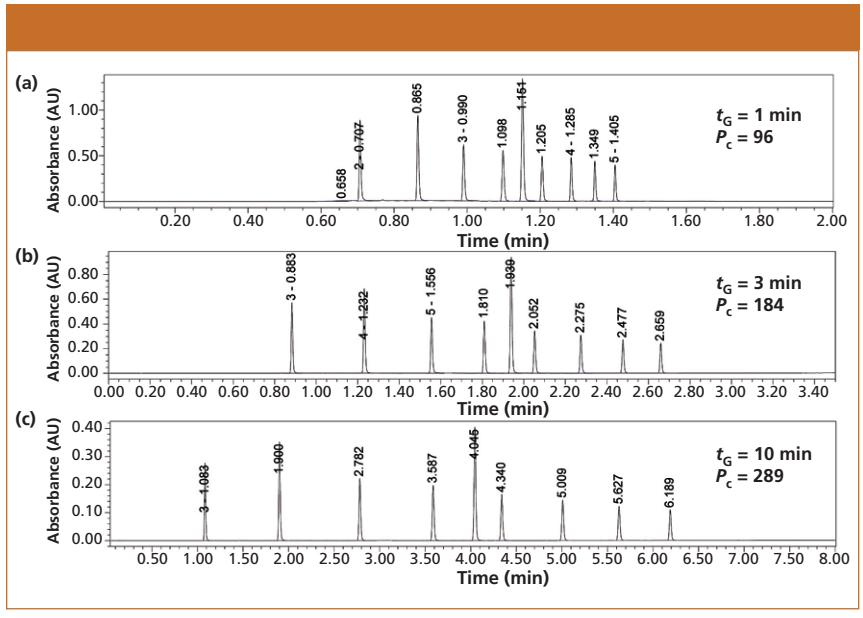

Figure 2: Comparative gradient UHPLC chromatograms obtained with gradient times of (a) 1 min, (b) 3 min, and (c) 10 min showing the effect on peak capacity. Column: 50 mm × 2.1 mm, 1.6-µm Cortecs C18+; mobile-phase A: 0.05% formic acid; mobile-phase B: acetonitrile; gradient: 30–100% B; flow rate: 1 mL/min; temperature: 40 °C; pressure: 12,000 psi; detection: UV at 254 nm; sample: a test mixture of nine alkylphenones. Peak capacities were measured by dividing the gradient time by the average measured base peak widths.

(balanced absorbance mobile-phases A and B) to provide a flatter baseline with UV detection at low wavelengths (such as <230 nm).

Gradient Conditions

A two-segment gradient program of 5–60% B and 60–95% B (rather than a single broad gradient of 5–95% acetonitrile) has been demonstrated to be more appropriate for most NCEs (4), which tend to be less hydrophobic and are typically designed according to the Lipinski's "rule of five" in drug discovery (15). A quick purging gradient is customarily needed to elute hydrophobic impurities such as dimers. A flow rate of 1.0 mL/min is optimum for this preferred column (2.7 µm, 50 mm × 3.0 mm) (14) and a column temperature of 30–40 °C is also quite standard. An initial operating pressure of 3300 psi is observed for this universal method, which renders it compatible with both HPLC and UHPLC equipment. UHPLC systems are preferable from the standpoints of lower dwell volume (shorter gradient delay times) and system dispersion (less extracolumn bandbroadening), leading to a faster sample turnaround time, taller and narrower peaks, and higher resolution (1,9).

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Table I: Data summary from the forced degradation study of the NCE

Number	Name	RT (min)	Base Peak	UV Max	t_0	6-day solution	6-day solid	6-day solution	6-day solution	3-h solution	3-h solution
					Fresh	20 °C	70 °C/100 RH	70 °C	Ambient Light	70 °C	70 °C
							Open Pan		Window Sill	0.1 N NaOH	0.1 N HCl
					% Area	% Area	% Area	% Area	% Area	% Area	% Area
1	M284	1.06	284.7	254.7							13.68
2	M293	1.16	293.1	251.8							70.31
3	M227	1.66	227.1	249.3	0.06	0.06	0.06		0.05	0.05	1.13
4	M516	3.01	516.1	210.3			0.10				
5	Cis	4.43	500.1	210.3	0.86	0.83	0.85	1.22	0.82	14.2	0.45
6	API	4.54	500.1	210.3	98.36	98.46	98.06	98.22	98.46	85.19	2.05
7	M516	4.68	516.1	210.3			0.13			0.10	
8	M265	4.81	265.1	210.3	0.44	0.43	0.43	0.44	0.44	0.38	
9	M338	6.48	338.3	246.9	0.04	0.04	0.10		0.04		0.07
10	M849	6.52	849.4	249.3	0.17	0.13	0.15	0.07	0.14	0.08	

Major degradation products are highlighted in orange colored boxes. t_0 = time zero or control, UV Max = λ_{max} in nm, RH =relative humidity, window sill = location placed to assess effect of light exposure.

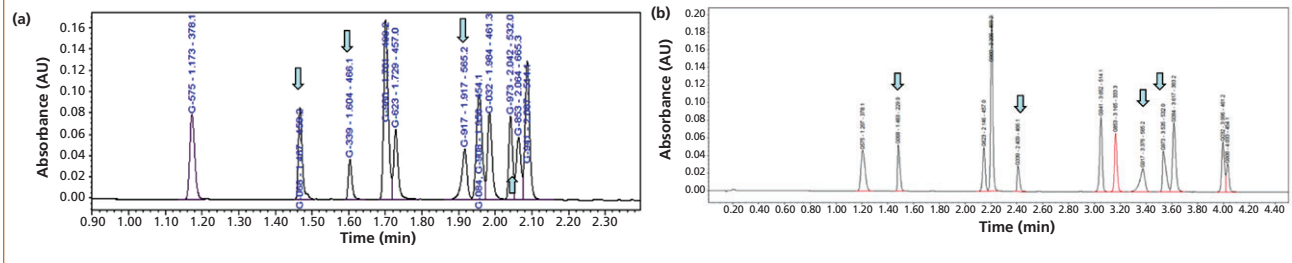

Figure 3: Chromatograms of the 12 NCE test mix obtained (a) using the same conditions as in Figure 1a but with 20 mM ammonium formate pH 3.7 as mobile-phase A and (b) after adjusting the gradient conditions. Conditions in (b): column: 50 mm × 2.1 mm, 2.7-µm Waters Cortecs C18+; mobile-phase A: 0.05% formic acid; mobile-phase B: acetonitrile; gradient: 5–40% B in 5 min, 40–95% B in 0.5 min; flow rate: 1.0 mL/min; temperature: 40 °C; pressure: 5300 psi; detection: UV absorbance at 254 nm and MS. Pressure in (a): 3700 psi. (Figure 3b is adapted with permission from reference 6.)

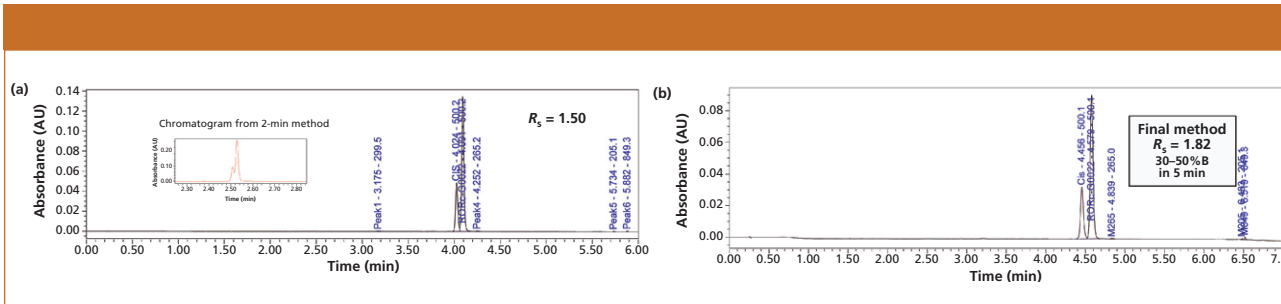

Figure 4: A case study on development of a stability-indicating method for an NCE with a cis isomer using the generic method approach: (a) Chromatogram of the NCE sample spiked with the cis isomer with a revised gradient program of 20–60% B in 5 min. The chromatogram in the inset shows the partial resolution (R_s = 0.8) of the two isomers using the 2-min generic method in Figure 1a. (b) Chromatogram of the final stability-indicating method with a total method development time of only 1 h. Column: 50 mm × 3.0 mm, 2.7-µm Waters Cortecs C18+; mobile-phase A: 0.05% formic acid; mobile-phase B: acetonitrile; gradient: 5–30% B in 0.5 min, 30–50% B in 5 min, 50–95% in 0.5 min; flow rate: 1.0 mL/min; temperature: 35 °C; pressure: 3700 psi; detection: UV absorbance at 254 nm and MS 150–999 amu ESI+.

Detection

A UV detection wavelength of 220 or 254 nm was typically used for this method and could be changed readily to match the maximum absorption wavelengths (λ_{max}) of the NCEs to maximize UV detection sensitivity (1,2). A typical MS scanning range of 150–1000 amu using electrospray ionization in the positive mode (ESI+) was found to work well for most small-molecule NCEs.

Resolving all NCEs in the Test Mixture Using a Modified Generic Method

As demonstrated by the chromatogram in Figure 1a, the proposed universal gradient method with a t_G of 2 min is capable of separating 10 peaks with excellent peak shapes in a sample test mixture of 12 NCEs. This fast, 2-min method may be adequate for potency assays for multiple NCEs or as a standard generic HPLC–UV–MS method for cleaning verification assay (4). As mentioned earlier, higher peak capacities are easily obtainable by increasing t_G and by adjusting the gradient range to separate any critical pairs in the sample. As was shown in the chromatogram in Figure 3b, all 12 NCEs could be near-baseline resolved with the same mobile phase and bonded phase used in Figure 1a by simple adjustments of a narrower gradient range and a longer t_G (5% to 40% B in 5 min) (5).

Can the Proposed Generic Method Be Modified for Stability-Indicating Assays?

This generic method appeared to serve well as a starting point for stability-indicating assays of many NCEs. To illustrate this approach, a challenging stability-indicating method was developed for an NCE with a cis-isomer (both an impurity and a degradant). The method development process using a sequence of method adjustments is shown in Figure 4. First, a particular NCE sample spiked with the cis-isomer was injected using the 2-min generic method shown in Figure 1a (5–60% B in 2 min) yielding a partial separation (R_s = 0.8) of the two isomers shown in the inset of Figure 4a. Next, a narrower gradient range of 20–60% B in 5 min was used to yield the chromatogram in Figure 4a showing R_s = 1.50 for the isomers. The chromatogram using final separation conditions (30–50% B in 5 min) is shown in Figure 4b, producing R_s = 1.82 for the isomers. This approach of maximizing the resolution around the main peak by keeping its elution near the end of a shallow gradient segment has been described elsewhere (5). A short first segment of 5–30% B in 0.5 min was added to retain any potential early-eluting impurities or degradation products. The entire method development process for this 6-min, three-segment gradient method took only 1 h in this case without the aid from any computer simulation software (1). Next, this 6-min method was successfully used to analyze a series of forced degradation samples to verify method specificity. This modified 6-min method separated all the impurities and degradation products (identified by their M+1 base peaks) and was subsequently used in a release testing of a pivotal API lot for toxicology evaluation.

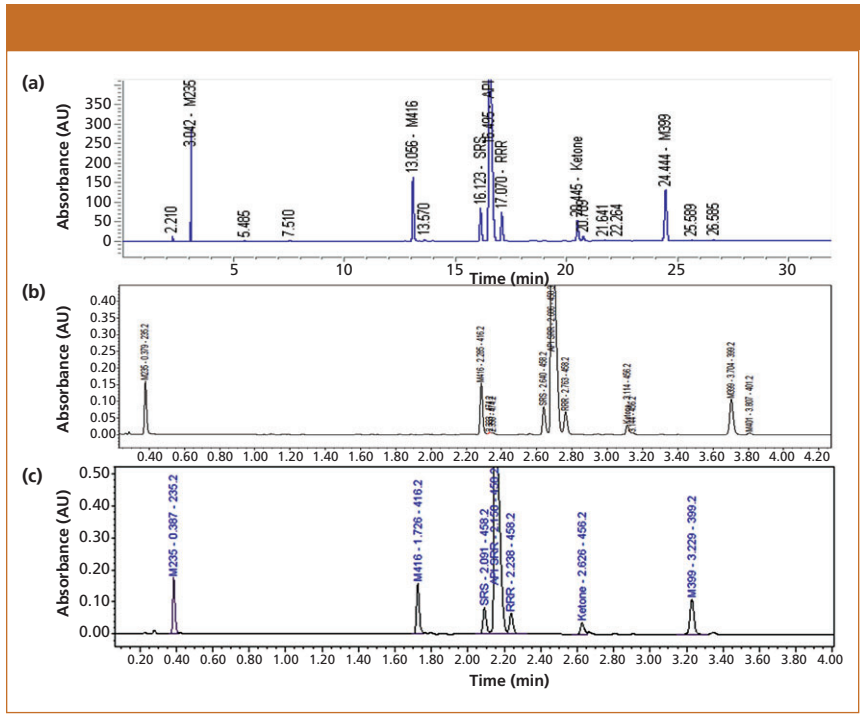

Figure 5: Chromatograms obtained using (a) the existing 42-min regulatory HPLC method for a complex NCE molecule with three chiral centers, (b) the 6-min, two-gradient segment method with the generic method approach using a Waters Cortecs C18+ column, and (c) the 6-min, two-gradient segment method with the generic method approach using an Agilent Poroshell HPH-C18 column. Conditions in (a): column: 150 mm × 4.6 mm, 3-µm ACE C18; mobile-phase A: 20 mM ammonium formate, pH 3.7; mobile-phase B: 0.05% formic acid in acetonitrile; gradient: 5–15% B in 5 min, 15–40% B in 25 min, 40–90% B in 10 min, 90% B in 3 min, 90–5% B in 0.1 min; flow rate: 1.0 mL/min; temperature: 30 °C; pressure: 3500 psi; detection: UV absorbance at 280 nm; sample: 10 µL of ~0.5-mg/mL API spiked with expected impurities. Conditions in (b): column: 50 mm × 3.0 mm, 2.7-µm Waters Cortecs C18+; gradient: 5–40% B in 4 min, 40–95% B in 1 min; temperature: 35 °C; sample: 2 µL of the retention marker solution; other conditions were the same as in (a). Conditions in (c): 50 mm × 3.0 mm, 2.7-µm Agilent Poroshell HPH-C18; pressure: 4000 psi; other conditions were the same as in (b). (The chromatogram in Figure 5a was adapted with permission from reference 16.)

The results from the forced degradation study (Table I) indicated that this NCE is relatively stable in solid form in the presence of heat and moisture, but it can degrade quickly in solution (50% acetonitrile in water), particularly at acidic or basic pHs.

This method development approach was also applied to a more complex drug molecule with three chiral centers. The performance of the existing 42-min regulatory HPLC method in the separation of a retention marker solution of the API spiked with expected impurities and degradation products (including the two expected diastereomers) is shown in Figure 5a and has been described in an earlier publication (16). The performance of a 5-min, two-segment gradient method developed using the generic method approach is shown in Figure 5b. The total method development (adjustment) process took about 1 h using the primary column with the same mobile phase A in the regulatory HPLC method (20 mM ammonium formate buffer at pH 3.7). The exact same method conditions were found to work well for a similar SPP column (50 mm × 3.0 mm, 2.7-µm Agilent Poroshell HPH-C18) as shown in Figure 5c.

An Alternate Column

Although the Cortecs C18+ 2.7 µm column worked well for the proposed 2-min generic method with acidic mobile phases, other sub-3-µm SPP columns from different manufacturers such as those from Advanced Chromatography Technologies (ACT), Agilent, Advanced Materials Technology (AMT), Supelco, Thermo Scientific, and Phenomenex may also be considered (7), although one may observe more peak tailing for very basic analytes when using low-ionic-strength mobile phases in some of the columns. Potential shortcomings of the Cortecs C18+ column are more peak tailing with acidic analytes and its incompatibility with high-pH mobile phases (1,17), which have the advantage of improving retention and peak shape for basic analytes, and are useful for the development of MS-compatible ICH-compliant purity methods for water-soluble basic drugs (17).

Potential alternative columns for high-pH mobile phase applications are the Waters CSH C18 (non-SPP charged surface hybrid) and Agilent Poroshell HPH-C18 (an SPP for high-pH applications) columns (18). Chromatograms demonstrating the performance of the Poroshell HPH-C18 column using a mobile-phase A consisting of 0.05% formic acid, formate buffer, and 0.05% ammonia are shown in Figures 6a–c. This column was also found to yield excellent peak shapes for very basic analytes with 0.05% formic acid mobile phase (Figure 6a) even though the bonded phase may not have a charged surface. Note that one serious issue for the use of high-pH mobile phases is the increased potential for analyte degradation as shown in Figure 6c (with one NCE [G-917] suffering severe degradation during analysis).

Strengths and Limitations of the Universal Gradient Method

The strengths of this modernized generic HPLC method are: its flexibility to provide good separations (P_c = 100–200); short run times (2–6 min); and good peak shapes for multiple NCEs. The generic method can be customized quickly for stability-indicating assays by simple adjustments of t_G and gradient range–segment. One limitation of the recommended 50-mm columns is their relatively low column efficiency (N = 12,000). Longer versions (100–150 mm) of these columns (capable of higher P_c of 200–400) are therefore more appropriate for the final regulatory stability-indicating methods for more-complex NCEs (19,20). Nevertheless, these short columns are excellent candidates for column and mobile phase screening studies and for purity assays of raw materials,

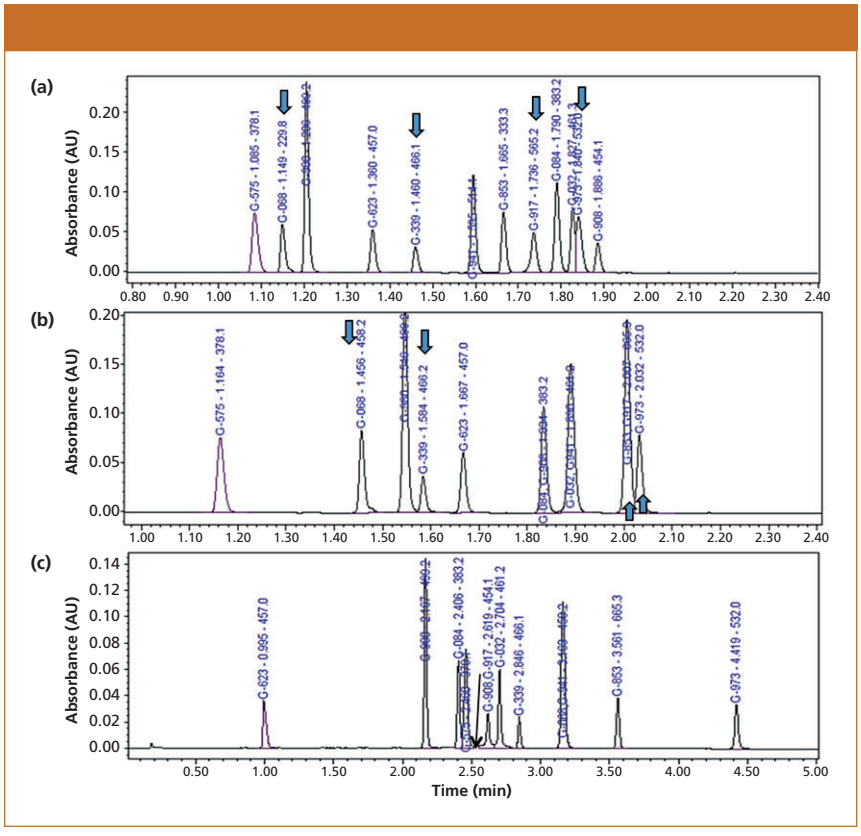

Figure 6: Chromatograms of the separation of the 12-NCE test mix obtained using the alternate column (50 mm × 3.0 mm, 2.7-µm Agilent Poroshell HPH-C18) and (a) the proposed generic method conditions with 0.05% formic acid mobile-phase A as in Figure 1a, (b) 20 mM formate buffer as mobile-phase A, and (c) 0.05% ammonia as mobile-phase A and a gradient program of 15–60% B in 5 min.

starting materials, and less complex NCEs (6,12,20).

Summary and Conclusion

A 2-min universal HPLC–UV–MS method for pharmaceutical analysis using modern SPP columns under optimized operating conditions is proposed. The advantages of this generic method are fast analysis, reasonable peak capacity (~100), and excellent peak shapes for many NCEs. This method may function as a general assay method for multiple NCEs and a standard method for cleaning verification. It also appears to serve well as a starting point for the development of stability-indicating assays of many drug molecules using a multigradient segment approach with a longer gradient time.

Acknowledgments

The authors would like to thank Naijun Wu of Celgene, Xiaotong Zhang of Allergan, Jacob Fairchild and Pam Iraneta of Waters Corporation, Hernan Fuertes of Gilead Sciences, Tom Waeghe of MAC-MOD Analytical, and Davy Guillarme and Szabolcs Fekete of the University Geneva for their editorial inputs and suggestions.

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Statistics for Analysts Who Hate Statistics, Part I: Collect and Examine Your Data

This new series of very short tutorials explains, in the simplest manner, how statistics can be both useful and accessible, even for chromatographers who normally find statistics difficult. Here in part I, we explain how to collect and examine your data.

Chromatographers drown under massive amounts of data. Modern separation methods produce multivariate data: either because numerous samples must be compared (because the operating parameters to optimize a method are numerous) or because many variables may explain the analytical result and must be identified, or even quantified. Statistical methods exploit and interpret these quantities of data in the most efficient and useful manner. Statistics may be used to select optimal operating conditions or to extract significant information from chromatographic data. Furthermore, statistics add value to your data because they provide information in the form of numbers and figures, which are helpful in conveying ideas.

One of the most useful tools I found to teach myself statistics was the papers written by Désiré Massart, Yvan Vander Heyden, and coworkers and published by *LCGC* in the early 2000s (1). However, while most chromatographers now need to learn about statistics, not all of them are willing to take the time to learn the mathematics behind the methods. There are several software programs available that make statistics much easier to practice, without the need to understand all the background mathematics.

My goal in this series of short tutorials is to show you, in the simplest manner, how statistics may be useful to you, even if you have only a minimal understanding of its features. The point is not to explain eigenvectors, matrix transposition, or anything of the sort. If you know nothing of statistics, and want to know just how helpful it could be in your everyday life as a chromatographer—to transform your data into understandable information—then read on!

Collect and Examine Your Data

Let's start this series of tutorials at the beginning. First, you must collect the data produced by your analysis. This is clearly the most important step in data analysis; statistics will never produce interesting information from poor data. Selecting appropriate data of good quality is an important task for a chromatographer. Whatever you do, remember that the data must be as precise and accurate as possible. Abnormal data may be eliminated at this stage, provided you have good reason to believe they are abnormal (for example, if they are caused by something like sample degradation or a system failure during the experiment). The decision to exclude some data can sometimes be made with the help of statistical tests (like Dixon's Q test). Not all atypical data must be excluded, but quantity is not quality, and I always find it preferable to reduce the set to the data I can trust. Outliers will have a bigger impact on small data sets. Chemical sense must guide you in selecting the best data set.

Second, before starting any data treatment, you need to get acquainted with your data. Embarking on a principal component analysis without knowing the structure of the input data would be inefficient, because you would not understand the results fully. In other words, the point is not to produce nice figures and graphics while ignoring the underlying reality that they are meant to represent.

The tools we can use at this stage belong to the category of "descriptive statistics." Rather than numbers, I often prefer a good figure. Usually the data and the question you are trying to answer will determine which type of figure is best. A few examples follow.

Pie Charts

If your data can be categorized into a finite number of classes, where no hierarchy (higher and lower levels) exists among the classes, a pie chart will help you visualize the proportion of each class. Pie charts may be used to visually compare sets of data, but they will be impractical to show how the data change with a variable. For instance, perhaps you analyzed extracts of natural products and quantified different families of compounds present in each sample (terpenes, phenols, alkaloids, steroids, and so on). Comparing a limited number of samples to see the relative proportion of each family of compounds can be easily achieved with pie charts (see an example in Figure 1). From such a figure, the differences are immediately clear and easier to read than in a data table.

Bar Chart

When a hierarchy exists between the classes and the classes can be ranked according to some variable, bar graphs may provide useful information about the shape of the distribution (possible Gaussian curve), if this information matters in your analysis.

Scatter Plots

Scatter plots allow you to see how the points are scattered along one variable. For instance, say you are developing a chromatographic method intended to be applicable to a large variety of ana-

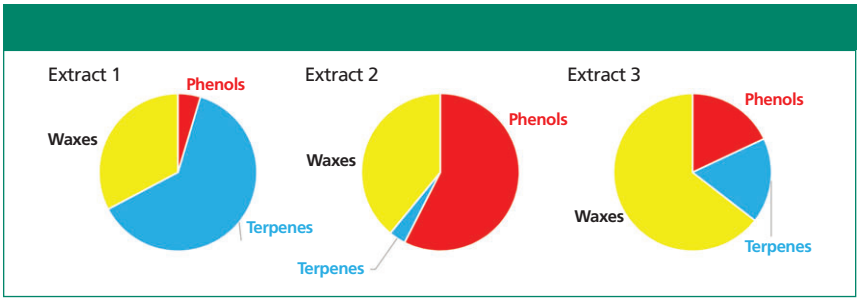

Figure 1: Pie charts illustrating the different proportions of analytes extracted from a single plant with varied extraction conditions.

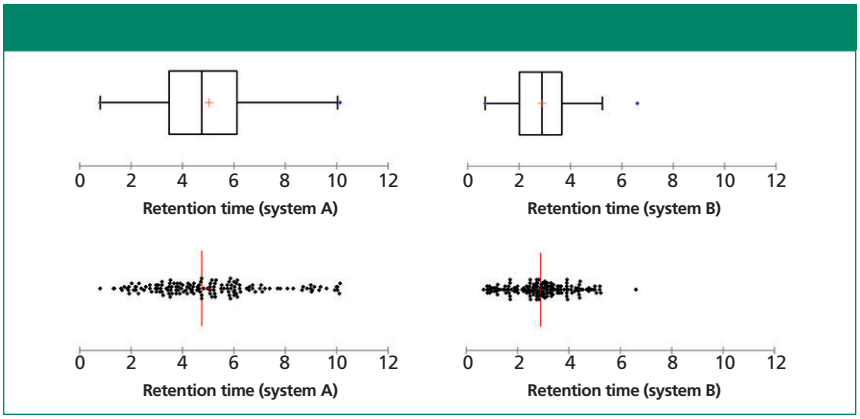

Figure 2: Scatter plots and box plots can help us observe chromatographic retention in different chromatographic systems for the same sample set.

lytes and you would like all the analytes to be well separated in your chromatographic system. In the example shown in Figure 2, the scatter plots make it clear that system A offers much better separation power for the set of analytes than system B. Box and whiskers plots (2), as shown in the upper section of Figure 2, will give similar views, with additional information on the position of the medians and quartiles.

Next: Linear Regression Analysis

In the next installment, I will discuss the simplest method of statistical analysis: linear regression analysis.

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Trends in Separation Science: Tackling Protein Analysis

Increasingly, separation scientists are turning their attention to the challenges of analyzing proteins, whether in pharmaceutical development or proteomics. Heading into the HPLC 2016 conference in San Francisco this June, we asked several leading analytical chemists, all of whom who will be speaking at the conference, to talk about their work in this area. Below, John Yates of the Scripps Research Institute, Mary Wirth of Purdue University, Andrew Alpert of PolyLC, and Alain Beck of the Pierre Fabre Immunology Center and Arnaud Delobel of Quality Assistance SA discuss approaches to improve the analysis of these large molecules.

Why Effective HPLC Separations Are Critical for the Analysis of High-Complexity Systems

John Yates III

Large-scale protein biochemistry, otherwise known as *proteomics*, was enabled by the protein sequence infrastructure created by genome sequencing. Tandem mass spectrometers create spectra of peptides that can be used to search sequence databases to assign amino acid sequences to the spectra. Such methods allow the analysis of protein complexes, organelles, and whole cells through a process that proteolytically digests proteins into a collection of peptides that can then be identified using tandem mass spectrometry (MS) and informatics. Intrinsic to this strategy is the creation of very large and complicated mixtures of peptides. Simultaneous electrospray ionization of large and diverse collections of peptides results in suppression of ion formation; consequently it is imperative to separate peptides sufficiently to relieve ion suppression and to allow the mass spectrometer sufficient time to collect tandem mass spectra of peptide ions. Thus, peptide separation methods are critical for proteomics studies. The challenges involved are great.

The digestion of the proteins from a human cell can produce many hundreds of thousands of peptides. The analysis of such a complex mixture by tandem MS is dependent on both the resolution of the separation and also on the scan speed of the tandem mass spectrometer. Separation resolution has to decrease the complexity of the mixture so the instrument can maximize collection of tandem mass spectra of peptide ions. The scan speeds of tandem mass spectrometers are increasing, allowing the collection of more spectra per unit time. New data acquisition strategies like data-independent data acquisition are threatening to further increase data acquisition rates. Clearly, this situation creates a challenging separation problem, especially if the goal is to also minimize the overall time required for the analysis.

Initial methods to resolve such complicated peptide mixtures used a two-dimensional liquid chromatography (2D LC) separation technique that combined a strong cation exchange separation with reversed-phase separation using a C18 column (1,2). While not perfectly orthogonal, the two modes provide excellent peak capacity, but do so with long analysis times to achieve high numbers of protein identifications. Various other 2D approaches to this problem have been developed, including a high-pH reversed-phase separation followed by a low-pH reversed-phase separation and methods that combine a strong anion exchange step with a reversed-phase step, analogous to the strong cation exchange–reversed phase combination (3).

Through continuous improvements to MS technology, it is now possible to analyze most of a mammalian cell proteome. In fact, a nearly complete analysis of the eukaryotic *Saccharomyces cerevisiae* proteome has been reported (4) with an analysis time under 90 min using reversed-phase high-performance LC (HPLC). By decreasing analysis times, higher throughput experiments can be designed that can examine more human patient samples or more sample conditions. The development of high-resolution single-dimension separations based on ultrahigh-pressure liquid chromatography (UHPLC) using a C18 column has offered a new direction, although the time required to achieve high numbers of peptide identifications is still 4–8 h for complex proteomes (5,6). Additionally, those analyses achieve nearly complete protein analyses but with very low numbers of identified peptides per proteins (in other words, low sequence coverage) and in fact most of the proteome is identified with one peptide per protein.

Another reason to increase the resolution of separations and peak capacities is to improve the dynamic range of the separation. Within a proteome there is at least an abundance range of 10^6 for expressed proteins and this range doesn't include post-translational modifications (PTMs). It remains to be seen if single-dimension UHPLC separations can be extended to encompass the entire proteome of a mammalian cell to capture all the subtleties and nuances of protein structure. Developing high-

speed 2D LC separations may provide a means to achieve this goal, but this type of method does present a challenge because of the slower analysis times often associated with separations in two dimensions.

As separation methods in combination with advanced MS technology bring us closer to the capability to characterize the complete human proteome, advancing these methods to achieve high sequence coverage will be essential. High sequence coverage in combination with good dynamic range will allow collection of information about isoforms and PTMs, information, which will be important to understand the function of proteins.

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New Materials for UHPLC Analysis of Monoclonal Antibody Drugs and Antibody–Drug Conjugates

Alexis Huckabee, Jonathan Yasosky, and Mary J. Wirth

The separation of intact proteins plays many roles in drug discovery and development: identification of drug targets, screening for drug candidates, quality control, and drug authentication. Various separation techniques are used, from immunoprecipitation to study a single protein of interest, through various types of column chromatography for detecting a handful of proteins at once, all the way to proteomics for studying hundreds to thousands of proteins. What all of these techniques and applications have in common is that the power of protein separations is limited by the fact that proteins are large, slowly diffusing, sticky molecules.

There is a demand for even higher performance. One critical need for better chromatography is in the development of antibody–drug conjugates (ADCs). Only a few ADCs have been approved by the US Food and Drug Administration (FDA), but many ADCs are under development and in all phases of clinical trials. An ADC directs a toxic small-molecule drug to cancer cells by covalently binding the toxic drug to a monoclonal antibody through a labile bond (1). The intention is that the labile bond breaks after the ADC is inside the cancer cell, but not before, to release the toxin where it is needed. ADCs have multiple binding sites, and each species potentially has different efficacy and toxicology. Therefore, it is necessary to determine to how many of these binding sites the drug has been attached. Hydrophobic interaction chromatography (HIC) uses columns packed with nonpolar polymer beads to separate ADCs based on how many drug molecules are conjugated to a given antibody (the drug–antibody ratio, or DAR) (2). The bands typically are too broad to fully separate all of the species, however, and some ADC candidates are too hydrophobic for current HIC columns. Decades of research and development (R&D) in the chromatography industry have been devoted to reversed-phase liquid chromatography (LC) because this mode of LC is so widely used for small-molecule drugs and for quality control of protein drugs. Creating better bonded phases for HIC, however, is a challenge for silica-based columns because HIC uses a fully aqueous mobile phase and neutral pH.

The bonded phase is a pervasive limitation to efficiency in monoclonal antibody separations. Another problem with monoclonal antibody separations is that silica particles having an average pore diameter of 300 Å do not allow full access to the surface area. The limited pore size also impedes intraparticle diffusion. Advances in the chromatographic materials need to consider all of these issues.

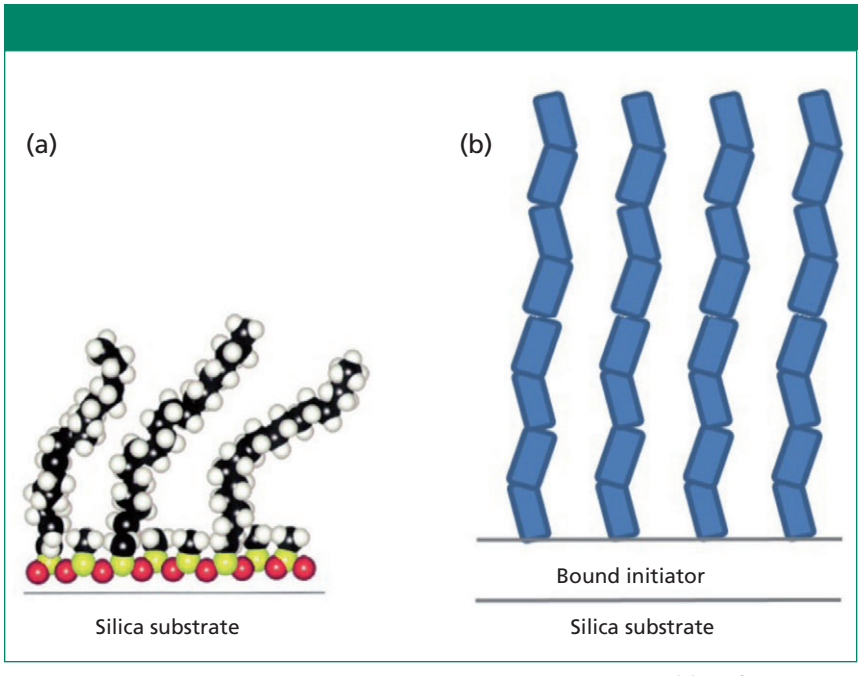

Figure 1: Two bonded phases that reduce silanol interactions: (a) self-assembled mixed silanes and (b) densely grown polymer chains tethered from silica. These images are not to scale; the polymer layer is typically about 10 times thicker than the C18 layer.

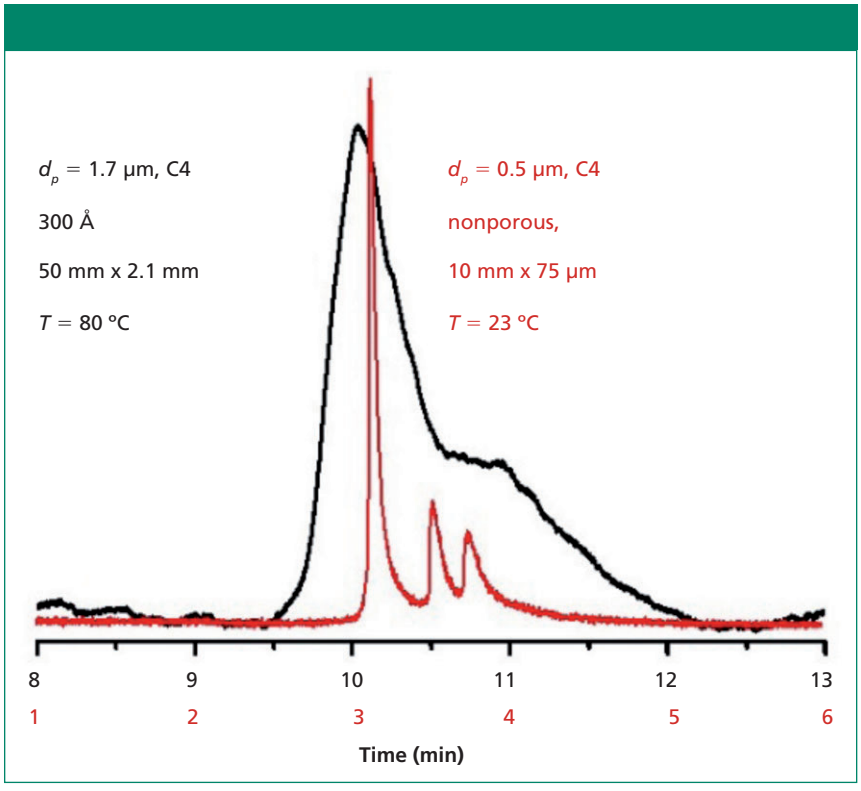

Figure 2: Reversed-phase LC data for a monoclonal antibody and aggregates for a commercial column of fully porous sub-2-µm particles (black) and a capillary packed with 0.5-µm particles (red), each with a C4 bonded phase. Adapted with permission from reference 14.

Bonded Phases with Less Protein Adhesion

There are two approaches to reducing silanol activity in HIC columns that our group has considered. One is self-assembled monolayers for reversed-phase separations, illustrated in Figure 1a. These monolayers form a dense, two-dimensional net over the surface to improve reversed-phase LC separations of proteins (3). Since coulombic interactions are long-range, any defects in the column produce the familiar tailing of mixed-mode chromatography techniques, which is mitigated by low pH. The other approach, surface-confined atom-transfer radical polymerization, alleviates the coulombic interactions by growing a dense layer of tethered polymer chains that is thicker than the Debye length, as illustrated in Figure 1b, reducing long-range attractions between proteins and silica (4). Nonporous polymer particles with tethered polymers as bonded phases (5) have been commercially available for some time. Despite their small surface area, these particles have been valuable for monoclonal antibody separations, including ion-exchange chromatography and HIC, as mentioned earlier. Our group found that the bonded phase depicted in Figure 1b gives excellent hydrophilic-interaction chromatography (HILIC), resolving the glycosylation states of ribonuclease B and providing partial resolution of their isomers (6). We are currently applying HILIC to monoclonal antibodies to decrease analysis time for probing glycosylation. We are also investigating whether the combination of self-assembly and tethered polymer chains on silica can result in a better HIC bonded phase for ADC analysis.

Morphologies to Decrease Diffusion Distance and Increase Accessible Surface Area

The most salient commercial advance in LC over the last 15 years has been the introduction of chromatographic particles that decrease the diffusion distances for mass transport. These include fully porous sub-2-µm particles (7), core–shell particles (8), and monolithic columns (9). Porous materials, regardless of morphology, will eventually approach the limit where the smaller particle size (or smaller shell thickness) approaches the diameter of the larger colloids needed to produce wider pore sizes (10). Sphere-on-sphere particles, which are core–shell particles with a single layer of large colloids (11), embody this idea. As this limit is approached for fully porous particles, it makes sense to consider using the colloids themselves as the packing material.

We have studied capillaries packed with 0.5-µm nonporous silica particles with C4 bonded phases, packed in capillaries. The accessible surface area for large proteins is quite high, falling in between those of core–shell particles and fully porous particles, each for a 300-Å pore size (12). The back pressure is alleviated by the presence of slip flow for reversed-phase LC (13), enabling column lengths of at least 10 cm for commercial high-pressure chromatography instruments. Colloidal particles pack more tightly than standard particles, obstructing the axial diffusion about twofold more. This tighter packing gives rise to a higher velocity inside the column, which makes the optimal volume flow rate slower, further alleviating back pressure. Uniform packing results in negligible eddy dispersion. With no intraparticle diffusion, lower obstructed diffusion, and lower eddy dispersion, all three terms in the van Deemter equation are thus smaller. Barring effects from strong adsorption of proteins, the van Deemter equation predicts submicrometer plate height for proteins.

We observed submicrometer plate heights for protein separations using 0.5-µm particles (12,13,15). Plate heights were as small as 15 nm and the plate number was in excess of 10^6 for a 2-min separation of proteins (13). In this case, self-assembly was used to produce a mixed C4–C1 bonded phase, injection was done by diffusing the protein into the head of the column to avoid contact between metal parts and protein, and on-column detection was used. For more-routine applications, Figure 2 shows an example separation where a commercial auto-injector was used, with the same bonded phase and on-column detection. An IgG4 monoclonal antibody drug and its two aggregates were resolved well in a short 1-cm separation length at room temperature,

whereas a 5-cm long column of fully porous particles did not resolve the three species.

HIC columns will likely enjoy a boost in flow rate from slip flow when used with submicrometer particles. The next goals are to extend the performance seen in Figure 2 to 2.1-mm i.d. stainless steel columns for reversed-phase LC, and combine this advance with a new polymeric bonded phase on silica for HIC of ADCs.

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Reconsidering HIC for Top-Down Proteomics

Andrew J. Alpert

Until recently, mass spectrometry (MS) was limited in the information it could supply regarding proteins larger than 40 kDa. The most recent instruments have broken through that limit, but proteins smaller than 40 kDa are still more easily detected in MS and can suppress the collection of data from larger proteins. This situation has created a demand for better separation of proteins upstream from the MS orifice to facilitate top-down proteomics. At present, though, this separation of proteins is something of a bottleneck. Methods such as reversed-phase liquid chromatography (LC) that involve mobile phases compatible with MS are not compatible with many proteins. With those mobile- and stationary-phase combinations, the proteins may be eluted in peaks 15-min wide or not at all (1,2). The conditions also tend to cause the loss of protein three-dimensional structure, precluding analysis of protein complexes.

Alternative modes of chromatography for protein analysis include the following:

- **Size-exclusion chromatography:** Size-exclusion chromatography is helpful in separating large proteins from small ones but is otherwise a low-resolution method.
- **Ion-exchange chromatography:** Ion-exchange chromatography is a high-resolution mode but generally requires more salt than a mass spectrometer can cope with.
- **Hydrophilic-interaction chromatography (HILIC):** HILIC has been used successfully for proteins that do not normally occur free in aqueous solution, such as histones (3), membrane proteins (4), and apolipoproteins (5). However, the high concentration of organic solvent required probably precludes its more general use.
- **Hydrophobic interaction chromatography (HIC):** HIC involves a gradient from a high to low concentration of salt, and the best-performing salts are nonvolatile. This condition would seem to eliminate it from consideration for protein separations online with MS. However, HIC separates proteins with high resolution, based on differences in the hydrophobicity of the surface of their tertiary structures. HIC is nondenaturing and extremely sensitive to differences in protein composition. Accordingly, we decided to take another look at HIC (6).

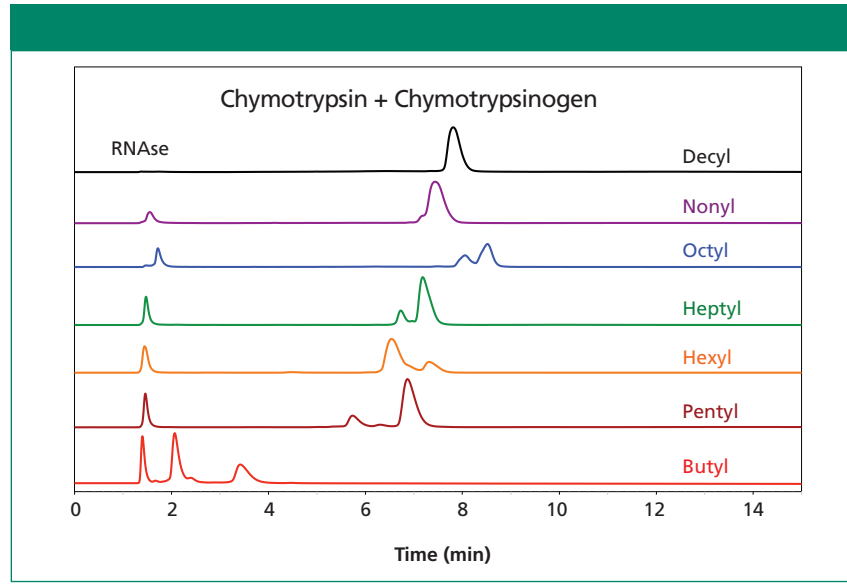

Figure 1: Retention of ribonuclease A (RNAse) and chymotrypsinogen (CHYGEN) on various columns. Columns: 100 mm × 4.6 mm, 3-µm d_p, 1500-Å (coating as shown); mobile-phase A: 1 M ammonium acetate; mobile-phase B: 20 mM ammonium acetate with 50% acetonitrile; gradient: 15-min linear, 0–100% B; flow rate: 1 mL/min; detection: 280 nm. (Adapted with permission from reference 6.)

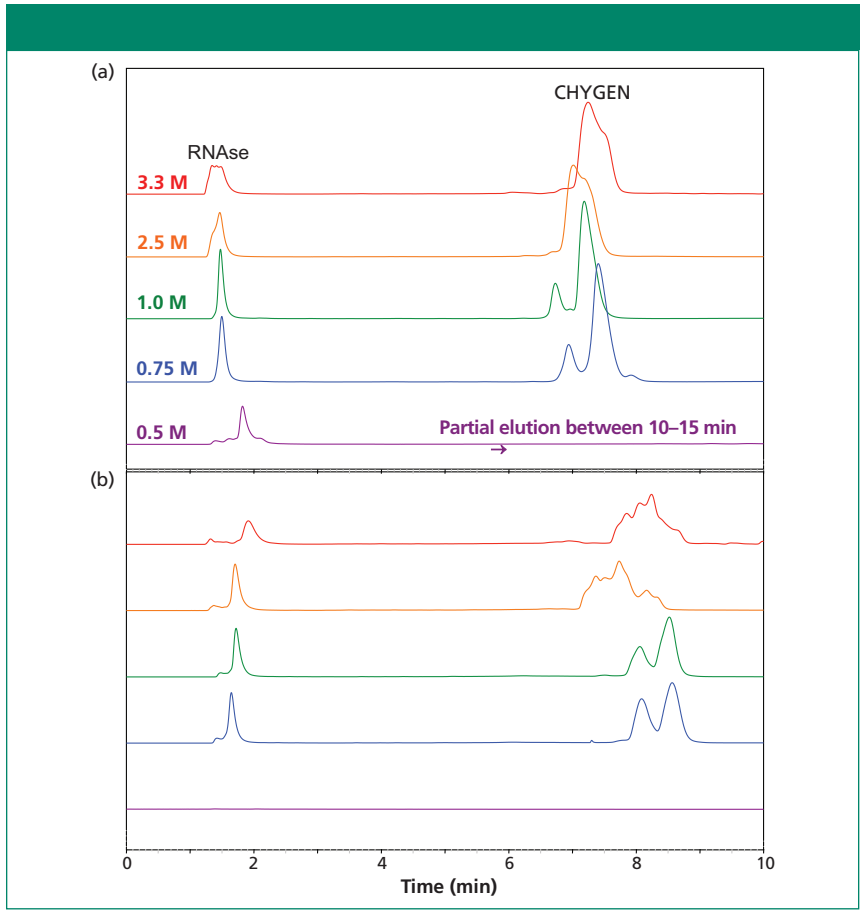

Figure 2: Retention of RNAse and CHYGEN as a function of initial salt concentration: (a) PolyHEPTYL A column, (b) PolyOCTYL A column. Mobile-phase A: ammonium acetate concentration as noted; mobile-phase B: 20 mM ammonium acetate with 50% acetonitrile; gradient: 15 min, 0–100% B, then 5 min at 100% B. Other running conditions were the same as in Figure 1. (Adapted with permission from reference 6.)

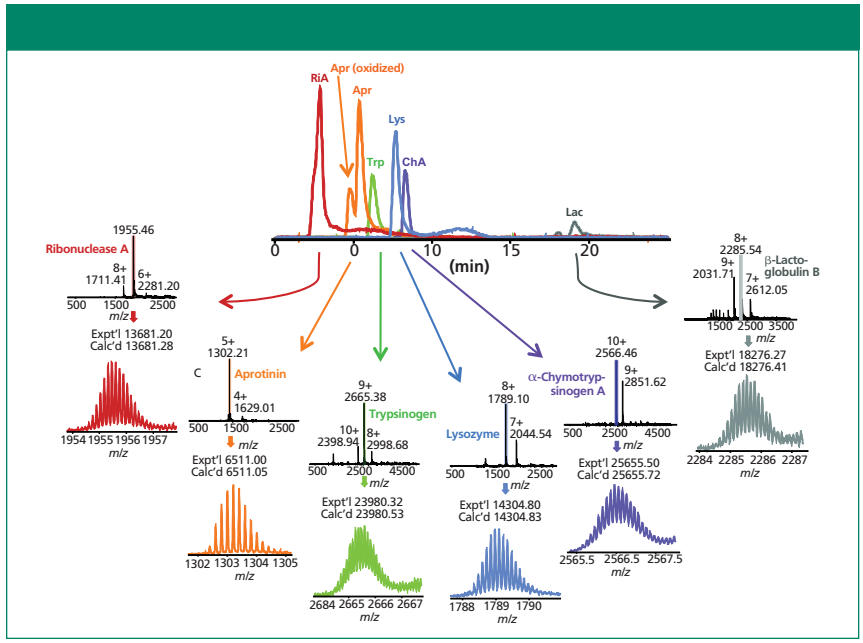

Figure 3: HIC separation of standard proteins and Q-TOF-MS analysis. Column: 100 mm × 0.2 mm, 3-µm d_p, 1500-Å PolyHEPTYL A capillary; mobile-phase A: 1 M ammonium acetate; mobile-phase B: 20 mM ammonium acetate with 50% acetonitrile; gradient: 15-min linear, 0–100% B, then 5 min at 100% B; flow rate: 2.4 µL/min; detection: Q-TOF-MS. (Adapted with permission from reference 6.)

The use of HIC would be practical if a volatile salt could be used. Suitable salts such as ammonium acetate are poor at promoting retention in the HIC mode. Literature on the subject has involved concentrations in the 3–4 M range, which is too high for a mass spectrometer. Now, the more hydrophobic a material is, the less salt is needed for retention in HIC. We decided to increase the hydrophobicity of the stationary phase systematically in an effort to produce materials that could retain proteins at concentrations of ammonium acetate that were practical for MS analysis. Increasing the length of the functional ligand in the coating from C_3 to C_4 to C_5 resulted in dramatic increases in protein retention, in keeping with past observations about HIC (7). However, lengthening the ligand from C_5 through C_{10} did not result in much change in protein retention times (Figure 1). Furthermore, in this range the retention of some of the protein standards was not directly proportional to the concentration of ammonium acetate (Figure 2). This behavior is normally associated with reversed-phase LC rather than with HIC. A concentration of 0.75–1 M ammonium acetate is still necessary in the starting mobile phase, but its function seems to be the maintenance of the tertiary structure of the proteins rather than promotion of binding. Mass spectrometers can handle such concentrations. The presence of some organic solvent in the final mobile phase was essential for elution of most proteins within the gradient. All in all, the behavior of these new HIC materials resembles that of reversed-phase LC as much as HIC. The distinction between the two modes seems to have been blurred if not erased.

Figure 3 demonstrates that standard proteins are eluted from the new materials with good peak shape. Direct elution into a mass spectrometer produced mass spectra characteristic of proteins with their native structures intact. Notwithstanding the high concentration of acetonitrile in the final mobile phase, the kinetics of the chromatography was evidently faster than the kinetics of denaturation. Figure 4 is a demonstration of direct HIC–MS of an extract of *E. coli* proteins. A protein as large as 206 kDa was identified.

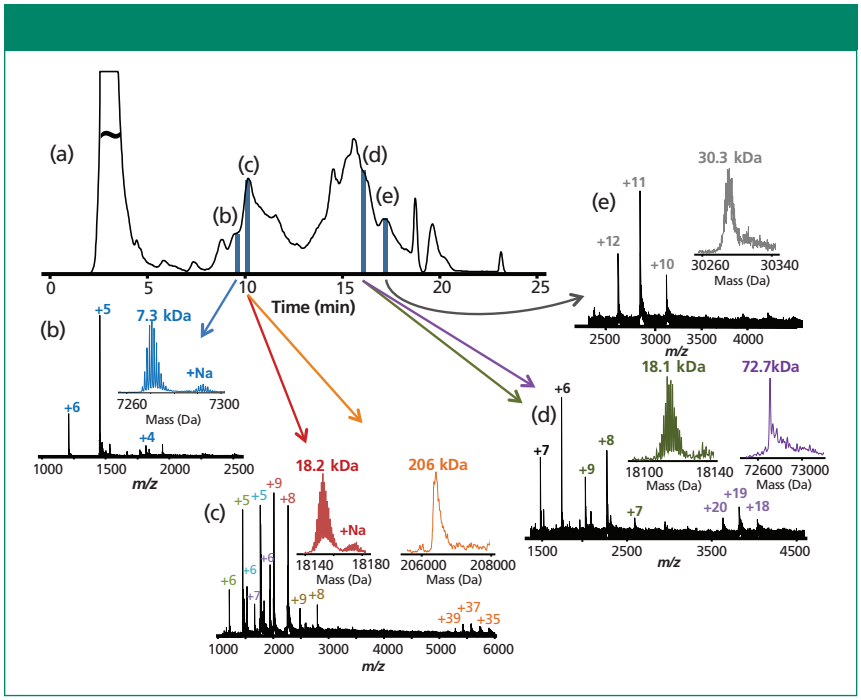

Figure 4: HIC separation of standard proteins and Q-TOF-MS analysis. Column: 100 mm × 0.2 mm, 3-µm d_p, 1500-Å PolyHEPTYL A capillary; mobile-phase A: 1 M ammonium acetate; mobile-phase B: 20 mM ammonium acetate with 50% acetonitrile; gradient: 15-min linear, 0–100% B, then 5 min at 100% B; flow rate: 2.4 µL/min; detection: Q-TOF-MS. (Adapted with permission from reference 6.)

This project seems to have been successful in adapting HIC to the requirements of MS for top-down proteomics. It should be possible to adapt ion-exchange chromatography for this purpose as well.

Acknowledgments

This work was performed in collaboration with Ying Ge of the University of Wisconsin-Madison, and the following members of her group: Bifan Chen, Ying Peng, Santosh G. Valeja, and Lichen Xiu.

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State-of-the-Art MS Methods for Structural Assessment of mAbs and ADCs: From the Research Lab to Routine Characterization

Alain Beck and Arnaud Delobel

Monoclonal antibodies (mAbs) are highly complex tetrameric glycoproteins that require extensive analytical and structural characterization to become drug candidates. This is also true for antibody–drug conjugates (ADCs). These immunoconjugates are based on highly cytotoxic small-molecule drugs covalently attached via conditionally stable linkers to mAbs and are among the most promising next-generation empowered biologics for cancer treatment. ADCs are more complex than naked mAbs, because the heterogeneity of the conjugates adds to the inherent microvariability of the biomolecules. The development and optimization of ADCs rely on improving their analytical and bioanalytical characterization by assessing several critical quality attributes, namely the distribution and position of the drug, the amount of naked antibody, the average drug-to-antibody ratio (DAR), and the proportions of residual small-molecule drug and drug-linker (1).

As a result of advances in multilevel (top, middle, bottom) state-of-the-art mass spectrometry (MS) methods, including native MS, ion mobility MS (2), capillary electrophoresis–electrospray ionization–MS (3,4), two-dimensional liquid chromatography–MS (2D LC–MS) (5), extended bottom-up (6), and top-down sequencing (7), combined with chromatographic and electrophoretic techniques (8) very precise characterization of biotherapeutics is now possible. Until recently, however, these techniques were considered suitable only for research use. With the advent of robust and user-friendly solutions (both hardware and software), these techniques are now amenable for routine use. Examples of their application to the characterization of mAbs and ADCs are discussed below.

Middle-Level Characterization of mAbs

During their biosynthesis or during their shelf life, mAbs can undergo many modifications, such as glycosylation, oxidation, deamidation, and C-terminal lysine clipping, to name a few. In most cases, these variants cannot be easily identified at the intact antibody level, because of limitations in chromatographic separation and MS resolution.

A middle-up approach using IdeS digestion (to yield Fab, Fc/2, and light-chain fragments) combined with a super macroporous reversed-phase column enables quick and efficient characterization of mAb variants (9). The relatively low molecular mass of the subunits (~25 kDa) allows accurate mass determination by high-resolution MS as well as top-down sequencing by electron

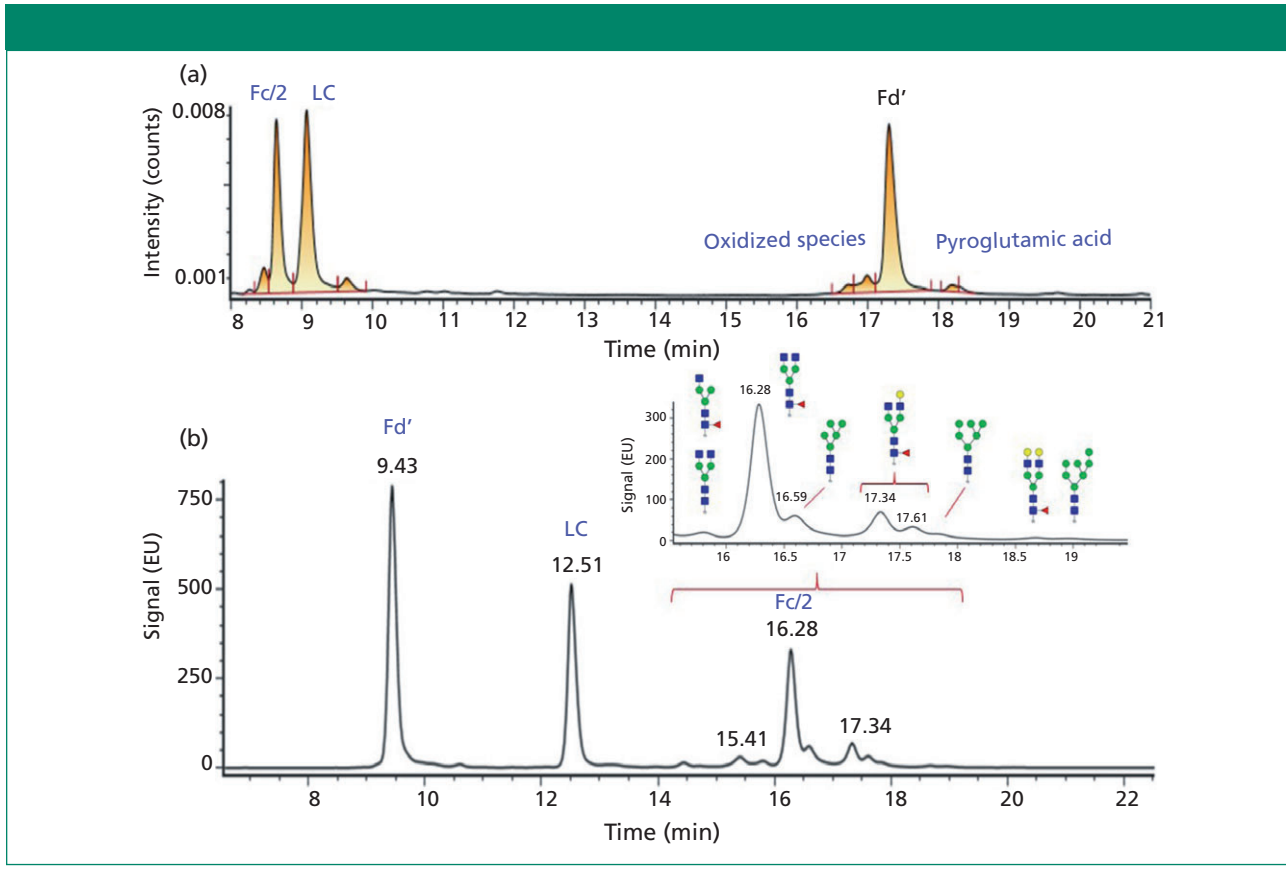

Figure 1: HPLC–UV chromatograms obtained for adalimumab sample digested with IdeS and analyzed on (a) a reversed-phase or (b) a HILIC column.

transfer dissociation (ETD). Using the same sample preparation, the glycoforms can also be separated and characterized using an approach such as hydrophilic-interaction chromatography (HILIC) with MS detection (10). An example of this approach is presented in Figure 1 for adalimumab.

Determination of Drug-to-Antibody Ratio on Intact ADCs

The number of cytotoxic molecules attached to an antibody (the drug-to-antibody ratio, or DAR) is a critical quality attribute of an ADC. It can be determined by UV spectrophotometry or hydrophobic interaction chromatography (HIC) with UV detection (11–13), but these methods are not universal and have some drawbacks. Mass spectrometry can be a universal tool to determine the DAR value, whatever the coupling chemistry or the nature of the drugs.

After N-deglycosylation, the ADC is desalted on-line using reversed-phase high performance LC (HPLC) or size-exclusion chromatography (SEC) (14) and detected by high-resolution electrospray ionization MS. The DAR can be automatically calculated by software after deconvolution of the multicharged electrospray spectrum. When working with cysteine-linked ADCs, native conditions must be used to avoid disruption of noncovalent interactions. In these methods, native SEC–MS is commonly used, which requires optimization of mobile phases and MS conditions.

Characterization of mAbs and ADCs by UHPLC–MS and HPLC–MS Peptide Mapping

Peptide mapping with MS detection is a common methodology for protein characterization. It can be used for the confirmation of the primary sequence, the quantification of post-translational modifications (PTMs), and the study of disulfide-bond scrambling. Thanks to commercially available software packages dedicated to biopharmaceutical analysis, the analysis of these MS data can be fully automated.

Applied to ADC characterization, peptide mapping is also a valuable tool to localize conjugation sites and determine site occupancy. ETD fragmentation can be used to localize conjugation sites for lysine-conjugated ADCs on peptides containing several lysine residues.

2D LC–MS Analysis of mAbs and ADCs

Two-dimensional LC with MS detection is widely used for the identification of proteins from complex proteome samples in many laboratories. Robust 2D-HPLC and 2D-ultrahigh pressure LC (UHPLC) systems are now commercially available, thus enabling the routine analysis of biopharmaceuticals with this technology.

Two-dimensional LC can be used to hyphenate MS-incompatible chromatographic separations to mass spectrometry detection: After a first dimension using mobile phases containing nonvolatile salts, the peak of interest is sent to a second dimension consisting of a reversed-phase column to desalt the sample and separate potentially coeluted species. This methodology (heart-cutting 2D LC–MS) can be routinely applied to identify the different species detected in size-exclusion and ion-exchange chromatography of monoclonal antibodies.

Another main application of 2D LC–MS is the rapid on-line structural elucidation of species observed in HIC distribution profiles of cysteine-conjugated ADCs (15,16).

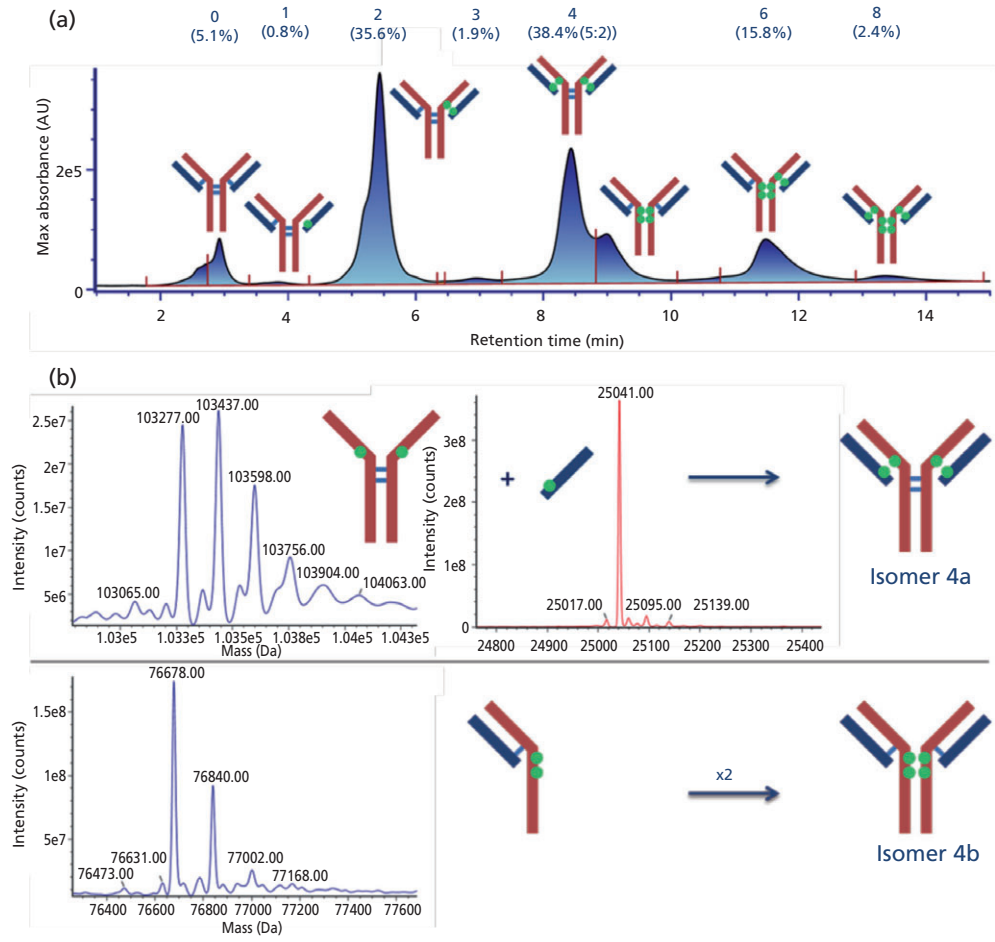

Figure 2: (a) HIC–UV chromatogram obtained for brentuximab vedotin and (b) deconvoluted mass spectra obtained by HIC–reversed-phase LC–MS for the two isomers of DAR4.

The identification of the different species is required to be able to determine the DAR value based on the HIC–UV profile as well as for the detection and the quantification of small-molecule drugs (17). The application of this methodology to brentuximab vedotin is presented in Figure 2.

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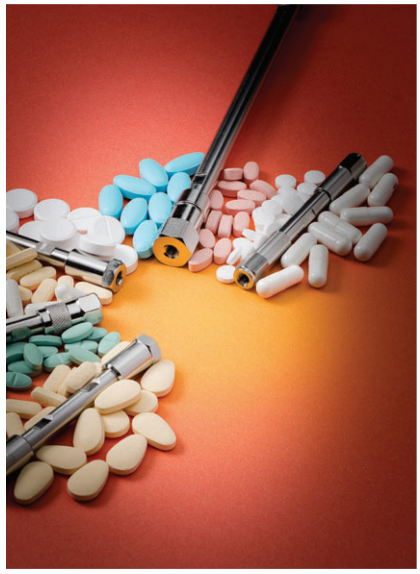
UHPLC Instrument Variations and Approaches to Ease the Method Transfer Process



Ultrahigh-pressure liquid chromatography (UHPLC) instruments from different manufacturers and instruments with different configurations can produce significant variations in chromatographic separation. The variety in instrument configuration increases the complexity of the method development process, which now requires a more thorough evaluation of the effect of instrument variations on the method. The studies presented here determined the typical interinstrument variations in dwell volume, extracolumn dispersion, and mixing efficiency as measured by mobile-phase compositional accuracy. Additionally, the dwell volume and extracolumn dispersion were independently and systematically varied to evaluate the resulting impact on resolution for a small-molecule test mixture during gradient elution. To account for these interinstrument variations, dwell volume and wash-out volume method translation and adjustment techniques were evaluated.

Laura E. Blue, Tawnya Flick, and David Semin

To support the need to get products to market more quickly and under tighter cost constraints, increased externalization of manufacturing and analytical development has occurred within the pharmaceutical and biotechnology industries (1,2). As activities are shifted to external laboratories, the diversity of instruments and configurations dramatically increases as compared to when all activities occurred within the same internal laboratory that typically had one instrument model and configuration. This instrument diversity has led to the observation of increased chromatographic separation variation.

This trend in conjunction with the shift from high performance liquid chromatography (HPLC) to ultrahigh-pressure liquid chromatography (UHPLC) platforms has driven the need to characterize the expected interinstrument variations (3). Although variations between HPLC instruments from different manufacturers or with different module configurations contribute to chromatographic differences, typically the impact is not significant because of the inherent total volume of the system and the efficiency of the columns (4–6). With the shift to higher efficiency columns, the instrumental variations have a significant impact on the chromatographic performance. For example, the dwell volume to void volume ratio (V_D/V_M) varies between 2.1 to 2.8 for a 100 mm × 3.0 mm, 3.5-µm d_p column on a binary versus quaternary pump HPLC. While using a 50 mm × 2.1 mm, 1.7-µm column on a binary versus quaternary UHPLC, the ratio can vary between 1.8 and 3.8. This difference illustrates the increased relative impact of instrumental variations on UHPLC methods.

Recommendations in the literature suggest adjustment of dwell volume, column temperature, and wash-out volume to produce equivalent chromatographic results on different instruments (5,7–10). While these suggestions are available, the amount of expected interinstrument variation and the tolerability of these variations have had

limited discussion. Additionally, the success rate of using these method translation and adjustment techniques has had limited unbiased evaluation, and the regulatory implications and required method validation studies to allow method adjustments require consideration.

The goals of this research are to understand the impact of instrumental parameters on the retention and resolution of analytes and better identify the cause of observed chromatographic differences between instruments for gradient separations. Additionally, method translation and adjustment techniques are evaluated with the goal of developing a framework to build quality into UHPLC methods to ease the method transfer process.

Experimental

All studies were performed using Waters H-Class instruments or Agilent 1290 instruments (binary and quaternary). All mobile phases were prepared using HPLC-grade solvents. The gradient test mixture was purchased and used as is (Waters gradient test mix as part of the Acquity UPLC Absorb Start-up solution). The gradient test mix was selected to represent a simple small-molecule mixture for which retention would span the typical range of a pharmaceutical method. The gradient test mix method used a 50 mm × 2.1 mm, 1.7-µm Waters Acquity BEH C18 column at 40 °C. The mobile phase, multistep gradient, and flow rate were as follows: mobile-phase A: water; mobile-phase B: acetonitrile; 0–0.25 min, 10% B; 0.25–2.5 min, 10–95% B; 2.5–2.6 min, 95% B; 2.6–3.0 min, 95–10% B; 3.0–5.0 min, 10% B; 0.6 mL/min. The pharmaceutical sample used was a small-molecule peak identification solution, which contained molecules varying in acidity or basicity and hydrophobicity. This sample was selected because of its complex nature and sensitivity to variations in method conditions.

For all experiments, a 50 mm × 2.1 mm, 1.7-µm column was used. This column was selected based on the majority of the methods that are used within our laboratory. Compared to other typical column dimensions used for UHPLC methods (excluding 1.0-mm i.d. columns), these column dimensions represent a worst-case scenario in terms of impact from instrumental parameters.

Dwell Volume

The interinstrument variation in dwell volume was assessed by using a gradient of water and 0.1% acetone in water (11). The gradient was a 0–100% B linear ramp over 10 min. The column was replaced with a 1000 cm × 0.018 cm i.d. piece of polyetheretherketone (PEEK) tubing and was accounted for (248 µL) in determining the dwell volume of the system. The intersection of the isocratic (zero slope) and gradient slope of the chromatogram was used as the dwell time. For each UHPLC system, the dwell time was determined in triplicate and the average dwell time was used for the determination of the dwell volume. The dwell volume was calculated as shown in equation 1, based on the measured dwell time.

$$\text{dwell volume} = \left(\text{dwell time (min)} \times \text{flow rate}\left(\frac{\text{mL}}{\text{min}}\right)\right) - \text{PEEK tubing volume (mL)} \quad [1]$$

To evaluate the impact of dwell volume on the retention time and resolution of the analytes in the gradient test mix, the dwell volume was physically modified by adding different lengths of 0.17-mm i.d stainless steel tubing between the pump and the injector. Under the method conditions stated above, the gradient test mix was injected in triplicate and the average retention time of each analyte and the average resolution for each pair was determined. The average change in resolution was plotted as a function of the total dwell volume of the system (system dwell volume and volume of tubing added) for each analyte pair. The average change in resolution as a function of the change in dwell volume was calculated based on the experimental data.

Extracolumn Dispersion

The column was replaced with a zero-dead-volume union, 50:50 water–acetonitrile was used as the mobile-phase, and 1 mL of 0.1% acetone in water was injected. The extracolumn dispersion was determined by measuring the 4σ peak width of the acetone peak (12). For one UHPLC system, the extracolumn dispersion (ECD) was measured at different flow rates between 0.4–1.0 mL/min. The variation in ECD as a function of flow rate was found to be less than 1 µL.

Therefore, only one flow rate was used for the measurement of ECD on the remaining instruments. For each UHPLC system, the measurement was determined in triplicate at 1.0 mL/min and the average ECD was calculated.

The effect of both precolumn and postcolumn ECD on the resolution of each analyte pair in the gradient test mix was evaluated. A known length of 0.12-mm i.d. PEEK tubing was placed between the injector and the column inlet to assess precolumn ECD and between the column outlet and the detector for postcolumn ECD. The same gradient test mix method conditions listed above were used for these experiments. The change in resolution for each analyte pair was plotted as a function of ECD added to the system. Based on this, the average change in resolution as a function of change in ECD was calculated for precolumn and postcolumn ECD.

Mixing Efficiency

For several instruments with different mixing volume and mixer type, the column was replaced with a zero-dead-volume union and a step gradient of 0.1% acetone in mobile-phase B was generated as described by Medvedovici and David (13). The following mobile-phase combinations were assessed: water–methanol, water–acetonitrile, and water–isopropanol. In all cases, %B was increased in increments of 10% and held at each level for 10 min. After the pump was delivering 100% mobile-phase B, the step gradient decreased in increments of 10% B until the pump was generating 0% mobile-phase B. The deviation from the theoretical gradient set point was determined by taking the difference between the instrumental set point and the $\%B_{plateau}$:

$$\%B_{plateau} = (\bar{A}_{plateau} - \bar{A}_{\%B=0}) \times \frac{100}{(\bar{A}_{\%B=100} - \bar{A}_{\%B=0})} \quad [2]$$

The average absorbance at the chromatographic plateau, $\bar{A}_{plateau}$, was corrected for the absorbance at 0% B, $\bar{A}_{\%B=0}$ and normalized against the difference between the absorbance at 100% B, $\bar{A}_{\%B=100}$ and the absorbance at 0% B. For each UHPLC system–mixer configuration and mobile-phase combination, the average percent deviation was calculated. The measurement of the mobile-phase compositional accuracy as determined above was deemed suitable to assess the mixing efficiency since larger variations were observed for different mixer types than when measuring baseline noise.

Method Translation

For method translation and adjustment by dwell volume, the initial isocratic hold time was increased when moving from a system with larger dwell volume to a system with smaller dwell volume. The required isocratic hold time was determined by calculating the interinstrument difference in dwell volume and dividing by the flow rate to produce the time required to generate the required dwell volume. When moving from a system with a smaller dwell volume to a system with a larger dwell volume, an injection delay was added to the method. Within the software, the dwell volume difference was entered and the required injection delay was automatically calculated based on the method flow rate. The gradient test mix and the pharmaceutical sample were assessed for retention time and resolution consistency between the two instruments under evaluation. Moving from a system with a smaller dwell volume

to a system with a larger dwell volume and vice versa was evaluated for each sample.

In addition to adjusting for dwell volume, the wash-out volume was also used as a method adjustment technique. A gradient of water and 0.1% acetone in acetonitrile from 0% to 100% B was generated with an additional isocratic hold at 100% B. The time required to transition from the gradient-slope region to the zero-slope region was used as the wash-out time for the system (7). The wash-out time and the flow rate were used to calculate the wash-out volume for each UHPLC system. The ratio of the wash-out volume for the two instruments of interest was used to scale every step in the gradient program. The gradient test mix was used to evaluate the consistency of retention time and resolution when adjusting for dwell volume and wash-out ratio simultaneously.

Results and Discussion

Instrument configuration variations can arise from differing pump type, mixer volume, detector cell volume, and tubing configuration—to name a few. When taken in combination, the potential for interinstrumental differences becomes quite high. Table I lists the common configurations available for several UHPLC systems currently on the market.

Dwell Volume

Dwell volume differences arise from instrumental parameters such as the pump configuration (binary or quaternary), the presence or absence of an in-line filter, mixer volume, and tubing configuration. The resulting dwell volume difference can affect the retention, selectivity, and resolution (14). The dwell volume and flow rate dictate the length of time required for the gradient to reach the column inlet, which affects the initial isocratic hold time experienced by the column and the speed of gradient change. Although dwell volume affects analytes throughout the entire separation space, variation in the dwell volume typically has a greater impact on weakly retained analytes; the elution of weakly retained analytes can vary from isocratic elution to gradient elution depending on the dwell volume of the system and the retention time of the analyte (9). Dwell volume can impact analytes throughout the retention window because of the resulting gradient shape dictated by the dwell volume to void volume ratio (10).

The dwell volume was measured for the UHPLC instruments within our laboratories to determine the amount of interinstrument variation that can be expected. The dwell volume for the instruments within our laboratories varied between 140 µL and 560 µL. For binary instruments, the dwell volume ranged from 140 µL to 220 µL and between 380 µL to 560 µL for quaternary instruments, depending on the configuration. To put this in perspective, for a method flow rate of 0.6 mL/min, the initial isocratic hold time would vary between 0.2 min and 0.9 min depending on the dwell volume of the system. Therefore, an analyte eluted between 0.2 and 0.9 min could be eluted under either isocratic or gradient conditions depending on the system used. Based on these potential variations, differences would be expected in the chromatographic separation between UHPLC instruments.

To evaluate the extent to which dwell volume affects analytes throughout the retention window, the dwell volume was physically modified by adding tubing between the pump and the injector. The resolution of the analyte pairs in the gradient test mix was measured as a function of system dwell volume (Figure 1). A change in resolution of 0.5 was chosen as an acceptable amount of variation. At this level of resolution variation, a dwell volume change up to 30 µL should not require method translation and adjustment. However, if the separation of interest cannot tolerate a resolution change of 0.5 between the critical pair, the amount of dwell volume variation acceptable would decrease as well.

As expected, instruments with the same configuration and from the same manufacturer typically will not require method adjustment because of dwell volume. However, variations in tubing internal diameter between the pump and injector can produce a dwell volume difference that would require method translation and adjustment to maintain the chromatographic separation. Based on these findings, it is recommended to measure the dwell volume of the UHPLC system in which the method will be run and define the dwell volume used during method development within the analytical method.

Extracolumn Dispersion

In addition to dwell volume, the extracolumn dispersion can be found to vary

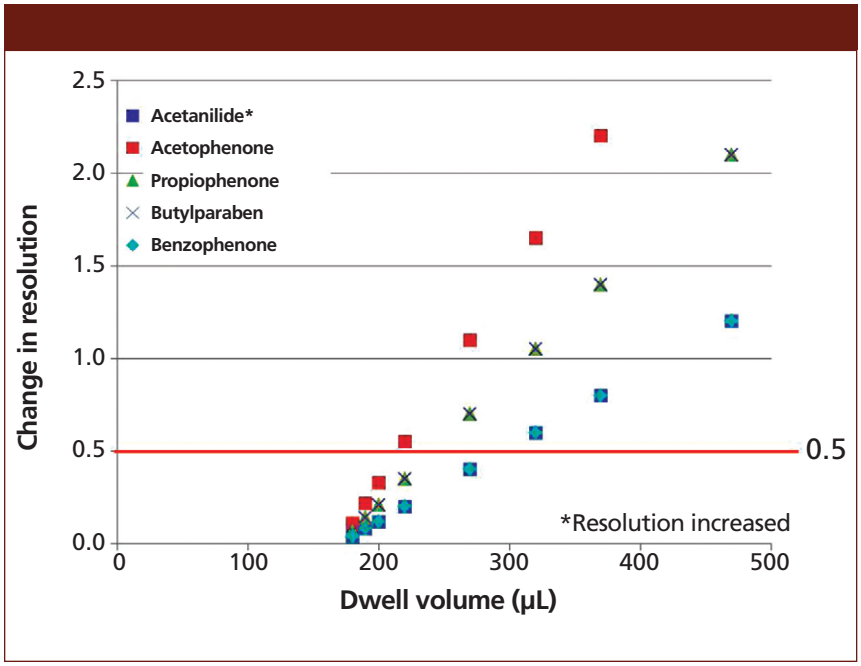


Figure 1: Effect of dwell volume on resolution.

between instruments producing differences in peak variance. The length and internal diameter of the connecting tubing and detector cell volume contribute to the ECD of the system (15). Although ECD contributes to band broadening in HPLC separations, as the variance related to the column decreases—as is the case with most UHPLC methods—the variance contributions from the system have an increased impact on the total peak broadening (14):

$$\sigma^2_{total} = \sigma^2_{ec,before} + \sigma^2_{column} + \sigma^2_{ec,after} \quad [3]$$

Although a large amount of research has addressed the effect of ECD on isocratic separations, few studies have addressed the impact on gradient separations. It is expected that the impact of the ECD will be less for gradient separations than for isocratic separations because of the gradient focusing that occurs, but this focusing cannot completely negate the contributions from the extracolumn dispersion (4,14).

The extracolumn dispersion for the UHPLC instruments within our laboratories varied between 12 µL and 50 µL. The majority of the instruments were found to have an ECD of 12–19 µL. A couple instruments were found to have an ECD of approximately 50 µL, which resulted from tubing modifications that were made after installation. This excessive ECD can be easily remedied by changing the tubing, but the variability in ECD should be expected when transferring methods to external

Table I: Summary of the available UHPLC instruments and configuration options

Parameter	Agilent 1290 Infinity (23–25)	Thermo Scientific Dionex UltiMate 3000 RSLC (26–29)	PerkinElmer Flexar UHPLC (30–31)	Shimadzu Nexera X2 (32–33)	Thermo Fisher Scientific Vanquish (34–36)	Waters UPLC (Acquity, H-Class, I-Class) (37–39)
Pump	Binary, quaternary	Binary, quaternary	Isocratic, binary, quaternary	Binary, quaternary	Binary, quaternary	Binary, quaternary
Mixer volume	35 µL, 100 µL, 380 µL	35 µL, 100 µL, 200 µL, 400 µL, 800 µL, 1550 µL	50 µL, 150 µL, 250 µL, 350 µL, 500 µL, 750 µL, 1000 µL	20 µL, 40 µL, 100 µL, 180 µL	25 µL (B), 200 µL (B), 400 µL (Q)	50 µL (A, I), 100 µL (H,I), 380 µL (I)
Detector cell volume	0.6–4 µL	2.5 µL, 13 µL	2.4 µL	2.5 µL, 12 µL	2 µL, 13 µL	0.5 µL
Tubing configurations	Default, ultralow dispersion	Default	Default	Default	Default	Default
Injection type	Flow through needle	In-line split loop (flow through)	Fixed loop	Flow through needle (30, 30ACMP) or fixed loop (30)	Split loop (flow through)	Flow through needle (H, I), fixed loop (A)
Injector volume	20–120 µL	25–500 µL	2–1000 µL	50 µL–2 mL	25–100 µL	10–1000 µL
Column heater	Peltier	Peltier with forced air	Peltier	Peltier with forced air or still air	Peltier with forced air or still air	Peltier

laboratories that may have many different system configurations.

Because the ECD was found to vary between the UHPLC instruments measured, the effect of ECD changes on resolution was evaluated. For both precolumn and postcolumn ECD, the change in resolution for the analyte pairs in the gradient test mix was measured as a function of added ECD (Figure 2). As expected, the change in resolution caused by additional precolumn ECD was less than the resolu-

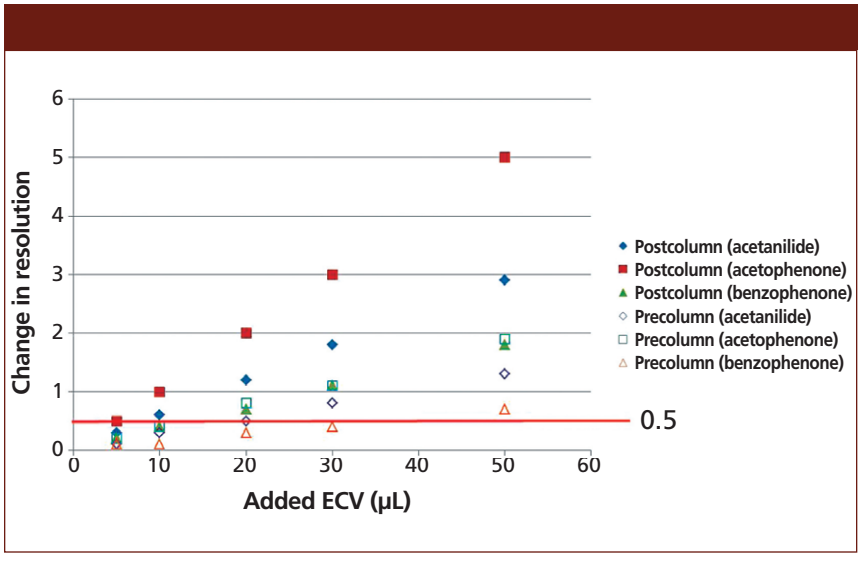


Figure 2: Effect of precolumn and postcolumn extracolumn dispersion on resolution.

tion change with added postcolumn ECD. Although the resolution change was more significant for postcolumn volume, the gradient focusing effect did not completely negate the effects of the precolumn ECD. Again, assuming that a resolution change of 0.5 is acceptable, changes of 10 µL and 4 µL are acceptable for the precolumn and postcolumn ECD, respectively. Therefore, based on the typical variation in ECD (7 µL) for the instruments measured, method translation and adjustment would not be required.

Comparing the effect of dwell volume and ECD on changes in resolution, the resolution change per volume change in ECD is more significant than that for dwell volume changes. However, the magnitude of interinstrument dwell volume variation is much greater than the ECD. Therefore, the dwell volume will have a more significant effect than the ECD on the separation.

Mobile-Phase Mixing

Historically, the mixing efficiency of high-pressure mixing (binary) instruments and low-pressure mixing (quaternary) instruments has been debated. Typically, greater mixing volumes or complex mixers are put in place to compensate for the mixing inefficiencies of high-pressure mixing instruments for solvents of differing viscosity (16). However, high-pressure mixing is more suitable if outgassing occurs during mixing (17). As technology has improved, mixing inefficiencies have decreased. In practice, concerns are still present, particularly in cases when mixing volume is decreased to accommodate fast separations.

The mobile-phase mixing efficiency was assessed by measuring the %B deviation from the theoretical set point (Figure 3). For binary and quaternary systems with the same mixing volume (100 µL), the %B

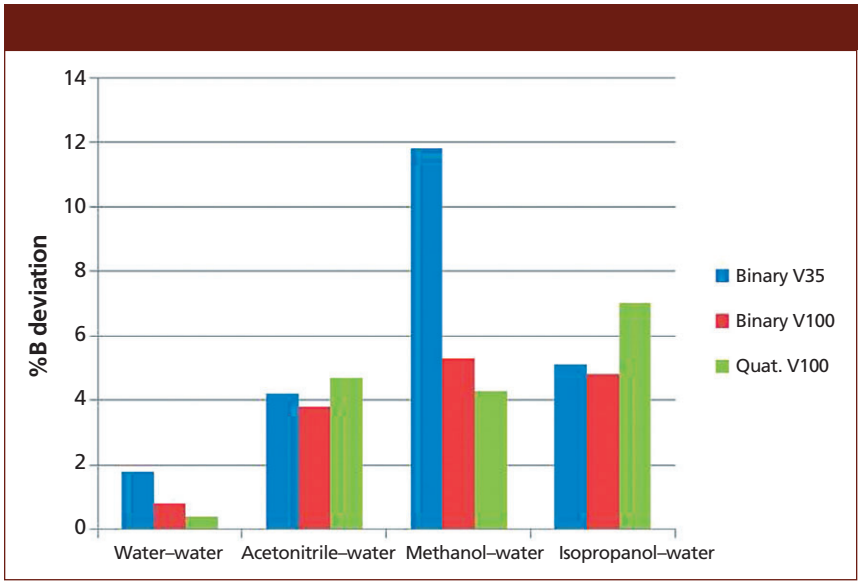


Figure 3: Comparison of mixing efficiency (compositional accuracy) for typical reversed-phase mobile-phase combinations.

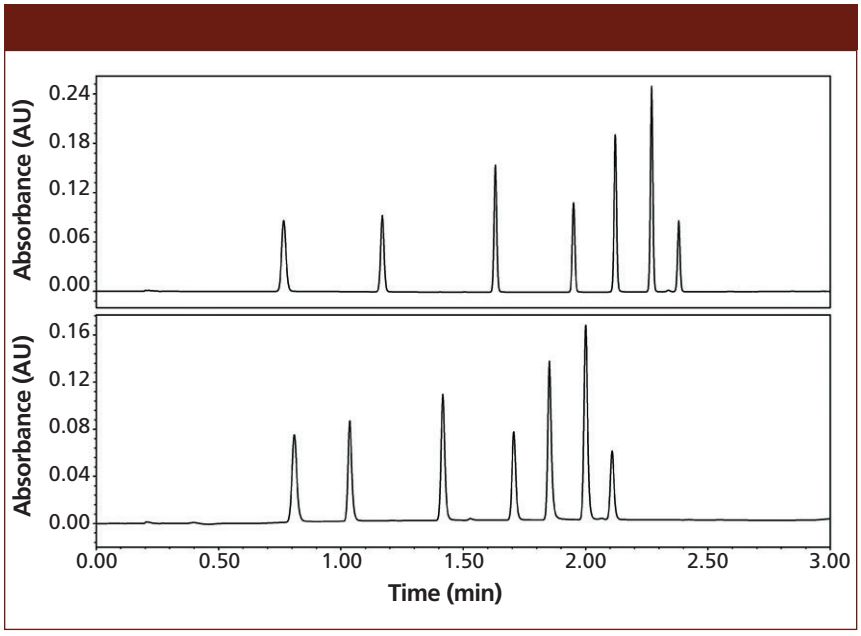


Figure 4: Comparison of chromatographic separation for instruments of differing configuration using the same method conditions.

deviation was similar and without a trend. Furthermore, in most cases similar mixing efficiency was achieved for a high-pressure mixing system with a 35-µL mixer and a 100-µL mixer on a low- or high-pressure mixing system. When mixing water and methanol, the 35-µL mixer on the high-pressure mixing system was not sufficient to give adequate mixing as measured from compositional accuracy. In cases when water and methanol will be used, the mixing volume for a binary system should be at least 100 µL. For separations on the 5-min time scale, the additional dwell volume due to the 100-µL mixer as compared to the 35-µL mixer does not hinder separation speed, but can ensure sufficient mixing. Very fast separations (1–2 min) can use the smaller mixing volumes, but they may lead to mixing inconsistencies in the case of water and methanol mixing.

Method Translation Techniques

Running the gradient test mix method on instruments with differing configurations was found to produce significantly different chromatography (Figure 4). Because of this variation, method translation and adjustment is required. As previously discussed, several method translation techniques have been suggested in the literature and by instrument manufacturers (5,7–10). One approach is to adjust the isocratic hold time or injection delay to account for the interinstrument differences in dwell volume. Using the gradient test mix and pharmaceutical samples, this method was found to produce equivalent chromatographic retention. Although the retention was comparable, in some cases the critical pair resolution was inconsistent (Figure 5). This resolution variation may be due to other system differences such as actual column temperature, column axial temperature gradient, and extracolumn dispersion, which are not accounted for by adjusting the dwell volume. For highly complex methods and methods that are highly sensitive to method conditions, the ability to translate or adjust the method by simple techniques becomes more difficult.

In addition to the dwell volume, analysts may need to account for the wash-out volume of the system. This volume is related to the mixer and manifests as a difference in the volume (time) over which the gradient changes (7). The difference results in an S-shaped transition or a Z-shaped transition depending on the wash-out volume. Upon determination of the wash-out volume ratio between the instruments of interests, the method was modified to account for the dwell volume differences and the wash-out volume ratio. Using the gradient test mix to assess this method translation technique, it was found that the interinstrument retention time deviation was greater than when using dwell volume alone (Figure 6). The wash-out volume ratio could be modified to align the chromatographic retention times on the two instruments, but this approach deviates from first principles of method translation and should be considered method development.

Based on the comparison of these method translation and adjustment techniques, the modification of the method based on dwell-volume differences has proven to be useful for the separations evaluated. To allow for dwell-volume adjustments in the method, an initial isocratic hold should be included in the method that can be adjusted to accommodate instruments with differing dwell volumes. Additionally, the dwell volume of the method development system should be included in the method to allow for accurate adjustments. This method does not account

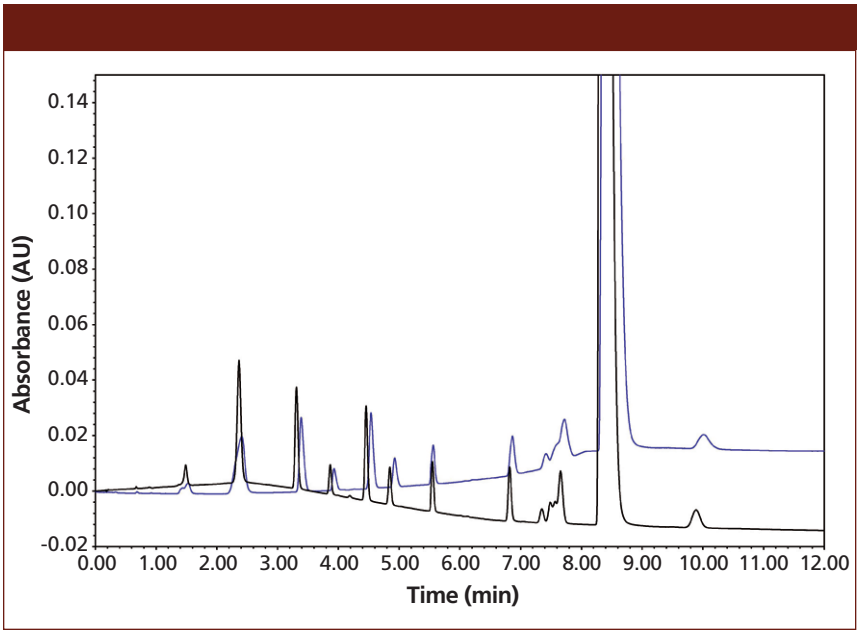


Figure 5: Comparison of chromatographic separation of a pharmaceutical sample on instruments of differing configuration with dwell volume adjustment. Blue trace: manufacturer 2, binary, original method; black trace: manufacturer 1, quaternary, injection delay equivalent to a 100-µL dwell volume.

for thermal differences, however, and they must be considered during the method validation studies if they are expected to affect the chromatographic separation.

Regulatory Considerations

Although method translation and adjustment can help ensure consistency of the chromatographic separation across differing UHPLC configurations, the method and modifications still need to comply with regulatory guidelines. According to *United States Pharmacopeia* (*USP*) <621>, the method can be adjusted for dwell volume and column temperature (±10 °C) (18). Although these modifications are acceptable, the adjustments should still be covered by the method validation robustness studies to allow for translation of the method during method implementation. Additionally, a method equivalency focus group as part of the IQ Consortium suggested that method modifications, including dwell volume, should be included in method validation robustness studies to allow for adjustments between instruments (19).

Additional method translation techniques have been developed by instrument manufacturers to compensate for instrumental differences and are promoted as not requiring additional robustness studies, but to date the acceptability by regulatory agencies is unknown (20–22). Although these translation techniques are based on first principles, their use without method validation robustness study coverage has yet

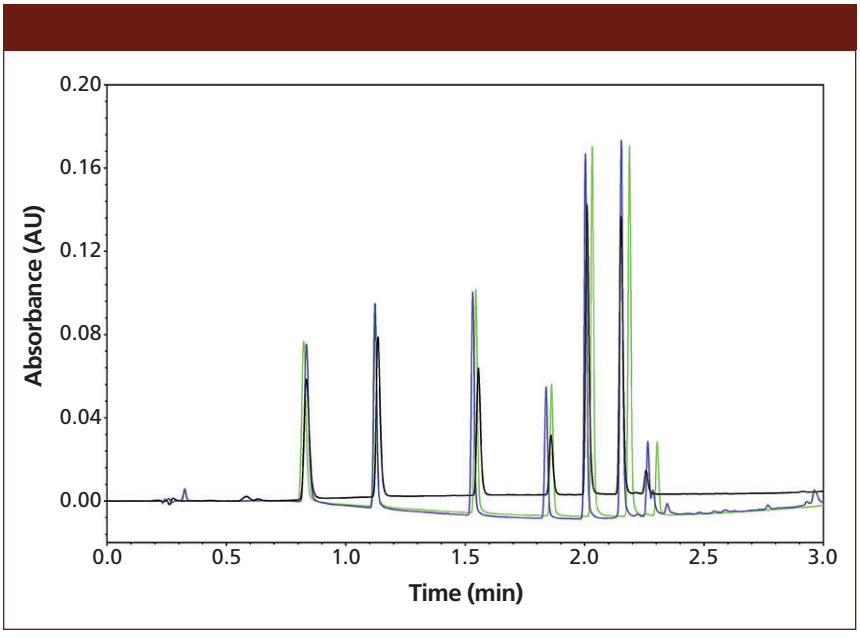


Figure 6: Comparison of chromatographic separation of gradient test mix on instruments with differing configuration. Black trace: system manufacturer 2, binary, original method; blue trace: manufacturer 1, quaternary, injection delay for dwell volume; green trace: manufacturer 1, quaternary, injection delay for dwell volume and gradient adjustment for wash-out volume.

to be accepted by the regulatory agencies. This lack of acceptance is not specifically because of the agencies disagreeing about the principles, but because of the hesitation of companies to submit methods that use this technology.

To stay within the regulatory guidelines and also allow method flexibility, the method should be evaluated over the entire range of expected dwell volumes. Within the method robustness studies, physically alter the dwell volume of the system and adjust the initial method isocratic hold time to maintain a constant isocratic step. Also, if a method is sensitive to the column temperature, assess the method at varying temperatures and include a temperature range within the method to allow for modifications to maintain the chromatographic performance specified within your system suitability criteria. Although most often the extracolumn dispersion should not vary by more than is tolerated by a typical UHPLC gradient separation, knowing the different system configurations that the method will be run on will allow the method developer to assess the potential impact.

Conclusions

The variation in dwell volume, extracolumn dispersion, and mixing efficiency were evaluated for the Waters H-Class and Agilent 1290 instruments present in our laboratories. For these method conditions,

the greatest interinstrument variation was found to be caused by the dwell volume. Variations in ECD and mixing efficiency were observed, but at the expected level of variation neither is expected to significantly affect gradient separations. A dwell-volume variation of less than 30 µL, a postcolumn ECD variation less than 4 µL, and a precolumn ECD variation less than 10 µL are not expected to require method translation or adjustment for the majority of gradient separations. Because the impact of these instrumental variations is dependent on the specific separation, these generalizations should be confirmed for the specific method.

In this study, the dwell volume and wash-out volume ratio method translation and adjustment methods were evaluated. The method found to be most useful for the methods evaluated was adjustment of dwell volume. To accommodate the need to adjust dwell volume, adding an initial isocratic hold in the method can ease the method translation–adjustment process. A further alternative is to develop methods on a column with a more modest column volume (that is, increased column internal diameter) to reduce the impact from instrumental parameters. Because of varying method complexity and analyte sensitivity to instrumental parameters, the specific method required for adjustment is expected to vary. To allow these types of method adjustments, the expected variations should be evaluated during the method validation robustness studies. The advantages in increased efficiency and shorter analysis time for UHPLC methods with columns of small volume and particle size are evident, but the time benefits of this technology have not been fully realized in a regulated environment because of the additional time required to assess method robustness. This missed opportunity points to the balance required between efficiency gains and increased impact from instrumental variations when using smaller column volumes.

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PRODUCTS & RESOURCES

Natural products applications notebook

An applications notebook from Waters Corporation reportedly describes applications with experimental conditions for the LC and LC–MS analysis of samples of botanical, traditional medicine, marine, and bacterial or fungal origins. According to the company, the application notebook is available for download on the company's website.

Waters Corporation,
Milford, MA.
www.waters.com/naturalscience

CCC and CPC systems

Countercurrent chromatography (CCC) and centrifugal partition chromatography (CPC) systems from AECS-QuikPrep reportedly are designed for ionic liquid usage. According to the company, the systems are suitable for the purification of precious metals, lanthanides and actinides, and natural product and synthetic organic compounds.

AECS-Quick Prep Ltd,
United Kingdom.
www.quattroprep.com

LC system

Agilent's 1260 Infinity II liquid chromatography system is designed to provide increased efficiency and ease of integration into existing laboratory environments. According to the company, the system's columns and supplies allow tracking of critical method information and offer ergonomic designs for system usability.

Agilent Technologies,
Santa Clara, CA.
www.agilent.com/chem/infinitylab

Automated SPE system

The TurboTrace ABN Parallel SPE system from FMS is designed to automate the manual steps in the sample preparation process. According to the company, the multicartridge, multifractionation system automatically extracts semivolatile analytes such as phenols, PAHs, phthalates, explosives, and pesticides in various matrices.

FMS, Inc.,
Watertown, MA.
www.fms-inc.com

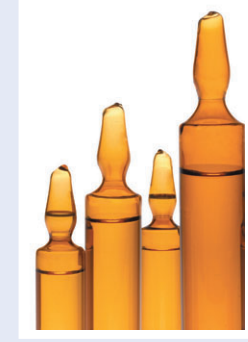
Thermal desorption instruments

The xr[1] thermal desorption instruments for GC–MS from Markes International are designed to preconcentrate organic vapors sampled using sorbent tubes, canisters, and on-line air streams. According to the company, features include the ability to quantitatively recover compounds from C_2 to C_{44}, a water-management module for on-line monitoring of humid air streams, and redesigned instrument control software.

Markes International, Llantrisant, UK. www.markes.com

CRMs for EPA Method 525.3

Certified reference materials (CRMs), developed specifically for EPA Method 525.3, are available from Restek. According to the company, the standards include the required analytes and no unnecessary compounds, and are formulated to provide stability in concentrations convenient for dilution.



Restek Corporation,
Bellefonte, PA.
www.restek.com

HPLC bioethanol analyzer

The BioEthanol Analyzer from Shimadzu is based on the company's Prominence-i HPLC system and is designed for real-time monitoring of the fermentation process in bioethanol production. According to the company, remote monitoring of the analyzer via the company's i-Series web interface or LabSolutions Direct software provides instrument status and chromatogram information using any smart device or PC.

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

Air samplers

Thermosorb-N air samplers from Ellutia are designed to monitor airborne nitrosamines in the workplace. According to the company, as air is drawn through the air sampler, it captures any nitrosamines present so that they can be analyzed, and levels can be reported.

Ellutia,
Charleston, SC.
www.ellutia.com

HPLC column

Machery-Nagel's NUCLEODOR C18 Gravity-SB HPLC column is designed for analytical separation of polar compounds such as antibiotics, water-soluble vitamins, and organic acids. According to the company, the column is a monomeric octadecyl modified phase that features hydrophobic and polar selectivity.

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

Active inlet replacement cartridge

The Opti-Max 600 bar active inlet replacement cartridge from Optimize is designed with 316 stainless steel, PEEK, and zirconia for compatibility in 400-bar and 600-bar applications. According to the company, the cartridge's zirconia ball travel is minimized, allowing the cartridge to exhibit low pulsation.

Optimize Technologies, Inc.,
Oregon City, OR.
www.optimizetech.com

Portable GC–MS analyzer

PerkinElmer's portable Torion T-9 gas chromatography–mass spectrometry analyzer is designed to be carried in the field. According to the company, the analyzer enables rapid screening of environmental volatiles and semivolatiles, explosives, chemical threats, and hazardous substances.

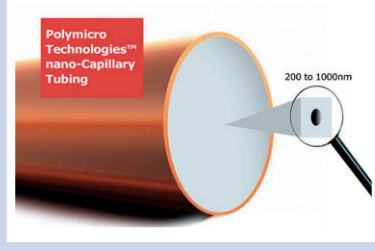
PerkinElmer,
Waltham, MA.
www.perkinelmer.com

Postcolumn derivatization system

Pickering's Pinnacle PCX Sigma Series is designed as an optimized HPLC postcolumn derivatization system for the analysis of samples such as amino acids, carbamates, mycotoxins, and antibiotics. According to the company, the system includes an electronic syringe pump and valves, a quick-change reactor cartridge, a column oven, inert flow paths, a liquid crystal display, and control software. The system reportedly works with all HPLC systems.

Pickering Laboratories,
Mountain View, CA.
www.pickeringlabs.com

Capillary tubing



Polymicro's nano-Capillary tubing is designed with internal diameters ranging from 200 to 1000 nm for scientific, medical, and industrial applications. According to the company, the tubing has a polyimide coating and can interface with existing 375-µm connectors.

Molex – Polymicro Technologies,
Lisle, IL.
www.molex.com

Mass spectrometer

The LCMS-8060 triple-quadrupole mass spectrometer from Shimadzu is designed to provide a scan speed of 30,000 u/s while maintaining mass accuracy and multiple reaction monitoring speeds of 555 ch/s. According to the company, the instrument has a polarity switching speed of 5 ms.

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

Elemental analyzer

Thermo Fisher Scientific's FLASH HT Plus EA-IRMS system is designed for the determination of C, N, S, O, and H isotopic signatures in geoscience, ecological, environmental, and food samples. According to the company, the system includes automation for five elements and dedicated features for isotopic determination.

Thermo Fisher Scientific,
San Jose, CA.
www.thermoscientific.com

Capillary GC columns

Watercol capillary gas chromatography (GC) columns from Supelco, a MilliporeSigma brand, reportedly contain ionic liquid stationary phases that produce a sharp peak shape for water, allowing measurement of water by GC. According to the company, narrow peak widths and optimal peak heights are also produced for other small polar analytes.

Supelco/Sigma-Aldrich,
Bellefonte, PA.
sigma-aldrich.com/watercol

Bioanalysis application note

An application note from Tosoh Bioscience titled "DAR Analysis of Antibody Drug Conjugates Using a TSKgel HIC Column" reportedly demonstrates the separation of unconjugated and drug conjugated trastuzumab samples with baseline resolution using the company's TSKgel Butyl-NPR column. According to the company, the baseline resolution enabled an integration and quantification of different drug payloads in ADC characterization.

Tosoh Bioscience, LLC,
King of Prussia, PA.
www.tosohbioscience.com

GC–MS system

Thermo Fisher Scientific's DFS GC–magnetic sector high-resolution MS system is designed for the analysis of dioxins and persistent organic pollutants. According to the company, the system provides worldwide full compliance with official dioxin, PCB, and PBDE methods (for example, EPA 1613, 1668, and 1614).

Thermo Fisher Scientific,
San Jose, CA.
www.thermoscientific.com/DFS

Solid-core analytical columns

Waters' CORTECS C8 and CORTECS Phenyl analytical columns are designed for HPLC and UHPLC separations. According to the company, the columns are available in 1.6- and 2.7-µm particles and are offered in 50 column configurations.

Waters Corporation,
Milford, MA.
www.waters.com/cortecs

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

Combustion ion chromatograph

Metrohm's combustion ion chromatograph (CIC) is designed to automate the determination of halogens and sulfur. According to the company, the system's autosampler can run both solid and liquid samples, and flame sensor technology is used to measure the light intensity from the pyrolysis oven during combustion.

Metrohm USA,
Riverview, FL.
www.metrohmusa.com/CIC

Headspace syringe

Hamilton's high-temperature headspace syringe is designed for PAL Combi-***xt*** headspace autosamplers from CTC Analytics. According to the company, a specially engineered needle attachment combined with a dynamic plunger tip enables use in applications requiring temperatures up to 200 °C.

Hamilton Company,
Reno, NV.
www.hamilton.com

Sample control system

MicroSolv's U-2D universal two-dimensional sample control system for chromatography is designed to allow instant visual inspection, thermal control, bubble formation control, and a second dimension of insert to vial use or storage. According to the company, the system is compatible with existing 96-well plate autosamplers and is available in deactivated and non-deactivated glass.

MicroSolv,
Eatontown, NJ.
www.mtc-usa.com

Syringe filters

EMD Millipore's Millex syringe filters are designed to prepare samples for analyses such as HPLC, UHPLC, or mass spectrometry. According to the company, the filters are manufactured to pharmaceutical standards with automated equipment and 100% visual and physical inspection, and in-process testing ensures that each filter is made correctly and that the membrane is fully sealed in the device.

EMD Millipore, Billerica, MA.
www.emdmillipore.com

CALENDAR

3–6 July 2016
18th International Symposium on Advances in Extraction Technologies (ExTech'2016) & 22nd International Symposium on Separation Sciences (ISSS'2016)
Torun, Poland
www.extech-isss2016.pl/

17–20 July 2016
PREP 2016—29th International Symposium on Preparative and Process Chromatography
Philadelphia, PA
www.prepsymposium.org

8–12 August 2016
National Environmental Monitoring Conference (NEMC)
Orange County, CA
nemc.us/index.php

20–26 August 2016
21st International Mass Spectrometry Converence (IMSC 2016)
Toronto, Canada
www.imsc2016.ca/

21–24 August 2016
New Zealand Institute of Chemistry Conference (NZIC-16)
Queenstown, New Zealand
www.nzic16.org/

28 August–1 September 2016
31st International Symposium on Chromatography (ISC 2016)
Cork, Ireland
www.isc2016.ie/

28 August–2 September 2016
36th International Symposium on Halogenated Persistent Organic Pollutants (Dioxin 2016)
Florence, Italy
dioxin2016firenze.org/

12–15 September 2016
NANOSTRUC 2016: The 3rd International Conference on Structural Nano Composites
Aberdeen, Scotland
www.nanostruc.info/

AD INDEX

THE ESSENTIALS

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

How to Optimize Key Variables in GC Analysis: Sample Introduction

Every analysis can benefit from the best injection possible. This involves selecting the ideal injection technique and optimizing it for every sample. This article covers which injection technique can be used for different sample and analysis types, which parameters should be optimized, and also some of the drawbacks of the inlet.

Split–Splitless Injection

Split injection is conventionally used for analyses in which the sample concentration is high and the user wishes to reduce the amount of analyte reaching the capillary column by performing an "on-instrument" dilution. However, why perform an on-instrument dilution rather than just diluting the sample further before injection? Capillary gas chromatography (GC) columns are limited to the amount of each analyte that can be introduced onto the column before peak shapes begin to deteriorate. Smaller inner diameter columns and thinner stationary-phase films have lower capacity, and analyte concentrations in the order of a few nanograms on column are typical; therefore, we require a reasonably dilute sample. Performing an on-instrument dilution is preferable because it results in sharper peaks. The split ratio is used to control the amount of analyte reaching the column, which ultimately affects peak width and sensitivity. Typical split ratios are in the range 1:20 to 1:400. When using columns with a thick stationary phase film (>0.5 µm) or a large inner diameter (0.533 mm i.d.) the sample capacity increases and lower split ratios are typical, 1:5 to 1:20. With very narrow GC columns (<100 µm i.d.) split ratios can be as high as 1:1000+.

Splitless injection is used for trace analysis as the entire sample is transferred to the analytical column. Analyte transfer from the inlet to the column is slow, which would result in broad analyte peaks; however, two focusing mechanisms occur that mitigate this problem and are a must for optimizing this injection technique. These involve setting the initial oven temperature 20 °C below the sample solvent boiling point, which ensures that condensation and reconcentration of the analyte band takes place in the column. The sample solvent and column polarity should also be matched—for example, by employing nonpolar hexane as the solvent when using a nonpolar poly(dimethylsiloxane) (PDMS) column. The splitless time should be optimized; too short a splitless time and high boiling analytes will be lost, too long a splitless time risks a large solvent peak. Typical splitless times are 20–90 s.

Sample discrimination and sample degradation occur with both split and splitless injection modes. Because of the vaporizing nature of the inlet solvent, backflash can occur, which can cause sample loss, poor resolution, peak shape problems, carry over, and ghost peaks if the injection volume is not optimized based on the particular sample solvent and inlet conditions (temperature, pressure, and liner volume).

Cool-on-Column Injection

Cool-on-column injection is particularly suited to mixtures containing high- and low-volatility analytes and for trace analysis where quantitative reproducibility is important. The sample solvent is deposited directly onto the column, thereby giving high sensitivity. The initial oven and inlet temperature should be set 10–20 °C below the sample-solvent boiling point to allow focusing of the analyte band, which also has the added advantage of reducing sample discrimination and thermal degradation. Matching the column and sample-solvent polarity is also essential to allow a homogeneous solvent film to be formed in the column; if the polarities are different, broad and often split peaks will be observed throughout the entire chromatogram. Because the entire sample is introduced onto the column, the use of a retention gap serves to protect the analytical column from involatile sample components and also allows increased injection volumes (2–5 µL) by providing a large surface area for solvent film formation. Regardless of column internal diameter, a wide-bore (0.53 mm) retention gap must be used. This technique can be prone to sample overload, is difficult to use with columns with inner diameters less than 0.25 mm, and is complex to automate.

Programmed Thermal Vaporizing

This inlet is best suited to the injection of large sample volumes (100 µL is possible, with injections of up to 1 mL having been demonstrated). In solvent vent mode, when carrying out multiple small injections, the time interval should be sufficiently long to allow almost the entire volume of sample solvent to evaporate. The optimum interval time is generally in the range of 2–20 s. For injection volumes below 10 µL use an unpacked baffled liner. For larger volumes use glass wool or beads packed in a straight liner so that the needle just touches the packing to help both solvent evaporation and analyte trapping. Liners with selective adsorption materials increase the range of components that can be trapped (improved trapping of volatiles). The best results are obtained when the boiling point difference between the sample solvent and analytes is at least 150 °C, with lower boiling solvents (<120 °C) being optimum. The initial inlet temperature should be set 30 °C below the solvent boiling point. Normal split ratios are used (50:1 to 200:1). In general, lower temperatures with higher flows are more desirable. Because of the number of parameters that must be optimized, this is the most complex injection technique, which also makes it expensive. If the parameters are not correctly optimized, there can be a loss of volatiles when using solvent vent mode.

More Online:

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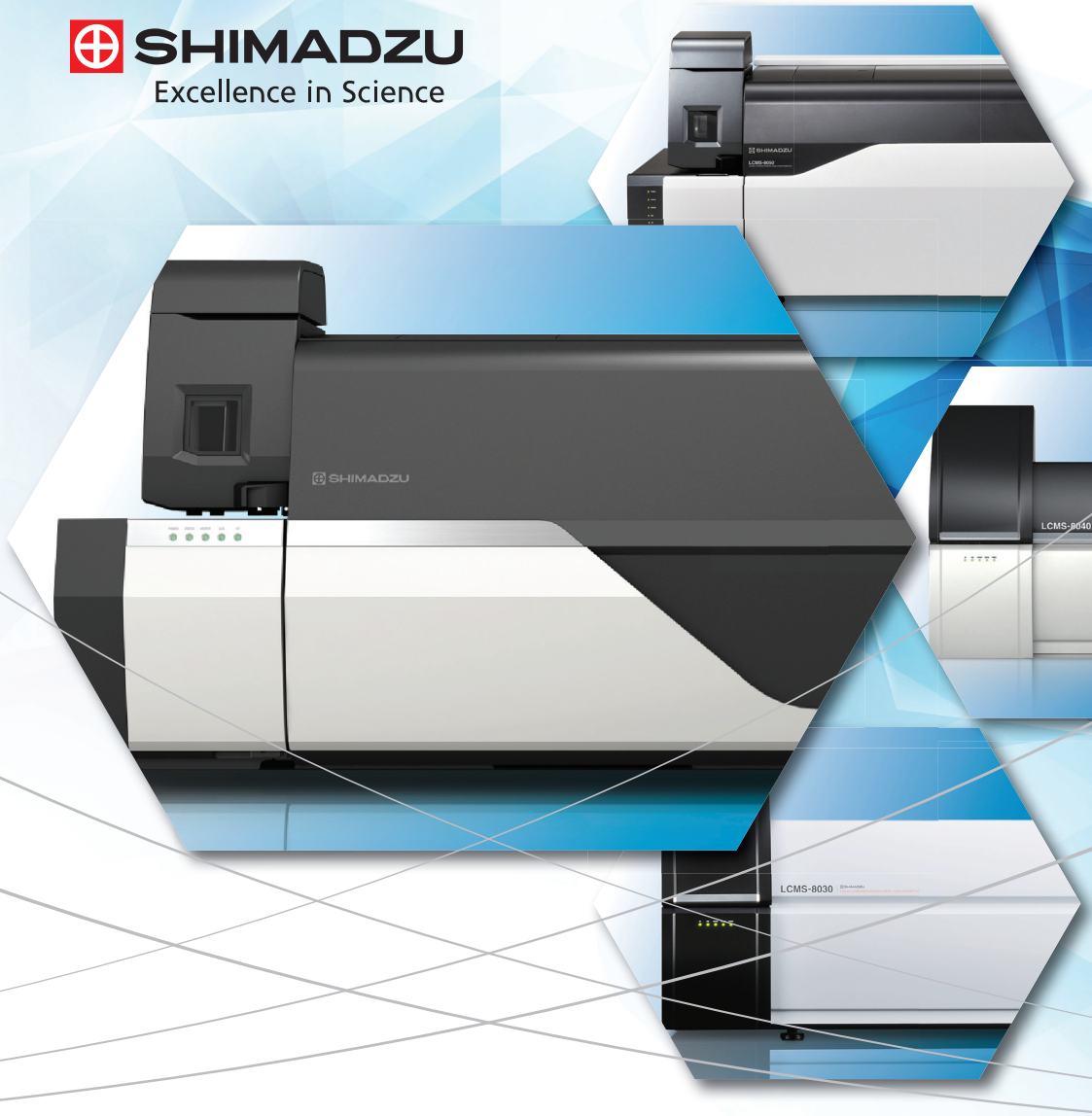

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