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September 2014

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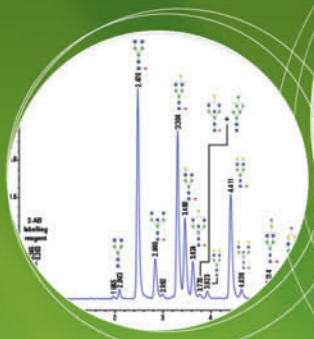

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on advances in mass spectrometry instrumentation that are opening up the field of metabolomics

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on the challenges of developing a HILIC-based SPE approach to analyze biological fluids

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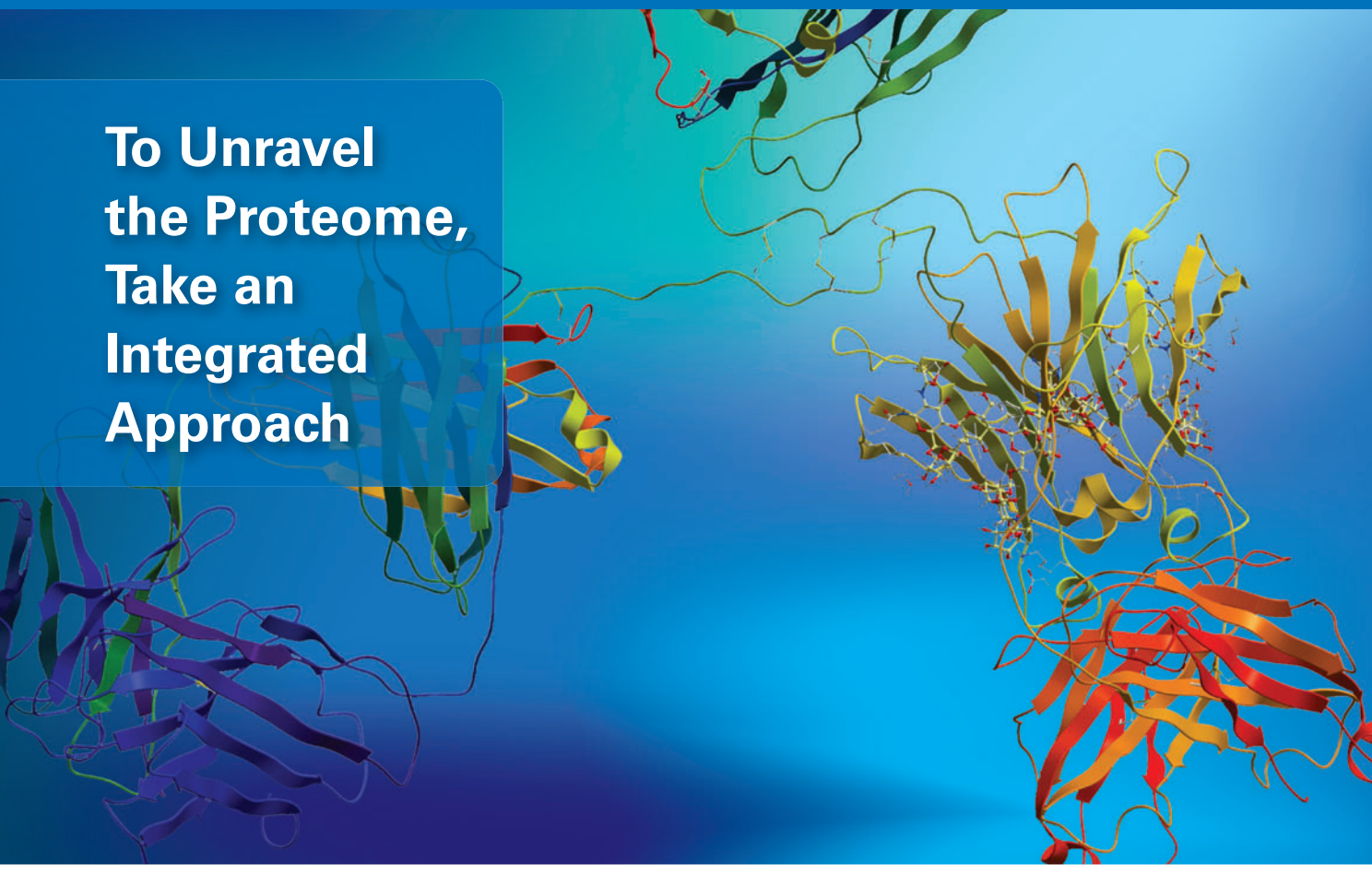

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PEAKS of Interest

André de Villiers Joins LCGC's Editorial Advisory Board

LCGC is pleased to announce the addition of André de Villiers to its editorial advisory board.

De Villiers, an associate professor of chemistry at Stellenbosch University in Stellenbosch, South Africa, is the winner of the *LCGC* 2014 Emerging Leader award. His research interests include fundamental studies that push the boundaries of the chemical characterization of complex mixtures using state-of-the-art techniques such as multidimensional liquid chromatography and gas chromatography combined with mass spectrometry and their applications, primarily to natural product analysis. He has published 50 papers in peer-reviewed journals, and his papers have been cited 925 times.

In addition to the *LCGC* Emerging Leader award, de Villiers has received a number of other awards from the separation science community, including the 2009 Csaba Horváth Memorial Award from the International Symposium on High-Performance Liquid Phase Separations and Related Techniques (HPLC) and the 2012 Chromatographer of the Year award from the Chromatographic Society of South Africa. He has also been invited to deliver lectures at prestigious international conferences, such as HPLC and the International Symposium on Hyphenated Techniques in Chromatography.

De Villiers is currently chairing the Western Cape board of the Chromatographic Society of South Africa. He was also responsible for the organization of two successful conferences that took place in Stellenbosch: the 39th National South African Chemical Institute convention in 2008 and Analitika 2010.

Dwight Stoll Wins Henry Dreyfus Teacher-Scholar Award

The Camille and Henry Dreyfus Foundation has awarded a Henry Dreyfus Teacher-Scholar Award to Dwight Stoll, an assistant professor of chemistry at Gustavus Adolphus College (St. Peter, Minnesota). The Dreyfus awards supports the research and teaching careers of talented young faculty in the chemical sciences at undergraduate institutions. Based on institutional nominations, the program provides discretionary funding to faculty at an early stage in their careers. The award is based on accomplishment in scholarly research with undergraduates, as well as a compelling commitment to teaching, and provides an unrestricted research grant of $60,000.

Stoll, a member of *LCGC's* editorial advisory board, plans to use a portion of the award money for activities related to the development of tools for learning separation science (such as the development of simulators). The remainder of the award money will go to research in fundamental studies related to the advancement of two-dimensional liquid chromatography. ■

LCGCtv New videos from *LCGC*

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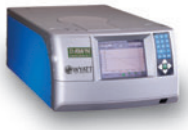

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COLUMN WATCH

Avoiding Reversed-Phase Chromatography Problems Through Informed Method Development Practices: Choosing the Stationary-Phase Chemistry

Many problems encountered executing high performance liquid chromatography (HPLC) methods are a result of decisions made during early method development. This installment of "Column Watch" discusses a critical variable in method development: the choice of stationary-phase chemistry. We discuss two stationary phase classes, embedded polar group (EPG) and perfluorophenyl (PFP), that are highly complementary to alkyl phases from a fundamental molecular interaction point of view. An understanding of the contrasting interactions that these different classes of stationary-phase chemistries provide then leads to more accurate decisions regarding what phases may be most appropriate for a given set of analytes.

Craig R. Aurand, Hugh Cramer, Jacinth McKenzie, and David S. Bell *are the guest authors of this installment.* ***Ronald E. Majors*** *is the editor of Column Watch*

In practice, most chromatographers will initially reach for their favorite C18 column when commencing method development (1). An analyst will possibly evaluate a column using a scouting gradient, adjusting parameters such as organic percentage, pH, and organic modifier type until the desired retention and selectivity are obtained. In cases where the desired results are not obtained, many scientists will then choose their second favorite C18 column and repeat this process. If the practice is unsuccessful, it becomes clear that use of a C18 stationary phase will not provide the necessary separation and the analyst is then faced with a choice: change the stationary phase or force the C18 phase to do something it does not inherently do.

Forcing a C18 phase to provide interactions that are not inherent to a significant degree, such as ion exchange, can be induced by such means as the addition of ion-pairing reagents. Although ion-pair chromatography usually works, it often comes at a significant price. Ion-pairing methods, because of the complexity of the interactions, tend to be difficult to reproduce, transfer, and troubleshoot (2). C18 systems can also be "made" to work by moving to highly complex mobile phases or extreme temperatures and pH levels. Again, such methods tend to lack robustness and ruggedness, causing extensive headaches later in the method lifetime.

A preferred approach is to use alternative stationary phases to obtain the desired retention and selectivity. This approach is often not chosen because of a lack of knowledge of interactions provided by the plethora of stationary phases available from many manufacturers. The objective of this work is to compare and contrast interactions provided by two main classes of alternative stationary phases to commonly used alkyl phases; namely, polar embedded phases and fluorinated aromatic phases. By understanding the interactions that are available on such column chemistries, method development analysts can readily identify phases most appropriate for a given sample. The use of the correct tools facilitates method development and validation. In addition, using the right column generates an increase in method robustness and ruggedness because of the simpler mobile phases that are generally required.

Molecular Interactions

Resolution in any chromatographic system is dependent on efficiency, retention, and especially selectivity. Although a lot of attention has been paid to efficiency in recent years (sub-2-µm and superficially porous particles, for instance), retention and selectivity are of equal or greater importance and are dependent on the inherent chemistry of the stationary phase. For example, if a classic fully porous C18 phase is not providing any resolution of a given critical pair, changing to a sub-2-µm or superficially porous C18 is not likely to provide the desired separation. If a change in selectivity is required, a different set of interactions needs to be invoked to better delineate the differences between the solutes. Interactions that contribute to retention and selectivity can be divided into three main categories: dispersive

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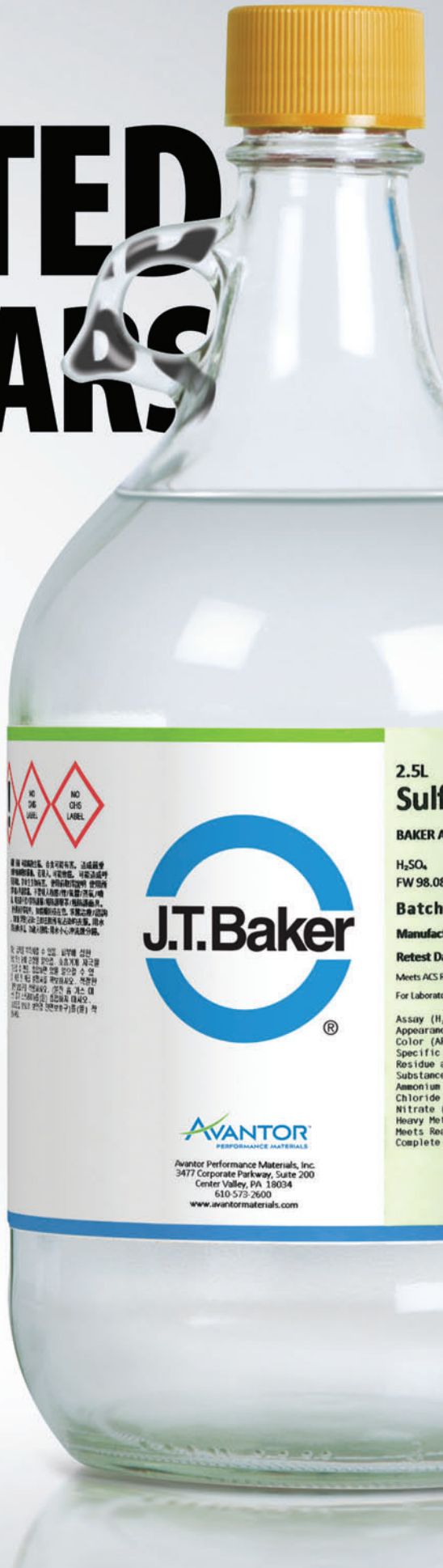

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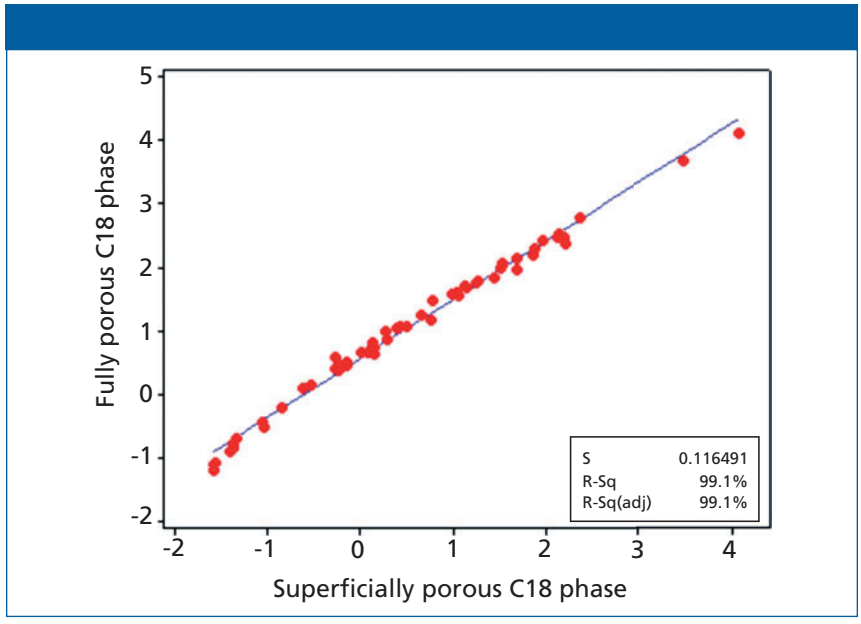

Figure 1: Plot of log *k* versus log *k* comparing fully porous C18 and superficially porous C18 phases. Fully porous phase column: 10 cm × 2.1 mm, 1.9-µm d_p Titan C18; superficially porous phase column: 10 cm × 2.1 mm, 2.7-µm Ascentis Express C18; mobile phase: 10 mM ammonium acetate (70:30 water–acetonitrile) adjusted to pH 4 with formic acid; flow rate: 0.3 mL/min; column temperature: 35 °C; injection volume: 2 µL; detection: MS; mode: ESI+; scan range: 100–1000 *m/z*. 60 component mixture of analytes at 300 ng/mL varying in Log P and pK_a values.

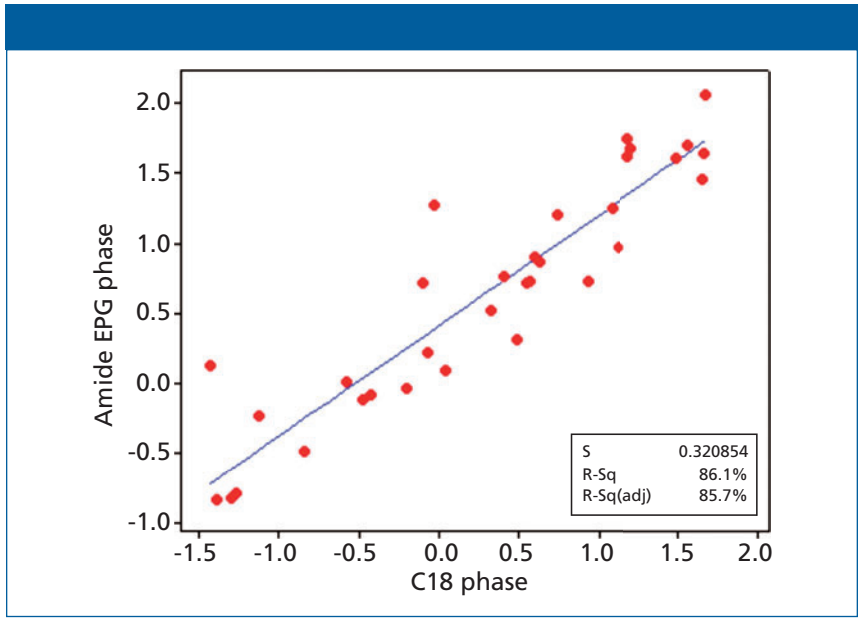

Figure 2: Plot of log *k* versus log *k* comparing C18 and amide EPG phases. Conditions same as in Figure 1. C18 phase column: 10 cm × 2.1 mm, 2.7-µm Ascentis Express C18; amide EPG phase column: 10 cm × 2.1 mm, 2.7-µm Ascentis Express RP-Amide.

interactions, polar interactions, and ionic interactions. Dispersive interactions are transient electronic interactions such as van der Waals forces or London forces. These forces are relatively weak on their own, but collectively are highly responsible for solubility and, thus, partitioning between mobile and stationary phases. Polar interactions such as the various dipole interactions are stronger than dispersive forces and also provide spatial selectivity. For example, if the stationary phase donates a hydrogen atom toward a hydrogen bond, the receiving analyte must be able to accept that hydrogen in a certain direction or range of directions. Such a system provides increased selectivity, not only from analytes that exhibit the ability to accept the hydrogen bond, but also analytes that differ in the spatial arrangement of the hydrogen bond donors. Full charge interactions or ionic interactions provide very strong, relatively long-range interactions and represent the third class of mechanisms.

Modern C18 stationary phases generally provide similar interactions (with few exceptions). The traditional approach of method development for using different C18 columns stems from earlier days when C18 columns from different manufacturers or brands were significantly different. Modern processes for both silica manufacturing and bonding chemistry have greatly lessened the differences between phases, making this practice obsolete in most cases. Plots of log *k* for a given set of test solutes on one column versus log *k* of the same analytes run under identical conditions on a second column are highly effective at illustrating selectivity similarities or differences (3,4). The selectivity difference due to the change in column chemistry is assessed by the degree of scatter in such a plot. A high degree of scatter means that the analytes react in different ways to the column change and thus show different chromatographic spacing or selectivity. Figure 1 shows such a plot comparing two modern C18 columns. Even in a case where a C18 column built with fully porous particles is compared to a C18 phase constructed on a superficially porous particle, little scatter is observed. The reason for this observation is that both phases exhibit the same fundamental chemistry and thus provide very similar interactions. To change selectivity, different sets of interactions are needed. Method development analysts may alter dominant interactions with a given stationary phase chemistry through changes in parameters such as percent organic, organic type, and pH. However, an often omitted variable, the stationary phase, can be more effective. Figure 2 shows a log *k* versus log *k* plot using the same set of analytes as in Figure 1, but here the comparison is between a polar embedded phase and a C18. Note the higher degree of scatter, demonstrating greater selectivity and a higher potential of obtaining different results.

Embedded Polar Group Phases

Embedded polar group (EPG) stationary phases are characterized as exhibiting an alkyl chain much like their C18

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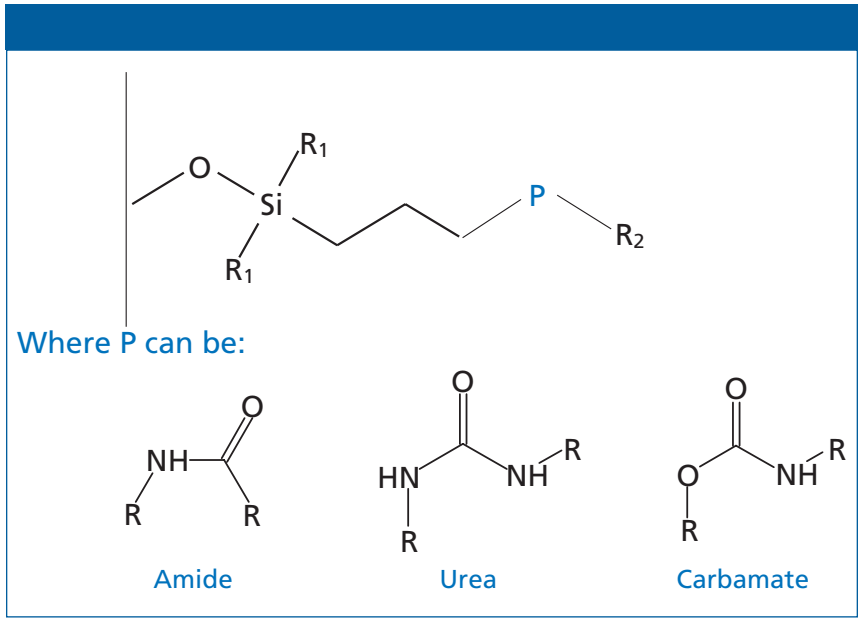

Figure 3: General structures for common nitrogen-containing polar embedded stationary phases.

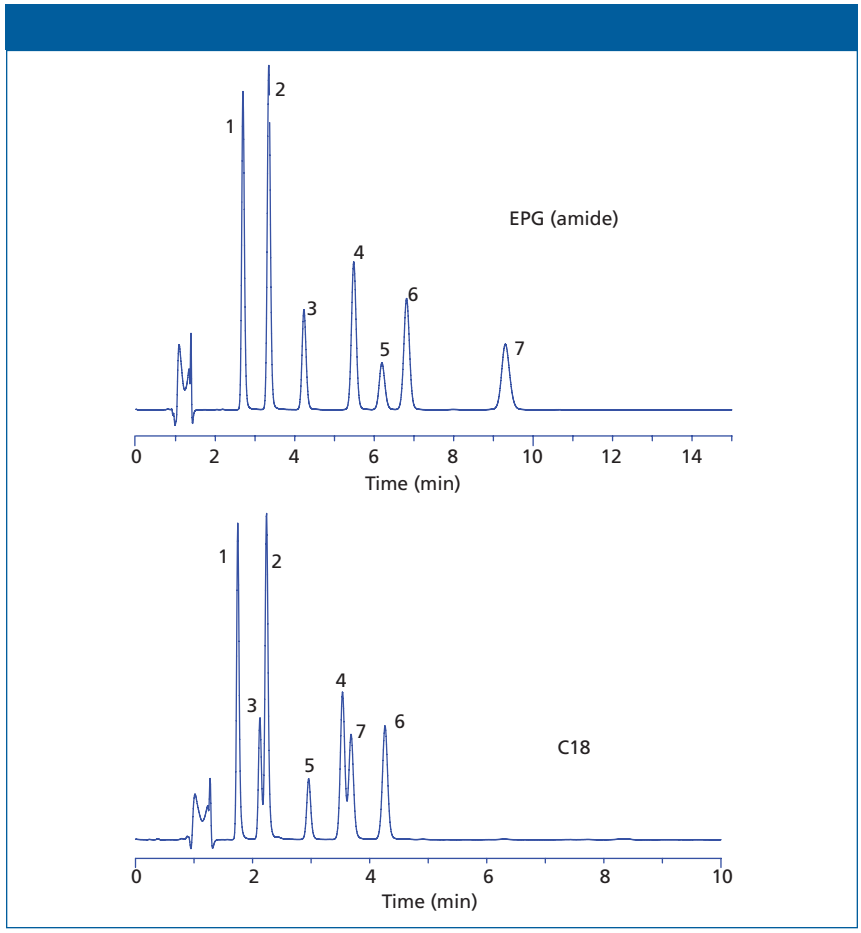

Figure 4: Comparison of retention on an embedded polar group phase versus a C18 phase. EPG phase column: 15 cm × 4.6 mm, 5-µm d_p Ascentis RP-Amide; C18 phase column: 15 cm × 4.6 mm, 5-µm d_p Ascentis C18; mobile phase: 75:25 20 mM phosphoric acid (pH 2.0 unadjusted)–acetonitrile; column temperature: 30 °C; flow rate: 1.5 mL/min; detection: UV absorbance at 270 nm; injection volume: 25 µL; sample: mixture of catecholamines and resorcinols at 50 µg/mL in 20 mM phosphate buffer (pH 2.0). Peaks: 1 = resorcinol, 2 = catechol, 3 = 2-methyl resorcinol, 4 = 4-methyl catechol, 5 = 2,5-dimethyl resorcinol, 6 = 3-methyl catechol, 7 = 4-nitro catechol.

counter-parts, but they include a polar group intrinsic to the chain. Figure 3 provides a generalized structure. For the present discussion only the so-called nitrogen-containing EPG phases such as those containing amide, carbamate, or urea functionalities will be discussed. Other phases such as phenyl ether and polar end-capped phases are often labeled as EPG phases; however, these phases tend to provide selectivities that more closely resemble alkyl phases than the nitrogen-containing EPG phases and are beyond the scope of this column installment (5,6).

Since the original EPG phases were first introduced (7), it has been shown that they provide different selectivity compared to classic C18 phases. To predict where and when a particular stationary-phase chemistry will be most effective, it is imperative to have some understanding of the interactions that contribute to different selectivity they provide.

In an effort to better understand the fundamental interactions that contribute to the differences between C18 and EPG phases, a linear solvation energy relationship (LSER) study was conducted. LSER studies provide a means of interrogating individual contributions of molecular interactions that are collectively manifested as retention and selectivity in chromatographic processes. Refer to reference 8 for an excellent review of LSER methodology. Although classic LSER procedures do not cover all potential interactions (9), the method provides good insight into contributions from hydrogen bonding, lone electron pair interactions, and polarizability. The LSER study presented in this work was conducted by comparing a C18 and an amide EPG phase built on the same silica substrate to emphasize the contribution related only to the surface chemistry. The results presented in Table I show that there are significant differences in the polarization and hydrogen bonding terms. The most significant difference between the phases is the ability of the EPG stationary phase to accept a hydrogen bond. Many analytes such as those containing phenolic and aniline groups can readily donate toward a hydrogen bond. If the stationary phase can accept such an interaction, the result should be observable as a difference in selectivity. Figure 4 shows a set of catechol and resorcinol compounds run on both an amide EPG phase and a corresponding C18 phase. Under the stated

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Table I: LSER comparison of C18 and amide EPG phases

Stationary Phase	Constant (c)	*v*	*e*	*s*	*a*	*b*
Ascentis RP-Amide	-0.496 ± 0.06	2.23 ± 0.07	0.145 ± 0.08	-0.385 ± 0.07	0.068 ± 0.05	-2.51 ± 0.13
Ascentis C18	-0.421 ± 0.09	2.30 ± 0.11	0.267 ± 0.1	-0.731 ± 0.10	-0.256 ± 0.08	-2.10 ± 0.18

- The molar volume (*v*) and electron lone pair (*e*) descriptors are shown to be statistically the same
- The polarization (*s*), hydrogen bonding acceptor (*a*), and hydrogen bond donator (*b*) terms are statistically different

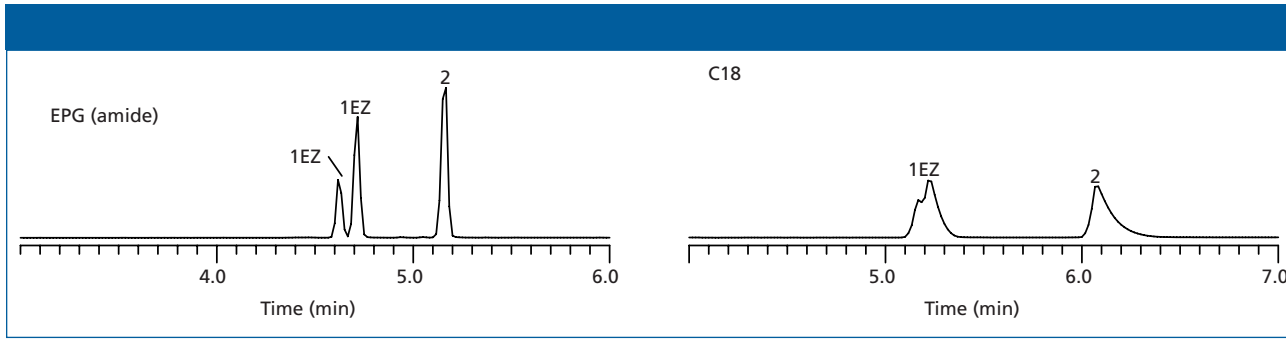

Figure 5: Comparison of peak shapes for hydrophobic basis on EPG and C18 phases. EPG phase column: 10 cm × 4.6 mm, 3-µm d_p Ascentis RP-Amide; C18 phase column: 10 cm × 4.6 mm, 3-µm d_p Ascentis C18; mobile-phase A: water; mobile-phase B: acetonitrile; mobile-phase C: 0.1% formic acid in water (unadjusted, pH 2.7), 0.1% ammonium formate in water (pH 3.0 with formic acid); gradient: 5–85% B over 8.5 min, hold at 85% B for 1.5 min, %C held constant at 10%; flow rate: 1.5 mL/min; column temperature: 40 °C; detection: UV absorbance at 220 nm; injection volume: 10 µL; sample: 50 µg/mL in 50:50 water–acetonitrile. Peaks: 1EZ = 4-hydroxytamoxifen (E and Z) isomers, 2 = tamoxifen.

Table II: Column classification data comparing a C18 and a PFP stationary phase

Column	k_{PB}	αCH_2	αTO	αCP	$\alpha BP_{7.6}$	$\alpha BP_{2.7}$
Discovery HS C18	6.68	1.49	1.55	0.40	0.38	0.10
Discovery HS F5	1.70	1.26	2.55	0.68	0.85	0.34

k_{PB} = retention of pentylbenzene; αCH_2 = hydrophobic selectivity; αTO = shape selectivity; αCP = hydrogen-bonding capacity; $\alpha BP_{7.6}$ and $\alpha BP_{2.7}$ = ion-exchange capacity at pH 7.6 and 2.7, respectively. Discovery HS C18, 15 cm × 4.6 mm, 3 µm, Discovery HS F5, 15 cm × 4.6 mm, 3 µm. For conditions see reference 5.

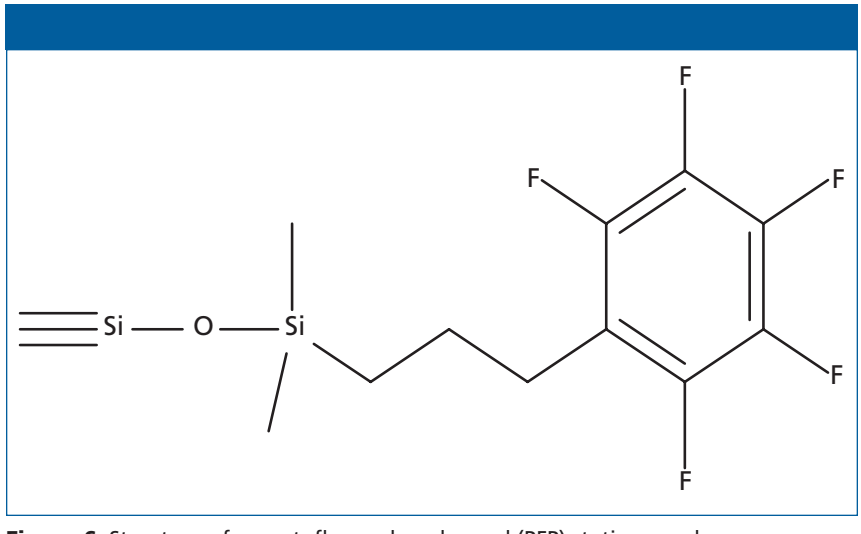

Figure 6: Structure of a pentafluorophenylpropyl (PFP) stationary phase.

conditions, the EPG phase provides superior selectivity that is likely because of the additional hydrogen bonding interactions that help differentiate the target analytes. Similar results are indeed observed in many cases where analytes exhibiting hydrogen-bond-donating moieties are present. This observation is consistent with other column classification studies involving nitrogen-containing EPG phases (5,6,10).

Classic LSER protocols omit ionic interactions as potential contributing mechanisms. An additional noted difference between alkyl and EPG phases is that the latter tend to attenuate ionic interactions with the silica surface. Hydrophobic bases are notorious for tailing issues when C18 phases are used. The tailing is a result of a small amount of accessible ionized (negatively charged) surface silanols that are present on any silica-based stationary phase. Because hydrophobic bases spend a good deal of residence time within the stationary phase, their probability of interacting with an ionized surface silanol group increases, especially at higher pH values. The presence of a polar group within the alkyl chain effectively masks the ionized silanol groups from the analytes through a number of proposed mechanisms (10,11), often resulting in improved peak shape for such analytes. The effect is especially evident at low pH levels. Figure 5 shows a set of hydrophobic bases chromatographed under the same conditions using an EPG and a C18 phase. As is most often the case, vastly improved peak shape is obtained with the EPG phase. When peak tailing is observed, method development scientists often resort to mobile-phase modifiers, such as ion-pairing reagents or surface ionization suppressors (for example, triethylamine) to attenuate the effect. Although these measures can

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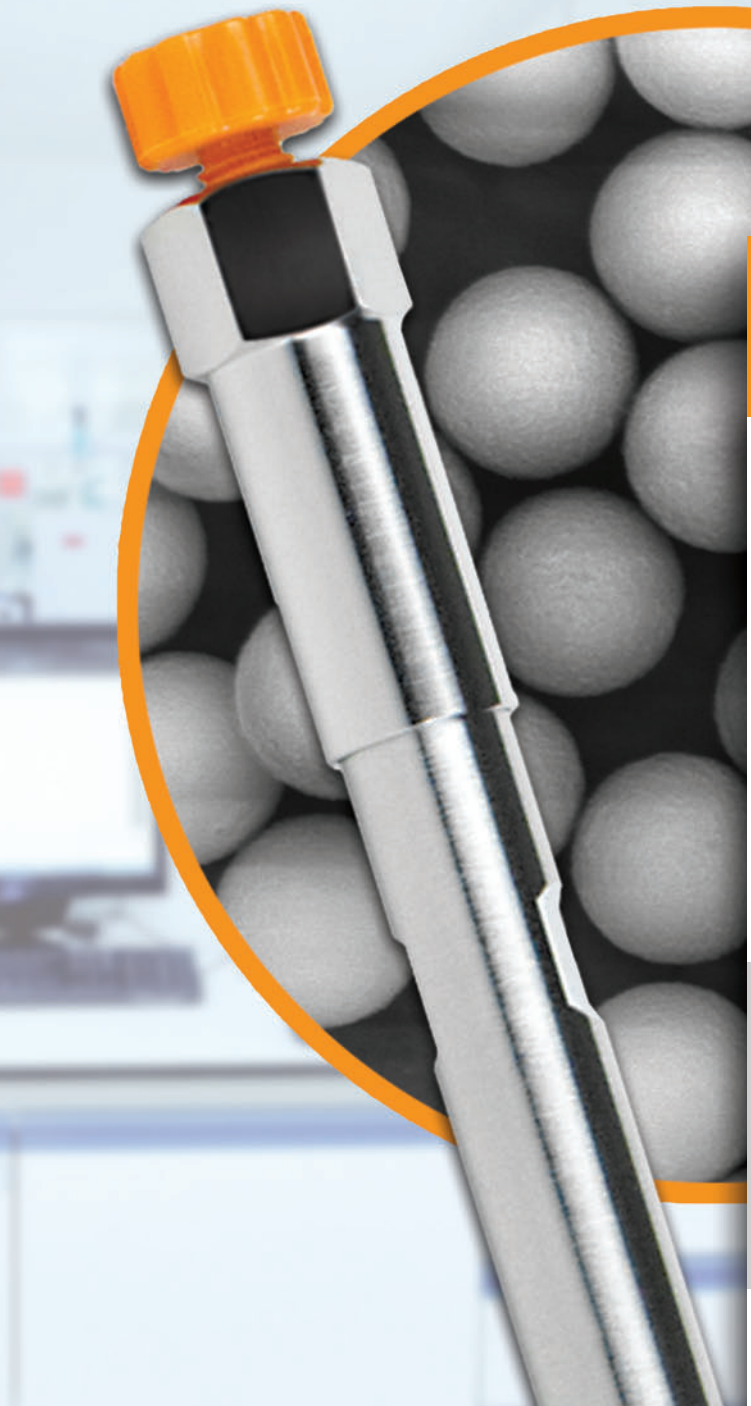

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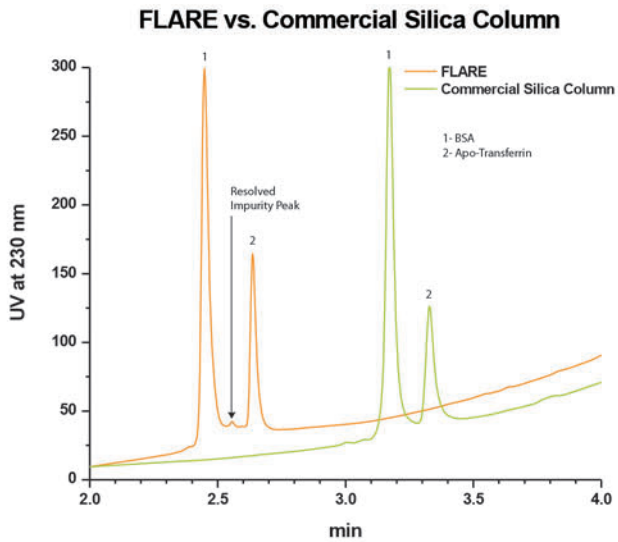

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Solvent B: 1000 ml ACN + 2 ml TFA
Flow Rate: 1 ml/min
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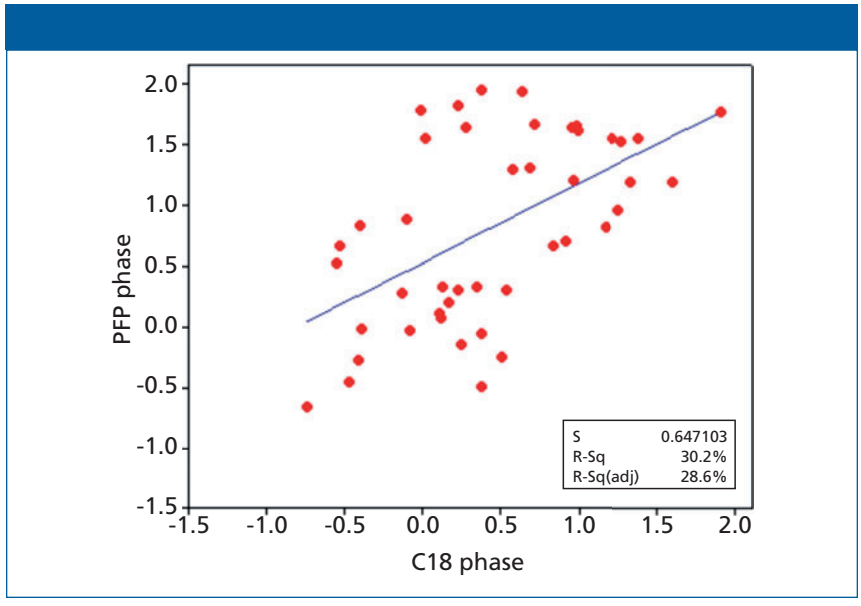

Figure 7: Plot of log *k* versus log *k* comparing C18 and PFP phases. Conditions are the same as Figure 1. C18 phase column: 10 cm × 2.1 mm, 2.7-µm Ascentis Express C18; PFP phase column: 10 cm × 2.1 mm, 2.7-µm Ascentis Express F5.

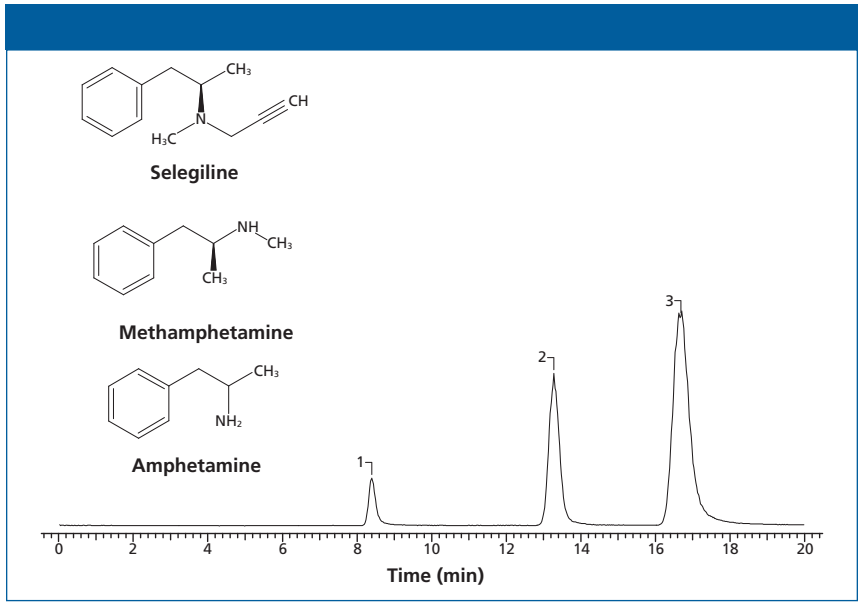

Figure 8: Selegiline and amphetamines retained and separated using a PFP stationary phase. Column: 10 cm × 4.6 mm, 2.7-µm Ascentis Express F5; mobile-phase A: 10 mM ammonium acetate, adjusted to pH 4.0 with acetic acid; mobile-phase B: acetonitrile; mobile-phase composition: 20:80 A–B; flow rate: 1.0 mL/min; column temperature: 35 °C; detection: MS, ESI (+), SIR *m/z* 136, 150, 188; injection volume: 2 µL; sample: 10 µg/mL each in methanol. Peaks: 1 = selegiline, 2 = amphetamine, 3 = methamphetamine.

be effective, they often lead to vastly more complicated chromatographic systems that can be difficult to validate, transfer, and troubleshoot. The use of EPG phases most often results in simpler mobile-phase compositions and improved robustness, ruggedness, and reliability.

As compared to alkyl phases, EPG phases provide additional polar interactions in the form of (predominantly) hydrogen bond acceptance and less ionic interactions. When an analyte mixture that contains hydrogen bond donors is not well separated on a C18 phase, an EPG phase will most often provide the needed selectivity. When hydrophobic bases exhibit tailing on a C18 phase, substitution of an EPG phase often results in improved peak shape. In addition to these two main attributes, EPG phases often provide significant differences in selectivity for polarizable compounds and can be used in 100% aqueous systems (5) without phase dewetting.

Aromatic Stationary Phases

Aromatic stationary phases have also become a popular alternative to alkyl phases (1). In particular, pentafluorophenyl (PFP) phases, because of their high orthogonality compared to C18 phases, have emerged as powerful tools in high performance liquid chromatography (HPLC) method development. In fact, many newer column lines are introduced with both the classical C18 and some version of a PFP phase. Figure 6 shows the general structure of a PFP stationary phase utilizing a propyl linkage. The strong carbon–fluorine dipoles provide significant dipole-dipole interaction potential. The electronegativity of the fluorine atoms generates a partial negative charge around the outside of the ring as well as a concurrent partial positive charge within the ring system providing the potential for charge transfer interactions. The potential for π-π interactions exists with the aromatic ring system as well as ionic interactions with the silica surface because of the increased spacing between bonded ligands relative to a C18 phase. Lastly, dispersive forces are present that may generate classic partitioning. A log *k* versus log *k* plot comparing a PFP and C18 phase is shown in Figure 7. The number of different interactions exhibited by the PFP phase is responsible for the high degree of scatter and thus orthogonal selectivity as compared to C18 columns. In short, the PFP phase provides potential for each of the three classes of interactions described earlier and thus orthogonal selectivity when compared to alkyl phases.

Table II shows a comparison of PFP and C18 phases constructed on the same silica substrate using a standard set of chromatographic conditions aimed at retention contributions from hydrophobicity, shape selectivity, hydrogen bonding capacity, and ion-exchange capacity (12). The largest selectivity differences between the phases are in the ionic and shape selectivity terms. These observations are consistent with other reports (12,13). PFP phases exhibit significantly more ion-exchange potential than their corresponding C18 phases (14,15). Figure 8 shows a chromatographic trace of selegiline, amphetamine, and methamphetamine using reversed-phase conditions. Note that the more polar

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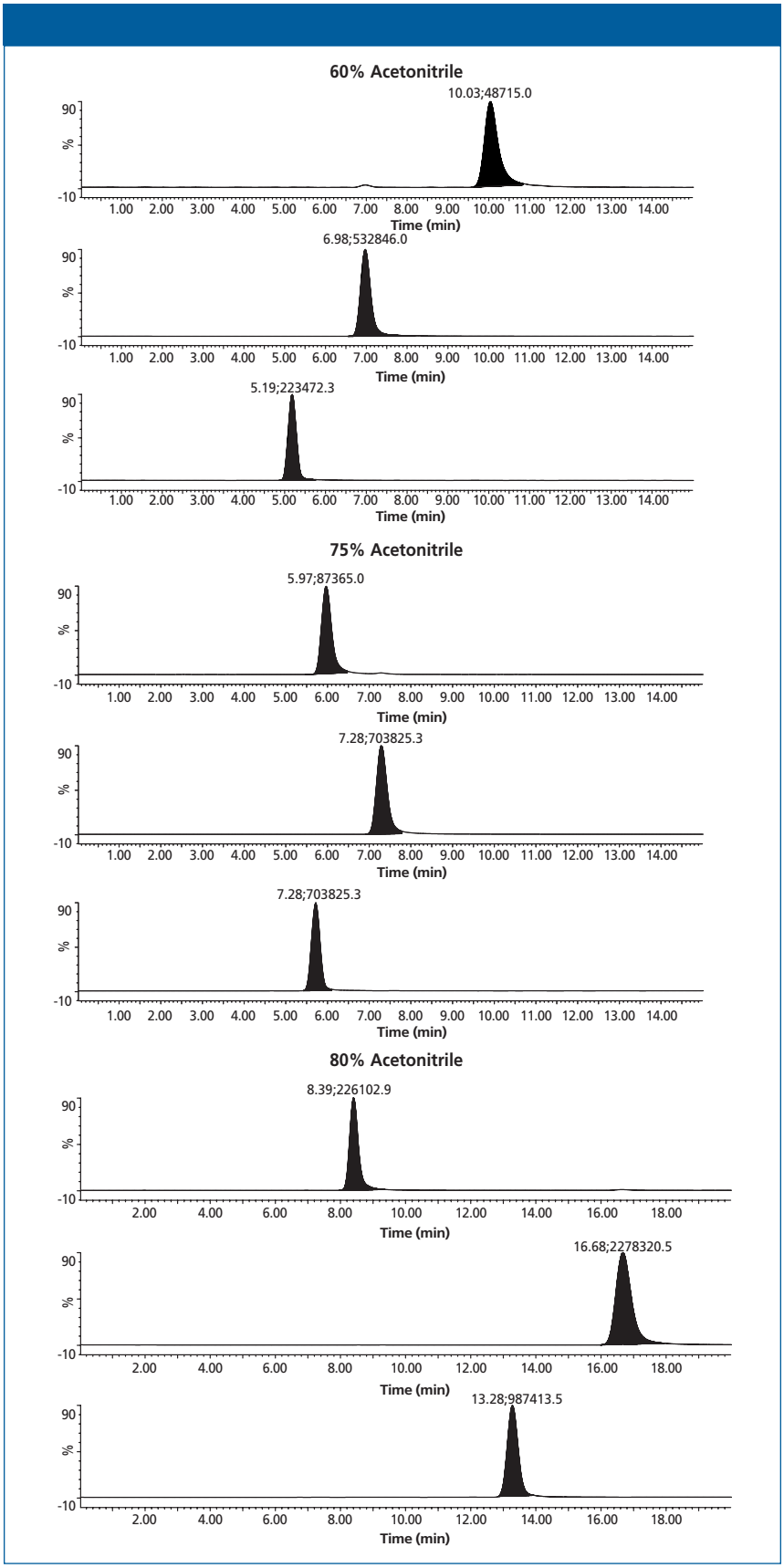

Figure 9: Altering retention and selectivity through manipulation of ion-exchange and percent organic components on a PFP phase. Same conditions as in Figure 8 with the exception of mobile-phase organic component percentage. In each case the analytes in the top, middle, and bottom chromatograms are selegiline, methamphetamine, and amphetamine, respectively.

amphetamines are retained longer than the relatively nonpolar selegiline solute. This is in contrast to what is expected in partition chromatography. The amphetamines, being stronger bases, are retaining predominantly via ion-exchange mechanisms, whereas selegiline is retaining predominantly by partition mechanisms. Because ion-exchange and partition interactions are dependent on different variables, the method developer can tailor the mobile phase to provide the desired elution. The power in this is illustrated in Figure 9, where by adjusting both the organic (partitioning) and the buffer concentration (ion exchange) it is possible to elute selegiline before, after, or even between the two amphetamines.

A common issue with alkyl phases is that they do not work well for analyte mixtures of highly variable polarity. The method development chemist is often confronted by metabolites or degradation products that are vastly more polar than an active pharmaceutical ingredient (API), for example. Figure 10 shows an example of this. A synthetic impurity and potential degradation product, 2-aminopyridine (2-AMP) is run along with its parent API, piroxicam, on a C18 and a PFP phase. Under isocratic conditions, retention of 2-AMP is not achieved on the C18 without extensive retention of the more hydrophobic parent API. It is possible to retain 2-AMP on the C18 through the addition of an ion-pair reagent, however a simpler, more robust, and repeatable system can be developed using the intrinsic ion-exchange properties of the PFP phase.

Another important property of the aromatic phases is their ability to discriminate based on so-called shape selectivity. Alkyl phases as well as EPG phases possess a high degree of freedom. The rigid structure of the aromatic phases presumably limits the freedom of ligand movement and thus how analytes can physically approach and interact with the stationary phase. An illustration of this is shown in Figure 11. Hydrocortisone and prednisolone differ only in one double bond. Their solubilities and functional groups are essentially identical. The largest difference between the analytes is the three dimensional shape. Where the C18 phase struggles to resolve them, the PFP phase provides good selectivity. Aromatic phases, such as PFP, often provide enhanced selectivity for fused-ring systems (steroids, terpenes, vitamins) (16).

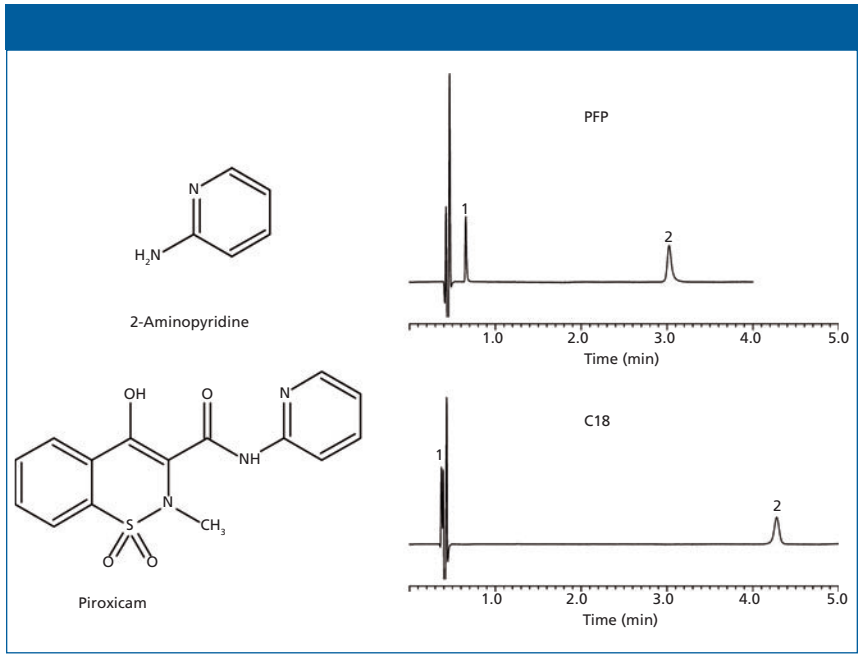

Figure 10: Comparison of retention for compounds of differing polarity on C18 and PFP phases. C18 phase column: 10 cm × 2.1 mm, 2.0-µm d_p Ascentis Express C18; PFP phase column: 10 cm × 2.1 mm, 2.0-µm d_p Ascentis Express F5; mobile-phase A: 10 mM ammonium formate adjusted to pH 3.0 with formic acid; mobile-phase B: acetonitrile; mobile-phase composition: 75:25 A–B; flow rate: 0.5 mL/min; column temperature: 35 °C; detection: UV absorbance at 250 nm; injection volume: 0.5 µL; sample: 5 µg/mL (2-aminopyridine) and 100 µg/mL (piroxicam) in 90:10 water–methanol. Peaks: 1 = 2-aminopyridine, 2 = piroxicam.

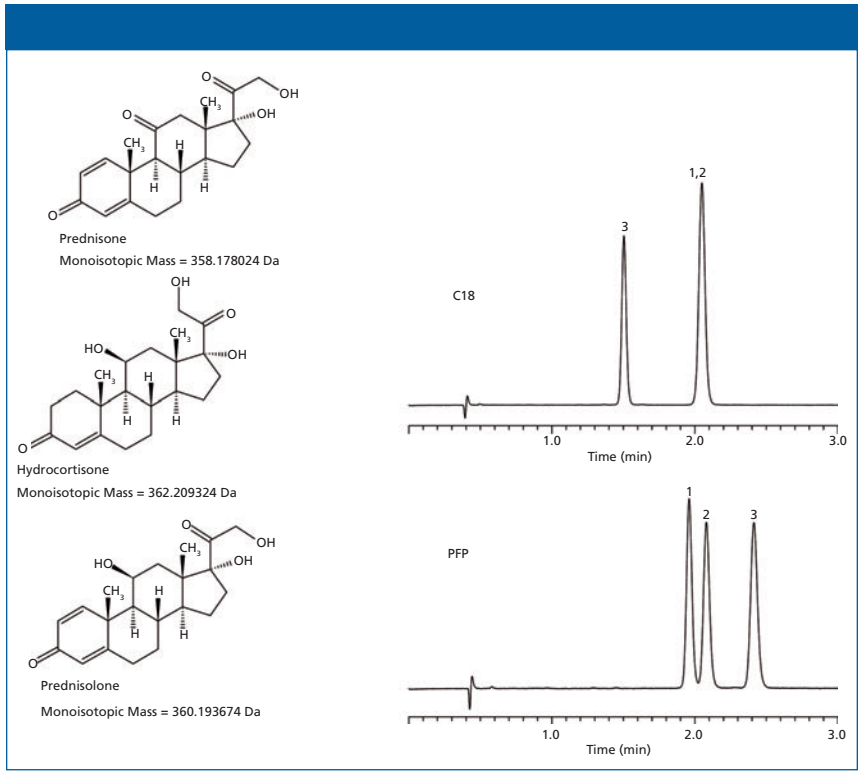

Figure 11: Apparent shape selectivity using C18 and PFP phases. C18 phase column: 10 cm × 2.1 mm, 2.0-µm d_p Ascentis Express C18; PFP phase column: 10 cm × 2.1 mm, 2.0-µm d_p Ascentis Express F5; mobile phase: 50:50 water–methanol; flow rate: 0.5 mL/min; column temperature: 35 °C, detection: UV absorbance at 240 nm; injection volume: 0.5 µL; sample: 50 µg/mL each component in 85:15 water–methanol. Peaks: 1 = hydrocortisone, 2 = prednisolone, 3 = prednisone.

Shape selectivity can also be extended to difficult pairs such as positional isomers.

Aromatic phases such as the PFP chemistry provide enhanced ion-exchange and shape selectivity as compared to their alkyl counterparts. In many cases, the largest differences in a critical pair may be in their ionization constants or shape resulting in incomplete resolution on alkyl phases. PFP and indeed many different aromatic stationary phases often provide the required interactions to discriminate such analytes. The method development scientist often faces a mixture of analytes possessing highly variable polarities. It is often possible to use the aromatic phases and independently adjust both partitioning and ion-exchange mechanisms to tailor the chromatography to a given set of needs. When fused-ring systems and positional isomers fail to separate using traditional alkyl phases, aromatic chemistries generally yield success.

Conclusion

Alkyl phases are excellent tools for sample mixtures that differ slightly to moderately in polarity, but often fall short when partition interactions are not enough. EPG phases have been shown to differ from alkyl phases mainly in terms of properties as well as a decrease in ion-exchange capacity because of silanol shielding effects. When analytes that can donate toward a hydrogen bond are present, EPG phases most often provide alternate selectivity and generally provide improved resolution as compared to alkyl phases. Where hydrophobic bases are present and exhibit tailing on C18 phases, the use of low pH on an EPG phase often provides a suitable solution without extensive use of mobile-phase modifiers.

Aromatic stationary phases provide additional ion-exchange capacity and shape selectivity as compared to C18 columns. When ion exchange is a desirable mechanism of interaction (analytes differ mostly in pK_a values) or when analytes differ mainly in their shape, aromatic phases such as the PFP often provide the necessary selectivity.

The most robust, rugged, and reliable systems are typically the simplest. An understanding of the interactions available from stationary phases other than C18 is paramount to make informed decisions during method development. By choos-

ing the right tools during method development, the simplest, most trouble free chromatographic conditions can be effectively achieved.

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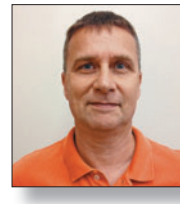
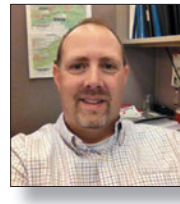


Jacinth A.M. McKenzie *is an analytical project management (Fellow) at Novartis Pharmaceutical Corporation. Jacinth holds a B.S. and MPhil from the University of the West Indies (Mona, Jamaica). In 1997, she relocated to Gainesville, Florida, and joined the cutting edge separation science research team of Robert Kennedy and received a PhD in Analytical Chemistry. She spent a short time at a generic pharmaceutical company and quickly determined that day-to-day stability testing and investigations were not attractive. In 2003, she joined the R&D group at Supelco and developed one of the first columns in the Ascentis family. Always seeking a challenge, she moved into the applications group providing technical support to sales, marketing, production groups, and pharmaceutical companies. She is currently an analytical project manager at Novartis Pharmaceutical managing analytical global activities to support various INDs and NDAs at various Novartis sites and third party contractors.*

Craig R. Aurand *is a principal research scientist at Supelco, providing applications development for both chromatographic separations along with sample preparation. Craig's primary role is in support of bioanalytical method development for small molecules, along with R&D product development for sample preparation with an emphasis in mass spectrometry. He is responsible for the development of sample preparation devices for bioanalytical applications at Supelco, with numerous presentations and publications focusing on novel chromatographic separations and sample preparation techniques. His most recent research has focused on the field of microsampling techniques in bioanalysis.*



David S. Bell *is a manager in pharmaceutical and bioanalytical research at Sigma-Aldrich/Supelco. With a B.S. degree from SUNY Plattsburgh and a PhD in Analytical Chemistry from The Pennsylvania State University, Dave spent the first decade of his career within the pharmaceutical industry performing analytical method development using various forms of chromatography and electrophoresis. During the past 15 years, working directly in the chromatography industry, Dave has focused his efforts on the design, development, and application of stationary phases for use in HPLC and hyphenated techniques. In his current role at Supelco, Dr. Bell's main focus has been to research, publish, and present on the topic of molecular interactions that contribute to retention and selectivity in an array of chromatographic processes.*
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LC TROUBLESHOOTING

Estimating Resolution for Marginally Separated Peaks

How can resolution be determined when peak width cannot be measured?

John W. Dolan
LC Troubleshooting Editor

I have had several reader inquiries lately regarding how to estimate resolution between two peaks in a liquid chromatography (LC) separation when the traditional calculation doesn't work. An example of this is shown for peak pairs A, B, and C in the chromatogram of Figure 1. In each case, the valley between the peaks does not dip below 50% of the height of the smaller peak, making it impossible to measure the peak width at the baseline or half-height. In this month's "LC Troubleshooting" installment, I would like to share a simple technique to estimate resolution that has been in use for many years (for example, see reference 1), but may not be well known because of our dependence on automatic data processing systems today.

Traditional Measurements

Most of us use the method of equation 1 or 2 to calculate the resolution, R_s, of a pair of peaks with retention times t_1 and t_2:

$$R_s = 2 \times (t_2 - t_1)/(w_{b1} + w_{b2}) \quad [1]$$

$$R_s = 1.18 \times (t_2 - t_1)/(w_{h1} + w_{h2}) \quad [2]$$

where w_{b1} and w_{b2} are the baseline peak widths between tangents drawn to the sides of the peaks, and w_{h1} and w_{h2} are the corresponding peak widths measured at half the peak height. That is, the resolution is the difference in retention times divided by the average baseline peak width (thus the factor of 2 in equation 1). The peak width at the baseline for a Gaussian peak is 4σ (4 standard deviations), whereas at the half-height, it is 2.354σ, so the factor in equation 2 is $(2 \times 2.354/4) = 1.18$. Because the half-height peak width is easier to measure (no tangent drawing involved), most data systems use the half-height method (equation 2) to calculate resolution.

The technique of equations 1 and 2 works well when the peaks are well separated, as with Figure 2a, where $R_s = 1.3$. When the resolution drops much below this, it will be difficult to measure the baseline peak width, but it may be possible to measure the half-height width. However, as the resolution drops, the valley between the two peaks rises, and at some point it becomes no longer possible to measure the half-width, either (for example, Figure 2b). Unfortunately, as the amount of peak overlap increases, measurement of resolution becomes more important. This is because it is more difficult to accurately measure peak areas when resolution drops below ~1.2, so system suitability tests often have minimum resolution requirements for partially separated peaks.

Modeling Peak Overlap

One way to estimate resolution for overlapping peaks is to measure the relative height of the valley between the peaks. The ideal chromatographic peak is Gaussian in shape, so we can generate Gaussian peaks (for example, in Excel) and sum their peak heights across a two-peak chromatogram. By changing the amount of resolution (overlap) and the peak heights, we can generate a simple tool to estimate resolution based on the relative height of the valley.

The Gaussian function is as follows:

$$y = (1/\sqrt{2\,\Pi})e^{-x^2/2} \quad [3]$$

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where y is the peak height at point x and x is the retention time measured from the band center (t = 0) in units of the standard deviation of the peak (σ). All the peaks I generated for Figures 2 and 3 range from times of -4σ to +9σ relative to the center of the first peak. Recall that the width of a peak between tangents drawn to baseline is 4σ. The chromatogram for the first peak of each pair is shown in red, the second in blue, and the sum of the two in green. In all cases, the second peak is of constant height, and the height of the first peak is varied (for example, in Figure 2, peak 1 is always twice the height of peak 2). Note that all the chromatograms of Figures 2–4 are computer-generated simulations; real peaks are likely to tail somewhat.

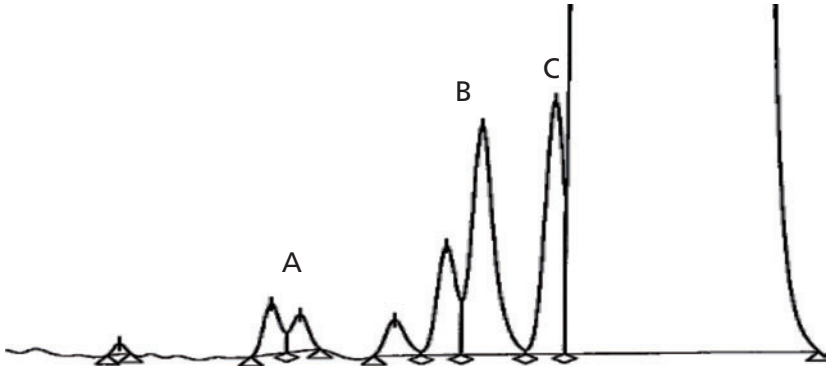

Figure 1: An example of poorly resolved peak pairs A, B, and C.

Table I: The valley-height technique of estimating resolution

	Valley Height*					
Peak-height ratio:	1:1	1.33:1	2:1	4:1	10:1	100:1
R_s						
1.4					11%	27%
1.3			10%	13%	19%	43%
1.2	11%	13%	16%	21%	31%	64%
1.1	18%	20%	25%	33%	47%	87%
1.0	27%	31%	38%	50%	68%	
0.9	40%	45%	54%	71%	91%	
0.8	55%	63%	75%	93%		
0.7	74%	84%	96%			
0.6	91%					

*Height of the valley as a percent of the height of the shorter peak

Now, I have the ability to generate chromatograms as in Figures 2 and 3 for any desired resolution and any relative peak height. I have used such data to construct Table I. The resolution (left-hand column) is known from the inputs. From the data in the spreadsheet (not shown) or the chromatograms, I can determine the peak height (which may be increased by overlap from the other peak) as h_1 and h_2 for the two peaks and the peak-height ratio (always h_1:h_2 in Figures 2 and 3 because the second peak is smaller). The height of the valley (green line) is h_v/h_2 (always the smaller of the two peaks). Each column of data is for one peak-height ratio, with the %-height of the valley shown below for each value of resolution. (I have done some rounding of numbers for presentation simplicity, so if you try to repeat my calculations, your results may vary slightly.) It should be obvious that if peak 2 is larger than peak 1, the data of Table I still apply — just remember to use the smaller of the two peaks for the valley-height ratio.

Application to Example Data

Next, let's see when it is appropriate to use Table I to estimate resolution and

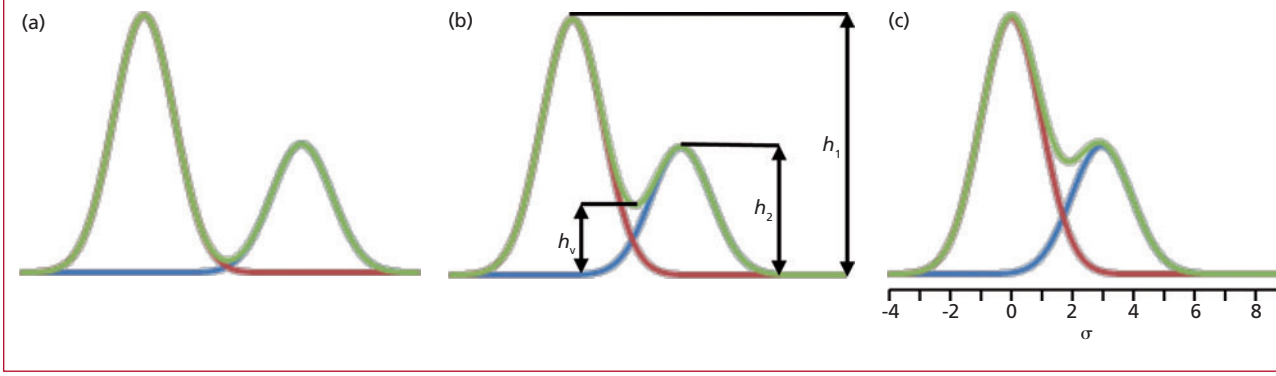

Figure 2: Simulated chromatograms for peak-height ratios of 2:1. (a) R_s = 1.3; (b) R_s = 0.9, h_1, h_2, and h_v are heights of the first peak, second peak, and valley, respectively; (c) R_s = 0.75.

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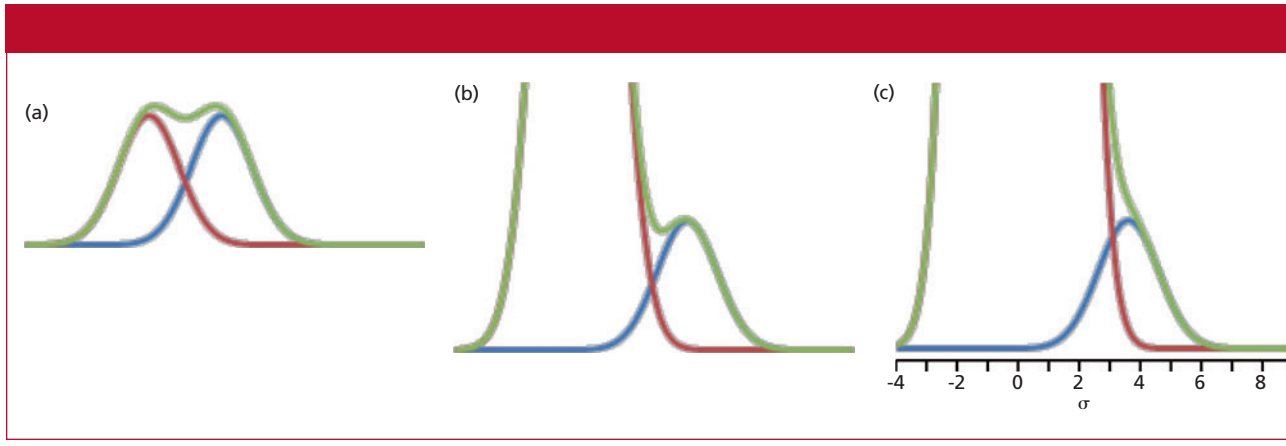

Figure 3: Simulated chromatograms for various peak-height ratios. (a) peak-height ratio = 1:1, R_s = 0.6; (b) ratio = 10:1, R_s = 0.9; (c) ratio = 100:1, R_s = 0.9.

when it is not very useful. First, consider the chromatogram of Figure 2a. In this case, R_s = 1.3 and the first peak is twice the height of the second (h_1:h_2 = 2:1). In this example, either the baseline or half-height peak widths can be measured easily, so there is no need to use Table I for help. A calculation using either equation 1 or equation 2 will give better results when one of them can be applied. You can see from Table I (R_s = 1.3, ratio = 2:1) that the height of the valley is 10%. My suggestion is not to use Table I if the valley is less than ~10%, so I have not even shown valley heights < 10%.

For the chromatogram of Figure 2b (R_s = 0.9, ratio = 2:1), the valley height is 54%. This means that it is not possible to measure the peak width, even at the half-height, so the method of Table I will be quite useful.

Table I works quite nicely when the peak-height ratios and the valley heights correspond to values in the table. When this is not the case, some interpolation will be necessary. In the example of Figure 2c, the peak-height ratio is 2:1 and valley is 86% of the height of the second peak. In the 2:1 ratio column of Table I, 86% falls roughly midway between a valley of 75% (R_s = 0.8) and 96% (R_s = 0.7), so we can assign R_s = 0.75 to this chromatogram (which is the resolution I selected to generate the data). Because of the rather coarse nature of the data intervals in Table I and other uncertainties, I suggest that you don't refine your estimates of resolution by more than 0.05 units using this technique.

Some Limitations to the Valley Ratio Technique

I mentioned above that whenever you can measure the peak width at baseline or half-height, you are better off using the traditional resolution calculation of equation 1 or 2. Let's look at some other examples where the valley-height technique is limited. In the chromatogram of Figure 3a, you can see that there is barely any valley between the peaks for the case of equal height peaks and R_s = 0.6 — the valley height is 91%. You can also see that the peak heights (green) of both peaks are greater than the individual peaks (red and blue) because of severe overlap. I don't think it is worthwhile trying to estimate the resolution with the valley-height technique if the valley height is more than ~90%. I have left a few values with valleys > 90% in Table I to help with interpolation with smaller valley ratios.

As the peak height difference between the two peaks gets larger, the valley-height technique still works, but a larger difference in retention times is required to obtain a reasonable valley. For example, in Figure 3b with a peak height ratio of 10:1, R_s = 0.9 is required to get the same 91% valley as with a 1:1 peak height ratio where resolution of only 0.6 was required (Figure 3a). When a peak height ratio of 100:1 is encountered (Figure 3c), R_s = 0.9 produces only a small bump on the tail of the first peak. At some point when the

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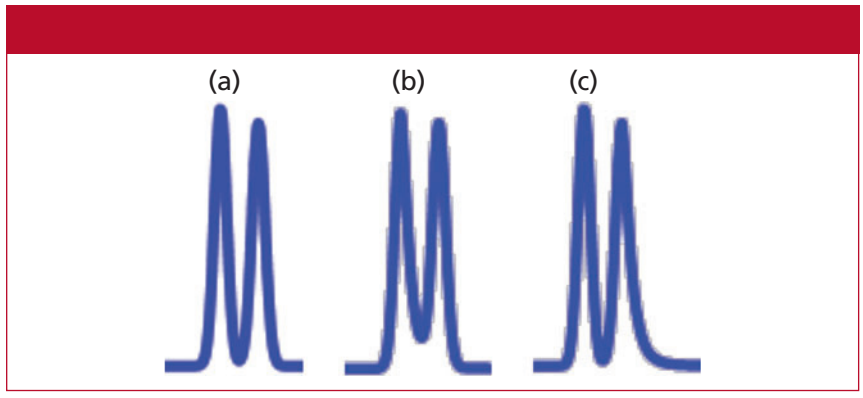

Figure 4: Simulated chromatograms showing effect of peak tailing factors (TF) (for peaks 1 and 2, respectively), on resolution for peak-height ratios of 1:1. (a) $TF_1 = TF_2 = 1.0$; (b) $TF_1 = 1.5$, $TF_2 = 1.0$; (c) $TF_1 = 1.0$, $TF_2 = 1.5$.

peak size ratio becomes too large, even with symmetric peaks, it will not be possible to observe two peaks, whereas with the same retention times and equal sized peaks, the separation would be obvious. Compare the chromatograms of Figures 2b, 3b, and 3c to see this trend for peaks with $R_s = 0.9$.

Peak tailing of the first peak of a peak pair will always reduce the resolution, whether the calculations of equations 1 or 2, or the valley-height technique of Table I is used. This is illustrated with Figure 4, where Figure 4a has $R_s = 1.5$ and both peaks are symmetric, so the tailing factor (TF) is 1.0. In Figure 4b, the peaks have the same retention times, but the first peak has TF = 1.5. My measurements show a valley height of 19%, so for a 1:1 peak height ratio this equates to $R_s \approx 1.1$. On the other hand, tailing only for the second peak, as in Figure 4c, will not affect the resolution. It is only the case where the peak tails into the valley that resolution is reduced.

A Real Example

All of the chromatograms in Figures 2–4 are simulated; so what happens when we try to apply the valley-height technique to a real example? Let's go back to the chromatogram of Figure 1, where the reader was unable to determine resolution between peak pairs A, B, and C using direct calculations. I expanded the chromatogram and measured the peak and valley heights for each pair. For peak pair A, the peak height ratio is 1.36:1 and the valley height is 64%. The height ratio is closest to the 1.33:1 data set in Table I, so I would estimate $R_s \approx 0.8$. For pair B, the height ratio is 2.17:1 and the valley is 50%. Using the 2:1 column in Table I, $R_s \approx 0.9$. For pair C, the peak height ratio cannot be determined, but my guess is that it is at least 100:1 and the valley is 69% of the smaller peak, so this gives $R_s \approx 1.2$. You could interpolate more carefully between the data points in Table I, but as I mentioned above, I would not try to get closer than ~0.05 resolution units using Table I. I don't know the nature of the sample in Figure 1, but it is typical of what might be observed for an impurities analysis of a pharmaceutical product. In such cases it may not be essential, or even possible, to separate all peaks to baseline, but it would be nice to be able to put a number on the resolution of the various peak pairs. Using the valley-height technique, we can do just that, when the traditional approach of equations 1 and 2 was not possible.

Conclusions

When chromatographic peaks are well separated, the traditional technique of calculating resolution based on retention time differences and average peak widths, as in equations 1 and 2, is the preferred way to determine resolution. This would generally apply over the range of peak height ratios of approximately 1:1 to 1:10 and resolution of at least 1.3. When resolution drops below ~1.3, it becomes more difficult to determine peak widths, so an estimate of resolution using the valley-height technique is more convenient. When the valley height exceeds ~40% of the height of the smaller peak, even the

width at half height cannot be determined with confidence, so the valley-height method is the best choice. In the real chromatogram of Figure 1, we were able to estimate the resolution of three peak pairs using the valley-height technique when it was not possible to make the corresponding calculations with equation 1 or 2.

Peak tailing for the first peak of a peak pair will always reduce resolution. In such cases, the valley-height technique may give more realistic values of resolution, as was illustrated with the chromatograms of Figure 4.

From a system suitability standpoint, the valley-height technique can be useful to assess separation quality when a marginal separation is the best that can be obtained, as in Figure 1 for peak pairs A, B, and C. For the example of Figure 2b, there is very little peak overlap at the peak centers, so peak-height measurements may be more appropriate for quantification; alternatively, peak-area measurements, using a perpendicular drop at the valley to separate the peak areas, should also give acceptable results for this example. You could specify in the system suitability requirements that $R_s > 0.9$ should be obtained. For routine work, however, it may be more convenient just to set a limit on the valley height that corresponded to $R_s = 0.9$. For example, you could require that the valley be no more than 55% of the height of the second peak and have the same result without the added step of looking up the resolution in Table I.

There is an interesting paradox here: As chromatographers, we usually are more concerned with peak separation when resolution drops below $R_s \approx 1.5$, at which point we must rely more on estimates than more exact calculations. Conversely, when $R_s > 1.5$, calculations become easier, but once baseline resolution is obtained, accurate measurements of resolution are of less interest. So when accuracy is important, we can't get it, and when it is not as important, it is easy to obtain.

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(1) L.R. Snyder and J.J. Kirkland, *An Introduction to Modern Liquid Chromatography, 2nd edition* (Wiley-Interscience, New York, 1979), Chapter 2.

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 John.Dolan@LCResources.com*

For more information on this topic, please visit www.chromatographyonline.com/dolan

MS – THE PRACTICAL ART

Quantifying Proteins by Mass Spectrometry

The quantification of proteins in a complex biological sample is an important and challenging task. Mass spectrometry (MS) is increasingly used for this purpose, not only to give a global survey of the components and their amounts, but also to precisely and accurately quantify specific target proteins. Here, we review the essential elements of MS approaches to protein quantification and critically compare the available options.

Mark W. Duncan, Alfred L. Yergey, and P. Jane Gale *are the guest authors of this month's installment.*
Kate Yu *is the editor of MS—The Practical Art.*

Proteins are the most abundant macromolecules in biological systems. Together with their smaller relatives, peptides, they are polymers comprising amino-acid building blocks joined through amide bonds. In contrast to the repeating units of other biopolymers (for example, polysaccharides and polynucleotides), the constituent amino acids are diverse in their chemical and physical properties. Consequently, the polymers derived from them are also a complex, chemically and physically diverse ensemble.

This structural diversity lends itself to extensive functional diversity. Proteins serve as antibodies, enzymes, messengers, structural components, and transport or storage molecules. For that reason, the majority of drug targets are proteins. Significantly, the genetic machinery of the cell is tasked with synthesizing proteins. Accordingly, one might argue that much of each cell, and therefore any organism in toto — its structure, function, reproduction, repair, and regulation — relies on proteins. Understanding biology — function or dysfunction, health or disease — is therefore about defining and understanding proteins.

Protein identification and quantification are thereby the two central objectives of many biological and biomedical studies. Historically, these tasks were performed on purified proteins that were exhaustively sequenced (such as Edman) or quantified by the immuno-based western blot or enzyme-linked immunosorbent assays (ELISAs). Today, however, because of advances in mass spectrometry (MS) and the development of a set of global, protein-analysis tools that some call the "proteomics toolbox," improved analytical strategies have evolved, and the objectives of researchers have changed. Typically, investigators now aim to study biological entities at the "systems" level; that is, they seek to resolve and identify a multitude of proteins simultaneously in a single sample and to quantify each in relative or absolute terms. (See definitions in Table I.)

Quantification is an important component of most studies. Defining differences or changes in protein abundances (or, more appropriately, the abundance of specific protein species including isoforms and post-translational variants) between two or more groups or states (such as control and test) is often at the heart of understanding function and regulation.

As the proteomics toolbox evolves, new approaches to protein quantification by MS are continually reported. These methods can be categorized into several major classes, all of which share features and performance characteristics. Furthermore, though some additional considerations are specific to protein quantification, it is also important to state that the process of quantification remains essentially the same, regardless of the nature of the analyte (that is, small molecules versus biopolymers). Consequently, the principles and practices that have guided the development and evaluation of quantitative methods (for example, replicate measurements to characterize the variance of a method) are no less applicable in this setting.

Non-MS Approaches to Protein Quantification

This column installment focuses on MS methods for protein quantification because of their growing importance. Yet it is important to acknowledge the existence of other strategies and that, moreover, in certain settings those alternatives may be the methods of choice. For example, the mainstay for targeted protein quantification for almost 50 years has been the western blot immunoassay,

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To ensure leak-free column connections to oxygen sensitive detectors, such as MSD or ECD where graphite/polyimide ferrules are recommended, use Agilent Self Tightening Column Nuts. If your application requires inertness, choose Agilent UltiMetal Plus Flexible Metal ferrules for connections in the flow path.

MSD's and ECD's are both oxygen sensitive detectors where polyimide/graphite ferrules are preferred due to cleanliness and limited permeability to gas infiltration, yet are a soft material reducing likelihood of mechanical damage to the fitting. Unfortunately, polyimide is a plastic material that expands and shrinks with repeated temperature cycles resulting in the need to be retightened. Self Tightening Column Nuts were specifically designed to keep a consistent spring-loaded tension on these ferrules as they expand with heat and contract with cooling during temperature programmed runs. With Self Tightening Column Nuts, the need to retighten polyimide/graphite ferrules to maintain a leak-free connection has been eliminated.

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For applications that require maximum inertness, UltiMetal Plus Flexible Metal ferrules with standard column nuts are the preferred choice. With a novel design which gently compresses around the column tubing forming a larger sealing surface, UltiMetal Plus Flexible Metal ferrules address the challenges of making and keeping reliable, robust column connections, without damage to the column or fitting.

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2 OVERTIGHTENING OF FITTINGS IS A COMMON OPERATOR ERROR

Knowing that leaks are damaging to the GC system, analysts commonly overtighten GC fittings attempting to ensure a leak-free system. Excessive tightening, unfortunately, causes more problems, including leaks. To avoid overtightening of column connections, the new Agilent Self Tightening Column Nut is installed by hand, without the use of wrenches to provide a leak-free fitting. Overtightening also occurs with the inlet septum nut, which if too tight can cause coring of the septum, creating both contamination and leaks.

Excessive force can deform ferrules such that they can no longer seal – or worse – extrude into the inlet causing chromatographic effects such as peak tailing and adsorption. In extreme cases, metal threads can be damaged to a point that they are no longer capable of sealing. On a GC/MS transfer line, this is an expensive repair. Tightened by hand, the Agilent Self Tightening Column Nut has a wing nut design to prevent overtightening, a common error when using a wrench. The inlet septum nut is another problem spot for overtightening. Here, cranking down on the soft septa material will cause it to core faster and leak sooner. To properly tighten the septum nut retainer on Agilent GCs, watch the C-ring clip on the septum nut and once it stops turning with the nut, tighten no further than a 3/4 turn.

3 INSTALL THE COLUMN AT THE CORRECT AND CONSISTENT HEIGHT

By measuring and pre-swaging metal or graphite ferrules onto the column with Agilent Column Installation Pre-Swaging tools, the correct length of column is maintained during the installation process, critical for accurate and reproducible results. Always visually inspect column ends using a magnifying loop to assure there are no burrs or jagged edges to interfere with the flow path.

The installation depth at the inlet and at the detector is critical in achieving optimal results. At the inlet the correct insertion depth assures smooth transfer of the sample to the head of the column. At the detector end, correct positioning maximizes signal response. Agilent Column Installation Pre-Swaging tools, for use with UltiMetal Plus Flexible Metal ferrules and graphite ferrules help simplify the process, outside of the oven, by providing clear marks for different inlets and detectors.

Always install the nut and ferrule onto the column first, make a clean straight cut, and inspect the cut under magnification before using the pre-swaging tool. Using these tools is a best practice for consistent performance with each column installation.

Agilent Column Installation Pre-Swaging tools

P/N G3440-80217 (works with graphite ferrules)

P/N G3440-80218 (works with metal ferrules)

4 CLEANLINESS MATTERS

Use an Agilent Gas Clean Filter System to remove oxygen, moisture, or other contaminants that can alter your analysis. UltiMetal Plus stainless steel tubing and fittings for carrier gas lines and GC system plumbing ensure optimized cleanliness. When performing a column installation, limit handling of the supplies, or wear gloves, to reduce oils or contaminants on parts in the flow path.

Contaminants in the carrier or detector gases can lead to a variety of deleterious chromatographic effects. Oxygen in the carrier can lead to irreversible column damage and excessive bleeding at temperatures above 250 °C resulting in short column lifetime and higher costs. Organic contaminants in the carrier can produce ghost peaks, baseline drift and the potential for analyte signal interference. Gas Clean filters, installed on each instrument, remove contaminants and provide a clear visual indication when the trap has saturated and needs to be changed.

Touchless packaging of UltiMetal Plus Flexible Metal Ferrules and Ultra Inert liners are additional examples of our commitment to keeping flow path components free of contamination. These are particularly helpful for operators who prefer to not wear gloves. Eliminating the need to touch the supplies during maintenance avoids the risk of transferring oils and contaminants from fingertips to the liner or ferrule, ensuring faster preventive maintenance (PM), less bakeout time, and better productivity.

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5 REDUCE AND ELIMINATE LEAKS AT THE MS INTERFACE

Agilent mass spec interface Self Tightening Column Nuts for graphite/polyimide ferrules provide assurance that your GC connections will last – cycle after cycle. Use a leak detector at all connections of the flow path to be certain that there are no leaks occurring throughout your system.

Air infiltration into a GC/MS system can lead to high column bleed, premature column demise, poor sensitivity, buildup of contaminants in the source, and in general more frequent maintenance. For years, a long polyimide/graphite ferrule has been recommended for use with a brass column nut at the mass spec transfer line. Unfortunately, the polyimide component of these ferrules shrinks with repeated heat cycling, resulting in leaks. Experienced GC/MS operators have learned to snug or retighten these ferrules after initial heat exposure, and periodically following additional heat cycles to maintain a leak-free system.

With Agilent Self Tightening Column Nuts, the need to retighten the column connections due to the elasticity of the ferrule has been eliminated. A series of springs maintain a constant pressure on the ferrule as it expands and contracts with heat cycles, thereby keeping the connections leak-free without user intervention.

Self Tightening Column Nut for MS transfer line
P/N 5190-5233

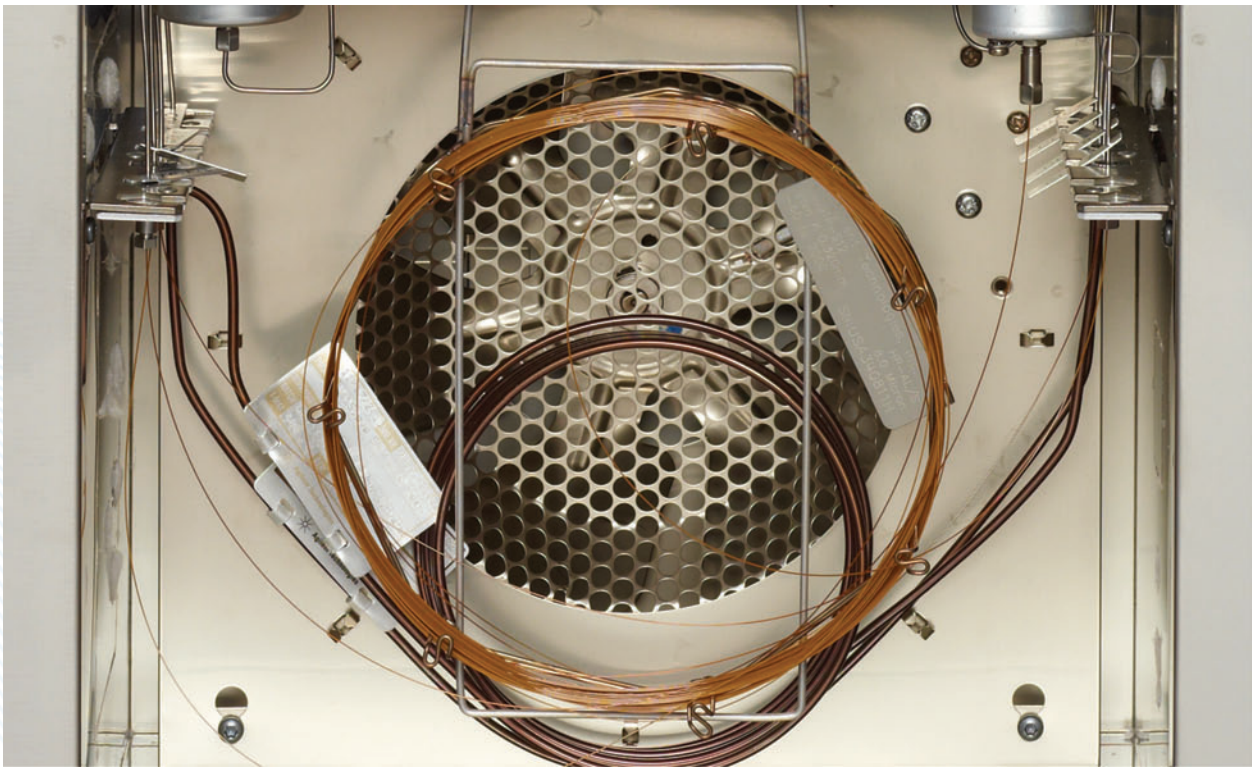
6 ADVANCED CONNECTIONS REQUIRE SPECIFIC SOLUTIONS

When additional complexity is necessary to achieve an analysis, using the proper supplies for that situation can make things easier. For example, use the Ultimate Union with UltiMetal Plus Flexible Metal ferrules for worry-free, inert guard column/retention gap connections.

Making inert and leak-free connections between retention gaps/guard columns and the analytical column had been a challenge in gas phase analysis. Agilent's solution is to use UltiMetal Plus Flexible Metal ferrules with an Ultimate Union.

These components are fully passivated for maximum inertness and designed to be easy to use. This same technology is integral to all of Agilent capillary flow devices – from a simple purged universal union used for column backflush, to a purged 3-way splitter capable of directing column effluent among 3 different columns while still providing backflush capability.

Deans switching is also greatly simplified by using an Agilent CFT Deans Switch with UltiMetal Plus Flexible Metal ferrules.



You can improve your GC flow path integrity – and lengthen periods of unattended operation – with easy-to-use, leak-free connections from Agilent. They allow all analysts to create reliable connections quickly, while making advanced flow path techniques (such as backflush, retention gaps, and column effluent switching) more routine.

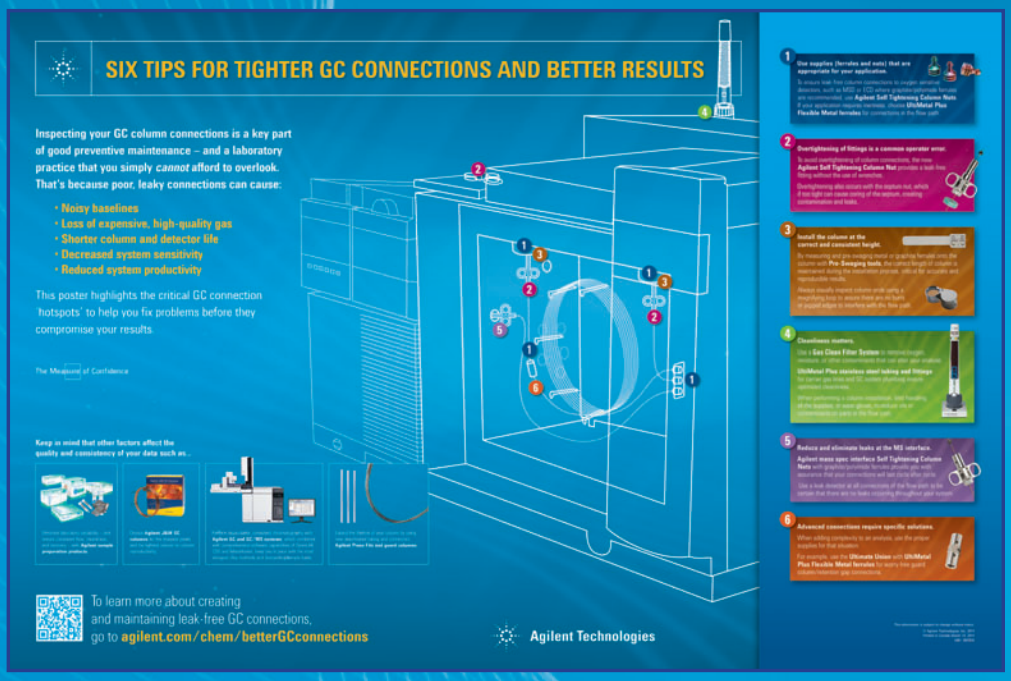


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Table I: Some essential definitions

Term	Definition
Relative quantification	Relative quantification strategies compare the levels of individual proteins in a sample to those in another sample. Results are typically expressed as a relative fold change, or percent change, of protein abundance.
Absolute quantification	Absolute quantification is the determination of the amount, in units of mass or concentration, of a protein in a sample. For example, protein X expressed in units of nanograms per milliliter of plasma.

in which antibodies are used to detect proteins transferred from polyacrylamide gels to nitrocellulose or polyvinylidene fluoride membranes. Refinements of the basic protocols yield detection limits in the attomolar range (1). Evolution of the principles underlying the western blot led to the development of the radioimmunoassay (RIA) and ELISA (2,3). In a clinical setting, specific proteins are commonly quantified by ELISA. Similarly, for several decades, quantification of multiple protein components in complex biological samples has relied on two-dimensional (2D) gel electrophoresis (discussed in some detail later). Additionally, a growing array of multiplexed, selective capture methods, including aptamer and antibody arrays, are increasingly used for protein quantification. Numerous reviews discuss these and related strategies (4–9).

MS Approaches to Protein Quantification

An Overview of the Approaches

We categorize protein quantification by MS into several groups. These are outlined below, represented in Figure 1, and discussed in more detail in the sections that follow.

Global, System-Wide (Multicomponent) Strategies

Here, hundreds or thousands of proteins are compared in two or more samples and quantification is typically relative. Nevertheless, with modification to the basic method, absolute quantification is sometimes possible.

Protein-Centric or Top-Down Strategies

These approaches involve resolution of a complex mixture of proteins (for example, by 2D gel electrophoresis) and quantification in their intact form. The approach often involves differential radio- or chemical-labeling of proteins in distinct samples (such as, difference gel electrophoresis [DIGE]). After tagging, the samples are combined, the proteins are resolved in two dimensions, and the relative amounts of the tagged proteins are measured. Protein spots are excised from the gel, digested, and identified by MS by means of peptide mass maps (that is, peptide masses), peptide sequences (that is, tandem mass spectrometry [MS-MS]), or a combination of both techniques.

Peptide-Centric, Bottom-Up, or Shotgun Strategies

Here, a complex mixture of proteins, an extracted proteome, is digested to peptides. The peptides, which serve as surrogates of the original intact proteins, are then separated, quantified, and identified. These strategies are further subdivided into label-free strategies and labeled strategies, depending on whether a label is incorporated:

Label-Free Strategies:

- Isolate proteins in a sample → proteolysis → separate peptides → sequence peptides → identify protein → repeat procedure for additional samples. We then compare either the number of peptides recovered for each protein (that is, spectral counts) or relative abundances of specific peptide ions (that is, peptide peak intensities by liquid chromatography–mass spectrometry [LC–MS]) to quantify proteins.

Labeled Strategies:

- This involves differential metabolic labeling with stable isotopes of proteins in two or more samples → combine samples → isolate total proteins → proteolysis → separate peptides → quantify differentially labeled peptides → sequence peptides and identify proteins (for example, stable isotope labeling by amino acids in cell culture [SILAC]).
- Differential chemical labeling with stable isotopes of proteins in two or more samples → combine samples → isolate total proteins → proteolysis → separate peptides → quantify differentially labeled peptides → sequence peptides and identify proteins (for example, isobaric tags for relative and absolute quantitation [iTRAC]). (Note the similarities of this approach to DIGE. The primary difference is that separation and quantification are performed on surrogate peptides, not intact proteins.)

Targeted (Single-Component or Several-Component) Quantification Strategies

In these approaches one or a few components are selectively isolated from a sample and quantified in relative or absolute terms. Approaches fall into two categories, top-down and bottom-up.

Top-Down Approach

Direct quantification by matrix-assisted laser desorption-ionization (MALDI) or protein isolation–concentration by an approach such as mass spectrometric immunoassay (MSIA, Thermo Fisher Scientific): Selective isolation of one or more proteins → determine protein abundances based on ion current. Protein identification or selectivity is derived from antibody and mass of target protein; amount is based on ratio of peak heights/areas for analyte and an internal standard. Absolute concentrations are determined referring to a calibration curve containing a fixed amount of internal standard (IS) and varying amounts of the intact target protein.

Bottom-Up Approach

Multiple-reaction monitoring (MRM) methods including stable isotope standard capture with anti-peptide antibodies (SISCAPA). Approaches selectively isolate target protein or proteins → digest proteins → quantify one or several peptides according to parent-ion (MS) or product-ion chromatograms (MS-MS).

General Considerations in Quantitative Proteomics by MS

An abundance of reviews discuss protein quantification by mass spectrometry, but most focus on instrumental considerations (10–13). Furthermore, most authors have

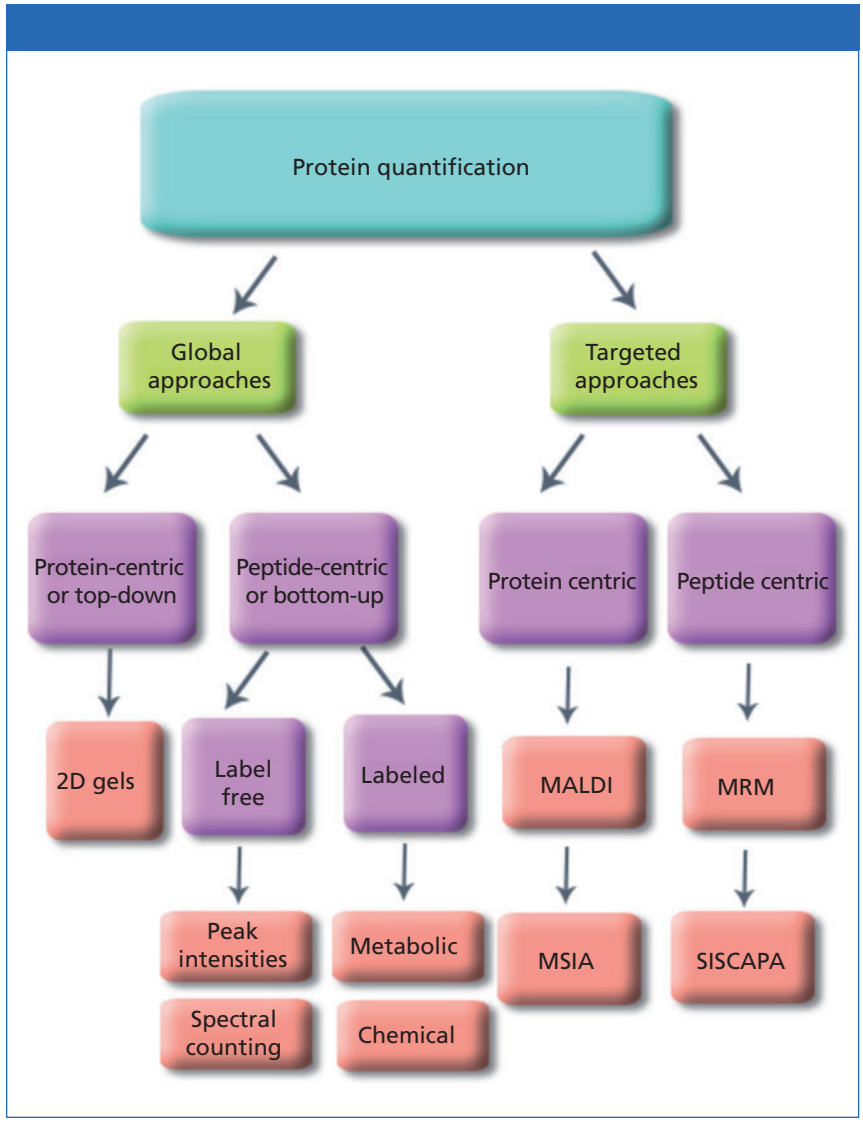

Figure 1: Diagrammatic representation of the common strategies for protein quantification and the relationships between them.

almost exclusively focused on electrospray ionization (ESI)-based approaches and have neglected the findings of precise and sensitive intact-protein quantification by MALDI-based methods. Our focus is on the overarching steps in system-wide protein quantification.

Sources of Inaccuracy and Imprecision

Multiple sample-manipulation steps are common before instrumental analysis (for example, protein precipitation or isolation, fractionation, selective depletion and enrichment, proteolysis, and tagging and labeling reactions). Each step is a source of pre-analytical sample variability that can compromise both precision and accuracy. For example, high-abundance proteins like albumin are sometimes removed from plasma samples by means of immunodepletion before analysis. However, that removal process introduces a risk of codepleting other components of interest because of nonspecific binding to both the antibodies used and to the albumin itself (by other sample components). Similarly, other steps such as protein precipitation and enzymatic digestion can introduce significant imprecision and inaccuracy because proteins are not recovered or digested quantitatively. Irreproducibility in other sample-handling steps, including chemical labeling, together with instrument perturbations (such as pressure and temperature fluctuations and tuning); the laboratory environment (for example, temperature and humidity); reagent variability; the presence of coeluted species or their levels; and analyte concentration can contribute to imprecision by altering the ionization process and thereby the measured signal intensity. Intensity comparisons are therefore compromised at a fundamental level. Studies that compare results across different analytical runs are most susceptible to these factors. Minimizing or carefully controlling key variables is critical. Yet even so, given the number of steps in the analysis and the number of species being measured, many potential sources of imprecision remain.

Validation of Proteomic Methods

It is important to remain mindful that the primary aim of any quantitative proteomics study is to provide timely, accurate, and reliable data that are fit for an intended purpose. Nevertheless, depending on the specific approach and the rigor with which the analysis is performed, the quality of the data will be variable and undefined. Quantifying thousands of components in a sample is a formidable challenge, to say the least. Defining specificity, linearity, accuracy, precision, range, detection limit, upper and lower limits of quantification, and robustness — all central considerations in the validation of a conventional quantitative analysis — is, given the scope of the task, empirically impossible for every protein species. Similarly, recovery and stability studies are not possible on each of the components. Consequently, in global proteomic studies, validation of the assay is typically perfunctory, and the resultant data are of uncertain and ill-defined reliability.

Although conventional validation is not practical, measures of precision and accuracy remain essential so that experimental findings can be put into context. Therefore, we must be confident that the measured differences are real and not merely an artifact of the method itself.

A method's assessed precision for a subset of analytes measured, at various concentrations, in one or more test samples and the derived data can be used to determine the method's suitability. These data can also help validate subsequent findings derived from the method. (14). Similarly, technical replicates (that is, repeat analyses of each of the samples in the study) provide additional support that a change is real, not an artifact of the analytical method itself. In the same vein, the issue of specificity must also be considered.

Accurate quantification cannot be assumed on the basis of one — or even a few — peptides simply because a single peptide defines only a single segment of any protein, and modifications elsewhere in the molecule are missed (14,15). Quantification based on a peptide common to multiple, related forms will always lead to an overestimate of the amount of any single variant whereas quantification based on a unique peptide fails to "recognize" and quantify closely related variants of that protein, even if they are significantly more abundant. Precise and accurate quantification of a specific protein variant is therefore achievable only when the targeted peptide or peptides are derived from a single precursor protein or, in the case of protein-centric methods, in instances in which we can resolve and quantify the specific (intact) protein species without interferences.

The situation, however, is not as bad as it might seem at first. Because a common objective in proteomics is to compare groups — for example, disease versus control or control versus test — absolute levels are not (necessarily) important. Defining percent change (or difference) is the overarching objective. Therefore, it is possible to take advantage of differential (isotopic) tags and the exquisite selectivity of mass detection to compare two (or more) samples worked up and assessed in the same experimental run under identical conditions. For example, in a typical experiment all proteins in a sample (such as control) are labeled with a chemical tag; separately, all of the proteins in a second sample (such as disease) are labeled with an isotopic variant of the same tag. The samples are then mixed and treated as one. Thereafter, each tagged protein and its isotopic variant behave in an identical manner during sample handling. Yet because of their difference in mass, they can be specifically detected and quantified by MS. While this detection and quantification strategy markedly reduces variance in one sense, such an approach is limited to "A versus B" comparisons. Numerous modifications of the basic strategy have been developed and will be discussed in more detail later in this installment.

Global or Proteome-Wide Protein-Centric Quantitative Tools

2D Gel-Based Methods

Using 2D gel-based methods is the most frequently adopted top-down strategy, and it is based on quantitative analyses of intact proteins resolved via 2D gel electrophoresis. Typically, the first step is protein separation by isoelectric focusing (IEF; first dimension [1D]) and then orthogonal separation of the proteins distributed on the 1D strip by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; second dimension). Protein spots (that is, discrete species) are visualized by dyes, fluorophores, or radioactive labels tagged. (These can be visible or fluorescent post-electrophoretic dyes, or fluorophores or radioactive labels that are tagged to proteins before resolution. Examples include silver stain, Pro-Q Diamond, 2,4-dinitrophenylhydrazine, or Coommassie blue.) The quantity of each protein is assessed by measuring the spot's density using a customized software package. Two-dimensional gels provide excellent sensitivity, precision, and linearity over a

wide dynamic range. Nevertheless, subsequent identification of proteins requires resecting, by hand or robot, the individual spots from the gel followed by MS (that is, a peptide mass map with or without additional MS-MS sequence information).

To minimize the influence of gel-to-gel variations, and to reduce the total number of gels required, variants of this general strategy involving differential labeling (or tagging) of the proteins in two or more samples have been developed (for example, with fluorophores or radioactivity labels). The objective of these approaches is to retain the physical properties of the proteins so that their mobility in each of the two dimensions of separation remains unchanged. Therefore, both can be run as a mixture on one gel, and each can be independently quantified, because of the tag.

The most common manifestation of this approach is DIGE (16). Here, three (or more) different protein-containing samples can be labeled with size-matched, charge-matched, spectrally resolvable fluorescent dyes (for example, Cy3, Cy5, and Cy2) before 2D gel electrophoresis. In this way, the number of gels to be resolved is reduced, precision is increased, and the time and cost of the comparison is also reduced. For example, running two samples on a single gel significantly increases reproducibility. Nevertheless, because three distinct "tags" are available, comparisons can also be made between multiple samples run across multiple gels by using a pooled internal standard (17). Composed of a mixture of all of the samples constituting the study, this standard is tagged and run on each gel. The pooled sample acts as an internal standard for every protein spot on each of the gels. As such, it is used to normalize all spots and to optimize inter-gel precision. Typically, reciprocal labeling (a dye-swap experiment) is performed to ensure the observed changes are not associated with dye-dependent interactions. Commercial software is used to detect differences and assign statistical confidence to them.

Critical Evaluation

With high precision, 2D gel electrophoresis and its variant, DIGE, allow relative abundance comparisons that detect modest changes of one to several thousand proteins in multiple samples. Provided they can be resolved, variant forms of the same protein (that is, protein variants or proteoforms) can also be independently quantified. Because each separation is visually represented, only proteins that differ in abundance need be resected from the gel and identified. Two-dimensional gel electrophoresis and DIGE have been successfully adopted by many investigators. Both methods have been thoroughly reviewed and have stood the test of time (18,19). However, the approach is cumbersome, labor-intensive, and difficult to fully automate. These limitations, especially the last, have dampened the enthusiasm of many to adopt 2D gel electrophoresis (and DIGE). While it is frequently suggested that the resolution of a 2D gel is limited, it is important to stress that no other approach rivals 2D gel electrophoresis for practical, intact protein separation. Comigration of multiple proteins to the same location on the gel is also often cited as a problem, but it is rarely an issue (20). Similarly, although large (>150 kDa) or small (<5 kDa) proteins are difficult to separate on gels, this difficulty is not a significant limitation because work-arounds are available, at least for peptides. DIGE is subject to variations in the extent of labels incorporated into the proteins, a potential source of variance in the measurements. Identifying proteins is sometimes problematic, especially with DIGE. Alignment problems can occur because identification requires running a separate (preparative) gel and then matching it to images for the set of analytical gels.

Other Top-Down Strategies

In this review, we forego discussion of instrument-intensive, top-down approaches simply because they are not yet practical for routine use. As alternatives to 2D gel, top-down proteomic approaches are, however, being developed by several groups. These alternatives involve introducing intact proteins into a mass spectrometer and fragmenting them directly. By working with the intact protein, complete sequence coverage is possible, and post-translational modifications (PTMs) are preserved. Until recently, however, top-down proteomic strategies were restricted to the analysis of purified proteins or simple mixtures. Over the last few years the approach has been extended to complex mixtures of proteins (21), but quantification remains a challenge nonetheless.

Global or Proteome-Wide Peptide-Centric Approaches

General Comments

Peptide-centric (bottom-up or shotgun) quantitative strategies are dominant because of their purported ability to quantify multiple components simultaneously in an automated or semiautomated manner. These strategies involve a common step: that is, site-specific cleavage of a mixture of isolated proteins (a proteome) to generate a substantially more complex mixture of peptides. Typically, peptide-centric applications utilize trypsin and quantify based on tryptic peptides. The selective cleavage of proteins to peptides is undertaken for these reasons: Peptides can be separated by LC better than proteins; most proteins generate one or more soluble peptides even if the antecedent protein is poorly soluble; peptides fragment better in a tandem mass spectrometer, giving spectra that can be sequenced; and peptides can be detected at much lower levels than their protein precursors. The peptides are then fractionated by LC and analyzed by MS-MS (14).

It is important to acknowledge, however, that all peptide-centric approaches to quantification are based on the assumption that when a protein is cleaved by a specific reagent, the reaction will go to completion, or at the very least, that the cleavage will be reproducible and predictable. Further, it is assumed that the target peptide or peptides are sufficient to define and selectively quantify the antecedent protein (that is, the target peptide is solely derived from a single antecedent protein). In practice, however, a target peptide or peptides may be degenerate and shared by multiple proteins. Through digestion, connectivity between the peptides and their antecedent protein is lost, a phenomenon referred to as the *protein inference problem* (22). In fact, multiple variants of a protein (variant protein species or proteoforms) are common, and unless peptides incorporating the specific, modified residue or residues (for example, the oxidized, reduced, nitrated, phosphorylated, glycosylated, or differentially "altered" amino acid) are targeted, quantification will be inaccurate. As a specific example, a single-point amino acid mutation may exist in a target protein, but if quantification is based on any tryptic peptide other than the one incorporating the modification, the variant will not be

detected. Similarly, other variants of the precursor protein including truncated or alternatively spliced forms are often misidentified (15). On the other hand, if the focus is on identifying specific modifications, and the correct peptide is targeted, the peptide-centric approach offers advantages. The influence of a modification on mass is more evident at the level of the peptides than it is at the protein level because the percent change in mass is greater.

Relative quantification by peptide-centric methods can involve the separate analysis of multiple samples by MS and their subsequent comparison (for example, label-free methods). Alternatively, tags (such as isotopic tags or stable isotopes) can be incorporated into proteins or their proteolytic peptides. These cause a shift in mass of the labeled protein or peptides in the mass spectrum. Differentially labeled samples can be combined and analyzed together, determining differences in the peak intensities of the isotope pairs. These intensity differences correlate with differences in the abundance of their antecedent proteins.

With these overarching issues in mind, the approach can be implemented in many, disparate ways. This review does not aim to be an encyclopedia of all available methods. Instead, it is a description of the basic divergent strategies and their strengths and weaknesses.

Label-Free Approaches

Label-free quantification is fast, cost-effective, and easy to implement. It is frequently used when stable isotope incorporation is impractical or cost-prohibitive. In these approaches, samples are analyzed separately and results from multiple runs are compared. The two main, label-free approaches each rely on proteolytic digestion of a sample followed by analysis by LC–MS or LC–MS-MS. Both strategies are used to make comparisons between two or more samples and to determine relative change in protein abundance (with the caveats noted above).

Spectral Counting

This is a practical, semiquantitative measure of protein abundance in proteomic studies. Relative quantification by spectral counting compares the number of identified spectra associated with the same protein between different samples — that is, the total number of tandem mass spectra that match peptides to a particular protein as a measure of protein abundance within a complex mixture. The approach is based on the finding that increasing protein abundance results in an increase in protein-sequence coverage. Therefore, abundant proteins produce more MS-MS spectra than less-abundant proteins, and their antecedent peptides are sampled more often in fragment-ion scans than those derived from low-abundance proteins. However, important caveats are associated with this approach. Low-mass proteins (that is, those generating fewer fragments on proteolysis) are problematic; the dynamic range of the approach is limited; precision is poor and, consequently, small changes in protein abundances are difficult to determine. Several modifications of spectral counting, including the normalized spectral abundance factor (NSAF) approach (23), have also been reported. NSAF corrects for the fact that larger proteins yield more peptides on digestion than shorter proteins and also accounts for sample-to-sample variations associated with replicate analysis. A modified spectral

counting strategy, absolute protein expression (APEX) profiling, has been used to measure the absolute protein concentration per cell after the application of several correction factors (24). Further refinements have also been made and the approach was recently reviewed (25).

Quantification Based on Peptide Peak Intensities as Determined by LC–MS

This approach to quantification is based on the observation that for a specific peptide separated and detected by LC–MS, the measured ion current increases with increasing concentration. Typically, ion chromatograms for each peptide of interest are extracted from an LC–MS run, and their peak areas are integrated over time. Peak areas for the same ion are then compared between different samples, to give relative quantification; absolute amounts can also be calculated by reference to a calibration curve. Most often, ion currents derived from the intact, protonated, peptide ions are monitored, but product ions generated by MS-MS can also be used for quantification. (Product-ion detection increases selectivity, but at the expense of sensitivity.) The approach and computational strategies to manage the data have been reviewed (26).

While the relationship between the actual amount of protein and generated ion current holds true for standard samples of limited complexity, in practice, the analysis of digests of complex biological samples is far more problematic. For example, variations in temperature, pressure, sample preparation, injection volume, retention time, and the presence of coeluted species can significantly compromise precision. (Studies often extend over weeks — or even months — and changes in column, mobile phase, instrument condition, and calibration begin to manifest themselves.)

Critical Evaluation of Label-Free Approaches

Label-free approaches are inexpensive and simple to implement, but the old aphorism, "You get what you pay for" may apply. They allow "semiquantitative" comparisons between samples, but precision and reliability is low, in large part because without an internal standard the measured ion current is susceptible to many factors when it is measured in many separate runs. In a recent study by The Association of Biomolecular Resource Facilities (ABRF), data generated from digests of parallel lanes of gel-separated proteins were supplied to several groups. The task was to "identify" the proteins in the sample and determine which were elevated or reduced in intensity relative to the adjacent lane. Not surprisingly, participants failed to agree, and there was no evidence that either approach — spectral counting or intensity-based quantification — could reliably address this question (27).

Labeled Approaches to Global Protein Quantification

General Comments

These approaches uniquely tag the proteins in two or more samples with a stable-isotope tag. The tagging can be done metabolically (that is, by adding enriched amino acids into cell culture medium) or chemically (that is, by covalently binding a labeled moiety to the proteins). The samples are then combined and analyzed in a single run. Precision is markedly improved because two or more samples are compared within one run, but at the expense of the time, cost, and complexity of the overall analysis. The earlier in the analytical process the label is incorporated into the proteins, the better, but its (global) incorporation is far from straightforward.

Metabolic Labeling

In this approach to relative quantification, the proteins in two or more samples are labeled with isotopically distinct forms of amino acids by growing cells in enriched culture medium (for example, SILAC). The first report of this approach was by Ong and colleagues in 2002 (28). Typically, two populations of cells are grown in separate cultures, one in standard medium and the other in medium containing stable-isotope-labeled amino acids. After the samples are combined, the mass difference between proteins and their proteolytic peptides in the two populations can be detected by MS. The ratio of peak intensities in the mass spectrum for the labeled versus unlabeled forms reflect the relative protein abundances in the two samples. This approach delivers the highest precision because the label is incorporated before any analytical steps are undertaken, and it therefore accounts for sample handling biases through the whole analytical process. These advantages are in part offset by the cost of the strategy and the fact that the metabolic labeling approach is far from widely applicable. For example, it cannot be applied to the assessment of protein differences in biological fluids collected from human subjects.

Chemical Labeling

Because metabolic labeling is often not feasible, if a stable-isotope label is to be used, it must be introduced later in the workflow by chemically tagging peptides or proteins. Two basic strategies are commonly adopted, as discussed below.

Isotopic Labeling

The many variants of this general strategy all aim to add isotopic atoms or isotope-coded tags to peptides or proteins. Some are simple in concept whereas others combine multiple elements to react with differentially tagged and selectively recovered peptides. Once again, two separate samples are differentially tagged with isotopic labels, mixed, and analyzed. Labeling strategies include enzymatic labeling with ^{18}O at the C-terminus of proteolytic peptides (29); global internal standard technology (GIST), in which deuterated acylating agents (for example, *N*-acetoxysuccinimide [NAS]) are used to label primary amino groups on digested peptides (30); and chemical labeling with formaldehyde in deuterated water, to label primary amines with deuterated methyl groups (31).

Commercial isotopic labeling reagents are also available. The best known commercial option is, perhaps, the isotope-coded affinity tag (ICAT) method (32). Several iterations of ICAT tags have emerged. The first generation of the reagent comprises three separate parts: a sulfhydryl-reactive chemical crosslinking group, a linker, and a biotin entity. The reagent's two versions are an unlabeled form and a heavy form incorporating eight deuterium atoms. The sulfhydryl-reactive group reacts with free thiols (that is, on cysteine residues); the biotin tag is used to selectively recover the tagged peptides (that is, through binding with avidin); and the linker provides the opportunity to differentially (mass) label two samples. Since not all proteins contain a cysteine residue, this approach is limited in that about 20% of the

proteome may be missed. Furthermore, the incorporation of deuterium as the label is suboptimal because of a discernible isotope effect, which manifests itself as differences in retention time. A variant ICAT reagent incorporating ^{13}C was reported several years later (33). A further refinement of the same basic strategy, isotope-coded protein labeling (ICPL), which tags lysine residues and the N-terminus on the intact proteins has also been reported (34). Importantly, ICPL allows the simultaneous comparison of three groups in a single experiment (that is, $[^2H_7]$, $[^2H_3]$, and $[^2H_0]$ forms).

Isobaric Labeling

These are the most commonly used isotope tags. *Isobaric labels* are a set of matched reagents designed to react with peptides to give products of identical masses and chemical properties. Significantly, these products can incorporate carefully selected combinations of heavy and light isotopes. Although many different manifestations of isobaric labels exist, they all comprise the same basic components. Those components are a reactive moiety that functionalizes groups such as primary amines or cysteines, a mass reporter with a unique number of isotopic substitutions, and a mass normalizer with a unique mass that balances or equalizes the mass of the tag. Each different tag is designed to be of equal mass when bound to a peptide, but to cleave on collision-induced dissociation (CID) at a specific linker location, thereby delivering different-sized tags (reporters) that can be quantified independently. In a typical workflow, the proteins in various samples are isolated, enzymatically digested to peptides, and labeled with different isobaric tags. The separately labeled samples are then mixed and analyzed as one. On LC–MS analysis, the peptides are separated, fragmented to produce sequence-specific product ions, to determine sequence, and the abundances of the reporter tags are used to determine the relative amounts of the peptides in the original samples. Commercially available isobaric mass tags (for example, TMT and iTRAQ) allow the simultaneous analysis of multiple samples in one run (such as 4, 6, or 8 mass-unit differences).

Critical Evaluation

Labeled approaches to global protein quantification offer relatively high precision and multiplexing capability, and they suit many sample types. Nevertheless, they are based on the assumption that analytes will be quantitatively — or at least uniformly — labeled in all samples. Because these strategies are based on measuring proteolytic peptides as surrogates of proteins, the general considerations raised previously (that is, the assumption of complete digestion and selection of diagnostic peptides) apply to all of these methods.

Targeted Protein Quantification

Approaches to targeted protein quantification similarly can be divided into two distinct groups: those that detect and quantify intact proteins (typically by MALDI) and those that quantify one or more surrogate peptides derived from each protein (typically by LC–MS-MS).

Intact Protein Quantification

Although numerous investigators have demonstrated the ability of MALDI as a

precise and accurate approach to protein quantification (35–37), the most powerful and widely adopted manifestation of MALDI protein quantification is the mass spectrometric immunoassay. Developed by Nelson and colleagues (38), this assay combines immunoaffinity column capture with MALDI detection and quantification to reduce the number of components in the sample. In contrast to a conventional ELISA, selectivity is achieved through both the antibody and mass-specific detection. In practice, the sample is passed through an immunoaffinity column; the column is washed, to remove other components; and the bound antigen is eluted directly onto a MALDI target, ready for MS.

For quantification by mass spectrometric immunoassay, fixed amounts of a modified form of the antigen, or a similar protein, are typically added to the sample early in the process, as an internal standard. Absolute quantification is possible by reference to a calibration curve prepared and run in concert with the samples. Mass spectrometric immunoassay offers high-throughput protein quantification. It is important to note that it can also provide details about PTMs and genetic variants. In fact, not only is it possible to identify protein heterogeneity, but the variant forms of the same protein can be independently quantified. Several different antibodies can be combined in a single column, to allow multiplexed antigen quantification. Although mass spectrometric immunoassay is most commonly combined with MALDI, ESI-based methods have also been developed (39).

Multiple Reaction Monitoring Approaches

Targeted quantification of proteins following their proteolysis to constituent peptides has increasingly become a routine task (40). With a few significant modifications, the process follows the same strategy, essentially, as that described earlier for "label-free methods." First, the target peptides are monitored in MRM mode. Then stable, isotope-labeled versions of the target proteolytic peptides are typically added as an internal standard (though the approach has also been used without incorporating an isotopic internal standard) (41). Monitoring more than one MS-MS transition for each target species provides a powerful approach to quantify a predetermined set of proteins for multiple samples, and it can potentially offer precise and accurate, absolute quantification. Each target protein is cleaved to yield peptides, many of which have a unique sequence (that is, signature, or "proteotypic" peptides). A stable, isotope-labeled version of each signature peptide, designed to be identical to the tryptic peptides generated during digestion, is added at a fixed concentration to each sample, to serve as an internal standard. Because the labeled peptides are coeluted with the target peptide, the internal standards enter the mass spectrometer at the same time as the sample-derived peptides, and therefore they can be concomitantly analyzed by MS-MS. Typically, this approach is performed on a triple-quadrupole mass spectrometer or a hybrid (for example, a quadrupole combined with time-of-flight [TOF] or orbital ion trap analyzer). The target peptide concentration is determined by measuring its observed signal response relative to that of the stable-isotope internal standard. Absolute concentrations can be calculated referring to a calibration curve prepared at the same time. (Calibration curves must be generated for each target peptide in the sample.) With thoughtful selection of the target peptides, it is possible to quantify a specific protein or even a modified form of that protein. No antibody is required, and the process can be performed simultaneously on multiple — even hundreds of — peptides. Therefore, multiple proteins can be quantified in a single LC–MS-MS run. A variant of this process, known as *parallel reaction monitoring* (PRM), allows simultaneous monitoring of all product ions of a target peptide, rather than only a few predetermined transitions (42).

Critical Considerations

Selection of the specific peptides is a central issue because they should be diagnostic of the full target protein; use of just one or two peptides can lead to overestimations of proteins (15). (As discussed previously, target peptides could be common to known variants of the same protein.) Other important considerations address the possibility of incomplete digestion and the fact that sensitivity can be limited in the case of low-abundance proteins without an isolation or enrichment step. A major benefit of this strategy is that cost-effective, precise, and accurate analysis is possible without access to immunoreagents. Yet the approach can prove costly because of the requirement for multiple, stable, isotope-labeled peptides for each target protein. It can also prove time-consuming, because of the need to analyze the potentially complex MRM data.

Stable-Isotope Standard Capture with Antipeptide Antibodies

Stable-isotope standard capture with antipeptide antibodies (SISCAPA) is essentially the same procedure as that described above, except that it incorporates a specific, antipeptide antibody capture step for the signature peptide and its companion internal standard (43). The additional step enriches the sample for the target peptide and stable isotope standard. At the same time, it provides an opportunity to deplete the sample of interferents, including other peptides generated during the digestion. Importantly, because the internal standard is a perfect mimic of the target peptide, the peptide-to-internal standard ratio is preserved throughout the workup process. Extensive washing can be undertaken, to remove other peptides and clean the sample, without introducing additional variability in the results. The sample is then resolved by a short reversed-phase LC separation and analyzed by LC–MS-MS. Ions characteristic of the target peptide and its corresponding internal standard are monitored in MRM mode. From the signature peptide-to-internal standard ratio, the concentration of the peptide can be calculated by reference to a calibration curve.

Critical Evaluation

Antibody quality is important. So, too, is the selection of the specific peptides. The use of a single peptide is fraught with the problems discussed above. The limit of detection is improved because of the opportunity to trap and enrich the target peptides, but the cost and complexity of the approach is increased by the inclusion of this step.

Conclusions

MS quantification is not a trivial undertaking, even for small molecules. When the

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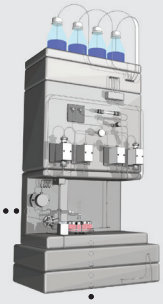

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task at hand is the quantification of hundreds to thousands of proteins in a single sample, the complexity of the analysis is even greater. Shortcuts compromise the process and lead to unsatisfactory and irreproducible results. Nevertheless, with proper care, reflection upon sources of variance, and attention to generating reproducible results, multicomponent protein quantification fit for specific purposes are possible.

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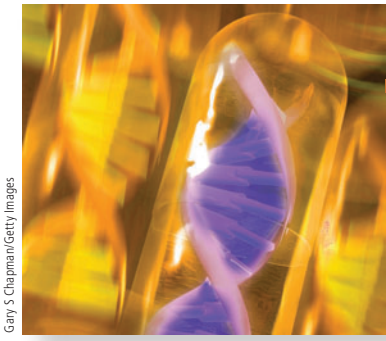

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BIOTECHNOLOGY TODAY

The Role of Elution Gradient Shape in the Separation of Protein Therapeutics

In this article, we discuss the role of the gradient in protein separations by reversed-phase and ion-exchange high performance liquid chromatography (HPLC). To illustrate the key points, we show data from two products. Granulocyte colony stimulating factor (GCSF) is a microbial protein that is expressed in *E. coli*. For this molecule, reversed-phase HPLC is examined for separation of the product-related variants. The other molecule is a biosimilar monoclonal antibody product and, in this case, ion-exchange HPLC is explored as a tool for analysis of the acidic, main, and basic variant species.

Varsha S. Joshi and Vijesh Kumar *are the guest coauthors of this installment.*

Anurag S. Rathore *is a coauthor and an editor of Biotechnology Today.*

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The number of therapeutic protein products available for use has radically increased in recent years. They include a wide variety of molecules such as recombinant human cytokines (for example, α and β interferon), cellular growth factors (such as granulocyte-macrophage colony-stimulating factor [GM-CSF]), hormones (such as glucagon), neuromuscular antagonists (for example, botulinum toxin), blood products (such as clotting factor VIII), and monoclonal antibodies (mAbs) (1). For protein therapeutics to be effective, they must be synthesized in their biologically active forms, with proper folding and post-translational modifications (2). However, these products are known to be associated with a variety of heterogeneities because of modifications such as glycosylation, deamidation, oxidation, and disulfide bond formation, which occur as a consequence of events during protein expression, purification, and storage (3). In view of these heterogeneities, thorough characterization using multiple orthogonal techniques is necessary for receiving regulatory approval for product commercialization. Of the many tools that are used, high performance liquid chromatography (HPLC) is the primary workhorse for analysis of biopharmaceutical proteins (4,5). The significant advantages that HPLC offers include high reproducibility, high sample throughput because of autosampling capabilities, high separation resolution, easy quantitation, high precision, and high robustness (6).

HPLC can further be classified into normal-phase, reversed-phase, ion-exchange, and gel filtration (size-exclusion) chromatography. Each mode is based on a different underlying mechanism and together make HPLC a powerful tool in the analytical arsenal. Since typical HPLC applications involve separation of product from product-related variants and impurities that have very similar physicochemical properties as compared to the product, the elution strategy has a significant impact on the quality of separation. The most commonly used strategies are isocratic elution and gradient elution. The latter technique can be implemented in linear, segmented, convex, and concave shapes (7). Elution is isocratic when the eluent strength is kept constant throughout the separation. Gradient elution implies that the mobile-phase composition will be varied during sample separation as per the chosen trajectory. Linear gradients are generally preferred because they are easy to create and are relatively robust. However, nonlinear gradients offer several distinct advantages, including reduced separation time, improved sample resolution, and higher detection sensitivity (8). In this installment, we primarily focus on reversed-phase HPLC and ion-exchange HPLC.

Separation by reversed-phase HPLC is achieved because of the interactions between the hydrophobic ligands covalently attached to the adsorbent and the hydrophobic patches of the species in the feed. Loading conditions are chosen such that the product binds strongly to the adsorbent. Thereafter elution is performed by using organic solvents such as acetonitrile, ethanol, or methanol. The molecules are eluted in the order of increasing hydrophobicity. More hydrophobic species are retained strongly and hence are eluted later, while the less hydrophobic species are eluted earlier. Several systematic approaches for reversed-phase HPLC method development have been described in the past (9–14). In most cases, an attempt is made to optimize sample retention (values of the retention factor, k), column efficiency (plate number, N), and selectivity (separation factor, α). Major emphasis is usually given to

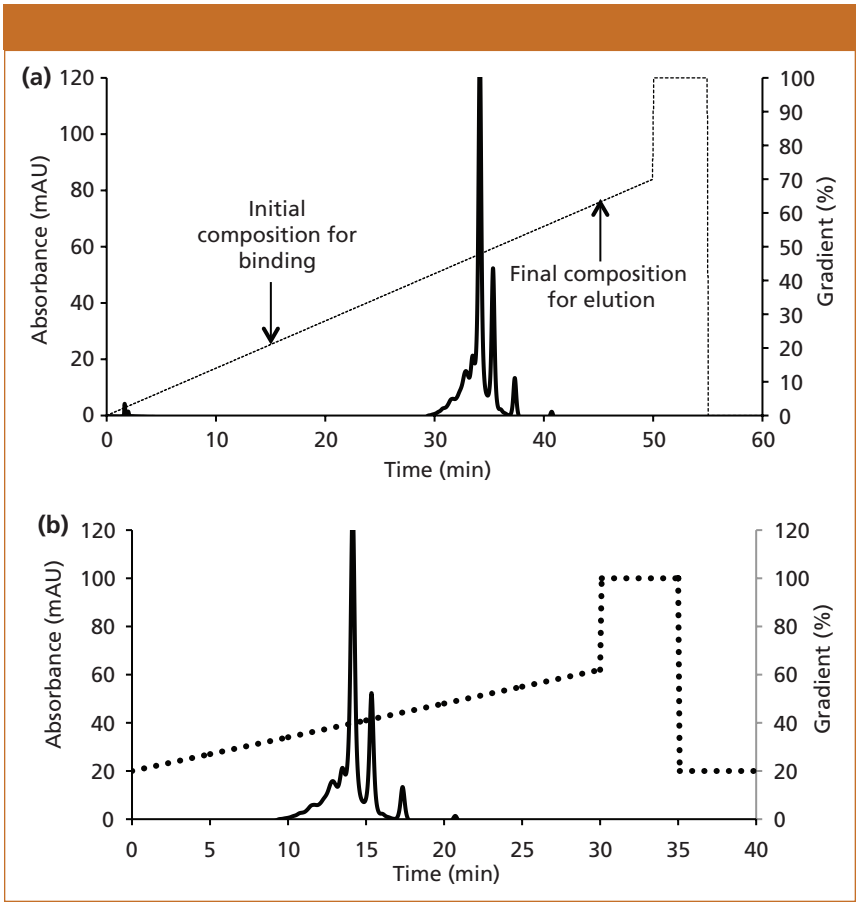

Figure 1: Chromatograms showing (a) determination of initial and final composition of eluent from the linear gradient for mAb and (b) a shorter method after determination of the initial and final eluent composition from the linear gradient for mAb.

the optimization of selectivity, often using a preselected series of experiments plus a computer program for predicting retention (k and α) as a function of one or more experimental variables. About four decades ago, the gradient elution was merely used for the prediction of isocratic behavior because the gradient run covers all binary compositions of possible interest to isocratic separation (14,15). In the past, favored method development strategies have been based on varying experimental conditions that are believed to have the largest effect on α; for example, solvent type, solvent strength (%B), and column type for neutral samples, or pH and ion-pair-reagent concentration for ionic samples. The use of gradient elution with temperature and gradient steepness as variable parameters for the optimization of selectivity and separation have been used as an approach for method development (13).

Ion-exchange HPLC is another popular, nondenaturing analytical method that is used for the separation of species based on their charge (16). Separation in this case is achieved by either changing the pH of the mobile phase or increasing the salt content in the mobile phase. Either of these alterations change the charge on the species and thus affect the interaction between the species and the stationary phase. The resolution of peaks is generally based on the differential retention of the protein on the column (17). There are several studies published on this topic that focus on the importance of development and validation of charge heterogeneity analysis of protein therapeutics using an ion-exchange method (18–24). Several approaches have been published in literature on the types of elution to resolve the charge variants of mAb, including salt gradient, pH gradient, and salt hybrid gradient. All of these types of gradients have advantages and disadvantages associated with them. The most popular of these is the formation of a salt gradient. In a salt gradient, apart from the routinely optimized parameters (column, pH, conductivity, temperature, and the type of salt),

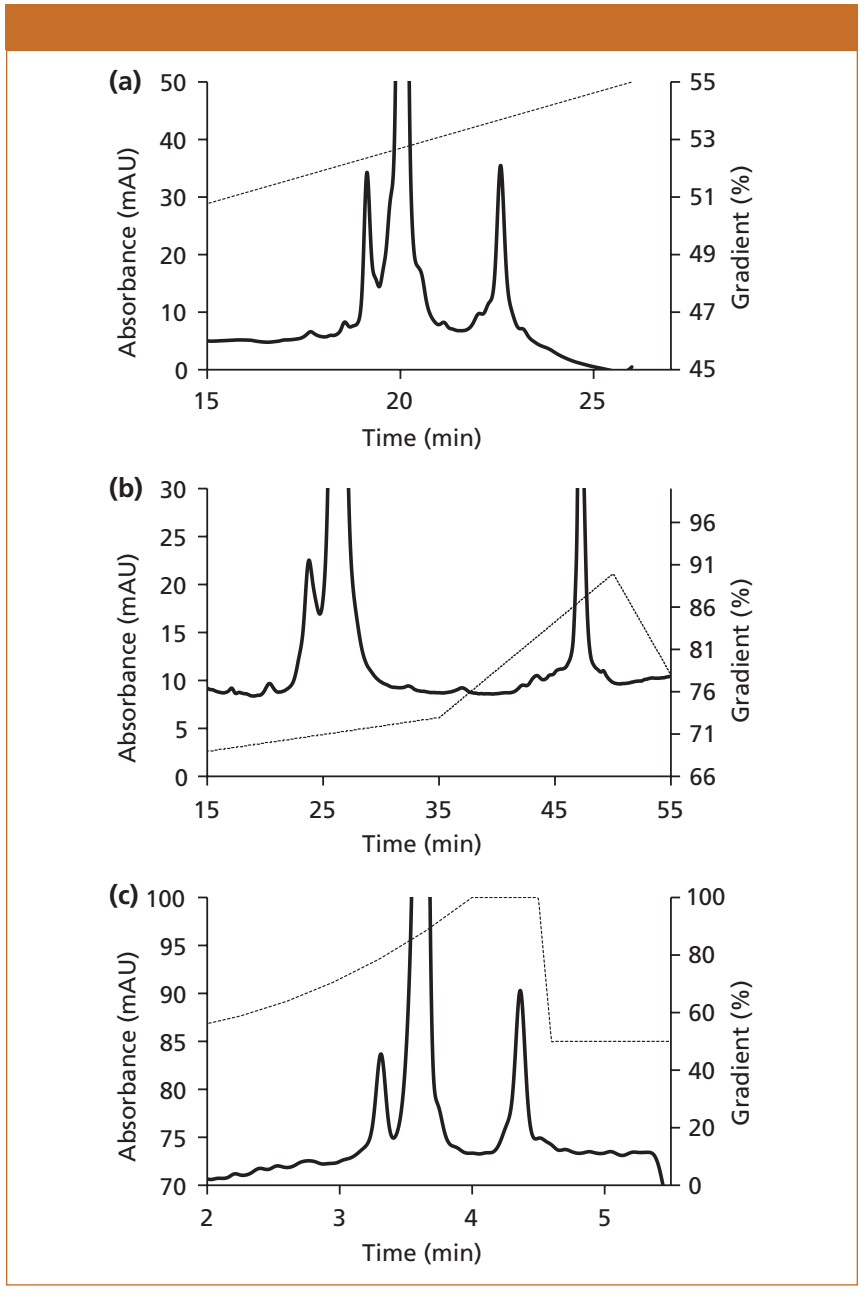

Figure 2: Comparison of gradient shapes for GCSF analysis: (a) linear, (b) segmented, and (c) concave.

the optimization of the shape of the elution gradient can have a significant impact on the resolution (25).

In this installment, we discuss the role of the gradient in separation by reversed-phase and ion-exchange HPLC. To illustrate the key points, we show data from two products. Granulocyte colony stimulating factor (GCSF) is a microbial protein that is expressed in *E. coli*. For this molecule, reversed-phase HPLC is examined for separation of the product-related variants (oxidized, main, and reduced species) in view of the difference in the hydrophobicity of these variants (24). The other molecule is a biosimilar monoclonal antibody product and in this case ion-exchange HPLC is explored as a tool for analysis of the charged variants (acidic, main, and basic species) because of the difference in the charges on these variants.

Materials and Methods

Protein Samples

The two therapeutic protein samples used in this study were recombinant human GCSF (*E. coli* derived) and an IgG1 mAb (CHO cell culture derived). Both were donated to us by major domestic biotech manufacturers.

Instrumentation and Columns

An Agilent 1200 series HPLC unit was used, consisting of a quaternary pump with degasser, an autosampler with a cooling unit, and a variable-wavelength detector.

Two ion-exchange columns were used in this study: a MAbPac SCX-10 strong-cation-exchange column and a 250 mm × 4.6 mm, 10-µm d_p MAbPac WCX-10 weak-cation-exchange column. Both columns were purchased from Dionex (now part of Thermo Fisher Scientific).

Three reversed-phase columns were used: a 100 mm × 4.6 mm Chromolith High Resolution RP-18 column from Merck, a 250 mm × 4.6 mm, 5-µm d_p C4 column from Phenomenex, and a 250 mm × 4.6 mm, 3.5-µm d_p X-bridge BEH300 C4 column from Waters.

Results and Discussion

Selection of Column Chemistry

Oxidized and reduced species of GCSF have a different hydrophobicity index. Thus, these species can be resolved from the product using any reversed-phase HPLC column with chemistry between C4 and C18. C18 columns are more hydrophobic than C4 and hence require strongly hydrophobic organic solvents for elution as compared to C4. In this study, we found that C4 columns yielded better resolution than C18 for GCSF. Therefore, a C4 column was selected for method development.

In the case of mAbs, a weak-ion-exchange column can be used for resolving the charged variants. Weak-ion-exchange columns offer more resistivity on pH transition over small changes in ionic strength, but require more time for equilibration than a strong-ion-exchange column. The two types may also offer differential selectivity toward acidic versus basic variants. Based on our preliminary investigation, we selected strong cation exchange as the chemistry for the analysis of mAb charged variants.

Selection of Solvent Composition

Solvent composition is known to have a high impact on column selectivity. Typical reversed-phase HPLC separations involve the use of mixtures of acetonitrile, methanol, isopropanol, and water along with trifluoroacetic acid (<1%). For ion-exchange HPLC, buffer pH and molarity are important. Based on preliminary experimentation, an acetonitrile–methanol–water system at

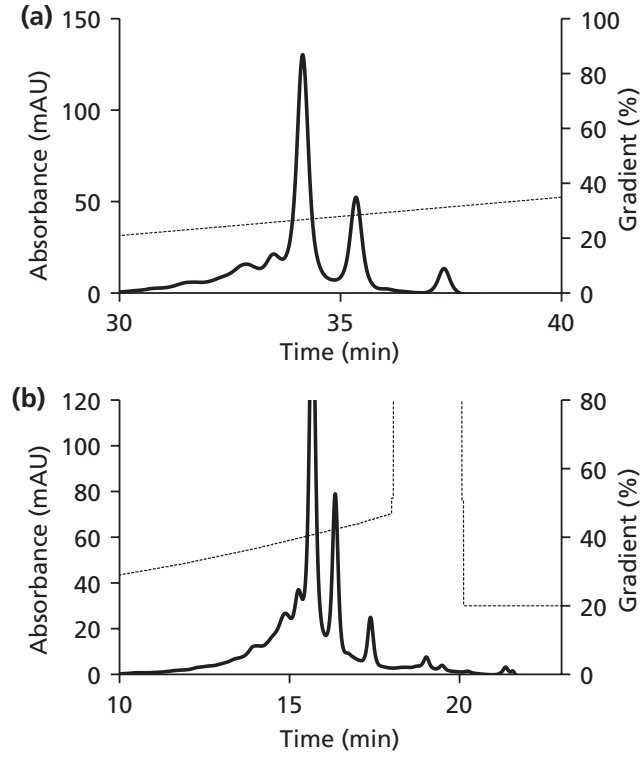

Figure 3: Comparison of gradient shapes for mAb analysis: (a) linear, (b) concave.

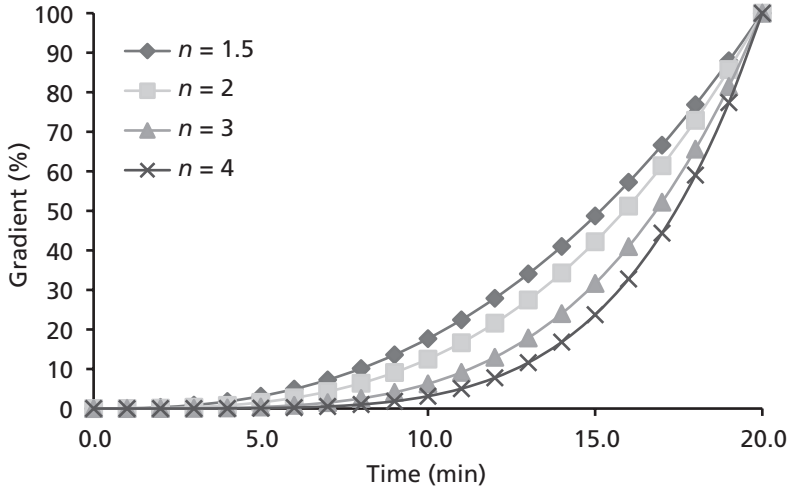

Figure 4: Overview of concave gradients with varying gradient steepness (*n*).

60 °C was chosen for reversed-phase HPLC and 15 mM phosphate at pH 6.8 and 28 °C with sodium chloride was used as the elution buffer for ion-exchange HPLC.

Gradient Optimization

Optimizing the Initial and Final Composition

First, a longer linear gradient (0–100%) was performed and from this chromatogram, the start and end conditions for a shorter gradient were obtained (Figure 1). The percentage of eluent at the retention time of the first eluted peak minus the time required for eluting one void volume was considered as the start percentage required for elution of the first peak protein. Similarly, the percentage of the eluent at the retention time of the last eluted peak minus the time required for eluting one void volume was considered the stop percentage required for elution of the last peak (Figure 1a). For GCSF, an initial mobile-phase B composition of 45% acetonitrile, 15% methanol, and 40% water and a final mobile-phase B composition of 80% acetonitrile, 15% methanol, and 5% water was found to be optimal. In the case of the mAb, the initial mobile-phase B (15 mM phosphate at pH 6.8 with 200 mM NaCl) composition of 20% and final mobile-phase B composition of 60% was chosen. The short linear gradient after selection of the initial and final mobile-phase compositions is shown in Figure 1b.

Selection of Linear, Convex, and Concave Gradient Shape

If t is time at which %B is desired, and t_f is the total time of gradient change, then for linear convex and concave gradients, %B can be approximated by

$$B(t) = t/t_f \times 100 \text{ for linear gradient} \quad [1]$$

$$B(t) = [1 - (1\text{-}(t/t_f)^n)] \times 100 \text{ for convex gradient} \quad [2]$$

$$B(t) = (t/t_f)^n \times 100 \text{ for concave gradient} \quad [3]$$

where n is the number controlling the steepness (convexity or concavity) of the gradient shape.

Depending on the peak distribution and spacing, a linear, convex, or concave shape can be selected. If the distribution of peaks and its spacing is uniform, then improvement in resolution or reduction in time is unlikely with a nonlinear gradient. However, if the spacing and distribution of peaks is nonuniform, then a convex or concave gradient shape may result in a better separation. The steepness of the curve can be increased where the peaks are widely spaced. The increase in steepness at the beginning of elution gradient results in a convex shape, but at the latter half it results in a concave shape. In our case, the spacing between the peaks was not uniform for both cases. The peaks after the main peak of interest were widely spaced and so a concave gradient was chosen. Otherwise,

if the peaks before the main peak were widely spaced then a convex gradient would have been chosen.

Three types of gradients were examined for GCSF, namely linear, segmented, and concave (Figure 2). All gradients were performed on a C4 column. As expected, the linear gradient resulted in evenly spaced peaks (Figure 2a). Further, the *European Pharmacopoeia*'s segmented gradient method was also performed (Figure 2b). This method has a shallow slope to resolve the oxidized impurities eluted before the main peak, followed by which the slope becomes steeper for elution of the reduced impurities after the main peak. The latter are easily resolved because of differences in hydrophobicities. Finally, a concave gradient was performed at high flow rates (3 mL/min) using a short monolithic column (Figure 2c). This approach resulted in optimal separation of the three components in a significantly reduced analysis time (1/6 of that with linear gradient and 1/14 of that with segmented gradient). Similarly, favorable results were obtained using a concave gradient for analysis of mAb charged variants (Figure 3) with the analysis time reduced from 45 min to 25 min.

Optimization of the Steepness Factor for a Gradient Curve

The concavity or convexity factor, *n*, which controls the steepness of the curve, was also examined. In most cases, this can be obtained by doing trials for *n* from 1.5 to 4.0 with a stepwise increase of 0.5 (Figure 4). The optimal value of *n* was found to be 2 for GCSF and 2.5 for mAb.

Final Length of Gradient

After an optimum value of gradient steepness has been determined, a decrease in interval between consecutive time points helps in reducing the gradient length thus compressing the peaks and making them sharp. This will also result in further reduction in time of analysis as shown above for GCSF (Figure 2c) and mAb (Figure 3b).

Conclusions

This installment aims to highlight the important role of gradient shape in HPLC analysis, in particular for biotech therapeutics. It should be noted that nonlinear gradients are likely to require more effort in optimization than the linear gradients. However, as illustrated in the case studies presented in this installment, a systematic optimization of these gradient shapes is likely to result in a significantly improved resolution along with a shorter analysis time.

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Comparison of Enantiomeric Separations and Screening Protocols for Chiral Primary Amines by SFC and HPLC

Supercritical (subcritical) fluid chromatography (SFC) was evaluated as an alternative to high performance liquid chromatography (HPLC) for the enantiomeric separation of primary amines on a cyclofructan-based chiral stationary phase. The effect of various organic modifiers, acidic and basic additives, as well as instrumentation-specific parameters such as column temperature, flow rate, and back pressure were evaluated. The results were compared to normal-phase and polar organic modes.

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Supercritical fluids were introduced as mobile phases in chromatographic separations by Klesper and colleagues in the 1962 (1). While capillary-based methods did not become widely utilized, packed-column supercritical (subcritical) fluid chromatography (SFC) has become increasingly common over the last 15 years (2–5). In the last decade, many of the hardware shortcomings have been improved and instrumentation for both analytical and preparative separations are readily available (6,7). The advantages of SFC are particularly pronounced in the field of chiral separations where many commonly used stationary phases provide optimal separations in the normal-phase mode (7–10). Now, with an impetus for "green separations" and high-throughput screening, SFC has become the platform of choice for many pharmaceutical companies, where speed is an essential aspect of method development (6,11,12). Higher flow rates without concomitant loss of column efficiency as well as lower solvent consumption are two of the major advantages of adopting SFC-based separations (5,6). Despite being commonly referred to as supercritical fluid chromatography, better separations are often obtained under subcritical conditions because of the improvements achieved by using a polar modifier such as methanol in combination with carbon dioxide (10,13,14). Regardless of the state of the carbon dioxide–modified mobile phase, separations utilizing SFC instrumentation with modified carbon dioxide mobile phases are most commonly referred to as SFC separations. Carbon dioxide has a polarity similar to pentane and can replace the nonpolar solvent in normal-phase methods, thus allowing them to be easily transferred to SFC instrumentation and vice versa (10,15,16). Short columns combined with high flow rates allow for rapid evaluation of multiple chiral stationary phases (CSPs) using multiple organic modifiers in a short period of time (17). When screening multiple CSPs and mobile-phase combinations, baseline resolution is not mandatory and analysis times can often be reduced to less than 10 min (17). By incorporating column and mobile-phase switching systems, what would typically take a technician days can often be reduced to hours. Advantages abound at the preparative scale as well since the major component of the mobile phase, which requires no evaporative step, and the low viscosity of carbon dioxide allows for high flow rates (18).

The use of crown-ether based chiral stationary phases to separate primary amine racemates was introduced in 1978 by Cram and colleagues (19). Since then, several crown ether–based CSPs have been developed and evaluated (20–24). These reversed-phase CSPs suffer from the need to operate under acidic aqueous conditions and are therefore

Table I: Effect of additives on enantiomeric separations*

	1,2-Napthylethylamine			α-Methyl-4-nitrobenzylamine			2-Amino-1-(4-nitrophenyl)-1,3-propanediol		
Additive†	t_r^1	α	R_s	t_r^1	α	R_s	t_r^1	α	R_s
TFA	1.9	1.20	1.0	2.53	1.15	1.0	2.48	1.19	0.8
TEA	3.47	1.09	1.0	2.60	1.00	0.0	3.40	1.10	0.5
NH_4OH	2.80	1.00	0.0	2.50	1.00	0.0	5.56	1.00	0.0
TFA, NH_4OH	1.60	1.04	0.0	1.72	1.06	0.0	2.60	1.05	0.0
TFA, TEA	2.7	1.13	1.5	4.00	1.12	1.1	5.75	1.18	1.7
AA, TEA	5.43	1.14	1.0	4.45	1.00	0.0	8.27	1.17	1.1
TFA, DEA	2.54	1.17	1.4	3.36	1.12	1.1	3.82	1.18	1.6
TFA, DIPA	2.40	1.13	1.2	2.96	1.12	1.1	1.40	1.26	1.5
TFA, BA	1.68	1.11	1.0	1.93	1.10	0.8	2.00	1.11	0.8

*Column: 150 mm × 4.6 mm, 5-µm particle diameter; mobile phase: 75:25 (v/v) carbon dioxide–methanol; acid concentration: 40 mM in methanol; base concentration: 15 mM in methanol; detection: UV at 254 nm; column temperature: 30 °C; flow: 4 mL/min
†TFA: trifluoroacetic acid, TEA: triethylamine, NH_4OH: ammonium hydroxide (37% w/w), AA: acetic acid, DEA: diethylamine, DIPA: diisopropylamine, BA: butylamine

not suitable for use with carbon dioxide mobile phases and are not advantageous for preparative scale separations.

A relatively new class of immobilized chiral selectors based on derivatized cyclofructans (CFs) have been shown to provide excellent selectivity toward a variety of racemic compounds (25–33). Cyclofructans also possess crown ether moieties with 6–8 pendant fructofuranose units. After it is derivatized with isopropylcarbamate groups, CF6 can separate a variety of primary amines without the need for aqueous mobile phases (25). However, no comprehensive study on its use under SFC conditions has been performed.

In this work, the Larihc CF6-P CSP was evaluated as a chiral selector under SFC conditions using 25 chiral primary amine probe analytes. These chromatographic results were compared to normal-phase conditions (hexane and ethanol) as well as polar organic conditions (acetonitrile and methanol). These three modes represent the most useful chromatographic conditions for many commonly used CSPs and a comparison of these modes will aid in developing future chiral methods using this CSP.

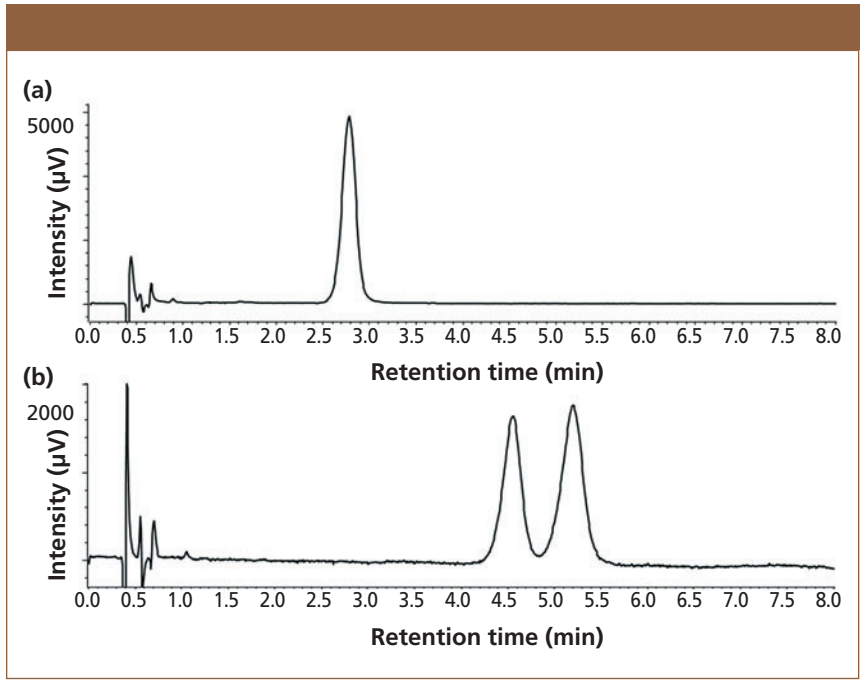

Figure 1: Effect of additives on retention and selectivity. Analyte: 1,2-naphthyethylamine; mobile phase: 80:20 (v/v) carbon dioxide–methanol; flow: 4 mL/min; (a) 0.2% (v/v) ammonium hydroxide; (b) 0.3–0.2% (v/v) trifluoroacetic acid–triethylamine.

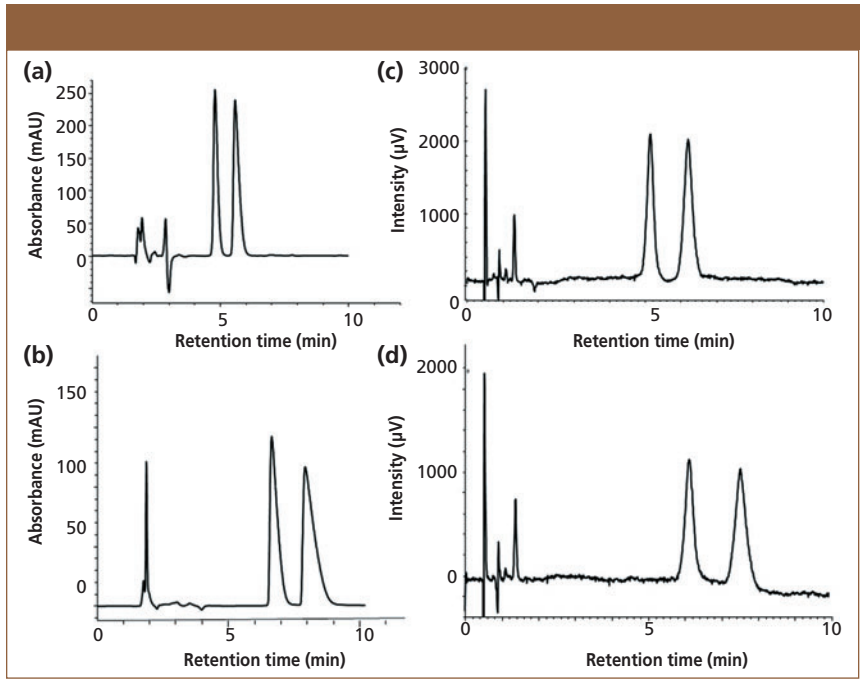

Figure 2: Effect of additive concentration on retention, selectivity, and peak symmetry. Analyte: (*RS/SR*) 2-amino-1,2-diphenylethanol; chromatograms (a) and (b): 90:10 acetonitrile–methanol; flow: 1 mL/min. (a) 0.3–0.2% (v/v) trifluoroacetic acid–triethylamine (overall), (b) 0.3–0.2% (v/v) trifluoroacetic acid–triethylamine (methanol only). Chromatograms (c) and (d): mobile phase 80:20 carbon dioxide–methanol; flow: 3 mL/min. (c) 0.3–0.2% (v/v) trifluoroacetic acid–triethylamine (overall), (d) 0.3–0.2% (v/v) trifluoroacetic acid–triethylamine (methanol only).

In SFC, the polar modifiers methanol, ethanol, and 2-propanol were evaluated using three probe analytes with short, intermediate, and long retention. Various acidic and basic additives and additive combinations were evaluated under similar mobile phase conditions. The advantages of using a combination of additives instead of individual acid or base additives include improved peak shapes, shorter retention times, and suppression of nonspecific interactions (34). Based on the collective data, recommended screening conditions are provided for SFC, normal-phase, and polar organic separations.

Experimental

Materials

High performance liquid chromatography (HPLC)-grade hexane, methanol, ethanol, 2-propanol, and acetonitrile were purchased from Fisher Scientific. ACS-grade acetic acid, ammonium hydroxide (37% w/w), trifluoroacetic acid, triethylamine, butylamine, diisopropylamine, and diethylamine were purchased from Sigma-Aldrich. A Larihc CF6-P HPLC column (150 mm × 4.6 mm, 5-µm particle diameter) was obtained from AZYP LLC.

Chiral Test Compounds

(*S*)-(-)-1-(2-Naphthyl)ethylamine, (*R*)-(+)-1-(2-naphthyl)ethylamine, (1*R*,2*R*)-(-)-2-amino-1-(4-nitrophenyl)-1,3-propanediol, (1*S*,2*S*)-(+)-2-amino-1-(4-nitrophenyl)-1,3-propanediol, (1*R*,2*S*)-(+)-*cis*-1-amino-2-indanol, (1*S*,2*R*)-(-)-*cis*-1-amino-2-indanol, (1*R*,2*R*)-(-)-*trans*-1-amino-2-indanol, (1*S*,2*S*)-(+)-*trans*-1-amino-2-indanol,α-methyl-4-nitrobenzylamine hydrochloride (±), α-methylbenzylamine (±),1,2-diphenylethylamine (±),norphenylephrine hydrochloride (±), DL-4-chlorophenylalaninol, normetanephrine hydrochloride (±), norephedrine hydrochloride (±), octopamine hydrochloride (±), *trans*-2-phenylcyclopropylamine hydrochloride (±), (1*S*,2*R*)-(+)-phenylpropanolamine, (1*R*,2*S*)-(-)-phenylpropanolamine, (*R*)-(-)-2-phenylglycinol, (*S*)-(+)-2-phenylglycinol, (*S*)-(-)-2-amino-3-phenyl-1-propanol, (*R*)-(+)-2-amino-3-phenyl-1-propanol, 1-(1-naphthyl) ethylamine (±), (1*S*,2*S*)-(+)-2-amino-1-phenyl-1,3-propanediol, (1*R*,2*R*)-(-)-2-amino-1-phenyl-1,3-propanediol, (*S*)-(-)-2-amino-1,1-diphenyl-1-propanol, (*R*)-(+)-2-amino-1,1-diphenyl-1-propanol, (1*R*,2*S*)-(-)-2-amino-1,2-diphenylethanol, (1*S*,2*R*)-(+)-2-amino-1,2-diphenylethanol, (*S*)-(-)-2-amino-3-methyl-1,1-diphenylbutane, (*R*)-(+)-2-amino-3-methyl-1,1-diphenylbutane, (*R*)-(+)-2-amino-4-methyl-1,1-diphenylpentane, (*S*)-(-)-2-amino-4-methyl-1,1-diphenylpentane, α-methyl-DL-phenylalanine methyl ester hydrochloride, (*S*)-(-)-1,1′-binaphthyl-2,2′-diamine, (*R*)-(+)-1,1′-binaphthyl-2,2′-diamine, (*R*)-(+)-2-amino-1,1′-binaphthalen-2-ol,

Table II: Effect of polar modifier on enantiomeric separations*

	1,2-Napthylethylamine				α-Methyl-4-nitrobenzylamine				2-Amino-1-(4-nitrophenyl)-1,3-propanediol			
Modifier	t_r^1	α	R_s	N	t_r^1	α	R_s	N	t_r^1	α	R_s	$N^\dagger$
Methanol	2.7	1.13	1.5	1800	4.0	1.12	1.1	2060	5.75	1.18	1.7	1700
Ethanol	4.7	1.17	1.2	1220	6.1	1.17	1.1	1150	9.1	1.22	1.6	950
2-Propanol	5.4	1.19	0.8	300	10.5	1.13	0.5	500	16.2	1.20	0.5	500

*Column: 150 mm × 4.6 mm, 5-µm particle diameter; mobile phase: 75:25 (v/v) carbon dioxide–modifier; additive, 0.3–0.2% (v/v) trifluoroacetic acid-triethylamine in modifier; detection: UV at 254 nm; column temperature: 30 °C; flow: 4 mL/min
†Number of theoretical plates per column

(*S*)-(-)-2-amino-1,1′-binaphthalen-2-ol, (*R*)-(+)-1,1-diphenyl-2-aminopropane, (*S*)-(-)-1,1-diphenyl-2-aminopropane, DL-alanine β-naphthylamide hydrochloride, methoxamine hydrochloride (±), 1-aminoindan (±) were purchased from Sigma-Aldrich.

HPLC Methods

All HPLC analyses were performed on an Agilent 1260 Infinity HPLC system that included a degasser, a quaternary pump, an autosampler, a column thermostat, and a diode-array detector. Data analysis was carried out using OpenLAB CDS Chemstation Edition Rev. C.01.04. Flow rates were held at 2 mL/min unless otherwise noted. Normal-phase separations were carried out using hexane with ethanol as a polar modifier in the range of 5–30% (v/v). Polar organic mode separations were carried out using acetonitrile with methanol as a polar modifier in the range of 5–20% (v/v).

SFC Methods

A Jasco 2000 series SFC (SFC-2000-7) equipped with a carbon dioxide pump (PU-2086), a modifier pump (PU-2086), a back-pressure regulator (BP-2080), an autosampler (AS-2059-SFC), a column oven (CO-2060), a variable-wavelength detector (UV-2075), and a makeup pump (PU-2080) supplying additional methanol to the back-pressure regulator was used for all SFC analyses. The carbon dioxide pump was chilled to -10 °C using a Julabo chiller. The back-pressure regulator was maintained at 60 °C. Instrument operation and data analysis was conducted using ChromNAV via an LC-NET II/ADC. The flow rate was held at 4 mL/min unless otherwise noted. Methanol, ethanol, and 2-propanol were used in the range of 2–40% (v/v). Acidic and basic additives were used in the range of 0.1–3% (v/v).

Universal Parameters

Samples were prepared in ethanol at 1 mg/mL. All injections were 5 µL. Column temperature was held at 30 °C unless otherwise noted. UV detectors were operated at 254 nm.

Results and Discussion

Effect of Additives

Table I provides data that allows comparison of the effects of various acidic and basic additives using three probe analytes and otherwise common chromatographic conditions.

Table III: Effect of instrument parameters on enantiomeric separations*

Parameter	1,2-Naphthylethylamine				α-Methyl-4-nitrobenzylamine				2-Amino-1-(4-nitrophenyl)-1,3-propanediol			
	t_r^1	α	R_s	N	t_r^1	α	R_s	N	t_r^1	α	R_s	$N^\dagger$
Temp., 25 °C	4.4	1.17	1.5	2000	5.9	1.15	1.5	2900	9.0	1.19	2.0	2000
Temp., 30 °C	4.6	1.17	1.5	1800	5.8	1.13	1.5	2800	9.0	1.18	1.9	1800
Temp., 35 °C	4.3	1.15	1.3	1700	5.7	1.13	1.4	2600	8.9	1.17	1.7	1800
Temp., 40 °C	4.2	1.14	1.2	1500	5.5	1.13	1.3	2600	8.8	1.16	1.5	1700
Flow, 1 mL/min	18.1	1.15	1.6	3000	24.3	1.12	1.8	5200	39.0	1.18	2.4	2900
Flow, 2 mL/min	8.9	1.15	1.6	2700	11.8	1.13	1.6	3800	18.8	1.18	2.1	2400
Flow, 3 mL/min	5.9	1.16	1.5	2200	7.9	1.13	1.5	3000	12.4	1.18	2.0	2100
Flow, 4 mL/min	4.5	1.17	1.5	1800	5.8	1.12	1.5	2400	9.0	1.19	1.9	1900
BPR‡, 80 bar	4.6	1.16	1.4	1900	6.0	1.12	1.5	2000	9.3	1.20	1.8	2000
BPR, 100 bar	4.5	1.17	1.5	1800	5.8	1.12	1.5	1800	9.0	1.19	1.9	1900
BPR, 120 bar	4.1	1.16	1.3	1800	5.7	1.13	1.3	1700	8.8	1.19	1.7	1800
BPR, 140 bar	3.9	1.15	1.3	1900	5.6	1.13	1.2	1600	8.7	1.20	1.8	1800

*Column: 150 mm × 4.6 mm, 5-µm particle diameter; mobile phase: 80:20 (v/v) carbon dioxide–methanol; additive: 0.3–0.2% trifluoroacetic acid–triethylamine; detection: UV at 254 nm
†Number of theoretical plates per column
‡ BPR: back pressure

Table IV: Chromatographic data for enantiomeric separations under SFC, polar organic (POM), and normal-phase (NP) screening conditions*

Analyte	Mode (% modifier)	t_r^1	k_1	α	R_s
1,2-Naphthylethylamine	SFC (20%)	4.50	10.25	1.17	1.5
	POM (5%)	5.20	4.78	1.09	1.2
	NP (20%)	5.70	5.33	1.17	1.8
2-Amino-1-(4-nitrophenyl)-1,3-propanediol	SFC (20%)	9.0	22.38	1.18	1.9
	POM (10%)	3.55	2.94	1.17	1.7
	NP (20%)	4.40	3.89	1.20	1.6
cis-1-Amino-2-indanol	SFC (20%)	5.48	12.69	1.07	0.5
	POM (10%)	3.00	2.33	1.14	0.8
	NP (20%)	8.40	8.33	1.00	0.0
trans-1-Amino-2-indanol	SFC (20%)	5.20	12.00	1.29	2.5
	POM (10%)	4.40	3.89	1.31	2.3
	NP (20%)	7.90	7.78	1.23	2.1
1-Aminoindan	SFC (20%)	9.44	22.60	1.11	1.5
	POM (10%)	3.50	2.89	1.19	1.9
	NP (20%)	5.80	5.44	1.12	1.5
α-Methyl-4-nitrobenzylamine	SFC (20%)	5.80	13.5	1.15	1.5
	POM (5%)	7.36	7.18	1.11	1.2
	NP (30%)	9.60	9.67	1.15	1.5
α-Methylbenzylamine	SFC (20%)	3.22	7.05	1.13	1.5
	POM (5%)	3.80	3.22	1.21	1.9
	NP (20%)	6.30	6.00	1.19	1.5
1,2-Diphenylethylamine	SFC (20%)	3.55	7.88	1.24	2.2
	POM (10%)	2.36	1.62	1.25	1.9
	NP (20%)	4.40	3.89	1.29	2.5
Norphenylephrine	SFC (25%)	11.10	26.75	1.16	1.5
	POM (10%)	5.80	5.44	1.18	1.5
	NP (30%)	18.95	20.06	1.18	1.5

The common SFC additive, ammonium hydroxide, was evaluated at 0.2% (v/v) in methanol, and absolutely no selectivity was observed (Figure 1). Indeed, it was clear from these studies that ammonium ion negates enantioselectivity and therefore should not be used with these stationary phases. When switching to 0.2% triethyamine, moderate selectivity was observed for two of the probe analytes, but none could be baseline separated. Under acidic conditions when using trifluoroacetic acid at 0.3%, excellent selectivity was observed, but significant peak asymmetry resulted in no baseline separations. By using a combination of trifluoracetic acid and triethylamine at 0.3%, 0.2% (40 mM trifluoracetic acid, 15 mM triethylamine in methanol, 8 mM trifluoracetic acid, 3 mM triethylamine overall) respectively, excellent selectivity and peak shapes were observed with two baseline separations. A likely explanation for the need for trifluoracetic acid is that chiral recognition is improved when analytes are ionized and interact more favorably with the chiral selector and by adding a competitive organic amine (triethylamine), mass transfer kinetics are improved and sharper peak profiles are obtained. Minor changes in retention and selectivity are observed when using alternate organic amines such as diethylamine, diisopropylamine, and butylamine. However, none provided greater resolutions than triethylamine. It appears that the smaller ammonium group most likely complexes too strongly with the chiral selector which inhibits chiral recognition between the derivatized cyclofructan and the probe analytes. Further evidence of this is provided by using a combination of trifluoracetic acid and ammonium hydroxide where retention is strongly attenuated and selectivities are very poor. When using a combination of acetic acid and triethylamine, retention times were increased and selectivities were lower compared to trifluoracetic acid and triethylamine at the same concentration. Previous studies using polar organic chromatographic conditions show that a 3:2 (v/v) acid:base ratio provides optimal separation conditions (30). This was confirmed for SFC by also testing 2:2 and 4:2 (v/v) acid:base combinations for the separation of three test analytes. Overall, selectivity and resolution values were always at a maximum value when the acid:base ratio was at 3:2 (v/v). For example, when 1,2-napthylethylamine was screened using the 3:2 acid:base ratio, selectivity and resolution values were 1.13 and 1.5, respectively. However, when 2:2 and 4:2 acid:base ratios were used to separate 1,2-napthylethylamine, selectivity and resolution decreased, in both cases, to 1.11 and 1.2, respectively. In SFC, the effect of holding the ratio constant and varying the total concentration of the additives was that increasing the amount of additives shortened retention and improved efficiencies, but with minimal improvement in selectivity or resolution (Figures 2c and 2d). This is not the case when operating under polar organic conditions where increasing the concentration of the additives improved peak symmetries and reduced analysis time with minimal loss of selectivity (Figures 2a and 2b). This is likely because of the higher diffusivity of carbon dioxide relative to acetonitrile and overall improved mass transfer kinetics relative to polar organic or normal phase conditions. Based on the combined results, the recommended additive for screening primary amines in SFC is 0.3–0.2% (v/v) in methanol. When operating under polar organic or normal phase conditions, it is possible to either premix the mobile phases

Table IV: Continued

Analyte	Mode (% modifier)	t_r[1]	k_1	α	R_s
DL-*p*-Chlorophenylalaninol	SFC (20%)	7.05	16.63	1.12	1.5
	POM (5%)	6.20	5.89	1.10	1.0
	NP (20%)	12.70	13.11	1.14	1.5
DL-Normetanephrine	SFC (40%)	5.40	12.50	1.16	1.5
	POM (10%)	6.10	5.78	1.17	1.6
	NP (30%)	28.30	30.44	1.20	1.5
Norephedrine	SFC 20%	5.60	13.00	1.13	1.5
	POM (5%)	6.00	5.67	1.18	1.5
	NP (20%)	8.30	8.22	1.18	1.9
DL-Octopamine	SFC (25%)	13.60	33.00	1.15	1.5
	POM (5%)	11.70	12.00	1.15	1.5
	NP (30%)	24.30	26.00	1.16	1.5
trans-2-Phenylcyclopropylamine	SFC 20%	10.20	24.50	1.04	0.4
	POM (5%)	9.20	9.22	1.02	0.4
	NP (20%)	10.20	10.33	1.06	0.6
Phenylpropanolamine	SFC 20%	4.65	10.63	1.11	1.5
	POM (5%)	6.40	6.11	1.16	1.7
	NP (20%)	8.20	8.11	1.18	1.9
2-Phenylglycinol	SFC (20%)	4.99	11.48	1.11	0.0
	POM (5%)	5.50	5.11	1.02	0.4
	NP (20%)	10.20	10.33	1.00	0.0
2-Amino-3-phenyl-1-propanol	SFC (20%)	5.70	13.25	1.17	1.5
	POM (10%)	3.90	3.33	1.15	1.5
	NP (20%)	9.70	9.78	1.13	1.6
1-(1-Naphthyl)ethylamine	SFC 20%	4.00	9.00	1.22	2.0
	POM (10%)	4.60	4.11	1.16	1.5
	NP (20%)	4.89	4.43	1.23	2.2
2-Amino-1-phenyl-1,3-propanediol	SFC (20%)	8.30	19.75	1.06	0.5
	POM (5%)	5.90	5.56	1.16	1.2
	NP (20%)	12.60	13.00	1.00	0.0
2-Amino-1,2-diphenylethanol	SFC (20%)	4.50	10.25	1.24	2.4
	POM (10%)	2.60	1.89	1.29	2.7
	NP (20%)	5.00	4.56	2.00	2.8
1,1′-Binaphthyl-2,2′-diamine	SFC (5%)	10.50	25.25	1.11	1.5
	POM (10%)	1.00	0.11	1.00	0.0
	NP (5%)	7.10	6.89	1.18	1.5
2-Amino-1,1′-binaphthalen-2-ol	SFC (5%)	8.60	20.50	1.09	1.4
	POM (10%)	1.00	0.11	1.00	0.0
	NP (5%)	6.30	6.00	1.11	1.4
1,1-Diphenyl-2-aminopropane	SFC (20%)	2.45	5.14	1.06	0.5
	POM (5%)	2.30	1.56	1.11	0.5
	NP (20%)	3.40	2.78	1.12	1.2
DL-Alanine-β-naphthylamide	SFC (20%)	10.77	25.93	1.00	0.0
	POM (10%)	4.30	9.75	1.00	0.0
	NP (20%)	9.20	9.22	1.06	0.6
Methoxamine	SFC (20%)	5.00	11.50	1.00	0.0
	POM (5%)	4.60	4.11	1.11	0.9
	NP (20%)	8.20	8.11	1.00	0.0

*Column: 150 mm × 4.6 mm, 5-µm particle diameter; polar modifier: methanol (SFC, POM), ethanol (NP); trifluoroacetic acid concentration: 0.3% (v/v) in modifier, triethylamine concentration: 0.2% (v/v) in modifier; flow: 4 mL/min (SFC), 2 mL/min (POM, NP)

or put additives in the separate mobile phase reservoirs (for example, in hexane and ethanol for normal-phase chromatography or in acetonitrile and methanol in the polar organic mode) and let the instrument mix the two solvents in the desired proportions. Regardless of one's approach, the overall additive concentration in the eluent should be 0.3–0.2% (v/v) trifluoracetic acid–triethylamine for normal phase or polar organic mode separations.

Effect of Polar Modifier

Different commonly used organic modifiers for SFC were evaluated at 25% (v/v) with 0.3%, 0.2% trifluoracetic acid, triethylamine and the results are reported in Table II. The general trend for SFC was that as larger alcohol modifiers were used, selectivities were often improved, but mass transfer kinetics were significantly diminished resulting in low plate numbers and pronounced peak tailing. Resolutions were lower for all probe analytes in all cases when changing the organic cosolvent from methanol. Plate numbers dropped precipitously when going from methanol to 2-propanol. Based on these results, methanol is recommended as the polar modifier of choice when screening primary amines using the Larihc CF6-P CSP in SFC. The added advantage of increased organic modifier volatility, when dealing with preparative separations, provides additional impetus for using methanol when developing chiral SFC methods.

Effect of Column Temperature, Flow Rate, and Back Pressure Under Subcritical Conditions

The same three probe analytes used in the "additive study" were used to evaluate the effect of column temperature at 25 °C, 30 °C, 35 °C, and 40 °C using 25% methanol with 0.3%, 0.2% trifluoracetic acid, triethylamine (Table III). As the column temperature was increased, the selectivity and resolution diminished without significant improvement in the analysis time. Interestingly, the highest temperature did not improve efficiency, indicating that under these mobile-phase conditions, mass transfer kinetics are not hindered by operating at subcritical temperatures. The loss of resolution observed by going from 30 °C to 40 °C was significant enough to merit operating at 30 °C. Given the necessity of having a considerable polar modifier to elute the analytes from the CSP (15–30% v/v) and the fact that resolutions were diminished at elevated temperatures, no effort was made to operate under true supercritical conditions as chromatographic performance would certainly be compromised at the temperatures and pressures necessary to reach the critical point.

Flow rates of 1-, 2-, 3-, and 4-mL/min were evaluated to study the effects of operating at higher linear mobile-phase velocities. The results also are presented in Table III. When going from 1 mL/min to 4 mL/min, plate counts were reduced by approximately 40% without a loss of selectivity. Resolutions were higher at 1 mL/min, but came with an obvious cost of analysis time. Because selectivities were not lower at higher linear velocities and plate counts were still acceptable, 4 mL/min is the recommended flow rate for screening.

A similar study was performed by analyzing the three probe analytes with the back-pressure regulator set at 80, 100, 120, and 140 bar. The effect was a moderate decrease in retention time at higher pressure with

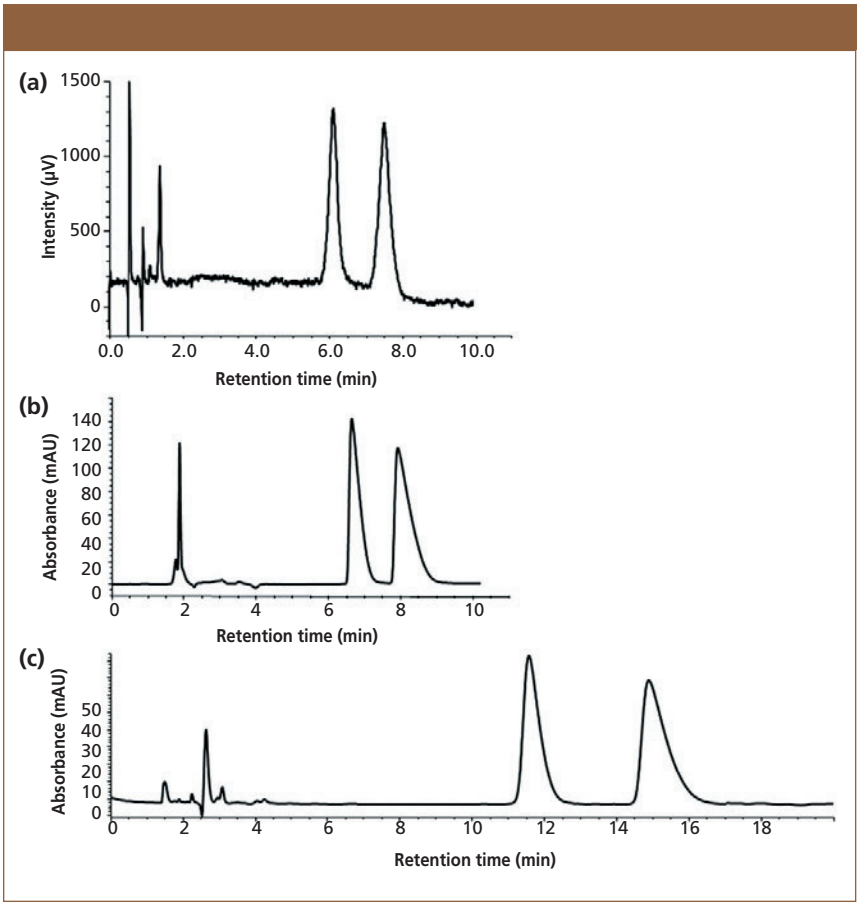

Figure 3: Comparison of SFC, polar organic, and normal-phase modes. Analyte: *RS*/*SR* 2-amino-1,2-diphenylethanol, chromatogram (a) mobile phase: 80:20 carbon dioxide–methanol; flow: 3 mL/min; (b) 90:10 acetonitrile–methanol, flow: 1 mL/min; (c) 80:20 hexane–ethanol; flow: 1 mL/min; all chromatograms 0.3–0.2% (v/v) trifluoroacetic acid–triethylamine (polar modifier only).

minor loss of selectivity and resolution. Minor losses in plate count were observed by increasing the column back pressure and thus a recommended back pressure of 100 bar seemed acceptable.

Comparison Between SFC, Normal-Phase, and Polar Organic Modes

Table IV gives the chromatographic results for the separation of 25 racemic primary amines under SFC, polar organic, and normal-phase modes. In general, SFC provided the best peak symmetries, while the polar organic mode provided the shortest analysis times and the normal phase provided the greatest resolutions at a cost of analysis time (Figure 3). Under SFC conditions, 16 out of 25 analytes were baseline separated in the screen while the polar organic and normal-phase modes provided 13 and 17 baseline separations, respectively. In total, the SFC screen showed enantioselectivity for all but three of the tested analytes. It should be noted that, for comparative purposes, trifluoracetic acid and triethylamine were only added to the polar modifier in the polar organic and normal-phase modes. In practice however, it is recommended to make the overall concentration 0.3–0.2% (v/v) trifluoracetic acid–triethylamine when using either the polar organic mode or normal phase. This will result in more baseline separations when using HPLC because of improved peak symmetries, particularly when operating under polar organic conditions (as discussed earlier and shown in Figure 2).

Acetonitrile provided the greatest eluotropic strength followed by hexane, with carbon dioxide having the lowest strength. Retention factors were always highest for SFC separations even when operating at a greater percentage of methanol relative to the polar organic mode. Because the normal-phase separations were conducted using ethanol instead of methanol as a polar modifier, direct comparisons of retention factors for normal phase and SFC are not possible, however, normal-phase retention factors under the tested conditions were always intermediate relative to SFC and polar organic modes.

Conclusions

SFC was evaluated as an alternative to HPLC for the separation of primary amines using the CF6-P chiral stationary phase. Using ammonium hydroxide as a basic additive destroys enantioselectivity. However, using methanol as a polar modifier with 0.3–0.2% (v/v) trifluoracetic acid–triethylamine as additives, excellent selectivity was observed with 18 baseline separations and 22 hits out of 25 probe analytes under general screening conditions. When compared to normal-phase chromatography and polar organic mode chromatography using the same CSP, SFC showed comparable selectivities and analysis times as well as improved peak symmetries. The recommended mobile phases for screening chiral primary amines in the polar organic and normal-phase modes are 90:10 acetonitrile–methanol and 80:20 hexane–ethanol, respectively, with an overall concentration of 0.3–0.2% (v/v) trifluoracetic acid–triethylamine. In general, additional optimization of the mobile-phase composition further enhances the selectivity and resolution of all analytes as compared to the screening solvent.

Acknowledgments

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PRODUCTS & RESOURCES

Compact benchtop tandem quadrupole mass spectrometer

The Xevo TQ-S micro compact benchtop tandem quadrupole mass spectrometer from Waters Corporation is designed to acquire data at accelerated rates for numerous analytes at varying concentrations. According to the company, the mass spectrometer is suitable for laboratory work in food, environmental, pesticides, pharmaceutical bioanalysis, and peptide screening.

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

Automated extraction system

The EconoPrep system from FMS, Inc., is designed to automate the manual processes involved in sample preparation for trace analysis. According to the company, the system offers kits for environmental and food matrices and automatically performs extraction, sample cleanup, and concentration, allowing for same-day results.

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

Flash and preparative chromatography

The PuriFlash MEU (MS-ELSD-UV) system from Interchim is designed for flash and preparative HPLC and provides up to six detector signals. According to the company, control of split flow to the MS and ELSD systems maximizes sensitivity from 1 to 250 mL/min, and a fume enclosure on the fraction collector allows users to operate the instrument on a laboratory bench.

Interchim Inc.,
Los Angeles, CA.
www.interchiminc.com

Preparative-scale SFC system

Jasco's Prep-2088 preparative SFC system is designed for chiral and achiral separations with column sizes ranging from 10 mm up to 30 mm. According to the company, a back-pressure regulator allows control of system pressure regardless of solvent composition and flow rate, and the system pumps deliver up to 120 mL/min of CO_2 and up to 80 mL/min of modifier and flush solvents at pressures up to 35 MPa (5000 psi).

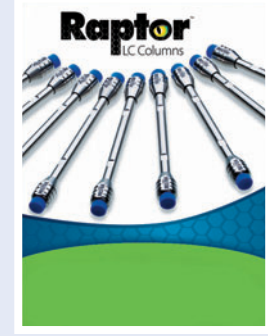
Jasco, Easton, MD. www.jascoinc.com

Chiral columns

RegisPack, RegisPack CLA-1, RegisCell, and Whelk-O 1 3.5-µm chiral columns from Regis are designed to provide a greater variety of analytical chiral separation capability. According to the company, multiple dimension sizes are available with ID sizing between 2.1 and 4.6 mm and lengths between 2 cm and 25 cm.

Regis Technologies, Morton Grove, IL.
www.registech.com

Core–shell columns



Raptor SPP core–shell columns from Restek are designed with 5-µm particles. According to the company, the particles provide benefits such as better efficiency, peak shapes, signal-to-noise ratios, and sensitivity over fully porous particles of the same dimension, but without the significant increase in pressure. The columns reportedly provide increased sample throughput on existing 400-bar HPLC systems.

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

Integrated HPLC systems

Shimadzu Scientific's Prominence-i and Nexera-i integrated HPLC systems are designed to provide a more efficient workflow for conventional to ultrahigh-speed analysis. According to the company, the data acquired by these systems via an interactive communication mode is sent to a laboratory's data center by the systems' LabSolutions network and managed uniformly by a server.

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

SEC monoclonal antibody analysis

An application note from Tosoh Bioscience titled "Analysis of Monoclonal Antibody Aggregates by SEC Using MS-Friendly Mobile Phases" describes the effective use of MS-compatible mobile-phase compositions in the analysis of monoclonal antibody aggregates using the company's TSKgel UltraSW Aggregate SEC Column.

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

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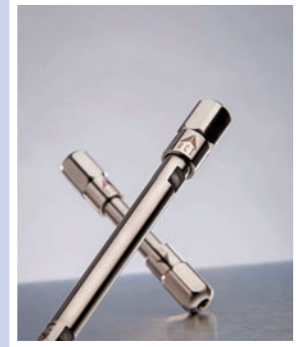
Published in USA, 5991-1198ENUC, 2013

Agilent Technologies

Diol HPLC column

The Selectra diol HPLC column from UCT is designed for normal-phase or HILIC Separations. According to the company, the columns are available with 1.8-, 3-, or 5-µm particles, and a protein- and peptide-friendly phase enables retention of biologically active compounds.



UCT, LLC,
Bristol, PA.
www.unitedchem.com

LC–MS column kits

YMC's Mariner LC–MS column kits are designed to simplify and accelerate method development. According to the company, the kits are available for HPLC and UHPLC, and customers may specify other combinations of phases in kits of three, four, or five columns. Preconfigured kits are also available.

YMC America, Inc.,
Allentown, PA.
www.ymcamerica.com

Sample preparation automation system

The AutoMate-Q40 system from Teledyne Tekmar is designed to automate the QuEChERS sample preparation workflow. According to the company, the system is configured "out of the box" to conduct two QuEChERS sample preparation methods: AOAC2007.01 and EN 15662.2008.

Teledyne Tekmar,
Mason, OH.
www.teledynetekmar.com/AutoMateQ40

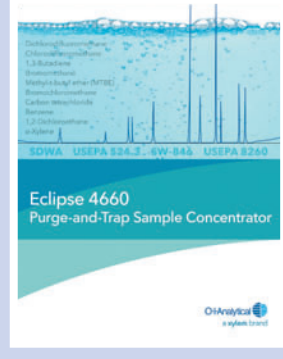
GC–MS system

Agilent's 7010 triple-quadrupole GC–MS system includes an electron ionization (EI) source designed to enable the system to attain attogram detection limits. According to the company, the source creates more than 20 times as many ions as the current generation of EI sources.

Agilent Technologies,
Santa Clara, CA.
www.agilent.com

Sample concentrator brochure

A brochure from OI Analytical reportedly details the design and performance characteristics of the company's Eclipse 4660 purge-and-trap sample concentrator for GC–MS analysis of volatile organic compounds. According to the company, the brochure contains instrument specifications and information about subcomponents that directly affect purge-and-trap efficiency.



OI Analytical,
College Station, TX.
www.oico.com

Capillary selection feature

The free on-line Capillary Configurator capillary tubing selection feature on the Polymicro Technologies website is designed to guide users in submitting specifications and application parameters for review by the Molex technical team to provide a quote on the company's capillary products. The feature reportedly also can be used for project planning and budgeting.

Polymicro Technologies, Phoenix, AZ.
www.polymicro.com

UHPLC system

Thermo Fisher Scientific's Vanquish UHPLC system is designed to be used as a standalone system or with the latest mass spectrometers. According to the company, the system uses the company's Accucore UHPLC columns, which have 1.5-µm solid-core particles, to take advantage of its 1500 bar (22,000 psi) maximum pump pressure and flow rate up to 5 mL/min.

Thermo Fisher Scientific,
San Jose, CA. www.thermofisher.com/vanquish

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. According to the company, the detector connects to its Optilab UT-rEX.

Wyatt Technology Corp.,
Santa Barbara, CA.
www.wyatt.com

Automated Solid-Phase Extraction for the Environmental Testing Laboratory

ON-DEMAND WEBCAST (originally aired Wednesday, September 24, 2014)

Register for free at www.chromatographyonline.com/solid_phase_extraction

EVENT OVERVIEW:

Today's analytical laboratory is faced with tight deadlines to produce results from testing environmental samples. Too often, solid-phase extraction (SPE) presents a bottleneck in the analytical testing process and may cause poor analyte recoveries and highly variable. Despite advances in analytical instrumentation, sample prep often relies on tedious, manual, and expensive techniques such as liquid-liquid extraction.

Sample preparation of environmental water samples can be automated, however. Use of automated sample preparation addresses the many challenges that laboratories face when preparing samples and can help improve sample processing turnaround times. The first part of the webinar will discuss how automated sample preparation can benefit analytical laboratories.

The second part of the webinar will present data from Orange County Water District (OCWD) where eight nitrosamines, 1, 4-dioxane and other emerging contaminants are monitored at sub parts per trillion levels. We will discuss the use of sample flow rate, elution time, drying time, and nitrogen gas flow rate as key operational parameters within the solid-phase extraction process to optimize the analytical step.

Key Learning Objectives:

- Strategies for streamlining method development and optimization.
- Review key applications for environmental testing.
- Learn how SPE can be automated Review key operational parameters for SPE, to optimize the analytical step and save time compared to Liquid-liquid extraction.

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PRESENTERS:

Aaron Kettle
Product Manager
Dionex ASE Systems, Dionex AutoTrace 280 SPE, and Rocket Evaporator Systems
Thermo Scientific

Mr. Lee Yoo
Laboratory Director
Orange County Water District

MODERATOR:

Laura Bush
Editorial Director
LCGC

Who Should Attend:

- Analysts performing solid-phase extraction of environmental waters.
- Laboratory managers who are looking to improve sample testing throughput and minimize laboratory error caused by sample preparation.
- Contract testing laboratories that are looking to reduce costs and time associated with sample preparation.
- Scientists that use or have access to an automated sample preparation instrument.

For questions, contact Kristen Moore at kmoore@advanstar.com

AD INDEX

H P L C INSTRUMENTS & SUPPLIES

THE ESSENTIALS

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

Critical Evaluation of HPLC Methods

The ability to properly develop, specify, and implement high performance liquid chromatography (HPLC) methods is critical for a successful separation, yet so many badly designed, badly specified, and poorly implemented methods exist. There are many parameters and variables that remain untouched between methods (the "lock and leave" syndrome) and are poorly understood in terms of their influence on a separation. Here, we describe important variables within a typical method to aid in understanding and highlight some of the commonly overlooked items when developing or implementing HPLC methods. For more information see the CHROMacademy webcast and Essential Guide Tutorial accompanying this article at www.chromacademy.com.

Acetonitrile is often chosen as the organic modifier for reversed-phase HPLC methods because of its low UV cut-off (190 nm) and relatively low viscosity (0.37 mPa·s). However, it is important to note that methanol, although not being so favorable in respect of UV cutoff (205 nm) or viscosity (0.6 mPa·s), has different physicochemical (solovophobic) properties that offer an alternative separation selectivity and can be particular useful when analyzing bases or less polar analytes. Isoeluograms can be used to obtain "equivalent" eutropic strengths when changing between modifiers, so that separations occur in a similar time frame but with an altered selectivity. This can be particularly useful when a suitable stationary phase for the separation cannot be easily identified; however, one should pay careful attention to the formation of azeotropic mixtures of methanol and water that lead to higher system back pressures, especially when using ultrahigh-pressure liquid chromatography (UHPLC) equipment. One should adjust the system pumping equipment to compensate for the viscosity differences of the various organic modifiers when performing on-line mixing.

When dealing with ionizable compounds, knowledge of the analyte pK_a values will allow the selectivity of separation to be "tuned," because retention in reversed-phase HPLC is related to analyte polarity and, therefore, the extent of ionization of functional groups. Acidic analytes have maximum retention in eluent systems at two pH units lower than the functional group pK_a and basic analytes in eluent systems at two pH units higher than the pK_a value. When dealing with analyte mixtures, the selectivity of the separation is altered by changing eluent pH until a suitable separation is obtained; however, one should note that separation robustness decreases with proximity to the analyte pK_a, where small changes in eluent pH give rise to larger changes in analyte retention. Recently, it has been fashionable to use eluent systems containing pH modifiers such as trifluoroacetic acid to adjust eluent pH well away from analyte pK_a values. For example, at pH 2.1, most acidic analytes will be fully neutral, most bases will be fully protonated (ionized), and modern stationary-phase chemistries can be chosen that will retain the more polar basic analytes. While this approach gives a robust method (eluent pH well away from the analyte pK_a values), we lose the ability to adjust the selectivity of the separation using eluent pH and rely solely on the nature of the organic modifier and the stationary-phase chemistry to optimize the band spacing.

One should note that trifluoroacetic acid is not a buffer and, as such, pH changes in the local environment as the sample is injected and enters the column may cause peak shape problems or retention time reproducibility issues. One should choose a true buffer (weak acid or base and its salt in cosolution such as trifluoroacetic acid and ammonium formate) whose pK_a is within one unit of the desired eluent pH value and is present at a high enough concentration to have the buffering capacity required (25 mM or less is typically sufficient with most common reversed-phase buffers).

Most modern HPLC stationary phases are made from spherical silica particles that are either fully porous or have a porous outer layer with a solid core. Smaller diameter particles are more efficient, but they will cause increased system back pressure in equivalent column dimensions. This increased efficiency can be used to effect high-resolution separations in a similar time frame to traditional particles or to obtain equivalent resolution in a reduced separation time frame. The larger the pore size within the stationary phase particle the lower the surface area, but the stronger the particle in terms of resistance to higher back pressures, and larger pore particles (300 Å) are typically used for biomolecule analysis to avoid exclusion of the large analyte from the pore system and hence poor retention. Some deactivation of the silica surface is usually necessary to obtain Gaussian peak shapes when analyzing polar or ionizable analytes to negate the interaction between these analytes and polar, lone (acidic) residual silanol groups on the silica surface. Analyses undertaken at low pH will help to reduce these interactions, which ultimately manifest themselves as tailing peaks, by operating at low eluent pH to help suppress the ionization of the acidic surface silanol species.

In general, short (circa 50 mm), narrow (2.1 or 3.0 mm i.d.) columns are used with smaller particles to generate high efficiencies in modern HPLC systems; however, these columns also highlight any problems with large extracolumn volumes in the HPLC system and all tubing and flow cell volumes need to be minimized to enjoy the benefits of the columns' increased efficiency.

When using UV detection, the data sampling rate and slit width must be optimized to realize the optimum sensitivity, acquisition wavelength, and bandwidth. Also, the reference wavelength and bandwidth need to be optimized to reduce baseline noise and drift in diode-array UV detection.

More Online:

Get the full tutorial at
www.CHROMacademy.com/Essentials
(free until October 20).

Next Generation UHPLC Technologies Change the Landscape in LC

LIVE WEBCAST: Thursday, September 25, 2014 at 8:00 am PDT/ 11:00am EDT/ 4:00pm BST/ 5:00pm CEST

Register for free at www.chromatographyonline.com/uhplc_technologies

EVENT OVERVIEW:

This webinar is all about UHPLC performance. How can new column technology, delivering the best separation power, be married with the best UHPLC hardware to ensure that the result is an outstanding one? How can we as chromatographers ensure that even very complex and unfamiliar samples are assayed with the highest scrutiny possible? During this webinar we will investigate:

1. How to get the most out of solid core column technology with the right UHPLC system
2. How the use of an extreme long column approach can be used for ultra-high resolution assays
3. The importance of robustness and retention time stability
4. How you can achieve the best of both worlds for your separation: speed versus resolution
5. How to manage and successfully process very data rich results

Who Should Attend:

- Analytical laboratories requiring fast and efficient separations of very complex sample sets
- Method developers
- High throughput or screening labs
- Laboratory managers and coordinators

Key Learning Objectives:

- How solid core columns technology and advances in UHPLC technology allow you to achieve the results you want in the time that you need
- How new UHPLC and solid core column technology provide robust and accurate results in a high throughput environment
- How complex data sets can be processed simply and effectively

Presenters:

Mike Oliver
Product Manager
Sample Preparation, Chromatography Consumables
Thermo Fisher Scientific

Rainer Bauder
Manager Pharma Solutions, Small Molecules
Thermo Fisher Scientific

Moderator:

Laura Bush
Editorial Director
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