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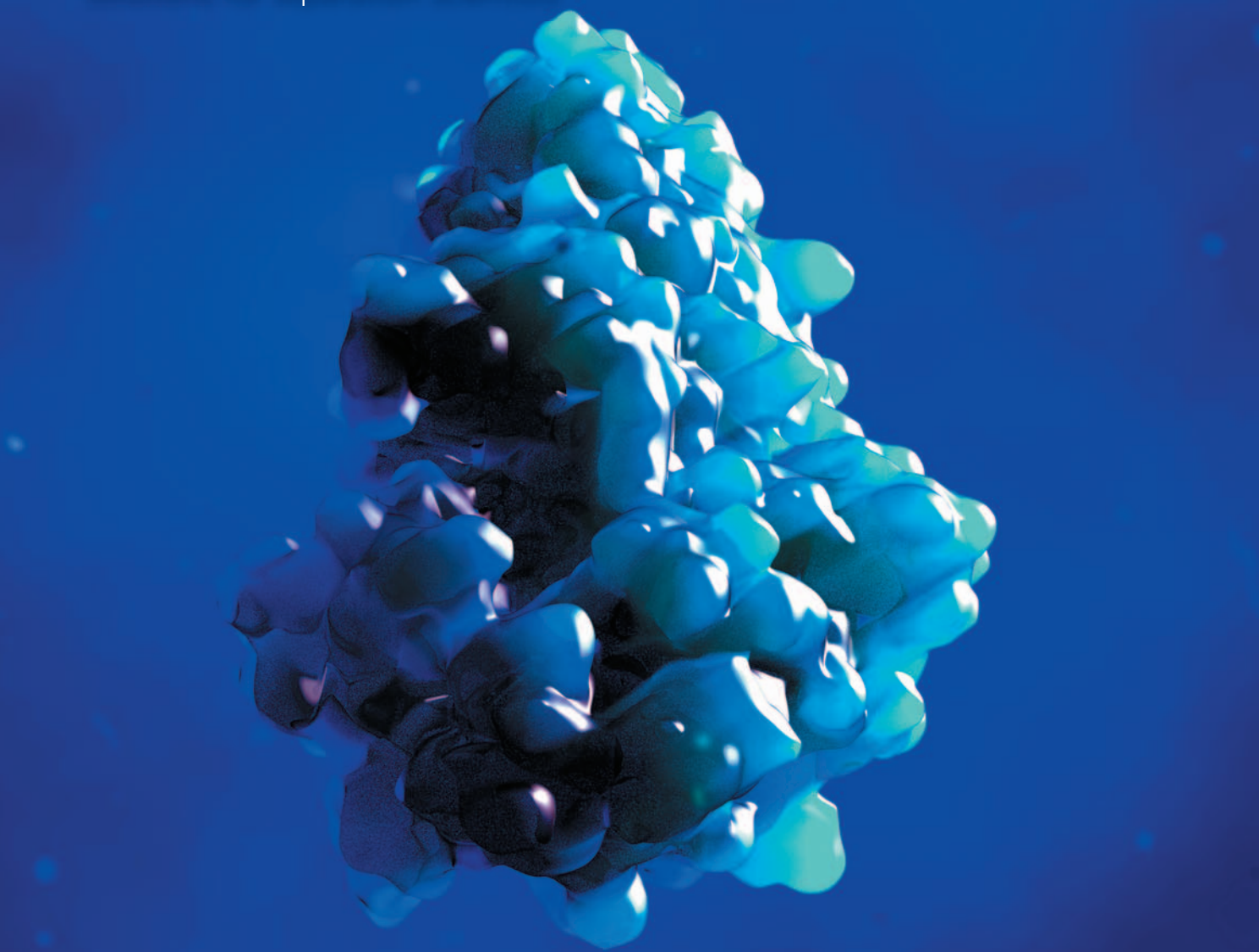

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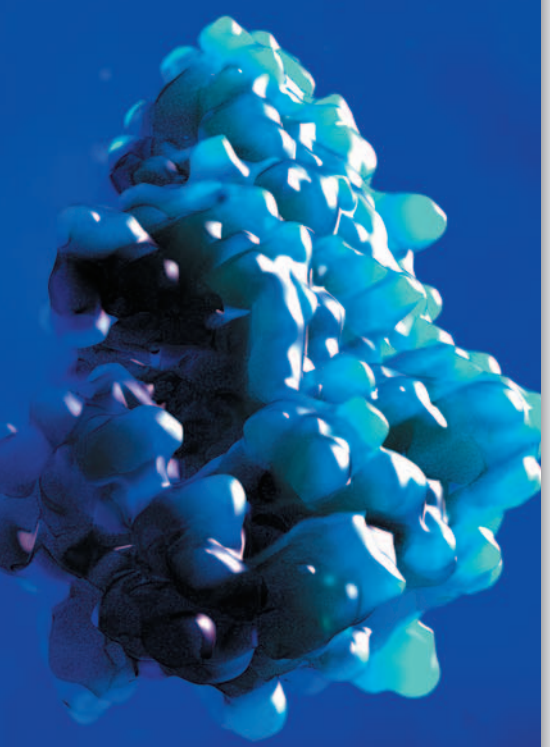

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Pressure Tuning: Increasing the Flexibility of **Comprehensive Two-Dimensional Gas Chromatography**

Mohammad Sharif Khan and Philip John Marriott, Australian Centre for Research on Separation Science, School of Chemistry, Monash University, Clayton, Victoria, Australia

Comprehensive two-dimensional gas chromatography (GC×GC) offers significant improvement for volatile chemical separation. Selecting suitable first (^{1}D) and second dimension (^{2}D) columns normally requires consideration of the chemical composition of a sample. Replacing one of these dimensions with a two-column ensemble (for example, $^1D_1$ + $^1D_2$ for the ^{1}D column), provided with a pressure tuning makeup gas between them, varies the relative retentions of compounds before the modulation step according to the junction pressure. This makes it possible to alter the apparent polarity of the ^{1}D ensemble, and this alters peak positions in the 2D GC×GC space. This article presents an account of studies that suggest this offers potential for improved operation for a GC×GC laboratory.

The general objectives of newly introduced stationary phases is to perform separations that achieve an improved or alternative selectivity, usually not possible on other phases. Ionic liquids, metal organic frameworks, and nanomaterials are a number of newer gas chromatography phase materials to emerge in the literature (1–3). Another valid approach for adjusting selectivity is to use coupled columns. This is an attractive and easy option to alter separation selectivity and was well-studied during the 1980s–90s (4). Pressure tuning (PT) at the mid-point between two coupled columns, 1 and 2, of different phases is able to achieve an intermediate selectivity of the serially-connected coupled columns, so that the overall result is neither that of column 1 nor of 2. By simply varying pressure, this approach is able to "tune" or adjust the fractional contribution of each column to the overall separation (5,6). This process is able to alter the relative elution orders of different compounds, which corresponds to making the coupled column (column 1 + column 2) to be more polar or less polar in nature, in effect it makes the coupled column more like column 1, or more like column 2. This is easily achieved on-line by simply adjusting the mid-point pressure. Sacks and co-workers were especially active in promoting the PT process, including various stop-flow methods, and multiple stages of PT during the gas chromatography (GC) analysis (7). It is possible that improved chromatographic selectivity (re)discovered by multidimensional and comprehensive two-dimensional (2D) chromatography platforms, and the increased dependence and innovations in mass spectrometry (MS), led to PT losing favour (8).

Pressure tuning of a coupled column arrangement as the first dimension (^{1}D) in GC×GC, termed PT-GC×GC, was recently proposed (see below). This innovation uses two first dimension columns—$^1D_1$ and $^1D_2$—with pressure tuning at their junction. This is followed by the modulator, which delivers analytes into the second dimension (^{2}D) column. The idea of "tuning" the ^{2}D in GC×GC has also been recently investigated by Gorecki's group, by using separately programmable temperature operation for the ^{2}D column, the goal of which is also to alter relative separation (9). This article will evaluate the scope and role of the PT-GC×GC process, discuss if it provides useful and controllable manipulation of compounds in the 2D separation, and the perspectives it could provide for practical application.

Selectivity Changes by Pressure Tuning

In a typical coupled column one-dimensional (1D) system with two different stationary phases, two columns are

KEY POINTS

- Pressure tuning at a ^{1}D junction between two ^{1}D columns in GC×GC effectively changes—tunes—the retention of compounds in ^{1}D.
- Changes in ^{1}D retention modifies the elution temperature (T_e) to the ^{2}D column, and changes the relative position of peaks in the 2D plot.
- Changing 2D peak positions by PT changes the overall separation orthogonality, and may be an effective strategy to more readily optimize separations.

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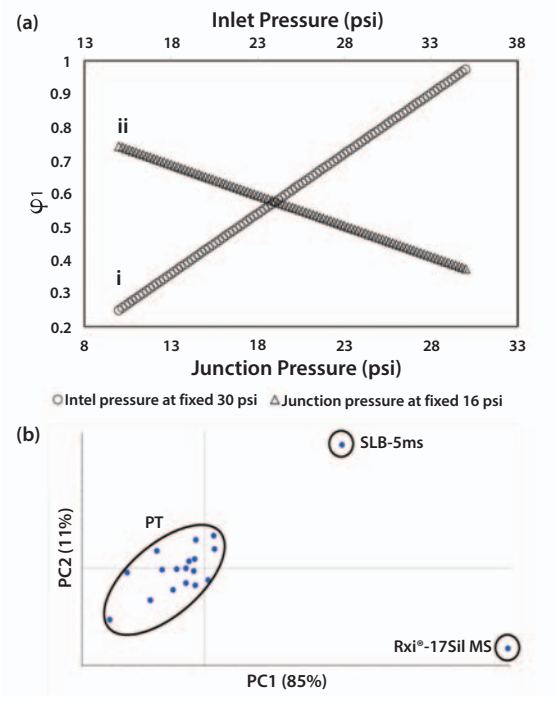
Figure 1: (a) Plot showing changes of 1st column contribution (φ_1) by either (i) a mid-point, or (ii) inlet pressure change; and (b) selectivity differences when using the PT process in terms of LSER descriptors in a PCA plot—the PT system is neither like the SLB-5ms phase, nor the Rxi-17 MS phase. Adapted with permission from reference 11.



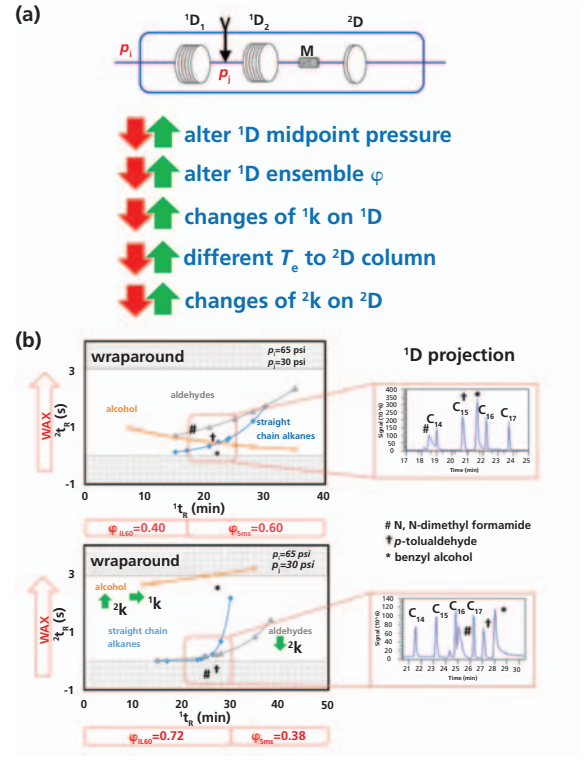
serially connected by, for example, a microfluidic splitter device (10). An analyte's interaction is different for each of these stationary phases, so this provides an intermediate retention property for the analyte. For example, if the analysis is conducted using polar–nonpolar coupled columns, the retention time will be the summation of the interaction time of the analyte in both the first and second columns. This is better expressed in terms of retention factors:

$$k_s = \frac{t_{R,1} + t_{R,2}}{t_{M,S}} \quad (1)$$

$$k_s = \varphi.(k_1 — k_2) + k_2 \quad (2)$$

where, $t_{R,1}$ and $t_{R,2}$ are analyte retention times on the first and second columns, k_s is the coupled column retention, and $t_{M,S}$ is the coupled column void time. The term φ is called the *relative retentivity* and measures the relative contribution of each column to the composite retention. It provides a straightforward numerical presentation of hold-up time and retention variation for a PT system. For isothermal analysis the overall retention will be a weighted average of the time that the analyte resides on each column. Peaks can shift relative to each other for different mid-point pressure settings, so careful adjustment of the pressure using this strategy allows an optimized 1D separation to be achieved.

Figure 2: (a) Illustration of features of PT-GC×GC; the arrows illustrate the parameters that will change according to variation in P_i and P_j settings; and (b) the changes in ^1k and ^2k for selected analytes as a result of change of mid-point pressure from 30 psi to 60 psi. Column set: 30 m × 0.25 mm, 0.20-µm ^{1}D SLB-IL60 phase (Supelco), 30 m × 0.25 mm, 0.25-µm SLB-5ms phase (Supelco), and 2 m × 0.10 mm, 0.10-µm ^{2}D Supelcowax10 (Supelco). The contour plot shows only a few experimental, and some extrapolated, data points with an increasing carbon skeleton of straight chain alkanes, aldehyde, and alcohol. M = modulator. See text for detail.



A change in the mid-point pressure (junction pressure) has a direct effect on the carrier gas flow, corresponding to changes in hold-up time for each column, that is, the t_M values for each column. At a given temperature, this determines the retention factor (k) of each solute on each column; changes in hold-up time can vary the retention properties of the analytes as a result of the PT process. It should be asked how much change in hold-up time is possible by PT, and how is this related to retention properties? The answer is based on (a) the analytes' properties, (b) the stationary phases used for the PT column ensemble, (c) the pressure setting, and (d) contribution of the stationary phase to separation on each column.

PT-GC×GC Mechanism for Adjustment of Retention

The relationship between relative column contributions and retention properties for different analytes for the decompression of carrier gas in each column in a coupled column system is documented in earlier work (11). Each column's contribution changes in a predictable manner with

pressure setting. Figure 1(a) shows a combined summary of changes in column 1 contribution (φ_1) by changing (i) the mid-point pressure at a fixed 30 psi inlet and (ii) the inlet pressure at a fixed 16 psi of mid-point pressure. Increasing the mid-point pressure at fixed inlet pressure increases the first column contribution by increasing the residence time on the $^1D_1$, and this, consequently, decreases the contribution of the second ($^1D_2$) column. Changes in inlet pressure can also provide a tuning opportunity if the mid-point pressure remains constant; mid-point pressure tuning seems more flexible. The overall solvation descriptors using the linear solvation energy relationship (LSER) in the case of PT are also described and compared to the single and coupled column descriptors. The possibility of applying PT for an intermediate selectivity (Figure 1[b]) is clearly demonstrated, indicating that variable retention properties can arise for PT, allowing peaks to shift in relative retention. Based on this point it is evident that the PT process could be an opportunity to alter ^1D selectivity in the first dimension of an on-line coupled column system in GC×GC, which can translate into different elution properties on the ^2D column.

The net retention of an analyte in PT-GC×GC involves a combination of steps that systematically change parameters, as shown in Figure 2(a). The first dimension in GC×GC is important to accomplish a preliminary first separation screen of the sample, and then the second dimension provides separation over a very short time with limited peak capacity. A viable separation strategy will ensure that the ^1D compound retentions are appropriate to then allow the ^2D column to complete the desired separation. PT in ^1D GC×GC could be an option to optimize the mid-point pressure to give best separation in the ^1D and then provide best separation in 2D space (12). For example, an increase of junction pressure from 30 to 60 psi in Figure 2(b) increases the first ^1D column contribution from 0.40 to 0.72; as a result the ^1D ensemble increases the relative retention for polar analytes, such as alcohols (yellow line), compared with straight chain alkanes (blue line).

The effect of PT on ^1D separation alters compound elution temperature (T_e) to the ^2D column and as a result the relative ^2D separation will be modified. For example, in Figure 2(b) the aldehyde (marked †: *p*-tolualdehyde) was coeluted with C_{15} alkane (t_R = 21 min) at 30 psi, but t_R ~ 27 min at 60 psi mid-point setting. This significantly changes the T_e (by 40 °C at 10 °C/min) and hence ^2t_R. In other words, in a temperature programmed run the T_e of this solute is increased when it enters the ^2D column. As a result the ^2D retention for this analyte was reduced from 1.015 to 0.226 s. This should be sufficient to resolve this compound from others that might have coeluted on the ^1D or the ^2D column. Similar observations can be made for other analytes. As a result of these changes in ^1D and ^2D retention of analytes by tuning the mid-point pressure, the overall separation orthogonality is also changed, as shown in a recently published work (12). Some wraparound—where wraparound causes ^2t_R to exceed the modulation period—did occur, which must be considered for the PT-GC×GC separation.

The changes in the ^2D can be predicted by estimating the elution temperature (T_e) for different PT programs. This change follows the isovolatility curve of analytes (progressive reduction in retention as *T* increases), with

Table 1: The relation between the T_e and ^2t_R for two analytes at different mid-point pressures in a PT-GC×GC system

p_i = 28 psi	Diacetone Alcohol			α-Terpineol		
p_j (psi)	^1t_R (min)	T_e (°C)	^2t_R (s)	^1t_R (min)	T_e (°C)	^2t_R (s)
15	7.33	103	2.87	13.17	161	3.90
16	7.42	104	2.69	13.33	163	3.57
17	7.58	105	2.54	13.50	165	3.36
18	7.83	108	2.24	13.75	167	2.97
20	8.50	115	1.66	14.50	175	2.09
23	10.67	136	0.33	17.08	200	0.66

data shown in Table 1 for two analytes. The ^2t_R value decreases as the T_e increases because of an increase of p_j for diacetone alcohol and α–terpineol. This relation can be used to determine the ^{2}D position of any analyte for different PT settings, which changes the analyte's retention position in 2D and therefore the overall separation orthogonality. Both of these analytes are polar, and so the effect of PT is not great. A nonpolar analyte will have much more of a variation.

Increasing flexibility of operation by using an "impulse" to tune the separation power of columns is not new in chromatography. Mommers and co-workers studied tuning the selectivity of GC×GC in both the ^{1}D and ^{2}D by using temperature (13,14). In terms of extending separation by use of multiple GC×GC arrangements, Savareear and Shellie proposed multiplexing column phases to give two independent GC×GC systems, and achieve more chromatographic information in reduced operation time (15). Synovec and co-workers recently demonstrated implementation of GC×GC×GC–time-of-flight (TOF)-MS, which offers many opportunities for additional column phase choices to maximize separation (16). The latter are "static" approaches that do not allow easily modified conditions permitted by pressure tuning of selectivity in GC×GC. For 1D approaches, temperature pulsing for liquid chromatography (LC) and a mid-point pressure tuning approach on supercritical fluid chromatography (SFC) have also been reported in the literature (17,18). These two studies demonstrate a degree of flexibility of operation in a 1D ensemble for LC and SFC.

The Multiple Benefits of PT-GC×GC System

Many strategies can be envisaged to alter the overall separation in a PT-GC×GC experiment.

(1) The first-dimension separation could be tuned by peak swapping as a result of the PT process.
(2) The second-dimension separation may be tuned by variation in T_e to the ^{2}D column.
(3) The overall separation orthogonality may be changed.
(4) A simple, on-line process of tuning selectivity by altering the mid-point pressure is possible.
(5) It may be possible to use a small set of ^{1}D ensemble and ^{2}D columns, but with PT of ^{1}D, to achieve the best separation for different sample types. By using a "good choice" of ^{1}D ensemble with an appropriate ^{2}D column, with specific PT of ^{1}D, it might be possible to use different PT set points, for example, set point 1 may be best for separation of sample type 1, set point 2 may be best for separation of sample type 2, and set point 3 may be best for separation of sample type 3.

Rapid Screening Method of a Wide Variety of Samples: Rapid screening of sample analytes to get an initial idea of sample contents for a wide variety of sample types—for example, essential oils, fatty acids, and so on—by using the tuning properties of PT-GC×GC is advantageous for any laboratory. Instead of accepting a less optimized result using a previously installed column set, an analyst can easily modify the selectivity of the ^{1}D using this PT mechanism, which would save time and resources and would increase throughput and output of the laboratory.

PT in ^{2}D of a GC×GC Separation: The second-dimension separation in GC×GC (GC×PTGC) may be investigated under incrementing isothermal conditions because of the very short time and fast separation on the ^{2}D. Optimization of GC×GC working parameters most often involves finding ways to adjust the ^{2}D column phase and its retention (^2t_R) for compounds eluted on the ^{1}D column. PT coupled columns in the ^{2}D (GC×PTGC) experiment have been reported recently to help in understanding the opportunities and limitations of ^{2}D PT (19). As in ^{1}D, the tunable residence time arising from differential pressure drop in each ^{2}D column ($^2D_1$ and $^2D_2$) results in a tunable fractional contribution of each column in the ^{2}D separation. However, the very short and fast retention times expected for the ^{2}D makes PT of the ^{2}D difficult.

Unknown Identification by PT Coupled Columns: Retention index data relate the retention of a solute to a reference series of homologous compounds, such as alkanes, on a specific stationary phase and at a given temperature, although temperature programming indices

are often also used. A number of sources include *I* data on commercial stationary phases. In PT, a first-dimension index—^1I—means that data for alkanes and the solute must be collected from two different phases at a given PT setting. This might be considered a difficult process to interpret, although the alkane and solute retentions are easily measured, just by noting their elution times from the ensemble. It is necessary to find a strategy to align the retention index data for the coupled column system in order to apply the index system to solute identification. By using isothermal data from individual columns and then fitting PT data, it is possible to align PT retention index data for the identification of library data of the analytes (20).

The On-Line Process of Selectivity Change: The PT process allows on-line selectivity changes in GC×GC. An investigation of first dimension selectivity by changing the ^{1}D composite column length ($^1D_1$ and $^1D_2$) has been reported in the literature (21). The authors used two contrasting columns (nonpolar and polar) to measure the effect of ^{1}D column selectivity on overall separation and orthogonality. They showed that the ^{2}D separation is affected by, and can be interpreted based on, the ^{1}D T_e to the ^{2}D column; data were simulated and agreed with the experiment. Changes in ^{1}D and corresponding ^{2}D retention changes closely followed the "isovolatility curve" of most compounds. The PT process simplifies this experiment by adjusting the selectivity of ^{1}D ensembles simply by changing the mid-point pressure setting as described above. In a similar vein, PT can tune the selectivity of the coupled column as discussed in that previous study, but without physically changing the column length.

Conclusion

Pressure tuning is apparently a straightforward process, effected by a mid-point pressure adjustment that is able to swap or alter peak positions in a chromatogram. In the GC×GC context, PT offers useful conceptual and potentially practical opportunities because of the tuning capability and consequent change of orthogonality in the ^{2}D space, offered simply by changing PT settings for many types of samples. The 1D PT ensemble probably has little attraction, particularly for complex samples, because it alters the relative retentions somewhat randomly, however for GC×GC, PT of the ^{1}D column offers a number of advantages largely based on the benefits of modulation and resultant 2D separation. The 2D plot quickly illustrates how PT alters separation, for example, by their overlay, and how by comparison of different PT settings a best result can be found. It might be, of course, that a non-PT arrangement is best for a specific sample. Ideally, the advantages of PT-GC×GC could be: (i) shorter downtime of the system because in cases where a ($^1D_1$–$^1D_2$) ×^{2}D column arrangement allowed acceptable tuned separation performance for a range of different samples, then dedicated column sets might not have to be installed and equilibrated for every different sample; (ii) extended column lifetime from less frequent column removal and installation; (iii) flexibility of operation because of on-line selectivity change and optimization, and (iv) reduced labour as a result of simpler column management. At this moment, PT-GC×GC is still a curiosity in terms of its full potential and application boundaries for practical process in GC×GC. Hopefully effective control of instrument performance, selection of suitable column chemistries, and demonstrated applications that are readily tuned through PT, and which offer fast screening of sample analytes, will be realized. A centralized laboratory relying on GC×GC to analyze many different samples might take advantage of this process by being able to provide tunable separations in GC×GC without having to frequently change column sets, simply by using PT to give suitable 2D separation.

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Tips, Tricks, and Troubleshooting for Separations of Biomolecules, Part 2: Contemporary Separations of Proteins by Size-Exclusion Chromatography

Szabolcs Fekete[1], Davy Guillarme[1], and Dwight R. Stoll[2], [1]University of Geneva, Geneva, Switzerland, [2]LC Troubleshooting Editor

Several new materials and columns have been introduced in recent years for size-exclusion separations of proteins. How do I know which one to choose, and which separation conditions will be the best for my protein separation?

In Part 1 of this series (1), we focused on reversed-phase separations of proteins. In recent years, many new materials and columns have been introduced that provide potential for substantially better separations compared with those from one or two decades ago. Although some things have stayed the same, much of the old conventional wisdom has been overturned with the development of better stationary-phase chemistries and new research that has provided deeper insights into why we observe some phenomena (for example, low recovery of proteins from reversed-phase materials under some conditions). This research has also led to new guidance for operating conditions that improve the likelihood of obtaining acceptable chromatographic results.

Over the past few years, we have seen tremendous expansion in commercially available offerings for size-based separations of proteins as well. These separations are most commonly referred to as size-exclusion chromatography (SEC), and we will use that term here. As with reversed-phase separations of proteins, the upside to having more commercially available columns to choose from is that we can more precisely tailor our column choices to the needs of our applications. However, the downside to more options is that we have to choose which one is the most suitable, and in some cases, this can be a challenging task in itself. On the other hand, recent research studies have added considerable insights to the existing knowledge base to support this decision-making process. Even if we don't fully understand why SEC materials behave the way they do in every situation (for example, see reference 2), we are in a much better position today to make good choices about columns and operating conditions than we were five years ago.

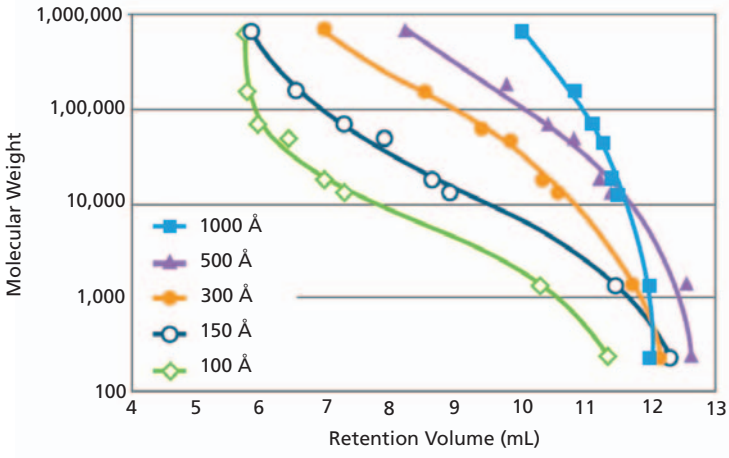
For this instalment of "LC Troubleshooting", I have asked two of my collaborators in the biomolecule application space, and genuine experts in SEC separations of proteins, to join me in sharing some of the details that we have found to be particularly important to successful SEC separations.

Dwight Stoll

Basics of SEC Separations

From a theoretical point of view, SEC is arguably the simplest of all chromatographic separation modes. In reversed-phase mode and other separation modes, we spend a lot of time thinking and talking about retention (that is, retention factors greater than zero are very important!), which is a function of differences between the strength of intermolecular interactions between analytes, mobile phase, and stationary phase. It is differences between the way one analyte interacts with the mobile and stationary phases compared to another analyte that give rise to differences in retention (that is, selectivity) and ultimately resolution of two analytes. In this way, resolution in reversed-phase and similar separation modes (sorptive modes) is inherently chemically driven. SEC, on the other hand, is completely different, at least in the ideal case. Here, resolution has a physical basis, rather than a chemical one, and in the ideal case, there is no retention of the analyte by the stationary phase (that is, retention factors are zero or apparently negative). Instead, separation arises from differences in the physical limitations that analytes of

Figure 1: Molecular weight vs. retention volume plots for SEC columns having particles with different average pore sizes. The smallest two molecules in the dataset are uracil (112 Da) and vitamin B12 (1350 Da), and the largest two molecules are gamma-globulin (158 kDa) and thyroglobulin (670 kDa). Adapted with permission from reference 6.



certain sizes experience preventing them from exploring the entire pore network of porous particles used in SEC columns. Very small analytes in a sample will be able to explore most of the pore network. On the other hand, larger analytes that are too big to explore all of the pores will travel through the column with a higher velocity, and be observed flowing from the column earlier than the small analytes. From the point of view of the large analytes, the mobile phase volume inside the column is effectively smaller. Under ideal circumstances (that is, no retention as a result of intermolecular interactions), very small analytes will be eluted at what we would normally refer to as the dead time (t_m) in reversed-phase separations. The mobile-phase volume associated with this time (that is, $t_m \times F$) is referred to as the inclusion volume (corresponding to the total porosity of the column). Larger analytes will elute at earlier times, before the inclusion volume.

Decision 1—Choosing the Column

Before we dive into the details here, we want to be clear about our intent for this instalment. A tremendous amount of very good information on the following topics has been published in recent years. Our discussion here is limited to a survey of highlights of that work. Readers interested in the details behind our discussion are strongly encouraged to engage the literature cited here to learn more.

Particle Size and Column Length

Before the advances in column technology for SEC in recent years, most SEC columns in use were relatively large—typically 7.8 mm in diameter,

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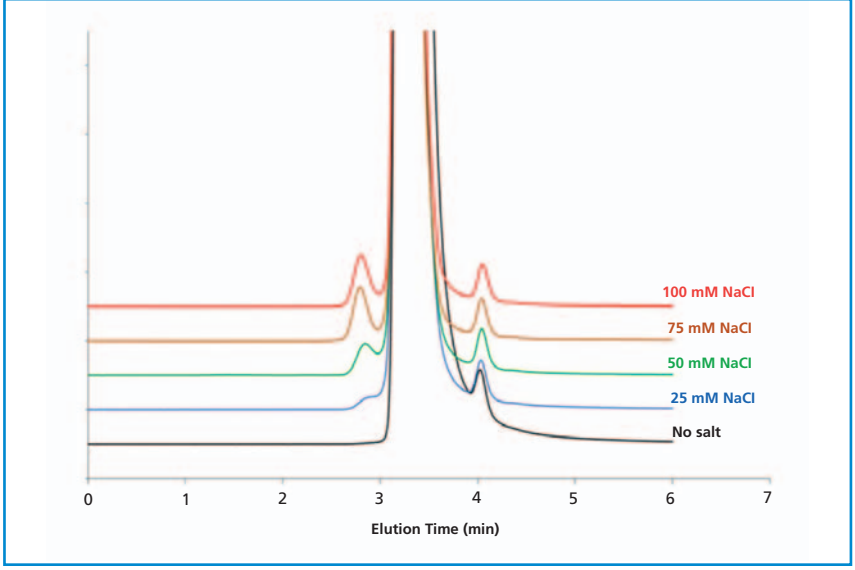
Figure 2: Impact of mobile phase salt concentration on detected concentration of aggregates in a sample of the therapeutic protein adalimumab. Conditions: Column: 150 mm × 4.6 mm, 2.7-µm Agilent AdvanceBioSEC; mobile phase: 100 mM phosphate buffer, pH 6.8, + indicated concentrations of sodium chloride; flow rate: 350 µL/min. Peaks in order of elution: Dimer, monomer, and fragment (3). Unpublished data from the laboratory of D. Guillarme.



and 150 to 300 mm in length. The long column lengths were required because of the large particles that were used, most of which did not have high mechanical strength and had to be used at relatively low pressures. The recent trend in column technology for SEC has been focused on the development of columns with smaller particles (<3 µm), in shorter columns (the standard now is 15 cm), and in smaller diameters (typically 4.6 mm). This trend has been supported by the development of particle chemistries that are both sufficiently mechanically stable to be used at the higher pressures that accompany the smaller particle sizes, and sufficiently inert toward biomolecules, to produce separations based mostly on molecular size. The move to smaller particle diameters also provides opportunities to improve separation speed by using higher flow rates through these columns. Whereas with larger particles, using high flow rates tends to result in decreases in efficiency (that is, plate number) and resolution, the price paid for doing so with smaller particles is not as severe.

Although we must be careful with generalizations, it is useful to think a bit about what the trend towards the use of small particles can do for us, in a practical sense. In rough terms the plate height scales with the particle diameter. So, upon moving from a 5-µm particle to a 2-µm particle, the plate height should decrease by about a factor of two (3). There are two main ways we can capitalize on this improvement in plate height—we can either improve resolution while using a column of the same length, or we can decrease analysis time while maintaining resolution. In the first case, if we use two columns of the same length—one with 5-µm particles and one with 2-µm particles—the plate number for the 2-µm particle should be approximately double that of the column with 5-µm particles. Since resolution scales with the square root of plate number, we should expect the resolution to improve by about 40%. In the second case, the plate number is directly proportional to column length, and inversely proportional to plate height. If the plate height decreases by a factor of two with the smaller particles, then we can decrease the column length by a factor of two, while maintaining the same plate number and resolution. If the same flow rate is used in both cases, we should expect this to immediately result in a 50% decrease in analysis time. This is a simple but useful view of these scenarios. There are a number of other factors to think about when considering the move to smaller particles, including the pressure limitations of the column and particles, and specifications of the instrument. More detailed discussions of the theory relevant to these considerations can be found elsewhere (4,5).

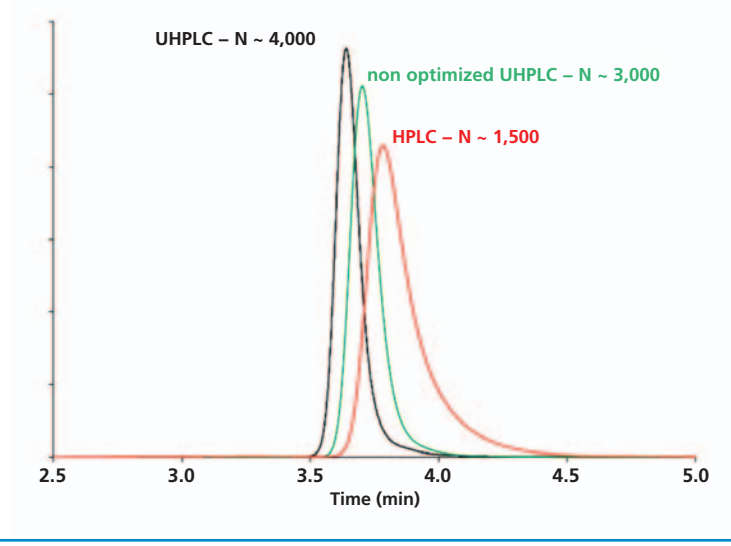
Average Pore Size and Distribution

As described above, the velocity of a particular molecule through a SEC column depends on the extent to which it can explore the pores of the particles. For particles with a well-defined pore size distribution, there is a range of molecular sizes for which a particular particle will be effective for size-based separations. The calibration curve shown in Figure 1 shows the selectivity (that is, difference in elution volume for a given change in molecular weight) for particles with different average pore diameters. We see that with small-pore columns there is good selectivity for small molecules, but the largest molecules will effectively be coeluted. On the other hand, the very large pore materials effectively separate the largest molecules, but the smallest molecules are coeluted. This type of plot can be used to decide which pore size will be most effective for the application at hand. For protein characterization, typical pore sizes between 150 and 500 Å are used. For common therapeutic proteins (MW ≈15–80 kDa), a pore size of 150–200 Å works well, while a 200–300 Å pore size is usually used for monoclonal antibodies (mAbs, MW ≈ 150 kDa). For very large proteins (MW > 200 kDa, for example, pegloticase or PEGylated proteins), typically the 500–1000 Å materials offer the most appropriate selectivity.

The pore size distribution has an impact on the slope of the calibration curves. The wider the pore size distribution, the steeper the curve is. Therefore, with a wide pore size distribution, the selectivity will be lower but the range of the analytes that can be separated will be broader. A narrow pore size distribution provides higher selectivity between species with slight differences in size, but only a limited size range of analytes can be separated.

The challenge in practice is that the only data that is readily available from column manufacturers is the nominal pore size. Unfortunately there is not broad agreement about how exactly to report pore size, and most of these measurements are based on gas adsorption/desorption measurements and may not be very meaningful for protein analyes. Thus, from the point of view of users of these columns, it is practically useful to experimentally determine the calibration curve by injecting a mixture of standard proteins in order to have a good sense for the selectivity that can be expected for a given protein sample.

Figure 3: Observed chromatograms for a mAb monomer on three different LC systems. Column: 150 mm × 4.6 mm, 1.8-µm; flow rate: 0.3 mL/min; temperature: ambient. Adapted with permission from reference 11.



Decision 2—Choosing the Mobile Phase

After choosing the column, the next most important decision involves choosing exactly what will go into the mobile phase. As described above, one of the basic tenets of SEC separations is that conditions should be chosen so that retention (in a chemical sense) is minimized. If achieved, this approach ensures that the elution volume is an indicator of molecular size (as in a calibration curve of the type shown in Figure 1) and nothing else. At first glance, this seems like it should be straightforward—we should just choose a stationary phase that does not interact strongly through specific types of interactions with the analyte, and choose a mobile phase in which the analyte has a high solubility and that is able to minimize analyte–stationary phase interactions. But, if we've learned anything from 50 years of liquid chromatography, one of the big lessons has been that apparently tiny changes in the chemistry or structure of stationary phase or analyte can lead to big changes in retention. Indeed, we often exploit these interactions to great effect in reversed-phase separations when developing a new method. However, implementing this approach also means that achieving the "no retention" condition in SEC separations of proteins can be quite difficult in practice. There is a rich literature describing studies that have explored the use of different mobile phase modifiers and conditions to minimize stationary phase–analyte interactions.

It has been our experience that many of the specific effects of different mobile-phase conditions are protein or stationary-phase specific (or both), and thus some amount of exploration of variables is a necessary part of method development when starting work with a new molecule. However, based on our experience and the literature available to date, we can provide some suggestions for starting conditions:

- **pH:** When the isoelectric point (pI) of the protein is known, the mobile-phase pH should be adjusted to approximately match the pI of the protein. If the pI is not known, pH 6.5 is a good starting point. One should ensure, either based on existing literature or by experiment, that the protein is both highly soluble and chemically stable at the pH that is planned for.
- **Salts:** Various additives have been tested as a means to reduce nonspecific interactions and retention of proteins under SEC conditions. For example, high concentrations (~0.2 M) of arginine have been used in the past (7). Arginine and other amino acids can interact with the protein and therefore decrease the accessible charges and possible electrostatic (ion-exchange) interactions. More commonly though, significant concentrations of sodium and potassium salts are used to suppress electrostatic interactions between the stationary phase and protein (8,9). An example of the effect of adding increasing levels of sodium chloride to a phosphate buffered mobile phase at pH 6.8 is shown in Figure 2 for the therapeutic protein adalimumab. Here, we see two major effects, both of which evidently result from decreased interactions between the protein and the stationary phase. First, the detected concentration of the mAb dimer (peak eluted before the monomer) increases dramatically (higher recovery) from barely detectable with no salt added, to easily detected at 100 mM sodium chloride added. Second, the elution volume of the dimer also decreases, again because interactions with the stationary phase are decreased, such that the resolution of the dimer and monomer increases.
- **Organic solvents:** Although most proteins are sufficiently hydrophilic that completely aqueous mobile phases will yield acceptable SEC results, hydrophobic proteins may require small additions of solvent to improve recovery and peak shape. In particular, antibody–drug conjugates (ADCs) are a class of molecules of current interest that may benefit from addition of organic solvent (10). In these cases, addition of 10–15% of isopropanol to the mobile phase is a good starting point.

And What About the Instrument?

There are at least two major issues we could discuss here—the impact of system dispersion on the performance of high-quality SEC separations, and the impact of instrument construction and the use of bioinert, biocompatible materials. The latter topic is complex and we will reserve that discussion for a later date. On the topic of system dispersion, we have to recognize that SEC separations are particularly prone to the negative effects of peak dispersion outside of the column (that is, extracolumn dispersion) because, again, the peaks are eluted with no retention or even before the inclusion volume. In separation modes where retention is desirable, the effects of extracolumn dispersion are less severe for peaks that are more retained, and in the case of gradient elution in many cases nearly all precolumn dispersion can be eliminated. Not so in SEC, because no peaks are retained, and all separations are isocratic.

As discussed above, until relatively recently most SEC columns in use were large in diameter (~7.8 mm) and long (300 mm). This resulted in separations where the peak volumes (that is, the peak width in time units, times the flow rate) were large enough in comparison to the injector-to-detector volumes of LC systems they were connected to. However, with the improved plate heights and smaller volumes of state-of-the-art columns, the peak volumes are small enough that extracolumn dispersion has become a very important issue again (11). Figure 3 shows a comparison of the detected peak for a monoclonal antibody monomer obtained on three different LC systems with different levels of extracolumn peak dispersion. Given that resolution is often very valuable in SEC separations, this comparison makes it clear that one should seriously consider the effect of extracolumn dispersion on the observed chromatography, particularly when using modern SEC columns with small volumes and small particles.

When working with a state-of-the-art 150 mm × 4.6 mm SEC column, for a small analyte that is eluted near the inclusion volume, only 25–60% of the intrinsic column efficiency can be attained on conventional high performance liquid chromatography (HPLC) systems. The situation is even worse with a partially excluded analyte. Optimized ultrahigh-pressure liquid chromatography (UHPLC) systems having very low extracolumn volumes (typically V_{ec} < 10 µL) have to be used to properly operate these columns. Therefore connector tubing volume and detector cell volume must be as low as possible. As most SEC separations are performed at ambient temperature, the mobile-phase preheater unit can also be removed to further gain in apparent efficiency. Another interesting finding is that conventional HPLC systems also have a big impact on the apparent elution time of proteins—and therefore on mass-calibration curve—when working with 150 mm × 4.6 mm columns. Under these conditions the resulting calibration data will not be reliable, except if corrected for extracolumn residence time.

Summary

Developing effective and high performing SEC separations for proteins requires attention to all facets of the method, including choices around stationary phase, particle size, and column dimensions, mobile-phase conditions, and instrument effects on chromatographic efficiency and resolution. Several research groups are continually contributing to our understanding of the effects of all of these decisions on separation performance. Although we certainly are very far from a complete understanding, we are in a better position than ever before to leverage the information we do have to develop the best methods possible today.

With this instalment of "LC Troubleshooting", I am approaching my first full year of writing monthly columns that address some of the pain points we experience as practitioners of liquid chromatography. As I have said many times already here, some new problems emerge as technology changes and we adapt to the new behaviours of instruments and columns, but there are also many problems that nominally remain the same over time. I will continue working to bring a mix of discussions of old and

new topics to the column, but I am also particularly interested to hear what you, as a regular consumer of the column, have to say about topics you would like to see addressed here. Are there topics that are emerging challenges that you have not seen addressed in the past? Are there "old" topics that you would like to see addressed in more depth? I'd love to hear your topic suggestions! Please send them along to LCGCedit@ubm.com

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Analyzing Phosphorylated N-Glycans with Full Recovery on Bio-Inert LC Systems and PEEK-Lined HILIC Columns

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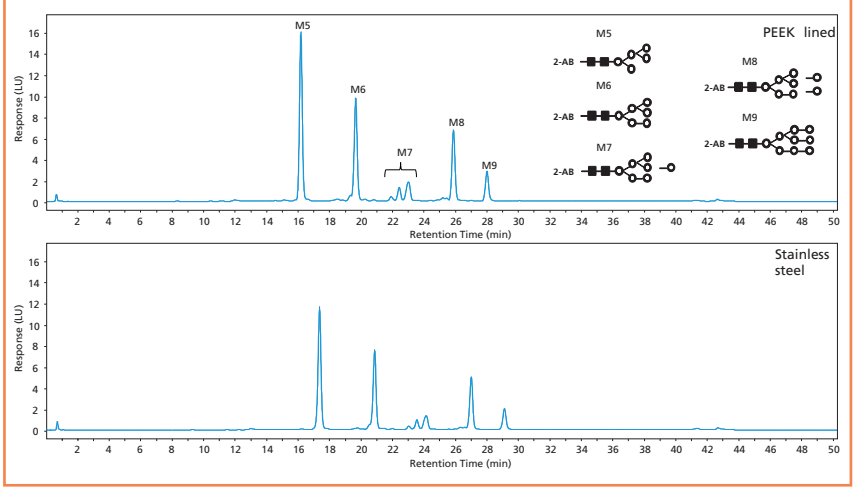
Glycosylation is a critical quality attribute (CQA) that can impact on product safety and efficacy of protein biopharmaceuticals. Characterization of N-glycans is therefore of paramount importance for the pharmaceutical industry. Hydrophilic interaction liquid chromatography (HILIC) combined with fluorescence detection (FLD) and 2-aminobenzamide (2-AB) labelling is the golden standard for the analysis of N-glycans enzymatically liberated from biopharmaceuticals. However, for phosphorylated N-glycans, that is, those attached on lysosomal enzymes, irreproducible data and recovery issues are observed on conventional liquid chromatography (LC) instrumentation and columns, which can be attributed to the interaction of the phosphate moieties with stainless steel components in the flow path. This article demonstrates the analysis of phosphorylated glycans with full recovery on a bio-inert LC system and PEEK-lined HILIC column.

Protein biopharmaceuticals are a rapidly growing class of therapeutics that are widely used for the treatment of various life-threatening diseases (1,2). Around 40% of protein biopharmaceuticals are glycosylated and total glycan mass can range from 2–3% for monoclonal antibodies (mAbs) and up to 50% for erythropoietin (EPO). Glycosylation can impact on product safety and efficacy and is therefore considered a critical quality attribute (CQA). For example, terminal sialic acid residues on complex N-glycans regulate the half-life of EPO in the blood stream, core fucosylation influences the effector function of mAbs, and mannose-6-phosphate moieties on therapeutic enzymes are essential for trafficking to the lysosomes where the enzymes need to be catalytically active (1,2). Characterizing these glycan structures is therefore an essential requirement. Glycosylation analysis of protein biopharmaceuticals can be performed at different levels, that is, at protein, peptide, and glycan levels (3).

Figure 1: HILIC–FLD chromatograms of 2-AB-labelled RNase B N-glycans on a PEEK-lined and on a stainless steel column installed on a bio-inert LC system.



Hydrophilic interaction liquid chromatography (HILIC) combined with fluorescence detection (FLD) and 2-aminobenzamide (2-AB) labelling is the gold standard for analysis of N-glycans enzymatically liberated from biopharmaceuticals (1,2,3). While this method has a proven track record on neutral and sialylated N-glycans, irreproducible data with poor recovery is often encountered upon analyzing phosphorylated N-glycans originating from therapeutic lysosomal enzymes. This is attributed to the interaction of the phosphate groups with stainless steel components in the flow path. This phenomenon has also been observed for nucleotides and phosphopeptides (4–9). For N-glycans analysis, this issue is often

Figure 2: HILIC–FLD chromatograms of 2-AB-labelled neutral and phosphorylated high mannose N-glycans originating from human acid alpha-glucosidase on a PEEK-lined and on a stainless steel column installed on a bio-inert LC system.

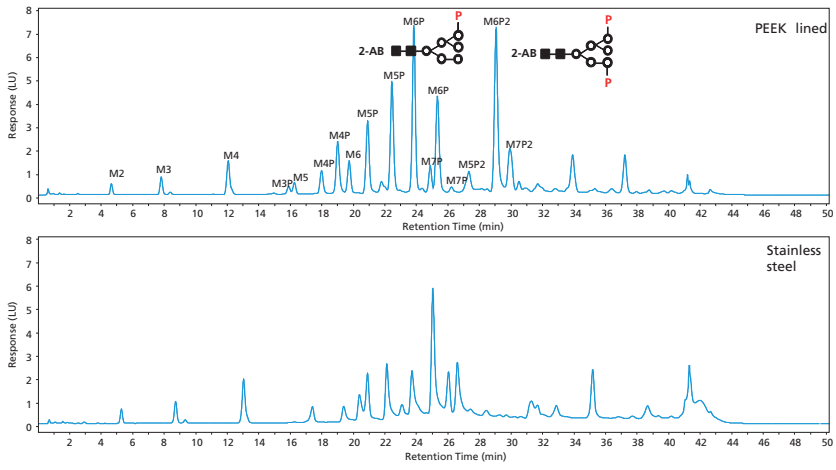
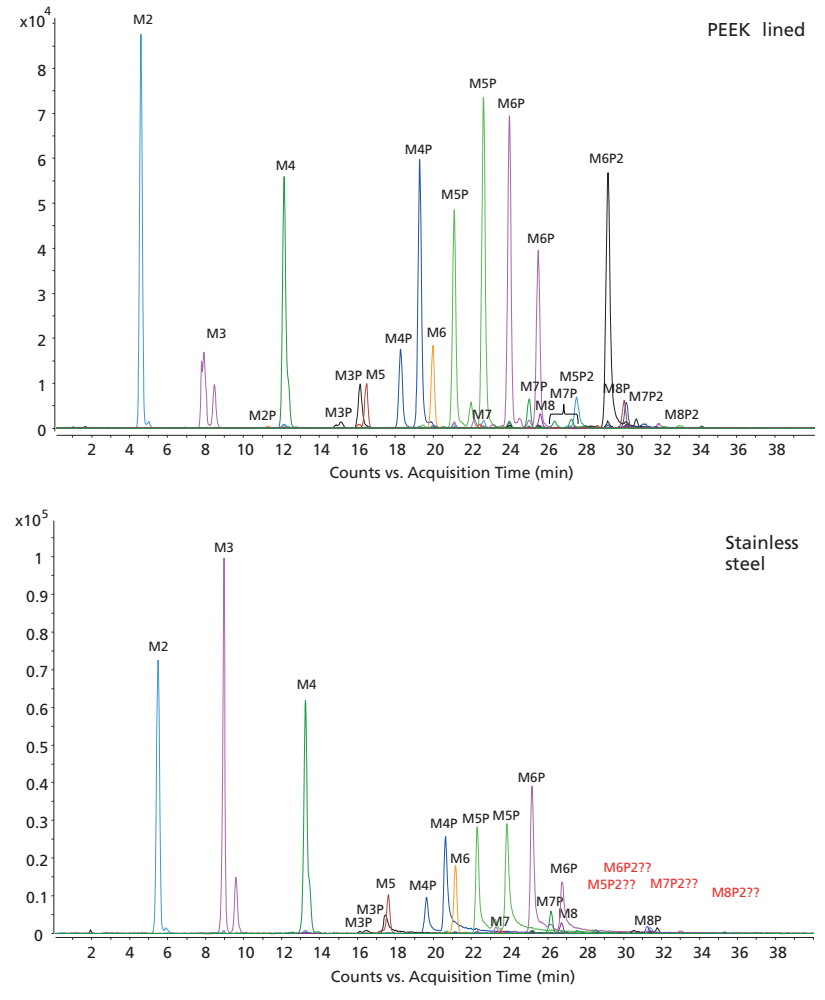


Figure 3: HILIC–ESI-MS chromatograms of 2-AB labeled neutral and phosphorylated high mannose N-glycans originating from human acid alpha-glucosidase on a PEEK-lined and on a stainless steel column installed on a bio-inert LC system. Shown are the overlaid extracted ion chromatograms (EIC) of the different glycans.



alleviated by using mobile phases containing an ion-pairing reagent such as triethylamine (10,11) or by performing separations at high pH (pH > 12) using high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (12,13).

This article demonstrates that phosphorylated glycans can be successfully analyzed by HILIC with the standard 2-AB-labelling method using the commonly applied mobile phase composition, that is, 100-mM NH4-formate pH 4.5 and acetonitrile, when the entire sample flow path is metal-free. To achieve this, the use of a bio-inert liquid chromatography (LC) system as well as a PEEK-lined HILIC column is mandatory.

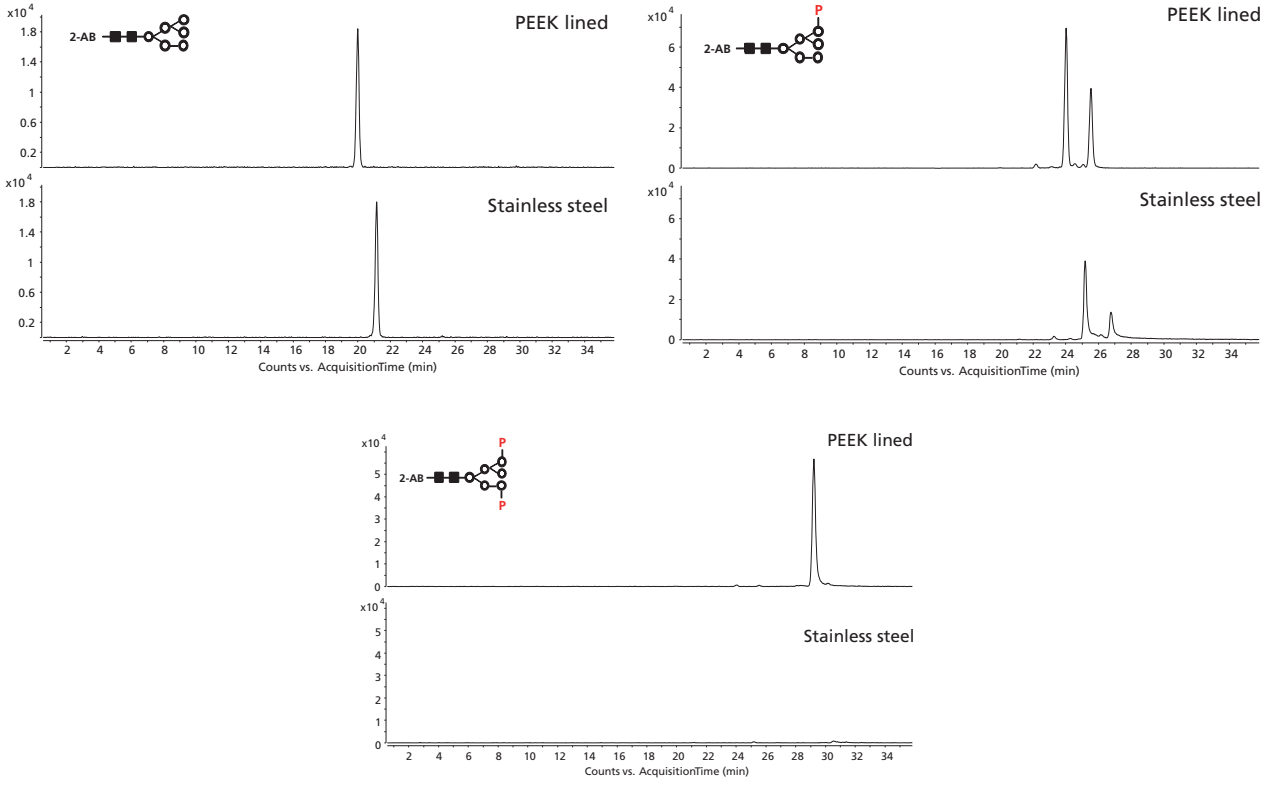
Around 40% of protein biopharmaceuticals are glycosylated and total glycan mass can range from 2–3% for monoclonal antibodies (mAbs) and up to 50% for erythropoietin (EPO).

Experimental

Materials: Acetonitrile and water were obtained from Biosolve. Ammonium formate was purchased from Sigma Aldrich. 2-AB-labelled RNase B and recombinant human acid alpha-glycosidase N-glycans (uncapped and capped) were provided by a local biotechnology company.

Methods: HILIC–FLD measurements were performed on an Agilent 1260 Infinity II Bio-Inert LC system equipped with a quaternary pump (G5654A), a multisampler (G5668A), a multicolumn thermostat (G7116A) with heat exchanger, and fluorescence detector (G7121B) with bio-inert flow cell. For LC–mass spectrometry (MS) experiments the above LC configuration was hyphenated to an Agilent QTOF LC–MS system (G6545A) equipped with JetStream electrospray ionization (ESI) source. LC and LC–MS data were acquired and analyzed using OpenLAB CDS version 2.1, MassHunter for

Figure 4: HILIC–ESI-MS ion extracted chromatograms of 2-AB labelled M6 (*m/z* 1517.539), M6P (*m/z* 1597.505), and M6P2 (*m/z* 1677.472) originating from human acid alpha-glucosidase on a PEEK-lined and on a stainless steel column installed on a bio-inert LC system.



instrument control (B06.01) and MassHunter for data analysis (B07.00) (Agilent Technologies), respectively.

Two columns were used in this study: a 2.1 mm × 100 mm, 2.7-µm AdvanceBio Glycan Mapping column (superficially porous particles—amide) (Agilent) in regular stainless steel housing and an equally dimensioned PEEK-lined column custom packed with identical superficially porous particles (Agilent). Elution was carried out with a linear gradient of (A) 100-mM NH_4-formate pH 4.5 and (B) acetonitrile from 80% to 60%B in 38 min. The flow was set at 0.4 mL/min, the column temperature at 40 °C, and the injection volume was 1 µL. Excitation and emission wavelengths of the FLD were 260 nm and 430 nm, respectively. The operational parameters for the QTOF source were drying gas temperature: 300°C, drying gas flow: 8 L/min, nebulizer pressure: 35 psig, sheath gas temperature: 350 °C, sheath gas flow: 8 L/min, nozzle voltage: 1000 V, capillary voltage: 3500 V, and fragmentor voltage: 150 V. QTOF data were collected in centroid mode at a rate of 1 spectrum per second and acquisition range was 500–3200 *m/z*. The system was operated in the extended dynamic range mode (2 GHz).

2-AB-labelled glycans elute based on their polymerization degree, that is, the higher the number of glycosidic bonds, the more retention.

Results and Discussion

HILIC with FLD and 2-aminobenzamide (2-AB) labelling is currently the method of choice for the analysis of neutral and sialylated N-glycans liberated from biopharmaceuticals (1,2,3). Figure 1 shows the analysis of 2-AB-labelled neutral high mannose RNase B N-glycans on a stainless steel and PEEK-lined amide HILIC column installed on a bio-inert LC system. Very similar chromatograms are obtained on both columns for these neutral glycans. 2-AB-labelled glycans elute based on their polymerization degree, that is, the higher the number of glycosidic bonds, the more retention. Moreover, sufficient selectivity differences exist to separate compounds with the same polymerization, that is, M7 isomers which differ in the positioning of the mannose residue on the glycan tree. Both profiles were obtained on the bio-inert LC system, but similar results could be produced on a conventional stainless steel HPLC system.

The HILIC–FLD and HILIC–ESI-MS analysis of 2-AB-neutral and

phosphorylated high mannose N-glycans originating from human acid alpha-glucosidase recombinantly expressed in yeast is shown in Figures 2 and 3, respectively. Human acid alpha-glucosidase catalyzes the hydrolysis of glycogen to glucose in the lysosomal compartment of the cell. Around 50,000 people worldwide have a deficiency of this enzyme, which leads to glycogen accumulation in the lysosomes causing muscle damage. These patients typically receive an enzyme replacement therapy with recombinant human acid alpha glucosidase (14). The presence of mannose-6-phosphate containing glycans is a CQA since these functionalities are responsible for targeting the enzyme to the lysosomes where it needs to break down glycogen (15).

In contrast to the observations on the neutral RNase B N-glycans, an enormous discrepancy is noticed when analyzing the 2-AB-labelled glycans on a stainless steel or on a PEEK-lined column. The neutral glycans M2-8 behave equally well on both columns. The monophosphorylated glycans M2-8P show tailing peaks on the stainless steel column while biphosphorylated glycans M5-8P2 are not recovered at all from the latter column as a result of interaction between metal ions and phosphate moieties. The same recovery principle is known to apply for nucleotides, that is, AMP > ADP > ATP. On the PEEK-lined column, perfect Gaussian shaped peaks are observed for both mono- (M6P, for example) and biphosphorylated (M6P2, for example) structures showing the importance of removing all metal parts from the flow path (instrument, column including frits). Isomeric monophosphorylated glycans are observed as well, which differ in the positioning of the phosphate group on the α1-3 or α1-6 branch of the glycan tree. This is further supported by the extracted ion chromatograms of M6, M6P, and M6P2 presented in Figure 4. Some relevant MS/MS spectra are shown in Figure 5.

It is important to note that the above mentioned phenomena related to adsorption of phosphorylated species on metal ions of the LC system and column only occurs for the phosphate mono-ester containing N-glycans $(PO_4—M)^{2-}$ and not for the phosphate di-ester containing N-glycans $(M—PO_4—M)^{1-}$. For the latter glycans, the profiles obtained on the stainless steel and PEEK-lined columns are exactly the same (Figure 6). Interestingly, these phosphate di-ester-carrying glycans are present on human acid alpha-glucosidase recombinantly produced in *Yarrowia lipolytica*, but the outer mannose residues are enzymatically removed during downstream processing to generate the active form of the enzyme, that is, carrying mannose-6-phosphate residues (15).

Conclusion

The HILIC analysis of phosphorylated N-glycans released from therapeutic enzymes using a bio-inert LC and PEEK-lined column has been described. It was demonstrated that these challenging structures can successfully be analyzed when the entire flow path is devoid of metal parts, that is, instrument and column inertness.

Figure 5: MS/MS spectra of the phosphorylated N-glycans eluting at 24.04 and 25.57 for M6P isomers and at 29.30 min for M6P2.

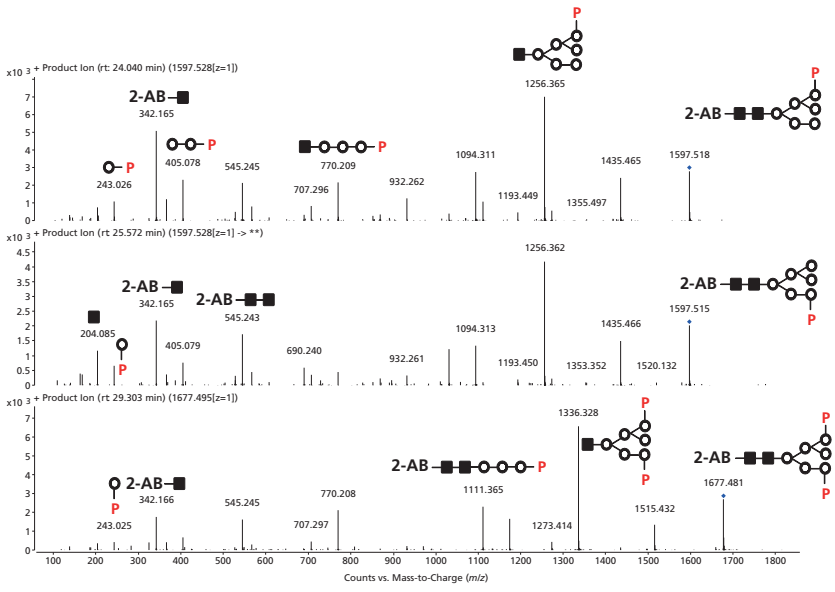
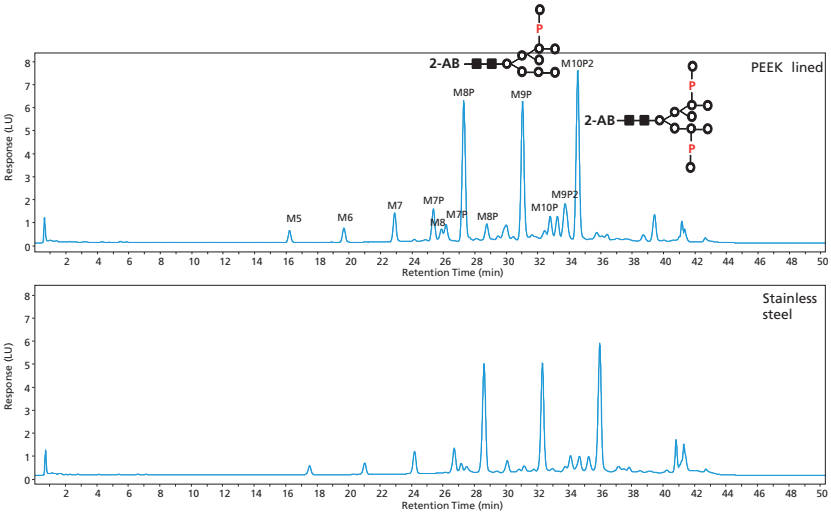


Figure 6: HILIC–FLD chromatograms of 2-AB labelled neutral and phosphate di-ester containing high mannose N-glycans on a PEEK-lined and on a stainless steel column installed on a bio-inert LC system.



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Highlights from the **HPLC 2018 Symposium**

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The 47th International Symposium on High Performance Liquid Phase Separations and Related Techniques (HPLC 2018), chaired by Norman Dovichi, was held from 29 July to 2 August in Washington, D.C., USA. This instalment of "Column Watch" covers some of the highlights observed at the symposium. In addition, trends and perspectives on future developments in HPLC noted from the conference are presented.

The 47th International Symposium on High Performance Liquid Phase Separations and Related Techniques, or HPLC 2018, was held in Washington, D.C., USA, at the Marriott Wardman Park Hotel and Conference Center from 29 July to 2 August 2018. This symposium, which continues to be the premier event bringing together leading scientists in the field of liquid chromatography and related techniques, attracted 870 delegates from 34 countries. The attendance for US-based venues continues to fall short of European sites; however, the US attendance has remained fairly consistent for a number of years (Table 1). The well-organized conference was chaired by Professor Norman Dovichi of the University of Notre Dame. As noted by the chair, the programme had a strong focus on separations in the pharmaceutical industry, including interesting sessions on continuous manufacturing and forward-looking pharmaceutical analysis. Other preconference highlights included presentations on three-dimensional (3D) printing, micropillar array technology development, and a special focus on the fast-rising cannabis industry.

The conference included 203 talks (of which 12 were tutorial presentations), 335 poster presentations, nine short courses, and nine vendor technical workshops. In this instalment of "Column Watch", observed highlights and trends from the conference are reported.

Highlights and Trends

In a similar fashion to the previous HPLC review articles (1,2), several colleagues in attendance at the symposium were asked for their insights regarding the most interesting topics they observed at the event. Much of what follows is a synopsis of their responses along with some personal views.

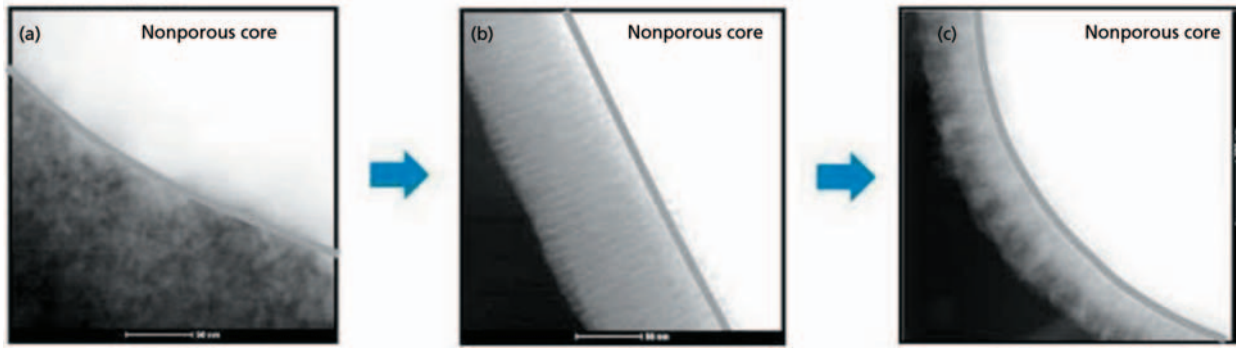
In reviewing highlights from the past several symposia, multiple areas of interest stood out, namely 3D printing, advances in large-molecule separations, multidimensional separations, and chiral and hydrophilic interaction liquid chromatography (HILIC). Reported progress in 3D printing seemed to wane in 2017; however, at the 2018 symposium, developments in 3D printing were again at the forefront of discussions. Attention towards large-molecule separations seemed to be even greater than in previous years, perhaps because of an overall conference focus on pharmaceutical analysis. Advances in HILIC and chiral chromatography continued to be presented this year, and general topics in separation fundamentals that promise to increase speed, generate higher throughput, and add selectivity continue to drive discussions at the symposium.

As indicated by the name of the conference—the International Symposium on High Performance Liquid Phase Separations and Related Techniques—liquid chromatography was not the only separation technology present at the symposium. Like many symposia before, the meeting often takes on the personality of its chair. It was not unexpected then that electrophoresis (CE) was a significant topic at HPLC 2018. Several sessions were dedicated to CE where much of the discussion centred on biomolecule separations.

Advances in Large-Molecule Separations

The characterization of large molecules requires a variety of different chromatographic approaches including reversed-phase, ion exchange, size exclusion, and hydrophobic interaction chromatography. HPLC 2018 included many presentations on techniques dealing with different aspects of large molecule separations. Matthew Lauber presented on a new polyphenyl column designed to improve efficiencies and resolution of targeted large molecules such as monoclonal antibodies (mAbs) and antibody–drug conjugates (ADCs) (3). Lauber demonstrated that the novel column chemistry is capable of performing separations of mAbs and ADCs at lower temperatures and with lower concentrations of ion-pairing reagents than existing columns, thus minimizing on-column degradation of biotherapeutics. The more "protein friendly" reversed-phase chromatography was stated to be a result of the high surface coverage

Figure 1: Progression of superficially porous particle technology. (a) Conventional SPPs. (b) First-generation pseudomorphic transformation SPPs. (c) Second-generation pseudomorphic transformation SPPs.



of the rigid phenyl moiety, which prevents access of the analyte to the underlying base silica particle.

Superficially porous particle (SPP) technology continues to be adopted for large molecule separations. Barry Boyes used a combination of C4, C18, and a new diphenyl stationary phase based on 1000-Å SPPs to develop methods for correct structure assignment of mAbs and ADCs (4).

Selectivity changes in large-molecule separations can be affected by mobile phases, as well as by alternative surface chemistry and particle technology. Kevin Schug showed how changes in mobile-phase components and pH may elicit different protein conformations, and thus producing alternative interactions with the stationary phase (5). The approach may have important implications in the realm of multidimensional separations of intact proteins.

Columns used for large-molecule separations are often characterized using traditional small-molecule probes and systems. Jennifer Field presented an interesting talk regarding the development of a column characterization protocol for large-molecule, reversed-phase columns (6). Field and colleagues developed a series of peptide probes and a set of conditions to characterize several commercially available, wide-pore reversed-phase columns. Field's protocol may lead to column characterization information that is much more useful to the large-molecule separation scientist than has previously been available.

Ion-exchange chromatography (IEX), size-exclusion chromatography (SEC), and hydrophobic interaction chromatography (HIC) all provide important separation capabilities that are complementary to reversed-phase separations. The high salt content often employed in these techniques, however, precludes the use of mass spectrometry (MS) for detection. Jonathan Bones presented the analysis of mAbs for charge variants and aggregates by ion-exchange–MS and SEC–MS using a novel, low-salt mobile phase that enables these modes of chromatography to be coupled directly to MS (7). The research demonstrated the ability to identify unique C-terminal lysine variants and mAb fragments that would have been missed using conventional IEX and SEC methods.

HIC is a widely used non-denaturing technique that separates analytes based on their surface hydrophobicity. Similarly to IEX chromatography, the traditional use of nonvolatile salt buffers in performing HIC have prevented direct coupling to MS. Bifan Chen reported on a novel combination of stationary-phase materials with volatile mobile phases based on ammonium acetate and some organic solvent (8). The resulting HIC–MS method was demonstrated with the analysis of intact proteins and more recently for mAbs. According to the authors, the mass spectra were characteristic of proteins with native structure, primarily featuring low charge states. For mAbs, the HIC–MS method allows for rapid determination of relative hydrophobicity, intact masses, and glycosylation profiles as well as sequence and structural characterization of the complementarity-determining regions in an online configuration. For ADCs, the same methods and conditions cannot be applied directly because of the fragile nature of the molecules. Method development becomes a balance between generating enough retention for the drug-to-antibody ratio (DAR) zero variant, but not so much as to denature the high DAR species (DAR6 and DAR8) by the stationary phase or organic solvent. Using an ADC mimic, the total ion chromatogram and the mass spectra demonstrated that different DAR species were separated successfully and, more importantly, remained intact during the chromatography. The group noted plans to submit their manuscript on the ADC analysis for publication soon.

Advanced Liquid Chromatography Technologies

Advances in particle designs continue to highlight HPLC symposia. Ta-Chen Wei gave a talk on the synthesis of an SPP that has a "dual-pore" architecture (9). In 2015, the group presented a new SPP with ordered elongated pores through a process called *pseudomorphic transformation (PMT)* (10), and later demonstrated the new particle provides 18–30% more efficiency than conventional SPPs for small-molecule separations (11). The improved efficiency is due to the anisotropic diffusion from the

Figure 2: Breakdown of the number of posters by session topic.

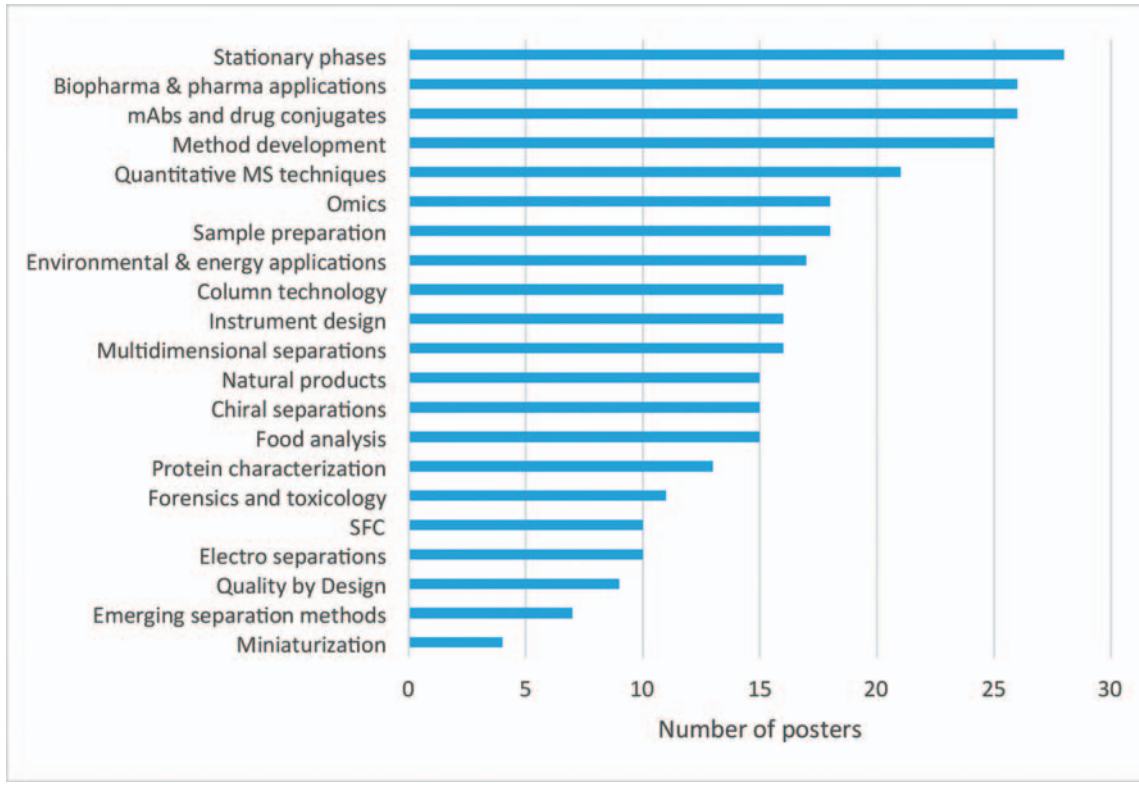


Table 1: Overview of posters and session topics since 2012

Conference	Number of Posters	Number of Topics
HPLC 2018: Washington DC	336	21
HPLC 2017: Prague	656	55
HPLC 2016: San Francisco	273	24
HPLC 2015: Geneva	560	27
HPLC 2014: New Orleans	425	25
HPLC 2013: Amsterdam	945	36
HPLC 2012: Anaheim	443	19

ordered pore structure, resulting in a much reduced B term. At HPLC 2018, Wei presented on a second generation of the PMT material that has dual pore structure on single particle (9). The inner region has ordered elongated straight pores for small-molecule separations, and the outer region has large conical pores for large-molecule separations. As a result, a single column packed with the new dual-pore SPPs can be used for both small- and large-molecule separations. In addition, the columns show improved efficiency and resolution over current conventional SPPs in their preliminary study. Figure 1 illustrates the progression of the PMT particle technology.

The quest for speed is ever present at the HPLC meetings. A much noted highlight was Dan Armstrong's presentation, "Practice and Ramifications of Ultrafast LC and SFC" (12). The talk centred on the idea that current practices in analytical chromatography will soon be obsolete as a result of combining fast chromatography (with appropriate columns) and peak processing. Armstrong believes that by performing HPLC in the optimal ultrafast mode and using columns above their peak capacity along with proper peak processing and resolution enhancement approaches, it may be possible to have 100 baseline or near baseline peaks in <60 s. Further, Armstrong predicted that identification (via retention time) and quantitation (via peak area measurement) using this approach will be comparable to, or better than, the typical best-practice separations today. The combination of advanced peak processing and fast LC is powerful. Armstrong went on to note that spectroscopy has been utilizing advanced processing techniques for decades and chromatography is behind the curve, but when the separations community catches up, the results will be impressive and profound.

James Grinias presented a talk on the transfer of standard

Table 2: Best poster awards

Award	Title	Name
First Place	A Novel Distance-Based Paper Device for DNA Measurement in Genomic Plant Cell Extraction	Boonta Chutvirasakul
Second Place	Developing Phage Endolysins as Novel Therapeutics for Multi-Drug Resistant Bacterial Infections	Sarah Gao
Third Place	Revealing the Ways of Manipulating Selectivity of Covalently Bonded Anion Exchangers for Ion Chromatography Toward Mono- and Polyvalent Organic Acids	Aleksandra Zatirakha
Honourable Mention	Liquid Chromatography as Sample Preparation Technique On-line Coupled to Comprehensive Two-Dimensional Gas Chromatography with Dual Detection for the Analysis Mineral Oil and Synthetic Hydrocarbons in Cosmetic Lip Products	Mariosimone Zoccali
Honourable Mention	Analysis of Drug Interactions with Alpha1-acid Glycoprotein Using High Performance Affinity Chromatography	Kyungah Suh
Honourable Mention	HILIC Method Development in Pharmaceutical Analysis	Dennis Asberg
Honourable Mention	A Critical Investigation into the Effects of Operating Temperature on Protein Retention in Hydrophobic Interaction Chromatography	Michael Menz
Honourable Mention	Native Reversed-Phase Liquid Chromatography: A Technique for LC–MS of Antibody–Drug Conjugates	Tse-Hong Chen
Honourable Mention	Altered Profiles and Metabolisms of L- and D-Amino Acids in Cultured Human Breast Cancer Cells	Siqi Du
Honourable Mention	A Systematic Approach for the Optimization and Validation of On-line Supercritical Fluid Extraction – Supercritical Fluid Chromatography – Mass Spectrometry for Polyaromatic Hydrocarbons in Soil	Alison Paige Wicker

methods to ultrahigh-pressure liquid chromatography (UHPLC) systems (13). Grinias investigated the effects of cyclic thermal gradients on retention reproducibility. Because of the pressure cycles in gradient chromatography, variable viscous heating results in column temperature cycles. The impact can be described as start-up effects in which the first few gradients are different from the later ones. Further details can be obtained from the Grinias group's published work (14). Grinias went on to discuss the transfer of HPLC methods to UHPLC methods as applied to the modernization of pharmacopoeial methods. Grinias concluded that modern UHPLC instrumentation and columns utilizing SPP technology can be used to greatly increase throughput of pharmaceutical analyses based on monograph methods.

Miniaturization continues to be a topic of interest in liquid chromatography. Milton Lee's (15) and Luke Tolley's (16) presentations on miniaturized LC instruments with high performance were considered highlights by many. Both talks centred on a promising new miniaturized LC instrument being developed by a company called Axcend. An interesting note from Lee's talk was that the mobility of an instrument may be as useful within a laboratory as it is for remote usage.

Among the highlights using microfabricated devices, Qun Fang presented on performing single-cell analysis using droplet-based microfluidic techniques (17). Fang and colleagues developed a device that can sample and load different solutions on a microplate. As an example of an application for this system, Fang showed the purification and detection of µRNA species that are easily bound to proteins. To remove proteins from the sample, Fang performed "in-droplet" tryptic digestion to unbind and remove protein from the µRNA prior to nano-LC–MS detection. The group intends to further develop the system using capillary electrophoresis–mass spectrometry (CE–MS).

Another notable highlight was a presentation given by Attila Felinger entitled, "Reversed-Flow Liquid Chromatography" (18). Felinger described a technique whereby flow is stopped such that the analyte is still within the column. The flow is then reversed either by physically turning the column around or by the use of a valve system. The technique allowed the researchers to investigate band broadening contributions from frits, understand trans-column dispersion effects, and probe the column sections for imperfections. Interestingly, it was found that the centre of the column was more efficient than either of the ends.

The topic of 3D printing columns was once again a highly discussed topic at HPLC 2018. At the forefront of the discussions were two presentations by Simone Dimartino, one of which was a tutorial (19,20). Dimartino described employing monomers with the functionality desired to construct the columns, rather than employing post printing chemical techniques to alter selectivity. Printed columns are still not capable of generating efficiencies as high as those of modern packed-bed columns, but the technology is progressing rapidly. In addition to being used to print columns, 3D printing has also been shown to be useful for device optimization. Theodora Adamopolou presented a talk showing computationally-derived 3D printed devices to aid in multidimensional separations (21).

HILIC and Chiral Separations

HILIC was once again a much discussed topic at the HPLC meeting. At the head of the line again was Andrew Alpert, who presented a talk on the importance of salt selection when developing HILIC or electrostatic repulsion–hydrophilic interaction chromatography (ERLIC) methods for phosphopeptides (22). Alpert provided examples in which the choice of both cation and anion elicited differential selectivity of phosphopeptides. The more hydrated the cation was, the more retained the negatively charged phosphopeptides were. Alpert also demonstrated evidence of molecular reorientation of peptides at the water–organic boundary based on salt type. This information could be used for applications involving glycopeptides and for intact proteins with different degrees of phosphorylation. A second HILIC talk that garnered attention was given by Jonathan Shackman. His talk, entitled "HILIC to the Rescue: Pharmaceutical Development Case Examples", showed that HILIC can be a useful and reliable technique for small-molecule pharmaceuticals when traditional methodologies such as reversed-phase LC fail to provide adequate results (23).

Advances in chiral separation technologies were once again a topic of discussion at HPLC. Ravindra Hegade presented the use of stationary phase optimized selectivity, or the coupling of stationary phases, as an approach to develop chiral separations in both LC and supercritical fluid chromatography (SFC) (24). In another highlight, Csaba Horváth Young Scientist Award winner Martina Catani discussed mass transfer phenomena and thermodynamic properties of modern porous particles for efficient enantioseparations (25). One interesting note from the talk was that advantages normally observed for SPP particles over fully porous particles in reversed-phase separations were not clearly evident in the chiral systems investigated.

Poster Session Highlights

As in years past, the poster session at HPLC 2018 was an important part of the overall symposium. There were 336 posters presented across 21 session topics. Compared to HPLC 2016 in San Francisco (the last time the conference was held in the United States), there was a 25% increase in the number of posters presented at the conference.

The number of posters presented in each category was fairly evenly distributed. A breakdown of the number of posters in each session topic is shown in Figure 2. The four most popular poster session topics, in terms of number of posters presented, were stationary phases, method development, biopharmaceutical applications, and characterization of monoclonal antibodies, drug conjugates, and other protein-based drugs; this ranking mirrors both the oral presentation topics at this year's conference and also the overall direction of growth in the LC markets. The popularity of the stationary phase and method development topics shows the ever present need to continue to improve and develop new separation media and analysis techniques. Within those two topics, and in several other session topics, there was an increased presence of posters on HILIC separations related to understanding and developing HILIC methods for a wide array of polar molecules that are difficult to retain on reversed-phase columns. One of the posters receiving an honourable mention in the poster awards competition was from Dennis Asberg on "HILIC Method Development in Pharmaceutical Analysis". Asberg outlined a comprehensive HILIC method development strategy to optimize the analysis of hydrophilic pharmaceutical compounds.

The twenty-one poster session topics were spread out across six different presentation sessions, two each day on Monday, Tuesday, and Wednesday. On Thursday morning, the finalists for consideration for poster awards presented their work again. The posters under consideration for awards were evaluated by a panel of international scientists and judged based on scientific contribution, originality of work, completeness of work, quality of experimental or theoretical execution, and lastly the readability and the presentation of the poster. The Best Poster Awards, sponsored by Agilent Technologies, were presented at the closing ceremonies of the conference. The winning posters, and those that received honourable mention, are listed in Table 2.

Conclusions

HPLC 2018 was a well-organized and lively symposium that engaged researchers interested in analytical science from around the globe. Many recent trends continued in 2018. A revival of progress in 3D printing of columns and devices was evident. There also seemed to be an additional surge of interest in innovative large-molecule separations. It is clear from the activity at the conference that research in the separations sciences is alive and well.

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Coverage of such a large symposium is impossible without a great amount of assistance. The authors would like to acknowledge invaluable assistance from Andrew Alpert, Robert Kennedy, Xiaoning Lu, Daniel Shollenberger, Ken Broeckhoven, Ta-Chen Wei, Matthew Lauber, Mariosimone Zoccali, and Daniel Armstrong for providing notes, text, insights, figures, and fruitful discussions regarding the content of various sessions.

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(19) S. Dimartino, "3D Printing in the Separations Sciences," presented at 47th International Symposium on High Performance Liquid Phase Separations and Related Techniques, Washington, DC, USA, (2018).
(20) S. Dimartino, "3D Print Your Own Chromatography Column," presented at 47th International Symposium on High Performance Liquid Phase Separations and Related Techniques, Washington, DC, USA, (2018).
(21) T. Adamopoulou, S. Deridder, S. Nawada, G. Desmet, and P.J. Schoenmakers, "Creating Devices for Multidimensional Separations based on Computational Insights," presented at 47th International Symposium on High Performance Liquid Phase Separations and Related Techniques, Washington, DC, USA, (2018).
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(23) J. Shackman, "HILIC to the Rescue: Pharmaceutical Development Case Examples," presented at 47th International Symposium on High Performance Liquid Phase Separations and Related Techniques, Washington, DC, USA, (2018).
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Cory E. Muraco is a Senior R&D Scientist in the Liquid Separations R&D group at MilliporeSigma, in Bellefonte, Pennsylvania, USA. Cory completed his graduate studies at Youngstown State University in 2013, focusing on the analysis and characterization of oxidized proteins. Upon graduation, Cory joined MilliporeSigma, first joining the chemical standards R&D group, then transferring to the liquid separations R&D group. Cory's current role at MilliporeSigma is to research, develop, and present on new particle technology for improved chromatographic separations of both small and large molecules and to develop new methodologies for characterizing biomacromolecules by several modes of chromatography. Cory has written about his research in several trade magazines and presented oral and poster presentations on his research at numerous conferences.

Connor Flannery is a Product Development Chemist in the HPLC R&D group at Restek, in Bellefonte, Pennsylvania. Connor has a Bachelor's of Science in chemistry, and joined Restek in 2014. Connor's work at Restek focuses on the development and characterization of new stationary phases and column-packing technologies.

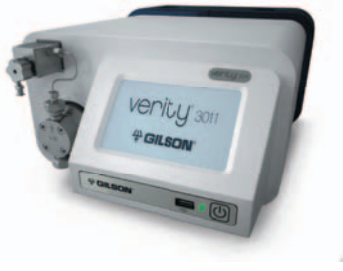
David S. Bell is a director of Research and Development at Restek. He also serves on the Editorial Advisory Board for *LCGC* and is the Editor for "Column Watch". Over the past 20 years, he has worked directly in the chromatography industry, focusing his efforts on the design, development, and application of chromatographic stationary phases to advance gas chromatography, liquid chromatography, and related hyphenated techniques. His undergraduate studies in chemistry were completed at the State University of New York at Plattsburgh (SUNY Plattsburgh). He received his Ph.D. in analytical chemistry from The Pennsylvania State University and spent the first decade of his career in the pharmaceutical industry performing analytical method development and validation using various forms of chromatography and electrophoresis. His main objectives have been to create and promote novel separation technologies and to conduct research on molecular interactions that contribute to retention and selectivity in an array of chromatographic processes. His research results have been presented in symposia worldwide, and have resulted in numerous peer-reviewed journal and trade magazine articles. Direct correspondence to: LCGCedit@ubm.com

Evaporation system

A six-position evaporation station, mVAP, is available for fully automated sample concentration at user-defined temperature and vacuum. The mVAP can evaporate solvents after SPE or liquid–liquid extraction including changing to a compatible solvent for the best possible chromatographic analysis and MS ionization. According to the company, significantly improved limits of detection are achieved. Injection to the GC–MS or LC–MS system is automated

www.gerstel.com
Gerstel GmbH & Co. KG, Mülheim an de Ruhr, Germany.

Isocratic pump



The Verity 3011 isocratic pump is a liquid delivery solution for chemical reaction monitoring in petroleum applications and for gel permeation chromatography (GPC). According to the company, the pump is highly accurate and delivers virtually pulse-free, stable solvent flow for a variety of liquids, including high viscosity solvents and allows for flow rates from 0.01 0 mL/min to 10 mL/min and pressures of up to 600 bar.

www.gilson.com/verity3011
Gilson, Middleton, Wisconsin, USA.

FID gas station

The VICI FID gas station combines the reliability of the VICI DBS hydrogen and zero-air generators into one compact and convenient package, according to the company. Available in high and ultra-high purity for all GC detector and carrier gas applications. The generator is available in two styles: flat for placement under a GC, or the Tower. Available in H_2 flow ranges up to 1 L/min and 10.5 bar.

www.vicidbs.com
VICI AG International, Schenkon, Switzerland.

MALS detector

The µDAWN is, according to the company, the world's first multi-angle light scattering (MALS) detector that can be coupled to any UHPLC system to determine absolute molecular weights and sizes of polymers, peptides, and proteins or other biopolymers directly, without resorting to column calibration or reference standards. The WyattQELS Dynamic Light Scattering (DLS) module, which measures hydrodynamic radii "on-the-fly", reportedly expands the versatility of the µDAWN.

www.wyatt.com
Wyatt Technology, Santa Barbara, California, USA.

Storage rack

For a safe storage of their ergonomic and electronic crimping tools, Macherey-Nagel offers a foam rack that is chemically resistant. According to the company, the crimping tools are properly organized and well protected in the rack. With their standing position they are kept handy for next usage. The storage rack completes the range of vials, caps, and chromatography accessories.

www.mn-net.com
Macherey-Nagel GmbH & Co. KG, Düren, Germany.

Fast GC kit

Designed with GC–MS users in mind, the GC Accelerator kit provides a simple way to speed up sample analysis, according to the company. By reducing oven volume, these inserts allow faster ramp rates to be attained, which reduces oven cycle time and allows for increased sample throughput and more capacity to process rush samples. When faster ramp rates are used, existing methods can be accurately scaled down to smaller, high-efficiency, narrow-bore columns using Restek's EZGC method translator.

www.restek.com/catalog/view/52293/23849
Restek Corporation, Bellefonte, Pennsylvania, USA.

Preparative system

Quattro countercurrent and centrifugal partition chromatographs and extractors are designed to work with, and complement, standard flash and HPLC laboratory and process instrumentation. When appropriate, replacing the solid–liquid columns with unique liquid–liquid instrumentation allows preparations from milligram to tonnes every year. No on-column adsorption or degradation will occur, according to the company. A mass-balance is the norm for CCC/CPC. Typically a 50–80% solvent saving occurs. Standard biphasic solvents, ionic liquids, liquid chiral selectors, and ion exchangers may all be used. According to the company, crude material that would poison standard columns can be injected without causing contamination.
www.quattroprep.com
AECS-QuikPrep Ltd., London, UK.

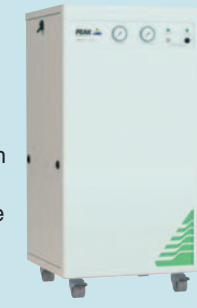
Chromatography software

Clarity enables the control of hundreds of different instruments from one environment. Its wide range of data acquisition interfaces allows connection to virtually any chromatograph. Clarity is multilingual; users can switch between six languages. With easy operation, outstanding user support, and optional extensions for various applications, such as PDA, MS, GPC, NGA, and many more. A free demo is available from DataApex's website.
www.dataapex.com
DataApex, Prague, The Czech Republic.

Microchip column

µPAC is PharmaFluidics' chip-based chromatography column for nano-liquid chromatography. Perfect order in the separation bed is achieved by etching a regular pattern of pillars into a silicon wafer using micromachining technology.
The column allows high-resolution separation of tiny, complex biological samples, with an unprecedented robustness. µPAC is suitable for lipidomic, metabolomic, and peptide profiling, according to the company.
www.pharmafluidics.com
PharmaFluidics, Ghent, Belgium.

Nitrogen generator

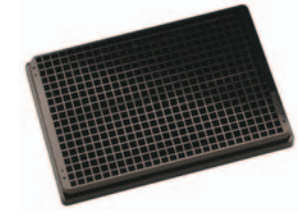


Peak Scientific's new Infinity XE 50 Series nitrogen generator system is designed to cater for numerous applications across a typical laboratory. Delivering a variable flow of nitrogen gas ranging from 11 L/min to 432 L/min at purities up to 99.5%, the system is ideally suited for a wide range of applications including LC–MS, ELSD, sample evaporation, NMR, FTMS, and gloveboxes.
www.peakscientific.com/infinityxe50
Peak Scientific Instruments Ltd, Glasgow, Scotland, UK.

MALS detector

The new Postnova PN3621 Maximum Angle MALS detector sets a high standard for precise multi-angle light scattering detection for size-exclusion chromatography (SEC) and field-flow fractionation (FFF), according to the company.
The detector simultaneously measures the scattering intensity at a maximum of 21 angles, which enables determination of absolute molecular weight and size of proteins, polymers, and nanoparticles.
www.postnova.com
Postnova Analytics GmbH, Landsberg, Germany.

Circular dichroism microplate



Porvair Sciences has introduced a new black Krystal UV Quartz bottomed microplate that offers optical transmission in the wavelength range of 185 nm to 1100 nm making them ideal for laboratories looking to make circular dichroism (CD) measurements. The microplates allow these measurements to be made in a convenient ANSI/SLAS compliant 96- or 384-well microplate footprint using next-generation CD spectrometers. The proprietary black polystyrene polymer mix ensures low cross-talk whilst the top-quality quartz bottom gives reduced birefringence, essential for good CD measurements.
www.porvair-sciences.com/krystal-uv-transparent
Porvair Sciences Ltd., Wrexham, UK.

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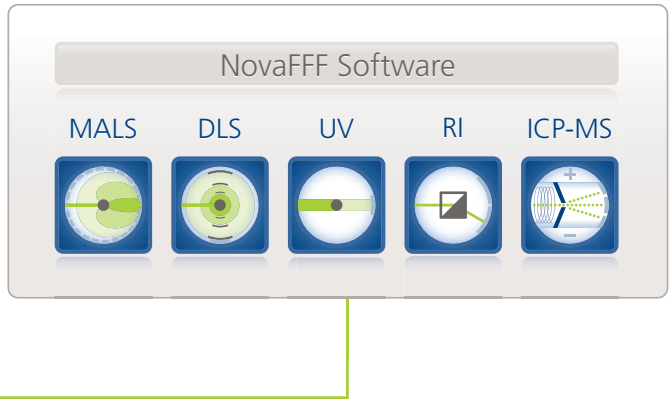

Contact us for more information: **www.postnova.com**

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