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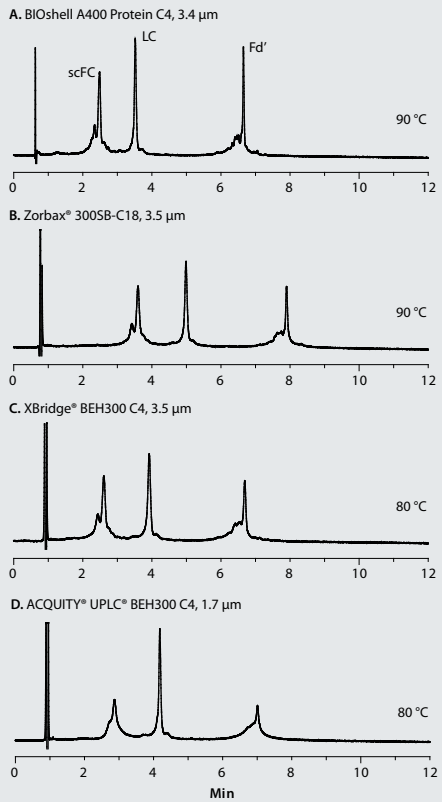

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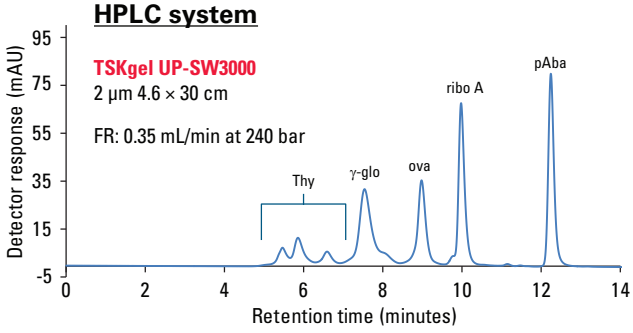

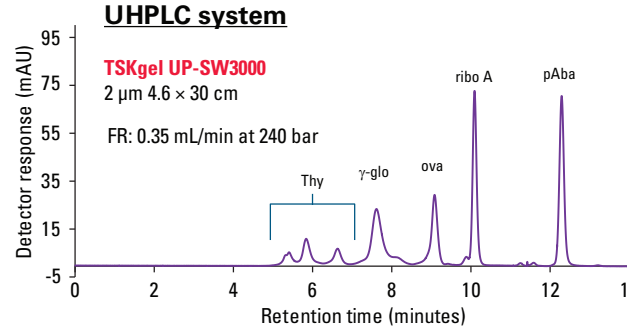

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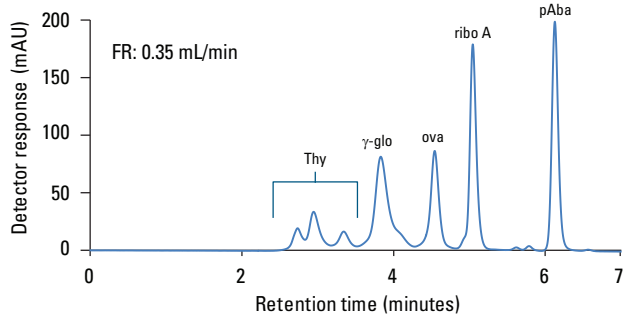

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Editorial
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LBush@advanstar.com
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Group Technical Editor ***Stephen A. Brown***
SBrown@advanstar.com
Associate Editor ***Cindy Delonas***
CDelonas@advanstar.com
Art Director ***Dan Ward***
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JANUARY 2016

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PEAKS of Interest

Mars to Open Food Safety Center in China

Mars, Incorporated (McLean, Virginia) has announced the opening of its Global Food Safety Center, a first-of-its-kind facility for pre-competitive research and training that aims to raise global food safety standards through collaboration. Located in Huairou, China, just north of Beijing, the center will feature analytical chemistry and microbiology laboratories, interactive training laboratories, and a conference auditorium to enhance knowledge sharing. The center is part of Mars' ongoing commitment to working with world-leading experts to ensure the safety and security of food.

The Mars Global Food Safety Center will leverage insights and expertise from over 60 Mars partnerships dedicated to innovative, sustainable, and responsible food safety practices. The World Food Programme (WFP), the Global Alliance for Improved Nutrition (GAIN), Partnership for Aflatoxin Control in Africa (PACA), and the IBM/Mars Consortium for Sequencing the Food Supply Chain are among the organizations Mars is partnering with to try to solve the challenge of feeding a global population expected to grow to nine billion by 2050.

Grant Reid, president and CEO of Mars, Incorporated, said "Food safety is a global issue that concerns us all—business, governments, academics, and the world's population. Working together across all disciplines is the only way we can truly advance efforts at scale, with the ultimate goal of increasing access to safe nutrition for billions of people around the world."

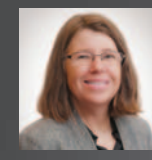
David Crean, vice president, Corporate R&D at Mars Incorporated, added "Unlike an R&D or innovation center focused on product development and improvement, the Mars Global Food Safety Center is a state-of-the-art facility dedicated to advancing food safety research through collaboration and the pre-competitive sharing of information."

Phenomenex Expands Method Development Services

PhenoLogix, Phenomenex's in-house analytical services group, has moved to an expanded, stand-alone facility. The 9000-ft^2 laboratory has been designed to develop methods for customers in ultrahigh-pressure liquid chromatography (UHPLC), LC, LC coupled to mass spectrometry (MS), and gas chromatography (GC), as well as sample preparation, impurity isolations, chiral screening and small- to medium-scale purifications. PhenoLogix provides support for the company's product line to customers in drug discovery and development, environmental testing, food safety and quality, forensics, and clinical research.

PhenoLogix was cultivated from Phenomenex's research and development department into a self-contained organization. The new facility features instruments from multiple manufacturers to address a range of applications. ■

LCGC*tv* NEW VIDEOS FROM LCGC



MARY J. WIRTH ON SLIP FLOW—HOW TO HARNESS IT

Mary J. Wirth of Purdue University explains the phenomenon of slip flow, how to harness it, and how the concept affects method development, the role of particle size, and future applications.

Other recent LCGC TV *interviews include:*

- Rudolph Krska on mycotoxin analysis using LC–MS-MS
- Luigi Mondello on the potential of mass spectrometry to replace chromatography
- Christian Wachsmuth on how GC–MS could be applied in a clinical setting and what developments are needed for this to happen.

Visit http://www.chromatographyonline.com/ to see these videos and more.

SPECIAL ISSUE HIGHLIGHTS

ADVANCING ENVIRONMENTAL ANALYSIS

Environmental science and analytical chemistry are intimately intertwined. This special issue, edited by Kevin Schug of the University of Texas at Arlington, provides a snapshot of emerging applications and solutions in environmental analytical chemistry.

Topics covered include:

- A new gas-phase chemiluminescence approach for analyzing and speciating waterborne arsenic
- Fourier transform molecular rotational resonance spectroscopy for VOC analysis
- Single-particle ICP-MS for the analysis of engineered nanoparticles in environmental water samples
- Flow-through solid-phase spectrometry
- GC–MS and UHPLC–MS-MS analysis of organic contaminants and hormones in whale earwax using selective pressurized liquid extraction
- Analytical efforts for monitoring groundwater in regions of unconventional oil and gas exploration

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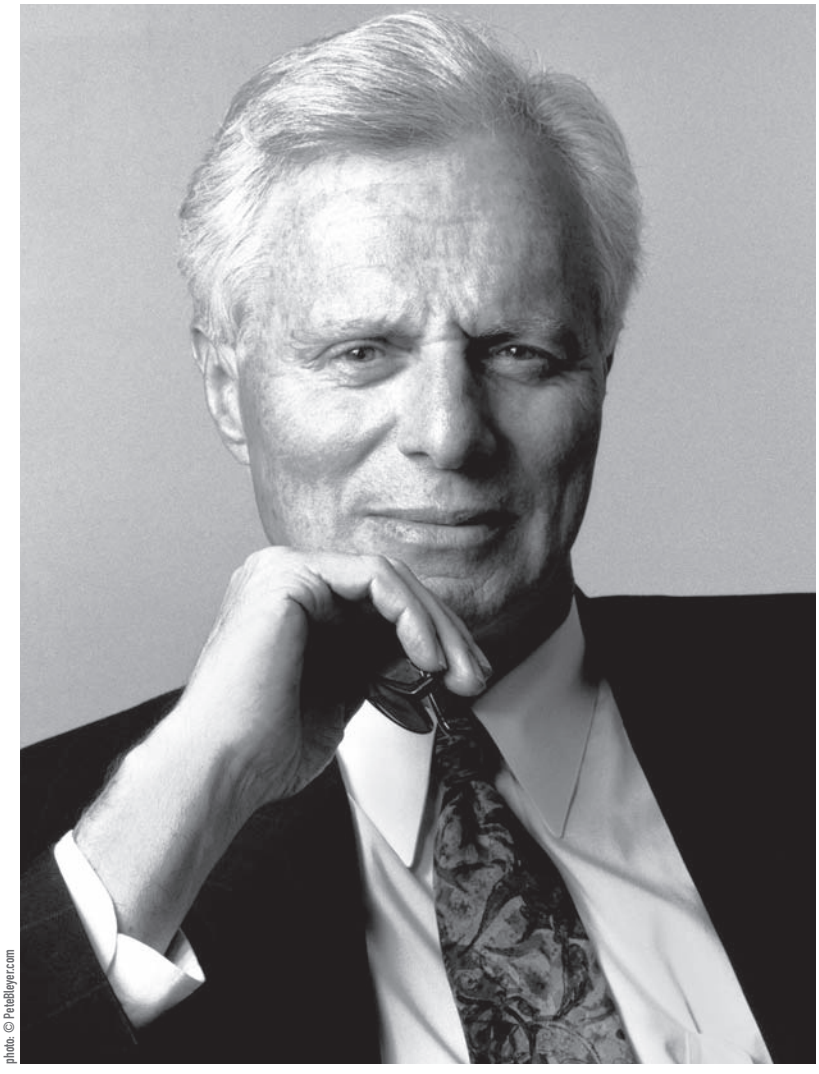

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SAMPLE PREP PERSPECTIVES

Surfactant-Mediated Extractions, Part I: Cloud-Point Extraction

Cloud-point extraction (CPE) manipulates temperature and surfactant concentration to move aqueous solutes into a micelle phase for separation. Although CPE has been around for some time, it is still considered an emerging technique. Much of the development, and most applications, of CPE have dealt with extraction and preconcentration of inorganic solutes. More recently, attention has turned to the use of CPE in the isolation of organic solutes. This month, we review how CPE works and focus on applications for extracting organics.

Douglas E. Raynie
Sample Prep Perspectives Editor

With the rapid development of sample preparation technologies since the mid-1970s, one sometimes overlooked technique that is getting renewed interest for chromatographic sample preparation is cloud-point extraction (CPE). CPE is the most widely used in a family of surfactant-based methods and was introduced in 1976 by Mitura, Ishii, and Watanabe (1). Initially, applications in the extraction of metal ions and, later, hydrophilic proteins seemed to predominate. But in the past several years, attention has shifted to small molecule organics, warranting a look at CPE as a promising, environmentally benign means for chromatographic sample preparation.

What Are Micelles?

Surfactant molecules generally possess a hydrophilic head group attached to a hydrophobic tail. At water–air or water–organic interfaces (or with other polar solvents besides water), these molecules align so that the hydrophilic portion is directed toward the aqueous component of the mixture. As concentration increases, the dispersed hydrophobic chains self-assemble into colloidal-sized clusters (2). When a concentration known as the critical micelle concentration (CMC) is reached, these colloidal aggregates are in dynamic equilibrium with surfactant monomers in the bulk aqueous solution and are called *micelles*. In these micellar colloids, water is the continuous phase and the aggregated surfactant molecules are the dispersed phase. Micelles typically are approximately spherical, but may also have other shapes depending on the nature of the surfactant and the solution properties. Reverse micelles, in which the hydrophobic chains are on the outside, are found with nonpolar solvents.

The degree of aggregation, or micelle size, is dependent on the nature of the surfactant (including the identity, or structure, of the hydrophilic and hydrophobic groups), solutes (especially electrolytes), nature of the solvent, temperature, and pH. Micelles will only form above the critical micelle temperature. Micelles in water-based solutions may solubilize hydrophobic compounds or those materials with limited water solubility to a much greater extent than in pure aqueous solutions. The hydrophobic solutes partition into the inner core of the micelle, as depicted in Figure 1. Similarly, reverse micelles dissolve hydrophilic substances into the micelle from a nonpolar organic solvent. Polar compounds may migrate to the center of the micelle, associate with the surfactant's polar head groups of the surfactant, or associate with the micelle hydrophobic chains. These compounds are solubilized through the polar region by electrostatic, π-ion, or hydrogen-bonding interactions.

For the "cloud-point system" in CPE, the phase transfer of nonionic surfactants from a clear homogenous solution to the cloudy colloidal system, and then a biphasic system upon increasing the temperature above the "cloud-point temperature" (CPT) of the surfactant is used (3). The term *cloud point* refers to the light scattering created by the formation of a colloidal system, the Tyndall effect.

Mechanism of Extraction

CPE is performed by adding a surfactant solution to the sample at levels exceeding the CMC, allowing the formation of micelles. As the analytes dissolve and partition into the micelles, two immiscible isotropic phases form. The first of these phases is the surfactant-rich

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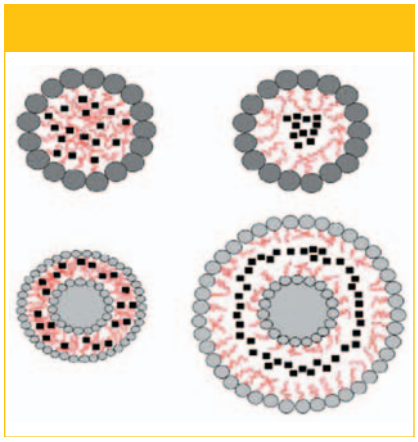

Figure 1: As particles solubilize into micelles they may either associate with the hydrophobic chains of the micelle (left) or form a separate phase inside the micelle. Adapted with permission from reference 2.

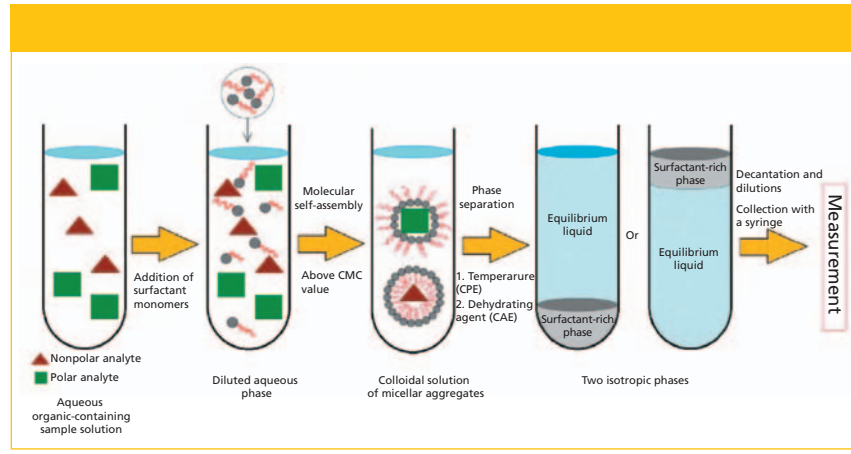

Figure 2: Comparison of the steps involved in cloud-point extraction and coacervative extraction. Adapted with permission from reference 2.

coacervative phase, which contains the extracted analyte. The bulk aqueous phase is in equilibrium with the coacervative phase. It is important to note that in CPE, only nonionic or zwitterionic surfactants are used in forming the colloidal micelles and temperature (the CPT) is used to induce the phase separation (2).

CPE is not to be confused with coacervative extraction (CAE), even though these terms are sometimes used interchangeably in the literature. The physical and chemical processes in CPE and CAE are similar, as diagrammed in Figure 2. In CPE, temperatures above the CPT are used to induce the phase separation, while in CAE, salts, organic solvents, pH, or other factors are used. Because of this difference in the conditions regarding how the phase separation occurs, CAE may use anionic or cationic surfactants. With the resulting charged micelles, temperature alone cannot overcome the electrostatic repulsions that prevent phase separation, and decreased temperature (rather than above the CPT) is used. CAE and other surfactant-based extractions, like aqueous surfactant two-phase (ASTP) extraction, which uses mixtures of ionic surfactants, will be the subject of a future "Sample Prep Perspectives" column.

CPE involves three fundamental steps:

- analytes present in the original matrix associate within the micellar aggregates;
- increasing the temperature to above the CPT, which causes separation of the coacervative phase from the bulk aqueous phase; and
- phase separation, often by centrifugation and decanting.

The nonionic surfactants used in CPE are often polyoxyethylene-based, and commonly used surfactants include

- Triton X-100 (CPT = 66 °C)
- Triton X-114 (CPT = 25 °C)
- Brij 30 (CPT = 2 °C)
- Brij 35 (CPT > 100 °C)
- Brij 56 (CPT = 69 °C)
- Brij 97 (CPT = 72 °C)
- Tween 80 (a sorbitol-based nonionic surfactant) (CPT = 65 °C).

CPT depends on the surfactant used and covers a broad temperature range, as shown above. Nonionic surfactants generally have a lower CPT than zwitterionic surfactants. The CPT also may vary with surfactant concentration and dissolved solutes, especially salts. The coacervative phase is usually cooled after the centrifugation step before decanting to collect the extract. Solute partitioning into the micelles from the bulk aqueous phase is primarily driven by hydrophobic interactions (van der Waals forces), with dipole–dipole and hydrogen bonding interactions playing separate roles and having secondary effects (3). Similar effects are observed in liquid–liquid extraction into nonpolar solvents. Extraction kinetics and achievement of the equilibrium of partitioning is rapid, as fast as 2 min (4). To accomplish such rapid extractions, with near 100% extraction efficiency, temperatures 15–20 °C greater than the CPT are used (5–7).

Advantages and Limitations of CPE

CPE involves just a few manual steps and uses standard glassware and equipment found in most laboratories—namely pipettes, flasks, heating plates, and a centrifuge. Hence, specialized instrumentation or extraction supplies are not needed. In most cases, cleanup of the extracted sample before chromatographic determination is not necessary. The surfactants are fairly inexpensive and have low flammability. As mentioned, quantitative yields are obtained in short times and several samples may be processed at once.

At the same time, there are some limiting factors to consider. The surfactants used may cause analytical interferences and issues with detection limits, especially if the analytes cannot be effectively isolated from the surfactants, depending on the analysis. Extraction efficiencies decrease with increasing solute polarity and with highly volatile or thermally unstable compounds.

Applications

Although the initial applications of CPE were directed toward metal ions and other hydrophilic compounds, recent attention has turned to small organic molecules, especially. The extraction of priority pollutants from water, solid, and biological samples by CPE has been established (4). These include phthalates, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, polychlorinated dibenzodioxins and dibenzofurans, chlorophenols, and organochlorine pesticides.

The CPE of triazine herbicides in milk was reported by Liu and colleagues (8) as an example of food analysis. Atrazine, cyanazine, simazine, and simetryn were isolated from 10 mL of milk mixed with Triton X-100, glacial acetic acid, and

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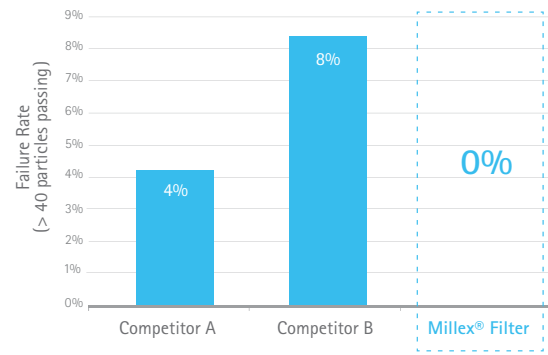

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sodium sulfate then diluted with water. Following centrifugation at 5 °C, the supernatant was filtered and the acidity was adjusted to pH 5. Following a 30-min incubation at 60 °C to cause phase separation, methanol was added to the coacervative phase, which was then filtered and characterized by high performance liquid chromatography with ultraviolet absorbance detection (HPLC–UV). Detection limits around 5–10 mg/L with recoveries of 70.5–96.9% and a linear range of 50–2000 mg/L was reported.

Other interesting applications are in drug-related studies. Benzodiazepines were extracted from medicinals with Triton X-114 at pH 6 at 40 °C and 30 min (9,10). Lorazepam was recovered at 52.7% and alprazolam at 67.0% with HPLC using diode-array detection (DAD). The preconcentration of β-lactam antibiotics with CPE was accomplished (11). Even more interesting are the myriad investigations into the determination of the active compounds in Chinese medicinal herbs (12–15).

Conclusion

Several review articles, including those that provided the basis for this column, establish the wide utility of CPE to the analytical extraction of small organic molecules (2–4,16–18). As greener, faster, and more efficient extraction methods are being investigated for the analytical laboratory, the use of CPE warrants a look.

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Douglas E. Raynie

"Sample Prep Perspectives" editor Douglas E. Raynie is an Associate Research Professor at South Dakota State University. His research interests include green chemistry, alternative solvents, sample preparation, high resolution chromatography, and bioprocessing in supercritical fluids. He earned his PhD in 1990 at Brigham Young University under the direction of Milton L. Lee.

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LC TROUBLESHOOTING

Detective Work, Part III: Strong Retention and Chemical Problems with the Column

Strongly retained sample compounds can cause various changes in the appearance of a chromatogram.

John W. Dolan
LC Troubleshooting Editor

This is the third "LC Troubleshooting" column in a series related to problems that we associate with liquid chromatography (LC) columns. In part I (1) we looked at the major causes and symptoms associated with failure of LC columns, as summarized in Table I. In last month's installment (2) we considered problems that are associated with physical problems with the column. This month is the first of several discussions looking at problems that are caused by chemical problems with the column. In Table I, we see that chemical problems (adsorbed sample and chemical attack) are associated primarily with changes in retention and selectivity (peak spacing). This month's topic is adsorbed samples that are strongly retained and may cause other changes in the appearance of the chromatogram.

Sample–Column Interactions

In liquid chromatography, adsorption of the sample plays a primary role in retention and separation. Consider that a sample molecule X can reside in the mobile phase, X_{mp}, or in the stationary phase, X_{C18} (assuming the stationary phase is C18). There is an equilibrium between these two conditions:

$$X_{mp} \rightleftharpoons X_{C18} \quad [1]$$

In the normal process of retention, the equilibrium is controlled by such factors as the mobile phase organic composition, type of organic, pH, temperature, and stationary-phase characteristics. If the equilibrium is shifted to the right, retention increases; to the left, it decreases. Another analyte, Y, will have similar behavior:

$$Y_{mp} \rightleftharpoons Y_{C18} \quad [2]$$

If the two equilibria of equations 1 and 2 are sufficiently different, X and Y will be separated as they travel down the column.

With reversed-phase LC, we often assume that all the retention is because of the C18 stationary phase, but in fact this is rarely the case. Most columns have additional interaction sites, the most common of which is interaction with unbonded silanol groups, SiOH, of the silica support material. Thus, we can have some of X attracted to these sites, as well:

$$X_{mp} \rightleftharpoons X_{SiOH} \quad [3]$$

For discussion purposes, let's consider a column with a total of 1000 interaction sites in which 20 of the sites will interact as in equation 3, and the remaining sites interact as in equation 1. Also, let's assume that the interaction in equation 3 is so strong that for practical purposes it can be expressed as follows:

$$X_{mp} \rightarrow X_{SiOH} \quad [4]$$

That is, molecules of X that interact with the silanol groups bind irreversibly.

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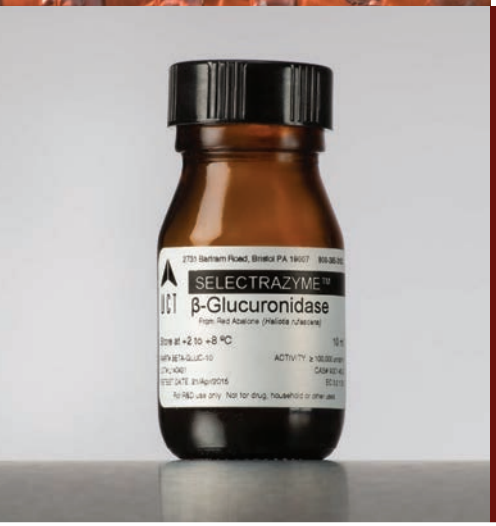

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Table I: Diagnosing column problems

Symptom	Cause				
	Pressure	Tailing	Plates	Selectivity	Retention
Blockage	XX	XX	X		
Voids		XX	XX		
Adsorbed sample			X	X	XX
Chemical attack		X	X	XX	X

X – commonly observed correlation
XX – strongly correlated

Now if we inject 100 molecules of X, 20 will be permanently bound to the silanols and the remaining 80 will be retained in the normal manner on the C18 sites. Next, make a duplicate injection and, because the silanol sites are already blocked by X bound from the previous injection, all 100 molecules of X in the second injection will be eluted. With this oversimplified model, you can see that the first injection will produce a smaller peak than the second and subsequent injections of X.

Peak Size or Retention Problems with the First Injection

It is common to observe that the first chromatogram of a run may have a different appearance from following chromatograms. This can occur with both isocratic and gradient methods. For example, the first injection might have a smaller peak area than a second, duplicate injection from the same sample vial. Or the retention time of the first injection may be a bit smaller or larger than subsequent injections. Sometimes peaks in the first injection will tail more than those of subsequent injections. This kind of behavior is more common with large molecules, such as proteins, than small molecules with molecular weights <1000 Da; however, it can occur with all types of samples.

The most likely cause of the above observations is that some portion of the sample is strongly adsorbed on the column, as discussed above. Although it is common to think of a reversed-phase column as having primarily C18 groups bonded to a silica surface, not all of the surface of the column packing behaves the same. Some portion of the surface interacts more strongly with certain molecules than others, and this interaction is usually attributed to the unbonded silanol groups on the surface of the silica particles. Some types of silanol groups are more acidic than others and tend to interact quite strongly with basic molecules in the sample—either analytes of interest or other basic compounds in the sample matrix. With the older, type A silica columns that were the standard before 1990 and are still used with many older methods, these silanol groups were responsible for strong sample retention and peak tailing. Today's high-purity, type B columns are much less likely to cause unwanted interactions with basic compounds.

If analyte molecules are attracted to these strong retention sites, some of the injected molecules can be strongly bound to the column, while other molecules are eluted in the normal manner. However, the strong retention sites quickly become saturated, so analyte molecules in the next injection will not interact with the sites that are "blocked" by molecules from the prior injection, or if they interact, there will be no net change in the concentration adsorbed molecules. The result will be a peak that is larger in the second injection and retention times may shift a bit, too. With the type A columns, such interactions were so common that many workers added triethylamine to the mobile phase at concentrations of 25 mM or more. The triethylamine interacted more strongly with the silanol sites than the analyte molecules, effectively blocking or diminishing the unwanted interactions, especially peak tailing. Type B columns are much less likely to have such problems, so triethylamine use is much less common today. When protein samples have problems of shifting retention of peak area for the first few injections, several injections of a high concentration of protein can accelerate the equilibrium. Often, such interactions are generic enough that another protein, such as bovine serum albumin, can be loaded onto the column to deactivate it for other proteins. Triethylamine or other small molecules tend to equilibrate quickly, so the appearance of the chromatogram usually stabilizes by the second or third injection, but these materials also wash out easily

during column flushing at the end of a batch of samples. Protein saturation, on the other hand, tends to be more permanent, so a single treatment may condition a column for all future uses.

This problem of reproducibility between the first and second injections is quite common. In most methods, the second and subsequent injections are stabilized relative to the first injection, so the simplest approach to solving this problem is to ignore the first injection. Just program the injection sequence so that the first sample is injected twice (or three times) and ignore the first injection.

Extra Peak in Second and Subsequent Injections

A variation on the theme discussed above is illustrated in the chromatogram shown in Figure 1. In this case, a peak appears in the chromatogram that is much broader than the neighboring peaks. As a general rule in both isocratic and gradient separations, peaks in the same part of the chromatogram should be approximately the same peak width. In Figure 1, the first and third peak fit this description. When a broad peak appears among narrow peaks, as in Figure 1, the most likely cause is late elution. In other words, the peak doesn't belong with the observed chromatogram; instead it is part of a prior injection, but the run time was not sufficiently long to allow it to be eluted with the proper sample. This is illustrated in the simulated chromatograms of Figure 2. In the top run of Figure 2, we see that the second peak, eluted at ~2.2 min, is broader than its neighbors. The simple way to verify that this peak is part of a previous injection is to extend the run time. In the bottom run of Figure 2, you can see that the broad peak is eluted at its normal retention time of ~7.2 min.

Usually the broad peak can be associated with the correct injection by extending the run time by a factor of two or three, as in the example of Figure 2. If this technique is not successful, you can estimate the true retention time in an isocratic method by comparing the peak widths. For isocratic runs, all the peaks in the run should have approximately the same column plate number, N:

$$N = 5.54\ (t_R/w_{0.5})^2 \qquad [5]$$

where t_R is the retention time of the peak and $w_{0.5}$ is the peak width at half the peak height. From equation 5, you can see that the ratio of the retention time to peak width should be constant if N is constant. Thus, for two peaks (subscripts 1 and 2),

$$t_{R,2} = (t_{R,1} \times w_{0.5,2})/w_{0.5,1} \qquad [6]$$

By measuring the retention and width of a normally eluted peak, plus the width of the broad peak, we should be able to estimate the true retention time of the broad peak. I did this for

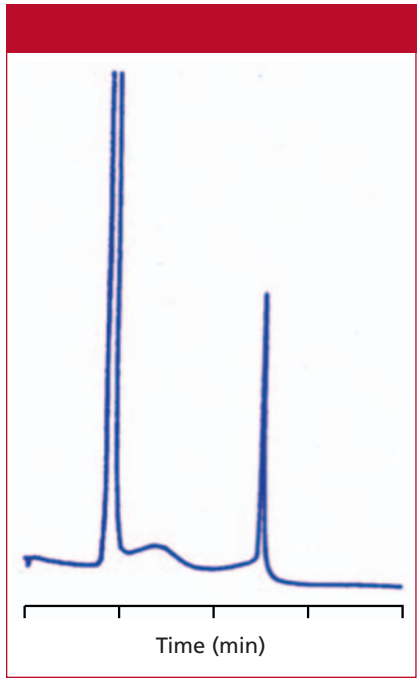

Figure 1: Chromatogram with normal elution for the first and third peaks. The second peak is a strongly retained peak originating from a prior injection.

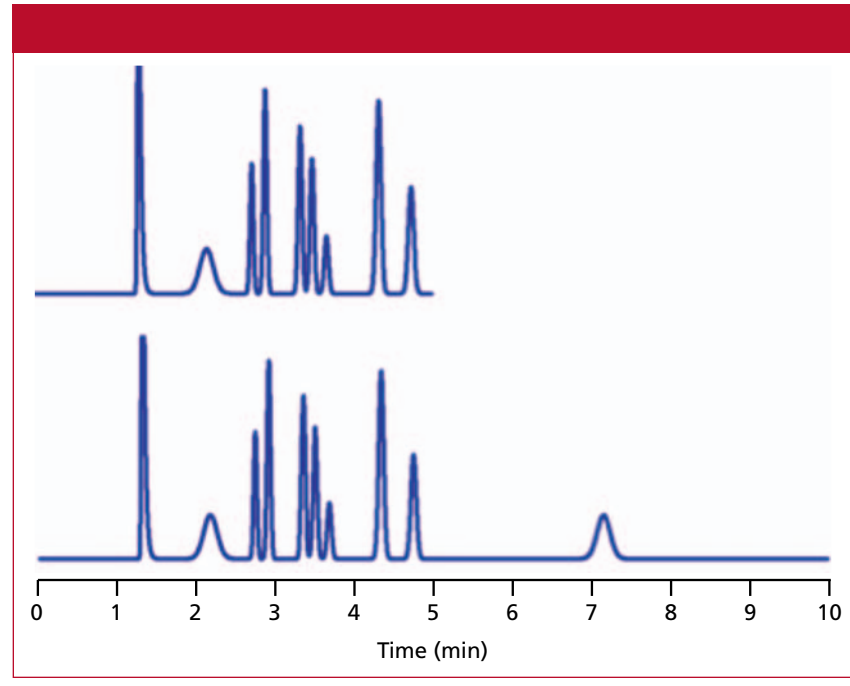

Figure 2: Simulation of a late-eluted peak. In the upper chromatogram, a broad peak at ~2.2 min appears out of place. In the lower chromatogram, extending the run time allows the peak to be eluted at its proper retention time of ~7.2 min.

the broad peak of Figure 1 and came up with a retention time of 26 min! (For this chromatogram, the measurements were somewhat subjective, so I expect this estimate may be good to perhaps ±20%.) In other words, the broad peak didn't come out from the immediately preceding injection, but from the sixth injection before the one we see in Figure 1.

There are several possible solutions to the problem of late elution, as in Figure 1 or 2. We could extend the run time of each run to allow the peak to be eluted normally. This might be practical for the method of Figure 2, but increasing the run time from 4 min to 26 min for the sample of Figure 1 wouldn't be practical. If we were interested in the broad peak, we could use a gradient method to reduce its retention to a reasonable time. If we were not interested in the broad peak, we could flush at 4 min with a strong solvent to clear the column or perhaps adjust the run time so that the broad peak would be eluted in a region of the chromatogram where it did not interfere with peaks of interest. Another option might be to modify sample pretreatment to remove the unwanted peak before injection.

Column Flushing

In the example of Figure 1, the broad peak was still recognizable, but you can imagine that for even more strongly retained peaks and at smaller concentrations, the peaks could be so broad that they wouldn't show up as peaks at all. Instead they would cause the baseline to wander or roll with a wavy appearance. Many real samples contain proteins, polymers, lipids, or other matrix components that are so strongly retained that by the time they are eluted, they are too broad to see. Under such conditions, the baseline will not be stable and the column chemistry may change sufficiently that the appearance of other peaks may be affected, as well. For this reason, you should flush the column after each batch of samples. Flushing with a strong solvent should remove these strongly retained materials and improve the appearance of the baseline.

Column flushing for reversed-phase columns is best accomplished using this procedure:

- Flush with buffer-free mobile phase
- Flush with 100% strong solvent
- Store or return to starting conditions

First, any buffer should be removed from the system. Usually, washing with five column volumes of unbuffered mobile phase is sufficient. (One column volume is ~1.5 mL for a 150 mm × 4.6 mm column and ~0.1 mL for a 50 mm × 2.1 mm column.) Thus, if the mobile phase were 50:50 methanol–buffer, you would flush with 50:50 methanol–water. Second, flush with 10 column volumes of 100% of the strong solvent, which is methanol in the present example. If you are finished with the column for the day, store the column in this solvent. If you want to put it back in service, return it to the original mobile phase. Remember that for column cleaning, it is the volume of solvent that is important, not the time, so you can reduce the flushing time by increasing the flow rate if the pressure is reasonable. Also, you probably can get by with using 100% water for the first flushing step, but it often will cause the column to dewet, which can require additional flushing in step two to rewet the column. (Dewetting occurs when the mobile phase, water, is so polar that it cannot enter the nonpolar pores of a reversed-phase column, so

the pores are not effectively washed. Rewetting requires sufficient organic—usually 5% is sufficient—to allow the mobile phase to repenetrate the pores and wet the surface of the packing.) Finally, it should be obvious that this kind of flushing primarily applies to isocratic methods; gradient methods usually incorporate a strong-solvent flush at the end of each run.

Summary

We have seen that components of injected samples may be strongly retained and change the appearance of the chromatogram. This change may appear as shifts in retention or peak area between the first and second injections of a run sequence. In other cases, peaks may be so strongly retained that they are eluted during a following injection. Real samples often contain matrix components that are so strongly retained that if they are eluted, they result in a wavy or undulating baseline, which can compromise quantification of small peaks. Because strong retention is fairly common for many methods, it is best to ignore the first injection and to flush strongly retained materials from the column after each sample batch or run sequence.

It should be obvious that some samples will contain components that will never be washed from the column. These may build up and eventually ruin the column, or they may just alter the chemistry of the column sufficiently that chromatograms for a used column may not be identical to a new column. For this reason, it is prudent to dedicate a particular column to an individual method. That is, even if the same brand and part number of column is used for two different methods, two separate columns should be used. You will find that fewer problems will be encountered by dedicating a specific column to each method than if the same column is shared between multiple methods.

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John W. Dolan

"LC Troubleshooting" Editor John Dolan has been writing "LC Troubleshooting" for LCGC *for more than 30 years. One of the industry's most respected professionals, John is currently the Vice President of and a principal instructor for LC Resources in Lafayette, 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*

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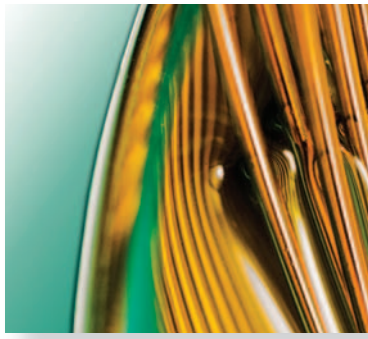

GC CONNECTIONS

Thermal Desorption Sampling

Thermal desorption sampling often provides a means for bringing otherwise intractable samples to a gas chromatography (GC) column for separation and detection. In this installment, John Hinshaw describes the principles of thermal desorption sampling in relationship to other analysis techniques for volatile solutes.

John V. Hinshaw
GC Connections Editor

When asked about common sampling techniques, gas chromatographers first envision a liquid inlet system such as what is found on most gas chromatography (GC) systems. Injection of a relatively clean liquid sample into a vaporizing inlet system often suffices to transfer analyte components from their physical state outside the GC column into a state suitable for GC analysis. A vaporizing inlet system heats the sample to vaporize the components of interest, mixes the sample vapor with carrier gas, and then transfers the sample into the GC column. Such injection techniques include direct, split, splitless, on-column, and programmed-temperature methods.

Many other samples present a different problem: They contain nonvolatile materials that can impair a vaporizing inlet's function when significant quantities of residue are deposited in the inlet or in the beginning of the column. The built-up residue interferes with subsequent analyte transport through the inlet system by causing peak-shape distortion, irreversible adsorption, and thermolytic decomposition. Other samples such as resins, powders, fibers, plastics, and biological fluids present a matrix that is simply incompatible with direct injection techniques.

Thermal Desorption

In such cases, gas chromatographers can call upon a number of related techniques that extract relatively volatile analytes from a less volatile sample matrix. Analyte concentration and focusing steps before transfer onto the GC column often accompany such procedures. Purge-and-trap, headspace, and thermal desorption sampling, plus solid-phase microextraction (SPME) add a number of sampling-stage steps that make them suitable for solid and liquid samples in difficult matrices.

Thermal desorption sampling refers to a number of related techniques that include one or more steps in which sample heating releases volatile analytes into the carrier-gas stream from an adsorbent or other involatile material. One or more adsorption–desorption steps serve to concentrate analytes as well as reduce their bandwidths to meet column injection requirements. Volatiles enter the desorption tube from a variety of sources, including directly from the atmosphere, from the headspace over a solid or liquid sample, by sparging a liquid sample with inert gas, or as the effluent from a chemical process or engine exhaust. A related technique, thermal extraction, heats a solid or semisolid sample directly in the carrier-gas stream using essentially the same equipment as thermal desorption. To distinguish it from pyrolysis injectors, thermal desorption operates at lower temperatures and does not intentionally cause sample thermolysis. For example, chromatographers can use thermal extraction to sample polymers, waxes, powders, pharmaceutical formulations, solid foods, and cosmetics for their volatile constituents.

The bandwidth or volume that analytes occupy as they approach an open-tubular column is often large enough to prevent the column from delivering all of its peak-resolving potential, especially for early-eluted peaks. Ancillary cold trapping or secondary focusing techniques help reduce analyte bandwidths appropriately. Other problems found in conventional liquid inlet systems from

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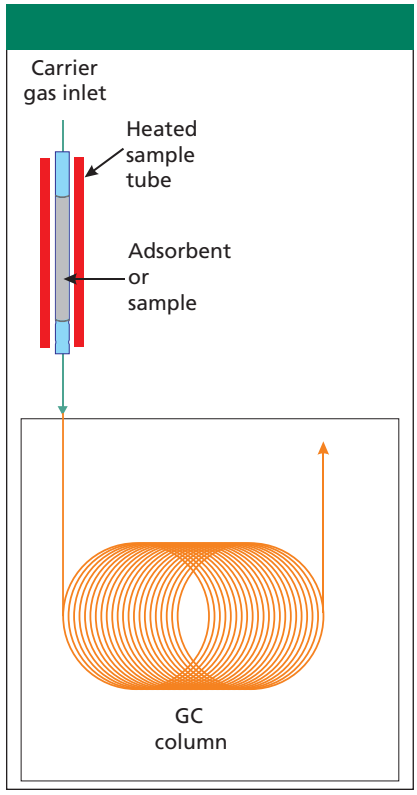

Figure 1: Direct thermal desorption.

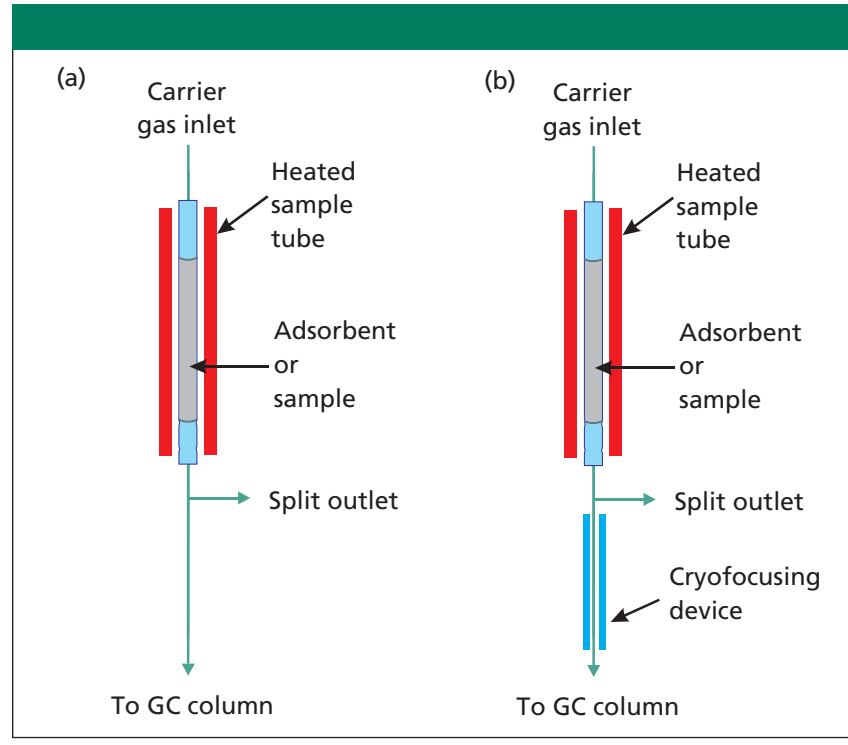

Figure 2: Thermal desorption with (a) stream splitting and with (b) stream splitting followed by a cryofocusing trap.

excessive amounts of solvent or nonvolatile residue deposition in the inlet are absent in volatiles extraction techniques such as thermal desorption. Water vapor from atmospheric or aqueous samples, however, may interfere in some cases, and may require additional sample management steps to prevent water-induced side effects.

Thermal Desorption Techniques

In its simplest form, thermal desorption involves heating a sample tube with carrier gas flowing through it. The emerging gas is conducted directly to the GC column. Figure 1 illustrates a simple thermal desorption setup. A tube containing an adsorbent or sample material (or both) is positioned in the carrier gas stream before the column inlet. After a short delay to flush out residual air, the tube is heated and analytes are desorbed or thermally extracted directly from the sample. Carrier flow conducts the released volatile compounds into the column. In practice, an existing inlet can provide a convenient base for a top-mounted desorption system, or it can act as the terminus of a heated transfer line for benchtop systems. After desorption has finished, the tube cools down and normal carrier-gas flow is restored.

This simple scheme suffers from a number of potential difficulties and generally is not used in practice without enhancements. Researchers have developed a variety of solutions, some of which are generally applicable to thermal desorption, while others are more useful for specific sample types. Thermal desorption problem areas include controlling the amount of sample amount entering the column, flow rates through the column and the sample tube, solute breakthrough volumes, and peak bandwidths. These areas are discussed in more detail below.

Sample Amount Entering the Column

Capillary GC columns tolerate a limited analyte mass beyond which column overloading occurs, characterized by peak distortion and broadening plus retention time shifts. Column sample capacity ranges from about 100 ng on a 0.25-mm i.d., 0.25-µm film column up to about 5 µg on a 0.53-mm i.d. column with a 5-µm stationary phase film. Analyte amounts released from thermal desorption or thermal extraction often exceed these levels, and serious performance losses could occur if all the analyte mass contained in a sample tube were permitted to enter the column.

Thermal desorption systems can use in-line sample splitting to control the analyte amounts that traverse the sampling system. A modified capillary column inlet splitter works well for top-mounted samplers, while benchtop systems often use self-contained splitters. Figure 2a shows an outlet splitter inserted after the sample desorption tube. A needle valve or electronic device (not shown) controls the outlet split flow and thus the split ratio from the desorption tube to the column. Split control with electronic pneumatics is possible when the in-line capillary GC inlet system supports it. For trace-level samples, direct transfer conducts all of the material onto the column. Sample splitting also helps to balance flow rates and reduce peak bandwidths by permitting faster tube flow rates and concomitantly faster desorption times.

Flow Rates

Thermal desorption tubes generally have inner diameters of 3–4 mm. With lengths of about 100 mm, tube internal volumes range from 2.8–5.0 cm^3. With a typical packing factor of about 0.4, tube void volumes are 1.1–2.0 cm^3. Desorption tubes are not packed

tightly with adsorbent from end to end, however, to allow space for glass wool or other materials that restrain the packing inside the tube within the heated zone. For this reason, typical void volumes are on the high side of this range. During desorption, when the tube is heated and analytes are no longer retained on the tube packing, a gas volume of at least 4–8 times the void volume should pass through the tube to transfer analytes to the column quantitatively. A desorption tube with the above dimensions needs at least 30 s to heat its contents to the desorption temperature. With these assumptions, a desorption volume of at least 10 cm^3 at flow rates of 20 cm^3/min or greater is suitable in most cases. If the tube packing slightly retains high-boiling analytes at elevated desorption temperatures, however, the gas volume and the time required for complete desorption increases significantly. In practice, longer desorption times help ensure quantitative transfer from the desorption tube.

For packed columns, desorption flow matches column flow well. However, a disparity exists between the desired desorption flow rate and optimum capillary column flows. At an average helium carrier-gas linear velocity of 40 cm/s and a temperature of 50 °C, the corrected outlet flow rate of a 25-m long, 0.25-mm i.d. capillary column is 1.75 cm^3/min, about five times less than the desired desorption flow. For a 25-m long, 0.53-mm i.d. column, the corresponding corrected outlet flow rate is 5.4 cm^3/min. This value is closer to the desorption tube flow rate, but still lower by a factor of 2. Without modification, direct connection to a capillary column would require excessively long desorption times to pass even a minimum amount of carrier gas through the tube: about 2–4 min for a wide-bore column and 6–12 min for a narrow-bore column.

A simple solution to this problem is to increase the column flow rate during desorption. This remedy is feasible for wide-bore columns, where the increase is limited to two or three times the optimum flow, and in many cases a wide-bore column can deliver good results with direct sample transfer. For narrow-bore columns, however, the needed increase in flow rate of greater than six times will affect analyte trapping efficiency and focusing at the head of the column, and may require more column inlet pressure than is available.

In-line stream splitting as shown in Figure 2a provides a better solution for nontrace samples with capillary columns. Flow from the desorption tube splits between the capillary column and the vent. Minimum desorption times remain in the 30-s range, and optimum flow persists in the capillary column. However, stream splitting alone may not suffice with trace-level samples for which a high split ratio would reduce analyte amounts below a minimum quantifiable limit. Alternative approaches such as secondary adsorbent trapping can be advantageous, as discussed below.

Peak Bandwidth

For peaks that are significantly retained at the initial column temperature, the time it takes for them to be desorbed from a sample tube and pass onto the

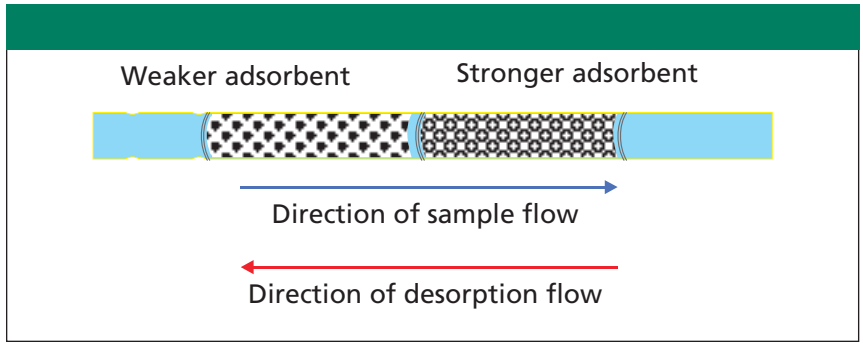

Figure 3: Dual-adsorbent sample tube.

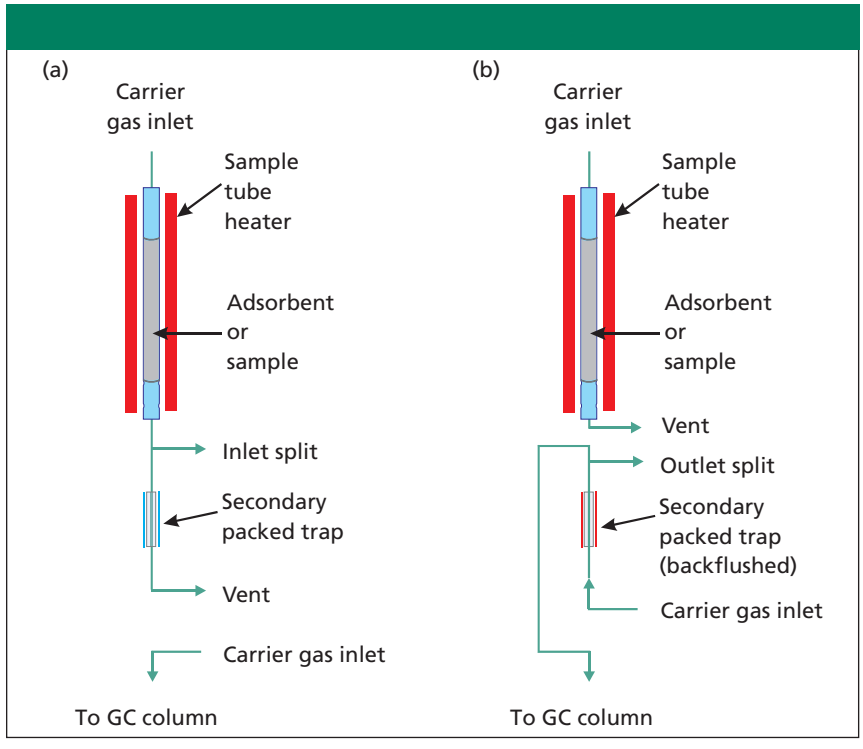

Figure 4: Thermal desorption with secondary trap: Desorption of (a) the primary sample tube and (b) the secondary trap.

column is not of too much concern. Such peaks will be focused at the head of the column by trapping in the stationary phase if their retention factors (k) are greater than about 10 at the initial temperature. Analytes that the column does not retain significantly at the initial temperature, however, hit the column and keep going. The column will not deliver the highest resolution possible for these peaks. Since the minimum desorption time is on the order of 30 s, thermal desorption sampling almost always requires some form of band focusing. Several solutions can help establish narrower initial peak widths for these early eluted analytes.

A cryogenic cooling device at the beginning of the column can effectively trap the more volatile analytes in a sample, along with those less volatile ones that would be trapped anyway, without such a device. Figure 2b shows a cryofocusing trap in a thermal desorption system, positioned just inside the column oven, after a stream splitter. Liquid nitrogen or carbon dioxide cools the cryofocusing zone to below 0 °C during sample tube desorption, and analytes from the sample tube, including the more volatile ones, are trapped there. After desorption is completed, cryogenic cooling is turned off and the cooled area is heated quickly to release the analytes trapped there.

A potential drawback of on-column cryofocusing is the accumulation of water at the head of the GC column when moist materials—for example, foodstuffs—are sampled by thermal extraction. Ice crystals can block the column, and ice on the column surface can affect trapping efficiency. Large amounts of water that are eluted from the column can cause detector problems, too.

Breakthrough Volume

An important characteristic of thermal desorption sampling is the GC column–like behavior of sample tubes packed with active adsorbents. As sample gas bearing analytes passes through an adsorbent bed, the analytes migrate along the length of the tube, just as they would in a GC column, and it is possible for them to traverse the length of the tube and emerge from its exit. The gas volume required for an analyte to do so is termed the *breakthrough volume*. It is expressed in units of gas volume per gram of adsorbent at a specific temperature, and is equivalent to the specific retention volume, V_g^0, on a packed column if the analyte were to be injected onto the adsorbent trap in a narrow plug. Breakthrough volumes help characterize adsorbents by determining not only the maximum gas volume when accumulating a sample, but also by indicating appropriate desorption temperatures.

If the breakthrough volume is too low at the sampling temperature, which is usually ambient temperature, then the trap will not adsorb the analyte quantitatively from a large sample gas volume. For a safety margin, the gas sample amount should not exceed half the breakthrough volume. If sample splitting is used, then the minimum quantifiable concentration increases accordingly.

After the sample compounds are adsorbed onto a sample tube, all of them must desorb at temperatures less than the maximum limit for the adsorbent. When an analyst is selecting a desorption temperature under the maximum, the highest-boiling or most strongly adsorbed compound determines the desired desorption temperature. The breakthrough volume decreases logarithmically with increasing temperature.

It is imperative to consider all the compounds of interest in a sample in terms of their breakthrough volumes. If lighter, less strongly adsorbed materials

are present, and sample collection cannot occur at subambient temperatures, then a second, stronger adsorbent material can augment the sample trapping scheme and extend the overall volatility range of thermal desorption sampling. Figure 3 illustrates a dual-adsorbent sampling tube. Sample gas passes through the weaker adsorbent first, and the higher-boiling, more strongly adsorbed components are trapped there. The lower-boiling, weakly adsorbed components break through the weak adsorbent into the stronger adsorbent where they are trapped. Care must be taken not to allow the higher-boiling components to break through the weak adsorbent; they will be permanently trapped on the strong adsorbent.

During thermal desorption the flow through a two-adsorbent trap must reverse and backflush the trap. If thermal desorption were to take place in the forward flow direction, then high-boiling materials from the weaker adsorbent would irreversibly encounter the strong adsorbent, just as if they had broken through to it during sampling. During backflush desorption, material from the strong adsorbent passes through the weak adsorbent without effect on its way out of the sample tube.

Multiple adsorbents extend the range of thermal desorption sampling to include compounds that quantitatively adsorb and desorb from either of two adsorbents in the same sample tube. The two adsorbents should be complementary in the range of suitable compounds. The maximum desorption temperature for a multiple-adsorbent sample tube is the lowest of the maximum temperatures for all included adsorbents. Good sources of information about adsorbents and trapping of specific compounds are available from the manufacturers of thermal desorption hardware and adsorbent tubes.

Secondary Trapping

With single or multiple adsorbents, forward- or back-flushed, the desorption time, volume, and flow rate from a conventional sample tube are not well matched to capillary column requirements. If the sample tube were much smaller, its desorption and flow characteristics would be a better match because it could be heated more rapidly, but its breakthrough volumes and sampling characteristics would not be suitable for collection of larger sample volumes. Nor would such a tube be well suited to direct thermal extraction. However, a small additional adsorbent trap positioned in-line between the sample tube and the column can serve both as a focusing device for desorbed analytes as well as a means to better balance the flow rates of the column and the sample tube. Figure 4 shows a small secondary adsorbent trap positioned between the sample tube and the column. Additional valving (not shown) creates the flow paths shown in Figures 4a and 4b. In Figure 4a, carrier gas flows directly into the column and isolates the two traps from the column. During sample desorption or thermal extraction, the sample tube heats up and desorption gas flow conducts released analytes past the first split point into the secondary adsorption trap, which is cooled to ambient temperature or below.

After desorption is completed, flow switches to the arrangement shown in Figure 4b. At that point, desorption

flow continues to purge the sample tube, while carrier gas flows through the secondary trap in the backflushed direction. The secondary trap is heated rapidly to desorb the analytes quickly. They pass through an optional second split point and then into the column.

The secondary trap contains only a small amount of an adsorbent, 10–20 mg, which can be the same single or multiple adsorbent material as used in the main sample tube. Adsorption breakthrough volumes on the secondary trap are concomitantly lower, but they are greater than the desorption volumes from the primary sample tube. Operation of the secondary trap at subambient temperatures also helps to increase the breakthrough volumes there.

The smaller inner diameter and shorter length of the secondary trap bring its desorption time, flow, and volume down to capillary column levels. A typical heating rate for the packing inside the secondary trap is 40 °C/s, so that it can reach a 200 °C desorption temperature in around 5 s. At elevated temperatures, the minimum desorption volume in such a trap is 1–2 mL, which is about 10 times less than for a primary sample tube. This lower volume opens the possibility for splitless transfer into a capillary column with trace-level samples, although some form of on-column focusing is still required at narrow-bore capillary column flow rates.

A secondary trap also helps with direct thermal extraction. With suitable hydrophobic adsorbents and trap temperatures, water from moist samples will not be adsorbed on the secondary trap, but will pass through the trap to vent. Water that does condense or freeze in the secondary trap is better tolerated there than at the head of the GC column; ice crystals or water droplets will not block gas flow. Subsequent stream splitting during secondary trap desorption reduces the amount of water actually entering the column.

The secondary trapping scheme is not without its drawbacks. It is considerably more complex than other systems and requires a more in-depth knowledge of thermal desorption principles for effective use. The mechanical implementation includes extra valves and tubing, all of which must be inert toward the sample materials and free of cold spots. But the additional complexity may be justified by the ability to handle difficult samples and the flexibility that comes along with secondary adsorbent trapping.

Conclusion

GC sampling techniques for volatiles are highly developed. Analysts can call upon a wide variety of methods for sampling volatiles from difficult matrices, including headspace, thermal desorption, solid-phase microextraction, and purge-and-trap sampling. Thermal desorption techniques present good methods for the concentration of gaseous samples from a variety of sources, including the headspace over solids and liquids, atmospheric samples, and process effluents. Direct thermal extraction of otherwise intractable samples is also possible with thermal desorption equipment.

Researchers have developed a variety of thermal desorption techniques. The need to accommodate capillary columns for large gas samples that span a wide volatility range has spurred development of ancillary methods for thermal desorption samplers, including on-column cryotrapping, sample stream splitting, multiple-adsorbent sample tubes, and secondary adsorbent traps. To be successful with thermal desorption sampling, chromatographers must pay careful attention to the relationships between the adsorption–desorption characteristics of trapping materials, the range of sample components and sample matrices to be analyzed, and the kind of equipment that will perform the sampling and analysis.

John V. Hinshaw

"GC Connections" editor John V. Hinshaw is a Senior Scientist at Serveron Corporation in Beaverton, Oregon, and a member of LCGC's editorial advisory board. Direct correspondence about this column to the author via e-mail: lcgcedit@lcgcmag.com

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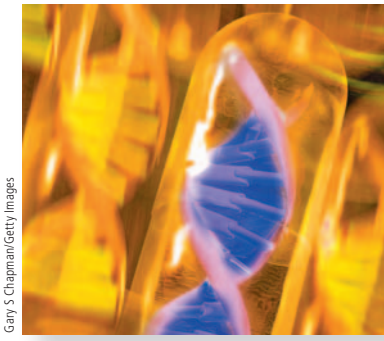

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BIOTECHNOLOGY TODAY

Glycosylation in mAb Therapeutic Products: Analytical Characterization and Impact of Process

Glycosylation of monoclonal antibody (mAb) therapeutics is widely recognized by the regulators and the industry as a critical quality attribute (CQA). Hence, it is necessary that glycosylation is measured and adequately controlled during production. This installment reviews the various process parameters and raw material attributes that affect glycosylation, as well as the different analytical tools that are used for characterization, with greater emphasis on the chromatographic methods of analysis. Key recent advancements that have occurred in the past five years are also discussed briefly. While significant progress has been made in the monitoring of glycosylation, its real time control has yet to be demonstrated.

Gunjan Narula, Vishwanath Hebbi, and Sumit Kumar Singh *are the guest coauthors of this installment.* ***Anurag S. Rathore*** *is a coauthor and an editor of Biotechnology Today.* ***Ira S. Krull*** *is an editor of Biotechnology Today.*

Biotherapeutics or biologics have emerged as potent treatments of a variety of diseases, including cancer and other immunological disorders. Since these products are produced in biological systems, they often exhibit a high degree of variability (physical and chemical modifications). These modifications are known to potentially impact the characteristics and biological activity of the therapeutic drug. Additionally, attributes such as drug clearance and half-life are also affected by these modifications. Regulatory authorities mandate manufacturers to make efforts to minimize these heterogeneities during process and formulation development, so as to ensure that the safety of patients is not jeopardized and that the drug efficacy stays intact. To this effect, the concept of quality by design (QbD) encourages designing quality into the product (1,2). Under this approach, any physical and chemical changes in the drug product that have the potential to affect the safety and efficacy are considered critical quality attributes (CQAs). And, manufacturing is accordingly designed so that these CQAs are consistently monitored and controlled (3–5).

Among the different CQAs (generally only a few for microbial products and more for mammalian products), a glycosylation profile of a product is well-recognized as a CQA that requires stringent monitoring and control. Glycosylation refers to a sequence of events that take place in the Golgi body and the endoplasmic reticulum of the cells of the expression system (predominantly mammalian) that lead to post-translational addition and processing of carbohydrate or glycan moieties to the protein backbone (usually serine- or threonine-linked glycosylation for O-linked glycans and asparagine-linked glycosylation for N-linked glycans) (6). The addition of a precursor 13-residue oligosaccharide moiety GlcNAc2Man8Gluc3 to the nascent polypeptide chain occurs in the endoplasmic reticulum, following which the assembly folds and acquires its secondary, tertiary, and quaternary structure. The folded structure gets transported via vesicles to the Golgi bodies (7). A cooperative and competitive interaction between multiple enzymes, cell lines, and cell culture conditions causes processing of the oligosaccharide structure during which some of the already attached moieties like mannose are trimmed to yield a five-residue core (Man3GlcNAc2), to which N-acetylglucosamine (GlcNAc), fucose, galactose, and N-acetylneuraminic acid (sialic acid or Neu5Ac) residues get added in a probabilistic fashion.

These moieties are critical in terms of their role in in-vivo safety and efficacy (pharmacodynamics and pharmacokinetics), protein folding, protein targeting and trafficking, ligand binding, stability, protein half-life regulation, and immunogenicity (8). For instance, researchers have shown that the fucosylation of monoclonal antibodies (mAbs) causes reduced antibody-dependent cell mediated cytotoxicity (ADCC). Similarly, some of the therapeutic mAbs with non-galactosylated

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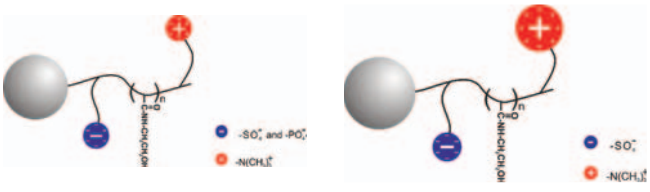

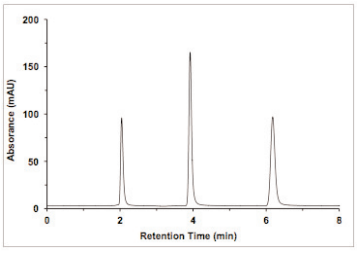

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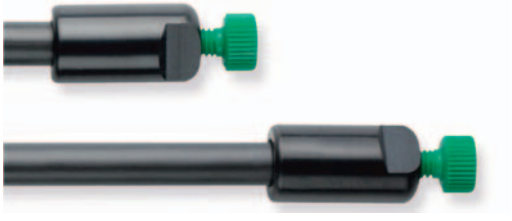

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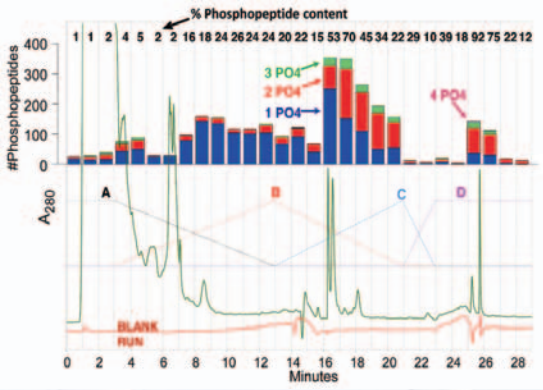
Detergent and Salt Removal From Polar Compounds

SEC, RPC, or HILIC - Eliminate Sample Losses

ERLIC-WAX ISOLATION MULTI PHOSPHORYLATED PEPTIDES



Sample: Tryptic Digest
Column: ERLIC-WAX microSPE p/n: SEM HIL-DE
Load in 85% ACN, 0.1% FA. Elute 10% ACN pH 2, 100mM Na-methylphosphonate
Desalt on MACROspin TARGA® C18 p/n: SMM SS18R
Analytical column: PolyWAX LP™ 4.6 x 100mm, 5µm, 300Å, p/n: P104WX0503

DISPLACEMENT CHROMATOGRAPHY VS. SELECTIVE ELUTION

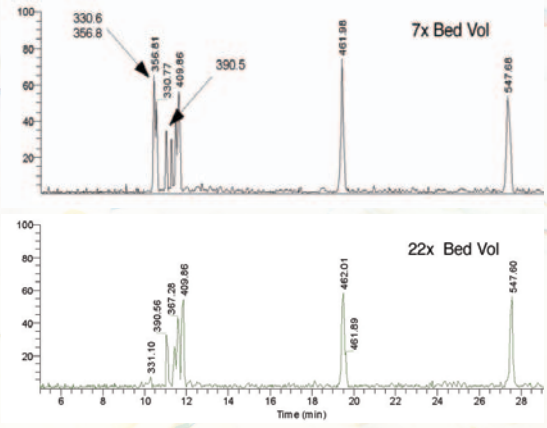
MASS RELATIVE TO COLUMN SIZE
Excess capacity; everything retained. Separation through selective elution.

MASS OVERLOADED SPE CARTRIDGE
Capacity limited; the most strongly-retained solutes displace those less strongly-retained. Results in a separation with selective retention, displacement chromatography.

EXAMPLE: ERLIC-CAT chromatography

VOLUME EFFECTS ON SPE TIPS

Desorption Increases From Excessive Sample & Wash Volumes



Column: TARGA® C18, 300µm x 30mm Trap
Sample Solvent: 0.1% FA water (volumes as shown). Trap Column in 0.1% FA water
Gradient: 98% water, 0.1% FA, 2% ACN to ACN, 0.1% FA

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MacroSpin™ Column	30-300µg	180µL	90µL	25-180µL
96-Well Spin Plate	10-100µg	75µL	35µL	7-75µL
96-Well MACROspin Plate	25-250µg	200µL	100µL	25-200µL
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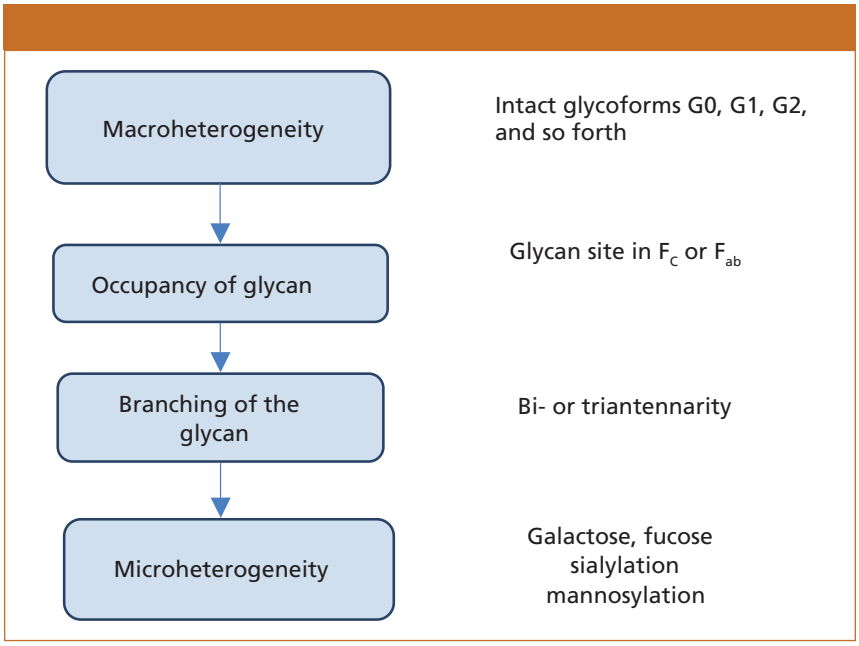

Figure 1: Framework for assessing changes in the glycan profiles for mAb therapeutics.

glycan profiles have shown less affinity toward the c1q component of the complement cascade, thus reducing the complement dependent cytotoxicity (CDC). A good number of mAb therapeutics that are used for treating autoimmune disorders have exhibited a strong and positive correlation between anti-inflammatory effects and the presence of the terminal sialic acid residues on the oligosaccharide structure attached to the protein polypeptide backbone (9). Thus, depending on the mechanism of action (MoA), CQA is defined for the therapeutic—that is, if ADCC is a desired therapeutic effect, fucosylation is selected as the CQA. Similarly, for CDC and immunomodulation, galactosylation and sialylation, respectively, are the CQAs (10).

Given the criticality of their role in exertion of a therapeutic protein's pharmacological effect, it is necessary to characterize detailed glycan structures, as well as those specific glycan subsets that are known or are likely to cause adverse events in the body. Notwithstanding the significant advancements in analytical characterization of these products, glycosylation analysis continues to be nontrivial and demands considerable resources in terms of analyst expertise as well as high-end characterization tools. In this installment, we review the various analytical tools that are used for characterization of mAb glycosylation, with a greater emphasis on the chromatographic methods of analysis. Key recent advancements that have occurred in the past five years are also discussed briefly.

Regulatory Perspective

Detailed workflow and regulatory expectations have already been laid out in the various regulatory guidance documents governing production, commercialization, and licensure of biologic drugs (11–14). The International Conference on Harmonization (ICH) guidelines mandate characterization of total carbohydrate contents (including neutral contents, amino sugars, and sialic acid), antennary profile, glycosylation sites interglycosidic linkages, and anomeric configuration (11). European Medical Agency (EMA) guidelines have an additional requirement of presenting data with regards to the presence or absence of glycosylation in light chain, degree of mannosylation, galactosylation, fucosylation, and sialylation (12). In the event that a product is found to contain any glycosylated variant that differs from the reference product, it is essential to establish that such differences are noncritical with respect to the safety and efficacy of the therapeutic using more comprehensive preclinical and clinical data. Figure 1 illustrates the framework for assessing the change in the glycan profiles of the biotherapeutic in question.

The use of orthogonal, high resolution, and innovative analytical tools that allow us to decipher product heterogeneity, as well as consistency at batch-to-batch levels is mandatory. Computational biology (bioinformatics) has further emerged as a source of information for assessing immunogenicity potential of a biotherapeutic (15). These activities allow for a rigorous and comprehensive review of the product development data by the regulatory bodies, based on scientific merit, and renders decisions that are data driven.

Process Parameters that Affect Glycosylation

Glycosylation is known to be affected by numerous process parameters, as well as genetic elements (16,17). However, in this column installment we focus our discussion only on important process parameters that either impact yield or the overall quality of the product (18–38).

A summary of important process variables affecting the glycosylation is presented in Table I. Based on literature review, it is evident that out of the various factors that affect glycosylation, the concentration of manganese, temperature, and concentration of ammonium chloride are recognized to have a major impact on glycosylation. Manganese supplementation under low glucose feed have resulted in higher levels of galactosylated and mannosylated glycoforms on mAbs expressed in Chinese hamster ovary (CHO) cells. Mn^{2+} is known to act as a cofactor to β-1,4-galactosyltransferase, which selectively up-regulates galactosylation of protein therapeutics. However, when glucose feed is substituted with other sugars such as galactose and fructose, specifically higher mannosylated species (M5 mannose glycans) have been observed, because of limited availability of nucleotide sugars. Fucosylated glycoforms remain consistent regardless of the manganese concentration in the culture media (23–26).

With regard to the effect of temperature, it is generally observed that

a downshift in temperature helps in lowering the content of acidic charge variant content in the mAb therapeutics (27). A recent study showed that good correlation exists between charge and glycosylation profiles of mAbs (28). However, an elaborate description of the mechanism of how such an affect manifests is not well-understood. Transcriptomic studies, to a large extent, provide certain insights into such an observation. It is shown that the downshift of temperature affects the gene expressions of enzyme systems (glycosyl transferases, mannosidase, and galactosyltransferase), which affects their accessibility to cause the necessary terminal site modification in mAbs (such as N-terminal pyroglutamate formation and C–terminal lysine clipping) and thus explains the varying glycosylation profiles observed among these variants (29,30,39). Apart from this, a negative correlation has been found to exist between terminal glycoforms (sialic acid) and DO levels in the culture (31,32).

Ammonium chloride is also recognized as a key component that is shown to play a critical role in the addition of terminal sugars (sialylation) during the glycosylation process (35–37). An ammonia concentration typically below 2 mM increases the intracellular pH, which in turn leads to an inhibition of galactosyl transferase and sialyl transferase, enzymes that mediate addition of the terminal sugars (38,40).

While pH has long been believed to be a critical process parameter during cell culture, the optimum pH range for cell culture is believed to be 6.9–8.2, with any perturbance beyond this range affecting glycosylation. Lower pH has been known to result in more galactosylation and hence production of a higher level of the corresponding species (33,34).

Various sugars, such as glucose, mannose, and galactose, have been known to affect mannosylation, fucosylation, and sialylation. It has been reported that addition of galactose to the culture medium improves sialylation. This improvement results from an increased availability of the binding sites (galactose moieties) for sialic residues (20). The ratio of mannose to glucose has also been known to affect mannosylation. A higher ratio (0.94) has been shown to result in increased levels of mannosylated species (18).

Characterization of mAb Glycosylation

Characterization of glycoproteins and released glycans involves the use of a large number of high resolution, orthogonal analytical tools, that together provide a complete glycan fingerprint at three levels. The first level identifies possible glycosylation sites (macro heterogeneity) present on the mAb (Cγ2 domains of Fc fragment, V_L domain and V_H domain or both). Reduction and acidification, papain digestion, or chemical digestion are usually performed to determine the oligosaccharide structures present on each peptide fragment (41,42).

The second level of information is with regards to the variation in the sequence of the sugars (microheterogeneity). This information is obtained either by cleaving the protein backbone and analyzing the glycopeptides or by enzymatically releasing the carbohydrate moieties from the protein

backbone (7). The first approach is accomplished by use of proteolytic enzymes like trypsin, lysyl endopeptidase (Lys-C), endoproteinase Glu-C, and pronase. In the second approach, enzymes such as PNGase F (releases all N-linked oligosaccharides), PNGase A (releases glycan with α1-3 core-fucosylation), and endoglycosidase H (releases high mannose like N-glycans) are used (10). Apart from these, two O-glycanases are also commercially available for cleaving the Galβ1-3GalNAc linked to serine or threonine residues, but the presence of sialic acid, fucose, GlcNAc, and GalNAc residues prevent this cleavage. The use of an enzymatic method is limited because of the variable structure of the O-glycans and the narrow substrate specificity of the available enzymes (43).

Both O- and N-glycans can be released by chemical hydrazinolysis. However, the drawback associated with this method is the degradation of reducing end monosaccharides (β-elimination) and destruction of noncarbohydrate substituents. Moreover, alditols obtained by β-elimination also prevent subsequent labeling of the oligosaccharide that hinders detection of glycans during separation and fractionation (44). The released glycans are recovered from the pool of peptides, salts, and other compounds by various methods based on the differences in the polarity and functionality of peptides and glycans. These methods include ethanol precipitation, zwitterionic hydrophilic interaction liquid chromatography (HILIC), solid-phase extraction (SPE) using graphitized carbon black (GCB), titanium, or anion-exchange chromatography using borate resin. This is followed by a step involving specific entrapment of the separated glycans. Furthermore, the separated glycans are then derivatized using one of several possible approaches, with the most popular being reductive amination with several types of aromatic amines. In this approach, the reducing-end of the oligosaccharides is labeled with either a fluorescence tag or a UV-absorbing tag for further detection and quantification. The labeling agent used generally depends on the reaction condition (aqueous or nonaqueous) and the analytical method used for further separation. Possible agents include 2-aminopyridine (PA), 2-aminobenzamide (2-AB), 2-aminobenzoic acid (2-AA), 8-aminopyrene-1,3,6-trisulfonate (APTS), and 1,2-diamino-4,5-methylene-dioxybenzene-dihydrochloride (DMB) (45). An alternative approach is permethylation of glycans to prevent loss of sialic acid during mass spectrometry (MS)-based analysis (45).

Reductively aminated derivatives are generally chemically stable and most of them, except for 2-aminopyridine and 8-aminonaphthalene-1,3,6-trisulfonate (ANTS), can be converted back to free oligosaccharides by treatment with hydrogen peroxide or acetic acid (46). Finally, the released, derivatized or un-derivatized glycans can be separated using a chromatographic technique (discussed in subsequent sections), such that the resolution and retention depend on the affinity of the glycan for the matrix used for separation followed by fluorescence or MS detection. It should be noted that analysis of native, unlabeled, and reduced

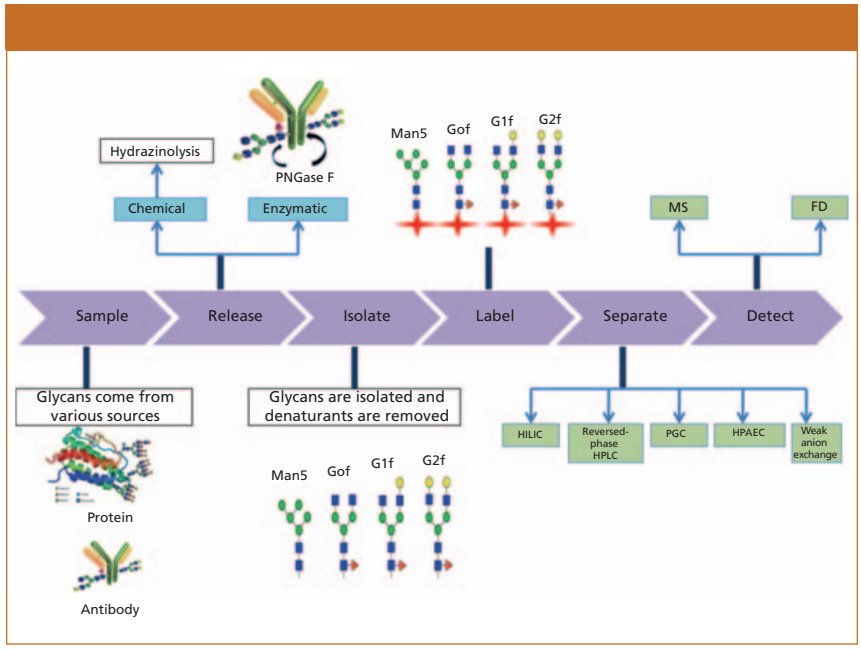

Figure 2: Workflow of glycan profiling. Glycans from various sources, such as proteins or mAbs are first released by chemical or enzymatic methods. This is then followed by isolation of the released glycans, and sample clean-up for removal of salts or denaturants. Labeling with appropriate reagents is performed so as to improve detectability. Various chromatographic tools, as shown in the figure, are used for separation of the glycans. Detection is generally done using a fluorescence detector or high-resolution MS.

(un-derivatized) glycans can also be performed but this analysis is restricted because of the limited resolution that the existing separation tools offer.

The third level of glycan structural analysis involves unraveling information regarding the intermolecular structures of oligosaccharides by characterization of the linkages between the constituting monosaccharides of glycans. A sequential enzyme-catalyzed reaction is performed on the basis of the bonds that occur between different monosaccharides, and the products are quantified using various analytical techniques to analyze the bond structure of the original glycan (7). The various chromatographic techniques used for the separation of released, purified, and labeled or unlabeled glycans are described below, and a workflow for glycan identification and characterization is illustrated in Figure 2.

Separation-Based Analysis of mAb Glycans

Like any other separation, oligosaccharide separation is principally done based on the properties of sample components such as size, polarity, and ionic strength. Predominantly, there are four separation-based techniques that are used for the characterization of mAb glycoproteins: reversed-phase high performance liquid chromatography (HPLC), HILIC, anion-exchange chromatography, and high performance anion-exchange chromatography.

Reversed-Phase HPLC

Reversed-phase HPLC refers to a mode of separation using nonpolar stationary phases and polar mobile phases. The polar nature of oligosaccharides causes minimal retention on reversed-phase columns resulting in their simultaneous elution and thus hampering the separation. To overcome this, glycans are derivatized into more hydrophobic forms by introducing a chromophore through the hydroxyl group (generally PA, 2-AB, or 2-AA) or by permethylation (7,47). However, labeling oligosaccharides with bulky hydrophobic tags has not been particularly successful, because it impairs separation based on structural differences among sugar moieties and causes the elution of oligosaccharides in the order of decreasing size. However, PA is still regarded as one of the most efficient labeling reagents because of its low hydrophobicity and minimal effect on retention time (48). Derivatization by permethylation has also emerged as a viable option for reversed-phase HPLC separations of sialylated glycans because of the increased stability, enhanced ionization efficiency, and predictable fragmentation when coupled to a mass spectrometer (49). However, a rigorous optimization of this step is necessary because it is prone to form various reaction by-products and impurities that may hinder the separation process (50).

The biggest advantage of reversed-phase HPLC is its compatibility for direct hyphenation to MS. This is because of the fact that the buffers that are generally used in reversed-phase separations allow for simultaneous separation during chromatography and ionization of the analyte during the MS. A major limitation of glycan separation using reversed-phase HPLC, however, is its limited ability to resolve glycan structural isomers. An alternative method is that of using porous graphitic carbon (PGC) as the stationary phase for chromatographic separation of labelled glycans,oligosaccharide alditols, and even permethylated glycans (44,51,52). This is a method of choice for direct liquid chromatography–mass spectrometry (LC–MS) of O-glycans released by β-elimination and is efficient in separating structural isomers (53). Further, for better separation and structural assignment, reversed-phase HPLC is often used together with HILIC and anion-exchange chromatography as a multidimensional strategy (44,51). Hydrophobic interaction chromatography (HIC) has also been used for the separation of glycoproteins on the basis of glycoforms (54).

HILIC

HILIC, in contrast to reversed-phase HPLC, uses a relatively polar mobile phase and a polar stationary phase (for example, silica derivatized with amide–amine groups). The polar nature of oligosaccharides enables them to interact with the polar stationary phase based on dipole–dipole interaction, electrostatic interactions, and hydrogen bonding. Therefore, HILIC separates sugar moieties as a function of their capacity to form hydrogen bonds with an amine–amide bonded-silica phase (7,43). Hydrophobic peptides are

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Table I: Summary of the process parameters that affect glycosylation according to current scientific literature

Process Parameter	Effect on Glycosylation	Operating Ranges Studied	Cell Line	References
Mannose to glucose ratio (carbon source)	High ratio (0.94) increases mannosylation	0.5–0.94	CHO	18
Glucose	GlcNAc occupancy and Man5 glycosylation behave differently in the two cell lines	5–8 g/L	CHO-1030 CH0-4384	19
Galactose	2-galactose glycans increases enhancing the monosialyation by 20.3% and increases FA2G1 and decreases FA2 and A2	0–40 mM 0–100 mM	CHO CHO-K1	20 21
Osmolality	Increases mannosylation	300–400 Osm/Kg	CHO	22
Manganese chloride	Elevates mannosylation in absence of glucose resulting in higher galactosylation, increases M6, M7, and M8 thereby increasing galactosylation and sialylation, improves galactosylation along with uridine and galactose resulting in increase in M5 and decrease in FA2	4 and 16 µM 0.4–40 µM 0–40 µM 1–40 µM	CHO CHO CHO CHO K1SV CHO-K1	23 24 25 26 21
Temperature	Decrease in temperature decreases the acid variants and fucosylation as well as increases galactose occupancy and lowers glycan processing with reduced G1F and G2F	33–37 °C, 30–39 °C 35.5–37 °C 32-36.5 °C	CHO Murine CHO	27,28 29 30
Dissolved oxygen	Increase in DO level from 0–100% increases galactosylation. At 100% DO, sialylation decreases in EPO. Oscillation in DO increases triantennary and sialylation levels.	0–100% 10–100% —	Murine CHO Murine	31 28 32
pH	Heterogeneity in glycoforms increases at pH outside 6.9–8.2	6.1–8.7		33,34
Ammonium chloride	Concentration >2.5 mM increased heterogeneity in EPO. Higher NH_4^+ concentration decreases sialylation in both N and O glycosylation and galactosylation and sialylation. Concentration >30 mM reduces sialyation in EPO.	2.5–40 mM 10 mM 0–15 mM 0–40 mM	CHO CHO CHO	35 36 37 38
Glutamate	Improves galactosylation in comparison with glutamine	6 mM	CHO	39
Glucosamine	Concentration >5 mM results in reduced sialylation and antennary in EPO	0–40 mM	CHO	38

GlcNAc = N-acetylglucosamine; Man = mannose, F = fucose, A = antennary, G = galactose, DO = dissolved oxygen, EPO = erythropoietin, CHO = Chinese hamster ovary

washed off with the organic mobile phase, while polar glycopeptides are retained on the HILIC phase, and these are eluted by increasing the water content of the mobile phase, thus separating glycosylated and nonglycosylated peptides (55).

Unlike reversed-phase HPLC, the purpose of labeling in HILIC is primarily for enhancing the sensitivity of detection. Because of the correlation between the glycan size (based on the addition of monosaccharide units to oligosaccharides) and its retention time, it is sometimes referred to as *size-based separation* (55). Oligosaccharides labeled with PA, 2-AB, 2-AA, and acridine derivatives are easily compatible with HILIC conditions, though with different retention times for derivatized and unmodified sugar structures (47). Some glycan structural isomers (G1Fa and G1Fb, differing in position of galactose residue) have been successfully resolved using HILIC (56). The structural variation of the individual glycans and changes in retention times can possibly be predicted by comparing the elution time of eluted glycans with a labeled glucose ladder (partial dextran hydrolysate or a mixture of oligosaccharides) (51).

HILIC is compatible with both fluorescence and MS detection methods (electrospray ionization [ESI] or matrix assisted laser desorption–ionization [MALDI]-time of flight [TOF]-TOF-MS-MS), where when combined with fluorescence detection of 2-AB labeled glycans, it is considered to be a gold standard for analyzing N-glycans of biotherapeutics (56). When coupled to MS, no tagging of glycans is needed, thereby yielding a much simpler procedure (47). For in-depth analysis of complex oligosaccharide mixtures, a multidimensional HPLC approach can be applied by combining HILIC with other chromatographic methods, such as anion exchange or weak anion exchange, that give comprehensive separation of complex N-glycans (57).

Anion Exchange and High-Performance Anion Exchange

Because carbohydrates are weak acids (pK_a > 11), they can be retained on a strong anion exchange column (pH > 13), and hence can be separated using high performance (high pH) anion-exchange chromatography. With this tool, glycan moieties are differentially adsorbed on a positively charged matrix, influenced by the number of acidic groups, molecular size, sugar composition, and monosaccharide linkage (43). Elution is often done by a salt gradient—that is, using concentration gradients of a sodium hydroxide–sodium acetate system. Because of the use of a high concentration of salt, the application of this system to MS is restricted (51). Detection is generally accomplished by pulsed amperometric detection (PAD), which is based on measuring

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the current generated via oxidation or reduction reactions on the electrode surface. The detection is highly sensitive, and concentrations of oligosaccharides can be measured by comparing its response with a calibration curve of reference compounds. However, only relative quantitation is possible because of the lack of reference compounds for generating the calibration curve (7).

Anion-exchange or weak anion-exchange HPLC can also separate some labeled (2-AB or PA) glycans, and these are generally performed from neutral to slightly alkaline pH. Separation is based on the negative charge of glycans, which in turn is determined by the number of sialic acids, carboxylic acids, glucoronic acids, sulfates, and phosphates (51). Elution is achieved via a salt gradient followed by fluorescence-based detection. Complex glycans on erythropoietin are generally characterized using this technique (58). Weak anion-exchange HPLC and high performance anion-exchange chromatography can also be combined to achieve a multidimensional separation (57).

Monitoring and Control of Glycosylation

As is evident from the aforementioned considerations, monitoring and control of glycosylation throughout the life cycle of a product, from cell line development to final product manufacturing, is of paramount importance (59,60). During cell line screening and early upstream process development, glycosylation analysis is required to select the best probable clone, as well as optimized culture conditions, from which a properly glycosylated product is obtained. Although there are very few reports on real time analysis of glycosylation (24,26,61), efforts are ongoing toward devising means or methods for achieving this. In view of the factors affecting glycosylation, monitoring can be performed either at the gene level, using commercially available gene chips, or at the protein level using the various techniques that have been described in detail in the previous sections. A major hurdle is the significant time that is spent on sample preparation. Over time, researchers have succeeded in shortening the analysis time and making the analysis automatic, using robotic sampling systems that offer high throughput glycan analysis and brings us closer to achieving real time monitoring and control of glycosylation (62–65).

A comparison of transcriptome and proteome of expressed genes (using gene chips, cDNA microarrays, real-time polymerase chain reaction [PCR] and two-dimensional [2D] gel electrophoresis–MS approaches), has led to the conclusion that the metabolic shift of the cells during culture is the combinatorial effect of biochemical reactions, as well as transcriptional events (61). Further, it has been observed that galactosylation of mAb N-glycans in CHO cell lines can be predicted accurately enough, and it can be controlled by varying supplements (such as uridine and manganese chloride) and cell culture conditions, using statistical modeling and measurement of cell surface glycans, so as to predict product glycosylation (26). These types of integrated, knowledge-based approaches are necessary and enable deeper understanding, monitoring, and control of glycosylation events.

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Although most researchers have taken into consideration the mesoscale (within the cytoplasm) and macroscale (within the reactor) variables to study their effect on glycosylation patterns, it is of prime importance to look at intracellular variables that affect glycosylation. As far as modeling is considered, a detailed understanding of all the micro-, meso-, and macroscale variables is a necessary prerequisite. An assessment of controllability of glycosylation, by modeling the effect of various intracellular variables such as enzymes, nucleotides, and sugar donors on the final pattern of glycosylation, has recently been published (66). The use of manganese chloride, ammonium chloride, and galactose as mesoscale input variables has shown that glycosylation control is possible during cell culture. Multivariate data analysis with parallel factor analysis (PARAFAC), principal component analysis (PCA), multiway PCA, partial least squares (PLS), and multiway PLS has been successfully used to optimize cell culture conditions (67,68). These models assist not only in cell line development but also enhance the speed and accuracy, which enables optimization of the media and cell culture conditions in a reproducible and consistent manner.

As already discussed above, understanding the dynamics of glycosylation requires real time glycosylation analysis. To this end, a new platform method has been described recently for real-time glycan analysis that comprises a sampling probe and an ultrahigh-pressure liquid chromatography system (μSI-Glycan map-UPLC, Waters Corporation) that allows automated sampling and sample preparation for glycan analysis in real time (24). Data can be collected every 12 h versus 1 day in the case of off-line analyzers for intact glycans, with varying degrees of mannosylation, fucosylation, and galactosylation.

Conclusions

Glycosylation is an important CQA, and hence it is necessary to monitor and control it throughout the product life cycle. Heterogeneity in glycosylation can be minimized by carefully controlling the various critical parameters like ammonium chloride, manganese, temperature, pH, and various sugars during cell culture. Orthogonal, analytical techniques must be used to analyze the heterogeneity at various levels of glycosylation. Current research focuses mainly on automating the process of monitoring and control of glycosylation in cell culture. In the future, this research may lead to a holistic approach to control this CQA, which will greatly help us in achieving consistency in product quality. While significant progress has been made in monitoring of glycosylation, its real-time control has yet to be demonstrated.

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Gunjan Narula *is a project fellow in the Department of Chemical Engineering at the Indian Institute of Technology in New Delhi, India.*

Vishwanath Hebbi *is a graduate student in the Department of Chemical Engineering at the Indian Institute of Technology.*

Sumit Kumar Singh *is a graduate student in the Department of Chemical Engineering at the Indian Institute of Technology.*

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

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

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From Sample to Vial

Monitoring Drug Behavior Using High Performance Affinity Chromatography

There is a growing demand in the pharmaceutical industry for fast and selective separation methods to monitor drug behavior in small-volume biological samples. David S. Hage from the University of Nebraska–Lincoln has recently developed a series of methods using affinity chromatography and related techniques for this purpose. *LCGC* interviewed him about this work.

In your view, what are the main challenges faced by scientists working in the pharmaceutical industry right now?

Hage: A great deal of past research in the creation of new separation techniques for pharmaceutical analysis has sought to improve the speed, efficiency, and information content of these methods. These are still ongoing needs as scientists in this field seek to work with increasingly complex agents or materials, such as biopharmaceuticals or samples from the areas of proteomics, glycomics, and metabolomics. Other growing needs call for fast and selective separation methods that can be used directly with small amounts of biological samples and that require minimal pretreatment. This last set of needs is where affinity chromatography and related separation methods can be especially valuable.

One strand of your research focuses on the development of affinity chromatography for characterizing drug–protein interactions. Why is this an important area of research in pharmaceutical analysis?

Hage: Most routine types of pharmaceutical analysis, as conducted by methods like reversed-phase chromatography and liquid chromatography–mass spectrometry (LC–MS), are concerned with measuring the amount of a drug or drug metabolite in a sample, or the change in this amount over time. The use of affinity chromatography to characterize drug–protein binding builds on these efforts by also looking at how a drug or its metabolites can interact with the proteins or other components in a biological system. This information provides a more complete picture of how the drug is behaving in an organism and how its pharmaceutical activity, rather than just its total concentration, may change over time or with a disease state. This type of information on drug–protein interactions also has great potential in the area of personalized medicine in that it may eventually allow the treatment of patients with pharmaceutical agents to be adjusted on an individual basis.

What are the basic principles of affinity chromatography and high performance affinity chromatography (HPAC)? What are the main applications of the technique?

Hage: Affinity chromatography is a type of liquid chromatography that is based on the selective and reversible interactions of many biological systems. The stationary phase in this method is an immobilized biological agent, such as an antibody or transport protein, that can be used to bind and retain a complementary target from an applied sample. High performance affinity chromatography (HPAC) is a type of affinity chromatography in which the immobilized biological agent is used within a high performance liquid chromatography (HPLC) column or HPLC system.

Affinity chromatography has been used for many years as a powerful tool for the selective purification or isolation of biological compounds. In recent years, affinity chromatography and HPAC have also seen considerable growth as methods for the analysis of specific compounds in biological samples and as tools for studying biological interactions. These separation methods can be used alone or in combination with other techniques, including reversed-phase chromatography and MS. Affinity-based separations have now been reported for a broad group of targets that have ranged from drugs, hormones, and other small biological molecules up to antibodies, enzymes, receptors, viral particles, and cells. In addition, HPAC and affinity columns can be used in flow-based biosensors and as part of miniaturized analytical devices.

You have recently published work using HPAC to analyze the interactions between glimepiride and human serum albumin (1). What led you to begin this work?

Hage: Human serum albumin (HSA) is an important serum transport for glimepiride and many other drugs. Glimepiride is a sulfonylurea drug, which is a common class of drugs used to treat type II diabetes. It is known that HSA can be modified by glucose in a process called "glycation," and that the extent of this process increases during diabetes. We have been interested in seeing how the modification of HSA by glycation affects the interactions of this protein with sulfonylurea drugs, including glimepiride, and in how these changes might then alter the amount of these drugs that will bind to HSA in patients with diabetes.

Did you experience any challenges and, if so, how did you overcome them?

Hage: Early in this type of work we had to develop and validate methods for preparing HPAC columns that contained either normal HSA or glycated HSA and that could be used as models for the soluble forms of these proteins. We have since identified several ways of making columns that contain these proteins and that give good agreement with the drug binding behavior that is seen for soluble HSA. This development led to the possibility of using HPAC and these columns as a biomimetic system for modeling drug–protein interactions in blood.

Our work with glimepiride in this approach met with several additional challenges. One such challenge was the relatively low solubility of this drug in aqueous solutions. The low solubility of this drug has in the past limited the types of conditions that could be used in other methods to examine binding by this drug with HSA.

However, we were able to identify experimental conditions and methods in HPAC that allowed us to examine these interactions and at concentrations over which glimepiride was soluble in a physiological buffer. We also found that this drug has multiple binding sites on HSA and that some of these sites can take part in allosteric interactions with this drug. By using several site-specific probes for our studies, we were able to obtain binding constants or information on the interactions for glimepiride at each of these sites. The result was a detailed picture of glimepiride binding with HSA and in how these various interactions were affected by the glycation of this protein.

At HPLC 2015 in Geneva, you presented a keynote lecture showing the use of microcolumns in HPAC to characterize drug interactions. Why is reducing column size of interest in HPAC?

Hage: The agent that is used as a stationary phase in HPAC usually has both strong and specific binding for its target. This combination means that short columns, or microcolumns, can often be used in HPAC to obtain a selective and high-resolution separation of this target from other sample components in a relatively short period of time. Other advantages of using these microcolumns include their need for only small amounts of the binding agent, their low back pressures, their low nonspecific binding, the potential of using them with other types of HPLC columns or detectors, and the possibility of using these microcolumns in some types of experiments that are not feasible with traditional HPLC columns or affinity systems.

What are the key factors to consider when using microcolumns in HPAC? Are there limitations?

Hage: The steps that are used to immobilize the binding agent within the microcolumns are important to consider because a large amount of binding agent for each unit volume is usually desirable to obtain high retention and resolution. The short amounts of time that samples spend in these columns and the relatively small binding capacities of these columns are other factors to keep in mind, because they can lead to kinetic effects that are not usually seen with larger columns. The same effects can also be used to provide some unique applications for affinity microcolumns when examining the binding strength or rates of drug–protein interactions.

In 2014, you coauthored a paper analyzing free-drug fractions of sulfonylurea drugs by ultrafast affinity extraction (2). Why did you develop this method? What advantages does it offer?

Hage: This was the next step in our work to see if the changes in drug–protein binding that we were observing as a result of glycation were also affecting the free drug fractions, or biologically active forms, of these drugs at clinically relevant concentrations. The approach that we developed based on ultrafast affinity extraction made it possible to directly measure these free fractions in a matter of minutes per experiment and using only microliter quantities of each sample. The results gave good correlation with those of the reference methods and with the changes that were predicted based on our previous studies of the binding by HSA or glycated HSA with these drugs (2). The same approach can be used for other drugs and is now being used with clinical samples, making it a possible tool that can be used in the future for personalized medicine.

Is there anything you would like to add?

Hage: I think the area of affinity separations and HPAC offers a great number of opportunities for pharmaceutical analysis. I also believe we are just now beginning to realize the potential of these techniques to measure drugs or proteins and to characterize the interactions between these agents. I think these possibilities, as well as the advances that are being made in supports, binding agents, and formats that can be used with affinity separations, will continue to provide great promise for these methods in the years to come.

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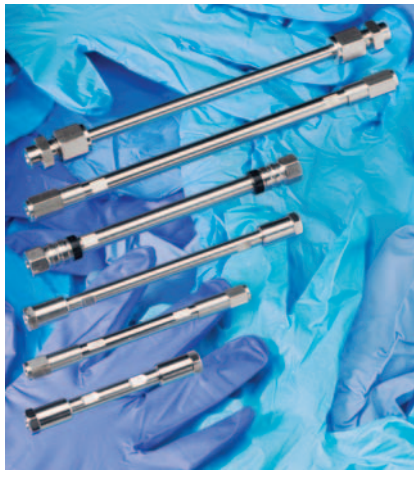
Ghost Peaks from Nitrile Glove Contamination in Reversed-Phase LC Drug Analysis



An unusual source of contamination was found in the reversed-phase liquid chromatography (LC) analysis of a 0.4% (w/w) drug product during the development stability studies at levels much greater than the 4 ppm limit of quantitation. System-related sources (mobile phase, injector carryover and contamination), formulation components (drug substance, excipients), and the sample-handling procedure were studied by a systematic approach. The evaluation of the sample-handling procedure identified extractables from the sample vial (different from that specified in the analytical method) and the rubber-lined vial cap, and incidental transfer of residue from commonly used nitrile gloves as the sources of unintended contamination in sample analysis.

Ghost peaks in liquid chromatography (LC) analyses have been reported in the literature (1–6). Their sources include instrumentation, solvents, glassware, and closure systems. It is important to address their occurrences in the early phases of drug development because they may be an indication of a lack of robustness of the analytical method. When unexpected new impurity peaks appear during stability studies, identifying whether the unexpected peaks are ghost peaks or degradation products is of highest priority. In both cases a thorough investigation needs to be performed. After the new peaks have been determined to be unrelated to stability, the investigation has to identify the source of contamination. When the usual sources are exhausted, then the unlikeliest of sources must be considered.

A validated, stability-indicating reversed-phase LC method used for the analysis of trace impurities in a dry powder inhaled formulation was adapted to support a lower dosage form containing the same excipients as the higher dose formulation. The main differences in the analytical method for the new formulation were the sample diluent used in the extraction and the sample concentration. For the old formulation (2.5% w/w drug load), samples were extracted with a diluent containing 80:20 (v/v) methanol–water followed by centrifugation, and the supernatant was analyzed with a 10-µL injection volume. For the new formulation (0.4% w/w drug load), samples were extracted with a diluent containing 10 mM didodecyldimethylammonium bromide (DDAB, a cationic surfactant) and 1.3 mM ammonium hydroxide (to ensure complete extraction and solubilization of drug substance and impurities) in 40:60 (v/v) methanol–water. The required limit of quantitation (LOQ) for the new formulation was 4 ppm relative to the overall formulation—that is, 0.1% of the 0.4% w/w drug content. After extraction, an equal volume of 1.3 mM ammonium hydroxide in water was added to precipitate the main formulation excipient (less soluble in aqueous media) and DDAB, thus, making the solution less viscous. After centrifugation, a 50-µL volume of the supernatant was injected to achieve approximately the same mass load of the active pharmaceutical ingredient (API) as the old formulation.

The same C18 reversed-phase column and gradient elution conditions were used for the old and new formulations. The binary gradient mobile phases were 0.1% (v/v) trifluoroacetic acid in water and 0.1% (v/v) trifluoroacetic acid in 90:10 (v/v) acetonitrile–methanol.

When this method was transferred to associates performing analysis on development stability samples of the new formulation, seven new degradation peaks (Figure 1) were detected that were out of trend (OOT) with the storage condition of the samples—

James Tam and Andrei Blasko

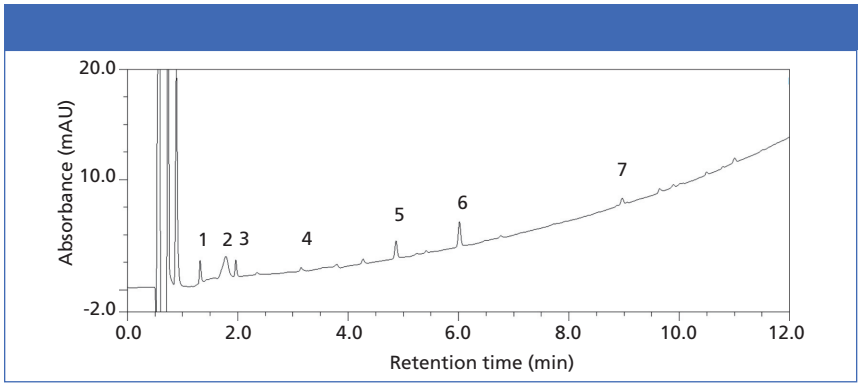


Figure 1: *T* = 0 (initial time point) development stability sample containing seven artifact peaks, labeled 1 to 7.

that is, samples stored at higher temperature and relative humidity or for longer duration had lower total impurities. Furthermore, replicate preparations of the same sample did not exhibit consistent degradation profiles.

To address the appearance of the new, ghost peaks, historical data were considered and the analyst-to-analyst variability was assessed. The high performance liquid chromatography (HPLC) method was used for more than two years both in research and development (R&D) and good manufacturing practice (GMP) testing of the old formulations by multiple analysts without incident with regards to ghost peaks, suggesting good precision. This article summarizes the investigation into the root cause of these new peaks and an unusual source of contamination—the nitrile gloves.

Investigation

Since the diluent blank injections prepared during system conditioning and throughout the stability analysis did not exhibit the same peaks as the samples in question, any system-related causality (such as carryover, mobile phase, or system contamination) was ruled out.

The investigation focused on the samples and sample preparation, since the extra peaks must be either inherent to the drug product samples or introduced through handling of the samples during preparation for injection.

Studies to Determine Whether Peaks Are of Drug-Product Origin

Drug-Substance Degradation Peaks

Forced-degradation studies of the drug substance had been conducted and comparison of the new peaks neither matched retention time or peak absorbance spectrum with peaks generated during forced degradation (Figure 2). These studies indicated that the anomalous peaks were unlikely caused by degradation of the drug substance during sample preparation.

Excipient Degradation Peaks

The new formulation samples being tested contained >99% excipient, predominately a phospholipid of high purity (>99%). This phospholipid can degrade via hydrolysis and generate a highly polar degradant (glycerophosphatidylcholine) and a nonpolar fatty acid. However, high temperature (80 °C for 1 day) stressing of the placebo and for-

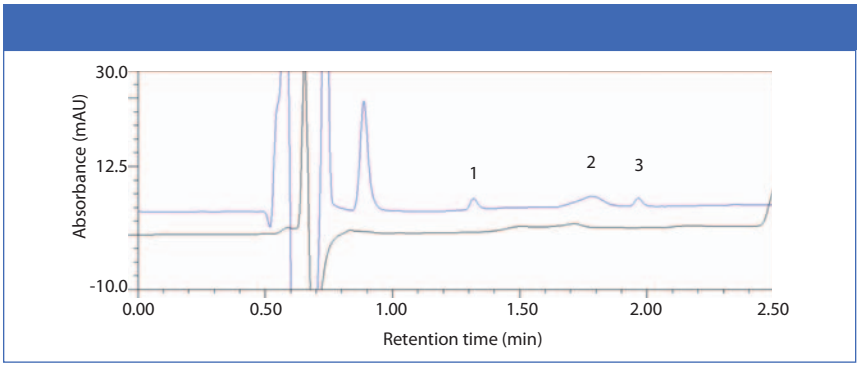


Figure 2: Top: formulation sample with artifact peaks 1, 2, and 3; bottom: photo-forced degraded API.

mulation did not generate the same peaks. Additionally, the expected degradants of the excipient were likely to be not detectable at the absorbance detection wavelength of 230 nm. Thus, degradation of the excipient was ruled out as a likely cause of the new peaks.

Studies to Determine Whether Peaks Are of Sample Handling Origin

Extractables from Sample Vials

After interviewing the analyst performing the stability samples testing, it was revealed that instead of 4-mL vials, 7-mL vials were used in the sample preparation. Originally the 4-mL vials were selected because the final sample solution volume (4 mL) allowed for efficient rinsing of the vial headspace and cap by agitation with vortexing, if any sample powder happened to deposit on those surfaces. The caps of the 4-mL vials are lined with rubber with an inert polytetrafluoroethylene (PTFE) coating. During method development and validation, the new peaks in question were not observed. It was discovered that the caps of the 7-mL vials contained only a rubber lining without PTFE coating. Thus, it was suspected that extractables from the rubber liner may have contributed to some of the spurious peaks. As a result, a study was performed whereby 4-mL and 7-mL empty vials were extracted with sample solvent while placed in an inverted position to maximize solvent–cap liner contact. Five out of the seven new peaks were present in the 7-mL vial extraction. A small, sixth peak (peak 4) was observed in the 4-mL vial extraction, despite the PTFE lining in that vial's cap (Figure 3). Thus, only one peak (peak 2), but the largest, of the new peaks remained a mystery.

Nitrile Glove Contamination

There was suspicion that contamination came from the nitrile gloves worn in the laboratory. It was observed that a slight foam formed on the gloves when they were wetted with a small amount of water and agitated by rubbing. Information from the glove manufacturer further indicated that the gloves' interiors are powder-free, chlorinated, and polymer coated (7,8), which further suggested the possibility of contamination from the glove residue. When a sample of the glove was extracted with the sample diluent, the predominant peak had the same retention time (Figure 4) and UV spectrum (Figure 5) as the unknown peak and the

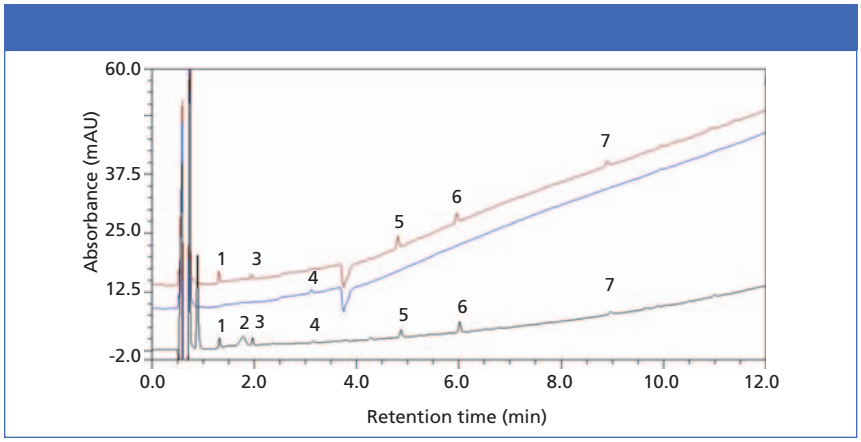


Figure 3: Top to bottom: Diluent in an inverted 7-mL vial with a rubber-lined cap; diluent in an inverted 4-mL vial with a PTFE-lined cap; and drug product sample in a 4-mL vial with a PTFE-lined cap. Peaks 1, 3, 5, 6, and 7 were attributed to the rubber cap liner from the 7-mL vial. Peak 4 was attributed to the PTFE cap liner from the 4-mL vial.

source of the last unknown peak was thus identified. The question then became, what was the contaminant?

Identification of Unknown Peak

A liquid chromatography–mass spectrometry (LC–MS) study of the nitrile glove extract yielded no ionizable peak in positive-mode electrospray ionization (ESI). In negative mode, a molecular ion of *m/z* 207 was observed (Figure 6). A prior nitrile glove extraction study using water (9) identified that naphthalene sulfonate, used as an emulsifying agent in nitrile rubber production, may be present. The structure of naphthalene sulfonate was corroborated by both published UV spectrum (10) with absorbance maxima at 227 nm and 275 nm and by mass spectral data. The initial observation that wet gloves produce foam is consistent with the presence of a surfactant residue. Finally, analysis of an authentic sample of naphthalene-2-sulfonate, matched both by retention time and by mass spectrum of the glove extract peak, confirmed the positional isomer structure. The final artifact peak of in the sample was thus identified.

Discussion

Why was the nitrile-glove peak not observed earlier, despite the method having been in use for more than two years in both R&D and GMP testing and the same type of nitrile gloves having been in use in the laboratory? First, the new drug-product formulation requires the use of a surfactant, didodecyldimethylammonium bromide (DDAB), to disrupt the phospholipid matrix to facilitate full extraction of the drug substance. The surfactant likely enhanced extraction of organic residue from all surfaces, including the nitrile gloves. Second, the new formulation method called for an injection volume of 50 µL instead of 10 µL. The fivefold increase in injection volume magnified the sensitivity to any contaminant peaks. Peaks that previously may have been at or below the detection limit were detected, reportable

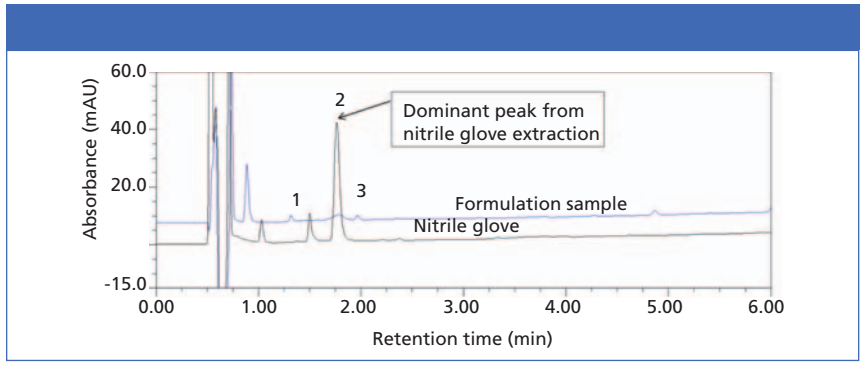


Figure 4: Top: drug product sample chromatogram with unknown peak 2; bottom: nitrile glove extracted with DDAB containing sample diluent.

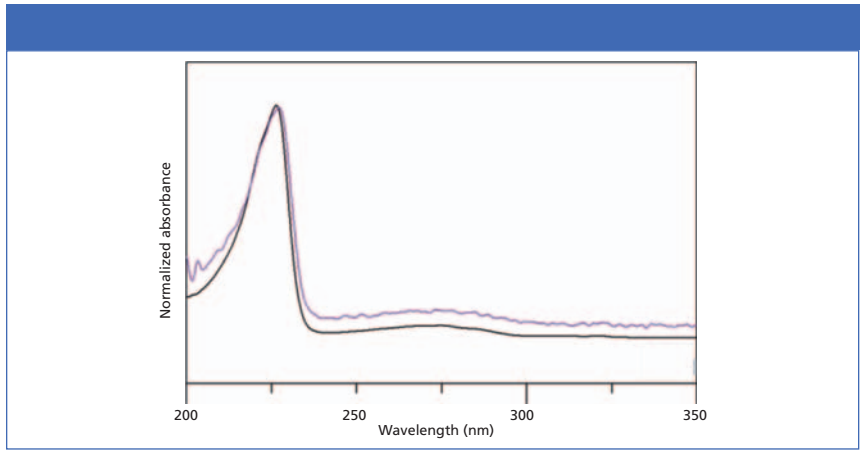


Figure 5: Top: UV spectrum of the 1.8-min retention time peak (peak 2) in the drug product sample; bottom: UV spectrum of nitrile glove extract peak at 1.8-min retention time.

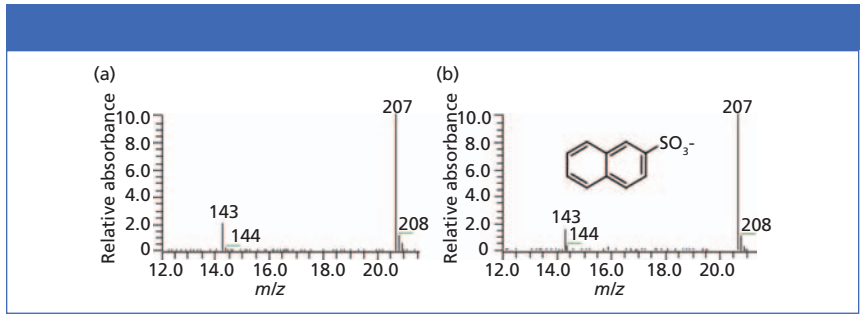


Figure 6: LC–MS negative-ion full-scan spectrum of unknown peak 2 at retention time of 1.8 min (left) and of authentic sodium naphthalene-2-sulfonate (right).

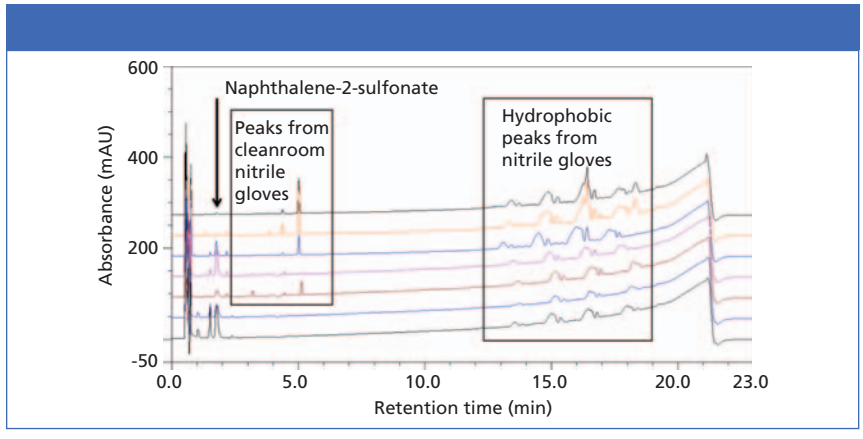


Figure 7: Reversed-phase LC chromatograms obtained from sample-solvent extractions of seven brands of nitrile gloves from various vendors.

peaks at the same concentrations. Third, the increase in water content in the new sample preparation procedure (80% water) compared to original procedure (20% water) probably had a sample peak focusing effect in reversed-phase gradient elution, especially since the artifact peak was eluted fairly early and close to the dead volume of the column. Finally, the analyst developing and validating the method had a practice of wiping the gloved hands with isopropyl alcohol wipes to remove static electricity before handling the powder samples. Serendipitously, this glove cleaning, which facilitated accurate quantitative sample transfer during weighing, also removed enough of the glove surface residue that contamination was not observed during method development. However, this glove wiping practice was not proceduralized and, hence, was not performed while preparing the stability samples. Thus, the sample contact surfaces (weighing paper, pipette tips, spatula) may have had incidental glove contamination.

Steps to mitigate the risk of glove contamination were evaluated. Although the predominant peak from the initial nitrile glove extract was the one observed in the formulation samples, a number of other extracted peaks were observed, particularly at higher retention times. Several other brands of nitriles gloves were extracted. Even cleanroom-grade, powder free, residue free, and chlorinated gloves gave similar peak profiles and additional hydrophobic peaks (Figure 7). Therefore, the best practice recommended is to take extra precaution while using nitrile gloves, which, when balanced with the protection they provide to laboratory workers for common organic solvent used in HPLC and sample preparation, is still the glove of choice for hand protection in the laboratory. Transferable residue from the nitrile gloves can be minimized by wiping them with isopropyl alcohol wipes before sample handling. This practice should not compromise the integrity and protection of the nitrile glove (11). An alternative would be to wear an inner nitrile glove and an outer latex glove, which had far fewer extractable peaks (Figure 8).

Conclusions

A systematic approach for evaluating all the possible sources was taken to isolate and identify the sources of the ghost peaks. Since injection of the sample solvent blank did not produce the ghost peaks of interest, system-related sources (for example,

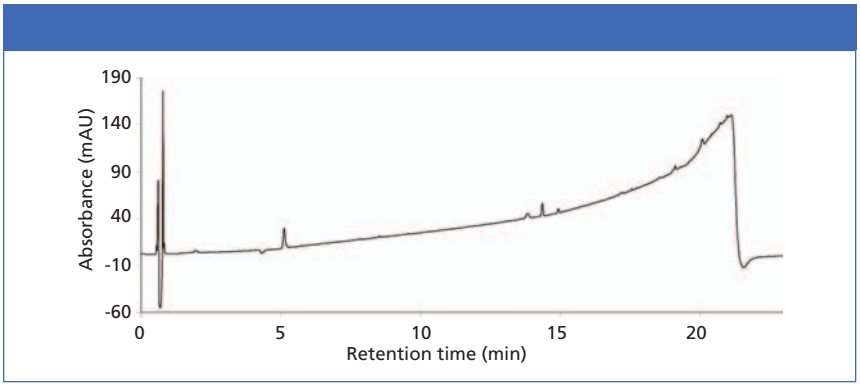


Figure 8: Latex glove (Microflex Safegrip, Microflex Corporation) extracted with DDAB sample diluent.

mobile-phase contamination, injector carryover, or contamination) were ruled out at the outset. Each component (drug substance or excipient) of the sample was studied and also ruled out. Finally, evaluation of the sample handling procedure identified extractables for both the (wrong) sample vial, the rubber-lined cap, and incidental transfer of residue from commonly used nitrile gloves as the sources of unintended contamination in sample analysis. Extra precaution in sample handling must be taken in trace impurities analysis and evaluated on a case-by-case basis, as we have shown that artifacts could come from the least expected of sources—the nitrile gloves used every day in the laboratory.

Acknowledgments

The authors would like to acknowledge Lennard Hachmann, Madhuri Nagadi, Jason Fleischauer, Betelham Guangul, Thomas Tarara, and Imad Haidar-Ahmad for their discussions and suggestions.

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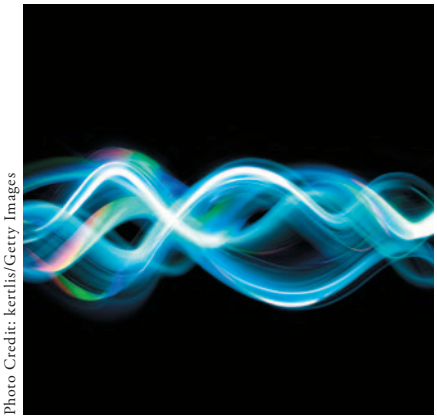
James Tam *is a Fellow at Novartis Pharmaceuticals Corporation in San Carlos, California.* ***Andrei Blasko*** *is a Senior Fellow at Novartis Pharmaceuticals Corporation. Direct correspondence to: james.tam@novartis.com* ■

Practical Applications of Asymmetrical Flow Field-Flow Fractionation: A Review



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Characterization of macromolecules and colloids is an area of considerable interest. Asymmetrical flow field-flow fractionation (AF4) has become a well-established method, but many potential users possess limited knowledge of its capabilities, or how it can provide additional information or serve as validation to the traditional analytical techniques. This review article highlights several practical applications where AF4 should be given special consideration, and discusses benefits and drawbacks of the different methods.

Mats Leeman, Matilda Ulmius Storm, and Lars Nilsson

It is almost 50 years since the late Professor Giddings (1) invented field-flow fractionation (FFF)—a family of separation techniques. A number of different subtechniques exist including centrifugal FFF (CFFF), thermal FFF (TFFF), and electrical FFF (EFFF), each with its own appropriate application area. However, the most widely spread and commonly used subtechnique is asymmetrical flow field-flow fractionation (AF4) (see Table I). From the first reports of AF4 in the 1980s (2) the technique has evolved to become a well-established method for size separation of macromolecules and particles in academia. The technique has also found its way into the industry, largely because of the availability of commercial systems in the latter half of the 1990s.

However, many potential users have still not heard about AF4 or refrain from using it because they have little experience with the technique. With the advent of improved instrumentation and an ever-increasing number of articles to help to select separation parameters, the relevance of AF4 is being re-evaluated. In this review article we highlight applications where AF4 is especially well suited in comparison to other common techniques.

Detection of Protein Aggregates

Studies of protein aggregation are common in both academia and industry. Protein aggregation can be of fundamental interest to understand the characteristics of a protein, but is also important to assess during product development and optimization, or to study the stability of the finished formulation. Size-exclusion chromatography (SEC) is a well-proven and excellent method for size separation of macromolecules, and is often used for size characterization of proteins including aggregation studies. However, it has been reported that SEC has limitations for the protein aggregates, indicating that the method is not always well suited for the separation and detection of higher aggregates (7–9), which can result in low recovery (10).

An example of differences that can be noticed in the results from SEC and AF4 analyses of an aggregated protein is presented in Figure 1. The

Measure Composition (SCB/1000C) and Concentration with the

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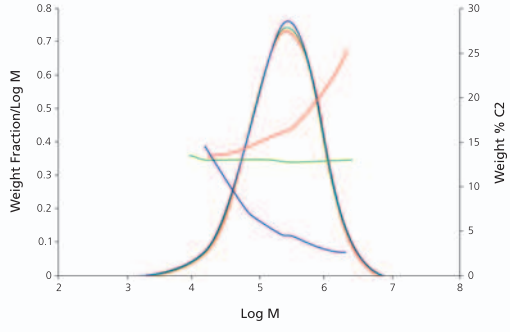

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Table I: About AF4: More detailed descriptions on how AF4 works can be found in other *LCGC* articles (3,4) and in several books on the subject (5,6). In short, AF4 is a size-separation technique for macromolecules, aggregates, particles, and other colloids that are dispersed in a solvent. The separation takes place in an open channel (no stationary phase) in which one wall consists of a semipermeable ultrafiltration membrane. The open channel provides a low surface area, low shear forces, and the ability to separate very large-sized components. In combination with the on-line coupling of light scattering detection, AF4 gives information on molar mass and size on the separated fractions directly without calibration standards. The parameters depending on detection are listed below.

Detection	Parameters
AF4	Hydrodynamic size distribution
AF4–MALS–dRI	As above + molar mass and r.m.s. radius distribution*, conformation, apparent density
AF4–DLS	Hydrodynamic size distribution (from both AF4 retention time and DLS)
SEC	Size relative standards
SEC–MALS–dRI	Molar mass and r.m.s. radius distribution*, conformation
DLS	Hydrodynamic size distribution
*For molecules larger than ~20 nm	

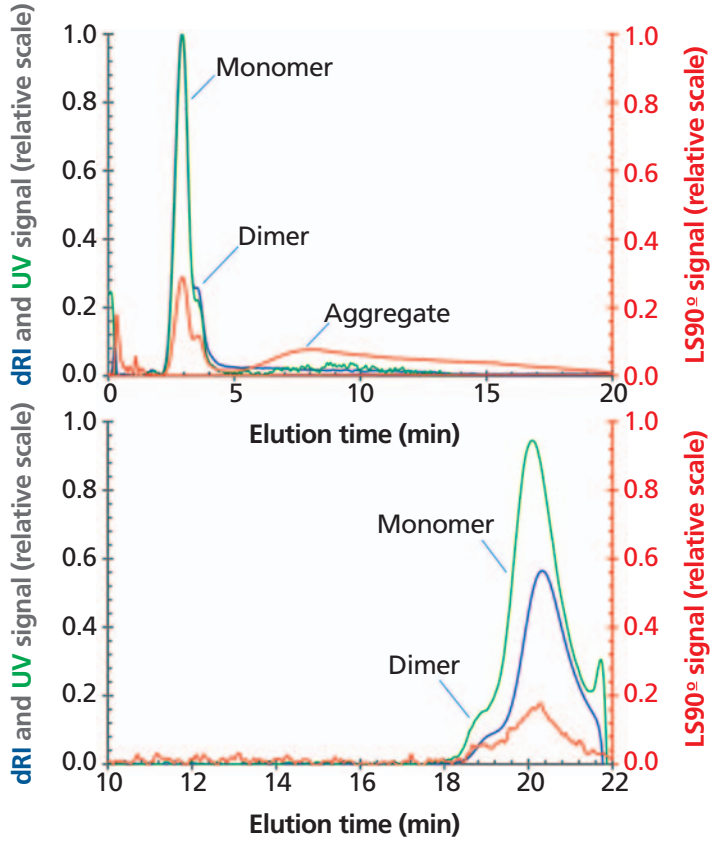

Figure 1: Separation of heat stressed IgG by (a) AF4–UV–MALS–dRI and (b) SEC–UV–MALS–dRI.

sample is a solution of IgG (molecular weight ~150 kDa) that is well-known to aggregate at elevated temperatures. For this illustration the solution was heated to ~65 °C for 5 min to promote the formation of aggregates. Elution of the protein monomer and dimer from AF4 was detected on both the differential refractive index (dRI) and multiangle light-scattering (MALS) detectors. The light-scattering detector, which is very sensitive to high molar mass compounds, also picked up the elution of much higher aggregates (not detected in the IgG solution before heat-stress). The amount of these aggregates is low compared to the monomer and dimer, representing less than 10% of the total protein. In contrast to the AF4 results, no higher aggregates were detected in the analysis by SEC. This is presumably a result of either shear degradation of the higher aggregates or that the aggregates are not eluted from the column.

The SEC column had a nominal pore size of 1000 Å, a nominal protein molecular weight separation range of 50–7500 kDa, and was operated at the relatively low flow rate of 0.5 mL/min (7.8 mm i.d. column). Hence, the protein and potential aggregates are expected to be well within column specifications. Nevertheless, the higher protein aggregates were not eluted from the column. Optimization of elution conditions such as the mobile-phase ionic strength and other additives (10 mM sodium nitrate in water was used) and testing of a variety of columns with different pore-sizes and chemistries could improve the situation. However, it is not certain that one would go through such an optimization procedure unless there is an indication that the current SEC method may fail to detect the aggregates. Great care should therefore be taken when estimating protein aggregation based on data from one method alone; the use of a complementary technique can be valuable for validation reasons.

Size Distribution of Nanoparticles

Nanoparticles are becoming more and more closely studied. One property of

high interest is the particle size, which is commonly analyzed with dynamic light scattering (DLS). This technique measures the fluctuations in scattered light from a sample (such as a particle dispersion) over time and provides information on the hydrodynamic diameter. DLS has several benefits such as compatibility with most solvents and is a relatively fast technique with low workload for the user. However, in some cases, it is difficult to obtain reliable size distribution data (11) on complex samples such as particles with multimodal (12) or broad size distributions (13), or when measuring small particles at low concentrations. This can be explained by the fact that an inherent property of DLS is that the measurements are performed on unfractionated samples, making the data processing and evaluation more complicated for samples with broad size distributions. For small particles, the limitation lies in the relatively low scattering intensity (the intensity of scattered light increases with particle size by the power of 6), which becomes especially challenging if the concentration is low.

The following example is from a recent project in which we participated where DLS was used to characterize the size of nanoparticles. Our DLS measurement gave an average hydrodynamic size of 73 nm and a polydispersity index of 0.25, which was in excellent agreement with the data given by the supplier; 72 nm and 0.25, respectively (also determined by DLS). Looking at the intensity graph from the DLS (Figure 2) it is obvious that the size distribution is broad (ranging from 10 to 400 nm) but there was otherwise no indication of anything remarkable. Possibly there is an indication of a shoulder on the size distribution curve at approximately 20–30 nm.

In contrast, AF4 analysis of the same sample gave a more detailed picture of the size distribution (Figure 3). The use of MALS-dRI detection gave molar mass and r.m.s. radius (a measure of [molecules or particles] size weighted by the mass distribution around its center of mass) information directly of the eluted fractions (independent of the separation and no standards necessary). The analysis detected three size populations on the refractive index and light scattering detectors. The first population (I) eluted from 2–5 min, the second (II) from 5–10 min, and the last (III) was visible mainly in the MALS-signal from 11–18 min. The size populations were incompletely resolved, partly as a result of band broadening, but a major factor is likely overlapping size distributions. This particle was produced in a two-step process, with a solid core that was coated. The core had a size of 5–10 nm as determined with transmission electron microscopy (TEM) prior to coating (likely population I in Figure 3). The second size population (II) probably corresponds to successfully coated particles and the third size population (III) to aggregated particles.

DLS is a rapid tool, excellent for fast screenings of well defined, low dispersity samples. However, this data

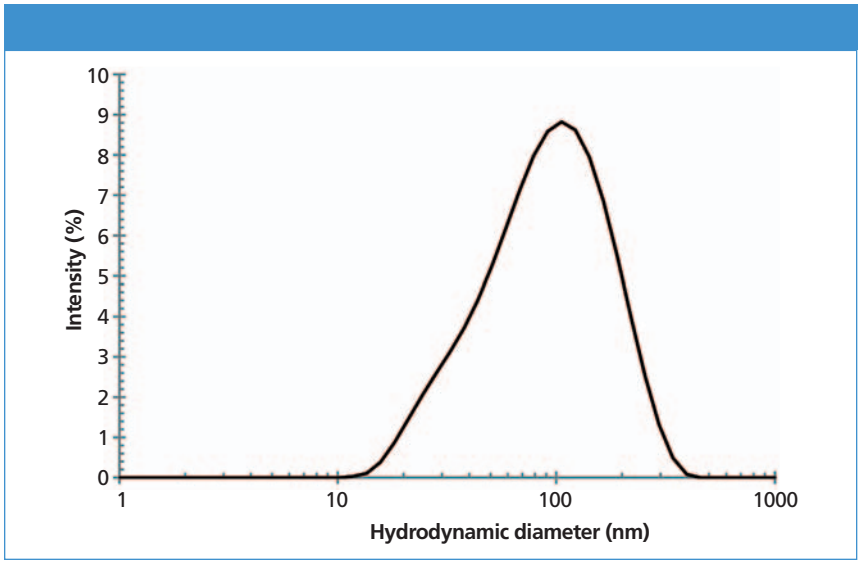

Figure 2: Size distribution of nanoparticles using DLS.

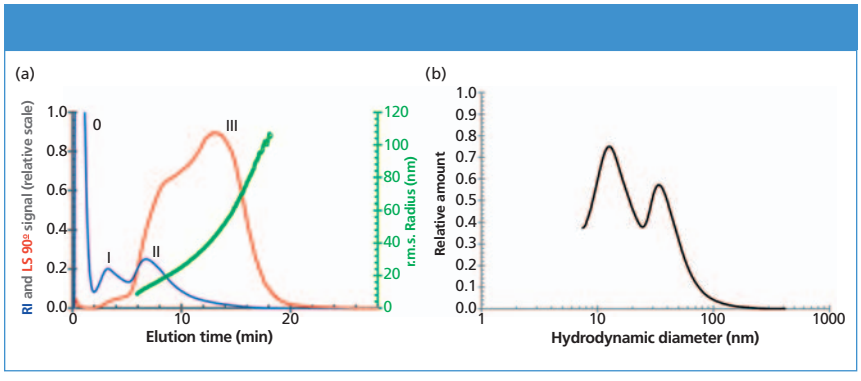

Figure 3: Size characterization of nanoparticles using (a) AF4 and (b) particle hydrodynamic diameter distribution obtained from the AF4 elution times, for comparison with Figure 2. (a) The blue trace is the dRI and red trace is the MALS 90° fractograms. Right axis is the root-mean-square (r.m.s.) radius of the eluted fractions (green circles) determined directly by MALS. Different size populations are indicated by I, II, and III. The early dRI peak (marked with 0) shows the elution of salts in the sample solution.

illustrates the difficulties involved in detecting different size populations in a complex sample using DLS and is one example when it could be suitable to characterize the sample with AF4. The use of a separation technique also offers the potential for an additional dimension because the size fractions can be collected and analyzed by other techniques to further characterize the sample.

Molar Mass and Size Determination of Large Polysaccharides

Polysaccharides are a class of compounds that are used in a wide variety of applications. Some of the polysaccharides, such as starch and cellulose, can be very large and are often used for rheological modification of products. However, the large sizes can also result in challenging size distribution analysis (14,15). Often only average properties are reported, or indirect measurements of the size such as viscosity. However, with AF4 it is possible to analyze very large polysaccharides (16–19) as well as supramolecular aggregates (20–22). Size analysis can be important because the molar mass distribution can be affected during functionalization or by processing conditions (23–28).

The analysis of amylopectin (one of the main constituents of starch) by AF4 is presented in Figure 4. Amylopectin is a branched polysaccharide of very large size and molar mass. Molar mass ranges from approximately ten million up to several hundred millions g/mol and r.m.s. radius ranges up to ~250 nm corresponding to a hydrodynamic diameter of 400–500 nm, which emphasizes the large size range over which AF4 can operate.

In this case AF4 was used to monitor dissolution conditions. Amylopectin is not easily dissolved in water, and the dissolution conditions can have a pronounced effect on the properties of the final amylopectin solution. In this example the dissolution temperature and its impact on the size distribution was investigated and compared to dissolution in dimethyl sulfoxide (DMSO) (Figure 5). The AF4 analysis clearly revealed the impact of high temperature dissolution conditions on the amylopectin (degradation) and provided guidance on how to choose the amylopectin processing conditions for the particular application.

Using SEC for such large compounds as in the example above would definitely be challenging. The size of the amylopectin would likely be excluded from the pores of the stationary phase. Furthermore, even if the pores are sufficiently large enough to allow the amylopectin to enter, the flow rate has to be kept very low to allow the sample time to diffuse in and out of the pores. In addition, shear degradation of amylopectin readily occurs as has been shown by Cave and colleagues (29).

Size Analysis of Vesicles/Liposomes

Liposomes as carriers for pharmaceutically active compounds have attracted both academic and commercial interest for several decades. One parameter that really matters in drug applications of liposomes is the size distribution because it has a considerable impact on the clearance of the liposomes from the blood stream (30–32).

AF4 is a technique very well suited for liposome characterization (33–38). The open channel reduces the risk of shear-induced changes to the sample and has a very low surface area (typically in the order of

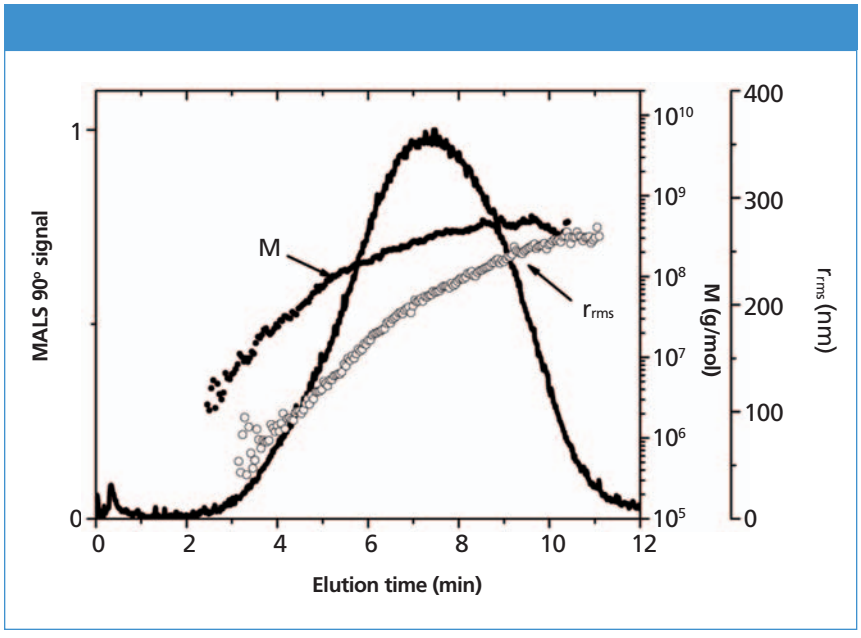

Figure 4: AF4–MALS fractogram showing molar mass (M, obtained from MALS–RI) and r.m.s. radius versus elution time for maize amylopectin obtained from dissolution in water at 140 °C (autoclave). Adapted and reproduced with permission from reference 28, ©Springer.

10^{-3}–10^{-2} m^2) compared to packed columns (at least 1–10 m^2/g stationary phase). An example of an AF4 analysis of liposomes is presented in Figure 6. The liposome size distribution was investigated after different processing steps for different liposome preparation procedures.

The AF4 separation demonstrated good recovery (98%) and was able to separate the relatively disperse systems, with liposome distributions spanning from ~25 to ~500 nm depending on production protocol. The use of a separation method also allowed different liposome size fractions to be collected after the AF4, and analyzed for their compositions by, for example, liquid chromatography–mass spectrometry (LC–MS).

The most commonly used techniques to analyze size and size distribution of liposomes are DLS and SEC (39). DLS suffers from the same limitations as was mentioned in the nanoparticle example above (difficulty to obtain accurate size distribution data for broad or complex size distribution), and this has also been noted for liposomes (35). SEC differs

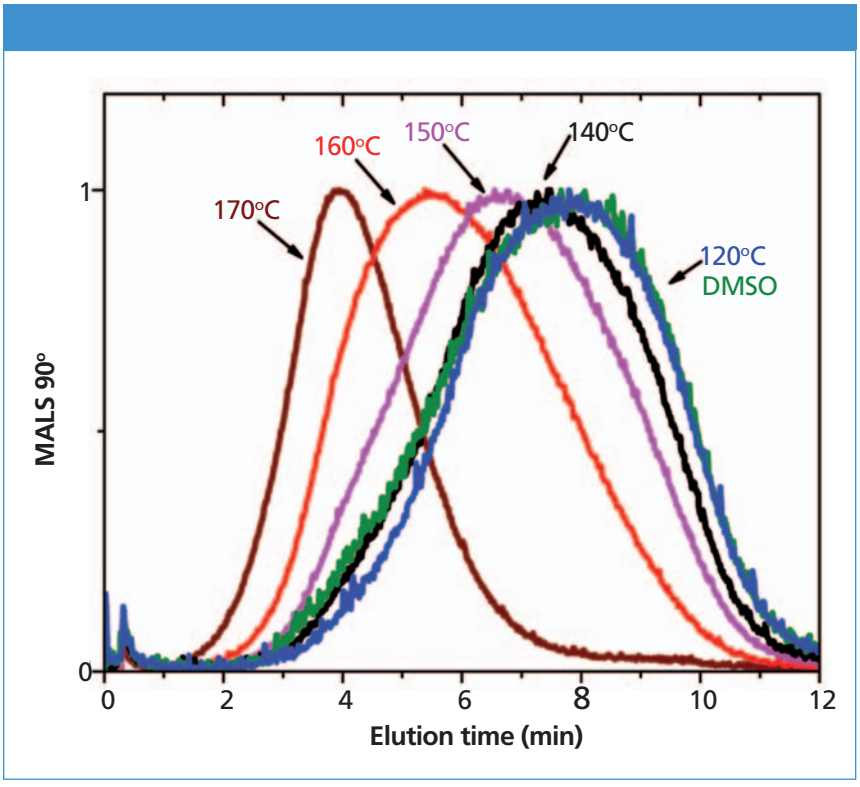

Figure 5: The effect of dissolution conditions (120–170 °C in water in an autoclave and in DMSO at 100 °C) on the elution time in AF4–MALS of maize amylopectin. AF4 analyses were performed under aqueous conditions. Adapted and reproduced with permission from reference 28, ©Springer.

from DLS in that it gives a physical separation of the differently sized liposomes, enabling direct measurement of the size distribution (40–43). However, lipid adsorption to the SEC column is often reported (41,43). To counter this, it is sometimes recommended to saturate (41) the column by several injections of lipid before the actual separation can be performed. Obviously, saturating the stationary phase before separation might affect column performance and retention properties. Therefore, using AF4 in such cases might be a better option.

Separation of Branched Polymers

Branched polymers are a widely used class of macromolecules. The amount of branching as well as the molar mass is of considerable interest since it can have a profound affect on the properties of the polymer in the application. Both parameters can be investigated by SEC when connected to viscosity and light-scattering detectors. However, it is not uncommon to see reports with anomalies in the elution behavior when analyzing branched polymers (44–51), making the evaluation challenging or even impossible. Several investigations point at the difficulties of separating branched high molar mass polymers. One suggested explanation has been anchoring of the branches in the SEC stationary phase pores (47).

For polymers of this complex character, AF4 is an attractive alternative. One illustrative example of using AF4 for separation of branched polymers can be seen in Figure 7 (including a comparison with SEC). The absence of a packing material in AF4 significantly reduces the risk for shear-induced degradation or branches interacting with a stationary phase.

When Not to Use AFFF—And What Not to Expect

Compounds smaller in size than the cut-off of the membrane used are not possible to analyze. The most commonly used membranes have a nominal molecular weight cut-off of 5 kDa, or 10 kDa, which gives an indication of the practical lower limit of AF4. SEC can be expected to exhibit better efficiency or fractionating power than FFF for low-molecular-weight compounds (~ 10^4 g/mol range and less) (52–54).

If the amount of sample is very limited, the use of AF4 might not be possible. The amount injected (in terms of mass) is comparable to what is used in SEC, usually 10–100 µg depending on detection. However, one benefit of AF4 is that if the sample concentration is low, larger volumes can be analyzed (thus reaching the required detection) without affecting resolution thanks to the initial focusing step when the analytes are relaxed in the channel.

The length of an AF4 separation varies depending on sample complexity. In general, total AF4 analysis time (injection, focusing, and elution) is in the 15–60 min range. Add to this the time for data evaluation, which can be very fast for a simple yes or no answer or take considerably more time if data from multiple detectors are to be evaluated and sample characteristics in terms of conformation, branching, and density are desired. Therefore, answers are not obtained as fast as with DLS; however, AF4 offers considerably shorter run times compared to analytical ultracentrifugation.

While AF4 does separate according to size, which in turn is related to mass, do not expect mass spectrometry-like data with isotope resolution. The resolution is simply not of that magnitude.

Conclusion

We have offered some examples where AF4 should be considered in comparison to other commonly used techniques, either because it can provide additional or complementary information or can serve as validation of other analytical techniques. Speaking in general terms, AF4 should be considered when the analytical task involves:

- aggregation-prone samples or when investigating aggregation and aggregate properties;
- broad or complex size distributions;
- large macromolecules;

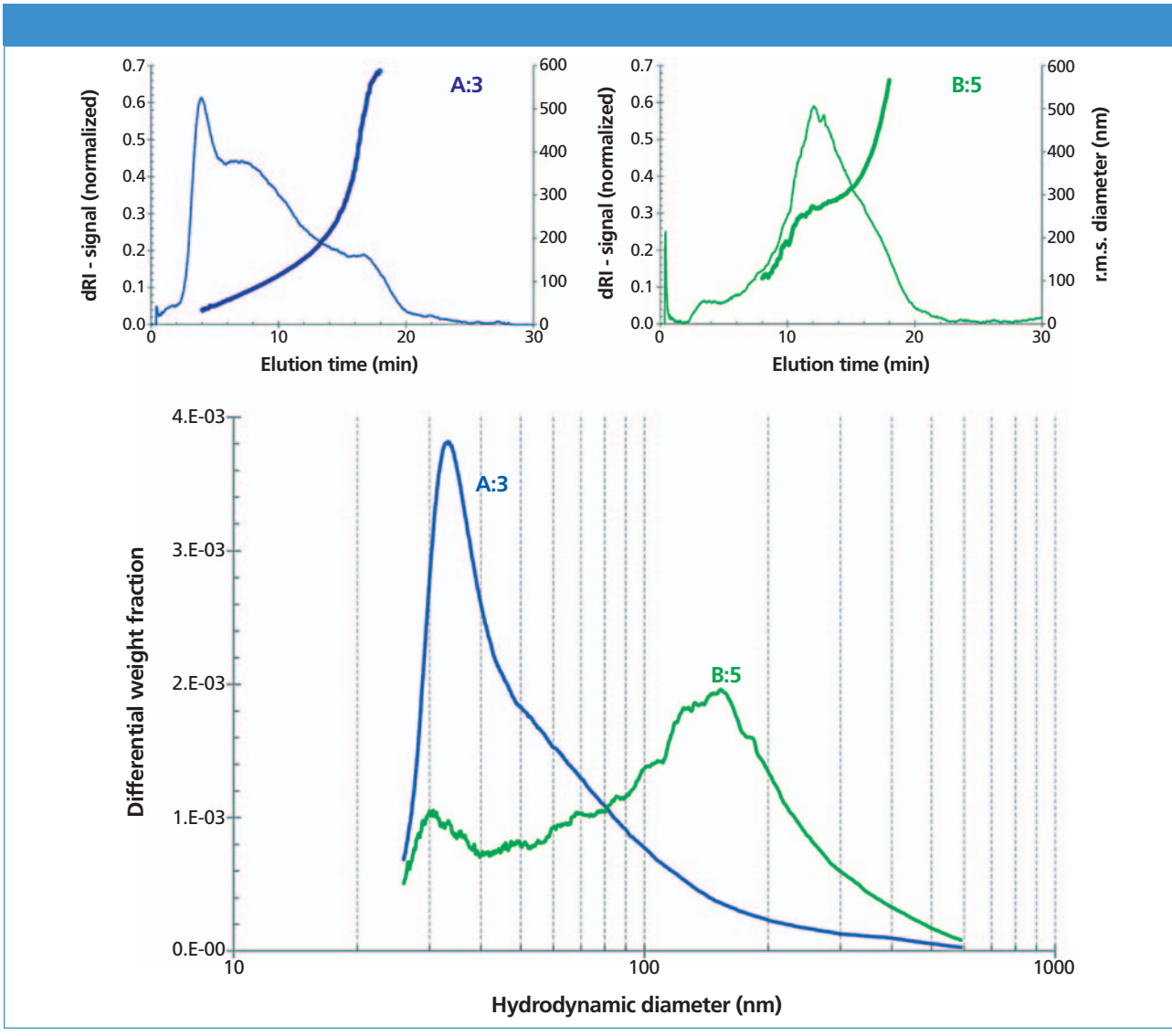

Figure 6: AF4 analysis of liposomes prepared by procedure A (top left graph) and B (top right graph), respectively, taken at different points in the respective procedure. Also plotted is the hydrodynamic diameter distribution of the liposomes (lower graph, log scale, obtained from the AF4 elution times).

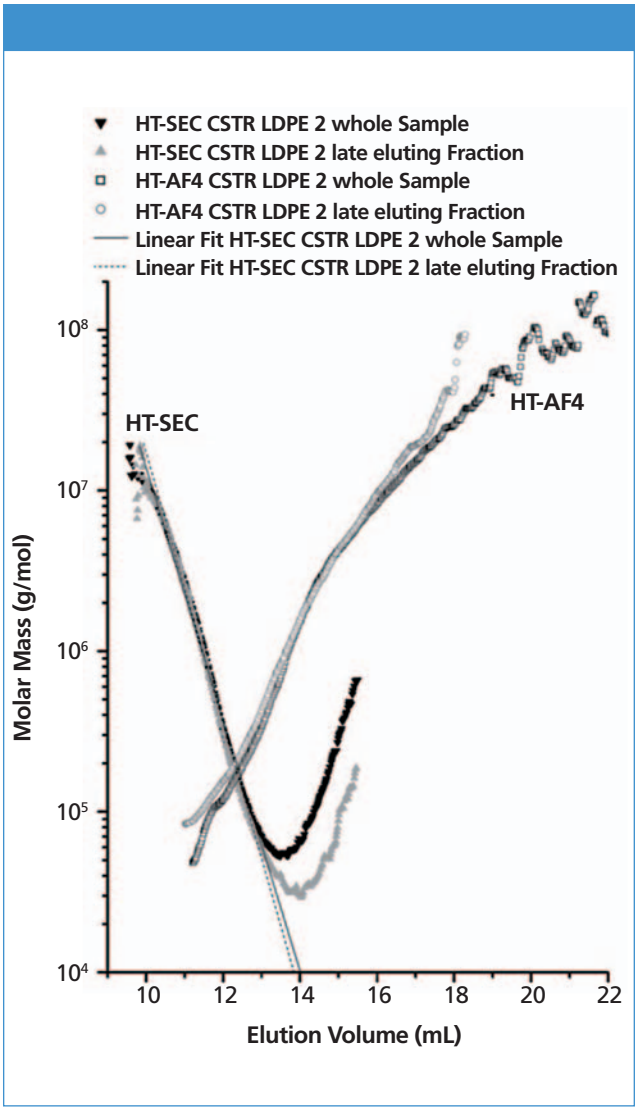

Figure 7: Analysis of polyethylene by AF4 and SEC showing molar mass as a function of elution volume. Note the inversion of the elution order from SEC at 14–16 mL elution volume range. Adapted and reproduced with permission from reference 49, ©Elsevier.

- shear sensitive samples;
- compounds that are difficult to analyze because of interactions with stationary phases.

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Mats Leeman *is senior scientist at SOLVE Research and Consultancy AB. Mats holds a PhD in Technical Analytical Chemistry from Lund University where he studied under the supervision of Professor K.-G. Wahlund. He has been using AF4 for more than 15 years for various applications within the pharmaceutical, specialty chemicals, and biotech industries.*

Matilda Ulmius Storm *is the CEO of SOLVE Research and Consultancy AB. SOLVE is a contract analysis company focusing on material characterization using AF4 among other techniques. The company is based in Lund, Sweden. Matilda holds a PhD in Biomedical Nutrition/Pure and Applied Biochemistry from Lund University where she studied soluble dietary fibers including AF4 to understand the properties and behavior of beta-glucans. She has experience in using AF4 technology for various applications including polysaccharides, proteins, and nanoparticles.*

Lars Nilsson *is currently Associate Professor in Food Technology at Lund University. He earned his PhD at Lund University in 2007 working on the adsorption of macromolecules during emulsification. After a brief time in the biotech industry, working on formulation and stability of foams, he returned full-time to Lund University in 2009. In 2010 Lars was awarded the AkzoNobel Nordic Prize in Surface and Colloid Chemistry for his work on macromolecular emulsifiers. Current research interests are field-flow fractionation techniques for macromolecules and colloidal dispersions, and structure/functionality relationships in macromolecules and their aggregates, emulsions, and foams as well as brewing technology.* ■

WEBCASTS

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Emma Marsden-Edwards, Nick Tomczyk, and Jason Wildgoose, Waters Corporation

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

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

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CryoXtract Instruments LLC, Boston, MA. tinyurl.com/cryoxtract-pdf

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The Hamilton Microlab 600 semi-automated liquid handler system is designed for tasks too small to automate or too large to reliably perform by hand. According to the company, the system is available as a single- or dual-syringe pump with a touchscreen interface to dilute and dispense fluids.

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

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Monolithic reflectron lenses from Photonis are designed for use in time-of-flight mass spectrometers. According to the company, its segmented monolithic reflectron lenses provide solid assembly replacements for a stacked ring assemblies and provide the same ability to alter ion flow. The monolithic lenses reportedly simplify the cleaning and assembly process.

Photonis, Inc., Sturbridge, MA. www.photonis.com

Capillary datasheets

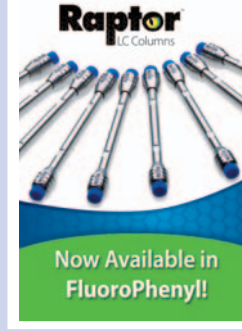
Polymicro's capillary datasheets are designed to provide users with detailed capillary product information and specifications. According to the company, the datasheets, documents, application notes, and literature are searchable.

Molex, LLC, Lisle, IL. www.molex.com

GC–MS system

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

LC columns



Restek's FluoroPhenyl Phase Raptor columns are designed to run in reversed-phase or hydrophilic interaction mode for analyzing a variety of compounds. According to the company, because of their efficiency with acidic mobile phases, the columns also are suitable for LC–MS.

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

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SEC columns

AdvanceBio size-exclusion chromatography columns from Agilent are designed to allow the analysis of protein aggregation using one column and one method instead of several. According to the company, the silica columns provide the resolution and stability to run shorter columns at higher flow rates.

Neta Scientific,
a distributor for Agilent Technologies,
Hainsport, NJ.
www.netascientific.com

Synthesis and screening system

SiliCycle's MiniBlock Micro Synthesis system is designed to allow solid-phase synthesis, solution-phase synthesis, and purification to be carried out on the same equipment. According to the company, the system is a complete platform for 6–48 parallel reactions (40 mL to 4 mL), with its own orbital shaker and heating (up to 120 °C) and cooling (down to -20 °C) capabilities.

SiliCycle, Inc., Quebec City, Quebec, Canada.
www.silicycle.com

Purified beta-glucuronidase formulas

Abalonase and Abalonase+ purified beta-glucuronidase formulas from UCT are designed to provide half the units of activity with the same conversion rate as a traditional abalone-derived enzyme. According to the company, within minutes of application, the purified beta-glucuronidase formula can deconjugate the major metabolites of interest including benzodiazepines, opioids, cannabinoids (naturally occurring and synthetic), and steroids.

UCT, LLC, Bristol, PA. www.unitedchem.com/catalog/abalonase

Sulfur and nitrogen chemiluminescence detectors

Agilent's model 8355 sulfur chemiluminescence detector and model 8255 nitrogen chemiluminescence detector are designed to provide complete integration of the gas chromatograph, detector, and software for low-level analyses. According to the company, these redesigned detectors have a simplified burner design with 50% fewer components.

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

HPLC and UHPLC columns

Tosoh's TSKgel UP-SW3000, 2-µm size-exclusion chromatography columns are designed for the analysis of monoclonal antibodies and other biopharma products and can be used on both HPLC and UHPLC systems. According to the company, the columns are packed with 2-µm silica-based beads shielded with a hydrophilic diol-type bonded phase that prevents the silica surface from interacting with protein samples.

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

Sample preparation kit

ProteinWorks, a family of five sample preparation kits from Waters, is designed for laboratories doing protein bioanalysis. According to the company, the kits facilitate a simplified, standardized, and reproducible path to LC–MS protein quantification in serum and plasma samples via the surrogate peptide approach.

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

Solid-phase extraction system

The EconoTrace parallel solid-phase extraction (SPE) system from FMS is designed with positive pressure pumping for delivery of the sample and conditioning, washing, and elution solvents. According to the company, up to eight extractions can run simultaneously and the system uses any size or type SPE cartridge.

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

Analytical SFC system

JASCO's SF-4000 analytical supercritical fluid chromatography system is designed with a range of column oven arrangements that can house as few as two columns for simpler configurations, and ovens that can accommodate up to 10 columns for more-complex configurations. According to the company, each oven has a separate temperature control for combining chiral and achiral SFC in a single system.

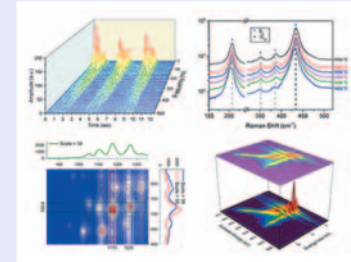
JASCO, Easton, MD. www.jascoinc.com

Sample control system

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

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

Data analysis and graphing software



Origin and OriginPro 2015 data analysis and graphing software from OriginLab add more than 100 new features and improvements. According to the company, enhancements include collapsible menus, project file search for string, thumbnail previews of graphs, and tooltips that display folder or window comments in Project Explorer.

OriginLab,
Northampton, MA.
www.originlab.com

Postcolumn derivatization system

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

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

HPLC columns

Shodex's HILICpak VG-50 HPLC columns are designed to support the analysis of saccharides and reducing sugars such as fructose, mannose, glucose, and sucrose. According to the company, applications are available from the company's database.

Showa Denko America, Inc.,
New York, NY.
www.shodex.net

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

Silicon oxide coating

SilcoTek's Dursan bio-inert coating is designed to improve the chemical inertness, corrosion resistance, lubricity, and hydrophobicity of stainless steels, glass, and ceramics. According to the company, the silicon oxide coating eliminates adsorptive effects from metals for improved trace-level measurements.

SilcoTek Corporation,
Bellefonte, PA.
www.silcotek.com/dursan

High-throughput plate system

MicroLiter's MPSS plate sampling system reportedly combines microplates, glass inserts, and a closure system to provide security for volatile samples. According to the company, the closure system is available for use with the company's µLmat or individual snap caps, providing options for septa or stoppers to optimize sample storage and processing requirements.

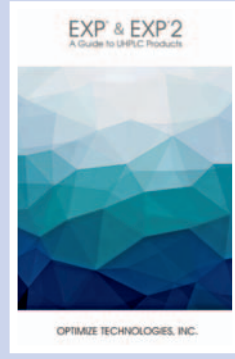
MicroLiter, A Wheaton Brand,
Millville, NJ.
wheaton.com/microliter

LC columns

YMC America's Triart ExRS HPLC and UHPLC columns for hydrophobic substances and isomers are designed with an organic and inorganic hybrid silica particle that uses polymeric bonding. According to the company, the columns are chemically stable at pH extremes (pH 1–12) and are mechanically stable at elevated pressures and temperatures.

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

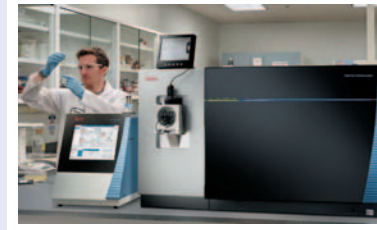
UHPLC products guide



Optimize's EXP and EXP2 brochure describes the company's ultrahigh-pressure liquid chromatography (UHPLC) hardware and consumables. According to the company, products include fittings, filters, and packed-bed products. The system design reportedly provides reusable hand-tight connections and hand-tight cartridge replacements for UHPLC applications.

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

Nano LC columns



Acclaim PepMap nanoLC columns from Thermo Scientific are designed to handle the 1200-bar pressure capability of the company's EASY-nLC 1200 systems. According to the company, the columns' 75-cm format provides a high peak capacity for increased sensitivity and resolution.

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

GC accessories brochure

Supelco's GC accessories brochure lists products used in gas chromatography preventative maintenance. The brochure contains common replacement items such as septa, liners, ferrules, solvents, syringes, vials, and purifiers.

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

Combustion ion chromatograph

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

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

CALENDAR

21–25 February 2016
MSACL 2016 US–The 8th Annual Conference on Clinical Mass Spectrometry
Palm Springs, CA
www.msacl.org

6–10 March 2016
The Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy (Pittcon)
Atlanta, GA
pittcon.org/

16–18 March 2016
Chemistry Conference for Young Scientists (ChemCYS 2016)
Blankenberge, Belgium
www.chemcys.be/

3–7 April 2016
32nd International Symposium on Microscale Separations and Bioanalysis
Niagara-on-the-Lake, Ontario, Canada
www.msb2016.org/registration/registration/registration.html

9–12 April 2016
26th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID)
Amsterdam, The Netherlands
www.eccmid.org/

24–28 April 2016
61st ISA Analysis Division Symposium
Galveston, TX
www.adsymposium.org/

2–4 May 2016
Minnesota Chromatography Forum (MCF) Spring Symposium: The Upper Midwest's Largest Chromatographic Instrumentation and Supplies Exhibition
Minneapolis, MN
www.minnchrom.com/welcome-to-the-mcf/spring-symposium

29 May–3 June 2016
40th International Symposium on Capillary Chromatography (ISCC) and 13th GCxGC Symposium
Riva del Garda, Italy
mytus.unime.it/slider.html

5–9 June 2016
64th ASMS Conference on Mass Spectrometry & Allied Topics
San Antonio, TX
www.asms.org/conferences/annual-conference/annual-conference-homepage

7–9 June 2016
IVT's 3rd Annual Microbiology Week
Philadelphia, PA
www.cbinet.com/conference/pi16056#.Vl4Ogt-rREI

16 June 2016
5th International IGC Symposium 2016
Munich, Germany
inverse-chromatography.com/

19–24 June 2016
44th International Symposium on High Performance Liquid Phase Separations and Related Techniques (HPLC 2016)
San Francisco, CA
www.hplc2016.org/

21–23 June 2016
14th Annual Product Complaints Congress for Life Sciences
Washington, DC
www.ivtnetwork.com/conference/pc16120

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

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

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

18–23 September 2016
23rd International Symposium On Electro- and Liquid-Phase Separation Techniques
Minneapolis, MN
www.cegss.ptchem.pl/itp-2016-23rd-international-symposium-electro-and-liquid-phase-separation-techniques

5–6 October 2016
SFC 2016 — 10th International Conference on Packed Column SFC
Vienna, Austria
www.greenchemistrygroup.org

7–9 November 2016
5th International Conference on Forensic Research & Technology
San Francisco, CA
forensicresearch.conferenceseries.com/

14–16 November 2016
2016 Eastern Analytical Symposium and Exposition
Somerset, NJ
eas.org

26–29 November 2016
14th Asia–Pacific Federation for Clinical Biochemistry and Laboratory Medicine Congress
Taipei, Taiwan
www.apfcbcongress2016.org/

AD INDEX

Call for Papers

LCGC invites researchers to submit their work for publication.

Readers interested in sharing the results of their research with more than 50,000 other chromatographers are encouraged to submit manuscripts for review and possible publication.

LCGC welcomes manuscripts that describe techniques and applications of all forms of chromatography and capillary electrophoresis and that are of immediate interest to users in industry, academia, and government.

Manuscripts are reviewed with the understanding that they have not been published previously and are not under consideration for publication elsewhere. Authors are responsible for all statements made in their work. All manuscripts are subject to peer review and copyediting. Authors of accepted papers will have an opportunity to review galleys. If illustrations or other material in a manuscript have been published previously, the author is responsible for obtaining permission to republish.

Papers accepted for publication in *LCGC North America* may be considered for publication in *LCGC Europe*, which is distributed mainly in Western Europe, and in *LCGC Asia Pacific*, which is distributed in Asia and Australia.

Types of Manuscripts

Review articles

Review articles survey recent developments and the state of the art of current techniques or emerging technologies. Manuscripts should be ~3500–4500 words long. We urge authors to submit a proposal to the editor before completing a manuscript.

Technical articles

Technical articles describe improved methods or improvements in techniques. Papers should be ~3500–4500 words long and should be of immediate relevance to chromatography users. Authors should not make comparisons between commercially available products from different manufacturers.

Manuscript Preparation

Manuscripts for regular issues should be approximately 3500–4500 words long, plus figures and tables as needed, including an abstract of approximately 150–200 words. Figures and tables, along with their captions, should appear at the end of the manuscript, and figures also must be sent as separate files, preferably in JPG, TIF, PNG, or XLS format.

References: Number the literature citations in the text consecutively in order of appearance and indicate them by Arabic numerals in parentheses. Number each reference separately. Group the references at the end of the manuscript in the order of their appearance in the text, not alphabetically.

Use Chemical Abstracts Service Source Index for journal abbreviations. Use the following format for references:

(1) J.L. Rafferty, J.I. Siepmann, and M.R. Schure, *J. Chromatogr. A* **1223,** 24–34 (2012).

(2) L.R. Snyder, J.J. Kirkland, and J.W. Dolan, *Introduction to Modern Liquid Chromatography*, 3rd Ed. (John Wiley & Sons, Hoboken, New Jersey, 2010), pp. 405–497.

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A number of supplemental issues are planned for 2016, for which we also invite contributions. Manuscripts for the supplement on and food and beverage analysis should be 2500–3000 words long, plus up to eight figures and tables, and should include an abstract of approximately 150–200 words.

Our supplement series on mass spectrometry appears four times a year: in March, May, July, and October. Manuscripts for this series should be approximately 3500–4500 words long, including an abstract of approximately 150–200 words, plus up to eight figures and tables.

We are still accepting submissions for the following issues:

Food & Beverage Analysis supplement, October 2016
Submission deadlines:
Abstracts: April 1, 2016
Manuscripts: June 8, 2016

Current Trends in Mass Spectrometry supplement, July 2016
Abstracts: March 1, 2016
Manuscripts: May 2, 2016

Current Trends in Mass Spectrometry supplement, October 2016
Abstracts: April 1, 2016
Manuscripts: June 8, 2016

Contact Us

For more information about contributing to *LCGC*, please contact

Laura Bush,
Editorial Director
LCGC
+1.732.346.3020
lbush@advanstar.com
www.chromatographyonline.com ■

THE ESSENTIALS

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

What Quadrupole GC–MS Operators Need to Know

Anyone operating a gas chromatography–mass spectrometry (GC–MS) instrument needs to pay attention to the special requirements that will allow you to get the most from this type of instrumentation. Here, we present a brief summary of a CHROMacademy webcast that deals with key variables and instrument setup requirements.

The ion sources of GC–MS instruments are susceptible to contamination by column bleed products, and the mass spectral background should be kept as low as possible to ensure good sensitivity and reduce spectral interferences. Furthermore, oxygen should be excluded from the carrier gas to prevent gas-phase oxidation of the analytes. For these reasons, it is important to install and regularly check the viability of high-capacity oxygen traps on the GC–MS carrier-gas lines.

Most manufacturers will produce GC column phases that are designated as "MS compatible," and these will either be the lowest bleed columns identified in quality control (QC) testing, or they may have modified phase chemistry to lower the amount of the bleed. Remember that more-polar, thicker-film columns will inherently bleed more.

Vespel–graphite ferrules are recommended for GC–MS work because they are less permeable to oxygen than the 100% graphite type, although they do suffer more from ferrule "creep" and it may be necessary to tighten the column inlet and detector connections from time to time to avoid leaks.

Take care with column flow rates, because the pumping capacity of most high-quality turbomolecular pumps will limit the column outlet flow to 2–4 mL/min. Also, don't forget to tell your data system that the pressure at the column outlet is "vacuum" rather than "atmosphere" otherwise the flow and linear velocity will be incorrectly set.

More Online:

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

The tuning and calibration intervals for GC–MS are often sources of disagreement. Tuning sets the voltages applied to the various electrostatic elements and lenses within the source and mass analyzer to optimize the instrument response toward the tune compound, which more often than not is perfluorotributylamine (PFTBA), a perfluorinated branched alkylamine. This tuning will give good sensitivity and ensure the correct spectral intensity ratios between fragment masses and their respective ^{13}C isotopes, which helps to improve the accuracy of library search results. The calibration routine will ensure that the mass-to-charge ratio (*m/z*) *x*-axis is calibrated across a wide mass range, which is obviously important for library searching, spectral interpretation, and compound identification for quantitative work.

It really is best to run the tuning algorithm before each analytical campaign because settings drift and the ion source and electrostatic elements become coated with less-volatile matrix materials and the required voltages will change, sometimes over surprisingly short periods of time. The repeller or pusher voltage is usually a good measure of source cleanliness, because it is this voltage that accelerates the ions from the ion volume through the lens stacks and into the mass analyzer. The dirtier this element becomes, the higher the voltage required to reach the ion abundance targets of the auto-tune routine. Many folks use electron multiplier (dynode) or channel plate amplification voltages as a measure of source cleanliness; however, this approach is unreliable because the detector active surfaces deplete over time and an increase in the required voltages here does not necessarily indicate a source clean is required.

It is highly recommended that users familiarize themselves with the manufacturers tune "target" values or specification ranges and ensure that these are manually checked after each tune and calibration. Of particular importance are the spectral peak widths, mass assignments, and fragment and isotopic ion abundance ratios as well the electron multiplier (detector) voltage and the repeller voltage. For more-advanced users, you may well wish to tune the quadrupole mass analyzer by optimizing the gain and offset values of the detector rods to optimize sensitivity and resolution. If the manufacturer offers several tune algorithms, be sure to select the most appropriate one for your analysis (high sensitivity and high quality spectra, or general).

Any leaks can also be detected from the PFTBA spectrum produced via the tune report by monitoring 18 (water) and 28 (air, nitrogen) ion abundance. Leaks can be detected by dynamically monitoring mass to charge 28 while spraying the susceptible instrument joints (column inlet, outlet, and the mass analyzer housing seals) with compressed air from a can.

It's important to ensure that the filaments within the ion source are switched off during the time that the solvent vapor passes through. This time is sometimes known as a solvent delay time, and switching off the filaments avoids burning them out by exposing them to the pressure increase when the relatively large solvent vapor volume passes through the ion source.

When setting up quantitative experiments, remember that the most unique ions for monitoring in selected ion mode (SIM) are higher mass or represent fragments resulting from larger mass losses from the parent ion (but above 50 *m/z*). Dwell times for ions should be equal unless there is strong justification, and remember to include qualifier ions when performing target analysis so that ion ratios can be checked to confirm identity and lack of interference.

Finally, remember that most quadrupole systems, while being nominally unit mass resolving, can be accurate to 0.1 *m/z* and there may be sensitivity gains to be had by specifying the ions for SIM mode to 0.1 *m/z*. That is, monitoring 273.3 *m/z* may give a significant improvement in sensitivity over monitoring the nominal unit mass of 273.0 *m/z*.

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