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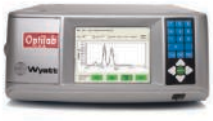

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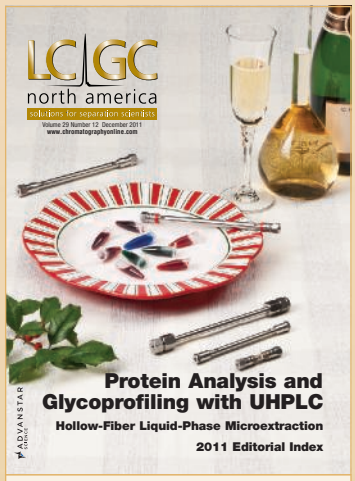

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PREPARATIVE CHROMATOGRAPHY

In a new roundtable, experts discuss preparative chromatography, which is widely used as a purification technique, particularly in the pharmaceutical industry.

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PEAKS of Interest

Spark Holland B.V. in Partnership with Axel Semrau GmbH & Co. KG

Spark Holland B.V. (Emmen, The Netherlands), a supplier of analytical instrumentation transferred its direct sales and service activities for its online solid-phase extraction (SPE) products and services in Germany to Axel Semrau GmbH & Co. KG (Sprockhövel, Germany). The transaction allows Axel Semrau to provide online SPE products to customers and markets in Germany.

Axel Semrau will become a "Value Added Partner" of Spark Holland, and as such, it will provide more value to the product offering along with a higher level of technical and customer support. The products supported are the online SPE systems, which include Spark's brand names Symbiosis Pico and Symbiosis Pharma. Product offerings also include consumables, such as SPE cartridges.

36th International Symposium on Capillary Chromatography

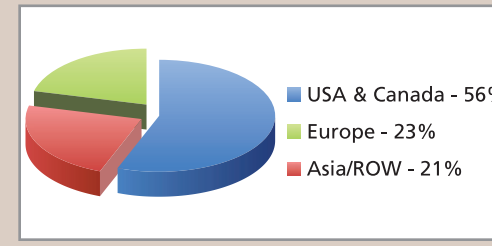
The 36th International Symposium on Capillary Chromatography (ISCC) will take place at the Palazzo dei Congressi in Riva del Garda, Italy, May 27–June 1, 2012. The four-day event will feature recent findings from leading academic and industrial experts in the form of lectures and posters. The conference will offer sessions on capillary gas chromatography, microcolumn liquid chromatography, electromigration methods, and microfabricated analytical systems. These are expected to cover lab-on-a-chip, column technology, coupled and multidimensional techniques, comprehensive techniques, hyphenated techniques, sampling and sample preparation, and trace analysis and automation.

At the meeting, the 2012 Marcel Golay Award will be presented in recognition of outstanding contributions in the field of separation science. Outstanding research work presented as oral or poster contributions by scientists 35 years and younger will be awarded the Leslie Ettre Award for research on capillary gas chromatography applied to environmental or food analyses.

The well-known "Riva Social Programme," which includes a welcome reception, a cocktail party, and a classical concert, will also take place. For more information, visit www.chromaleont.it/iscc. ■

Chromatography Market Profile

GC–MS



Regional distribution of Sdi's survey of GC and GC–MS users.

Gas chromatography–mass spectrometry (GC–MS) combines a gas chromatographic front-end separation with a mass spectrometer. For the most part, the gas chromatographs and mass spectrometers used are modular in design and are relatively easily to separate. GC–MS is the most widespread tandem technique in the analytical instrumentation industry. The systems are employed in many different industries, particularly for environmental, chemical, and toxicological applications.

The mass analyzers used in GC–MS include quadrupole, ion trap, and time-of-flight (TOF) analyzers. Quadrupole mass analyzers consist of four parallel rods. By simultaneously changing both the dc and rf amplitudes applied to the rods, ions of various sizes (mass-to-charge ratios are able to pass through the quadrupole to the detector.

Ion traps use an electric field that is generated by a sandwich geometry in which a space is bounded in three dimensions by ring and cap electrodes on each end. Ions of selected m/z range are trapped in the space bound by the electrodes, and the electric field is varied to eject ions of increasing m/z for detection. Ion traps can perform multiple MS-MS dissociations as well.

In TOF mass analyzers, which operate in a pulsed mode rather than a continuous mode, all the ions are accelerated to the same kinetic energy and are pulsed into the field-region of the flight tube. Ions with different m/z values arrive at the detector at different times. Lighter atoms with higher velocities arrive before the heavier atoms.

In a recent survey of nearly 400 GC and GC–MS users conducted by Strategic Directions International (SDi), the end users were asked to rate a variety of instrument parameters according to how important they were when selecting a GC–MS instrument. Overall, system quality and reliability, sensitivity, and post-sales service and support were the highest-rated factors by the survey participants.

The accompanying figure shows the regional distribution of respondents to the survey. Participants from the United States and Canada represented the largest number of respondents, followed by Europe, Asia, and the rest of the world.

The foregoing data were extracted from SDi's Tactical Sales and Marketing (TSM) report entitled *GC and GC–MS: Global Insight into Market Trends and End-User Attitudes*. For more information, contact Glenn Cudiamat, VP of Research Services, Strategic Directions International, Inc., 6242 Westchester Parkway, Suite 100, Los Angeles, CA 90045, tel. (310) 641-4982, fax (310) 641-8851, email: cudiamat@strategic-directions.com

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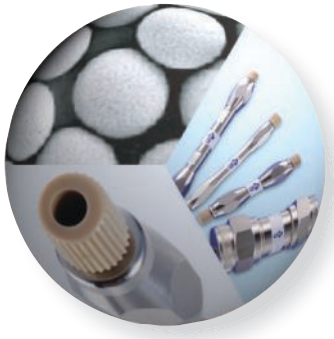

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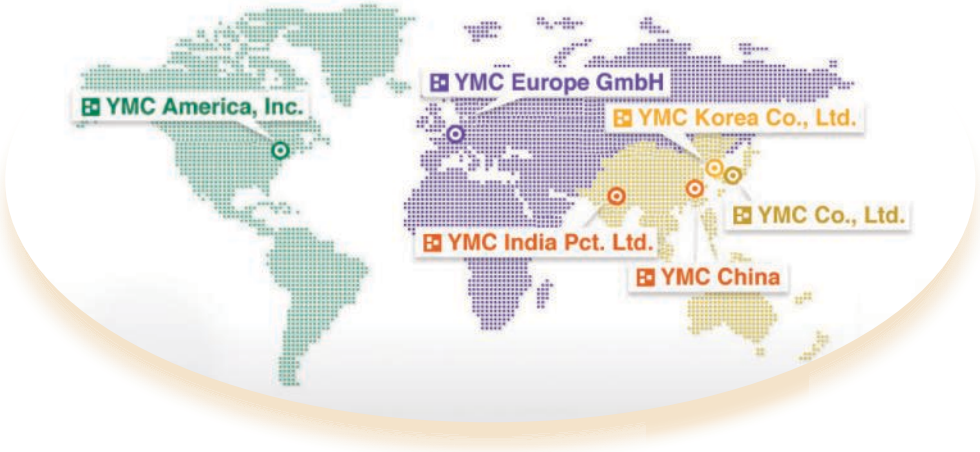

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SAMPLE PREP PERSPECTIVES

Hollow-Fiber Liquid-Phase Microextraction in the Three-Phase Mode — Practical Considerations

In this installment of "Sample Prep Perspectives," Norwegian authors from the University of Oslo describe the practical aspects of hollow fiber liquid-phase microextraction in the three-phase mode (HF^{3}LPME). The guest authors highlight important practical issues related to the supported liquid membrane, the hollow fiber, and the extraction itself. They also discuss practical work with electromembrane extraction (EME), which is related to HF^{3}LPME but uses an electrical potential as the driving force for the extraction.

Astrid Gjelstad, Hamidreza Taherkhani, Knut Einar Rasmussen, *and* ***Stig Pedersen-Bjergaard*** *are the guest authors of this month's column.*

Ronald E. Majors *is the Column Watch Editor*

This column installment describes practical aspects of hollow-fiber liquid-phase microextraction in the three-phase mode (HF^{3}LPME). HF^{3}LPME is a microscale sample preparation technique (1) in which target analytes are extracted from an aqueous sample through a supported liquid membrane (SLM) that is immobilized in the pores of a porous polymeric material and into a volume of acceptor solution (typically, 10–30 µL). In this context, the porous polymeric material is a hollow fiber. Here, we highlight important practical issues related to the SLM, the hollow fiber, and the extraction itself, as these issues are important for successful HF^{3}LPME. We also discuss practical work with electromembrane extraction (EME), which is related to the HF^{3}LPME device but uses an electrical potential as the driving force for the extraction (2).

How Does HF^{3}LPME Work?

HF^{3}LPME can be used for extraction of basic or acidic analytes from aqueous samples. Figure 1 illustrates a setup for HF^{3}LPME. The sample is contained in a sample vial and the pH is adjusted in the sample before extraction to keep the analytes in their uncharged state. For basic analytes, the sample is made alkaline, and for acidic analytes, the sample is acidified. A small piece of a porous hollow fiber, typically made of polypropylene, is closed in one end and dipped in an organic solvent immiscible with water. In a few seconds, this organic solvent is immobilized in the pores in the wall of the hollow fiber by capillary forces, forming an SLM. A 10–30 µL volume of aqueous acceptor solution is then injected into the lumen of the hollow fiber. For basic analytes, the acceptor solution is acidic, whereas it is alkaline for acidic analytes. The hollow fiber is finally placed into the sample and the whole assembly (sample vial and hollow fiber) is agitated for typically 15–45 min. During this time, analyte molecules are extracted in their uncharged state from the sample into the SLM, and further into the acceptor solution. In the acceptor solution, the analyte molecules become ionized, which prevents them from re-entering the SLM. After extraction, the acceptor solution is collected and analyzed directly by high performance liquid chromatography (HPLC), capillary electrophoresis (CE), mass spectrometry (MS), or other related analytical techniques.

The major advantages of HF^{3}LPME can be summarized as follows:

- High enrichment (up to 25,000-fold) (3)
- Excellent sample cleanup
- Direct compatibility with HPLC, CE, and MS
- Low solvent consumption (10–30 µL of solvent per extraction)

Advantages, as well as limitations, of HF^{3}LPME have been discussed substantially in the literature and several reviews discussed a broad range of applications (4–11).

Which Hollow Fibers Are Used for HF^{3}LPME?

The porous hollow fibers used for HF^{3}LPME typically are made of polypropylene (4). Most work published in the literature has been accomplished with a polypropylene hollow fiber from

Membrana (Wuppertal, Germany) termed "Q3/2" that has an internal diameter of 600 µm, a wall thickness of 200 µm, and a pore diameter of 0.2 µm (4). The hollow fiber is connected to a precut pipette tip or a medical syringe needle. The pipette tip or syringe needle serves as a guide tube to facilitate the injection and withdrawal of the acceptor solution, as illustrated in Figure 2. The fiber can be arranged either in the loop configuration with connections in both ends (Figure 2b) or in the rod configuration with a connection in one end and the other end closed (Figure 2a). To close the hollow fiber, mechanical pressure with a pair of pincers can be used — no heat or glue is required. In our laboratory, we use pipette tips for the connections. In this case, we carefully heat the connection between the tip and the fiber with a soldering iron to prevent disruption. Alternatively, the hollow fiber also can be attached directly to the needle of a microsyringe for easy injection and withdrawal of the acceptor solution, as illustrated in Figure 2c (12). Alternative fiber dimensions also can be used, but generally the thickness of the wall of the fiber should not exceed 200–300 µm because the extraction speed and the recovery are dependent on the thickness of the SLM. Fibers with internal diameters less than 600 µm have been reported to speed up HF^{3}LPME with small volumes of acceptor solution for high enrichment (3), and hollow fibers with internal diameters larger than 600 µm have been used for easier injection and withdrawal of the acceptor solution (13,14).

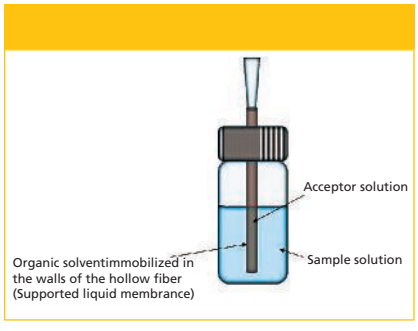

Figure 1: Illustration of a typical hollow-fiber liquid-phase microextraction in the three-phase mode (HF^{3}LPME) setup.

What Solvents Can Be Used as the SLM?

In HF^{3}LPME, the SLM is an intermediate extraction medium. Analytes should be extracted rapidly and efficiently into the SLM, but transport out of the SLM and into the acceptor phase also should be efficient to avoid substantial trapping of analytes in the SLM itself. Substantial trapping in the SLM is undesirable because it reduces the extraction recovery. In other words, finding the optimum SLM solvent for the application is an important step.

For HF^{3}LPME, dihexyl ether and 1-octanol have been the most popular SLM solvents (4). As seen in Table I, these two solvents have a high boiling point (>195 °C), and when the hollow fiber is dipped in the solvents, little or almost no evaporation of the SLM is observed during 2 min of air exposure (Table I). This observation is important because the hollow fiber with the immobilized SLM is normally exposed for a short time (<2 min) to open air before it is placed in the sample. After the hollow fiber with the SLM is inserted in the sample in a capped vial, evaporation is no longer an issue because the SLM is protected by the aqueous sample and the system is closed. Volatile solvents such as toluene and 1-chloropentane may be difficult to use in HF^{3}LPME because they evaporate quickly and give an unstable SLM (see Table I). In general, it is recommended not to use solvents with a boiling point below 190–200 °C for HF^{3}LPME.

In addition to the volatility of the solvent, the water solubility also is important. For dihexyl ether, the water solubility is low (<110 µg/mL), therefore, SLMs made from this solvent are very stable during extraction. With 1-octanol, the water solubility is higher (1200 µg/mL) and this solvent may leak into the sample (and the acceptor solution) in significant amounts. As seen in Table I, about 11% of an 1-octanol SLM may theoretically leak into 1 mL of sample based on the water solubility, resulting in a significant reduction of the SLM. This level of SLM leakage has been verified experimentally in our laboratory by analyzing the sample solution after HF^{3}LPME with gas chromatography–mass spectrometry (GC–MS) to determine traces of 1-octanol. This loss may be even greater if the sample volume is increased or if surfactants and other emulsifying agents are present in the sample. In general, it is preferred not to use solvents exceeding 200–400 µg/mL in terms of water solubility. In addition to dihexyl ether, solvents such as 1-decanol, dodecyl acetate, 2-nitrophenyl octyl ether, and 1-nonanol fulfill the criteria discussed above and have been used in HF^{3}LPME studies (Table I) (4). Some new solvents that have been tested recently in our laboratory for HF^{3}LPME also are included in Table I and may be interesting SLM candidates for the future.

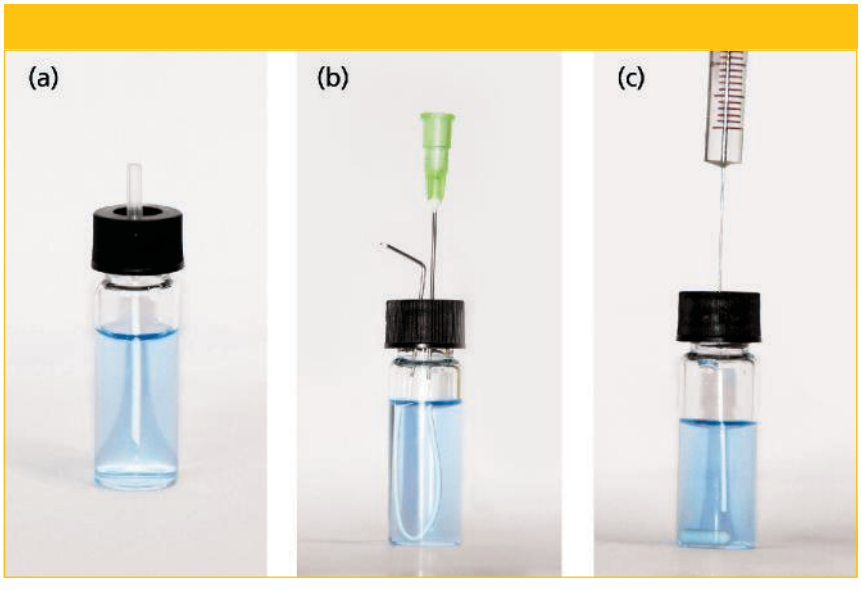

Figure 2: Different configurations for hollow-fiber liquid-phase microextraction in the three-phase mode (HF^{3}LPME): (a) rod configuration, (b) loop configuration, and (c) hollow fiber attached directly to the needle of a microsyringe.

Table I: Boiling point, evaporation, water solubility, and dissolution data for various solvents for hollow-fiber liquid-phase microextraction in the three-phase mode (HF^{3}LPME)

	Boiling point* (°C)	Evaporation of SLM from Fiber After 2 min in Open Air† (%)	Water Solubility‡ (µg/mL)	Potential Dissolution of SLM from Fiber into Sample§ (%)
Frequently Used Solvents				
Dihexyl ether	223	0.0	110	1.0
1-Octanol	195	0.8	1200	11
Less Frequently Used Solvents				
1-Chloropentane	107	40.6	180	1.6
1-Decanol	228	0.0	120	1.1
Dodecyl acetate	265	0.0	20	0.2
2-Nitrophenyl octyl ether	351	0.0	6	<0.1
1-Nonanol	212	0.0	390	3.5
2-Octanone	173	1.6	2300	21
Toluene	111	34.5	320	2.9
Future Alternative Solvents				
2,2-Dimethyl-1-propylbenzene	209	1.6	1.9	<0.1
2-Hexyl-1-decanol	304	0.0	0.039	<0.1
Isopentyl benzene	193	0.7	2.5	<0.1
Nitrostyrene	239	0.0	300	2.7

*Data obtained from SciFinder
†Measured with an analytical balance
‡Data obtained from SciFinder, at 25 °C and pH 10
§Calculated from the water solubility data based on a sample volume of 1 mL

How Much Does the SLM Affect the Extraction?

Table II (basic drugs as model analytes) and Table III (acidic drugs as model analytes) illustrate recent examples from our laboratory in which the extraction recovery in HF^{3}LPME was strongly affected by the type of solvent used in the SLM. For the basic model analytes, the highest recoveries were obtained with dodecyl acetate, 2-octanone, and isopentyl benzene as the SLM solvent. For the acidic drugs, dodecyl acetate, isopentyl benzene, and 1-decanol were the top three SLM solvent candidates. A closer look at the results in Tables II and III indicate several important aspects. First, recoveries were generally higher for the acidic model analytes than for the basic model analytes. The reason for this result was probably that the selected acidic model analytes were slightly more polar than the basic ones. From earlier experience (15), it is well known that extraction recoveries in HF^{3}LPME are highest for analytes with log P values in the 2–4 range, whereas the extractability decreases somewhat for analytes with log P values exceeding 4. The basic model analytes in Table II where highly hydrophobic (log P in the 3.1–5.3 range), and extraction from the organic SLM and into the aqueous acceptor phase was somewhat limited by partition. Because of this, selection of the solvent was very important for the basic model analytes. Second, the extraction performance of each of the solvents was checked against the Snyder solvent selectivity classification system (16). The two top solvents, namely dodecyl acetate and 2-octanone, were both class VI solvents (aliphatic ketones and esters) and the next two solvents were both class VII solvents (aromatic hydrocarbons). Although relatively different in terms of chemical structure, class VI and VII solvents are close to each other in terms of solvent selectivity properties with relatively strong proton acceptor and dipole characteristics.

A somewhat different picture was observed for the acidic model analytes in Table III. Because these analytes were less hydrophobic, with log P values in the 2.9–4.3 range, they were more easily extracted from the organic SLM and into the aqueous acceptor phase. Therefore, the selectivity of the solvent was less critical for these analytes. Thus, the five top solvents, all of which provided average recoveries of 70% or more, belonged to Snyder classes I, II, VI, and VII. These solvents have substantial differences in terms of solvent selectivity, proton acceptor, proton donor, and dipole characteristics.

In general, solvent selection in HF^{3}LPME has been carried out mainly by trial and error, testing a limited number of candidates including dihexyl ether and 1-octanol. Most likely, more systematic approaches will be developed in the

Table II: Hollow-fiber liquid-phase microextraction in the three-phase mode (HF^{3}LPME) performance ranking of solvents for basic model analytes

Solvent Rank	Recovery (%) (*n* = 3)					
	Droperidol	Haloperidol	Nortriptyline	Clomipramine	Clemastine	Average*
Dodecyl acetate	70	64	65	48	62	62
2-Octanone	85	54	54	41	49	57
Isopentyl benzene	55	65	68	41	43	54
2,2-Dimethyl-1-propylbenzene	39	58	69	40	41	49
Dihexyl ether	29	48	50	48	59	47
2-Nitrophenyl octylether	28	57	64	41	41	46
3-Nitrostyrene	65	46	47	21	18	39
1-Nonanol	68	21	38	15	10	30
1-Octanol	67	19	35	22	14	29
2-Hexyl-1-decanol	21	26	40	28	25	28
1-Decanol	65	20	32	11	8	27

*Average of recoveries reported for droperidol, haloperidol, nortriptylin, clomipramine, and clemastine

Table III: Hollow-fiber liquid-phase microextraction in the three-phase mode (HF^{3}LPME) performance ranking of solvents for acidic model analytes

Solvent Rank	Recovery (%) (*n* = 3)				
	Ibuprofen	Naproxen	Ketoprofen	Gemfibrozil	Average*
Dodecyl acetate	83	82	83	79	82
Isopentyl benzene	83	83	61	83	78
1-Decanol	74	77	71	70	73
3-Nitrostyrene	71	71	74	69	71
1-Octanol	70	72	69	69	70
2,2-Dimethyl-1-propyl benzene	70	70	60	70	67
1-Nonanol	68	69	66	58	65
2-Nitrophenyl octylether	68	67	48	71	63
2-Hexyl 1-decanol	60	56	34	55	51
Dihexyl ether	8	39	6	2	14
2-Octanone	nd	nd	nd	nd	nd

*Average of recoveries reported for ibuprofen, naproxen, ketoprofen, and gemfibrozil

future that will take further solvent properties into consideration. It also should be mentioned that HF^{3}LPME is best suited for analytes with log P > 2. For analytes with log P < 2, extraction is more challenging and requires the addition of a carrier such as an ion-pair reagent either to the sample or the SLM (15).

How Should the Dry Hollow Fiber Be Handled?

The dry hollow fibers for HF^{3}LPME typically are purchased as bundles from the manufacturer. No hollow fibers are currently manufactured specifically for HF^{3}LPME; instead they are industrial products for totally different applications. We recommend storing the hollow fibers in a closed bag protected from light, because air and light exposure over long time periods might degrade the mechanical stability of the hollow fiber and make it more fragile. Before use, the hollow fiber needs to be cut to form pieces of appropriate length. We recommend using

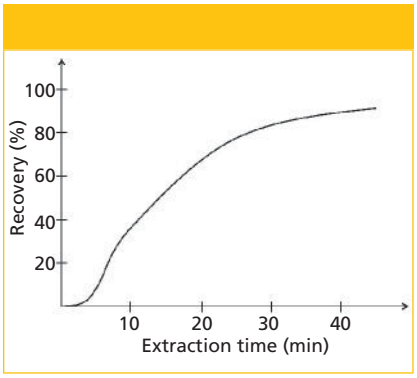

Figure 3: Recovery versus extraction time for haloperidol. Supported liquid membrane: 2-nitrophenyl octylether; sample: 1 mL of 25 mM ammonia buffer pH 10 containing 1 µg/mL haloperidol; acceptor solution: 25 µL of 10 mM HCl.

gloves when cutting the fiber to avoid contaminating it. It is important that each piece be cut to exactly the same length. If fibers are of different lengths, the volume of the SLM will vary from extraction to extraction, and this may give some variation in the results. In two-phase hollow-fiber liquid-phase microextraction it is common to wash the hollow fiber with acetone before extraction to remove additives in the polymeric material (17). This is less important in HF^{3}LPME because the acceptor solution is aqueous and most polymer additives are not soluble in aqueous solution. However, for extraction of very hydrophobic analytes, recoveries may be slightly improved if the hollow fiber is prewashed with acetone (18). Each piece of hollow fiber is for single use and should always be discarded after extraction to avoid carryover from one extraction to another.

How Should the SLM Be Prepared?

The SLM normally is prepared by dipping the hollow fiber into the organic solvent for 5–10 s. The organic solvent is immediately immobilized in the pores in the wall of the hollow fiber by capillary forces. This procedure is very simple, but the exact amount of organic solvent is unknown, and excess organic solvent may be located on the surface of the hollow fiber. In such cases, it is recommended to remove excess solvent from the hollow fiber. This can be accomplished either by wiping the fiber with a medical wipe or exposing the fiber and the SLM to ultrasonification in a water bath for 5–10 s. The former method (medical wipe) is recommended, as this procedure has been reported to yield the most reproducible SLM (18).

Alternatively, the organic solvent can be injected into the lumen of the hollow fiber using a microsyringe. In this procedure, the SLM is coated from the inside of the hollow fiber. The advantage here is that the volume of the SLM solvent is controlled more exactly; this approach may be interesting for future automation of HF^{3}LPME. The typical volume of the SLM solvent in one piece of hollow fiber is 10–30 µL.

It is recommended to immobilize the SLM solvent in the hollow fiber in the shortest amount of time possible before the extraction. This is done to avoid partial evaporation of the SLM solvent and so that the SLM solvent is not gradually swelled into the polymer itself. With solvents like dihexyl ether and 1-octanol, swelling may totally interrupt the SLM after a few days of storage. Other solvents have been found to be highly stable as the SLM solvent, and they may be immobilized for up to 60 days before use. These solvents include silicone oil AR 20 (polyphenyl-methylsiloxane), 2-nitrophenyl octyl ether, and dodecyl acetate (18).

How Should the Acceptor Solution Be Loaded?

When the SLM is prepared, the acceptor solution has to be injected into the lumen of the hollow fiber. This is accomplished with a microsyringe. Loading exact and constant volumes of acceptor solution from extraction to extraction is important to obtain the highest repeatability. Make sure that injection of the acceptor solution is performed slowly. Rapid injection of the acceptor solution into the narrow hollow fiber may cause air bubbles, which results in small segments of the hollow fiber containing no active acceptor solution. Air bubbles in the hollow fiber from rapid injection can affect the results significantly and sacrifice repeatability (18).

How Should the Actual Extraction Be Performed?

Extraction is initiated at the time when the hollow fiber, containing both the SLM and the acceptor solution, is placed in the sample. Exact timing of the extraction is important, and the time for each extraction should be measured from the point when the fiber is placed in the sample. Immediately, the whole assembly (sample plus the hollow fiber) should be transferred to an agitator. Agitation (or stirring) is important to facilitate extraction and constantly replenish the sample in close contact with the SLM. Normally, we recommend agitating the entire extraction unit (sample vial and hollow fiber) at 800–1200 rpm, but stirring with small magnetic stir bars also may be used. Normally, HF^{3}LPME is accomplished at room temperature with no external temperature control.

How Should the Extraction Be Finished?

When the extraction has been completed at an exact stop time, the hollow fiber should be removed immediately from the sample to stop the extraction. This is especially important if extractions are not carried out to equilibrium as shown in Figure 3. Additionally, the acceptor solution should be removed immediately from the hollow fiber to avoid partial back-extraction into the SLM and loss of analyte. The acceptor solution normally is removed with a microsyringe and transferred to a sample vial for the final analysis by HPLC or CE. Because the acceptor solution volumes typically used are low, the acceptor solution should be protected from evaporation. Therefore, storage at low temperature in a closed vial is highly recommended. When the acceptor solution is removed from the hollow fiber, it also is important to check the volume of the acceptor phase. Occasionally, the volume collected after extraction is different compared to what was injected into the hollow fiber before extraction; this volume difference is a clear indication of leakage in the system. In such cases, the acceptor solution should be discarded.

What About pH Effects?

In HF^{3}LPME of basic and acidic compounds, the pH of the sample and the acceptor solution is highly important. For basic analytes, the pH of the sample should be high to suppress ionization of the basic substances and promote their extraction into the SLM, whereas the pH of the acceptor solution should be low to ionize the basic substances upon arrival

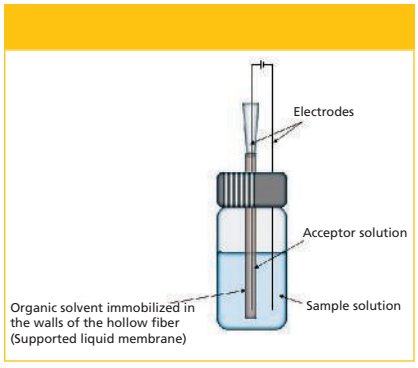

Figure 4: Illustration of a typical electromembrane extraction (EME) setup.

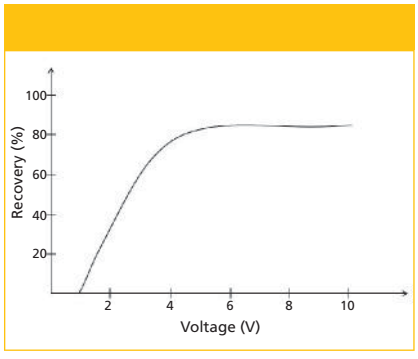

Figure 5: Recovery versus voltage for haloperidol. Supported liquid membrane: 1-isopropyl-4-nitrobenzene; sample: 1 mL of 10 mM HCl containing 1 µg/mL haloperidol; acceptor solution: 25 µL of 10 mM HCl; extraction time: 5 min.

in the acceptor solution. The latter effect also prevents the analytes from re-entering the SLM. The strong pH-gradients across the SLM serve as the driving force for the extraction. For basic analytes, we normally recommend adjusting the pH 1–3 units above the pK_a values of the analytes in the sample and 1–3 units below their pK_a values in the acceptor solution. Typically, the sample is made alkaline with sodium hydroxide, whereas hydrochloric acid (10 mM) or formic acid (LC–MS friendly) is used as the acceptor solution (4). For acidic substances, the pH gradient is reversed, with acidic conditions in the sample and alkaline conditions in the acceptor solution. Usually, hydrochloric acid is used to acidify the sample, whereas 10 mM sodium hydroxide or ammonia solution (LC–MS friendly) is used as the acceptor solution (4).

What About EME?

Electromembrane extraction (EME) also is an extraction method for basic or acidic analytes from aqueous samples. Figure 4 illustrates the setup of EME. The setup and procedure are very similar to those used for HF^{3}LPME. In EME, electrodes are inserted into both the sample and the acceptor solution and the electrodes are connected to a dc electrical power supply. In EME, an electrical potential of typically 5–100 V is applied over the electrodes, creating an electrical field over the SLM. This electrical field is the principal driving force for extraction in EME. For EME of basic analytes, the anode is located in the sample, whereas the cathode is placed in the acceptor solution. The sample has to be acidified to make sure that the basic analytes are ionized in the sample. Thus, the basic analytes are extracted as protonated species from the sample, through the SLM, and into the acceptor solution. The acceptor solution also is acidic to support the electrokinetic transfer and to avoid back-extraction into the SLM. For acidic analytes, the direction of the electrical field is reversed, and alkaline conditions are used in the sample and the acceptor solution to maintain the analytes in their charged configuration. The advantages discussed above for HF^{3}LPME are more or less the same for EME. However, EME is faster than HF^{3}LPME because the driving force is an electrical potential rather than a pH gradient. EME often can be finished after 5 min. Several reviews have been published summarizing current applications of EME (19–23).

The practical details discussed above for HF^{3}LPME are also valid, more or less, for EME, but the following differences are important:

- EME is performed with other solvents for SLM as compared to HF^{3}LPME.
- EME is performed with pH conditions different from those used in HF^{3}LPME.
- The extraction voltage should be selected with care in EME.
- The current flowing in the extraction system should be measured in EME.

For EME of basic substances, solvents such as 2-nitrophenyl octyl ether (NPOE), 1-ethyl-2-nitrobenzene, and 1-isopropyl-4-nitrobenzene are typically used (2,24–28). For extraction of more polar substances, an ion-pair or another modifier is added to these solvents to facilitate the mass transfer of analytes across the SLM. Typical examples are di-(2-ethylhexyl) phosphate (DEHP) and tris-(2-ethylhexyl) phosphate (TEHP) (24,29,30). Acidic compounds have been extracted only a few times by EME and, in those cases, 1-octanol was used as the principal SLM (31,32).

The pH conditions in the sample and in the acceptor solution should be selected to ensure ionization of the analytes and promote their electrokinetic migration across the SLM. Extraction of basic analytes is carried out under acidic conditions, typically using dilute hydrochloric acid or formic acid in both the sample and the acceptor solution. However, several basic drugs have been extracted from physiological pH (pH 7.4) solutions when they are still ionized. Acidic analytes are extracted with alkaline conditions in the sample or acceptor solution, typically obtained with dilute sodium hydroxide or ammonia solution.

In EME, the driving force for the extraction is the electrical potential, and this parameter must be optimized. Normally, extraction recoveries increase with increasing voltage up to a certain level until there is no further gain in recovery, as illustrated in Figure 5. The optimal voltage must be established by experimental optimization, as this voltage is dependent on both the analytes and the composition of the SLM. Usually, voltages in the range of 5–100 V are used. During EME, the exact timing of the extraction is important for repeatable data, and we strongly recommend measuring the current flowing in the system. This is accomplished by a microammeter coupled in series with the cable from the power supply. We suggest not operating the system at currents higher than 100 µA because higher currents may cause bubble formation in both the sample and the acceptor solution because of excessive electrolysis.

Conclusion

This column installment focuses on practical considerations regarding HF^{3}LPME and describes the most important issues for a successful extraction. The first step in the development of a new HF^{3}LPME application is the choice of the hollow fiber, including

the material, size, and configuration. When deciding on the SLM, important factors to consider are the capability of the organic solvent to act as an intermediate extraction medium, the boiling point, and the water solubility. Some new experimental data regarding the leakage of the SLM into the aqueous samples are included in this column installment; likewise the suggestion of some new organic solvents that are usable in HF^{3}LPME are mentioned. The practical steps in an HF^{3}LPME procedure are covered in detail, including the handling of the dry hollow fiber, preparation of the SLM, loading of the acceptor solution, convection of the sample during the extraction, and finishing the extraction procedure. The importance of correct pH in the sample solution and in the acceptor solution is discussed. The central issues mentioned also are highly relevant in the procedure of EME, which has been introduced as a faster alternative to HF^{3}LPME.

Interest in HF^{3}LPME has been growing for the last decade, although ready-to-use equipment is still not commercially available. We hope that further work in this direction by instrument manufacturers will help HF^{3}LPME become a viable extraction method. Likewise, automation of the various steps described in this paper will establish HF^{3}LPME as a useful and robust sample preparation method in the future.

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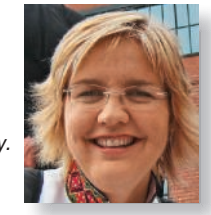

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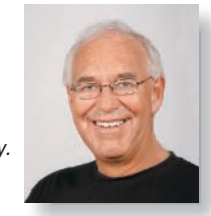

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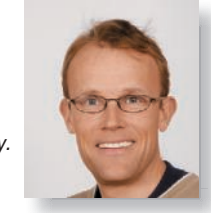

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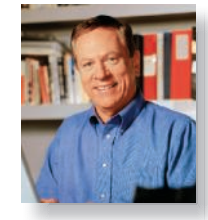

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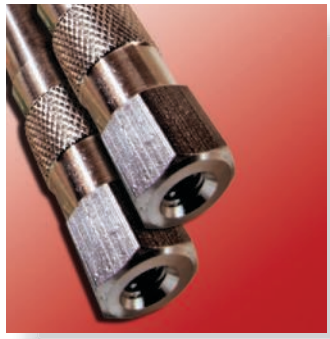

LC TROUBLESHOOTING

Troubleshooting Basics, Part III: Retention Problems

What causes peaks to appear where they don't belong?

John W. Dolan
LC Troubleshooting Editor

This is the third installment in a series focusing on some of the basic principles of troubleshooting liquid chromatography (LC) methods. First, we looked at some general practices for troubleshooting any LC problem (1). Then we looked at problems whose symptoms are related to pressure changes (2). This month, we'll concentrate on problems exhibited as abnormal retention times. As a means to organize the discussion, let's look at situations where retention times are too long, too short, or inconsistent.

What Controls Retention?

Before we look at specific problems, let's take a moment to consider the things that influence retention. We can categorize these as the mobile phase, the stationary phase (column), the hardware, the environment, and the sample. Let's simplify this discussion and assume that nothing has happened to the sample, such as degradation or other chemical changes.

The mobile phase can change because of an error in formulating it, such as a mistake in volumetric measurement or adjustment of the pH. If an error in formulating the mobile phase is suspected, it is best to make a new batch to see if it fixes the problem. Some mobile phases can change over time because of chemical degradation, selective evaporation of one component, or microbial growth in highly aqueous mobile phases. Again, reformulation is the best way to verify this problem source. Most of us use on-line mixing to prepare isocratic mobile phases. An error in instrument settings or hardware failure can be the cause of errors in on-line mixing. Check for proper degassing and pump operation, as well as the correct control-program settings. Sometimes hand-mixed mobile phases can be used as a check for on-line mixing, or alternate mixing channels can be used for both isocratic and gradient methods (for example, use the C and D solvent reservoirs instead of A and B in a four-solvent LC system).

The stationary phase in the column has a finite lifetime, generally in the 500–2000 sample range (or more), depending on the nature of the sample. Every column will die eventually, and some methods are harder on columns than others. For example, mobile phases outside the pH 2–8 region accelerate the degradation of silica-based columns. Some column types have shorter lifetimes than others. For example, cyano and amino columns are unlikely to last as long as C8 or C18 columns, which tend to be quite robust. In addition to changes in retention, column failure usually is accompanied by a rise in system pressure and an increase in peak tailing. Replacement of a suspect column with a new one is the easiest way to check for column-related problems.

System hardware problems that generate symptoms of changed retention most commonly are associated with pump malfunctions or leaks. Pump problems can be checked with a simple flow-rate measurement with a stop watch and volumetric flask. A secondary symptom of pump problems may be high, low, or fluctuating pressure. Low flow may be associated with faulty check valves, worn pump seals, air bubbles in the pump, or errors in pump settings. Cleaning, component replacement, or degassing should correct such problems. High flow rates usually are a result of improper settings.

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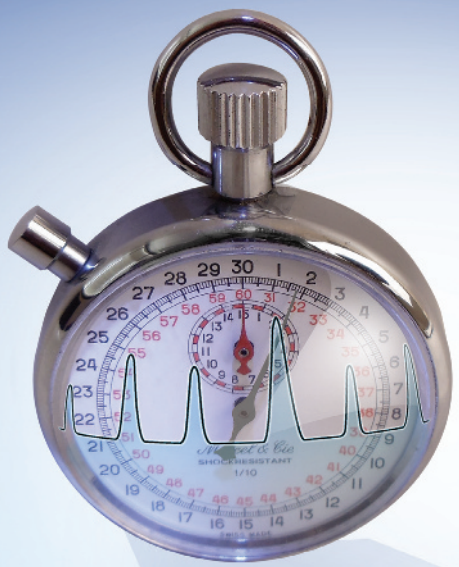

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The most common environmental cause of retention changes is a change in column temperature. This effect is common if the column oven is not used or is not working properly. Methods that operate under ambient conditions are highly susceptible to failure, especially if the laboratory temperature is not well controlled. In my travels, I have encountered laboratory temperatures ranging from 10 °C (central China in January) to 35 °C (Tel Aviv in June). If we use the rule of thumb that retention can change by 2% with each 1 °C change in temperature, you can imagine the result if the same method were run in both of those laboratories under ambient conditions! Use the column oven and make sure that it is operating properly.

Two Important Measurements

One tool that can be very useful in diagnosing the source of retention problems is the retention factor (also called the capacity factor, k'). Recall that the retention factor, k, is calculated as

$$k = (t_R - t_0)/t_0 \quad [1]$$

where t_R is the retention time and t_0 (sometimes abbreviated as t_M) is the column dead time, usually measured by the first disturbance in the baseline (the "solvent front"). Another useful calculation is the selectivity, or relative retention, α,

$$\alpha = k_2/k_1 \quad [2]$$

where k_1 and k_2 are the k-values for the first and second peaks of an adjacent peak pair, respectively.

Notice that changes in flow rate, whether intentional or due to a leak, will change both t_0 and t_R proportionally, so k will remain constant for such changes. On the other hand, chemical changes will change only t_R, so the k value is changed, too. Generally, when the k value is changed it does not change exactly the same for all peaks in the chromatogram. One way to confirm chemical changes in the system is to check the α value; if α changes, a chemical source of the problem is most likely. Because k and α are so useful in distinguishing between flow-related and chemical changes, it is a good idea to make these measurements a part of the process for troubleshooting retention-time problems.

Excessive Retention

When retention times increase and k-values stay constant, a flow-rate problem is indicated. Double-check the flow-rate setting to be sure you didn't make a mistake. Leaks and pump problems are the two most common remaining causes. Check for leaks throughout the system; these may or may not be accompanied by low system pressure, depending on the magnitude of the leak. Several possible problems related to the pump could be sources of increased retention. Air bubbles in the pump will also cause pressure fluctuations; thorough degassing of the mobile phase and purging the pump should correct such problems. If problems persist, check to be sure the frit in the mobile phase reservoir is not restricting flow to the pump. Faulty check valves or pump seals also can result in low flow and long retention times. Sonication of check valves will usually restore their function. Pump seal leakage often is accompanied by liquid dripping from the drain hole just behind the inlet check valve on most pumps. Check the maintenance records — if the pump seal has been in use for a year or more, replace it.

When a change in k values (and often α) is observed, you have evidence that a change in system chemistry is responsible for an increase in retention. The easiest way to check this is to make a new batch of mobile phase. If this does not correct the problem, replace the column.

A final possible source of increased retention is a drop in the column temperature. As mentioned above, a 2% increase in retention for a 1 °C drop in temperature is common. Based on the magnitude of the retention change, you can estimate the temperature change and see if it is a reasonable cause of retention. Has the oven failed, did you forget to turn it on, or are you relying on ambient operating conditions? Any of these sources can account for increased retention.

Retention Is Too Small

When retention times are smaller than they normally appear, a flow-rate change is highly unlikely, unless you made an error in one of the settings. This is because decreases in flow due to leaks or other malfunctions are not uncommon, but there are no corresponding causes that result in higher-than-normal flow rates that are necessary for reduced retention.

As with retention times that are too long, do a stepwise elimination of problem sources by first making up a new batch of mobile phase. If this approach doesn't fix the problem, replace the column. Temperatures that are higher than normal will cause a drop in retention; the sources of temperature problems are the same as for excess retention.

Fluctuating Retention Times

Usually, an increase or decrease in retention will not be an abrupt change. If it is, the cause is likely related to operator intervention, such as improper formulation of a new batch of mobile phase, installing the wrong column, or changing a column-oven setting. More commonly, retention will gradually increase or decrease over tens, hundreds, or thousands of samples, or it cycles over a 24-h period. Cycling retention is commonly correlated with ambient methods and a laboratory temperature that changes throughout the day and night.

Retention drift that occurs over hundreds or perhaps thousands of injections is most likely because of normal column aging. Drift from column aging usually will be accompanied by a gradual increase in pressure and an increase in peak tailing. Often, a shift in relative retention also will be observed when α-values are calculated. If the column is suspected, replace it to see if the problem is corrected.

Shorter-term retention drift may be caused by the mobile phase. Although fairly rare, it is possible to selectively evaporate a volatile component of the mobile phase, especially if helium sparging is used for degassing. Retention drift resulting from a pH shift can occur if the buffer is outside its optimal buffering region, generally ±1 pH unit from its pK_a. The use of volatile buffers, as is

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common with LC–mass spectrometry (MS) mobile phases, may shorten the stable lifetime of the mobile phase, so daily reformulation may be a wise practice. Make up a new batch of mobile phase if the mobile phase is suspected, and be sure to adjust the pH before any organic solvent is added.

Problem Prevention

To avoid retention problems, make sure to keep the instrument in good operating condition by servicing it regularly. A routine preventive maintenance session should be done on an annual basis at a minimum, and more often for heavily used LC systems.

Because column temperature changes can have such a profound influence on retention time, it is wise to always use a column oven. Many ovens do not control the temperature well near room temperature; a good practice is to use a minimum operating temperature of 30–35 °C so that good temperature control is ensured. It may take 30 min or longer for the column oven to reach a stable temperature. Be sure to use the solvent preheater that is included with most column ovens. The most common preheater implementation is a piece of capillary tubing that is embedded in the aluminum block of the oven.

Columns usually will last for more than 1000 injections. When this number of samples has been analyzed, the cost-per-sample for the column is less than 5% of the overall per-sample cost of analysis. My feeling is that at this point it isn't worth my time to do anything more than flush the column with strong solvent (for example, acetonitrile or methanol); if this doesn't restore the column, replace it. Guard columns or sample preparation both will extend the column life, but they have their associated costs, which may make the economics of their use questionable. A 0.5-µm in-line filter between the autosampler and column will help keep particulate matter from blocking the column inlet frit, but it will not influence retention-related problems. Another good practice for extending column life is to use a single column for each method. When the same column is used for multiple methods, sometimes unimportant sample components from one method will interfere with another method.

Mobile phases have finite lifetimes, too. My recommendation is to replace any buffer at least once a week and organic-based mobile-phase components at least monthly. It is a good idea to replace the reservoir with a clean one whenever the mobile phase is replaced so that prior contamination doesn't get transferred to the new mobile phase.

If you pay close attention to patterns in retention changes, correlations with mobile-phase use, and column history, you can establish expected replacement cycles for each component of each method. After such patterns are defined, you can put in place preventive-maintenance and component-replacement practices that will help you avoid most retention-related problems. Armed with an understanding of which variable most strongly influences retention in your particular method, you'll be able to more quickly identify and correct problems when they occur.

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(2) J.W. Dolan, *LCGC* **29**(9), 818–824 (2011).

John W. Dolan

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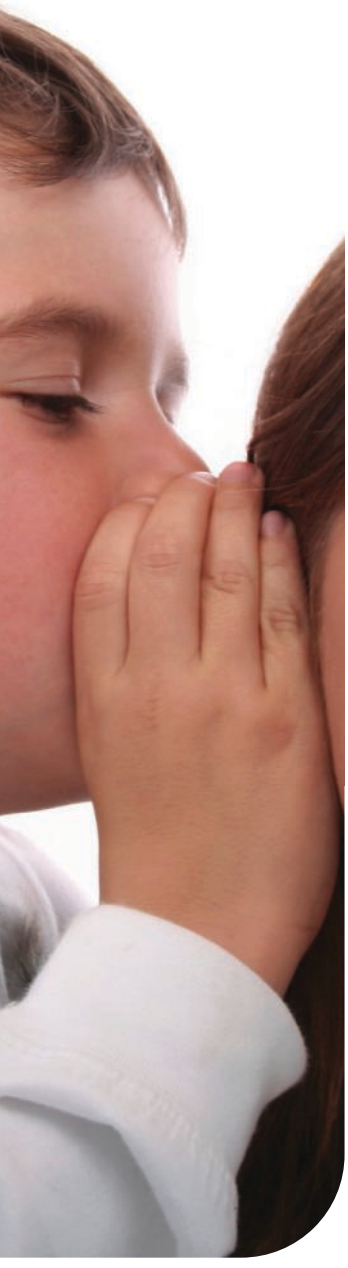

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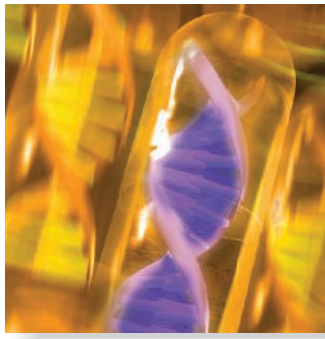

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BIOTECHNOLOGY TODAY

Current Applications of UHPLC in Biotechnology, Part II: Proteins and Glycans

As mentioned in part I of this series, there are four major applications areas of ultrahigh-pressure liquid chromatography (UHPLC) for biotechnology: peptide mapping, amino acid analysis (AAA), intact protein and antibody analysis, and glycan analysis or glycoprofiling. The first two of these areas were extensively covered in Part I. This installment will emphasize intact protein–antibody analysis and glycan analysis or glycoprofiling and why they are used.

Thomas E. Wheat *is a guest coauthor of this month's column.* ***I.S. Krull*** *and* ***A. Rathore*** *are the editors of Biotechnology Today.*

In part I of this two-part series on the current usage of ultrahigh-pressure liquid chromatography (UHPLC) in biotechnology, we introduced the fundamentals of performing UHPLC and discussed specific applications for peptide mapping and amino acid analysis (AAA) (1). Readers are encouraged to read part I before part II. There are four major applications areas where UHPLC has become important for biotechnology: peptide mapping, amino acid analysis, intact protein characterization, and glycan analysis or glycoprofiling. These applications are essential analytical challenges in biopharmaceutical development, in which UHPLC has proven valuable (2–6). The first two topics were discussed in part I; here, we will focus on the latter two (6,7).

Using much smaller particle diameter packing materials, and shorter or narrower columns, has improved virtually all chromatography for larger proteins or antibodies, as well as for their smaller cousins. Such trends will, of course, continue into the future. When using UHPLC for biotechnology applications, perhaps the very first areas of emphasis have been intact proteins, especially mixtures of protein variants in a drug substance (DS), or antibody variants, isoforms, or glycoforms.

The structure of intact proteins presents a difficult analytical problem because the pharmacological activity of these large molecules is altered by small chemical changes to the protein. The modifications affect a tiny fraction of the chemical properties, so it is necessary to use multiple modes of separation to detect and measure them. The common approaches of reversed-phase chromatography, size-exclusion chromatography (SEC), and ion-exchange chromatography (IEC) are now available in UHPLC.

The reversed-phase high performance liquid chromatography (HPLC) of intact proteins, especially large molecules such as antibodies, is usually characterized by broad, diffuse, and poorly resolved peaks, with low plate counts and often large asymmetry values. These molecules are the "bad actors" of HPLC because their high molecular weights, slow mass transfer, and low diffusion coefficients lead to large peak volumes. Specific chemical interactions also degrade the analysis through mixed modes of separation (hydrophobic and hydrophilic patches and ionic binding), as well as poor solubility in most HPLC solvents. When UHPLC materials were being developed for proteins, it was efficient to consider both implementation of small particles with shorter diffusion distances and optimized particle chemistry for reduced chemical interactions. As illustrated in some of the figures in part I, this combination has facilitated using UHPLC for proteins or antibodies. For example, Figure 1 in this installment compares two different columns with the same base particle, bonded phase, and bonding chemistry, operated under identical conditions in two different particle sizes: 3.5 µm and 1.7 µm. It is a controlled comparison between conventional HPLC (3.5-µm particles) and UHPLC (1.7-µm particles). The relative retentions for all of the peaks are the same in the two chromatograms, but more resolution is apparent with the smaller particles. The sample consists of light chains

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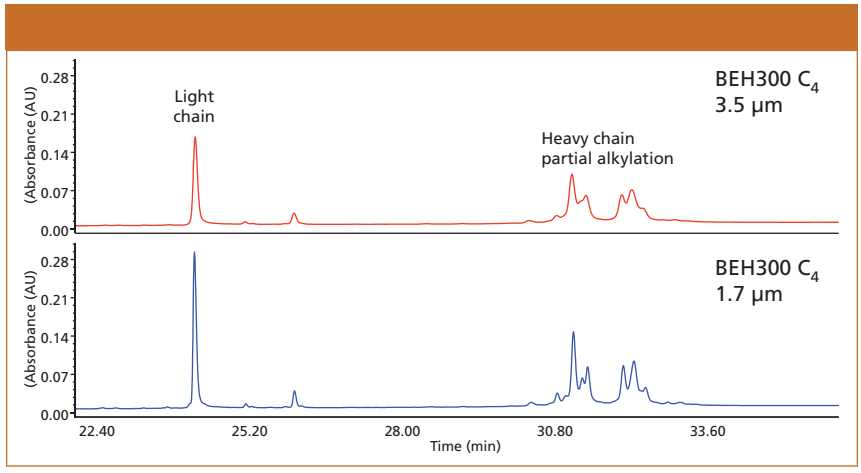

Figure 1: UHPLC separation of light and heavy chains of a reduced and partially alkylated monoclonal antibody (IgG). (Reprinted with permission from reference 8.)

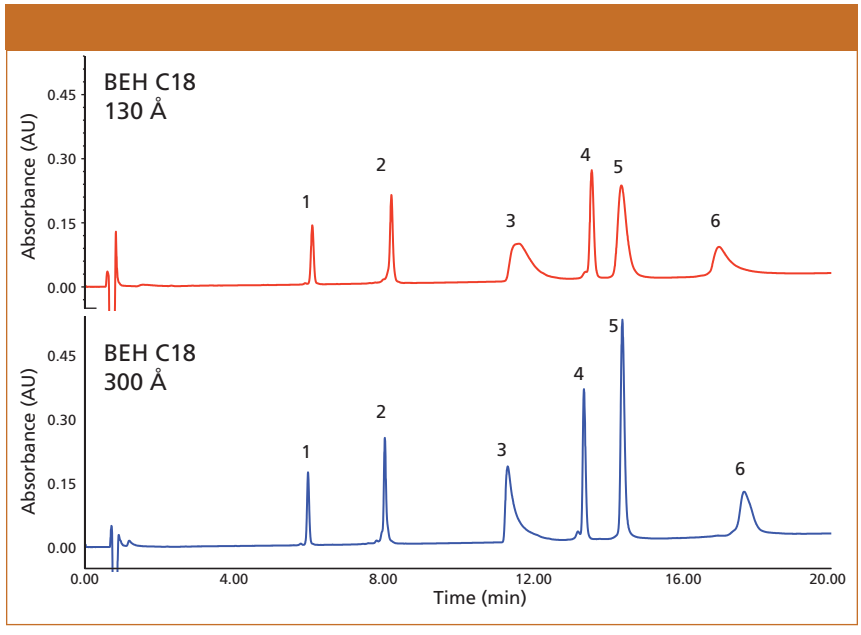

Figure 2: In reversed-phase UHPLC, there is an effect of pore size on resolution and peak shapes possible for a typical mixture of proteins, as indicated. Peaks: 1 = ribonuclease, 2 = cytochrome *c*, 3 = bovine serum albumin (BSA), 4 = β-lactoglobulin, 5 = enolase, 6 = phosphorylase b. (Reprinted with permission from reference 8.)

(LC) and heavy chains (HC) of an antibody (immunoglobulin, or IgG) with the heavy chains having different degrees of glycosylation or modifications (post-translational modifications, or PTMs). In addition, the sample was reduced and intentionally alkylated only partially to further increase the sample heterogeneity as a test of chromatographic resolving power. The improved resolution with the UHPLC packing material and instrumentation is apparent. It also should be noted that the run time could be reduced by using different dimensions of the columns. Thus, the area of intact proteins remains one of the four most important applications of UHPLC in use today. It will surely remain so in the future.

Intact protein profiling via UHPLC serves several functions in regulatory submittals. It provides a chromatographic profile of the number of variants present and their relative ratios (percent peak areas), and it helps to define lot-to-lot variations among different production batches. It is important that each peak in such a DS profile be uniform, homogeneous, and a single variant, if possible. Such intact protein profiling then defines a "typical" production batch, as well as batch-to-batch variations and their limitations. It also is a very important and reliable application for comparing biosimilars and proprietary drug substances.

The other major application area we will emphasize in this installment is glycan analysis or glycoprofiling. The sugars that are attached to proteins have profound effects on the biological properties of proteins, including binding specificity, stability, affinity, and potential immunogenicity. The analysis of oligosaccharides derived from glycoproteins or antibodies is, therefore, a fundamental required characterization test. The biosynthesis of pharmaceutical proteins yields a mixture of proteins with the same amino acid sequence, but with variable glycans attached. Because the vast majority of biotechnology-derived DSs today contain glycoforms as the variants, it has become *de rigueur* for any regulatory submittal to define the nature of glycans found in the preparation of glycoproteins. This characterization includes determining the distribution of all the glycans found in the sample, the proportions of each protein glycoform, and the location or position of attachment of the glycan to specific amino acids in the protein backbone. The proportion of the glycoforms is most often measured using intact-protein liquid chromatography–mass spectrometry (LC–MS), and points of attachment are characterized as part of peptide mapping, as discussed in part I of this column. *Glycan analysis* or *glycoprofiling* really refers to describing all the oligosaccharides or monosaccharides (if any) that are found on a total mixture of glycoproteins, as well as their relative or absolute amounts.

Each oligosaccharide must be structurally defined or sequenced, often versus authentic reference standards, and chromatograms must be provided in a submittal that shows the glycoprofile of the glycoproteins versus authentic reference standards of the glycans found. A glycan is an oligosaccharide, often composed of different monosaccharides and exhibiting extensive branching. These PTMs can be N-linked or O-linked, depending on the protein and on the cell system used for synthesis. A recombinant protein to

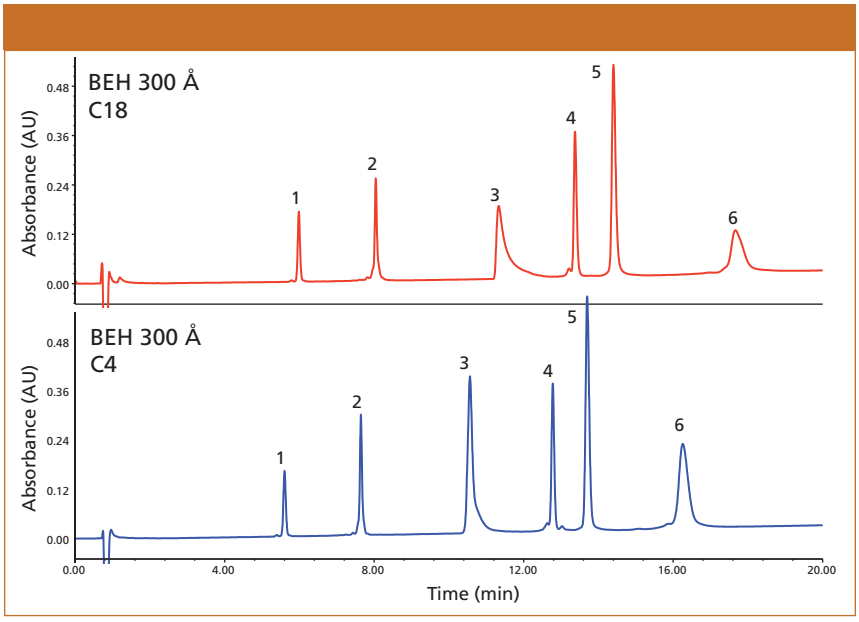

Figure 3: Effect of varying bonded phase chain length in reversed-phase UHPLC separation of a mixture of standard proteins, as indicated. Peaks: 1 = ribonuclease, 2 = cytochrome *c*, 3 = BSA, 4 = β-lactoglobulin, 5 = enolase, 6 = phosphorylase b. (Reprinted with permission from reference 8.)

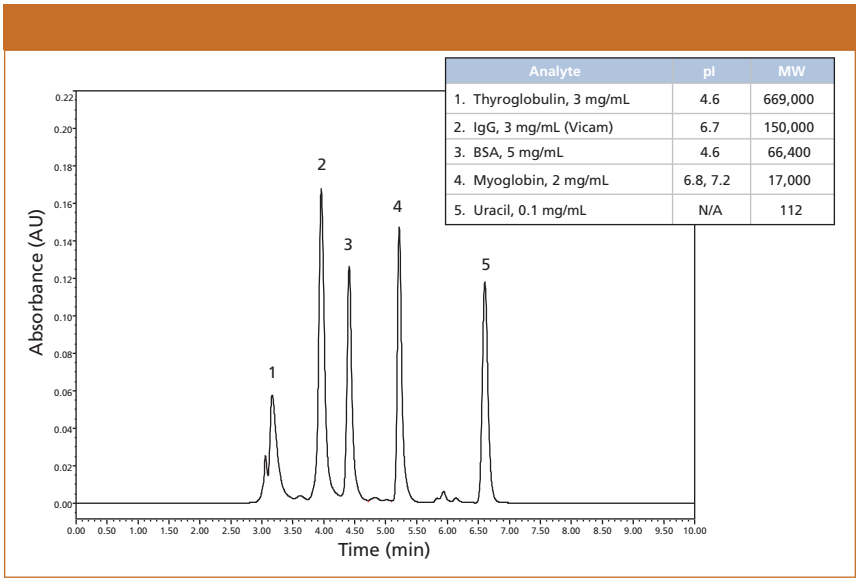

Figure 4: Size-exclusion chromatography of standard proteins in UHPLC. (Reprinted with permission from reference 8.)

be used as a biotherapeutic will always be a mixture of glycoforms reflecting the heterogeneity of the attached glycans. For analysis, the sugars are released by chemical or enzymatic methods. The released glycans are then qualitatively and quantitatively analyzed. There are numerous methods now available for glycoprofiling, but two have become more popular than others. The two popular, or common, techniques are high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD) and hydrophilic liquid interaction chromatography (HILIC) with fluorescence detection of derivatized sugars. Often, these and other techniques are applied to initially derivatized glycans.

As with intact protein profiling, glycoprofiling serves several functions in regulatory submittals. It defines the nature of the glycan pool that is present, as another way to structurally define the mixture of glycoproteins or others. It helps to demonstrate chemical equivalency, lot-to-lot, for release testing, and it can be very useful when comparing biosimilars to innovator glycoprotein DSs or drug products (DPs). It also provides a demonstration that the drug production process is within certain tolerance limits of variabilities. If the glycoprofiling finds a certain mixture of glycans present, then these also must be found on one or more of the glycoproteins in the DS. It is often possible to define the exact amino acid sequence, as well as glycan and glycan locations on every variant in a glycoprotein DS. These must agree, batch-to-batch, or else something is amiss in the production process.

Intact Protein Analysis

As mentioned in part I and above, there are serious challenges for successful protein separations. In general, reversed-phase HPLC applications have been less than ideal, in terms of final peak shapes, efficiencies, resolutions, and peak capacities. Success requires the detection of small chemical differences, often between quite large molecules (molecular weight, size, and shape). Successful UHPLC now employs a variety of analytical techniques that are sensitive to different properties of the proteins (hydrophobic, hydrophilic, ion exchange, hydrogen bonding, and others). Currently, the most popular techniques are IEC for changes in net charges of the proteins (salt or pH gradients are popular); SEC for changes in size or aggregation; and reversed-phase chromatography for detecting a wide range of small changes in the proteins. Success in each of these modes depends on choosing the ideal packing material, particle size, pore size, length of ligand (C18 versus C4), mobile phase, gradients, flow rates, temperature, and other variables available in UHPLC. In developing reversed-phase UHPLC protein separations, it was not sufficient to just use sub-2-µm particles. It also was necessary to re-examine the properties of the base particle, the pore size, the bonded phase, and the bonding chemistry (9–11).

Figure 2 illustrates the chromatographic differences as a consequence of

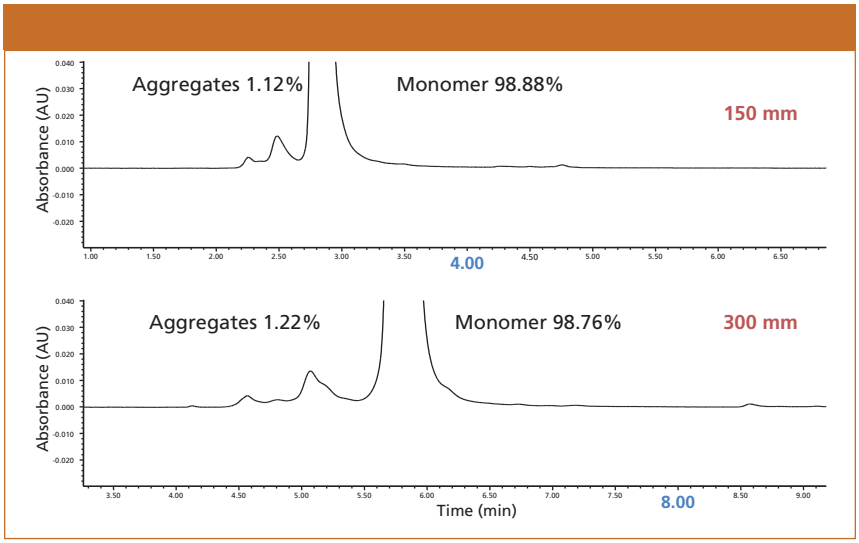

Figure 5: Size-exclusion UHPLC of antibody aggregates, as a function of column length. (Reprinted with permission from reference 8.)

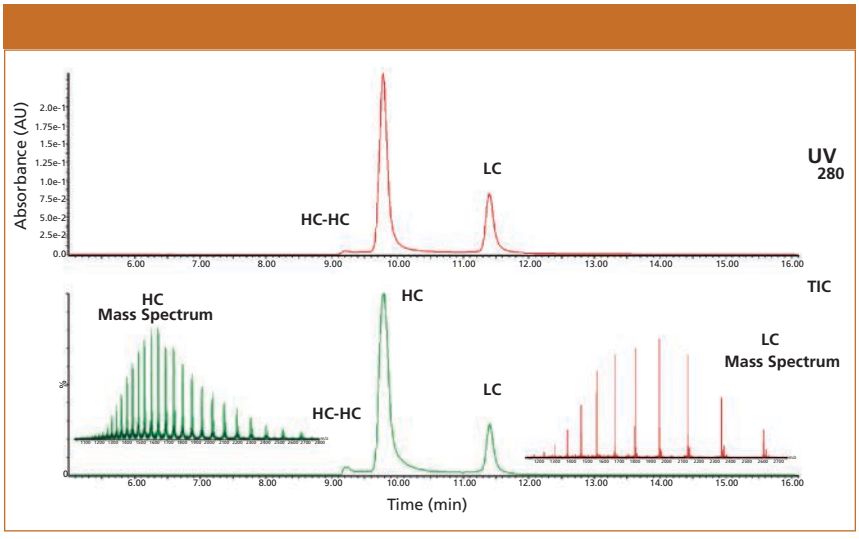

Figure 6: Size-exclusion chromatography–UV-MS analysis of a reduced and alkylated monoclonal antibody, showing both UV and MS (total ion chromatogram) chromatograms and mass spectra for both heavy chains and light chains (12).

pore size in the packing material for the same mixture of proteins and mobile phase conditions (8). The larger pore size leads to improved peak shapes and narrower peaks, with minimal effect on selectivity. However, some proteins still do not give the sharp symmetrical peaks expected for UHPLC. For example, peak 3 in Figure 2 is bovine serum albumin (BSA), for which the separation includes several variants that are coeluted under this one, broadened peak. This is not a characteristic of the UHPLC conditions, but rather a reflection of the limitations of reversed-phase mechanisms to discriminate among small chemical changes on a very large molecule. However, larger pore sizes generally allow the proteins to diffuse more freely and rapidly in and out of the pores, where the majority of the interactions with the bonded phase occur. Differences in distribution coefficients and mass transfer of the proteins can thereby effect overall improved peak shapes and improved separations. It is really a matter of the proteins being able to approach equilibrium interaction with the surface ligands of the bonded phase. Unfortunately, it is not possible to suggest a molecular weight limit in which the separation must be done on 300-Å pore packings. The protein assumes a three-dimensional structure that is usually larger than the native protein (but different for every protein sequence) because of the disordering of the protein structure at low pH in relatively high concentrations of organic solvents.

A similar set of experiments examined the effect of varying the bonded phase chain length (C18 versus C4), again in reversed-phase-UHPLC, on the peak shapes for a mixture of standard proteins (Figure 3) (8). In this particular illustration, all peak shapes, peak narrowness (asymmetry factor), peak heights, resolutions, and plate counts are improved by going to the smaller C4 chain length (all other particle and mobile-phase conditions were identical). With large proteins, their interactions with very hydrophobic ligands, such as C18, lead to slower mass transfer, stronger hydrophobic–hydrophobic interactions with the proteins, and thus peak tailing, loss of peak shape, and loss of efficiency, as well as overall decreased resolutions. With much smaller peptides, C18 is usually the stationary phase of choice, but for larger proteins, C4 or even C3 is preferred for all of the reasons stated earlier. It is important, however, to recognize that there is no obvious cutoff molecular weight whereby analysts should automatically choose the shorter chain bonded phase. As with pore size, this observation is related to the sequence-dependent disordering of protein structures.

An additional operational parameter to consider is that mass transfer is often improved at higher temperatures. The kinetics of equilibrium between the mobile and stationary phase are faster because of a reduced viscosity and resistance to flow, leading to improved mass transfer effects. Recoveries tend to be improved at elevated temperatures. For these reasons, it is often suggested that reversed-phase separations be performed at 70 °C. However, some proteins show worse peak shapes at the higher temperature. The chromatographic behavior of proteins at low pH and with organic solvents reflects a complicated interplay among mass transfer, solubility, and the equilibrium of disordered structures. Good practice seems to favor testing each sample at both low and

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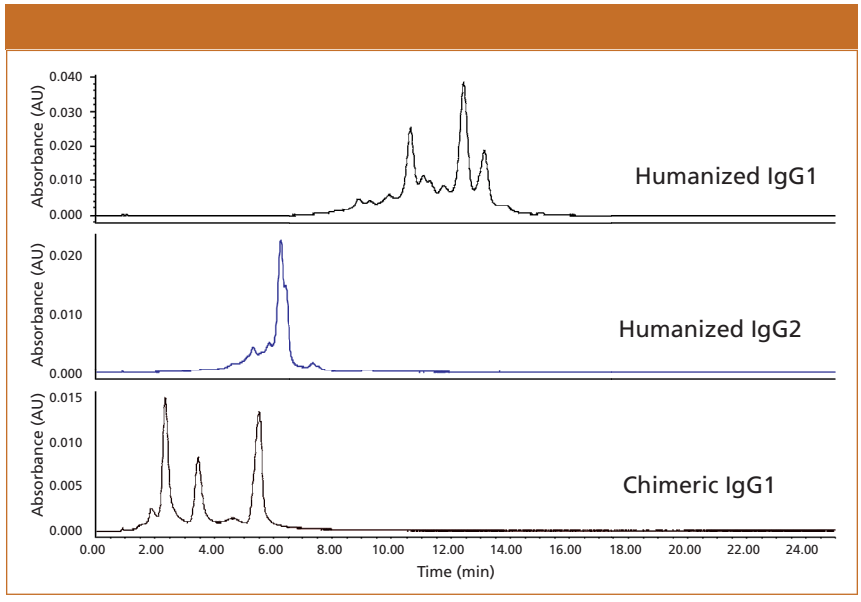

Figure 7: Comparison of three different antibody samples by ion-exchange chromatography using larger particles that mimic superficially porous materials (13).

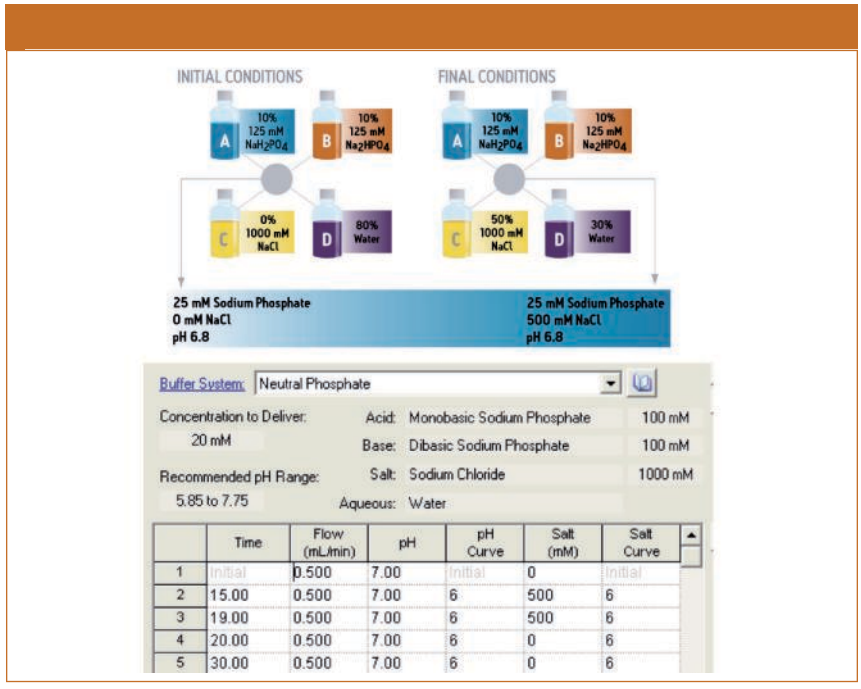

Figure 8: The use of Auto-Blend Plus software allows programming of a four-solvent pumping system directly in units of pH and salt concentrations in ion-exchange UHPLC of proteins or antibodies (14).

high temperatures, perhaps 45 °C and 75 °C, to identify the range to be used for optimizing the final separations.

Several other operational parameters are of importance in the reversed-phase-UHPLC analysis of proteins. Acidic mobile phase modifiers (formic acid, trifluoroacetic acid, and others) are generally used and higher concentrations of these reagents lead to better peak shapes and resolutions. Trifluoroacetic acid is preferred for the best peak shape and resolution, and formic acid gives better sensitivity and spectral quality with MS detection.

The effects of flow rate and column length also can be useful and have the expected effects on resolution. In the case of column length, longer columns usually lead to improved or better resolution of proteins. Lower flow rates improve peak shapes and resolution because the large protein molecules diffuse slowly in and out of the pores. This effect has been underutilized in developing protein separations because the run times increase significantly. It has often been observed, however, that a shorter column at lower flow rates will outperform a longer column at scaled flow rates that give the same run time. Computerized method development software routines, usually commercially available today, can also be useful for systematically optimizing UHPLC conditions (9–11).

SEC has traditionally been a critical tool for the analysis of biopolymers. UHPLC columns for this separation mechanism are just now becoming available. Perhaps the earliest players in biopolymer separations were packings such as Sephadex or Sepharose, polysaccharides, that were used in open-column, low-pressure biopolymer separations on semipreparative and preparative scales. Analytical SEC became popular at least 40 years ago, with the introduction of HPLC columns with hydrophilic coatings or bonded phases on silica particles. More recently, packings have been introduced at the UHPLC scale that are able to withstand high back pressures, higher temperatures, and higher flow rates, and they can resolve proteins, aggregates, antibodies, and fragments in one analysis. Although SEC has traditionally been a low-resolution technique because of the size and slow mass transfer of these analytes (often with extensive band broadening), modern size-exclusion UHPLC gives substantially better resolution in shorter run times. Figure 4 illustrates a typical separation of four proteins, ranging in molecular weight from 17,000 to 669,000 Da, along with a completely included, low-molecular-weight analyte, uracil. The four proteins are all baseline resolved in under 5.50 min, which is considerably less than what has been possible with conventional size-exclusion HPLC, for the very same proteins. Peak shapes are excellent with very low asymmetry factors, high plate counts, and baseline resolution in under 5.50 min. This is truly excellent

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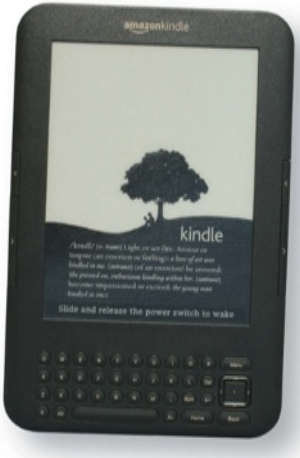

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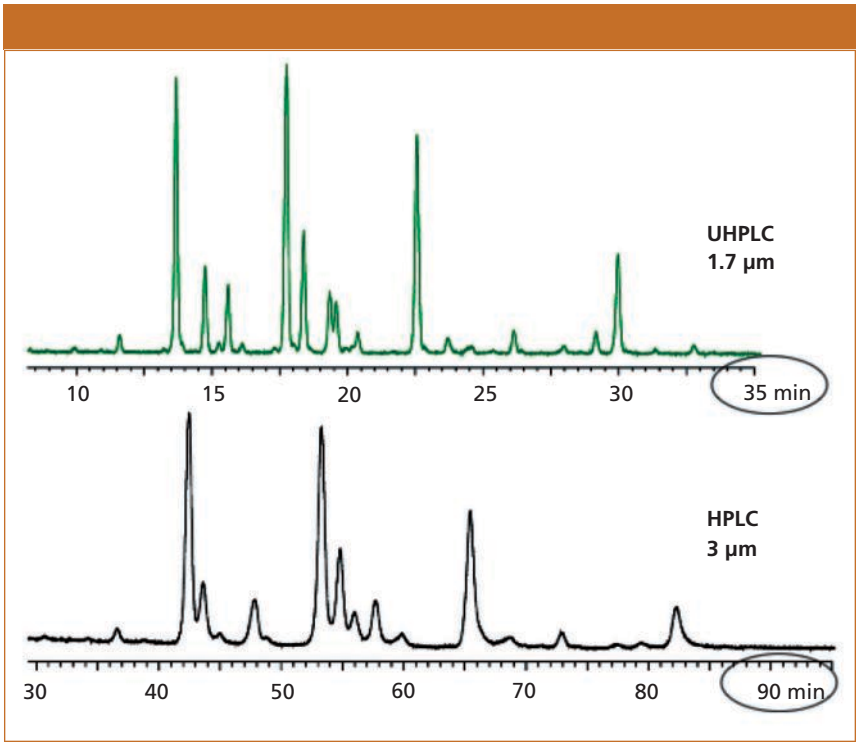

Figure 9: Comparison of a conventional 3-µm HPLC column with a 1.7-µm UHPLC column for the analysis of 2-AB labeled glycans from human IgG. Column: Waters BEH glycan (HILIC). (Reprinted with permission from reference 21.)

size-exclusion UHPLC, perhaps the very best ever demonstrated and far superior to conventional size-exclusion HPLC.

SEC has become a very important technique in biotechnology, in part, because it is able to resolve high-molecular-weight aggregates of proteins and especially of antibodies (see Figure 5). Aggregates (also termed associates), in general, are noncovalent clusters of a monomer, which are usually formed in equilibrium with the monomer as a function of temperature, time, solvent conditions, and even pressure. Figure 5 illustrates the ability of modern size-exclusion UHPLC to resolve fully to the baseline all aggregates present, even at 1.12–1.22% composition versus the monomer. These are almost all baseline resolved. Aggregates can be dimers, trimers, and higher order species of the monomer, or mixed aggregates with various combinations of heavy and light chains (IgG) present. These are usually considered impurities of the DS, often being immunogenic. Regulatory agencies want to know how many and how much of these aggregates are present in the final DP and if they are immunogenic in humans. They also can ask to have such aggregates removed before the DP can go to market (12).

For characterization of the peaks observed in SEC, both multiple angle light scattering (MALS) and MS, readily interfaced with UHPLC, can provide molecular weight information (15). When considering the use of information-rich detectors with SEC, it is important to remember that the technique measures the size and shape of a protein in solution. It has the great advantage that the separation can be conducted under the conditions where the native, biologically active structure is maintained. However, those separation conditions may be inconsistent with the best performance of the detector. And, of course, the optimal conditions for detection may disturb the protein's structure. This is particularly relevant for MS detection, which performs best in a volatile mobile phase at low pH with relatively high concentrations of organic solvent. SEC can be performed under these conditions, but the observed elution volume will no longer reflect the structure of the protein as it existed in its native, biologically active state.

Despite this, there is value in SEC-MS. As shown in Figure 6, the SEC separation of a reduced and alkylated monoclonal antibody can be executed in a mobile phase that is optimal for electrospray ionization (ESI) (12). The heavy chain, light chain, covalent dimers, and clips are conveniently separated, and the mass of each is measured. This analysis is very useful for high-throughput assays such as reaction monitoring or fraction screening. There is no requirement for gradient re-equilibration and no need to develop methods for specific samples in this approach. Although SEC–MS is not a direct path to characterizing structural variants, it is still a source of valuable information about the protein sample (12).

The fundamental question in biopharmaceutical analysis is the composition of the original sample, with respect to protein three-dimensional structure, and especially aggregation. Several approaches to this problem, alone or in conjunction with SEC, are available. Perhaps in a future "Biotechnology Today" column we will discuss at greater length the advantages of using MALS, SEC–MALS, analytical ultracentrifugation, and field-flow fractionation for both protein monomer and aggregate studies.

IEC is the third significant chromatographic separation mode applied to biopharmaceutical characterizations. To date, true UHPLC packing materials suitable for protein separations have not become commercially available. New materials, however, have been introduced by several manufacturers that give higher resolution chromatography than was available even a few years ago. These materials represent recent advances in surface chemistry that maximize protein selectivity and minimize secondary interactions. These materials also exhibit the reduced band-broadening characteristic of UHPLC on sub-2-µm particles. But all of the materials use large particles that mimic superficially porous materials by one of several, proprietary mechanisms. These modern packings do offer improved resolution of complex protein samples, as shown in Figure 7 (13).

Table I: Advantages of UHPLC for biopharmaceutical analysis
Improved molecular diffusion and mass transfer
Improved peak efficiencies (*N*) and plate counts
Lower HETP values
Sharper and narrower peaks, narrower bandwidths
Improved peak symmetry values
More symmetrical peak shapes
Greater peak capacity
Improved baseline peak resolutions
Faster sample throughput
Shorter analysis times
Greater productivity (number of samples per hour)
Shorter retention times
Reduced solvent and sample usage
Reduced instrumentation time per sample
Reduced analysis costs per sample
Ability to perform faster and improved separations in all types of chromatographic separations — SEC, IEC, reversed-phase chromatography, HILIC, and others

It also is interesting to observe that IEC analysis of proteins has benefited from the recent developments in instrument design and control that began as refinements to meet the requirements of UHPLC separation mechanisms. Dispersion in the sample fluid path was minimized and more exact control of mobile-phase delivery was established. This has been extended to method programming tools that are specific to protein chromatography today. Because protein separations are most effectively adjusted by optimizing pH and ionic strength, it proved useful to develop algorithms (Auto-Blend Plus Technology, Waters Corporation, Milford, Massachusetts) that allow programming of four solvent pumping systems, directly in units of pH and salt concentration, as shown in Figure 8 (14).

We have now considered three ways to analyze proteins. Each is based on different properties of the molecules, so all are employed to help ensure complete characterization of the different kinds of variation that can occur in protein structures. Now, let's move on to describe the analysis of one of the most important kinds of PTMs of biopharmaceutical proteins today — the attachment of glycans.

Glycoprofiling (Glycan Analysis)

As mentioned above, a key analytical technique that has become required in virtually all regulatory submittals of glycoproteins involves total glycan and monosaccharide analyses. In general, glycoproteins contain glycans, or oligosaccharides (sugars), and usually do not contain attached monosaccharides. Characterization of any glycoprotein requires the determination of the sugars that are present, measurement of their configurations as glycans, determination of the site or sites of attachment on the protein, and finally, the distribution of glycoforms (also known as *variants, PTMs,* or *isoforms*) of the protein within the sample.

One of the quality control and characterization methods available today first releases all bound glycans (or just N-linked glycans first), and then digests or hydrolyzes the freed glycans into their monosaccharide constituents. Then, the monosaccharides are monitored by a variety of accepted techniques, including HPAEC–PAD; fluorescence derivatization of monosaccharides followed by HPLC with UV and fluorescence detection; or permethylation followed by gas chromatography–mass spectrometry (GC–MS) analysis of the derivatized sugars (16,17). The qualitative and quantitative analyses for these monosaccharides then become lot-release and comparative assays to demonstrate consistency of production of the glycoprotein DS. It also serves to confirm the nature of the components in the DS, because any changes in specific glycoprotein components would change the nature of the monosaccharide profiling. Today, monosaccharide analysis is a routinely used method to confirm lot-release consistency for individual glycoproteins or mixtures.

To obtain more-complete information on the biological properties of the glycans, it is necessary to describe how the monosaccharides are assembled into the oligosaccharides on the surface of the protein. This description ultimately specifies the various compositions, sequences, chain lengths, linkages, and branching. This complicated analysis, true glycoprofiling (also known as *glycan analysis*), typically combines several kinds of information for complete characterization. The process begins with release of the N- or O-glycans by either chemical or enzymatic means. All glycans can be released together using base-catalyzed hydrolysis of intact glycoproteins or by hydrazinolysis. For characterizing biopharmaceuticals, N-linked glycans are usually the focus of analysis, and they are commonly released using specific enzymes, particularly PNGase F or G.

There are numerous methods for identifying these released glycans and, then, generating a glycoprofile. These now-routine assay methods are like other chromatographic assays in that the sample in question can often be compared to an authentic standard of pure, characterized glycans at known concentrations. As with all assays, a more elaborate validation process, including multiple kinds of information, supports the standard in use and the identification of the components derived from the glycoproteins. Several separation techniques have by now proven suitable for assaying biopharmaceutical glycoproteins, as explained below.

HPAEC–PAD was the first routine assay method developed several years ago. More recently, techniques involving HPLC and UHPLC or high

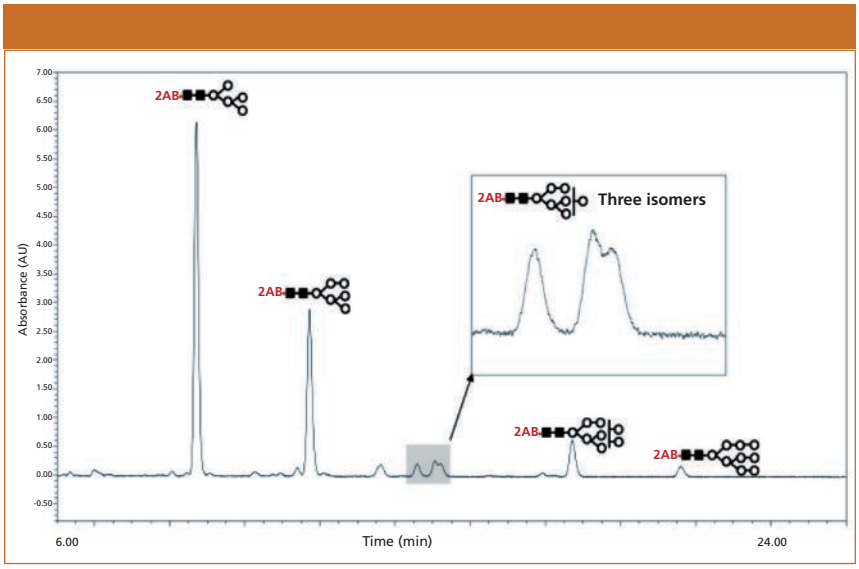

Figure 10: UHPLC analysis of 2-AB glycans derived from ribonuclease B glycans. Peak identification was done using UHPLC with electrospray ionization MS detection under the same gradient conditions. (Reprinted with permisison from reference 21.)

performance capillary electrophoresis (HPCE) have become common and accepted. There is significant literature describing HPCE of glycans that can be located through the Beckman Coulter (Indianapolis, Indiana) web site (18). Other analytical instrument vendors also offer HPCE instrumentation and applications for glycoprofiling (for example, Agilent Technologies in Santa Clara, California).

However, the prevailing analytical methods invoked by most biotechnology firms involve some form of tagging the released glycans with UV- or fluorescence-active reagents, followed by appropriate UHPLC separations (reversed-phase chromatography, IEC, or HILIC) (19,20). In general, there is a great deal of literature on HPLC methods for providing a glycoprofile, usually with some form of organic tagging before separation and detection (16,17). Perhaps the most common reagent in vogue today is 2-aminobenzamide, or 2-AB. 2-AB and other commonly used reagents are compatible with fluorescence detection for best sensitivity, which is why UHPLC with fluorescence detection is rapidly becoming the standard method for glycoprofiling (Figure 9). Again, UHPLC conditions provide a reduced total elution time compared with conventional HPLC, improved resolution, improved peak symmetry and shapes, higher peak capacity, and the other attributes indicated in Table I.

There are several ways to identify the individual glycans in a chromatogram, as illustrated in Figure 9. One approach is to inject a known mixture of tagged glycan standards that are expected or known to be found in the specific sample, and then compare elution times and peak shapes. Peak identification can be confirmed by coupling the separation with both UV–fluorescence and ESI-MS detection. The MS system would provide the molecular weight of each 2-AB glycan, from which the parent glycan is readily derived, and this is then compared with the molecular weights of the known, standard glycans. Unequivocal identification of the peaks is not always possible, because many of the biologically significant structural variations have isobaric linkage and positional isomers. However, usually the high-resolution MS fragmentation patterns, especially cross-ring glycan fragmentations, are different for isobaric structural variations and they can be differentiated. Fragmentation patterns using collisionally induced dissociation (CID) or electron transfer dissociation–electron capture dissociation (ETD–ECD) of the intact 2-AB glycans do not always distinguish these isobaric isomers. The MS data can be described as consistent with a proposed glycan structure, but that must be combined with other analytical determinations to provide absolute confirmation of their structures.

Many techniques are commonly used for complete determination of glycan structure as a part of validating the routine assay. This topic really ranges beyond the scope of this review, but we can briefly mention some of the common choices. One of the most powerful techniques is enzymatic (exoglycosidase) digestion of the tagged glycan, releasing one end-group monosaccharide at a time, and determining the shifts in elution times and molecular weights (with online ESI-MS) for the original glycan. By using a combination of enzymes with different specificity, both the sequence and the linkages can be deduced. Naturally, MS is a convenient and very popular tool for the characterization. It is used in combination with suitable databases and fragmentation patterns of standard, known glycans that are already well derived. Both matrix-assisted laser desorption–ionization time-of-flight mass spectrometry (MALDI TOF-MS) with in-source decay (ISD) off-line and HPLC–ESI-MS-MS have by now been well developed to enable sequencing and absolute identification of all known glycans found in natural or recombinant glycoproteins or antibodies. Ultimately, however, the description of the glycan profile is based on a knowledge of the enzymes present in the cell that synthesized the protein, enzymatic digestion, and often isolation of the glycan, followed by MS and nuclear magnetic resonance (NMR) spectroscopy.

UHPLC techniques have brought improved resolution and reliability to the assay of released glycans. As shown in Figure 9, the methods are better than comparable HPLC techniques. It should be noted that this useful assay is based on HILIC rather than reversed-phase chromatography. To achieve this performance, it was not sufficient to just use smaller particles. Rather, a new packing material was synthesized to be compatible with the small particles and higher pressure operation, while having improved selectivity for the important glycans. The percent peak areas or their ratios,

in Figure 9, can then be used to characterize a specific glycoprofile for the released glycans that were first derived. This, then, becomes characteristic of that individual or mixture of glycoproteins and is suitable for lot-to-lot (batch-to-batch) comparisons and demonstration of chemical equivalencies of biosimilars, in part.

Figure 10 illustrates a different mixture of 2-AB glycans, these coming from ribonuclease B protein (21). The open circles and dark squares represent different monosaccharides linked together to yield the glycans indicated. There are any number of other monosaccharides possible in glycans derived from other glycoproteins. Some glycans are biantennary, some are triantennary, and some are higher order, branched chains. The inset figure in Figure 10 illustrates three distinct glycans for the three isomers possible for this triantennary glycan.

There are innumerable arrays of possible glycoprofiles possible for other glycoproteins, mixtures of glycoproteins, mixture of antibodies, fusion proteins, and others. And, each such glycoprofile, as shown in Figures 9 and 10, then becomes unique for that specific glycoprotein or any mixture of other glycoprotein variants. It is not only an issue of qualitative identification of each glycan present on the original DS, but also the relative percent peak areas of each such glycan, that then characterizes the original DS. And, that is what really becomes extremely useful in demonstrating batch-to-batch consistency of production or isolation, as well as showing that the expression system and production purification processes remain constant, lot-to-lot. These same techniques are proving extremely useful in comparing biosimilars with proprietary DS or DP. These are crucial points to make in any submittal to a regulatory agency.

Acknowledgments

We indicate our sincere appreciation to numerous colleagues within Waters Corporation who, over many years, have provided us with copies of journal publications, magazine articles, application notes, poster papers, and related materials dealing with UHPLC applications in biopharmaceuticals. We are especially indebted for several figures being used in this article, as provided by Tom Wheat and Ken Fountain at Waters, as well as for interesting discussions as we planned the content for these two columns.

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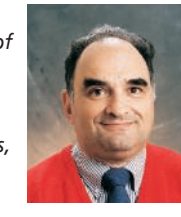

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Analysis of Phenazepam in Whole Blood Using Solid-Phase Extraction and LC–Tandem Mass Spectrometry

In this study, a solid-phase extraction (SPE) procedure is described for the analysis of phenazepam in whole blood. Extraction was performed using a mixed-mode SPE column. Samples of whole blood were diluted with aqueous phosphate buffer (pH 6). After loading the diluted sample onto the SPE column, the sorbent was washed with deionized water, acetic acid, and methanol. After drying the SPE columns, the analytes were eluted from the SPE column with 3 mL of an elution solvent consisting of methylene chloride, isopropanol, and ammonium hydroxide. The eluates were collected, evaporated to dryness, and dissolved in mobile phase (100 µL) for analysis by liquid chromatography–tandem mass spectrometry (LC–MS-MS). Chromatography was performed in gradient mode using a C18 column and a mobile phase consisting of acetonitrile and 0.1% aqueous formic acid. The total run time for each analysis was 5 min. The limits of quantitation and detection for this method were determined to be 1.0 ng/mL and 0.5 ng/mL, respectively. The method was found to be linear from 1.0 ng/mL to 100 ng/mL ($r^2 > 0.995$). Recoveries of the phenazepam were found to be greater than 90%.

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Phenazepam (7-bromo-5-[2-chlorophenyl]-1,3-dihydro- 2H-1,4-benzodiazepin-2-one) (Figure 1) is a benzodiazepine-type drug that was developed in the former Soviet Union and is now produced in Russia and some other countries (1). Phenazepam is used in the treatment of neurological disorders such as epilepsy, alcohol withdrawal, and insomnia (2), but it is now becoming a drug of interest to the forensic community because of its reported misuse (3). It can be used as a premedication before surgery because it augments the effects of anesthetics and reduces anxiety. Phenazepam is available as a 0.5-mg tablet, and the maximum daily dosage should not exceed 10 mg (2). The possible side effects of using phenazepam include dizziness, loss of coordination, and drowsiness, along with anterograde amnesia that can be quite pronounced in high doses (4). As with other benzodiazepines, in case of abrupt discontinuation following prolonged use, severe withdrawal symptoms may occur including restlessness, anxiety, insomnia, and convulsions (5). The metabolism of phenazepam in several species of mammals including humans has been known since the 1980s, when it was reported (6) that after oral administration (human) peak blood concentrations of the parent drug were achieved in 4 h and had a half life ($t_{1/2}$) of 60 h. The authors of the study observed that the conversion of phenazepam to the metabolite 3-hydroxy-phenazepm is not significant in humans; thus phenazepam is the main analyte of interest for forensic toxicologists because its use and misuse is becoming prevalent (3). Phenazepam has been determined in biological fluids by gas chromatography–mass spectrometry (GC–MS) (7) and GC using nitrogen specific detection (NPD) (8) as well as liquid chromatography–tandem

mass spectrometry (LC–MS-MS) (9), following liquid–liquid extraction (LLE).

This article is (to our knowledge) the first report on the continent of North America of phenazepam in a drugs-and-driving case employing mixed-mode solid-phase extraction (SPE) and LC–MS-MS. A recent report has been published in Europe for the analysis of this drug in Finland (10).

Experimental

Chemicals and Reagents

Phenazepam was obtained from Lipomed (Cambridge, Massachusetts) as a 1-mg/mL methanolic solution. The internal standard (diazepam-d_5) was purchased from Cerilliant (Round Rock, Texas) as a 100-µg/mL methanol solution. Acetonitrile, acetic acid (glacial), concentrated ammonium hydroxide solution (32% by volume), formic acid, isopropanol, methanol, and methylene chloride were obtained from Fisher Scientific (Pittsburgh, Pennsylvania). The SPE columns (CSDAU206) were obtained from UCT Inc. (Bristol, Pennsylvania). Deionized (DI) water was laboratory grade and was generated in the Massachusetts State Police Crime Laboratory (MSPCL). The water was produced by passing water through mixed-bed ion-exchange filters followed by ultraviolet light radiation; the resulting deionized water had 18-MΩ resistance. All chemicals were of ACS grade.

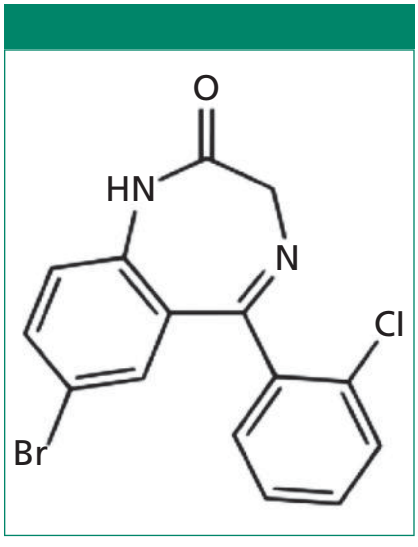

Figure 1: The structure of phenazepam.

Acetic acid was prepared as a 1.0 M solution by diluting glacial acetic acid (58.0 mL to 500 mL), making it up to 1 L with DI water, and mixing well. Formic acid was prepared as a 0.1% (v/v) solution by adding 1 mL of the acid to 900 mL of DI water and diluting to 1 L. Acetonitrile containing 0.1% formic acid (v/v) was prepared by adding 1 mL of formic acid to 900 mL of acetonitrile and diluting to 1 L. Phosphate buffer (pH 6, 0.1 M) was purchased from Fisher Scientific as a ready-to-use solution.

Chromatographic Analysis

Analysis was performed using an API 3200 Q-Trap instrument supplied by Applied Biosystems (Foster City, California). The chromatographic system consisted of a Shimadzu CBM 20 A controller, two Shimadzu LC 20 AD pumps including degasser, a Shimadzu SIL 20 AC autosampler, and a Shimadzu CTO AC oven (set at 10 °C) (Shimadzu Scientific Instruments, Columbia, Maryland). The instrument was fitted with a 50 mm × 2 mm, 5 µm Imtakt US-C18 column from Silvertone Sciences (Philadelphia, Pennsylvania), which was attached to a Unison US-C18 guard column (5 mm × 2 mm) obtained from the same supplier. The LC system's column oven was maintained at 40 °C throughout the analyses. The injection volume was 10 µL. The mobile phase consisted of solvent A, DI water containing 0.1% formic acid, and solvent B, acetonitrile containing 0.1% formic acid. The mobile phase was delivered at a flow rate of 0.5 mL/min. The mobile-phase gradient was programmed as follows: 5–90% B in 4.0 min, then the proportion of solvent B was returned to 5.0%. The instrument was ready for reinjection after 5.1 min.

The mass spectrometry was performed on an API 3200 QTRAP system using multiple reaction monitoring mode (MRM). The following transitions were monitored (quantification ions underlined): *m/z* 350.8 → 206.3, 104.4, for phenazepam. The internal standard (diazepam-d_5) was monitored at the following transitions: *m/z* 290.1 →198.3, 154.3. Tandem mass spectrometry was performed under the following conditions: curtain gas setting, 15; collision gas setting, medium; ion spray voltage setting, 5000 V; temperature setting, 650 °C; ion source gas 1 setting, 50; ion source gas 2 setting, 50. Tandem mass spectrometer conditions are shown in Table I. The analytical data were collected using Analyst Software Version 1.5 supplied by Applied Biosystems.

The retention times for phenazepam and the internal standard (diazepam-d_5) were 3.49 and 3.54 min, respectively (Figure 2).

Sample Preparation for Analysis

Calibrators and Controls

A solution of phenazepam was prepared at a concentration of 1 µg/mL by the dilution of 10 µL of stock solution with acetonitrile to 10 mL in a volumetric flask. A solution of the internal standard (diazepam-d_5) was prepared by diluting 100 µL of the stock solution (100 µg/mL) to 10 mL with acetonitrile in a volumetric flask. The choice of internal standard was based on the fact that deuterated analogs of phenazepam are not currently available and that an isotopically labeled analog of a benzodiazepine (which shares structural similarities to phenazepam) would not be observed in a case sample.

Calibrators were prepared by the addition of 0.5, 1.0, 10.0, 50, and 100 ng of phenazepam into 1.0-mL samples of drug-free whole blood. Then, 50 ng of the internal standard was added to these samples. Control samples were repared by the addition of 4 ng of phenazepam to 1.0

Table I: Tandem mass spectrometry conditions

Compound	Q1	Q3	Time (ms)	DP (volts)	EP (volts)	CXP (volts)	CE (volts)
Phenazepam (1)	350.799	206.3	250	56	10.5	4	49
Phenazepam (2)	350.799	104.1	250	56	10.5	4	83
Diazepam-d_5 (1)	290.162	198.3	250	56	4.5	4	43
Diazepam-d_5 (2)	290.162	154.3	250	56	4.5	4	39

Time = dwell time; DP = declustering potential; EP = exit potential; CXP = collision cell exit potential; and CE = collision energy

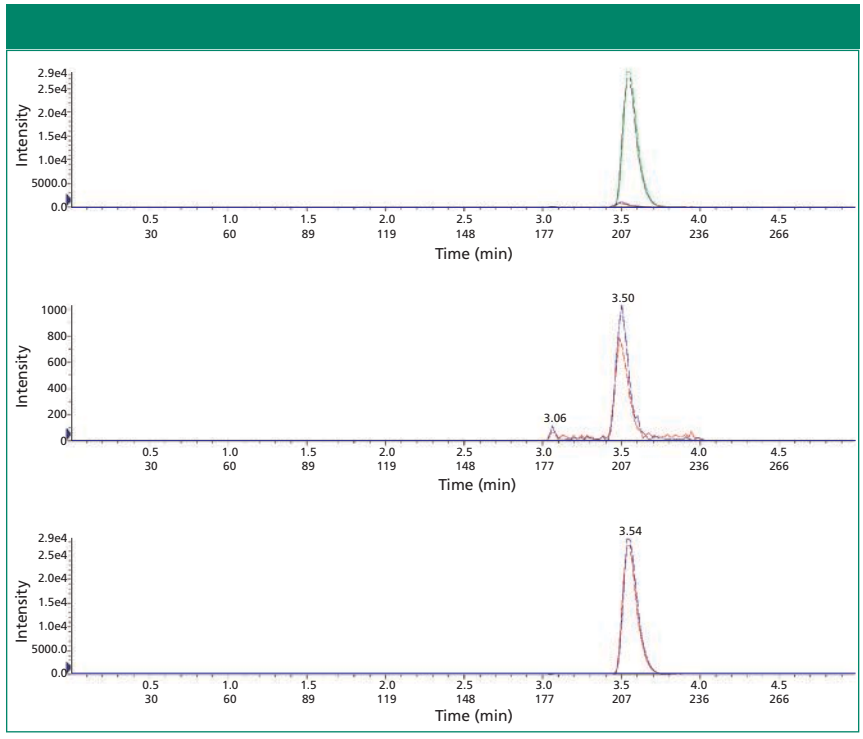

Figure 2: Chromatogram of a blood extract containing phenazepam at LOQ (1.0 ng/mL) showing total ion chromatogram (TIC) (upper), phenazepam (middle), and internal standard (lower).

mL samples of drug-free whole blood in addition to 50 ng of the internal standard. A negative control sample was prepared by adding only the internal standard (50 ng) to a sample of drug-free whole blood (1.0 mL). To each of the calibrators, control, and test samples was added 5 mL of pH 6 buffer. These were then well mixed on a vortex mixer (1 min) and centrifuged at 3000 rpm for 10 min before application on individual SPE columns. All determinations were performed in duplicate.

To assess the performance of the procedure, calibration curves were constructed twice daily over five consecutive days using the spiked controls; we obtained intraday and interday values from these data.

Solid-Phase Extraction

Solid-phase extraction columns were conditioned by the sequential addition of 1 × 3 mL of methanol, 1 × 3 mL of DI water, and 1 × 1 mL of 0.1 M phosphate buffer (pH 6). Each liquid was allowed to percolate through the sorbent using gravity without allowing the sorbent to dry out between steps.

Following the passage of the methanol, DI water, and 0.1 M phosphate buffer (pH 6) through the SPE columns, each diluted sample (that is, calibrator, control, and case item) was loaded on to an individually marked SPE tube, and allowed to pass through the sorbent using gravitational flow. The columns were then washed with 1 × 3 mL of DI water, 1 × 3 mL of 1.0 M acetic acid, and 1 × 3 mL of methanol, respectively. The SPE columns were then dried by applying a vacuum to the SPE manifold at 15 in. of mercury pressure with the aid of an electric vacuum pump connected to the vacuum manifold.

The analytes were eluted from the SPE columns by the addition of 1 × 3 mL of a 78:20:2 methylene chloride–isopropanol–ammonium hydroxide solution. This solution was prepared daily by adding 2 mL of concentrated ammonium hydroxide solution to 20 mL of isopropanol and mixing well. Finally, 78 mL of methylene chloride was added to this solution and the resultant solution was transferred to a clean screw-top glass bottle for use. A screw-top bottle ensures that the basicity of the solution remains high by eliminating any loss of ammonia from the bottle. The elution solvent was allowed to flow through the SPE sorbent with the aid of gravity and was collected in separate glass tubes (75 mm × 125 mm). Glass tubes were chosen because they are standard laboratory materials within this toxicology laboratory.

The eluate from each SPE column was evaporated to dryness using a gentle stream of nitrogen at 35 °C, after which the samples were dissolved in 100 µL of a solution consisting of 95% mobile-phase A and 5% mobile-phase B for LC–MS-MS analysis.

Recovery Studies

To determine the recovery values across the dynamic range of the analysis, the results of the SPE extractions of the whole blood extracts (as duplicate analyses) were compared to the values obtained from unextracted standards at corresponding concentrations. The unextracted standards were prepared by evaporation of acetonitrile solutions containing phenazepam (including 50 ng of the internal standard). The dried residues were dissolved in mobile phase (100 µL) before analysis by LC–MS-MS.

Matrix Effects

Studies into the matrix effects were performed according to procedures described by Matuszewski and colleagues (11). In this process, samples of drug-free whole blood (1 mL) were spiked with phenazepam before analysis using the SPE methodology. A second set of drug-free whole extracts was analyzed according to the SPE method. Following elution from the SPE columns, the extracts were spiked with phenazepam. Both sets of samples were evaporated to dryness under a gentle stream of nitrogen at 35 °C, and the residues were dissolved in 100 µL of a solution consisting of 95% mobile-phase A and 5% mobile-phase B, the samples were combined for analysis by LC–MS-MS.

Phenazepam solutions (each with a concentration of 50 ng/mL) were infused into the tandem mass spectrometer using the on-board syringe pump (controlled by Analyst 1.5 software) via a 1-mL Hamilton syringe (model 1001TLL, supplied by Fisher Scientific) at a flow rate of 5 µL/min. At the same time as the phenazepam solution was flowing into the mass spectrometer, a 10-µL aliquot of the SPE-extracted whole blood matrix (drug-free whole blood, free of phenazepam) was injected using the autosampler syringe on the Shimadzu liquid chromatograph using Analyst 1.5 software. The liquid

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2. Understand different options that are available
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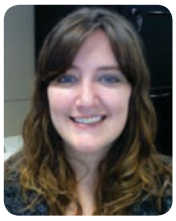
- Lab Personnel responsible for qualification



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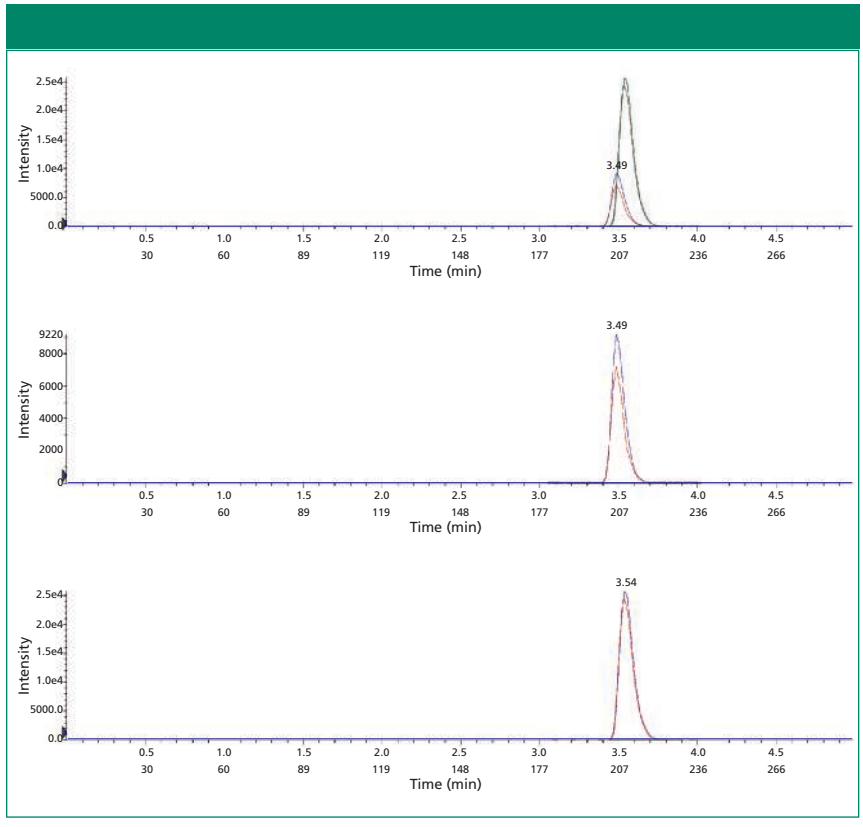

Figure 3: Chromatogram of an actual blood extract containing phenazepam showing total ion chromatogram (TIC) (upper), phenazepam (middle), and internal standard (lower).

chromatograph and mass spectrometer were arranged so that samples from the liquid chromatograph were mixed into the flow of phenazepam via a three-port T-section before the total flow entered the mass spectrometer. Any suppression effects on the phenazepam could be monitored at the MRMSs for the noted drugs.

Selectivity

When analyzing samples of biofluids such as blood via SPE and LC–MS-MS, it is essential to ensure that the interfering effects of other drug compounds can be eliminated. In this procedure, samples of drug-free whole blood (1 mL) were spiked with 49 drugs at a concentration of 100 ng/mL (bupropion, lidocaine, methadone, amitriptyline, nortriptyline, thioridazine, trazodone, mesoridazine, pethidine, diphenhydramine, phenyltoloxamine, imipramine, desipramine, benztropine, trimethoprim, diltiazem, haloperidol, strychnine, morphine, codeine, 6-acetylmorphine, oxycodone, oxymorphone, hydrocodone, noroxycodone, hydromorphone, diazepam, nordiazepam, oxazepam, temazepam, alprazolam, α-hydroxyalprazolam, lorazepam, triazolam, α-hydroxytriazolam, flunitrazepam, 7-amino-flunitrazepam, chlordiazepoxide, midazolam, α-hydroxymidazolam, flurazepam, desalkyl-flurazepam, cocaine, ecgonine methyl ester, ecgonine ethyl ester, benzoylecgonine, cocaethylene, clonazepam, and 7-amino-clonazepam) and were extracted according to the SPE method. The interfering effect of these compounds was not found to be significant.

Results and Discussion

Recovery

The recovery of phenazepam from drug-free whole blood was 98% (±2%). This result is an excellent indicator for the efficiency of the extraction procedure of phenazepam using whole blood as a matrix. The procedure was performed twice daily during a period of five days.

Imprecision of Analysis

The spiked control samples (4 ng/mL) were determined to have concentrations of 3.9 ng/mL (±0.2 ng/mL). This value was determined during a period of five days.

Intraday variation and interday variation for the analysis of phenazepam were found to be less than 5% and less than 8%, respectively. Ion suppression studies revealed that suppression of monitored ions was less than 2%. This method was found to be linear ($r^2 > 0.995$) throughout the 1.0–100 ng/mL dynamic range for phenazepam.

LOD and LOQ

The *limit of detection* (LOD) of a particular method can be defined as the level at which the signal-to-noise ratio for the particular analyte is greater than or equal than 3:1. The *limit of quantification* (LOQ) for the method is the level at which the signal-to-noise ratio for a particular analyte is greater than or equal to 10:1. In this study, LOD values were determined empirically by analyzing extracted samples of drug-free whole blood fortified with phenazepam by LC–MS-MS according to the SPE method. This analysis was performed until the lowest level at which each of the respective analytes just failed the signal-to-noise ratio of 3:1. This was observed to be 0.5 ng/mL. In terms of LOQ, samples of drug-free blood were spiked with phenazepam at concentrations below 10 ng/mL and extracted according to the SPE procedure until the analytes just failed a signal-to-noise ratio of 10:1; this value was found to be 1.0 ng/mL.

Solid-Phase Extraction

As noted earlier, phenazepam is a relatively new compound of interest to forensic toxicologists. The use of mixed-mode SPE offers toxicologists in forensic laboratories a very clean sample to analyze. The sample is loaded onto the sorbent as a diluted solution at pH 6, and it is cleaned and concentrated on the SPE column. The use of an ion-exchange moiety allows coextracted materials to rinse off the sorbent while the drug of interest is retained in a clean condition. In this situation, the drug can be eluted using a mid-polarity solvent mixture that is easily evaporated for further analysis. This combination of hydrophobic and ion-exchange chemistries is a powerful tool for producing clean samples for chromatographic analyses.

Tandem Mass Spectrometry

This project was aimed at introducing new methodology to the forensic

community involved in the analysis of phenazepam in biological samples, with selectivity and sensitivity in mind. In other words, the ability to detect, confirm, and quantify a compound such as phenazepam in a complex mixture at low levels is a highly desired quality in a new procedure, especially if it can lead to a fast turnaround time and an increase in laboratory efficiency.

Conclusion

Phenazepam is quickly becoming a drug of interest in forensic laboratories in the United States, the United Kingdom, and Europe (3,12), and analysts will be asked to test for it on a routine basis. With that in mind, this new procedure using SPE and LC–MS-MS will offer forensic toxicology laboratories the ability to perform the analysis of phenazepam in biological fluids, such as blood, quickly and efficiently. When this new method was applied to a genuine case sample taken from a driver operating a motor vehicle, the whole blood sample was found to contain 9 ng/mL of phenazepam (Figure 3).

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Effective & Efficient Application of GC-MS in Food Testing, Environmental, & Pharmaceutical Applications

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EVENT OVERVIEW:

The detection and analysis of potentially harmful volatile organic compounds such as pesticides and residual solvents in foods, pharmaceuticals and the environment are of critical concern. Many countries have set strict regulatory requirements for the detection of these contaminants well below safety threshold levels.

To meet these analytical goals requires highly sensitive and selective methods to effectively measure these compounds. These analyses are further complicated by a large and ever expanding list of compounds to screen for, as well as sometimes very difficult and complex sample matrices to test from.

In this presentation, the application of a new GC-MS platform and it's resulting performance to a number of applications such as pesticide testing in a series of food and water samples, as well as residual solvent analysis in pharmaceuticals and nutritional supplements will be covered in great detail. The results from these studies indicate that this new GC-MS system capable of delivering results necessary to meet regulatory requirements in terms of performance, reliability and robustness.

KEY LEARNING OBJECTIVES:

- To learn about the performance characteristics of a new GC-MS platform and how it meets the requirements for accurate analysis of volatile organic compounds
- To learn some of the latest methods for pesticide residue analysis in Food and Water samples
- To learn the latest methods in residual solvent analysis for pharmaceutical and nutritional products

WHO SHOULD ATTEND:

- Analysts in Food Testing Laboratories
- Analysts in Environmental Testing Laboratories
- Pharmaceutical Development Scientists and Managers
- Analytical Product QC Scientists
- Process Development Scientists
- Analytical Chemists in Chemistry Support Groups

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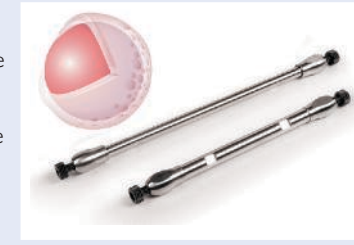
PRODUCT RESOURCES

HPLC columns

The XSelect HSS Cyano and HSS PFP columns from Waters are designed to offer scientists an alternative to traditional C18 column chemistries. The columns reportedly offer more control over the resolving power of HPLC separations, reducing time and method development costs. **Waters Corporation,** Milford, MA. www.waters.com

HPLC–UHPLC columns



Aeris core–shell HPLC–UHPLC columns from Phenomenex are designed for the analysis of proteins and peptides. The Widepore columns (3.6-µm pores) reportedly are optimized for the separation of intact proteins and polypeptides and are available in three selectivities: XB-C18, XB-C8, and C4. The Peptide columns (3.6- and 1.7-µm pores) are available in the XB-C18 selectivity and are intended for the separation of low-molecular-weight peptides and for peptide mapping. **Phenomenex, Inc.,** Torrance, CA. www.phenomenex.com

Centrifuge tubes

UCT's Enviro-Clean PAH-certified centrifuge tubes are designed for performing PAH analysis using QuEChERS, AOAC, or other methods that require the use of 50-mL centrifuge tubes. The model ECPAHFR50CT polypropylene tubes are supplied with plug-seal caps. **UCT, Inc.,** Bristol, PA. www.unitedchem.com

SPE cartridge columns and plates

SPE cartridge columns and 96-well plates from Thermo Fisher Scientific are designed for high-throughput sample preparation for drugs-of-abuse testing. The Servo cartridge columns and plates are intended for total drug screening and specific testing for THC, opiates, amphetamines, PCP, and cocaine. The Servo+ cartridge columns and plates are designed to provide greater selectivity, higher loading capacity, and increased robustness. **Thermo Fisher Scientific,** Waltham, MA. www.thermoscientific.com/servo

Automated headspace analyzer

The Versa automated headspace analyzer from Teledyne Tekmar is designed for traditional static headspace analysis. The analyzer includes a 20-position autosampler, built-in pressure control, an automated leak check and benchmark function, a method optimization mode, and sample heating to 200 °C. **Teledyne Tekmar,** Mason, OH. www.teledynetekmar.com

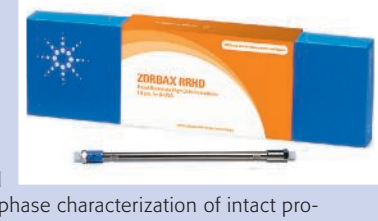
Syringe selection guide

Supelco's 44-page syringe selection guide is designed to help users choose the correct syringe for a given application. The guide lists autosampler, manual, and gastight syringes, including color-coded and digital syringes. The guide also includes a syringe selection table. **Supelco/Sigma-Aldrich,** St. Louis, MO. www.sigmaaldrich.com/syringes

Mass spectrometer

Shimadzu's LCMS-8030 triple-quadrupole mass spectrometer is designed to complement UHPLC systems, offering power and speed in the detection of target analytes. According to the company, the system features multiple reaction monitoring (MRM) transitions that enable data acquisition of as many as 500 channels/s, 15-ms polarity switching, and mass spectrum measurement speeds of 15,000 u/s. The instrument reportedly accelerates ions out of the collision cell by forming a pseudo-potential surface, producing high-efficiency collision-induced dissociation (CID) and high-speed ion transport. **Shimadzu Scientific Instruments, Inc.,** Columbia, MD; www.ssi.shimadzu.com

Reversed-phase UHPLC column



Agilent's Zorbax RRHD 300SB-C18 1.8-µm column for UHPLC separations is a rapid resolution, high definition silica reversed-phase column. According to the company, the column is suited for higher-order reversed-phase characterization of intact proteins and protein digests. The column reportedly is stable at pH values as low as 1 and at temperatures as high as 90 °C. **Agilent Technologies,** Santa Clara, CA. www.agilent.com

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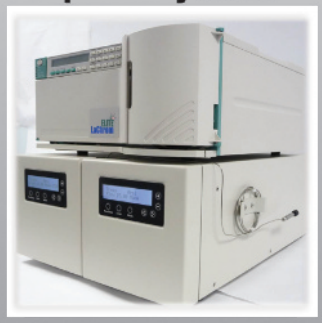

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