Column Watch

Stationary-Phase Technology in Separation Science

Ron Majors looks at recent stationary-phase developments, including molecular-imprint polymers and monolithic phases. He also discusses column stability, temperature as a separation variable, ultrahigh-pressure LC, and retention mechanisms.

Ronald E. Majors
Column Watch Editor

Even though separations in the liquid phase have been recognized as a science since the original work of Tswett in 1906, and even though high performance liquid chromatography (HPLC) itself is more than 30 years old, the development of novel and improved stationary phases continues. The advent of new separation technologies such as capillary electrochromatography (CEC), microseparation technology such as packed fused-silica capillary chromatography, and nanoseparation technology such as laboratory-on-a-chip systems has spurred new approaches and attempts to place a nonextractable retentive stationary phase into a different column format.

Occasionally, I review some of these developments so that casual workers in the field can see the exciting research going on in liquid-phase separation techniques. Earlier in the year, I discussed some of the new developments in HPLC column materials based on packing morphology and the resulting improvements in column efficiency (1). Partly based on findings unveiled at HPLC 2000 in Seattle, Washington, in June 2000, this installment of "Column Watch" will discuss work in new stationary-phase developments with regard to phase design, column packing stability, and temperature effects in separations. I'll also look at some studies of retention mechanisms that elucidate how these stationary phases interact with molecules. Those studies can help chromatographers predict the optimum stationary phases and column configurations for specific separations.

As mentioned above, the HPLC column is an area of ongoing investigation. The most active areas of current research are monolithic and molecular-imprint polymer columns; the development of more-stable phases that allow chromatographers to use temperature as a true separation variable; a renewed interest in micro-, capillary, or nano-liquid chromatography (LC) or separations on columns 1 mm and smaller in inner diameter; and fast LC columns driven by high-throughput requirements and LC–mass spectrometry (MS) and LC–MS–MS, which sometimes is accomplished by using very small particles (~1 µm) at very high pressures (as high as 75,000 psi). I will examine some of these developments in this installment of "Column Watch."

Studies on Column Stability

Despite all of the work that has been performed on novel packings, silica gel has been and still is the major packing used in bonded HPLC columns. To effectively use silica gel–based bonded phases, chromatographers must understand how the mobile-phase environment influences the separation characteristics, as well as the stability of packed columns. Silica gel is soluble in water, and its solubility increases with temperature and pH, as depicted in Figure 1. At low pH values, the base silica gel itself is fairly stable to dissolution, but the acid-catalyzed hydrolysis of the siloxane bond of the chemically bonded phase causes a deterioration of the column performance by the loss of bonded phase (2). On the acid side, longer chain bonded phases (C8 and C18) (3) and sterically protected functional groups (2) can yield better long-term per-

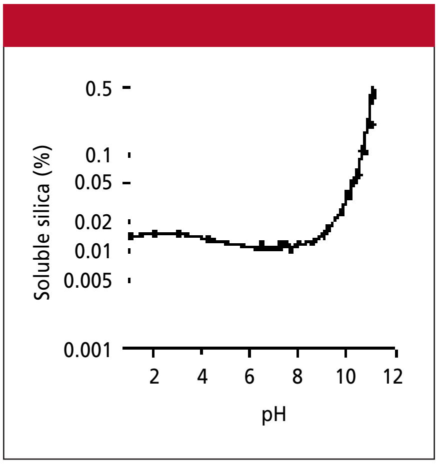

Figure 1: Solubility of silica gel in water as a function of pH (1). Temperature: 30 °C.

formance. At neutral and high pH, dissolution of the underlying silica gel through attack by the hydroxide ion causes a loss of column performance. Often, a column void is formed as the base silica gel is solubilized, which results in peak doublets, peak tailing, and other undesirable characteristics. To suppress the dissolution of silica in typically used mobile phases, analysts must prevent the hydroxide ion in the mobile phase from reaching the underlying silica. One approach to blockage is to coat the silica with a polymer that can protect the underlying silica against dissolution, provided that the mobile phase doesn't penetrate the coating and begin the dissolution process underneath the polymer shell. The polymer coating also can cause a loss of chromatographic efficiency because diffusion in polymeric layers generally is poorer than the diffusion that occurs in silica-based monolayer bonded phases.

For high-pH work, another approach that also delays the dissolution of silica is covering the silica gel base with a high density of chemically bonded phase. The rate of silica dissolution is determined by the bonding characteristics, type of bond, type of silica, presence of certain salts and buffers, buffer concentration, mobile-phase pH, and other experimental parameters (4). Hybrid silica–organic materials offer another approach to preventing dissolution of the packing, especially at basic pH values (5).

Of course, other alternative packings for high-pH work exist. For example, polymeric packings such as polystyrene–divinylbenzene (PS–DVB) and polymethacrylates provide a wider range of pH stability, but their efficiencies generally are lower, and swelling and shrinking can still be problems with certain types of polymers, especially those with low cross-linking. Newer packings such as graphitized carbon, zirconia, polymer-coated zirconia, and titania add a new dimension to the stability story because they have low solubility in water and can resist high-pH mobile phases. These packings also show good performance at high temperatures, but they often must be used with different mobile-phase additives to suppress various undesirable surface interactions.

Using Temperature as a Separation Variable

The effect of temperature on LC separations has been well studied (6). In reversed-phase chromatography, analysts can use temperature to control selectivity, reduce column back pressure (by lowering mobile-phase viscosity), and increase the recovery of certain analytes such as hydrophobic peptides and proteins, as depicted in Figure 2 (7). However, in practice, temperature seldom is used as a variable in method development to optimize chromatographic resolution. Compared with an unthermostated column at room temperature, a column thermostated slightly above ambient temperature in an oven or heater block provides more-reproducible retention.

For ion-exchange chromatography on polymeric materials or size-exclusion chromatography of high molecular weight compounds such as polymers, higher temperatures are a necessity for better mass transfer or for compound dissolution, respectively. However, at neutral-to-high pH and high temperatures, bonded silica gels generally are avoided because of the known solubility of silica gel in an aqueous environment. At low pH, Kirkland and Henderson (2) demonstrated that certain silica bonded phases can be used at temperatures as high as 90 °C.

New reports of the development and use of high-temperature HPLC phases, as well as the use of temperature as a separation variable, are beginning to appear. Peter Carr and co-workers (8) from the University of Minnesota (Minneapolis) have reported the use of polystyrene-coated porous zirconia particles at temperatures as high as 200 °C, and they observed no problems of column instability. Their work on high-temperature ultrafast LC showed that column efficiency at high velocity improves at higher temperatures, especially of well-retained solutes. In addition, the reduced plate height (h) versus reduced velocity (ν) curve flattens out significantly as the temperature increases.

At 150 °C with a flow rate of 15 mL/min in a 5 cm × 4.6 mm, 3-µm d_p column, long-chain barbiturates were well resolved, and the analysis time was decreased compared with that of a separation performed at room temperature. The separation of barbiturates is depicted in Figure 3. Superheated water, which has interesting solvation properties at these higher temperatures, was used as an eluent. This solvent provides lower toxicity, flammability, cost, and background absorbance in UV detection when compared with typical water–organic solvent mixtures.

As a side note, several other research groups have studied the use of superheated pure water as a mobile phase for HPLC

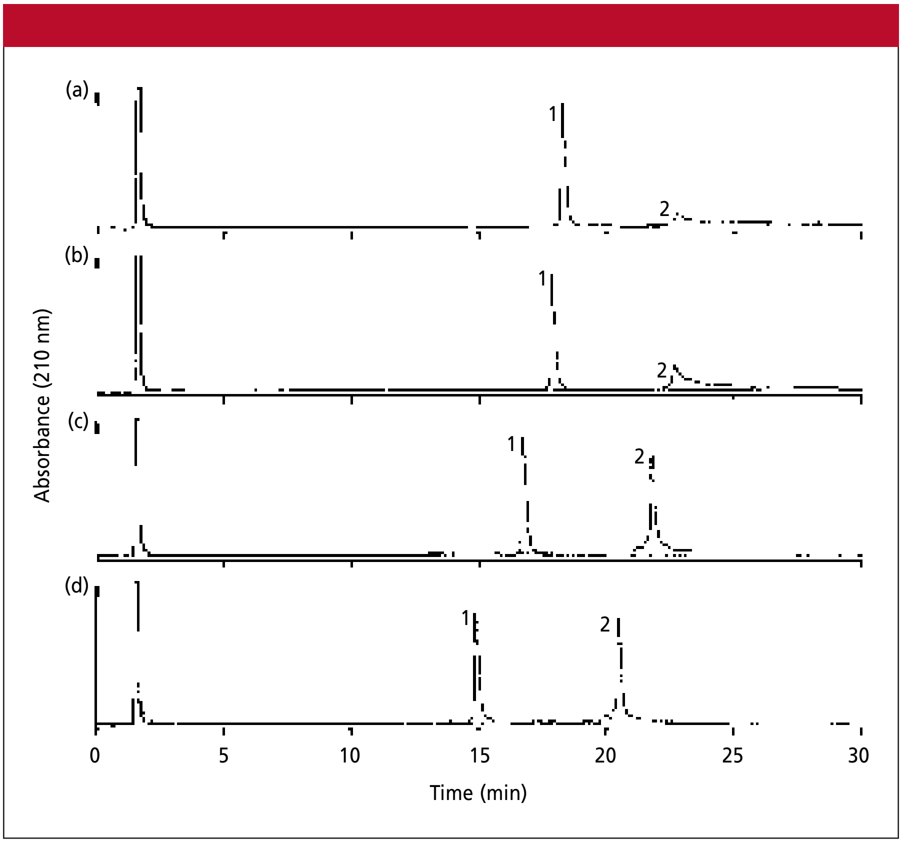

Figure 2: Recovery of β-amyloid peptides at (a) 25 °C, (b) 40 °C, (c) 60 °C, and (d) 80 °C (7). Column: 150 mm × 4.6 mm Zorbax 300 Stablebond-C18; mobile phase: A = 0.1% trifluoroacetic acid in water, B = 0.09% trifluoroacetic acid in acetonitrile; gradient: 20–45% B in 35 min; flow rate: 1.0 mL/min; sample: 10 µL (5 µg) peptide in 6 M urea–5% acetic acid. Peaks: 1 = peptide 1–38, 2 = peptide 1–43. Recovery of peptide 1–43: (a) less than 10%, (d) greater than 70%.

(9–13). Ian Wilson and co-workers (11–13) from Astra Zeneca (Alderley, United Kingdom) have used superheated deuterium oxide as a mobile phase in conjunction with high-field nuclear magnetic resonance spectroscopy detection. In this type of application, the ability to avoid the use of organic modifiers is especially useful in that it eliminates the need to remove potentially interfering signals caused by protons from the organic modifier. In addition, the use of deuterium oxide as a mobile phase is a less-expensive alternative to deuterated organic solvents. With pure water, chromatographers no longer have the ability to modify selectivity by changing the organic modifier; therefore, Wilson (14) recently investigated a series of different packing materials for their suitability to provide stationary-phase selectivity enhancements for the separation of drugs in biological fluids using pure superheated deuterium oxide as the mobile phase.

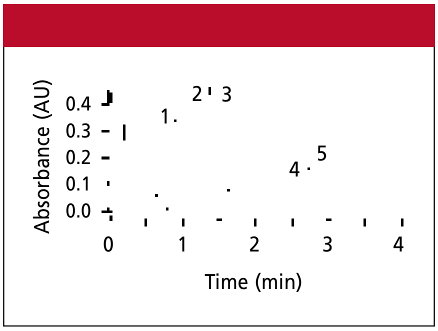

Figure 3: High-temperature separation of barbiturates on a bonded-phase zirconia column using 100% water as mobile phase. Column: 50 mm × 4.6 mm DiamondBond-C12 (dodecylbenzene covalently bonded to carbon-clad zirconia phase); mobile phase: 100% water; temperature: 200 °C; detection: UV absorbance at 220 nm; sample volume: 5 µL. Peaks: 1 = barbital, 2 = methbarbital, 3 = butethal, 4 = hexobarbital, 5 = mephobarbital. (Courtesy of Zirchrom Separations, Anoka, Minnesota.)

Compared with its use in gas chromatography, temperature programming is seldom used in HPLC. A group from Selerity Technologies (North Salt Lake, Utah) led by Brian Jones showed that temperature programming could simplify HPLC method development (15). They developed silica C8 and C18 bonded phases that exhibited good lifetimes at high temperatures beyond 100 °C when used in aggressive solvent conditions.

Tyge Greibrokk and colleagues (16,17) from the University of Oslo (Norway) advocated the use of temperature programming — not with conventional columns because their large inner diameters prevent rapid temperature variations, but with capillary columns that equilibrate much faster. In aqueous and nonaqueous mobile-phase systems, temperature programming may be a substitute for gradient elution in a narrow range of elution strength. At temperatures lower than ambient, resolution sometimes can be improved (for example, with chiral compounds), and larger injection volumes can be used with a focusing effect at the column inlet. This research group demonstrated separations at temperatures from ambient to 200 °C with band focusing. They packed their capillaries using supercritical carbon dioxide as a slurry packing solvent and demonstrated excellent column robustness and performance at temperatures in the 90–100 °C range.

J.A. Ooms and co-workers (18) from Spark Holland (Emmen, The Netherlands) demonstrated temperature-assisted, on-line solid-phase extraction (SPE). By heating the SPE cartridge during the desorption step, these researchers obtained significantly reduced desorption times and desorption volumes, which provided a faster SPE cycle time. Elevated temperature also was used successfully as an alternative to increasing the organic modifier content in the wash solvent during the wash step for the selective removal of early eluted matrix peaks.

In a practical study of temperature effects, S. Linde and co-workers (19) from Novo Nordisk (Bagsvaerd, Denmark) found that they had to use very precise temperature control in the 50 °C range to separate certain polypeptides by reversed-phase HPLC. They found that some types of column heaters had insufficient accuracy and precision to allow resolution of monoglycosylated insulin from several minor impurities, even when calibrated within ±2 °C.

Because of the advent of newer stable phases, more workers will use temperature as a separation parameter. Y. Mao and co-workers (20) from the University of Minnesota introduced a new concept for HPLC separations based on the thermally tuned tandem column concept. Using two columns with distinctly different chromatographic selectivity connected in series but independently thermally controlled, analysts could adjust and optimize chromatographic band spacing faster and better than with a single column using mobile-phase optimization. Using a computer-assisted optimization method based on a window diagram, the researchers easily determined optimum conditions based on four or five trial chromatograms.

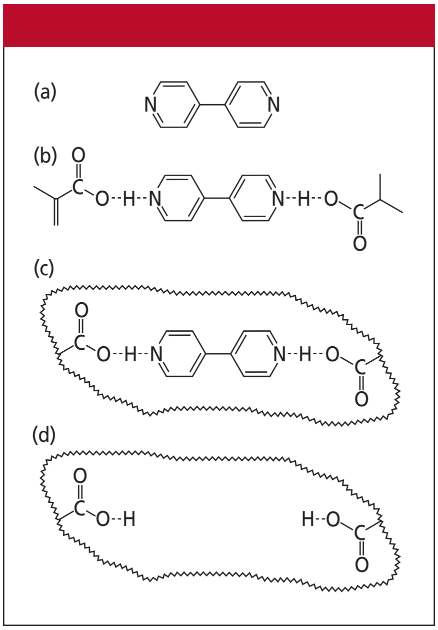

Figure 4: Typical synthesis of molecular-imprint phases, in which analysts (a) choose a template molecule such as 4,4′-bipyridyl, (b) allow the functional monomer to form complexes with the template, (c) polymerize the monomers with a cross-linker while maintaining the complexes, and (d) wash the polymer to reveal recognition sites that can specifically bind the template.

Ultrahigh-Pressure LC

Most commonly used HPLC columns have packings of 3 or 5 μm in average particle diameter. Using even smaller particles is attractive because many more theoretical plates can be generated, but the high pressure required to push solvent through these columns becomes problematic. Workers have investigated columns packed with sub-3-μm porous and nonporous particles, but these particles have not gained widespread acceptance because of their high cost, the fact that many instruments are not designed to handle them, and the high back pressure and plugging problems that can occur. Nevertheless, studies of small particle columns continue to receive attention.

James Jorgenson and co-workers (21) from the University of North Carolina (Chapel Hill) have extended their work on the use of particles as small as 1 μm that require ultrahigh pressure. Conventional pumps cannot handle these columns, so the researchers must use special high-pressure pumps capable of generating pressures in excess of 5000 bar (75,000 psi). These small-particle columns can provide 250,000 plates in less than 1 h. Jorgenson and colleagues reported an ultrahigh-pressure system that can be used for gradient elution. To instruct analysts about using these systems routinely, Milton Lee and co-workers (22) from Brigham Young University (Provo, Utah) devoted their attention to solving some of the practical concerns of ultrahigh-pressure systems. They paid particular attention to the designs of injection valves with respect to injection reproducibility, injection time, maximum operating pressure, sample amount injected, and the valves' impact on system efficiency. These researchers also used supercritical carbon dioxide as a packing solvent.

Molecular-Imprinted Polymers

The investigation of molecular-imprinted polymers continues to receive attention. As depicted in Figure 4, these phases are constructed by preparing a packing material, usually a polymer such as polymethacrylate, in the presence of a template that leaves its molecular impression on the surface as the polymer forms around it. After extracting the original molecule, the final product shows a greater molecular recognition factor for the analyte than an unimprinted material prepared in a similar manner but without the imprinting template. Indeed, adsorption of analyte molecules with similar molecular structure is diminished relative to the template, suggesting a lock-and-key relationship. This lock-and-key phenomenon was first proposed and then thoroughly investigated by Frank Dickey (23), who was a graduate student of the late Linus Pauling, in 1955 and later by a host of other scientists. Because unextractable original template molecules often remain behind in finished molecular-imprinted polymers, researchers have debated that the recognition may involve some sort of interaction with the unextracted template rather than being an actual molecular imprint. Various studies have revealed that polar interactions between analyte functionalities and topographically imprinted functional groups on the polymer surface play a role in the selectivity of molecular-imprinted polymers. There is no doubt that the pockets — fixed in shape, size, and chemical functionality — formed by the template in the polymeric materials are part of the molecular recognition.

Both inorganic polymers such as silica gel and organic polymers have been imprinted with various template molecules. Imprinted monolithic structures also have been investigated. Molecular-imprinted polymers have been used in several areas of analytical chemistry, including sensors, immunoassays, sample preparation, and chromatography. In a way, molecular-imprinted polymers mimic antibodies, but, compared with immunosorbents, they offer some distinct advantages — they are less expensive to produce, more chemically stable, and present more potential for selective phases. Molecular-imprinted polymers generally are prepared in organic media, but many of the potential applications put them in an aqueous environment. The polymer does not necessarily retain its original shape when exposed to this environment. Shrinking or swelling may result in reduced affinity.

The research group of Klaus Mosbach and co-workers (24) of Lund University (Lund, Sweden) has been studying imprinted polymers for several years. They have prepared various configurations, including membranes, beads, microspheres, and composite materials (25). A study by Vincent Remcho and co-workers (26) from Oregon State University (Corvallis) revealed that polar interactions between polymer moieties such as acid sites on polymethacrylates with the amine group of a series of similar tricyclic antidepressants were involved in the molecular recognition process. The potential utility of molecular-imprinted polymer–based chromatographic sorbents packed into micro-LC columns for combinatorial library screening was a goal of their investigations. Another study by the Oregon State University group involved nonsteroidal anti-inflammatory drugs as templates for molecular-imprinted polymers used as packings for LC and CEC (27).

Molecular-imprinted polymers usually are prepared by bulk polymerization, which means that the block polymers obtained must be crushed, ground, and sieved to produce irregular packing materials. These materials are less suitable for HPLC than spherical packings. Thus, J. Haginaka and co-workers (28) from Mukogawa Women's University (Nishinomiya, Japan) set out to synthesize spherical molecular-imprinted polymers. Using a multiple-step swelling and thermal polymerization method, they were able to obtain uniformly sized and monodispersed spherical polymethacrylate-based materials. They synthesized chiral molecular-imprinted polymer phases and used them to baseline resolve acidic and basic drug enantiomers by studying optimum synthetic conditions. Steffan Nilsson and colleagues (29) also investigated chiral molecular-imprinted polymers. They synthesized these molecular-imprinted polymer monoliths in situ in capillary columns and used them in CEC.

Even though they exhibit good recognition for small molecules and are stable, the organic polymers used for molecular-imprinted polymers sometimes cause a heterogeneous distribution of binding sites, slow mass transfer kinetics resulting in broad and asymmetrical peaks, and the above-mentioned swelling-and-shrinking phenomenon. To overcome these problems, B. Sellergren and colleagues (30) from Johannes Gutenberg University (Mainz, Germany) investigated polymer-grafted porous and nonporous silica with a well-defined pore system and pore-size distributions. Silica gel is more rigid, provides better chromatographic efficiency than typical polymeric supports, and does not swell or shrink with solvent changes. Because of the shape factor and the flexibility of the template in molecular imprinting, rigid and planar molecules tend to provide good imprints, and small bulky molecules give poor imprints.

T. Kubo and co-workers (31) from Kyoto University's polymer science department (Japan) have developed a new approach to imprinting using the *fragment imprinting effect* for the smaller molecules. They take a fragment of the real template molecule. For example, they might use biphenyl as a fragmental template for the coplanar polychlorinated biphenyls (PCBs) and cyclohexyl benzene for PCBs that have ortho substitutions. With a partially imprinted surface, a lock-and-key relationship with molecules of similar structure still occurs, and the researchers noted a higher degree of specificity than that provided by polymeric phases prepared without the fragment template.

The favorable molecular recognition properties of molecular-imprinted polymers make them attractive as sample preparation phases when analysts want to remove a specific analyte or groups of analytes from a complex mixture. However, the molecular-imprinted polymers do not provide efficient molecular recognition when determining drugs in aqueous biofluids, because proteins unspecifically and irreversibly bind to molecular-imprinted polymer adsorbents and interfere with the recognition process.

Karl-Siegfried Boos and C.T. Fleischer (32) of the University Hospital Grosshandern (Munich, Germany) have developed a four-step, highly selective column switching scheme for the on-line cleanup and analysis of drugs in biological fluids. Initially, a restricted-access media column separates the macromolecular matrix components and the low molecular weight extract. The latter undergoes a solvent exchange (aqueous to organic) and is directed into a second column packed with a tailor-made molecular-imprinted polymer adsorbent. Finally, the column-switching valve directs the effluent to an HPLC column to complete the automated analysis. Because the proteinaceous material never comes in contact with the molecular-imprinted polymer, the material is never fouled, the separation takes place in a more favorable organic medium, and its true molecular recognition properties are observable. A set of experiments with and without the molecular-imprinted polymer column showed the advantages of this multistep approach. J. Jodlbauer and co-workers (33) from the University of Vienna's Institute of Analytical Chemistry (Austria) used molecular-imprinted polymer extraction columns in their investigation of ochratoxin A analysis in foodstuffs and other matrices followed by LC–MS–MS analysis.

Monolithic Phases

Monolithic silica-gel and monolithic polymeric phases are of great interest in HPLC column technology. Monolith columns differ from the conventional packed HPLC columns in that they are solid continuous columns of porous stationary phase rather than individual particles. The phase is *cast* inside a column rather than packed. The columns appear to be relatively inexpensive to make, and the efficiency of monolithic columns rivals conventional packed columns. These monolithic beds have flowthrough pores (macropores) and diffusive pores (mesopores) and exhibit greater permeability than packed-bed columns. Thus, they can be used at higher flow rates that make them attractive for high-throughput applications. In addition, they appear to offer some advantages in CEC because they don't require frits, which can be a source of problems such as generating band broadening and erratic fluid movement, if the cast bed is tightly held inside the capillary. With the development of nanoseparation devices such as the laboratory-on-a-chip, casting monolithic phases inside the tiny microfluidic passages presents a more attractive alternative than attempting to pack them with conventional particles.

At HPLC '98 in St. Louis, the introduction of the first commercial monolith silica rod column (34), now called Chromolith (Merck KGaA, Darmstadt, Germany), brought much fanfare. Since then, researchers have conducted a great deal of experimental and published work but little in the way of additional commercialization of silica-based monoliths. However, Matt Przybyciel (35) of ES Industries (West Berlin, New Jersey) recently introduced commercial derivatized silica monoliths for both small and macromolecules.

A number of research groups are studying monolithic phases. These studies, conducted mostly by Japanese and German scientists, have investigated silica monolith phases for HPLC columns as well as for coatings inside capillaries and silicon chips for use in CEC. N. Tanaka and co-workers (36) of the Kyoto Institute of Technology (Japan) and Kyoto University compared the practical operational parameters of micro-HPLC and CEC to generate 100,000 theoretical plates per column using monolithic silica in a 50-μm i.d. fused-silica capillary column configuration. For the monolith column in HPLC, they demonstrated the generation of 100,000 plates on a 130-cm column with a pressure drop of less than 6 bar at a linear velocity of 1 mm/s in 80% acetonitrile at room temperature. In CEC, they were able to achieve 70,000–80,000 plates in a 25-cm column with an electroosmotic velocity of approximately 1 mm/s with a low-surface-coverage stationary phase and an electric field of 30 kV.

The generation of high plate counts in CEC was more difficult with high-coverage stationary phases, probably because of the slower electroosmotic flow associated with the monolithic silica material of high purity. Presumably, making a controlled, but less pure, monolithic phase by doping could result in an improved CEC column, and I am sure that work is proceeding in this direction.

Merck researchers and their Japanese colleagues have continued to investigate the silica monoliths. Merck's E. Muller and A. Seiler (37), working with groups from Kyoto University and Kyoto Institute of Technology, have demonstrated that the silica rod monoliths display higher performance in HPLC compared with polymeric monoliths of similar dimensions. One defining feature of the silica monoliths is the fairly flat h versus ν curves that allow them to operate at higher flow rates without a huge loss in efficiency. Because the mass transport properties of the monolith columns seem to be determined by convection rather than by diffusion, higher molecular weight molecules such as proteins and peptides (for example, tryptic digests) can be separated more efficiently with monolithic columns than with packed capillary columns. The Japanese group also reported the development of wide mesopore silica monolith rods, which can separate high molecular weight biomolecules such as polypeptides (38).

The other monolith school advocates polymeric-based materials. Last year in *LCGC,* Iberer and colleagues (39) reported about state-of-the-art polymeric monoliths. Several commercial products have been introduced in the polymeric monolith arena. Several research groups currently are studying polymeric monoliths for micro-HPLC and CEC. The well-known work of J.M.J. Fréchet and F. Svec (40) of the University of California at Berkeley was extended by T. Jiang and co-workers (41) of Eindhoven University of Technology (Eindhoven, The Netherlands). They prepared micromonolithic CEC columns by in-situ polymerization. Using the copolymerization of ethylene glycol dimethacrylate, butyl methacrylate, and 2-acryl-amido-2-methyl-1-propane sulfonic acid in the presence of various porogens inside 100-μm i.d. fused-silica capillaries, these authors were able to obtain column efficiencies of as much as 140,000 plates/m by optimizing the porogen volume fraction. They compared their monolithic columns with packed commercial octadecylsilane columns and found similar efficiency but different selectivity, as might be expected.

The Fréchet and Svec group from Berkeley have collaborated with Isco (Lincoln, Nebraska) (42) to prepare weak anion exchangers based on poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) monolith modified with diethylamine. These phases are suited for the high-throughput HPLC separation of biomacromolecules in both analytical and preparative scale. They demonstrated good stability, dynamic loading capacity, and protein recovery even after a large number of repetitive injections. The Berkeley group also collaborated with Wolfgang Lindner's group from the University of Vienna (Austria) in developing monolithic chiral stationary phases based on quinidine carbamate (43).

M. Lammerhofer, formerly with Lindner and now with the Berkeley group, synthesized macroporous phases by copolymerizing a quinidine-functionalized methacrylate derivative, either 2-hydroxyethyl methacrylate (HEMA) or glycidyl methacrylate (GMA), and ethylene dimethacrylate (EDMA) as a cross-linker in the presence of 1-dodecanol–cyclohexanol as a porogenic mixture inside fused-silica capillaries. By varying the porogen composition, the researchers were able to vary the porosity of the monolith. In CEC, the quinidine carbamate residues played a double role: they controlled the electroosmotic flow and provided the chiral recognition factor. These chiral monoliths provided good enantioselectivity towards chiral acids when separated by CEC. In addition, Lammerhofer generated 50,000–250,000 plates/m in approximately 30 min.

R. Shediac and co-workers (44) from Sandia National Laboratories (Livermore, California) developed novel acrylate-based UV-initiated hydrophobic microporous polymers for performing reversed-phase CEC of amino acids and peptides. They incorporated charged functionalities such as sulfonic acids and quaternary amines to influence the generation of electroosmotic flow. The monolithic columns were cast in situ with a polymerization time of less than 5 min, and the authors claimed that the columns were very reproducible. The Sandia group also investigated these monoliths for chip electrochromatography to allow development of miniaturized integrated analysis systems for biological compounds.

R. Freitag and D. Hoegger (45) of ETH (Lausanne, Switzerland) have extended their monolithic polymer work reported at HPLC '99 by investigating hydrophilic acrylamide monomers polymerized inside the fused-silica capillary in water or aqueous buffer. By adding salt, they found that hydrophobic interactions between the polymer chains occur and cause a lower degree of cross-linking than in the case of hydrophobic polymers produced in organic solvents. The resulting monolith has sufficient structural rigidity, and, because of the in-situ formation of the macroporous polymer, pressure injection or hydrodynamic flushing can be used with a commercial CE system.

With the large amount of research being conducted in silica and polymeric monoliths, scientists will continue to make great strides in synthesis and application. If the silica rods can be fabricated to be tightly enclosed in a rigid column configuration and the structure of the polymerized beds can be controlled reproducibly, the future for monoliths looks very bright. They will become truly disposable analytical tools as inexpensive, easily replaced, low-pressure-drop, high-efficiency HPLC columns, and stationary phases for capillaries and chip channels.

Retention Mechanisms

Liquid chromatographers always want to understand the mechanisms of retention so they can more easily predict how to approach future separation problems. Thus, studies on elucidating retention mechanisms always are popular. Y. Kazakevich and R. LoBrutto (46) of Seton Hall University (South Orange, New Jersey) conducted an impressive study. Using typical water–organic modifier mobile phases, the authors studied the effect of the adsorbed organic layer on the surface of reversed-phase packings. Surprisingly, they found that with an acetonitrile–water eluent the thickness of the adsorbed layer exceeded the hypothetical thickness of the bonded layer of alkyl chains; the thickness showed little dependence on the length of the bonded alkyl ligands. The retention process may be threefold: analytes partition from the binary eluent into the adsorbed organic liquid phase; analytes then adsorb on the hydrophobic side of the bonded alkyl chain; and analytes partition between bonded alkyl chains. Kazakevich and LoBrutto developed a mathematical model that helps to explain the complex mechanism for retention of analytes on reversed-phase columns.

A similar quantitative study using sorption isotherms for model compounds conducted by Natalia Felitsyn and Frederick Cantwell (47) of the University of Alberta (Edmonton, Alberta, Canada) revealed that the mechanism of sample sorption depended upon the volume percentage of organic modifier in the mobile phase. For example, when the volume percent of organic modifier 1-propanol was less than 15% in the 1-propanol–water mobile phase, the sample sorption (1-hexanol) occurred nearly exclusively in the stationary phase (C18), but at concentrations of 15–30% (v/v), sample sorption occurred in both the stationary phase and the sorbed organic modifier from the mobile phase.

More often, reversed-phase chromatography is used with organic–aqueous solvent mixtures in the 30–70% range. With lower and higher concentrations of organic modifier, retention mechanisms may change and actual molecular interactions may be different. P. Jandera and co-workers (48) from the University of Pardubice (Czech Republic) investigated reversed-phase chromatography retention behavior at these compositions. When using reversed-phase chromatography to analyze lipophilic substances with high percentages of organic solvent in the mobile phase, the character of the stationary phases, such as the chemistry of the bonded moieties and the number and shielding of residual silanol groups, affects the separation much more significantly than it does in more water-rich mobile phases. Furthermore, preferential solvent adsorption on the surface of the stationary phase affects the solvation of larger molecules. In addition to the presence of the silanol groups, this effect causes interesting differences in retention for geometrical isomers and oligomers. On the other side of the composition curve, many ionic compounds can be separated in mobile phases that contain ionic additives in pure water or low concentrations of an organic modifier. Under these conditions, the residual silanol groups often are partially ionized and interact by attractive or repulsive forces with ionized sample solutes. Again, the number, shielding, and spacing of the silanol groups and the chemistry of the bonded phase affect the separation selectivity significantly. Often, chromatographers can achieve surprisingly good separations of geometrical isomers with ionic character. The separations usually are sensitive to the character of the mobile-phase additive and pH, even in the range of complete dissociation of the sample solutes. Jandera and colleagues showed several examples of separations at both extremes of mobile-phase composition.

Mary J. Wirth and co-workers (49) from the University of Delaware (Newark) have long been active in using spectroscopy to probe the surfaces of chromatography stationary phases. Their latest contribution is directed at understanding the adsorption to active silanols on chemically modified fused silica. Adsorption to silanols has been a long-standing problem in HPLC, especially with basic compounds. Using single-molecule probing with a cationic dye and investigating the surfaces by fluorescence imaging and single-molecule fluorescence spectroscopy, the authors found that active silanols are located at nanometer indentations, which are consistent with the steric inaccessibility of active silanols to the chlorodimethyloctadecylsilane (C18) reagent. Adsorption to active silanols was stronger when acetonitrile–water, rather than pure water, was used as the mobile phase. With single-molecule spectroscopy, they used the kinetics of adsorption and desorption and the fluorescence lifetime of the adsorbate to investigate the properties of active silanols and determine why the silanols are so strongly adsorptive and why acetonitrile enhances their adsorptive strength.

Joel M. Harris and co-workers (50) from the University of Utah (Salt Lake City) used internal reflection spectroscopy to study the selective detection of interfacial species, a useful method for investigating possible retention mechanisms in chromatography. Their approach was to deposit thin films of silica onto optically transparent substrates and then study the nature of the silica-solution interface, the transport of molecules within the silica, and the nature of the molecular interactions at the silica–solution interface. Using a similar approach, J.E. Pemberton and co-workers (51) from the University of Arizona (Tucson) used Raman spectroscopy to understand the degree of conformational order and disorder in conventional alkylsilane-based stationary phases in reversed-phase chromatography. They anticipated this technique would be useful to understand the stationary phase–analyte relationships and to create a series of designer stationary phases for specific compound classes.

Georges Guiochon and co-workers (52) from the University of Tennessee (Knoxville) and University of Veszprem (Hungary) investigated column-to-column and batch-to-batch reproducibility of retention times and retention factors for C18 reversed-phase packings. The parameters that influenced the reproducibility of the retention times were identified as the diameter of the column tube and the packing density. Principal component analysis confirmed this identification and allowed a detailed study of the effects of the two parameters. The reproducibility of the retention factors depended upon the phase ratio and secondary retention mechanisms. In all cases, principal component analysis confirmed that two factors are sufficient to characterize the variability of retention times or retention factors.

John Dolan and co-workers (53) from LC Resources (Walnut Creek, California), Linfield College (McMinnville, Oregon), and Florida State University (Tallahassee) have made it their goal to understand the differences between reversed-phase columns from different sources, as well as from different column batches from the same source, and then perhaps to correct for the differences in retention and separation on the different columns. Using many acidic, basic, and neutral test solutes for a range of experimental conditions, they developed an equation that correlates their data and allows them to modify column selectivity for the purposes of either choosing a column for a maximum difference in selectivity or a column from two different batches to give equivalent retention for any sample or set of experimental conditions.

K. Kimata and co-workers (54) from Nacalai Tesque (Kyoto, Japan), Kyoto Institute of Technology, and NEOS (Shiga, Japan) conducted an interesting study of the effect of polarizability of the stationary phase on retention properties in reversed-phase HPLC. They designed nonpolar stationary phases with aromatic, aliphatic, highly polarizable, and nonpolarizable organic moieties. Then they measured methylene group selectivity of these stationary phases using alkylbenzenes in common reversed-phase mobile phases. They also looked at a series of halogen-substituted alkylbenzenes and showed that highly polarizable compounds will preferentially be retained on polarizable stationary phases. As a result of this study, the authors were able to synthesize specialty phases for specific classes of closely related compounds that could not be separated on simple alkyl reversed-phase chromatography materials. In other words, they were able to custom design HPLC packing materials.

Conclusions

Clearly, investigations of new stationary phases for HPLC, CEC, sample preparation, and laboratory-on-a-chip nanotechnology will continue unabated as workers search for optimum separation performance and phase stability. Molecular-imprinted polymers offer an approach for extremely selective phases. Monoliths allow the production of miniature separation beds and easily replaceable conventional columns, and they may be particularly attractive for preparative LC because of their greater permeabilities. Temperature as a separation variable in HPLC should receive more attention now that packings that can withstand higher temperatures, especially at high pH values, are available. As chromatographers continue to learn more about separation mechanisms, they should be able to predict chromatographic retention for conventional phases as well as design custom stationary phases for difficult separations.

References

(1) R.E. Majors, *LCGC* **18**(6), 586–598 (2000).

(2) J.J. Kirkland and J.W. Henderson, *J. Chromatogr. Sci.* **32,** 473–480 (1994).

(3) J.J. Kirkland, J.L. Glajch, and R.D. Farlee, *Anal. Chem.* **61,** 2 (1988).

(4) H.A. Claessens, M.A. van Straten, and J.J. Kirkland, *J. Chromatogr.* **728,** 259 (1996).

(5) U.D. Neue, J. Carmody, Y.-F. Cheng, J. O'Gara, B.A. Alden, T. Walter, E.S.P. Bouvier, and R. Crowley, "Packings with Minimal Silanol Activity," paper L-048 presented at the 23rd International Symposium on High Performance Liquid Chromatography and Related Techniques — HPLC '99, Granada, Spain, 30 May–4 June 1999.

(6) L.R. Snyder, J.J. Kirkland, and J.L. Glajch, *Practical HPLC Method Development* (John Wiley & Sons, Inc., New York, 1997), p. 251.

(7) Barry E. Boyes and Doug G. Walker, "Reversed-Phase Separation of β-Amyloid Peptides," poster 505M presented at the Protein Society Meeting, San Jose, California, 5 May 1996.

(8) Bingwen Yan, James S. Brown, Peter W. Carr, "High Temperature Ultrafast Liquid Chromatography and Its Applications," paper L-1008 presented at the 24th International Symposium on High Performance Liquid Chromatography and Related Techniques — HPLC 2000, Seattle, Washington, 24–30 June 2000.

(9) R.M. Smith and R.J. Burgess, *Anal. Commun.* **33,** 327–329 (1996).

(10) D.J. Miller and S.B. Hawthorne, *Anal. Chem.* **69,** 623–627 (1997).

(11) R.M. Smith and R.J. Burgess, *J. Chromatogr. A* **785,** 49–55 (1997).

(12) R.M. Smith, O. Chienthavorn, I.D. Wilson, and B. Wright, *Anal. Commun.* **35,** 261–263 (1998).

(13) R.M. Smith, O. Chienthavorn, I.D. Wilson, B. Wright, and S.D. Taylor, *Anal. Chem.* **71,** 4493–4497 (1999).

(14) I.D. Wilson, private communication, October 2000.

(15) B. Jones, N. Porter, W.D. Felix, and D. Knowles, "High Temperature HPLC: Extended Life Columns Under Aggressive Conditions," paper L-0911 presented at the 24th International Symposium on High Performance Liquid Chromatography and Related Techniques — HPLC 2000, Seattle, Washington, 24–30 June 2000.

(16) T. Greibrokk, E. Lundanes, P. Molander, R. Trones, T. Andersen, K. Haugland, and A. Holm, "Temperature Programming in HPLC," paper L-0807 presented at the 24th International Symposium on High Performance Liquid Chromatography and Related Techniques — HPLC 2000, Seattle, Washington, 24–30 June 2000.

(17) T. Andersen, P. Molander, T. Greibrokk, R. Trones, D. R. Hegna, and J. Paus, "Applications of Temperature-Programmed Packed Capillary Liquid Chromatography," poster P-1409 presented at the 24th International Symposium on High Performance Liquid Chromatography and Related Techniques — HPLC 2000, Seattle, Washington, 24–30 June 2000.

(18) A.R. Duinkerken, O. Halmingh, G.J.M. Van Gils, A. Schellen, and J.A. Ooms, "Temperature Assisted On-Line SPE (TASPE) for HPLC and LC-MS," paper L-1313 presented at the 24th International Symposium on High Performance Liquid Chromatography and Related Techniques — HPLC 2000, Seattle, Washington, 24–30 June 2000.

(19) S. Linde, L. Hørlyck, and J.K. Jacobsen, "Temperature Critical RP-HPLC Polypeptide Separations: An Approach Towards Control," poster P-1322 presented at the 24th International Symposium on High Performance Liquid Chromatography and Related Techniques — HPLC 2000, Seattle, Washington, 24–30 June 2000.

(20) Y. Mao and P.W. Carr, "The Thermally Tuned Tandem Column Concept: A New Way to Do HPLC," paper L-0912 presented at the 24th International Symposium on High Performance Liquid Chromatography and Related Techniques — HPLC 2000, Seattle, Washington, 24–30 June 2000.

(21) J.W. Jorgenson, K. Patel, J. Sousa, L. Tolley, and A. Jerkovich, "Gradient Elution Separation in Ultra-High Pressure Liquid Chromatography," paper L-0901 presented at the 24th International Symposium on High Performance Liquid Chromatography and Related Techniques — HPLC 2000, Seattle, Washington, 24–30 June 2000.

(22) M.L. Lee, N. Wu, J.A. Lippert, and B. Xin, "Practical Aspects of Ultra-High Pressure Capillary Liquid Chromatography," paper L-0405 presented at the 24th International Symposium

on High Performance Liquid Chromatography and Related Techniques — HPLC 2000, Seattle, Washington, 24–30 June 2000.

(23) F.H. Dickey, *J. Phys. Chem.* **59,** 695 (1955).

(24) L. Ye, P.A.G. Cormack, and K. Mosbach, *Anal. Commun.* **36,** 35–38 (1999).

(25) K. Mosbach, K. Haupt, L. Ye, and E. Yilmaz, "Various Configurations and Their Use of Molecularly Imprinted Polymers," paper L-0002 presented at the 24th International Symposium on High Performance Liquid Chromatography and Related Techniques — HPLC 2000, Seattle, Washington, 24–30 June 2000.

(26) P.T. Vallano, G. Chirica, and V.T. Remcho, "Development of Materials that Exhibit — and Methods that Harness — Molecular Recognition," paper L-0409 presented at the 24th International Symposium on High Performance Liquid Chromatography and Related Techniques — HPLC 2000, Seattle, Washington, 24–30 June 2000.

(27) M.M. Degen, G.S. Chirica, P.T. Vallano, and V.T. Remcho, "Development of Molecular Imprint Polymers as Stationary Phases for Microscale Separations of Non-Steroidal Anti-Inflammatory Drugs," poster P-1511 presented at the 24th International Symposium on High Performance Liquid Chromatography and Related Techniques — HPLC 2000, Seattle, Washington, 24–30 June 2000.

(28) J. Haginaka, H. Sanbe, and C. Kagawa, "Uniform-Sized Molecularly Imprinted Polymers as HPLC Chiral Stationary Phases," paper L-1206 presented at the 24th International Symposium on High Performance Liquid Chromatography and Related Techniques — HPLC 2000, Seattle, Washington, 24–30 June 2000.

(29) L. Schweitz, L.I. Andersson, and S. Nilsson, "Soap CEC on MIPs," paper L-0909 presented at the 24th International Symposium on High Performance Liquid Chromatography and Related Techniques — HPLC 2000, Seattle, Washington, 24–30 June 2000.

(30) C. Sulitzky, B. Ruckert, B. Sellergren, and K.K. Unger, "Synthesis and Characterization of New Molecularly Imprinted Polymer Coated Inorganic Supports," poster P-1531 presented at the 24th International Symposium on High Performance Liquid Chromatography and Related Techniques — HPLC 2000, Seattle, Washington, 24–30 June 2000.

(31) K. Hosoya, T. Kubo, S. Hisai, T. Ikegami, N. Tanaka, and K. Kaya, "Fragment Imprinting Effect," poster P-0202 presented at the 24th International Symposium on High Performance Liquid Chromatography and Related Techniques — HPLC 2000, Seattle, Washington, 24–30 June 2000.

(32) K.-S. Boos and C.T. Fleischer, "Highly Selective Solid-Phase Extraction (SPE) of Biofluids Using Restricted Access Materials (RAM) in Combination with Molecular Imprinted Polymers (MIPs)," paper L-1303 presented at the 24th International Symposium on High Performance Liquid Chromatography and Related Techniques — HPLC 2000, Seattle, Washington, 24–30 June 2000.

(33) J. Jodlbauer, P. Zöllner, N.M. Maier, and W. Lindner, "Determination of Ochratoxin in Biological Samples with Molecularly Imprinted Polymers for Sample Preparation," poster P-1703 presented at the 24th International Symposium on High Performance Liquid Chromatography and Related Techniques — HPLC 2000, Seattle, Washington, 24–30 June 2000.

(34) K. Cabrera, G. Wieland, D. Lubda, K. Nakanishi, N. Soga, H. Minakuchi, and K.K. Unger, *Trends Anal. Chem.* **17**(1), 50–53 (1998).

(35) M. Przybyciel, "The Development and Evaluation of Porous Silica Monolith Technology for Liquid Chromatography," paper L-0507 presented at the 24th International Symposium on High Performance Liquid Chromatography and Related Techniques — HPLC 2000, Seattle, Washington, 24–30 June 2000.

(36) N. Tanaka, H. Kobayashi, H. Nagayama, T. Ikegama, K. Hosoya, N. Ishizuka, H. Minakuchi, and K. Nakanishi, "Comparison of Practical Operational Parameters Between Micro-HPLC and CEC to Generate 100,000 Theoretical Plates per Column by Using a Monolithic Silica in Capillary," paper L-1401 presented at the 24th International Symposium on High Performance Liquid Chromatography and Related Techniques — HPLC 2000, Seattle, Washington, 24–30 June 2000.

(37) E. Müller, A. Seiler, H. Minakuchi, and K. Nakanishi, "Performance Properties of Silicarod Capillary Columns," poster P-0626 presented at the 24th International Symposium on High Performance Liquid Chromatography and Related Techniques — HPLC 2000, Seattle, Washington, 24–30 June 2000.

(38) N. Ishizuka, H. Minakuchi, K. Nakanishi, K. Hirao, K. Hosoya, and N. Tanaka, "Preparation and Evaluation of Monolithic Silica with

Wide Mesopores for Polypeptide Separation," poster P-0537 presented at the 24th International Symposium on High Performance Liquid Chromatography and Related Techniques — HPLC 2000, Seattle, Washington, 24–30 June 2000.

(39) G. Iberer, R. Hahn, and A. Jungbauer, *LCGC* **17**(11), 998–1005 (1999).

(40) E.C. Peters, M. Petro, F. Svec, and J.M.J. Fréchet, *Anal. Chem.* **70,** 2296–2302 (1998).

(41) T. Jiang, H.A. Claessens, J. Jiskra, and C.A. Cramers, "Preparation and Characterization of Columns with Monolithic Polymer Stationary Phases for Capillary Electrochromatography and Micro-HPLC," poster P-0106 presented at the 24th International Symposium on High Performance Liquid Chromatography and Related Techniques — HPLC 2000, Seattle, Washington, 24–30 June 2000.

(42) S. Xie, P. Wagner, J. Lin, R.W. Allington, F. Svec, and J.M.J. Fréchet, "High Throughput Bioseparations in Monolithic Ion Exchangers," paper L-1413 presented at the 24th International Symposium on High Performance Liquid Chromatography and Related Techniques — HPLC 2000, Seattle, Washington, 24–30 June 2000.

(43) M. Lammerhofer, F. Svec, J.M.J. Fréchet, and W. Lindner, "Macroporous Monolithic Chiral Stationary Phases Based on Quinidine Carbamate for Enantioselective Capillary Electrochromatography," paper L-0506 presented at the 24th International Symposium on High Performance Liquid Chromatography and Related Techniques — HPLC 2000, Seattle, Washington, 24–30 June 2000.

(44) R. Shediac, S.M. Ngola, D.J. Throckmorton, D.S. Anex, A.K. Singh, and T.J. Shepodd, "In Situ Porous Polymer Monoliths for Reverse-Phase Capillary Electrochromatography of Amino Acids and Peptides," poster P-0108 presented at the 24th International Symposium on High Performance Liquid Chromatography and Related Techniques — HPLC 2000, Seattle, Washington, 24–30 June 2000.

(45) R. Freitag and D. Hoegger, "Monolithic Hydrophilic Stationary Phases for CEC," paper L-0504 presented at the 24th International Symposium on High Performance Liquid Chromatography and Related Techniques — HPLC 2000, Seattle, Washington, 24–30 June 2000.

(46) Y. Kazakevich and R. Lobrutto, "Formation of an Adsorbed Phase of the Organic Eluent Component on the Surface of RP Packings: Its Effect on the Analyte Retention," paper L-0811 presented at the 24th International Symposium on High Performance Liquid Chromatography and Related Techniques — HPLC 2000, Seattle, Washington, 24–30 June 2000.

(47) N. Felitsyn and F.F. Cantwell, "Stationary Phase Contribution of the Organic Modifier to Sample Sorption on an ODS-Bonded Phase," paper L-0802 presented at the 24th International Symposium on High Performance Liquid Chromatography and Related Techniques — HPLC 2000, Seattle, Washington, 24–30 June 2000.

(48) P. Jandera, S. Buncekova, and L. Kolarova, "Reversed-Phase Retention Behavior in Mobile Phases with Low Concentrations of Water or of the Organic Modifiers," paper L-0808 presented at the 24th International Symposium on High Performance Liquid Chromatography and Related Techniques — HPLC 2000, Seattle, Washington, 24–30 June 2000.

(49) M.J. Wirth, D.J. Swinton, and M.D. Ludes, "Single-Molecule Probing of Adsorption to Active Silanols in Reversed Phase HPLC," paper L-0801 presented at the 24th International Symposium on High Performance Liquid Chromatography and Related Techniques — HPLC 2000, Seattle, Washington, 24–30 June 2000.

(50) J.M. Harris, D. Rivera, and K. McCain, "Internal Reflection Spectroscopy Studies of Retention Mechanisms and Chromatographic Interfaces," paper L-0809 presented at the 24th International Symposium on High Performance Liquid Chromatography and Related Techniques — HPLC 2000, Seattle, Washington, 24–30 June 2000.

(51) J.E. Pemberton, M.W. Ducey, M. Ho, J. Turner, and K. Harwell, "Chromatographic Stationary Phase Structure-Function Relationships: Understanding Conventional Stationary Phases and Exploring Unconventional Stationary Phases," paper L-0810 presented at the 24th International Symposium on High Performance Liquid Chromatography and Related Techniques — HPLC 2000, Seattle, Washington, 24–30 June 2000.

(52) A. Felinger, M. Kele, and G. Guiochon, "Factors Influencing the Reproducibility of Retention Data," paper L-0201 presented at the 24th International Symposium on High Performance Liquid Chromatography and Related Techniques — HPLC 2000, Seattle, Washington, 24–30 June 2000.

(53) J.W. Dolan, L.R. Snyder, N.S. Wilson, M.D. Nelson, R.G. Wolcott, M. Hemberger, and J.G. Dorsey, "Characterization of HPLC Column Selectivity and Its Modification by Changes in Separation Conditions," paper L-1403 presented at the 24th International Symposium on High Performance Liquid Chromatography and Related Techniques — HPLC 2000, Seattle, Washington, 24–30 June 2000.

(54) K. Kimata, T. Morimoto, M. Turowski, T. Monde, T. Ikegami, K. Hosoya, and N. Tanaka, "Effect of the Polarizability of the Stationary Phase on the Retention Properties in Reversed-Phase Liquid Chromatography," poster P-0510 presented at the 24th International Symposium on High Performance Liquid Chromatography and Related Techniques — HPLC 2000, Seattle, Washington, 24–30 June 2000.

Ronald E. Majors

"Column Watch" editor Ronald E. Majors is business development manager, consumables and accessories business unit, Agilent Technologies, Wilmington, Delaware, and is a member of LCGC*'s editorial advisory board. Direct correspondence about this column to "Column Watch,"* LCGC*, 859 Willamette Street, Eugene, OR 97401, e-mail lcgcedit@lcgcmag.com.*