Watch

Columns for Reversed-Phase LC Separations in Highly Aqueous Mobile Phases

Ronald E. Majors and Matthew Przybyciel

Last month's "Column Watch" discussed the phenomenon of phase collapse and ways to solve the problem and successfully separate polar analytes using mobile-phase systems with a very high percentage of water. In this month's column, Majors and Przybyciel describe columns that can deal with this difficult situation and suggest some commercial columns for separating polar compounds in highly aqueous environments.

Ronald E. Majors
Column Watch Editor

Reversed-phase chromatography is by far the most widely used mode in high performance liquid chromatography (HPLC) (1). It allows chromatographers to manipulate the mobile phase by changing organic solvent type, solvent composition, and pH; by adding modifiers such as surfactants, chiral reagents, competing bases, and ion-pair reagents; or by adjusting experimental conditions such as flow rate and temperature. Originally, researchers believed that they would need only a few stationary phases to achieve virtually any separation they encountered. Indeed with a C18 column and the ability to adjust the myriad parameters, chromatographers have achieved many successful separations. Still, sometimes analysts can neither find the necessary selectivity nor obtain a rugged and reproducible separation easily, no matter how many parameters they adjust.

Early Modified Surfaces in Reversed-Phase Chromatography

New and modified reversed-phase chromatography stationary phases have been introduced throughout the years to provide more separation power. In reversed-phase chromatography, basic compounds frequently can interact with unreacted silanols on the silica gel because it is virtually impossible, at least with monomeric bonding, to remove or cover all of the silanols due to steric reasons, especially with long alkyl chain phases such as octadecylsilane (C18). This interaction is most problematic when the packing is used at intermediate pH values of pH 4–8 at which silanols and many basic compounds are partially ionized.

Twenty years ago, the low-purity silica gel used for most HPLC columns caused high surface acidity and a tailing problem with basic compounds. The first modified stationary phases were the so-called base-deactivated bonded silicas. These base-deactivated phases were not deactivated with base, but the term implied that the column had been treated to provide minimal interaction and tailing with strongly basic compounds. Among the treatments were acid washing of the silica before bonding, single- or multiple-reactant endcapping, covering or encapsulating the surface with polymeric phase, and other unspecified proprietary methods. Later, researchers developed Type B silicas, which had less acidic surfaces, a more homogeneous distribution of silanol groups, and more-reproducible and denser bonding of alkyl chains. Most silica-based columns today are built on Type B base materials, and the most popular phases of those are based on C8 and C18 alkylsilane chemistries. When separating basic compounds, the base-deactivated phases also are popular, and many of them are available on the market (2).

Conventional Reversed-Phase Chromatography in Highly Aqueous Mobile Phases

Even with these improved alkylsilane surfaces, certain experimental conditions can cause separation or stationary-phase problems. For example, both very high pH (higher than pH 10) and low pH (lower than pH 2) conditions can present problems with stationary-phase and column stability (3). For very polar analytes, other problems can arise. For solubility reasons, many polar compounds prefer a highly aqueous mobile phase and can be retained only with a minimal concentration of organic modifier, sometimes less than 5%.

In last month's "Column Watch," we demonstrated and discussed the phenomenon of phase collapse and mentioned

approaches to solve the problem and successfully separate polar analytes using mobile-phase systems with a very high percentage of water, even as great as 100% water (4). In this month's column, we will present ways to deal with this difficult situation. In addition, we will suggest typical commercial columns that chromatographers can use to separate polar compounds in highly aqueous environments.

Several aspects of the design of stationary phases can help retain polar analytes under highly aqueous conditions, including

- nonendcapped, short-chain alkyl phases;
- hydrophilic, polar-endcapped, and polar-enhanced stationary phases;
- polar-embedded alkyl phases;
- long-chain alkyl phases; and
- wide-pore-diameter phases.

Nonendcapped, Short-Chain Alkyl Phases

When unreacted surface silanol groups are present on an alkyl bonded silica gel, they impart a degree of polarity that sometimes can be useful but sometimes can be detrimental. Their utility comes when the alkyl function alone provides insufficient separation selectivity, and the presence of silanols can cause polar interactions with polar functionality on analytes. The resulting mixed mechanisms can yield improved separations, but the packings might be difficult to reproduce batch-to-batch unless the ligand-to-silanol ratio is exactly the same.

Surface silanol groups also interact with water (hydrogen bonding), so the available alkyl surface is determined by the high density of the bonded phase in some cases, especially for short-chain alkyl phases (smaller than C4). In those situations, little free volume exists between bonded chains, and little shielding of the silanols occurs. As Kasakevich and co-workers (5) pointed out, these short-chain phases present little risk for phase collapse, and the predominant separation mechanisms are adsorption (not partitioning) onto the alkyl phase and the polar interactions of the analyte with silanols. The phase-bonding densities are lower for chains longer than C4, and they have a greater degree of conformational freedom. Partitioning (not adsorption) into the alkyl chains can occur if the chains are highly solvated and extend away from the surface. When the amount of organic solvent is decreased to less than 5–10% with some of these long-chain packings, the phase solvation decreases and the phases tend to collapse and cause the problems discussed in last month's column (4).

On the detrimental side, these unreacted silanols can ionize at intermediate-to-high pH values (pH 5 and greater) and can undergo ion-exchange interactions that cause severe tailing for basic compounds and nonrugged separation methods in which pH and temperature must be carefully controlled (3).

Hydrophilic, Polar-Endcapped, and Polar-Enhanced Stationary Phases

Using polar or hydrophilic endcapping along with bonding of longer alkyl chains such as C18 is a successful development approach for stationary phases that can retain polar analytes reproducibly under highly aqueous conditions. As with the low-bonding-density packings discussed earlier, these polar or hydrophilic endcapping chemicals allow the silica surface to be wetted with water and allow the full interaction with the longer alkyl chains.

Table I lists several commercially available columns that use polar or hydrophilic endcapping techniques. Unfortunately, we found few references for the exact nature of these polar or hydrophilic endcapping reagents. Researchers generally believe that these reagents can be used in a manner similar to that of classical endcapping reagents such as trimethylchlorosilane. Trimethylchlorosilane is a reactive chemical used to deactivate residual silanol groups after bonding an alkyl moiety such as C18. The bonding and endcapping process for this

Table I: Examples of reversed-phase chromatography columns for use in highly aqueous mobile phases

Product Name	Supplier	Functional Group	Comments
Alltima AQ	Alltech Associates	C_{18}	Hydrophilic endcapping
Aqua	Phenomenex	C_{18}	Polar endcapping
AquaSep	ES Industries	Hydrocarbon ether	Single-step synthesis; no endcapping
AquaSep Basic C18	ES Industries	C_{18}	Single-step bonding without hydrophilic endcapping
AquaSep-WP	ES Industries	Hydrocarbon ether	Large pores allow for chromatography of larger molecules
Aquasil C18	Thermo Hypersil-Keystone	C_{18}	Hydrophilic endcapping
Chemcobond ODS W	DyChrom	C_{18}	
Genesis AQ	Argonaut Technologies	C_{18}–short alkyl	Short chains, non-trimethylsilane
HydroBond PS	Mac-Mod Analytical	C_8, C_{18}	Hydrophilic endcapping
Hydrosphere C18	YMC	C_{18}	Surface-enhanced polar selectivity
MetaSil Aq	Ansys Technologies	C_{18}	Polar endcapping
Multospher 120 RP 18-AQ	CS-Chromatographie Service	C_{18}	Full endcapping
Nucleosil 100-5 Nautilus	Macherey-Nagel	C_{18}	Modified C18 with mixture of hydrophilic and hydrophobic groups
Polarity dC18	Waters	C_{18}	Difunctional bonding, partial endcapping
PrincetonSpher C-27	Princeton Chromatography	C_{27}	Larger particle sizes are available upon request
ProntoSIL C18 AQ Plus	Bischoff Chromatography	C_{18}	Hydrophilic endcapping
Synergy Hydro-RP	Phenomenex	C_{18}	Bulk packing available
YMC AQ	YMC	Unspecified	
Zorbax StableBond AQ	Agilent Technologies	Proprietary	Sterically protected chemistry

type of reversed-phase packing minimally is a two-step process. In the first step, a monochloroalkylsilane (for example, C8 or C18) is bonded to silica. A second bonding step uses the specific endcapping reagent. Polar or hydrophilic endcapping chemicals could be short-chain trimethoxy- or triethoxysilanes, which can be hydrolyzed after bonding to produce silanol groups. These silanol groups near the surface would provide a high degree of polar character to the final alkyl bonded phase, but they have a lower acidity than residual silanols found on the surface of bonded silicas.

Companies Mentioned in this Column

Advanced Chromatography Technologies, Aberdeen, United Kingdom
Agilent Technologies Inc., Wilmington, Delaware
Alltech Associates, Inc., Deerfield, Illinois
Analytical Sales and Services, Inc., Pompton Plains, New Jersey
Ansys Technologies, Inc., Lake Forest, California
Argonaut Technologies, Foster City, California
Bischoff Chromatography, Leonberg, Germany
Cluzeau-Info-Labo, Sainte-Foy-La-Grande, France
CS-Chromatographie Service GmbH, Langerwehe, Germany
DyChrom, San Jose, California
Eka Chemicals, Inc., Marietta, Georgia
ES Industries, Berlin, New Jersey
GL Sciences, Tokyo, Japan
HPLC Technology Co. Ltd., Welwyn Garden City, United Kingdom
Macherey-Nagel, Easton, Pennsylvania
Mac-Mod Analytical Inc., Chadds Ford, Pennsylvania
The Nest Group, Inc., Southborough, Massachusetts
Nomura Chemical Co. Ltd., Seto, Japan
Phenomenex Inc., Torrance, California
Princeton Chromatography, Inc., Cranbury, New Jersey
Restek Corp., Bellefonte, Pennsylvania
Serva Electrophoresis GmbH, Heidelberg Germany
Supelco, Inc., Bellefonte, Pennsylvania
Thermo Hypersil-Keystone, Bellefonte, Pennsylvania
Waters Corp., Milford, Massachusetts
Whatman, Inc., Clifton, New Jersey
YMC Co. Ltd., Kyoto, Japan

Another approach to modifying polar or hydrophilic surfaces involves the use of polymeric bonding reagents such as octadecyltrimethoxysilane. The silica first is bonded with the octadecyltrimethoxysilane. Then, water reacts with any remaining trimethoxy groups to form additional silanols (6). The resulting highly aqueous mobile phase can penetrate the silica pores of the polar-enhanced surface.

Another way to achieve a polar-enhanced surface is to incorporate polar groups or polar-embedded groups in alkyl chains. We will discuss this technique extensively in the following section. We also should note that ether groups have been used effectively as polar-enhancing agents (7). The ether group can be incorporated in the alkyl chain near the silica attachment point. This modification is polar enough to allow water to enter the pores of the silica. This modification has the inherent advantage of being a single-step bonding process without any type of endcapping. Without endcapping, many of these alkyl ether phases can be used at low pH levels.

The purine and pyrimidine base separation shown in Figure 1 is a unique application using a highly aqueous mobile phase. The separation is unique because uracil is retained and eluted after 5-fluorocytosine and cytosine. Uracil typically is used as void volume marker because it is not retained with most reversed-phase columns. However, the uracil was retained on an alkyl ether phase with 100% aqueous mobile phase, as shown in Figure 1. Uracil also was retained on a hydrophilic endcapped column with the same conditions as described in Figure 1. The separation of purine and pyrimidine bases is one of many applications that have been performed with columns engineered to operate with highly aqueous mobile phases. Other applications include the separation of low molecular weight organic acids, catecholamines, and water-soluble vitamins (7).

Polar-Embedded Alkyl Phases

Another successful method for applications with highly aqueous mobile phases is using polar-embedded alkyl stationary phases, sometimes called polar-linked phases. Table II is a partial list of many of the commercial products introduced during the past several years. With the incorpora-

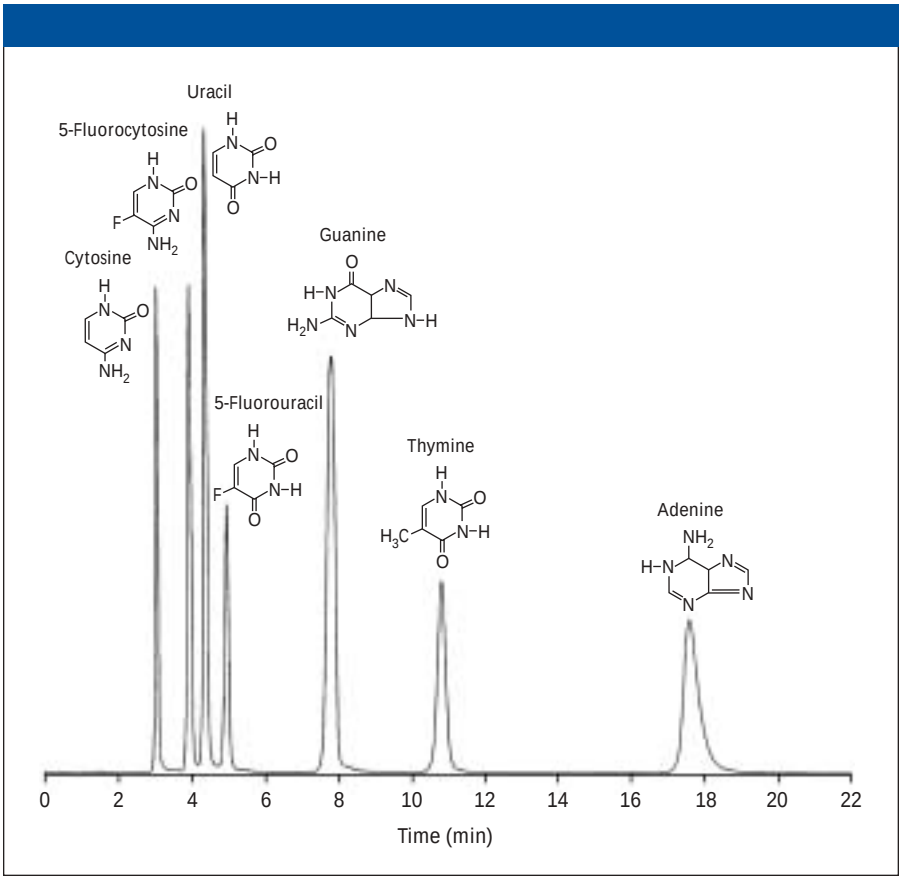

Figure 1: Separation of purine and pyrimidine bases on a hydrophilic stationary phase. Column: 150 mm × 4.6 mm, 5-µm d_p AquaSep; mobile phase: 0.05 M sodium acetate (pH 4.6); flow rate: 1.0 mL/min; detector: UV at 254 nm. (Courtesy of ES Industries.)

Table II: Typical polar-embedded reversed-phase columns

Product Name	Supplier	Functional Group	Embedded Polar Group	Comments
Ace AQ	Advanced Chromatography Technologies	C_{18}	Unspecified	
Chromegabond ODS-PI	ES Industries	C_{18}	Urea	
Discovery Amide	Supelco	C_{16}	Amide	Prepared by one-step process
HydroBond AQ	Mac-Mod Analytical	Unspecified	Unspecified	
Inertsil Embedded Polar	GL Sciences	Unspecified	Unspecified	
Kromasil Amide C8	Eka Chemicals	C_8	Amide	Endcapped
Maccel AQPS C18	Nest Group	C_{18}	Unspecified	
Nucleosil 100-3 Protect I	Macherey-Nagel	Unspecified	Unspecified	
Pinnacle IBD	Restek	C_{18}	Unspecified	IBD = intrinsically base-deactivated
Polaris C18- and C8-Ether	Ansys Technologies	C_8, C_{18}	Ether	
Polaris-A	Ansys Technologies	C_8, C_{18}	Unspecified	
Prism RP18	Thermo Hypersil-Keystone	C_{18}	Unspecified	Endcapped; Prism RPN is nonendcapped version
ProntoSIL C18 ACE-EPS	Bischoff Chromatography	C_{18}	Unspecified	
ProTec	ES Industries	C_8, C_{18}	Amide	
Quest Advance	Thermo Hypersil-Keystone	C_8	Amide	
RTF C18	Whatman	C_{18}	Amide	
Stability-BS-C23	Cluzeau-Info-Labo	C_{18}	Amine	Endcapped and nonendcapped versions
Supelcosil ABZ+	Supelco	C_{18}	Amide	One-step synthesis
SymmetryShield RP18	Waters	C_8, C_{18}	Carbamate	
Zorbax Bonus-RP	Agilent Technologies	C_{14}	Amide	Triple endcapped, bulky sterically protected bonded phase

tion of a polar functional group in the alkyl ligand close to the surface of the silica gel, the phase remains solvated by water at low percentages of organic modifier and even with 100% water. Under these conditions, the alkyl chains maintain their conformational freedom and can interact with polar analytes. Researchers also believe that the presence of the polar functionality close to the surface shields the effects of unreacted silanol groups.

Nomura and co-workers (8) made the first report of this type of bonded phase. They acylated an aminopropyl bonded phase to form an amide linkage, as depicted in Figure 2. Later, Ascah and Feibush (9) used similar chemistry to develop Supelcosil ABZ (Supelco), which was the first commercial polar-embedded phase. Others have prepared these amide phases with *R* groups varying from C_8 to C_{18}. Because this approach has two individual bonding steps, the possibility of mixed derivatized and underivatized amino groups led to potential mixed modes of separation. By incorporating a carbamate functional group into the silane, Neue and co-workers (10) were able to develop a one-step synthesis method that resulted in a second type of embedded polar group, and the product was named SymmetryShield RP 8 (Waters Corp.). Since then, developers have incorporated several other specific functional groups, including urea, ether, mixed amide and carbamate, and amine, into the alkyl chain. As Table II shows, many manufacturers do not specify the nature of the polar-embedded group and treat the phases as proprietary.

The polar-embedded concept has many advantages:

- The stationary phase maintains a reversed-phase character.
- The phases provide a different selectivity compared with alkyl phases, particularly with polar analytes (see Figure 3).
- The phases can be used in low percentages of organic solvent and even in 100% water without dewetting. This feature is especially useful for polar compound retention and leads to improved chromatographic performance (stable and reproducible retention) and faster gradient regeneration.

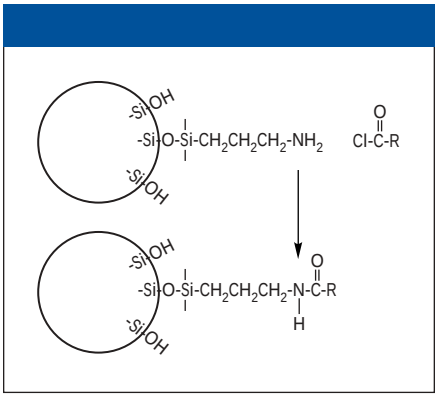

Figure 2: Synthesis of an amide polar-embedded phase starting with an aminopropyl bonded phase.

- Silanol activity is suppressed, which leads to better peak shape and decreased tailing of basic compounds, particularly at intermediate pH values.

The mechanism for the improved performance of polar-embedded alkyl phases has not been studied well nor have any systematic comparisons of the various types of polar-embedded functionalities been pub-

lished. Researchers engage in considerable speculation about how they work relative to standard alkyl phases (11). In general, relative to their alkyl counterparts, the polar-embedded phases show reduced retention factors for polar and basic analytes, and nonpolar analytes are less affected. Hydrogen bond donors can interact through hydrogen bonding with the polar-embedded groups, and this interaction might explain the increased retention for this class of compounds compared with conventional alkyl phases (12). One proposed mechanism involves the interaction of the phases' polar functionalities with the residual silanols on the surface of the silica-based packing through hydrogen bonding and an increase in the water concentration on the surface because of the hydrogen-bonding ability (13). A weakening of the interactions between basic analytes and residual silanols occurs as a result of this surface layer of water (14). In highly aqueous mobile phases, these polar-embedded phases certainly wet more easily because of their hydrogen-bonding ability with water, and the contact angle between the surface and water could drop to less than 90° at which the water could penetrate the porous surface freely (15).

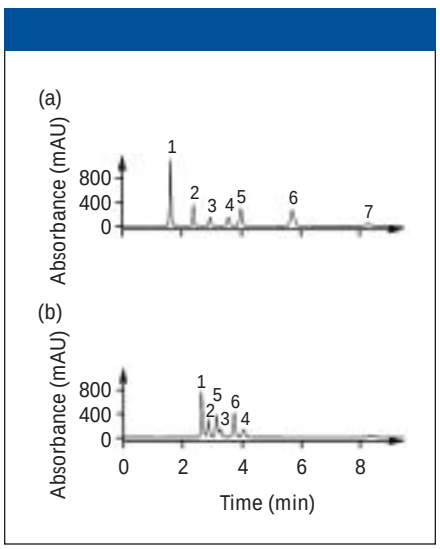

Figure 3: Comparative separations of triazine pesticides on (a) Zorbax Bonus-RP and (b) Alkyl C8 columns. Column dimensions: 150 mm × 4.6 mm; mobile phase: 70:30 (v/v) methanol–0.1% trifluoroacetic acid; flow rate: 1.0 mL/min; temperature: ambient; injection volume: 2 µL; detection: UV absorbance at 254 nm. Peaks: 1 = prometryn, 2 = tebuthiuron, 3 = atrazine, 4 = propazine, 5 = diuron, 6 = propanil, 7 = dimethyl tetrachloroterephthalate. (Chromatograms courtesy of Agilent Technologies.)

Long-Chain Stationary Phases

As discussed above, some analytes are so polar that even 5% or less of organic solvent in the mobile phase can prevent sufficient retention on a C18 column. Increasing the hydrophobicity of the stationary phase can generate a sufficient amount of hydrophobic interaction between the stationary phase and cause the hydrophobic portions of the analytes to increase the retention of polar analytes. Table III lists some of the long-alkyl-chain columns — from C27 to C30 — that are commercially available. Generally, these phases are more retentive for polar and nonpolar analytes than are most polar-embedded and even high-coverage C18 phases. Because of a higher degree of surface shielding, long-chain phases also offer greater pH stability than do C8 and C18 phases.

Surprisingly, the long-chain phases also are more resistant to phase collapse under high aqueous conditions than are C18 phases. This behavior could be related to conformation changes in the stationary phase with temperature. The melting point of $C_{18}H_{38}$, which is the functional portion of a typical C18 phase, is 29–30 °C; however, the melting point of $C_{30}H_{62}$ is 68–69 °C. Therefore, C30, the triacontyl ligand, is in a solid state at 30–40 °C, which is the typical operating temperature of a reversed-phase column, and its ligand does not move or collapse under highly aqueous mobile-phase conditions (16). On the other hand, the C18 phase is in a liquid state, and its ligand moves easily or collapses under highly aqueous mobile-phase conditions. Of course, this scenario assumes that the bonded phase behaves in a manner similar to that of the liquid hydrocarbon state, but solid-state nuclear magnetic resonance work for C30 phases showed that as the temperature increased, the ligand chains became more mobile and more disordered with an increase in kinks and bends (17).

Figure 4 shows an example of a long-chain hydrocarbon phase in a buffered totally aqueous mobile phase under isocratic conditions in which good retention and selectivity were observed for a series of nucleotides. Note the symmetrical peaks generated using the C30 phase. A C18

Table III: Long-chain reversed-phase packings

Product Name	Supplier	Functional Group
Advantage C30 Altocarb	Analytical Sales and Services	C_{30}
Develosil C30-UG-5	Nomura Chemical	C_{30}
Hicarbosphere	HPLC Technology	C_{30}
MetaChem C30	Ansys Technologies	C_{30}
PrincetonSpher C-27	Princeton Chromatography	C_{27}
ProntoSIL C30	Bischoff Chromatography	C_{30}
Triacontyl-Si 100	Serva Electrophoresis GmbH	C_{30}
YMC Pack C30	YMC	C_{30}

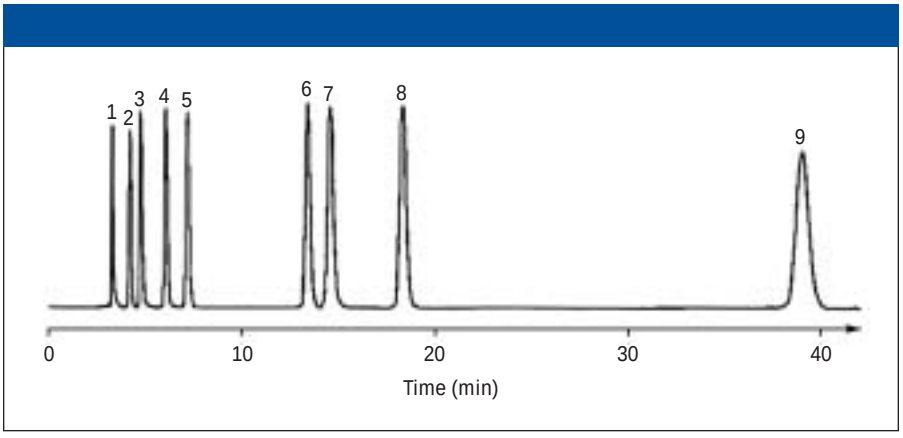

Figure 4: Use of 100% aqueous mobile phase with a C30 bonded phase. Column: 250 mm × 4.6 mm, 5-µm d_p Develosil RP-Aqueous; mobile phase: 0.1 M dibasic potassium phosphate with potassium hydroxide (pH 6.0); flow rate: 1.0 mL/min; temperature: 30 °C; detection: UV absorbance at 260 nm; sample volume: 4 µL. Peaks: 1 = 5′-CTP, 2 = 5′-CDP, 3 = 5′-CMP, 4 = 5′-GTP, 5 = 5′-GDP, 6 = 5′-GMP, 7 = 5′-ATP, 8 = 5′-ADP, 9 = 5′-AMP. (Chromatogram courtesy of Nomura Chemical Co. Ltd.)

phase run under similar conditions resulted in a total loss of retention after 35 h of run time (16). Interestingly, Nomura Chemical Co. recently received a U.S. patent in which the claims cover the use of C30 stationary phases with a mobile phase of not less than 97% water by volume (18). This patent may negate the opportunity for users to take full advantage of these long-chain phases in highly aqueous media.

Wide-Pore Diameter Phases

If the pore diameter of a silica-gel packing is widened, then the bonded phase is less densely packed and dewetting can be delayed. However, the lower surface area of wide-pore silicas results in lower retention, so this approach is of less interest as a means of increasing the retention of polar compounds in highly aqueous environments (19).

Conclusions

We have suggested several approaches to handle difficult separations of polar analytes by reversed-phase chromatography under highly aqueous separation conditions. Compared with the use of alkyl bonded phase columns, most of these approaches prevent dewetting of the alkyl bonded phases, provide increased retention for polar analytes, give good peak shapes, and allow higher sample throughput because of faster mobile-phase reequilibration. Although the exact nature of the mechanisms of operation is not entirely understood from a theoretical viewpoint, these columns could provide solutions for users experiencing difficulty using regular reversed-phase columns with polar analytes and highly aqueous mobile phases. Often, these specialty columns also perform well in regular reversed-phase chromatography but provide a different selectivity than normal C8 and C18 phases.

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