An Efficient Approach to Column Selection in HPLC Method Development

The base-particle characteristics and bonded-phase chemistries of silica-based high performance liquid chromatography (HPLC) columns have astonishing diversity. Chromatographers often must use a time-consuming trial-and-error approach to column selection when developing new methods. In this article, the authors present an efficient, informed approach to HPLC column selection based upon complementary decisions regarding bonded-phase and particle physical characteristics. First, the authors used knowledge of the sample components, particularly molecular structure information, as a guide in choosing bonded-phase characteristics. Second, they used an analysis of the separation goals in choosing column and particle physical characteristics.

Craig S. Young and Raymond J. Weigand
Alltech Associates, Inc.,
2051 Waukegan Road, Deerfield, Illinois 60015

Address correspondence to R.J. Weigand.

High performance liquid chromatography (HPLC) method development can be laborious and time-consuming. Chromatographers have taken many approaches to trimming the time required for this task, including experimental design techniques such as a systematic evaluation of mobile-phase chemistries (1,2), scouting gradients (3), chemometric software aids such as liquid chromatography (LC) simulation and prediction software (4), and specialized hardware for unattended switching of mobile-phase streams or columns (5).

These approaches certainly are valuable. However, a commonly overlooked and vitally important aspect of method development can be undertaken in the planning stage, when chromatographers can perform a knowledgeable evaluation of appropriate column chemistries and packed-bed physical characteristics (2). An evaluation of column physics and chemistry as they relate to a separation can lead to the selection of an HPLC column that is uniquely suited to a method's goals. This approach requires that analysts know something about the sample chemistry and that they give the formulation of realistic method goals adequate attention. Addressing these criteria represents a small initial time investment, but it can result in the selection of an optimum column to begin method development and a time saving in the laboratory.

An Overview of the Column Selection Approach

In our approach, we base column selection upon complementary decisions regarding bonded-phase and particle physical characteristics. First, we use our knowledge of the sample components, particularly molecular structure information, as a guide in choosing bonded-phase characteristics such as bonding chemistry, bonding mode, endcapping, and carbon load. Recognizing that sample retention on a column is a product of chemical interactions, we can select a column chemistry that is optimum for separating the molecular structures in question. Second, we use an analysis of the separation goals to choose column and particle physical characteristics, including column-bed dimensions, particle shape, particle size, surface area, and pore size.

Our discussion is limited to silica-based packings. Modern bonded-silica columns can be suitable throughout a pH 1–10

range with carefully chosen, nonaggressive, nonphosphate buffers. This limitation is a reflection of column manufacturers' efforts to improve column stability at pH extremes. Traditionally, a pH 2–7.5 range is cited for silica-based columns because prolonged exposure to pH extremes causes hydrolysis of the bonded phase (acidic extremes) or degradation of the base silica (basic extremes). Some samples can benefit from pH extremes used as a selectivity tool; for example, using pH 10–11 buffer to maintain organic amines in neutral form for enhanced retention. Other samples can possess solubilities that dictate a pH of less than pH 2 or greater than pH 7.5. In either case, a sample could be analyzed on nonsilica (zirconia or graphitized carbon) or polymer-based (poly[styrene–divinylbenzene], divinylbenzenemethacrylate, polyvinyl alcohol, or vinyl alcohol) columns. Silica-based columns typically exhibit higher efficiencies than their nonsilica counterparts. Chromatographers also benefit from a much larger knowledge base for silica-based packings because these packings have been used for many years in diverse applications.

The Planning Stage

Chromatographers' knowledge of samples influences their choice of column bonded-phase characteristics. The more analysts know about the makeup of their samples, the more successful they will be in predicting optimum HPLC column chemistry. Analysts should ask and attempt to answer questions such as: How many sample components are present? What is the sample matrix? What are the structures of sample components? What are the pK_a values of sample components? Is UV spectral information available? What is the sample concentration range? What is the molecular weight range? What do the solubility data suggest? The answers to these questions also give preliminary indications of appropriate detection type (for example, UV detection for sample molecules that have spectroscopic chromophores) and initial mobile phase (normal or reversed phase).

Goals for method influence the choice of a column's particle physical characteristics. Assessing the most important method goals is in order. Analysts should ask and attempt to answer questions such as: Is maximum resolution of all sample components necessary? Is fast analysis desirable? Is low solvent usage important? Are column stability and lifetime important issues? Is maximum detector sensitivity important?

Choosing the Bonded Phase

Step 1: Draw the molecular structures for all known sample components. Identify the two structures that are the most similar. In this example, the pair are the steroids, prednisone and prednisolone, as shown in Figure 1a. We can use these structures to guide our selection of an optimum bonded-phase chemistry.

Step 2: Next, circle any structural features that differ (see Figure 1b). The differences allow the stationary phase to discriminate between the two molecules. Analyte retention occurs as the functional groups in question interact with the stationary and mobile phases and promote resolution of the pair by a process of differential migration. The intermolecular forces to be considered here are van der Waals (hydrophobic interaction), dipole–dipole (polar interaction), hydrogen-bonding (strong intermolecular force between a proton and electronegative atoms) and π–π interactions (π orbital overlap and π electron donor–acceptor considerations) (6).

Step 3: Use the results of the structure comparison to choose a bonded phase (or no bonded phase) that is likely to discriminate between the pair based upon the functional groups' interaction with the stationary phase. In this example, an analyst might consider using a normal-phase silica column (no bonded phase) for its ability to retain and differentiate prednisone and prednisolone through hydrogen bonding interactions (7). Specifically, silica–silanol groups (Si–OH) can contribute hydrogen-bonding interactions that separate prednisone and prednisolone to the degree that the functional groups circled in Step 2 differ.

An examination of traditional bonded-phase alternatives for normal- and reversed-phase HPLC columns is integral to understanding solute–stationary phase interactions.

C18, C8, and C4: C18, C8, and C4 are nonpolar phases (see Figure 2). Retention is

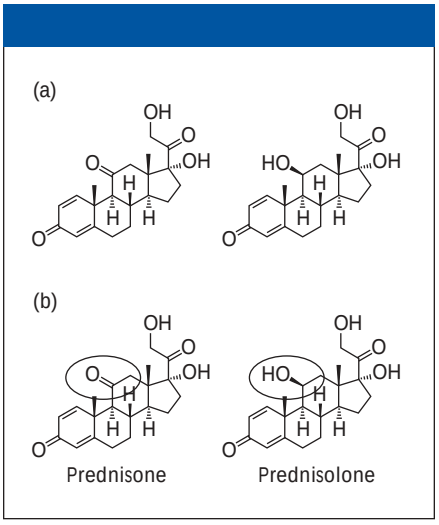

Figure 1: Structures used to guide the selection of an optimum bonded-phase chemistry: (a) the steroids prednisone and prednisolone, (b) the steroids with structural differences circled.

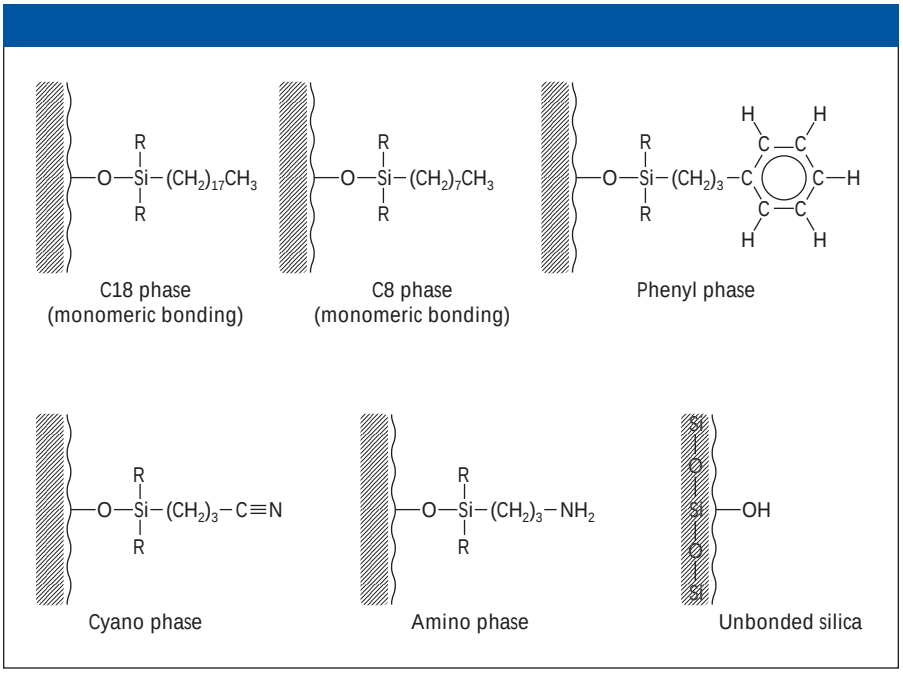

Figure 2: Structures of various bonded phases.

Table I: Column Selection Chart

	Particle Size (μm)			Column Length (mm)			Column Inner Diameter (mm)		
What Do You Need?	**3**	**5**	**10**	**30**	**150**	**300**	**2.1**	**4.6**	**22.5**
Default column*		✓			✓			✓	
High efficiency	✓								
High capacity									
Low back pressure			✓	✓					
High resolution	✓					✓			
High sample loadability									✓
Capability to analyze samples with molecular weights greater than 2000									
High stability									
High sensitivity				✓			✓		
Fast analysis				✓					
Low mobile-phase consumption				✓			✓		
Stability at pH extremes									
Fast equilibration				✓					

*A default column is good for most applications.

based upon van der Waals interactions with hydrophobic compounds. Because the C8 phase has approximately 40–50% of the carbon loading of a C18 phase, its hydrophobicity and hence its hydrophobic resolving power are less than that of a C18 phase.

Phenyl: Phenyl phases also are nonpolar (see Figure 2). Retention is a mixed mechanism of hydrophobic and π–π interactions. The overall hydrophobic retention of a phenyl phase is similar to that of a C8 bonded phase, but unique selectivity rests in its π-orbital interaction with analyte electron-deficient functional groups.

Cyano: Cyano phases have intermediate polarity (see Figure 2). Retention is a mixed mechanism of hydrophobic, dipole–dipole, and π–π interactions. These phases are best used for analyzing polar organic compounds, and they are versatile enough for use in both normal- and reversed-phase modes.

Amino: The amino phase is a polar phase that can be used in both normal and ion-exchange modes (see Figure 2). Retention is caused by dipole–dipole interactions or acid–base interactions. Amino phases commonly are used for carbohydrate analyses, but they also can be used for analyzing both organic and inorganic ions. Samples and mobile phases containing aldehydes or ketones should be avoided when using amino phases because they can react with the amino functionality of the packing.

Unbonded silica: Unbonded silica is very polar (see Figure 2) and is used for normal-phase chromatography. Retention results from hydrogen bonding interactions with suitable solutes.

Each bonded phase has unique selectivities for certain sample types. For example, if the two most difficult compounds to separate are toluene and ethyl benzene (different by only one CH_2 unit), then a C8 or C18 bonded phase is a logical choice to accomplish the separation. These bonded phases interact with the region of the sample molucules that makes them different. If an important method goal is maximum resolution, then the decision should be narrowed further to a particular packing material that shows good retention of hydrophobic compounds (that is, a high-surface-area base silica and high bonded-phase coverage or carbon load) to maximize the separation. Issues of base silica surface area and carbon loading will be discussed below in more detail.

Surface Area (m^2/g)		Pore Size (Å)			Carbon Load (%)			Bonding Type		Particle Shape	
200	300	60	100	300	3	10	20	Monomeric	Polymeric	Spherical	Irregular
✓			✓			✓		✓		✓	
								✓		✓	
	✓	✓					✓		✓		✓
										✓	
	✓						✓				
	✓	✓					✓		✓		
				✓							
									✓	✓	
✓					✓						
									✓		
✓											

Assessing the Role of Base Silica Chemistry

Most of the surface of any bonded-phase packing is made of unreacted silica silanols (Si–OH). Silica silanols offer unique selectivity through hydrogen-bonding interactions with polar analytes (8). Traditionally, silanols have been problematic because of the presence of metal impurities that cause them to become acidic or active. This result causes tailing peaks for certain polar analytes such as amine bases. Thus, the conventional wisdom of silanol avoidance is based upon years of experience with silica that was insufficiently pure to prevent tailing. Approaches used to combat the effects of active silanols include the addition of mobile-phase modifiers, base deactivation of the silica before bonding, endcapping, and silica alternatives such as polymers, zirconium, and hybrids. When based upon sufficiently clean silicas, column packings that possess little or no endcapping actually can exhibit unique selectivity for polar analytes and maintain good peak shape.

Choosing the Particle Physical Characteristics

Table I is useful for discovering how column format and particle physical characteristics affect chromatography. The chart uses a default column as a starting point (first row). This profile represents an average analytical column that is good for most applications: 150 mm × 4.6 mm, 5-µm d_p, 100-Å pore size, 200-m^2/g surface area, 10% carbon load, monomeric bonding, and spherical particles.

To use the chart effectively, match the method goals with individual particle physical characteristics. Change only those physical parameters that are affected by specific method goals. For example, a goal of fast equilibration is best achieved by using a short 30–50 mm column with a silica surface area of 200 m^2/g. Assessing the most important method goals will lead to important decisions regarding the physical aspects of the column and conclude with the selection of an optimal column. Finally, recognize the optimum column as a possible compromise of method goals. For example, the optimum column for highest resolution of all sample components could sacrifice speed of analysis. This result is because resolution (R_s) is dependent in part upon the number of theoretical plates, which in turn is affected by column length and particle efficiencies (9).

The following lists better describe the column and particle physical characteristics and how they affect chromatography. These effects are summarized in Table I.

Column dimensions: The column dimensions are the length and inner diameter of the packing bed.

- *Short:* Short columns are 30–50 mm in length. They provide short run times and low back pressure.
- *Long:* Long columns are 250–300 mm in length. They provide higher resolution and longer run times.
- *Narrow:* Narrow-bore columns have inner diameters smaller than 2.1 mm. They provide higher detection sensitivity.
- *Wide:* Wide-bore columns have 10–22 mm inner diameters. They enable the loading of large samples.

Particle shape: Particles are either spherical or irregular.

- Spherical particles have reduced back pressures and provide longer column life when used with viscous mobile phases such as 50:50 (v/v) methanol–water.
- Irregular particles have higher surface areas and higher carbon loads, and they generally produce higher capacity factors for potentially greater resolution.

Particle size: The particle diameter range is 1.5–20 μm.

- Smaller particles offer higher efficiency. Choose 1.5- or 3-μm particles for resolving complex, multicomponent samples. Otherwise, choose 5- or 10-μm d_p packings.

Surface area: The surface area is the sum of particle outer surface and interior pore surface in square meters per gram.

- High surface areas generally provide greater retention, capacity, and resolution for separating complex, multicomponent samples.

Pore size: The pore size is the average size of the particles' pores or cavities. They range in value from 60 Å to 10,000 Å.

- Larger pores allow larger solute molecules to be retained through maximum exposure to the surface area of the particles. Choose a pore size of 150 Å or less for samples with molecular weights less than 2000. Choose a pore size of 300 Å or greater for samples with molecular weights greater than 2000.

Bonding type: The bonding type is the attachment mode of each bonded-phase strand to the base silica.

- *Monomeric:* Monomeric phases have single-point attachments of bonded-phase molecules. Monomeric bonding provides faster equilibration and higher column efficiency.
- *Polymeric:* Polymeric phases have multiple-point attachments of bonded-phase molecules. Polymeric bonding offers increased column stability, particularly when used with highly aqueous mobile phases. Polymeric bonding also enables columns to accept higher sample loading (see Figure 3).

Carbon load: The carbon load is the amount of bonded phase attached to the base material, expressed as the percentage of carbon.

- High carbon loads generally offer greater resolution and longer run times for hydrophobic samples.
- Low carbon loads shorten run times and often show different selectivity.

Endcapping: Endcapping is the capping of exposed silanols with short hydrocarbon chains after the primary bonding step.

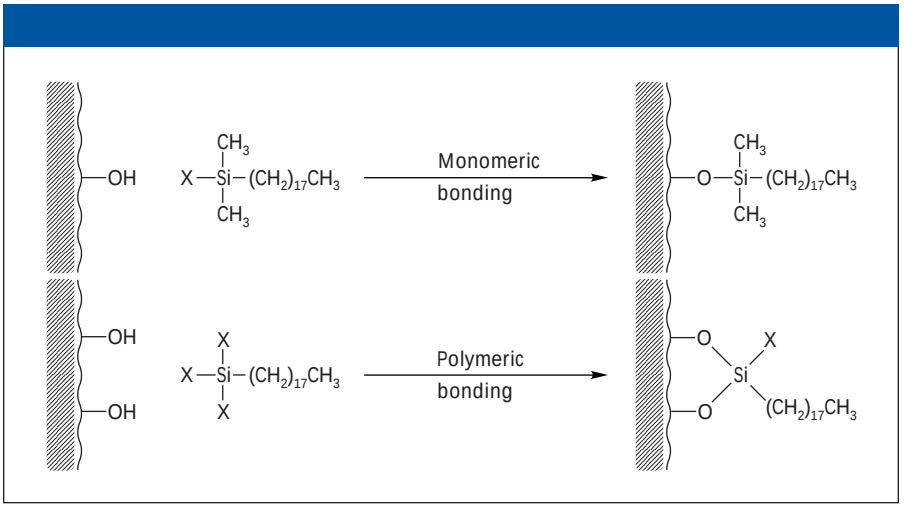

Figure 3: Simplified diagrams of monomeric and polymeric bonded phases.

- Endcapping reduces peak tailing of polar solutes that interact excessively with the otherwise-exposed silanols. Nonendcapped packings provide a different selectivity than that of endcapped packings, especially for polar samples.

A Real Example

We'll use an example to illustrate this approach's effectiveness in column selection. In this scenario, the most important method goals were rapid analysis and high resolution. In some instances, these goals are mutually exclusive as highest resolution can result in longer run times. Our sample was a mixture of naturally occurring flavonoids that contained quercitin, rutin, myricetin, naringenin, apigenin, and catechin. Figure 4 shows the structures of these flavonoids.

Based upon an analysis of the functional groups, we determined the most similar pair was naringenin and apigenin.

Next, we circled the structural difference between the pair (Figure 5). The naringenin structure exhibits π-electron density delocalized among three carbon atoms and two oxygen atoms, whereas the apigenin structure shows delocalization among five carbon atoms and two oxygen atoms. This structure suggests that a phenyl or cyano bonded phase would be a good choice for analysis because they offer mixed-mode retention mechanisms (hydrophobic and π–π). The π cloud of the bonded-phase functional group can interact with apigenin and naringenin to different degrees based upon their differing degrees of π-electron delocalization.

When referring to Table I, the goal of fast analysis is best satisfied by using a short column packed with a low-surface-area silica and a low carbon loading. The goal of high resolution is best met by considering a long column packed with small particles of high-surface-area silica and a high carbon loading. Although it might seem that the goals are mutually exclusive, we might be able to find a compromise that satisfies both. The goal of fast analysis could be met by using a short column (~50 mm), and the use of small particles (3-μm d_p), high-surface-area base silica, and high bonded-phase coverage will ensure enough separation to achieve the desired resolution. Based on this reasoning, we selected a 53 mm × 7 mm Rocket HPLC column packed with 3-μm d_p Alltima phenyl bonded phase (both from Alltech Associates, Deerfield, Illinois). The column dimensions were chosen because they provided good analysis speed and maintained good peak shape and resolution. The bonded phase was chosen for its high efficiency 3-μm particle size and its 350-m^2/g base silica surface area, which provided high bonded-phase coverage with a 16% carbon load.

Three experiments are represented in Figure 6 with their chromatographic conditions.

- The column packed with the 3-μm d_p phenyl phase separated all six flavonoids in 2.5 min.
- As a comparison, a similar column packed with 3-μm d_p C18 media was unable to resolve apigenin and naringenin in a similar time frame.
- With the C18 column, the mobile phase was weakened to achieve the separation of the difficult pair. The concomitant run time increased to more than 12 min. Unexpectedly, we lost the resolution of the early eluting rutin and catechin.

The experimental results demonstrated that the column of choice, based upon a prediction made using the philosophy outlined in this article, was optimal for achieving the method goals. We achieved the separation of six flavonoids rapidly and with good peak shape and resolution. This result is significant. Our initial tendency might have been to try a short C18 column for this separation. With enough effort at mobile-phase selection and optimization, and perhaps some trial-and-error work regarding column format, the C18 column could have worked. However, our experiments showed that this approach would have been problematic in achieving the method goals; namely, high resolution in a minimum amount of time. Although the phenyl column is not necessarily the only column capable of meeting these goals, we avoided laboratory trial-and-error processes and saved much time by starting the investigation with a column that was optimal for our needs.

Conclusion

In this approach to HPLC column selection, chromatographers choose a bonded-phase chemistry on the basis of an analysis of sample component structures. The physical characteristics of a column are chosen according to an analysis of the goals for the separation method. This approach succeeds in predicting unique, optimum, bonded-phase chemistries and particle-bed physical characteristics that are likely to meet the

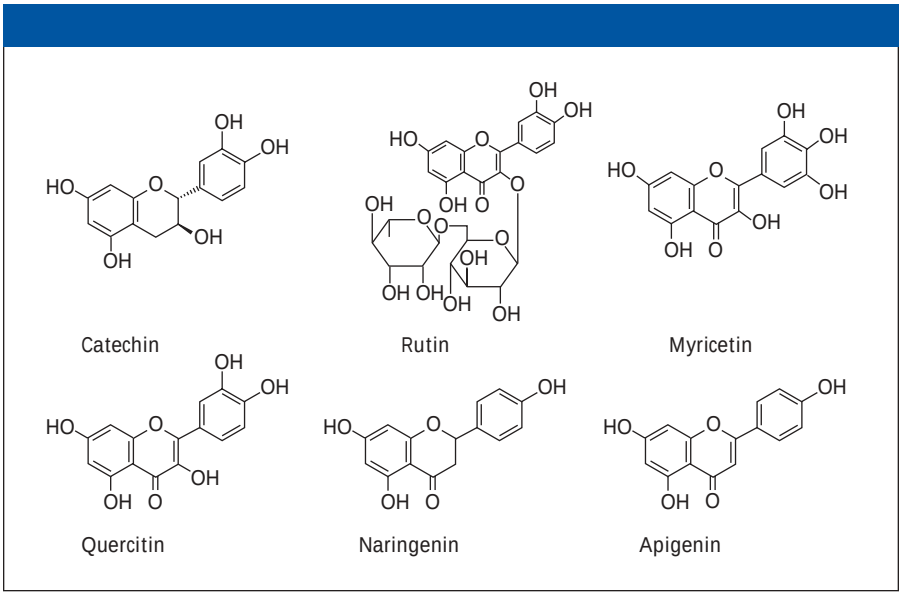

Figure 4: Structures of naturally occurring flavonoids in the sample mixture.

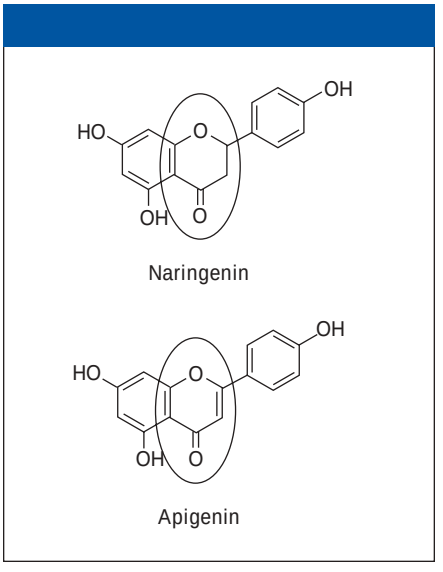

Figure 5: Structures of naringenin and apigenin with the structural differences circled.

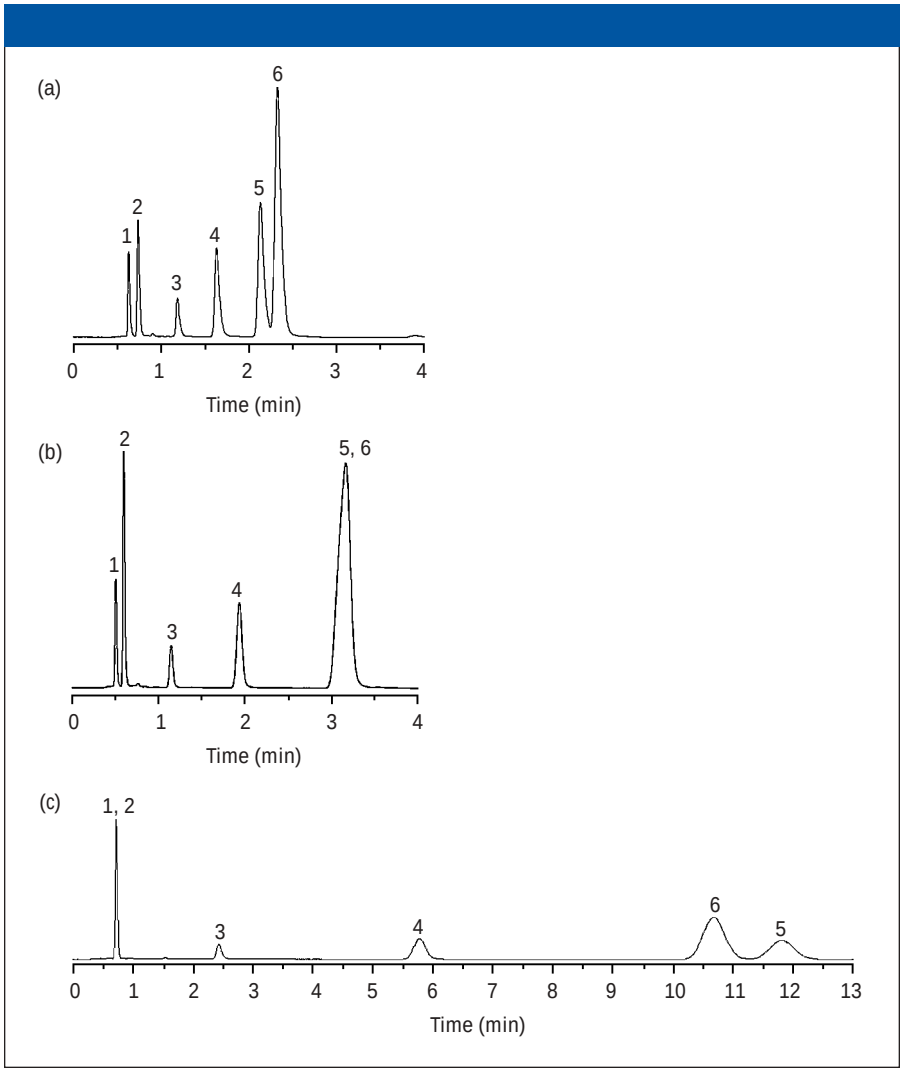

Figure 6: Isocratic separation of a flavonoid mixture using (a) a phenyl column and (b, c) a C18 column. (a) Column: 53 mm × 7 mm, 3-µm d_p Rocket Alltima Ph; mobile phase: 35:65 (v/v) acetonitrile–0.2% trifluoroacetic acid. (b) Column: 53 mm × 7 mm, 3-µm d_p Rocket Alltima C18; mobile phase: 35:65 (v/v) acetonitrile–0.2% trifluoroacetic acid. (c) Column: 53 mm × 7 mm, 3-µm d_p Rocket Alltima C18; mobile phase: 25:75 (v/v) acetonitrile–0.2% trifluoroacetic acid. Flow rate: 2.5 mL/min. Detection: UV absorbance at 280 nm. Peaks: 1 = rutin, 2 = catechin, 3 = myricetin, 4 = quercitin, 5 = apigenin, 6 = naringenin.

goals of a separation method. It also succeeds in saving time by suggesting to analysts a column that is better suited to their work before they begin their experiments.

References

(1) L.R. Snyder, J.J. Kirkland, and J.L. Glajch, *Practical HPLC Method Development* (John Wiley & Sons, New York, 2nd ed., 1997).

(2) J.W. Dolan, *LCGC* **17**(12), 1094–1097 (1999).

(3) J.W. Dolan, *LCGC* **18**(5), 478–487 (2000).

(4) L.R. Snyder and J.W. Dolan, *Chem. Anal. (Warsaw)* **43,** 495 (1998).

(5) C.H. Jersild, "Combining Short, High Speed HPLC Columns with Automated Column Selection for Rapid HPLC Method Development," poster presented at the Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy — Pittcon 2000, New Orleans, Louisiana, 12–17 March 2000.

(6) R.T. Morrison and R.N. Boyd, *Organic Chemistry* (Prentice Hall, Upper Saddle River, New Jersey, 6th ed., 1992).

(7) V. Garg and W.J. Jusko, *J. Chromatogr. Biomed. App.* **567,** 39–47 (1991).

(8) S.C. McGee, R.W. Weigand, and I. Chappell, "Extended Polar Selectivity for the Analysis of Peptides and Proteins by Reversed Phase HPLC," poster presented at the 22nd International Symposium on High Performance Liquid Phase Separations and Related Techniques — HPLC 1998, St. Louis, Missouri, 3–8 May 1998.

(9) L.R. Snyder, J.J. Kirkland, and J.L. Glajch, *Practical HPLC Method Development* (John Wiley & Sons, New York, 2nd ed., 1997). ■