PERSPECTIVES IN MODERN HPLC

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Superficially Porous Particles: Perspectives, Practices, and Trends

Columns packed with superficially porous particles (SPPs) have created considerable excitement over the last few years. Indeed, this column technology manifests the advantages of fully porous material (loading capacity, retention) and some beneficial properties of nonporous particles (kinetic performance). This review provides an updated overview of the theory behind the success of SPP technology, trends, benefits, and limitations. It also summarizes the latest developments of sub-2-µm SPPs and instrumental constraints associated with their use. Finally, it describes several applications to illustrate the performance and the universal applicability of these newly engineered particles.

Szabolcs Fekete and Davy Guillarme *are the guest coauthors of this installment.* ***Michael W. Dong*** *is the editor of Perspectives in Modern HPLC.*

Superficially porous particles (SPPs) (also called core–shell, fused-core shell, partially porous, pellicular, or solid-core) are made of a solid, nonporous silica core surrounded by a porous shell layer with similar properties to those of the fully porous materials used in conventional high performance liquid chromatography (HPLC) columns. The "fused-core" terminology was originally introduced by Jack Kirkland to describe the manufacturing procedure that "fuses" a porous silica layer onto a solid silica particle (1).

The very high efficiency achieved on columns packed with sub-3-µm SPPs, combined with convenient operating conditions (modest back pressures and the ability to use conventional HPLC instruments) has generated significant interest in the chromatographic community and widespread applications in many fields (2). Columns packed with sub-3-µm SPPs rival the efficiency of columns packed with sub-2-µm fully porous particles, but the former generate only half the back pressure. As a result, practitioners can use such columns on regular HPLC equipment, leading to the initial interest in these materials and their successful application. Moreover, further performance improvement is possible with very fine SPPs (1.3–1.7 µm), though the use of ultralow-dispersion ultrahigh-pressure liquid chromatography (UHPLC) systems (for example, extracolumn peak variance $\sigma_{ec}^2 < 3\ \mu L^2$) is mandatory. Faster analysis and higher efficiency is always desirable in liquid chromatography (LC), particularly to pharmaceutical scientists or researchers in life science wishing to attain higher productivity in the laboratory or more accurate analysis (higher resolution) of very complex samples. Reducing analysis time while maintaining resolution requires high kinetic performance (more separation power per unit time) using smaller particles, better particle morphology, or both.

The initial intent of applying SPPs was to enhance kinetic performance in the analysis of large biomolecules such as therapeutic proteins. The rationale behind this concept was to improve column efficiency by shortening the diffusion path that molecules have to travel and, thus, improve their mass transfer kinetics (3,4). Shell-type (pellicular) particles were first developed by Horváth and colleagues in the late 1960s for the analysis of large molecules in ion-exchange mode (5). Shortly afterward, Kirkland demonstrated that superficially porous particles (pellicular materials) with 30–40 µm diameters could provide much better separations than totally porous ones (6). In 2001, a new column for fast protein or peptide analysis was introduced that was packed with a 5-µm SPP with a shell thickness of 0.25 µm.

In 2007, a revolution started with the commercialization of a new generation of sub-3-µm SPPs adapted for separation of small and large molecules (7) by Advanced Materials Technology. This material possesses a 1.7-µm solid core covered by a 0.5-µm-thick shell of porous silica. It combined the advantages of both fully porous and nonporous particles. In particular, this improved particle design solved the problem of the low

Table I: A current list of HPLC columns made with commercially available SPP materials including the newest sub-2-µm SPPs

Provider	Name	Particle Diameter, d_p (µm)	Shell Thickness (µm)	Ratio Core/d_p (ρ)	Available Stationary Phases
Advanced Chromatography Technologies	ACE UltraCore	2.5 5.0	0.45 0.70	0.64 0.72	SuperC18, SuperPhenlHexyl (pH 1.5 to 11)
Agilent Technologies	Poroshell 300	5.0	0.25	0.90	SB-C18, SB-C8, SB-C3, Extend-C18
	Poroshell 120	2.7	0.50	0.63	SB-AQ, SB-C18, SB-C8, EC-C18, EC-C8, phenylhexyl, EC-CN, Bonus-RP, Peptide-mapping, HILIC, PFP, HPH-C18, HPH-C8 (high-pH phases up to pH 11)
Advanced Materials Technology	Halo	2.7	0.50	0.63	C18, C8, phenylhexyl, Amide, PFP, CN, HILIC, Penta-HILIC
	Halo-5	4.7	0.60	0.74	C18, C8, phenylhexyl, Amide, PFP, CN, HILIC, Penta-HILIC
	Halo Peptide-ES 160 A	2.7	0.50	0.63	C18, CN
	Halo Protein 400 A	3.4	0.20	0.88	C4, C18
Diamond Analytics	Flare Diamond Coreshell	3.6	0.10	0.94	C18, C18-WAX, HILIC
Fortis	SpeedCore	2.6	0.40	0.69	C18, diphenyl, PFP, HILIC
Knauer	Blueshell	2.6	0.50	0.62	C18, C18A, PFP, phenylhexyl, HILIC
Macherey-Nagel	Nucleoshell	2.7 5.0	0.50 0.60	0.63 0.76	C18, phenylhexyl, PFP, HILIC
Nacalai Tesque	Cosmocore	2.6	0.50	0.62	C18
PerkinElmer	Brownlee SPP	2.7	0.50	0.63	C18, C8, phenylhexyl, RP-amide, PFP, ES-CN, HILIC
	Brownlee SPP Peptide-ES	2.7	0.50	0.63	C18, C8, phenylhexyl, RP-amide, PFP, ES-CN, HILIC
Phenomenex	Kinetex	5.0 2.6 1.7 1.3	0.60 0.35 0.23 0.20	0.76 0.73 0.73 0.69	C18, C8, phenylhexyl, PFP, HILIC, biphenyl
	Aeris Peptide	3.6 1.7	0.50 0.23	0.72 0.73	C18
	Aeris Widepore	3.6	0.20	0.89	C18, C8, C4
Restek	Raptor	2.7	0.50	0.63	Biphenyl
Sigma-Aldrich (Supelco)	Ascentis Express	2.7	0.50	0.63	C18, C8, phenylhexyl, Amide, F5, CN, HILIC, OH5
	Ascentis Express 5 µm	4.7	0.60	0.74	C18, C8, phenylhexyl, Amide, F5, CN, HILIC, OH5
	Ascentis Express Peptide160 A	2.7	0.50	0.63	C18, CN
	BIOshell 400 A	3.4	0.20	0.88	C4
Sunniest	SunShell	2.6 4.6	0.50 0.60	0.62 0.74	C18, C8, PFP
Thermo Scientific	Accucore XL	4.0	0.50	0.75	C30, C18, C8, C4, phenylhexyl, PFP, HILIC, RP-MS, AQ, Amide-HILIC
	Accucore	2.6	0.50	0.62	C30, C18, C8, C4, phenylhexyl, PFP, HILIC, RP-MS, AQ, Amide-HILIC
Waters	Cortecs	1.6	0.25	0.70	C18, C18+, HILIC
	Cortecs	2.7	0.40	0.70	C18, C18+, HILIC
YMC	Meteoric Core	2.7	0.50	0.63	C18, C18 Bio, C8

loading capacity of early pellicular particles because an approximately 75% volume fraction of these particles is still porous. Since then, many providers commercialized SPPs with particle sizes ranging between 1.3 and 5 µm. Table I provides a current list of commercially available columns packed with SPP materials, their pertinent properties, and available bonded phases.

Column Performance: The Impact of Shell Thickness

In 1956, the famous van Deemter equation was proposed to describe column performance (plate height) as a function of linear velocity (8). Since then, several plate height and rate models have been derived for LC by numerous researchers. Knox suggested a three-term equation to describe the dependency of the theoretical plate height (H) of a column as a function of linear velocity (u) (9):

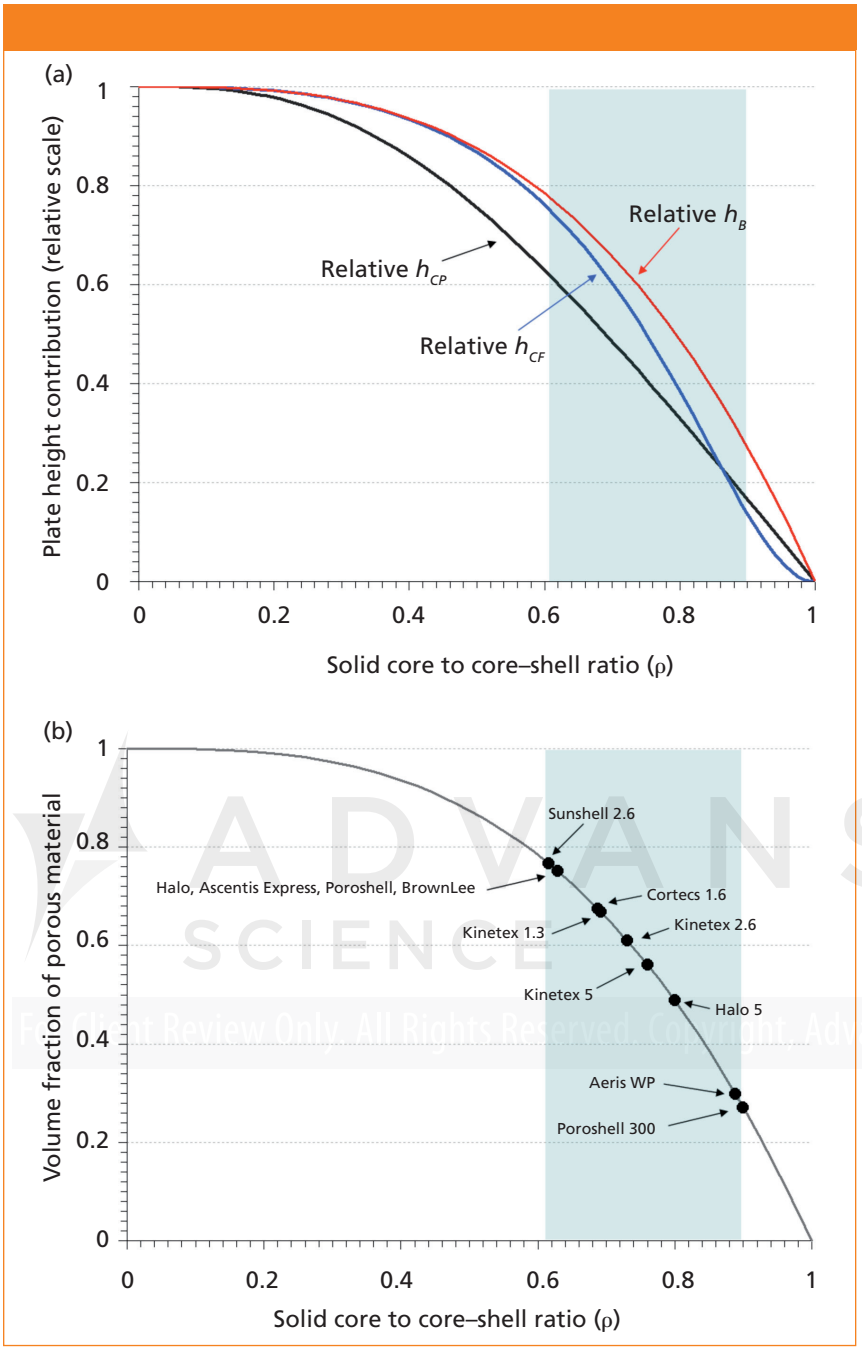

Figure 1: The theoretically expected reduction of (a) reduced plate height contributions and (b) the volume fraction of the porous shell as a function of particle structure (ρ). The light blue zones indicate the ρ range of commercially available SPP materials.

$$H = Au^{1/3} + (B/u) + Cu \quad [1]$$

where A, B, and C are constants, determined by the magnitude of band broadening because of eddy dispersion, longitudinal diffusion, and mass transfer resistance, respectively. In practice, the C term is the sum of contributions from resistance to both trans-particle mass transfer (C_p) and external film mass transfer (C_F).

The application of reduced parameters is common in engineering to compare the performance of columns in normalized units. It is useful to convert H to a dimensionless parameter such as reduced plate height ($h = H/d_p$), where d_p is particle diameter. The advantage of this approach is the ability to compare the performance of columns packed with particles of different sizes or structures (*morphology*). With the use of the reduced parameters, equations similar to equation 1 can be written. Therefore, the total reduced plate height (h) can be written as the sum of at least four different contributions:

$$h = h_A + h_B + h_{Cp} + h_{CF} \quad [2]$$

where h_A, h_B, h_{Cp}, and h_{CF} are the reduced plate height contributions from eddy dispersion, longitudinal diffusion, trans-particle mass transfer, and external film mass transfer, respectively.

The initial idea of preparing SPPs was to increase column efficiency by reducing mass transfer resistance across particles (C_p). However, it turned out that trans-particle mass transfer resistance is far from being the main contributor to band broadening in LC for small molecules (especially for modern columns packed with small particles) (3). The enhanced performance of SPP materials clearly lies in contributions from other factors.

Theoretically, the *intraparticle diffusivity* (the diffusion speed inside the particle pores) of an SPP depends on the ratio of the diameter of the solid core to that of the SPP (d_p). As this ratio (ρ) increases, the mass transfer kinetics becomes faster through the particles. Similarly, the external mass transfer (C_F) process depends on the particle's structure. It has been demonstrated that mass transfer kinetics is mainly affected by the external film mass transfer resistance across the thin layer of the mobile phase surrounding the external surface area of the particles (3). The trans-particle mass transfer resistance is important for large molecules when the diffusion time inside the particles is much longer, but not so critical for small molecules. As far as diffusion time is concerned, the 2.7-µm Halo (Advanced Materials Technology) and Poroshell 120 (Agilent Technologies) phases are equivalent to a 1.8-µm fully porous packing material, while the 2.6-µm Kinetex material (Phenomenex) is equivalent to a 1.3-µm fully porous stationary phase (10).

Additionally, the presence of a solid core within an SPP has a direct impact on longitudinal diffusion (the B term of the van Deemter equation). It decreases the B term contribution to the plate height by about 20% when the ratio of the core to the particle diameter (ρ)

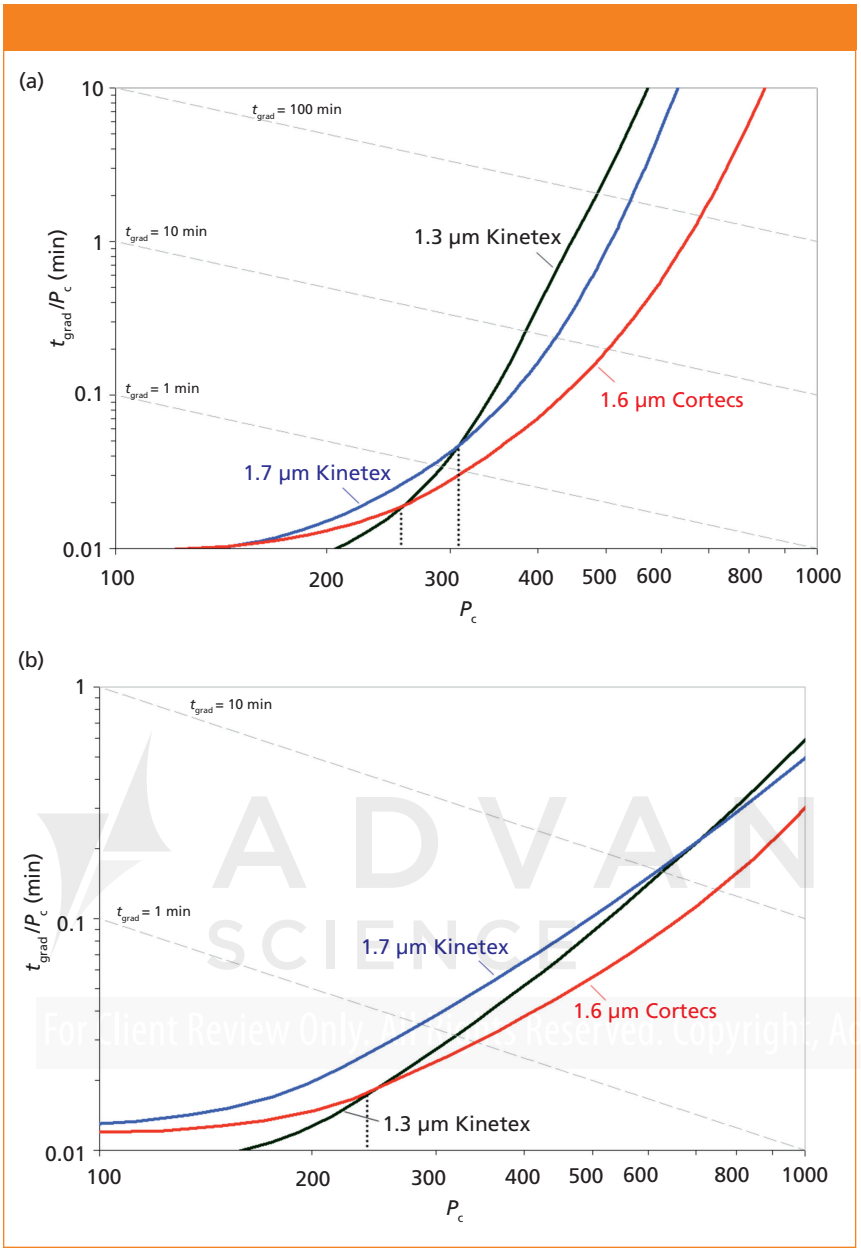

Figure 2: Gradient kinetic plot comparison of columns packed with 1.3-, 1.6-, and 1.7-µm SPPs for the separation of (a) small molecules and (b) peptides. Maximum operating pressure: ΔP_{max} = 1200 bar for Cortecs and ΔP_{max} = 1000 bar for Kinetex columns, $\Delta\Phi$ = 0.9 (5–95% B), S = 6 for butylparaben and S = 14.8 for decapeptide. Adapted from reference 22 with permission.

is 0.63 (for example, Halo [Advanced Materials Technology], Ascentis Express [Sigma-Aldrich], BrownLee SPP [PerkinElmer]) (3,4). However, the improvement in overall efficiency is not only because of the reduced internal particle porosity of SPPs: It was experimentally determined that the solid core reduced longitudinal diffusion by about 30% in comparison with fully porous particles (11). This reduction in longitudinal diffusion produces only a ~10% increase in total overall column efficiency compared with columns packed with fully porous particles.

Figure 1a shows the theoretical reduction in reduced plate heights (h) on a relative scale for trans-particle mass transfer (h_{Cp}), external film mass transfer (h_{CF}), and longitudinal diffusion (h_B) as a function of particle structure (ρ) (4). Theoretically, on average about ~40–60% lower h values are expected of current SPPs compared to fully porous materials of the same diameters. Please note that Figure 1a does not say anything about the relative contribution of the three different dispersive phenomena to the total h.

Finally, according to several experimental results, eddy dispersion (the A term in the van Deemter equation) in SPP columns is significantly smaller (~30–40%) than that of the columns packed with fully porous particles of similar size (12–14). This is unexpected because particle structure should not affect the multipath dispersion of solutes when traveling through the column. When modern SPP columns are operated at their optimum linear velocity, 50% of the band dispersion results from long-range packing heterogeneity (12–14). However, it is still unclear whether this significant improvement in efficiency is because of narrower particle size distribution (PSD) of SPPs or their higher density, which enables them to form very homogeneous, efficient packed beds. Some recent studies have indeed indicated that SPPs displaying a very narrow PSD can lead to unprecedented low minimal plate heights (3,4). Another explanation proposed was that some SPPs appear to have rougher surfaces than fully porous particles. This might also have a positive influence on packing quality, apart from the narrowness of the PSD.

Based on Figure 1a, a thinner shell is advantageous for efficiency, but it also reduces sample loading capacity and analyte retention. Therefore, the optimum shell thickness is a compromise between efficiency, sample loading capacity, and analyte retention. The sample loading capacity and expected retention factor of a given solute are proportional to the stationary phase volume. Therefore, sample loading and retention are expected to be somewhat lower on SPPs than on fully porous particles. Figure 1b shows the volume fraction of the porous layer of some typical SPP structures. Please note that nominal particle size and shell thickness values were considered for Figure 1; however, in practice, the average and nominal values could be slightly different.

As can be seen in Figure 1b, the commercially available SPP materials present a huge variety in terms of volume fraction of porous material, between 27% and 75%. SPPs with low volume fractions correspond to relatively large wide-

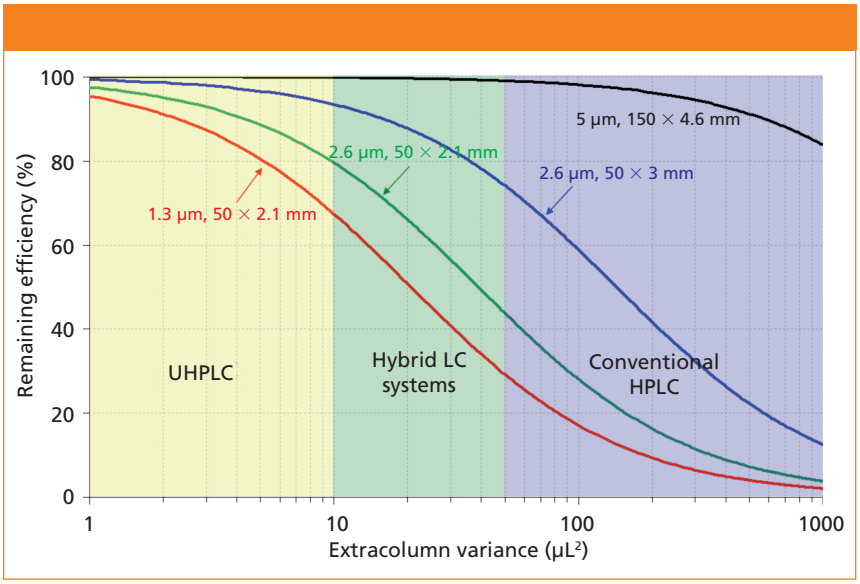

Figure 3: Effect of the extracolumn variance on the remaining (observed) column efficiency for columns packed with 1.3-, 2.6-, and 5-µm SPPs (50 mm × 2.1 mm, 50 mm × 3 mm, and 150 mm × 4.6 mm). A retention factor of k = 5 was assumed for the model calculations. The yellow area corresponds to <10 µL^2 extracolumn peak variance (optimized UHPLC systems), the green area represents 10–50 µL^2 extracolumn peak variance (hybrid systems), and the blue zone indicates the variance typical of conventional LC systems.

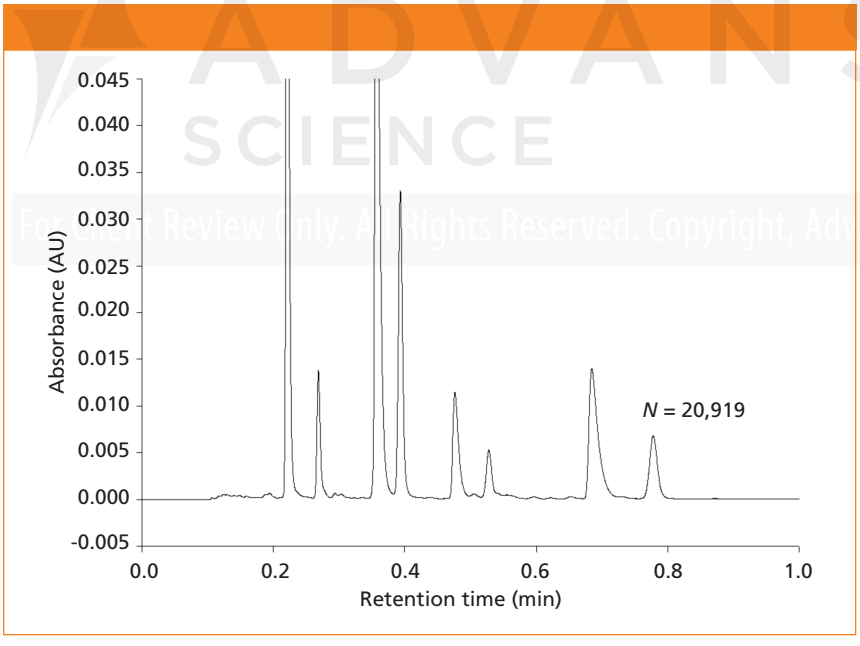

Figure 4: Isocratic separation of cashew nut extract performed on 50 mm × 2.1 mm 1.3-µm SPP column (Kinetex C18). Mobile-phase composition: 14:86 (v/v) water–acetonitrile; temperature: 25 °C; flow rate: 0.8 mL/min; detection: 280 nm (80 Hz); injected sample volume: 0.3 µL sample; observed pressure: 781 bar.

pore particles (for example, 3.6-µm Aeris WP [Phenomenex] or 5-µm Poroshell 300 [Agilent Technologies]) developed for large molecules. In practice, the loadability of most commercially available sub-3-µm SPP materials seems to be similar to that of fully porous particles of the same diameter (15,16). This result may be explained partly by the relatively high surface area of the silica in the porous shell. It is also conceivable that sample molecules do not penetrate appreciably into the center of totally porous particles (15). Similar to loadability, it appears that the retention characteristic of sub-3-µm SPP materials may not be significantly different from that of their totally porous counterparts when operating under identical conditions (16).

Latest Developments in SPP Technology: Decreasing the Particle Size

The last decade has seen significant enhancements in LC performance brought about by the development of new columns packed with sub-2-µm fully porous particles and improved UHPLC instrumentation. This new level of performance originates from the achievement of lower height equivalent of a theoretical plate (HETP) values, which is manifested in practice as narrower peaks eluted in a very short time and with superior resolution.

As described above, the exceptional performance of columns packed with SPPs with low-molecular-weight analytes is related to a reduction of eddy dispersion and longitudinal diffusion. Because of these unexpected, but beneficial features, SPPs are likely to have better morphology for further performance optimization via particle miniaturization (17). Therefore, column providers have introduced very fine sub-2-µm SPPs. In 2009, 1.7-µm particles with ρ = 0.73 appeared on the market and showed quite impressive kinetic performance. Minimal HETP values between 2.6 and 4.3 µm were observed with low-molecular-weight solutes, resulting in some very efficient separations of peptides (18).

The real breakthroughs in column performance came in 2013, when two providers introduced 1.3- and 1.6-µm SPP materials. The 1.3-µm packing (ρ = 0.69) provided exceptionally low minimal plate heights of ~2 µm, corresponding to a plate count of up to 500,000 plates/m (19,20). It was shown that the 1.3-µm SPP material provides the shortest analysis time among existing stationary phases for efficiencies below 30,000 plates. Despite its excellent chromatographic performance, it is evident that the efficiency of this column is limited by current instrumentation in terms of upper pressure limit and extracolumn band broadening. Even at 1000 bar, it is hard to reach optimal linear velocity, thanks to its low permeability (K_v = 1.7 × 10^{-11} cm^2) (19). The effect of extra-

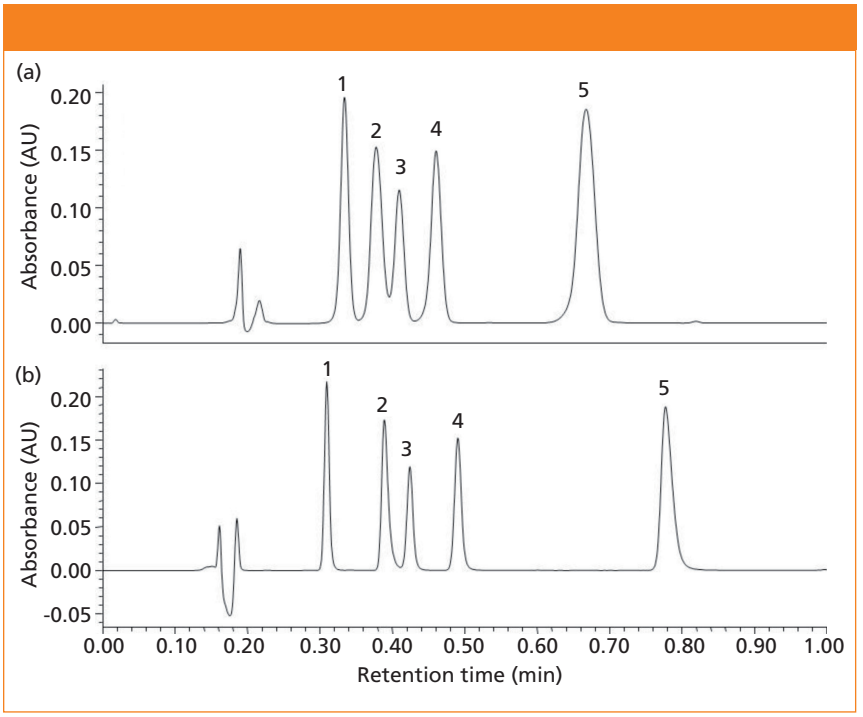

Figure 5: Comparative separations of five local anesthetics by HILIC using columns packed with (a) totally porous and (b) SSP materials. Columns: (a) 50 mm × 2.1 mm, 1.7-µm Acquity BEH HILIC and (b) 50 mm × 2.1 mm, 1.6-µm Cortecs UPLC HILIC (both from Waters). Mobile-phase A: 50:50 acetonitrile–10 mM ammonium formate with 0.125% formic acid; mobile-phase B: 90:10 acetonitrile–10 mM ammonium formate with 0.125% formic acid; flow program: isocratic at 99.9% B for 1 min, then gradient from 99.9– 0.1% B in 1.6 min as a washing step; flow rate: 0.8 mL/min; detection: 245 nm; injected amount: 5 µL; temperature: 30 °C. System used: Waters Acquity UPLC I-Class. Chromatograms adapted and reprinted with permission from Waters Corporation.

column band broadening of current UHPLC equipment still has a major impact on the apparent kinetic performance when using short narrow-bore columns (50 mm × 2.1 mm) packed with a 1.3-µm SPP material. In spite of these limitations, this 1.3-µm material seems very promising for ultrafast separation of small molecules and peptides in both isocratic and gradient elution modes (19,21).

Very recently, a column packed with 1.6-µm SPPs (ρ = 0.70) also appeared on the market. The kinetic performance of 1.3-, 1.6-, and 1.7-µm materials was compared in a recent study (22). It was found that the 1.3-µm phase outperforms the two comparators in ultrafast separations. Conversely, the 1.6-µm packing seemed to be the better size for high-resolution chromatography, in isocratic and gradient modes for small molecules and peptides. This exceptional performance was attributed to its more favorable permeability (K_v = 3.5 × 10^{-11} cm^2) and somewhat higher mechanical stability of the columns (ΔP_{max} of 1200 bar). Figure 2 shows the comparison of these three packings using gradient kinetic plot representation obtained from experimental data for small molecules and peptides (21). When constructing these plots, the maximum achievable peak capacity values (P_c) at any flow rate were considered for a given gradient time (t_{grad}) to estimate the highest achievable performance for a defined gradient time (21). For these calculations, a generic scouting gradient run from 5% B to 95% B (Δφ = 0.9) was assumed. The ratio of gradient time and achievable maximum peak capacity (t_{grad}/P_c) is plotted against the maximum peak capacity (P_c). In this representation, the minimal analysis time to achieve a certain peak capacity can be discussed and compared. As shown here, the 1.3-µm material is most useful for ultrafast separations requiring a peak capacity of less than 250. If higher efficiency is required, the best choice is the 1.6-µm material. The 1.6-µm material outperforms the 1.3-µm packing for small molecule separations when P_c > 300. Please note that these plots show the maximum achievable performance when operating the columns at their maximum pressure capability (kinetic performance limit).

These sub-2-µm SPP materials were also successfully applied to the separation of tryptic digests of proteins generating 0.5–2 kDa peptides (21,22). With 50 mm × 2.1 mm columns, peak capacities between 200 and 300 were reached within 10 min (depending on the operating conditions).

Instrumental Considerations for Using Columns Packed with SPPs

Pressure Requirement

Commercially available SPP materials can be classified into three groups based on their particle sizes and operating pressure requirements: relatively large particles of 3.6, 4, 4.6, and 5 µm; sub-3-µm particles (2.5, 2.6, and 2.7 µm); and sub-2-µm materials (1.3, 1.6, and 1.7 µm). The columns packed with 3.6–5 µm SPPs can be operated in the same pressure range as conventional 3–5 µm fully porous particles; therefore these materials can be operated on any conventional HPLC systems.

The 2.5–2.7 µm SPP packings can operate at one-half or one-third of the pressure compared to fully porous sub-2-µm particles; therefore it is theoretically possible to use these columns on conventional HPLC instruments. In generic conditions, when using an acetonitrile–water mobile phase, there is little need to go beyond 400 bar when using shorter columns packed with 2.5–2.7 µm particles. Indeed, the maximum viscosity of acetonitrile-water mixture is around 1 cP at 25 °C, while the viscosity of methanol–water and isopropanol–water mixtures can reach 1.6 and 2.9 cP at 25 °C, respectively. The nominal mechanical stability of some 2.5–2.7 µm SPP columns is 600 bar, whereas the pressure capability of conventional HPLC systems is limited to 400 bar. The pressure capability of conventional HPLC systems seems to be appropriate for columns packed with 2.5–2.7 µm SPPs, but the full benefits cannot be attained with columns of standard dimensions. Indeed, operating these columns at a pressure higher than 400 bar not only provides faster

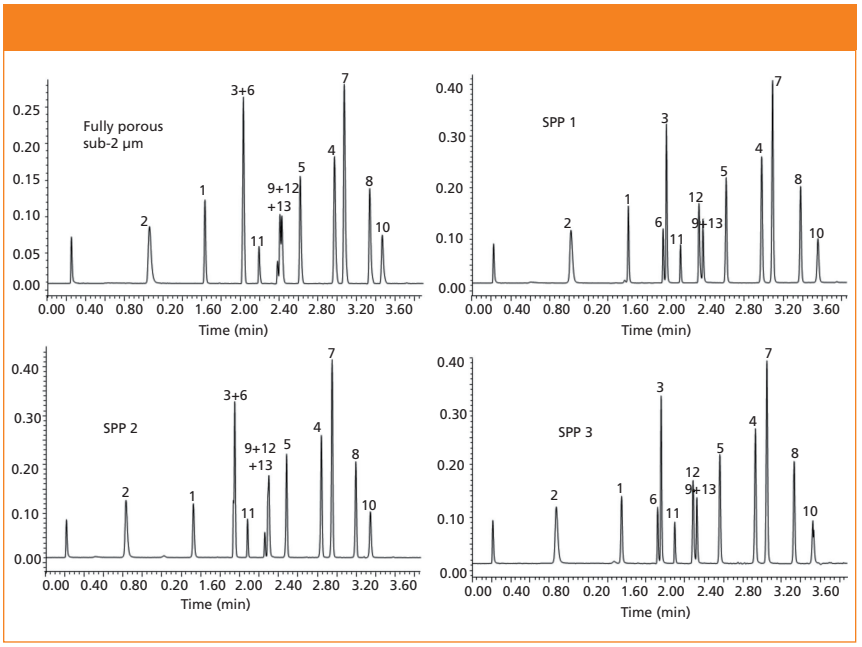

Figure 6: Fast separations of 13 pharmaceutical compounds on 50 mm × 2.1 mm columns packed with fully porous sub-2-µm (Waters Acquity BEH C18, 1.7 µm) and SPP sub-3-µm materials (SPP 1 = Poroshell C18 [Agilent Technologies], 2.7 µm, SPP 2 = Kinetex C18 2.6 µm [Phenomenex], SPP 3 = Halo C18, 2.7 µm [Advanced Materials Technology]). Mobile phase: phosphate buffer (20 mM, pH 6.85) modified with acetonitrile; gradient profile: 5% acetonitrile for 1 min, then 5–95% acetonitrile in 3 min; flow rate: 500 µL/min; temperature: 40 °C; injected volume: 2 µL; detection wavelength: 230 nm. Peaks: 1 = morphine, 2 = atenolol, 3 = codeine, 4 = lidocaine, 5 = prilocaine, 6 = acebutolol, 7 = bupropion, 8 = bupivacaine, 9 = propanolol, 10 = trimipramine, 11 = ketoprofen, 12 = flurbiprofen, 13 = ibuprofen. Adapted from reference 25 with permission.

separations, but also allows for the use of alternative (more viscous) solvents to adjust selectivity.

Sub-2-µm SPP materials can be operated optimally only on UHPLC equipment, since they require operating pressures higher than 400 bar. Such columns are generally stable up to 1000–1300 bar, and their real benefits can be observed only beyond 800 bar. Because of their low permeability, very high pressure is required to operate these columns at reasonable flow rates for fast separations (19).

System Dispersion

The currently available commercial HPLC instruments can be classified into three groups according to their system dispersion: optimized UHPLC systems for fast separations with very low dispersion (σ^2_{ec} <10 µL^2); hybrid HPLC or UHPLC systems recommended by vendors for both fast and conventional separations (σ^2_{ec} = 10–50 µL^2); and conventional HPLC systems with extracolumn variances over 50 µL^2 (23). The extracolumn peak variances of several commercially available instruments were reported in the literature. As expected, the impact of system variance on apparent column efficiency could be critical, particularly for shorter columns in 2.1-mm i.d. format. Some model calculations were performed to illustrate the importance of system dispersion, based on experimental efficiency data observed on columns packed with SPPs of different dimensions. Figure 3 shows the effect of extracolumn variance on the percentage of remaining (observed) column efficiency for 1.3-µm (50 mm × 2.1 mm), 2.6-µm (50 mm × 2.1 mm and 50 mm × 3 mm), and 5-µm (150 mm × 4.6 mm) SPP columns (assuming an analyte with a moderate retention factor of k = 5).

Clearly, when using the standard bore column (4.6 mm), no significant impact on column efficiency is observed; however, it is always advantageous to use a system with very low dispersion. When working with a 3-mm i.d. column, a significant percentage of its intrinsic column plate numbers can be lost when this column is used on conventional systems (for example, ~40% loss for a 100-µL^2 system variance). It becomes even more critical with narrow-bore, 2.1-mm i.d. columns. These small, very efficient columns can only be used on dedicated UHPLC systems. When working with the most efficient 1.3-µm particles, only ~70% of their intrinsic column efficiency can be achieved on a system with a 10-µL^2 variance.

To take full advantage of these very efficient 2.1- and 3-mm i.d. columns under isocratic conditions, optimized UHPLC systems with very low dispersion are mandatory. Often, the system dispersion of an HPLC or UHPLC system can be minimized by a reduction of all system volumes in the sample fluidic flow path. It is recommended to replace the injector sample loop with a lower-volume loop, the standard detector flow cell with a lower-volume one, and the standard tubing with 0.0025–0.005 in. i.d. tubing; the tubing length should be shortened as well. Some additional suggestions are that any column switching valves should be bypassed and the detector sampling rate and time constant should be optimized. Note that a lower system dispersion is generally a good thing for maximum performance for demanding applications, but it may lead to some sacrifices in system convenience and flexibility for routine analysis when using conventional HPLC methods with larger volume columns, higher flow rates, or larger injection volumes (24). A possible way to negate precolumn band broadening is to use a weak solvent for the sample so that the sample can be focused at the head of the column.

In gradient mode, most of the effects of precolumn system dispersion (loop volume, precolumn connection tubing) are much less important. However, the effect of system dwell volume could become critical when performing fast analysis on short narrow-bore columns. Conventional HPLC systems have a 1–3 mL dwell volume, which is clearly unacceptable for fast gradient separations. Similar to extracolumn band dispersion, the system dwell volume also has to be minimized. In this regard, high-pressure mixing pumps with small-volume mixers (<100 µL) are generally preferred. Vendors typically offer mixers of different volumes for different applications. Some of the mixers designed for peptide

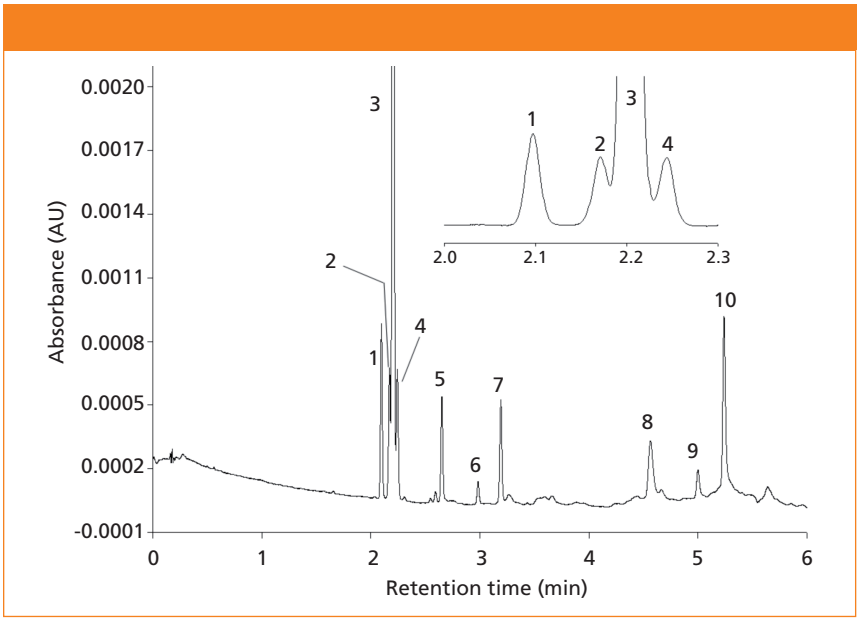

Figure 7: Separation of atorvastatin's related impurities. Column: 50 mm × 2.1 mm, 1.6-µm Cortecs C18; mobile-phase A: 20 mM phosphate buffer (pH 3.34); mobile-phase B: acetonitrile; flow rate: 0.6 mL/min; gradient: 30–75% B in 5 min; column temperature: 30 °C; detection wavelength: 210 nm. Peaks: 1 = impurity-A, 2 = impurity-B, 3 = atorvastatin, 4 = impurity-C, 5 = atorvastatin lactam, 7 = impurity-D, 6,8 = unknown impurities or degradation products, 9,10 = system peaks. Further method development efforts including additional column or mobile phase screening may be required to improve the resolution of several critical pairs in the chromatogram.

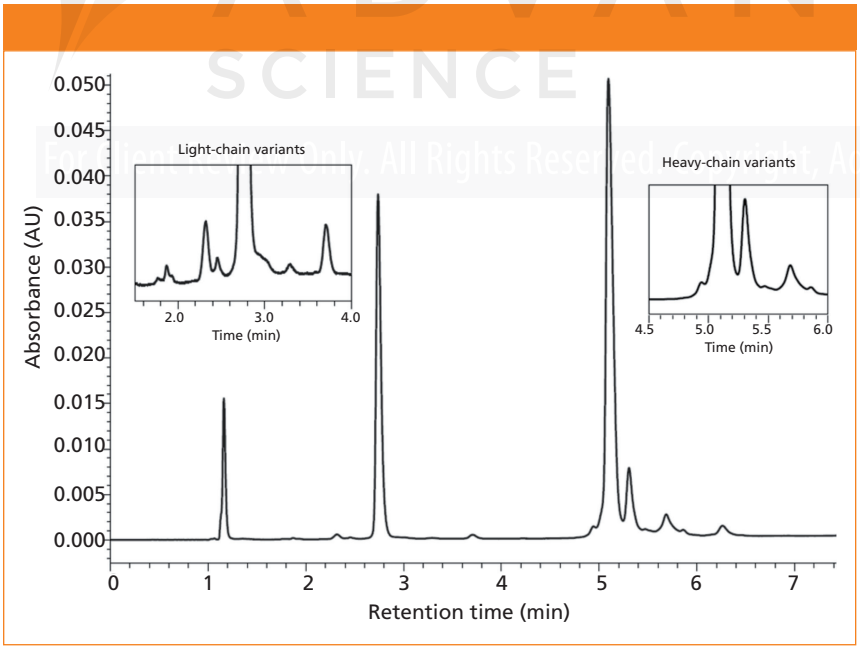

Figure 8: Representative chromatogram of reduced IgG monoclonal antibody (50- and 25-kDa fragments). Column: 150 mm × 2.1 mm, 3.6-µm Aeris WP (Phenomenex); mobile-phase A: 0.1% trifluoroacetic acid in water; mobile-phase B: 0.1% trifluoroacetic acid in acetonitrile; flow rate: 250 µL/min; gradient: 30–37% B in 8 min; temperature: 75 °C; injected volume: 0.5 µL; detection wavelength: 280 nm. Adapted from reference 28 with permission.

mapping (a demanding application requiring very efficient mixing to prevent baseline perturbation of ultraviolet [UV] detection from poor pump blending) may need to be as large as 450 µL for UHPLC gradient separations.

Applications Using Columns Packed with SPPs

SPP technology is an improved extension of totally porous HPLC packing materials, allowing higher kinetic performance through the modification of particle morphology. Because the fundamentals of stationary phase chemistry and the interaction with mobile phases remain the same, the fields of applications are similar. Not surprisingly, SPP materials are universally amenable to diversified applications, including the following:

- Determination of drugs and metabolites in biological fluids and tissues (such as oral fluid, human plasma, blood, and urine)
- Analysis of drug substances and drug products for new pharmaceutical development or quality control (such as impurity profiling, stability-indicating assays, and cleaning validation)
- Characterization of large biomolecules such as proteins or monoclonal antibodies therapeutics in their intact or digested form (for example, purity assays, variant analysis, and peptide mapping)
- Analysis of plant extracts or natural materials (such as quality control of plant samples, profiling and fingerprinting for comparison of plant species, and dereplication)
- Determination of various types of contaminants in food stuff (for example, drugs, pesticides, and herbicides) or environmental samples (such as surface water, ground water, waste water, and soil)
- Proteomics, metabonomics, and other life science investigations and research

A few examples are selected here to illustrate the possibilities of current SPP technology.

Figure 4 demonstrates a sub-1-min isocratic separation of a cashew nut extract on a 50 mm × 2.1 mm, 1.3-µm SPP column (19). The dried seeds of *Annacardium occidentalli* were powdered and successively extracted with solvents of increasing polarity (hexane, dichloromethane, and methanol). The dichloromethane extract contained a mixture of anacardic acids, cardols, and cardonols and was selected for further analysis. An isocratic separation with a 0.8-mL/min flow rate and a mobile phase of 14:86

(v/v) water–acetonitrile at 25 °C enabled a sub-1-min separation of the extracted components. The observed pressure under these conditions was 781 bar (11,348 psi). A 25-min long separation of the same extract had been reported in the literature using a conventional 150 mm × 4.6 mm, 5 µm column. This gain in analysis time clearly accentuates the advantage of SPP technology. In the SPP separation, the last peak was eluted at a very high efficiency of almost 21,000 plates. This example confirms the utility of sub-2-µm SPPs for high throughput separations with elevated plate counts.

Figure 5 shows the comparative separations of five local anesthetics using hydrophilic-interaction chromatography (HILIC) with columns packed with 1.7-µm fully porous materials and 1.6-µm SPPs, respectively. Identical mobile phase and operating conditions were used. Note that the resolution performance of the 1.6-µm SPP column is significantly higher because of different selectivity and substantially higher column efficiency.

Figure 6 shows gradient separations of 13 pharmaceutical compounds on 50 mm × 2.1 mm columns packed with a fully porous sub-2-µm C18 material and three different sub-3-µm SPP C18 materials (25). The test mixture included basic drugs with pK_a ranging between 7.2 and 9.5 as well as acidic compounds with pK_a ranging between 4.1 and 4.4. Identical generic broad gradient conditions using the same mobile phases were used. Similar elution order, selectivity, and peak capacity were observed on all the columns. However, the column pressure drops were 2–3 times lower on the columns with the sub-3-µm SPP materials versus that on the fully porous sub-2-µm material. The favorable permeability, morphology, and larger particle size of sub-3-µm SPP particles allow the operation of these SPP columns at higher flow rates for faster analysis time or in longer column lengths for higher overall resolution.

Figure 7 demonstrates the rapid separation of atorvastatin and its related impurities in the stability-indicating assay of this common drug product (22). While the *British Pharmacopoeia* (26) suggests an 85-min long method using a 250 mm × 4.6 mm, 5-µm column, based on the *European Pharmacopoeia* (*Ph. Eur.*) monograph 2191 (27), reasonable resolution between the critical peak pairs is demonstrated here using a short column (50 mm × 2.1 mm) packed with 1.6-µm SPP material with a 5-min gradient from 30% to 75% B.

Figure 8 shows the rapid separation of heavy and light chain variants of an IgG1 antibody (28) using a column packed with 3.6-µm wide-pore SPPs. The monoclonal antibody (mAb) was reduced to break up the heavy and light chains, and chromatographic conditions were optimized to resolve as many variants as possible within a reasonable analysis time. A relatively steep (0.88% B/min) and fast gradient (8 min) at elevated temperature (75 °C) provided an efficient separation of the mAb fragments. This type of rapid method can be useful for the quality control in variants analysis (heterogeneity) of many mAbs used as therapeutics.

The Future of SPPs in HPLC

Limitations of early SPP products such as reduced retention and sample capacity appear to be have been resolved by the introduction of sub-3-µm particles and improved particle morphology. Other limitations such as pressure limit (to 600 bar), temperature limit (to 60 °C), pH range (pH 2–8), and the limited availability of bonded phases are becoming less of an issue with newer introduction of SPP products by numerous manufacturers. Our last count of SPP column manufacturers totaled 16 vendors. We expect this trend of improving product quality, applicability, and diversity (different particle sizes and bonded phases) to continue as SPPs become a mainstream technology. As previously pointed out, a low-dispersion UHPLC is needed for columns packed with sub-2-µm SPPs. However, such a requirement is mainly driven by the small particle size and column dimension and not related to particle morphology.

Conclusion

In the last decade, a remarkable improvement in HPLC column technology occurred with the introduction of SPPs and, more recently, with the 5-µm and sub-2-µm SPP versions. Today, it is becoming clear that the kinetic advantage of SPPs is related to the reduction of the effects of eddy dispersion (*A* term) and longitudinal diffusion (*B* term), rather than a reduction of mass transfer resistance (*C* term) for small molecule analysis. Recent studies have shown that the selectivity, retention, loading capacity, and peak shape performance achieved with SPP phases are comparable to those on fully porous particles. As shown in this paper, further gains in kinetic performance can be attained using sub-2-µm SPP phases with efficiencies of 400,000–500,000 plates/m on very low dispersion UHPLC systems. Whereas longer 4.6-mm i.d. columns packed with 5-µm SPPs are fully compatible with conventional HPLC systems, UHPLC equipment is mandatory for practitioners wishing to achieve the highest performance attainable with sub-2-µm SPPs.

As SPP technology is getting universal endorsements by all column manufacturers to deliver higher kinetic performance through improved particle design, we will no doubt witness a continued increased use of SPPs in all HPLC applications formerly dominated by totally porous silica particles.

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References

(1) J.J. Kirkland, T.J. Langlois, and J.J. DeStefano, *Am. Lab.* **39,** 18–21 (2007).
(2) J.J. Kirkland, S.A. Schuster, W.L. Johnson, and B.E. Boyes, *J. Pharm. Anal.* **3,** 303–312 (2013).
(3) G. Guiochon and F. Gritti, *J. Chromatogr. A* **1218,** 1915–1938 (2011).
(4) S. Fekete, E. Oláh, and J. Fekete, *J. Chro-*

matogr. A **1228,** 57–71 (2012).
(5) Cs. Horváth, B.A. Preiss, and S.R. Lipsky, *Anal. Chem.* **39,** 1422–1428 (1967).
(6) J.J. Kirkland, *Anal. Chem.* **41,** 218–220 (1969).
(7) F. Gritti, A. Cavazzini, N. Marchetti, and G. Guiochon, *J. Chromatogr. A* **1157,** 289–303 (2007).
(8) J.J. van Deemter, F.J. Zuiderweg, and A. Klinkenberg, *Chem. Eng. Sci.* **5,** 271–289 (1956).
(9) G.J. Kennedy and J.H. Knox, *J. Chromatogr. Sci.* **10,** 549–556 (1972).
(10) A. Felinger, *J. Chromatogr. A* **1218,** 1939–1941 (2011).
(11) S. Deridder and G. Desmet, *J. Chromatogr. A* **1218,** 46–56 (2011).
(12) F. Gritti and G. Guiochon, *J. Chromatogr. A* **1252,** 31–44 (2012).
(13) F. Gritti and G. Guiochon, *J. Chromatogr. A* **1252,** 45–55 (2012).
(14) F. Gritti and G. Guiochon, *J. Chromatogr. A* **1252,** 56–66 (2012).
(15) M.M. Fallas, S.M.C. Buckenmaier, and D.V. McCalley, *J. Chromatogr. A* **1235,** 49–59 (2012).
(16) S. Fekete, R. Berky, J. Fekete, J.L. Veuthey, and D. Guillarme, *J. Chromatogr. A* **1252,** 90–103 (2012).
(17) A.C. Sanchez, G. Friedlander, S. Fekete, J. Anspach, D. Guillarme, M. Chitty, and T. Farkas, *J. Chromatogr. A* **1311,** 90–97 (2013).
(18) S. Fekete, K. Ganzler, and J. Fekete, *J. Pharm. Biomed. Anal.* **54,** 482–490 (2011).
(19) S. Fekete and D. Guillarme, *J. Chromatogr. A* **1308,** 104–113 (2013).
(20) F. Gritti and G. Guiochon, *J. Chromatogr. A* in press (2014).
(21) S. Fekete and D. Guillarme, *J. Chromatogr. A* **1320,** 86–95 (2013).
(22) B. Bobály, D. Guillarme, and S. Fekete, *J. Sep. Sci.* **37,** 189–197 (2014).
(23) S. Fekete, I. Kohler, S. Rudaz, and D. Guillarme, *J. Pharm. Biomed. Anal.* **87,** 105–119 (2014).
(24) M.W. Dong, *LCGC North Am.* **31**(10), 868–880 (2013).
(25) J. Ruta, D. Zurlino, C. Grivel, S. Heinisch, J.L. Veuthey, and D. Guillarme, *J. Chromatogr. A* **1228,** 221–231 (2012).
(26) *British Pharmacopoeia* 2014 on-line (http://www.pharmacopoeia.co.uk), monograph: Atorvastatin calcium trihydrate.
(27) *European Pharmacopoeia* on-line 8.2 (http://online6.edqm.eu/ep802/), monograph: Atorvastatin calcium trihydrate
(28) S. Fekete, R. Berky, J. Fekete, J.L. Veuthey, and D. Guillarme, *J. Chromatogr. A* **1236,** 177–188 (2012).

Szabolcs Fekete *holds a PhD degree in analytical chemistry from the Technical University of Budapest, Hungary. He worked at the Chemical Works of Gedeon Richter Plc at the analytical R&D department for 10 years. Since 2011, he has been working at the University of Geneva in Switzerland. He contributed 50 journal articles and authored several book chapters. His main interests include liquid chromatography, column technology, pharmaceutical and protein analysis and method development.*

Davy Guillarme *holds a PhD degree in analytical chemistry from the University of Lyon, France. He is a senior lecturer at the University of Geneva in Switzerland. He authored 100+ journal articles related to pharmaceutical analysis and was an editor of a recent book. His expertise includes HPLC, UHPLC, HILIC, LC–MS, analysis of proteins and mAbs, and SFC. He is an editorial advisory board member of* LCGC North America.

Michael W. Dong *is a senior scientist in Small Molecule Drug Discovery at Genentech in South San Francisco, California. He is responsible for new technologies, automation, and supporting late-stage research projects in small molecule analytical chemistry and QC of small molecule pharmaceutical sciences. He holds a PhD in analytical chemistry from the City University of New York and a certificate in Biotechnology from U.C. Santa Cruz. He has conducted numerous courses on HPLC/UHPLC, pharmaceutical analysis, HPLC method development, drug development process, and drug quality fundamentals. He is the author of 100+ journal articles,* Modern HPLC for Practicing Scientists, *and a co-editor of* Handbook of Pharmaceutical Analysis by HPLC. *He is a member of the editorial advisory board of* LCGC North America.

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