by Richard A. Henry and Stephanie A. Schuster

Reprinted from *American Laboratory* June/July 2017

How to Avoid Size Mismatch Between Solutes and Column Pores for Optimum HPLC Performance

Improved large-molecule separations are achieved when samples are matched with the correct column pore sizes. During large-molecule separations, overlap of size-exclusion chromatography (SEC) with phase interaction modes (such as reversed-phase chromatography [RPC], normal-phase chromatography [NPC], and hydrophilic interaction chromatography [HILIC]) cannot be avoided; therefore, a better understanding of how SEC impacts high-resolution HPLC is needed. Data from these authors and others suggest that the diameter of target molecules should be no larger than 10% of the mean pore diameter to minimize pore crowding, maintain adequate retention, and avoid significant loss of efficiency and resolution. This article describes a new approach for selecting optimum column pores for separating large molecules while maintaining high performance.

Introduction

While superficially porous particles (SPPs) and fully porous particles (FPPs) using silica substrates have been enhanced for small-molecule HPLC analysis, optimum particle designs and modifications are still evolving for analyzing large molecules. Depending on the application, SPP silica can be optimized by changing pore diameter of the porous layer, layer thickness, and overall particle diameter. The ability to control and optimize porous layer thickness and particle size independently is attractive for large-molecule separations.

A 3.4-µm, 400-Å SPP-type particle developed by Kirkland for proteins and other large molecules is shown in *Figure 1B* next to a 2.7-µm 90-Å SPP (Figure 1*A*). Figure 1*C* shows pore-size characteristics of several commercial SPPs used for HPLC separations. Some porosity is necessary in HPLC to provide adequate surface area for loading stationary phase and creating a viable two-phase distribution system within the column, but pores should also be large enough to allow access to the stationary phase by larger solutes. Commercial silica columns typically have a wide pore distribution range to permit a single column to separate a range of different molecular sizes. When molecular size varies significantly within the sample, some molecules may show performance loss due to mismatch of larger solutes with smaller-particle pores. For maximum performance, method development for different-sized molecules requires agreement between column pore-size range and the molecular sizes within the sample. Ideally, all solutes should be able to enter the pores, diffuse rapidly, and not interfere in any way with the separation process.

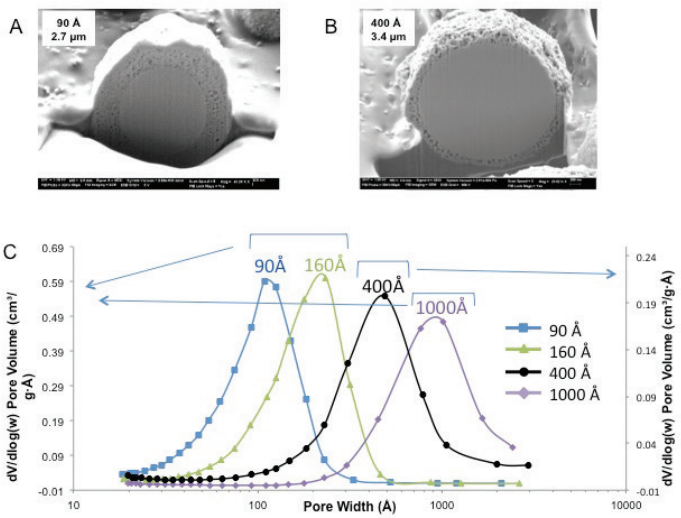

Figure 1 – Differences in morphology for Fused-Core particles (Advanced Materials Technology, Inc., Wilmington, DE) optimized for A) small molecules, 2.7 µm; 90 Å, and B) large molecules, 3.4 µm; 400 Å. C) Pore-size ranges of Fused-Core particles for different-sized solutes.

Small molecules typically have diameters of 5–10 Å that can fit into most particles with 90-Å average pore diameter. Pores in the 90–120 Å range do not normally confine small molecules, allowing them to diffuse freely within the pore space. Pore structure is seldom considered during small-molecule method development and is not deemed important for separation; yet pore size and distribution may become key performance factors if small molecules in the sample significantly exceed 10 Å.

A particle with an average pore size of 160 Å is shown in Figure 1*C*. For sample molecules in the 2000-Da range, it might be necessary to move

from 90 Å to a particle with larger-pore diameter to relieve confinement and regain high performance. Pores in the 150–200 Å range usually work well up to about 15,000 Da before performance loss is observed. Particles with mean pore diameters of 400 Å and 1000 Å should be ideal for solutes with molecular weight (MW) between 15,000 and 500,000 Da or larger. The effects of column pore size mismatch were described by Kirkland et al.,[1–3] regarding performance of 400-Å and 1000-Å SPP particles.

Molecular-weight data is readily available, but molecular size and shape are more important in determining solute behavior toward pore structure and for selecting optimum HPLC column pore size. If samples contain a wide-solute MW and size range, screening different RP columns with a range of pore sizes can establish the point at which significant retention loss and peak broadening occurs for the largest molecule. If pore size can be optimized for the largest target molecule in the sample, it should be adequate for smaller molecules if retention is adequate as well. Determining which solute is the largest may not be easy for unknown samples, but this is where SEC experiments can be helpful.

Size-exclusion chromatography

Much can be learned about solute size and how much access molecules have to the pore environment by performing size-exclusion experiments before conducting analytical experiments. SEC can also be valuable for troubleshooting after poor RP column performance has been observed. For this, an SEC column with a pore size similar to the high-resolution analytical column should be chosen.

The principles of SEC are described by the simple equations shown below. Additional details are available in Ref. 4.

$$V_M = V_0 + V_P \quad (1)$$

$$V_R = V_0 + K_{Size} V_P \quad (2)$$

Total column volume, V_M, is the sum of the column volume outside the particles, V_0, added to the volume inside the pores, V_P. Eq. [2] introduces a size constant, K_{Size}, which ranges from one for small molecules to zero for large molecules. Thus, the observed elution volume, V_R, can be equal to or less than V_M, but should never be greater unless solute is retained by the stationary phase. Small molecules should be free to move within all available pore space and therefore elute very near the total liquid volume of the column (V_M), which is referred to in small-molecule HPLC as the solvent front.

A low-MW marker is normally included in SEC calibrations to establish V_M. As molecules increase in MW and size, they gradually become more confined until they are totally excluded from all pores and elute at V_0, the excluded volume. V_0 is also called interstitial volume or the volume around the outside of the column particles. Nothing should elute sooner than V_0, and everything should elute within the total volume of the column. SEC columns are defined by a calibration curve that typically shows a linear range of about two orders of magnitude, depending on the pore-size distribution of the column particles. Only solutes that elute within the linear range of the calibration plot should be used for size estimation.

Klein and Treichel[5] used the cylindrical pore-size model to arrive at the geometric equation shown in *Figure 2* to describe how solute-radius to pore-radius (a/r) affects the (pore) distribution constant, K_{OC}. A small value for a/r (radius-ratio) is desirable for HPLC because it allows adequate stationary-phase access. Klein's occupational constant is closely related to K_{Size} in Eq. [2]. Small molecules such as uracil would have radius-ratio values approaching zero and a K_{Size} approaching one for almost any column pore size. They can be employed as markers for total column volume because they are free to travel nearly anywhere mobile phase is located and have access to the stationary phase for optimum retention. On the other hand, large molecules such as monoclonal antibodies have much larger radius-ratio values and may lose contact with the stationary phase unless a larger-pore column is installed. A radius-ratio value of 0.1 is shown at the vertical line in Figure 2, where Klein's equation estimates that about 80% of the stationary phase would be accessible.

Figure 3 shows the SEC calibration plot obtained by injecting individual peptide and protein standards on a small-pore 150-Å column. SEC plots like this supply specific size information relative to the pore size of the column and can help users select HPLC columns that should provide adequate pore access and allow high-performance separations. Uracil (MW 112, point 12) elution volume estimates total column volume, and a monoclonal antibody (mAb) (MW 150 kDa, point 2) estimates excluded column volume. Since thyroglobulin (MW 667 kDa, point 1) lies outside the linear range, its elution volume is not an accurate indicator of size. The linear range of the semi-log plot estimates column pore volume. If the SEC calibration curve covers about two orders of magnitude, the 80% phase access point (a/r = 0.1) should be located near the midpoint of the linear plot, which intersects the calibration line at about MW 10,000. This is in agreement with the largest MW recommended earlier for HPLC on a 160-Å column. For the standards and column shown in Figure 3, larger molecules to the left of the midpoint would see less than 20% of the available stationary phase, while the smaller molecules to the right would see more than 80% of the available stationary phase on a 150-Å pore column. A larger-pore RP-HPLC column should be used for any sample component that lies to the left of the midpoint in Figure 3.

Giddings[6] theorized that peak broadening occurs for larger molecules because of pore crowding (radius-ratio of 0.1–1.0). He employed thermodynamic relationships to describe phase distribution in a bed of porous (or superficially porous) particles, where large molecules encounter wall constraints, gradually lose freedom of movement (decreasing entropy) and finally become excluded entirely from the pores. According to Giddings, this gradual exclusion process interferes with retention equilibria, slows mass transfer rate, and creates peak broadening. Wagner et al.[3] reported the effect of 1000-Å pores for separations of mAbs and other large proteins and provide supporting evidence that diffusion rate (efficiency) of large molecules drops rapidly within the radius-ratio region of 0.1–1.0.

Results for large-molecule HPLC

Large-molecule RP-HPLC separations and loss of retention and efficiency in the hindered diffusion region are referred to as the exclusion effect. Serious performance problems usually result when solute size exceeds about one-tenth of the mean pore diameter.

The 160-Å C4 SPP column in *Figure 4* is recommended for solutes up to about 15,000 D, so it would not be expected to perform well for a monoclonal antibody (150 kDa). The peak is too broad and too close to the exclusion limit of this 160-Å column. A calibration plot with a 200-Å SEC column would likely predict this result; this was confirmed by doing the same separation on a 400-Å C4 column. Note that peak width has become much narrower with the larger-pore column, which shows that pore crowding has been greatly reduced for the mAb, allowing better mass transfer and a much sharper peak. Although surface area is much smaller for the larger-pore column, retention is comparable, showing there is much more room in the larger pores for phase access.

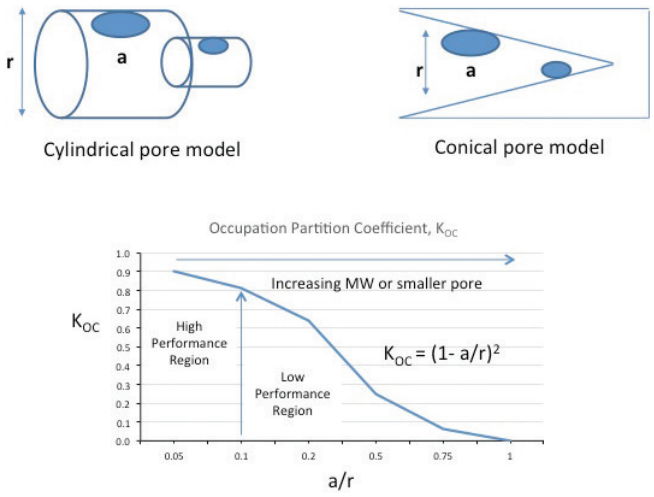

Figure 2 – How the ratio of solute radius (a) to pore radius (r) impacts pore occupancy, K_{OC}, in pure SEC.[5]

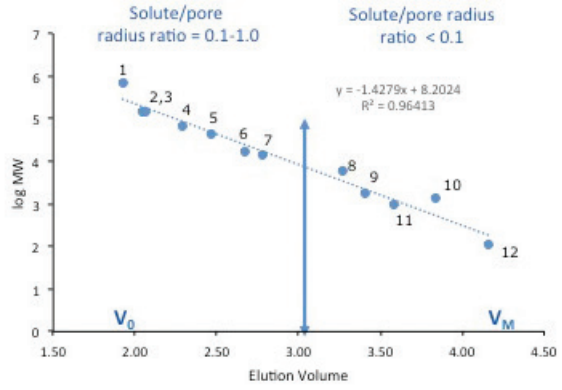

Figure 3 – SEC calibration curve for peptides and proteins. Conditions—column: Zenix SEC-150 (Sepax Technologies, Newark, DE), 4.6 × 300 mm; 3 µm; mobile phase: 0.2 M *potassium phosphate, pH 7.0; flow rate: 0.25 mL/min; temperature: 25 °C; injection volume: 0.5 µL; instrument: Nexera (Shimadzu Scientific Instruments, Columbia, MD); detection: photodiode array (PDA), 215 nm and 280 nm. Peak identities: 1) thyroglobulin, 2) Sigma MAb, 3) IgG, 4) bovine serum albumin (BSA), 5) ovalbumin, 6) myoglobin, 7) ribonuclease A, 8) bovine insulin, 9) neurotensin, 10) Vitamin B12, 11) angiotensin II, 12) uracil.*

Figure 5 shows a monoclonal antibody separation on a 1000-Å SPP C4 column and a 300-Å FPP C4 column. The larger-pore SPP particle exhibits greater retention and higher efficiency for the mAb. This is expected for the 1000-Å particle, because the radius-ratio would be about 0.1 compared to only about 0.3 for the 300-Å particle, and peak broadening arises from slower mass transfer, described earlier by Giddings. Although calibration curve results are not shown for 300-Å and 1000-Å SEC columns, mAbs should lie in the low-performance region for a 300-Å SEC column and in the high-performance region for a 1000-Å SEC column (see Figure 2).

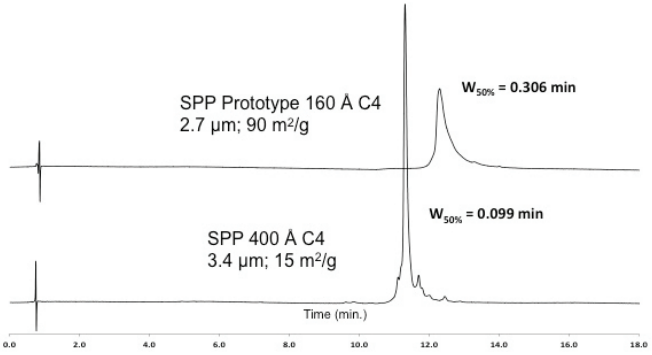

Figure 4 – Example of monoclonal antibody that is too large for a 160-Å-pore column, showing peak broadening corrected by changing to a 400-Å-pore column, which greatly improves efficiency and allows important peaks to be detected. Conditions—columns: 2.1 × 150 mm; mobile phase A: water/0.1% difluoroacetic acid; mobile phase B: ACN (acetonitrile)/0.1% difluoroacetic acid; gradient: 27–37% B in 20 min; flow rate: 0.4 mL/min; temperature: 80 °C; injection volume: 2 µL of 0.5 mg/mL Sigma MAb; instrument: Nexera; detection: PDA, 280 nm.

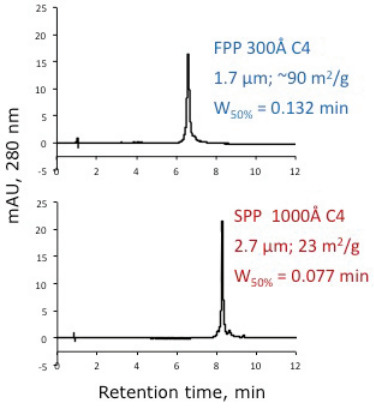

Figure 5 – Comparison of a partially excluded large-molecule (Sigma MAb) on different large-pore columns. Conditions—columns: 2.1 × 150 mm; mobile phase A: water/0.1% TFA (trifluoroacetic acid); mobile phase B: ACN/0.1% TFA; gradient: 32–38% ACN in 12 min; flow rate: 0.4 mL/min; temperature: 80 °C; injection volume: 2 µL of 0.5 mg/mL; instrument: Nexera; detection: PDA, 280 nm; backpressure: FPP, 322 bar; Fused Core, 221 bar.

Conclusion

Changing particle pore diameter is not usually a primary consideration for improving column performance when developing small-molecule HPLC methods, but it can be the most important variable for developing high-resolution HPLC methods for the separation of large molecules. Although other methods may be more accurate for collecting size information about sample molecules, SEC has been proposed as a fast and convenient technique that uses readily available HPLC systems. Screening samples with appropriate SEC columns to identify possible size mismatch with analytical columns should be easily automated. Even if standards are not available for every sample component, a solute exclusion profile of the sample will still be valuable for estimating whether any components might be too large for high-resolution performance and help to locate analytical columns with larger pores to move solutes into the high-performance region.

References

1. Schuster, S.A.; Wagner, B.M. et al. *J. Chromatogr. A* **2013**, *1315*, 118–26.
2. Wagner, B.M.; Schuster, S.A. et al. *LC·GC N. Am.* Nov **2015**, *33*(11), 856–65.
3. Wagner, B.M.; Schuster, S.A. et al. *J. Chromatogr. A* **2017**, *1489*, 75–85.
4. Snyder, L.R.; Kirkland, J.J. et al. *Introduction to Modern Liquid Chromatography*, 3rd ed. John Wiley & Sons: Hoboken, NJ, 2010.
5. Klein, J. and Treichel, K. *Chromatographia* **1977**, *10*, 604–10.
6. Giddings, J.C. *Unified Separation Science*, Chapter 2. Entropy Effects in Porous Media, Wiley: New York, NY, 1991.

Richard A. Henry is independent consultant, 983 Greenbriar Dr., State College, PA 16801, U.S.A.; e-mail: hyperLC@comcast.net. Stephanie A. Schuster is senior chemist, Advanced Materials Technology, Inc., Wilmington, DE, U.S.A.; www.advanced-materials-tech.com