Impact of Pore Exclusion on Reversed-Phase HPLC Column Performance

Richard A. Henry, Technical Consultant, 983 Greenbriar Drive, State College, PA 16801 (hyperLC@comcast.net)
Stephanie Schuster, AMT, Inc., Silverside Road, Wilmington DE 19810 (SSchuster@advanced-materials-tech.com)
Two Main HPLC and UHPLC Particle Types

Totally Porous Particle (TPP)

Superficially Porous Particle (SPP)

300 Å pores

Shell with 90 Å pores
Surface-Layer Porosity in 400Å Superficially-Porous Silica$^{1, 2}$

- Large solutes diffuse 10-100 times more slowly than small ones.
- Unique performance and separation speed of SPP particles arise from a combination of large pores with short diffusion paths.
- Proven SPP advantages, including speed, for interactive modes (RP, NP, HILIC) may extend to the size exclusion mode (SEC).
Common Pore-Size Ranges for Fused-Core Silica (SPP)

- Small drugs: 2.0 Å
- Small peptides (< 5000 Da): 5-10 Å
- Small peptides (< 2000 Da): 20-30 Å
- Large peptides/small proteins (< 15,000 Da): 40-50 Å
- Large proteins (15,000 to 500,000 Da): 100-200 Å

Pore Width (Å)

- 90 Å
- 160 Å
- 400 Å

Water (18 Da)

12-2016 LCGC 2016 RAH
How Pore-Size Impacts Column Performance

- Porous and superficially porous particles are required in HPLC columns to create adequate surface area and retention.
- Small solutes have essentially full access to mesopores in 90Å-130Å particles to allow rapid phase-solute interaction; however, even partial exclusion can adversely affect column separation when solute size approaches the pore diameter.
- When too confined, solutes lose freedom to diffuse and interact with the surface. Both equilibrium constants and (interaction) kinetics may be impacted.
- Ideally, one should know MW and size for all sample solutes and particle pore structure for HPLC columns (and match them carefully) in order to develop reproducible methods with optimum separation.
Retention Equation for Pure Size Exclusion HPLC\textsuperscript{4,5}

\[ V_M = V_0 + V_P \quad (1) \]

\[ V_R = V_0 + K_{\text{Size}} V_P \quad (2) \]

\( K_{\text{Size}} \) varies from zero for total pore exclusion to one for total pore occupation. Separation by a pure SEC mechanism correlates well with solute hydrodynamic radius and pore geometry.

The subject of this study is the point on the curve where solute size adversely affects column performance.
Retention Equation for Pure Sorption HPLC Modes

Retention by stationary phase interaction:

\[ V_R = V_M + K_{Sorp} V_S = V_0 + V_P + K_{Sorp} V_S \]  (3)

\( K_{Sorp} \) can vary from 0 to infinity, and \( V_S \) stationary phase volume which is related to surface area. Equations (2) and (3) are usually employed separately under conditions where only one mode dominates.

SEC mode behaves differently compared to retention modes such as RPC and can be very useful for analysis of complex samples by multi-dimensional techniques.
Retention Equation When SEC and Sorption Modes Overlap

When both large and small molecules are present, they will not experience equal access to the pores, therefore retention must be predicted by combining equations:

\[ V_R = V_0 + K_{\text{Size}} (V_P + K_{\text{Sorp}} V_S) \]  (4)

Equation (4) reduces to Equation (3) for small molecules \( K_{\text{Size}} = 1 \). When molecules become large enough to become partly excluded from pores \( K_{\text{Size}} < 1 \), retention factors will be reduced and efficiency (rate of mass transfer) may also be reduced.

Rule of thumb: To eliminate undesirable size exclusion effects, select a column having an average pore diameter that is 10X the diameter of the largest solute in the sample.
Klein defined an occupational partition coefficient \( K_{OC} \) that is essentially equivalent to \( K_{size} \). The occupational partition coefficient impacts \( V_S \) and solute retention in a complex fashion. Mode overlap regularly occurs between size exclusion and stationary phase retention modes of HPLC with microporous column particles.

A complete understanding of this process is needed to optimize any HPLC method, especially one involving large-molecule separations. Klein used a cylindrical pore model and equation to explain the steric exclusion process based on solute-to-pore radius ratio.

\[
K_{OC} = (1 - a/r)^2 = K_{Size}
\]  

(5)

where \( K_{OC} \) is the occupation partition coefficient, \( a \) is the solute radius and \( r \) is the pore radius. Both \( a \) and \( r \) are variables for different samples and columns, respectively.
Impact of Size Exclusion on Solute Retention

Pore radius \( (r) \) varies within a range for porous particles; an average is usually compared.

Solute radius \( (a) \) increases with MW; an optimum ratio \( (a/r) \) exists for each sample component.

As solutes become larger, they are excluded from pores to create the calibration curve, but they also lose access to surface area.

\[
K_{OC} = (1 - \frac{a}{r})^2
\]

Using this formula, solutes having 1/10 of the pore diameter can access ca. 80% of the pore volume and allow phase interaction to dominate.
Effect of Pore-Size on Efficiency for High MW Solute

High efficiency is the key to high peak capacity; the larger 160Å pore particle shows higher efficiency and peak capacity for insulin and other large peptides compared to 90Å. Giddings predicted this but more explanation is needed.

Based on SEC-150 column calibration, Bovine Insulin is < 50% excluded from the 160Å column pores, but likely is > 50% excluded from 90Å column pores.

Columns: 4.6 x 100 mm; Mobile phase: 31.5% ACN/68.5% Water/0.1% TFA; Flow rate: 1.0 mL/min; Temperature: 60°C; Sample: Bovine Insulin, MW = 5733; Injection volume = 10 µL
90 Å Pore-Size Column - Too Small for Many Peptides

1. Ribonuclease A (13,700 g/mol)
2. Bovine Insulin (5733 g/mol)
3. Human Insulin (5808 g/mol)
4. Cytochrome c (12,400 g/mol)
5. Lysozyme (14,300 g/mol)

• Retention, efficiency and sensitivity are lost with a 90Å column due to pore-crowding for the larger peptides
• The 160Å column is preferred for peptides.

Columns: 100 mm x 4.6 mm; mobile phase: A: water/0.1% TFA; B: acetonitrile/0.1% TFA; gradient: 25–42% B in 10 min; flow rate: 1.5 mL/min; temperature: 30 °C; detection: 215 nm; Peak widths in minutes above each peak.

Surface areas
2.7 um 90 A  135 m²/g
2.7 um 160 A  90 m²/g
Impact of Size Exclusion on Zone Broadening

Sources of solute zone spreading in packed-bed liquid chromatography have been widely studied. Schure has recently submitted a comprehensive analysis of HPLC zone broadening that includes a description of hindered diffusion inside pores (relative to free mobile phase diffusion) as solute size approaches average pore diameter.

Hindered diffusion in cylindrical pore as function of ratio of molecule radius to pore radius.

Although phase interaction is still the dominant mode at a radius ratio of 0.1, the pore diffusion coefficient has already dropped by almost 50% according to Schure.
Calibration Curve for Size Exclusion Chromatography

Chromatographic Conditions

- **columns:** Sepax Zenix SEC-150, 30 cm x 4.6 mm I.D., 3 µm
- **mobile phase:** 0.2 M potassium phosphate, pH 7.0
- **flow rate:** 0.25 mL/min
- **pressure:** 66 bar
- **column temp.:** 25 °C
- **detector:** UV 215, 280 nm
- **injection:** 0.5 µL
- **samples:** listed below

<table>
<thead>
<tr>
<th>Analyte</th>
<th>MW (Da)</th>
<th>Size (Å)</th>
<th>$K_{\text{size}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 thyroglobulin</td>
<td>667000</td>
<td>200</td>
<td>0.00</td>
</tr>
<tr>
<td>2 SigmaMab</td>
<td>150000</td>
<td></td>
<td>0.06</td>
</tr>
<tr>
<td>3 IgG</td>
<td>150000</td>
<td>100</td>
<td>0.05</td>
</tr>
<tr>
<td>4 BSA</td>
<td>66400</td>
<td></td>
<td>0.16</td>
</tr>
<tr>
<td>5 ovalbumin</td>
<td>45000</td>
<td>40</td>
<td>0.24</td>
</tr>
<tr>
<td>6 myoglobin</td>
<td>17000</td>
<td>40</td>
<td>0.33</td>
</tr>
<tr>
<td>7 ribonuclease A</td>
<td>13700</td>
<td></td>
<td>0.38</td>
</tr>
<tr>
<td>8 bovine insulin</td>
<td>5700</td>
<td></td>
<td>0.60</td>
</tr>
<tr>
<td>9 neurotensin</td>
<td>1700</td>
<td></td>
<td>0.66</td>
</tr>
<tr>
<td>10 vitamin B12</td>
<td>1350</td>
<td>20</td>
<td>0.85</td>
</tr>
<tr>
<td>11 angiotensin II</td>
<td>1000</td>
<td></td>
<td>0.74</td>
</tr>
<tr>
<td>12 uracil</td>
<td>112</td>
<td></td>
<td>1.00</td>
</tr>
</tbody>
</table>

* Unpublished data by AMT Applications Lab
160 Å Pore-Size Column - Too Small for Large Proteins

- Efficiency and sensitivity are lost with the 160Å column due to pore-crowding for the larger proteins.
- The 400Å column is preferred for proteins.

Peak Identities:
1. Ribonuclease A 13.7 kDa
2. Cytochrome c 12.4 kDa
3. Lysozyme 14.3 kDa
4. α-Lactalbumin 14.2 kDa
5. Catalase 60 kDa (subunit)
6. Enolase 46.7 kDa

Columns: 4.6 x 100 mm
Gradient: 23-50 %B in 15 minutes
A: water/0.1% TFA
B: ACN/0.1% TFA
Flow rate: 1.5 mL/min
Detection: 215 nm
Temp: 60 C
Injection: 5 µL

Peak widths in minutes above each peak.

Surface areas:
- 2.7 µm 160 Å 90 m²/g
- 3.4 µm 400 Å 15 m²/g
Large Protein Comparison on 160 Å and 400 Å Columns

- Efficiency is higher on 400Å pore size particle and retention is equal, even with much lower surface area.
- The 400Å column is preferred for Myosin.

Myosin cellular protein >200 kDa

![Graph showing absorbance](image)

- Conditions: columns= 2.1 × 100 mm; temperature= 80 ºC; flow rate = 0.45 mL/min. Gradient 35–65% B in 15 min: A = 0.1% trifluoroacetic acid; B = acetonitrile with 0.1% trifluoroacetic acid. Detection = 215 nm; injection volume = 1 µL; sample = myosin, 400 kDa initial, but denatured in mobile phase.

Surface areas
- 2.7 µm 160 Å: 90 m²/g
- 2.7 µm 400 Å: 29 m²/g
SILU Lite SigmaMAb Comparison on 160Å and 400Å

- Solute size of MAb is now approaching the average pore diameter of the 160Å column.
- The 400Å column is preferred.

Columns: 150 mm x 2.1 mm; mobile phase: A: water/0.1% difluoroacetic acid; B: 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; detection: 280 nm
Wide-Pore Column Comparisons for Small Molecules

Chromatographic Conditions
- columns: Fused-Core C4, 15 cm x 2.1 mm I.D., 3.4 µm
  - Acquity UPLC Protein BEH C4, 15 cm x 2.1 mm I.D., 1.7 µm
- mobile phase: [A] water; [B] acetonitrile; (75:25, A:B)
- flow rate: 0.3 mL/min
- pressure: 552 bar (BEH); 177 bar (Fused-Core)
- column temp.: 25 °C
- detector: 254 nm
- injection: 0.2 µL
- sample: uracil, phenol, propiophenone, 1-chloro-4-nitrobenzene

Either column would work for smaller molecules.
Column Comparisons for Monoclonal Antibody

BEH 300Å C4

1.7 µm; ~90 m²/g

Fused-Core 400Å C4

3.4 µm; 15 m²/g

- **Fused-Core 400Å** has more retention, at lower surface area, and much higher efficiency than Acquity 300Å.
- **Fused-Core 400Å** column would be preferred for this MAb molecule.

**Chromatographic Conditions**

- columns: Fused-Core C4, 15 cm x 2.1 mm I.D., 3.4 µm
- Acquity UPLC Protein BEH C4, 15 cm x 2.1 mm I.D., 1.7 µm
- mobile phase A: 0.1% DFA in water
- mobile phase B: 0.1% DFA in acetonitrile
- flow rate: 0.4 mL/min
- gradient: 29 to 35% B in 12 min
- initial pressure: 322 bar (BEH); 115 bar (Fused-Core)
- column temp.: 80 °C
- detector: 280 nm
- injection: 2 µL
- sample: SILu™Lite SigmaMAb; 0.5 mg/mL
Recognizing a Column Pore-Exclusion Problem

A pore exclusion problem might exist:

1. When high MW solutes elute close to or even before low MW solutes, under either isocratic or gradient conditions.
2. When high MW solutes show much lower efficiency than expected compared to lower MW solutes.
3. When higher MW solutes show improved retention and efficiency after a larger pore column is installed.

Larger pore-size analytical columns should be included in column screening, especially when sample composition is unknown. The use of SEC columns for early screening is recommended. Analytical columns should be screened initially in RP or another retention mode with a low or moderate MW test mixes to establish expected retention and efficiency performance.
Heavy and Light Chain Reduced Fragments of SigmaMAb

Peak Identities:
1. Light Chain (x2) 23 kDa
2. Heavy Chain (x2) 50 kDa

Reduced SigmaMAb
0.5 µg/2 µL (0.1% TFA)
20-40% AcN/0.1% Formic acid in 20 min
0.5 mL/min; 80°C
2.1 mm x 150 mm

• Both columns perform well for the light chain fragment but the lower surface area 400Å has better retention.

• The 400Å column is preferred because of both narrow peaks and more retention for the fragments.
Using SEC Columns to Estimate Solute Size

• Select SEC columns with similar pore size to high resolution analytical columns available. Two or more SEC columns are useful.
• Select standards with known size for calibrating target solutes; samples are often injected separately since SEC is a low resolution technique. Solute markers near $V_0$ and $V_M$ are very important.
• Pore exclusion would not be expected for target solutes that elute from SEC columns close to $V_M$ (solute/pore radius <0.1).
• Performance loss would be expected for solutes eluting with $K_{size} < 0.5$ (solute/pore radius 0.1-1.0) due to poor phase access and hindered pore diffusion within the analytical column.
• Solutes of interest should never be retained by phase interaction under optimum SEC conditions. Retention by stationary phase makes solutes appear too small. Nothing should elute after $V_M$ in an SEC experiment. Under certain conditions, RP and other columns may be useful with strong solvents for estimating solute size\(^{16}\).
**Conclusions**

- Column retention and efficiency can both be lost for larger sample components when size exclusion mode significantly overlaps with stationary phase retention modes to partially exclude large solutes from stationary phase and interfere with diffusion processes.
- Guidelines have been proposed that solutes should be less than ca. 10% of the column exclusion limit for optimum performance. For example, if the exclusion limit is 150,000 Da, largest solute size to avoid significant peak retention and efficiency loss would be 15,000 Da.
- Preliminary sample screening by SEC can save valuable time by identifying solutes that might be subject to pore exclusion and require evaluation of larger pore columns.
- When data on solute sizes and average column pore size is not available for selecting optimum radius ratios in advance, the use of larger pore columns is recommended for screening unknown samples.
References

16. Private communication from Joe Foley.
Acknowledgements

• Jack Kirkland and Barry Boyes of AMT for general column discussions.
• Mark Schure of Kroungold Analytical, Inc. for conversations about particles, pores and peak broadening.
• Hillel Brandes of Supelco Division of MilliporeSigma for discussions about preparing SEC calibration plots.
• Will Miles and Bob Moran of AMT for chromatographic data acquisition.

Trademarks:
Fused-Core is a registered trademark of Advanced Materials Technology, Inc.
Acquity is a registered trademark of Waters Corporation.
SigmaMAB is a trademark of Sigma-Aldrich Co. LLC.
Zenix is a trademark Sepax Technologies, Inc.