

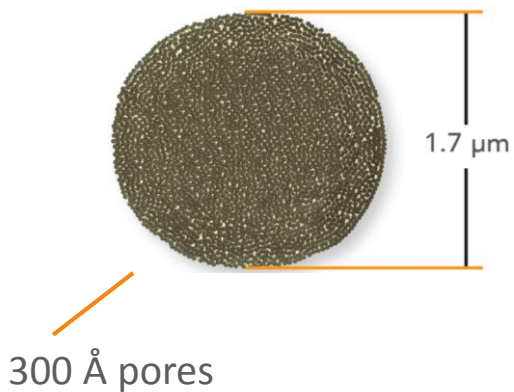
# Impact of Pore Exclusion on Reversed-Phase HPLC Column Performance

Richard A. Henry, Technical Consultant, 983 Greenbriar Drive,  
State College, PA 16801 ([hyperLC@comcast.net](mailto:hyperLC@comcast.net))

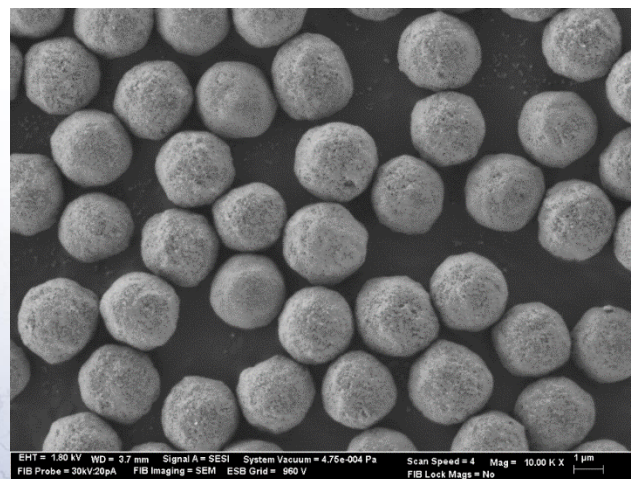
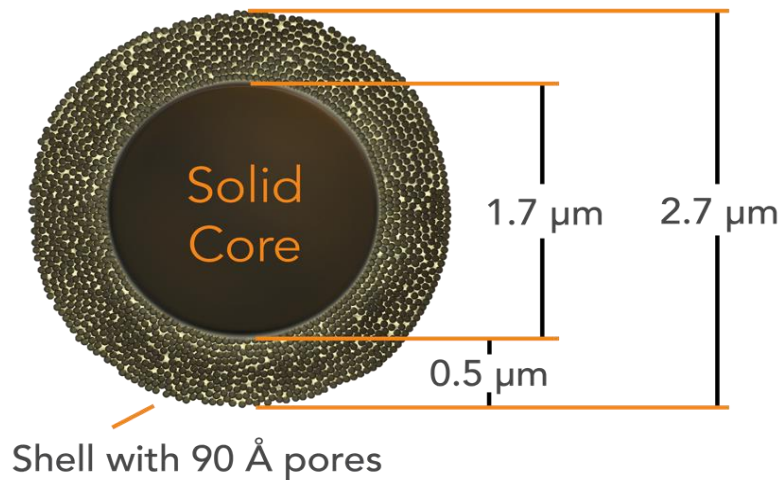
Stephanie Schuster, AMT, Inc., Silverside Road, Wilmington DE  
19810 ([SSchuster@advanced-materials-tech.com](mailto:SSchuster@advanced-materials-tech.com))

# Two Main HPLC and UHPLC Particle Types

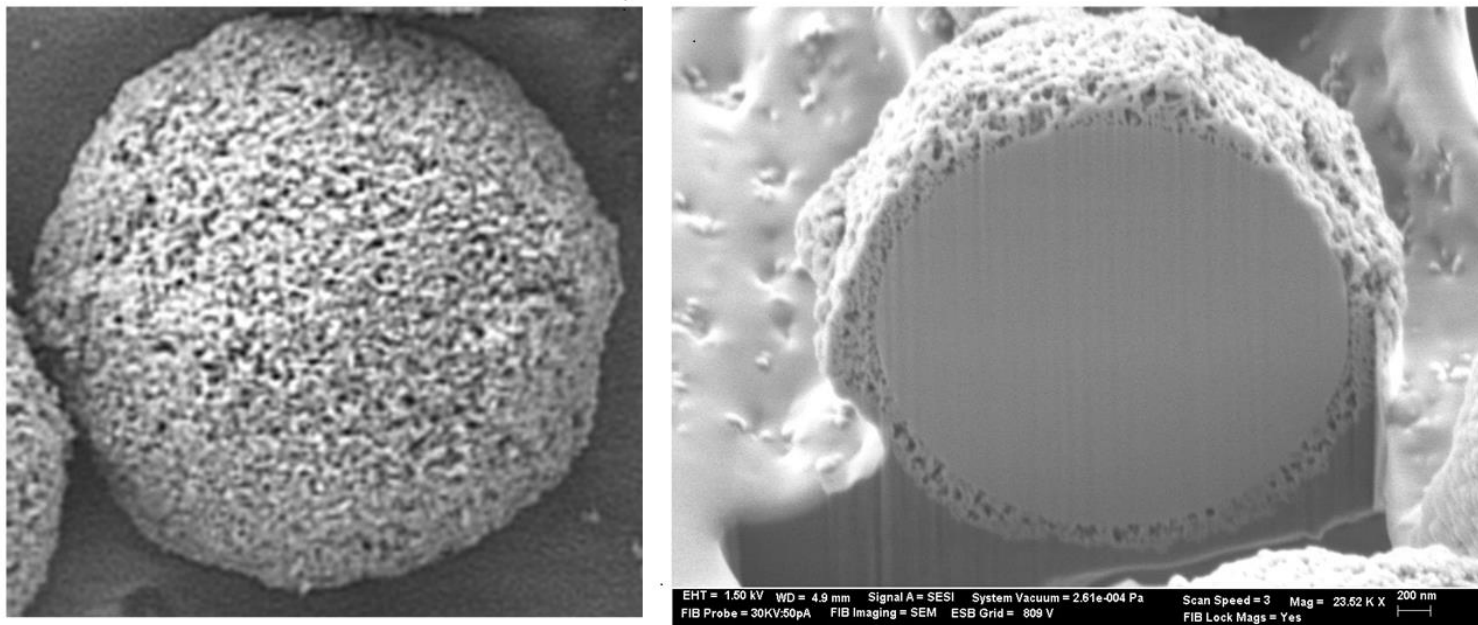
## Totally Porous Particle (TPP)



## Superficially Porous Particle (SPP)

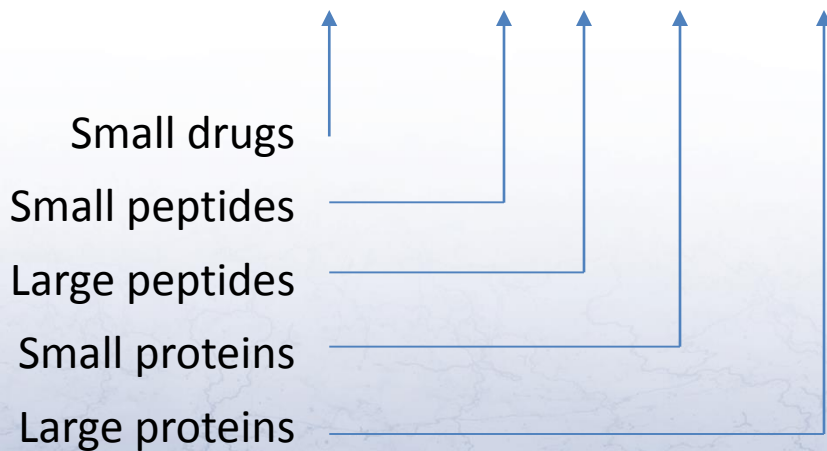
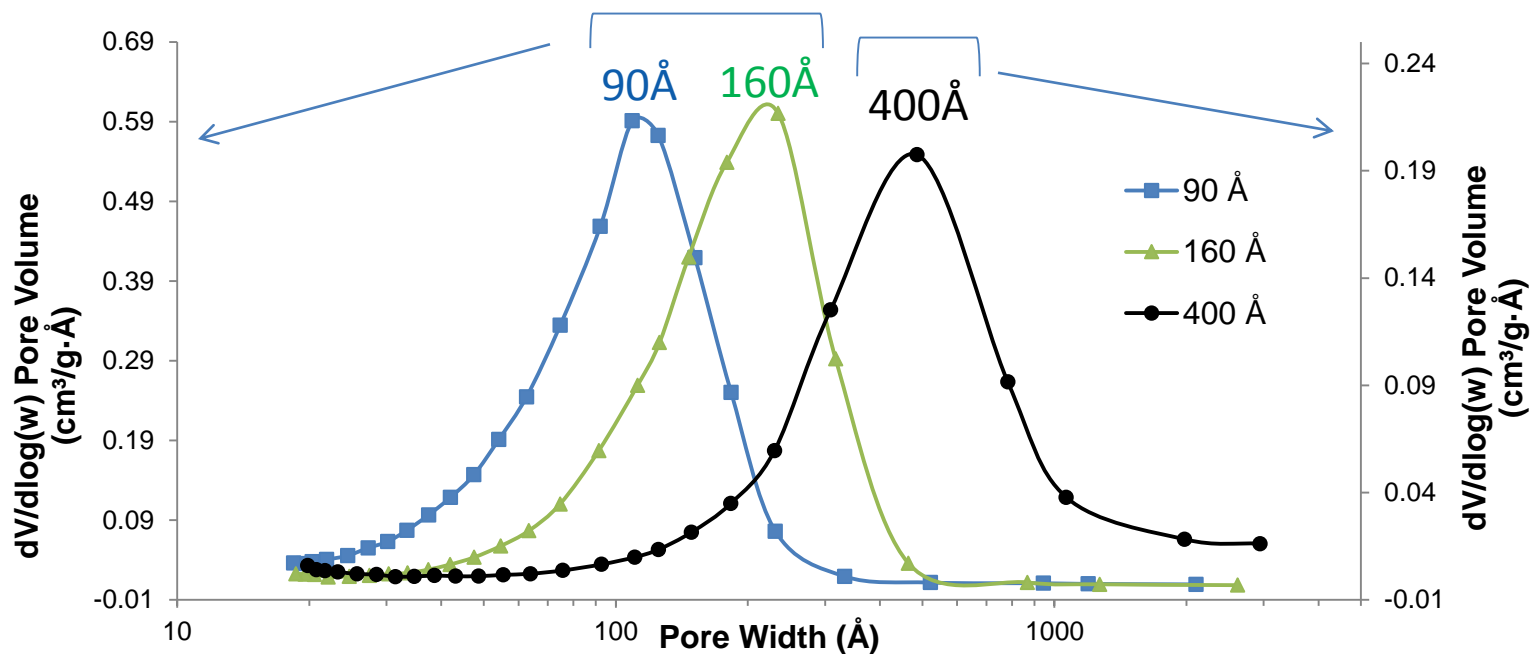


## Surface-Layer Porosity in 400Å Superficially-Porous Silica<sup>1, 2</sup>



- 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)



Water (18 Da)	2.0 Å
Small drugs (< 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

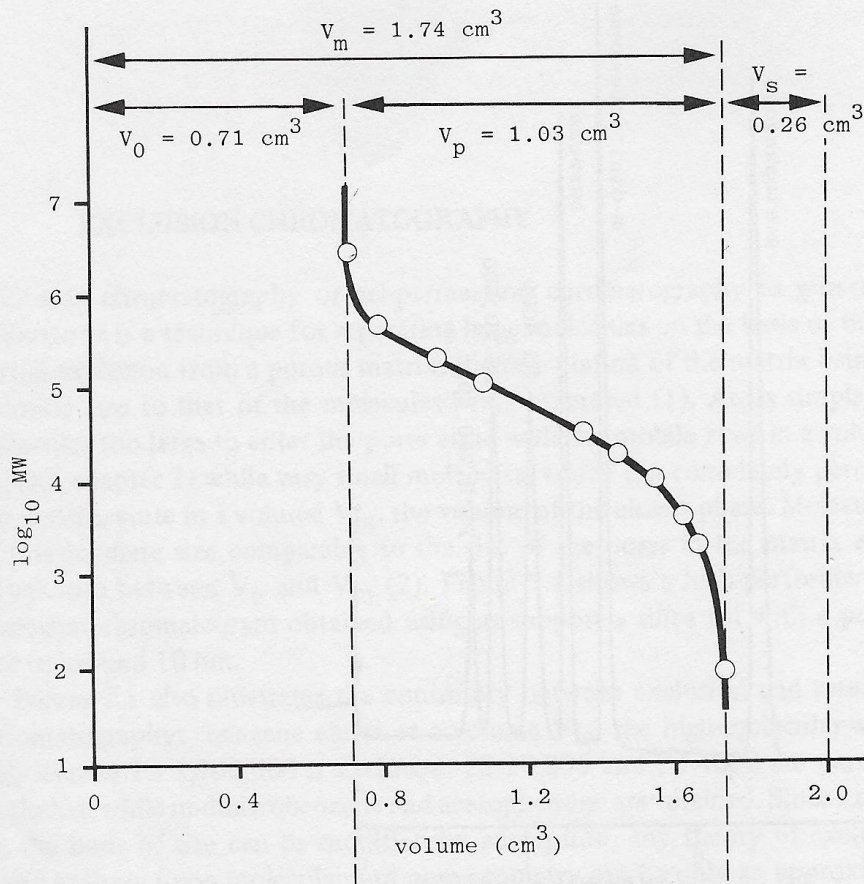
## How Pore-Size Impacts Column Performance<sup>3</sup>

- 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<sup>4,5</sup>

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

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



$K_{\text{Size}}$  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:

$$V_R = V_M + K_{\text{Sorp}} V_S = V_0 + V_P + K_{\text{Sorp}} V_S \quad (3)$$

$K_{\text{Sorp}}$  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 Overlaps<sup>6-10</sup>

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:

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

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.



# SEC Mode Overlap Impacts Column Performance

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.

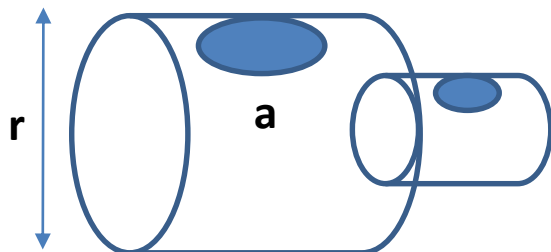
A complete understanding of this process is needed to optimize any HPLC method, especially one involving large-molecule separations.

Klein used a cylindrical pore model and equation to explain the steric exclusion process based on solute-to-pore radius ratio.

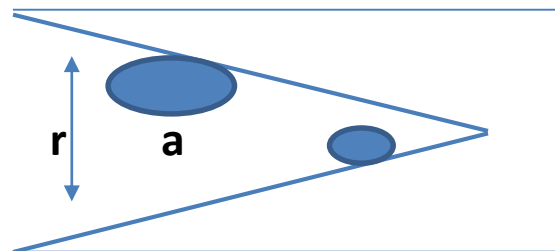
$$K_{OC} = (1 - a/r)^2 = K_{Size} \quad (5)$$

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<sup>6</sup>



Cylindrical pore model

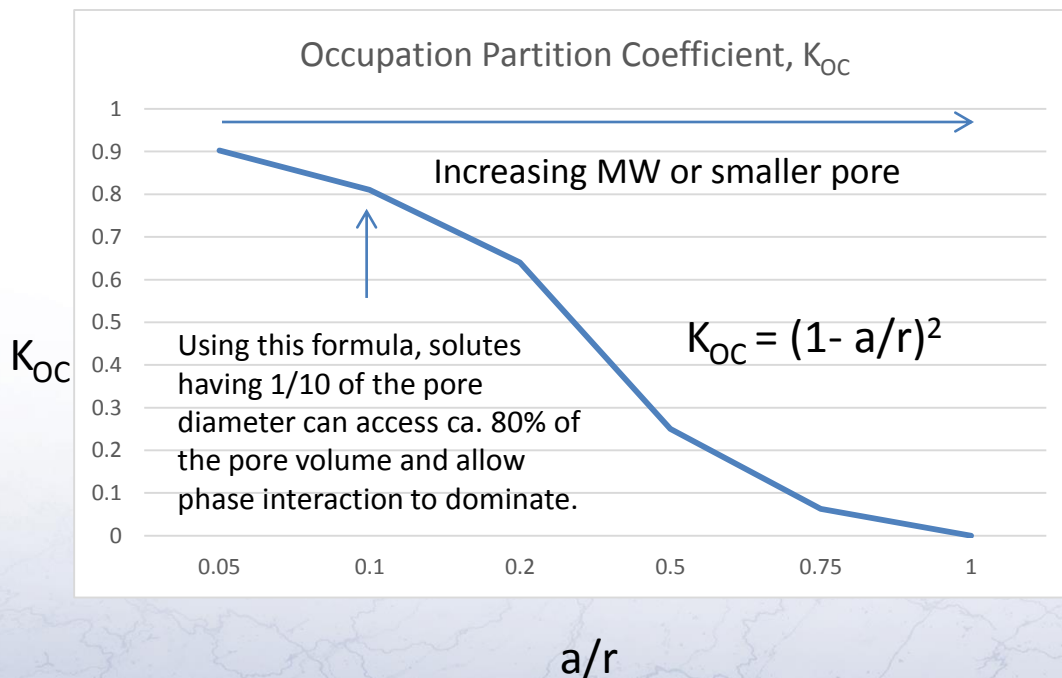


Conical pore model

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

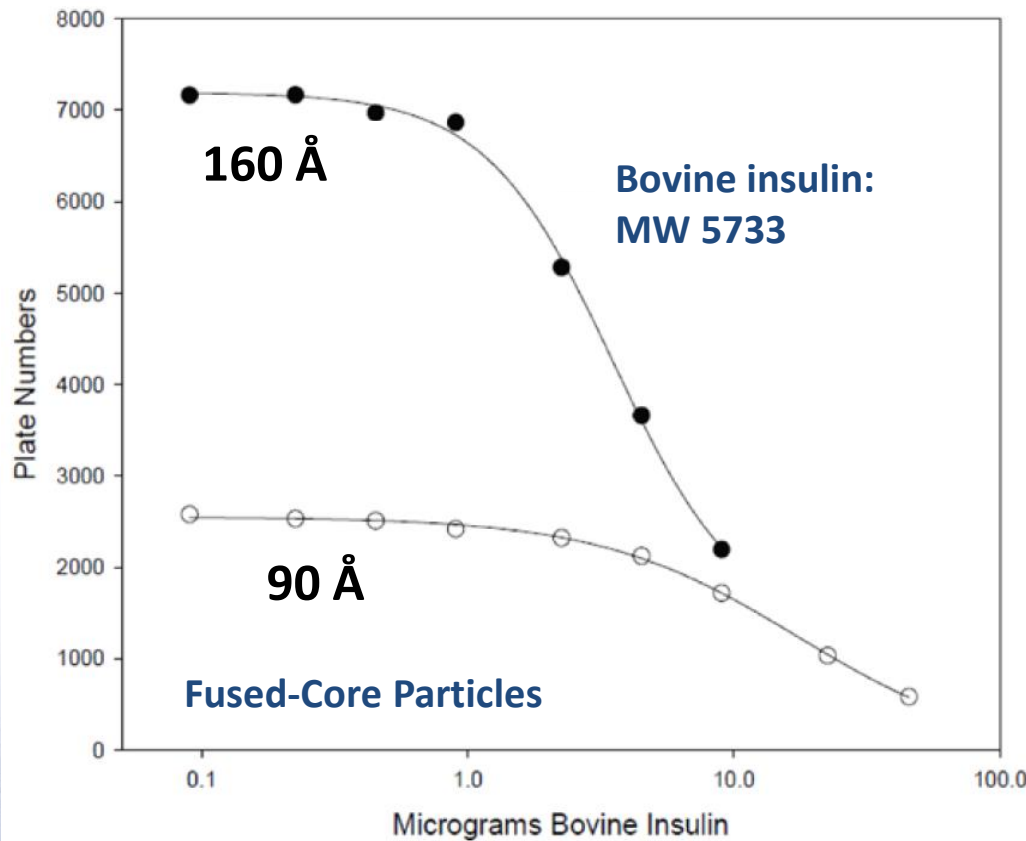
Solute radius (**a**) increases with MW; an optimum ratio (**a/r**) exists for each sample component.

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



# Effect of Pore-Size on Efficiency for High MW Solute<sup>11</sup>

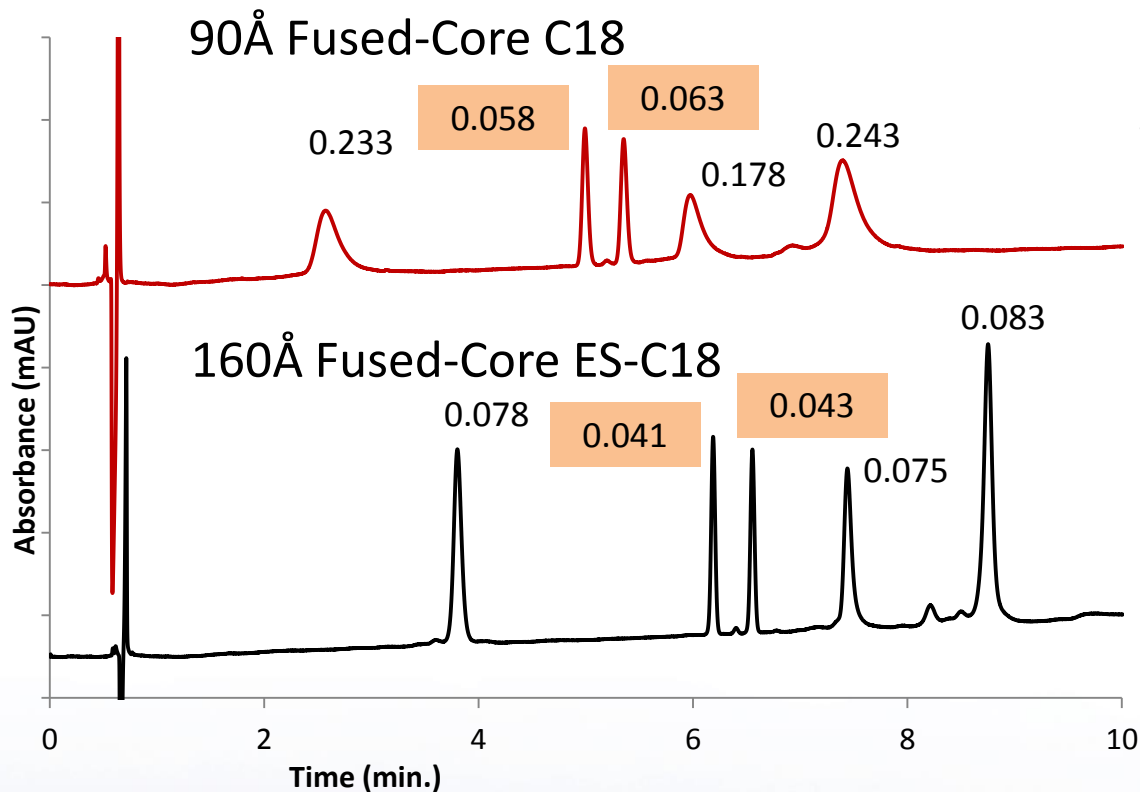
Columns: 4.6 x 100 mm; Mobile phase: 31.5% ACN/68.5% Water/0.1% TFA; Flow rate: 1.0 mL/min; Temperature: 60°C; Sample: Bovine Insulin, MW = 5733; Injection volume = 10  $\mu$ L



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.

# 90 Å Pore-Size Column - Too Small for Many Peptides<sup>12</sup>



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.**

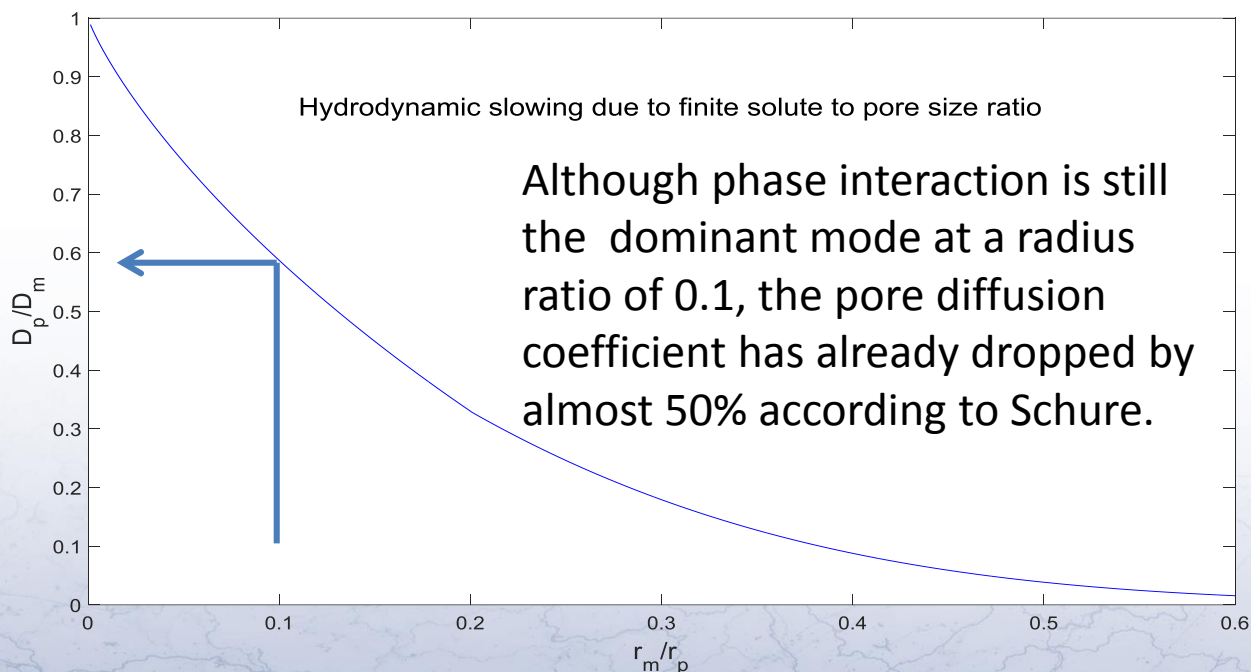
## Surface areas

2.7 um 90 A 135 m<sup>2</sup>/g  
2.7 um 160 A 90 m<sup>2</sup>/g

## Impact of Size Exclusion on Zone Broadening<sup>13</sup>

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.

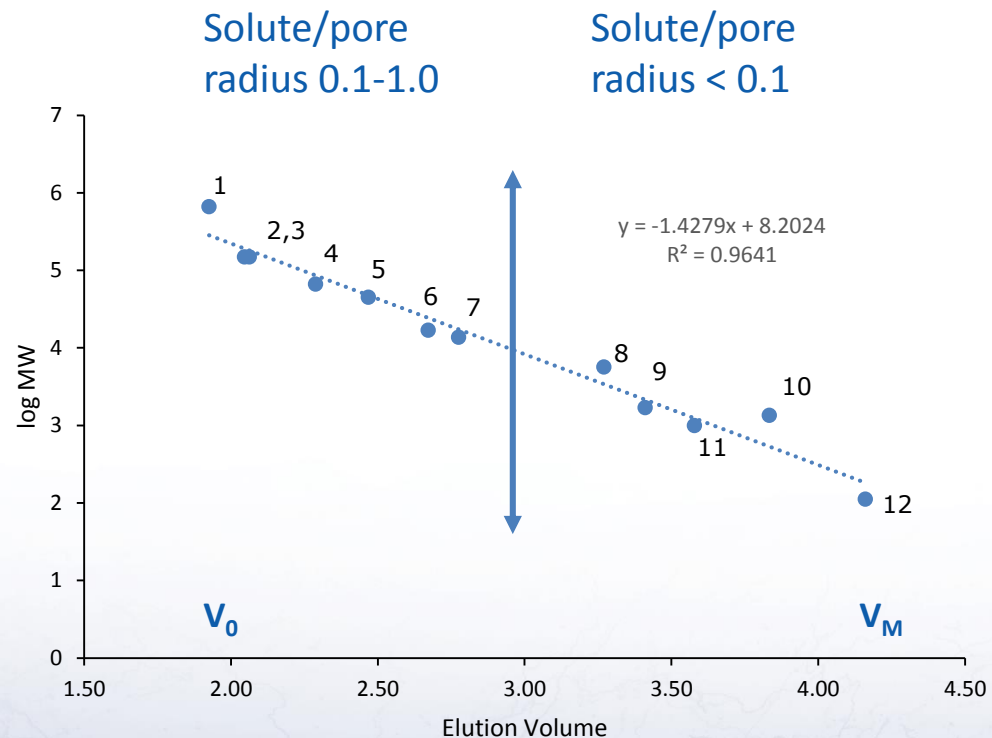


# Calibration Curve for Size Exclusion Chromatography\*

## Chromatographic Conditions

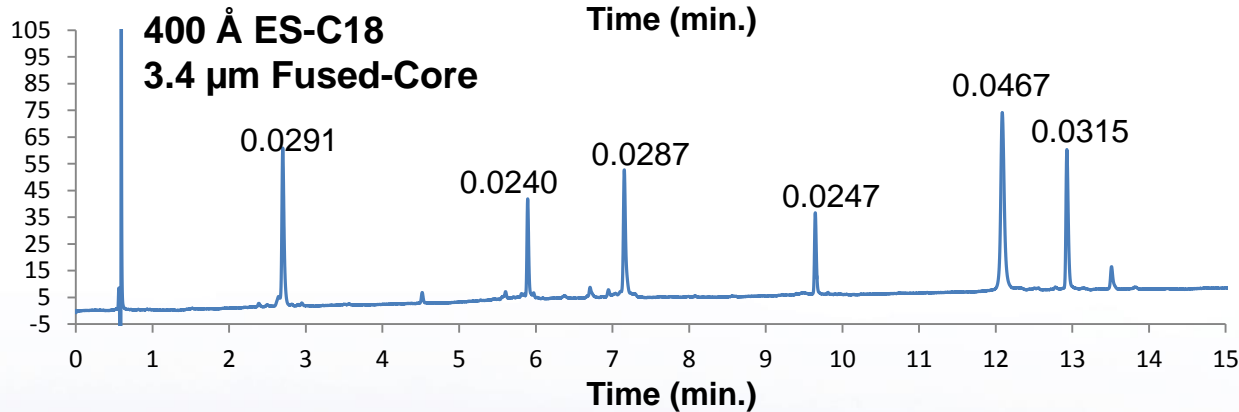
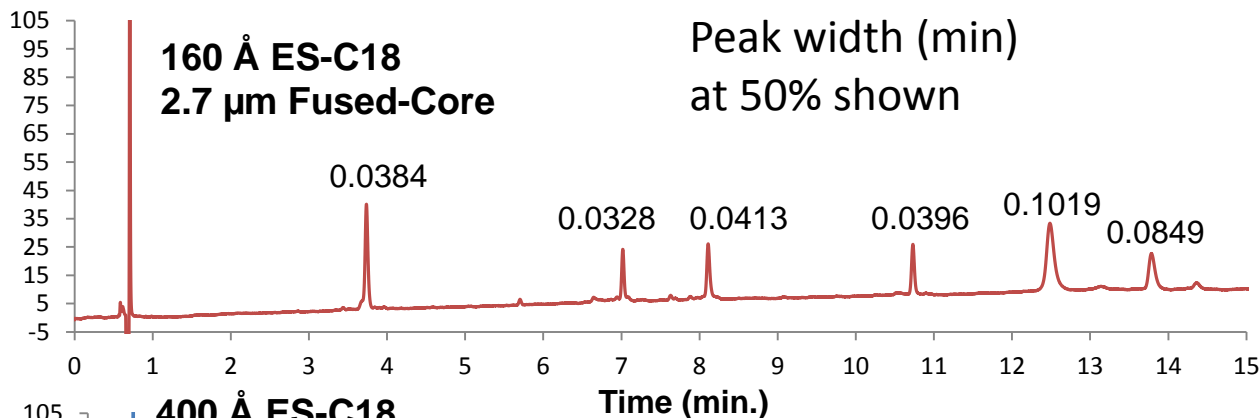
columns: Sepax Zenix SEC-150, 30 cm x 4.6 mm I.D., 3  $\mu$ m  
 mobile phase: 0.2 M potassium phosphate, pH 7.0  
 flow rate: 0.25 mL/min  
 pressure: 66 bar  
 column temp.: 25  $^{\circ}$ C  
 detector: UV 215, 280 nm  
 injection: 0.5  $\mu$ L  
 samples: listed below

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



\* Unpublished data by AMT Applications Lab

# 160 Å Pore-Size Column- Too Small for Large Proteins<sup>15</sup>



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

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

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

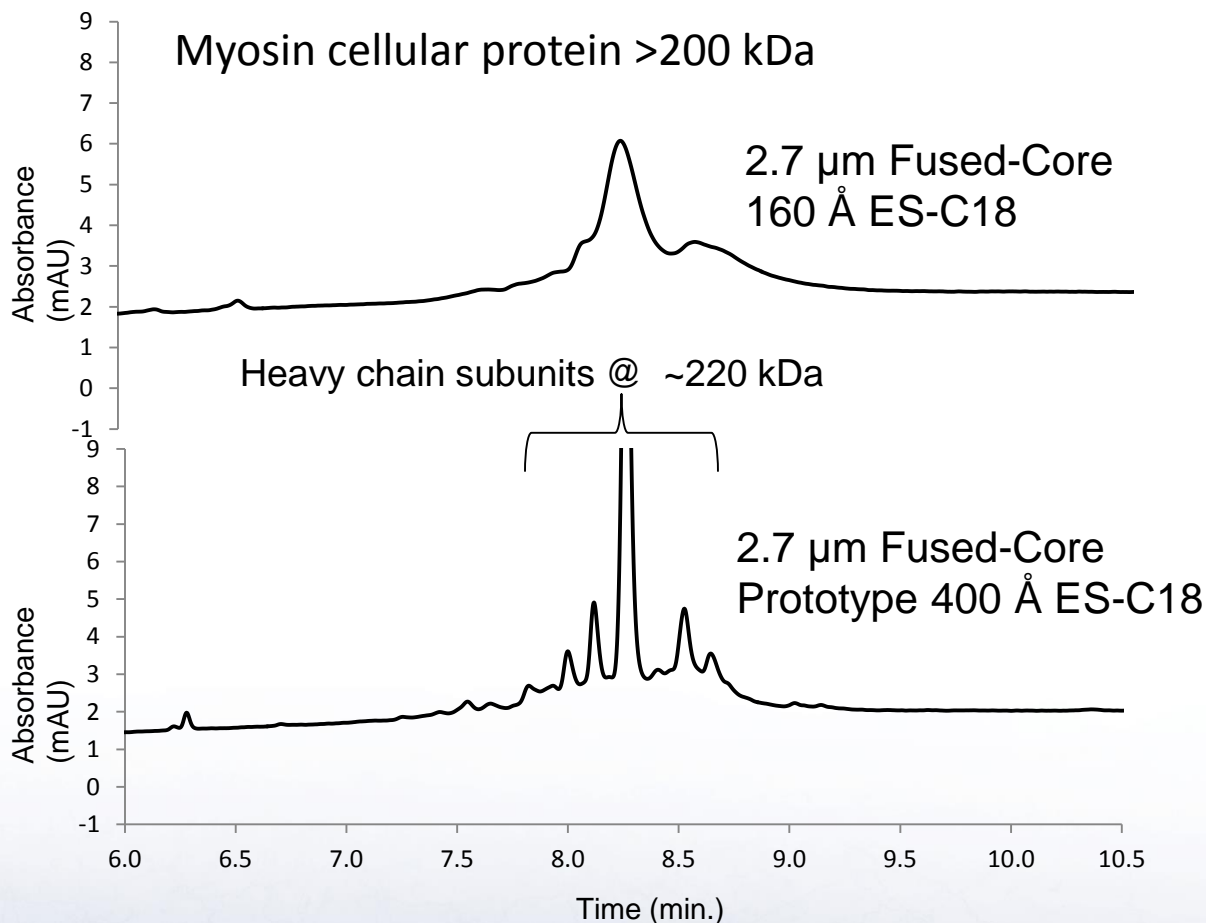
**Peak widths in minutes above each peak.**

## Surface areas

2.7 um 160 A 90 m<sup>2</sup>/g

3.4 um 400 A 15 m<sup>2</sup>/g

# Large Protein Comparison on 160 Å and 400 Å Columns<sup>14</sup>



- 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.**

## Surface areas

2.7  $\mu\text{m}$  160 Å 90 m<sup>2</sup>/g

2.7  $\mu\text{m}$  400 Å 29 m<sup>2</sup>/g

Conditions: columns= 2.1  $\times$  100 mm; temperature= 80 °C; 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  $\mu\text{L}$ ; sample = myosin, 400 kDa initial, but denatured in mobile phase.



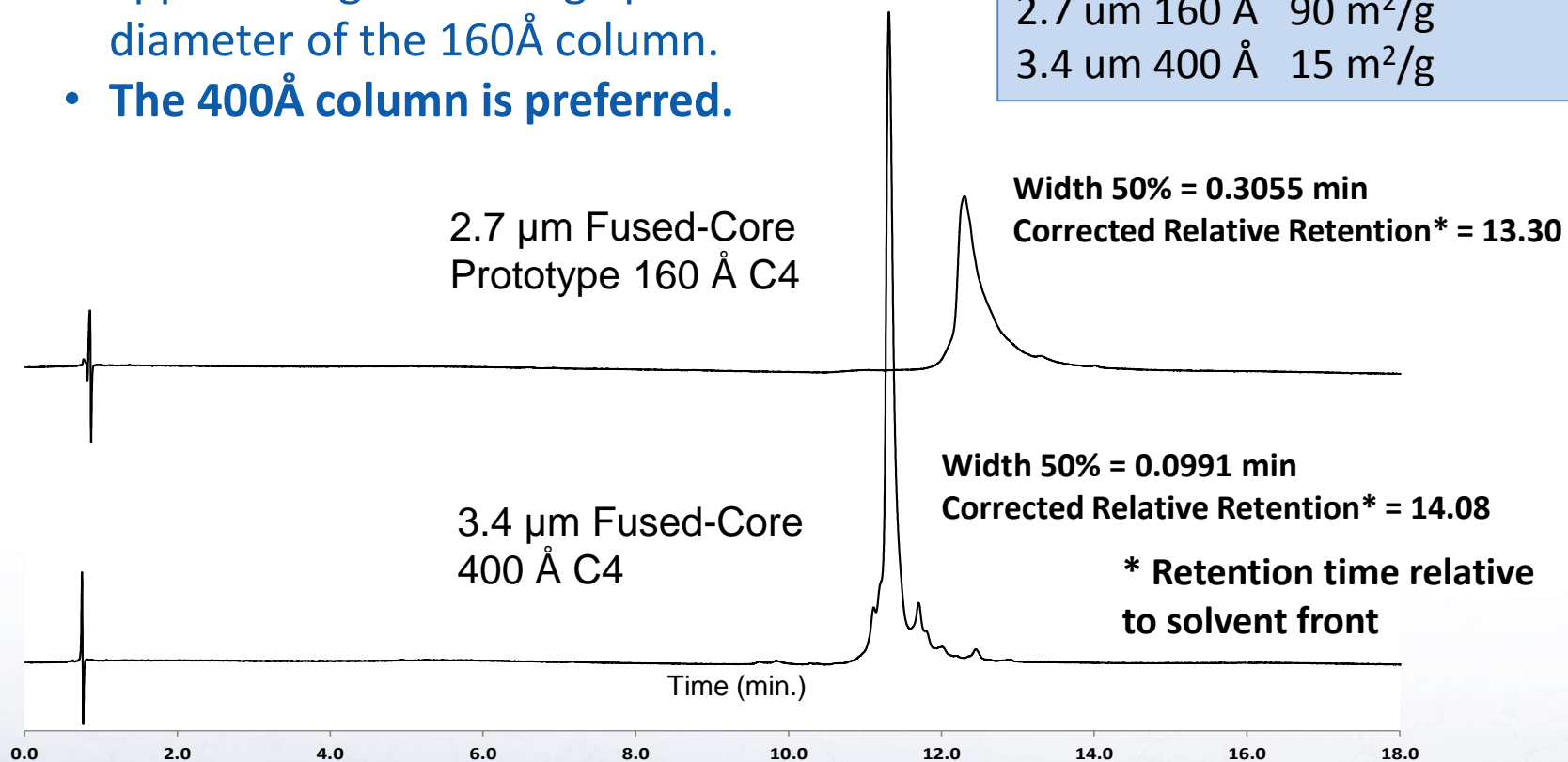
# SILU Lite SigmaMAb Comparison on 160Å and 400Å<sup>15</sup>

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

## Surface areas

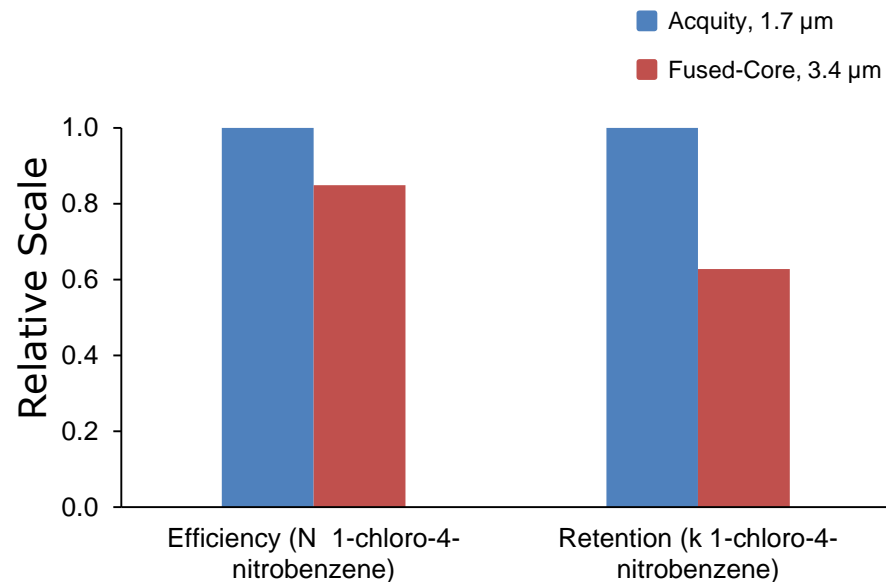
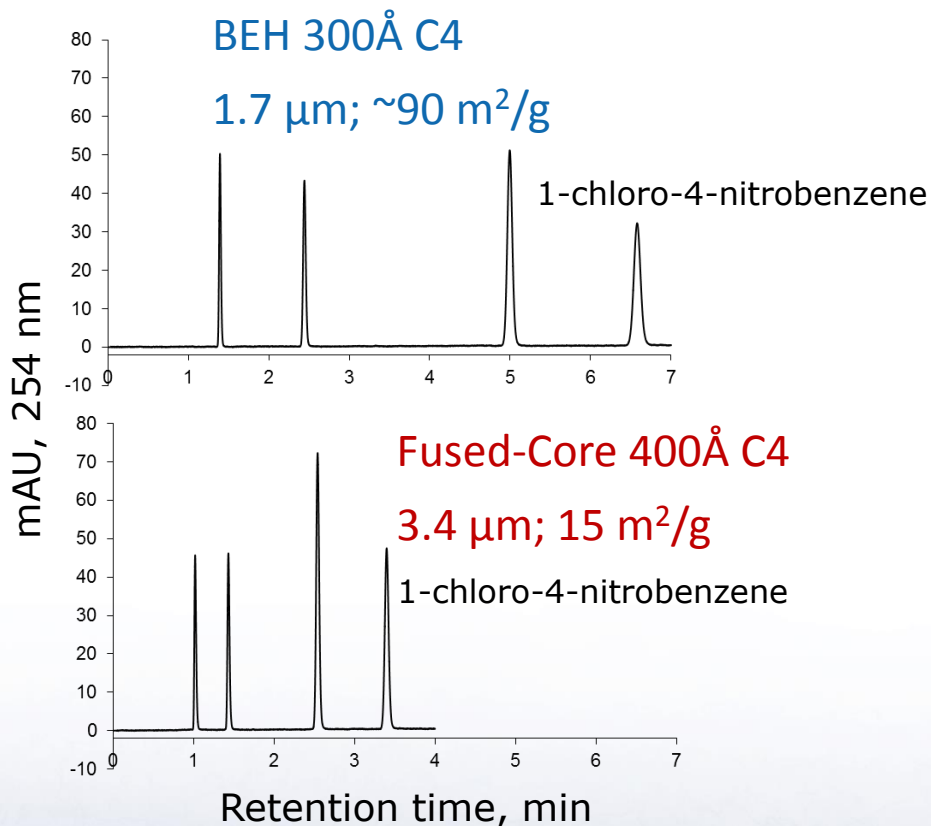
2.7  $\mu\text{m}$  160 Å 90  $\text{m}^2/\text{g}$

3.4  $\mu\text{m}$  400 Å 15  $\text{m}^2/\text{g}$



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  $\mu\text{L}$ ; detection: 280 nm

# Wide-Pore Column Comparisons for Small Molecules<sup>15</sup>



- **Either column would work for smaller 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)**

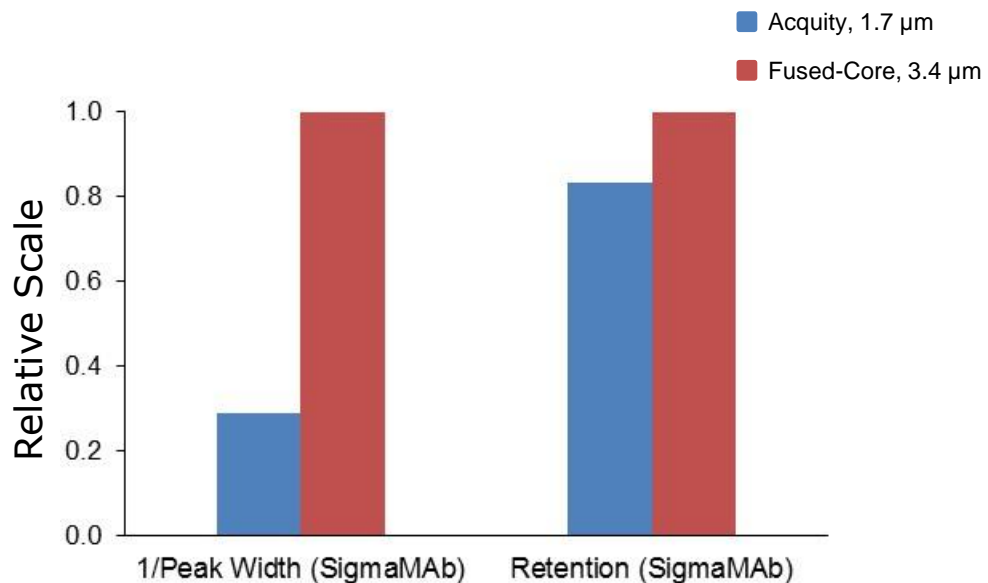
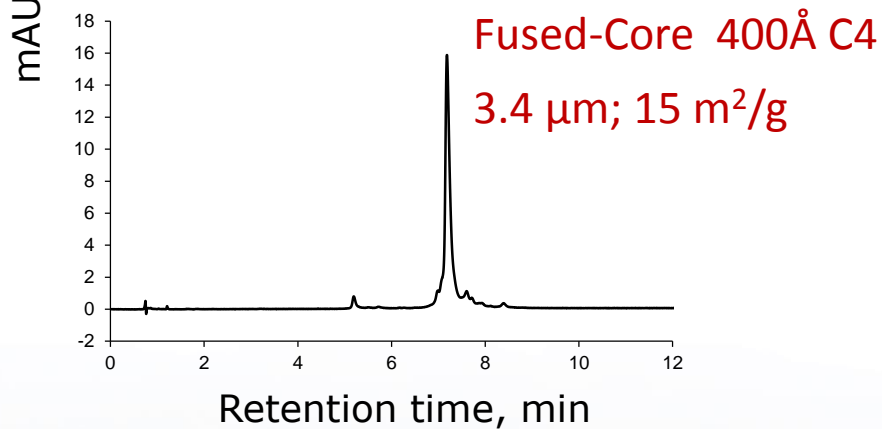
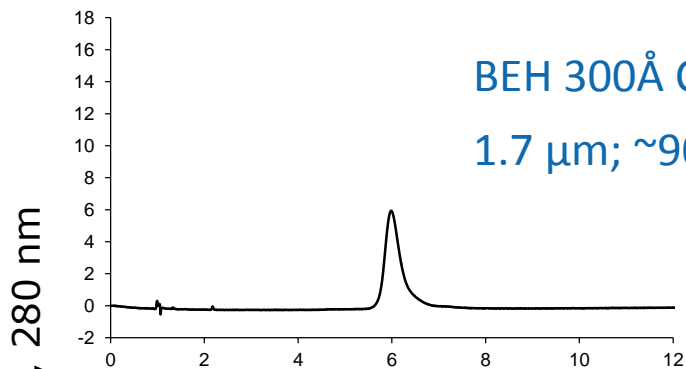
column temp.: 25 °C

detector: 254 nm

injection: 0.2 μL

sample: uracil, phenol, propiophenone, 1-chloro-4-nitrobenzene

# Column Comparisons for Monoclonal Antibody<sup>15</sup>



## 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

- 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.

## Recognizing a Column Pore-Exclusion Problem

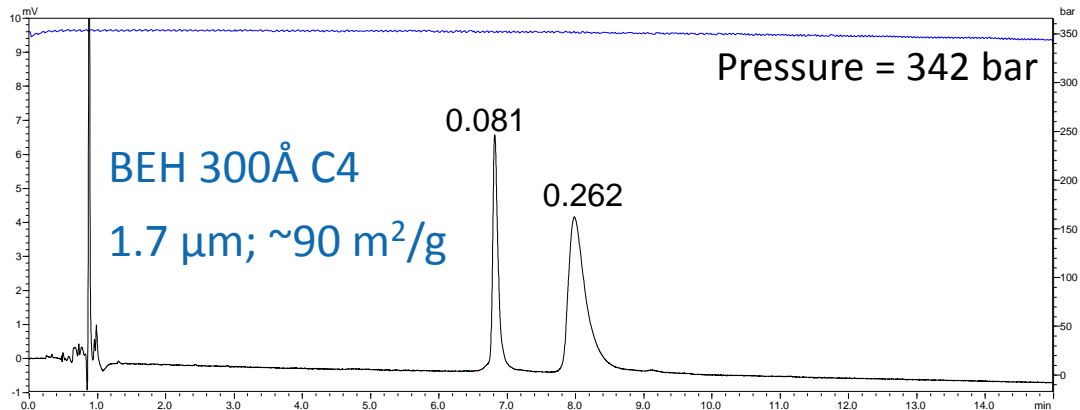
A pore exclusion problem might exist:

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<sup>15</sup>



Peak Identities:

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

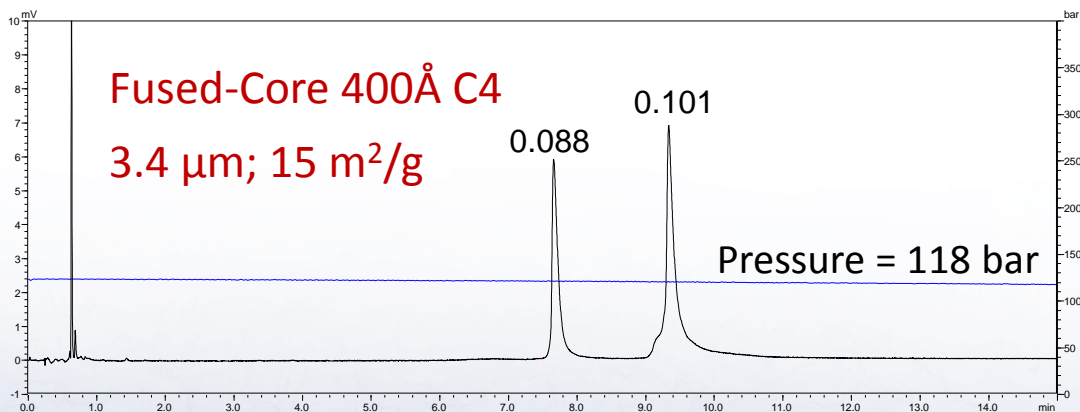
Reduced SigmaMAb

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<sup>16</sup>.

## 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.

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15. Unpublished data by AMT Applications Lab (2016).
16. Private communication from Joe Foley.



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