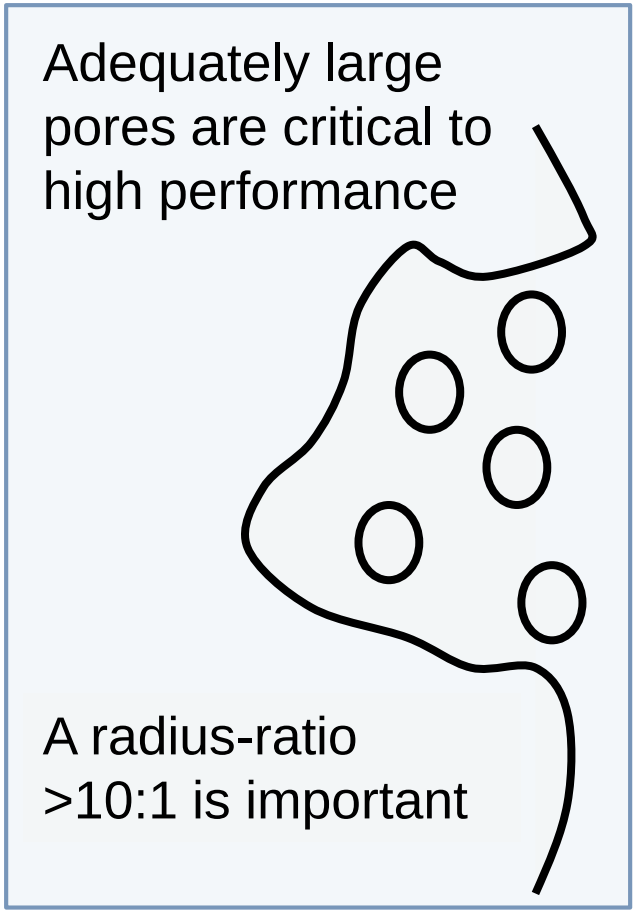
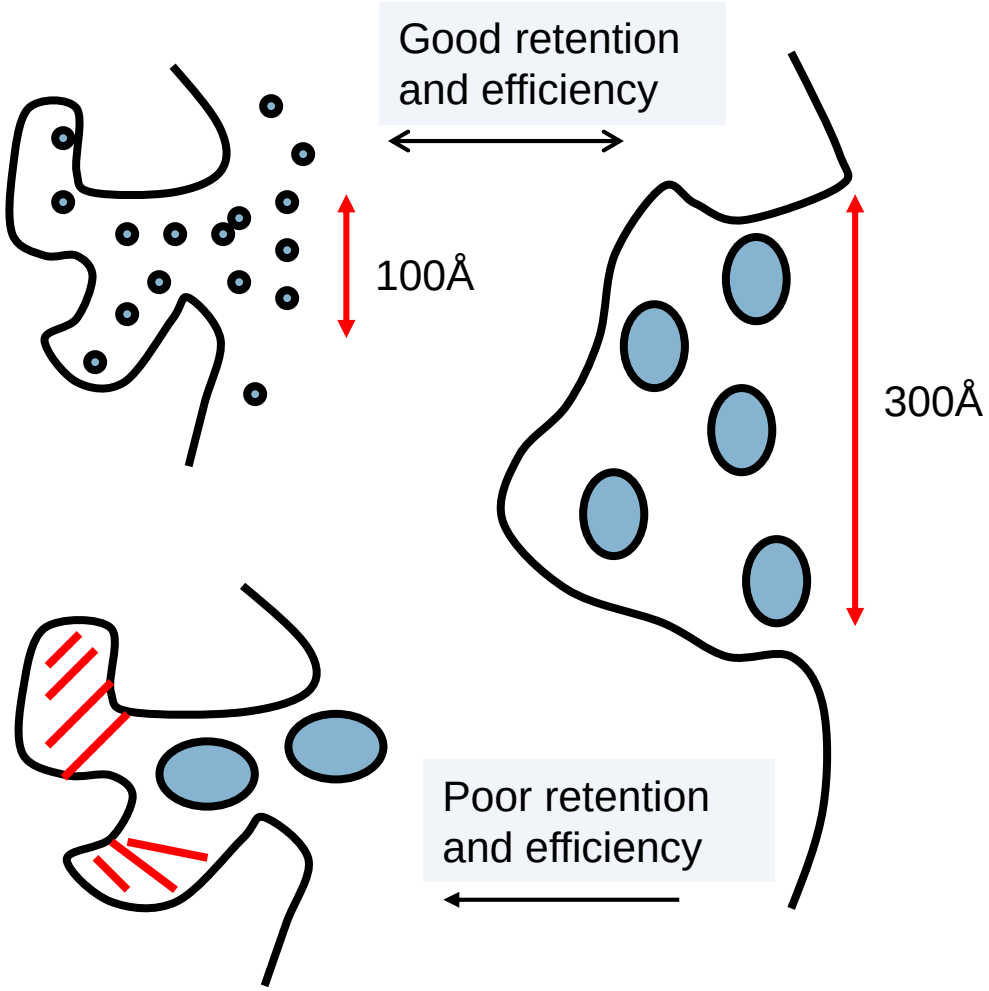
Improve HPLC Separations by Carefully Matching Column Pore-Size to Solute-Size

Richard A. Henry, Consultant
983 Greenbriar Drive
State College, PA 16801
HyperLC@comcast.net

Stephanie A. Schuster, Sr. Chemist
Advanced Materials Technology, Inc.
Wilmington, DE 19810
sschuster@advanced-materials-tech.com



Surface Area and Pore Structure Control Retention



- Although the greater surface area of small-pore silica can provide more retention and resolution for small molecules, larger column pores are needed to match larger molecules.
- Full surface area of a 100Å silica is not accessible to molecules such as BSA which is only ca. 1/2 of the average pore diameter.
- For BSA, a better choice would be ca. 400-500Å where the analyte/pore radius ratio is almost 1/10.

Angiotensin II: MW 1.0 kDa, Stokes radius < 10Å

BSA: MW 66 kDa, Stokes radius 35.5Å (71Å diameter)

Column Selection and Method Development for HPLC of Large Molecules

- High speed separations of large molecules with any particle or HPLC mode depends strongly upon selecting a particle pore-diameter that is “adequate” for the largest analytes in the sample.
- Giddings introduced the idea of pore-confinement, where solutes lose freedom to diffuse and interact with the surface. Both equilibrium constants (entropy) and kinetics can be impacted (1,2).
- Unger cited evidence that pore-openings should be 10 times larger than analyte diameter in order to achieve unrestricted diffusion and high performance defined as speed and resolution (3-5).
- Kirkland, et al. recently described a 1000Å solid-core particle that shows the best results yet for large protein and other analytes (6).
- Matching pores to analytes may be the first and most important step in large molecule method development as convenient experiments become readily available.

Pore Diameter- A New HPLC Resolution Variable

Column variables (controlled by suppliers):

- Substrate (usually silica)
- Bonded phase (substrate *should* have minor impact)
- Pore-size (adequately large pores needed to maximize retention, efficiency and selectivity for large molecules)

Mobile phase variables (controlled by users):

- Solvent type: ACN, MeOH and Water for RPLC or HILIC
- Solvent strength (% organic)
- pH (controls ionic state of substrate and phase)
- Additive type and concentration
- Temperature

* Selectivity variables originally proposed by John Dolan, LC Resources, MCF 2009.

** Suppliers must be willing and able to provide full details on column variables.

Fundamentals of HPLC Retention & Separation

Retention by the stationary phase:

$$V_{R1} = V_M + K_{Sorp}\, V_S = V_0 + V_P + K_{Sorp}\, V_S \qquad (1)$$

Separation by size exclusion:

$$V_{R2} = V_0 + K_{Size}\, V_P \qquad (2)$$

K_{Sorp} can vary from 0 to infinity, and K_{Size} can vary from 0 for a large molecule to 1 for a small molecule.

These equations are usually employed separately when either the retention mode or the exclusion mode dominates.

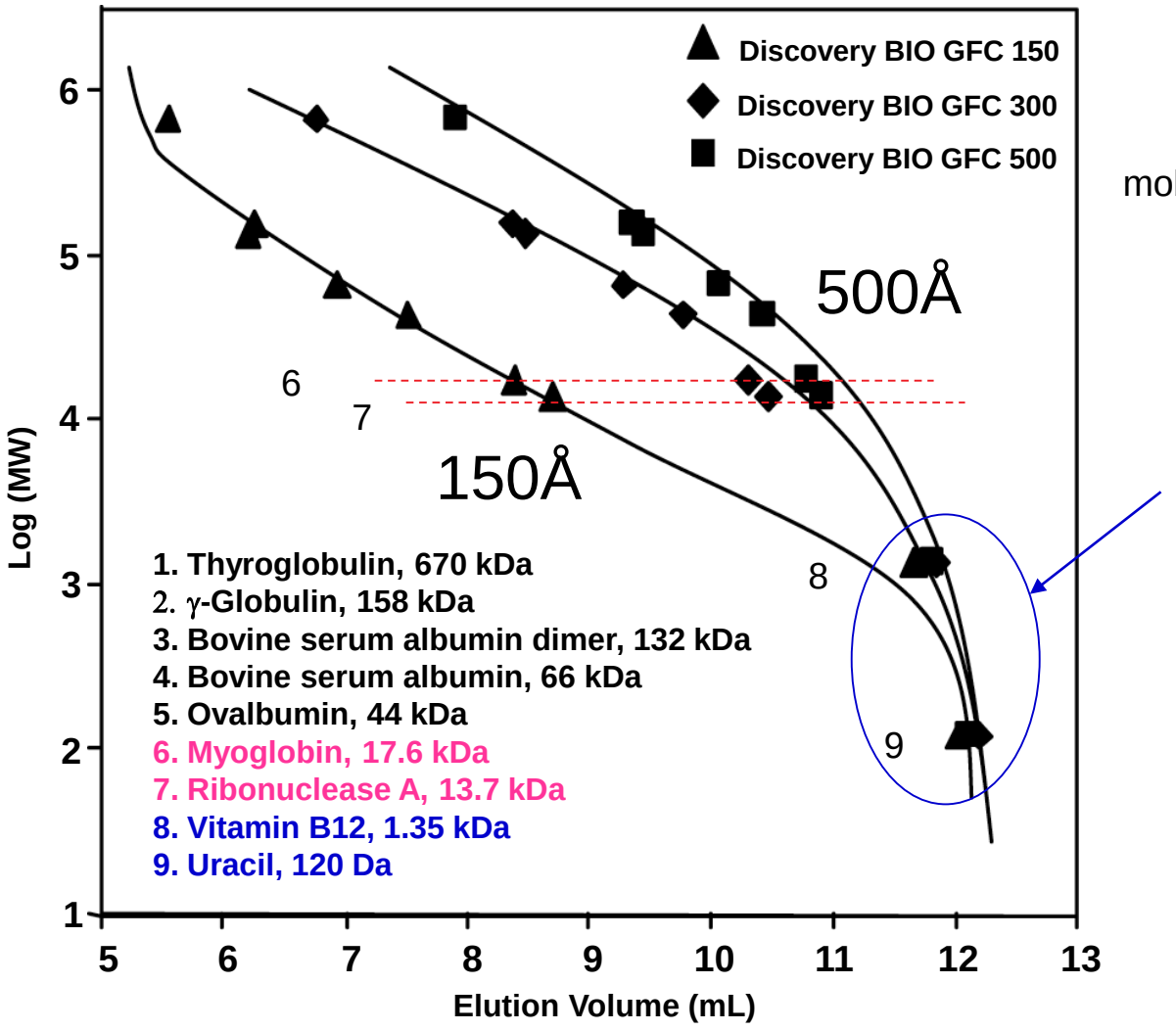
Combining Equations When Modes Overlap

When both large and small molecules are present they cannot all access pores equally, therefore both equations must be considered to interpret retention.

$$V_R = V_0 + K_{Size} (V_P + K_{Sorp} V_S) \qquad (3)$$

- Equation (3) more completely describes retention and separation and simply applies the pore-size constant to everything that goes on within the pores, where most of the stationary phase interaction occurs.
- For the simple case of small molecules ($K_{Size} = 1$), retention equation (3) reduces to equation (1). Maximum retention is possible for small molecules where $K_{Size} = 1$ and $K_{Sorp} > 0$.
- Lightest retention at the excluded volume is observed for large molecules (larger than particle pores) where $K_{Size} = 0$.
- Separating and analyzing large and small molecules together in a single column may be the most challenging HPLC experiment.

Learning About Size From SEC Calibration Curves

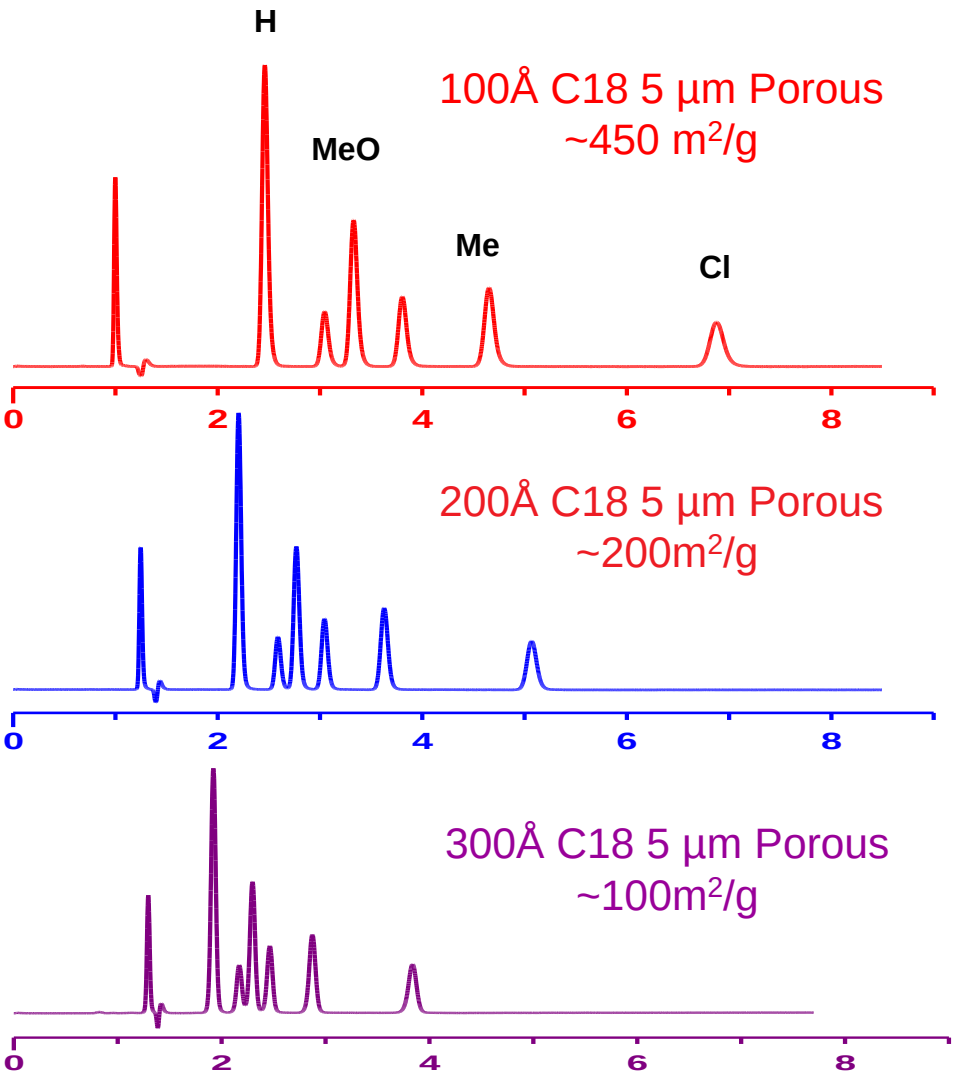


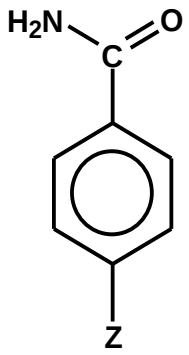
column:	Aqueous SEC columns, 30 cm x 7.8 mm I.D., 5 µm particles
mobile phase:	150 mM phosphate buffer, pH 7.0
flow rate:	1.0 mL/min
det.:	UV at 214 nm
inj.:	10 µL

- Note that molecules 8, 9 with MW ≤1,350 can move freely within particle pores.
- Molecules 6, 7 with MW ≥10,000 are excluded from about half of the pore volume of a 150Å column.
- Molecules 1-5 require wide-pore columns for retention modes (RP, etc.)

* Data provided by Supelco

Small Molecule Retention Behavior



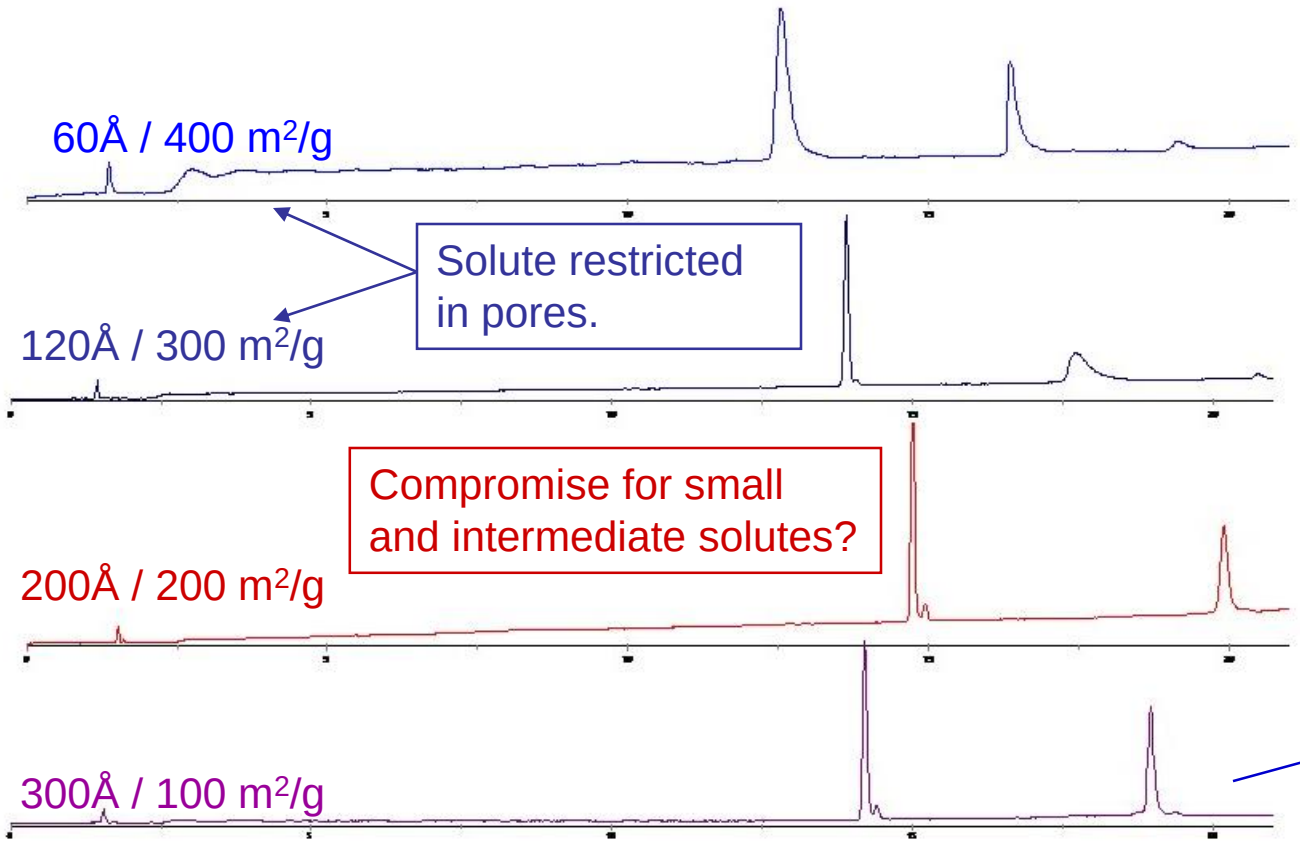


Retention of Low MW Solutes Varies Inversely with Pore Size

Pore Diameter	Surface Area
60	500
100	450
120	300
180	200
200	200
300	100
500	50
1000	25

* Data provided by Supelco

Large Molecule Retention Behavior



Available surface area impacts retention and performance of larger molecules :

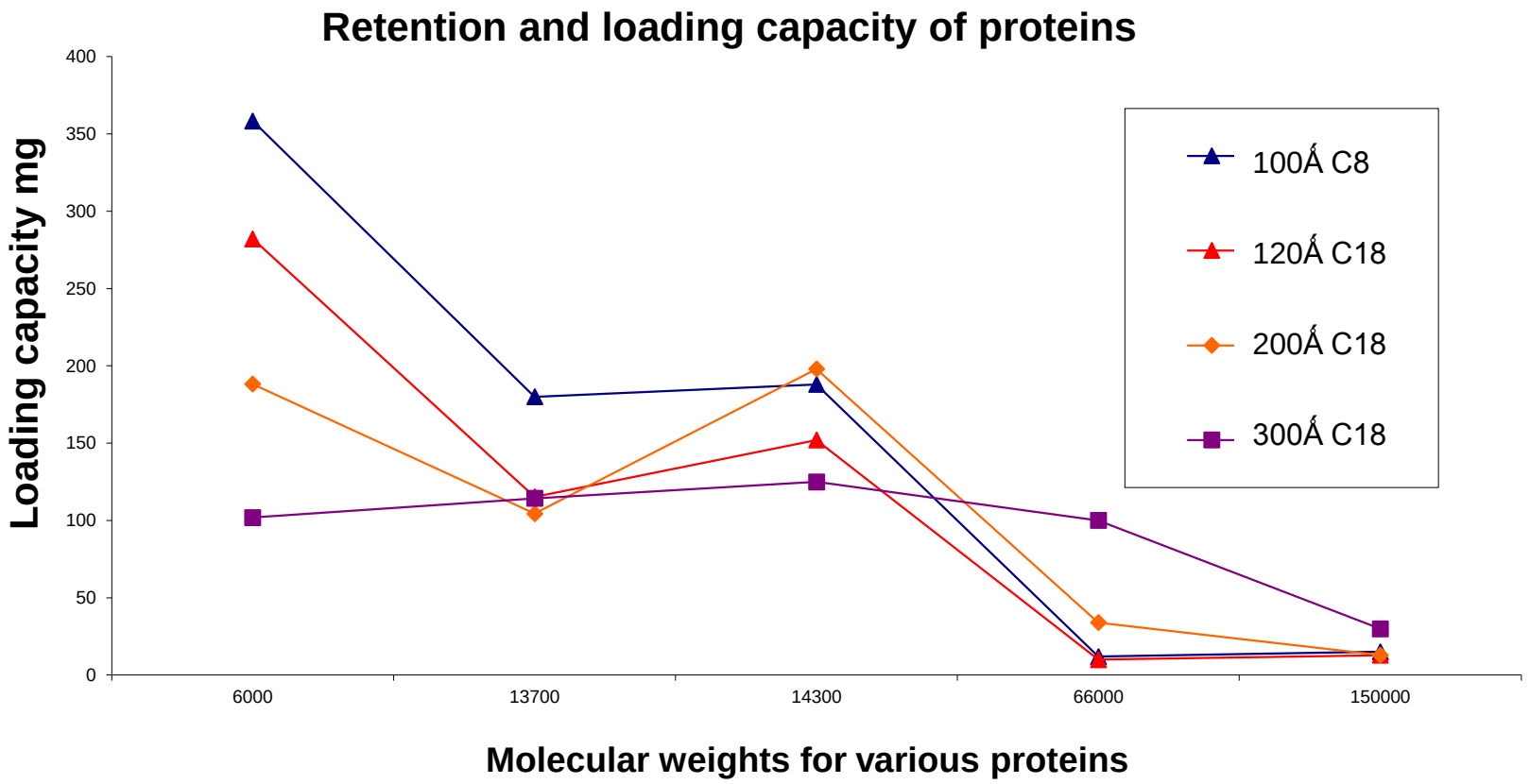
- Retention
- Efficiency
- Resolution
- Recovery

Porous C18 Columns; 150 x 4.6mm
Mobile Phase: A: 10% ACN / 90% HOH / 0.1% TFA
B: 70% ACN / 30% HOH / 0.09% TFA
Flow: 1.5mL/min; Detection: 220nm;
Protein Concentration: 1μg/μL each; Injection: 10μL

* Data provided by Supelco

Retention Decreases as Large Molecule MW Increases

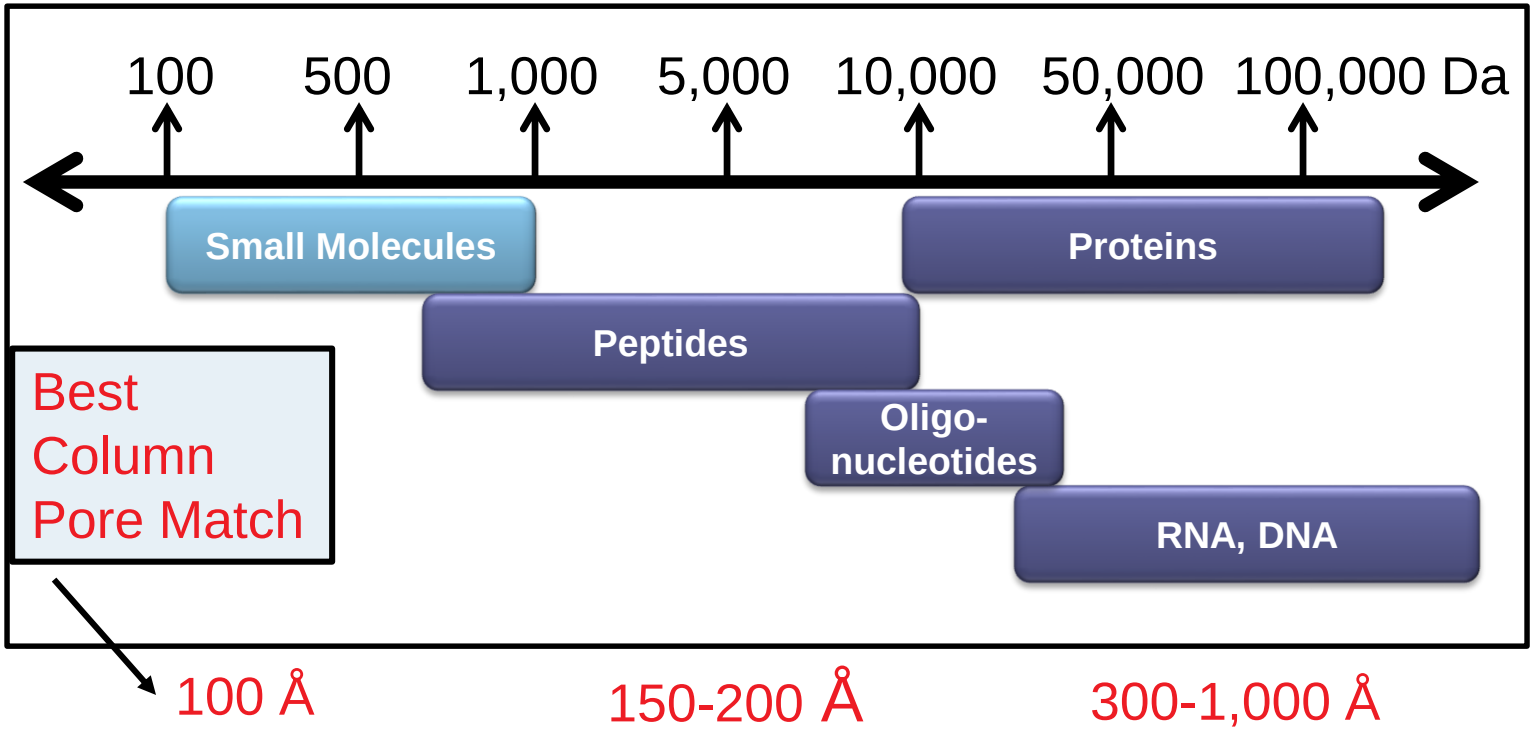
Note that 300Å C18 shows better overall retention for small and large molecules when they are present in the same sample. Wide-pore columns should be evaluated for this application.



* Data provided by Supelco

Biomolecules Often Exceed Particle Pore Diameters

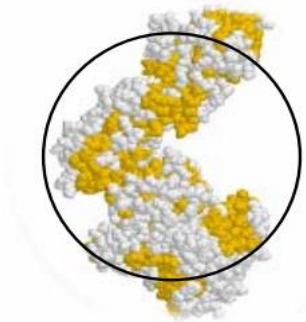
- Biomolecules are important to living organisms. They can range in size from amino acids or small lipids to large proteins such as antibodies or polynucleotides such as DNA or RNA.
- New HPLC/UHPLC product designs must rely on knowledge of how pore size and shape affects separation of large and small molecules



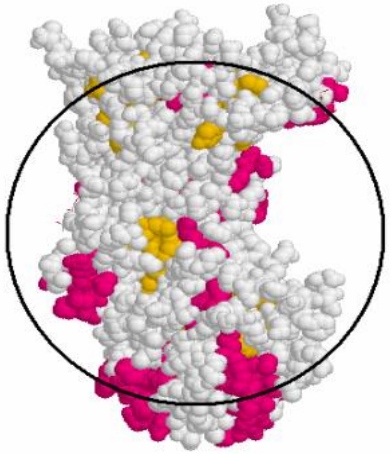
Size Comparison of Biomolecules to Pore Openings

Lysozyme
M_W=14.5 kDa
R_H=1.9 nm

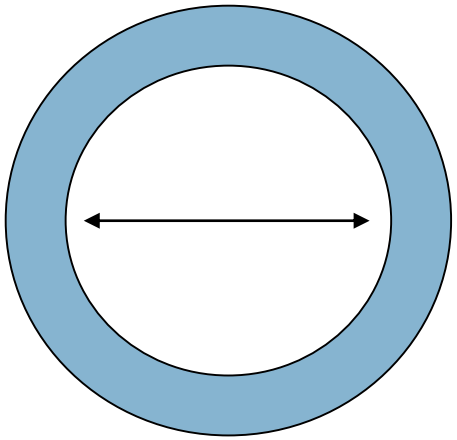
Insulin - pH 7
M_W=34.2 kDa
R_H=2.7 nm



Immunoglobulin G
M_W=160 kDa
R_H=7.1 nm



Thyroglobulin
M_W=650 kDa
R_H=10.1 nm



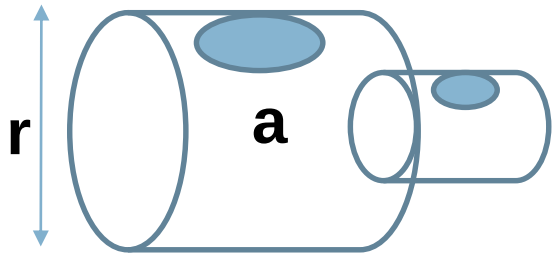
100 Å (10 nm) pore diameter

approximate values

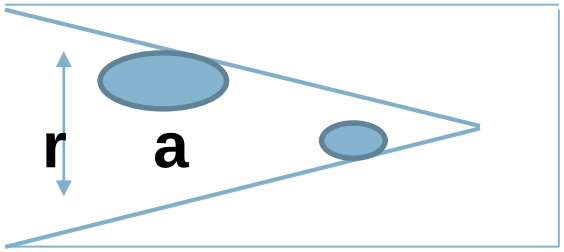
10 nm = 100 Å

While small pore particles may be adequate for drugs and small peptides, larger 300-1000 Å pores are needed for proteins and large biomolecules.

Effect of Radius Ratio (a/r) on SEC Retention



Cylindrical pore model

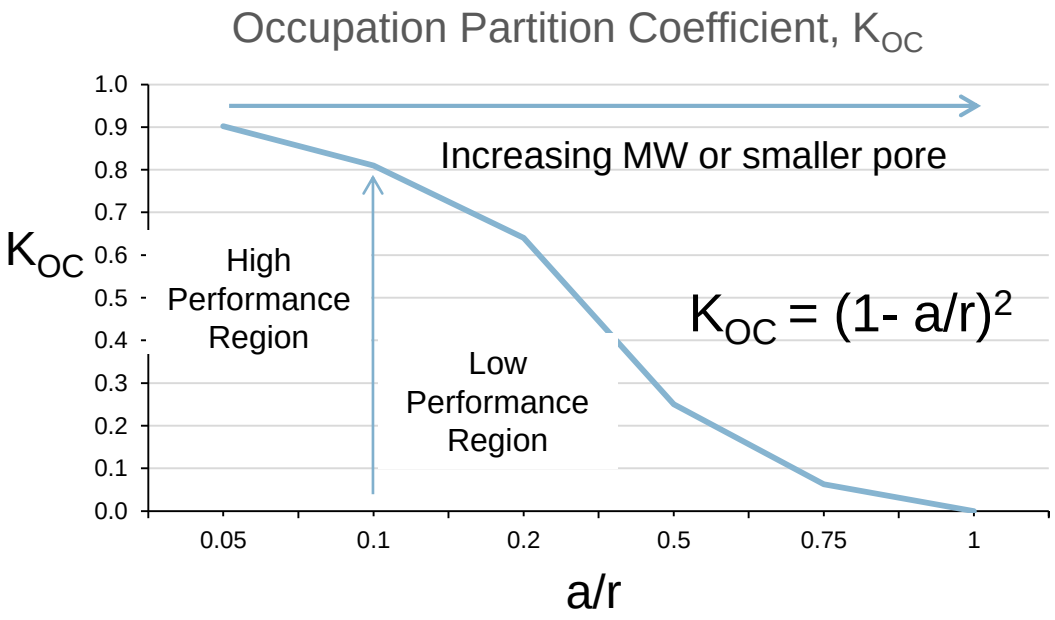


Conical pore model

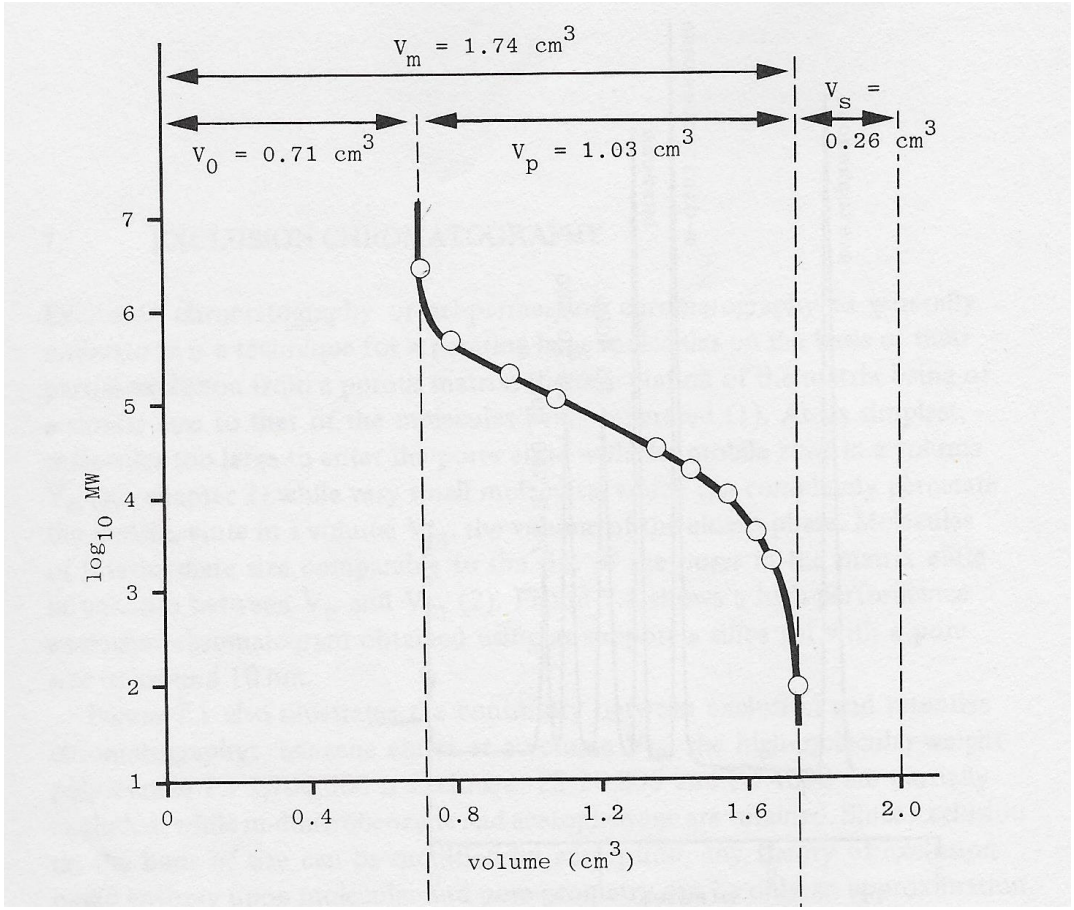
Pore radius (**r**) varies within a range for porous particles; an average is usually reported.

Analyte radius (**a**) increases with MW; a specific ratio (**a/r)** exists for each sample component (7).

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



Theoretical Curve for Pure Size Exclusion HPLC



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

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

K_{Size} varies from zero for total pore exclusion to one for total pore occupation. Separation by a pure SEC mechanism correlates with solute hydrodynamic radius and pore geometry (8).

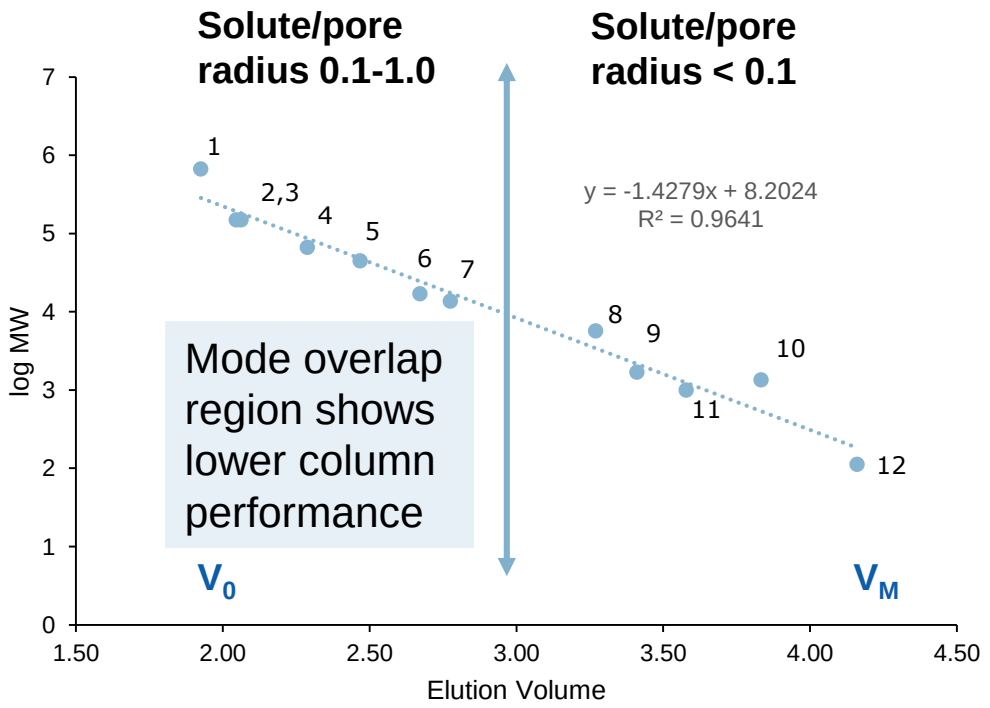
The subject of this study is to identify the point on the curve where solute size begins to significantly impact column performance.

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

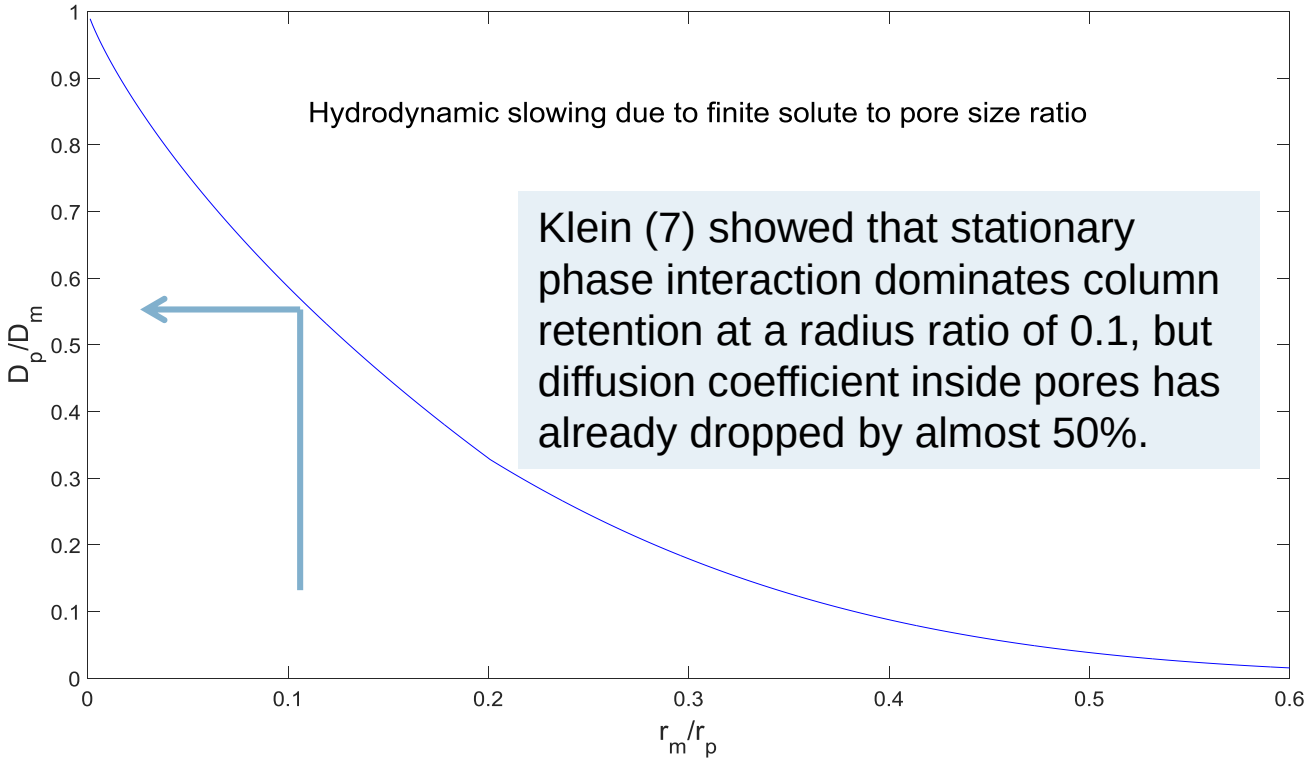
	Analyte	MW (Da)	Size (Å)	K_{size}
1	thyroglobulin	667000	200	0.00
2	SigmaMab	150000		0.06
3	IgG	150000	100	0.05
4	BSA	66400		0.16
5	ovalbumin	45000		0.24
6	myoglobin	17000	40	0.33
7	ribonuclease A	13700		0.38
8	bovine insulin	5700		0.60
9	neurotensin	1700		0.66
10	vitamin B12	1350	20	0.85
11	angiotensin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



Data provided by AMT

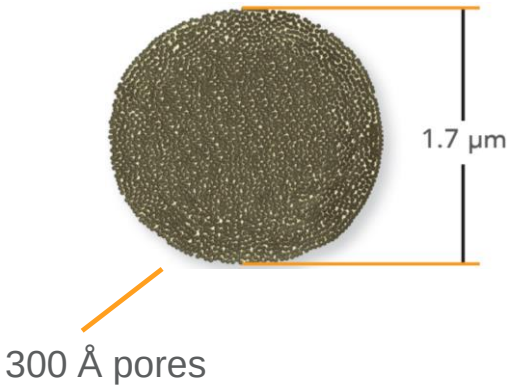
Impact of Size Exclusion on Band Broadening

Sources of solute zone spreading in packed-bed liquid chromatography have been widely studied. Schure (6) recently described another type of band broadening by estimating lower diffusion rates inside crowded pores (D_p) relative to free diffusion in mobile phase outside pores (D_m) and plotting it against radius ratio (molecule/average pore).

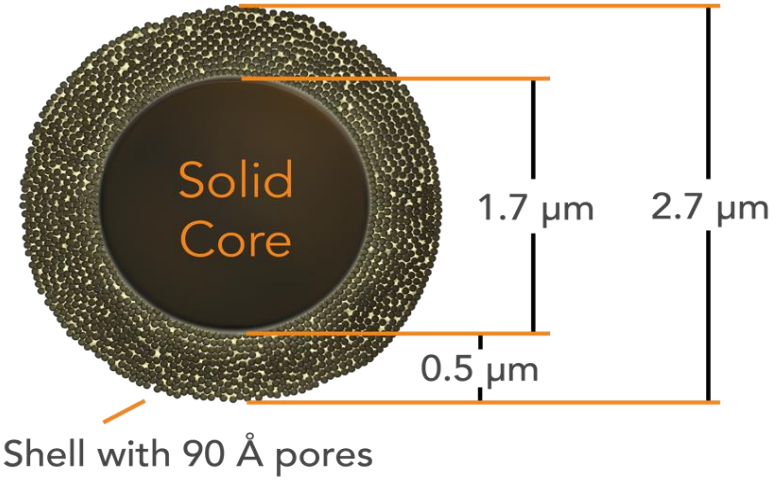


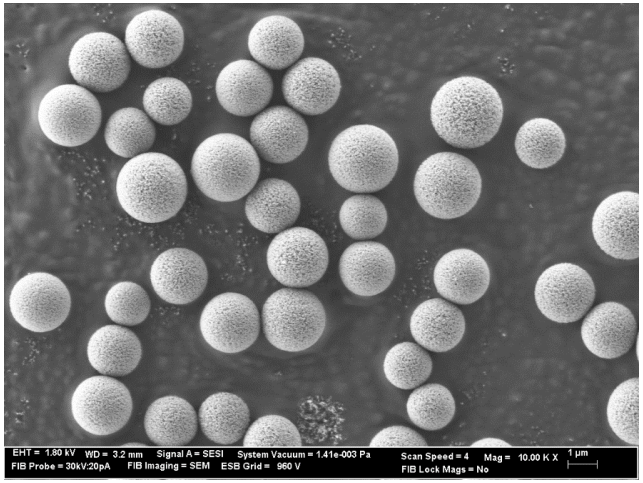
Two Important Porous Particle Designs

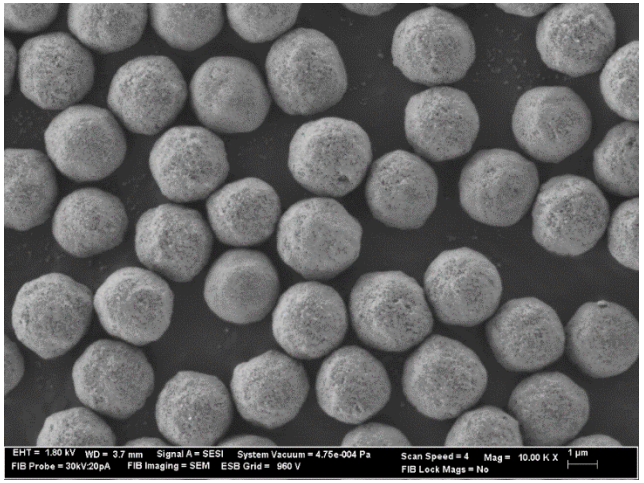
Totally Porous Particle (TPP)



Superficially Porous Particle (SPP)

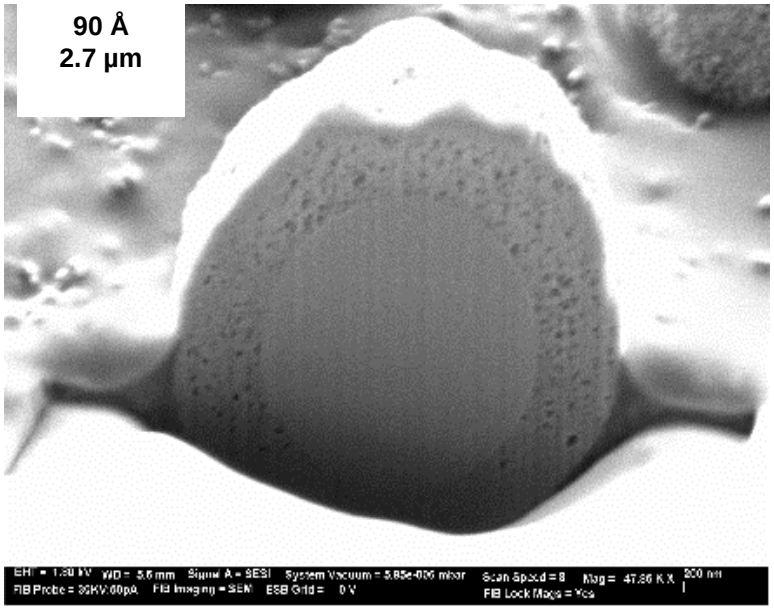


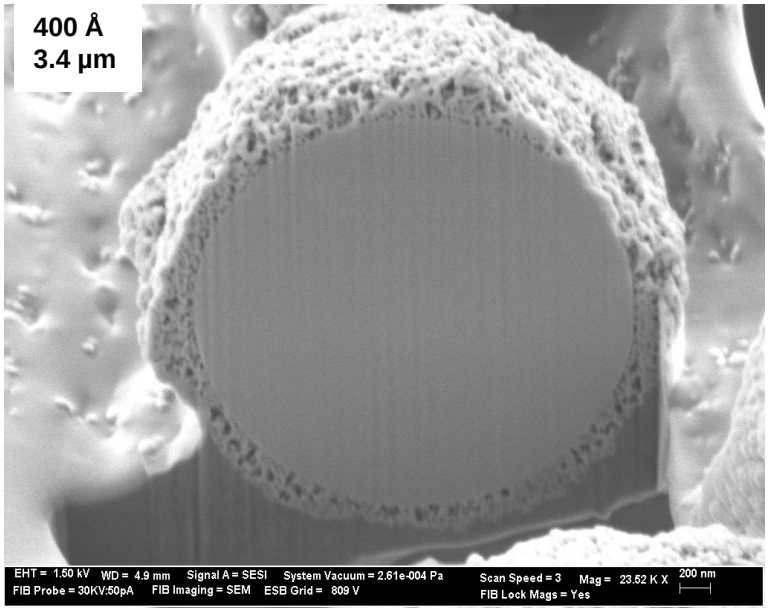




* Data provided by AMT

Examples of Halo Silica Surface-Layer Porosity

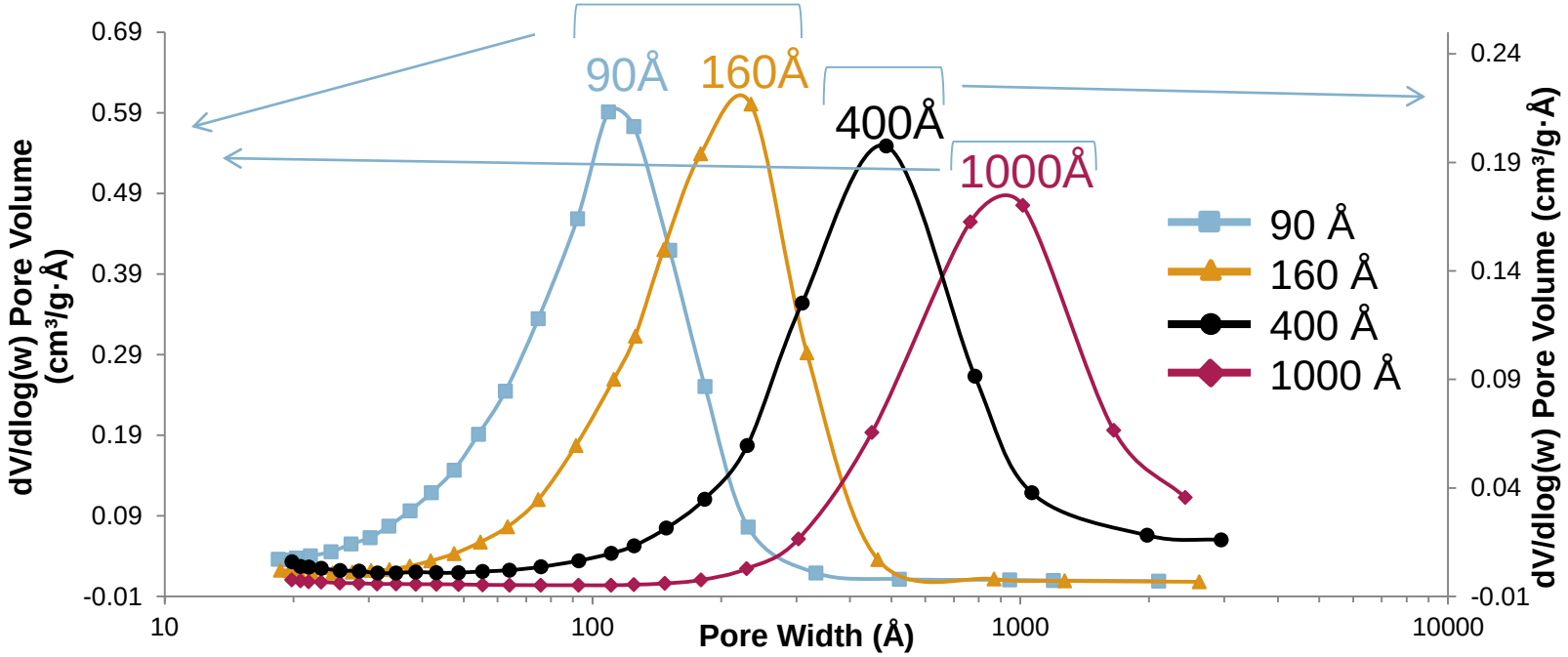




- Unique speed and efficiency of superficially porous particles arise from the ability to use larger particles with shorter diffusion paths at lower pressure.
- Large molecules need larger pores and diffuse 10-100 times slower than small ones, benefitting more from the short diffusion paths of SPP columns.
- SPP advantages for interactive modes (RP, HILIC, NP) increase for large molecules and may extend to SEC mode; pores up to 1000Å are, available.

* Data provided by AMT

Pore-Sizes of Halo Silica for Different Molecules



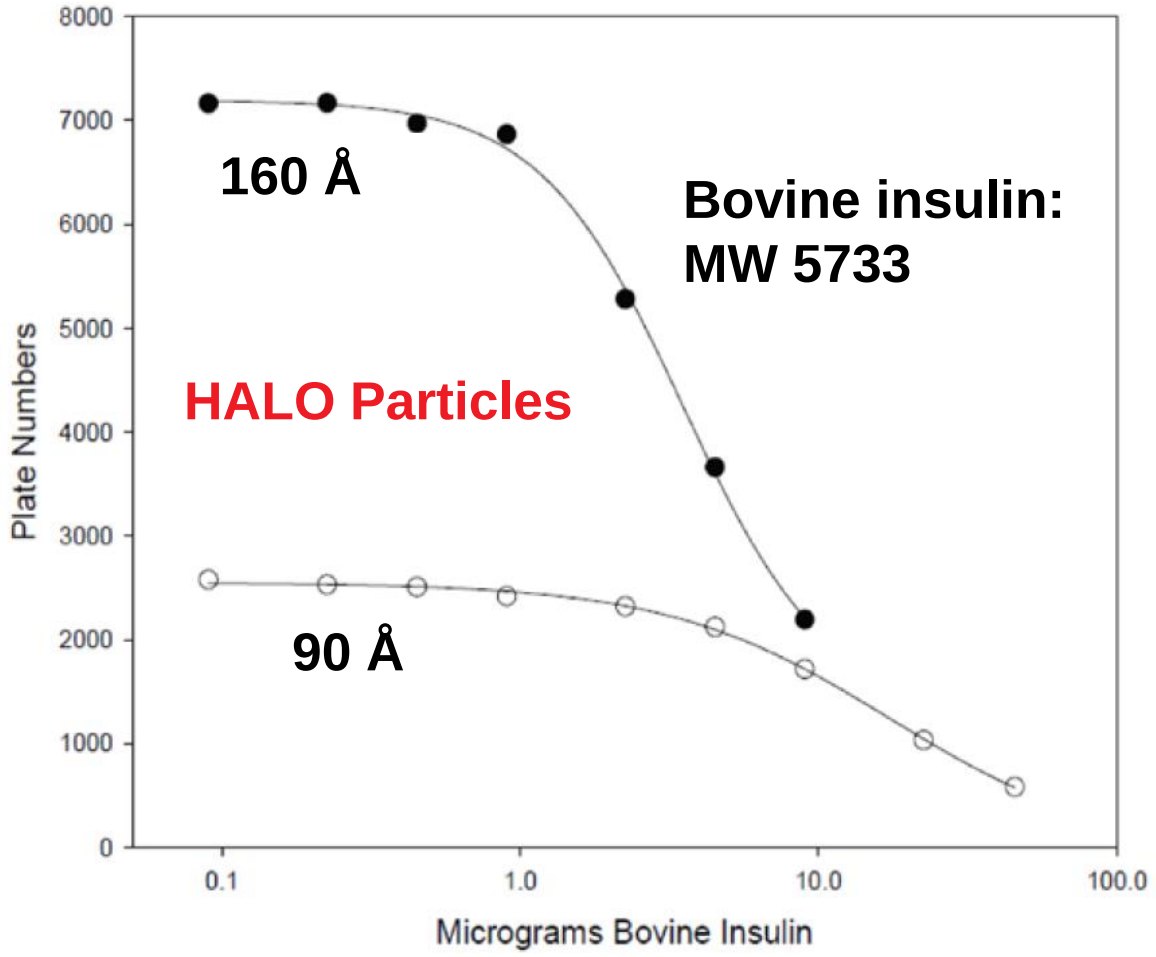
Small drugs
Small peptides
Large peptides
Small proteins
Large proteins

Molecule	Diameter
Small drugs	5-20 Å
Small peptides	20-30 Å
Large peptides	30-50 Å
Small proteins	50-100 Å
Large proteins	100-200 Å

* Data provided by AMT

Pore-Crowding Lowers Efficiency for High MW Solute

Column: 4.6 x 100 mm Halo Peptide ES-C18; T= 60 °C
Sample: Bovine insulin, MW= 5733; Inj vol = 10 µL
Mobile Phase: 31.5% ACN/68.5% Water/0.1% TFA, Flow Rate: 0.5 mL/min



High efficiency is the key to high peak capacity; only the larger pore particle will have high peak capacity for bovine insulin.

From a previous slide, we see that insulin has a diameter of about 50Å.

* Data provided by AMT

Halo 90 Å: Pores Too Small for Large Peptides

Columns: 100 mm x 4.6 mm HALO C18 (90 Å pores) and 100 mm x 4.6 mm HALO ES-C18 (160 Å pores); mobile phase: A: water/0.1% trifluoroacetic acid; B: acetonitrile/0.1% trifluoroacetic acid; 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.

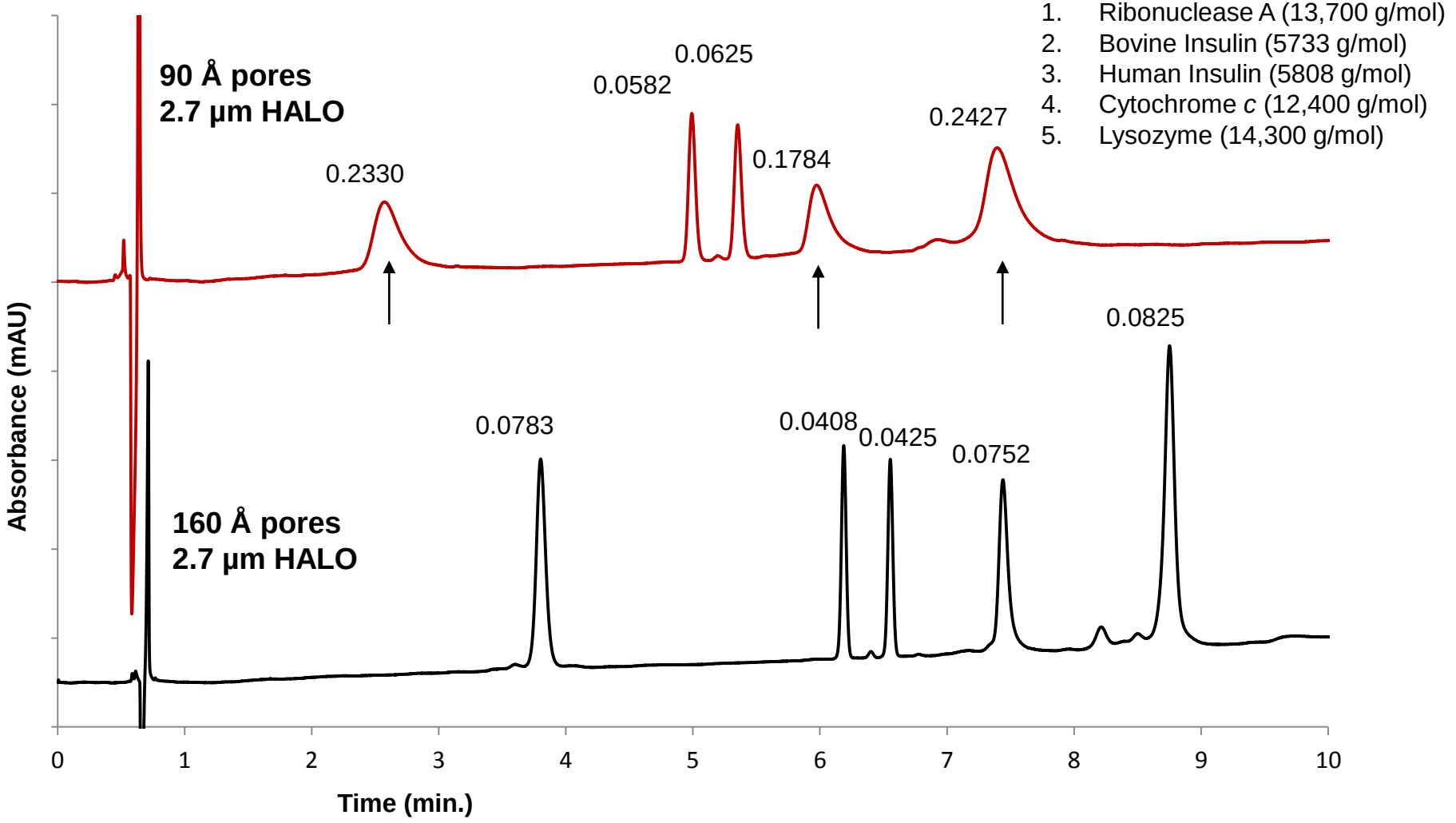


Fig.3 from J. Pharm. Anal. 2013;3(5):303–312

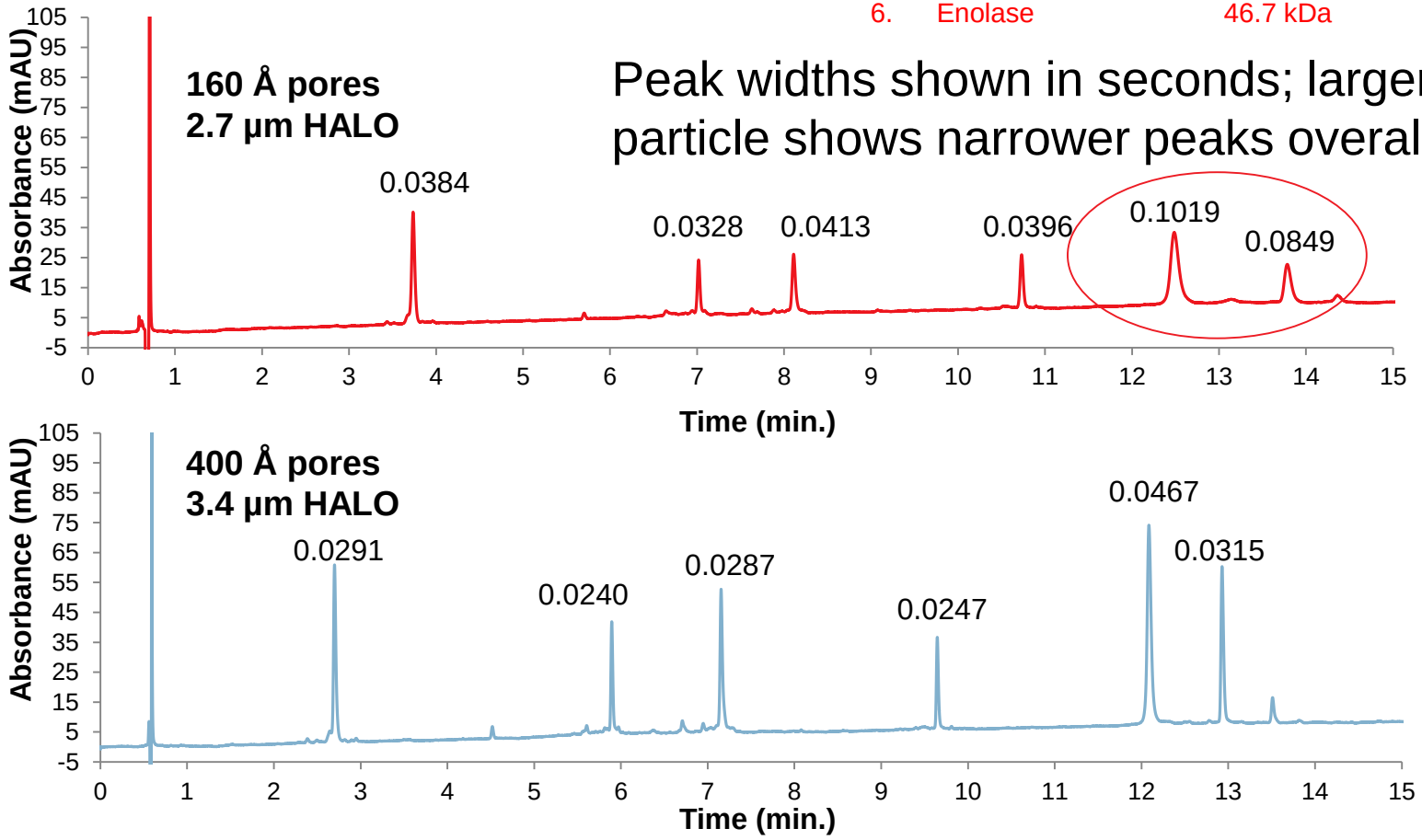
HALO 160 Å: Pores Too Small for Large Proteins

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 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	tetramer of ~ 60 kDa each
6.	Enolase	46.7 kDa

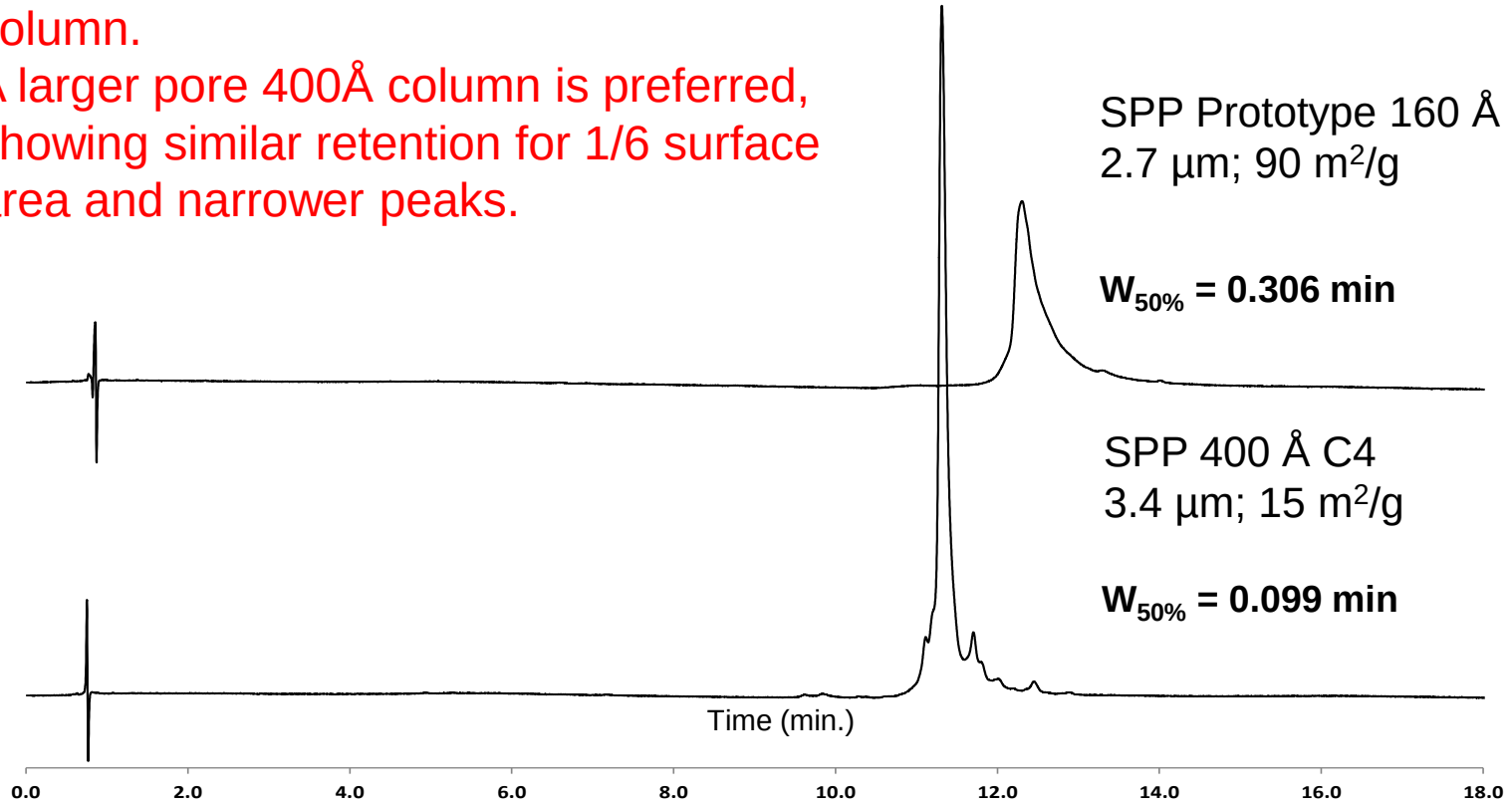


MCF 2017 RAH

* Data provided by AMT

Light Chain MAb Fragment on Halo 160Å and 400Å

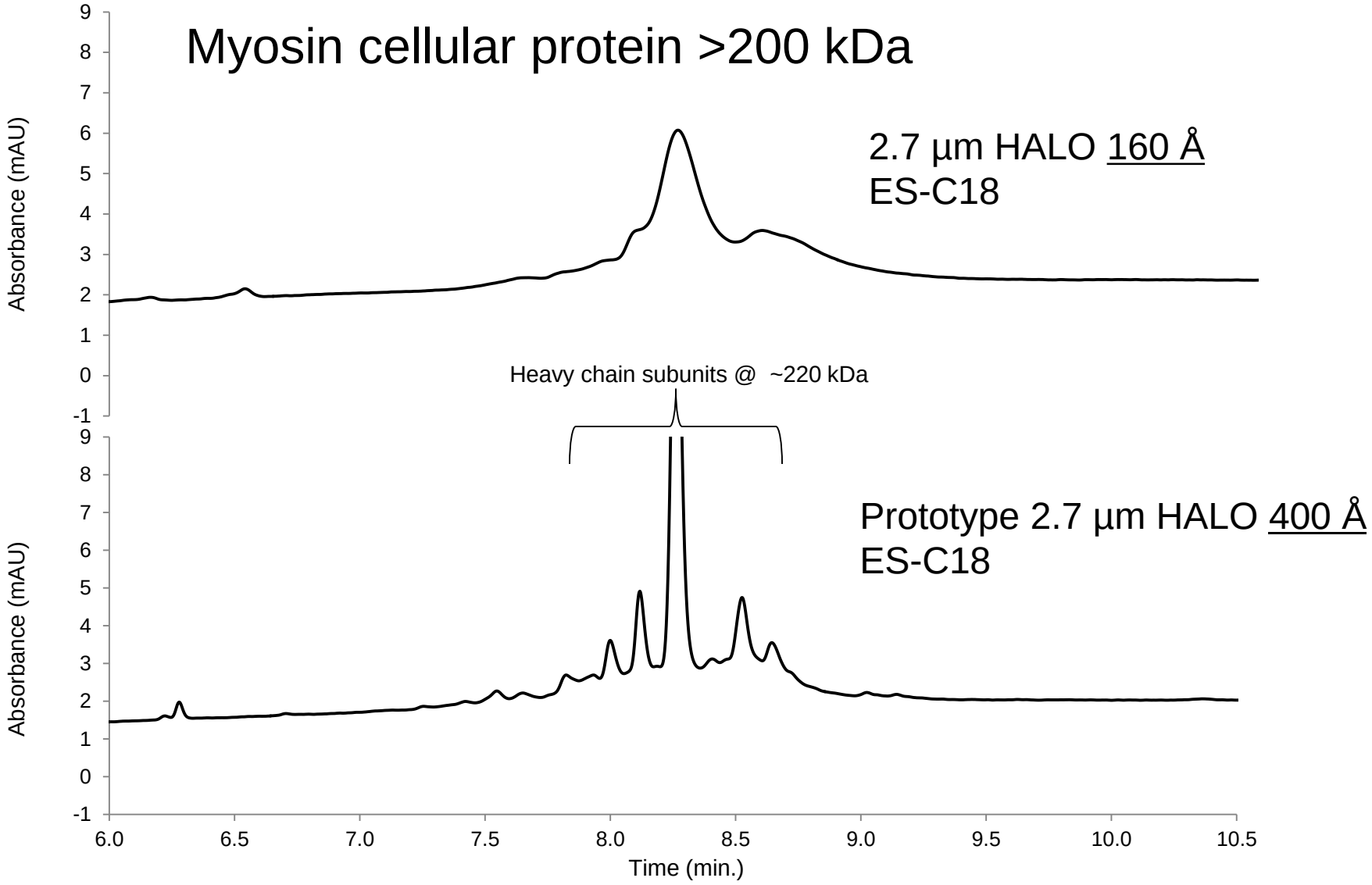
- Light chain fragment (25 kDa) of SigmaMAb is too large for the 160Å column.
- A larger pore 400Å column is preferred, showing similar retention for 1/6 surface area and narrower peaks.



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

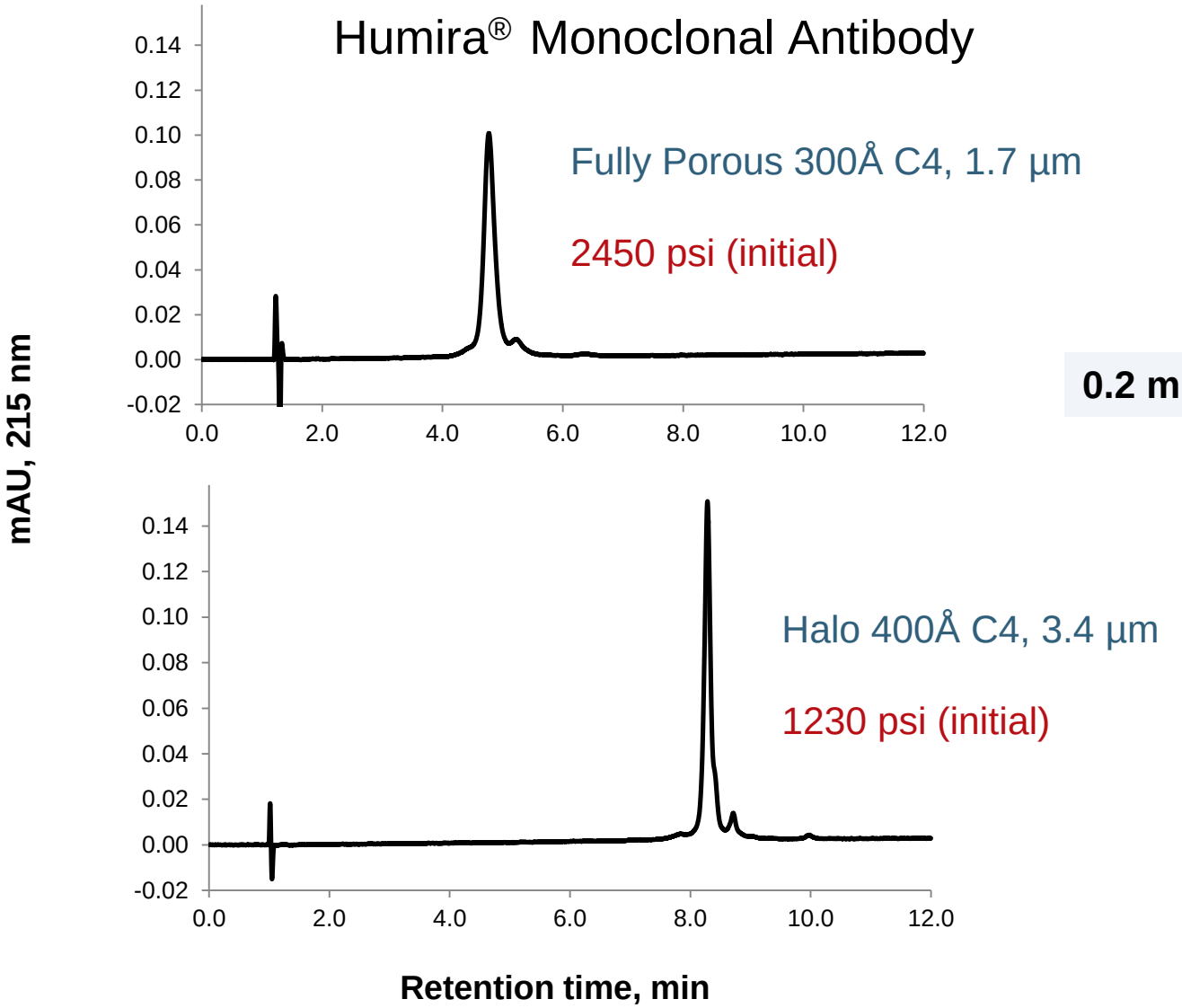
* Data provided by AMT

Comparison of 160Å and 400 Å on Large Proteins



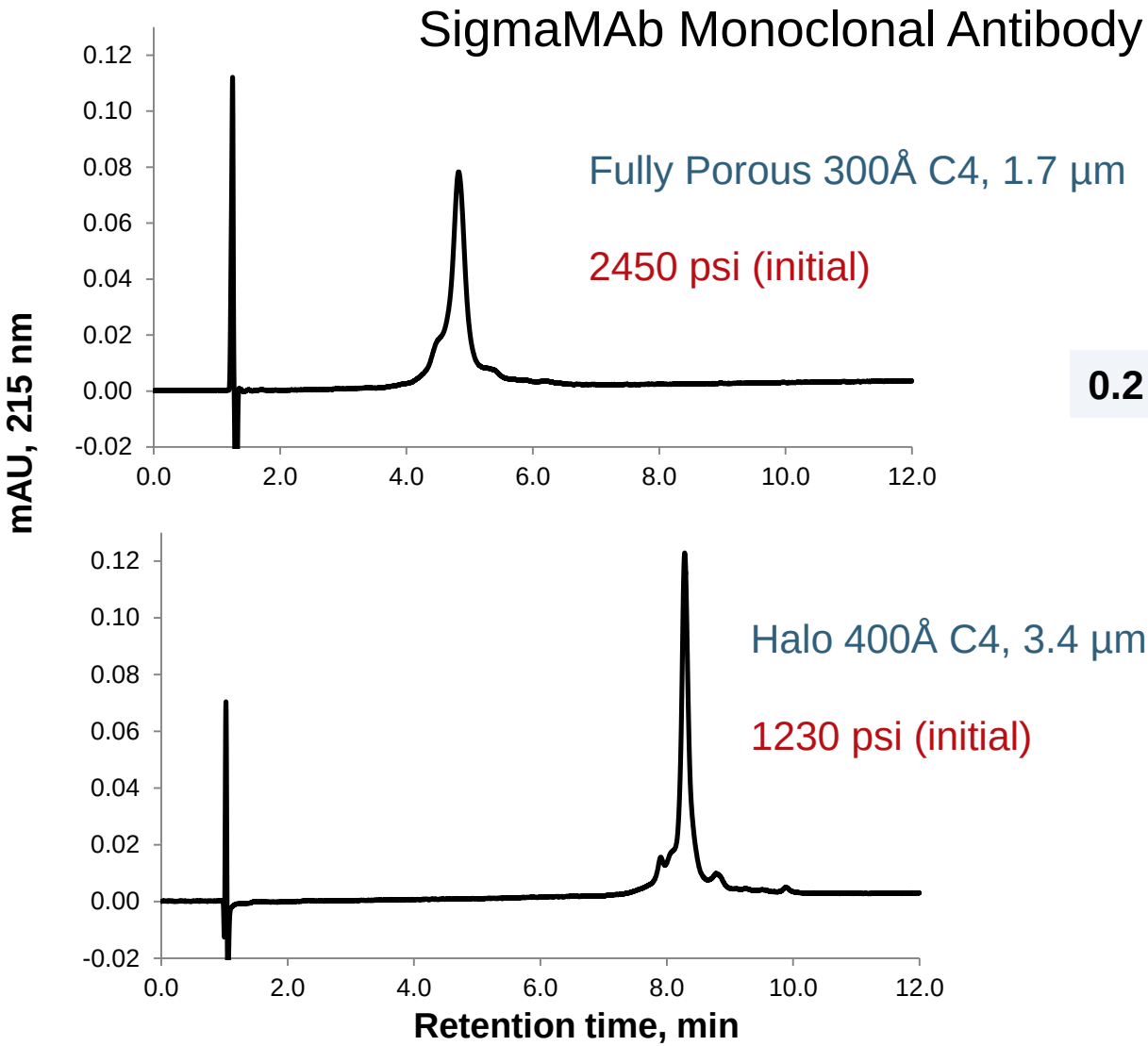
* Data provided by AMT

Humira® (adalimumab): Too Large for 300Å Columns



* Data provided by Supelco

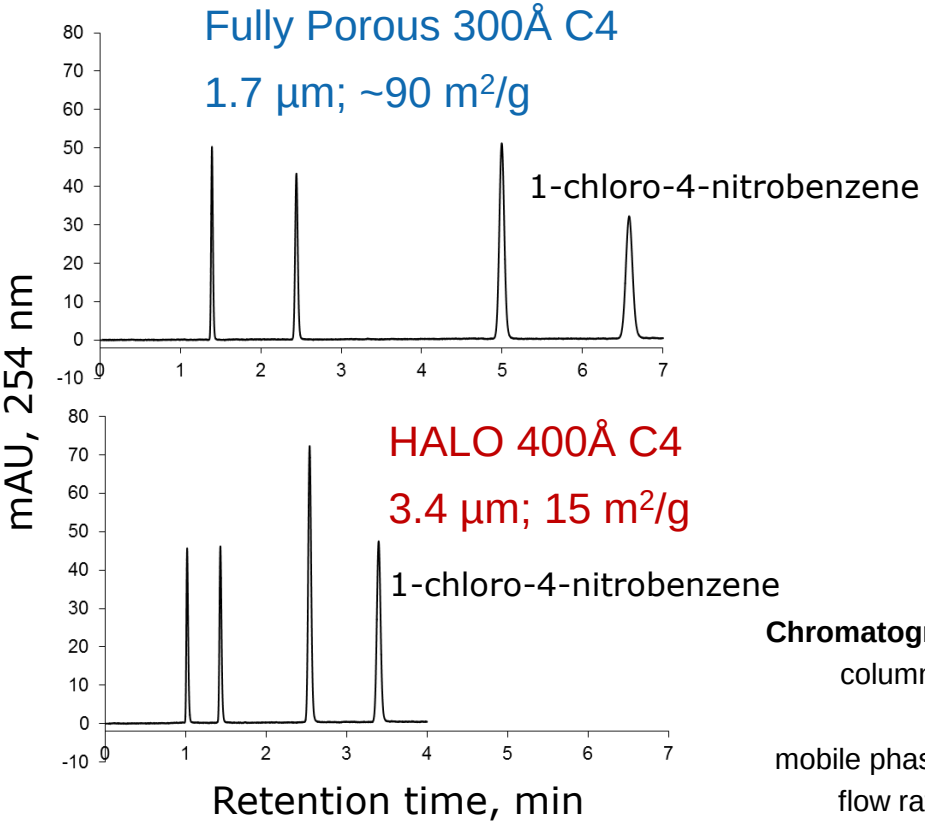
SigmaMAb: Too Large for 300Å Columns

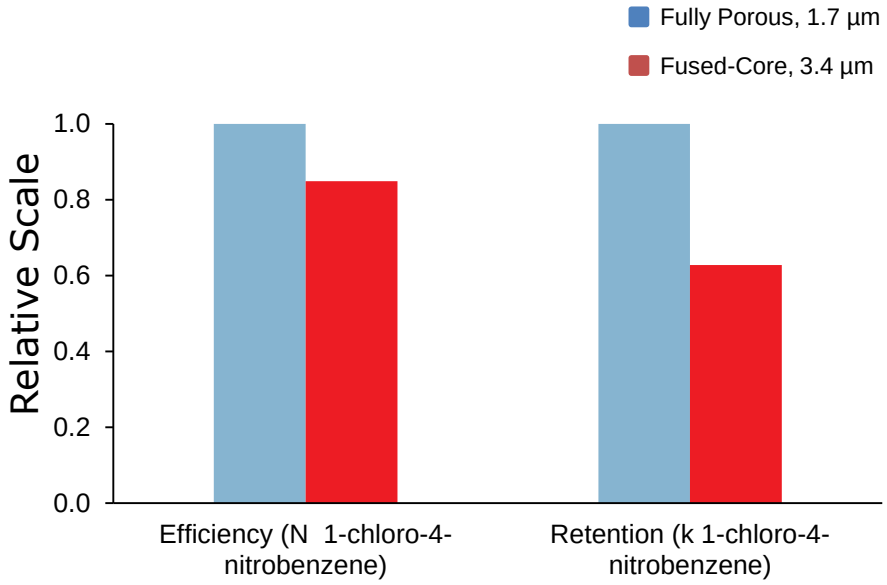


0.2 mL/min each

* Data provided by Supelco

Small Molecules Confirm Relative Pore-Sizes and Surface Areas for 300Å and 400Å Columns





Chromatographic Conditions

columns: HALO C4, 15 cm x 2.1 mm I.D., 3.4 µm
Fully Porous 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 (Fully Porous); 177 bar (HALO)

column temp.: 25 ° C
detector: 254 nm
injection: 0.2 µL
sample: uracil, phenol, propiophenone, 1-chloro-4-nitrobenzene

* Data provided by AMT

Conclusions

- Column retention and efficiency can both be lost for larger sample components when size exclusion mode significantly overlaps with retention modes and partially exclude large molecules and interfere with normal diffusion processes.
- Guidelines have been proposed that solutes should be smaller than ca. 10% of the column exclusion limit for optimum performance (radius ration of 0.1). For example, if exclusion limit is ca. 150,000 Da, largest solute size to avoid significant peak retention and efficiency loss might be estimated at 15,000 Da.
- Preliminary sample screening by SEC can save valuable time by identifying solutes that might be subject to excessive pore exclusion and require evaluation with larger pore columns.
- If data on solute sizes and average column pore size is not available to select optimum radius ratios in advance, screening larger pore columns is recommended for unknown samples.

References

1. J. C. Giddings, Unified Separation Science, Chapter 2, Entropy Effects in Porous Media, Wiley (1991).
2. J. C. Giddings, Dynamics of Chromatography: Principles and Theory, CRC Press, Boca Raton, FL (2002).
3. K. Unger, Porous Silica, Chapter 9 Size-Exclusion, Elsevier (1979).
4. F. Eisenbeis and S. Ehlerding, Kontakte 1/78 (1978) 22-29.
5. G. Barka and P. Hoffman, J. Chromatogr., 389 (1987) 273-278.
6. B. M. Wagner, S. A. Schuster, B. E. Boyes, T. J. Shields, W. L. Miles, M. J. Haynes, R. E. Moran, J. J. Kirkland and M. R. Schure, J. Chromatogr. A, 1489 (2017) 75-85.
7. J. Klein and K. Treichel, Chromatographia, 10 (1977) 604-610.
8. High Performance Liquid Chromatography, J. H. Knox, ed. Edinburgh University Press (1978).

L.R. Snyder, J.J. Kirkland, J.W. Dolan, Introduction to Modern Liquid Chromatography, 3rd ed., John Wiley & Sons, Hoboken, NJ, 2010.

Acknowledgements and Trademarks

Thanks to Stephanie Schuster, Joe DeStefano and Jack Kirkland of Advanced Materials Technology for data and helpful discussions; Mark Schure also contributed helpful information.

Thanks also to William Betz, Hillel Brandes, Stacy Squillario and Roy Eksteen of Supelco Division of MilliporeSigma who also provided data.

www.advanced-materials-tech.com
www.mac-mod.com