Column Pore Size- An Underutilized Variable for Optimizing HPLC Separation

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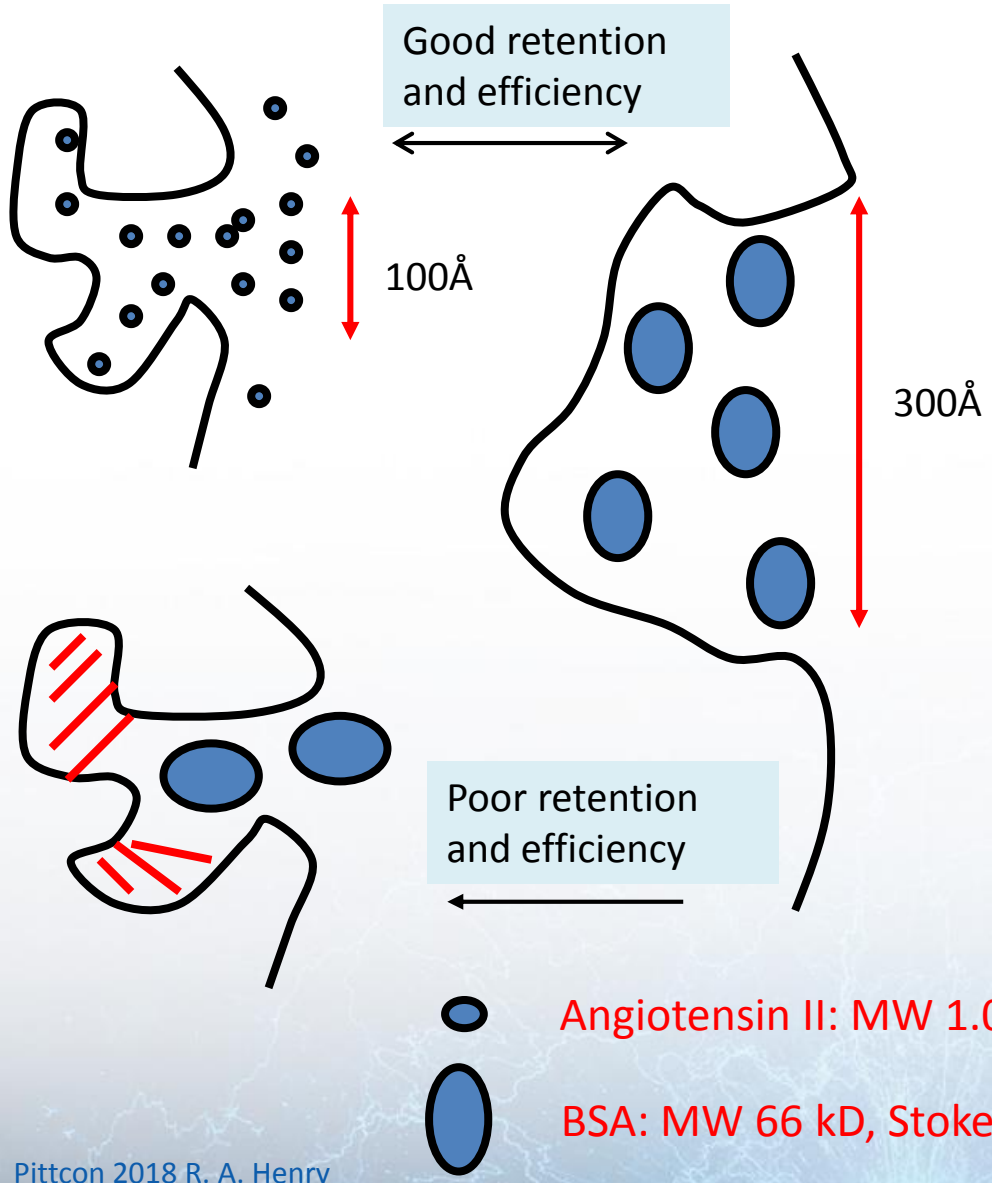
Surface Area and Pore Structure Control Retention



- 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 about 1/10.

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

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

Entropy Effects in Porous Media

"…entropy plays a significant role (in phase distribution) when one of the phases has a porous structure, providing the mean pore diameter is of the same order of magnitude as the diameter of the partitioning species."

J. Calvin Giddings

Entropy Effects in Porous Media, pg 31

Unified Separation Science, 1991 John Wiley & Son.

- Analytes should be about an order of magnitude smaller than the average particle pore for high performance results!

Silica as Packing in Size Exclusion Chromatography

"In pure SEC, solutes elute with decreasing MW, whereas in LSC (including RP), the reverse elution order is obtained. In mixed mechanisms, LSC can contribute only within pores accessible to the solute. Therefore, total retention cannot be the sum of K_{SEC} and K_{LSC}."

Klaus K. Unger

Chapter 9, pg 287, Porous Silica (…as a support in column LC), Elsevier Scientific 1979.

- Avoid overlap between SEC and LSC/RP modes for high LC column reproducibility and performance.

Silica as Packing in RP and Other HPLC Modes

"….solutes should encounter at least 10-15 times larger mean pore diameter for fast mass transfer into particles and efficient interaction with stationary phases."

J. Jack Kirkland

Personal conversation, 2016.

- When analytes become too confined within the pores, column retention, efficiency and peak capacity will be reduced.

Pore Diameter- A New HPLC Resolution Variable

Column variables (controlled by suppliers)

- Substrate (usually silica)
- Bonded phase (ideally, substrates *should* have no impact)
- Pore-diameter (adequately large pores needed to maximize retention, efficiency and selectivity for larger molecules)

Mobile phase variables (controlled by instruments and users)

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

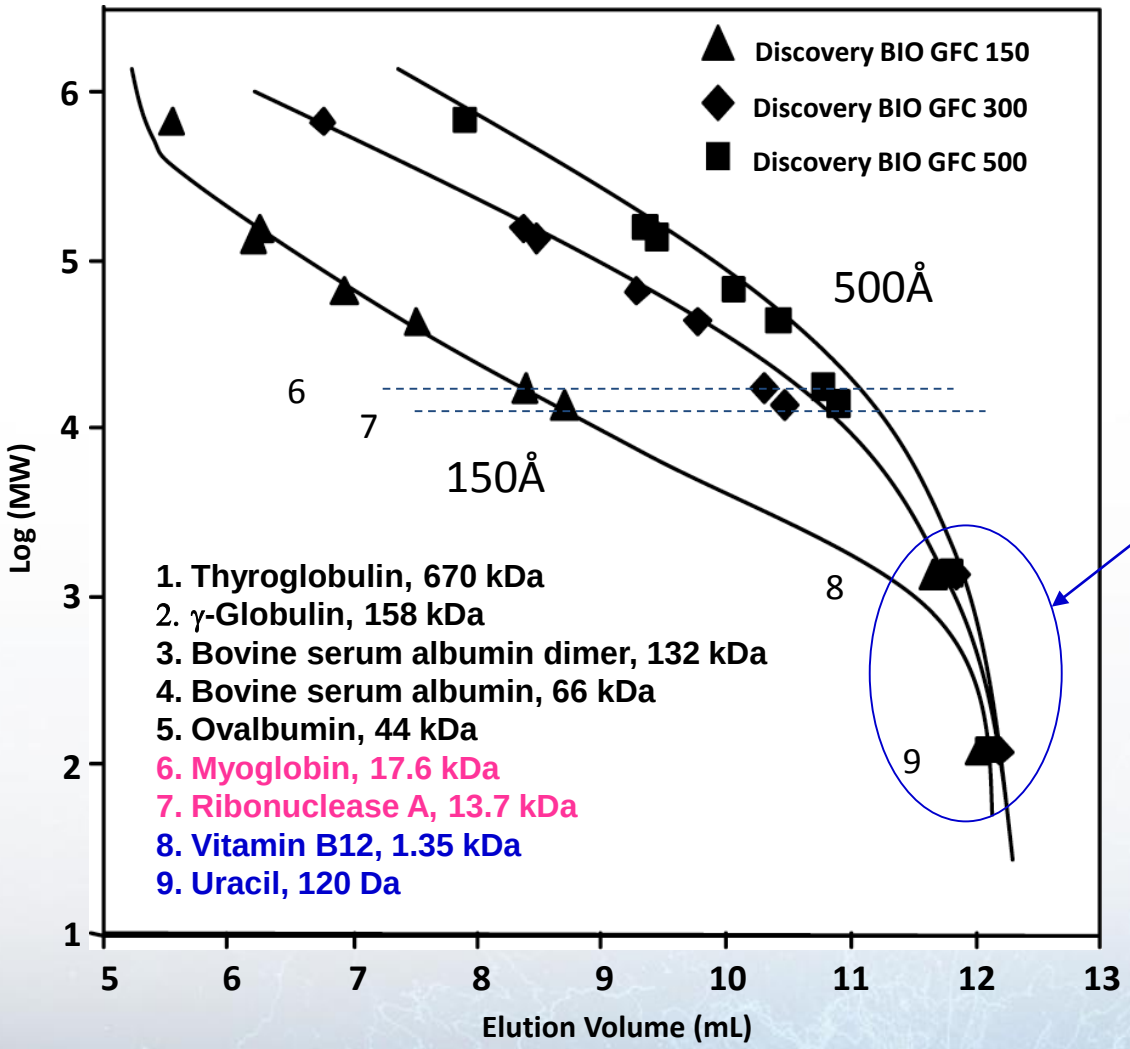
Sample variables (uncontrolled)

- Chemistry
- MW (size and shape)

* Presentation by John Dolan, LC Resources, MCF 2009.

** Suppliers should provide full details on column variables.

Some Mode Overlap Occurs in All Porous Particles



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.)

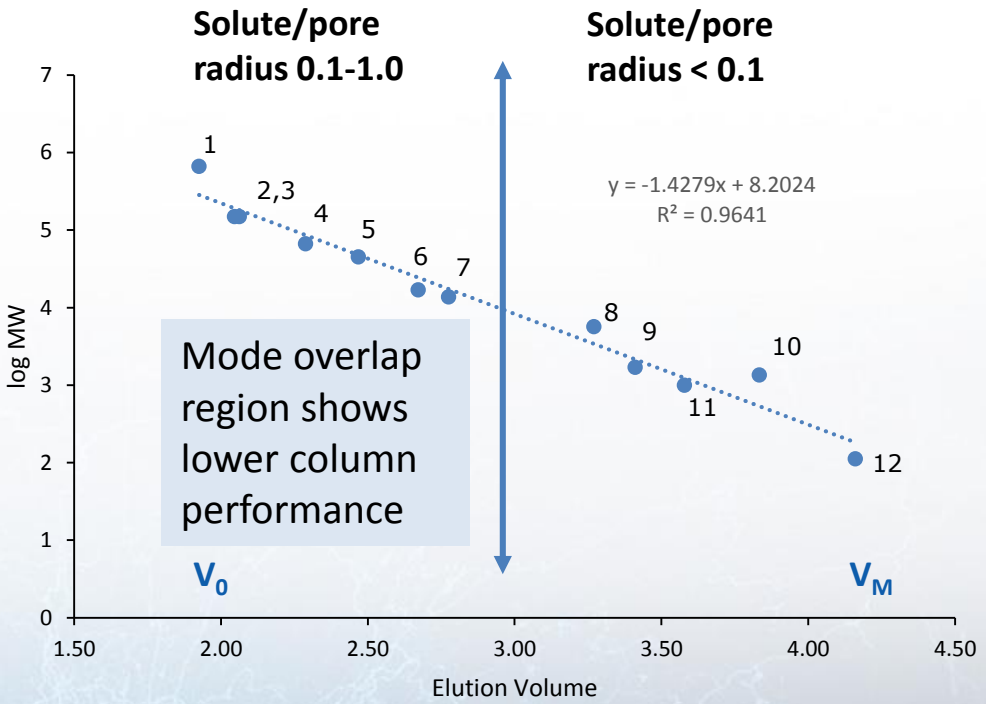
* Data provided by Supelco

Size Exclusion Chromatography Calibration Plot

Chromatographic Conditions

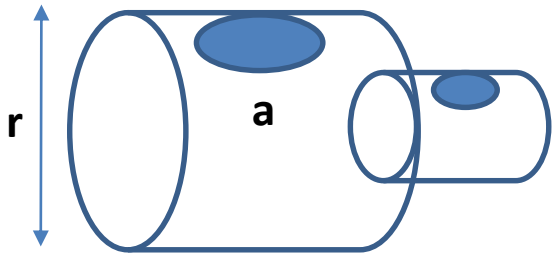
columns: Aqueous SEC-150, 30 cm x 4.6 mm I.D., 3 µm
mobile phase: 0.2 M potassium phosphate, pH 7.0
flow rate: 0.25 mL/min
pressure: 66 bar
column temp.: 25 °C
detector: UV 215, 280 nm
injection: 0.5 µL
samples: listed below

	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

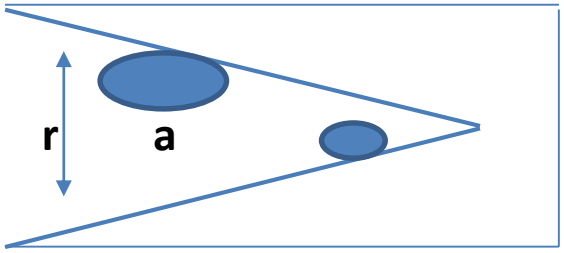


Data provided by AMT

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



Cylindrical pore model

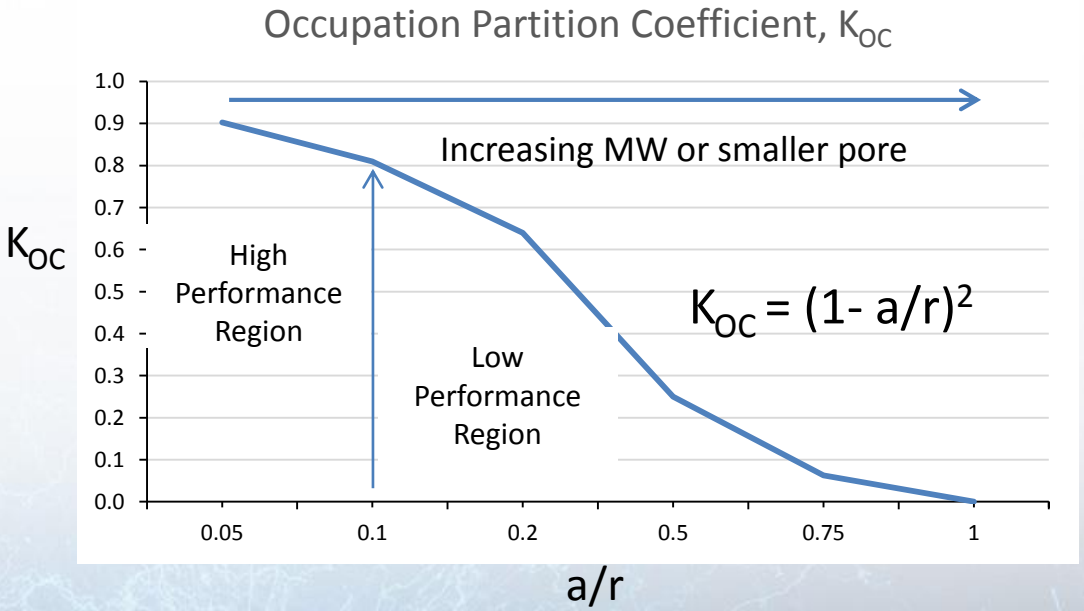


Conical pore model

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

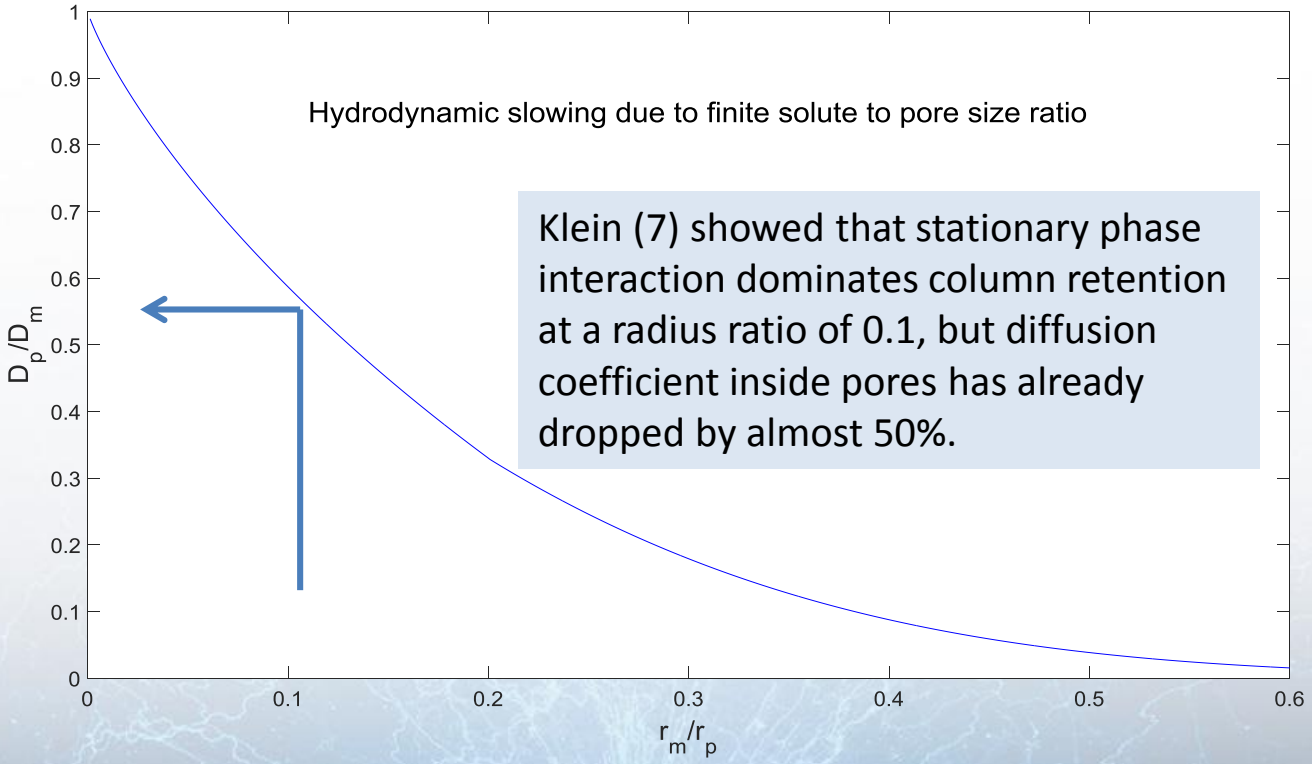
Analyte radius (**a**) increases with MW; a specific ratio (**a/r)** exists for each sample component (7).

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

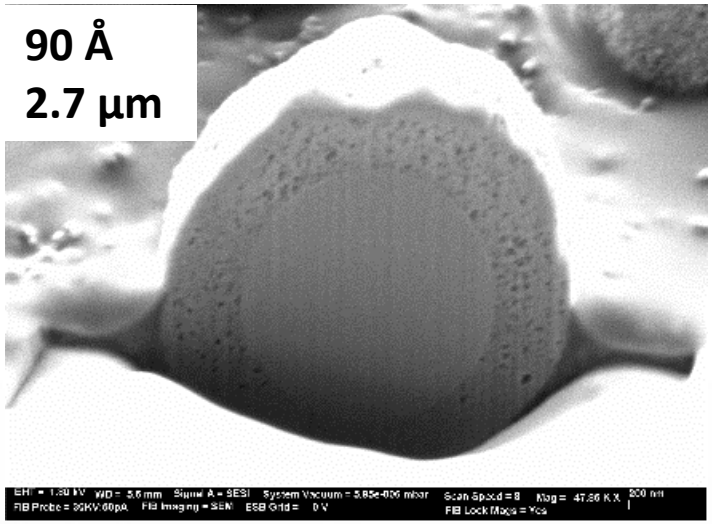


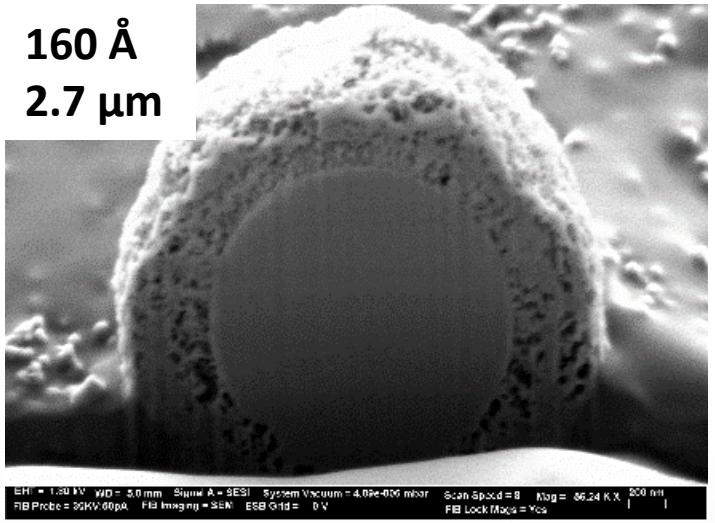
Impact of Size Exclusion on Band Broadening

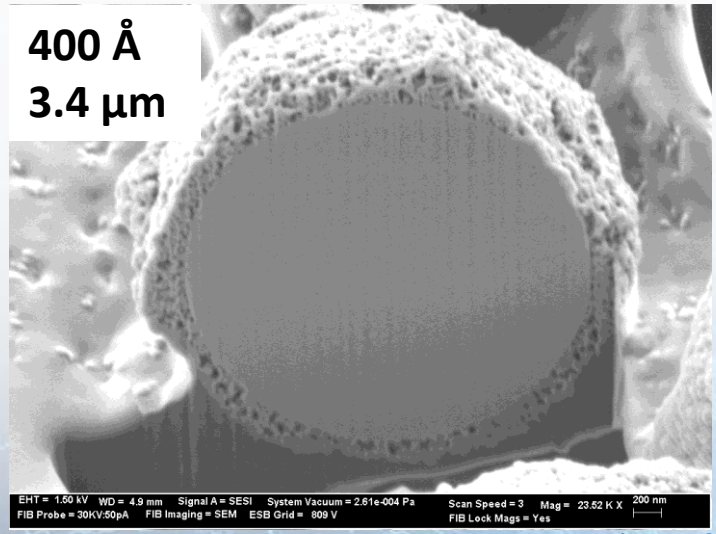
Schure (6) recently described another type of band broadening by estimating reduced diffusion rates inside crowded pores (D_p) relative to free diffusion in mobile phase outside pores (D_m) and plotting it against radius ratio (molecule/average pore).

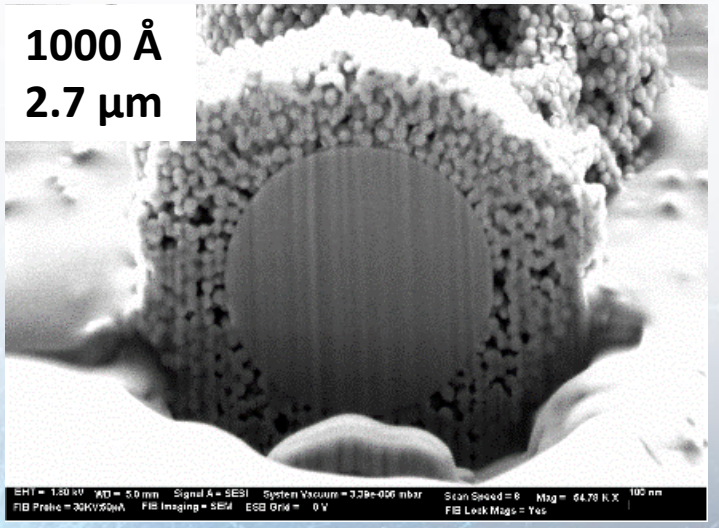


Range of Superficially-Porous HALO® Silica Particles

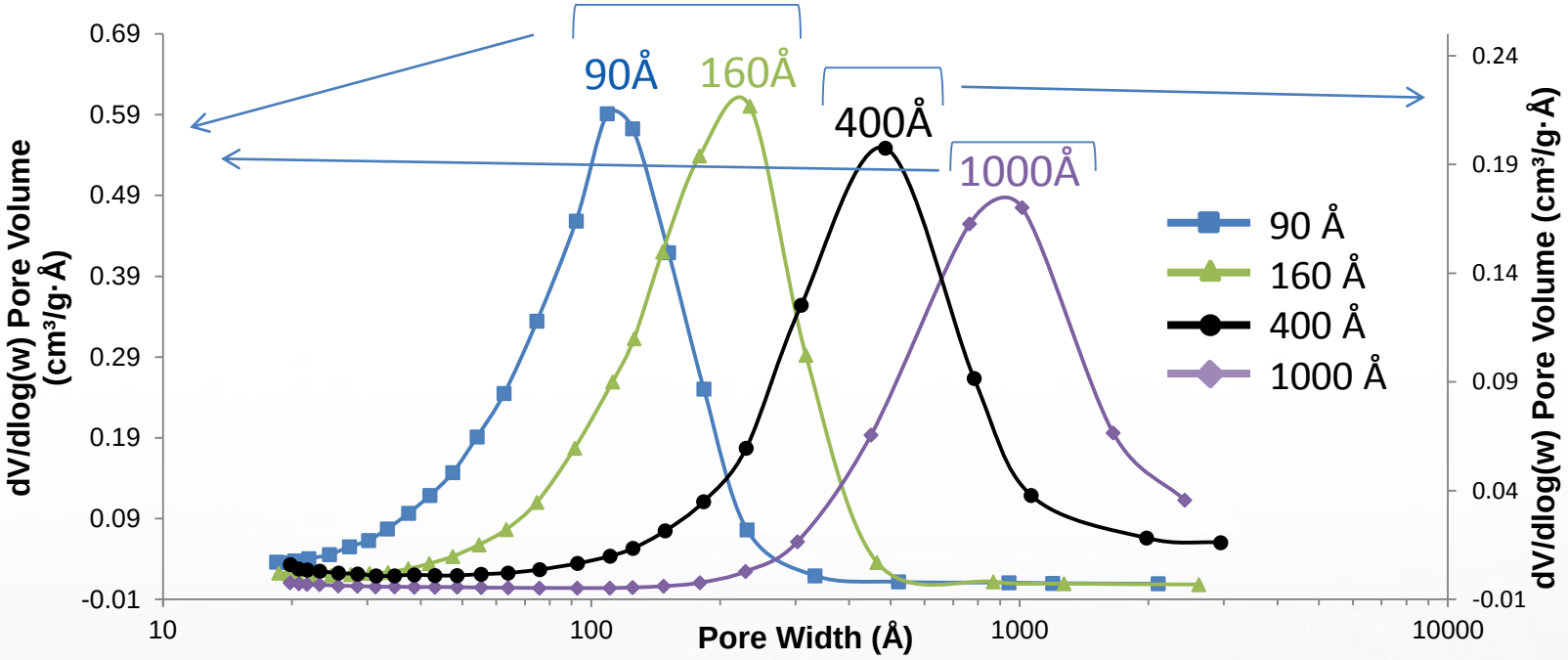








Pore-Sizes of Halo Silica for Different Molecules



Small drugs

Small peptides

Large peptides

Small proteins

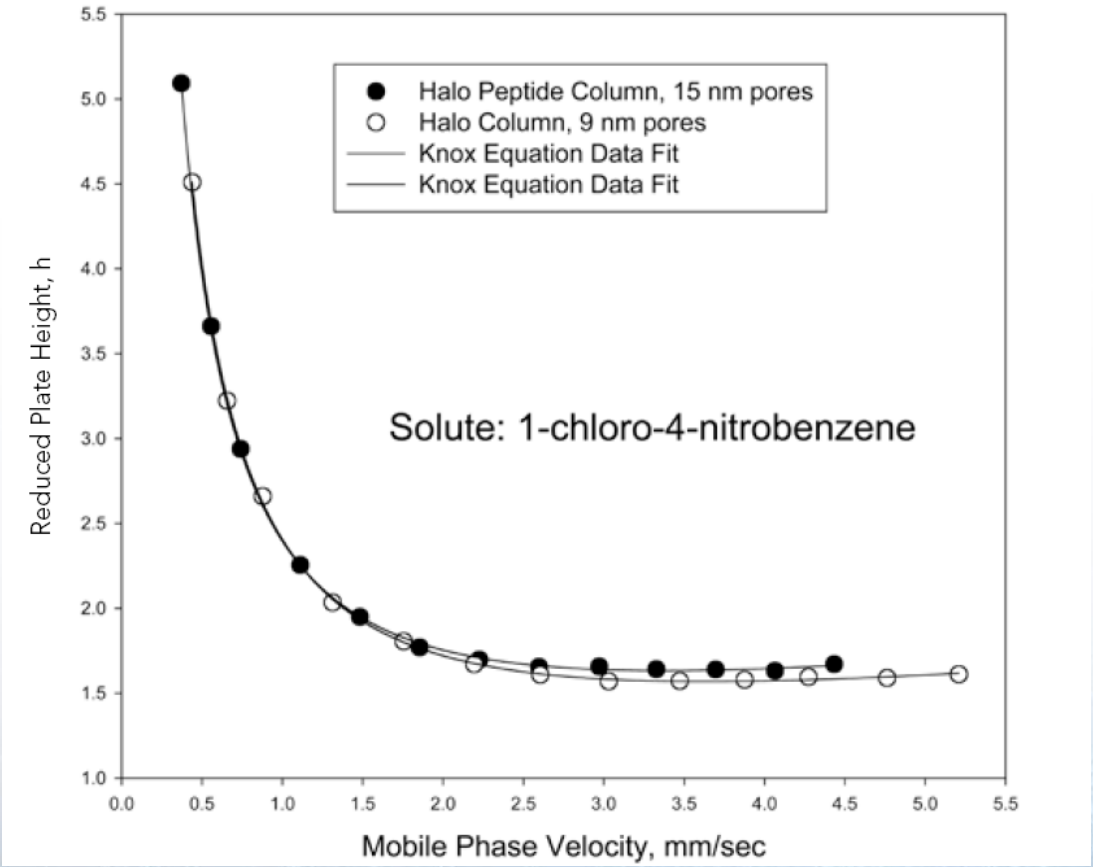
Large proteins

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

Effect of Pore-Size on "Small Analyte" Efficiency

Columns: 4.6 x 100 mm; Particle size: 2.7 µm
Mobile phase: 50% acetonitrile/50% water; 25 °C
Agilent 1100 with autosampler



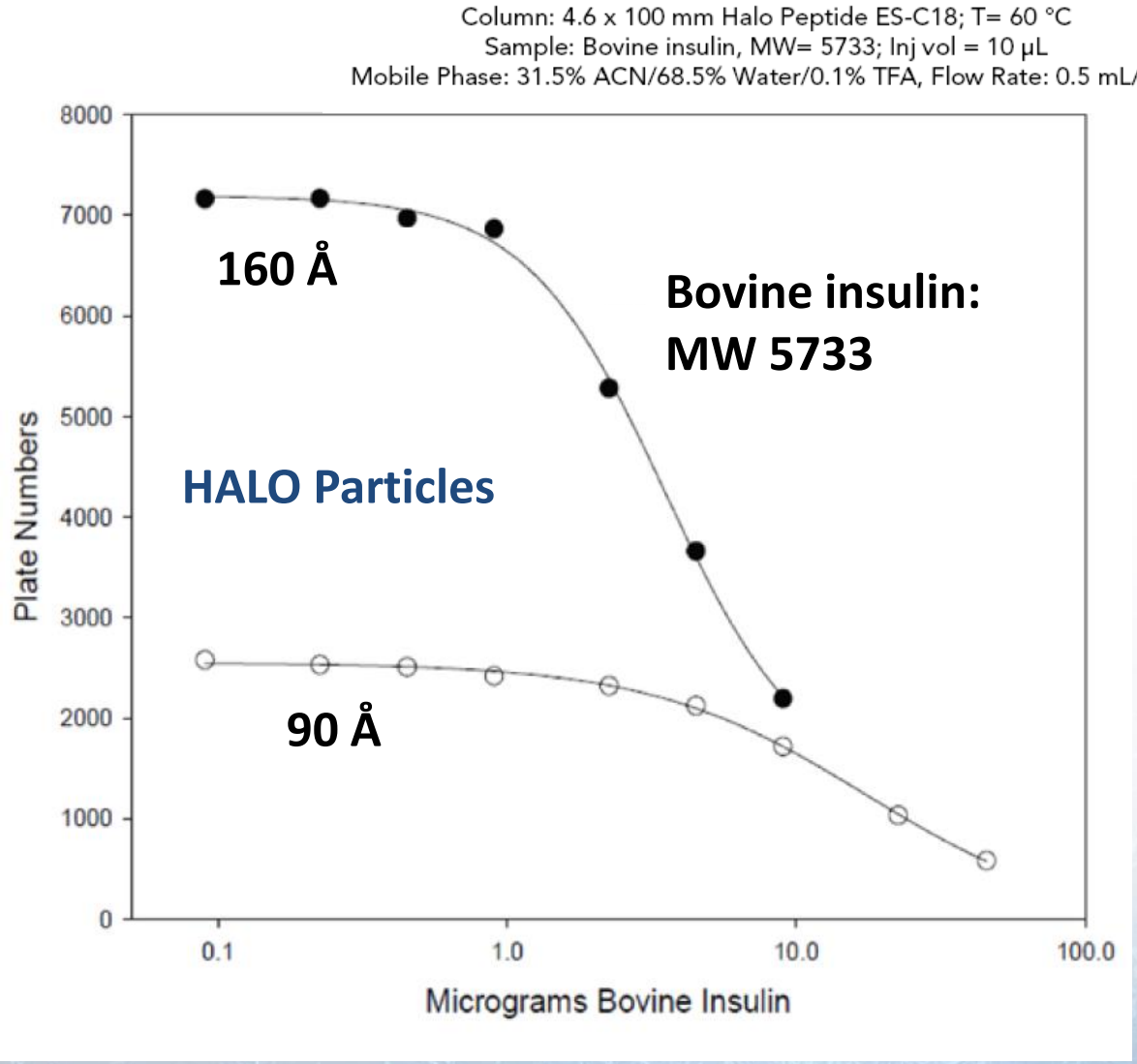
High efficiency is critical to high resolution; both particles show high peak capacity for small molecules.
This is an indication of minimal crowding within the pores

HALO C18, 90 Å pores

HALO ES-C18, 160 Å pores

Data provided by AMT

Pore-Crowding Reduces Efficiency of Larger Solutes



High efficiency is the key to high resolution and peak capacity; a larger pore particle is needed for high efficiency with bovine insulin.

Insulin has a diameter of about 50Å. Even peptides of 1000 MW may show lower performance. Pore crowding is not limited to peptides.

* 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.

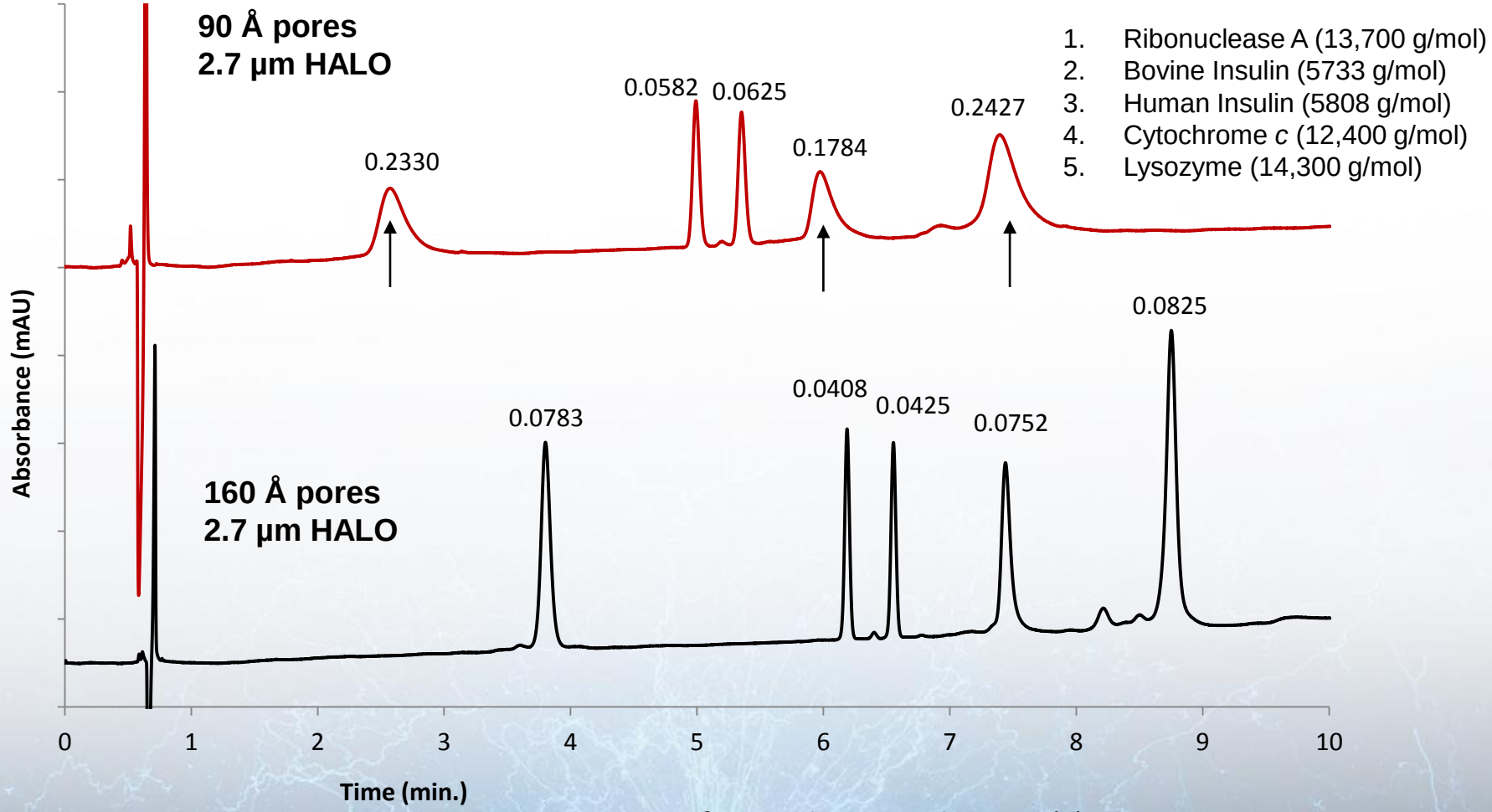
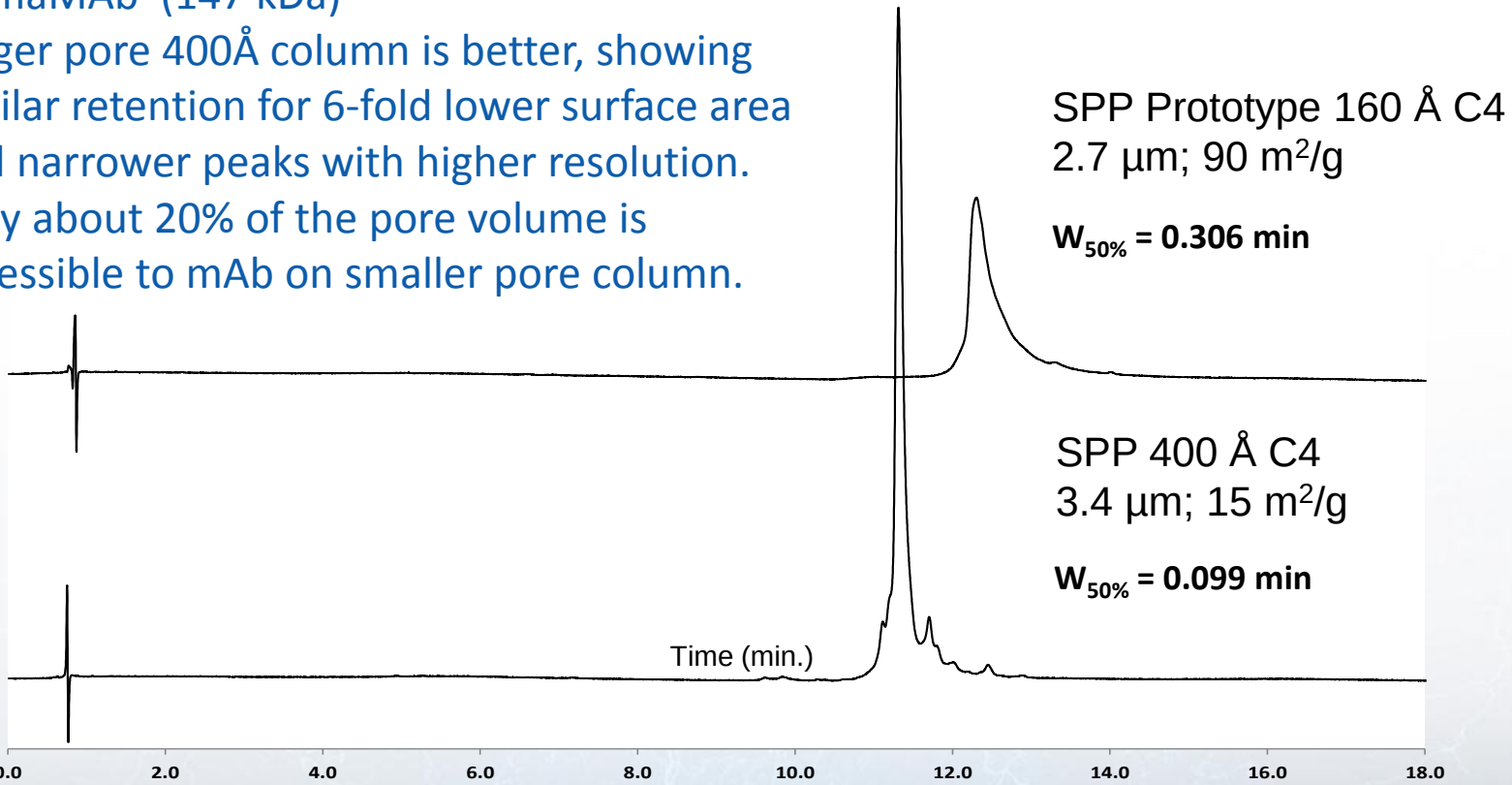


Fig.3 from J. Pharm. Anal. 2013;3(5):303–312

SILU Lite SigmaMAb on Halo 160Å and 400Å

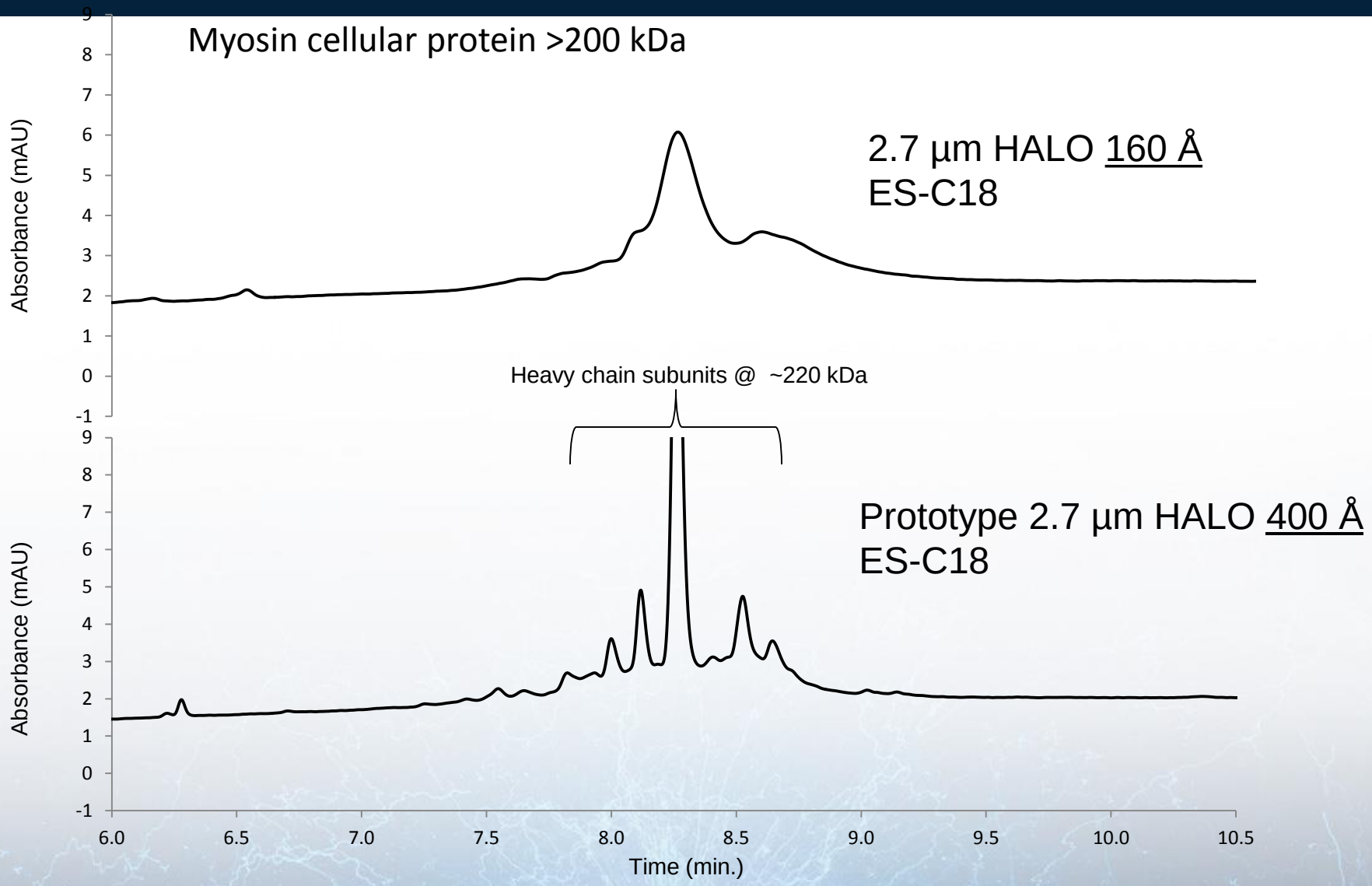
- 160Å pore size column is much too small for SigmaMAb (147 kDa)
- Larger pore 400Å column is better, showing similar retention for 6-fold lower surface area and narrower peaks with higher resolution.
- Only about 20% of the pore volume is accessible to mAb on smaller pore column.



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

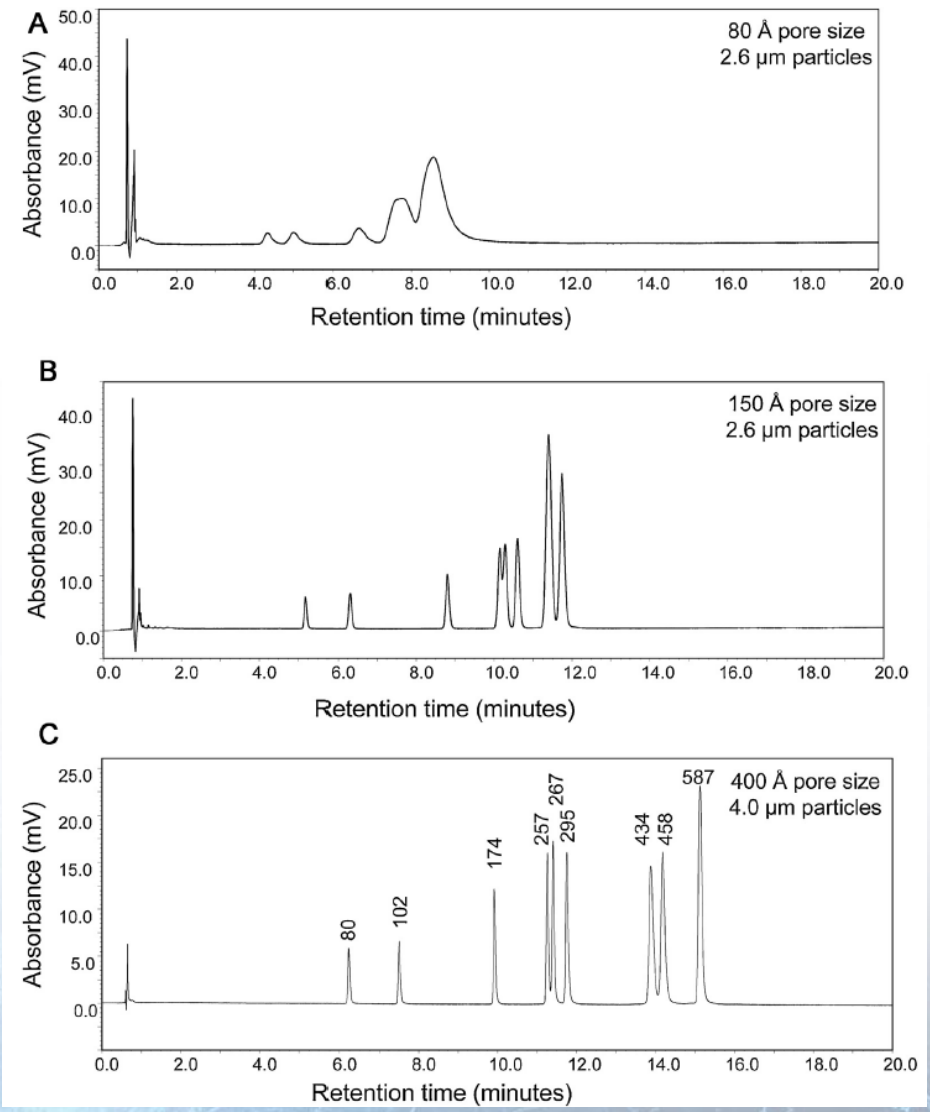
Large Protein on 160Å and 400 Å C18



* Data provided by AMT

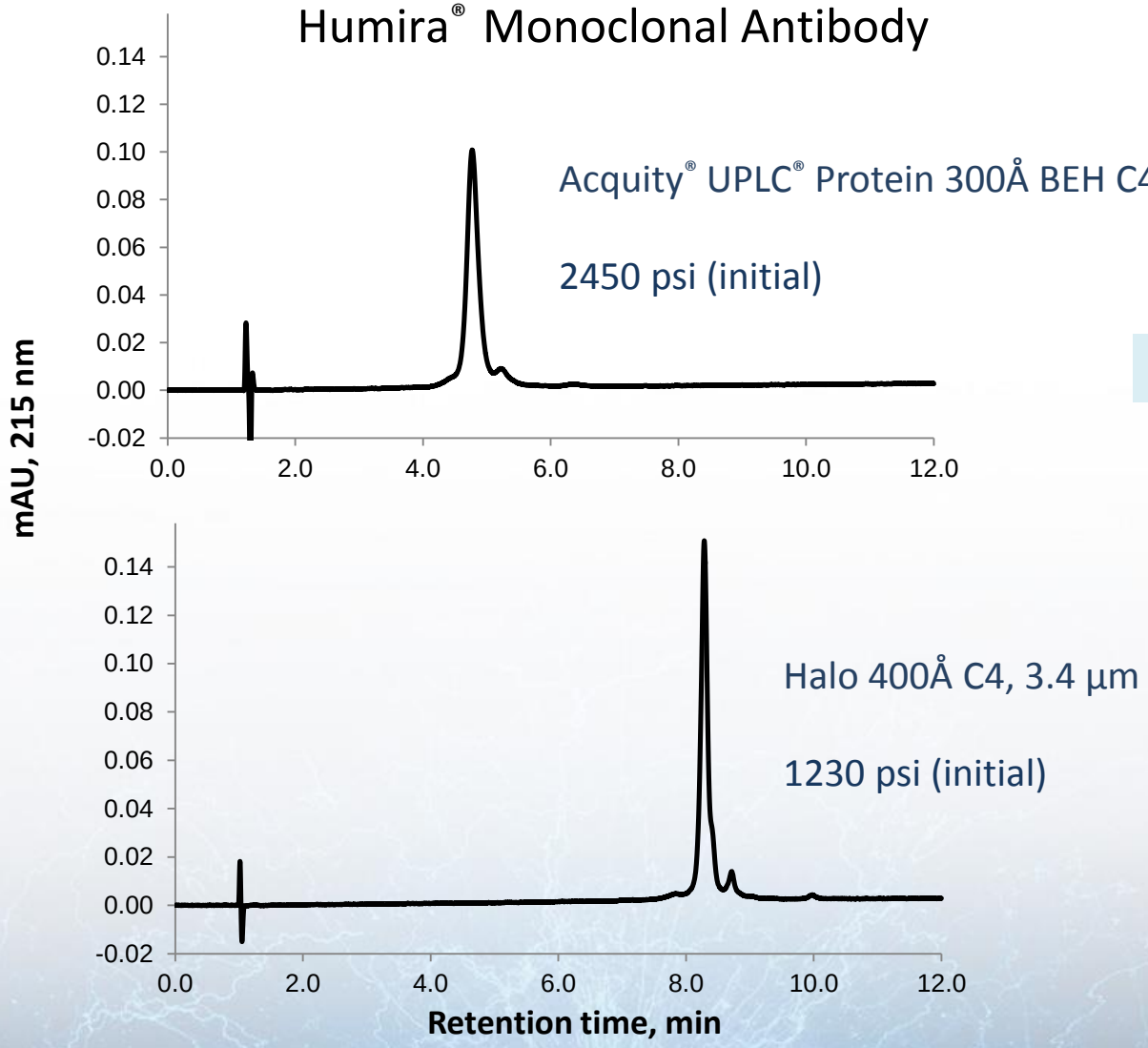
Pore-Size Effects on IP RP HPLC of DNA Digest

- Same DNA restriction digest analyzed on columns with different pore openings.
- Restriction digest base pairs too large for 80Å pore size column.
- Increased retention and sharper peaks observed on 150Å pore size column.
- Sharpest peaks and highest retention observed on 400Å pore size column.



Adapted from Fig. 3 in Journal of Chromatography A, 1440 (2016) 135–144.

Adalimumab: Too Large for 300Å Columns

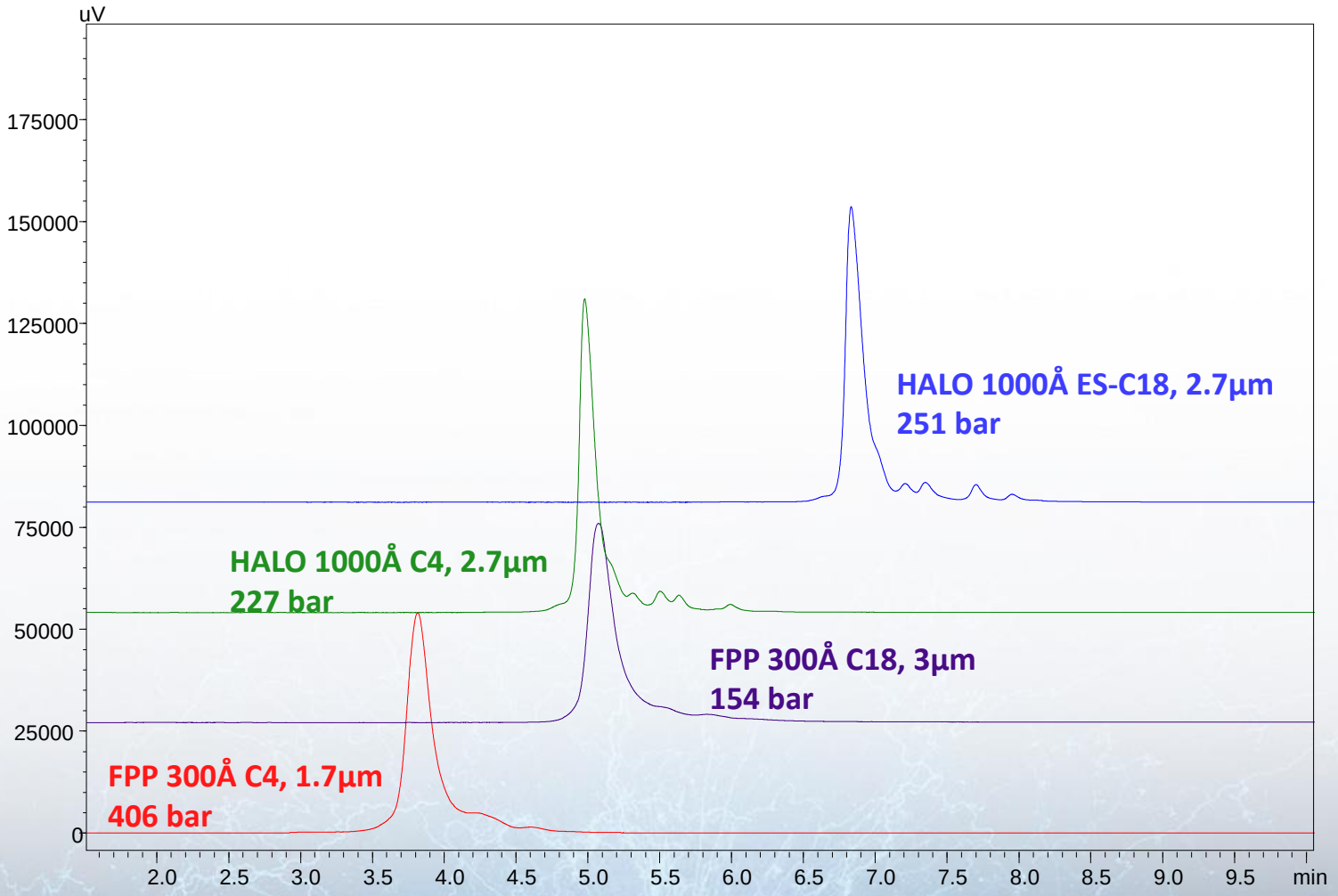


0.2 mL/min each

* Data provided by Supelco

Effect of Pore Size on RP Separation of Trastuzumab

2.1 x 150 mm, A = water/0.1% TFA, B = ACN/0.1% TFA, 34-42% B in 16 min, 0.4 mL/min, 60 °C, 2 µl @ 2mg/mL in 0.1% TFA, 280 nm



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; partial exclusion of large molecules interferes with stationary phase access and normal diffusion processes.
- Guidelines have been proposed that solutes should be smaller than ca. 10% of the column exclusion limit for optimum performance (radius ratio 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 with larger pore columns is recommended for unknown samples.

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