Optimizing HPLC Separation Performance for Peptides and Other Mid-Size Molecules

……one size does not fit all; exploring the relationship between pore size and separation retention and efficiency

Richard A. Henry, Independent Consultant, State College, PA 16801.

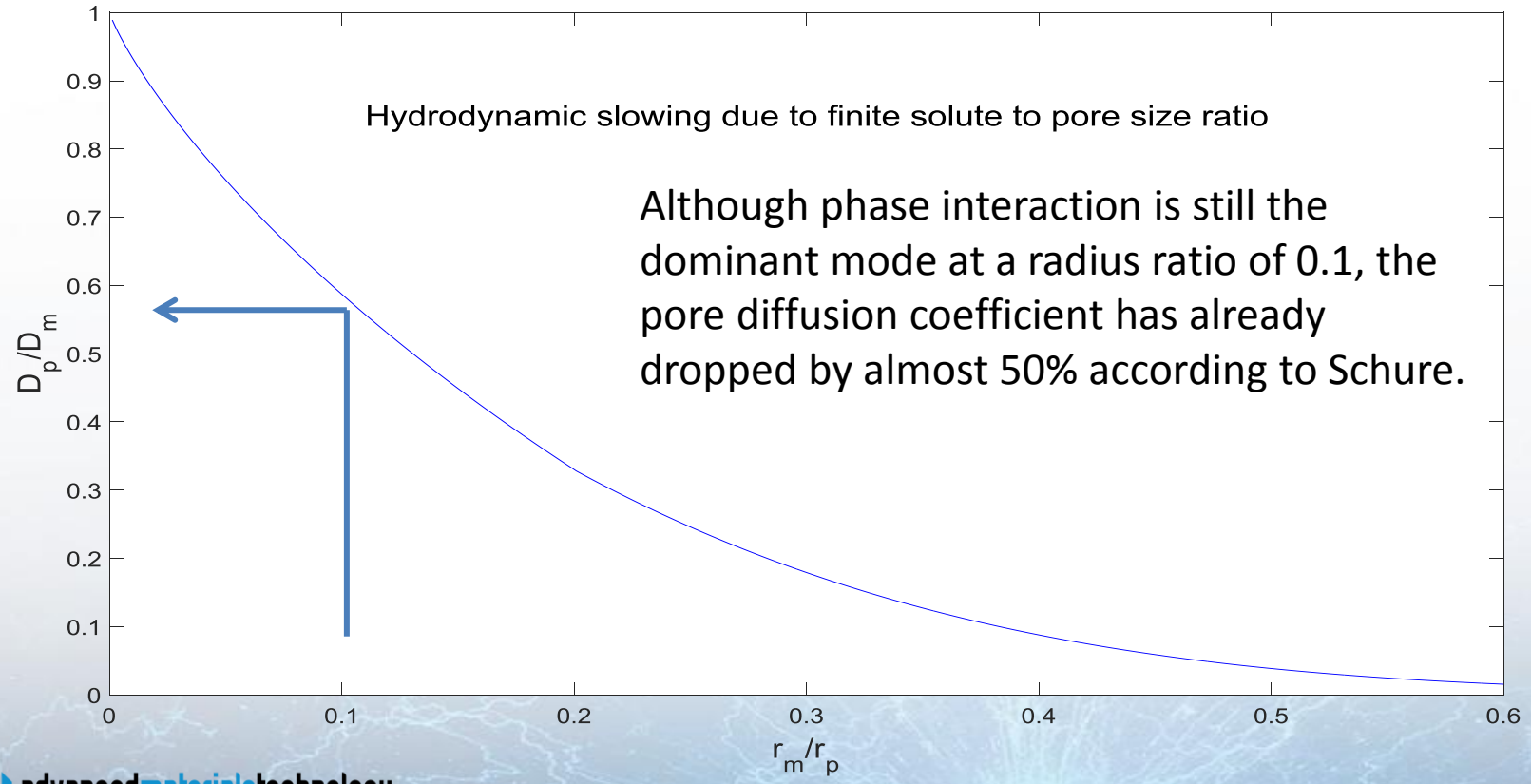
Justin M. Godinho and Joseph J. DeStefano, Advanced Materials Technology, Wilmington, DE 19810.

The HPLC Column Pore Environment

- Choosing the correct HPLC column often centers around identification of a stationary phase to magnify resolution for certain classes of compounds. While gains in resolution are most affected by phase selectivity, enhancements can also be made with intentional selection of the packing material's microstructure. This has been effectively demonstrated for peptides by Schuster, et. al.[1]
- The pore variable is often overlooked for larger molecules for making potentially significant performance gains. Increased efficiency, especially when operating at above optimum flow rates, can be observed if the pore size is significantly larger than the solvated analyte[2-4].
- Reduced access to pore structure due to physical hindrance (partial exclusion) also limits retention, as the vast majority of surface area and bonded phase exists within the particle. While partial exclusion is often tolerated, it may not be the most reproducible place to operate.

Hindered Diffusion in Cylindrical Pore as Function of Ratio of Molecule Radius to Pore Radius.

Schure[3] has recently described HPLC zone broadening that includes a description of hindered diffusion inside pores (compared to free mobile phase diffusion) as solute size approaches average pore diameter (pore-crowding).



- Y axis is diffusion in pore divided by diffusion in mobile phase, which are not always the same.
- X axis is ratio of average solute size to pore size. As the diameter ratio approaches unity, movement inside the pore slows down and efficiency drops.

Experimental Conditions

Analyte	MW	Mobile Phase (in water)
Naphthalene	128	50% Acetonitrile
Lorazepam	321	30% Acetonitrile
Angiotensin 1-12	1509	30% Acetonitrile + 0.1% TFA
Bombesin	1619	21% Acetonitrile + 0.1% TFA
Insulin Chain B Oxidized	3496	28% Acetonitrile + 0.1% TFA
Insulin	5777	30% Acetonitrile + 0.1% TFA
Ribonuclease A	13700	22% Acetonitrile + 0.1% TFA

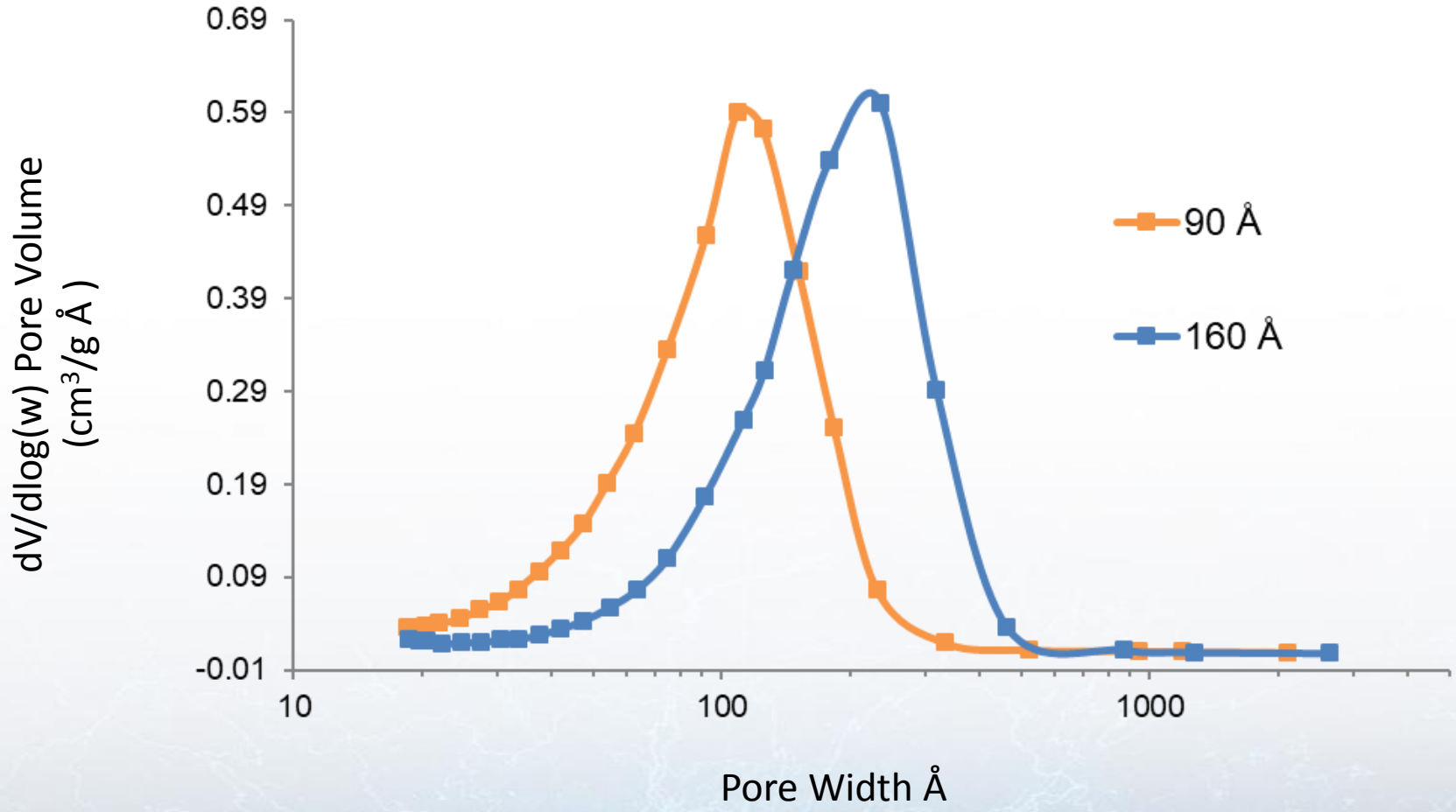
- Instrument and Columns: Nexera X2, HALO 90 Å Phenyl-Hexyl, 2.7 µm, 2.1 x 50 mm and HALO 160 Å Phenyl-Hexyl, 2.7 µm, 2.1 x 50 mm
- Temperature: 60 °C
- Flow Rate: 0.05 to 1.75 mL/min
- Mobile Phase: Premixed and pumped through a single pump under isocratic conditions for van Deemter calculations

HALO® Superficially Porous Particle Characteristics

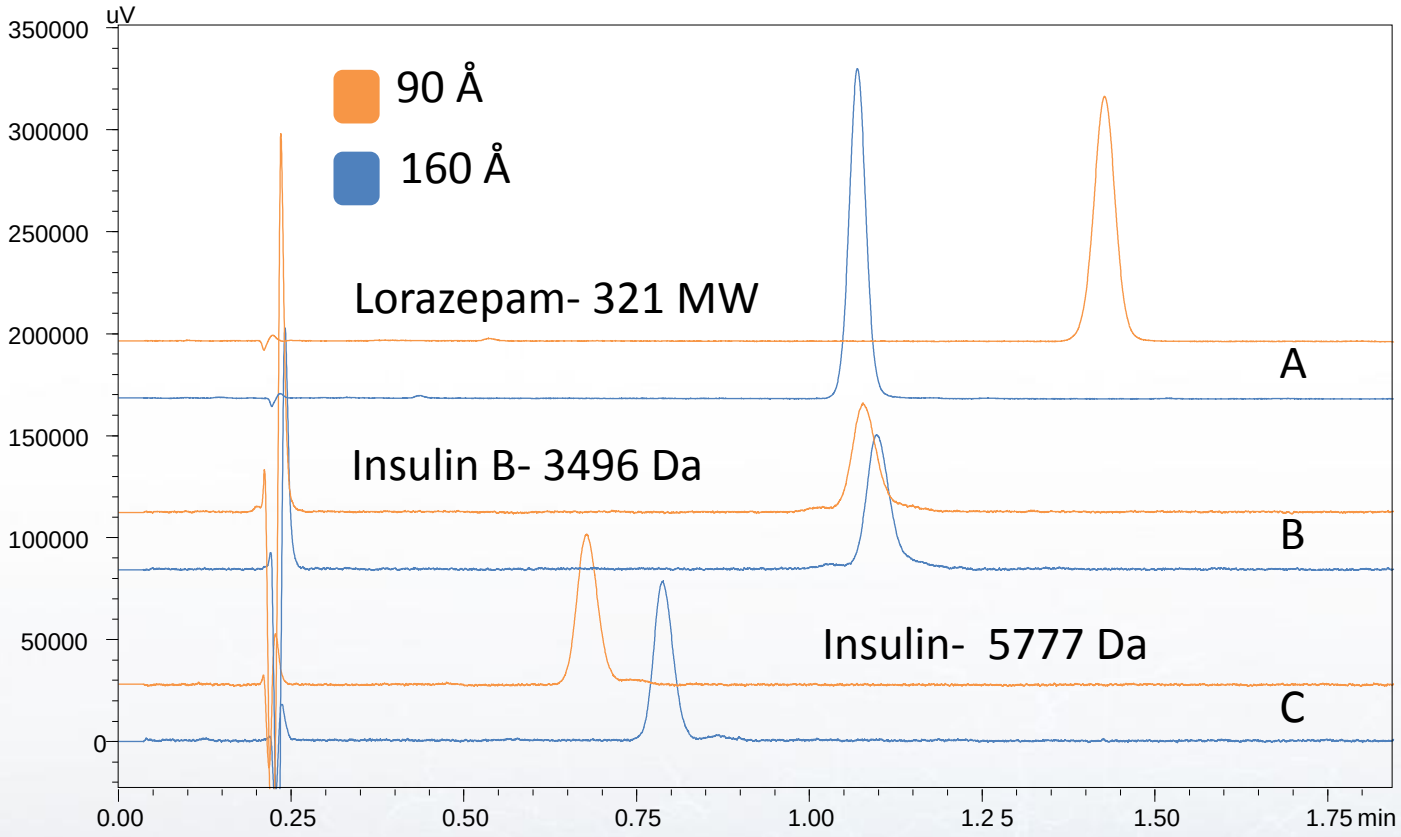
Shell with 90 Å pores
2.7 µm particle diameter
1.7 µm core
135 m^2/g

Shell with 160 Å Pores
2.7 µm particle diameter
1.7 µm core
90 m^2/g

Comparison of Pore Volume for 90 Å and 160 Å Columns



Isocratic Comparison on 90Å and 160Å Columns



Analytes: (A) Lorazepam (321 MW), (B) insulin chain B oxidized (3496 MW) and (C) Insulin (5777 MW). Conditions: 0.5 mL/min (≈4 mm/sec). Retention order and performance shifts as MW increases and analytes are restricted from stationary phase in smaller pore materials.

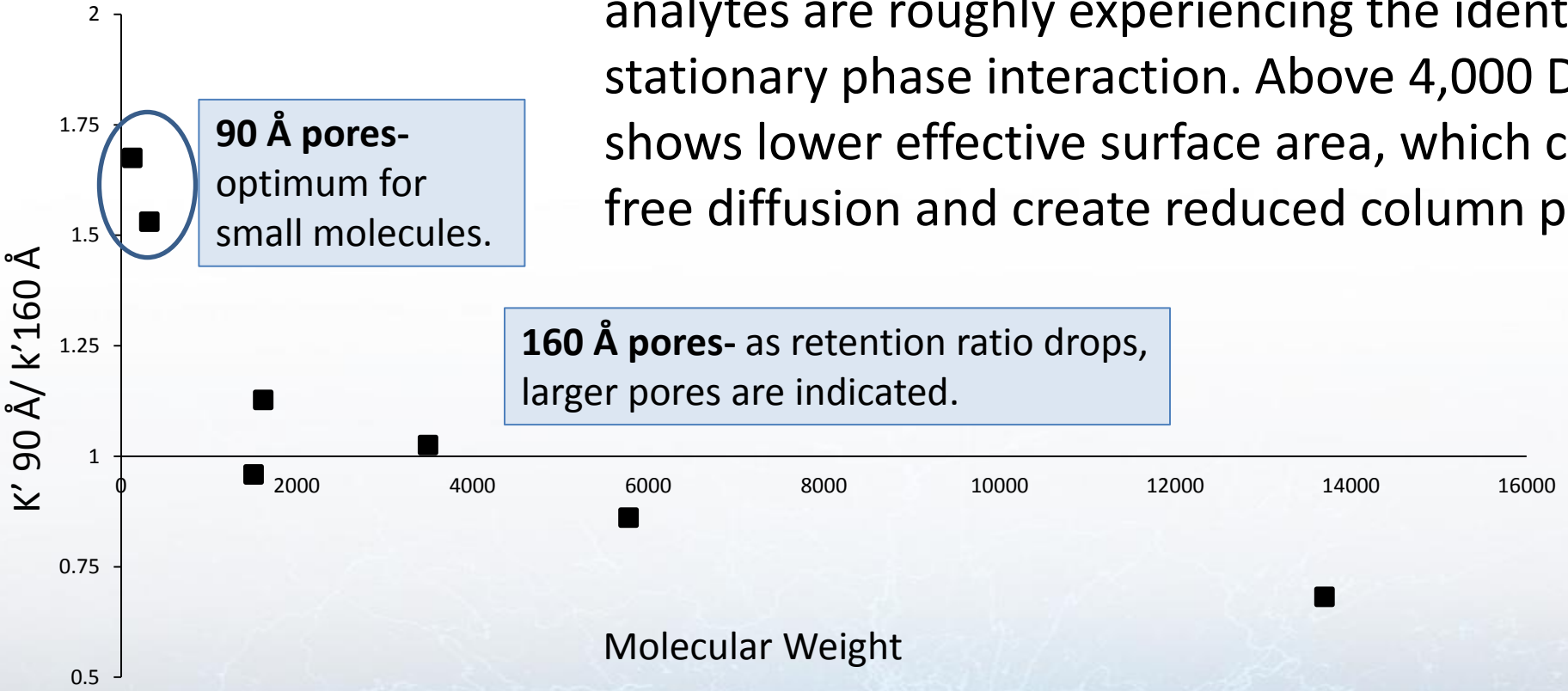
A. **<u>Small molecule</u>**: *Greater retention on smaller pore materials.*
- Retention ratio dominated by relative surface areas. Free diffusion in pores.

B. **<u>Medium molecule</u>**: *Similar retention on both materials.*
- Beginning of restricted access into 90 Å pores; limiting effective surface area and stationary phase access.

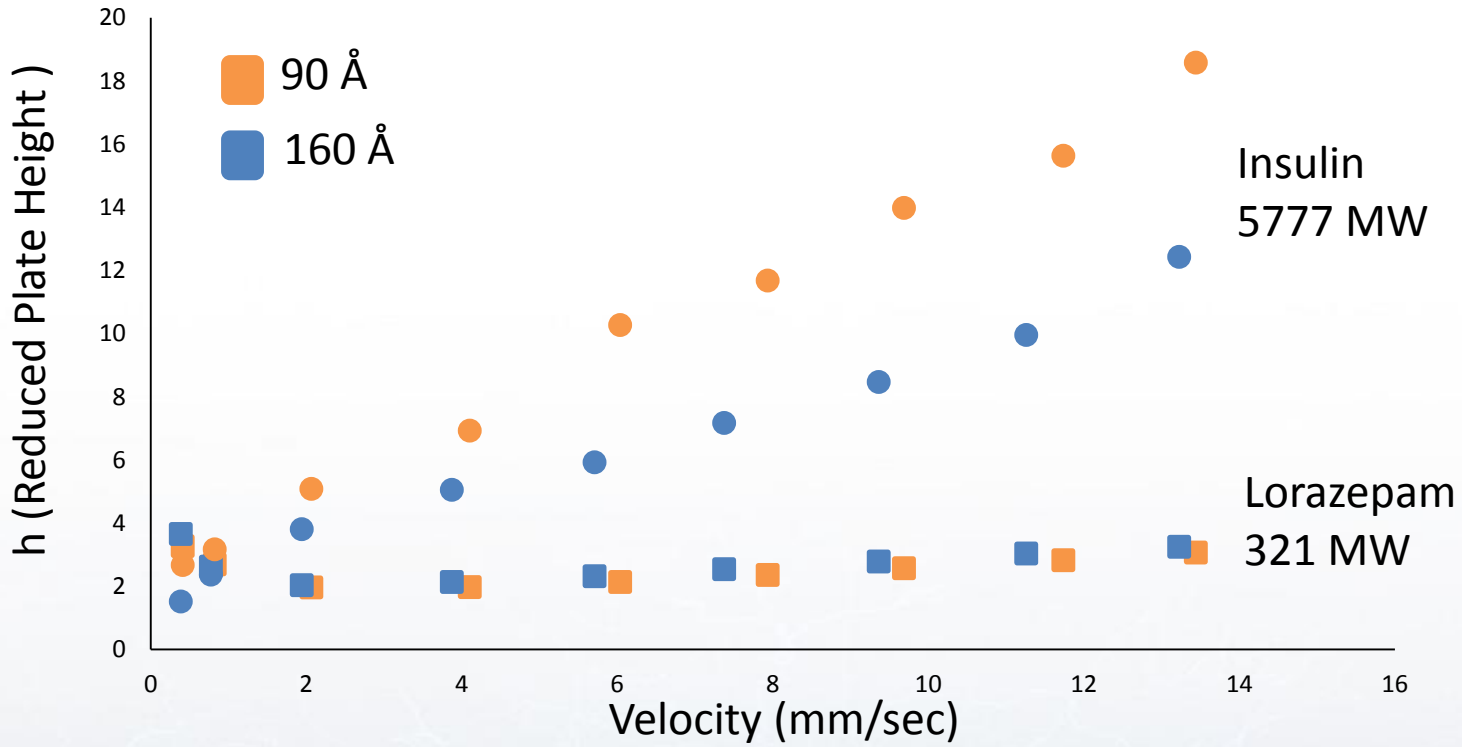
C. **<u>Larger molecule</u>**: *More retention on larger pore materials.*
- Surface area does not dominate retention; restricted access to bonded phase volume on the 90 Å pore material

Comparing Ratio of Relative Retention for 90 Å/160 Å

Measuring k' on two different columns (with same phase) is a valuable diagnostic. When retention factor ratio reaches one, analytes are roughly experiencing the identical amount of stationary phase interaction. Above 4,000 Da, the 90Å column shows lower effective surface area, which can interfere with free diffusion and create reduced column performance.



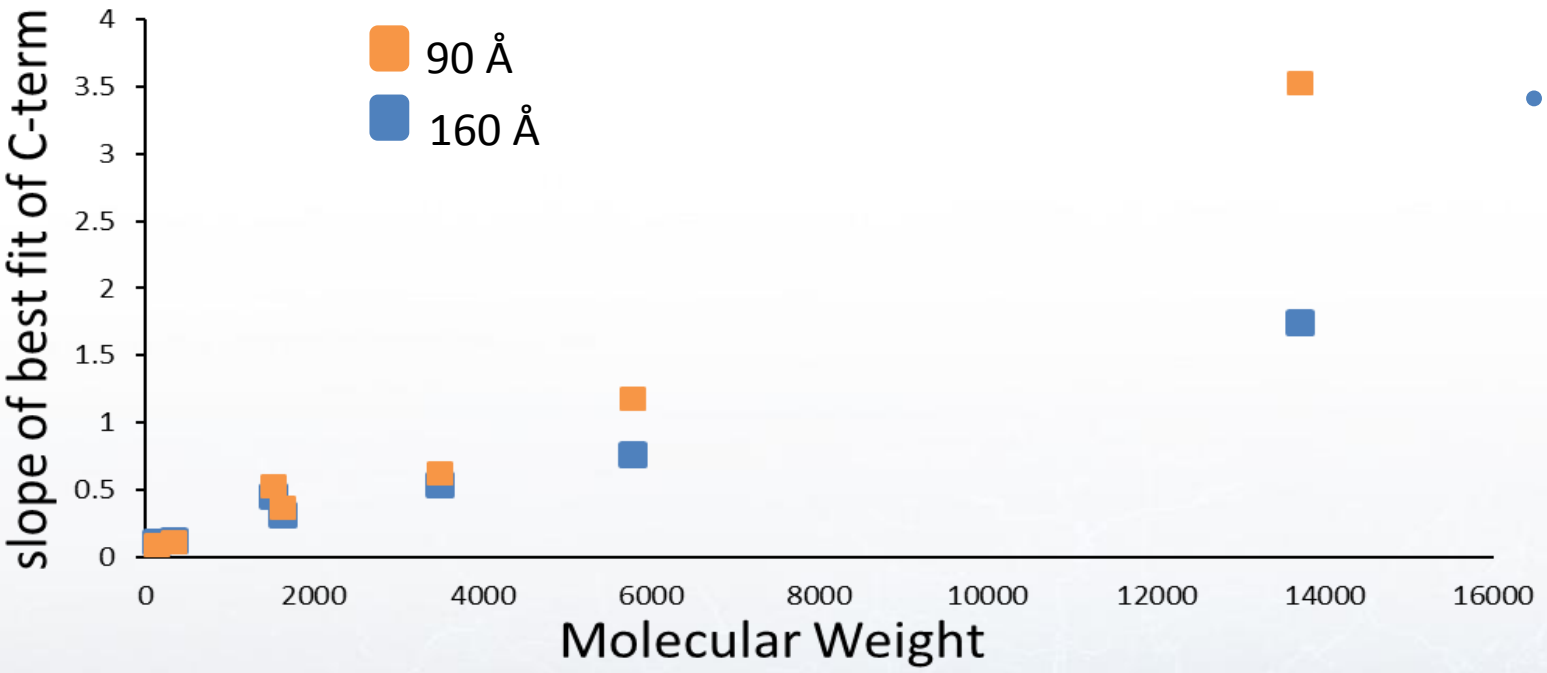
Exemplary van Deemter Plots



Plot of reduced plate height vs velocity for Insulin and Lorazepam. Nearly identical performance is seen for lorazepam (321 MW) while the 160 Å column shows distinct advantages for the larger molecule Insulin (5777 MW).

$$h = H(\mu m)/dp(\mu m)$$

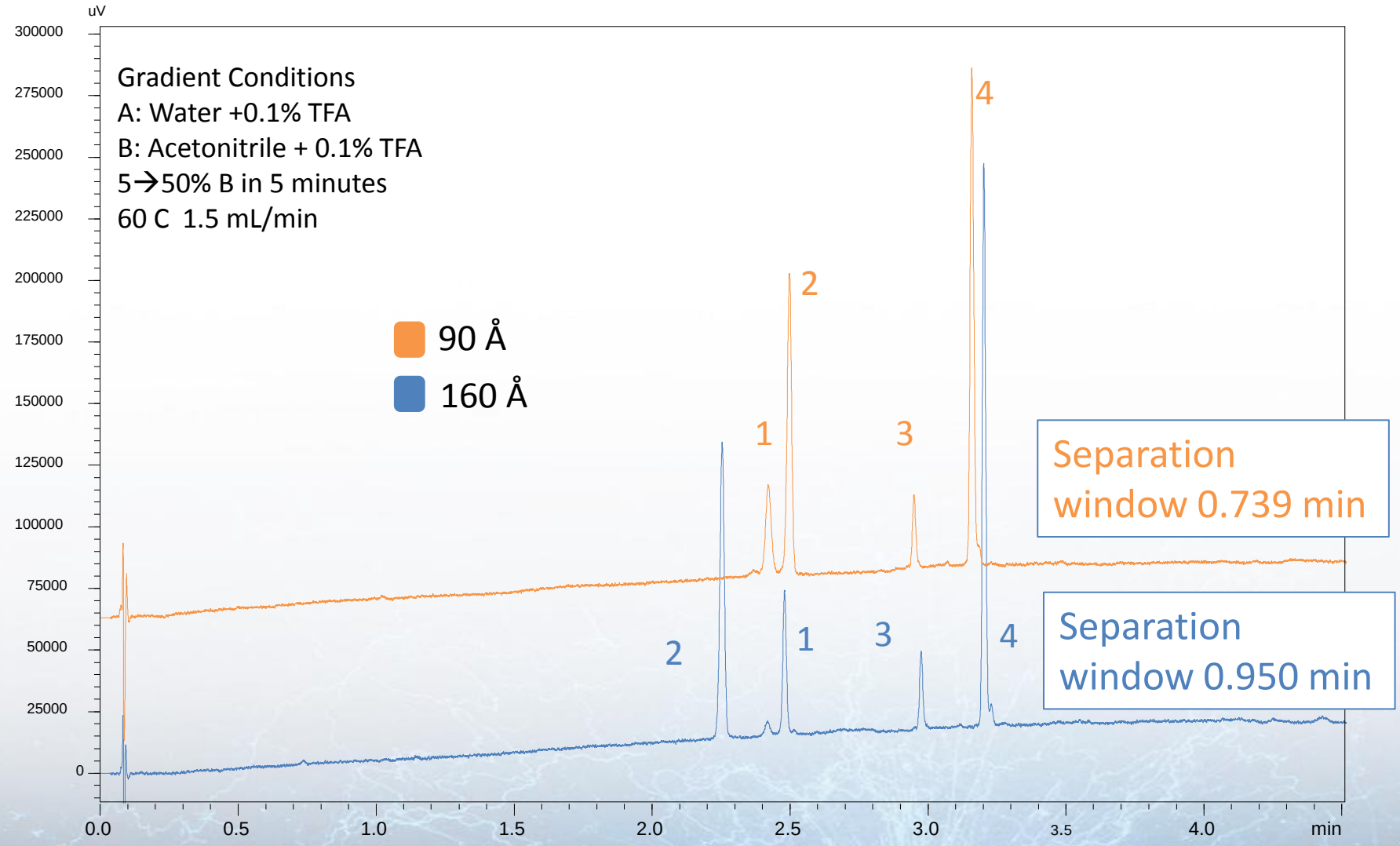
Comparing C-Term Region Slope for 90 Å/160 Å



- Significant efficiency deviation begins to occur at MW > 4000 for these compounds and particle geometries.

Plot of the slope of the best fit for the C-term vs MW. Nearly identical performance is seen at very low MW. As MW increases, performance differences become exaggerated. Significant deviation occurs at a MW > 4000 for these compounds.

Pore Mismatch Affects Gradient Performance.



Compound (MW or Da)

1. Ribonuclease A, 13,700
2. Lorazepam, 321
3. Insulin Chain B, 3496
4. Insulin Chain B, 5777

- HALO 160 Å Phenyl-Hexyl produces greater peak capacity for this sample mixture.

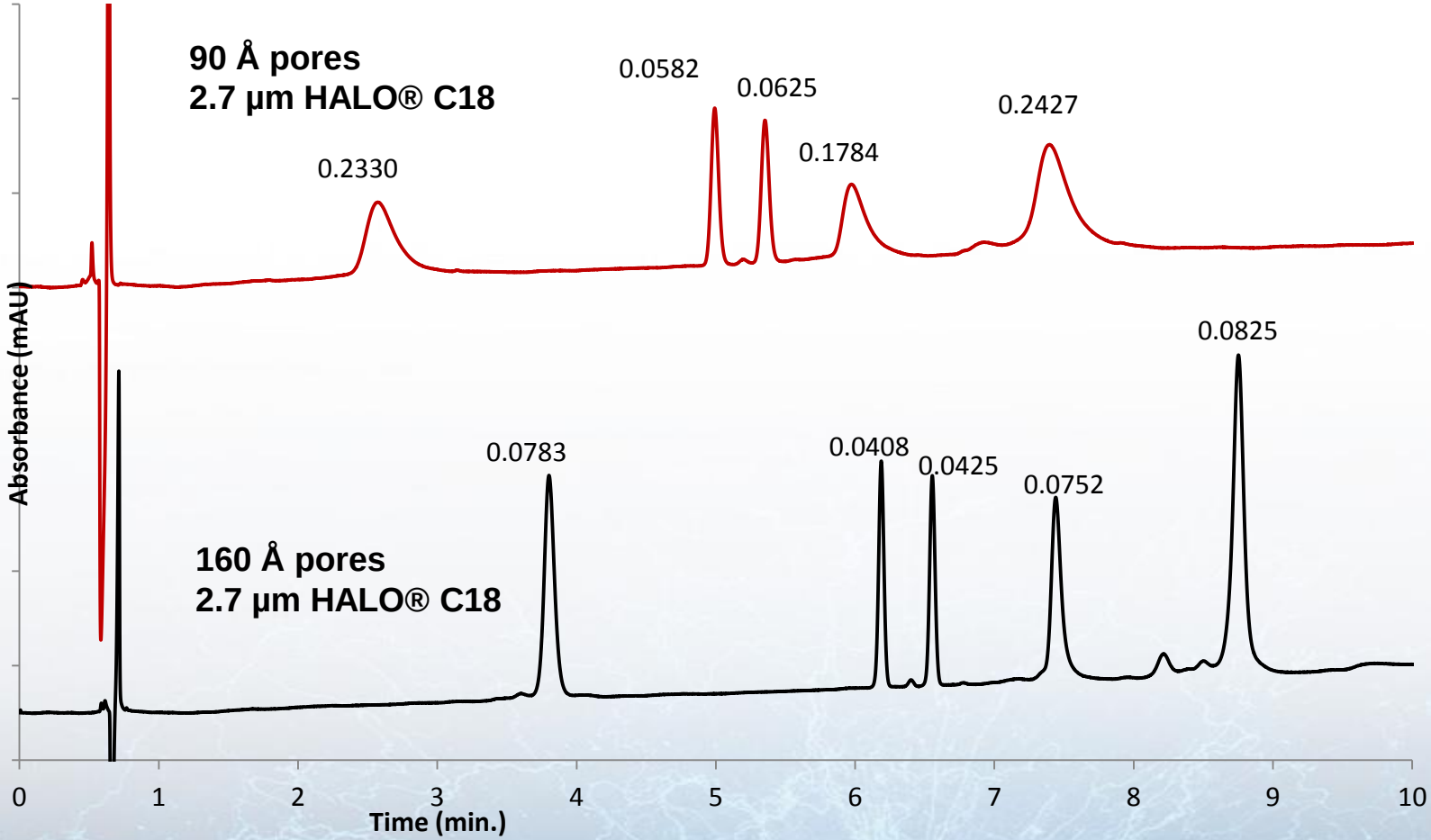
Summary of Gradient Performance When MW Varies Within Sample

			90 Å			160 Å		
			Retention	USP		Retention	USP	160 Å Percent
Compound	MW	Peak #	Time	Width	Peak #	Time	Width	Improvement
Ribonuclease A	13,700	1	2.421	0.038	2	2.480	0.023	+ 39
Lorazepam	321	2	2.497	0.030	1	2.254	0.030	0
Insulin Chain B	3496	3	2.949	0.022	3	2.976	0.023	- 5
Insulin Chain B	5777	4	3.160	0.025	4	3.204	0.022	+ 12
Average Peak Width				0.029			0.025	+ 14
Separation Window			0.739			0.950		+ 29
Peaks/Window		26			38			+ 46

Peak capacity can be estimated by dividing total separation window by average peak width.

Example of HALO 90 Å Pores Being Too Small for Large Peptides[3]

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.



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)

- HALO 160 Å ES-C18 produces greater peak capacity for this sample mixture.

Conclusions

- The C-term increases as analyte size increases, exacerbated when smaller pore materials are used with larger MW analytes. Larger pore materials can mitigate performance loss. Retention properties transition from surface-area-dominated to exclusion-dominated as MW increases, a different separation mechanism.
- Choosing larger pore-size can increase the resolution window and peak capacity under all conditions, when samples are too large or vary widely in MW.
- An optimum region exists for each sample and HPLC column where pores are sufficiently large to permit *full access* to stationary phase. This maximum resolution window of separation opportunity can be confirmed by introducing small molecules, including a marker like uracil, with the largest molecule in the sample and screening candidate columns with a fast gradient from low to high organic in RPLC.

References and Acknowledgements

References:
1. S.A. Schuster et al., J. Chromatogr. Sci., 48 (2010) 566-571.
2. B.M. Wagner et al., J. Chromatogr. A, 1489 (2017) 75-85.
3. J.J. Kirkland et al., J. Pharm. Anal. 3 (2013) 303–312.
4. R.A. Henry, S.A. Schuster, American Lab., June/July (2017) 1-4.

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