Improving Biomolecule Separations with Superficially Porous Particles of Silica

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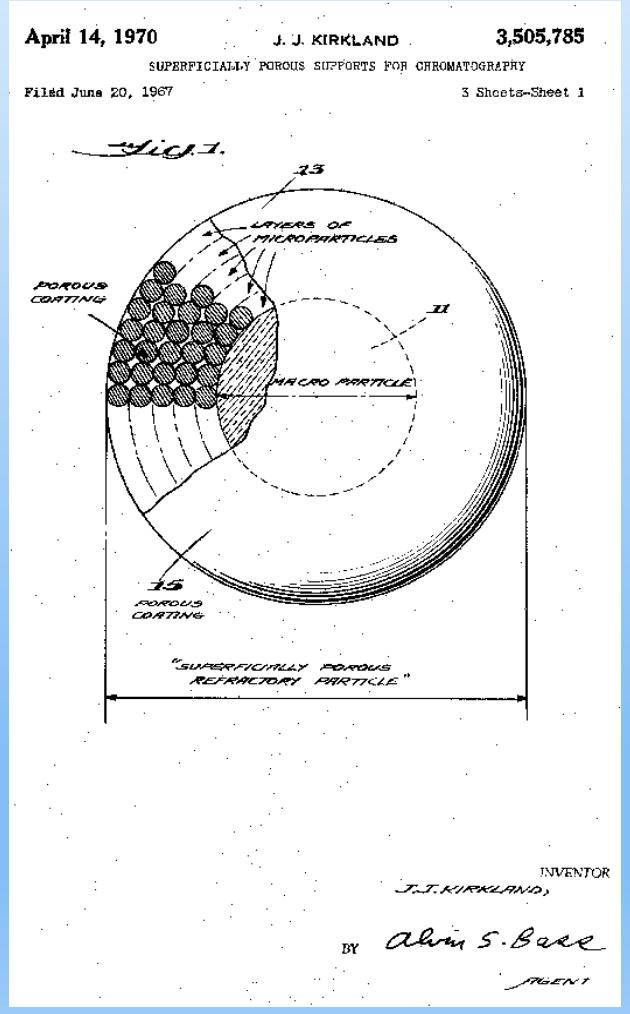
The Early Days -Conceptual



3,505,785

SUPERFICIALLY POROUS SUPPORTS FOR CHROMATOGRAPHY

Joseph J. Kirkland, Wilmington, Del., assignor to E. I. du Pont de Nemours and Company, Wilmington, Del., a corporation of Delaware

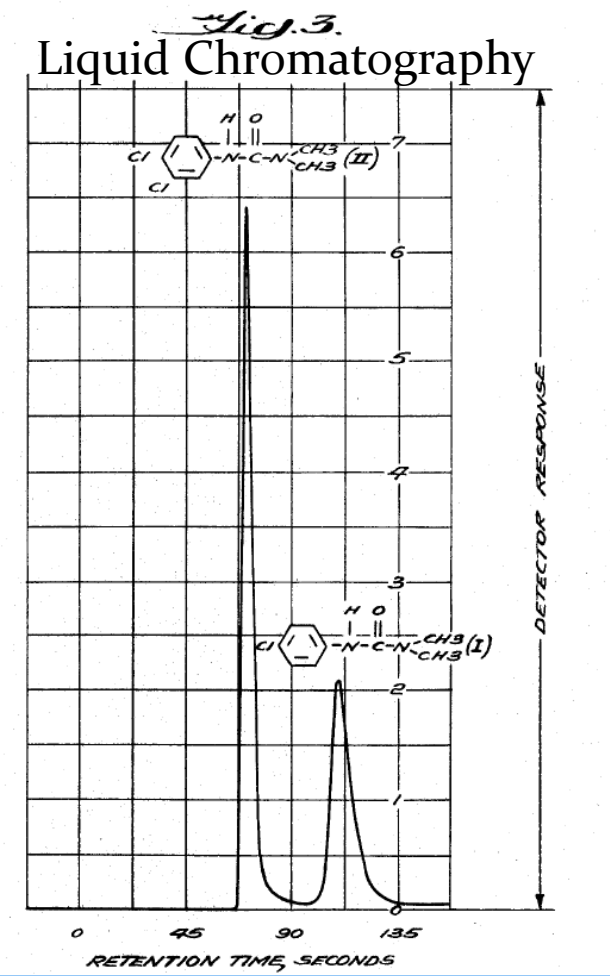
Filed June 20, 1967, Ser. No. 647,506
Int. Cl. B01d *15/08*
U.S. Cl. 55—67 8 Claims

ABSTRACT OF THE DISCLOSURE

This invention relates to an improvement in chromatography and chromatographic columns. A novel packing of superficially porous refractory particles for use in chromatography has been prepared consisting of a plurality of discrete macroparticles with impervious cores and having irreversibly joined thereto a coating of a series of sequentially adsorbed like monolayers of like colloidal inorganic microparticles. The coating is characterized by being uniform and of predetermined thickness. In preferred embodiments, the cores would be ceramics, preferably glass spheres, and the coating would consist of monolayers of colloidal refractory particles, preferably silica, in a structure of predetermined thickness and porosity.

The Early Days - Practice

Liquid Chromatography

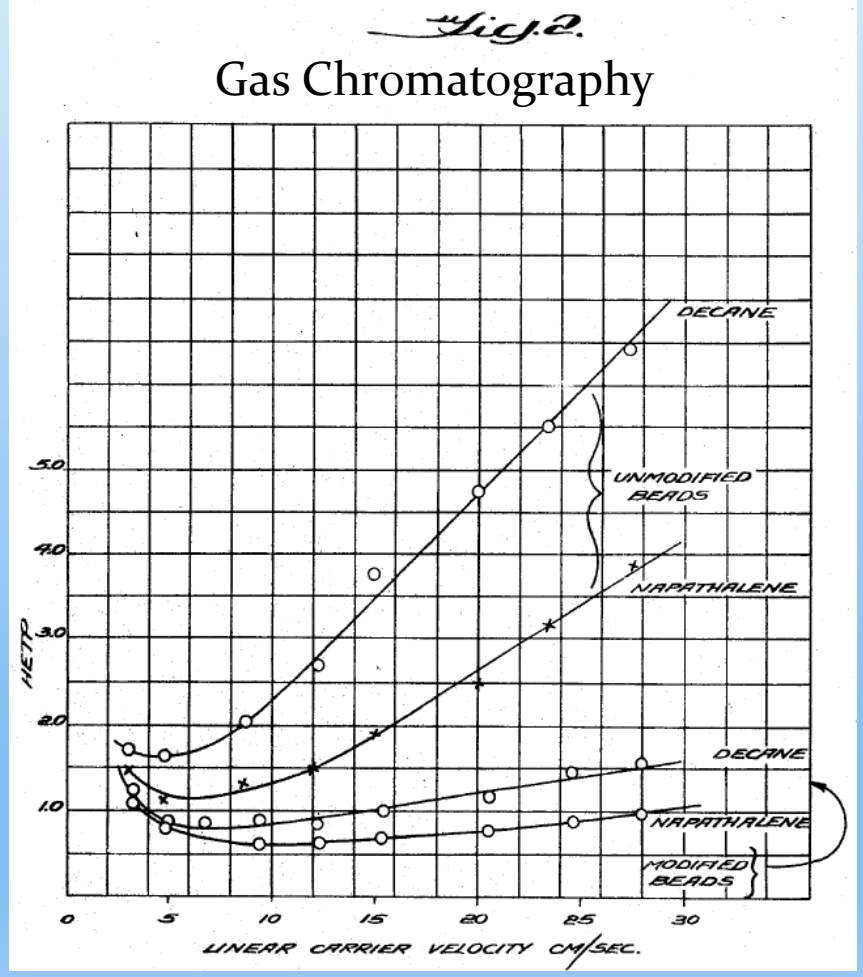


Diuron

Monuron

1.4 mm x 500 cm

Gas Chromatography



The Early Days – Practice at E.I. DuPont Company

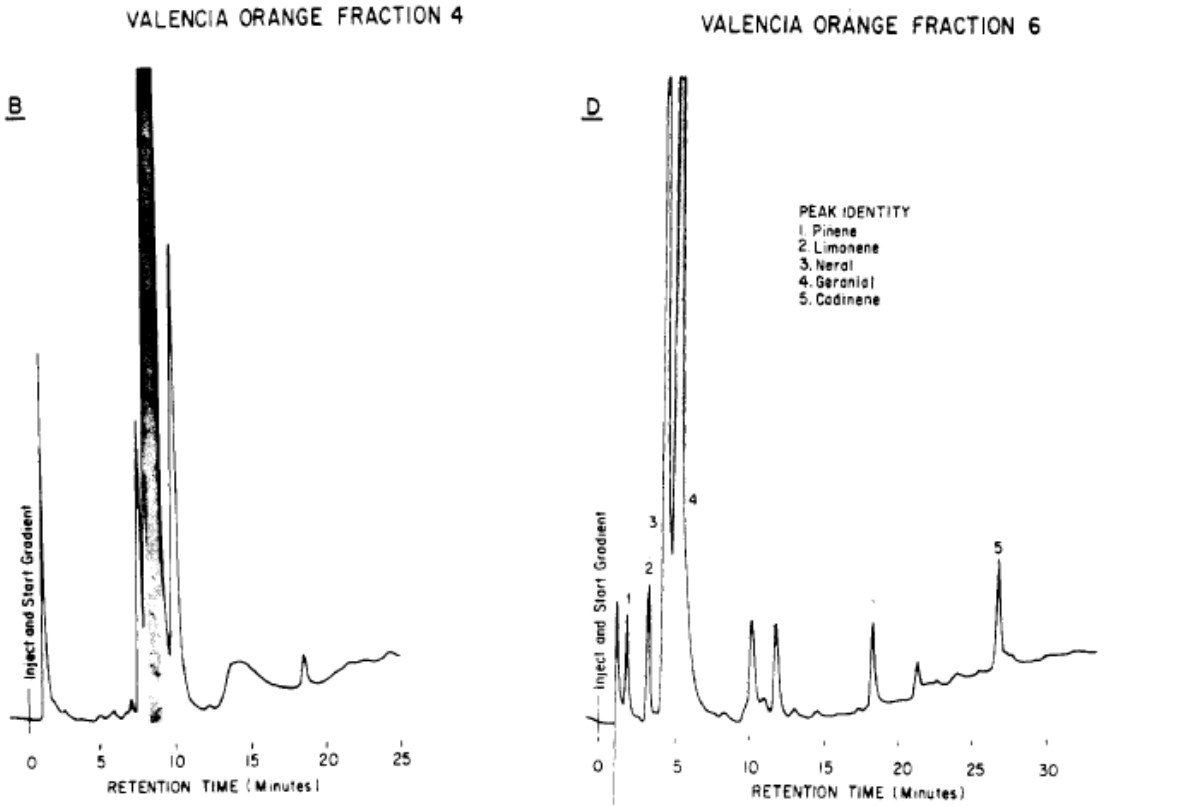


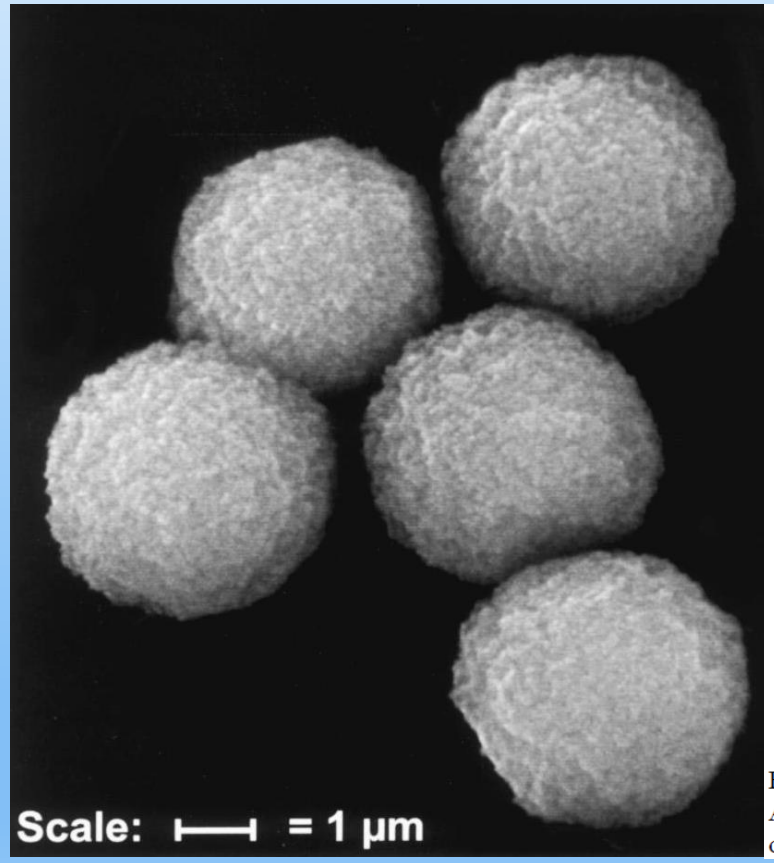
Figure 3. Reversed-phase liquid chromatographic separations of the total cold-pressed oil and collected fractions. Operating conditions: column, 1 m × 2.1 mm; Permaphase ODS mobile phase, linear gradient from 5% MeOH/95% H_2O to 100% MeOH at 3%/min; column temp, 50°; flow rate, 1.5 ml/min; detector, uv photometer.

J. Agr. Food Chem., Vol. 21, No. 4, 1973 **553**

PermaPhase ODS Reversed Phase
2.1 x 1000 mm; 50°C; 1.5 mL/min
5-100% MeOH @3%/min

JA Schmit, RC Williams, RA Henry
J.Agr.FoodChem. 21 (1973) 551-556.

The Middle Days – “Best is the enemy of better.”



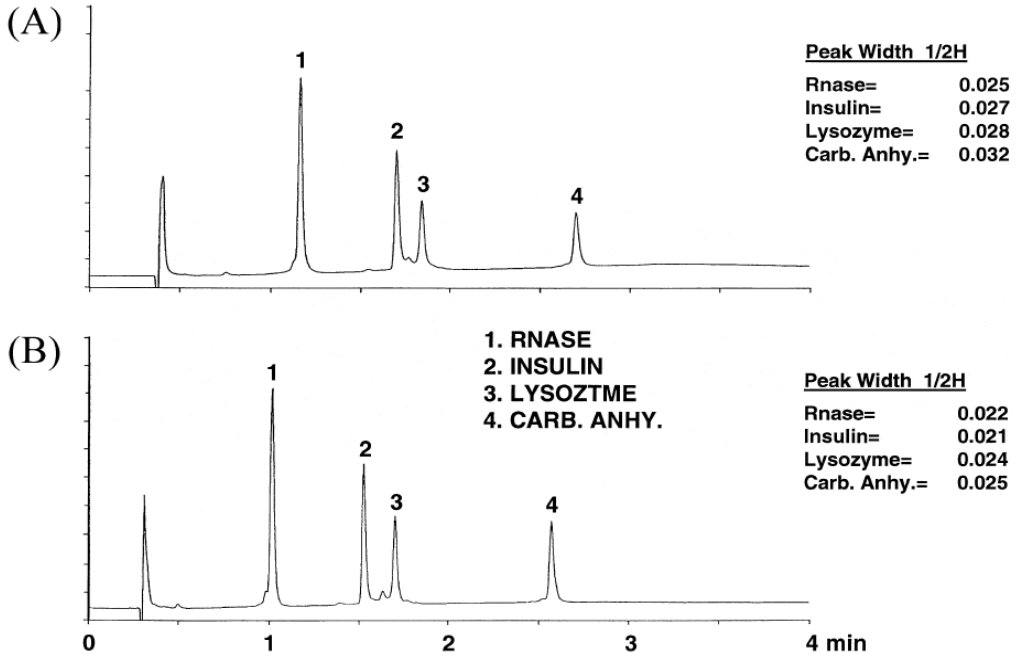
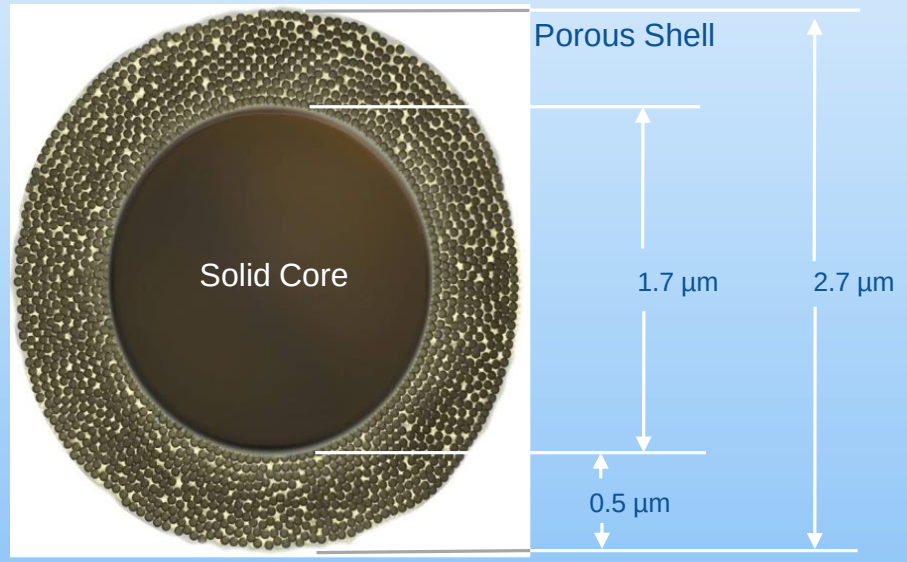
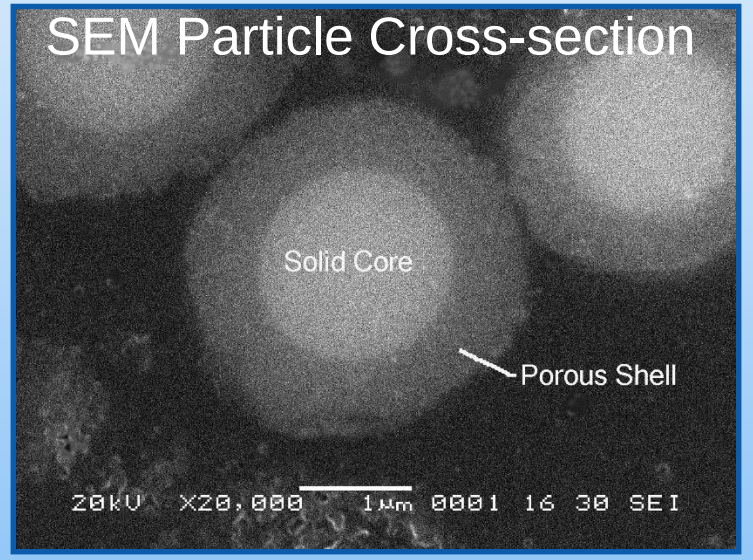


Fig. 6. Effect of porous shell thickness on protein separations. Columns: 150×4.6 mm, 5-µm Poroshell 300 SB-C_{18}; mobile phases: A=0.1% aqueous trifluoroacetic acid, B=0.09% aqueous trifluoroacetic acid; gradient: 23–53% B in 2.5 min; flow rate: 4.0 ml/min; UV detector: 215 nm; temperature: 60°C. Upper plot: 1-µm porous shell; lower plot: 0.25-µm porous shell.

Superficially Porous Particles (SPP/90Å): 2006/7



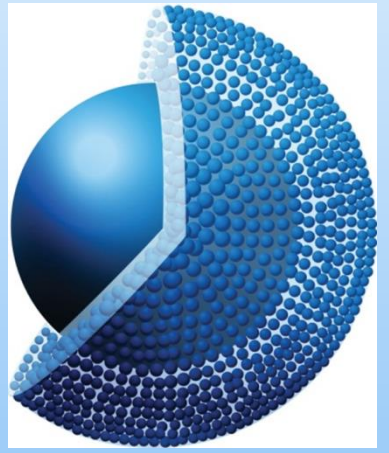


- Low back pressure due to the particle design (solid core with a porous shell)
- No need for specialized HPLC equipment
- Not necessary to filter samples and mobile phase since frits are not as small as needed for sub-2-µm
- High resolution is maintained at high flow rates (flat C-term in van Deemter plot)

J.J. Kirkland, T. Langlois, J. DeStefano, Fused core particles for HPLC columns, Am. Lab. 39 (2007) 18–21.

Wide Pore SPP Can Fit the Needs for Protein Science

What is needed for high performance separations of larger (Bio) molecules?

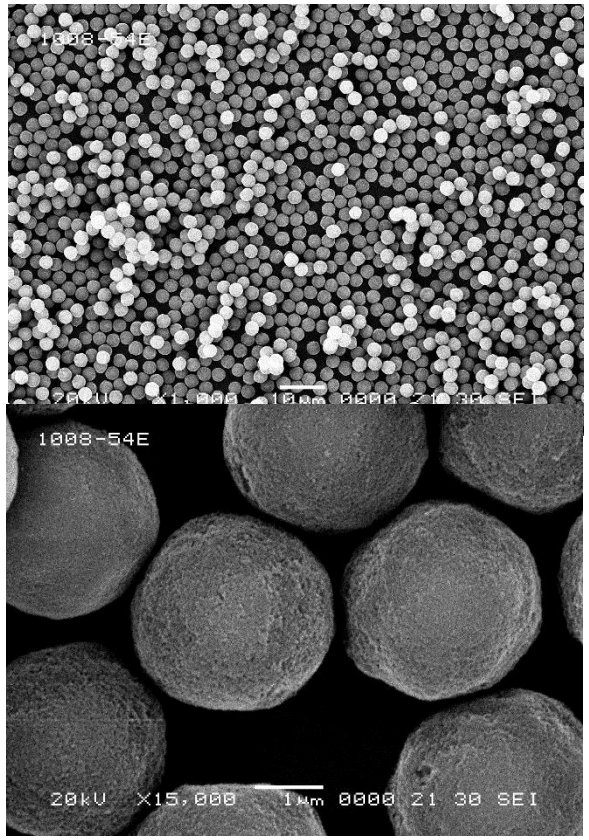


- **Pore size must "fit" molecule size**
  - **Restricted diffusion limits efficiency and load capacity**
  - **Peak capacity effects by kinetic and retention limitations**
- **Particle morphology must optimize surface area/volume**
  - **Shell thickness determines diffusion path and surface area**
  - **Must have "Right" size and desirable particle distribution**
- **Surface chemistry appropriate to samples**

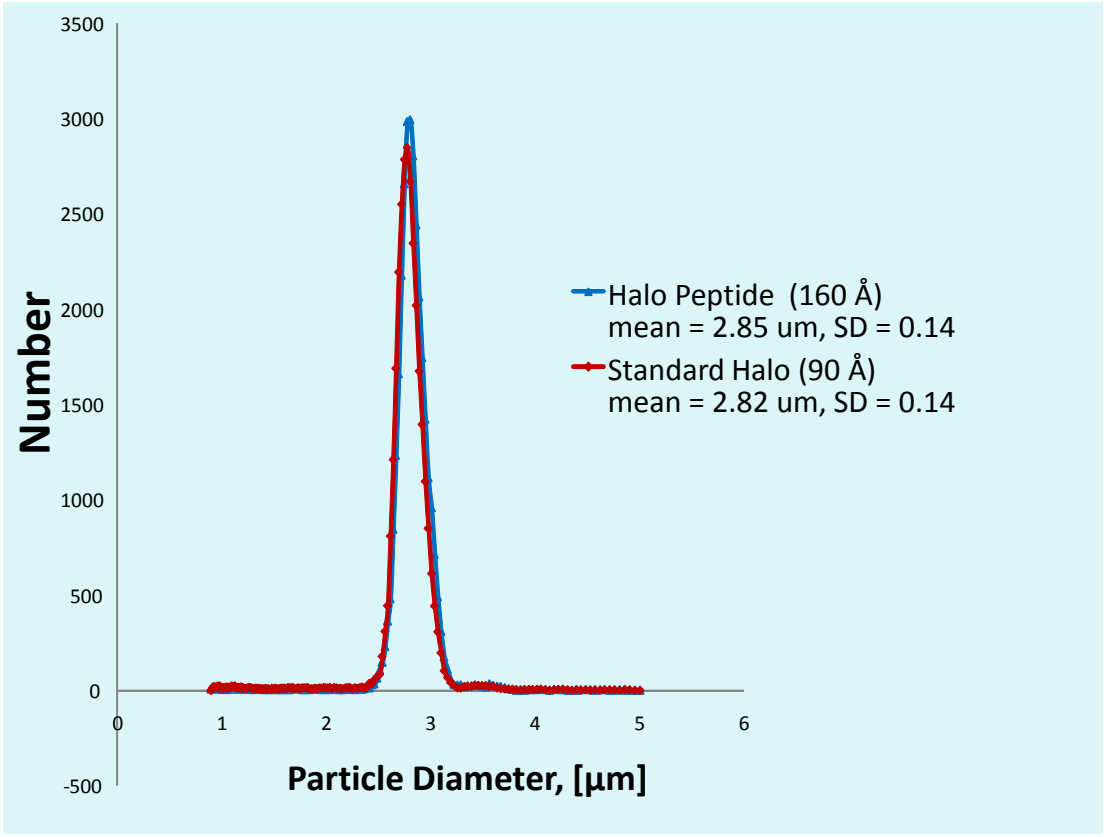
"Everything is a compromise."

Halo Peptide Fused-Core Particle Analysis

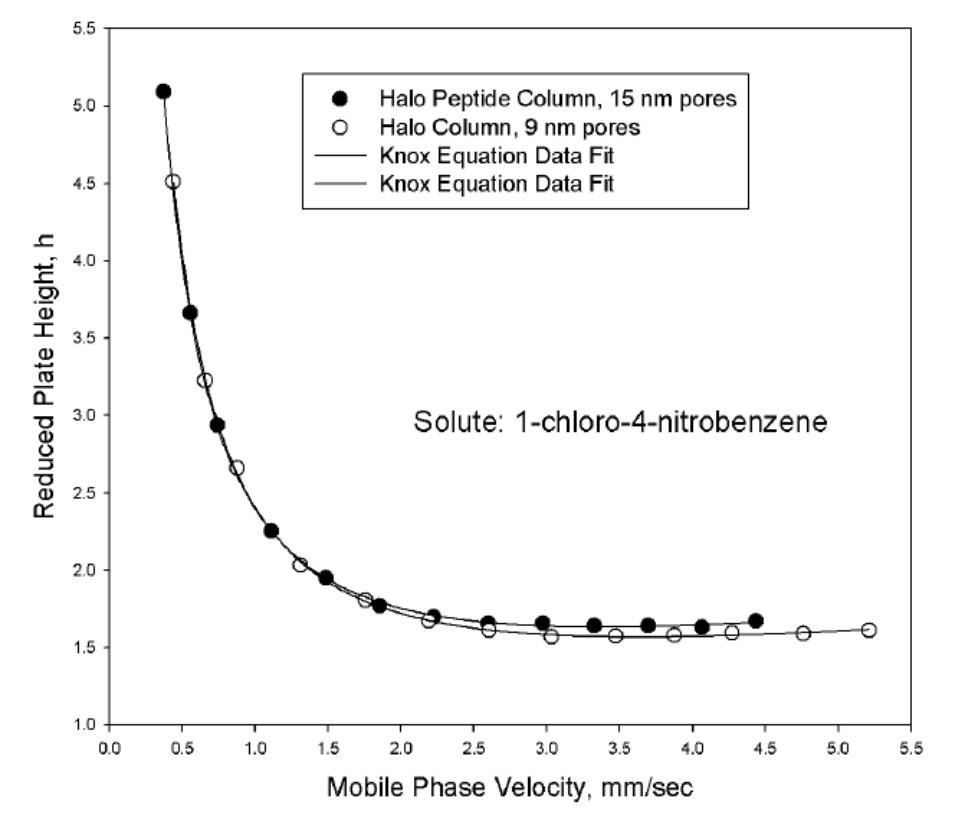
Electron Micrographs of Halo Peptide



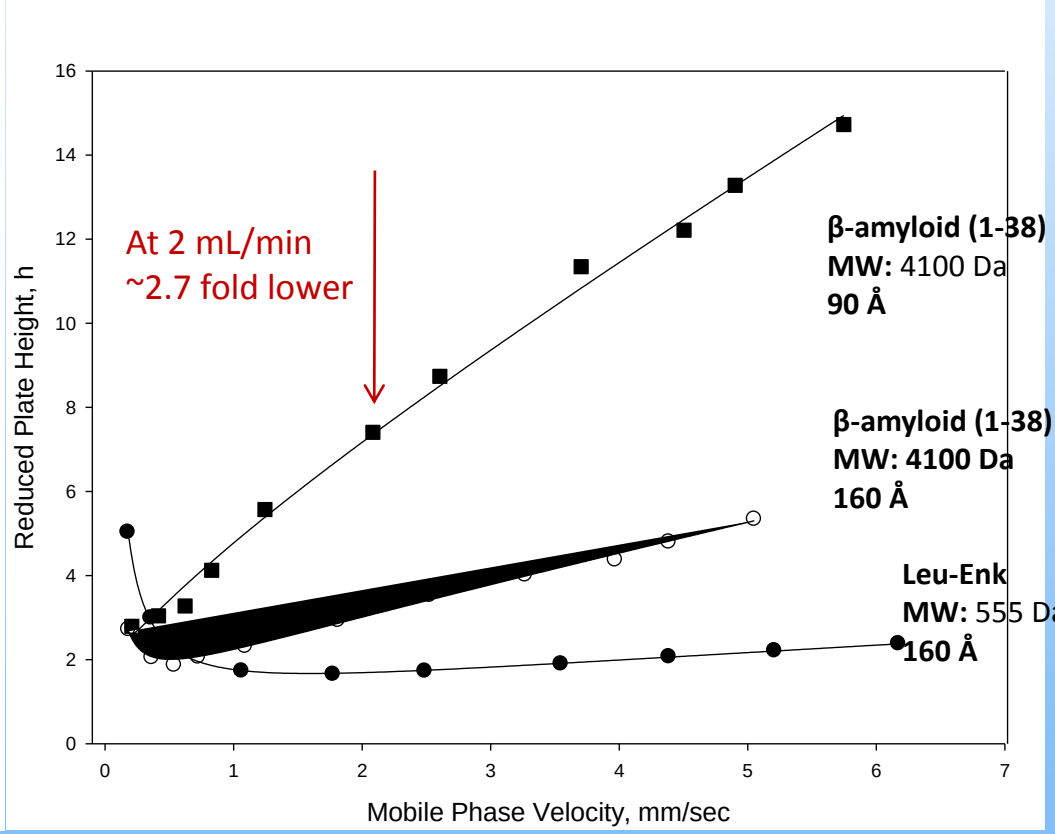
Standard Halo vs. Halo Peptide



Halo Peptide Column Efficiency



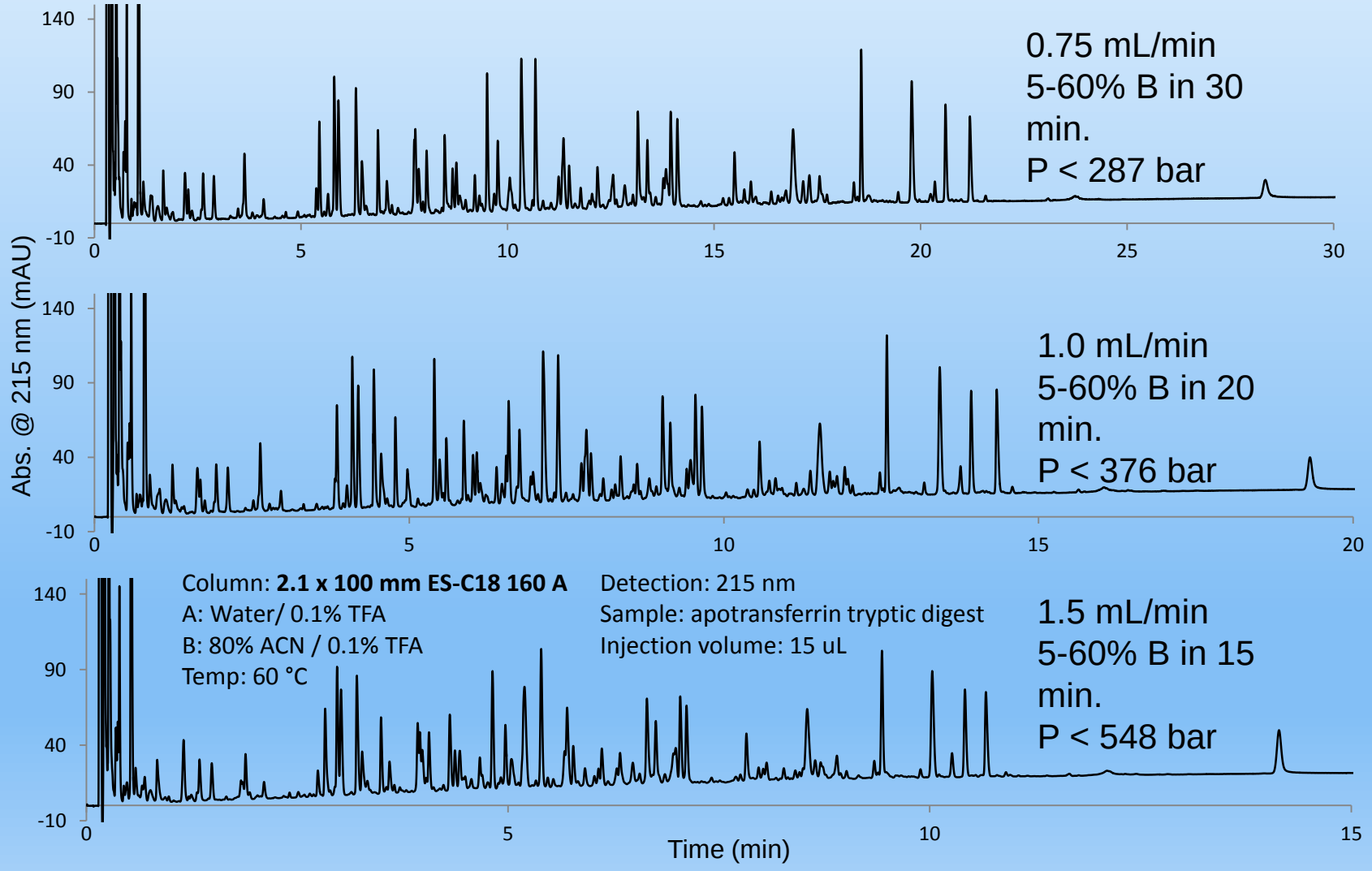
Columns: 4.6 x 100 mm; Particle size: 2.7 µm
Mobile Phase: 50% ACN/50% water/0.1% TFA



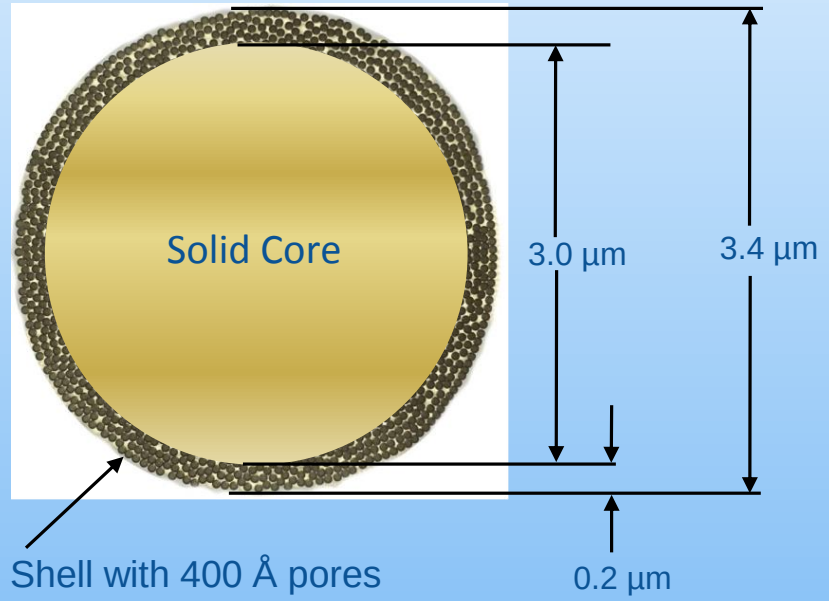
Columns: 4.6 x 100 mm; Particle size: 2.7 µm;
Mobile Phase: Leu-Enk: 21% ACN/79% Water/0.1% TFA
ß-amyloid (1-38) 160 Å : 29% ACN/71% Water/0.1% TFA
ß-amyloid (1-38) 90 Å : 27% ACN/73% Water/0.1% TFA

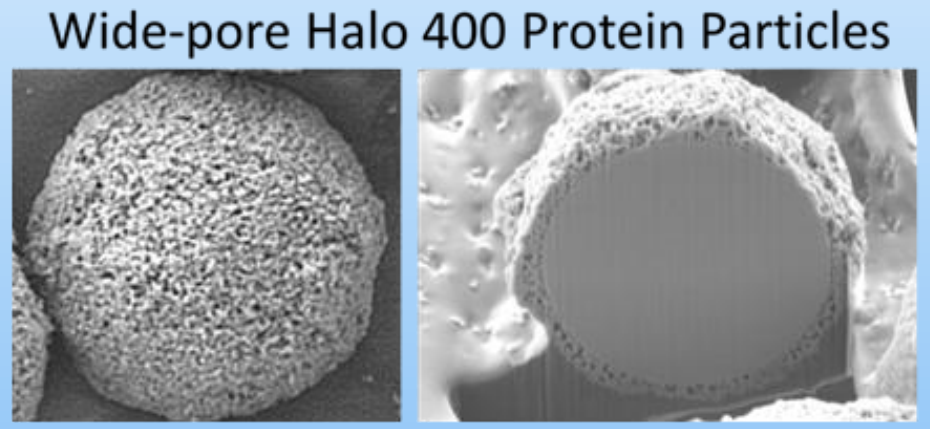
S.A. Schuster, B.M. Wagner, B.E. Boyes, Kirkland, J.J Wider pore superficially porous particles for peptide separations by HPLC, J. Chromatogr. Sci. 48 (2010) 566–571.

High Speed Separation of apo-Transferrin Tryptic Digest



Superficially Porous (Fused-Core®) Wide Pore Particles: 400 Å

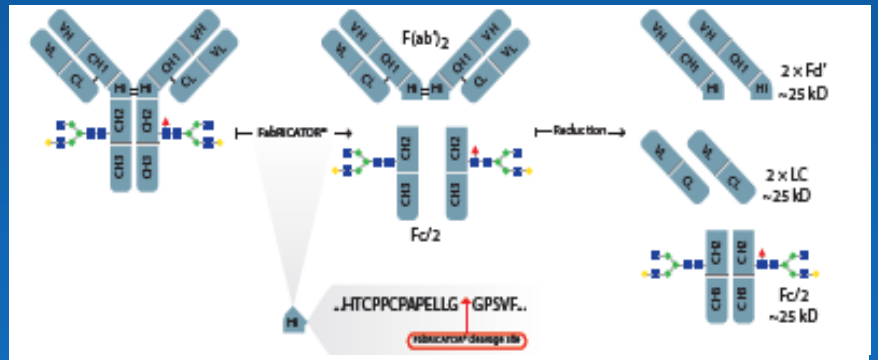




- Example above is 3.4 µm particle/400 Å pore size
- Many variations in shell thickness, pore size and particle size have been studied
- Theory to support “best properties” is complex, with limited tests using proteins, particularly with larger proteins
- Look for compromise in diffusion path for high MW molecules (to maintain small C-term), load tolerance, usability, speed and efficiency

S.A. Schuster, B.M. Wagner, B.E. Boyes, J.J. Kirkland, Optimized superficially porous particles for protein separations, J. Chromatogr. A 1315 (2013)118–126.

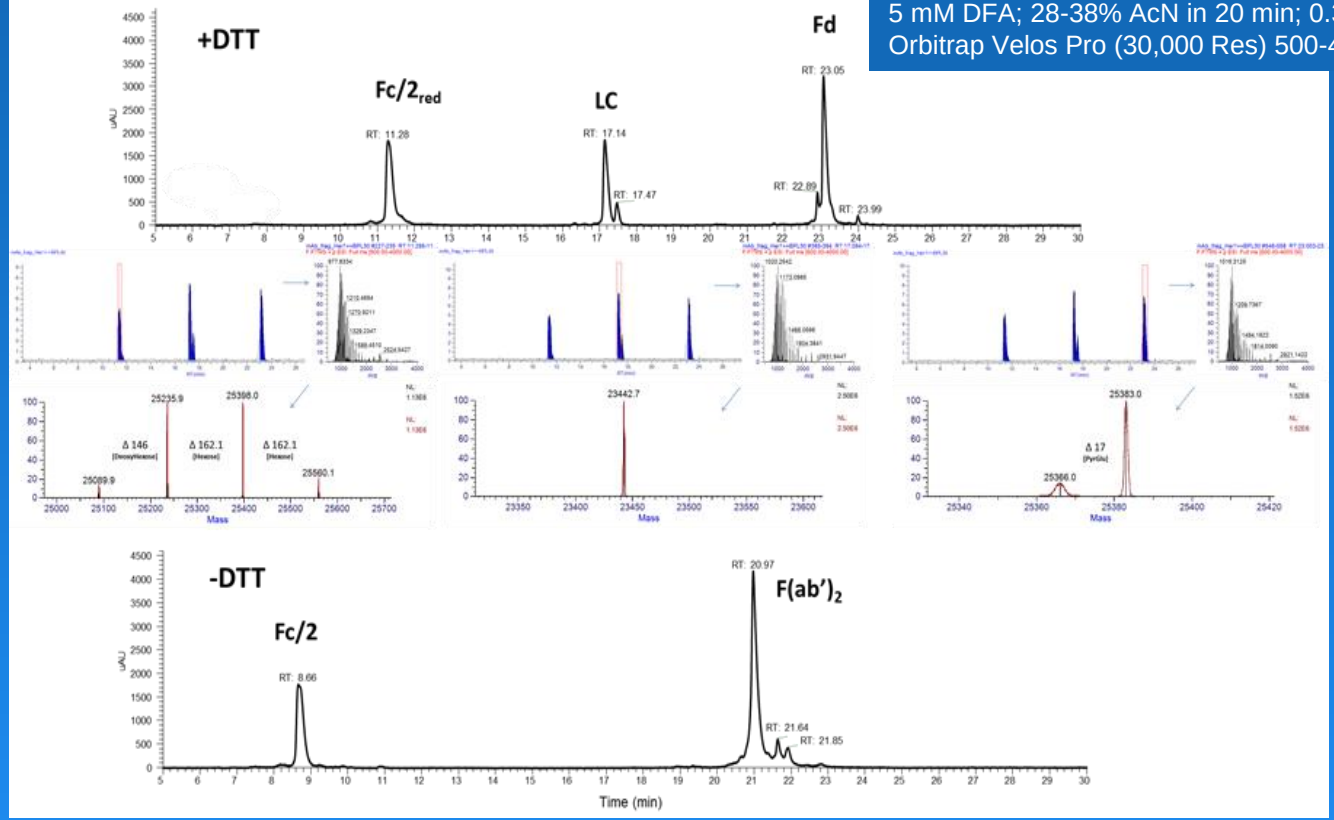
Fragments for mAb Structure: IdeS Digest



http://www.genovis.com/fabricator

An, Zhang, Mueller, Shameem & Chen (2014) A new tool for monoclonal antibody analysis, mAbs, 6:4, 879-893, DOI: 10.4161/mabs.28762

Halo Protein C4 400 Å, 2.1 mm ID x 150 mm;
5 mM DFA; 28-38% AcN in 20 min; 0.35 mL/min, 80 ºC;
Orbitrap Velos Pro (30,000 Res) 500-4000 m/z, +3.8 kV, 275 ºC capillary



High Resolution Separations for Protein LC/MS. ASMS 2016 556
B Boyes, B Libert, S Schuster, B Wagner, W Miles, J Kirkland

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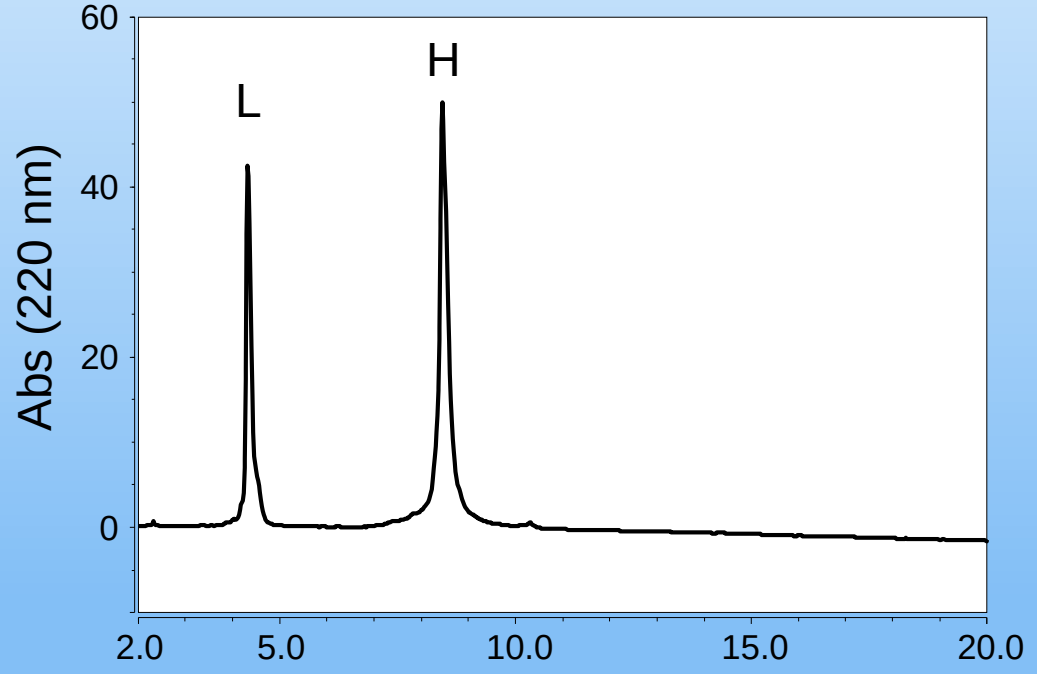
IgG H and L Chain Separations

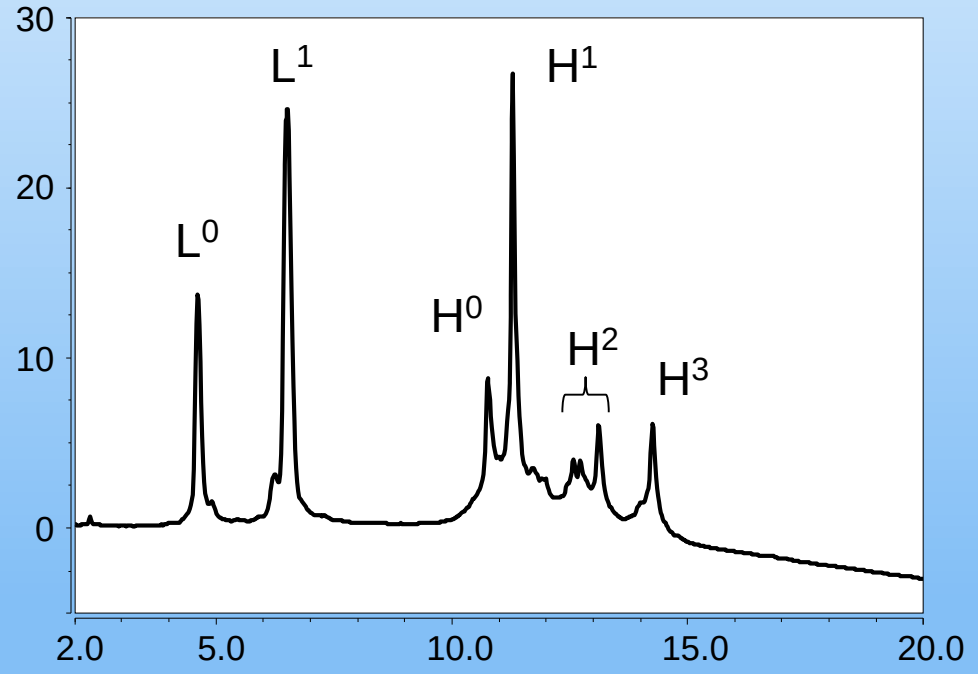
Column: HALO 400Å C4, 2.1 x 150 mm; Flow rate: 0.4 mL/min; Temp: 75 °C
Mobile Phase A: water/10 mM DFA; Mobile Phase B: AcN/ 10 mM DFA;
Gradient: 28.5-31.2%B 8 min; 31.2-45.8% in 12min
Instrument: Shimadzu Nexera/Abs (220nm); Orbitrap Velos Pro, 15k Res, ESI 3.8 kV
Injection Volume: 10 µL of mAb (5 µG) in 0.1% TFA Reduced and IAm alkylated Cys



Trastuzumab

L – 23,728 Da

H – 49,997 Da + Glycans (G_o, G_oF, G_1F, G_2F)

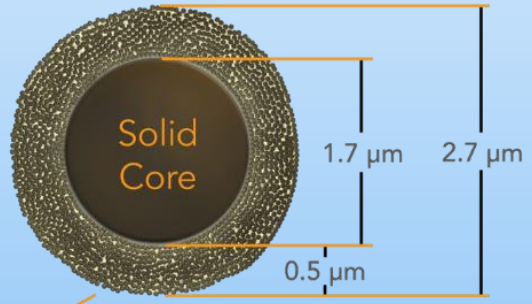


S/M MSQ8 ADC Mimic

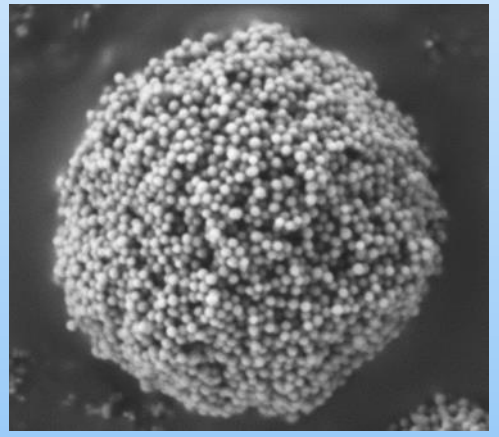
L^0 – 23,284 Da; $L1^1$ – 23,895

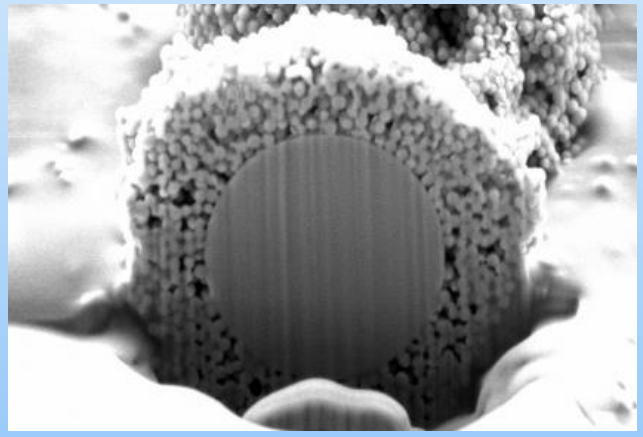
H^n – 49,585 Da + Glycans (G_0F, G_1F) + n(611 Da)

Superficially Porous (Fused-Core®) Wide Pore Particles: 1000 Å



Shell with 1000 Å pores


SEM


Section analysis by FIB-SEM

- 2.7 µm particle with 0.5 µm thick shell and 1000 Å pores
- Densely bonded C4 phase with endcapping
- Outstanding high temperature and low pH stability
- Surface area ~ 22 m^2/g
- Designed for larger proteins

Wagner, Schuster, Boyes, Shields, Miles, Haynes, Kirkland, and Schure. Superficially porous particles with 1000 Å pores for large biomolecule high performance liquid chromatography and polymer size exclusion chromatography J. Chromatogr. A 1485 (2017) 75–85.

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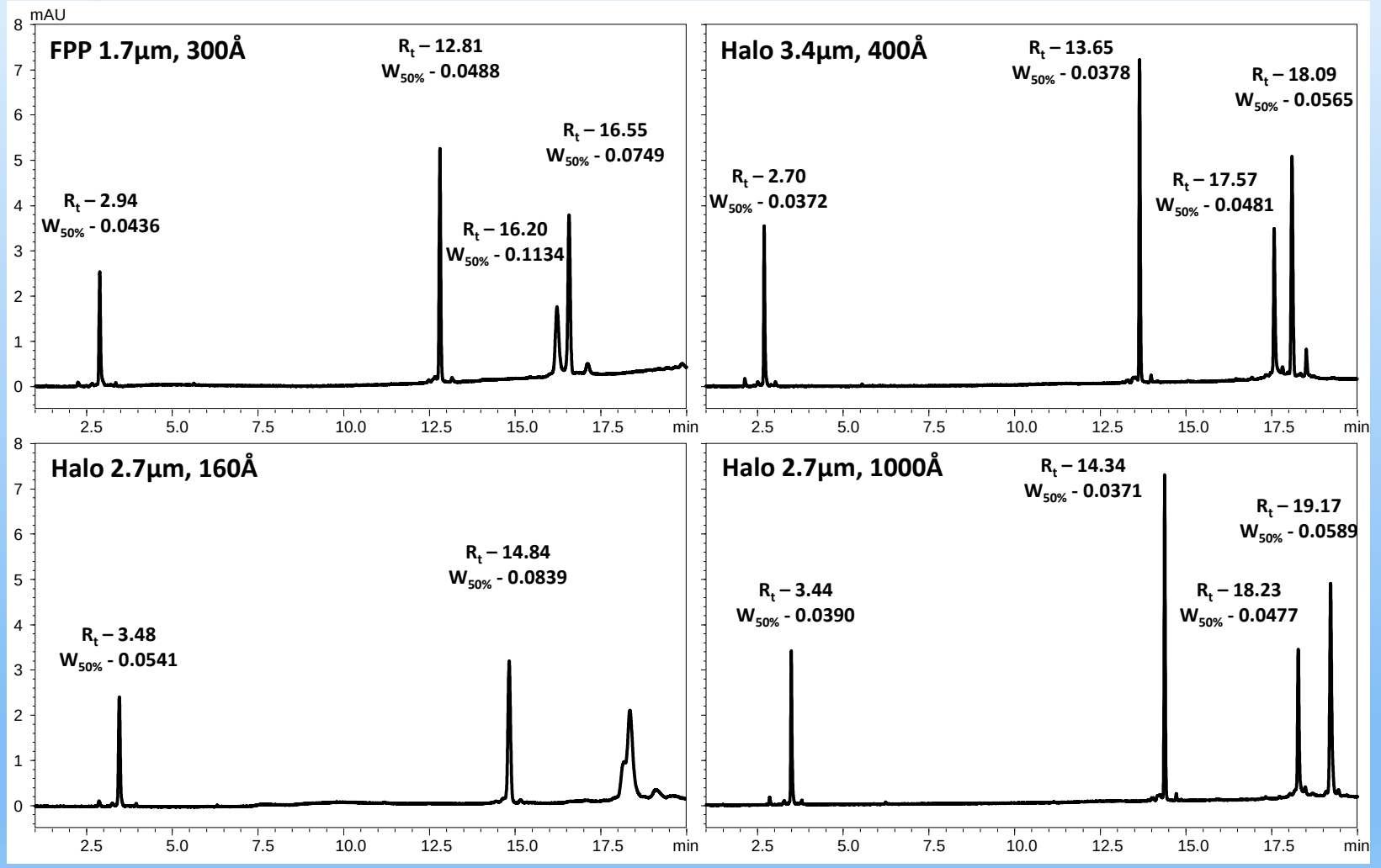
Protein Separation on Wide Pore SPP vs FPP

2.1 mm ID x 150 mm C4 columns
20-50% AcN/0.1% DFA in 24 min
Flow: 0.5 mL/min
Temp: 60°C
1.5 µL (0.15-0.2 ug each)

1. RNase A 13.7 kDa
2. α-Lactalbumin 14.2 kDa
3. Enolase 93.1 kDa
4. Carbonic Anhydrase 30.0 kDa



- Improvement in peak width and retention with larger pore SPP
- As protein size increases, peak widths decrease with increasing pore size
- Similar results in TFA and DFA as mobile phase acidic modifiers

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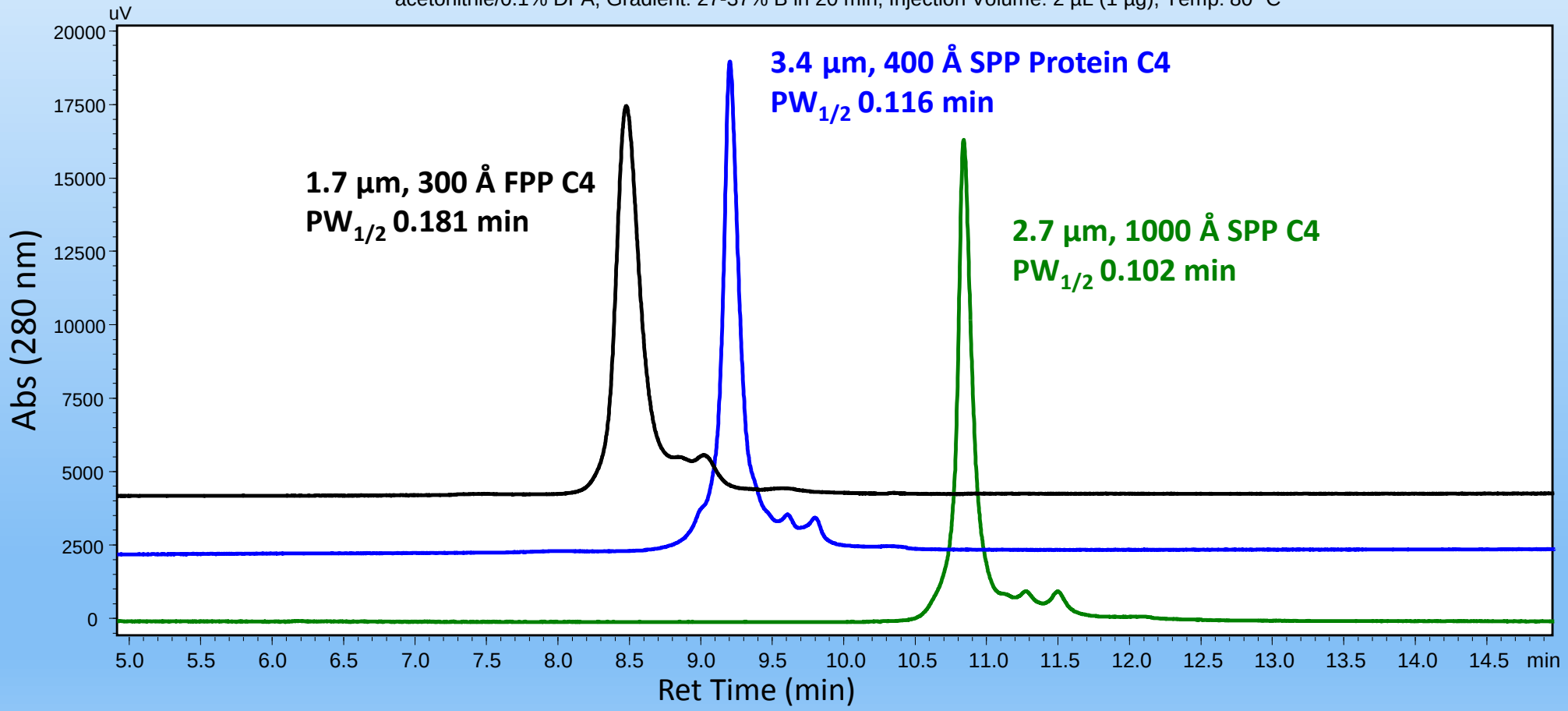
mAb IgG Separation on Wide Pore SPP vs FPP

High Efficiency Separation of Trastuzumab

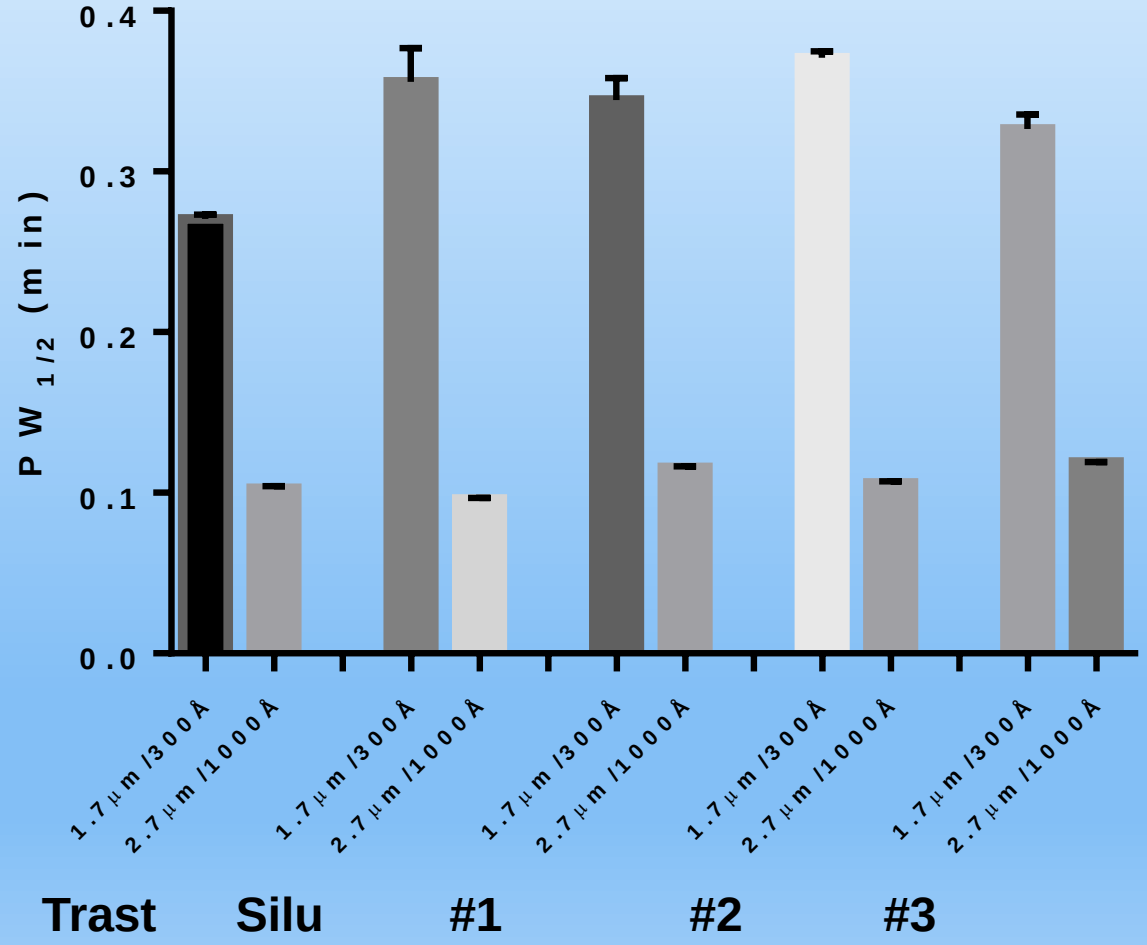
Columns: 2.1 x 150 mm; Flow rate: 0.4 mL/min; Mobile Phase A: water/0.1% DFA; Mobile Phase B: acetonitrile/0.1% DFA; Gradient: 27-37% B in 20 min; Injection Volume: 2 µL (1 µg); Temp: 80 °C



- Large improvement in peak width and *increased* retention with pore size for SPP, moderate additional improvement in peak width with 1000 Å pores

mAb IgGs Separation on Wide Pore SPP vs FPP

Columns: 2.1 x 150 mm; Flow rate: 0.4 mL/min; Mobile Phase A: water/0.1% DFA; Mobile Phase B: acetonitrile/0.1% DFA; Gradient: 27-37% B in 20 min; Injection Volume: 2 µL (1 µg); Temp: 80 °C



FPP : 0.334 min
SPP : 0.108 min

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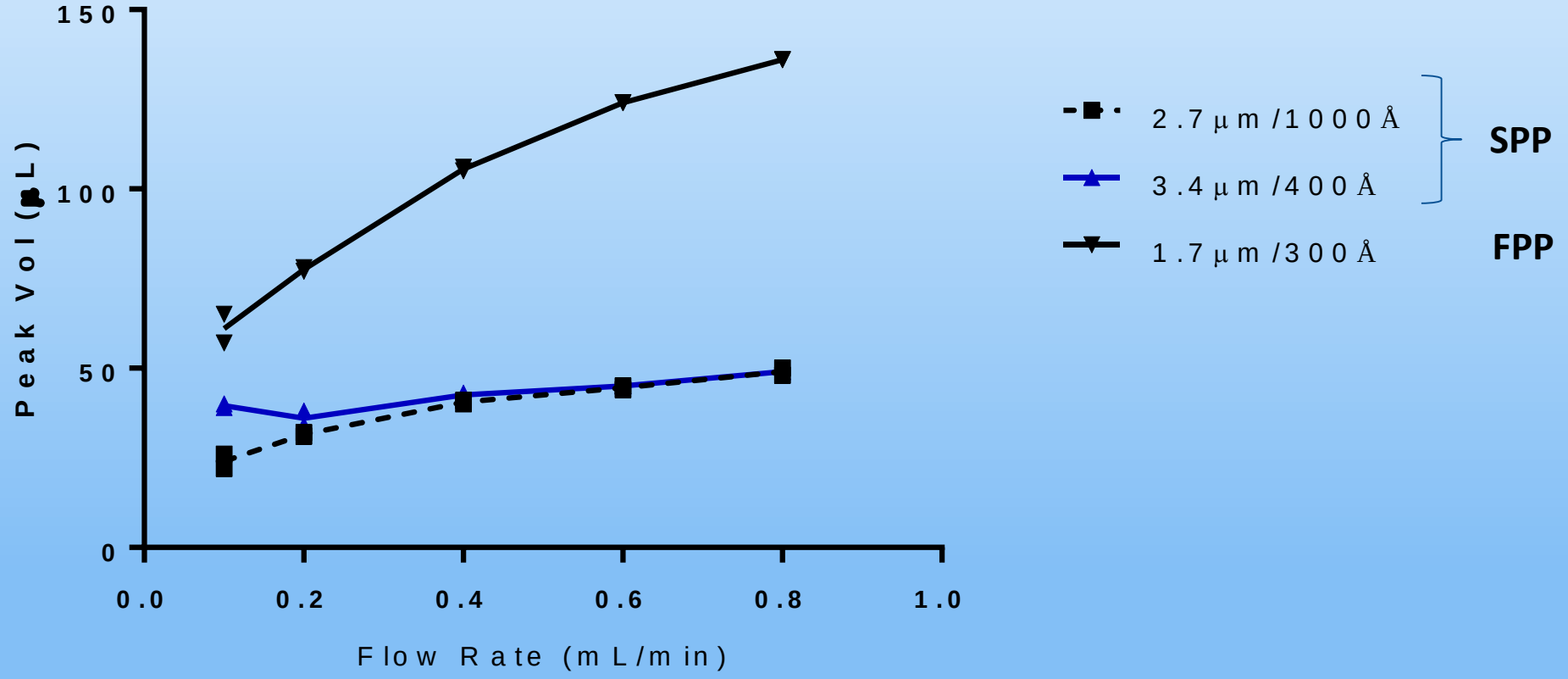
Flow Rate Effects on Peak Volume for mAb IgG

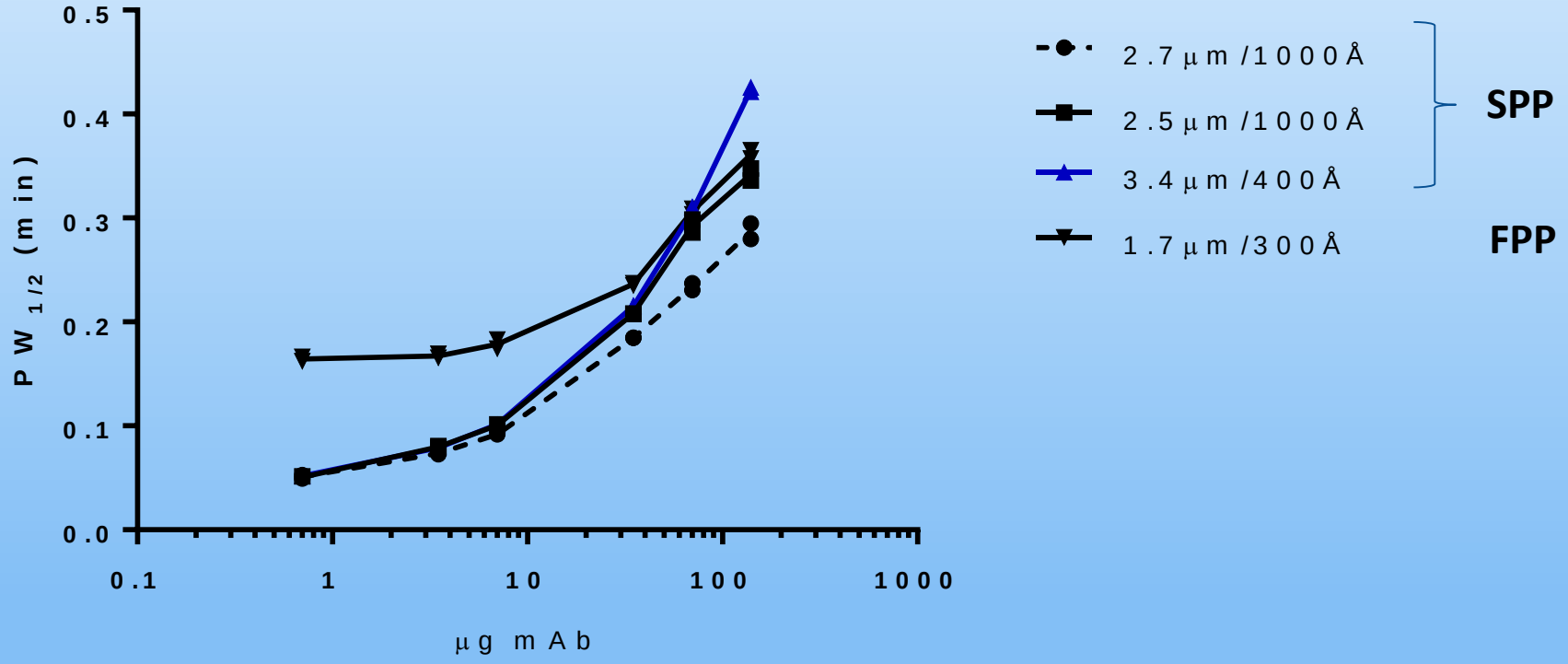
Fixed Volume Gradient Conditions (4.8 mL); Peak Volume = $PW_{1/2}$ x Flow Rate
Trastuzumab 0.5 µg; 29-35% AcN in 0.1% DFA; 80°C;



- Mass transfer is improved for the large pore SPP particles with higher MW protein.
- Trastuzumab and Silumab exhibited similar results

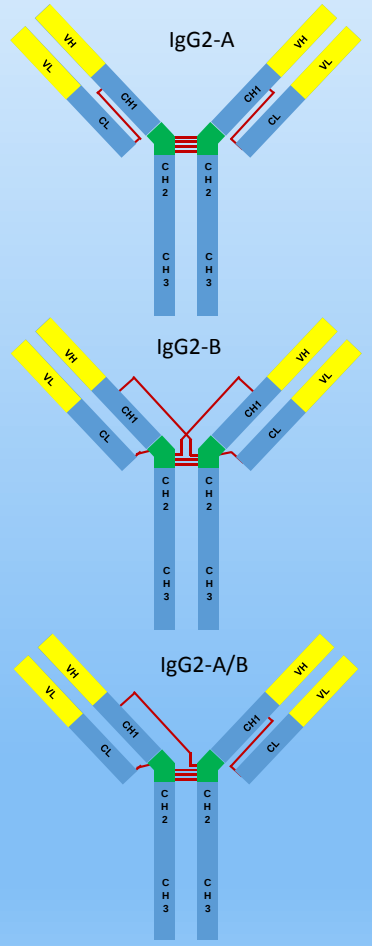
Load Effects on Peak Width for SPP and FPP for mAb IgG

2.1 mm ID x 150 mm C4 columns; Trastuzumab 0.7 – 140 µg;
27-37% AcN (0.1% DFA) in 10 min; 80°C

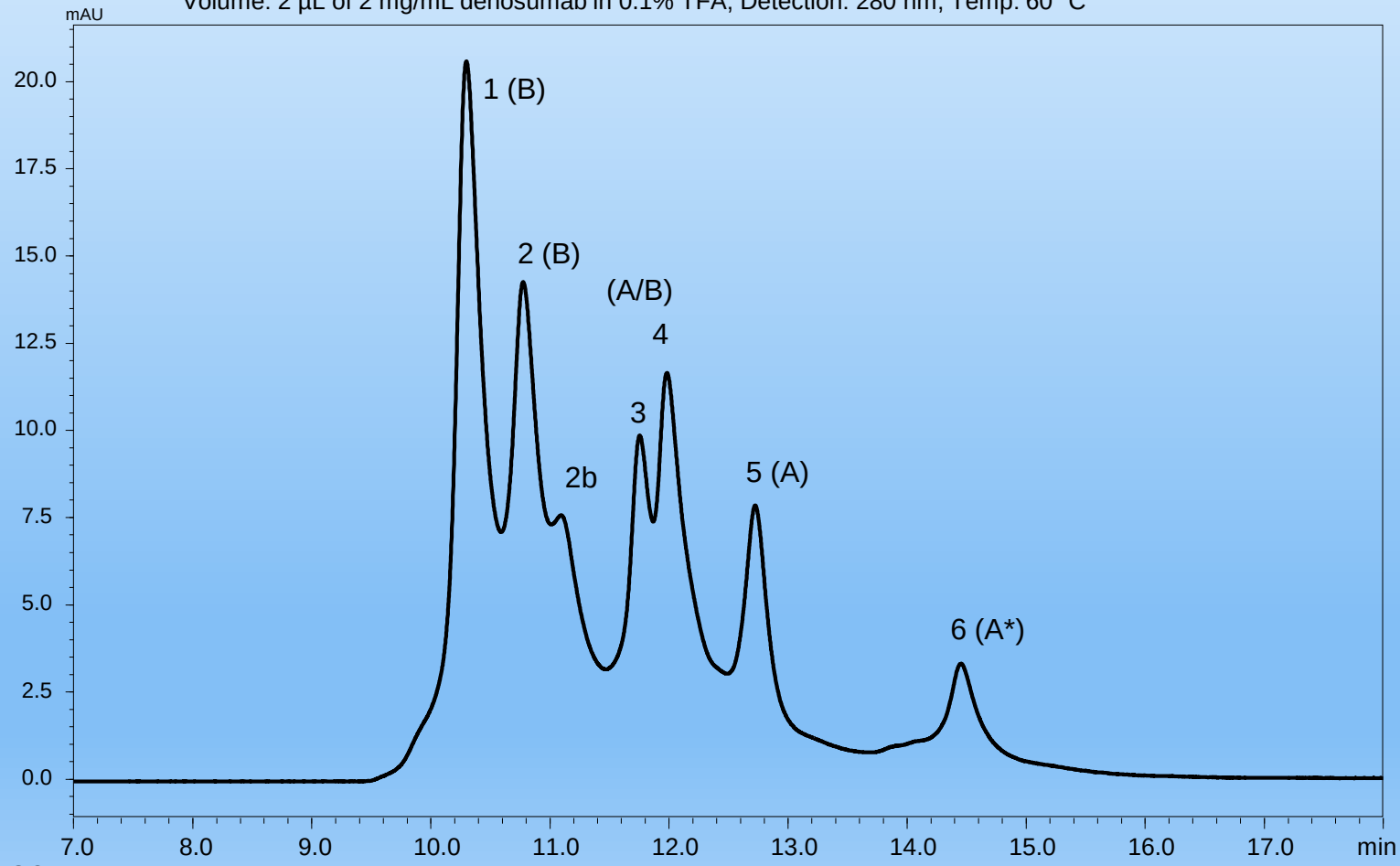


- For larger molecules, large pore SPP particles tolerate large sample masses effectively.
- Performance loss is progressive, occurring around 20-50 µg on column
- At all load levels 1000Å pore size SPP performed best for this mAb

IgG2 Disulfide Variant Separation



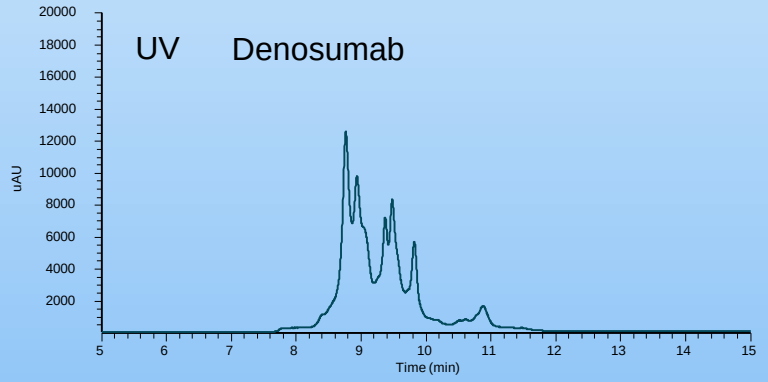
Column: HALO 1000Å C4, 2.1 x 150 mm; Flow rate: 0.2 mL/min; Temp: 60 °C
Mobile Phase A: 88/10/2 water/AcN/n-propanol/0.1% TFA; Mobile Phase B: 70/20/10 n-propanol/AcN/water/0.1% TFA; Gradient: 20-28% B in 32 min; Instrument: Shimadzu Nexera; Injection Volume: 2 µL of 2 mg/mL denosumab in 0.1% TFA; Detection: 280 nm; Temp: 60 °C

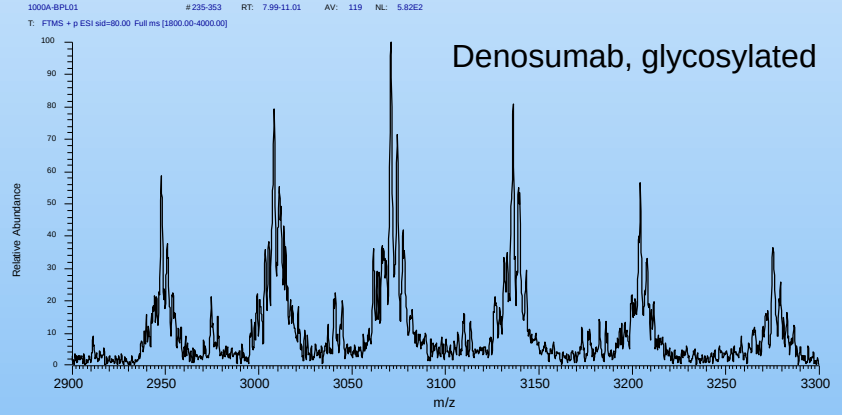


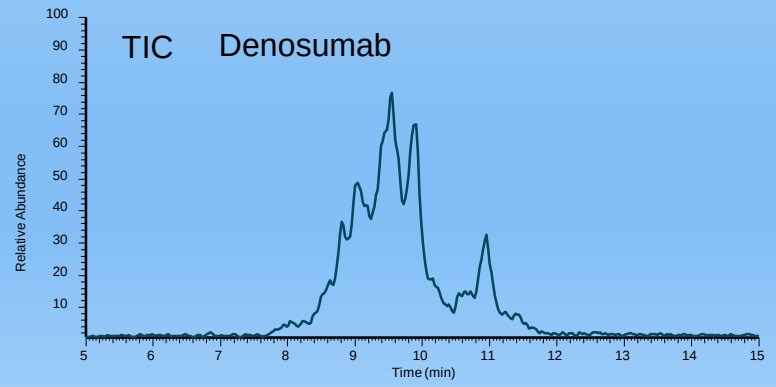
Wypych, et al., J. Biol. Chem. 283 (2008) 16194–205.
Dillon, et al., J. Biol. Chem. 283 (2008) 16206-205.

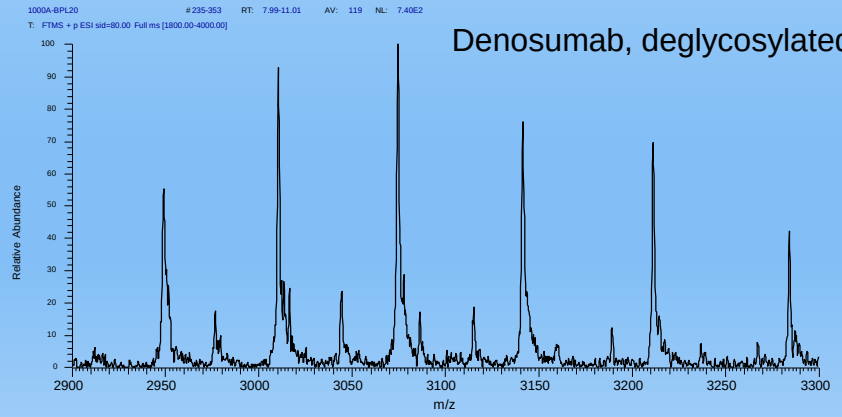
IgG2 Separation

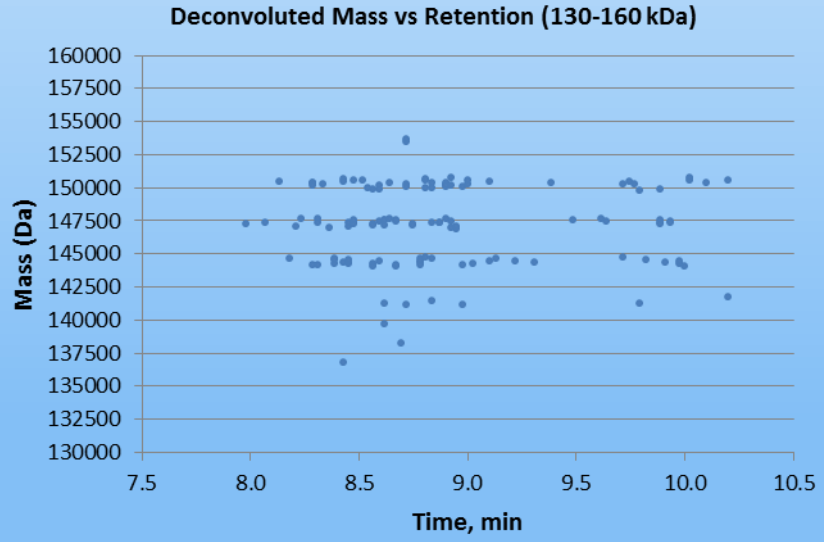
Column: 2.1 x 150 mm; Flow rate: 0.4 mL/min; Injection Volume: 4 µL of 0.5 mg/mL mAb; Detection: 280 nm; Temp: 80 ^{o}C
Mobile Phase A: 95/5 water/N-propanol/0.1% DFA; Mobile Phase B: 70/20/10 N-propanol/AcN/water/0.1% DFA;
Gradient: 14-24% B in 20 min; Instrument: Shimadzu Nexera, Velos Pro Orbitrap



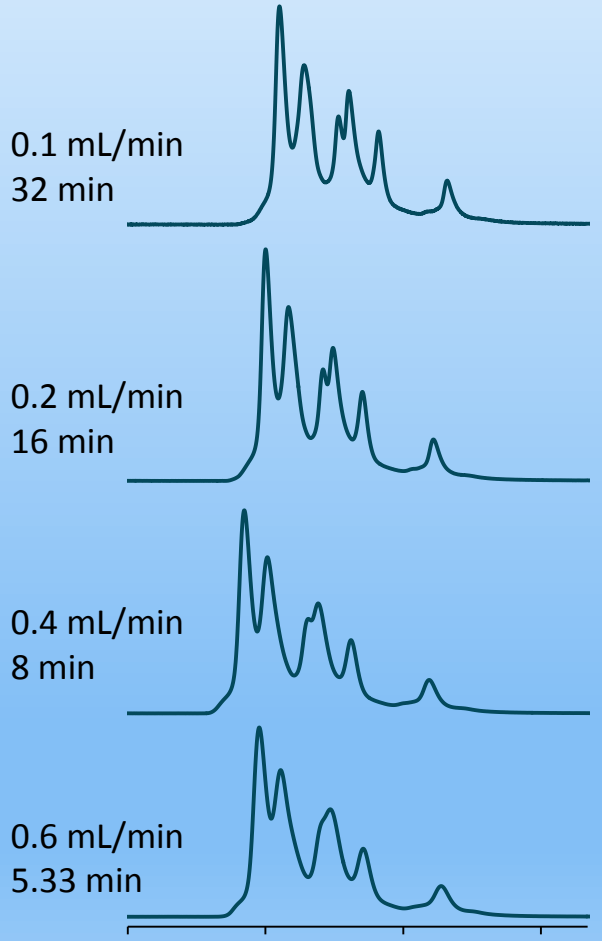




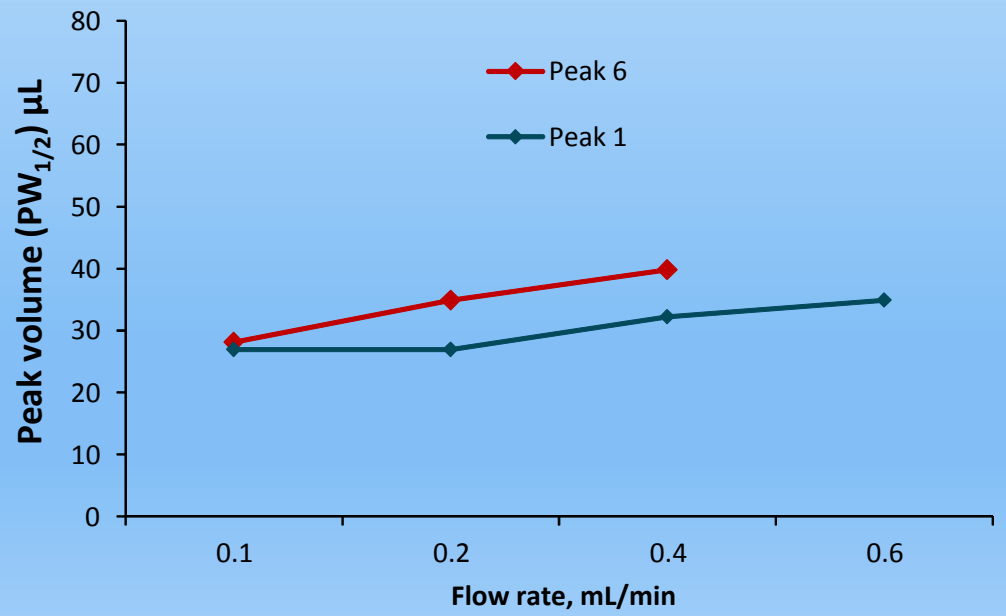




IgG2 Separation



Column: HALO 1000Å C4, 2.1 x 150 mm; Flow rate: 0.1, 0.2, 0.4, or 0.6 mL/min; Mobile Phase A: 88/10/2 water/AcN/n-propanol/0.1% TFA; Mobile Phase B: 70/20/10 n-propanol/AcN/water/0.1% TFA; Gradient: 20-28% B in time scaled to flow rate; Instrument: Shimadzu Nexera; Injection Volume: 2 µL of 2 mg/mL denosumab in 0.1% TFA; Detection: 280 nm; Temp: 60 ^{o}C



Summary and Future Work

- Improving protein separations is both materials and chemistry.
- Superficially porous particle silica packing materials have met the promise of supplying superior separations for large (and small) molecules. Fused-Core with enlarged pore sizes (400 and 1000 Å) have particular utility for protein analyses, are highly robust, and routinely allow *faster* protein separations with *higher* efficiency.
- Patience and persistence can pay off, eventually. Dr. Kirkland demonstrates this well with this technology, which required significant effort between concept and practice.
- We continue to build on this legacy, developing new materials and methods (MP and SP) to enable larger biomolecules (>100 kDa) LC and LC/MS analysis, and to improve materials targeted to lower molecular weight analytes, using a variety of LC modes.

"Every experiment tells you something."

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Thank you Jack Kirkland!

“You Sell the Sizzle, not the Steak”

“You Biology Guys need a lot of Help”

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