Improving Larger Protein Separations Using 1000 Å Superficially Porous Particles

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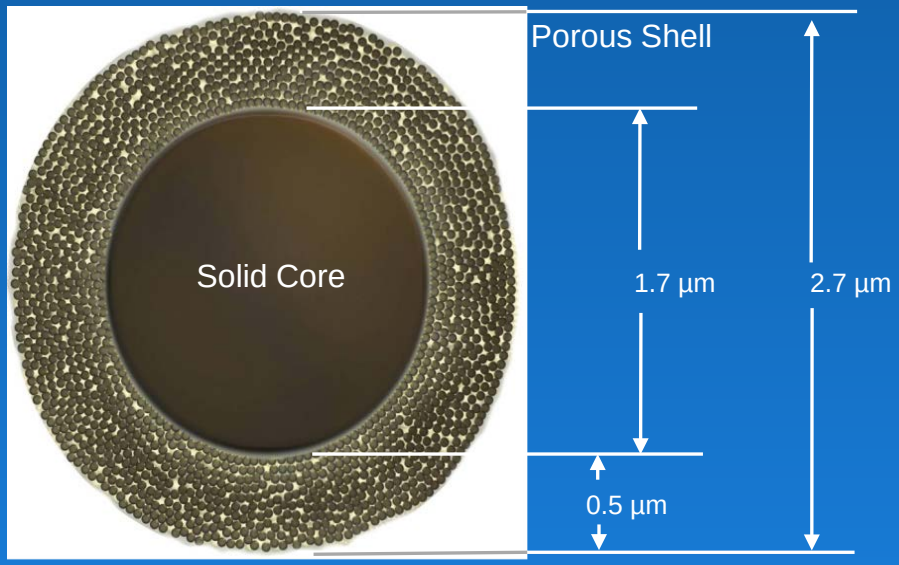
Agenda

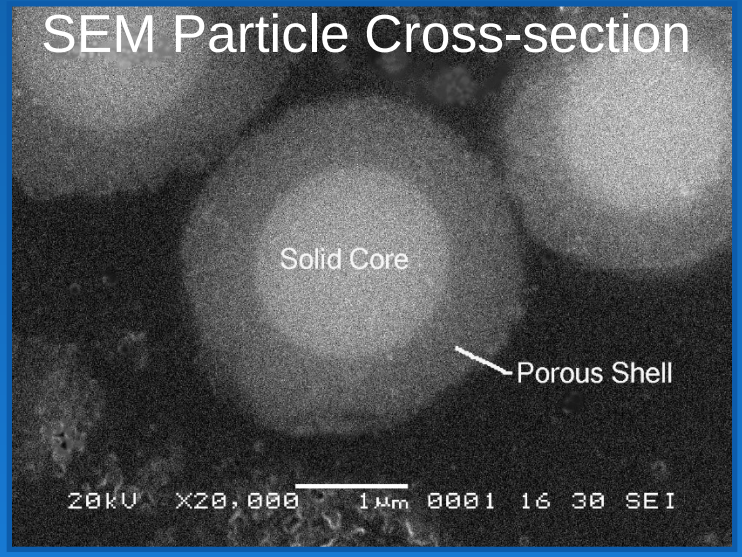
- Fused-Core Particles for highly efficient separations
- Conditions for RP Peptide and Protein Separations
  - Acidic Conditions
  - Acetonitrile Gradients, sometimes with Alcohol Mixes
  - Appropriate Pore Sizes for Samples of Interest
- Large Pore SPP: 400 Å and NEW 1000 Å for Proteins
- Recent Application Examples

Faster HPLC Separations

- Smaller Particle Packed Beds
  - Totally (Fully) Porous (including flow through)
  - Not Porous (Pellicular)
  - Partly Porous (Superficially Porous) 
- Monolithic Materials
- Open Tubular Columns (channels)

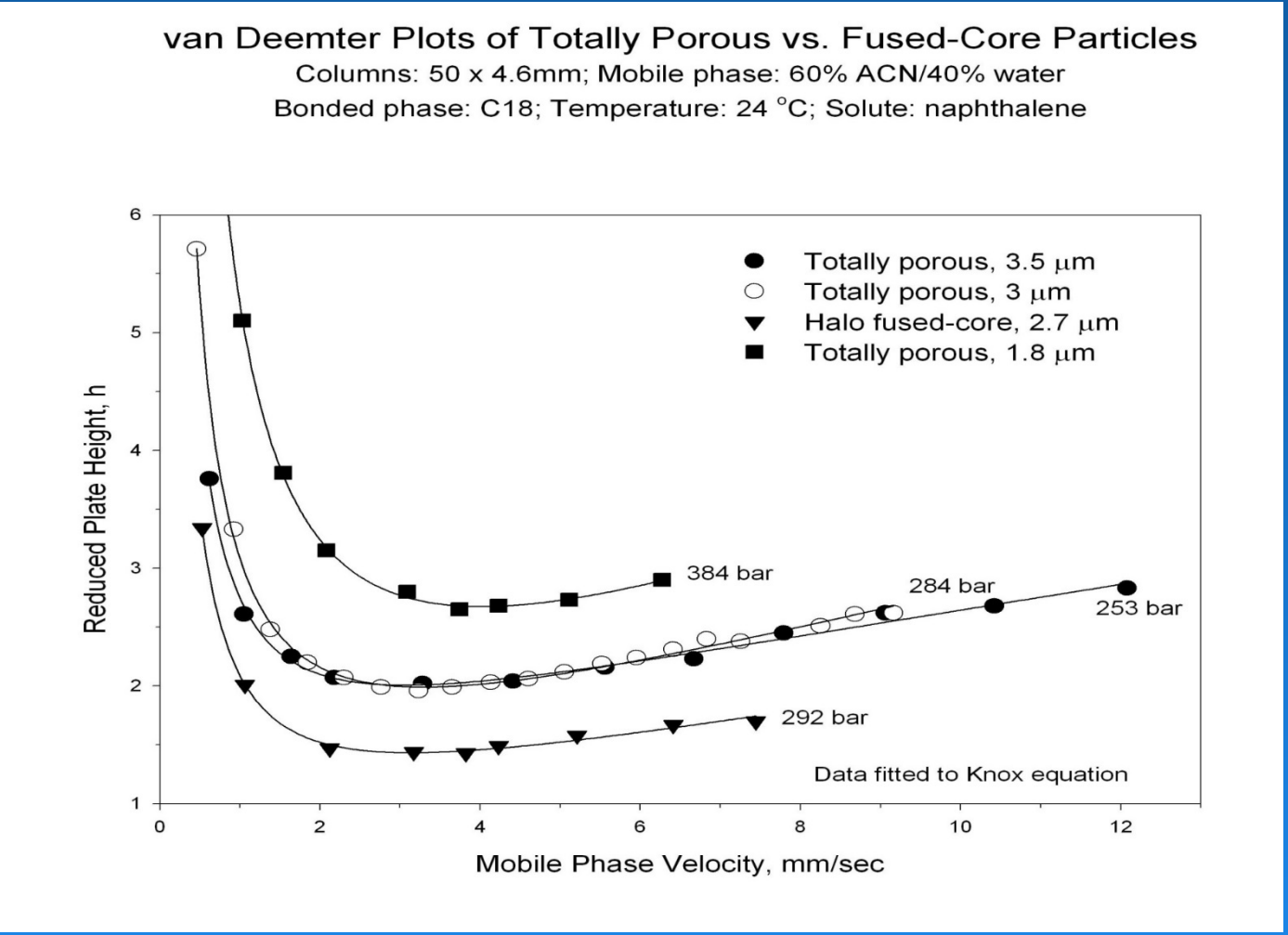
Superficially Porous Particles: Halo Fused-Core®





- 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)

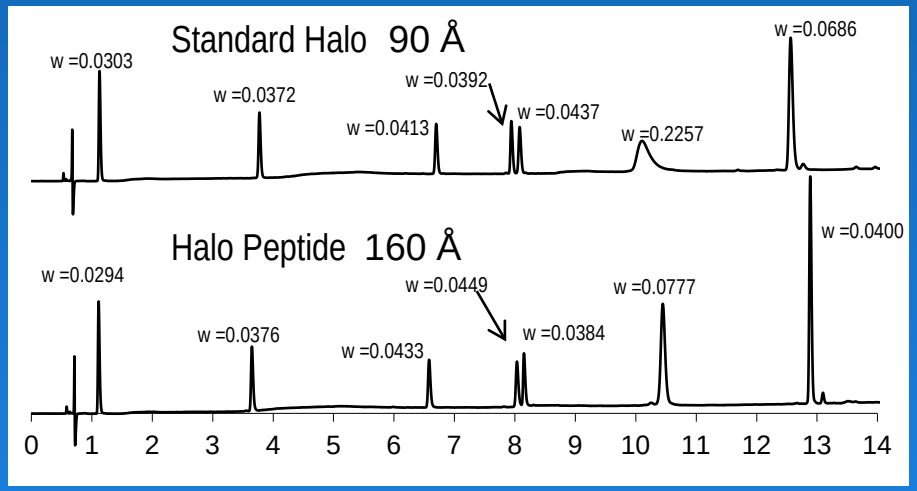
Initial Proof Of Principle Results

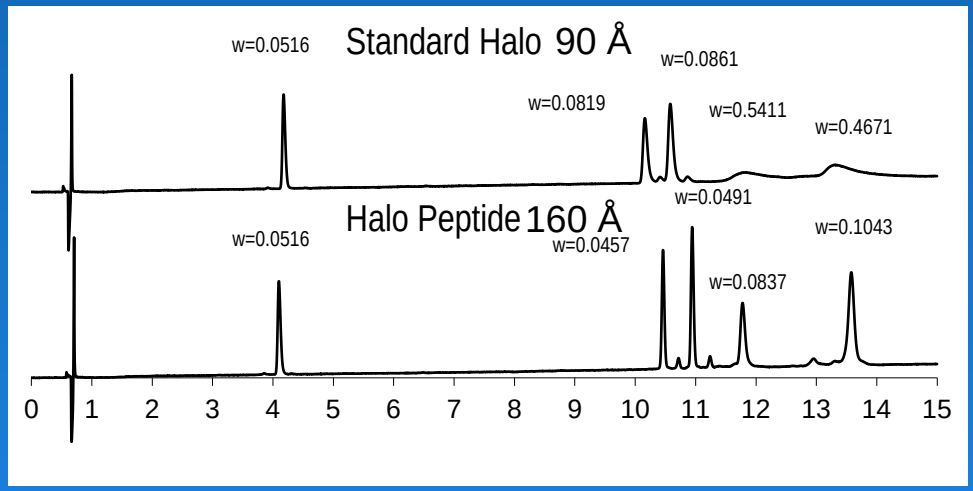


J. Kirkland, T. Langlois, J. DeStefano, Fused core particles for HPLC columns, Am. Lab. 39 (2007) 18–21.

Halo Peptide ES-C18 Separations: Pore Size Matters

Column: 4.6 x 100 mm; Flow rate: 1.5 mL/min; Temperature: 30° C
A: 0.1% TFA/10% ACN, B: 0.1% TFA/70% ACN
Gradient: 0% to 50% B in 15 min.; Injection volume: 5 µL





Sample 1
Gly-Tyr, Val-Tyr-Val, Met-enk, Angiotensin II, Leu-enk
Ribonuclease, Porcine Insulin

Sample 2
Leu-enk
Bovine Insulin, Human Insulin, Cytochrome C, Lysozyme

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

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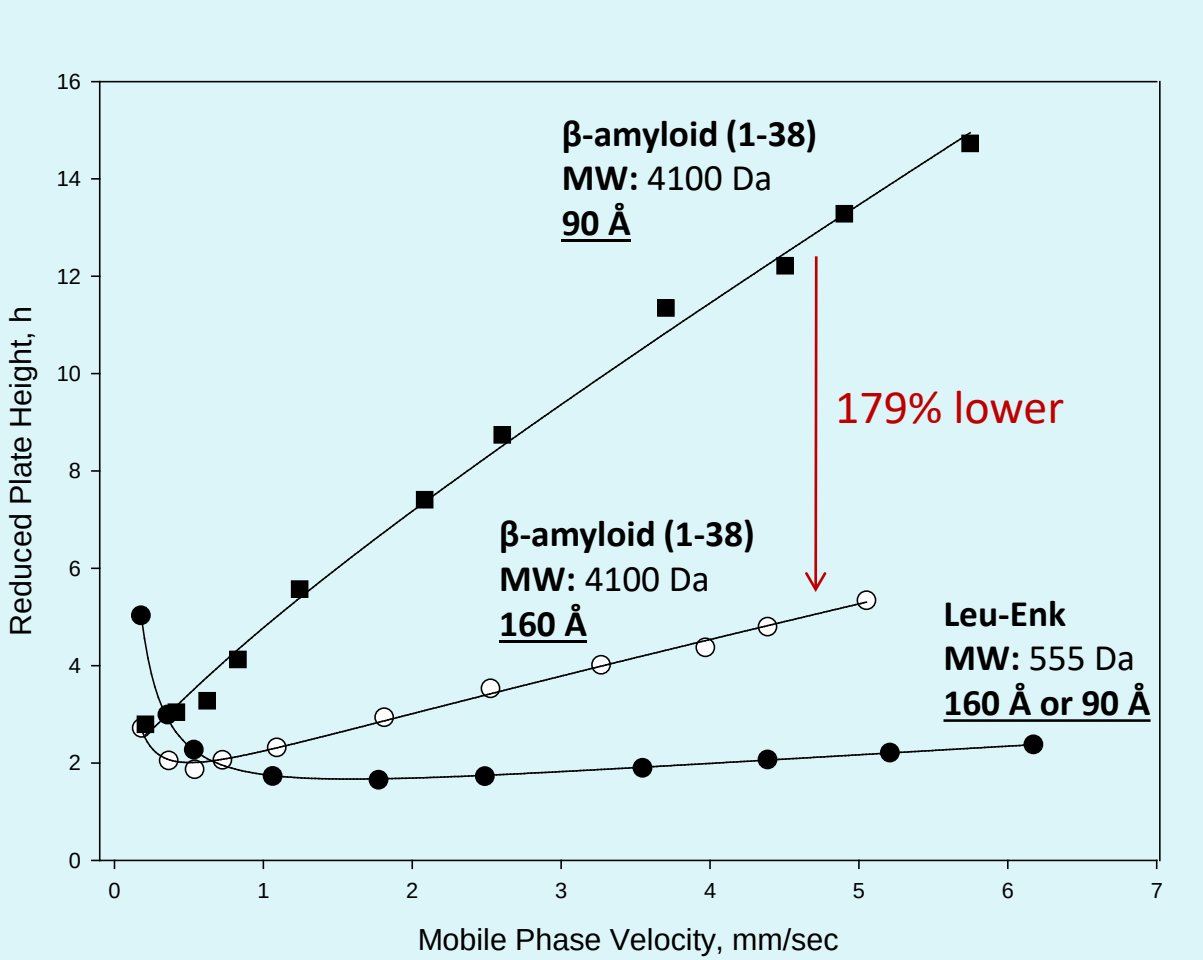
Peptide Separation: Effect of Pore Size on Column Efficiency



Columns: 4.6 x 100 mm
HALO C18, 2.7 µm, 90 Å
HALO Peptide ES-C18, 2.7 µm, 160 Å

Mobile Phase:
Leu-Enk: 21% ACN/0.1% TFA
β-amyloid (1-38) 160 Å : 29% ACN/0.1% TFA
β-amyloid (1-38) 90 Å : 27% ACN/0.1% TFA

Temperature: 60 °C
Detection: 215 nm

Schuster, Boyes, Wagner, et al., Fast high performance liquid chromatography separations for proteomic applications using Fused-cores silica particles, J.Chromatogr.A 1228 (2012) 232–241.

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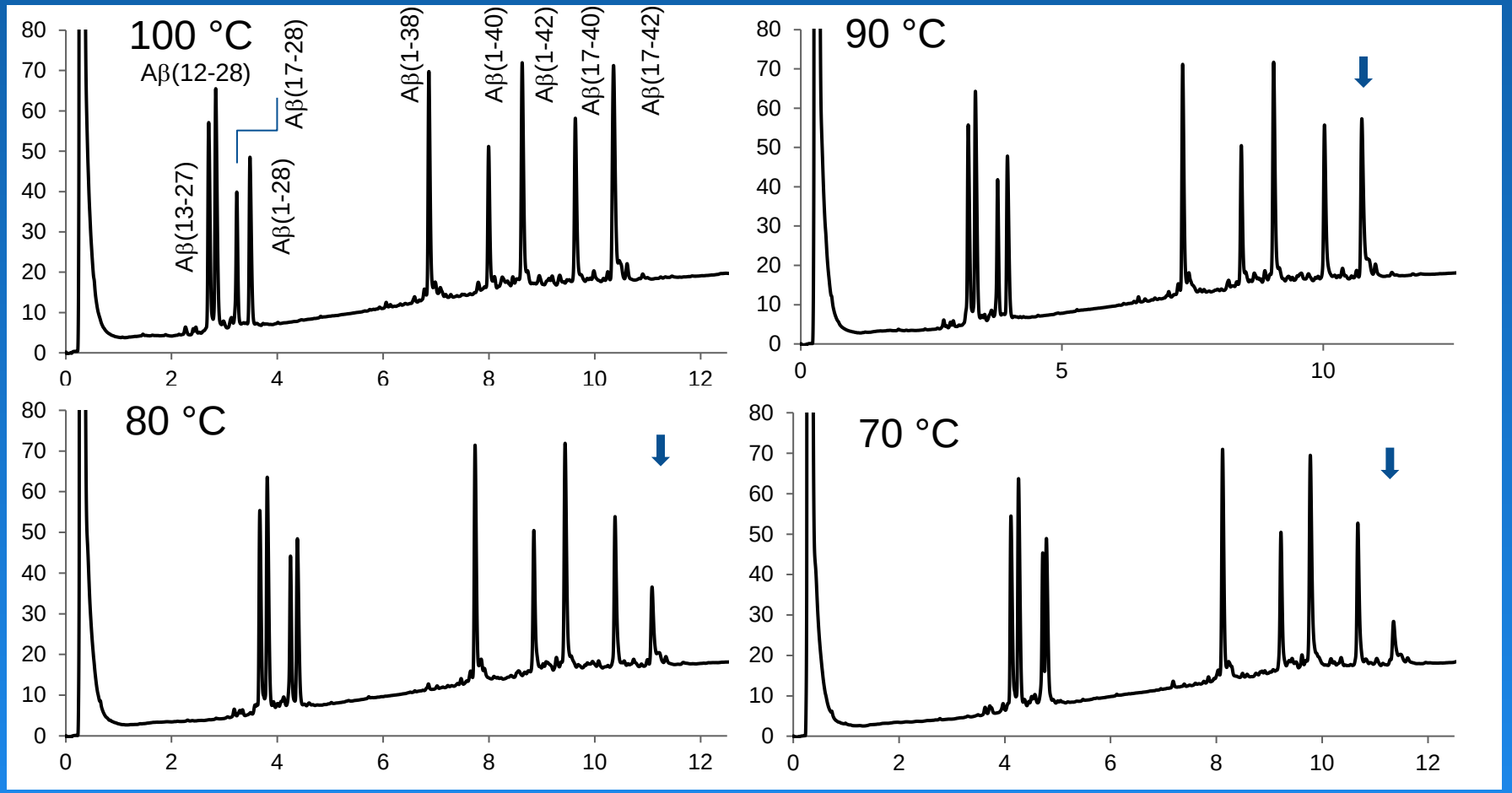
Rapid Separation at High Temperature

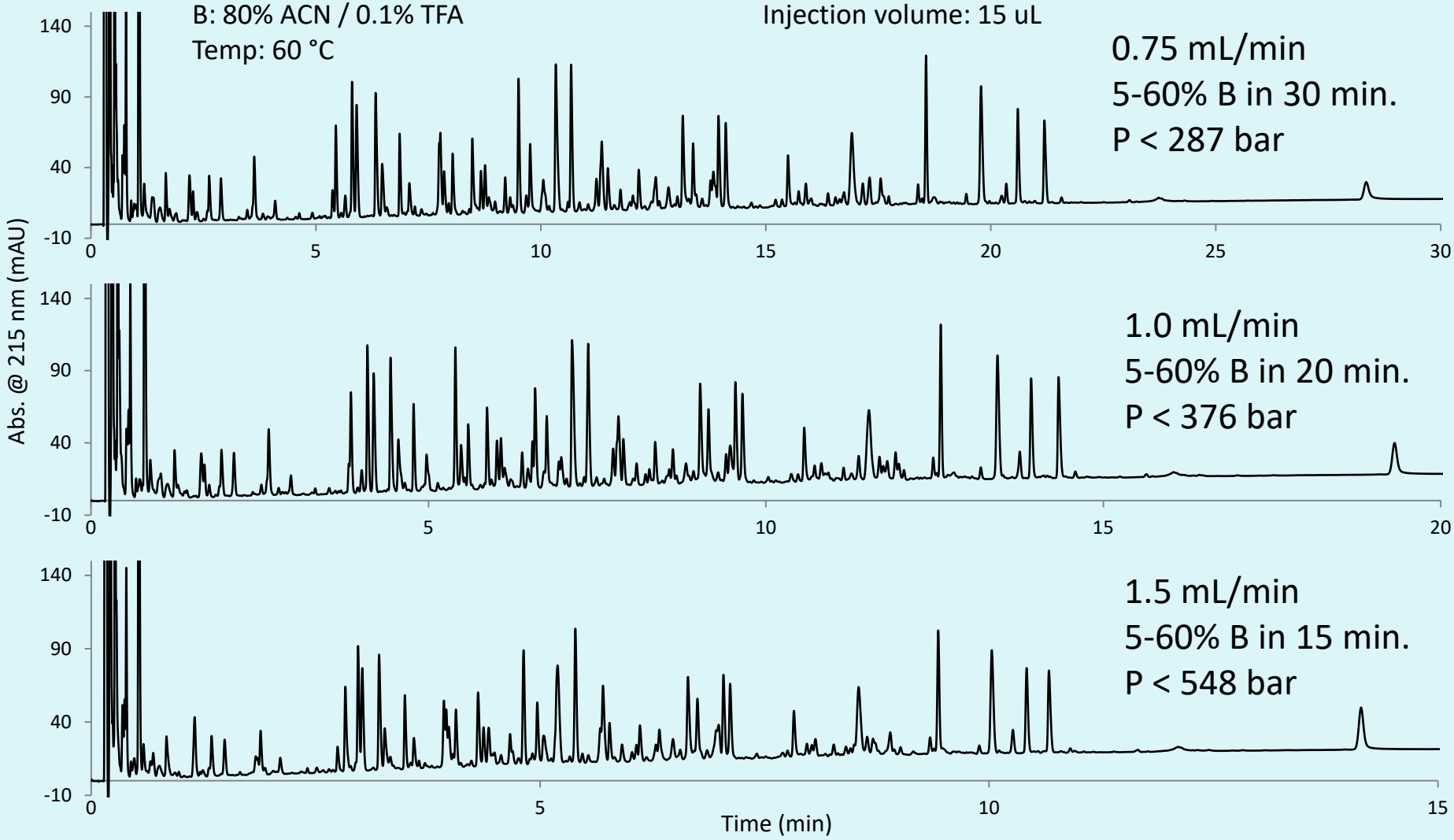
Column: 2.1 x 50 mm Halo Peptide ES-C18; Flow: 0.5 mL/min; A: 0.1% TFA; B: 0.1% TFA/80% AcN;
Gradient: 15-50% B in 12.5 min.; Sample: 5 µL (250-500 ng) Aβ Peptides



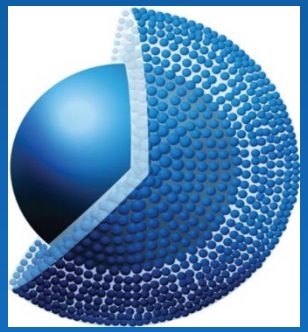
Ultra Fast High Resolution Separation of apo-Transferrin Digest

Column: **2.1 x 100 mm Peptide ES-C18 160 A**
A: Water/ 0.1% TFA
B: 80% ACN / 0.1% TFA
Temp: 60 °C

Detection: 215 nm
Sample: apotransferrin tryptic digest
Injection volume: 15 uL



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 Geometry must Optimize Surface Area/Volume

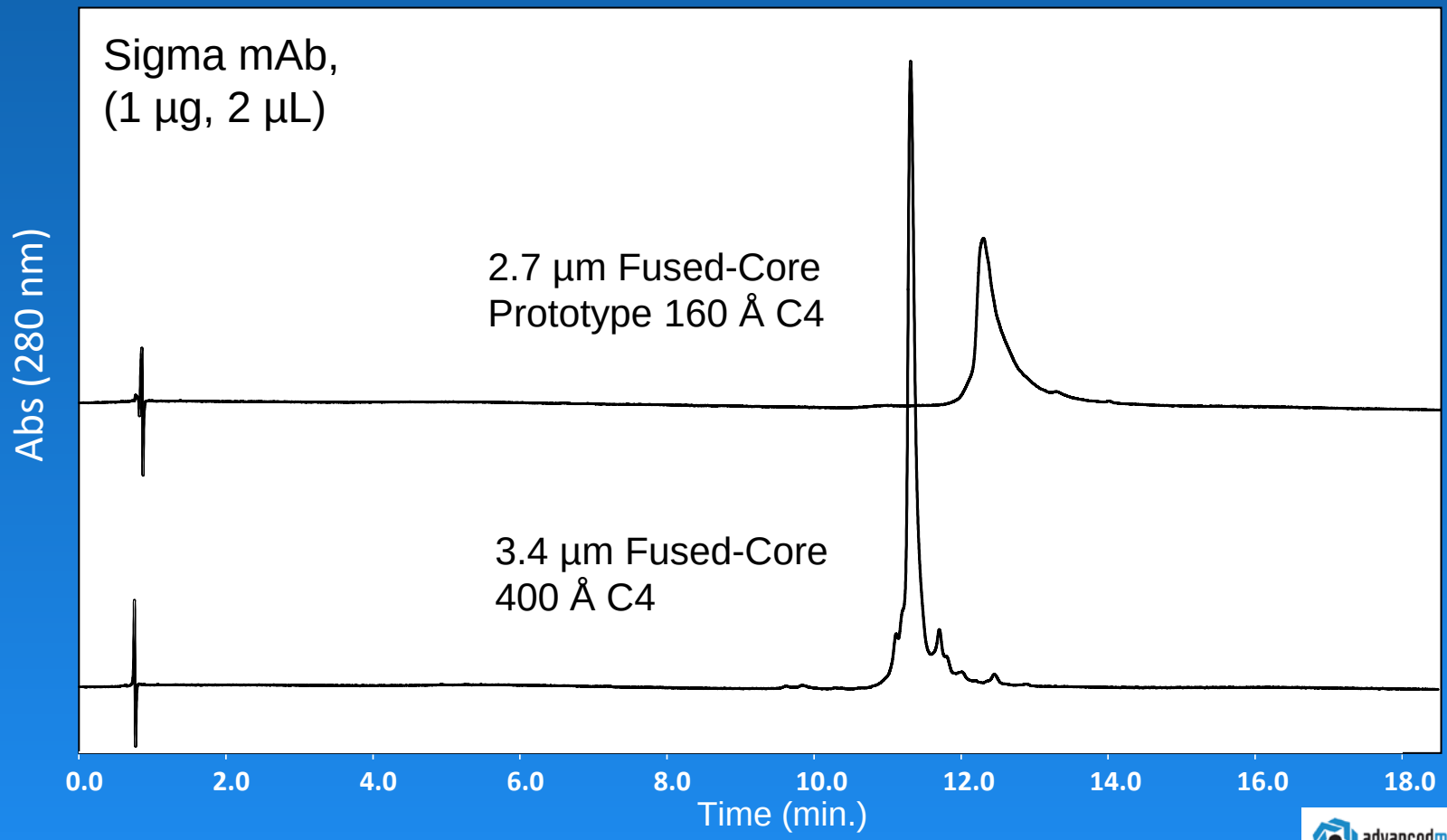
- Shell thickness determines diffusion path and Surface Area
- Must have “Right” size AND desirable particle distribution

Chemistry appropriate to Samples

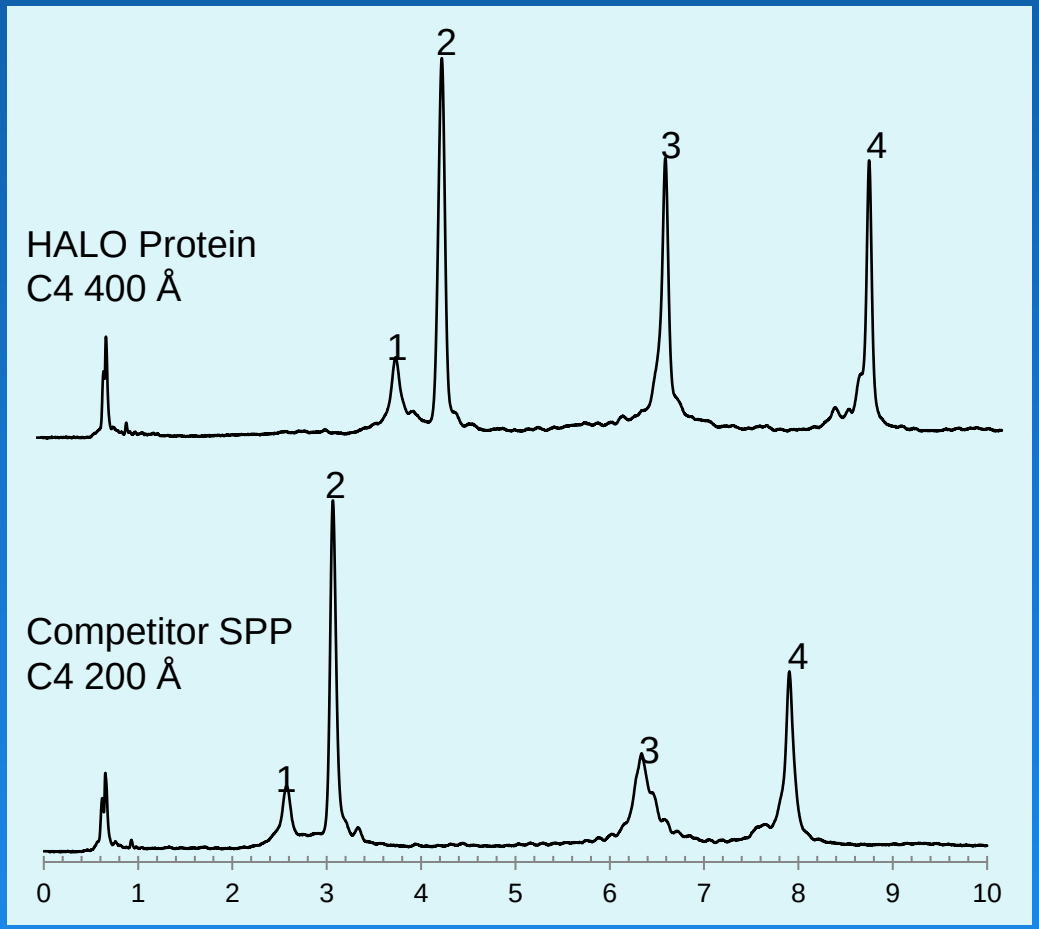
- Both Surface and Mobile Phase Properties

Defining the Needed Pore Size and Shell Thickness for SPP of Larger Proteins

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; Temp: 80 °C



Effect of Pore Size on Column Efficiency: Protein Separations



Columns: 2.1 x 100 mm
Injection Volume: 1 µL
Temperature: 60 °C

Mobile Phase A: water/0.1% TFA
Mobile Phase B: 80/20 ACN/water/0.1% TFA
Gradient: 40-47% ACN in 10 min.
Flow rate: 0.3 mL/min

Peak Identities:

1.	Catalase	250 kDa [~60 kDa subunit]
2.	α-Chymotrypsinogen A	25.0 kDa
3.	β-Galactosidase	465 kDa [116 kDa subunit]
4.	β-Amylase	200 kDa [~50 kDa subunit]

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.

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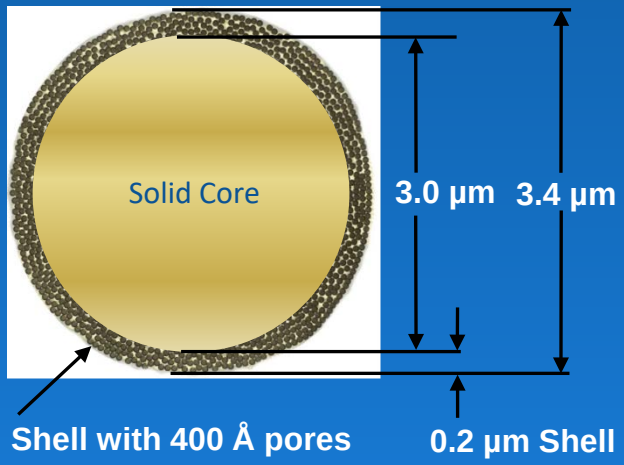
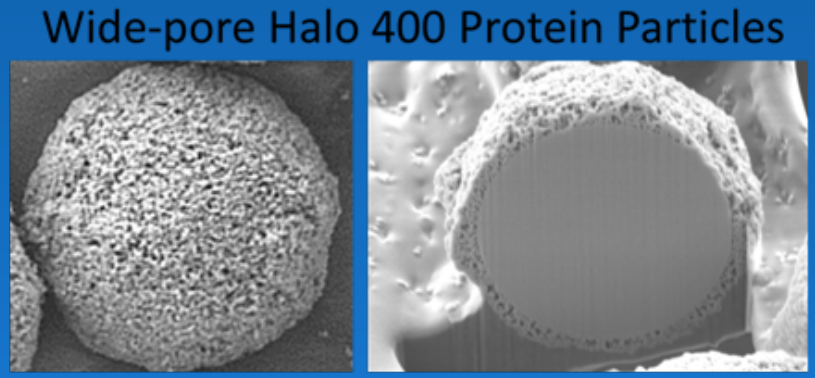
Superficially Porous (Fused-Core®) 400 Å Pore Particles for Protein Separations





- Many variations in shell thickness, pore size and particle size have been studied
- All variations will allow narrow particle size distribution
- 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.

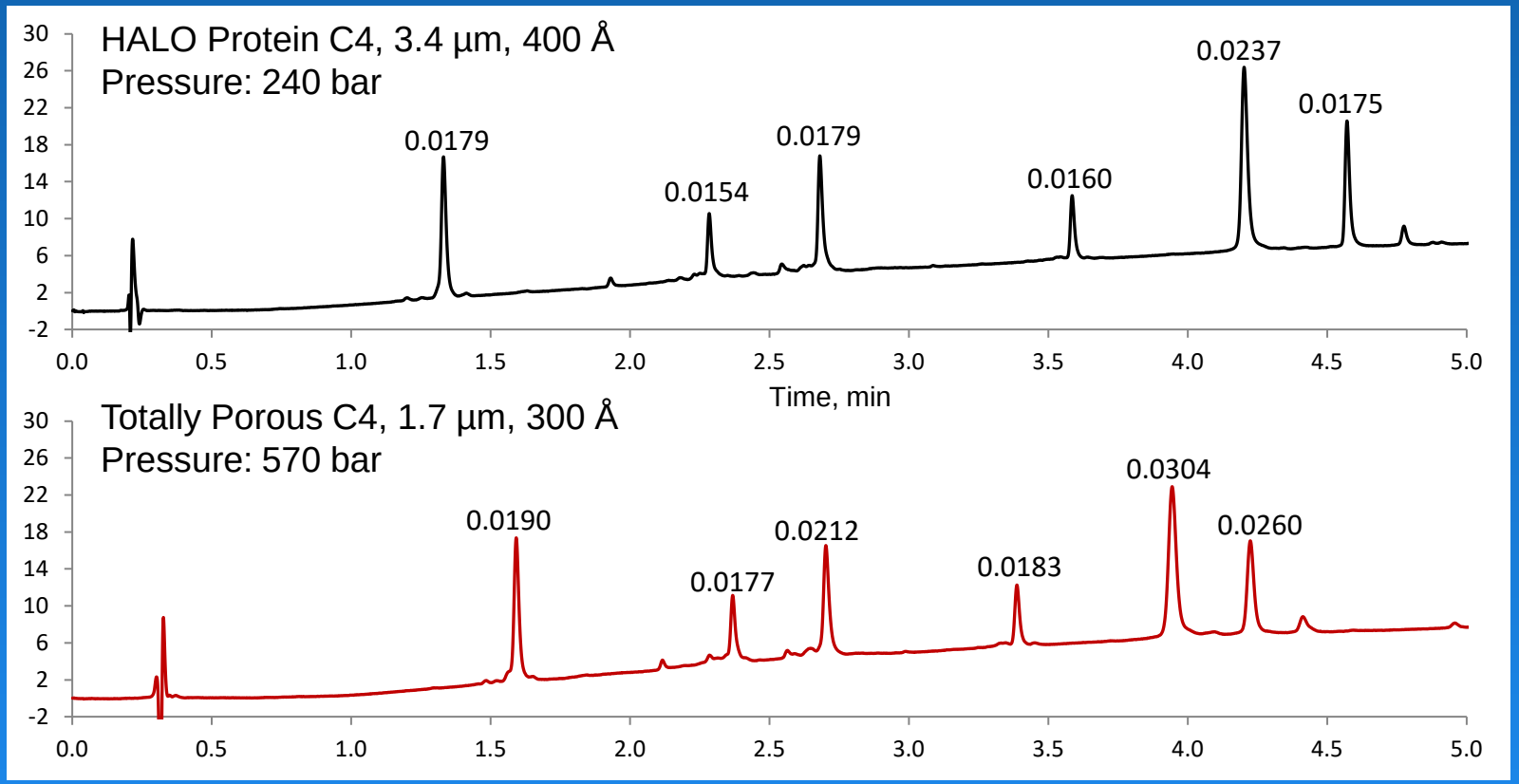
Protein Separations: SPP vs sub-2 µm FPP

Columns: 2.1 x 100 mm
Injection Volume: 1 µL
Detection: 215 nm
Temperature: 60 °C

Flow rate: 1.1 mL/min
Mobile Phase A: water/0.1% TFA
Mobile Phase B: acetonitrile/0.1% TFA
Gradient: 23-52% B in 5 min

Peak Identities:

1. Ribonuclease A
2. Cytochrome *c*
3. Lysozyme
4. α-Lactalbumin
5. Catalase
6. Enolase



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.

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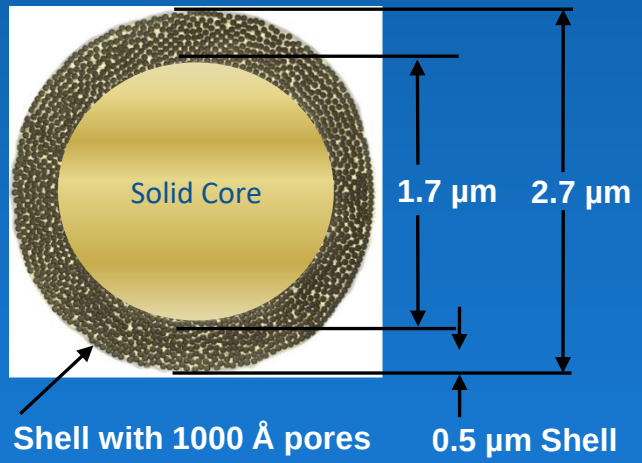
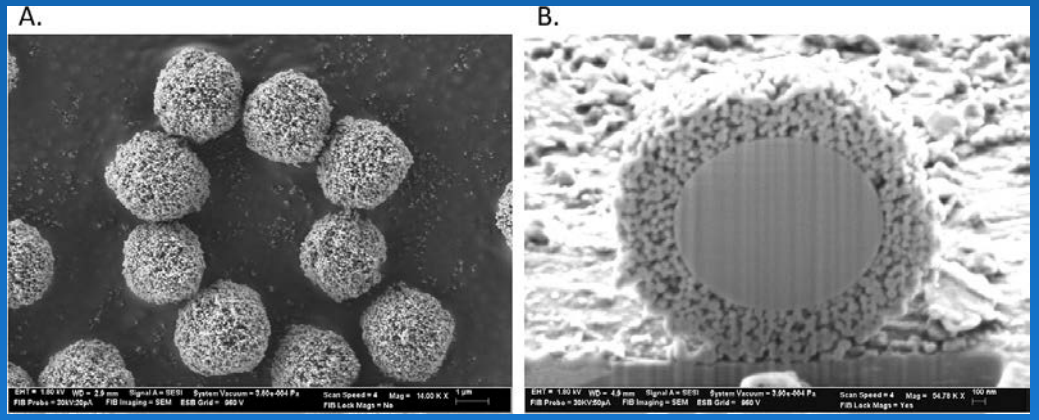
Introducing NEW Fused-Core® 1000 Å Pore Particles for Protein Separations

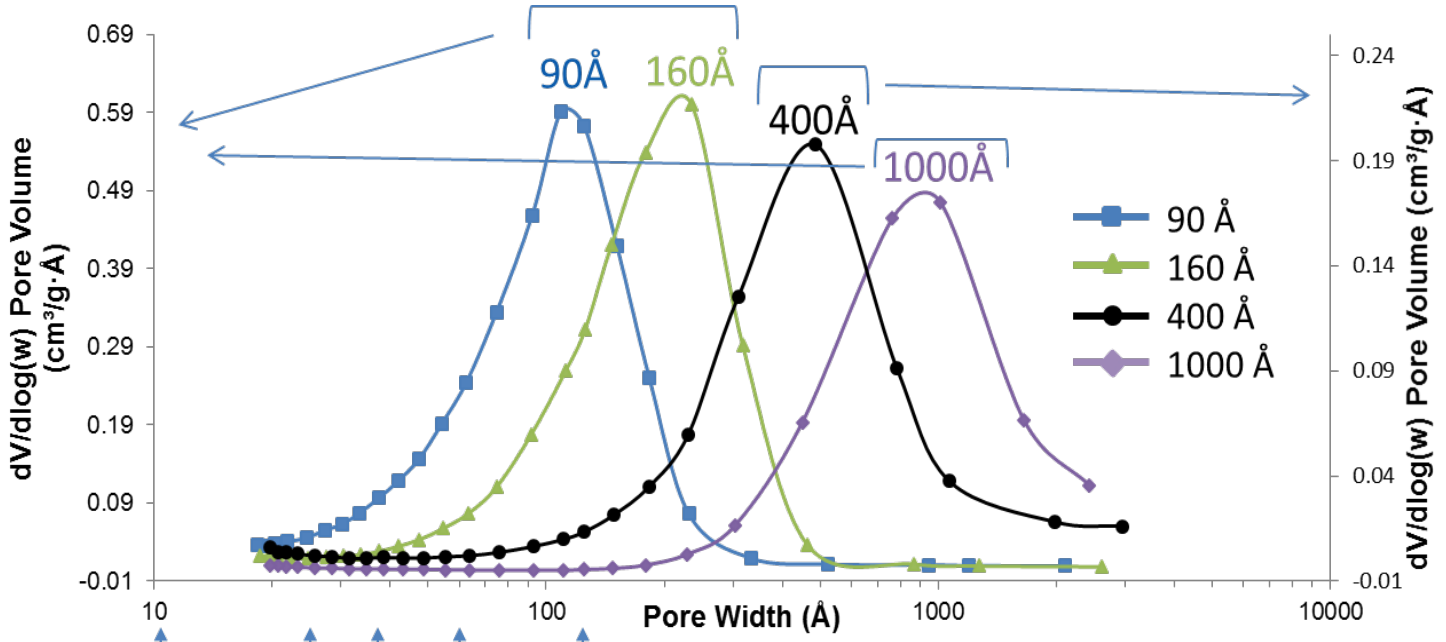




- Many variations in shell thickness, larger pores and particle sizes
- Theory to support “best properties” is complex, with end-points using mostly larger and complex proteins
- Again observe high quality particles, with excellent distribution of particle sizes
- Important compromises between load, efficiency and reproducibility for protein separations

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.

Halo Fused-Core® Family



Small drugs

Small peptides

Large peptides

Small proteins

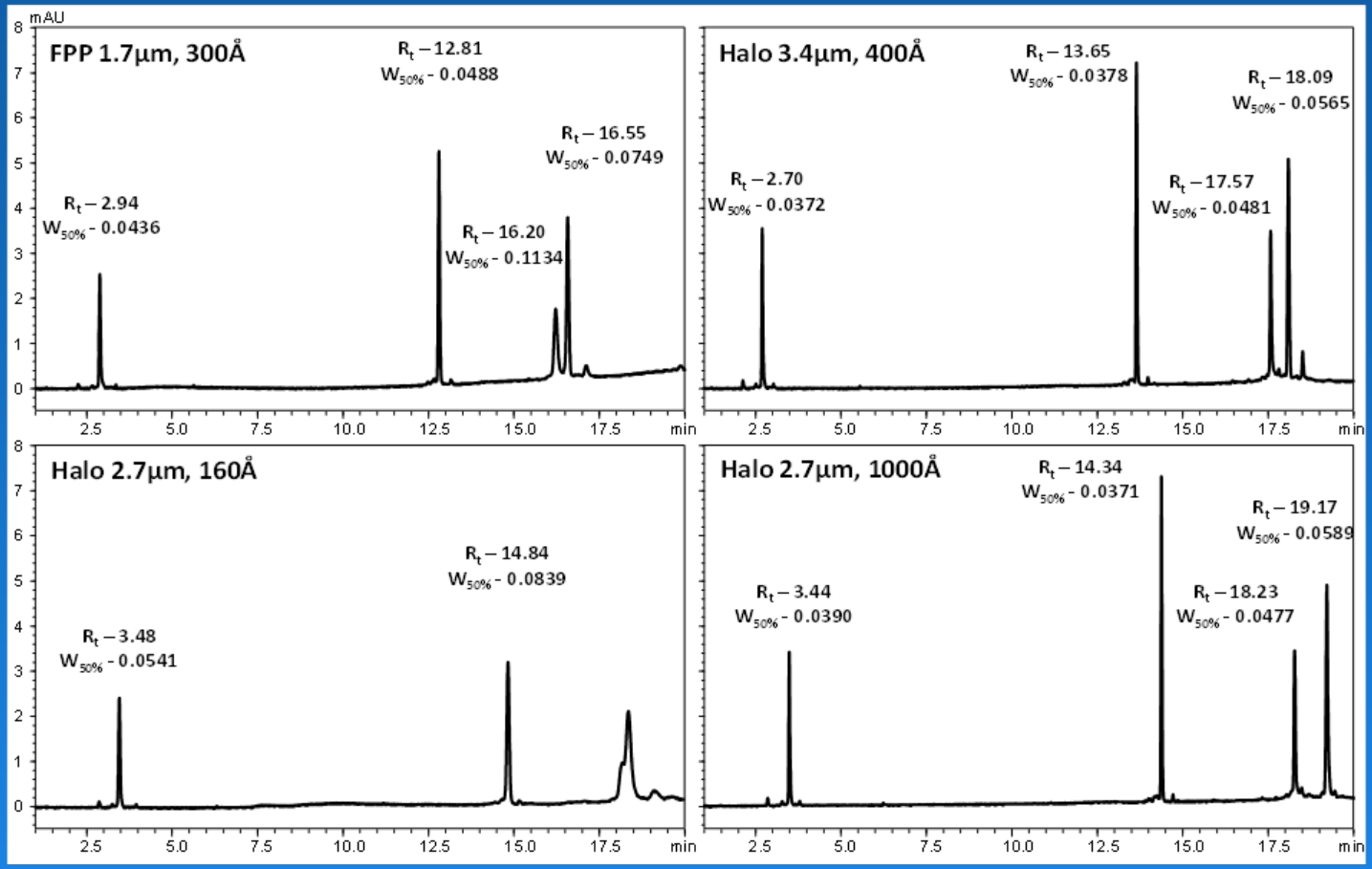
Large proteins

Small drugs	5-10 Å
Small peptides	20-30 Å
Large peptides	30-40 Å
Small proteins	40-100 Å
Large proteins	100-200 Å

Mid-size Protein Separations on Wide Pore SPP

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, Resolution and higher Retention with Larger Pore SPP
- Mid-sized proteins (> 15 kDa) require increasing pore size (>160 or 300 Å)
- Similar results in TFA, DFA and FA as mobile phase acidic modifiers

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Rise of the mAbs!

Biotechnology and Applied Biochemistry

Industry and regulatory experience of the glycosylation of monoclonal antibodies

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Abstract.

We surveyed 23 antibody-related marketing applications for glycoform analytical and functional information. Our database analysis shows a clear trend of increasing sophistication of analytical methods used to identify and quantify glycans. These have revealed a high degree of complexity and heterogeneity of glycans attached to antibody products. The nature of the complexity is influenced by product type and expression system, and may be associated with functional consequences in some but not all cases.

Published 2011. This article is a U.S. Government work and is in the public domain of the USA. Volume 58, Number 4, July/August 2011, Pages 213–219 •
E-mail: kurt.brorson@fda.hhs.gov

Keywords: glycosylation, monoclonal antibodies

1. Introduction

The number of licensed therapeutic monoclonal antibodies (mAbs) has been increasing over the past few years, with hundreds more already undergoing clinical study for indications for a variety of therapeutic applications, including cancer and inflammatory diseases [1]. Most of these products are produced in conventional bioreactor-based mammalian cell culture [*e.g.*, Chinese hamster ovary (CHO) or murine myeloma transfectomas], although a few are produced by other expression systems (*e.g., Escherichia coli*) [2]. Therapeutic antibodies must be demonstrated to meet applicable quality requirements to ensure continued safety, purity, and potency to convince regulators to allow marketing as a drug product. Part of the demonstration of product quality is an intensive biochemical characterization of the antibody itself, which includes a thorough examination of glycan distribution and potential impacts of glycoform on function [3]. This characterization is conducted in two major stages, (a) a complete glycan distribution characterization of reference standard or conformance lots of the antibody glycoprotein and (b) abbreviated testing of all subsequent batches to establish manufacturing consistency and comparability with the reference material. The tests used in these analyses span a wide range of analytical methodologies, which have grown more sophisticated over the years [4].

For the most part, glycans on commercial antibodies are attached at asparagine residues at or near position 297 (N297) within the Fc portion of the protein [5]. Mammalian cell culture-produced antibodies typically possess N-linked complex biantennary structures, with heterogeneous levels of terminal galactosylation and fucosylation of the core N-acetylglucosamine [6]. To a lesser degree, terminal sialylation and bisecting N-acetylglucosamine are also present. Although these glycans do not directly impact the antigen-binding function of the antibody protein, they can impact effector functions such as antibody-dependent cellular cytotoxicity (ADCC) or complement binding and activation (also known as CDC or complement-dependent cytotoxicity) [7]. Examples of documented impacts of glycosylation on antibody functionality include, but are not limited to, (a) an inverse correlation between ADCC activity on core fucosylation [8], (b) an increase in CDC activity with increased galactosylation [9], and (c) a positive correlation between anti-inflammatory activity and increased sialylation [10]. A subset of antibody-like products, Fc fusion proteins, possesses more complex glycan distributions, including O-linked glycans. Thus, glycoform variation can impact the potency or *in vivo* distribution/clearance of therapeutic antibodies and needs to be characterized and controlled. As part of glycan characterization, the impact of glycan distribution on the product mechanism of action (MoA; *e.g.*, cancer cell destruction, down-modulation of inflammatory activity) is commonly evaluated by firms wishing to market antibody-based medicinal products.

Over the past 25 years, almost 40 antibody products have been approved for marketing by US Food and Drug Administration (FDA). The licensure decision is based on information submitted in the marketing dossier including the above

Abbreviations: α-gal, α-galactosyl residues; ADCC, antibody-dependent cellular cytotoxicity; BLAs, Biological License Applications; CE, capillary electrophoresis; CHO, Chinese hamster ovary; CDC, complement-dependent cytotoxicity; exo, exoglycosidase; MS, mass spectrometry; MoA, mechanism of action; mAbs, monoclonal antibodies; OP, oligosaccharide profiling; G0F, G1F and G2F, outer arm non, mono or bi-β1-galactosylated variant of core fucosylated biantennary N-linked glycans; FDA, US Food and Drug Administration.

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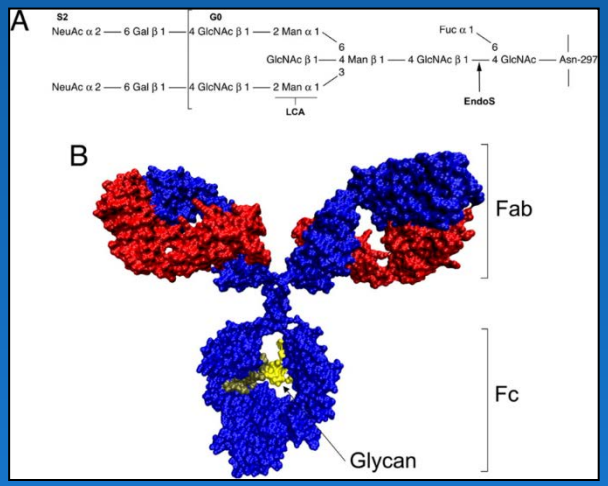
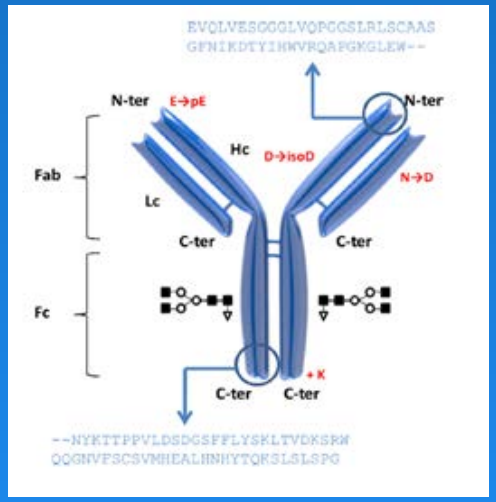
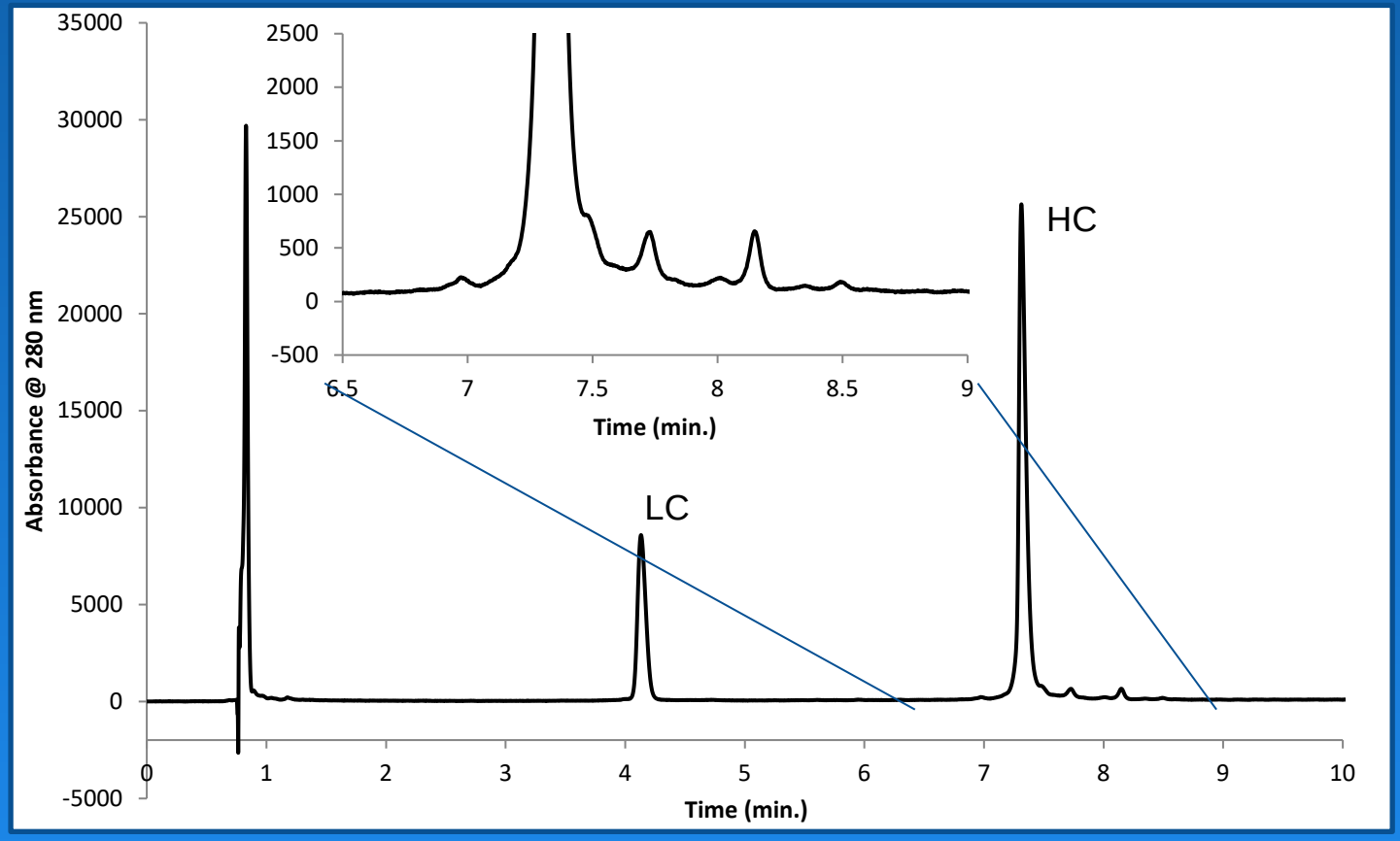




Reduced IgG2-B in TFA mAb Separation

Column: 2.1 x 100 mm HALO Protein 400 C4
Sample: 0.5 mg/mL IgG2-B treated with 100 mM DTT in 8 M Guanidine HCl at 50 °C for 35 min.

Mobile Phase A: water/0.1% TFA
Mobile Phase B: 80/20 ACN/water/0.1% TFA
Gradient: 33-40% B in 10 min.; Flow rate: 0.25 mL/min
Temperature: 80 °C



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.

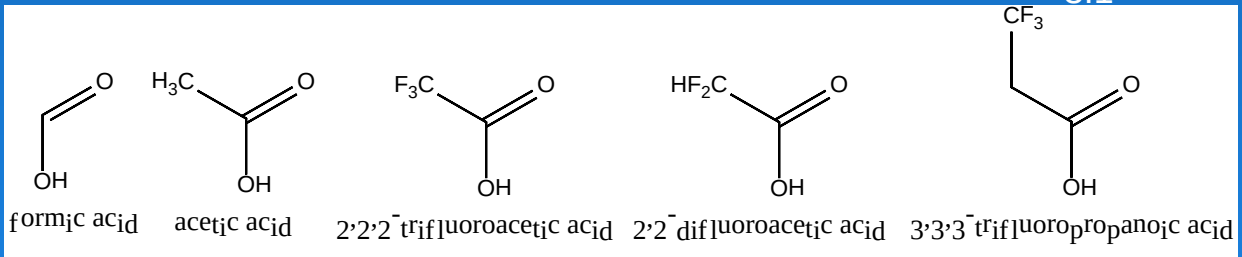
Mobile Phases for Improved Protein LC/MS

Selection and testing in LC/MS indicated some candidates with promise:

Required features:

volatility, lower pKa, high protein solubility, moderate ion pairing

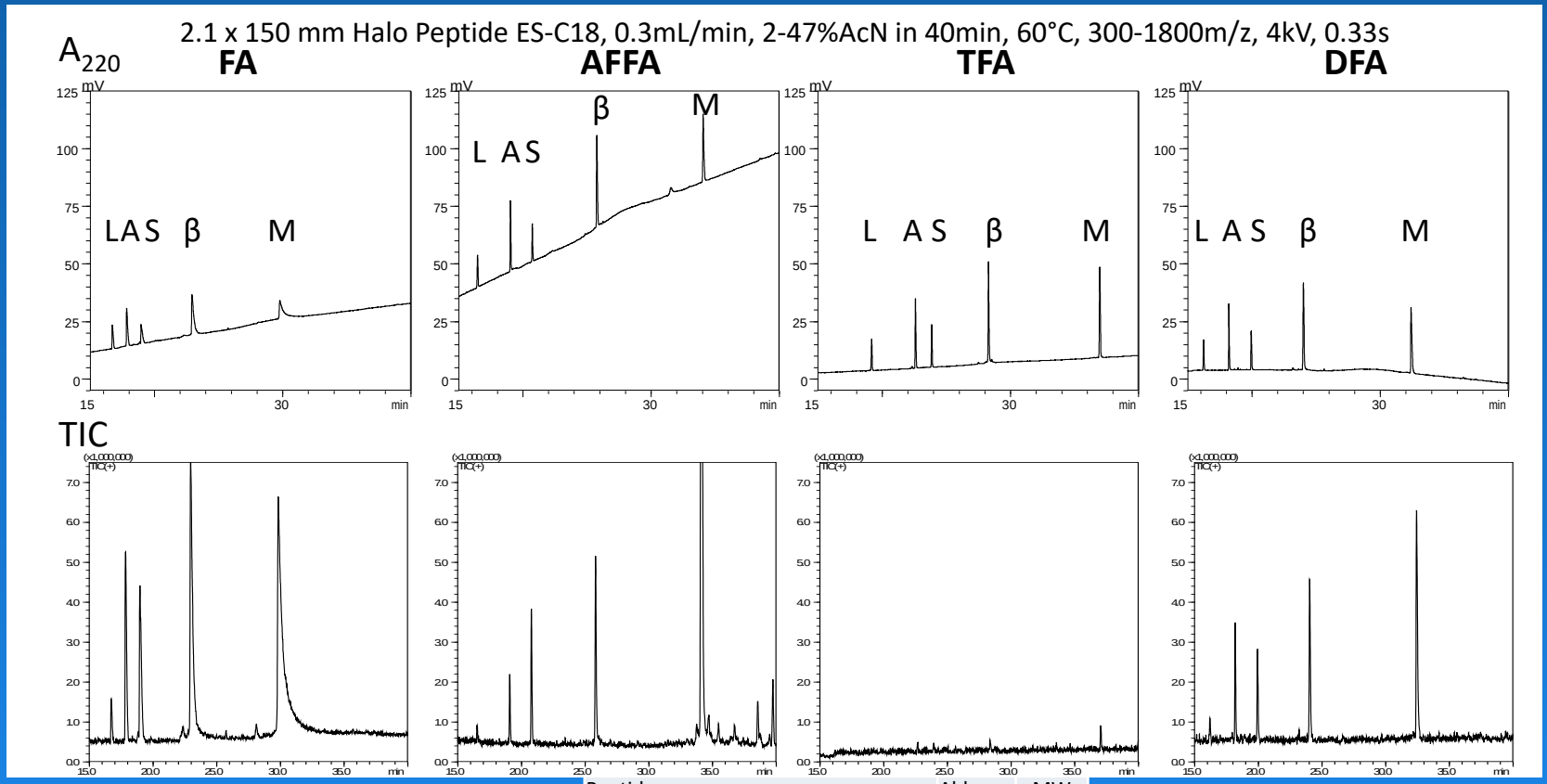
FA	HOAc	TFA	DFA	3FPA
3.8	4.8	0.3-1.6	1.3-1.4	3.1



formic acid | acetic acid | 2,2,2-trifluoroacetic acid | 2,2-difluoroacetic acid | 3,3,3-trifluoropropanoic acid

Synthetic Peptide Mixture LC/MS in Several Acidic Modifiers

10 mM Acid; 50pmol 5 peptide mix



Peptide	Abbrev.	MW
[Leu5]-enkephalin	L	555.6
angiotensin I, human acetate hydrate	A	1297
substance P acetate salt hydrate	S	1348
Melittin, honey bee venom	M	2847
beta-endorphin, human	β	3465

Mobile Phases for Improved Protein LC/MS

2.1 x 100 mm
HALO Protein C4 400Å
15-55% AcN in 30 min

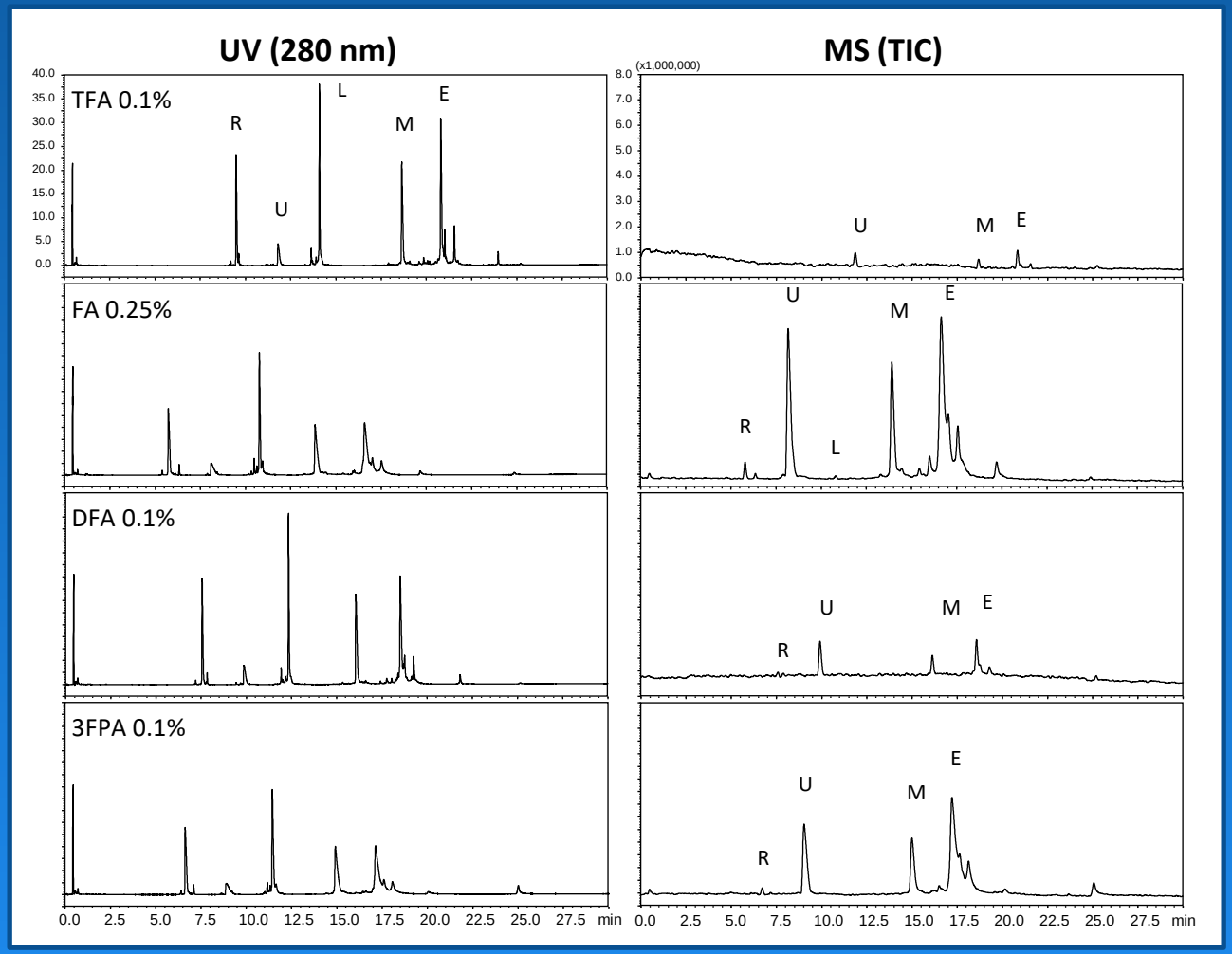
0.35 mL/min; 50ºC

25 pmol each protein

R – Ribonuclease
U – rec. Ubiquitin
L – Lysozyme
M – apo-Myoglobin
E – Enolase

Nexera LC system

MS-2020 Single Quad
400 – 2000 m/z 3 pps
3.8 kV ESI



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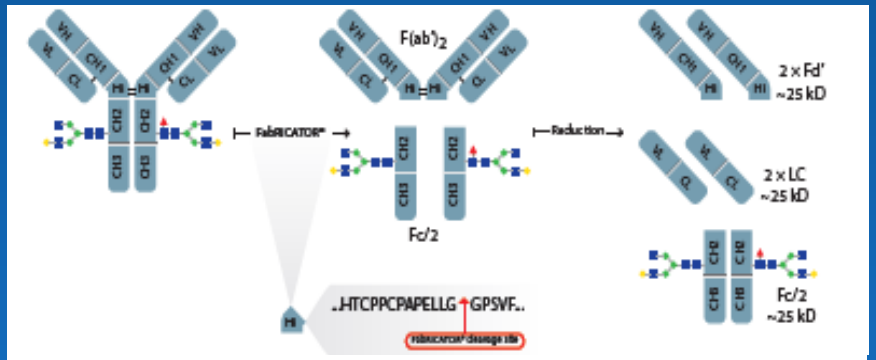
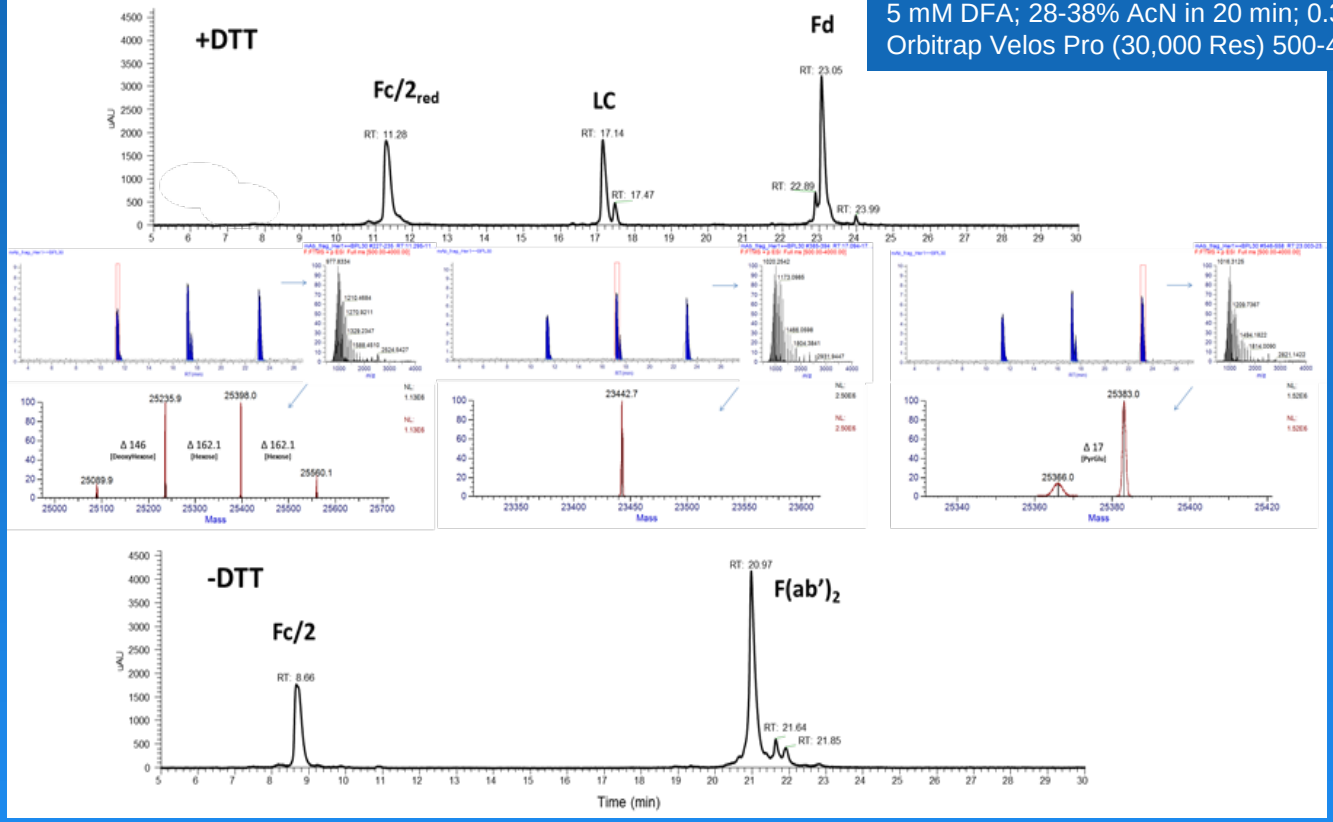
Fragments for mAb Structure: IdeS Digest



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

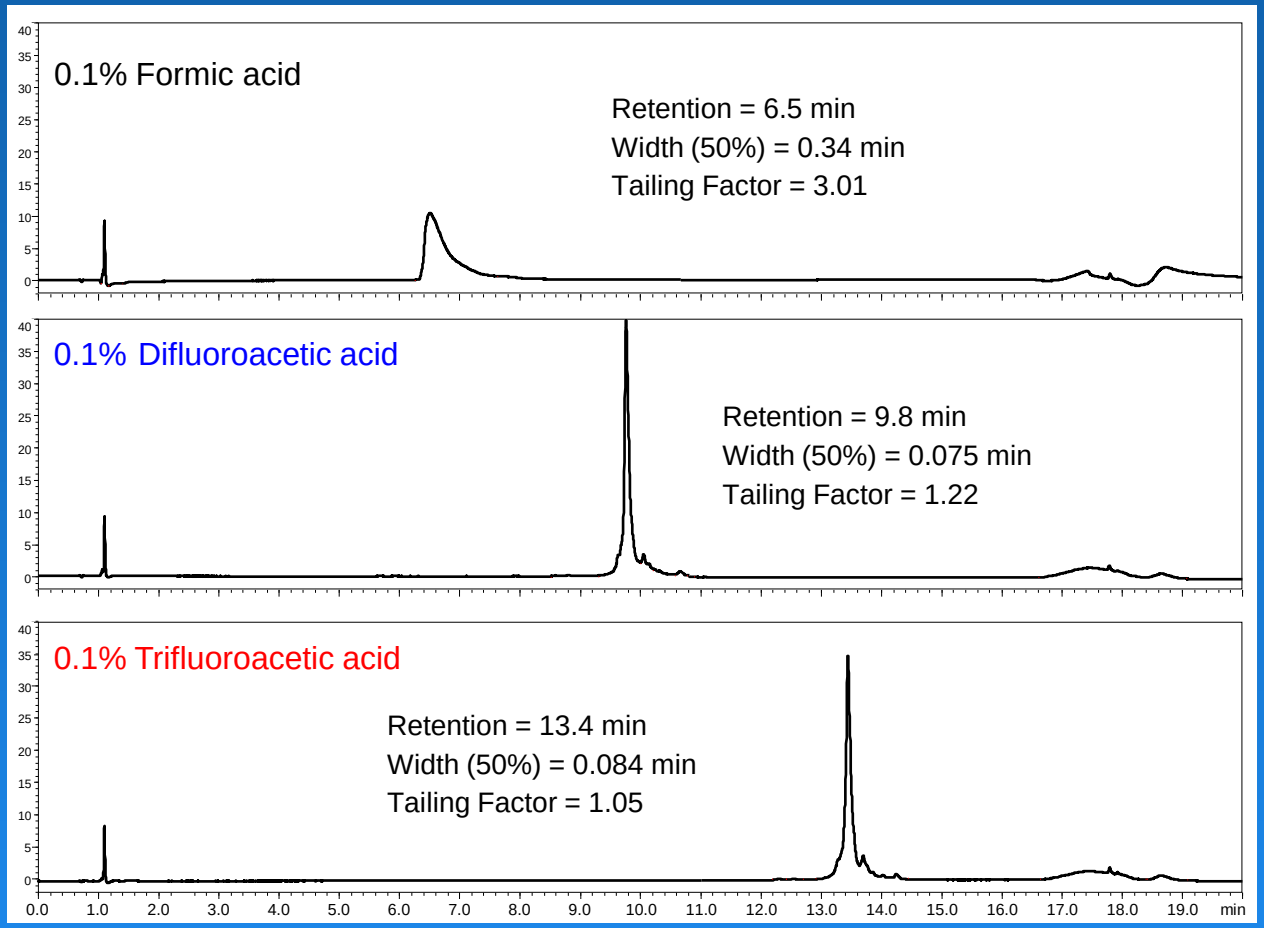
Halo Protein C4 400 Å, 2.1 mm ID x 150 mm;
5 mM DFA; 28-38% AcN in 20 min; 0.35 mL/min, 80 ºC;
Orbitrap Velos Pro (30,000 Res) 500-4000 m/z, +3.8 kV, 275 ºC capillary



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

Mobile Phases for mAb IgG Analysis

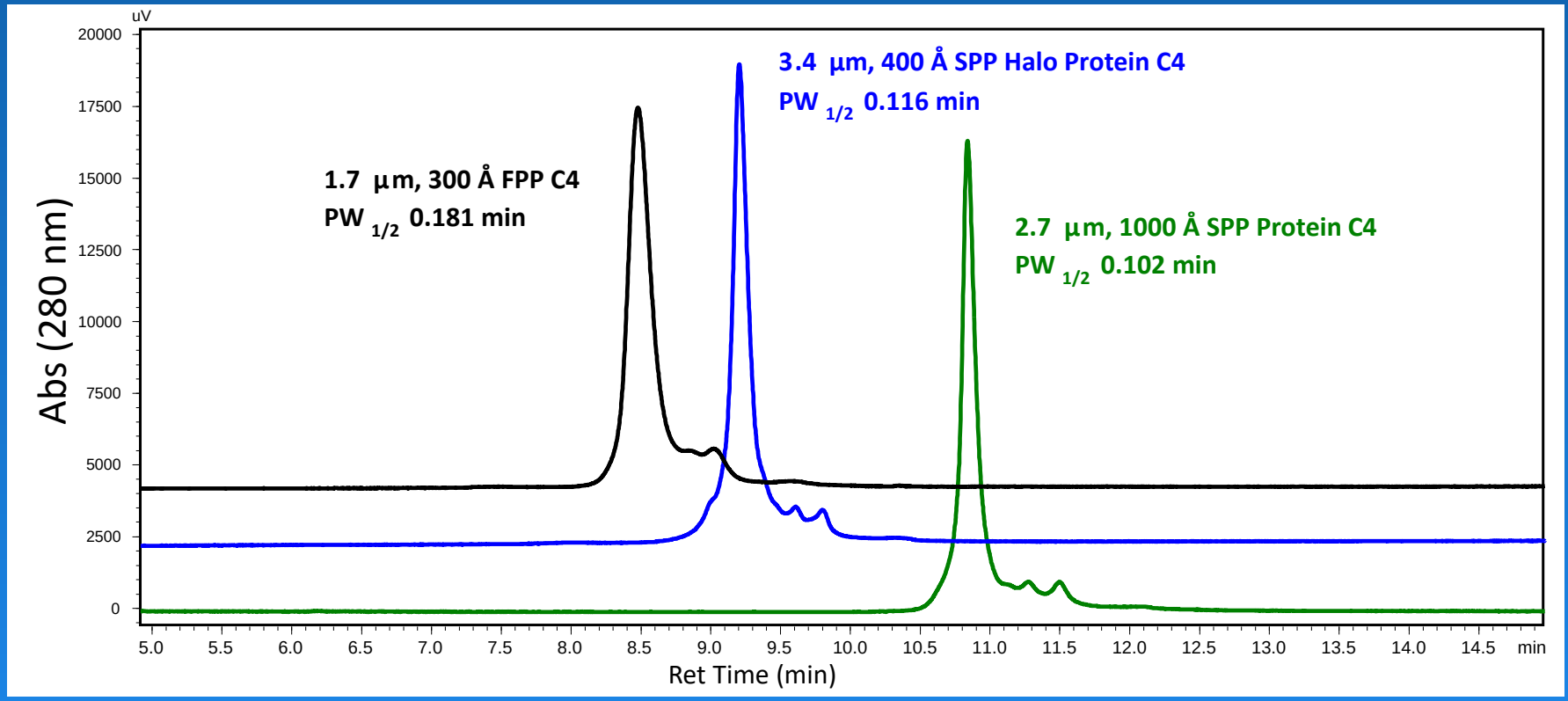
2.1 x 150 mm Halo Protein 400 C4; Gradient: 28-38% AcN/0.1% acid as indicated 15 min
Flow: 0.3 mL/min; Temp: 80ºC; Sample: 2 µL of Intact SILu™Lite SigmaMAb - 0.5 µg/µL (H_2O)



mAb IgG Separation on Wide Pore SPP and 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 ^{o}C



- Large improvement in Peak Width and *increased* Retention with Larger Pore SPP, moderate additional improvement in Peak Width with Larger Pores

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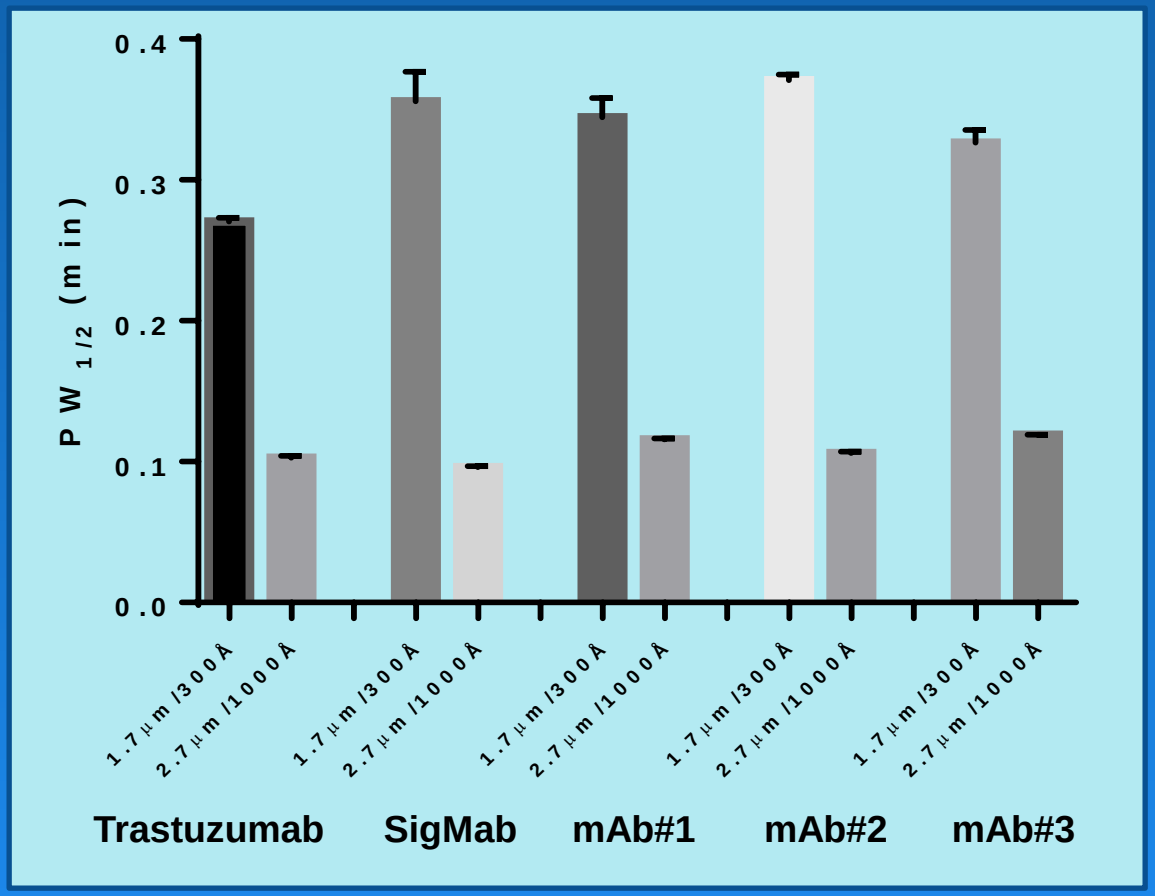
mAb Separations: 1000 Å SPP vs 300 Å FPP

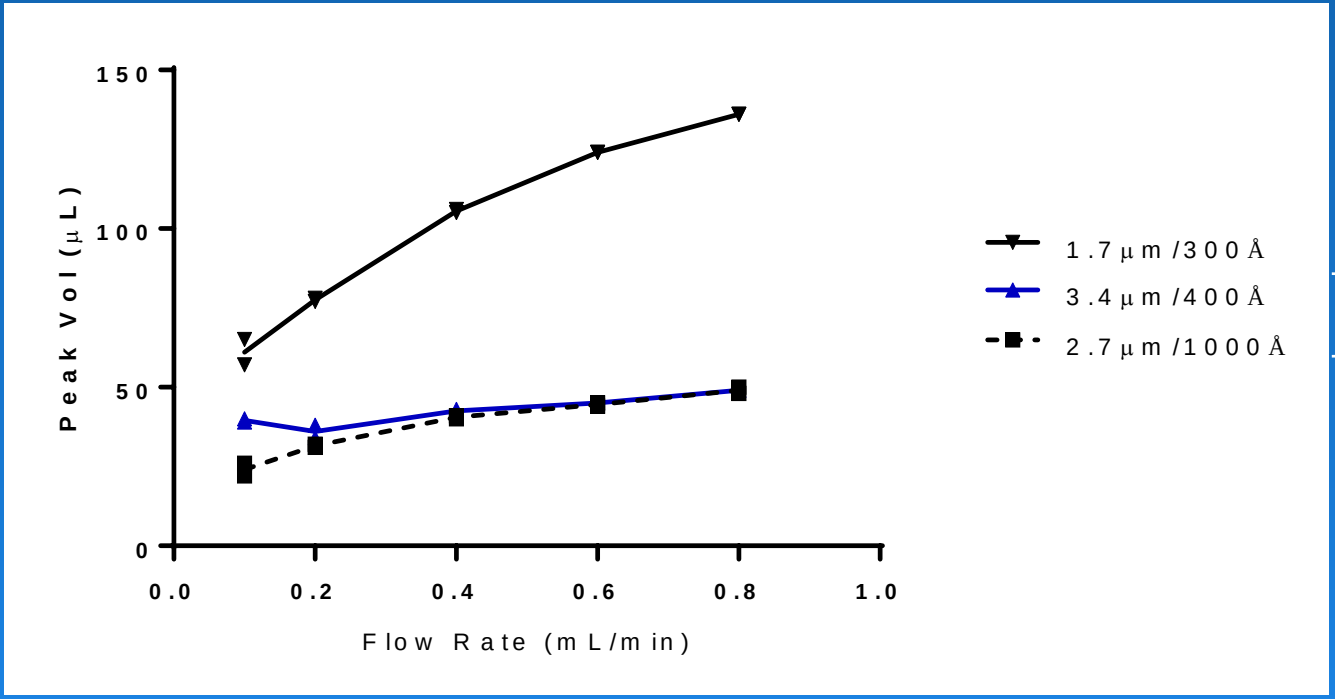
Columns: 2.1 x 150 mm; Flow rate: 0.4 mL/min; Mobile Phase A: water/0.1% DFA; Mobile Phase B: acetonitrile/0.1% DFA; Gradient: 27-37% B in 20 min; Injection Volume: 2 µL (1 µg); Temp: 80 °C



FPP : 0.334 min
SPP : 0.108 min

Flow Rate Effects on Peak Volume for mAb IgG

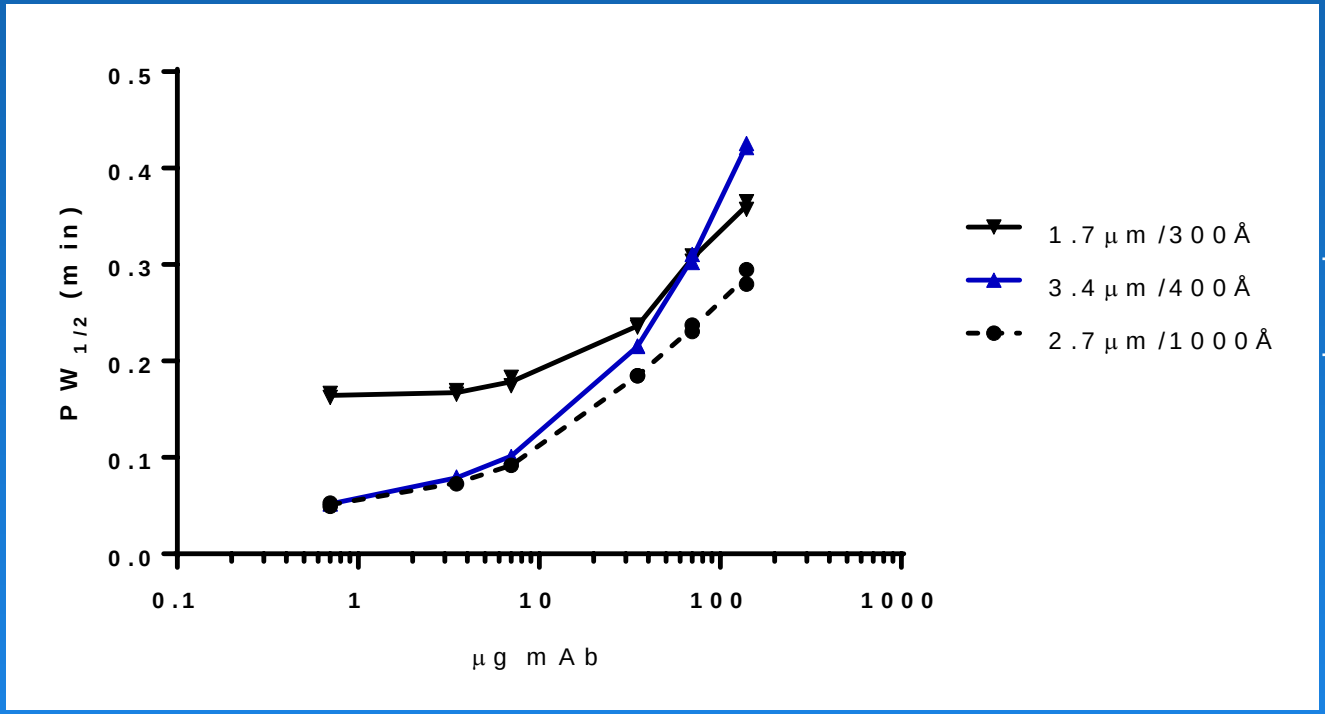
Fixed Volume Gradient Conditions (4.8 mL); Peak Volume = $PW_{1/2}$ x Flow Rate
Trastuzumab 0.5 µg; ΔG = 29-35% AcN in 0.1% DFA; 80°C;



- Under these conditions, smaller Peak Volume reflects better Mass Transfer
- Large pore SPP particles are best at all flow rates and do not show decrement with increasing flow rates.

Load Effects on Peak Width for mAb IgG

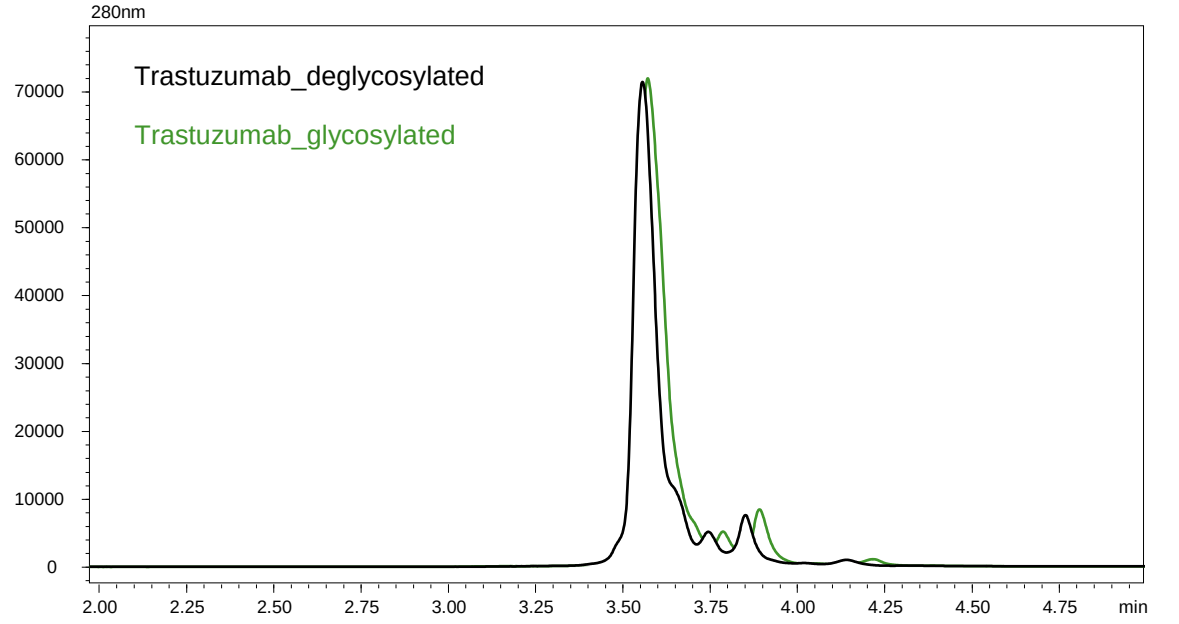
2.1 mm ID x 150 mm C4 columns; Trastuzumab 0.7 – 140 µg;
16-35% AcN (0.1% DFA) in 10 min; 80°C; 0.4 mL/min



- For larger molecules, larger 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 load tolerance

Glycosylation of mAbs by RP-LC/MS

2.1 x 100 mm Halo HALO 1000Å C4, 2.7 µm,
0.35mL/min, 0.1% DFA 28-38%AcN in 10min, 80°C, 1µg



	Go/GoF		GoF/GoF		G1F/GoF		G1F/G1F, G2F/GoF		Deglycosylated PNGase F	
	Theoric (Da)	Measured (Da)	Theoric (Da)	Measured (Da)	Theoric (Da)	Measured (Da)	Theoric (Da)	Measured (Da)	Theoric (Da)	Measured (Da)
Trastuzumab	147911	147909	148057	148056	148219	148218	148381	148385	145167	145173
ΔMass (glyc) Trastuzumab	2744	2736	2890	2883	3052	3045	3214	3212		6

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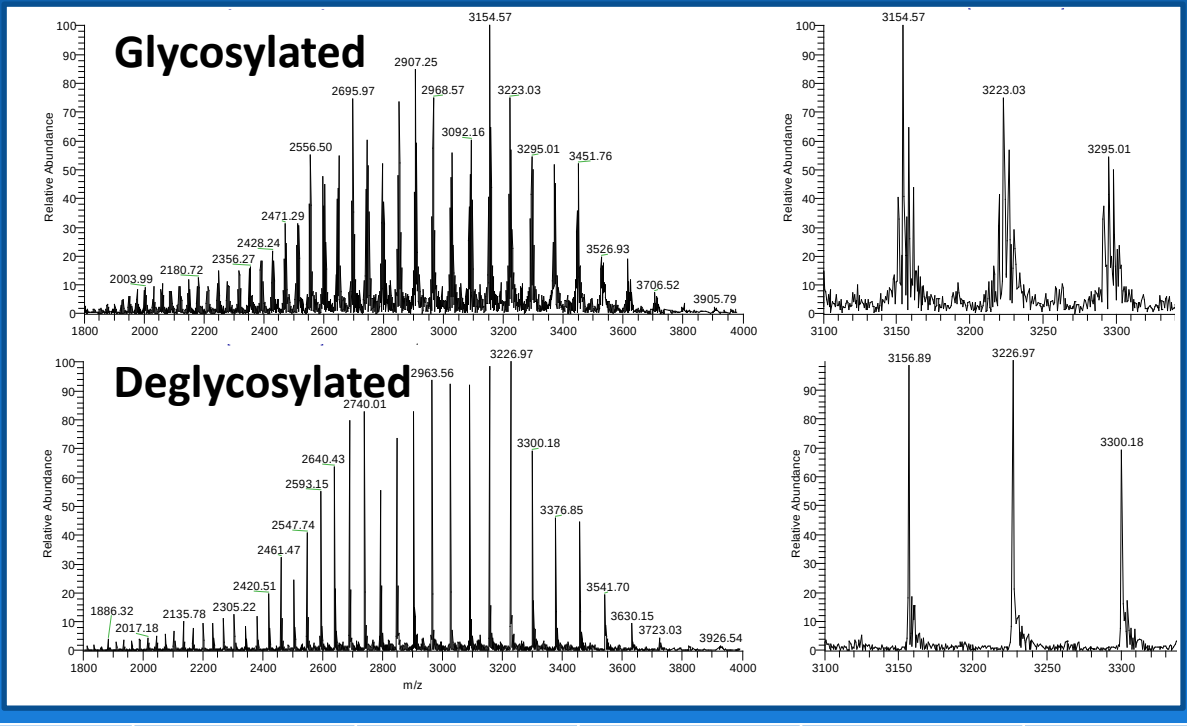
Glycosylation of mAbs by RP-LC/MS

Orbitrap VelosPro, Low flow HESI-2, Capillary 275 °C, 80V Source



	Go/GoF		GoF/GoF		G1F/GoF		G1F/G1F, G2F/GoF		Deglycosylated PNGase F	
	¹Theoric (Da)	Measured (Da)	Theoric (Da)	Measured (Da)	Theoric (Da)	Measured (Da)	Theoric (Da)	Measured (Da)	Theoric (Da)	Measured (Da)
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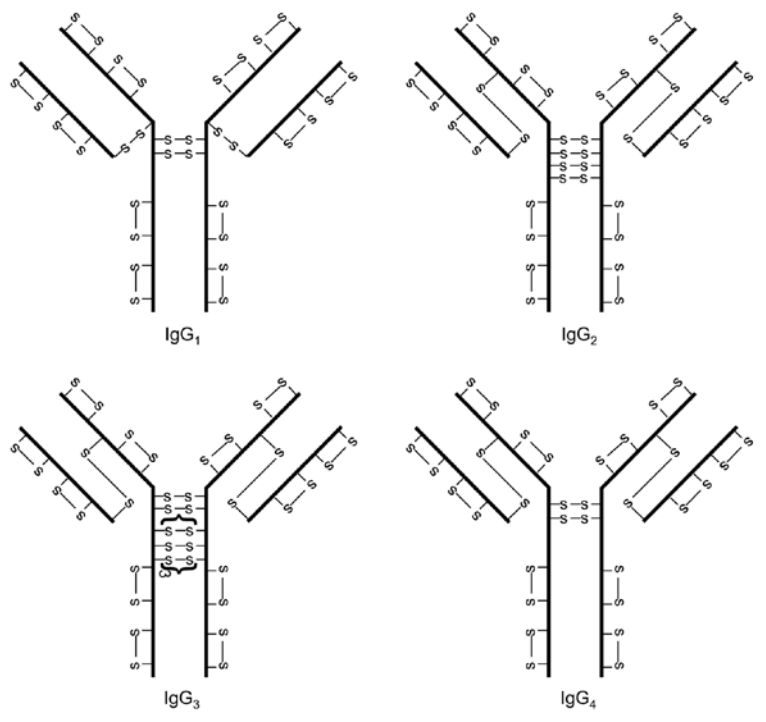
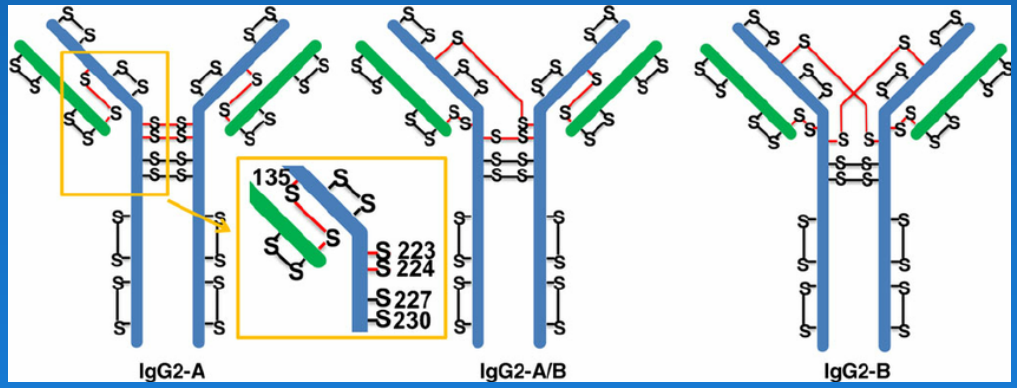
IgG Structures

IgG Isotype Subclasses



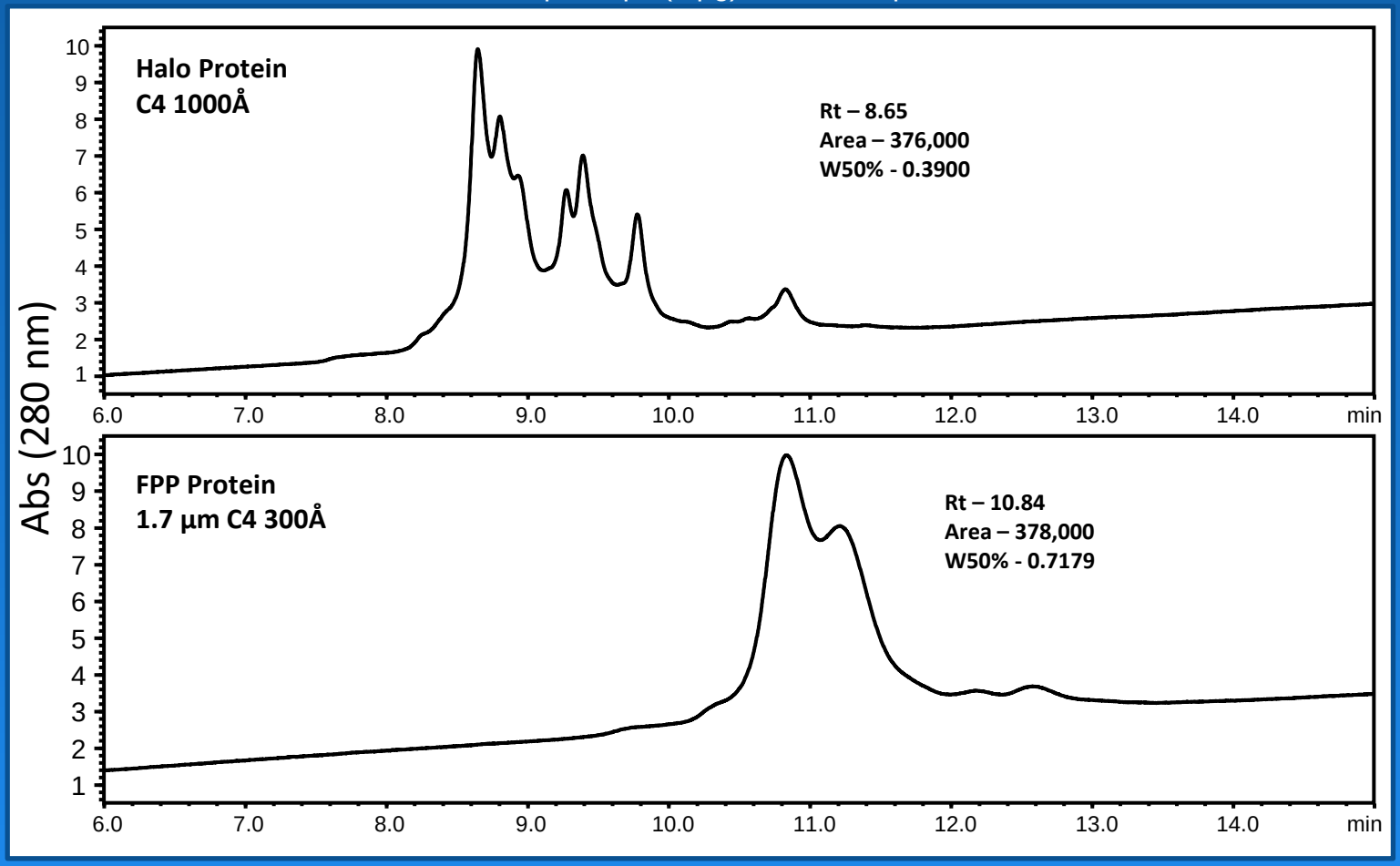
IgG_2 Isoforms



These structural variants occur in all IgG2 preparations, including therapeutic formulations

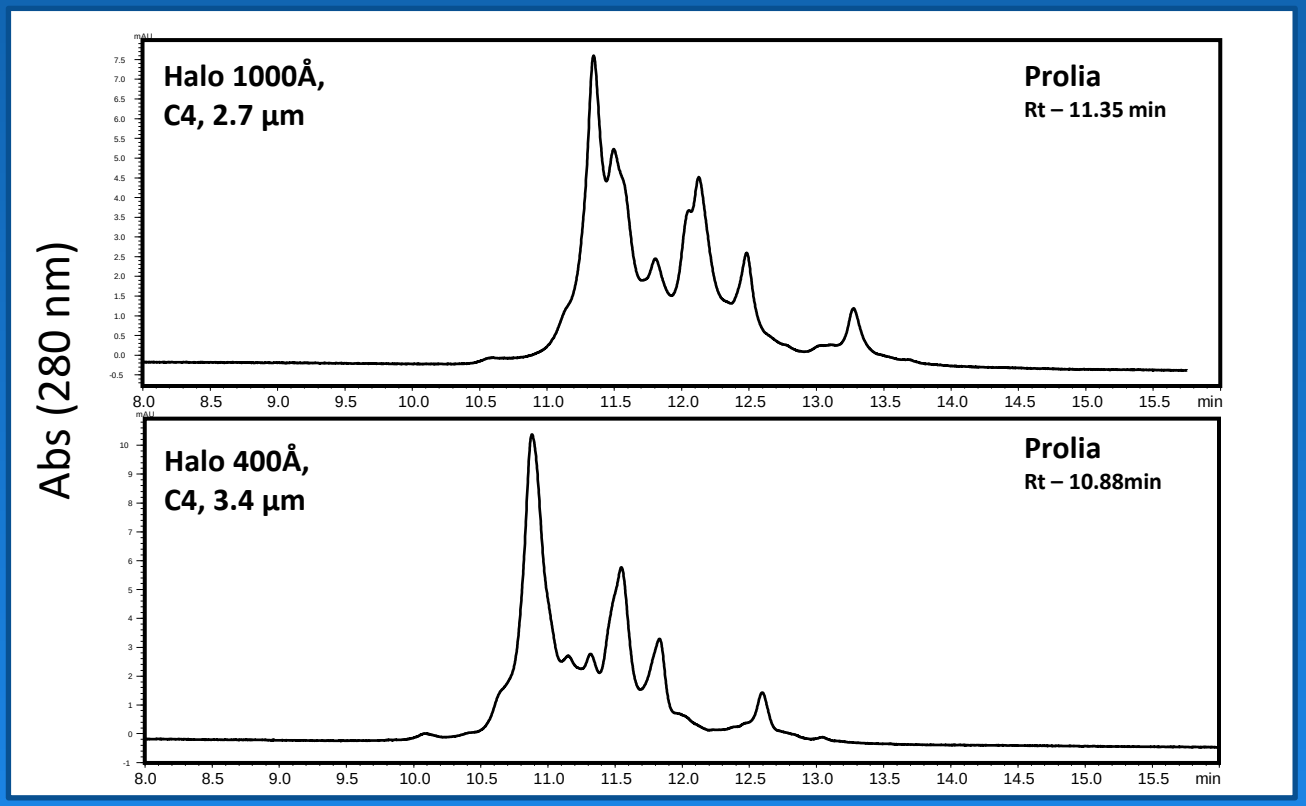
mAb IgG2 (Prolia) Separation

Columns: 2.1 x 150 mm; Flow rate: 0.4 mL/min; Mobile Phase A: water/ 5% n-PrOH/0.1%DFA; Mobile Phase B: 70% n-PrOH/20% AcN/10% water/0.1% DFA; Gradient: 14-24% B in 20 min; Sample: 4 µL (2 µg) Prolia; Temp: 80 ^{o}C



mAb IgG2 Separation on Wide Pore SPP

Columns: 2.1 x 150 mm; Flow rate: 0.4 mL/min; Mobile Phase A: water/ 5% n-PrOH/0.1%TFA; Mobile Phase B: 70% n-PrOH/20% AcN/10% water/0.1% TFA; Gradient: 16-36% B in 20 min; Injection Volume: 4 µL (2 µg); Temp: 80 °C



- Larger Pore supports different resolution of IgG2 structure variants for this therapeutic mAb.

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Conclusions

- Fused-core silica packing materials have proven high utility for biomolecule separations, without need of very high pressure operation.
- Fused-Core with enlarged pore sizes (400 Å and 1000 Å) have particular utility for protein S/F analyses, and are highly robust to allow *faster* protein separations. 1000 Å pore materials are targeted specifically for large intact protein analysis.
- IgGs are comparatively large molecules, and high performance analysis requires matching particle characteristics with sample features, particularly the increased size of the analyte.

Acknowledgements

Thank you for your Attention!

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  - Dr. Joe DeStefano, Dr. Stephanie Schuster, William Miles, Ben Libert, Mark Haynes, and Bob Moran, Dr. Mark Schure and Taylor Shields.
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