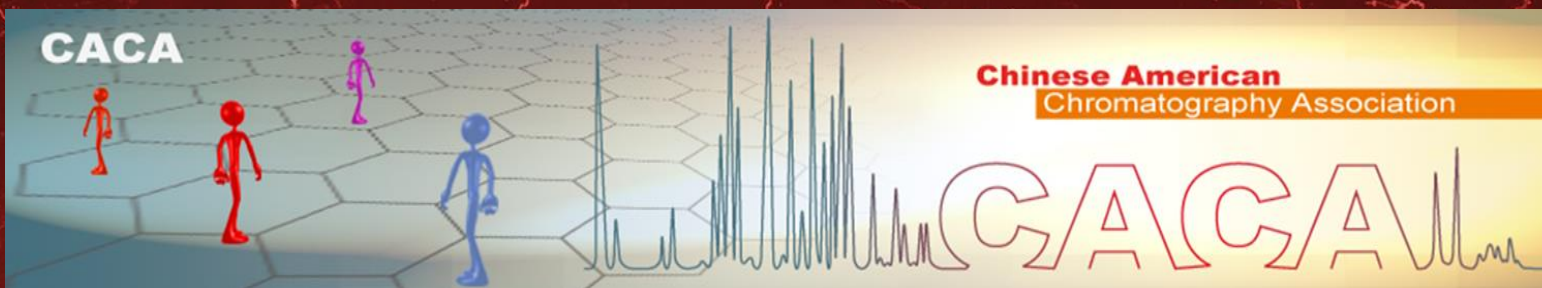
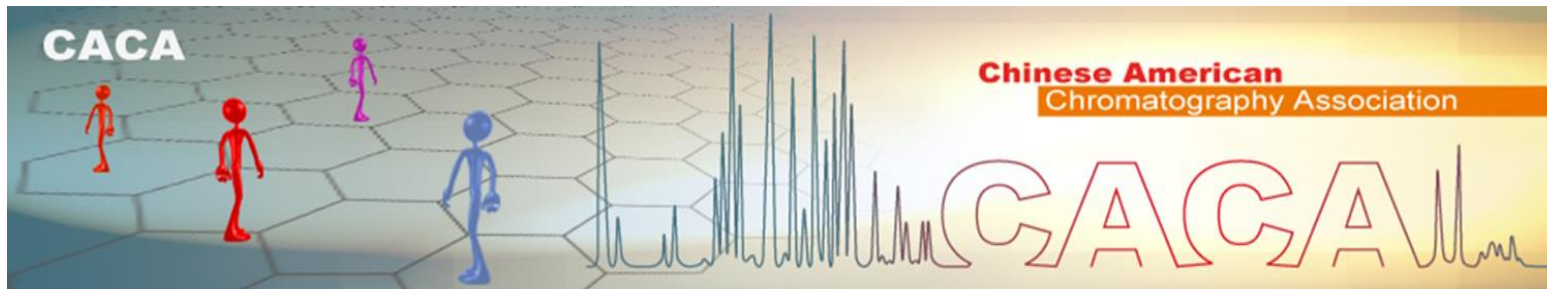




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**A Bioanalysis Webinar:
LC and LC-MS of Intact Protein Biotherapeutics and their
Variants and Panel Discussion with Industry Experts!**



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MAC-MOD Analytical – Who we are

Who we are:

- A leading supplier of Chromatography Columns and Consumables to the US Market
- 34 Years of Operating as a Chromatography Solution Provider
- Currently represent a multitude of exemplary cutting-edge manufacturing partners in the HPLC, UHPLC, LC-MS Chromatography Accessories and Safety Product Lines

Capabilities:

- Excellent manufacturing network to solve your chromatography challenges
- Technically focused sales and support staff to ensure we match the right products with your analytical needs
- Provide up-to-date, accurate technical catalogs, technical reports, webinars and white papers from industry-leading scientists to keep you informed about new technologies and application development
- Maintain excellent inventory to supply products in an expedient manner
- Consultative Sales Approach to partner with you to solve your difficult chromatography challenges



Speakers for the talks today:



Dr. Lijuan Kang
Senior Scientist
Janssen Pharmaceutical
Company



Dr. Barry Boyes
Vice President of R&D
Advanced Materials
Technology, Inc.



Dr. Lijuan Kang



Dr. Lijuan Kang
Senior Scientist
Janssen Pharmaceutical
Company

Dr. Kang is a Sr. Scientist at Janssen Pharmaceutical Companies of Johnson & Johnson. Lijuan obtained her B.Sc. in Chemistry at the Zhong Shan (Sun Yat-Sen) University, then she received her Ph.D. in Chemistry and Chemical Biology at Rutgers, the State University of New Jersey. Lijuan joined Frontage Laboratories after graduation and worked on bioanalysis method development and validations on both small and large molecules as well as regulated study support. In 2015, she joined Janssen and has been supporting preclinical and clinical bioanalysis activities since then. In addition to regular bioanalytical support to various studies, Lijuan's research interests include using advanced LC-MS methodologies to support novel therapeutics such as peptide-protein conjugates, Fc-fusion proteins, oligonucleotides, as well as bioanalysis of small and large molecule biomarkers. Lijuan has published a number of high-impact articles, including intact protein analysis on Analytical Chemistry and a recent review paper about LC-MS bioanalysis of intact proteins and peptides.

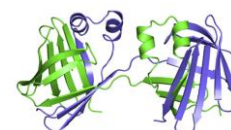
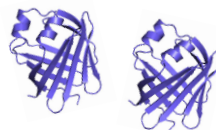
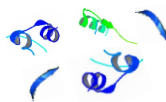
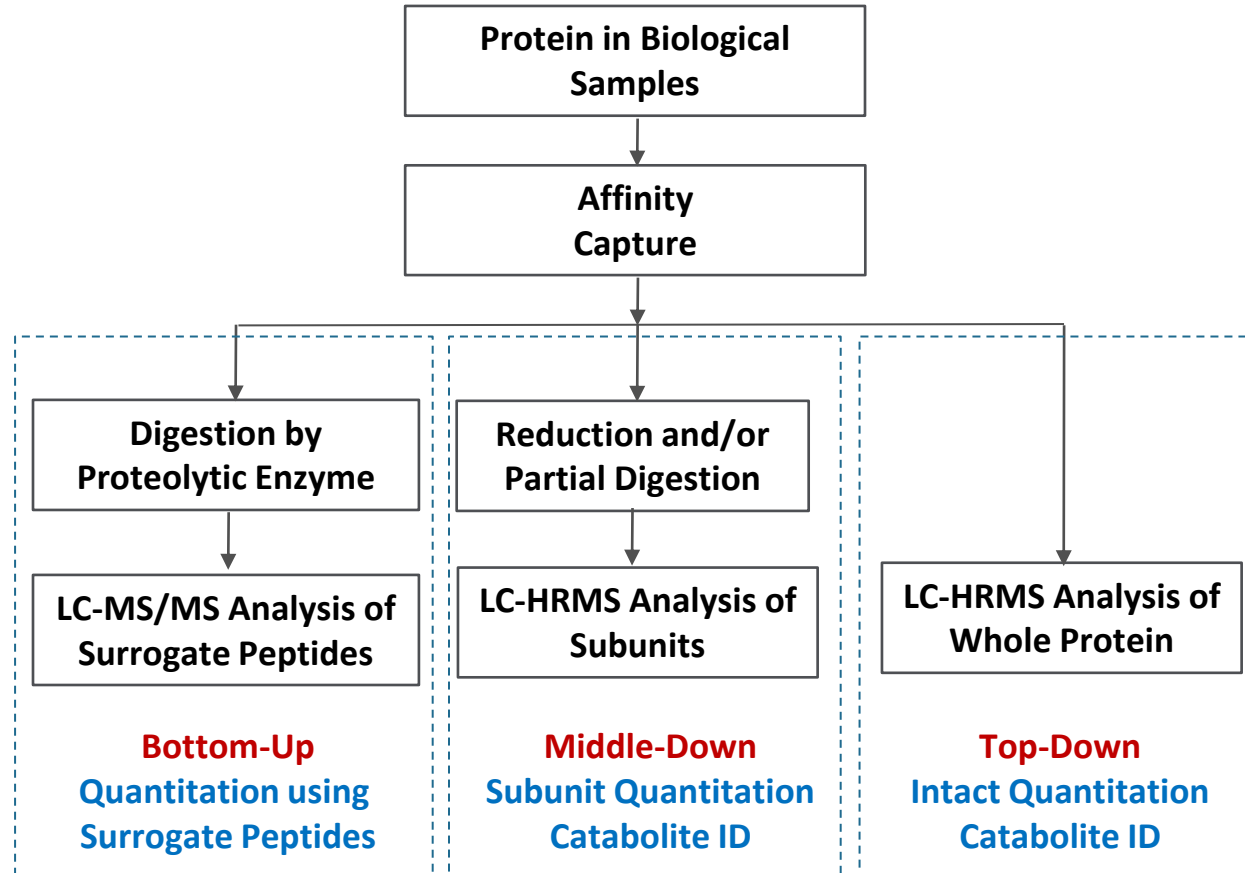


Current Chromatography Developments in Intact Protein (Top-Down) Therapeutics Bioanalysis by LC-HRMS

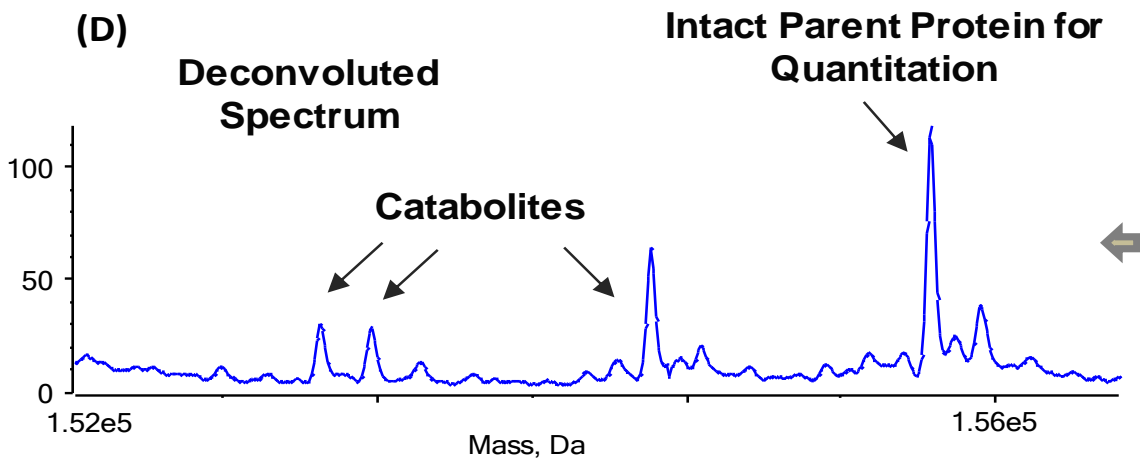
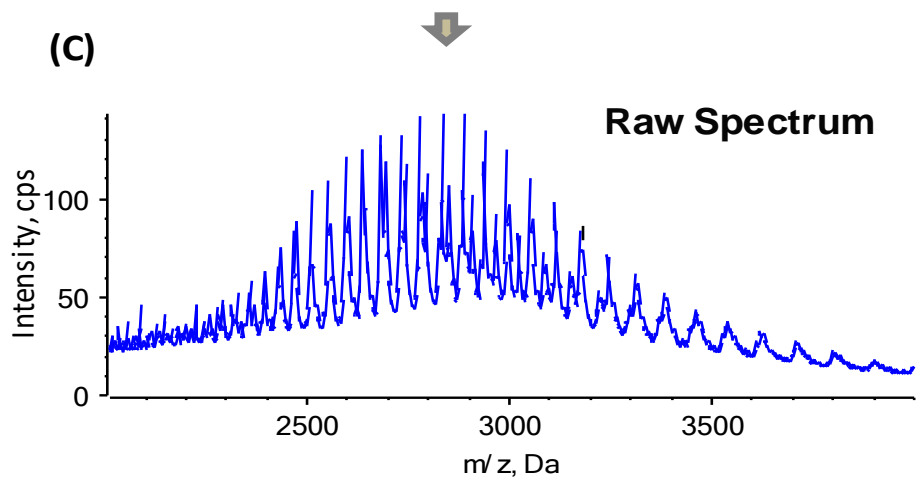
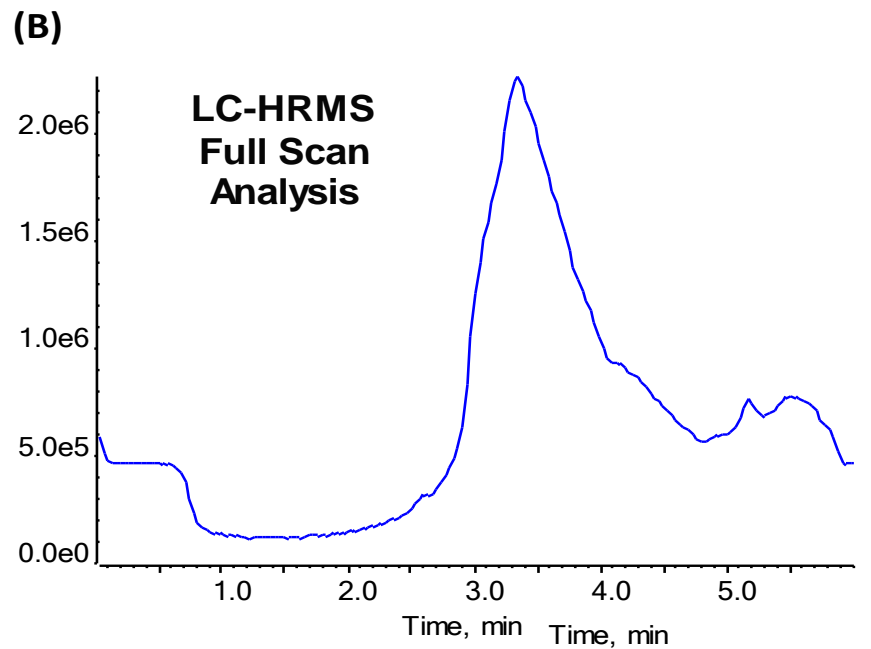
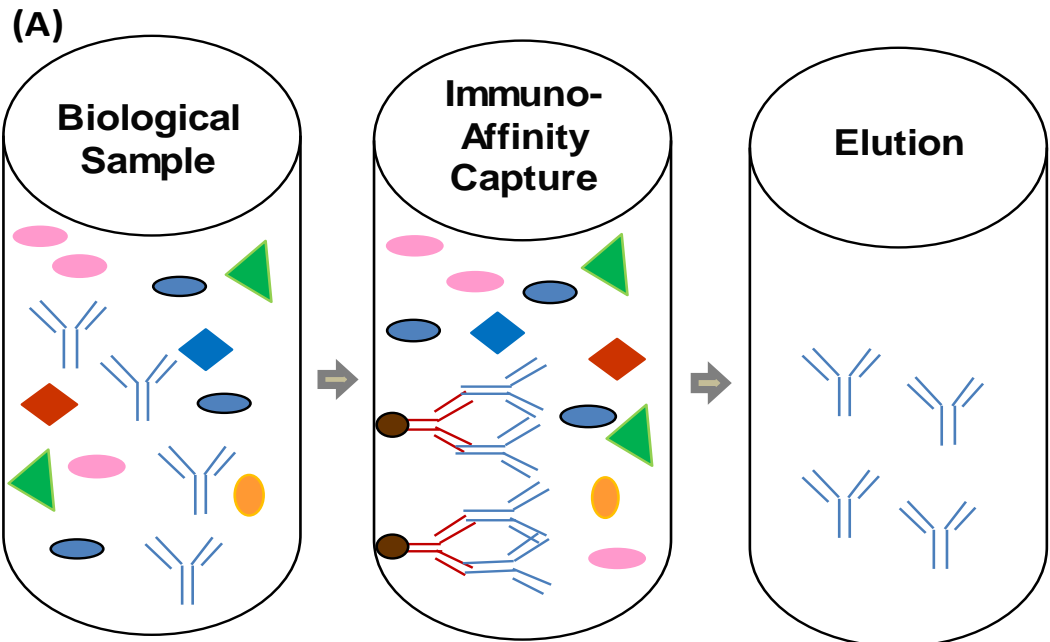
Lijuan Kang, Ph.D.
Janssen Research & Development

**CACA Webinar
21-May-2020**

Overview of Protein Bioanalysis by LC-MS

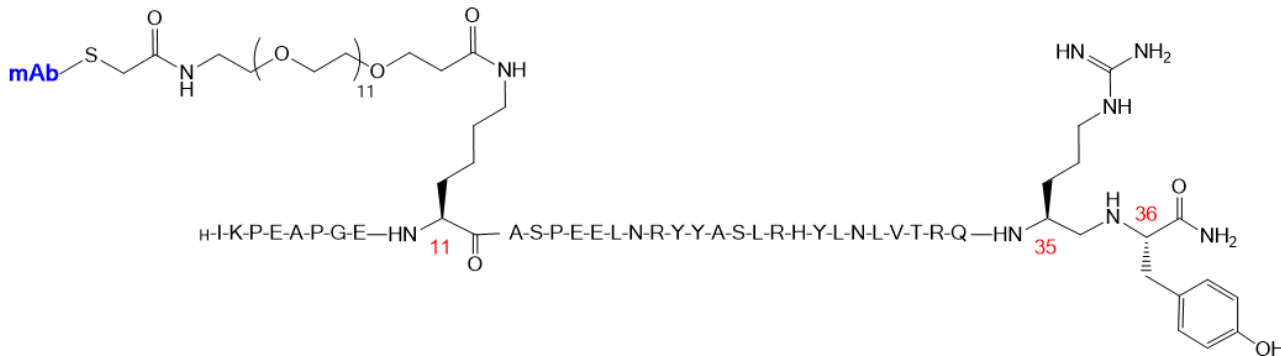
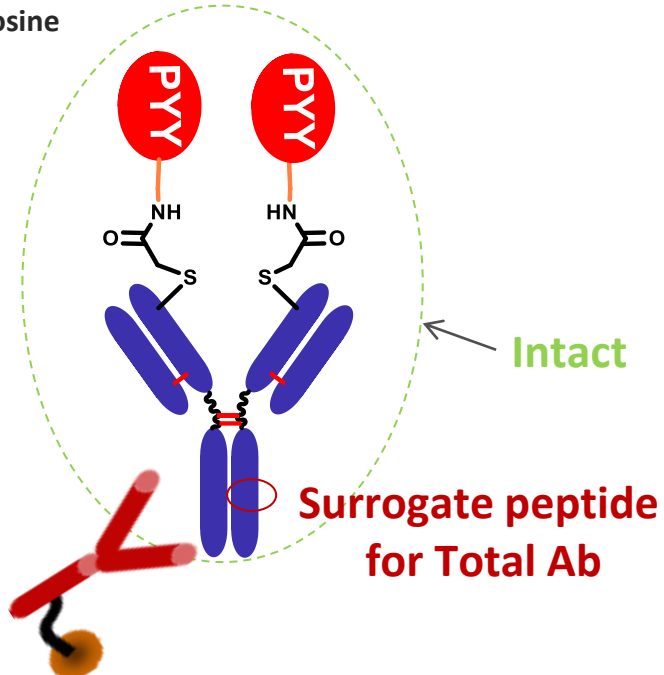


Workflow for Intact Protein Bioanalysis Using HRMS



Application Case Study – Antibody-peptide Conjugate

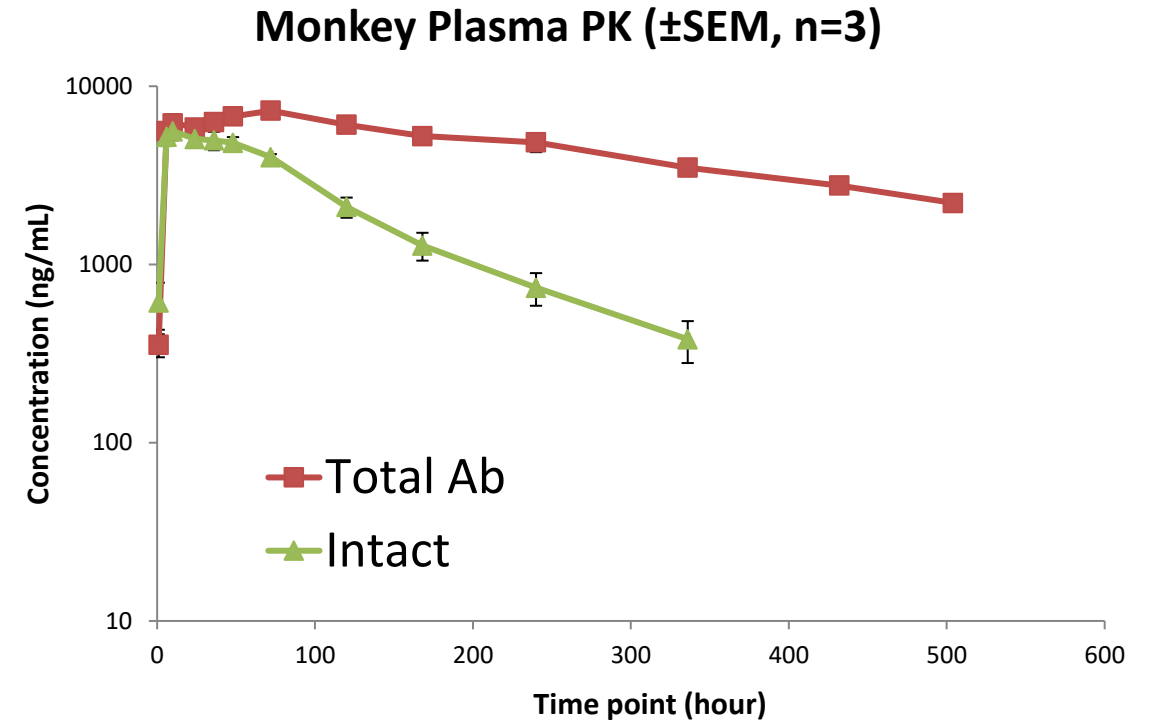
PYY: Peptide Tyrosine-Tyrosine
Gut hormone regulating food intake



One example of PYY peptide structure on the PYY-mAb conjugate

Reason for intact bioanalysis:

- No suitable surrogate peptide to represent intact molecule
- Immuno-assay not available due to lack of suitable antibody for PYY peptide with chemical modifications
- To understand catabolism/soft spots on PYY peptide

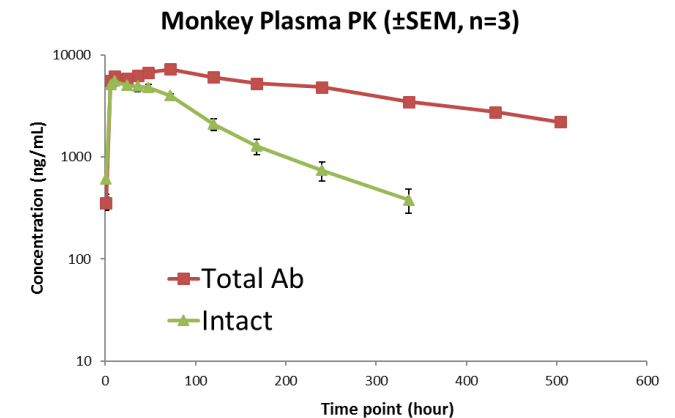


Intact Protein Bioanalysis Challenges

- Reverse phase LC (RPLC) is the most widely used chromatography coupling with MS.
- Challenges of RPLC separation of intact protein
 - Different proteoforms have similar physico-chemical properties --- Hard to separate
 - The diffusion coefficient of protein is smaller than small molecule --- Peak broadening
- Unique feature of intact protein bioanalysis using IA LC-HRMS
 - HRMS can differentiate protein variants by molecular weight
 - Immunoaffinity capture specifically enriched target protein
 - Challenge: sensitivity

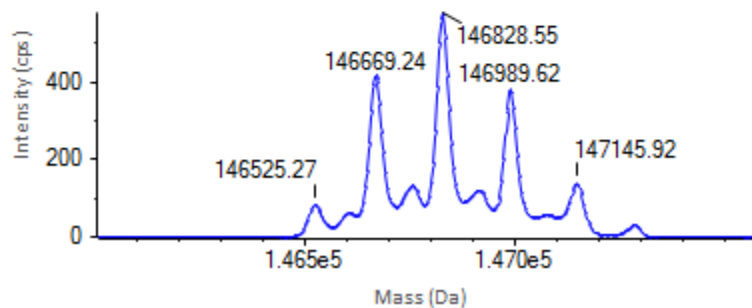
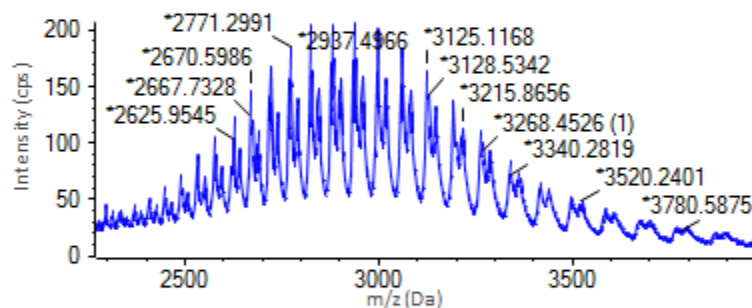
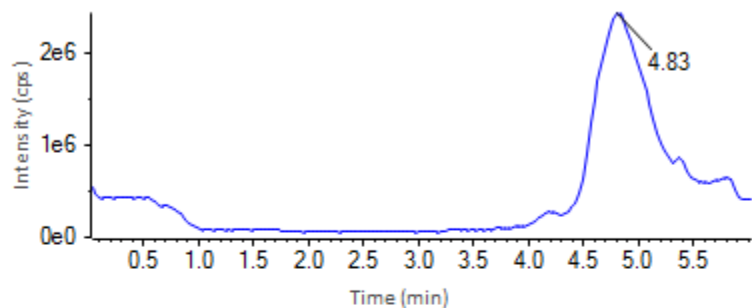
Intact Protein Bioanalysis Challenges

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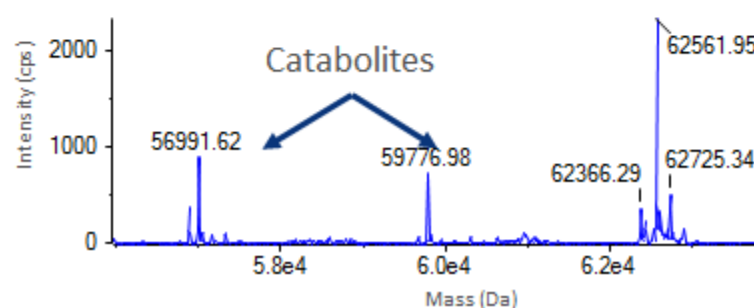
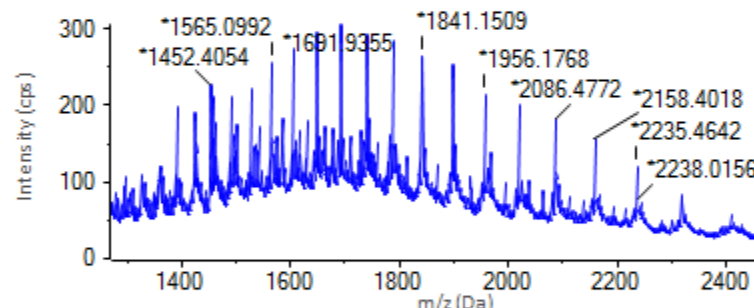
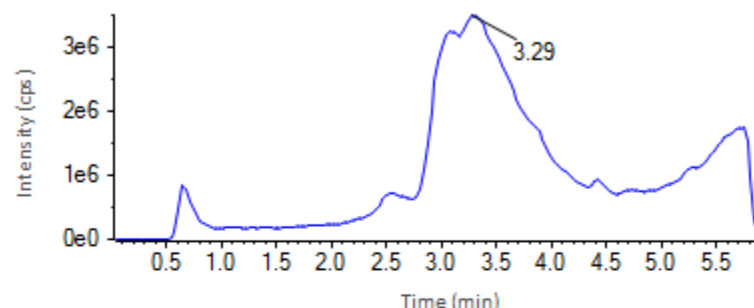


Example Chromatogram and Spectrum

Human IgG1 (~142 kDa)



Dulaglutide (~62 kDa)



Mobile Phases

A: 0.1% FA in H₂O

B: 0.1% FA in ACN

Column:

C4; 50 mm x 2.1mm; 3.6 μm

Flow Rate: 0.4 mL/min

Temperature: RT

Gradient:

Time	Module	Events	Parameter
0.15	Pumps	Pump B Conc.	10
4.50	Pumps	Pump B Conc.	90
4.80	Pumps	Pump B Conc.	90
4.95	Pumps	Pump B Conc.	10
6.00	System Controller	Stop	

Review of recent publication on intact protein bioanalysis

Analyte	MW (kDa)	LLOQ (ng/mL)	IS	Sample Volume (μL)	Injection Volume (μL)	Sample Preparation	LC (Sorbent; L * id ; particle size)	Data Processing	%Bias and %CV	Reference
SILu™ Lite mAb (Universal Human IgG1)	~146	1000	SILu™ mAb Stable-Isotope Labeled mAb	100	60	IAC - Bead Based	C4; 50 mm x 2.1mm; 3.6 μm	Mass Deconvolution	within 20%	Jian et al. (2016)
Dulaglutide (GLP1-Fc fusion protein)	~62	50	SILu™ mAb Stable-Isotope Labeled mAb	100	60	IAC - Bead Based	C4; 50 mm x 2.1mm; 3.6 μm	Mass Deconvolution	within 20%	Kang et al. (2017)
SILu™ Lite mAb (Universal Human IgG1)	~146	50	None	100	60	IAC - Bead Based	C4; 50 mm x 2.1 mm; 3.6 μm	Mass Deconvolution/ XIC	within 20%	Qiu et al. (2018)
Trastuzumab emtansine (ADC)	~151	5000	None	100	5	IAC - Bead Based	C4; 50 mm x 2.1 mm; 1.7 μm	Mass Deconvolution/ XIC	within 15%	Jin et al. (2018)
IgG1	~142	100	[¹³ C]-lysine/ arginine labeled version IgG1	50	60	IAC - Tip Based	RP-4H; 250 mm x 1 mm; monolith	XIC	within 20%	Lanshoeft et al. (2018)
BMS-986192 (protein-drug conjugate)	~11	20	None	150	30	IAC - Bead Based	C8; 50 mm x 2.1 mm; 1.7 μm	XIC	within 10%	Zhao et al. (2017)
mAb1	~150 digested and reduced to ~25	100 (Lc and Fd), 250 (1/2 Fc)	anti-idiotypic capture antibody	NA	3	IAC - Plate Based	C4; 50 mm x 150 μm; 1.7 μm	XIC	within 20%	Kellie et al. (2016)
dAb	~12	10	N ¹⁵ -labeled dAb	100	3	IAC - Plate Based	C4; 50 mm x 150 μm; 1.7 μm	XIC	within 20%	Kellie et al. (2017)
mAb2	~150	25000	In-house mouse mAb	100	3	IAC - Plate Based	C4; 50 mm x 150 μm; 1.7 μm	XIC	within 20%	
SILu™ Lite mAb (Universal Human IgG1)	~146	200	SILu™ mAb Stable-Isotope Labeled mAb	400	20	IAC - Bead Based	SEC; 100 mm x 4.6 mm; 3 μm; RP; 150 mm x 2.1 mm; 8 μm	Mass Deconvolution/ XIC	within 15%	Zhang et al. (2018)
SILu™ Lite mAb (Universal Human IgG1)	~146	5000	SILu™ mAb Stable-Isotope Labeled mAb	100	20	IAC - Bead Based	SEC; 100 mm x 4.6 mm; 3 μm; WCX; 10 mm x 1 mm; 5 μm	Mass Deconvolution/ XIC	NA	
MK8226 (mAb)	~145	14000	SILu™ mAb Stable-Isotope Labeled mAb	30	20	IAC - Bead Based	SEC; 100 mm x 4.6 mm; 3 μm; WCX; 10 mm x 1 mm; 5 μm	Mass Deconvolution/ XIC	within 20%	
Transtuzumab	~150	500	None	30	2	IAC - Bead Based	Diphenyl; 50 mm x 2.1 mm; 1.8 μm; Or PLRP-S, 50 mm x 2.1 mm; 5 μm	XIC	Within 20%	Vasicek et al. (2019)

RPLC Conditions --- LC

Analyte	MW (kDa)	LC (Sorbent; L * id ; particle size)	Mobile Phases	Reference
SILu™ Lite mAb (Universal Human IgG1)	~146	C4; 50 mm x 2.1mm; 3.6 µm	A: 0.1% FA in H2O B: 0.1% FA in ACN	Jian et al. (2016)
Dulaglutide (GLP1-Fc fusion protein)	~62	C4; 50 mm x 2.1mm; 3.6 µm	A: 0.1% FA in H2O B: 0.1% FA in ACN	Kang et al. (2017)
SILu™ Lite mAb (Universal Human IgG1)	~146	C4; 50 mm x 2.1 mm; 3.6 µm	A: 0.1% FA in H2O B: 0.1% FA in ACN	Qiu et al. (2018)
Trastuzumab emtansine (ADC)	~151	C4; 50 mm x 2.1 mm; 1.7 µm	A: 0.1% FA in H2O B: 0.1% FA in ACN	Jin et al. (2018)
IgG1	~142	RP-4H; 250 mm x 1 mm; monolith	A: 0.1% FA in H2O B: 0.1% FA in ACN	Lanshoeft et al. (2018)
BMS-986192 (protein-drug conjugate)	~11	C8; 50 mm x 2.1 mm; 1.7 µm	A: 0.1% FA, 0.1% TFA in H2O B: 0.1% FA, 0.1% TFA and 5% DMSO in ACN	Zhao et al. (2017)
mAb1	~150 digested and reduced to ~25	C4; 50 mm x 150 µm; 1.7 µm	A: 0.1% FA in H2O B: 0.1% FA in ACN:IPA (6:4)	Kellie et al. (2016)
dAb	~12	C4; 50 mm x 150 µm; 1.7 µm	A: 0.1% FA in H2O B: 0.1% FA in ACN	Kellie et al. (2017)
mAb2	~150	C4; 50 mm x 150 µm; 1.7 µm	A: 0.1% FA in H2O B: 0.1% FA in ACN	
Transtuzumab	~150	Diphenyl; 50 mm x 2.1 mm; 1.8 µm; Or PLRP-S, 50 mm x 2.1 mm; 5 µm	A: 0.1% FA in H2O B: 0.1% FA in ACN	Vasicek et al. (2019)

- Packing support and bonded phases:
 - Silica based: C4, C8, Diphenyl
 - Polymer based: PLRP-S, Monolith
- Particle
 - Pore Size
 - Particle size
 - Core Shell /Superficially Porous Particle (SPP)
- Column temperature
- Column dimension
 - Micro-flow LC-MS

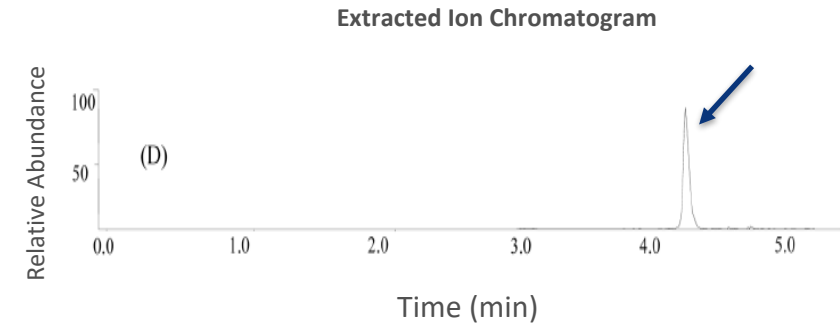
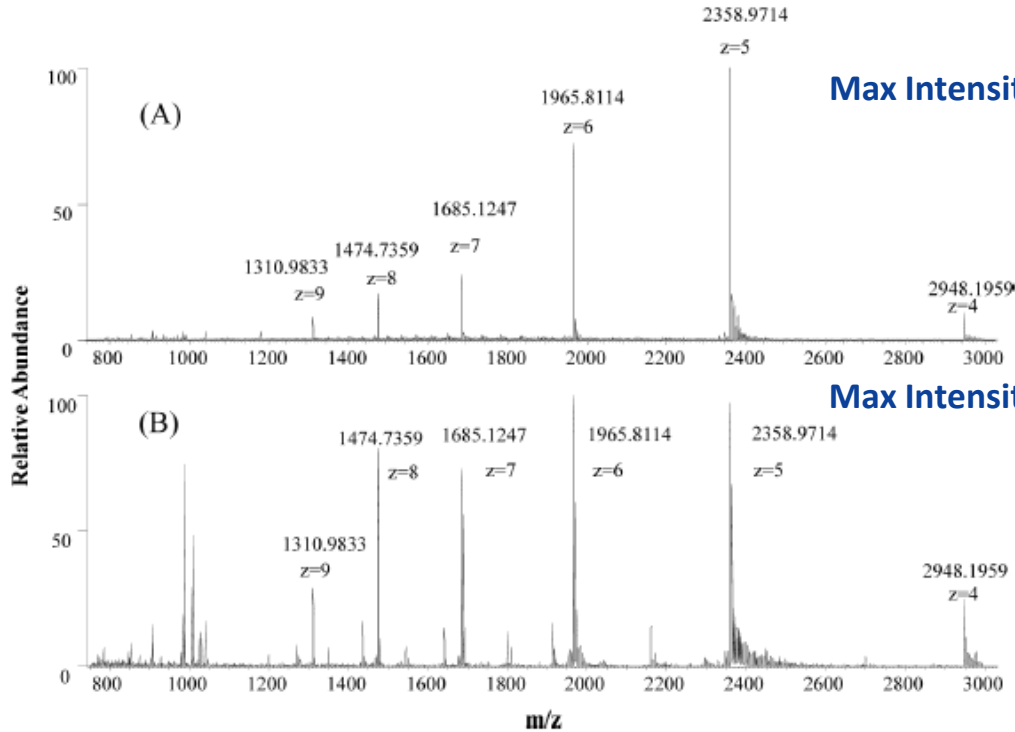
RPLC Conditions --- Mobile Phases

Analyte	MW (kDa)	LC (Sorbent; L * id ; particle size)	Mobile Phases	Reference
SILu™ Lite mAb (Universal Human IgG1)	~146	C4; 50 mm x 2.1mm; 3.6 μm	A: 0.1% FA in H ₂ O B: 0.1% FA in ACN	Jian et al. (2016)
Dulaglutide (GLP1-Fc fusion protein)	~62	C4; 50 mm x 2.1mm; 3.6 μm	A: 0.1% FA in H ₂ O B: 0.1% FA in ACN	Kang et al. (2017)
SILu™ Lite mAb (Universal Human IgG1)	~146	C4; 50 mm x 2.1 mm; 3.6 μm	A: 0.1% FA in H ₂ O B: 0.1% FA in ACN	Qiu et al. (2018)
Trastuzumab emtansine (ADC)	~151	C4; 50 mm x 2.1 mm; 1.7 μm	A: 0.1% FA in H ₂ O B: 0.1% FA in ACN	Jin et al. (2018)
IgG1	~142	RP-4H; 250 mm x 1 mm; monolith	A: 0.1% FA in H ₂ O B: 0.1% FA in ACN	Lanshoeft et al. (2018)
BMS-986192 (protein-drug conjugate)	~11	C8; 50 mm x 2.1 mm; 1.7 μm	A: 0.1% FA, 0.1% TFA in H ₂ O B: 0.1% FA, 0.1% TFA and 5% DMSO in ACN	Zhao et al. (2017)
mAb1	~150 digested and reduced to ~25	C4; 50 mm x 150 μm; 1.7 μm	A: 0.1% FA in H ₂ O B: 0.1% FA in ACN:IPA (6:4)	Kellie et al. (2016)
dAb	~12	C4; 50 mm x 150 μm; 1.7 μm	A: 0.1% FA in H ₂ O B: 0.1% FA in ACN	Kellie et al. (2017)
mAb2	~150	C4; 50 mm x 150 μm; 1.7 μm	A: 0.1% FA in H ₂ O B: 0.1% FA in ACN	
Transtuzumab	~150	Diphenyl; 50 mm x 2.1 mm; 1.8 μm; Or PLRP-S, 50 mm x 2.1 mm; 5 μm	A: 0.1% FA in H ₂ O B: 0.1% FA in ACN	Vasicek et al. (2019)

- Mobile Phases:
 - A: 0.1% FA in H₂O
 - B: 0.1% FA in ACN
- Modifiers:
 - Improving peak shape: Trifluoroacetic acid (TFA)
Difluoroacetic acid (DFA)
 - Supercharging reagent: DMSO
 - Post column addition of Triethanolamine (TEA)

Supercharging Reagent Effect

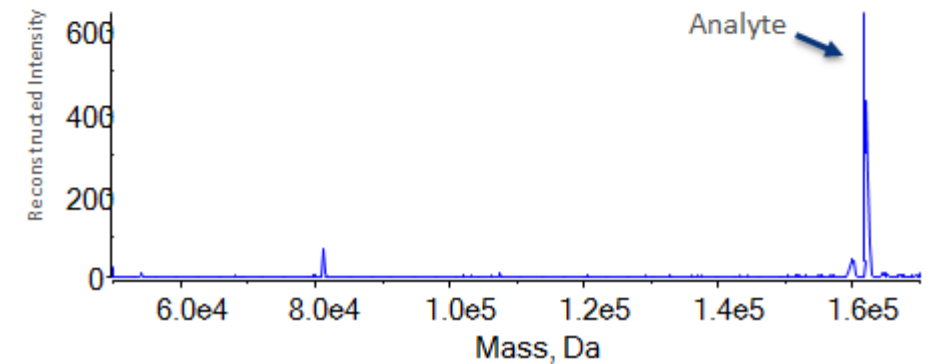
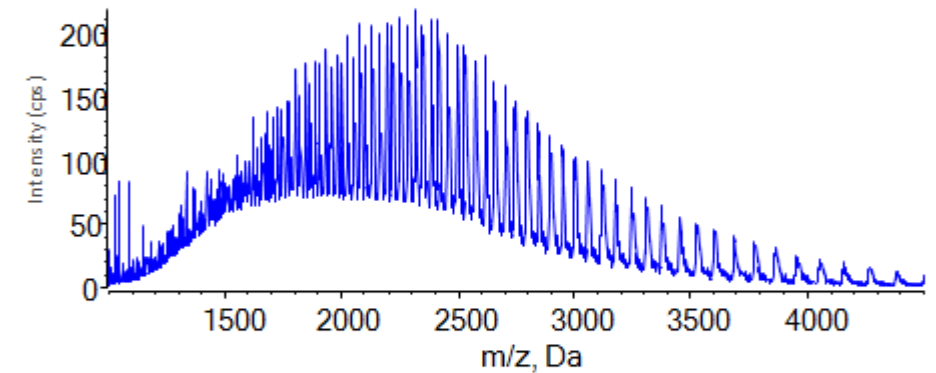
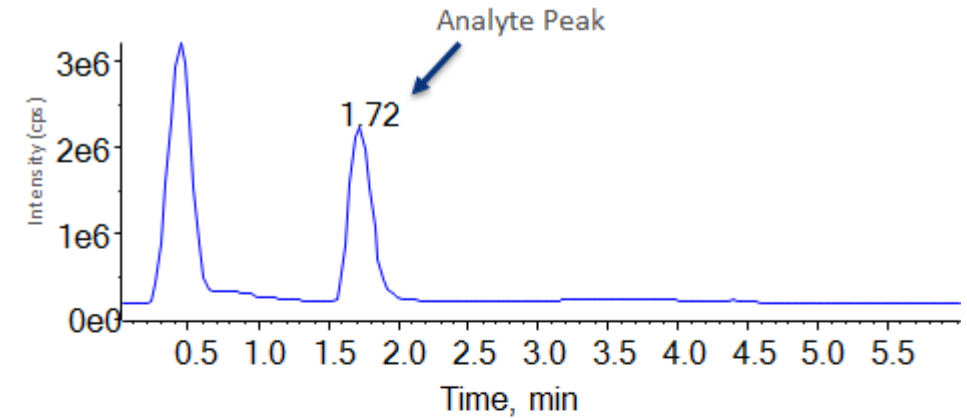
Analyte	MW (kDa)	LC (Sorbent; L * id ; particle size)	Mobile Phases	Reference
BMS-986192 (protein-drug conjugate)	~11	C8; 50 mm x 2.1 mm; 1.7 μ m	A: 0.1% FA, 0.1% TFA in H ₂ O B: 0.1% FA, 0.1% TFA and 5% DMSO in ACN	Zhao et al. (2017)



- Addition of 5% DMSO lead to charge states coalescence to lower charge state.
- Charge states 5 and 6 which were used for quantitation, the XIC peak intensity was increased.

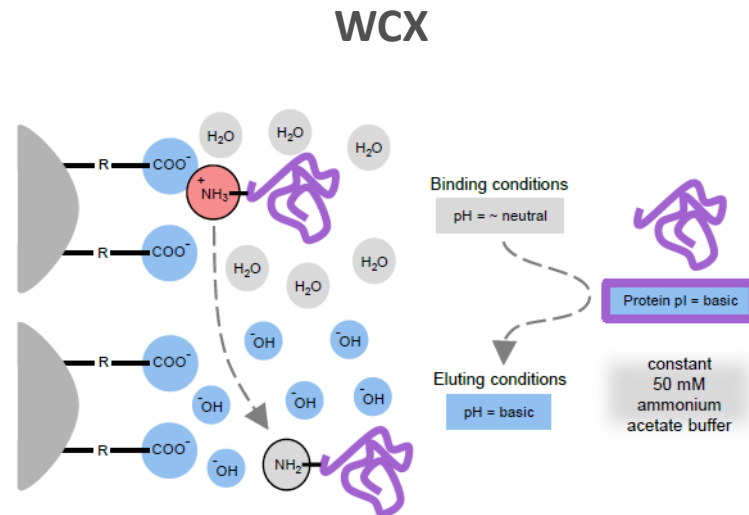
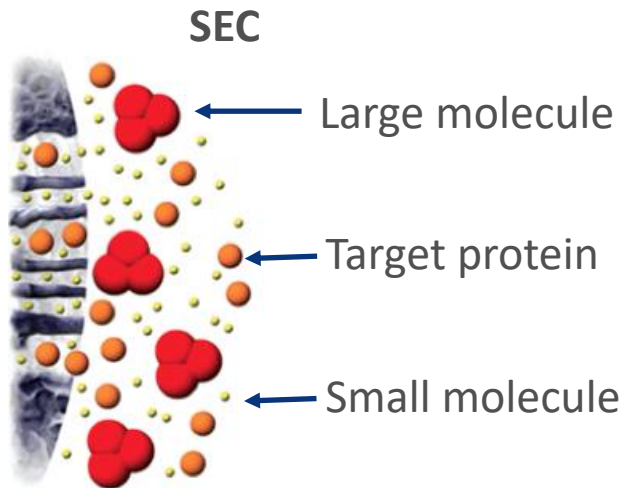
Post-column addition of TEA

- No signal was observed without post-column addition of TEA.
- Signal was observed with addition of post-column addition of TEA.
- The post column addition of amines is known to reduce the charge complexity of large protein and PEGylated proteins.
- LC mobile phase conditions:
 - Flow rate: 0.4 mL/min
 - A: 0.1% formic acid in water
 - B: 0.1% formic acid in ACN
 - C: 0.2% TEA in 50% ACN added by post-column T at 0.05 mL/min



Native vs. Denatured Condition

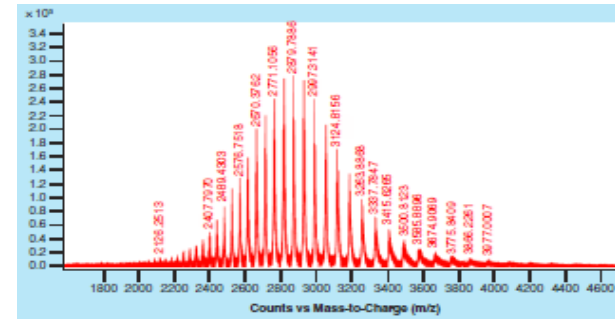
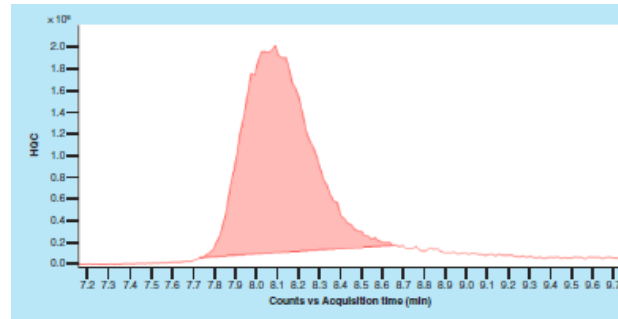
Analyte	MW (kDa)	LLOQ	LC (Sorbent; L * id ; particle size)	Mobile Phases	Reference
SILu™ Lite mAb (Universal Human IgG1)	~146	200	SEC; 100 mm x 4.6 mm; 3 μm; RP (PLRP-S); 150 mm x 2.1 mm; 8 μm	SEC: H ₂ O:ACN:FA (90:10:0.1) RP: A: 0.1% FA in H ₂ O B: 0.1% FA in ACN	Zhang et al. (2018)
SILu™ Lite mAb (Universal Human IgG1)	~146	5000	SEC; 100 mm x 4.6 mm; 3 μm; WCX; 10 mm x 1 mm; 5 μm	SEC: 100mM Ammonium Acetate, pH 7; WCX: A:50 mM Ammonium Acetate, pH 6.5	
MK8226 (mAb)	~145	14000	SEC; 100 mm x 4.6 mm; 3 μm; WCX; 10 mm x 1 mm; 5 μm	B: 50mM Ammonium Acetate, pH9.5	



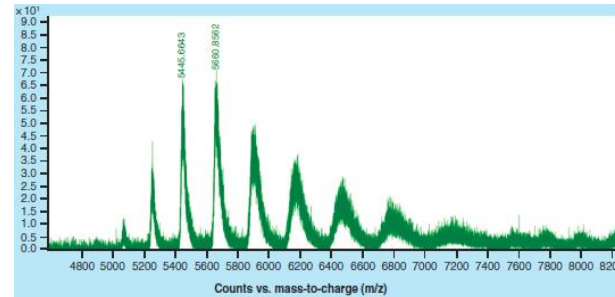
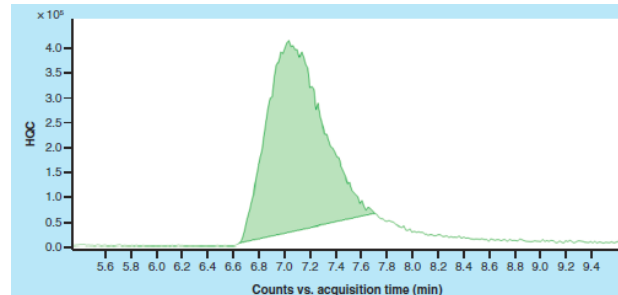
- Size exclusion chromatography (SEC): separate protein by size.
- Weak cation exchange (WCX): separate protein by charge variant.
- Native LC-MS mobile phases
 - Volatile salt buffer

Native vs. Denatured Condition

Denatured



Native

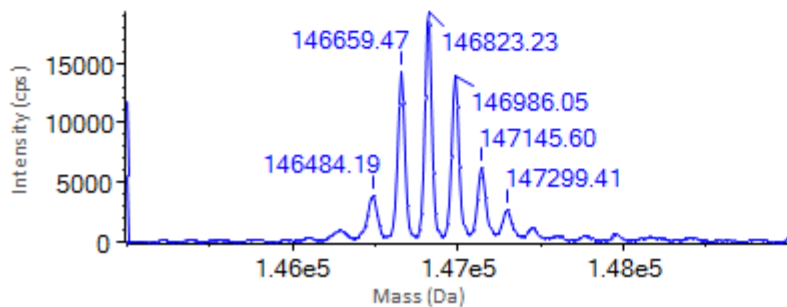
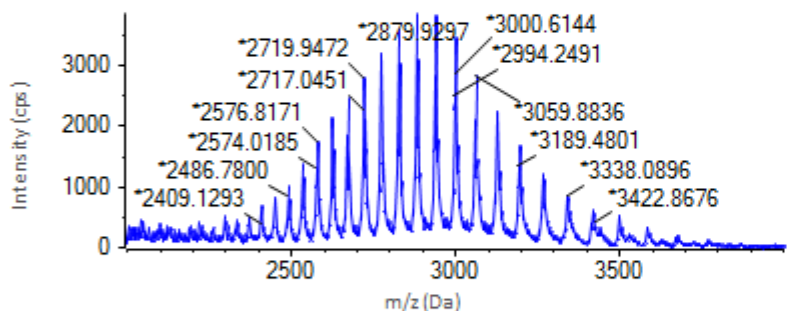
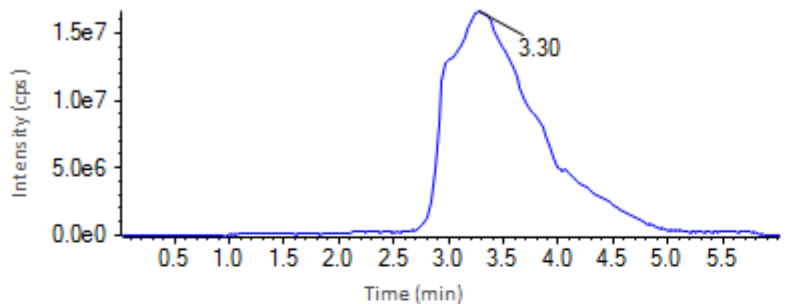


- The work successfully quantified antibody in native form using 2D LC-HRMS.
- Under native condition, the sensitivity is much lower than in denatured condition.
 - Suboptimal electrospray solvent

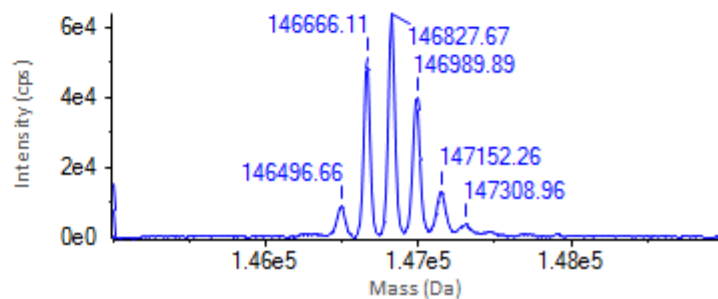
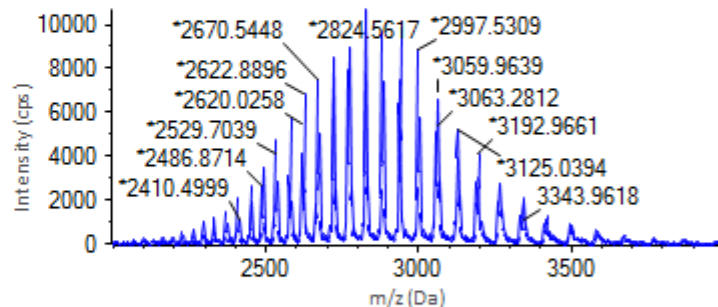
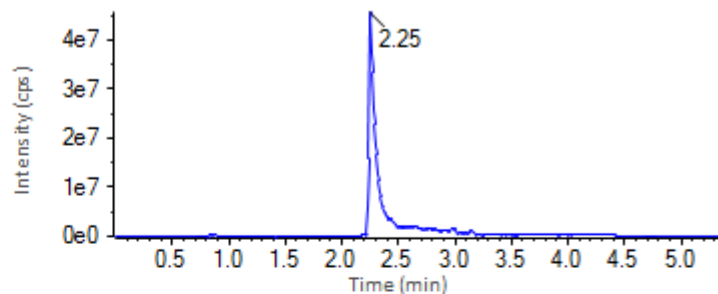
Recent chromatography development of intact protein bioanalysis in our lab

Spectrum of 10 µg/mL IgG1 in mouse plasma

Original FPP C4



Current SPP Diphenyl



Current LC Condition

Mobile Phases
 A: 0.1% FA in H₂O
 B: 0.1% FA in ACN

Column:
 Halo Diphenyl Column; 50 mm
 x 2.1mm; 2.7 µm; 1000 Å

Flow rate: 0.4 mL/min

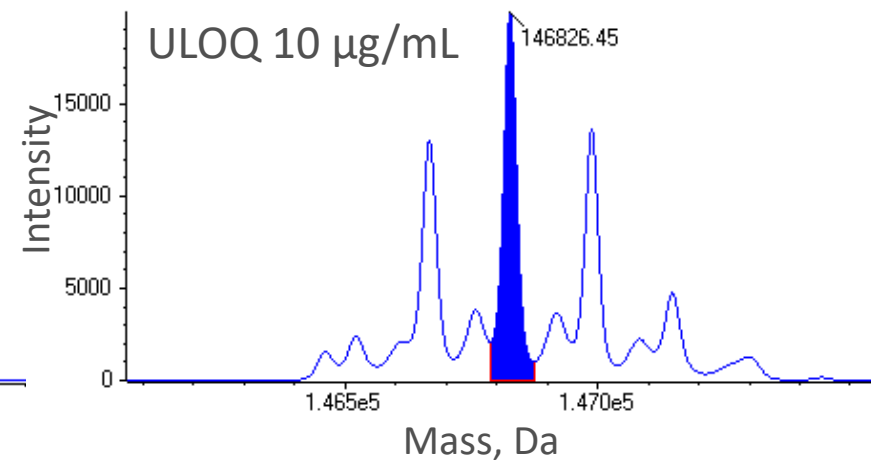
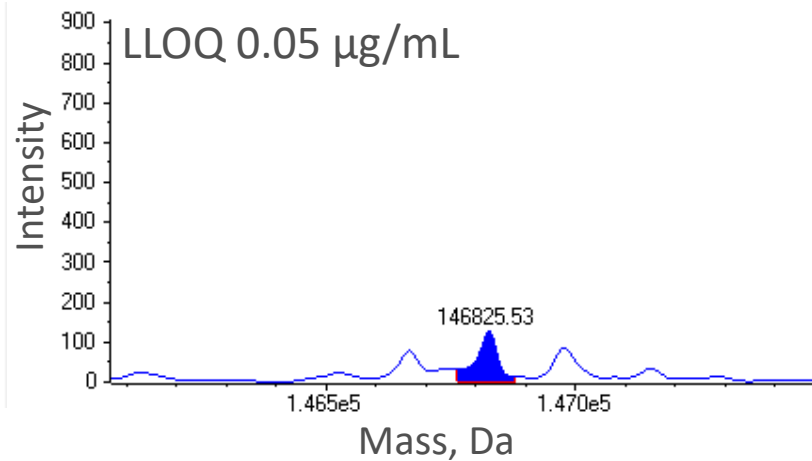
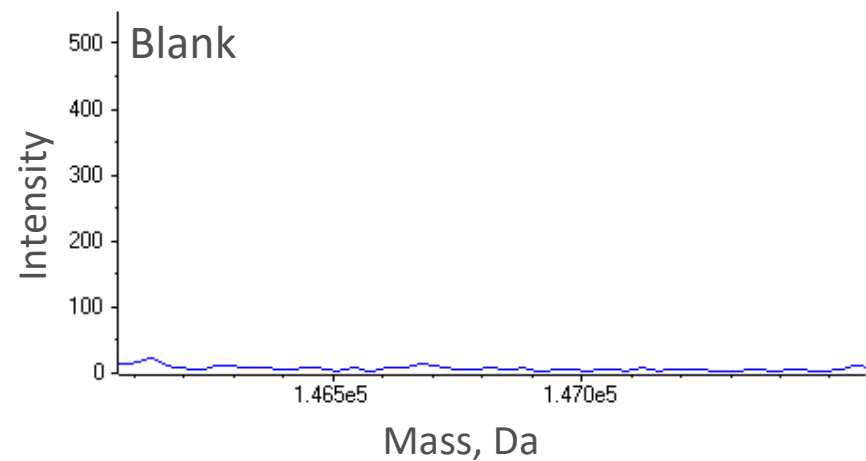
Column Temperature: 60 °C

Gradient:

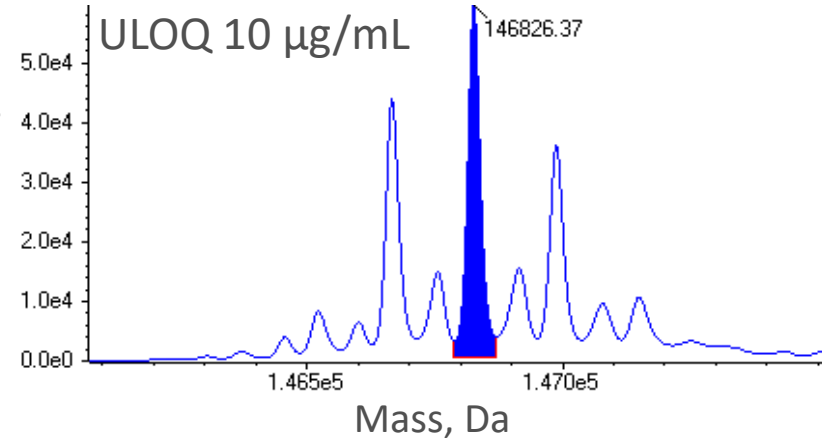
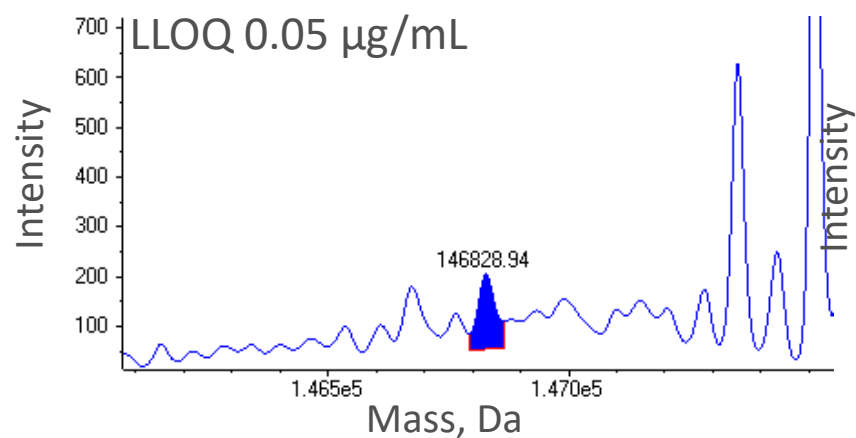
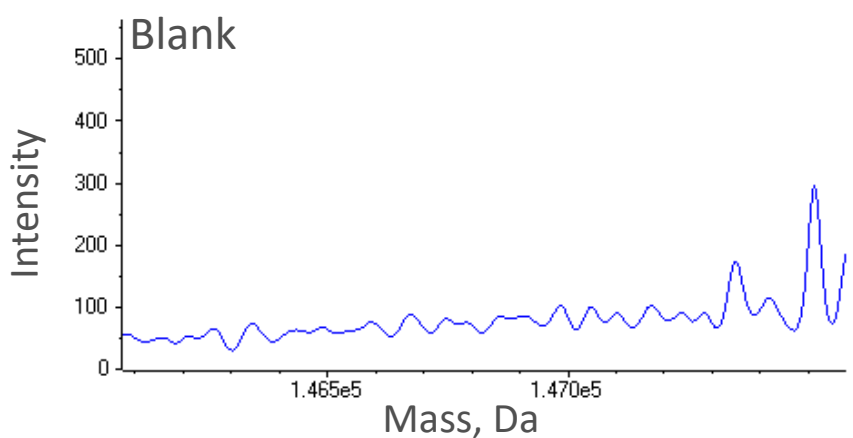
Time	Module	Events	Parameter
0.50	Pumps	Pump B Conc.	20
3.50	Pumps	Pump B Conc.	90
4.20	Pumps	Pump B Conc.	90
4.50	Pumps	Pump B Conc.	20
5.40	System Controller	Stop	

Deconvoluted spectrum of IgG1 spiked in mouse plasma

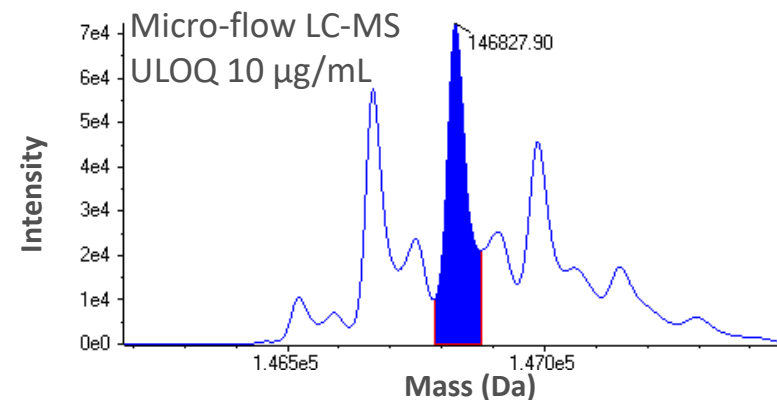
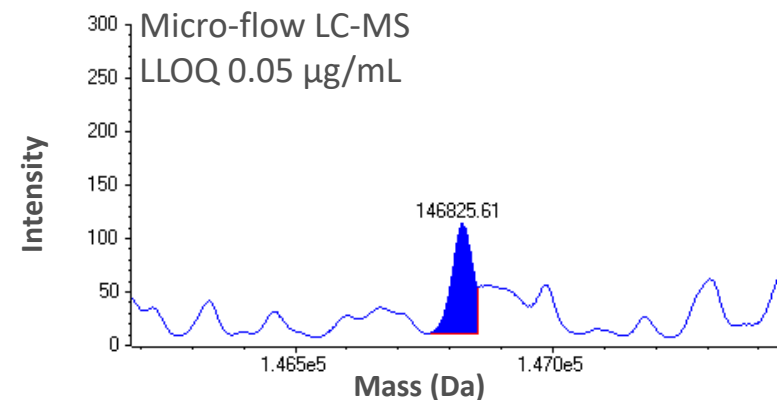
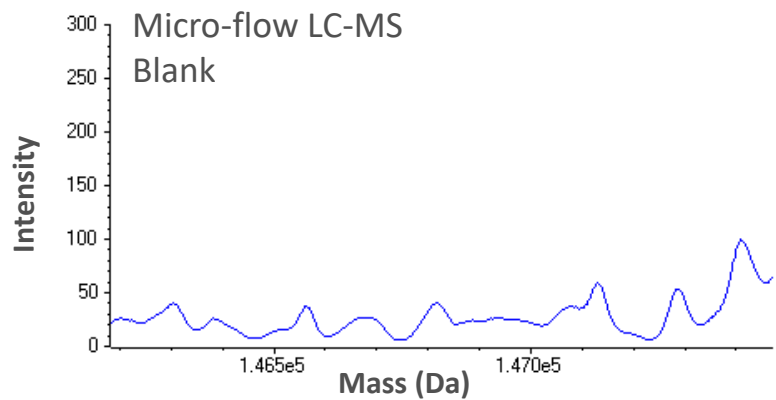
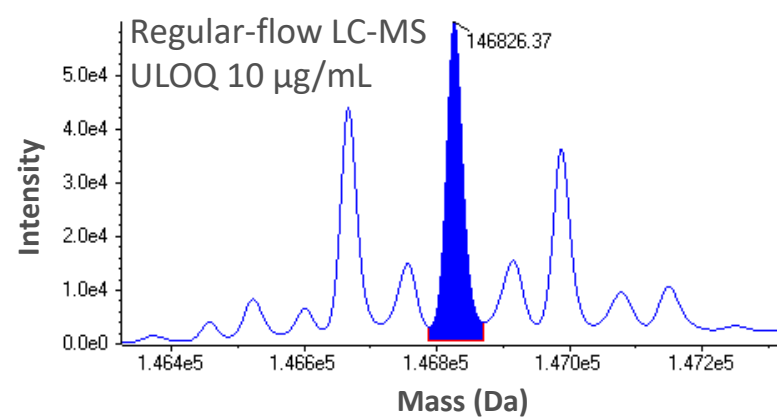
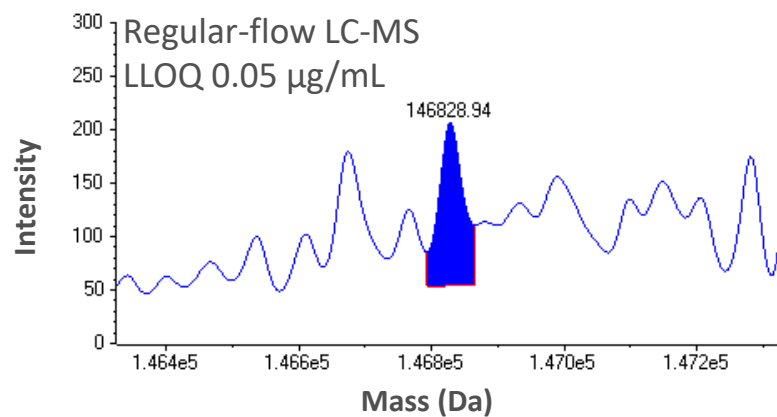
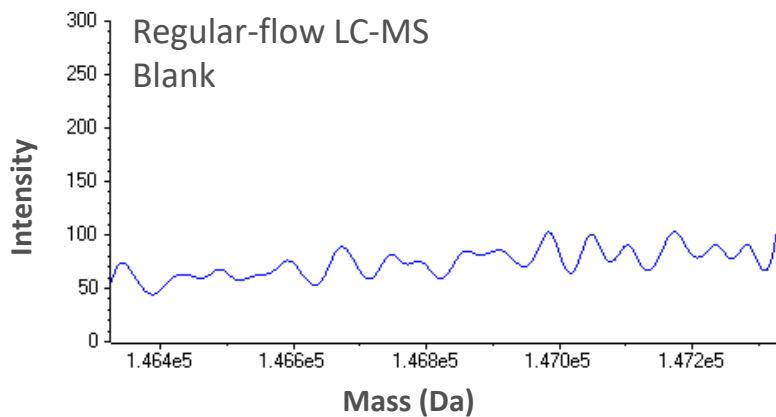
Original



Current



Deconvoluted spectrum of IgG1 spiked in mouse plasma



Conclusion

- LC-HRMS intact protein bioanalysis can provide intact protein quantitation as well as the important high-level structure and biotransformation information.
- Challenging for intact protein bioanalysis is limited sensitivity.
- Continue the effort on microflow-LC with development of compatible traps/columns, improved instrument configuration for intact analysis.

Acknowledgment

- Janssen DMPK
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 - Yi Zhang
 - Michael Merriman
 - John Hevko
- Advanced Materials Technology
 - Stephanie Rosenberg
- Mac-Mod Analytical
 - Eddie Faden

Questions and Answers for Dr. Lijuan Kang



Dr. Barry Boyes

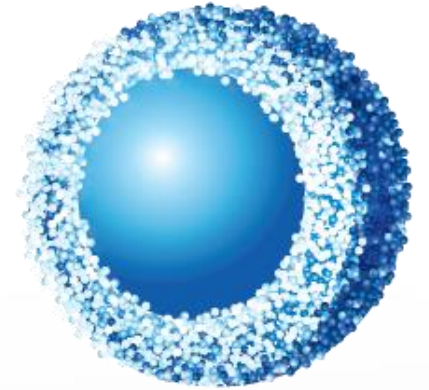


Dr. Barry Boyes
Vice President of R&D
Advanced Materials
Technology, Inc.

Dr. Boyes is the Vice President of R&D at Advanced Materials Technologies (AMT), Inc., in Wilmington, DE, USA. Barry completed a B.Sc. in Biochemistry at the University of Alberta, then Ph.D. in Neuroscience at UBC (Vancouver, B.C.), under the supervision of Prof. Edith G. McGeer. Barry worked at DuPont Central Research, then Rockland Technologies Inc. (ZORBAX columns), developing a variety of products and technologies for biomolecule separations. HP acquired Rockland Technologies in 1997, which later became part of Agilent Technologies. At HP and Agilent, Barry served in a variety of roles in R&D and Product Development, becoming a Sr. R&D Manager for Separations Consumables and Services, then the Bioreagents business. In 2006, he became the VP of R&D at the Edgewood, MD site of Smiths Detection, developing and commercializing field-ready biothreat detectors. In 2009, Barry returned to developing separations technologies at AMT, using the new superficially porous particles (SPP) pioneered by Dr. Jack Kirkland. Barry has published more than 75 peer-reviewed papers, reviews, patents and applications, and was an Adjunct Professor of Chemistry at UGA (Athens, GA), and continues collaborations on protein modification analysis, CNS disease investigations, and contributions to teaching Proteomics and Separation Science at UGA.



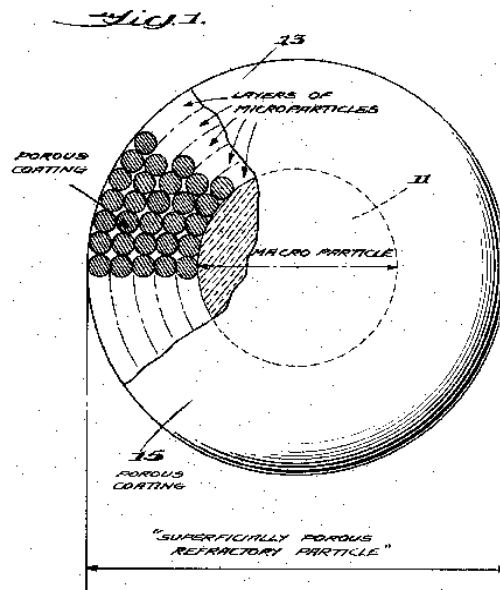
Protein Variant Separation Improvements Using Superficially Porous Particles and Operating Condition Manipulations



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Vice President, R&D
Advanced Materials Technology, Inc.
Wilmington, Delaware, USA
bboyes@advanced-materials-tech.com

The Early Days -Conceptual

April 14, 1970 J. J. KIRKLAND 3,505,785
 SUPERFICIALLY POROUS SUPPORTS FOR CHROMATOGRAPHY
 Filed June 20, 1967 3 Sheets-Sheet 1



INVENTOR
 J. J. KIRKLAND,
 BY *Alvin S. Ball*
 AGENT

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

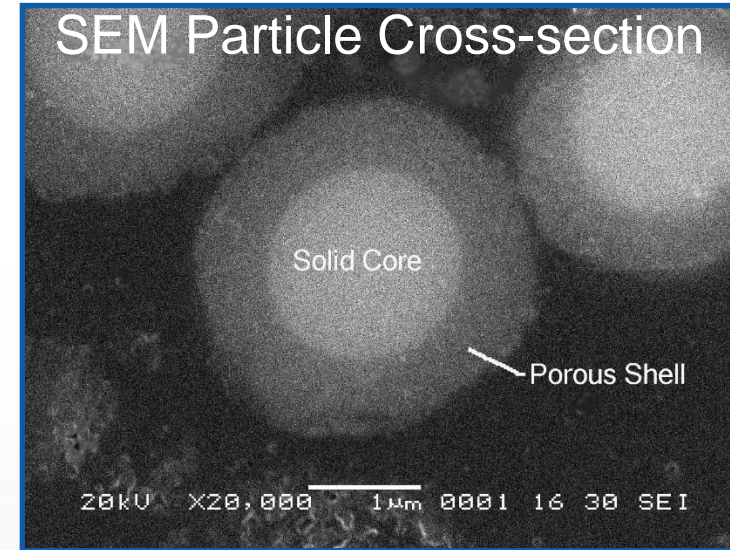
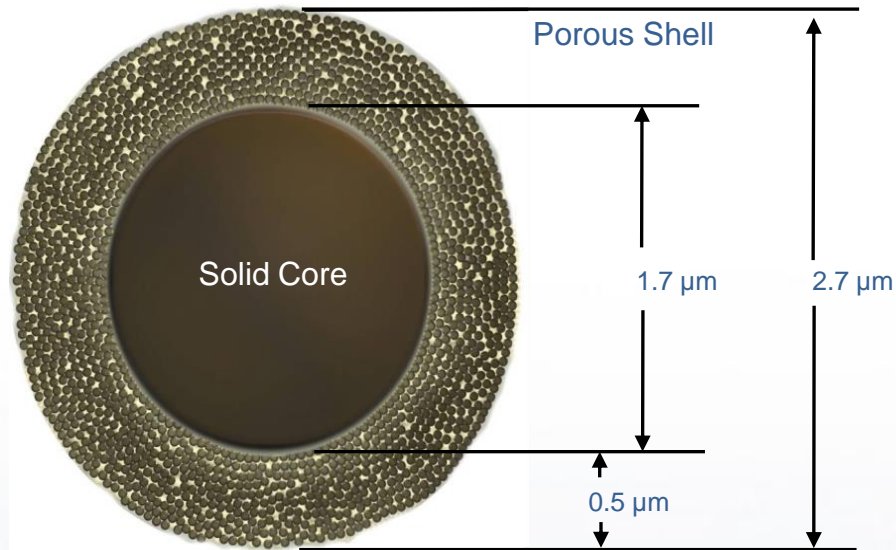
U.S. Cl. 55—67

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.

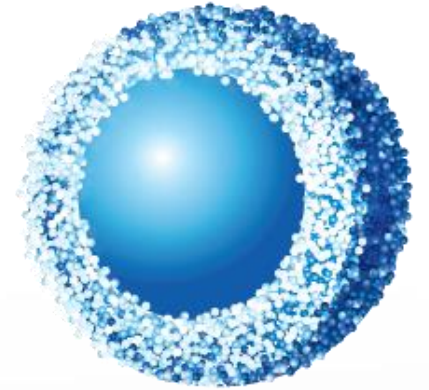
Superficially Porous Particles (SPP-90 Å): 2006



- 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)

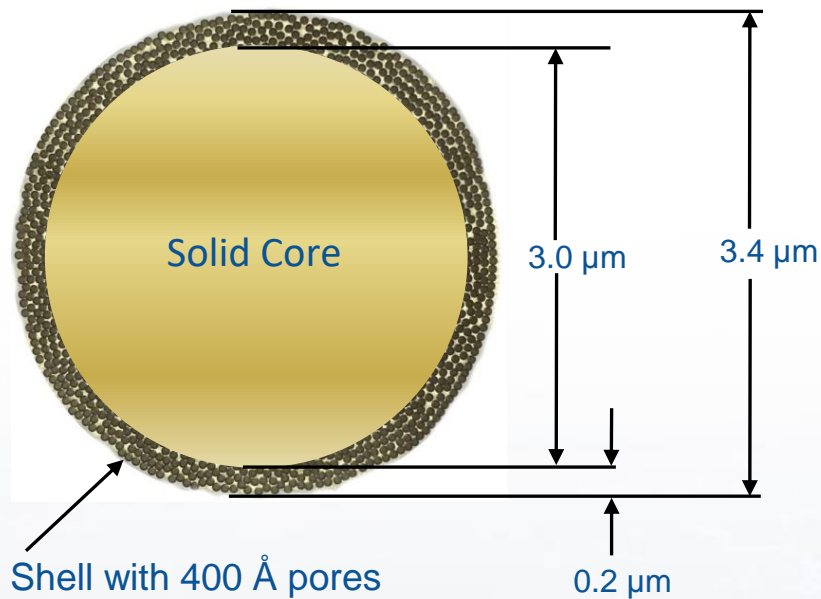
What is needed for high performance separations of larger (Bio) molecules?

- **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**

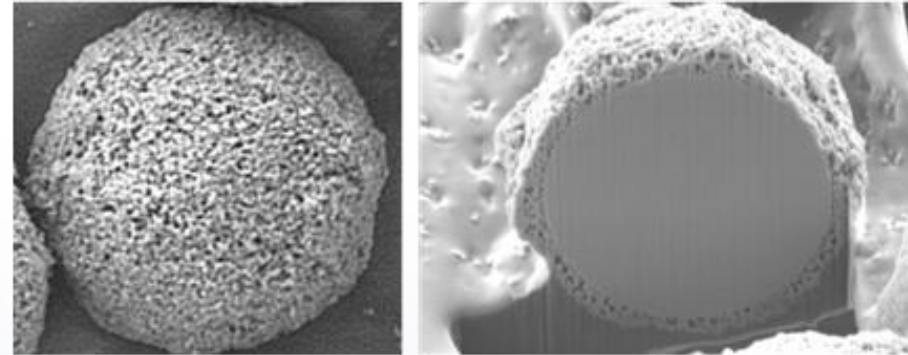


Very Large Pore SPP

Surface Chemistry Options

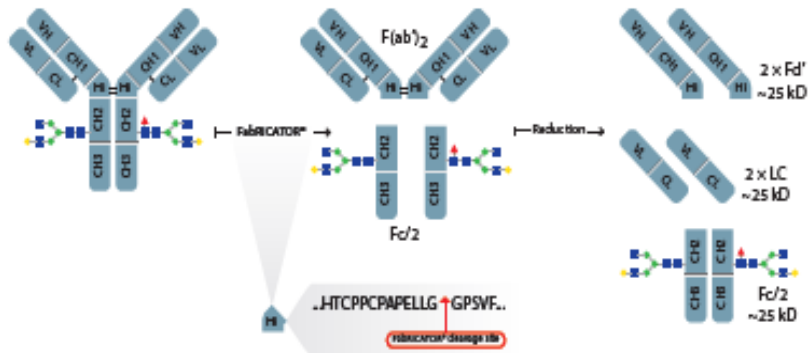
Superficially Porous (Fused-Core[®]) Wide Pore Particles: 400 Å

Wide-pore Halo 400 Protein Particles

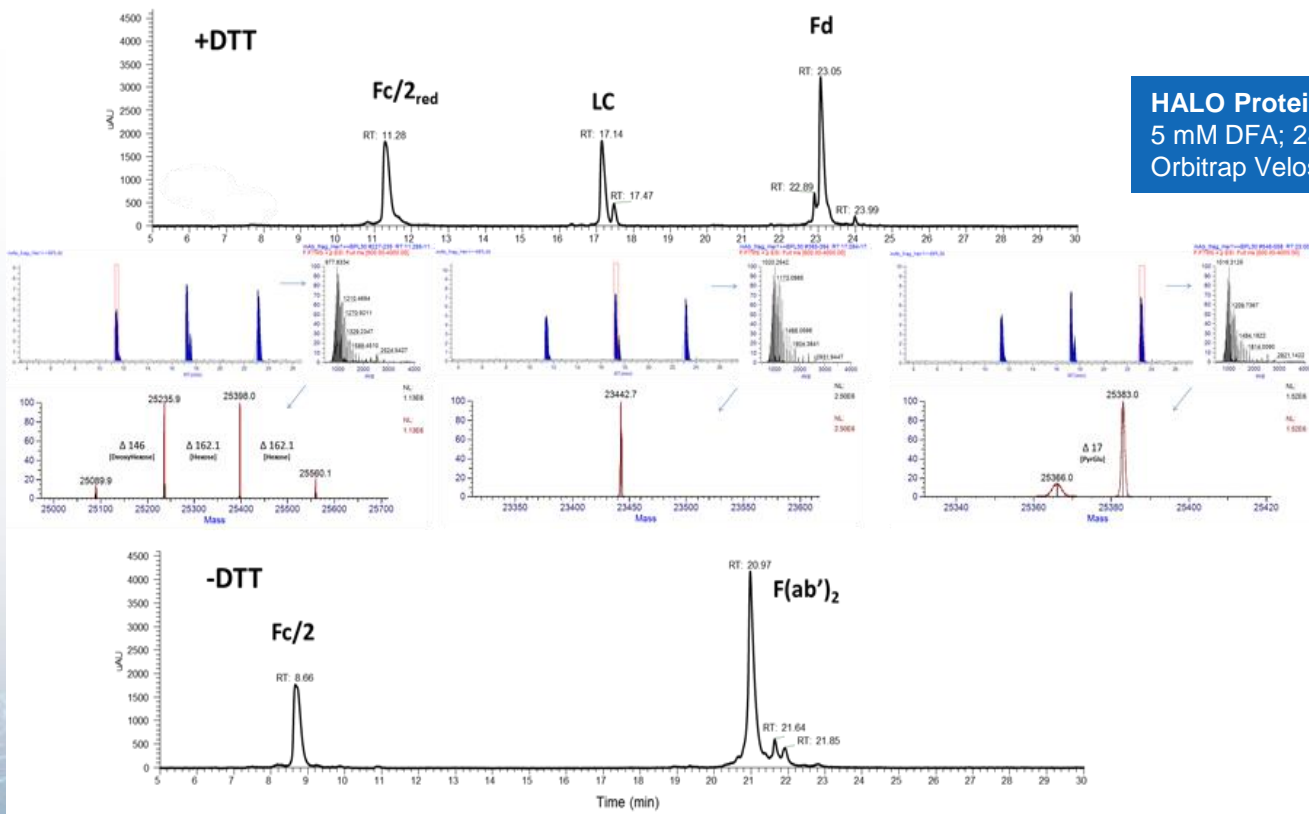


- Example above is 3.4 μm particle/400 Å pore size
- Many variations in shell thickness, pore size and particle size have been studied
- Theory to support “best properties” is complex, with limited tests using proteins, particularly with larger proteins
- Look for compromise in diffusion path for high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

Fragments for mAb Structure: IdeS Digest



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



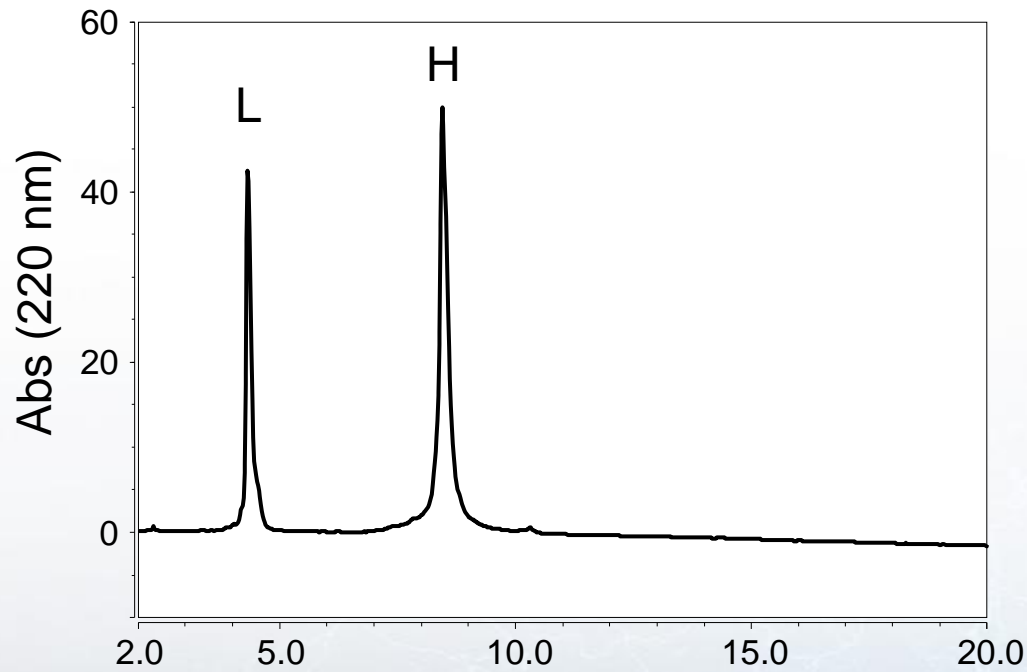
HALO Protein C4 400 Å, 2.1 mm ID x 150 mm;
5 mM DFA; 28-38% AcN in 20 min; 0.35 mL/min, 80 °C;
Orbitrap Velos Pro (30,000 Res) 500-4000 m/z, +3.8 kV, 275 °C capillary

Why protein chemists like fast HRMS!

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

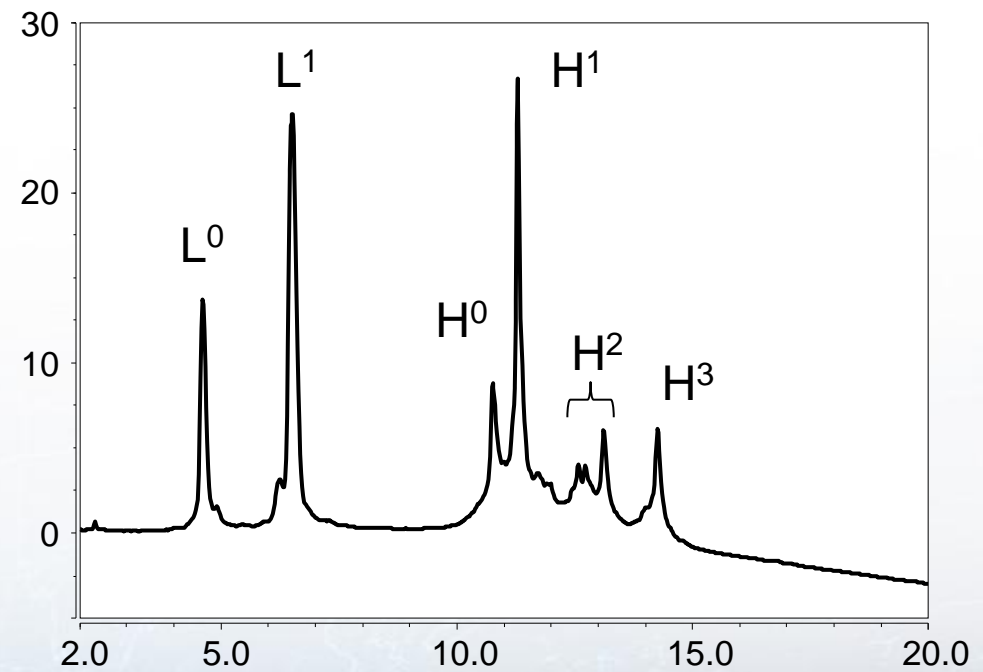
IgG H and L Chain Separations

Column: HALO 400 Å 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 (G₀, G₀F, G₁F, G₂F)



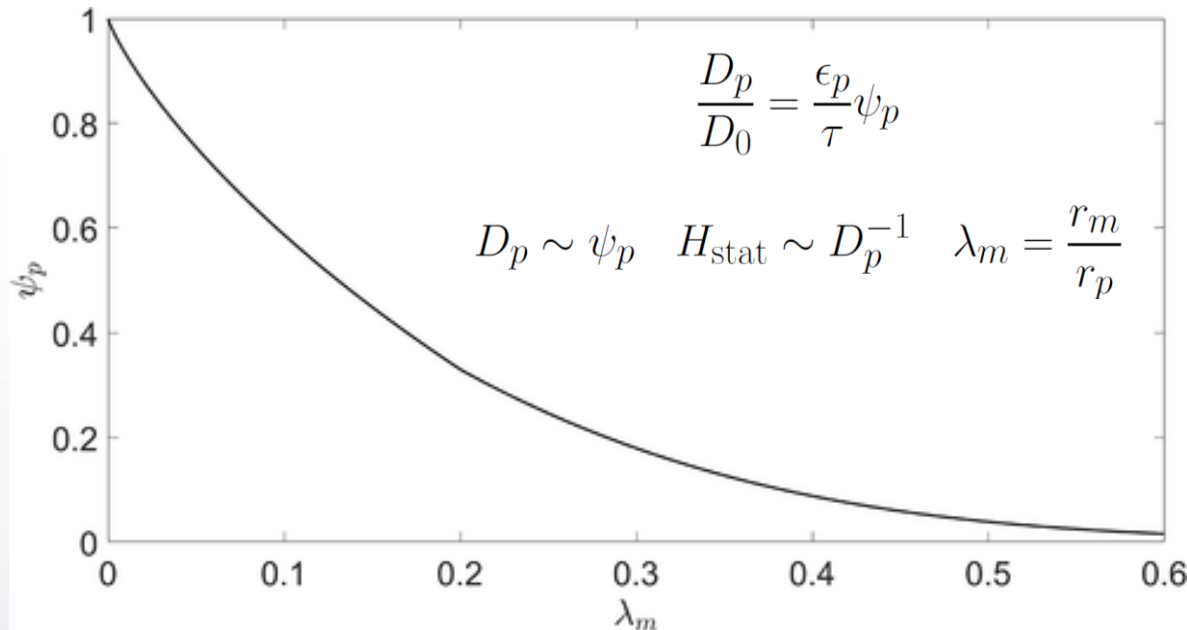
S/M MSQ8 ADC Mimic

L⁰ – 23,284 Da; L¹ – 23,895
 Hⁿ – 49,585 Da + Glycans (G₀F, G₁F) + n(611 Da)

Restricted diffusion: Why you need larger pores than the size of the solute

It has long been recognized that diffusion of molecules in pores is slower than diffusion in bulk liquid. This leads to more zone broadening through the resistance to mass transport within the stationary phase term of the plate height equation.

(Efficiency)



Theories have been developed which account for this effect in idealized pore shapes (cylinders, slabs) as shown to the left.¹

Diffusion in more realistic particle geometries shows a similar effect.²

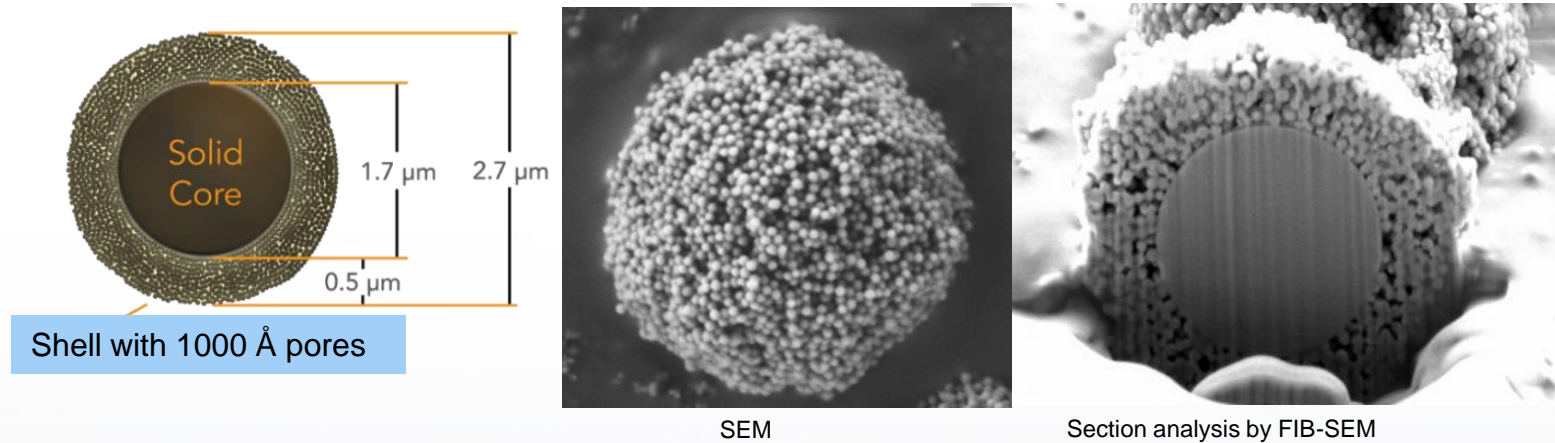
Ongoing efforts examine the fluid mechanics and transport properties of SPPs in packed beds.³

¹P. Dechadilok, W.M. Deen, Hindrance factors for diffusion and convection in pores, *Ind. Eng. Chem. Res.* 45 (2006) 6953–6959.

²R. S. Maier, M. R. Schure, Transport properties and size exclusion effects in wide-pore superficially porous particles, *Chem. Eng. Sci.* 185 (2018) 243-255.

³M. R. Schure, R. S. Maier, T. J. Shields, C. M. Wunder, B. M. Wagner, Intraparticle and interstitial flow in wide-pore superficially porous and fully porous particles, *Chem. Eng. Sci.* 174 445–458 (2017).

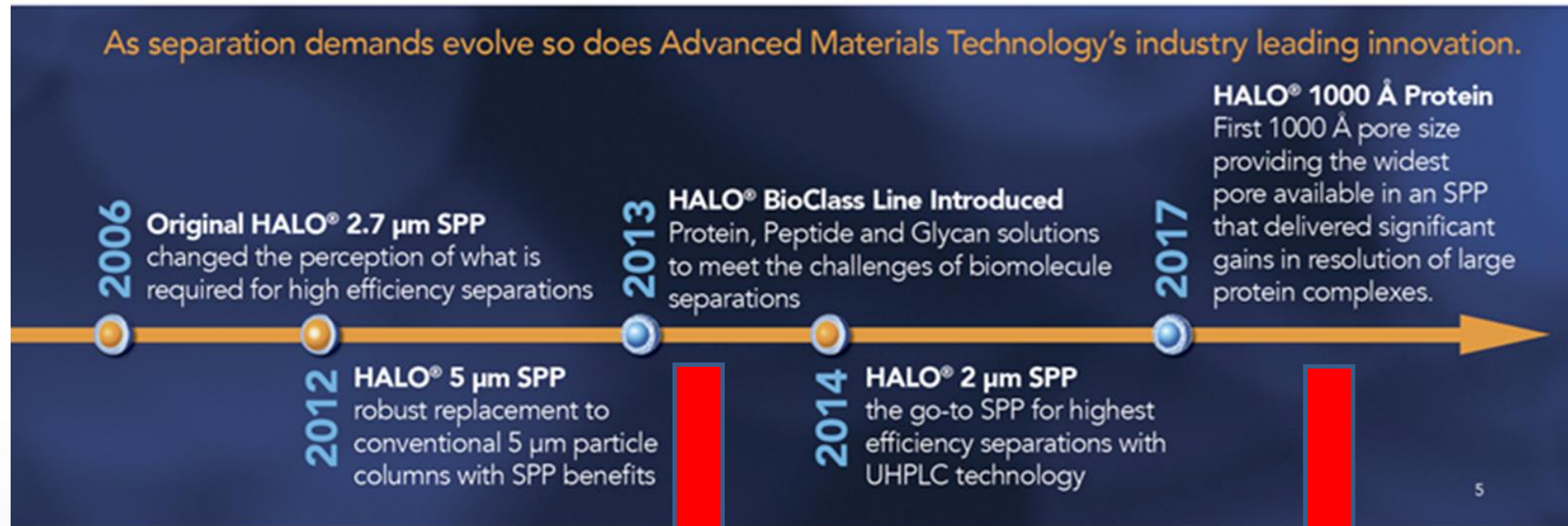
Superficially Porous (Fused-Core[®]) Wide Pore Particles: 1000 Å



- 2.7 μm particle with 0.5 μm thick shell and 1000 Å pores
- Surface area ~ 22 m²/g
- Designed for larger proteins
- Densely bonded C4 phase with end-capping
- High temperature and low pH stable

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.

Evolution of Protein Columns



400 Å

- Began demonstration of the need for increased pore size; utility of SPP
- Good for small and mid size proteins
- Can also be used for larger proteins

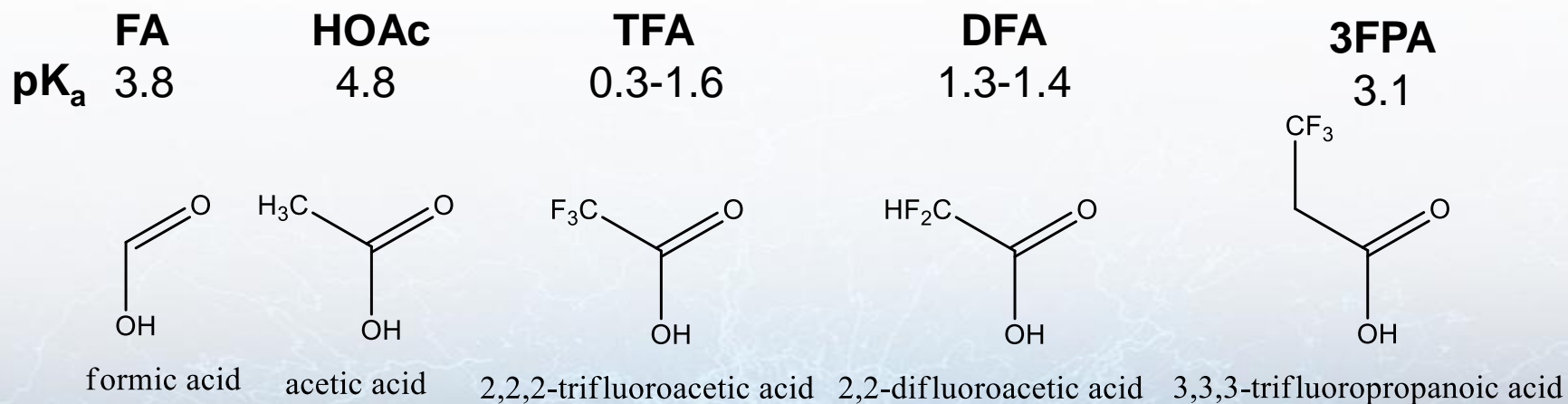
1000 Å

- Best in Class
- Thicker shell, larger surface area for increased resolution of very large proteins (i.e. mAbs)
- **Highest resolution and new method development**

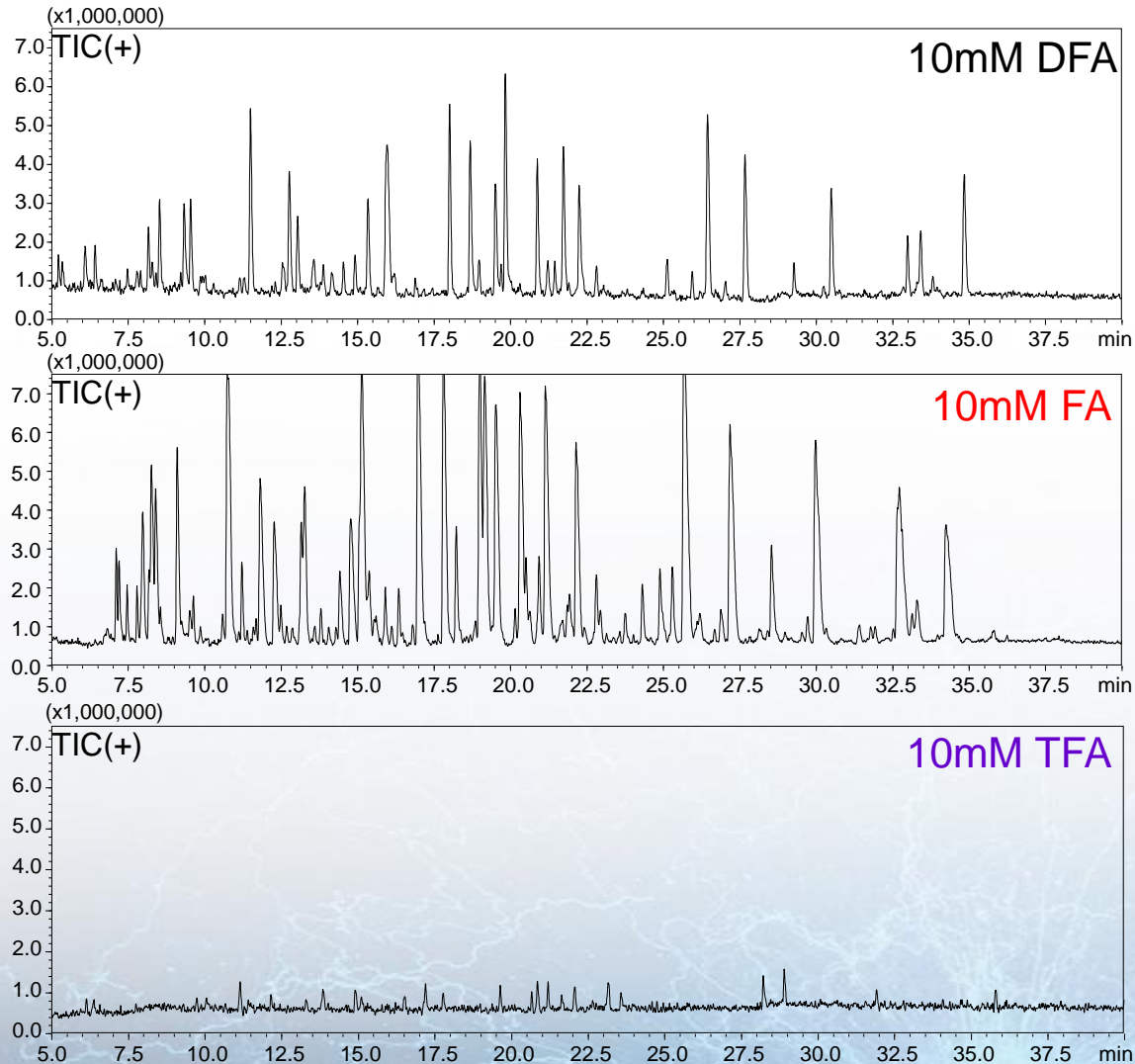
Improved Protein LC/MS Mobile Phases: Properties That May Help

Initial selection and testing indicated some candidates with promise:

Share required features of volatility, lower pKa, but variable protein solubility



10 mM acid in mobile phases; 2.1 x 150 mm HALO 160 Å ES-C18, 0.3mL/min, 2-47%AcN in 40min, 60°C, 300-1800m/z, 4kV, 0.33s



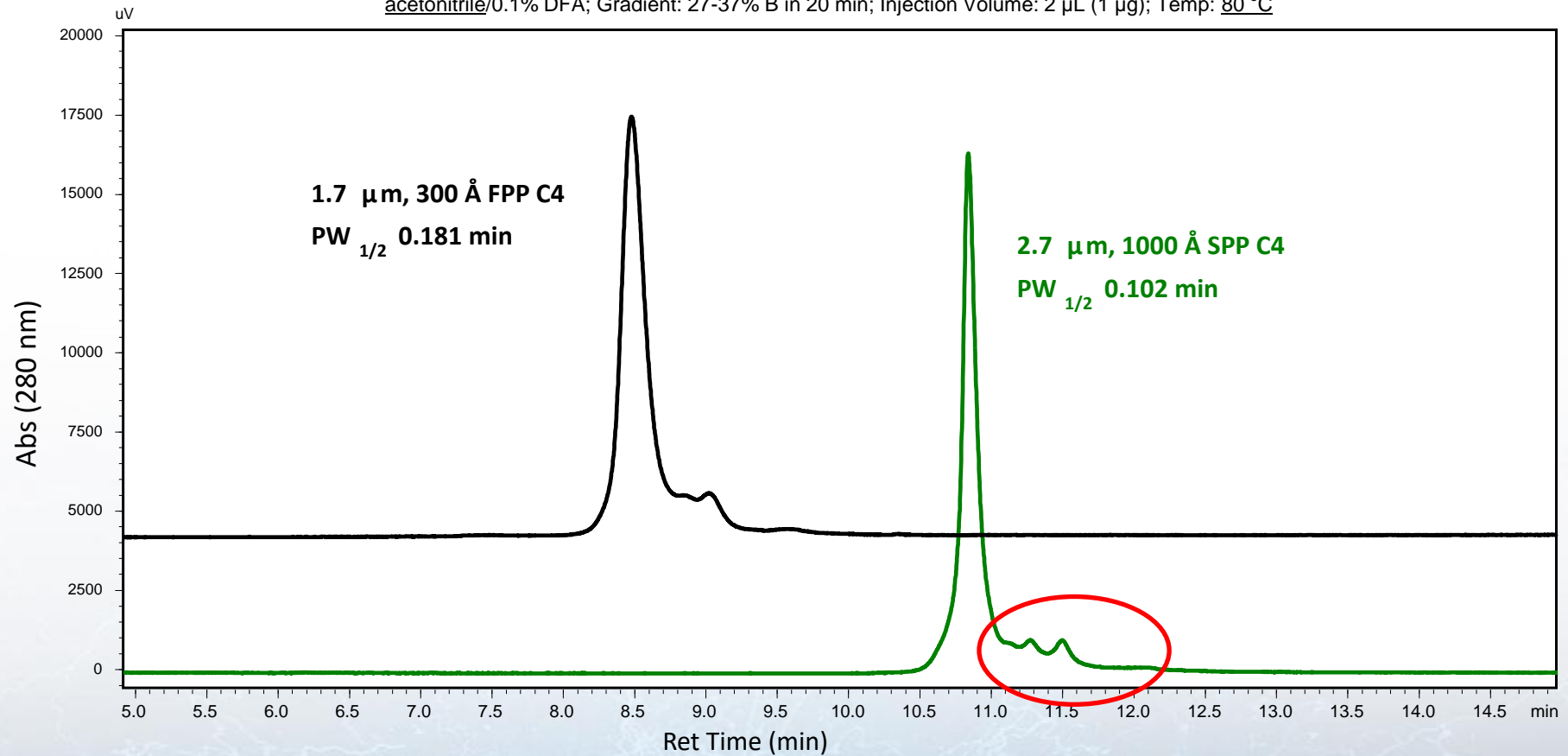
50pmol Reduced and Alkylated Enolase Digest Peptides

- DFA is slightly less retentive than TFA, some small selectivity differences (IP effect)
- DFA beneficial effects for MS are greatest for peptides, smaller for proteins
- DFA is much, much easier to get out of an LC/MS than is TFA (wash out)
- I never put TFA in my Orbitraps, but DFA is fine, and has caused no problems (>4 years)

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 μ L (1 μ g); Temp: 80 $^{\circ}$ C

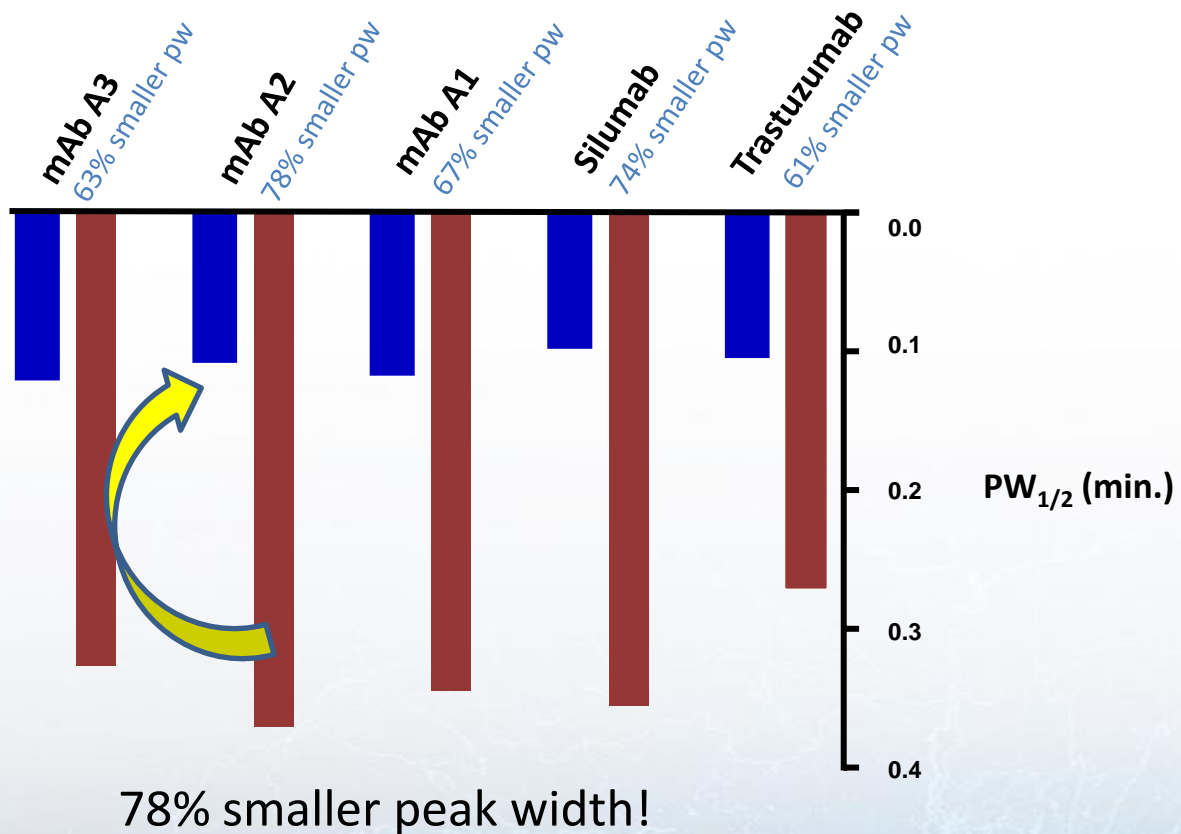


- Large improvement in peak width and increased retention with pore size for SPP, additional improvement in peak width with 1000 \AA pores

mAb IgGs Separation on Wide Pore SPP vs FPP

Smaller Peak Widths for Various mAbs

■ 2.7 μm , HALO 1000 \AA SPP, C4
■ 1.7 μm , 300 \AA FPP, C4

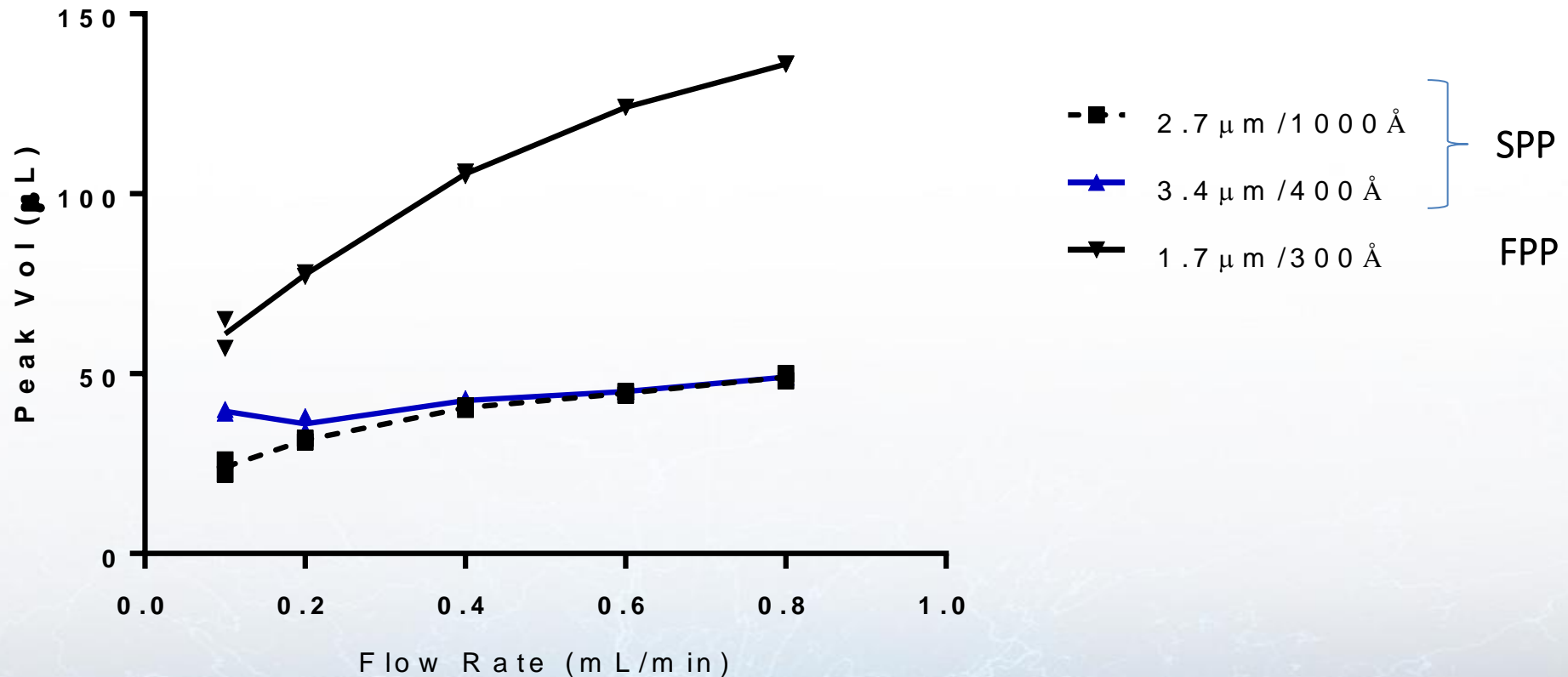


- The SPP advantage is not unique to one mAb.
- Advantage is greater at higher flow rates.

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; Instrument:
 Shimadzu Nexera; Injection Volume: 2 μL (1 μg); Detection: 280 nm; Temp: 80 $^{\circ}\text{C}$

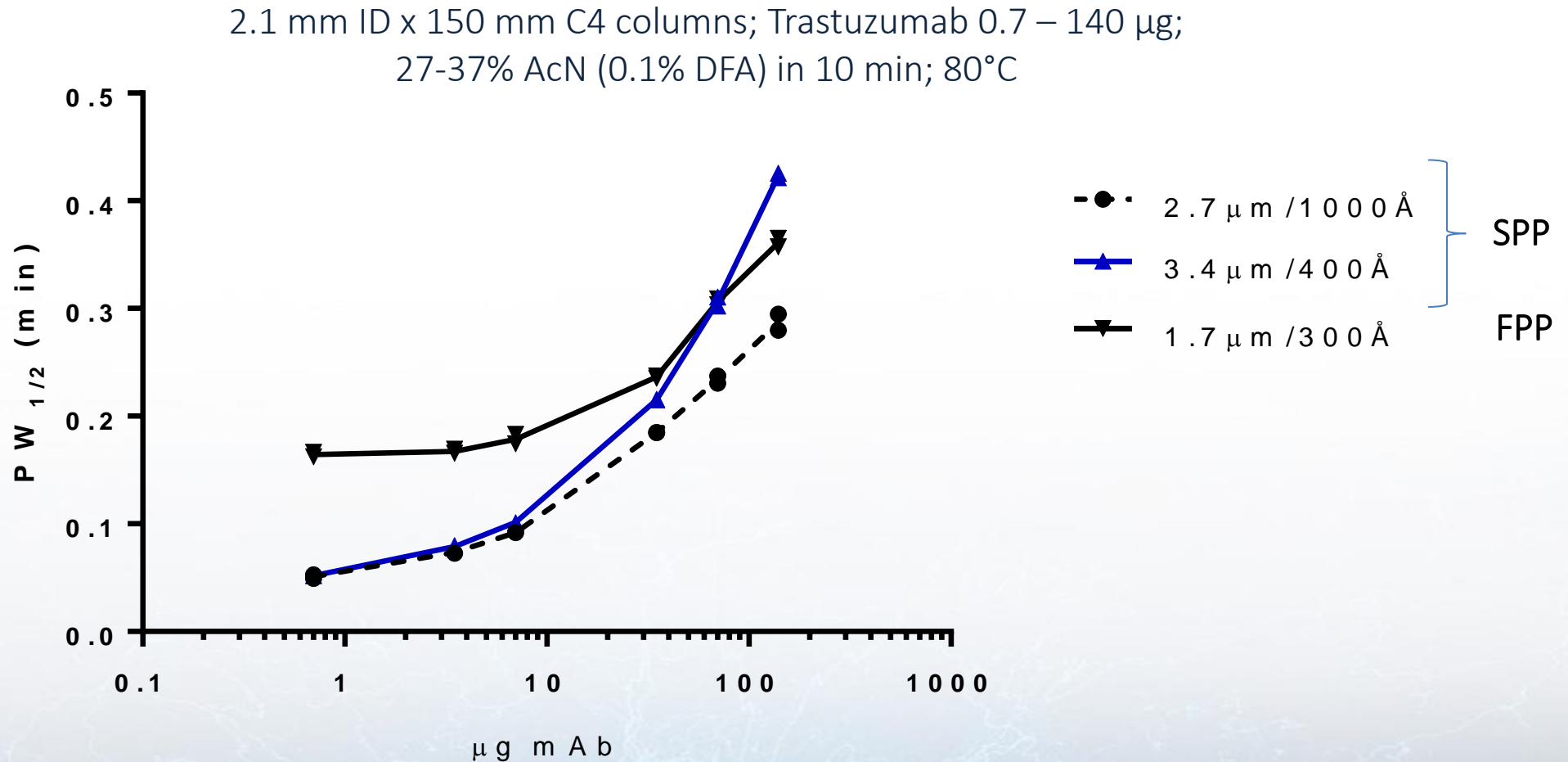
Flow Rate Effects on Peak Volume for mAb IgG

Fixed Volume Gradient Conditions (4.8 mL); Peak Volume = $PW_{1/2} \times \text{Flow Rate}$
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; TFA and DFA show same results

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



- 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 1000 \AA pore size SPP performed best for this mAb

Schemes for Improving Protein Separations by Reversed Phase

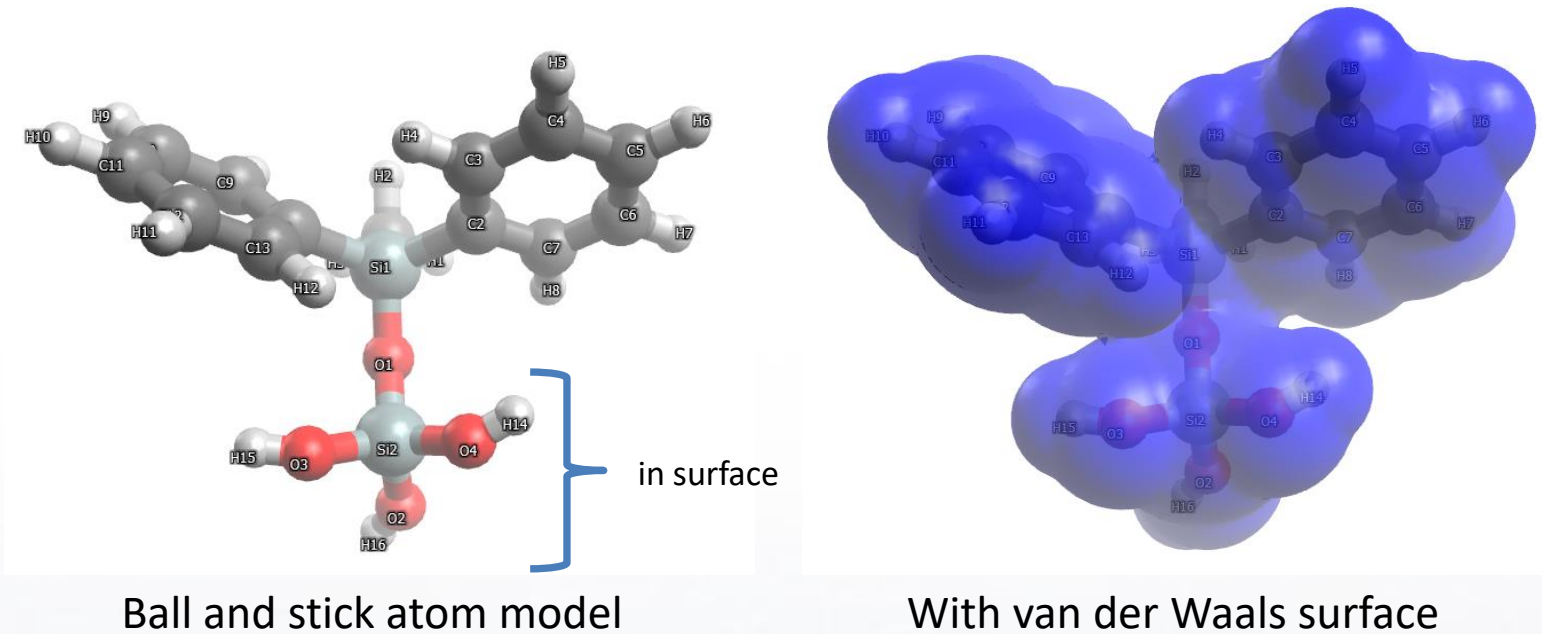
Limited choices of favorable conditions: low pH, elevated T_{col} , UV transparent, productive for ESI-MS detection, high recovery of proteins of varying natures

Many biomolecules of interest are inherently heterogeneous, and RP will not resolve all variants (eg., glycosylation)

Parameters readily altered for RP selectivity/recovery optimization

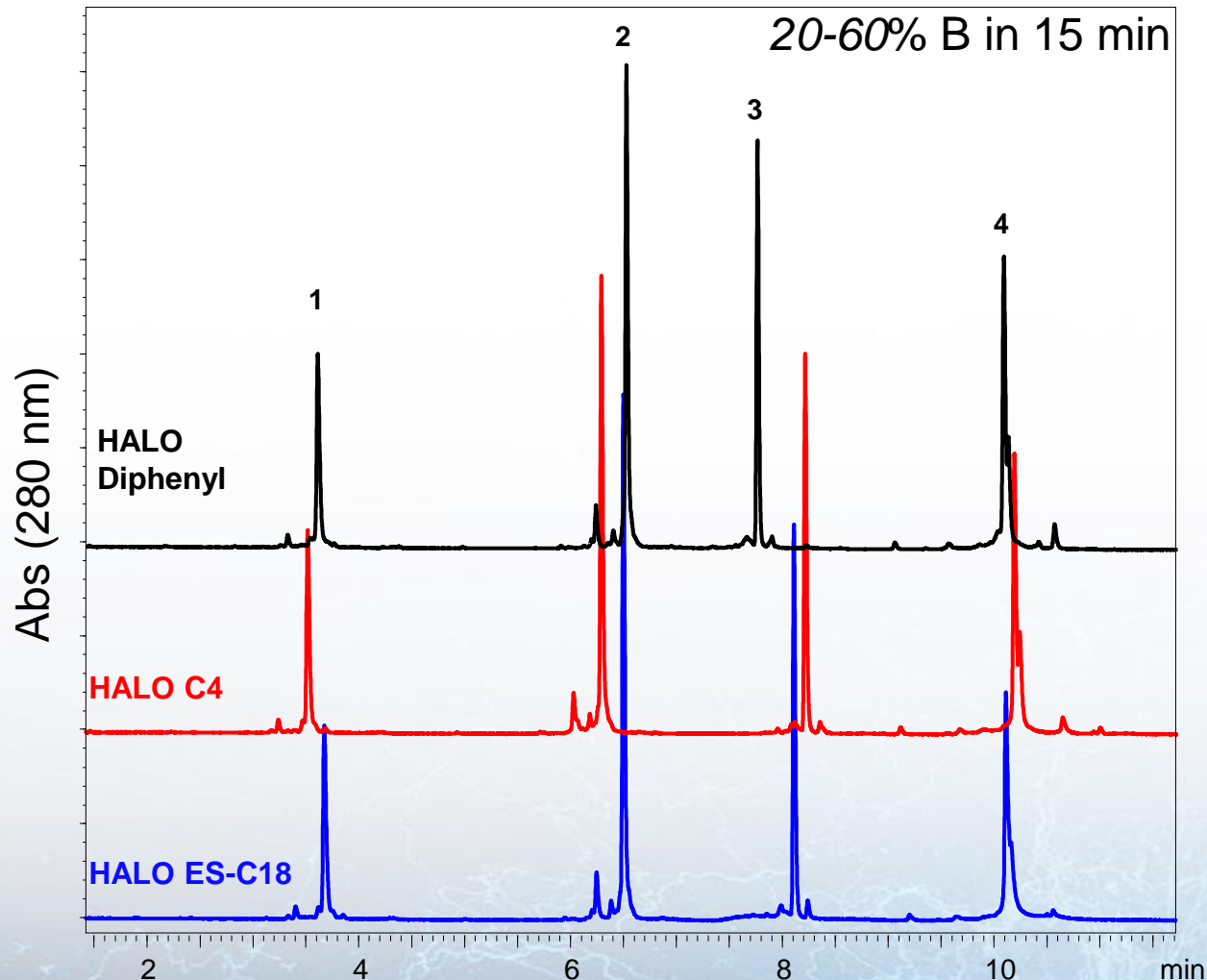
- Bonded Phase Manipulations
 - C4 is not the only option
- Mobile Phase Manipulations
 - Acids (FA, TFA, DFA, AF) and Organic Modifiers (AcN, short chain alcohols)
- Operational Temperatures
 - 40-90°C is a reasonable window

Diphenyl methyl sil(ane)oxane



- Phenyl groups are not coplanar
- Phenyl groups form a nice van der Waals surface (a pocket) for large molecule interaction.
- Rotation around C2-Si1 and C8-Si1 bonds accommodate large molecule fragments.
 - These rotations, when not sterically crowded, don't cost much energy.
 - As with C₁₈, these groups will accommodate the solute through bending and rotation.
- 1000 Å HALO SPP surface reaction is 2.7 μmol/m² (5.4 phenyl)

Effect of Bonded Phase on Protein Separations using HALO 1000 Å

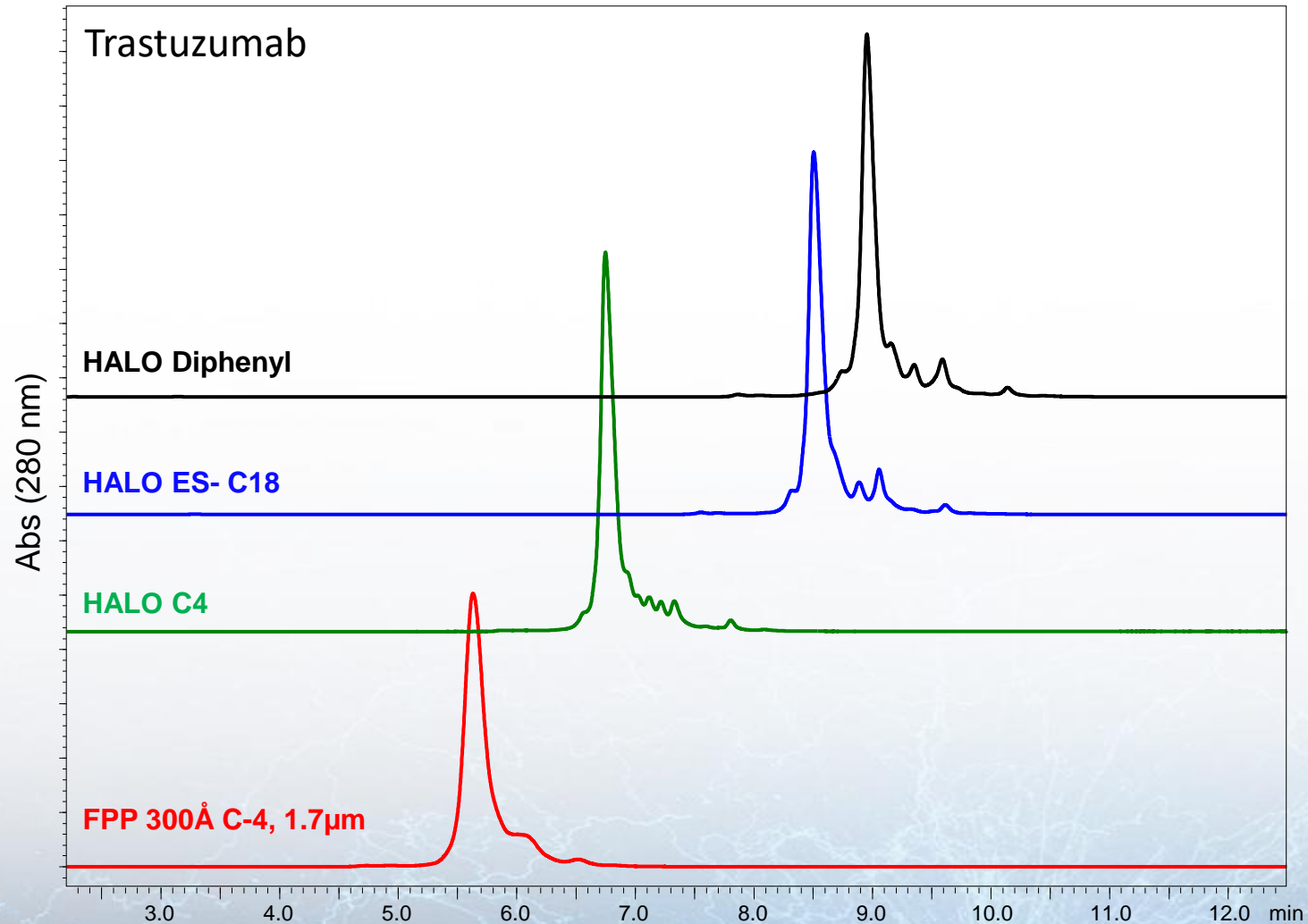


Columns: 2.1 x 150 mm; Flow rate: 0.4 mL/min; Mobile Phase A: H₂O/0.1% TFA; Mobile Phase B: ACN/0.1% TFA; Gradient: 20-60 %B in 15 min; Instrument: Shimadzu Nexera; Injection Volume: 2 µL; Detection: 280 nm; Temp: 80 °C

1. Ribonuclease A
2. Lysozyme
3. α-Lactalbumin
4. Enolase

- Retention of proteins across bonded phase columns:
 - not correlated to small molecule retention
 - not a global pattern of retention
- Selectivity differences allow separations choices.
- Similar peak widths for these proteins with each bonded phase

Effect of Bonded Phase on mAb Separations using HALO 1000 Å



Columns: 2.1 x 150 mm; Flow rate: 0.4 mL/min;
Mobile Phase A: H₂O/0.1% TFA; Mobile Phase B: ACN/0.1% TFA; Gradient: 32-40 %B in 16 min; Instrument: Shimadzu Nexera; Injection Volume: 2 µL; Detection: 280 nm; Temp: 80°C

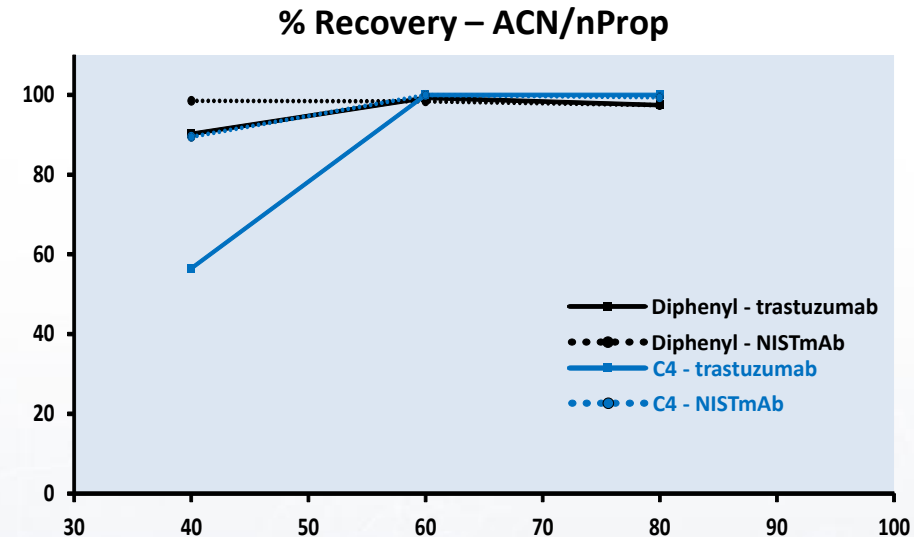
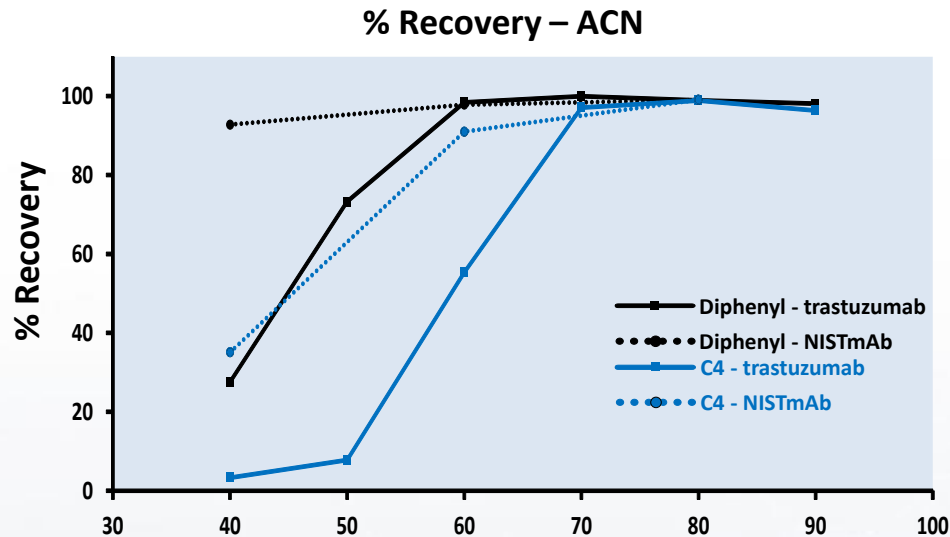
- Retention of mAb is often:
DP>C18>C4
- Selectivity differences observed with variants
- Similar peak widths for this mAb with each bonded phase

Temperature-dependent Recovery of mAbs: Bonded Phase/Mobile Phase Effects

Columns: 2.1 x 150 mm HALO 1000; Flow rate: 0.4 mL/min, Recovery as %Maximum Area

A – H₂O/0.1% TFA, B – ACN/0.1% TFA: 30-45%B in 15min
4 μL at 2 mg/mL (8 μg)

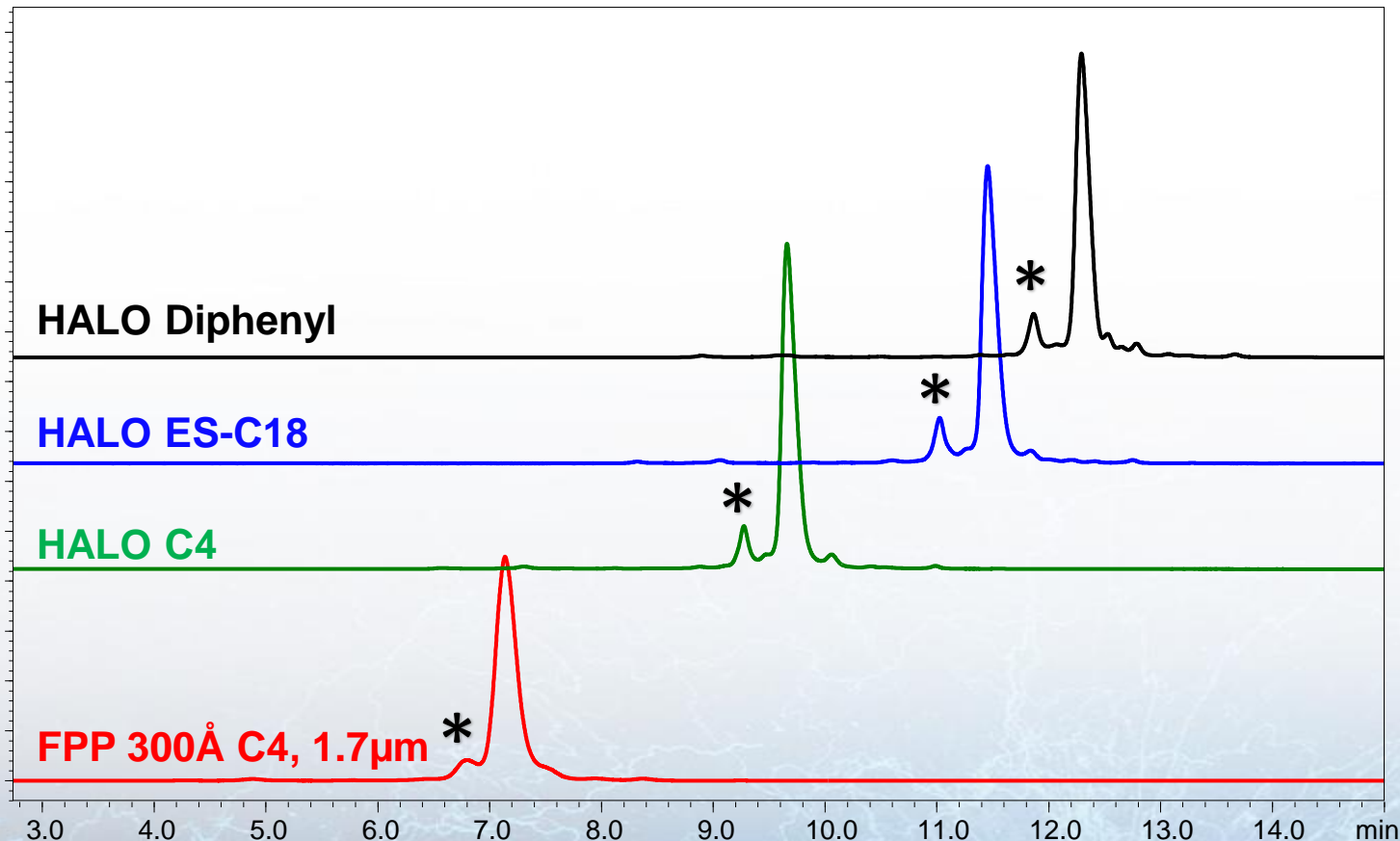
A – H₂O/0.1% TFA, B – (50/50 ACN/nPropanol)/0.1% TFA: 28-43%B in 15min
4 μL at 2 mg/mL (8 μg)



- ES-C18 yields similar results to C4 bonded phase for recoveries; at maximum recovery columns show the same area counts
- Diphenyl exhibits a lower temperature for full recovery of many mAbs
- Highest recovery in AcN for many mAbs T>70° with alkyl bonded phases
- Many mAbs show ↓T for high recovery using the AcN/n-Propanol mixture (c.10-15°C)
- Mixtures of propanol (i- and n-) and AcN between 80/20 and 20/80 have similar effects on recovery
- Similar patterns of recovery are observed for 2 addnl IgG1 and 2 IgG2 mAb examined to date

Effect of Temperature on mAb Separation: **DANGER**

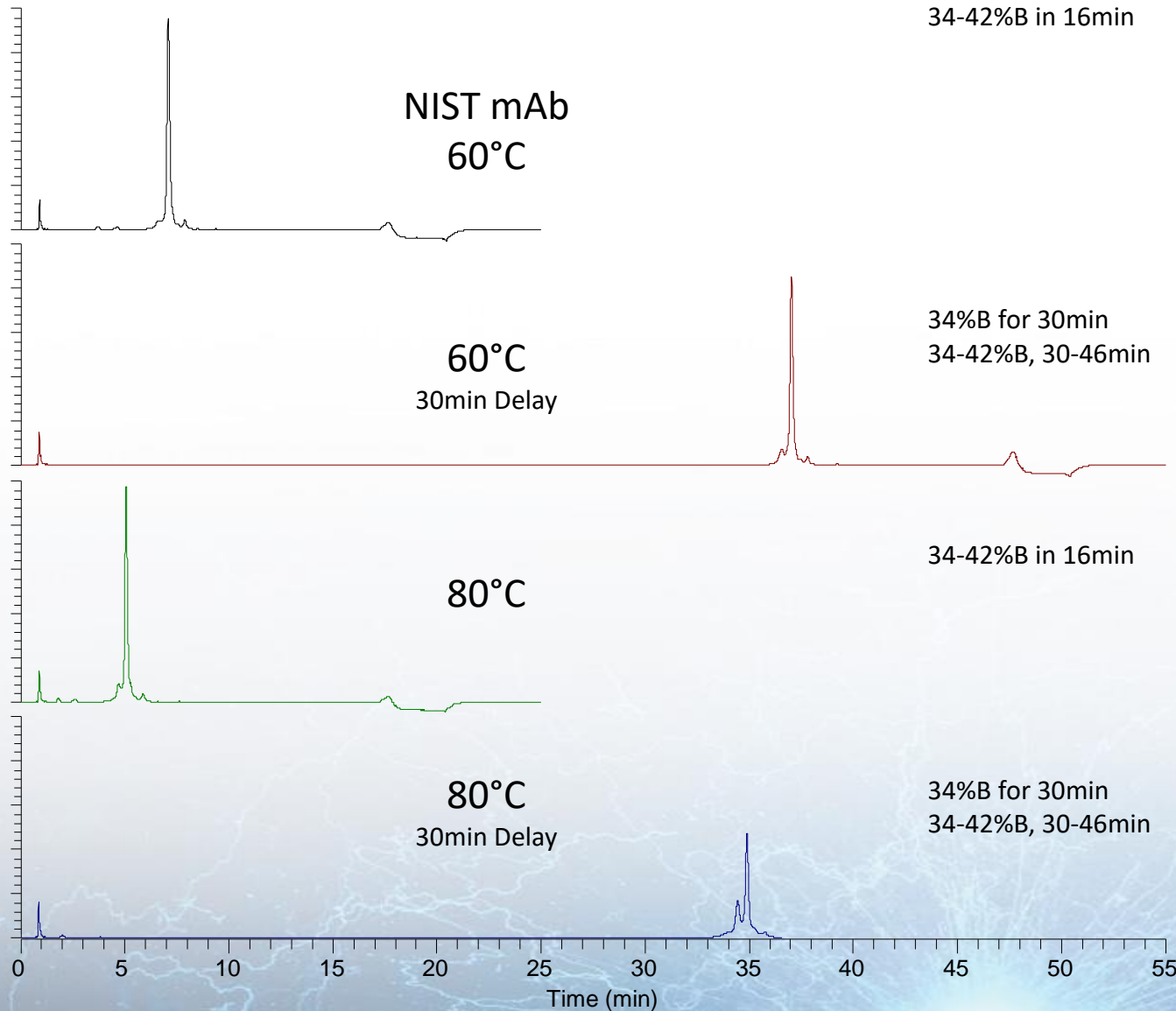
NISTmAb



Columns: 2.1 x 150 mm; Flow rate: 0.4 mL/min; Mobile Phase A: H₂O/0.1% TFA; Mobile Phase B: ACN/0.1% TFA; Gradient: 34-42 %B in 16 min; Instrument: Shimadzu Nexera; Injection Volume: 2 µL; Detection: 280 nm; Temp: 80°C

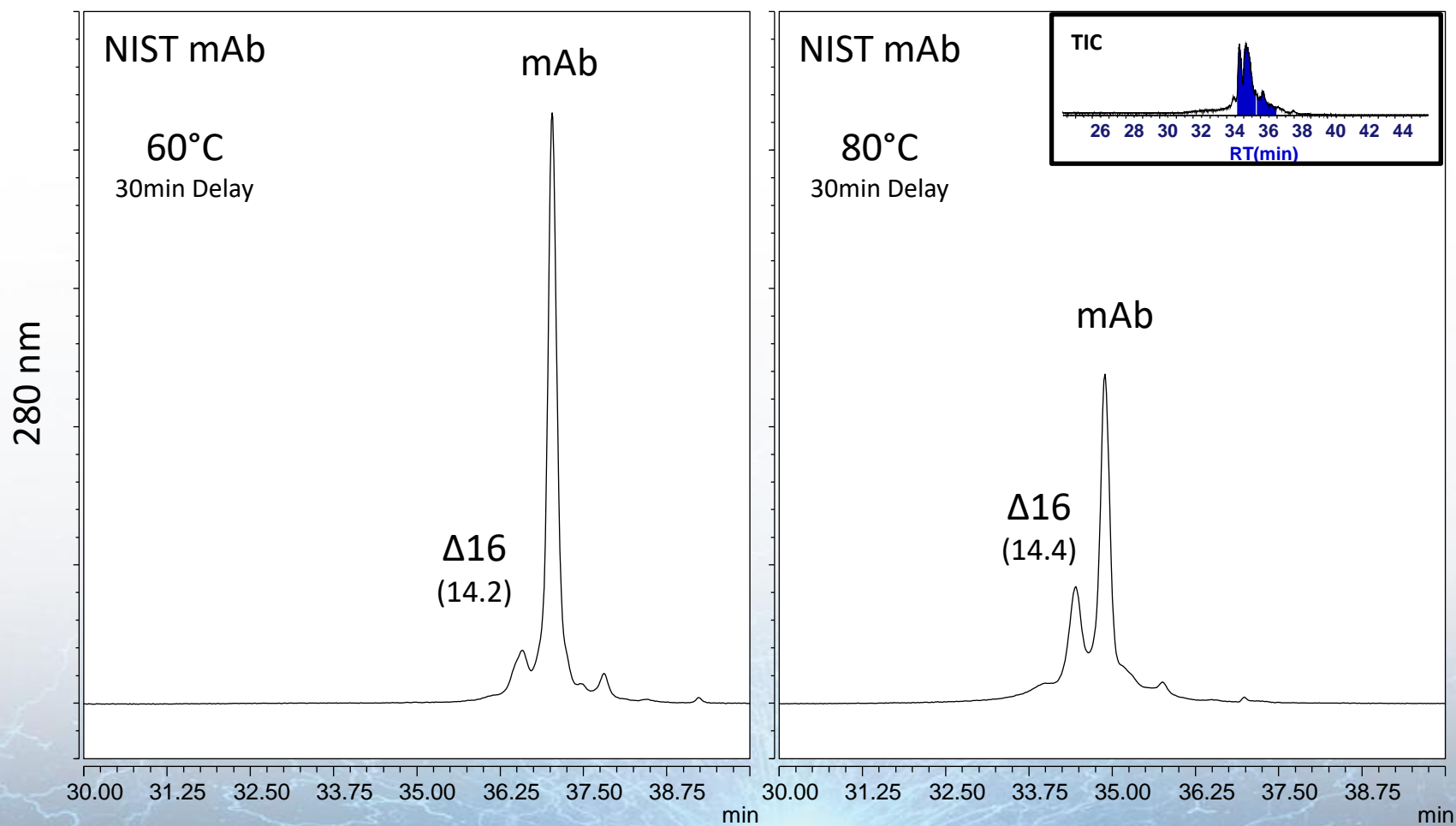
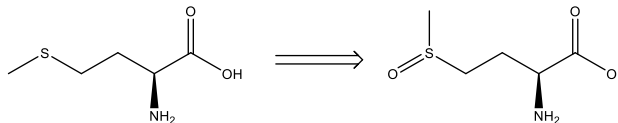
- * denotes a high temperature artifact
- Reinjection of main peak generates this; the artifact remains a single peak (irreversible)
- Is absent at or below 60°C
- Forms at higher temperature, with all columns and mobile phases

Effect of Temperature on mAb Separation: **DANGER**



Columns: 2.1 x 150 mm; Flow rate: 0.4 mL/min;
Mobile Phase A: H₂O/0.1% DFA; Mobile Phase B:
ACN/0.1% DFA; Gradient: 34-42 %B
Instrument: Shimadzu Nexera; Injection Volume: 2 µL;
Detection: 280 nm;

- Time on column effects early peak production
- DFA or TFA shows same results
- Intermediate times show intermediate conversion

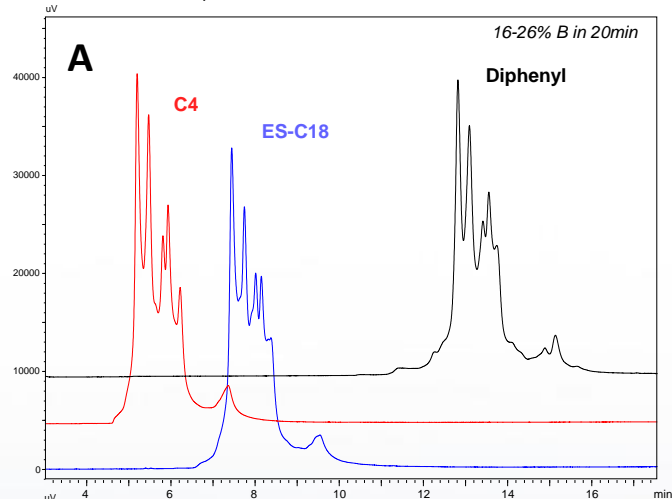
Effect of Temperature on mAb Separation: **DANGER**

Method Development Approaches: BP, MP, T

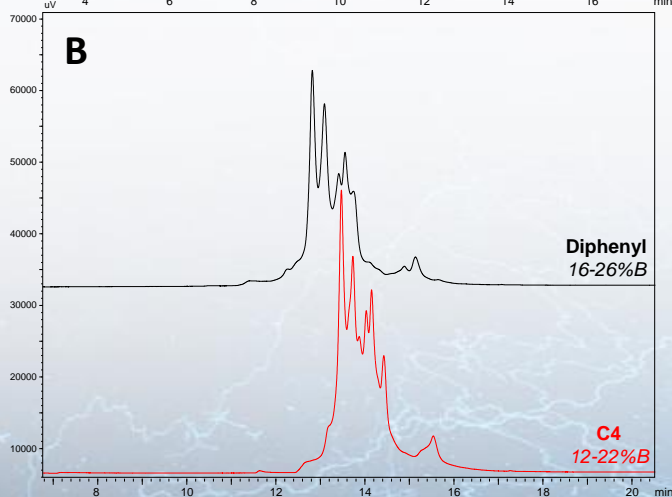
Column Based Approach

Columns: 2.1 x 150 mm; Flow rate: 0.2 mL/min; Mobile Phase A: 88/10/2 H₂O/ACN/nProp + 0.1% DFA; Mobile Phase B: 70/20/10 nProp/ACN/H₂O + 0.1% DFA; Gradient: 16-26 %B in 20 min; Instrument: Shimadzu Nexera; Injection Volume: 2 μL; Detection: 280 nm; Temp: 80 °C

} AcN/nProp mix
Based on lit.



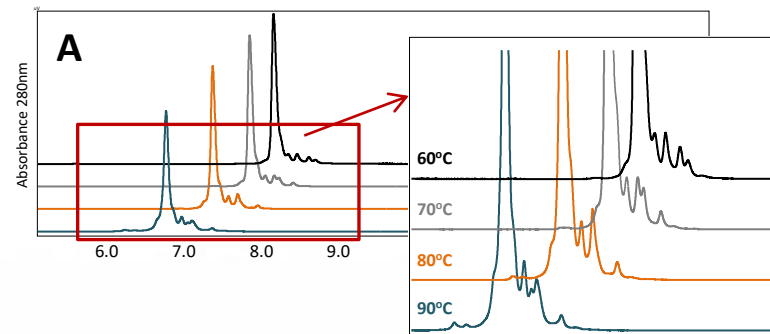
BP compared
Hi/Low T scan



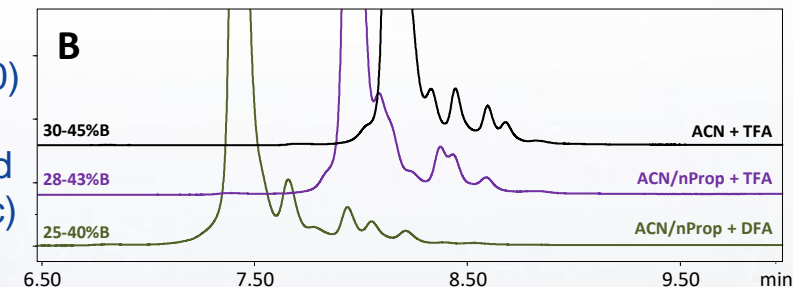
Rt adjusted
C4 selected
T and ΔG adjusted

T and MP Composition

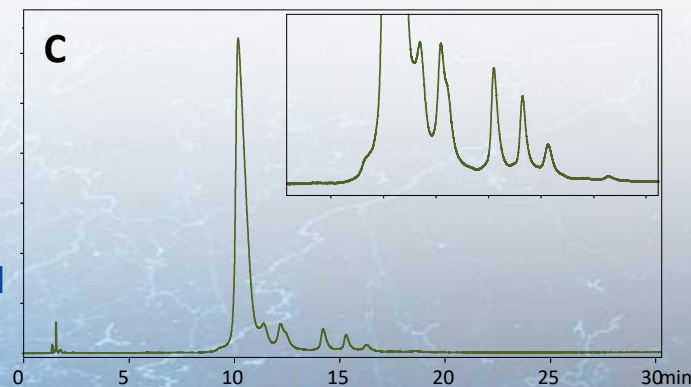
Columns: 2.1 x 150 mm HALO 1000 Diphenyl; Flow rate: 0.4 mL/min; A H₂O/0.1% TFA; B: ACN/0.1% TFA; Gradient: 30-45 %B in 15 min; Instrument: Shimadzu Nexera; Injection Volume: 2 μL; Detection: 280 nm;



BP fixed
T Compared

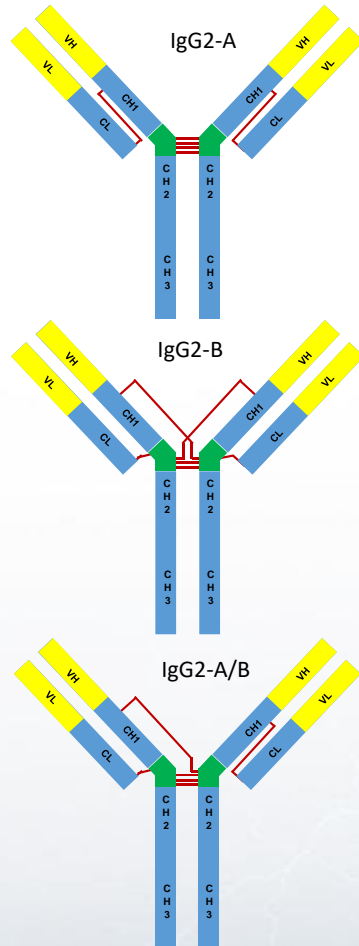


T selected (60)
MP Compared
(acid, Organic)



MP selected
AcN/n-Prop/DFA
ΔG, flow
and Time adjusted

IgG2 Disulfide Bridge Variant Separation

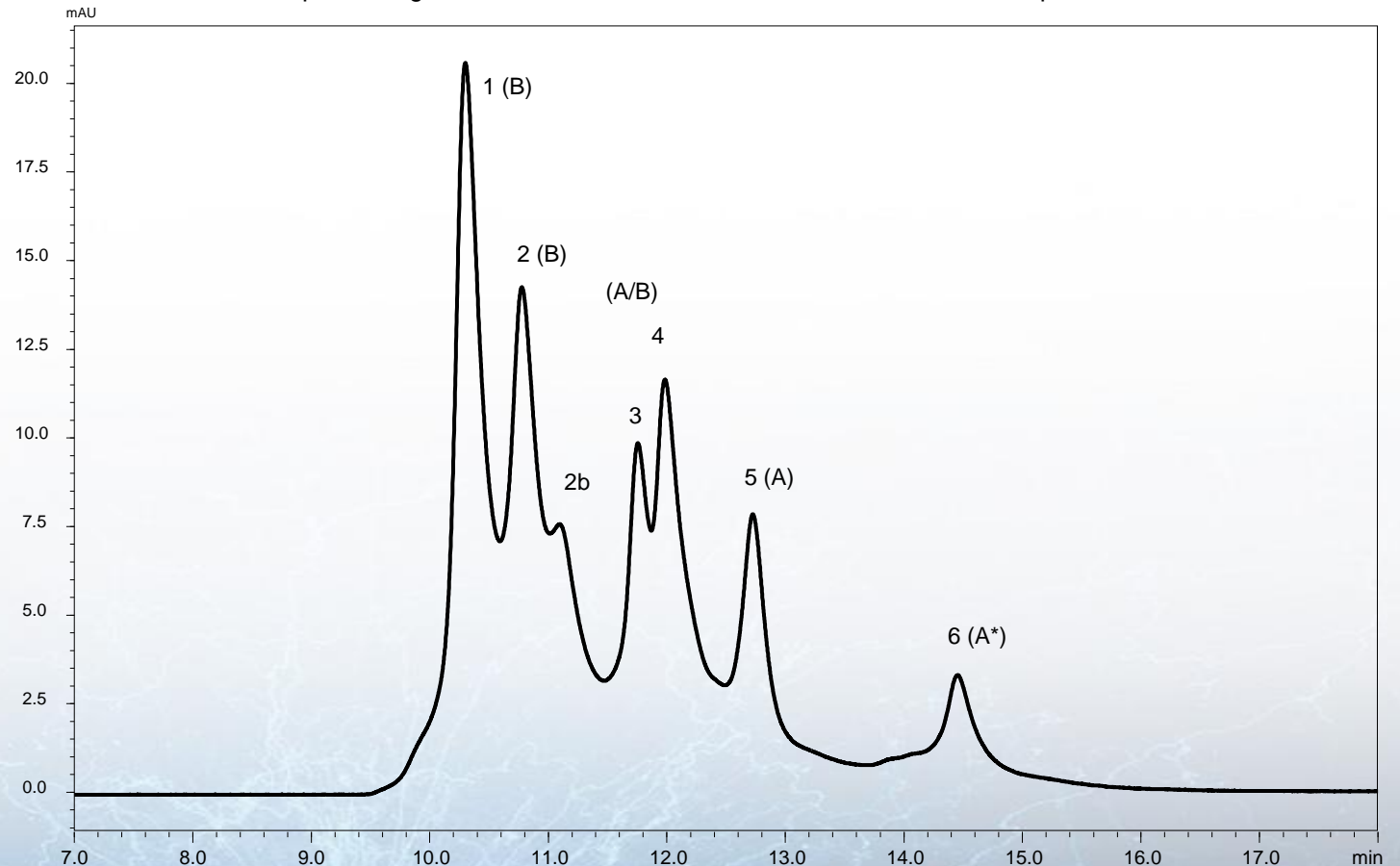


Wypych, et al., J. Biol. Chem. 283 (2008) 16194–205.

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

Wei, Zhang, Boyes, and Zhang. J. Chromatogr. A 1526 (2017) 104–111.

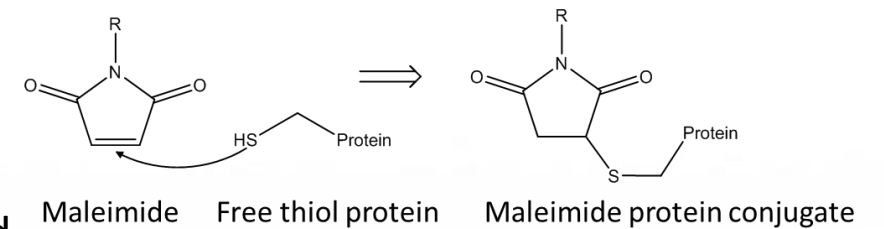
Column: HALO 1000 Å C4, 2.1 x 150 mm; Flow rate: 0.2 mL/min; Temp: 60 °C
 Mobile Phase A: 88/10/2 water/AcN/n-propanol/0.1% TFA; Mobile Phase B: 70/20/10 n-propanol/AcN/water/0.1% TFA; Gradient: 20–28% B in 32 min; Instrument: Shimadzu Nexera; Injection Volume: 2 µL of 2 mg/mL denosumab in 0.1% TFA;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



IgG Disulfide Bridge and Free Thiol Variant Analysis

Most proteins possess disulfide bridges and may also have free thiol groups (R-SH) present. In IgG1, these may be considered problematic, or at least must be monitored during bioprocessing, and in therapeutic formulations. In the case of IgG2, free thiols are formed during conversion of IgG2 disulfide variants - A, B, A/B isoforms. Conversions of R-S-S-R to (R-HS)₂ can occur, but exhibit a small mass shift, and are challenging for analysis.

Maleimides are convenient reagents for attaching a label at free thiols: R groups used can be manipulated to effect greater retention shift.



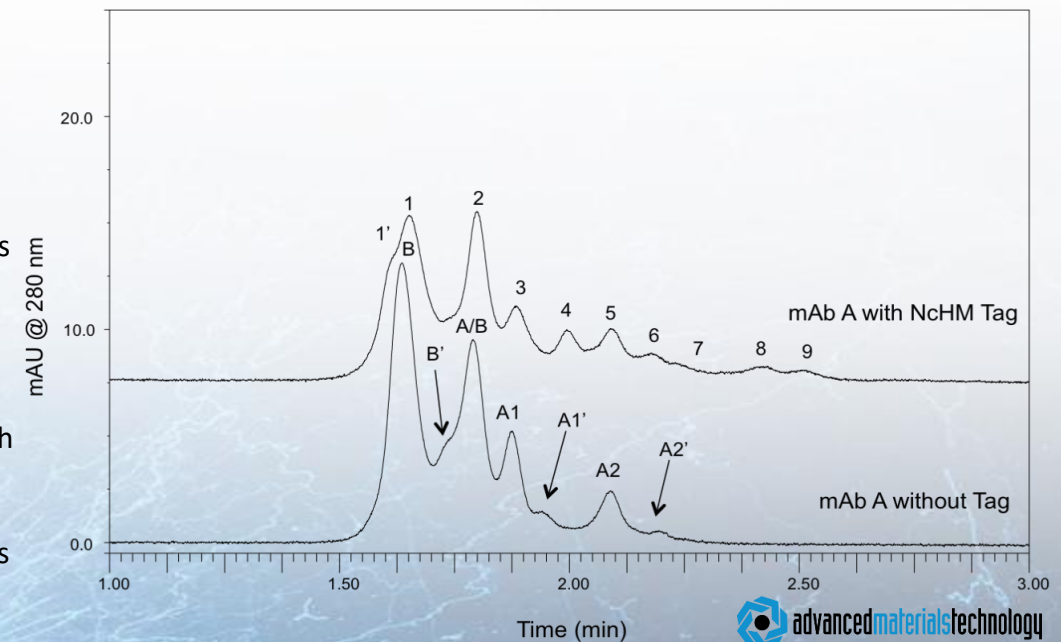
This approach has recently been described for IgG1, IgG1 constructs, and IgG2 mAbs recently by a number of investigators.

Zhang, Zhang, Hewitt, Tran, Gao, Qiu, Tejada, Gazzano-Santoro, and Kao. Identification and Characterization of Buried Unpaired Cysteines in a Recombinant Monoclonal IgG1 Antibody. *Anal Chem.* 84 (2012) 7112–7123.

Wei, Zhang, Boyes, and Zhang. Reversed-phase chromatography with large pore superficially porous particles for high throughput immunoglobulin G2 disulfide isoform separation. *J. Chromatogr. A* 1526 (2017) 104-111.

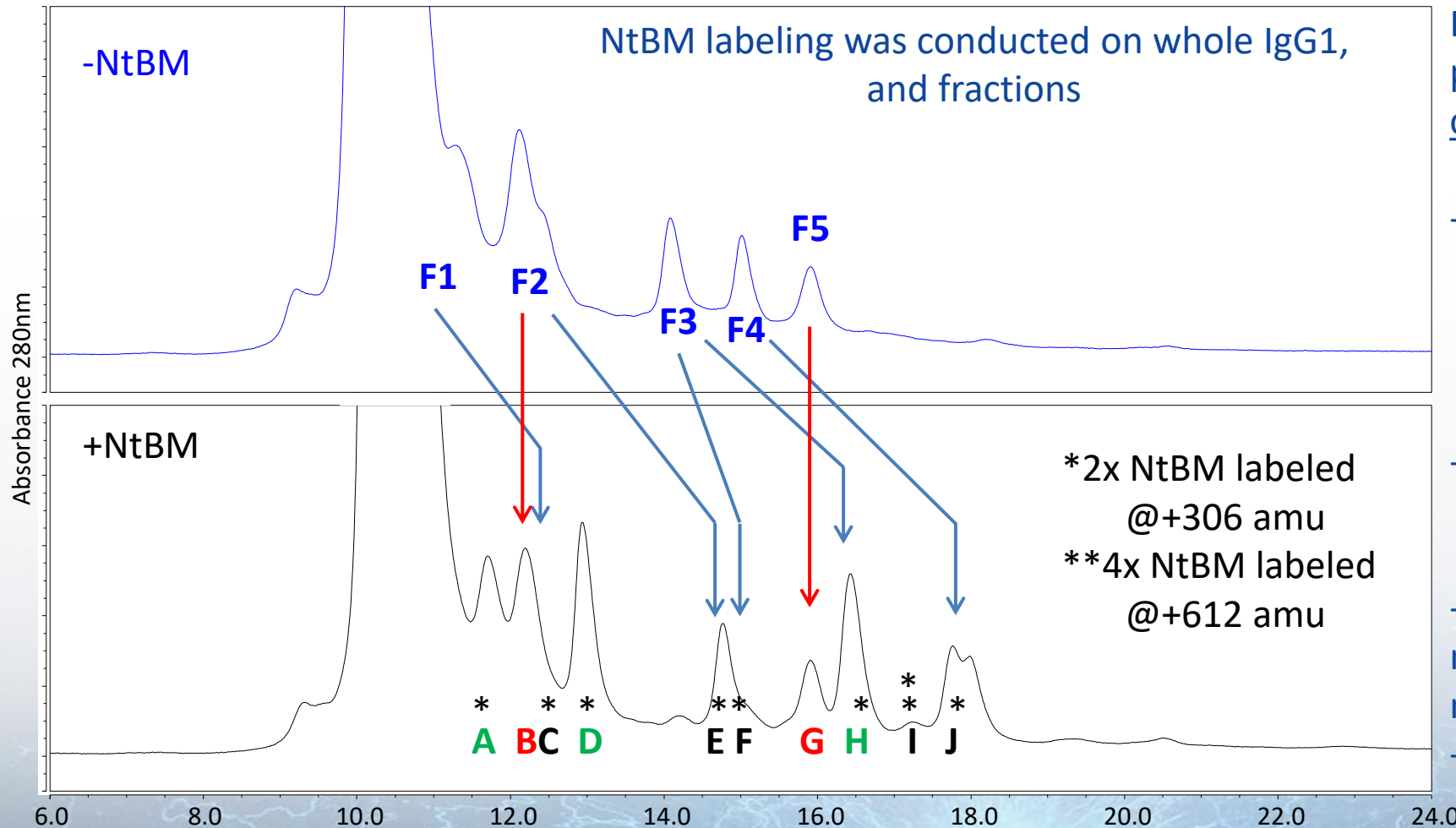
Welch, Dong, Hewitt, Irwin, McCarty, Tsai, and Baginski. Facile quantitation of free thiols in a recombinant monoclonal antibody by reversed-phase high performance liquid chromatography with hydrophobicity-tailored thiol derivatization. *J Chromatogr. B* 1092 (2018) 158-167.

Liu, Chen, Tsui, Wei, Yang, Yu, Cornell. Predictive *In Vitro* and Serum Models and Methods to Assess Thiol-related Quality Attributes in Protein Therapeutics. *Anal Chem*, (2020), *in Press*.



Resolution Has it Costs: What are those Resolved Peaks (trastuzumab)?

Column: 2.1 x 150 mm HALO 1000 Diphenyl; Flow rate: 0.25 mL/min; A H₂O/0.1% DFA; B: ACN/nPropanol (50/50)/0.1% DFA; Gradient: 29-33 %B in 30 min; 60°C; Injection Volume: 2 µL; Detection: 280 nm; MS Analysis in Orbitrap Velos Pro, 15,000 Rs, 3.8 kV, 275°C; NtBM labeling in 3.6 M GuHCl/100 mM NaOAc (pH 5.3)



NtBM labeling was conducted on whole IgG1, and fractions

Literature suggests resolved “late peaks” are free thiols. This is mostly confirmed.

-Direct LC/MS reveals “late peaks” are within 3-10 amu of full bridged IgG1 (PNGase +/-).

-Main peak shows no free thiols +/- after fractionation

-Two of the components (B/G) are not NtBM labeled, and assumed to not have free thiols (↓)

-All others Rt and mass shifted.

*2x NtBM labeled @+306 amu
 **4x NtBM labeled @+612 amu

Summary and Future Work

- Improving protein separations is both particle and chemistry (SP and MP and protein).
- Superficially porous particle silica packing materials have met the promise of supplying superior separations. Fused-Core with enlarged pore sizes (400 and 1000 Å) have particular utility for protein analyses, are robust, and routinely allow faster protein separations with higher efficiency.
- Subtle, but useful, differences in selectivity are available with additional bonded phases available on 1000 Å pore size materials (C4, ES-C18, DP).
- For protein analysis, temperature optimization is crucial to maximize recovery and selectivity, but diligence is required to avoid artifacts.
- Work continues on optimizing pore size and geometry for silica SPP. The end points will be defined by careful analysis of resolved protein samples, with limited guidance guaranteed from small molecule analysis.
- The more resolution gained with these newer RP materials, the greater detail that can be obtained on subtle structure variations in WCBPs.

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Thank you for your Attention

Questions and Answers for Dr. Barry Boyes



Panel Discussion:

Expert Panel



Dr. Naidong Weng
Scientific Director
Janssen Fellow
Janssen Pharmaceutical
Company



Dr. Thomas Waeghe
Senior Scientist
MAC-MOD Analytical



Dr. Michael Dong
Principal
MWD Consulting



Dr. Lijuan Kang
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US Sales Director
MAC-MOD Analytical

