Protein Separations Using High Performance Large Pore Superficially Porous Particles: Optimization Strategies for mAb Variant Resolution

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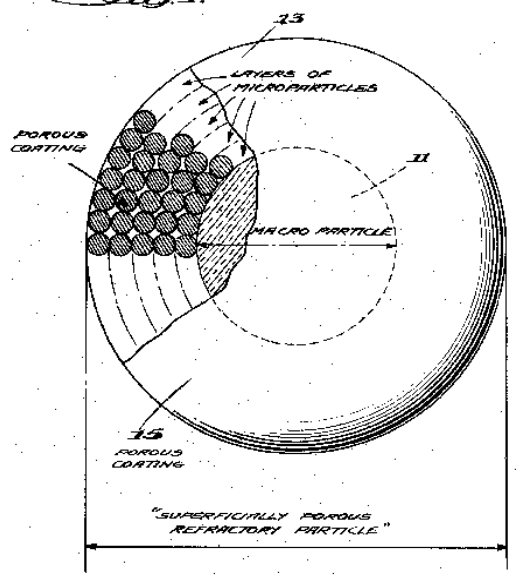
The Early Days -Conceptual

April 14, 1970 J. J. KIRKLAND 3,505,785

SUPERFICIALLY POROUS SUPPORTS FOR CHROMATOGRAPHY

Filed June 20, 1967 3 Sheets-Sheet 1

Fig. 1.



INVENTOR
J. J. KIRKLAND,
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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

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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.

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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**

Very Large Pore SPP **Surface Chemistry Options**

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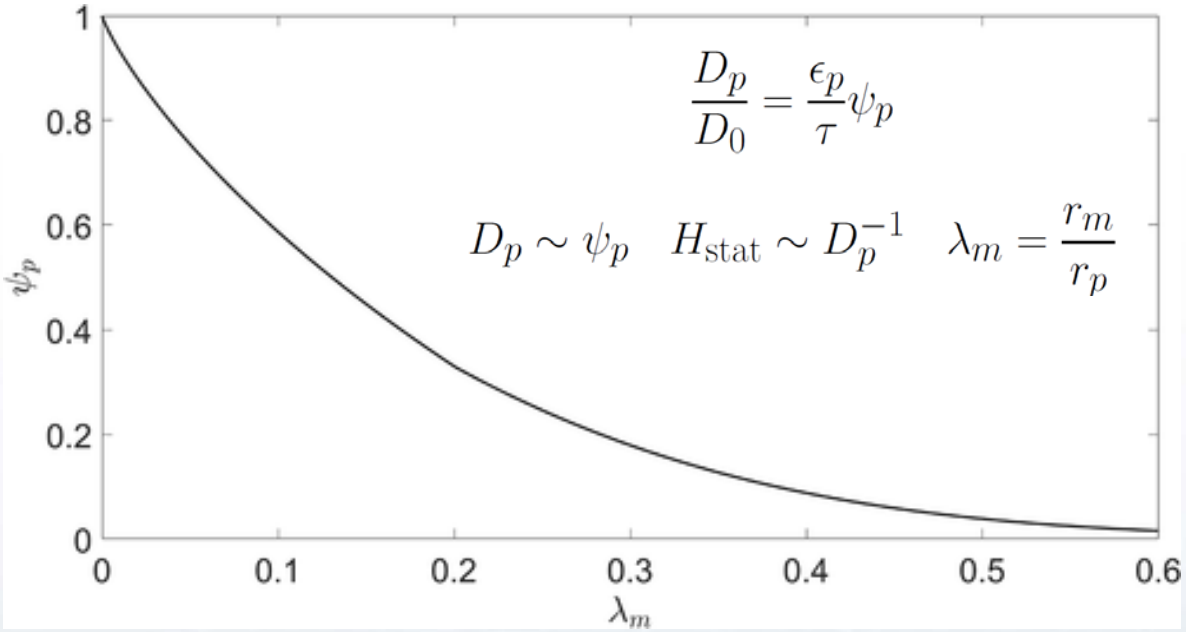
Restricted diffusion: Why you need larger pores than the size of the solute

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.



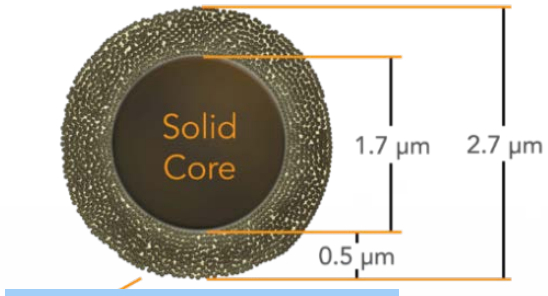
Theories have been developed which account for this effect in idealized pore shapes (cylinders, slabs) as shown to the left.[1]

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

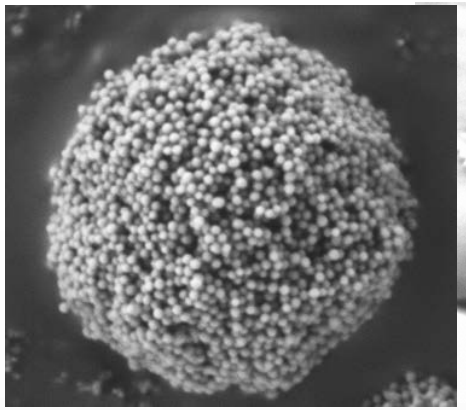
Ongoing efforts examine the fluid mechanics and transport properties of SPPs in packed beds.[3]

[1]P. Dechadilok, W.M. Deen, Hindrance factors for diffusion and convection in pores, Ind. Eng. Chem. Res. 45 (2006) 6953–6959.
[2]R. S. Maier, M. R. Schure, Transport properties and size exclusion effects in wide-pore superficially porous particles, Chem. Eng. Sci. 185 (2018) 243-255.
[3]M. 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).

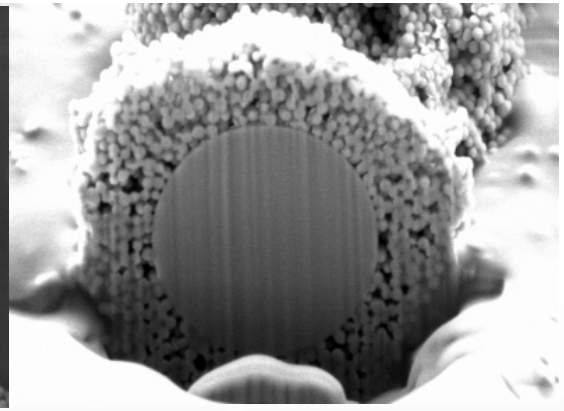
Superficially Porous (Fused-Core®) Wide Pore Particles: 1000 Å



Shell with 1000 Å pores



SEM

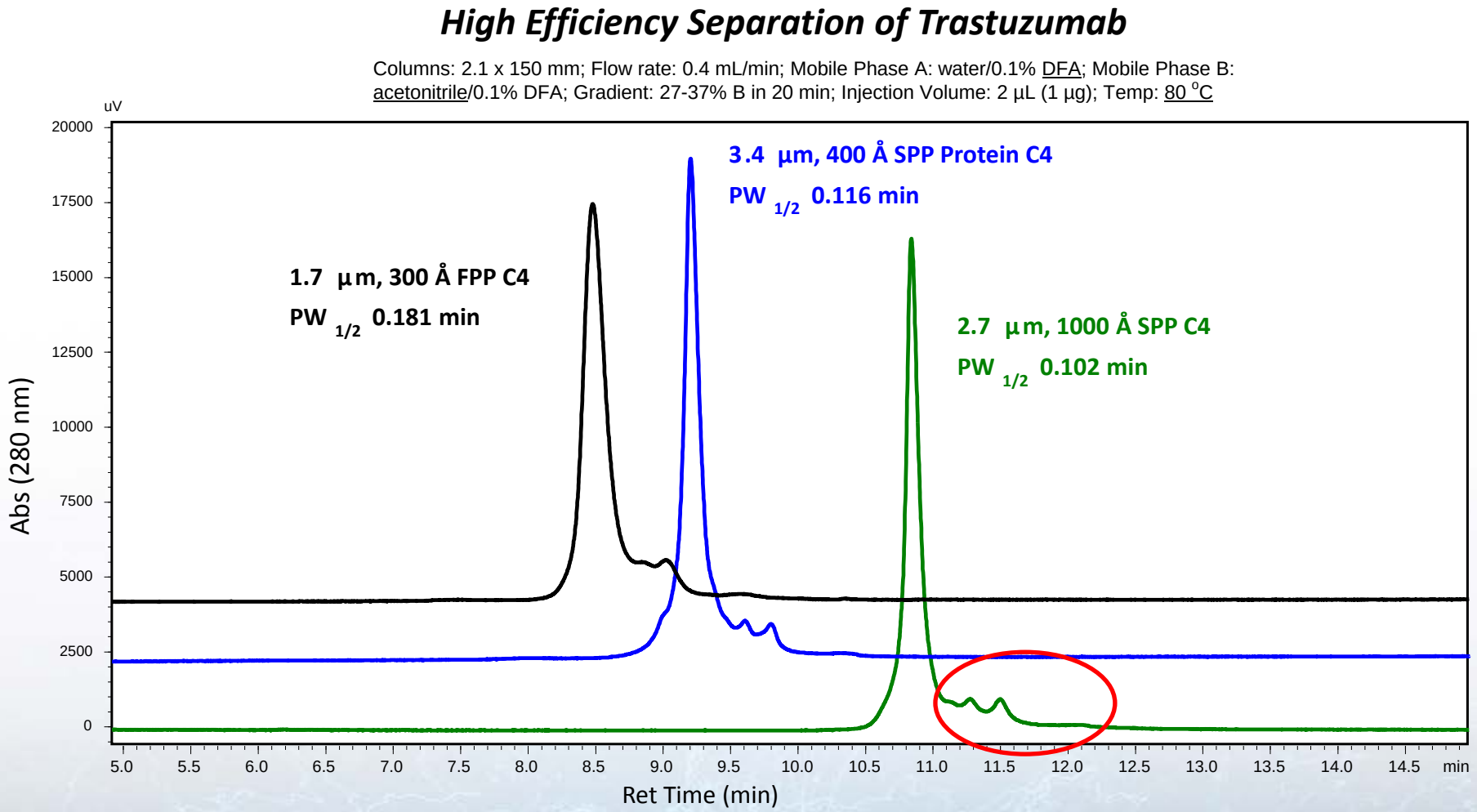


Section analysis by FIB-SEM

- 2.7 µm particle with 0.5 µm thick shell and 1000 Å pores
- Surface area ~ 22 m^2/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.

mAb IgG Separation on Wide Pore SPP vs FPP



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

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**

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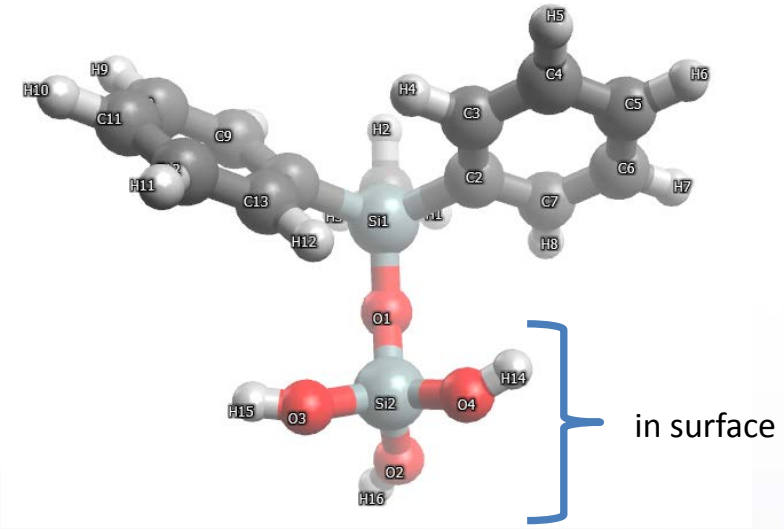
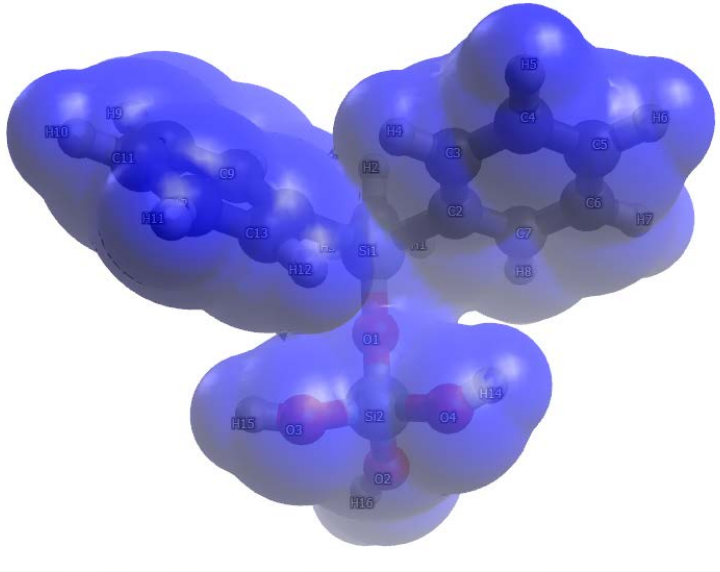
Diphenyl methyl sil(ane)oxane



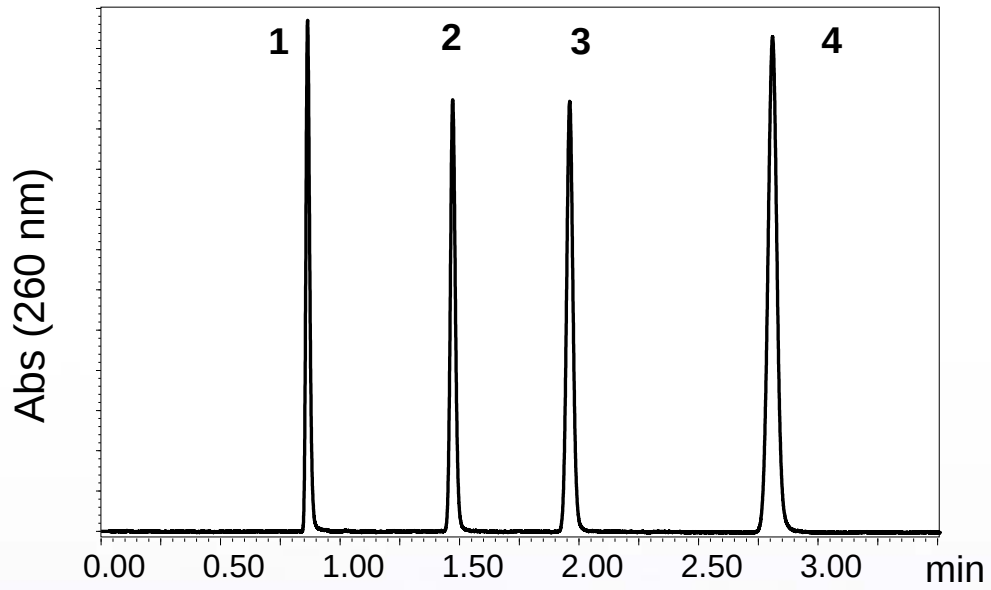
Ball and stick atom model



With van der Waals surface

- 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 C_{18}, these groups will accommodate the solute through bending and rotation.
- 1000 Å HALO SPP surface reaction is 2.7 µmol/m^2 (5.4 phenyl)

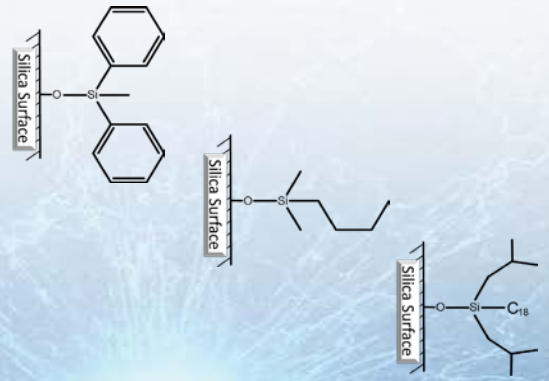
Effect of Bonded Phase on RP Separations of Small Probes using HALO 1000 Å SPP (2.7 µm; 0.5 µm shell)



Columns: 2.1 x 150 mm; Flow rate: 0.4 mL/min; Mobile Phase H_2O/AcN; 50/50 Instrument: Injection Volume: 2 µL; Detection: 260 nm; 25°C

1 - Uracil 60 µg/ml
2 - Hexanophenone 130 µg/ml
3 - Octanophenone 200 µg/ml
4 - Decanophenone 360 µg/ml

Bonded Phase	Retention Factor k'_4	Selectivity $\alpha_{4,3}$
Diphenyl	2.25	1.77
C4	4.95	2.13
ES-C18	17.92	2.81



- Highly similar N, Tf, different k' and α
- Retention for small molecules is: DP<C4<<C18

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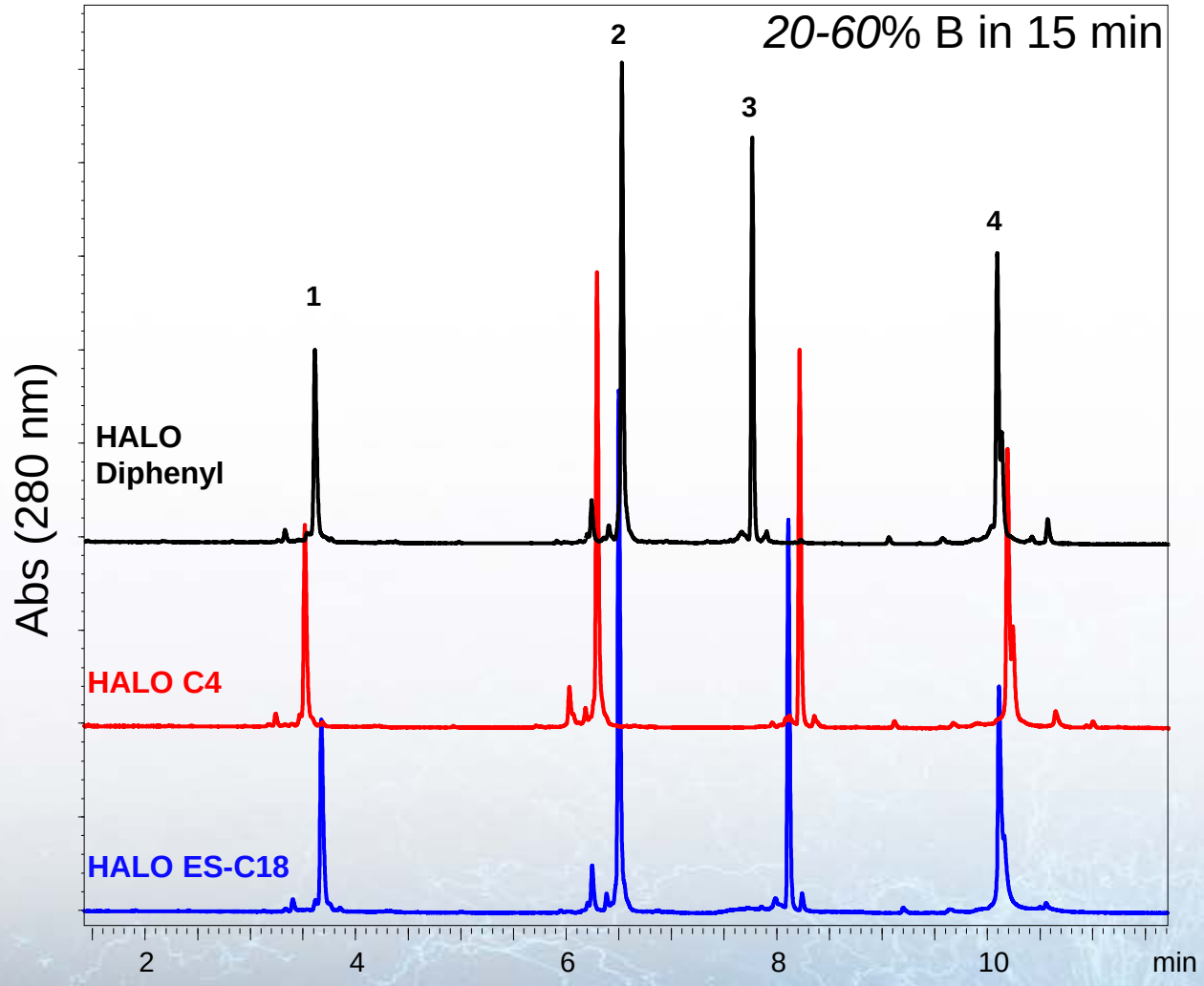
Effect of Bonded Phase on Protein Separations using HALO 1000 Å



Columns: 2.1 x 150 mm; Flow rate: 0.4 mL/min; Mobile Phase A: H_2O/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 ^{o}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

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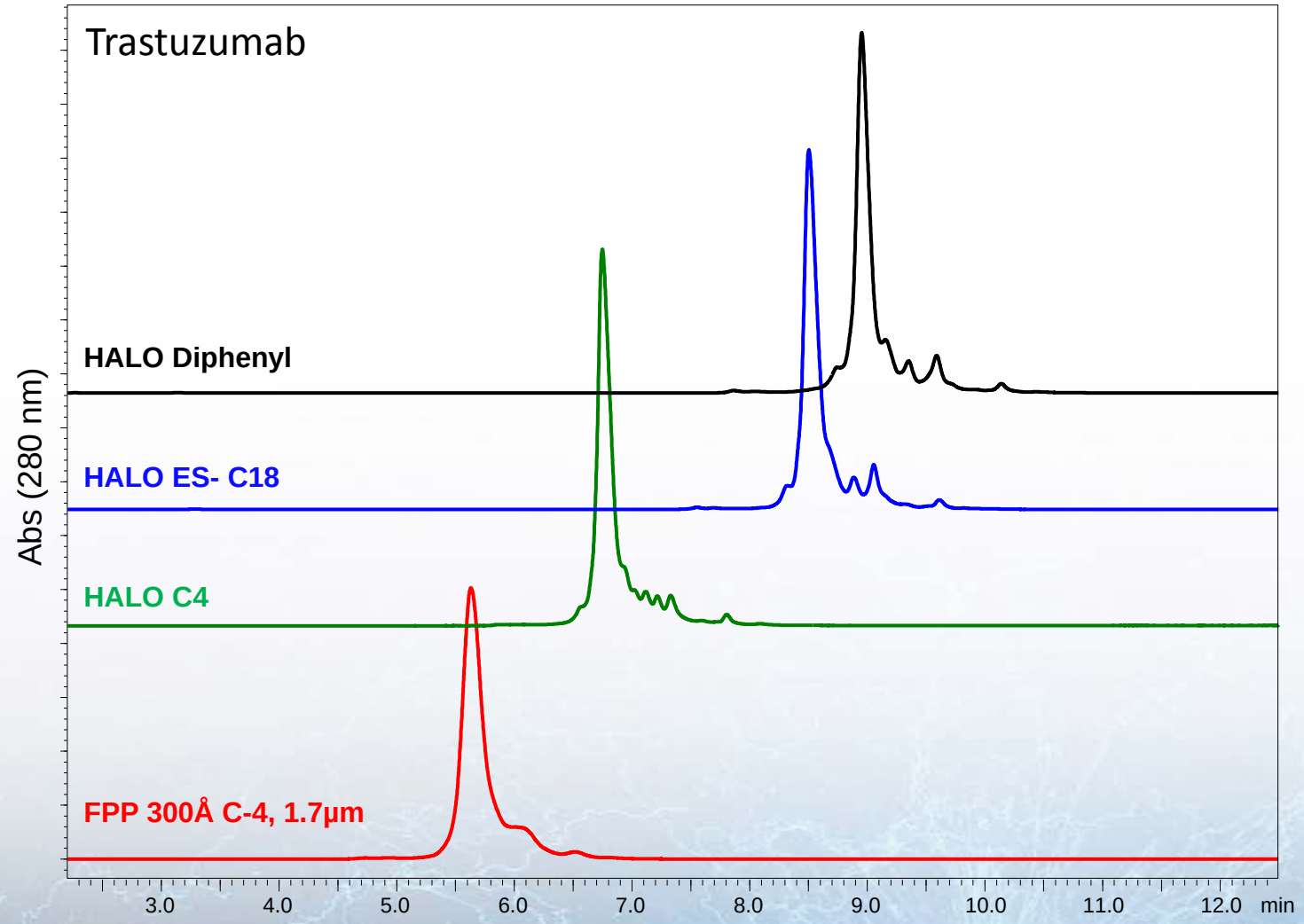
Effect of Bonded Phase on mAb Separations using HALO 1000 Å



Columns: 2.1 x 150 mm; Flow rate: 0.4 mL/min; Mobile Phase A: H_2O/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

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Improved Protein LC/MS: Manipulations That May Help Methods

Volatility

- **Necessary but not sufficient for additives. Must NOT mess up our ESI interface or block capillaries, or foul ion paths!**

Low pKa Acids

- **Low pH and dissociation of acid; sufficient ionic strength appears beneficial for separation needs, while effect on ESI suppression must be managed**
- **DFA is a viable alternative to TFA for larger proteins**

Organic Modifier

- **Mixture of Protic (small alcohols) and Aprotic (Acetonitrile) have become favored**

Favor Peptide and Protein Solubility/Recovery

- **Acidic (usually). Fluorinated? Polar? Chaotropic?**
- **Elevated Temperature**

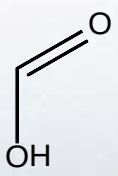
Bonded-phases

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

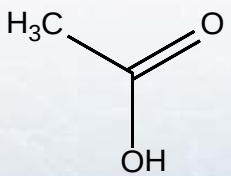
Initial selection and testing indicated some candidates with promise:

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

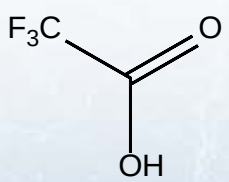
	FA	HOAc	TFA	DFA	3FPA
pK_a	3.8	4.8	0.3-1.6	1.3-1.4	3.1



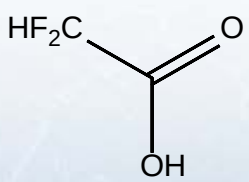
formic acid



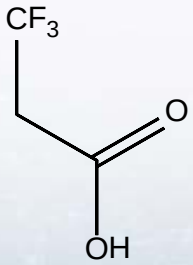
acetic acid



2,2,2-trifluoroacetic acid



2,2-difluoroacetic acid



3,3,3-trifluoropropanoic acid

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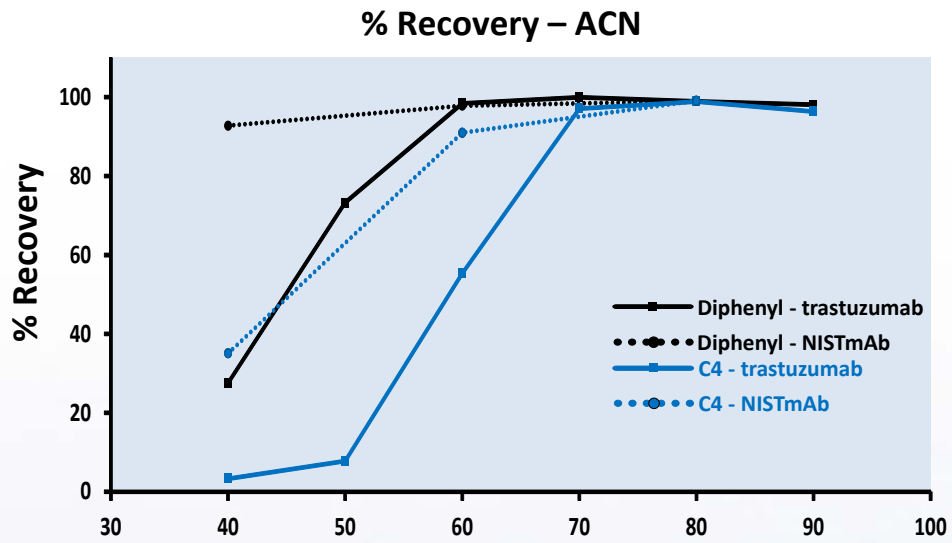
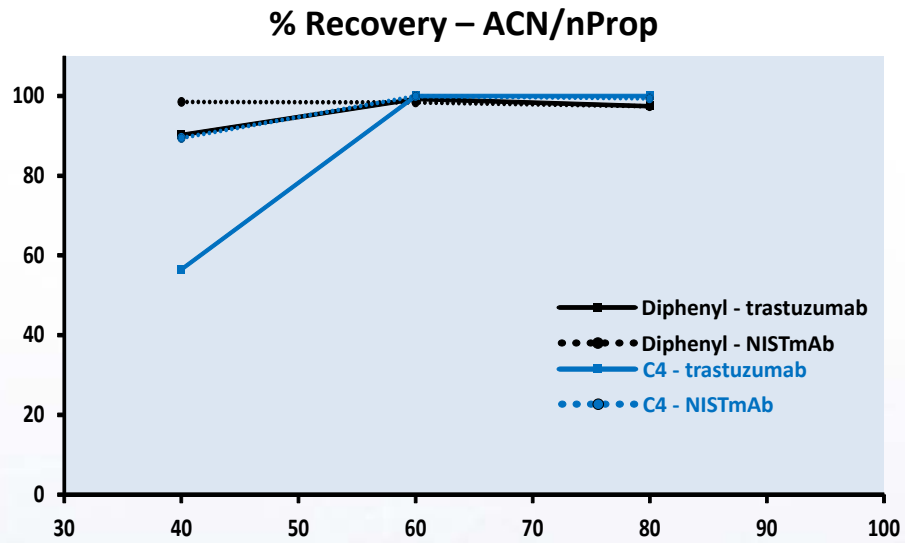
Temperature-dependent Recovery of mAbs: Bonded Phase/Mobile Phase Effects

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

A – H_2O/0.1% TFA, B – ACN/0.1% TFA: 30-45%B in15min
4 µL at 2 mg/mL (8 µg)



A – H_2O/0.1% TFA, B – (50/50 ACN/nPropanol)/0.1% TFA: 28-43%B in15min
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

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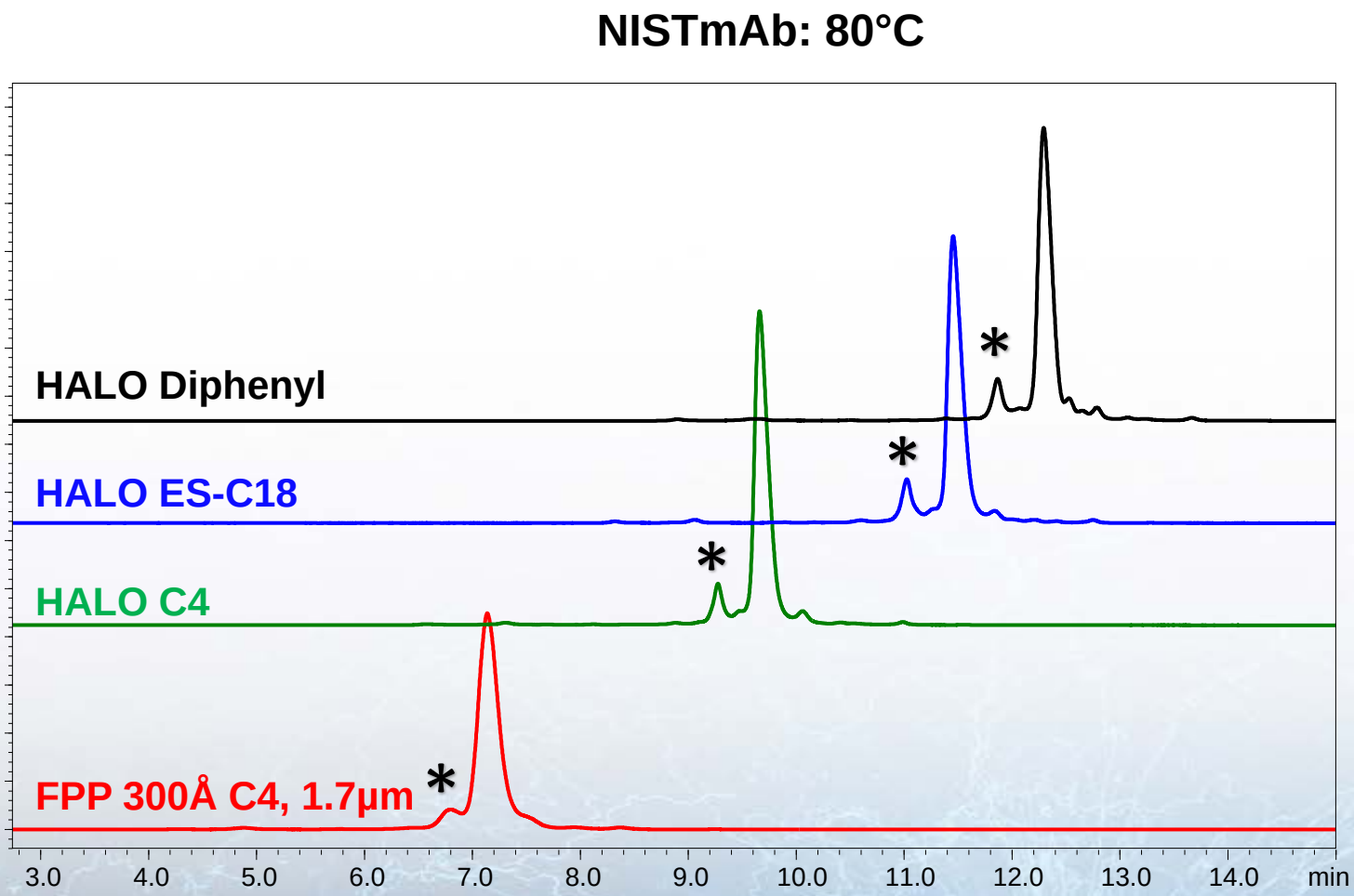
Effect of Temperature on mAb Separation: DANGER



Columns: 2.1 x 150 mm; Flow rate: 0.4 mL/min; Mobile Phase A: H_2O/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

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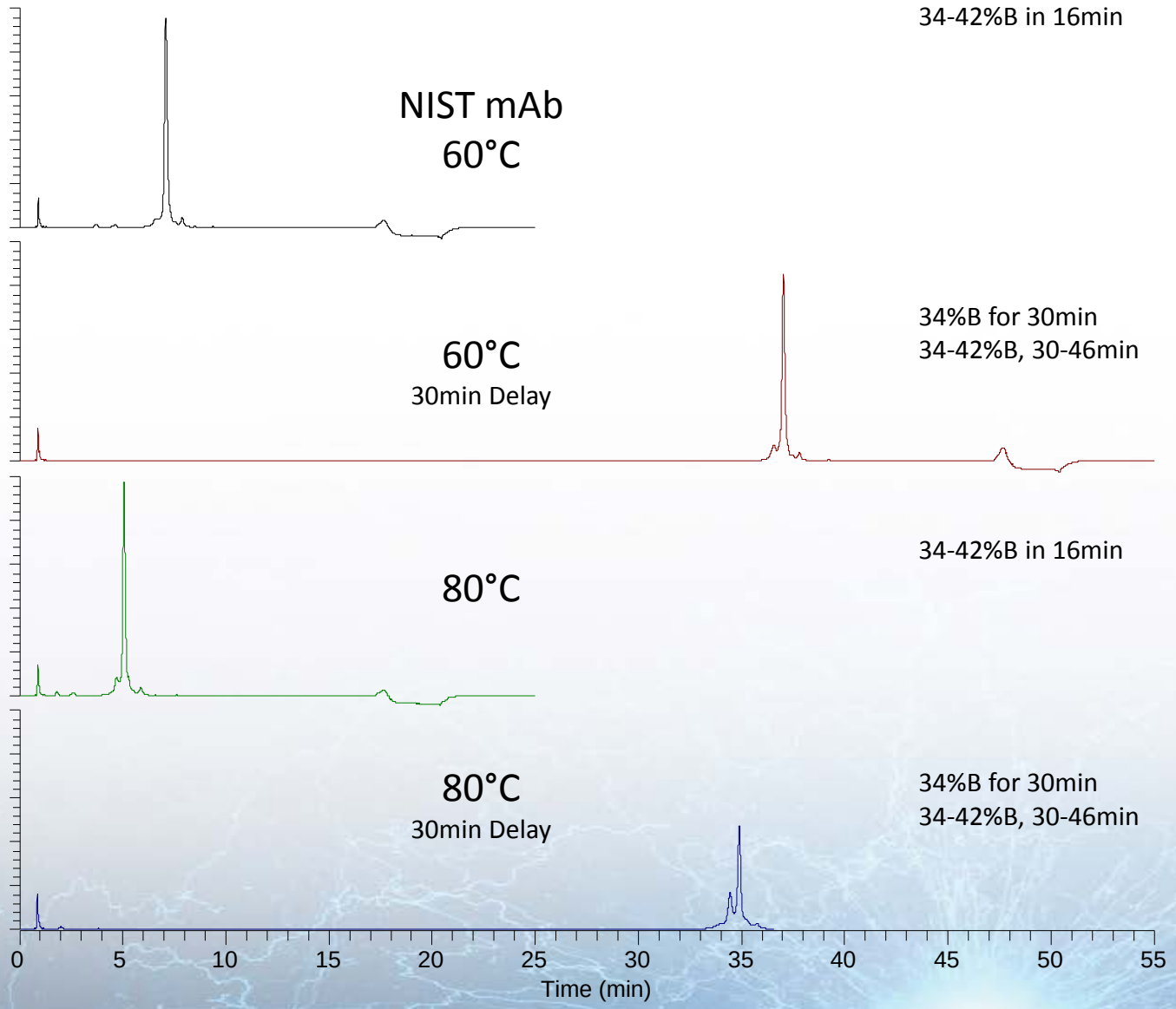
Effect of Temperature on mAb Separation: DANGER



Columns: 2.1 x 150 mm Halo Diphenyl 1000Å
Flow rate: 0.4 mL/min;
Mobile Phase A: H_2O/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

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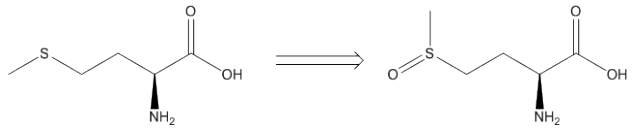
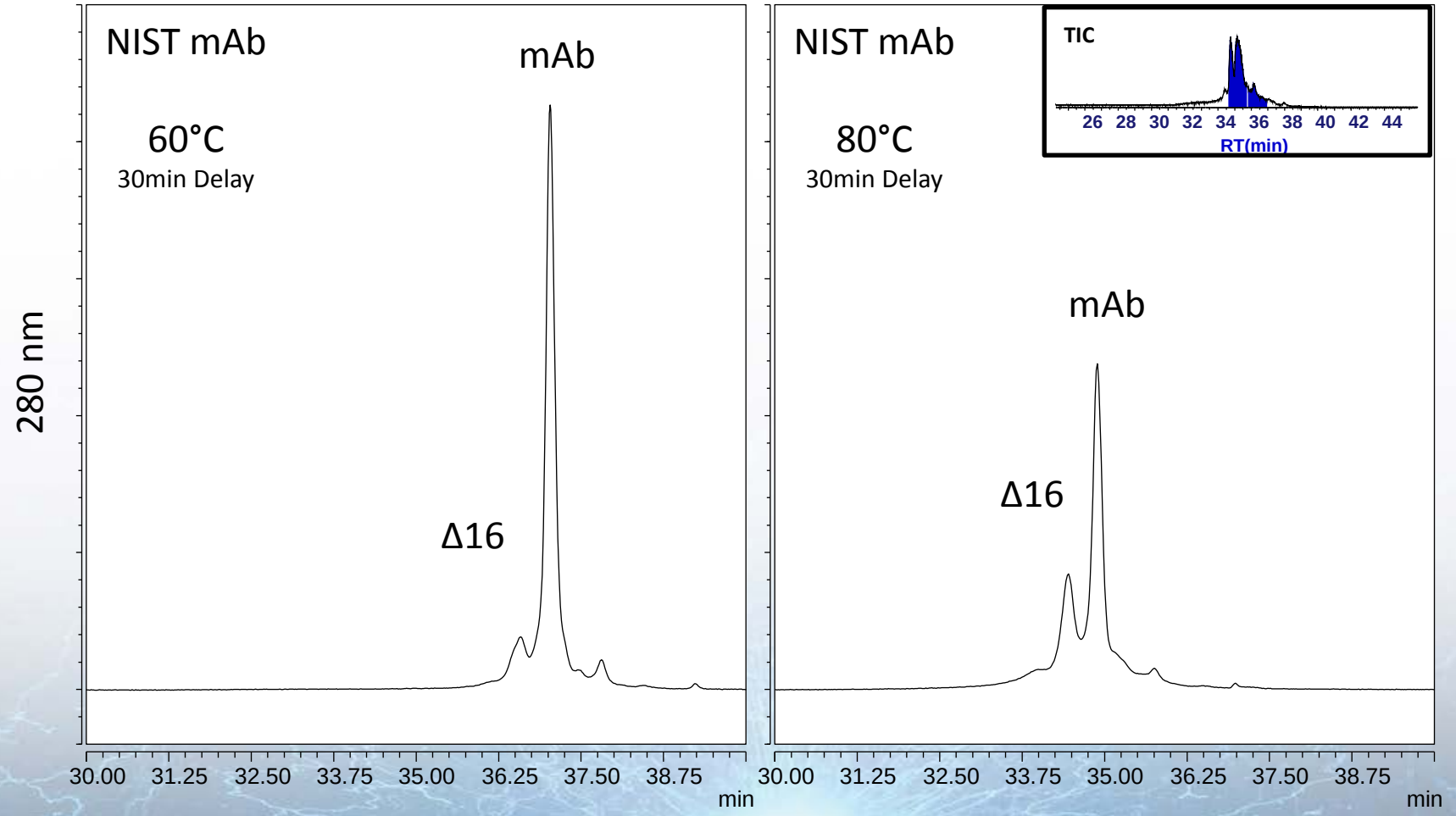
Effect of Temperature on mAb Separation: DANGER





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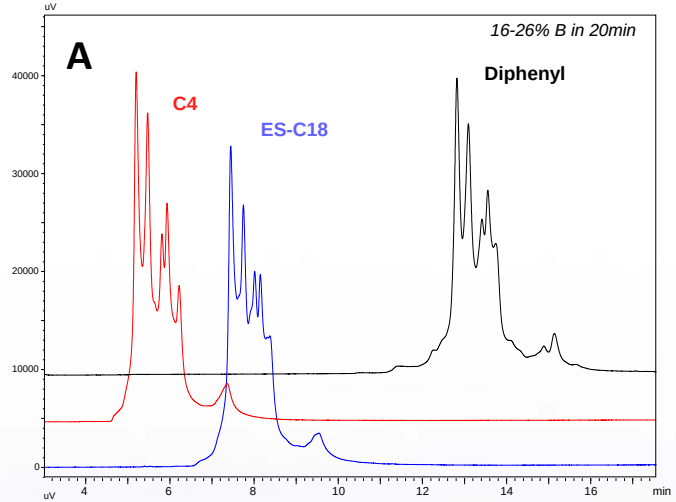
Method Development Approaches: BP, MP, T

Column Based Approach

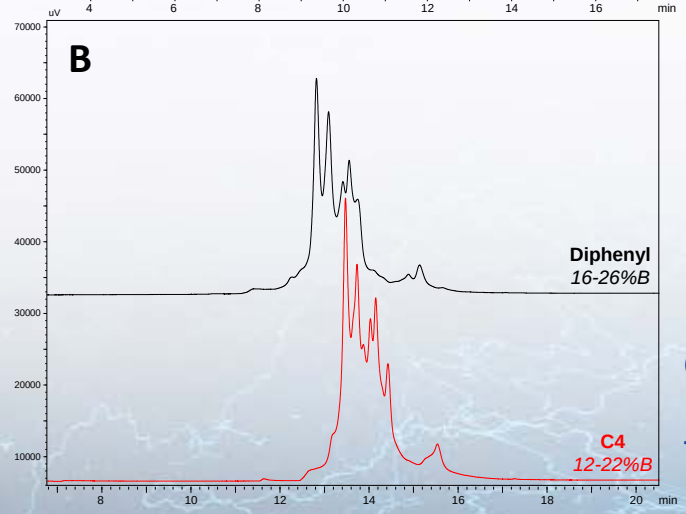
Columns: 2.1 x 150 mm; Flow rate: 0.2 mL/min; Mobile Phase A: 88/10/2 H_2O/ACN/nProp + 0.1% DFA; Mobile Phase B: 70/20/10 nProp/ACN/H_2O + 0.1% DFA; Gradient: 16-26 %B in 20 min; Instrument: Shimadzu Nexera; Injection Volume: 2 µL; Detection: 280 nm; Temp: 80 °C

AcN/nProp mix
Based on lit.



BP compared

Hi/Low T scan



Rt adjusted

C4 selected

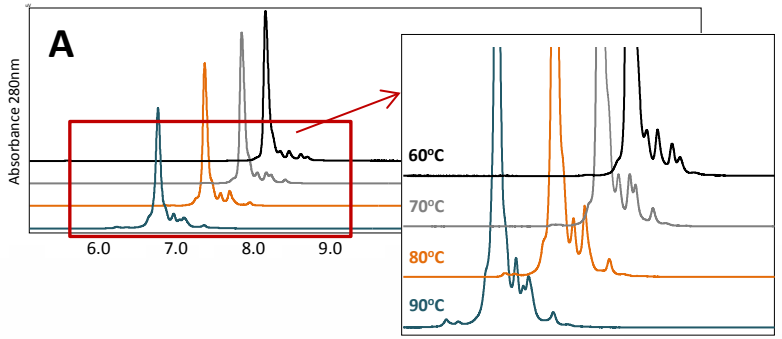
T and ΔG adjusted

T and MP Composition

Columns: 2.1 x 150 mm HALO 1000 Diphenyl; Flow rate: 0.4 mL/min; A H_2O/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;

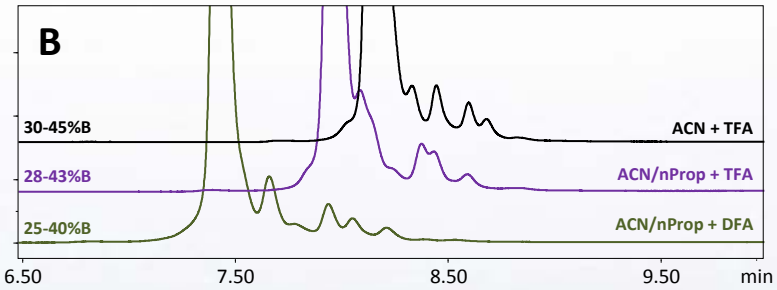
BP fixed

T Compared



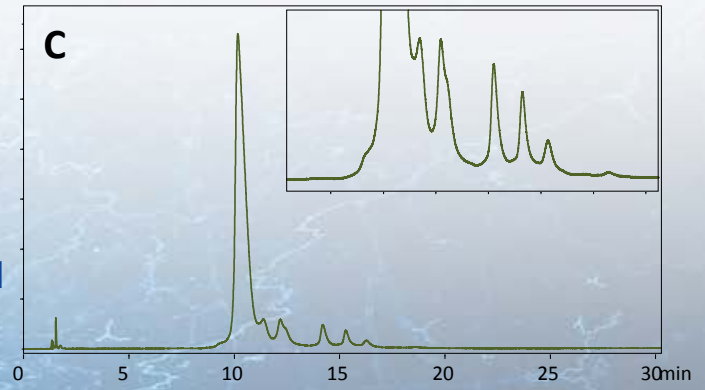
T selected (60)

MP Compared
(Acid, Organic)

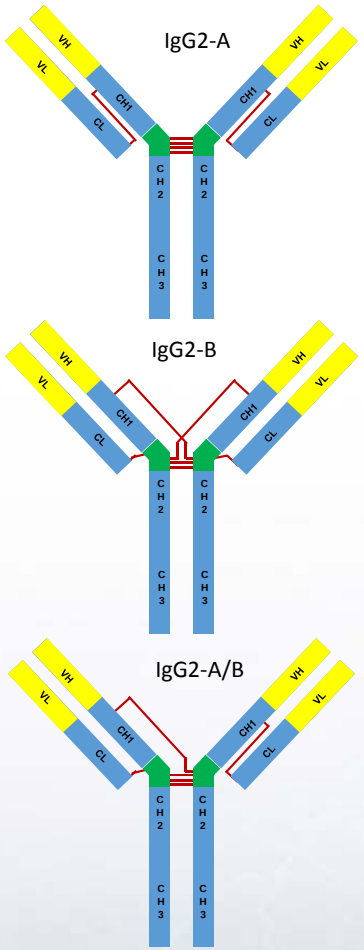


MP selected
AcN/n-Prop/DFA

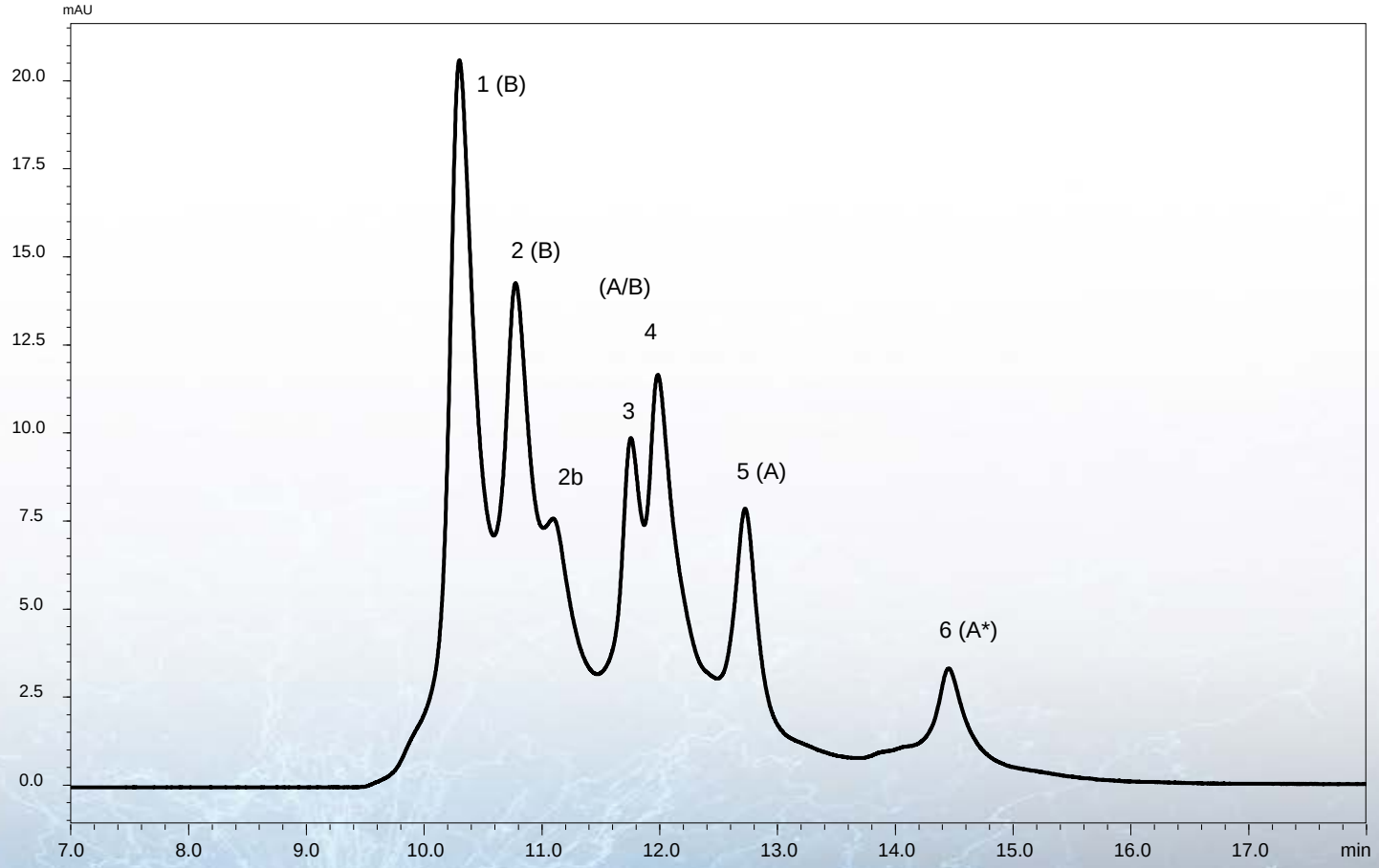
ΔG, flow
and Time adjusted



IgG2 Disulfide Bridge Variant Separation



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



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.

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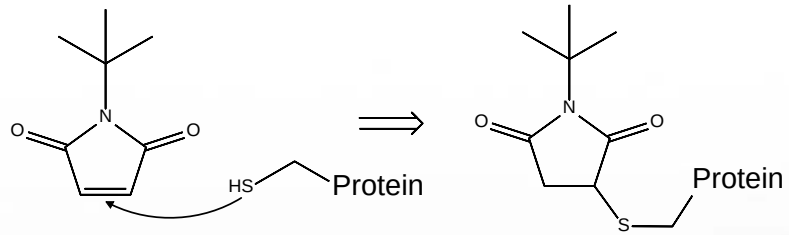
IgG Disulfide Bridge and Free Thiol Variant Analysis

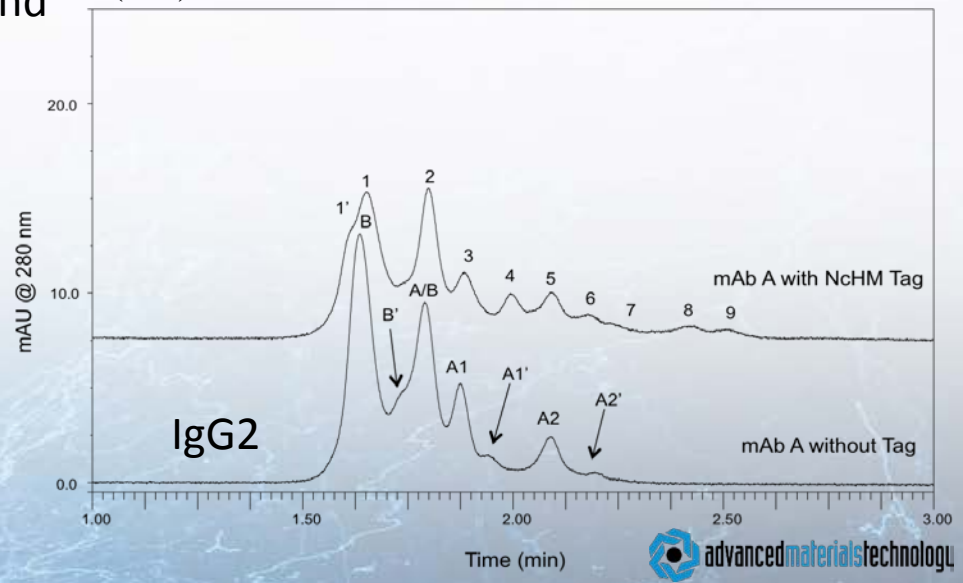
Many proteins possess disulfide bridges and may also have free thiol groups (R-SH) present. In IgG1, these may be considered problematic, or at least must be monitored during bioprocessing, for therapeutic formulations. In the case of IgG2, free thiols are formed during conversion of IgG2 disulfide variants - A, B, A/B isoforms. Conversions of R-S-S-R to $(R\text{-}HS)_2$ 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.



N-t-butylmaleimide (NtBM): 153.08 amu

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.

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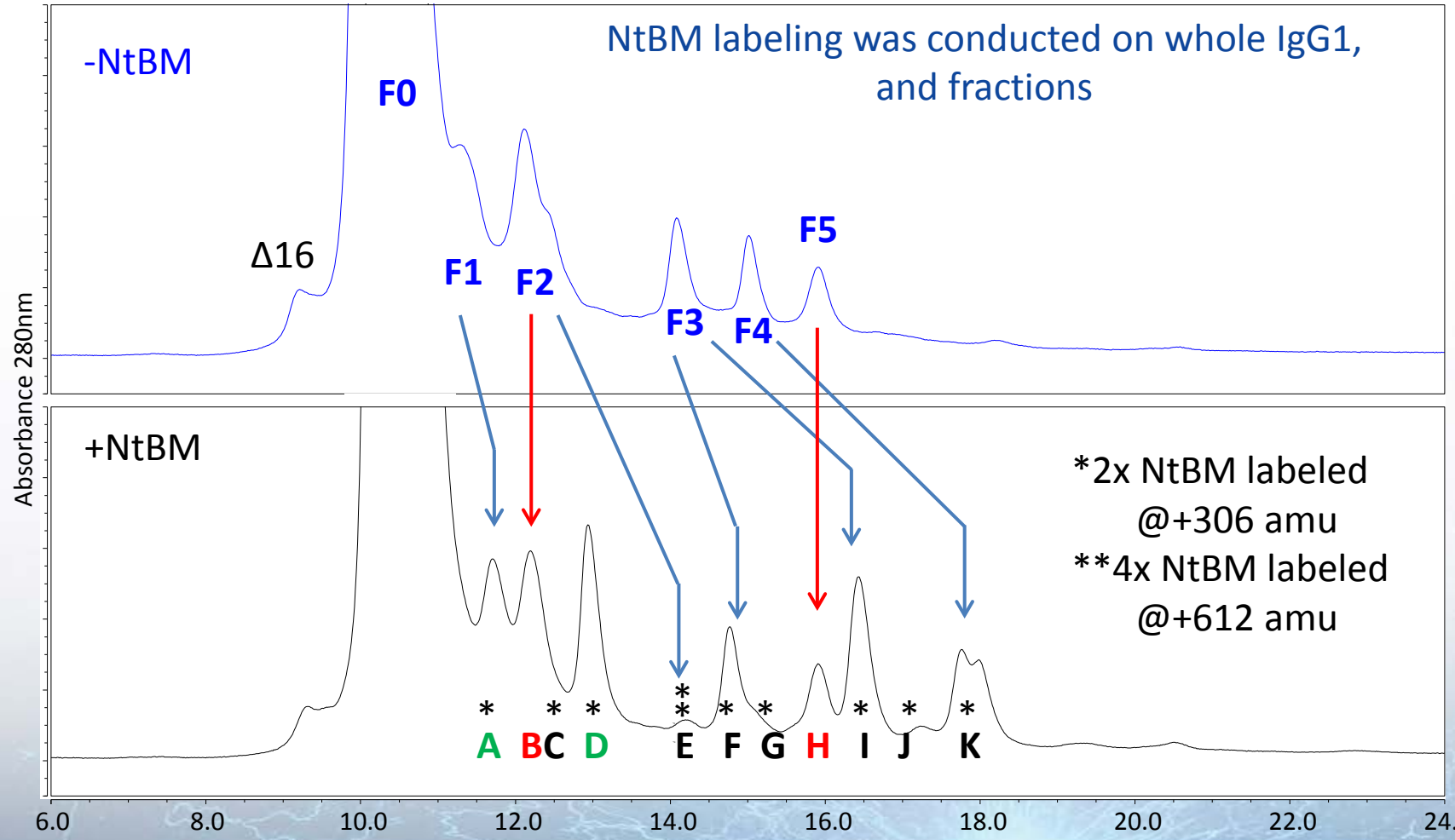
Free Thiol Variants in Intact IgG1 mAbs (trastuzumab)

Column: 2.1 x 150 mm HALO 1000 Diphenyl; Flow rate: 0.25 mL/min; A H_2O/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)



Literature suggests resolved "late peaks" are free thiol variant proteins. <u>This is mostly confirmed.</u>

-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/H) are not NtBM labeled, and assumed to not have free thiols (↓)
-All others Rt and mass shifted.

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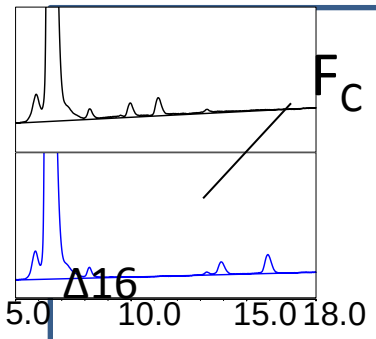
Domain Resolved Sulfhydryls: IdeS Digest

IgG1

IdeS Digest (Fabricator)

+/- NtBM Label

LC/MS
Fc/2 and $(Fab)_2$

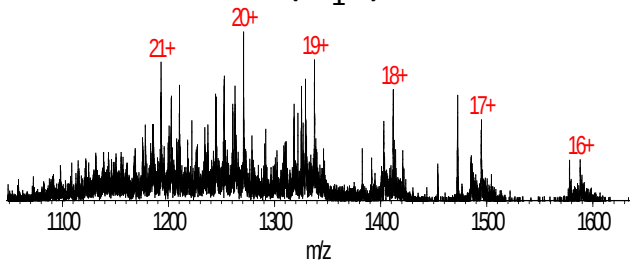


*

*+

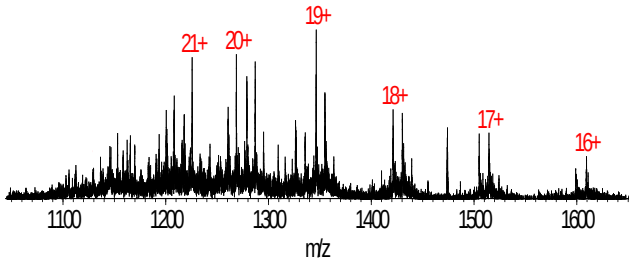
-NtBM

* 25,396 Da
(G_1F)



+NtBM

*+ 25,702 Da
Δ 306



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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 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, using novel materials science approaches.
- The more resolution gained with 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

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