



Enhanced Sensitivity for Intact Monoclonal Antibody Analysis via LCMS using a Novel UHPLC Column Design

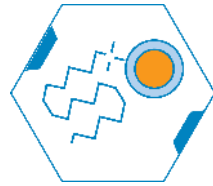
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Parameters that Influence Sensitivity

- **Column type**

- Particle Type (SPP vs FPP)
- Pore size
- Particle size
- Stationary Phase



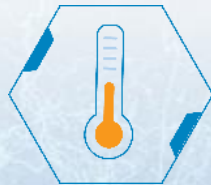
- **Mobile phase**

- Acidic Modifiers



- **Temperature**

- Recovery Studies



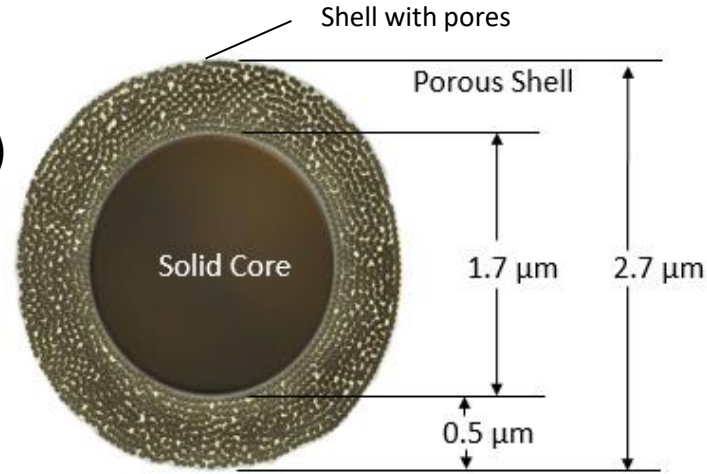
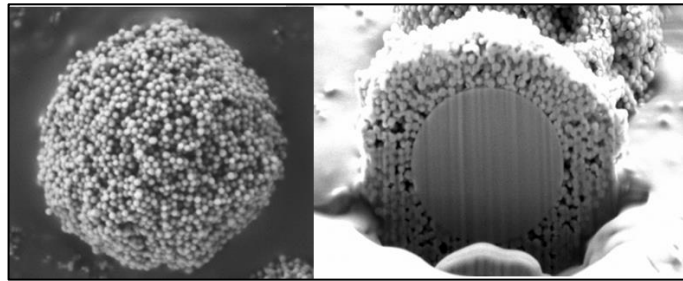
- **Column Dimension**

- 1.5 mm i.d

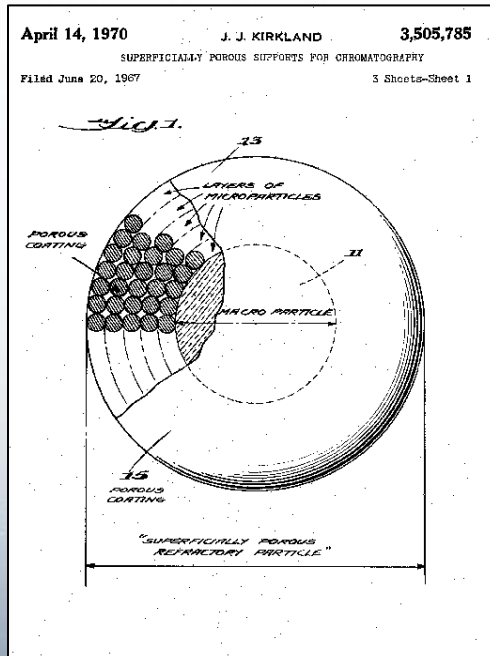
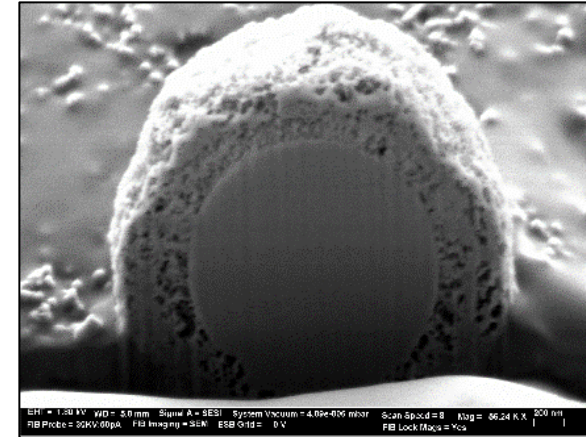


Superficially Porous Particle Technology (SPP)

- High Purity Silica Particles (2, 2.7, 3.4, 5 μm)
- Bonded Phase Shell Fused to Solid Core
- Shell Consists of Different Pore Sizes (90, 160, 400, 1000 \AA)



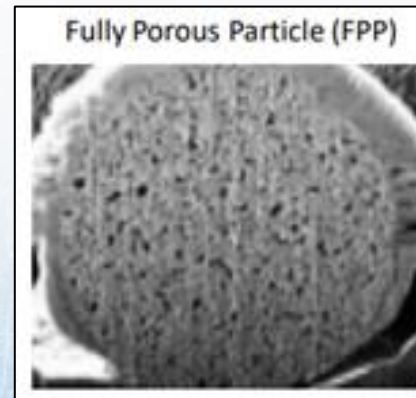
SEM Particle Cross-section



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.



50
years of HPLC
 A look back at the particles, pressures, and other advances that led to the technique's success
P.28

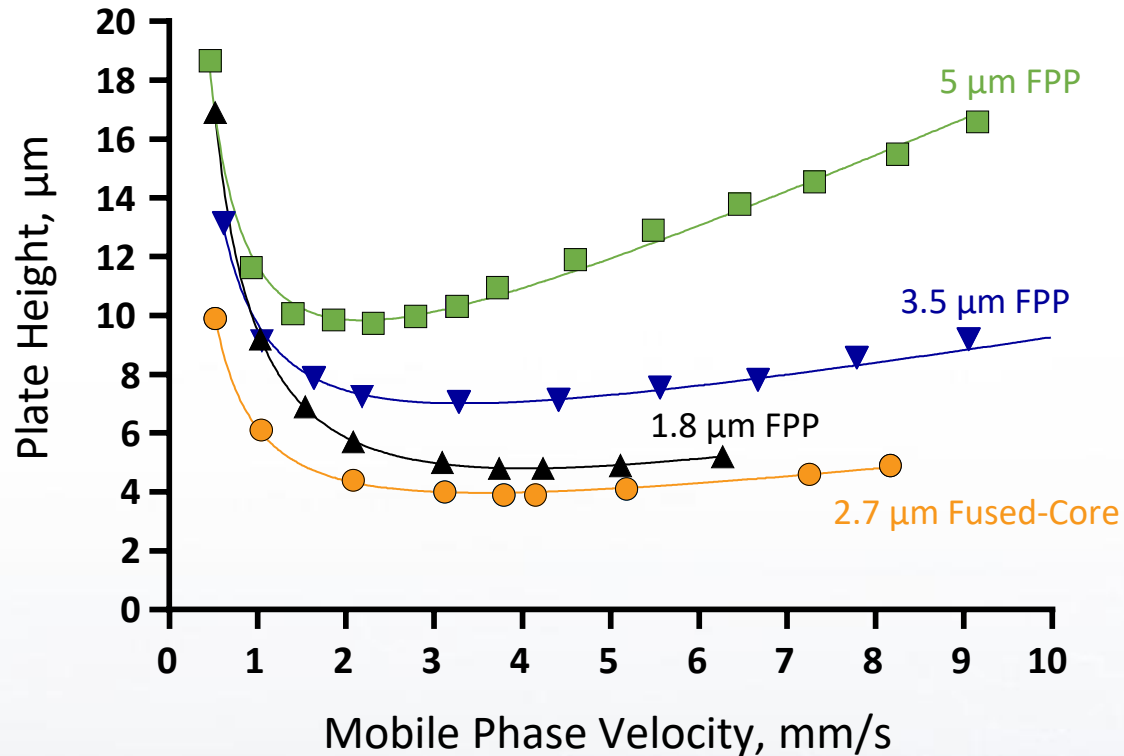
British chemistry braces for possible 'Brexite' from the EU
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How materials chemistry shaped the record industry
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How SPP Benefits Separations?

Speed and Efficiency



J.J. DeStefano, T.J. Langlois, & J.J. Kirkland, *J. Chromatogr. Sci.*, 2008, 46(3), 254-260

Effect of Particle Size and Type

Columns: 4.6 x 50 mm
 5 µm FPP C18
 3.5 µm FPP C18
 1.8 µm FPP C18
 2.7 µm HALO C18

Solute: naphthalene
 Mobile phase: 60% ACN/40% water
 Temperature: 24 °C

van Deemter Equation

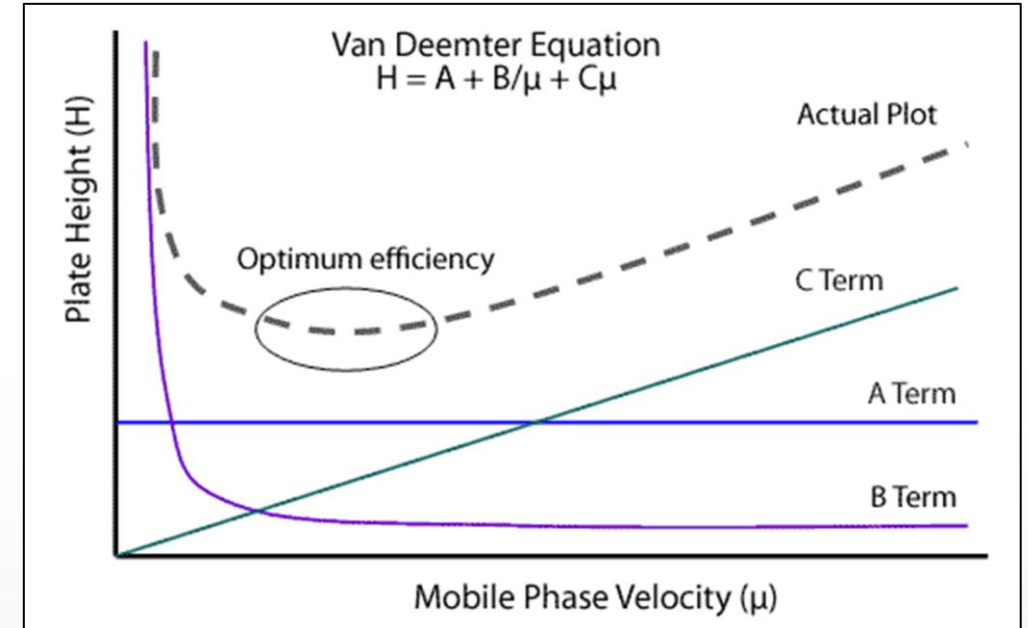
H = height equivalent to theoretical plate

A = eddy diffusion term (particle size and how well bed was packed) **30 - 40% smaller**

B = longitudinal diffusion term **25 - 30% smaller**

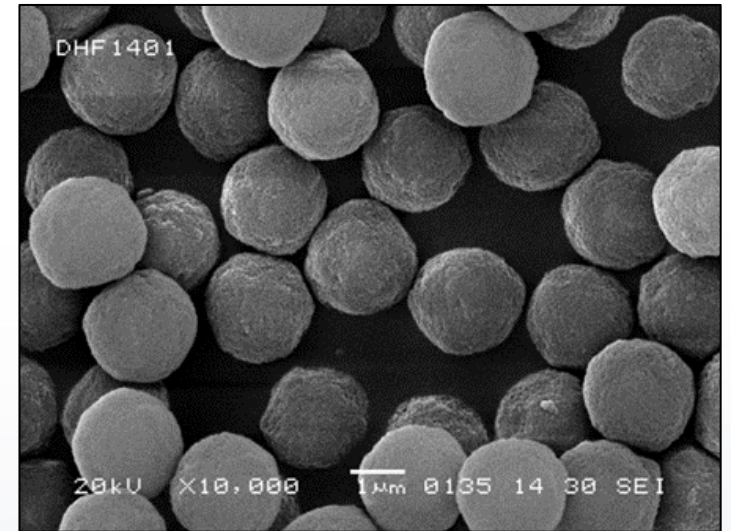
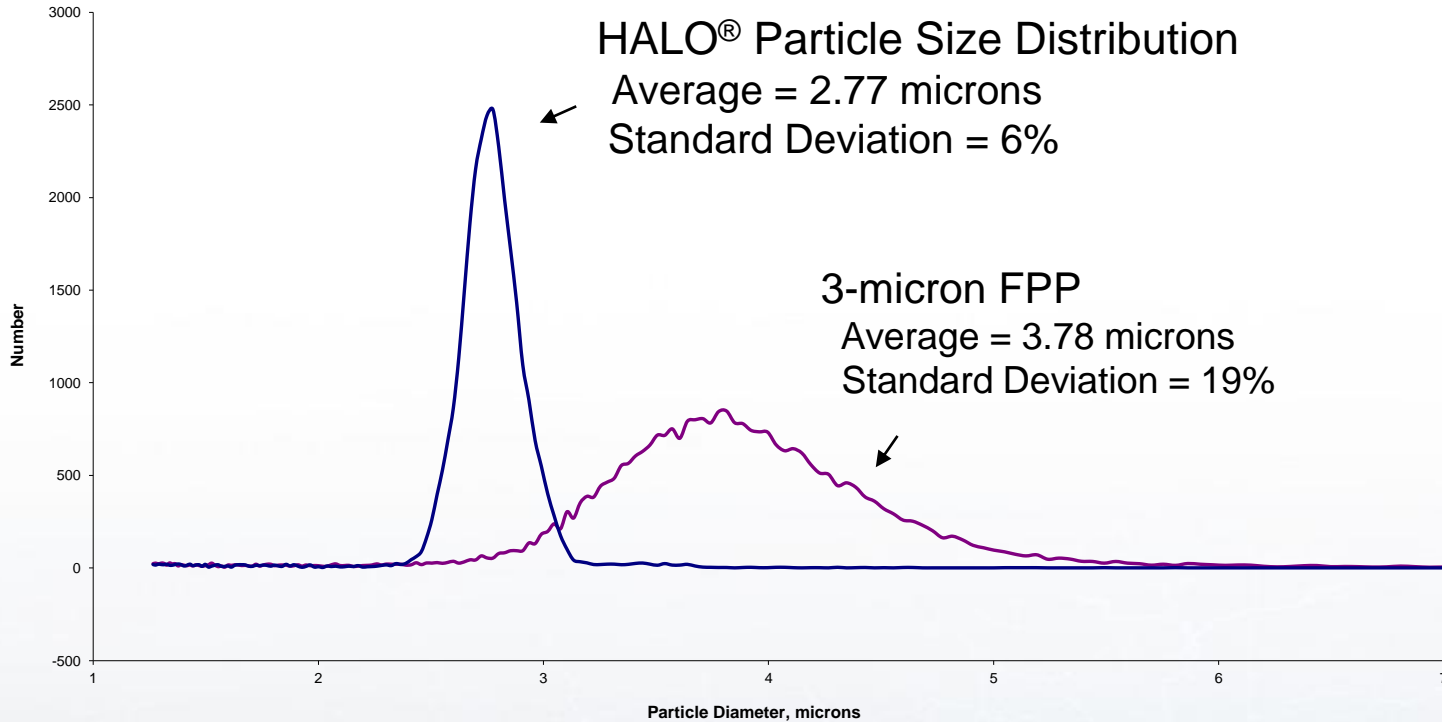
C = resistance to mass transfer term (kinetics of the analyte b/w mobile phase and stationary phase)

µ = mobile phase linear velocity (L/t₀)



$$H = A + \frac{B}{\mu} + C\mu$$

High Efficiencies



“This particle lets you do “UHPLC-like” separations on a standard system or do ultrafast HPLC on a UHPLC system”

-Customer Comment

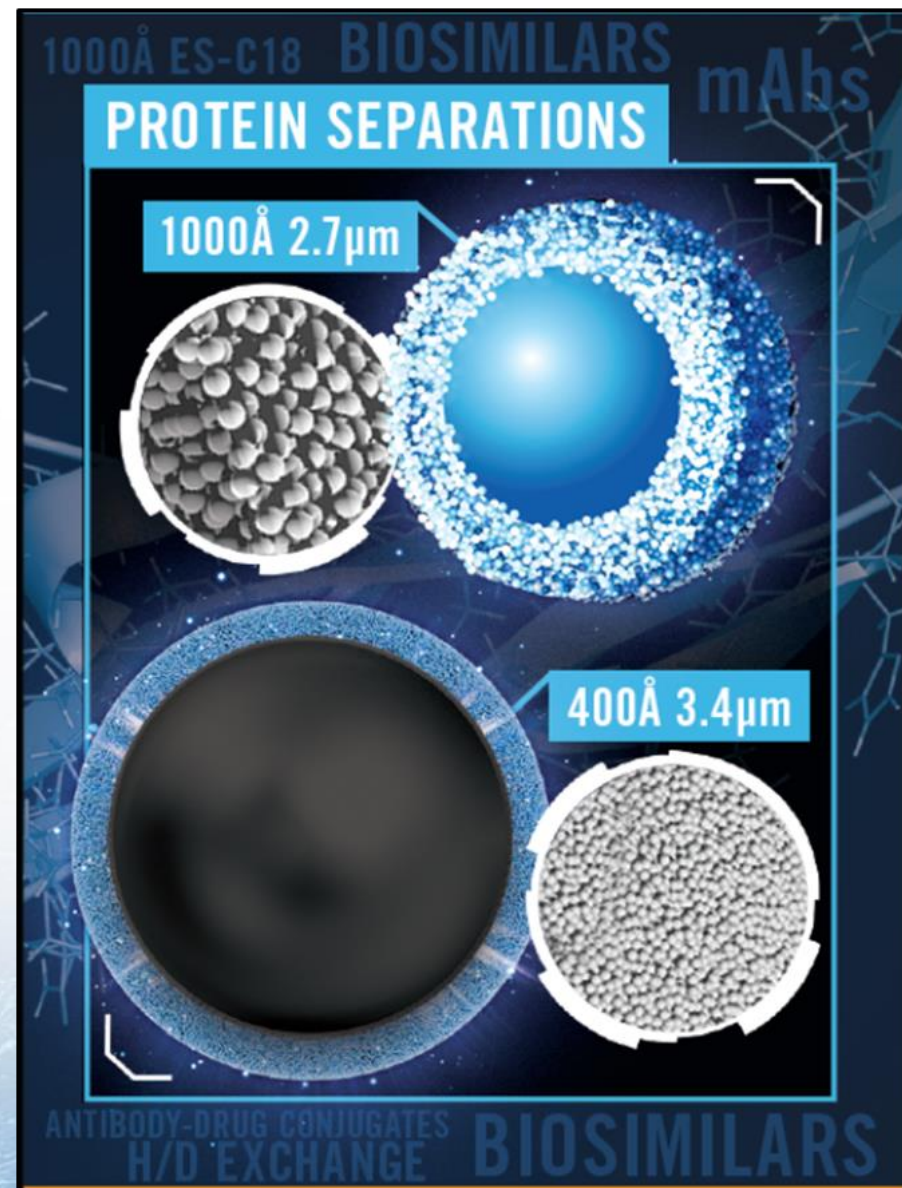
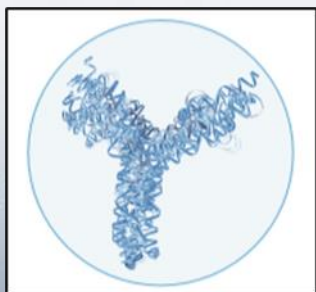
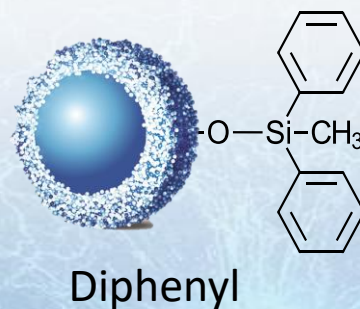
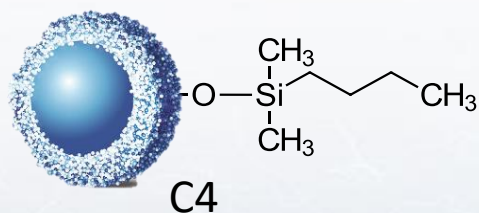
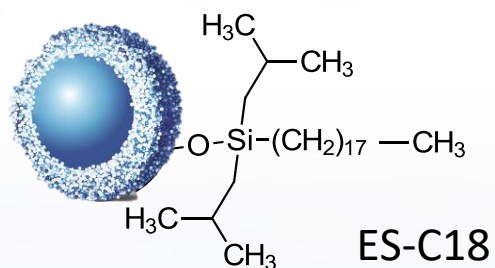
HALO[®] BioClass-Protein Analysis

- Two particle designs for protein analysis:

1000 Å, 2.7 μm particle

400 Å, 3.4 μm particle

- 1000 Å particle is used for the ultimate resolution of mAbs and other large proteins



1000Å ES-C18 BIOSIMILARS mAbs

PROTEIN SEPARATIONS

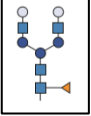

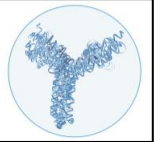
1000Å 2.7μm

400Å 3.4μm

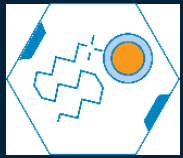
ANTIBODY-DRUG CONJUGATES H/D EXCHANGE BIOSIMILARS



Column Type: Pore Size

	Molecule Size	Pore Size (Å)	Application	Particle Sizes (µm)	Column Family
	SMALL (<5000 Da)	90	Small Molecules	2, 2.7, 5.0	HALO
	SMALL (< 20 kDa*)	90	Glycans	2.7	HALO BIOCLASS
	MEDIUM (100 Da < MW < 15 kDa)	160	Peptides	2, 2.7, 5.0	
	LARGE (2 kDa < MW < 500 kDa)	400	Proteins	3.4	
	LARGE (> 50 kDa)	1000		2.7	
* for glycan, glycopeptide, glycoproteins					

- Large molecules cannot be well separated on small pore packings
- Small molecules can be separated on either small or large pore packings



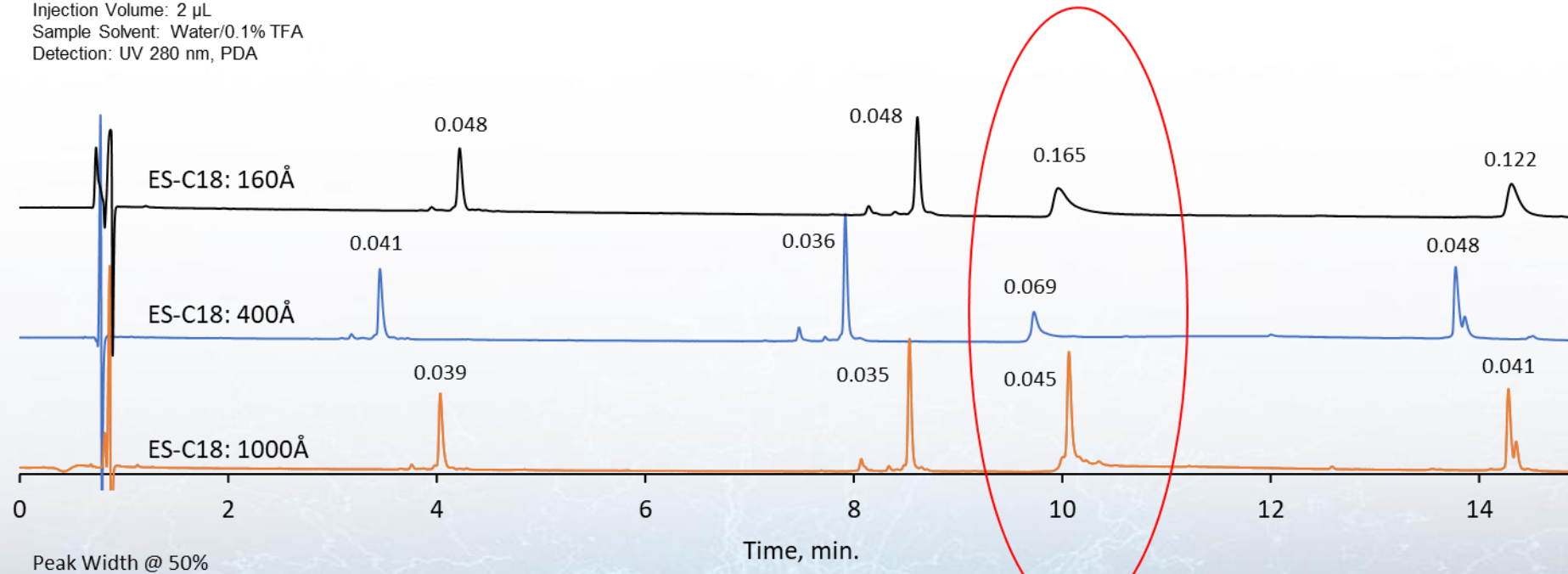
Why Pore Size Matters

TEST CONDITIONS:

Column: HALO 1000Å ES-C18, 2.7 μ m, 2.1 x 150 mm
Part Number: 92712-702
A= Water, 0.1% TFA
B= 80/20 ACN/ Water, 0.085% TFA
Gradient:
Time (min.) %B
0.00 27
15.00 60
Flow Rate: 0.4 mL/min.
Pressure: 268 bar
Temperature: 60 °C
Injection Volume: 2 μ L
Sample Solvent: Water/0.1% TFA
Detection: UV 280 nm, PDA

PEAK IDENTITIES:

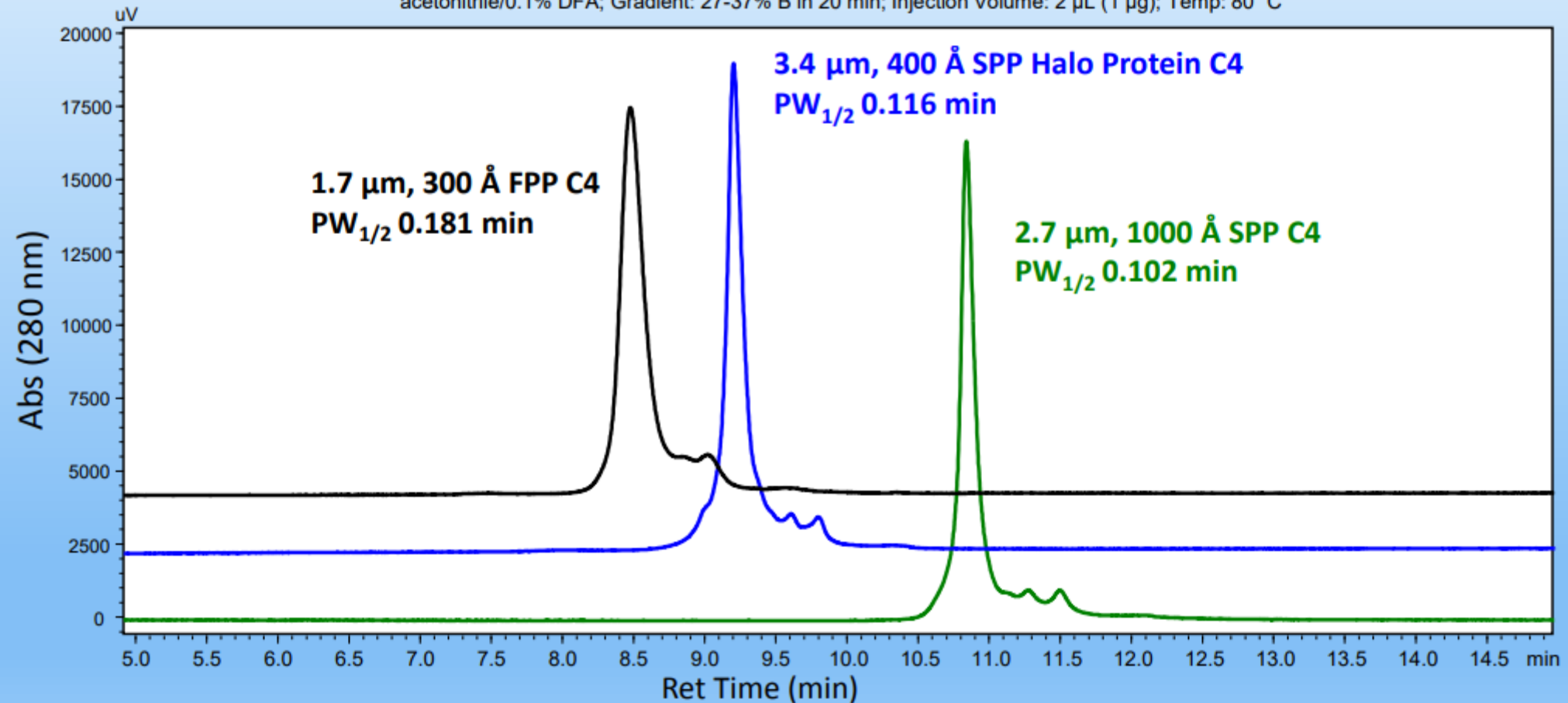
1. Ribonuclease A 13.7 kDa
2. Lysozyme 14.3 kDa
3. SigmaMAb ~150 kDa
4. Enolase 46 kDa monomer



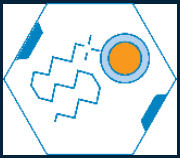
mAb IgG Separation on Wide Pore SPP vs FPP

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



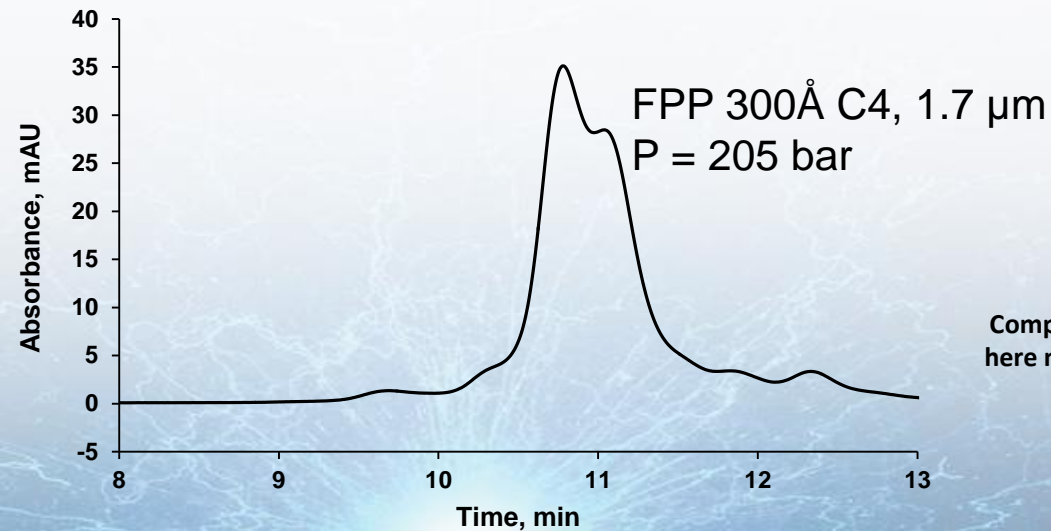
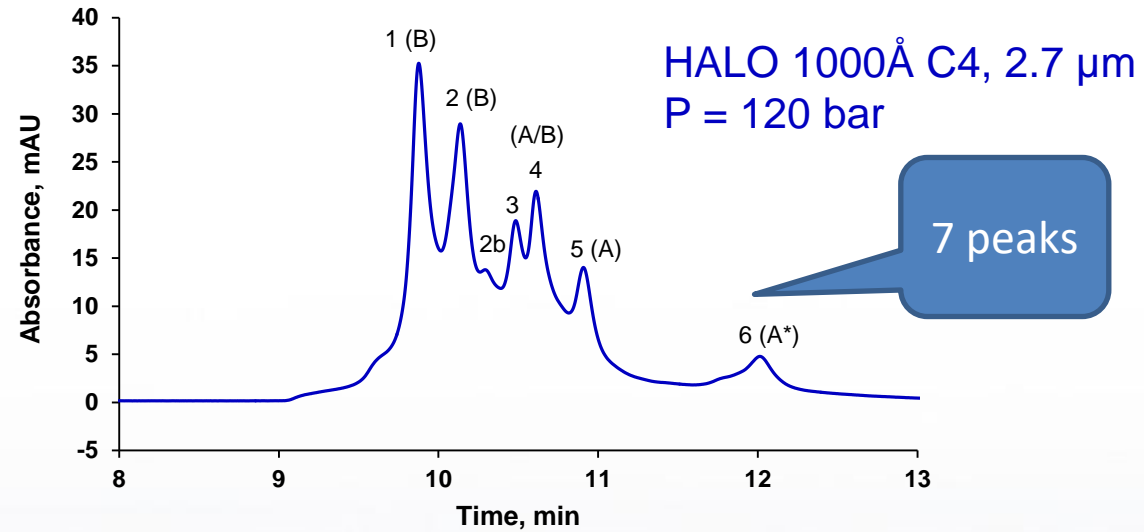
- Large improvement in Peak Width and *increased* Retention with Larger Pore SPP, moderate additional improvement in Peak Width with Larger Pores



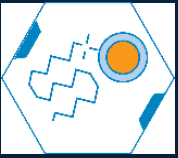
Improved Resolution of IgG2 Variants

Columns: 2.1 x 150 mm; Flow rate: 0.2 mL/min; Mobile Phase A: 88/10/2 H₂O/ACN/n-Propanol + 0.1% DFA; Mobile Phase B: 70/20/10 n-Propanol/ACN/H₂O + 0.1% DFA; Gradient: 14-24% B in 20 min; Injection Volume: 2 μ L of 2 mg/mL denosumab in water + 0.1% DFA; Temp: 80 °C; Detection: PDA at 280 nm

Twice the peaks
Almost half the back pressure



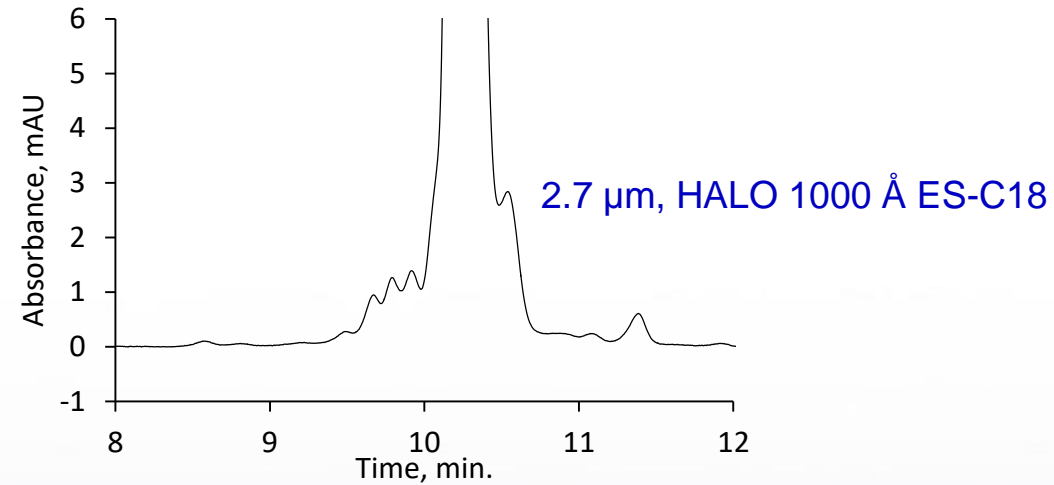
Comparative results presented here may not be representative for all applications



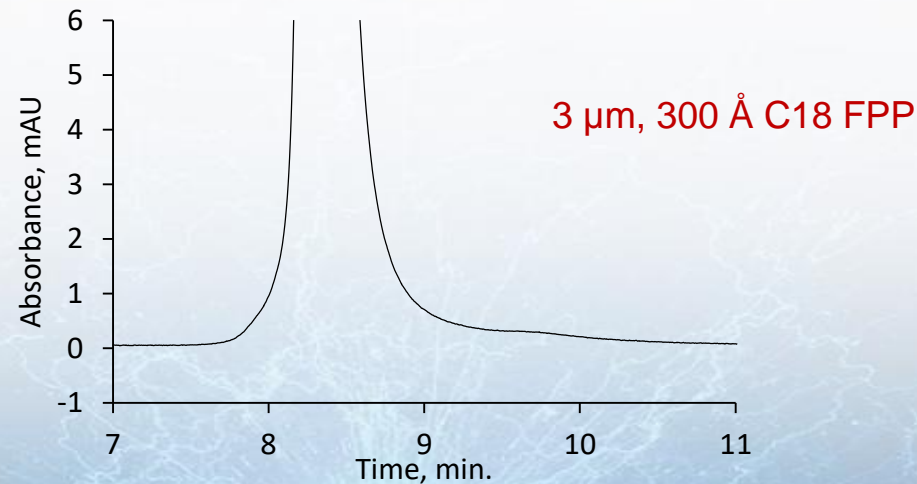
mAb Separation: 1000Å Fused-Core vs. 300Å FPP

Columns: 2.1 x 150 mm; Flow rate: 0.4 mL/min; Mobile Phase A: water/0.1% TFA; Mobile Phase B: ACN/0.1% TFA; Gradient: 36-44% B in 16 min; Injection Volume: 2 µL of 2 mg/mL NISTmAb in water/0.1% TFA; Temp: 60 °C; Detection: PDA at 280 nm

Enhanced resolution with the 1000Å for NISTmAb



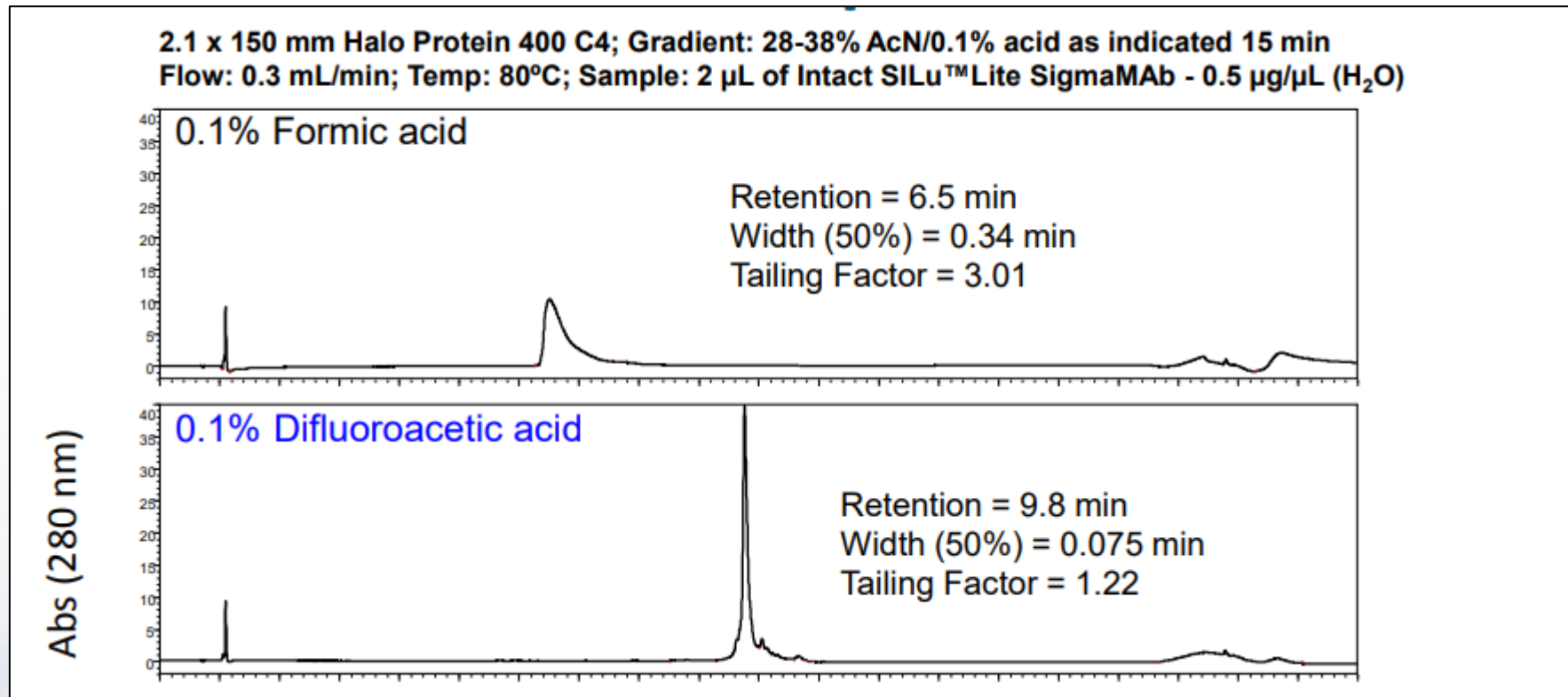
The NISTmAb material is a recombinant humanized IgG1k expressed in murine suspension culture. It is an »150 kDa homodimer of two identical light chains and two identical heavy chains linked through both inter- and intra-chain disulfide bonds.



Comparative results presented here may not be representative for all applications

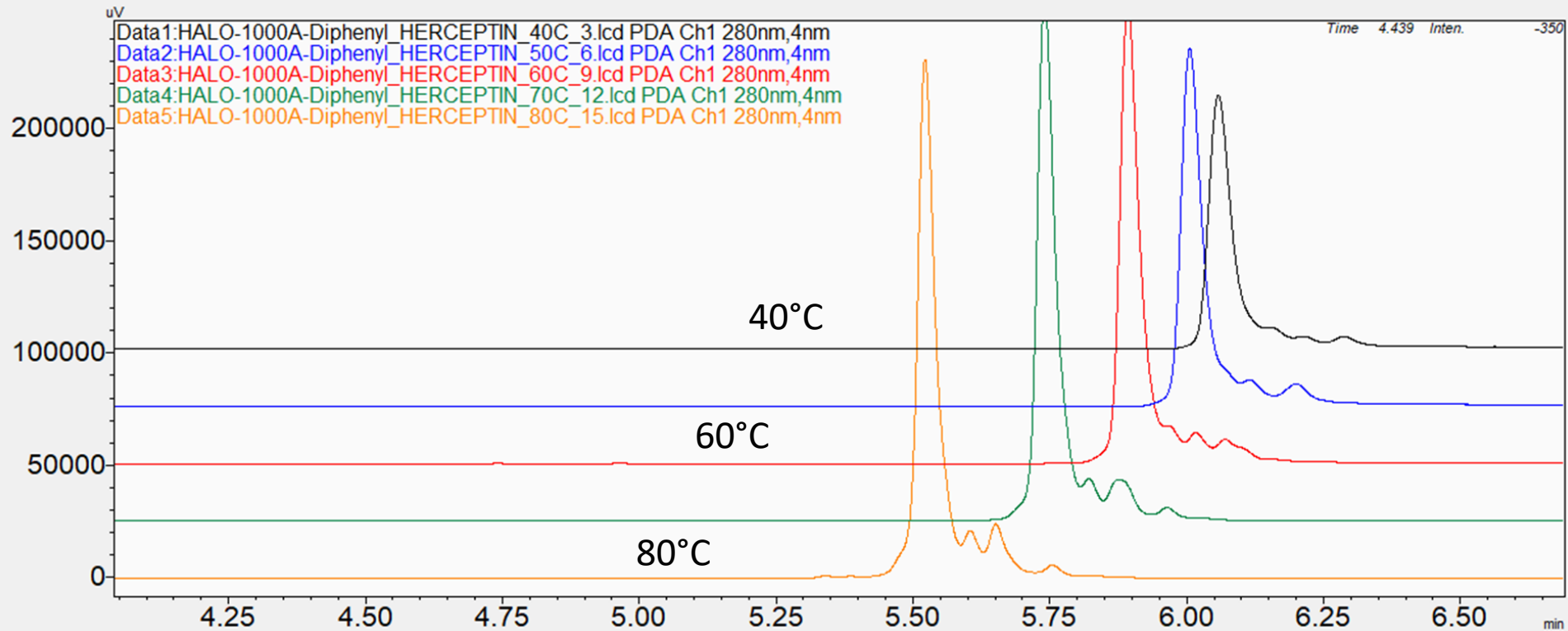


Mobile Phases for Improved mAb LC





Temperature Effects: Recovery



TEST CONDITIONS:

Column: HALO 1000Å Diphenyl, 2.7µm, 2.1x150mm
Part Number: 92712-726

A= Water, 0.1% TFA

B= Acetonitrile, 0.1% TFA

Gradient	Time (min.)	%B
	0.00	25
	8.00	45

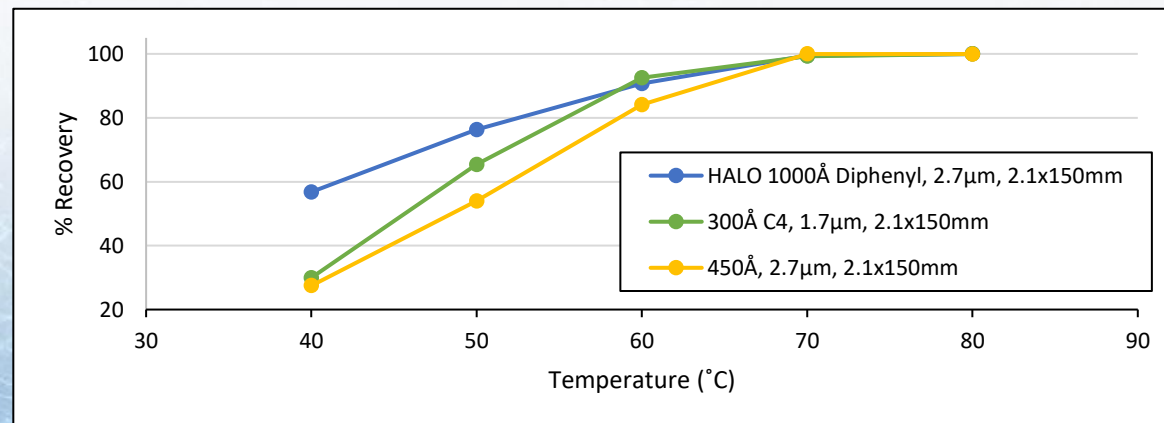
Flow Rate: 0.5 mL/min.

Temperature: 40,50,60,70,80 °C

Injection Volume: 2 µL (2mg/mL Trastuzumab)

Sample Solvent: Water

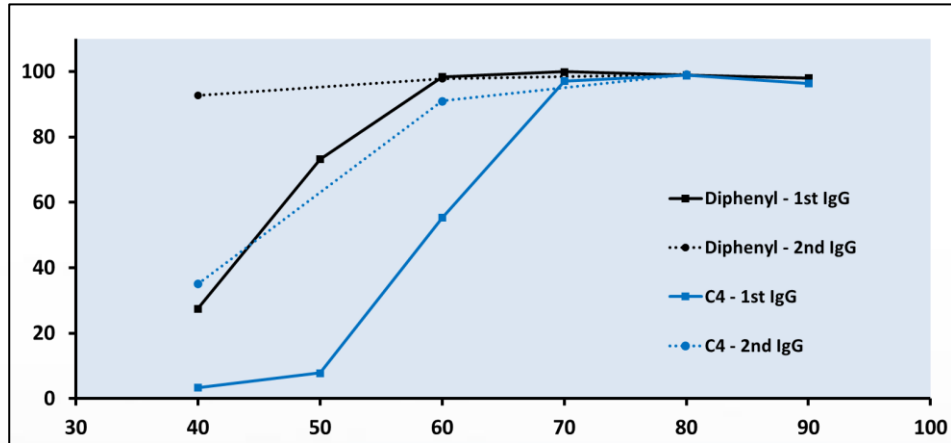
Detection: UV 280 nm, PDA



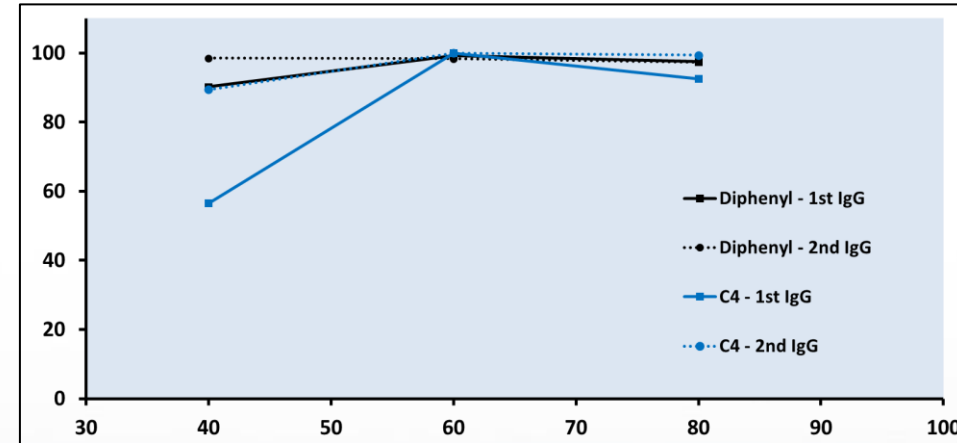


Temperature Dependence of IgG Recovery

% Recovery- ACN



% Recovery- ACN/ nProp



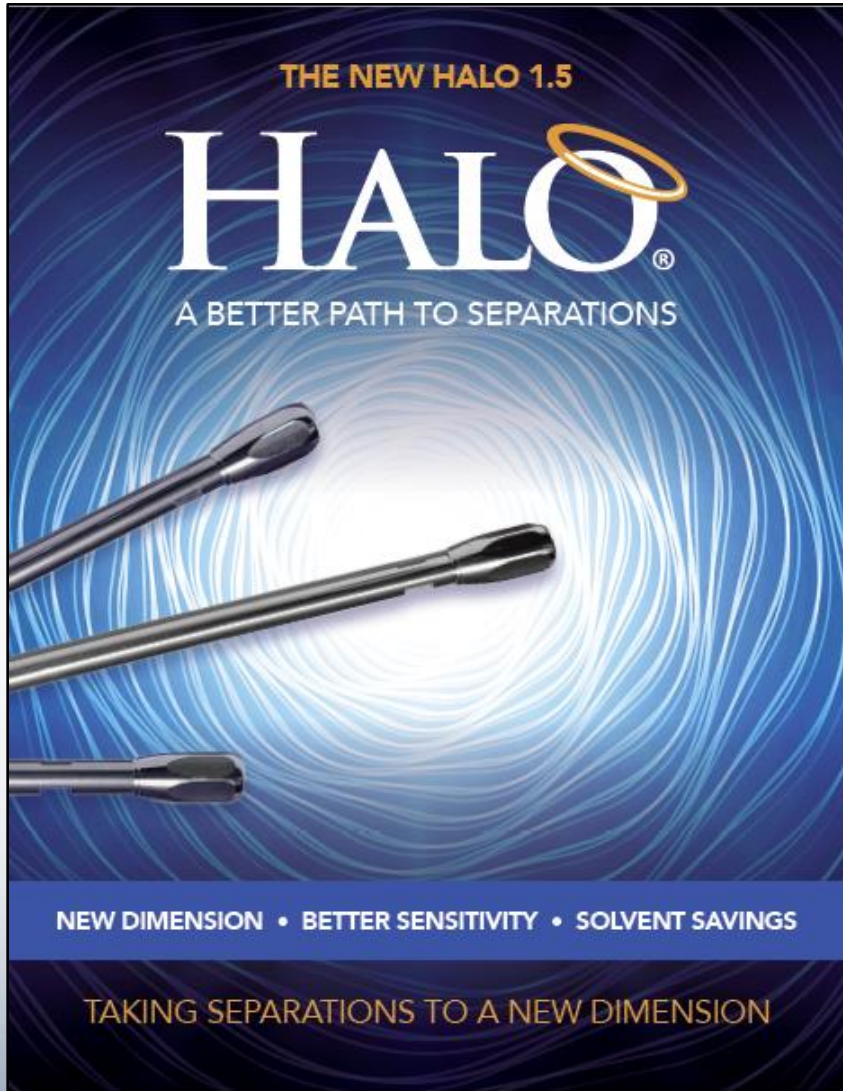
Temperature, °C

Left figure MP A – $H_2O + 0.1\% TFA$, MP B – $ACN + 0.1\% TFA \rightarrow 30-45\%B$ in 15min,
Right figure MP A – $H_2O + 0.1\% TFA$, MP B – $50/50 ACN/nProp + 0.1\% TFA \rightarrow 28-43\%B$ in 15min

Diphenyl increases recovery of IgG1s and IgG2 (data for denosumab not shown) at lower temperatures compared to C4 and ES-C18 (data not shown). Different IgG analytes require different temperatures for high recovery, even IgGs of the same isotope. Simple changes to the mobile phase like the addition of n-propanol can shift high recovery to lower operating temperatures than observed for ACN. NISTmAb shows an artifact lower k' peak at $T > 70^\circ C$, but high overall recovery.

A NEW DIMENSION IN SEPARATIONS

MORE PERFORMANCE FROM UHPLC AND LCMS SYSTEMS



More sensitivity from conventional UHPLC systems



Higher ionization efficiencies from LCMS systems

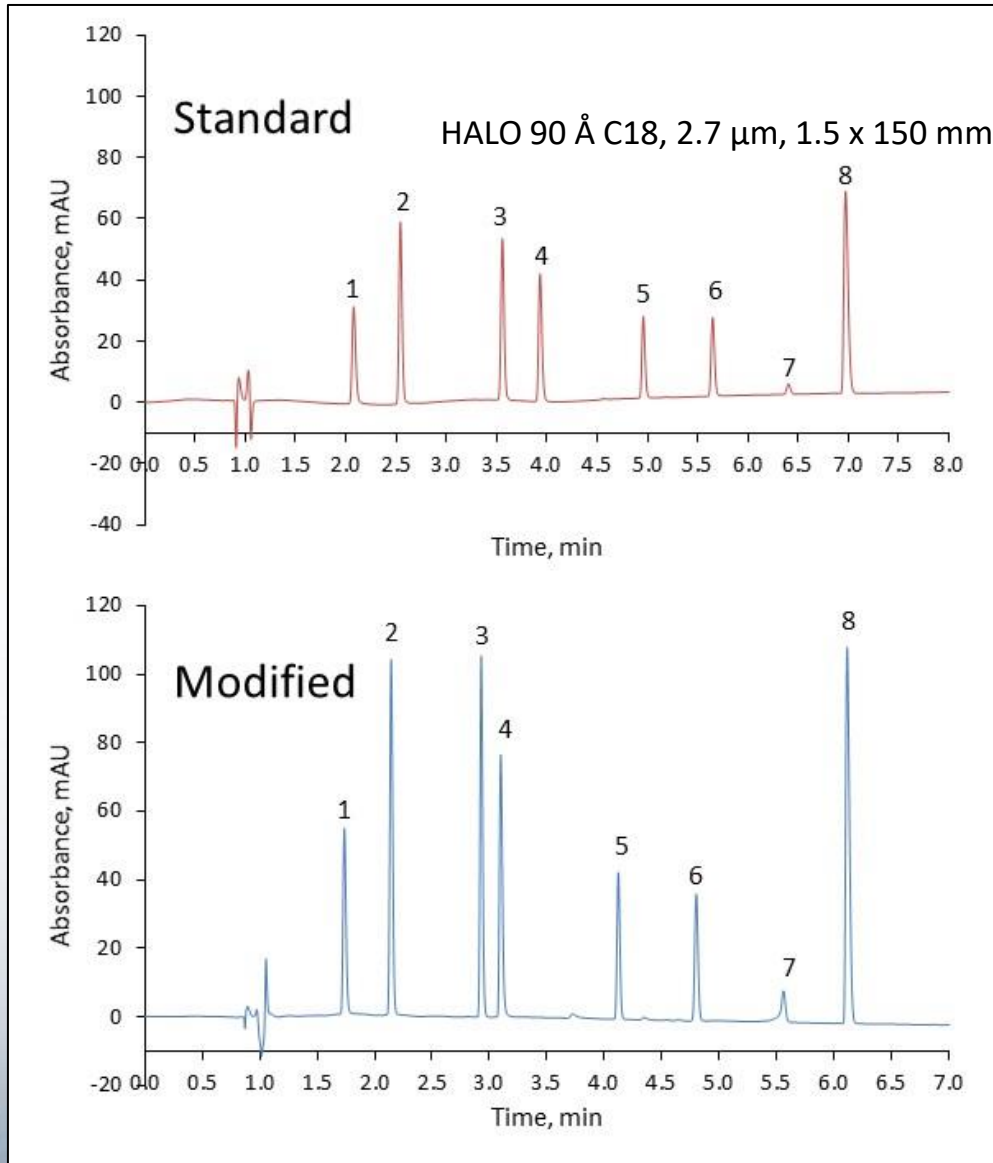


Reduced solvent consumption compared to 2.1 mm id columns (and greater)

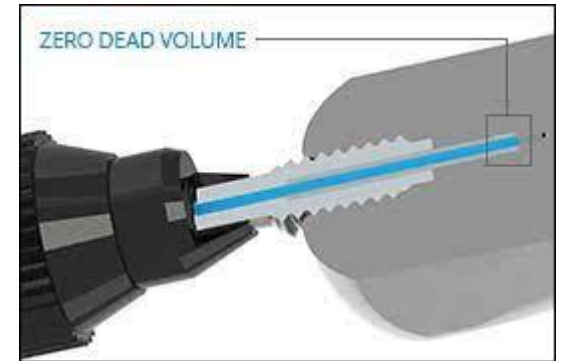


Easy to implement microflow solution

Q: What happens if I don't optimize?



- Peaks heights will be shorter
- Peak widths will be broader

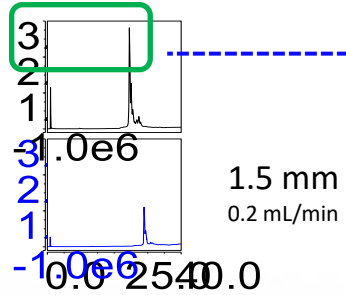


	Standard	Modified
Mixer	100 μL	20 μL
Tubing from injector to column	0.1 mm x 800 mm 6.3 μL	75 μm x 350 mm 1.5 μL
Tubing from column to Detector	0.1 mm x 509 mm 4 μL	60 μm x 707 mm 2 μL
Flow cell (μL)	1	1
Dispersion (μL ²)	14	2



Higher ionization efficiencies from LCMS Systems

Intact Trastuzumab using HALO 1000 Å Diphenyl

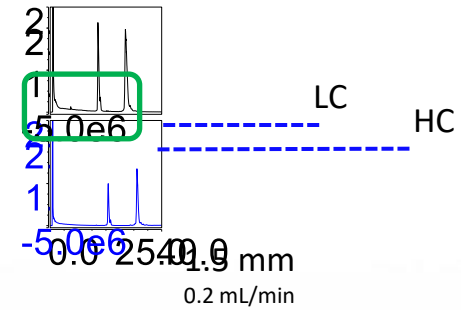


Area is 2.9x larger and peak height is increased

2.1 mm
0.4 mL/min



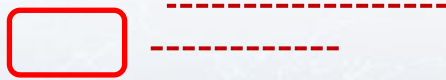
Reduced and Alkylated Trastuzumab using HALO 1000 Å Diphenyl



Heavy Chain (HC)
Area is 2.3x larger

Light Chain (LC)
Area is 2.7x larger

2.1 mm
0.4 mL/min



TEST CONDITIONS
 Column: HALO 1000 Å Diphenyl, 2.7 µm, 1.5 x 150 mm
 Column: HALO 1000 Å Diphenyl, 2.7 µm, 2.1 x 150 mm
 Mobile Phase A: Water/0.1% DFA
 Mobile Phase B: 50% Acetonitrile/50% n-Propanol/0.1% DFA
 Gradient: 27-36 %B in 40 min
 Flow Rate: 0.2 mL/min for 1.5 mm ID, 0.4 mL/min for 2.1 mm ID
 Back Pressure: 252 bar (1.5 mm), 272 bar (2.1 mm)
 Temperature: 60 °C
 Injection Volume: 3 µL of 1.0 mg/mL reduced and alkylated trastuzumab
 Sample Solvent: water/0.1% TFA
 LC System: Shimadzu Nexera X2
 MS System: ThermoFisher Q Exactive

- Sales, Technical and Marketing Materials:

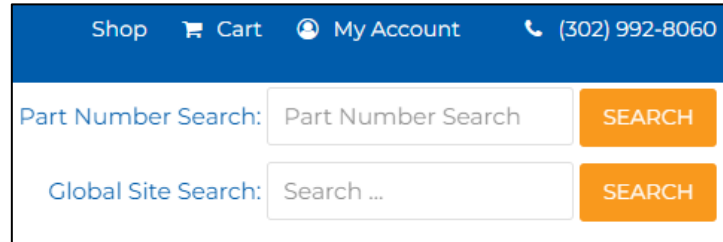
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