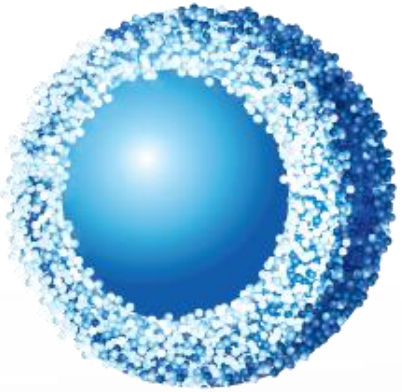
HALO®

Wide Pore Superficially Porous Particles with Various Bonded Phases for High Resolution Protein Chromatography



William Miles, Barry Boyes, Ben Libert, Stephanie Schuster, Brian Wagner, Conner McHale
Associate Scientist, R&D
Advanced Materials Technology, Inc.
Wilmington, Delaware, USA
wmiles@advanced-materials-tech.com

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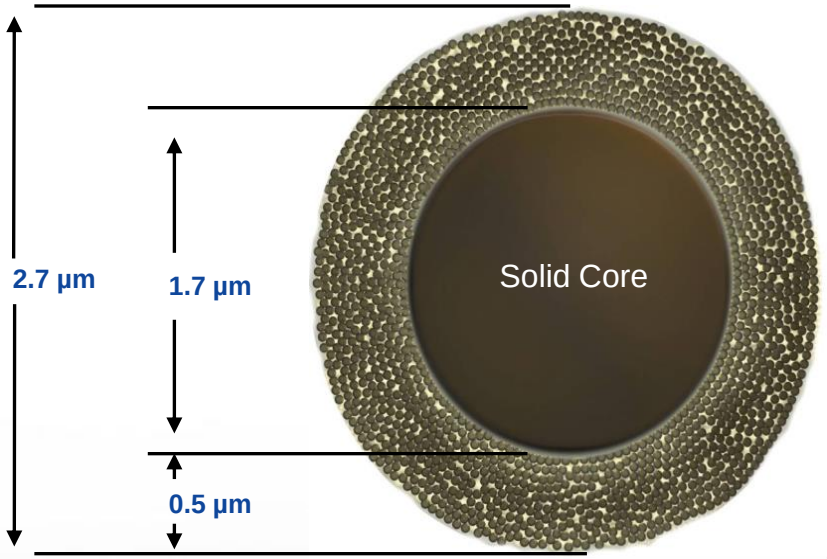
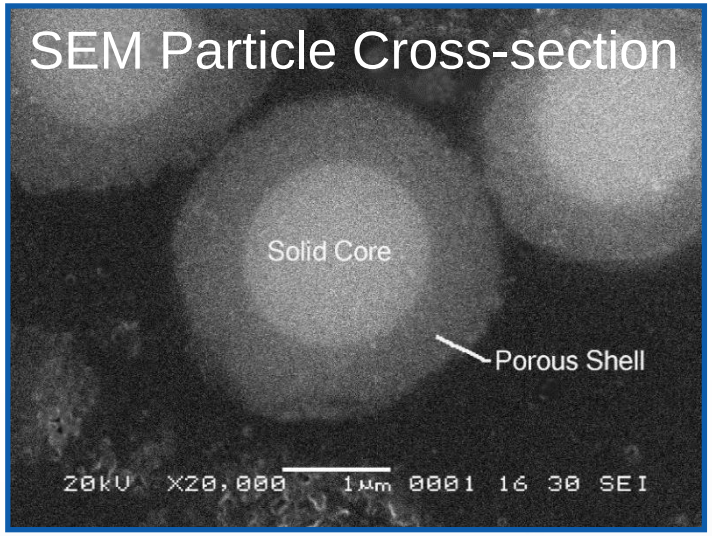
Superficially Porous Particles (90 Å): 2006



Porous Shell
90 Å

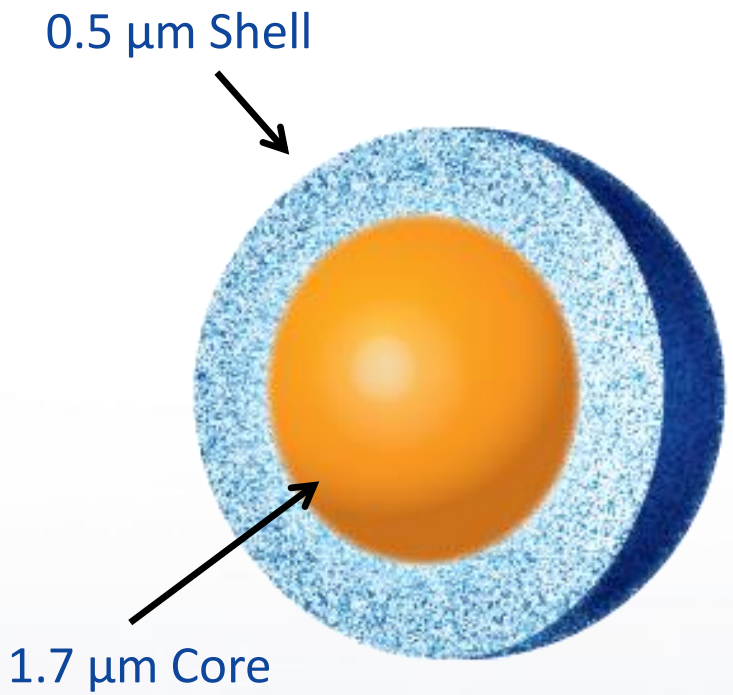


Fully Porous Particles (FPP) vs. Superficially Porous Particles (SPP)

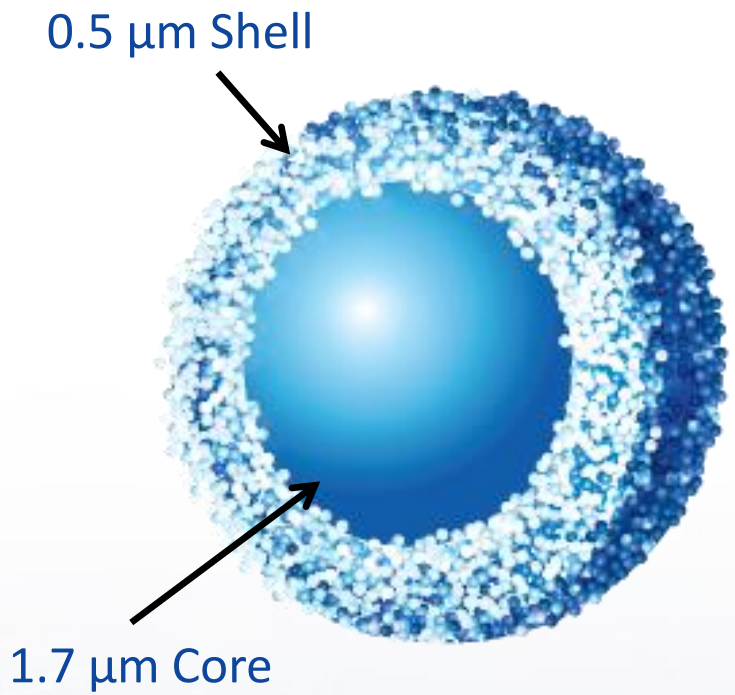
- Lower back pressure
- Higher efficiency / resolution:
  - Narrow particle size distribution
  - Improvements in mass transfer
- Maintains high resolution at high flow rates, flat C-term in van Deemter plots

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

Developments in HALO® Fused-Core® Pore Size



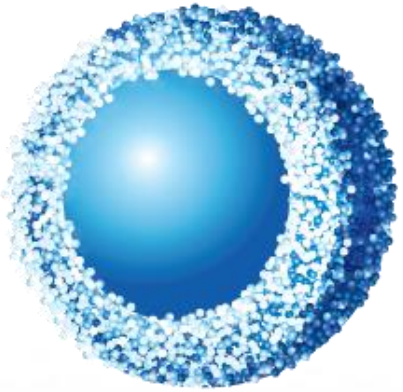
90 Å, 2.7 µm
135 m^2/g
2006



1000 Å, 2.7 µm
22 m^2/g
2017

Wide Pore SPP Benefits Protein Science

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



HALO
1000 Å, 2.7µm

- **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**

Experimental: HPLC Methods

Reversed-Phase Liquid Chromatography (RPLC) separations of proteins and mAbs

Standard Protein RPLC methods

- Gradient elution *30-45% ACN in 15 minutes*
- Strong Solvent *acetonitrile (ACN), n-propanol (nProp*)
- Weak Solvent *(H_2O)*
- Ion Pair Reagent *trifluoroacetic acid (TFA) or difluoroacetic acid (DFA)*
- Columns *2.1x150mm*
- Flow Rate *0.2-0.6 mL/min with 2.1mm ID*
- High column temperature *60-90°C*
- 280nm UV absorbance

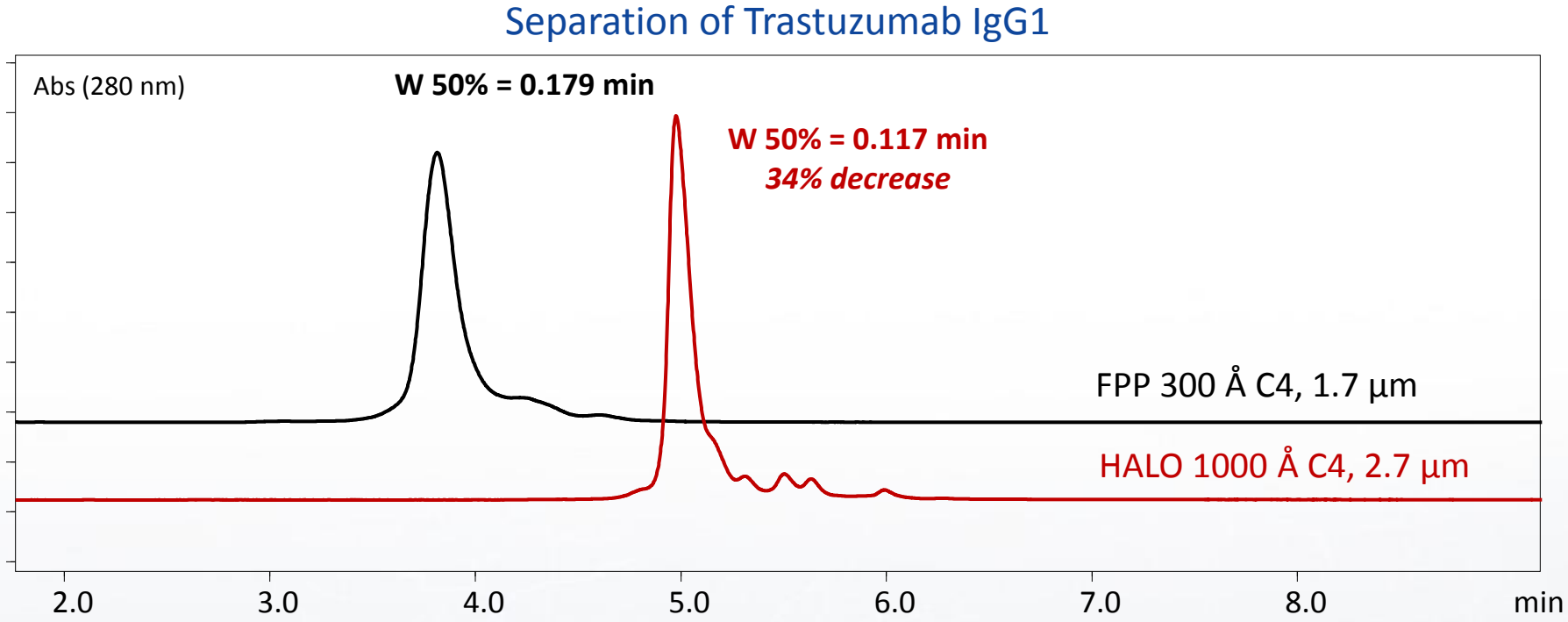
IgG1 Separation on HALO 1000 Å vs FPP 300 Å

Peak Width

- Larger improvement for HALO 1000 Å

Retention

- Increase for HALO 1000 Å



MP A – aq. 0.1% TFA; MP B – ACN + 0.1% TFA; 34-42% B in 16 min
Flow: 0.4 mL/min; Temp: 60°C; Inj.Vol: 2 µL (4 ug)

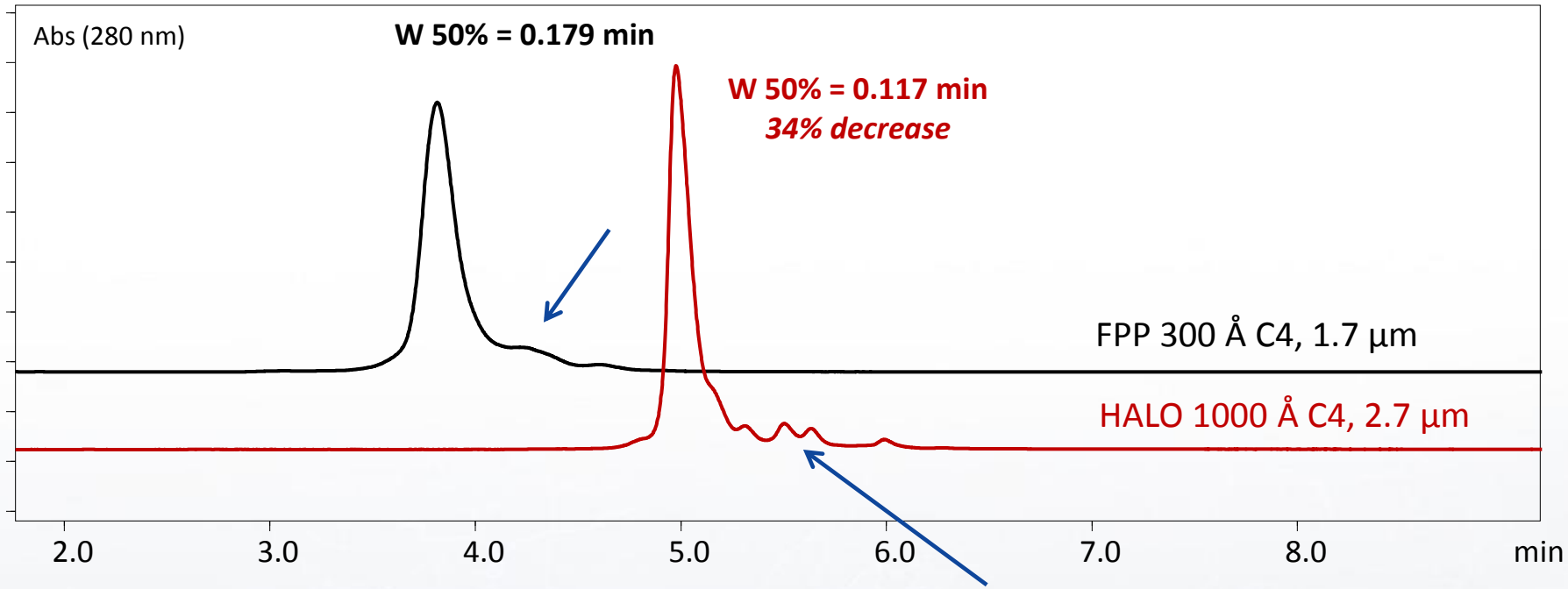
IgG1 Separation on HALO 1000 Å vs FPP 300 Å

Peak Width
- Larger improvement for HALO 1000 Å

Retention
- Increase for HALO 1000 Å

Resolution
- Increase in R_s for later eluting minor variant peaks on HALO 1000 Å

Separation of Trastuzumab IgG1



Resolution improvement for variant peaks

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Flow: 0.4 mL/min; Temp: 60°C; Inj.Vol: 2 µL (4 ug)

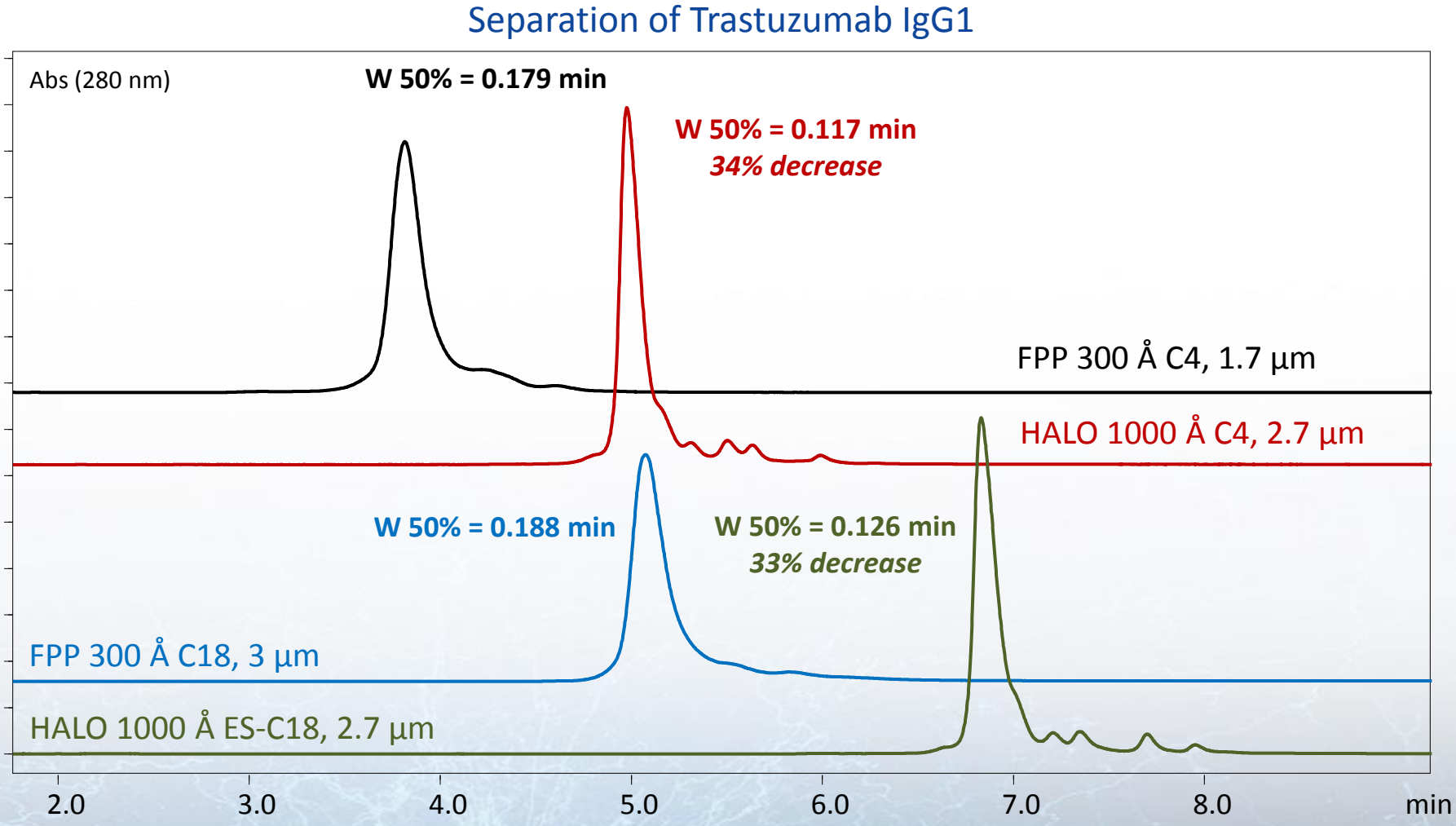
IgG1 Separation on HALO 1000 Å vs FPP 300 Å

Peak Width
- > 30% decrease in width for both 1000 Å phases

Retention
- Increase for HALO 1000 Å

Resolution
- Increase in R_s for later eluting minor variant peaks on HALO 1000 Å

Improvements seen on both C4 and ES-C18 bonded-phase comparisons



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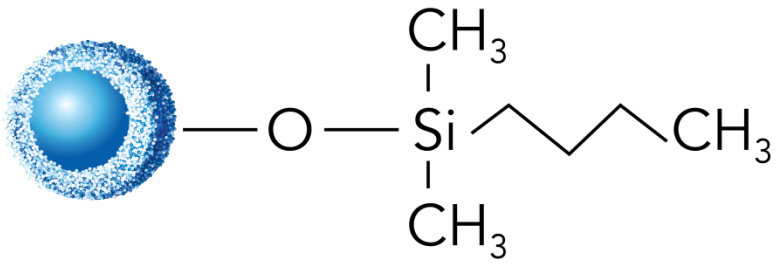
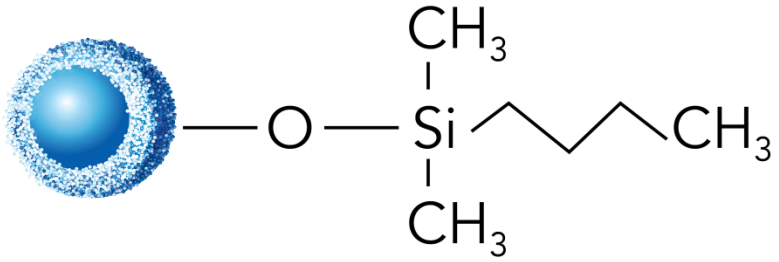
HALO 1000 Å Bonded-Phases



C4

- Traditional alkyl phase for protein separations
- 4.1 µmol/m^2 (0.65% C)

HALO 1000 Å Bonded-Phases



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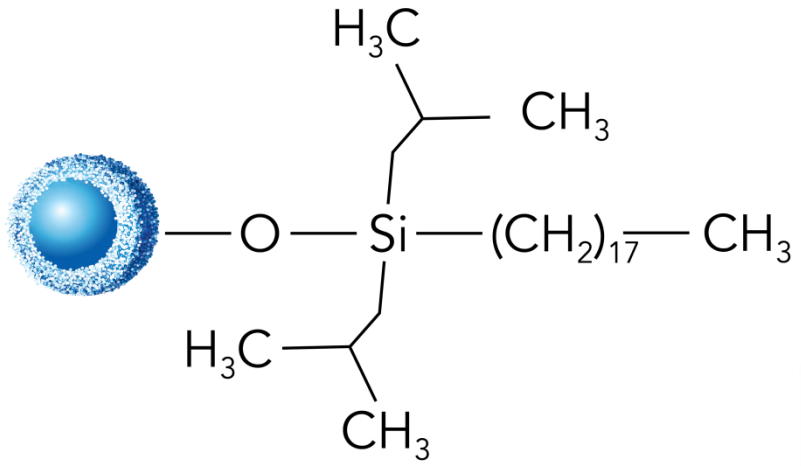
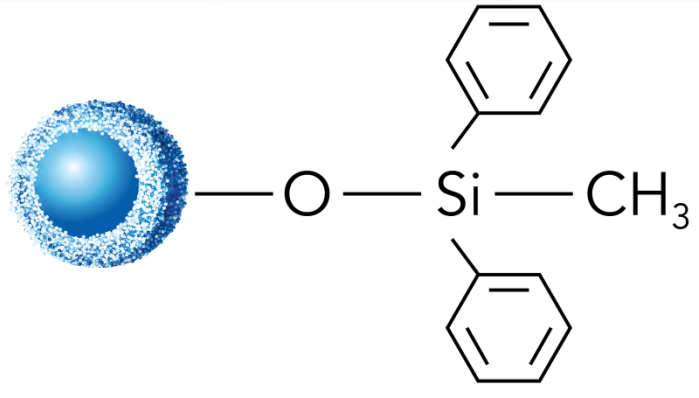


ES-C18

- Sterically-protected, long chain alkyl phase
- 1.9 µmol/m^2 (1.4% C)



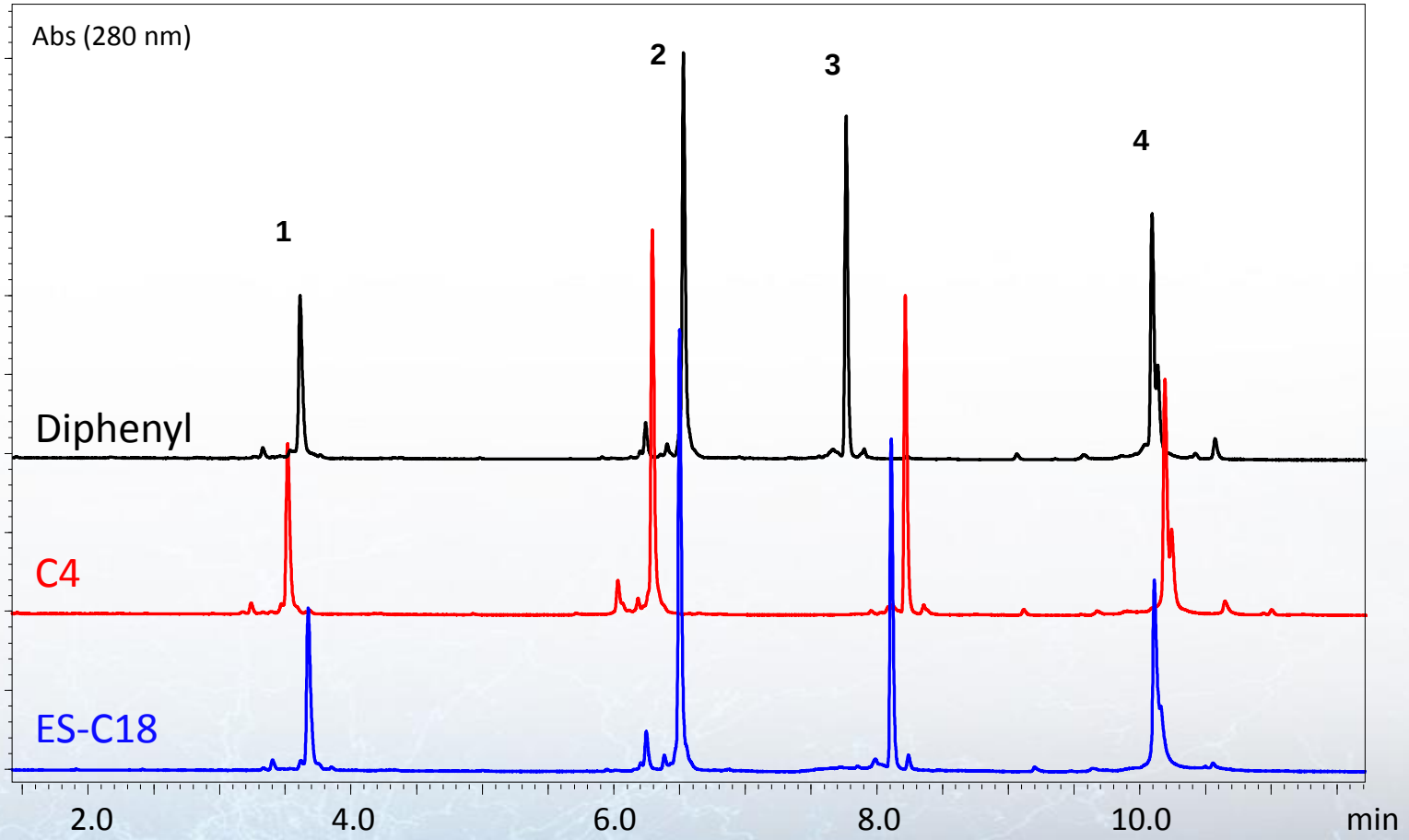
Diphenyl

- Aromatic phenyl phase
- Selectivity differs from alkyl phases
- 2.7 µmol/m^2 (1.0% C)

Effect of Bonded-Phase on Retention/Selectivity

- Hydrophobic, Small Molecule Retention
  - Diphenyl < C4 << ES-C18
- Protein Retention
  - No global retention pattern
  - Differences in individual protein retention time

Separation of 4 Protein Mixture on HALO 1000 Å Bonded-Phases



1. RNase A – 13.7 kDa
2. Lysozyme – 14.3 kDa
3. α-Lactalbumin – 14.2 kDa
4. Enolase – 46.6 kDa

MP A: aq. 0.1% TFA; MP B: ACN + 0.1% TFA; Gradient: 20-60 %B in 15 min;
Injection Volume: 2 µL; Flow: 0.40mL/min; Temp: 80 °C

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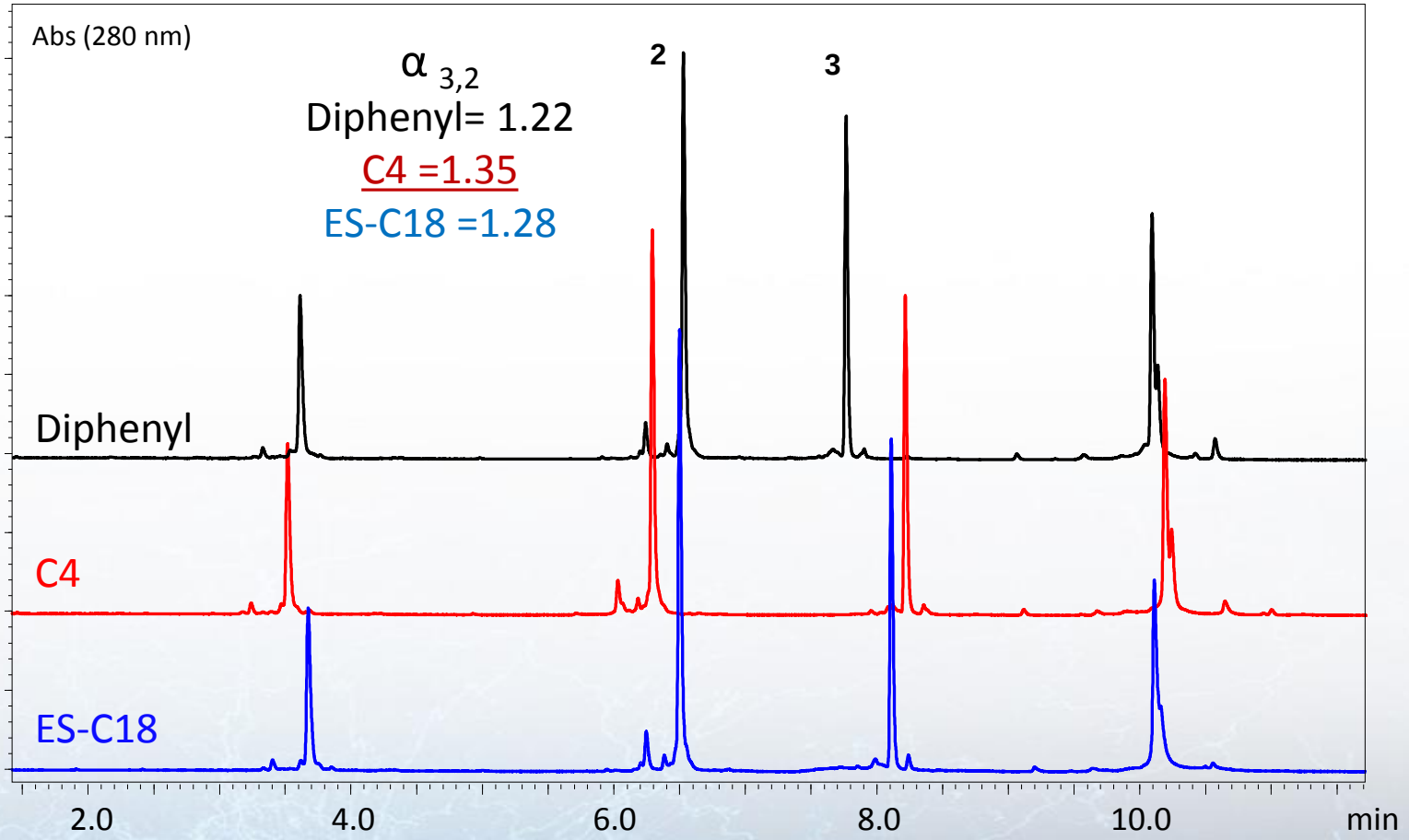
Effect of Bonded-Phase on Retention/Selectivity

- Hydrophobic, Small Molecule Retention
  - Diphenyl < C4 << ES-C18
- Protein Retention
  - No global retention pattern
  - Differences in individual protein retention time
- Protein Selectivity
  - Subtle but useful changes

Separation of 4 Protein Mixture on HALO 1000 Å Bonded-Phases



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  - Diphenyl < C4 << ES-C18
- Protein Retention
  - No global retention pattern
  - Differences in individual protein retention time
- Protein Selectivity
  - Subtle but useful changes
- Protein Peak Width 50%
  - Average width nearly identical between phases

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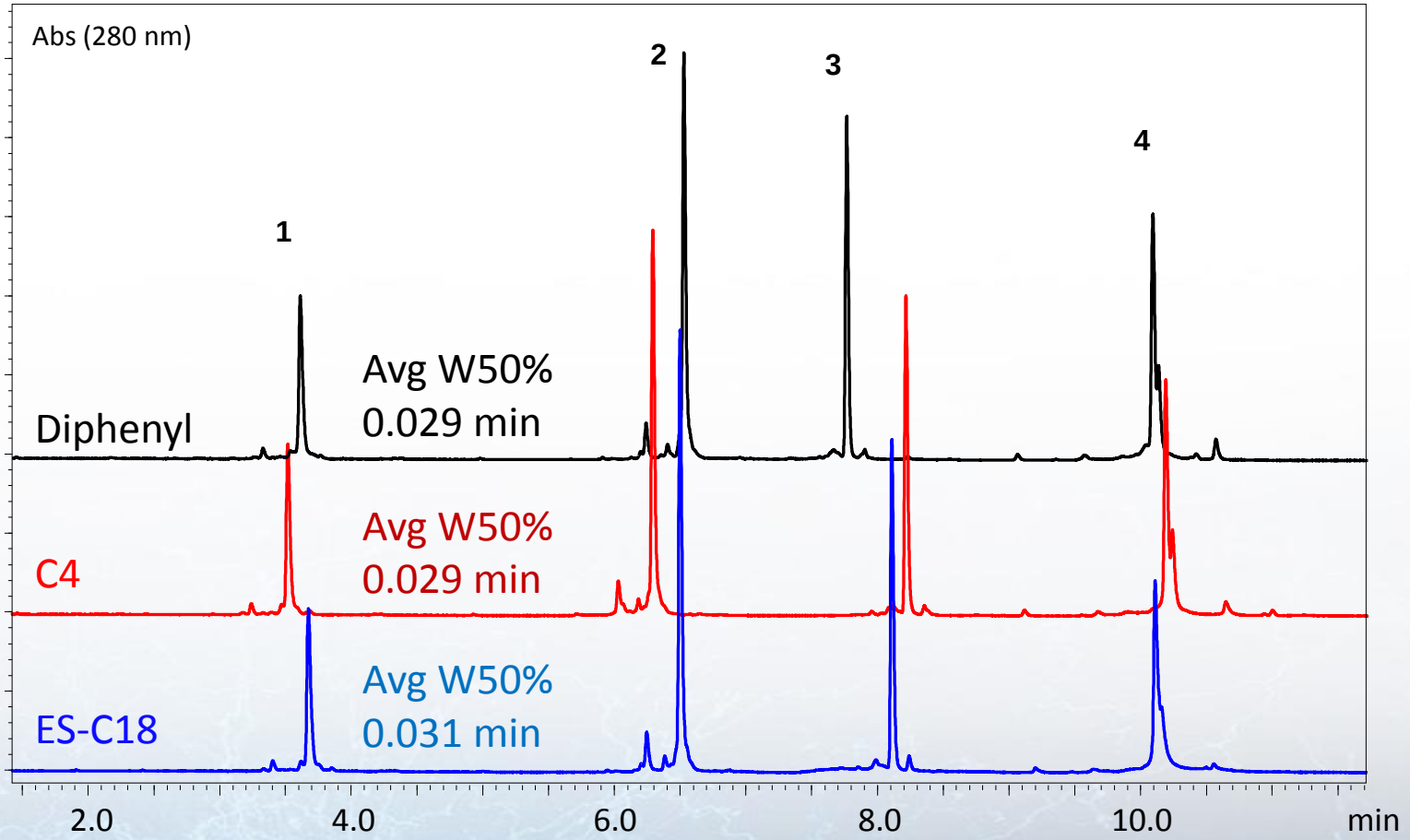
Separation of 4 Protein Mixture on HALO 1000 Å Bonded-Phases



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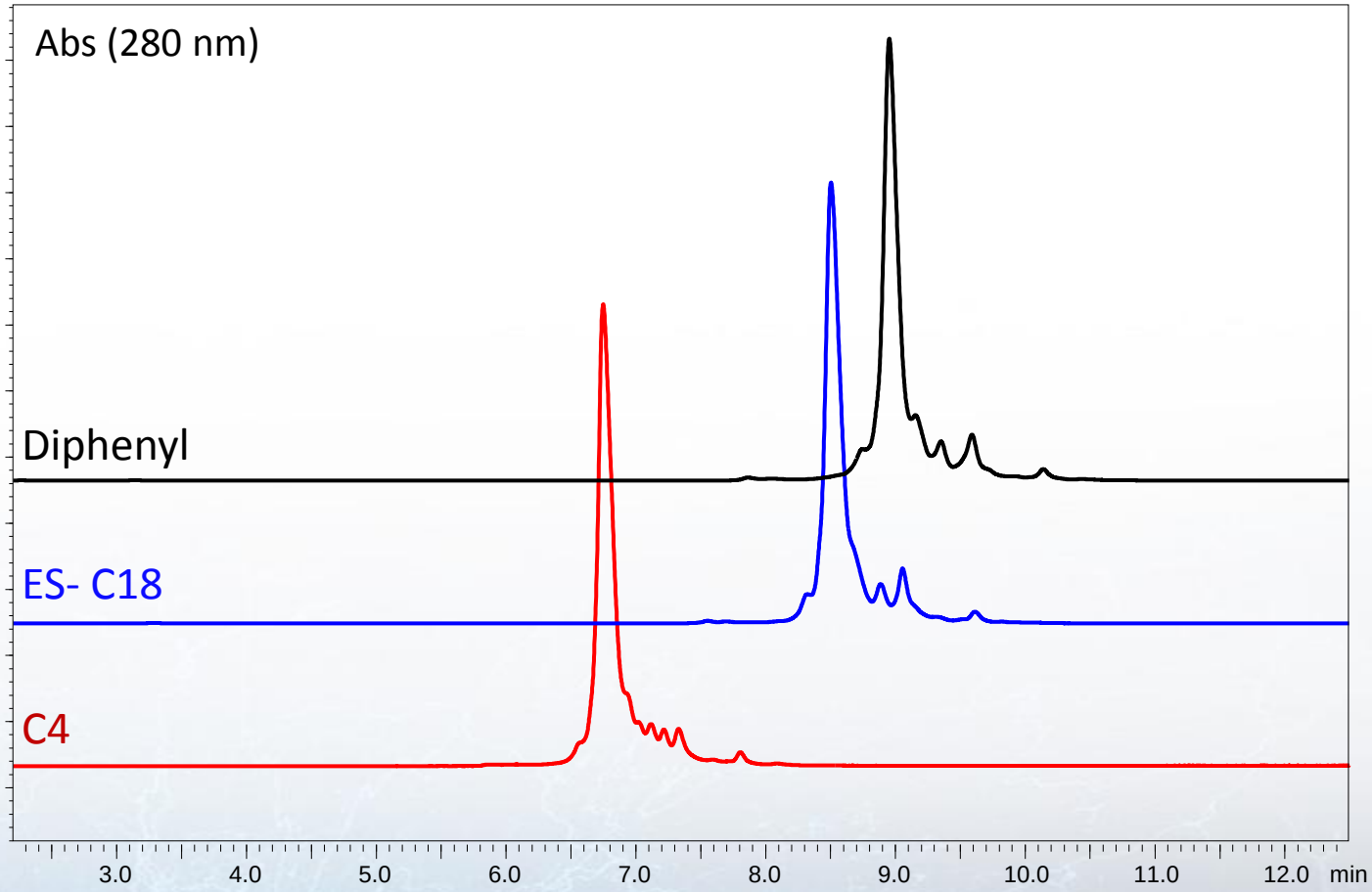
Effect of Bonded-Phase on Retention/Selectivity

- mAb Retention
  - C4 < ES-C18 < Diphenyl
  - Increased retention for ~150 kDa M.W. mAb with Diphenyl bonded-phase

Separation of Trastuzumab IgG1 on HALO 1000 Å Bonded-Phases



MP A: aq. 0.1% TFA; MP B: ACN + 0.1% TFA; Gradient: 32-40 %B in 16 min;
Flow 0.40mL/min; Inj Vol 2 µL; Temp: 80˚C

Effect of Bonded-Phase on Retention/Selectivity

- mAb Retention
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  - Differences observed with later eluting variant peaks

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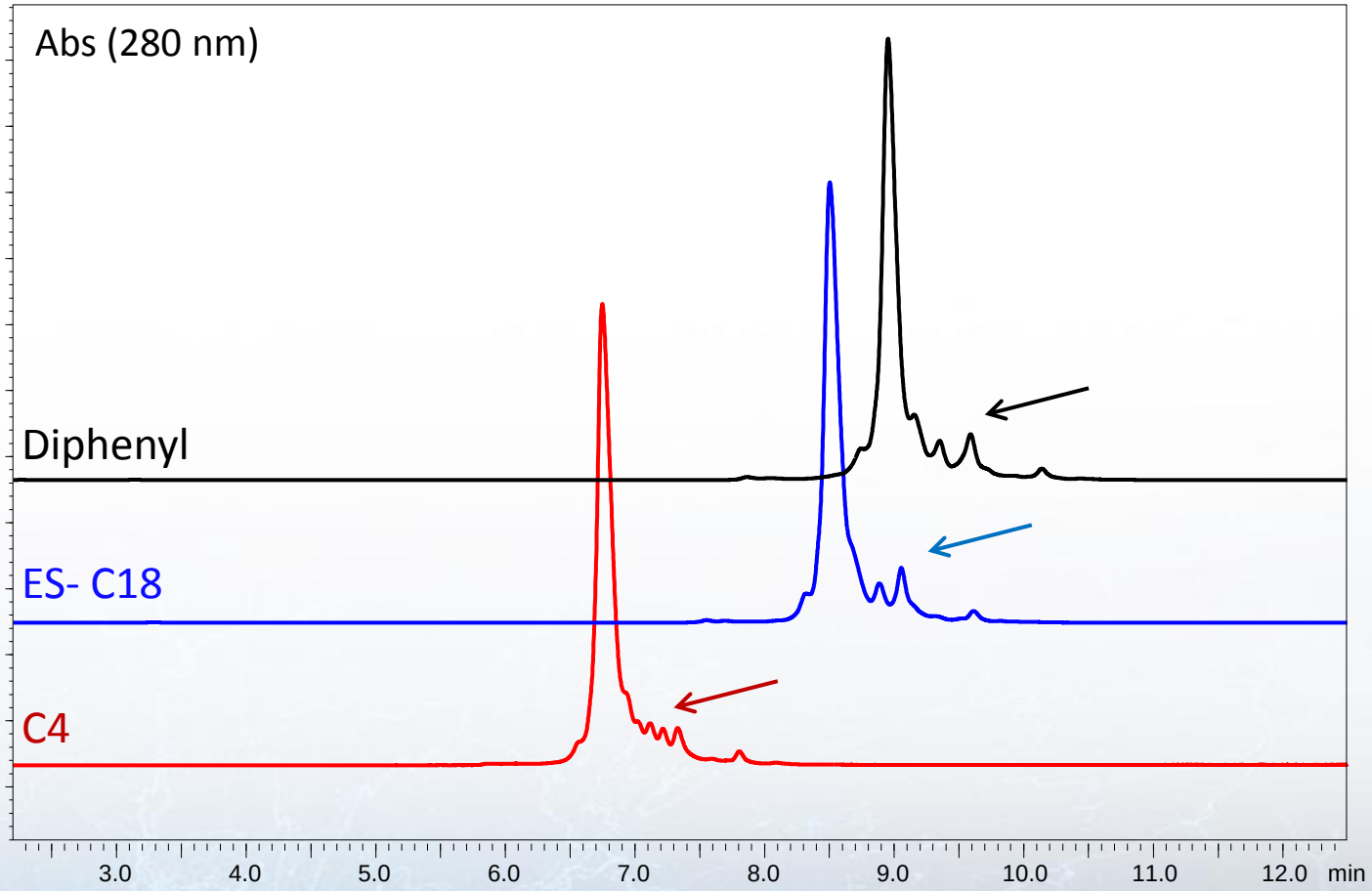

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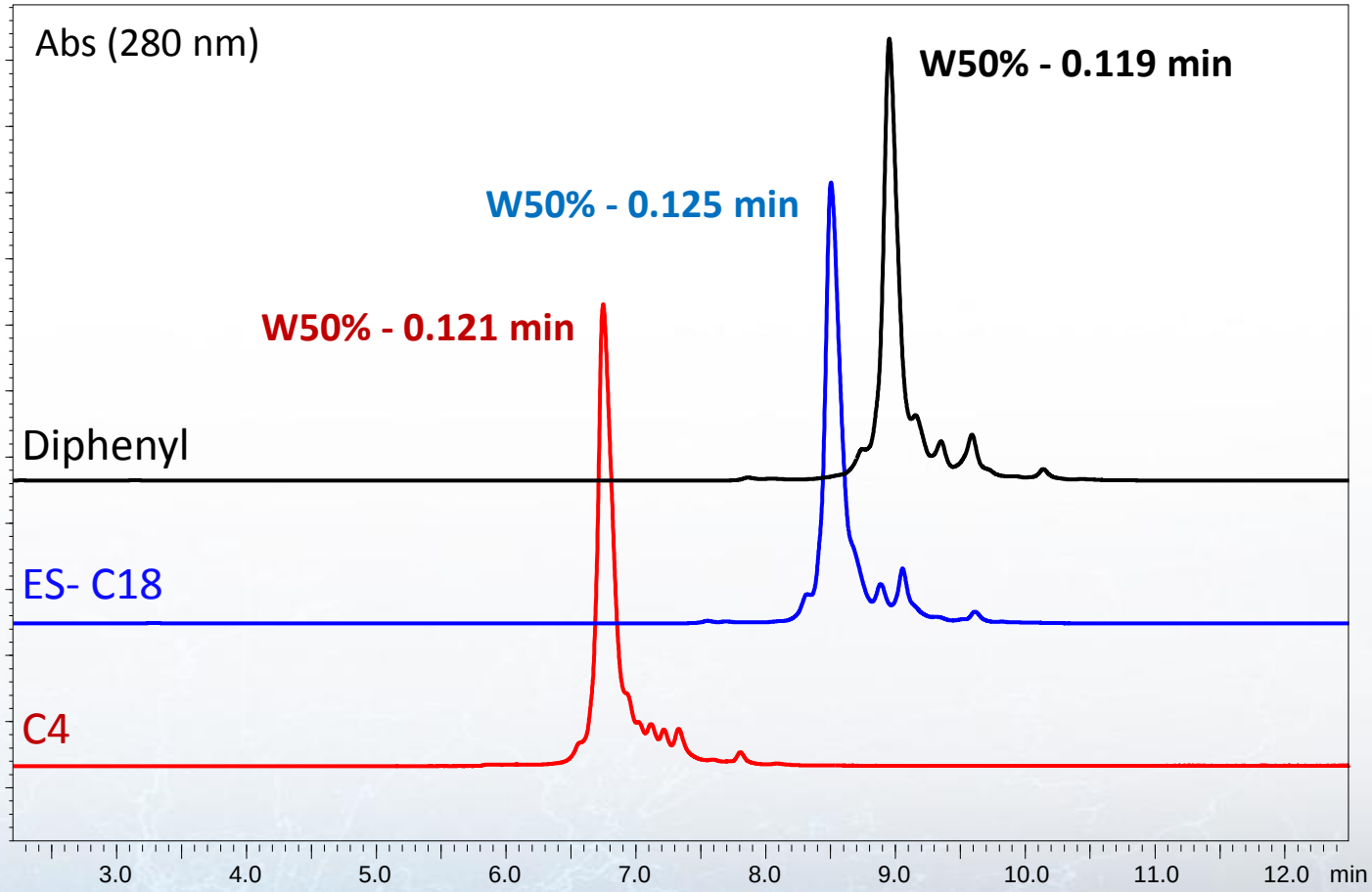
Effect of Bonded-Phase on Retention/Selectivity

- mAb Retention
  - C4 < ES-C18 < Diphenyl
  - Increased retention for ~150 kDa M.W. mAb with Diphenyl bonded-phase
- mAb Selectivity
  - Differences observed with later eluting variant peaks
- mAb Peak Width 50%
  - Nearly identical for each phase

Changes in bonded-phase chemistry preserve efficiency of the separation, while providing useful changes in retention and selectivity

Separation of Trastuzumab IgG1 on HALO 1000 Å Bonded-Phases



MP A: aq. 0.1% TFA; MP B: ACN + 0.1% TFA; Gradient: 32-40 %B in 16 min;
Flow 0.40mL/min; Inj Vol 2 µL; Temp: 80˚C

Selectivity Manipulation in RPLC of mAbs

1. Bonded-Phase
   - C4, ES-C18, and Diphenyl offer changes in retention and selectivity

Easy to change between columns

2. Gradient Elution Variables
   - Temperature
   - Mobile Phase Strong Solvent
   - Ion Pairing Reagent
   - Gradient Slope and Time
   - Flow Rate

More in-depth method development

Gradient Elution Variables: IgG1 Example

Bonded-Phase
- **Diphenyl**

Temperature
- Varied

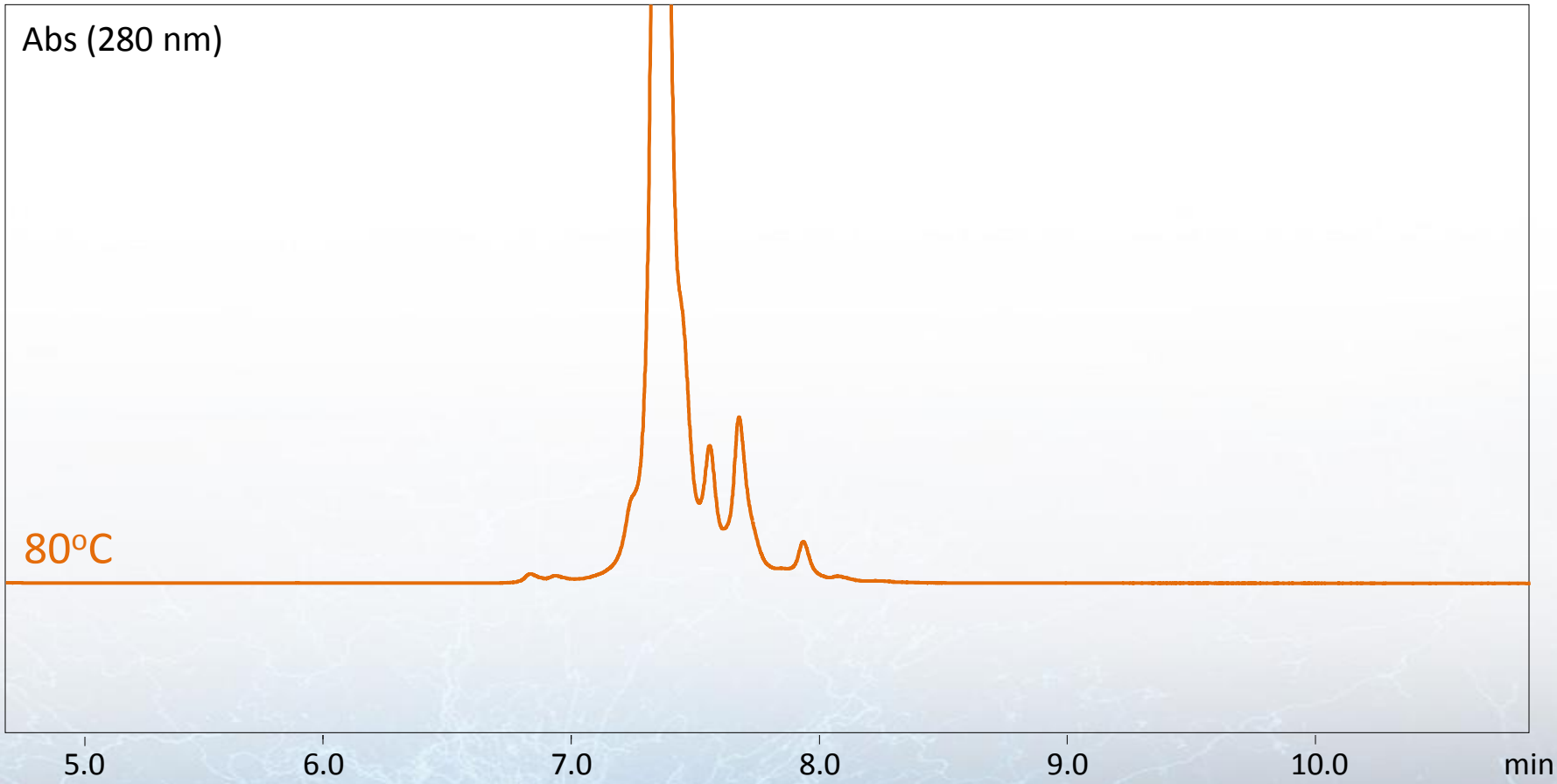
Mobile Phase Strong Solvent
- ACN

Ion Pairing Reagent
- 0.1% TFA

Gradient
- 30-45% B in 15 min

Flow
- 0.40 mL/min

Sample = trastuzumab IgG1



Separation on HALO 1000 Å Diphenyl, 2.7µm, 2.1x150mm
MP A: aq. 0.1% TFA; MP B: ACN + 0.1% TFA; Inj Vol 2 µL (4µg)

Gradient Elution Variables: IgG1 Example

Bonded-Phase
- **Diphenyl**

Temperature
- Varied

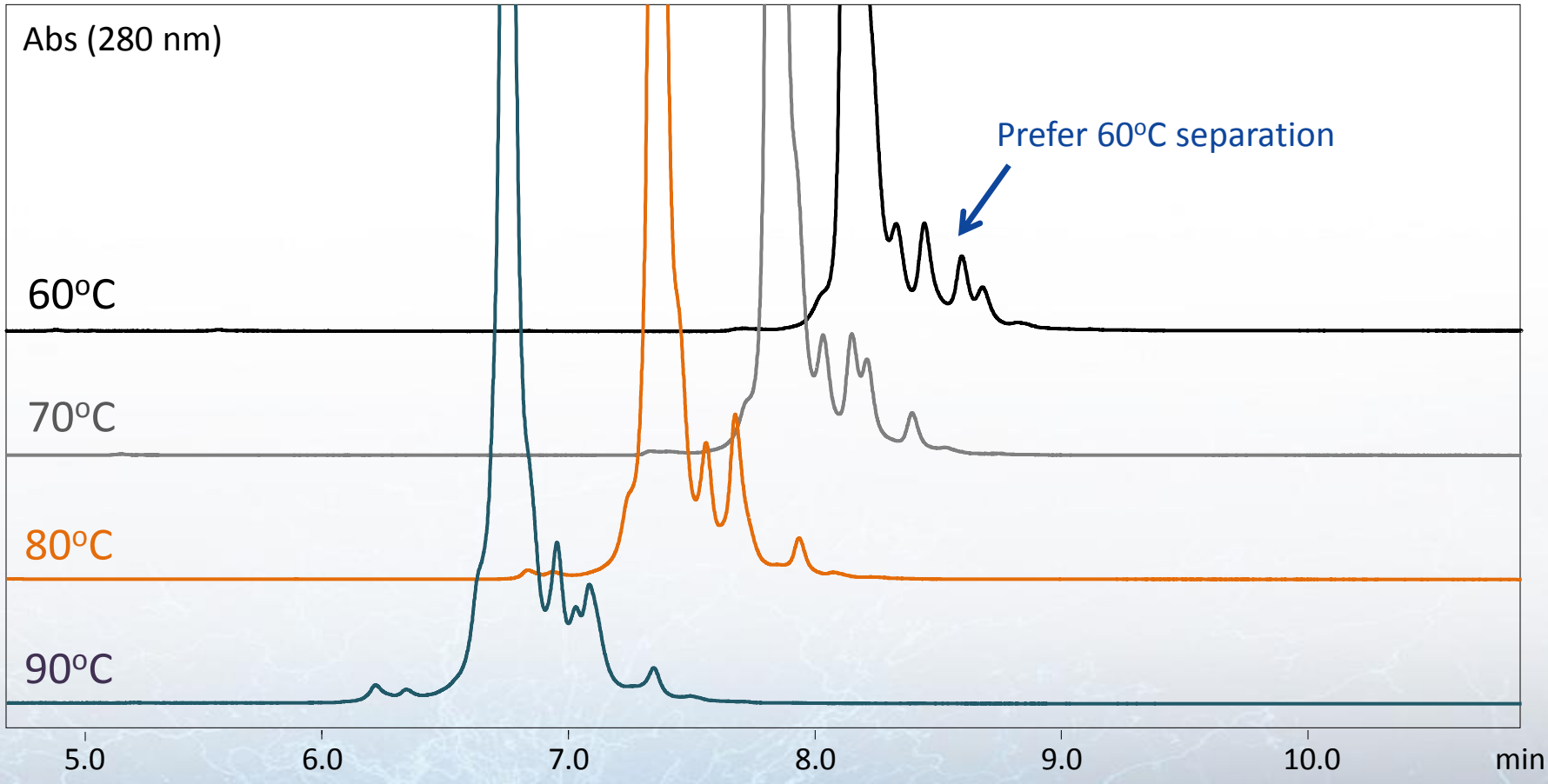
Mobile Phase Strong Solvent
- ACN

Ion Pairing Reagent
- 0.1% TFA

Gradient
- 30-45% B in 15 min

Flow
- 0.40 mL/min

Sample = trastuzumab IgG1



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MP A: aq. 0.1% TFA; MP B: ACN + 0.1% TFA; Inj Vol 2 µL (4µg)

Gradient Elution Variables: IgG2 Example

Bonded-Phase
- **C4**

Temperature
- 80°C

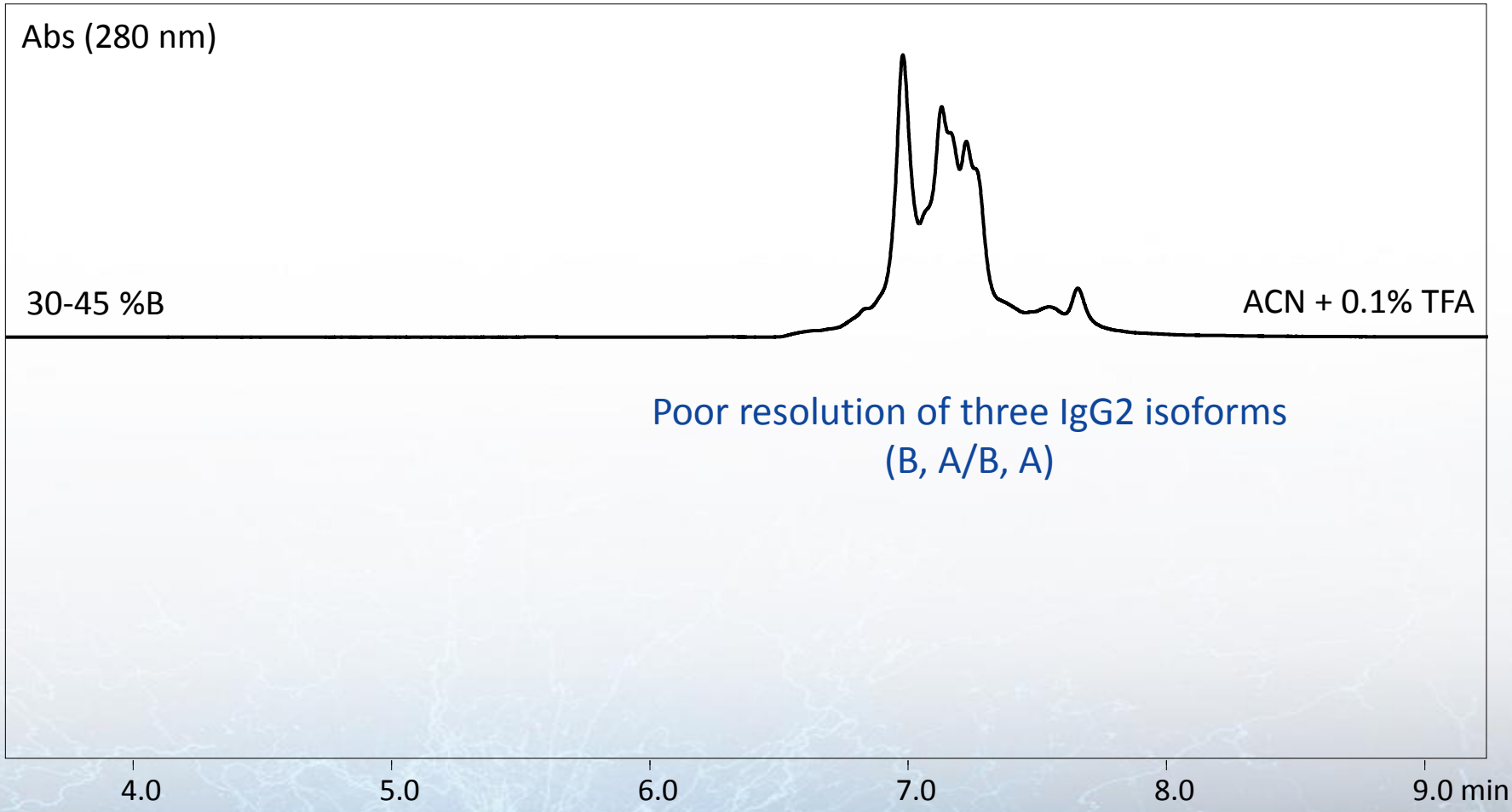
Mobile Phase Strong Solvent
- ACN

Ion Pairing Reagent
- 0.1% TFA

Gradient
- 15% B in 15 min

Flow
- 0.40 mL/min

Sample = denosumab IgG2



Separation on HALO 1000 Å C4, 2.7µm, 2.1x150mm
MP A: aq. 0.1% *specified acid*; MP B: *specified + 0.1% acid*; Gradient: 30-45%B for ACN/TFA,
Temp: 80°C; Inj Vol 2 µL (4µg)

Gradient Elution Variables: IgG2 Example

Bonded-Phase
- **C4**

Temperature
- 80°C

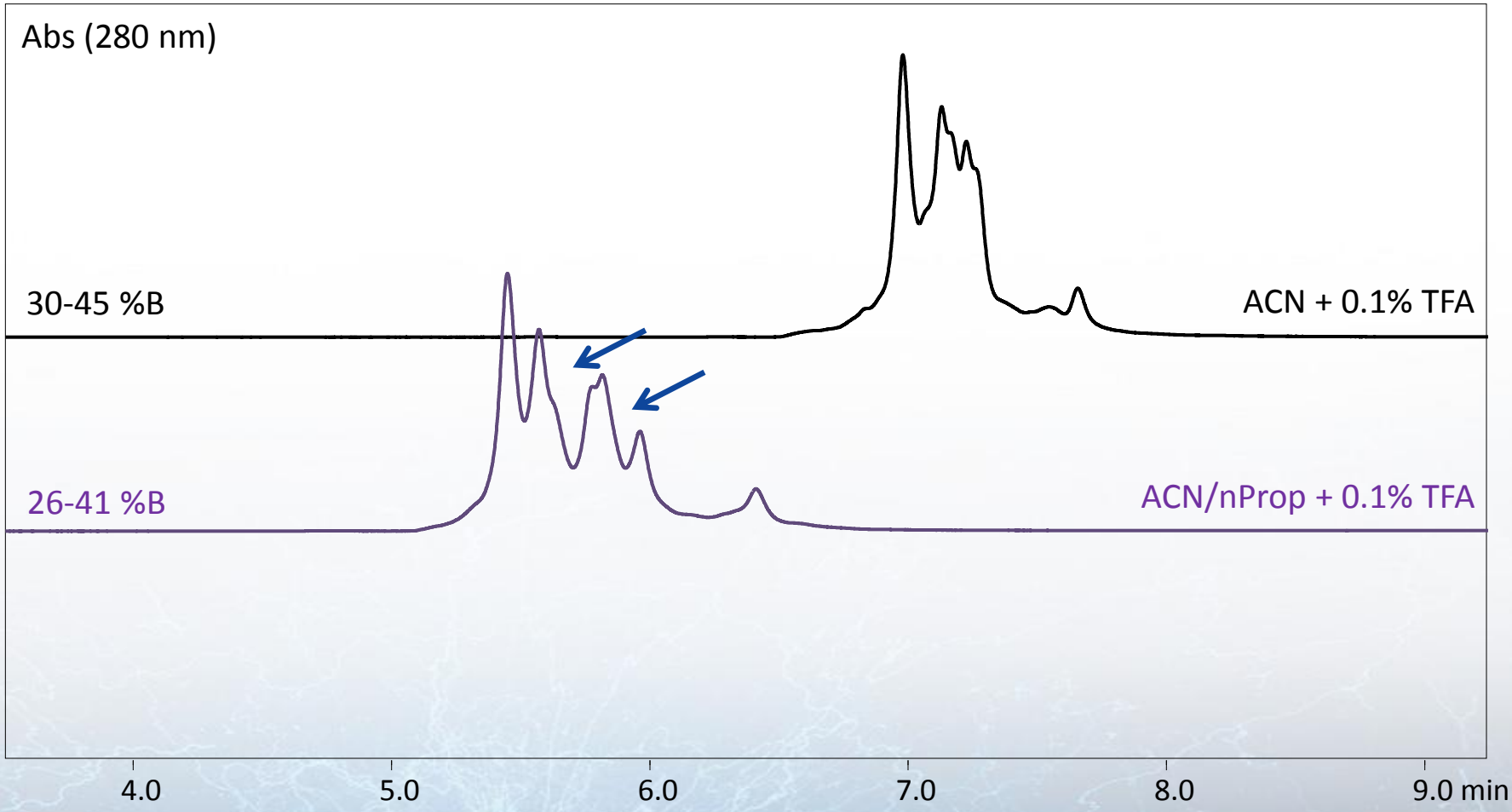
Mobile Phase Strong Solvent
- ACN
- 50/50 ACN/nProp

Ion Pairing Reagent
- 0.1% TFA

Gradient
- 15% B in 15 min

Flow
- 0.40 mL/min

Sample = denosumab IgG2



Separation on HALO 1000 Å C4, 2.7µm, 2.1x150mm
MP A: aq. 0.1% *specified acid*; MP B: *specified + 0.1% acid*; Gradient: 30-45%B for ACN/TFA, 26-41%B for (ACN/nProp/TFA); Temp: 80°C; Inj Vol 2 µL (4µg)

Gradient Elution Variables: IgG2 Example

Bonded-Phase
- **C4**

Temperature
- 80°C

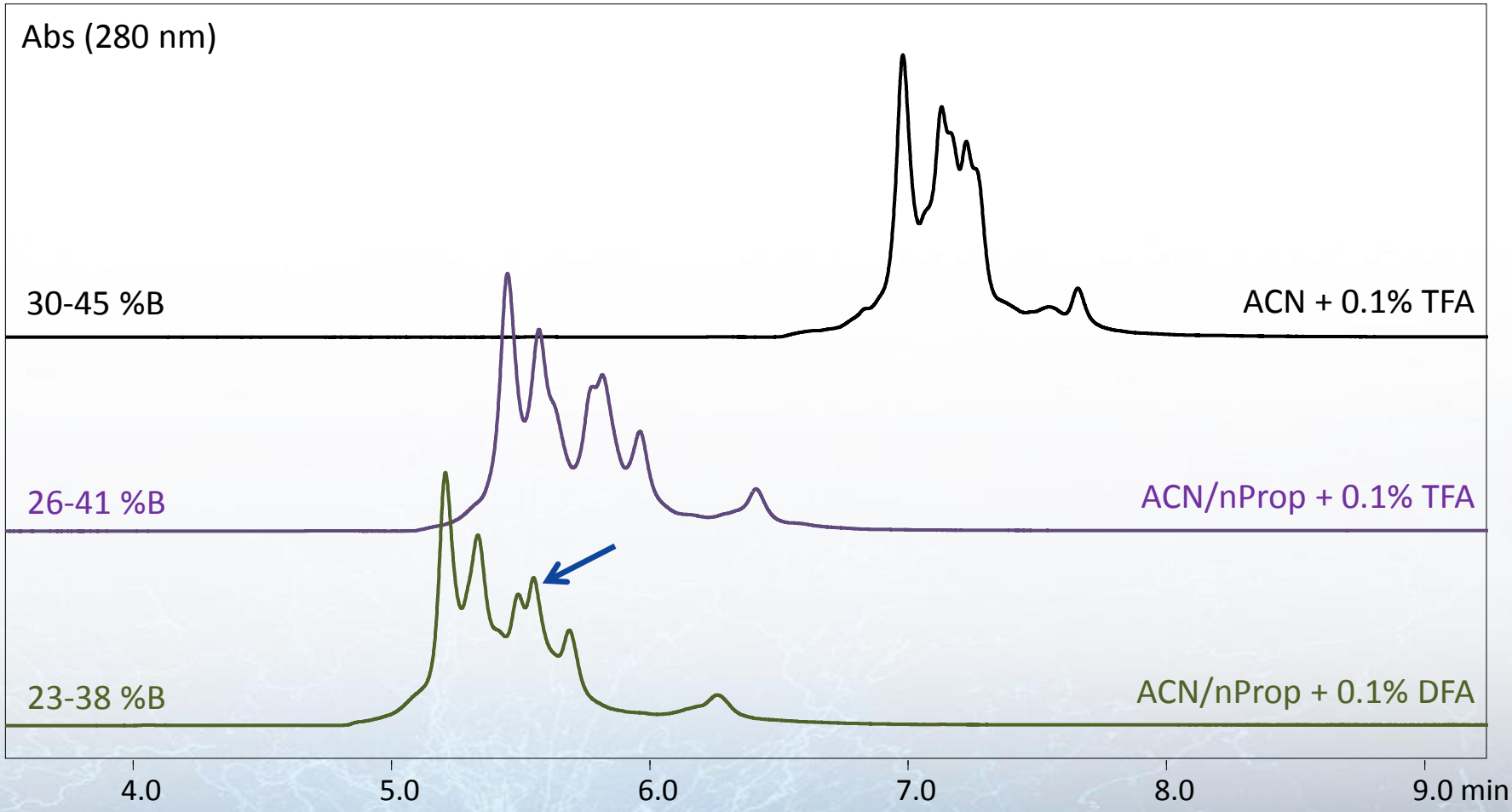
Mobile Phase Strong Solvent
- ACN
- 50/50 ACN/nProp

Ion Pairing Reagent
- 0.1% TFA
- 0.1% DFA

Gradient
- 15% B in 15 min

Flow
- 0.40 mL/min

Sample = denosumab IgG2



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MP A: aq. 0.1% *specified acid*; MP B: *specified + 0.1% acid*; Gradient: 30-45%B for ACN/TFA, 26-41%B for (ACN/nProp/TFA); 23-38%B for (ACN/nProp/DFA); Temp: 80°C; Inj Vol 2 µL (4µg)

Summary and Future Work

- **Improving protein separations is combination of both particle morphology and bonded-phase chemistry**
- **Wide pore HALO 1000Å columns demonstrate superior biomolecule separations**
- **Subtle, but useful, differences in selectivity are available through changes in bonded-phase**
- **Gradient elution variables can alter selectivity and resolution of complex mAbs**
- **Work continues on optimizing pore size and geometry for silica SPP**
- **The resolution gained by these new technologies provides a greater level of detail in the structural differences of well characterized biotechnology products**

Acknowledgements

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Thank you for your Attention