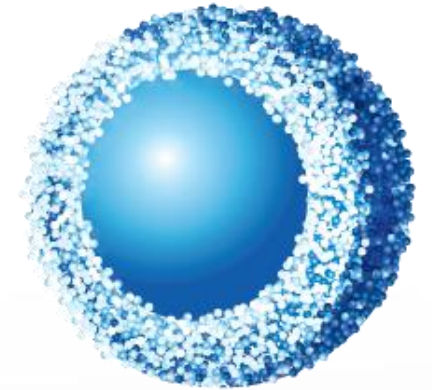


## Wide Pore Superficially Porous Particles with Various Bonded Phases for High Resolution Protein Chromatography



William Miles, Barry Boyes, Ben Libert, Stephanie Schuster, Brian Wagner, Conner McHale

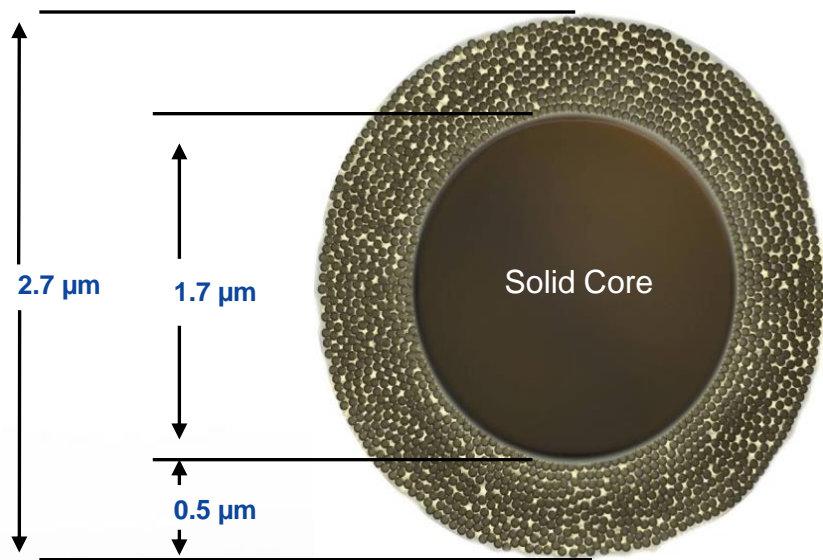
Associate Scientist, R&D

Advanced Materials Technology, Inc.

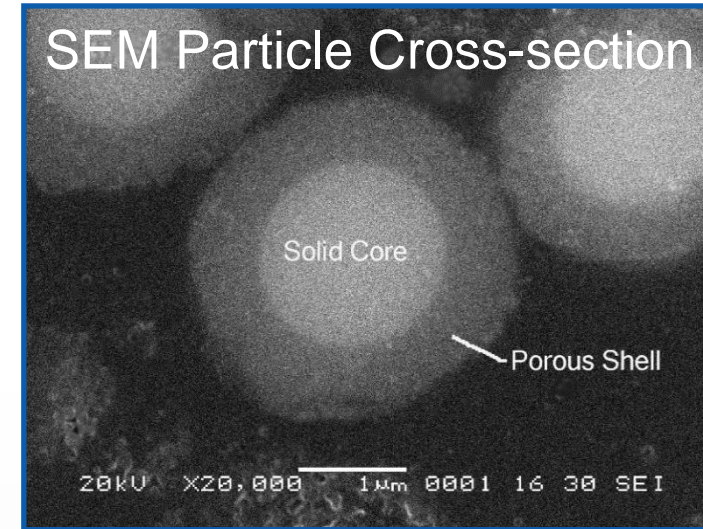
Wilmington, Delaware, USA

[wmiles@advanced-materials-tech.com](mailto:wmiles@advanced-materials-tech.com)

# Superficially Porous Particles (90 Å): 2006



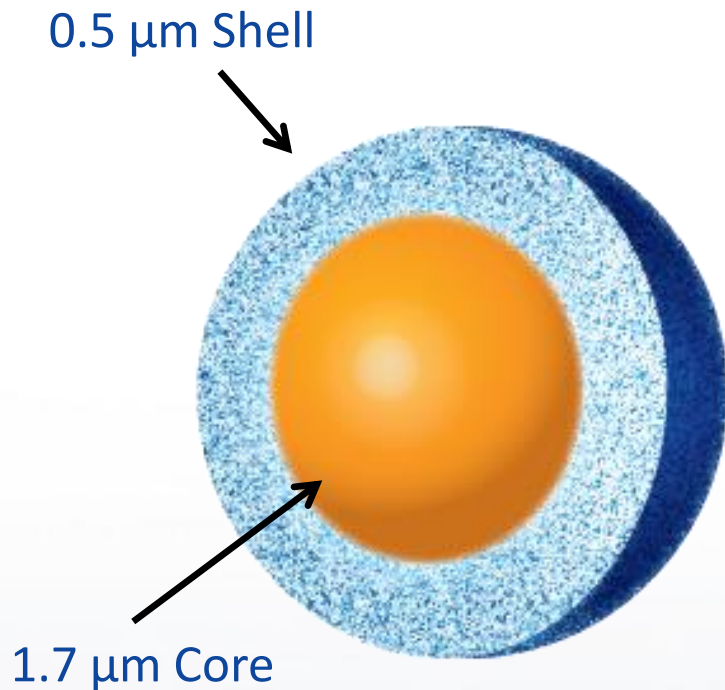
Porous Shell  
90 Å



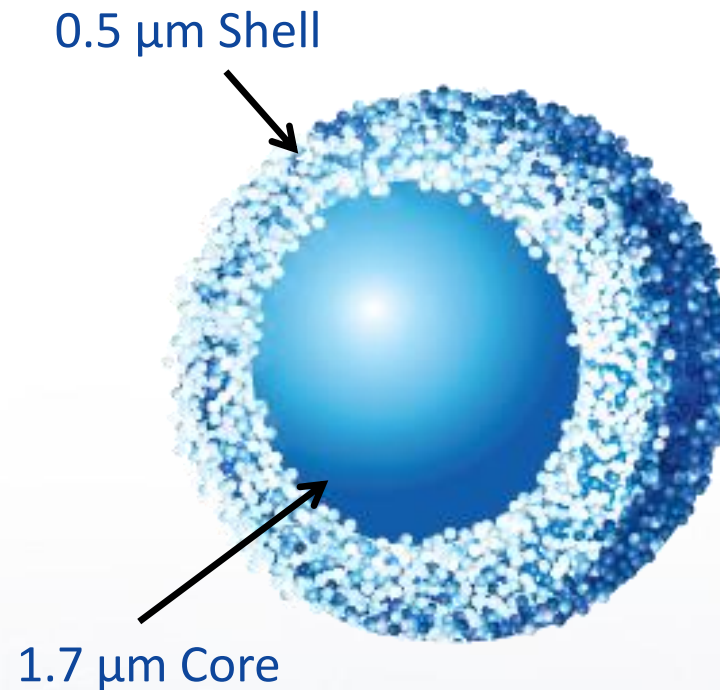
## Fully Porous Particles (FPP) vs. Superficially Porous Particles (SPP)

- 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

# Developments in HALO<sup>®</sup> Fused-Core<sup>®</sup> Pore Size



90 Å, 2.7 μm  
135 m<sup>2</sup>/g  
2006

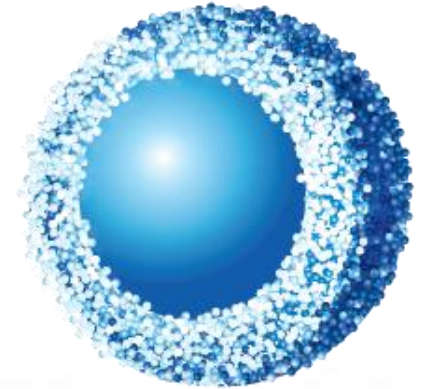


1000 Å, 2.7 μm  
22 m<sup>2</sup>/g  
2017

# Wide Pore SPP Benefits 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**



HALO  
1000 Å, 2.7μm

**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

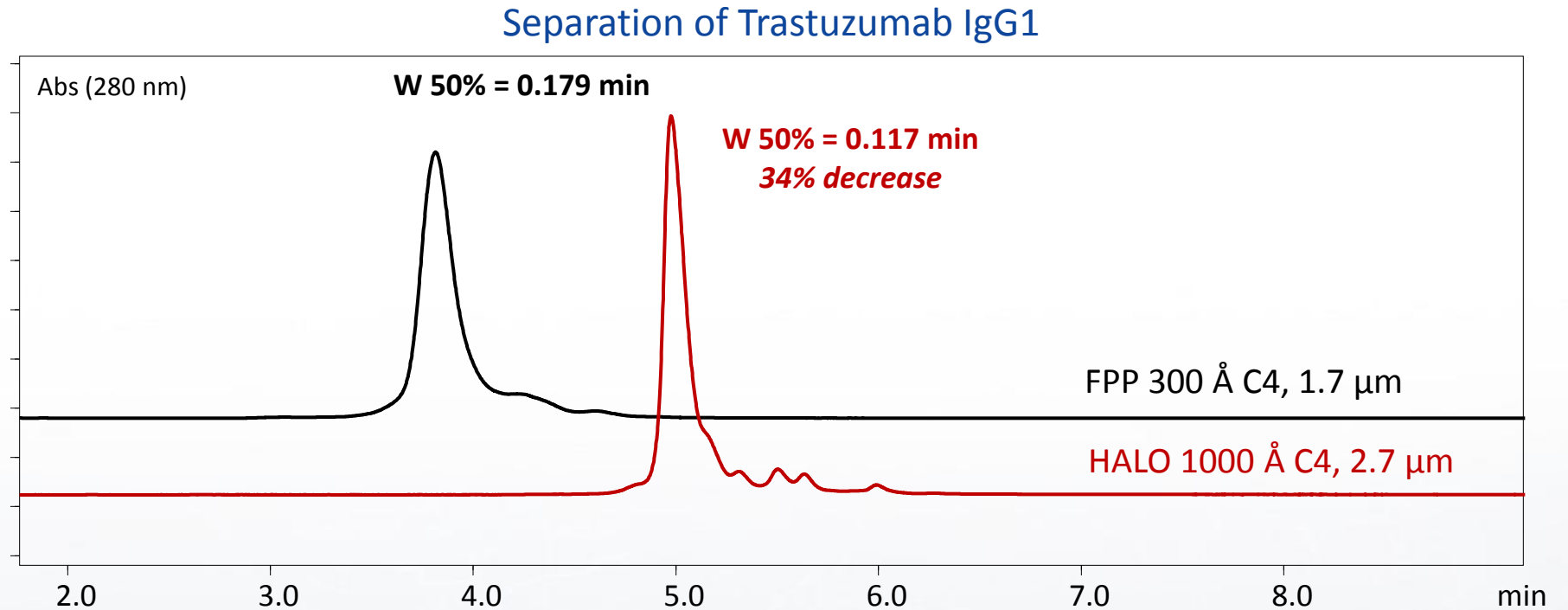
# IgG1 Separation on HALO 1000 Å vs FPP 300 Å

## 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

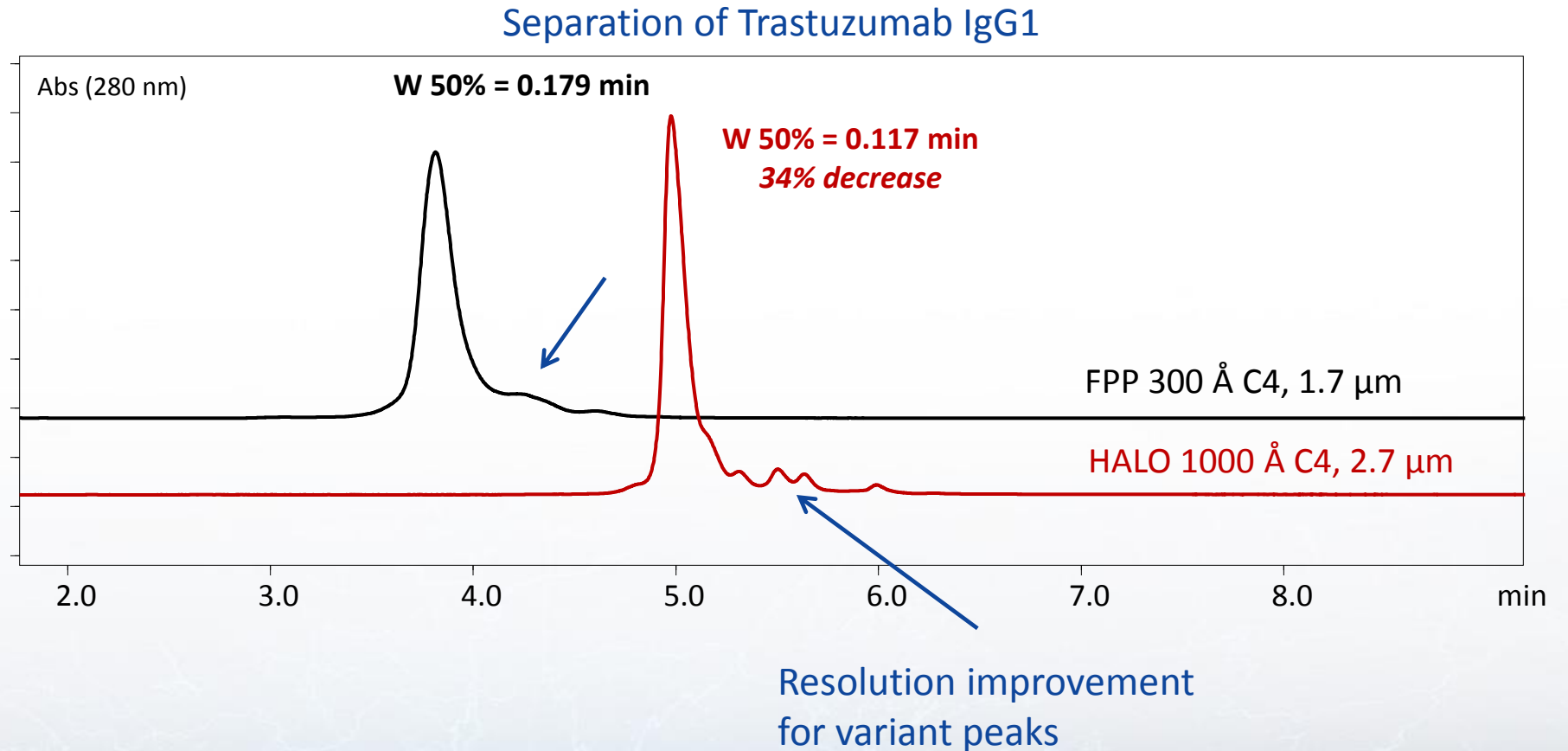
- Larger improvement for HALO 1000 Å

## Retention

- Increase for HALO 1000 Å

## Resolution

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



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

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

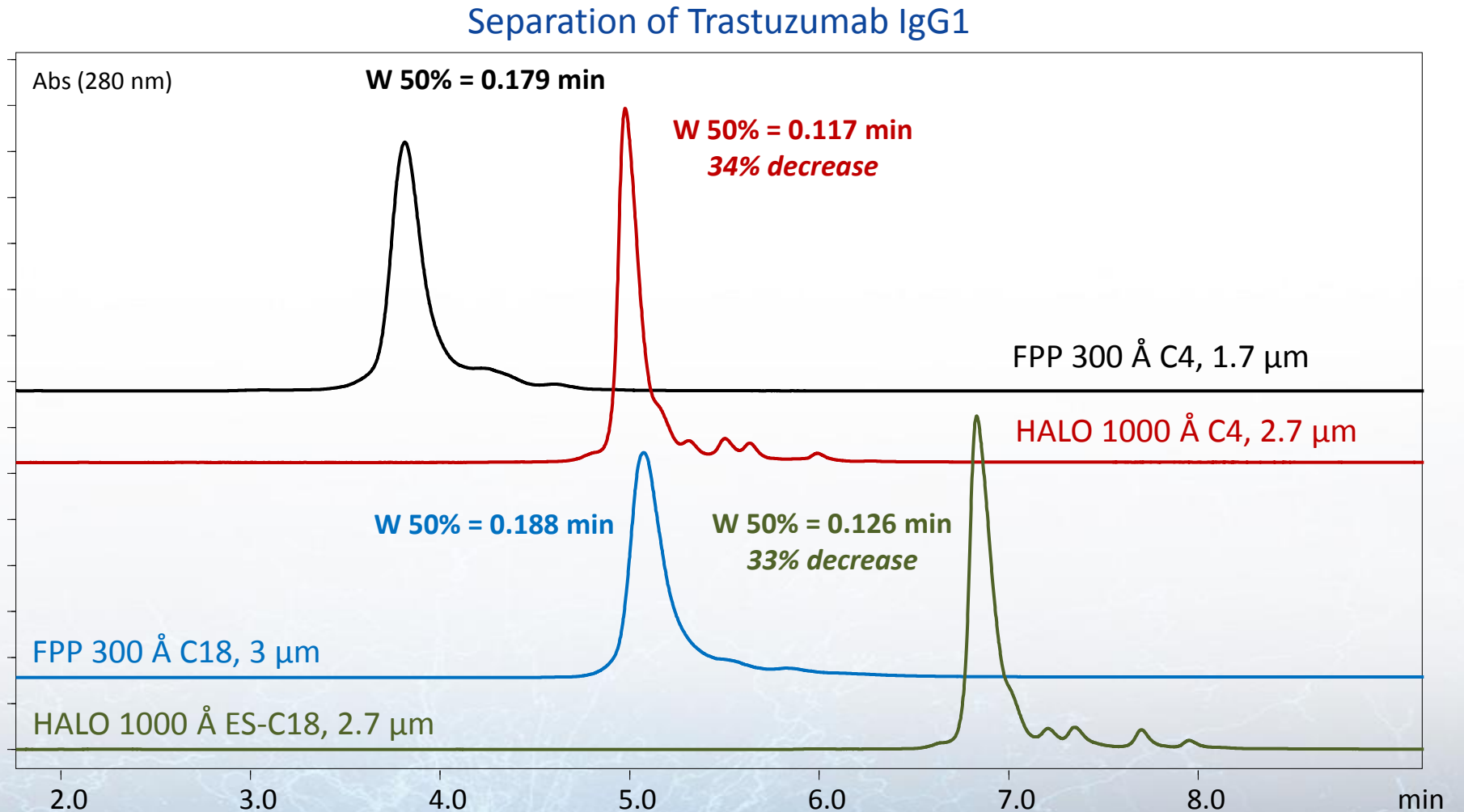
## Retention

- Increase for HALO 1000 Å

## Resolution

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

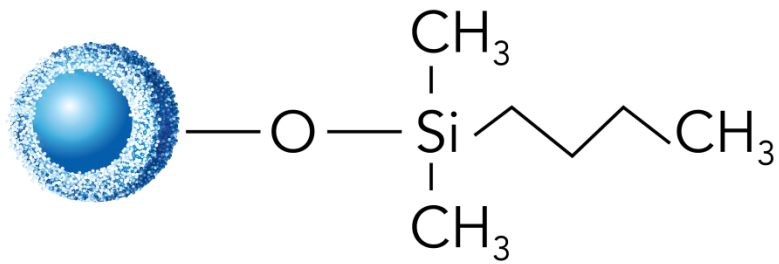
## Improvements seen on both C4 and ES-C18 bonded-phase comparisons



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)



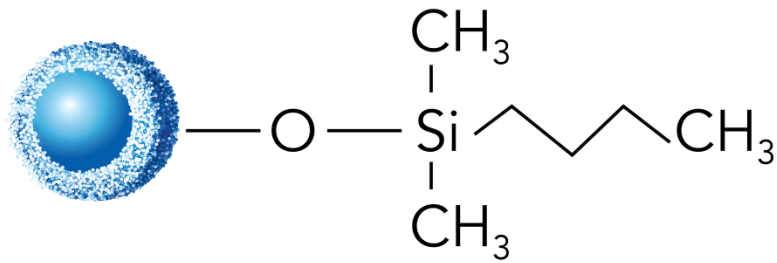
# HALO 1000 Å Bonded-Phases



## C4

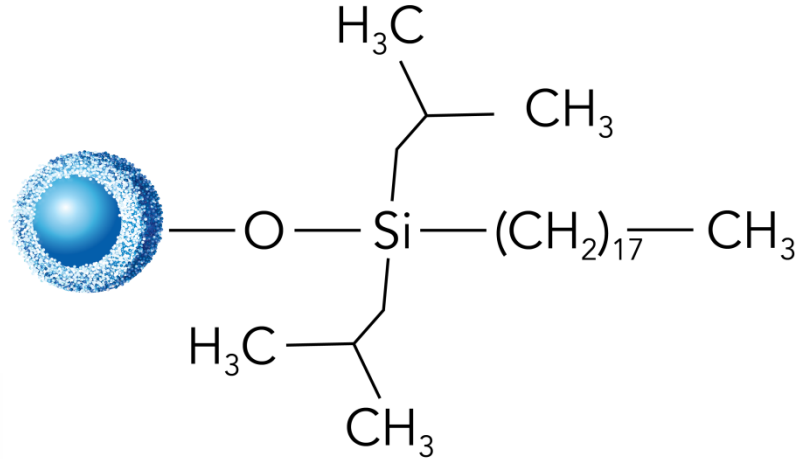
- Traditional alkyl phase for protein separations
- 4.1  $\mu\text{mol}/\text{m}^2$  (0.65% C)

# HALO 1000 Å Bonded-Phases



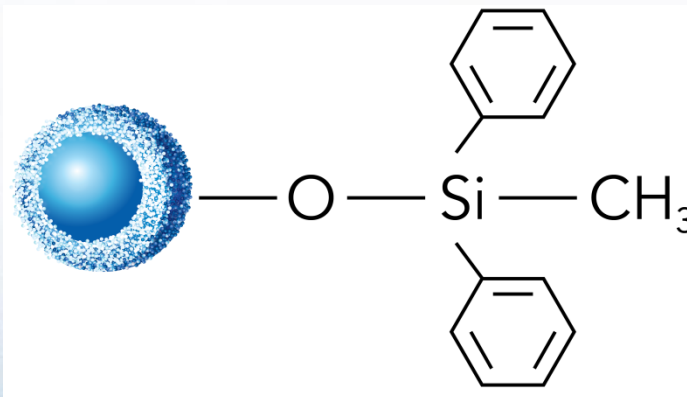
## C4

- Traditional alkyl phase for protein separations
- 4.1  $\mu\text{mol}/\text{m}^2$  (0.65% C)



## ES-C18

- Sterically-protected, long chain alkyl phase
- 1.9  $\mu\text{mol}/\text{m}^2$  (1.4% C)



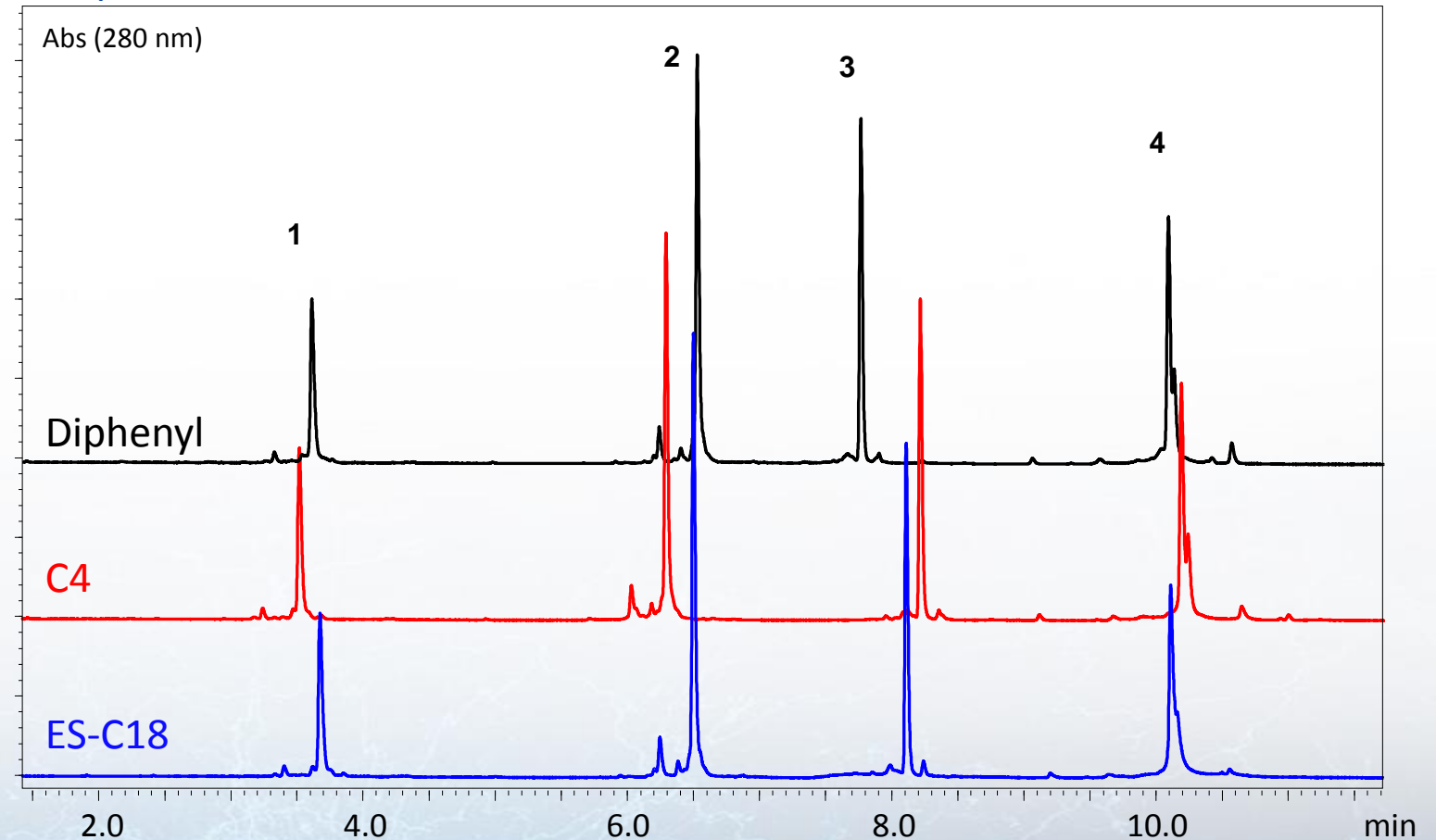
## Diphenyl

- Aromatic phenyl phase
- Selectivity differs from alkyl phases
- 2.7  $\mu\text{mol}/\text{m}^2$  (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.  $\alpha$ -Lactalbumin – 14.2 kDa
4. Enolase – 46.6 kDa

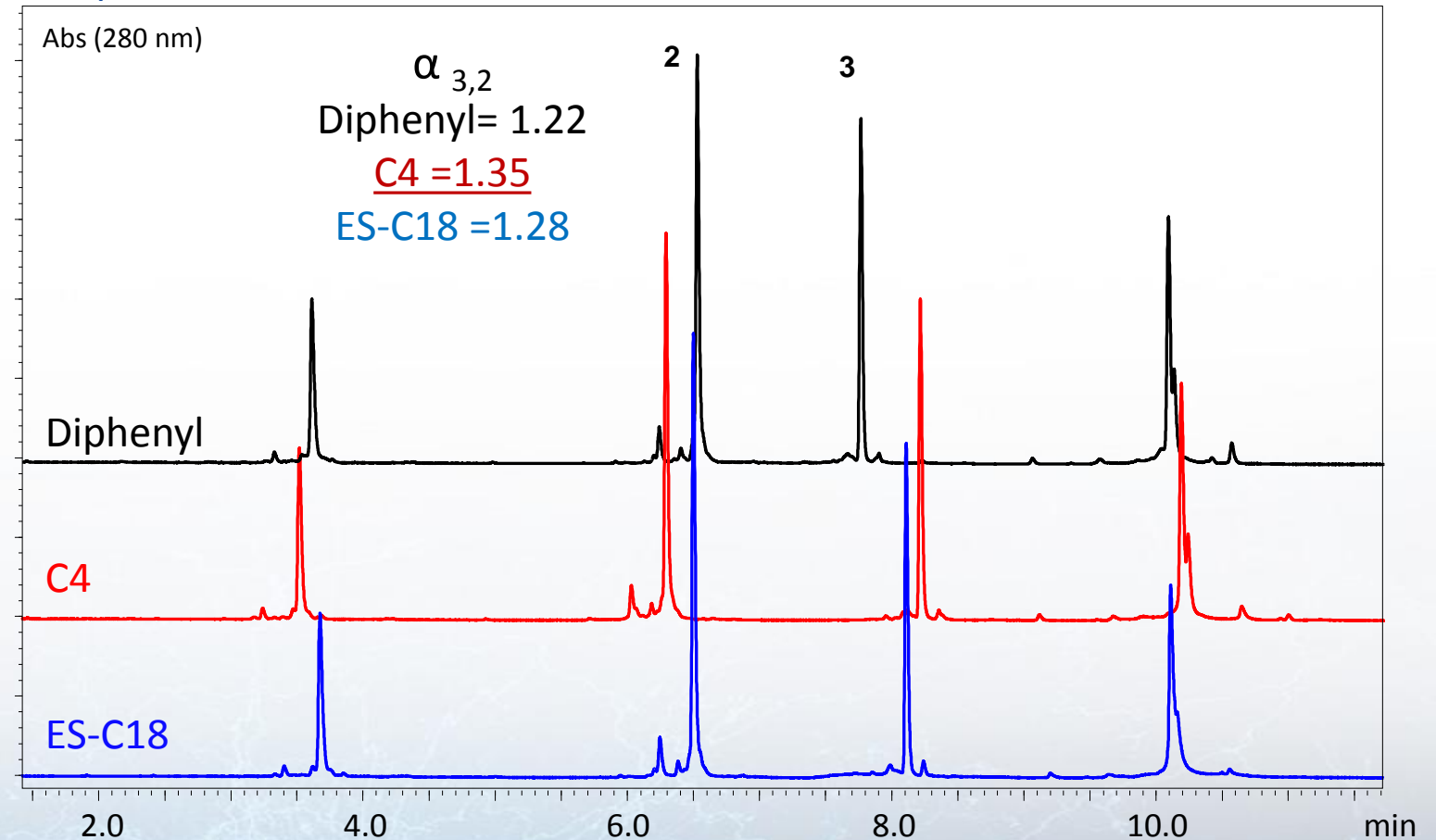
MP A: aq. 0.1% TFA; MP B: ACN + 0.1% TFA; Gradient: 20-60 %B in 15 min;  
Injection Volume: 2  $\mu$ L; Flow: 0.40 mL/min; Temp: 80  $^{\circ}$ C

# 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
- Protein Selectivity
  - Subtle but useful changes

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

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



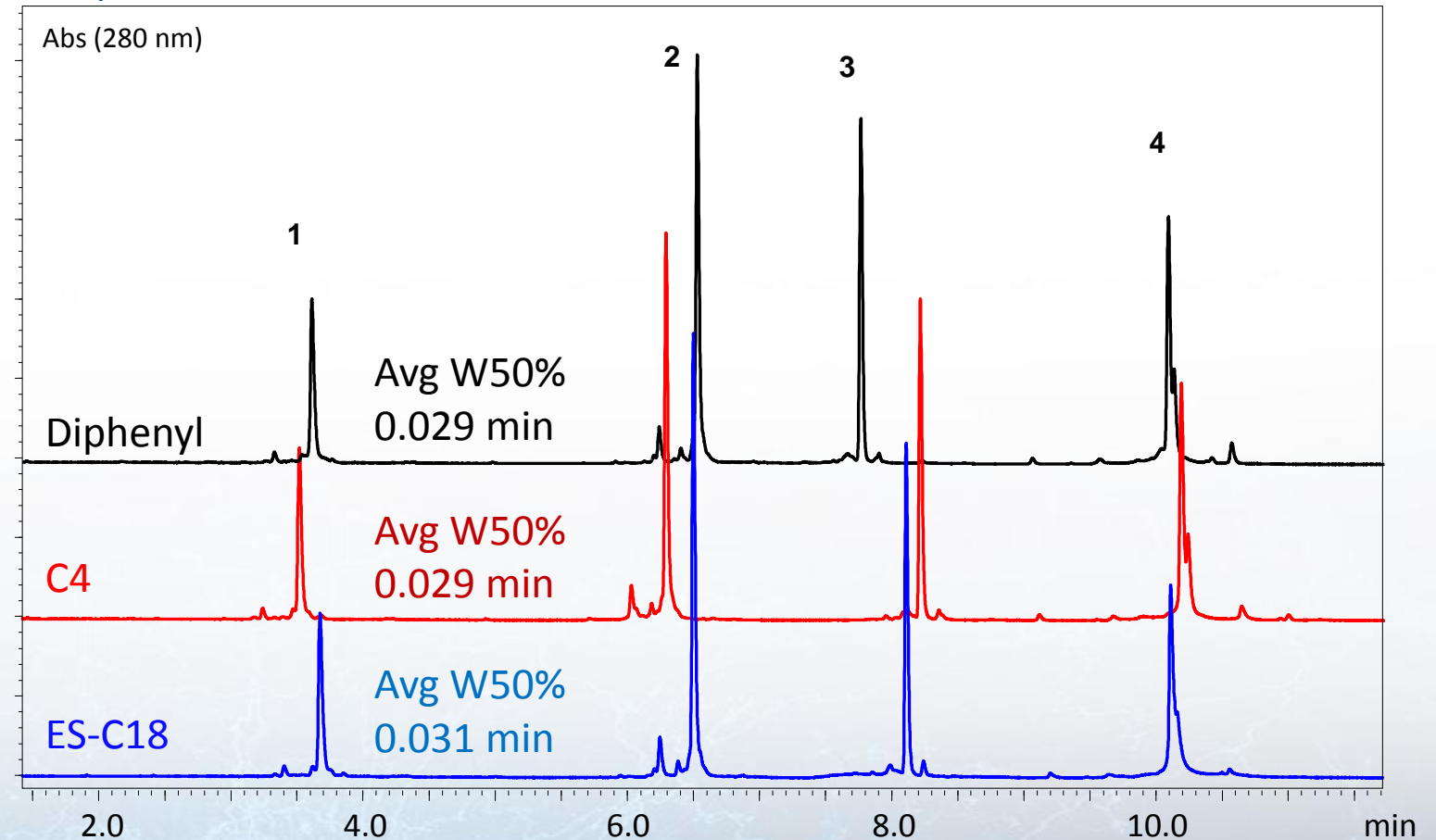
MP A: aq. 0.1% TFA; MP B: ACN + 0.1% TFA; Gradient: 20-60 %B in 15 min;  
Injection Volume: 2  $\mu$ L; Flow: 0.40 mL/min; Temp: 80  $^{\circ}$ C

# 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
- **Protein Selectivity**
  - Subtle but useful changes
- **Protein Peak Width 50%**
  - Average width nearly identical between phases

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

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



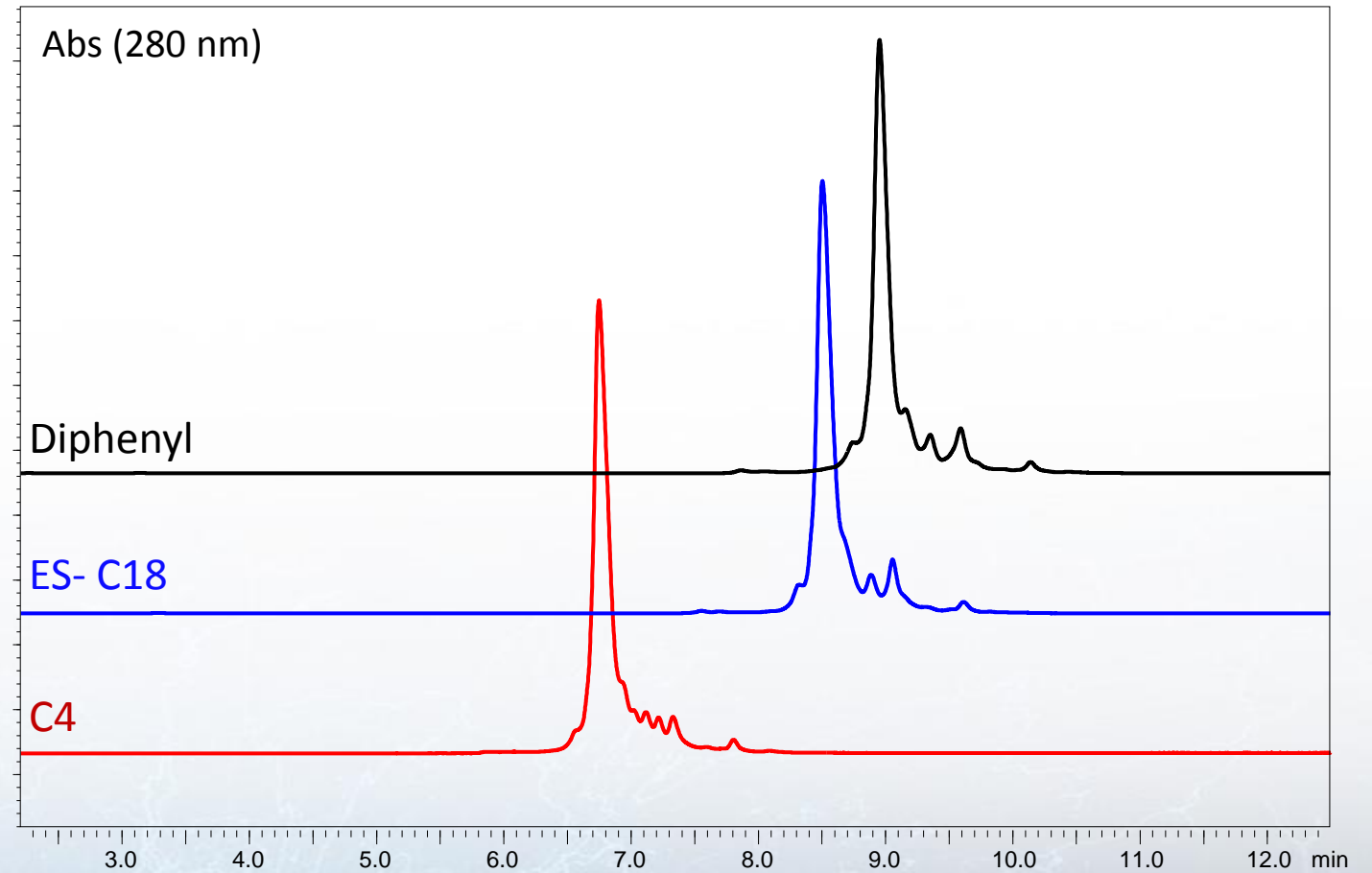
MP A: aq. 0.1% TFA; MP B: ACN + 0.1% TFA; Gradient: 20-60 %B in 15 min;  
Injection Volume: 2  $\mu$ L; Flow: 0.40mL/min; Temp: 80  $^{\circ}$ C

# 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

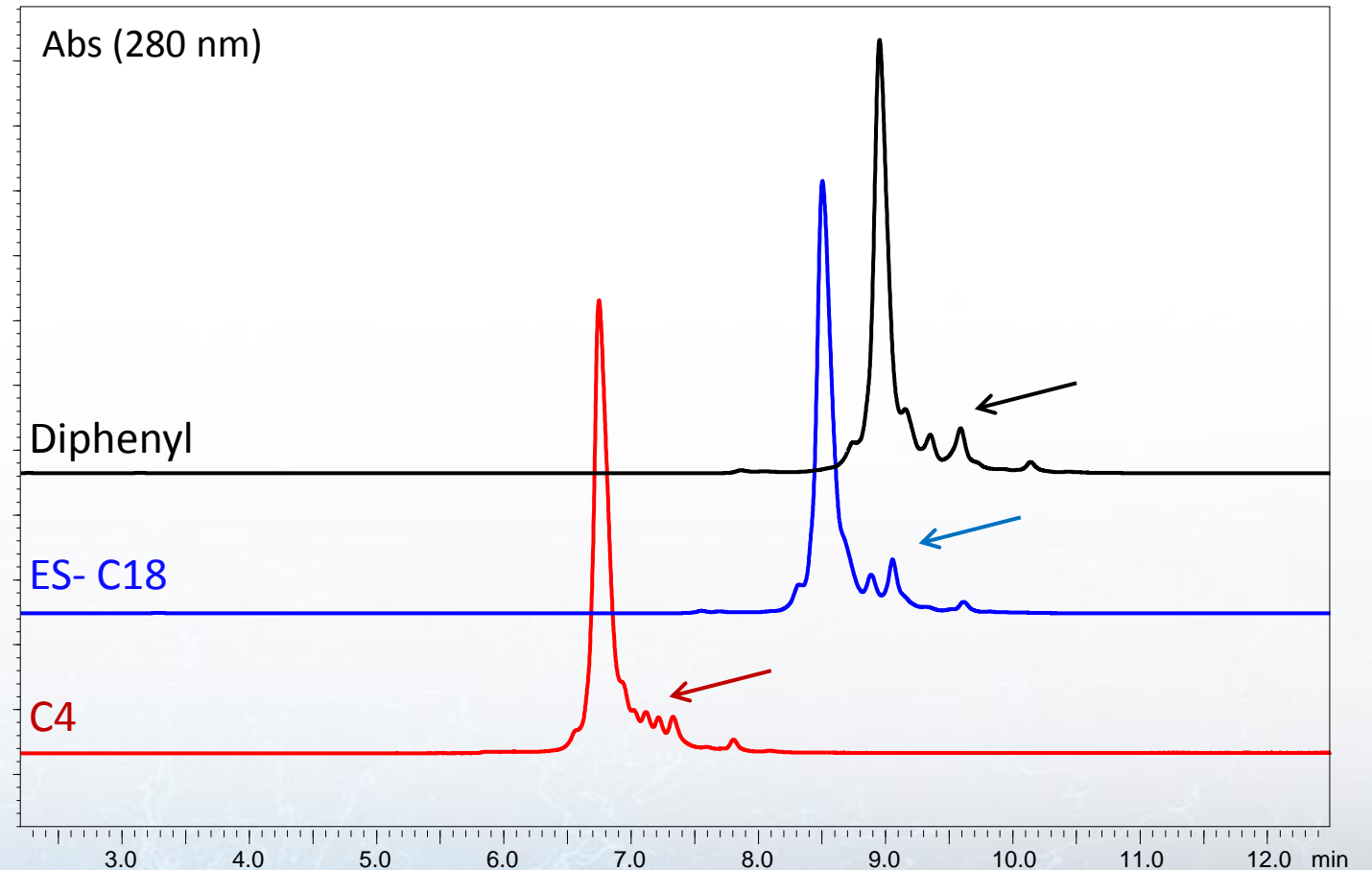


MP A: aq. 0.1% TFA; MP B: ACN + 0.1% TFA; Gradient: 32-40 %B in 16 min;  
Flow 0.40mL/min; Inj Vol 2 µL; Temp: 80°C

# Effect of Bonded-Phase on Retention/Selectivity

- mAb Retention
  - 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

Separation of Trastuzumab IgG1 on HALO 1000 Å Bonded-Phases



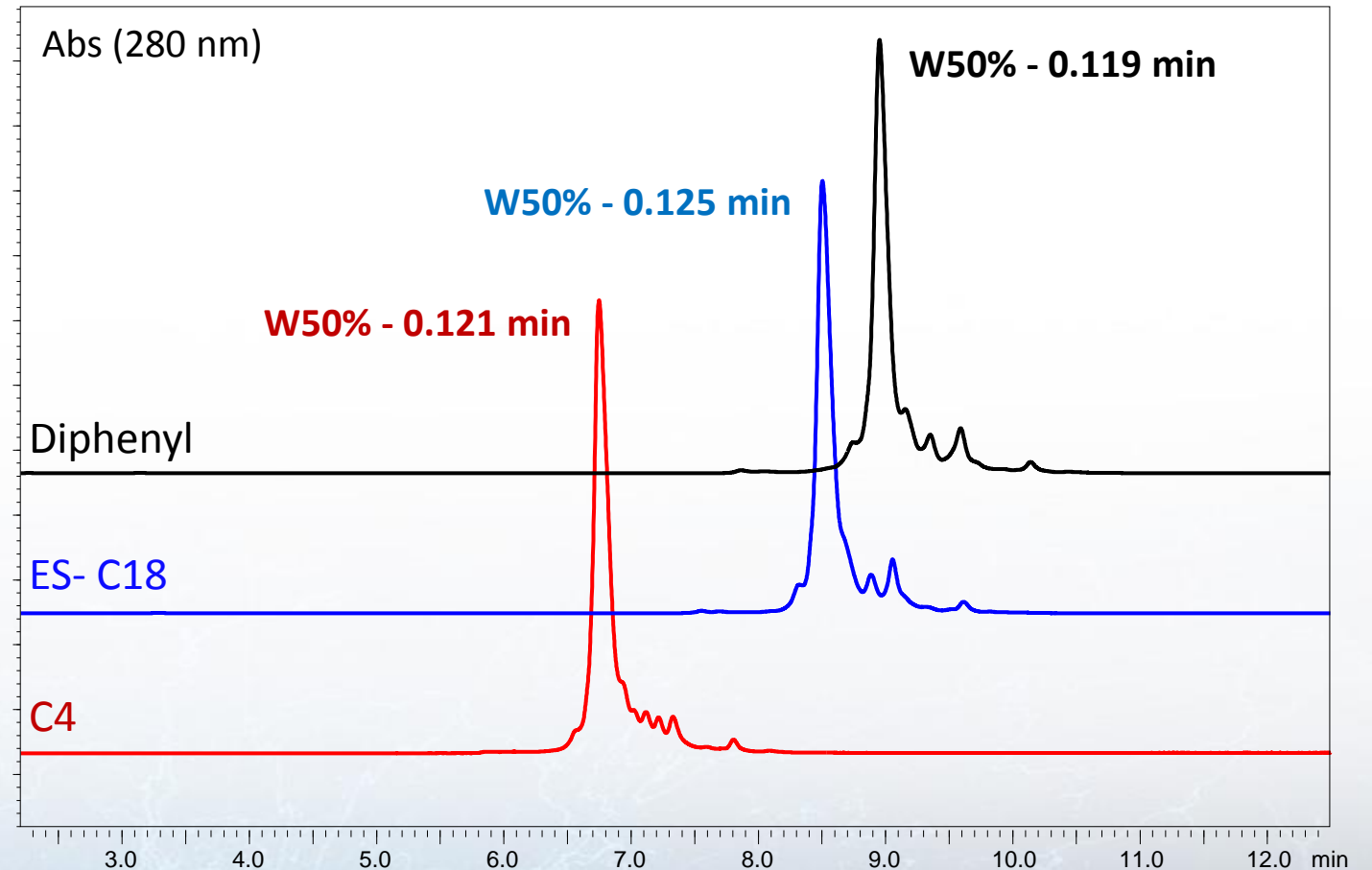
MP A: aq. 0.1% TFA; MP B: ACN + 0.1% TFA; Gradient: 32-40 %B in 16 min;  
Flow 0.40mL/min; Inj Vol 2 µL; Temp: 80°C

# Effect of Bonded-Phase on Retention/Selectivity

- mAb Retention
  - 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

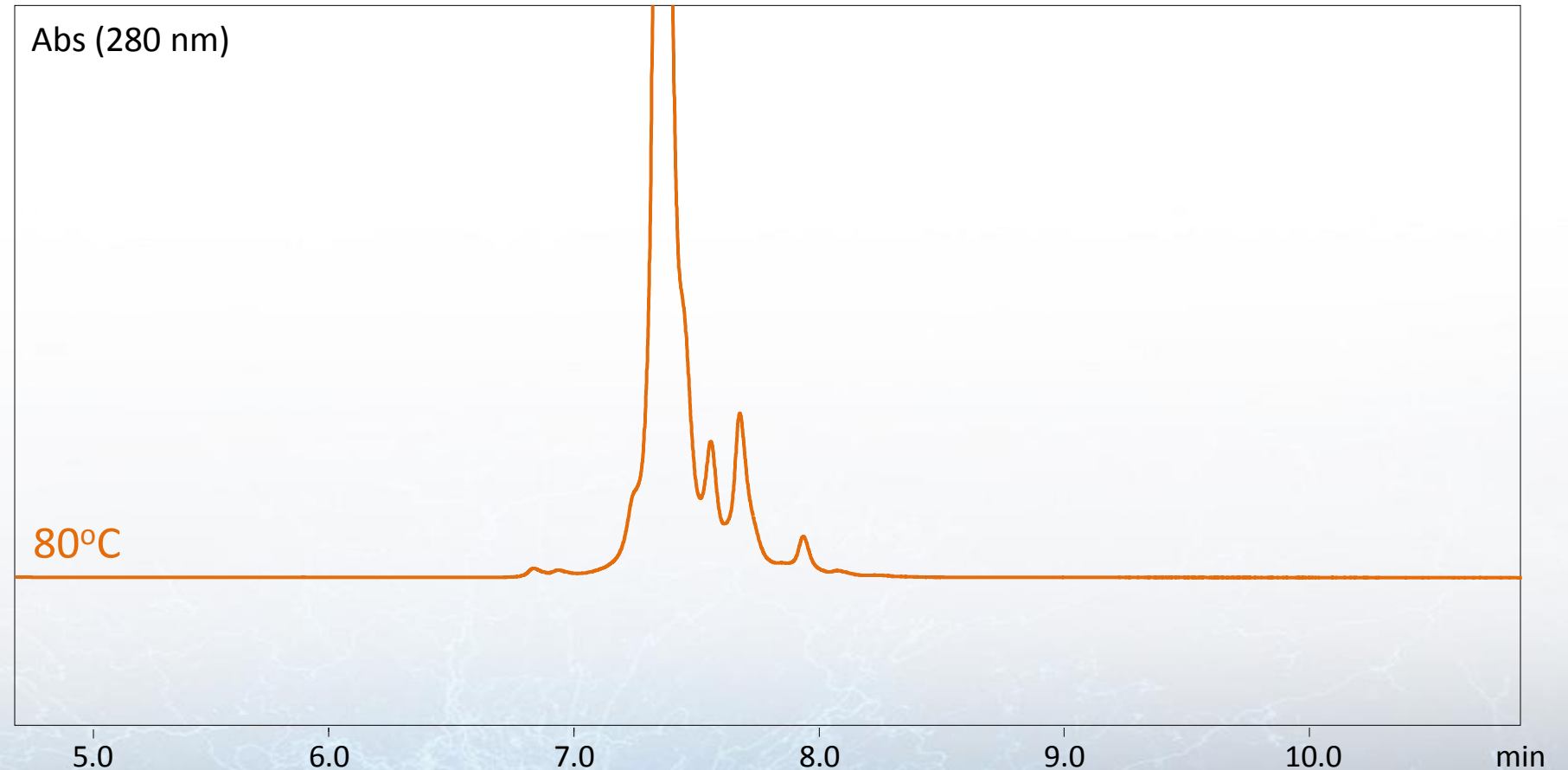
## Gradient

- 30-45% B in 15 min

## Flow

- 0.40 mL/min

Sample = trastuzumab IgG1



Separation on HALO 1000 Å Diphenyl, 2.7 $\mu$ m, 2.1x150mm  
MP A: aq. 0.1% TFA; MP B: ACN + 0.1% TFA; Inj Vol 2  $\mu$ L (4 $\mu$ g)

# Gradient Elution Variables: IgG1 Example

## Bonded-Phase

- Diphenyl

## Temperature

- Varied

## Mobile Phase Strong Solvent

- ACN

## Ion Pairing Reagent

- 0.1% TFA

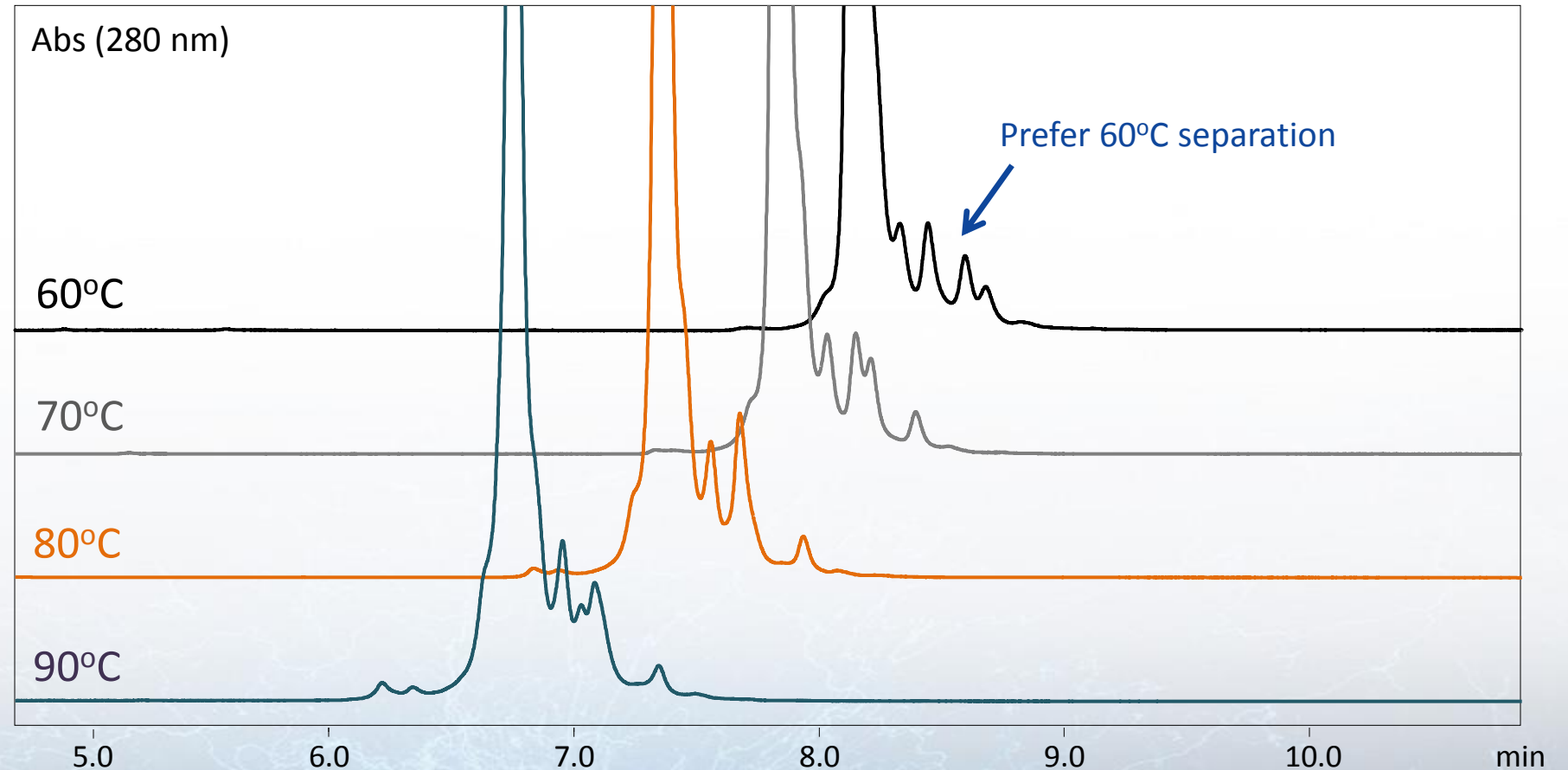
## Gradient

- 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: IgG2 Example

## Bonded-Phase

- C4

## Temperature

- 80°C

## Mobile Phase Strong Solvent

- ACN

## Ion Pairing Reagent

- 0.1% TFA

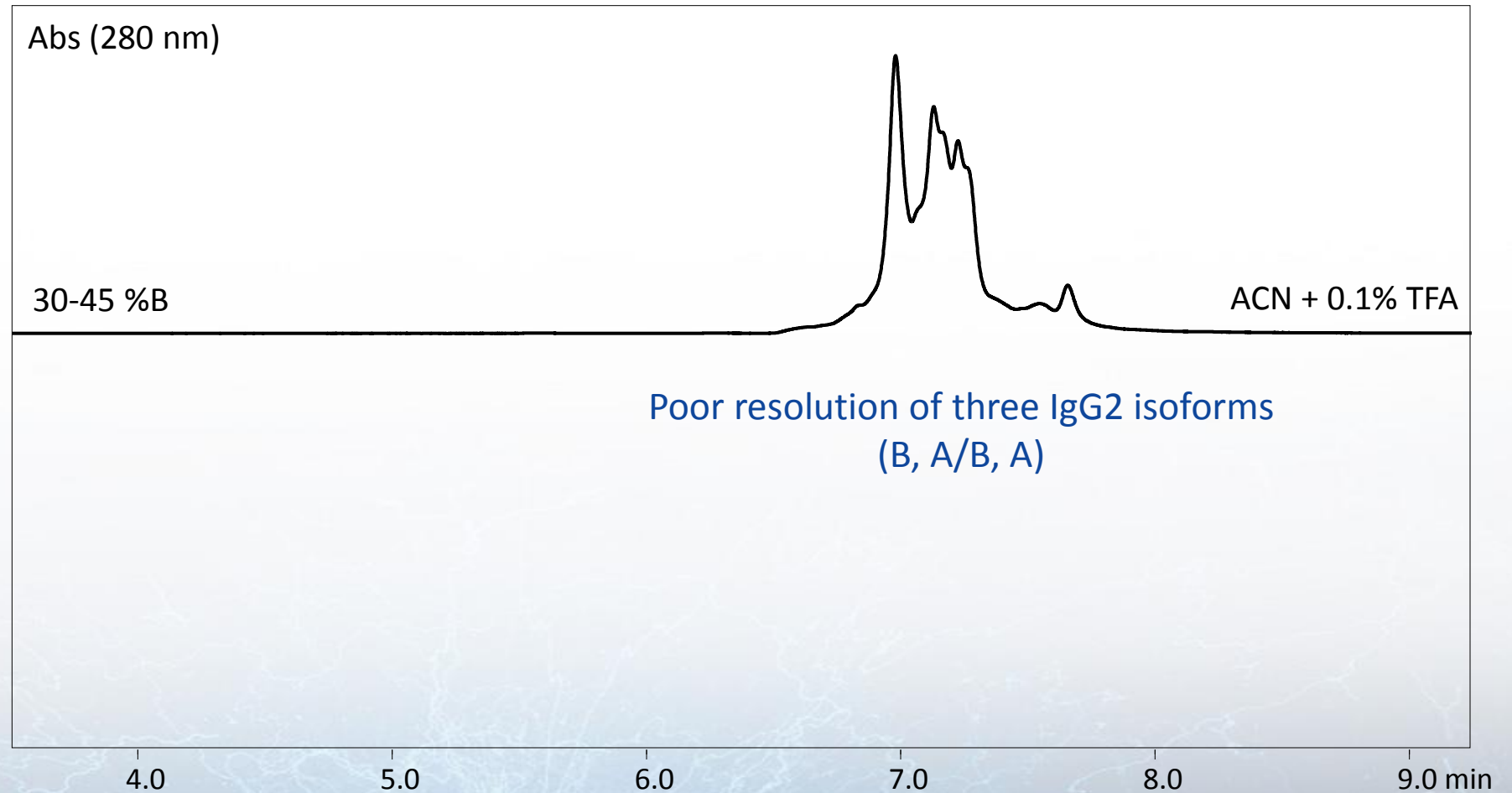
## Gradient

- 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

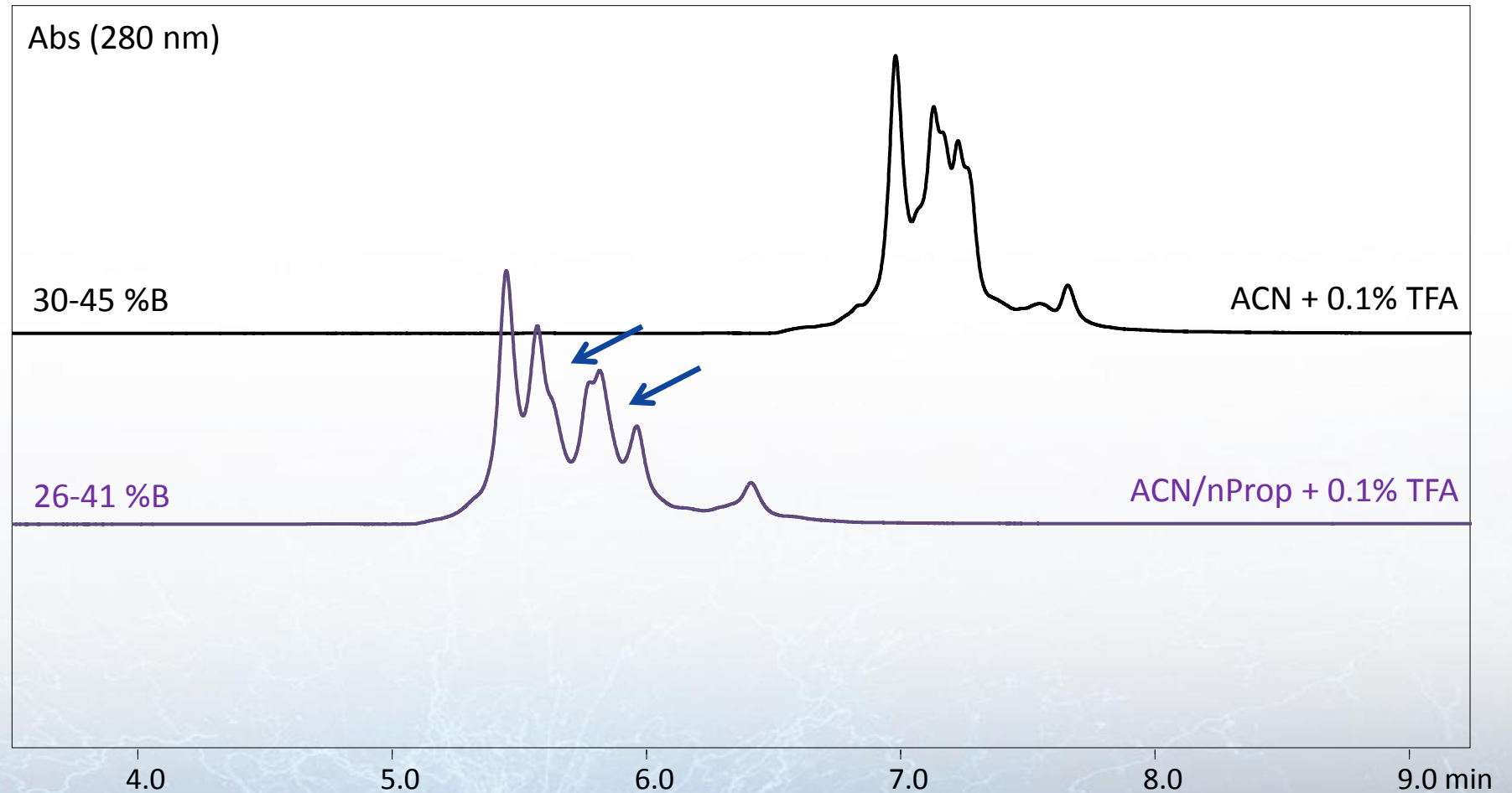
## Gradient

- 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

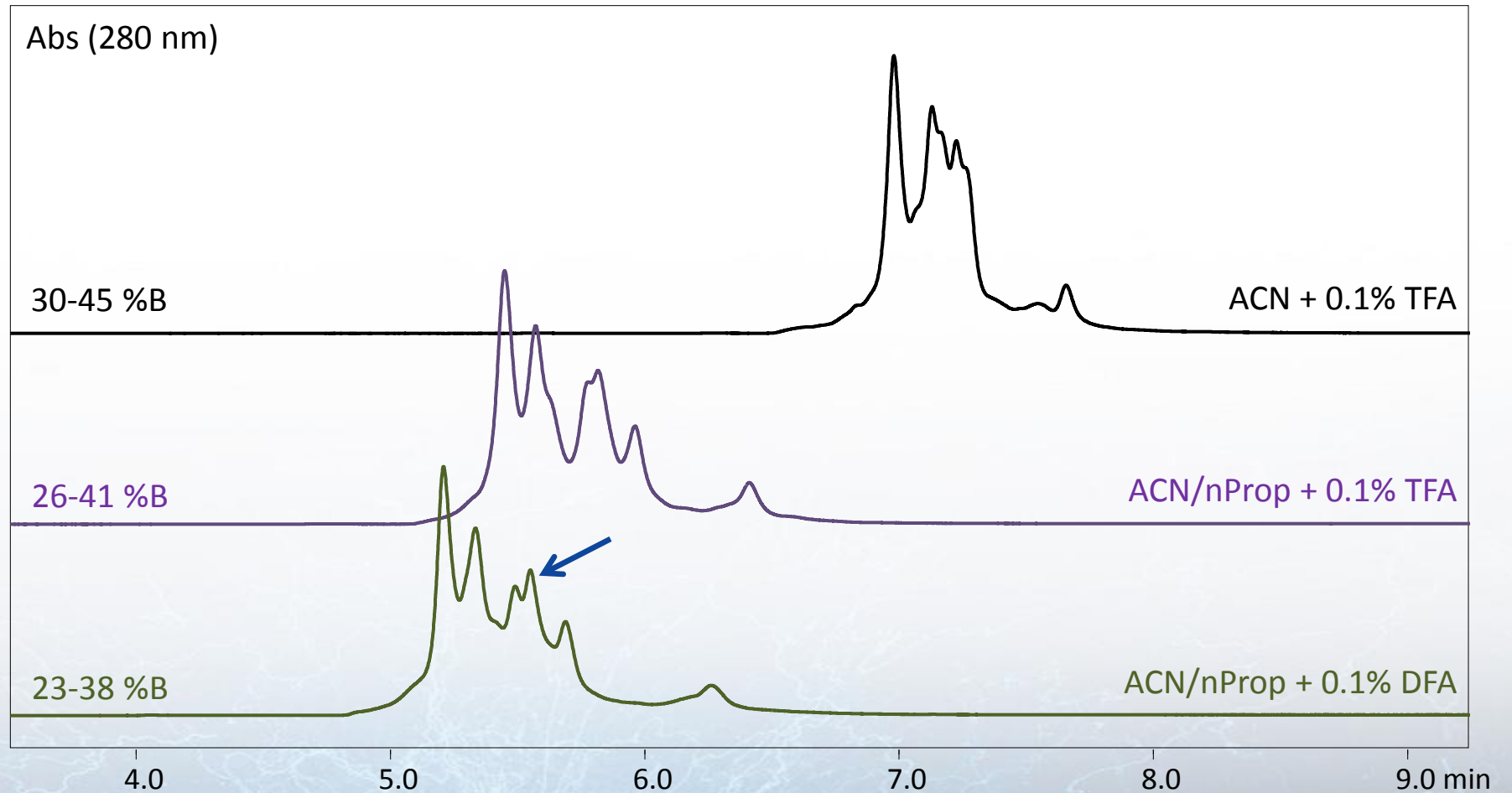
## Gradient

- 15% B in 15 min

## Flow

- 0.40 mL/min

Sample = denosumab IgG2



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); 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

- The Research and Development team at Advanced Materials Technology including, Justin Godinho and Bob Moran (AMT) are thanked for advice, technical assistance, and samples.
- AMT management including Joe DeStefano and Tim Langlois for ongoing support on protein analytical science.
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**Thank you for your Attention**