# Superficially Porous Silica Particle Technology Developments for Pharma and Biopharma Applications

Barry Boyes[1,2], Joseph DeStefano[1], Tim Langlois[1], and Joseph J. Kirkland[1]

[1]Advanced Materials Technology, Inc.
Wilmington, DE USA
[2]Complex Carbohydrate Research Center
University of Georgia, Athens, GA USA
bboyes@advanced-materials-tech.com

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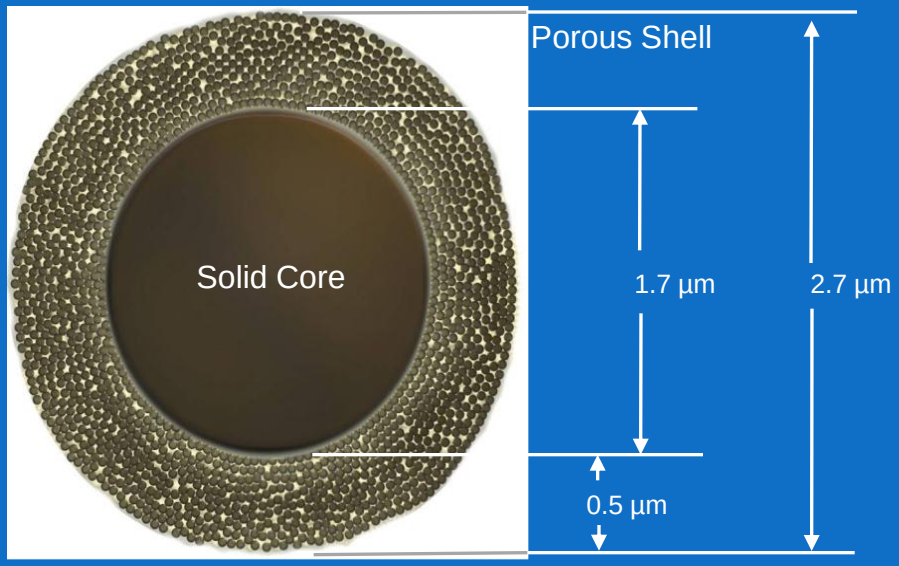
# Agenda

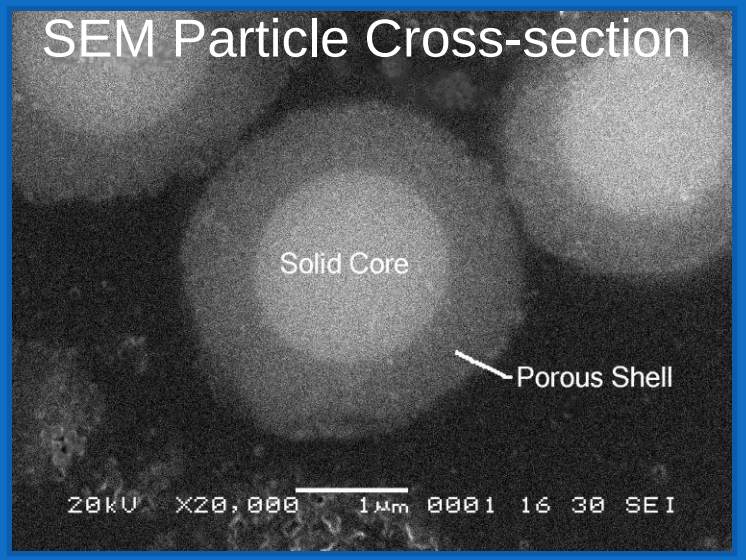
- The Brief Logic Fused-Core (SPP) Particles
- Recent Developments for Small Molecules
  - Particle Size Developments: Smaller and Larger
  - Expansion of Bonded Phase Choices
- Recent Developments for Larger Molecules
  - 160 Å pore RP for peptides
  - 400 Å pore RP for proteins
  - Penta-HILIC for glycans and glycopeptides

# Faster HPLC Separations

- Smaller Particle Packed Beds
  - Totally Porous (including flow through)
  - Not Porous (Pellicular)
  - Partly Porous (Superficially Porous)
- Monolithic Materials
- Open Tubular Columns (channels)

# Superficially Porous (Fused-Core®) Particles



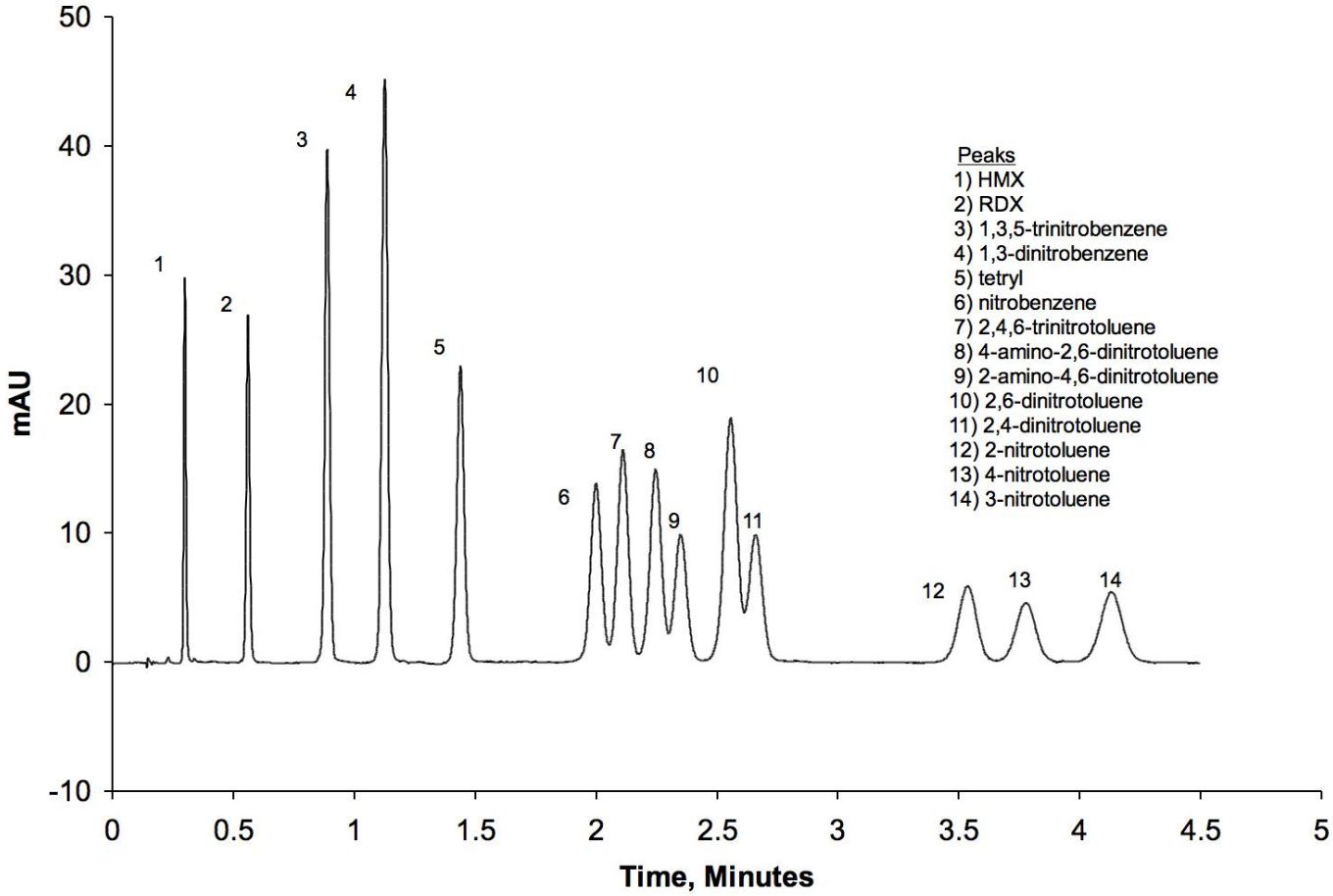




*Why 2.7µm?* $P \cong 2500L\eta F/(d_p^2 d_c^2)$ $d_p \sim (2500L\eta F/Pd_c^2)^{1/2}$

- Not as necessary to filter samples and mobile phase since frits are not as small as needed for sub-2-µm: practical compromise.
- With 0.5 µm shell high resolution is maintained at high flow rates (flat C-term in van Deemter plot).

# Separation of Explosives (c. 2007 EAS)

Column: 4.6 x 50 mm Halo C18; Mobile phase: 27% Methanol/73% Water; Temperature: 40ºC; Flowrate: 3.3 ml/min; Detector: UV @ 254 nm; Pressure: 343 bar; Agilent 1100



# Recent Developments for Small Molecules

- Particle Size Developments: Smaller and Larger

  2.7 µm Halo: high efficiency, preferred analytical choice

  *5 µm Halo: lower back pressures, ~3 µm porous particle efficiency*

  *2 µm Halo: higher efficiency (higher pressures)*

- Expansion of Bonded Phase Options

  C18, C8, Phenyl-hexyl, RP-Amide, Pentafluoropropyl (PFP), ES-CN

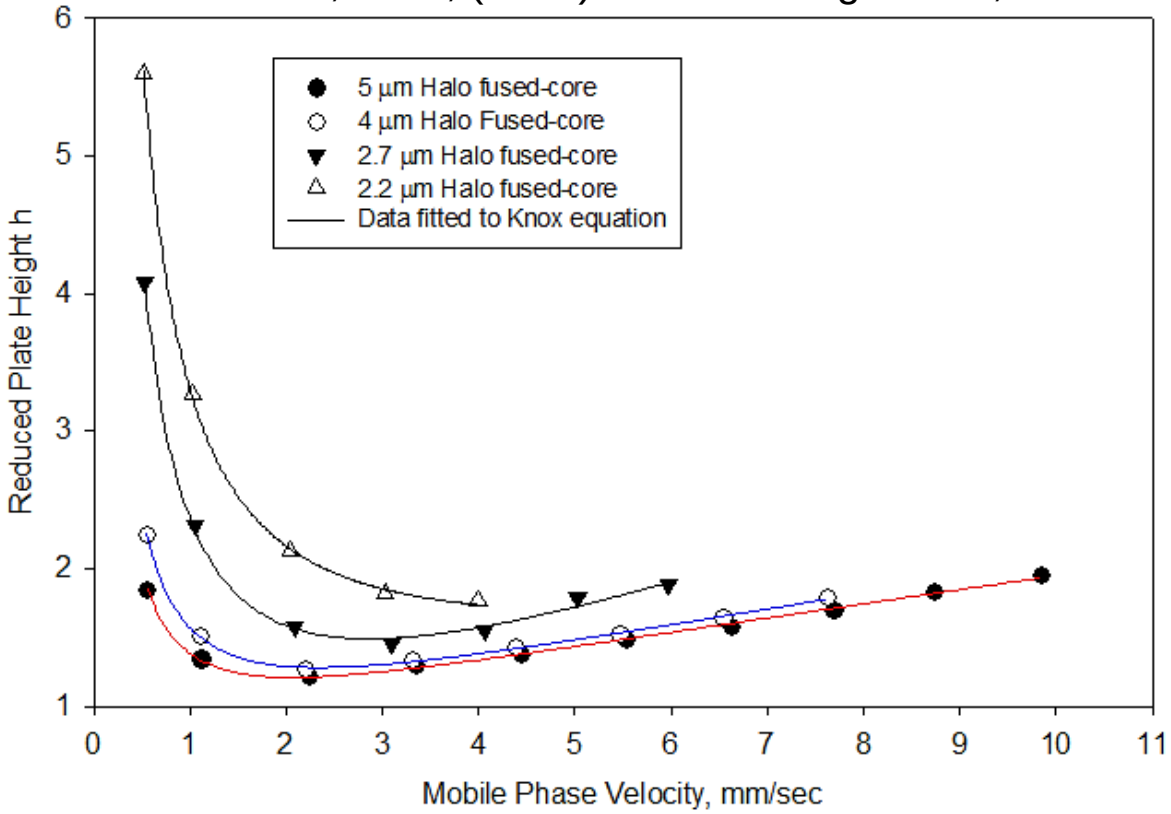
  *Penta-HILIC*

# 5 µm Halo Particle Utility

Effect of Particle Size on Reduced Plate Height

Columns: 4.6 x 150 mm; Temperature: 30 °C
Mobile phase: 50% acetonitrile/50% water
Solute: 1-Cl-4-nitrobenzene: Injection: 1 µL
Instruments: <400 bar, Agilent 1100; >600 bar, Agilent 1200

DeStefano, et al., (2012) J. Chromatogr. 1258, 76-83




h values lower for 5 µm HALO (h = 1.2) - more homogeneously packed bed structure

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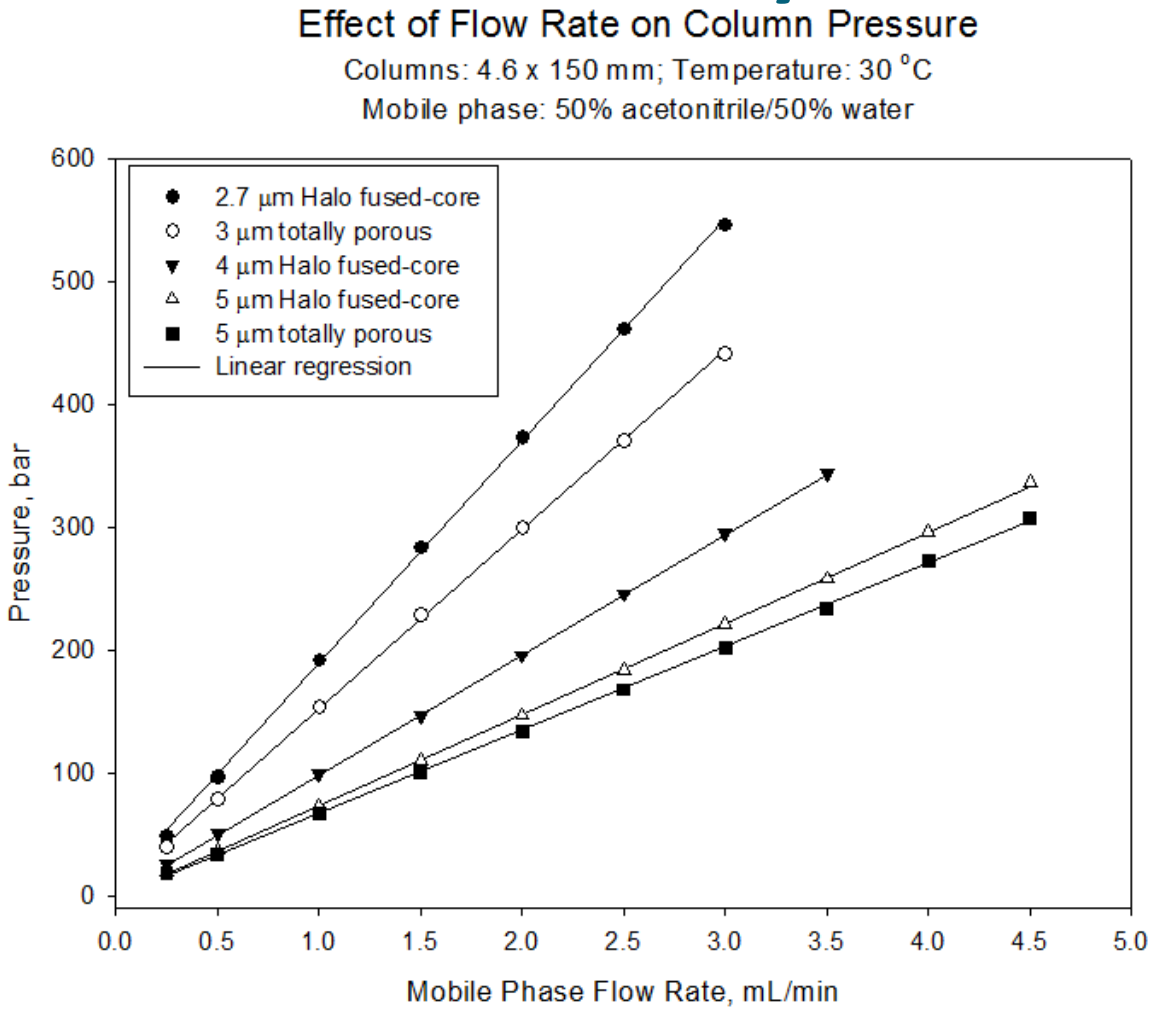
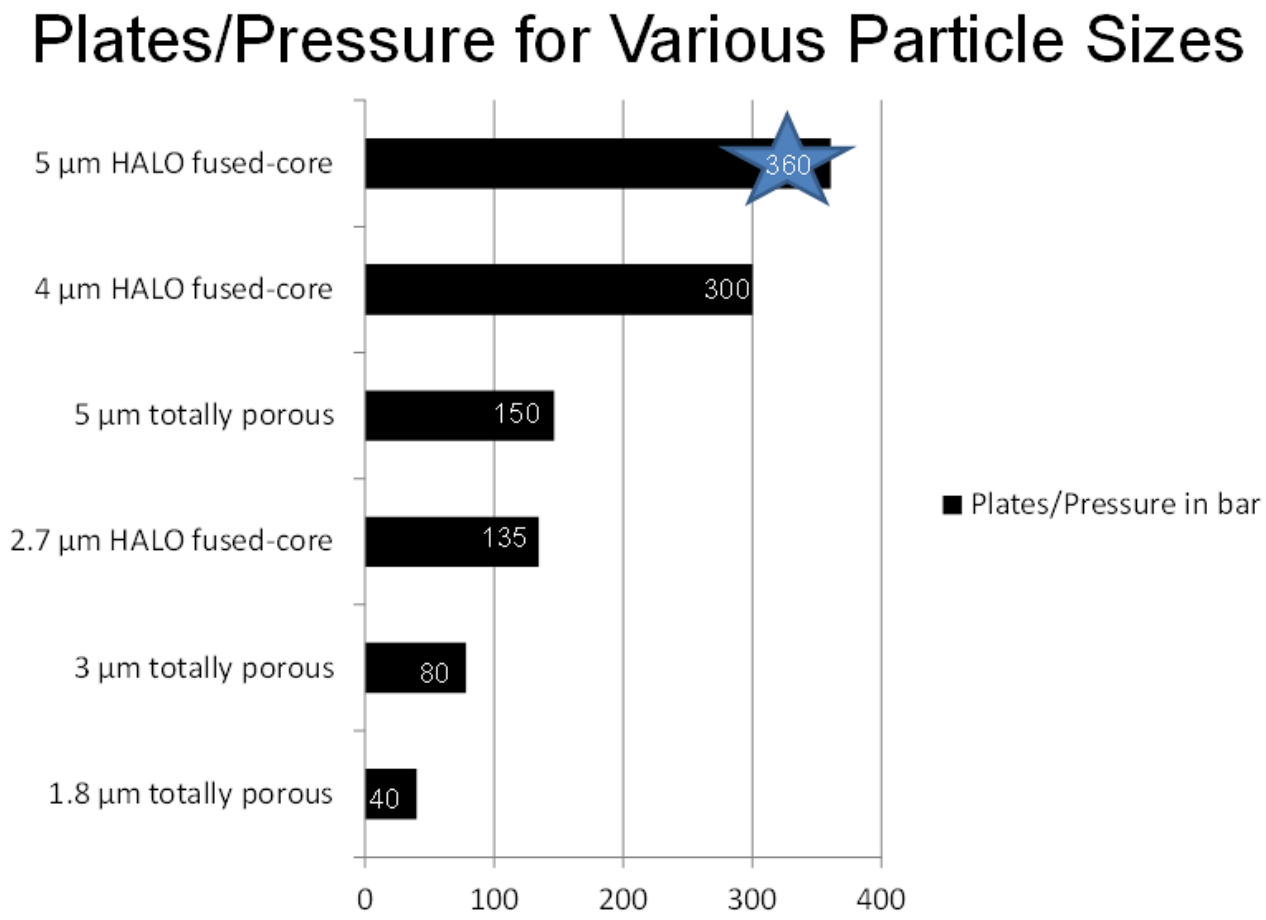
# 5 µm Halo Particle Utility




Pressure increases with $\left(\frac{1}{d_p}\right)^2$; small particles require high pressure

# 5 µm Halo Particle Utility

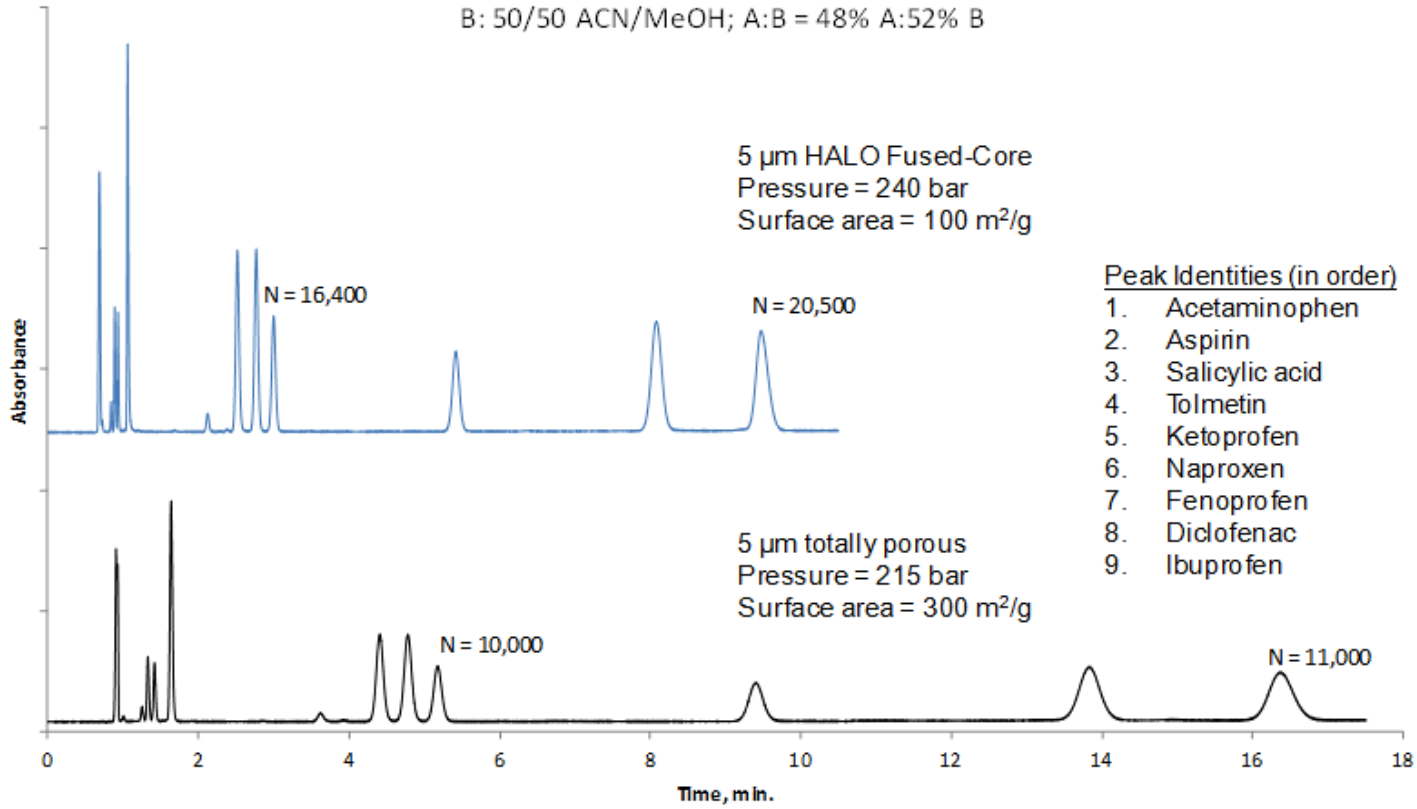



The 5 µm HALO fused-core particle has more than double the number of plates/pressure of the 5 µm totally porous particles and four times the number of plates/pressure of the 3 µm totally porous particles. Data on 4.6 x 150 mm columns at the plate height minimum, except for 1.8 µm particle (estimated).

# 5 µm Halo Particle Utility

## 5 µm HALO Fused-core vs. 5 µm Totally Porous: NSAIDs

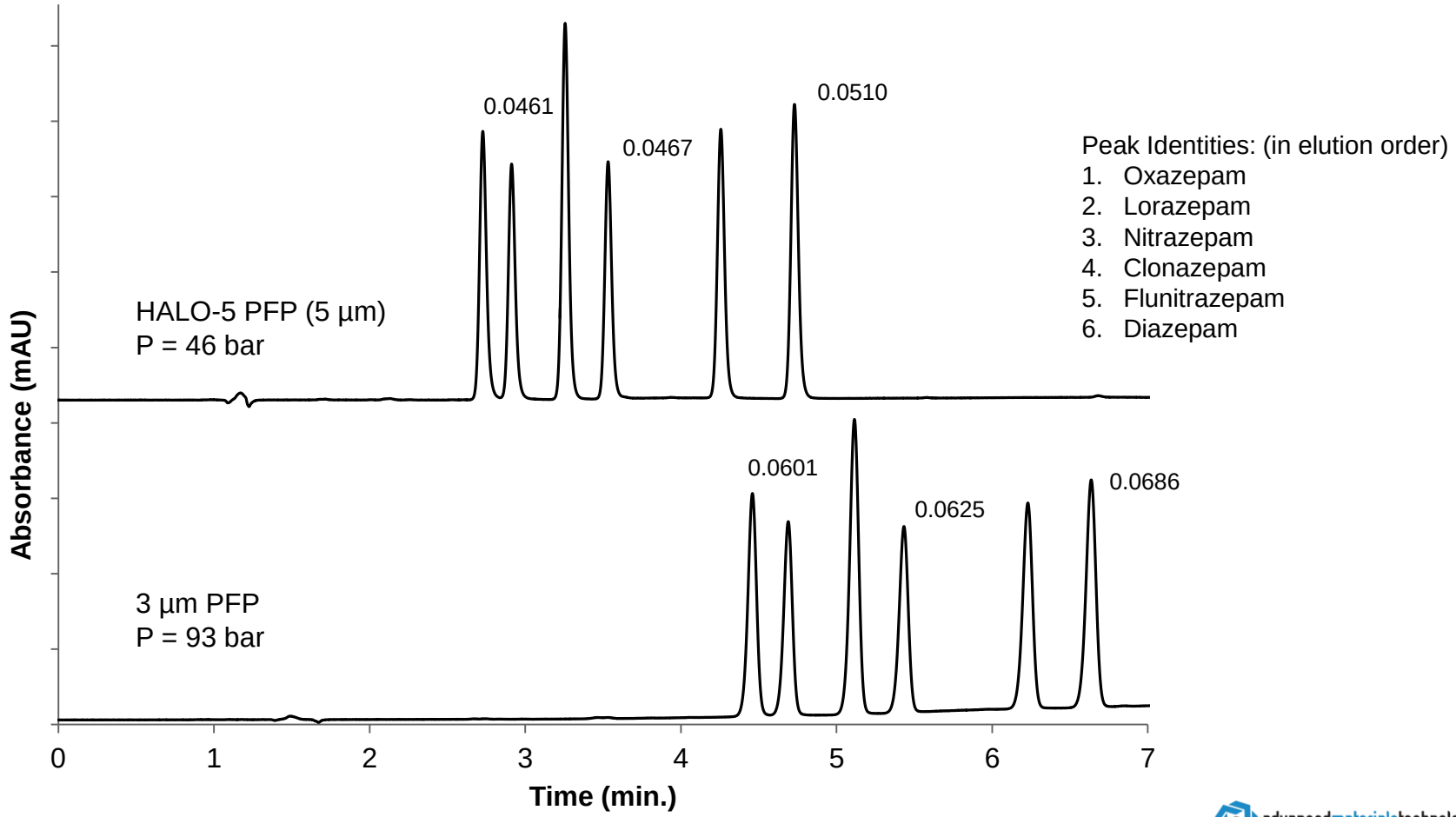
Columns: 4.6 x 150 mm Instrument: Shimadzu Prominence UFLC XR
Flow rate: 2.0 mL/min, Injection Volume: 2 µL,
Detection: 254 nm; Temperature = 35 °C
Mobile Phase: A: 20 mM pH 2.5 Potassium Phosphate
B: 50/50 ACN/MeOH; A:B = 48% A:52% B



# Separation of TCAs on PFP Phases

Column: 4.6 x 100 mm; Temperature: 35ºC; Flow: 0.75 ml/min; Detector: UV @ 254 nm; 1 µL inj.

Mobile Phase: A- 25 mM Ammonium Acetate, pH 5.5, B- AcN; 36-65% B in 7 min.

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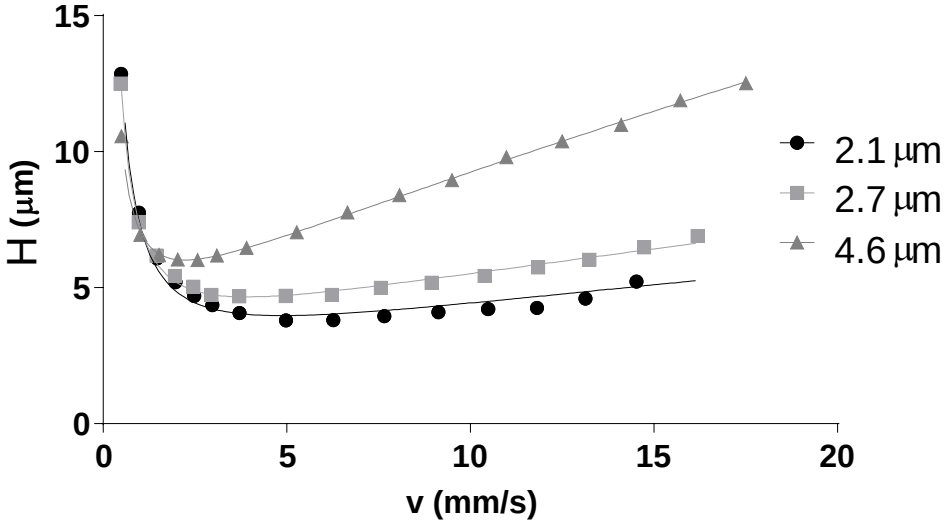
# 2 µm Halo Particle Utility

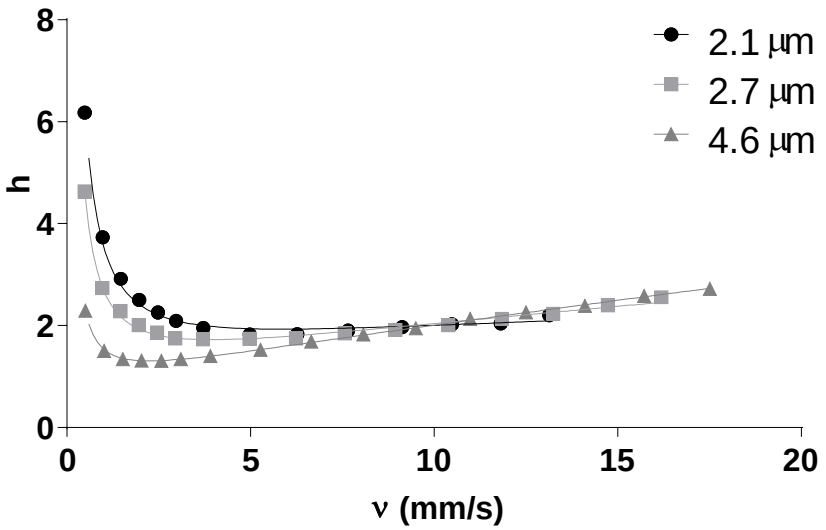
Column: 4.6 x 50 mm Halo C18; Mobile phase: 50%AcN/50% Water; Temperature: 25ºC; Detector: UV @ 260 nm; Nexera, PDA, Measurements for 1-Cl-4-nitrobenzene

1.4 µm Core, 0.33 µm Radial Shell, 90 Å pore size

**Column Efficiency Comparisons Varying Particles**




**Column Reduced Efficiencies Fitted to Knox**



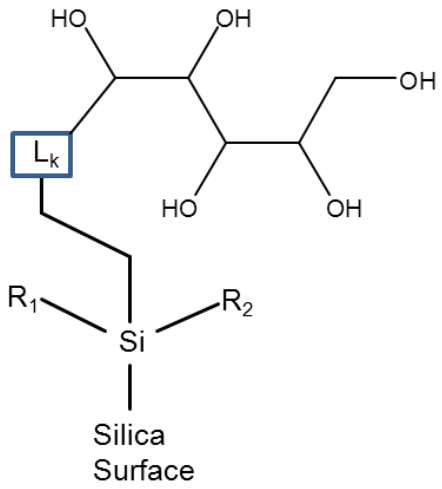
# Halo Penta-HILIC

## Opportunities

- HILIC may be an attractive alternative for difficult polar analyte separations
- HILIC separation speed and robustness may be improved
- New bonded phases may broaden applications served by HILIC

## Development and Characterization of Fused-Core HILIC BP

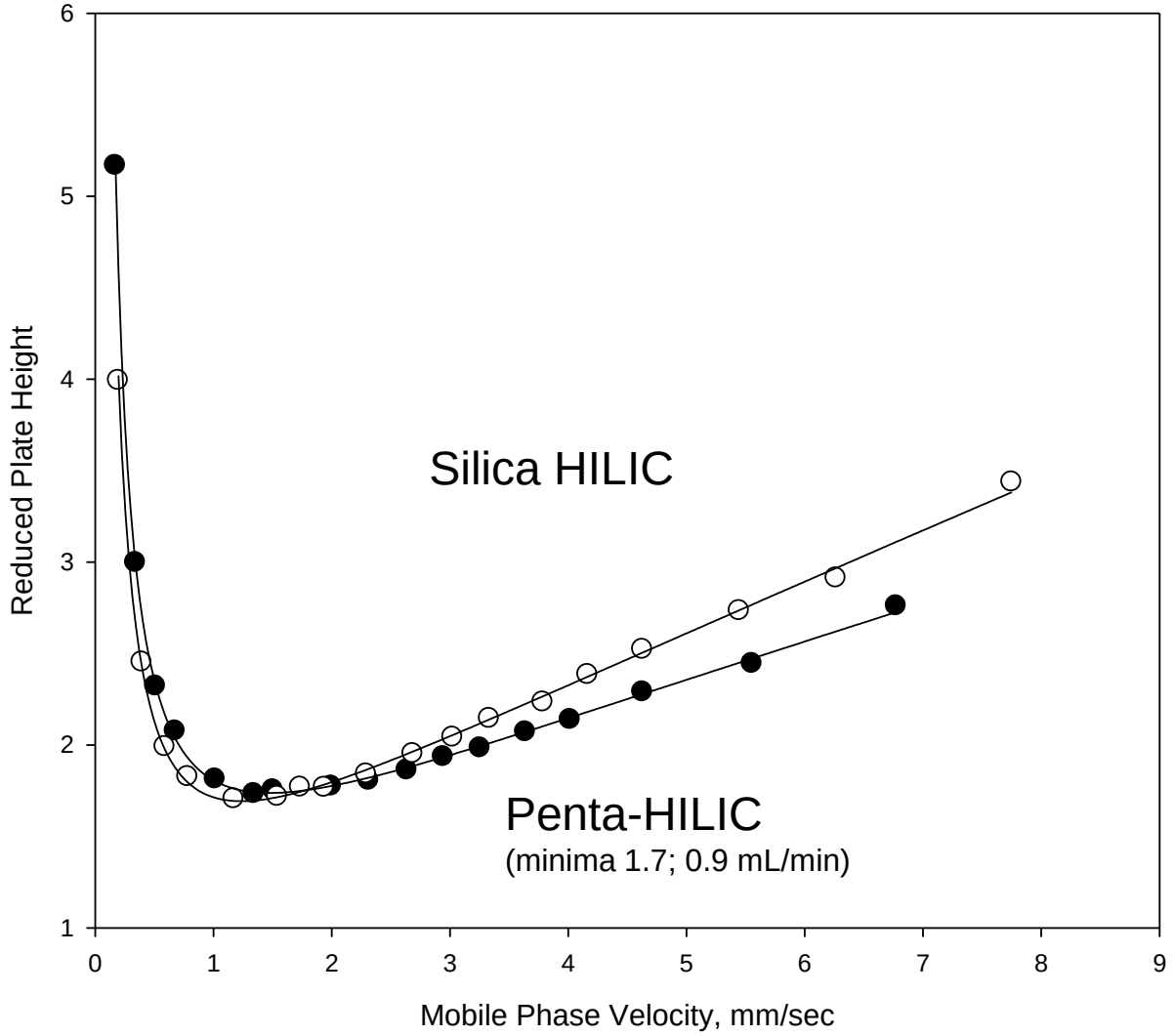
- HILIC retention is complex
- Highly hydroxylic bonded phase is appealing
- Polymeric bonded phases are likely to limit mass transfer kinetics – prefer use of a monolayer silane



# *Effect of Linear Velocity on Penta-HILIC Column Efficiency*

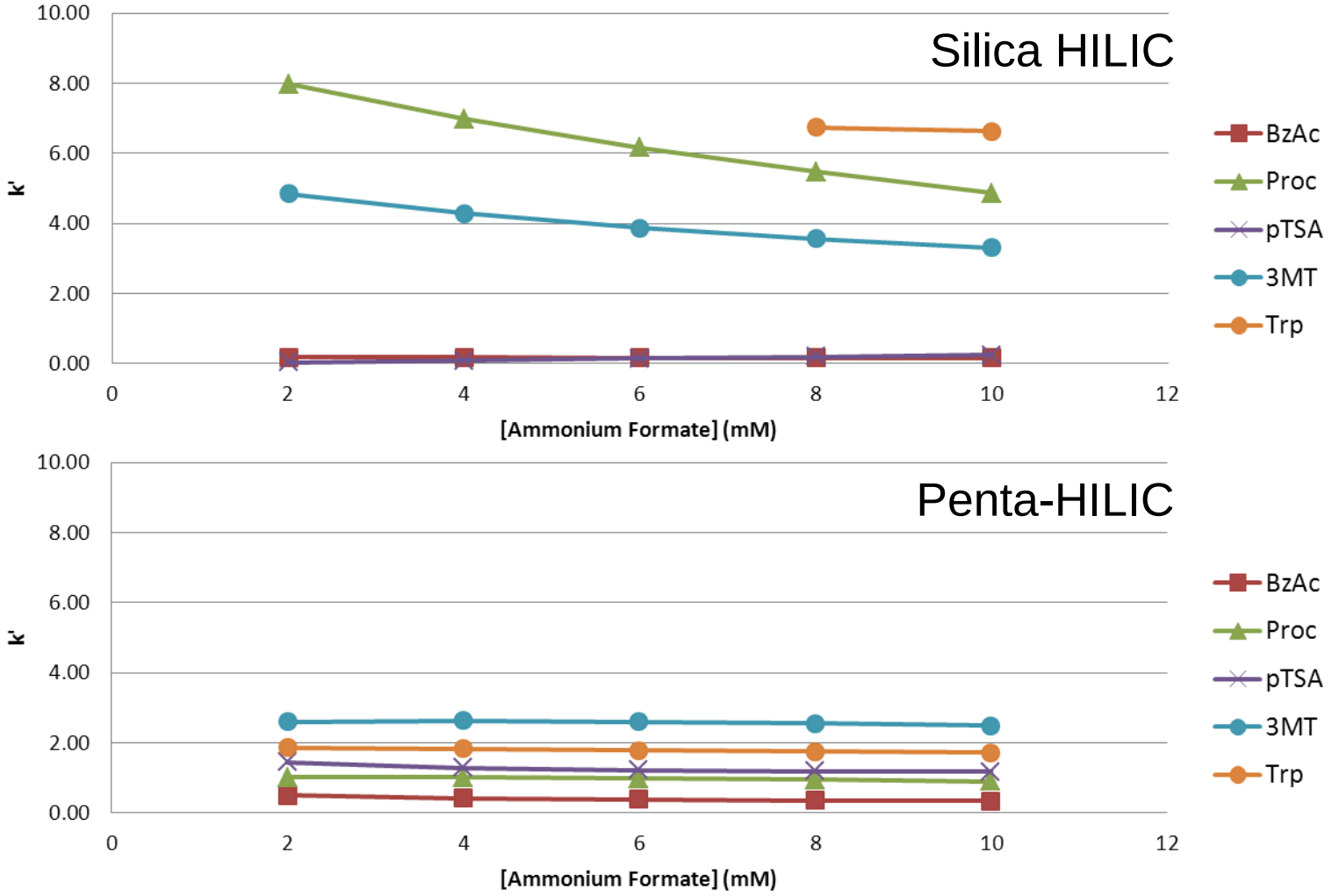
***4.6 mm ID x 50 mm; 90% AcN/10 mM $NH_4$Form 3.0, 25 °C; 1 μL, 50 ng Adenosine***

Data fitted to Knox Equation



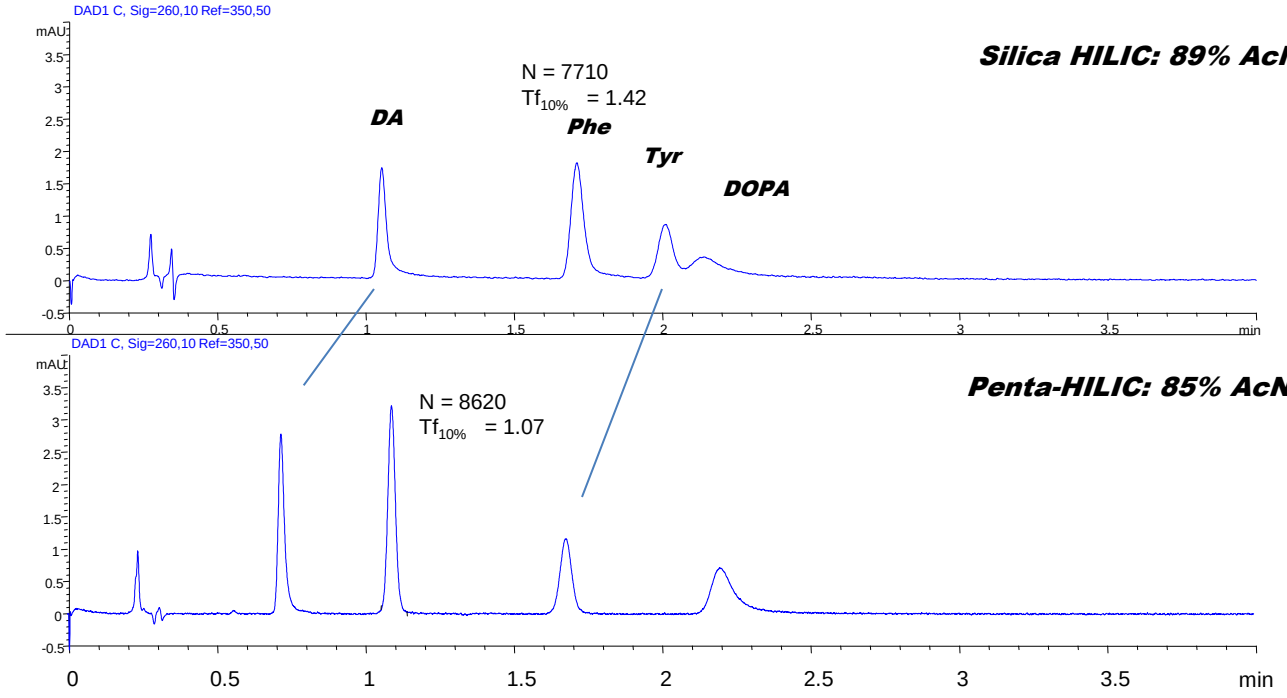
# *Effect of Buffer Concentration on HILIC Separations*

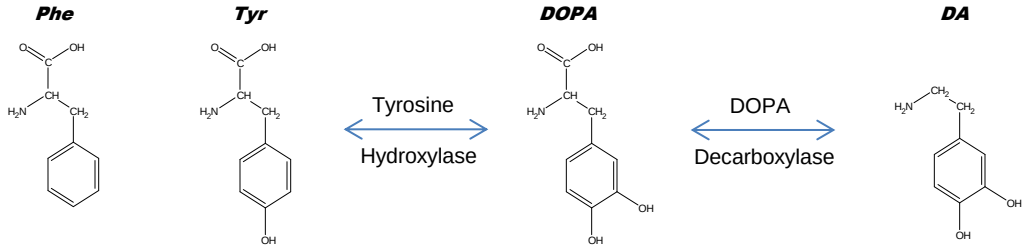
## *90% Acetonitrile/$NH_4$Form, pH 3.0: 2.1 mm ID x 100 mm, 25 °C, 0.5 mL/min*



# *High Speed HILIC Separation of Catecholamines and Amino Acids*

***4.6 mm ID x 50 mm; 2 mL/min., 85% AcN/10 mM $NH_4$Form 3.0, 25 °C; 3 μL inj***



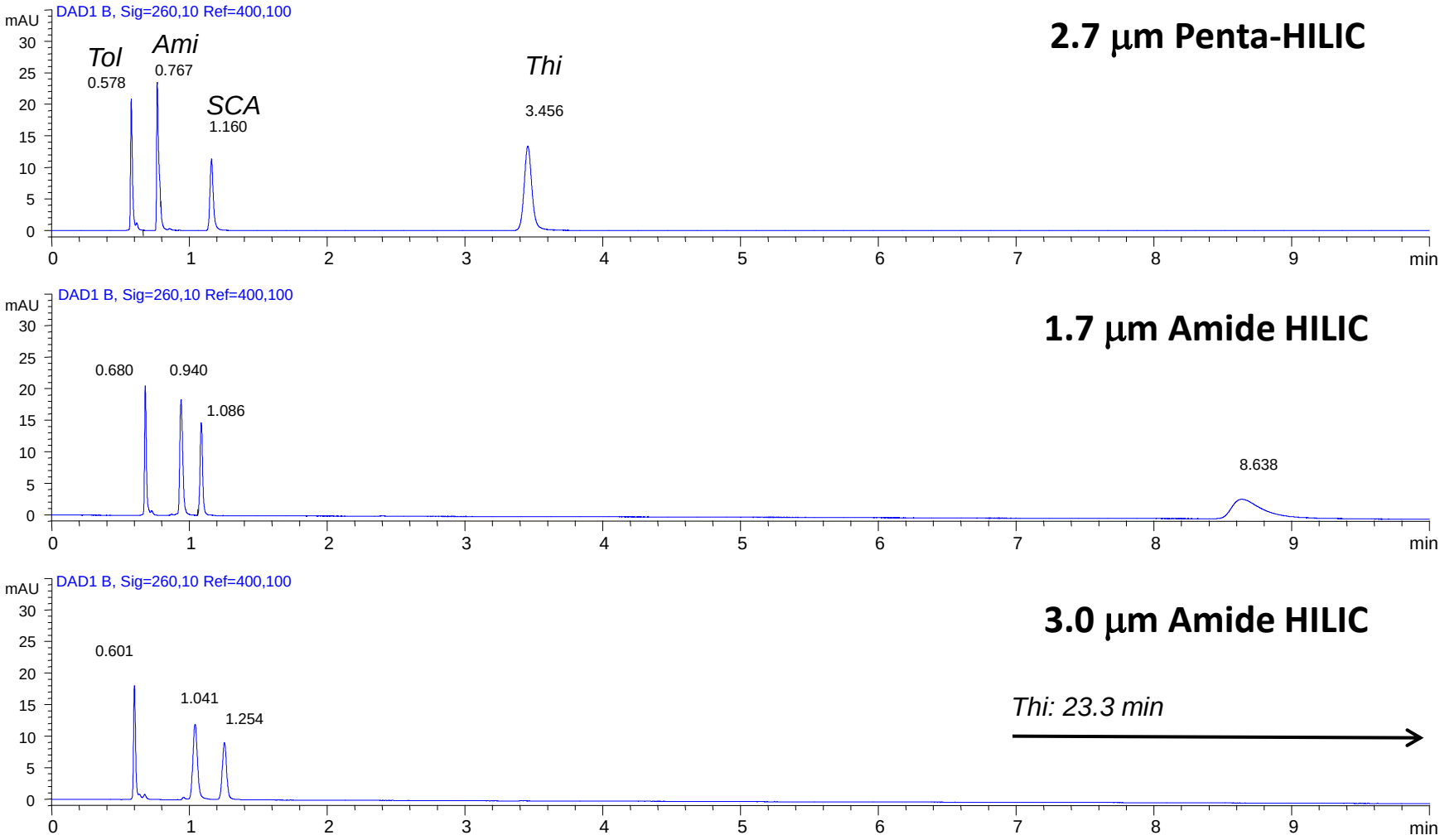

*Note: at 85% AcN all k' lower on Silica than on Penta-HILIC.*

# *Comparative Retention of HILIC Columns*

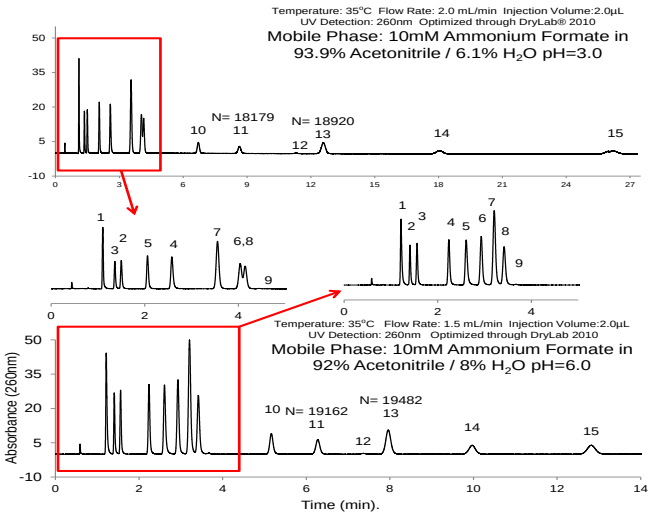
***2.1 mm ID x 150 mm***

***Test: 90% AcN/10 mM $NH_4$Form pH 3.0, 0.5 mL/min, 23 °C***

***Toluene/Amitriptyline/Salycic Acid/Thiamine***

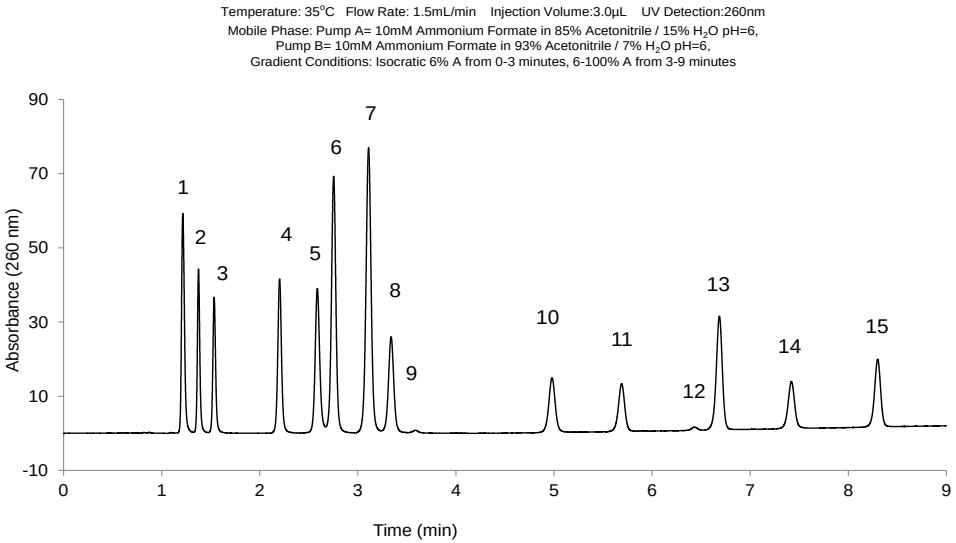


# Halo Penta-HILIC Separation of Nucleosides and Nucleobases




| | Compounds |
|---|---|
| 1 | Thymine |
| 2 | Uracil |
| 3 | Thymidine |
| 4 | 2'-Deoxyadenosine |
| 5 | Adenine |
| 6 | Uridine |
| 7 | Adenosine |
| 8 | Hypoxanthine |
| 9 | Xanthine |
| 10 | Cytosine |
| 11 | 2'-Deoxycytidine |
| 12 | Guanine |
| 13 | 2'-Deoxyguanosine |
| 14 | Cytidine |
| 15 | Guanosine |

LC-MS Compatible Gradient with Ammonium Formate pH=6




4.6 mm ID x 100 mm Penta-HILIC

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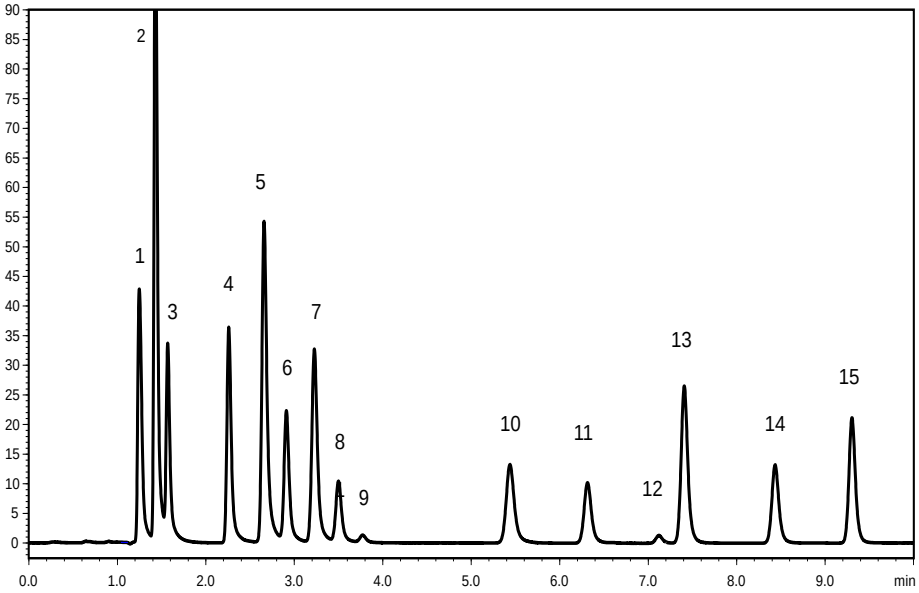
# Halo Penta-HILIC Separation of Nucleosides and Nucleobases

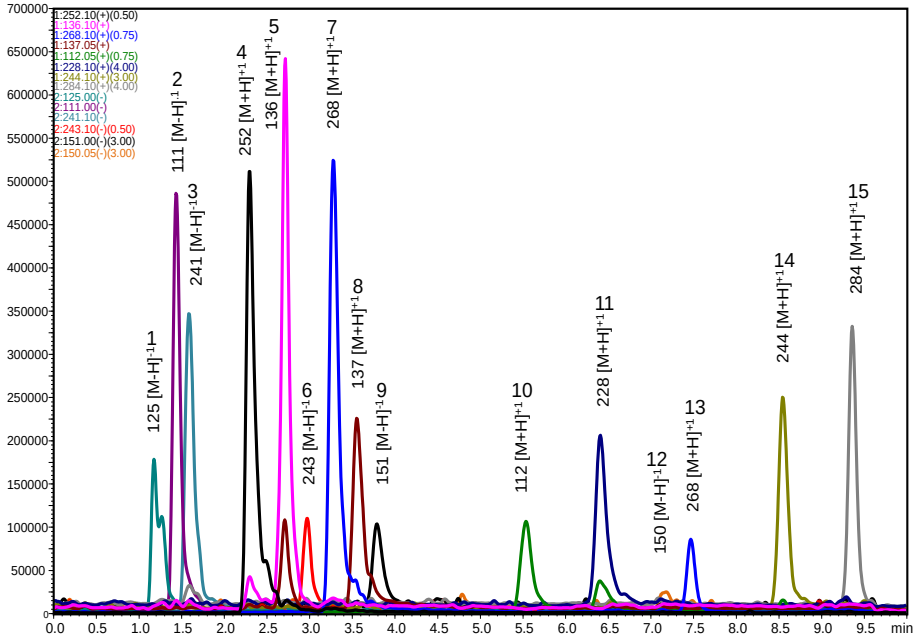
HILIC Separation with Coupled UV and MS Detection of Nucleosides and Nucleobases

Shimadzu Nexera LC: 2.1 mm ID x 100 mm Halo Penta HILIC column; 35°C ; Abs. 260 nm
Gradient: 6% B for 3.5 min, 65-100% B 3.5 to 9.5 min., hold 100% B for 0.5 min;
A – 10mM Ammonium Formate in 93% AcN/7% $H_2O$, pH 6.0; B – A at 85% AcN
Flow 0.31 mL/min 0-10 min, 1.0 mL/min (6%B) 10-16 min, return to initial
Sample: 2 uL of 15 purine and pyrimidine bases and –sides (as above)



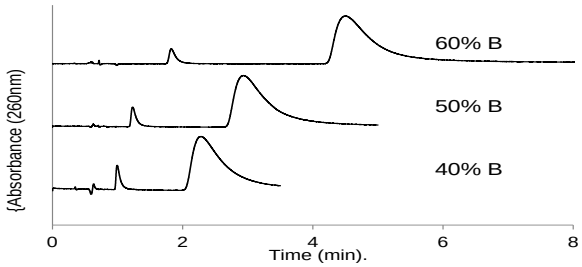

HILIC Separation with LC-ESI/MS Detection of Nucleoside and Nucleobase Molecular Ions

Shimadzu LCMS-2020 Single Quadrupole: ESI (+) 4.5 kV, (-) 3.5 kV; SIM 1 amu; 0.25 sec each SIM Event
(2 points/sec sample rate); 1.2 LPM $N_2$ neb; 12.5 LPM $N_2$ dry gas; Cap DL 300°C; Heat Block, 400°C



# Halo Penta-HILIC Separation of Nucleotides

Temperature: 35°C Flow Rate: 1.5 mL/min Injection Volume: 10.0µL UV Detection: 260nm
Mobile Phase: Pump A= 100mM Ammonium Formate pH=6.0
Pump B= Acetonitrile

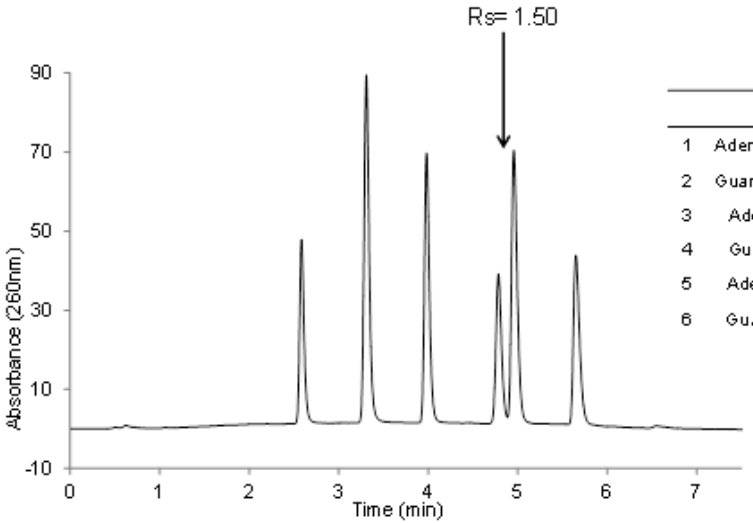



ATP

| % B | Tailing Factor 10% | Width Half-Height (min) |
|---|---|---|
| 60 | 3.062 | 0.576 |
| 50 | 2.782 | 0.523 |
| 40 | 2.417 | 0.480 |

## Ammonium Phosphate Mobile Phase

4.6 mm ID x 100 mm; Flow Rate: 1.5 mL/min; Temp. 35°C; Abs (260nm); 1.0 uL Inject
A- 12.5 mM Amm Phos (pH 6.0) in 50% AcN, B- 6.25 mM Amm Phos (pH 6.0) in 25% AcN
Gradient 90%-40% B over 8 min.




| Elution Order | | Tailing Factor 10% |
|---|---|---|
| 1 | Adenosine Monophosphate | 1.265 |
| 2 | Guanosine Monophosphate | 1.225 |
| 3 | Adenosine Diphosphate | 1.244 |
| 4 | Guanosine Diphosphate | 1.198 |
| 5 | Adenosine Triphosphate | 1.294 |
| 6 | Guanosine Triphosphate | 1.397 |

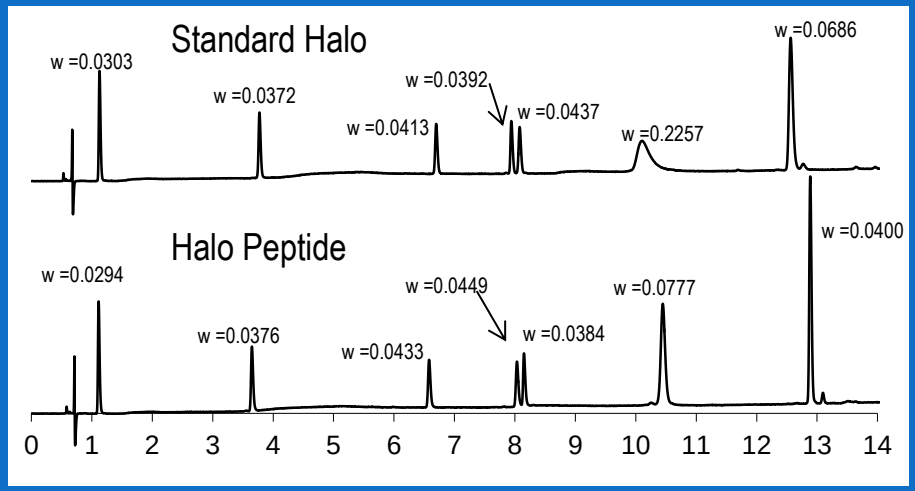
Retention of nDP's and nTP's overlap. Above is an example for limiting resolution for GDP and ATP (Rs=1.5). Overall the ammonium phosphate buffer shows excellent peak shape and allows resolution between the purine mono- ,di-, and trinucleotides.

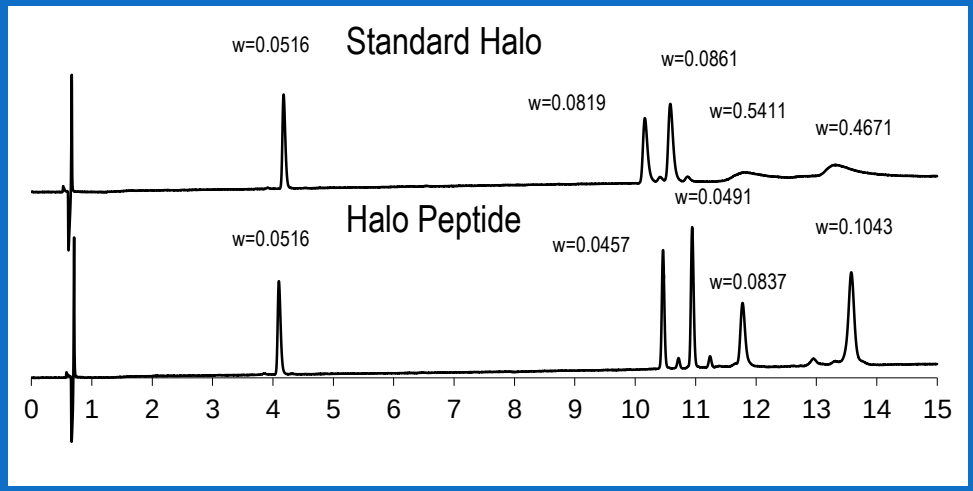
# Recent Developments for Larger Molecules

- 160 Å pore size Reversed-Phase for peptides and small proteins
  - Typical TFA Conditions
  - LC/MS for Proteomics
    - Ammonium Formate Conditions
    - Utility for Protein ID Workflow
- 400 Å pore size RP for Intact Proteins
  - Definition
  - Advantage for Larger Proteins
- Penta-HILIC for N-Linked Glycan and Glycopeptide Analysis

# Halo Peptide ES-C18
# Protein and Peptide Separations

Column: 4.6 x 100 mm; Flow rate: 1.5 mL/min; Temperature: 30° C
A: 0.1% TFA/10% ACN, B: 0.1% TFA/70% ACN
Gradient: 0% to 50% B in 15 min.; Injection volume: 5 µL

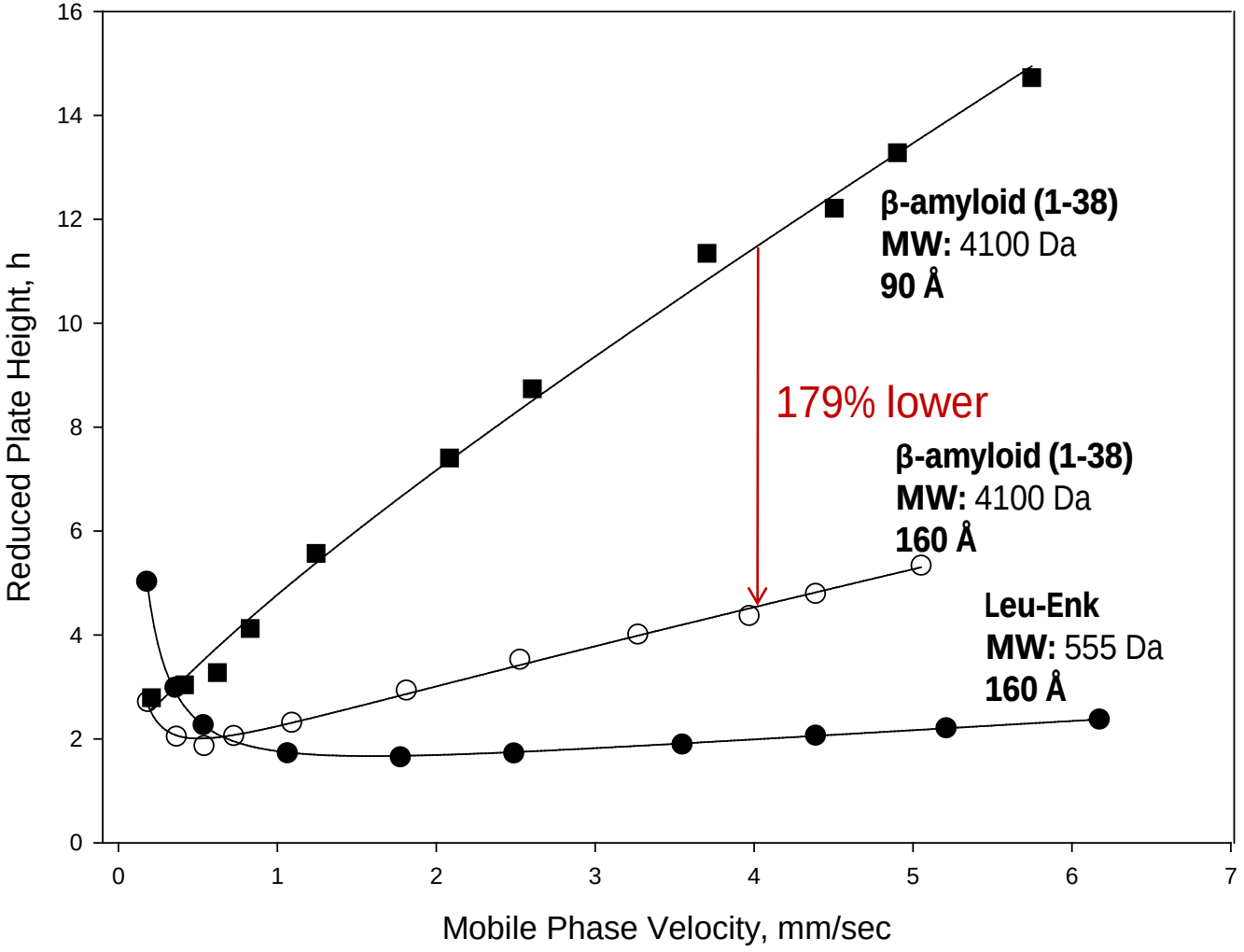



**Sample 1**
Gly-Tyr, Val-Tyr-Val, Met-enk, Angiotensin II, Leu-enk
Ribonuclease, Porcine Insulin

**Sample 2**
Leu-enk
Bovine Insulin, Human Insulin, Cytochrome C, Lysozyme

# Effect of Pore Size on Efficiency



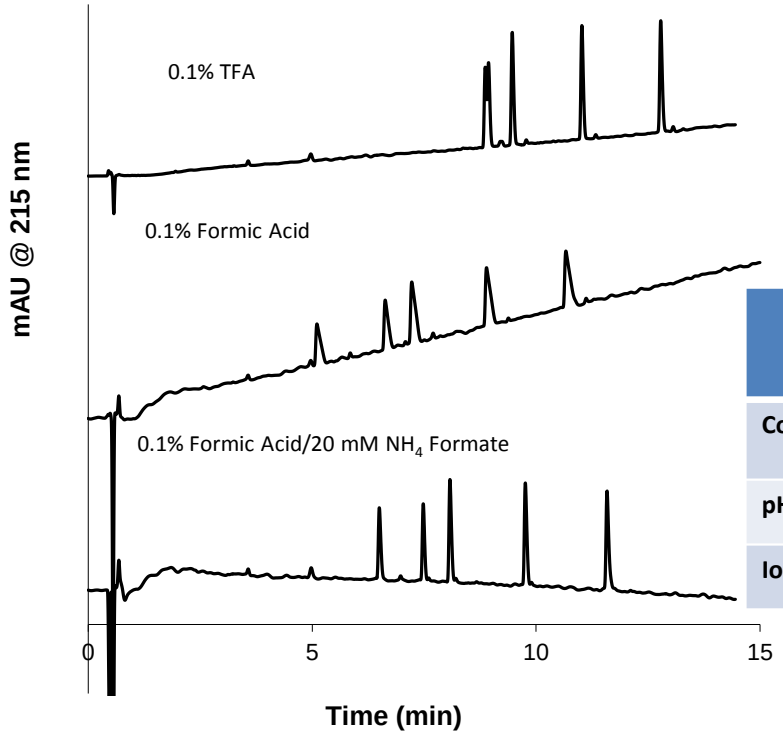

**Columns:** 4.6 x 100 mm HALO C18, 2.7 µm, 90 Å
4.6 x 100 mm HALO Peptide ES-C18, 2.7 µm, 160 Å

**Mobile Phase:** Leu-Enk: 21% ACN/79% Water/0.1% TFA
β-amyloid (1-38) 160 Å : 29% ACN/71% Water/0.1% TFA
β-amyloid (1-38) 90 Å : 27% ACN/73% Water/0.1% TFA

**Temperature:** 60 °C
**Detection:** 215 nm

# Recent Developments for Larger Molecules

## Improving Retention and Peak Shape Using Ammonium Formate




Column: Halo Peptide ES-C18, 4.6 x 100 mm; Flow rate: 2.0 mL/min; T= 30 C;
A: Water/acid modifier; B: ACN/0.1% TFA or Formic Acid
Gradient: 1.5% to 26% B in 15 min.; Injection: 8 µL (800 ng) of synthetic peptides S1-S5

**Synthetic Peptide Retention Standards:**

S1 RGAGGLGLGK-Am
S2 Ac-RGGGGLGLGK-Am
S3 Ac-RGAGGLGLGK-Am
S4 Ac-RGVGGLGLGK-Am
S5 Ac-RGVVGLGLGK-Am

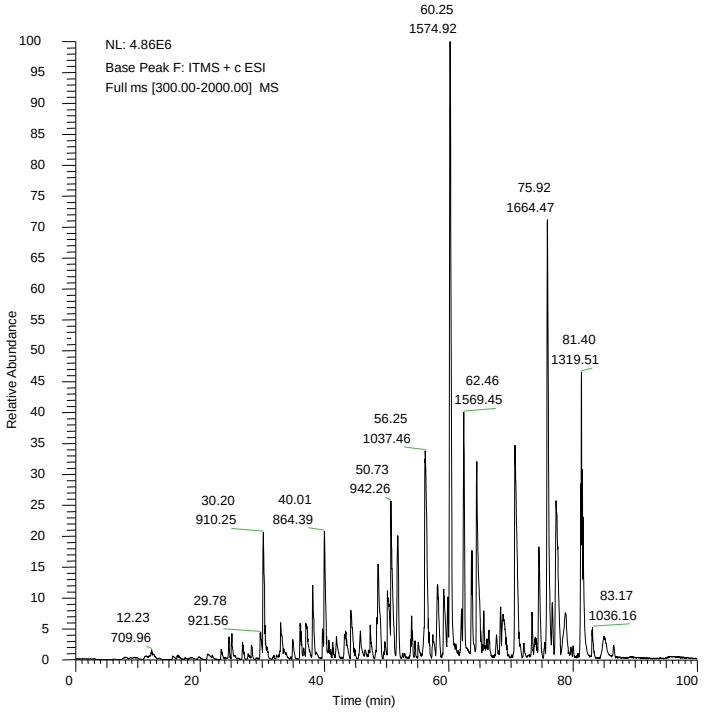
| | TFA 0.1% | Formic Acid 0.1% | Ammonium Formate | AF/FA |
|---|---|---|---|---|
| **Concentration** | **0.013 M** | **0.026 M** | **0.020 M** | **20 mM AF 26 mM FA** |
| **pH** | **1.8** | **2.7** | **c. 7** | **3.3** |
| **Ionic strength** | **26 mM** | **4.4 mM** | **40 mM** | **c. 44 mM** |

McCalley, D. V., Effect of buffer on peak shape of peptides in reversed-phase high performance liquid chromatography. *J Chromatogr* **2004,** *1038* (1-2), 77-84.
Schuster, S. A.; Boyes, B. E.; Wagner, B. M.; Kirkland, J. J., Fast high performance liquid chromatography separations for proteomic applications using Fused-Core® silica particles. *J Chromatogr* **2012,** 1228, 232-241.

# Recent Developments for Larger Molecules

## Efficient Proteomic Analysis using Halo Peptide ES-C18

Column: 0.2 x 150 mm Halo Peptide ES-C18; Flow: 4 µL/min
Gradient: 2 - 56% B in 85 min; Pmax - 320 bar;
A: 0.1% formic acid/10 mM AF/water; B: 80% acetonitrile/A;
Sample: 5 pmol transferrin, carbonic anhydrase, and apomyoglobin digest mixture
Detection: Thermo LTQ Ion Trap MS/Michrom ESI interface




### Canine Prostate: Identification of Soluble Proteins

| Mobile Phase Modifier | Protein IDs | Matched Spectra | Peptide IDs | Spectra/Peptide ID |
|---|---|---|---|---|
| 0.1% Formic Acid | 70 | 1142 | 359 | 3.18 |
| 0.1% FA, 10mM Ammonium Formate | 118 | 2028 | 538 | 3.77 |

≈ 50%

| Mobile Phase Modifier | X Bar Peak Width (s) | Standard Deviation | Measured Peak Capacity |
|---|---|---|---|
| 0.1% Formic Acid | 16.11 | 2.98 | 76.30 |
| 0.1% FA, 10mM Ammonium Formate | 13.99 | 2.35 | 99.55 |

≈ 30%

- Protein IDs: validated using 5% protein false discovery rate
- Peptide IDs: validated using 5% peptide false discovery rate

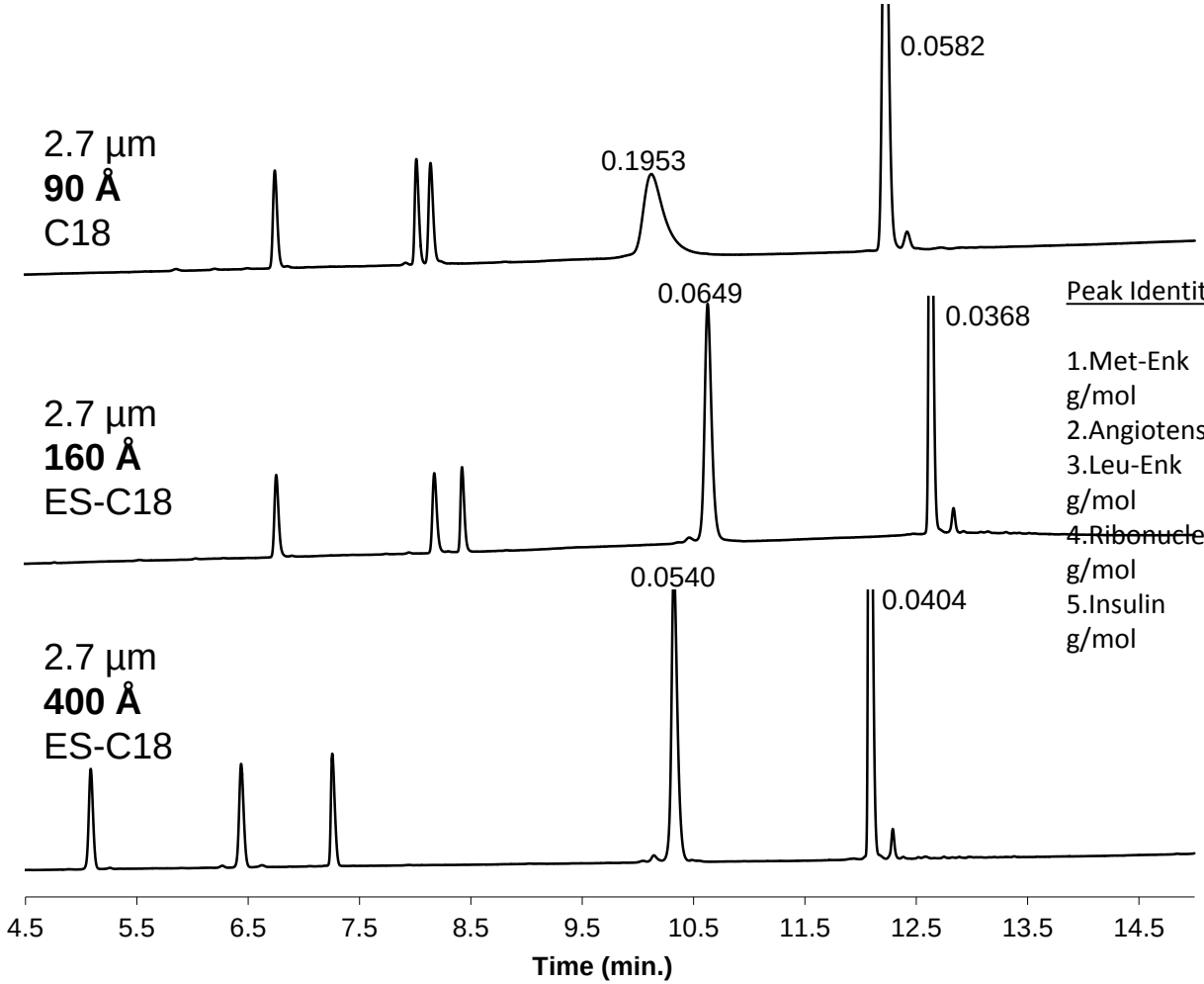
# Recent Developments for Larger Molecules

| Fused-Core Particle | Particle Size, µm | Pore Size, Å | BET Surface Area, $m^2/g$ | Shell Thickness, µm | % Porosity | Pore volume, $cm^3/g$ |
|---|---|---|---|---|---|---|
| Halo | 2.7 | 90 | 135 | 0.5 | 75 | 0.26 |
| Halo Peptide | 2.7 | 160 | 80 | 0.5 | 75 | 0.29 |
| Wide-pore | 2.7 | 400 | 30 | 0.35 | 59 | 0.23 |
| Wide-pore | 3.4 | 400 | 15 | 0.2 | 31 | 0.11 |

Wagner, Schuster, Boyes, Kirkland (2012) J. Chromatogr. 1264, 22-30.

# Recent Developments for Larger Molecules

Columns: 4.6 x 100 mm; Instrument: Agilent 1100; Flow rate: 1.5 mL/min; Injection Volume: 5 µL; Detection: 220 nm;
Mobile Phase: A: 10% ACN / 0.1% TFA; B: 70% ACN / 0.1% TFA; Gradient: 0% B to 50% B in 15 min.; Temperature = 30 $^{o}$C




Peak Identities: In order

1. Met-Enk MW = 573.7 g/mol
2. Angiotensin II MW = 1046.2 g/mol
3. Leu-Enk MW = 555.6 g/mol
4. ~~Ribonuclease~~ A MW = 13700 g/mol
5. Insulin MW = 5800 g/mol

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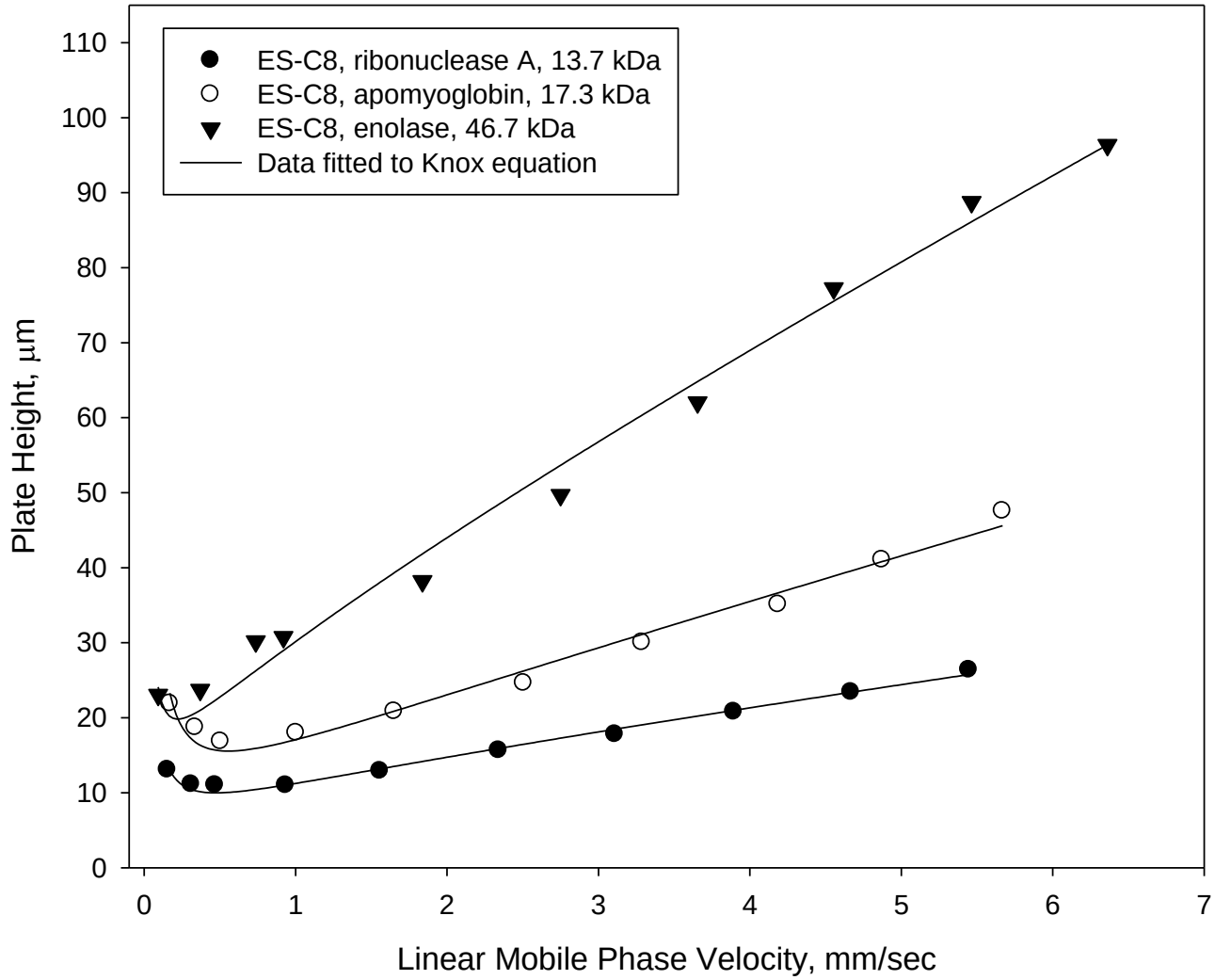
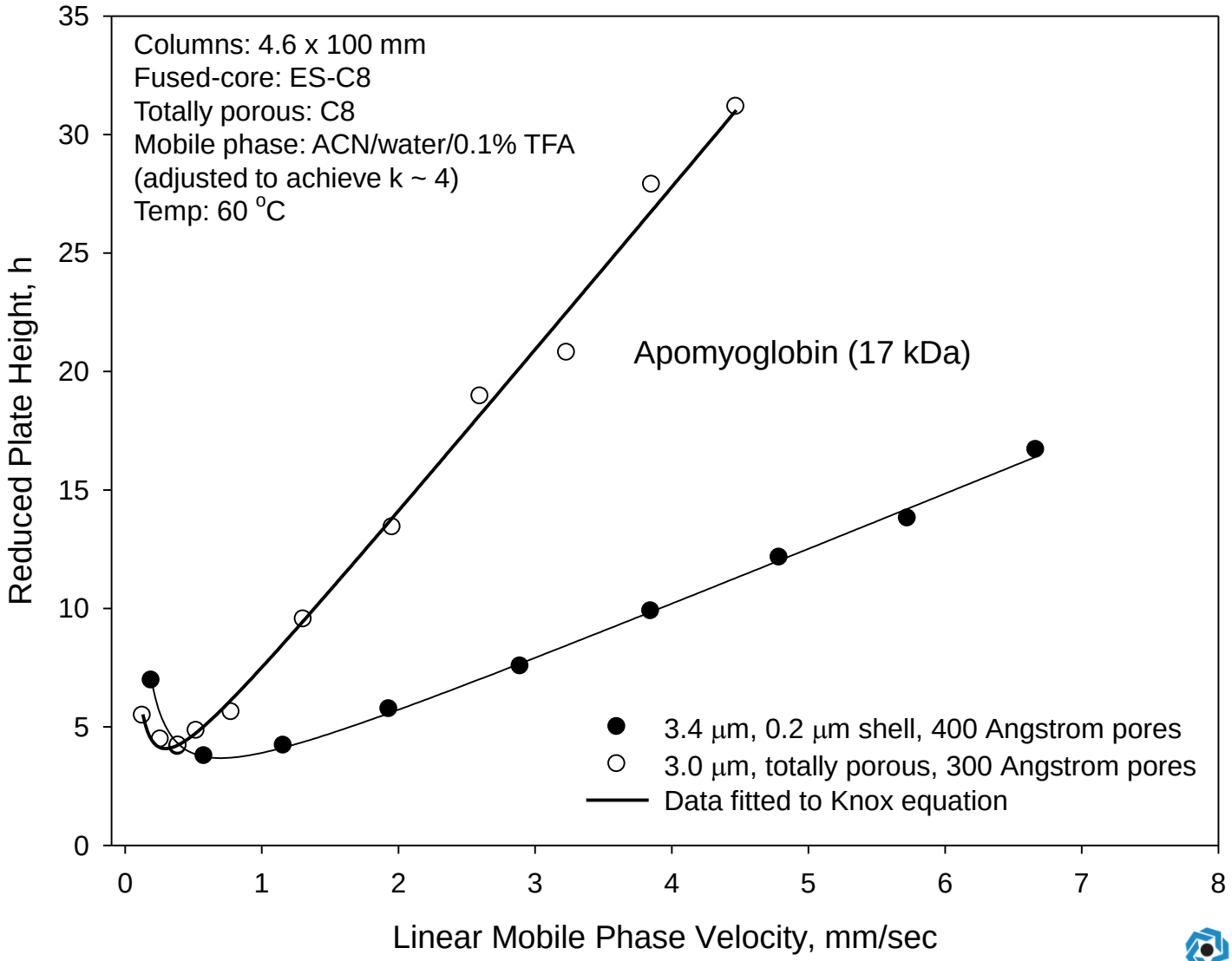
# Mass Transfer Dependence on Protein Size

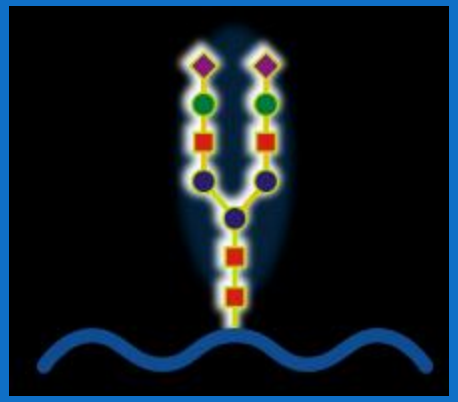
Column: 4.6 x 100 mm , ES-C8, 400 Å, 2.7 µm; Mobile phase: ACN/water/0.1% TFA (adjusted to achieve k ~ 3); Temp: 60 $^{o}$C



# Improved Mass Transfer for Halo 400



# *Protein N-linked Glycan Analysis*



- Release of glycoprotein Asn-linked glycans by PNGase F is a well established approach, allow analysis of complex mixtures of glycans by a variety of methods.

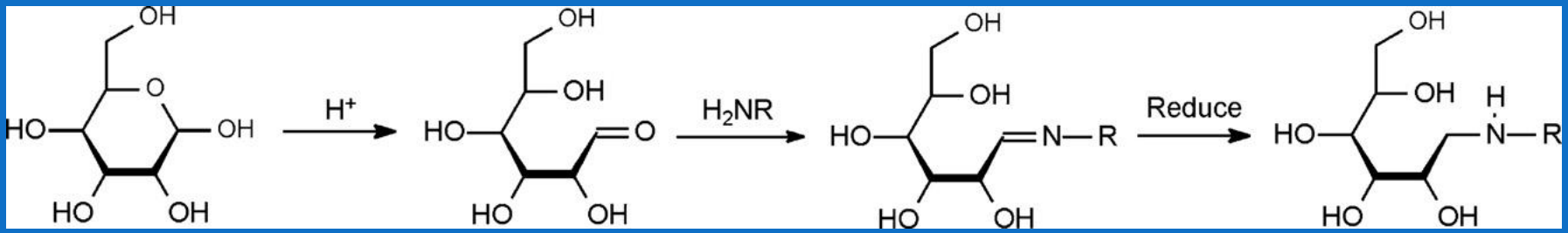
- Various additional exoglycosidases and endoglycosidases can be helpful in structure elucidation.

- Considerable work has been accomplished for N-linked analysis of "native" glycans, as well as permethylated glycans using MS (ToF and ESI), and MS coupled to high resolution separation methods, particulary CE and LC.

- Glycoprofiling of N-linked glycans, using HILIC (NP) combined with fluorescence detection and/or MS is becoming a standard for biopharmaceutical glycoproteins, including antibodies.

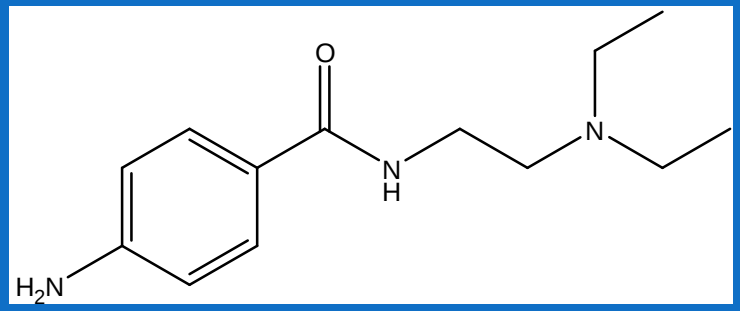
# *Analysis of PNGase Released and Labeled N-Glycans*

Release of protein N-linked glycans using PNGase F releases oligosaccharides with a free reducing terminus (alditol) that is readily labeled by amines via formation of a Schiff's base, which can be reduced readily.




Many amines have been applied to labeling glycans, (Harvey, 2011, J. Chrom. 879).
In the current work Procainamide is favored due to reported improvements in ESI-MS detection (Klapoetke, et al., 2010, J. Pharm. Biomed. Anal. 53)

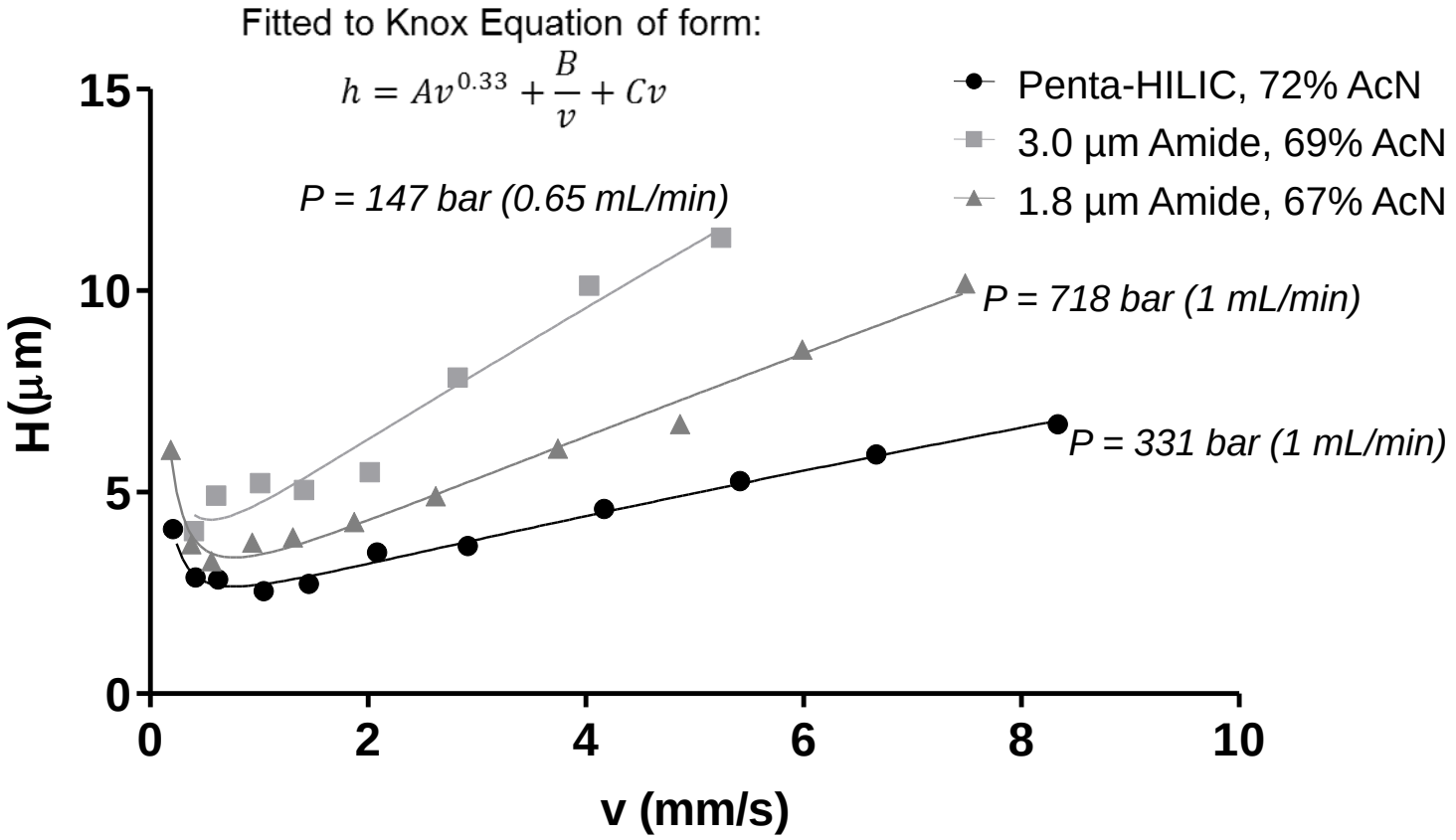
Typical Labeling Conditions
Glycan in water (up to 10% volume)
90+% volume of:
0.4 M procainamide
1M sodium cyanoborohydride
in 30% acetic acid/70% DMSO
12-16 hr reaction at 37°C
SEC cleanup on Sephadex G-10 minicolumn
Absorbance Detection 300 nm or Fluorescence Ex 330/Em 380 nm




Mass: glycan + 219.32

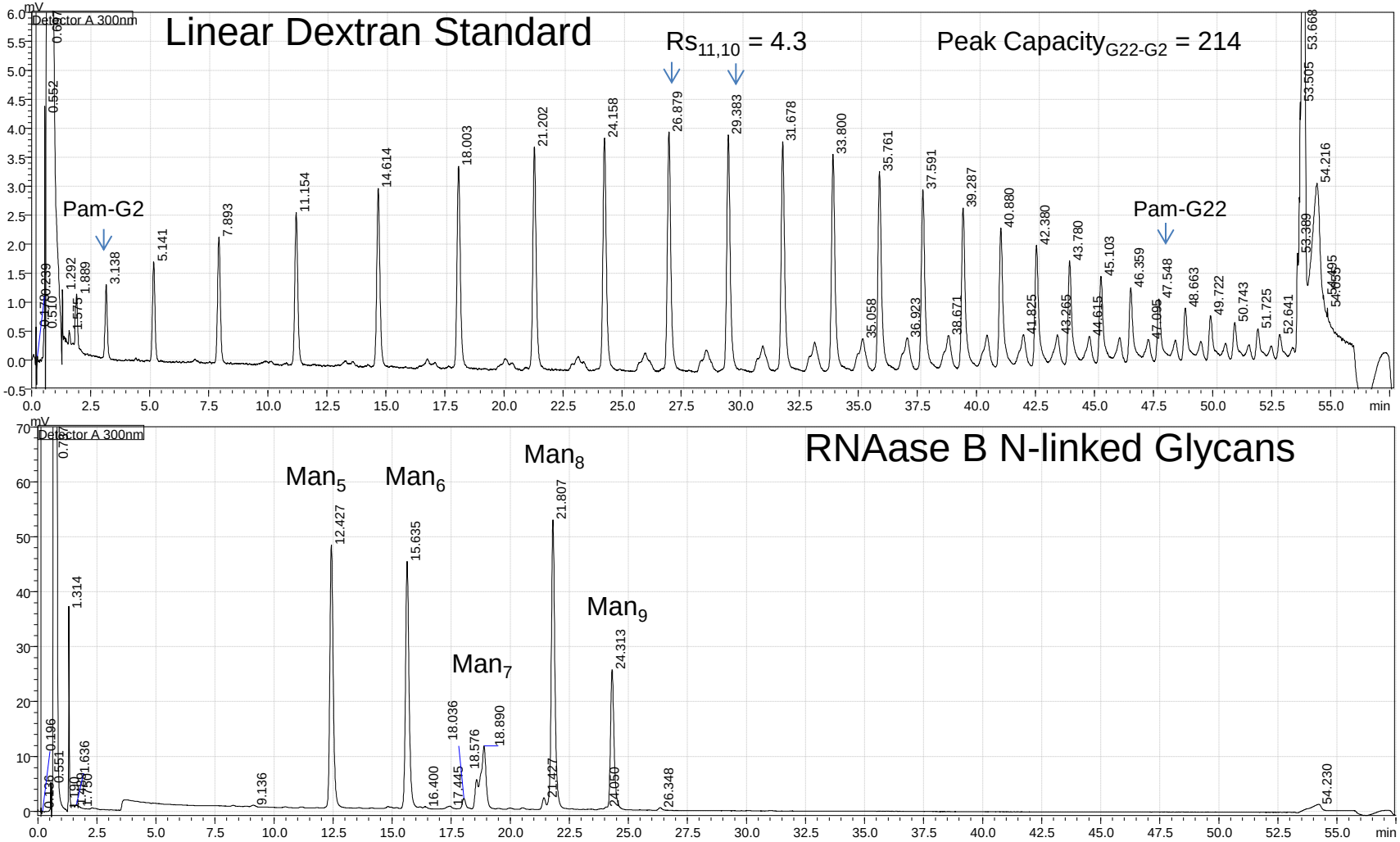
# *Column Efficiency Comparisons using Pam-$G_5$*

***2.1 (2.0 mm) ID x 150 mm, 60°C, k' ≈ 6,***
***50 mM Ammonium Formate Aqueous, pH 4.4***
***0.5 uL Injection (50 pmol), Abs. 300 nm***



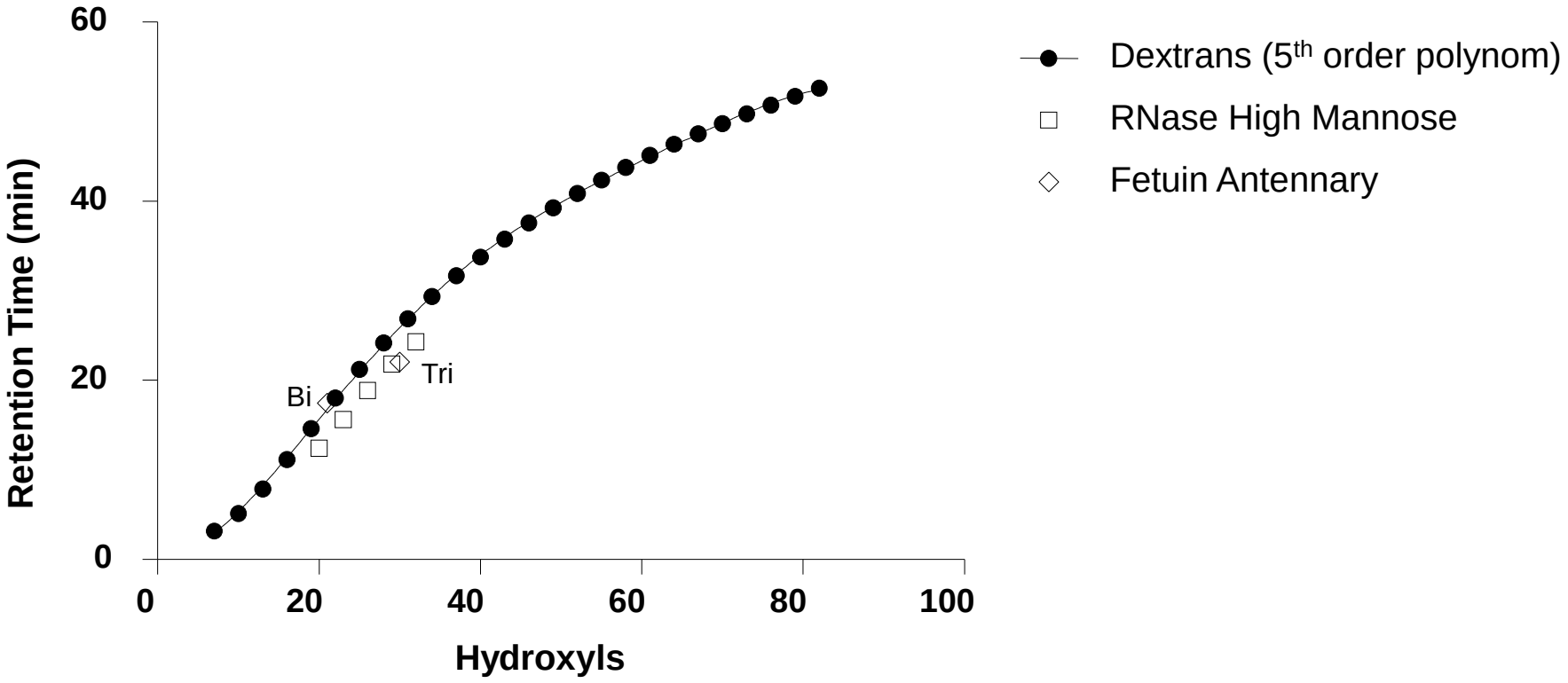
# *Penta-HILIC Separations of Labeled Oligosaccharides and Glycans*

***2.1 mm ID x 150 mm; 50 mM Ammonium Formate, pH 4.4, 77.5-56.5% AcN (B) in 52.5 min., 60°C; 600 µL/min.***
***Detection: 300 nm Abs; ESI-MS (MS-2020, (+) 4.2 kV, 400-2000 with SIM)***



# *Penta-HILIC Retention of Labeled Oligosaccharides and Glycans*

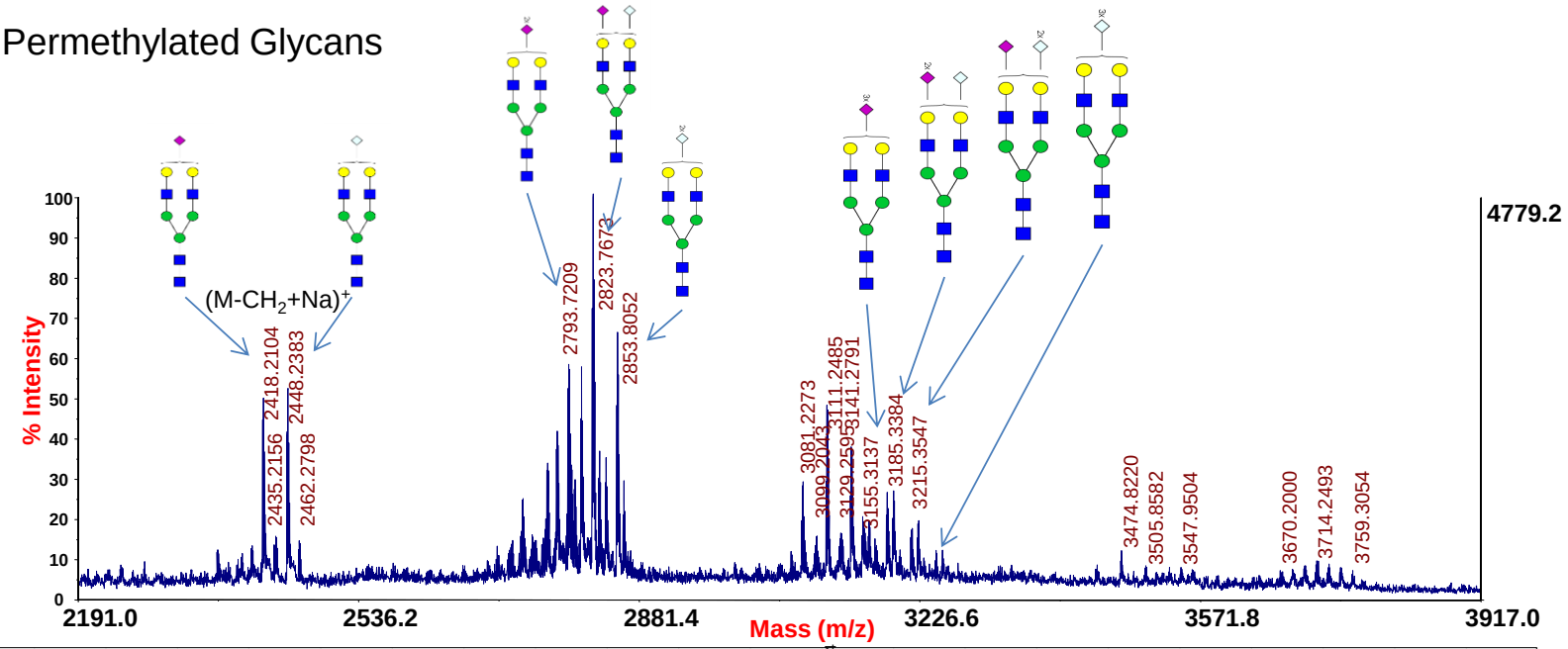
***2.1 mm ID x 150 mm; 50 mM Ammonium Formate, pH 4.4, 77.5-56.5% AcN (B) in 52.5 min., 60°C; 600 µL/min.***
***Detection: 300 nm Abs; ESI-MS (MS-2020, (+) 4.2 kV, 400-2000 with SIM)***

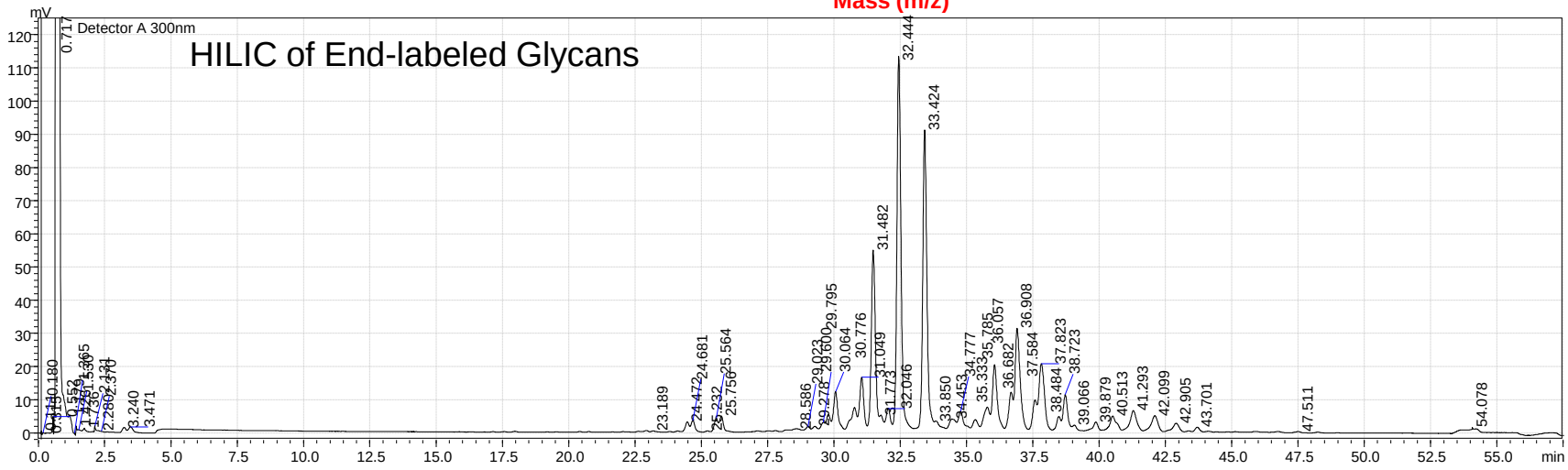


# *Penta-HILIC Separations of Abundant bov. a1-AGP N-glycans*

***2.1 mm ID x 150 mm; 50 mM Ammonium Formate, pH 4.4, 77.5-56.5% AcN (B) in 52.5 min., 60°C; 600 µL/min.***

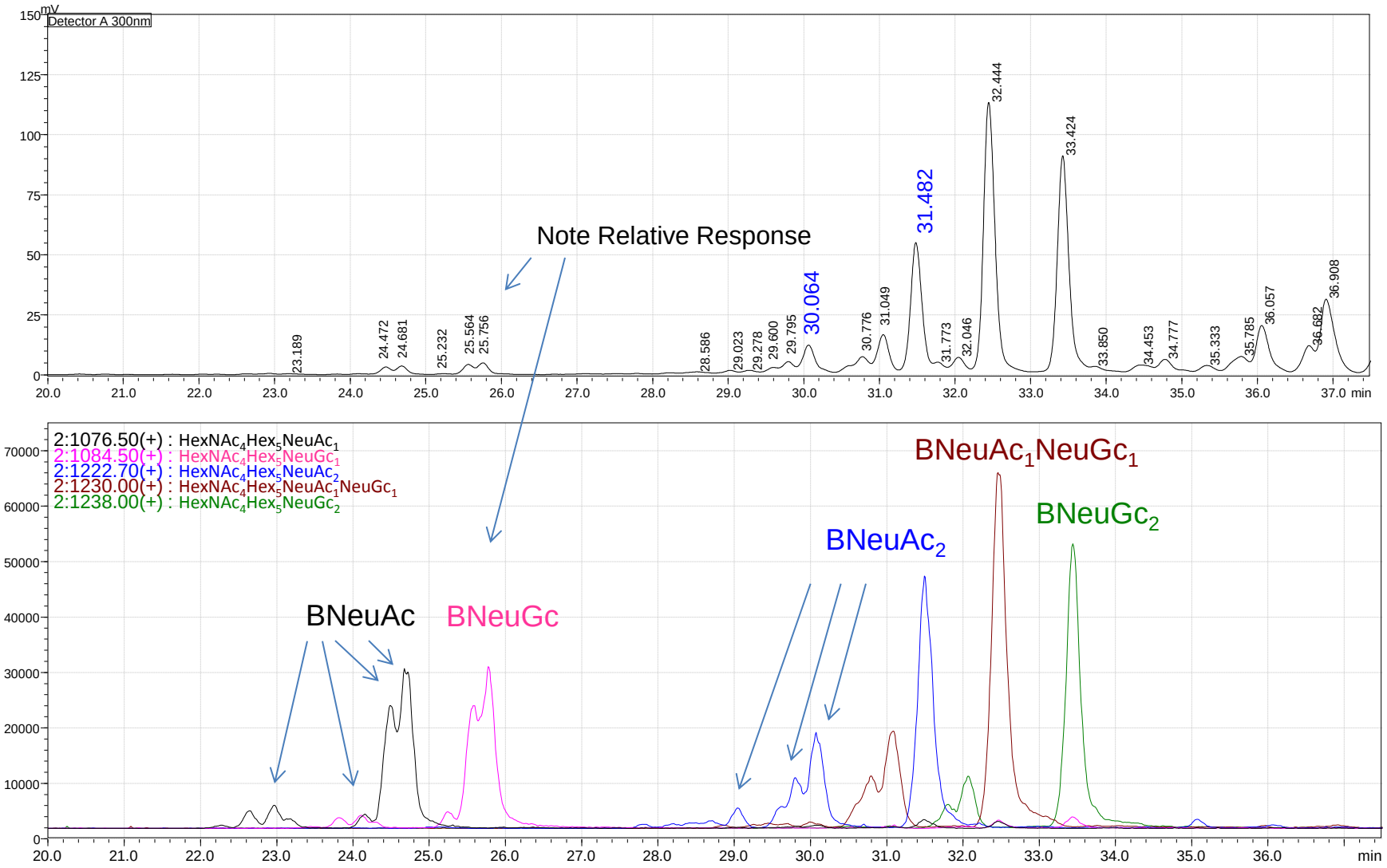
MALDI of Permethylated Glycans



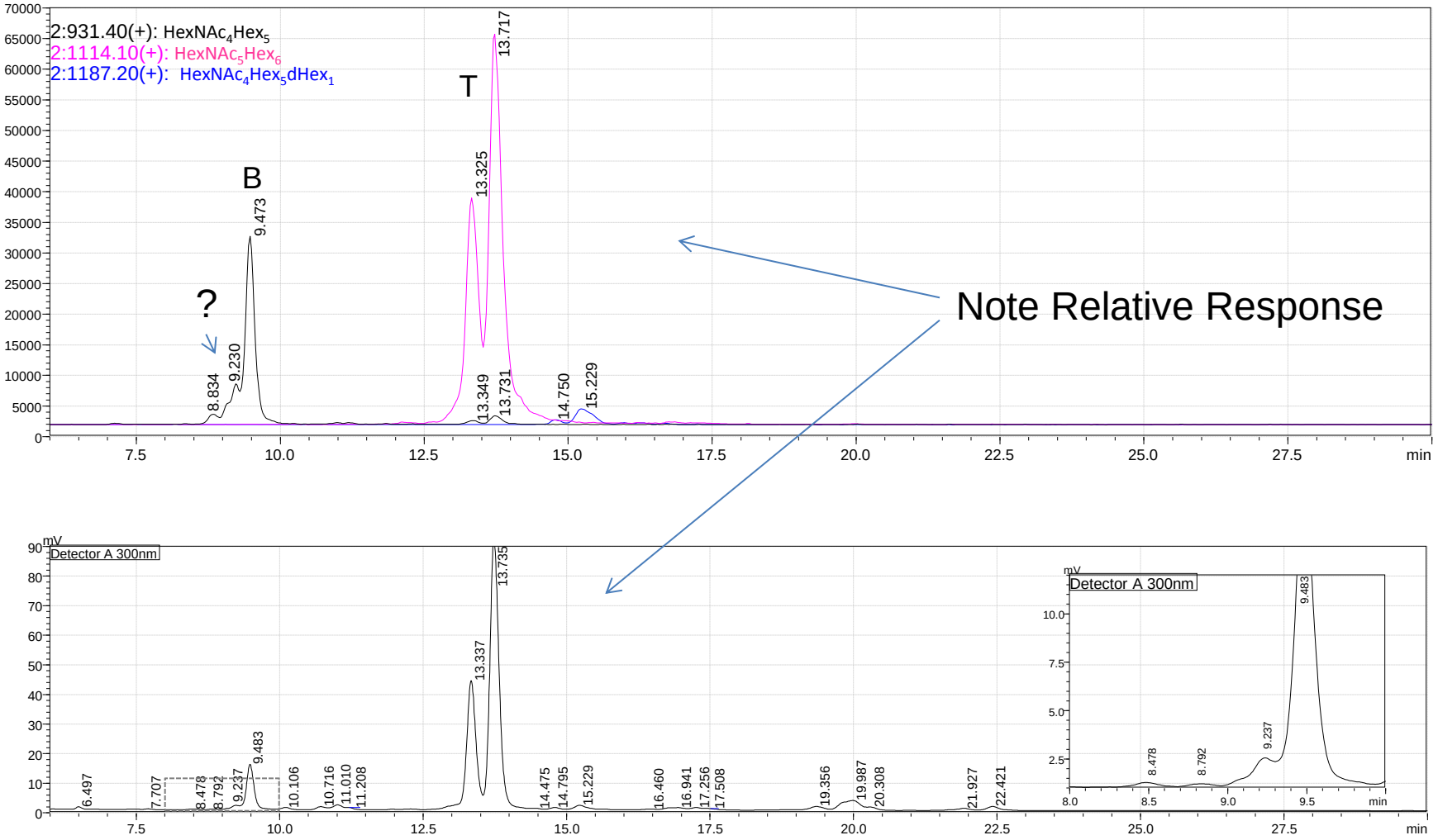
# *HILIC Separations of a1-AGP N-glycans*

***2.1 mm ID x 150 mm; 50 mM Ammonium Formate, pH 4.4, 77.5-56.5% AcN (B) in 52.5 min., 60°C; 600 µL/min.***
***Detection: 300 nm Abs; ESI-MS ((+) MS-2020, 4.2 kV, 400-2000 with SIM)***



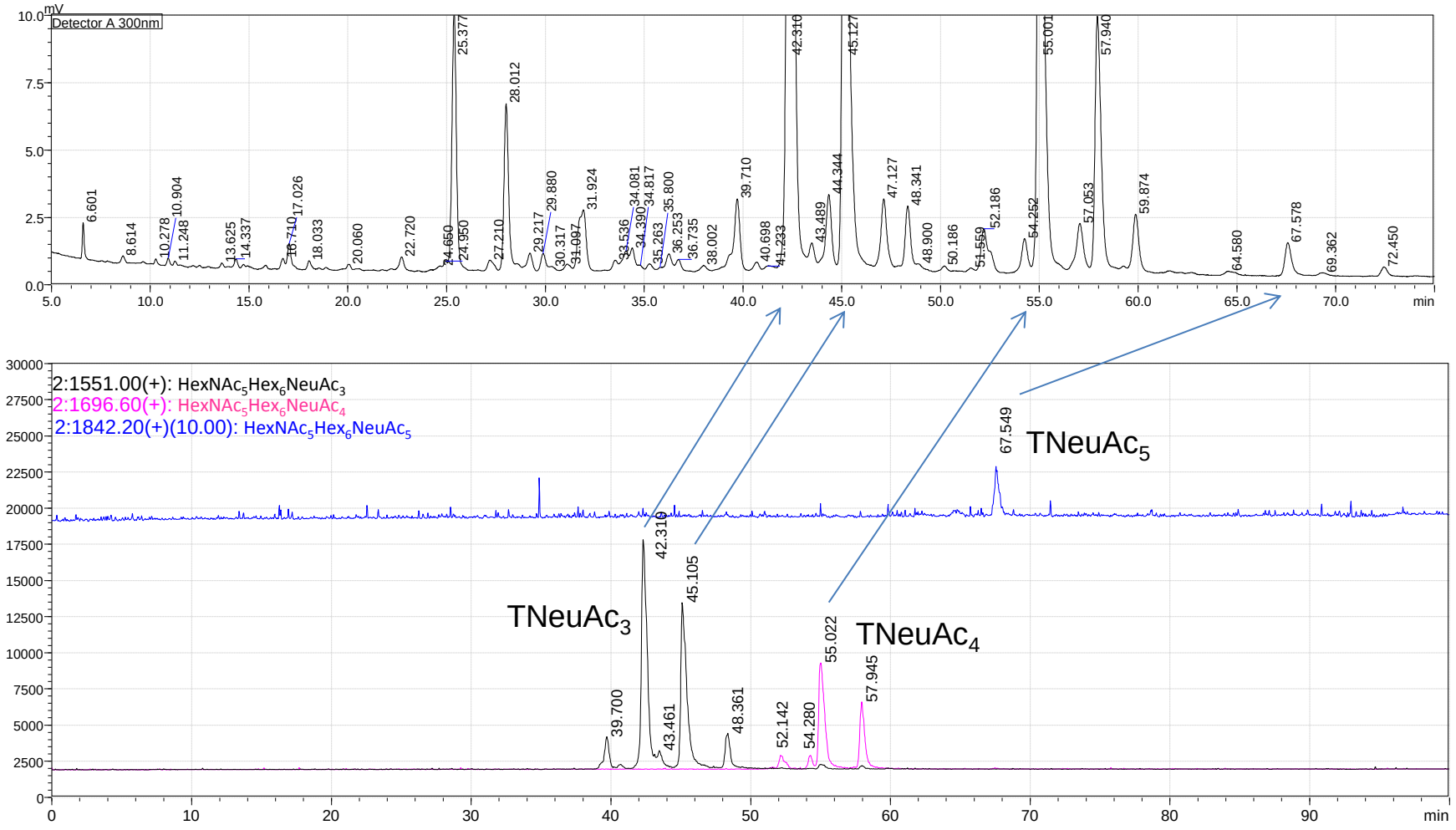
# *High Resolution HILIC Separations of asialo-Fetuin N-glycans*

***2.1 mm ID x 300 mm; 50 mM Ammonium Formate, pH 4.4, 70-55% AcN (B) in 90min., 60°C; 600 µL/min.***
***Detection: 300 nm Abs; ESI-MS ((+) MS-2020, 4.2 kV, 400-2000 with SIM)***



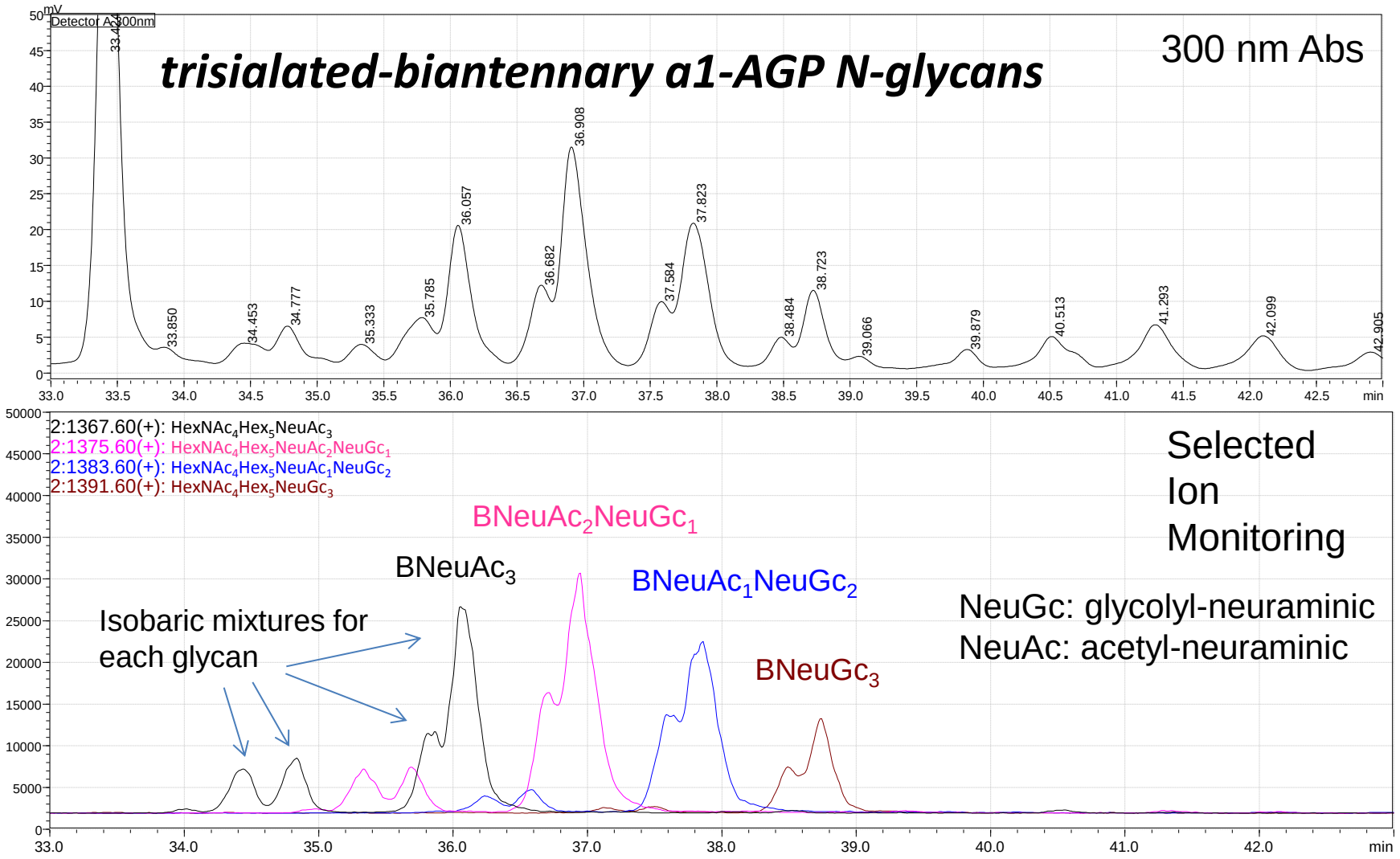
# *High Resolution HILIC Separations of Fetuin N-glycans*

***2.1 mm ID x 300 mm; 50 mM Ammonium Formate, pH 4.4, 70-55% AcN (B) in 90min., 60°C; 600 µL/min.***
***Detection: 300 nm Abs; ESI-MS ((+) MS-2020, 4.2 kV, 400-2000 with SIM)***



# *HILIC Separations of a1-AGP N-glycans*

***2.1 mm ID x 150 mm; 50 mM Ammonium Formate, pH 4.4, 77.5-56.5% AcN (B) in 52.5 min., 60°C; 600 µL/min. Detection: 300 nm Abs; ESI-MS ((+) MS-2020, 4.2 kV, 400-2000 with SIM)***



# Summary of N-Glycan Analysis

- The HILIC material and method described can be operated in high resolution mode, permitting analysis of complex mixtures of released N-linked protein glycan samples to be resolved, or in very high speed mode, to enable high throughput glycan analysis.
- High resolution HILIC resolves isobaric glycans, which are abundant (and complex).
- Variations in isobaric glycan profiles between proteins is very possible.
- *Quantitation by end-labeling using UV or Fluorescence detection only could be problematic, detection by MS only will not easily allow quantification of complex glycans (reference problem).*

# Summary of Recent Developments

- Fused-Core superficially porous particle selection continues to grow, with expansion of particle size and pore size availability.
- Bonded phase availability for these particles is expanding, particularly for RP and HILIC modes of operation.
- Research to apply these materials in useful ways to small and large molecules continues, resulting in additional opportunities for particle construction and improvement.
- *Improvement in these LC column packing materials is closely tied to application needs that are emerging in pharma and biopharma.*

# Acknowledgements


## Thank you for your Attention!

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