Novel Superficially Porous Particle HILIC Separation Material Applied to N-Linked Glycan Analysis by LC/MS

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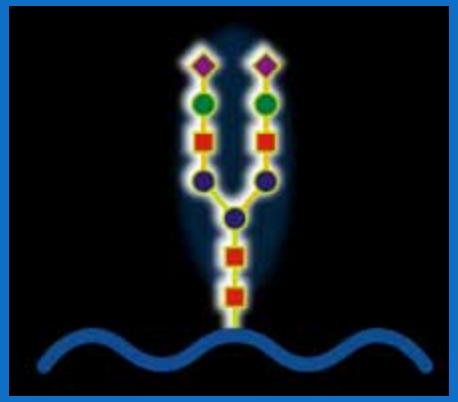
Agenda

- Protein N-glycan analysis by release, labeling and HILIC
  - General workflow
- Fused-Core Particles for highly efficient separations
- New polar bonded phase for HILIC
- Analysis of N-Glycans by Penta-HILIC
  - Standards and separation optimization strategy
- Complexity in action: isobaric glycan separations
- Implications of Complexity on Analysis of Released Glycans

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.

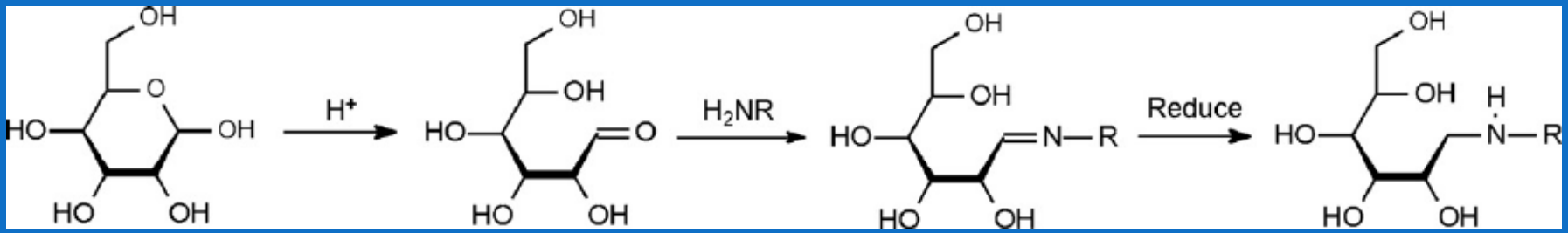
- Various additional exoglycosidases and endoglycosidases can be helpful in structure elucidation.

- 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.

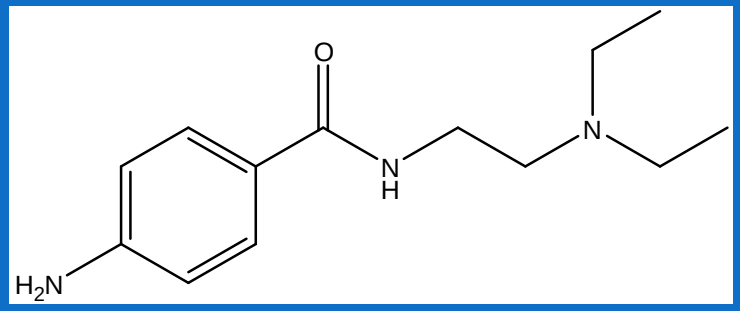
Analysis of PNGase Released and Labeled N-Glycans

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.



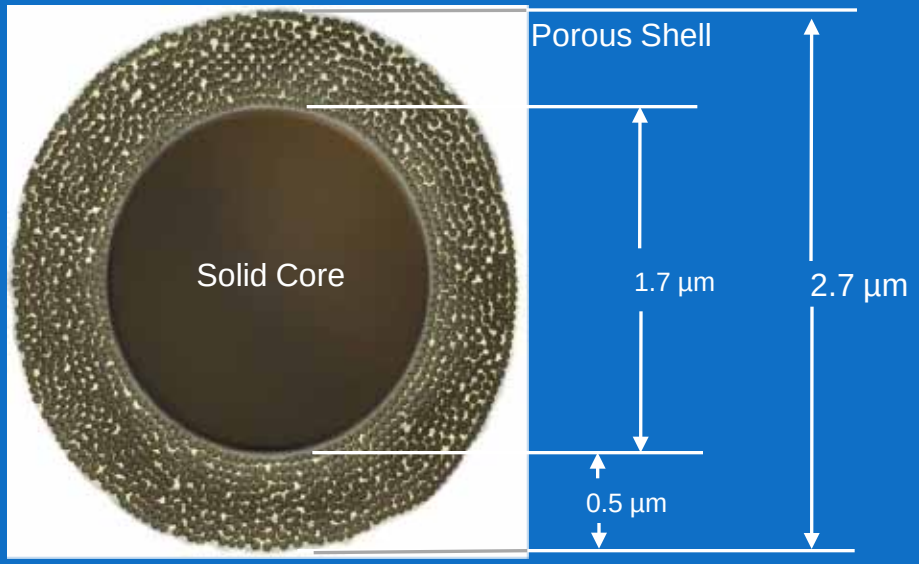
Many amines have been applied to labeling glycans, (Harvey, 2011, J. Chrom. 879).
In the current work Procainamide is favored due to reported improvements in ESI-MS detection (Klapoetke, et al., 2010, J. Pharm. Biomed. Anal. 53)

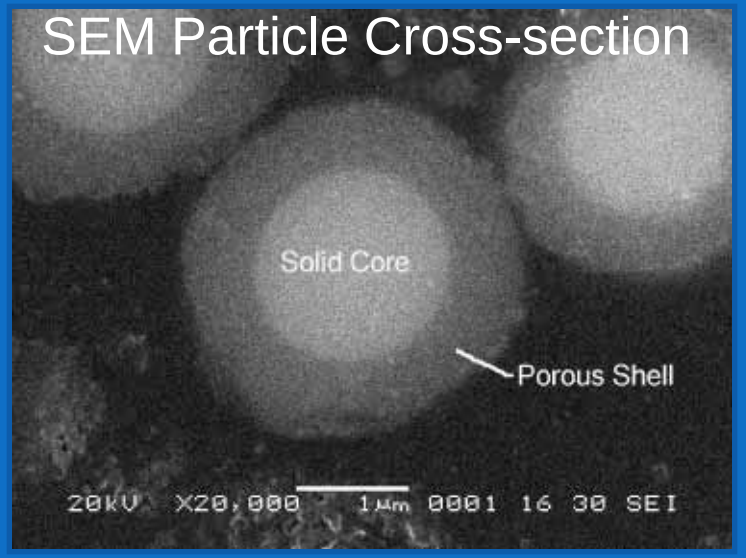
Typical Labeling Conditions
Glycan in water (up to 10% volume)
90+% volume of:
0.4 M procainamide
1M sodium cyanoborohydride
in 30% acetic acid/70% DMSO
12-16 hr reaction at 37°C
SEC cleanup on Sephadex G-10 minicolumn
Absorbance Detection 300 nm or Fluorescence Ex 330/Em 380 nm



Mass: glycan + 219.32

Superficially Porous Particles (Fused-Core®)

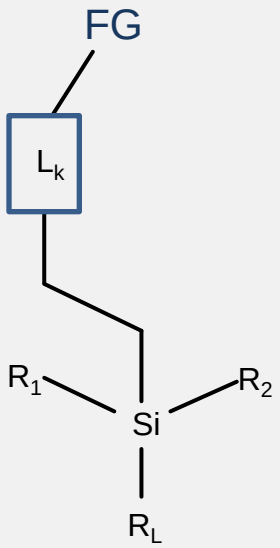




- Low back pressure due to the particle design (solid core with a porous shell)
- Specialized high pressure HPLC equipment is optional
- Not necessary to filter samples and mobile phase since 2 µm frits are not as small as needed for sub-2-µm
- High resolution is maintained at high flow rates (flat C-term in van Deemter plot)

Silane Surface Modifications with Polar Functional Groups

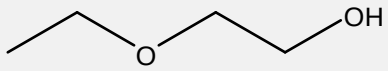
- Support effective HILIC retention
- Exhibit desired kinetic advantages of SPP morphology
- Reduce unfavorable ionic/coloumbic interactions



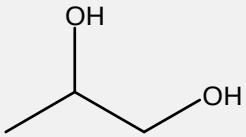
R_L = silanol reactive leaving
R_1 = R_2 or $R_1 \neq R_2$, R_1 = R_L
Eg. -Me, -OMe, -Cl, -DiiPr

Hydroxylic Functional Groups

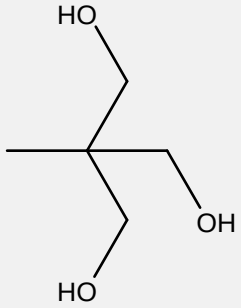
Mono-OL



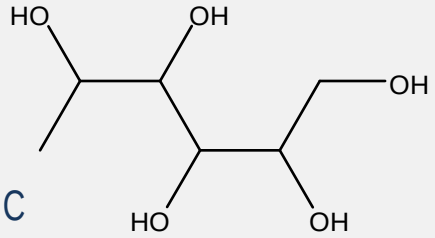
Di-OL



Tri-OL



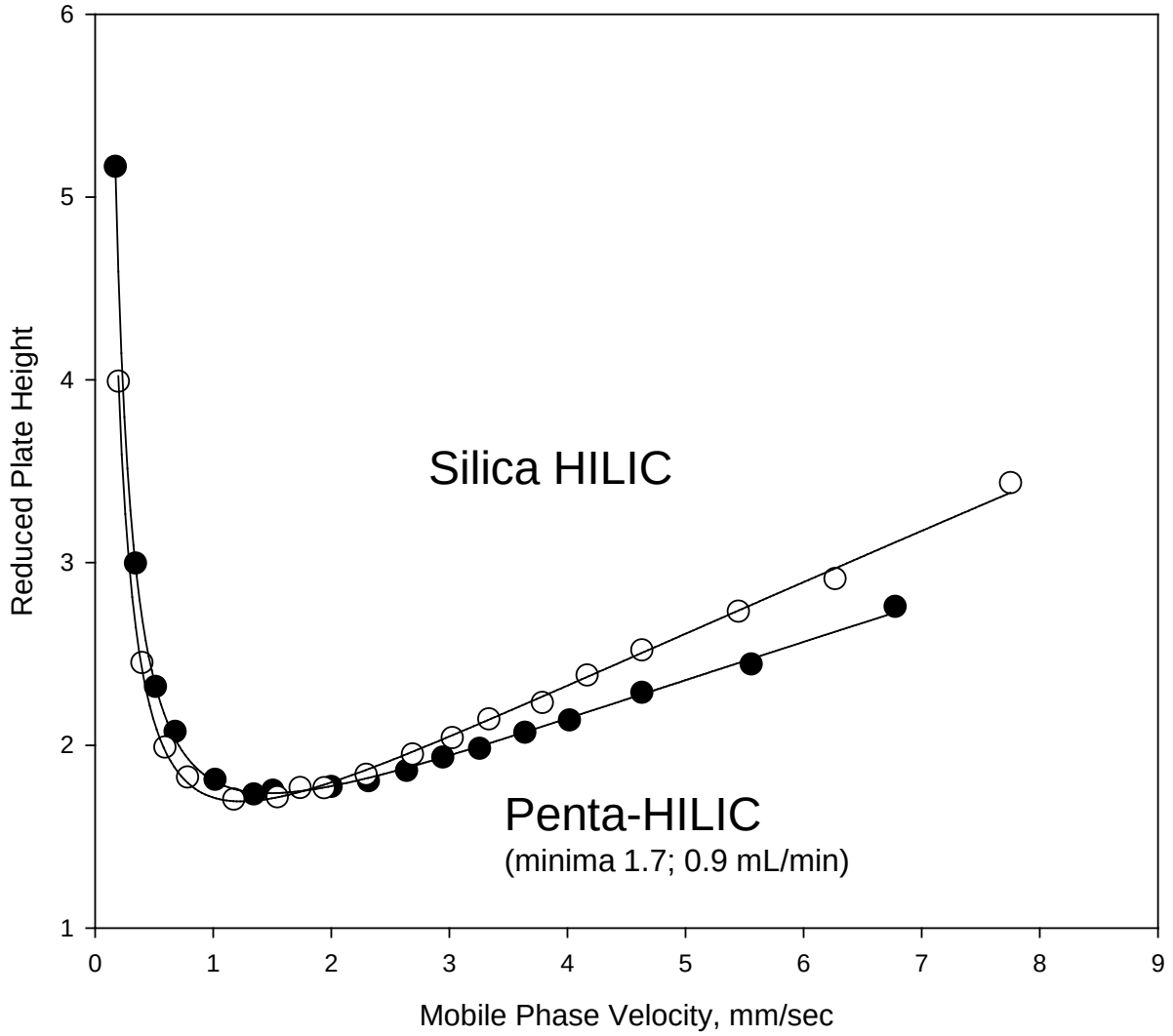
Pentan-OL
AKA
Halo Penta-HILIC



Effect of Linear Velocity on Penta-HILIC Column Efficiency

4.6 mm ID x 50 mm; 90% AcN/10 mM NH_4Form 3.0, 25 °C; 1 mL, 50 ng Adenosine

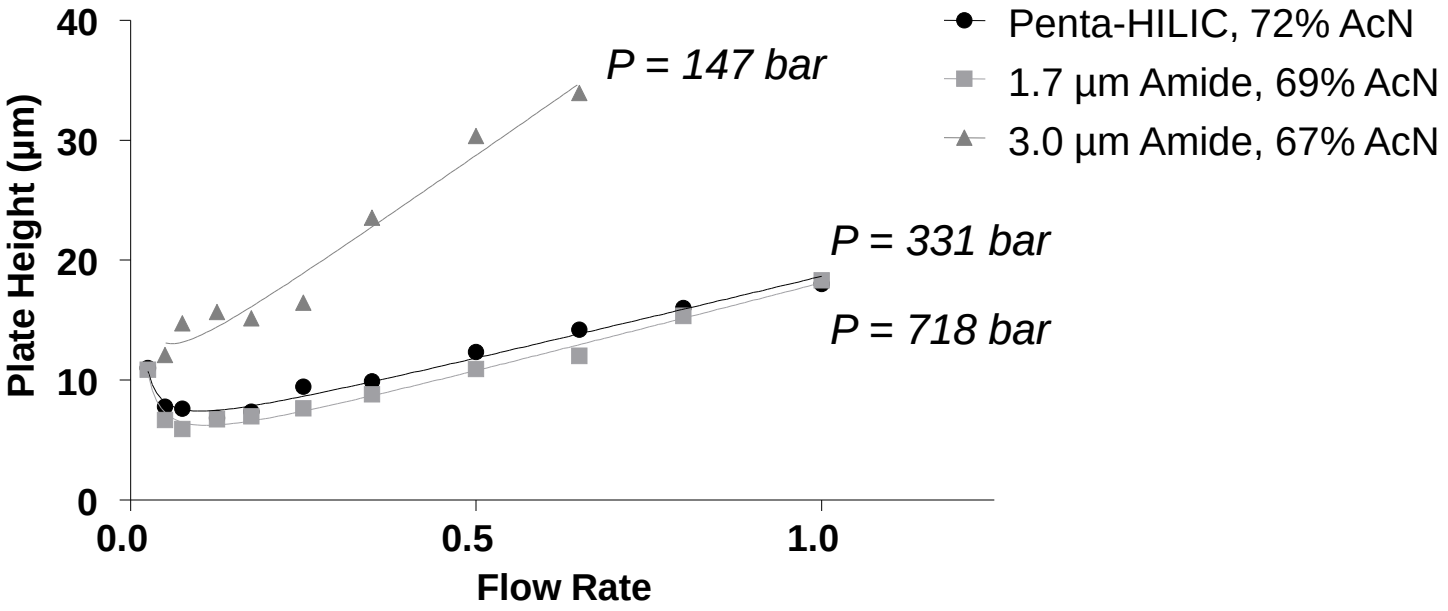
Data fitted to Knox Equation



Column Efficiency Comparisons Pam-G5

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

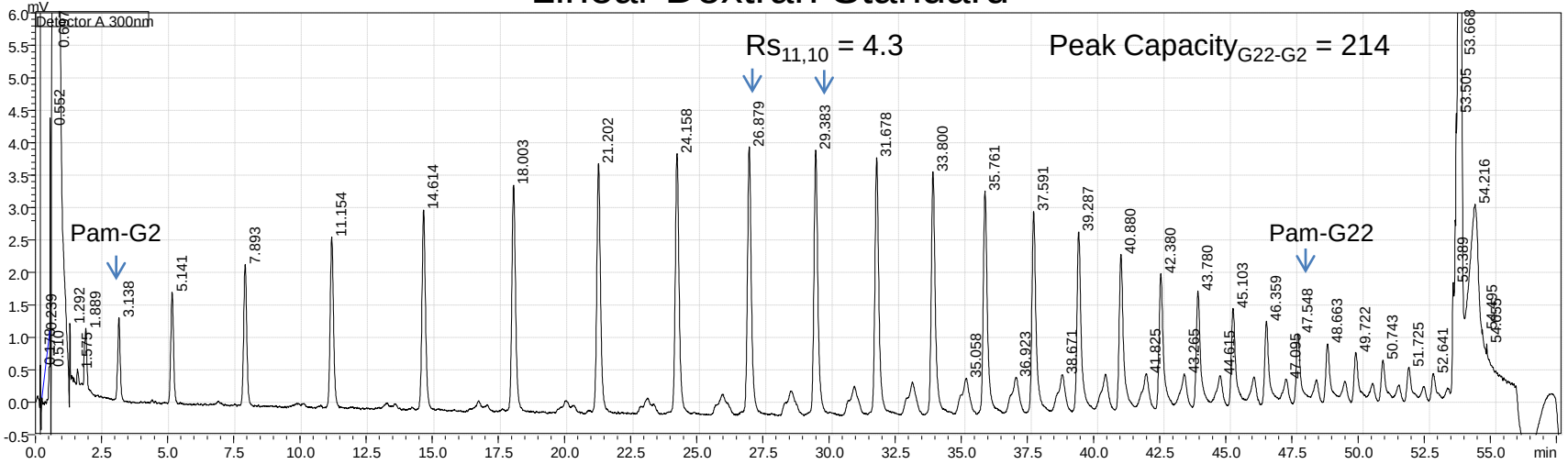
$$H = A + \frac{B}{u} + Cu$$



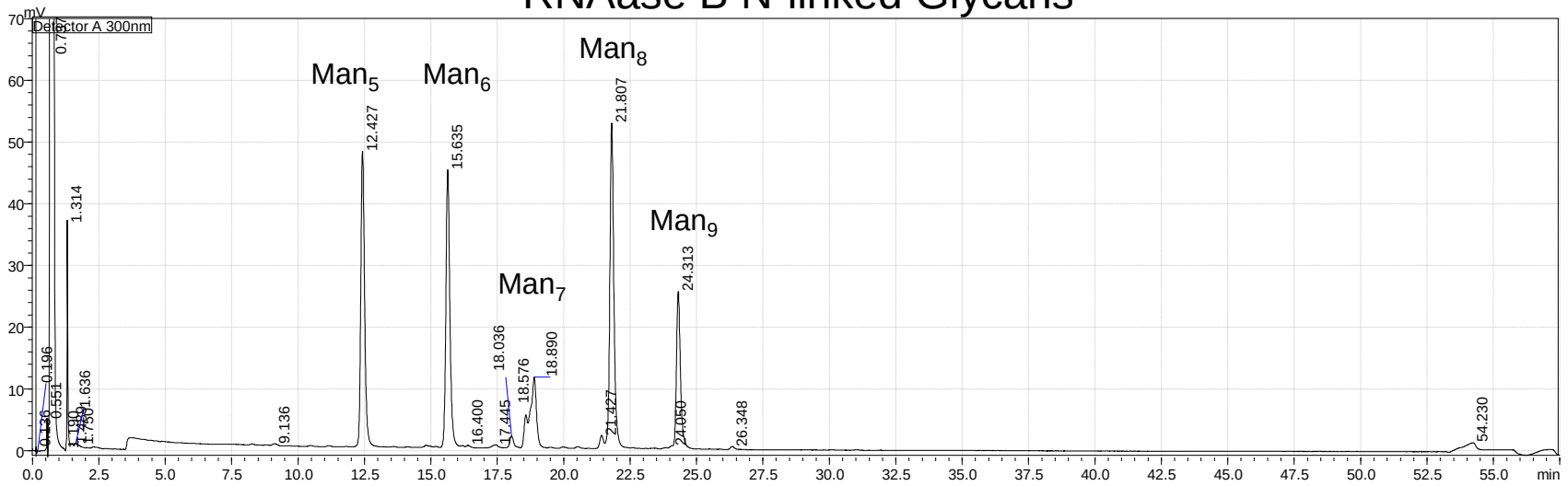
Penta-HILIC Separations of Labeled Oligosaccharides and Glycans

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 mL/min.
Detection: 300 nm Abs; ESI-MS (MS-2020, (+) 4.2 kV, 400-2000 with SIM)

Linear Dextran Standard

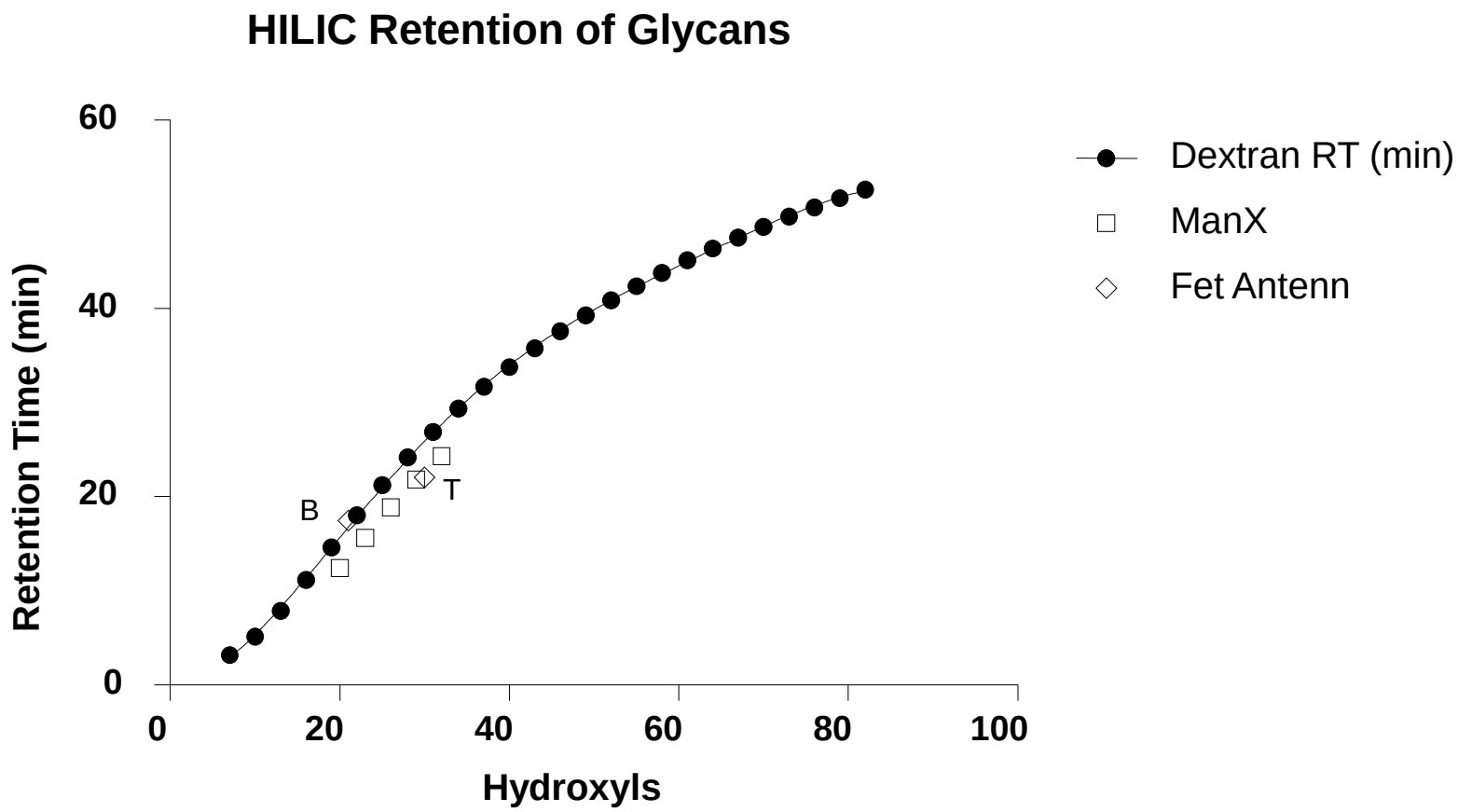


RNAase B N-linked Glycans



Penta-HILIC Retention of Labeled Oligosaccharides and Glycans

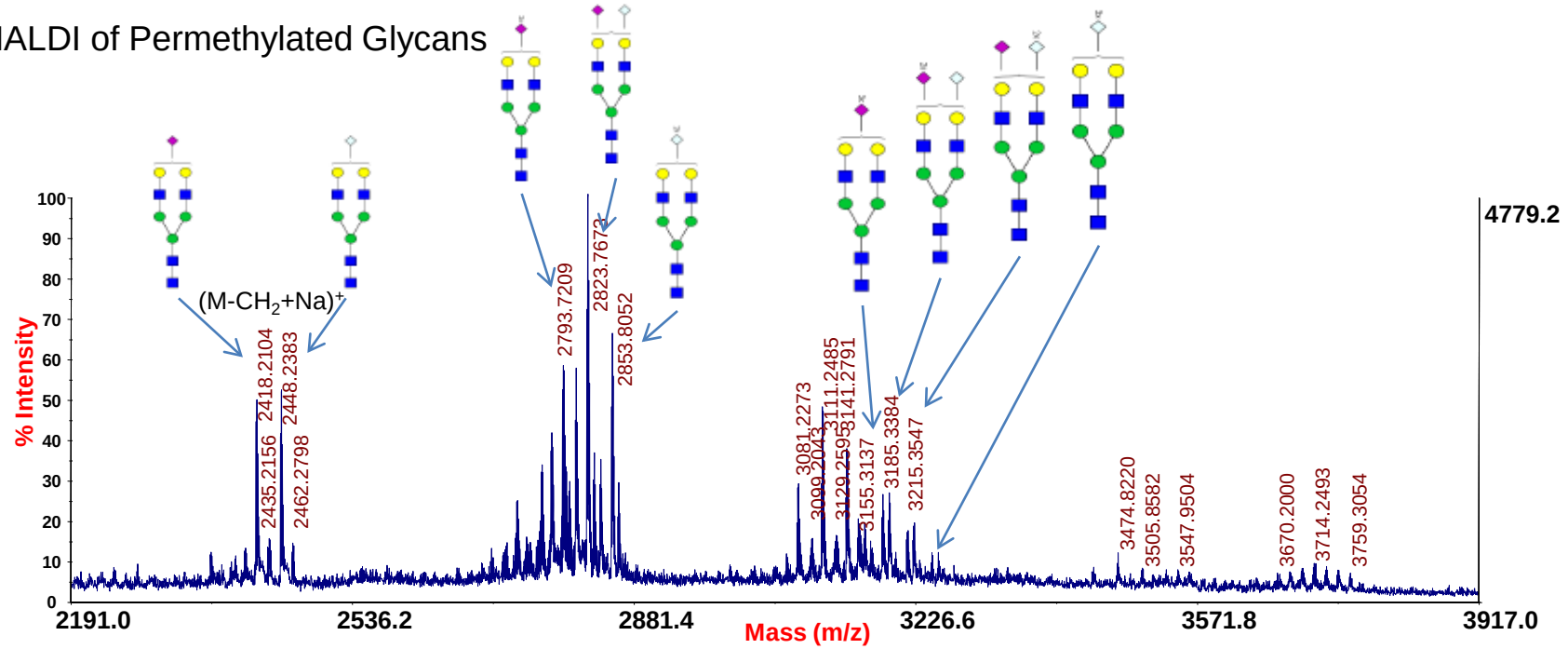
2.1 mm ID x 150 mm; 50 mM Ammonium Formate, pH 4.4, 77.5-56.5% AcN (B) in 52.5 min., 60°C; 600 mL/min. Detection: 300 nm Abs; ESI-MS (MS-2020, (+) 4.2 kV, 400-2000 with SIM)



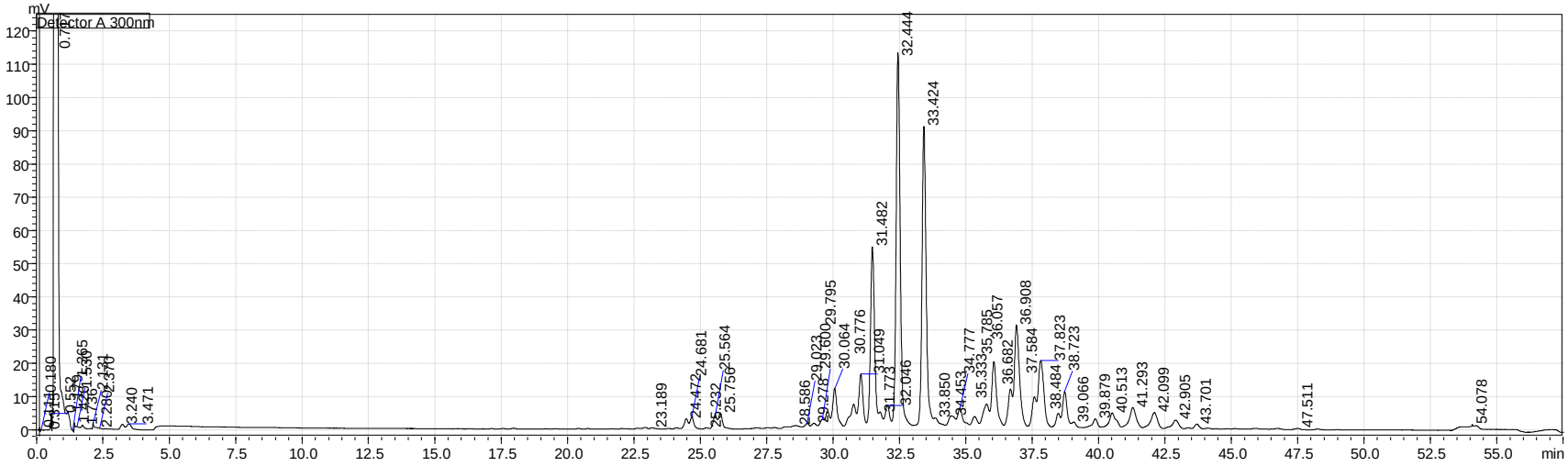
Penta-HILIC Separations of Abundant bov. a1-AGP N-glycans

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 mL/min.

MALDI of Permethylated Glycans

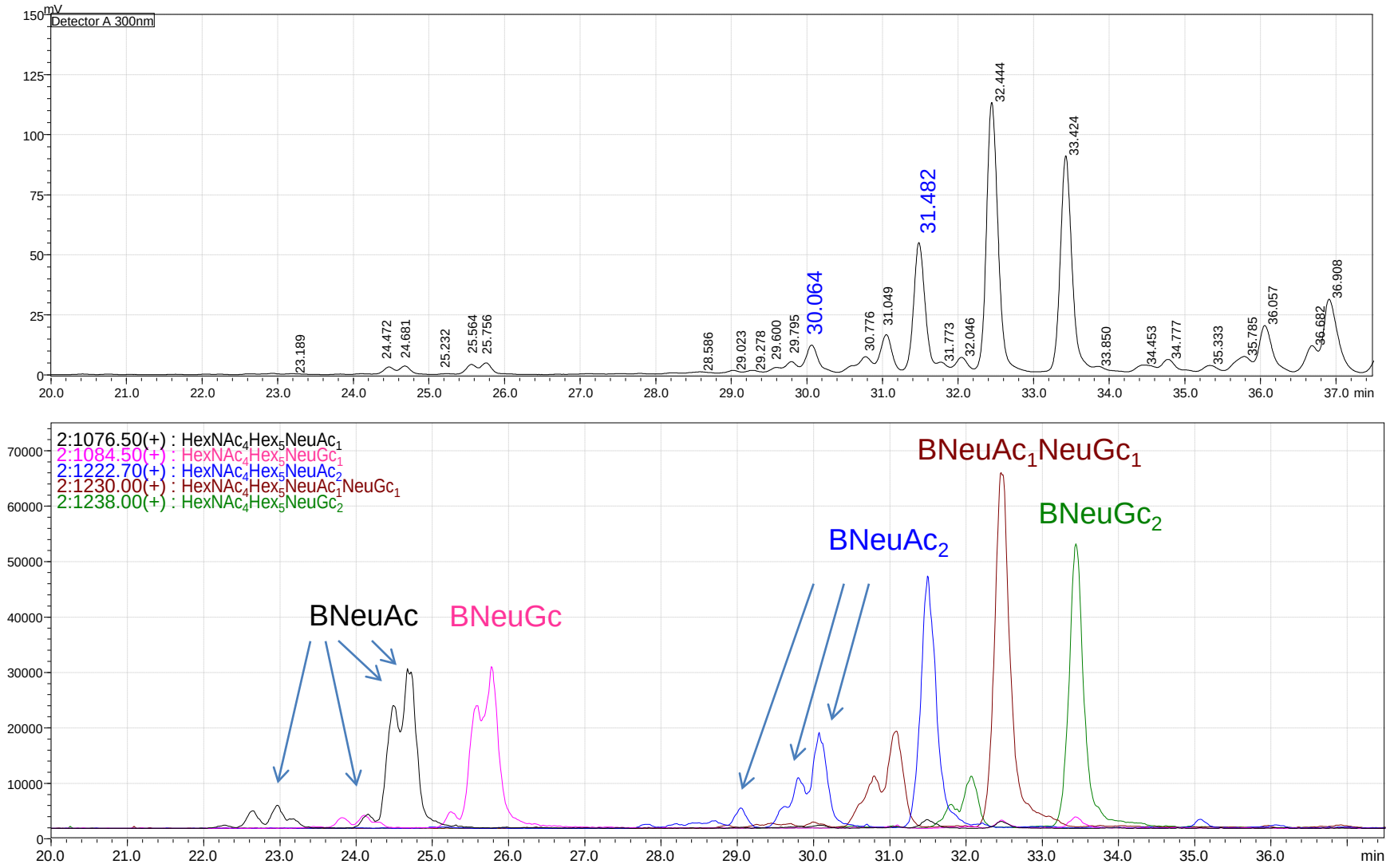


HILIC of End-labeled Glycans



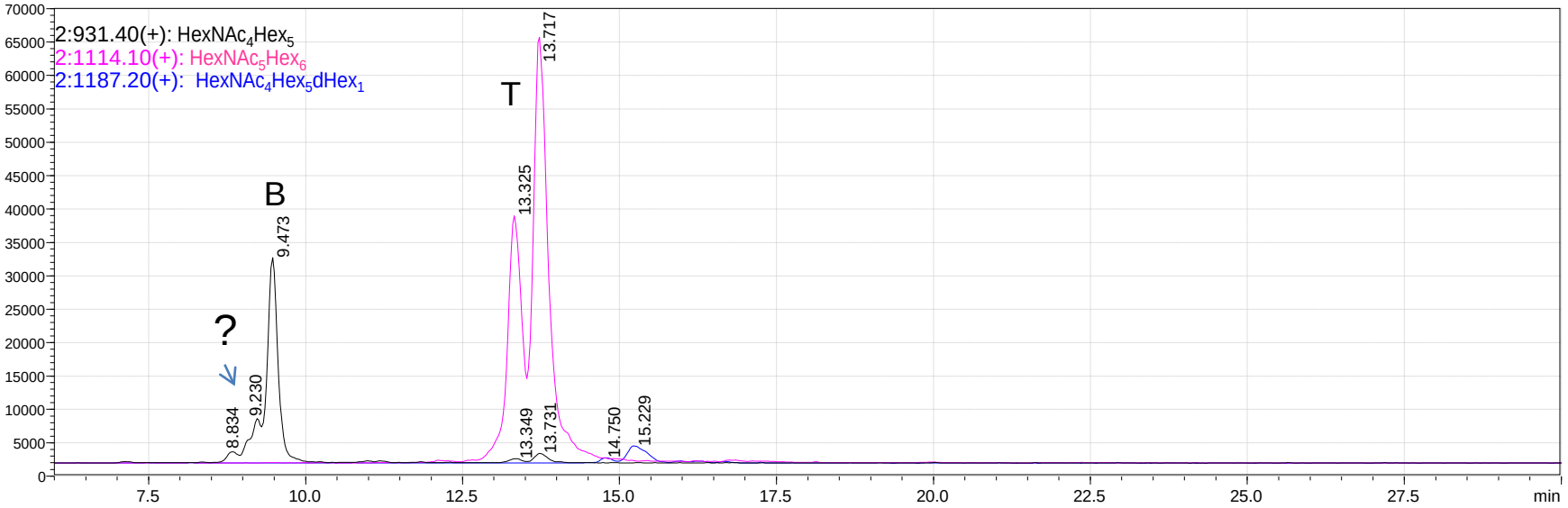
HILIC Separations of a1-AGP N-glycans

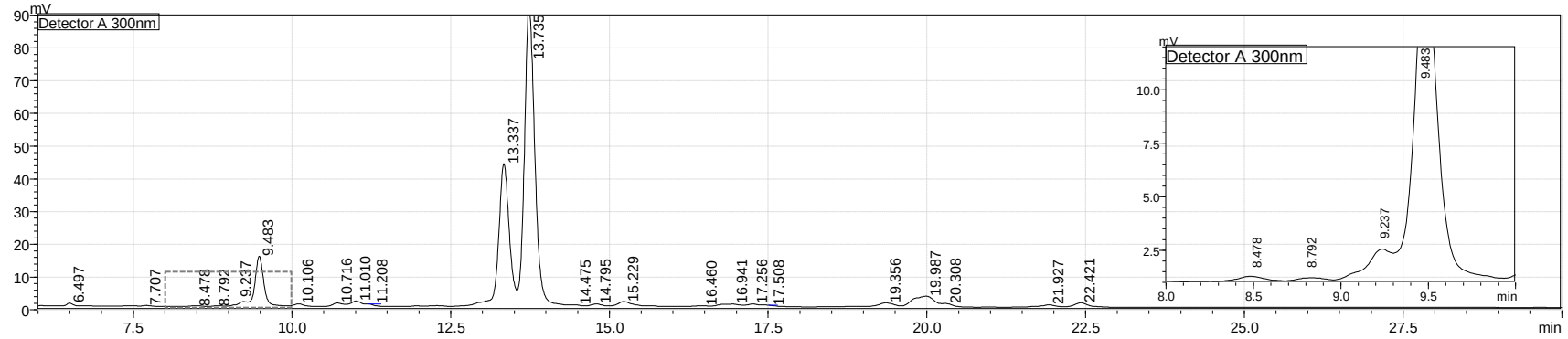
2.1 mm ID x 150 mm; 50 mM Ammonium Formate, pH 4.4, 77.5-56.5% AcN (B) in 52.5 min., 60°C; 600 mL/min.
Detection: 300 nm Abs; ESI-MS ((+) MS-2020, 4.2 kV, 400-2000 with SIM)



High Resolution HILIC Separations of asialo-Fetuin N-glycans

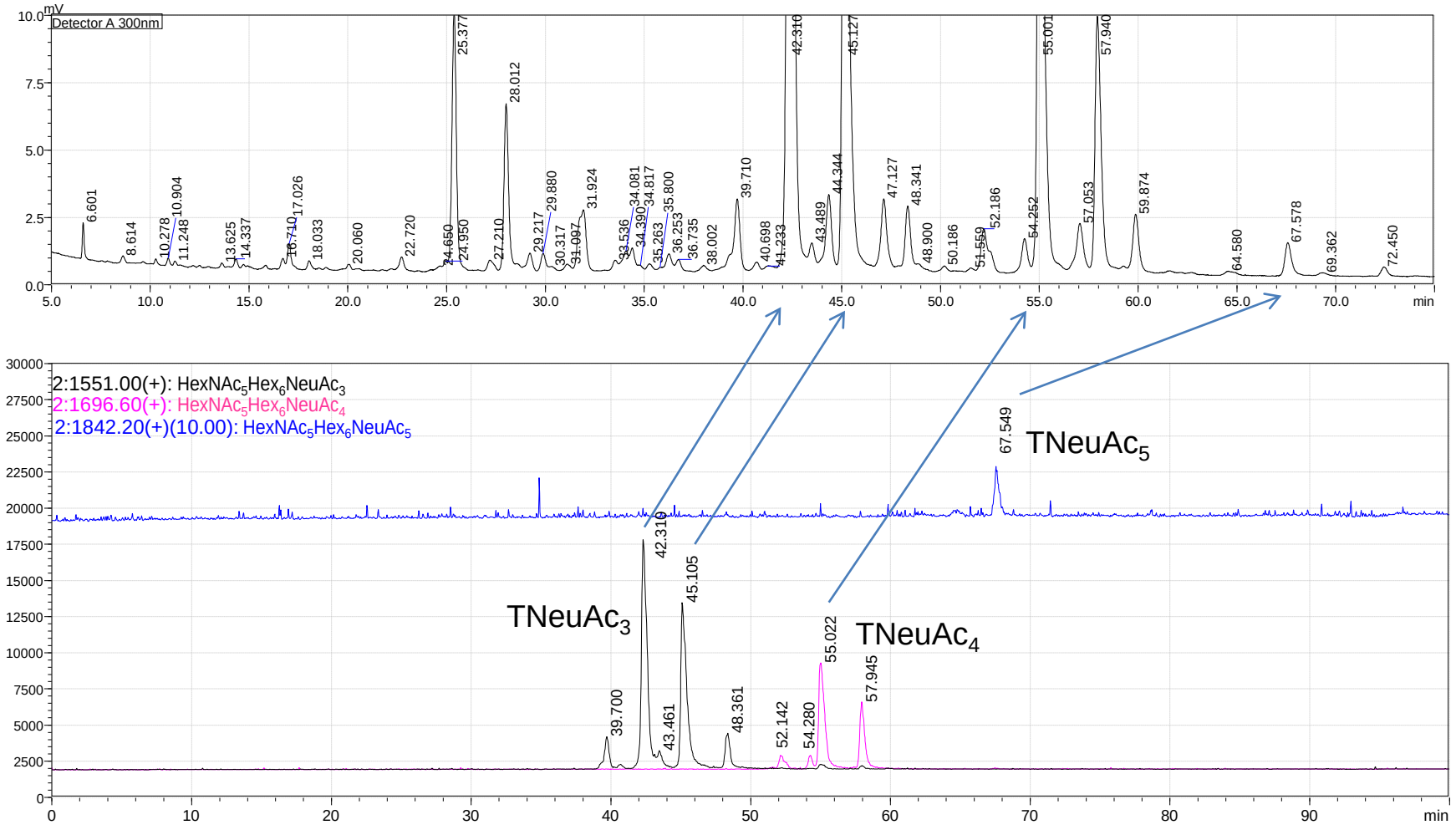
2.1 mm ID x 300 mm; 50 mM Ammonium Formate, pH 4.4, 70-55% AcN (B) in 90min., 60°C; 600 mL/min.
Detection: 300 nm Abs; ESI-MS ((+) MS-2020, 4.2 kV, 400-2000 with SIM)





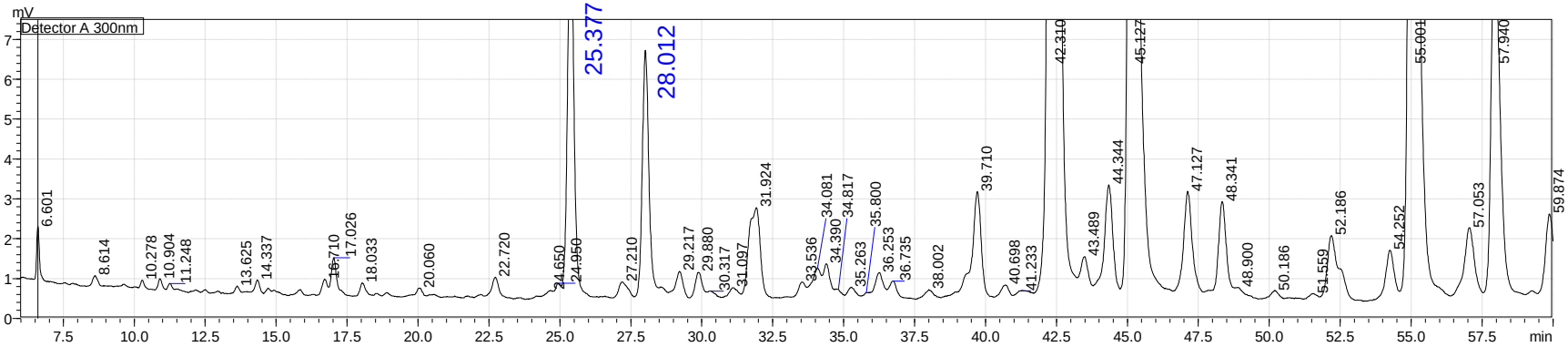
High Resolution HILIC Separations of Fetuin N-glycans

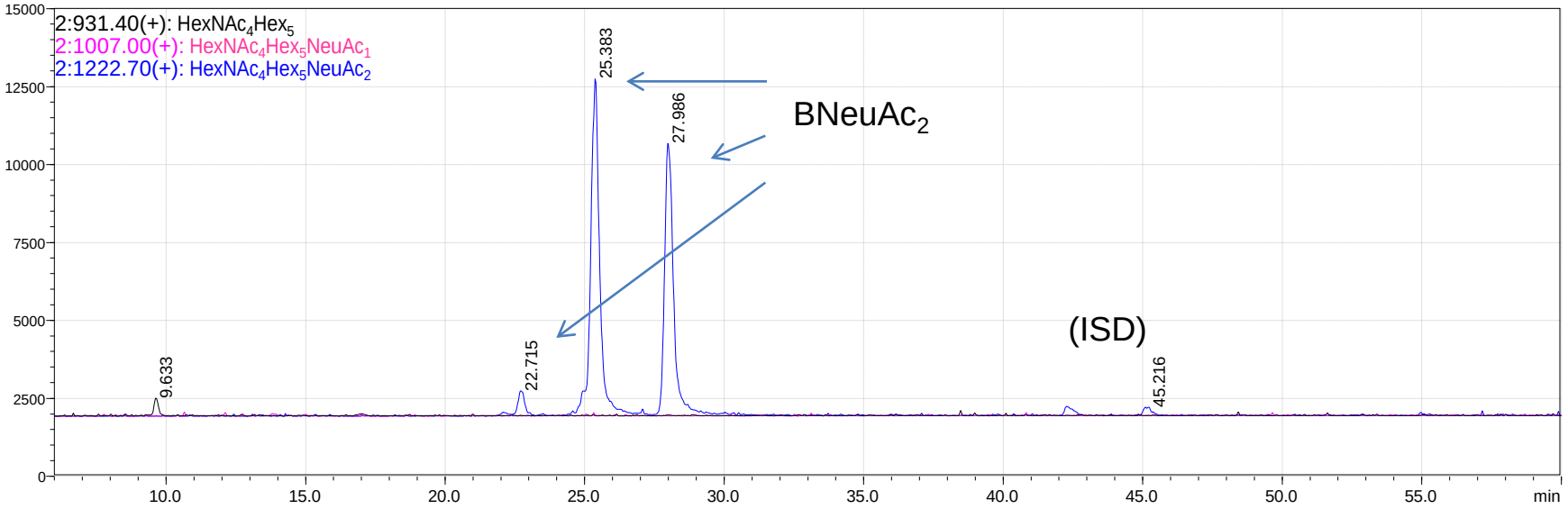
2.1 mm ID x 300 mm; 50 mM Ammonium Formate, pH 4.4, 70-55% AcN (B) in 90min., 60°C; 600 mL/min.
Detection: 300 nm Abs; ESI-MS ((+) MS-2020, 4.2 kV, 400-2000 with SIM)



High Resolution HILIC Separations of Fetuin N-glycans

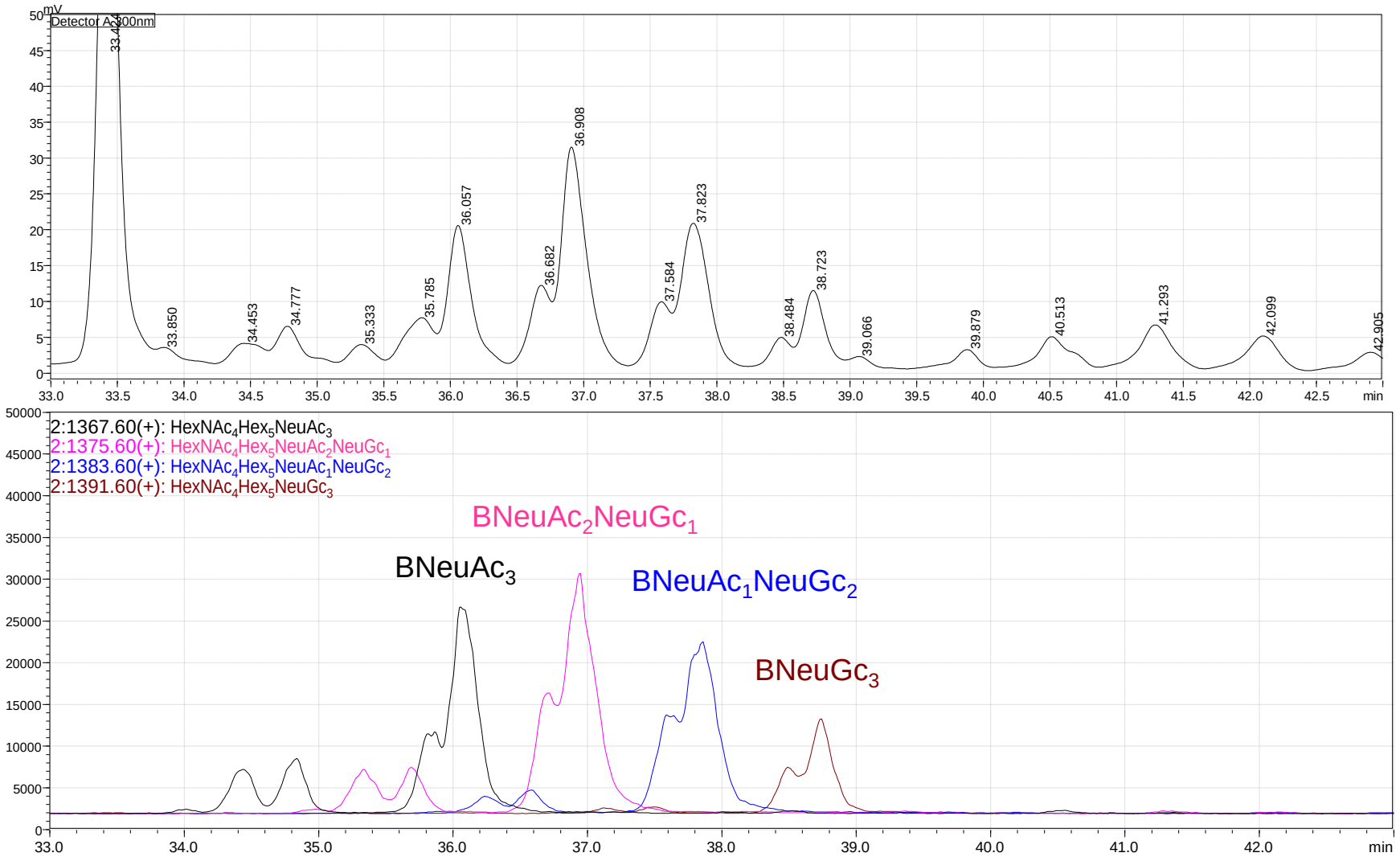
2.1 mm ID x 300 mm; 50 mM Ammonium Formate, pH 4.4, 70-55% AcN (B) in 90min., 60°C; 600 mL/min.
Detection: 300 nm Abs; ESI-MS ((+) MS-2020, 4.2 kV, 400-2000 with SIM)





HILIC Separations of bov. a1-AGP N-glycans (tri-sialated)

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 mL/min.
Detection: 300 nm Abs; ESI-MS ((+) MS-2020, 4.2 kV, 400-2000 with SIM)



Conclusions

- A novel hydroxylated Fused-core silica HILIC packing material has high utility for end-labeled Glycan separations.
- 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 reveal isomers.

Acknowledgements

Thank you for your Attention!

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