

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

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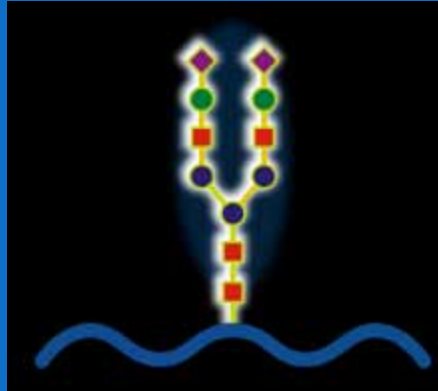
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Agenda

- 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

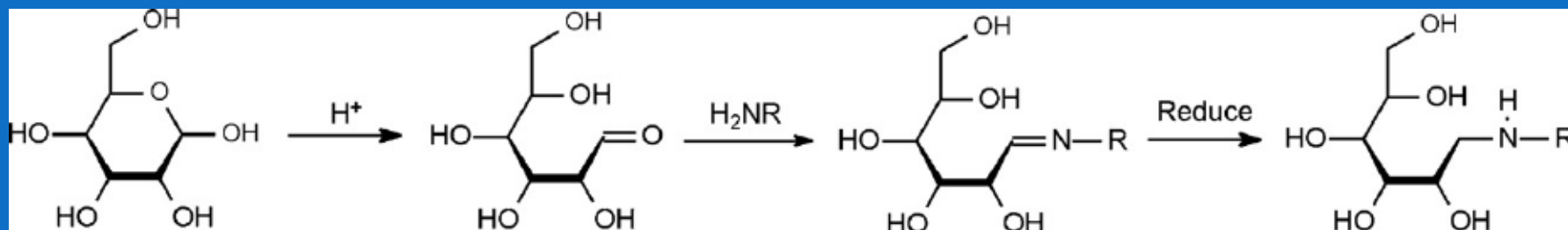
Protein N-linked Glycan Analysis



- 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, particularly 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

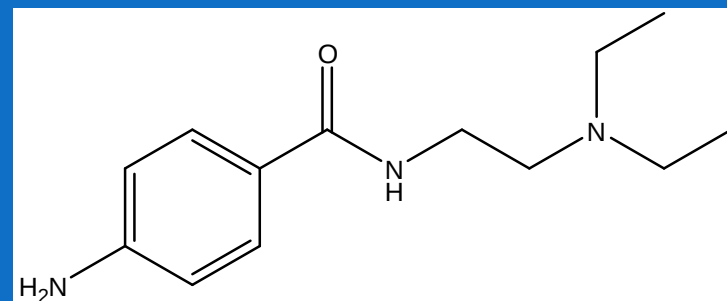
1M sodium cyanoborohydride

in 30% acetic acid/70% DMSO

12-16 hr reaction at 37°C

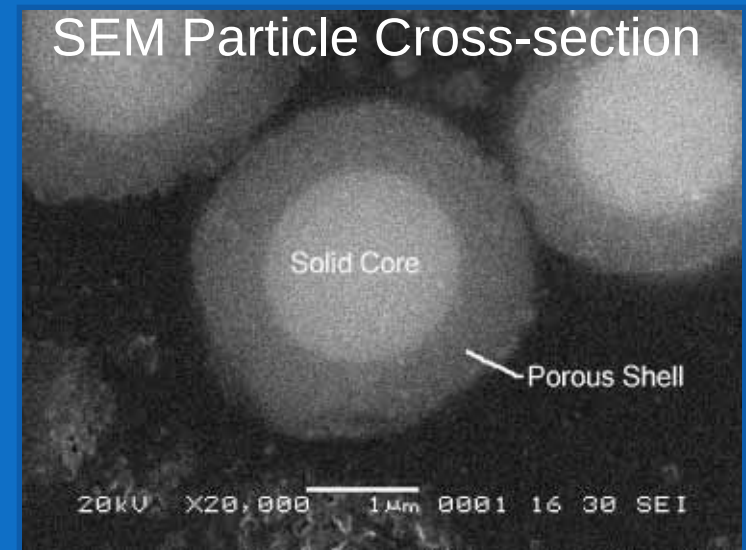
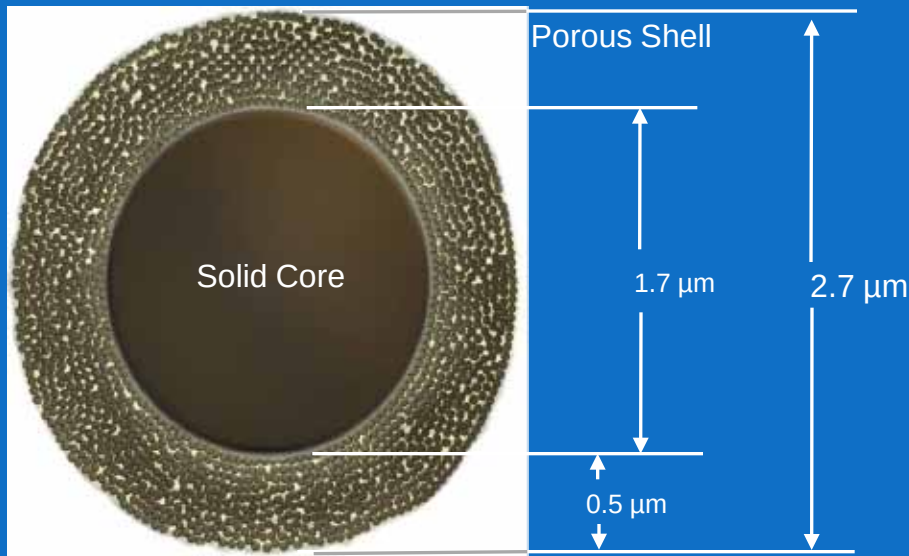
SEC cleanup on Sephadex G-10 minicolumn

Absorbance Detection 300 nm or Fluorescence Ex 330/Em 380 nm



Mass: glycan + 219.32

Superficially Porous Particles (Fused-Core®)

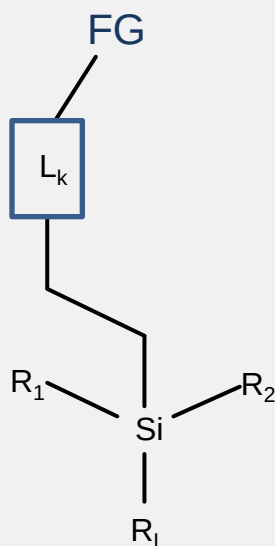


- Low back pressure due to the particle design (solid core with a porous shell)
- Specialized high pressure HPLC equipment is optional
- Not necessary to filter samples and mobile phase since 2 μm frits are not as small as needed for sub-2- μm
- High resolution is maintained at high flow rates (flat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

Silane Surface Modifications with Polar Functional Groups

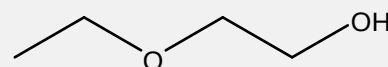
- Support effective HILIC retention
- Exhibit desired kinetic advantages of SPP morphology
- Reduce unfavorable ionic/coulombic interactions

Hydroxylic Functional Groups

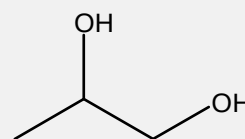


R_L = silanol reactive leaving
 $R_1 = R_2$ or $R_1 \neq R_2$, $R_1 = R_L$
 Eg. -Me, -OMe, -Cl, -DiiPr

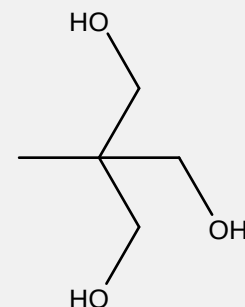
Mono-OL



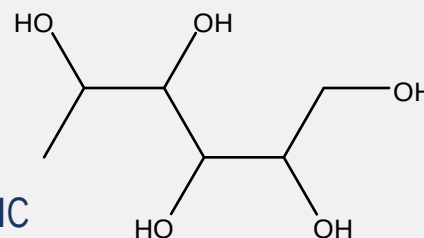
Di-OL



Tri-OL



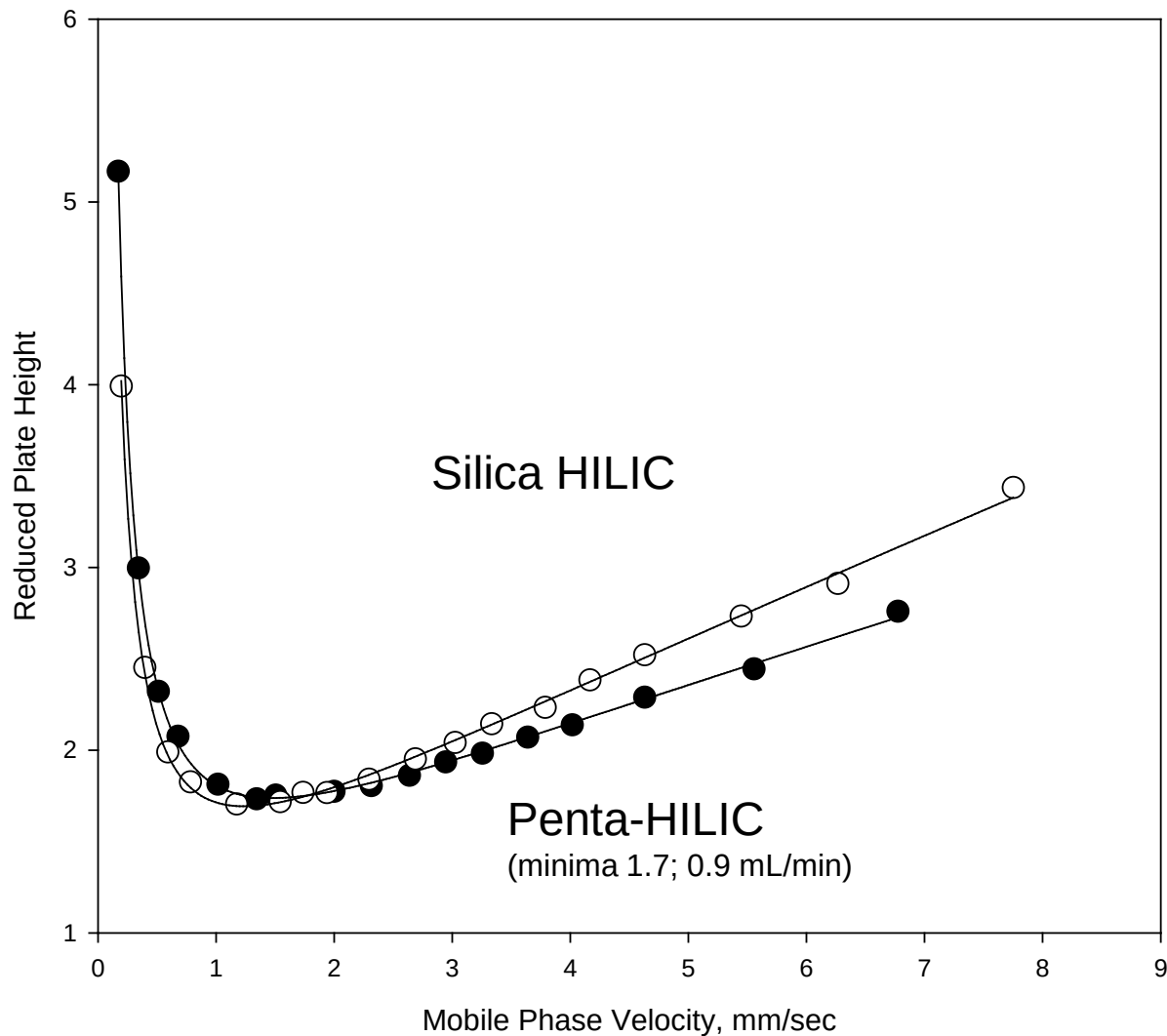
Pentan-OL
 AKA
 Halo Penta-HILIC



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 μL , 50 ng Adenosine

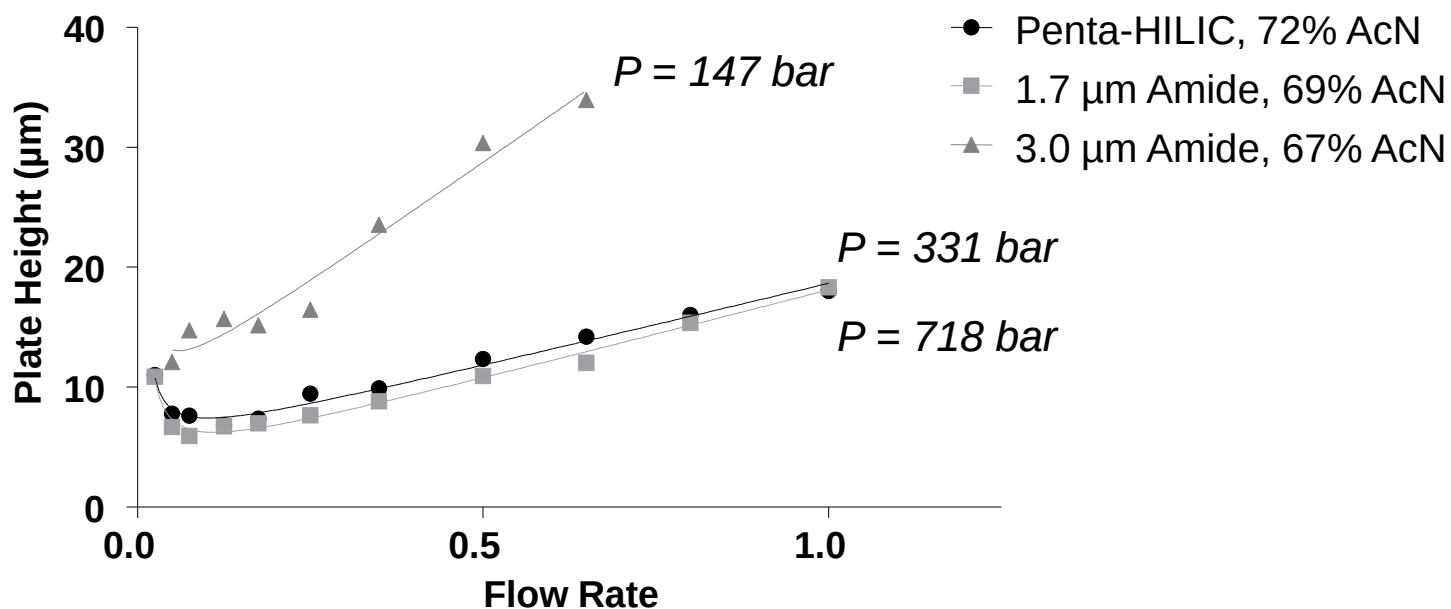
Data fitted to Knox Equation



Column Efficiency Comparisons Pam-G5

2.1 (2.0 mm) ID x 150 mm, 60°C, $k' \approx 6$,
50 mM Ammonium Formate Aqueous, pH 4.4
0.5 μ L Injection (50 pmol), Abs. 300 nm

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

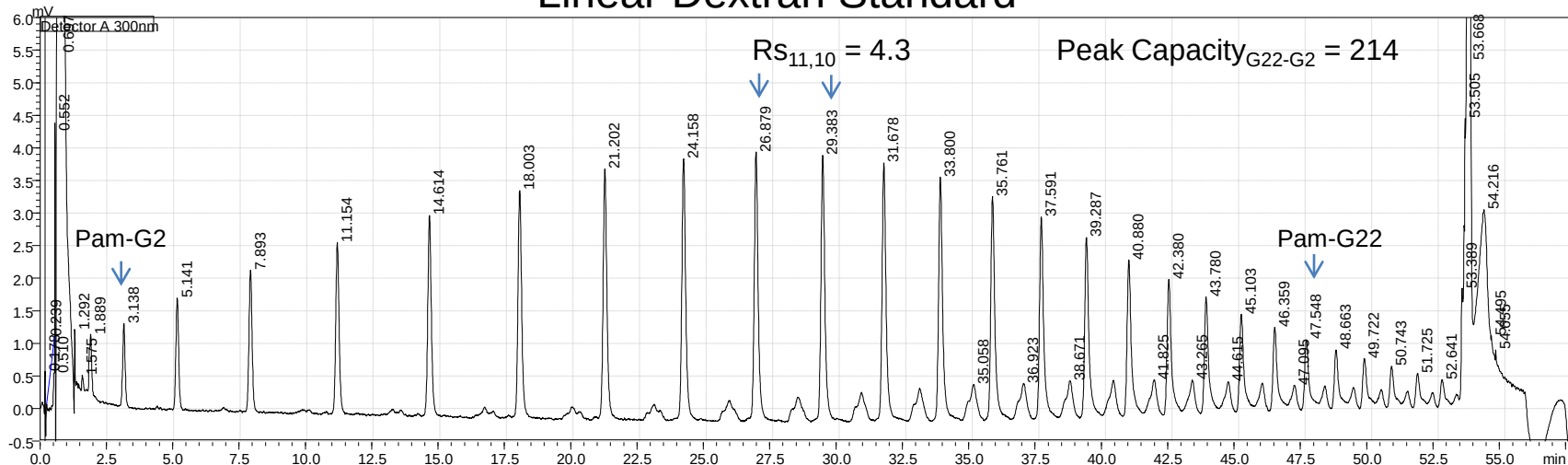


Penta-HILIC Separations of Labeled Oligosaccharides and Glycans

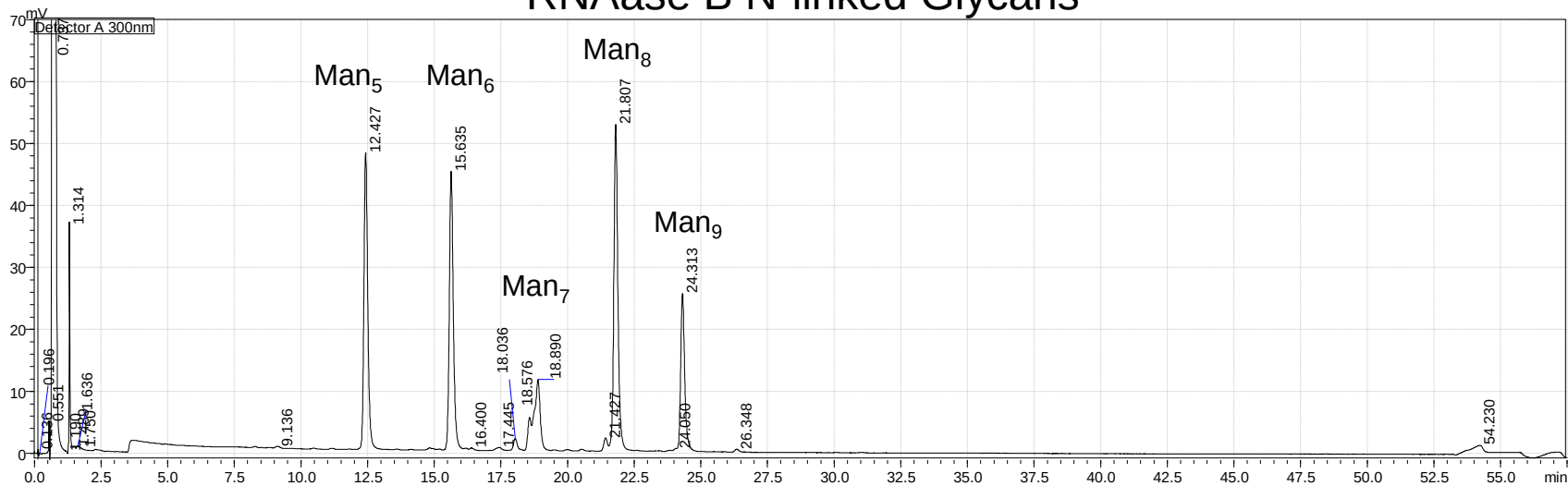
2.1 mm ID x 150 mm; 50 mM Ammonium Formate, pH 4.4, 77.5-56.5% AcN (B) in 52.5 min., 60°C; 600 mL/min.

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

Linear Dextran Standard



RNAase B N-linked Glycans

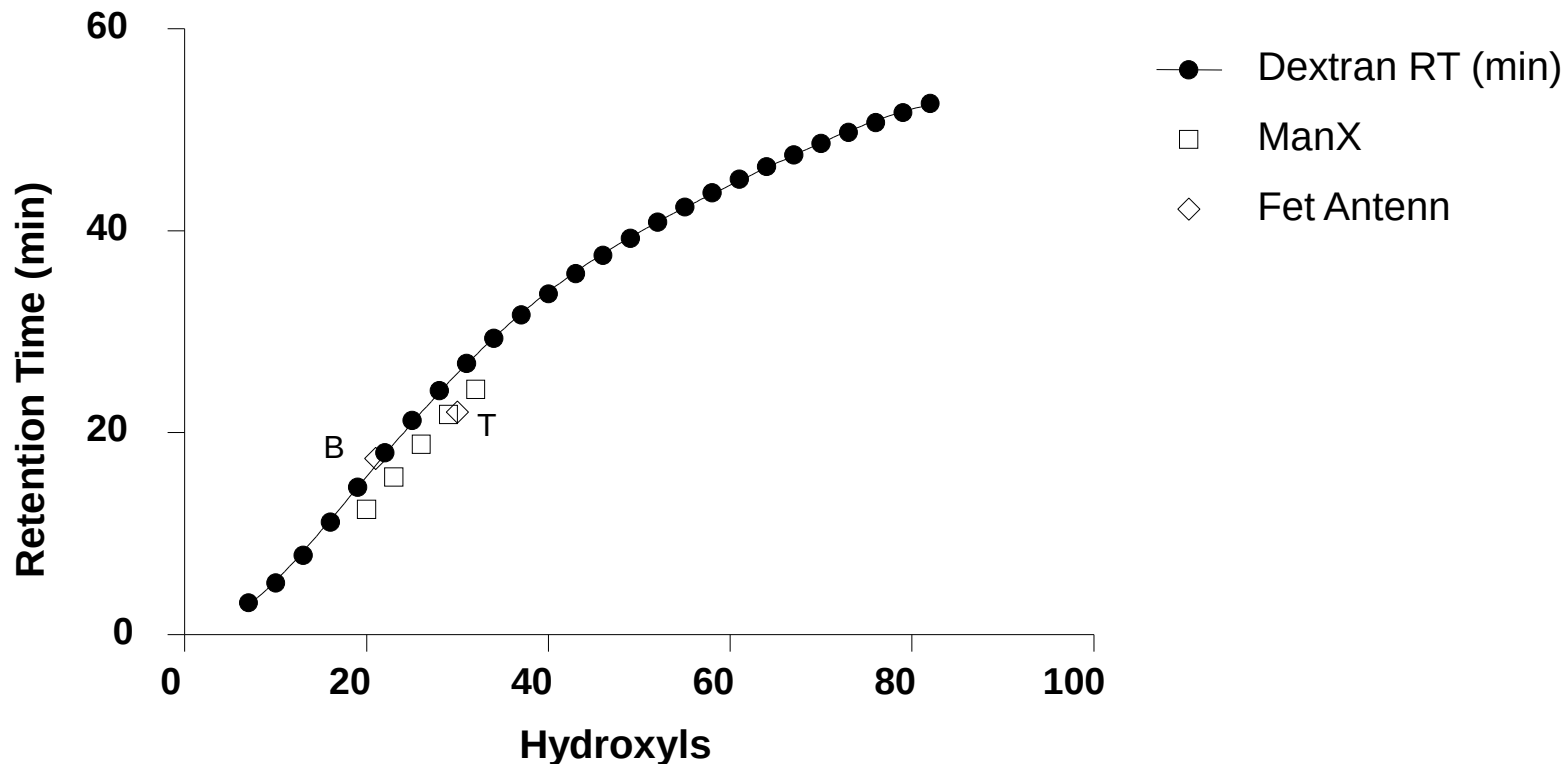


Penta-HILIC Retention of Labeled Oligosaccharides and Glycans

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 nL/min.

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

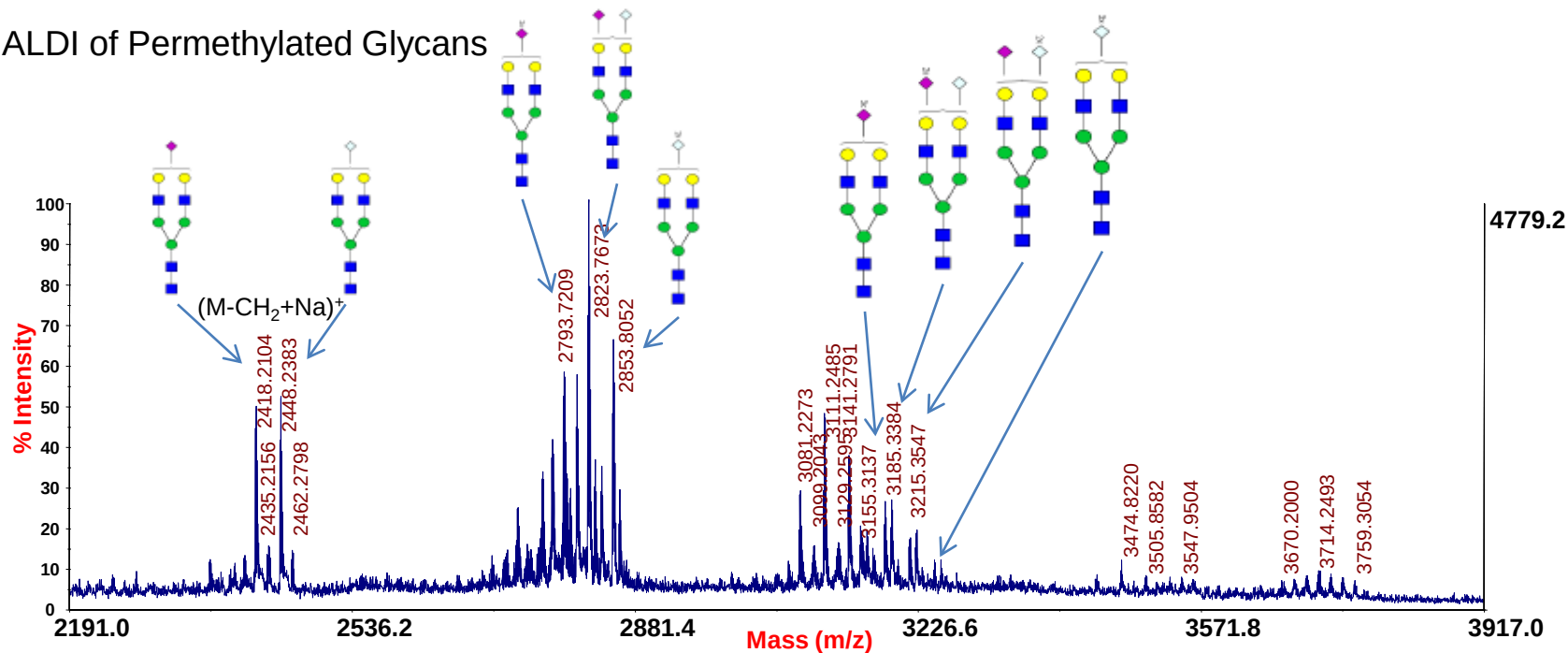
HILIC Retention of Glycans



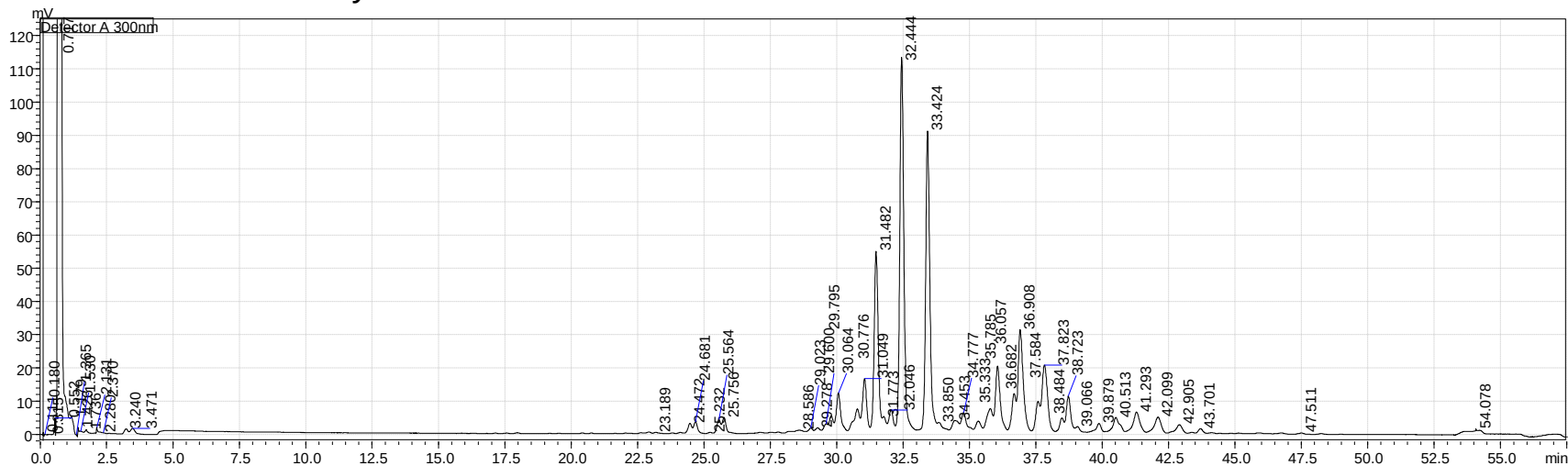
Penta-HILIC Separations of Abundant bov. a1-AGP N-glycans

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



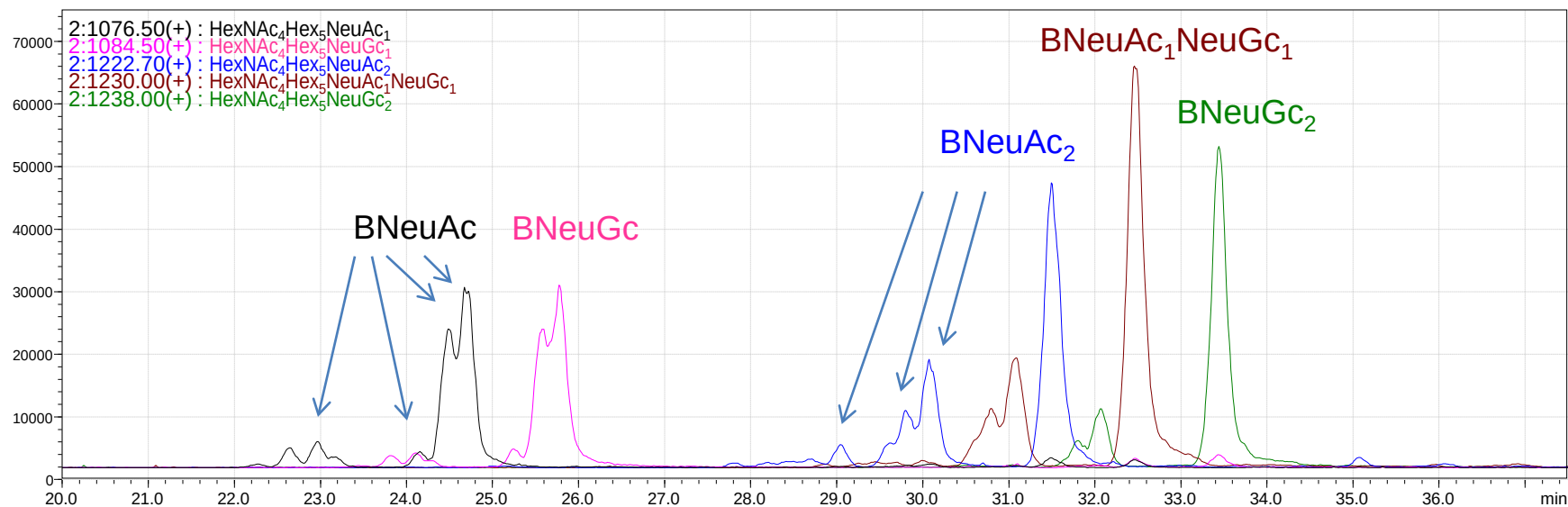
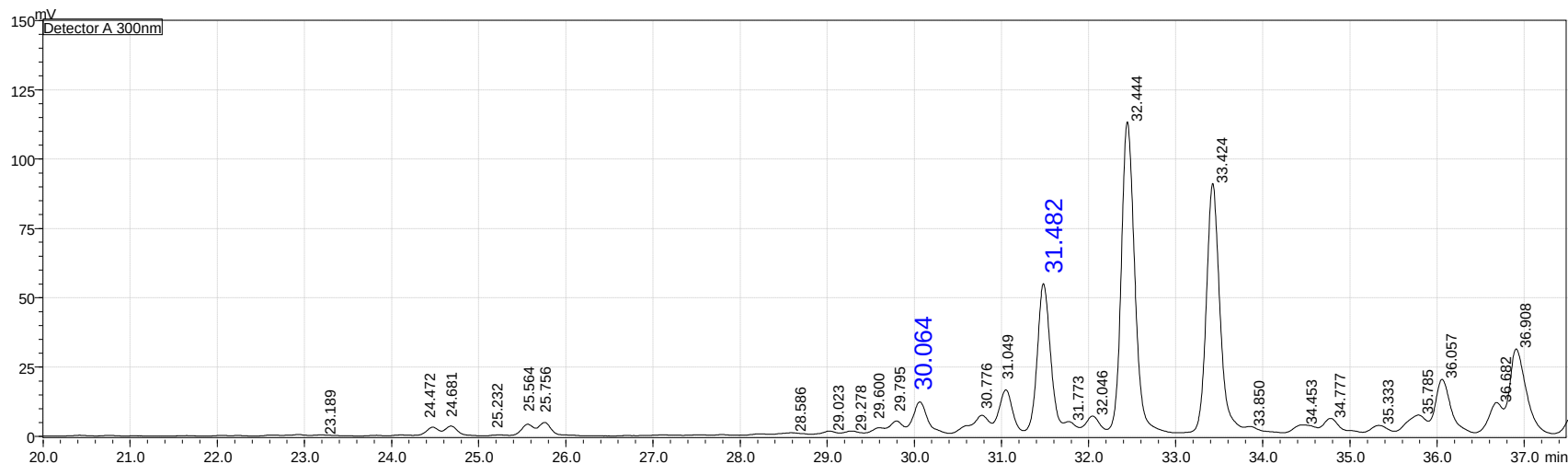
HILIC of End-labeled Glycans



HILIC Separations of α 1-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 μ l/min.

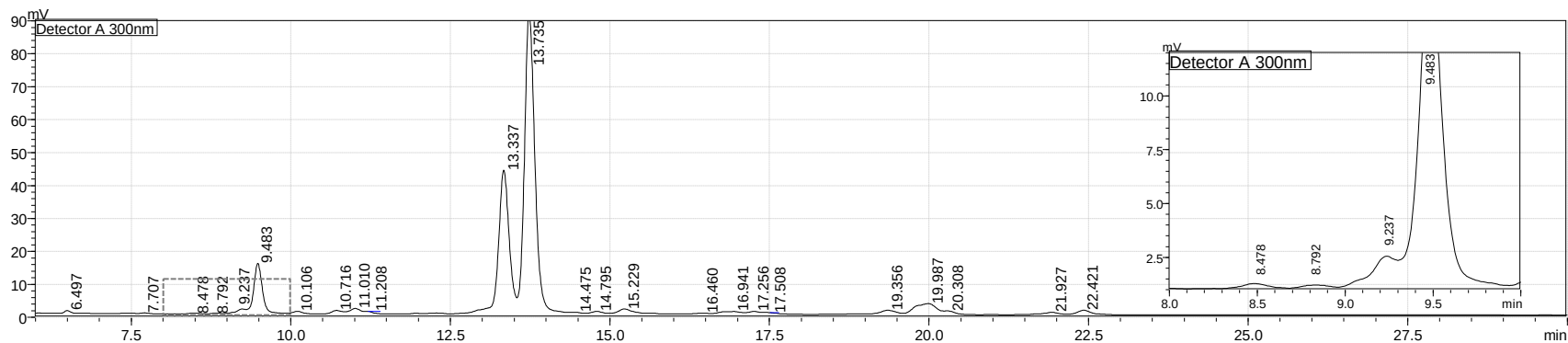
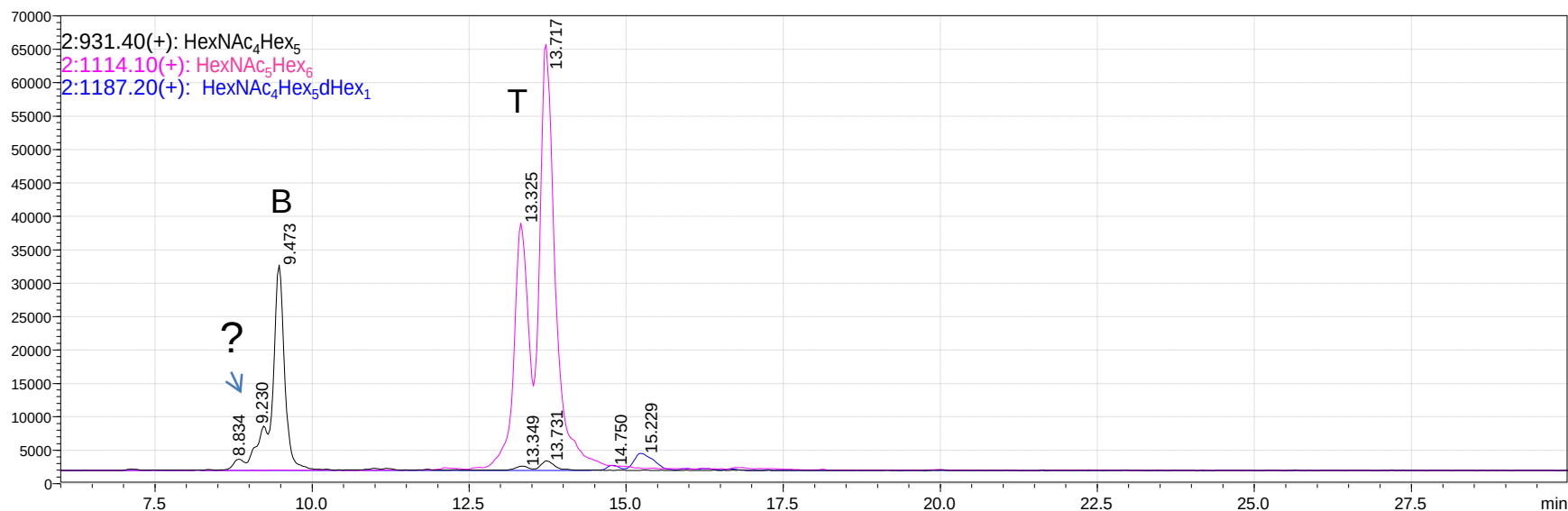
Detection: 300 nm Abs; ESI-MS ((+)) MS-2020, 4.2 kV, 400-2000 with SIM



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 nL/min.

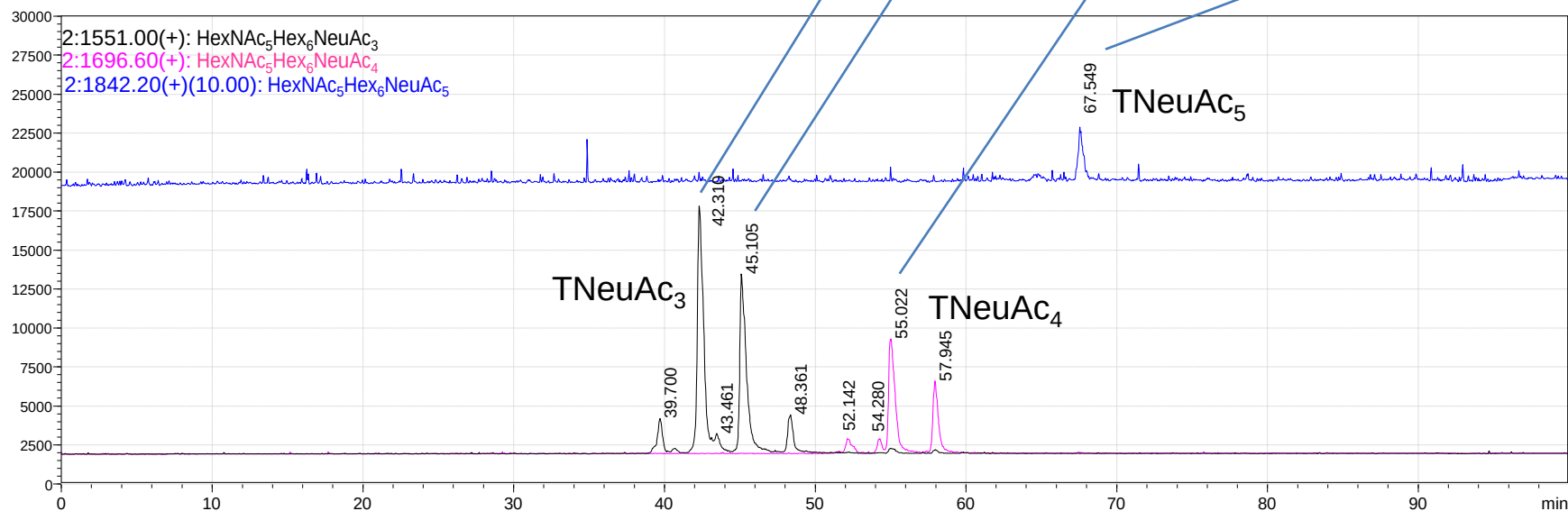
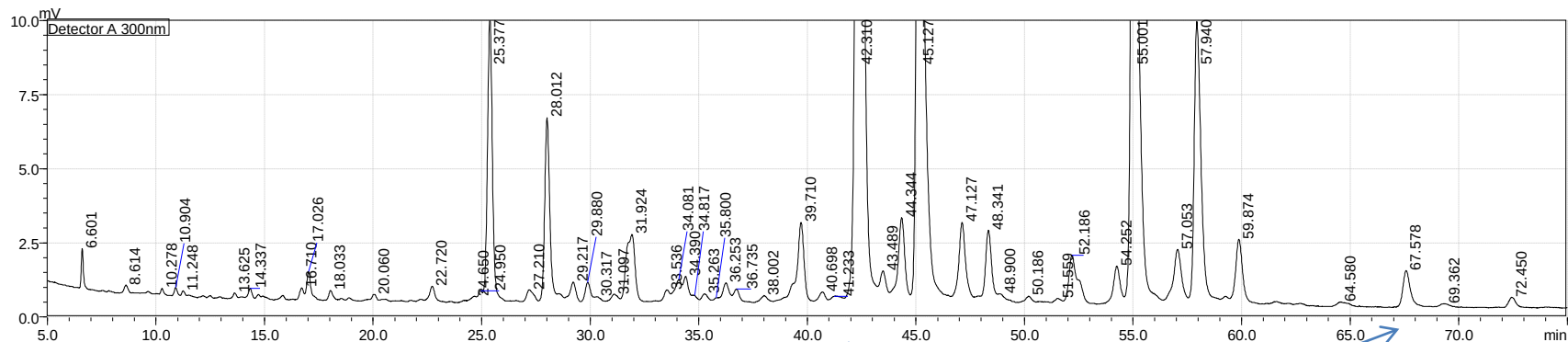
Detection: 300 nm Abs; ESI-MS ((+) MS-2020, 4.2 kV, 400-2000 with SIM)



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 nL/min.

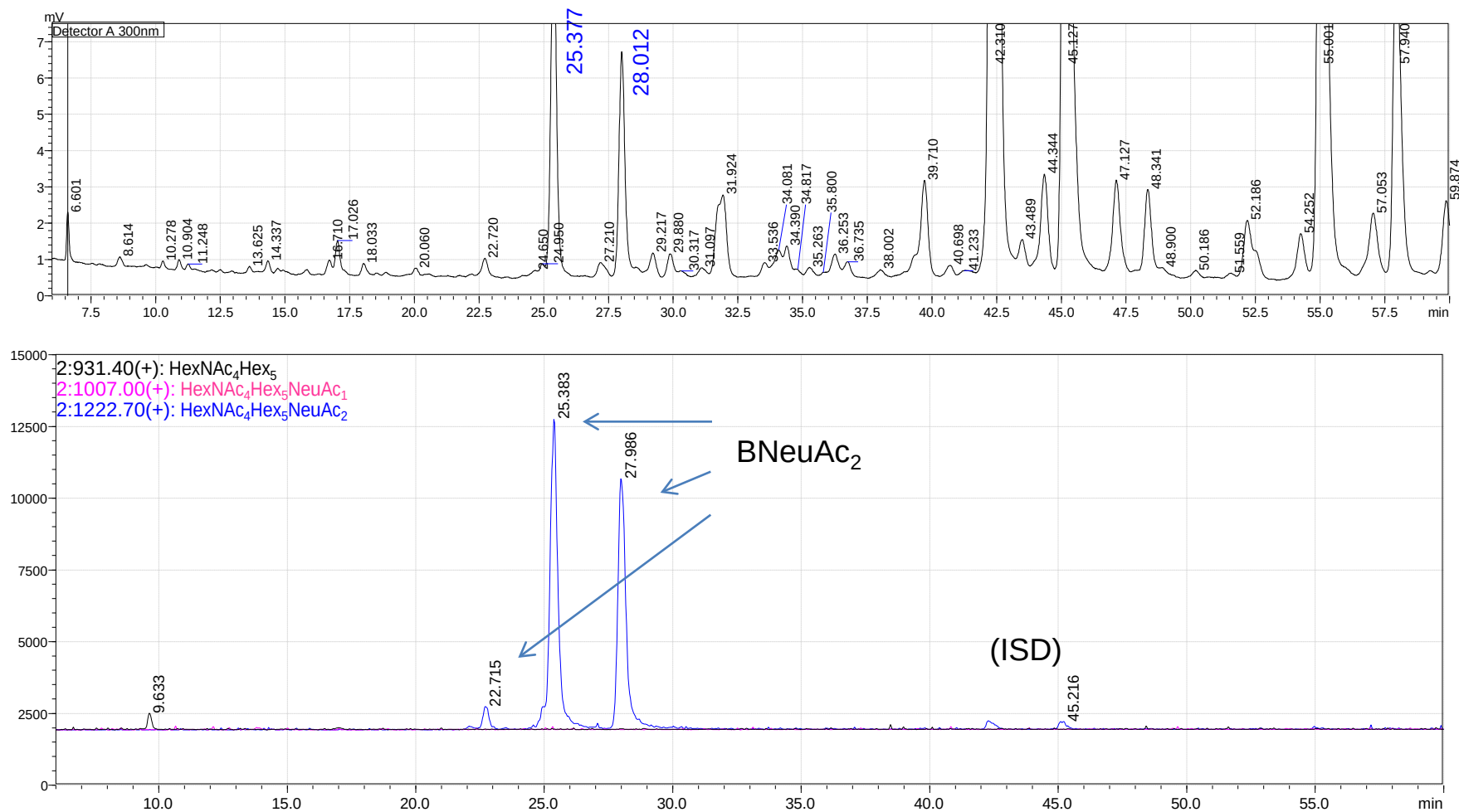
Detection: 300 nm Abs; ESI-MS ((+) MS-2020, 4.2 kV, 400-2000 with SIM)



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 nL/min.

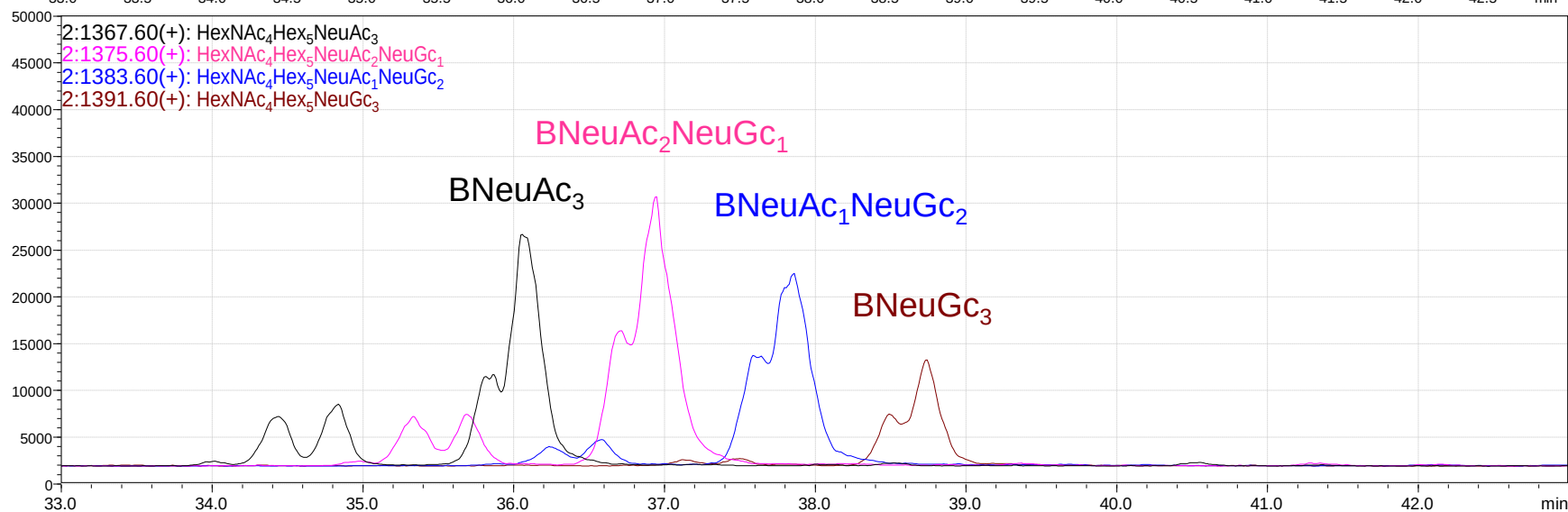
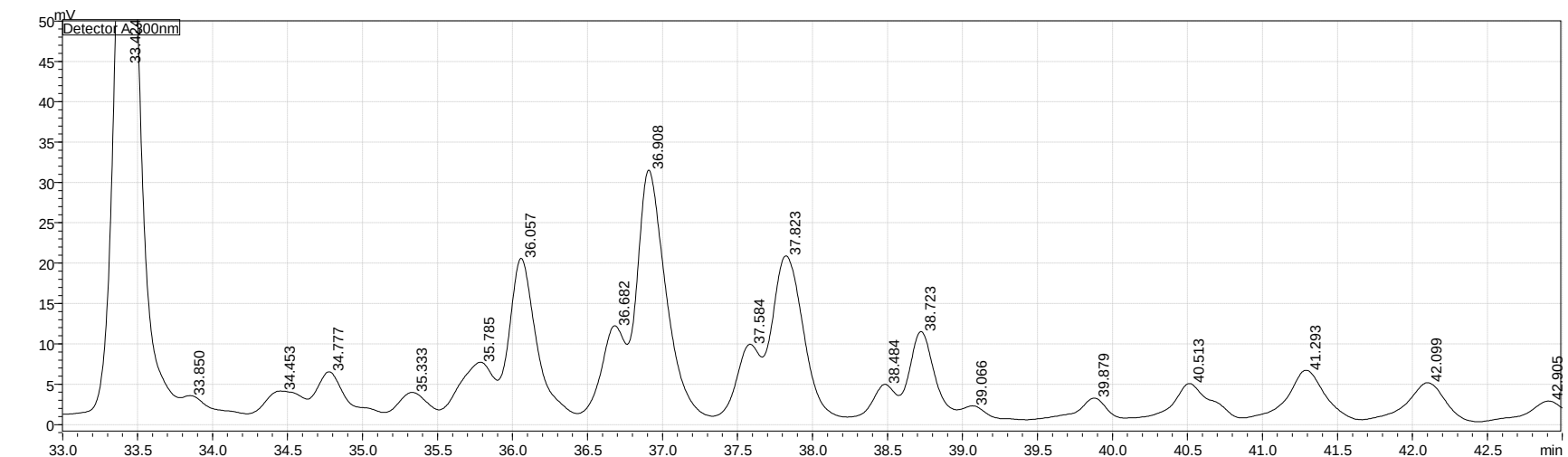
Detection: 300 nm Abs; ESI-MS ((+) MS-2020, 4.2 kV, 400-2000 with SIM)



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 nL/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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