Intact Protein and Glycoprotein Separations by HILIC

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Agenda

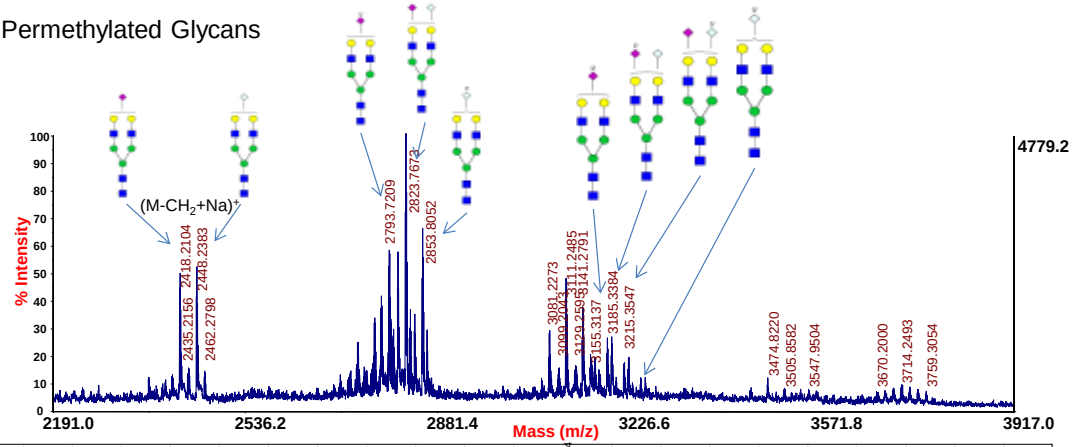
- HILIC separations have utility in glycoscience applications
- Polar interactions for glycans, glycopeptides and glycoproteins lead to useful separations
- Materials and methods of use for such separations are improving, but use remains complex
- Will show examples of protein separations based on Glycoform structure

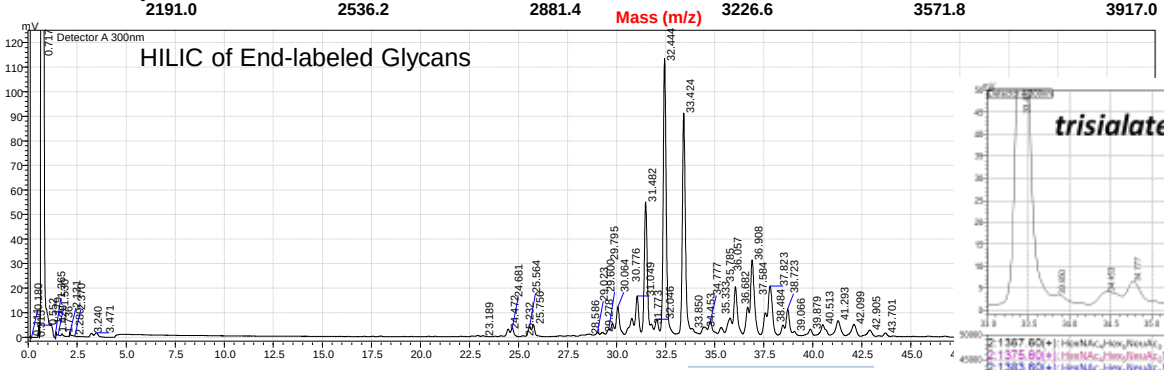
HILIC for Glycoscience
Utility for Oligosaccharide Profiling

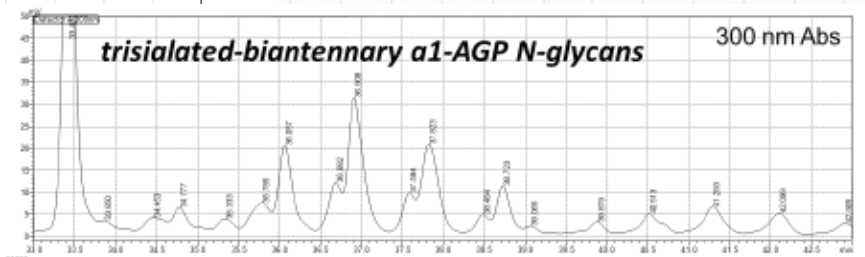
Penta-HILIC Separations of Abundant bov. a1-AGP N-glycans

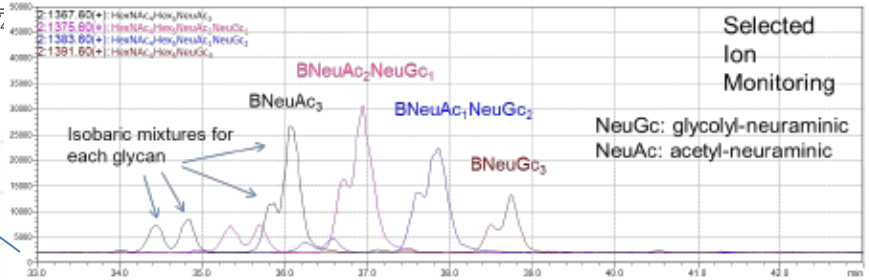
2.1 mm ID x 150 mm; 50 mM Ammonium Formate, pH 4.4, 77.5-56.5% AcN (B) in 52.5 min., 60°C; 600 nL/min.

MALDI of Permethylated Glycans









Peptide Separations by HILIC

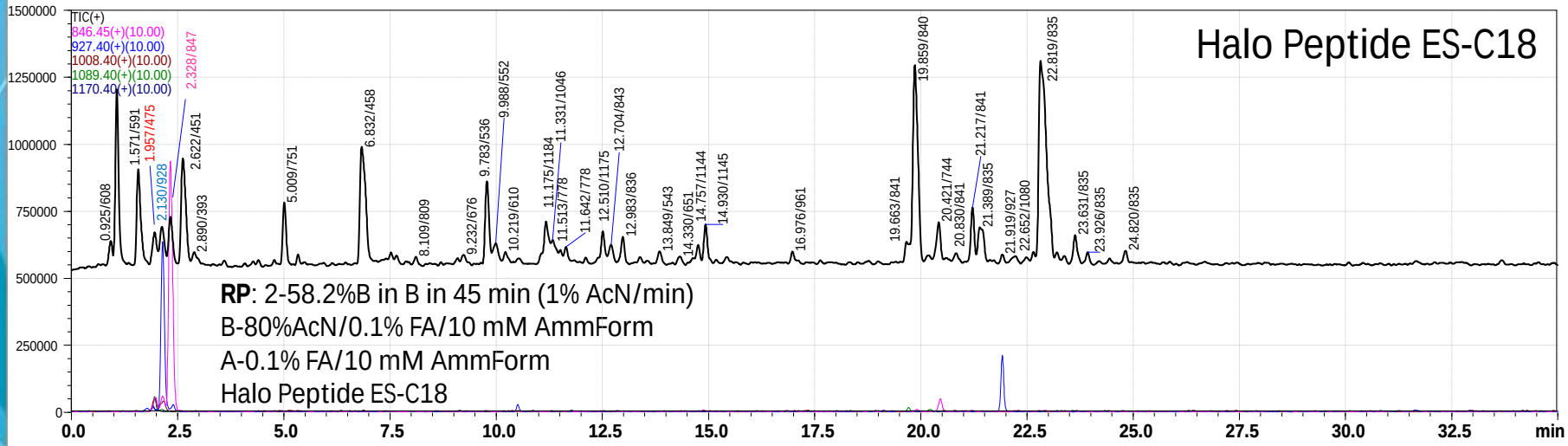
Generalities:

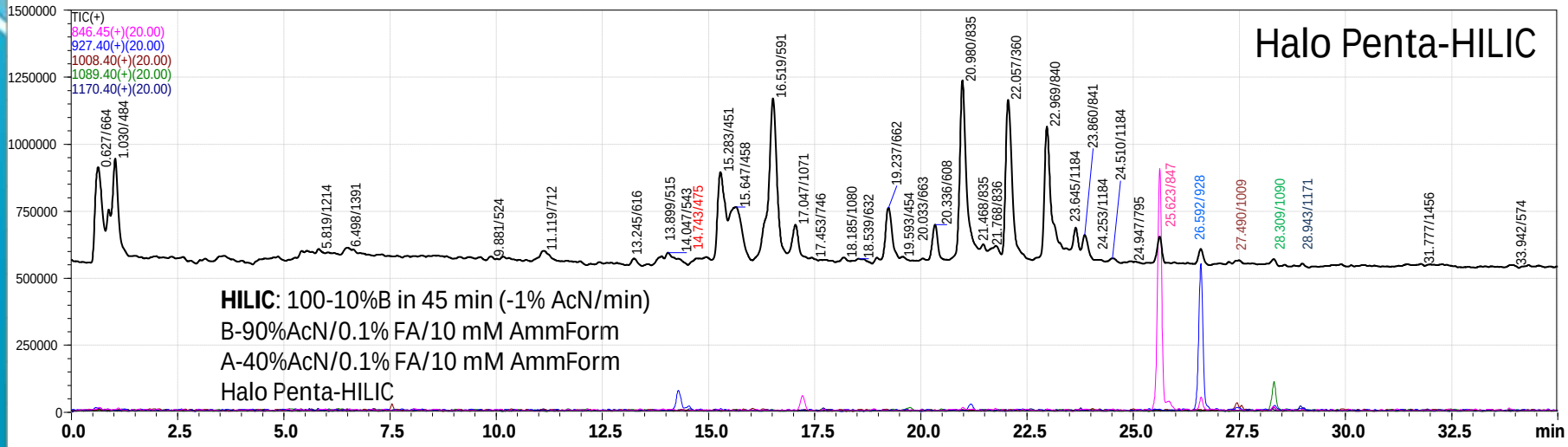
- acidic conditions (TFA, FA, HOAc, Phosphoric, etc.)
- can exhibit orthogonality in retention, relative to RP
  - M. Gilar, et al., Anal. Chem. 82 (2010) 265, and others
  - Boyes, et al., ISPPP 2013
- does not have to be at a disadvantage for efficiency
- can be very MS friendly
- can show some solubility problems
- additional "polar modes" of operation are possible, eg., ERLIC, HILIC/SALT (Mant, et al., J. Chromatogr. 1277 (2013))
- *can allow separations that are very difficult using RP*

Penta-HILIC Strongly Retains N-linked Glycopeptides

2.1 mm ID x 100 mm, 0.35 mL/min, 40 C, MS: SQ TIC (+ 300-2000 m/z) @ 0.35/s

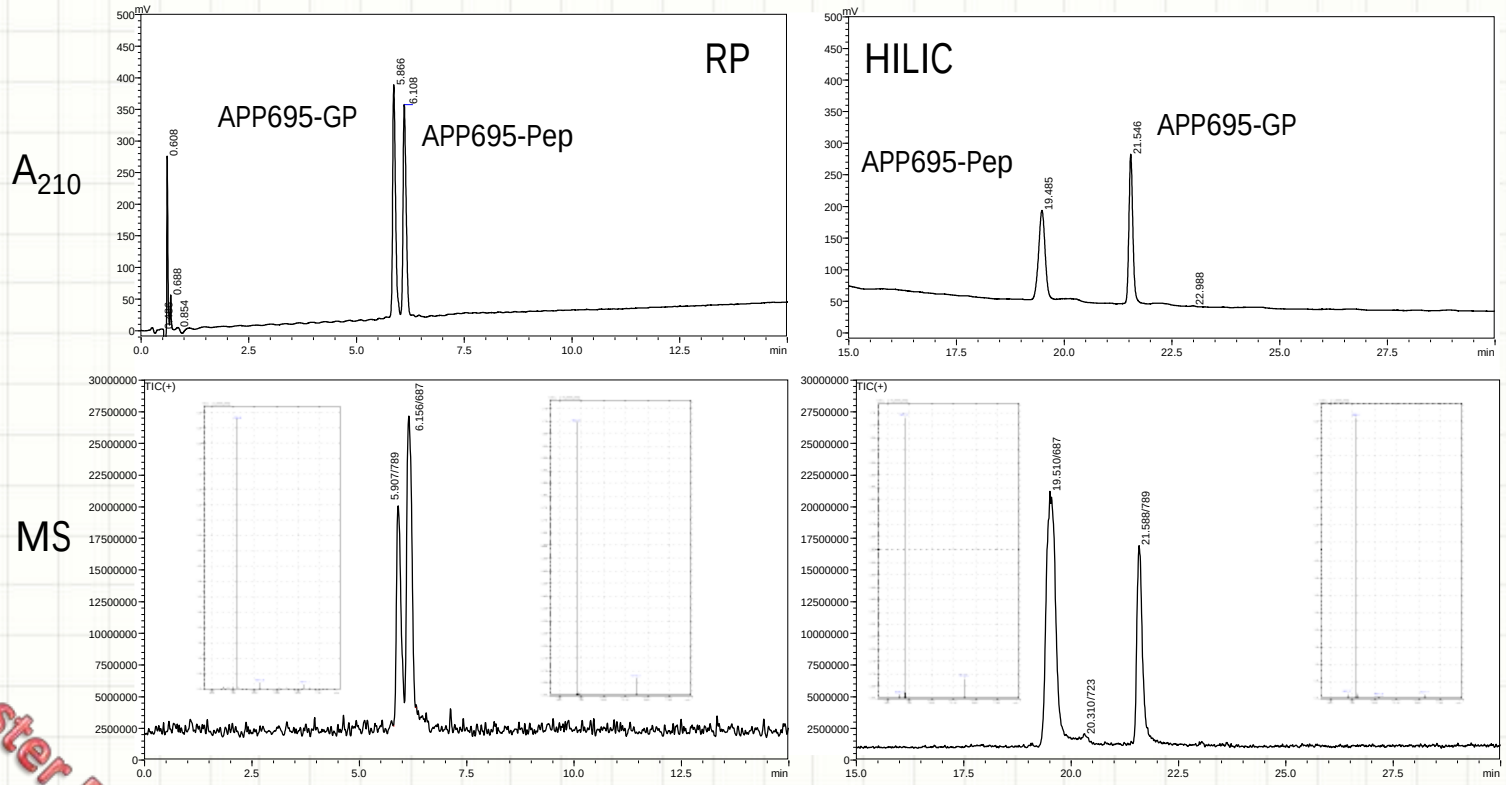
20 µg Bovine Ribonuclease B tryptic digest (CAM)





LC/MS of O-GlcNAcylated Peptides

2.1 x 100 mm Halo Penta-HILIC, 0.4 mL/min at 60°C.
Gradient conditions: A – 0.1% formic acid/10 mM ammonium formate; B – 90% Acetonitrile in A.
500 pmol, MS operated +4.5 kV, with 0.45 s scan from 500 to 2000 m/z (TIC)
RP Gradient - 4% to 34% AcN/30 min (1%/min); HILIC Gradient - 90% to 60% AcN/30 min (-1%/min).



APP695-14GPep	VPTT(OGlcNAc)AASTPDAVDK
APP695-14Pep	VPTTAASTPDAVDK

See Poster P-W-2304

Protein Separations by HILIC

- Limited use relative to RP
  - Variety of examples by Alpert and collaborators
  - Tetaz, et al., J. Chromatogr. 1218 (2011) 5892.
  - Carrol, et al., P.N.A.S. U.S.A. 103 (2006) 16170.
  - Focused on membrane proteins, histone and lipoprotein PTM modifications
- Expected to show very different selectivity than Reversed Phase separations of proteins
- Challenge of using high organic mobile phases for proteins
- Not clear what features are needed to improve intact protein separations – mobile phase, additives, stationary phases, or combinations of all

This could be a longer term project!

Operational Considerations

- Mobile phases components:
  - TFA, Formic Acid, FA/AF (0.05%-1%)
  - Acetonitrile, *i*-propanol, *n*-propanol
  - HFIP, TFE, others
  - Solubility is always a consideration for both salts and proteins

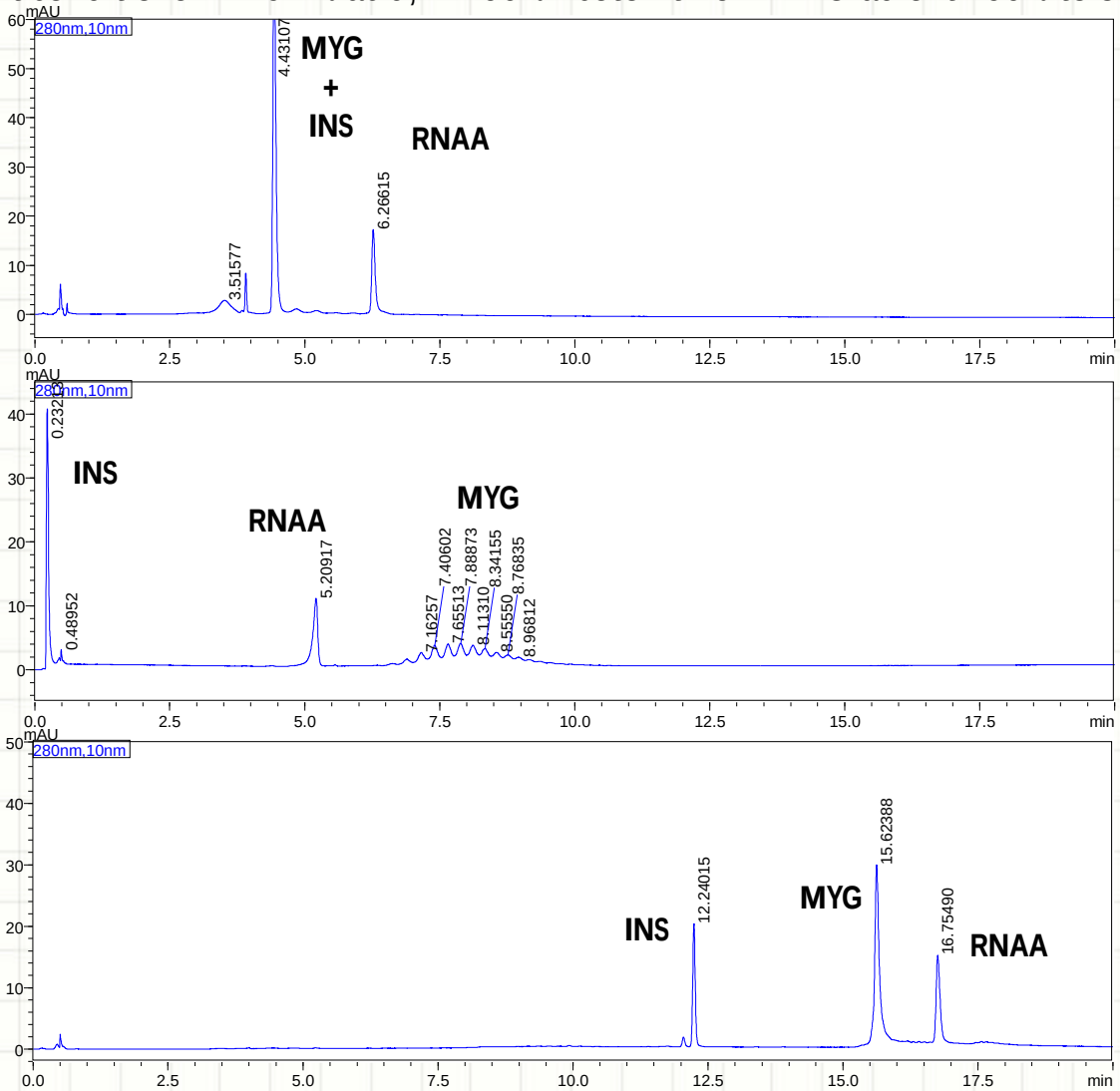
- Stationary phase characteristics:
  - 2.7 µm Halo Penta-HILIC
    - hydroxylated neutral HILIC bonded phase
    - Fused-Core particle with 0.5 um SPP layer
  - 90 Å and 160 Å pore size

- Aqueous soluble proteins and glycoproteins
  - Samples adjusted to 1-3% Formic Acid in High Organic MP

- Combination of absorbance and MS detection, where possible

Effect of Acid Modifier

2.1 x 100 mm Halo Penta-HILIC 90 Å pore size, 0.5 mL/min at 50°C.
Gradient conditions: A – 0.1% acid; B – 90% Acetonitrile in A. Gradient - 90% to 50% AcN/20 min (-2%/min).



0.1% TFA

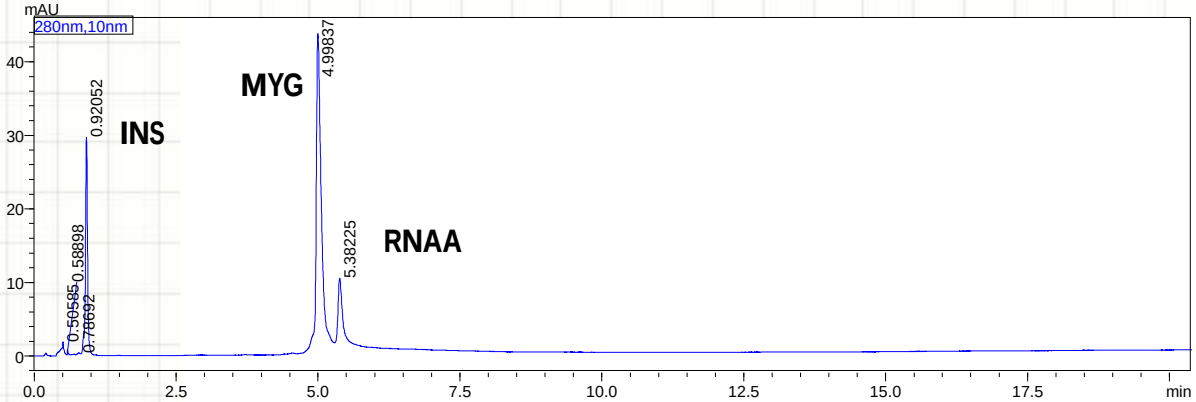
0.1% FA

0.1% FA/10 mM AF

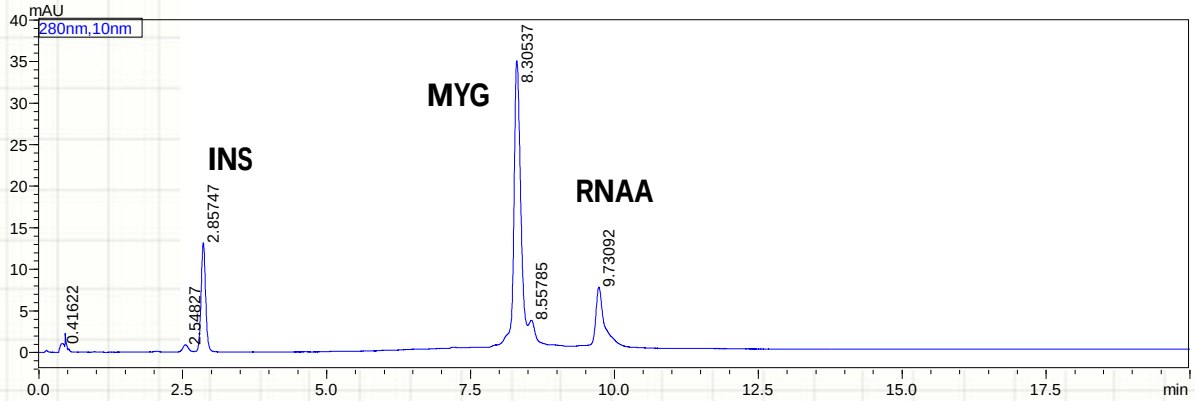
Current Consensus MP & Pore Size

- FA/AF buffer (0.2%/20 mM) with 0.5% HFIP (does not HURT!)
- Mixture of Acetonitrile/*i*-Propanol (40%/35%) to 10% iPA
- Elevated column temperature: 40-60°C
- Pore Size is not a clear factor for proteins
- Adjusting sample solvent is crucial – addition of FA to 1-2% resolves many solubility problems

2.1 x 100 mm Halo Penta-HILIC, 0.5 mL/min at 50°C; Gradient conditions: B – 0.2% FA/20 mM AF/40% AcN/35% IPA/ 0.5% HFIP; A – 0.2% FA/20 mM AF/10% IPA Gradient - 90% to 55% B in 20 min.



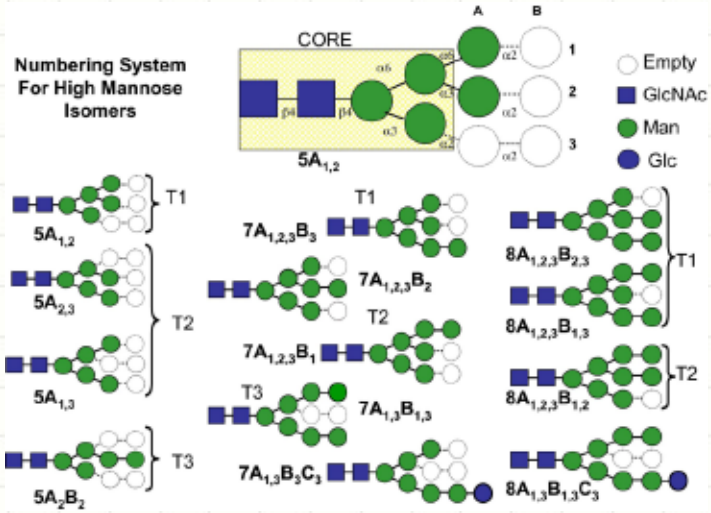
160 Å pore
- **Lower Rt**
- **Slightly Lower PW**



90 Å pore

Bovine Ribonuclease as a Glycoprotein

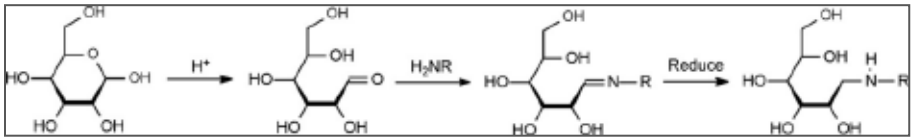
- Composition:
  - RNase A - 124 aa residues, 4 disulfide bridges, 13.7 kDa
  - RNase B – Asn^{34} is the site of glycosylation, with 5 high mannose structures M5-M9 , leading to enzyme heterogeneity, 14.9-15.5 kDa
  - In addition to compositional heterogeneity, branch variants of the high mannose oligosaccharides M5, M7, M8 are indicated

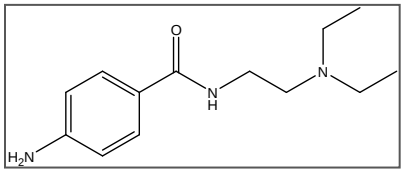


Prien, et al., J. A.S.M.S. 20 (2009) 539

RNase B N-Linked Glycans

Release of protein N-linked glycans using PNGase F yields 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.

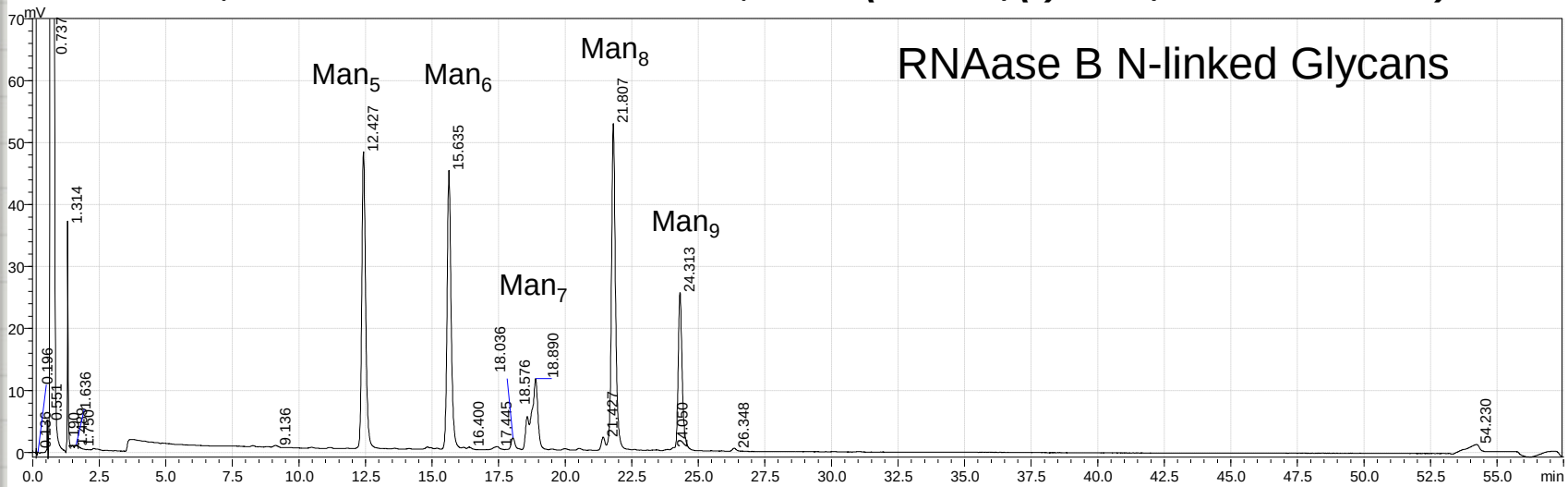




Procainamide
Abs: 300 nm
Fl: Ex 330/Em 380 nm
Mass: glycan + 219.32

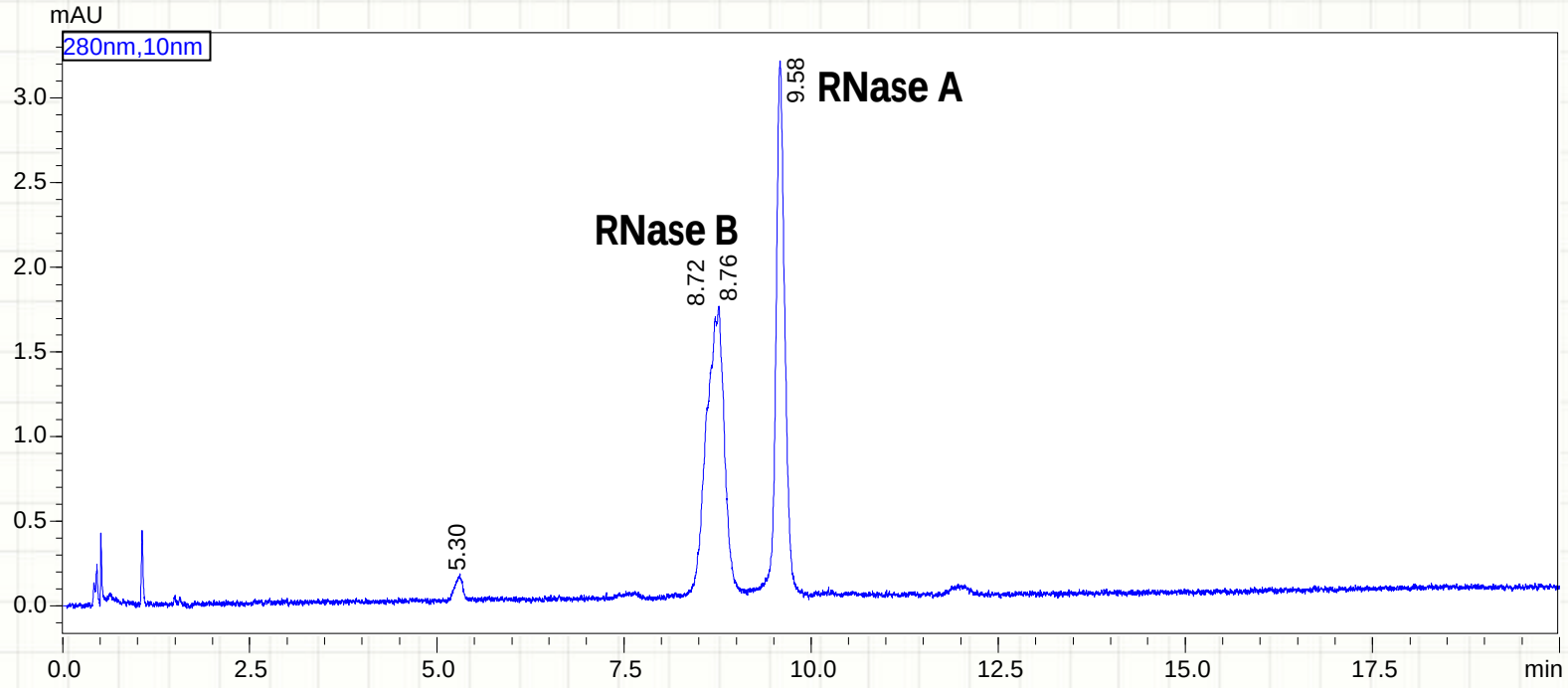
Many amines have been applied to labeling glycans, (Harvey, 2011, J. Chrom. 879).
Procainamide is favored due to improvements in ESI-MS detection (Klapoetke, et al., 2010, J. Pharm. Biomed. Anal. 53), and favorable fragmentation conditions in CID.

2.1 mm ID x 150 mm Halo Penta-HILIC; 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)



Separation of RNases by RP

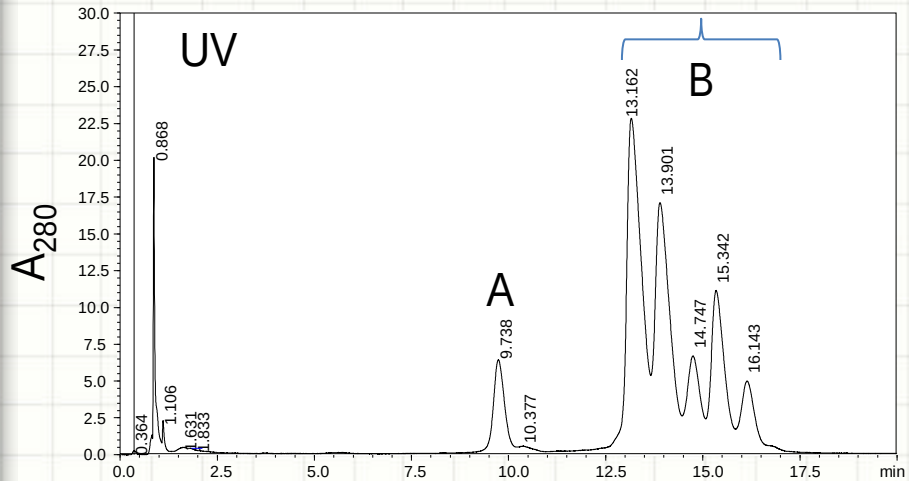
2.1 x 100 mm Halo Peptide ES-C18, 0.5 mL/min at 50°C; Gradient conditions: A – 0.1% TFA/water, B – 0.1% TFA in 80% AcN; Gradient - 30% to 40% B in 20 min (0.4% AcN/min), 0.5 µg each.

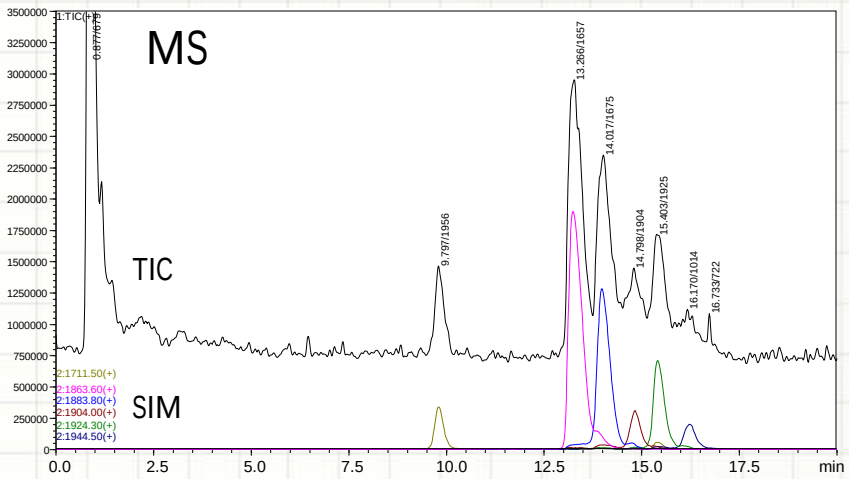


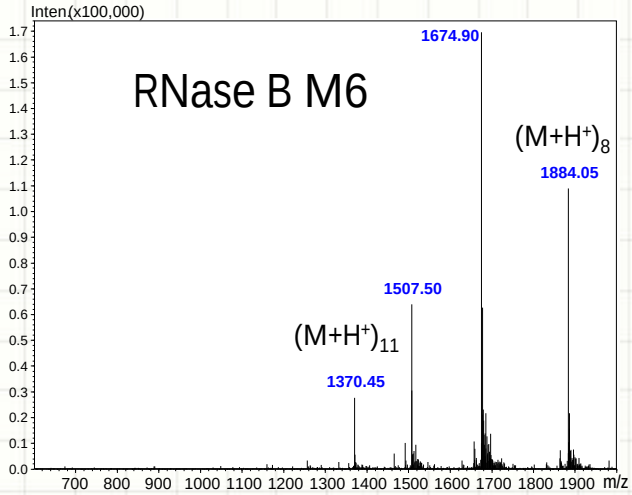
RP allows Resolution of A/B, little resolution of B glycoforms

HILIC Separation of RNase B Glycoforms

2.1 x 100 mm Halo Penta-HILIC, 0.35 mL/min at 50°C; Gradient conditions: B – 0.2% FA/20 mM AF/40% AcN/35% IPA/ 0.5% HFIP; A – 0.2% FA/20 mM AF/10% IPA Gradient - 85% to 75% B in 20 min.



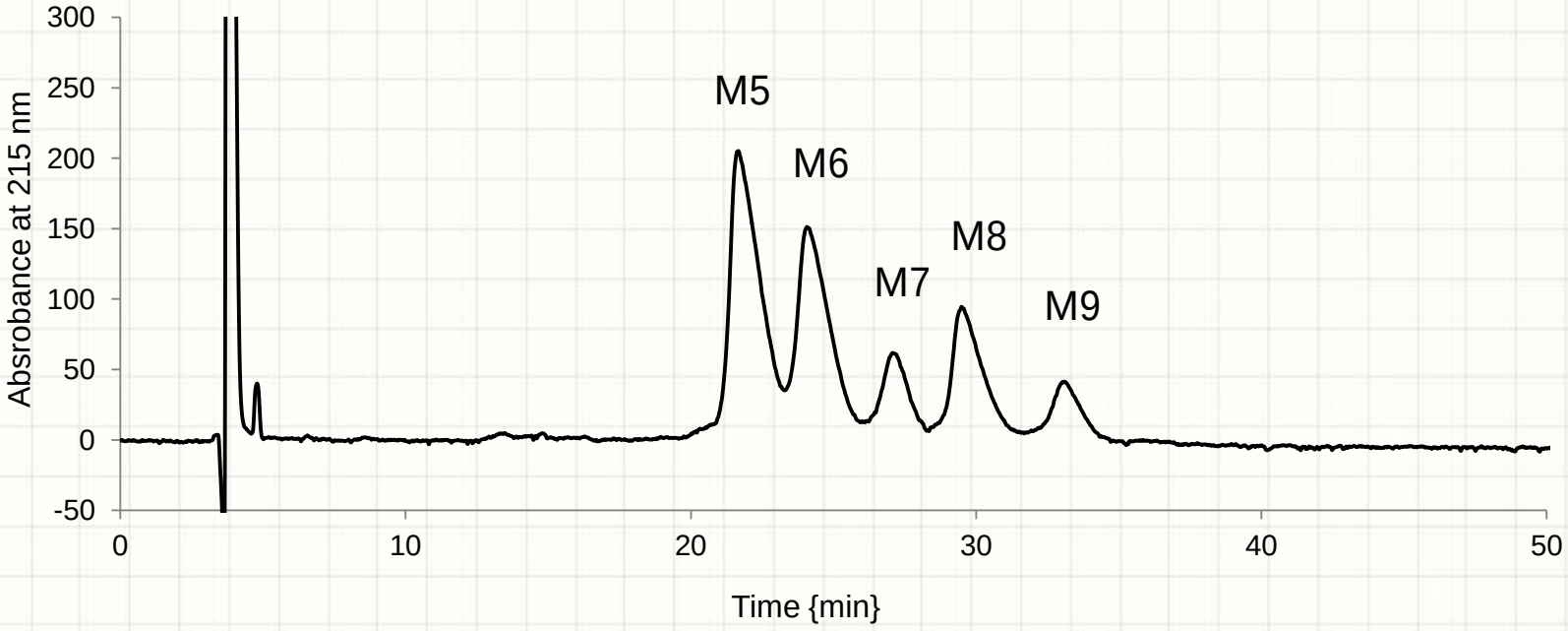




ID	Mass (Da) Theory	Mass (Da) Observed
RNA A	13,681	13,686
RNA B M5	14,898	14,903
RNA B M6	15,060	15,068
RNA B M7	15,222	15,227
RNA B M8	15,384	15,390
RNA B M9	15,564	15,550

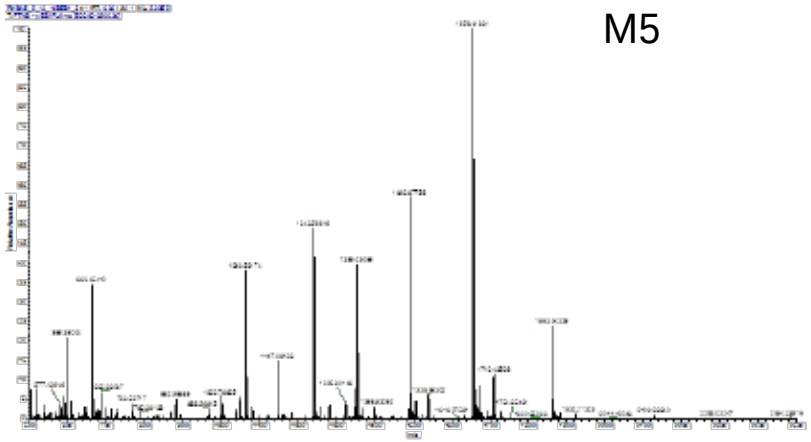
Isolated RNase B Glycoforms

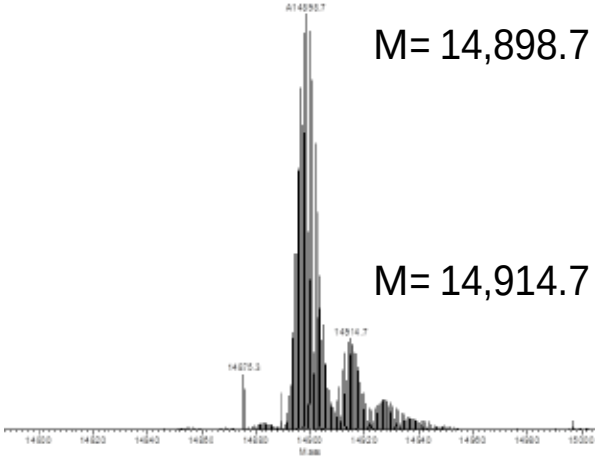
Separation Conditions: 4.6 x 250 mm Penta HILIC 2.7 µm 90Å, 60 C, 0.4 mL/min, 215 nm, 78-75 % B over 60 min, B – 40% AcN/35% IPA/0.2%FA 20 mM AF/0.5% HFIP



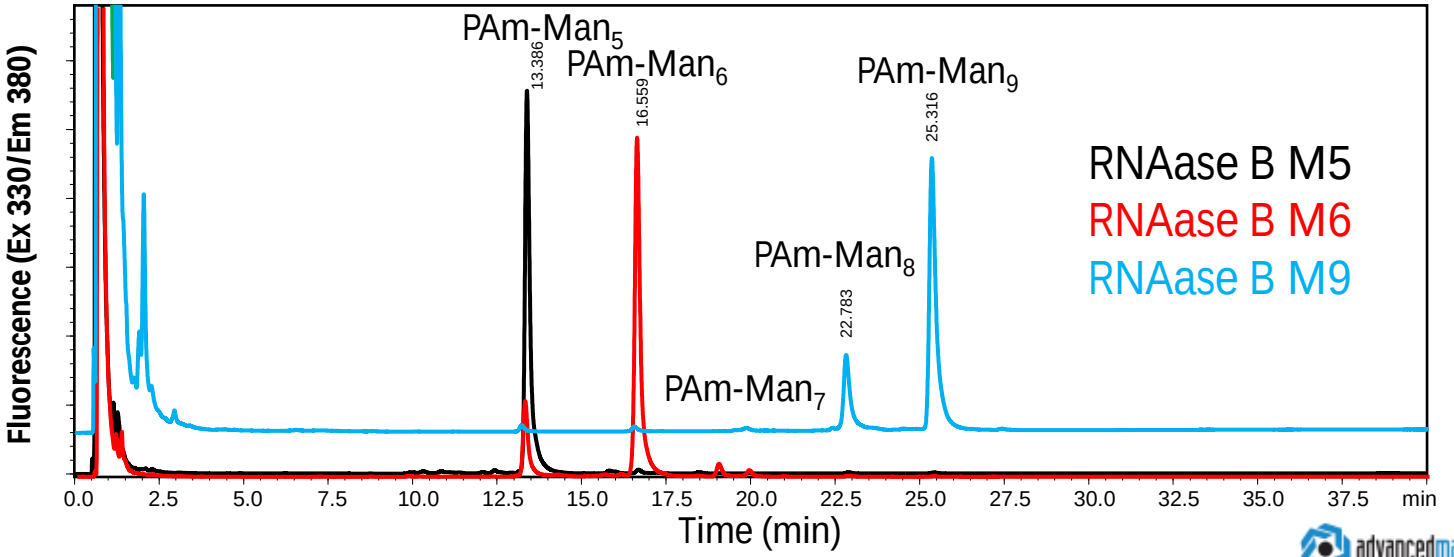
Characterization of Resolved RNase B Glycoforms

High Resolution ESI/MS (Orbitrap) Infused in 0.5% FA/50% AcN



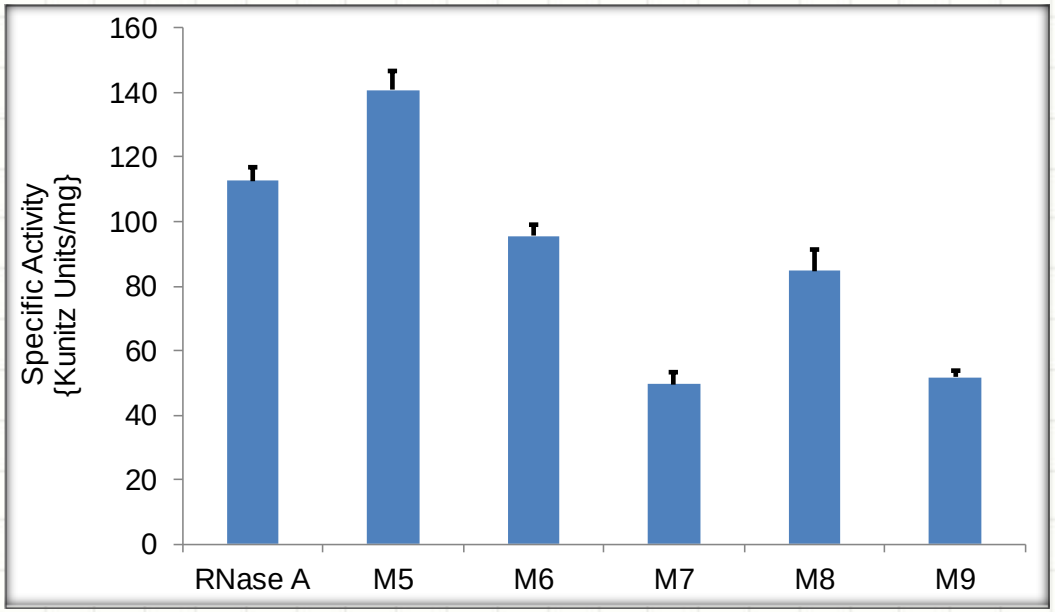


Analysis of PNGase F released glycans from M5, M6 and M9 Proteins



Isolated RNase B Glycoforms

Assay Conditions: 50 mM NaOAc, pH 5.0, 25°C, 0.5 mg/mL RNA, ΔA300



More on Glycoproteins

- Protein structure contributes to retention
- Mass and nature of glycans will contribute to retention
- Not all glycoproteins are as simple as RNase B
- LC/MS resolution requirements increase a lot with size
- MS Sensitivity is a problem as size and glycosylation rise

TABLE 3

Characteristics of Glycoproteins Chosen to Test the Organic Solvent Precipitation/Extraction Methodology

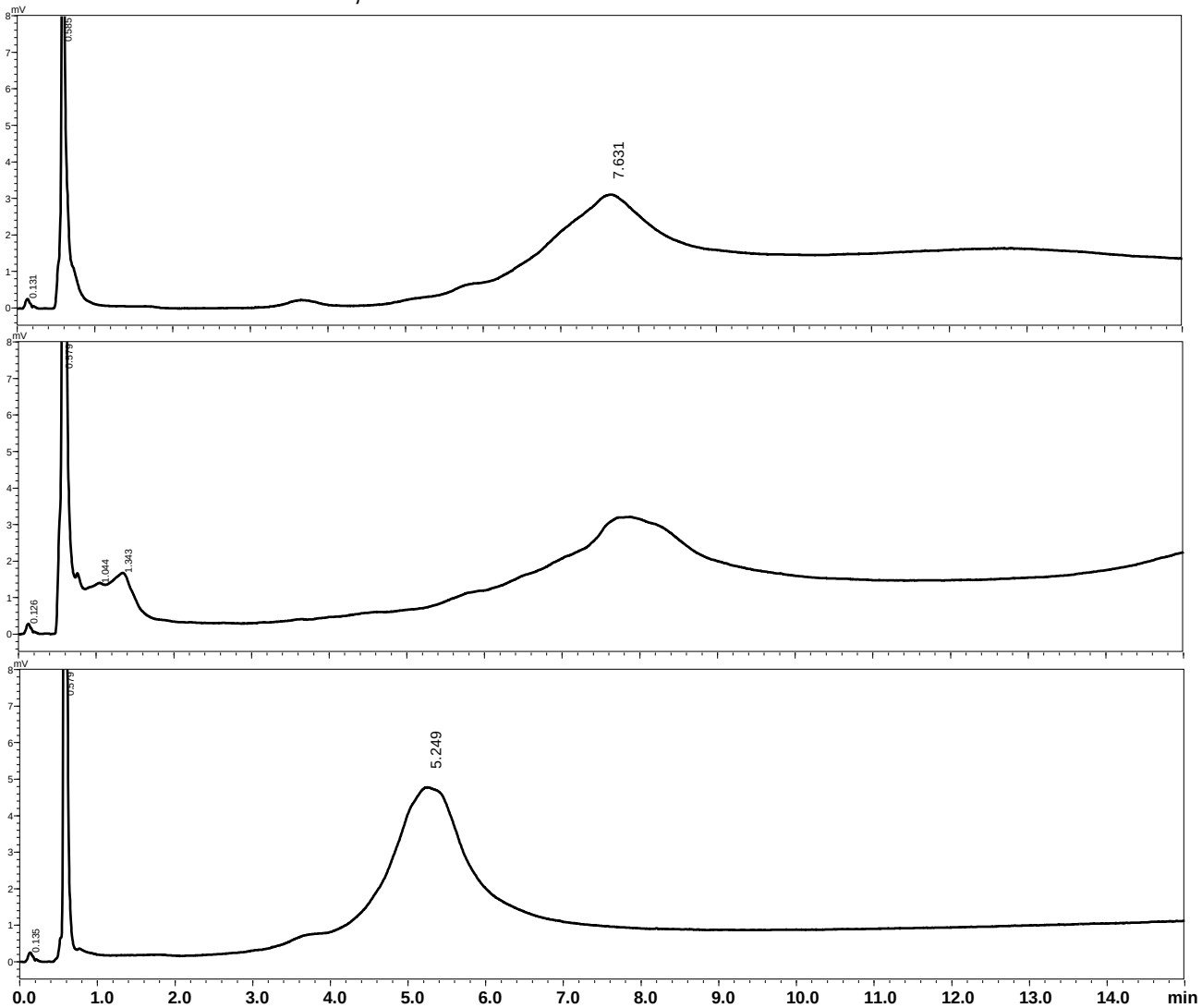
Glycoprotein	Source	Monomer (kDa)	Native form	CH_2O[a] Content (g/100 g)	Oligosaccharides/monomer: N-linked: High mannose	Oligosaccharides/monomer: N-linked: Complex	Oligosaccharides/monomer: O-linked	Amount treated (mg)
RNase B	Bovine	13	Monomer	11	1	0	0	1, 20
Ovalbumin	Hen	43	Monomer	3	1	0	0	5 to 60
Invertase	Wt yeast	60	Octamer	50	9–10	0	0	20
	Sec18 yeast			28	9–10	0	0	10
Thyroglobulin	Bovine	330	Dimer	9	5–6	13–14	0	20, 40
Fetuin	Bovine	48	Monomer	23	0	3	3	40
BSSL	Human/*P. pastoris*	76.3	Dimer	16	0.7	0	27	65, 80, 100

[a] Carbohydrate.

Verostek, et al., Anal. Biochem. 278 (2000) 111.

Real Glycoprotein HILIC HUMPograms

2.1 x 100 mm Halo Penta-HILIC, 0.4 mL/min at 50°C; Gradient conditions: B – 0.2% FA/20 mM AF/40% AcN/35% IPA/ 0.5% HFIP; A – 0.2% FA/20 mM AF/10% IPA. Gradient - 90% to 50% B in 15 min.



Intact Fetuin

Asialo-Fetuin

PNGase F Fetuin (O-glycosylated)

Conclusions

- HILIC separations methods have impact in glycobiology at the glycan, glycopeptide and glycoprotein structure levels.
- Results with intact protein and glycoprotein LC/MS separations of smaller proteins performed well, but expanding to a broader range of proteins requires further effort on mobile phase and use conditions (sample solvent dependencies).
- Selectivity for glycan separations, when attached to peptide and protein structures, appears excellent.
- RNase B glycoforms can be well resolved, under conditions that retain activity.

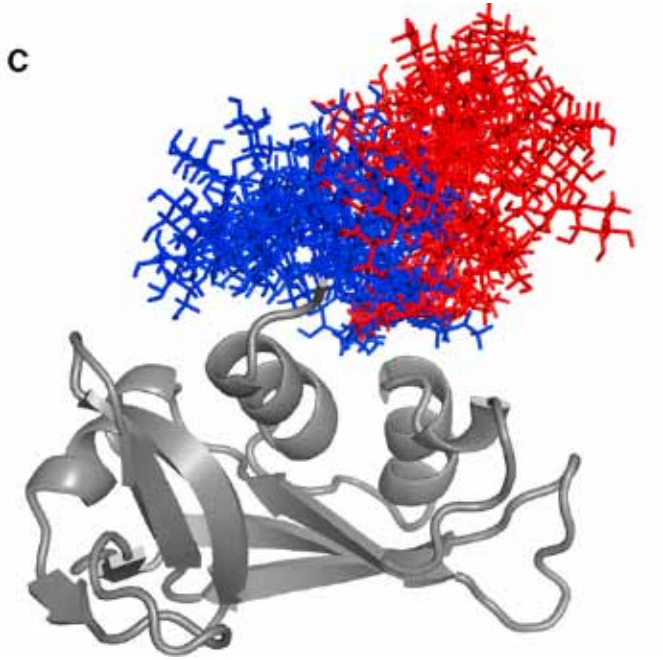
Thank you for your attention!

Also thanks to:

- AMT – Jack Kirkland, Stephanie Schuster, William Miles and Bob Moran for advice and technical assistance.
- NIH for Financial Support - GM093747

Protein and Glycoprotein Surfaces

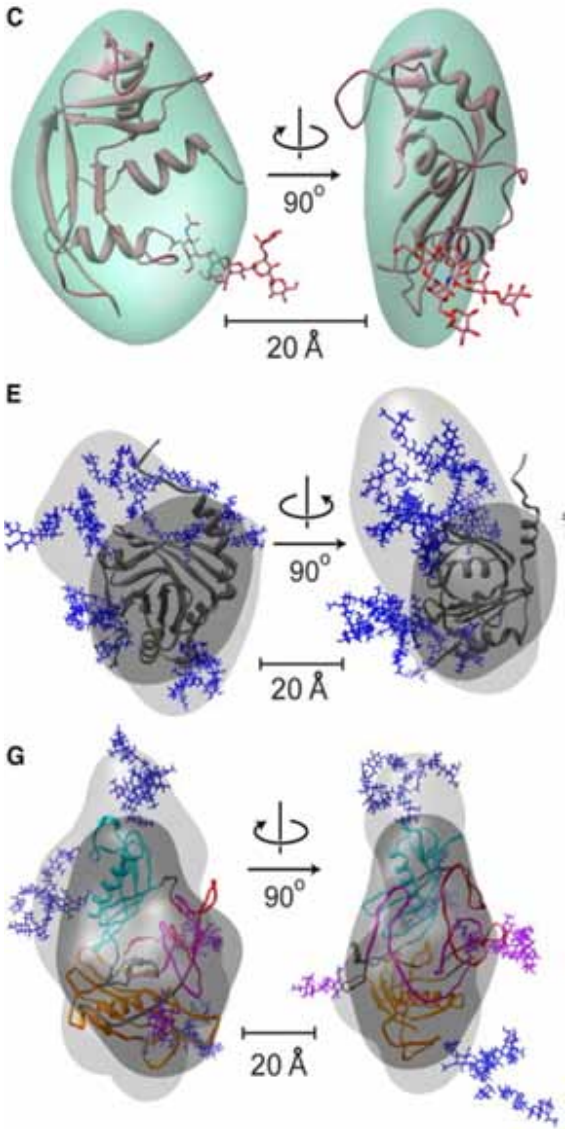
Glycan positions are shown for the ten best (red) and poorest (blue) fitting models.



RNaseB

a1AGP

Fetuin



Guttman, et al., Structure 21 (2013) 321.

Separation of RNase B PNGase F Released N-Linked Glycans

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