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Oligosaccharide Analysis by High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection

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Agenda

- Introduction to HPAE-PAD
- Carbohydrate analysis by HPAE-PAD
- Fundamentals of HPAE-PAD oligosaccharide analysis
- HPAE-PAD oligosaccharide analysis applied to glycoproteins
- Comparison to other techniques and other topics
- Conclusions

HPAE-PAD

High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection

HPAE-PAD: An Established Technique

- First described in the early 1980s
- First applied to glycoproteins in 1986-1987
- Well over 1000 peer-reviewed publications using HPAE-PAD
- Used in >10 United States Pharmacopeia official monographs

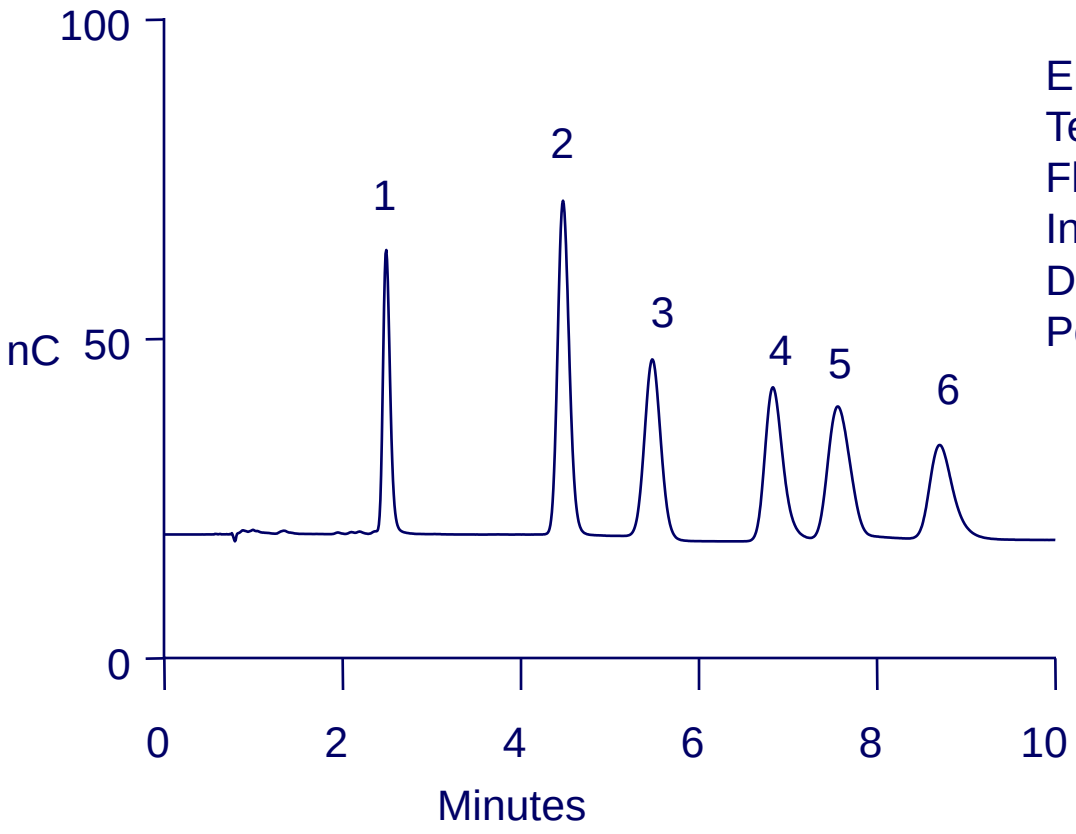
Basics of HPAE

- Carbohydrates are separated as oxyanions at high pH (>12).
- These separations require hydroxide eluents.
- If the carbohydrate is charged, acetate or another strong eluent must be added to the hydroxide eluent to elute the carbohydrate.

Basics of PAD

- Carbohydrates are detected on a gold working electrode (WE) at high pH by PAD.
- PAD applies a series of potentials (a waveform) to a WE and the carbohydrate is detected by its oxidation at 1 potential.
- The waveform is applied at a frequency of 2 Hz, i.e. two times a second.

Glycoprotein Monosaccharides



Column:	Thermo Scientific™ Dionex™ CarboPac™ PA20	
Eluent:	10 mM Sodium hydroxide	
Temp:	30 ºC	
Flow Rate:	0.5 mL/min	
Inj. Volume:	10 µL	
Detection:	PAD (Waveform A TN21)	
Peaks:	1. Fucose	100 pmol
	2. Galactosamine	100
	3. Glucosamine	100
	4. Galactose	100
	5. Glucose	100
	6. Mannose	100

What is Required for HPAE-PAD?

- A non-metallic high-pressure liquid chromatography system
- High-quality deionized water
- Autosampler capable of injecting low microliter volumes
- High-performance anion-exchange column designed for carbohydrates
- Electrochemical detector and cell capable of executing the waveforms used for carbohydrate determinations

Why HPAE-PAD is an Established Technique

- No sample derivatization required for separation or detection – Direct detection
- Sensitive detection
- High-resolution separations
- High capacity – can observe a small amount of one carbohydrate in the presence of a large amount of other
- One technique able to determine a wide range of carbohydrates
- One separation reveals both products and substrates – including labeled products

Popular HPAE-PAD Applications for Glycobiology

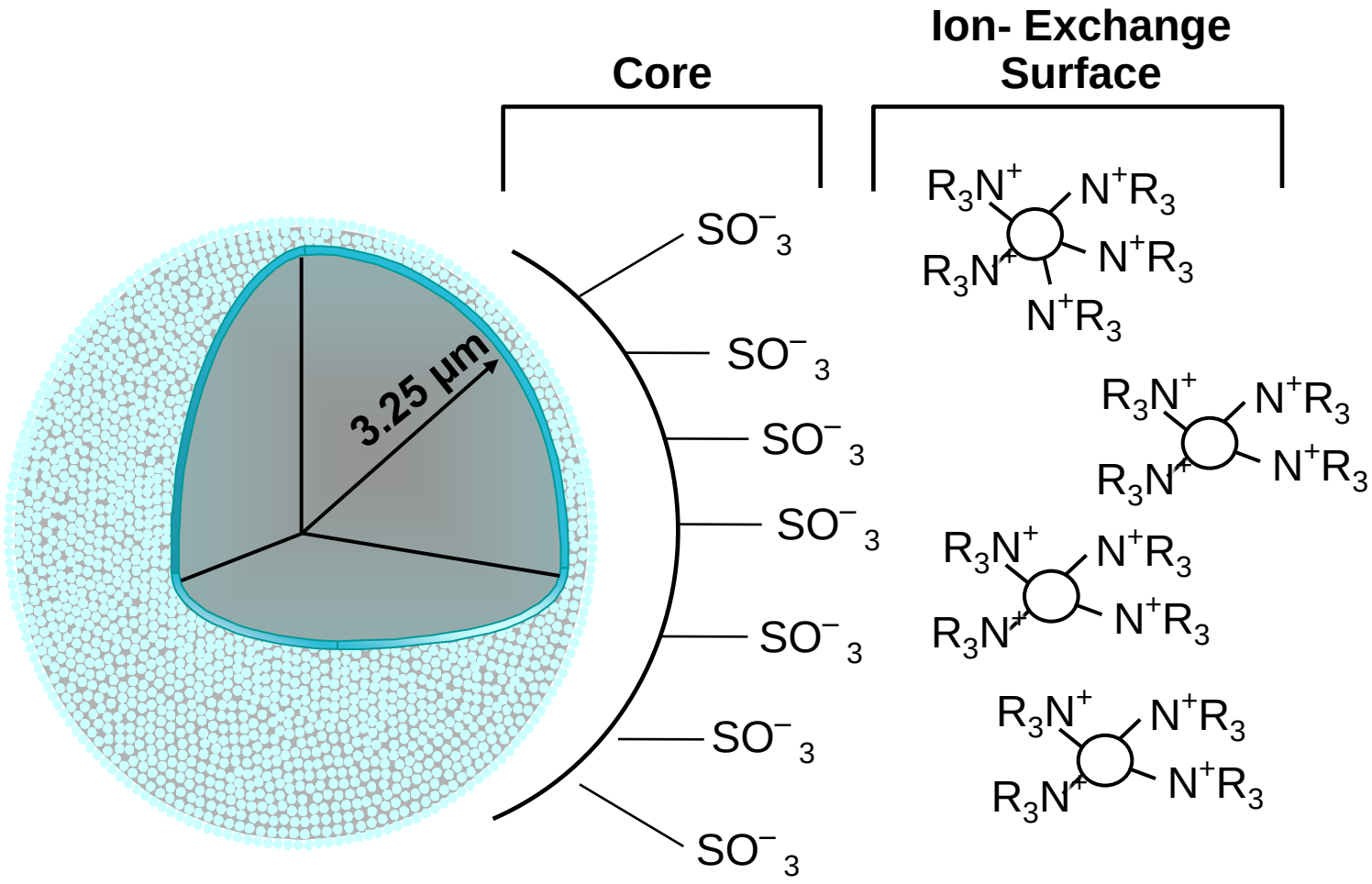
- Monosaccharide compositional analysis
- Sialic acid compositional analysis
- Mannose-6-phosphate analysis
- Glycoprotein oligosaccharide analysis

General HPAE-PAD Conditions for Glycoprotein Oligosaccharide Analysis

Column:	Dionex CarboPac PA200 (3 x 250 mm) or Dionex CarboPac PA100 or Dioenx CarboPac PA1 (4 X 250 mm)*
Eluent:	100 NaOH with a sodium acetate gradient up to as high as 500 mM
Flow Rate:	0.5 mL/min (1.0 mL for PA1/100)
Detection:	PAD (Au) (See Technical Note 21)
Samples:	In water

*9 X 250 mm and 22 X 250 mm have been used for preparative work.

Basic Thermo Scientific Dionex CarboPac Construction



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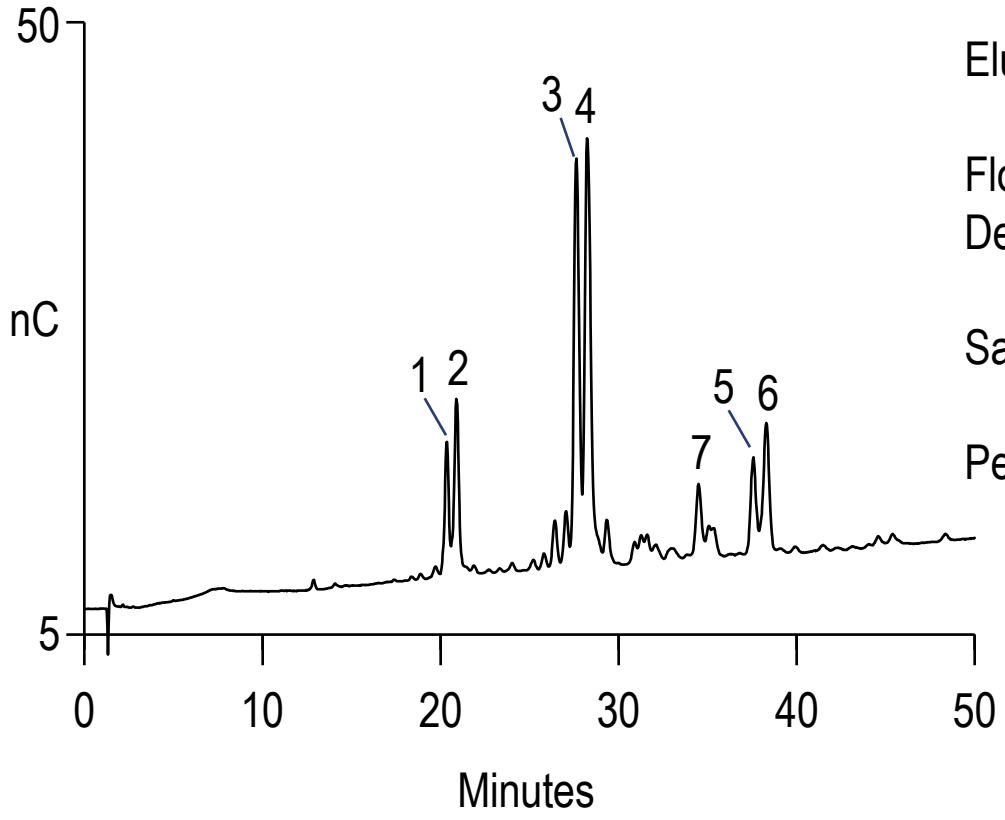
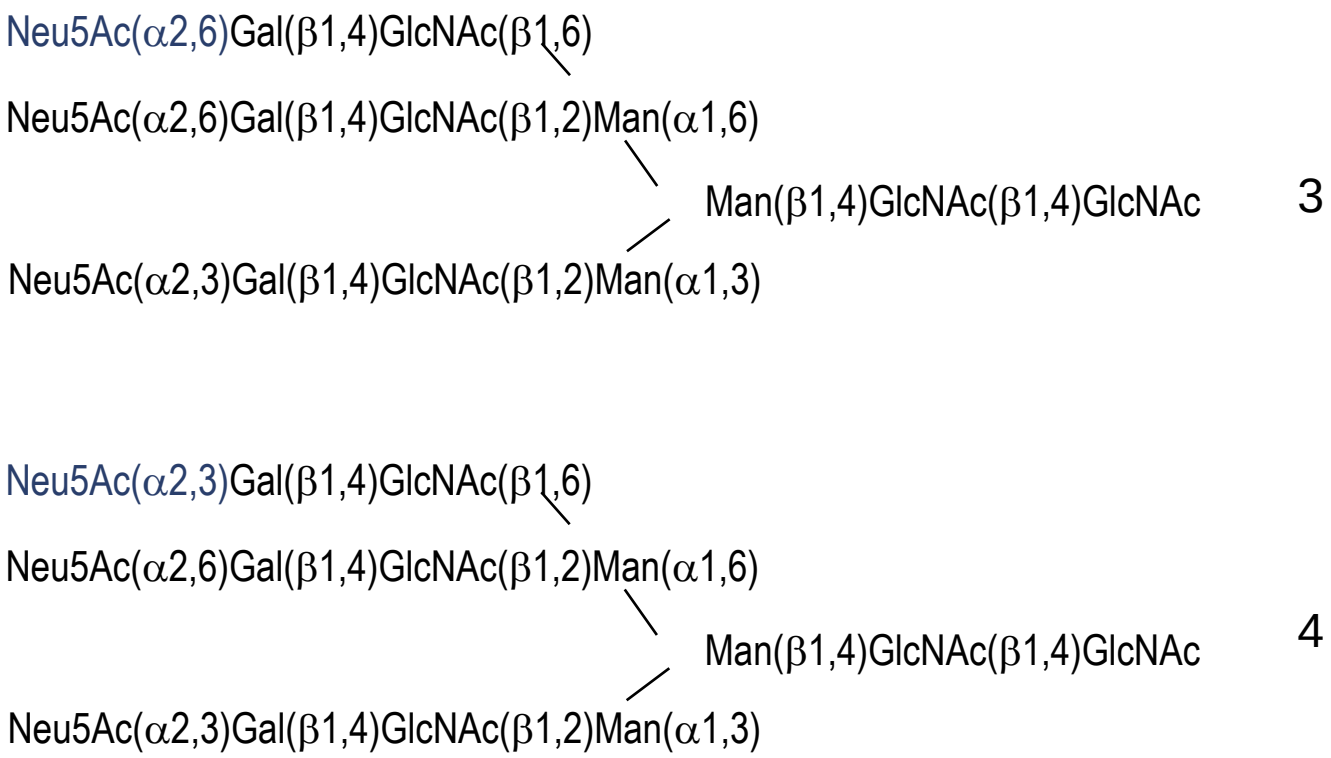
Glycoprotein Oligosaccharide Analysis: Fetuin *N*-Linked Oligosaccharide Alditols

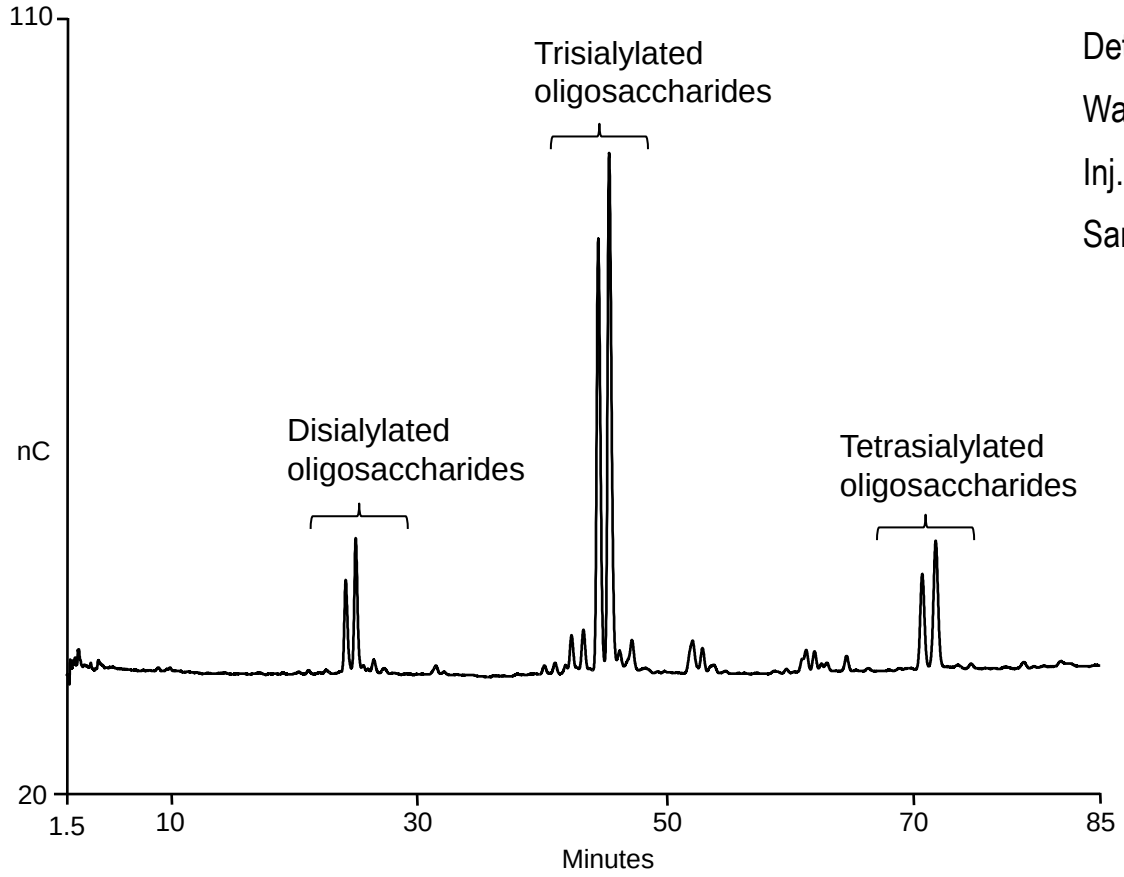


Column:	Dionex CarboPac PA100 and guard
Eluent:	Sodium acetate gradient in 100 mM Sodium hydroxide
Flow Rate:	1.0 mL/min
Detection:	Pulsed amperometry, gold electrode
Sample:	Fetuin oligosaccharide alditol standard
Peaks:	1. Disialylated, triantennary 2. Disialylated, triantennary 3. Trisialylated, triantennary 4. Trisialylated, triantennary 5. Tetrasialylated, triantennary 6. Tetrasialylated, triantennary 7. Trisialylated, triantennary

Sialylated Oligosaccharide Linkage Isomer Separation



Highly Sialylated Oligosaccharide Alditols



Column:	Dionex CarboPac PA200 (3 x 250 mm) Guard (3 x 50 mm)
Temp.:	30 C
Flow:	0.5 mL/min
Detection:	PAD, Au on PTFE electrode
Waveform:	Carbohydrate (standard quad)
Inj. Volume:	20 µL (full loop)
Samples:	Fetuin oligosaccharide alditol standard

HPAE-PAD of Glycoprotein Oligosaccharides

- Oligosaccharides must first be released from the protein.
  For asparagine (*N*-linked) oligosaccharides –
  PNGase F

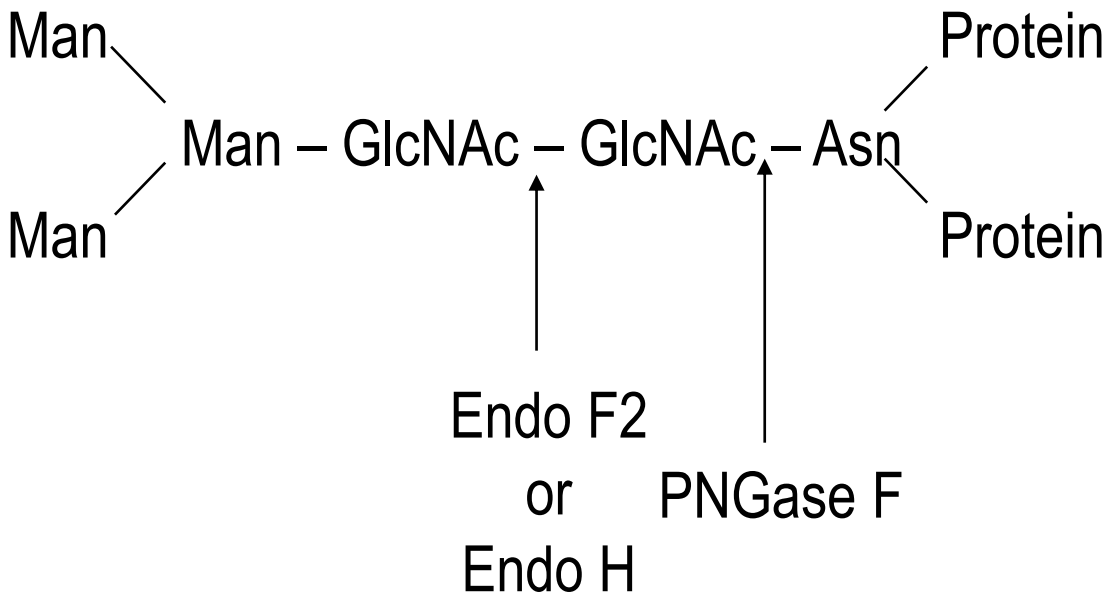
- For serine/threonine oligosaccharides –
  Reductive β-elimination.

- Some samples can be directly injected into the HPAE-PAD system

General Conditions for PNGase F Digestion

- 20-30 mg glycoprotein in 100-mL 50mM Sodium phosphate pH 7.6 10mM EDTA, 0.15% Triton X-100 (reduced) and 1 unit glycerol-free PNGase F.
- Include 10mM beta-mercaptoethanol (BME), if needed.
- Include 10mM BME and 10mM SDS, if needed.
- Inject less than 50-mL.
- BME can be removed by drying the sample.
- SDS can be removed by cold EtOH precipitation.
- REVIEW OF CONDITIONS FOR HPAE-PAD in AU176

Enzyme Cleavage Sites– PNGase F & Endos F2 and H

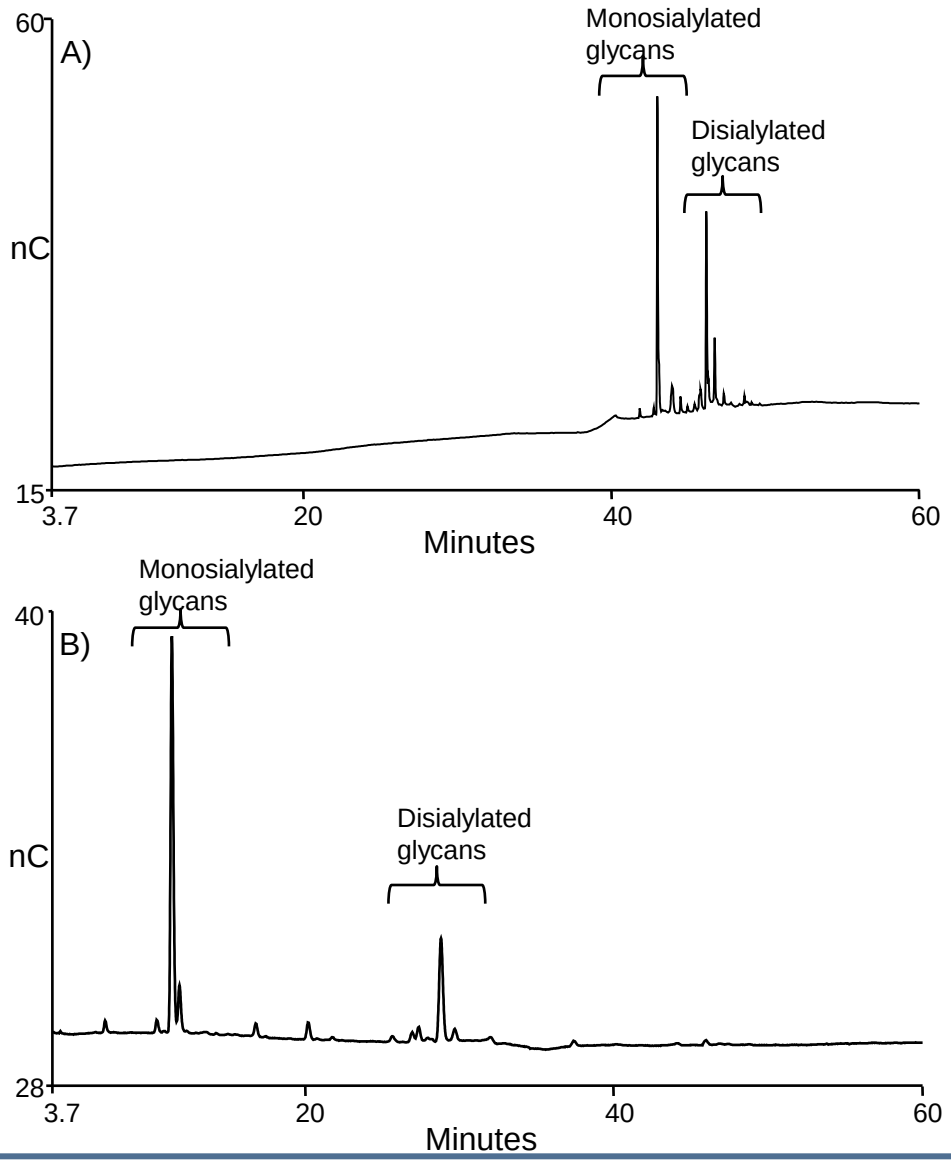


General Conditions for Beta-Elimination*

- Treat 20 mg protein with 100 mL1N $NaBH_4$ in 0.1N NaOH.
- Incubate for 24 h at 37 ^oC.
- Neutralize with acetic acid.
- Desalt (BioGel P2, 0.7 x 10 cm).

* - Anderson, D.C. et al. (1994) Glycobiology 4, 459-467.

Screening Total and Charged Oligosaccharides



Columns: Dionex CarboPac PA200, 3 × 250 mm and Dionex CarboPac PA200 Guard 3 × 50 mm

Sample: PNGase F digestion of human transferrin.

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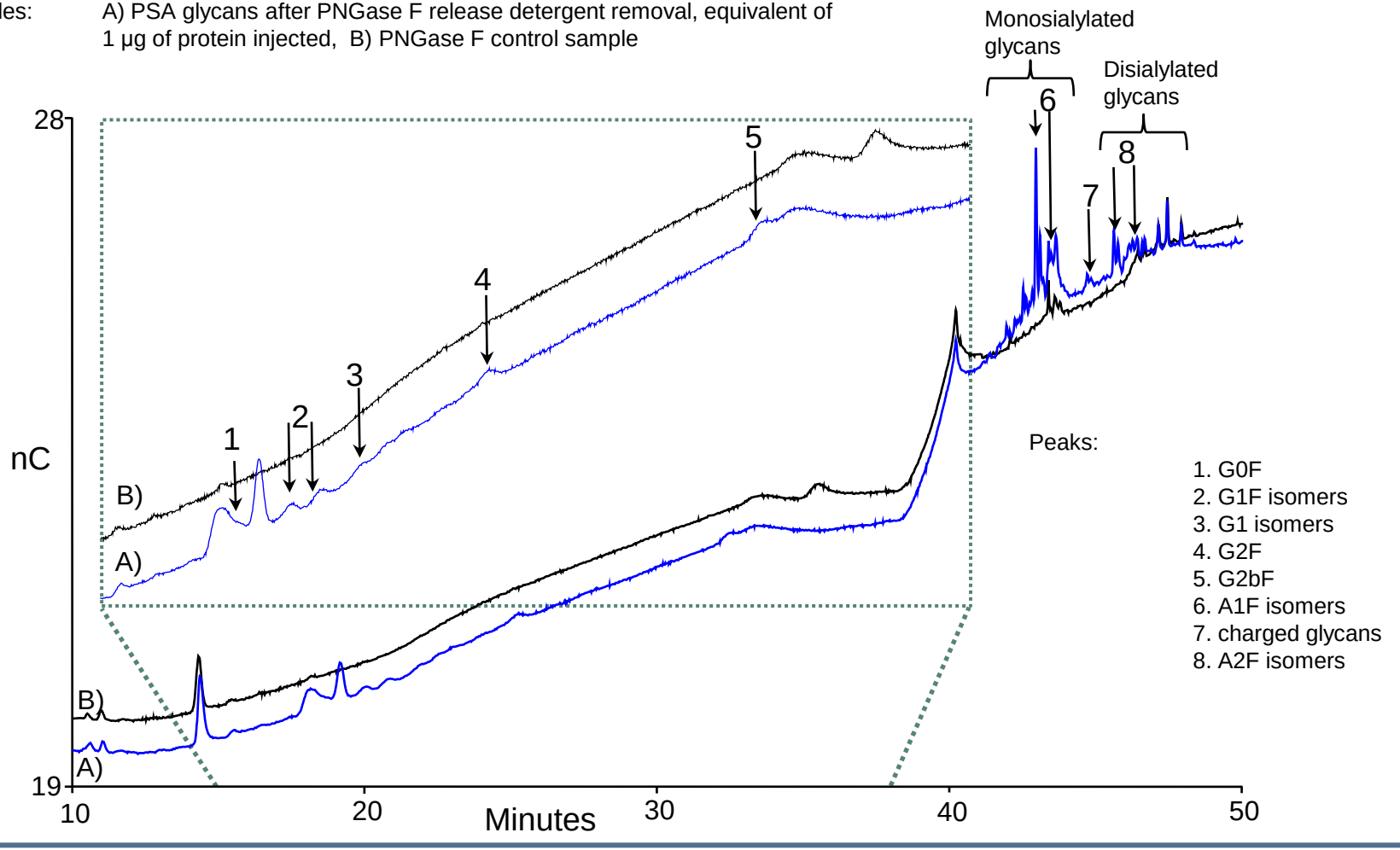
Low Level Oligosaccharide Analysis

Inj. Vol.: 5 µL
Temp.: 30 °C
Samples: A) PSA glycans after PNGase F release detergent removal, equivalent of 1 µg of protein injected, B) PNGase F control sample



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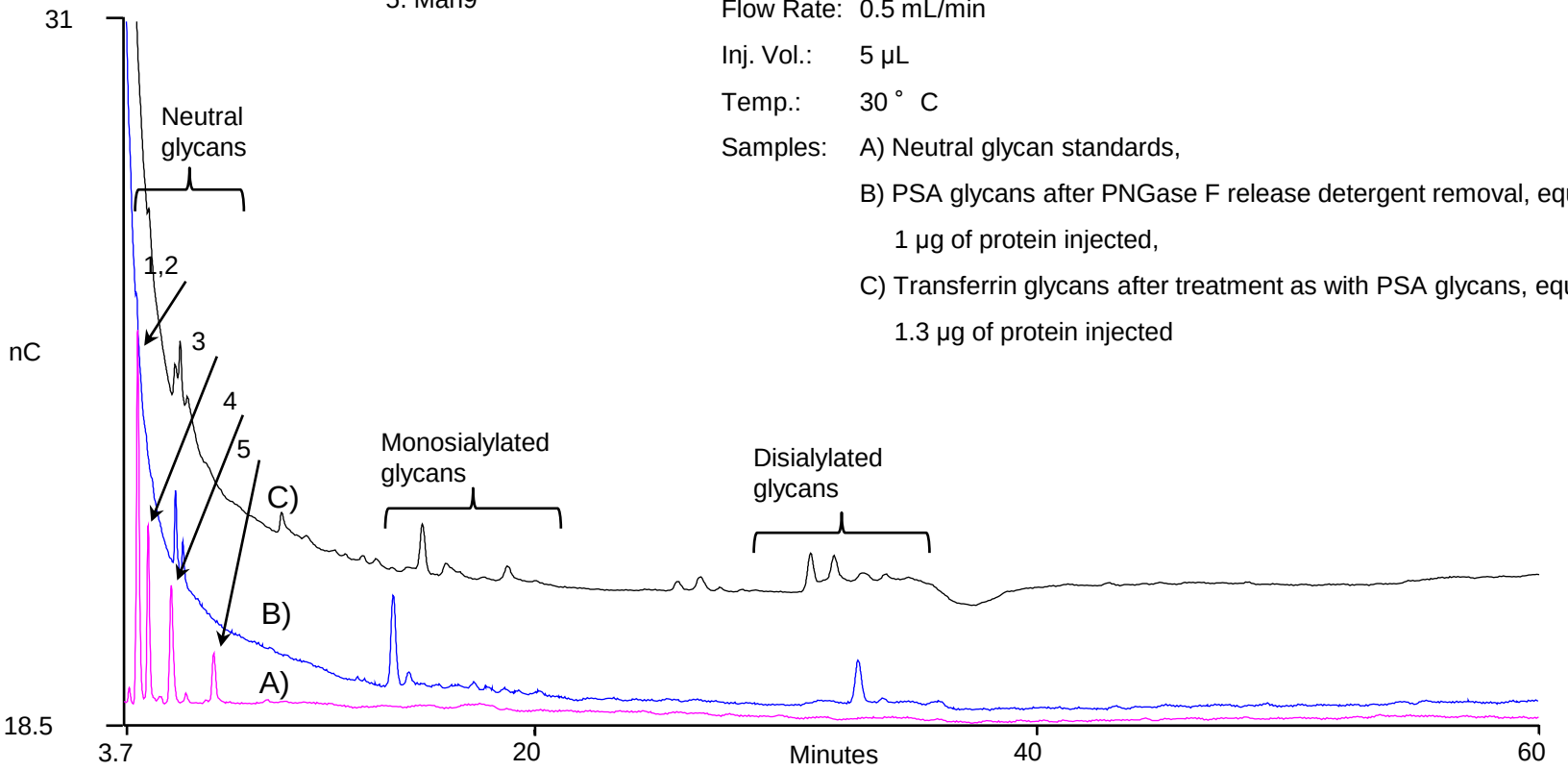
Low Level Oligosaccharide Analysis -Charged

Peaks:

1. G0
2. G1F isomers
3. Man6
4. G2bF
5. Man9



Columns: Dionex CarboPac PA200, 3 × 250 mm and Dionex CarboPac PA200 Guard 3 × 50 mm

Eluent: A) 100 mM NaOH

B) 1M Sodium Acetate in 100 mM NaOH

Gradient: 20-150 mM Sodium Acetate in 100 mM NaOH from 0- 75 min, 20 mM Sodium Acetate in 100 mM NaOH from 75.1-100 min

Flow Rate: 0.5 mL/min

Inj. Vol.: 5 µL

Temp.: 30 ° C

Samples: A) Neutral glycan standards,

B) PSA glycans after PNGase F release detergent removal, equivalent of 1 µg of protein injected,

C) Transferrin glycans after treatment as with PSA glycans, equivalent of 1.3 µg of protein injected

AN 1050

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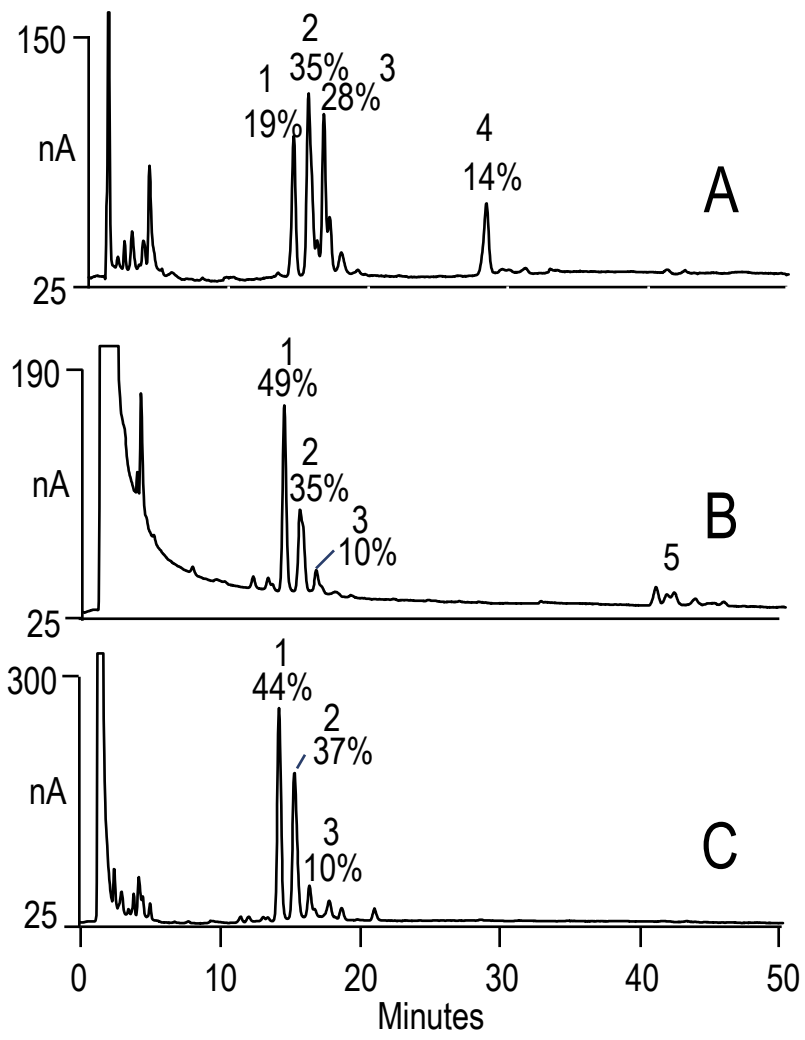
Profiling and Using Exoglycosidases To Assist in Identification of Structures

N-Linked Oligosaccharides from Three IgG Preparations



Column: Dionex CarboPac PA-100

Eluent: 0 to 250 mM NaOAc over 110 min in 100 mM NaOH

Flow Rate: 1 mL/min

Detector: Pulsed amperometry (Gold)

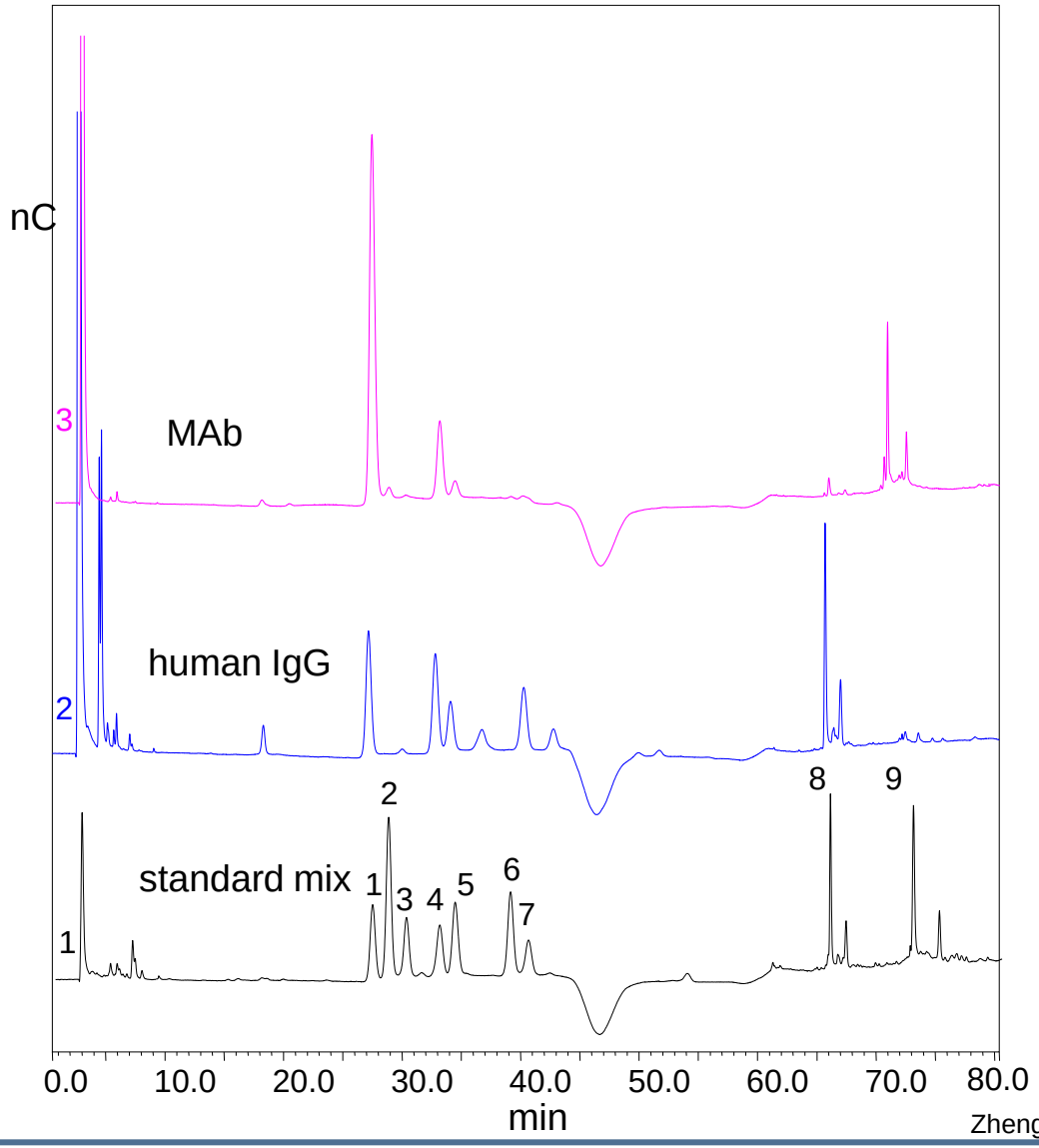
Chromatograms:

A. PNGase F digest of human polyclonal IgG

B. PNGase F digest of murine MAB MY9-6

C. PNGase F digest of humanized MAB M115

Profiling *N*-linked Oligosaccharides from hIgG and a MAb



Column: Dionex CarboPac PA 200 with guard
Eluents: A 100 mM NaOH
B 50 mM NaOAc in 100 mM NaOH
C H_2O
D 0.5 M NaOAc in 100 mM NaOH

Gradient:

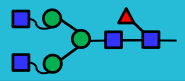
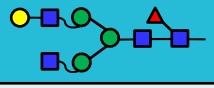
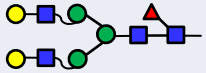
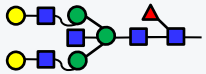
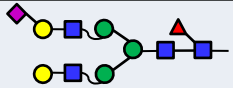
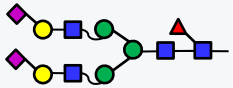
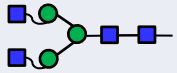
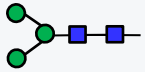
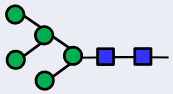
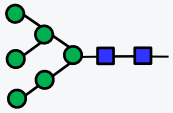
Time (min)	Flow rate (ml/min)	%A	%B	%C	%D
-10	0.35	48%	2%	50%	0%
0	0.35	48%	2%	50%	0%
50	0.35	37%	13%	50%	0%
51	0.35	45%	0%	50%	5%
80	0.35	24%	0%	50%	26%
81	0.45	0%	0%	0%	100%
91	0.45	0%	0%	0%	100%
92	0.45	48%	2%	50%	0%
100	0.45	48%	2%	50%	0%

Temp: 30 °C
Injection Volume: 10 µL
Sample Conc.: ~5 µM
Peaks: 1. G0F
2. Man5
3. G0
4,5. G1F
6. Man6
7. G2F
8. A1F
9. A2F

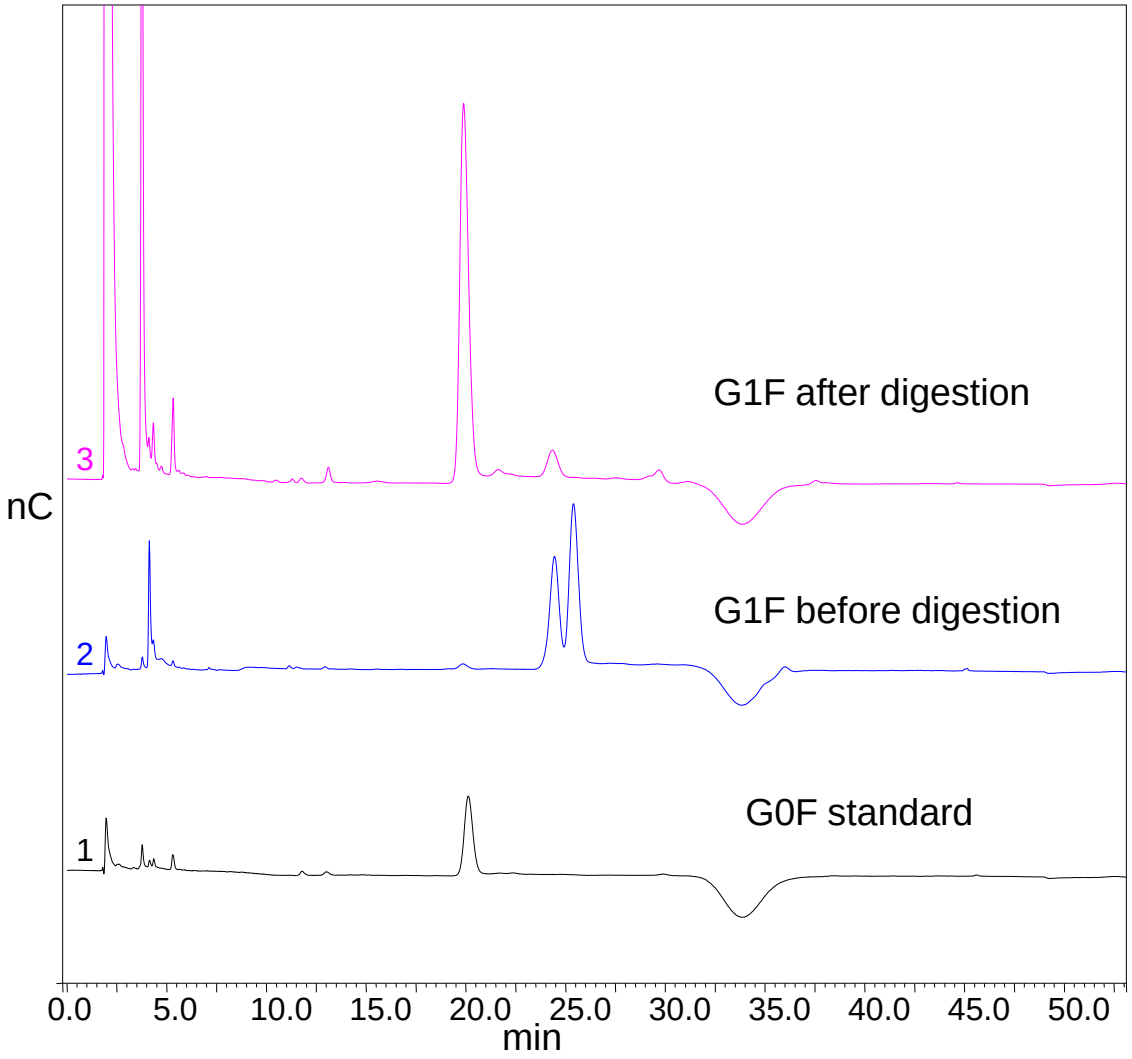
Zheng, T., Rohrer, J., Rao, S. (2010) *Genetic Engineering News* 30(10), 42-43.

Common *N*-linked Oligosaccharides found on IgG

- *N*-acetylglucosamine (GlcNAc)
- Fucose (Fuc)
- Mannose (Man)
- Galactose (Gal)
- *N*-acetylneuraminic acid (Neu5Ac)

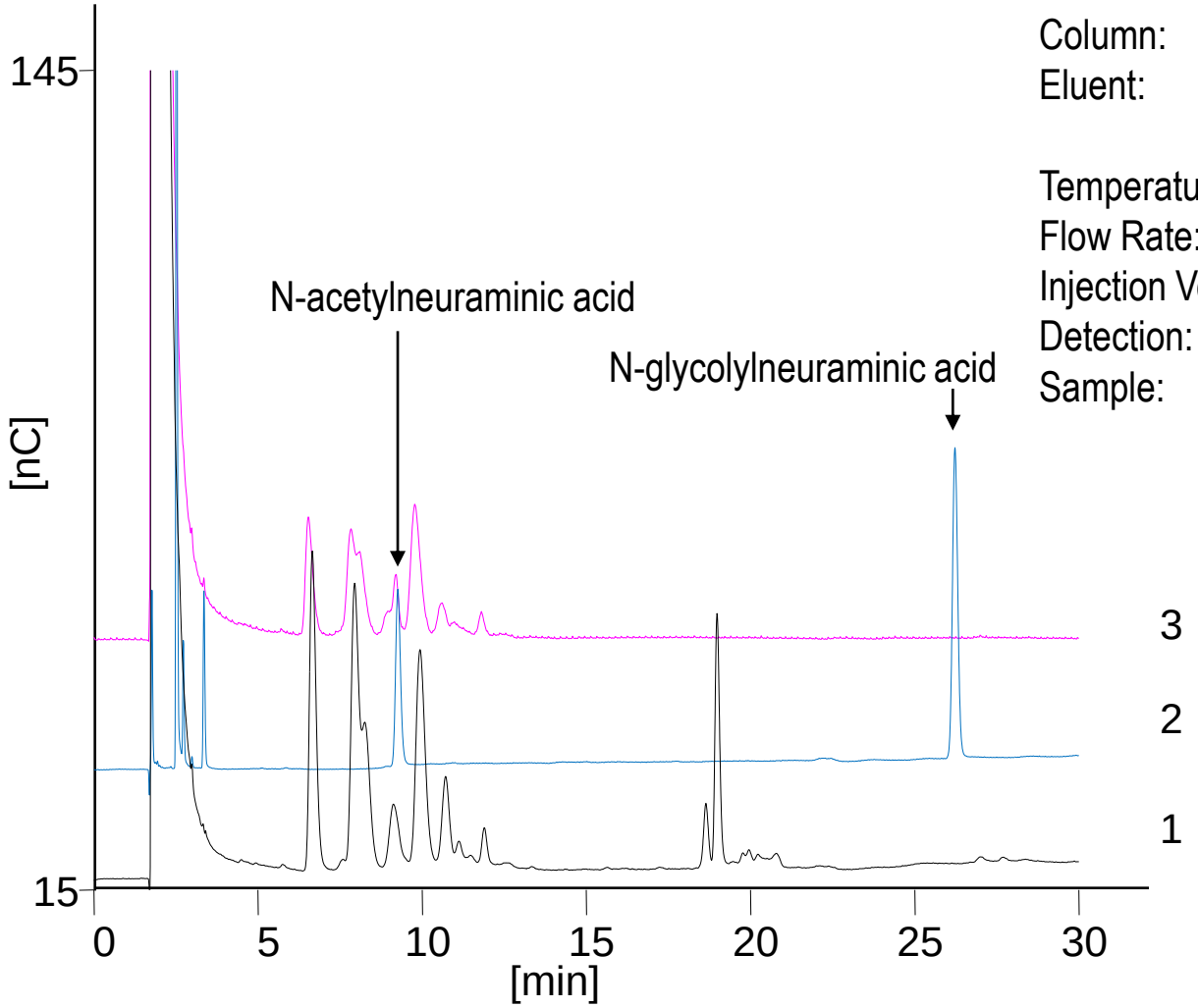
Glycan (Oxford)	mAb acronym	Structure
NGA2F (FA2G0)	G0F	
NA2G1F (FA2G1)	G1F	
NA2F (FAG2)	G2F	
NA2FB (FABG2)	G2bF	
G2FA1 (FA2G2S1)	A1F	
G2FA2 (FAG2S2)	A2F	
NGA2 (A2G0)	G0	
Man3	M3	
Man5	M5	
Man6	M6	

G1F Treated with Beta-Calactosidase



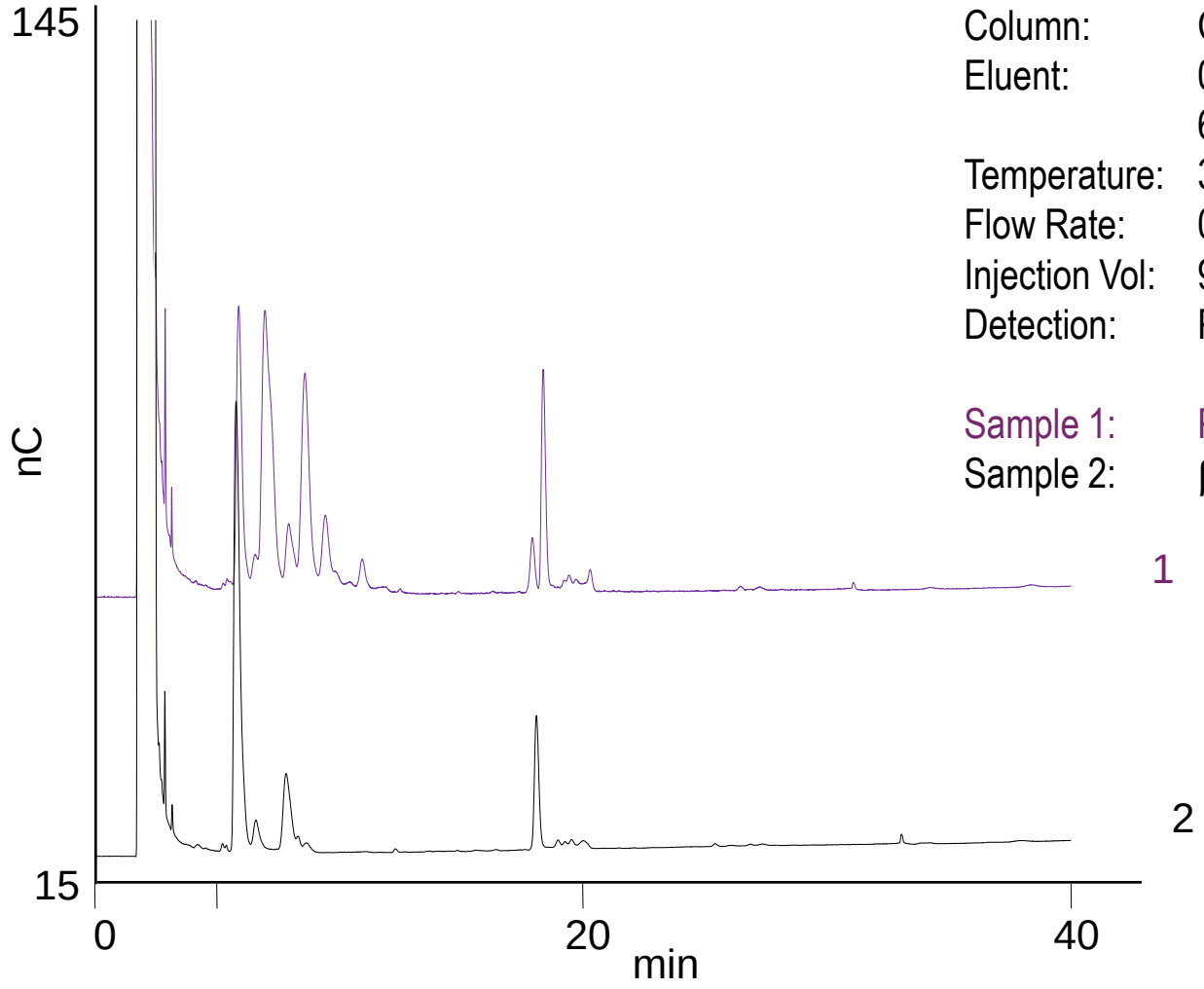
Column: CarboPac PA 200 with guard
Gradient: 0-5 mM NaOAc in 50 mM NaOH from 0-40 min.
Flow rate: 0.5 mL/min
Temperature: 30 °C
Injection Volume: 15 µL
Sample Conc.: ~5 µM
Samples: 1. G0
2. G1
3. G1 after digestion

Monitoring Sialic Acid Release from hIgG *N*-Linked Oligosaccharides



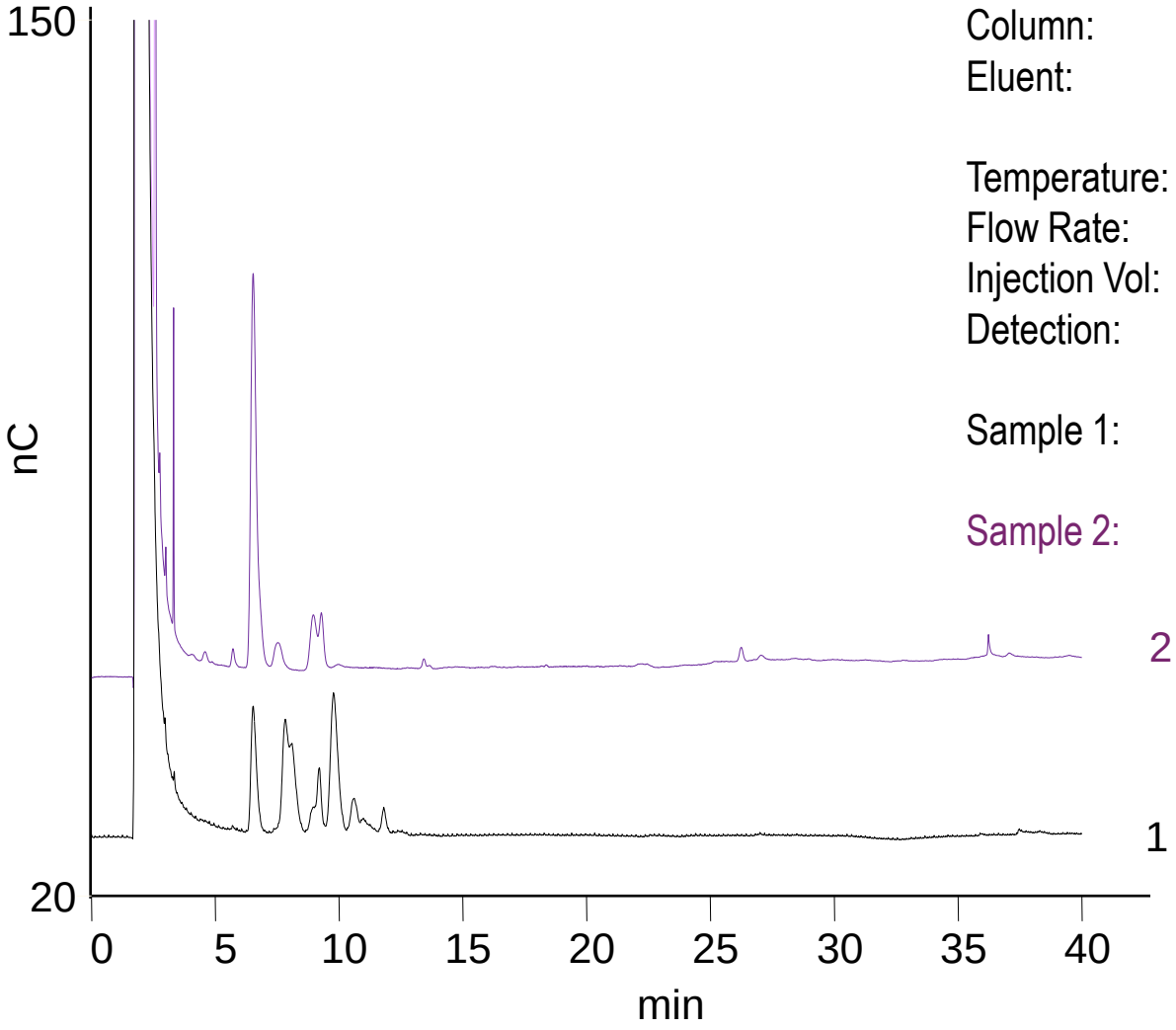
Column:	CarboPac PA200 and guard
Eluent:	0-5 min: 100 mM NaOH/5 mM NaOH
	60 min: 100 mM NaOH/180 mM NaOH
Temperature:	30 °C
Flow Rate:	0.5 mL/min
Injection Vol:	9 µL from 10-µL loop
Detection:	PAD (Au) Waveform A (TN 21)
Sample:	(1) PNGase-F digest of human IgG
	(2) Sialic acids standard
	(3) Neuraminidase digest (1)

Degalactosylated *N*-Linked Oligosaccharides from h.IgG



Column:	CarboPac PA200 and guard
Eluent:	0-5 min: 100 mM NaOH/5 mM NaOAC
	60 min: 100 mM NaOH/180 mM NaOAc
Temperature:	30 °C
Flow Rate:	0.5 mL/min
Injection Vol:	9 μL from 10-μL loop
Detection:	PAD (AU) Waveform A (TN21)
Sample 1:	PNGase-F of human IgG
Sample 2:	β-galactosidase digest of sample 1

Desialylated-Degalactosylated hIgG Oligosaccharides



Column:	CarboPac PA200 and guard
Eluent:	0-5 min: 100 mM NaOH/5 mM NaOAC
	60 min: 100 mM NaOH/180 mM NaOAc
Temperature:	30 °C
Flow Rate:	0.5 mL/min
Injection Vol:	9 μL from 10-μL loop
Detection:	PAD (AU) Waveform A (TN21)
Sample 1:	human IgG digested in turn by PNGase-F and neuraminidase
Sample 2:	β-galactosidase of sample 1

Empirical Relationships of *N*-Linked Oligosaccharide Structure to Retention on the Dionex CarboPac PA1, Dionex CarboPac PA100, and Dionex CarboPac PA 200 Columns

1. The larger the oligosaccharide, the later it elutes. (e.g. the greater the number of mannose residues in a high-mannose oligosaccharide, the later it elutes.)

2. Increased negative charge (sialylation, phosphorylation, sulfation), increased retention.

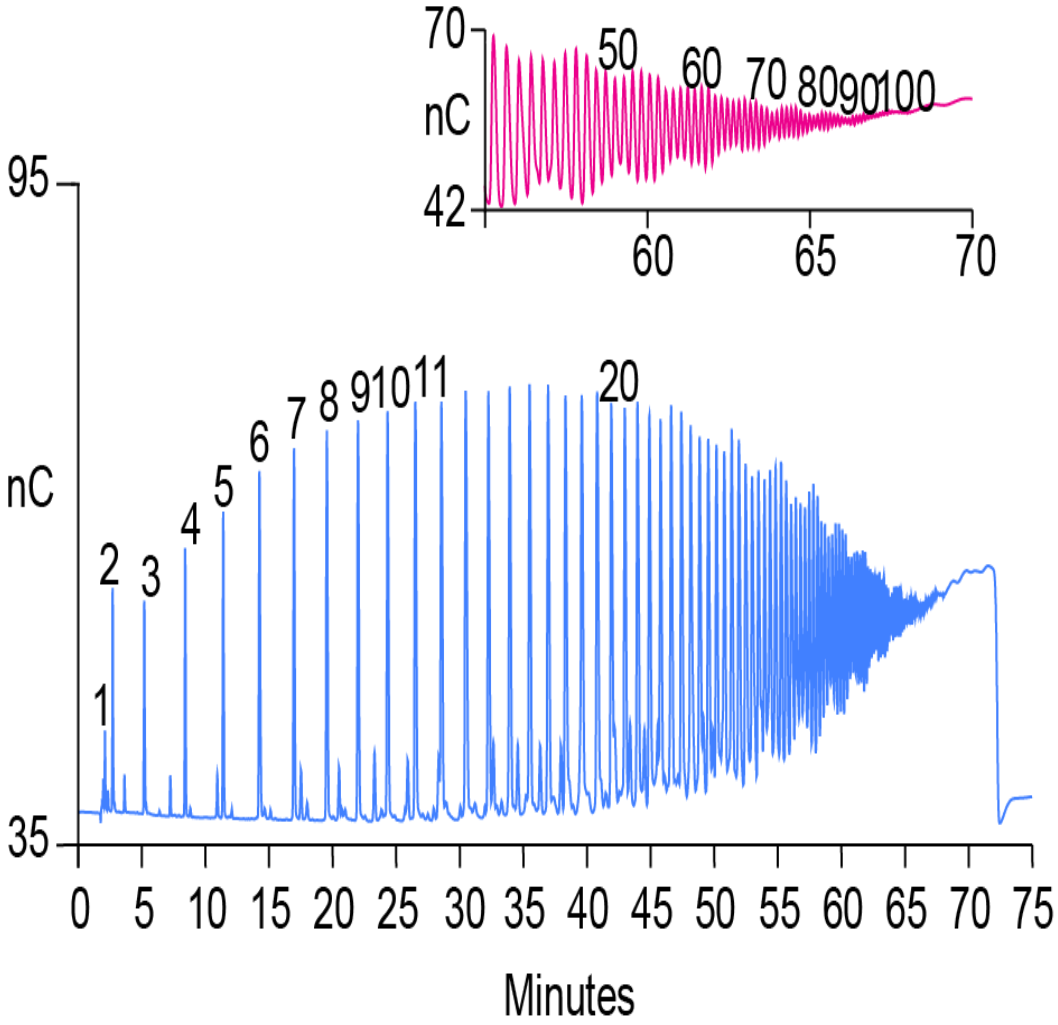
3. Addition of fucose to an oligosaccharide reduces its retention.

4. An oligosaccharide with a Neu5Acα2$\rightarrow$3Gal elutes later than the same oligosaccharide with a Neu5Acα2$\rightarrow$6Gal.

4 of 12 Rules from Rohrer, J. (1995) *Glycobiology* 5, 359-360.

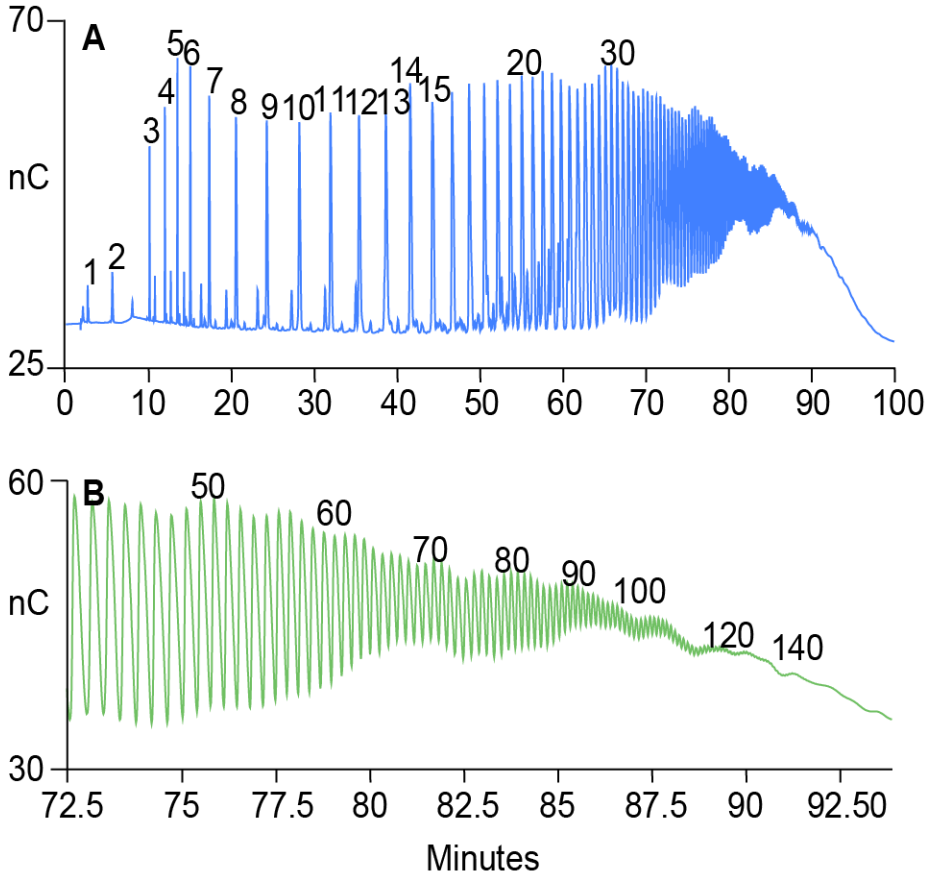
Polysialic Acids

Separation of Colominic Acid with an Acetate Gradient



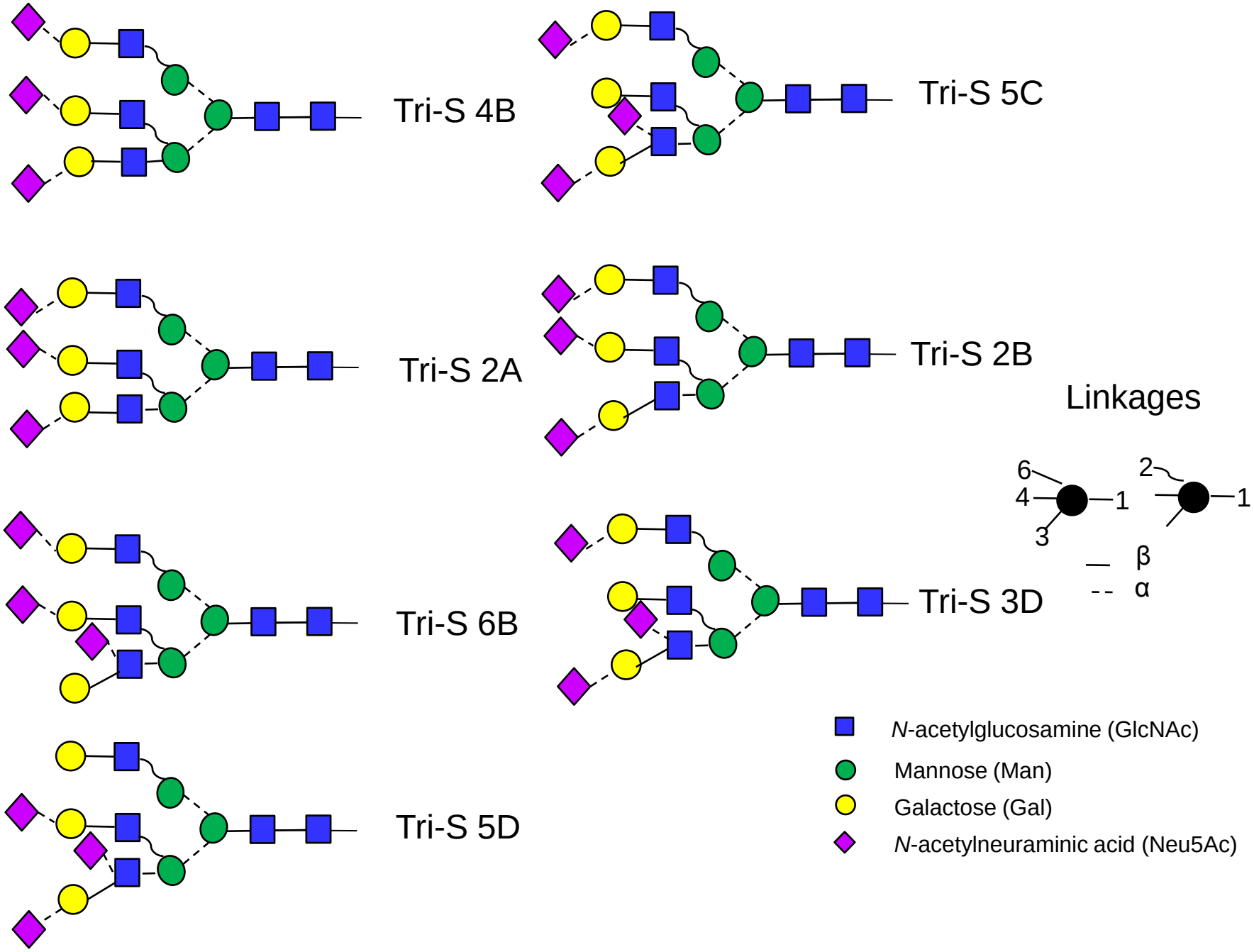
Columns:	Dionex CarboPac PA200 Guard and Analytical
Eluent:	100 mM NaOH with 200 mM to 1000 mM NaOAc in 69 min (Curve 4)
Flow Rate:	0.5 mL/min
Inj. Volume:	10 µL
Temperature:	30 °C
Detection:	PAD (Au)

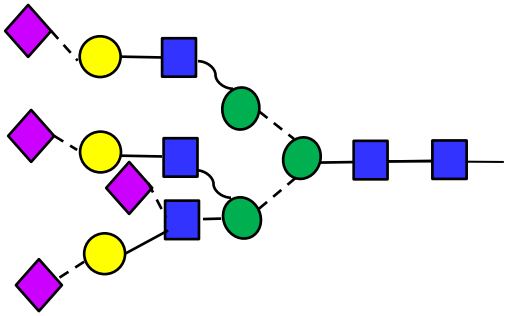
Separation with a Nitrate Gradient (Stronger Eluent)



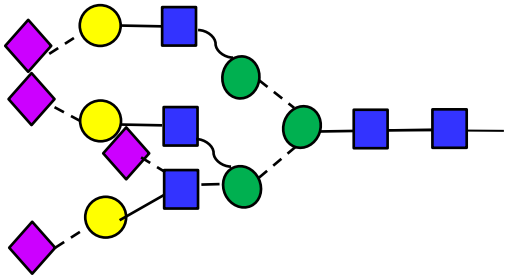
Columns:	Dionex CarboPac PA200 Guard and Analytical
Eluent:	100 mM NaOH with 20 mM $NaNO_3$ for 2 min, 20 to 100 mM $NaNO_3$ in 7 min, 100 to 160 mM $NaNO_3$ in 30 min, 160 to 310 mM in 60 min, and 10 min equilibration at 20 mM $NaNO_3$ (curve 5)
Flow Rate:	0.5 mL/min
Inj. Volume:	10 µL
Temperature:	30 °C
Detection:	PAD (Au)

What About Electrochemical Response?

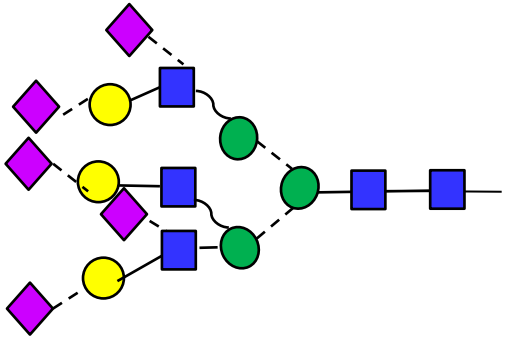

Tri-S 4B
Tri-S 5C
Tri-S 2A
Tri-S 2B
Tri-S 6B
Tri-S 3D
Tri-S 5D
Linkages
6
4
3
1
2
1
β
α
N-acetylglucosamine (GlcNAc)
Mannose (Man)
Galactose (Gal)
N-acetylneuraminic acid (Neu5Ac)



Tetra-S 5

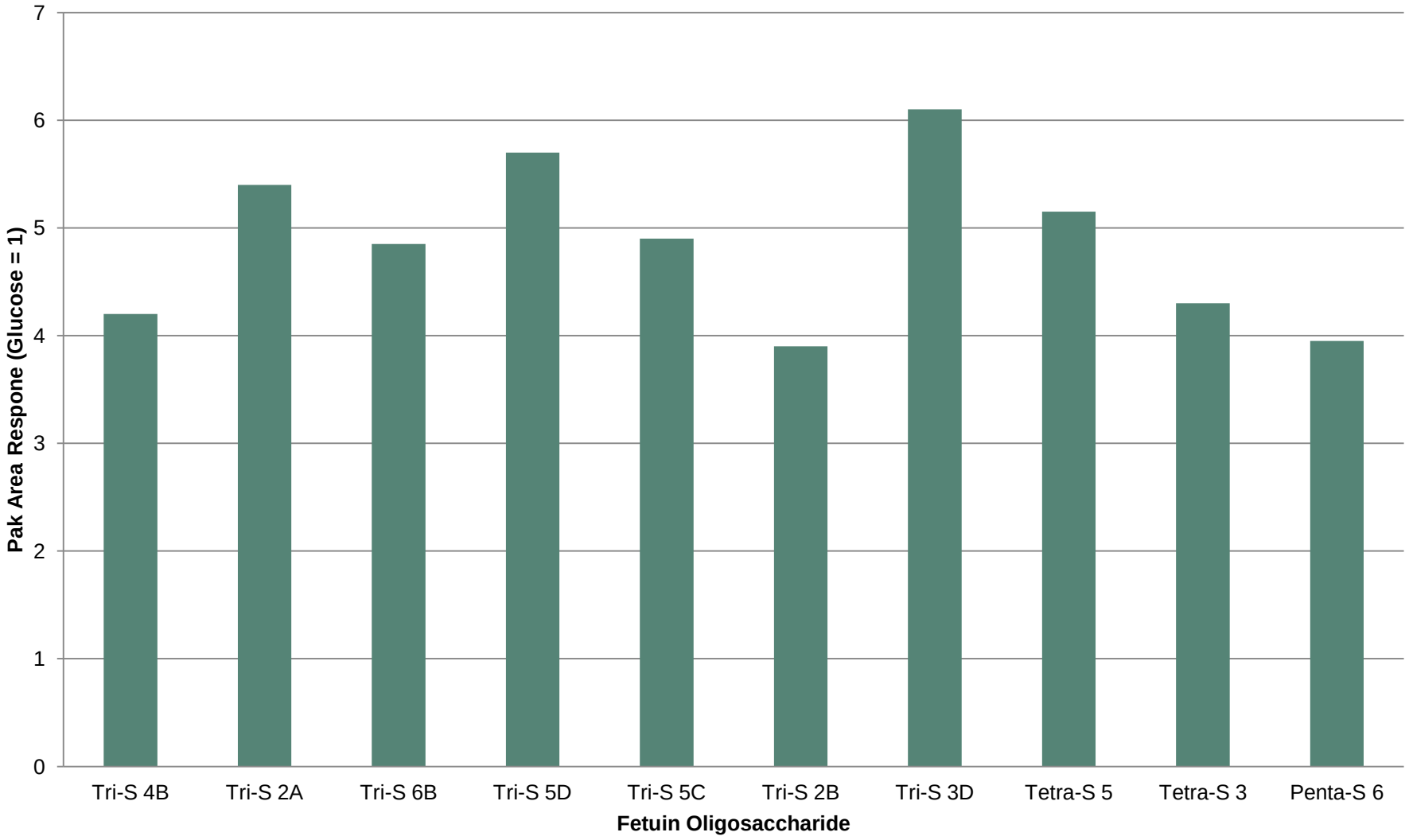


Tetra-S 3

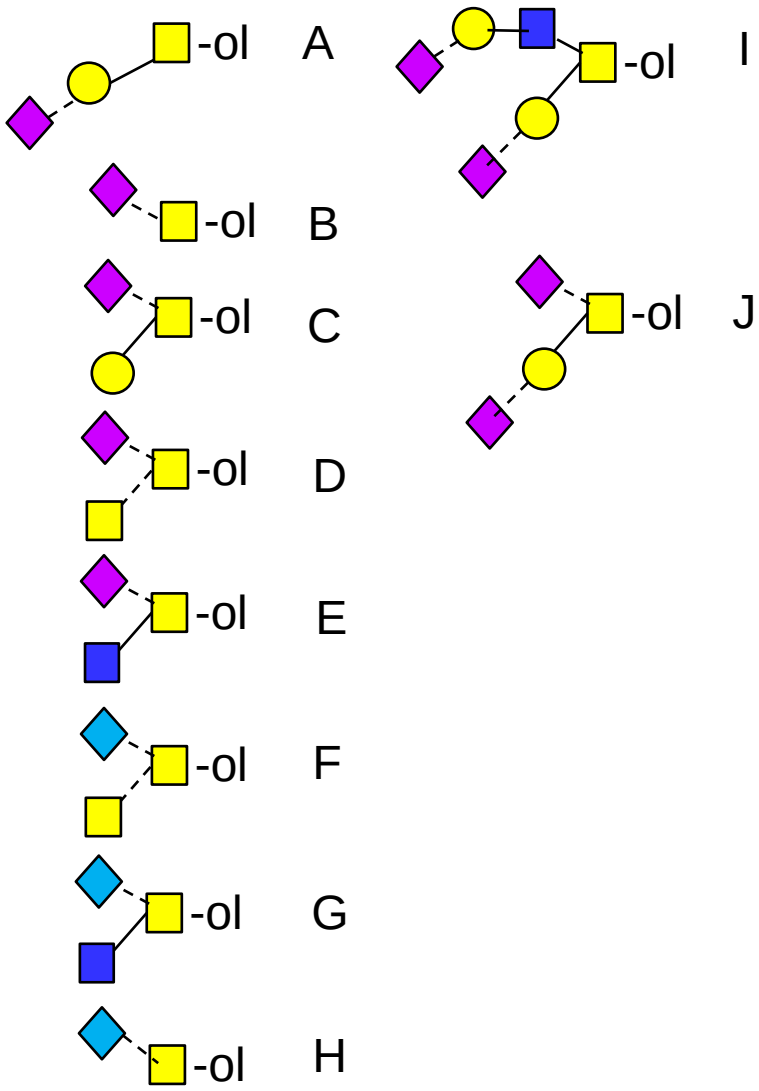


Penta-S 6

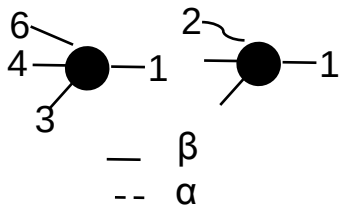
HPAE-PAD Peak Area Response of Fetuin Oligosaccharides



Townsend, R. R.; et al. *Anal. Biochem.* ***1988, 174, 459–470.***



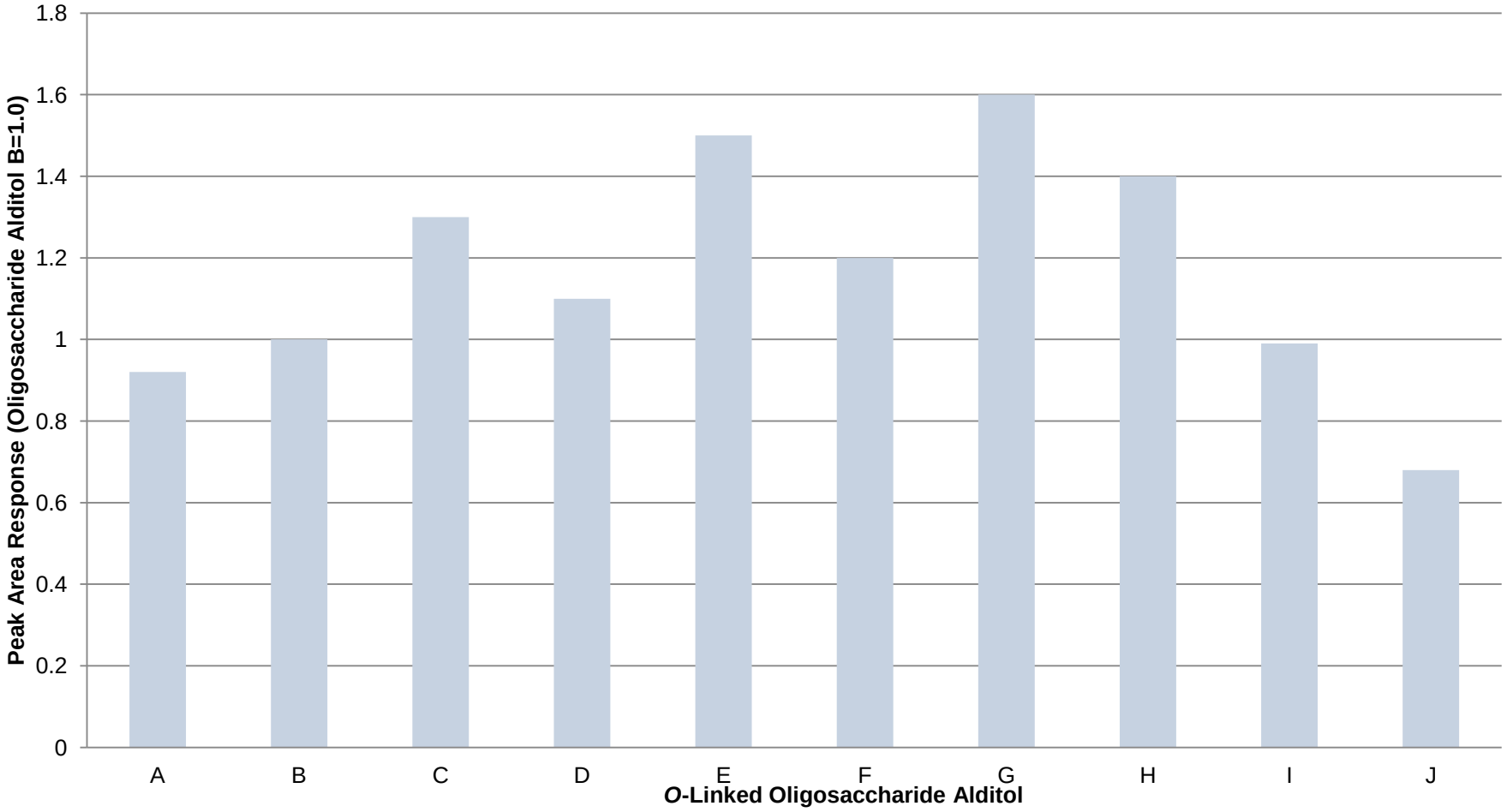
Linkages



Sugars

- *N*-acetylglucosamine (GlcNAc)
- Mannose (Man)
- Galactose (Gal)
- *N*-acetylneuraminic acid (Neu5Ac)
- *N*-acetylgalactosamine (GalNAc)
- *N*-glycolylneuraminic acid (Neu5Gc)

HPAE-PAD Peak Area Responses of *O*-Linked Oligosaccharides



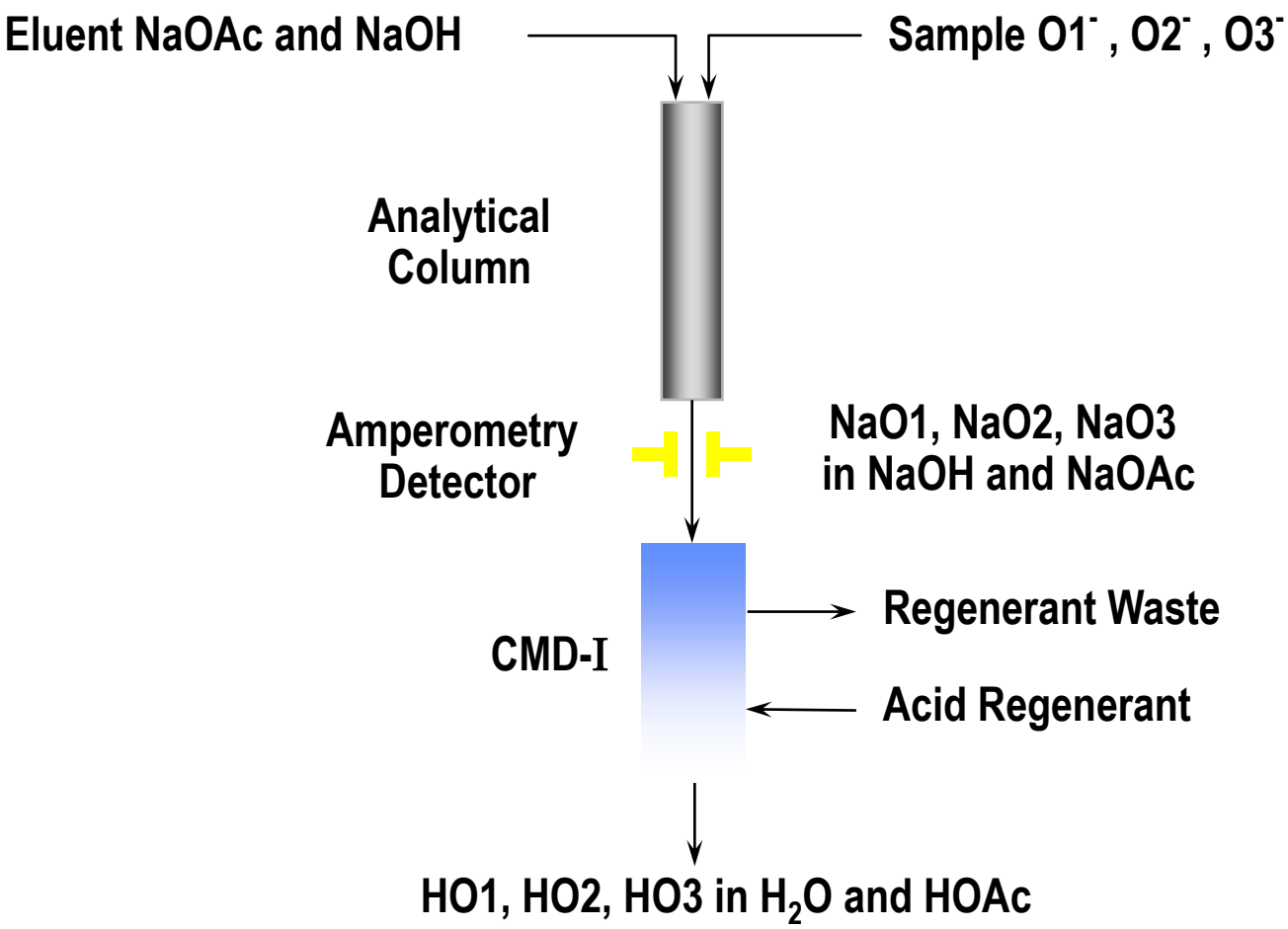
Hayase, T.; et al. *Anal. Biochem.* ***1993, 211, 72–80.***

Purifying for or Interfacing to Mass Spectrometry

The Carbohydrate Membrane Desalter

- Simple device automates the removal of sodium hydroxide and sodium acetate
- Combines acidic regenerant and electrolysis to replace Na^+ with hydronium ions
- Delivers oligosaccharides in a weak acetic acid solution
- No dialysis required

In-line Carbohydrate Desalting with the Carbohydrate Membrane Desalter



O1, O2, O3 = Oligosaccharides

Brief Comparison to Other Techniques

HPAE-PAD Compared to MALDI-TOF

- Field, M.; et al.*Anal. Biochem.* ***1996, 239, 92–98.*** – quantification of oligosaccharides with both techniques yielded similar values.

- Zhou, Q.; et al. *Anal. Biochem.* ***2004, 335, 10–16.*** **–** Found that the two techniques yielded similar results

- Gray, J. S.; et al. *Glycobiology* ***1996, 6, 23–32.*** **–** Again found the two techniques yielded similar results except for isomers

- Grey, C.; et al. *J. Chromatogr., B* ***2009, 877, 1827–1832.*** **–** Relative glycan response similar for both methods.

HPAE-PAD Oligosaccharide Analysis Compared to HILIC-FLD and CE-LIF

- HPAE-PAD is orthogonal to CE-LIF.
- HPAE-PAD has some orthogonality to HILIC-FLD.
- Working sensitivity is similar.
- HPAE-PAD requires no sample derivatization.
- HPAE-PAD may require more time per analysis.
- HPAE-PAD has better separation of charged oligosaccharides.
- HPAE-PAD preserves the oligosaccharides sialylation.

Other Carbohydrate Analytes – HPAE-PAD

- Sugar phosphates, including Man-6-P, small inositol phosphates
- Sugar alcohols, e.g. mannitol
- Small polysaccharides, e.g. maltodextrins to DP50
- Sulfated sugars, e.g. Gal-3-S
- Sugar acids, e.g. galacturonic acid
- Glycolipid oligosaccharides, including GPI anchors
- Aminoglycosides (e.g. neomycin)
- Sucralose
- Fluorodeoxyglucose
- Nucleotides and Nucleosides

Samples Analyzed by HPAE-PAD

- Glycoproteins, glycopeptides, glycolipids
- Cane sugar
- Serum and urine
- Fermentation broths
- Enzymatic reactions
- Foods and beverages
- Vaccines, pharmaceutical preparations
- River and sea waters
- Wood pulp

Conclusions

- HPAE-PAD provides high resolution separations of both *N*-linked and *O*-linked oligosaccharides derived from glycoproteins.
- The same HPAE-PAD system can be used for determinations of monosaccharides, sialic acids, sugar phosphates, and other carbohydrates.
- HPAE-PAD can be used to prepare pure oligosaccharides from NMR or MS, or can be interfaced directly with MS.

Thank you for your attention!