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Analytical Strategies for Studying Glycosylation of Biopharmaceuticals

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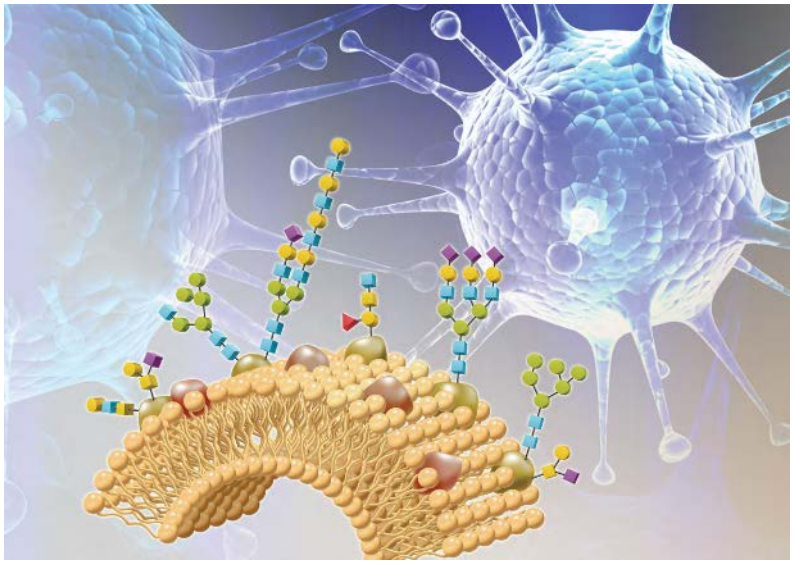
Vendor Seminar, ISC 2016, Cork, Ireland

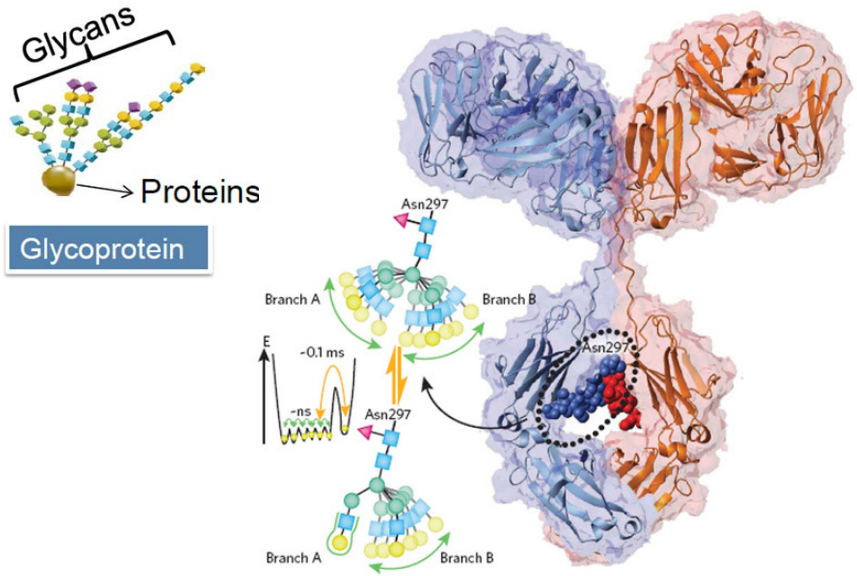
Outline

- Introduction
- Glycan workflows
  - Monosaccharides and sialic acids
  - Labeled glycans
  - Labeled glycans – high throughput
  - Unlabeled glycans
  - Glycopeptides
  - Intact glycoproteins
- Conclusions

What Role do Glycans Play in Biotherapeutics?

- **70%** of protein drug candidates in clinical development are glycosylated
- Many host-pathogen interactions occur using glycans (recognition, degradation, etc.)
- Glycosylation affects:
  - Biological activity
  - Pharmacokinetics
  - Stability
  - Immunogenicity
- Glycosylation is the most common post-translational modification (PTM) studied in biopharmaceuticals





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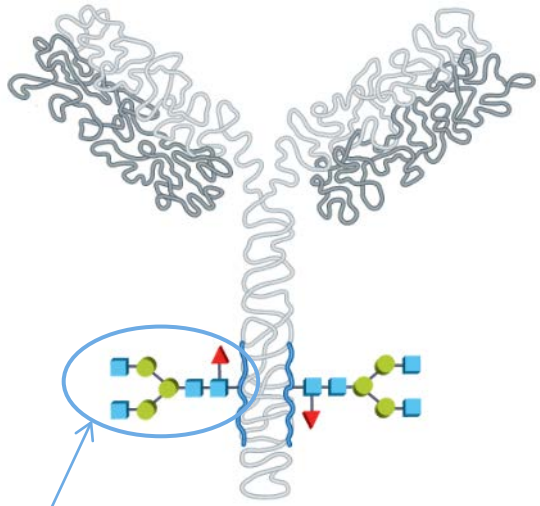
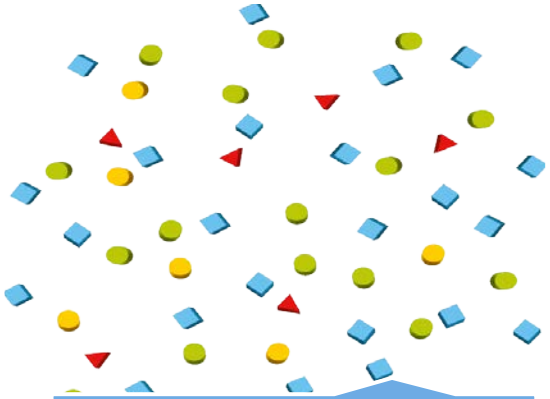
Characterization and Confirmation of Biological Products

ICH (Q6B) recommended six test approaches for characterization and confirmation of biological products:

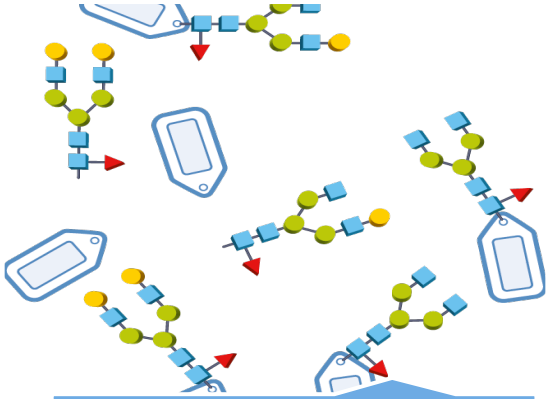
- Amino acid sequence
- Amino acid composition
- Terminal amino acid sequence
- Peptide map
- Sulfhydryl group(s) and disulfide bridges
- **Carbohydrate structure**
  - *"For glycoproteins, the carbohydrate content and structure (neutral sugars, amino sugars, and sialic acids) is determined."*



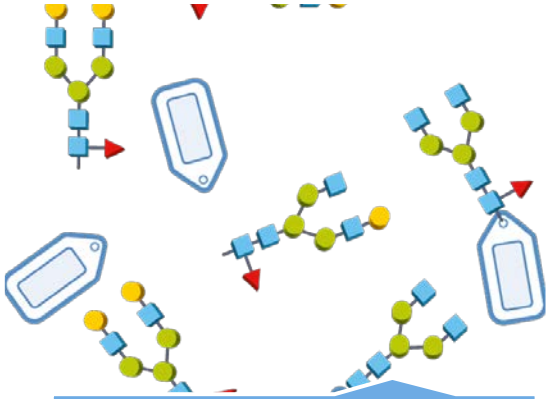
Glycan Workflows



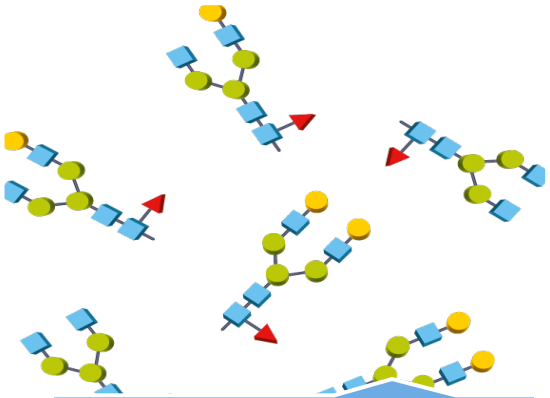
Monosaccharides & Sialic Acids



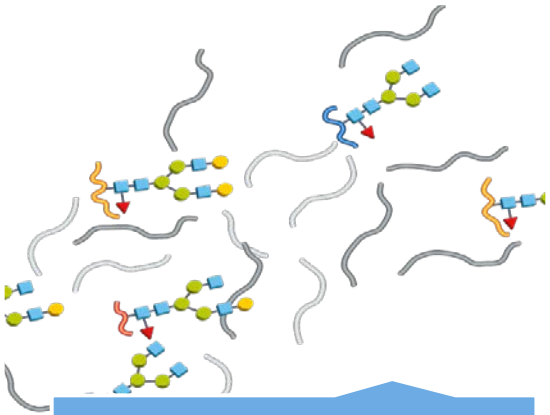
Labeled Glycans



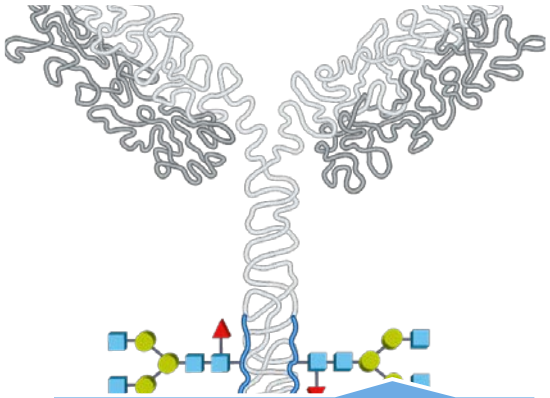
Labeled Glycans – High throughput



Unlabeled Glycans

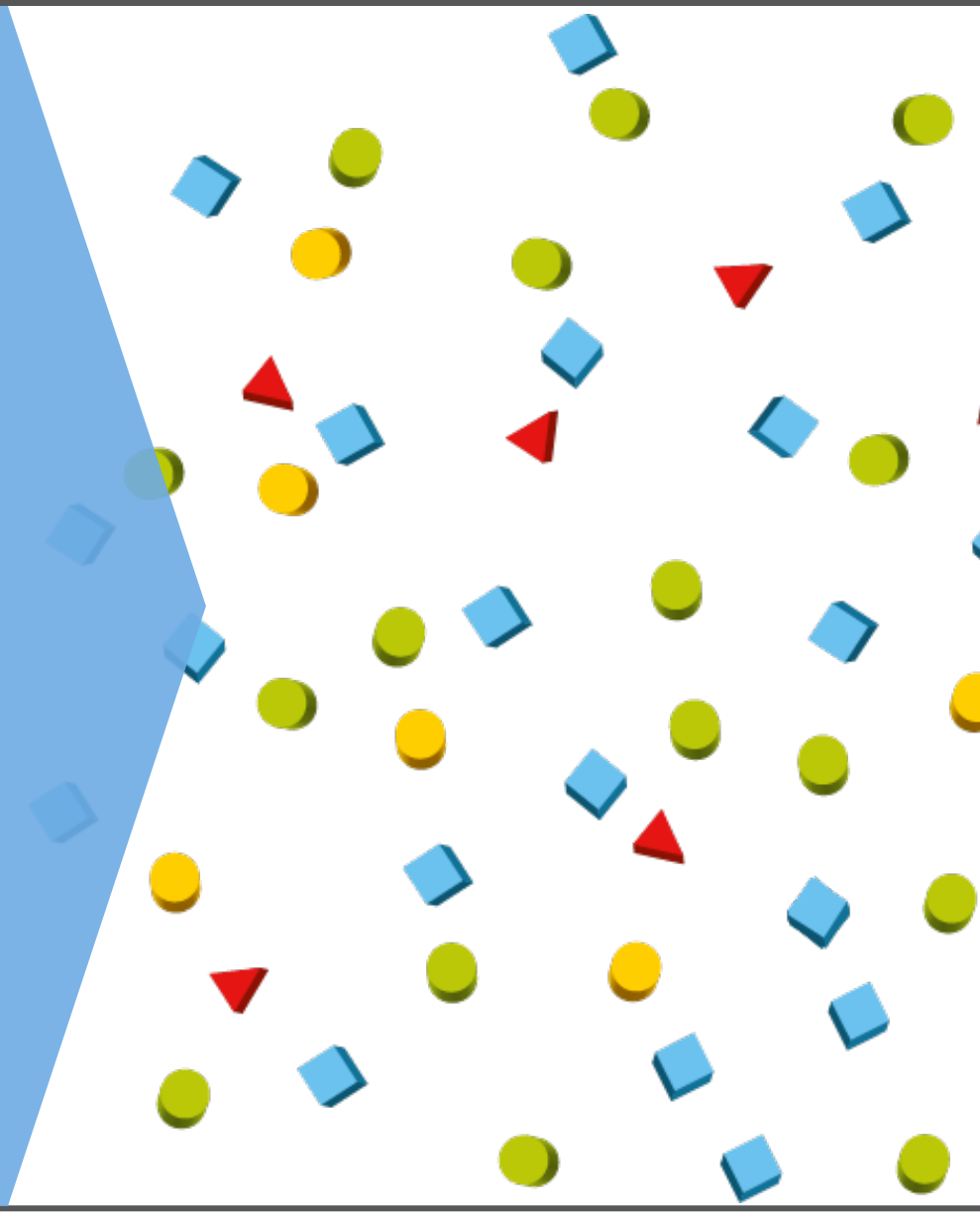


Glycopeptides

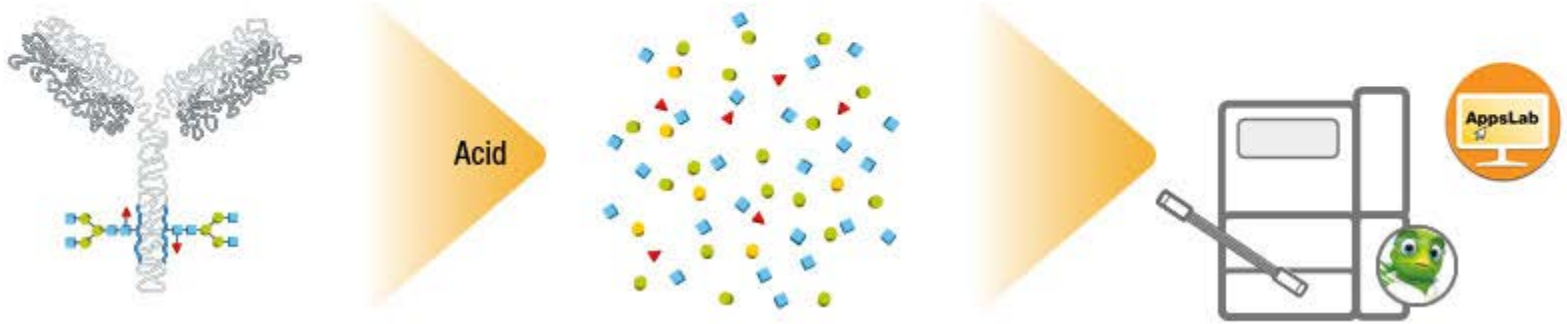


Intact Glycoprotein

Monosaccharides & Sialic Acids



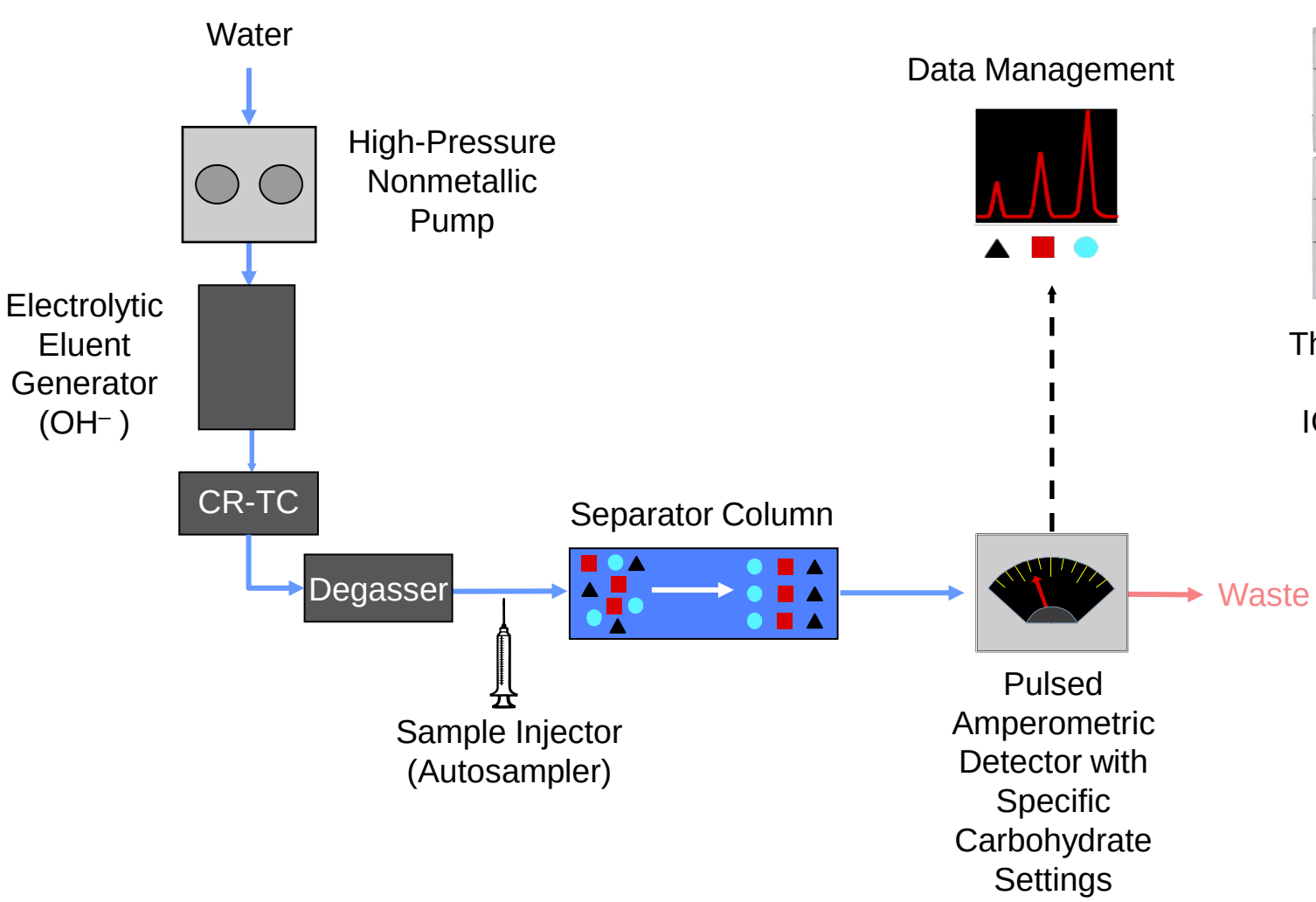
Monosaccharide Analysis Workflow



Glycoprotein | Monosaccharides | HPAE-PAD

- Monosaccharide composition can screen for changes in glycosylation
- Allows measurement of **total sugars** and amounts of specific **monosaccharides**
- Workflow using **HPAE-PAD** (anion-exchange chromatography) – specific carbohydrate chromatography and detection

HPAE-PAD Glycoprotein Monosaccharide Systems



Thermo Scientific ™ Dionex™ ICS-5000⁺ HPIC™

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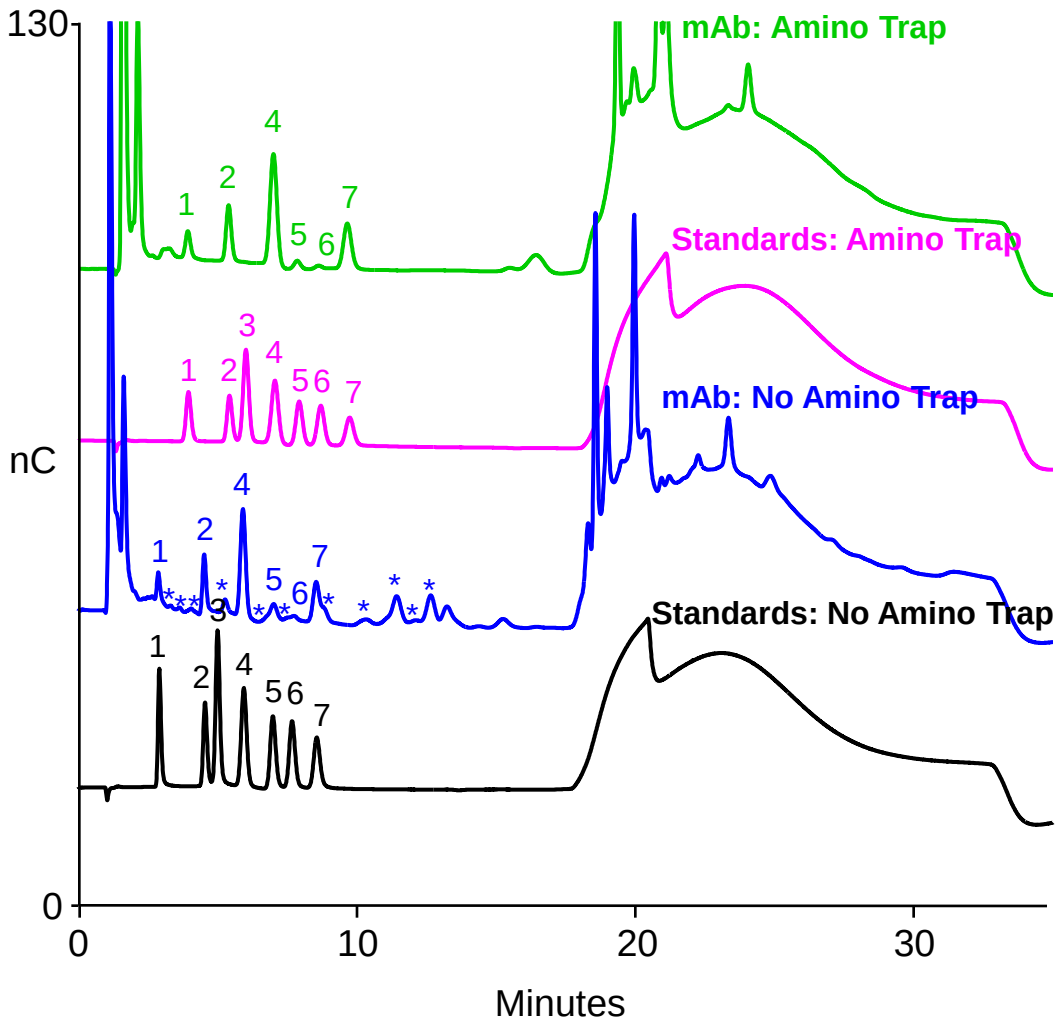
Monosaccharide Compositional Analysis of Human IgG



Column:	Thermo Scientific™ Dionex™ CarboPac™ PA 20
Dimensions:	150 mm × 0.4 mm i.d.
Temperature:	30 ° C
Eluent:	KOH (EG)
Gradient:	12 mmol/L for15 min, then to 100 mmol/L for 15 min
Flow rate:	9 µL/min
Inj. volume:	0.4 µL
Detection:	IPAD (carbohydrate quadruple waveform) on a gold electrode
Sample:	Standards (10 µM)

Peaks:
1. Fucose
2. Deoxyglucose (internal standard)
3. Galactosamine
4. Glucosamine
5. Galactose
6. Glucose
7. Mannose

* Amino acids

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Sialic Acid Analysis Workflow

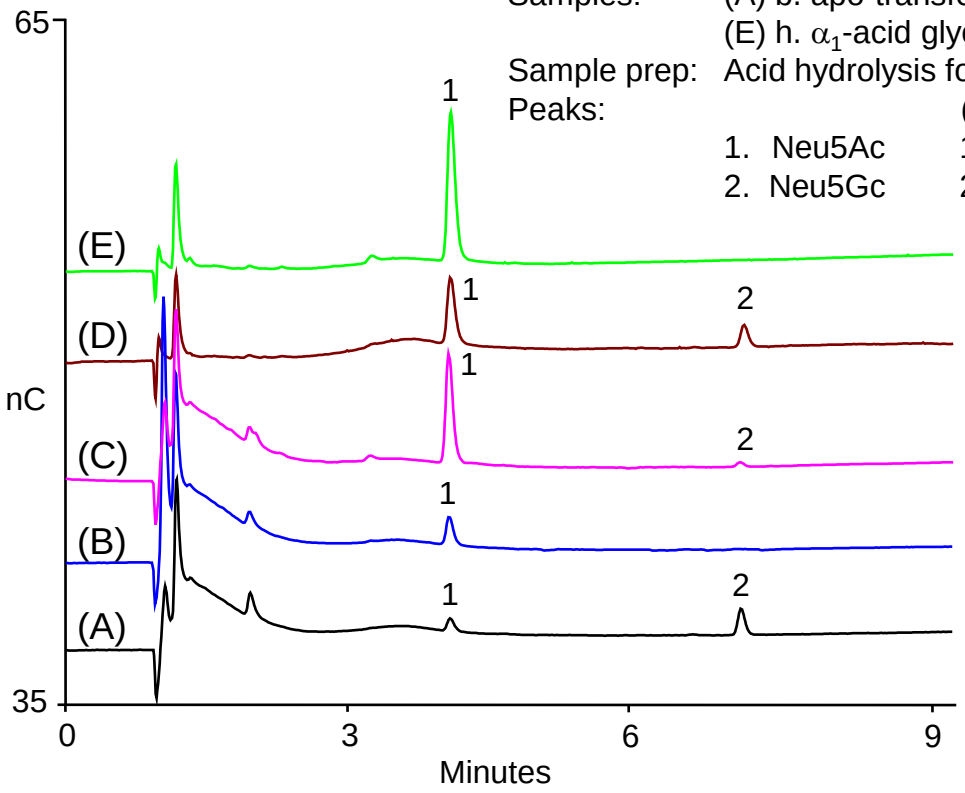
- Sialic acids are released from glycoproteins by either mild acid hydrolysis or by treatment with a neuraminidase
- Samples are then dried to remove the acid
- Samples are injected onto the HPAE-PAD system
- For neuraminidase digestions the sample is either injected or diluted and injected

Traditional Gradient Separation of Glycoprotein Hydrolysates

Column:	Dionex CarboPac PA20 with guard
Dimensions:	150 mm × 3 mm i.d.
Eluent:	70-300 mmol/L NaOAc in 100 mmol/L NaOH from 0-7.5 min, 300 mmol/L NaOAc in 100 mmol/L NaOH from 7.5-9.0 min
Temperature:	30ºC
Flow rate:	0.5 mL/min
Inj. volume:	10 µL
Detection:	IPAD, disposable Au electrode
Samples:	(A) b. apo-transferrin, (B) h. transferrin, (C) fetuin, (D) s. α_1-acid glycoprotein, (E) h. α_1-acid glycoprotein
Sample prep:	Acid hydrolysis followed by lyophilization and dissolution

Peaks:

	(A)	(B)	(C)	(D)	(E)	
1. Neu5Ac	1.7	4.4	18	15	37	pmol
2. Neu5Gc	2.1	ND	0.4	2.6	ND	



A 10% signal offset has been applied.
ND = Not Detected

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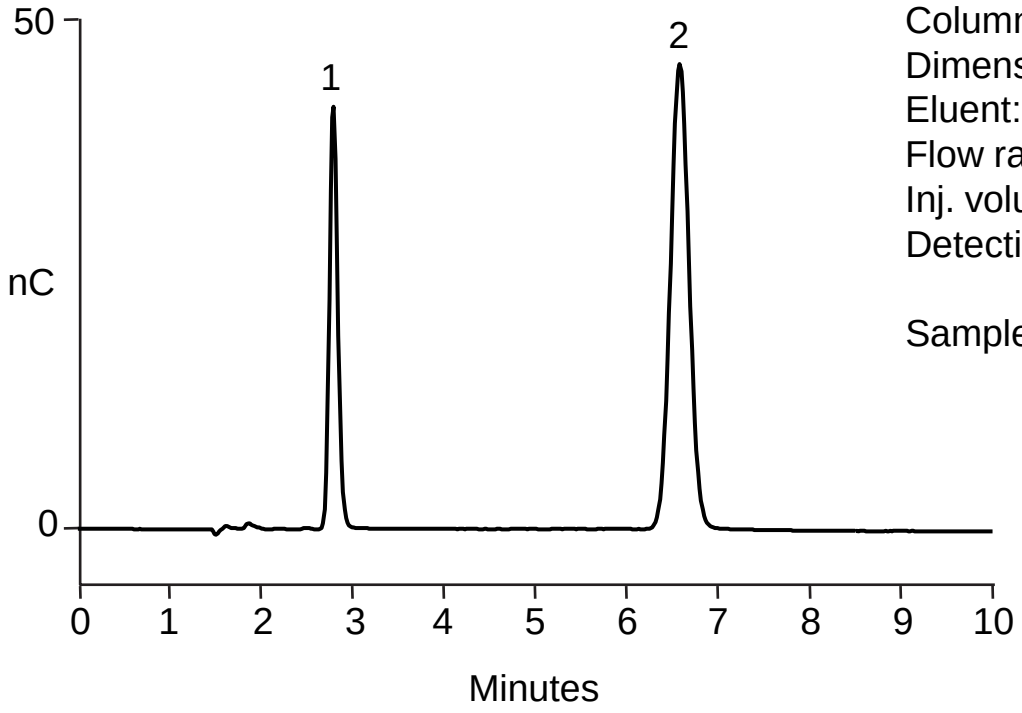
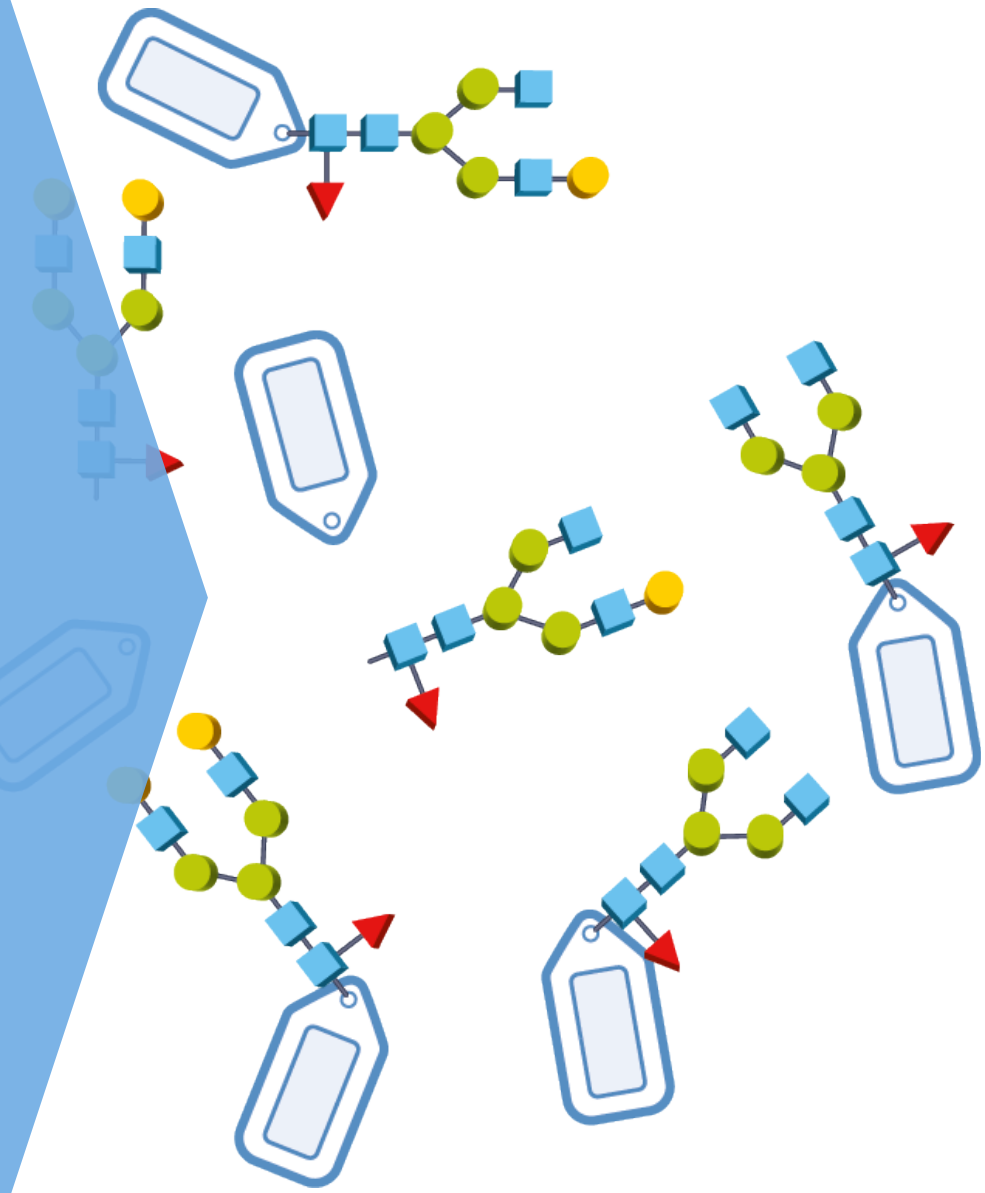
Rapid Isocratic Separation of Sialic Acids



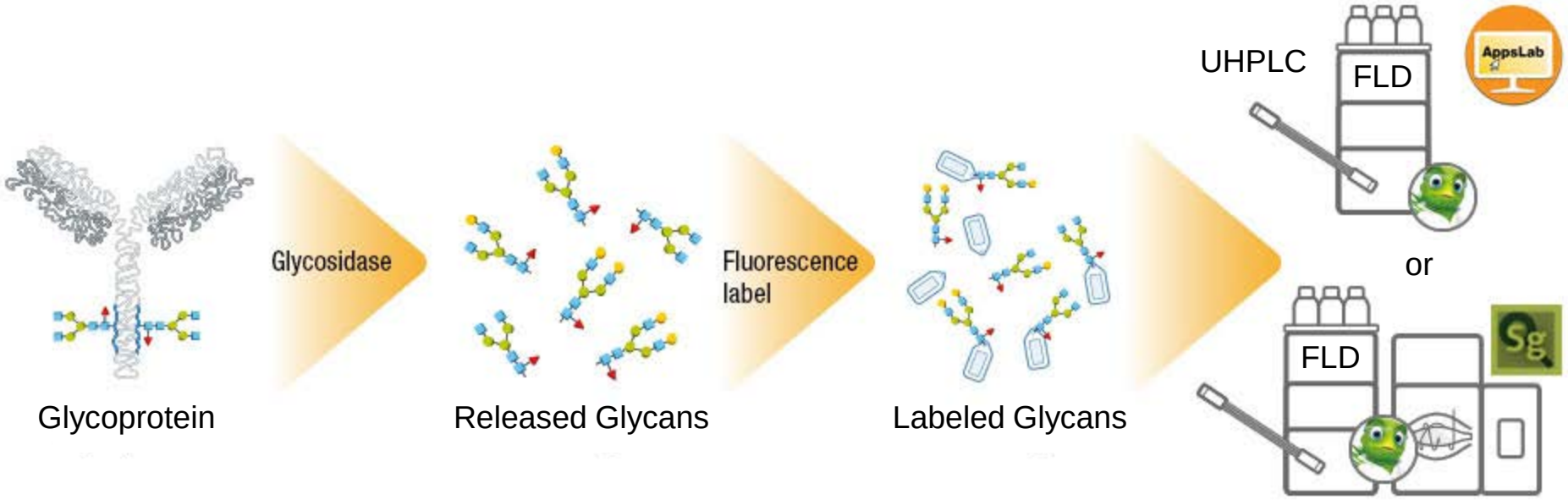
Column:	Dionex CarboPac SA10
Dimensions:	250 mm × 4 mm i.d.
Eluent:	0.2 mol/L NaOH
Flow rate:	1.5 mL/min
Inj. volume:	10 µL
Detection:	IPAD, Au electrode

Sample:	1. *N*-acetylneuraminic acid	50 µmol
	2. *N*-glycolylneuraminic acid	50

Labeled Glycans

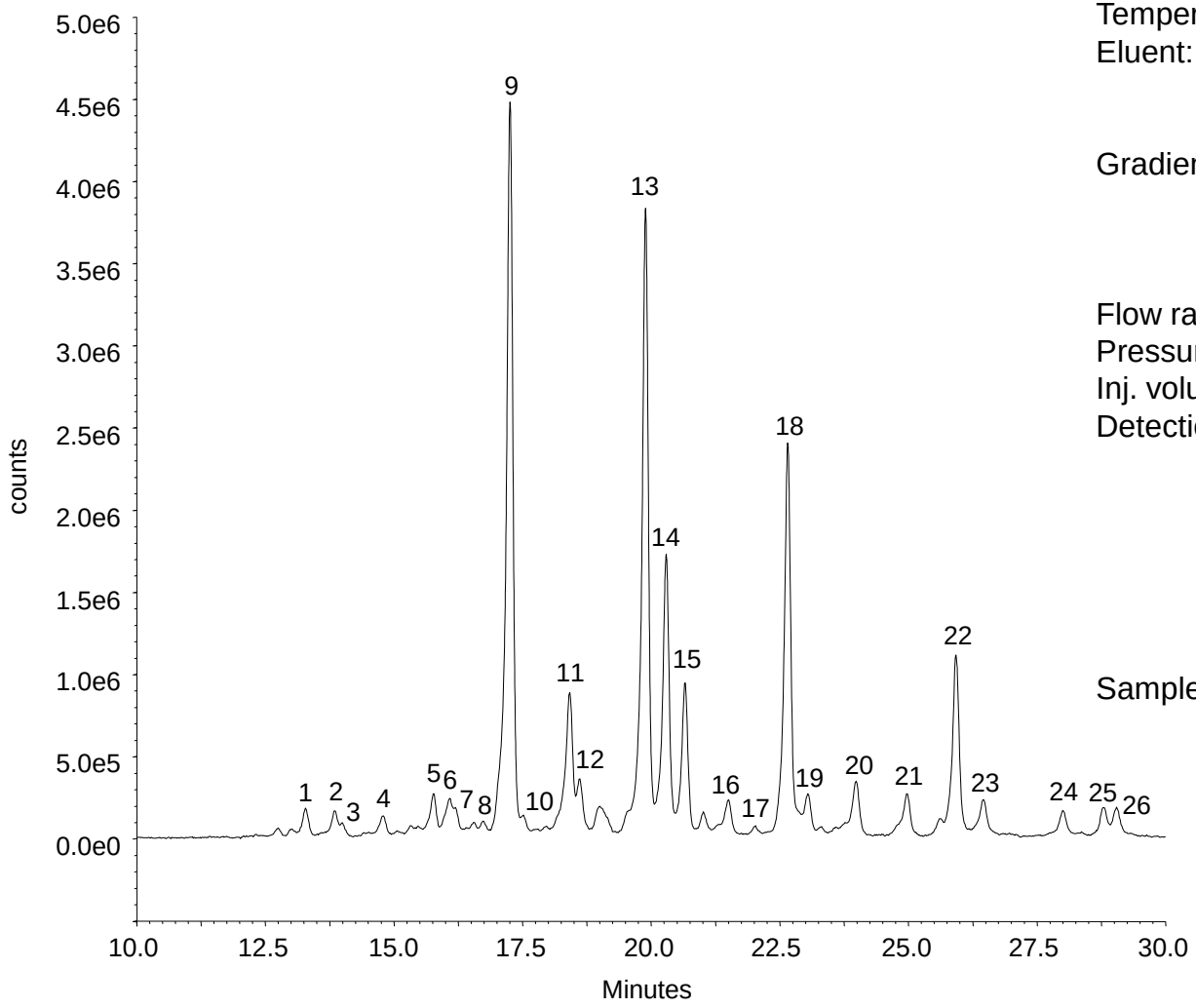


Labeled Glycans – Quantification and Qualification



- Glycan-specific separations:
  - Thermo Scientific™ GlycanPac™ AXH-1
  - Thermo Scientific™ GlycanPac™ AXR-1
  - Thermo Scientific™ Accucore™ 150-Amide-HILIC
- Trace **quantification using new fluorescence detector** for Thermo Scientific™ Vanquish™ Flex UHPLC
- Qualitative **released glycan structure analysis** can be confirmed using HRAM MS and SimGlycan® software (PREMIER Biosoft)

HILIC Separation of 2AB Labeled Glycans from Human IgG



Column:	Accucore 150 Amide HILIC 2.6 µm
Dimensions:	150 mm × 2.1 mm i.d.
Temperature:	50 ºC, still air
Eluent:	A. MeCN B. 50 mmol/L ammonium formate, pH 4.4
Gradient:	0-30 min from 20 to 42% B, 30-30.5 min to 50% B, 30.5-32 min 50% B, 32-32.5 min to 20% B, 32.5-60 min 20% B (equilibration)
Flow rate:	400 µL/min
Pressure:	210 bar (max.)
Inj. volume:	5 µL
Detection:	Fluorescence Excitation wavelength: 320 nm Emission wavelength: 420 nm Lamp mode: HighPower Sensitivity: 8 Data collection rate:5 Hz Response time: 1 s
Sample:	1 nmol/mL 2-AB labeled *N*-glycans Immunoglobulin G library

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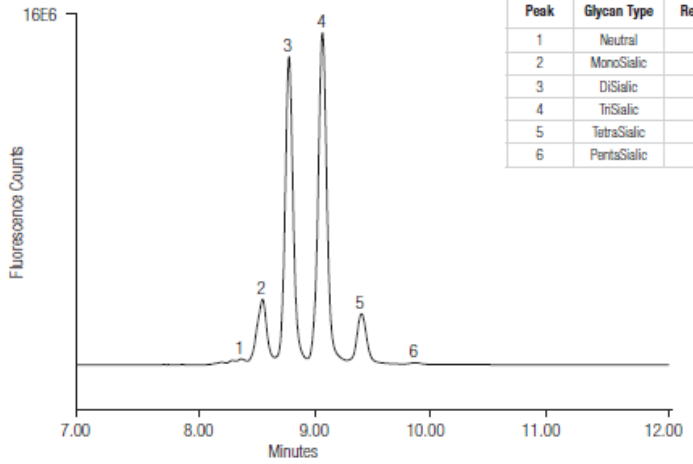
Charge-based/HILIC Separation on GlycanPac AXH-1



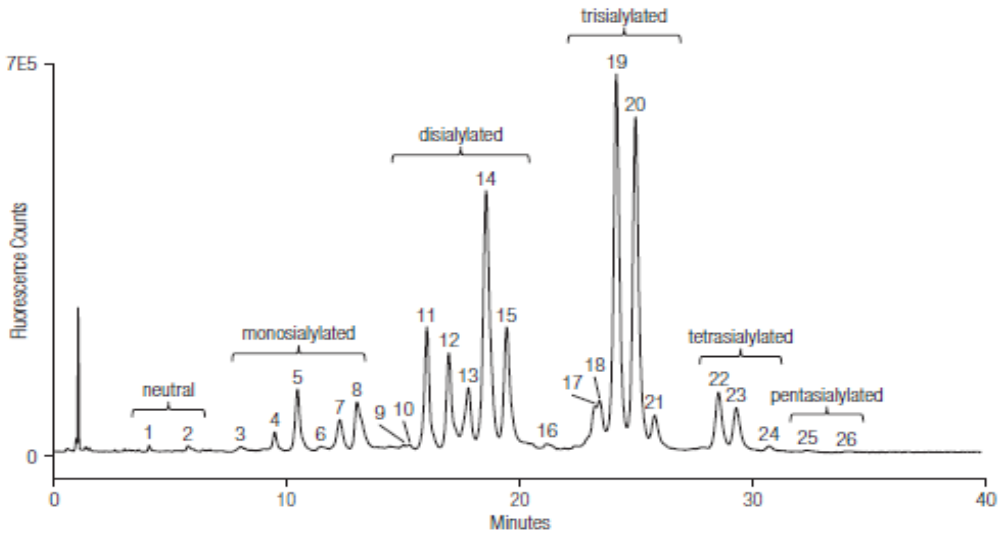
Peak	Glycan Type	Relative %
1	Neutral	0.4
2	MonoSialic	8.6
3	DiSialic	38.4
4	TriSialic	45.4
5	TetraSialic	7.0
6	PentaSialic	0.2

Column: **GlycanPac AXH-1 (1.9 µm)**
Dimension: 2.1 × 150 mm
Mobile Phase A: Acetonitrile
Mobile Phase B: Ammonium formate (50 mM, pH = 4.4)
Mobile Phase C: Water

Time (min)	% A	% B	% C	Flow (mL/min)
-5	90	10	0	0.4
0	90	10	0	0.4
6	50	20	30	0.4
12	50	20	30	0.4

Flow Rate: 0.4 mL/min
Injection Volume: 40 pmole
Temperature: 30 °C
Detection: Fluorescence at 320/420 nm
Sample: 2AB Labeled *N*-glycans from bovine fetuin

After enzymatic digestion with sialidase S and sialidase A



Column: **GlycanPac AXH-1 (1.9 µm)**
Dimension: 2.1 × 150 mm
Mobile Phase A: Acetonitrile (100%)
Mobile Phase B: Water
Mobile Phase C: Ammonium formate (100 mM, pH = 4.4)
Flow Rate: 0.4 mL/min
Injection Volume: 50 Pmoles
Temperature: 30 °C
Detection: Fluorescence at 320/420 nm
Sample: 2AB labeled *N*-glycan from bovine fetuin

Time (min)	% A	% B	% C	Flow (mL/min)	Curve
-10	78	20	2	0.4	5
0	78	20	2	0.4	5
30	70	20	10	0.4	5
35	60	20	20	0.4	5
40	50	20	30	0.4	5

Separation based on charge, size, and shape

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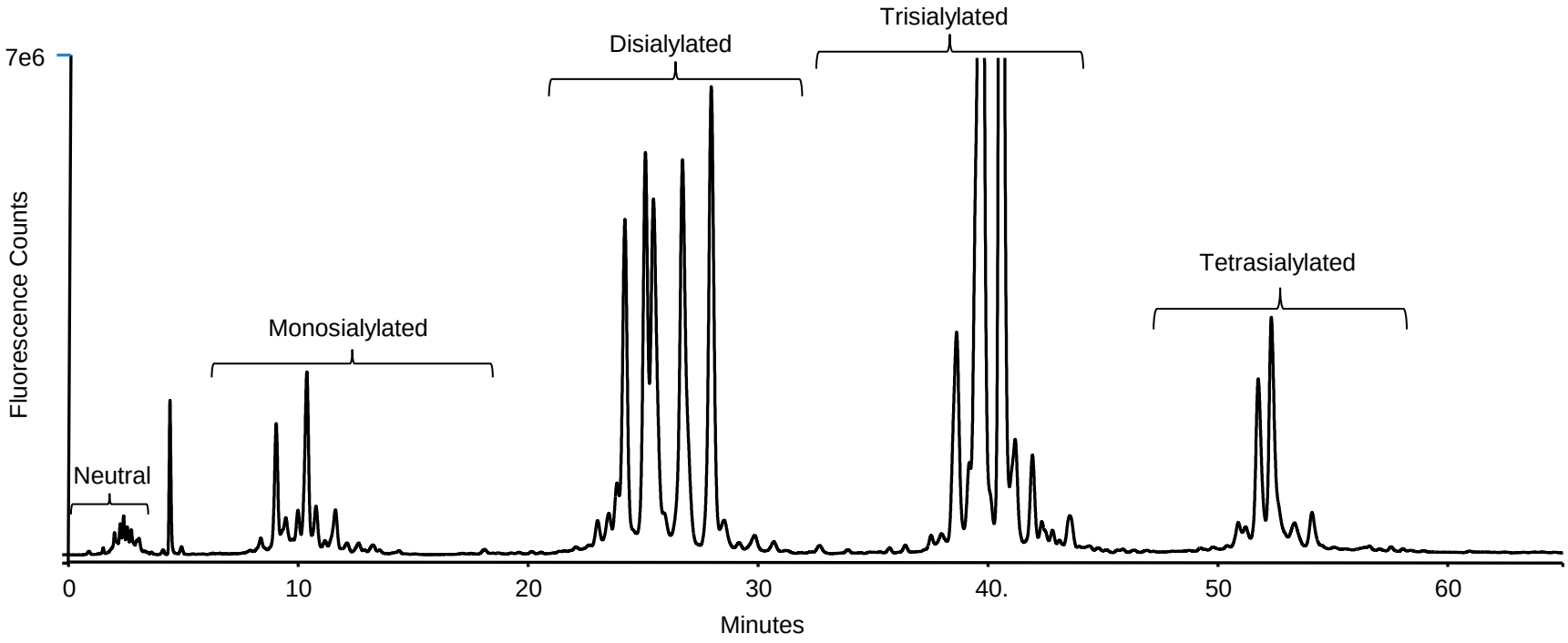
Charged-based/RP Separation on GlycanPac AXR-1

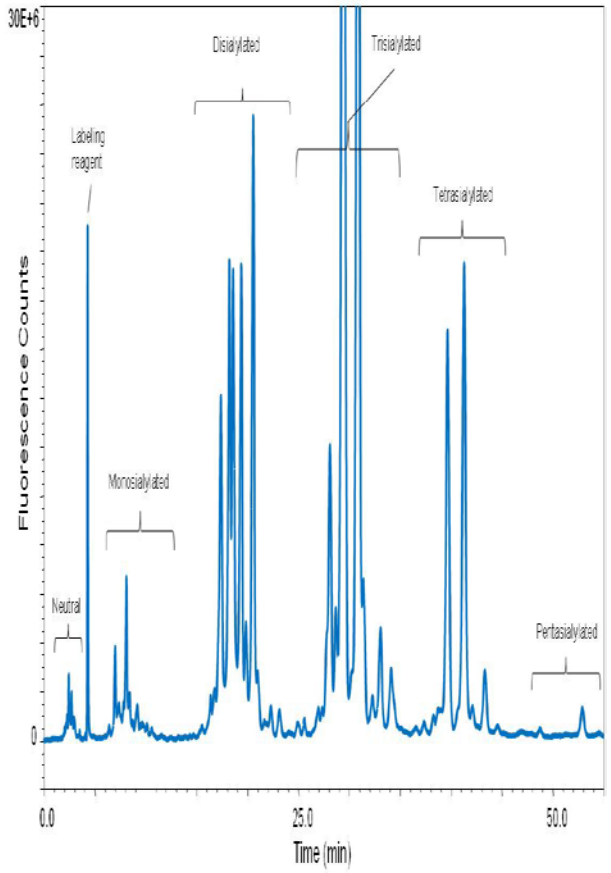
- WAX functionality: separates glycans into different “clusters” in order of increasing charge
- RP functionality: facilitates further separation within each “cluster” to achieve high-resolution separations for glycans of the same charge according to their isomerism and size



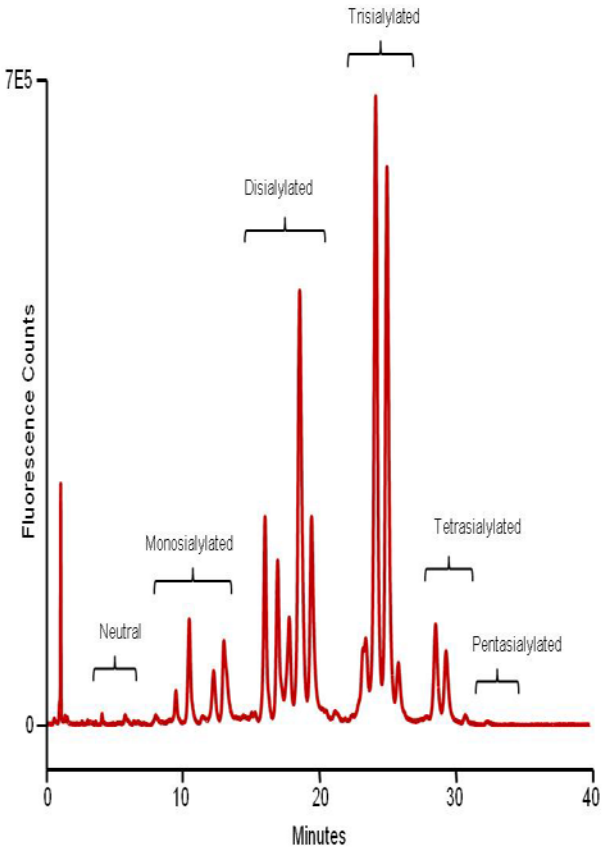
~ 80 resolved peaks

Selectivity Comparison of GlycanPac and Amide HILIC columns

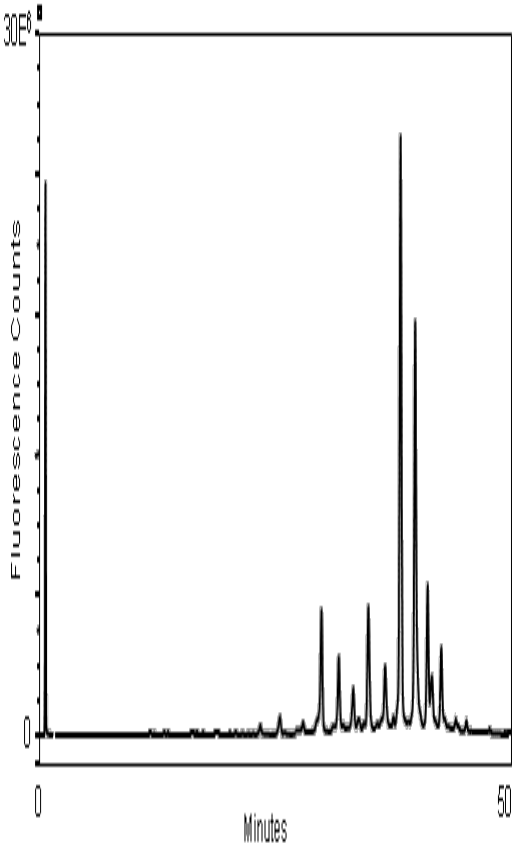
GlycanPac AXR-1 (1.9 µm)
(>100 peaks resolved)



GlycanPac AXH-1 (1.9 µm)
(>60 peaks resolved)



Amide HILIC (1.7 µm)
(>40 peaks resolved)



When to use which column?

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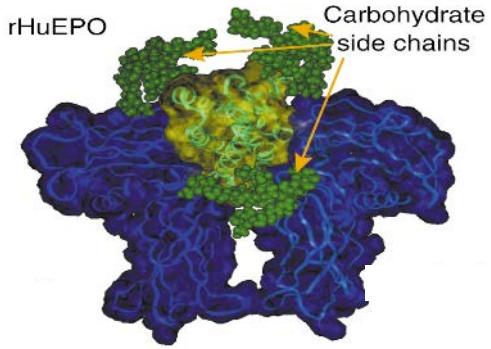
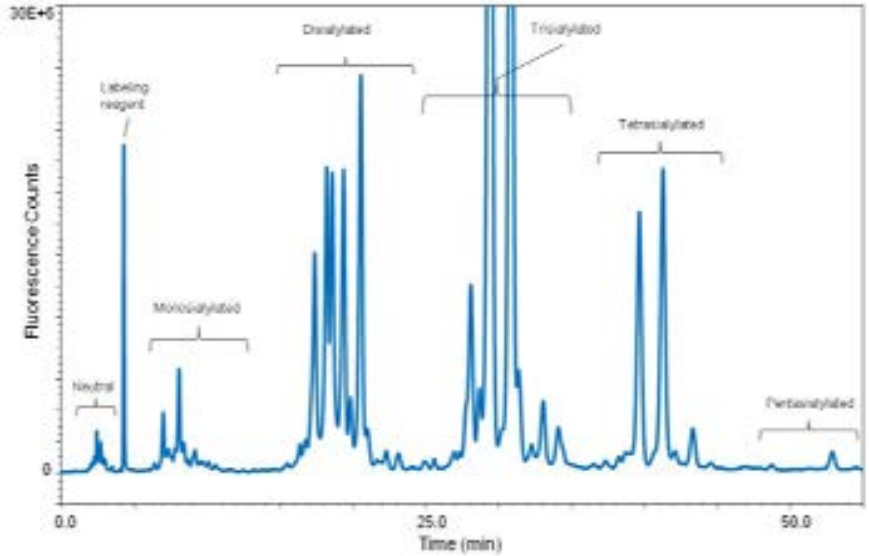
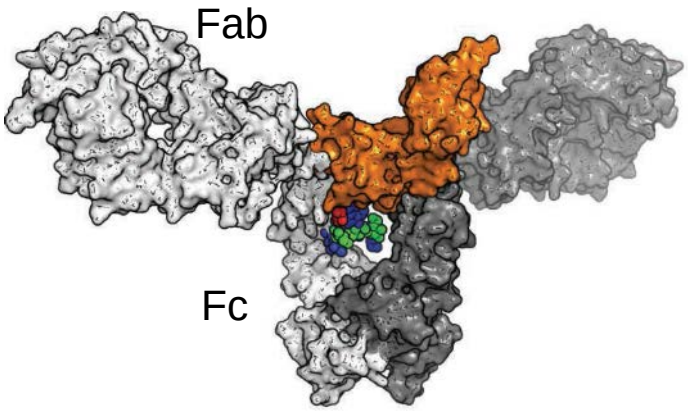
Glyco-Biopharmaceuticals

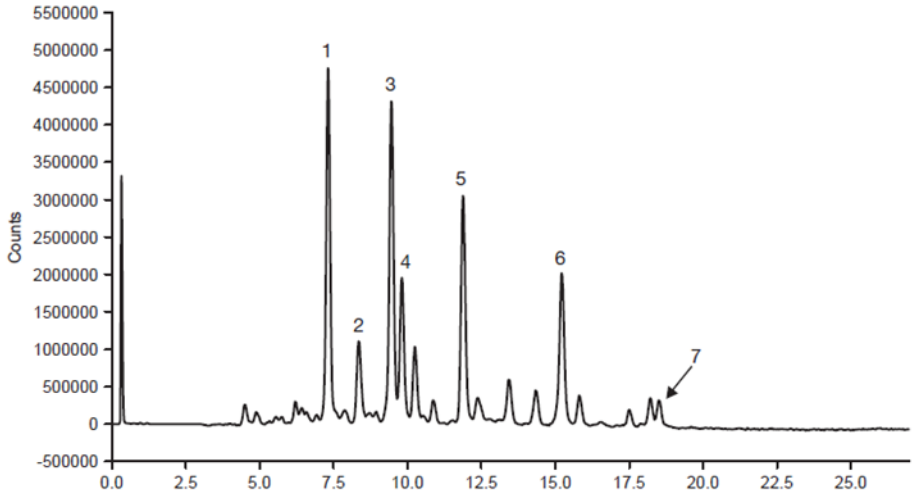
EPO





Therapeutic Antibodies





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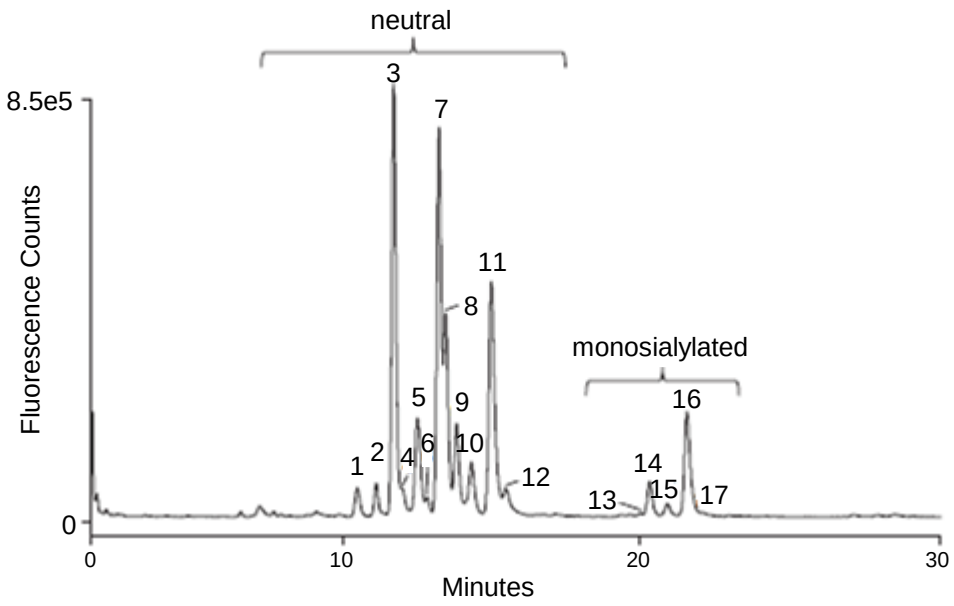
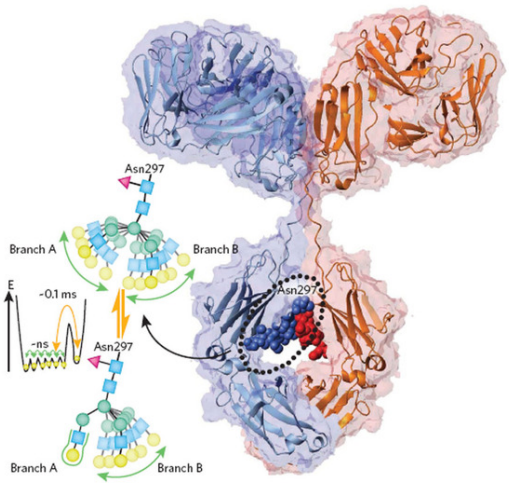
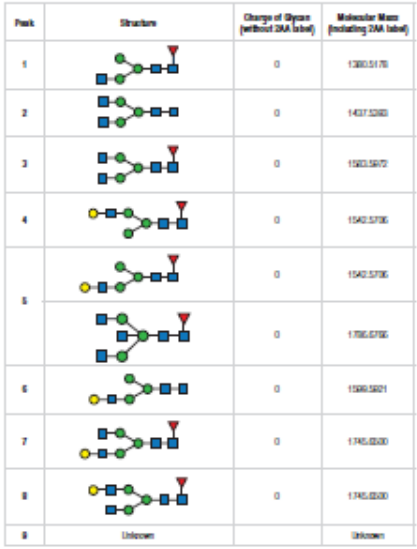
2AA Labeled *N*-Glycans from Human IgG

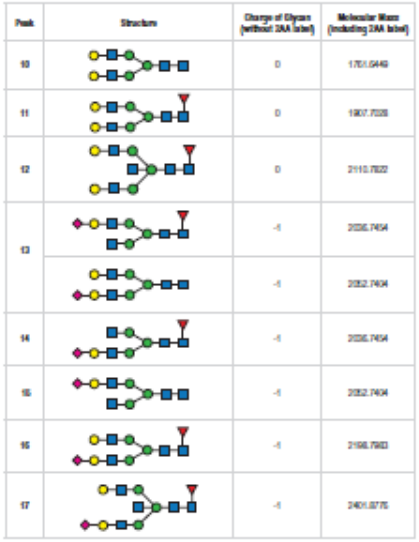


Column:	GlycanPac AXH-1, 1.9 µm
Dimensions:	150 mm × 2.1 mm i.d.
Temperature:	30 ºC
Eluent:	A. MeCN/water (80:20 v/v) B. 80 mmol/L ammonium formate, pH 4.4
Gradient:	1% B to 12.5% B in 30 min
Flow rate:	0.4 mL/min
Injection:	20 pmoles
Detection:	Fluorescence Excitation wavelength: 320 nm Emission wavelength: 420 nm
Sample:	2-AA labeled *N*-glycans from human IgG



- *N*-Acetyl glucosamine (GlcNAc)
- Mannose (Man)
- Galactose (Gal)
- *N*-Acetyl neuraminic acid (Neu6Ac)
- L-Fucose (L-Fuc)





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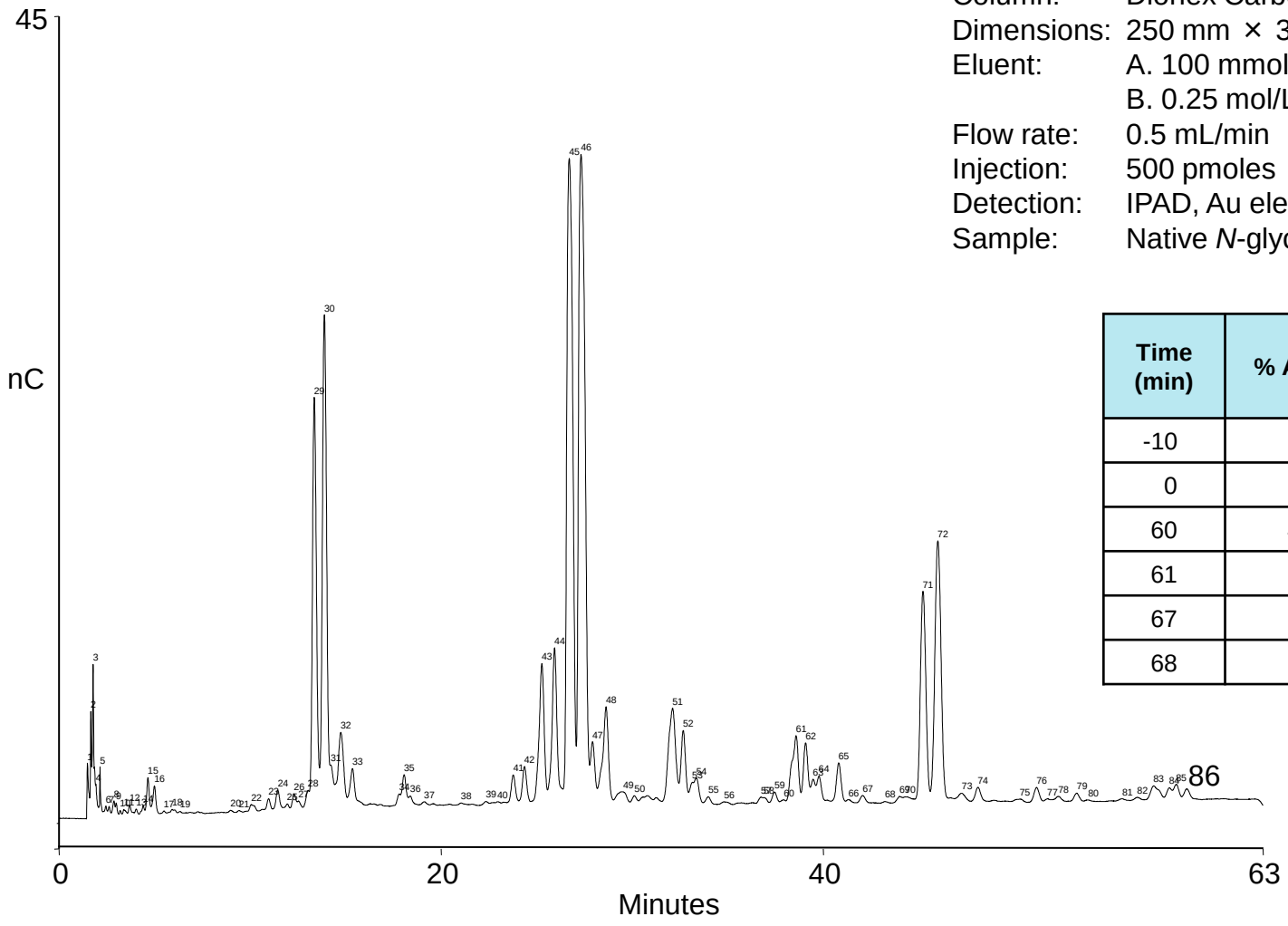
Anion-Exchange Separation of Native *N*-Glycans from Fetuin



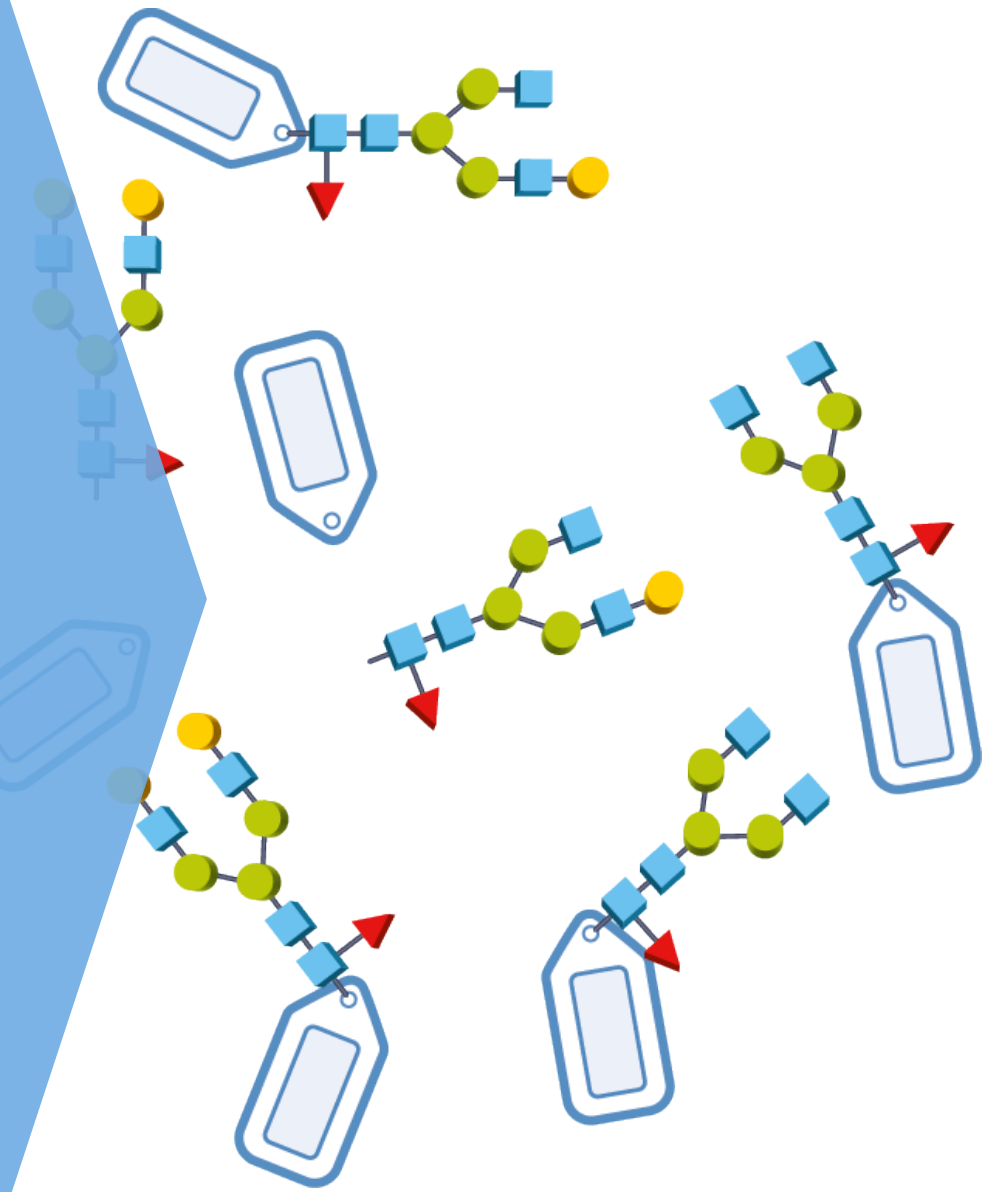
Column:	Dionex CarboPac PA200
Dimensions:	250 mm × 3 mm i.d.
Eluent:	A. 100 mmol/L NaOH
	B. 0.25 mol/L NaOAc in 100 mmol/L NaOH
Flow rate:	0.5 mL/min
Injection:	500 pmoles
Detection:	IPAD, Au electrode
Sample:	Native *N*-glycan from bovine fetuin

Time (min)	% A	% B	Flow rate (mL/min)
-10	90	10	0.5
0	90	10	0.5
60	40	60	0.5
61	0	100	0.5
67	0	100	0.5
68	90	10	0.5

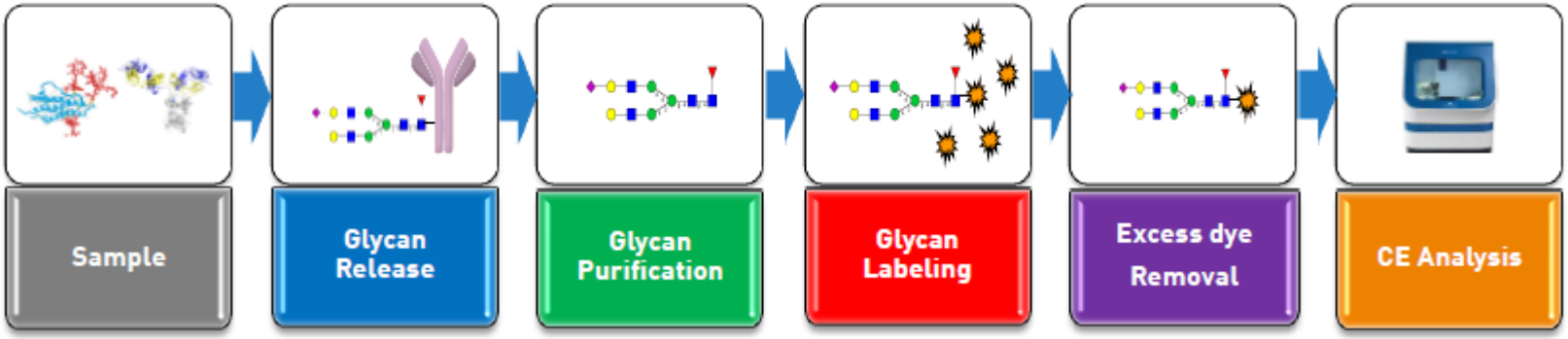
Upstream High-Throughput Glycan Screening

Labeled Glycans

- High throughput
- Early discovery



Workflow



Glycoprotein	PNGase-F 1 h	Magnetic Beads 30 min	APTS – 2 h Teal – 30 min Turquoise – 30 min	Magnetic Beads 30 min	24 Capillary CE Analysis 3 h

Hands-on time < 3 h

Time to results: 7 – 9 h* (96 samples)

*Depends on the dye used for glycan labeling

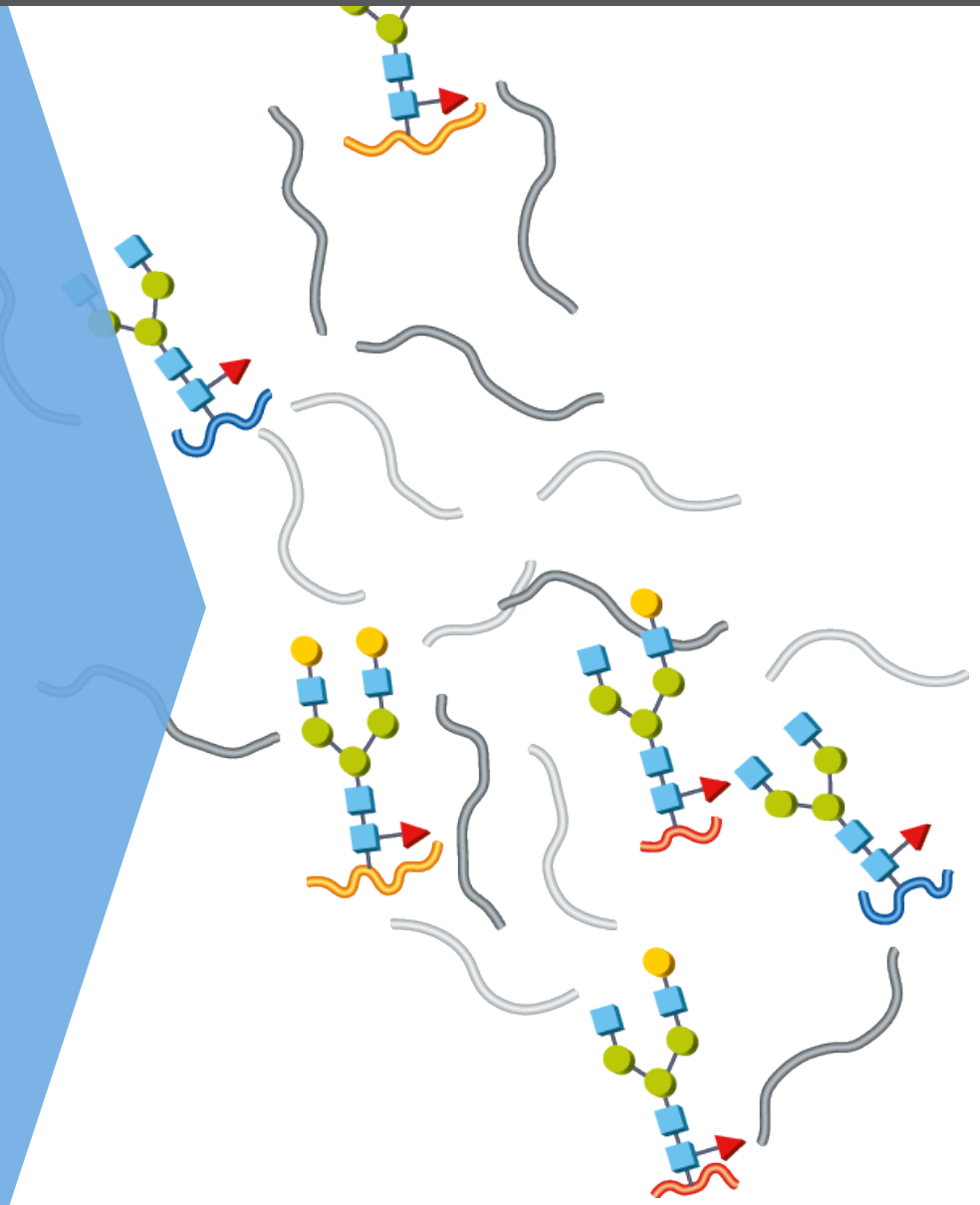
CE Instrumentation

Applied Biosystems® 3500 XL Genetic Analyzer

24 Capillary Array (60 cm)

- Industry „gold standard“ CE instrument
- Parallel analysis of 8 or 24 samples in 45 min
- Multi-color capability
- Calibration across capillaries using internal standard
- RFID-tagged capillary array & CE consumables for automated tracking

Glycopeptides



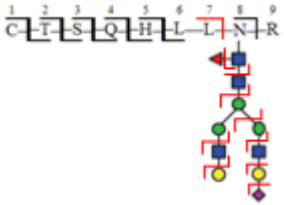
Glycopeptide Workflow

Glycoprotein | SMART Digest Kits | Glycopeptides | UHPLC Orbitrap MS

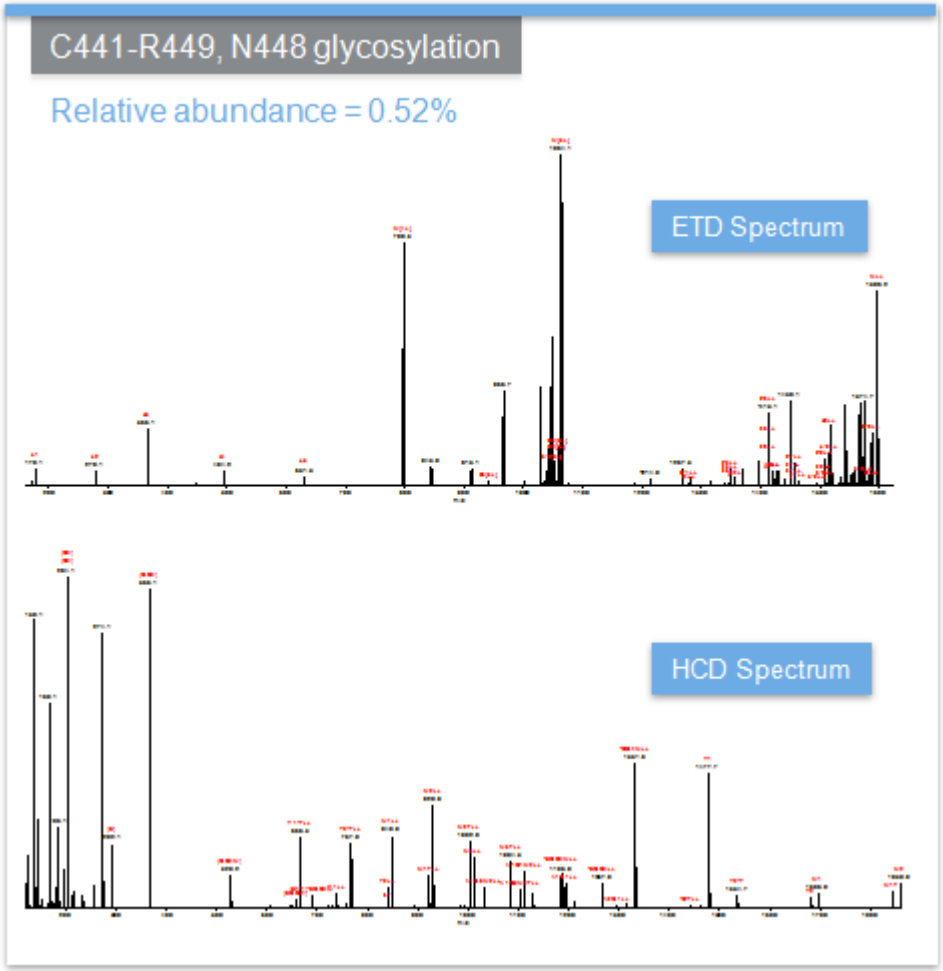
- Important for site profiling of PTMs including glycosylation
  - A variety of fragmentation techniques can be used
  - **ETD, HCD** or CID
- **Robustly digest in 1 h** using Thermo Scientific™ SMART Digest™ kits
- Bioinformatics tools are extremely valuable for data interpretation and glycosite profiling
  - Thermo Scientific™ BioPharma Finder™ software

Complete Characterization of Glycopeptides Using HCD and ETD

- Unique HCDpdETD method features on-the-fly identification of glycopeptides using diagnostic fragment ions from carbohydrate fragmentation.
- A high quality HCD spectrum is generated for each peptide.
- An additional ETD spectrum is generated for each glycopeptide.
- For each glycopeptide, ETD provides information of peptide sequence and site of glycosylation while HCD provides information of glycan structure and additional peptide sequence.

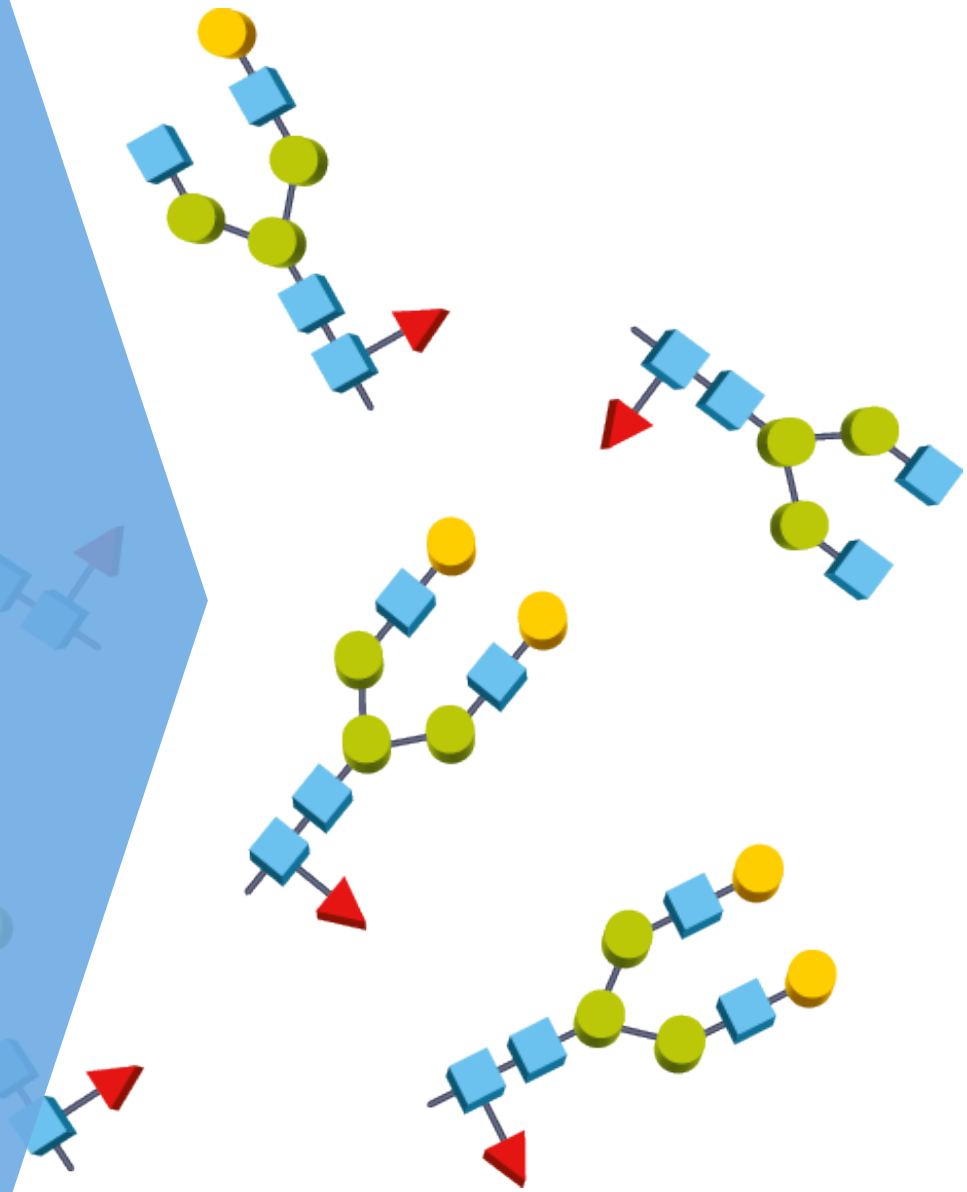


ETD Fragmentation
HCD Fragmentation

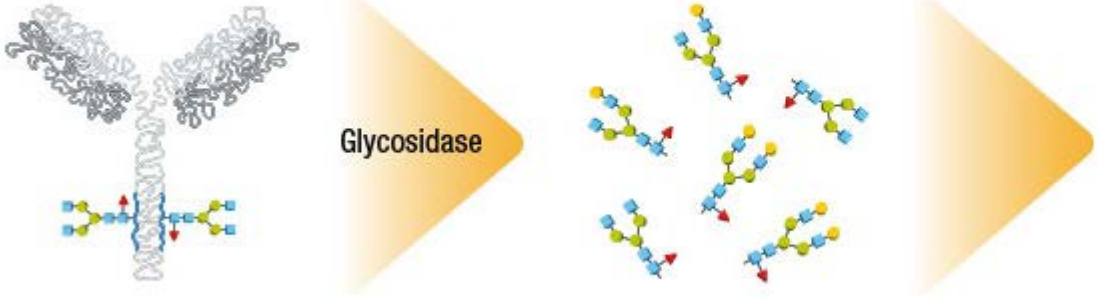


Zhiqi Hao et al. 2014 ASMS TP264

Unlabeled *N*-Linked Glycans

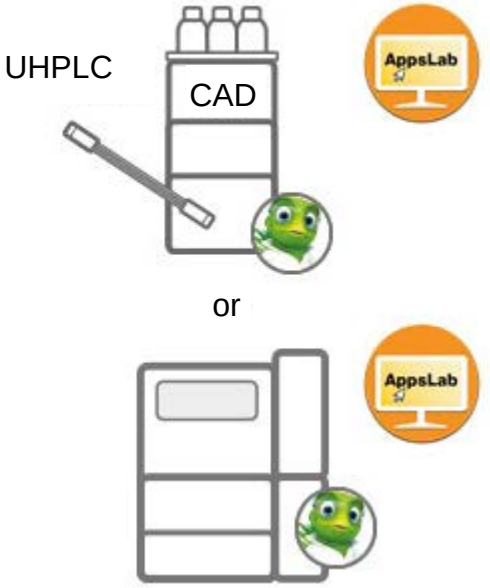


Charged Aerosol Detection for Unlabeled Glycans



Glycoprotein

Released Glycans



HPAE-PAD

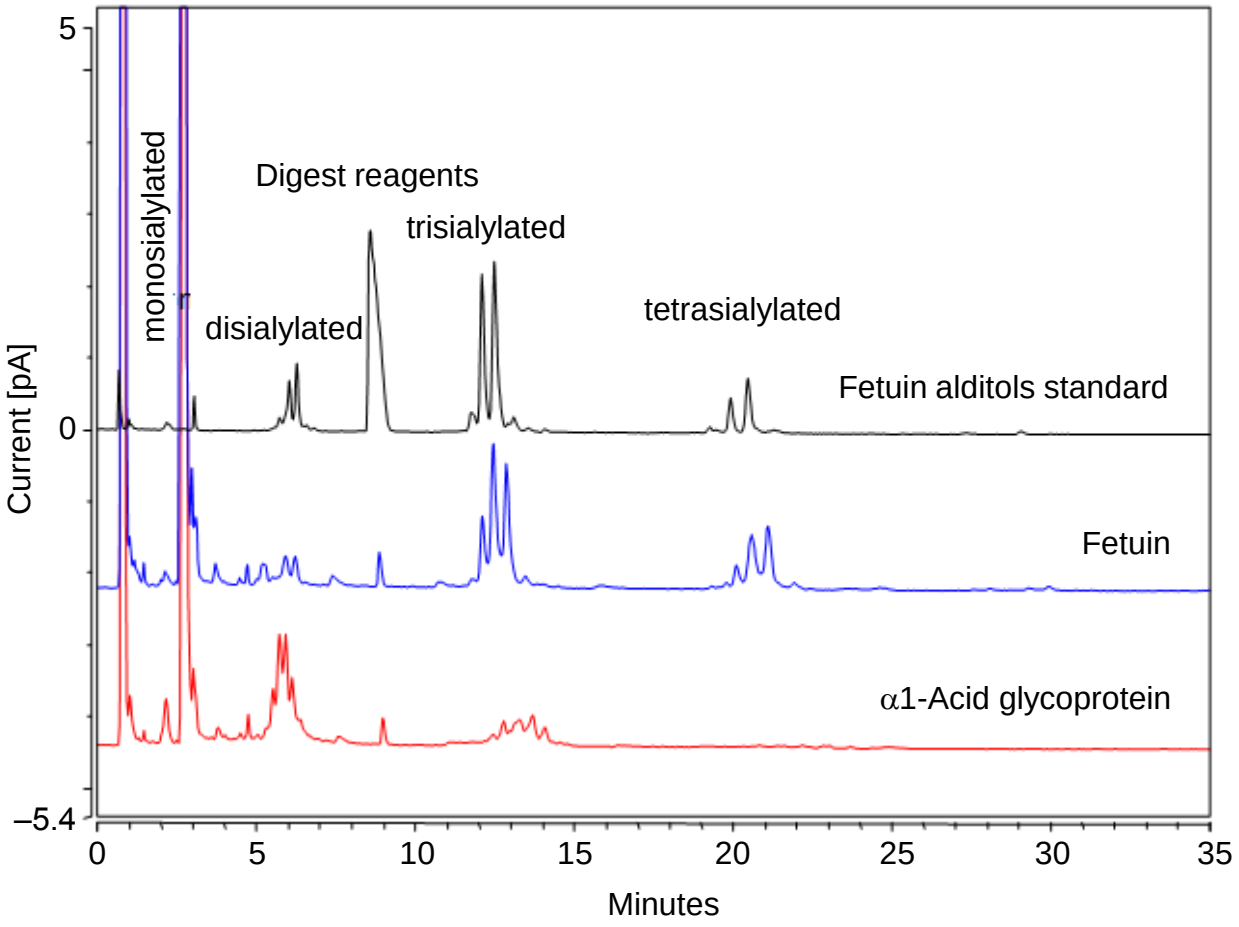
- No requirement for labeling
- Near-universal detection
- Quantitative response without individual standards
- Orthogonal detection to MS

Released 2015

Thermo Scientific™ Vanquish™ charged Aerosol detector

Full integration with Thermo Scientific™ Vanquish™ UHPLC platform, slide-in module design, reduced flow path for optimum operation

Label-Free Analysis of *N*-linked Glycans by UHPLC-CAD

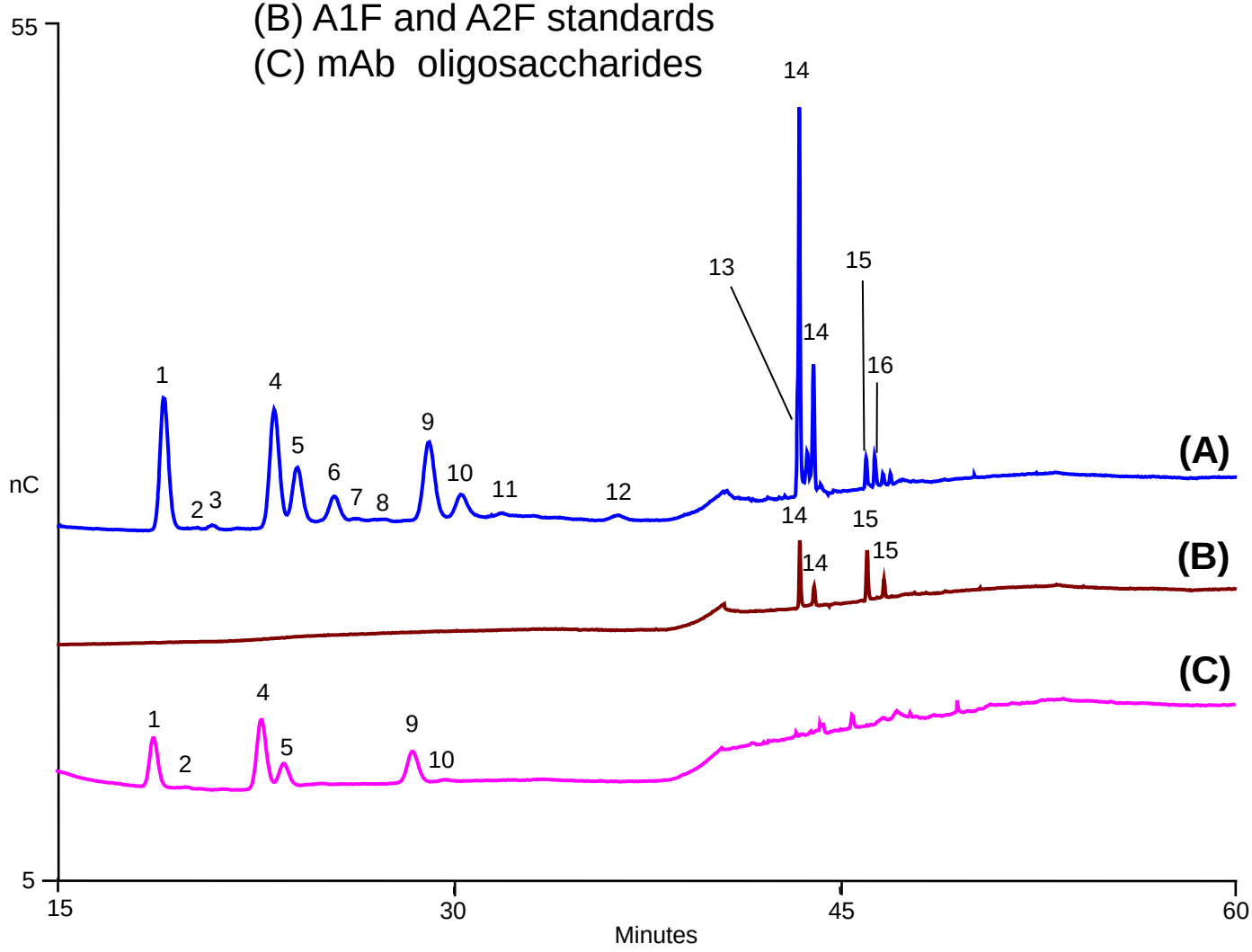


System:	Vanquish UHPLC
Column:	GlycanPac AXR-1, 1.9 µm
Dimensions:	150 m × 2.1 mm i.d.
Temperature:	30ºC
Eluent:	A. Deionized water B. 100 mmol/L ammonium formate, pH 4.4
Gradient:	4–39 % B in 35 min
Flow rate:	0.4 mL/min
Inj. volume:	2 µL
Detection:	Charged Aerosol Detection (50ºC, PF 1.0, 10 Hz, 5 s)

PNGase F Digest – No Labeling Required

HPAE-PAD of *N*-Linked Oligosaccharides from IgG and a mAb

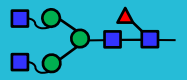
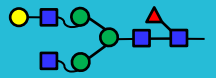
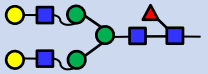
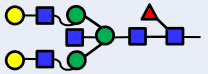
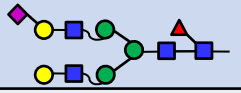
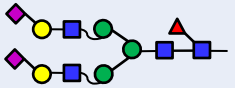
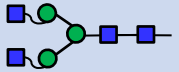
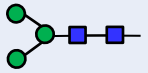
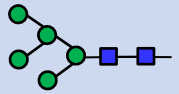
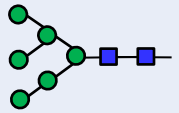
Samples: (A) Polyclonal IgG oligosaccharides
(B) A1F and A2F standards
(C) mAb oligosaccharides



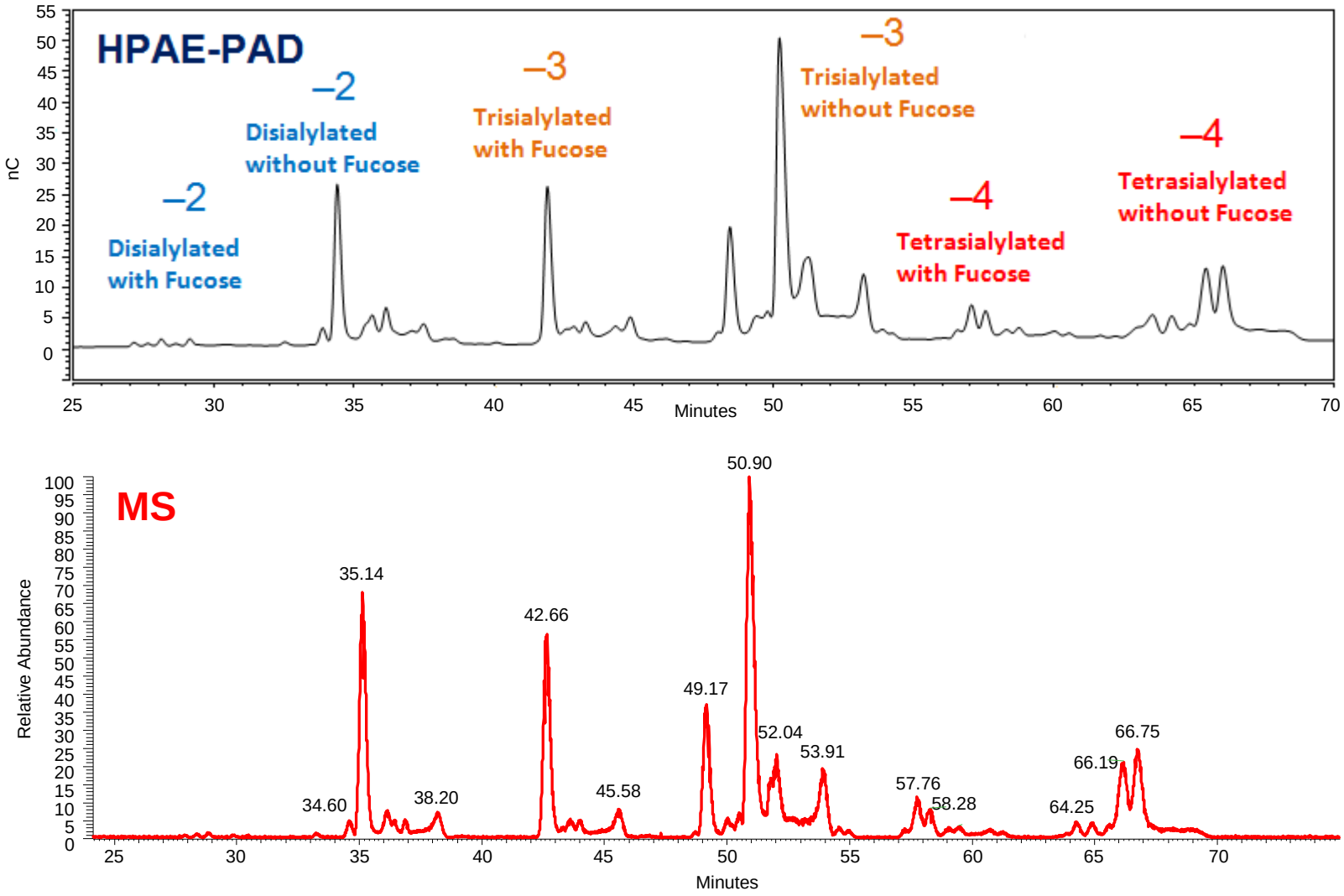
Peaks:
1. G0F
2. Man5
3. G0
4. G1F (1-6)
5. G1F (1-3)
6. G0bF, G1 (1-6)
7. G1 (1-3)
8. G0b
9. G2F
10. G1bF
11. G2
12. G2bF
13. FA2G1S1
14. A1F (1-3)
15. A2F
16. A2

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	

Label-Free Analysis of *N*-linked Glycans by HPAE-PAD-MS



HPAE-PAD is able to separate highly complex glycan mixtures based on charge, linkage & fucose

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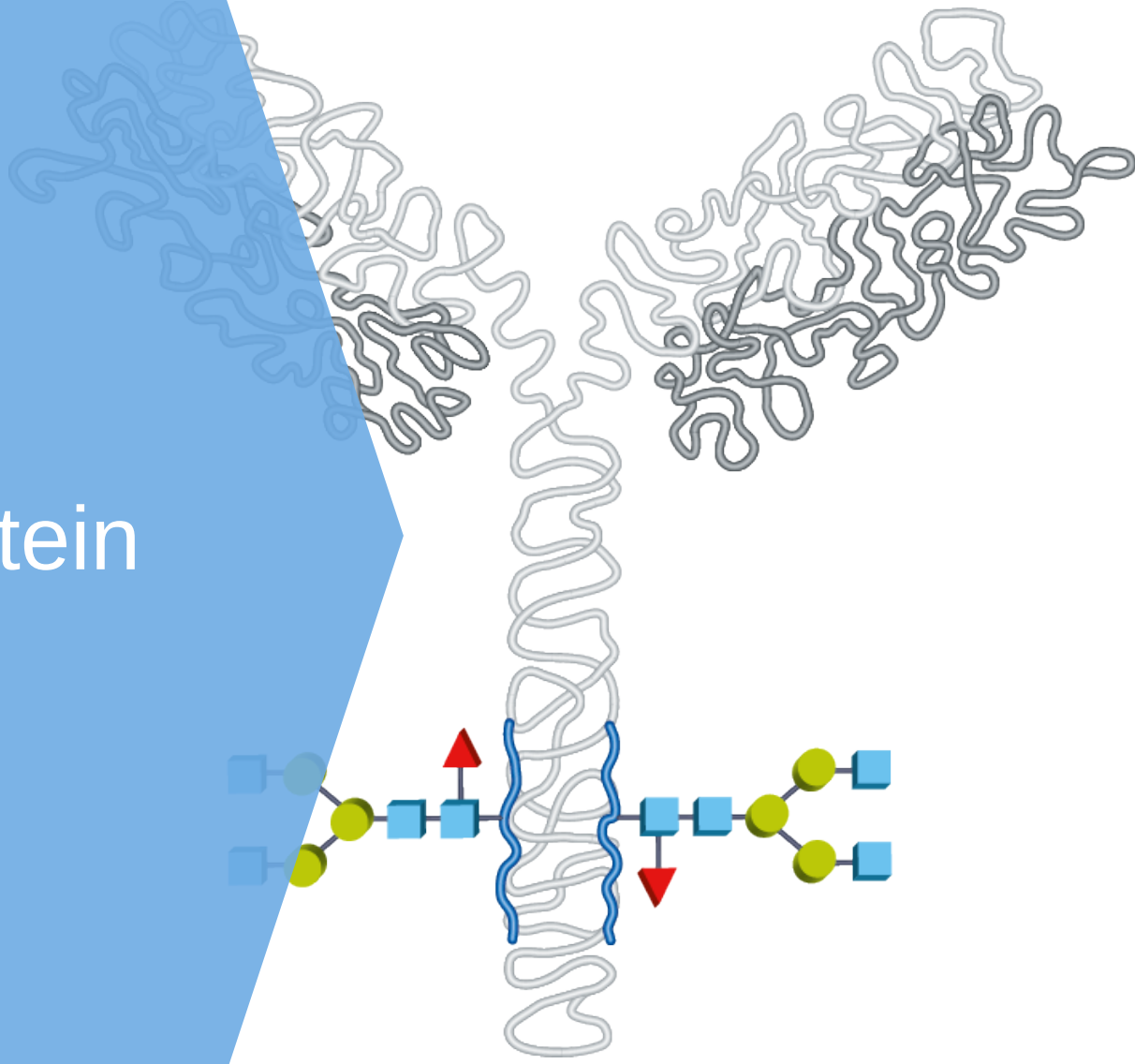
Intact Glycoprotein



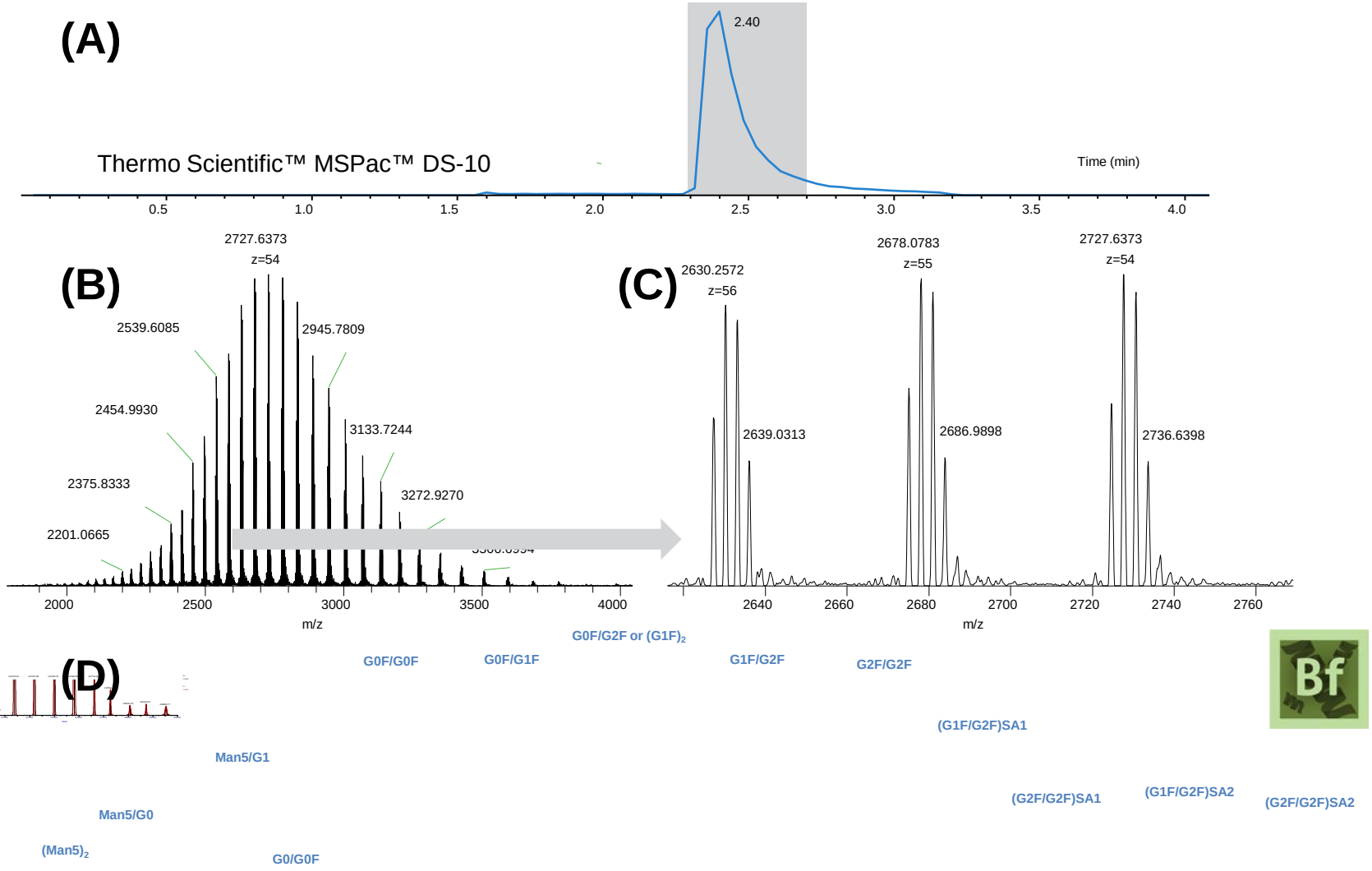
Intact Glycoform Workflow

Protein or reduced protein

UHPLC Orbitrap MS

- **Fast analysis** of the protein in “intact” form is important for biotherapeutic development
- A legal requirement to characterize the intact form and determine heterogeneity
- Due to the variations in structure, charge, etc. Of the attached glycans, the highest resolution and most accurate mass MS is required for precise quantification.

Glycan Analysis of Rituximab

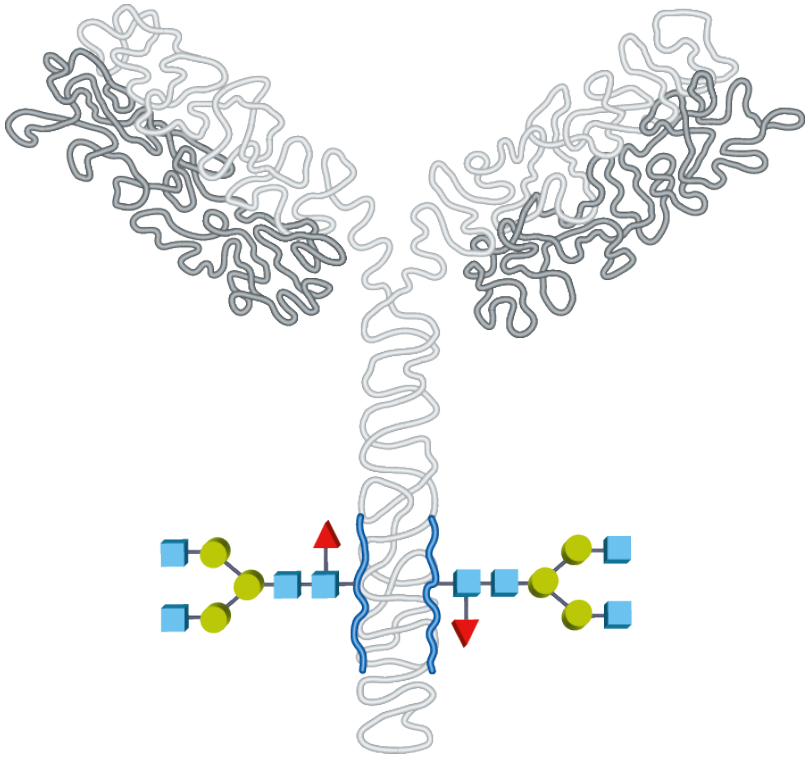


Application Note 21465: Fast online desalting of mAbs using a reversed-phase desalting cartridge for LC-MS analysis

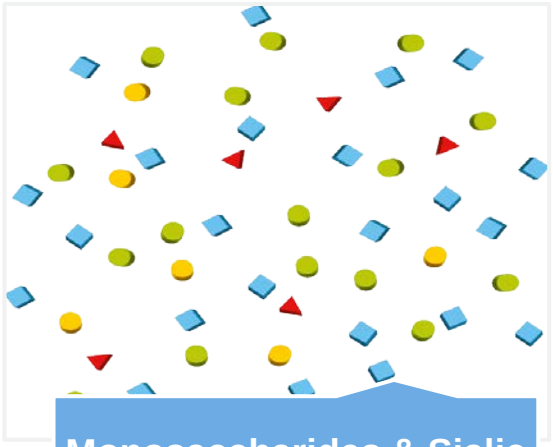
ThermoFisher SCIENTIFIC

Intact Glycoprotein Characterization

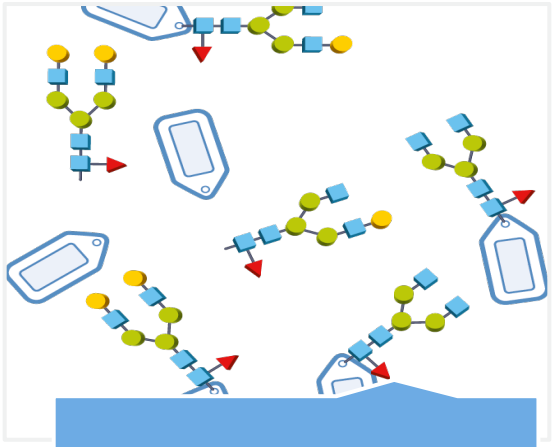
- A **fast 4 min** desalting method for high-throughput characterization
- Intact Mab mass and the relative **glycoform abundance within 5 min**
- In-depth characterization for glycoforms detection **below 1% relative intensity**
- Single software for all data processing
  - BioPharma Finder software



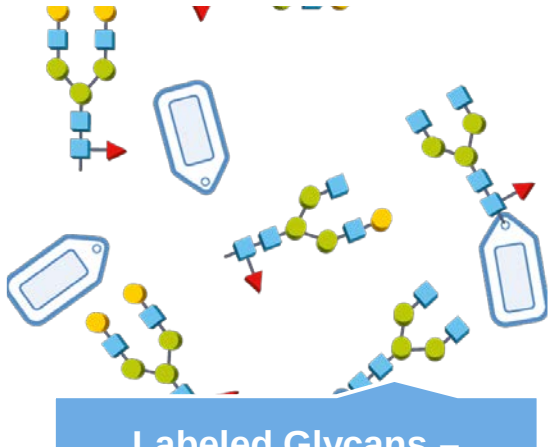
Summary – Glycan Workflows



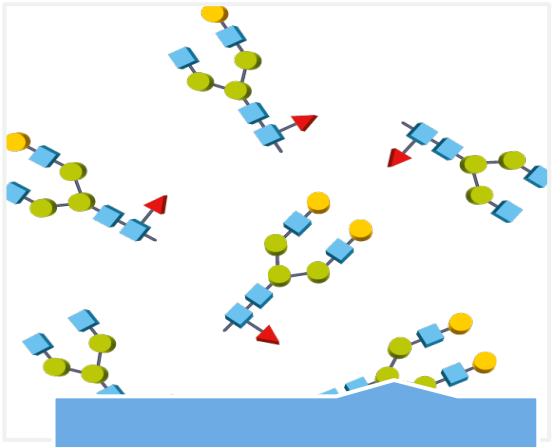
Monosaccharides & Sialic Acids



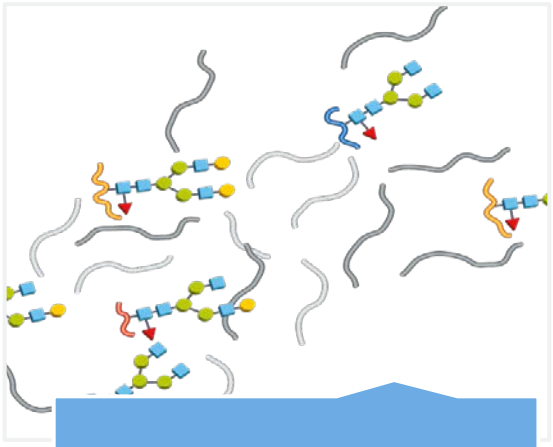
Labeled Glycans



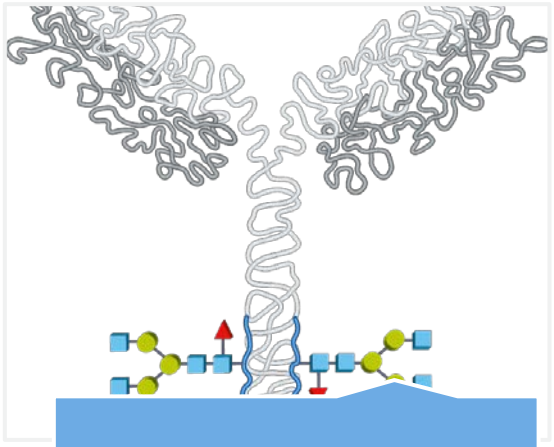
Labeled Glycans – High throughput



Glycans



Glycopeptides



Intact Glycoprotein

Thank You for Your Kind Attention

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