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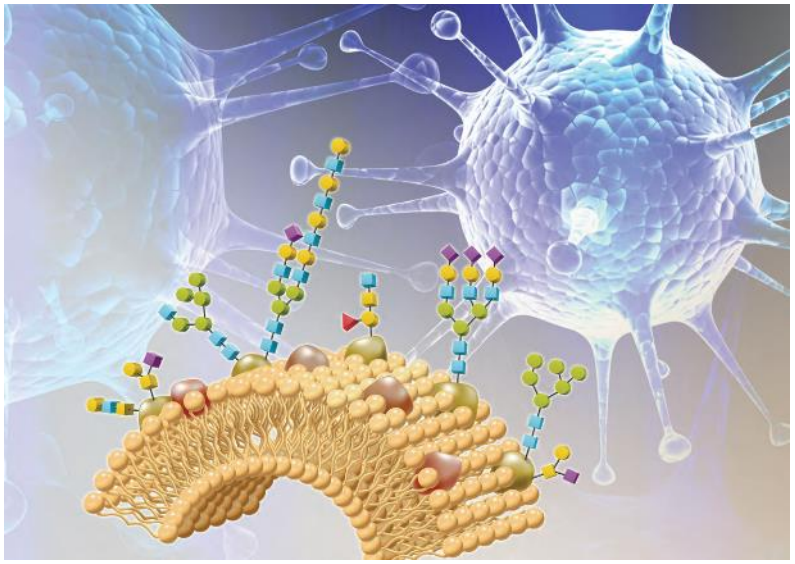
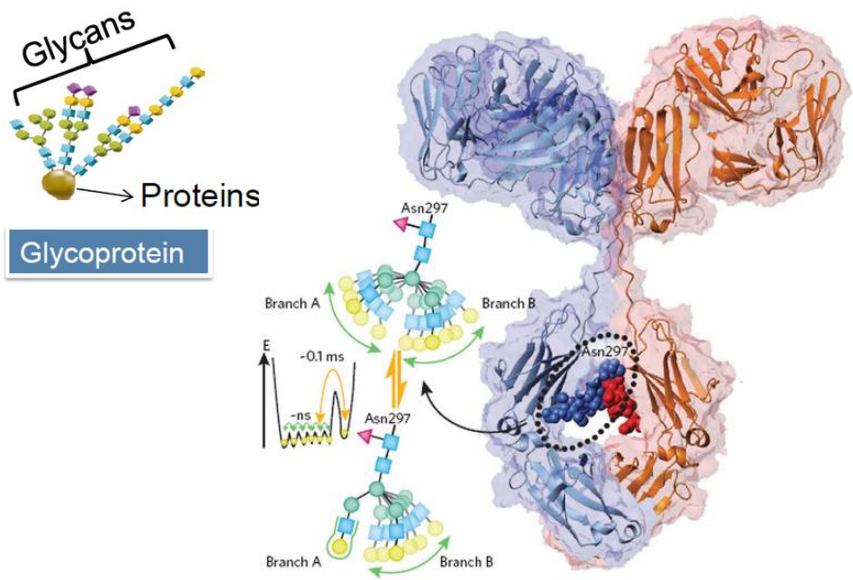
Six analytical strategies for studying glycosylation of biopharmaceuticals

Global Pharma Tour 2016

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 PTM (post translational modification) studied in biopharmaceuticals





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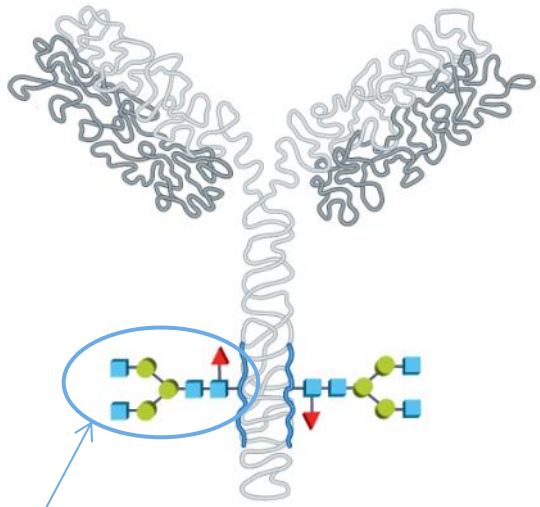
Characterization and Confirmation of Biological Products

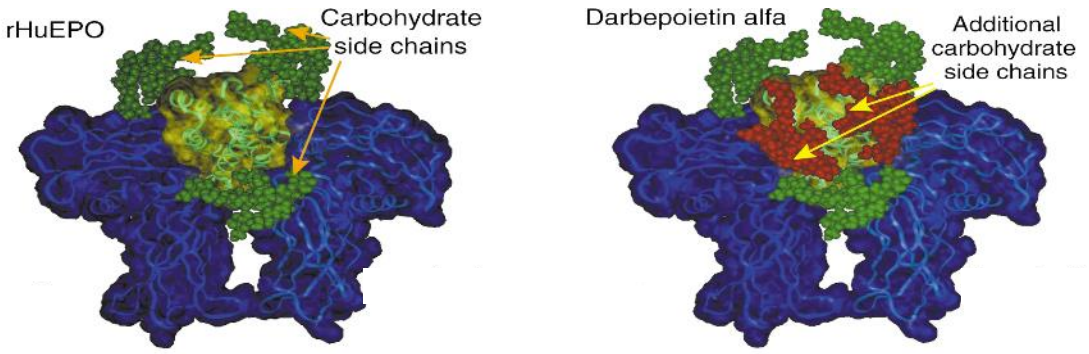
ICH (Q6B) recommended 6 test approaches for characterization and confirmation of biological products:

- 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."*



Glyco-engineering to improve biopharmaceuticals

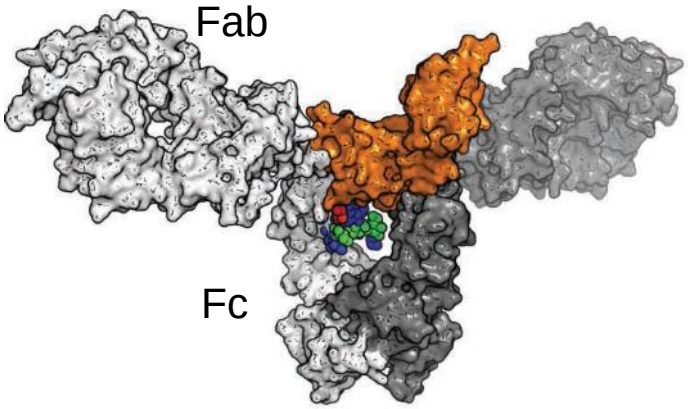
EPO:

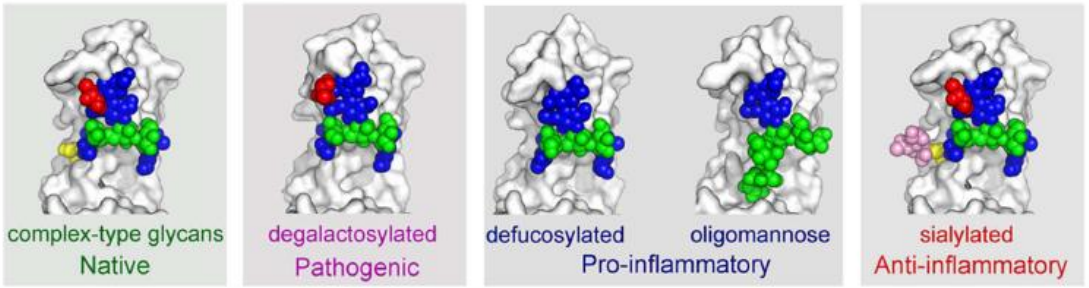


$T_{1/2}$ = 19h

$T_{1/2}$ = 32h

Therapeutic antibodies: Fc glycans determine function





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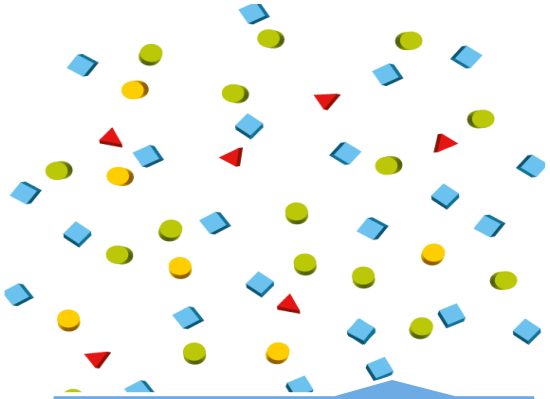
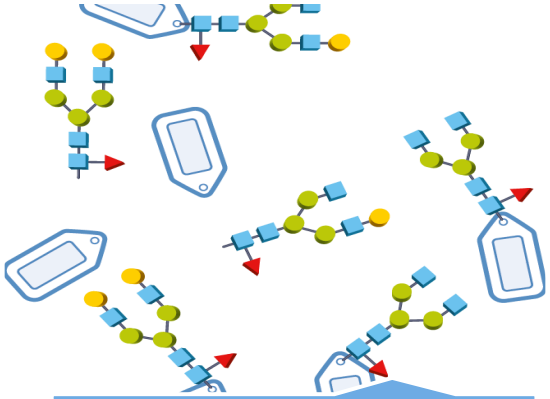
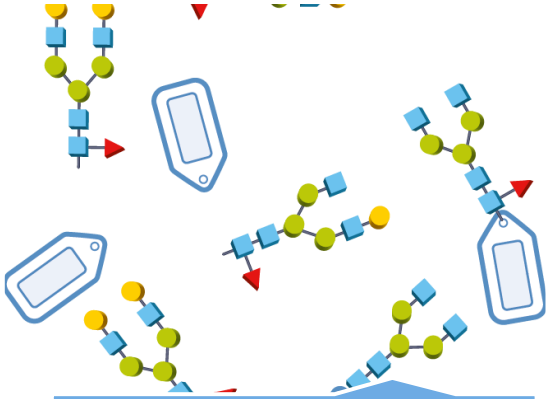
Glycan workflows



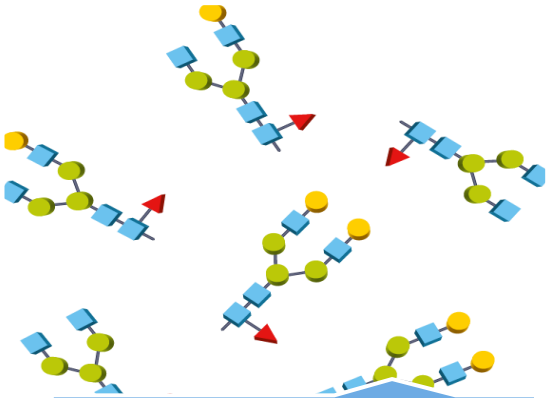
Monosaccharides & Sialic Acids



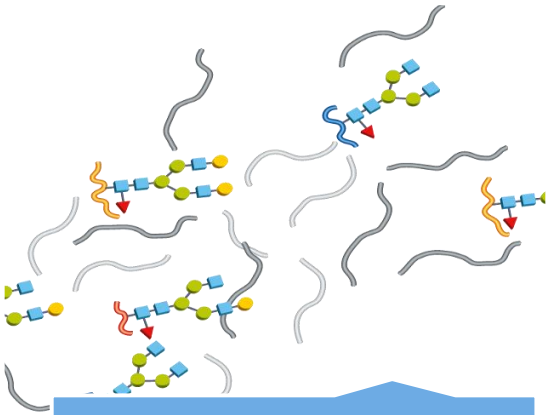
Labeled Glycans



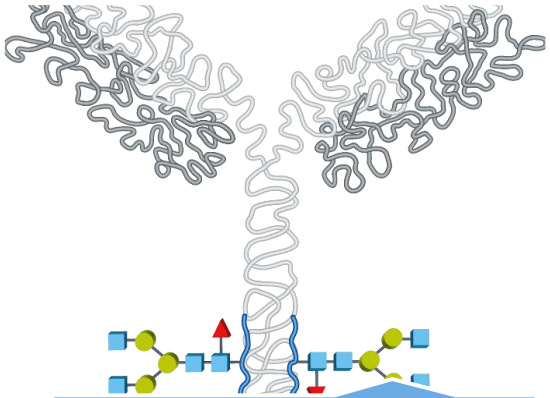
Labeled Glycans – High throughput



Unlabeled Glycans

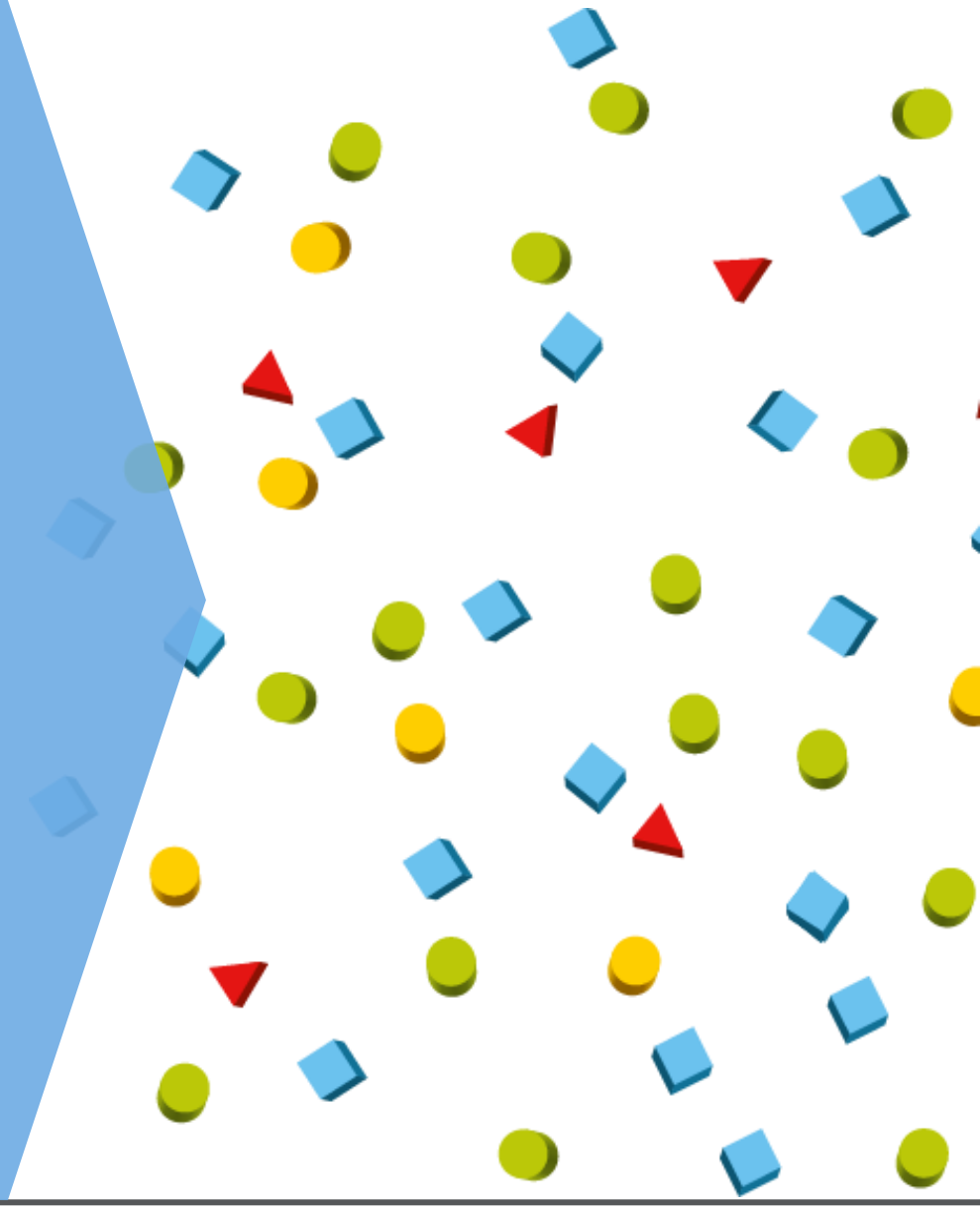


Glycopeptides

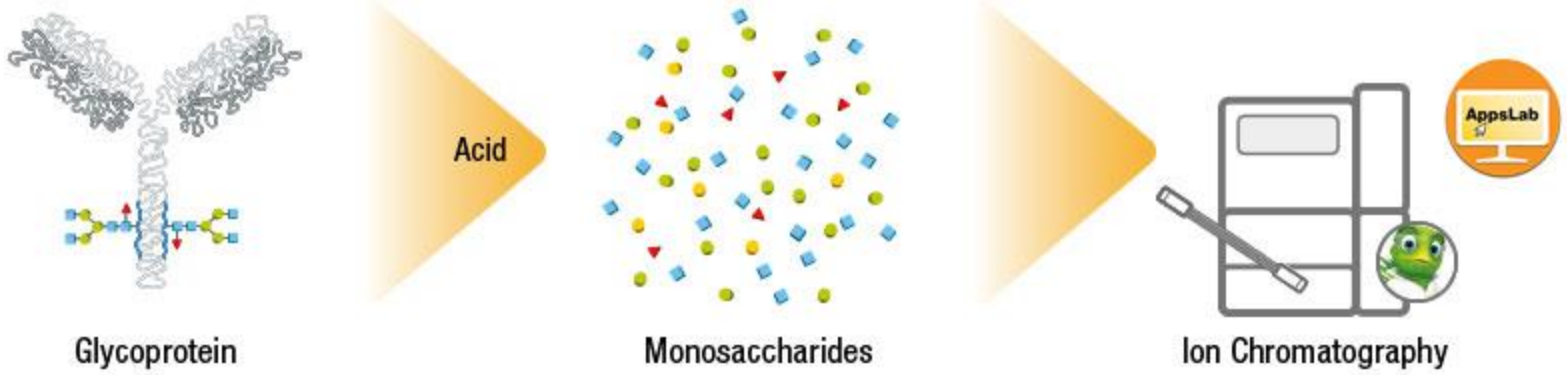


Intact Glycoprotein

Monosaccharides & Sialic Acids

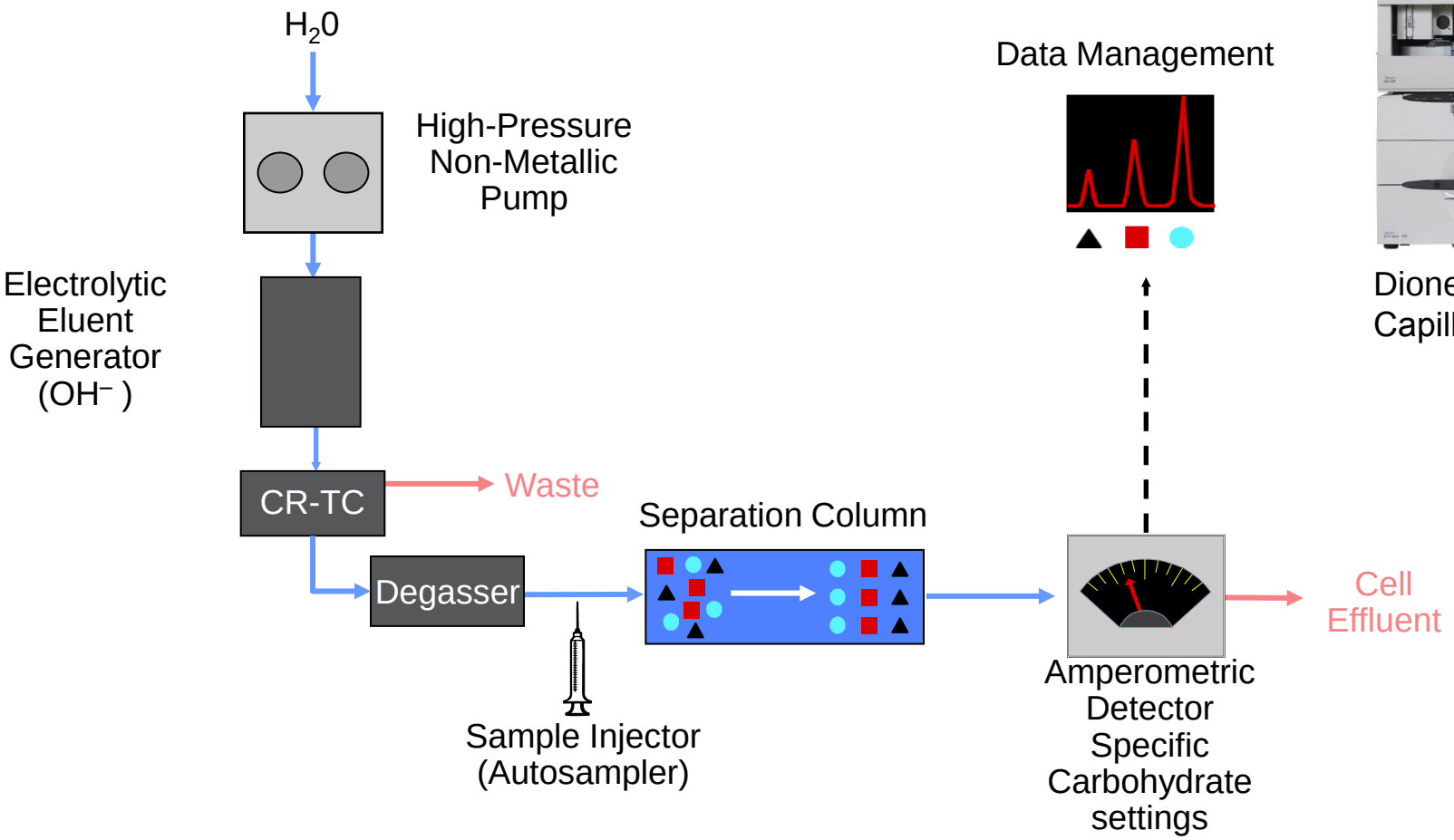


Monosaccharide workflow



- Monosaccharide composition can screen for changes in glycosylation
- Allows measurement of **total sugars** and amounts of specific **monosaccharides** & **sialic acids**
- Workflow using **HPAE-PAD** (ion chromatography) - Specific Carbohydrate Chromatography and Detection

HPAE-PAD Glycoprotein Monosaccharide System



Dionex™ ICS-5000+ Capillary HPIC™

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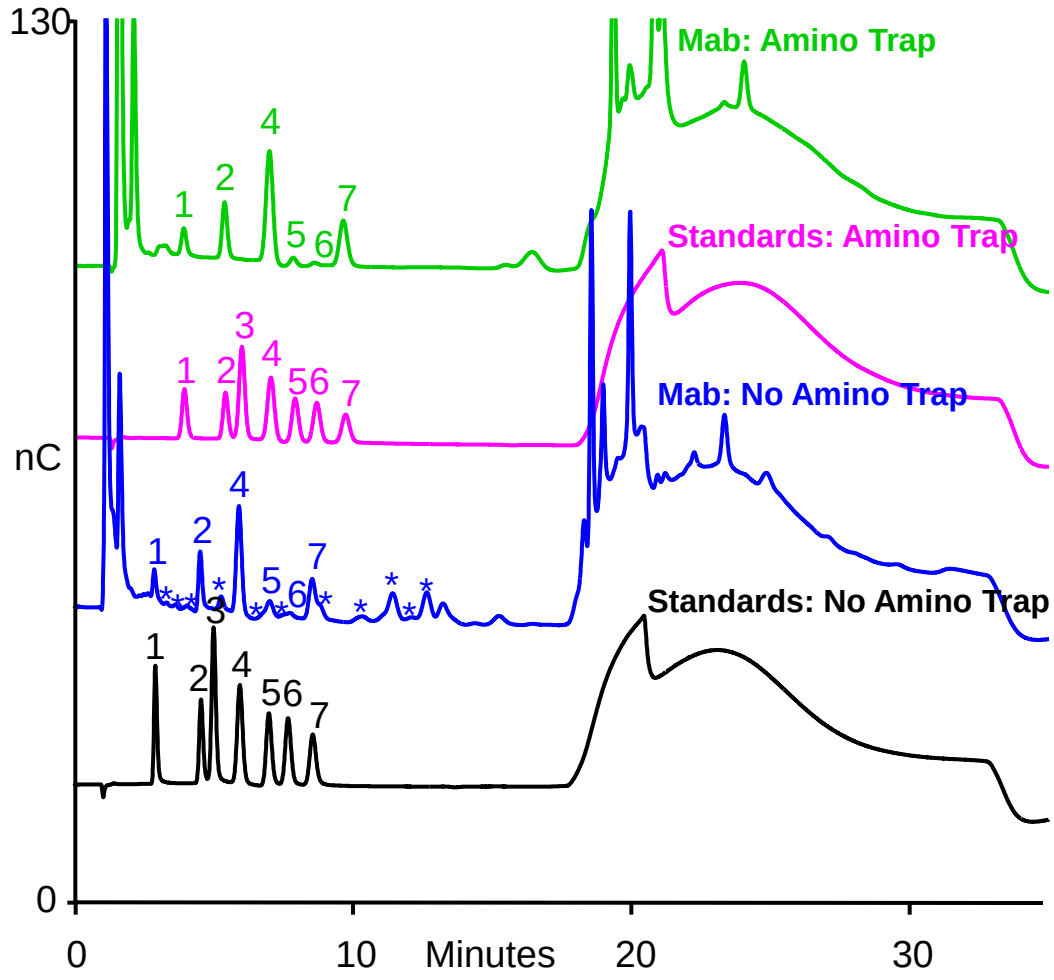
Monoclonal Antibody Hydrolysate with and without Amino Trap



Column: Thermo Scientific™ CarboPac® PA 20 (0.4 × 150 mm)

Temperature: 30 ° C

Eluent: 12 mM KOH (15 min) / 100 mM KOH (15 min) / 12 mM KOH (20 min) (EG)

Flow Rate: 9 µl/min

Inj. Volume: 0.40 µL

Det. Method: PAD (carbohydrate quadruple waveform)

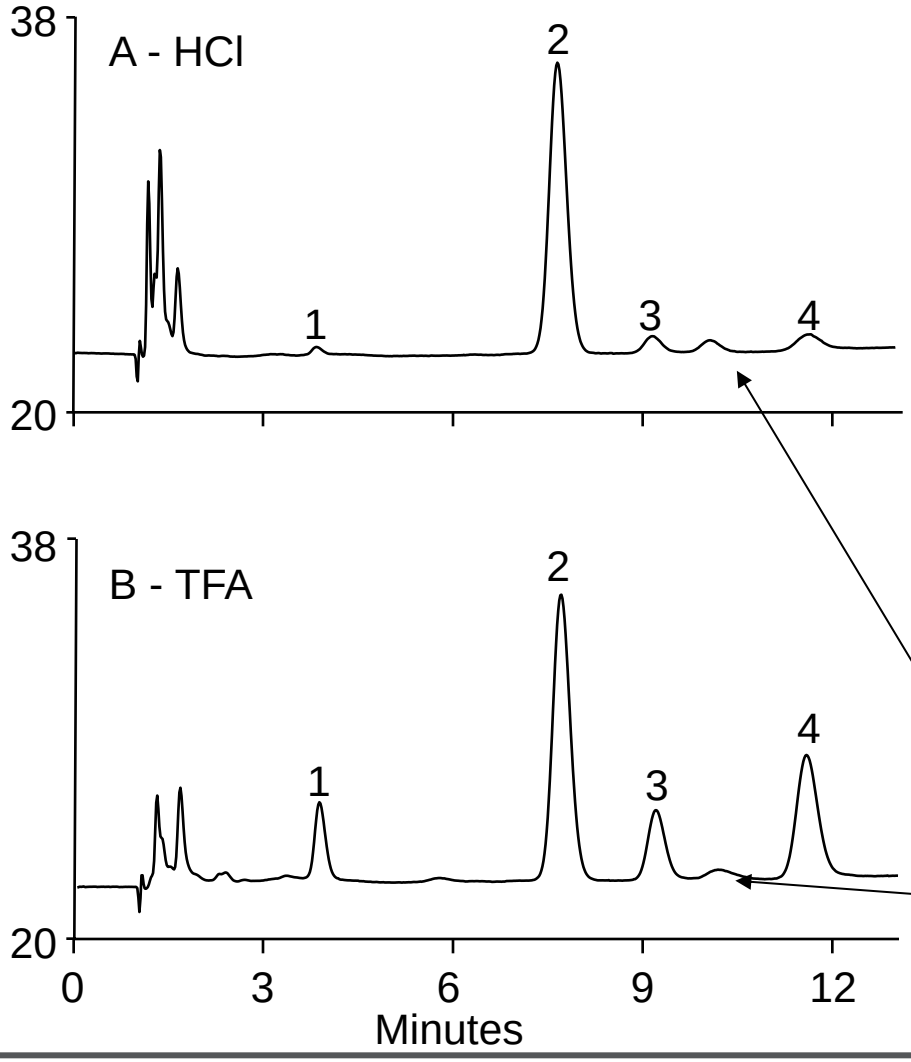
Electrode: Au

Sample: Standards (10 µM)

Peaks:
1. Fucose (Fuc)
2. DeoxygLucose (dGlc, internal standard)
3. Galactosamine (GalN)
4. Glucosamine (GlcN)
5. Galactose (Gal)
6. Glucose (Glc)
7. Mannose (Man)

* Amino acids

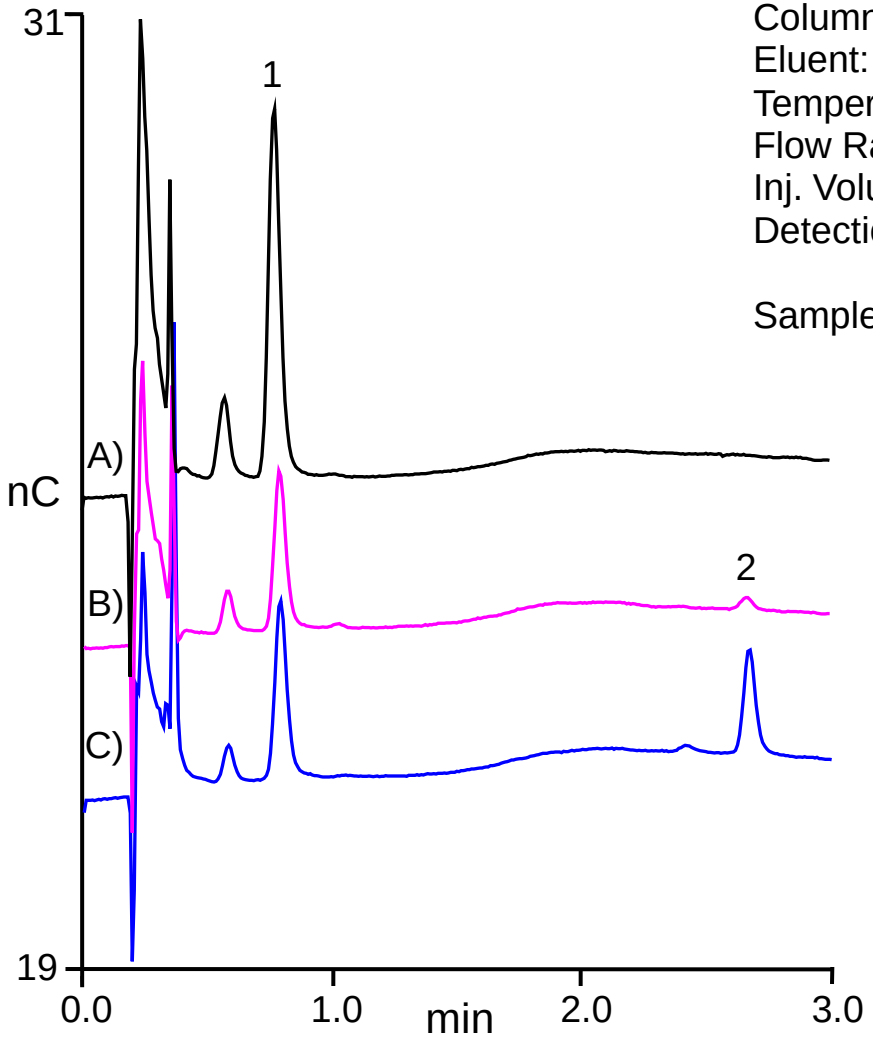
Monosaccharide Compositional Analysis of hIgG



Column:	Thermo Scientific™ CarboPac™ PA20 + Thermo Scientific™ AminoTrap™
Eluent:	10 mM KOH
Flow Source:	EG50 + CR-ATC
Flow Rate:	0.5 mL/min
Inj. Volume:	10 µL (**2 µg**)
Detection:	PAD (Au) Disposable Waveform A (TN21)
Temperature:	30 ° C
Sample:	A) 6N HCl hydrolyzate B) 4N TFA hydrolyzate
Peaks:	1. Fucose 2. Glucosamine 3. Galactose 4. Mannose

Glucose impurity

Separation of Sialic Acids



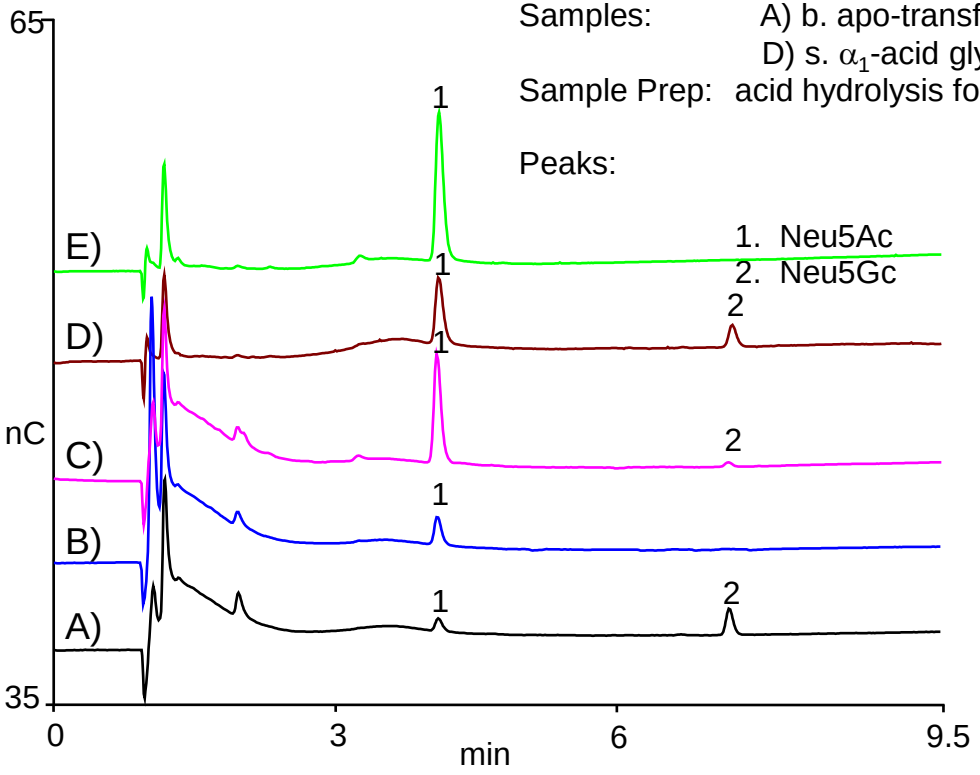
Column: CarboPac™ PA20 Fast Sialic Acid, 3 x 30 mm
Eluent: 70-300 mM acetate in 100 mM NaOH
Temperature: 30 ° C
Flow Rate: 0.5 mL/min
Inj. Volume: 4.5 µL (full loop)
Detection: PAD

Samples: A) human α_1-acid glycoprotein, 23 ng protein
B) fetuin, 18 ng protein
C) s. α_1-acid glycoprotein, 7.9 ng protein

Peaks:		A)	B)	C)
1.	Neu5Ac	13	5.6	6.1 pmol
2.	Neu5Gc	----	0.20	1.2

Separation of Glycoprotein Acid Hydrolyzates

Column:	CarboPac™ PA20 guard, 3 x 30 mm CarboPac PA20, 3 x 150 mm
Eluent:	70-300 mM acetate in 100 mM NaOH from 0-7.5 min, 300 mM acetate in 100 mM
NaOH from	7.5-9.0 min, 300-70 mM acetate from 9.0-9.5 min. 7 min of equilibration at 70 mM
acetate in 100	mM NaOH
Temperature:	30 ° C
Flow Rate:	0.5 mL/min
Inj. Volume:	10 µL
Detection:	PAD, Au (Disposable)
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	0.39	2.6	ND

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

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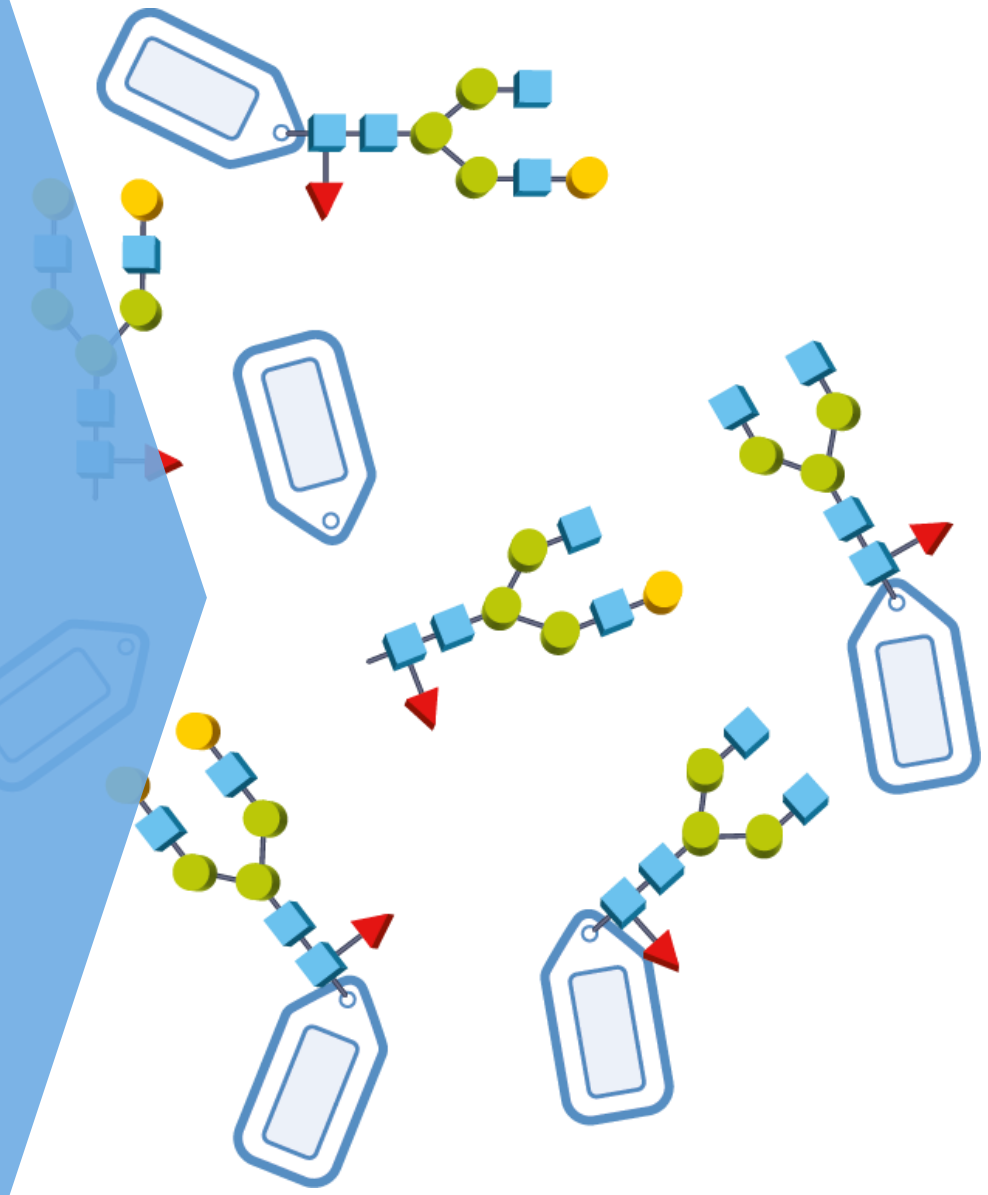
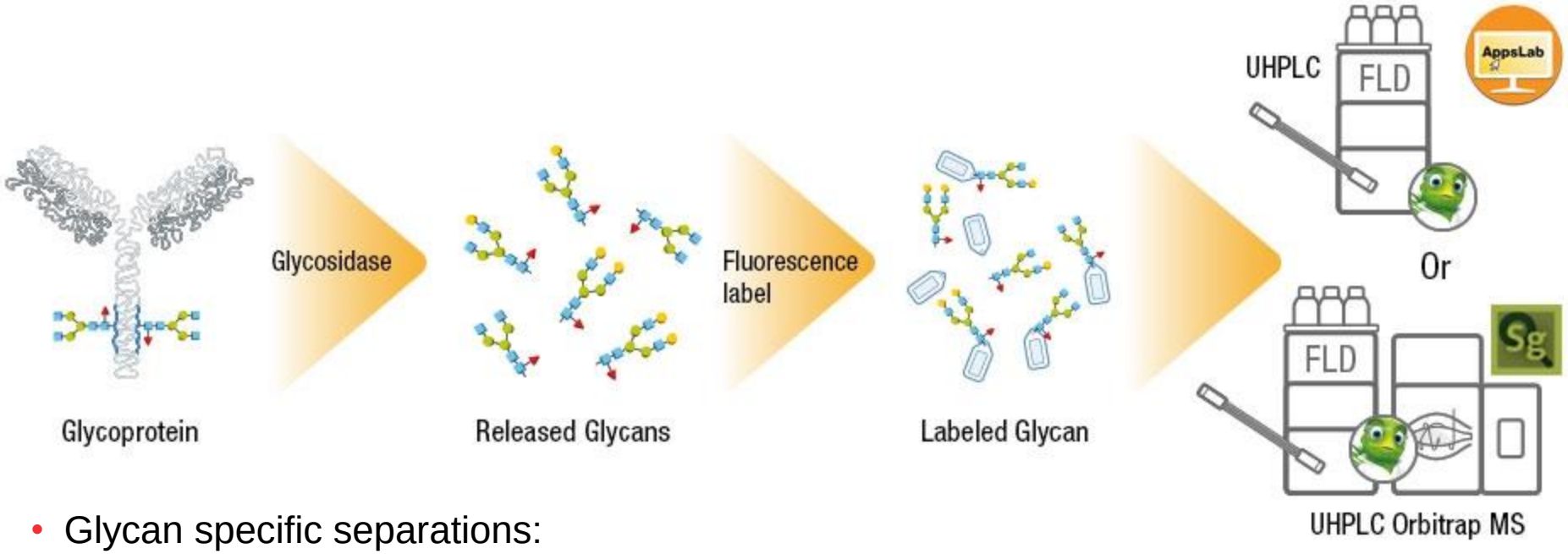
Labeled glycans



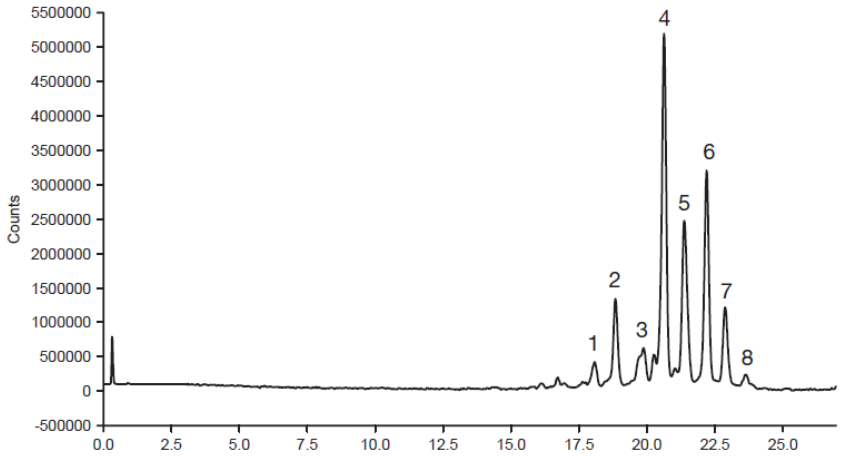
Labeled glycans – quantification and qualification



- Glycan specific separations:
  - Thermo Scientific™ GlycanPac™ AXH-1
  - Thermo Scientific™ GlycanPac™ AXR-1
  - Thermo Scientific™ Accuore™ 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 PREMIER Biosoft SimGlycan® software

2AB bovine fetuin glycans on Accuore150-Amide-HILIC

Traditional HILIC chromatography of Released Glycans



Peak Number	Glycan
1, 2	A3G2S2, A3G3S1, A3G3S2
3	A3G3S2, A3G2S3
4	A3G3S3, A3G3S4
5, 6	A3G3S3, A3G2S4
7	A3G3S3, A3G3S4
8	A3G3S3, A3G3S4

Separation Conditions				Part Number
Instrumentation:	Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC HPLC system equipped with a Thermo Scientific Dionex FLD fluorescence detector			
Column:	Accucore 150-Amide-HILIC, 2.6 µm, 100 × 2.1 mm			16726-102130
Mobile phase A:	Acetonitrile			
Mobile phase B:	50 mM ammonium formate, pH 4.4 (prepared from LS-N-BUFFX40, Ludger Ltd)			
Gradient:	Time (min)	% B	Flow rate (mL/min)	
	0	20	1.0	
	26	40	1.0	
	27	50	1.0	
Column temperature:	60 °C			
Backpressure:	300 bar			
Injection details:	5 µL in water, 50 µL loop			
Injection wash solvent:	Acetonitrile / water (78:22 v/v)			
Excitation wavelength:	330 nm			
Emission wavelength:	420 nm			

Accuore-150-Amide-HILIC – 2.6µm superficially porous silica particles modified with polyamide

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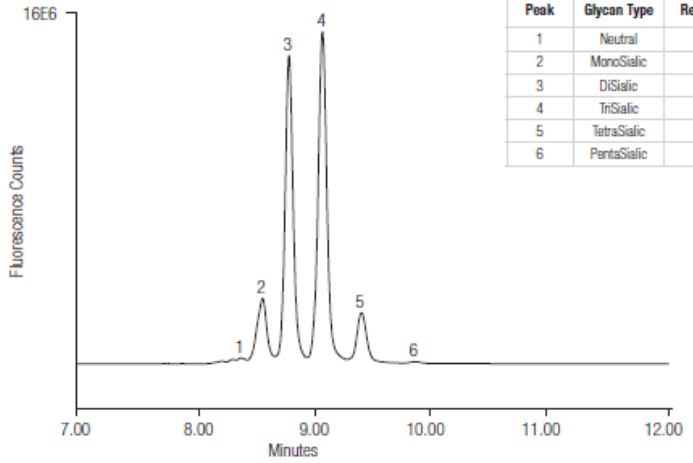
Charge-based / HILIC separation **GlycanPac AXH-1**

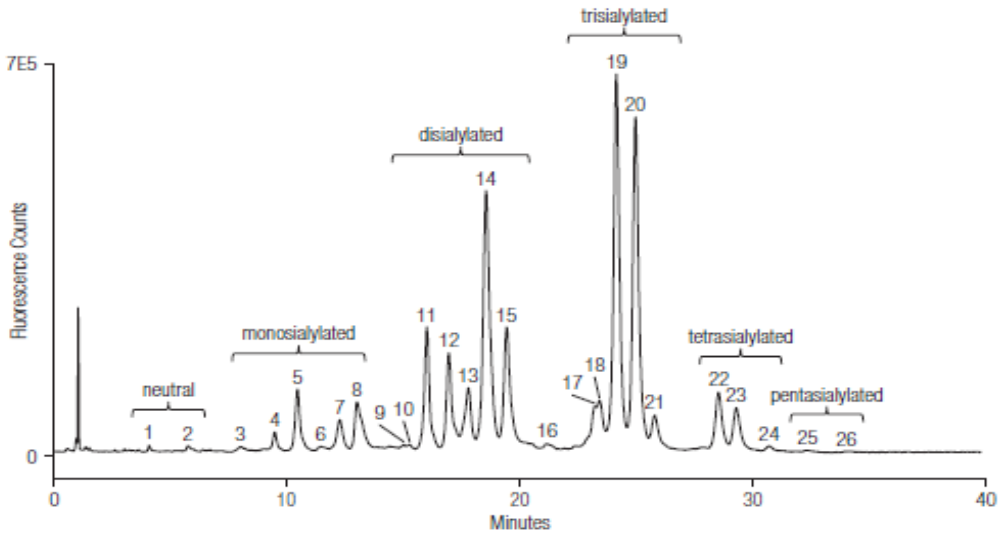


Peak	Glycan Type	Relative %
1	Neutral	0.4
2	MonoSialic	8.6
3	DiSialic	38.4
4	TriSialic	45.4
5	TetraSialic	7.0
6	PentaSialic	0.2

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



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

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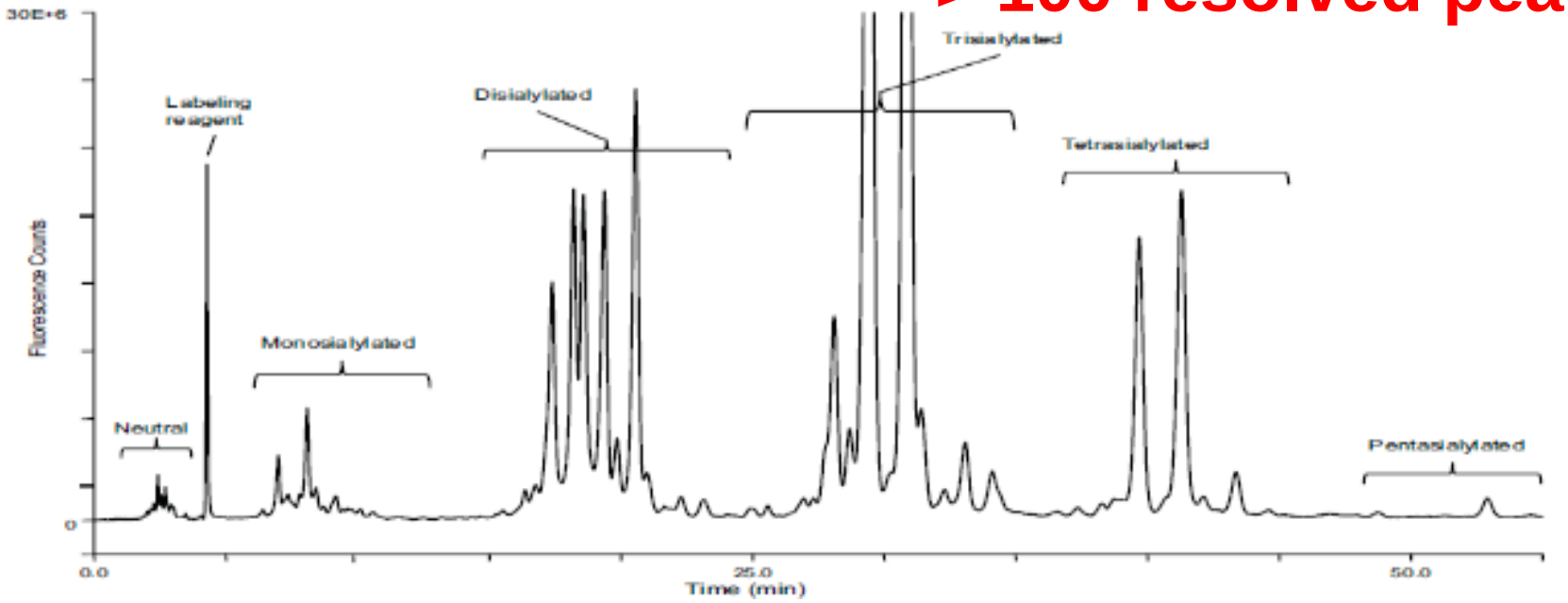
RP / Charged based Separation - **GlycanPac AXR-1**

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

> 100 resolved peaks



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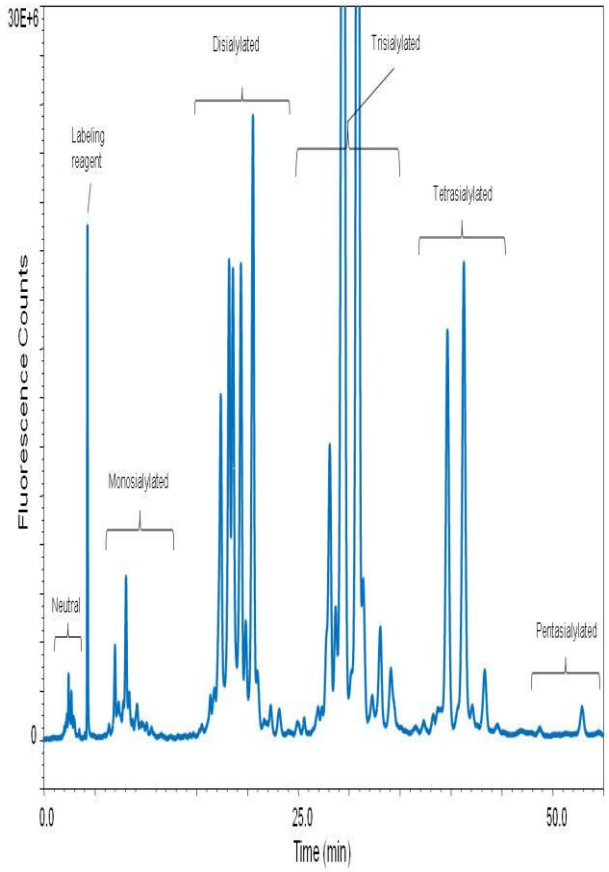
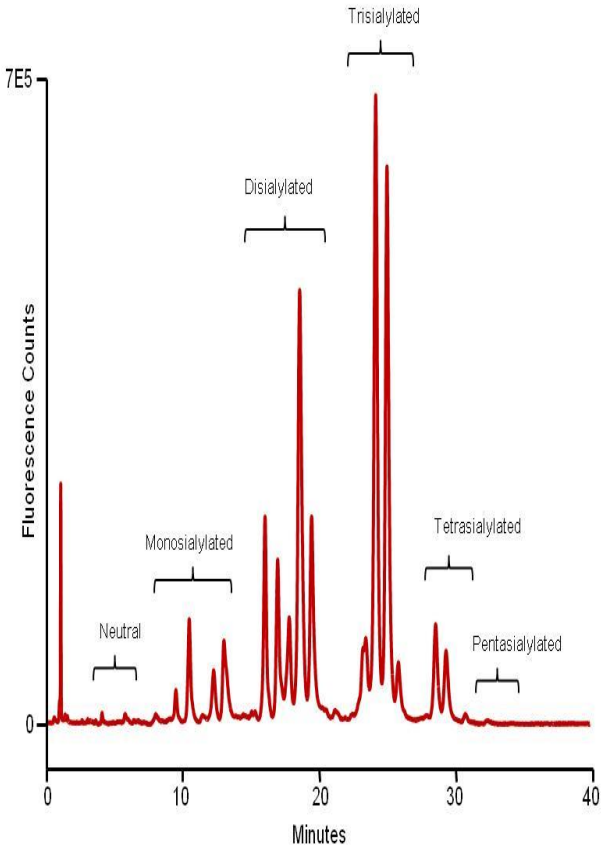
GlycanPac columns and Amide HILIC column

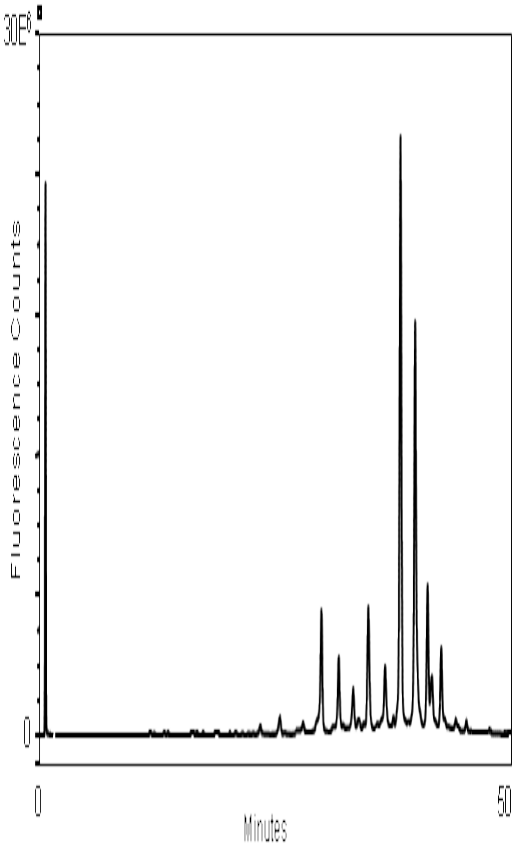
GlycanPac AXR-1 (1.9µ)
(>100 peaks resolved)



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



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



When to use each column?

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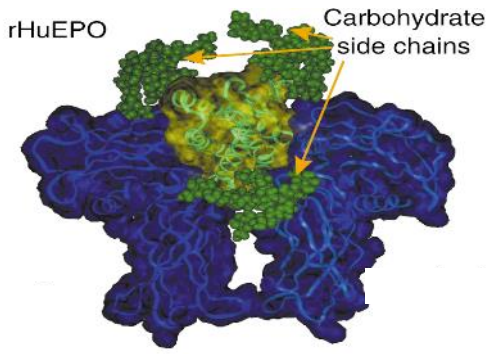
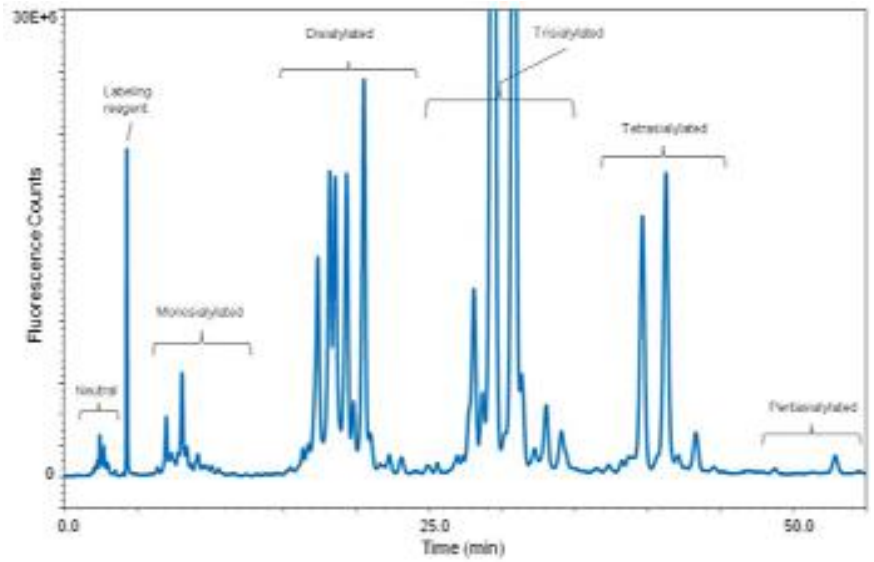
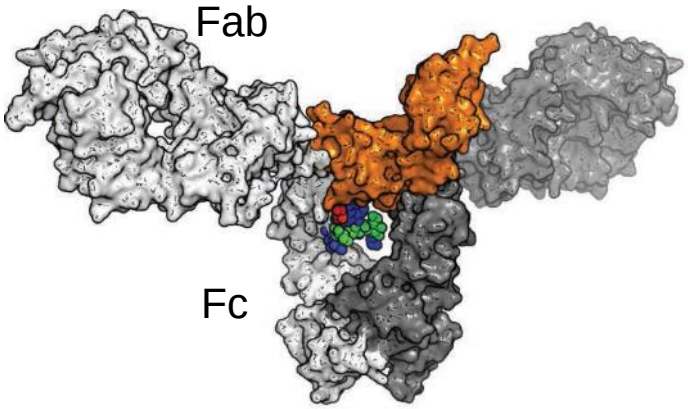
Glyco-biopharmaceuticals

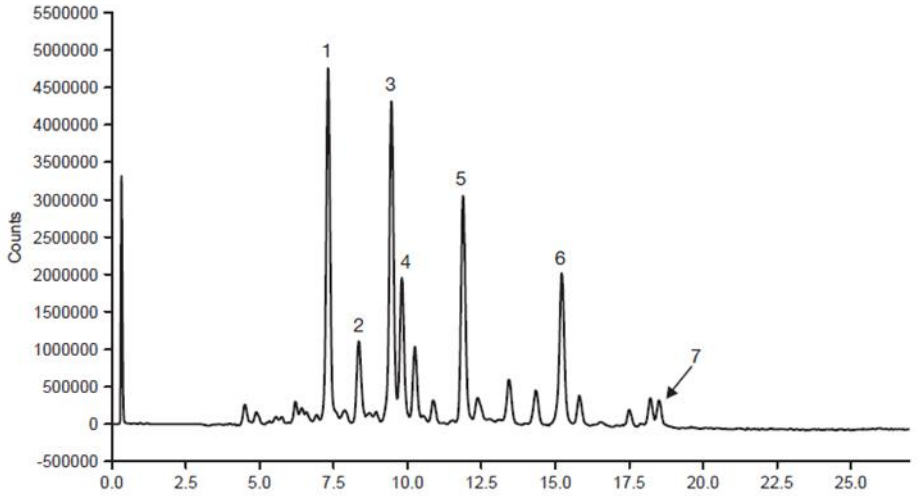
EPO:





Therapeutic antibodies





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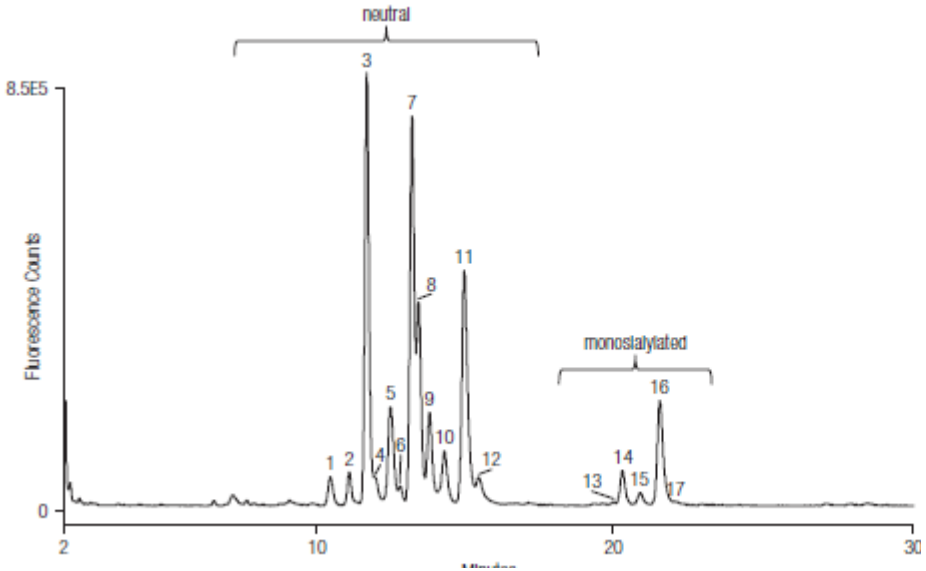
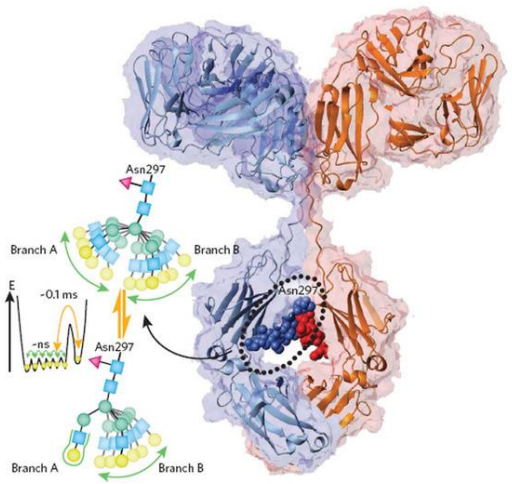
2AA labeled *N*-glycans from human IgG



Column:	**GlycanPac AXH-1 (1.9 µm)**
Dimension:	2.1 × 150 mm
Mobile Phase A:	Acetonitrile (80%) + water (20%)
Mobile Phase B:	Ammonium formate (80 mM, pH = 4.4)
Flow Rate:	0.4 mL/min
Injection Volume:	20 Pmoles
Temperature:	30 °C
Detection:	Fluorescence at 320/420 nm
Sample:	2AA labeled *N*-glycan from human IgG

Time (min)	% A	% B	Flow (mL/min)	Curve
-10	99	1.0	0.4	5
0	99	1.0	0.4	5
30	87.5	12.5	0.4	5



Peak	Structure	Charge of Glycan (without 2AA label)	Molecular Mass (including 2AA label)
1		0	1380.5178
2		0	1437.5393
3		0	1543.5872
4		0	1542.5735
5		0	1542.5735
		0	1705.6258
6		0	1588.5821
7		0	1745.6300
8		0	1745.6300
9	Unknown		Unknown

Peak	Structure	Charge of Glycan (without 2AA label)	Molecular Mass (including 2AA label)
10		0	1701.6449
11		0	1907.7028
12		0	2110.7822
13		-1	2036.7454
		-1	2052.7404
14		-1	2036.7454
15		-1	2052.7404
16		-1	2198.7983
17		-1	2401.8775

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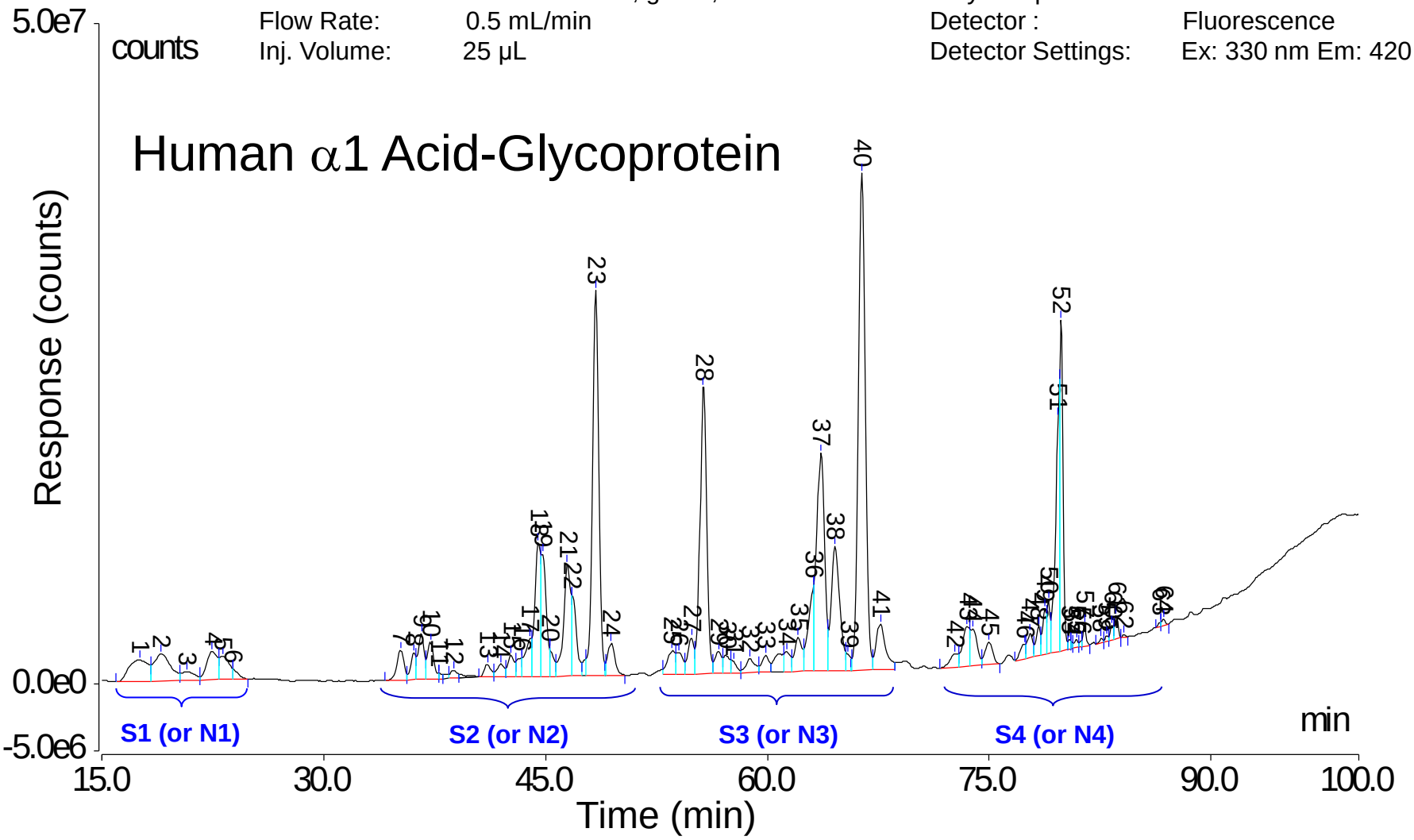
USP 212 Proposed Method: 2AB-Labeled glycans by IC-FLD

Columns:	CarboPac PA1, analytical, 4 × 250 mm	Column Temperature:	25 ° C
	CarboPac PA1, guard, 4 × 50 mm	Tray Temp:	4 ° C
Flow Rate:	0.5 mL/min	Detector :	Fluorescence
Inj. Volume:	25 µL	Detector Settings:	Ex: 330 nm Em: 420 nm



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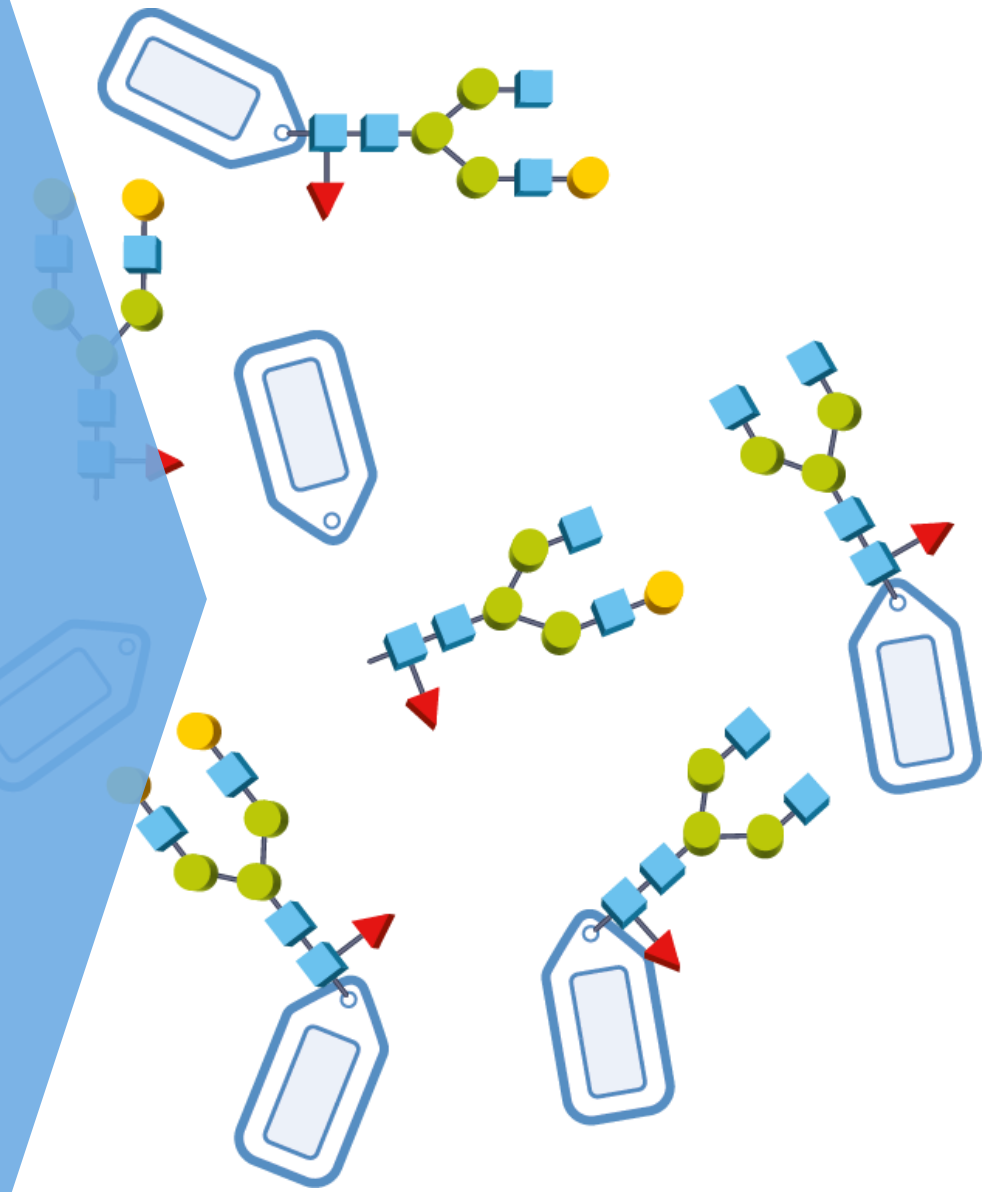
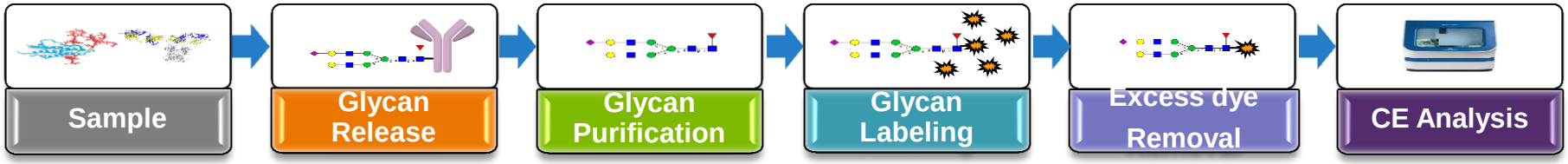
Upstream high-throughput Glycan screening

Labeled glycans

- High throughput
- Early discovery



Thermo Scientific™ GlycanAssure™ Workflow



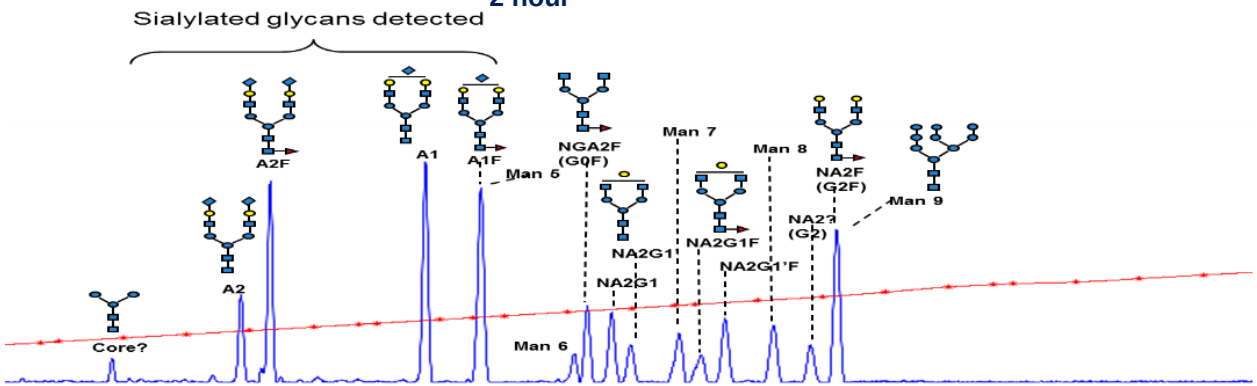
PNGase-F
1 hour

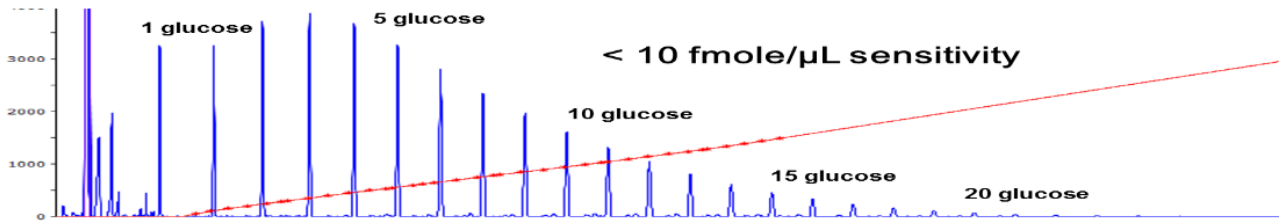
Magnetic Beads
30 min

APTS
Teal™
Turquoise™
2 hour

Magnetic Beads
30 min

24 Capillary
CE Analysis
3 hour





- High throughput
- Parallel analysis or 8 or 24 arrays

Hands on Time < 3hr

Time to Results: 7 - 9hrs* (96 samples)

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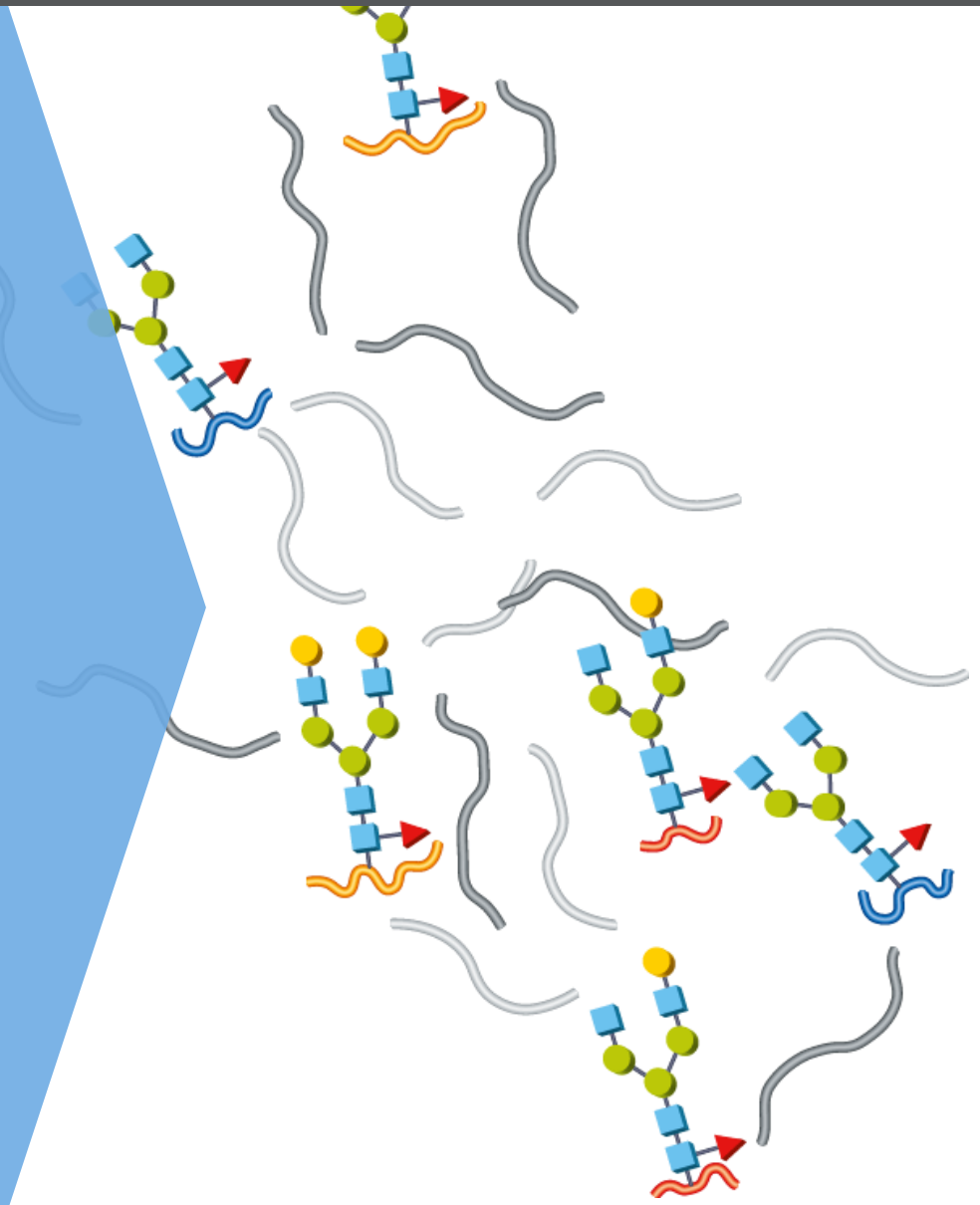
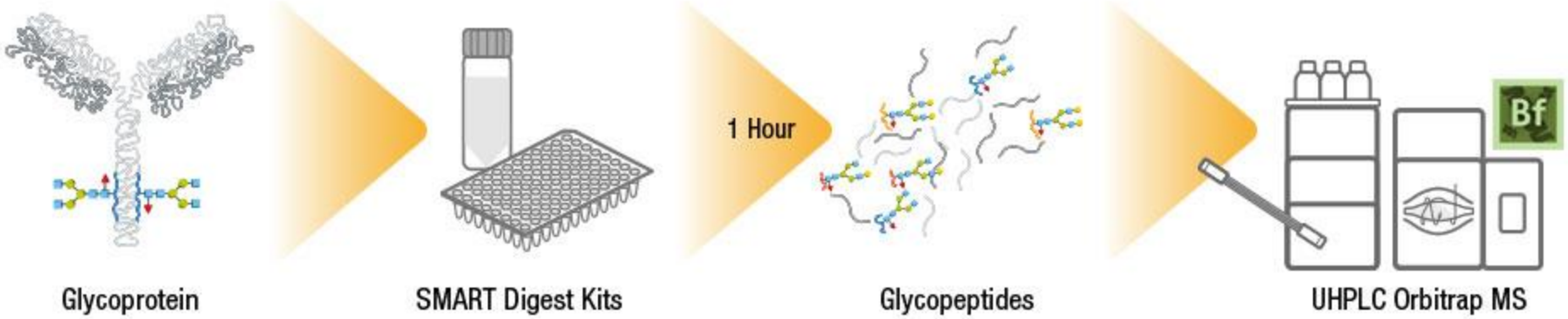
Glycopeptides



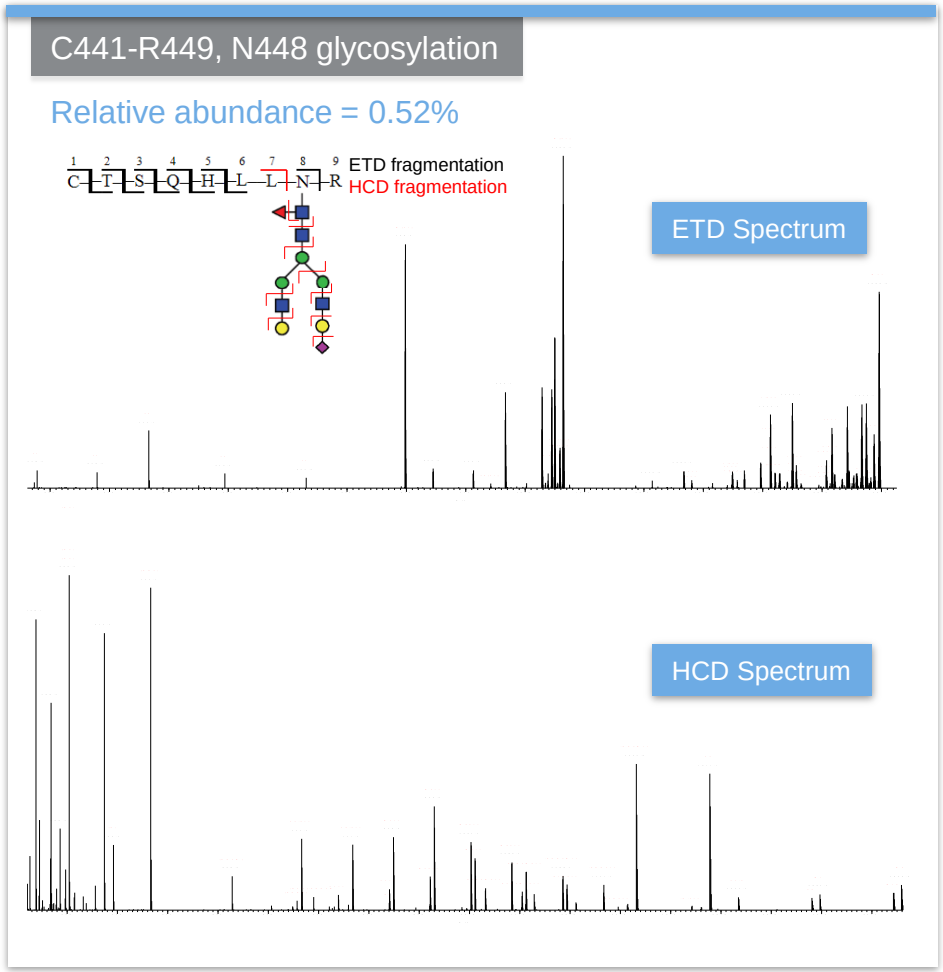
Glycopeptide workflow



- Important for site profiling of PTMs including glycosylation
  - A variety of fragmentation techniques can be used
  - **ETD, HCD** or CID
- **Robustly digest in 1 hour** 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

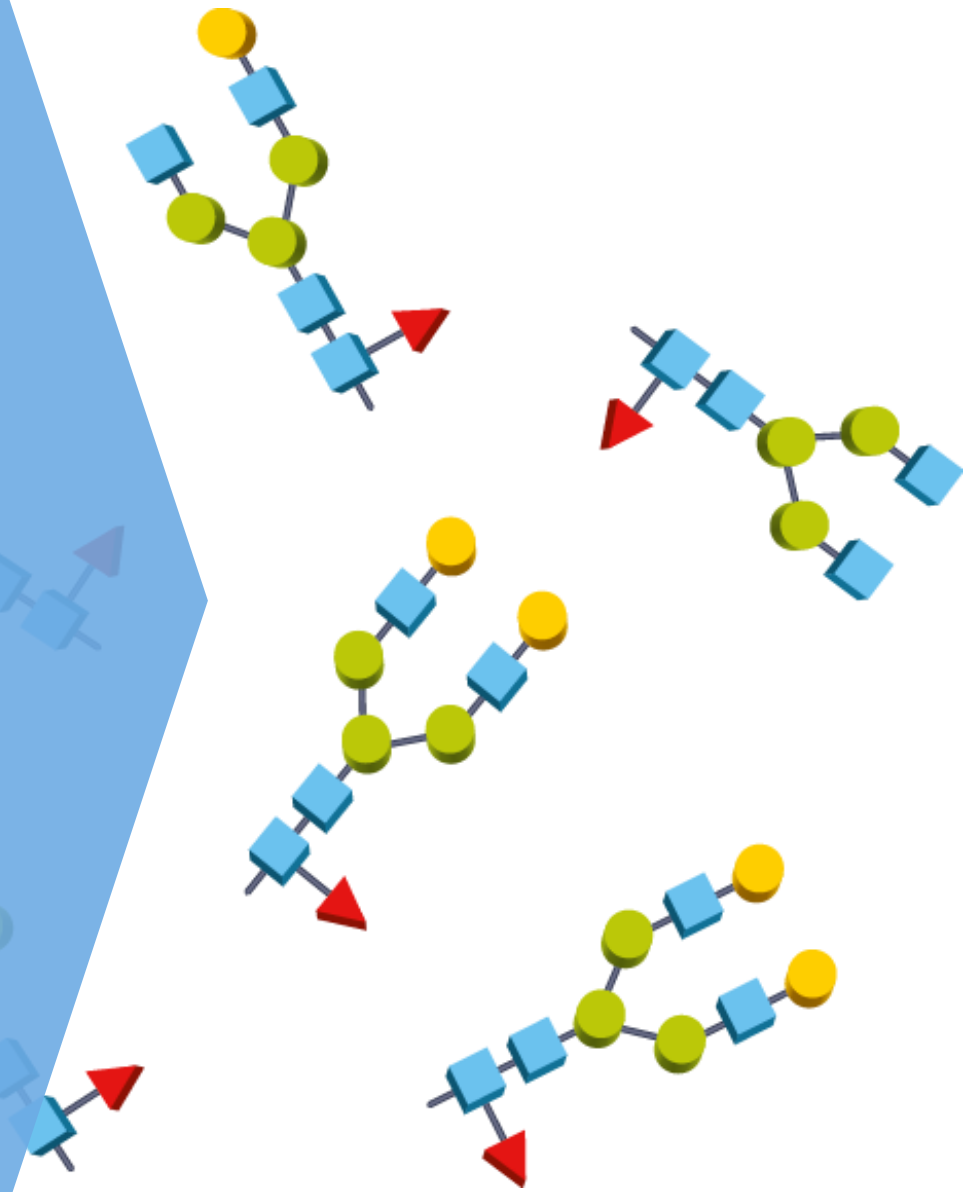
- Unique HCDpdETD method features on-the-fly identification of glycopeptides using diagnostic fragment ions from sugar fragmentation.
- A high quality HCD spectrum is generated for each peptide.
- An additional ETD spectrum is generated for each glycopeptide.
- For each glycopeptide, ETD provides information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



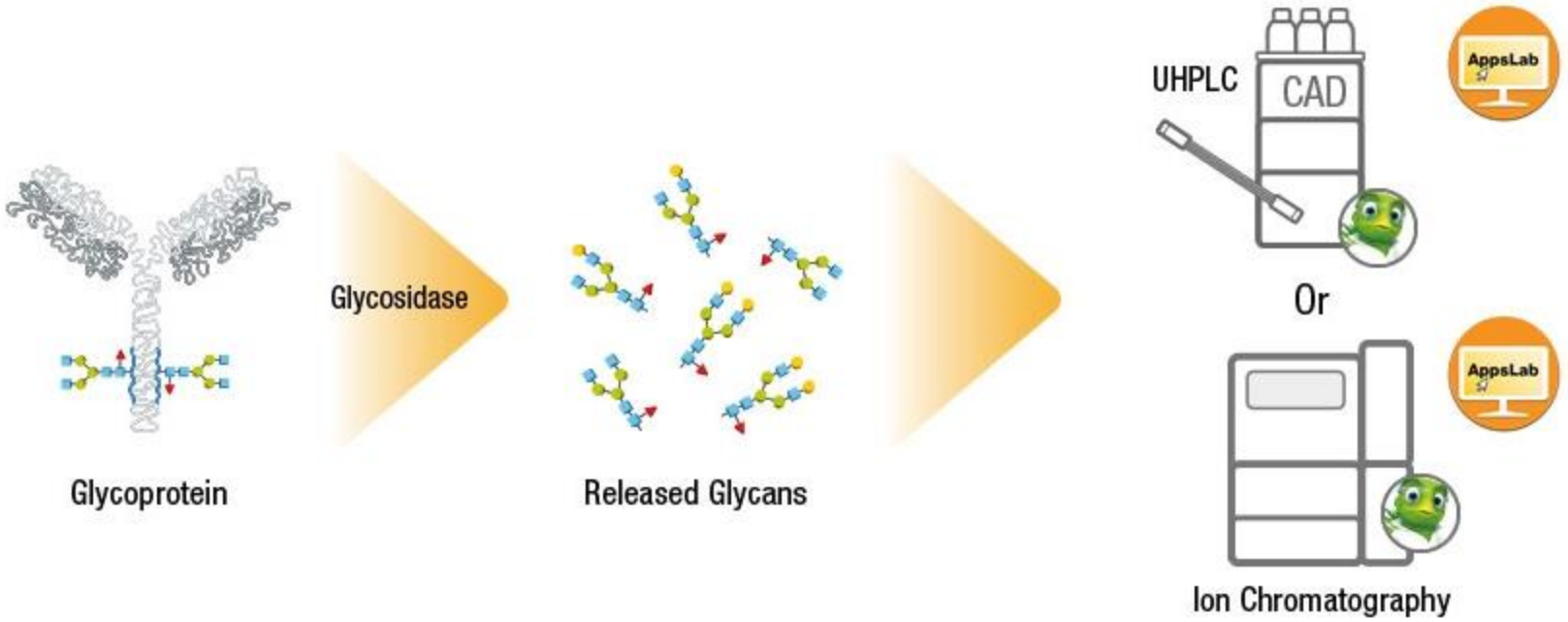
Zhiqi Hao et al 2014 ASMS TP264

Unlabeled glycans

- O-linked & N-linked



Charged Aerosol Detection for Unlabeled Glycans



- 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

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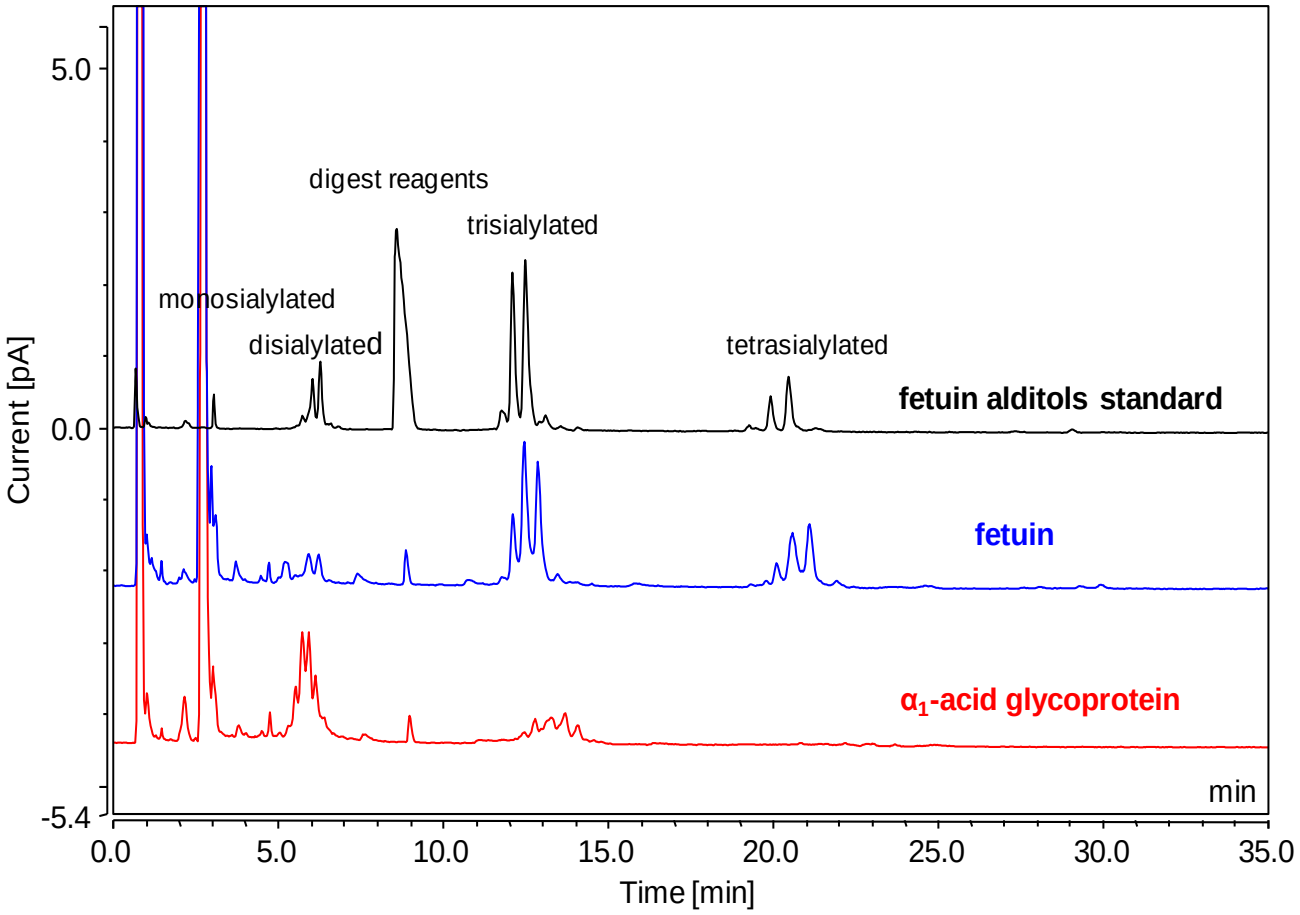
Label-free Analysis of N-linked Glycans by UHPLC-CAD



System:	Thermo Scientific™ Vanquish™ UHPLC
Column:	GlycanPac™ AXR-1 1.9 µm x 2.1x 150 mm
MPhase A:	Deionized water
MPhase B:	100 mM Ammonium formate, pH 4.4
Gradient:	4 % B to 39% B in 35 min
Flow Rate:	0.4 mL/min
Inj. Volume:	2 µL
Col. Temp:	30° C
Detector:	Vanquish Charged Aerosol Detector H
Settings:	50ºC, PF 1.0, 10 Hz, 5s

PNGase F Digest - No Labeling Required

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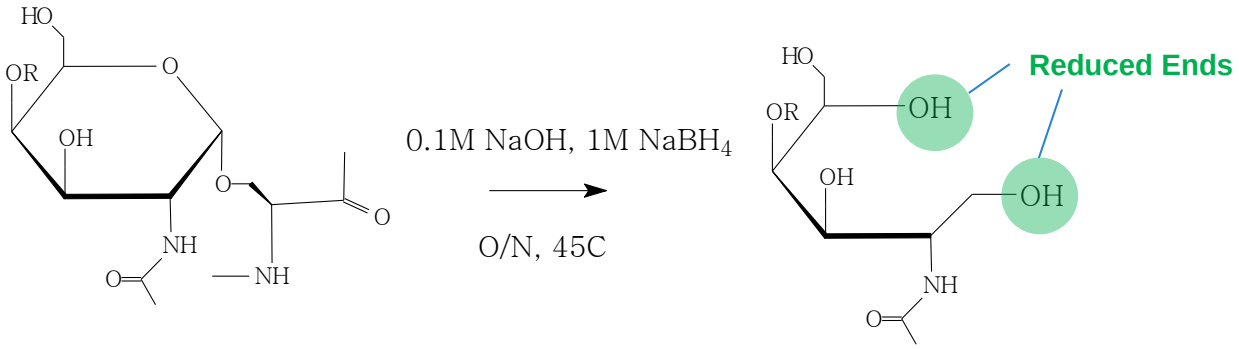
Label-free O-glycan Analysis by HPLC-CAD

- Problems with O-linked glycan analysis:

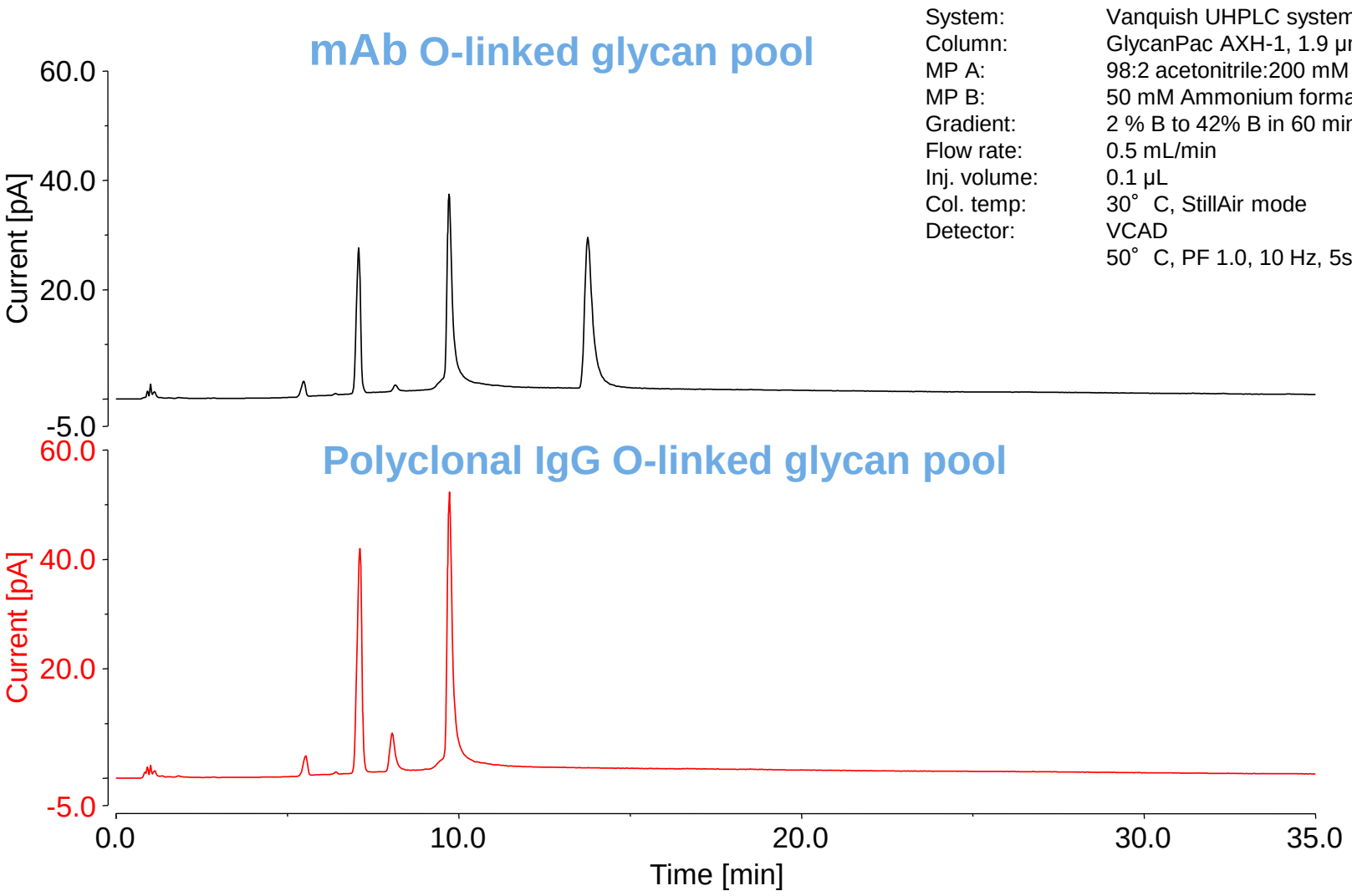
1. **Released glycans degrade by peeling reaction if not reduced**
2. **Reduced alditols cannot be labeled for enhanced detection**

Alditols produced by reductive β-elimination cannot be labeled:



Glycan Labeling is not required with UHPLC-CAD

Label-free Analysis of O-linked Glycans by HPLC-CAD



System:	Vanquish UHPLC system
Column:	GlycanPac AXH-1, 1.9 µm, 2.1 × 150 mm
MP A:	98:2 acetonitrile:200 mM NH4OFa
MP B:	50 mM Ammonium formate, pH 4.4
Gradient:	2 % B to 42% B in 60 min
Flow rate:	0.5 mL/min
Inj. volume:	0.1 µL
Col. temp:	30° C, StillAir mode
Detector:	VCAD
	50° C, PF 1.0, 10 Hz, 5s

Reductive Beta Elimination - No Labeling Required

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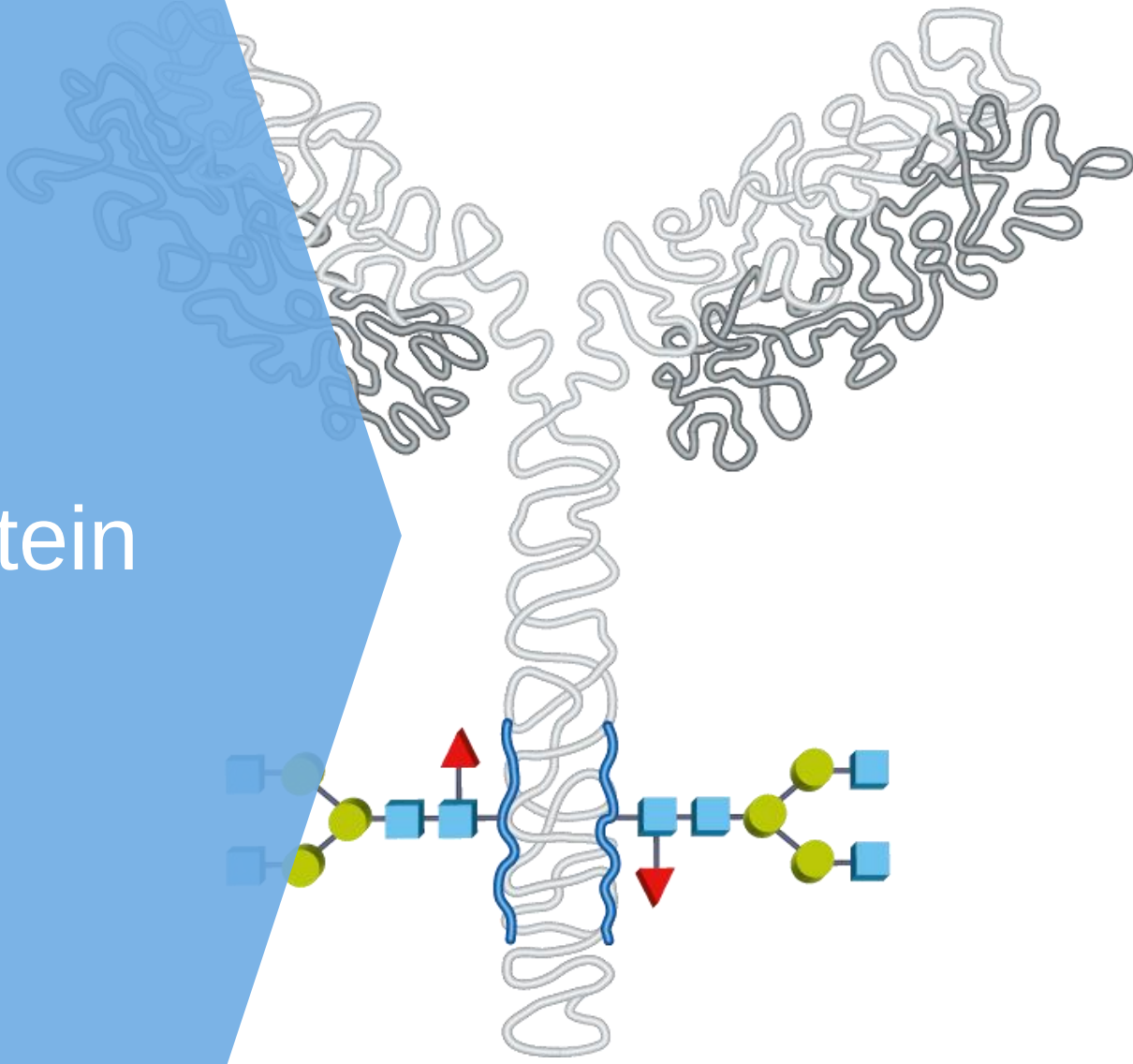
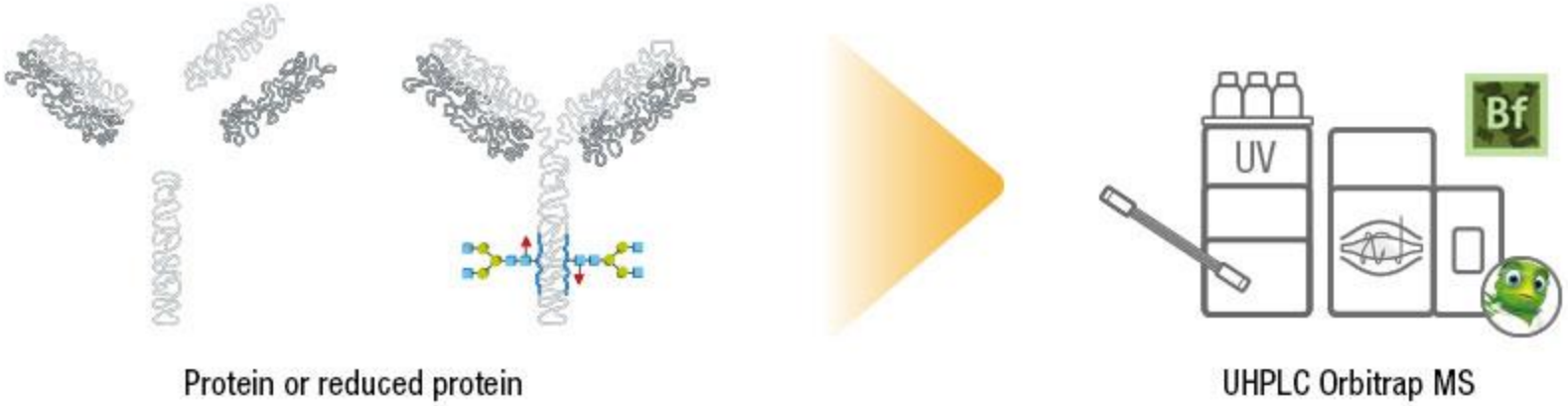
Intact Glycoprotein

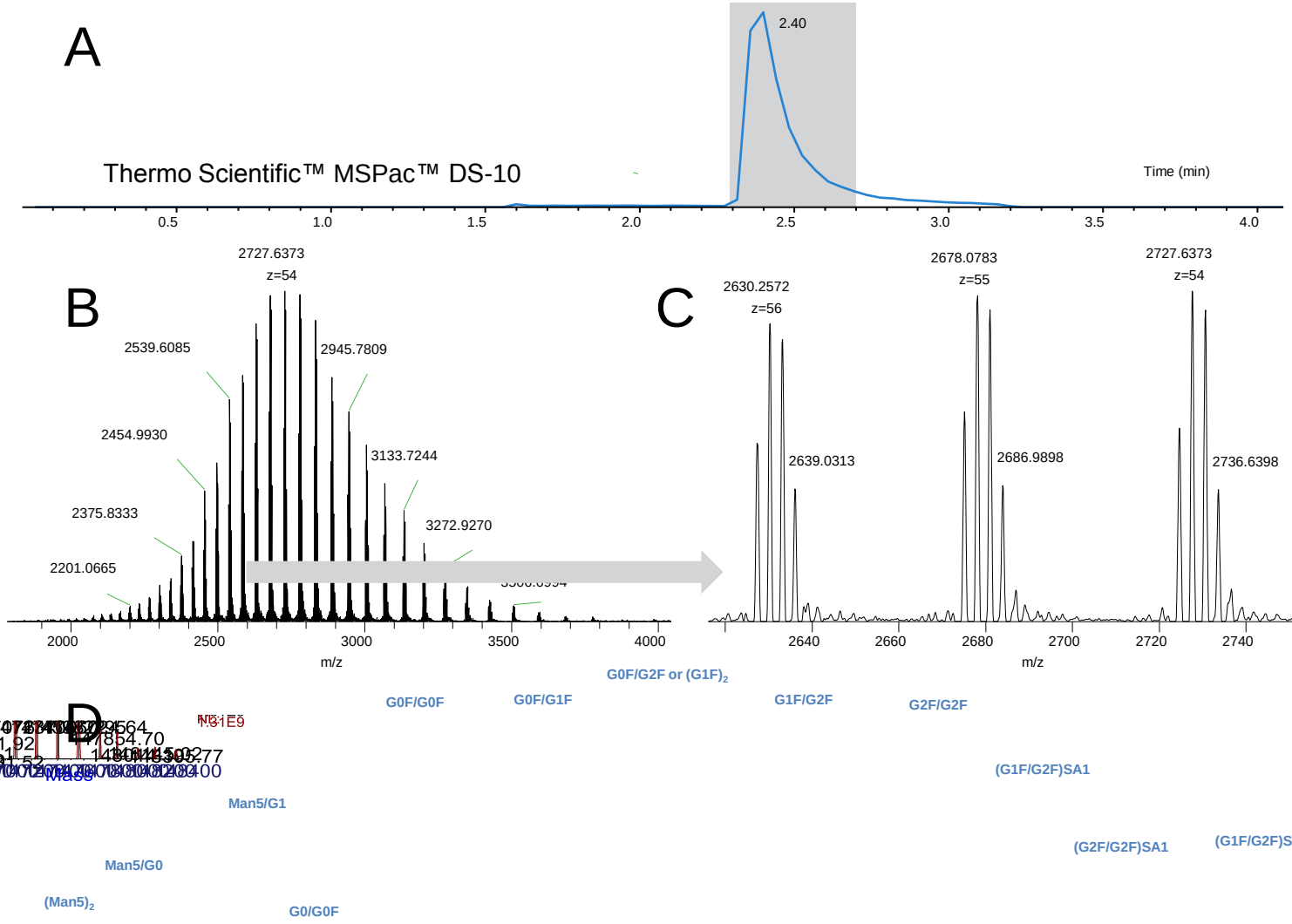


Intact glycoform workflow



- **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, attached glycans, charge etc, the highest resolution and most accurate mass MS is required for precise quantification.

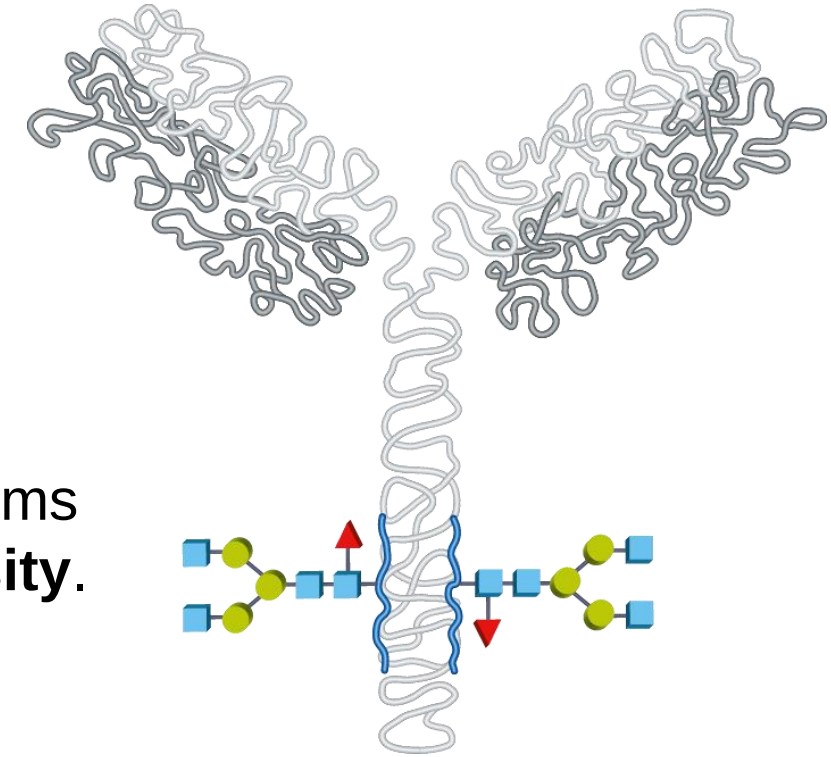
Glycan Analysis of Rituximab



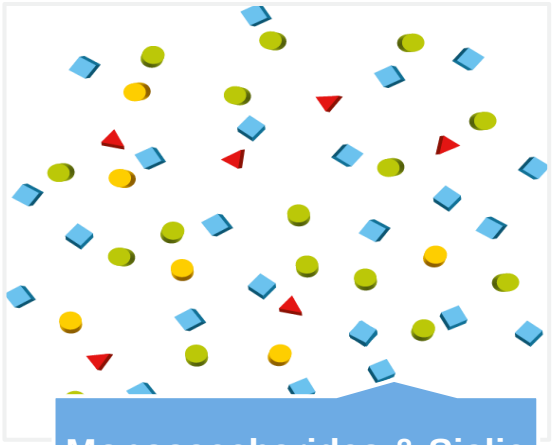
Download Application Note 21465: Fast online desalting of mAbs using a reversed phase desalting cartridge for LC-MS analysis

Intact glycoprotein characterization

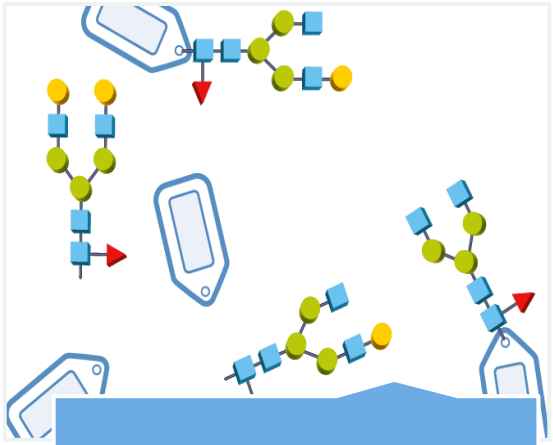
- A **fast 4 minutes** desalting method for high-throughput characterization.
- Intact Mab mass and the relative **glycoform abundance within 5 minutes**.
- In-depth characterization for glycoforms **detection below 1% relative intensity**.
- Single software for all data processing
  - Thermo Scientific™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



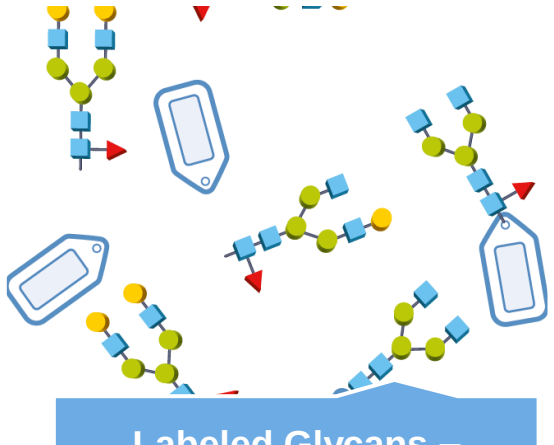
Summary – Six Glycan workflows



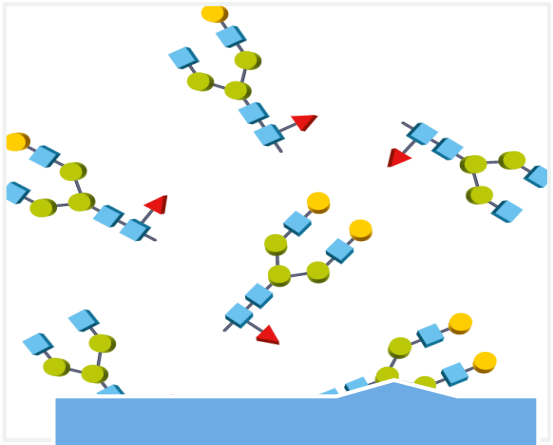
Monosaccharides & Sialic Acids



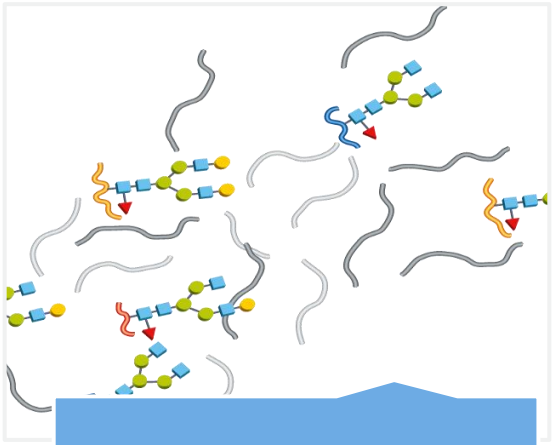
Labeled Glycans



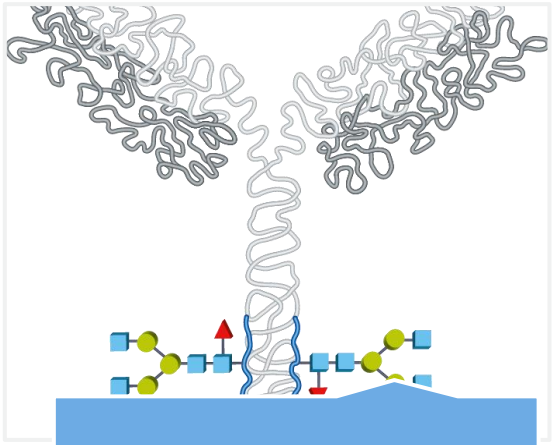
Labeled Glycans – High throughput



Glycans

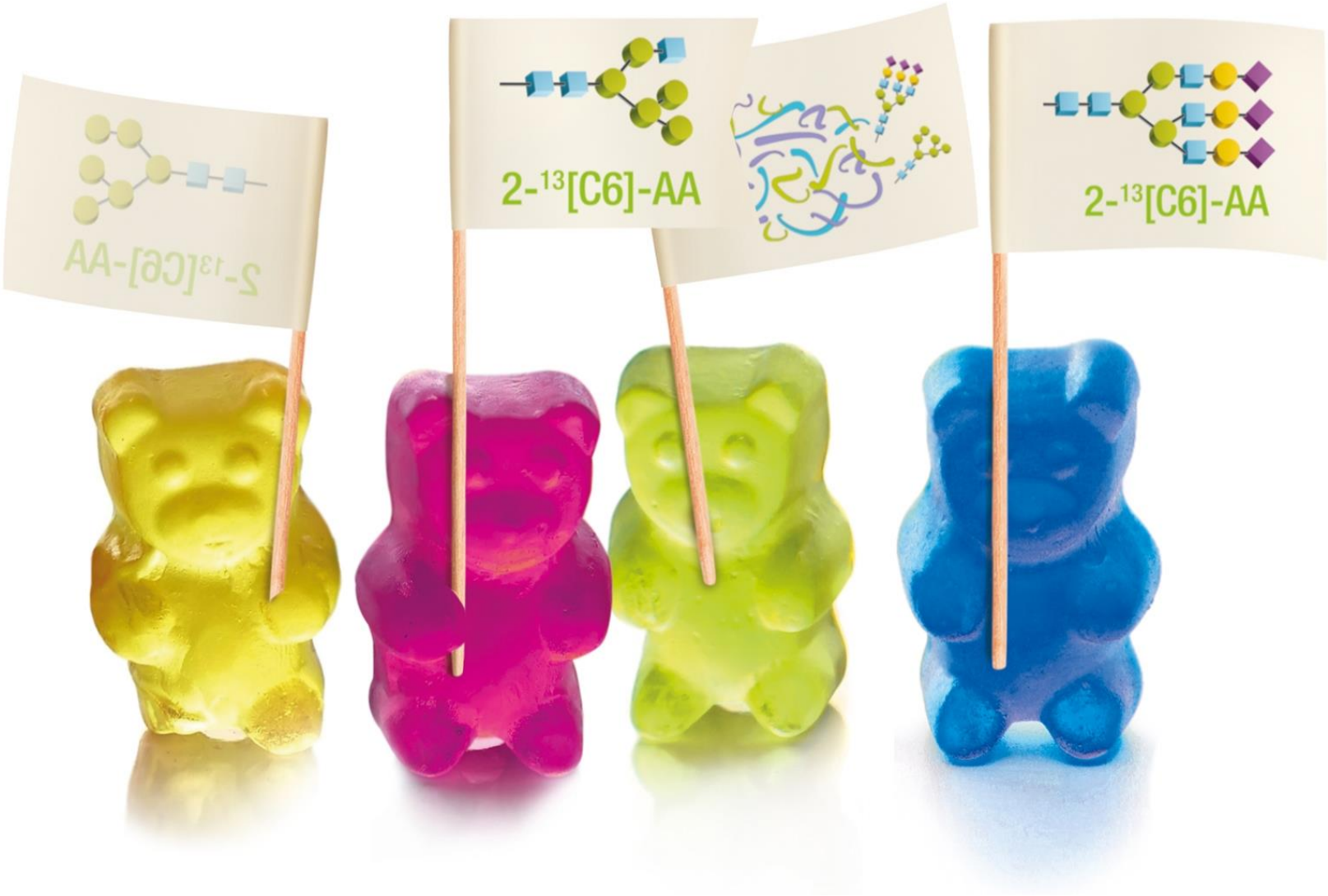


Glycopeptides



Intact Glycoprotein

Thank you



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