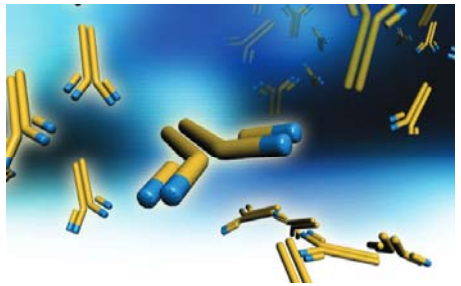
ThermoFisher SCIENTIFIC



New Innovations in UHPLC and LC-MS Workflows for the Characterization of Glycan structure on Biotherapeutics

Ken Cook

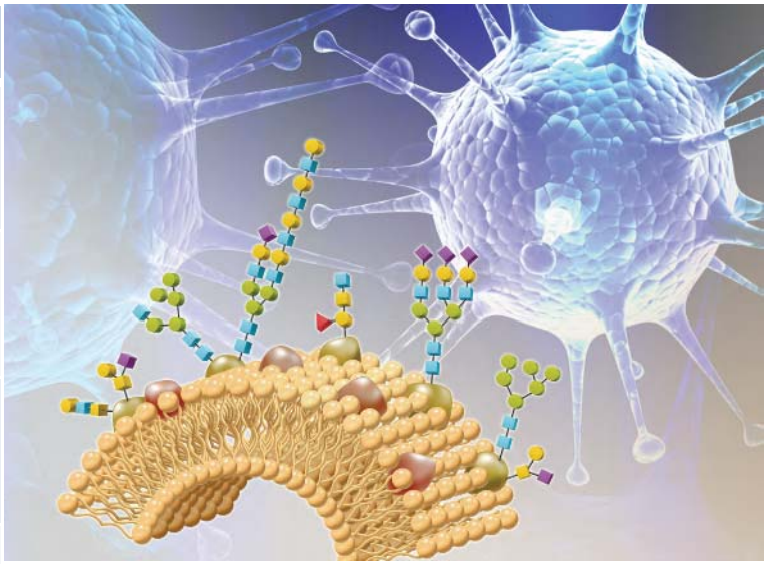
EU Bio-Separations Support Expert

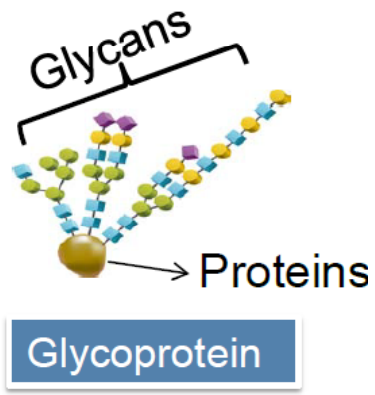
April 2015

The world leader in serving science

Why Study Glycans?

- Most diseases affecting humans involve glycans
- Many host-pathogen interactions occur using glycans (recognition, degradation, etc)
- Glycans are often key biomarkers for disease state
- Altered glycosylation is a universal feature of cancer contributing to pathogenesis and progression
- Many vaccines are glycan based (Tamiflu®)
- Most protein therapeutics must be glycosylated to work effectively (and not have nasty side effects!)

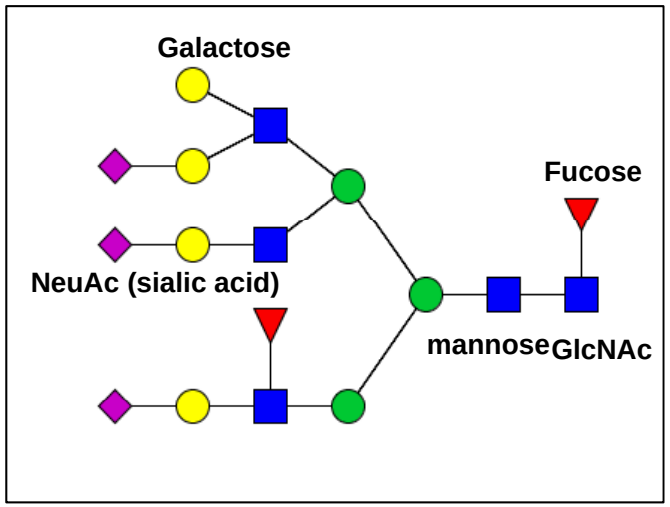




Tamiflu is a registered trademark of Gilead Sciences

Important Parameters for Glycan Characterization

- Monosaccharide composition
- Size
- Charge (# of sialic acid)
- Linkage and branch isomerism



Due to the branching of the chains and post-translational modifications, their structures are very complex and difficult to characterise

What Role do Glycans Play in Biotherapeutics?

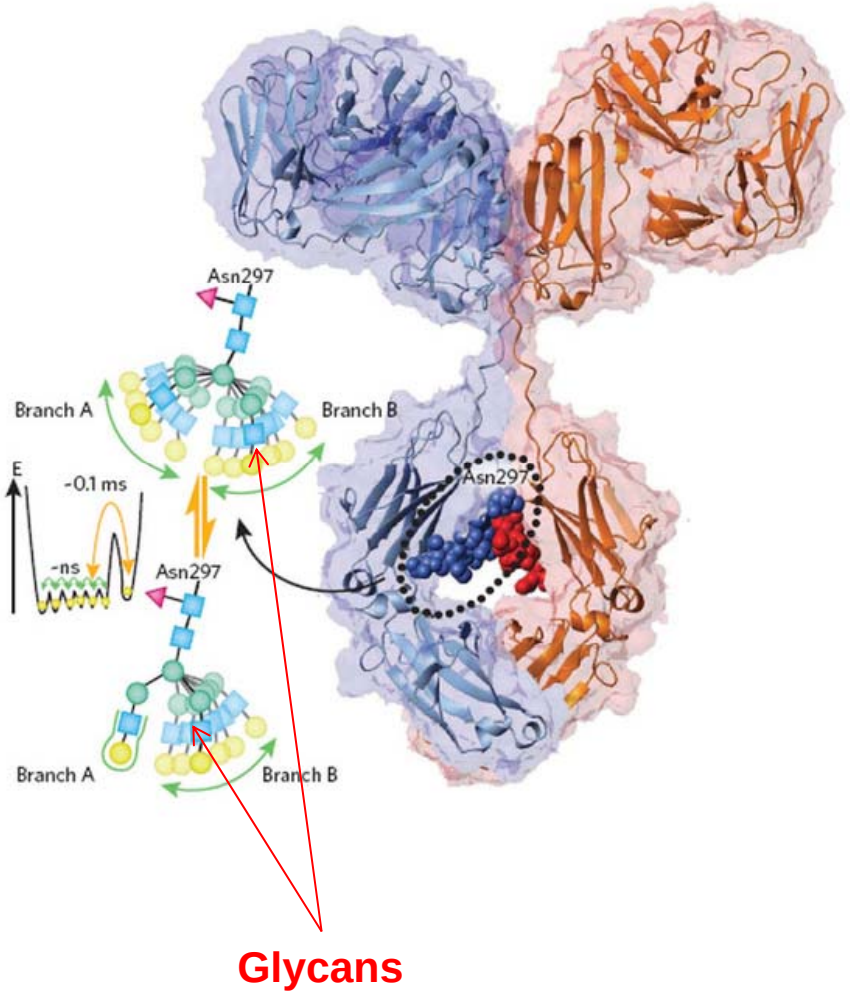
They can have a critical role in biotherapeutics

70% of protein drug candidates in preclinical and clinical development are glycosylated

This includes monoclonal antibodies (mAbs) and antibody drug conjugates (ADCs)

Glycosylation in therapeutic proteins affects: biological activity, pharmacokinetics, stability, immunogenicity

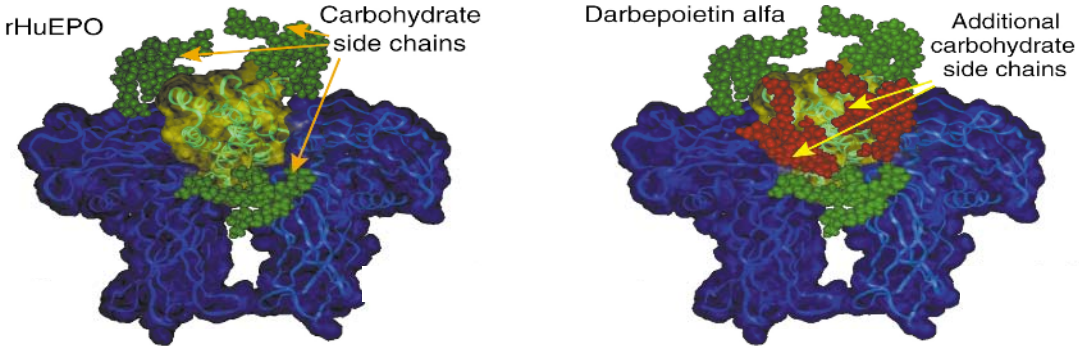
Glycosylation is the most common PTM (post translational modification) in biopharmaceuticals



ThermoFisher SCIENTIFIC

Glyco-Engineering to Improve Biopharmaceuticals

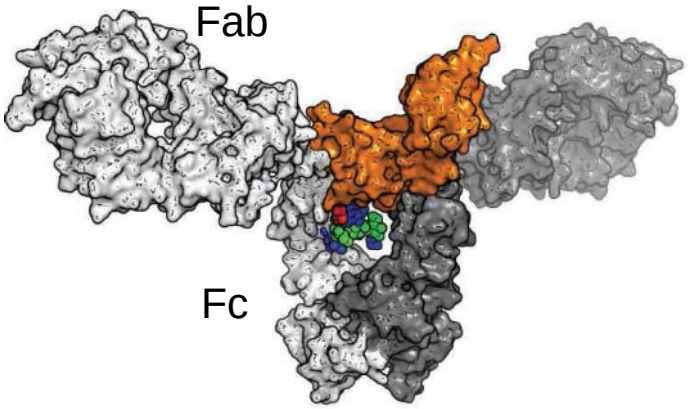
EPO:

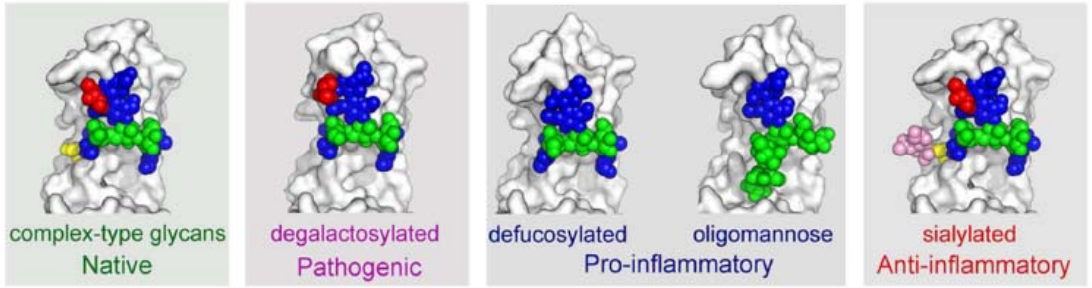


$T_{1/2}$ = 19h

$T_{1/2}$ = 32h

Therapeutic antibodies: Fc glycans determine function





ThermoFisher SCIENTIFIC

Current Legislation

Legislation ensures glycans / glycoslyation is well characterized

FDA (in US) and EMA (in Europe) define the regulatory requirements

Biotherapeutic manufacturers are legally obliged to comply with these

US FDA, Nov 3, 2010

Many complex biologics have sugar chains that impact their efficacy and safety, and industry experts have noted that the FDA has made several statements over the years suggesting a primary concern that biosimilars have the same sugar structure for interchangeability and those sugar structures do not change with time.

EMEA Guideline 2009

On the development, production, characterization and specifications for monoclonal antibodies and related products 'glycan structures should be characterized, and particular attention should be paid to their degree of mannosylation, galactosylation, fucosylation and sialylation'.

European Medicines Agency, EMEA/CHMP/BWP/157653/2007 (2009).

Current Legislation and Biosimilars

This also applies to biosimilars

As monoclonal antibodies come off patent, biosimilars will appear

The FDA and EMA consider the degree of glycosylation to be a critical factor in determining the degree of “similarity” to the original approved product

• **Similar**: Further information is needed to determine if the product is highly similar to the reference product. Additional analytical data or other studies are necessary to determine if observed differences are within an acceptable range to consider the proposed biosimilar. For example, the agency says that “*glycosylation plays an important role in the PK of certain protein products. Manufacturing process conditions may impact glycosylation. Comparative PK and PD studies of the proposed biosimilar product and the reference product help resolve that some differences in glycosylation identified in the analytical studies would be within an acceptable range to consider the proposed biosimilar product to be highly similar to the reference product*”;

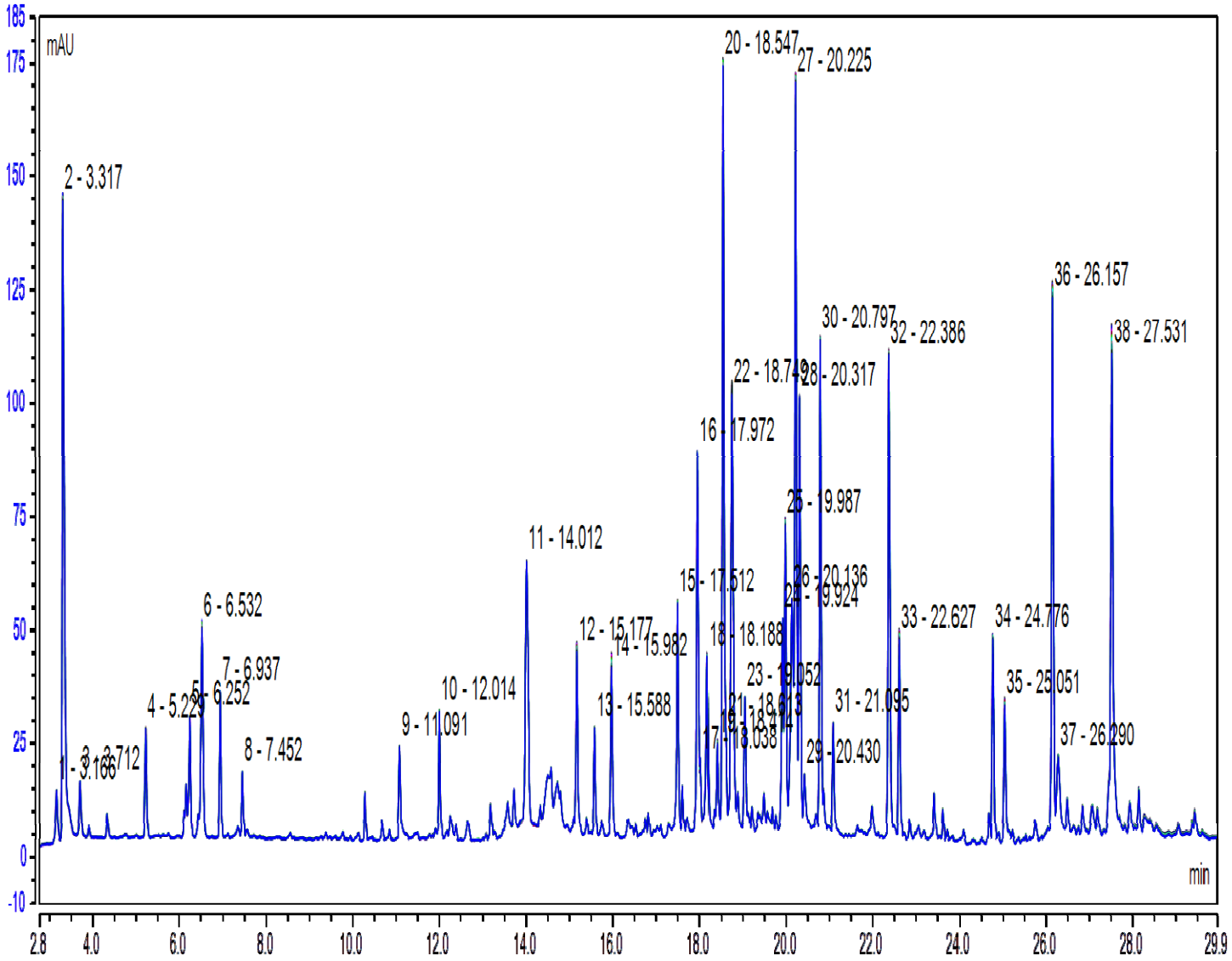
New Benchmark for Bio-Separations

Thermo Scientific™ Vanquish™ UHPLC System

- Integrated modularity
- Viper-based, tool-free fluidic connections
- Biocompatible, iron-free flow path
- Support for latest innovations in column technology
- New column thermostatting technology with up to 3 column compartments in one system
- Enhanced LC-MS support and integration
- Portable system control with tablet PC
- Reduced system height
- Revolutionary module drawer system for repairs
- Removable doors for easy access

Overlay of 10 Runs of a Trypsin Digest mAb with Retention Time Precision

peak #	RT (min)	RT-RSD (%)
3	3.315	0.082
9	5.231	0.065
14	6.532	0.017
15	6.937	0.023
19	10.290	0.021
23	12.013	0.012
31	14.011	0.013
39	15.177	0.012
42	15.589	0.010
51	17.511	**0.007**
55	17.969	0.011
61	18.546	0.010
83	20.798	0.010
85	21.095	0.012
87	22.386	**0.009**
96	24.774	0.012
103	26.155	**0.009**
106	26.155	**0.009**
109	27.529	0.010



ThermoFisher SCIENTIFIC

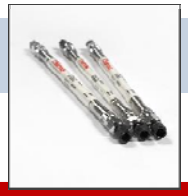
Folie 9

JH1 Please format with title-case text.
Jim Hegarty, 15.10.2014

Glycan Analysis – The Workflows in Brief

Digestion to monosaccharides

The protein is digested to allow analysis of monosaccharide and sialic acids. Analysis is often done by ion chromatography (HPAE-PAD) or HPLC



Release of glycans

Glycans are separated from the mAb host for profiling by HPLC. Ideally they should be separated by mass, polarity and charge; column choice is critical

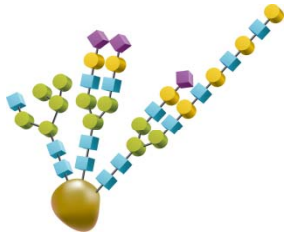
Digestion to glycopeptides

Proteins are digested to the constituent glycopeptides, i.e small glycosylated fragment. Structure and site analysis is performed using LC-MS

Intact mAb characterization

Intact mAbs are characterized fully intact, with the glycans in-situ. Accurate mass MS is required to identify glycan types, numbers and positions; critical for biotherapeutic efficacy

Released Glycan Analysis Workflow



PNGase F Digestion

Native Glycans

2AB/2AA labeling

Labeled Glycans

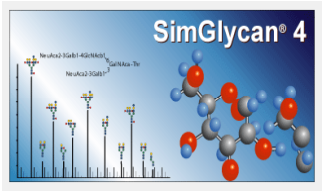
Thermo Scientific™ GlycanPac ™ AXR-1 and GlycanPac AXH-1 Columns

GlycanPac AXH-1
GlycanPac AXR-1
Accucore 150-Amide-HILIC Columns

Thermo Scientific™ Dionex™ Ultimate™ 3000 UHPLC System

Separation of glycans

LC-MS analysis



Simglycans

Data analysis

Glycan structures

Thermo Scientific™ Q Exactive ™ or OrbiTrap Fusion™ MS Instruments

Separation of glycans

LC-FLD

Qualitative & quantitative analysis

FLD3400RS

*LC/MS of **native** or **labeled** glycans*

*FLD for **labeled** glycans*

ThermoFisher SCIENTIFIC

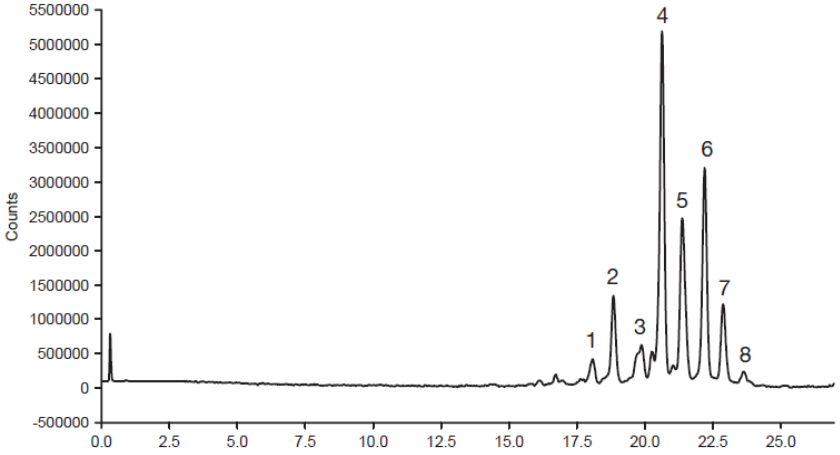
HILIC is Most Commonly Used for Glycan Separation

- HILIC separates glycans by hydrogen bonding – size-based separation
- Commonly used HILIC columns are amide based HILIC
- Routine approach for 2-AB labeled glycans, ***however .....***
- Glycans of different charge states are intermingled in the separation envelope

Mixed-Mode Columns

- Definition
  - Hydrophobic (or hydrophilic) interaction + ion-exchange interaction
- Benefits
  - Adjustable selectivity for optimal separation
  - Simplified mobile phase (no need for ion-pairing reagents)
  - Simultaneous separation of different types of analytes
- Types useful for Glycan analysis
  - Anion-exchange (AEX)/reversed-phase (RP)
  - Anion-exchange (AEX)/ HILIC

2AB Bovine Fetuin Glycans on an Accuore150-Amide-HILIC Column



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			

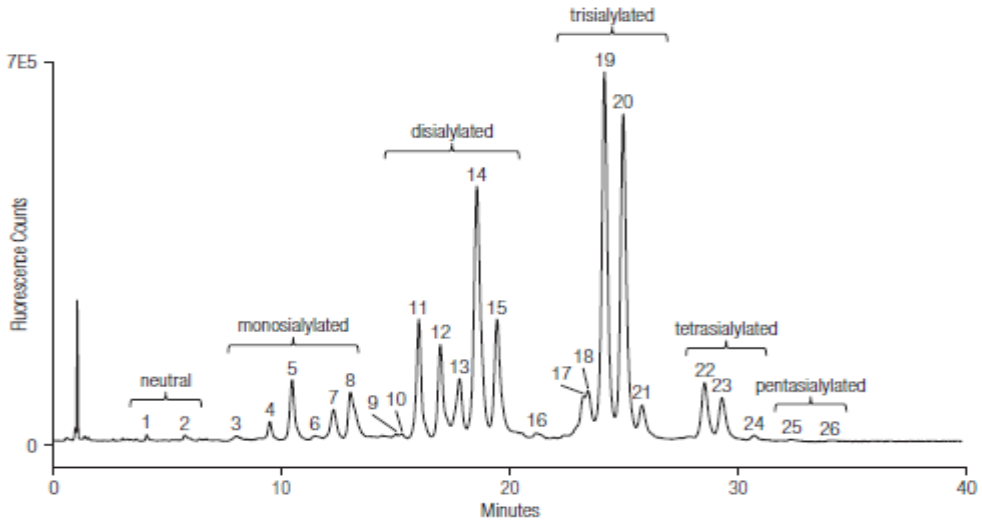
Thermo Scientific™ Accuore™ 150-Amide-HILIC Column

2.6 µm superficially porous silica particles modified with polyamide

ThermoFisher SCIENTIFIC

GlycanPac AXH-1 Column – Separation Mechanism

- WAX functionality: groups glycans into different “clusters” in the order of increasing charge
- HILIC functionality: within each “cluster” facilitates hydrogen bonding interaction, resulting in size- and composition-based separation for glycans of same charge

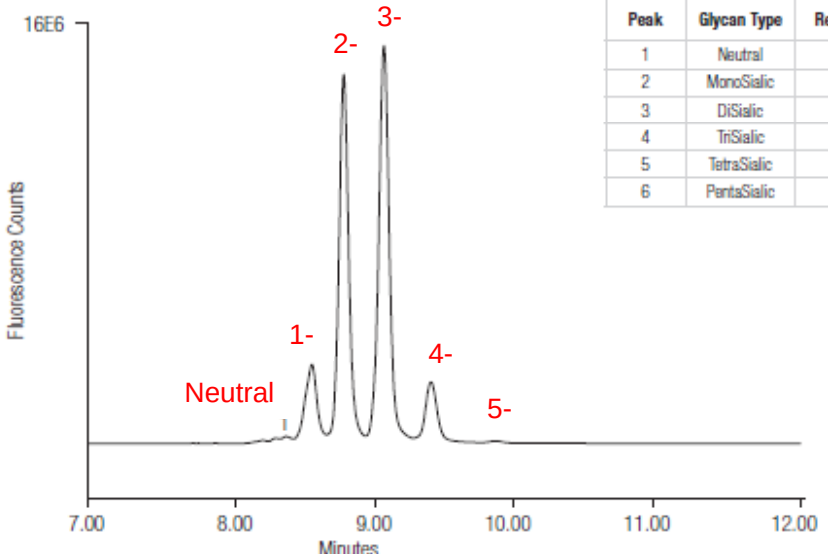


Column:	**GlycanPac AXH-1 (1.9 µm)**
Dimension:	2.1 × 150 mm
Mobile Phase A:	Acetonitrile (100%)
Mobile Phase B:	Water
Mobile Phase C:	Ammonium formate (100 mM, pH = 4.4)
Flow Rate:	0.4 mL/min
Injection Volume:	50 Pmoles
Temperature:	30 °C
Detection:	Fluorescence at 320/420 nm
Sample:	2AB labeled *N*-glycan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

ThermoFisher SCIENTIFIC

Charge-Based Separation

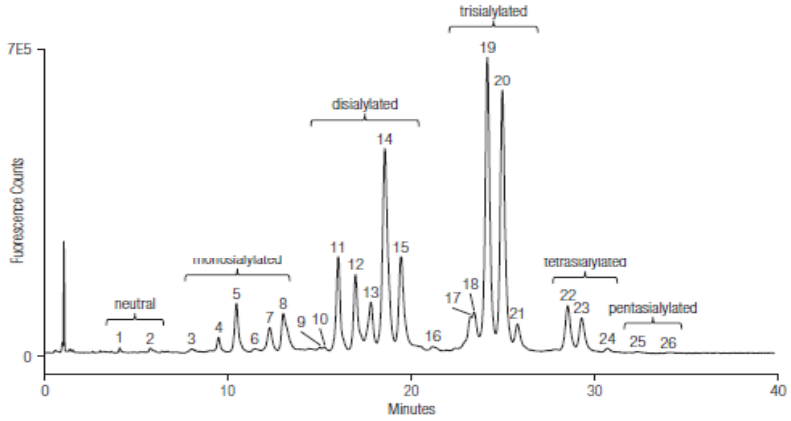


Peak	Glycan Type	Relative %
1	Neutral	0.4
2	MonoSialic	8.6
3	DiSialic	38.4
4	TriSialic	45.4
5	TetraSialic	7.0
6	PentaSialic	0.2

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

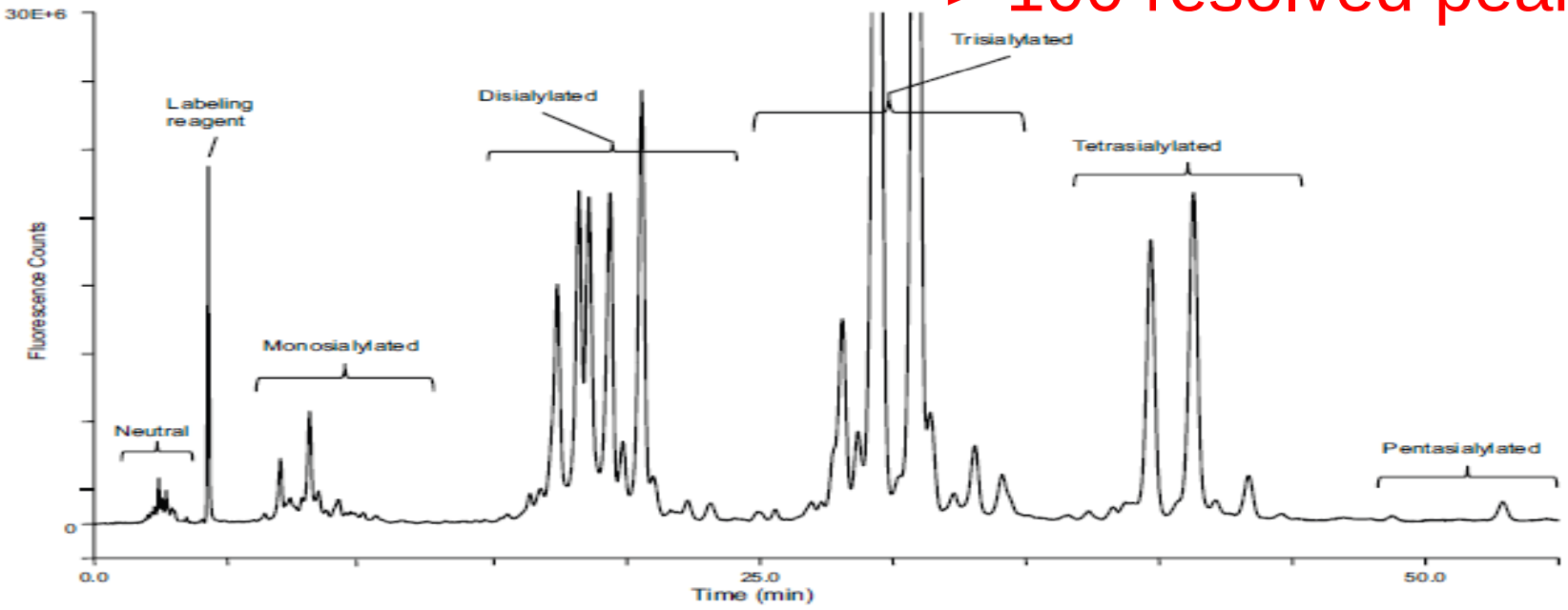
Time (min)	% A	% B	% C	Flow (mL/min)	Curve
-10	78	20	2	0.4	5
0	78	20	2	0.4	5
30	70	20	10	0.4	5
35	60	20	20	0.4	5
40	50	20	30	0.4	5

ThermoFisher SCIENTIFIC

GlycanPac AXR-1 Column – Separation Mechanism

- 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

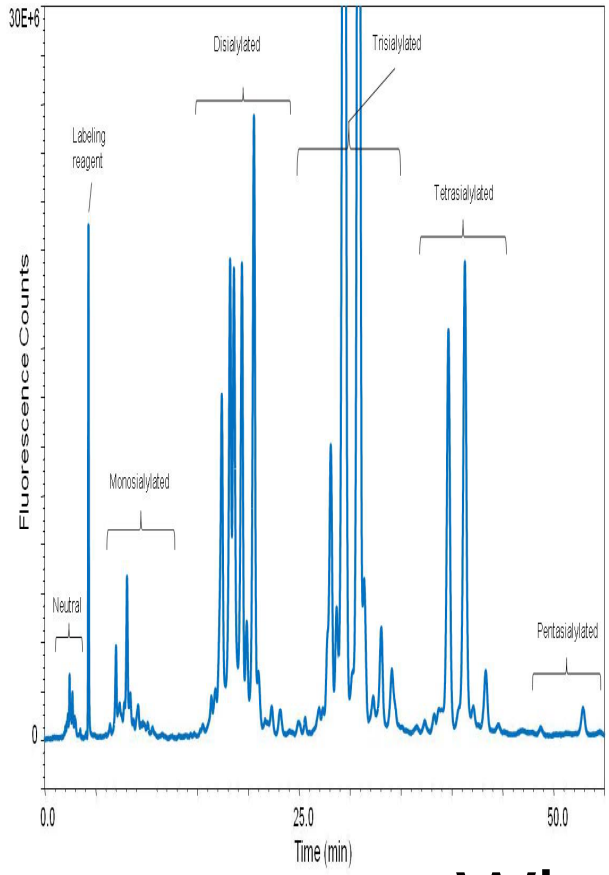
> 100 resolved peaks



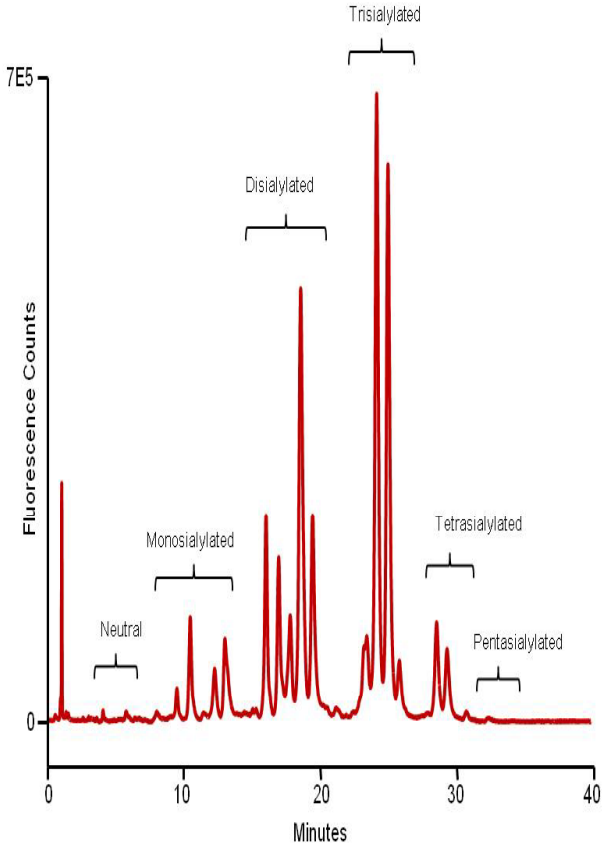
ThermoFisher SCIENTIFIC

GlycanPac Columns and Amide HILIC column

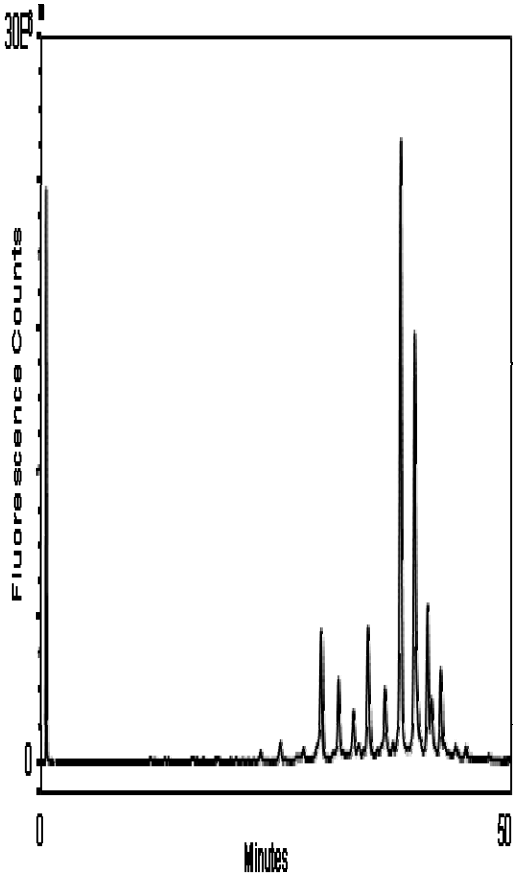
GlycanPac AXR-1 Column (1.9µ)
(>70 peaks resolved)



GlycanPac AXH-1 Column (1.9µ)
(>40 peaks resolved)



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

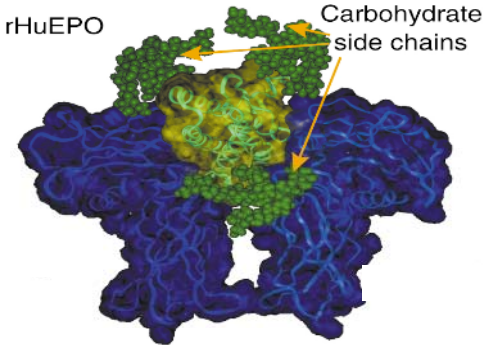


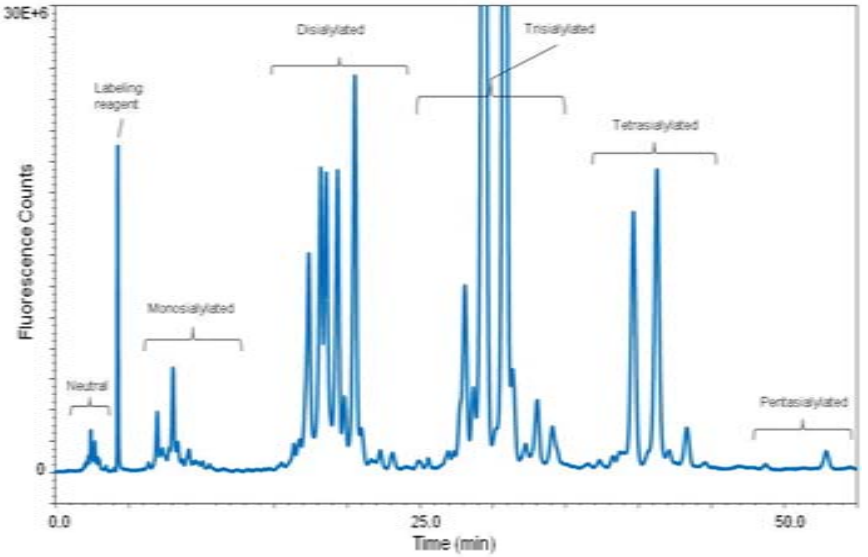
When to use each column?

ThermoFisher SCIENTIFIC

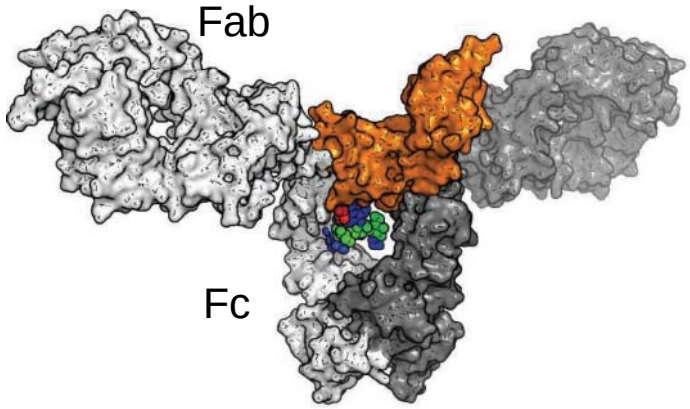
Glyco-Biopharmaceuticals

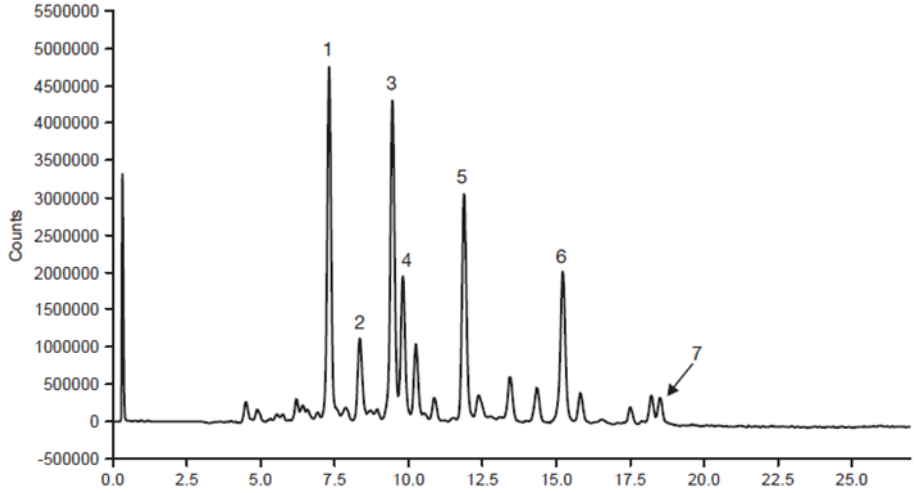
EPO:





Therapeutic antibodies

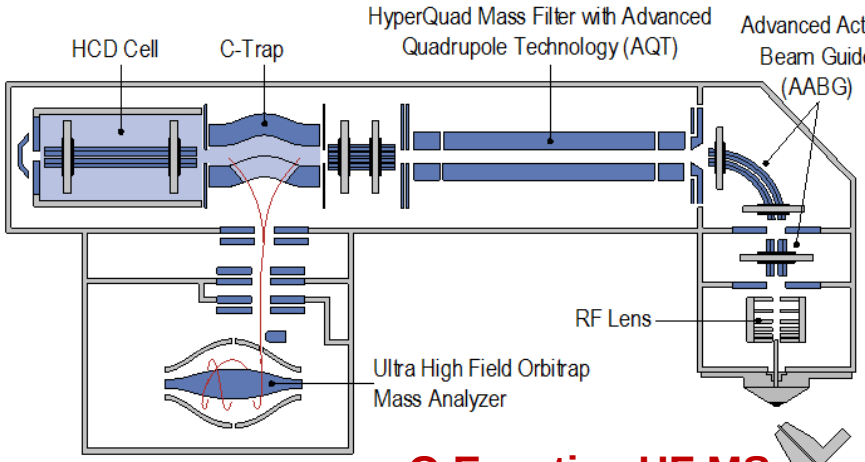




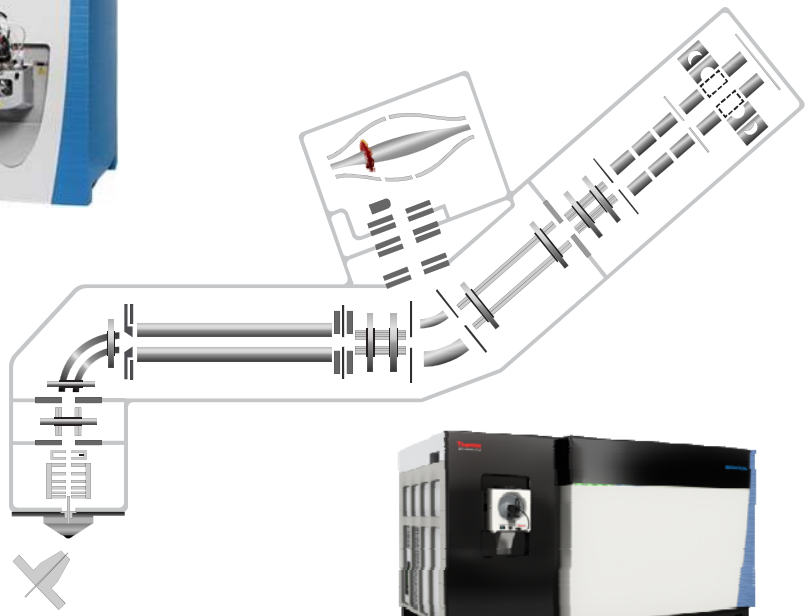
ThermoFisher SCIENTIFIC

NEW MEMBERS OF THE THERMO SCIENTIFIC™ ORBITRAP™ FAMILY

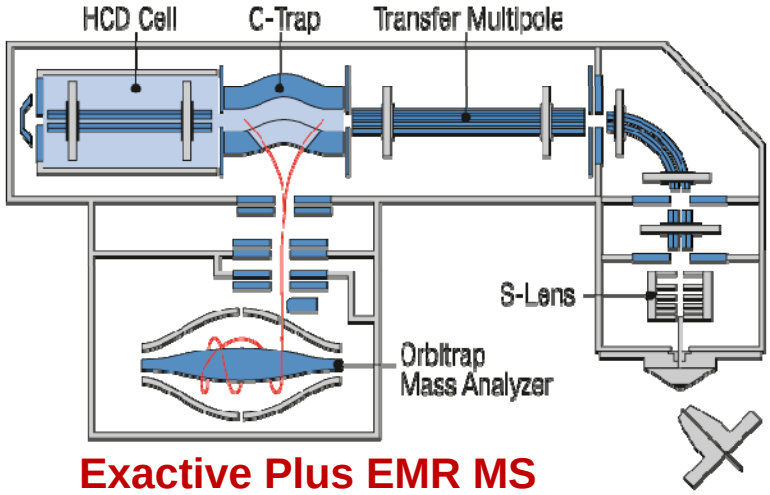
MS Tools for Major Biopharma Characterization Workflows



Q Exactive HF MS



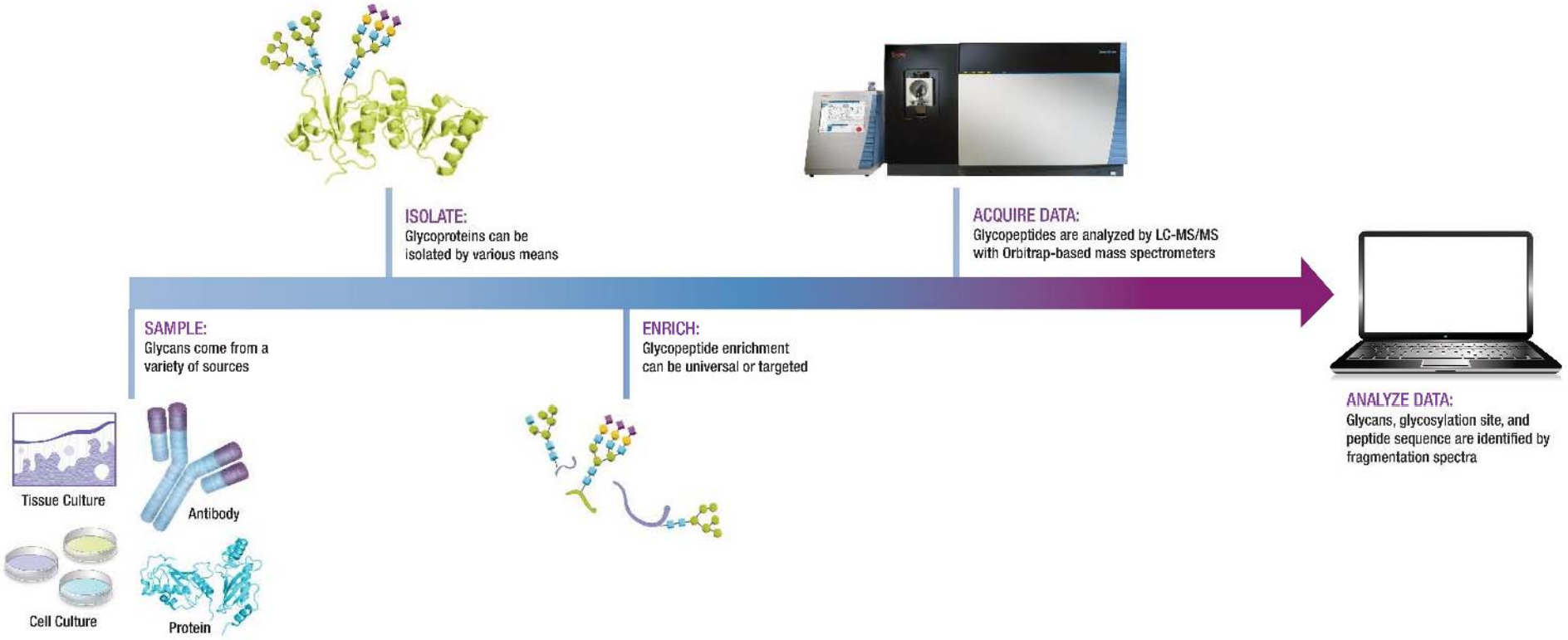
Orbitrap Fusion Tribrid MS



Exactive Plus EMR MS

ThermoFisher SCIENTIFIC

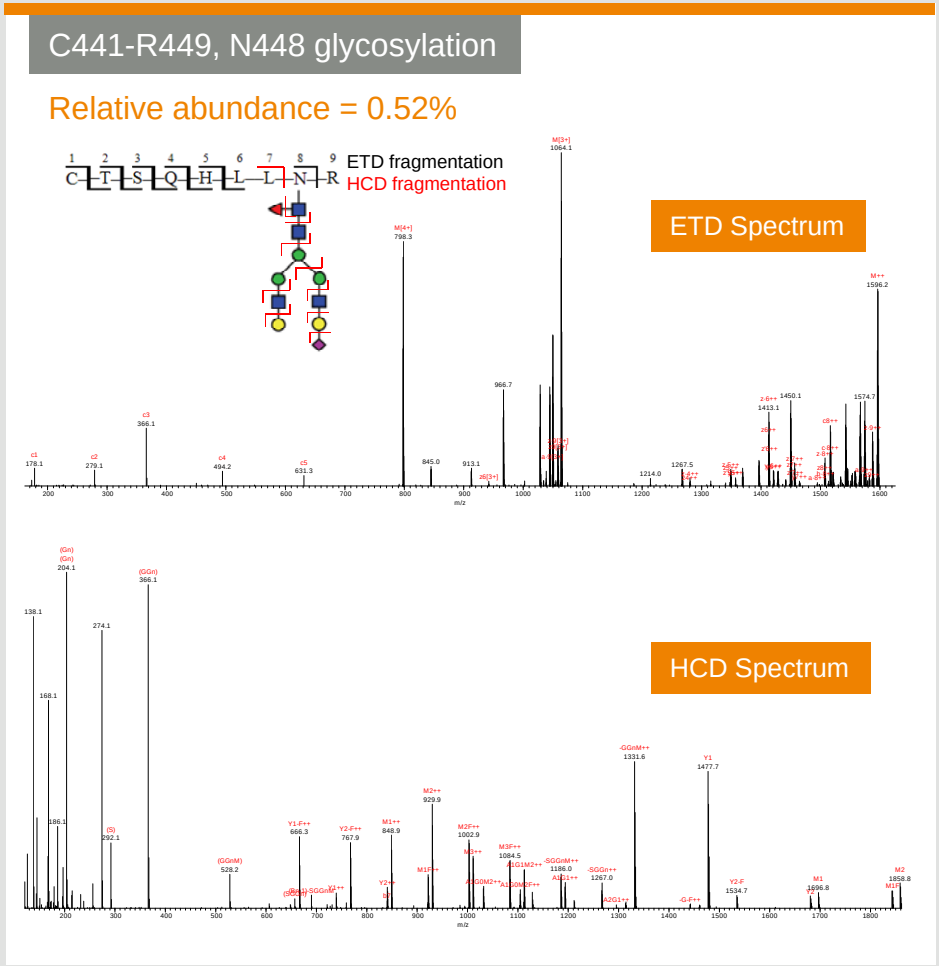
Workflow Detail – Glycopeptides



- Profiling at the peptide level is important for site profiling
- Enrichment is used to reduce sample complexity – isolating certain glycopeptides
- A variety of fragmentation techniques may be used (e.g. HCD or CID with ECD)
- Bioinformatics tools (Thermo Scientific™ SimGlycan™ Software) are extremely valuable for data interpretation.

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.



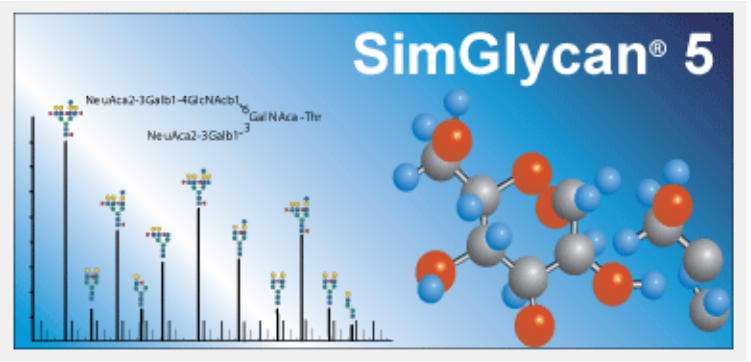
Zhiqi Hao et al 2014 ASMS TP264

ThermoFisher SCIENTIFIC

Product: SimGlycan Software

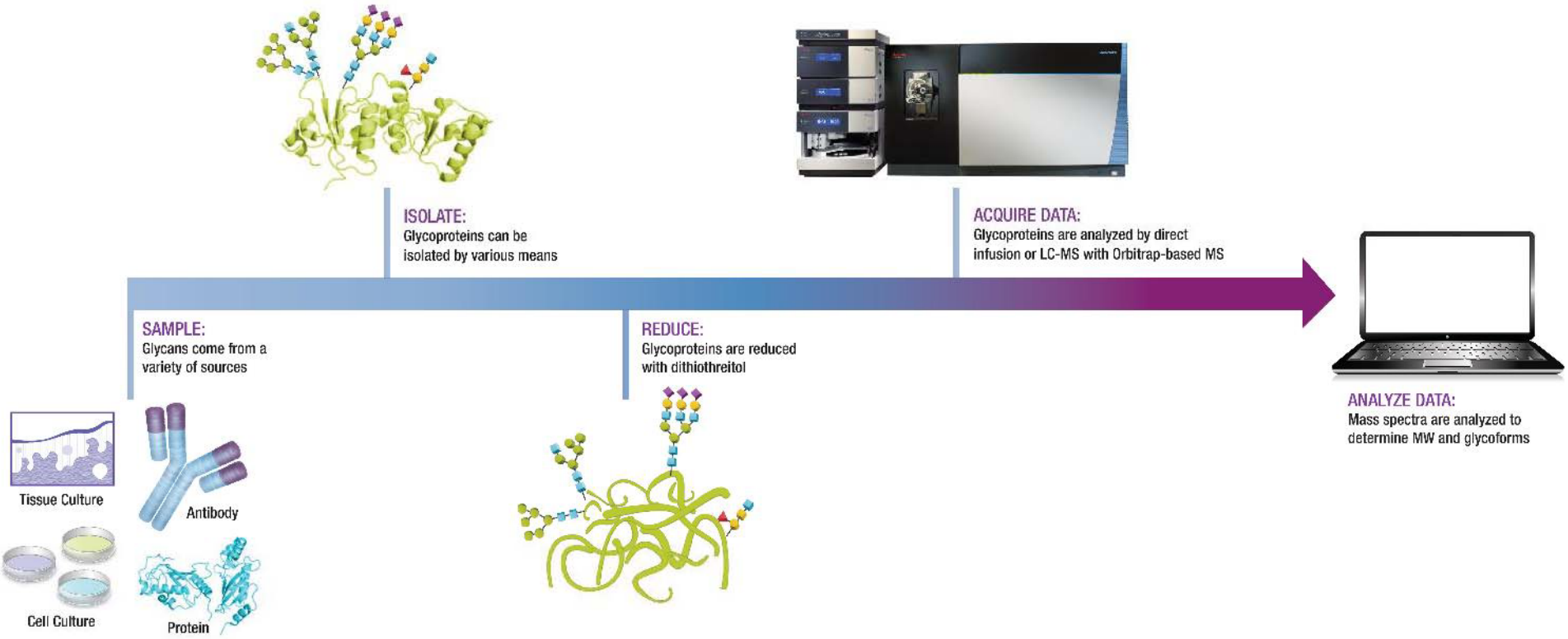
What is this product used for?
Software for automated structural analysis & elucidation of glycans from MS/MS spectra
For every probable glycan structure, SimGlycan software provides glycan fragments, structure, sequence, composition, glycan mass, class, reaction, pathway and enzyme
Summary: *The most comprehensive software application for MS/MS and MS^n data analysis*

Benefit	Detail
Largest commercial fragment database	Over 22500 glycans to process and to verify glycan structures
Direct support for all iontrap and Orbitrap instruments	Including LTQ / Orbitrap, LTQ Velos / Orbitrap, MALDI LTQ Orbitrap & Q Exactive range plus many more
Denovo sequencing	Useful for novel glycans
Batch processing & spectra labeling	Up to 1000 profiles



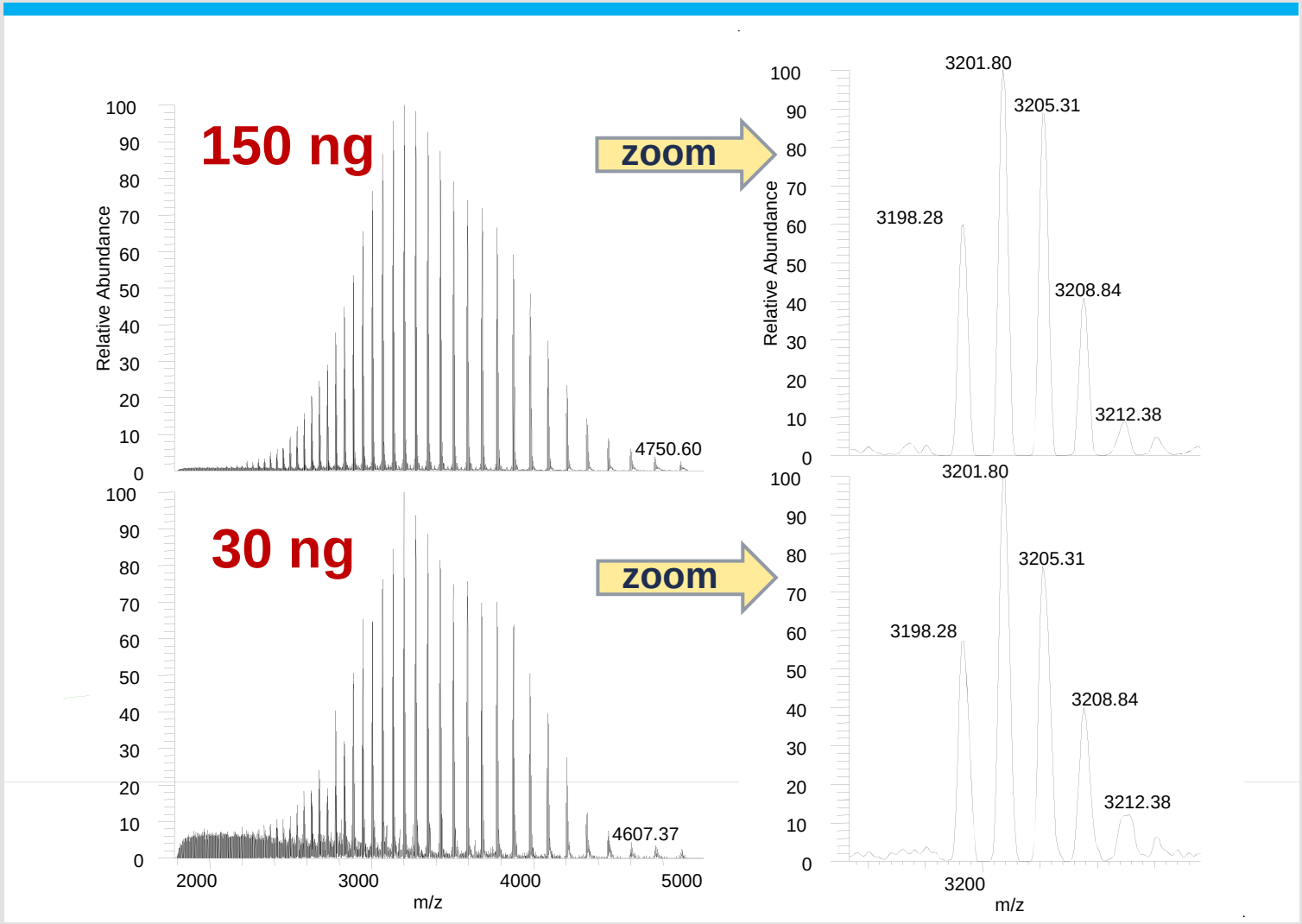
ThermoFisher SCIENTIFIC

Workflow Detail – Intact Glycoprotein Analysis



- 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.

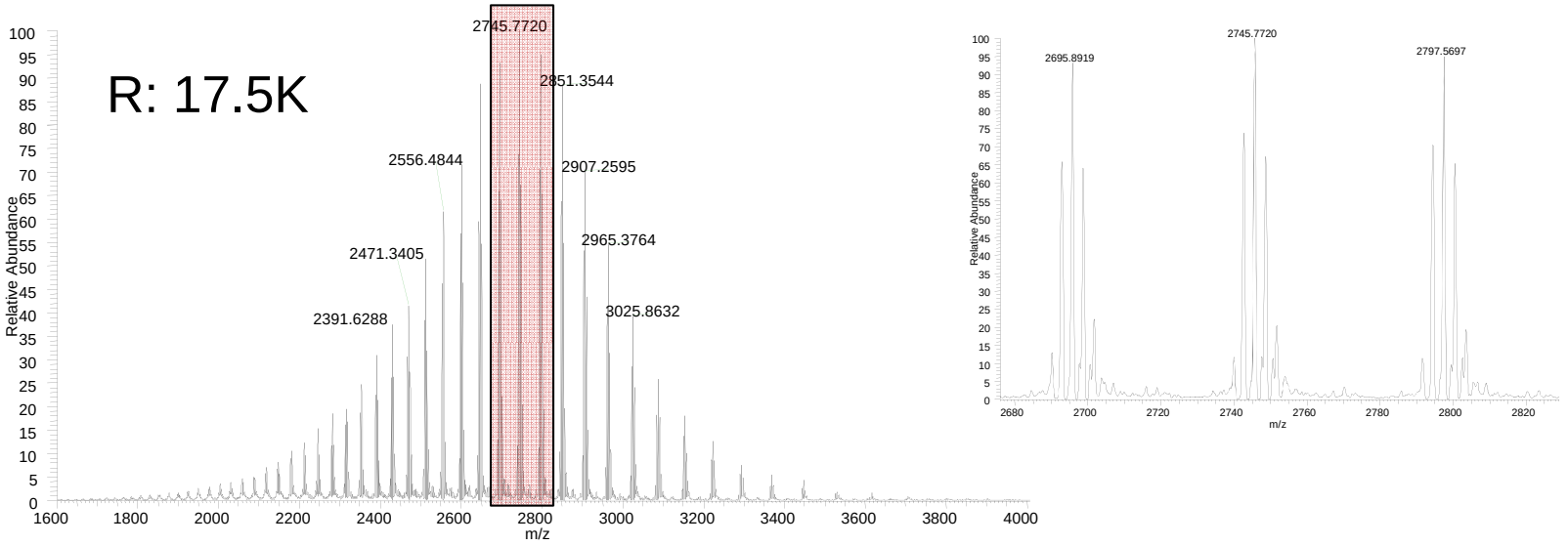
1 x 50 mm ProSwift Column, Intact Rituximab®

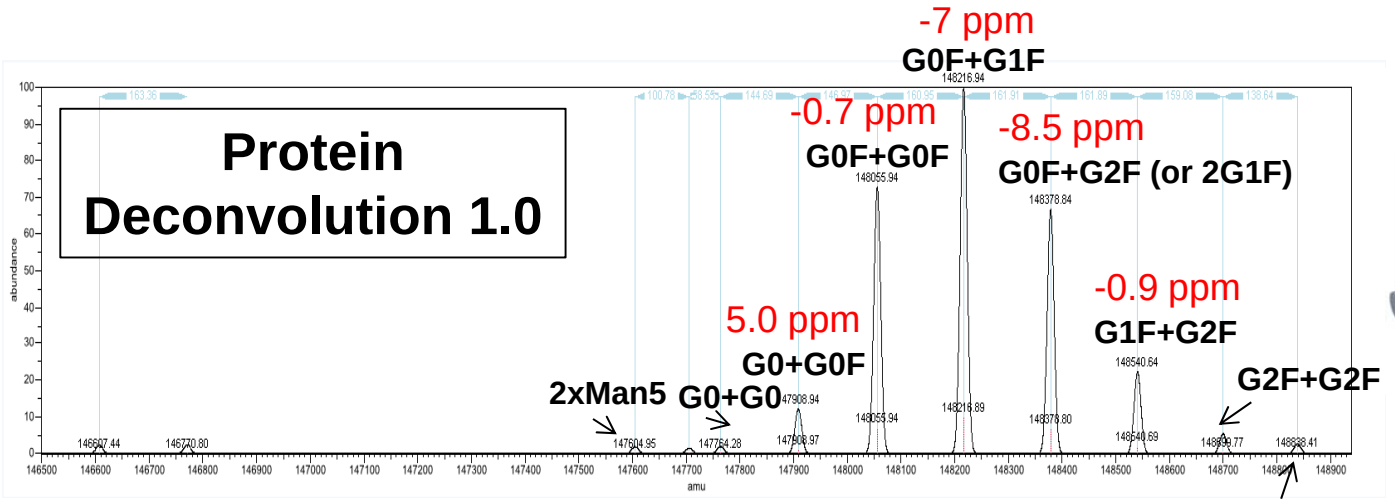


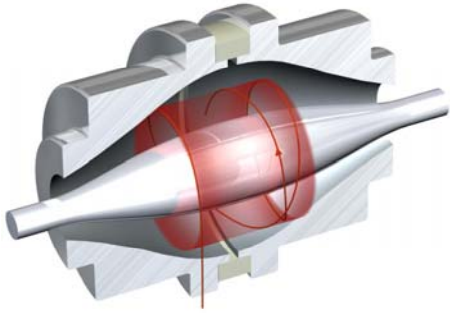
Rituximab is a registered trademark of Genentech, Inc.

ThermoFisher SCIENTIFIC

Trend Toward HR/AM MS for Intact IgG Mass Measurement e.g. Glycoform Analysis







Q Exactive Orbitrap MS

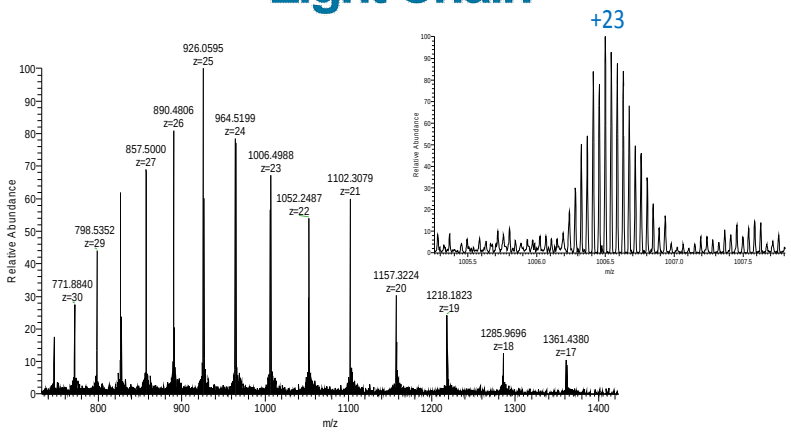
In-depth characterization, comparability studies

ThermoFisher SCIENTIFIC

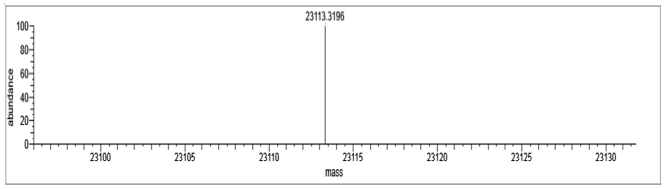
Accurate Monoisotopic Mass Measurement Using Ultra High Resolution

LC-MS of Antibody Light Chain and Heavy Chain

Light Chain

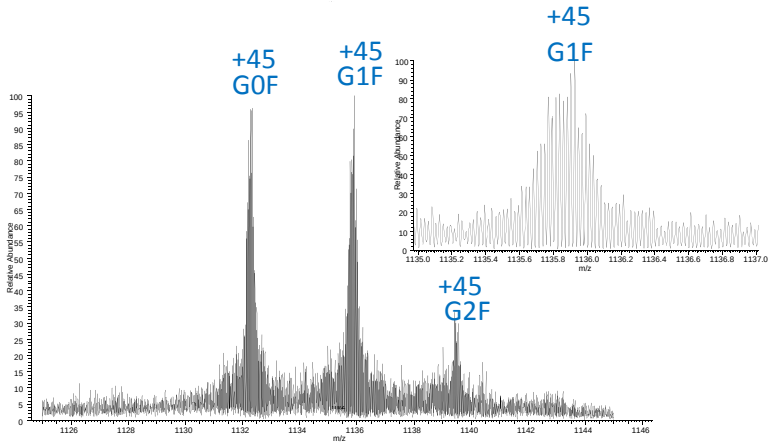


0.7 ppm



Q Exactive Plus MS, 140K

Heavy Chain

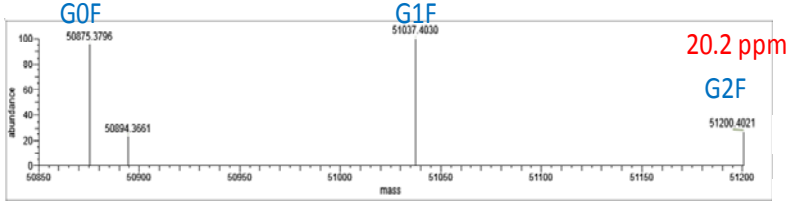


2.3 ppm

G0F

1.8 ppm

G1F



20.2 ppm

G2F

Q Exactive Plus MS, 280K, protein mode

ThermoFisher SCIENTIFIC