**Thermo Scientific**
Glycan Analysis for Biotherapeutics

## Join the sweet revolution in

# biopharmaceuticals

- Intact Glycoproteins
- Glycopeptides
- Free Glycans
- Monosacccharides

Thermo SCIENTIFIC

# MEETING THE CHALLENGES of Glycan Characterization

Biotherapeutic proteins, which include antibodies, are becoming the fastest growing category of drugs due to their efficacy for patient health. The complex nature of these large molecules brings major new challenges to the pharmaceutical scientist. The industry is undergoing a scientific revolution as it adapts to meet the challenges of characterizing biotherapeutics.

## Glycosylation of biotherapeutic medicines plays a critical role in the efficacy of the drug.

In-depth characterization of these glycans (complex sugars) bound to the drug entity is mandatory and challenging. To overcome this challenge, we have developed a series of four simple workflow solutions based on leading technology platforms.

**1**

### WHAT IS MY GLYCOSYLATION PATTERN?

Intact Glycoprotein analysis

**2**

### WHERE DO MY GLYCANS RESIDE?

Glycopeptide profiling

**3**

### WHICH GLYCANS ARE IN MY DRUG AND WHAT IS THEIR CONCENTRATION?

Glycan structure determination

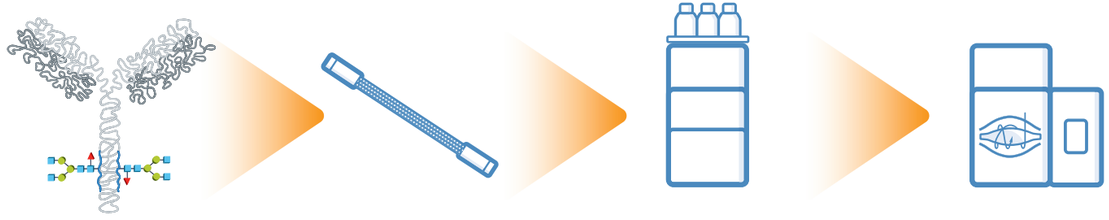
**4**

### WHAT IS THE COMPOSITION OF SUGARS IN MY DRUG ENTITY?

Monosaccharide analysis

# WHAT IS MY Glycosylation Pattern?

## Intact Glycoprotein Analysis Workflow



**INTACT GLYCOPROTEIN**
Isolated antibodies and large proteins are often chemically reduced prior to analysis.

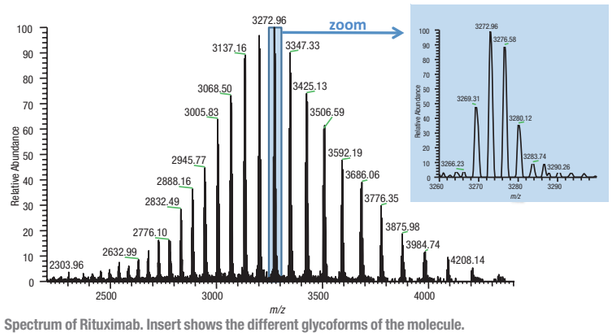
**SEPARATE GLYCOPROTEINS**
Advanced ion exchange or hydrophobic interaction chromatography columns allow full separation of antibody fragments.

**LIQUID CHROMATOGRAPHY**
Reproducible, bio-compatible chromatography is required to simplify variants.

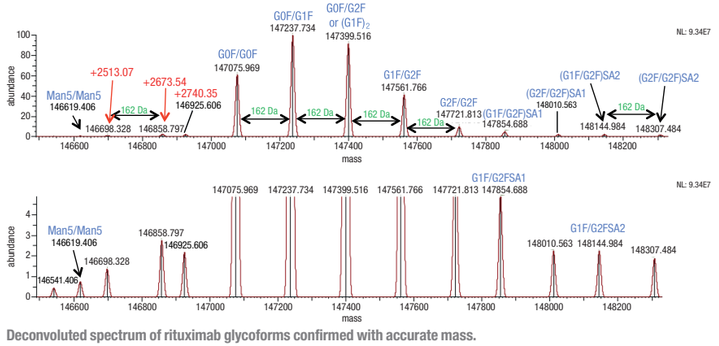
**CONFIRM MASS OF GLYCOPROTEIN**
High resolution and mass accuracy are required together with deconvolution software to interpret spectra.

## Analyze with Mass Spectrometry

Intact glycoprotein profiling is used to ascertain the pattern and degree of glycosylation. Due to the heterogeneity of the attached glycan moieties, intact glycoprotein profiling is best performed using high resolution/accurate mass (HR/AM) mass spectrometry together with chromatographic separation to gain full insight into the various glycoforms present on a protein or antibody.




Spectrum of Rituximab. Insert shows the different glycoforms of the molecule.

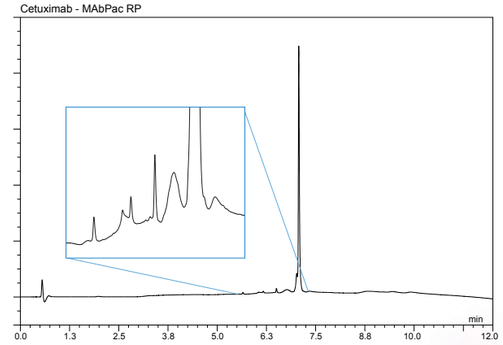



Deconvoluted spectrum of rituximab glycoforms confirmed with accurate mass.



## Separate Antibody Variants & Fragments

Thermo Scientific™ MAbPac™ columns are designed for the separation of monoclonal antibodies (mAbs) and related biologics by hydrophobic interaction (HIC), ion exchange (IEC), size exclusion (SEC) and reverse phase (RP) chromatography. This suite of columns have unique chemistries to provide high resolution, excellent bio-compatibility, and complementary selectivity suitable for a broad range of assays for mAbs and related substances.




Separation of Cetuximab variants using a MAbPac RP column.

## Deconvolute Protein Spectra

Screen, identify, and characterize intact proteins with higher productivity and confidence using Thermo Scientific™ Protein Deconvolution software. The software uses not one, but two deconvolution algorithms. One algorithm to take full advantage of the high-quality, high-resolution accurate-mass (HRAM) data produced by Thermo Scientific™ Orbitrap™ based mass spectrometers. The second algorithm is for lower resolution spectra where the mass spectral peaks are not isotopically resolved.

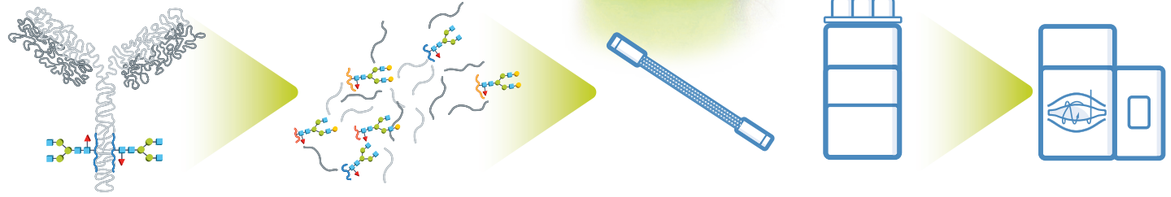

Extended mass range (EMR) of up to m/z 20,000 and improved detection of high-mass ions.

## Analyze Intact Proteins

Many biopharmaceutical and biological research applications require analysis of proteins in their native (intact) state. The Thermo Scientific™ Exactive™ Plus EMR mass spectrometer combines unsurpassed high-resolution accurate-mass analysis with an extended mass range (EMR) to create an outstanding tool for investigating the structure, topology, and architecture of native-like tertiary and quaternary protein structures. A superb tool for high-performance screening of monoclonal antibodies, antibody-drug conjugates (ADC), PEG-ylated proteins, oligomerized protein-based drugs and glycoforms.

# WHERE DO MY Glycans Reside?

## Glycopeptide Mapping Workflow



### INTACT GLYCOPROTEIN
Isolate antibody or glycoprotein.

### DIGEST TO GLYCOPEPTIDES
Reproducible, fast, digestion in less than one hour.

### SEPARATE GLYCOPEPTIDES
Glycopeptides can be separated faster using advanced UHPLC columns.

### ANALYZE & MAP
Routine peptide mapping provides protein sequence incluiding glycopetides. Advanced MS fragmentation provides exact glycopeptide location information.

Glycan analysis can also be performed at the peptide level, with the goal of obtaining both glycan composition and peptide sequence at the site of glycosylation.

### DIGEST

Thermo Scientific™ SMART Digest™ kits are designed for biopharmaceutical applications that require highly reproducible, sensitive and fast analysis, often in high throughput workflows. The kit uses unique optimized heat-stable immobilized trypsin technology.

SMART Digest allows complete antibody digestion in just 60 minutes; a saving of up to 23 hours versus in-solution digestion methods.

### SEPARATE PEPTIDES

The glycopeptides can then be separated in a few minutes with the highest speed and resolution using the biocompatible Thermo Scientific™ Vanquish™ UHPLC system together with Thermo Scientific™ Accucore™ C18 or Acclaim 120 RS UHPLC columns.

### ANALYZE AND MAP

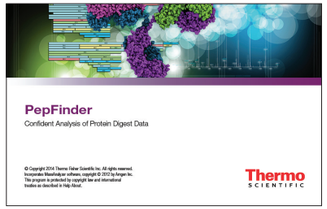
Glycopeptide mapping can be performed rapidly and accurately with the combination of any of our Thermo Scientific™ Q Exactive™ family of Orbitrap powered mass spectrometers, combined with the latest software for comprehensive mapping of biotherapeutic proteins - PepFinder.

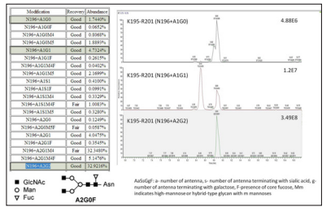
For more advanced characterization of an intact glycopeptide, multiple fragmentations are required: HCD Higher-energy collisional dissociation provides information about the glycan composition and ETD (electron transfer dissociation) provides extensive fragmentation along the peptide batckbone to enable sequencing of the peptide while preserving attachment of the glycans, allowing localization of the glycosylation sites. ETD is available on Thermo Scientific™ Orbitrap Fusion Lumos™ Tribrid™ MS.

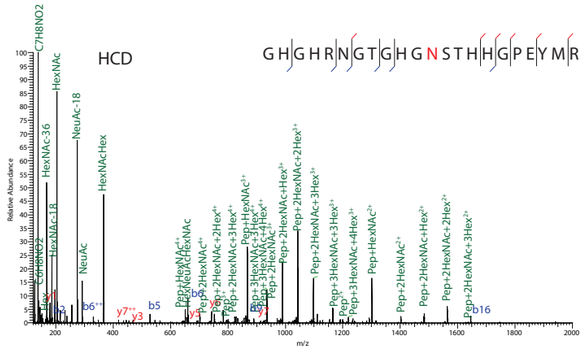


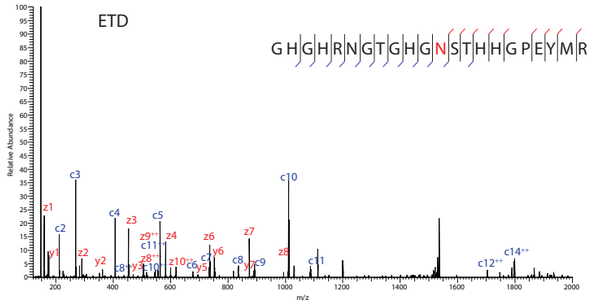
## Simple, Advanced Peptide Mapping Software

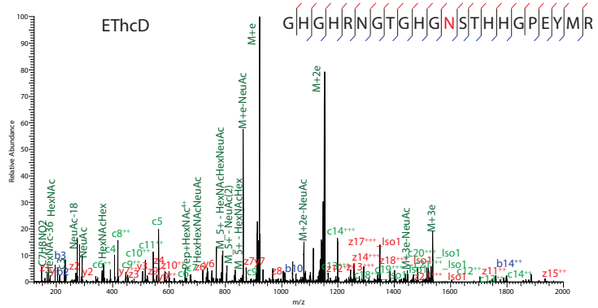
Thermo Scientific™ PepFinder™ software provides accurate identification, in-depth characterization, and relative quantitation of biotherapeutic and other proteins from mass spectrometric data. It provides an automated workflow for glycopeptide identification, disulfide bond mapping, and quantification of PTMs. PepFinder software automates previously time-consuming manual processes, processing complex data and integrating the results into concise, informative reports.




Multiple fragmentation techniques allow unambiguous assignment of glycosylation site.

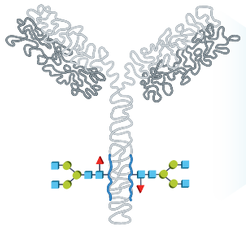
Resolution up to 500,000 FWHM and exceptional <1 ppm mass accuracy provides absolute confidence in glycopeptide identification.

## Determine the Exact Location of Glycosylation

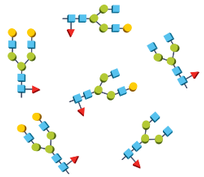
The Orbitrap Fusion Lumos Tribrid MS is the most sensitive Orbitrap instrument available and can perform a wide variety of analyses, from in-depth discovery experiments to characterization of complex PTMs to comprehensive qual/quan workflows. The availability of multiple fragmentation techniques - CID, HCD, ETD; combined ionization spectra such as EThcD; and also product dependent acquisition (such as HCDpdETD), which generates a pair of HCD and ETD spectra. At any stage of MSn, with fragment ion detection in either the ion trap or Orbitrap mass analyzer, offers a new level of versatility and performance for the most challenging applications. Such as, the identification and location of glycosylated peptides.

# WHICH GLYCANS ARE IN MY DRUG AND what is their concentration?

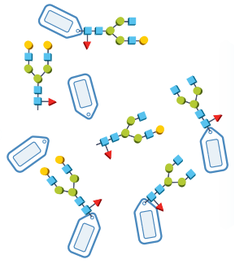
## Free Glycan Analysis Workflow



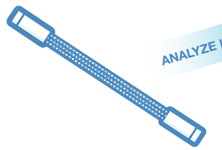
**INTACT GLYCOPROTEIN**
Isolate antibody or glycoprotein.



**RELEASE GLYCANS**
Glycans are released enzymatically using glycosidase.



**LABEL GLYCANS**
Chemically react to add either a fluorophore or aminoxy tandem mass tag (TMT).

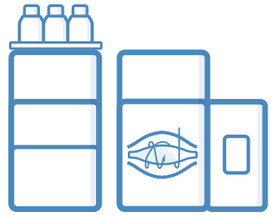


**SEPARATE**
Selective chemistries using combined HIC/WAX phases provide exceptional glycan separations.

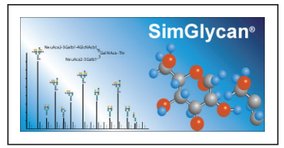
ANALYZE LC

**ANALYZE & QUANTIFY**
Separate and detect fluorescence labelled glycans.

ANALYZE LC-MS

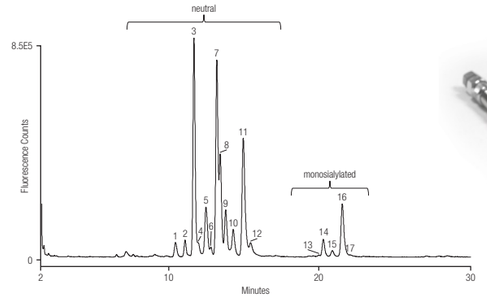


**ANALYZE & QUANTIFY**
Separate and detect unlabeled, fluorescence labelled or glycans.




## Characterize Free Glycans

You can predict the structure of released labelled or unlabelled glycans using tandem mass spectrometry together with SimGlycan® (PREMIER Biosoft). The software automatically matches experimental mass spectra against a comprehensive database and generates a scored list of candidate structures.




**Separation of labeled N-glycans from human IgG using a GlycanPac AXH-1 column.**

## GlycanPac AXH-1 & AXR-1 Columns

Thermo Scientific™ GlycanPac™ AXR-1 and GlycanPac AXH-1 columns are high-performance, silica-based HPLC columns designed for high-resolution glycan separations. They provide industry-leading resolution with unique selectivities for biologically important glycans, either labeled or native, using fluorescence and/or mass spectrometry (MS) detection. Both columns separate glycans by charge and size. Additionally the GlycanPac AXR-1 columns are superior for separating glycan isomers. The GlycanPac AXH-1 columns are also used for fast, accurate, and simple quantification of glycans by charge.

Monitoring of specifc glycan species or determination of relative quantities of a particular set of glycans provides important information for the development of biotherapeutics. Due to the complexity of glycan structures, quantiation and identification is performed upon release of glycans from the protein. N-linked glycans are released by enzymatic treartment, whereas O-linked glycans needs to be relased by chemical method as no enzyme exists for this purpose.

Glycans have no chromophore and have a poor response with conventional LC-UV detection. Glycans can be labelled with fluorescent tags prior to high sensitivity analysis by LC-Fluorescence detection. The most common label being 2-aminobenzamide (2-AB). Mass spectrometry has emerged as one of the most powerful tools for glycan structure elucidation. However, as most glycans do not ionize efficiently 2-AB labelling can also be performed to improve sensitivity. Thermo Scientific™ aminoxyTMT™ (Tandem Mass Tag™) reagents can also be used for labeling proteins or released glycans. This enables multiplexing experiments to be performed for quantitation of glycans by mass spectrometry.

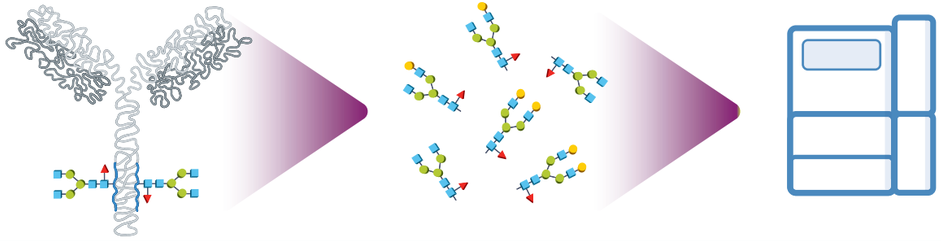
## Revolutionize Your UHPLC Experience

The Vanquish UHPLC system takes biomolecule separations to a new level. The system offers better, faster, separations than previously possible. The system has exceptional control and stability giving you unrivalled reproducibility for meaningful answers.

- Maximized separation power, flow and gradient precision
- Controlled separations with reproducible thermostatting
- Analyze more samples with Vanquish Charger module
- Compliant Thermo Scientific™ Chromeleon™ CDS software

# WHICH TYPES OF COMPLEX GLYCANS ARE attached to my drug?

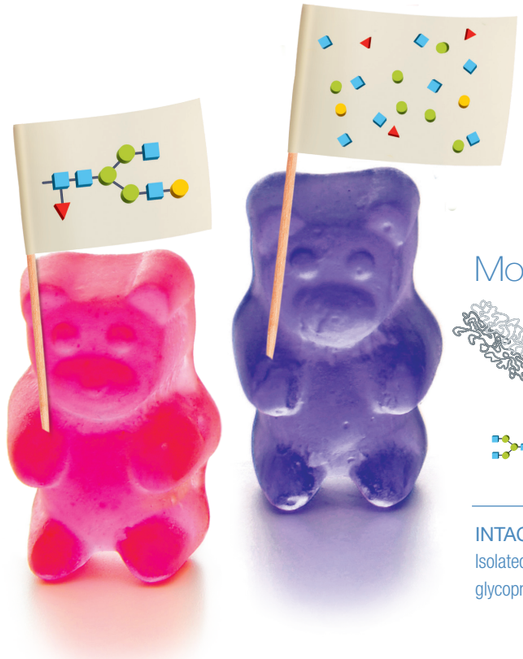
## Label-free Glycan Workflow



**INTACT GLYCOPROTEIN**
Isolated antibody or glycoprotein.

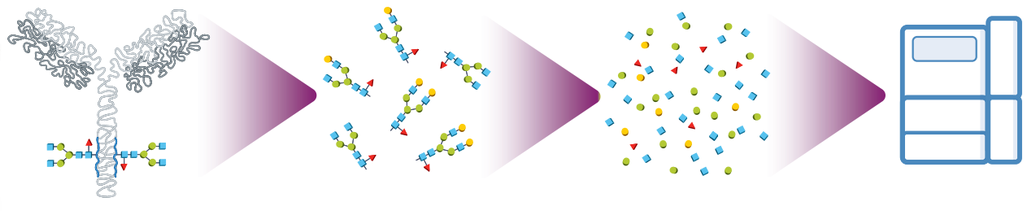
**RELEASE GLYCANS**
Glycans are released enzymatically using glycosidase.

**ANALYZE USING ION CHROMATOGRAPHY**
Simple, sensitive glycan analysis without labelling.



# WHAT IS THE COMPOSITION OF sugars in my drug entity?

## Monosaccharide Workflow



**INTACT GLYCOPROTEIN**
Isolated antibody or glycoprotein.

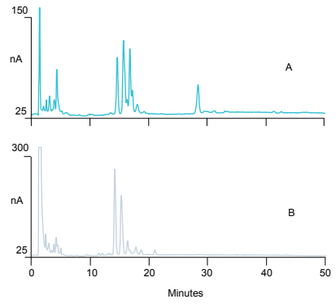
**RELEASE GLYCANS**
Glycans are released enzymatically using glycosidase.

**RELEASE MONOSACCHARIDES**
Glycans are released enzymatically using glycosidase. Glycans are then hydrolyzed to monosaccharides using acid.

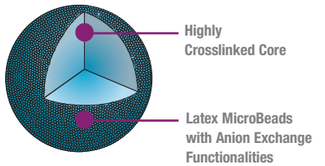
**ANALYZE USING ION CHROMATOGRAPHY**
Ultra sensitive monosaccharide and sialic acid analysis.

## HPAE-PAD

High performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) is a well-established analysis method to characterize released Glycans. The separation is exceptionally selective for sugars and resolves glycans based on charge, size, composition, isomers and linkages. It is even possible to separate glycans based on sialic acid linkage, providing valuable information not only about the sugar sequence of a glycan, but also the subtle linkage differences that may indicate disease states.

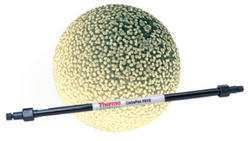


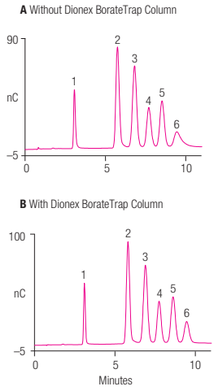

**Free unlabelled N-Linked Oligosaccharides from Monoclonal Antibody Preparations. Chromatograms:**
**A: PNGase F digest of human polyclonal IgG**
**B: PNGase F digest of humanized MAB M115**




## Unique CarboPac Chemistry

Resins of Thermo Scientific™ CarboPac™ columns consist of nonpourous beads covered witrh a fine latex of functionlized Thermo Scientific™ Dionex™ MicroBead™. This pellicular resin structure permits excellent mass transfer, resulting in high resolution chromatography and rapid reequilibration.






Monosaccharide composition, namly Fucose, Galactosamine, Glucosamine, Galactose, Glucose and Mannose, is routinly determined as their number and composition bound to the protein can impact efficacy of biotherapeutics. Monosaccharides are weak acids and can be separated by anion-exchange chromatography under basic conditions. Samples are acid hydrolyzed to release monosaccharides and analyzed by HPAE-PAD after chromatographic separation with CarboPac columns.

## High Performance Ion Chromatography™ for HPAE-PAD

The Thermo Scientific™ Dionex ICS-5000+ and ICS-4000 HPIC™ systems are ideal for HPAE PAD ion chromatography of mono-, di-, polysaccharides and sialic acids. With completely metal free flowpaths we eliminate the possibility of metal contamination and improve robustness for biomolecules. The analysis can be performed using Reagent-Free™ IC (RFIC™) systems with eluent generation that only require deionized water to electrolytically generate the eluent.

HPAE-PAD only detects those compounds that contain functional groups that become oxidized at the detection voltage employed. Detection is sensitive and highly selective for sugars, because many potentially interfering species cannot be oxidized or reduced, and are not detected.

## Join the Sweet Revolution in Biopharmaceuticals visit **www.thermoscientific.com/sweetrevolution**

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