Structural Analysis of Native *N*-Glycans Released from Proteins Using a Novel Mixed-Mode Column and a Hybrid Quadrupole-Orbitrap Mass Spectrometer

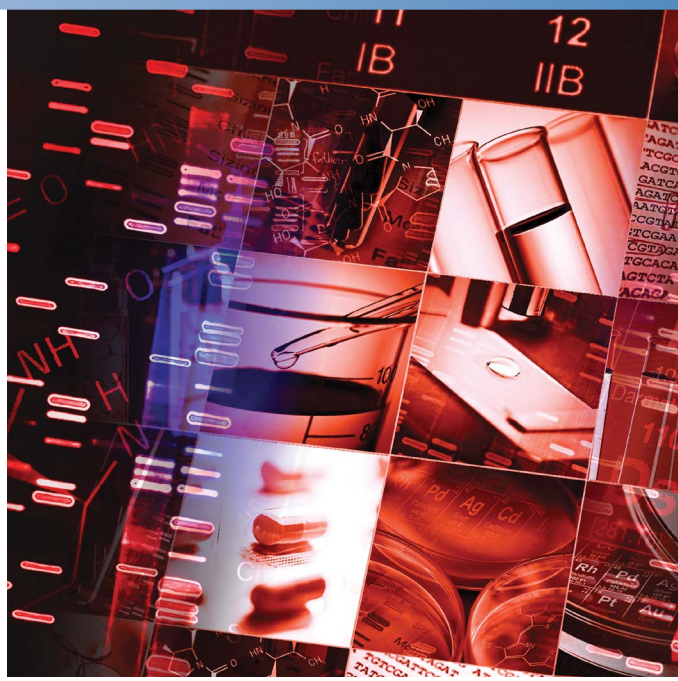
Udayanath Aich[1], Julian Saba[2], Xiaodong Liu[1] and Chris Pohl[1]
[1]Thermo Fisher Scientific, Sunnyvale, CA, USA; [2]Thermo Fisher Scientific, Mississuaga, ON, Canada

Application Note 20827

Key Words
Native *N*-glycans, 2AB labeled *N*-glycan, GlycanPac AXH-1, column chromatography, HILIC, weak anion exchange (WAX), mixed-mode, glycans, UltiMate 3000, Q Exactive, LC-MS/MS

Abstract
This application note compares two approaches for the structural characterization of *N*-glycans released from proteins. Analysis was carried out using a novel Thermo Scientific™ GlycanPac™ AXH-1 column and a Thermo Scientific™ Q Exactive™ hybrid quadrupole-Orbitrap™ mass spectrometer.

Differences are observed when analyzing native (unlabeled) and 2AB labeled *N*-glycans. Analysis of unlabeled glycans not only eliminates an extra reaction step and cumbersome cleanup methods during labeling, but also retains the original glycan profiles without adding further ambiguity imposed by labeling reactions.



Introduction
Glycans are polysaccharides or oligosaccharides present in natural systems, either in the free state or as conjugated species with other bio-molecules such as proteins or lipids. They play important roles in clinical, biological, and physiological processes, including recognition and regulatory functions, cellular communication, gene expression, cellular immunity, growth, and development [1].

The efficacy and safety of protein-based drugs, for example recombinant proteins and monoclonal antibodies (mAb), are often dependent on the structures and types of glycans attached [2]. Understanding, measuring, and controlling glycosylation and oligosaccharide content of glycoprotein products, as well as thorough characterization of biosimilars, has become increasingly important [3].

Traditionally, native glycans have been analyzed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD), which delivers high-sensitivity, high-efficiency separations of linkage and positional isomers based on charge and size [4]. However, the commonly used eluents for these separations are incompatible with most nebulizer-based detectors [e.g., mass spectrometer (MS), evaporative light scattering detector (ELSD), charged aerosol detector (CAD).

GlycanPac AXH-1 HPLC and UHPLC columns are specifically designed for structural analysis of glycans released from proteins. These columns are based on innovative mixed-mode surface chemistry combining both weak anion exchange and hydrophilic interaction liquid chromatography (HILIC) retention mechanisms. The weak anion exchange functionality provides retention and selectivity for negatively charged glycans while the HILIC mode facilitates the separation of glycans of the same charge according to their polarity and size.

MS detection methods were used for the simultaneous detection and identification of glycans released from proteins. It should be noted that other common detection technologies such as CAD can be used; however, these require larger quantities of glycan samples [5].

Thermo SCIENTIFIC

In this application note the differences observed when analyzing native (unlabeled) and 2AB labeled *N*-glycans derived from bovine fetuin are described. A GlycanPac AXH-1 column was used to separate the glycan species and a Q Exactive hybrid quadrupole-Orbitrap mass spectrometer was used for their detection and structural analysis.

Experimental Details

Consumables	Part Number
Deionized (DI) water, 18.2 MΩ-cm resistivity	
Fisher Scientific™ acetonitrile (CH3CN), HPLC grade	AC610010040
Fisher Scientific LC-MS grade formic acid	A117-50
Fisher Scientific ammonium formate	AC40115-2500
Thermo Scientific Premium 2 mL vial convenience kit	60180-600
Thermo Scientific™ HyperSep™ Hypercarb™ 6 mL SPE column	60106-403
Fisher Scientific trifluoracetic acid	28904
PNGase F and bovine fetuin were sourced from an accredited supplier	

Equipment	Part Number
Thermo Scientific™ Dionex™ UltiMate™ 3000 BioRS system, including pump: LPG-3400RS; thermal compartment: TCC-3000RS; pulled-loop well plate auto sampler: WPS-3000TRS; fluorescence detector with Dual-PMT: FLD3400RS; and 2 µL micro flow cell: 6078.4330	
Q Exactive hybrid quadrupole-Orbitrap mass spectrometer	
Thermo Scientific™ Savant™ SPD131DDA SpeedVac™ Concentrator	
Thermo Scientific Lyophilizer (Labconco® FreeZone® -105 °C 4.5 L benchtop freeze dry system)	16-080-207
Thermo Scientific 24-Port SPE vacuum manifold	60104-233

Buffer Preparation

Ammonium formate (80 mM, pH 4.4): Dissolve 5.08 ± 0.05 g of ammonium formate (Crystal) and 0.60 g of formic acid in 999.6 g of DI water. Sonicate the resulting solution for 5 minutes.

Sample Preparation

Release unlabeled *N*-glycans from glycoproteins with PNGase F enzyme and purify by HyperSep Hypercarb SPE cartridge (6 mL) under vacuum using a 24-Port SPE vacuum manifold.

Dissolve 2AB labeled *N*-glycan from fetuin (5000 pmol) in 25 µL DI water in a 250 µL autosampler vial.

Add 75 µL acetonitrile to the same vial and mix thoroughly.

Note: Store the standard at -20 °C.

Separation Conditions					Part Number
Column:	GlycanPac AXH-1, 2.1 × 150 mm, 1.9 µm				082472
Mobile Phase A:	Acetonitrile / water (80:20 v/v)				
Mobile Phase B:	Ammonium formate (80 mM, pH 4.4)				
Flow rate:	400 µL/min				
Column temperature:	30 °C				
Sample volume:	10 µL				
Sample amount:	500 pmol				
Mobile phase gradient:	Time (min)	% A	% B	Flow (mL/min)	Curve
	-10	97.5	2.5	0.4	5
	0	97.5	2.5	0.4	5
	30	87.5	12.5	0.4	5
	35	75.0	25.0	0.4	5
	40	62.5	37.5	0.4	5

MS Conditions	
MS instrument:	Q Exactive hybrid quadrupole-Orbitrap
Ionization mode:	Negative ion mode
MS scan range:	380-2000 *m/z*
Resolution:	70,000
AGC target	1×10^6
Max IT:	60 ms
dd-MS2 resolution:	17,500
MS/MS AGC target	2×10^5
MS/MS max IT:	250–1000 ms
Isolation window:	2 *m/z*
Dynamic exclusion:	90 s

Data Processing & Software	
Chromatographic software:	Thermo Scientific™ ChromQuest™ 5.0 Chromatography Data System
MS data acquisition:	Thermo Scientific™ Xcalibur™ 2.2 SP1.48
MS/MS data analysis:	SimGlycan® software (PREMIER Biosoft)

Results

A GlycanPac AXH-1 column was used for structural analysis of uncharged (neutral) and charged glycans present in proteins. Figure 1 shows the separation of native *N*-glycans from bovine fetuin on the GlycanPac AXH-1 column using MS detection. The combined HILIC and anion exchange retention mechanisms deliver unique selectivity. The separation of glycans is dependant on charge state: the neutral glycans elute first, followed by the negatively charged unlabeled *N*-glycans in increasing charge state from monosialylated to pentasialylated species. Within each charge envelope the glycans were further separated based on their size, isomeric structure, and polarity. The consequence of this is that a greater number of unique glycans are separated enabling more definitive characterization.

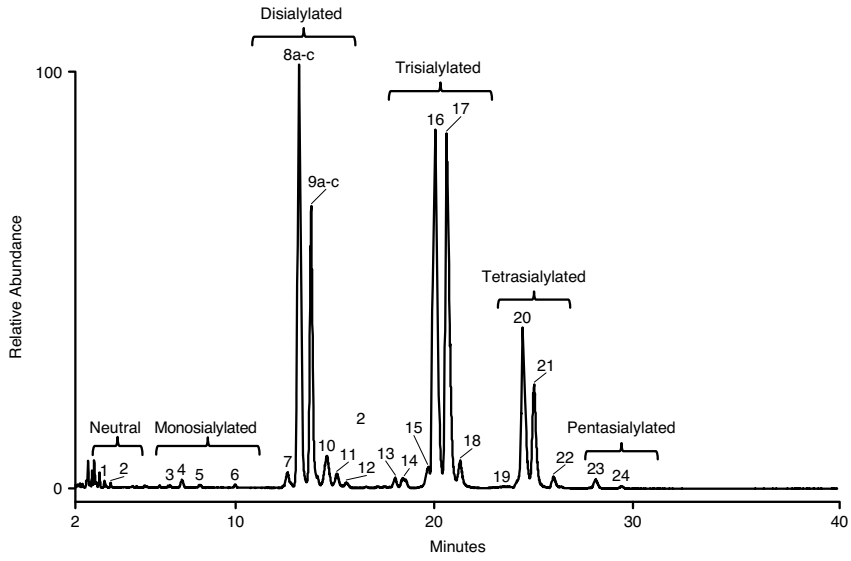


Figure 1: LC-MS analysis of native N-glycans released from bovine fetuin using the GlycanPac AXH-1 column

The structure of glycans was determined by LC-MS/MS. Data-dependant higher-energy collisional dissociation (HCD) MS/MS spectra were acquired on all precursor ions (z< 2). Raw data files from the Q Exactive mass spectrometer were directly input into SimGlycan software where the glycan structure was elucidated with database searching and scoring techniques [6]. HCD MS/MS spectra contain peaks relating to glycosidic and cross-ring fragments, thereby increasing confidence in identification. The detailed structural information obtained (Table 1) from the MS/MS data for native *N*-glycans from fetuin (a representative MS/MS spectra is shown in Figure 3) further validated the ability of the GlycanPac AXH-1 column to separate glycans based on charge, size, isomers, and polarity.

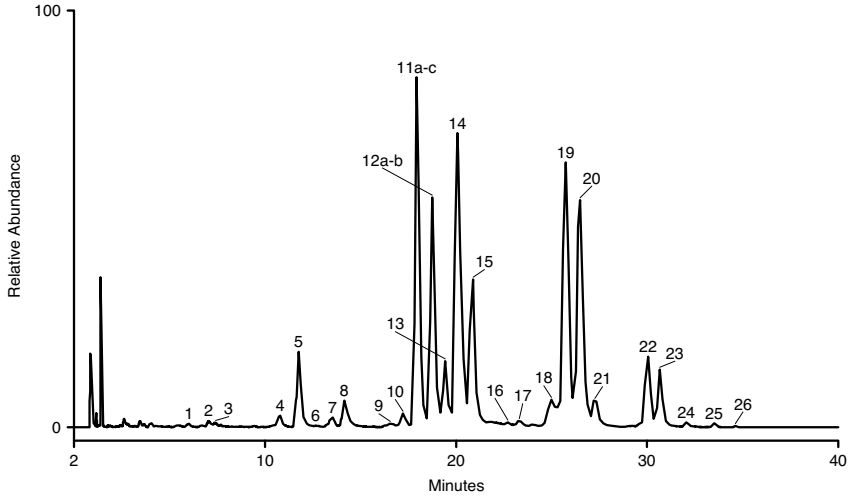


Figure 2: LC-MS analysis of 2AB labeled *N*-glycans from bovine fetuin using the GlycanPac AXH-1 column

Chromatographic profiles for native *N*-glycans are different from the profile of fluorescently (2AB) labeled *N*-glycans, especially for mono and disialylated glycans. The profiles of the native *N*-glycans within the disialylated charge envelope (peaks 8 and 9, Figure 1) are similar to peaks 11 and 12 of the 2AB labeled glycans (Figure 2).

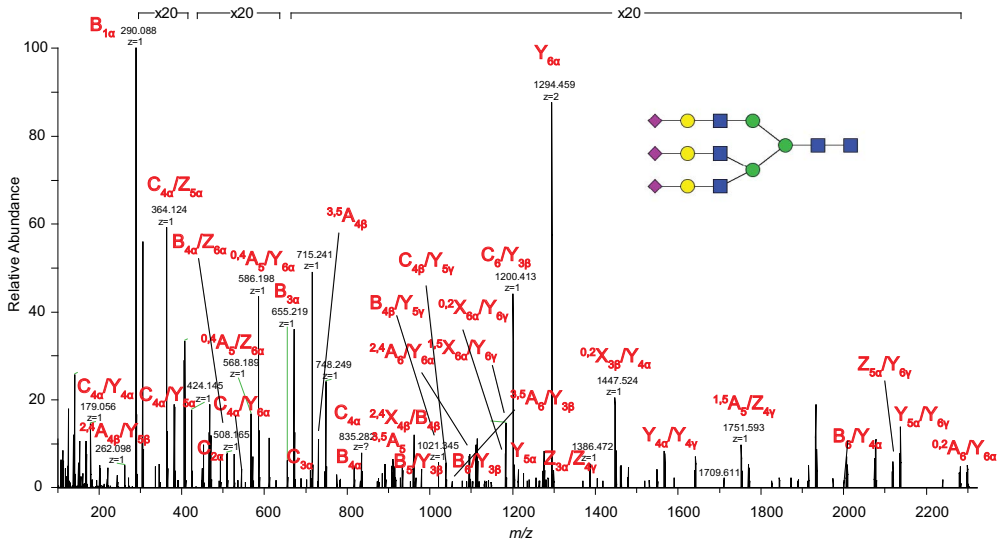
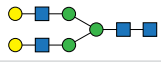
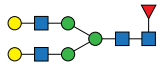
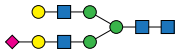
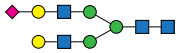
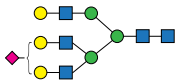
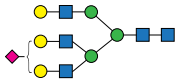
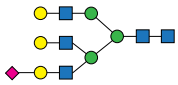
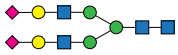
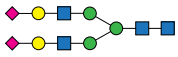
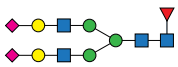
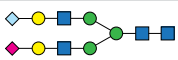
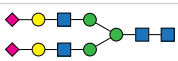
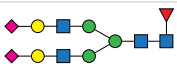
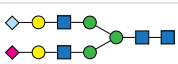
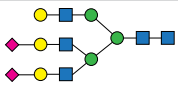
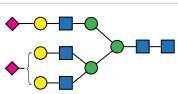
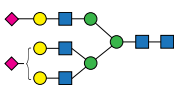
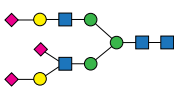


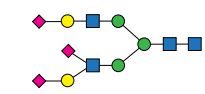
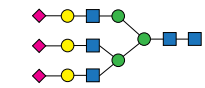
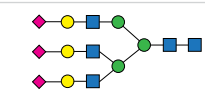
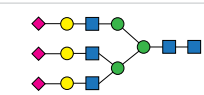
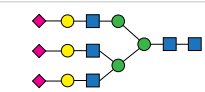
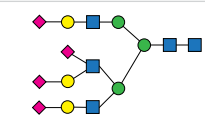
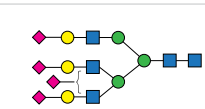
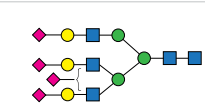
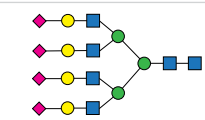
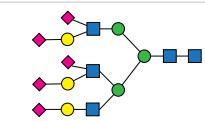
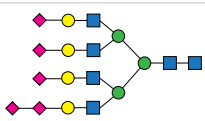
Figure 3: MS/MS spectra for a native trisialylated triantennary *N*-glycans released from bovine fetuin

Another observable difference is that in the case of native glycans, peaks 11 and 12 (Figure 1) are minor peaks as compared to the major peaks 14 and 15 for labeled glycans (Figure 2). However, the identity of glycans represented by peaks 10 to 12 in the native *N*-glycan sample is the same as in peaks 13 to 15 in the labeled glycan.

The observed differences between 2AB labeled and unlabled glycans can be attributed to artifactual additional peaks being present after the derivatization, or enhanced concentrations of lower-charged glycans being present. This is due to some of the trisialylated and tetrasialylated charge state glycans being converted to lower charge states (for example, disialylated glycans).

When possible, analyzing unlabeled glycans eliminates the extra reaction step and the time consuming cleanup methods required during labeling and retains the original glycan profile without the changes that the labeling reaction can introduce. However the GlycanPac AXH-1 column is useful for the analysis of both native and labeled *N*-glycans allowing greater flexibility.

Peak	Compound Stucture
1	
2	
3	
4	
5a	
5b	
5c	
6	
7a	
7b	
7c	
8a	
8b	
8c	
9	
10	
11	
12	

Peak	Compound Stucture
13	
14	
15	
16	
17	
18	
19	
20	
21	
22	
23	

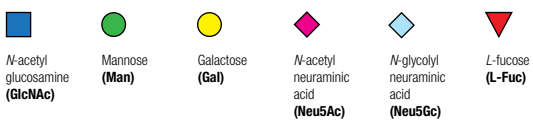


Table 1: Structural characterization of glycans present in each peak by the separation of native *N*-glycans from bovine fetuin using GlycanPac AXH-1 column

Conclusion

- Structural analysis of native *N*-glycans from proteins was successfully achieved using GlycanPac AXH-1 column.
- The GlycanPac AXH-1 column separates glycans with unique selectivity based on charge, size, and polarity, which helps in glycan structure identification by mass spectrometry.
- LC/MS or LC-MS/MS analysis of native glycans from proteins was demonstrated using GlycanPac AXH-1 columns.
- The Q Exactive hybrid quadrupole-Orbitrap mass spectrometer provides excellent MS/MS fragmentation information, allowing characterization of the structure of native *N*-glycans.
- Characterization of native *N*-glycans retains the original glycan profile that can be altered by labeling reactions.

References

[1] Varki, A. Biological Roles of Oligosaccharides: All the Theories are Correct. *Glycobiology* **1993**, *3*, 97–130.

[2] Bertozzi, C. R.; Freeze, H.H.; Varki, A. and Esko, J.D. Glycans in Biotechnology and the Pharmaceutical Industry, Essentials of Glycobiology. 2nd edition. Chapter 51 Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2009.

[3] Guidance for Industry, Scientific Considerations in Demonstrating Biosimilarity to a Reference Product, 2012, U.S. (www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM291128.pdf).

[4] CarboPac PA 200 data sheet (http://www.dionex.com/en-us/webdocs/7087-DS_CarboPac_PA200_25Oct06_LPN1618-03.pdf).

[5] Hvizd, M. G.; Bailey, B.; Crafts, C., Plante, M. and Acworth, I. (2011) Simple Separation and Detection Techniques for the Analysis of Carbohydrates (Application note:110521-PO-IC-Carbohydrates-21Nov2011-LPN2954-01.pdf).

[6] Apte, A and Meitei, N.S., Bioinformatics in Glycomics: Glycan Characterization with Mass Spectrometric Data using SimGlycan® software, *Meth. in Mol. Biol.*, **2010**, *600*, 269-81.

thermofisher.com/glycan

USA and Canada +1 800 332 3331
Australia 1300 735 292 (free call domestic)
China 800 810 5118 (free call domestic)
400 650 5118
France +33 (0)1 60 92 48 34
Germany +49 (0) 2423 9431 20 or 21
India +91 22 6742 9494
+91 27 1766 2352
Japan 0120 753 670 (free call domestic)
0120 753 671 fax
United Kingdom +44 (0) 1928 534 110
New Zealand 0800 933 966 (free call domestic)
Singapore +65 6289 1190
All Other Enquiries +44 (0) 1928 534 050

Technical Support
For advice and support, please visit our website:
www.thermoscientific.com/chromexpert

Thermo SCIENTIFIC
Part of Thermo Fisher Scientific

AN20827_E 07/16S