Structural Analysis of Labeled *N*-Glycans from Proteins by LC-MS/MS Separated Using a Novel Mixed-Mode Stationary Phase

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Abstract
This application note describes the liquid chromatography-mass spectrometry (LC/MS) analysis of fluorescently labeled *N*-glycans released from proteins. The chromatographic separation is carried out with a novel Thermo Scientific™ GlycanPac™ AXH-1 (1.9 µm, 2.1 × 150 mm) column for high-resolution and high-throughput analysis of glycans. This column possesses unique selectivity that provides separation of glycans based on charge, size, and polarity. MS and MS/MS analyses are performed using a Thermo Scientific™ Q Exactive™ hybrid quadrupole-Orbitrap™ mass spectrometer in negative ion mode to provide detailed structural information of *N*-glycans released from proteins.

Introduction

Glycans are involved in a wide range of biological and physiological processes, including recognition and regulatory functions, cellular communication, gene expression, cellular immunity, growth, and development [1]. Glycans are commonly investigated as important species in therapeutic protein drug development because there is strong evidence that bioactivity and efficacy are affected by glycosylation [2]. Commonly, both the structure and types of glycans attached to the proteins are examined. Understanding, measuring, and controlling glycosylation in glycoprotein-based drugs, glycan content of glycoprotein products, as well as thorough characterization of biosimilars has become increasingly important.

The structures of glycans are highly diverse, complex, and heterogeneous due to post-translational modifications. This makes it challenging to comprehensively characterize glycan profiles and determine their structures [3]. It is therefore essential to separate all isomeric, charge, and branching glycan variations to understand the detailed structure of the glycans by LC-MS/MS methods.

Various HPLC separation modes have been used for the analysis of glycans, including normal phase (NP) or hydrophilic interaction (HILIC) chromatography, ion-exchange (IEX) chromatography, and reversed-phase (RP) chromatography. Because they are highly hydrophilic, polar substances, neutral glycans are commonly separated using amide HILIC columns, such as the Thermo Scientific™ Accucore™ 150-Amide-HILIC column [4], which separates glycans by hydrogen bonding, resulting in a size and composition-based separation. This type of column is particularly useful for the separation of glycans released from monoclonal antibodies, of which the majority are neutral [5].

Based on novel mixed-mode surface chemistry, the GlycanPac AXH-1 column combines both weak anion-exchange (WAX) and HILIC retention mechanisms for optimal selectivity and high resolving power [6]. The WAX 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. As a result, the GlycanPac AXH-1 column

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provides unparalleled capabilities for glycan separations. In addition, this column has the flexibility to be used in a purely ion-exchange, charge-based separation mode for the separation of various glycans without discrimination of size and polarity. This makes it a suitable tool for accurate quantification of glycans based on charge, which cannot be achieved with any other HPLC/UHPLC columns on the market. The GlycanPac AXH-1 column is designed for use with LC-fluorescence detection and LC/MS applications using volatile aqueous buffers (e.g., ammonium acetate or ammonium formate) and acetonitrile. The substrate of the GlycanPac AXH-1 column is based on high purity 1.9 µm and 3.0 µm spherical silica for UHPLC and standard HPLC applications, respectively.

In this application note we highlight the use of a GlycanPac AXH-1 column and Q Exactive hybrid quadrupole-Orbitrap mass spectrometer for the structural analysis of a 2-aminobenzamide (2AB) labeled *N*-glycans from bovine fetuin.

Experimental Details

Consumables	Part Number
GlycanPac AXH-1, 1.9 µm, analytical column (2.1 × 150 mm)	082472
Deionized (D.I.) water, 18.2 MΩ-cm resistivity	
Fisher Scientific™ HPLC grade acetonitrile (CH_3CN)	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
New England Biolabs® PNGase F	P0705L
Bovine fetuin from a commercial source	
Thermo Scientific™ Hypercarb™ 6 mL cartridge	60106-403
Fisher Scientific trifluoracetic acid	28904
Fisher Scientific sodium cyanoborohydride	AC16855-0500
Fisher Scientific anthranilamide (2AB)	AC10490-5000
Fisher Scientific glacial acetic acid	AA36289AP

Equipment	Part Number
Thermo Scientific™ Dionex™ UltiMate™ 3000 Bio-RS system, consisting of LPG-3400RS pump, TCC-3000RS thermal compartment, WPS-3000TRS pulled-loop well plate autosampler, FLD3400RS fluorescence detector with Dual-PMT, and a 2 µL micro flow cell	6078.4330
Q Exactive hybrid quadrupole-Orbitrap mass spectrometer	
Thermo Scientific™ SpeedVac™ system	
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 D.I. water. Sonicate the resulting solution for 5 minutes.

Sample Preparation

1. Native *N*-glycans are released from glycoproteins with PNGase F enzyme and purified by a Hypercarb cartridge (6 mL) with the help of 24-port SPE vacuum manifolds under vacuum. The released glycans are conjugated with a 2-amino benzamide (2AB) label group using the reported procedure of Bigge et al. [7].

2. Dissolve 2AB labeled *N*-glycan from fetuin (5,000 pmol) in 25 µL D.I. water in a 250 µL autosampler vial.

3. Add 75 µL acetonitrile to the same vial and mix till uniformity.

Note: Store the standard at -20 °C.

Separation Conditions					
Column:	GlycanPac AXH-1, 2.1 x 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)				
Column temperature:	30 °C				
Sample volume:	1 µL				
Gradient:	Time (min)	%A	%B	Flow Rate (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 MS
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:	1000 ms
Isolation window:	2 *m/z*
Dynamic exclusion:	90 s

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

Results

Glycan Separation by Charge, Size and Polarity

Figure 1 shows the separation of neutral and acidic 2AB labeled *N*-glycans from bovine fetuin using a GlycanPac AXH-1 (1.9 µm, 2.1 × 150 mm) column. The glycan elution profile consists of a series of peaks grouped into several clusters in which the neutral glycans elute first, followed by monosialylated, disialylated, trisialylated, tetrasialylated, and finally pentasialylated species. Analytes in each cluster represent glycans of the same charge. Within each cluster, the glycans having the same charge are further separated according to their sizes and polarity by HILIC interaction. The structure of the glycans present in each peak was determined in an LC-MS/MS study as shown in the following section.

Structural elucidation

The 2AB labeled *N*-glycans from bovine fetuin were separated on the GlycanPac AXH-1 column based on the separation conditions using a two eluent system and analyzed on a Q Exactive benchtop mass spectrometer. The total ion chromatogram (TIC) is shown in Figure 1. For structural elucidation, data dependant MS/MS spectra were acquired on all precursor ions ($z \leq 2$) and SimGlycan software from PREMIER Biosoft was used for data analysis [8]. The detailed structural information obtained (Table 1) from the MS/MS data further validated the ability of GlycanPac AXH-1 columns to separate glycans based on charge, size, isomers, and polarity. These results also confirmed that the GlycanPac AXH-1 column would be ideal for MS use.

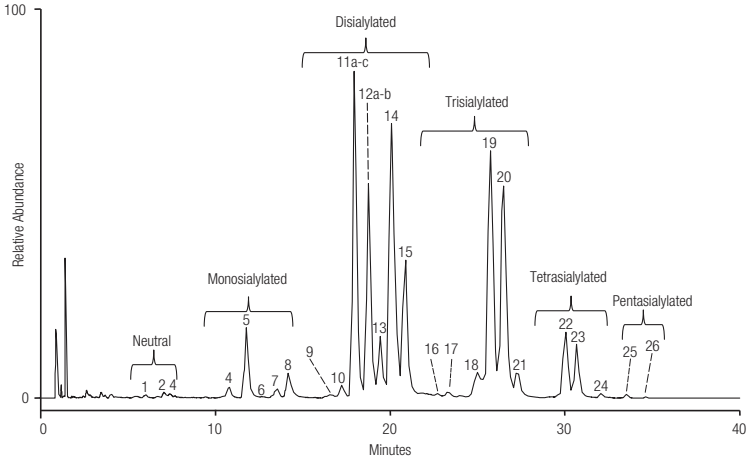

Figure 1: LC/MS analysis of 2AB labeled *N*-glycans from bovine fetuin using the GlycanPac AXH-1 column

Peak	Compound Stucture (2AB labeling are not shown)
1	
2	
4	
5	
6	
7	
8	
9	
10	
11a	
11b	
11c	
12a	
12b	
13	
14	
15	
16	
17	
18	
19	
20	
21	
22	
23	
24	
25	
26	

N-acetyl glucosamine **(GlcNAc)**

Mannose **(Man)**

Galactose **(Gal)**

N-acetyl neuraminic acid **(Neu5Ac)**

N-glycolyl neuraminic acid **(Neu5Gc)**

L-fucose **(L-Fuc)**

Table 1: Structural characterization of glycans present in each peak by the separation of 2AB labeled *N*-glycans from bovine fetuin using a GlycanPac AXH-1 column

Conclusion

The GlycanPac AXH-1 column is a high-performance, silica-based HPLC column for simultaneous separation of glycans by charge, size, and polarity. It is designed for high-resolution and high-throughput analysis with unique selectivity for biologically important glycans. We have demonstrated that this column provides unique selectivity and excellent resolution for glycans released from fetuin.

LC-MS/MS analysis of 2AB labeled *N*-glycans derived from glycoproteins are demonstrated using GlycanPac AXH-1 columns. The Q Exactive hybrid quadrupole-Orbitrap instrument provides excellent MS/MS fragmentation information to enable characterization of glycan structures.

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