Separation of 2AB-Labeled *N*-Linked Glycans from Bovine Fetuin on a Novel Ultra High Resolution Mixed-Mode Column

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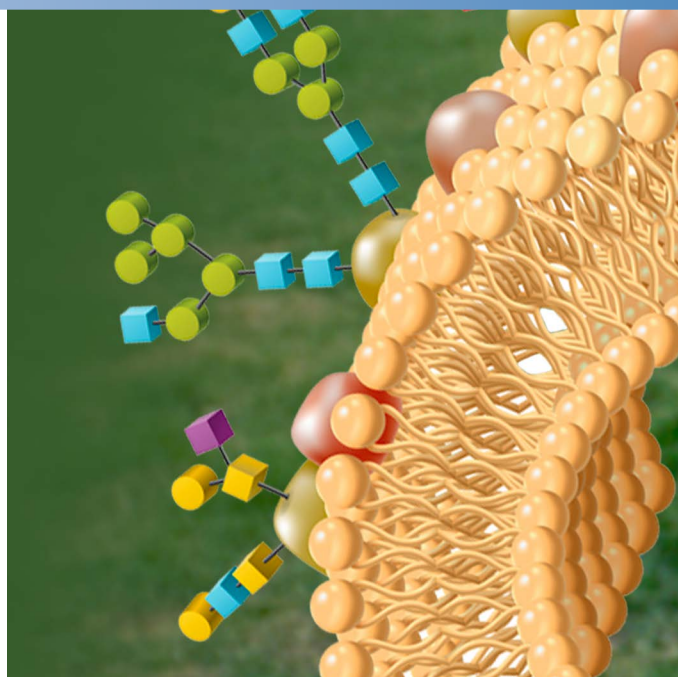
Key Words

GlycanPac AXR-1, charge based separation, isomeric separation, mixed-mode chromatography, 2AB-labeled *N*-linked glycans, bovine fetuin, glycan analysis

Abstract

This application note demonstrates the separation of 2AB-labeled *N*-linked glycans released from proteins (bovine fetuin) by reversed phase (RP) / weak anion exchange (WAX) mixed-mode chromatography. The separation is carried out with both 1.9 µm and 3 µm Thermo Scientific™ GlycanPac™ AXR-1 columns using fluorescence detection. The method exhibits an excellent separation based on charge, isomeric structure and size.



Introduction

Glycans are involved in a wide range of biological and physiological processes including cell and pathogen recognition, regulatory functions, cellular communication, gene expression, cellular immunity, growth and development. The functions of proteins are often dependent on the structure and types of their attached oligosaccharides. *N*-linked glycans are commonly investigated as important species in therapeutic protein drug development because there is strong evidence that bioactivity and efficacy are altered by glycosylation. Understanding, measuring and controlling glycosylation in glycoprotein- based drugs, glycoprotein products and biosimilars is increasingly important. However, glycan structures are highly diverse, complex and heterogeneous due to branch and linkage isomers that make comprehensive glycan characterization challenging [1].

Various HPLC separation modes have been developed for glycan analysis, including normal phase (or hydrophilic interaction, HILIC), ion-exchange and reversed-phase [2,3]. Because glycans are very hydrophilic (polar) a common separation mode utilizes amide HILIC columns; as exemplified by the Thermo Scientific™ Accucore™ 150-Amide-HILIC column [4], that resolves glycans based on hydrogen bonding, resulting in size and composition-based separations. Amide HILIC columns are particularly useful for the separation of 2AB-labeled *N*-linked glycans released from antibodies, for example MAbs, in which the majority of glycans harbor no charge. However, Amide HILIC amide columns do not provide adequate separations when glycans harbor 2 or more charge states (e.g., neutral and mono- or di-sialylated *N*-linked glycans) because glycan isoforms with different charge states are intermingled in the separation envelope.

Recently we developed a mixed-mode column (Thermo Scientific™ GlycanPac™ AXH-1) with both weak anion-exchange (WAX) and hydrophilic interaction (HILIC) properties [5] which separates *N*-linked glycans based on charge, polarity, and size. The GlycanPac AXH-1 provides improvements to the amide HILIC phases due to its charge-based separation. This column enjoys broad applicability for qualitative, quantitative and structural analysis of both labeled (2AB- and 2AA-) and native *N*-linked glycans from proteins using fluorescence and/or mass spectrometry (MS) detection [6]. Here we describe the new GlycanPac AXR-1 mixed-mode column that further improves separations by resolving glycans into different charge groups, and also separates glycans within

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each charge group based on isomerization and size, producing substantially increased resolution of *N*-linked glycans.

The GlycanPac AXR-1 column is based on novel mixed-mode column chemistry, combining the retention mechanisms of both WAX and reversed phase (RP) properties for optimal selectivity and resolution. The WAX functionality provides retention and selectivity for negatively charged glycans, while the reversed phase mode facilitates the separation of glycans of the same charge according to their isomeric structure, and size. As a result the GlycanPac AXR-1 column provides industry-leading resolution of charged 2AB-labeled *N*-linked glycans. The GlycanPac AXR-1 column is designed for, and tested with, LC-fluorescence detection and LC-MS applications using volatile aqueous buffers (e.g., ammonium acetate or ammonium formate) with, or without acetonitrile. The substrate of the GlycanPac AXR-1 column is a modified high-purity spherical silica. The column is available in both 1.9 µm particle size for UHPLC and 3.0 µm particle size for HPLC applications.

Experimental Details

Consumables	Part Number
Deionized water, 18.2 MΩ-cm	
Fisher Scientific HPLC grade acetonitrile	AC610010040
Fisher Scientific LC-MS grade formic acid	A117-50
Fisher Scientific ammonium formate	AC401152500
Unlabeled non-reduced glycans are released from glycoproteins with PNGase F enzyme (New England BioLabs, P0705L). The released glycans are conjugated with the 2-amino benzamide (2-AB) label group with slight modification from the reported procedure of Bigge et. al. [7]	
Fisher Scientific 2-Amino benzamide	AC 10490-1000
Fetiun *N*-linked glycan library, labeled with 2AB	Prozyme GKSB-002

Buffer Preparation

Ammonium formate (100 mM, pH 4.4): Dissolve 6.35 ± 0.05 g ammonium formate and 0.70 ± 0.05g formic acid in 999.6 g of D.I. water. Confirm pH to 4.4 using a pH meter. Mix this solution well and filter through a 0.2 µm pore filter.

Sample Preparation

Dissolve 2AB-labeled *N*-linked glycans from fetuin or individually labeled standards (approximately 5000 pmol each) in 100 µL D.I. water in a 250 µL autosampler vial. Samples are ready for injection. Inject 1–5 µL.

Note: store the standard at -20 oC

Instrumentation

Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC Analytical LC system consisting of DGP-3600RS pump, TCC-3000RS thermal compartment, WPS-3000TRS autosampler, FLD3400RS fluorescence detector (with Dual-PMT) and a biocompatible 2 µL micro flow cell (6078.4330)

Separation Conditions for Figure 1

Column:	GlycanPac AXR-1 (1.9 µm, 150 × 2.1 mm)
Mobile Phase:	A: D.I. water
	B: Ammonium formate (100 mM, pH 4.4)
Flow rate:	400 µL/min
Initial pressure:	~300 bar with new column
Column temperature:	30 °C
Sample amount:	100 pmoles
Fluorescence detector:	λ_{Ex} = 320 nm & λ_{Em} = 420 nm

Separation Conditions for Figure 2	
Column:	GlycanPac AXR-1 (1.9 µm, 250 × 2.1 mm)
Mobile Phase:	A: Acetonitrile
	B. D.I. water
	C: Ammonium formate (100 mM, pH 4.4)
Flow rate:	400 µL/min
Initial pressure:	~500 bar with new column
Column temperature:	30 °C
Samples amount:	100 pmoles
Fluorescence detector:	λ_{Ex} = 320 nm & λ_{Em} = 420 nm

Data Processing	
Software:	Thermo Scientific™ Dionex™ Chromeleon™ 6.8 Chromatography Data System

Results

The GlycanPac AXR-1 column is designed for high-resolution separation of charged and neutral glycans present in glycoproteins, glycolipids and glycopolymers. It is noted that the GlycanPac AXR-1 works exceptionally well for charged *N*-linked glycan species.

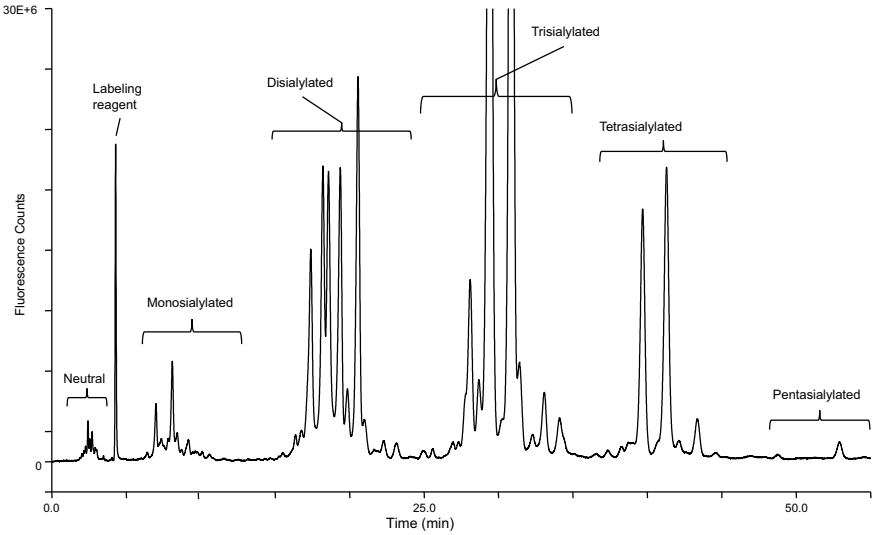

Figure 1: Separation of 2AB labeled *N*-linked glycans from bovine fetuin by charge, size, polarity and isomeric structure using the GlycanPac AXR-1 (1.9 µm, 150 × 2.1 mm) column and a binary gradient

Time (min)	%A	%B
-10	93	7
0	93	7
80	0	100
84	0	100

Table 1: Binary gradient for GlycanPac AXR-1 150 × 2.1 mm column (1.9 µm)

Figure 1 shows the separation of 2AB-labeled *N*-linked glycans from bovine fetuin using a GlycanPac AXR-1 (1.9 µm; 150 × 2.1 mm) column with a binary gradient (Figure 1, Table 1). The glycan elution profile consists of a series of peaks grouped into several clusters with neutral glycans eluting first, near the void, followed by monosialylated, disialylated, trisialylated, tetrasialylated and finally pentasialylated species. Peaks in each cluster represent the glycans of the same charge, grouped by ion exchange interaction. Within each cluster glycans with the same charge are further separated according to their isomeric structures and size by reversed phase interaction. *N*-linked glycan structures present in each peak were identified using LC-MS/MS (data not shown). Using the binary gradient the 150 × 2.1 mm GlycanPac AXR-1 (1.9 µm) resolved more than 70 components from the 2AB-labeled bovine fetuin *N*-linked glycans.

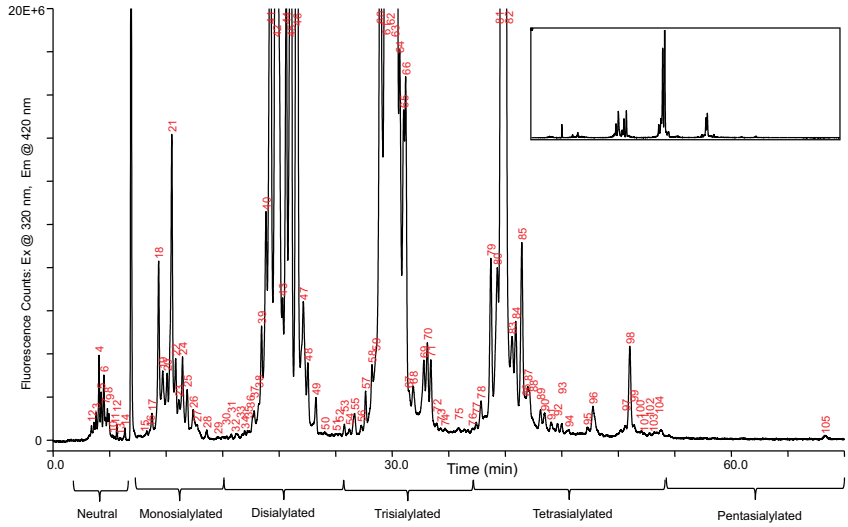

Figure 2: Improvements to resolution of fetuin 2AB-labeled *N*-linked glycans on a longer, 250 × 2.1 mm GlycanPac AXR-1 column. Inset is full Chromatogram

Time (min)	%A	%B	%C
-10	0	93	7
0	0	93	7
90	25	0	75
90.5	0	0	100

Table 2: Ternary gradient for 250 × 2.1 mm GlycanPac AXR-1 column (1.9 µm)

The effect of column length and a ternary gradient program (Table 2) are shown in Figure 2, where the 250 × 2.1 mm GlycanPac AXR-1 revealed at least 105 glycan-containing peaks in less than 70 minutes. In the 250 mm column, acetonitrile starts at 0% and increases over 90 minutes to 25 %. The longer column also results in more efficient peaks, thus increasing detection sensitivity for glycans.

Conclusion

- The GlycanPac AXR-1 is designed to provide high- resolution separations for biologically important complex glycans based on charge, isomeric structure and size.
- The GlycanPac AXR-1 column provides exceptional selectivity and resolution for 2AB-labeled *N*-linked glycans from bovine fetuin.
- The GlycanPac AXR-1 accomodates injection of fully aqueous samples as native or 2AB-derivatized *N*-linked glycans.

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