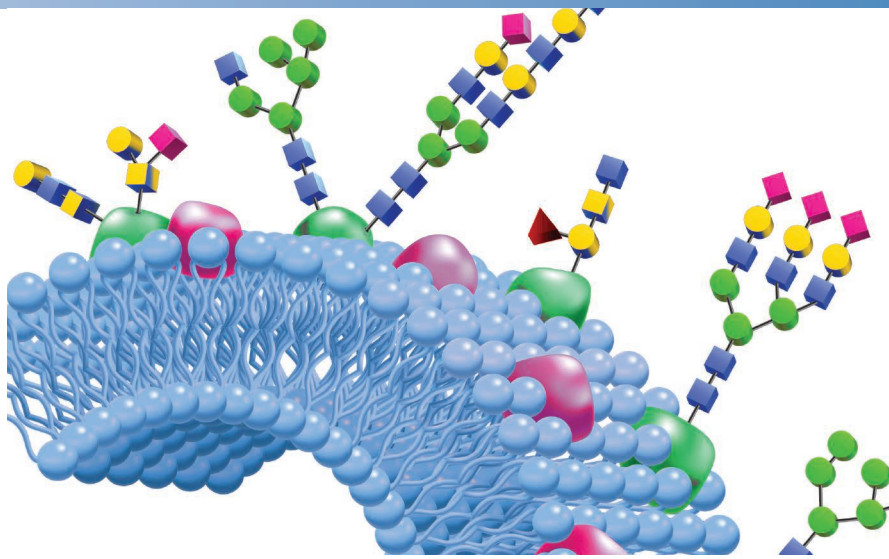
GlycanPac AXH-1 Column

High-Resolution Columns for Glycan Analysis

The Thermo Scientific™ GlycanPac™ AXH-1 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, either labeled or native, by LC-fluorescence and LC-MS methods.

Product features include:

- Unique glycan selectivity based on charge, size and polarity
- Excellent resolution for both native and labeled glycans
- Useful for both high-resolution glycan profile characterization and easy quantification of glycans based on charge
- Compatible with fluorescence and MS detection methods
- High chromatographic efficiency and excellent column stability



Introduction

Glycans are widely distributed in biological systems in “free state” as well as conjugated forms such as glycoproteins, glycolipids, and proteoglycans. They 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. The functions of glycans are often dependent on the structure and types of oligosaccharides attached to the proteins. The structures of glycans are highly diverse, complex and heterogeneous due to post-translational modifications and physiological conditions. Thus, it is highly challenging to comprehensively characterize glycan profiles and determine their structures.

The biological functions of glycans are dependent on the structure, conformation and the types of oligosaccharides attached to the proteins or lipids. Oligosaccharides are covalently attached to proteins through two structural motifs. Those attached to asparagine via an *N*-linkage are referred to as *N*-linked glycans. Glycans may also be attached to proteins through serine or threonine via an *O*-linkage and are referred to as *O*-linked glycans. Both types of glycans are commonly investigated as important species in therapeutic protein drug development, as there is strong evidence that bioactivity and efficacy are affected by glycosylation. Understanding, measuring, and controlling glycosylation in glycoprotein-based drugs, the oligosaccharide content of glycoprotein products, as well as thorough characterization of biosimilars has become increasingly important.

Various modes of HPLC separation have been adopted for the analysis of glycans, including normal phase (NP) or hydrophilic interaction (HILIC) chromatography, ion-exchange (IEX) chromatography and reversed-phase (RP) chromatography. Because glycans are highly hydrophilic and polar substances, they are commonly separated using an amide HILIC column which separates glycans mainly by hydrogen bonding, resulting in size and composition-based separation. However, one limitation of this approach is that glycan identification and quantitation become highly challenging because glycans of different charge states are intermingled in the separation envelope. The GlycanPac AXH-1 column overcomes these limitations and can separate glycans based on charge, size and polarity. In addition, this column allows for easy quantitation of glycans solely based on charge without discrimination of the size and polarity of glycans. The GlycanPac AXH-1 column provides greater selectivity, higher resolution and faster analysis compared to other HPLC columns for glycan analysis.

Column Technology

Based on innovative mixed-mode surface chemistry, the GlycanPac AXH-1 column combines both weak anion-exchange (WAX) and HILIC retention mechanisms for optimal selectivity and resolution power. 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 the result, the GlycanPac AXH-1 column provides unparalleled capabilities for glycan separations. In addition, this column offers charge-based separations for various glycans without discrimination of size and polarity, making it a suitable tool for accurate quantification of glycans based on the charge, which cannot be achieved with other HPLC column. The GlycanPac AXH-1 column is designed and tested for LC-fluorescence detection and LC-MS applications using volatile aqueous buffers (e.g., ammonium acetate or ammonium formate) and acetonitrile. Because the surface chemistry is customized, both neutral and charged glycans can be eluted at low buffer concentrations to achieve optimal MS sensitivity. While several chromatographic conditions are provided as the starting point for method development, further optimization can be undertaken by adjusting mobile phase buffer concentration, pH, temperature, solvent content, and gradients.

The GlycanPac AXH-1 column's silica substrate are spherical and high purity and are available in both 1.9 µm and 3.0 µm sizes for UHPLC and standard HPLC applications, respectively. The GlycanPac AXH-1 columns are available in various dimensions for applications of different resolution, throughput and instrument requirements.

Applications

Glycan Analysis by LC/Fluorescence

The GlycanPac AXH-1 column can be used for qualitative, quantitative, and structural characterization of uncharged and charged glycans present in biological molecules (e.g. proteins). Figure 1 shows the separation of neutral and acidic 2AB labeled *N*-glycan 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 retained by anion exchange interactions represent the 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. This interpretation is substantiated by injecting 2AB labeled glycan standards with different charges under the same conditions (Figure 2) and with the same MS results.

Figure 3 demonstrates the separation obtained for the same sample on a GlycanPac AXH-1 column packed with 3 µm particles. Compared to its 1.9 µm counterpart, the 3 µm GlycanPac AXH-1 column operates at 50% lower backpressure with excellent resolution. Thus, it is compatible with standard HPLC systems.

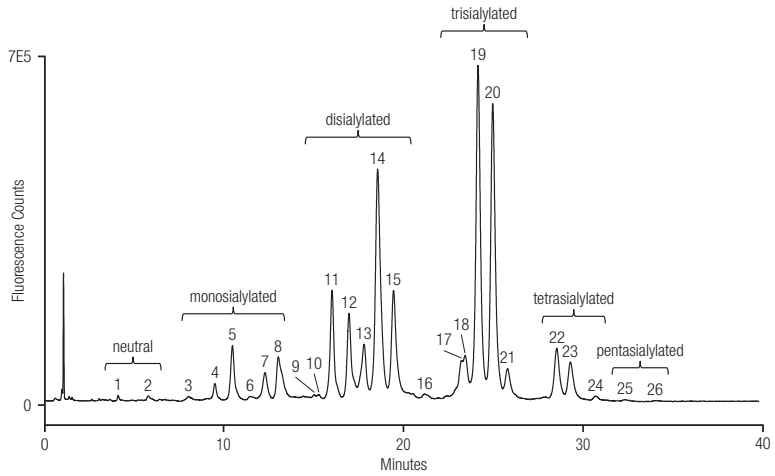


Figure 1: Separation of 2AB labeled *N*-glycans from bovine fetuin by charge, size and polarity.

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

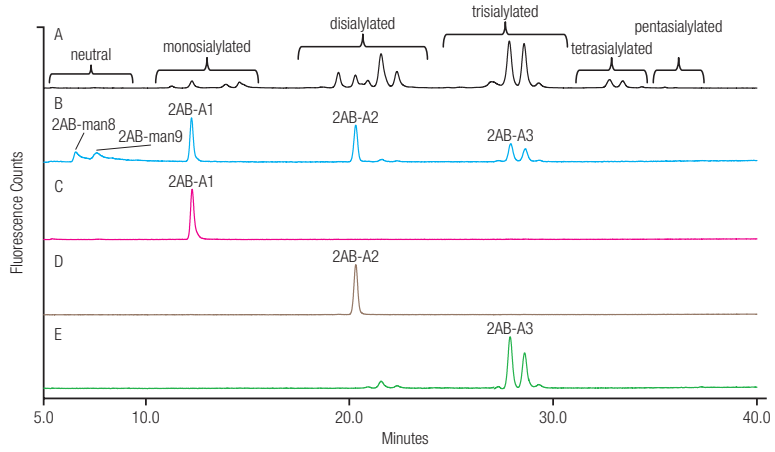


Figure 2: Comparison of 2AB labeled *N*-glycans standards and 2AB labeled *N*-glycans from bovine fetuin.

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:	10 Pmoles of each glycans
Temperature:	30 °C
Detection:	Fluorescence at 320/420 nm
Sample:	2AB-man8, 2AB-Man9, 2AB-A1, 2AB-A2, 2AB-A3 and 2AB labeled *N*-glycans 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

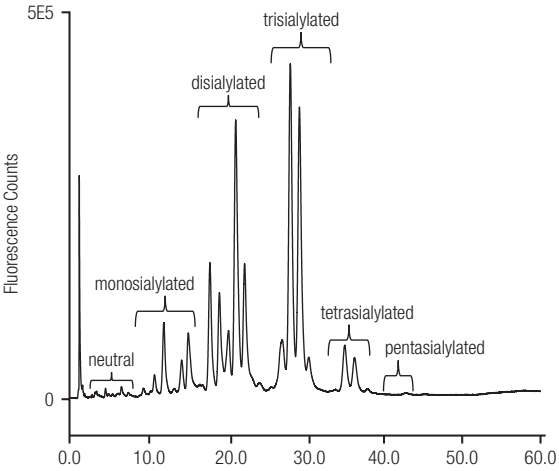


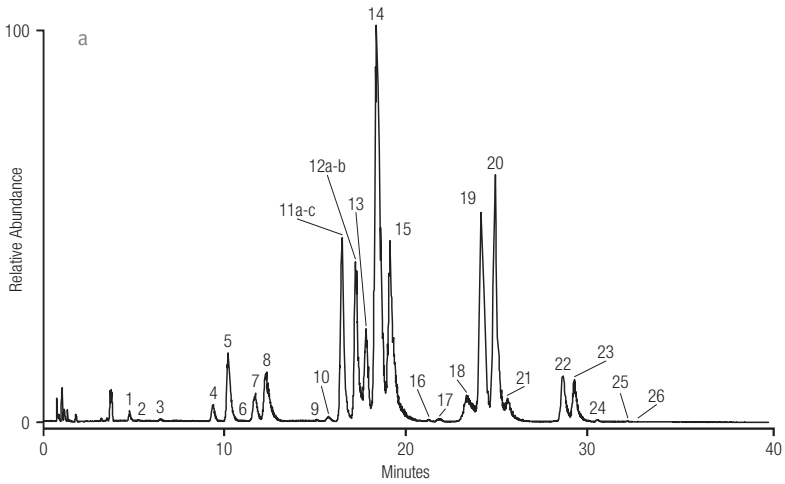
Figure 3: Separation of 2AB labeled *N*-glycans from bovine fetuin using a 3 µm GlycanPac AXH-1 column.

Column:	**GlycanPac AXH-1 (3.0 µm)**
Dimension:	2.1 × 150 mm
Mobile Phase A:	Acetonitrile (100%)
Mobile Phase B:	Ammonium formate (50 mM, pH = 4.4)
Flow Rate:	0.4 mL/min
Injection Volume:	50 pmole
Temperature:	30 °C
Detection:	Fluorescence at 320/420 nm
Sample:	2AB labeled *N*-glycans from bovine fetuin

Time (min)	% A	% B	Flow (mL/min)	Curve
-10	77	23	0.4	5
0	77	23	0.4	5
5	77	23	0.4	5
55	65	35	0.4	5

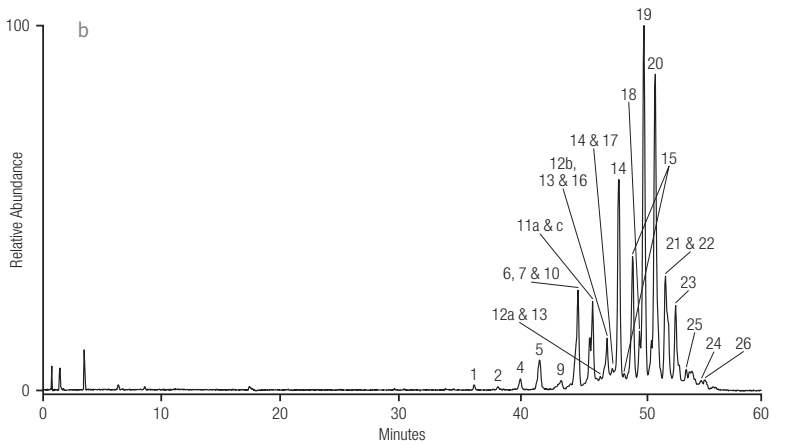
Glycan Analysis by LC/MS

The capability of separating glycans based on charge, size and polarity makes the GlycanPac AXH-1 a powerful tool for accurate glycan analysis by LC/MS. Figure 4 provides a comparison between a 1.9 µm GlycanPac AXH-1 column and a commercial 1.7 µm amide HILIC column for the separation of 2AB labeled *N*-glycans from bovine fetuin detected by MS in negative mode (mass scan range 400-2200 Daltons). Both columns are run under respective recommended conditions. The structural characterization of each identified glycan is determined by MS/MS fragmentation data using structural analysis SimGlycan® software. The list of glycans identified in bovine fetuin is shown in Table 1. The data clearly show that the GlycanPac AXH-1 column provides superior separation in terms of selectivity and resolution compared to the 1.7 µm commercial amide HILIC column, on which glycans with various charges elute with significant co-elutions.



Column:	**GlycanPac AXH-1 (1.9 µm)**
Dimension:	2.1 × 150 mm
Mobile Phase A:	Acetonitrile (80%) + water (20%)
Mobile Phase B:	Ammonium formate (80 mM, pH = 4.4)
Flow Rate:	0.4 mL/min
Injection Volume:	50 Pmoles
Temperature:	30 °C
Detection:	MS detector
Sample:	2AB labeled *N*-glycans from bovine fetuin
MS Instrument:	Thermo Scientific™ Q-Exactive™
MS Mode:	Negative
FT-MS Range:	m/z = 380-2000

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



Column:	**Amide-HILIC column (1.7 µm)**
Dimension:	2.1 × 150 mm
Mobile Phase A:	Acetonitrile (100%)
Mobile Phase B:	Ammonium formate (100 mM, pH = 4.4)
Flow Rate:	0.4 mL/min
Injection Volume:	50 pmole
Temperature:	30 °C
Detection:	MS detector
Sample:	2AB labeled *N*-glycans from bovine fetuin
MS Instrument:	Q-Exactive
MS Mode:	Negative
FT-MS Range:	m/z = 380-2000

Time (min)	% A	% B	Flow (mL/min)	Curve
-10	75.0	25.0	0.4	5
0	75.0	25.0	0.4	5
5	75.0	25.0	0.4	6
55	55.0	45.0	0.4	6
63	50.0	50.0	0.4	6

Figure 4a: LC-MS analysis of 2AB labeled *N*-glycans from bovine fetuin by GlycanPac AXH-1 (1.9 µm) column with MS detection.

Figure 4b: LC-MS analysis of 2AB labeled *N*-glycans from bovine fetuin by competitor amide-HILIC column (1.7 µm) with MS detection.

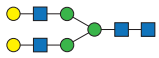
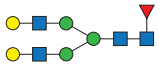
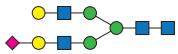
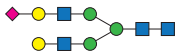
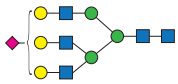
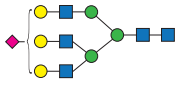
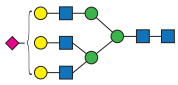
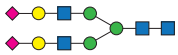
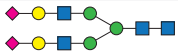
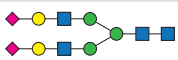
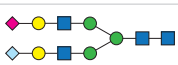
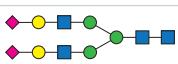
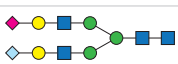
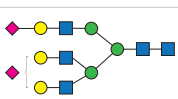
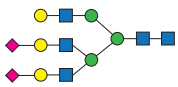
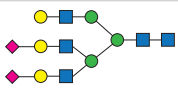
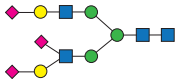
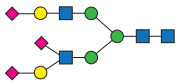
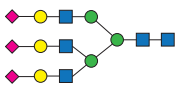
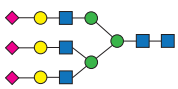
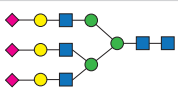
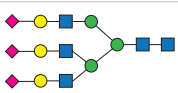
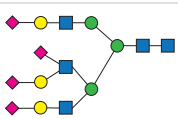
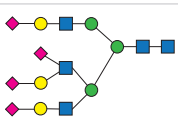
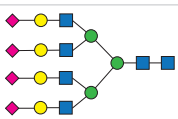
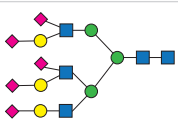
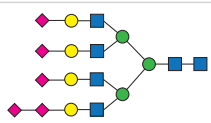
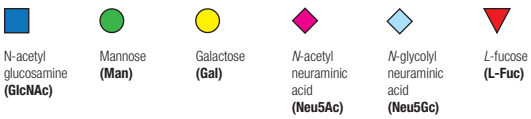
Peak	Structure	Charge of Glycans (without 2AB label)	Molecular Mass (including 2AB label)
1		0	1760.6609
2		0	1906.7188
Unknown	Unknown	Unknown	Unknown
4		-1	2051.7563
5		-1	2051.7563
6		-1	2416.8885
7		-1	2416.8885
8		-1	2416.8885
9		-2	2342.8518
10		-2	2342.8518
11a		-2	2342.8518
11b		-2	2488.9097
11c		-2	2358.8467
12a		-2	2342.8518
12b		-2	2358.8467
13		-3	2707.9839
14		-3	2707.9839
15		-3	2707.9839
16		-3	2633.9472
17		-3	2633.9472
18		-3	2999.0794
19		-3	2999.0794
20		-3	2999.0794
21		-3	2999.0794
22		-4	3290.1748
23		-4	3290.1748
24		-4	3655.3070
25		-5	3581.2702
26		-5	3946.4024

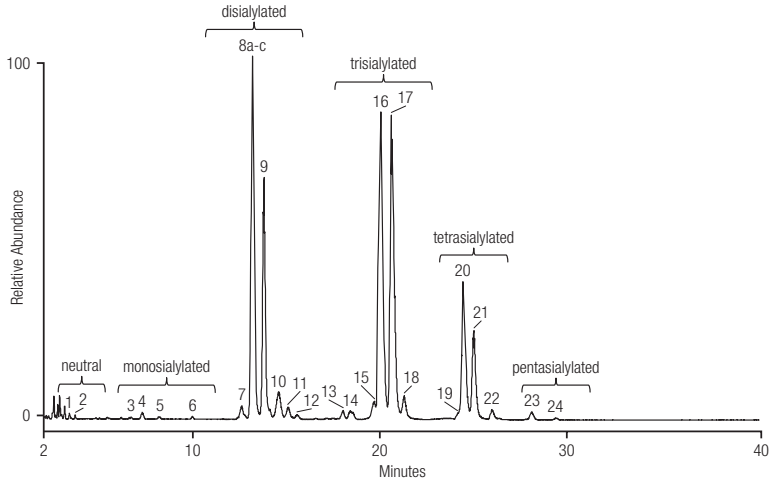
Table 1: Structural characterization of 2AB labeled *N*-glycans from bovine fetuin.



Separation of Native Glycans

Analyzing native glycans directly without fluorescence labeling is desirable because it eliminates the time-consuming derivatization and cleanup steps for labeling, while also retaining the original glycan profile without adding further ambiguity imposed by labeling reactions. The GlycanPac AXH-1 column is well suited for separating native glycans from monoclonal antibodies and other proteins. Figure 5 shows the LC/MS analysis of native *N*-glycans from bovine fetuin on a GlycanPac AXH-1 column (1.9 µm, 2.1 × 150 mm) using MS-compatible conditions. The excellent separation allows for accurate structural information based on FT-MS and MS/MS fragmentation data.

Note that the profile of native glycans is significantly different from that of fluorescently labeled glycans, indicating that the glycan information may be distorted by the labeling process.



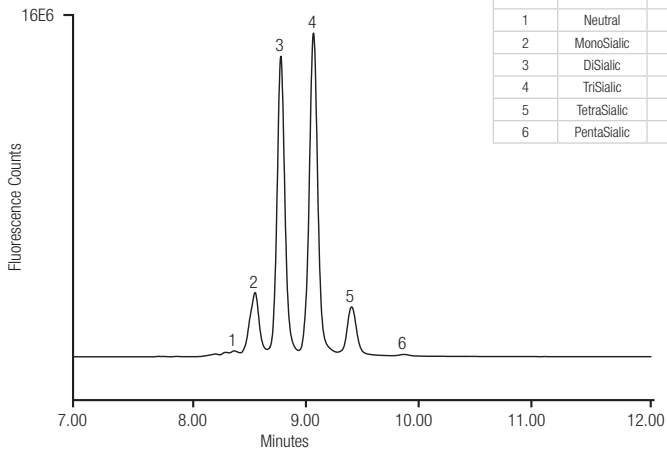
Column:	**GlycanPac AXH-1 (1.9 µm)**
Dimension:	2.1 × 150 mm
Mobile Phase A:	Acetonitrile (80%) + water (20%)
Mobile Phase B:	Ammonium formate (80 mM, pH = 4.4)
Flow Rate:	0.4 mL/min
Injection Volume:	500 pmole
Temperature:	30 °C
Sample:	Native *N*-glycan from bovine fetuin
MS Mode:	Negative
FT-MS Range:	m/z = 380-2000

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

Figure 5: LC-MS analysis of native *N*-glycans from bovine fetuin.

Charge Based Quantitative Determination of Glycans

Accurate and easy quantitative analysis of glycans of each charge state is essential for assessing glycan variation in different protein batches and to compare diseased cell glycosylation profiles to normal ones. In addition, quantitative analysis of glycans based on charge state provides a unique tool for determining the relative amounts of glycans based on the number of sialic acid linkages after enzymatic digestion with sialidase S and sialidase A. Figure 6 shows an example of quantitative analysis of 2AB labeled *N*-glycans based on charge on a GlycanPac AXH-1 column (1.9 µm, 2.1 × 150 mm) with fluorescence detection. A standards curve was created using the data from the chromatographic analysis of 2AB-A2 glycan standard injected with different concentrations (from 0.1 pmoles to 5 pmoles).



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

Figure 6: Charge-based quantitative separation of 2AB labeled *N*-glycans from bovine fetuin.

LC-MS Analysis of Antibody Glycans

Antibody research has gained significant interest as a part of the development of protein biotherapeutics. There is a large amount of research and development currently underway to create monoclonal antibodies for the treatment of numerous serious diseases. Glycosylation of antibodies is a major source of product heterogeneity with respect to both structure and function. Variation in glycosylation is one of the main factors in product batch-to-batch variation, affecting product stability *in vivo*, and significantly influencing Fc effector functions *in vivo*. Based on FDA and European regulations, it is essential to understand the glycan profiles in these proteins because glycans can have a profound influence on the safety and effectiveness of a biopharmaceutical product.

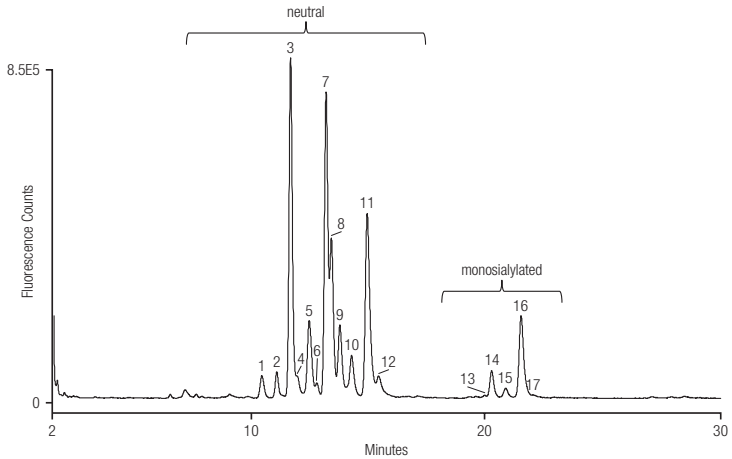
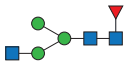
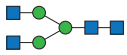
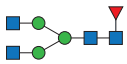
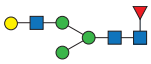
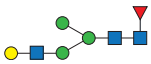
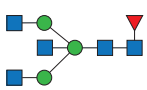
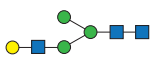
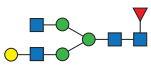
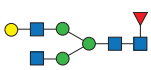


Figure 7: Separation of 2AA labeled *N*-glycans from human IgG.

Column:	**GlycanPac AXH-1 (1.9 µm)**
Dimension:	2.1 × 150 mm
Mobile Phase A:	Acetonitrile (80%) + water (20%)
Mobile Phase B:	Ammonium formate (80 mM, pH = 4.4)
Flow Rate:	0.4 mL/min
Injection Volume:	20 Pmoles
Temperature:	30 °C
Detection:	Fluorescence at 320/420 nm
Sample:	2AA labeled *N*-glycan from human IgG

Time (min)	% A	% B	Flow (mL/min)	Curve
-10	99	1.0	0.4	5
0	99	1.0	0.4	5
30	87.5	12.5	0.4	5

Figure 7 gives an example of LC/MS analysis of 2AA labled glycans released from human IgG using the GlycanPac AXH-1 column (1.9 µm). MS characterization data of each identified glycan is listed in Table 2. Three different glycan charge states were found in this human IgG with dominating amount of neutral glycans and minor amount of monosialylated ones.

Peak	Structure	Charge of Glycan (without 2AA label)	Molecular Mass (including 2AA label)
1		0	1380.5178
2		0	1437.5393
3		0	1583.5972
4		0	1542.5706
5		0	1542.5706
		0	1786.6766
6		0	1599.5921
7		0	1745.6500
8		0	1745.6500
9	Unknown		Unknown

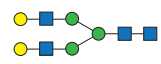
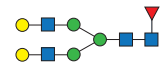
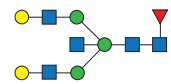
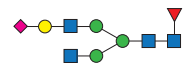
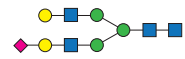
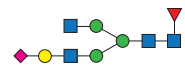
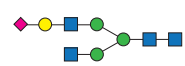
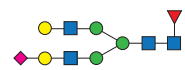
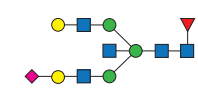
Peak	Structure	Charge of Glycan (without 2AA label)	Molecular Mass (including 2AA label)
10		0	1761.6449
11		0	1907.7028
12		0	2110.7822
13		-1	2036.7454
		-1	2052.7404
14		-1	2036.7454
15		-1	2052.7404
16		-1	2198.7983
17		-1	2401.8776

Table 2: Structural characterization of glycans present in each peak by the separation of 2AA labeled *N*-glycans from Human IgG on the GlycanPac AXH-1 column (1.9 µm).

N-acetyl glucosamine **(GlcNAc)**

Mannose **(Man)**

Galactose **(Gal)**

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

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

Reproducible Manufacturing

Each GlycanPac AXH-1 column is manufactured to strict specifications to ensure column-to-column reproducibility. Each column is individually tested and shipped with a qualification assurance report.

Physical Data

	GlycanPac AXH-1 column (3 µm)	GlycanPac AXH-1 column (1.9 µm)
Column Chemistry	WAX and HILIC Mixed-Mode	WAX and HILIC Mixed-Mode
Silica Substrate	Spherical, high purity, porous	Spherical, high purity, porous
Particle Size	3 µm	1.9 µm
Surface Area	300 m^2/g	220 m^2/g
Pore Size	120 Å	175 Å

Specifications and Operational Parameters

Column Particle Size	Dimension (mm)	P/N	Maximum Pressure (psi)	pH Range	Temperature Limit (°C)	Solvent/ Aqueous Compatibility	Recommended Flow Rate (mL/min)	Maximum Flow Rate (mL/min)
1.9 µm	2.1 × 100 mm	082473	7,000	2.0–8.0	< 60	Compatible with 0–90% aqueous Compatible with acetonitrile as organic solvents	0.1–0.4	0.5
1.9 µm	2.1 × 150 mm	082472	10,000	2.0–8.0	< 60		0.1–0.4	0.5
1.9 µm	2.1 × 250 mm	082521	15,000	2.0–8.0	< 60		0.1–0.4	0.5
3 µm	4.6 × 150mm	082468	6,000	2.0–8.0	< 60		0.6–1.2	1.5
3 µm	3.0 × 150 mm	082469	6,000	2.0–8.0	< 60		0.3–0.6	0.75
3 µm	2.1 × 150 mm	082470	6,000	2.0–8.0	< 60		0.1–0.4	0.5

Ordering Information

Description	Part Number
GlycanPac AXH-1, Analytical 3 µm, 4.6 × 150 mm	082468
GlycanPac AXH-1, Analytical 3 µm, 3.0 × 150 mm	082469
GlycanPac AXH-1, Analytical 3 µm, 2.1 × 150 mm	082470
GlycanPac AXH-1, Analytical 1.9 µm, 1.0 × 150 mm	164863
GlycanPac AXH-1, Analytical 1.9 µm, 2.1 × 250 mm	082521
GlycanPac AXH-1, Analytical 1.9 µm, 2.1 × 150 mm	082472
GlycanPac AXH-1, Analytical 1.9 µm, 2.1 × 100 mm	082473
GlycanPac AXH-1, Guard 3 µm, 4.6 × 10 mm	082474
GlycanPac AXH-1, Guard 3 µm, 3.0 × 10 mm	082475
GlycanPac AXH-1, Guard 3 µm, 2.1 × 10 mm	082476

thermoscientific.com/chromatography

Thermo Fisher Scientific, Sunnyvale, CA USA is ISO 9001:2008 Certified.

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
A Thermo Fisher Scientific Brand

PS20695_E 05/14S