

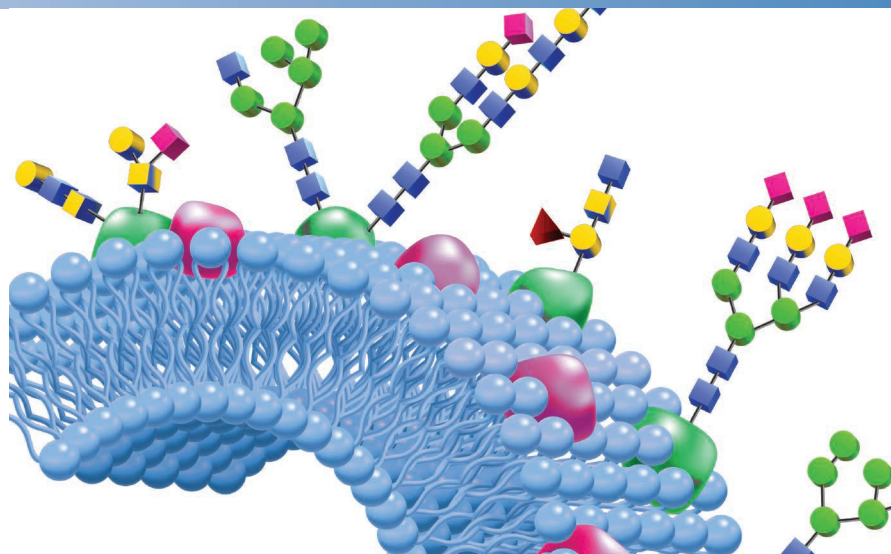
GlycanPac AXH-1 Column

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 \times 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.

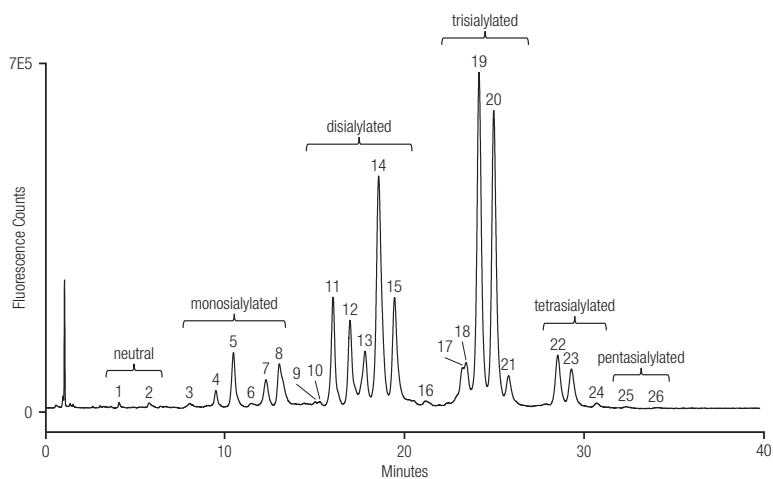


Figure 1: Separation of 2AB labeled *N*-glycans from bovine fetuin by charge, size and polarity.

Column: **GlycanPac AXH-1 (1.9 μ m)**
 Dimension: 2.1 \times 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 $^{\circ}$ 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 |

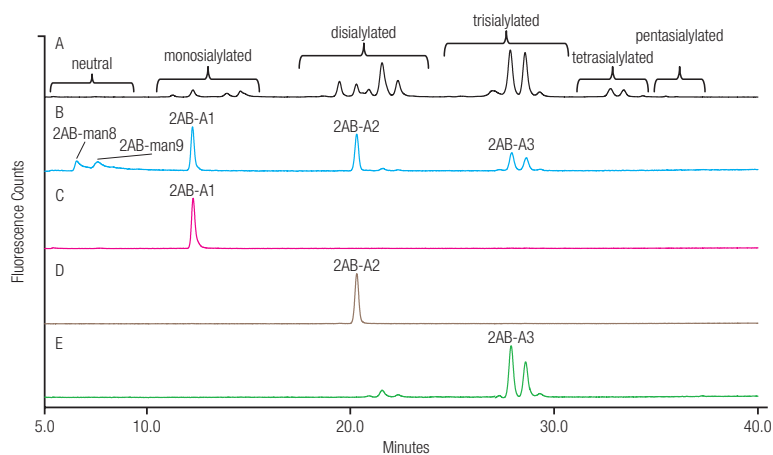


Figure 2: Comparison of 2AB labeled *N*-glycans standards and 2AB labeled *N*-glycans from bovine fetuin.

Column: **GlycanPac AXH-1 (1.9 μ m)**
 Dimension: 2.1 \times 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 $^{\circ}$ 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 |

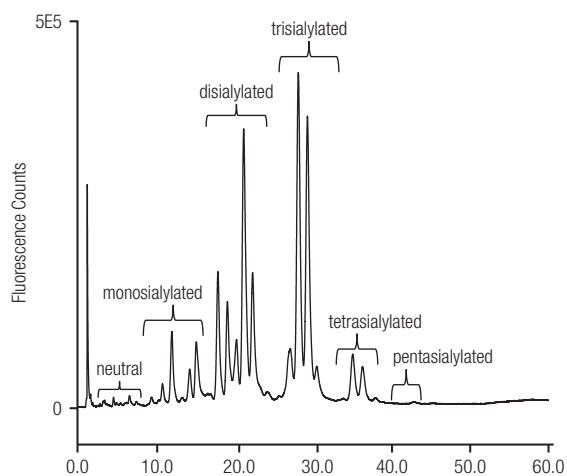


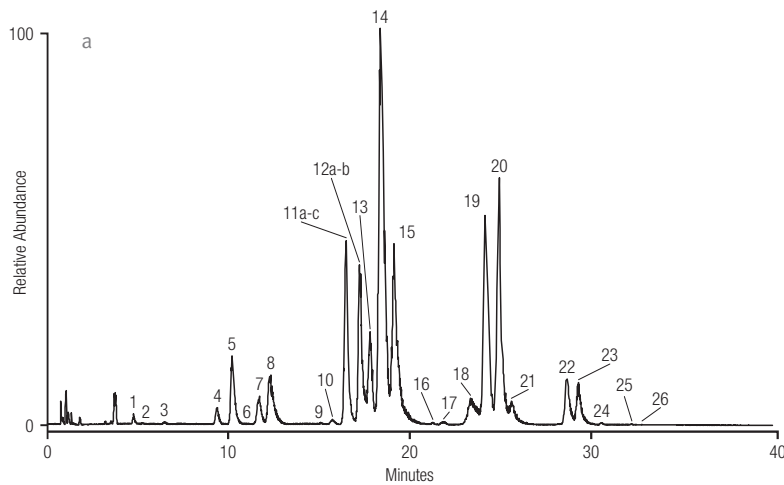
Figure 3: Separation of 2AB labeled *N*-glycans from bovine fetuin using a 3 μ m GlycanPac AXH-1 column.

Column: **GlycanPac AXH-1 (3.0 μ m)**
 Dimension: 2.1 \times 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 $^{\circ}$ 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 |

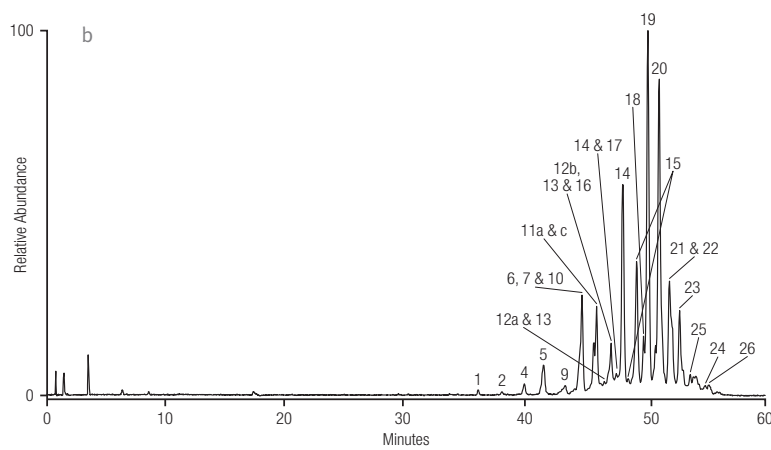
Glycan Analysis by LC/MS

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 \times 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 $^{\circ}\text{C}$
 Detection: MS detector
 Sample: 2AB labeled *N*-glycans from bovine fetuin
 MS Instrument: Thermo Scientific[™] Q-Exactive^{™™}
 MS Mode: Negative
 FT-MS Range: $m/z = 380\text{-}2000$

| 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 \times 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 $^{\circ}\text{C}$
 Detection: MS detector
 Sample: 2AB labeled *N*-glycans from bovine fetuin
 MS Instrument: Q-Exactive
 MS Mode: Negative
 FT-MS Range: $m/z = 380\text{-}2000$

| 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 |

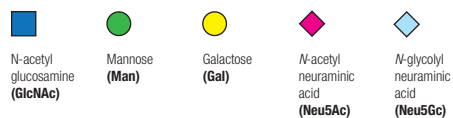
Figure 4a: LC-MS analysis of 2AB labeled *N*-glycans from bovine fetuin by GlycanPac AXH-1 (1.9 μm) column with MS detection.

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 |

| Peak | Structure | Charge of Glycans (without 2AB label) | Molecular Mass (including 2AB label) |
|------|-----------|---------------------------------------|--------------------------------------|
| 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 |

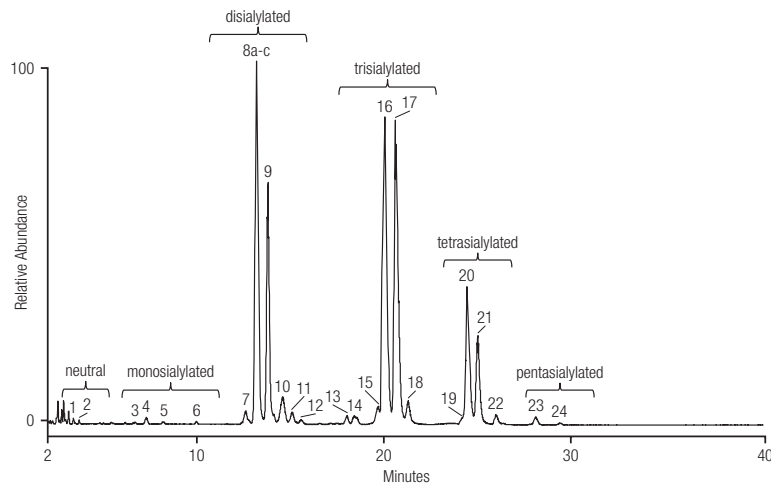
Table 1: Structural characterization of 2AB labeled *N*-glycans from bovine fetuin.



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 μ m, 2.1 \times 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.



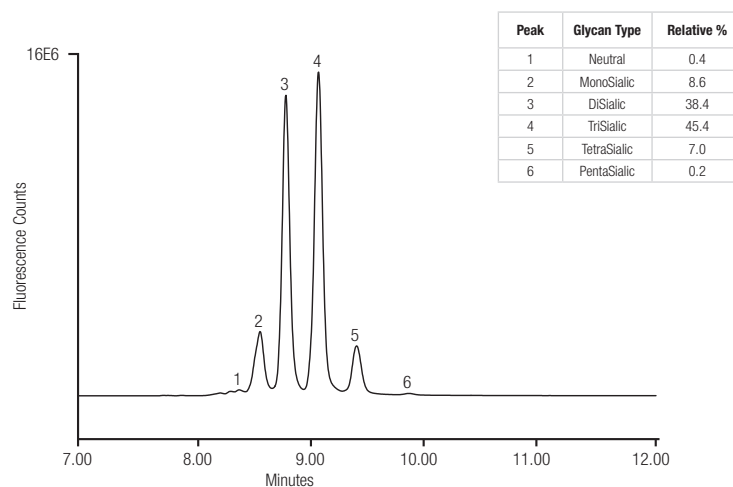
Column: **GlycanPac AXH-1 (1.9 μ m)**
 Dimension: 2.1 \times 150 mm
 Mobile Phase A: Acetonitrile (80%) + water (20%)
 Mobile Phase B: Ammonium formate (80 mM, pH = 4.4)
 Flow Rate: 0.4 mL/min
 Injection Volume: 500 pmole
 Temperature: 30 $^{\circ}$ C
 Sample: Native *N*-glycan from bovine fetuin
 MS 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 μ m, 2.1 \times 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).



Column: **GlycanPac AXH-1 (1.9 μ m)**
 Dimension: 2.1 \times 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 $^{\circ}$ 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.

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.

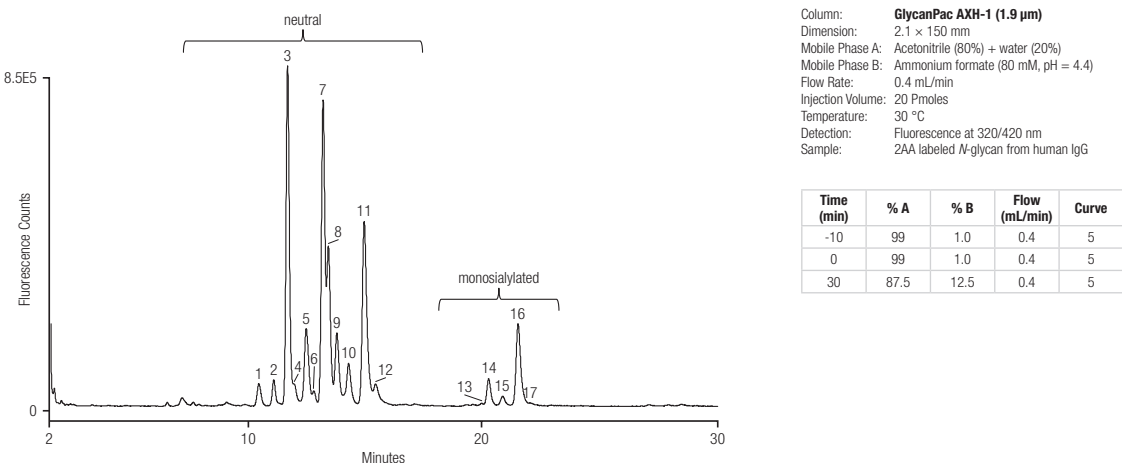


Figure 7: Separation of 2AA labeled N-glycans from human IgG.

Figure 7 gives an example of LC/MS analysis of 2AA labeled 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 |
| 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).



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 × 150 mm | 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 |

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