Thermo Scientific

GlycanPac AXH-1 Columns

Product Manual

P/N: 065479-01 January 2013

Thermo SCIENTIFIC

Product Manual

for

GlycanPac AXH-1 Columns

GlycanPac AXH-1, 1.9 µm, Analytical, 2.1 x 100 mm (P/N 082473)
GlycanPac AXH-1, 1.9 µm, Analytical, 2.1 x 150 mm (P/N 082472)
GlycanPac AXH-1, 1.9 µm, Analytical, 2.1 x 250 mm (P/N 082521)

GlycanPac AXH-1, 3µm, Analytical, 4.6 x 150 mm (P/N 082468)
GlycanPac AXH-1, 3µm, Analytical, 3.0 x 150 mm (P/N 082469)
GlycanPac AXH-1, 3µm, Analytical, 2.1 x 150 mm (P/N 082470)

GlycanPac AXH-1, 3 µm, Guard, 4.6 x 10 mm (P/N 082474)
GlycanPac AXH-1, 3 µm, Guard, 3.0 x 10 mm (P/N 082475)
GlycanPac AXH-1, 3 µm, Guard, 2.1 x 10 mm (P/N 082476)

Revision History:

Revision 01, January, 2013, Original Publication;

For Research Use Only. Not for use in diagnostic procedures.

Safety and Special Notices

Make sure you follow the precautionary statements presented in this guide. The safety and other special notices appear in boxes.

Safety and special notices include the following:

 **SAFETY**	***Indicates a potentially hazardous situation which, if not avoided, could result in death or serious injury.***
 **WARNING**	***Indicates a potentially hazardous situation which, if not avoided, could result in damage to equipment.***
 **CAUTION**	***Indicates a potentially hazardous situation which, if not avoided, may result in minor or moderate injury. Also used to identify a situation or practice that may seriously damage the instrument, but will not cause injury.***
 **NOTE**	***Indicates information of general interest.***
IMPORTANT	***Highlights information necessary to prevent damage to software, loss of data, or invalid test results; or might contain information that is critical for optimal performance of the system.***
Tip	***Highlights helpful information that can make a task easier.***

Contents

1. Introduction

GlycanPac AXH-1 columns are designed to separate glycans based on charge, size and polarity. Structural, qualitative and quantitative analysis of glycans can be performed faster than other commercially available columns. GlycanPac AXH-1 columns are mixed-mode weak anion exchange and HILIC dual phase column optimized for the high resolution separation of fluorescently labeled or native glycans. The GlycanPac AXH-1columns are available in 3 and 1.9 µm particle sizes. Both particle size columns provide high resolution separation of glycans with unique selectivity. The HILIC mode of separation is used to retain very polar compounds. Weak anion exchange mode separates compounds based on charge. The GlycanPac AXH-1column is based on high-purity, spherical, porous silica gel that is covalently modified with a proprietary hydrophilic layer.

GlycanPac AXH-1, 1.9 µm particle size columns provides better resolution with smaller peak width and requires UHPLC compatible system to operate at the higher flow rate . GlycanPac AXH-1, 3 µm particle size column is recommended for the separation of glycans using a standard HPLC system with low back pressure tolerance. Both the columns and eluents are highly compatible with MS instruments.

1.1 The Main Features of the GlycanPac AXH-1 Columns 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

1.2 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

1.3 Specifications and Operational Parameters

Particle size	Column Dimension	P/N	Maximum Pressure (psi)	Recommended Flow Rate (mL/min)	Maximum Flow Rate (mL/min)
1.9 µm	2.1 x 250 mm	082251	15,000	0.1 – 0.4	0.5
	2.1 x 150 mm	082472	10,000	0.1 – 0.4	0.5
	2.1 x 100 mm	082473	8,000	0.1 – 0.4	0.5
3 µm	4.6 x 150 mm	082468	6,000	0.6 – 1.2	1.5
	3.0 x 150 mm	082469	6,000	0.3 – 0.6	0.75
	2.1 x 150 mm	082470	6,000	0.1 – 0.4	0.5
3 µm guards	4.6 x 10 mm	082474	6,000	0.6 – 1.2	1.5
	3.0 x 10 mm	082475	6,000	0.2 – 0.6	0.75
	2.1 x 10 mm	082476	6,000	0.1 – 0.4	0.5

pH Range: 2.0 – 8.0 (**always use and store the column with a buffered mobile phase)**
Temperature Limit: < 60 °C
Solvent Compatibility: 0 – 90% aqueous buffer; 10 – 100% acetonitrile or alcohols.

2. Step-By-Step User Guide

Thermo Fisher Scientific recommends that you perform an efficiency test on your GlycanPac AXH-1column before use. The purpose of column performance validation is to ensure no damage has occurred during shipping. Test the column using the conditions described on the Quality Assurance Report (QAR) enclosed in the column box. Repeat the test periodically to track the column performance over time.

NOTE

Slight variations may be obtained on two different HPLC systems due to system electronic, plumbing, operating environment, reagent quality, column conditioning, and operator technique.

2.1 Step 1 – Visually Inspect the Column

Report any visual damage to Thermo Fisher Scientific.

2.2 Step 2 – Mobile Phase Preparation

Obtaining reliable, consistent and accurate results require mobile phases that are free of ionic and spectrophotometric impurities. Chemicals, solvents and de-ionized water used to prepare mobile phase should be of the highest purity available. Maintaining low trace impurities and low particle levels in mobile phases helps to protect your columns and system components. Thermo Fisher Scientific cannot guarantee proper column performance when the quality of the chemicals, solvents and water used to prepare the mobile phase has been compromised.

To analyze glycans with GlycanPac AXH-1 columns, the mobile phase system usually consists of an organic solvent (e.g. acetonitrile) and a buffer (e.g. ammonium acetate or ammonium formate). Both pre-mixed and proportioning mobile phases give satisfactory results. The use of proportioning valve provides better flexibility in method optimization, while the pre-mixed mobile phase provides more reproducible results.

Preparation of 100 mM ammonium formate buffer, pH4.4

1. Dissolve 6.35 g of ammonium formate salt (Sigma, Cat. No. 516961-100) in 999.0 g of D.I. water using a 1-L eluent bottle.
2. Add 0.70 g of formic acid (Fisher Scientific, Cat. No. A117-50) to above solution.
3. Sonicate for 5 min.

Solvents

The solvents used must be free from ionic, fluorescent and UV-absorbing impurities. Use of ultrahigh purity solvents, HPLC grade, will usually ensure that your chromatography is not affected by impurities in the solvent.

De-ionized Water

The de-ionized water used to prepare the mobile phase should be ultra pure water with 18-megohm-cm water or Type 1 Reagent Grade water or HPLC Grade water. The de-ionized water should be free of ionized impurities, organics, microorganisms and particulate matter larger than 0.2 µm. Many commercial water purifiers are designed for HPLC applications and are suitable for these applications.

NOTE

Degas the aqueous component of the mobile phase and then add the solvent component. Avoid excessive purging or degassing of mobile phases containing solvents, if possible, since the volatile solvent can be 'boiled' off from the solution.

Mobile Phase for Column Performance Test (QA test):

Mobile phase can be generated either by pre-mixing or by using a proportioning valve, both give satisfactory results. The use of proportioning valve provides better flexibility in method optimization, while the pre-mixed mobile phase provides less baseline noise. Ammonium formate buffer and acetonitrile are used for QAR test

1. Mobile phase preparation for 1.9 µm column performance test: mix 550.2 g of acetonitrile (Fisher Scientific, Cat. No. A996), 100 g of 100 mM ammonium formate (pH 4.4) buffer and 200 g of D.I. water. Sonicate for 5 min.
2. Mobile phase preparation for 3.0 µm column performance test: mix 550.2 g of acetonitrile (Fisher Scientific, Cat. No. A996) and 200 g of 100 mM ammonium formate (pH 4.4) buffer and 100 g of D.I. water.. Sonicate for 5 min.
3. Must use HPLC/MS grade acetonitrile and water for all the analysis.
4. Preparation of column performance test standard
    1. Dissolve 200 pmole of 2AB-A2 (GKSB 311, Prozyme) in 50 µL of D.I. water in the original container.
    2. Transfer it solution to a 250 µL sample vial.
    3. Add 150 µL acetonitrile to the vial and mixed properly by pipette to obtain column performance test mix.

2.3 Step 3 – Set up the LC System

Use a standard LC system (HPLC/UHPLC) equipped with a LC pump, a column oven, a fluorescence detector (FLD) (or mass spectrometer (MS), or charged-aerosol detector (CAD) depending on the application), and an injector (or an autosampler). The system should be thoroughly primed before use. To obtain quality results, cares should be taken to minimize extra column volume in the system.

2.4 Step 4 – Condition the Column

Each new column is shipped in the solution containing 70% acetonitrile with 30% ammonium formate buffer. Before use, the column should be washed thoroughly with the mobile phase (~20 to 50 column volumes depending on the aqueous content in the mobile phase) before any injection is made.

When switching to a different mobile phase, make sure that the new mobile phase is compatible with the existing mobile phase in the column to avoid column clogging due to precipitation.

2.5 Step 5 – Reproduce the Chromatogram in the Quality Assurance Report

Perform the column QA test using the conditions described in the Quality Assurance Report (QAR), and compare the result with the reported values. The column should be fully equilibrated before any injection. At least five injections should be made until reproducible results are obtained.

NOTE

Due to various reasons, such as difference of LC systems, mobile phases, oven temperature control, etc, you may observe somewhat different separation from that in the report.

2.6 Step 6 – Real Sample Analysis

Once you are satisfied with the column performance report result, the column is ready for your application.

NOTE

Avoid any precipitation of glycan samples due to excesses organic solvent. Make sure both organic and aqueous phases mix together (are miscible) without any floating particles.

3. Considerations in Method Development

3.1 Selection of Organic Solvents

Acetonitrile is the preferred solvent for glycan analysis using the GlycanPac AXH-1 column. Always use high quality (HPLC/MS) grade acetonitrile (e.g. Fisher Scientific, A996) for all the analysis.

3.2 Buffer Types

The selection of buffer depends on the detection method and pH requirement.

1. Ammonium acetate and ammonium formate buffers are the preferred buffer systems because of their compatibility with FLD, CAD, ELS and MS detector, high solubility in organic solvent, and familiarity to most chromatographers.
2. Volatile organic acids, such as acetic acid and formic acid can be used to adjust mobile phase pH..
3. Other buffers, such as phosphate buffers may also be used. However, phosphate buffers tend to precipitate in high organic concentrations. Thus special attention is required for glycan analysis applications. Unless required do not use phosphate buffer for the analysis.

3.3 Mobile Phase pH

Mobile phase pH needs to be controlled for optimal and reproducible results of charged glycans. Depending on the nature of analytes, different mobile phase pHs are needed. Ammonium acetate or ammonium formate in the pH range of 4 to 6 is a good starting point in most cases.

3.4 Isocratic or Gradient Method

Isocratic methods are suitable for simple and/or well-defined applications. When dealing with unknown samples, or a sample consisting of molecules with dramatically different hydrophilicity or/and charges, a gradient method is often advantageous. Contrary to reversed-phase application, In HILIC mode organic solvent is the weak eluting mobile phase and aqueous buffer is strong eluting one. Thus, for a gradient method, the mobile phase organic solvent should start from the higher level and gradually decrease to a low level. When using a gradient method, the column should be fully equilibrated with at least 10 column volumes before any injection is given.

3.5 Injection Volume

It is highly recommended that the injection sample be dissolved in the mobile phase (or the starting mobile phase in the case of a gradient method) or a diluent that is weaker (containing higher organic solvent) than the mobile phase. Too strong of a diluent will cause peak distortion or retention time variation. If the sample contains strong diluent, the injection volume should be minimized to a fraction of the normal injection volume. For a 2.1 x 150-mm column, the typical injection volume is 0.2 to 2 µL.

3.6 Guard Columns

Use guard columns for real sample analysis to protect the analytical columns. The guard columns should be replaced in time depending on the nature of the sample.

4. Column Care

4.1 Column Storage

All GlycanPac AXH-1columns must be used and stored in buffered mobile phase. The column can be stored in the mobile phase for short-term storage (< 24 hours). For long-term storage (> 24 hours), store the column in a solution containing 90% acetonitrile and 10% ammonium formate buffer (100 mM, pH 4.4). If an aggressive mobile phase (pH below 3 or above 7) is used, the column should be stored in a long-term storage solution when not in use.

4.2 Operating pH Range: pH 2.0 to 8.0

The column lifetime depends heavily on the chromatographic condition. To obtain better column lifetime, it is recommended to use "silica friendly" mobile phases, such as using a buffer at a pH between pH 3 to 6 for the aqueous portion in the mobile phase.

4.3 Operating Temperature Limit: 60 ºC

Based on our experimental data, this column can be used up to 60 °C. The typical operating temperatures for most applications are between 20 and 40 °C.

4.4 Pressure Limit

It is extremely important not to impose a sudden column pressure surge. The maximum pressure ratings for continuous service are listed in Section 1.3.

4.5 Flow Rate

Use the column within the flow rate limit (refer to Section 1.3. for detail).

NOTE

The column must be operated within its flow and pressure limits at the same time.

4.6 Column Cleaning Procedure

During use, column contamination will occur due to the contaminants from samples, LC system, and mobile phase. Among all, metal contamination can be witnessed quite often because most LC system and column hardware are made from stainless steel which will bleed out iron over time. When this happens, charged glycans elute as broad and tailing peaks. Apply following cleaning procedure to restore the performance of the column (in 2.1x150-mm format). For other column dimensions, the flow rate and washing time should be adjusted proportionally.

Time (min)	Acetonitrile (%)	50 mM sodium pyrophosphate in 100 mM ammonium formate, pH 4.4 (%)	D.I. water (%)	100 mM Ammonium formate buffer, pH 4.4 (%)	Flow (mL/min)
0	50	0	0	50	0.25
5	20	0	0	80	0.25
10	20	80	0	0	0.25
35	20	80	0	0	0.25
36	20	0	0	80	0.25
50	20	0	0	80	0.25
60	80	0	0	20	0.4

NOTE

Use the column cleaning procedure when peak tailing is observed for charged glycans.

The time for pyrophosphate buffer wash can vary from 25 min (shown above) to 120 min, depending on the severity of contamination.

5. Frequently Asked Questions

5.1 What is the Difference Between the GlycanPac AXH-1 Column and Other Columns for Glycan Analysis?

Unlike other LC columns for glycan analysis that have covalently bonded hydrophilic surfaces, such as amine, amide, zwitterionic functionality, the GlycanPac AXH-1column is based on unique mixed mode chemistries with both weak anion exchange and HILIC properties, and designed for the high resolution separation of fluorescently labeled or native carbohydrates (glycans). Its HILIC functional group is used to retain very polar compounds, and weak anion exchange property separates based on charge. As the result, the GlycanPac AXH-1column exhibits unique selectivity and high resolution compared to any other LC columns for glycan analysis.

5.2 Why Do I Need a GlycanPac AXH-1 Column?

The GlycanPac AXH-1 column offers the following features designed for a wide range of applications:

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

In addition, the column chemistry is unique and different from the majority of glycan columns in the market. Thus you will have a unique high-quality glycan column in your method development toolbox.

5.3 When Do I Need a GlycanPac AXH-1 Column?

When you are analyzing charged glycans, you will benefit from the GlycanPac AXH-1 column because of the desired features listed in Section 5.2. native

5.4 What Factors Should I Consider for Method Development Using this Column?

During method development, the following factors should be considered:

1. Type of glycan samples
2. Nature of glycans of interest
3. Detection method
4. Solvent content
5. Isocratic or gradient
6. Mobile phase composition
7. Mobile phase pH
8. Temperature

5.5 What Mobile Phases Should I Use with this Column?

This new column is compatible with any mobile phase commonly used for HILIC or Normal-Phase separations. For a typical HILIC application, consider 80 to 90% acetonitrile with 20 to 10% ammonium formate buffer as the starting point.

Please refer to "Section 3 Considerations in Method Development" for more details.

5.6 What Should I Do Before Starting to Use the GlycanPac AXH-1 Column?

Read the Product Manual carefully, and contact Thermo Fisher Technical Support if you have any questions regarding the use of this column.

5.7 How to Store the Column?

Refer to "Section 4.1 Column Storage" for details.

5.8 Do I Need a Guard Column?

Guard columns must be used to protect the analytical column against fouling by dirty samples or contaminated mobile phases. Guard cartridges are rated to 6,000 psi. Guard cartridges require the holder P/N 069580, sold separately.

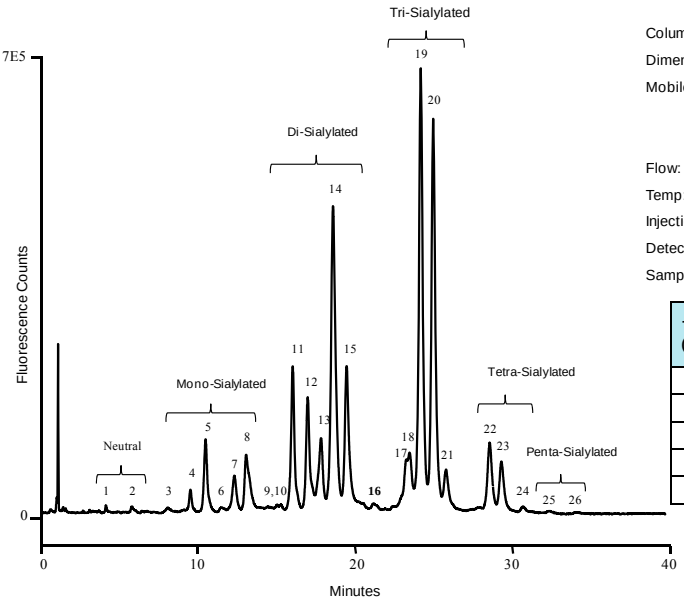
6. Applications

6.1 Separation of N-glycans by GlycanPac AXH-1(1.9 um) Column Based on Charge, Size and Polarity

The GlycanPac AXH-1 column can be used for qualitative, quantitative, structural analysis and characterization of uncharged and charged glycans present on proteins. For example, one of the most important parameters of N-glycan analysis is in the separation of neutral and negatively charged fluorescently labeled glycans. Figure 1 shows the separation of neutral and acidic 2AB labeled N-glycans from Bovine fetuin using the GlycanPac AXH-1 (1.9 µm, 2.1x150 mm) column using a ternary gradient. The glycan separation and elution is based on charge: the neutral glycans elute first, followed by mono-sialylated, di-sialylated, tri-sialylated, tetra-sialylated and finally penta-sialylated species. Glycans of each charge state are further separated based on their size and polarity. The retention time of each glycan charge state was confirmed using 2AB labeled glycans standards (as shown in Figure 2). Separation of glycans based on charge, size and polarity provides important structural and conformational information.

GlycanPac AXH-1 column, packed with a 3 µm particles, can also be used for this separation. A larger particle size provides lower back pressure separations (as shown in Figure 3) using binary gradient conditions making it compatible with all standard HPLC systems. Both particle size columns (1.9 µm and 3 µm) provide high resolution and unique selectivity with baseline separations.

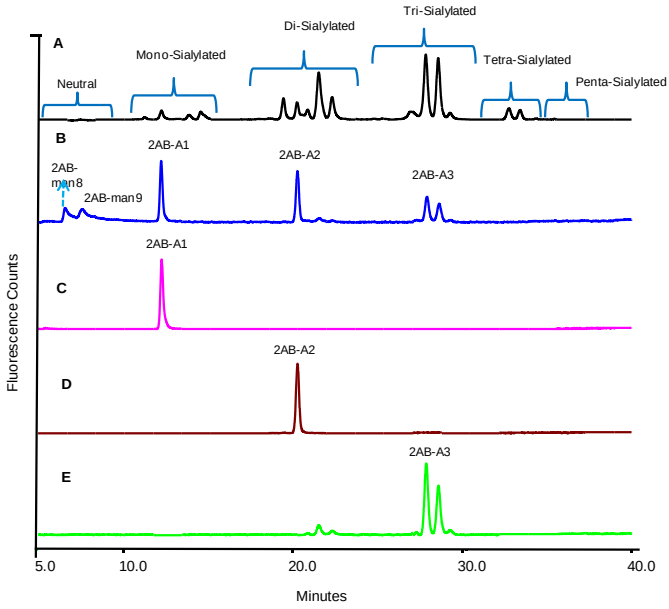
Figure 1 Separation of 2AB labeled N-glycans from bovine fetuin by charge, size and polarity



Column:	**GlycanPac AXH-1 (1.9 µm)**
Dimension:	2.1x150 mm
Mobile phase:	A: Acetonitrile (100 %)
	B: water
	C: Ammonium formate (100 mM, pH =4.4)
Flow:	0.4 mL/min
Temp:	30 ºC
Injection:	50 Pmoles
Detection:	Fluorescence at 320/420 nm
Sample:	2AB labeled N-glycan from bovine fetuin

Time (min)	% A	% B	% C	Flow Rate (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

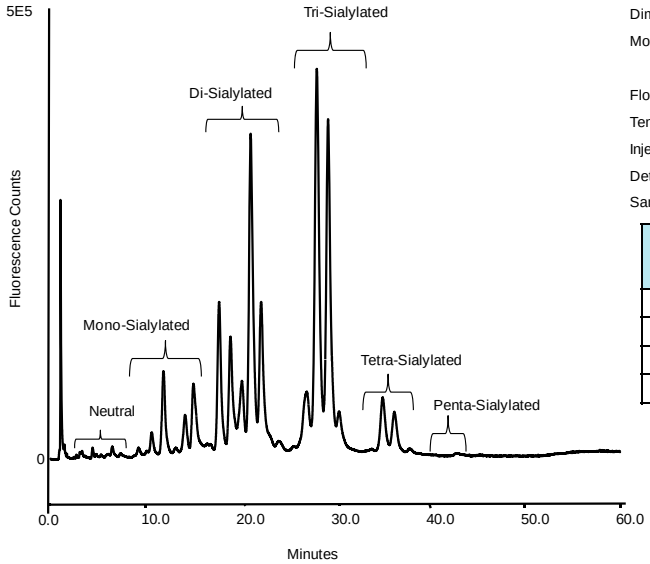
Figure 2 Comparison of 2AB labeled N-glycans standards and 2AB labeled N-glycans from bovine fetuin



Column:	**GlycanPac AXH-1 (1.9 µm)**
Dimension:	2.1x150 mm
Mobile phase:	A: Acetonitrile (100 %)
	B: water
	C: Ammonium formate (100 mM, pH =4.4)
Flow:	0.4 mL/min
Temp:	30 °C
Injection:	10 Pmoles of each glycans
Detection:	Fluorescence at 320/420 nm
Sample:	2AB-man8, 2AB-Man9, 2AB-A1, 2AB-A2, 2AB-A3 and 2AB
labeled	N- glycan from bovine fetuin

Time (min)	% A	% B	% C	Flow Rate (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



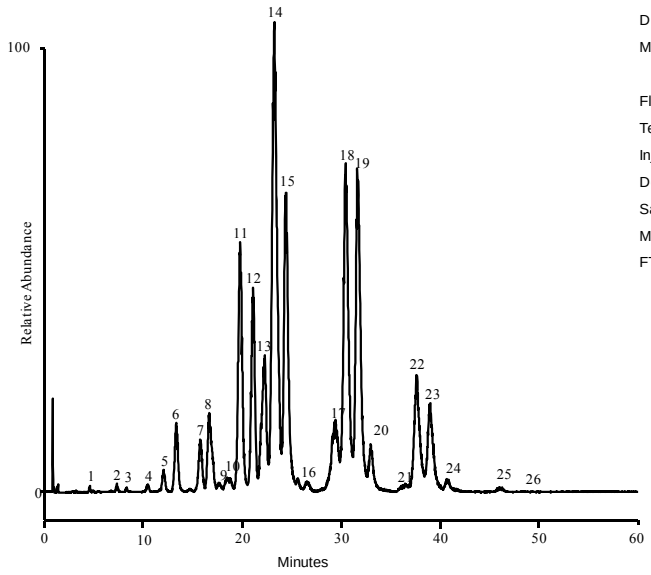
Column:	**GlycanPac AXH-1 (3.0 µm)**
Dimension:	2.1x150 mm
Mobile phase:	A: Acetonitrile (100 %)
	B: Ammonium formate (50 mM, pH =4.4)
Flow:	0.4 mL/min
Temp:	30 °C
Injection:	50 pmole
Detection:	Fluorescence at 320/420 nm
Sample:	2AB Labeled N-glycan from Bovine fetuin

Time (min)	% A	% B	Flow Rate (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

6.2 LC/MS Glycan Separations

The capability of the GlycanPac AXH-1 to separate glycans based on charge, size and polarity results in more accurate data analysis. For example, 2AB labeled N-Glycans from Bovine fetuins were separated using the GlycanPac AXH-1 column (3µm in Figure 4 and 1.9 µm in Figure 5A) and detected by MS. The FT-MS of all the peaks separated by LC were estimated, and structural characterization of the glycans present in each peak were determined from the MS/MS fragmentation data by using structural analysis "Simglycan" software (Table 1). As compared to our column, the competitor HILIC column doesn't provide separation based on the charge, which makes difficulty in characterizing minute quantity of different charge state glycans because those species are co eluted with other major peaks in competitor columns (Figure 5B).

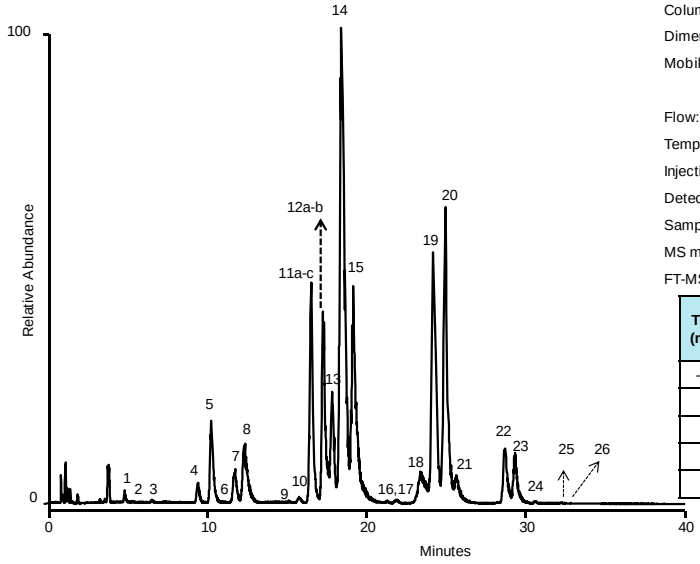
Figure 4 LC-MS analysis of 2AB labeled N-glycans from Bovine fetuin by GlycanPac AXH-1 (3 µm) column with MS detection



Column:	**GlycanPac AXH-1 (3.0 µm)**
Dimension:	2.1x150 mm
Mobile phase:	A: Acetonitrile (100 %)
	B: Ammonium formate (50 mM, pH =4.4)
Flow:	0.4 mL/min
Temp:	30 °C
Injection:	50 pmole
Detection:	MS detector
Sample:	2AB Labeled N-glycan from Bovine fetuin
MS mode:	negative
FT-MS range:	m/z= 380-2000

Time (min)	% A	% B	Flow Rate (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

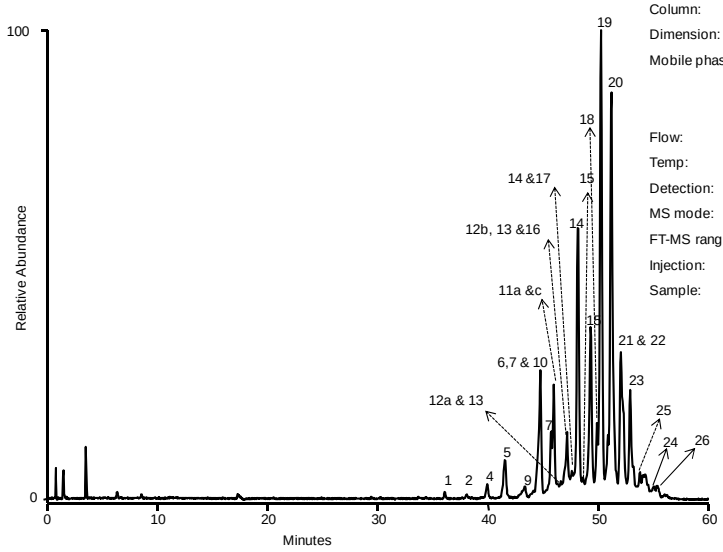
Figure 5A LC-MS analysis of 2AB labeled N-glycans from Bovine fetuin by GlycanPac AXH-1 (1.9 µm) column with MS detection



Column: **GlycanPac AXH-1 (1.9 µm)**
Dimension: 2.1x150 mm
Mobile phase: A: Acetonitrile (80 %) +20 % water
B: Ammonium formate (80 mM, pH =4.4)
Flow: 0.4 mL/min
Temp: 30 °C
Injection: 50 Pmoles
Detection: MS detector
Sample: 2AB Labeled N-glycan from Bovine fetuin
MS mode: negative
FT-MS range: m/z= 380-2000

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

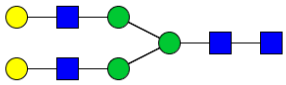
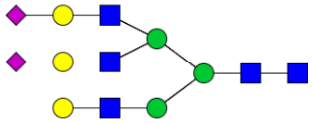
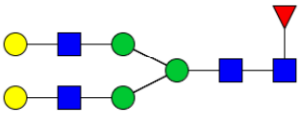
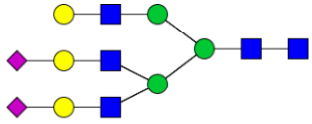
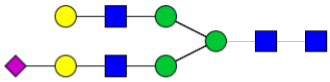
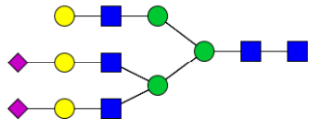
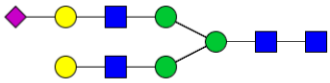
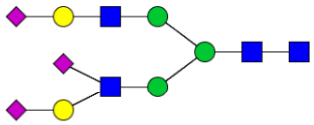
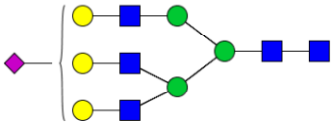
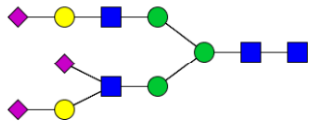
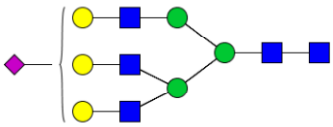
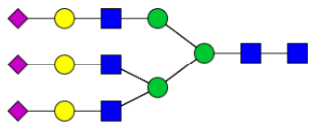
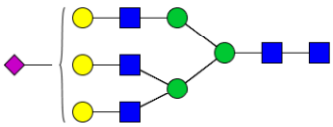
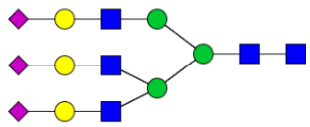
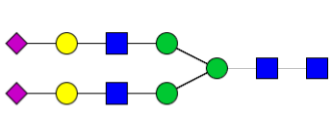
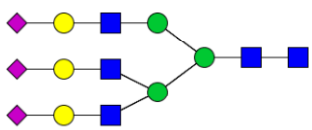
Figure 5B LC-MS analysis of 2AB labeled N-glycans from Bovine fetuin by competitor amide-HILIC column (1.7 µm) with MS detection

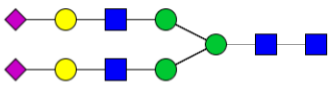
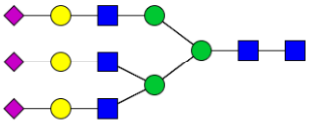
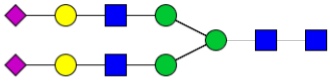
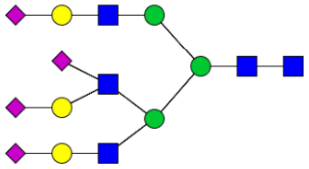
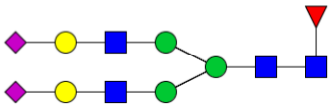
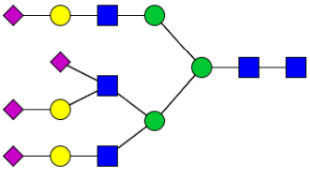
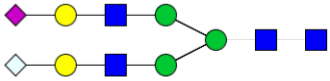
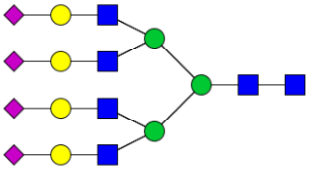
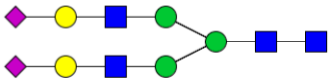
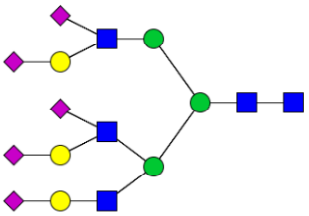
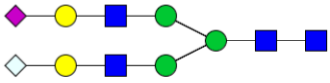
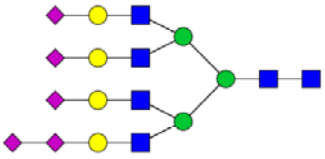
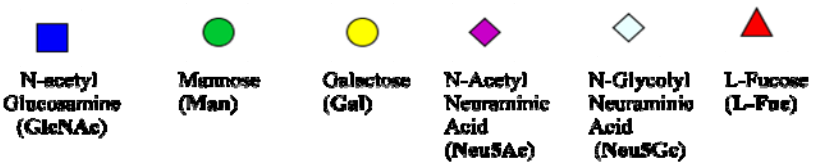


Column: **Competitor amide-HILIC column (1.7 µm)**
Dimension: 2.1x150 mm
Mobile phase: A: Acetonitrile (100 %)
B: Ammonium formate (100 mM, pH =4.4)
Flow: 0.4 mL/min
Temp: 30 °C
Detection: MS detector
MS mode: Negative
FT-MS range: m/z= 380-2000
Injection: 50 pmole
Sample: 2AB Labeled N-glycan from bovine fetuin

Time (min)	% A	% B	Flow Rate (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

Table 1 Structural characterization of glycans present in each peak by the separation of 2AB labeled N-glycans from Bovine fetuin using GlycanPac AXH-1 (1.9 µm, 2.1x150 mm) column

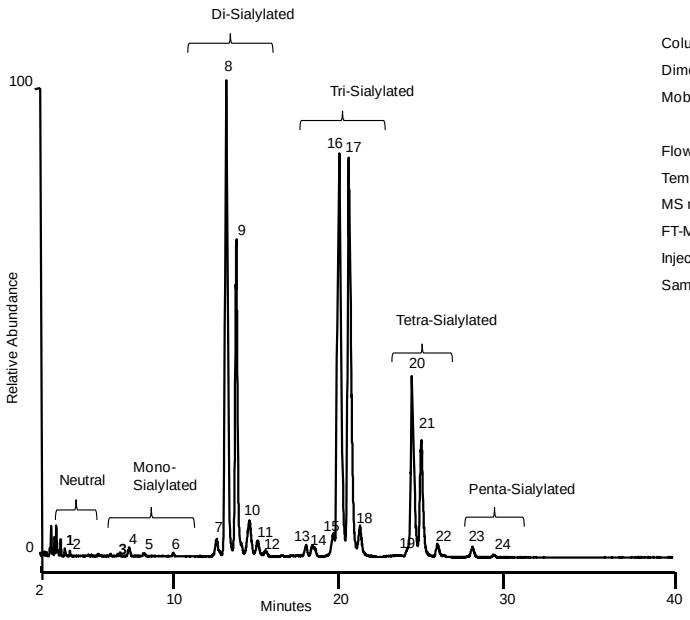
Peak No.	Compound Structure (2AB labeling are not shown)	Peak No.	Compound Structure (2AB labeling are not shown)
1		13	
2		14	
4		15	
5		16	
6		17	
7		18	
8		19	
9		20	

10		21	
11a		22	
11b		23	
11c		24	
12a		25	
12b		26	
			

6.3 Separation of native glycans

The GlycanPac AXH-1 column is well suited for high performance LC/MS separation and analysis of native reduced glycans from monoclonal antibodies and other proteins. Analyzing native glycans not only eliminates an extra step of reaction step and cumbersome cleanup methods during labeling, but also retains the original glycan profile without adding further ambiguity imposed by labeling reactions.Figure 6 shows the LC/MS analysis of native native N-glycans from Bovine fetuin using the GlycanPac AXH-1 column (1.9 µm). The native glycans were separated based on charge, size and polarity. Using an ammonium formate / acetonitrile gradient highly compatible with MS detection, the separation enables excellent FT-MS and MS/MS fragmentation data for accurate confirmation of the glycan structure of each chromatographic peak. The maximum amount of ammonium formate (30 mM) used for the separation is significantly less than what is normally when using other commercially available HILIC or normal phase columns.

Figure 6 LC-MS analysis of native N-glycans from bovine fetuin



Column:	**GlycanPac AXH-1 (1.9 µm)**
Dimension:	2.1x150 mm
Mobile phase:	A: Acetonitrile (80 %) +20 % water
	B: Ammonium formate (80 mM, pH =4.4)
Flow:	0.4 mL/min
Temp:	30 °C
MS mode:	Negative
FT-MS range:	m/z= 380-2000
Injection:	500 pmole
Sample:	Native N-glycan from bovine fetuin

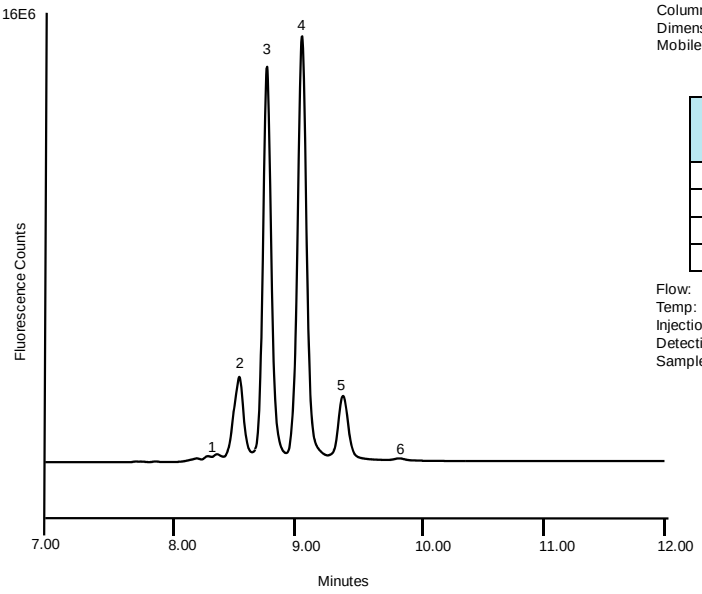
Time (min)	% A	% B	Flow Rate (mL/min)	Curve
-10	97.5	2.5	0.4	5
0	97.5	2.5	0.4	5
30	87.5	12.5	0.4	5
35	75.0	25.0	0.4	5
40	62.5	37.5	0.4	5

Native glycan profiles are significantly different from the profile of fluorescently labeled glycans, especially higher sialic acid glycans. However, MS/MS fragmentation for labeled glycans generally provides better sensitivity. The GlycanPac AXH-1 column is useful for the analysis of both native and labeled N-glycans depending on amount of sample available. If the amount sample is not extremely limited, analysis of native glycans using the GlycanPac AXH-1 is generally feasible.

6.4 Quantitative determination of glycans based on charge

Quantitative analysis of each glycan charge state is essential for quick assessment of glycan variation in protein batch comparisons as well as for comparison of diseased cell glycosylation profiles to normal cell profiles. In addition, quantitative analysis of glycans based on charge state also provide a tool for calculating the relative amounts of different sialic acid linkages after enzymatic digestion with sialidase S and sialidase A. Figure 7 shows the quantitative analysis of 2AB labeled N-glycans based on charge using GlycanPac AXH-1 column (1.9 µm) with fluorescence detection. The relative amount of each charge state glycan was estimated using a standard curve (A standards curve was drawn using the data from the chromatographic analysis of 2AB-A2 glycan standard, with the injection of different amount of samples, start from 0.1 pmoles to 5 pmoles).

Figure 7 Charge-based quantitative separation of 2AB labeled N-glycan from bovine fetuin



Column: **GlycanPac AXH-1 (1.9 µm)**
Dimension: 2.1x150 mm
Mobile phase: A: acetonitrile
B: water
C: ammonium formate (50 mM, pH =4.4)

Time (min)	% A	% B	%C	Flow Rate (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: 0.4 mL/min
Temp: 30 °C
Injection: 40 pmole
Detection: Fluroscence at 320/420 nm
Sample: 2AB Labeled N-glycan from bovine fetuin

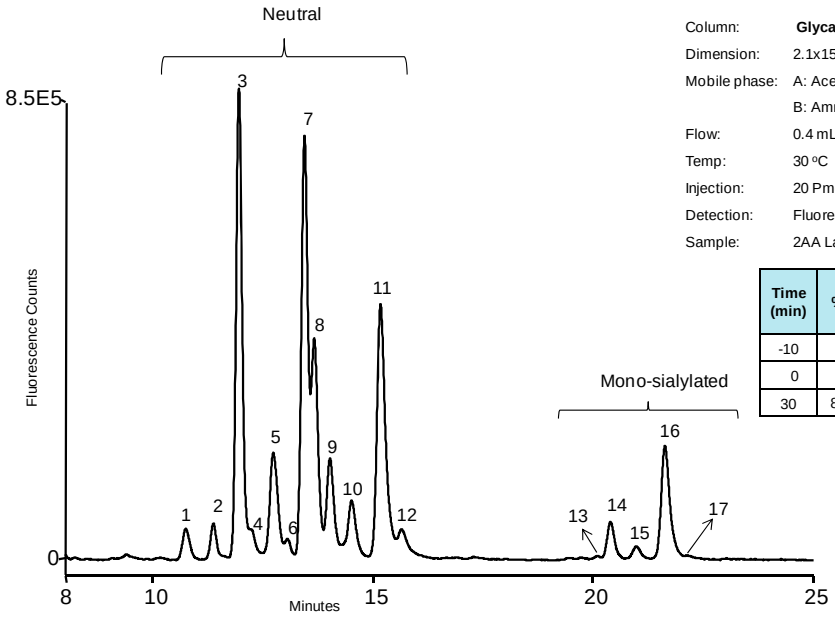
Peak No.	Peak Type	% AUC from Total
1	Neutral	0.4
2	Monosialic	8.6
3	Disialic	38.4
4	Trisialic	45.4
5	Tetrasialic	7.0
6	Pentasialic	0.2

6.5 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 effecter 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.

A representative example of the chromatographic separation of antibody glycans is shown in Figure 8, where 2AA labeled N-glycan from IgG were separated using the GlycanPac AXH-1 column (1.9 µm). Characterization of each glycan peak was determined by LC-MS and shown in Table 2. Three different glycan charge states were found in this human IgG, the majority of glycans are neutral or mono-sialylated with minor amounts of di-sialylated glycans. Separation of glycans based on charge provides advantages compared to other commercially available HILIC columns. The separation was completed in less than 30 minutes.

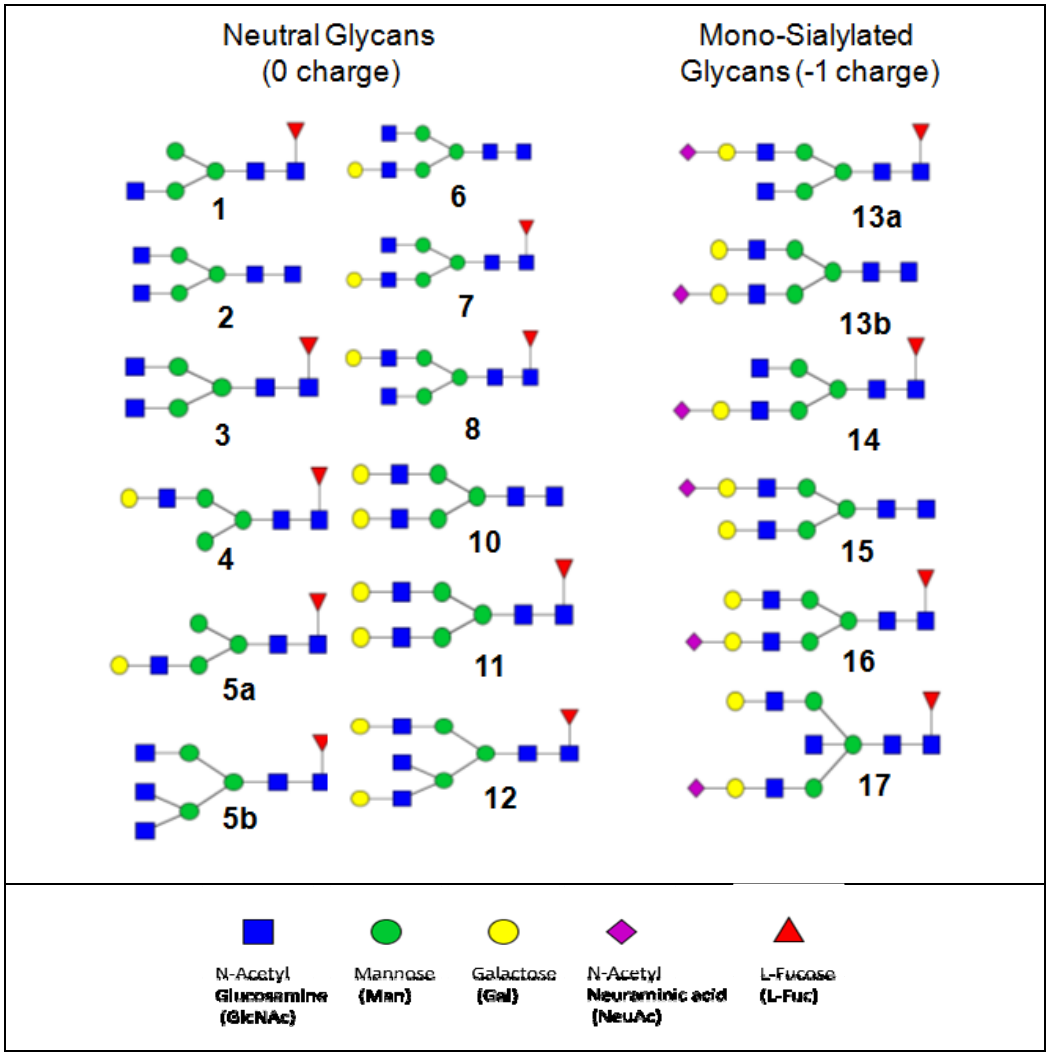
Figure 8 Separation of 2AA labeled N-glycans from human IgG



Column:	**GlycanPac AXH-1 (1.9 µm)**
Dimension:	2.1x150 mm
Mobile phase:	A: Acetonitrile (80 %) + water (20 %)
	B: Ammonium formate (80 mM, pH =4.4)
Flow:	0.4 mL/min
Temp:	30 °C
Injection:	20 Pmoles
Detection:	Fluorescence at 320/420 nm
Sample:	2AA Labeled N-glycan from Human IgG

Time (min)	% A	% B	Flow Rate (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

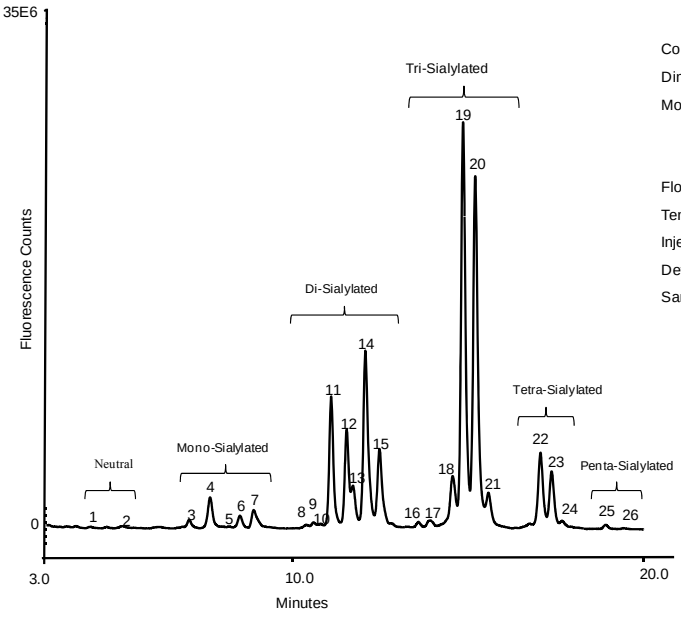
Table 2 Structural characterization of glycans present in each peaks by the separation of 2AA labeld N-glycans from Human IgG using GlycanPac AXH-1 column (1.9 µm, 2.1x150 mm)



6.6 High throughput analysis of 2AB labeled N-glycan

High-throughput analysis of glycan is important. Figure 9 shows a fast analysis of 2AB labeled N-glycan from bovine fetuin on a 1.9-µm 2.1x100-mm GlycanPac AXH-1 column; compared to the 2.1x150-mm column, similar glycan profile can be obtained on a 2.1x100-mm column with largely reduced analysis time (20 min vs. 40 min).

Figure 9 High throughput separation of 2AB labeled N-glycan from Bovine fetuin



Column:	**GlycanPac AXH-1 (1.9 µm)**
Dimension:	2.1x150 mm
Mobile phase:	A: Acetonitrile (100 %)
	B: water
	C: Ammonium formate (100 mM, pH =4.4)
Flow:	0.4 mL/min
Temp:	30 °C
Injection:	50 Pmoles
Detection:	Fluorescence at 320/420 nm
Sample:	2AB labeled N-glycan from bovine fetuin

Time (min)	% A	% B	% C	Flow Rate (mL/min)	Curve
-10	75	20	5	0.4	5
0	75	20	5	0.4	5
1	75	20	5	0.4	5
20	60	20	20	0.4	5