Thermo Scientific

GlycanPac AXR-1

Column Product Manual

P/N: 065580-01 January 2014

Part of Thermo Fisher Scientific

Thermo SCIENTIFIC

Product Manual

for

GlycanPac AXR-1 Columns

GlycanPac AXR-1, 1.9 µm, Analytical, 2.1 x 150 mm (P/N 088136)
GlycanPac AXR-1, 1.9 µm, Analytical, 2.1 x 250 mm (P/N 088135)

GlycanPac AXR-1, 3µm, Analytical, 4.6 x 150 mm (P/N 088255)
GlycanPac AXR-1, 3µm, Analytical, 3.0 x 150 mm (P/N 088252)
GlycanPac AXR-1, 3µm, Analytical, 2.1 x 150 mm (P/N 088251)

GlycanPac AXR-1, 3 µm, Guard, 4.6 x 10 mm (P/N 088260)
GlycanPac AXR-1, 3 µm, Guard, 3.0 x 10 mm (P/N 088259)
GlycanPac AXR-1, 3 µm, Guard, 2.1 x 10 mm (P/N 088258)

© 2014 Thermo Fisher Scientific Inc. All rights reserved.

All trademarks are the property of Thermo Fisher Scientific Inc. and its subsidiaries.

Thermo Fisher Scientific Inc. provides this document to its customers with a product purchase to use in the product operation. This document is copyright protected and any reproduction of the whole or any part of this document is strictly prohibited, except with the written authorization of Thermo Fisher Scientific Inc.

The contents of this document are subject to change without notice. All technical information in this document is for reference purposes only. System configurations and specifications in this document supersede all previous information received by the purchaser.

Thermo Fisher Scientific Inc. makes no representations that this document is complete, accurate or error free and assumes no responsibility and will not be liable for any errors, omissions, damage or loss that might result from any use of this document, even if the information in the document is followed properly.

This document is not part of any sales contract between Thermo Fisher Scientific Inc. and a purchaser. This document shall in no way govern or modify any Terms and Conditions of Sale, which Terms and Conditions of Sale shall govern all conflicting information between the two documents.

For Research Use Only. Not for Use in Diagnostic Procedures.

Revision History:

Revision 01, January, 2014, Original Publication;

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.. 7

1.1 The Main Features of the GlycanPac AXR-1 Columns Include: 7

1.2 Physical Data 7

1.3 Specifications and Operational Parameters...7

2. Step-By-Step User Guide 8

Step 1 – Visually Inspect the Column 8

Step 2 – Mobile Phase Preparation...8

Step 3 – Set Up the LC System 9

Step 4 – Condition the Column 9

Step 5 – Reproduce the Chromatogram in the Quality Assurance Report..9

Step 6 – Real Sample Analysis...9

3. Considerations for Method Development.. 10

3.1 General Considerations 10

3.2 Selection of Organic Solvents...10

3.3 Buffer Types 10

3.4 Mobile Phase pH...10

3.5 Isocratic or Gradient Methods...10

3.6 Injection Volume 10

3.7 Guard Cartridges..11

3.8 Other Factors...11

4. Column Care 12

4.1 Column Storage 12

4.2 Operating pH Range: pH 2.5 to 7.0 12

4.3 Operating Temperature Limit: 60 ºC 12

4.4 Upper Pressure Limit 12

4.5 Flow Rates 12

4.6 Column Washing and Cleaning Procedure 13

5. Example Applications.......... 14

5.1 Separation of 2AB-labeled *N*-Linked glycans by the GlycanPac AXR-1 (1.9 um) column based on charge, size and structural isomers using fluorescence detection, and influence of particle diameter on resolution 14

5.1.1 Elution of 2AB-labeled Fetuin *N*-linked glycans on a 2.1 x 150 mm column packed with 1.9µm particles 14

5.1.2 Elution of 2AB-labeled Fetuin *N*-linked glycans on a 3 x 150 mm column packed with 3µm particles 16

5.2 Separation of 2AA derivatized N-glycans released from IgG by GlycanPac AXR-1 (1.9 um) column; Analysis of *neutral* glycans on the GlycanPac AXR-1 column 17

5.3 Extending Resolution by Increasing Gradient Time 18

5.4 Extending Resolution by Increasing Column Length 19

5.5 GlycanPac Analyses with High-Resolution, Accurate Mass (HRAM) Detection 20

1. Introduction

The Thermo Scientific™ GlycanPac™ AXR-1 columns are high-performance mixed-mode (anion-exchange reversed-phase) HPLC/UHPLC columns for the separation of glycans by charge, size and isomerism. They are designed for industry-leading resolution by providing excellent selectivity for biologically important complex glycans, including *N*- and *O*-Linked glycans derived from protein therapeutics. These columns resolve labeled or native glycans using fluorescence and/or MS detection methods. The GlycanPac AXR-1 columns are available in 3 and 1.9 µm particle diameters. Both particle sizes provide high resolution separations of glycans with exceptional selectivity. The AXR-1 surface chemistry comprises a mixed-mode stationary phase: weak anion exchange separates glycans based on numbers of charges, and reversed-phase resolves glycans based on size and isomerism.

The GlycanPac AXR-1 column employs high-purity, spherical, porous silica that is covalently modified with a proprietary ligand. The columns and eluents are fully compatible with MS instruments.

1.1 The Main Features of the GlycanPac AXR-1 Columns Include:

- Unique glycan selectivity based on charge, branch/linkage isomerism, and size
- Exceptional resolution for native and derivatized charged glycans
- Utility for high-resolution glycan structural characterization (MS^n) and glycan quantification.
- Compatibility with both mass spectrometric and fluorescence detection methods.
- Reliable column performance.
- Available in HPLC (3µm) and UHPLC (1.9µm) formats.

1.2 Physical Data

	GlycanPac AXR-1 column (3 µm)	GlycanPac AXR-1 column (1.9 µm)
Column chemistry	WAX and RP Mixed-Mode	WAX and RP Mixed-Mode
Silica substrate	Spherical, high-purity, porous	Spherical, high-purity, porous
Particle size	3 µm	1.9 µm
Surface area	220 m^2/g	220 m^2/g
Pore size	175 Å	175 Å

1.3 Specifications and Operational Parameters

Column Particle size	Column Dimension	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 x 150 mm	10,000	2.0 – 8.0	< 60	Compatible with 0 – 100% aqueous and common HPLC solvents (except acetone)	0.2 - 0.4	0.5
	2.1 x 250 mm	15,000	2.0 – 8.0	< 60		0.2 – 0.4	0.5
3 µm	4.6 x 150mm	6,000	2.0 – 8.0	< 60		0.8 – 1.2	1.5
	3,0 x 150 mm	6,000	2.0 – 8.0	< 60		0.3 – 0.6	0.75
	2.1 x 150 mm	6,000	2.0 – 8.0	< 60		0.1 – 0.3	0.4

2. Step-By-Step User Guide

Thermo Fisher Scientific recommends that you perform a qualification test on your GlycanPac AXR-1 column before use. The purpose of column performance validation is to ensure no damage has occurred during shipping, and to verify operation of your chromatographic system. Test the column using the conditions described on the Quality Assurance (QA) Report enclosed in the column box. Repeat the test periodically to track the column performance over time.

The sample analyte is a 2AB-labeled, *N*-linked disialylated galactosylated biantennary oligosaccharide (2AB-A2, available from Prozyme PN GKSB-312).

NOTE

Slight variations in retention and resolution may be expected on different HPLC systems due to system plumbing, operating environment, reagent quality, column conditioning, and program execution.

Step 1 – Visually Inspect the Column

Immediately report any visual damage to Thermo Fisher Scientific.

Step 2 – Mobile Phase Preparation

Reliable, consistent and accurate results require mobile phases without ionic, particulate and spectrophotometric impurities. Chemicals, solvents and de-ionized water used to prepare mobile phases should be of the highest purity available. Use of reagents with low trace impurities and free of particulates helps protect your columns and system from premature wear. Thermo Fisher Scientific cannot guarantee proper column performance when the quality of the chemicals, solvents and water used to prepare the mobile phase is not of the highest available quality.

Depending on the specific application, mobile phase systems consist of an organic solvent (e.g. usually acetonitrile) and an aqueous eluent (e.g. ammonium acetate or ammonium formate). Gradient elution using proportioning valve generated programs give satisfactory results. The use of multiple proportioning valves (3-4 eluent mixing) provides better flexibility for method optimization, while binary gradients with high-pressure mixing may produce more reproducible results at the expense of flexibility.

<u>Solvents</u>

The solvents used must be free from ionic, fluorescent and UV-absorbing impurities. Use of ultrahigh purity solvents will usually ensure predictable, easy-to-interpret chromatography without baseline upsets.

<u>De-ionized Water</u>

De-ionized water used to prepare the mobile phase must be 18.2-megohm-cm (Type 1 Reagent 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 because the volatile solvent will be preferentially removed, altering the eluent composition.

Mobile Phase for Column Performance Test (QA test):

Ammonium formate buffer and acetonitrile are used for QAR test

Ammonium formate buffer is prepared by mixing ammonium formate salt in D.I. water and adjust the pH to 4.4 using formic acid and ammonium hydroxide.

1. Ammonium formate (100 mM, pH 4.4):
   a. Dissolve 6.35 ± 0.05 g of ammonium formate (Fisher Scientific AC-401152500)
   b. Add 0.70 ±0.05 g of formic acid (Fisher Scientific A117-50) in 999.6 ±0.05 g of 18.2-megohm-cm D.I. water. Thoroughly mix the resulting solution.
2. Use high-purity HPLC/MS grade acetonitrile and water for all Glycan-Pac AXR-1 column eluents.

Step 3 – Set Up the LC System

Use a standard HPLC or UHPLC system equipped with an LC pump, a column oven, a fluorescence detector, and an injector or autosampler. The system should be thoroughly primed before sample analysis.

Step 4 – Condition the Column

Each new column is shipped in 10 mM ammonium acetate containing 90% acetonitrile and should be conditioned before sample analysis. To condition the column, wash thoroughly with the mobile phase (~20 column volumes) before any injection is made.

When switching to alternate mobile phases, ensure that the new mobile phase is miscible with the existing mobile phase to avoid precipitation and column clogging. Ensure all lines and autosampler fluidics are purged and primed.

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. Four to six injections should be made to ensure complete column equilibration and that reproducible results are produced.

NOTE

Due to small differences in LC system plumbing, mobile phase preparation, and connecting tubing, slight differences in column performance between the test report and your evaluation may be expected.

Step 6 – Real Sample Analysis

Once you are satisfied with the column performance, the column is ready for your samples.

NOTE

To Avoid precipitation of glycan samples with excess organic solvent, ensure that the sample matrix and the organic and aqueous phases are all miscible.

3. Considerations for Method Development

3.1 General Considerations

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

3.2 Selection of Organic Solvents

Because it generally produces lower pressure deviations, acetonitrile is the preferred solvent for the GlycanPac AXR-1 column. However, the AXR-1column is compatible with most common HPLC solvents. When considering other solvents and buffer systems, ensure all components are miscible with one another.

3.3 Buffer Types

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

1. Ammonium acetate and ammonium formate buffers are the preferred eluents due to their compatibility with fluorescence and MS detectors, high solubility in organic solvents, and familiarity to chromatographic scientists.
2. Volatile organic acids, such as acetic acid and formic acid, can be used to control the mobile phase pH and enjoy benefits similar to ammonium acetate (or formate) buffers. TFA is not recommended for GlycanPac LC/MS applications.
3. Phosphate may be used in applications that require low UV background, as it contributes little near-UV absorbance. However, phosphate buffers tend to precipitate in high solvent concentrations.

3.4 Mobile Phase pH

Mobile phase pH needs to be controlled for reproducible results. Ammonium acetate (pK_a 4.76) and ammonium formate (pK_a = 3.75) offer good starting buffer components. Adjusting ammonium formate buffer pH can be done using formic acid and ammonium hydroxide.

3.5 Isocratic or Gradient Methods

Isocratic methods are suitable for resolution of simple or closely-related glycans. When evaluating unknown or complex samples, gradient methods are generally required.

3.6 Injection Volume

For analyses on the GlycanPac AXR-1 column, the sample must be dissolved in D.I. water. When using 2.1 x 150-mm columns, the typical injection volume is 0.5 to 15 µL.

3.7 Guard Cartridges

Guard cartridges help protect analytical columns when samples from complex or biological matrices are chromatographed. They also protect against fouling by contaminated mobile phases. The guard cartridges are available in 2.1, 3.0 and 4.6 mm diameters, and employ 3µm diameter particles. These guard cartridges are recommended for use only with analytical columns packed with 3µm packing material because they are rated to 6000 psi.

The guard cartridges require the cartridge holder P/N 069580. Part numbers for these cartridges are provided on the front page of this manual.

3.8 Other Factors

- The type, or nature, of glycans present: The GlycanPac AXR-1 is designed to accommodate the highest complexity glycan analyses.
- The detection sensitivity of the method: fluorescence and/or mass spectrometric detection methods are needed when glycan concentrations are low. However, if glycan concentration is very high, CAD, Evaporative light scattering or even RI may suffice.
- The complexity of the glycans present; when the glycan mixture to be resolved is simple and composed of very similar glycans, isocratic elution may be preferred. When complex or dissimilar glycan structures and glycans of different charge states are present, gradient elution will very likely be necessary.
- Fully aqueous mobile phases promote the best glycan separation. Addition of solvent during gradient elution may help improve MS detection sensitivity, and where elution profiles comprise few, but very well separated peaks, gradients of increasing solvent can help reduce run time while still supporting good resolution.
- Mobile phase pH: in some cases, changes in pH can produce selectivity changes for glycans with subtle structural differences. Remember to maintain the buffer pH within the recommended operational limits (see section 1.3).
- Temperature: Changes in temperature can influence resolution slightly between closely-spaced glycan peaks, and can also influence column back pressure. Generally, glycans with charges of 0-2 will elute earlier as the temperature increases. However, the effect of temperature on glycans with higher charges is not as predictable. Glycan peak widths tend to be lower as temperatures increase, so higher temperature operation tends to support improved resolution, but may also promote premature column deterioration. Hence, Thermo Scientific recommends operation between 25 and 40 °C.

4. Column Care

4.1 Column Storage

It is highly recommended that the GlycanPac AXR-1 column be stored in 90% acetonitrile, 10 m*M* Ammonium formate, pH 4.4 when not in use..

4.2 Operating pH Range: pH 2.0 to 8.0

The column lifetime depends on the chromatographic conditions. To obtain better column lifetime, mobile phases with pH values between 3 and 7 are recommended.

4.3 Operating Temperature Limit: 60 ºC

Based on user experience, the GlycanPac AXR-1 column may be used at temperatures up to 60 °C. However, the recommended operating temperatures are between 25 and 40 °C.

4.4 Upper Pressure Limit

Sudden pressure surges can quickly damage chromatographic columns. Therefore, it is important not to introduce sudden pressure surges. These may occur when switching between organic solvents and aqueous eluents. Thermo Scientific advises that a pump upper-pressure limit (e.g., 200 bar), and lower than normal flow-rates (e.g., 0.2 mL/min for the 2.1 mm I.D. columns) be applied when switching between organic and aqueous eluents, or when converting from one eluent system to another. The maximum pressure ratings for continuous service are listed in Section 1.3. Operating pressures under normal conditions of mobile phase and flow rate should, initially, be significantly lower than the maximum rating.

4.5 Flow Rates

Flow rates between 0.2 and 0.4 mL/min are typical for 2.1 mm I.D. columns. Higher flow rates may be used for analyses with larger ID columns, provided that their pressure limits are not exceeded. The typical operating flow range for 3.0-mm I.D. columns is between 0.3 and 0.6 mL/min, that and for 4.6 mm I.D. columns is between 0.8 and 1.2 mL/min.

NOTE

The column must be operated within both flow and pressure limits applied.

4.6 Column Washing and Cleaning Procedure

All samples should be filtered or centrifuged at high force (e.g., 10,000 g) prior to injection onto the GlycanPac AXR-1 column. In the event that column washing/cleaning is needed, the following procedure serves as a guideline:

1. Wash the column with 0.1 M ammonium formate /acetonitrile v/v 90/10 for 30 column volumes at flow rates of 0.2 (2.1 mm I.D.), 0.4 (3.0 mm I.D.) or 0.9 (4.6 mm I.D.) mL/min.
2. Next, wash the columns with 0.1 M ammonium formate /acetonitrile v/v 10/90 for 30 column volumes at flow rates of 0.2 (2.1 mm I.D.), 0.4 (3.0 mm I.D.) or 0.9 (4.6 mm I.D.) mL/min.
3. Finally, before any injection is made, the column should be equilibrated with the mobile phase thoroughly for 20 min at the flow rate to be used for analysis.

5. Example Applications

The GlycanPac AXR-1 column supports quantitative analyses of glycans after release from glycoproteins using fluorescence and/or mass spectrometric detection, and offers structural characterization with MS^n detection.

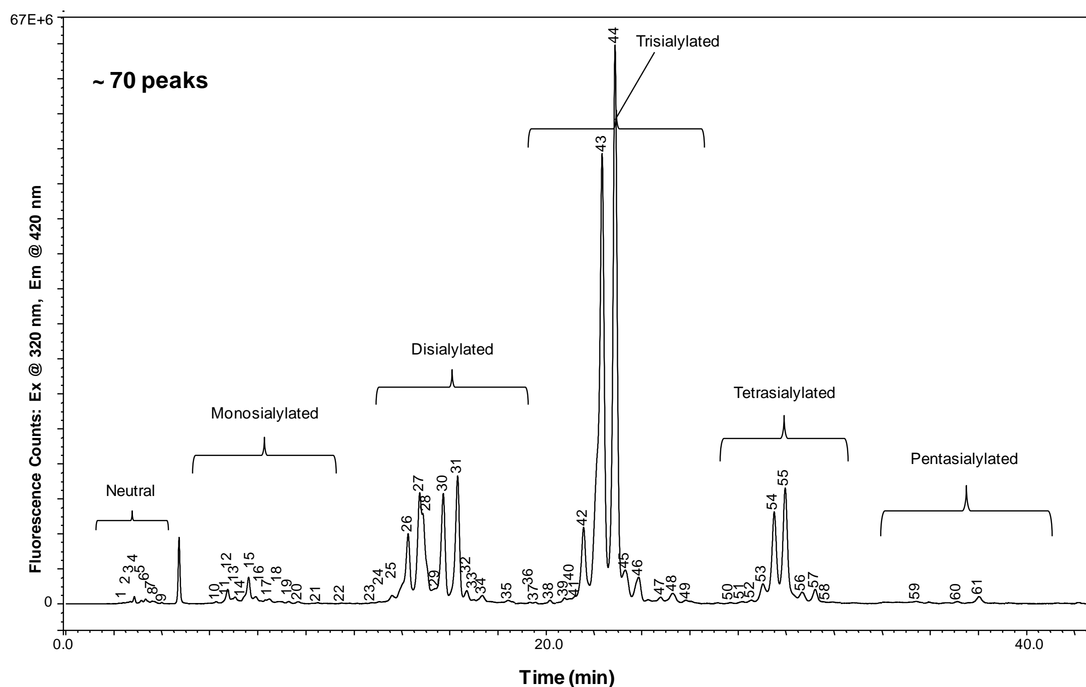
5.1 Separation of 2AB-labeled *N*-Linked glycans by the GlycanPac AXR-1 column based on charge, size and structural isomers using fluorescence detection, and influence of particle diameter on resolution.

One of the most important options for glycan analysis is the analysis of negatively charged fluorescently labeled glycans. The GlycanPac AXR-1 can resolve sialylated (or other negatively charged) glycans including N- and O-linked glycans from glycoproteins. Here the AXR-1 column is applied to the separation of 2AB-labeled *N*-linked glycans released from bovine fetuin.

5.1.1 Elution of 2AB-labeled Fetuin *N*-linked glycans on a 2.1 x 150 mm column packed with 1.9µm particles

Figure 1 shows the separation of neutral and acidic 2AB-labeled N-glycans from bovine fetuin using the GlycanPac AXR-1 (1.9 µm, 2.1x150 mm) column using a ternary gradient. The glycan elution profile is based primarily on charge: the neutral glycans eluting first, followed by mono-sialylated, di-sialylated, tri-sialylated, tetra-sialylated and finally penta-sialylated structures. Glycans of each charge state are further separated based on their size and isomeric structure. In all more than 60 glycan-containing peaks were observed to elute in less than 40 minutes. This separation can also be approximated using a binary gradient using 5 mM ammonium formate, pH 4.4 as eluent A, and 85 mM ammonium formate, pH 4.4 containing 15% acetonitrile as eluent B. To produce an elution profile equivalent to the ternary program of Figure 1, the binary gradient program would run from 0 to 100% over 70 minutes.

Figure 1 Separation of 2AB-labeled Fetuin *N*-Linked glycans by charge, isomers, and size using a GlycanPac AXR-1 (1.9µm) column



Column: **GlycanPac AXR-1 (1.9 µm)**
Dimensions: 2.1x150 mm
Mobile phase: A: Acetonitrile
B: D.I H_2O
C: 100 mM Ammonium Formate, pH 4.4
Flow: 0.4 mL/min
Temp: 30 °C
Injection: 100 pmoles
Detection: Fluorescence at 320/420 nm
Sample: 2AB-labeled Fetuin *N*-linked glycans

Time (min)	%A	%B	%C
-10	0	93	7
0	0	93	7
70	15	0	85
74	15	0	85

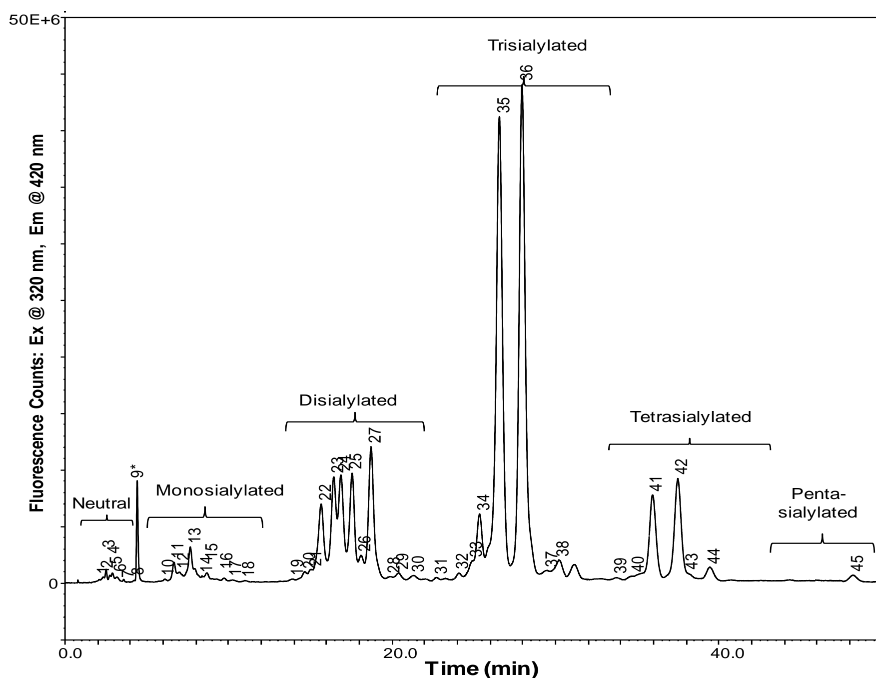
5.1.2 Elution of 2AB-labeled Fetuin *N*-linked glycans on a 3 x 150 mm column packed with 3µm particles

In Figure 2, the same sample is chromatographed on a 3x150 mm column packed with 3µm diameter AXR-1 stationary phase. For this comparison two variables are changing:

1. The column diameter has increased from 2.1 to 3.0 mm. Since the linear velocity changes with the cross-sectional area (XSA), and the ratio of XSA values is 2.0, the flow rate is doubled to accommodate this change.
2. The column particle diameter is also increased from 1.9 to 3 µm. This should not require significant changes to the gradient.

To exemplify the use of *binary* gradients, only two eluents were used to generate this glycan separation; they are: 5 m*M* ammonium formate, pH 4.4 as eluent A, and 100 m*M* ammonium formate, pH 4.4 as eluent B. For this example, acetonitrile is absent from both eluents. Hence, in this binary gradient the eluent composition changes from 0 to 100% over 70 minutes, and the last glycan peak elutes before 50 minutes. This demonstrates the increased ammonium formate gradient slope (0-100 m*M* here, vs. 0 – 85 m*M* in figure 1) and the lack of acetonitrile in this example (0-% here and 0-15% acetonitrile in figure 1). Since Figure 1 was generated with the 1.9 µm diameter particles and Figure 2 with 3 µm particles, the relative peak widths in figure 2 are greater and produce less resolution. The increase in peak width results in lower peak heights that also limit detection sensitivity. At 0.4 mL/min, the 1.9 µm particle column generates pressure in excess of that supported by standard HPLC systems. However, reducing the flow rate by 50% and doubling the gradient time would produce peak resolution close to that of the UHPLC separation shown in figure 1, but with slightly lower detection sensitivity, and decreased sample throughput.

Figure 2 Separation of 2AB-labeled Fetuin *N*-linked glycans on a GlycanPac AXR-1, 3µm, 3 x 150 mm column



Column:	**GlycanPac AXR-1 (3 µm)**
Dimensions:	3.0 x 150 mm
Mobile phase:	A: DI H_2O
	B: 100 mM Ammonium Formate,,pH 4.4
Flow:	0.8 mL/min
Temp:	30 °C
Injection:	100 pmoles
Detection:	Fluorescence at 320/420 nm
Sample:	2AB-labeled Fetuin *N*-linked glycans

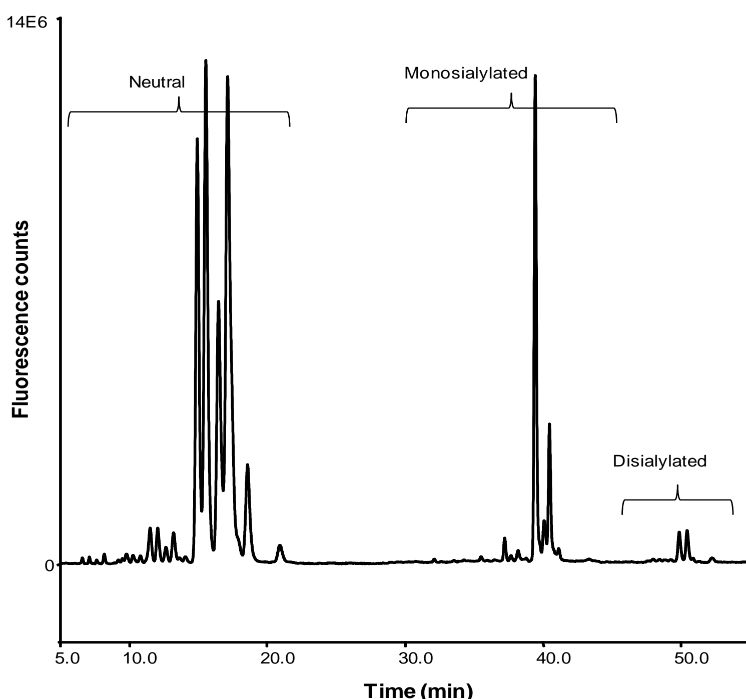
Time (min)	%A	%B
-10	93	7
0	93	7
70	0	100
74	0	100

* This peak (9) represents a residual 2AB labeling reagent contaminant.

5.2 Separation of 2AA derivatized N-glycans released from IgG by GlycanPac AXR-1 (1.9 um) column; Analysis of *neutral* glycans on the GlycanPac AXR-1 column

The GlycanPac AXR-1 column tends not to retain *neutral* oligosaccharides very well. However, by derivatizing with a different fluorophore, 2AA (2-amino benzoic acid; related to 2AB) introduction of an additional formal negative charge to each glycan can be accomplished. Hence, neutral glycans become charged to the level of monosialylated glycans, thus increasing their retention. An important example of predominantly neutral glycans is the glycan suite released from human IgG proteins. As monoclonal antibodies (MAbs), these are often developed as protein *therapeutics,* so their characterization, and stability from lot-to-lot, must be determined and controlled. Glycan labeling with 2AA employs essentially the same process as that for 2AB, so 2AA derivatives can be readily prepared using the 2AB derivatization process. Figure 3 shows chromatography of 2AA-labeled *N*-linked glycans released by PNGase-F from a human IgG. This chromatogram mirrors that of figures 1 and 2, in that "neutral" (now singly charged by 2AA labeling) glycans elute first, followed by monosialylated, then disialylated glycans. In this case, the "neutral" glycans elute during an isocratic section of the method, while the monosialylated and disialylated glycans elute during ammonium formate and acetonitrile gradient development.

Figure 3 Separation of 2AA-labeled *N*-Linked glycans from human IgG by charge, isomers and size using a GlycanPac AXR-1 (1.9µm) column



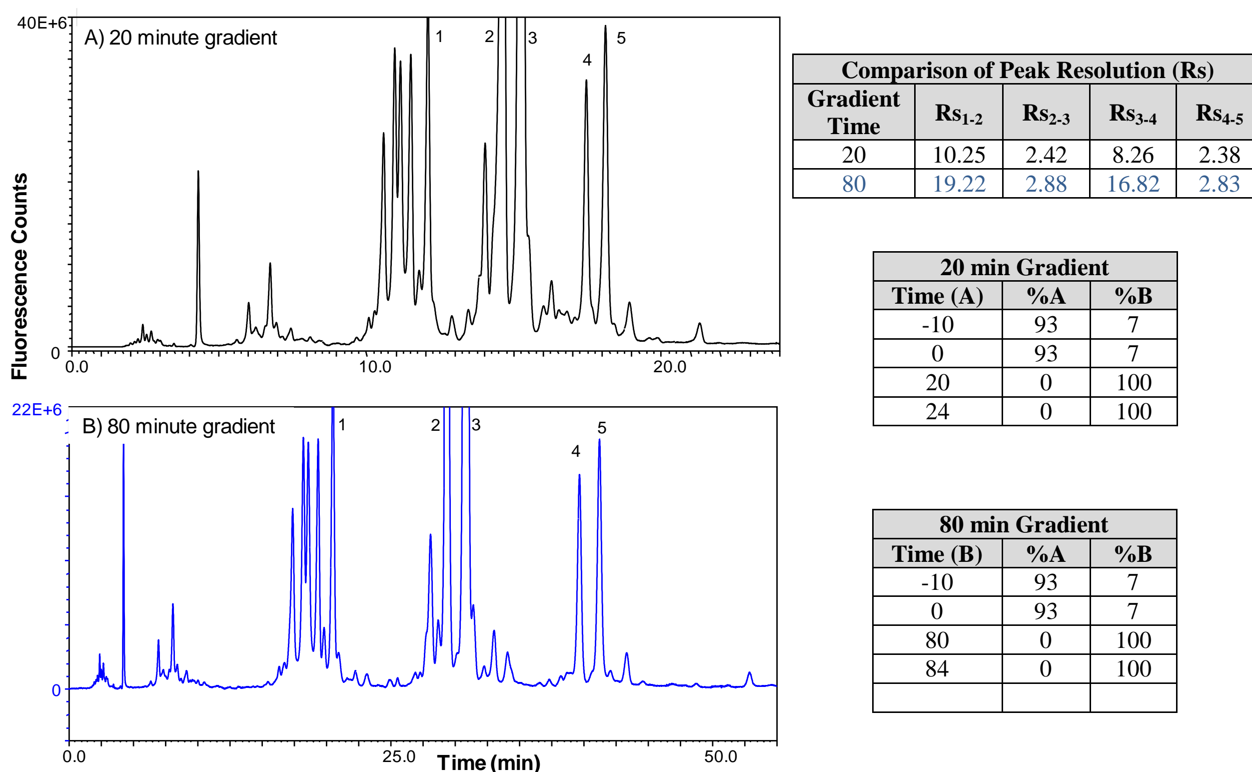
Column: **GlycanPac AXR-1 (1.9 µm)**
Dimensions: 2.1 x 150 mm
Mobile phase: A: Acetonitrile
B: Water
C: 100 mM Ammonium Formate, pH 4.4
Flow: 0.3 mL/min
Temp: 30 °C
Injection: 100 pmoles
Detection: Fluorescence at 320/420 nm
Sample: 2AA-labeled *N*-Linked glycan from human IgG

Time (min)	%A	%B	%C
-10	0	92	8
0	0	92	8
1	0	92	8
25	0	92	8
26	0	50	50
60	10	40	50

5.3 Extending Resolution by Increasing Gradient Time

As with all columns designed for gradient use, peak capacity and resolution improve with increasing gradient time. This also applies to the GlycanPac AXR-1. Figure 4 shows an example where gradients of 20 and 80 minutes are compared. This example shows that increasing gradient time produces dramatic improvements to resolution of some, and modest improvement to resolution of other, glycans in the fetuin 2AB-labeled fetuin *N*-linked glycan mixture. Dramatic improvements are noted between peaks 1 and 2 and between peaks 3 and 4. The first of each of these peak pairs harbor a different *charge* than the glycan from which it is resolved. Between these pairs, the presence of several minor peaks is observed in the bottom chromatogram, demonstrating the advantage of the longer gradient. The peak pairs that show little improvement represent isomers *within* a given charge set, so less profound improvements to resolution are expected. For glycans from proteins with less extensive glycosylation than Fetuin, shorter gradients may prove useful to improve throughput of glycan characterization.

Figure 4 Effect of gradient time on glycan resolution on the GlycanPac AXR-1 column. Comparison of 20 min and 80 min gradients on 2.1 x 150mm formats



Comparison of Peak Resolution (Rs)				
Gradient Time	Rs_{1-2}	Rs_{2-3}	Rs_{3-4}	Rs_{4-5}
20	10.25	2.42	8.26	2.38
80	19.22	2.88	16.82	2.83

20 min Gradient		
Time (A)	%A	%B
-10	93	7
0	93	7
20	0	100
24	0	100

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

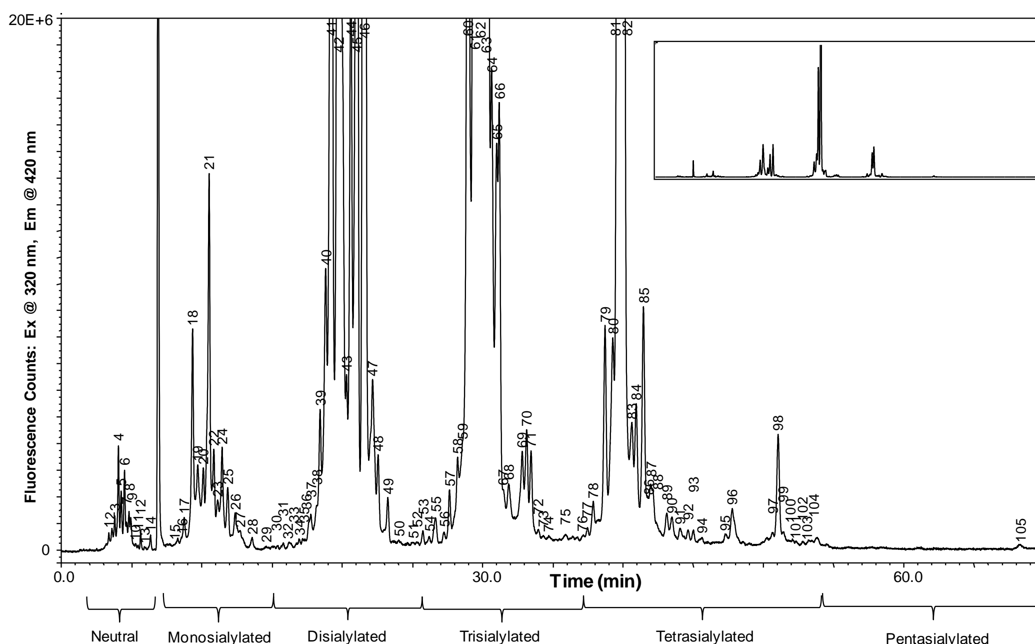
Column: **GlycanPac AXR-1 (1.9 µm)**
Dimensions: 2.1 x 150 mm
Mobile phase: A: D.I. H_2O
B: 100 mM ammonium formate, pH 4.4
Flow: 0.4 mL/min
Temp: 30 °C
Injection: 100 pmoles
Detection: Fluorescence at 320/420 nm
Sample: 2AB-labeled Fetuin *N*-linked glycan

5.4 Extending Resolution by Increasing Column Length

As with all columns, peak capacity and resolution also improve with increasing column length. Hence, longer GlycanPac columns will also produce improved resolution. This will maximize separation efficiency, but with commensurately longer gradient times and decreased throughput. Figure 5 shows the separation of the 2AB-labeled fetuin *N*-linked glycans on a 2.1x250mm GlycanPac AXR-1 column.

With this format, the 90 minute gradient reveals 105 labeled glycans in less than 70 minutes, a dramatic improvement over the 150mm long GlycanPac AXR-1 columns depicted in Figures 1 and 2. Increasing column length also tends to improve (reduce) peak width. Hence, detection *sensitivity* may also improve. For the most thorough glycan characterization using LC/MSn methods, the 250 mm GlycanPac columns are recommended. This same gradient can be developed using a *binary* eluent system where eluent A = 7 m*M* Ammonium formate, pH 4.4, and eluent B = 75 m*M* ammonium formate, pH 4.4 with 25% acetonitrile. This binary gradient would run from 0 to 100% Eluent B in 90 minutes.

Figure 5 Improvement in resolution of fetuin 2AB-labeled *N*-linked glycans on a longer, 2.1 x 250 mm GlycanPac AXR-1 column. Inset is full Chromatogram



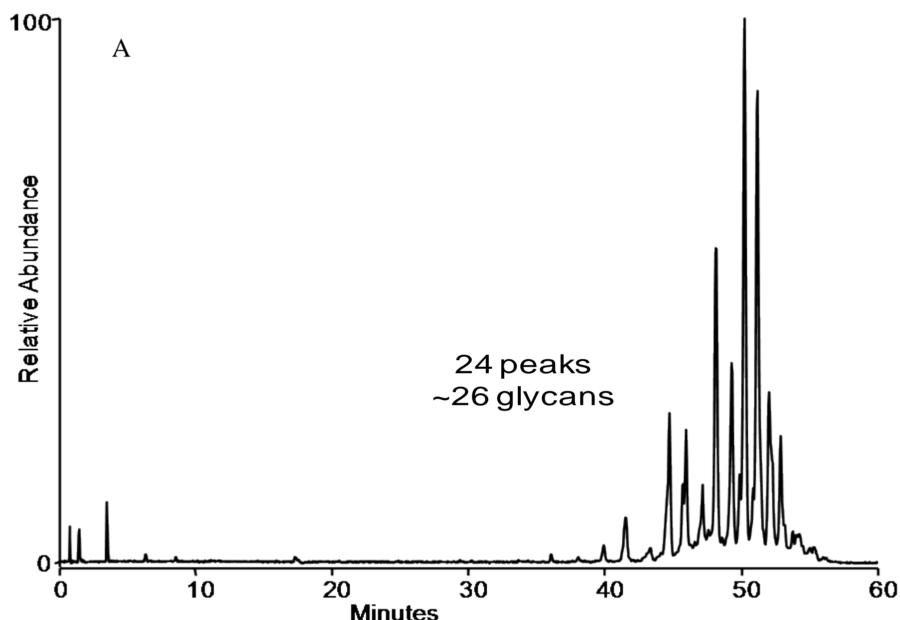
Column: **GlycanPac AXR-1 (1.9 µm)**
Dimensions: 2.1 x 250 mm
Mobile phase: A: Acetonitrile
B: D.I. H_2O
C: 100 mM ammonium formate, pH 4.4
Flow: 0.4 mL/min
Temp: 30 ^oC
Injection: 100 pmoles
Detection: Fluorescence at 320/420 nm
Sample: 2AB-labeled Fetuin *N*-linked glycans

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

5.5 GlycanPac Analyses with High-Resolution, Accurate Mass (HRAM) Detection

The separation of glycans based on charge, isomerism and size makes the GlycanPac AXR-1 column a powerful tool for accurate glycan analysis by LC/MS. In the comparison that follows, 2.1 x 150 mm columns are used. While 250 mm long columns would produce better resolution, the commercial 1.7 µm particle column was not available in a 250 mm length. Figure 6 provides a comparison between a a commercial 1.7 µm amide HILIC column (A) and a 1.9 µm GlycanPac AXR-1 column (B) designed for the separation of 2AB-labeled *N*-glycans. In this figure, 2AB-labeled N-linked glycans released from bovine fetuin are chromatographed and detected by MS in negative-ion mode, and both columns are run under respectively optimized conditions. The structural characterization of each identified glycan on the GlycanPac AXR-1 was determined by MS/MS fragmentation data using structural analysis SimGlycan® software. The list of glycans identified in bovine fetuin using the GlycanPac AXR-1 column is shown in Table 1. This table contains several oligosaccharides structures that appear identical by m/z and charge, but that elute at slightly different positions in the gradient. In many cases the MS^3 results fully describe the isomers, but in others there is insufficient MS data to fully elucidate linkage and/or branch isomer structure. The fact that they appear in the MS with identical high-resolution, accurate-mass (HRAM) m/z values, but elute at different times indicates that they are isomers. The data clearly show that the GlycanPac AXR-1 column (with 73 peaks containing 135 independent glycans) resolves, and allows MS identification of many more glycans than the 1.7 µm commercial amide HILIC column where differently charged glycans often co-elute. This clearly demonstrates the advantages of the GlycanPac AXR-1 column.

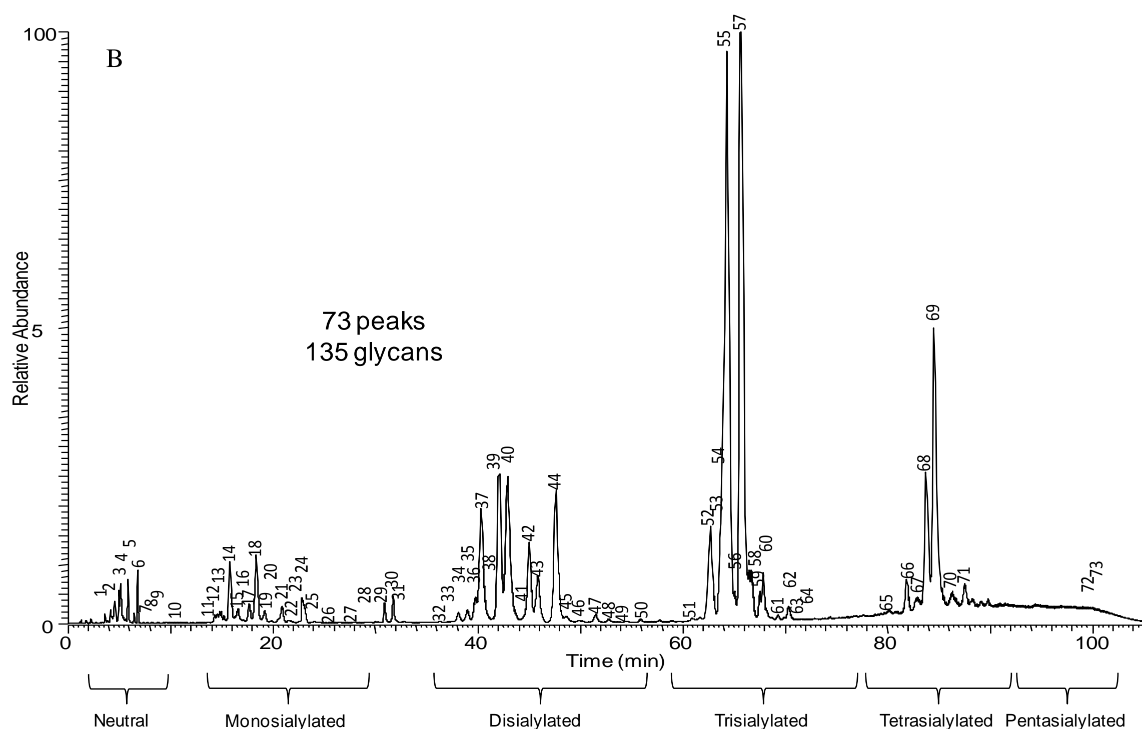
Figure 6 Comparison of fetuin 2AB-labeled N-Linked glycan LC/MS analyses on Amide-HILIC (A) and GlycanPac AXR-1 columns (B)



Column: Commercial Amide-HILIC column (1.7 µm)
Dimensions: 2.1x150 mm
Mobile phase: A: Acetonitrile (100 %)
B: 100 mM Ammonium Formate, pH 4.4
Flow: 0.4 mL/min
Sample: 2AB-labeled Fetuin *N*-Linked glycans
MS detector: Q-Exactive, FT-MS range: m/z= 400-2200
Temp: 30 °C
MS mode: Negative
Injection: 50 pmole

Time (min)	%A	%B	Curve
-10	75	25	5
0	75	25	5
5	75	25	6
55	55	45	6
63	50	50	6

Figure 6 Comparison of fetuin 2AB-labeled N-Linked glycan LC/MS analyses on Amide-HILIC (A) and GlycanPac AXR-1 columns (B)



Column: **GlycanPac AXR-1 (1.9 µm)**, 2.1 x 150 mm
Mobile phase: A: Acetonitrile
B: 100 mM ammonium formate pH 4.4
C: DI H_2O
Flow: 0.2 mL/min
Injection: 100 pmoles
Sample: 2AB-labeled Fetuin *N*-linked glycans
MS detector: OrbiTrap Fusion
Temp: 30 °C
MS mode: Negative
Injection: 50 pmole

Time (min)	%A	%B	%C	Curve
-10	0	10	90	5
0	0	10	90	5
1	0	10	90	5
45	1	50	49	5
90	20	50	30	5
100	20	60	30	5