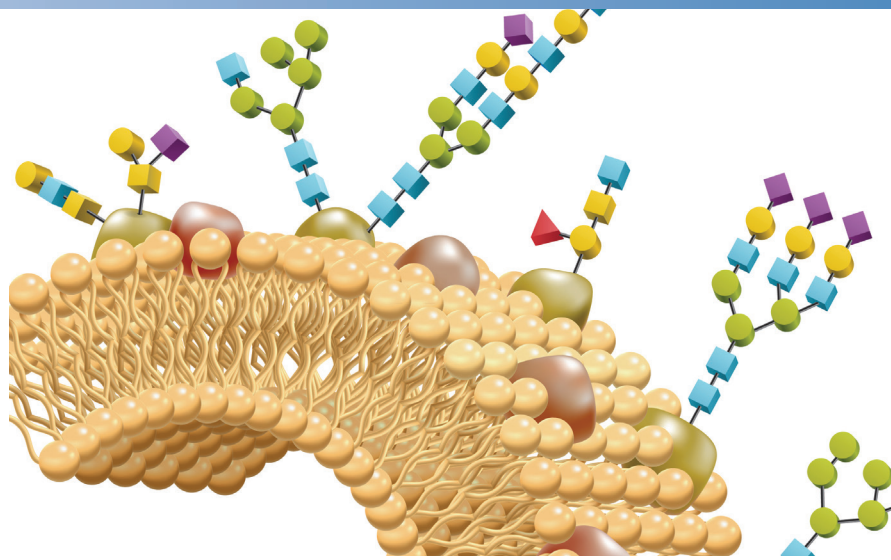
GlycanPac AXR-1 Columns

For High Resolution Glycan Analysis

The Thermo Scientific™ GlycanPac™ AXR-1 columns are high-performance, silica-based HPLC columns for simultaneous separation of glycans by charge, isomer structure and size. They provide industry-leading resolution with unique selectivity for biologically important glycans, either labeled or native, using either fluorescence or MS detection.

Product features include:

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



Introduction

Complex glycans are widely distributed in biological systems in “free state”, as conjugates to proteins and lipids, and as proteoglycans. They mediate a wide range of biological and physiological processes including cell recognition, physiological regulation, cellular communication, gene expression, cellular immunity, growth and development, and are often perturbed in diseased tissues. The functions of proteins are dependent on the structure and types of their oligosaccharide attachments. Glycan structures are highly diverse, complex and heterogeneous due to post-attachment modifications and these depend on physiological and culture conditions. Thus, comprehensive characterization of glycans is very challenging.

Oligosaccharides are covalently attached to proteins through two structural motifs. Those attached to the amide nitrogen of asparagine residues are referred to as “*N*-linked” glycans. Glycans may also be attached to proteins through hydroxyl moieties on serine or threonine residues (“*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 influenced by glycosylation. Hence, characterizing, measuring, and controlling the specific glycosylation of glycoprotein-based therapeutics, including biosimilars, is of fundamental importance.

Several chromatographic separation modes have been adopted for glycan analysis, including normal phase (NP, or hydrophilic interaction liquid chromatography, HILIC), ion-exchange (IEX) and reversed-phase (RP). Because glycans are both polar and hydrophilic, they are commonly separated using amide HILIC phases that separate glycans primarily by hydrogen bonding, resulting in separations based on size and polarity. One limitation of this approach is that glycans of different charge states are intermingled in the separation window, making identification and quantification very challenging, even with MS, or MS^2 detection. In contrast, the GlycanPac AXR-1 column harbors a unique surface chemistry to overcome these limitations by separating glycans based on charge, isomerism and size. As a result, this column supports direct glycan quantification of many more isoforms than can be quantified using other column types. In addition, the eluents employed for these separations are directly compatible with mass spectrometry, providing opportunity to verify the identity of eluted glycans and detection of impurities. The GlycanPac AXR-1 column delivers greater selectivity and higher resolution compared to other HPLC (or UHPLC) columns for glycan analysis.

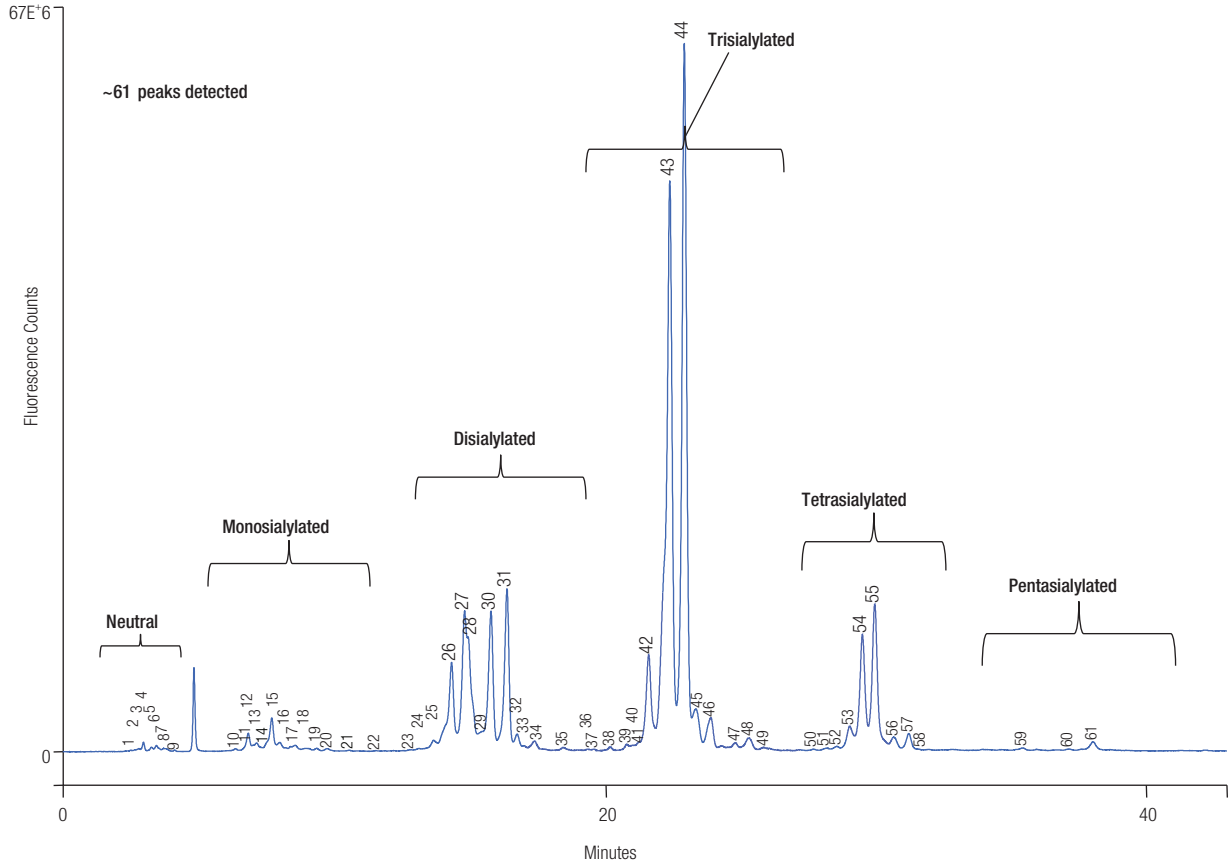
Column Technology

Based on innovative mixed-mode surface chemistry, the GlycanPac AXR-1 column combines both weak anion-exchange (WAX) and reversed-phase interaction mechanisms producing exceptional glycan selectivity and industry-leading resolution. The WAX functionality provides retention of negatively charged glycans that elute in order of increasing charge, and the reversed-phase mode facilitates resolution of glycans of the same charge according to their isomerism and size. These attributes produce unparalleled glycan separations. The GlycanPac AXR-1 column is designed for HPLC and UHPLC methods using either fluorescence or MS detection, and uses volatile aqueous buffers (e.g., ammonium acetate or ammonium formate) and acetonitrile, presenting the eluting glycans ready for introduction into MS instruments. The GlycanPac AXR-1 column chemistry is customized to elute both neutral and charged glycans at eluent concentrations compatible with high MS detection sensitivity, and supports MS^n applications. Chromatographic condition tables are provided. These may be used for further method development using adjustments to buffer concentration, pH, temperature and solvent content. The GlycanPac AXR-1 stationary phase is based on high-purity spherical silica, and is available in both 1.9 µm and 3 µm particle diameters for UHPLC or HPLC applications. The columns are available in different dimensions to support applications requiring different resolution, throughput or instrumental requirements. Figure 1 shows the separation of 2AB-labeled *N*-linked glycans from fetuin on the 1.9 µm particles in a 2.1 × 150 mm column. This shows resolution of approximately 61 glycan peaks in less than 40 minutes.

Applications

Glycan Analysis by LC/Fluorescence

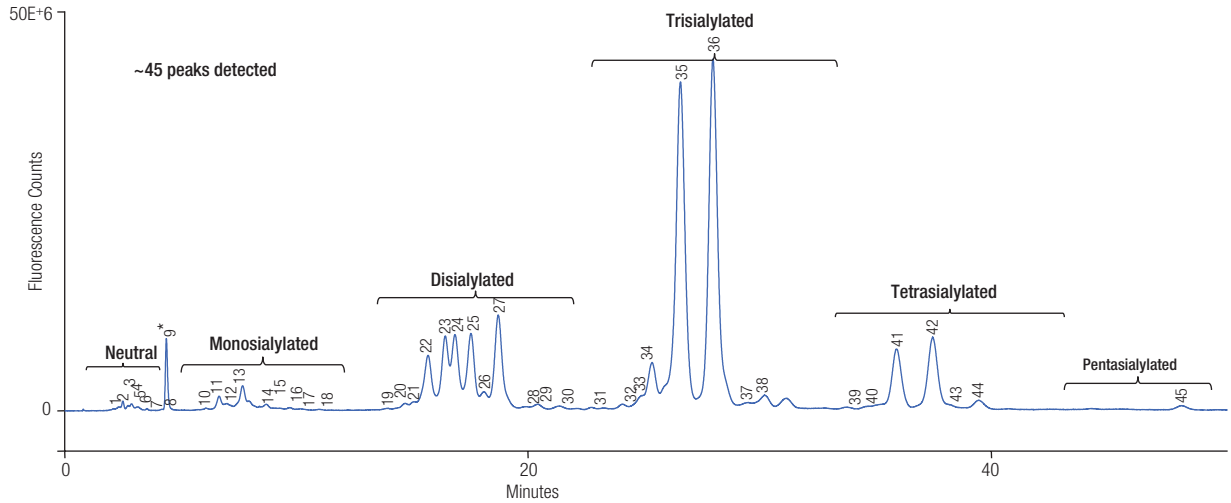
The GlycanPac AXR-1 column can be used for qualitative, quantitative, and structural characterization of uncharged and charged glycans released from biological molecules (e.g. proteins). Figure 2 shows the separation of neutral and acidic 2AB-labeled *N*-Linked glycans from bovine fetuin using a GlycanPac AXR-1 (3 µm, 3.0 × 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 pentasialylated forms. Glycan clusters are retained by anion exchange interactions and represent glycans of the same charge. Within each cluster, the glycans having the same charge are further separated according to their branch— or linkage-isomers, and size, by reversed-phase interactions, revealing at least 44 putative glycans (in Figure 2, and 61 in Figure 1). This interpretation is substantiated by independent UHPLC-MS^n studies. Compared to the 1.9 µm counterpart, the 3 µm GlycanPac AXR-1 column depicted in Figure 2 operates at 50% lower backpressure but still offers excellent resolution using standard HPLC systems, especially with longer gradient time.



Column: **GlycanPac AXR-1 (1.9 µm)**
Dimension: 2.1 × 150 mm
Mobile Phases: A) Acetonitrile
B) D.I. H_2O
C) 100 mM Ammonium formate, pH 4.4
Flow Rate: 0.4 mL/min
Injection Volumes: 100 pmoles
Temperature: 30 °C
Detection: Fluorescence at 320/420 nm
Sample: 2AB-labeled fetuin *N*-linked glycans

Time (min)	% A	% B	% C
–10	0	7	93
0	0	7	93
70	15	0	85
74	15	0	85
74.5	0	7	93
85.0	0	7	93

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



Column: **GlycanPac AXR-1 (3 µm)**
Dimension: 3.0 × 150 mm
Mobile Phases: A) D.I. H_2O
B) 100 mM Ammonium formate, pH 4.4
Flow Rate: 0.8 mL/min
Injection Volume: 100 pmoles
Temperature: 30 °C
Detection: Fluorescence at 320/420 nm
Sample: 2AB-labeled fetuin *N*-linked glycans

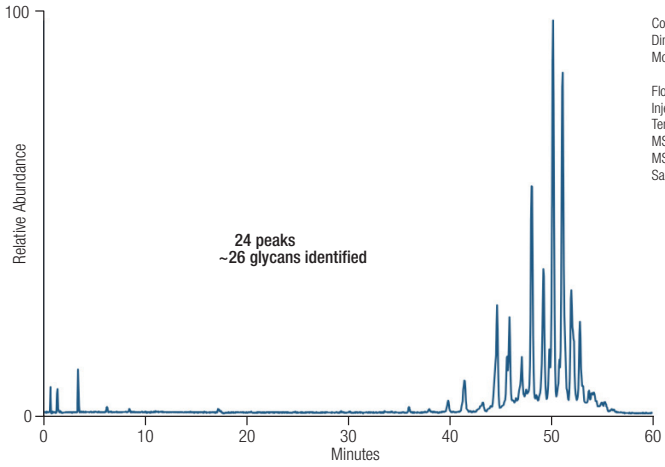
Time (min)	% A	% B
-10	93	7
0	93	7
70	0	100
74	0	100

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

Figure 2: Separation of 2AB-labeled fetuin *N*-linked glycans on a GlycanPac AXR-1, 3 µm 3.0 × 150 mm columns.

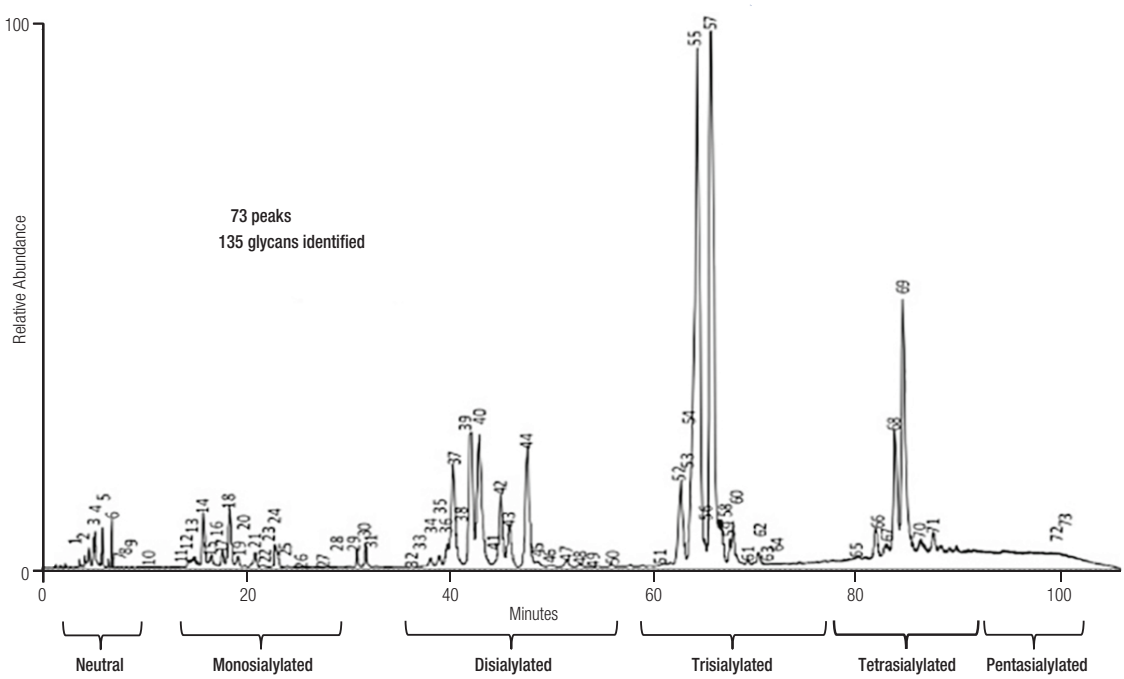
Glycan Analysis by LC/MS

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. Figure 3 provides a comparison between two 2.1 × 150 mm columns. One, a 1.9 µm GlycanPac AXR-1 column and the other a commercial 1.7 µm amide HILIC column designed for the separation of 2AB-labeled *N*-glycans. In this figure, 2AB-labeled glycans from bovine fetuin are chromatographed and detected by MS in negative-ion mode (mass scan range 400-2200 Daltons), 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 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 isomers. 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 glycans) resolves and allows MS identification of many more glycans than the 1.7 µm commercial amide HILIC column that resolves many fewer glycan-containing peaks, where differently charged glycans often co-elute.



Column:	**Commercial Amide-HILIC (1.7 µm)**
Dimension:	2.1 × 150 mm
Mobile Phases:	A) Acetonitrile
	B) 100 mM Ammonium formate, pH 4.4
Flow Rate:	0.4 mL/min
Injection Volume:	50 pmoles
Temperature:	30 °C
MS Mode:	Negative
MS Detector:	Q-Exactive, FT-MS range: *m/z*= 400-2200
Sample:	2AB-labeled fetuin *N*-linked glycans

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

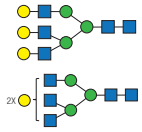
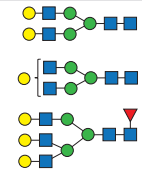
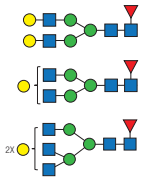
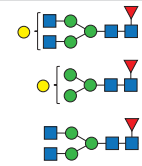
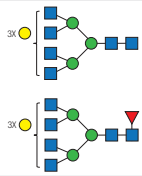
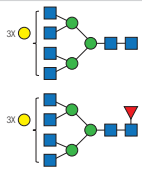
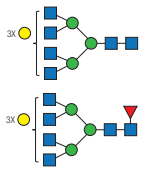
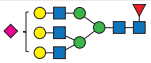
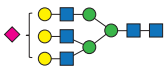


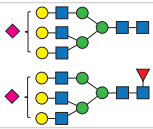
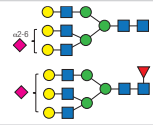
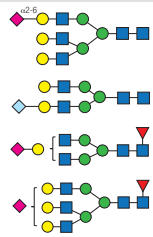
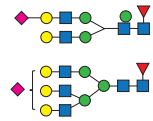
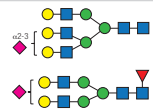
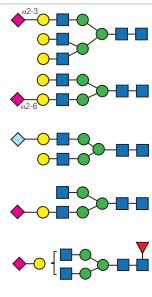
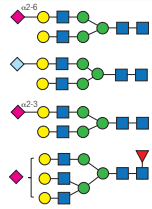
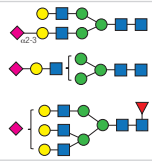
Column:	**GlycanPac AXR-1 (1.9 µm)**
Dimension:	2.1 × 150 mm
Mobile Phases:	A) Acetonitrile
	B) 100 mM Ammonium formate, pH 4.4
	C) D.I. H_2O
Flow Rate:	0.2 mL/min
Injection Volume:	100 pmoles
Temperature:	30 °C
MS Mode:	Negative
MS Detector:	OrbiTrap Fusion
Sample:	2AB-labeled fetuin *N*-linked glycans

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

Figure 3: Comparison of 2AB-labeled fetuin glycan LC/MS analyses on Amide-HILIC and GlycanPac AXR-1 columns.

Table 1: Glycan structures associated with peaks in the GlycanPac AXR-1 column elution pattern shown in Figure 3. Note that a few peaks do not exhibit *m/z* values consistent with glycans, so no structures are presented for those peaks.

Peak	Structure	Ret. Time (min)	*m/z*	Charge
1		3.64 3.64	1061.892 980.865	0
2		4.16 4.30 4.36	879.324 798.297 1134.918	0
3		5.00 5.22 5.24	952.352 871.326 1053.892	0
4		5.44 5.70 5.65	871.326 769.786 790.299	0
5	No glycan *m/z*			
6	No glycan *m/z*			
7		7.77 7.77	1163.429 1236.458	0
8		8.21 8.21	1163.429 1236.458	0
9		8.50 8.50	1163.429 1236.458	0
10	No glycan *m/z*			
11		13.65	1280.466	1
12		14.17	1280.466	1

Peak	Structure	Ret. Time (min)	*m/z*	Charge
13		14.98 14.61	1207.437 1280.466	1
14		15.81 15.48	1207.437 1280.466	1
15		16.60 16.89 16.87 16.28	1207.437 1032.868 1016.873 1280.466	1
16		17.34 17.13	1097.900 1280.466	1
17		17.77 18.11	1207.437 1097.900	1
18		18.39 18.39 18.34 18.39 18.60	1207.437 1024.871 1032.868 943.845 1016.873	1
19		19.19 19.33 19.30 18.96	1024.871 1032.868 1097.900 1280.466	1
20		19.95 20.59 19.67	943.845 842.305 1280.466	1

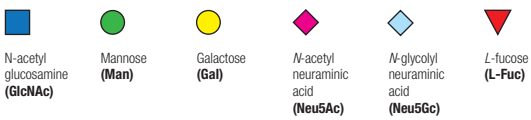
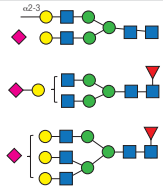
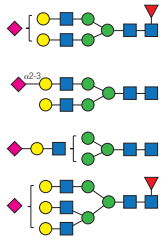
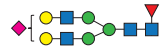
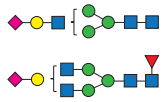
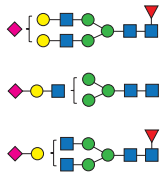
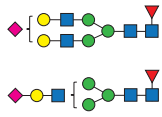
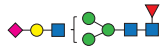
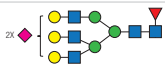
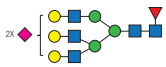
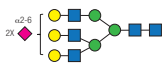
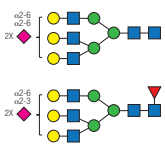
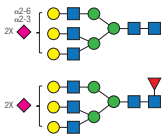


Table 1: Continued.

Peak	Structure	Ret. Time (min)	*m/z*	Charge
21		20.92 20.96 20.96	1024.871 1016.873 1280.466	1
22		21.72 21.89 21.73 21.59	1097.900 943.845 842.305 1280.466	1
23		22.40	1097.900	1
24		22.90 22.90	842.305 1016.873	1
25		24.07 24.17 24.26	1097.900 842.305 915.334	1
26		24.57 25.37	1097.900 915.334	1
27		26.87	915.334	1
28		28.24	915.334	1
29	No glycan *m/z*			
30	No glycan *m/z*			
31	No glycan *m/z*			
32		36.30	1426.014	2
33		37.26	1426.014	2
34		38.16	1352.985	2
35		39.02 38.88	1352.985 1426.014	2
36		39.84 40.02	1352.985 1426.014	2

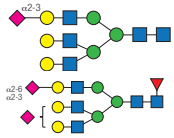
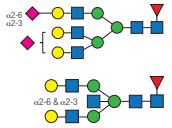
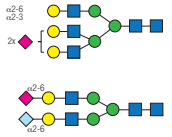
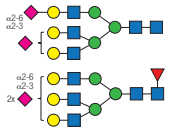
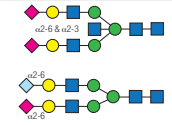
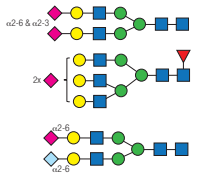
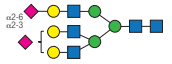
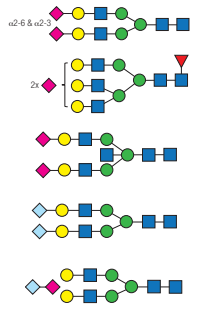
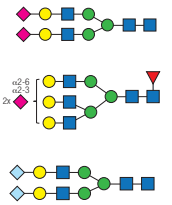
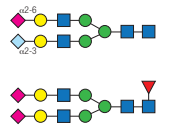
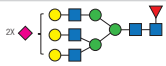
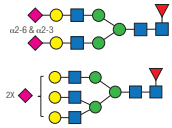
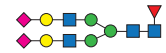
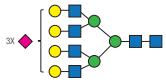
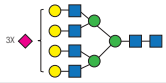
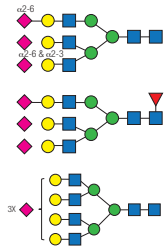
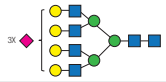
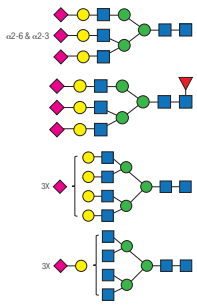
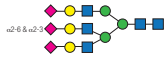
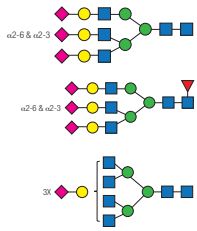
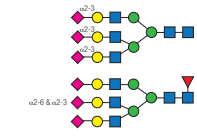
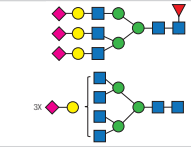
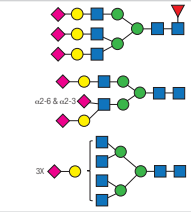
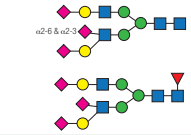
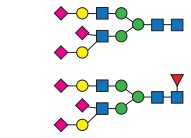
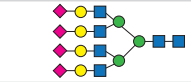
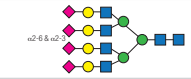
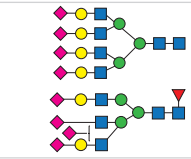
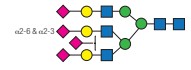
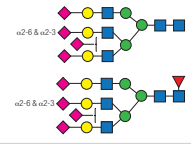
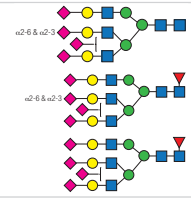
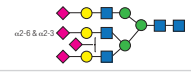
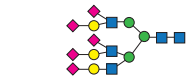
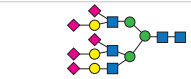
Peak	Structure	Ret. Time (min)	*m/z*	Charge
37		40.31 40.31	1352.958 1426.014	2
38		40.99 41.64	1426.014 1271.958	2
39		42.19 42.48	1352.958 1178.958	2
40		42.91 43.54	1352.958 1426.014	2
41		44.53 43.63	1271.958 1178.416	2
42		45.03 45.03 44.84	1170.419 1426.014 1178.416	2
43		45.91	1352.958	2
44		47.67 47.52 47.35 47.83 47.67	1170.419 1426.014 1271.958 1186.414 1178.416	2
45		48.64 48.21 48.69	1170.419 1426.014 1186.414	2
46		51.54 49.87	1170.419 1243.448	2

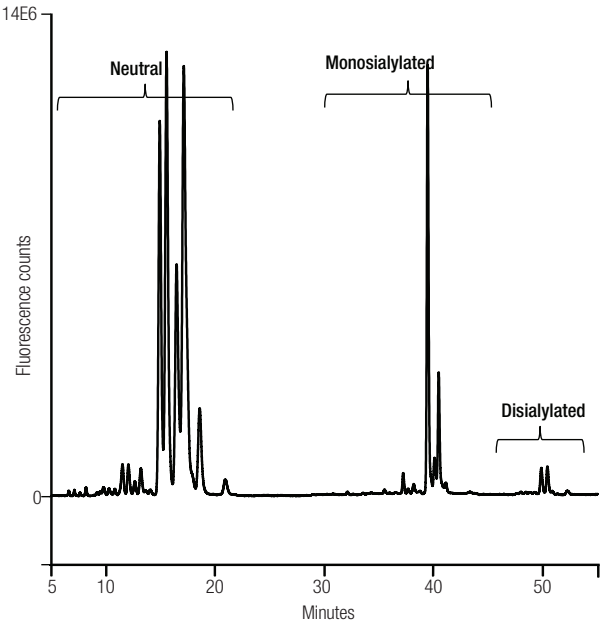
Table 1: Continued.

Peak	Structure	Ret. Time (min)	*m/z*	Charge
47		51.38	1426.014	2
48		52.80 52.86	1243.448 1426.014	2
49	No glycan *m/z*			
50		55.89	1243.448	2
51		60.43	1120.397	3
52		61.57	1120.397	3
53		62.74 63.01 62.41	998.686 1047.372 1120.397	3
54		63.09	1120.397	3
55		64.27 64.74 64.27 65.00	998.686 1047.372 1120.397 1066.379	3
56		65.68	998.686	3
57		66.76 66.37 66.25	998.686 1047.372 1066.379	3
58		67.09	1315.9663	3
59		67.83 67.53	998.686 1047.372	3

Peak	Structure	Ret. Time (min)	*m/z*	Charge
60		68.19 67.83	1047.372 1066.379	3
61		69.20 69.28 69.03	1047.372 1315.966 1066.379	3
62		70.31 70.19	1315.966 1388.995	3
63		71.46 71.14	1315.966 1388.995	3
64		72.14	1315.966	3
65		80.19	1217.428	4
66		81.96	1217.428	4
67		83.23 83.17	1217.428 1144.404	4
68		83.74	1095.718	4
69		84.54 84.18	1095.718 1144.404	4
70		86.26 85.67 86.39	1095.718 1144.404 1144.404	4
71		87.54	1095.718	4
72		99.50	1192.750	5
73		99.89	1192.750	5

Analysis of 2AA-labeled Antibody Glycans

Unlike 2AB, 2AA-labeling introduces a formal negative charge to each glycan. This promotes greater binding to the GlycanPac AXR-1 column, thus improving retention of both neutral and negatively charged glycans. Antibodies are the most common proteins developed for therapeutics, and are under development for the treatment of numerous diseases. However, antibody glycosylation is a major source of heterogeneity with respect to both structure and therapeutic function. Glycosylation variants are primary factors in batch-to-batch antibody variation, altering product stability in vivo, and significantly influencing Fc effector functions in vivo. Both the U.S. FDA and European regulations require understanding of glycan profiles in these proteins because of their profound influence on safety and efficacy of biopharmaceuticals. Figure 4 shows the separation of neutral and acidic 2AA-labeled *N*-linked glycans from a human IgG using a GlycanPac AXR-1 (1.9 µm, 2.1 × 150 mm) column. As with the fetuin sample in the previous figures, the IgG-derived glycan elution profile consists of clusters of peaks in which the neutral glycans elute first, followed by monosialylated and disialylated forms. Analytes in each cluster represent the glycans of the same charge. Within each cluster, the glycans having the same charge are further separated according to their isomerism and size by reversed phase interactions. As shown in Figure 4, 2AA-labeled neutral glycans elute between 5 and 22 minutes, 2AA-labeled monosialylated glycans elute between 30 and 45 min and 2AA-labeled disialylated glycans elute between 45 and 55 minutes. More than 40 peaks are identified from the separation of 2AA-labeled *N*-glycans from this human IgG.



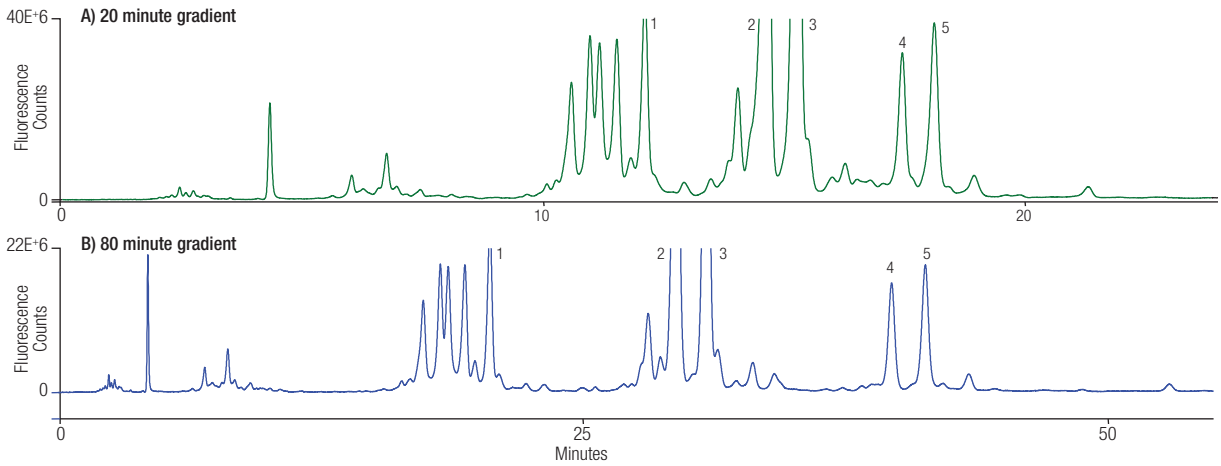
Column:	**GlycanPac AXR-1 (1.9 µm)**
Dimension:	2.1 × 150 mm
Mobile Phases:	A) Acetonitrile B) D.I. H_2O C) 100 mM Ammonium formate, pH 4.4
Flow Rate:	0.3 mL/min
Injection Volume:	100 pmoles
Temperature:	30 °C
Detection:	Fluorescence at 320/420 nm
Sample:	2AA labeled *N*-glycan from human IgG

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

Figure 4: Separation of 2AA-labeled *N*-linked glycans from human IgG by charge, isomers and size using a GlycanPac AXR-1 (1.9 µm) column.

Options for Improved Resolution or Throughput

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 5 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-*N*-linked mixture. Overall, relatively little resolution loss is observed with shorter gradient times. For glycans from proteins with less profound glycosylation than fetuin, shorter gradients may prove useful for glycan characterization with improved throughput.



Column:	**GlycanPac AXR-1 (1.9 µm)**
Dimension:	2.1 × 150 mm
Mobile Phases:	A) D.I. H_2O B) 100 mM Ammonium formate, pH 4.4
Flow Rate:	0.4 mL/min
Injection Volume:	100 pmoles
Temperature:	30 °C
Detection:	Fluorescence at 320/420 nm
Sample:	2AB-labeled fetuin *N*-linked glycans

(A) 20 Minute Gradient

Time (min)	% A	% B
-10	93	7
0	93	7
20	0	100
24	0	100
24.5	93	7

(B) 80 Minute Gradient

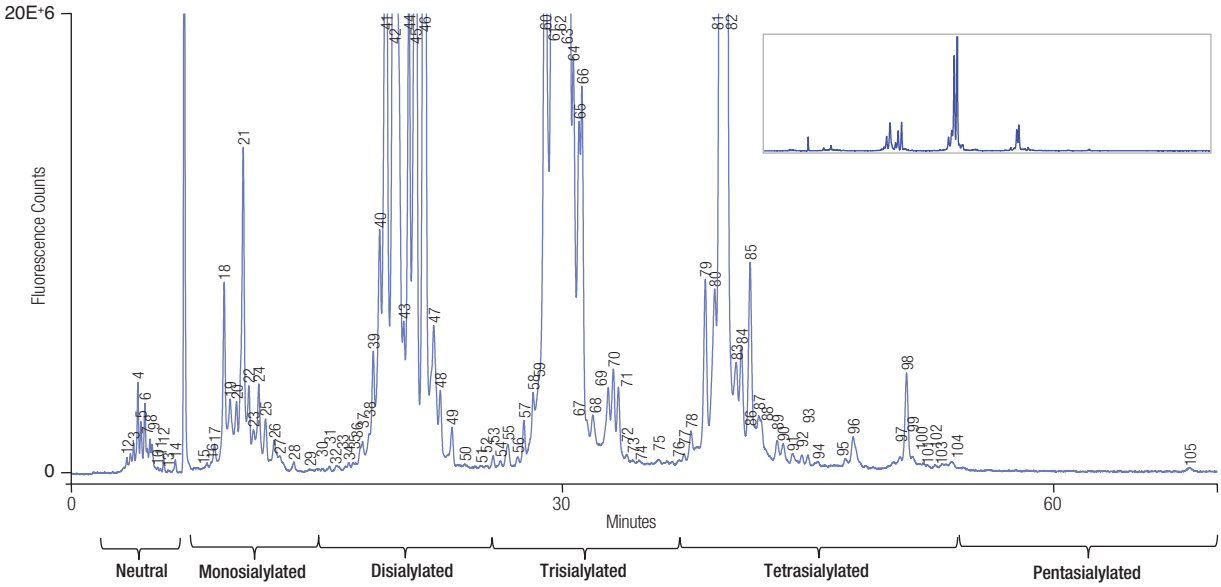
Time (min)	% A	% B
-10	93	7
0	93	7
80	0	100
84	0	100
84.5	93	7

Comparison of Peak Resolution

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

Figure 5: Effect of gradient time on glycan resolution on the GlycanPac AXR-1 column. Comparison of 20 min and 80 min gradients on 2.1 × 150 mm formats.

Longer GlycanPac columns also increase resolution, maximizing separation efficiency, but with commensurately longer gradients, or lower throughput. Figure 6 shows the separation of the 2AB-labeled fetuin *N*-linked glycans on a 2.1 × 250 mm 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 150 mm long GlycanPac AXR-1 column.



Column:	**GlycanPac AXR-1 (1.9 µm)**
Dimension:	2.1 × 250 mm
Mobile Phases:	A) Acetonitrile B) D.I. H_2O C) 100 mM Ammonium formate, pH 4.4
Flow Rate:	0.4 mL/min
Injection Volume:	100 pmoles
Temperature:	30 °C
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
90.5	0	0	100
95.5	0	0	100
105	0	93	7

Figure 6: Significant improvement of fetuin 2AB-labeled *N*-linked glycans on a longer, 2.1 x 250 mm GlycanPac AXR-1 column. Inset is full Chromatogram.

Reproducible Manufacturing

Each GlycanPac AXR-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 AXR-1 Column
Column Chemistry	WAX and RP mixed-mode
Silica Substrate	Spherical, high purity, porous
Particle Size	3 µm, 1.9 µm
Surface Area	220 m^2/g
Pore Size	175 Å

Specifications and Operational Parameters

Column Particle Size	Dimension (mm)	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 × 150 mm	10,000	2.0–8.0	<60	Compatible with 0–100% aqueous and common HPLC solvents	0.2–0.4	0.5
	2.1 × 250 mm	15,000	2.0–8.0	<60		0.2–0.4	0.5
3 µm	4.6 × 150 mm	6,000	2.0–8.0	<60		0.8–1.2	1.5
	3.0 × 150 mm	6,000	2.0–8.0	<60		0.4–0.6	0.75
	2.1 × 150 mm	6,000	2.0–8.0	<60		0.2–0.3	0.4

Ordering Information

	Particle Size	Length (mm)	2.1 mm ID	3.0 mm ID	4.6 mm ID
GlycanPac AXR-1 Analytical Column	1.9 µm	150	088136		
		250	088135		
	3 µm	150	088251	088252	088255
GlycanPac AXR-1 Guard Columns: used only with 3 µm columns; require Guard cartridge holder P/N 069580	3 µm	10	088258	088259	088260

www.thermoscientific.com/columns

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+91 27 1766 2352
Japan 0120 753 670 (free call domestic)
0120 753 671 fax
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