Integrated LC/MS Workflow for the Analysis of Labeled and Native N-Glycans from Proteins Using a Novel Mixed-Mode Column and a Q Exactive Mass Spectrometer

Udayanath Aich[1], Julian Saba[2], Rosa Viner[2], Xiaodong Liu[1], Srinivasa Rao[1], Yury Agroskin[1], Andreas Huhmer[2] and Chris Pohl[1]
[1]Thermo Fisher Scientific, Sunnyvale, CA; [2]Thermo Fisher Scientific, San Jose, CA

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Key Words
GlycanPac AXH-1, HILIC, WAX, glycomics, glycoproteins, glycopeptides, glycans, labeled *N*-glycans, Q Exactive, SimGlycan software

Goal
Develop a comprehensive method for the structural characterization of released glycans from proteins. The described integrated method covers sample preparation, separation, mass spectrometry data acquisition, and analysis.

Introduction
Glycans are widely distributed in biological systems in 'free state' as well as conjugated forms such as glycoproteins, glycolipids, and proteoglycans. They play significant roles in many biological and physiological processes, including recognition and regulatory functions, cellular communication, gene expression, cellular immunity, growth, and development.[1] Glycans can affect efficacy and safety of protein based drugs. For example, recombinant proteins and monoclonal antibodies (mAb) are often dependent on the structure and types of glycans attached to the proteins.[2] The structures of glycans are diverse, complex, and heterogeneous due to post-translational modifications (PTMs) and physiological conditions. Minor changes in glycan structure can result in striking differences in biological functions and clinical applications. The structural characterization of glycans is essential in bio-therapeutics and bio-pharmaceutical projects.[3] In addition to the characterization of the sugar sequence, the analysis must elucidate linkages and separate all isomeric, charge, and branching variations of glycans.

Liquid chromatography (LC) coupled to mass spectrometry (MS) has emerged as one of the most powerful tools for the structural characterization of glycans. Hydrophilic interaction liquid chromatography (HILIC) columns based on amide, amine, or zwitterionic-based packing materials are often used for glycan analysis. These HILIC columns separate glycans mainly by hydrogen bonding, resulting in size and composition-based separation. A limitation of this approach is that identification of the glycan charge state is not possible due to the fact that glycans of different charge states are intermingled in the separation envelope.

The Thermo Scientific™ GlycanPac™ AXH-1 column is a high-performance HPLC/UHPLC column specifically designed for structural analysis of glycans, either labeled or native, by LC-fluorescence or LC/MS methods.The GlycanPac AXH-1 column is based on innovative mixed-mode surface chemistry combining both weak anion-exchange (WAX) and HILIC retention mechanisms. The WAX functionality provides retention and selectivity for negatively charged glycans, while the HILIC mode facilitates the separation of glycans according to their charge, polarity, and size. As a result, the GlycanPac AXH-1 column provides unparalleled separation capabilities for glycans.

LC-MS/MS analysis of glycans requires the processing of large sets of data. The incorporation of SimGlycan® software (PREMIER Biosoft) alleviates this issue, thus enabling the development of a true high-throughput workflow.

This application note presents a step-by-step method for the release, labeling, separation, and structural elucidation of *N*-glycans from proteins by LC-MS/MS.

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Experimental Conditions

Chemicals and Reagents

- Deionized (DI) water, 18.2 MΩ-cm resistivity
- Acetonitrile (CH_3CN), HPLC grade (Fisher Scientific™, AC610010040)
- LC/MS grade formic acid (Fisher Scientific, A117-50)
- Ammonium formate (Fisher Scientific, AC40115-2500)
- Thermo Scientific Premium 2 mL vial convenience kit, 60180-600
- PNGase F (New England BioLab, P0705L)
- Bovine fetuin (Sigma-Aldrich®, F2379)
- Thermo Scientific™ Hypercarb™ cartridge, 6 mL, 60106-403
- Trifluoracetic acid (Fisher Scientific, 28904)
- Sodium cyanoborohydride (Fisher Scientific, AC16855-0500)
- Anthranilamide (2AB) (Fisher Scientific, AC10490-5000)
- Glacial acetic acid (Fisher Scientific, AA36289AP)
- Dimethylsulfoxide (DMSO) (Fisher Scientific, D128500LC)
- Sodium hydroxide (NaOH) (Fisher Scientific, S318-100)
- Ammonium acetate (Fisher Scientific, A637-500)
- SEC column, 0.9 x 50 cm Sephadex® (GE Healthcare, G-10-120)
- GlykoClean™ G Cartridges, Prozyme, GC250
- 2-mercaptoethanol (Fisher Scientific, O3446I-100)

Equipment

- Thermo Scientific™ Dionex™ UltiMate™ 3000 system, including pump: LPG-3400RS, thermal compartment: TCC-3000RS, pulled-loop well plate auto sampler: WPS-3000TRS, fluorescence detector with Dual-PMT: FLD3400RS, and 2µL micro flow cell: 6078.4330
- Q Exactive hybrid quadrupole-Orbitrap mass spectrometer
- Thermo Scientific™ SpeedVac™ Concentrator
- Thermo Scientific Lyophilizer (Labconco® FreeZone® -105 °C 4.5 L benchtop freeze dry system) 16-080-207
- Thermo Scientific 24-Port SPE vacuum manifold, 60104-233

Buffer Preparation

- Ammonium formate (80 mM, pH 4.4):
  Dissolve 5.08 ± 0.05 g of ammonium formate (crystal) and 0.60 g of formic acid in 999.6 g of DI water. Sonicate the resulting solution for 5 min.
- 0.1 M sodium phosphate buffer, pH 7.25:
  Add 102.24 mg of Na_2HPO_4 and 38.14 mg of NaH_2PO_4 to 10 mL of DI water. Vortex to mix the solid completely. Verify that the pH of the solution is 7.25 ± 0.02.

Release of *N*-Glycans from Proteins

1. Dissolve 1 mg of the bovine fetuin protein in 500 µL of 0.1 M sodium phosphate buffer, pH 7.2 ± 0.05, in an Eppendorf tube.
2. Add 0.5 µL of 2-mercaptoethanol to this solution.
3. Finally, add 50 U (units) of PNGase F and incubate total solution at 37 °C water bath for 18 h.
4. Cool to room temperature and purify the released glycans as described in the next section.

Purification of *N*-Glycans

Purify free glycans after digestion using a Hypercarb cartridge as follows:

1. Attach a single Hypercarb cartridge per reaction to a designated port in the SPE manifold.
2. Slowly, and with a consistent flow rate, pre-treat each cartridge with the following volumes of reagents in the order described: 15 mL of 1M NaOH, 15 mL of HPLC grade water, 15 mL of 30% acetic acid, 15 mL of HPLC grade water.
3. Prime the cartridge with 15 mL of 50% acetonitrile/0.1% trifluoroacetic acid (TFA), followed by 15 mL of 5% acetonitrile/0.1% TFA.
4. Load the entire sample volume into the cartridge and let it permeate into the resin by pulsing the vacuum on and off quickly.
5. Rinse the reaction tube with ~50 µL of HPLC grade water, transfer into the cartridge, and pulse the vacuum again.
6. Wash the cartridge with 15 mL of HPLC grade water, followed by 15 mL of 5% acetonitrile/0.1% TFA.
7. Elute the glycans with 4 x 2.5 mL of 50% acetonitrile/ 0.1% TFA into a labeled 15 mL conical tube.
8. Immediately freeze samples on dry ice and then lyophilize to dryness (16–24 h).
9. After lyophilization, dissolve the solid in 1 mL of water, dry the samples again in a 1.5 mL Eppendorf tube, and store at -20 °C.

2AB Labeling Reaction

Carry out the labeling reaction using a modified reported procedure.[4]

1. Prepare the 2AB labeling reagent (100 µL): Dissolve 2-aminobenzamide (4.6 mg) in 70 µL of DMSO.
2. Add 30 µL of glacial acetic acid (100%) to the mixture.
3. Transfer the complete solution to a black or light-protected, screw-cap, 1.5 mL Eppendorf tubes containing 6.4 mg of sodium cyanoborohydride.
4. Incubate the solution at 60 °C for 10 min to dissolve sodium cyanoborohydride completely. Occasionally vortex the solutions. When all the solids are completely dissolved, the 2AB labeling reagent is ready to use for the labeling reaction.
5. Add 20 µL of 2AB labeling reagent to 50 µg of free glycans and vortex to mix the solution. Then, incubate the mixture at 60 °C for 3 h.

Clean Up of Labeled Glycans

1. After completion of the 2AB reaction, add 250 µL of acetonitrile to the vial at room temperature.
2. Purify the samples using a GlykoClean G cartridge; pre-equilibrate the column with the following solutions in the order they appear: wash with 3 mL of deionized water, 3 mL acetonitrile, 3 mL of 96% acetonitrile.
3. Add the labeled glycans to the pre-equilibrated column.
4. Wash with 96% acetonitrile.
5. Elute the glycans with 5 mL of DI water.
6. Lyophilize the solution to dryness.
7. Upon dryness, dissolve the sample in 500 µL of water.
8. Further purify the labeled glycans using a size-exclusion chromatography (SEC) Sephadex® column to get highly pure labeled oligosaccharides.
9. Inject the samples onto an SEC column connected to a UV detector. Equilibrate the column with 10 mM ammonium acetate at a flow rate of 0.35 mL/min until a steady baseline of 205 nm is achieved.
10. Run the column with 10 mM ammonium acetate for 90 min and collect glycan containing fractions using UV detection at 205 nm.
11. Dry the combined fractions by lyophilization, re-suspend with 1 mL of DI water. Quantify the glycans[5] and then store the remaining sample at -20 °C for future use.
12. Ready for use as 2AB labeled *N*-glycan from fetuin.

Sample Preparation for Injection

1. Mix 25 µL of purified labeled glycans at 0.2 nmol/µL in DI water with 75 µL of acetonitrile.
2. Transfer the total solution to the auto sampler vial for analysis.

Note: Store the standard at -20 °C.

Separation Conditions

Column	GlycanPac AXH-1, 2.1 x 150 mm, 1.9 µm
Mobile phase	A: acetonitrile + water (80:20, v/v)
	B: ammonium formate (80 mM, pH 4.4)
Flow rate (µL/min)	400
Column temperature (°C)	30
Sample volume (injected) (µL)	1
Mobile phase gradient	Refer to Table 1

Table 1. Mobile phase gradient

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

MS Conditions

MS instrument	Q Exactive hybrid quadrupole-Orbitrap MS
Source	HESI-II probe
Ionization mode	Negative ion
Full MS	
MS scan range (*m/z*)	380–2000
Resolution	70,000
Microscans	1
AGC target	1×10^6
Max IT (ms)	60
dd-MS2	
dd-MS2 resolution	17,500
Microscans	3
MS/MS AGC target	2×10^5
MS/MS max IT (ms)	250–1000
Isolation window (*m/z*)	2
NCE	35
Stepped NCE	8%
Dynamic exclusion (s)	90

Source Conditions

Source position	C
Sheath gas flow rate (arb units)	20
Auxillary gas flow rate (arb units)	5
Sweep gas flow rate	0
Spray voltage (kV)	3.30
Capillary temperature (ºC)	275
S-lens RF level	50
Heater temperature (ºC)	300

Data Processing and Software

Chromatographic software	Thermo Scientific™ Chromquest™ v 5.0 Chromatography Data System
MS data acquisition	Thermo Scientific™ Xcalibur™ software v 2.2 SP1.48
MS/MS data analysis	SimGlycan software v 4.5

SimGlycan Search Parmeters

Ion mode	Negative
Adduct	H
Chemical derivatization	Underivatized
Match fragment ion for charge state	< Precursor *m/z* charge state
Precursor ion *m/z*	10 ppm
Fragment ion	0.05 Da
Modification	2AB
Class	Glycoprotein
Sub class	*N*-glycan (Intact Core)
Biological source	Bovine Fetuin
Pathway	Unknown
Search structure	All
Glycan type	All
% of evident glycosidic linkages	2
Fragmentation pattern	Specify Expected Fragments in the Spectra
Glycosidic	B: Yes; C: Yes; Y:Yes; Z:Yes
Cross-ring	A:Yes; X:Yes
Glycosidic/Glycosidic	Z/Z: Yes; Y/Y: Yes; B/Y or Y/B: Yes; C/Z or Z/C: No; Z/Y or Y/Z: No; B/Z or Z/B: No; C/Y or Y/C: Yes
Cross-ring/Glycosidic	A/Y or Y/A: Yes; A/Z or Z/A:Yes; X/Y or Y/X: Yes; X/Z or Z/X: No; X/B or B/X: Yes; X/C or C/X: Yes

Results and Discussion

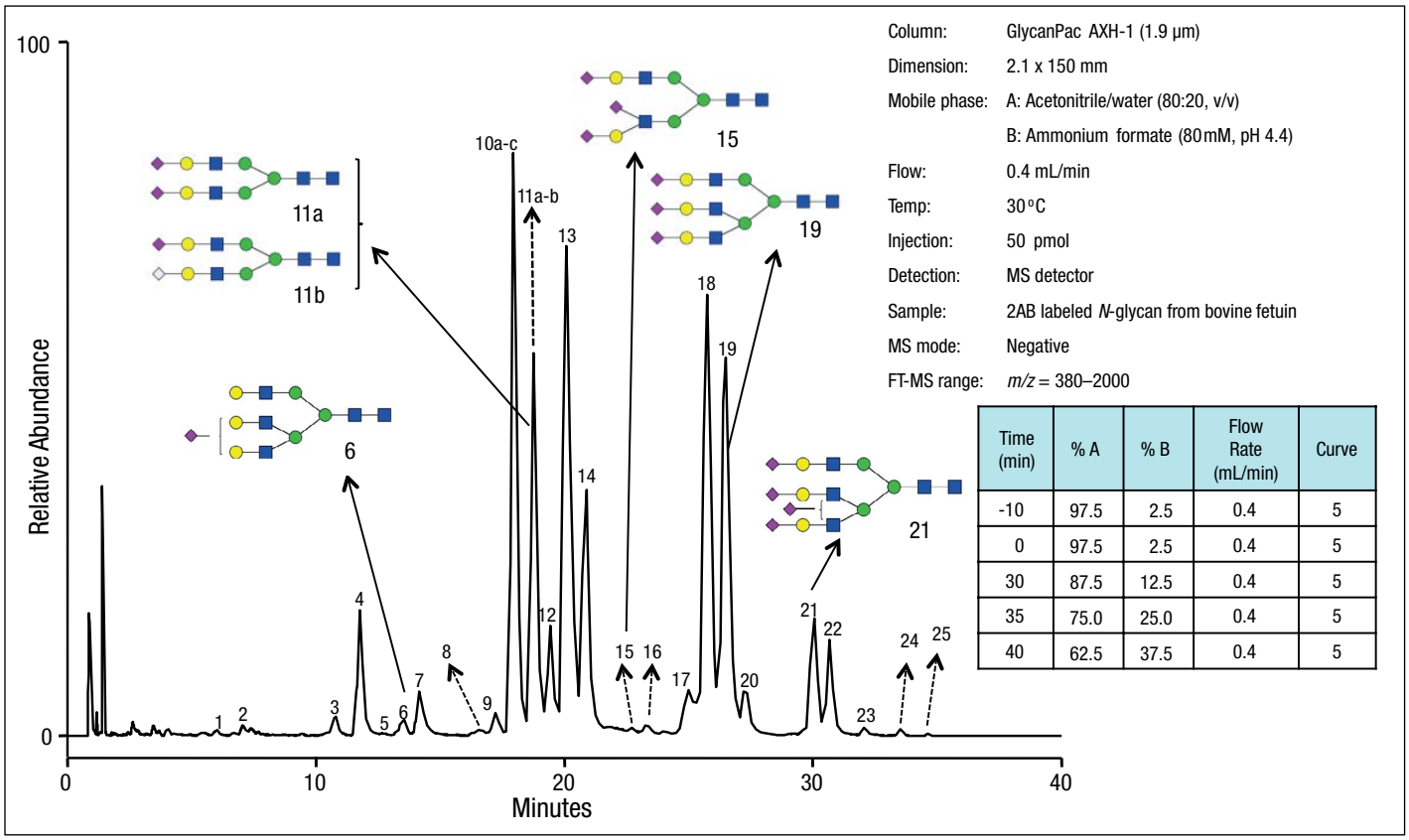
The protocol outlined in this application note yields detailed information on the set of glycans present in proteins including mAbs. The protocol describes a fully integrated workflow that combines novel column technology (GlycanPacAXH-1 column), mass spectrometry (Q Exactive mass spectrometer), and a bioinformatics tool (SimGlycan software). This fully integrated workflow is demonstrated for *N*-glycans released from bovine fetuin glycoprotein, but can be used for released *N*-glycans from any glycoprotein.

The GlycanPac AXH-1 column described in this application note can be used for qualitative and quantitative characterization of neutral and charged glycans present on proteins. The elution of glycans is based on charge: the neutral glycans elute first, followed by the separation of acidic glycans from mono-sialylated to penta-sialylated species. Glycans of each charge state are further separated based on their size and polarity. Separation of glycans based on charge, size, and polarity–combined with MS–provides complete structural and quantitative information.

2AB labeled *N*-linked glycans from bovine fetuin were separated on the GlycanPac AXH-1 column and analyzed on a Q Exactive mass spectrometer (Figure 1). Data-dependant MS/MS spectra were acquired on all precursor ions ($z \geq 2$), and SimGlycan software was used for structural elucidation. A representative example of the analysis is shown in Figure 2. The Q Exactive mass spectrometer was selected for these experiments because of its 140,000 FWHM resolution at *m/z* 200, high scan speeds at all resolution settings, and sensitivity. All of these contribute to the detection of minor glycan species and generation of high-quality MS/MS spectra even for low-abundance glycans.

Additionally, the Q Exactive mass spectrometer has the ability to generate higher-energy collisional dissociation (HCD) with high-resolution, accurate-mass (HR/AM) fragment ions. This allows for differentiation of near-mass fragment ions, which were observed to be useful for correctly assigning branching and linkage. The variation of collision energy can provide different fragment ions within the mass spectrometer. To maximize both glycosidic and cross-ring fragments, normalized stepped collision energy (NSCE) was incorporated. This provided optimum conditions for generation of a maximum number of both cross-ring and glysodic cleavages in a single spectrum, thereby increasing confidence in the identification (Figure 2). The detailed structural information obtained from the MS/MS data shown in Table 2 further validated the ability of the GlycanPac AXH-1 column to separate glycans based on charge, size, and polarity.

The use of LC-MS/MS for glycan analysis increases the complexity of data analysis due to the large number of MS/MS spectra generated. SimGlycan software was incorporated to simplify data analysis.[6,7] SimGlycan software predicts the structure of a glycan from the MS^n data. It accepts the raw MS^n files, matches them with its own database of theoretical fragmentation of over 22,000 glycans, and generates a list of potential glycan structures. Each proposed structure is assigned a score to reflect how closely it matches with the experimental data.



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 1. LC-MS analysis of 2AB labeled *N*-glycans from bovine fetuin by GlycanPac AXH-1 column with MS detection

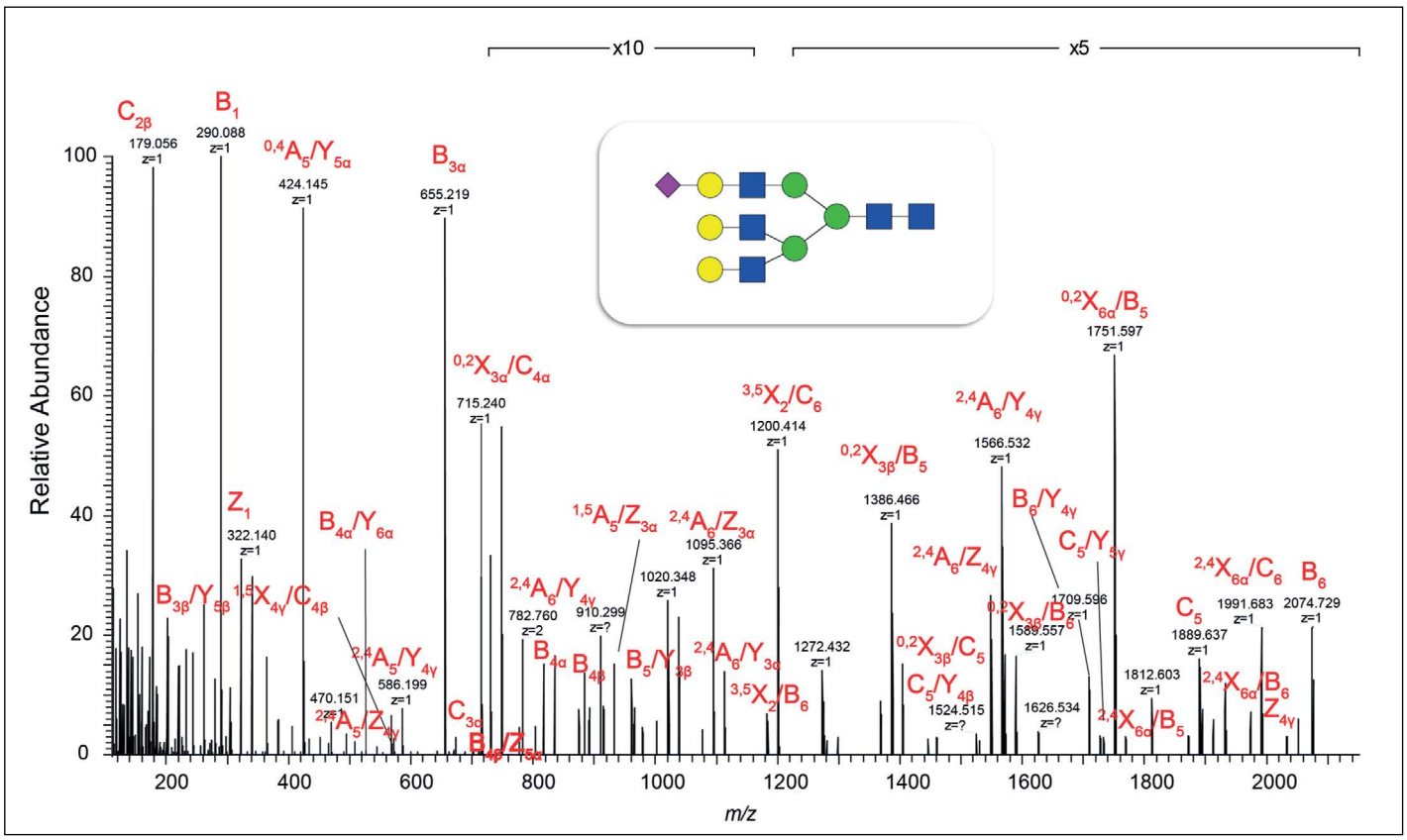
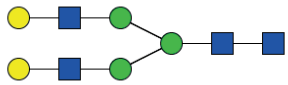
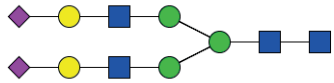
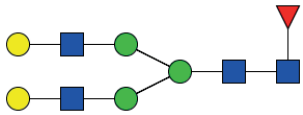
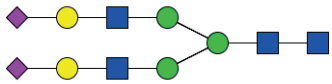
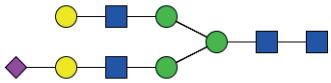
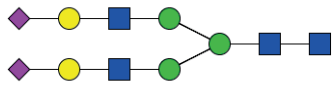
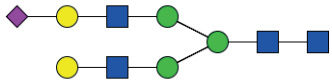
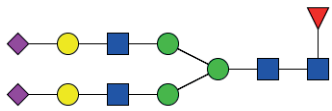
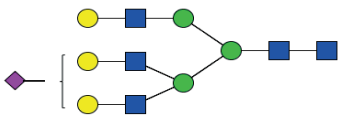
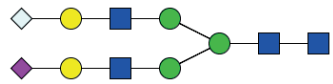
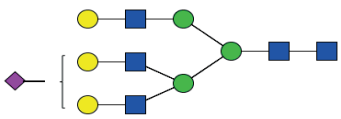
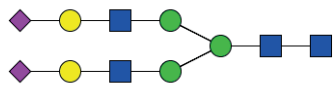
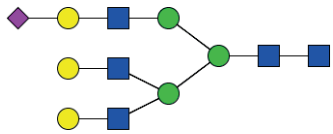
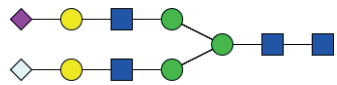
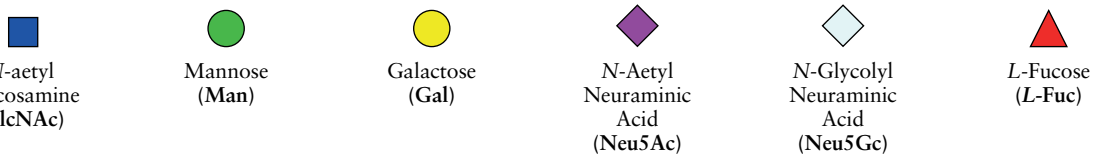
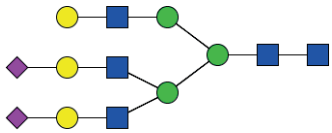
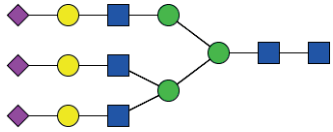
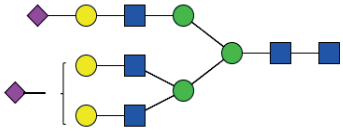
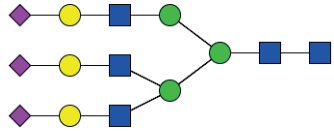
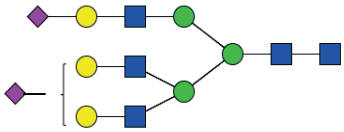
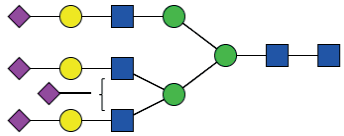
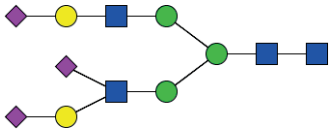
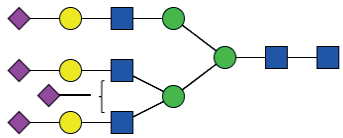
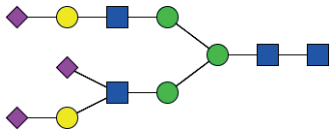
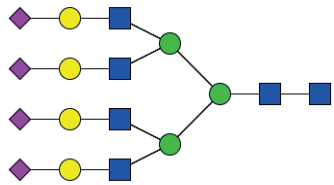
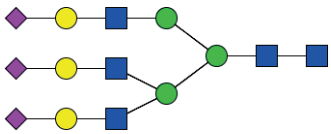
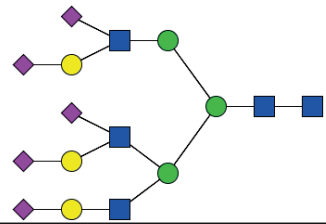
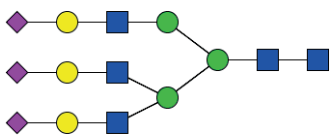
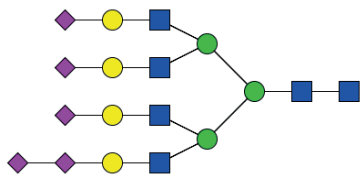


Figure 2. HCD MS/MS spectrum of a 2AB-labeled monosialylated triantennary *N*-glycan from bovine fetuin

Table 2. Structural identification of glycans present in each peak by the separation of 2AB labeled *N*-glycans from bovine fetuin using GlycanPac AXH-1 column and Q Exactive mass spectrometer

Peak (Figure 1)	Compound structure (2AB labeling is not shown)	Peak (Figure 1)	Compound structure (2AB labeling is not shown)
1		8	
2		9	
3		10a	
4		10b	
5		10c	
6		11a	
7		11b	

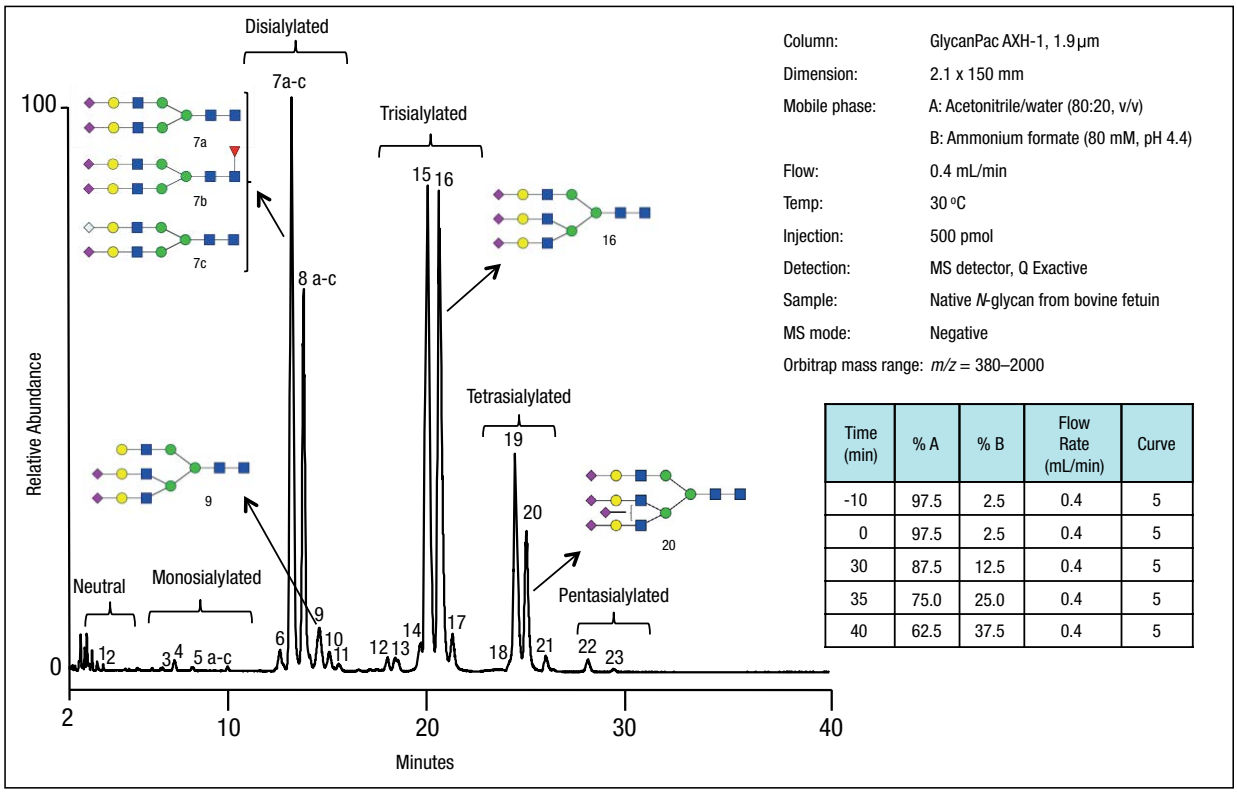


12		19	
13		20	
14		21	
15		22	
16		23	
17		24	
18		25	

LC-MS Analysis of Native *N*-Glycans Released from Proteins

The GlycanPac AXH-1 column is also suitable for analysis of native glycans. Analyzing unlabeled glycans not only eliminates the extra reaction step and cleanup methods during labeling, but also retains the original glycan profile without adding further ambiguity imposed by the labeling reaction.

Figure 3 shows the LC/MS analysis of native *N*-glycans from bovine fetuin using the GlycanPac AXH-1 column. Detailed information is in Table 3. A representative MS/MS spectrum for a trisialylated triantennary glycan is shown in Figure 4.



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 3. LC/MS analysis of native *N*-glycan from bovine fetuin

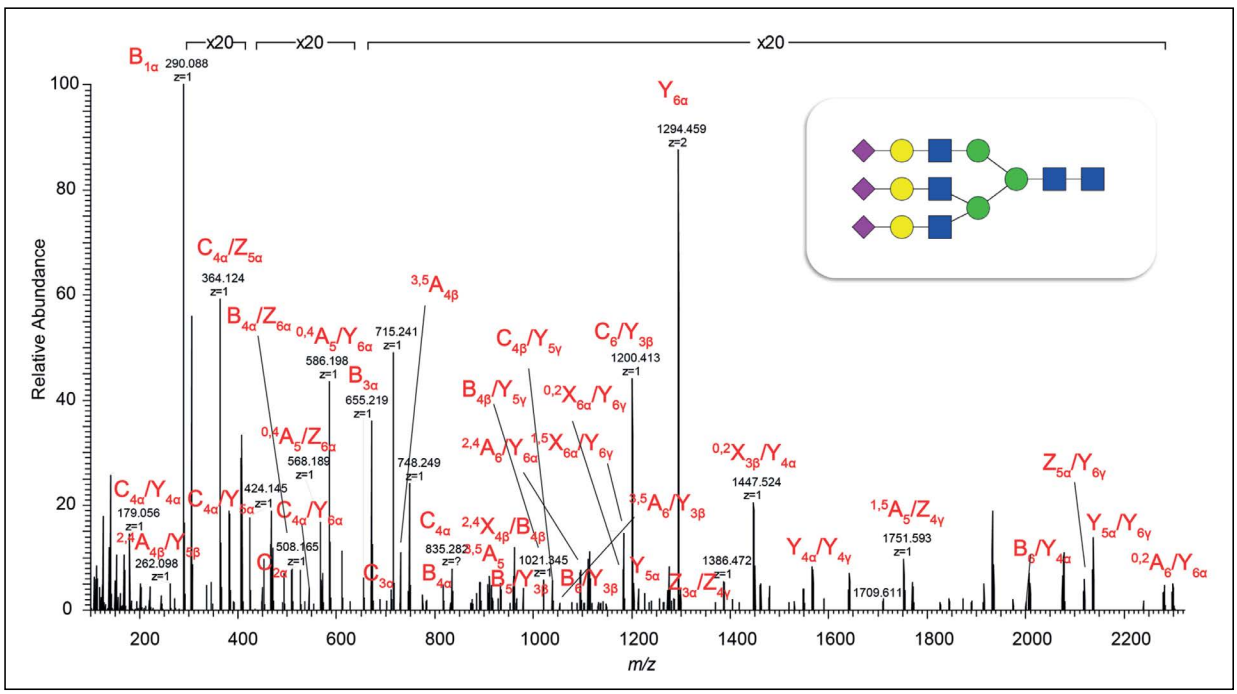
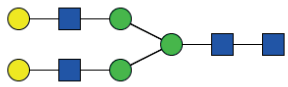
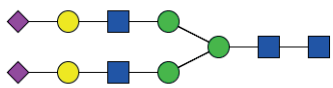
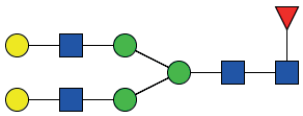
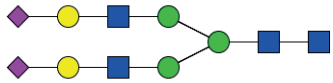
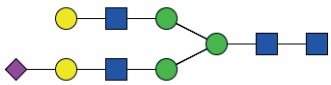
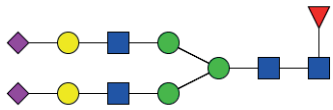
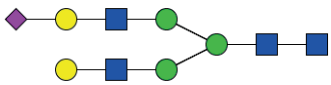
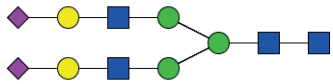
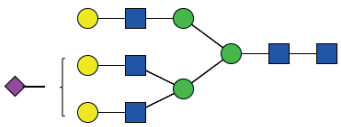
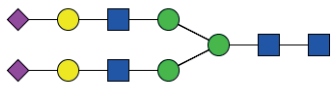
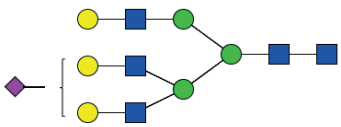
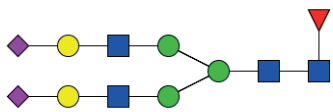
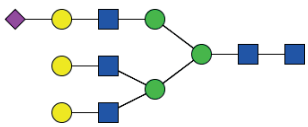
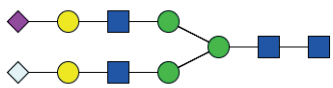
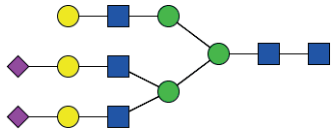
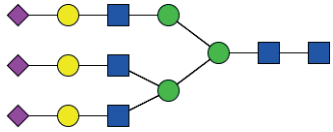
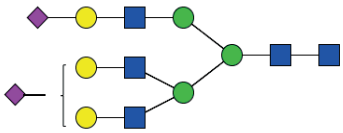
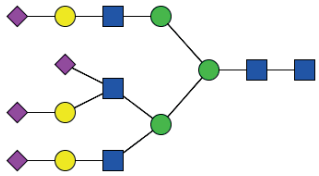
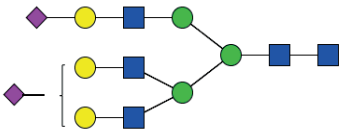
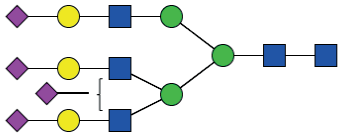
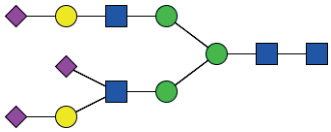
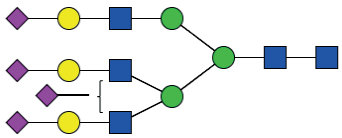
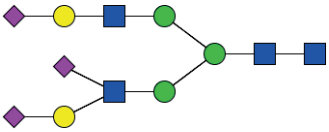
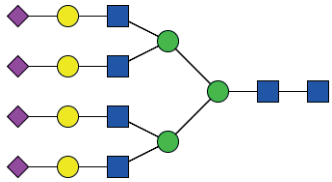
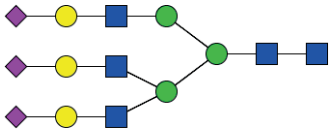
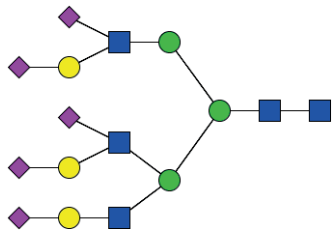
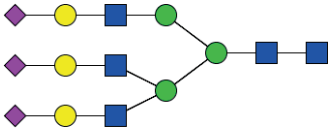
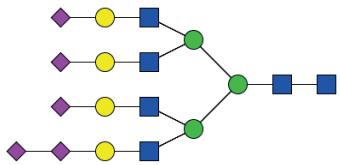
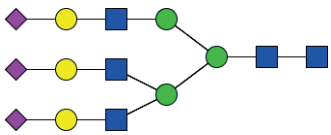


Figure 4. MS/MS spectra for a native trisialylated triantennary *N*-glycan released from bovine fetuin

Table 3. Structural identification of glycans present in each peak by the separation of native *N*-glycans from bovine fetuin using GlycanPac AXH-1 column and Q Exactive mass spectrometer

Peak (Figure 1)	Compound structure	Peak (Figure 1)	Compound structure
1		6	
2		7a	
3		7b	
4		7c	
5a		8a	
5b		8b	
5c		8c	

9		17	
10		18	
11		19	
12		20	
13		21	
14		22	
15		23	
16			

Native glycan profiles are significantly different from the profile of fluorescently labeled glycans, especially for glycans containing multiple sialic acids (Figure 3). However, labeled glycans require smaller amounts (10 times) of samples for MS analysis as compared to native glycans. Thus, the GlycanPac AXH-1 column is useful for the analysis of biologically relevant glycans including glycans from antibodies, either labeled or native, by LC-fluorescence or LC-MS methods. If the amount of the sample is not extremely limited, analysis of unlabeled glycans using the GlycanPac AXH-1 is highly feasible.

Conclusion

- A fully integrated workflow for structural characterization of native and fluorescently labeled *N*-glycans released from proteins was demonstrated successfully.
- Novel GlycanPac AXH-1 column demonstrated excellent separation of released N-glycans especially forsilalylated species. It allowed for their sensitive detection by the Q Exactive mass spectrometer and identification by SimGlycan software.
- This LC-MS integrated technology is also useful for the separation and structural characterization of reduced *O*-linked glycans from proteins, mucins, and the analysis of charged and neutral glycosylaminoglycans and glycolipids.

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