O-glycan profiling using HPAE-PAD hyphenated with a high-resolution accurate mass (HRAM) mass spectrometer

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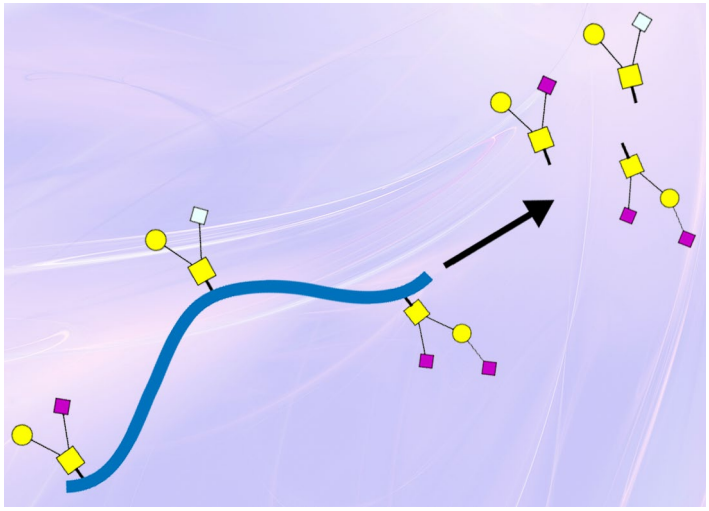


Goal

To evaluate and demonstrate an HPAE-PAD/MS workflow for profiling O-glycans from four different glycoproteins

Introduction

Glycosylation is an important post-translational modification (PTM) that occurs on nitrogen and oxygen atoms of amino acid residues of proteins. Glycosylation plays an essential role in physiological and pathological cellular functions. Atypical and abnormal glycosylation is linked with different diseases, e.g., cancer, rheumatoid arthritis, diabetes, and cardiac diseases.[1,2] Thus, it is important to characterize protein glycosylation in biological and clinical specimens. It has been estimated recently that more than 50% of all proteins are glycosylated.[3,4] Two main types of protein glycosylation have been distinguished: N-glycosylation and O-glycosylation. N-glycosylation is the most abundant PTM in which oligosaccharides (glycans) are attached via an N-glycosidic bond to asparagine (Asn) in the consensus sequence Asn-Xaa-Ser/Thr (Ser, serine; Thr, threonine; Xaa, any amino acid except proline). O-glycosylation is another type of protein glycosylation. For O-glycosylation, the oligosaccharide is linked via an oxygen of serine (Ser) or threonine (Thr). In humans, the most common sugars linked to Ser or Thr are N-acetylglucosamine (GlcNAc) and N-acetylgalactosamine (GalNAc).[7]

Contrary to N-glycosylation, O-glycosylation is not sequence-specific, and thus O-glycan attachment is not easily predicted from the protein's sequence. There is also an absence of a common glycan core. GalNAc-linked glycans are the most common type of O-glycosylation. These are often called mucin-type O-glycans and are abundant on many extracellular and secreted glycoproteins.[5] O-glycosylation is often quite challenging to study, not only because of the lack of a universal enzyme for O-glycan release, but also because of the absence of a common glycan core. Furthermore, the heterogeneity of glycans, both in composition and in linkage, complicates their analysis and structural elucidation. Good O-linked glycan profiling requires high-resolution separation and reliable identification of released glycans. Therefore, high-resolution chromatographic separations are important for subsequent MS detection. High-performance anion-exchange chromatography coupled with pulsed amperometric detection and mass spectrometric detection (HPAE-PAD/MS) is a well-established and powerful technique for glycan analysis.[6-8]

In this application note, we demonstrate a workflow combining chemical release of O-linked glycans, followed by sample cleanup and analysis by HPAE-PAD hyphenated to a Thermo Scientific™ Q Exactive™ HF Hybrid Quadrupole-Orbitrap™ mass spectrometer. We used a recently introduced Thermo Scientific™ Dionex™ CarboPac™ PA300-4µm column. This column enables simultaneous separation of neutral and charged glycans without the need for derivatization. HPAE separation employs high-pH eluents containing high sodium levels, which cannot be directly injected into the mass spectrometer. Therefore, we used a post-column desalting device to remove sodium from the eluent. High-resolution MS data and tandem MS/MS spectra with diagnostic fragment ions provide highly reliable structural annotations of heterogeneous glycans. We applied HPAE-PAD/MS to analyze O-glycan structures released from four different glycoproteins. The possible glycan structures were first identified by SimGlycan™ software (PREMIER Biosoft, Palo Alto, CA) high-throughput search and score function. The structures were confirmed by annotating diagnostic fragmentation patterns observed in MS^2 spectra. The approach described here identified O-glycan structures in good agreement with structures previously reported for the four glycoproteins. In fact, for thyroglobulin, we believe that this is the first instance of direct experimental demonstration of the presence of an O-glycan structure and its composition.

Experimental

Equipment

- Thermo Scientific™ Dionex™ ICS-5000+ system, including:
  - DP Dual Pump
  - DC Detector/Chromatography Compartment
  - Conductivity Detector (P/N 061830)
  - ED electrochemical detector (No cell, P/N 072042)
  - ED cell (no reference or working electrode; P/N 072044)
  - ED cell polypropylene support block for use with disposable electrodes (P/N 062158)
  - Gold on PTFE disposable electrodes (Pack of 6, P/N 066480)
  - pH-Ag/AgCl reference electrode (P/N 061879)
- Thermo Scientific™ Dionex™ ERD 500 desalter, 2 mm (P/N 085089)
- Thermo Scientific™ Dionex™ AS-AP autosampler (P/N 074926) with cooling tray option (recommended)
- Q Exactive HF Hybrid Quadrupole Orbitrap mass spectrometer with HESI-II probe
- 1.5 mL polypropylene autosampler vials, with caps and split septa (P/N 079812)
- Centrifuge (Eppendorf™ 5400 series)
- Thermo Scientific™ Nalgene™ Rapid-Flow™ 0.2 µm filter units, 1,000 mL, nylon membrane, 90 mm diameter (P/N 164-0020)
- Autosampler vials with septum, 0.3 mL capacity (P/N 055428)
- Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software, version 7.2.9
- Thermo Scientific™ Foundation 3.0 software
- Thermo Scientific™ Xcalibur™ software
- SimGlycan software, version 5.0

Consumables

- Thermo Scientific™ Dionex™ HPAE-PAD/MS Assembly Kit (P/N 302854)
- Nitrogen, ultrahigh purity, UHP 300 (Airgas, P/N UN1066)
- 10 µL PEEK™ sample loop (P/N 042949)
- Thermo Scientific™ Dionex™ Borate Trap inline trap column (P/N 047078)
- Thermo Scientific™ Nalgene™ syringe filters, PES, 0.2 µm (Fisher Scientific, P/N 09-740-61A)
- AirTite™ All-Plastic Norm-Ject™ syringes, 5 mL, sterile (Fisher Scientific, P/N 14-817-28)
- Vial kit, 10 mL polypropylene with caps and septa (P/N 055058)
- Thermo Scientific™ Nalgene™ Rapid-Flow™ sterile disposable filter units with nylon membrane (1,000 mL, 0.2 µm pore size, Fisher Scientific P/N 09-740-46)
- Thermo Scientific™ Dionex™ IC PEEK Viper fitting kit for 2 mm Dionex ICS-6000 system with electrochemical detector (P/N 302966)

Reagents and standards

- Deionized (DI) water, Type I reagent grade, 18 MΩ·cm resistivity or better
- Mucin from porcine stomach, Type III (Sigma-Aldrich, P/N M1778-10G)
- Bovine fetuin (Sigma-Aldrich, P/N F2379)
- Bovine thyroglobulin (Sigma-Aldrich, P/N T1001)
- Bovine fibrinogen (Sigma-Aldrich, P/N F8630)
- Thermo Scientific™ Dionex™ sodium acetate salt, electrochemical grade (P/N 059326)
- Sodium hydroxide, 50% w/w (Fisher Chemical™, P/N SS254-500)
- Sodium borohydride (Fisher Chemical™, P/N S678-25)
- Formic acid, 97%, ACS reagent (ARCOS Organics™, P/N AC423755000)
- Methanol, Optima™ for HPLC (Fisher Chemical™, P/N A454-4)
- Acetonitrile, Optima™ LC/MS Grade (Fisher Chemical™, P/N A955-4)
- Trifluoroacetic Acid (TFA), LC-MS Grade (Thermo Scientific™ Pierce™, P/N PI28901)
- Thermo Scientific™ HyperSep™ Hypercarb™ filter plate, 40 µL bed volume (P/N 60110-504)
- 96 well PCR plate (Fisherbrand™, P/N 14230237)

Table 1A. Experimental conditions, separation

HPAE separation	
System	Dionex ICS-5000⁺ HPIC system
Columns	Dionex CarboPac PA300-4µm guard column (2 × 50 mm, P/N 303347) Dionex CarboPac PA300-4µm analytical column (2 × 250 mm, P/N 303346)
Column temp.	30 °C
Eluent A	Milli-Q™ water
Eluent B	200 mM NaOH
Eluent C	25 mM sodium acetate in 50 mM NaOH
Eluent D	250 mM sodium acetate in 100 mM NaOH
Flow rate	0.25 mL/min
Eluent program	

Time	%A	%B	%C	%D	Comments
-1.0	78.5	19.5	2	0	Equilibration
0.0	78.5	19.5	2	0	Load/inject
4.0	78.5	19.5	2	0	
20.0	20	20	60	0	
50.0	0	0	0	100	Regeneration
59.9	0	0	0	100	
60.0	78.5	19.5	2	0	Equilibration
75.0	78.5	19.5	2	0	End

Injection volume	10 µL (Full Loop)
Loop-overfill factor	2
Desalter	Dionex ERD 500 2 mm, using DI water as regenerant
Regenerant flow rate	3.5 mL/min (using pump 2 of the Dionex ICS 5000⁺ DP)
Desalter current	250 mA

Table 1B. Experimental conditions, detection 1

Detection 1: Pulsed amperometry	
Working electrode	Carbohydrate certified disposable gold working electrode with 0.002 inch (50.8 µm) gasket
Reference electrode	Ag/AgCl reference electrode
Waveform (TN21)[9]	Time / Potential (V) integration 0.00 / +0.1 0.20 / +0.1 Begin 0.40 / +0.1 End 0.41 / –2.0 0.42 / –2.0 0.43 / +0.6 0.44 / –0.1 0.50 / –0.1
Typical background	20–25 nC

Table 1C. Experimental conditions, detection 2

Detection 2: MS	
MS instrument	Q Exactive HF Hybrid Quadrupole-Orbitrap
Ion source	Heated Electrospray Ionization (HESI-II) probe
Ionization mode	Negative ionization
Spray voltage	3.2 kV
Capillary temperature	300 °C
Sheath gas flow rate	25 arbitrary units
Auxiliary gas flow rate	10 arbitrary units
Scan range	400–2,000 *m/z*
Resolution	120,000
AGC target	3×10^6
Maximum IT	100 ms
dd-MS^2 resolution	30,000
dd-MS^2 AGC target	1×10^5
dd-MS^2 Maximum IT	50 ms
TopN	5
Isolation window	4 *m/z*
(N)CE	30

Preparation of solutions and reagents

Eluent preparation

It is essential to use high-quality deionized (DI) water of high resistivity (18 MΩ·cm) as free of dissolved carbon dioxide as possible. It is important to minimize contamination by carbonate, a divalent anion at high pH that binds strongly to the columns, causing loss of chromatographic resolution and efficiency. Commercially available sodium hydroxide pellets are covered with a thin layer of sodium carbonate and should not be used. A 50% (w/w) sodium hydroxide solution is much lower in carbonate and is the preferred source for sodium hydroxide.

200 mM NaOH

To prepare 1 L of 200 mM sodium hydroxide, either pipette 10.4 mL or weigh 16.0 g 50% (w/w) sodium hydroxide into a plastic 1 L volumetric flask containing approximately 800 mL degassed DI water. If pipetting, use a plastic 5 or 10 mL sterile serological pipette with 0.1 mL gradations. Briefly stir this solution (15–30 s) and then bring to volume. Immediately transfer this solution to the plastic eluent bottle on the HPAE-PAD system and blanket it with nitrogen at 34 to 55 kPa (5 to 8 psi).

25 mM sodium acetate in 50 mM NaOH

To prepare 1 L of 50 mM NaOH/25mM sodium acetate, dissolve 2.051 g of high-purity anhydrous sodium acetate into approximately 800 mL DI water. Vacuum filter this solution through a 0.2 µm nylon filter to remove particles from the sodium acetate that can damage parts of the pump. This filtration is often slow, as the insolubles in the sodium acetate will gradually clog the filter. After filtration, transfer the solution into a plastic 1 L volumetric flask, add 2.6 mL or 4.0 g of 50% NaOH (for 50 mM), and bring to volume. Immediately transfer this solution into a plastic eluent bottle on the HPAE-PAD system and blanket it with nitrogen at 34 to 55 kPa (5 to 8 psi). For additional details on mobile phase preparation, refer to Thermo Scientific Technical Note 71 (TN71).[10]

250 mM sodium acetate in 100 mM NaOH

Follow the same method as described above but use 20.51 g of high-purity anhydrous sodium acetate and 5.2 mL or 8 g of 50% NaOH to prepare 1 L of 100 mM NaOH/250 mM sodium acetate.

Sample preparation

O-linked glycans are released from mucin glycoproteins by reductive β-elimination. Reductive β-elimination is a common approach to liberate O-glycans from glycoproteins due to its relative simplicity and safety. Glycans are released under alkaline conditions (typically in 1 M NaOH) with an incubation time generally between 16 and 40 h. To prevent “peeling” (a degradation of the glycan), the reducing ends of the carbohydrates are reduced into base-stable alditols by adding a reducing agent, such as

sodium borohydride, to the reaction mixture. The reaction is quenched by acid addition. Then, glycans are purified with porous graphitized carbon resin (HyperSep Hypercarb filter plates, 40 µL). The detailed sample preparation steps are shown in Scheme 1. The purified O-glycan mix is stored at -30 °C and resuspended in 50 µL DI water for analysis.

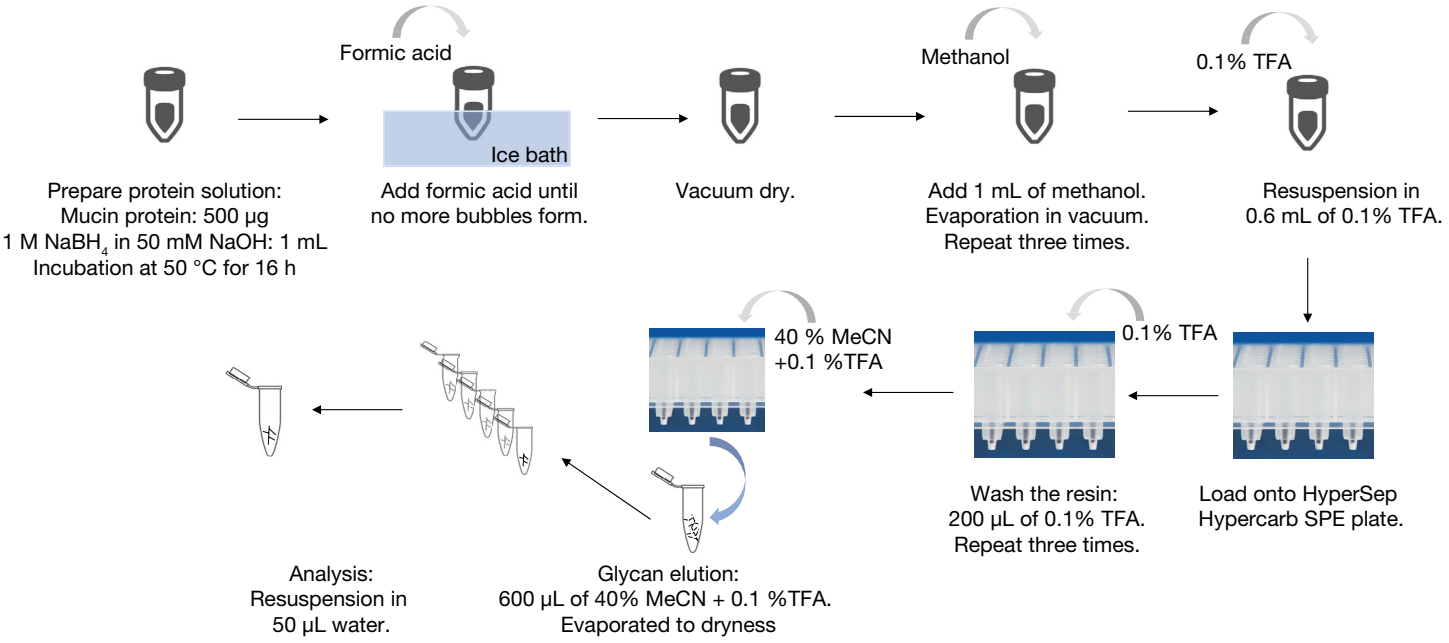
Note: For mucin type III and bovine fetuin proteins, we used 10 mg/mL solutions. For bovine fibrinogen and bovine thyroglobulin, we used 25 mg/mL solutions.

Instrument setup

Open the configuration program (gear symbol), select the SII module, select configure, and then add the IC modules. Three modules are added to this instrument configuration: Dionex AS-AP autosampler, Dionex ICS-5000⁺ system, and Q Exactive HF hybrid quadrupole-Orbitrap mass spectrometer. For creating an instrument method and running a sample sequence in Xcalibur software, create a new instrument method using the Xcalibur instrument set up wizard by entering values from Table 1. Save the instrument method. For running the sequence, open the sequence setup wizard, and then choose an appropriate instrument method. Input the sample name and a sample volume consistent with the sample loop installed.

System setup

The Dionex ICS 5000⁺ system is configured for electrochemical detection, operating under high pressure conditions up to 5,000 psi. To install this application, connect the Dionex AS-AP autosampler, Dionex ICS-5000⁺ system modules, and Q Exactive HF hybrid quadrupole-Orbitrap mass spectrometer as shown in Figure 1. Two pumps are used – one for the eluent system for IC separations and another for water regeneration of the downstream desalter. In addition, using a tee, a Dionex ERD 500 device is plumbed in the system, as shown in Figure 1. The “tee” is placed after the column to split the flow, thus enabling PAD and MS detections from the same separation. One half of the flow goes to the ED detector. The oxyanions of carbohydrates are detected by measuring the electrical current generated by their oxidation at the surface of a gold working electrode. The other half of the flow goes to the MS after passing through a Dionex ERD 500 device. The Dionex ERD 500 device is placed between the tee and conductivity detector and exchanges the sodium cation with hydronium, thus converting the NaOH to water and the sodium acetate to acetic acid. This prevents salts from going into the MS. (Note that it is optional to have a CD cell in the HPAE-PAD/MS set up.) If a CD cell is not used, the Dionex ERD 500 device is directly connected to the MS.



Scheme 1. Sample preparation and purification workflow for O-glycans

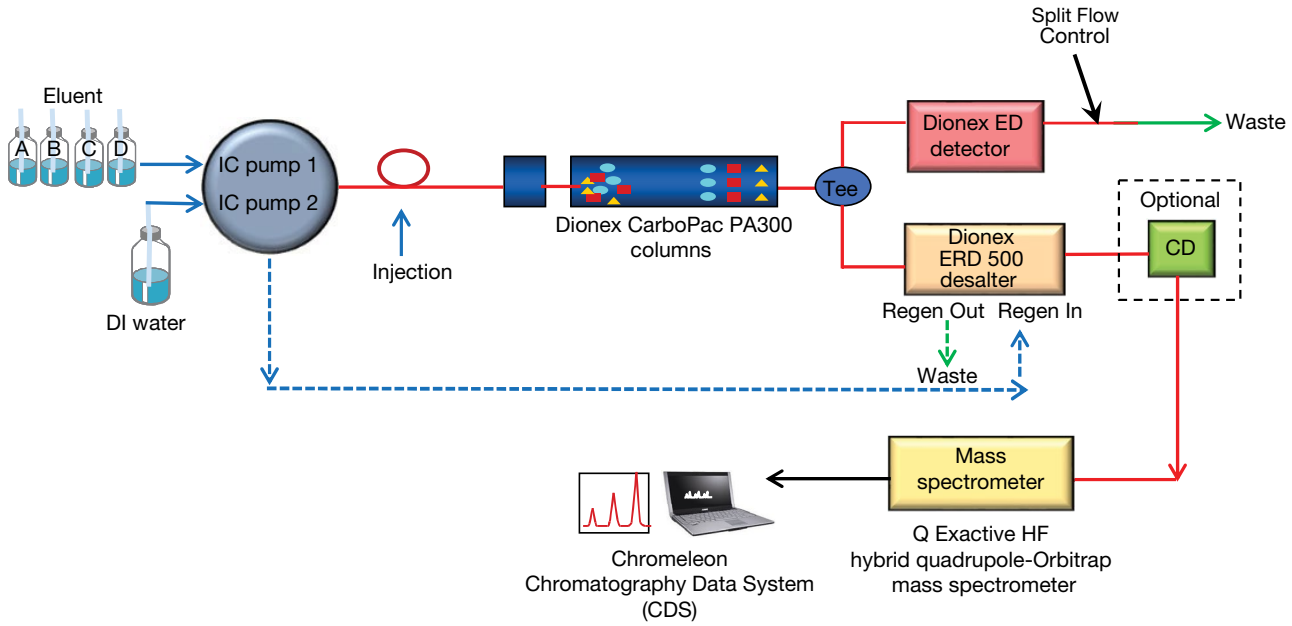


Figure 1. Flow diagram for HPAE-PAD/MS setup

It is important to determine the flow to the MS through the following steps:

1. Set the pump flow at the initial eluent conditions to 0.25 mL/min and measure the flow coming out of the suppressor eluent channel over a 5 min period by capturing it in a tared vial.

2. Subtract the tare weight (mg) from the collected weight (mg) to obtain the flow in µL/min (each mg is equal to a µL).

3. If the flow is less than 50% of the total flow (i.e., <125 µL/min), stop the pump and exchange the 180 mm tubing (0.005 × 0.062 × 70.9'' PK, RED) for the 190 mm (0.005 × 0.062 × 74.8'' PK, RED) tubing at the ED cell outlet. If the flow is more than 60% of the total flow (>150 µL/min), stop the pump and exchange the 180 mm tubing for the 170 mm (0.005 × 0.062 × 66.9'' PK, RED) tubing at the ED cell outlet.

4. Repeat steps 1 and 2 to verify that the eluent flow through the suppressor is between 140 and 150 µL/min.

For detailed instruction on system configuration, see Technical Note 72478 (TN72478).[8]

O-glycan structural annotation

Xcalibur software, version 4.1.31.9, was used for data acquisition, and Chromeleon software, version 7.2.9, was used for processing. MS/MS data were analyzed using SimGlycan software, version 5.0. All proposed glycan structures* were identified using the monoisotopic *m/z* of the precursor mass and accompanying MS/MS product ion spectra that were generated with higher energy collision induced dissociation (HCD). (* All annotations are tentative as we did not run pure glycan standards.)

Results and discussion

We followed the same workflow as described in application note AN74042.[11] The protocol in that application note details a sensitive and informative workflow for identifying and characterizing the O-glycans released from porcine gastric mucin glycoproteins. We applied the HPAE-PAD/MS workflow described here to the analysis of O-glycans released from four glycoproteins. The glycoproteins used were porcine gastric mucin type III, bovine fetuin, bovine fibrinogen, and bovine thyroglobulin. The glycans eluting from the column were detected simultaneously by PAD and HRAM mass spectrometry.

The workflow starts with a simple sample preparation devoid of derivatization. O-glycans are released from glycoproteins using reductive β-elimination under reducing conditions. This process converts the GalNAc at the reducing end into its alditol form. The glycan alditols are purified using porous graphitized carbon, and the samples are then ready for analysis by HPAE-PAD-MS. The workflow presented here does not require specific enrichment or derivatization steps before analysis. The label-free analysis eliminates extra reaction and cleanup steps. Moreover, it retains the native glycan profile without losing structures or adding ambiguous information with extraneous labeling reactions. This workflow can be applied with ease for free oligosaccharides or for released O-glycans or N-glycans from glycoproteins. But reductive beta-elimination will not effectively release all N-glycans.

After the sample preparation, the released glycans were separated using the Dionex CarboPac PA300-4µm column. This column was recently introduced, with unique selectivity that provides high-resolution separations of neutral, sialylated, and sulfated glycans. The column improves the separation of neutral glycans that elute at the beginning of the gradient program relative to older Dionex CarboPac columns. The eluent gradient starts with a low concentration of sodium hydroxide and sodium acetate, facilitating the separation of small, neutral glycans. With the elevated concentration of hydroxide and acetate, larger neutral glycans elute from the column, followed by charged glycans, such as sialylated and sulfated glycans. The column effluent containing separated glycans is then passed through a desalter to remove sodium ions in the effluent prior to mass spectrometric analysis. A Q Exactive HF mass spectrometer used in negative electrospray mode was coupled to the ion chromatography system. Fragmentation of glycans in the negative mode by HCD provided information-rich MS^2 spectra dominated by glycosidic and cross-ring fragments, which frequently revealed linkage information. The possible glycan structures were first identified by SimGlycan high-throughput search and score function. The structures were confirmed by annotating the diagnostic fragmentation patterns observed in MS^2 spectra.

Porcine gastrin mucin type III

Mucins are highly glycosylated proteins that are found in the mucous membranes of animals and humans. They mainly contain O-linked glycans that are linked to the protein through GalNAc, and are composed of varying amounts of GlcNAc, fucose, galactose, and sialic acid (Neu5Ac, N-acetylneuraminic acid).[12] Figure 2A depicts the HPAE-PAD profiles, while the trace in Figure 2B shows the corresponding base peak chromatogram (BPC) acquired for the same injection. MS detection has a delay compared to PAD due to the eluent going through the Dionex ERD 500 device, so there is a slight delay between the ED and MS detectors (approximately 0.6 min) independent of glycan type. The resolution of the peaks representing the glycans in the BPC remains the same as that of the PAD chromatogram, yet the presence of minor peak broadening/tailing indicates the potential of post-column peak dispersion caused by the electrolytically regenerated desalter. There is also a difference in the peak response for the PAD chromatogram and the BPC. The response provided by the two different detection methods for each glycan species is mostly dependent on the glycan structures. The O-linked glycans released from porcine gastric mucin type III protein include a wide range of diverse structures, varying in size, linkage position, stereochemistry, monosaccharide composition, and sulfate groups. Detailed information of the proposed glycan compositions, their observed and theoretical *m/z*, and the mass accuracy is shown in Table 1. The mass accuracy of all the detected structures was less than 5 ppm, ensuring high confidence peak annotation. As shown in Figure 2 and Table 2, peaks eluting from 4.2 min to 30.0 min are small, neutral glycans. Peaks eluting from 33.1 to 35 mins are sialylated glycans. Charged, sulfated glycans elute after 35 min.

For more detailed information on mucin O-glycan structures, see AN74042.[11]

Bovine fetuin

Bovine fetuin, which contains N-glycosylation sites and a number of O-glycosylation sites, has been frequently used as a model glycoprotein to test analytical workflows in glycoproteomics.[13,14] As annotated in Figure 3 and Table 3, the glycans released from bovine fetuin included sialylated N-glycans and O-glycan structures. The majority of the O-linked structures are of the sialylated tri- and tetrasaccharide type based on the type 1 core (Galβ1-3GalNAcα1-Ser/Thr). The O-glycans on the protein include a monosialylated core-1 O-glycan ([Neu5Ac] [Hex] [HexNAc]) and a disialylated tetrasaccharide ([Neu5Ac]2 [Hex] [HexNAc]) and a disialylated hexasaccharide ([Neu5Ac]2 [Hex]2 [HexNAc]2). These data are consistent with data reported by other research groups.[15,16]

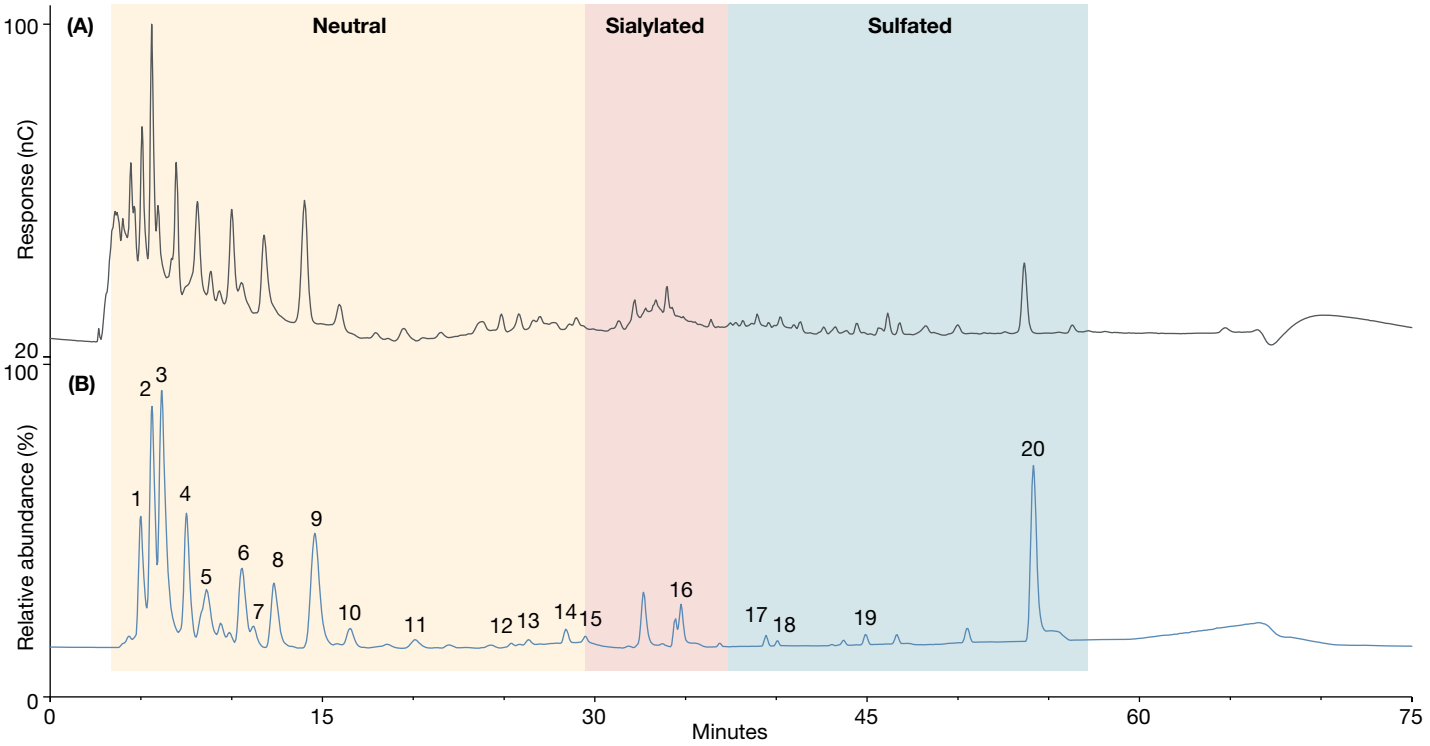


Figure 2. Comparison of (A) PAD chromatogram and (B) MS base peak chromatogram of O-linked glycans released from porcine gastric mucin type III

Table 2. Compositional annotation of glycans released from porcine gastric mucin (type III)

Peak #	RT (min)	Observed *m/z*	Theoretical *m/z*	Charge stage	Mass accuracy (ppm)	Composition*
1	4.96	733.2885	733.2884	1	0.1	Hex Fuc $[HexNAc]_2$
2	5.57	587.2350	587.2353	1	0.5	Hex [HexNAc]2
3	6.17	530.2086	530.2090	1	0.8	Hex Fuc HexNAc
4	7.46	790.3103	790.3147	1	5.6	Hex [HexNAc]3
5	8.34	733.2891	733.2884	1	1.0	Hex Fuc $[HexNAc]_2$
6	10.67	1098.4191	1098.4206	1	1.4	$[Hex]_2$ Fuc $[HexNAc]_3$
7	11.34	895.3408	895.3412	1	0.5	$[Hex]_2$ Fuc $[HexNAc]_2$
8	12.61	1041.3983	1041.3992	1	0.9	$[Hex]_2$ [Fuc]2 $[HexNAc]_2$
9	14.8	1155.4409	1155.4421	1	1.0	$[Hex]_2$ [HexNAc]4
10	16.93	1098.4213	1098.4206	1	0.6	$[Hex]_2$ Fuc$[HexNAc]_3$
11	19.12	1203.4508	1203.4520	1	1.0	$[Hex]_3$ $[Fuc]_2$ $[HexNAc]_2$
12	26.03	958.3557	958.3571	2	1.5	$[Hex]_4$ $[Fuc]_3$ $[HexNAc]_4$
13	26.92	1317.4933	1317.4949	1	1.2	$[Hex]_3$ $[HexNAc]_4$
14	27.67	986.8673	986.8678	2	0.5	$[Hex]_4$ $[Fuc]_2$ $[HexNAc]_5$
15	30.11	1040.3793	1040.3788	1	0.5	Neu5Ac $[Hex]_2$ $[HexNAc]_2$
16	34.52	821.3050	821.3045	1	0.6	Neu5Ac Hex Fuc HexNAc
17	38.22	852.2811	852.2848	2	4.3	N-glycan, hybrid, sulfated
18	39.76	669.7120	669.7115	2	0.8	$[Hex]_3$ Fuc $[HexNAc]_3$-SO_3^-
19	46.13	1121.3558	1121.3559	1	0.1	$[Hex]_2$ $[Fuc]_2$ $[HexNAc]_2$-SO_3^-
20	53.34	813.2461	813.2452	1	1.1	Hex Fuc $[HexNAc]_2$-SO_3^-

*Hex: Hexose; Fuc: Fucose; HexNAc: N-Acetylhexosamine; Neu5Ac: N-Acetylneuraminic acid; Neu5Gc: N-Glycolylneuraminic acid

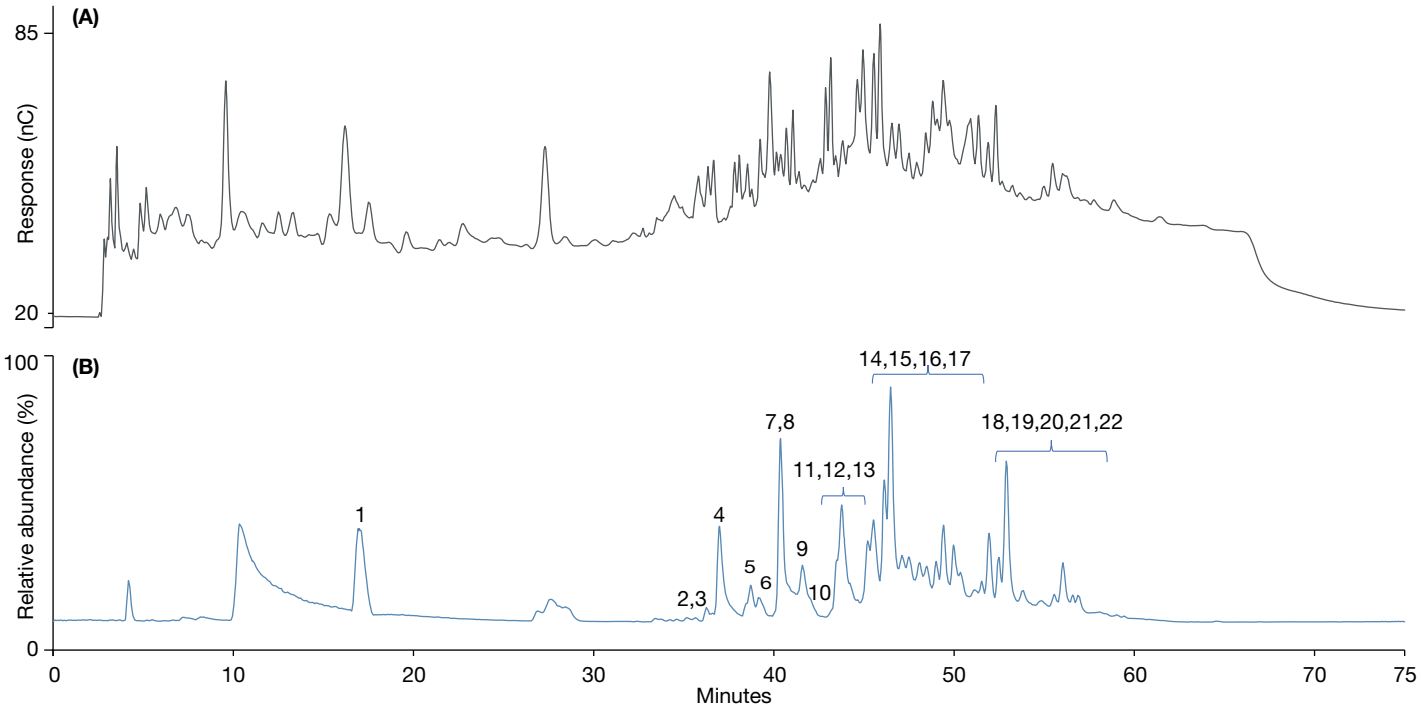


Figure 3. Comparison of (A) PAD chromatogram and (B) MS base peak chromatogram of O-linked glycans released from bovine fetuin

Table 3. Compositional annotation of glycans released from bovine fetuin

Peak	RT (min)	Observed *m/z*	Theoretical *m/z*	Mass accuracy (ppm)	Charge state	Composition	Type
1	18.04	675.2480	675.2514	4.3	1	[Neu5Ac] [Hex] [HexNAc]	O-glycan
2	36.30	965.8547	965.8516	3.2	2	[Neu5Ac] [Hex]2 [HexNAc]4 [Man]3	N-glycan
3	36.70	691.2552	691.2538	2.0	1	[Neu5Gc] [Hex] [HexNAc]	O-glycan
4	37.17	774.7787	774.7804	2.2	2	[Neu5Gc] [Neu5Ac] [Hex]2 [HexNAc]3	O-glycan
5	38.52	1160.932	1160.934	0.9	2	[Neu5Ac] [Hex]1 [Fuc]1 [HexNAc]6 [Man]3	N-glycan
6	38.74						
7	40.24	665.2376	665.2407	4.7	2	[Neu5Ac]2 [Hex]2 [HexNAc]2	O-glycan
8	40.53	482.6726	482.6746	4.1	2	[Neu5Ac]2 [Hex] [HexNAc]	O-glycan
9	41.58	1111.395	1111.399	3.7	2	[Neu5Ac]2 [Hex]2 [HexNAc]4 [Man]3	N-glycan
10	42.23	1293.971	1293.965	4.3	2	[Neu5Ac]2 [Hex]3 [HexNAc]5 [Man]3	N-glycan
11	43.44	993.0312	993.0368	5.6	3	[Hex]4 [HexNAc]9 [Man]3	N-glycan
12	43.69						
13	44.17						
14	45.16	959.3405	959.342	1.6	3	[Neu5Ac]3 [Hex]3 [HexNAc]5 [Man]3	N-glycan
15	45.43						
16	46.03						
17	46.37						
18	51.88	1056.370	1056.374	3.8	3	[Neu5Ac]4 [Hex]3 [HexNAc]5 [Man]3	N-glycan
19	52.41						
20	52.84						
21	53.75						
22	56.03						

Bovine fibrinogen

Figure 4 shows a separation of bovine fibrinogen glycans. The glycans released from bovine fibrinogen protein include mainly N-glycan structures with few O-glycan structures. Out of the 16 annotated glycan structures, three were identified as O-glycan structures. Interestingly, a recent study by Zauner et al. also showed O-glycosylated sites lying within certain regions in human fibrinogen protein.[17] In their study, they identified seven novel O-glycosylated regions within the fibrinogen α- and β-chains, while no O-glycosylation was observed on the γ-chain. All glycopeptides identified in their study carry the same O-glycan with composition ([Neu5Ac] [Hex] [HexNAc]).

As annotated in Table 4, bovine fibrinogen primarily contains afucosylated, biantennary, mono-, and disialylated

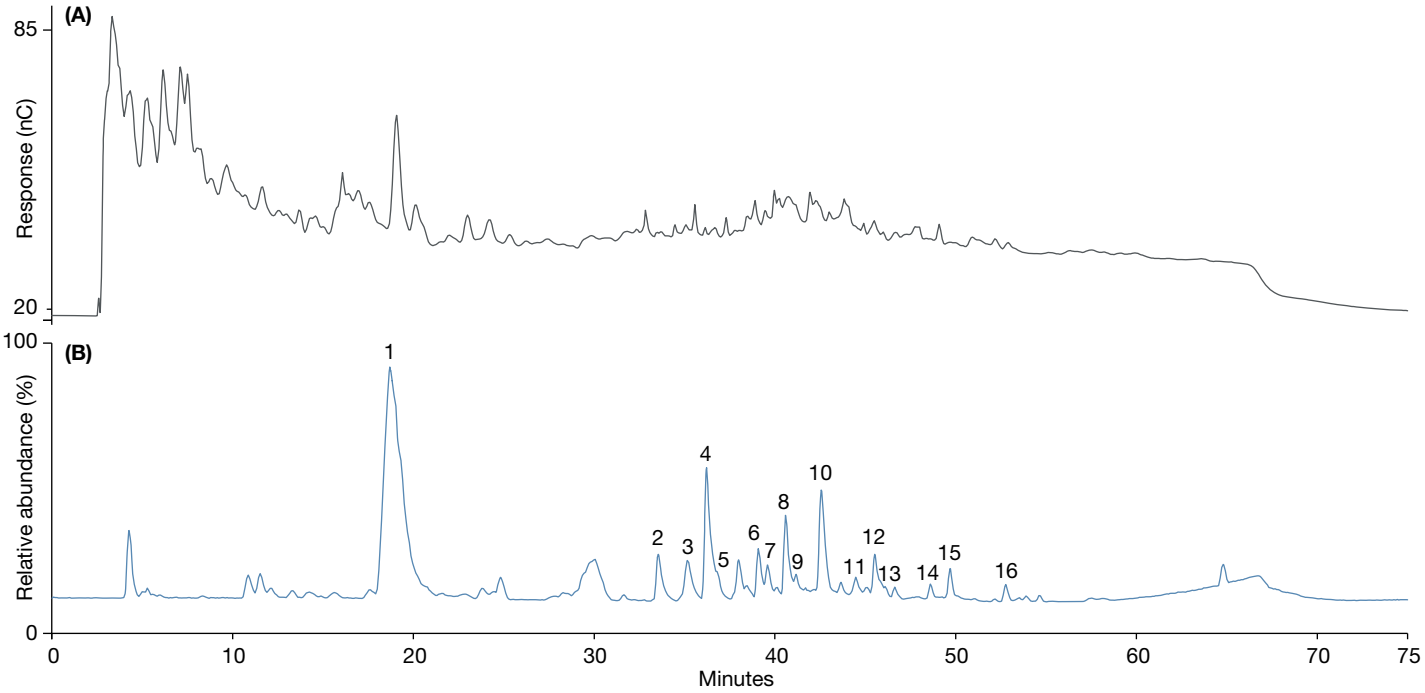


Figure 4. Comparison of (A) PAD chromatogram and (B) MS base peak chromatogram of O-linked glycans released from bovine fibrinogen

Table 4. Compositional annotation of glycans released from bovine fibrinogen

Peak	RT (min)	Observed *m/z*	Theoretical *m/z*	Mass accuracy (ppm)	Charge state	Composition	Type
1	18.48	675.2480	675.2514	5.0	1	[Neu5Ac] [Hex] [HexNAc]	O-glycan
2	35.15	965.8452	965.8468	1.7	2	[Neu5Ac] [Hex]2 [HexNAc]4 [Man]3	N-glycan
3	36.18	965.8452	965.8468	1.7	2	[Neu5Ac] [Hex]2 [HexNAc]4 [Man]3	N-glycan
4	36.31	691.2442	691.2463	3.0	1	[Neu5Gc] [Hex] [HexNAc]	O-glycan
5	37.99	1022.3575	1022.3518	5.6	2	[Hex] [HexNAc]2 [Man]9	N-glycan
6	38.46	1160.9277	1160.9286	0.8	2	[Neu5Ac] [Hex]1 [Fuc]1 [HexNAc]6 [Man]3	N-glycan
7	39.09	982.3525	982.3518	0.7	2	[Hex]4 [HexNAc]4 [Man]3	N-glycan
8	40.11	1111.3998	1111.3945	4.8	2	[Neu5Ac]2 [Hex]2 [HexNAc]4 [Man]3	N-glycan
9	40.54	482.6676	482.6697	4.4	2	[Neu5Ac]2 [Hex] [HexNAc]	O-glycan
10	40.61	973.8424	973.8442	1.9	2	[NeuGc] [Hex]2 [HexNAc]4 [Man]3	N-glycan
11	41.17	1111.3954	1111.3945	0.8	2	[Neu5Ac]2 [Hex]2 [HexNAc]4 [Man]3	N-glycan
12	44.49	1119.3932	1119.3919	1.2	2	[Neu5Gc] [Neu5Ac] [Hex]2 [HexNAc]4 [Man]3	N-glycan
13	45.52						
14	48.61						
15	49.70	1127.8989	1127.8995	0.5	2	[Neu5Gc]2 [Hex]2 [HexNAc]4 [Man]3	N-glycan
16	52.16						

glycans terminated by Neu5Ac and N-glycolylneuraminic acid (Neu5Gc). The identified O-glycans include mono-, di-, and trisialylated structures containing Neu5Ac, Neu5Gc, or both. We confirmed the annotated O-glycan structures using MS/MS data. The Q Exactive HF mass spectrometer has the capacity to generate HCD fragment ions and detect them with high resolution and mass accuracy. This allows for identification and differentiation of fragment ions with similar *m/z* ratios and is useful for branching annotation and linkage assignment. Figure 5 (A-C) shows MS/MS spectra of annotated O-glycan structures of *m/z* 675.2480, 691.2414, and 482.6678. All three structures are based on the type 1 core. The peak at *m/z* 675.2480 represents monosialylated core-1 O-glycan terminated by Neu5Ac. Two glycosidic fragments (*m/z* 290.0884 and *m/z* 384.1518) were observed in MS/MS spectra. The loss of Neu5Ac is characterized by a peak at *m/z* 290.0884 (Figure 5A). Similarly, a peak at *m/z* 691.2414 is also a monosialylated core-1 O-glycan terminated by Neu5Gc instead of Neu5Ac. Compared to Neu5Ac, Neu5Gc has an extra oxygen, presented as the hydroxyl in the N-glycolyl group at C-5. The loss of Neu5Gc is characterized by a peak at *m/z* 306.0832 (Figure 5b). The peak at *m/z* 482.6678 is a doubly charged disialylated core-1 O-glycan terminated by two Neu5Ac.

Bovine thyroglobulin

Bovine thyroglobulin contains 10% carbohydrate by weight.[18] As annotated in Figure 6 and Table 5, only one O-glycan structure with composition ([Neu5Ac] [Hex] [HexNAc]) was identified. As discussed above, the same O-glycan structure is also observed in bovine fetuin and

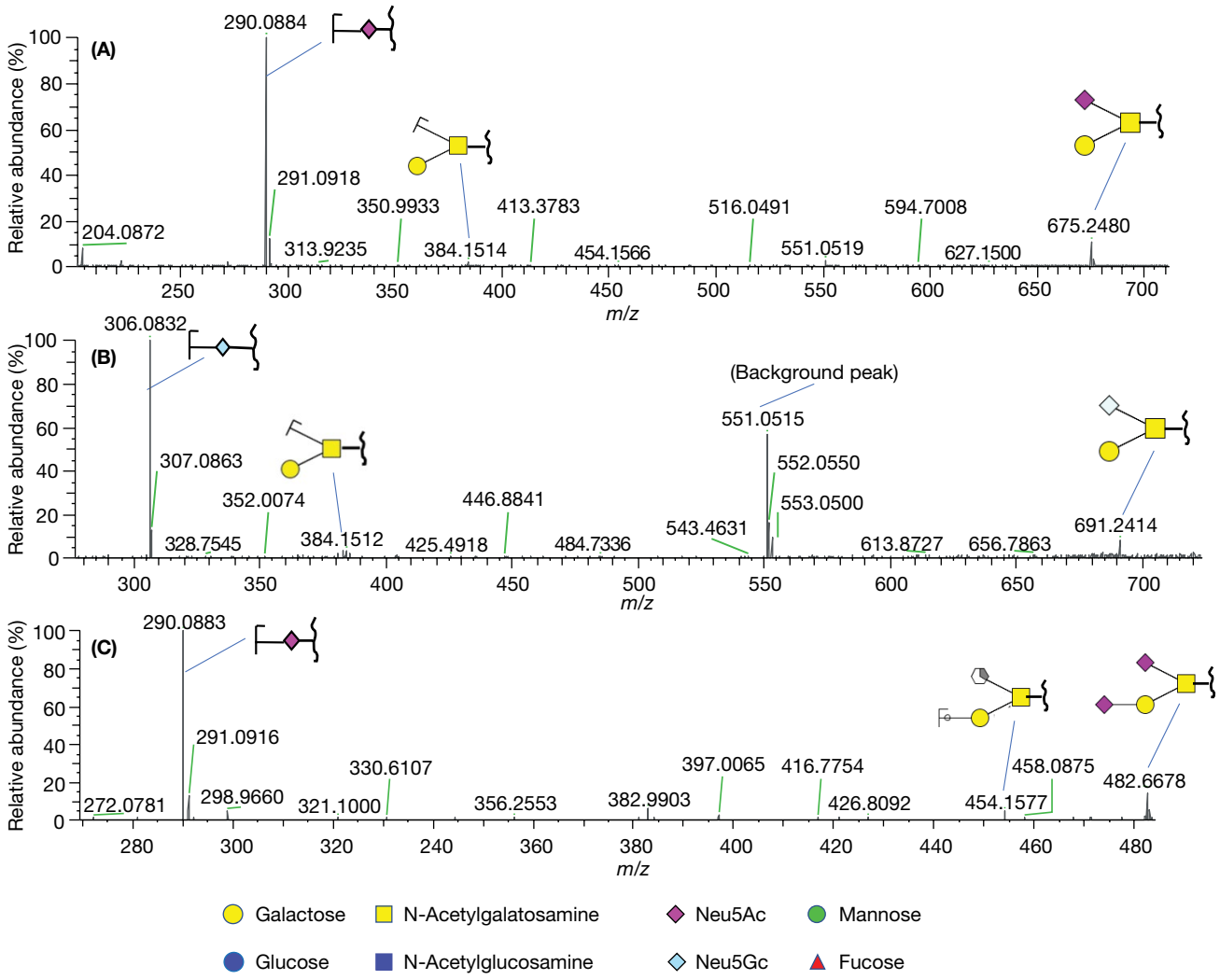


Figure 5. MS/MS spectra of three O-glycans released from bovine fibrinogen

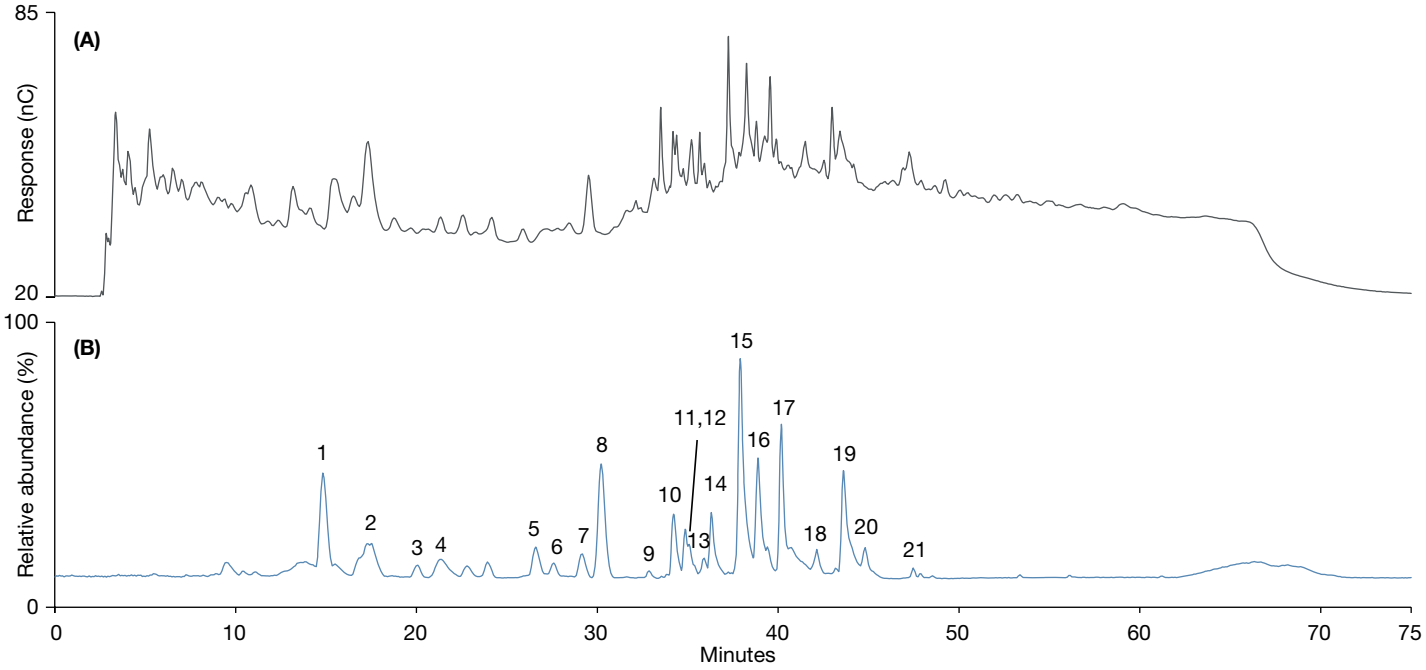


Figure 6. Comparison of (A) PAD chromatogram and (B) MS base peak chromatogram of O-linked glycans released from bovine thyroglobulin

Table 5. Compositional annotation of glycans released from bovine thyroglobulin

Peak	RT (min)	Observed *m/z*	Theoretical *m/z*	Mass accuracy (ppm)	Charge	Composition	Type
1	15.71	617.2312	617.2322	1.6	2	[HexNAc]2 [Man]5	N-glycan
2	18.85	675.2480	675.2514	4.3	1	[Neu5Ac] [Hex] [HexNAc]	O-glycan
3	21.15	698.2442	698.2460	2.6	2	[HexNAc]2 [Man]6	N-glycan
4	23.89	779.2709	779.2725	2.1	2	[HexNAc]2 [Man]7	N-glycan
5	24.91	779.2705	779.2725	2.6	2	[HexNAc]2 [Man]7	N-glycan
6	27.31	860.2963	860.2989	3.0	2	[HexNAc]2 [Man]8	N-glycan
7	28.27	860.2963	860.2989	3.0	2	[HexNAc]2 [Man]8	N-glycan
8	30.7	941.3223	941.3253	3.2	2	[HexNAc]2 [Man]9	N-glycan
9	32.34	986.3582	986.3600	1.8	2	[Neu5Ac] [Hex]1 [HexNAc]5 [Man]3	N-glycan
10	34.26	1169.4355	1169.4363	0.7	2	[Hex]2 [Fuc]2 [HexNAc]5[Man]3	N-glycan
11	34.87	941.3199	941.3253	5.7	2	[HexNAc]2 [Man]9	N-glycan
12	35.05	1119.3959	1119.3919	3.6	2	[Neu5Gc] [Neu5Ac] [Hex]2 [HexNAc]4 [Man]3	N-glycan
13	35.84	1015.3529	1015.3519	1.0	2	[Hex]1 [Fuc]1 [HexNAc]6 [Man]3	N-glycan
14	36.19	1119.3913	1119.3919	0.5	2	[Neu5Gc] [Neu5Ac] [Hex]2 [HexNAc]4 [Man]3	N-glycan
15	37.82	1236.4381	1236.4289	7.4	2	[Neu5Ac]3 [Hex]3 [HexNAc]3 [Man]3	N-glycan
16	38.76	1184.4304	1184.4234	6.0	2	[Neu5Ac]2 [Hex]2 [Fuc]1 [HexNAc]4[Man]3	N-glycan
17	40	1184.4304	1184.4234	6.0	2	[Neu5Ac]2 [Hex]2 [Fuc]1 [HexNAc]4[Man]3	N-glycan
18	41.97	934.6525	934.6575	5.4	3	[Neu5Ac]4 [Hex]2 [HexNAc]4 [Man]3	N-glycan
19	43.52	1184.4081	1184.4040	3.5	2	[Neu5Ac]2 [Hex]2 [Fuc]1 [HexNAc]4[Man]3	N-glycan
20	44.7	1184.4081	1184.4040	3.5	2	[Neu5Ac]2 [Hex]2 [Fuc]1 [HexNAc]4[Man]3	N-glycan
21	47.36	1008.0244	1008.0223	2.1	3	[Neu5Ac]3 [Hex]3 [Fuc]1 [HexNAc]5 [Man]3	N-glycan

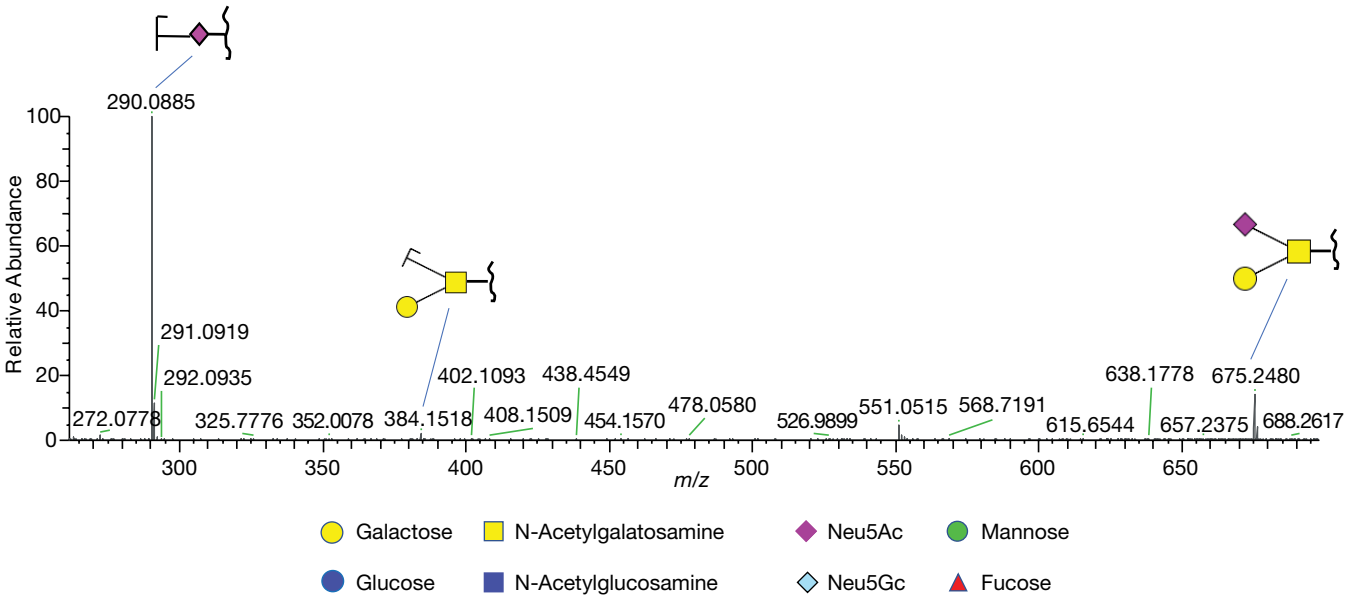


Figure 7. MS/MS spectrum of an O-glycan released from bovine thyroglobulin

bovine fibrinogen glycan profiles. MS/MS spectra (Figure 7) of the peak at *m/z* 675.2480 shows two glycosidic fragments at *m/z* 290.0885 and *m/z* 384.1518. The presence of an O-glycan structure in bovine thyroglobulin has not been reported before, but in the case of human thyroglobulin, limited O-linked glycosylation of two types has been reported.[19]

The N-glycan profile of bovine thyroglobulin revealed high-mannose, complex, and hybrid structures (Table 4). The N-glycan set also has several minor glycans terminated with Neu5Ac, Neu5Gc, and both Neu5Ac and Neu5Gc. The data are consistent with previous studies that thyroglobulin oligosaccharides include high-mannose and complex-type N-linked glycans.[6]

Conclusion

In this application note, we used the recently introduced Dionex CarboPac PA300 column that enables simultaneous separation of neutral and charged glycans without derivatization. We evaluated an HPAE-PAD/MS workflow for O-glycan profiling of four different glycoproteins: porcine gastric mucin type III, bovine fetuin, bovine fibrinogen, and bovine thyroglobulin. The O-glycans released from porcine gastric mucin type III protein include a wide range of diverse structures, varying in size, linkage position, stereochemistry, monosaccharide composition, and sulfate groups. The glycans released from bovine fetuin included sialylated N-glycans and O-glycan structures. Most of the O-glycans released from bovine fetuin are of the sialylated tri- and tetrasaccharide type based on the type 1 core. The identified O-glycans in bovine fibrinogen include mono-, di-, and trisialylated structures containing Neu5Ac, Neu5Gc, or both. For thyroglobulin, this is the first instance of direct experimental demonstration of the presence of an O-glycan structure. The mass accuracy of all the detected structures was less than 5 ppm, ensuring high confidence peak annotation. The structures were confirmed by annotating diagnostic fragmentation patterns observed in MS^2 spectra. The approach described here identified O-glycan structures that were in good agreement with structures previously reported for the four glycoproteins.

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