

A 3D molecular model of glycoproteins, showing complex, branched structures in shades of red, blue, and white. The structures are set against a blurred background of green and white, suggesting a biological or laboratory environment.

## Improved profiling of sialylated *N*-linked glycans by ion chromatography-Orbitrap mass spectrometry

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HPAE-MS, HRAM, CarboPac  
PA200 column

### Goal

To describe an HPAE-MS method for improved glycan profiling

### Introduction

Protein glycosylation is a key intracellular process that involves interactions of various enzymes and substrates.<sup>1</sup> Glycoproteins are central to several important biological processes.<sup>2,3</sup> Carbohydrates, especially those linked through an asparagine side chain (*N*-linked), play a critical role in these biological activities. Due to increased understanding of the biological significance of carbohydrates, an increasing number of glycoprotein therapeutics are being developed as treatments for various pathological conditions.

Manufacturing glycoproteins in high volume is complex, not only due to the nature of the molecules, but also because it involves a cell-based process. Protein glycosylation is heavily dependent on the manufacturing process. Various factors that influence protein glycosylation include cell type, cell age, and cell culture conditions. Maintaining batch-to-batch consistency of glycoprotein therapeutics requires close attention to quality control. Hence, there is an increased demand for methods to characterize glycoprotein carbohydrates.

Good profiling of a recombinant glycoprotein's asparagine-linked (*N*-linked) glycans requires high-resolution separation and reliable identification of released glycans. High-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) is a well-established, powerful technique for glycan analysis. HPAE-PAD is especially effective for separating sialylated glycans, but there is opportunity to increase resolution and improve glycan identification. By implementing changes to commonly used HPAE-PAD conditions we can achieve improved resolution.<sup>4</sup>

Given the interest in glycoproteins as therapeutics, there have been attempts<sup>5,6</sup> to use HPAE-PAD to create a glycan database. A technique suitable for building such a database needs to be convenient and cost effective. HPAE-PAD fits both criteria as it enables derivatization-free high-resolution separation with sensitive detection. Even with a good separation there is still a need for fast determination of glycan structure. Here, we have coupled a HPAE chromatography system to a Thermo Scientific™ Orbitrap™ mass spectrometer to harvest synergy between these two techniques to facilitate rapid structure determination.

The methods proposed here use a Thermo Scientific™ Dionex™ CarboPac™ PA200 column for glycan separation. The Dionex CarboPac PA200 column is a nonporous, high-efficiency, polymeric anion exchange column that provides the highest resolution available for oligosaccharide mapping and analysis by HPAE. The Dionex CarboPac PA200 includes smaller particle-size packing material (5.5 μm) than is used in the Thermo Scientific™ Dionex™ CarboPac™ PA1 column (10 μm). The pellicular resin structure of the Dionex CarboPac PA200 column permits excellent mass transfer, resulting in high-resolution chromatography and rapid reequilibration.

After separation by HPAE, the glycans pass through a desalting device that removes sodium present from the eluent. Fragmentation of glycans in the negative mode by higher-energy collisional dissociation (HCD) provides information-rich MS<sup>2</sup> spectra dominated by glycosidic and cross-ring fragments that frequently reveal linkage information. 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 the diagnostic fragmentation patterns observed in MS<sup>2</sup> spectra. This allowed comparison of structures identified

under different sets of conditions to test whether glycan resolution can further be improved. Moreover, changes in elution conditions allowed correlation of glycan structure with observed elution behavior. Here we show that in some cases, changed elution conditions can be used to resolve different glycan structures. This approach may be an effective way to quickly screen the impact of changes in cell culture conditions on sialylation.

## Experimental Equipment

- Thermo Scientific™ Dionex™ ICS-5000+ High-Pressure Ion Chromatography (HPIC™) system, an integrated ion chromatograph that includes:
  - SP single pump module (P/N 061707) or DP dual pump (P/N 061712) with degas option
  - DC detector compartment (P/N 061767) with single-temperature zone
  - Electrochemical detector (P/N 061719) and cell (P/N 061757)
  - pH-Ag/AgCl reference electrode (P/N 061879)
  - Carbohydrate disposable Au working electrode, pack of 6 (two 2.0 mil gaskets included) (P/N 066480)
  - 20 μL sample loop
- Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software, version 7.2.9
- Thermo Scientific™ Q Exactive™ Hybrid Quadrupole Orbitrap™ Mass Spectrometer with HESI-II probe
- Thermo Scientific™ Dionex™ AS-AP autosampler (P/N 074926) with cooling tray option (recommended)
- Thermo Scientific™ Dionex™ ERD 500 desalter (P/N 085089)
- 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, 1000 mL, nylon membrane, 90 mm diameter (P/N 164-0020)
- Autosampler vials with septum, 0.3 mL capacity (P/N 055428)
- HPAE-PAD/MS Assembly Kit (P/N 302854)

## HPAE -PAD conditions

Columns:	Dionex CarboPac PA200, 3 × 250 mm analytical column (P/N 062896) and Dionex CarboPac PA200 Guard 3 × 50 mm guard column (P/N 062895)
Flow rate:	0.5 mL/min
Injection volume:	10 µL (push_partial_LS)
Column temp.:	25, 30, or 35 °C
Sampler tray temp.:	4 °C
Eluents:	A) 0.1 M sodium hydroxide, B) 0.25 M sodium acetate in 0.1 M sodium hydroxide or A) 0.15 M sodium hydroxide, B) 0.25 M sodium acetate in 0.15 M sodium hydroxide
Elution gradient:	Table 1
Detection:	Pulsed Amperometric, Gold on PTFE disposable working electrode (P/N 066480) using a 2 mil gasket (P/N 060141) , Waveform A Technical Note 21

**Table 1. Gradient program for elution**

Time (min)	Solution A (%)	Solution B (%)	Elution
0	97.6	2.4	Initial condition
70	24	76	6 to 190 mM NaOAc, isocratic 0.1 M NaOH
70.1	20	80	200 mM NaOAc, isocratic 0.1 M NaOH
75.1	20	80	200 mM NaOAc, isocratic 0.1 M NaOH
75.1	97.6	2.4	Re-equilibrium

\*Note: For the high hydroxide concentration eluent condition, solutions A and B are prepared in 0.15 M rather than 0.1 M sodium hydroxide.

## Reagents and standards

- Thermo Scientific™ Dionex™ sodium acetate salt, electrochemical-grade (P/N 059326)
- Sodium hydroxide, 50% w/w (Fisher P/N SS254-500)
- Alpha-1-acid glycoprotein (Sigma P/N G9885)
- Bovine fetuin (Sigma P/N F2379)
- Bovine thyroglobulin (Sigma P/N T1001)
- Bovine fibrinogen (Sigma P/N F8630)
- Oligosaccharide mixtures enzymatically released from, human alpha-1-acid glycoprotein, bovine fetuin, bovine thyroglobulin, and bovine fibrinogen using PNGase F
- Fetuin alditol standard (P/N 043064)
- PNGase F, 15,000 units, 500,000 U/mL, a Unit is defined as the amount of enzyme required to remove >95% of carbohydrate from 10 µg of denatured RNase B in 1 h at 37 °C in a total reaction volume of 10 µL (New England BioLabs®, P/N P0705S). Enzyme is supplied with: 10× glycoprotein denaturing buffer (5% SDS, 10% β-mercaptoethanol), 10× G7 buffer (0.5 M sodium phosphate, pH 7.5 at 25 °C), and 10% NP-40.

## Mass spectrometry software

- Thermo Scientific™ Foundation 3.0 software
- Thermo Scientific™ Xcalibur™ software or Thermo Scientific™ TraceFinder™ software with SII for Xcalibur software
- SimGlycan software, version 5.0

## Eluent preparation

- It is essential to use high-quality water of high resistivity (18 MΩ·cm) as free of dissolved carbon dioxide as possible. It is extremely important to minimize contamination by carbonate, a divalent anion at high pH that binds strongly to the columns, causing a 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. For additional details on mobile phase preparation refer to Thermo Scientific Technical Note 71 (TN71).<sup>7</sup>

- Sodium hydroxide: To 900 mL of deionized (DI) water, previously filtered through a 0.2 µm Nalgene 1 L nylon filter and degassed, add 5.2 mL (0.1 M) or 7.8 mL (0.15 M) 50% (w/w) sodium hydroxide solution, mix gently, and immediately install on the system under UHP grade nitrogen or helium gas cover at 34 to 55 kPa (5 to 8 psi).
- Sodium acetate in sodium hydroxide: Dissolve 20.5 g (0.25 M) anhydrous sodium acetate in 800 mL of previously degassed DI water. Vacuum filter this solution through a 0.2 µm Nalgene 1 L nylon filter to remove particles from the sodium acetate that can damage parts of the pump. Transfer the solution to a 1 L volumetric flask, add 5.2 mL (0.1 M) or 7.8 mL (0.15 M) 50% (w/w) NaOH and bring to volume, mix gently, and immediately install on the system under UHP grade nitrogen or helium gas cover at 34 to 55 kPa (5 to 8 psi).

## Methods

### PNGase F digestion

Add 20 µL of 1:10 dilution of PNGase F enzyme preparation to 200 µL protein (8 mg/mL) and add 200 µL DI water. Incubate the mixture at 37 °C for 20 h. Dilute this sample 1:1 with DI water prior to chromatography. For the digestion control, substitute the 200 µL protein with 200 µL DI water (i.e., 400 µL total DI water).

### High performance anion exchange chromatography

The glycans were separated on a Thermo Scientific™ Dionex CarboPac™ PA200 BioLC™ analytical column (3 × 250 mm) attached to a Dionex ICS-5000+ HPIC dual IC system. The system was equipped with a Dionex ERD 500 electrolytically regenerated desalter. Desalting is accomplished with a Dionex ERD 500 desalter using 350 mA current and 3.5 mL/min regenerant water flow. The column flow is split immediately post column and ~250–300 µL/min (50–60% of the total flow) is diverted to the suppressor and into the MS.

### Coupling HPAE to MS

The Dionex ICS-5000+ HPIC system is configured for electrochemical detection, operating under high-pressure conditions up to 5000 psi. To install this application first connect the Dionex AS-AP autosampler, the Dionex ICS-5000+ HPIC system, Dionex ERD 500 desalter, and Q Exactive mass spectrometer modules using the HPAE-PAD/MS Assembly Kit. For detailed instruction on system configuration see Technical Note 72478 (TN72478).<sup>8</sup>

### Configuring the Dionex ERD 500 desalter

The Dionex ERD 500 desalter (ERD 500) removes sodium ions from the column effluent, before passing into the mass spectrometer. A properly operating Dionex ERD 500 desalter will exchange ≥99.5% of sodium ions present (up to 0.35M) under the following conditions.

Eluent flow:

- 4 mm column: ≤1 mL/min
- 2 mm column: ≤0.25 mL/min

Regenerant flow:

- 4 mm column: DI water at ≥7 mL/min
- 2 mm column: DI water at ≥2 mL/min

(Note: The method described here uses a 3 mm column for which a 2 mm Dionex ERD 500 desalter was used. The eluent flow through to the device was ~0.3 mL/min and regenerant (DI water) flow rate was 3.5 mL/min.)

The Dionex ERD 500 desalter is rated to 150 psi total post-desalter backpressure. Adjust tubing lengths accordingly to maintain the backpressure on the device below 150 psi. Refer to the Dionex ERD 500 desalter manual (Thermo Scientific P/N 031761-05) for more details.

## Mass spectrometry conditions

A Q Exactive Orbitrap mass spectrometer used in negative ion electrospray mode was coupled to the IC system. The spray voltage was 3.2 kV and the capillary temperature was set to 320 °C. The probe heater temperature was 200 °C. The sheath and auxiliary gas flows were 30 and 10, respectively (arbitrary units). MS spectra were acquired at  $m/z$  400–2000 over the chromatographic separation at 60,000 FWHM resolution at  $m/z$  200. The MS automatic gain control (AGC) target was set to  $1 \times 10^5$  and the maximum injection time was 200 ms. Data-dependent MS/MS experiments for the top 10 ions were performed using a normalized collision energy (NCE) of 30 eV. For MS/MS experiments the AGC target value was  $1 \times 10^5$ , the maximum injection time was 300 ms, and resolution was 17,500 FWHM at  $m/z$  200. The number of microscans in both MS and MS/MS experiments was 1, and the quad isolation window was 1.5  $m/z$ . The underfill ratio and intensity threshold values were set at 5% and 2 $\times$ , respectively. Xcalibur software version 4.1.31.9 was used for data acquisition, and Thermo Scientific™ Chromeleon™ software, version 7.2.9, was used for processing. MS/MS experiments were evaluated using SimGlycan software, version 5.0.

## Configuring the modules in Xcalibur software with SII for Xcalibur software

To configure the IC system, first close all Xcalibur programs. 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 ICS5000+ HPIC system DP pump and DC detector modules and Dionex AS-AP Autosampler.

## Creating an instrument method and running sample sequence in Xcalibur software

To create a new instrument method using the Xcalibur Instrument Set Up wizard, enter the values from the HPAE-PAD and Mass Spectrometry Conditions sections. Save the instrument method. For running the sequence open Sequence Setup wizard, and then choose appropriate instrument method.

## Sample analysis

Equilibrate the column with initial mobile phase conditions for a minimum of 15 min. Inject 10  $\mu$ L of DI water blank sample and run the gradient program at least once to equilibrate the column and system before starting glycan sample analysis.

## Results and discussion

### Setting up an initial HPAE-MS experiment

Here we have used HPAE-PAD coupled to Orbitrap mass spectrometry to test improvements in resolution. We evaluated changes to commonly used HPAE-PAD conditions to improve resolution using *N*-linked glycans released from four different glycoproteins by PNGase F. The glycoproteins used were bovine fetuin, bovine thyroglobulin, bovine fibrinogen, and human alpha-1-acid glycoprotein (AGP). We first tested HPAE-PAD separation of released *N*-linked glycans on a Dionex CarboPac PA200 column, using a typical sodium hydroxide concentration and temperature, i.e. 100 mM with a gradient of sodium acetate at 30 °C. The column effluent containing separated glycans was passed through a desalter to remove sodium ions contained in the effluent prior to mass spectrometry. A Q Exactive Orbitrap 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<sup>2</sup> spectra dominated by glycosidic and cross-ring fragments that 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<sup>2</sup> spectra.

### Overall resolution under high NaOH concentration conditions

Potential gain in neutral glycan resolution observed with increased hydroxide concentration has been reported.<sup>4,9</sup> At the same time, other studies have indicated lower hydroxide concentration to be useful for improving neutral as well as sialylated glycan resolution.<sup>6</sup> Moreover, systematic analysis of glycan structure and its effect on observed resolution has not been performed. We

have previously shown the benefit of higher hydroxide concentration and lower temperature on glycan resolution.<sup>4</sup> In continuation of that work, an effort to correlate glycan structure with elution conditions is attempted here.

Four glycoproteins that are highly sialylated were chosen for this study. This enabled availability of diverse glycan structures for the correlation. High sialylated glycan content from these proteins simplifies sample preparation. Glycosylation patterns for these glycoproteins have also been studied using HPAE-MS,<sup>8,10</sup> simplifying the identification of structures. This provides an opportunity to systematically study the effect of glycan structure on elution behavior.

Using PNGase F-released glycans for all four glycoproteins, effects of higher hydroxide concentration as well as temperature were studied on glycan separation. Figure 1 shows HPAE chromatograms for fetuin glycans under different elution conditions. Higher NaOH concentration appears to improve overall glycan resolution both at 25 °C and 35 °C. Moreover, lower temperature appears to be more favorable than the higher temperature.

### Positional and structural isomers

Next, we wanted to correlate the observed improved resolution with glycan structure. All major and detectable minor peaks were annotated for all four glycoproteins. Figure 2 shows glycan structures corresponding to major glycan peaks for fetuin. It appears that overall improvement in resolution achieved using higher NaOH concentration is attributed to improved resolution of isomeric glycan peaks. Figure 3 shows another example of improved resolution of structural isomers using higher NaOH concentration for two fibrinogen glycans.

### Fucosylated glycans

Fucosylated glycans are known to elute earlier than their non-fucosylated counterparts.<sup>5,11</sup> Here, we discover that fucosylated glycans are better resolved from their non-fucosylated counterparts at 100 mM eluent concentration as compared to 150 mM NaOH irrespective of the temperature. For example, Figures 4 and 5 show two examples of separation of a fucosylated glycan from non-fucosylated analogue for fetuin and AGP respectively. Both these pairs are not resolved using 150 mM NaOH.

[Note: Normally, fetuin does not contain fucosylated glycans, but they are sometimes present as contamination.]

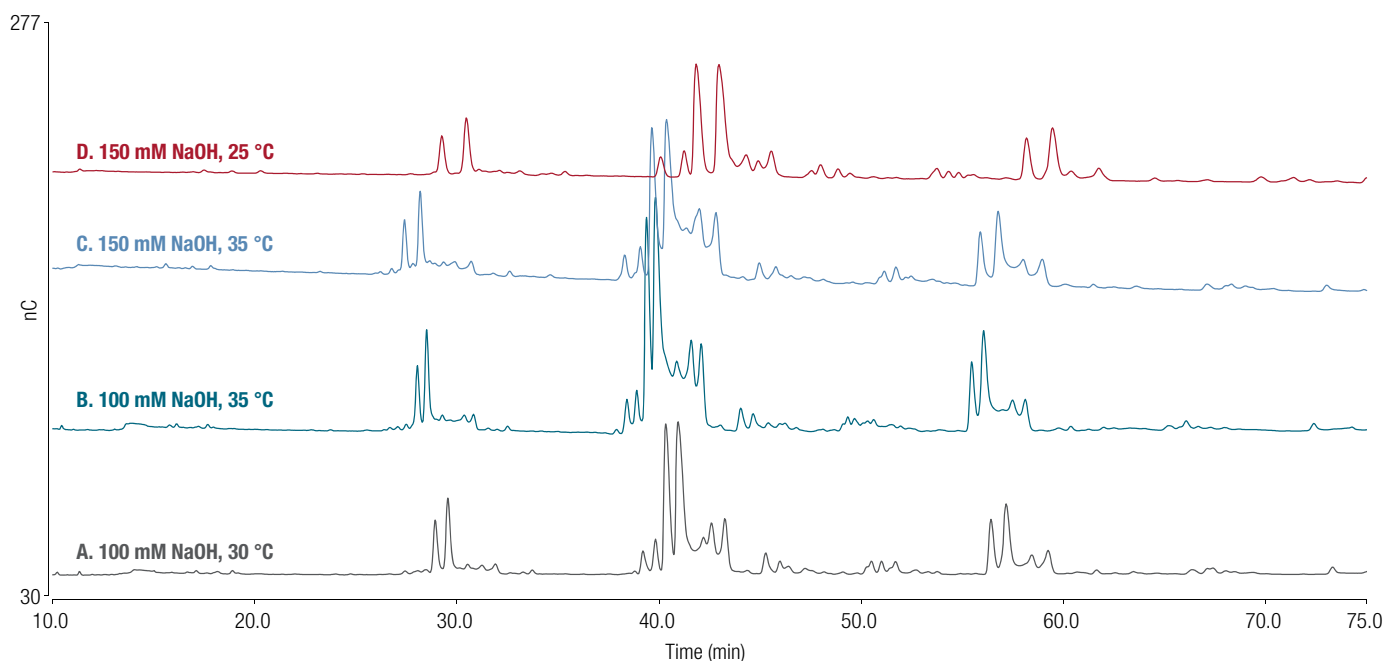
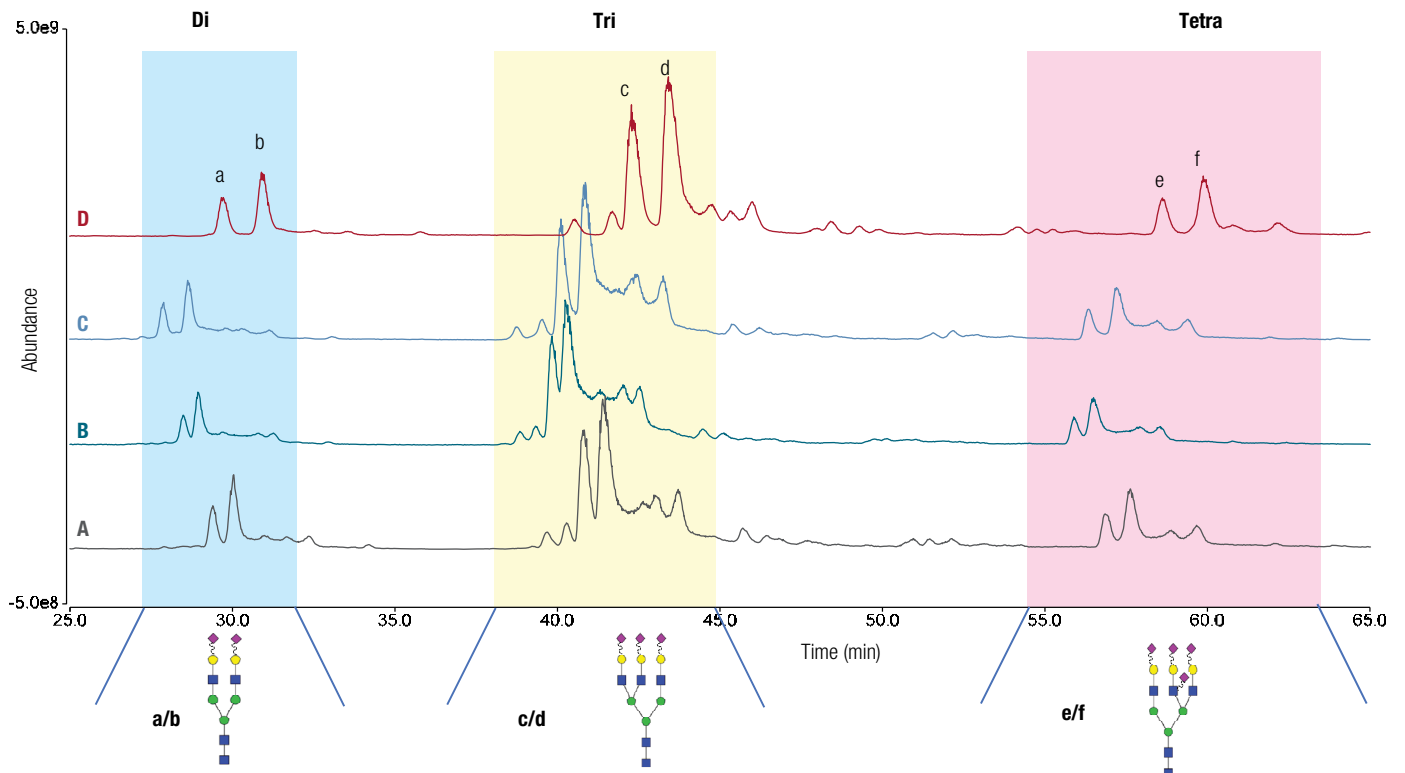


Figure 1. Separation of fetuin glycans under different elution conditions



**Glycan structure legend:**

- *N*-Acetyl glucosamine (GlcNAc)
- Galactose (Gal)
- ◇ *N*-Glycolyl neuraminic Acid (Neu5Gc)
- Mannose (Man)
- ◆ *N*-Acetyl neuraminic Acid (Neu5Ac)
- ▲ *L*-Fucose (L-Fuc)

Note: Serpentine lines linking sialic acids indicate presence of either  $\alpha 2 \rightarrow 3$  or  $\alpha 2 \rightarrow 6$  linkage

Figure 2. Extracted ion chromatogram showing resolution of major fetuin sialylated glycan structures under different elution conditions

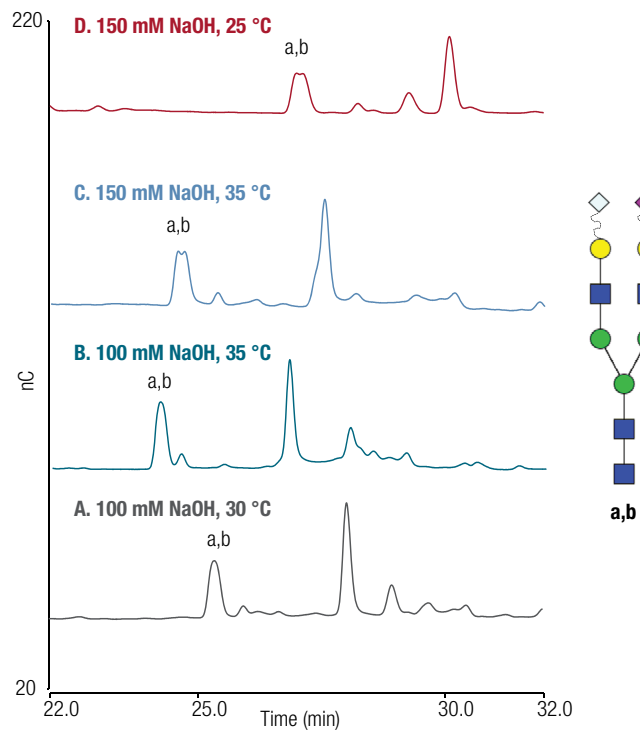


Figure 3. Resolution of a fibrinogen biantennary disialylated glycan under different elution conditions

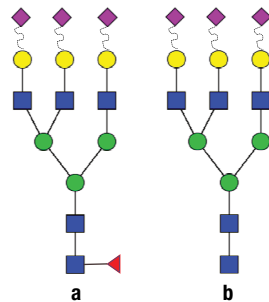
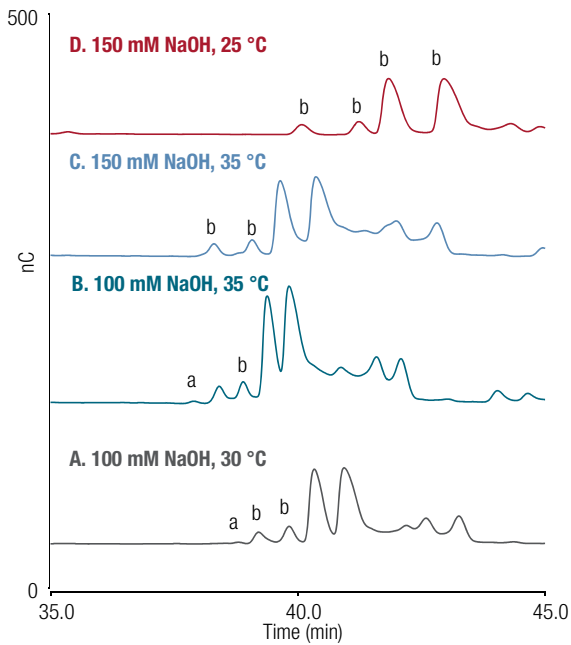


Figure 4. Separation of fucosylated and non-fucosylated fetuin glycans tested at different elution conditions

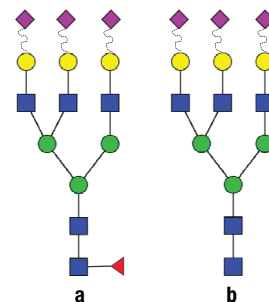
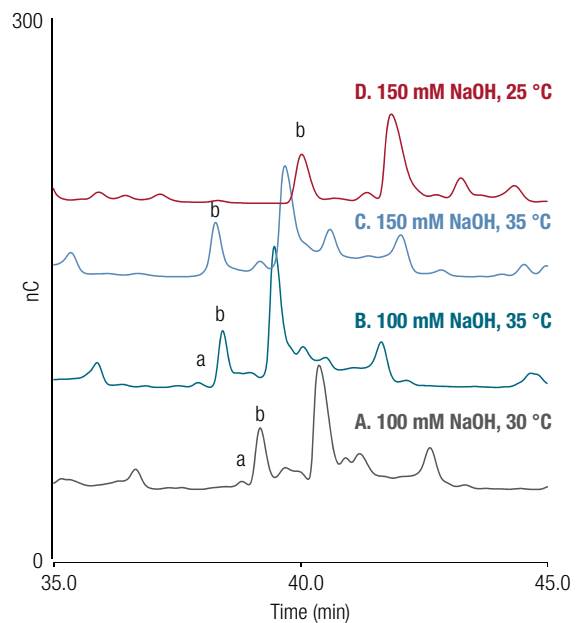


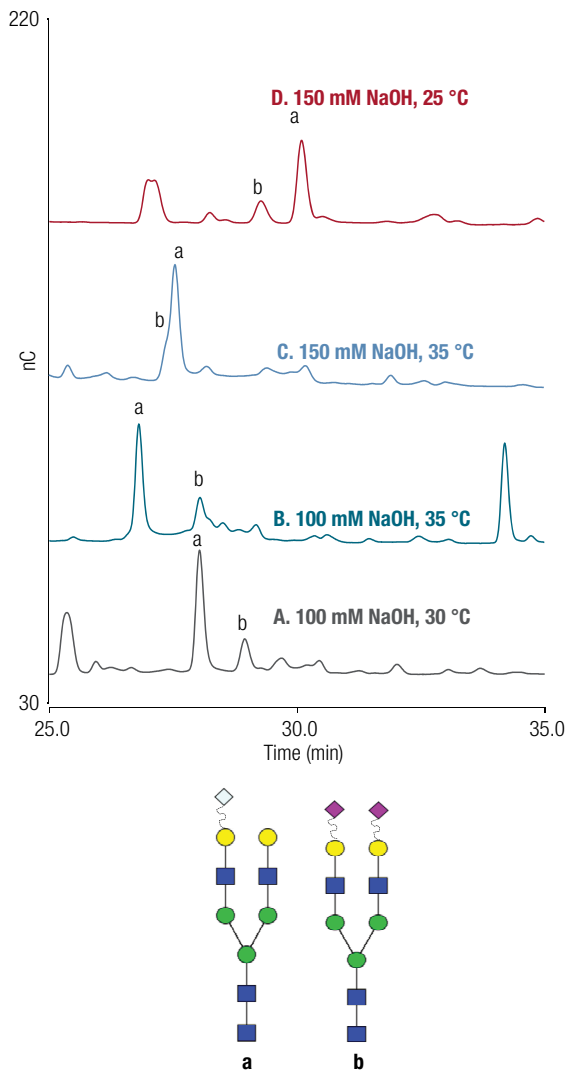
Figure 5. Separation of fucosylated and non-fucosylated AGP glycans tested at different elution conditions

### Degree of sialylation

Generally, in an HPAE separation glycans of a homologous series elute in order,<sup>11,12</sup> although exceptions to this rule have also been observed.<sup>5,12</sup> Sialylated glycans elute in the increasing order of sialylation due to increased binding<sup>11</sup> because of increased charge. So, for sialylated glycans the elution order would be, asialo < monosialylated < disialylated < trisialylated < tetrasialylated glycan, indicating increased retention with charge. Here we report that at least in some cases the elution order changes at higher sodium hydroxide, for example as shown in Figure 6, in case of fibrinogen glycans, a biantennary disialylated glycan changes

elution order under high hydroxide condition. Using 100 mM NaOH the disialylated glycan elutes before the monosialylated glycan. This observation is interesting as it is another example of exception to the homologous series elution order rule. Generally, a monosialylated glycan is expected to elute earlier than a disialylated glycan.<sup>5</sup> However, at high NaOH the elution order is reversed and a single Neu5Gc leads to increased retention compared to two Neu5Ac residues. Under typical conditions a single Neu5Gc on an oligosaccharide is more tightly bound to the column (i.e. retained longer) than the same oligosaccharide with a Neu5Ac.





**Figure 6. Change in elution order of a fibrinogen biantennary sialylated glycan pair under different elution conditions**

## Conclusion

Excellent separation of many different glycans based on their charge, isomerism, fucosylation, and sialylation was achieved using HPAE. This work presents an improved approach for further increasing coverage in glycan analysis using HPAE-PAD coupled to HRAM mass spectrometry. The effect of increased hydroxide concentration and temperature on glycan resolution

was studied. The change in hydroxide concentration and column temperature can be exploited for increasing glycan coverage. We find that higher hydroxide concentration leads to improved overall resolution resulting from improved separation of glycan isomers. Lower hydroxide concentration helps resolve certain structural features such as fucosylation.

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