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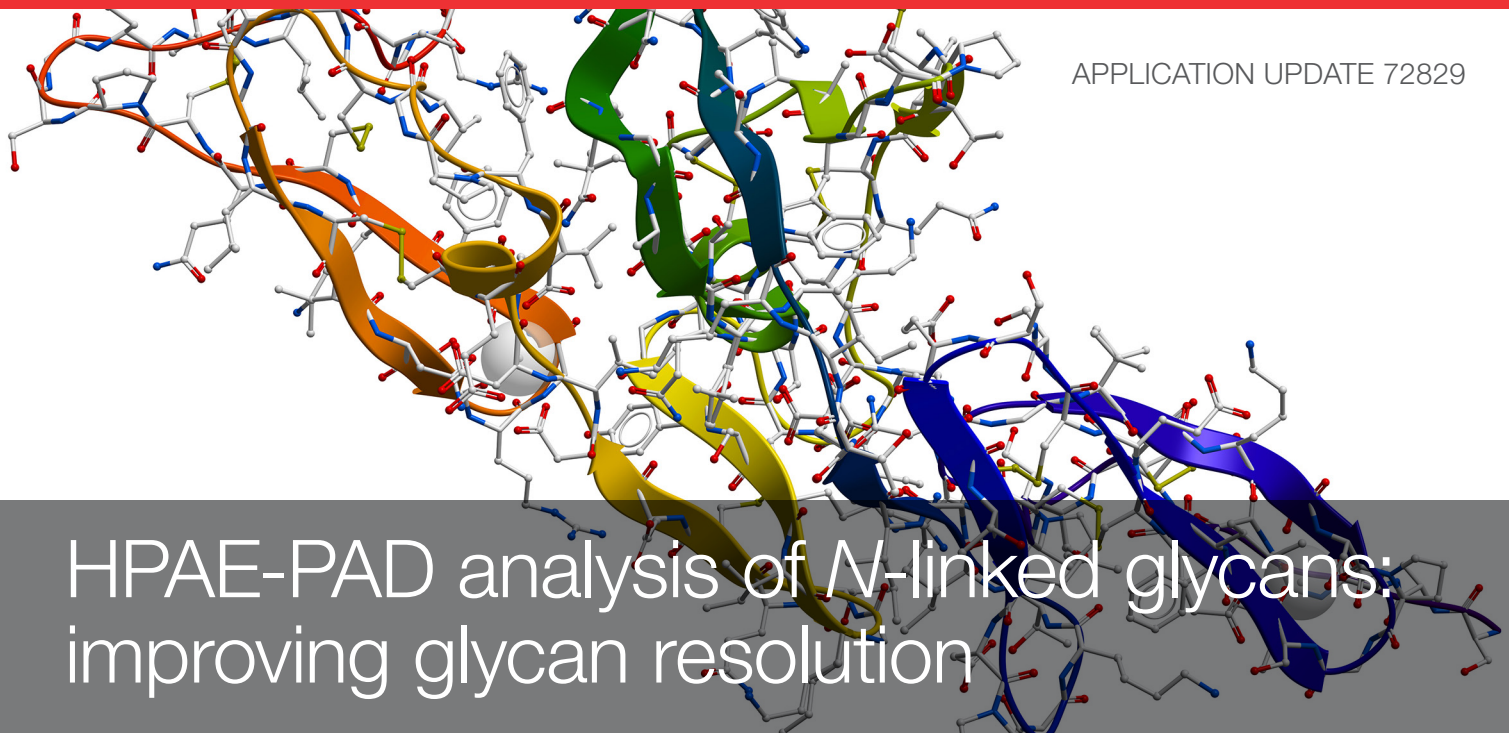
APPLICATION UPDATE 72829



HPAE-PAD analysis of *N*-linked glycans: improving glycan resolution

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Keywords

Biopharmaceuticals, Protein glycosylation, CarboPac PA200 column, HPAE-PAD, Sialylated glycans

Goal

To demonstrate conditions for improved resolution of sialylated *N*-glycans on Thermo Scientific™ Dionex™ CarboPac™ PA200 columns

Introduction

Glycosylation is an important post-translational modification[1] (PTM) that plays a key role in biological processes such as protein folding[2] and many recognition events[3]. When producing recombinant glycoprotein therapeutics, the process of protein glycosylation can be affected by various factors, including cell type, cell age, cell culture conditions, and the manufacturing process. Therefore, close product quality control of glycoprotein therapeutics is important to minimize batch-to-batch variability. This has resulted in an increased demand for methods to characterize these carbohydrates.

Profiling the *N*-linked oligosaccharides of a glycoprotein is one of the important analyses used for glycoprotein characterization. This is especially true when a glycoprotein is being produced as a human therapeutic. High-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) is one of the techniques commonly used for in-depth analyses of *N*-linked oligosaccharides in glycoproteins.[4,5] HPAE-PAD separates native carbohydrates (i.e., underivatized) at high pH (>12) and detects them by pulsed amperometry on a gold working electrode.

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This application update demonstrates improved resolution of sialylated *N*-glycans on Thermo Scientific™ Dionex™ CarboPac™ PA200 columns. Starting with a recently described method,[6] changes to elution conditions were tested to improve resolution of sialylated *N*-glycans enzymatically released from four different glycoproteins. Separations were first evaluated on the analytical format (3 × 250 mm column). Next, the possibility that a shorter column, such as a guard column, would allow significantly improved throughput was evaluated. Finally, a narrow bore format 1 × 250 mm column was tested. The use of this column not only allows reduced eluent consumption but also makes the method more amenable to mass spectrometric oligosaccharide analysis. Results from all three different formats are discussed here.

Experimental

Equipment

- A Thermo Scientific™ Dionex™ ICS-5000+ Reagent-Free™ Ion Chromatography (RFIC™) system is 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 and 0.4 µL (P/N 00110-03-0041) sample loops (Note: the 0.4 µL sample loop is internal)
- Thermo Scientific™ Dionex™ AS-AP autosampler (P/N 074926) with cooling tray option (recommended)
- 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 (Thermo Scientific P/N 164-0020)
- Vials with septum, 0.3 mL capacity (P/N 055428)

Conditions

Flow Rate:	0.5 mL/min (analytical and guard), 0.063 mL/min (1 × 250 mm format)
Injection Volume:	10 µL (analytical), 2.5 µL (guard), 0.4 µL (1 × 250 mm format)
Column Temp.:	25, 30, or 35 °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:	See Table 1
Detection:	Pulsed Amperometry (PAD), Gold on Polyester disposable working electrode (P/N 060139) with 2 mil (50.8 µm) gasket (P/N 060141), Gold on PTFE disposable working electrode (P/N 066480) using the same gasket
Columns 1:	Dionex CarboPac PA200, 3 × 250 mm analytical column (P/N 062896), Dionex CarboPac PA200 Guard 3 x 50 mm guard column (P/N 062895)
Columns 2:	Dionex CarboPac PA200, 1 × 250 mm analytical column (P/N 302861), Dionex CarboPac PA200 Guard 1 × 50 mm guard column (P/N 302862)

Table 1. Gradient program for elution using Dionex CarboPac PA200 columns*

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-equilibrate

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

Reagent 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: 10x glycoprotein denaturing buffer (5% SDS, 10% β-mercaptoethanol), 10x G7 buffer (0.5 M sodium phosphate, pH 7.5 at 25 °C), and 10% NP-40.

Preparation of solutions and standards

- Sodium hydroxide: To 900 mL of deionized (DI) water, add 5.2 mL (0.1 M) or 7.8 mL (0.15 M) of 50% (w/w) sodium hydroxide solution. Filter solution through an alkaline-resistant nylon membrane with pore size of not more than 0.45 µm and degas before use.
- Sodium acetate in sodium hydroxide: Dissolve 20.5 g (0.25 M) or 82.0 g (1 M) of anhydrous sodium acetate, in 800 mL of 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) of 50% (w/w) NaOH, and bring to volume. Degas before use.
- For additional details on mobile phase preparation refer to Thermo Scientific Technical Note 71.[7]

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 solution with 200 µL DI water (i.e., 400 µL total DI water).

Sample analysis

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

Results and discussion

A recently described 90-minute method[6] was set up and tested for separation of *N*-linked glycans. Then two different column temperatures, 25 °C and 35 °C, were tested to evaluate the impact of temperature on resolution. As shown in Figure 1, for bovine fetuin *N*-linked oligosaccharide alditols, the resolution was improved at 25 °C, whereas 35 °C resulted in lower resolution. In this experiment, we found that peak area and signal-to-noise ratio was highest at 30 °C compared to 25 °C. Next, a higher hydroxide concentration (150 mM vs. 100 mM used previously) was tested. This has been reported to improve oligosaccharide resolution,[8] which is what we observed (not shown). Lower temperature and higher sodium hydroxide concentration when tested together improved resolution (Figure 2), which allowed new peaks that were not observed previously to be identified. The new peaks are indicated by arrows.

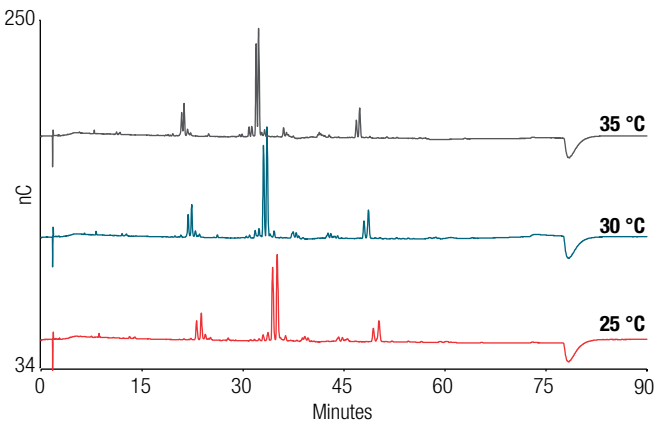

Figure 1. Fetuin alditols separated at different temperatures

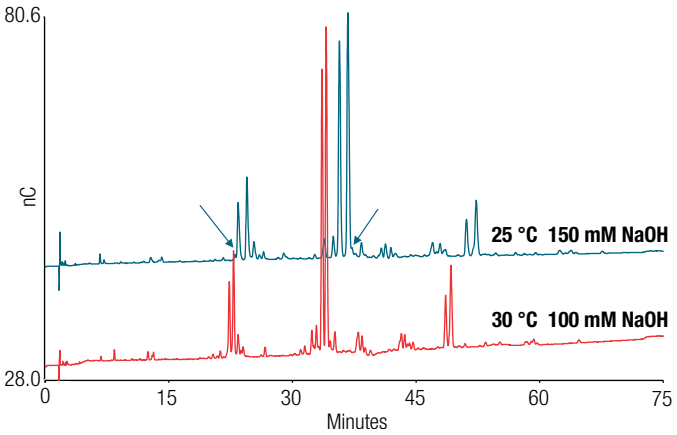

Figure 2. Fetuin alditols separated at high [NaOH]-low temperature conditions

Next, to potentially achieve reduced run time and significantly improved sample throughput, the same separations were tested on a Dionex CarboPac PA200 guard column (3 × 50 mm). As expected, with the shorter column the overall resolution is lower than that achieved with the standard-length analytical column (Figure 3). However, the chromatogram does show separation of the major glycan peaks. For the separation on this short column the high [NaOH], low temperature method yields significant resolution improvement.

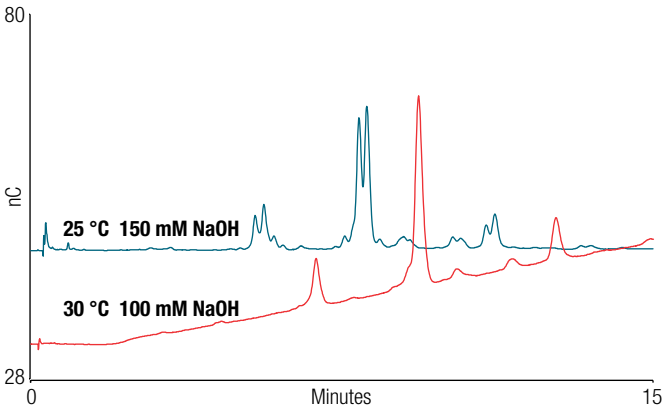

Figure 3. Fetuin alditol separation on a Dionex CarboPac PA200, 3 × 50 mm guard column at high [NaOH]-low temperature conditions

Finally, the oligosaccharide separation method designed here was tested on a Dionex CarboPac PA200 column in a 1 × 250 mm format. As shown in Figure 4, for fetuin oligosaccharide alditols, the separation on the 1 × 250 mm column runs significantly longer than the 3 × 250 mm column. It is not clear why, but the longer run time may be due to variable column geometry leading to potential higher relative capacity for the 1 × 250 mm column.

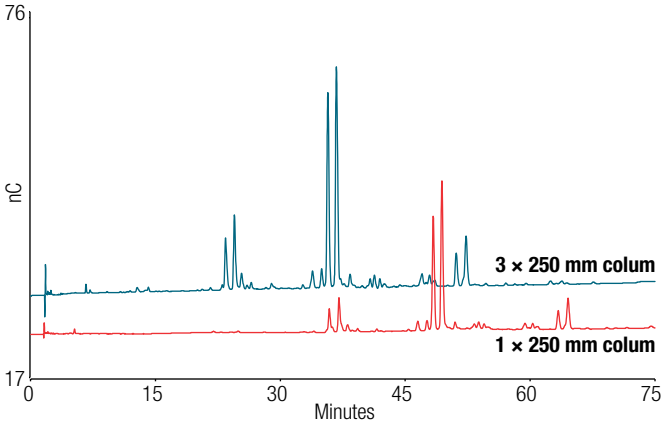

Figure 4. Comparison of fetuin alditol separation on a Dionex CarboPac PA200, 3 × 250 mm column with Dionex CarboPac PA200, 1 × 250 mm column at high [NaOH]-low temperature conditions

The high [NaOH], low temperature condition was also tested with the PA200 1 × 250 mm column. The resultant separation is improved relative to 100 mM NaOH and 30 °C column temperature conditions, but no new peaks are apparent for fetuin oligosaccharide alditols. The same separation was also tested for AGP, fibrinogen, and thyroglobulin glycans. Figures 5 through 7 show that potential new peaks can be identified for each glycoprotein under these conditions along with general resolution improvement.

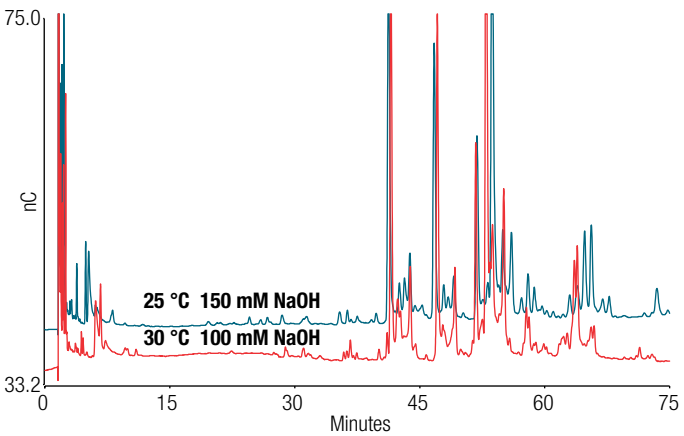

Figure 5. AGP glycan separation using high [NaOH]-low temperature conditions on a Dionex CarboPac PA200, 1 × 250 mm column

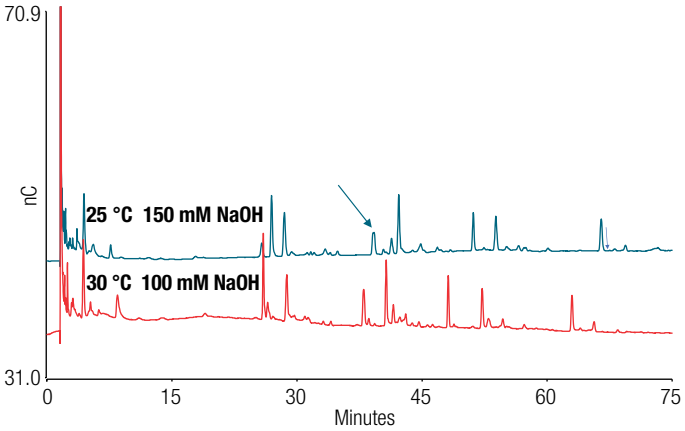

Figure 6. Fibrinogen glycan separation using high [NaOH]-low temperature conditions on a Dionex CarboPac PA200, 1 × 250 mm column

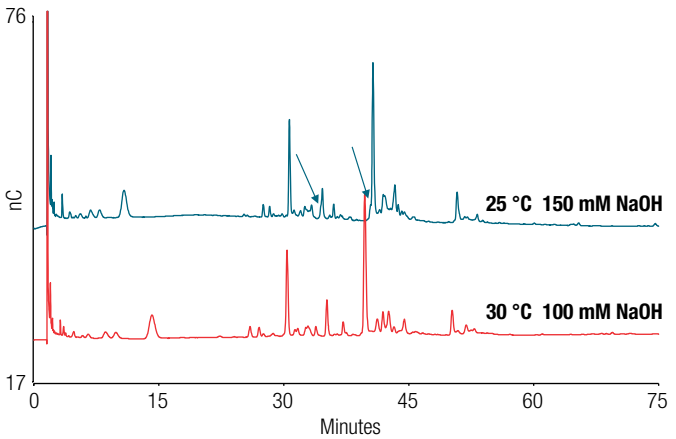

Figure 7. Thyroglobulin glycan separation using high [NaOH]-low temperature conditions on a Dionex CarboPac PA200, 1 × 250 mm column

Conclusions

Improved separation of glycoprotein sialylated *N*-glycans was achieved using a lower temperature combined with a higher sodium hydroxide concentration on Dionex CarboPac PA200 columns. The results were consistent across all three column formats and all four proteins studied here. The resolution achieved on the guard column may satisfy some application needs such as a quick check on overall oligosaccharide sialylation.

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