APPLICATION UPDATE



# Improved Separation of Aminobenzamide (2-AB)-Labeled *N*-glycans from Human α1 Acid-Glycoprotein for Analysis by HPAE-FLD

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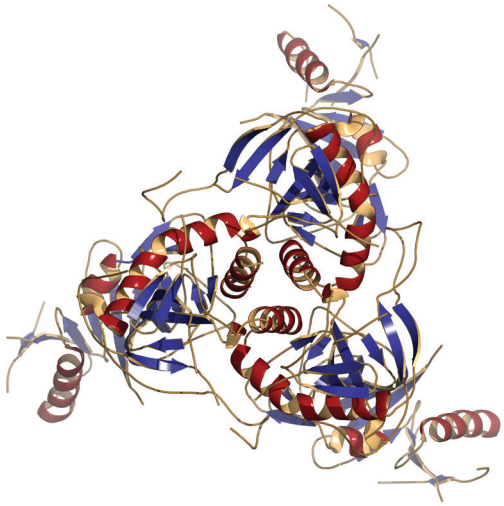

## Goal

To demonstrate improved chromatographic separation achieved using a Thermo Scientific™ Dionex™ CarboPac™ PA200 column for the HPAE-FLD method described in proposed United States Pharmacopeia (USP) General Chapter <212> to separate 2-AB-labeled *N*-linked oligosaccharides from human α1 acid-glycoprotein

## Introduction

Glycosylation is a key intracellular process that involves interactions of various enzymes and substrates.[1] Glycoproteins are central to several important biological processes.[2,3] Carbohydrates, especially those linked through an asparagine side chain (*N*-linked), play a critical role in these biological activities. The role of carbohydrates linked through serine or threonine side chains, however, is not as clear. 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 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, cell culture conditions, etc. 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.

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Thermo Scientific Application Note 1130 (AN1130)[4] demonstrated the use of high-performance anion-exchange (HPAE) chromatography combined with fluorescence detection (HPAE-FLD) for selective and sensitive analysis of *N*-linked glycans released from glycoproteins and labeled with the fluorophore 2-AB.

AN1130 used a Thermo Scientific™ Dionex™ ICS-5000+ HPIC system equipped with a fluorescence detector to perform the HPAE-FLD method described in USP General Chapter <212>[5] to analyze *N*-linked glycans from glycoprotein therapeutics. Oligosaccharides released from human α1 acid-glycoprotein and bovine fetuin, two of the proposed glycoprotein standards for <212> were labeled with a fluorophore, (2-AB), purified, and analyzed using HPAE-FLD. Two different methods for purification of the labeled oligosaccharides from unbound labeling reagent were tested along with two different elution conditions. The data showed that neither of the variations significantly affected the retention time profiles, and that the method described was suitable for routine labeling and profiling of oligosaccharides released from glycoproteins.

The method proposed in USP <212>[5] and AN1130[4] uses a Thermo Scientific™ Dionex™ CarboPac™ PA1 column for *N*-glycan separation. In this application update, the column is changed to a Thermo Scientific™ Dionex™ CarboPac™ PA200 column. 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 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. In addition, the Dionex CarboPac PA200 column requires less sodium acetate to elute a given oligosaccharide as compared to the Dionex CarboPac PA1 column. This, in part, allows for a significantly shorter method. The 3 × 250 mm format of the Dionex CarboPac PA200 column, as compared to the 4 × 250 mm format of the Dionex CarboPac PA1 column, provides fast separations that result in significant savings in eluent consumption.

## Equipment

- A Thermo Scientific™ Dionex™ ICS-5000+ Ion Chromatography system. The Dionex ICS-5000+ system is an integrated ion chromatograph that includes:
  - SP single pump module (P/N 075924) or DP Dual Pump (P/N 079975) with degas option
  - DC detector compartment (P/N 075945) with single-temperature zone
  - Electrochemical detector (P/N 072042) and cell (P/N 072044)
  - 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)
  - 25 µL sample loop
- Thermo Scientific™ Dionex™ AS-AP autosampler (P/N 074926) with cooling tray option (recommended)
- Thermo Scientific™ Dionex™ FLD-3400RS Rapid Separation Fluorescence Detector with Dual-PMT (P/N 5078.0025) with analytical flow cell, 8 µL volume (P/N 6078.4230)
- Sterile assembled micro-centrifuge tubes with screw cap, 1.5 mL (Sarstedt P/N 72.692.005)
- 1.5 mL polypropylene autosampler vials, with caps and split septa (P/N 079812)
- GlycoClean S SPE Cartridge (Prozyme P/N GKI-4726)
- Macro Spin G-10 mini SEC columns (Harvard Apparatus P/N 743900)
- Centrifuge (Eppendorf 5400 series)
- Nalgene Rapid-Flow 0.2 µm filter units, 1000 mL, nylon membrane, 90 mm diameter (P/N 164-0020)

## Conditions

| | |
|---|---|
| Columns | Dionex CarboPac PA1, analytical, 4 × 250 mm (P/N 035391) and Dionex CarboPac PA1 Guard, 4 × 50 mm (P/N 043096)<br><br>Dionex CarboPac PA200, 3 × 250 mm (P/N 062896), Dionex CarboPac PA200 Guard 3 × 50 mm (P/N 062895) |
| Flow Rate | 0.5 mL/min |
| Injection Volume | 25 µL |
| Column Temperature | 25 °C |
| Tray Temperature | 4 °C |
| Fluorescence Detector Wavelengths | excitation: 330 nm,<br>emission: 420 nm |
| Eluents | Dionex CarboPac PA1 column<br>A) Water; B) 0.5 M sodium acetate in 0.05 M sodium hydroxide; C) 0.5 M sodium hydroxide<br><br>Dionex CarboPac PA200 column<br>A) 0.1 M sodium hydroxide B) 1 M sodium acetate in 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)
- Signal™ 2-AB labeling kit (Prozyme P/N GKK-404)
- AB-labeled standards A1, A2, and A3 (Prozyme P/Ns GKSB-311, GKSB-312, GKSB-314)
- Oligosaccharide mixtures released from human $\alpha$1 acid-glycoprotein using PNGase F (Received from the USP as a part of a collaborative project)

## Preparation of Solutions and Standards

- Sodium acetate: Dissolve 41.0 g (0.5 M) or 82.0 g (1 M) of anhydrous sodium acetate in 900 mL of 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, bring to the volume with water, and degas before use.
- Sodium hydroxide: To 900 mL of water, add 5.2 mL (0.1 M) or 26 mL (0.5 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 41.0 g (0.5 M) or 82.0 g (1 M) of anhydrous sodium acetate, in 800 mL of 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 2.6 mL (0.05 M) or 5.2 mL (0.1 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[6] (TN71).

## Methods

Experiments in this work consisted of two parts:

1. **Sample Preparation:** A vial containing 20 µg of released and dried oligosaccharides as received from the USP, was used for each 2-AB labeling reaction. The labeled oligosaccharides were purified using two different methods—size exclusion chromatography (SEC) and solid phase extraction (SPE). Data obtained using SEC-purified samples are shown here. Refer to Thermo Scientific Application Note 1130[4] for the detailed sample preparation protocol.
2. **Chromatographic Analysis:** Two different elution conditions and fluorescence detection were used for separation and detection of oligosaccharides.

### Samples for chromatographic analysis:

- ***Sample solution:*** Reconstitute the dried 2-AB labeled oligosaccharides (obtained from the step of *Removal of Free 2-AB* in AN1130)[4] with 500 µL of water.

Note: Store the reconstituted sample solution at 2–8 °C, protect from light, and use within two weeks.

- ***Blank solution: Water***

Two different gradient programs were used for elution from the Dionex CarboPac PA1 and PA200 columns as shown below in Tables 1 and 2 respectively.

Note. Equilibrate the column with initial mobile phase conditions for a minimum of 15 min. Inject 25 µL of water and run the gradient program at least once to equilibrate the column and system before starting sample analysis.

**Table 1. Gradient program for elution using the Dionex CarboPac PA1 column.**

| Time (min) | Solution A (%) | Solution B (%) | Solution C (%) | Elution |
|---|---|---|---|---|
| 0 | 80 | 10 | 10 | Initial condition |
| 15 | 80 | 10 | 10 | 50 to 150 mM NaOAc, isocratic 0.05 M NaOH |
| 70 | 62 | 30 | 8 | 150 to 450 mM NaOAc, isocratic 0.05 M NaOH |
| 94 | 8.9 | 90 | 1.1 | NaOAc wash, no gradient |
| 99 | 8.9 | 90 | 1.1 | |
| 105 | 0 | 10 | 90 | NaOH wash, no gradient |
| 110 | 0 | 10 | 90 | |
| 111 | 80 | 10 | 10 | Re-equilibration |
| 130 | 80 | 10 | 10 | |

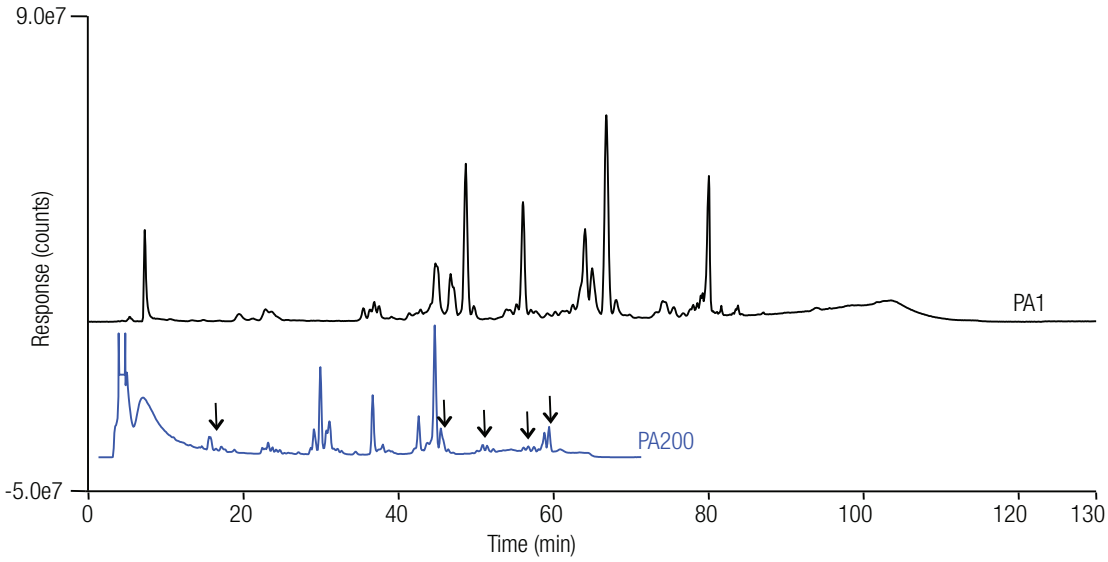
**Table 2. Gradient program for elution using the Dionex CarboPac PA200 column.**

| Time (min) | Solution A (%) | Solution B (%) | Elution |
|---|---|---|---|
| 0 | 98 | 2 | Initial condition |
| 15 | 85 | 15 | 20 to 150 mM NaOAc, isocratic 0.1 M NaOH |
| 60 | 85 | 15 | 150 mM NaOAc, isocratic 0.1 M NaOH |
| 60.001 | 98 | 2 | Re-equilibrium |
| 70 | 98 | 2 | |

## Results

A comparison of elution behavior of 2-AB labeled oligosaccharides from human α1 acid glycoprotein was made using two different columns, the Dionex CarboPac PA1 and the Dionex CarboPac PA200 columns. Figure 1 shows the separation of 2-AB labeled *N*-linked oligosaccharides released from human α1 acid glycoprotein separated on both columns.

The higher resolution and better peak efficiencies of the Dionex CarboPac PA200 column are apparent in these chromatograms. All the areas where a notable gain in resolution is observed using the Dionex CarboPac PA200 column are highlighted by arrows. In addition to improved resolution, the method using the Dionex CarboPac PA200 column requires significantly less time, improving process economics.




| | | | |
|---|---|---|---|
| Columns: | CarboPac PA1, analytical, 4 × 250 mm | Column Temperature: | 25 °C |
| | CarboPac PA1, guard, 4 × 50 mm | Tray Temp: | 4 °C |
| | CarboPac PA200, analytical, 3 × 150 mm | Detector : | Fluorescence |
| | CarboPac PA200, guard, 3 × 50 mm | Detector Settings: | Ex: 330 nm Em: 420 nm |
| Flow Rate: | 0.5 mL/min | | |
| Inj. Volume: | 25 µL | | |

**Figure 1. Comparison of human α1 acid glycoprotein oligosaccharide analysis on Dionex CarboPac PA1 and PA200 columns. Areas of improved resolution achieved with CarboPac PA200 are highlighted. (Note: Two different sample preparations were used for both chromatograms shown here. Hence, even if the injection volumes are the same, the amounts of oligosaccharides injected onto each column is different).**

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## Conclusion

The Dionex ICS-5000+ system, equipped with a fluorescence detector and the Dionex CarboPac stationary phases, is a powerful tool for the analysis of 2-AB-labeled glycans. Using this configuration, 2-AB-labeled oligosaccharides were separated using two different columns, the Dionex CarboPac PA1 and Dionex CarboPac PA200 columns.

The Dionex CarboPac PA200 column, due to its smaller particle size as compared to PA1 column, delivers more efficient peaks and hence offers better oligosaccharide resolution and shorter run times.

The improved resolution is a significant advantage when performing oligosaccharide fingerprinting. The shorter run times make the method convenient and economical. Moreover, less waste is generated using this method. The results indicate that the Dionex CarboPac PA200 column is a valuable tool for the separation of 2-AB-labeled *N*-linked oligosaccharides using HPAE with either PAD or fluorescence detection.

## References


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## Acknowledgements


The original method, using the Dionex CarboPac PA1 column, as well as the released oligosaccharide samples and the 2-AB-labeled standards used in this work, were provided by the USP as a part of the collaborative study.


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