HPAE-FLD Method for Separation of 2-Aminobenzamide (2-AB)-Labeled Oligosaccharides from Human α1 Acid-Glycoprotein and Bovine Fetuin

Sachin Patil, John Guajardo, and Jeff Rohrer
Thermo Fisher Scientific, Sunnyvale, CA

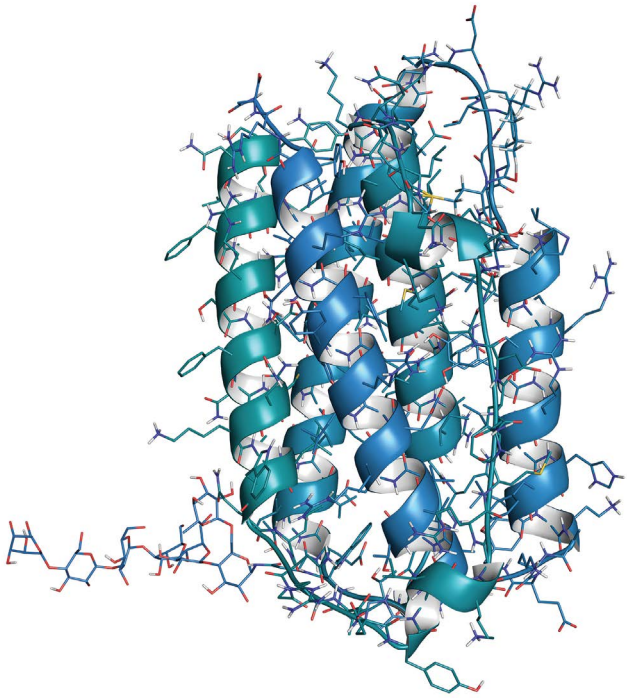
Application Note 1130

Key Words

N-linked Glycans, Carbohydrates, USP General Chapter <212>, High-performance Anion-exchange, Pulsed Amperometric Detection, Dionex ICS-5000+ System, Dionex CarboPac Column

Goal

To demonstrate the HPAE-FLD method described in the proposed USP General Chapter <212> to separate 2-AB-labeled *N*-linked oligosaccharides from human α1 acid-glycoprotein and bovine fetuin.



Introduction

Glycosylation plays an important role in protein structure and function. It is a post-translational modification (PTM) that requires interaction of various enzymes and substrates.[1] Glycoproteins play a key role in important biological processes such as protein folding[2] and a large number of recognition events.[3] Today it is understood that the carbohydrates, especially those that are 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 is less clearly understood. Consistent with their biological significance, an increasing number of glycoprotein therapeutics are being developed to target various pathological conditions.

Protein glycosylation is affected by various factors, including cell type, cell age, cell culture conditions, etc. It can also be affected by the manufacturing process. These factors can lead to significant batch-to-batch variability, and therefore close product quality control of glycoprotein therapeutics is important. This has resulted in an increased demand for methods to characterize these carbohydrates.

High-performance anion-exchange (HPAE) chromatography is used to separate anionic analytes such as carbohydrates that are or can be ionized at high pH values (> pH 12). Therefore, HPAE uses hydroxide-based eluents at high pH to produce anions from analytes that would not be anionic at neutral pH. HPAE combined with fluorescence detection (HPAE-FLD) offers an effective tool for selective and sensitive analysis of *N*-linked glycans released from glycoprotein therapeutics and labeled with the fluorophore 2-AB. In the work presented here, an HPAE-FLD method for analyzing *N*-linked glycans that is described in the proposed USP General Chapter <212>[4] is used to separate the 2-AB-labeled *N*-linked glycans from human α1 acid-glycoprotein and bovine fetuin. This method is easily executed on a Thermo Scientific™ Dionex™ ICS-5000+ system with a FLD detector. If the Dionex ICS-5000+ system is equipped with the dual pump and an electrochemical detector (ED), one half of the system can be used for this method while the other half can be used for traditional HPAE-PAD, including separation of the unlabeled released glycans, monosaccharide analysis, and sialic acid analysis. HPAE combined with pulsed amperometric detection (HPAE-PAD) is widely used for carbohydrate analysis.[5]

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Here, oligosaccharides released from two glycoproteins are labeled with a fluorophore followed by their purification and HPAE-FLD analysis. Two different methods for purification of the labeled oligosaccharides from unbound labeling reagent were tested along with two different elution conditions. The data show that the variations tested here do not significantly affect the retention time profiles and that the method described here is suitable for routine labeling and profiling of oligosaccharides released from glycoproteins.

Equipment

- A Dionex ICS-5000+ Reagent-Free Ion Chromatography (RFIC) system was used in this work. The Dionex ICS-5000+ system is a modular 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
  - Thermo Scientific™ Dionex™ UltiMate™ 3000 RS fluorescence detector (P/N 5078.0025) with analytical flow cell, 8 µL volume (P/N 6078.4230)
  - 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)*
  - Thermo Scientific Dionex AS-AP autosampler (P/N 074926) with cooling tray option (recommended)
  - 25 µL sample loop
- Sterile assembled micro-centrifuge tubes with screw cap, 1.5 mL (Sarsted P/N 72.692.005)
- 1.5 mL polypropylene autosampler vials, with caps and split septa (Dionex P/N 061696)
- Glyko 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 (Thermo Scientific P/N 164-0020)

*Not required for the method described in this application note, but needed if the system will run HPAE-PAD.

Conditions	
Columns:	Thermo Scientific™ Dionex™ CarboPac™ PA1, analytical, 4 × 250 mm (Dionex P/N 035391) and guard, 4 × 50 mm (Dionex P/N 043096)
Flow Rate:	0.5 mL/min
Injection Volume:	25 µL
Column Temperature:	25 °C
Tray Temp:	4 °C
Fluorescence Detector Wavelengths:	330 nm excitation wavelength and 420 nm emission wavelength
Eluents:	Unmodified conditions: A: Water; B: 0.5 M Sodium acetate and C: 0.5 M Sodium hydroxide
	Modified conditions: A: Water; B: 0.5 M Sodium acetate and 0.05 M Sodium hydroxide; and C: 0.5 M Sodium hydroxide

Reagent and Standards

- Sodium acetate, anhydrous, electrochemical grade (Dionex 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/N GKSB-311, GKSB-312, and GKSB-314)
- Oligosaccharide mixtures released from bovine human α1 acid-glycoprotein and fetuin using PNGase F (Received from the USP as a part of a collaborative project)

Preparation of Solutions and Standards

- 0.5 M Sodium acetate: Dissolve 41.0 g of sodium acetate anhydrous 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.
- 0.5 N Sodium hydroxide: To 900 mL of water, add 26 mL of 50% (w/w) sodium hydroxide solution. Filter solution through an alkaline-resistant nylon membrane with pore size of NMT 0.45 µm and degas before use.
- 0.5 M Sodium acetate and 0.05 N sodium hydroxide: Dissolve 41.0 g 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 of 50% (w/w) NaOH, and bring to volume. Degas before use.
- For additional details on mobile phase preparation refer to TN 71.[6]

Methods

The experiments in this work consisted of two different parts:

1. **Sample Preparation:** Detailed labeling and labeled oligosaccharide purification using two different methods-size exclusion chromatography (SEC) and solid phase extraction (SPE)
2. **Chromatographic Analysis:** Using two different elution conditions and fluorescence detection

1. Sample Preparation

Detailed protocol for 2-AB labeling of oligosaccharides is described below.

a. Sample labeling: Oligosaccharides released from the proteins used in this study were labeled using a Signal 2-AB labeling kit (Prozyme).

Reagents needed: Glacial acetic acid, dimethyl sulfoxide (DMSO), 2-aminobenzamide (2-AB), sodium cyanoborohydride

Procedure:

- Prepare the labeling solution by following the instructions accompanying the labeling kit.
- Add 10 µL of labeling solution to the bottom of the vials containing oligosaccharides and mix well.
- Immediately incubate at 37 °C for 16–18 h or at 65 °C for 3 h.
- Allow to cool at room temperature for 10 min.

b. Removal of free 2-AB:

Note: One labeled sample is purified by Method 1 and the other sample is purified by Method 2.

Method 1: Using size exclusion chromatography (SEC) spin column:

Procedure:

- Prepare suitable G-10 microcentrifuge spin columns by tapping the column gently to ensure the dry resin is settled at the bottom.
- Remove the caps and place the column in a 2-mL collection tube. Add 0.5 mL of water to the resin and let it swell for at least 15 min. Longer swelling time (up to 24 h) is acceptable, if maintained at 2–8 °C.
- Centrifuge the column at maximum speed for 5–10 s. Remove the water from the collection tube.
- Wash the resin by adding 0.5 mL of water and centrifuge the column.
- Repeat the washing step one more time.
- After removing the water from the final wash step, spin at maximum speed for 10 s to remove residual water from the resin. Residual water is sufficiently removed if the resin appears white.
- Place the column in a new, labeled collection tube.
- Add 100 µL of water to the *2-AB-labeled oligosaccharides*, and then apply the entire sample to the center of the washed G-10 column.
- Place the G-10 column in the microcentrifuge and spin at approximately 200 × g for 1 min.
- Apply the flow-through (approximately 90 µL to 120 µL) to the center of the second, un-used but previously washed G-10 column and spin at approximately 200 × g for 1 min.
- Transfer the repeat flow-through (approximately 60 µL to 100 µL) to a 0.5-mL micro-centrifuge tube.
- Dry the eluates by centrifugal evaporation with no heat.

Method 2: Using solid phase extraction (SPE) cartridge

Note: If choosing to use the commercial kit, please follow the instruction accompanying the kit to remove free 2-AB.

Reagents needed: 30% (v/v) acetic acid solution, acetonitrile, 96% (v/v) acetonitrile

Procedure:

- Prepare suitable SPE cartridges by washing with 1.0 mL of water, followed by 5 x 1.0 mL of 30% (v/v) acetic acid solution, and then 1.0 mL of acetonitrile. (Note: Allow each wash to drain completely before adding the next.)
- Apply the oligosaccharide solutions to the center surface of the cartridge disc.

  Note: Make sure the disc is still wet with acetonitrile. If the disc has dried out, it can be re-wetted by washing with 0.5 mL of acetonitrile.
- Allow the solution to incubate on the disc for 15–20 min.

  Note: For maximal recovery, each sample vial can be rinsed with 100 µL of acetonitrile and the rinse applied to the corresponding cartridge disc.
- Wash each disc with 1 mL of acetonitrile, followed by 6 × 1.0 mL of 96% (v/v) acetonitrile, allowing each aliquot to drain before the next is applied. Each wash is discarded.
- Elute 2-AB-labeled sample with 3 × 0.5 mL of water, allowing each aliquot to drain before the next is applied.
- Dry the eluates by centrifugal evaporation with no heat.

c. Samples for chromatographic analysis:

Sample solutions: Reconstitute the dried *2-AB-labeled oligosaccharides* (obtained from the step of Removal of free 2-AB) with 500 µL of water.

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

Standard solutions: Reconstitute 2-AB-labeled A1, A2, and A3 glycans with 100 µL of water, respectively. Store the reconstituted *Standard solutions* at 2–8 °C, protect from light and use within two weeks.

Blank solution: Water

2. Chromatographic Analysis

Two different elution methods were tested. The gradient time programs used were as shown below in Tables 1 and 2.

Table 1. Gradient elution using unmodified conditions.

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 N NaOH
70	60	30	10	150 to 450 mM NaOAc, isocratic 0.05 N NaOH
94	0	90	10	NaOAc wash, no gradient
99	0	90	10	
105	0	10	90	NaOH wash, no gradient
110	0	10	90	
111	80	10	10	Re-equilibrium
130	80	10	10	

Table 2. Gradient elution using modified conditions.

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 N NaOH
70	62	30	8	150 to 450 mM NaOAc, isocratic 0.05 N 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-equilibrium
130	80	10	10	

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 the system.

- Analyze two preparations of each *Sample solution* (two injections for each preparation). Integrate peaks in the resulting chromatogram. A base line is drawn from the first to the last peaks eluting within a peak group.
- Identify peaks corresponding to S1, S2, S3, and S4 in the chromatogram from the *Sample solutions* by referencing the retention times of the Standards A1, A2, and A3 and provided example chromatogram.
- Report retention times and average retention times of S1, S2, S3, and S4 peaks from *Sample solutions*.
- Report the relative retention times (RRT) and average RRT, with respect to S4 from *Sample solutions*.

Results and Discussion

This work describes a method for qualitative analysis of protein glycosylation through profiling of glycans released through enzymatic hydrolysis. The method involves labeling of the released oligosaccharides with a fluorescence dye, 2-AB, followed by chromatographic analysis using HPAE-FLD. The goal of this work is to demonstrate that the procedural variations tested in these two steps do not significantly affect the elution behavior of labeled oligosaccharides. The labeled oligosaccharides are purified from the unbound dye using either of two methods: SEC or SPE. It needs to be shown that the two methods used for purification of labeled oligosaccharides from unbound labeling reagent do not change their elution profile.

After purification of the labeled oligosaccharides from the unbound labeling reagent, the AB-labeled oligosaccharides can be easily separated using a Thermo Scientific™ Dionex™ CarboPac™ PA1 column. Here, the oligosaccharides are separated using two different gradient elution methods. The first "unmodified" method uses the gradient program shown in Table 1. Figure 1A and 1B show chromatograms obtained using this method for analysis of oligosaccharides derived from both the proteins used in this study, i.e. human α1 acid-glycoprotein and bovine fetuin. Figure 1C shows separation of three commercial AB-labeled standards analyzed using the unmodified method. The second "modified" elution method involves slight modification of sodium acetate solution used in the gradient. This solution contains 0.05 N sodium hydroxide, which is not present in the unmodified conditions. This modification is introduced because sodium acetate without sodium hydroxide in eluent can cause microbial contamination of the system.[6] Table 2 shows the gradient program used for the modified elution. In theory, this very minor change should not significantly impact the elution of *N*-glycans, but lab work is still needed to confirm that the glycan elution profile will not change with such modification. Figures 2A and 2B show the separation of AB-labeled oligosaccharides from both proteins using modified elution. Figure 2C shows separation of three commercial AB-labeled standards using modified method.

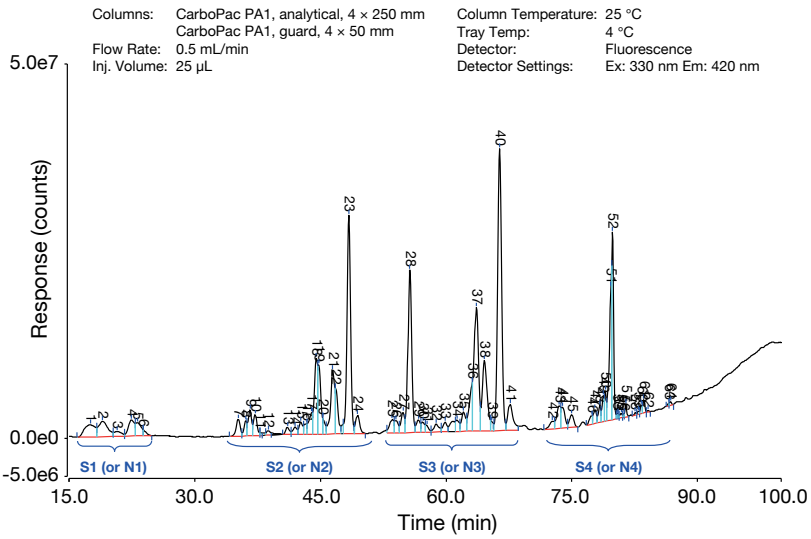

Figure 1A. AB-labeled oligosaccharides from human α1 acid-glycoprotein analyzed using unmodified elution.

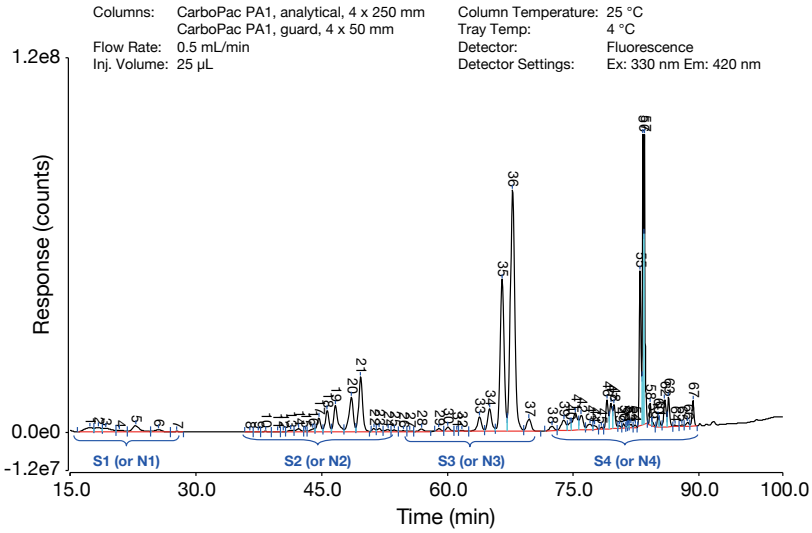

Figure 1B. AB-labeled oligosaccharides from bovine fetuin analyzed using unmodified elution.

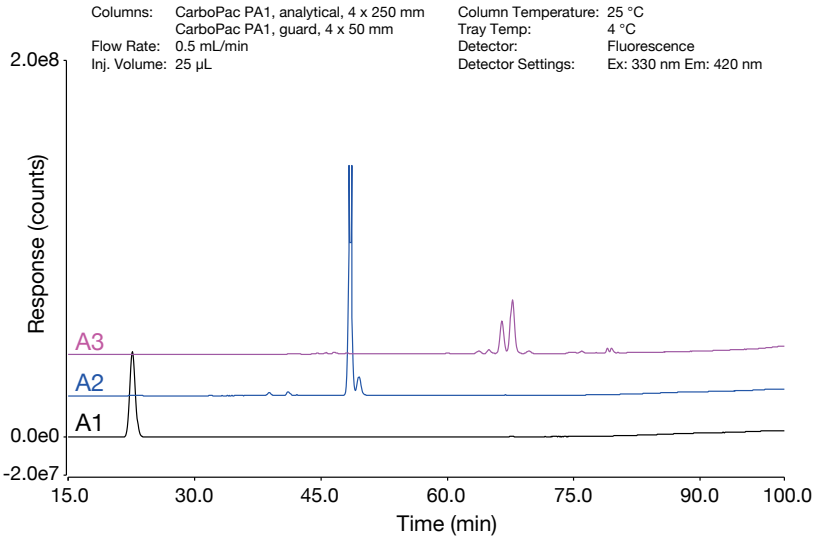

Figure 1C. AB-labeled A1, A2, and A3 oligosaccharides standards analyzed using unmodified elution.

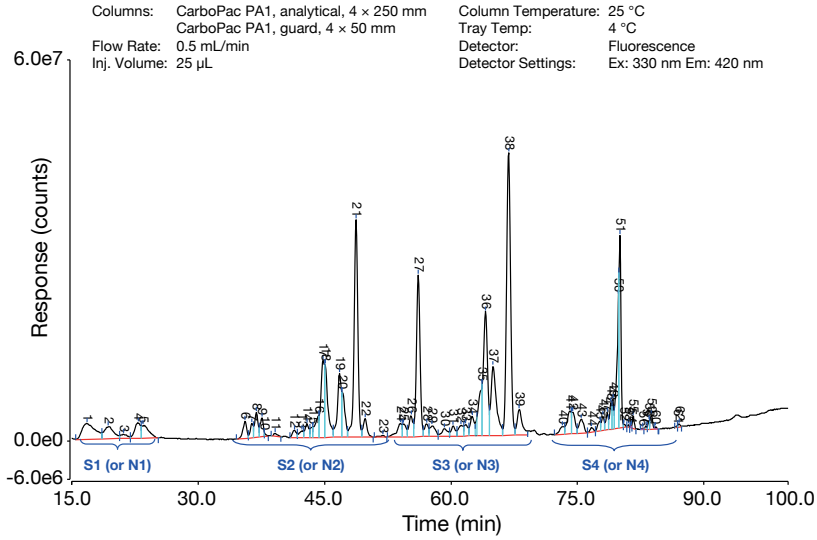

Figure 2A. AB-labeled oligosaccharides from human α1 acid-glycoprotein analyzed using modified elution.

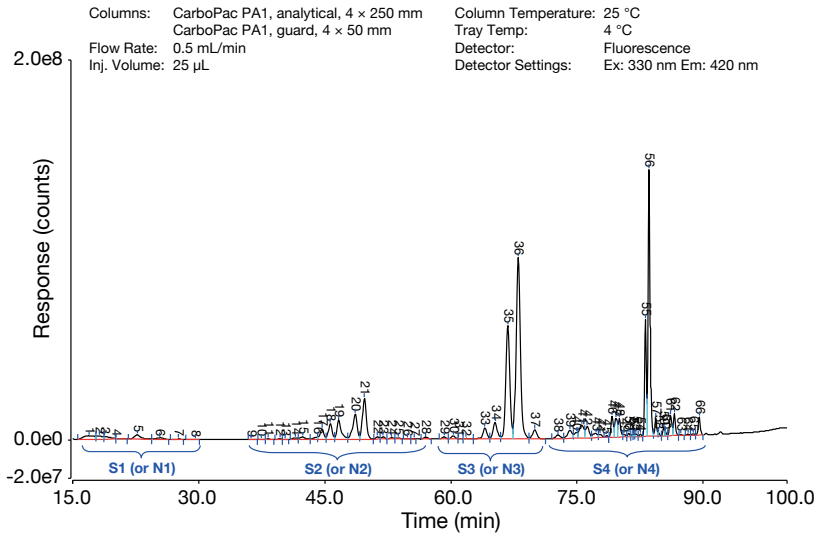

Figure 2B. AB-labeled oligosaccharides from bovine fetuin analyzed using modified elution.

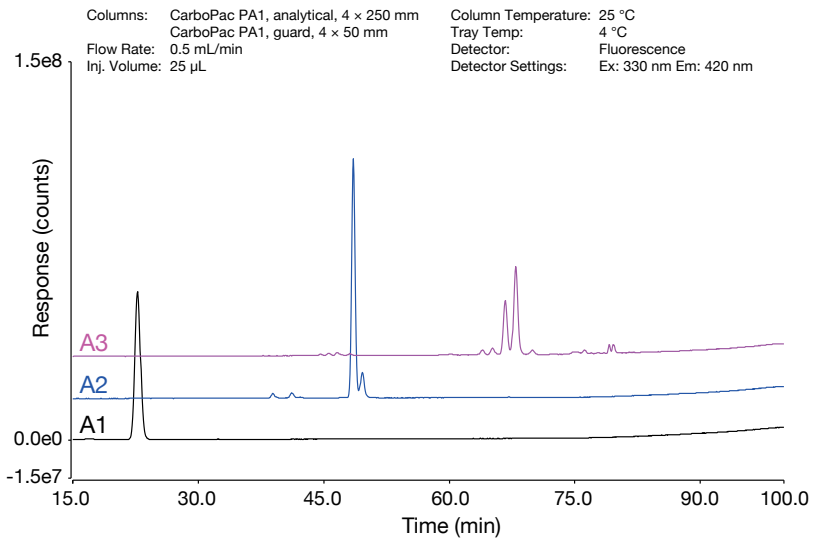

Figure 2C. AB-labeled A1, A2, and A3 oligosaccharides standards analyzed using modified elution.

The peak groups corresponding to S1, S2, S3, and S4 (mono-sialylated, di-sialylated, tri-sialylated and tetra sialylated, respectively) *N*-glycans were determined using the retention times of the AB-labeled standards. Then, the average retention time and average relative retention against S4 *N*-glycans present in the samples were calculated. Retention time data contained in Tables 3 and 4, for the oligosaccharides derived from both the proteins used in this work, shows only minor difference between the two purification methods as well as the elution methods. This result confirms that both the method variations tested in this work do not significantly affect the elution behavior of the oligosaccharides.

Table 3. Average and relative retention times for 2-AB labeled *N*-glycans in oligosaccharides released from human α1 acid-glycoprotein under unmodified as well as modified elution conditions.

N-glycans in Sample	Average Retention Time (min)				Average Relative Retention Time			
	Unmodified Elution		Modified Elution		Unmodified Elution		Modified Elution	
	GlycoClean SPE Cartridge	G10 Spin Column	GlycoClean SPE Cartridge	G10 Spin Column	GlycoClean SPE Cartridge	G10 Spin Column	GlycoClean SPE Cartridge	G10 Spin Column
S1	22.17	21.13	20.84	20.70	0.27	0.26	0.26	0.25
S2	42.35	42.25	42.30	42.35	0.52	0.52	0.52	0.52
S3	60.26	60.17	59.91	59.94	0.75	0.75	0.74	0.74
S4	80.02	80.02	80.09	80.09	1.00	1.00	1.00	1.00

Table 4. Average and relative retention times for 2-AB labeled *N*-glycans in oligosaccharides released from bovine fetuin under unmodified as well as modified elution conditions.

N-glycans in Sample	Average Retention Time (min)				Average Relative Retention Time			
	Unmodified Elution		Modified Elution		Unmodified Elution		Modified Elution	
	GlycoClean SPE Cartridge	G10 Spin Column	GlycoClean SPE Cartridge	G10 Spin Column	GlycoClean SPE Cartridge	G10 Spin Column	GlycoClean SPE Cartridge	G10 Spin Column
S1	21.32	21.68	23.06	22.39	0.26	0.26	0.28	0.27
S2	46.29	46.4	46.45	46.6	0.57	0.57	0.57	0.57
S3	63.04	63.11	63.23	63.28	0.77	0.78	0.78	0.77
S4	81.12	80.89	81.0	81.30	1.00	1.00	1.00	1.00

Conclusion

The Dionex ICS-5000+ system hyphenated with the FLD-3400RS fluorescence detector is a powerful tool for the analysis of 2-AB-labeled glycans with Dionex CarboPac column stationary phases. Using this configuration, separation of 2-AB-labeled oligosaccharides was tested with two variations to the method. First, separation of labeled oligosaccharides from unbound labeling reagent before chromatographic analysis was achieved using SEC as well as SPE methods and no major effect on retention profiles was observed. Second, elution using two different methods resulted in similar retention times and relative retention time profiles as well. This indicates that both the methods used here are equally suitable for analyzing the 2-AB-labeled oligosaccharides.

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Africa +43 1 333 50 34 0
Australia +61 3 9757 4300
Austria +43 810 282 206
Belgium +32 53 73 42 41
Brazil +55 11 3731 5140
Canada +1 800 530 8447
China 800 810 5118 (free call domestic) 400 650 5118
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