Guidelines for Successful Use of Thermo Scientific Dionex AminoTrap Columns

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Introduction

The recommended technique to determine the monosaccharide content of a glycoprotein is to hydrolyze it in acid (e.g., 2 N trifluoroacetic acid [TFA]) to break the glycoprotein's oligosaccharide into monosaccharides and also to release the oligosaccharide from the protein. In addition to producing monosaccharides, this procedure produces amino acids and small peptides, which can interfere with monosaccharide quantification by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD).[1] Using HPAE-PAD, the monosaccharides can be separated on a strong anion-exchange column with a hydroxide eluent and then detected on a gold on polytetrafluoroethylene (PTFE) disposable electrode.

The interference is due to the detection of the amino acids and peptides by PAD on the gold on PTFE disposable electrode. Because the detection potential sequence (waveform) is optimized for carbohydrates, the amino acids and peptides are detected—although with ~100-fold less sensitivity—and their oxidation products are inefficiently removed from the working electrode surface. This inefficient removal leads to inhibition of detector response for monosaccharides.

To eliminate this problem, the Thermo Scientific™ Dionex™ AminoTrap™ column was developed.[2] The Dionex AminoTrap column is an in-line pretreatment column that replaces the Thermo Scientific Dionex CarboPac™ PA10/PA20 Guard columns in HPAE-PAD monosaccharide analysis. The Dionex AminoTrap column delays the elution of amino acids and small peptides so that they elute from the Dionex CarboPac PA10/PA20 Analytical carbohydrate columns after all the monosaccharides have eluted; hence there is no interference for monosaccharide quantification of glycoprotein hydrolysates or other amino acid-containing samples.[3,4] The amino acids and small peptides elute during the column wash (regeneration) step at a higher base concentration and are completely removed from the working electrode surface before the next sample injection.

The 4 × 50 mm Dionex AminoTrap column is recommended for use with the Dionex CarboPac PA10 Analytical column. The 3 × 30 mm Dionex AminoTrap column is recommended for use with the Dionex CarboPac PA20 Analytical column. The addition of the Dionex AminoTrap columns results in slight peak broadening and longer retention times, compared to those obtained with the Dionex CarboPac PA10/PA20 Guard/Analytical column sets.

A failing or damaged Dionex AminoTrap column can cause issues with HPAE-PAD monosaccharide analysis that may be misdiagnosed as a failing Dionex CarboPac Analytical column or failing PAD detection. This work—intended as a guide for troubleshooting in such instances—provides guidelines for typical Dionex AminoTrap column performance as well as tests to assess that performance.

Goal

To develop testing guidelines for Dionex AminoTrap columns that enable diagnosis of failing or damaged columns

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Equipment

- Thermo Scientific Dionex ICS-5000 system,* including:
  - SP Single Pump or DP Dual Pump, Gradient or Isocratic, with the EG40/EG50 Vacuum Degas Conversion Kit (P/N 055431) option installed
  - EG Eluent Generator
  - Potassium Hydroxide Eluent Generator Cartridge (EGC III KOH) (P/N 074532)
  - DC Detector/Chromatography Compartment
  - ED Electrochemical Detector, without cell (P/N 072042)
  - ED Cell—no reference electrode or working electrode (P/N 072044)
  - Gold on PTFE Disposable Electrode (P/N 066480)
  - pH, Ag/AgCl Reference Electrode (P/N 061879)
- Thermo Scientific Dionex AS or AS-AP Autosampler
- Thermo Scientific Dionex Chromeleon™ Chromatography Data System (CDS) software
- Thermo Scientific Dionex EO Eluent Organizer, including 2 L plastic bottles and pressure regulator
- Vial Kit, 0.3 mL, Polyprop with Caps and Septa (P/N 055428)
- Polypropylene vials with detachable caps (1.5 mL, Sarstedt P/N 72.692.005 or equivalent)
- Nalgene 1000 mL 0.2 µm nylon filter units (P/N 09-740-46)
- Tubing, PEEK™, Yellow, 0.003″ i.d., per in. (P/N 049715)

* A Dionex ICS-5000+ system can also be used for this application.

Reagents and Standards

- Deionized (DI) water, Type I reagent grade, 18 MΩ-cm resistivity or better, filtered through a 0.2 µm filter immediately before use
- Thermo Scientific Dionex MonoStandard™, Mixture of Six (P/N 043162), containing the following monosaccharides: fucose, galactosamine, glucosamine, galactose, glucose, and mannose
- D-Glucose, monohydrate (Fisher Scientific P/N D16500)
- Lysine, monohydrate (Fisher Scientific P/N AC17373-0250)

Conditions

Columns:	Dionex CarboPac PA20 Analytical, 3 × 150 mm (P/N 060142) with a Dionex AminoTrap Column, 3 × 30 mm (P/N 060146) Or Dionex CarboPac PA10 Analytical, 4 × 250 mm (P/N 046110) with a Dionex AminoTrap Column, 4 × 50 mm (P/N 046122)
Eluent Source:	Thermo Scientific Dionex EGC III KOH Eluent Generator Cartridge with Dionex CR-ATC Continuously Regenerated Anion Trap Column
Inj. Volume:	10 µL
Column Temp:	30 °C
Cell Temp:	30 °C
Backpressure:	2200 psi
Detection:	PAD
Working Electrode:	Gold on PTFE Disposable Electrode
Reference Electrode	
Mode:	Ag/AgCl mode
Noise:	10–30 pC

Carbohydrate 4-Potential Waveform for the ED

Time (s)	Potential (V)	Gain Region*	Ramp*	Integration
0.00	+0.1	Off	On	Off
0.20	+0.1	On	On	On
0.40	+0.1	Off	On	Off
0.41	–2.0	Off	On	Off
0.42	–2.0	Off	On	Off
0.43	+0.6	Off	On	Off
0.44	–0.1	Off	On	Off
0.50	–0.1	Off	On	Off

*Settings required for Dionex ICS-5000/3000 systems but not used with older Dionex systems

Gold Integrated PAD (IPAD) 1 Waveform

Time (s)	Potential (V)	Gain Region	Ramp	Integration
0.00	–0.2	Off	On	Off
0.04	–0.2	Off	On	Off
0.21	0.0	On	On	On
0.22	+0.22	On	On	On
0.46	+0.22	On	On	On
0.47	0.0	On	On	On
0.56	0.0	Off	On	Off
0.57	–2.0	Off	On	Off
0.58	–2.0	Off	On	Off
0.59	+0.6	Off	On	Off
0.60	–0.2	Off	On	Off

Preparation of Solutions and Reagents

Eluent Solution

Generate the potassium hydroxide (KOH) eluent on line by pumping high-quality degassed DI water through the Dionex EGC III KOH cartridge. Biological contamination must be absent. Chromeleon CDS software tracks the amount of KOH used and calculates the remaining cartridge lifetime. Eluents can be prepared manually if needed, but reproducibility will be compromised because consistent preparation of a 10 mM hydroxide eluent is difficult due to variable carbonate contamination. The impact of carbonate contamination is significant when using low-concentration hydroxide eluents. If eluents must be prepared manually, use NaOH rather than KOH and prepare according to the general instructions for hydroxide eluents in Dionex (now part of Thermo Scientific) Technical Note 71.[5] Keep the eluent water blanketed with 8–10 psi of nitrogen at all times to reduce diffusion of atmospheric carbon dioxide into the eluent water.

Glucose and Lysine Solutions

Dissolve solid standards in DI water to prepare 100 mg/L stock solutions for glucose and lysine. Maintain the stock solutions at -20 °C until needed. Dilute these stock solutions with DI water to prepare a glucose (5 mg/L)–lysine (10 mg/L) solution.

Carbohydrates Standards

Dissolve the contents of one Dionex MonoStandard, Mixture of Six 100 nmol vial in 1.0 mL of DI water and mix to prepare a stock standard solution containing 0.1 M (100 pmol/µL) of each monosaccharide. Immediately freeze the unused stock standard at < -10 °C. Avoid repeated freeze/thaw cycles. Deterioration has been observed at room temperature within 24–48 h.

Results and Discussion

Figure 1A shows a typical separation of a 10 mM Dionex MonoStandard, Mixture of Six on a Dionex CarboPac PA20 Analytical column; Figure 1B shows the separation on the same CarboPac PA20 Analytical column preceded by a CarboPac PA20 Guard column. The six monosaccharide peaks are baseline resolved and elute within a window of 12 min. When the Dionex AminoTrap column is substituted for the Dionex CarboPac PA20 Guard column (Figure 1C), slight peak broadening and longer retention times are observed as compared to those obtained with the Dionex CarboPac PA20 Guard/Analytical set (Figure 1B). Good resolution of the six monosaccharides is still observed.

Similar results are obtained with the 4 × 50 mm Dionex AminoTrap and the Dionex CarboPac PA10 Analytical column. In this case, the six monosaccharides elute in 18 min (Figure 2).

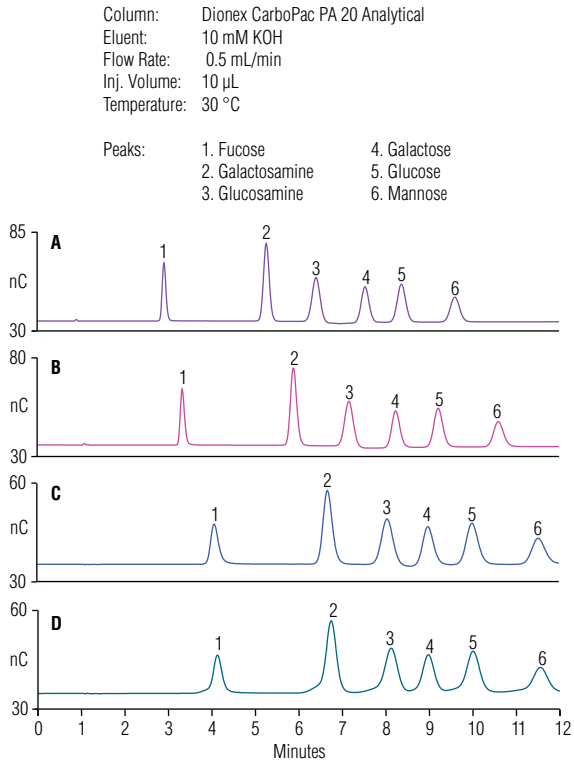

Figure 1. Monosaccharide standards on a Dionex CarboPac PA20 Analytical column (A), a Dionex CarboPac PA20 Guard/Analytical column set (B), a Dionex CarboPac PA20 Analytical column with a 3 × 30 mm Dionex AminoTrap column (C), and a Dionex CarboPac PA20 Analytical column with a damaged 3 × 30 mm Dionex AminoTrap column (D).

Figure 1D shows an example of peak fronting that has been occasionally reported in applications using a Dionex AminoTrap column. The asymmetry (As) of a peak is the measure of peak fronting (or tailing). Asymmetry is defined by both the USP and European Pharmacopeia as As = (RW5% + LW5%)/(2 x LW5%), where RW5% and LW5% are right and left peak width at 5% of the peak height. The asymmetries of the six monosaccharide peaks for a properly functioning Dionex AminoTrap/CarboPac column set (Figure 1C, Table 1) are >1.0. A failing column set exhibits peak fronting with peak(s) asymmetry values <0.95 (Figure 1D and Table 1).

Table 1. Peak asymmetries for the monosaccharides shown in Figures 1C and 1D.

Peak No.	Analyte	Figure 1C	Figure 1D
1	Fucose	1.33	0.94
2	Galactosamine	1.15	0.83
3	Glucosamine	1.03	0.82
4	Galactose	1.19	0.98
5	Glucose	1.10	0.83
6	Mannose	1.07	0.83

Typically, when peak fronting is observed, the analytical column is thought to be the problem and is often replaced. In instances of peak fronting, individually test both the Dionex AminoTrap column and the analytical column to identify the cause of peak fronting. Test the Dionex CarboPac Analytical column first. In the example shown in Figure 1D, when the Dionex AminoTrap column was removed, the CarboPac PA20 Analytical column met the specifications described in the quality assurance (QA) test report for the separation of the Dionex MonoStandard, Mixture of Six (data not shown). This indicated that the analytical column was not the cause of the observed peak fronting.

At this point, simply replace the Dionex AminoTrap column. To verify Dionex AminoTrap column failure, install it without the Dionex CarboPac Analytical column.

Note: to achieve a backpressure of ~2000 psi (with only the Dionex AminoTrap column in place), thus ensuring that the EG can function efficiently, install backpressure tubing (Tubing, PEEK, Yellow, 0.003″ i.d., per in.) in the eluent line before the injection valve. Installation of backpressure tubing can be omitted if using manually prepared eluents.

Test the Dionex AminoTrap column by injecting a mix of glucose and lysine (5 and 10 mg/L, respectively) and eluting with 60 mM hydroxide. The glucose–lysine profile on a properly functioning Dionex AminoTrap column is shown in Figure 3A.

For this analysis, use the gold IPAD 1 waveform (as defined in Chromeleon CDS software). As a best practice, use a new disposable electrode for this analysis due to the difference in waveform. The gold IPAD 1 waveform is used because the four-potential waveform typically used for carbohydrate analysis gives a poor peak shape for lysine.

The retention time on the Dionex AminoTrap column was 1.03 min for glucose and 2.80 min for lysine. The peak efficiencies were 356 and 270, respectively. The peak asymmetries are listed in Table 2. Figure 3B shows the chromatogram for the glucose–lysine mix on the Dionex AminoTrap column that exhibited fronting with the analytical column (Figure 1D). On this Dionex AminoTrap column, the glucose peak shows fronting (asymmetry of <1), compared to a properly functioning Dionex AminoTrap column (for which the asymmetry ranges from 1.0–1.5). This confirms that the Dionex AminoTrap column was the cause of the fronting peaks.

Independent testing provided the information needed to determine which column to replace; however, if the Dionex CarboPac Analytical column passes its test, analysts can save time by replacing the Dionex AminoTrap column without testing it.

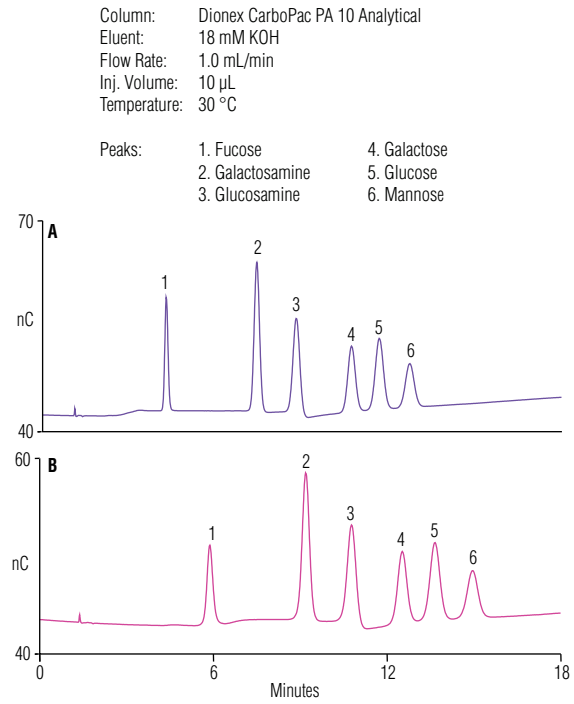

Figure 2. Separation of monosaccharide standards on a Dionex CarboPac PA10 Guard/Analytical column set (A) and a Dionex CarboPac PA10 Analytical column with a 4 × 50 mm Dionex AminoTrap column (B).

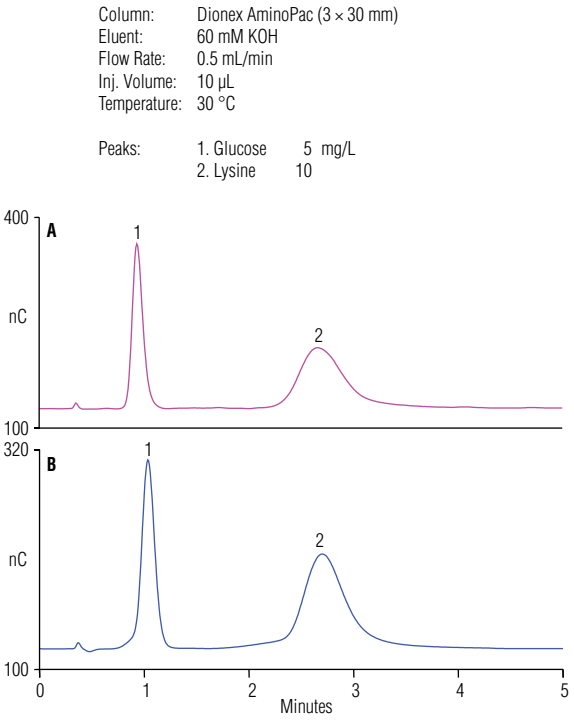

Figure 3. Separation of glucose and lysine mix on a Dionex AminoTrap column (A) and a damaged Dionex AminoTrap column (B).

Table 2. Peak asymmetries for glucose and lysine on the Dionex AminoTrap columns shown in Figure 3.

Chromatogram	Glucose	Lysine
A	1.23	1.45
B	0.94	1.11

Peak fronting usually results from a physical problem with a column. It is hypothesized that pumping water through the Dionex AminoTrap column can occasionally cause irreversible column damage, which manifests as peak fronting. Therefore, do not pump water through a Dionex AminoTrap column. If the Dionex AminoTrap column is exposed to water, try to regenerate it by pumping 200 mM base for 30 min, followed by re-equilibration with the eluent.

The glucose–lysine mix can also be used to test the 4 × 50 mm Dionex AminoTrap column (Figure 4). Use a flow rate of 1.0 mL/min for this column; other parameters are the same as those used for the 3 × 30 mm Dionex AminoTrap column. The dip in the baseline at approximately 3.5 min is due to dissolved oxygen. The position of the dip varies from column to column.

For applications using Dionex AminoTrap columns, monitor the relative response of the monosaccharides. If the peak response of early eluting monosaccharides is normal while the response for those eluting after galactosamine is low, this could be an indication of lysine breaking through the Dionex AminoTrap column. The test results shown in Figure 3 (and Figure 4 for the 4 × 50 mm column) will demonstrate if lysine elution is properly delayed by the Dionex AminoTrap column.

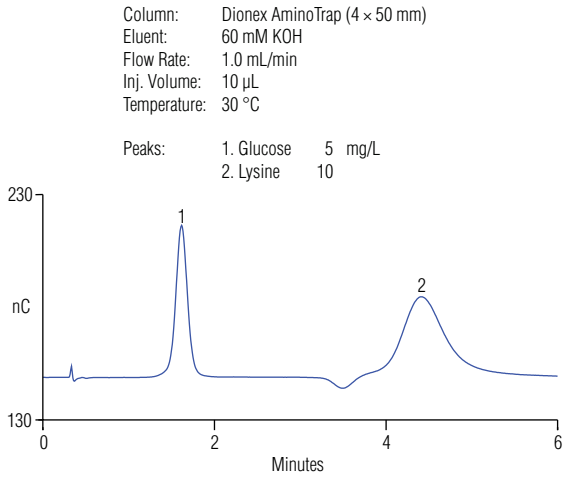

Figure 4. Glucose and lysine mix on a Dionex AminoTrap column.

Conclusion

These general troubleshooting guidelines are intended for Dionex AminoTrap columns used in carbohydrate analyses. To ensure that the column and system are operating correctly, test a new analytical column by itself, not connected to the Dionex AminoTrap or a guard column, using the conditions described in the QA test report. During troubleshooting, use a combination of two tests—the glucose–lysine mix on the Dionex AminoTrap column and the Dionex MonoStandard, Mixture of Six (i.e., the QA test) on the analytical column—to ensure both columns are functioning correctly.

References

1. Rohrer, J.; Thayer, J.; Avdalovic, N.; Weitzhandler, M. HPAEC/PAD Analysis of Monoclonal Antibody Glycosylation. *Techniques in Protein Chemistry* **1995**, *6*, 65–73.
2. Weitzhandler, M.; Slingsby, R.; Rohrer, J.; Narayanan, L.; Pohl, C.; Avdalovic, N. Eliminating Amino Acid and Peptide Interference in HPAE-PAD Glycoprotein Monosaccharide Analysis. *Anal. Biochem.* **1996**, *241*, 128–134.
3. Thermo Scientific Technical Note 40: Glycoprotein Monosaccharide Analysis Using HPAE-PAD with Eluent Generation. Sunnyvale, CA, 2012. [Online] www.dionex.com/en-us/webdocs/5052-TN40-IC-Glycoprotein-Monosaccharide-23May2012-LPN1632-01.pdf (accessed Oct. 15, 2012).
4. Dionex (now part of Thermo Scientific) Technical Note 53: Determination of Glycoprotein Monosaccharide Composition by HPAE-PAD Using On-Line Electrolytically Generated Eluents. Sunnyvale, CA, 2002 [Online] www.dionex.com/en-us/webdocs/6513-TN53_LPN1405-01.pdf (accessed Oct. 15, 2012).
5. Dionex (now part of Thermo Scientific) Technical Note 71: Eluent Preparation for High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection. Sunnyvale, CA, 2009 [Online] www.dionex.com/en-us/webdocs/58087-TN71-Eluent-Prep-HPAE-PAD-16Sept2009-LPN1932-02.pdf (accessed Oct. 15, 2012).
6. Dionex (now part of Thermo Scientific) Product Manual for Amino Trap Columns, Document No. 031197-06, Sunnyvale, CA, 2011 [Online] www.dionex.com/en-us/webdocs/4343-Man-031197-06-AminoTrap-Sep11.pdf (accessed Oct. 15, 2012).

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