

Determination of Glycoprotein Monosaccharide Composition by HPAE-PAD Using On-Line Electrolytically Generated Eluents

INTRODUCTION

High-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) at a gold electrode is a convenient and sensitive technique for carbohydrate analysis. HPAE-PAD provides picomole-level detection with no sample derivatization and simple isocratic elution. However, carbonate contamination of manually prepared hydroxide eluents inevitably causes retention time shifting that degrades performance. To ensure reproducibility, the column must be regenerated with 200 mM NaOH after every injection to elute strongly retained carbonate.

This Technical Note describes the setup and typical performance of an HPAE-PAD system for the monosaccharide analysis of glycoproteins that uses a high-purity eluent generated on-line with the EG40 Eluent Generator.

L-Fucose, D-galactosamine, D-glucosamine, D-galactose, D-glucose and D-mannose are separated in 20 min on a CarboPac™ PA10 column by using an isocratic 18 mM KOH eluent. The column is regenerated by stepping to 100 mM KOH for 5 min and equilibrated for 15 min, for a cycle time of 40 min. The carbonate-free hydroxide regenerant produced by the EG40 accomplishes regeneration in less time than manually prepared hydroxide and eliminates the need for users to prepare the alkaline eluents.

This Technical Note describes general hydrolysis procedures to release monosaccharides from glycoproteins, and provides guidelines to optimize the hydrolysis procedures for success with most proteins. Several different proteins are analyzed, ranging from light to heavy glycosylation and spanning a wide range of monosaccharide composition.

EQUIPMENT

Dionex DX-600 chromatography system consisting of:

GP50 Gradient Pump with vacuum degas option

EG40 Eluent Generator

EGC-KOH cartridge (P/N 053921)

EG40 Vacuum Degas Conversion Kit (P/N 055431)

ED40/50 Electrochemical Detector

ED40/50 Electrochemical Amperometry Cell with Au Working Electrode (P/N 055290)

AS50 Automated Sampler with thermal compartment

Stainless steel sampling needle assembly (P/N 054273)

PeakNet® 6.1 Chromatography Workstation

Screw Cap Micro Tubes (Sarstedt #163.204)

Autosampler vials (P/N 055428)

SpeedVac Evaporator System (SpeedVac SVC100)

Heating Block (VWR 13259-005)

REAGENTS AND STANDARDS

Trifluoroacetic acid (Pierce 53102 HPLC-grade TFA)

Hydrochloric acid (Pierce 24309 constant-boiling 6 N HCl)

MicroBCA Protein Assay (Pierce 23235)

MonoStandard® Mixture of Six (Dionex P/N 043162)

SAMPLES

Fetuin, fetal bovine serum (Calbiochem 341506)
IgG, from human serum (Sigma I 4506)
Mucin, Type II from porcine stomach (Sigma M 2378)
Ribonuclease B, Type III-B from bovine pancreas (Sigma R 5750)
Thyroglobulin, bovine (Sigma T 1001)
Human serum transferrin, (Sigma T 0519 and Boehringer Mannheim 14877000)

CHROMATOGRAPHIC CONDITIONS

Columns: CarboPac PA10 Analytical 4 x 250 mm (P/N 046110)
BorateTrap, 4 x 50 mm (P/N 047078)
AminoTrap, 4 x 50 mm (P/N 046122)
ATC-1 4mm (P/N 037151, 2 each)
Eluent: KOH (EG40 as the source)
Flow Rate: 1.0 mL/min
Program:

Time (min)	mM KOH	Comment
0.00	100	
5.00	100	
5.10	18	
21.0	18	Inject
40.0	18	End

Temperature: 30 °C
Injection: 10 µL partial loop with 10 µL cut volume from 25 µL sample loop
Detection: Pulsed amperometry
Background: ~25–35 nC
Noise: ~30–100 pC peak-to-peak
Backpressure: ~2800 psi
Run Time: 40 min

PREPARATION OF SOLUTIONS AND REAGENTS

Reagent Water

Distilled or deionized water of specific resistance $\geq 17.5 \text{ M}\Omega\text{-cm}$, preferably UV irradiated, filtered through a 0.2-µm filter immediately before use.

Eluent Solution

Generate 18 mM KOH eluent on-line by pumping reagent water through the EG40/EGC-KOH. Sparge the water prior to use with helium or sonicate under vacuum for 10 min. Maintain 3–5 psi head pressure of helium on the water reservoir to reduce contamination from atmospheric carbon dioxide.

Stock Standard Solutions

Dissolve the contents of one Dionex MonoStandard Mix of Six 100 nmol vial in 1.0 mL of reagent water and mix to prepare a stock standard solution containing 0.1 mM (100 pmol/µL) of each monosaccharide. Immediately freeze unused stock standard at $< -10 \text{ }^\circ\text{C}$. Avoid repeated freeze-thaw cycles. Deterioration has been observed at room temperature within 24–48 h.

Working Standard Solutions

Use reagent water to prepare appropriate dilutions of the stock standard for calibration as needed.

Sample Preparation

Estimate the appropriate amount of protein to inject by considering the degree of glycosylation of the protein. As a guideline, the amount of protein to inject = $(10 \text{ }\mu\text{g}/x)$, where $x = \%$ glycosylation of the protein. For example, if the protein is 3% glycosylated, $(10 \text{ }\mu\text{g}/3) = 3.3 \text{ }\mu\text{g}$ protein. To inject 3.3 µg of hydrolyzed protein by using a 10-µL injection requires a sample concentration of 0.3 µg hydrolyzed protein/µL (i.e., 0.3 mg/mL).

It is convenient to prepare a stock standard that is nominally 10X this protein concentration: $10 \times 0.3 \text{ mg/mL} = 3 \text{ mg/mL}$. Transfer $0.030 \pm 0.001 \text{ g}$ protein to a small bottle on a tared analytical balance. Record the mass, remove from the balance, and add 10.0 mL of reagent water. Dissolve by gentle swirling to avoid denaturing the protein.

Next, because protein preparations typically include an unknown amount of water and buffer salts, determine the actual protein concentration by performing the MicroBCA assay on a portion of the solution.

Dilute the stock standard as necessary with reagent water to prepare a 0.3 mg/mL solution for 2 N TFA hydrolysis. For 6 N HCl hydrolysis, either use the 3 mg/mL stock standard, or dry an aliquot of the dilute sample in the hydrolysis tube before adding 6 N HCl, as discussed below.

Hydrolysis Procedures

TFA hydrolysis for neutral monosaccharides: Add 200 μL of a protein solution to a clean Sarstedt vial. Add 140 μL reagent water. Add 60 μL of neat TFA from a freshly opened 1-mL ampoule. Cap, mix, and place vial in a heating block at 100 °C for 4 h. Include at least one blank, substituting reagent water for sample, each time the TFA hydrolysis procedure is performed.

HCl hydrolysis for amino monosaccharides: Add 20 μL of a concentrated protein solution to a clean Sarstedt vial. (Or, add 200 μL of a dilute protein solution and evaporate to dryness in a SpeedVac). Add 400 μL of 6 M HCl from a freshly opened 1-mL ampoule. Cap, mix, and place vial in a heating block at 100 °C for 4 h. Include at least one blank, substituting reagent water for sample, each time the HCl hydrolysis procedure is performed.

After 4 h, remove the vials from the heating block and let them cool before opening. Microfuge the vials and unite the condensate with the bulk liquid. Evaporate to dryness at ambient temperature in a SpeedVac. Drying time may range from 4 hours to overnight. Reconstitute by adding 200 μL of reagent water to each vial, vortex 30 s to mix, and centrifuge at 5000 rpm for 5 min. Transfer supernatant to a limited volume autosampler vial. Cap the vial and inspect the vial for bubbles; dislodge any bubbles that might interfere with injection.

These are the specific procedures followed for this Technical Note. The procedures may be adjusted as described in “Results and Discussion” on page 5.

SYSTEM PREPARATION AND SETUP

Figure 1 shows the placement of each component. Use 0.01-in. ID PEEK tubing to plumb 4-mm systems (0.005-in. tubing for 2-mm systems). Cut tubing square with a tubing cutter and avoid excess lengths of tubing to minimize extra column band broadening. Use the following installation sequence to ensure that contaminants flushed from each new column do not contaminate the next column in line.

Condition two ATC-1 columns with 2 N KOH or 2 N NaOH as described in the *Installation Instructions and Troubleshooting Guide for the IonPac ATC-1 Anion Trap Column* (Document No. 032697). Place one conditioned ATC-1 column between the pump and the EG40 to scavenge carbonate from the source water. Connect the Pump Out end of the black PEEK tubing to this ATC-1 and the EGC In end to the inlet of the EGC-KOH.

Connect the tubing labeled EGC Out that originates from the bottom front of the EG40 Degas Module to the EGC Out port of the EGC-KOH. For carbohydrate applications, modify the EG40 Degas Module to further reduce hydrogen gas generated by the EG40 at high KOH concentrations to the low level required for sensitive amperometric detection. Connect the EG40 Degas Module to the GP40/50 Vacuum Degas system by using the Degas Conversion Kit (P/N 055431) as directed in Document No. 031521. Turn on the pump and go to the Degas Options screen (<Menu> <4>). Set the start-up duration to at least 5 min, the cycle duration to at least 60 s, and the time between cycles to 5 min or less. Set the pump front control panel to “Degas Status” and monitor the degas reading to ensure that there are no leaks in the high-vacuum side of the Degas Module. A major vacuum leak will cause both a rapid drop in the degas reading after the vacuum pump cycles off and baseline oscillations in the chromatogram

with a period corresponding to the vacuum pump cycle. A minor leak will cause a smaller drop in the vacuum reading and smaller baseline oscillations.

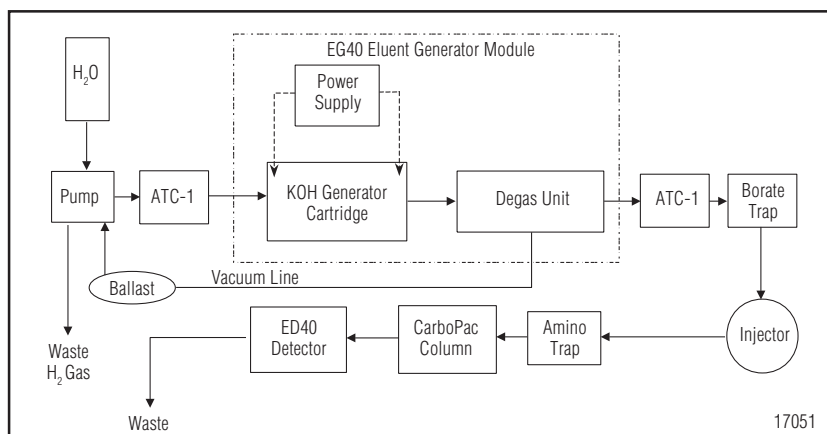


Figure 1. HPAE-PAD system using EG40-generated eluent.

Connect the EG40 to the LAN and configure it with the PeakNet chromatography data system, then condition the EluGen® cartridge as directed in the EG40 manual. Cut and temporarily couple a 24-in. piece of 0.003-in. PEEK tubing to the Injection Valve In tubing exiting the bottom rear of the Vacuum Degas Module. Trim the length to provide backpressure of 2000 psi at 1 mL/min. Perform the following gradient to condition the EluGen cartridge: 1 to 60 mN KOH in 20 min, then 60 mN for 40 min at 1 mL/min.

Remove the 0.003-in. PEEK backpressure tubing temporarily installed during conditioning of the EluGen cartridge and connect the tubing labeled Injection Valve In to the second ATC-1 column. Connect the second ATC-1 column to the BorateTrap column, and connect the BorateTrap to port 2 of the injection valve. The BorateTrap column will scavenge borate from the source water. Borate is one of the first ions to break through water purification systems and will cause peak tailing, especially for mannose. Typical peak asymmetry for a well performing system is 1.0 ± 0.3 . Replace the BorateTrap column when tailing of the mannose peak exceeds this limit.

Install an AminoTrap™ column after the injection valve and condition as directed in the AminoTrap instructions by flushing with 100 mN KOH at 1 mL/min for 1 hour. The AminoTrap delays the elution of amino acids and small peptides found in glycoprotein hydrolysates and is used in place of a guard column before the CarboPac PA10. **Do not pump pure water through the AminoTrap column; it may damage the column irreversibly.**

Install a 4 x 250 mm CarboPac PA10 immediately after the AminoTrap column. With the second ATC-1, the AminoTrap, and the CarboPac PA10 columns now installed, monitor the system pressure displayed by the pump when 18 mM KOH is delivered at 1 mL/min. The EG40 Vacuum Degas Module requires at least 2000 psi backpressure to efficiently remove hydrolysis gas from the eluent. If necessary, install the backpressure coils supplied with the EG40 shipkit to bring the system pressure to between 2300

and 2800 psi (2300 psi accounts for about 300 psi contributed by the ATC-1 and the EGC-KOH). Condition the CarboPac PA10 by flushing the column with 100 mN KOH at 1 mL/min for 1 h as directed in the column manual. Program the EG40 to deliver the gradient program shown in the “Conditions” section and run the gradient, monitoring the backpressure during the entire run. Trim the backpressure coil if necessary to maintain backpressure less than 3000 psi. Caution: Because the backpressure can rise over time, trim the backpressure coil as needed to keep the backpressure under 3000 psi. **Do not exceed 3000 psi or the EG40 Degas Module tubing may rupture.**

Assemble and install the ED40/50 Electrochemical Detector Amperometry Cell with a gold working electrode. From the front control panel of the ED40/50, go to the Detail screen for the Integrated Amperometry mode (<Menu> <2>). Select the Ag/AgCl reference electrode in the REF field.

Program the data acquisition software to deliver the gradient shown in “Chromatographic Conditions”. In the program, use the quadruple potential waveform in Table 1 and program a 2-Hz data acquisition rate.¹ Make replicate injections of a reagent water blank until two successive runs resemble the blank run shown in Figure 4A. An equilibrated, contaminant-free system has a background signal of 25–35 nC, with peak-to-peak noise of 30–100 pC and no significant peaks in the 22–40 min window.

Table 1 Waveform for the ED40/ED50

Time (sec)	Potential (V vs. Ag/AgCl)	Integration
0.00	+0.1	
0.20	+0.1	Begin
0.40	+0.1	End
0.41	-2.0	
0.42	-2.0	
0.43	+0.6	
0.44	-0.1	
0.50	-0.1	

AUTOSAMPLER QUALIFICATION

Peak area precision and accuracy depend on autosampler performance. For best performance, inspect the AS50 sample syringe and its tubing daily for bubbles. Remove any bubbles by following the instructions in the AS50 manual. Replace the water in the flush reservoir daily with freshly filtered and degassed water or apply 5 psi helium pressure to the AS50 syringe reservoir. Use a stainless steel sampling needle assembly (P/N 054273) for mono-saccharide analysis to reduce adsorptive loss of amino-sugars caused by worn Teflon[®]-coated sampling needles.

The precision and accuracy of the AS50 will vary depending on the mode of injection. The most accurate and precise injections can be made with a calibrated sample loop in the Full Loop injection mode. For loop sizes less than 17 μL , 2.5 times the loop volume plus 25 μL is delivered to the valve. Peak area precision for full loop injections of 25 μL is typically 0.3% RSD. A 10- μL Full Loop injection uses 50 μL of sample, allowing three injections from a 200 μL sample.

To conserve sample, use either the Partial Loop or the Partial Loop, Limited Sample (Partial LS) injection mode. In the Partial Loop mode, the AS50 draws the volume to be injected from the sample vial, plus two times the cut segment volume. (The cut segment volume is a portion of the sample that is discarded from each end of the aspirated sample to improve accuracy.) The middle portion of the sample is positioned in the loop and injected. For the best precision and accuracy when using one of the partial loop injection modes, install a sample loop that is at least two times the injected volume. Peak area precision for Partial Loop injections of 10 μL is typically 1% RSD. A 10- μL Partial Loop injection with a cut volume of 10 μL uses 30 μL of sample, allowing six injections from a 200- μL sample.

The AS50 in Partial LS mode wastes no sample and usually provides precision of 1–2% RSD. See the AS50 reference manual for a complete discussion of the different injection modes.

In this Technical Note, we describe the use of an AS50 in the Partial Loop injection mode, which allows six 10- μL injections from a 200- μL glycoprotein hydrolysate sample with good precision and accuracy. Be sure to enter the correct sample loop size and sample syringe volume in the AS50 Plumbing Configuration screen. Reconstitute the

MonoStandard Mix of Six with 1.00 mL of reagent water, dilute 1:1 with reagent water, and inject 10 μL from a 25- μL sample loop with the cut volume set to 10 μL . Confirm that the resulting chromatogram resembles that shown in Figure 2.

Qualify the autosampler before proceeding by injecting three more replicates of the 2X dilution of the MonoStandard Mix of Six. The relative standard deviation of the peak area of the four replicates should be $\leq 2\%$. See the autosampler reference manual for troubleshooting techniques if the autosampler fails this test.

RESULTS AND DISCUSSION

Chromatography

Figure 2 shows a typical separation of a 2X dilution of the MonoStandard Mix of Six containing L-fucose, D-galactosamine, D-glucosamine, D-galactose, D-glucose, and D-mannose at 500 pmol each. The peaks are baseline resolved and elute within a window of 15 min. The total run time is extended to 40 min to allow complete stabilization of the baseline after the column regeneration step.

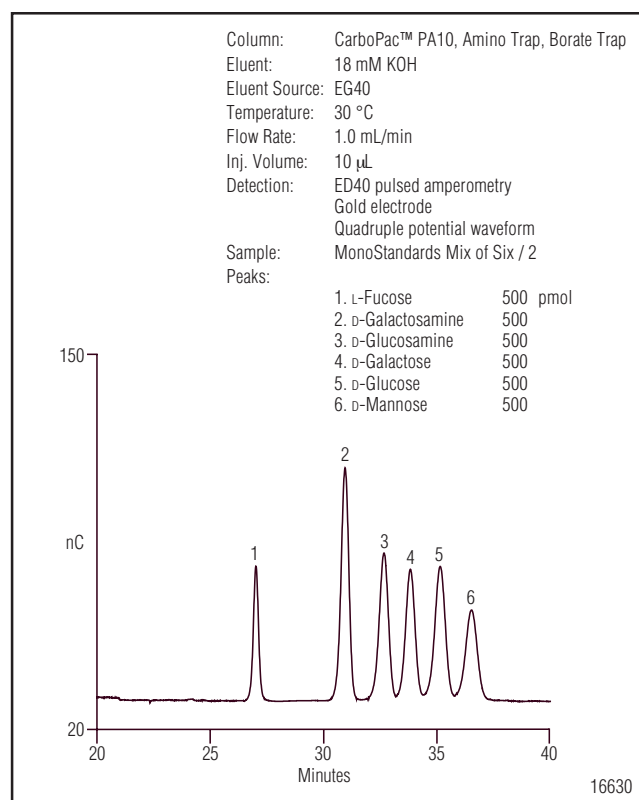


Figure 2. 500 pmol monosaccharide standard.

In the blank runs in Figure 4, the large flat-topped peak eluting between 2 and 10 min results from the column cleanup step. The height and appearance of this peak will vary depending on the amount of material from the previous injection that is flushed off the column. A trough that lasts until $t = 25$ min may follow this peak, whereupon the baseline should increase to its normal, stable value. (A second broad hump may elute between 10 and 20 min as hydrogen gas, a byproduct of eluent generation, elutes from the column.) The sample is injected at $t = 21$ min so that the peaks of interest elute on a stable baseline.

Performance

Table 2 summarizes the calibration data and method detection limits (MDLs) obtained in this study for the six monosaccharides. The MDL for each analyte is established by making seven replicate injections of a monosaccharide standard with peak height for each analyte of three to five times the estimated baseline noise. We used seven replicates of a 0.75 pmol standard. Pulsed amperometric detection with the quadruple waveform allows quantification of these monosaccharides at the low picomole level if a 10- μ L sample is injected. It is possible to lower the detection limit by injecting a larger volume of sample, depending on the ionic strength and nature of the sample matrix.

EG40 eluent generation is highly reproducible. Actual performance of the EG40 is demonstrated in Figure 3, a summary of the retention time of monosaccharide standards injected over a period of seven days. The variation in retention time of each monosaccharide is less than 0.3% RSD for over 200 injections. When retention times become significantly shorter or more variable, recondition

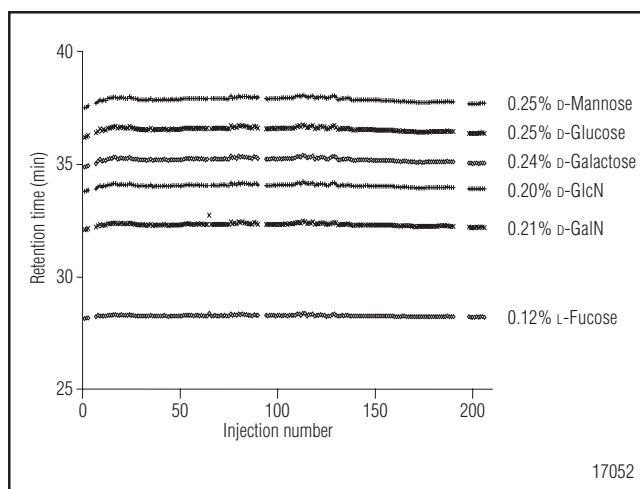


Figure 3. Long-term reproducibility (% RSD) of monosaccharide standards.

the ATCs as described in the *Installation Instructions and Troubleshooting Guide for the IonPac ATC-1 Anion Trap Column* (Document No. 032697). The EGC-KOH cartridge lifetime under the conditions used here is approximately 2000 injections, or 1300 hours of continuous operation. PeakNet 6 software tracks the amount of KOH used and calculates the remaining lifetime. Be prepared to replace the EGC-KOH cartridge when the remaining lifetime is less than 10%.

The next several figures illustrate the performance of this method with various glycoprotein hydrolysates, beginning with the TFA and HCl hydrolysis blanks depicted in Figure 4. The hydrolysis acid, the reagent water, and the labware used in the hydrolysis procedure can contaminate the sample with monosaccharides or coeluting interferants. The level of contamination will affect the detection limit. A reasonable guideline for the detection limit is 10 times the background level of contaminating monosaccharides, so for trace level determinations significant sources of contamination must be eliminated. The hydrolysis procedure blanks are an important means of assessing contamination and qualifying the reagents. The level of glucose contamination in Figure 4B is typical and is acceptable for the glycoproteins analyzed here (none of which is known to contain glucose). However, for analysis of glycoproteins that contain glucose, removal of contaminating glucose may be important.

Table 2 Linear Range and MDLs for Monosaccharides

Monosaccharide	Range (pmol)	r ²	MDL Sample (pmol)	Calculated MDL* (pmol)
L-Fucose	0.5–500	0.9989	0.75	0.17
D-Galactosamine	0.5–500	0.9996	0.75	0.21
D-Glucosamine	0.5–500	0.9999	0.75	0.30
D-Galactose	0.5–500	0.9992	0.75	0.34
D-Glucose	0.5–500	0.9997	0.75	0.34
D-Mannose	0.5–500	0.9992	0.75	0.25

*MDL = $(t) \times (S)$ where t = Student's t value for a 99% confidence level and a standard deviation estimate with $n - 1$ degrees of freedom [$t = 3.14$ for seven replicates of the MDL Standard] and S = standard deviation of the replicate analysis.

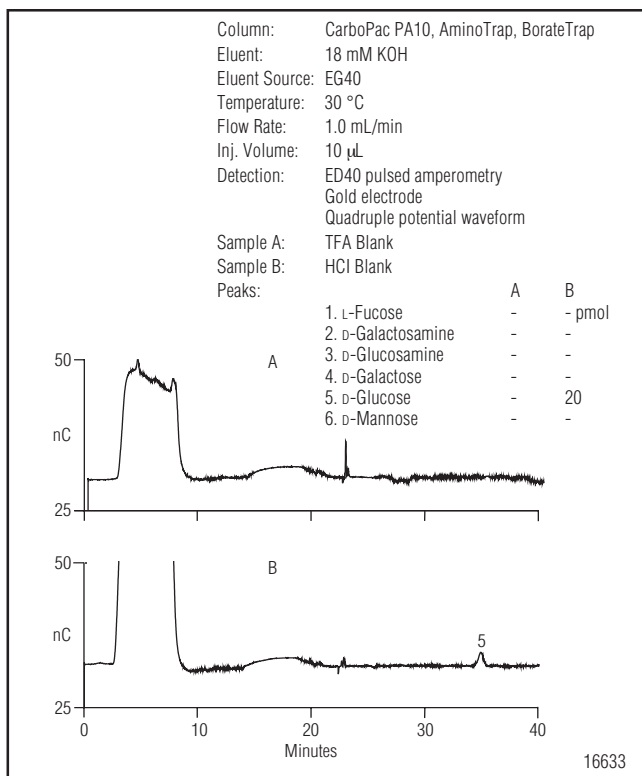


Figure 4. Hydrolysis procedure blanks: deionized water.

Figure 5 shows the HPAE-PAD chromatogram of a fetal bovine fetuin hydrolysate. The monosaccharide peaks are baseline-resolved and elute well away from the early baseline disturbances caused by the column cleanup step, even though the amount of protein injected was about 10X the recommended amount. The neutral monosaccharides are seen at higher concentrations in the TFA hydrolysate, and the amino sugars at higher concentrations in the HCl hydrolysate, as expected.

When injecting glycoprotein hydrolysates, strongly retained hydrolysis products can accumulate on the column and impair its performance. Signs of a fouled column include an increase in unidentified peaks, baseline disturbances, peak tailing, and shortened retention times. This method includes a step change to 100 mM KOH for 5 minutes to flush strongly retained material off the column. Table 3 highlights the efficacy of the column cleanup step to prevent column fouling when glycoprotein hydrolysates are analyzed. Every six injections of a glycoprotein hydrolysate sample were followed by three injections of the MonoStandard Mix of Six containing 500 pmol of each monosaccharide. Compare the retention time reproducibility of monosaccharide standards alone to that obtained when the standards are interspersed with injections of various protein hydrolysates. The good RT stability of the

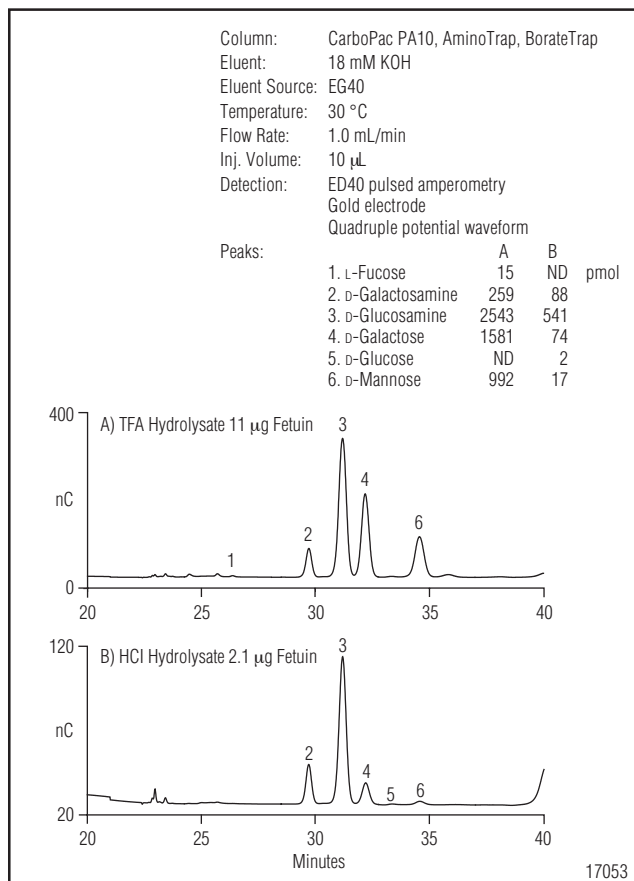


Figure 5. Glycoprotein hydrolysates: fetuin.

Table 3 Retention Time Precision (% RSD) of Monosaccharide Standards Interspersed with Glycoprotein Hydrolysates

Sugar	Standards Alone	Standards with 3 µg IgG	Standards with 30 µg IgG	Standards w/ Various Proteins
L-Fucose	0.10	0.14	0.09	0.08
D-Galactosamine	0.19	0.19	0.08	0.12
D-Glucosamine	0.20	0.17	0.09	0.14
D-Galactose	0.15	0.17	0.11	0.18
D-Glucose	0.18	0.17	0.11	0.19
D-Mannose	0.19	0.17	0.12	0.19

standards alone is maintained not only with typical protein hydrolysates (3 µg IgG), but also with injections of more concentrated protein hydrolysates (30 µg IgG) that challenge the method. Good RT stability is also observed with a mix of glycoproteins representing a range of glycosylation types and amounts, including transferrin, fetuin, ribonuclease B, thyroglobulin, and mucin.

The column cleanup step, together with the AminoTrap guard column, helps maintain the stability of the electrode response. Protein hydrolysis products such as amino acids and peptides, which can poison the gold working electrode, are strongly retained by the AminoTrap column until they are eluted by the 100 mM KOH cleanup step. Under these conditions, working electrode performance is maintained.

Stable electrode response is demonstrated in Table 4, which summarizes the peak area reproducibility of monosaccharide standards injected alone or interspersed with injections of various glycoprotein hydrolysates. Although the peak area variability does increase when glycoprotein hydrolysates are analyzed compared to standards alone, even under the most challenging conditions peak area reproducibility remains acceptable (i.e., less than 10% RSD).

Table 4 Peak Area Precision (% RSD) of Monosaccharides Standards Interspersed with Glycoprotein Hydrolysates

Sugar	Glycoprotein Hydrolysates			
	Standards Alone	Standards with 3 μ g IgG	Standards with 30 μ g IgG	Standards w/ Various Proteins
L-Fucose	1.74	2.35	4.28	5.53
D-Galactosamine	1.17	1.72	2.56	3.24
D-Glucosamine	1.56	1.95	2.34	6.82
D-Galactose	1.17	2.31	1.34	3.72
D-Glucose	1.09	2.71	1.68	3.34
D-Mannose	1.91	4.36	4.18	4.90

Hydrolysis Procedures

Two different hydrolysis procedures are available to release monosaccharides from glycoproteins. Hydrolysis with 2 N TFA is recommended for neutral sugars, but is too mild to release completely the amino sugars galactosamine and glucosamine. Hydrolysis with 6 N HCl is recommended for the amino sugars, but partially degrades the neutral sugars; both procedures must be used in tandem to accurately measure the monosaccharide composition of a glycoprotein.

To hydrolyze with 2 N TFA, dilute neat (13 N) TFA with a combination of protein solution and reagent water. Here we add 60 μ L of neat TFA to 200 μ L of protein solution plus 140 μ L of reagent water. The final TFA concentration is calculated to be: $(13 \text{ M TFA}) * (60 \mu\text{L TFA}/400 \mu\text{L total}) \approx 2 \text{ M TFA}$. Different ratios of protein solution/reagent water can be used, depending on the concentration of the protein solution, to produce 2 M TFA.

Evaporate the hydrolysate to remove residual TFA and water and then reconstitute the hydrolysate with reagent water. Here we add 200 μ L to provide a sufficient volume for replicate injections. Vortex to mix, centrifuge, and transfer the supernatant to an autosampler vial for analysis.

When hydrolyzing with 6 N HCl, use a smaller volume of protein solution than with 2 N TFA to minimize dilution of the 6 N HCl by the sample. For example, we use 20 μ L of protein solution and 400 μ L of 6 N HCl for a total volume of 420 μ L. The final HCl concentration is: $(6 \text{ N HCl}) * (400 \mu\text{L 6 N HCl}/420 \mu\text{L total}) \approx 6 \text{ N HCl}$. (If the protein solution is too dilute, a larger volume can be added and then dried prior to the addition of HCl.)

Evaporate the hydrolysate to remove residual HCl and water. Then reconstitute the hydrolysate with reagent water, vortex to mix, centrifuge, and transfer the supernatant to an autosampler vial for analysis.

These are good general hydrolysis conditions for determining the monosaccharide content of a glycoprotein. The hydrolysis procedure may be optimized for any specific glycoprotein that is going to be analyzed routinely. Fan et al. have provided a good framework for optimizing glycoprotein hydrolysis conditions for monosaccharide analysis.²

Variations in hydrolysis reproducibility add to the overall variability of this analysis, along with such factors as water quality, autosampler performance, column condition, and electrode performance. We evaluated hydrolysis reproducibility by comparing the variability of replicate injections from a single hydrolysate vial to the variability between vials of replicate hydrolysates of the same protein sample. Results are summarized in Table 5 (TFA hydrolysis) and Table 6 (HCl hydrolysis) for several different glycoproteins.

Two trends are evident in Tables 5 and 6. First, the peak area variability (% RSD) of injections from a single hydrolysate is less than the variation from vial to vial. Single-vial precision of 1–2% is what we expect for a well performing autosampler. However, several factors can cause higher variability between hydrolysates. For example, it was difficult to get uniform samples from the mucin solution, which was viscous and contained suspended solids. Because the hydrolysis procedure is an important source of variability, we recommend performing triplicate hydrolyses for each protein. Second, the peak area variability increases as analyte amounts approach the minimum detection limits of the method. Peak area RSD of about 10–15% is expected for a peak with S/N = 3. The peak area reproducibility for replicate hydrolyses is good enough to assure us that we have a robust hydrolysis technique and to allow us to calculate the relative amount of each monosaccharide that was present in the original glycoprotein.

Table 5 Peak Area Precision of Replicate Injections of Glycoprotein TFA Hydrolysates

Sugar	% RSD for Replicate Injections ^a			% RSD of replicate Hydrolyses ^b
	Vial 1	Vial 2	Vial 3	
Thyroglobulin				
L-Fucose	1.65	1.86	1.84	6.64
D-Galactose	0.92	0.51	0.80	3.76
D-Mannose	1.08	2.34	1.63	2.13
Ribonuclease B				
L-Fucose	ND	ND	ND	ND
D-Galactose	7.03	14.0	7.68	10.8
D-Mannose	2.37	2.8	0.70	3.45
Mucin				
L-Fucose	1.44	0.55	0.86	1.59
D-Galactose	1.28	0.45	1.00	0.82
D-Mannose	6.97	5.54	4.67	26.0

^aNumber of injections was six

^bNumber of hydrolyses was three

Table 6 Peak Area Precision of Replicate Injections of Glycoprotein HCl Hydrolysates

Sugar	% RSD for Replicate Injections ^a			% RSD of Replicate Hydrolyses ^b
	Vial 1	Vial 2	Vial 3	
Thyroglobulin				
D-Galactosamine	ND	ND	ND	ND
D-Glucosamine	2.33	0.99	0.80	1.80
Ribonuclease B				
D-Galactosamine	ND	ND	ND	ND
D-Glucosamine	1.50	2.34	1.80	2.73
Mucin				
D-Galactosamine	0.51	0.55	1.02	4.87
D-Glucosamine	0.37	0.42	0.46	5.71

^aNumber of injections was six

^bNumber of hydrolyses was three

Composition Analysis

Calculate the monosaccharide content of a glycoprotein in mol monosaccharide/mol glycoprotein as follows. First calculate the amount of protein analyzed by HPAE-PAD. Begin with the actual protein concentration of the solution that was hydrolyzed and account for dilution when reconstituted. (It is very important to measure the actual protein content of the solution hydrolyzed; otherwise, only the relative proportion of each sugar can be determined, and not the actual monosaccharide composition or percent glycosylation. An assay such as the BCA assay will help account for an unknown amount of water, salts, or other components present in the solid protein.)

For example, the fetuin sample concentration determined by using the BCA assay was 2.1 g/L. The fetuin HCl hydrolysate was diluted tenfold upon reconstitution. Therefore, the mass of hydrolyzed fetuin injected was:

$$(2.1 \text{ g/L}) * (20 \text{ }\mu\text{L}/200 \text{ }\mu\text{L}) * (10 \times 10^{-6} \text{ L}) = 2.1 \times 10^{-6} \text{ g}$$

The number of moles of fetuin injected was:

$$(2.1 \times 10^{-6} \text{ g}) / (48,000 \text{ g fetuin/mol}) = 44 \times 10^{-12} \text{ mol}$$

Next, determine the amount of each monosaccharide found in the glycoprotein hydrolysate. Use the results from the 2 N TFA hydrolysate for neutral sugars and the 6 N HCl hydrolysate for the aminosugars. For example, the amount of D-galactosamine found in the fetuin 6 N HCl hydrolysate was 93×10^{-12} mole. The D-galactosamine content of fetuin is:

$$\frac{(93 \times 10^{-12} \text{ mol D-galactosamine})}{44 \times 10^{-12} \text{ mol fetuin}} = \frac{2.1 \text{ mol D-galactosamine}}{\text{mol fetuin}}$$

Monosaccharide composition analysis of the various glycoprotein hydrolysates (Table 7) yields monosaccharide ratios consistent with expected ranges. For example, the monosaccharide ratios obtained for bovine fetuin are consistent with the ranges determined by a multicenter study of quantitative carbohydrate analysis.³ The slightly low values obtained for galactose and mannose may be a result of injecting about 10X more fetuin than the recommended amount.

Table 7 Glycoprotein Monosaccharide Composition				
Protein	Protein Injected (μg)	Monosaccharide	Amount Found (pmol)	mol Sugar/mol Protein
Transferrin				
	13.5	L-Fucose	24	0.1
	13.5	D-Galactosamine		
	13.5	D-Glucosamine	1827	5.4 (6.8)*
	13.5	D-Galactose	645	1.9 (2.4)*
	13.5	D-Mannose	816	2.4 (3.0)*
BM Transferrin				
	15.3	L-Fucose	33	0.1
	15.3	D-Galactosamine		
	15.3	D-Glucosamine	2196	5.2 (6.5)*
	15.3	D-Galactose	740	1.9 (2.4)*
	15.3	D-Mannose	912	2.4 (3.0)*
Fetuin				
	10.7	L-Fucose	30	0.1
	2.1	D-Galactosamine	926	2.1
	2.1	D-Glucosamine	5549	12.4
	10.7	D-Galactose	3147	7.1
	10.7	D-Mannose	1962	4.4
Thyroglobulin				
	12.4	L-Fucose	320	8.5
	6.2	D-Galactosamine		
	6.2	D-Glucosamine	2469	65.5
	12.4	D-Galactose	896	23.7
	12.4	D-Mannose	1848	49.0
Ribonuclease B				
	7.8	L-Fucose		
	7.8	D-Galactosamine		
	7.8	D-Glucosamine	2362	1.0
	7.8	D-Galactose	94	0.0
	7.8	D-Mannose	5882	2.6
Mucin				
	0.5	L-Fucose	3930	9198
	0.5	D-Galactosamine	5999	14040
	0.5	D-Glucosamine	11507	26931
	0.5	D-Galactose	6439	15070
	0.5	D-Mannose	283	662

*Normalized to mannose = 3

SUMMARY

HPAE-PAD using an EG40-generated hydroxide eluent simplifies and improves monosaccharide composition analysis of glycoproteins. It can provide reproducible retention times and detector response for hundreds of samples over several days. Pulsed amperometric detection provides sensitivity at the low-picomole level and a linear range of 0.5–500 pmol for monosaccharides commonly found in mammalian glycoproteins.

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