Glycoprotein Monosaccharide Analysis Using HPAE-PAD with Eluent Generation

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Key Words
Carbohydrate Analysis, High Throughput, Bovine Fetuin, Human Immunoglobulin (IgG), Gold on PTFE Disposable Electrode

Goal
This study is intended as a guide for investigators interested in validating a high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD)-based method for monosaccharide composition analysis. Described here are the configuration, typical performance, and precautions pertaining to use of an HPAE-PAD system for the monosaccharide analysis of glycoproteins. A Thermo Scientific Dionex ICS-5000 system with electrolytically generated eluent and a disposable gold on polytetrafluoroethylene (Au on PTFE) working electrode are used. Reproducibility, linearity, detection limits, and accuracy using common glycoprotein monosaccharides are evaluated.

Introduction
Characterization of glycoproteins routinely involves carbohydrate analysis. Minor variations in glycosylation can affect the efficacy of protein therapeutics.[1] Monosaccharide composition analysis can detect variations in glycosylation, and support quality control for process development and manufacturing procedures. There are over 30 approved glycoprotein-based biodrugs on the market and the number is increasing rapidly. Agencies such as the the U.S. FDA and the European Medicines Agency have increased pressure on biopharmaceutical manufacturers to demonstrate satisfactory programs for understanding, measuring, and controlling glycosylation in glycoprotein-based drugs.

Studies show that HPAE-PAD is a simple and effective way to determine glycoprotein monosaccharide composition without derivatization. In a typical method, monosaccharides are separated on a Thermo Scientific Dionex CarboPac PA10 or PA20 anion-exchange column, preceded by a Thermo Scientific Dionex AminoTrap guard column, using a hydroxide eluent prepared manually or generated by an eluent generator.[2]

Disposable Au on PTFE electrodes have longer lifetimes and can operate at higher hydroxide concentrations compared to earlier-generation disposable working electrodes. They also provide greater system-to-system reproducibility and require no laborious electrode polishing.

Equipment
- Dionex ICS-5000 Ion Chromatography system including:
  - SP Single Pump or DP Dual Pump, Gradient or Isocratic, with Vacuum Degas option
  - EG Eluent Generator Module
  - Potassium Hydroxide Eluent Generator Cartridge (Thermo Scientific Dionex EGC III KOH) (P/N 074532)
  - DC Detector/Chromatography Compartment
  - ED Electrochemical Detector (P/N 072042)
  - Gold on PTFE Disposable Electrode (6 pack, P/N 066480)
  - pH, Ag/AgCl Reference Electrode (P/N 061879)
  - AS or AS-AP Autosampler
  - EO Eluent Organizer and Accessories, including 2 L plastic bottles and pressure regulator
- Thermo Scientific Dionex Chromeleon Chromatography Data System software
- Vial Kit, 0.3 mL Polyprop with Caps and Septa, 100 each (P/N 055428)
- Polypropylene vials with detachable caps (1.5 mL, Sarstedt® P/N 72.692.005 or equivalent)
- Thermo Scientific Nalgene 250 mL HDPE Narrow-Mouth Bottle (P/N 2002-0008)
- Nalgene™ 1000 mL HDPE Narrow-Mouth Bottle (P/N 2002-0032)

Thermo SCIENTIFIC

- Nalgene 250 mL 0.2 µm nylon filter units (P/N 153-0020)
- Nalgene 1000 mL 0.2 µm nylon filter units (P/N 154-0020)
- Thermo Scientific SpeedVac Lyophilization System
- Heating Block (VWR® P/N 13259-005)

Reagents and Standards

- Deionized (DI) water, Type I reagent grade, 18 M -cm resistivity or above, filtered through a 0.2 µm filter immediately before use
- Thermo Scientific Dionex MonoStandard, Mixture of Six (P/N 43162) containing the following monosaccharides (in order of elution): fucose (Fuc), galactosamine (GalN), glucosamine (GlcN), galactose (Gal), glucose (Glc), and mannose (Man)
- IgG from human serum (Sigma-Aldrich® P/N I-4506)
- Fetuin from fetal calf serum (Sigma-Aldrich P/N F2379-250MG)
- Thermo Scientific Pierce Trifluoroacetic Acid (TFA), sequencing grade for making 0.1% v/v TFA solutions (P/N 28904)
- Thermo Scientific Pierce Hydrochloric Acid (P/N 24308)
- Thermo Scientific Pierce Micro BCA Protein Assay Kit (P/N 23235)

Conditions

Method

Columns:	Dionex CarboPac™ PA20 Analytical, 3 × 150 mm (P/N 060142) Dionex AminoTrap™, 3 × 30 mm (P/N 060146)
Eluent:	10 mM KOH
Eluent Source:	Thermo Scientific Dionex CR-ATC Continuously Regenerated Anion Trap Column EGC III KOH
Flow Rate:	0.5 mL/min
Inj. Volume:	10 µL (partial loop injection mode with a 4 µL cut volume)
Column Temp.:	30 °C
Cell Temp.:	30 °C
Backpressure:	2200 psi
Detection:	PAD
Background:	30–50 nC
Working Electrode:	Carbohydrate PTFE Disposable Au Working Electrodes
Reference Electrode:	Mode: Ag/AgCl mode Noise: 10–30 pC

Carbohydrate Waveform

Carbohydrate four-potential waveform for the ED:

Time (s)	Potential (V)	Gain	Ramp* Region*	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 in the Dionex ICS-3000/5000 systems but not used in older Dionex systems

Preparation of Solutions and Reagents

Eluent Solution

Generate the potassium hydroxide (KOH) eluent on line by pumping high-quality degassed DI water (no biological contamination) through the Dionex EGC III KOH cartridge. Chromeleon™ software tracks the amount of KOH used and calculates the remaining cartridge lifetime. Although eluents can be manually prepared if needed, 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 (TN) 71.[3] For this application, electrolytic eluent generation delivers superior performance. Keep the eluent water blanketed under 8–10 psi of nitrogen at all times to reduce diffusion of atmospheric carbon dioxide into the eluent water.

Carbohydrates Standards

Dissolve the contents of one Dionex MonoStandard™ 100 nmol vial in 1.0 mL of DI 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 °C. Avoid repeated freeze/thaw cycles. Deterioration can occur within 24–48 h at room temperature.

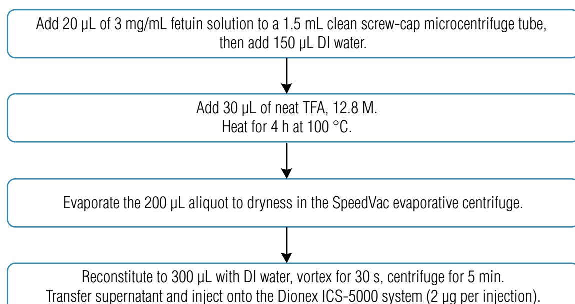
Working Standard Solutions

Use DI water to prepare appropriate dilutions of stock standard for calibration as needed. For example, prepare the 10 µM standard by diluting the stock (0.1 mM) 10-fold.

Glycoprotein Acid Hydrolysates

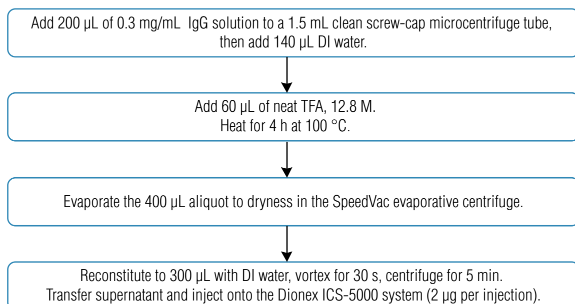
Determine the protein concentration by performing the Micro BCA™ assay.[4] The TFA hydrolysis is optimized for neutral sugar (Fuc, Gal, Glc, Man) recovery, whereas the HCl hydrolysis is optimized for amino sugar (GalN, GlcN) recovery.[5] Estimate the appropriate amount of protein to inject by considering the degree of glycosylation. As a guideline, the amount of protein to inject = (10 µg/x), where x = % glycosylation of the protein. For example, if the protein is 3% glycosylated, (10 µg/3) = 3.3 µg of protein. To inject 3.3 µg of hydrolyzed protein 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 10 times this protein concentration (3 mg/mL). Once sample chromatography has been established, the best long-term results are achieved by injecting the lowest amount protein that yields monosaccharide peaks safely above the limit of quantification.

Schemes 1 and 2 describe the hydrolysis steps for fetuin and IgG. Prepare TFA hydrolysates of fetuin by combining 20 µL of 3 mg/mL fetuin solution, 150 µL DI water, and 30 µL of TFA (12.8 M) in a 1.5 mL microcentrifuge tube. For HCl fetuin hydrolysates, add 6 M HCl. Prepare HCl hydrolysates for IgG by combining 400 µL of 6 M HCl with 20 µL of 3 mg/mL IgG. Prepare TFA hydrolysates of IgG by combining 200 µL of 0.3 mg/mL IgG, 140 µL of DI water, and 60 µL of neat TFA. Heat the solutions for 4 h at 100 °C and then dry in a SpeedVac™ concentrator for ~3 h. Reconstitute each vial with 300 µL of DI water, vortexed for 30 s and centrifuged for 5 min. Inject 10 µL of the supernatant into the ion chromatography system.



Note: 6 M HCl also can be used for hydrolyzing fetuin. Combine 400 µL of 6 M HCl with 20 µL of 3 mg/mL fetuin solution. This is recommended for amino sugars and can improve their recovery by 30-57%; however, fetuin can potentially degrade neutral sugars.

Scheme 1.



Note: 6 M HCl also can be used for hydrolyzing IgG. Combine 400 µL of 6N HCl with 20 µL of 3 mg/mL IgG.

Scheme 2.

Guidelines and Precautions

Columns

To ensure that the column and system are operating correctly, the Dionex CarboPac PA20 Analytical column must be tested using the same conditions as described in the quality and reliability test report. Maintain the column set under a controlled temperature to reduce variations in peak area and retention times.

EG Module

To function properly, the EG module requires backpressure in the range of 2000 to 3000 psi.

Dionex CR-ATC Continuously Regenerated Anion Trap Column

The detector baseline signal typically will be between 30 and 50 nC at 0.5 mL/min of 10 mM KOH with the system described here. A high baseline may be due to malfunction or improper configuration of the degas module, or the presence of electrochemically active impurities in the water. Change the Dionex CR-ATC column when the KOH cartridge is changed.

Working Electrodes and Electrochemical Response

Replace the reference electrode every three to six months. Indications of a failed reference electrode are a pH reading >12.5 or the absence of any reading for 10 mM KOH eluent and the inability to calibrate the reference electrode. Disposable electrodes must always be replaced when they have been used with a failed reference electrode. Upon installation of a new working electrode, allow 1–2 h for the background to stabilize. Refer to Dionex (now part of Thermo Scientific) TN 110[6] for lifetime, background, and noise characteristics of the disposable Au on PTFE working electrodes.

System conditions resulting in low peak area (i.e., lower method sensitivity) can be due to contaminated eluents, a fouled electrode, a failed reference electrode, or incorrect waveform. Other factors that are not related to the detection can also cause lower response, such as incorrectly prepared standards or a malfunctioning autosampler.

Dionex AminoTrap Column

The Dionex AminoTrap column delays the elution of amino acids and small peptides found in glycoprotein hydrolysates and is used in place of a guard column before the Dionex CarboPac PA20 column. Install the Dionex AminoTrap column after the injection valve and condition by flushing with 100 mM KOH at 0.5 mL/min for 2 h. Although slight peak broadening and longer retention times are expected with the addition of the Dionex AminoTrap column (compared to those obtained with the analytical column), good resolution of the six monosaccharides still will be observed. *Do not pump water through the Dionex AminoTrap column; it will damage the column irreversibly.*

Acid Hydrolysis

Include at least one blank, substituting reagent water for protein sample, each time the hydrolysis procedure is performed.

Autosampler

Dionex ICS-5000 autosamplers have three modes of injection. For the highest peak area or height precision, the Full-Loop mode of injection is recommended; however, sample use is higher (e.g., 50 µL for a 10 µL injection) than the Partial-Loop mode of injection. For highest conservation of sample, use the Partial-Loop Limited-Sample mode of injection (e.g., 10 µL sample for a 10 µL injection). All results presented here were obtained using the Partial-Loop mode of injection with a 4 µL cut volume. When Partial-Loop mode of injection is chosen, the sample loop volume must be at least twice the injection volume for best accuracy. These modes of injection are available with the Dionex ICS-5000 AS and AS-AP autosamplers.

To prevent baseline disturbances and electrode fouling, do not use organic solvents in the wash reservoir. Replace rinse water (DI water) frequently. The injector syringe and the transfer line must be free of bubbles to ensure accurate injection volumes.

Results and Discussion

Separation

Figure 1 shows a typical separation of a 10 µL injection of the Dionex MonoStandard containing Fuc, GalN, GlcN, Gal, Glc, and Man at 10 µM each. The peaks are baseline resolved and elute within a window of 13 min. The total run time is 24 min to allow for stabilization of the baseline after the column regeneration step. When protein hydrolysate samples are analyzed, the Dionex CarboPac PA20 and AminoTrap columns must be regenerated with 100 mM KOH at 0.5 mL/min for ≥3 min following each run. A periodic 2 h wash with 100 mM KOH at 0.5 mL/min removes the more strongly retained compounds expected in these samples. In certain cases, complete restoration of column capacity will require the use of stronger premade eluents (for example, 200 mM NaOH for 2 h) in an off-line system mode.

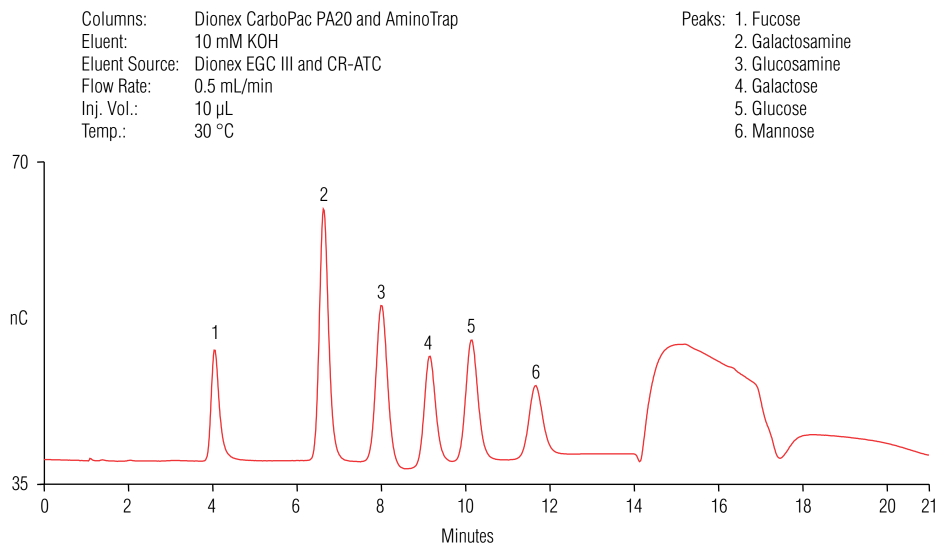


Figure 1. Monosaccharide standards on the Dionex CarboPac PA20 and AminoTrap columns

Figure 2 presents the HPAE-PAD chromatograms of bovine fetuin acid hydrolysates. The monosaccharide peaks are baseline resolved, the neutral monosaccharides are seen at higher concentrations in the TFA hydrolysate, and the amino sugars at a higher concentration in the HCl hydrolysate, as expected. These examples have much more protein injected than is needed. Five- to 10-fold less protein can be injected with good results.

Figure 3 shows a typical injection of IgG acid hydrolysates (TFA and HCl). Human serum IgG has lower carbohydrate content relative to most mammalian glycoproteins. The low concentration of carbohydrate makes monosaccharide determination more challenging due to the higher concentration of peptides and amino acids in the acid hydrolysate relative to glycoproteins with higher percentages of glycosylation.

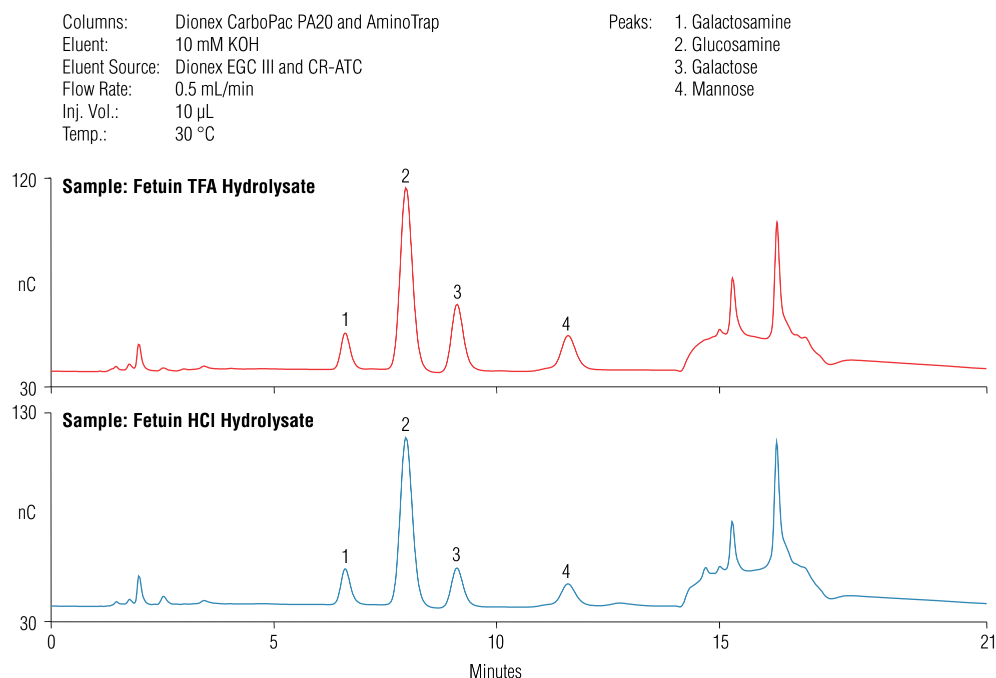


Figure 2. Monosaccharide composition analysis of bovine fetuin: the HCl hydrolysis condition was milder than Scheme 1 (20 µL of 3 mg/mL bovine fetuin with 30 µL of 6 M HCl).

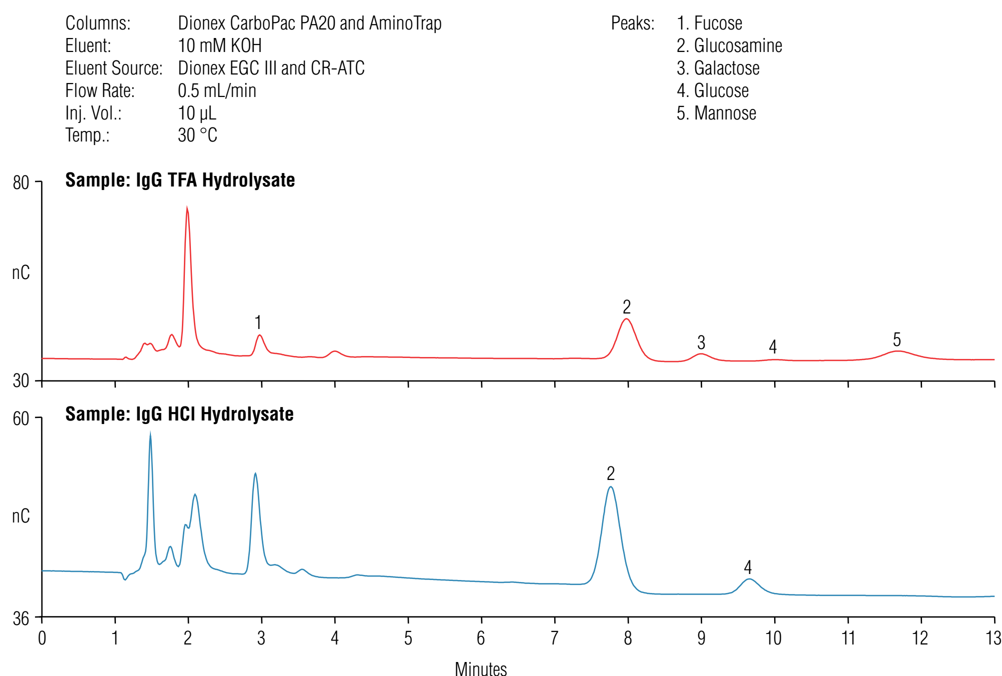


Figure 3. Monosaccharide composition analysis of human serum IgG

Reproducibility with Interspersed Samples

The ruggedness of the system was tested by evaluating the peak area and retention time stability of each monosaccharide in a 10 µM injection of the Dionex MonoStandard interspersed with ten injections of hydrolysates (2 µg protein load per injection). The sequence of one injection of 10 µM Dionex MonoStandard, five injections of fetuin TFA hydrolysates, five injections of fetuin HCl hydrolysates, and one injection of 10 µM Dionex MonoStandard was repeated for seven days (Figure 4). Figures 4 and 5 show selected chromatograms of the monosaccharide standard from the seven-day analysis interspersed with 2 µg protein/injection of fetuin and IgG acid hydrolysates, respectively.

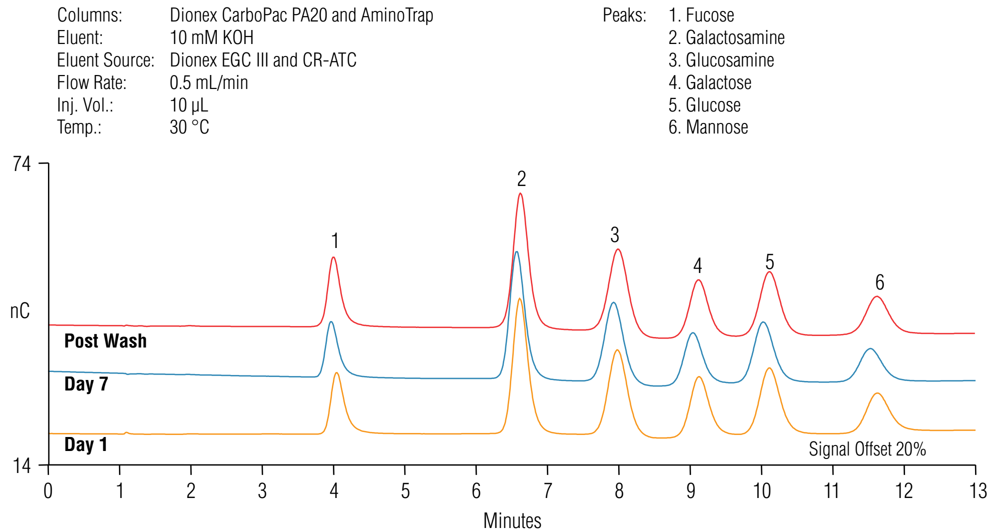


Figure 4. Monosaccharide standards: seven-day analysis interspersed with 2 µg/injection load of fetuin acid hydrolysates

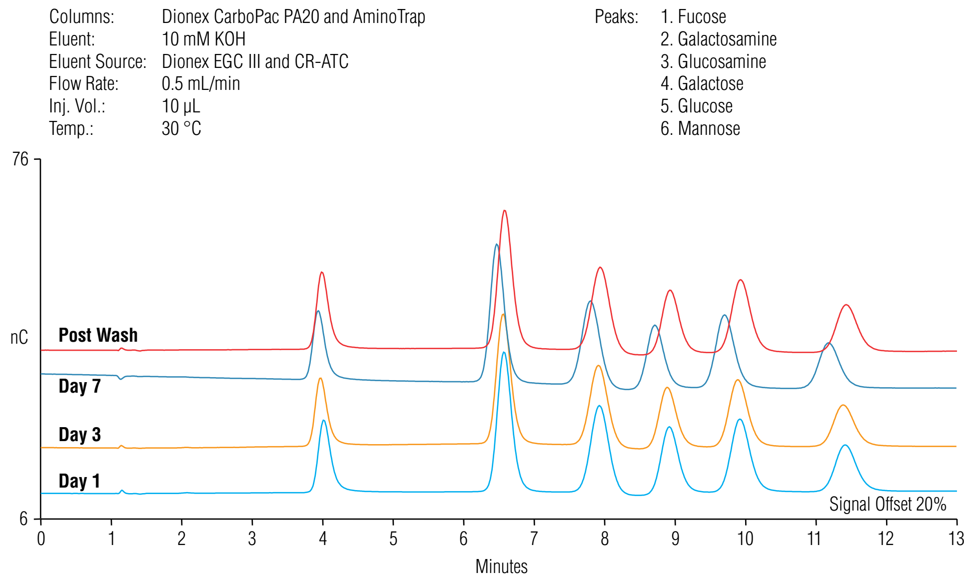


Figure 5. Monosaccharide standards: seven-day analysis interspersed with 2 µg/injection load of IgG acid hydrolysates

Table 1. Precision and percent change of retention time over seven days of fetuin hydrolysate analysis

	Retention Time RSD			Retention Time (min)			% Change
Analyte	Day 1	Day 7	Over 7 Days	Day 1	Day 7	After Wash on Day 7	After Wash[a]
Fucose	<0.01	0.15	0.69	4.04	3.95	4.00	-1.04%
Galactosamine	0.09	0.09	0.35	6.61	6.55	6.62	0.09%
Glucosamine	0.07	0.08	0.35	7.98	7.90	7.99	0.10%
Galactose	0.06	0.07	0.49	9.13	8.99	9.11	-0.15%
Glucose	0.06	0.06	0.44	10.11	9.97	10.11	-0.02%
Mannose	0.05	0.05	0.45	11.63	11.46	11.62	-0.09%

[a]2 h 100 mM KOH

Table 2. Precision and percent change of peak area over seven days of fetuin hydrolysate analysis

	Peak Area RSD			Peak Area			% Change
Analyte	Day 1	Day 7	Over 7 Days	Day 1	Day 7	After Wash on Day 7	After Wash[a]
Fucose	0.80	3.30	4.43	2.80	2.68	2.90	3.57%
Galactosamine	1.66	0.45	2.15	6.81	6.90	7.09	4.10%
Glucosamine	1.14	0.32	2.42	5.26	5.42	5.53	5.13%
Galactose	1.49	0.93	3.46	3.46	3.38	3.54	2.47%
Glucose	1.13	0.35	2.57	4.20	4.23	4.29	2.25%
Mannose	1.90	0.22	4.27	2.80	2.64	2.99	6.68%

[a]2 h 100 mM KOH

Tables 1 and 2 summarize the precision and percent change of retention time and peak area data over seven days of a protein load of 2 µg/injection of fetuin hydrolysates. Decrease in retention time ranged from -1.0 to -2.2% over time. The loss of up to 2.2% of retention time is extremely low and all peaks were separated and easily quantified, even after over 300 injections. Single-day and seven-day retention time RSDs are presented, but with the note that their value is limited because of the slight negative trend in the data. Peak area changes ranged from 0.65 to -5.82%. The peak area RSD ranged from 2.15 to 4.43%. Figure 4 shows chromatography from the reproducibility studies with fetuin hydrolysates, including a chromatogram after a 2 h 100 mM KOH column wash that was executed to restore the retention time. As noted in the previous archived version of TN 40, partial retention time restoration is achieved with a 100 mM base wash, and complete restoration of column capacity will require the use of premade eluents in an off-line system mode.

Table 3. Precision and percent change of retention time over seven days of IgG hydrolysate analysis

	Retention Time RSD			Retention Time (min)			% Change
Analyte	Day 1	Day 7	Over 7 Days	Day 1	Day 7	After Wash on Day 7	After Wash[a] on Day 7
Fucose	0.24	0.00	0.62	3.99	3.93	3.99	0.08%
Galactosamine	0.15	0.00	0.76	6.58	6.45	6.59	0.08%
Glucosamine	0.12	0.00	0.80	7.94	7.77	7.94	0.04%
Galactose	0.14	0.63	1.02	8.95	8.67	8.93	-0.16%
Glucose	0.15	0.61	0.80	9.95	9.65	9.93	-0.16%
Mannose	0.11	0.60	0.98	11.45	11.12	11.43	-0.19%

[a]2 h 100 mM KOH

Table 4. Precision and percent change of peak area over seven days of IgG hydrolysate analysis

	Peak Area RSD			Peak Area			% Change
Analyte	Day 1	Day 7	Over 7 Days	Day 1	Day 7	After Wash on Day 7	After Wash[a] on Day 7
Fucose	0.60	2.90	3.94	3.06	2.96	3.20	4.67%
Galactosamine	0.29	1.24	2.50	6.47	6.41	6.75	4.28%
Glucosamine	0.56	0.89	2.92	5.16	5.15	5.17	0.06%
Galactose	0.89	0.89	4.43	3.76	3.58	3.64	-3.23%
Glucose	0.81	0.76	1.13	4.45	4.46	4.58	2.83%
Mannose	0.35	0.01	3.80	3.14	3.14	3.33	5.86%

[a]2 h 100 mM KOH

Tables 3 and 4 summarize the precision and percent change of retention time and peak area data over seven days of a protein load of 2 µg/injection of IgG hydrolysates. Similar to the fetuin data, retention time decrease ranged from -1.5 to -3.0% over the seven days. Peak area changes ranged from 0.9 to -4.8%. The peak area RSD ranged from 1.13 to 4.43%. As noted for fetuin, single-day and seven-day retention time RSDs are presented, but with the note that their value is limited because of the slight negative trend in the data.

In both hydrolysate experiments (fetuin and IgG), retention time gradually decreased over seven days. This could be due to the accumulation of a portion of the hydrolysate sample matrix on the column, resulting in a slight loss of column capacity. Decreases in retention times ranged from 1 to 3%. As shown in Tables 1 and 3, the largest actual increase in retention time was 0.33 min (mannose in the IgG experiment), which did not result in any peak coelution (Figure 5).

Overall, the data is similar to or better than data reported in the previous archived version of TN 40, which was collected using a Dionex ICS-3000 system. Note that the Au on PTFE disposable working electrodes provide the advantage of increased lifetime compared to the previously used disposable Au on polyester (P/N 060139) working electrode. Disposable Au working electrodes on the polyester substrate are limited to 100 mM hydroxide as the maximum eluent strength, and have a lifetime of two weeks. After two weeks of use, there is a possibility of delamination of the electrode from the substrate, resulting in a loss in response. Gold on PTFE electrodes do not delaminate from the substrate at hydroxide concentrations >100 mM and have consistent response for at least four weeks and excellent electrode-to-electrode reproducibility.[6]

Ruggedness

The variance due to different columns was tested by comparing results from columns from two different lots. Both column sets showed similar precisions and trends in retention time and peak area.

Linearity

Response curves were generated for the six monosaccharides between 1 and 700 µM (Figure 6). The upper limit of linearity for each monosaccharide is shown in Table 5. Fucose and galactosamine are linear through 170 µM, glucosamine is linear up to 210 µM, galactose up to 420 µM, glucose up to 210 µM, and mannose up to 420 µM. Above these concentrations, a >10% decrease in response (relative to response predicted by the other concentrations) was observed. These concentrations reflect the upper limits found when a mix of monosaccharides was tested. The upper limits result due to exceeding either the column capacity or the limit of available electrolytic sites on the Au electrode.

Detection Limits

Lower limits of detection were determined for monosaccharides by injecting small quantities (0.025, 0.25, 0.5, and 1 µM) of Dionex MonoStandard. At 0.08–0.17 µM, 10 µL injection (i.e., at 0.8–1.7 pmol), signal-to-noise ratios for the monosaccharides were at least 3:1. Detection limits may be improved through larger injection volume (provided the column is not overloaded).

Conclusion

The HPAE-PAD-based method on a Dionex ICS-5000 chromatography system for monosaccharide composition analysis is fast and capable of providing reproducible retention time and detector response for hundreds of samples over several days. The disposable Au working electrode contributes to the reproducibility of PAD between electrodes and between laboratories. Overall, the method has high sample throughput, high precision, and performance ruggedness for glycoprotein monosaccharide analysis.

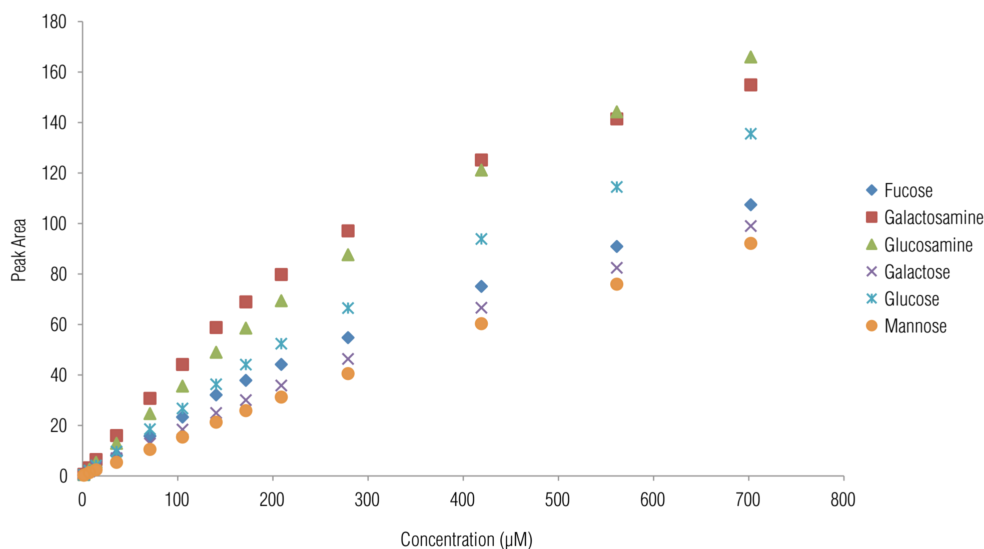


Figure 6. Plot of concentration vs response for the monosaccharides

Table 5. Method lower limit of detection and upper limit of linearity

	Lower Limit of Detection (µM)	Upper Limit of Linearity (µM)
Fucose	0.17	170
Galactosamine	0.08	170
Glucosamine	0.10	210
Galactose	0.14	420
Glucose	0.11	210
Mannose	0.16	420

Suppliers

VWR, 1310 Goshen Parkway, West Chester, PA 19380, U.S.A., Tel: 800-932-5000.

Sigma-Aldrich Co., P.O. Box 2060, Milwaukee, WI 53201, U.S.A., Tel: 800-558-9160.

Sarstedt Inc., 1025 St. James Church Road, P. O. Box 468, Newton, NC 28658-0468, U.S.A., Tel: 828-465-4000.

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