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Glycoprotein monosaccharide analysis using HPAE-PAD with manually prepared eluent

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Keywords

Glycoprotein, Monosaccharide, HPAE-PAD, Manually prepared eluent, Dionex CarboPac columns, Dionex AminoTrap column

Goal

To present an accurate method of determining monosaccharides in glycoproteins using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) and manually prepared eluent

Introduction

Glycosylation plays an important part in protein structure and function.[1] Factors such as cell age, culture conditions, and purification affect the nature of protein glycosylation. As understanding of the role glycoproteins play in many biological processes[2,3] increases, the number of protein-based therapeutics will likely grow rapidly. In the near future, patents will be expiring for several key biotherapeutic glycoproteins. This will create an increased emphasis on the production of biosimilars[4] and need for methods to characterize protein glycosylation for quality control.

Two major types of protein glycosylation are observed in nature. The first type is *N*-linked glycosylation, which involves attachment of oligosaccharide chains to a side-chain nitrogen of the amino acid asparagine. The second type of glycosylation is *O*-linked, involving attachment through the hydroxyl-containing side chains of amino acids like serine and threonine. Overall, *N*-linked glycosylation is more clearly understood than *O*-linked glycosylation.

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Determination of the monosaccharide composition of a glycoprotein pharmaceutical is a typical quality control assay in the pharmaceutical industry. High-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) is a well-established method for glycoprotein carbohydrate analysis. HPAE-PAD separates carbohydrates with specific interactions between their hydroxyl and carboxyl groups based on charge, size, composition, isomerism, and linkages. Therefore, HPAE-PAD is the best method for determining monosaccharides, sialic acids, and other carbohydrates. It allows for direct detection without sample derivatization, thereby reducing analyst time, expense, and exposure to hazardous chemicals. It is a selective technique with sensitive detection. Fast separations can be performed without loss of resolution using Thermo Scientific™ Dionex™ CarboPac™ PA20 columns.

The goal of this work is to describe an HPAE-PAD method for monosaccharide composition analysis using manually prepared eluent. Monosaccharide analysis using electrolytically generated eluent has been described before.[5] Here, three commercially available proteins, IgG, fetuin, and alpha-1-acid glycoprotein (AGP), were individually subjected to two hydrolysis conditions using 1) HCl, for the amino sugars galactosamine and glucosamine, and 2) TFA, for the neutral sugars mannose, glucose, and galactose. Results for method linearity, robustness, and accuracy for monosaccharide quantification are discussed here.

Experimental

Conditions

Columns:	Dionex CarboPac PA20, 3 × 150 mm (P/N 060142), Thermo Scientific™ Dionex™ AminoTrap™ 3 × 30 mm column (P/N 060146)
Column Temperature:	30 °C
Compartment Temperature:	30 °C
Flow Rate:	0.5 mL/min
Eluent:	A) DI water B) 0.2 M sodium hydroxide
Working Electrode:	Gold disposable on PTFE (P/N 066480)
Sampler Tray Temperature:	4 °C
Injection Volume:	10 µL (push_partial_LS)
Typical Backpressure:	2600 psi
Sample Loop Size:	20 µL
Elution Conditions:	10 mM NaOH for 12 min, 200 mM NaOH for 10 min, 10 mM NaOH for 10 min (Table 1)

Table 1. Elution conditions.

Time (min)	Solution A (%)	Solution B (%)	Elution
0	95	5	10 mM NaOH
12	95	5	10 mM NaOH
12.001	0	100	200 mM NaOH
22	0	100	200 mM NaOH
22.001	95	5	Start re-equilibration
32	95	5	End

Equipment

- A Thermo Scientific™ Dionex™ ICS-5000+ Reagent-Free Ion Chromatography (RFIC™) system was used in this work. The Dionex ICS-5000+ HPIC system is a modular ion chromatograph that includes:
  - DP dual pump module (P/N 079975) with degas option
  - DC standard bore detector compartment (P/N 075943) with dual temperature zones, two injection valves
  - Electrochemical detector (P/N 072042) and Cell (P/N 072044)
  - pH-Ag/AgCl reference electrode (P/N 061879)
  - Carbohydrate disposable Au working electrode, pack of 6 (two 2.0 mil gaskets included) (P/N 066480)
- AS-AP autosampler (P/N 074926) with cooling tray option (recommended)
- Sterile assembled microcentrifuge tubes with screw cap, 1.5 mL (Sarstedt® P/N 72.692.005)
- Nalgene Rapid-Flow 0.2 µm filter units, 1000 mL, nylon membrane, 90 mm diameter (Thermo Scientific P/N 164-0020)

Table 2 describes the carbohydrate four-potential waveform for the electrochemical detector.

Table 2. Carbohydrate four-potential waveform for the ED.
Reference electrode used in Ag mode (Ag/AgCl reference).

Time (s)	Potential (V)	Gain	Ramp Region	Integration
0	0.1	Off	On	Off
0.2	0.1	On	On	On
0.4	0.1	Off	On	Off
0.41	-2	Off	On	Off
0.42	-2	Off	On	Off
0.43	0.6	Off	On	Off
0.44	-0.1	Off	On	Off
0.5	-0.1	Off	On	Off

Reagents and standards

- Deionized (DI) water, Type I reagent grade, 18 MΩ·cm resistivity or better
- Sodium hydroxide, 50% w/w (Fisher Scientific™ P/N SS254-500)
- Human serum IgG (Sigma® P/N I4506)
- Bovine serum fetuin (Sigma P/N F2379)
- Alpha-1-acid glycoprotein from human plasma (Sigma P/N G9885)
- Thermo Scientific™ Pierce™ Micro BCA™ Protein Assay Kit (P/N 23235)
- Thermo Scientific Pierce Trifluoroacetic acid (TFA), sequencing grade (P/N 28904)
- Thermo Scientific Pierce Hydrochloric acid (P/N 24308)

Preparation of eluent and reagents

200 mM sodium hydroxide eluent

Dilute 10.4 mL of a 50% (w/w) sodium hydroxide solution into 1 L of DI water to prepare a 0.2 M sodium hydroxide solution. After preparation, keep the eluent blanketed under UHP-grade nitrogen (5.0) or UHP-grade helium (5.0) at 34 to 55 kPa (5 to 8 psi) at all times. Note: Hydroxide eluents for HPAE-PAD should be prepared only from commercial 50% (w/w) sodium hydroxide solutions. Please see Technical Note 71[6] for more information on preparing eluents for HPAE-PAD.

Carbohydrate standards

Dissolve the contents of one Thermo Scientific™ 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.

Methods

TFA and HCl hydrolysis

1. Human serum IgG
   - HCl hydrolysates: Prepare HCl hydrolysates for IgG by combining 400 µL of 6 M HCl with 20 µL of 3 mg/mL IgG in a 1.5 mL microcentrifuge tube.
   - TFA hydrolysates: 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 in a 1.5 mL microcentrifuge tube.
2. Bovine fetuin and AGP
   - TFA hydrolysates: Prepare TFA hydrolysates of fetuin and AGP by combining 20 µL of 3 mg/mL protein solution, 150 µL DI water, and 30 µL of neat TFA in a 1.5 mL microcentrifuge tube.
   - HCl hydrolysates: Combine 400 µL of 6 M HCl with 20 µL of 3 mg/mL fetuin solution in a 1.5 mL microcentrifuge tube.
3. Heat the solutions for 4 h at 100 °C and then dry for 3 h at room temperature in a Thermo Scientific™ Savant™ SpeedVac™ concentrator equipped with an acid trap.
4. Reconstitute each vial with 300 µL of DI water.
5. Vortex for 30 s and centrifuge for 5 min. Inject 10 µL of the supernatant (2 µg protein per injection) into the ion chromatography system.

Dionex BorateTrap column

Borate is a known contaminant in laboratory water supplies. In chromatography, borate contamination of HPLC eluents can come from degrading (i.e. poorly maintained) deionized water systems or it may come from leaching from glass eluent bottles, which should not be used for HPAE-PAD. We have found that if borate is present in the eluent, it forms anionic complexes with carbohydrate analytes. Because the carbohydrate-borate complex is less efficiently eluted by hydroxide from the anion exchanger than the carbohydrate itself, peak tailing occurs. Analytes with vicinal hydroxyl groups, such as sugar alcohols and mannose, show severe chromatographic tailing when borate is present in the eluents. This tailing causes the peak to differ from a Gaussian distribution (where peak asymmetry = 1), making it difficult to identify and quantify the carbohydrate analytes. If peak tailing of mannose (or alditols) is observed, the Thermo Scientific™ Dionex™ BorateTrap™ Inline Trap column may be used to remove borate from the eluent stream. The Dionex BorateTrap column should be installed between the HPLC pump and the sample injector. After installation, the mannose peak should appear symmetric. With prolonged use, if the capacity of the Dionex BorateTrap is exceeded, peak tailing of mannose may become apparent. If this occurs, the Dionex BorateTrap should be replaced.

Dionex AminoTrap column

The Dionex AminoTrap column delays the elution of amino acids and small peptides found in glycoprotein hydrolysates. The Dionex AminoTrap column 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), the six monosaccharides will be well resolved. Please see Technical Note 125[7] for guidance on successful use of Dionex AminoTrap columns.

Note: Do not pump water through the Dionex AminoTrap column; it will cause irreversible damage to the column.

Results and discussion

Separation

Separation of monosaccharides was achieved using a Dionex CarboPac PA20 column (3 × 150 mm) with a Dionex AminoTrap guard column using isocratic elution conditions, followed by a step change to higher eluent concentration that was used to remove contaminant species, including carbonate, still bound to the column. The Dionex AminoTrap column delays the elution of the amino acids and small peptides from protein acid hydrolysis that could interfere with monosaccharide peak integration and response. Figure 1 shows a typical separation of a 10 µL injection of the Dionex MonoStandard, containing fucose, galactosamine, glucosamine, galactose, glucose, and mannose, each at 10 µM concentration (100 pmol each). The peaks are baseline resolved and elute within a window of 13 min. The total run time is 32 min to allow for washing and re-equilibration after the column regeneration step. The chromatogram shows not only the region where the monosaccharides elute, but also the column wash and re-equilibration regions.

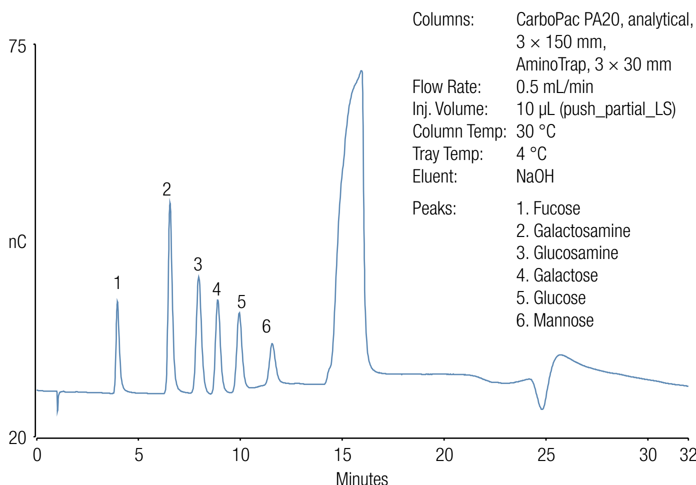

Figure 1. Separation of a 10 µM Dionex MonoStandard injection containing 10 µM each of the six monosaccharides.

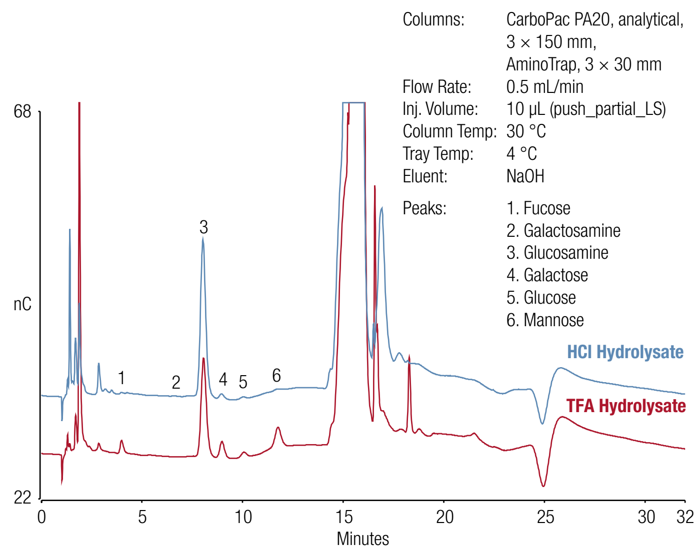

Figure 2. Analysis of human serum IgG TFA and HCl hydrolysates.

For each of the three proteins used in this study, hydrochloric acid (HCl) and trifluoroacetic acid (TFA) hydrolysates were prepared and injected directly after drying. Each injection contained 2 µg protein. Figure 2 shows typical injections of human serum IgG TFA as well as HCl hydrolysates. Human serum IgG has lower carbohydrate content compared to most mammalian glycoproteins.

The low concentration of carbohydrate makes monosaccharide determination more challenging because there is a higher concentration of peptides and amino acids in the acid hydrolysate, relative to glycoproteins when there is more glycosylation. However, the proposed method is sensitive enough to determine monosaccharides in this sample without derivatization. The TFA hydrolysis is done to determine the neutral sugars, fucose, galactose, and mannose. The yield of the amino sugars, galactosamine and glucosamine, is not 100% (commonly estimated to be 95%), but many scientists use these or similar hydrolysis conditions to determine amino sugars.

To improve amino sugar accuracy, some scientists use HCl hydrolysis, as we have done here. HCl hydrolysis conditions destroy a majority of the neutral sugars. While glucose is observed in the hydrolysates, it is nearly always a contaminant as it is not typically present in glycoprotein oligosaccharides. Note the difference in the column cleaning section of the chromatogram compared to the chromatogram of the standard in Figure 1.

Figure 3 presents the HPAE-PAD chromatograms of bovine fetuin TFA and HCl 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. As fetuin is more glycosylated than IgG, note the increase in the ratio of the size of the monosaccharide peaks to the peaks in the column cleaning section of the chromatogram compared to human serum IgG.

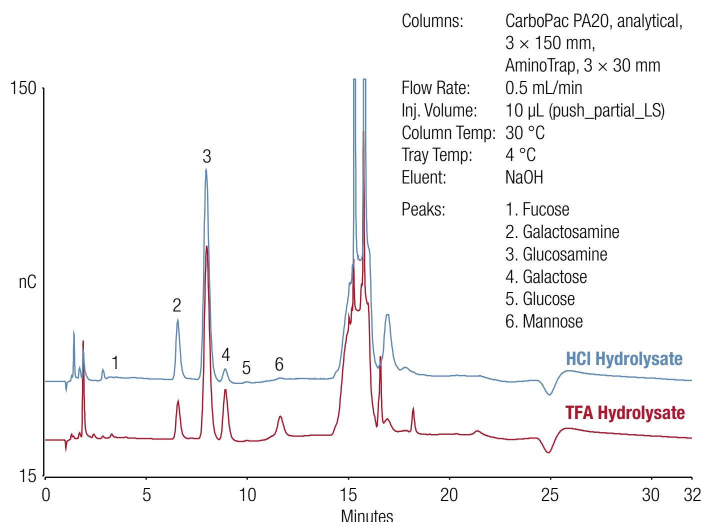

Figure 3. Analysis of bovine fetuin TFA and HCl hydrolysates.

Figure 4 shows HPAE-PAD chromatograms from human alpha-1-acid glycoprotein (AGP) hydrolysates. AGP has the highest level of glycosylation among the three proteins studied here. Both TFA and HCl hydrolysates show well-resolved amino and neutral sugars.

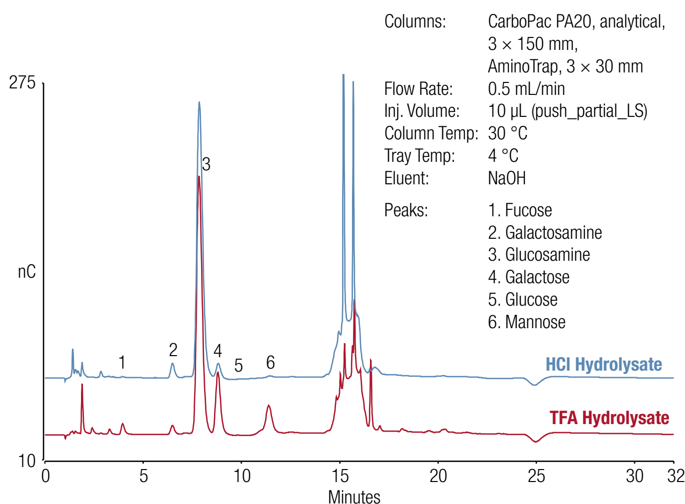

Figure 4. Analysis of AGP TFA and HCl hydrolysates.

Linearity and precision

The linearity of monosaccharide determination was studied by generating peak area response curves for all six monosaccharides using a monosaccharide standard mix containing 1.56 to 300 µM of each of the six monosaccharides, except galactosamine for which the linearity range was from 1.56 to 50 µM. The results included in Table 3 show that the coefficients of determination ranged from 0.983 to 0.999 for all six monosaccharides. A linear curve fit was used for five of the six monosaccharides, and a second order polynomial curve fit was used for glucosamine. Figure 5 shows calibration plots obtained for each of the six monosaccharides.

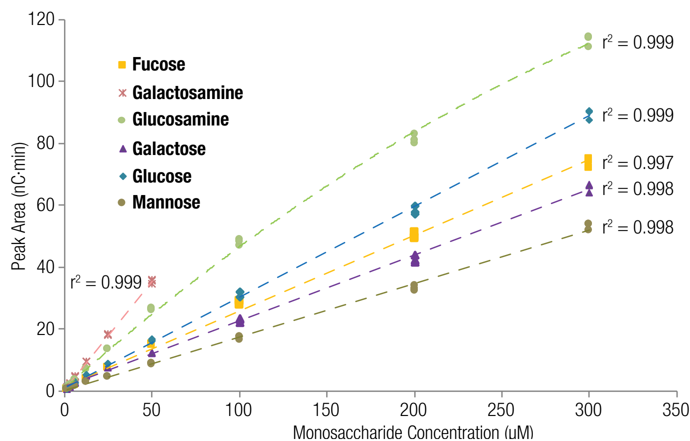

Figure 5. Calibration plots generated for each of the six monosaccharides.

The method precision was determined in two ways. First, method repeatability was determined at three concentrations of the six Dionex MonoStandard mix with three replicates of each sample to give nine total injections. The results included in Table 4 show excellent peak area as well as retention time precision for all three concentration levels tested with all RSD values below 2%. The intermediate precision was determined by assaying eight replicate injections of the 10 µM Dionex MonoStandard each day for three consecutive days. The results of this experiment contained in Table 5 show excellent retention time as well peak area precision. The highest RSDs for retention time and peak area were 0.58% and 3.39%, respectively.

Table 3. Calibration data for monosaccharides (n=3).

Peak No.	Peak Name	Retention Time (min)	Concentration Range (µM)	Levels	Coefficient of Determination
1	Fucose	3.91	1.56-300	9	0.999
2	Galactosamine	6.48	1.56–50	6	0.999
3	Glucosamine	7.87	1.56–300	9	0.983
4	Galactose	8.83	1.56–300	9	0.997
5	Glucose	9.87	1.56–300	9	0.998
6	Mannose	11.42	1.56–300	9	0.998

Table 4. Method precision determined at three concentrations (n=3).

Standard Conc	Retention Time and Peak Area RSDs											
	Fucose		Galactosamine		Glucosamine		Galactose		Glucose		Mannose	
	RT	Peak Area	RT	Peak Area	RT	Peak Area	RT	Peak Area	RT	Peak Area	RT	Peak Area
3.12 µM	0.12	1.14	0.07	1.84	0.01	1.57	0.01	1.78	0.05	1.89	0.07	0.73
12.5 µM	0.13	1.12	0.00	0.91	0.06	0.92	0.01	1.06	0.05	1.08	0.05	0.95
100 µM	0.00	1.67	0.00	1.46	0.06	1.42	0.01	1.22	0.00	1.33	0.01	1.07

Table 5. Intermediate precision determined using 10 µM Dionex MonoStandard samples over three days (n=8).

Days	Retention Time and Peak Area RSDs											
	Fucose		Galactosamine		Glucosamine		Galactose		Glucose		Mannose	
	RT	Peak Area	RT	Peak Area	RT	Peak Area	RT	Peak Area	RT	Peak Area	RT	Peak Area
1	0.29	2.08	0.41	2.21	0.49	2.09	0.34	1.91	0.45	2.6	0.58	1.93
2	0.09	3.39	0.06	3.37	0.07	3.4	0.06	2.92	0.04	3.01	0.06	3.43
3	0.1	3.02	0.09	2.63	0.05	2.55	0.08	1.81	0.06	2.59	0.05	2.12

Detection limits

The monosaccharide detection limits for this HPAE-PAD assay under the conditions described was set at monosaccharide concentrations that resulted in a signal-to-noise ratio of 10:1. A series of monosaccharide standards were prepared and analyzed. The signal-to-noise ratios were calculated using the peak height for each monosaccharide; the noise level was calculated from a stable portion of the baseline where no peak elutes. Table 6 contains limits of detection for all six monosaccharides determined in this study.

Table 6. Method sensitivity determination (n=3).

Monosaccharide	Detection Limit (µM)	Amount Injected (pmoles)	S/N Ratio
Fucose	0.25	2.5	13.4
Galactosamine	0.1	1.0	10.9
Glucosamine	0.2	2.0	9.7
Galactose	0.25	2.5	13.7
Glucose	0.25	2.5	11.7
Mannose	0.2	2.0	9.5

Accuracy

Accuracy of the assay was determined by spiking a known amount of monosaccharides into each of the dried and reconstituted acid hydrolysates prepared for the three glycoproteins used in the study. For each monosaccharide, 20% to 150% spike levels based on calculated endogenous monosaccharide concentration were used. The monosaccharides present below the lowest calibration standard were not quantified and hence were not spiked. The results in Table 7 show excellent recoveries of the spiked monosaccharides with all the recoveries falling between 80% and 120%.

Table 7. Recovery of monosaccharide spikes in to the acid hydrolysates prepared for all three glycoproteins used in this study (n=3).

Sample	Parameter	AGP HCl Digest	AGP TFA Digest	Fetuin HCl Digest	Fetuin TFA Digest	IgG HCl Digest	IgG TFA Digest
Fucose	Base Conc. (µM)	-	5.3	-	-	-	-
	Spike%	-	37.5	-	-	-	-
	%Recovery	-	120	-	-	-	-
Galactosamine	Base Conc. (µM)	3.2	1.9	6.7	4.7	-	-
	Spike%	62.1	104	89.3	129	-	-
	%Recovery	98.3	94.5	87.4	76.9	-	-
Glucosamine	Base Conc. (µM)	162.5	142.9	45.9	44.9	11.0	6.2
	Spike%	49.0	55.0	130.8	66.8	72.9	129.03
	%Recovery	90.1	102	93.2	102	79.3	98.9
Galactose	Base Conc. (µM)	8.6	58.3	-	20.1	-	-
	Spike%	23.2	137	-	150	-	-
	%Recovery	84.7	104	-	94.1	-	-
Mannose	Base Conc. (µM)	2.1	55.3	-	21.7	-	7.5
	Spike%	94.0	145	-	138	-	107
	%Recovery	113	108	-	97.8	-	105

Robustness

Assay robustness was determined on three columns, two new columns from the same lot and a 6-month-old column from a different lot. Figure 6 shows representative chromatograms of the 10 µM monosaccharide standard on all three columns used. The robustness was studied by introducing ±10% variation in common chromatographic parameters. The parameters varied in this study were: initial eluent concentration, final eluent concentration, column temperature, and flow rate. Method performance under these conditions was evaluated by calculating percent difference in three key chromatographic parameters: retention time, peak asymmetry, and resolution.

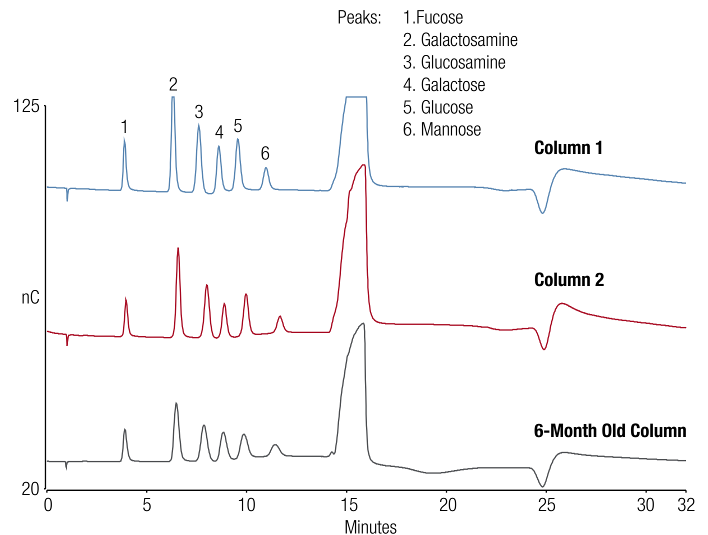

Figure 6. Separation of a 10 µM Dionex MonoStandard injection containing 10 µM each of the six monosaccharides on three different columns used for robustness.

The results are included in Tables 8, 9, and 10 for column 1, column 2, and column 3 (the 6-month-old column), respectively. For the first two columns, none of the experimental variations tested here resulted in significant disruption of the three target chromatography parameters. The highest impact was observed when the column temperature was reduced to 27 °C, which resulted in approximately 18% reduction in resolution between glucosamine and galactose. Even under these conditions, the resolution between these two peaks was 1.7 for column 1 and 1.9 for column 2. This level of resolution remains good for quantitative analysis. In the robustness studies using the 6-month-old column, the highest impact was also observed for the reduced-temperature condition. This condition resulted in the highest percent difference of 16.6 as compared to the non-stressed method. The lowest resolution between glucosamine and galactose is 1.33 for the low temperature condition, which is not ideal for peak quantification. The impact of lower temperature is more severe for the older column because it has reduced retention of the target monosaccharides at the start of the experiment compared to columns 1 and 2, as can be observed in Figure 6. This difference is due to the natural loss of column capacity with injection of samples that can sometimes be reversed with an aggressive column cleaning. This cleaning was not done for column 3 before the start of the robustness experiment. Refer to Dionex CarboPac PA20 column manual[8] section 5.3.1 for column cleaning methods.

Table 8. Results of robustness study performed on column 1 using 10 µM Dionex MonoStandard samples containing 10 µM of each monosaccharide (n=3).

Condition	Percent Difference (%)																
	Retention Time						Asymmetry						Resolution				
	Fuc	GalN	GlcN	Gal	Glc	Man	Fuc	GalN	GlcN	Gal	Glc	Man	Fuc	GalN	GlcN	Gal	Glc
0.5 mL/min, 10 mM/200 mM NaOH, Column Temp. 30 °C																	
0.5 mL/min, 9 mM/200 mM NaOH, Column Temp. 30 °C	1.5	2.7	3.3	2.5	3.0	4.0	3.5	0.9	1.6	0.6	-2.1	10.3	2.3	2.4	-6.5	4.6	7.1
0.5 mL/min, 11 mM/200 mM NaOH, Column Temp. 30 °C	-0.7	-1.7	-2.2	-1.5	-1.9	-2.6	3.0	2.4	2.9	1.8	1.8	-7.5	-2.0	-2.4	6.8	-3.3	-4.3
0.5 mL/min, 10 mM/200 mM NaOH, Column Temp. 27 °C	2.2	5.6	5.9	4.1	4.9	5.0	1.7	3.0	0.0	2.8	1.2	-7.5	3.9	-2.1	-18.5	5.9	-0.5
0.5 mL/min, 10 mM/200 mM NaOH, Column Temp. 33 °C	-2.7	-6.2	-6.5	-4.5	-5.3	-5.7	5.0	6.3	5.3	3.1	2.1	-8.6	-6.5	0.0	21.7	-6.5	-0.3
0.45 mL/min, 10 mM/200 mM NaOH, Column Temp. 30 °C	12.0	12.4	12.7	12.4	12.6	13.1	5.0	6.6	5.9	5.6	2.4	-9.4	2.4	3.4	0.1	4.0	7.2
0.55 mL/min, 10 mM/200 mM NaOH, Column Temp. 30 °C	-8.4	-8.3	-8.06	-8.3	-8.1	-7.8	4.2	5.4	3.6	3.4	4.9	-7.5	-2.4	-1.3	-5.5	-0.4	0.8
0.5 mL/min, 10 mM/180 mM NaOH, Column Temp. 30 °C	0.2	-0.0	0.00	-0.06	-0.03	0.02	5.5	7.2	5.6	4.0	4.6	-0.5	-1.4	-0.2	-0.9	0.1	0.6
0.5 mL/min, 10 mM/220 mM NaOH, Column Temp. 30 °C	0.3	0.2	0.32	0.1	0.2	0.3	6.7	7.6	4.9	3.7	3.9	1.6	-1.2	0.1	-2.3	0.8	1.3

Table 9. Results of robustness study performed on column 2 using 10 µM Dionex MonoStandard samples containing 10 µM of each monosaccharide (n=3).

Condition	Percent Difference (%)																
	Retention Time						Asymmetry						Resolution				
	Fuc	GalN	GlcN	Gal	Glc	Man	Fuc	GalN	GlcN	Gal	Glc	Man	Fuc	GalN	GlcN	Gal	Glc
0.5 mL/min, 10 mM/200 mM NaOH, Column Temp. 30 °C																	
0.5 mL/min, 9 mM/200 mM NaOH, Column Temp. 30 °C	1.3	2.5	3.1	2.3	2.7	3.7	3.7	2.1	-0.3	0.2	-1.1	5.2	1.4	2.7	-7.1	4.6	5.5
0.5 mL/min, 11 mM/200 mM NaOH, Column Temp. 30 °C	-1.1	-2.3	-2.9	-2.1	-2.6	-3.4	0.0	1.6	1.5	0.5	0.5	-0.5	-3.5	-3.3	5.2	-4.9	-6.7
0.5 mL/min, 10 mM/200 mM NaOH, Column Temp. 27 °C	2.0	5.5	5.8	3.9	4.8	4.9	-0.6	0.0	-1.5	0.5	-0.8	-0.2	3.8	-2.2	-18.0	6.1	-1.1
0.5 mL/min, 10 mM/200 mM NaOH, Column Temp. 33 °C	-2.6	-5.9	-6.3	-4.5	-5.3	-5.5	1.7	5.4	6.3	2.8	1.4	1.9	-8.6	-1.8	15.7	-7.3	-2.4
0.45 mL/min, 10 mM/200 mM NaOH, Column Temp. 30 °C	11.0	11.0	11.1	11.0	11.0	11.1	5.1	7.9	5.1	4.3	3.3	0.2	-2.0	-0.4	-0.9	-0.4	-0.3
0.55 mL/min, 10 mM/200 mM NaOH, Column Temp. 30 °C	-9.2	-9.6	-9.6	-9.7	-9.6	-9.7	-2.4	1.6	1.8	1.7	0.0	-0.5	-4.1	-3.1	-3.9	-2.1	-2.8
0.5 mL/min, 10 mM/180 mM NaOH, Column Temp. 30 °C	-0.3	-0.7	-0.8	-0.9	-0.9	-1.0	2.0	4.3	3.6	4.0	1.9	4.4	-4.0	-2.6	-2.5	-1.6	-2.8
0.5 mL/min, 10 mM/220 mM NaOH, Column Temp. 30 °C	-0.5	-1.1	-1.2	-1.4	-1.4	-1.4	1.5	5.4	3.0	4.0	3.9	1.6	-4.9	-2.7	-4.6	-1.9	-2.8

Table 10. Results of robustness study performed on column 3 (old column) using 10 µM Dionex MonoStandard containing 10 µM of each monosaccharide (n=3).

Condition	Percent Difference (%)																
	Retention Time						Asymmetry						Resolution				
	Fuc	GalN	GlcN	Gal	Glc	Man	Fuc	GalN	GlcN	Gal	Glc	Man	Fuc	GalN	GlcN	Gal	Glc
0.5 mL/min, 10 mM/200 mM NaOH, Column Temp. 30 °C																	
0.5 mL/min, 9 mM/200 mM NaOH, Column Temp. 30 °C	1.3	2.8	3.5	2.5	3.1	4.2	-0.7	-1.9	-1.5	2.6	-3.6	1.3	2.5	2.9	-8.1	5.7	7.3
0.5 mL/min, 11 mM/200 mM NaOH, Column Temp. 30 °C	-1.1	-1.8	-2.3	-1.7	-2.1	-2.7	-0.9	-1.3	1.5	0.0	0.5	-3.2	-1.7	-2.6	4.8	-3.4	-4.1
0.5 mL/min, 10 mM/200 mM NaOH, Column Temp. 27 °C	2.1	5.7	6.0	4.1	4.9	5.1	-3.9	-2.7	-5.7	0.6	-2.2	-5.6	5.4	0.1	-16.6	6.9	0.2
0.5 mL/min, 10 mM/200 mM NaOH, Column Temp. 33 °C	-3.0	-6.2	-6.6	-4.7	-5.5	-5.8	2.3	2.9	6.3	1.1	1.4	-0.5	-8.2	-3.8	16.4	-8.8	-2.7
0.45 mL/min, 10 mM/200 mM NaOH, Column Temp. 30 °C	10.9	10.9	10.8	15.2	7.0	10.8	2.1	2.4	2.7	2.0	-1.9	1.3	-1.4	-1.8	-0.2	9.9	-9.5
0.55 mL/min, 10 mM/200 mM NaOH, Column Temp. 30 °C	-9.1	-9.1	-9.1	-9.2	-9.2	-9.2	-2.7	-1.3	0.3	1.1	0.0	-1.3	-2.2	-2.3	-3.7	-1.9	-1.6
0.5 mL/min, 10 mM/180 mM NaOH, Column Temp. 30 °C	-0.3	-0.4	-0.4	-0.4	-0.4	-0.5	0.9	1.6	1.5	0.6	1.1	0.3	-2.1	-2.2	-1.9	-1.9	-2.7
0.5 mL/min, 10 mM/220 mM NaOH, Column Temp. 30 °C	-0.9	-1.3	-1.4	-1.7	-1.7	-1.7	1.6	0.8	1.2	2.0	1.4	0.3	-2.7	-2.5	-4.0	-1.3	-1.9

Conclusion

This study describes an HPAE-PAD assay for determination of the monosaccharide composition of a glycoprotein. A single injection provides analysis of six monosaccharides. This assay for monosaccharide quantification was validated according to the analytical performance characteristics outlined in USP General Chapter <1225>, Validation of Compendial Procedures.[9] The results show that the method can provide baseline separation for all the monosaccharides in HCl as well TFA hydrolysates. Even for IgG, which has low glycosylation and, therefore, a greater protein-to-carbohydrate ratio compared to many other glycoproteins, the separation is good. The method shows excellent precision for retention time as well as peak area. The method was shown to accurately measure the monosaccharide concentration in complex matrices such as acid-hydrolyzed proteins. Moreover, the method is robust to experimental condition variations that may occur during routine use.

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