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APPLICATION NOTE 72580

An improved HPAE-PAD method for glycoprotein monosaccharide determination

Authors
Sachin Patil and Jeffrey Rohrer
Thermo Fisher Scientific,
Sunnyvale, CA, USA

Keywords
Dionex ICS-5000+, RFIC system, carbohydrates, fetuin, alpha-1-acid glycoprotein, Dionex CarboPac PA20-Fast-4µm column, biopharmaceutical

Goal

To demonstrate an improved HPAE-PAD assay for determination of the monosaccharide composition of a glycoprotein

Introduction

Determination of the monosaccharide composition of a glycoprotein pharmaceutical is a key quality control assay for glycoprotein-based therapeutics. High-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) is a well-established method for glycoprotein carbohydrate analysis.[1] This work demonstrates an improved HPAE-PAD method using a recently introduced Thermo Scientific™ Dionex™ CarboPac™ PA20 column with 4 µm particle size to simultaneously quantify six major monosaccharides present in glycoprotein acid digests. The total run time is 20 min as compared to 32 min with the method using a 6.5 µm particle size column.[2] The smaller particle size of this column offers higher peak efficiencies, leading to high-resolution separations and allowing a shorter column format and, ultimately, significantly shorter run times.

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The shorter column format also results in lower eluent consumption, thereby improving overall process economics.

Here, two different commercially available proteins, bovine fetuin and alpha-1-acid glycoprotein (AGP), were individually subjected to two different sets of hydrolysis conditions—using HCl, which favors release of amino sugars like galactosamine and glucosamine, and using TFA, which favors the release of neutral sugars like mannose, glucose, and galactose. The more efficient peaks also allow the injection of less sample for routine monosaccharide quantification. The method described here requires 0.5 µg injected protein compared to 2 µg used in the method previously described.[2] This assay was validated according to the analytical performance characteristics outlined in USP General Chapter <1225>, Validation of Compendial Procedures.[3] The method shows excellent retention time and peak area precision and provided accurate determinations of the monosaccharide content of the two test glycoproteins. Moreover, the method is robust to experimental condition variations that occur during routine use.

Experimental

Equipment

- A Thermo Scientific™ Dionex™ ICS-5000+ Reagent-Free™ Ion Chromatography (RFIC™) system was used in this work. The Dionex ICS-5000+ system is an integrated ion chromatograph that includes the following:
  - SP single pump module (P/N 061707) or DP Dual Pump (P/N 061712) with degas option
  - DC detector compartment (P/N 061767) with single-temperature zone
  - Electrochemical detector (P/N 061719) and cell (P/N 061757)
  - pH-Ag/AgCl reference electrode (P/N 061879)
  - Carbohydrate disposable Au working electrode, pack of 6 (six 2.0 mil gaskets included) (P/N 066480)
- Thermo Scientific™ Dionex™ AS-AP autosampler (P/N 074926) with cooling tray option (recommended)
- Sterile assembled micro-centrifuge tubes with screw cap, 1.5 mL (Sarstedt® P/N 72.692.005)
- Thermo Scientific™ Nalgene™ Rapid-Flow™ 0.2 µm filter units, 1000 mL, nylon membrane, 90 mm diameter (P/N 164-0020)

Software

- Thermo Scientific™ Chromeleon™ Chromatography Data System software version 7.2, SR4

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)
- Bovine serum fetuin (Sigma® P/N F2379)
- Alpha-1-acid glycoprotein from human plasma (Sigma P/N G9885)
- Thermo Scientific Pierce trifluoroacetic acid (TFA), sequencing grade (P/N 28904)
- Thermo Scientific Pierce hydrochloric acid (P/N 24308)
- Thermo Scientific™ Dionex™ MonoStandard™ mix of six carbohydrate standards (P/N 043162)

Preparation of eluent and reagents

Eluent solution

Generate the potassium hydroxide (KOH) eluent online by pumping high-quality degassed DI water (no biological contamination) through the Dionex EGC 500 KOH cartridge. Thermo Scientific™ 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.[4] 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.

Carbohydrate 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.

Conditions

Columns:	Dionex CarboPac PA20-Fast-4µm, 2 × 100 mm column (P/N 302749) Dionex CarboPac PA20 -Fast-4µm 2 × 30 mm guard column (P/N 302750)
Column Temp.:	30 °C
Compartment Temp.:	30 °C
Flow Rate:	0.22 mL/min
Eluent:	KOH
Eluent Source:	Thermo Scientific™ Dionex™ EGC 500 KOH (P/N 075778)
Working Electrode:	Gold disposable on PTFE (P/N 066480)
Sampler Tray Temp.:	4 °C
Inj. Volume:	2.5 µL (full loop)
Typical Backpressure:	3800 psi
Elution Conditions:	10 mM KOH for 8 min, 100 mM KOH from 8.01 to 14 min, 10 mM KOH from 14.01 to 20 min

Time (min)	*Eluent Concentration*
0	10 mM KOH
8	10 mM KOH
8.01	100 mM KOH
14	100 mM KOH
14.01	10 mM KOH, Re-equilibrium
20	10 mM KOH, End

Methods

TFA hydrolysis

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 hydrolysis

Combine 400 µL of 6 M HCl with 20 µL of 3 mg/mL fetuin or AGP solution in a 1.5 mL microcentrifuge tube. Heat the solutions for 4 h at 100 °C and then dry at room temperature in a Thermo Scientific™ Savant™ SpeedVac™ concentrator equipped with an acid trap for ~3 h. Reconstitute each vial with 300 µL of DI water. Vortex for 30 s and centrifuge for 5 min. Inject 2.5 µL of the supernatant (0.5 µg protein per injection) into the ion chromatography system.

Dionex CarboPac PA20-Fast-4µm column cleaning

The Dionex CarboPac PA20-Fast-4µm column can be readily cleaned by rinsing the column with ~60 column volumes of 100 mM KOH or NaOH. For more stubborn contamination problems refer to Dionex CarboPac PA200-Fast-4µm column manual[5] (Appendix A.4) for column cleaning methods. Cleaning with eluents stronger than 100 mM hydroxide will require manual preparation of eluents.

Dionex BorateTrap column

Because this work uses electrolytic eluent generation using a Dionex EGC 500 KOH cartridge, a Thermo Scientific™ Dionex™ BorateTrap™ column is not required. When using manually prepared eluent it may be necessary to install a Dionex BorateTrap column if poor water quality is suspected. Borate is

Table 1. Carbohydrate 4-potential waveform for the ED.

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

Reference electrode used in Ag mode (Ag/AgCl reference).

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 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 is observed, the Dionex BorateTrap column may be used to remove borate from the eluent stream. The Dionex BorateTrap column should be installed between the pump and the sample injector. After installation, the mannose peak should appear symmetric. With prolonged use, if the capacity of the Dionex BorateTrap column is exceeded, peak tailing of mannose may become apparent. If this occurs, the Dionex BorateTrap column should be replaced.

Results and discussion

Separation

A typical separation achieved using a Dionex CarboPac PA20-Fast-4µm column (2 × 100 mm format) is shown in Figure 1. The method includes initial 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. A 2.5 µL injection of the Dionex MonoStandard mix containing fucose, galactosamine, glucosamine, galactose, glucose, and mannose, each at 10 µM concentration (25 pmol), results in baseline-resolved peaks that elute within a window of 6 min. The total run time is 20 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.

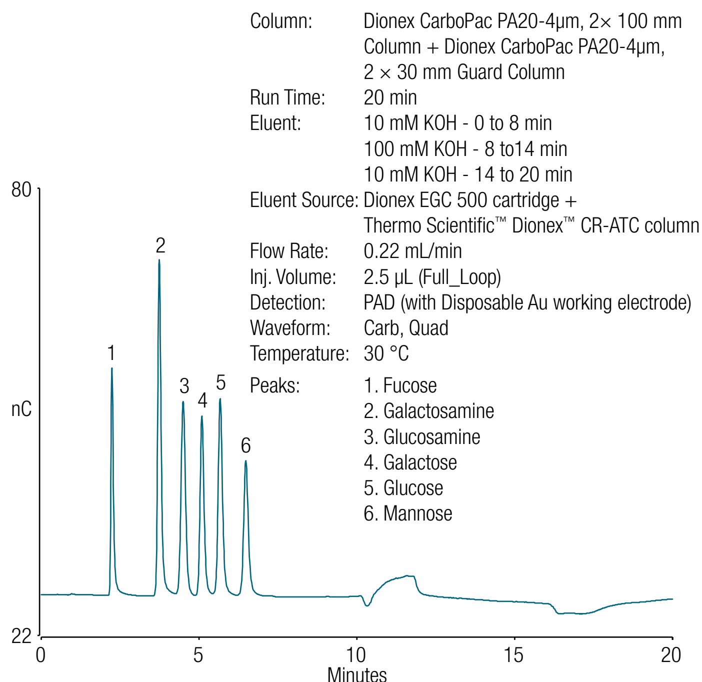

Figure 1. Monosaccharide separation on a Dionex CarboPac PA20-Fast-4µm, 2 × 100 mm column.

For each of the two proteins used in this study, hydrochloric acid (HCl) and trifluoroacetic acid (TFA) hydrolysates were prepared and injected directly, after drying in a SpeedVac concentrator and suspending in water as described above. Each injection contained 0.5 µg protein. Figure 2 shows typical injections of bovine fetuin TFA as well as HCl hydrolysates. 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 the amino sugars too. 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.

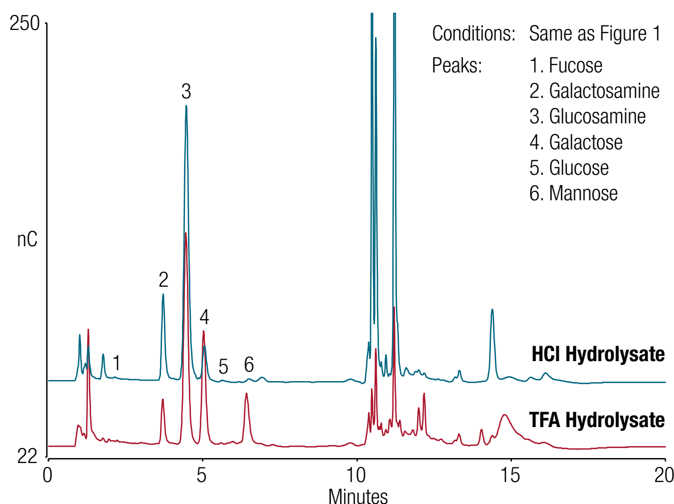

Figure 2. Analysis of bovine fetuin TFA and HCl hydrolysates.

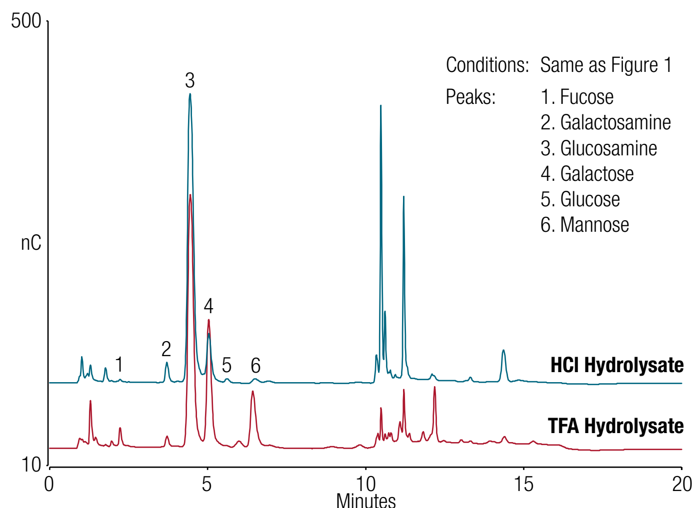

Figure 3. Analysis of AGP TFA and HCl hydrolysates.

Figure 3 presents the HPAE-PAD chromatograms of human alpha-1-acid glycoprotein (AGP) hydrolysates. The monosaccharide peaks are baseline-resolved. As expected, 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 AGP is more glycosylated than fetuin, 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 fetuin. Both figures suggest that even less protein could be injected for accurate quantification.

Linearity and precision

The linearity of monosaccharide determination was studied by generating response curves for all six monosaccharides using a monosaccharide standard mix containing 1.56 to 300 µM of each of the six monosaccharides. Table 2 contains the calibration range and coefficients of determination for each of the six monosaccharides. A linear curve fit was used for four of the six monosaccharides, and a second order polynomial curve fit was used for galactosamine and glucosamine.

The method precision was determined at three concentrations of the six monosaccharide standard mix with three replicates of each standard concentration to give nine total injections. The results included in Table 3 show excellent peak area as well as retention time precision for all three concentration levels tested with all RSD values below 3%.

Table 2. Calibration data for monosaccharides (n=3).

Peak Number	Peak Name	Retention Time (min)	Concentration Range (µM)	Levels	Coefficient of Determination
1	Fucose	2.24	1.56–50	6	0.995
2	Galactosamine	3.68	1.56–50	6	1.000
3	Glucosamine	4.46	1.56–300	9	1.000
4	Galactose	5.09	1.56–100	7	0.995
5	Glucose	5.67	1.56–50	6	0.993
6	Mannose	6.48	1.56–100	7	0.994

Note: Linear curve fits were used for all monosaccharides, except galactosamine and glucosamine for which a second order polynomial curve fit was used.

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

Standard Conc. (µM)	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
1.56	0.22	4.53	0.26	1.09	0.22	2.86	0.19	1.71	0.17	1.26	0.22	1.71
12.5	0.01	2.20	0.00	1.99	0.00	1.54	0.00	1.55	0.00	1.48	0.00	2.31
50	0.22	0.90	0.00	1.30	0.11	1.36	0.09	1.75	0.08	2.06	0.00	2.43

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 both the glycoproteins used in the study. A 20 µM of monosaccharide concentration spike was used for each monosaccharide. The monosaccharides present below the lowest calibration standard were not quantified and hence were not spiked. The spike levels based on calculated endogenous monosaccharide concentration are included in Table 4 along with calculated recoveries. The results show excellent recoveries of the spiked monosaccharides with all the recoveries falling between 82% and 94%.

Table 5 contains monosaccharide composition data for both proteins as moles of monosaccharide per mole protein. These calculations used the protein molecular weight provided by the supplier and protein concentration calculated at A_{280}. The monosaccharide composition obtained here is consistent with previous results considering that the protein samples and method of protein quantification are different.[2] These results for fetuin and AGP, two highly glycosylated proteins, are consistent with previous results that used the Thermo Scientific™ Dionex™ AminoTrap™ column in place of the guard column. This is likely due to injecting far less protein and therefore suppression of electrochemical signal does not occur. We have found a small impact on galactosamine signal for the lightly glycosylated human serum IgG.

Table 4. Recovery of monosaccharide spikes into the acid hydrolysates prepared for glycoproteins used in this study (n=3).

		Fucose	Galactosamine	Glucosamine	Galactose	Mannose
AGP HCl Extract	Base Amount Present (µM)	-	4.02	188	-	-
	%Spike	-	497	10.6	-	-
	% Recovery	-	91.6	89.9	-	-
AGP TFA Extract	Base Amount Present (µM)	4.55	-		70.9	47.9
	%Spike	439	-	-	28.2	41.7
	% Recovery	94.5	-	-	92.2	93.7
Fetuin HCl Extract	Base Amount Present (µM)	-	8.73	53.7	-	-
	%Spike	-	229	37.2	-	-
	% Recovery	-	91.6	89.9	-	-
Fetuin TFA Extract	Base Amount Present (µM)	-	-	-	28.1	18.0
	%Spike	-	-	-	71.0	110
	% Recovery	-	-	-	82.6	88.2

Table 5. Monosaccharide compositions of glycoproteins determined using methods described here.

Protein	Monosaccharide	mol/mol		
		Literature[6]	TN72225[2]	This Work
Fetuin (Mol. Wt. 48,400)	Galactosamine	1.9	1.6	2.3
	Glucosamine	15.0	11.2	13.3
	Galactose	13.0	4.9	6.87
	Mannose	7.7	5.3	4.4
AGP (Mol. Wt. 40,000)	Fucose	2–4	1.07	0.92
	Glucosamine	22–28	32.8	38.0
	Galactose	14–25	11.8	14.3
	Mannose	11–14	11.2	9.68

Robustness

Assay robustness was determined on two columns. 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. The results are included in Tables 6 and 7 for column 1 and column 2, respectively. For both 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 19% reduction in resolution between glucosamine and galactose. Even under these conditions, the resolution between these two peaks was 2.6 for column 1 and 1.9 for column 2. This level of resolution remains good for quantitative analysis.

Table 6. Results of robustness study performed on column 1 using 10 µM Dionex MonoStandard mix 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
0.22 mL/min, 10 mM/200 mM KOH, Column Temperature 30 °C																
0.22 mL/min, ***9*** mM/100 mM KOH, Column Temperature 30 °C	1.05	2.45	3.09	2.34	2.88	3.72	-1.67	0.00	-3.51	2.52	-1.10	0.55	2.31	3.39	-7.07	5.16	6.87
0.22 mL/min, ***11*** mM /100 mM KOH, Column Temperature 30 °C	-1.05	-2.08	-2.56	-1.87	-2.28	-3.20	-2.09	1.02	-0.58	0.00	0.00	3.30	-2.31	-2.29	5.52	-3.94	-6.67
0.22 mL/min, 10 mM /100 mM KOH, Column Temperature ***27*** °C	2.11	5.43	5.80	4.21	5.10	5.13	-0.63	-1.02	-3.51	1.40	-2.47	1.92	4.26	-2.89	-18.10	6.11	-0.83
0.22 mL/min, 10 mM /200 mM KOH, Column Temperature ***33*** °C	-1.66	-4.89	-5.28	-3.34	-4.14	-4.50	1.88	4.06	2.92	-0.28	-0.27	4.95	-4.66	2.19	19.38	-5.43	-1.46
0.2 mL/min, 10 mM /200 mM KOH, Column Temperature 30 °C	10.24	10.69	10.55	10.97	10.74	10.63	2.93	1.52	0.00	2.24	1.92	0.55	1.62	2.59	2.97	1.09	1.77
0.24 mL/min, 10 mM /200 mM KOH, Column Temperature 30 °C	-8.28	-8.24	-8.29	-8.23	-8.34	-8.38	-0.21	0.51	-1.75	0.28	0.55	0.55	-1.76	-1.69	-3.25	-0.95	-1.35
0.22 mL/min, 10 mM /***90*** mM KOH, Column Temperature 30 °C	0.45	0.09	0.23	0.40	0.42	0.26	-3.77	2.54	-2.05	0.00	-1.37	3.85	-0.33	0.80	-0.28	0.54	-0.83
0.22 mL/min, 10 mM /***110*** mM KOH, Column Temperature 30 °C	0.00	-0.18	-0.23	-0.07	-0.06	-0.26	-0.42	2.03	-1.46	1.12	-0.82	1.92	-0.73	0.00	0.99	-0.14	-1.46

Table 7. Results of robustness study performed on column 2 using 10 µM Dionex MonoStandard mix 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
0.22 mL/min, 10 mM/200 mM KOH, Column Temperature 30 °C																
0.22 mL/min, ***9*** mM/100 mM KOH, Column Temperature 30 °C	1.11	2.26	2.72	1.87	2.49	3.42	-3.96	-0.93	0.28	1.50	0.25	4.18	2.03	2.49	-5.75	4.52	6.48
0.22 mL/min, ***11*** mM /100 mM KOH, Column Temperature 30 °C	-1.11	-2.50	-3.25	-2.61	-3.00	-3.82	-3.76	1.17	2.56	0.50	0.50	1.23	-2.71	-3.04	3.83	-3.45	-6.59
0.22 mL/min, 10 mM /100 mM KOH, Column Temperature ***27*** °C	1.66	5.17	5.37	3.46	4.43	4.66	-0.40	-0.23	0.00	-0.25	0.50	-0.25	4.23	-3.50	-15.76	6.24	0.41
0.22 mL/min, 10 mM /200 mM KOH, Column Temperature ***33*** °C	-2.36	-5.65	-6.10	-4.42	-5.24	-5.50	0.40	1.17	5.11	0.00	4.77	3.93	-5.75	0.83	14.91	-6.77	-1.42
0.2 mL/min, 10 mM /200 mM KOH, Column Temperature 30 °C	9.85	9.85	9.69	9.76	9.77	9.72	-1.19	0.23	3.69	1.75	1.51	0.00	1.66	1.47	2.45	1.86	1.42
0.24 mL/min, 10 mM /200 mM KOH, Column Temperature 30 °C	-8.88	-9.04	-9.09	-9.30	-9.21	-9.14	-0.79	0.93	1.99	-1.50	1.26	0.25	-2.57	-2.67	-3.62	-1.20	-1.52
0.22 mL/min, 10 mM /***90*** mM KOH, Column Temperature 30 °C	-0.42	-0.40	-0.66	-0.74	-0.56	-0.62	-1.78	-1.63	2.27	-0.50	-0.50	1.23	-0.44	-0.74	-0.75	0.66	-0.41
0.22 mL/min, 10 mM /***110*** mM KOH, Column Temperature 30 °C	-0.97	-1.13	-1.26	-1.13	-1.07	-1.15	0.40	-1.17	1.99	0.50	0.25	0.25	-1.05	-1.47	0.00	-0.27	-1.22

Conclusion

An improved HPAE-PAD assay for determination of the monosaccharide composition of a glycoprotein is described in this study. Run time for a single injection that provides analysis of six monosaccharides is 20 min, which is a significant time savings over the previous method.[1] The results show that the method can provide baseline separation for all the monosaccharides in HCl as well as TFA hydrolysates.

This assay for monosaccharide quantification was validated according to the analytical performance characteristics outlined in USP General Chapter <1225>, Validation of Compendial Procedures.[3] The method shows excellent precision for retention time as well as peak area. Spike recovery studies show high recoveries indicating good method accuracy. The method is able to withstand matrix effects and can 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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