

Determination of glycoprotein sialic acid composition using HPAE-PAD in dual eluent generation cartridge mode

Authors: Jingli Hu and Jeffrey Rohrer
Thermo Fisher Scientific, Sunnyvale, CA

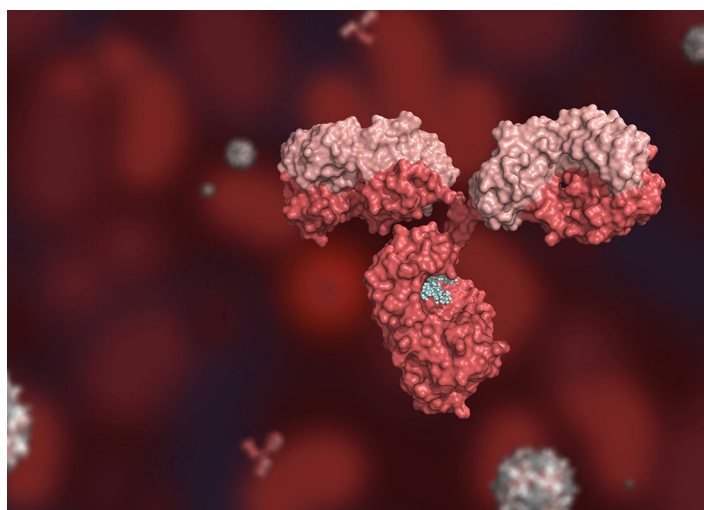
Keywords: Dionex CarboPac PA20-1 mm column, Dionex ICS-6000 HPIC system, electrochemical detection, Dual EGC Mode, *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc)

Goal

To demonstrate that the glycoprotein sialic acid determination method in Thermo Scientific Application Update (AU) 180 can be executed with a Thermo Scientific™ Dionex™ CarboPac™ PA20-1 mm column using KOH/KMSA eluent produced electrolytically using dual eluent generation cartridge (Dual EGC) mode

Introduction

Sialic acids are derivatives of the nine-carbon sugar neuraminic acid. The two important sialic acids are *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc), and these can be found in many biomolecules including glycoproteins, proteoglycans, and glycolipids. Sialic acids play several vital physiological functions due to their negative



charge and hydrophilicity.¹ For recombinant therapeutic glycoproteins, sialylation can maintain the properties of the native glycoprotein, such as circulatory half-life, biological activity, and solubility, and sometimes these properties can be improved when the protein is engineered to have increased sialylation.² The presence of Neu5Gc in a therapeutic glycoprotein can potentially cause an immune response in humans because humans generally do not produce Neu5Gc.³ Therefore, it is important to determine the sialic acid content of a glycoprotein when assaying for therapeutic efficacy and safety.

Sialic acids can be determined by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) after releasing them from the glycoprotein by acid hydrolysis or enzymatic digestion. HPAE-PAD features direct detection, and therefore, eliminates errors associated with analyte derivatization. AU 180 demonstrated the determination of sialic acids in five representative glycoproteins by acid hydrolysis followed by HPAE-PAD using a Thermo Scientific™ Dionex™ CarboPac™ PA20-3 mm column with manually prepared sodium hydroxide/sodium acetate eluents.⁴

In this application note, the AU 180 method was evaluated with a Dionex CarboPac PA20-1 mm column using HPAE-PAD in Dual EGC mode. The 1 mm column requires a flow rate about nine times less than the 3 mm column, significantly reducing eluent consumption. Dual EGC mode operation avoids the manual preparation of the sodium hydroxide/sodium acetate eluents. This mode uses a methanesulfonic acid (MSA) eluent generation cartridge (EGC) and a potassium hydroxide (KOH) EGC in series to generate an extremely reproducible and accurate KOH/KMSA eluent gradient needed for the sialic acid separation. Key performance parameters were evaluated, including separation, linearity, limits of detection, accuracy, and precision. The sialic acid contents of three glycoprotein samples were determined.

Experimental

Equipment

- Thermo Scientific™ Dionex™ ICS-6000 HPIC system including:
 - Thermo Scientific™ Dionex™ ICS-6000 DP Pump module or SP Pump module
 - Thermo Scientific™ Dionex™ ICS-6000 EG Eluent Generator module
 - Thermo Scientific™ Dionex™ ICS-6000 DC Detector/Chromatography module with ED Electrochemical Detector
 - Thermo Scientific™ Dionex™ AS-AP Autosampler with sample tray cooling (P/N 074926)
 - 4-port valve rebuild kit (P/N 074699), which includes a 0.4 µL injection loop
 - Thermo Scientific™ Dionex™ Dual EG Eluent Degasser (P/N 22181-60951)

- Thermo Scientific™ Dionex™ ICS-6000 ED Electrochemical Detector Cell (P/N 072044)
- Gold on PTFE Disposable working electrode with 1 mil gasket, Pack of 6 (P/N 303397)
- Reference electrode, pH, Ag/AgCl (P/N 061879)
- Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software, version 7.2.9
- Thermo Scientific™ SpeedVac™ SPD 140DDA Vacuum Concentrator
- Thermo Scientific™ Nanodrop™ 2000c Spectrophotometer
- Thermo Scientific™ Barnstead/Thermolyne™ Dri-Bath™ Block

Assemble the cell following the Dionex ICS-6000 Operator's Manual⁵ and Dionex ED User's Compendium for Electrochemical Detection.⁶

Consumables

- Thermo Scientific™ Nalgene™ Rapid-Flow 0.2 µm filter units, 1,000 mL, nylon membrane, 90 mm diameter (P/N 164-0020)
- Microcentrifuge tube, 2 mL (Fisher Scientific P/N 05-408-138)
- Nitrogen, ultrahigh purity grade from Airgas
- Thermo Scientific™ Dionex™ EGC 400 KOH Potassium Hydroxide Eluent Generator Cartridge (P/N 302766)
- Thermo Scientific™ Dionex™ EGC 400 MSA Methanesulfonic Acid Eluent Generator Cartridge (P/N 302767)
- Thermo Scientific™ polypropylene autosampler vials, 1.5 mL (Fisher Scientific P/N NC0380837)

Reagents and standards

- Deionized (DI) water, Type I reagent grade, 18 MΩ·cm resistivity or better
- *N*-Acetylneuraminic acid (Neu5Ac), (Sigma-Aldrich P/N 19023-10MG)
- *N*-Glycolylneuraminic acid (Neu5Gc), (Sigma-Aldrich P/N 362000-10MG)
- Acetic acid, Glacial (Fisher Scientific P/N A38S-500)
- Thermo Scientific™ Micro BCA™ Protein Assay Kit, (P/N 23235)

Samples

Three glycoproteins were selected for analysis.

- Sample #1: Bovine apo-transferrin (b. apo-transferrin), (Sigma-Aldrich P/N T1428)
- Sample #2: Calf fetuin (fetuin), (Sigma-Aldrich P/N F2379)
- Sample #3: Human α_1 -acid glycoprotein (h. AGP), (Sigma-Aldrich P/N G9885)

Chromatographic conditions

Parameter	Value
Columns	Dionex CarboPac PA20 Guard, 1 x 30 mm (P/N 303370) Dionex CarboPac PA20 Separation, 1 x 150 mm (P/N 303369)
Eluent	Gradient (Table 1)
Flow rate	0.056 mL/min
Column temperature	30 °C
Injection volume	0.4 μ L
Autosampler temperature	5 °C
Reference electrode	Ag/AgCl
Working electrode	Gold disposable electrode with a 1 mil (25.4 μ m) gasket
Detection	Pulsed Amperometric Detector (Electrochemical Detection)
Detection compartment temperature	30 °C
Detection waveform	Gold, Carbohydrates, 4-Potential (Table 2)
System backpressure	~3,700 psi (100 psi = 0.6894 MPa)
Run time	30 min

Table 1. Eluent gradient

Time (min)	KMSA (mM)	KOH (mM)
0	19	100
4	100	100
13	100	100
13	19	100
30	19	100

Table 2. Carbohydrate, 4-potential waveform

Time (s)	Potential (V) vs. Ag/AgCl	Integration
0	0.1	Off
0.2	0.1	On
0.4	0.1	Off
0.41	-2.0	Off
0.42	-2.0	Off
0.43	0.6	Off
0.44	-0.1	Off
0.5	-0.1	Off

System installation and precautions

Install the system according to Figure 1 and the Dual EGC mode installation guide.⁷ An operational vacuum degasser pump (part of the analytical pump) is important to the success of this and other electrochemical detection methods. This pump can be accidentally turned off during a system restart or instrument configuration. If the vacuum degasser pump is not running, poor baseline performance and loss of column capacity can be observed.

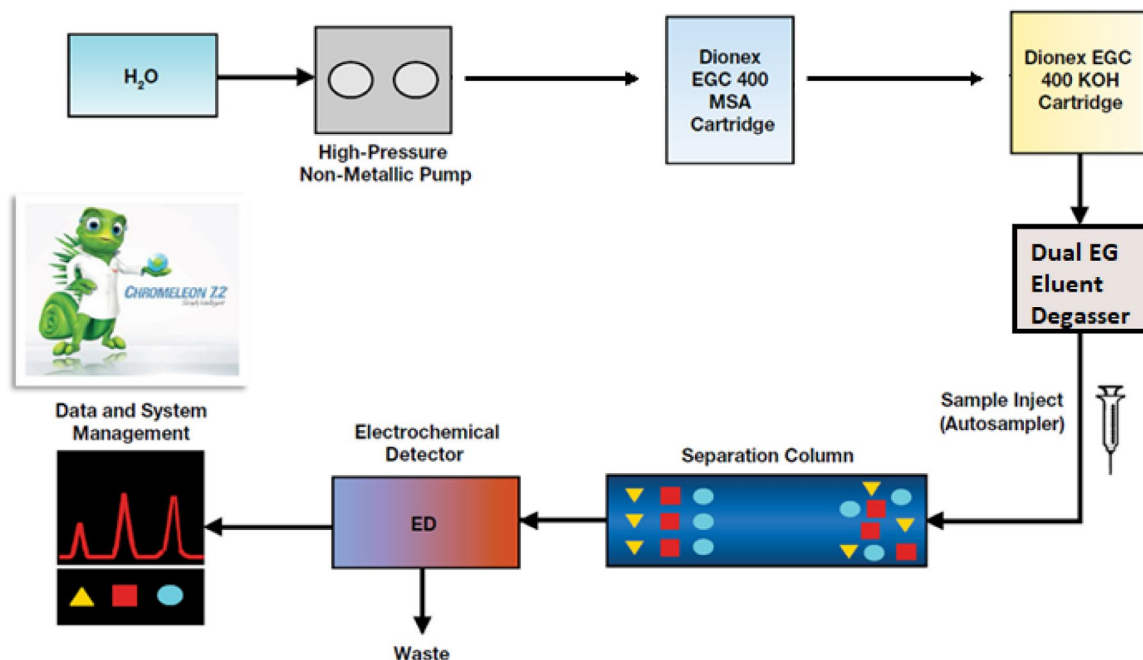


Figure 1. Dionex ICS-6000 HPIC system in Dual EGC mode

Always ensure that the vacuum degas pump is on before running a sequence. To do that, press F8 or click the “gear” command button on the main Chromeleon CDS instrument panel and locate the pump and then the degasser status. Choose “Expert” mode from the three modes available “Normal, Advanced, Expert.” Make sure the degasser is “On”, and “DegasserVacuum” is “OK”. If it is “OFF” and “NOT OK”, turn on the degasser by selecting “On” from the drop-down menu and wait until “DegasserVacuum” turns to “OK”. If the value reads NOT OK, check for leaks at all the connections necessary for vacuum. Install PEEK backpressure tubing (Item #22181-20031) as needed to achieve an instrument pressure value above 3,000 psi. To ensure the best system performance, only turn on the EGC power when the system pressure exceeds 3,000 psi. Fill a 2 L eluent bottle with degassed DI water. Connect the eluent bottle to the pump and keep the eluent under an insert gas (helium or nitrogen) at 5–8 psi, to minimize absorption of atmospheric carbon dioxide. Turn on the pump and pump DI water through the Dionex EGC 400 MSA cartridge for 15 min at a flow rate of 1 mL/min. Then condition the Dionex EGC 400 MSA cartridge for 30 min using 100 mM MSA at a flow rate of 0.1 mL/min. Connect the Dionex EGC 400 KOH cartridge and pump DI water through it for 15 min at a flow rate of 1 mL/min. Then condition the Dionex EGC 400 KOH cartridge for 30 min using 100 mM KOH at a flow rate of 0.1 mL/min. After conditioning the EGCs, install the Dionex CarboPac PA20-1 mm column set. Set KMSA and KOH to the desired concentrations for the application and keep the flow on at 0.056 mL/min for 60 min.

Preparation of solutions and reagents

Acetic acid, 4 M

Dilute 22.5 mL of glacial acetic acid to 100 mL with DI water. Use this solution to release sialic acids from glycoproteins.

Standards

Stock standard solutions

Dissolve 5 mg of Neu5Ac in 10 mL DI water and dissolve 5 mg of Neu5Gc in 10 mL of DI water. This results in 1.62 mM and 1.54 mM stock solutions, respectively. Add 619 μ L of 1.62 mM Neu5Ac and 65 μ L of 1.54 mM Neu5Gc to a polyethylene vial containing 9.316 mL of DI water to prepare a combined stock of 100 μ M Neu5Ac and 10 μ M Neu5Gc. Store at around -40 °C.

Working standard solutions

Prepare eight working standards by serially diluting the combined stock standards (100 μ M Neu5Ac and 10 μ M Neu5Gc) with DI water (Table 3). Prepare levels 6-8 by diluting the combined stock standards. Prepare levels 3-5 by diluting levels 6-8. Prepare levels 1-2 by diluting level 3. For example, mix 250 μ L of the combined stock standards with 750 μ L of DI water in a vial to yield the level-8 standard containing 25 μ M Neu5Ac and 2.5 μ M Neu5Gc. Mix 100 μ L of the level-8 standard with 900 μ L of DI water in a vial to yield the level-5 standard containing 2.5 μ M Neu5Ac and 0.25 μ M Neu5Gc. Prepare working standards daily from the stock solutions stored at around -40 °C.

Sample preparation

Protein stock solutions, 4.0 mg/mL nominal

Dissolve 3.78 mg of sample #1 (b. apo-transferrin) in 0.945 mL of DI water to prepare a 4 mg/mL solution.

Table 3. Sialic acid calibration standards

Level	Neu5Ac concentration (μ M)	Neu5Gc concentration (μ M)	Neu5Ac amount (pmol) (0.4 μ L)	Neu5Gc amount (fmol) (0.4 μ L)
1	0.1	0.01	0.04	4
2	0.25	0.025	0.1	10
3	0.5	0.05	0.2	20
4	1	0.1	0.4	40
5	2.5	0.25	1	100
6	5	0.5	2	200
7	10	1	4	400
8	25	2.5	10	1,000

Gently swirl to thoroughly mix the solution. Prepare 200 μL aliquots of the solution in microcentrifuge vials to minimize freeze/thaw cycles when the stock is needed. Store all protein solutions at around $-40\text{ }^{\circ}\text{C}$. Repeat this process as follows. Dissolve 3.78 mg of sample #2 (fetuin) in 0.945 mL of DI water. Dissolve 4.31 mg of sample #3 (h. AGP) in 1.078 mL of DI water. Protein may be lost both during freeze/thaw cycles and by adsorption to surfaces. Therefore, it is essential to measure the working stock protein concentrations before hydrolysis using a colorimetric BCA protein assay kit. Values listed in Table 4 are results from the BCA assay of the working stock solutions using a Nanodrop 2000c Spectrophotometer.

Acetic acid hydrolysis of proteins

Add 138 μg of b. apo-transferrin, 93 μg of fetuin, and 91 μg of h. AGP to individual 2 mL microcentrifuge tubes to produce a total volume of 200 μL sample in 2 M acetic acid, as detailed in Table 4. For example, pipet 35 μL of the b. apo-transferrin stock, 65 μL of DI water, and 100 μL of 4 M acetic acid to prepare the solution for hydrolysis. Hydrolyze the protein solutions for 3 h at $80\text{ }^{\circ}\text{C}$ on a Dri-Bath (i.e., heat block). After hydrolysis, dry the sample hydrolyzate in a SpeedVac equipped with an acid trap and then resuspend the dry hydrolyzate in 2 mL of DI water. Please note that this acid hydrolysis method may not be optimized for complete release of all sialic acids without degradation of the free sialic acids. Optimization of the hydrolysis conditions for a given sample and analysis method is highly recommended. Additional hydrolysis conditions may be found in Thermo Scientific Technical Note 41.⁸ To avoid underestimating the sialic acid content due to acid-catalyzed degradation, perform analysis of samples prepared by dilution within 24 h of hydrolysis.

Table 4. Protein hydrolysate concentrations

Sample (S#)	Protein	BCA measured working solution conc. (mg/mL)	Volume protein (μL)	Amount of protein (μg)	Volume DI water (μL)	Volume 4 M acetic acid (μL)	Protein conc. ($\mu\text{g}/\mu\text{L}$)
1	b. apo-Transferrin	3.94	35	138	65	100	0.690
2	Fetuin	2.66	35	93.0	65	100	0.465
3	h. AGP	2.60	35	91.0	65	100	0.455

Results and discussion

Separation

The Dionex CarboPac PA20 column has been specially developed to provide high-resolution separation of monosaccharides and disaccharides.⁹ It is also the recommended column for determining sialic acids.^{4,10-12} Thermo Scientific AU 180 uses a Dionex CarboPac PA20-3 mm column with manually prepared sodium hydroxide/sodium acetate eluents. Table 5 shows the AU 180 separation conditions. The gradient from 70 to 300 mM NaOAc in 100 mM NaOH (0–7.5 min) separates Neu5Ac and Neu5Gc, 300 mM NaOAc in 100 mM NaOH (7.5–9 min) washes the column, and then there is a return to starting conditions (9.5–16.5 min) to re-equilibrate the column before the next injection.

Table 5. Eluent gradient of AU 180

Time (min)	NaOAc (mM)	NaOH (mM)
0	70	100
7.5	300	100
9	300	100
9.5	70	100
16.5	70	100

Dual EGC mode replaces the manual preparation of the sodium hydroxide/sodium acetate eluents. This mode uses a MSA EGC and a KOH EGC in series to generate an extremely reproducible and accurate KOH/KMSA eluent gradient needed for separating complex carbohydrates. A Dionex CarboPac PA20 column in a 1 mm diameter format was recently launched for use with the Dual EGC mode. KMSA is about two to three times stronger than sodium acetate. To match the strength of 300 mM sodium acetate,

150 mM KMSA is needed at 7.5 min. Dual EGC mode limits the total concentration of KOH+ KMSA to <200 mM. Therefore, we need to develop new Dual EGC mode eluent conditions to achieve a similar separation. We developed the gradient given in Table 1 to separate Neu5Gc and wash the column, followed by column re-equilibration. Figure 2 shows the separation of Neu5Ac and Neu5Gc using a Dionex CarboPac PA20-1 mm column set. The peaks are well separated and easily quantified. Additionally, the Neu5Ac peak is well separated from the void, which is an important consideration when analyzing acid hydrolyzed samples that may contain additional poorly retained compounds. The retention time of Neu5Ac matches with the AU 180 method, and of Neu5Gc elutes about 7 min later than reported in the AU 180 method. The column re-equilibrium is 10 min longer than in the AU 180 method. To confirm how effective the 9 min column rinsing step using 100 mM KOH + 100 mM KMSA is for this application, retention time stability was monitored. The retention times of Neu5Ac and Neu5Gc were stable for 15 days of continuous injections of samples and standards. If over longer analysis times retention times shorten, column cleaning with stronger manually prepared eluents may be necessary.

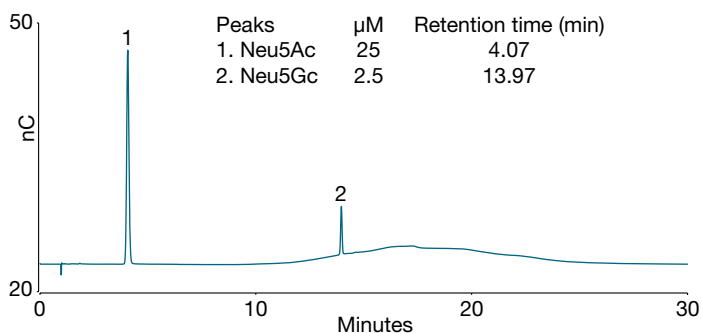


Figure 2. Separation of sialic acid standards using a Dionex CarboPac PA20-1 mm column set

Calibration, limit of detection (LOD), and limit of quantification (LOQ)

A calibration curve with eight concentration levels (Levels 1–8) was constructed for Neu5Ac, and a calibration curve with seven concentration levels (Levels 2–8) was constructed for Neu5Gc. In both cases, the response was linear for the range studied. Table 6 shows the calibration results for Neu5Ac and Neu5Gc. Figure 3 shows the two calibration curves. The calibration curves showed linearity in the selected calibration ranges with coefficients of determination (r^2) greater than 0.999.

The determination of LOD was based on the signal-to-noise ratio (S/N). The S/N is determined by comparing the measured signal from a standard with a low concentration of analyte with those of a blank and establishing the minimum concentration at which the analyte can be reliably detected. A S/N = 3 is used for estimating LOD, and a S/N = 10 is used for estimating the quantification limit (LOQ).¹³ In this study, the baseline noise was first determined by measuring the peak-to-peak noise in a representative 1 min segment of the baseline where no

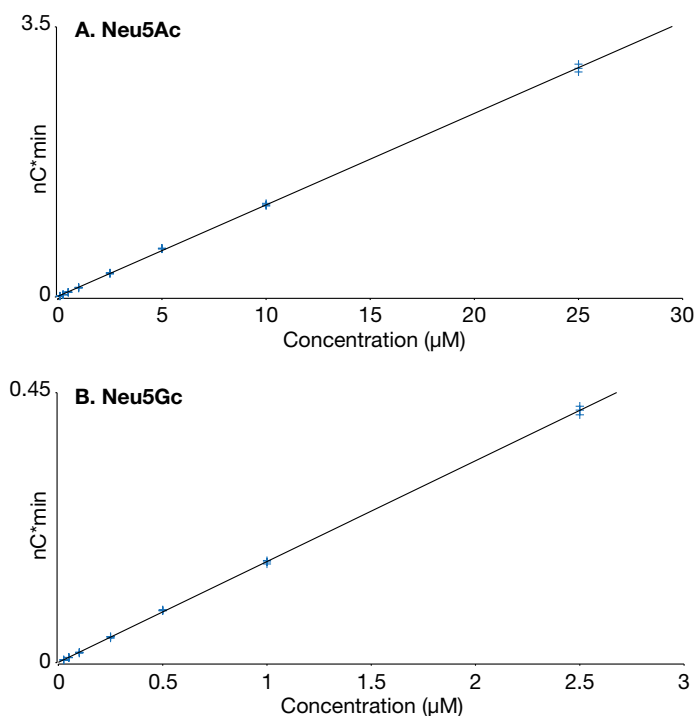


Figure 3. Sialic acid calibration curves

Table 6. Linearity, LOD, and LOQ

Standard	Concentration range (μM)	Amount range (μmol)	Coeff. of determination (r^2)	LOD conc. (nM)	LOQ conc. (nM)	LOD amount (fmol)	LOQ amount (fmol)
Neu5Ac	0.1–25	0.04–10	0.9996	20.9	69.7	8.36	27.9
Neu5Gc	0.025–2.5	0.01–1	0.9996	10.3	34.4	4.13	13.8

peaks elute but close to the peak of interest. The signal was determined from the average height of three injections of Neu5Ac at 25 nM and Neu5Gc at 25 nM. Table 6 summarizes the LOD and LOQ results. The calculated LOD concentrations of Neu5Ac and Neu5Gc were 20.9 nM and 10.3 nM, respectively. The injection volume was 0.4 μ L. Therefore, the LOD amounts of Neu5Ac and Neu5Gc correspond to 8.36 fmol and 4.13 fmol, respectively.

Sample analysis

Three glycoproteins were analyzed in this study. Triplicate acid hydrolysis was performed for each sample. Figure 4 shows the separation of sialic acids in three acid-hydrolyzed protein samples. Neu5Ac is well separated from early eluting components of the hydrolyzed samples. Neu5Ac is present in all three glycoproteins as expected; Neu5Gc is not detected in human forms of the glycoproteins (Sample #3) as expected. Table 7 shows the calculated results. The amounts of sialic acids determined in the protein samples are consistent with AU 180 and AU 181. The three replicate RSDs ranged from 0.37 to 3.82%.

Note: The vendor reports the MW of b. apo-transferrin is 76–81 kDa. An average MW of 79 kDa was used to calculate the moles of this protein. Likewise, the vendor shows the MW of fetuin is 51–67 kDa. An average MW of 59 kDa was used to calculate the moles of this protein.

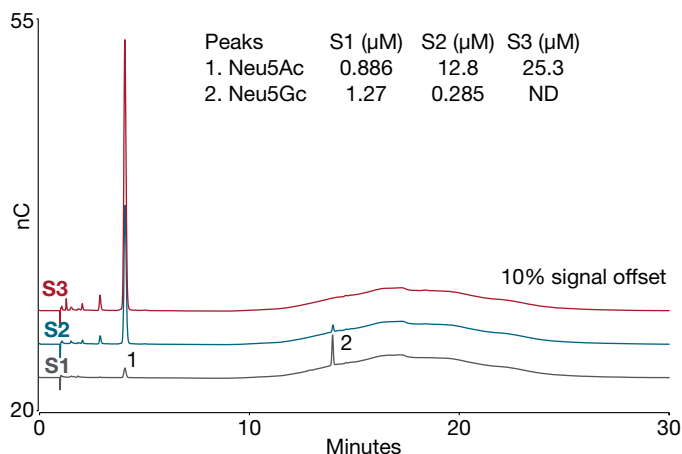


Figure 4. Sialic acid determination of three glycoprotein acid hydrolyzates

Method accuracy and precision

Method accuracy was evaluated through spike recovery studies. Sialic acids were spiked into each sample hydrolyzate solution at 50 to 100% of the original amount. The recovery for sialic acids in the three samples ranged from 95.1 to 105% (Table 8). While this does not evaluate the accuracy of the sialic acid release from the glycoproteins, which has already been evaluated by comparing to published results, it shows that the method facilitates accurate measurements of sialic acids without column or detector overload. The inter-day precision was determined by triplicate injection of the level-6 calibration standard on three separate days. The calculated peak area precision varied from 0.83 to 1.04%, with retention time precision <0.2% for all target sialic acids. The high performance is consistent with results obtained with a system using eluent generation.

Table 7. Sialic acid determination in three glycoprotein acid hydrolyzates

Sample (S#)	Protein	Protein MW (kDa)	Neu5Ac		Neu5Gc	
			(mol analyte/mol protein)	RSD (n=3)	(mol analyte/mol protein)	RSD (n=3)
1	b. apo-Transferrin	79	1.02	0.37	1.42	2.12
2	Fetuin	59	15.7	2.65	0.349	3.04
3	h. AGP	44	25.6	3.82	ND	N/A

Table 8. Spike recovery of sialic acids in glycoprotein hydrolyzates

Sample (S#)	Protein	Neu5Ac			Neu5Gc		
		Amount Found (μ M)	Amount added (μ M)	Recovery (%)	Amount Found (μ M)	Amount added (μ M)	Recovery (%)
Blank	Reagent blank	ND	2.5	95.1	ND	0.25	98.9
1	b. apo-Transferrin	0.888	0.5	95.3	1.24	1.00	98.6
2	Fetuin	12.4	10	99.6	0.275	0.25	99.1
3	h. AGP	26.5	20	105	ND	0.25	101

Conclusion

This application note demonstrated that the AU 180 method for sialic acid determination of glycoproteins could be successfully modified for use with a Dionex CarboPac PA20-1 mm column and HPAE-PAD in dual eluent generation cartridge mode. The separation, linearity, sensitivity, accuracy, and reproducibility were excellent. Comparison with a traditional HPAE-PAD separation of sialic acids using manually prepared sodium hydroxide/sodium acetate eluents showed that the Dual EGC method delivered a similar resolution of sialic acids, simplified operation (no eluent preparation), and improved retention time precision.

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