

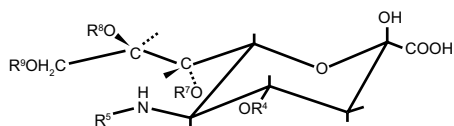
# Rapid Glycoprotein Sialic Acid Determination by High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection

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## Introduction

Due to their critical role in protein chemistry, sialic acids are commonly determined in therapeutic protein products. These carbohydrates are important in controlling glycoprotein bioavailability, function, stability, and metabolism. Because the final glycoprotein sialylation amount and identity varies by expression cell line and growth conditions for that cell line, expression experiments and production optimization have the potential to generate large numbers of samples. Supporting this process requires high-throughput analyses to allow quick evaluation and decision making during therapeutic protein expression optimization. Although over 50 natural sialic acids have been identified, two forms are commonly determined in glycoprotein products: *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc).

FIGURE 1. Sialic acids (neuraminic acids).



R <sup>5</sup>	R <sup>4,7,8,9</sup>
$\begin{array}{c} -C-CH_3 \\    \\ O \end{array}$	- H (4, 7, 8, 9)
$\begin{array}{c} -C-CH_2 \\    \quad   \\ O \quad OH \end{array}$	$\begin{array}{c} -C-CH_3 \\    \\ O \end{array}$ (4, 7, 8, 9)
$\begin{array}{c} -C-CH_2 \\    \quad   \\ O \quad O \\   \\ C=O \\   \\ CH_3 \end{array}$	$\begin{array}{c} -C-CH-CH_3 \\    \quad   \\ O \quad OH \end{array}$ (9) - CH <sub>3</sub> (8) - SO <sub>3</sub> H (8) - PO <sub>3</sub> H <sub>2</sub> (9)

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Determination of these two sialic acids is frequently performed in two steps. Acid hydrolysis is typically used to release the sialic acids, which is then followed by a chromatographic method to determine the amounts of Neu5Ac and Neu5Gc and the total sialic acid content. Among the chromatographic methods for determination are direct detection methods, such as high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD), and those that require analyte derivatization for detection, such as fluorescent labeling followed by ultrahigh performance liquid chromatography (UHPLC). This work describes an HPAE-PAD sialic acid assay with example results from five model glycoproteins: calf fetuin, bovine apo-transferrin (b. apo-transferrin), human transferrin (h. transferrin), sheep  $\alpha_1$ -acid glycoprotein (s. AGP), and human  $\alpha_1$ -acid glycoprotein (h. AGP). The direct and fast (<5 min) HPAE-PAD analyte determination makes this method appropriate for rapid sample screening while avoiding the labeling steps common in other methods.

## Experimental

Thermo Scientific Dionex ICS-3000 or 5000 ion chromatography system including:

- DP Dual Pump module
- DC Detector/Chromatography module
- AS Autosampler
- ED Electrochemical Detector
- Electrochemical Cell
- Disposable Gold Working Electrode
- Reference Electrode (Ag/AgCl)

The Thermo Scientific Dionex Chromeleon™ Chromatography Data System was used for system control and data processing.

Column: Thermo Scientific Dionex CarboPac™ PA20 Fast Sialic Acid, 3 × 30 mm

Eluent A: 100 mM Sodium hydroxide

Eluent B: 1.0 M Sodium acetate in 100 mM sodium hydroxide

Gradient: 70–300 mM Acetate in 100 mM NaOH from 0–2.5 min, 300 mM acetate in 100 mM NaOH from 2.5–2.9 min, 300–70 mM acetate from 2.9–3.0 min; 1.5 min of equilibration at 70 mM acetate in 100 mM NaOH

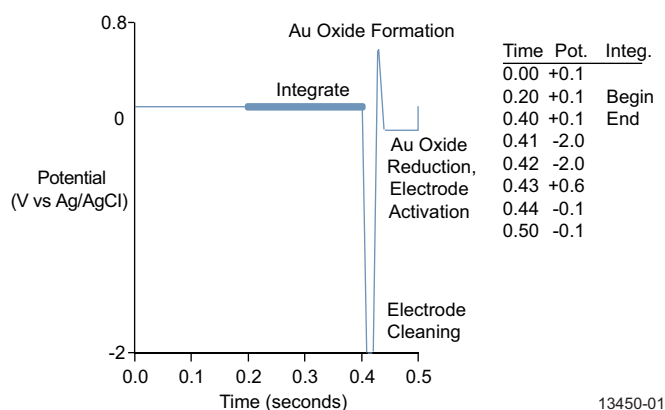
Flow Rate: 0.5 mL/min

Inj. Volume: 4.5 µL (full loop)

Temperature: 30 °C

Detection: PAD, Au on PTFE disposable electrode, 2 mil gasket

**FIGURE 2. Automated four-potential waveform as used for sialic acids.<sup>1</sup>**



## Sample Preparation

Protein hydrolysis: Calf fetuin (80 µg), h. transferrin (144 µg), b. apo-transferrin (175 µg), h. AGP (100 µg), and s. AGP (35 µg) were each added to individual 1.5 mL microcentrifuge vials along with 200 µL of 2 M acetic acid. The protein solutions were hydrolyzed by the method of Varki et al.<sup>2</sup>

It is strongly recommended that the hydrolysis conditions be optimized for each protein. Recommendations for developing experiments to optimize hydrolysis conditions can be found in the work of Fan et al.<sup>3</sup> A review of hydrolysis and sample preparation conditions suitable for HPAE-PAD sialic acid analysis has been previously published.<sup>4</sup>

Dilute the protein hydrolyzate 100 fold before HPAE-PAD analysis.

For greater long-term stability of the hydrolyzate or for extended storage at -40 °C, lyophilize a 50 µL aliquot of hydrolyzate and then dissolve it in 500 µL DI water.<sup>5</sup>

## Results and Discussion

The chromatogram below illustrates the separation of Neu5Ac and Neu5Gc within 3 min. Neu5Ac is well retained past the void, which is important when analyzing hydrolyzate samples. Neu5Gc elutes in <3 min, allowing a rapid method. A 1.5 min equilibration returns the column to the initial conditions.

**FIGURE 3. Rapid sialic acids determination method on the Dionex CarboPac PA20 Fast Sialic Acid column.**

Column: Dionex CarboPac PA20 Fast Sialic Acid, 3 × 30 mm

Eluent: 70–300 mM acetate in 100 mM NaOH from 0–2.5 min, 300 mM acetate in 100 mM NaOH from 2.5–2.9 min, 300–70 mM acetate from 2.9–3.0 min; 1.5 min of equilibration at 70 mM acetate in 100 mM NaOH

Temperature: 30 °C

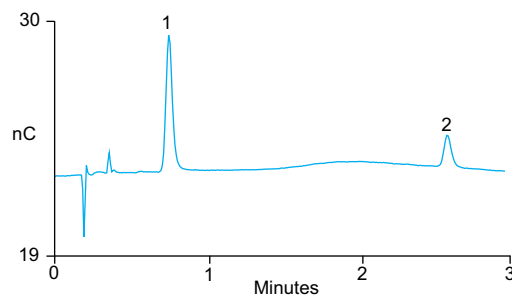
Flow Rate: 0.5 mL/min

Inj. Volume: 4.5 µL (full loop)

Detection: PAD, Au on PTFE, 2 mil gasket

Samples: Neu5Ac and Neu5Gc standard

Peaks: 1. Neu5Ac 11 pmol  
2. Neu5Gc 1.1



Response is linear between 0.27–68 pmol for Neu5Ac and 0.23–11 pmol for Neu5Gc with coefficients of determination  $\geq 0.9995$  (Table 1). The correlation may vary from day to day or if different concentration ranges are selected. As with any method, the detection limits can vary with system performance.

**Table 1. Limits of Quantitation (LOQs), Limits of Detection (LODs), and Linearity**

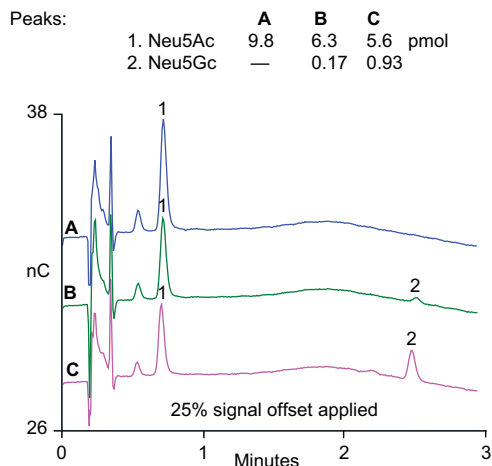
Analyte	LOQs <sup>a</sup> (pmol)	LODs <sup>b</sup> (pmol)	Linear Range (pmol)	Coeff. of Determination (r <sup>2</sup> )
Neu5Ac	0.34	0.11	0.27–68	0.9995
Neu5Gc	0.18	0.058	0.23–11	0.9997

<sup>a</sup>LOQs were determined as the concentration that gave a peak ten times the signal-to-noise (S/N) ratio.

<sup>b</sup>LODs were determined as the concentration that gave a peak three times the S/N ratio.

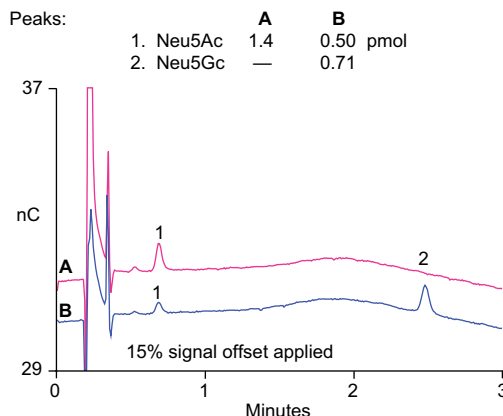
**FIGURE 4. Separation of fetuin, h. AGP, s. AGP hydrolyzates (1:100 dilution).**

Column: Dionex CarboPac PA20 Fast Sialic Acid, 3 × 30 mm  
 Eluent: 70–300 mM acetate in 100 mM NaOH from 0–2.5 min,  
 300 mM acetate in 100 mM NaOH from 2.5–2.9 min,  
 300–70 mM acetate from 2.9–3.0 min;  
 1.5 min of equilibration at 70 mM acetate in 100 mM NaOH  
 Temperature: 30 °C  
 Flow Rate: 0.5 mL/min  
 Inj. Volume: 4.5 µL (full loop)  
 Detection: PAD, Au on PTFE, 2 mil gasket  
 Samples: A) h. α<sub>1</sub>-acid glycoprotein,  
 1:100 dilution (23 ng protein)  
 B) calf fetuin hydrolyzate,  
 1:100 dilution (18 ng protein)  
 C) s. α<sub>1</sub>-acid glycoprotein hydrolyzate,  
 1:100 dilution (7.9 ng protein)



**FIGURE 5. Separation of h. and b. apo-transferrin hydrolyzates (1:100 dilution).**

Column: Dionex CarboPac PA20  
 Fast Sialic Acid, 3 × 30 mm  
 Eluent: 70–300 mM acetate in 100 mM NaOH from 0–2.5 min,  
 300 mM acetate in 100 mM NaOH from 2.5–2.9 min,  
 300–70 mM acetate from 2.9–3.0 min;  
 1.5 min of equilibration at 70 mM acetate in 100 mM NaOH  
 Temperature: 30 °C  
 Flow Rate: 0.5 mL/min  
 Inj. Volume: 4.5 µL (full loop)  
 Detection: PAD, Au on PTFE, 2 mil gasket  
 Samples: A) h. transferrin hydrolyzate, 1:100 dilution (32 ng protein)  
 B) b. apo-transferrin hydrolyzate, 1:100 dilution (39 ng protein)



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Method precision, as measured with seven sequential injections of a mid-range standard, shows excellent peak area precision (RSD <2) and good retention time precision (RSD <1) (Table 2). Due to the short elution times, the retention time standard deviation is shown along with the retention time RSD. The retention times are stable within 0.008 min.

**Table 2. Precision of the Method, n = 7 (11 pmol Neu5Ac, 1.1 pmol Neu5Gc)**

Retention Time (min)	Retention Time RSD (%)	Retention Time σ (min)	Peak Area (nC*min)	Peak Area RSD (%)
0.745	0.88	0.007	0.3206	1.63
2.58	0.32	0.008	0.0865	1.38

Acid hydrolyzates of five glycoproteins were analyzed by this method. Each glycoprotein was hydrolyzed, diluted in DI water 1:100, and injected within 24 h. As expected, Neu5Gc was not detected in human proteins. In each case Neu5Ac is well resolved from other hydrolyzate components as illustrated in Figures 4 and 5.

Assay precision and accuracy were both evaluated with glycoprotein hydrolyzate samples. Example data for one day of analysis is presented below. Retention time precision is similar to that of standards, with peak area precision typical for PAD detection. Interday precision for analysis of three samples of each protein was within 11%, however variability of up to 20% has been observed. Acid hydrolysis is a balance between carbohydrate release from the glycoprotein and subsequent acid-catalyzed degradation. Because of this, variability in sample replicates can be expected (Table 3).

**Table 3. One-Day Sample Analysis Precision (n = 3)**

Protein	Analyte	Amount (pmol)	Retention Time (min)	RT Precision (Triplicate Injections) (RSD)	Peak Area Precision (Triplicate Injections) (RSD)	Intraday Precision (Triplicate Sample Hydrolyzates) (RSD)
Fetuin	Neu5Gc	0.18	2.58	0.19	3.35	7.15
	Neu5Ac	5.8	0.74	0.65	2.59	8.83
h. Transferrin	Neu5Gc	ND				
	Neu5Ac	1.4	0.72	0.67	2.62	6.66
b. apo-Transferrin	Neu5Gc	0.91	2.51	<0.01	2.75	2.35
	Neu5Ac	0.61	0.70	0.68	3.23	3.51
h. AGP	Neu5Gc	ND				
	Neu5Ac	13	0.72	1.16	0.98	10.6
s. AGP	Neu5Gc	1.0	2.52	0.57	1.18	6.25
	Neu5Ac	5.8	0.71	0.68	2.33	6.35

ND = none detected

Method accuracy was tested by spiking Neu5Ac and Neu5Gc into diluted glycoprotein hydrolyzates. Spiked and unspiked samples were analyzed as soon as possible to reduce acid-catalyzed sialic acid decomposition. Overall, recoveries ranged from 81–96% for Neu5Ac and 82–106% for Neu5Gc, suggesting method accuracy (Table 4).

**Table 4. Accuracy of Analysis as Measured by Spiking (n = 3)**

Protein	Analyte	Average Native Amount (pmol)	Added Amount (pmol)	Recovery (%)
Hydrolyzate blank	Neu5Ac	ND	2.2	94 ± 5.8
	Neu5Gc	ND	0.22	92 ± 7.1
Fetuin	Neu5Ac	5.8	2.2	84 ± 1.0
	Neu5Gc	0.18	0.22	86 ± 2.2
h. Transferrin	Neu5Ac	1.6	2.2	95 ± 4.1
	Neu5Gc	ND	0.22	94 ± 2.4
b. apo-Transferrin	Neu5Ac	0.35	1.8	87 ± 5.0
	Neu5Gc	0.45	0.90	95 ± 3.0
h. AGP	Neu5Ac	4.5	3.6	91 ± 1.5
	Neu5Gc	ND	0.36	89 ± 3.4
s. AGP	Neu5Ac	5.8	4.5	94 ± 1.9
	Neu5Gc	1.0	0.45	98 ± 6.9

ND = none detected

The proposed method results were compared to two other methods: HPAE-PAD with a 150 mm anion-exchange column and guard column, and sample derivatization followed by UHPLC on a C18 column with fluorescence detection. Results from all three methods are very similar, and largely within experimental error (Table 5).

**Table 5. Proposed Method Gives Similar Results Compared to Two Other Methods**

Protein	Analyte	Mol Analyte/ Mol Protein, Day 1	Mol Analyte/ Mol Protein, Day 2	Mol Analyte/ Mol Protein, Day 3	Mol Analyte/ Mol Protein, HPAE-PAD Method <sup>5</sup>	Mol Analyte/ Mol Protein, UHPLC Method <sup>6</sup>
Fetuin	Neu5Gc	0.33 ± 0.02	0.30 ± 0.03	0.35 ± 0.04	0.32 ± 0.04	0.46 ± 0.05
	Neu5Ac	14 ± 1.4	16 ± 1.8	14 ± 0.77	14 ± 1.5	20 ± 2.4
h. Transferrin	Neu5Gc	ND	ND	ND	ND	ND
	Neu5Ac	3.7 ± 0.27	2.7 ± 0.80	2.9 ± 0.22	3.4 ± 0.38	4.8 ± 0.92
b. apo- Transferrin	Neu5Gc	1.4 ± 0.02	1.3 ± 0.12	1.3 ± 0.14	1.6 ± 0.18	1.9 ± 0.13
	Neu5Ac	1.0 ± 0.02	1.1 ± 0.17	1.3 ± 0.11	1.2 ± 0.13	1.9 ± 0.12
h. AGP	Neu5Gc	ND	ND	ND	ND	ND
	Neu5Ac	24 ± 3.0	30 ± 5.0	32 ± 4.0	25 ± 3.2	25 ± 4.3
s. AGP	Neu5Gc	5.1 ± 0.36	4.5 ± 0.71	4.4 ± 0.10	4.5 ± 0.54	4.0 ± 0.41
	Neu5Ac	29 ± 2.0	25 ± 3.8	24 ± 0.71	26 ± 3.5	24 ± 2.6

ND = none detected

Table 6 illustrates the total time necessary for analyzing a set of samples by both this high-throughput method and by derivatization followed by UHPLC with fluorescence detection. Sample analysis time is similar, with drastically reduced sample preparation time for the HPAE-PAD method, saving both time and reagent costs (Table 6).

**Table 6. Comparison of Method Analysis Times (h) for Proposed HPAE-PAD and Derivatization Followed by UHPLC-Fluorescence**

Method Step	Proposed HPAE-PAD Method	Derivatization Followed by UHPLC- Fluorescence Detection Method
Lyophilization	1.0 (Optional)	Not needed
Derivatization reagent preparation	N/A	0.5
Sample/standard derivatization and preparation	N/A	2.5+0.5
Chromatographic run time, one injection	0.08 (5 min)	0.17 (10 min, 5 min for very clean samples)
Total time posthydrolysis	0.08–1.08	3.67

## Conclusion

This <5 minute assay allows rapid, direct, and accurate quantification of sialic acids in glycoprotein acid hydrolyzates, providing a convenient screening method. The described method is stable with good recoveries, good precision, and linear detection for Neu5Ac and Neu5Gc in the ranges specified. This method allows high-throughput results without costly and time-consuming derivatization steps required for UHPLC fluorescence detection methods.

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