

Rapid Screening of Sialic Acids in Glycoproteins by HPAE-PAD

Deanna Hurum and Jeff Rohrer

Introduction

Glycoprotein sialylation has been shown to be critical to bioavailability, stability, metabolism, and immunogenicity of therapeutic proteins.¹⁻⁴ As a result, such proteins are routinely analyzed to determine sialylation amount and identity. Although over 50 forms of sialic acid have been identified,⁵⁻⁶ two forms of this carbohydrate are routinely determined, *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc). Of these, Neu5Gc is generally not found in human proteins.⁷ Due to this lack of Neu5Gc in healthy human tissue and the natural occurrence of antibodies against Neu5Gc, this sialic acid has the potential to cause an immune response in patients when present in a glycoprotein therapeutic.⁸

Many therapeutic proteins are produced via expression of the protein in a cell line chosen to maximize protein yield. Because the final glycoprotein sialylation amount and identity depend on the expression cell line and growth conditions for that cell line,⁹⁻¹¹ expression experiments and production optimization have the potential to generate large numbers of samples requiring analysis. In this case, high-throughput screening assays are valuable for quick product evaluation relative to expression cell lines and growth conditions.

Sialic acid determination can be performed by many methods. Typically, sialic acids are released from glycoproteins by acid hydrolysis or by enzymatic digestion before analysis. Once the sialic acids are liberated, there are multiple options for quantification. Numerous spectroscopic methods exist, although interferences in these methods can overestimate the sialic acid concentrations in many samples, and therefore, chromatographic methods that separate the sialic acids from potentially interfering compounds are preferred.¹² Among the chromatographic methods, some require further sample derivatization for analyte detection, such as fluorescent labeling followed by high-performance liquid chromatography (HPLC). Others use direct detection methods, such as high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD).¹³ Of these two methodologies, HPAE-PAD offers the advantage of direct analysis without sample derivatization.

In this work, sialic acids are determined in five representative glycoproteins by acid hydrolysis followed by HPAE-PAD. Sialic acid determination by HPAE-PAD using the Thermo Scientific Dionex CarboPac PA20 Fast Sialic Acid column is specific and direct, eliminating the need for sample derivatization after hydrolysis. The use of a disposable gold on polytetrafluoroethylene (PTFE) working electrode simplifies system maintenance while providing consistent response with a lifetime of four weeks. The rapid gradient method discussed separates Neu5Ac and Neu5Gc in <3 min with a total analysis time of 4.5 min. By using the Dionex CarboPac™ PA20 Fast Sialic Acid column, total analysis time is reduced, per sample eluent consumption and waste generation are reduced, and sample throughput is improved.

Equipment

Thermo Scientific Dionex ICS-3000 or Dionex ICS-5000 Ion Chromatography system including:

- SP Single Pump or DP Dual Pump module
- DC Detector/Chromatography module
- AS Autosampler

Electrochemical Detector (P/N 079831)

Electrochemical Cell (P/N 061757)

Disposable Gold Electrode, Au on PTFE (P/N 066480)

Reference Electrode (P/N 061879)

Thermo Scientific Dionex Chromeleon 7
Chromatography Data System software

Polypropylene injection vials with caps, 0.3 mL
(P/N 055428)

Polypropylene injection vials with caps, 1.5 mL
(P/N 079812)

Nalgene™ 1000 mL, 0.2 µm nylon filter units
(Fisher Scientific P/N 09-740-46)

Polypropylene microcentrifuge screw cap tubes, 1.5 mL
(Sarstedt P/N 72.692.005)

Dry block heater (VWR P/N 13259-005)

- Neuraminic Acids
- Anion Exchange
- Pulsed Amperometric Detection
- CarboPac PA20 Fast Sialic Acid Column
- High-Throughput Analysis

Reagents and Standards

Deionized (DI) water, Type I reagent grade, 18 M Ω -cm resistivity or better

Sodium hydroxide, 50% (w/w) (Fisher Scientific P/N SS254-500)

Sodium acetate, anhydrous (P/N 059326)

Acetic acid (JT Baker P/N 9515-03)

N-Acetylneuraminic acid (Neu5Ac, NANA)
Ferro Pfanstiehl

N-Glycolylneuraminic acid (Neu5Gc, NGNA)
Ferro Pfanstiehl

Micro BCA™ Protein Assay Kit, (Thermo Scientific P/N 23235)

Samples

Five glycoproteins were selected for analysis:

Calf fetuin (Sigma P/N F2379)

Bovine apo-transferrin (b. apo-transferrin)
(Sigma P/N T1428)

Human transferrin (h. transferrin) (Sigma P/N T8158)

Sheep α_1 -acid glycoprotein (s. AGP)
(Sigma P/N G6401)

Human α_1 -acid glycoprotein (h. AGP)
(Sigma P/N G9885)

Conditions

Column: Dionex CarboPac PA20 Fast Sialic Acid Column, 3 × 30 mm (P/N 076381)

Eluent 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

Eluents: A: 100 mM NaOH
B: 1.0 M sodium acetate in 100 mM NaOH

Flow Rate: 0.5 mL/min

Inj. Volume: 4.5 μ L, (full loop)

Temperature: 30 °C (column and detector compartments)

Detection: Pulsed amperometric, disposable Au on PTFE working electrode

Background: 18–25 nC (using the carbohydrate waveform)

Noise: ~15–30 pC

Sys. Backpress.: ~750 psi

Carbohydrate 4-Potential Waveform for the ED

Time(s)	Potential (V)	Gain Region*	Ramp*	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, but not used in older Dionex systems. Reference electrode in Ag mode (Ag/AgCl reference). See Thermo Scientific (formerly Dionex) Application Note 141 and Technical Note 41 for more information regarding sialic acid determination.^{14,15}

Preparation of Standards and Samples

Eluent Solutions

Prepare 1 L of 100 mM sodium hydroxide by adding 5.2 mL of 50% (w/w) NaOH to 994.8 mL of degassed DI water.

Prepare 1 L of 1 M sodium acetate in 100 mM sodium hydroxide by dissolving 82.0 g of anhydrous sodium acetate in ~800 mL of DI water. Filter and degas the acetate solution through a 0.2 μ m nylon filter unit. Transfer the solution to a 1 L volumetric flask, add 5.2 mL of 50% (w/w) NaOH, and fill the flask with degassed DI water.

See Thermo Scientific (formerly Dionex) Technical Note 71 for detailed information on eluent preparation for HPAE-PAD applications.¹⁶

Acetic Acid, 4 M

Transfer 22.5 mL of glacial acetic acid to a polyethylene bottle containing 77.5 mL of DI water.

Stock Standard Solutions

Dissolve 149.8 mg of Neu5Ac in 50 mL DI water and 41.0 mg Neu5Gc in 50 mL of DI water. This results in 9.68 mM and 2.52 mM stock solutions, respectively. Add 20 μ L 9.68 mM Neu5Ac to 949 μ L DI water to prepare a 0.20 mM solution, and 8.0 μ L 2.52 mM Neu5Gc to 992 μ L DI water to prepare a 0.020 mM solution of Neu5Gc. Add 500 μ L of 0.20 mM Neu5Ac and 500 μ L of 0.020 mM Neu5Gc to a 1.5 mL cryogenic storage vial to prepare a combined stock of 0.10 mM Neu5Ac and 10 μ M Neu5Gc and store at -40 °C.

Working Standard Solutions

Prepare calibration standards by diluting the stock standard solution as detailed in Table 1. For example, add 5.0 μ L of the stock solution to 195 μ L of DI water to prepare a calibration standard of 2.5 μ M Neu5Ac and 0.25 μ M Neu5Gc (11 pmol Neu5Ac and 1.1 pmol Neu5Gc per 4.5 μ L injection). Prepare working standards daily from the stocks stored at -40 °C.

Volume of Combined Stock Standard (µL) Diluted to 200 µL	Neu5Ac Concentration (µM)	Neu5Gc Concentration (nM)	Neu5Ac Amount (pmol/4.5 µL)	Neu5Gc Amount (pmol/4.5 µL)
0.5	0.25	25	1.1	0.11 [*]
1.0	0.5	50	2.3	0.23
5.0	2.5	250	11.0	1.1
10.0	5.0	500	23.0	2.3
20.0	10.0	1000	45.0	4.5
30.0	15.0	1500	68.0	6.8
40.0 [†]	20.0	2000	90.0	9.0

*Not used for routine Neu5Gc calibration

† Not used for Neu5Ac calibration

Table 1: Sialic acid standards used for sample analysis.

Protein	Working Solution Conc (mg/mL)	Volume Protein (µL)	Amount of Protein (µg)	Volume DI Water (µL)	Volume 4 M Acetic Acid (µL)	Protein Conc (µg/µL)
Fetuin	2.3	35	80	65	100	0.40
s. AGP	1.0	35	35	65	100	0.18
h. AGP	2.9	35	100	65	100	0.51
b. apo-Transferrin	5.0	35	180	65	100	0.88
h. Transferrin	4.1	35	140	65	100	0.72

Table 2: Protein hydrolyzate concentrations.

Protein Stock Solutions, 4.0 mg/mL Nominal

Dissolve 2.4 mg of sheep α_1 -acid glycoprotein in 400 µL of DI water to prepare a 4 mg/mL solution. 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 -40 °C. Repeat this process as follows. Dissolve 8.8 mg (b. apo-transferrin) and 8.6 mg (h. transferrin) in individual 2 mL aliquots of DI water. Dissolve 19.0 mg of fetuin in 4.75 mL of DI water. Dissolve 2.2 mg of h. AGP in 0.60 mL of DI water. Protein may be lost both during freeze/thaw cycles and by adsorption to surfaces. Therefore, it is important to measure the working stock protein concentrations before hydrolysis using a colorimetric BCA protein assay kit. Values listed in Table 2 are results from BCA assay of the working stock solutions.

Acetic Acid Hydrolysis of Proteins

Add 80 µg fetuin, 140 µg h. transferrin, 175 µg b. apo-transferrin, 100 µg h. AGP, and 35 µg s. AGP to individual 1.5 mL microcentrifuge vials with a total of 200 µL of 2 M acetic acid, as detailed in Table 2. For example, pipet 35 µL of the fetuin 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 °C.¹⁷ After hydrolysis, dilute the hydrolyzate 1:100 with 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 (formerly Dionex) Technical Note 41.¹⁵

Precautions

The Dionex CarboPac PA20 Fast Sialic Acid column has been tested for glycoprotein hydrolyzates only. More complex matrixes may not separate acceptably with the conditions presented here. For greater sensitivity and sample stability, lyophilization followed by dissolution in DI water is recommended. See Thermo Scientific (formerly Dionex) Application Update 180 for more information on stability of lyophilized hydrolysates that have been dissolved in DI water.¹⁸ To avoid underestimation of sialic acid content due to acid catalyzed degradation, perform analysis of samples prepared by dilution within 24 h of hydrolysis.

Absolute mass detection limits will depend on the sample injection volume. For this reason, a calibrated injection loop was used. Prepare a 4.5 µL sample loop by measuring approximately 3.7 in of 0.010 in. i.d. tubing. Verify the volume of the loop by first weighing the empty tubing, fill the tube with DI water, then reweigh the filled tube and calculate the volume. The total sample volume should be ~4.5 µL. Due to the high-throughput nature of this method, service requirements on the autosampler and injection valves will increase. If replicate injections show poor precision, check the autosampler needle assembly, transfer line, and the injection valve to ensure each is in good condition.

Analyte	Range (pmol)	Coeff of Determination (r ²)	Retention Time (min)	Retention Time Precision ^a (RSD)	Peak Area Precision (RSD)	LOQ ^b (pmol)	LOD (pmol)
Neu5Ac	0.27–68	0.9995	0.745	0.88	1.36	0.34	0.11
Neu5Gc	0.23–11	0.9997	2.58	0.32	1.38	0.18	0.058

^aPrecision was measured by seven injections of 11 pmol Neu5Ac and 1.1 pmol Neu5Gc.

^bLOD and LOQ are confirmed by injections at the concentrations listed and measuring response at 3× and 10× the noise, respectively.

Table 3: Linearity, limit of detection (LOD), limit of quantification (LOQ), and precision of sialic acid determination.

Results and Discussion

Figure 1 shows the separation of Neu5Ac and Neu5Gc on the Dionex CarboPac PA20 Fast Sialic Acid column with a 70–300 mM acetate gradient in 100 mM NaOH. The peaks are well separated and easily quantified. Additionally, the Neu5Ac peak is well separated from the void, which is an important consideration because a large void volume peak can interfere with quantification. Neu5Gc elutes in <3 min under these conditions, allowing a short run time.

Linear Range, Limit of Quantification, Limit of Detection and Precision

Table 3 shows the calibration results for Neu5Ac and Neu5Gc. In both cases, response is linear for the range studied. The LOD and LOQ were confirmed by standard injections that resulted in a response of 3× and 10× the noise, respectively. Neu5Ac had an LOD of 0.11 pmol on column and an LOQ of 0.34 pmol. Similarly, Neu5Gc limits were 0.058 pmol and 0.18 pmol. Retention time and peak area precisions of standards were determined by seven injections of a mid-range standard. In both cases, precision was excellent, with an RSD of <0.9 and a standard deviation of <0.008 min for retention time for both sialic acids and peak area RSDs of 1.36 and 1.38 for Neu5Ac and Neu5Gc, respectively.

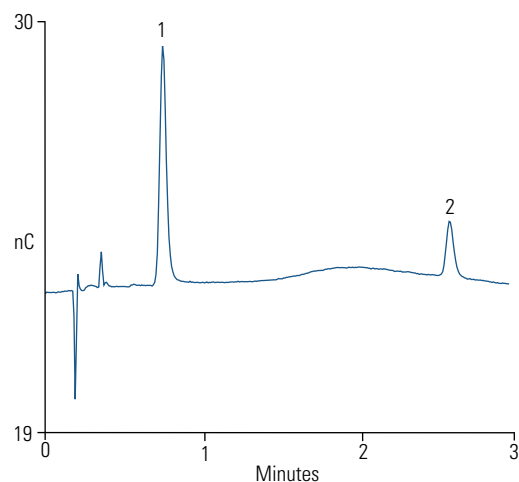
Sample Analysis, Precision, and Accuracy

Figures 2 and 3 illustrate the separation of sialic acids from acid hydrolyzed and diluted protein samples. In each case, Neu5Ac is well separated from early eluting components of the hydrolyzed sample and, as expected, Neu5Gc is not detected in the human glycoproteins. The amount of protein necessary for sialic acid determination depends on the individual protein. For glycoproteins that are highly sialylated, such as α_1 -acid glycoproteins, the amount of protein that is hydrolyzed easily can be reduced. In the example of s. AGP, 35 μ L of 0.18 μ g/ μ L protein solution are hydrolyzed, which is equivalent to 7.9 ng of protein per injection. The average determined amount for a single day of triplicate sample analysis in the hydrolyzate for s. AGP is 6.1 pmol of Neu5Ac and 1.1 pmol of Neu5Gc. Based on the determined LOQs for Neu5Ac and Neu5Gc, and because of the high degree of sialylation, the concentration of s. AGP can be reduced by a factor of 5 before reaching the LOQ of Neu5Gc and a factor of 17 before reaching the LOQ of Neu5Ac.

Conversely, b. apo-transferrin (39 ng per injection), as

Column: Dionex CarboPac PA20 Fast Sialic Acid, 3 × 30 mm
 Eluent: 70–300 mM sodium acetate in 100 mM NaOH from 0–2.5 min, 300 mM acetate in 100 mM NaOH from 2.5–2.9 min, 300 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 μ L (Full loop)
 Temperature: 30 °C
 Detection: PAD, Au on PTFE, 2 mil gasket
 Samples: Neu5Ac and Neu5Gc standard

Peaks: 1. Neu5Ac 11 pmol
 2. Neu5Gc 1.1



28840

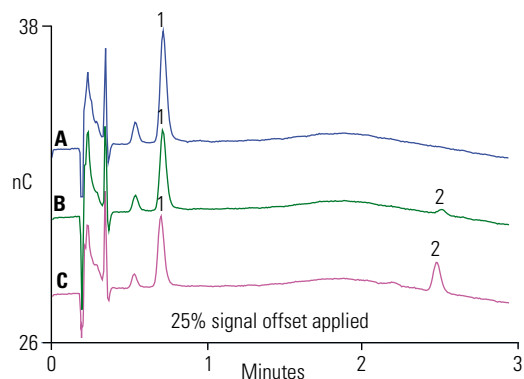
Figure 1: Separation of sialic acid standards on the Dionex CarboPac PA20 Fast Sialic Acid column.

shown in Figure 3, contains less total sialic acid, and lower hydrolysis amounts are not recommended. Depending on the degree of sialylation, the amount of protein hydrolyzed can be reduced to low-ng/ μ L concentrations and still allow efficient sialic acid determination. Designing experiments that release amounts of analyte routinely near the LOQ is not recommended. However, this evaluation highlights both the sensitivity of the method and the importance of considering the approximate protein sialylation amount when designing acid hydrolysis experiments.

Table 4 presents the results from one day of triplicate

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_1 -acid glycoprotein,
 1:100 dilution (23 ng protein)
 B) calf fetuin hydrolyzate,
 1:100 dilution (18 ng protein)
 C) s. α_1 -acid glycoprotein hydrolyzate,
 1:100 dilution (7.9 ng protein)

Peaks:	A	B	C	
1. Neu5Ac	9.8	6.3	5.6	pmol
2. Neu5Gc	—	0.17	0.93	



28865

Figure 2: Separation of fetuin, h. AGP, s. AGP hydrolyzates (1:100 dilution) on the Dionex CarboPac PA20 Fast Sialic Acid column.

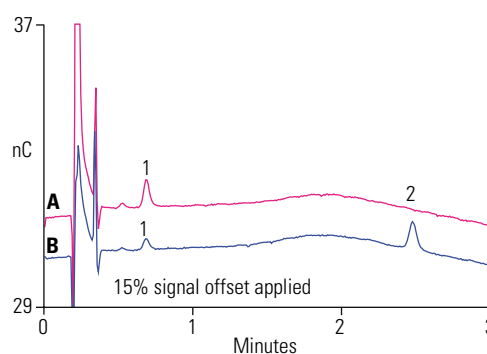
sample analysis. Retention time precision is similar to that determined by injecting standards, with retention time RSDs ranging from <0.01–1.18. Differences in the absolute retention time can be expected, depending on the eluent preparation. Peak area precision for triplicate injections, as measured by RSD, is generally good, ranging from 0.13–6.14. As expected, larger peak area RSD is observed near the LOQ.

Table 5 lists the calculated results of sialic acid determination for the studied proteins, as well as both intraday precision for one day of analysis and between-day precision for three days of triplicate analysis. The amounts of sialic acids determined in the protein samples are generally consistent with literature results for the glycoproteins.^{19–24} However, as shown in Table 5, sample replicate precision RSDs can be greater than chromatographic precision, with intraday RSDs ranging from 2.4–11 and between-day RSDs ranging from 3.9–17. For this reason, optimization of the acid hydrolysis for individual glycoproteins is highly recommended.

Acid hydrolysis is a complex balance between release of

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)

Peaks:	A	B	
1. Neu5Ac	1.4	0.50	pmol
2. Neu5Gc	—	0.71	



28866

Figure 3: Separation of h. and b. apo-transferrin hydrolyzates (1:100 dilution) on the Dionex CarboPac PA20 Fast Sialic Acid column.

the sialic acids from the glycoprotein and degradation of the released analytes. The efficiency of the hydrolysis depends on the hydrolysis temperature, acid concentration, type of sample being hydrolyzed, and the relative concentrations of acid and the sample. Because of these interdependent factors, which can impact the hydrolysis, variability between sample preparations must be expected. For the best accuracy, either an optimized acid hydrolysis or neuraminidase digestion is recommended. For methodology to optimize acid hydrolysis, see Fan et al.²⁵

Method accuracy was investigated by spiking protein acid hydrolyzates with known amounts of Neu5Ac and Neu5Gc in similar concentration as the determined amounts (Table 6). For human glycoproteins, which lack Neu5Gc, 0.22 pmol of Neu5Gc was added. Recoveries for Neu5Ac ranged from 81–96% and recoveries for Neu5Gc were similar, ranging from 82–106%.

Sample (replicate #)	Analyte	Amount (pmol)	Retention Time (min)	Retention Time Precision (RSD)	Peak Area Precision (RSD)
Fetuin (1)	Neu5Gc	0.18*	2.58	0.19	3.35
	Neu5Ac	5.78	0.74	0.65	2.59
Fetuin (2)	Neu5Gc	0.17*	2.58	0.19	5.25
	Neu5Ac	6.31	0.75	0.65	1.23
Fetuin (3)	Neu5Gc	0.18*	2.58	<0.01	6.14
	Neu5Ac	7.44	0.74	0.65	0.99
h. Transferrin (1)	Neu5Gc	ND			
	Neu5Ac	1.42	0.72	0.67	2.62
h. Transferrin (2)	Neu5Gc	ND			
	Neu5Ac	1.52	0.72	0.67	1.75
h. Transferrin (3)	Neu5Gc	ND			
	Neu5Ac	1.64	0.72	0.67	2.50
b. apo-Transferrin (1)	Neu5Gc	0.91	2.51	<0.01	2.75
	Neu5Ac	0.61	0.70	0.68	3.23
b. apo-Transferrin (2)	Neu5Gc	0.91	2.51	0.19	1.49
	Neu5Ac	0.63	0.71	<0.01	2.93
b. apo-Transferrin (3)	Neu5Gc	0.88	2.51	0.19	2.23
	Neu5Ac	0.61	0.71	<0.01	2.14
h. AGP (1)	Neu5Gc	ND			
	Neu5Ac	15	0.72	1.34	1.14
h. AGP (2)	Neu5Gc	ND			
	Neu5Ac	13	0.72	1.16	0.98
h. AGP (3)	Neu5Gc	ND			
	Neu5Ac	12	0.72	0.67	0.95
s. AGP (1)	Neu5Gc	1.0	2.52	0.57	1.18
	Neu5Ac	5.8	0.71	0.68	2.33
s. AGP (2)	Neu5Gc	1.2	2.52	0.57	1.70
	Neu5Ac	6.6	0.71	1.18	2.64
s. AGP (3)	Neu5Gc	1.0	2.52	0.33	0.13
	Neu5Ac	5.9	0.71	0.68	2.52

*Neu5Gc calibration range extended from 0.11 pmol–11 pmol, $r^2 = 0.9995$.

Table 4: Sialic acid determination from five glycoprotein acid hydrolyzates.

Sample	Analyte	Acid Hydrolysis Average (mol analyte/mol protein)	Intraday Precision Between Replicates (RSD)	Between-Day Precision (RSD)
Fetuin	Neu5Gc	0.33	7.2	7.3
	Neu5Ac	15	8.8	7.0
h. Transferrin	Neu5Gc	ND	—	—
	Neu5Ac	3.1	6.7	17
b. apo-Transferrin	Neu5Gc	1.4	2.4	3.9
	Neu5Ac	1.1	3.5	13
h. AGP	Neu5Gc	ND	—	—
	Neu5Ac	29	11	14
s. AGP	Neu5Gc	4.7	6.2	8.7
	Neu5Ac	26	6.4	9.0

Table 5: Triplicate sample analysis results of between-day precision over three days.

Sample	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

Table 6: Accuracy of analysis as measured by recovery (n = 3).

Conclusion

In this work, sialic acids are determined in five representative glycoproteins by acid hydrolysis release followed by HPAE-PAD. The method is both specific and direct, eliminating the need for sample derivatization common in other chromatographic methods. Good recoveries, precision, and linear detection for Neu5Ac and Neu5Gc are demonstrated, indicating the method is appropriate for glycoprotein analysis. Using the Dionex CarboPac PA20 Fast Sialic Acid column, this rapid method separates Neu5Ac and Neu5Gc with a total analysis time of <5 min, providing high-throughput sample analysis while reducing eluent consumption and waste generation.

Suppliers

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

Thermo Fisher Scientific, One Liberty Lane, Hampton, NH 03842, U.S.A., Tel: 800-766-7000. www.fishersci.com

Sigma-Aldrich, P.O. Box 14508, St. Louis, MO 63178, U.S.A., Tel: 800-325-3010. www.sigma-aldrich.com

Ferro Pfanstiehl, 1219 Glen Rock Avenue, Waukegan, IL, 60085, U.S.A., Tel: 800-383-0126. www.ferro.com

References

- Byrne, B.; Donohoe, G.G.; O’Kennedy, R. Sialic Acids: Carbohydrate Moieties that Influence the Biological and Physical Properties of Biopharmaceutical Proteins and Living Cells. *Drug Discovery Today* **2007**, *12*, 319–326.
- Jenkins, N.; Curling, E.M. Glycosylation of Recombinant Proteins: Problems and Prospects. *Enzyme Microb. Technol.* **1994**, *12*, 354–364.
- Kaneko, Y.; Nimmerjahn, F.; Ravetch, J.V. Anti-Inflammatory Activity of Immunoglobulin G Resulting from Fc Sialylation. *Science (Washington, DC, U.S.)* **2006**, *313*, 670–673.
- Du, J.; Meledeo, M.A.; Wang, Z.; Khanna, H.S.; Paruchuri, V.D.P.; Yarema, J.K. Metabolic Glycoengineering: Sialic Acid and Beyond. *Glycobiology* **2009**, *19*, 1382–1401.
- Klein, A.; Diaz, S.; Ferreira, I.; Lamblin, G.; Roussel, R.; Manzi, A.E. New Sialic Acids from Biological Sources Identified by a Comprehensive and Sensitive Approach: Liquid Chromatography-Electrospray Ionization-Mass Spectrometry (LC-ESI-MS) of SIA Quinoxalinones. *Glycobiology* **1997**, *7*, 421–432.
- Angata, T.; Varki, A. Chemical Diversity in the Sialic Acids and Related α -Keto Acids: An Evolutionary Perspective. *Chem. Rev. (Washington, DC, U.S.)* **2004**, *102*, 439–469.
- Varki, A. Glycan-Based Interactions Involving Vertebrate Sialic-Acid-Recognizing Proteins. *Nature (London, U.K.)* **2007**, *446*, 1023–1029.
- Padler-Karavani, V.; Yu, H.; Cao, H.; Chokhawala, H.; Karp, F.; Varki, N.; Chen, X.; Varki, A. Diversity in Specificity, Abundance, and Composition of Anti-Neu5Gc Antibodies in Normal Humans: Potential Implications for Disease. *Glycobiology* **2008**, *18*, 818–830.

9. Hokke, C.H.; Bergwerff, A.A.; van Dedem, G.W.K.; van Oostrum, J.; Kamerling, J.P.; Vliegthart, J. F.G. Sialylated Carbohydrate Chains of Recombinant Human Glycoproteins Expressed in Chinese Hamster Ovary Cells Containing Traces of N-Glycolylneuraminic Acid. *FEBS Lett.* **1990**, *275*, 9–14.
10. Hossler, P.; Khattak, S.F.; Li, Z.J. Optimal and Consistent Protein Glycosylation in Mammalian Cell Culture. *Glycobiology* **2009**, *19*, 936–949.
11. Bork, K.; Reutter, W.; Weidemann, W.; Horstkorte, R. Enhanced Sialylation of EPO by Overexpression of UDP-GlcNAc 2-epimerase/ManAc Kinase Containing a Sialuria Mutation in CHO Cells. *FEBS Lett.* **2007**, *581*, 4195–4198.
12. Lacomba, R.; Salcedo, J.; Algeria, A.; Lagarda, M.J.; Barbera, R.; Mantencio, E. Determination of Sialic Acid and Gangloisides in Biological Samples and Dairy Products. *J. Pharm. Biomed. Anal.* **2010**, *51*, 346–357.
13. Rohrer, J.S. Analyzing Sialic Acids Using High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection. *Anal. Biochem.* **2000**, *283*, 3–9.
14. Thermo Fisher Scientific, Inc. (formerly Dionex Corp.). *Improved Long-Term Stability of N-Acetylneuraminic Acid and N-Glycolylneuraminic Acid Peak Area Responses Using Waveform A, a Quadruple Potential Waveform*. Application Update 141, LPN 1225, 2000, Sunnyvale, CA.
15. Thermo Fisher Scientific, Inc. (formerly Dionex Corp.). *Analysis of Sialic Acids Using High-Performance Anion-Exchange Chromatography*. Technical Note 41, LPN 0931-02, 2000, Sunnyvale, CA.
16. Thermo Fisher Scientific, Inc. (formerly Dionex Corp.). *Eluent Preparation for High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection*. Technical Note 71, LPN 1932-01, 2007, Sunnyvale, CA.
17. Varki, A.; Diaz, S. The Release and Purification of Sialic Acids from Glycoconjugates: Methods to Minimize the Loss and Migration of O-Acetyl Groups. *Anal. Biochem.* **1984**, *137*, 236–247.
18. Thermo Fisher Scientific, Inc. (formerly Dionex Corp.). *Direct Determination of Sialic Acids in Glycoprotein Acid Hydrolyzates by HPAE-PAD*. Application Update 180, LPN 2831, 2011, Sunnyvale, CA.
19. Spiro, R. G.; Bhoyroo, V. D. Structure of the O-Glycosidically Linked Carbohydrate Units of Fetuin. *J. Biol. Chem.* **1974**, *249*, 5704–5717.
20. Townsend, R. R.; Hardy, M. R.; Cumming, D. A.; Carver, J. P.; Bendiak, B. Separation of Branched Sialylated Oligosaccharides Using High-pH Anion-Exchange Chromatography with Pulsed Amperometric Detection. *Anal. Biochem.* **1989**, *182*, 1–8.
21. Richardson, N. E.; Buttress, N.; Feinstein, A.; Stratil, A.; Spooner, R. L. Structural Studies on Individual Components of Bovine Transferrin. *Biochem. J.* **1973**, *135*, 87–92.
22. Spik, G.; Bayard, B.; Fournet, B.; Strecker, G.; Bouquelet, S.; Montreuil, J. Studies on Glycoconjugates. LXIV. Complete Structure of Two Carbohydrate Units of Human Serotransferrin. *FEBS Lett.* **1975**, *50*, 296–299.
23. Friedrich, M.; Berger, E.G. Effect of Neuraminidase Treatment of IgG and Alpha 1-Acid Glycoprotein on Their Intestinal Transport in Suckling Rats in Vivo. *J. Ped. Gastroent. Nutrition* **1982**, *1*, 395–399.
24. Kuster, B.; Hunter, A.P.; Wheeler, S.F.; Dwek, R.A.; Harvey, D.J. Structural Determination of N-linked Carbohydrates by Matrix-Assisted Laser Desorption/Ionization-Mass Spectrometry Following Enzymatic Release within Sodium Dodecyl Sulphate-Polyacrylamide Electrophoresis Gels: Application to Species-Specific Glycosylation of α_1 -Acid Glycoprotein. *Electrophoresis* **1998**, *19*, 1950–1959.
25. Fan, J. Q.; Namiki, Y.; Matsuoka, K.; Lee, Y. C. Comparison of Acid Hydrolytic Conditions for Asn-Linked Oligosaccharides. *Anal. Biochem.* **1994**, *219*, 375–378.

In addition to these offices, Thermo Fisher Scientific maintains a network of representative organizations throughout the world.

Dionex Products:
(408) 737-0700

North America:
U.S./Canada
(847) 295-7500

South America:
Brazil
(55) 11 3731 5140

Europe:
Austria
(43) 616 51 25

Benelux
(31) 20 683 9768
(32) 3 353 4294

Denmark
(45) 36 36 90 90

France
(33) 1 39 30 01 10

Germany
(49) 6126 991 0

Ireland
(353) 1 644 0064

Italy
(39) 02 51 62 1267

Sweden
(46) 8 473 3380

Switzerland
(41) 62 205 9966

United Kingdom
(44) 1276 691722

Asia Pacific:
Australia
(61) 2 9420 5233

China
(852) 2428 3282

India
(91) 22 2764 2735

Japan
(81) 6 6885 1213

Korea
(82) 2 2653 2580

Singapore
(65) 6289 1190

Taiwan
(886) 2 8751 6655

www.thermoscientific.com

Legal Notices: ©2011 Thermo Fisher Scientific Inc. All rights reserved. Micro BCA is a trademark of Pierce Biotechnology, Inc. All other trademarks are the property of Thermo Fisher Scientific Inc. and its subsidiaries. This information is presented as an example of the capabilities of Thermo Fisher Scientific Inc. products. It is not intended to encourage use of these products in any manners that might infringe the intellectual property rights of others. Specifications, terms and pricing are subject to change. Not all products are available in all countries. Please consult your local sales representative for details.



Thermo Fisher Scientific,
San Jose, CA USA is ISO Certified.

AU181_E 11/12SV LPN2892-01