

Improved Long-Term Stability of N-Acetylneuraminic Acid and N-Glycolylneuraminic Acid Peak Area Responses Using Waveform A, a Quadruple Potential Waveform

N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc) are sialic acids that occupy terminal positions on many mammalian glycoprotein and glycolipid oligosaccharides. When a glycoprotein loses sialic acid residues it has a reduced serum half-life, and in some cases reduced activity.¹ Therefore it is important to know the sialic acid content of a glycoprotein when assaying its function or its efficacy as a pharmaceutical therapeutic. In Dionex Technical Note 41, “Analysis of Sialic Acids Using High-Performance Anion-Exchange Chromatography”, we showed that the sialic acid content of a glycoprotein could be accurately and reproducibly determined by HPAE-PAD.² That Note showed the separation of Neu5Ac, Neu5Gc, and the internal standard 3-deoxy-D-glycero-D-galacto-2-nonulosonic acid (KDN), and demonstrated the linear range and minimum detection limits that can be expected when using a new working electrode and a triple potential waveform. Furthermore, it was shown that with triple potential waveform use there is a recession of the working electrode surface that causes a gradual reduction in peak area response, a rise in the minimum detection limits, and a change in the linear ranges for each analyte.³ When this triple potential waveform was used for 14 consecutive days, the measured decrease in peak area response for Neu5Ac and Neu5Gc was 22% and 32%, respectively.⁴ Despite the changes caused by electrode recession, the use of an

internal standard and external standards allows the accurate determination of the sialic acid content of glycoproteins regardless of the state of the working electrode.³ Since the publication of Technical Note 41 and Reference 3, a pulsed electrochemical waveform for carbohydrate analysis that does not cause electrode recession was developed.⁴ This waveform is a quadruple potential waveform (Waveform A) that was described and compared to two triple potential waveforms for carbohydrate analyses (Waveforms B and C) in Dionex Technical Note 21.⁵ These waveforms are depicted in Figure 1. In this Application Update we show that Waveform A offers 48-h peak area reproducibility (peak area RSDs < 5%) for sialic acids analyzed by HPAE-PAD similar to that reported in Technical Note 41, and improved reproducibility of peak area response with long-term (> 48 h) use.

EQUIPMENT

Dionex DX-500 chromatography system consisting of:

- GP50 Pump with degas option
- LC25 or LC30 Chromatography Module
- ED40 Electrochemical Detector and cell with gold working electrode
- AS3500 Autosampler equipped with Tefzel® rotor seal and stainless steel needle
- PeakNet™ Chromatography Workstation

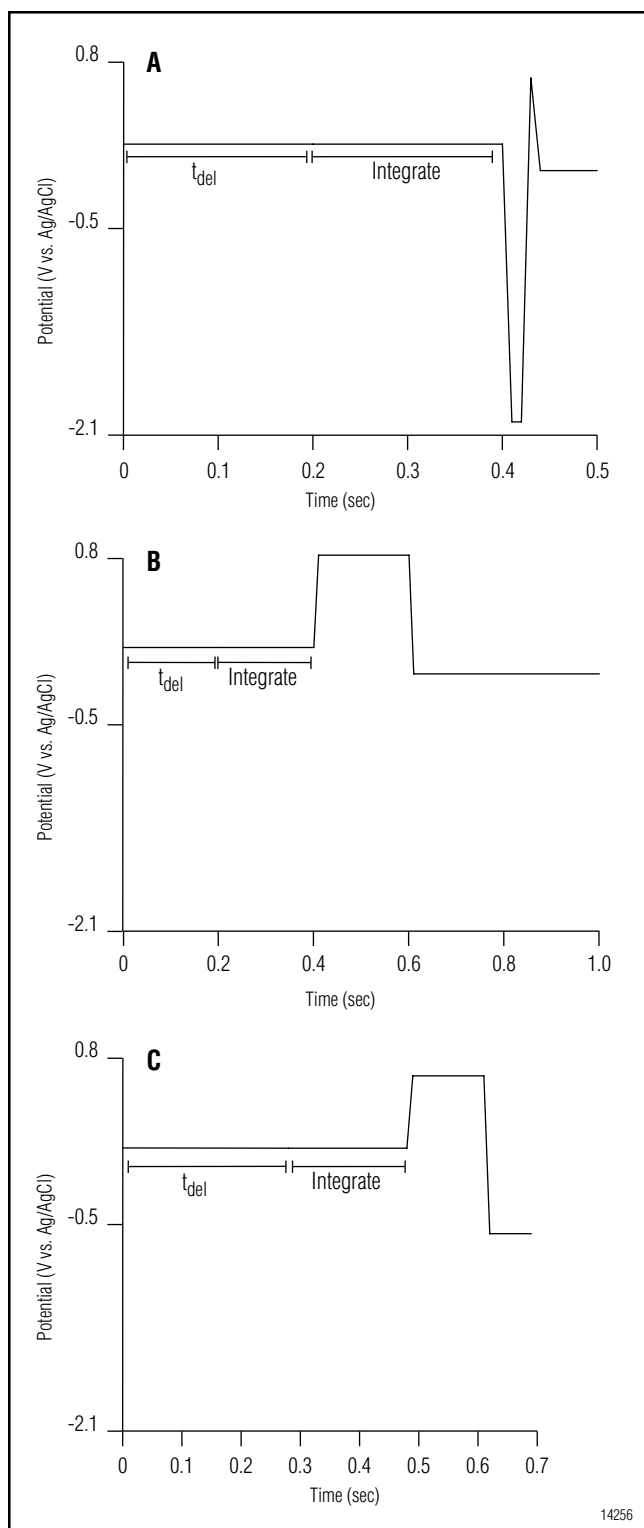


Figure 1. Pulsed amperometry waveforms for carbohydrate analysis.

MATERIALS

Sodium acetate (Fluka, Ronkonkoma, NY)
 50% NaOH (Fisher, Pittsburgh, PA)
 Neu5Ac and Neu5Gc (Pfanstiehl, Waukegan, IL)
 6 N HCl (ABI-PE, Foster City, CA)
 KDN (Toronto Chemicals, Toronto, Canada)
 Human transferrin, partial Fe (Boehringer Mannheim, Indianapolis, IN)
Arthrobacter ureafaciens neuraminidase (Boehringer Mannheim)
 Autosampler vials, caps, and septa (Sun Brokers, Wilmington, NC; part numbers, 500-114, 200-288, 200-396)

CONDITIONS

Column: CarboPac™ PA-10 analytical column (0.4 x 25 cm) with guard
 Flow Rate: 1.0 mL/min
 Detection: ED40 Electrochemical Detector (gold electrode)

Injection Loop: 100 μ L

Waveform:	Time (sec)	Potential (V)	Integration
	0.00	+0.1	
	0.20	+0.1	Begin
	0.40	+0.1	End
	0.41	-2.0	
	0.42	-2.0	
	0.43	+0.6	
	0.44	-0.1	
	0.50	-0.1	

Eluents: A: 100 mM sodium hydroxide
 B: 100 mM sodium hydroxide, 1 M sodium acetate

Method:	Time (min)	A (%)	B (%)
	Initial	93	7
	0.0	93	7
	10.0	70	30
	11.0	70	30
	12.0	93	7

Autosampler
 Cycle Time: 27 min
 Oven
 Temperature: 30 °C

ELUENT AND STANDARDS PREPARATION

Eluents A and B and the sialic acid standards were prepared as described in Technical Note 41.

RELEASE OF SIALIC ACIDS FROM HUMAN TRANSFERRIN

Human transferrin was digested with 0.1 N HCl or treated with *Arthrobacter ureafaciens* neuraminidase to release sialic acids as described in Technical Note 41.

RESULTS AND DISCUSSION

Good reproducibility of this or any other automated chromatography method is highly dependent on a properly functioning autosampler. A useful autosampler test involving replicate measurements of different volumes of glucose is described in Technical Note 41. A new injection valve rotor seal and a new autosampler needle were installed just prior to conducting the experiments described in this Application Update. The gold working electrode used in these experiments was used exclusively with Waveform A for approximately one year prior to the start of these experiments and showed no visible signs of recession from the electrode block. To determine reproducibility, 200 pmol each of Neu5Ac, KDN, and Neu5Gc (20 μ L) were analyzed repetitively for 48 h (107 injections). Figure 2 shows a typical separation of Neu5Ac, KDN, and Neu5Gc from this study. Figure 3A shows that peak area reproducibility over 48 h using Waveform A (Neu5Ac, KDN, and Neu5Gc peak area RSDs of 2.1, 2.7, and 2.7%, respectively) is similar to that found using Waveform B in Technical Note 41 (2.4, 2.3, and 2.8%, respectively). The peak area RSDs for Neu5Ac and Neu5Gc were lowered when their areas were adjusted for changes in the internal standard (KDN) response (Figure 3B). Though not shown here, when analyzing sialic acids after a period of instrument inactivity or activity with another application, peak areas sometimes increase for up to 8 h before stabilizing. To investigate whether the injection of samples (HCl or neuraminidase digests of glycoproteins) causes any loss of response, sample and standard injections were interspersed. Once again, peak area RSDs (1.8, 3.2, and 2.4%, respectively; see Figure 4) were similar to those found in Technical Note 41 (4.1, 4.5, and 4.0%, respectively). In this example internal standard correction provided no improvement in the RSDs of Neu5Ac and Neu5Gc standard peak area responses. Waveform A was also shown to be tolerant of glycoprotein samples hydrolyzed for neutral and amino sugar analysis.⁴

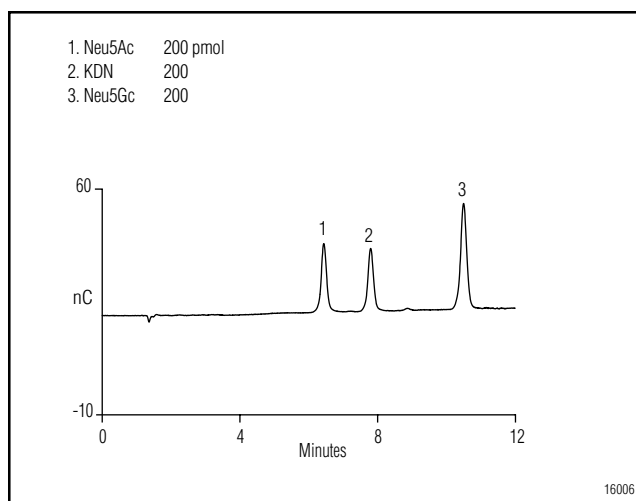


Figure 2. Separation of Neu5Ac, KDN, and Neu5Gc.

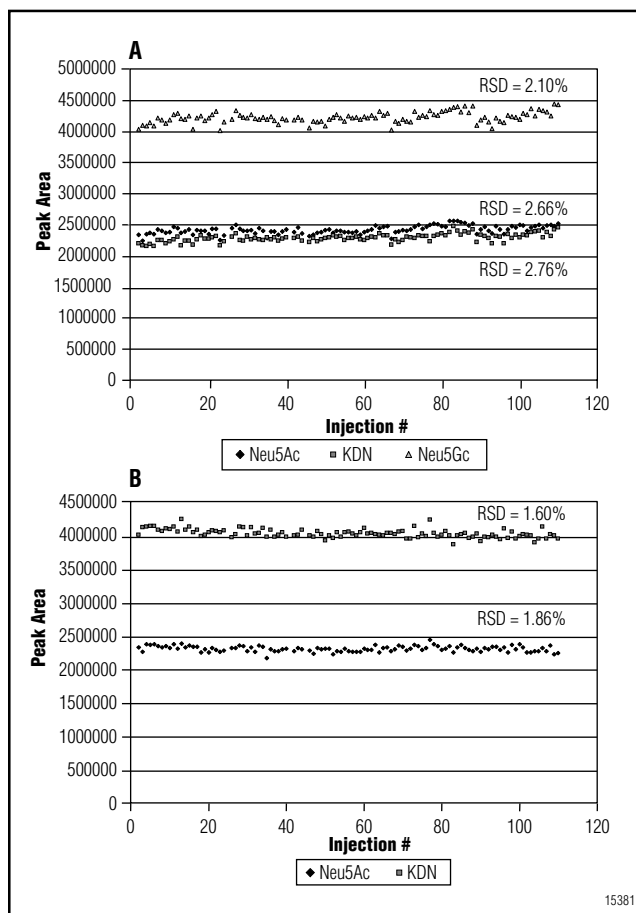


Figure 3. Peak area reproducibility of Neu5Ac, KDN, and Neu5Gc using Waveform A. Each injection (20 μ L) contained 200 pmol of each compound. Panel A shows the peak area for each compound from each injection. The peak area RSD for each compound is displayed on the plot. Panel B shows the peak areas for Neu5Ac and Neu5Gc after adjustment for changes in the internal standard (KDN).

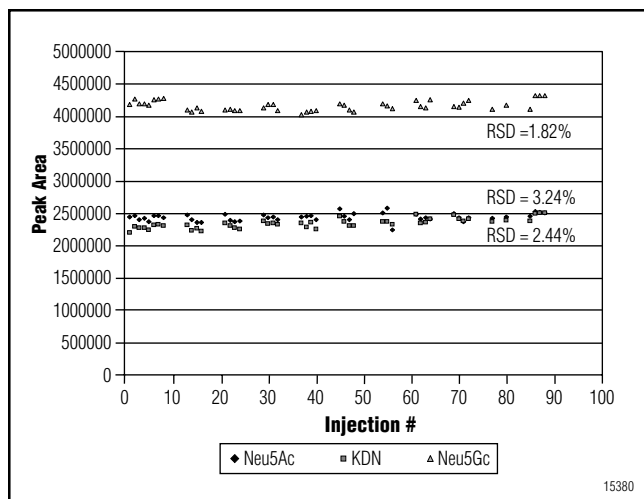


Figure 4. Peak area reproducibility of Neu5Ac, KDN, and Neu5Gc with interspersed sample injections using Waveform A. Standard injections were the same as in Figure 1. Samples were either acid hydrolysates or neuraminidase digests of human transferrin.

To assess the long-term reproducibility of peak area response, we recorded the average peak areas for the 200-pmol standard injected intermittently over a 1.5-month period (Table 1). Note that there is no decrease in peak area over the course of the study. A similar study conducted over only a 2-week period using Waveform B showed an 18.0–21.5% decrease in peak area for the three standards.³ Rocklin et al. performed a 2-week study in which Neu5Ac and Neu5Gc were analyzed

continuously using Waveform B; it showed 22% and 32% losses in Neu5Ac and Neu5Gc peak areas.⁴ The same experiment using Waveform A showed a 2.2% increase in Neu5Ac peak area and a 6.6% loss in Neu5Gc peak area. All these studies show that Waveform A, a quadruple potential waveform, provides better long-term peak area reproducibility than Waveform B, a triple potential waveform.

Although good results can be obtained when assaying Neu5Ac and Neu5Gc using HPAE-PAD with Waveform B,^{2,3} Waveform A clearly offers better long-term reproducibility. We recommend that new assays for Neu5Ac and Neu5Gc be developed and validated using Waveform A as instructed in Technical Note 21.⁵

REFERENCES

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2. Dionex Technical Note 41, 1997.
3. Rohrer, J. S.; Thayer, J.; Weitzhandler, M.; Avdalovic, N. *Glycobiology* **1998**, *8*, 35–43.
4. Rocklin, R. D.; Clarke, A. P.; Weitzhandler, M. *Anal. Chem.* **1998**, *70*, 1496–1501.
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Table 1 Long-Term Sialic Acid Peak Area Reproducibility (200 pmol each) with Waveform A

Experiment Number	Number of Injections ^a	Average Neu5Ac Area	Neu5Ac Area RSD	Average KDN Area	KDN Area RSD	Average Neu5Gc Area	Neu5Gc Area RSD
1 ^b	107	2429321	4.29	2329543	5.50	4116421	5.00
2	53	2323541	2.64	2240904	4.20	4024646	2.89
3	110	2421353	2.66	2291496	2.76	4226660	2.10
4	45	2443536	2.44	2349556	3.24	4166784	1.82

^aExperiments with lower injection numbers included analyses of 0.1 N HCl and neuraminidase digests of human transferrin.

^bFor this experiment, seven individual standards were prepared rather than one larger volume standard that was then distributed into seven vials. We believe this accounts for the higher than expected area RSDs.



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