Application Note 253

HPAE-PAD Determination of Infant Formula Sialic Acid

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Introduction

Dietary sialic acids are important for infant development, serving both immune system and cognitive development roles.[1] Although these functionalized neuraminic acids are present in all mammalian milk, the proportions vary significantly according to the species. Even though many neuraminic acids have been identified in human milk, sialyl-conjugates contain *N*-acetylneuraminic acid (Neu5Ac) but not *N*-glycolylneuraminic acid (Neu5Gc). In comparison, bovine milk has primarily Neu5Ac, but also a small proportion of oligosaccharides possessing Neu5Gc.[1]

In addition to containing different forms of sialic acids, bovine milk has been shown to contain less than 25% of the total sialic acid content of human milk.[2] Therefore, unfortified infant formulas made from bovine milk have a lower sialic acid content than human milk. Because of the critical role these carbohydrates play in infant development, manufacturers have begun enriching infant formulas with sialic acids to supplement the base of the formula and more closely mimic human milk.

Determination of sialic acids in a complex matrix, such as a dairy product, presents many challenges. The majority of sialic acids are found as part of a glycoconjugate rather than in the free form. In human milk, ~73% of sialic acids are bound to oligosaccharides, while some infant formulas have been shown to contain sialic acids primarily bound to glycoproteins.[2] In order to determine the sialic acids, they must first be released from the glycoproteins, glycolipids, and oligosaccharides. In dairy products, this is typically accomplished by a dilute (25 to 100 mM) acid digestion at 80 °C.[3] Many acid hydrolysis methods have been published. While sulfuric acid is commonly used, other

acids have been evaluated including acetic acid, TFA, and HCl.[3,4] TFA and HCl have the advantage of being volatile and easily removed by lyophilization, depending on the needs of further sample-preparation steps.

Following sample hydrolysis, many sialic acid determination methods exist. Numerous spectroscopic methods have been previously reviewed.[3] Interferences in these methods can overestimate the concentration of sialic acids in complex samples; therefore, chromatographic methods that separate the sialic acids from potentially interfering compounds are preferred. Among the chromatographic methods, some require further sample derivatization for analyte detection, such as fluorescent labeling, followed by high-performance liquid chromatography (HPLC). Direct detection methods, such as high-performance anion-exchange with pulsed amperometric detection (HPAE-PAD), offer the advantage of direct analysis without sample derivatization.

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In the work shown here, sialic acids are determined in infant formulas following acid hydrolysis. Two sample preparation methods are presented: one uses ion-exchange and the other uses enzyme digestion. Each method has advantages for a specific type of sample, allowing options for sample-preparation optimization. Both methods remove many potentially interfering compounds present in a complex matrix such as infant formula. Subsequent sialic acid determination by HPAE-PAD on a Thermo Scientific™ Dionex™ CarboPac™ PA20 column is specific and direct, eliminating the need for sample derivatization after sample preparation.

Equipment

Thermo Scientific Dionex ICS-3000 Ion Chromatography System* including:

- Thermo Scientific Dionex SP Single Pump or Thermo Scientific Dionex DP Dual Pump module
- Thermo Scientific Dionex DC Detector/Chromatography module (single- or dual-temperature zone configuration)
- Thermo Scientific Dionex AS Autosampler
- Thermo Scientific Dionex Electrochemical Detector
- Thermo Scientific Dionex Electrochemical Cell
- Thermo Scientific Dionex Disposable Gold Electrode, Carbohydrate Certified
- Thermo Scientific Dionex Reference Electrode
- 10 µL PEEK Sample Injection Loop
- Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System (CDS) Software, Workstation 7
- Polypropylene injection vials with caps, 0.3 mL
- Polypropylene injection vials with caps, 1.5 mL
- 1000 mL 0.2 µm nylon filter units
- Polypropylene screw-cap tubes, 7 mL
- Syringe filters, 0.2 µm, 25 mm
- Thermo Scientific™ Dionex™ OnGuard™ IIA Cartridge, 2.5 cc
- Dionex OnGuard Sample Prep Station
- Polymethylpentene (PMP) volumetric flasks, 500 mL, Class A
- Dry block heater

**Equivalent or improved results can be achieved using the Thermo Scientific Dionex ICS-5000+ system.*

Reagents and Standards

- Deionized (DI) water, Type I reagent-grade, 18 MΩ-cm resistivity or better
- Sodium hydroxide, 50% (w/w)
- Sodium acetate, anhydrous
- Sulfuric acid
- *N*-Acetylneuraminic acid (Neu5Ac, NANA)
- *N*-Glycolylneuraminic acid (Neu5Gc, NGNA)
- Amyloglucosidase

Samples

Three brands of infant formula were purchased for analysis. A soy-based formula was chosen for use as a matrix blank. Because this formula is dairy-free, it is expected to contain no sialic acids.

Brand A: Dairy-based infant formula.
Brand B: Dairy-based infant formula containing added maltodextrins.
Brand C: Soy-based infant formula.

Conditions

Columns:	Dionex CarboPac PA20, 3 × 150 mm Dionex CarboPac PA20 Guard, 3 × 30 mm
Eluent A:	100 mM NaOH
Eluent B:	400 mM sodium acetate in 100 mM NaOH
Eluent Gradient:	10 to 200 mM acetate in 100 mM NaOH from 0 to 15 min, 200 mM acetate in 100 mM NaOH from 15 to 20 min, 10 mM acetate in 100 mM NaOH from 20 to 25 min
Flow Rate:	0.5 mL/min
Temperature:	30 °C (column and detector compartments)
Inj. Volume:	10 µL
Detection:	Pulsed amperometric, disposable carbohydrate certified gold working electrode
Background:	16–25 nC (using the carbohydrate waveform)
Noise:	~20 to 50 pC
System Backpressure:	~2900 psi

Carbohydrate 4-potential waveform for the Dionex ED detector:

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 and Thermo Scientific Dionex ICS-5000 systems but not used in older Thermo Scientific Dionex systems.*

Reference electrode in Ag mode (Ag/AgCl reference). See Dionex (now part of Thermo Scientific) Application Update (AU) 141: I*mproved Long-Term Stability of N-Acetylneuraminic Acid and N-Glycolylneuraminic Acid Peak Area* for more information.[5]

Preparation of Reagents and Standards

Eluent Solution

Prepare 1 L of 100 mM sodium hydroxide by adding 5.2 mL of 50% NaOH to 994.8 mL degassed DI water. Prepare 1 L of 400 mM sodium acetate in 100 mM sodium hydroxide by dissolving 32.8 g anhydrous sodium acetate in ~800 mL DI water. Filter and degas the acetate solution through a 0.2 µm nylon filter unit. Transfer the solution to a 1 L volumetric flask, add 5.2 mL of 50% NaOH, and bring to volume with degassed DI water.

See Thermo Scientific Technical Note (TN) 71: *Eluent Preparation for High-Performance Anion-Exchange Chromatography with Pulsed Amperometic Detection* for detailed information on manual eluent preparation for HPAE-PAD applications.[6]

50 mM Sulfuric Acid for Sample Digestion

Prepare 1 L of 50 mM sulfuric acid by adding 2.8 mL concentrated sulfuric acid to a 1 L polypropylene volumetric flask that contains ~500 mL DI water. Bring to volume with DI water and mix thoroughly.

1000 U/mL Amyloglucosidase Stock Solution

On the day of analysis, prepare a stock solution of 1000 U/mL amyloglucosidase. The exact weight of amyloglucosidase will vary by the activity of the lot of enzyme purchased. For example, if the enzyme contains 57.7 U/mg, add 86.7 mg amyloglucosidase to 5.0 mL DI water and gently swirl to dissolve the enzyme.

Standard Stock Solutions

Prepare sialic acid stock solutions by dissolving Neu5Ac (149.8 mg in 50 mL DI water) and Neu5Gc (41.0 mg in 50 mL DI water). This results in 9.68 mM and 2.52 mM stock solutions, respectively. In dairy samples, ~95% of sialic acids are Neu5Ac. For this reason, prepare a mixed stock of 0.10 mM Neu5Ac and 6.8 µM Neu5Gc by diluting 500 µL of 9.68 mM Neu5Ac and 130 µL of 2.52 mM Neu5Gc to 48.4 mL total. Place aliquots of this solution into 1.5 mL cryogenic storage vials and store at -40 °C.

Standard Solutions

Calibration standards are prepared by diluting the stock standard solution as detailed in Table 1. For example, 10 µL of stock solution are added to 990 µL DI water to prepare a calibration standard of 1.0 µM Neu5Ac, or 10 pmol/10 µL injection. Prepare standards daily from stocks stored at -40 °C.

Ion-Exchange Cartridge Preparation

For best recoveries, convert a 2.5 cc Dionex OnGuard IIA cartridge from carbonate to chloride form by washing it with 15 mL DI water and then 15 mL of 100 mM NaCl. Even hydration of the resin is necessary and can be done using a slow and controlled flow of the initial water wash and the subsequent NaCl wash. Recommended cartridge washing steps and methods are further described in the Dionex OnGuard II cartridges product manual.[7] An Dionex OnGuard workstation can be used to control flow rate through the cartridges when simultaneously preparing multiple samples.

Table 1. Sialic acid standards used for sample analysis.

Stock Standard Volume Diluted to 1000 (µL)	Neu5Ac Conc. (nM)	Neu5Gc Conc. (nM)	Neu5Ac Amount (pmol/10 µL)	Neu5Gc Amount (pmol/10 µL)
1	100	6.8	1	<LOD*
2.5	250	17	2.5	<LOD*
5	500	34	5	0.34
10	1000	68	10	0.68
25	2500	170	25	1.7
50	5000	340	50	3.4
75	7500	510	75	5.1
100	10000	680	100	6.8

** Not used for Neu5Gc calibration*

Powdered Infant Preparation, Acid Hydrolysis, and Maltodextrin Removal

Prepare powdered infant formulas by suspending 0.750 g of formula in 10.0 mL DI water. Use a vortexing mixer to ensure even mixing of the samples. Hydrolyze this solution by adding 900 µL formula to 5.0 mL of 50 mM sulfuric acid in a 7 mL polypropylene screw-cap vial. Heat the capped vial in a heat-block maintained at 80 °C for 1 h. After 1 h, remove samples and allow to cool to room temperature (~10 min). Before further treatment, centrifuge samples to separate fats and proteins suspended in the sample. To remove maltodextrins by anion exchange, prepare an Dionex OnGuard IIA cartridge. Skim off fat from the centrifuged sample with a pipet tip and pour the acid-hydrolyzed sample directly into the cartridge reservoir, taking care to leave precipitated proteins in the digestion tube. After loading the sample on the cartridge, wash the cartridge with 10 mL DI water to remove any residual uncharged compounds from the resin. Elute the bound sialicacids with 25 mL of 50 mM NaCl. Before the samples are injected, filter them through an IC syringe filter (0.2 µm, 25 mm) and dilute 1:2.5 with DI water to minimize retention-time shifting of Neu5Gc due to chloride.

Maltodextrins in hydrolyzed samples were removed by two independent methods. The first method tested was anion exchange and the second was enzymatic digestion. To remove maltodextrins by enzyme digestion, dilute the acid-hydrolyzed sample with DI water to nearly 500 mL in a 500 mL PMP volumetric flask. Add 500 µL of amyloglucosidase to the solution and dilute to 500 mL. Mix gently and allow the sample to digest for a minimum of 1 h at ambient temperature.

Precautions and Considerations

Labware: Avoid using glass volumetric flasks for dilution of samples and standards. Class A PMP flasks are recommended, although polypropylene is acceptable. Similarly, use polypropylene (rather than glass) digestion vials and injection vials.

When filling PMP or polypropylene labware, remove bubbles from the surface by gently swirling the solution in the volumetric flask while it is approximately three-quarters full. Bubbles on the walls of the flask cause dilution errors. Make the final dilution by gently adding water down the side of the flask. Similarly, bubbles in injection vials can lead to inconsistent injections and must be removed.

Results and Discussion

Figure 1 shows separation of Neu5Ac and Neu5Gc on the Dionex CarboPac PA20 column with a 10 to 200 mM acetate gradient in 100 mM NaOH. The peaks are well separated and easily quantified. For samples that have few interfering compounds, the gradient can easily be shortened by eluting with a gradient of 20–200 mM acetate in 100 mM NaOH and reducing the gradient time. In dairy samples, however, numerous other carbohydrates are present that can potentially interfere with sialic acid quantification. Infant formula, for example, contains added lactose, maltodextrins, and cereal starches. After acid hydrolysis, these carbohydrates interfere with sialic acid determination. While sample preparation steps minimize these interfering compounds, they may still be present and detected by HPAE-PAD. By using a shallower gradient, other carbohydrates in the sample will be resolved from the sialic acids.

Column:	Dionex CarboPac PA20 guard, 3 × 30 mm Dionex CarboPac PA20, 3 × 150 mm
Eluent:	10–200 mM acetate in 100 mM NaOH from 0–15 min, 200 mM acetate in 100 mM NaOH from 15–20 min, 10 mM acetate in 100 mM NaOH from 20–25 min
Temperature:	30 °C
Flow Rate:	0.5 mL/min
Inj. Volume:	10 µL
Detection:	PAD, Au (Disposable)
Samples:	Neu5Ac and Neu5Gc standards
Peaks:	1. Neu5Ac 25 pmol 2. Neu5Gc 1.7

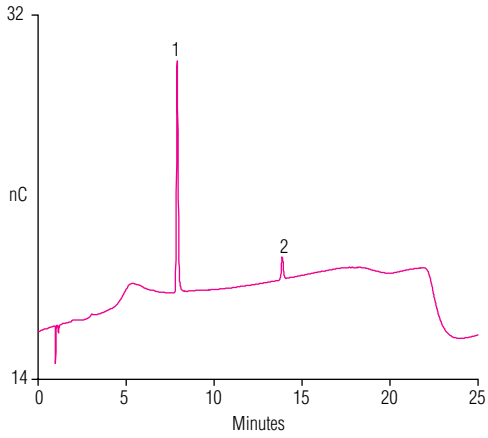

Figure 1. Separation of sialic acids on the Dionex CarboPac PA20 column.

Linear Range, Limits of Quantification and Detection, and Precision

Table 2 shows the calibration results for Neu5Ac and Neu5Gc. In both cases, response is linear for the range studied. Two calibration ranges for Neu5Ac were investigated. The first calibration range covers concentrations of Neu5Ac present when determined by acid hydrolysis and OnGuard IIA sample preparation; the second calibration range is extended to include lower concentrations of Neu5Ac present when enzyme digestion is used after acid hydrolysis. In both cases, the response is linear. Neu5Gc is a minor component of infant formula and is derived from the bovine dairy source. The calibration range used is the same for both sample preparation methods.

The limit of detection (LOD) and limit of quantification (LOQ) were confirmed by standard injections that resulted in a response of 3× and 10× the noise, respectively. Neu5Ac was determined to have an LOD of 0.24 pmol on column and an LOQ of 0.80 pmol. Similarly, Neu5Gc limits were found to be 0.21 pmol and 0.70 pmol.

Table 2. Linearity, LOD, LOQ, and precision.

Analyte	Range (pmol)	Corr. Coef. (r^2)	RT (min)	RT Precision (RSD)	Peak Area Precision[a] (RSD)	LOQ[b] (pmol)	LOD (pmol)
Neu5Ac	5.0–100	0.9995	7.89	0.05	0.98	0.8	0.24
Neu5Ac	1.0–75	0.9995					
Neu5Gc	0.34–6.8	0.9997	13.86	0.05	1.98	0.7	0.21

[a] *Precision is measured by seven injections of 25 pmol Neu5Ac, 1.7 pmol Neu5Gc.*
[b] *LOD and LOQ are confirmed by injections at the concentrations listed with response measured at 3× and 10× the noise, respectively.*

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 retention time RSD of 0.05 for both sialic acids and peak area RSDs of 0.98 and 1.98 for Neu5Ac and Neu5Gc, respectively.

Sample Analysis

Before choosing a sample-preparation method, a number of methods were investigated to remove interfering compounds. Because sialic acids are charged at neutral pH, ion-exchange resins will trap the sialic acids on the resin and neutral compounds will not be retained.[8] The sulfuric acid matrix of samples loaded onto the cartridge does not affect Neu5Ac and Neu5Gc binding to the resin. This was confirmed experimentally with standards in sulfuric acid. The retained sialic acids may then be eluted using a stronger eluent, such as formate, acetate, or chloride. Each of these eluents was tested to evaluate resin loading and recovery of standards on an Dionex OnGuard IIA cartridge. Of the eluents tested, the best condition determined was elution with 50 mM sodium chloride followed by a 1:2.5 dilution of the sample in DI water. This process yields the highest recoveries and offers the simplest preparation before sample injection. Chromatographic results of this preparation are shown in Figure 2. Neu5Ac is well resolved from interfering peaks under these conditions, and peaks from neutral maltodextrins are minimized.

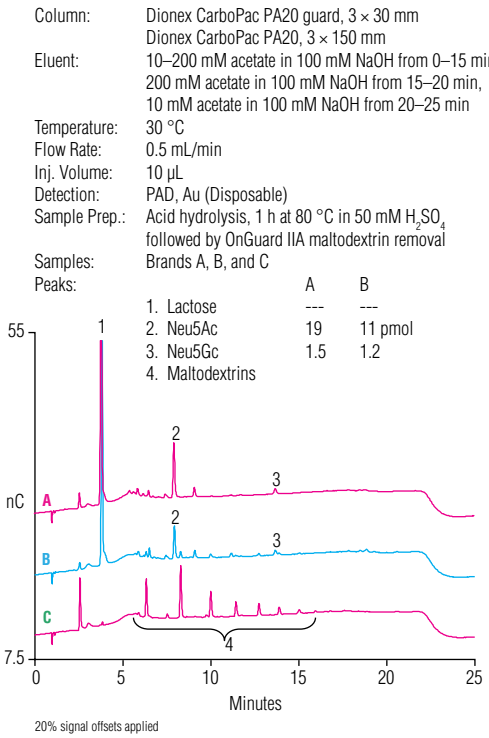

Figure 2. Separation of anion-exchange resin prepared infant formula samples based on A) dairy, B) dairy with added maltodextrins, and C) soy with added maltodextrins.

Sample Analysis Precision and Accuracy

Precision was evaluated over three days of triplicate sample analysis. Representative results for one day of triplicate sample analysis are presented in Table 3. Table 4 shows data collected after three days of analysis. Sample C, the soy-based infant formula, did not contain detectable Neu5Ac or Neu5Gc and was used as a blank matrix for comparing recovery of spiked sialic acids.

Table 3. Sample Analysis precision data, day 2.

Sample	Analyte	Amount (pmol)	mg/100 g of Sample	Peak Area Precision (RSD)	RT Precision (RSD)	Analysis Precision (RSD)
Brand A, Replicate #1	Neu5Ac	22	85	4.25	0.06	0.59
	Neu5Gc	1.6	6.3	3.51	0.04	8.4
Brand A, Replicate #2	Neu5Ac	23	86	2.96	0.06	
	Neu5Gc	1.5	5.9	2.89	0.04	
Brand A, Replicate #3	Neu5Ac	22	85	2.39	0.12	
	Neu5Gc	1.3	5.4	2.44	0.13	
Brand B, Replicate #1	Neu5Ac	14	54	5.50	<0.01	15.7
	Neu5Gc	1.3	5.0	2.23	0.06	11.0
Brand B, Replicate #2	Neu5Ac	13	48	5.52	<0.01	
	Neu5Gc	1.1	4.4	5.08	<0.01	
Brand B, Replicate #3	Neu5Ac	10	40	8.52	0.11	
	Neu5Gc	1.0	4.0	4.81	0.13	

Table 4. Between-day sample analysis precision.

Day	Sample	Analyte	Average Amount (pmol)	mg/100 g of Sample	Intraday Precision (RSD)	Between-day Precision (RSD)
1	**Brand A**	Neu5Ac	16	62	20.6	18
		Neu5Gc	1.2	4.7	23.5	11
	Brand B	Neu5Ac	11	44	6.8	11
		Neu5Gc	1.3	5.2	4.0	8.9
2	**Brand A**	Neu5Ac	22	86	0.59	
		Neu5Gc	1.4	5.8	8.4	
	Brand B	Neu5Ac	12	47	15.7	
		Neu5Gc	1.1	4.5	11.0	
3	**Brand A**	Neu5Ac	17	64	5.3	
		Neu5Gc	1.3	5.3	1.4	
	Brand B	Neu5Ac	10	38	13.6	
		Neu5Gc	1.1	4.5	10.3	

When corrected for dilution during the sample preparation process, the prepared samples of Brands A and B contained 86 and 47 mg Neu5Ac in 100 g of sample, respectively. Retention time precision was similar to that determined by injecting standards, with RSDs ranging from <0.01 to 0.13. Peak area precision RSDs ranged from 2.23 to 8.52.

Variability between sample replicates of dairy samples may be large, as shown in Table 5; therefore, optimization of digestion and sample-preparation methods for individual infant formulas is highly recommended.

Method accuracy was investigated by spiking infant formula acid hydrozylates with known amounts of Neu5Ac and Neu5Gc and evaluating recovery of the amended sample through the sample-preparation procedure. Recoveries for Neu5Ac ranged from 80 to 109% for three different formulas treated by anion-exchange sample preparation (Table 5). Recoveries for Neu5Gc were similar, ranging from 78 to 111%.

Maltodextrin Removal by Enzymatic Digestion

Amyloglycosidase was chosen to remove maltodextrins from the samples without the need for ion-exchange sample cleanup. This enzyme was chosen for its broad activity against glycosidic linkages (α1–2, 1–6, and 1–4) as well as its optimal activity at low pH.[9] Because amyloglucosidase is active at pH 3, samples may simply be diluted after acid hydrolysis without the need to further adjust pH prior to adding enzyme. When samples were digested by this method, maltodextrins were significantly reduced (Figure 3B). However, as shown in Figure 3A, in some formulas there are other potential interferences in addition to maltodextrins.

For samples that contain maltodextrins, this sample-preparation method is useful; however, conditions must be customized for each sample type. For samples that do not contain significant amount of maltodextrins, this method will not reduce interfering compounds. For example, results for Brand A are highly elevated compared to the results found when using the anion-exchange sample-preparation method (Tables 5 and 6). This brand does not have additional maltodextrins added to the formula. Results for Brand B are similar for both sample-preparation methods. In both cases, recoveries of standards spiked into the sample digest are good. Recoveries from infant formulas treated by this method range from 89.4 to 95%. For soy formulas, the enzyme did not sufficiently remove the putative maltodextrins after 24 h of digestion; therefore, the method is not recommended for these formulas. The polysaccharides added to the soy-based formula tested are likely linked by different glycosidic linkages that are not easily digested by amyloglycosidase. The enzyme and the conditions for digestion may be improved, depending on additives in a specific infant formula sample.

Column:	Dionex CarboPac PA20 guard, 3 × 30 mm Dionex CarboPac PA20, 3 × 150 mm		
Eluent:	10–200 mM acetate in 100 mM NaOH from 0–15 min, 200 mM acetate in 100 mM NaOH from 15–20 min, 10 mM acetate in 100 mM NaOH from 20–25 min		
Temperature:	30 °C		
Flow Rate:	0.5 mL/min		
Inj. Volume:	10 µL		
Detection:	PAD, Au (Disposable)		
Sample Prep.:	Acid hydrolysis, 1 h at 80 °C in 50 mM H_2SO_4 followed by enzyme digestion of maltodextrins		
Samples:	Brands A and B		
Peaks:		A	B
	1. Neu5Ac	9.1	3.0 pmol
	2. Neu5Gc	0.44	0.41

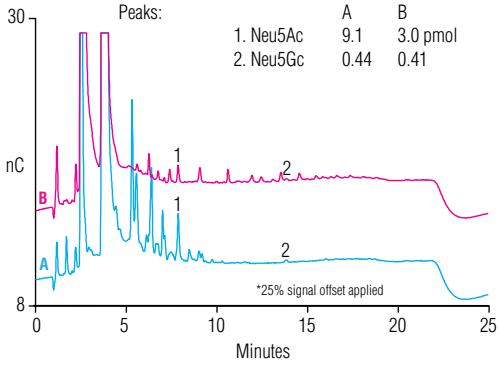

Figure 3: Separation of enzyme digestion prepared infant formula samples based on A) dairy, B) dairy with added maltodextrins. Soy-based formula maltodextrins are not well digested by amyloglycosidase and a chromatogram is not shown.

Table 5. Recovery data for three types of infant formulas.

Day	Sample	Analyte	Amount Spiked into 5.9 mL Hydrozylate (nmol)	Theoretical Concentration of Spiked Sample (pmol)	Measured Concentration (pmol)	% Recovery
1	Brand A	Neu5Ac	75.0	25	27	108
		Neu5Gc	5.1	1.8	2.0	111
	Brand B	Neu5Ac	75.0	20	20	100
		Neu5Gc	5.1	1.9	1.8	94.7
	Brand C	Neu5Ac	75.0	8.9	8.5	95.6
		Neu5Gc	5.1	0.6	0.61	100
2	Brand A	Neu5Ac	75.0	31	28	90.3
		Neu5Gc	5.1	2.0	1.7	85
	Brand B	Neu5Ac	75.0	21	20	96.6
		Neu5Gc	5.1	1.7	1.8	102
	Brand C	Neu5Ac	75.0	8.7	8.1	93.1
		Neu5Gc	5.1	0.59	0.54	91.5
3	Brand A	Neu5Ac	75.0	26	23	88.5
		Neu5Gc	5.1	1.9	1.6	84.2
	Brand B	Neu5Ac	75.0	19	20	105
		Neu5Gc	5.1	1.7	1.6	94.1
	Brand C	Neu5Ac	75.0	8.9	7.1	80.1
		Neu5Gc	5.1	0.6	0.47	78.3

Table 6. Sialic acid content determined after enzyme digestion of infant formula acid hydrozylates.

Sample	Analyte	Average Concentration of Sialic Acid (pmol) (n=3)	Sialic Acid Amount (mg/100 g of Sample)
Brand A	Neu5Ac	5.97	131
	Neu5Gc	0.39	9
Brand B	Neu5Ac	1.96	45
	Neu5Gc	<LOD	

Conclusion

Sialic acids in infant formulas are accurately determined by HPAE-PAD using the Dionex CarboPac PA20 column following acid hydrolysis and maltodextrin removal using one of two sample-preparation methods. HPAE-PAD provides reliable determination of sialic acids in acid-hydrolyzed infant formula samples without sample derivatization. This method may be used to quantify sialic acids in formulas that have been enriched with sialic acids.

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