DIONEX

Application Note 266

Determination of Sialic Acids Using UHPLC with Fluorescence Detection

INTRODUCTION

Dietary sialic acids play an important role in infant development, serving both immune system and cognitive development roles.[1] Many neuraminic acids have been identified in human milk, however *N*-acetylneuraminic acid (Neu5Ac) is predominant, and *N*-glycolylneuraminic acid (Neu5Gc) is generally absent. In comparison, bovine milk contains approximately 5% 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] The sialic acid content in unfortified infant formulas is dependent on the sialic acids from bovine milk. As such, these formulas have lower sialic acid contents and different sialic acid proportions compared to human milk. Because of the critical role these carbohydrates play in infant development, many manufacturers enrich infant formulas with sialic acids to more closely mimic human milk.

Sialic acid determination in a complex matrix, such as a dairy product, presents many challenges. The majority of milk sialic acids are found as part of a glycoconjugate rather than as the free acid. In human milk, ~73% of sialic acid is bound to oligosaccharides, but 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] These acids have the advantage of being volatile and easily removed by lyophilization, which could be important, depending on the needs of further sample preparation steps.

Following sample hydrolysis, many options are available for determination of sialic acids. Numerous spectroscopic methods exist. However, interferences in these methods can overestimate the concentration of sialic acids and, therefore, chromatographic methods that separate the sialic acids from potentially interfering compounds are preferred. Both direct and indirect chromatographic methods such as High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAE-PAD, direct) or fluorescent labeling followed by HPLC (indirect) have been published.[3,4] One common fluorescent labeling method, using 1,2-diamino-4,5-methylenedioxybenzene dihydrochloride (DMB) to label the sialic acids, was first published by Hara, et al.[5-6] This method has previously been modified to determine sialic acids in infant formulas.[7-8] Although the fluorescent labeling method determines sialic acids indirectly, the chromatographic conditions are less likely to change the O-acetylation of the sialic acids, allowing identification of a wider range of sialic acids.[9]

In this work, N-acetylated sialic acids are determined and O-acetylated sialic acids are identified by HPLC with fluorescence detection following acid hydrolysis and DMB derivatization of infant formula samples. By using a water:acetonitrile gradient, high resolution of the sialic acids was obtained in a 20 min analysis time, compared to the common 40 min isocratic method. The described assay uses a short format Acclaim® RSLC 120 C18 column that allows fast run times and requires less acetonitrile than other published methods by using a lower flow rate and having a shorter run time. The sensitivity of fluorescence detection easily allows determination of sialic acids in the infant formula which are present in the pmol range. The sensitivity provides for simple determination of Neu5Ac, Neu5Gc, and O-acetylated sialic acids in the derivatized samples.

EQUIPMENT

Dionex UltiMate® 3000 RSLC system including:

SRD-3600 Solvent Rack and Degasser (Dionex P/N 5035.9230)

HPG-3400RS Binary Pump with a 350 µL mixer (Dionex P/N 5040.0046)

WPS-3000TRS Well Plate Sampler, Thermostatted (Dionex P/N 5840.0020)

Sample loop, 25 µL (Dionex P/N 6820.2415)

TCC-3000RS Thermostatted Column Compartment (Dionex P/N 5730.0000)

Precolumn Heater (Dionex P/N 6722.0530)

Viper UHPLC Fingertight Fitting and Capillary Kit, RSLC Systems, SST (Dionex P/N 6040.2301)

FLD-3400RS Fluorescence Detector with dual PMT (Dionex P/N 5078.0025)

Chromeleon® 7.0 Chromatography Workstation

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

7 mL Polypropylene screw cap tubes (Sarstedt P/N 60.550)

IC Acrodisc® syringe filters, 0.2 µm, 25 mm (Pall Corporation P/N 4583T)

OnGuard® IIA, 2.5 cc Cartridges (Dionex P/N 057092)

OnGuard Sample Prep Workstation (Dionex P/N 039599)

1.5 mL Microcentrifuge tubes (Sarstedt P/N 72.692.005)

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

REAGENTS AND STANDARDS

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

Acetonitrile (Honeywell, P/N 015-4)

Formic acid (Fluka P/N 06440)

Sulfuric acid (JT Baker P/N 9673-00)

N-Acetylneuraminic acid (Neu5Ac, NANA) Ferro Pfanstiehl

N-Glycolylneuraminic acid (Neu5Gc, NGNA) Ferro Pfanstiehl

Glyko® Sialic Acid Reference Panel (ProZyme P/N GKRP-2503)

Glacial acetic acid (JT Baker P/N 9515-03)

2-Mercaptoethanol (Aldrich P/N M6250)

Sodium hydrosulfite (Sigma P/N 157953)

1,2-Diamino-4,5-methylenedioxybenzene dihydrochloride (DMB) (Sigma P/N D4784)

SAMPLES

Three brands of commercially available infant formula were purchased for analysis. A soy-based formula was chosen for use as a matrix blank, because sialic acids are not expected in this nondairy product.

Brand A: Dairy-based infant formula

Brand B: Dairy-based infant formula with maltodextrins

Brand C: Soy-based infant formula

CONDITIONS

Column:	Acclaim RSLC 120 C18, 2.2 µm, 2.1 × 100 mm
Gradient:	5% B from 0–5 min, 5%–20% B from 5–13 min, 20–40% B from 13–15 min, 40% B from 15–20 min, 3 min equilibration at 5% B before injection
Flow Rate:	0.42 mL/min
Inj. Volume:	5 µL
Temperature:	45 °C (column compartment)
Detection:	Excitation λ, 373 nm Emission λ, 448 nm
Noise:	~2000 counts
System Backpressure:	~300 bar (~4350 psi)
Run Time:	20 min

Table 1. Sialic Acid Standards Preparation						
Combined Stock Standard (µL)	2 M Formic Acid (µL)	DI Water (µL)	Neu5Ac (µM)	Neu5Gc (µM)	Neu5Ac in 5 µL Injection (pmol)	Neu5Gc in 5 µL Injection (pmol)
10	500	490	1.0	0.78	5.0	0.39
25	388	363	3.2	2.5	16	1.3
50	400	350	6.3	4.9	31	2.4
100	500	400	10.0	7.8	50	3.9
100	375	275	13.0	1.0	67	5.2
100	300	200	17.0	1.3	83	6.5
100	250	150	20.0	1.6	100	7.8
100	200	100	25.0	2.0	130	9.8
200	200	0	50.0	4.0	260	20.0

PREPARATION OF SOLUTIONS AND REAGENTS

Mobile Phases A and B

Mobile Phase A: DI water, Type I reagent grade, 18 MΩ-cm resistivity or better.

Mobile Phase B: Acetonitrile, HPLC grade or better.

Reagents

Formic acid, 1 M

Add 42.5 mL concentrated formic acid to a 1 L volumetric flask containing ~500 mL DI water. Fill the flask to the mark with DI water, cap the flask, and invert to mix the solution.

Formic acid, 2 M

Add 21.25 mL concentrated formic acid to a 250 mL volumetric flack containing ~150 mL DI water. Fill the flask to the mark with DI water, cap the flask, and invert to mix the solution.

Sulfuric acid, 100 mM

Add 540 µL of concentrated sulfuric acid to 99.46 mL (g) of DI water. Mix well.

Standard Stock Solutions

Dissolve 149.8 mg dried Neu5Ac in 50 mL of deionized water to prepare a 9.68 mM stock solution. Similarly, dissolve 41.0 mg dried Neu5Gc in 50 mL of deionized water to prepare a 2.52 mM stock solution. In dairy samples, ~95% of the sialic acids are Neu5Ac. Replicate this proportion of sialic acids in the samples by diluting 250 µL of 9.68 mM Neu5Ac and 75 µL of 2.52 mM Neu5Gc in 24.23 mL total volume. This combined stock standard solution contains 0.10 mM Neu5Ac and 7.8 µM Neu5Gc. Aliquot this solution into 1.5 mL cryogenic storage vials and store at -40 °C. Avoid repeated freeze–thaw cycles.

Standard Solutions

Both the stock solution described above and a sialic acids standard mixture containing Neu5Gc, Neu5Ac, Neu5,7Ac2, Neu5Gc9Ac, Neu5,9Ac2, and Neu5,7,(8),9Ac3 were used to identify sialic acids in infant formulas. Dissolve the contents of the standard mixture vial in 25 µL DI water to prepare the panel for derivatization.

Prepare calibration standards by diluting the combined stock solution as shown in Table 1. For example: Pipet 100 µL combined stock solution into a 1.5 mL microcentrifuge tube. Pipet an additional 100 µL DI water and 200 µL of 2 M formic acid to prepare a standard of 25 µM Neu5Ac and 2.0 µM Neu5Gc in 1 M formic acid. It is critical that the standards are in the same matrix as the samples. If the standards are not prepared in formic acid, the derivatization reaction efficiency will not be the same for both standards and samples, resulting in a potentially large systematic error in the quantification of the samples.

Powdered Infant Formula Preparation, Acid Hydrolysis, and Maltodextrin Removal

Prepare powdered infant formulas by suspending 0.75 g in 10.0 mL DI water. Mix using a vortexing mixer to ensure an even suspension. Hydrolyze this solution by adding 2.5 mL formula suspension to 2.5 mL of 100 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 the samples and cool to room temperature (~10 min). Before further treatment, centrifuge the hydrolysates at 5000 rpm and 5 °C for 10 min to separate the fats and proteins suspended in the sample.

To remove maltodextrins by anion exchange, prepare an OnGuard II A cartridge as described in the manual.[10] Skim the fat off the top of the centrifuged sample with a pipet tip and pour the acid-hydrolyzed sample directly into the cartridge reservoir, taking care to leave the precipitated proteins in the digestion tube. Load the sample onto the anion-exchange cartridge and wash the cartridge with 10 mL DI water. This step washes off the neutral carbohydrates. Elute the sialic acids with 20 mL of 1 M formic acid. After elution, filter the sample with a 0.2 µm IC syringe filter. Promptly derivatize this sample as described below.

DMB Derivatization Reagent

Prepare the DMB reagent in the following order. Add 1.5 mL of DI water to a glass vial. To this solution add 172 µL of glacial acetic acid. Mix well. To this solution add 112 µL of 2-mercaptoethanol. Mix the solution well. Add 4.9 mg of sodium hydrosulfite to the solution and mix. The solution may become cloudy in appearance. Lastly, add 3.5 mg of DMB hydrochloride and 200 µL DI water and mix the solution well. Prepare the reagent fresh each day of analysis. The reagent is light sensitive and should be stored at -20 °C in the dark when not in use. Best results are obtained in this work with fresh derivatization reagent. As the DMB reagent ages, additional peaks that are unrelated to carbohydrate derivatization were observed in reagent blanks.

Derivatization Conditions

Derivatize samples and standards by adding 50 µL of the derivatization reagent to 50 µL of sample in a 1.5 mL screw cap microcentrifuge vial. Transfer the vials to a heating block and incubate for 2.5 h in the dark at 50 ± 2 °C.

Samples, standards, and controls must be derivatized at the same time with the same preparation of derivatization reagent. After 2.5 h of incubation, freeze the solutions at -40 °C to slow the reaction. Thaw the samples and transfer to 0.3 mL injection vials. Best results are obtained within 24 h of derivatization. Derivatized samples degrade with exposure to light and oxygen and should be analyzed as soon as possible.

Precautions

Perform derivatization reagent preparation, sample derivatization, and sample transfers to injection vials in a fume hood. Analyze samples promptly. Derivatized samples will degrade faster on exposure to light. It is strongly recommended that a temperature-controlled autosampler be set to 4 °C and the samples be kept in the dark by use of amber vials or by keeping the autosampler cover closed. When filling low-volume conical vials, it is important to ensure that all air is removed from the cone of the vial. If bubbles are present, peak area precision will be poor.

As noted by Hara et al., the concentration of acid will affect the efficiency of the reaction. It is important for the sample conditions to be mimicked in the standards that are derivatized to avoid systematic error due to different derivatization efficiency. For example, standards that were derivatized in 750 mM acetic acid showed 57% of the peak area for Neu5Ac as compared to the same concentration standards that were derivatized in 750 mM acetic acid in addition to 500 mM formic acid. Furthermore, sodium chloride strongly impacts the derivatization reaction efficiency. Samples containing high amounts of sodium chloride (50 mM) will degrade during the derivatization incubation time, leading to peak areas of <35% of those without the added salt. Standards in formic acid containing 5 mM of sodium chloride exhibited decreased peak areas of 12–13% compared to those without. This effect is reduced compared to 50 mM sodium chloride, and is similar to the between-day variability observed in standards. For best accuracy, the standards should be derivatized in a matrix as similar to the samples as possible, including both the concentration of acid and salts. Optimization of derivatization conditions is highly recommended.

The commonly reported isocratic method using water/methanol/acetonitrile is not recommended for these samples. Backpressure was found to increase after multiple sample injections.

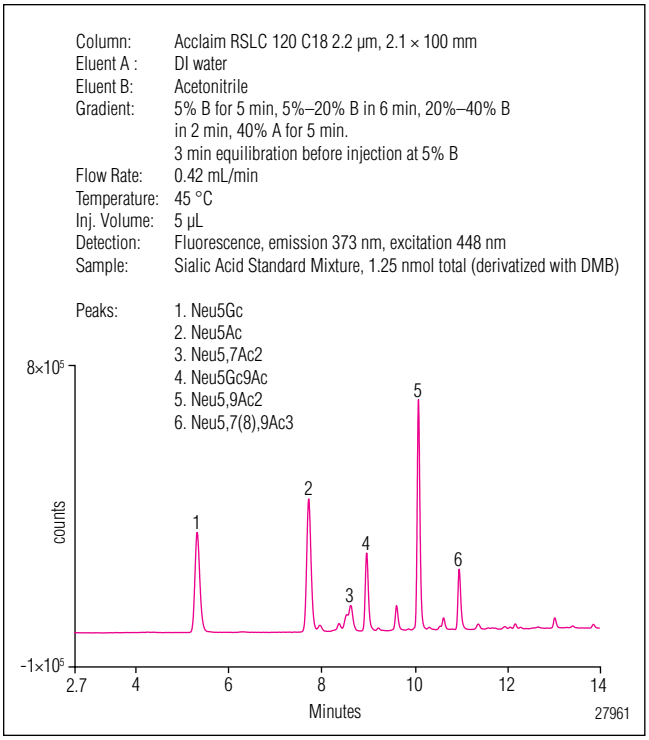

Figure 1. Separation of a derivatized sialic acid standard mixture on the Acclaim RSLC 120 C18 column.

The implication of this is that components of the samples are not eluted from the column. With continued injections, the efficiency of the column will decrease. The gradient method described in this work is recommended for best column performance during routine analysis. Direct injection of sample hydrolysates on to column is not recommended because it may result in lipids and other materials accumulating on the column.

RESULTS AND DISCUSSION

Figure 1 shows the separation of a sialic acid reference standard mixture on the Acclaim RSLC C18 column. As can be seen, Neu5Gc and Neu5Ac are well separated from one another. The O-acetylated sialic acids are also present in this standard with Neu5,7Ac2, Neu5Gc9Ac, Neu5,9Ac2, and Neu5,7(8),9Ac3 identified. In the case of Neu5,7Ac2, a reagent peak can interfere. The intensity of this reagent peak will vary with the derivatization reagent preparation. The sialic acids of interest are separated in under 15 min. However, to maintain column performance, a column wash step is added after each injection. Separation of the reference standards was evaluated on the Acclaim PA and PA2 columns. The shortest run time for standards was obtained with the PA column; however, when injecting samples, the best resolution was found with the C18 column.

Table 2. Linearity, LOD, and LOQ for Sialic Acids

Analyte	Range (pmol)	Correlation Coefficient (r^2)	LOD (pmol)	LOQ (pmol)
Neu5Ac	5–260	0.9952	0.06	0.17
Neu5Gc	0.2–9.8	0.9940	0.08	0.23

Table 3. Peak Area Reproducibility for Multiple Days of Derivatization (n = 3)

Analyte	Day	RT (min)	RT Precision* (RSD)	Peak Area (counts*min)	Peak Area Precision (RSD)
Neu5Ac	1	7.69	0.04	1088000	0.55
Neu5Gc		5.29	0.03	80650	0.76
Neu5Ac	2	7.69	0.02	1229000	0.35
Neu5Gc		5.28	0.05	90430	0.79
Neu5Ac	3	7.69	0.03	1096000	0.96
Neu5Gc		5.28	0.05	81060	1.16
Neu5Ac	4	7.70	0.06	895000	1.45
Neu5Gc		5.29	0.08	66010	1.69

*A standard of 67 pmol Neu5Ac and 5.2 pmol Neu5Gc was used for determination of retention time (RT) and peak area precisions.

The effect of temperature was investigated between 35 and 50 °C. At 50 °C, the peak areas were reduced compared to 40 °C, indicating on-column decomposition. At 45 °C, the overall run time was shortest, with no detectable decomposition of the standards compared to 40 °C.

Linear Range, Limit of Quantification (LOQ), Limit of Detection (LOD), and Precision

Table 2 shows the calibration range, correlation coefficients, and precisions for several days of sialic acid standard preparations. The efficiency of the derivatization reaction impacts the standard peak area from day to day. Preparing standards along with samples limits the effects of this variability; however, between-day peak areas were observed to vary by 13% for both Neu5Ac and Neu5Gc, as detailed in Table 3. Similarly, the LOQ and LOD may vary between analysis days. Using the conditions described, the LOQ and LOD were determined to be 0.17 pmol and 0.06 pmol, respectively, for Neu5Ac. The LOQ and LOD for Neu5Gc were 0.23 and 0.08 pmol, respectively.

In addition to variability of the determined LOQ and LOD based on the derivatization conditions, the detection settings of the fluorescence detector must be considered. In this work, the photomultiplier tube (PMT) was set to the least sensitive collector voltage setting of 1 and the lamp set to the standard flash lamp rate. If greater sensitivity is required, the flash lamp frequency can be increased and sensitivity settings can be changed to further increase the sensitivity. It should be noted that even without optimizing the detector conditions, the method discussed has ample sensitivity to determine sialic acids in infant formulas.

Determination of Sialic Acids in Infant Formulas

The separation of sialic acids in infant formulas is shown in Figure 2. As expected, the dominant sialic acid present in dairy-based formulas is Neu5Ac. Neu5Gc is present to a lesser extent. Brand A also contains minor amounts of Neu5,7Ac2 and both Brands A and B contain a small amount Neu5,9Ac2. Hydrolysis conditions are not optimal for determining these sialic acids; however, they are present. As expected, Brand C, a soy-based formula, does not contain the identified sialic acids. However, it should be noted that under the gradient conditions described here, there is a small unknown peak that elutes near Neu5Gc and could potentially interfere with determination of this sialic acid. Different gradients and use of an isocratic method (8:7:85 CH_3OH:CH_3CN:water) did not fully resolve this peak from Neu5Gc. Previously published work did not observe this peak under isocratic conditions and it is likely dependent on the specific ingredients of the soy infant formula.[7] However, the RT is consistently shorter than Neu5Gc and in spiked samples it is evident there are two components eluting.

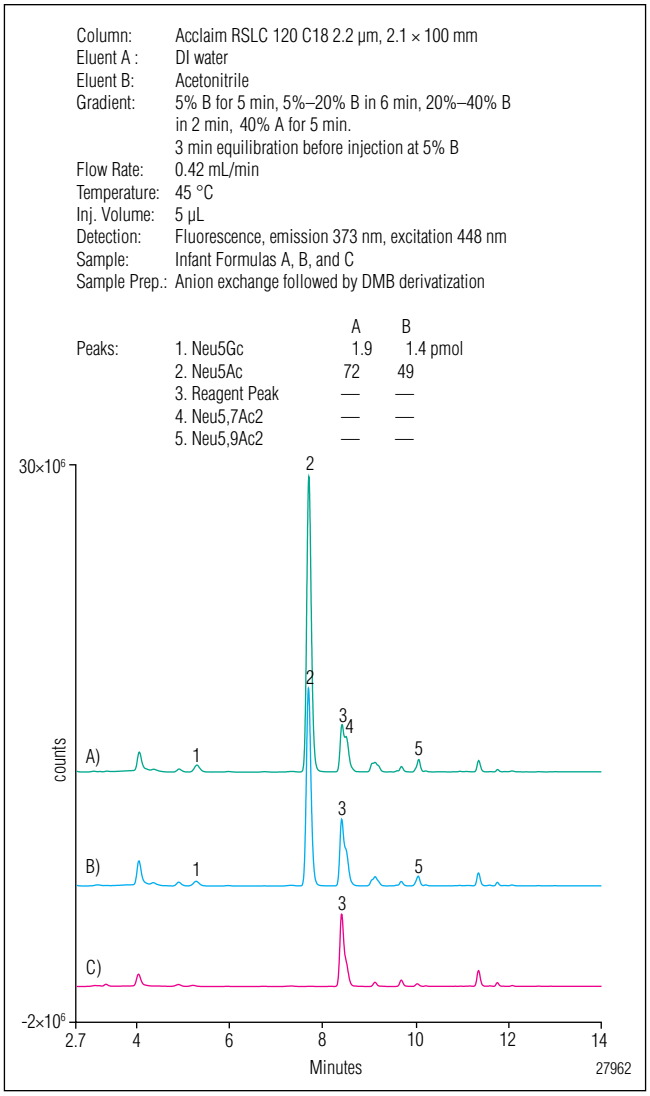

Figure 2. Determination of sialic acids in infant formulas on the Acclaim RSLC 120 C18 column.

Table 4. Sample Analysis Results, Triplicate Infant Formula Sample Preparations								
Sample	**Analyte**	**RT (min)**	**RT Precision (RSD)**	**Peak Area (counts*min)**	**Peak Area Precision (RSD)**	**Measured Concentration (pmol)**	**mg/100 g of Sample**	**Sample Analysis Precision (RSD)**
A replicate #1	Neu5Ac	7.69	0.05	1925000	3.95	70.2	91	7.7
	Neu5Gc	5.29	0.07	55900	3.99	1.86	2.5	8.0
A replicate #2	Neu5Ac	7.69	0.05	1931000	0.40	70.4	92	—
	Neu5Gc	5.28	0.09	57110	1.16	1.9	2.6	—
A replicate #3	Neu5Ac	7.69	0.05	2198000	0.28	80.1	100	—
	Neu5Gc	5.28	0.06	64640	1.67	2.14	2.9	—
B replicate #1	Neu5Ac	7.69	0.08	1060000	1.59	38.7	50	12
	Neu5Gc	5.27	0.14	31590	1.97	1.06	1.4	16
B replicate #2	Neu5Ac	7.69	0.03	1346000	1.47	49.1	63	—
	Neu5Gc	5.27	0.02	40790	1.85	1.36	1.8	—
B replicate #3	Neu5Ac	7.69	0.05	1161000	1.84	42.4	56	—
	Neu5Gc	5.28	0.06	30790	2.25	1.03	1.4	—

Precision and Accuracy of Determination

Samples were analyzed in triplicate to evaluate the precision of the assay. Table 4 details the results for one day of analysis. Peak area RSDs for Neu5Ac are generally <2, with the exception of replicate #1 of Brand A, which had a single injection with consistently lower peak areas than the other injections. RTs were stable, indicating that under these gradient components nonpolar sample components elute from the column and do not impact subsequent analyses. The analysis precision (RSD) for triplicate samples was 7.7 for Neu5Ac and 8.0 for Neu5Gc for Brand A.

Replicates of Brand B were more variable, with RSDs of 12 and 16 for Neu5Ac and Neu5Gc, respectively. Between-day precision was evaluated by repeating sample analysis. When comparing the average determined amounts, between-day precision (RSD) was 1.3 and 1.0 for Neu5Ac and 6.6 and 8.9 for Neu5Gc in infant formula Brands A and B, respectively. This is exceptional, considering the precision when comparing replicates within a day can vary widely. Expecting to routinely achieve such low values for between-day precision is unrealistic.

Table 5. Recoveries of Sialic Acids from Infant Formula Samples						
Sample	**Analyte**	**Amount (Unspiked) (pmol)**	**Amount Spiked into Hydrolysate (pmol)**	**Theoretical Spiked Concentration (after Sample Prep.) (pmol)**	**Measured Amount (Spiked) (pmol)**	**Recovery (%)**
Brand A	Neu5Ac	63.5	225	28.5	92.4	100
	Neu5Gc	0.52	18	2.22	3.20	120
Brand B	Neu5Ac	47.5	170	21.3	72.8	120
	Neu5Gc	1.13	13	1.66	3.20	120
Brand C	Neu5Ac	<LOD	75	9.52	9.00	95
	Neu5Gc	<LOD	5.8	0.74	0.70	95
Blank	Neu5Ac	<LOD	75	9.41	8.62	92
	Neu5Gc	<LOD	5.8	0.73	0.65	89

Accuracy was evaluated by spiking the sample hydrolysates before sample preparation by anion exchange with known amounts of Neu5Ac and Neu5Gc to approximately double the amount present in the samples (Table 5). This spiking was also done in a reagent blank and soy formula for comparison. Recoveries range from 89 to 120%. Recoveries were higher in dairy-based infant formulas compared to the soy infant formula and reagent blank control samples. Accuracy can be highly impacted by the efficiency of the derivatization, which, as noted in the precautions section, can by affected by the matrix of the derivatization reaction.

Sample Preparation Comparison to HPAE-PAD Analysis

Previous work illustrates the application of HPAE-PAD in the analysis of these samples.[11] Some comparisons can be made to this work. HPAE-PAD is a direct method that does not require derivatization; however, typical strong base elution conditions do not allow for determination of O-acetylated sialic acids. If only the total amount of Neu5Ac and Neu5Gc are of interest, both methods are appropriate as the O-acetylated sialic acids will degrade in base to the parent Neu5Ac or Neu5G. The time required to prepare samples for the two methods are dramatically different. Both methods require the same sample hydrolysis optimization and anion-exchange sample preparation for consistent sample analysis. These steps will take approximately 4 h in total for a set of three triplicate samples and three controls (12 digestions total). In addition to the sample preparation time, derivatization for fluorescence detection will require 2.5 h for the reaction with an additional 1 h to stop the reaction and prepare the samples for injection after the derivatization is complete.

CONCLUSION

In this work, N-acetylated sialic acids were determined and O-acetylated sialic acids are identified by HPLC with fluorescence detection following acid hydrolysis and DMB derivatization of infant formula samples. By using a water:acetonitrile gradient, high resolution of the sialic acids was obtained in a 20 min analysis time, including a column-wash step to maintain method performance. The sensitivity of the fluorescence detector easily allows determination of sialic acids in the infant formula that are present in the pmol range. The sensitivity provides for simple determination of Neu5Ac, Neu5Gc, and O-acetylated sialic acids in the derivatized samples.

SUPPLIERS

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

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

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www.ferro.com

ProZyme, 1933 Davis Street, Suite 207, San Leandro, CA 94577, U.S.A. Tel: 800-457-9444.
www.prozyme.com

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