

Sialic Acid Determination in Infant Formulas: Comparing Two Liquid Chromatography Methods

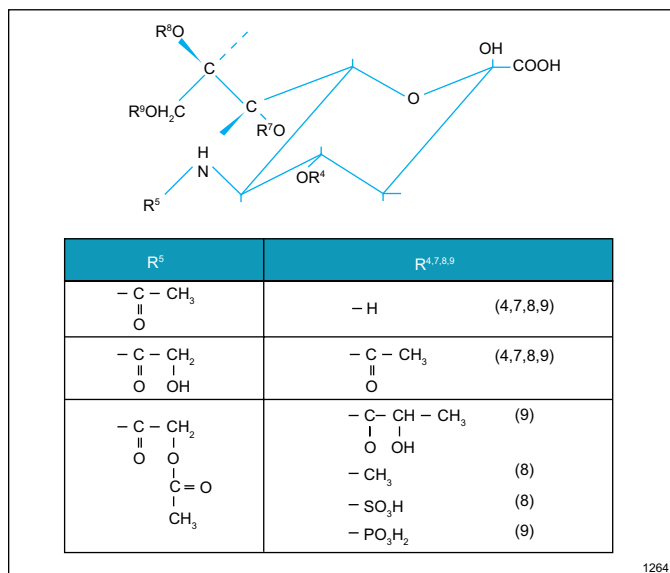
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Introduction

Sialic acids have been identified as important factors in infant immune system and cognitive development.¹ Many neuraminic acids have been identified in human milk, however *N*-acetylneuraminic acid (Neu5Ac) is predominant, and *N*-glycolylneuraminic acid (Neu5Gc) is usually absent. In comparison, bovine milk contains approximately 5% Neu5Gc. In addition to containing Neu5Gc, 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 both lower total sialic acid content and different sialic acid proportions compared to human milk. Because of the role these carbohydrates play in infant development, many manufacturers enrich infant formulas with sialic acids to more closely mimic human milk.

Infant formula samples, and food samples in general, present a challenging matrix. They are not homogenous solutions, but suspensions of lipids, proteins, and carbohydrates. Determination of sialic acids in this complex matrix presents many challenges, including efficient sialic acid release from glycoconjugates, sample preparation to remove interfering components in the matrix, and analyte determination. Among the chromatographic determination methods, there are those which require further sample derivatization for analyte detection, such as fluorescent labeling followed by UHPLC, and others which detect analytes directly, such as high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD). This work describes the development of two assays for sialic acids in infant formula with a dilute sulfuric acid hydrolysis followed by anion-exchange sample preparation and analyte determination by either HPAE-PAD or fluorescent labeling followed by UHPLC (UHPLC-FLD).

FIGURE 1. Sialic acids (neuraminic acids).



This poster will focus primarily on the *N*-acetylated and *N*-glycosylated (position R⁵) neuraminic acids (Figure 1). However, there are many other sialic acids which may be present. *O*-acetylation may occur at other positions in the molecule.

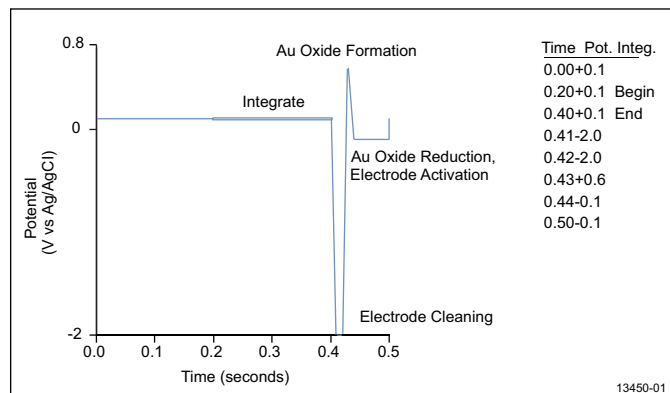
Experimental Conditions

Method 1

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

- DP Dual Pump module
- DC Detector/Chromatography module
- AS Autosampler
- ICS-3000 ED Electrochemical Cell Disposable Gold Electrode
- Electrochemical Cell Disposable Gold Electrode Reference Electrode
- Four potential Waveform (illustrated in Figure 2)

FIGURE 2. Automated four potential waveform as used for sialic acids.³



Method 2

Thermo Scientific Dionex UltiMate™ 3000 RSLC System including:

- SRD-3600 Solvent Rack
- HPG-3400RS Pump with 350 µL mixer
- WPS-3000TRS Autosampler
- TCC-3000RS Column Compartment
- FLD-3400RS Fluorescence Detector with dual PMT
- Pre-column heater
- Thermo Scientific Dionex Viper™ capillary kit, RS system

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

Samples and Sample Preparation

Three samples were analyzed in this work: Brand A, a dairy-based formula; Brand B, a dairy-based formula with added maltodextrins; and Brand C, a soy-based formula.

Samples are prepared as follows:⁴⁻⁶

For all samples

- Suspend 0.75 g of infant formula in 10 mL of DI water.
- Hydrolyze samples in sulfuric acid for 1 h at 80 °C (0.9 mL of sample in 5 mL of 50 mM H₂SO₄).
- Load hydrolyzed samples onto a Thermo Scientific Dionex OnGuard™ II A anion-exchange cartridge.

HPLC-PAD preparation

- Wash neutral components off the resin with DI water and elute the charged sialic acids with 50 mM NaCl. Dilute the eluted samples and inject.
- No derivatization or lyophilization is needed prior to analysis.

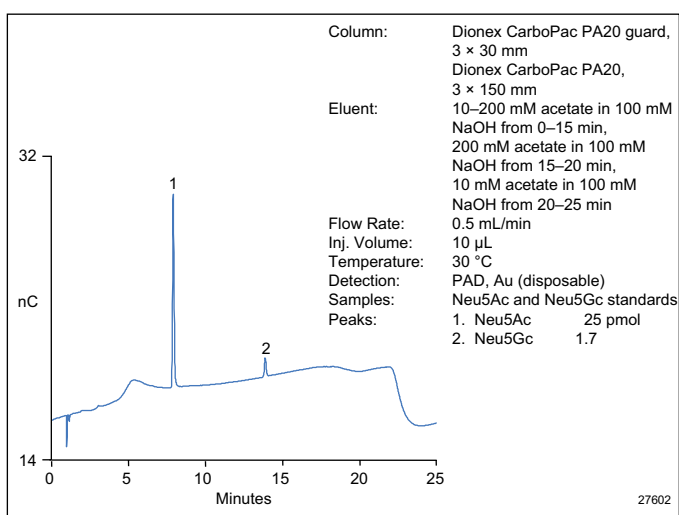
UHPLC-FLD preparation

- Elute sialic acids with 20 mL of 1 M formic acid.
- Filter eluted samples through a 0.2 µm IC syringe filter. Derivatize samples for 2.5 h at 50 °C with DMB reagent.

Results

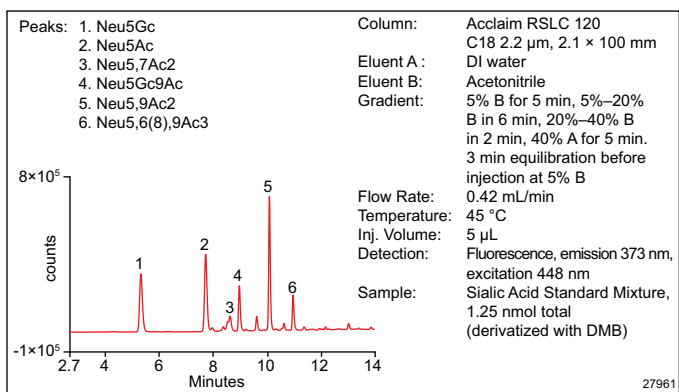
The HPAE-PAD gradient method was developed to resolve the sialic acids from other components in the infant formulas.⁵ Neu5Ac and Neu5Gc were well resolved from each other and pmol amounts are easily quantified (Figure 3)

FIGURE 3. Sialic acid separation using the Thermo Scientific Dionex CarboPac™ PA20 column.



The UHPLC-FLD gradient method similarly resolved Neu5Ac and Neu5Gc from each other, and allowed determination of O-acetylated sialic acids as well. However, residual derivatization reagent can lead to additional interfering peaks. A shorter run-time isocratic method was also tested, but the gradient method increased column lifetime by removing strongly retained compounds, (Figure 4).

FIGURE 4. Derivatized sialic acids separation using the Thermo Scientific Acclaim™ RSLC 120 C18 column.



Two concentration ranges were investigated for Neu5Ac and Neu5Gc, to match the amounts determined in samples. In both cases the calibration was linear. Standards prepared by derivatization may show some increased deviation from linearity depending on the derivatization reaction efficiency of a given standard. Both methods demonstrated sufficient sensitivity to determine sialic acids in infant formula, (Table 1).

As shown in Figure 5, the HPAE-PAD method separated Neu5Ac well from residual carbohydrates in the samples. Neu5Ac and Neu5Gc were identified in Brands A and B. Brand C, the soy-based formula, contains no added sialic acids. None were determined in the sample. However, this soy-based formula included probable maltodextrins that carried through the sample preparation process due to non-specific binding.

Sampling the infant formula can be a challenge. As listed in Table 2, determined amounts in replicates can be similar as in Brand A, or highly variable as in Brand B. The formulation of the infant formula affects the performance of the sample preparation. Amounts of fats and proteins impact the sample preparation, therefore optimization of the sample preparation is recommended for each sample type.

FIGURE 5. Separation of infant formula samples using the Dionex CarboPac PA20 column: HPAE-PAD method.

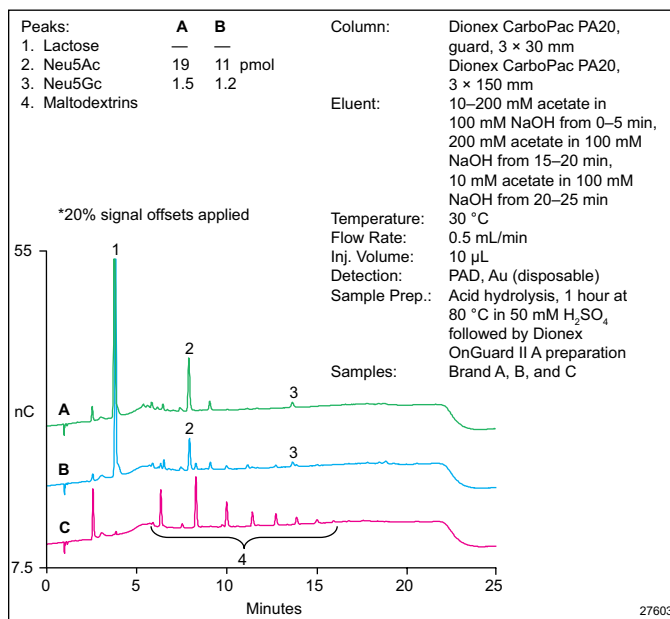


Table 1. Linearity, LOD, and LOQ, of Sialic Acid Determination

Analyte	Method	Range (pmol)	Coeff. of Determination (r ²)	LOQ (pmol)	LOD (pmol)
Neu5Ac	HPAE-PAD	5.0–100	0.9995	0.80	0.24
	UHPLC-FLD	5–260	0.9952	0.17	0.06
Neu5Gc	HPAE-PAD	0.34–6.8	0.9997	0.70	0.21
	UHPLC-FLD	0.2–9.8	0.9940	0.23	0.08

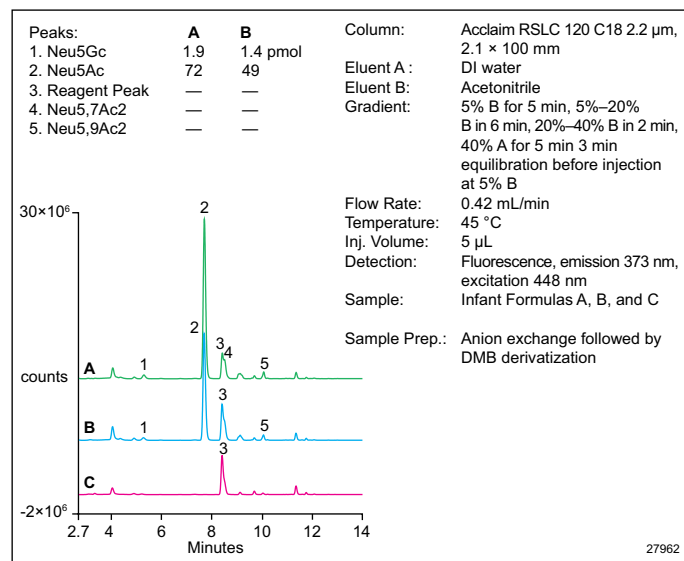
Table 2. HPAE-PAD Assay Sample Analysis Precision Data, n=3

Sample	Analyte	RT (min)	RT Precision (RSD)	Average Determined Amount (pmol)	mg/100 g of Sample	Peak area Precision (RSD)	Analysis Precision (RSD)
Brand A	Neu5Ac	7.92	0.06	22	85	4.25	0.59
	Neu5Gc	13.7	0.04	1.5	5.9	3.51	8.4
Brand B	Neu5Ac	7.93	<0.01	12	47	5.50	15.7
	Neu5Gc	13.7	0.06	1.1	4.5	2.23	11.0

RT= retention time

By UHPLC-FLD Neu5Ac and Neu5Gc were well resolved, though a peak that is present in reagent blanks can interfere with Neu5,7Ac2 (Figure 6). As expected, and consistent with the HPAE-PAD data, no sialic acids were detected in the soy-based formula, Brand C.

FIGURE 6. Separation of infant formula samples using the Acclaim RSLC 120 C18 column: UHPLC-FLD method.



Assay results, as shown in Table 3, and sample precision between replicates is similar to that of HPAE-PAD. For the most consistent results, sample preparation should be optimized for each sample formulation, regardless of the method used for analyte determination.

Recovery experiments were performed by spiking the hydrolyzate prior to sample preparation. Recoveries for Neu5Ac, when determined by HPAE-PAD, ranged from 80–109% for three different formulas treated by anion-exchange sample preparation on three different days. Recoveries for Neu5Gc were similar, ranging from 78–111% (Table 4).

Table 4. Sialic Acid Recovery in Three Infant Formulas

Sample	Analyte	HPAE-PAD Recovery (%)	UHPLC-FLD Recovery (%)
Brand A	Neu5Ac	108	100
	Neu5Gc	111	120
Brand B	Neu5Ac	100	120
	Neu5Gc	95	120
Brand C	Neu5Ac	96	95
	Neu5Gc	100	95

Recoveries for the two sialic acids, when determined by UHPLC-FLD, ranged from 95 to 120%. Recovery varied depending on the sample matrix and the ability to match that matrix when preparing standards.⁶

The sialic acid quantification differences between the two methods are similar to the analysis variability by either method. There is a bias towards higher quantification results for UHPLC determination of Neu5Ac (Table 5). There

Table 3. UHPLC-FLD Assay Sample Analysis Results, Triplicate Sample Preparations

Sample	Analyte	RT (min)	RT Precision (RSD)	Average Determined Amount (pmol)	mg/100 g of Sample	Peak Area Precision (RSD)	Sample Analysis Precision (RSD)
Brand A	Neu5Ac	7.69	0.05	74	94	1.54	7.7
	Neu5Gc	5.29	0.07	2.0	2.7	2.27	8.0
Brand B	Neu5Ac	7.69	0.05	43.4	56	1.63	12
	Neu5Gc	5.27	0.07	1.15	1.5	2.00	16

may also be a tendency for higher results for Neu5Gc by HPAE-PAD. Brand B exhibits an interfering compound eluting closely with Neu5Gc. However, Brand A showed no signs of this interference.

Overall, the sample analysis time is faster for HPAE-PAD. The UHPLC method requires significant additional time for the derivatization step, as detailed in Table 6.

Conclusions-HPAE-PAD

The developed HPAE-PAD method is capable of determining Neu5Ac and Neu5Gc in complex sample matrices such as infant formula.

Recoveries for the assay range from 79 to 111%. This is a good result for a challenging matrix with a multistep sample preparation method.

The method allows direct determination of sialic acids. No derivatization steps are required.

For routine analysis, HPAE-PAD is faster and more straightforward.

Conclusions-UHPLC-FLD

The developed UHPLC-FLD method is also capable of determining Neu5Ac and Neu5Gc in complex sample matrices such as infant formula.

For O-acetylated sialic acids, UHPLC-FLD is a better option.

However, some precautions must be acknowledged:

- Standards and samples must be derivatized in the same matrix for accurate results.
- The derivatization efficiency may not be the same for standards as for samples.
- The fluorescence response may not be the same for each of the derivatized sialic acids.

Table 5. Comparative Analysis, Triplicate Samples

Sample	Analyte	HPAE-PAD Amount (mg/100 g of Formula)	Precision (RSD)	UHPLC-FLD Amount (mg/100 g of Formula)	Precision (RSD)
Brand A	Neu5Ac	86	0.59	94	7.7
	Neu5Gc	5.9	8.4	2.7	8.0
Brand B	Neu5Ac	47	15.7	56	12
	Neu5Gc	4.5	11.0	1.5	16

Table 6. Analysis Time (h)

Method Step	HPAE-PAD	UHPLC-FLD
Sample Hydrolysis	1	1
Sample Cleanup after Hydrolysis	1 (Six Samples)	1 (Six Samples)
Lyophilization	Not Needed	Not Needed
Derivatization Reagent Preparation	N/A	0.5
Sample/Standard Derivatization	N/A	2.5 + 0.5
Chromatographic Run Time, One Injection	0.42 (25 min)	0.35 (21 min)
Total Time (h)	2.42	5.85

References

1. Wang, B.; Brand-Miller, J. The Role and Potential of Sialic Acid in Human Nutrition. *Eur. J. Clin. Nutr.* **2003**, *57*, 1351.
2. Wang, B.; Brand-Miller, J.; McVeigh, P.; Petocz, P. Concentration and Distribution of Sialic Acid in Human Milk and Infant Formulas. *Am. J. Clin. Nutr.* **2001**, *74*, 510.
3. Dionex Corporation. Application Update 141: Improved Long-Term Stability of *N*-Acetylneuraminic Acid and *N*-Glycolylneuraminic Acid Peak Area Responses Using Waveform A, a Quadruple Potential Waveform, LPN 1225. Sunnyvale, CA, 2000.
4. Martín, M.J., Vázquez, E., Rueda, R. Application of a Sensitive Fluorometric HPLC Assay to Determine the Sialic Acid Content of Infant Formulas. *Anal. Biomed. Chem.* **2007**, *387*, 2943.
5. Dionex Corporation. Application Note 253: HPAE-PAD Determination of Infant Formula Sialic Acids, LPN 2561-01. Sunnyvale, CA, 2010.
6. Dionex Corporation. Application Note 266: Determination of Sialic Acids Using UHPLC with Fluorescence Detection, LPN 2662-02. Sunnyvale, CA, 2011.

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