Determination of Sialic Acid in Milk and Products Using High Performance Anion-Exchange Chromatography Coupled with Pulsed Amperometric Detection

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Abstract: A resolved succinct environmental-friendly chromatography method was developed for determination of *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc) in bovine milk and products using high performance anion-exchange chromatography coupled with pulsed amperometric detection. The conditions affecting separation, detection, and sialic acid release from milk were optimized. The separation was finished on CarboPac PA20 anion-exchange column using 100 mM NaOH and 20–300 mM NaAc elution in 20 min at a flow rate of 0.5 ml min⁻¹. The separation and detection temperatures were both at 30°C. The detection was carried out on gold working electrode. The detection limits (25 µl injection, three times of the baseline noise) were 3.0 and 1.8 µg l⁻¹, and the linear range of 5–500 µg l⁻¹ was obtained. The spiked recovery of Neu5Ac and Neu5Gc in real samples was 88%–115%.

Key Words: High performance anion-exchange chromatography; Pulsed amperometric detection; Sialic acid; Milk

1 Introduction

Sialic acid is the generic name given to a family of acylated derivatives of a 9-carbon carboxylated monosaccharide. Sialic acids are acidic monosaccharides, which are among the most important molecules of higher animals and occur in some microorganisms. Sialic acids are bound to complex carbohydrates and occupy prominent positions, especially in cell membranes. Their structural diversity is high and, correspondingly, the mechanisms for their biosynthesis are complex. General functions of sialic acid residues at the cell surface, like binding and transport of positively charged molecules, attraction/repulsion of cells and molecules, as well as their antiproteolytic effect in glycoproteins are attributed to their physicochemical properties, *i.e.* electronegativity and hydrophilicity. Due to the surface location of the acidic molecules, sialic acids shield macromolecules and cells from enzymatic and immunological attacks. But sialic acids also

represent recognition sites for various physiological receptors, as well as for toxins and microorganisms, and thus allow their colonization. Many viruses use sialic acids for the infection of cells. As sialic acids also play a decisive role in tumor biology, they prove to be rather versatile molecules that modulate cell biological events in a sensitive way. It is discussed that their evolution may have stimulated evolution and rendered organisms less vulnerable to environmental attacks. However, disturbance of their metabolism may cause diseases. Substantial evidence now shows that sialic acid is a possible risk factor for tumor, cancer, diabetes, cardiovascular diseases, and their thrombotic complications^[1,2].

Recently, as sialic acid should be added to different kinds of food product for better health, “SA” program was launched by academic nutrition institute in global food market, especially infant milk products. According to the research, sialylated compounds in breast milk could help infants keeping away from intestine infection and improve their

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digestion and intelligence. However, bovine milk, the substitute of breast milk, has much less sialic acid than breast milk actually. That is why the “SA” program was initiated^[3].

Substituents on the C-5 can be either an amino, an acetamido, a glycolyl, or a hydroxyl group, thus defining the four major types of sialic acids: neuraminic acid, *N*-acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc), and deaminoneuraminic acid (KDN). Neuraminic acid does not exist in nature: only the other forms exist. Neu5Ac and Neu5Gc are the most abundant forms of sialic acids^[4].

Utilizing biological characters of sialic acid, spectrophotometric and enzymic reactions, antibodies, lectins, and viruses have all been used to determine either the total sialic acid content or specific sialic acid types^[4,5]. Common limitations of these procedures are their inability to differentiate the types of sialic acid and the requirement for purified samples to avoid interference from other lipid contaminants^[4,5]. With chemical characters of sialic acid, steady refinements of analytical techniques, such as high performance liquid chromatography (HPLC) and gas-chromatography (GC) combined with mass spectrometry (MS), nuclear magnetic resonance (NMR) spectrometry, and recent capillary electrophoresis (CE), have allowed for more definite elucidation of the structures of sialic acids with different acylation patterns and more sensitive determination^[3,6,7]. However, in general, carbohydrates are usually hydrophilic, neutral, and lack satisfactory chromophores for UV detection, which is similar to that of sialic acid. High performance liquid chromatography, GC, and CE require derivatization of the hydroxyl groups used to improve sensitivity and chromatographic resolution, which become more complex, labor-intensive, and prone to contamination. MS-based methods are expensive and complex, and GC-MS also needs derivatization to quantify.

Nowadays, a method of high-performance anion-exchange chromatographic-pulsed amperometric detection (HPAEC-PAD) was developed to analyze sialic acids^[8,9] because of its prominent advantages of performance. In HPAEC-PAD, the use of alkaline eluents causes sialic acid to be ionized, which is fit for separation by anion-exchange mechanisms. Then, the detection occurs by measuring the current generated when the carbohydrate compounds are oxidized on a gold working electrode. Compared with the methods mentioned above, the HPAEC-PAD eliminates derivatization, costly reagents, and toxic waste production and also improves safety; moreover, it provides accurate, reproducible, and effective analysis of sialic acid. The limit detection of sialic acid is below $\mu\text{g l}^{-1}$.

In this study, an original method was developed for determination of common sialic acid in bovine milk and products for *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc). Furthermore, this method was applied to analyze fifteen brands of bovine milk and products.

2 Experimental

2.1 Chromatography system and instruments

This study was performed on an ICS-3000 system (Dionex, Sunnyvale, CA). The system is composed of three modules. Dual pump (DP) module includes one isocratic pump and one gradient pump. The gradient pump with on-line degassing was used for sialic acid separation. Detection/chromatography (DC) module contains injection valve, column, and detector. The temperature in this part was maintained constant at 30 °C. Electrochemical detector consisted of Au working electrode, Ag/AgCl reference electrode, and Ti counter electrode. AS module is one autosampler used for injection, and AS auto-sampler with a stainless-steel injection needle and 25- μl injection loop. Chromeleon chromatography software (Dionex) was used for the system control and data analysis. CarboPac PA20 (Dionex, 150 mm $\times$ 3 mm), an anion-exchange column, was installed in DC module, which was maintained at 30°C. The ED cell waveform was 0.1 V from 0.00 to 0.40 s, then -2.0 V from 0.41 to 0.42 s, and a ramp -2.0 – -0.6 V from 0.42 to 0.43 s, followed by -0.1 V from 0.44 to 0.50 s (end of cycle). The integration region was from 0.2 s to 0.4 s. The eluent was kept blanketed under 34–55 kPa of nitrogen gas all times to reduce carbonate buildup and biological contamination. It was pumped at a flow rate of 0.5 ml min^{-1} . The run time was 20 min per sample.

Milli-Q system (USA) was used to provide 18.2 M Ω ·cm water.

2.2 Chemicals

N-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc) were purchased from Sigma-Aldrich, with 97%–99% purity. Solution of 50% (w/w) sodium hydroxide ($\geq 99\%$, Alfa Aesar Co.), sodium acetate anhydrous (Fluka, $\geq 99.5\%$), sodium azide ($> 99\%$, Sigma) and hydrochloric acid (36%–38%, Beijing Chemical Reagent Co.) were used in the experiment. All solvents, chromatography eluent, and standards were prepared using 18.2 M Ω ·cm deionized water, free of electrochemical active impurities.

2.3 Preparation of standards and eluents

2.3.1 Standard solution

To keep standards stable and prevent biological contamination, each standard was dissolved in 20 mg l^{-1} NaN_3 water solution to 1000 mg l^{-1} concentrations and was refrigerated at -20 °C until required. During the experiment, the standards were combined and serially diluted in 20 mg l^{-1} NaN_3 water solution to yield concentrations ranging from 5 to 500 $\mu\text{g l}^{-1}$.

2.3.2 NaOH eluent

Hydroxide eluent (250 mM) was prepared by diluting 13.1 ml of 50% sodium hydroxide to 1000 ml with water. The eluent was kept blanketed under 34–55 kPa of nitrogen gas all times to reduce carbonate buildup and biological contamination.

2.3.3 NaAc eluent

Sodium acetate anhydrous (82.0 g) was accurately weighed and diluted to 1000 ml using purified water. After filtered through 0.22- μm nylon membrane, 1 M NaAc eluent was kept blanketed under nitrogen gas all times.

2.4 Preparation of samples

Fifteen bovine milk products were purchased from native supermarket, including pure milk, powdered milk, and yogurt.

Pure milk: Sample was accurately measured by 100 μl and hydrolyzed using 900 μl 0.1 M HCl at 80 $^{\circ}\text{C}$ for 1 h, then centrifuged at rate of 6000 rpm for 15 min. Transparent extraction was diluted 10-fold in 20 mg l^{-1} NaN_3 solution for injection.

Yogurt: One hundred milligram of yogurt was accurately weighed and hydrolyzed using 900 μl of 0.1 M HCl at 80 $^{\circ}\text{C}$ for 1 h. The remaining procedure was the same as above.

Powdered milk: according to drink method on product pack, five hundred milligrams of powder was accurately weighed and dissolved in 5 ml of water. The mixture was swirled to liquid milk. The remaining procedure was the same as above.

3 Results and discussion

3.1 Method development and optimization

Because sialic acid is acidic compound, which is prone to be ionized in alkaline eluent, anion-exchange column offers good retention. It is hard for medium elution ability NaOH to separate sialic acid effectively. Moreover, after hydrolysis, desialylated oligosaccharide and glycoproteins in milk are more difficult to be eluted than sialic acid. Considering both of them, choosing a column with low capacity and effective eluent are both important for chromatography optimization to separate compounds in short time. First of all, CarboPac PA20 was chosen from the four types of sugar-analyzing columns, including CarboPac MA1, CarboPac PA1, CarboPac PA20, and CarboPac PA100. CarboPac PA20 column has less capacity and retention for oligosaccharide, so it is good at separating monosaccharide and disaccharide. All of these are perfect for milk component and sample background. Second, high concentration of acid or alkali can cause destruction of sialic acid, so choosing more effective

and light alkaline eluent such as NaAc is much better than increasing NaOH concentration to elute and separate sialic acid earlier. In sum of considering resolution, analytical time, and sample, eluent programs were optimized to isocratic eluent of 100 mM NaOH and 20 mM NaAc for 5 min, then slope gradient eluent of 100 mM NaOH and 20–300 mM NaAc for 10 min, followed by the regeneration of the column with 200 mM NaOH for 2 min, and finally kept in initial concentration for 5 min to equilibrate. Finally, column temperature at 30 $^{\circ}\text{C}$, inject volume of 25 μl , and flow rate at 0.5 ml min^{-1} were selected.

3.2 Detection condition

For the aim of detecting sialic acid and avoiding interference from sample background, pulsed amperometric detection can be carried out as it has the same strong alkaline condition with separation, without derivatization, 0.1 V low and exclusive detect potential, and clean potential to avoid electrode poisoning. The use of an appropriate detection potential makes this a very selective technique for sialic acid of milk. The ED cell waveform was 0.1 V from 0.00 s to 0.40 s, then -2.0 V from 0.41 s to 0.42 s, and a ramp from -2.0 V to 0.6 V during 0.42 s to 0.43 s, followed by -0.1 V from 0.44 s to 0.5 s (end of cycle). The integration region was from 0.2 s to 0.4 s.

Under the optimized chromatographic conditions, separation and detection of Neu5Ac and Neu5Gc was demonstrated in Fig. 1.

3.3 Method linearity, reproducibility, and detection limit

Table 1 shows that the method is linear for Neu5Ac and Neu5Gc over the range of 5 to 500 $\mu\text{g l}^{-1}$ for a 25- μl injection. The detection limit for 25- μl injections of Neu5Ac and Neu5Gc were calculated from injected volume required to produce a peak height signal-to-noise ratio of 3. Peak area and retention time RSDs were determined for continuous 7 replicate injections of 100 $\mu\text{g l}^{-1}$ Neu5Ac and Neu5Gc. RSDs of peak area are 3.8% and 4.2%, respectively, and both RSDs of retention time are blow 0.07%.

Fig.1 Standard chromatogram of 200 $\mu\text{g l}^{-1}$ sialic acid
1. *N*-acetylneuraminic acid (Neu5Ac); 2. *N*-glycolylneuraminic acid (Neu5Gc)

Table 1 Limits of detection and linearity of sialic acid

Analytes	Detection limit ($\mu\text{g L}^{-1}$)	Coeff. det.	Offset	Slope	Linear range ($\mu\text{g L}^{-1}$)
Neu5Ac	3.0	0.9990	0.069	0.019	5–500
Neu5Gc	1.8	0.9975	0.085	0.049	5–500

3.4 Sample analysis

3.4.1 Liberation of sialic acid

In spite of the variety of analytical methods available for sialic acids, their analysis in biological samples remains a difficult task. Critical step in sialic acid analysis is their liberation from the biologic sources, which entails isolation of the sialylated macromolecules and/or removal of the sialic acid residues from the glycoconjugates.

Liberation of sialic acids from the purified samples was achieved either by hydrolytic procedures or treatment with specific enzymes^[10]. Despite their profound advantages of enzymes, the action of sialidases was limited to terminal residues and was dependent on several factors ranging from the substitution pattern to the sample composition, which were not easily controlled in biological samples. Therefore, in this experiment, the low cost, ease, and effectiveness of hydrolytic procedures were established, even though they always require optimization of hydrolysis time and acidity control because these were associated with some destruction of these carbohydrates.

The liberation effect of hydrochloric acid, acetic acid, trifluoroacetic acid, and vitriolic acid was compared for the same sample, with the control of acidity, time, and temperature. Experiment results indicate that hydrolysis using 0.1 M HCl at 80 °C for 1 h ensures the hydrolytic cleavage of all sialic acid linkages, deacylation, and decarboxylation of these residues, good reproducibility, most amount of sialic acid, and least interference from sample background.

3.4.2 Accuracy and precision

To testify reliability of acidic hydrolysis, the results of 100 $\mu\text{g L}^{-1}$ sialic acid standard before and after the hydrolysis were compared. The concentration changed slightly below 5%, which ensure stable existence of sialic acid during acidic hydrolysis.

In general, for the best precision and reproducibility, hydrolyzed sample should be injected in 2 h after sample handling at room temperature. Sialic acid in milk sample after hydrolysis is relatively stable; for example, consider sample No. 7 the concentration for 6 h after sample handling sialic acid decreased by 4% and decreased by 10% over one night.

To determine the accuracy, three representative samples of Nos. 7, 8 and 9 products were spiked with known amount of internal standards and were subjected to the same sample hydrolysis and cleanup procedures. The recoveries of Neu5Ac were found from 108% to 115% and the recoveries of Neu5Gc were from 88% to 101%.

3.4.3 Analysis result

This method was successfully applied for the analysis of 15 samples, 1–6 samples were yogurt, 7–14 samples were liquid milk, and No. 15 sample was powdered milk. Figure 2 displays chromatogram of one representative sample.

Table 2 shows the results of determination. The most type of sialic acid in milk products is Neu5Ac, the range of its content is 8–25 mg L^{-1} , less Neu5Gc ranging between 0.1 and 10 mg L^{-1} .

Actually, it was reported that 300–1500 mg L^{-1} sialic acid is present in breast milk^[4]; there is much difference between the amount of sialic acid present in bovine milk and breast milk.

Fig.2 Chromatogram of milk
1. Neu5Ac; 2. Neu5Gc

Table 2 Content analysis results of milk samples

Sample No.	Neu5Ac		Neu5Gc	
	Dilution ^a ($\mu\text{g L}^{-1}$)	Sample (mg L^{-1})	Dilution ($\mu\text{g L}^{-1}$)	Sample (mg L^{-1})
1	130.53	13.05	101.82	10.18
2	122.94	12.29	25.02	2.50
3	87.25	8.72	45.87	4.59
4	198.07	19.81	104.25	10.43
5	144.27	14.43	12.22	1.22
6	134.96	13.50	6.93	0.69
7	161.89	16.19	10.18	1.02

(To be continued)

Table 2 (Continued)

Sample No.	Neu5Ac		Neu5Gc	
	Dilution ^a ($\mu\text{g l}^{-1}$)	Sample (mg l^{-1})	Dilution ($\mu\text{g l}^{-1}$)	Sample (mg l^{-1})
8	121.07	12.11	28.79	2.88
9	164.44	16.44	1.77	0.18
10	124.59	12.46	22.78	2.28
11	248.66	24.87	40.52	4.05
12	111.55	11.15	1.60	0.16
13	152.09	15.21	65.12	6.51
14	130.62	13.06	17.72	1.77
15	133.90	13.39	20.24	2.02

^a: 100 fold diluted sample.

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