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Determination of Six Human Milk Oligosaccharides in Milk Powder Using Ion Chromatography

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Abstract [**Objectives**] To develop a method for the quantification of six human milk oligosaccharides (HMOs) in milk powder utilizing ion chromatography. [**Methods**] The sample was dissolved in water and deproteinized through acetonitrile precipitation, followed by freezing and centrifugation. The resulting supernatant was then diluted and filtered before being subjected to separation using a PA1 analytical column. The sample was analyzed using ion chromatography coupled with an amperometric detector and quantified by the external standard method. [**Results**] The limits of detection (LOD) for difucosyllactose (DFL), 2'-fucosyllactose (2'-FL), lacto-N-neotetraose (LNnT), lacto-N-tetraose (LNT), 3'-sialyllactose (3'-SL), and 6'-sialyllactose (6'-SL) were 8, 100, 15, 15, 10, and 6 mg/100 g, respectively. Correspondingly, the limits of quantification (LOQ) were 15, 300, 50, 50, 30, and 10 mg/100 g, respectively. The method exhibited excellent linearity over the concentration range of 2 - 20 μg/mL (R^2 > 0.999). Spiked recoveries ranged from 82.5% to 104.9%, with relative standard deviations (*RSD*) between 0.44% and 2.78%. [**Conclusions**] The method demonstrates excellent selectivity, precision, repeatability, and stability, thereby offering a reliable analytical method for quantifying six HMOs in milk powder.

Key words Human milk oligosaccharides (HMOs), Ion chromatography, Milk powder

0 Introduction

Human milk oligosaccharides (HMOs) are a group of structurally and functionally diverse oligosaccharides found in human milk. They are present in significant quantities, representing the third most abundant solid component after lactose and lipids[1], and account for approximately 20% of the total carbohydrates in human milk[2]. HMOs are composed of 3 to 14 monosaccharide units linked in either linear or branched configurations. Theoretically, more than 1 000 distinct HMOs structures may exist[3]. The content and compound structure of HMOs exhibit significant individual variability. Since infant formula is typically derived from animal milk sources, such as bovine milk, its oligosaccharide profile differs notably from that of human milk[4]. It is thus evident that HMOs represent a critical gap that must be bridged in the pursuit of humanizing infant formula[5]. Therefore, to facilitate the gradual humanization of infant formula and meet the nutritional needs of infants and young children, regulations permitting the use of HMOs as nutritional fortifiers have been approved in multiple countries and regions, including China[6-7]. Accordingly, approved HMO ingredients are progressively being incorporated into infant formula products. To ensure standardized application and product quality, numerous studies have reported methods for detecting HMOs in infant formula. The primary methods employed include high-performance liquid chromatography (HPLC)[8-9], ion chromatography[10-11], and liquid chromatography-mass spectrometry (LC-MS)[12-13]. However, these methods present certain limitations, such as complex sample preparation, high instrument costs, and a limited scope of detectable HMOs species. In this study, complex milk powder (without added oligosaccharides such as galactooligosaccharides and fructooligosaccharides) was selected as the test substrate. A rapid, highly selective, and high-throughput detection method was established, providing a reliable analytical tool to support the development, quality control, and market regulation of related HMOs dairy products.

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1 Materials and methods

1.1 Reagents and materials Difucosyllactose (DFL, 20768-11-0), 2'-fucosyllactose (2'-FL, 41263-94-9), lacto-N-neotetraose (LNnT, 13007-32-4), lactose-N-tetraose (LNT, 14116-68-8), 3'-sialyllactose (3'-SL, 128596-80-5), 6'-sialyllactose (6'-SL, 157574-76-0), and acetonitrile (HPLC grade) were obtained from ANPEL Laboratory Technologies (Shanghai) Inc. Laboratory water used in the experiment was required to have a resistivity of no less than 18.2 MΩ.

1.2 Instruments and equipment The following instruments were utilized: an ion chromatograph (Thermo Fisher Scientific), an AB265-S analytical balance (METTLER-TOLEDO), a Sorvall Biofuge Stratos high-speed freezing centrifuge (Thermo Fisher Scientific), a Millipore Q water purifier (Millipore), and an IKA KS shaker (IKA).

1.3 Methods

1.3.1 Sample preparation. Approximately 0.5 g of the sample (excluding additives such as galactooligosaccharides or fructooligosaccharides) was weighed into a 10 mL volumetric flask and thoroughly dissolved in water at 60 ℃. Subsequently, 2 mL of acetonitrile was added, and then the mixture was vortexed before

being diluted to the calibration mark with water. The resulting solution was vortexed for 30 min and then centrifuged at 0 ℃ and 12 000 rpm for 5 min. 0.5 mL of the supernatant was precisely transferred to a 10 mL volumetric flask, diluted to the calibration mark with water, and mixed thoroughly. Prior to analysis, the solution was filtered through a 0.22 μm membrane filter.

1.3.2 Instrument operating conditions. Chromatographic columns utilized included the CarboPac™ PA1 analytical column (250 mm × 4 mm) and the CarboPac™ PA1 preparative column (4 mm × 50 mm). Detection was performed using an amperometric detector equipped with an Au working electrode, an Ag/AgCl reference electrode, and a standard sugar four-potential waveform. Both the column temperature and detection cell temperatures were maintained at 30 ℃. The injection volume was set to 10 μL. Gradient elution conditions are shown in Tables 1 – 2.

Table 1 Gradient elution program for DFL, 2'-FL, LNnT, and LNT

Time//min	Flow rate//mL/min	Ultrapure water//%	500 mmoL/L NaOH//%	300 mmoL/L NaOAc//%
0	1	88	12	0
12.00	1	88	12	0
20.00	1.3	87	13	0
38.00	1.3	87	13	0
38.01	1.3	68	30	2
42.00	1.3	68	30	2
43.00	1	73	25	2
54.00	1	73	25	2
54.01	1	0	20	80
59.00	1	0	20	80
59.01	1	88	12	0
65.00	1	88	12	0

Table 2 Gradient elution program for 3'-SL and 6'-SL

Time//min	Flow rate mL/min	Ultrapure water//%	500 mmoL/L NaOH//%	300 mmoL/L NaOAc//%
0	1	57	30	13
28.50	1	57	30	13
28.51	1	0	20	80
31.00	1	0	20	80
31.01	1	57	30	13
35.00	1	57	30	13

1.3.3 Preparation of standard solutions. (i) Standard stock solution. An appropriate amount of the HMOs reference standard was accurately weighed to the nearest 0.000 01 g and transferred into a volumetric flask. The standard was dissolved and diluted to the calibration mark with water, then mixed thoroughly to prepare a standard stock solution with a concentration of 1 mg/mL. The solution was stored at 4 ℃ in a refrigerator for up to one month. (ii) Analytical working solution. 30, 50, 80, 100, 200, 500, and 1 000 μL of the standard stock solution were accurately pipetted, diluted to 1 mL with water, and mixed thoroughly. Analytical working solutions with concentrations of 0.3, 0.5, 0.8, 1, 2, 5, and 10 μg/mL were subsequently prepared.

1.4 Calculation The content of each HMO was calculated according to Equation (1):

$$X = \frac{\rho \times D}{M} \times 10^{-1} \quad (1)$$

where X represents the content of each HMO in the sample, mg/100 g; ρ denotes the solution concentration of the measured component in the HMOs, determined using the external standard curve, μg/mL; D refers to the volume multiplied by the dilution factor; M indicates the sample weight, g; and 10^{-1} is the conversion factor.

2 Results and analysis

2.1 Standard curves, limits of quantification (LOQ) and limits of detection (LOD) As shown in Table 3, the concentrations of the six components ranged from 0.2 to 20 μg/mL, with the correlation coefficients (R^2) of the standard curves exceeding 0.99, thereby demonstrating excellent linearity. The LOD, determined at an $R_{S/N}$ of 3, were 8, 100, 15, 15, 10, and 6 mg/100 g for DFL, 2'-FL, LNnT, LNT, 3'-SL, and 6'-SL, respectively. Correspondingly, the LOQ, determined at an $R_{S/N}$ of 10, were 15, 300, 50, 50, 30, and 10 mg/100 g for these compounds, respectively.

2.2 Results of accuracy and precision tests A spiked recovery experiment was conducted on milk powder samples at three concentration levels (low, medium, and high), each with six replicates. Recovery rates and relative standard deviations (*RSD*) were calculated, and the results are presented in Table 4. The recovery rates ranged from 82.5% to 104.9%, while the *RSD* values varied between 0.44% and 2.78%. These results comply with the relevant requirements specified in GB 5009.295-2023 *National Food Safety Standard—General Guidelines for Validation of Chemical Analysis Methods*, indicating that the method possesses satisfactory accuracy and precision.

Table 3 Standard curves, LOQ and LOD

Compound	Linearity range//μg/mL	Linear equation	Correlation coefficent (R^2)	LOD//mg/100 g	LOQ//mg/100 g
DFL	0.2 – 20	$Y = 0.875\,6x - 0.106\,7$	0.999 9	8	15
2'-FL	0.2 – 20	$Y = 0.968\,9x - 0.019\,6$	0.999 9	100	300
LNnT	0.5 – 20	$Y = 0.496\,3x - 0.048\,6$	0.999 9	15	50
LNT	0.5 – 20	$Y = 0.870\,1x + 0.021\,0$	0.999 9	15	50
3'-SL	0.2 – 20	$Y = 0.680\,1x - 0.023\,4$	0.999 9	10	30
6'-SL	0.2 – 20	$Y = 1.087\,1x - 0.039\,8$	0.999 9	6	10

Table 4 Recovery and precision results

Matrix	Compound name	Spiked amount//mg/100 g	Recovery range//%	Average recovery rate//%	*RSD*//%
Milk powder	DFL	15	95.6 – 101.6	97.5	1.71
		30	88.0 – 92.6	90.4	1.65
		50	82.5 – 88.6	85.2	2.26
	2'-FL	300	90.4 – 94.2	92.4	1.96
		500	91.2 – 95.1	93.2	1.60
		1 000	90.6 – 94.8	93.0	2.03
	LNnT	50	102.3 – 104.8	103.9	1.09
		100	103.1 – 104.5	104.1	0.57
		250	102.7 – 104.3	103.3	0.74
	LNT	50	98.2 – 103.9	100.8	2.04
		100	101.5 – 104.9	103.1	1.62
		250	100.9 – 104.9	103.6	1.37
	3'-SL	30	100.6 – 104.8	103.1	0.44
		60	99.7 – 104.9	102.4	1.17
		150	95.7 – 103.2	100.2	1.85
	6'-SL	10	92.4 – 101.8	97.2	1.85
		20	95.3 – 104.7	99.3	2.78
		50	95.8 – 98.8	97.5	0.95

2.3 Specificity experiment According to General Rule 0512 of the 2015 edition of the *China Pharmacopoeia* on the resolution requirements for high-performance liquid chromatography, the resolution should exceed 1.5. The resolutions between the actual target substance and adjacent peaks were as follows: DFL, 2.32; 2'-FL, 2.57; LNnT, 3.78; LNT, 1.84; 6'-SL, 7.01; 3'-SL, 2.35. The minimum resolution observed was 1.84, which surpasses the threshold of 1.5. This result satisfies the resolution criteria and indicates that the method demonstrates good selectivity.

3 Conclusions

The efficient separation and accurate quantification of six HMOs components in milk powder (without added galactooligosaccharides, fructooligosaccharides, or similar ingredients) were achieved through protein precipitation using acetonitrile, coupled with two optimized elution conditions. The LOD for DFL, 2'-FL, LNnT, LNT, 3'-SL, and 6'-SL were 8, 100, 15, 15, 10, and 6 mg/100 g, respectively. Corresponding LOQ were 15, 300, 50, 50, 30, and 10 mg/100 g, respectively. The recovery rates ranged from 82.5% to 104.9%, while the *RSD* values varied between 0.44% and 2.78%. These results indicate that the method demonstrates satisfactory precision, repeatability, and stability, thereby providing a reliable analytical approach for the quantitative determination of DFL, 2'-FL, LNnT, LNT, 6'-SL, and 3'-SL in milk powder.

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