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Determination of sugars and sugar alcohols in infant formula by high performance liquid chromatography with evaporative light-scattering detector

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ABSTRACT

A method was established for the simultaneous determination of five sugars (fructose, glucose, sucrose, lactose, maltose) and five sugar alcohols (erythritol, xylitol, sorbitol, mannitol, maltitol) in infant formula by high performance liquid chromatography-evaporative light scattering detector. After the samples were extracted with acetonitrile–water solution, precipitated by acetic acid, and purified with solid phase extraction cartridge, ALLChrom Rocksil Carbohydrate ES column was adopted for separation, and isocratic elution was conducted at the flow rate of 1.0 mL/min with acetonitrile-0.04 % ammonia solution as the mobile phase. The analytes were detected by an evaporative light-scattering detector, and quantified by external standard method. The linear ranges of the 10 components were 0.04–4.0 g/L with the correlation coefficients greater than 0.999, and the limits of quantification (S/N = 10) of the method were 0.08–0.4 g/100 g. The relative standard deviation of the lactose parallel samples reached 1.29 %, and the recoveries of the other 9 components ranged from 80.4 % to 99.4 % with the relative standard deviation of 2.8 %-7.1 %. The method performs well in sensitivity and separation, which is suitable for the simultaneous quantitative determination of sugars and sugar alcohols in infant formula.

1. Introduction

Sugar is the most important source of energy required for human body and one of the necessities for the growth and development of infants. Infant formula is made from milk and lactoprotein products with reference to the composition of breast milk, which is rich in nutrients, with monosaccharides and functional oligosaccharides as important components. The types and contents of sugars and relevant compounds in infant formula are closely related to metabolic diseases such as lactose intolerance and galactosemia in infants. GB 10765–2021 National Food Safety Standard Infant Formula [1] stipulates that fructose and sucrose should not be used as a source of carbohydrates in infant formula, and glucose polymers may be added as appropriate. For milk-based infant formula, lactose is preferred as the source of carbohydrates (lactose content shall account for ≥ 90 % of total carbohydrates). However, some profit-driven enterprises may add fructose, sucrose and other sugar substances that should not be used. Therefore, it is necessary to develop a method for the detection of sugars and related compounds in infant formula, which is of great significance for improving the quality of infant formula.

The main methods currently used for sugar analysis are gas chromatography-vacuum ultraviolet spectroscopy (GC-VUV) [2], gas chromatographic-mass spectrometry (GC–MS)[3], enzymolysis [4], ion chromatography-pulse amperometric detector (IC-PAD)[5–9], high-performance liquid chromatography-refractive index detector (HPLC-RID) [10,11], high-performance liquid chromatography-evaporative

Abbreviations: VUV, vacuum ultraviolet spectroscopy; ELSD, Evaporative light scattering detector; RID, Refractive Index Detector; RSD, relative standard deviation; SPE, solid phase extraction; GC, gas chromatography; GC–MS, Gas chromatographic-mass spectrometry; IC-PAD, ion chromatography- pulse amperometric detector; HPLC, high-performance liquid chromatography; LOQ, limit of quantitation.

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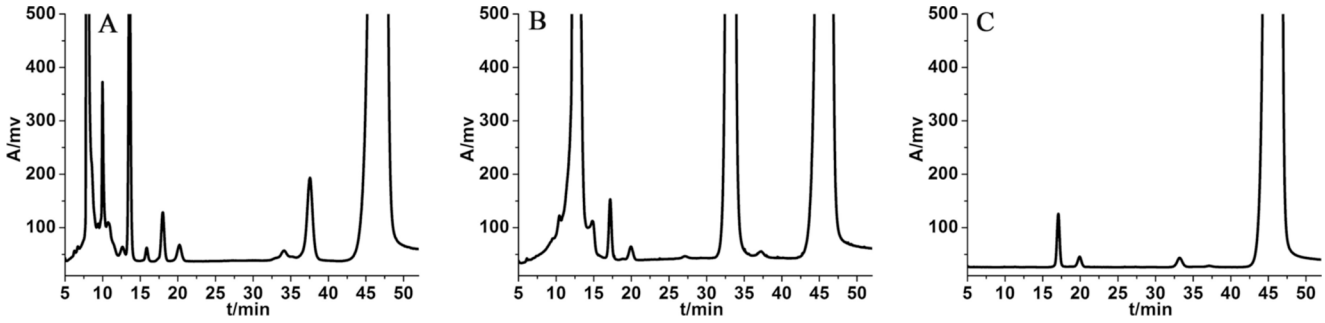

Fig. 1. Chromatograms of real sample with different protein precipitation methods. A. potassium ferricyanide and zinc acetate methods; B. pH adjustment method; C. acetic acid method.

Table 1
Recoveries of the ten analytes spiked in blank milk samples with different SPE columns ($n = 6$).

Compound	EMR cartridge recovery/ (%)	RSD (%)	HLB cartridge recovery/ (%)	RSD (%)	RP and H cartridge recovery/ (%)	RSD (%)
Erythritol	89.0	6.5	100	6.1	96.2	6.8
Xylitol	78.0	6.6	95.0	7.0	94.6	5.9
Fructose	86.0	5.9	96.0	6.2	94.6	6.7
Sorbitol	88.0	6.4	96.0	6.5	95.2	6.2
Mannitol	86.0	6.6	95.0	6.4	96.2	7.3
Glucose	89.0	6.5	100	6.3	94.4	6.8
Sucrose	79.0	6.3	100	5.7	100	6.3
Maltitol	88.5	6.7	100	5.9	100	6.5
Lactose	88.9	5.8	100	6.6	90.0	5.8
Maltose	88.7	7.1	95.0	6.3	91.6	7.0

light scattering detector (HPLC-ELSD) [12–18], and high-performance liquid chromatography- diode array detector (HPLC-DAD) [19]. GC and GC–MS are effective and sensitive in separation, but complicated in sample pretreatment due to the low volatility and difficulty in gasification of sugar, which generally requires silylation or esterification of the target analytes before detection. Enzymolysis, despite its high specificity, is limited by the high detection limit and the singleness of detection components, and its detection results are often influenced by the purity and activity of the enzyme. IC has been widely applied in the determination of sugar content, with high sensitivity, but it is unsatisfactory in accuracy due to the redox reaction of sugars on the electrode surface. HPLC-RID is less sensitive and does not support gradient elution, thus making it not applicable to the simultaneous separation and detection of multiple sugars. HPLC-ELSD, which is characterized by a stable baseline, high sensitivity, good separation performance, simple sample pretreatment, is an ideal method for the separation and quantitative analysis of sugar substances, and can be applied to the simultaneous determination of multiple sugars and sugar alcohols.

In this study, the pretreatment method and the main parameters of instrumental chromatographic separation conditions for 10 sugars and sugar alcohols in infant formula were optimized. An analytical method was firstly established that allows rapid and simultaneous analysis of 10 sugars and sugar alcohols present in infant formula by HPLC-ELSD technology. Additionally, the content and methodological study of sugars and sugar alcohols were determined in 10 infant formulas commercially available by the newly developed analysis method. The method is rapid, efficient, and reproducible, enabling accurate analysis of sugars and sugar alcohols.

Table 2
The chromatographic conditions of three kinds of chromatographic columns.

Chromatographic column	Mobile phase	Chromatographic elution condition	Proportion of mobile phase/(v/v)	Flow rate / (mL/min)	Column temperature (°C)
ALLChrom Rocksil Carbohydrate ES	Acetonitrile-0.04 % ammonia solution	Isocratic elution	82:18	1.0	25
ZORBAX original NH_2	Acetonitrile-water	Isocratic elution	80:20	1.0	30
XBridge@HILIC	Acetonitrile-water	Isocratic elution	90:10	0.8	40

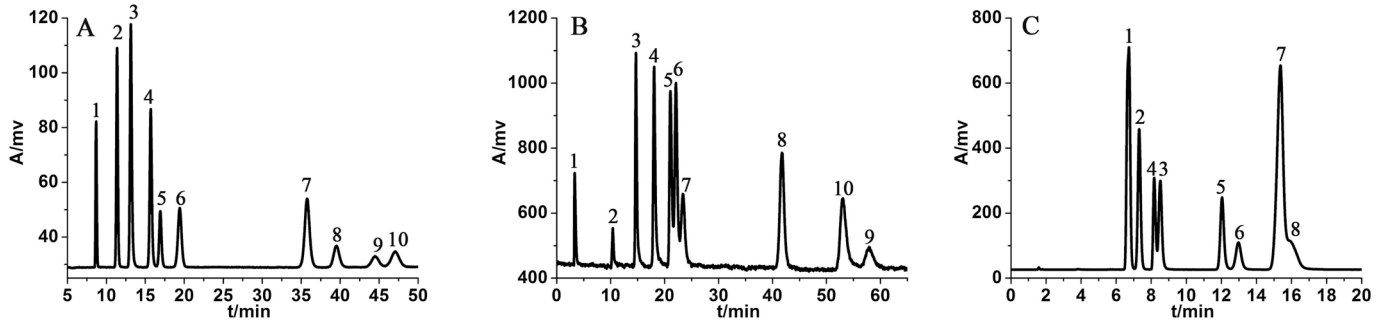

Fig. 2. Chromatograms of the ten analytes with different chromatographic columns. A. ALLChrom Rocksil Carbohydrate ES chromatographic column; B. ZORBAX original NH_2 chromatographic column; C. XBridge@HILIC chromatographic column Peaks: 1. Erythritol; 2. Xylitol;3. Fructose;4. Sorbitol;5. Mannitol;6. Glucose;7. Sucrose;8. Maltitol;9. Lactose;10. Maltose.

Table 3
Linear range, linear equation, R^2 and LOQ of each compound.

Compound	Linear range/(g/L)	Linear equation	R^2	LOQ/(g/100 g)
Erythritol	0.1 – 4.0	$Y = 1.6077X + 1.7762$	0.9997	0.4
Xylitol	0.04 – 1.0	$Y = 1.6046X + 2.6452$	0.9999	0.08
Fructose	0.04 – 1.0	$Y = 1.3522X + 2.6508$	0.9999	0.08
Sorbitol	0.04 – 1.0	$Y = 1.3386X + 2.7870$	0.9997	0.08
Mannitol	0.04 – 1.0	$Y = 1.3740X + 2.7936$	0.9990	0.08
Glucose	0.04 – 1.0	$Y = 1.2834X + 2.3049$	0.9998	0.08
Sucrose	0.04 – 1.0	$Y = 1.1936X + 2.4883$	0.9997	0.08
Maltitol	0.04 – 1.0	$Y = 1.3767X + 2.0415$	0.9998	0.08
Lactose	0.1 – 4.0	$Y = 1.0543X + 1.4730$	0.9997	0.2
Maltose	0.1 – 4.0	$Y = 1.1174X + 1.9119$	0.9998	0.2

2. Materials and methods

2.1. Instruments, materials and reagents

HPLC(equipped with ELSD) was supplied from Thermo Fisher Scientific Corporation of the USA; P300H ultrasonic cleaner was supplied from Elmasonic Corporation of Germany; Multifuge X1R High-speed Refrigerated Centrifuge was supplied from Thermo Company of the USA; MMS-3020 Oven Controlled Oscillator was supplied from EYELA Company of Japan; AE260 electronic scale was supplied from METTLER TOLEDO Company of Switzerland; WH-861 vortex mixer was supplied from Taicang Hualida Instrument Factory; Synergy185 ultrapure water system was supplied from Millipore Company of the USA; Microporefilm Filtering Equipment (0.22 μm, organic phase); ALLChrom Rocksil Carbohydrate ES (250 mm × 4.6 mm, 5 μm) was purchased from Shanghai Yipang Experimental Equipment Co., ltd.; ZORBAX original NH_2 column (250 mm × 4.6 mm, 5 μm) was purchased from Agilent Technologies (China) Co., ltd.; XBridge@HILIC column (250 mm × 4.6 mm, 5 μm) was purchased from Waters Technologies (Shanghai) Limited.

Acetonitrile, *n*-Hexane was purchased from Merck Company of Germany and and used with chromatographically pure; aqueous ammonia was purchased from Hangzhou Gaojing Fine Chemical Co., ltd. and used with analytically pure; ethanoic acid was purchased from Shanghai Lingfeng Chemical Reagents Co., ltd. and used with analytically pure; petroleum ether was purchased from Shanghai Lingfeng Chemical Reagents Co., ltd. and used with analytically pure; zinc acetate dihydrate was purchased from Guangdong Guanghua Sci-Tech Co., ltd. and used with analytically pure; potassium hexacyanoferrate(II) trihydrate was purchased from Xilong Chemical Co., ltd. and used with analytically pure; Captiva EMR solid phase extraction (SPE) cartridge (6 mL/600 mg, no activation was required before use, Agilent Technologies Co., ltd.), IC Guard RP cartridge (1 mL, activate with 5 mL of methanol and 15 mL of water before use, CNW), IC Guard H cartridge (1 mL, activate with 15 mL of water before use, CNW), HLB polymer SPE cartridge (6 mL/200 mg, activate sequentially with 3 mL of methanol and 3 mL of water before use, Oasis), ultrapure water, other reagents used in the experiment were all analytically pure unless otherwise specified.

Standard substances: erythritol, maltitol, mannitol were supplied by ANPEL Co., ltd. (purity ≥ 96.2 %); xylitol, sorbitol, lactose were supplied by Dr Ehrenstorfer GmbH of Germany Co., ltd. (purity ≥ 99.4 %); fructose, glucose, sucrose, maltose were supplied by Bepure Co., ltd. (purity ≥ 99.8 %).

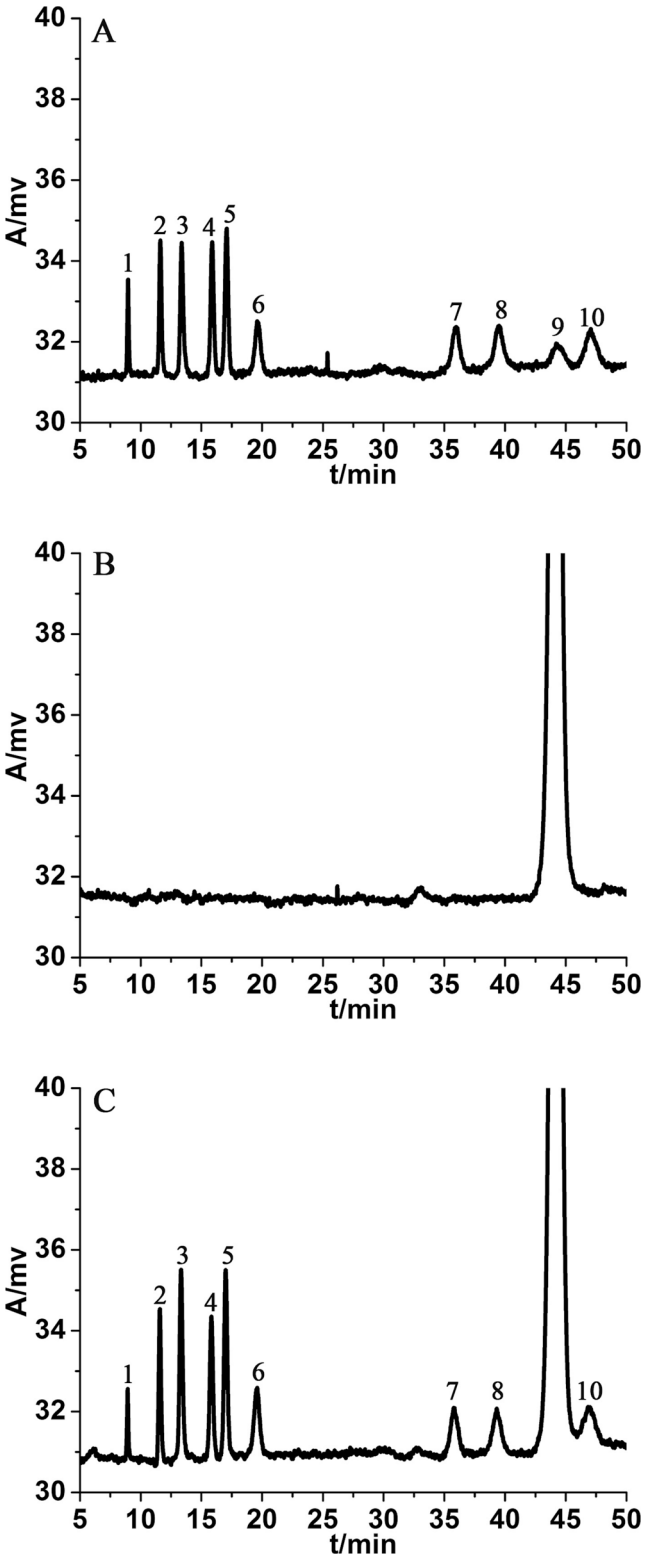

Fig. 3. Chromatograms of standard solution, blank milk sample and spiked recovery, A. Standard solution; B. Blank milk sample; C. Spiked recovery. Peaks: 1. Erythritol; 2. Xylitol;3. Fructose;4. Sorbitol;5. Mannitol;6. Glucose;7. Sucrose;8. Maltitol;9. Lactose;10. Maltose.

2.2. Stock and preparation of working standard solutions

0.1 g (accurate to 0.1 mg) of the above standard substances were precisely weighed and dissolved in a 10-mL volumetric flask with water

Table 4
Recoveries and RSDs of the nine analytes spiked in blank milk samples ($n = 6$).

Compound	Spiked/ (g/100 g)	Blank milk sample	
		Recovery (%)	RSD (%)
Erythritol	0.4	80.4–95.1	6.7
	0.8	86.4–95.9	6.6
	4.0	87.4–96.2	2.8
Xylitol	0.08	81.6–98.6	6.8
	0.16	81.3–95.3	5.5
	0.8	85.7–97.4	3.4
Fructose	0.08	81.8–98.1	6.8
	0.16	81.5–94.2	5.5
	0.8	85.7–96.2	3.6
Sorbitol	0.08	81.8–98.2	6.5
	0.16	81.3–94.7	5.7
	0.8	84.8–96.1	3.7
Mannitol	0.08	84.7–98.1	6.6
	0.16	81.3–94.2	5.5
	0.8	84.8–96.2	3.6
	0.08	82.7–99.4	6.5
Glucose	0.16	81.6–95.3	5.7
	0.8	85.6–96.5	3.6
Sucrose	0.08	81.6–98.4	6.6
	0.16	81.5–94.2	5.7
	0.8	86.8–96.5	3.6
Maltitol	0.08	83.7–97.3	7.1
	0.16	82.4–94.8	5.3
	0.8	84.8–96.2	3.6
Maltose	0.2	81.5–97.2	6.9
	0.4	82.4–94.6	5.9
	2.0	86.8–96.3	3.8

to prepare 10 g/L standard stock solution, and stored them at 4 °C.

Preparation of mixed standard working solution: The above standard stock solutions were accurately pipetted and diluted to the following series of standard working solutions with acetonitrile: water (4:6, v/v) solution: 0.04, 0.08, 0.1, 0.2, 0.4, 1.0 g/L (xylitol, fructose, sorbitol, mannitol, glucose, sucrose, maltitol), 0.1, 0.2, 0.4, 1.0, 2.0, 4.0 g/L (erythritol, lactose and maltose), and stored at 1 °C – 4 °C in the refrigerator and protected from light, with a quality guarantee period of 6 months.

2.3. Sample preparation

1 g sample was weighed (accurate to 0.0001 g) and dissolved in a 10-mL volumetric flask with 6 mL water and mixed with a vortex mixer. The solution was put in water bath at 85–90 °C for 25 min, cooled to room temperature. Then 0.5 mL of 5 % (v/v) acetic acid aqueous solution was added, extracted by ultrasonic treatment for 10 min, placed at room temperature. Acetonitrile was added to 10 mL, and mixed. The solution was placed in the refrigerator for 1 h. An appropriate amount of solution was transferred to a 15 mL stoppered centrifuge tube, and centrifuged at 8,500 r/min for 5 min. 2.5 mL of the supernatant after centrifugation was pipetted to a new 15 mL stoppered centrifuge tube, added with 2.5 mL water and then mixed with a vortex mixer for purification.

The solution to be purified was placed in the activated RP cartridge and H cartridge. After the sample was loaded, the first 3 mL of purified solution was discarded and the remaining 2 mL of purified solution was collected and filtered through a 0.22 μm filter membrane for determination.

2.4. Chromatographic conditions

The HPLC analysis was performed using an ALLChrom Rocksil Carbohydrate ES column. The isocratic elution (eluent A: acetonitrile, eluent B: 0.04 % ammonia solution) involved 18 %B. The column temperature was maintained at 25 °C. The flow rate was 1.0 mL/min. ELSD drift tube temperature was set to 85 °C. The flow rate of N_2 was 2.0 L/min. The injection volume was 10 μL.

3. Results and discussion

3.1. Optimization of extraction conditions

Due to the complex composition of infant formula, there are many types of proteins, fats and other nutrients, which may cause irreversible damage to the column and detector, and interfere with the target chromatographic peaks. Therefore, the proteins need to be completely removed during the sample pre-treatment process. Based on the literature, several protein precipitation methods include potassium ferricyanide-zinc acetate methods [20,21], pH regulation method [22,23] and acetic acid method [24], etc. The results showed that the protein was not completely precipitated and the extract was not clear enough, which affected the subsequent purification on the cartridge (see Fig. 1A and 1B), when potassium ferricyanide-zinc acetate method and pH adjustment method were used. The protein was completely precipitated and the extract was clear and transparent, with satisfactory extraction effect (see Fig. 1C), when 5 % (v/v) acetic acid aqueous solution was adopted. Therefore, 5 % (v/v) acetic acid aqueous solution was chosen as the protein precipitant in this experiment.

3.2. Optimization of purification conditions

In order to reduce the interference of lipids and lipid-soluble substances on chromatographic separation, the purification effects of EMR [25], RP and H cartridge [26], HLB [27] and other commonly used SPE cartridges were compared. After the three samples were extracted by the optimal method, they were purified by the above three kinds of SPE cartridges and detected by HPLC. The purification steps of the three SPE cartridges were as follows: EMR cartridge, RP and H cartridge: receiving directly after loading the sample; HLB cartridge, washing with water and eluting with methanol after loading the sample. When EMR or HLB cartridge were taken as the purification column, there were both some matrix interference peaks in the chromatogram of the ten kinds of sugars and sugar alcohols, and the recoveries ranges of the ten target compounds were 78.0 %-89.0 % and 95.0 %-100 % respectively (see Table 1). RP and H cartridges were adopted for purification that can remove the matrix interference to the target peak, and the recoveries ranged from 90.0 % to 100 %. Therefore, this experiment eventually adopted RP and H cartridge to purify the extraction solution of infant formula samples.

3.3. Influence of chromatographic column

The commonly used columns for sugar detection include sugar column, NH_2 column and HILIC column, etc. In this experiment, three chromatographic columns, named ALLChrom Rocksil Carbohydrate ES, ZORBAX original NH_2, and XBridge@HILIC were selected to investigate the resolution of the ten sugars and sugar alcohols. The chromatographic

Table 5
Repeated determination of lactose content of infant formula ($n = 6$) .

Compound	Content(g/100 g)						Average value (g/100 g)	RSD(%)
	1	2	3	4	5	6		
Lactose	50.8	50.6	50.9	51.5	49.5	50.7	50.7	1.29

conditions of three chromatographic columns were shown in Table 2. The results showed that the chromatographic peaks of several target compounds partially overlapped when separated by NH_2 and HILIC chromatographic columns (see Fig. 2B and 2C). In contrast, the separation factor was good and the chromatographic peak shape was sharp when the ALLChrom Rocksil Carbohydrate ES column was used for separation (see Fig. 2A). Therefore, the ALLChrom Rocksil Carbohydrate ES column was chosen for the separation of ten analytes in this experiment.

3.4. Influence of mobile phase

The sugar molecules contain polar group, therefore the solvents with greater polarity such as acetonitrile, methanol, and ethanol were selected as the mobile phase [28,29]. The acetonitrile was applied in this experiment, because the methods reported in many literatures usually [10–13,20,29] adopted acetonitrile as the mobile phase. The solubility of sugar in acetonitrile is low, so a mixture of acetonitrile and water was used as the mobile phase. The experiment studied the impacts of mobile phase acetonitrile–water (75:25, 80:20, 82:18, 85:15, 90:10,v/v) with different volume ratios on the separation of 10 target compounds. The results showed that increasing the proportion of aqueous phase could shorten the analysis time, but reduce the degree of separation, resulting in the overlap of some chromatographic peaks. With the increase of acetonitrile ratio, the degrees of separation of 10 analytes were improved, but the corresponding analysis time was lengthened and some chromatographic peaks widened remarkably. In contrast, when acetonitrile–water (82:18, v/v) was employed for mobile phase, the separation factors and the peak shape of most target compounds were good. However, the resolutions of chromatographic peaks of sorbitol and mannitol, sucrose and maltitol were poor. The addition of a little ammonium hydroxide to the mobile phase could increase the efficiency of separation and peak shape. There are some literatures [30–32] about that different volume ratios of ammonia solution (0.04 %, 0.1 %, 1 %) were added to the mobile phase to improve the peak shape. When acetonitrile-0.04 % ammonia solution was taken as the mobile phase, the chromatographic peaks of 10 target compounds were completely separated within 50 min. Therefore, acetonitrile-0.04 % ammonia solution was adopted as the mobile phase in this experiment.

3.5. Standard curve, linear range and limit of quantitation

10 g/L standard solutions of each sugar and sugar alcohol were diluted in turn to prepare the standard working solutions at the mass concentrations of 0.04, 0.08, 0.1, 0.2, 0.4, 1.0 g/L (xylitol, fructose, sorbitol, mannitol, glucose, sucrose, maltitol), and 0.1, 0.2, 0.4, 1.0, 2.0, 4.0 g/L (erythritol, lactose and maltose). According to the above stated chromatographic conditions, the prepared series of standard solutions were sequentially detected by HPLC, and the linear regression was performed with the mass concentrations of sugars and sugar alcohols as the horizontal coordinates (X) and the peak areas as the vertical coordinates (Y), and the obtained linear equations, linear ranges, correlation coefficients (R^2) and limit of quantitations (LOQs) (S/N = 10) are shown in Table 3.

3.6. Recovery

The spiked recoveries and the precisions of the method were determined by adding standard solutions to the blank milk samples without sugars and sugar alcohols, respectively. The spiked levels of erythritol were 0.4, 0.8, 4.0 g/100 g, xylitol, fructose, sorbitol, mannitol, glucose, sucrose and maltitol were 0.08, 0.16, 0.8 g/100 g, and maltose were 0.2, 0.4, 2.0 g/100 g, respectively. The spiked recoveries and relative standard deviations (RSDs) were calculated by six parallel determinations, and the related chromatograms are shown in Fig. 3, and the results are shown in Table 4. The recoveries of the nine analytes ranged from 80.4 % to 99.4 %, and the RSD values ($n = 6$) ranged from 2.8 % to 7.1 %. The recovery and precision met the requirements of SANTE/12682/2019 [33] and the analytical requirements for infant formula. Hence, this method can be applied to daily analysis and detection of sugars and sugar alcohols.

3.7. Precision

As shown in Table 5, the relative standard deviation of the precision of the lactose assay was 1.29 %. The precision met the requirements of SANTE/12682/2019 [33] and the analysis requirements for infant formula, which can be used for the daily analysis of lactose.

3.8. Real samples analysis

The method developed in this experiment was used to detect 10 sugars and sugar alcohols in 10 different brands of infant formula commercially available. The results showed that no fructose and sucrose that are not allowed to be used were detected in any of the 10 infant formula collected in this experiment, which met the requirements of GB 10765–2021 National Food Safety Standard Infant Formula [1] that infant formula should not use fructose and sucrose as a source of carbohydrates.

4. Conclusion

In this paper, a method was developed for the simultaneous separation and determination of 10 sugars and sugar alcohols in infant formula using the above stated analysis system. The samples were extracted with acetonitrile–water solution, cleaned up by RP and H cartridge, separated by ALLChrom Rocksil Carbohydrate ES column, eluted with acetonitrile-0.04 % ammonia solution as mobile phase isocratic, and quantified by external standard method. The LOQs (S/N = 10) ranged from 0.08 to 0.4 g/100 g, the RSD of the lactose parallel samples reached 1.29 %, the recoveries of the other 9 components ranged from 80.4 to 99.4 %, and the RSD reached 2.8–7.1 %. The method was adopted to determine the content of target compounds in 10 market-purchased infant formula samples, and no fructose and sucrose were detected in these samples in this experiment. The method is characterized by high detection sensitivity, great separation capacity, easy and rapid operation, which is applicable to routine analysis, thus providing technical support for the quality control and safety evaluation of infant formula.

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CRediT authorship contribution statement

Yaqin Zhang: Methodology, Validation. **Wenhua Zhang:** Methodology, Validation, Writing – original draft. **Jianbo Hou:** Methodology, Validation. **Jianmin He:** Validation, Formal analysis. **Ke Li:** Validation, Formal analysis. **Yi Li:** Validation, Formal analysis. **Dunming Xu:** Methodology, Validation, Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used is confidential.

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