Chapter

Analysis of Additives in Milk Powders with SPE-HPLC or 2D-HPLC Method

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Abstract

Dairy products are beneficial to human health, especially for formula-fed newborns. According to the regulation of FDA and China national food safety standard, food additives such as benzoic acid, sorbic acid, natamycin, lysozyme, saccharin sodium, and aspartame are not permitted to be added to milk powder. So, the establishment of accurate and convenient methods for the analysis of these food additives in milk powder is critical to people's health. For the reason of the complex matrix of infant milk powders, we compared six sample pretreatment methods (liquid-liquid extraction, organic precipitation, heavy precipitation, and three different solid-phase extraction (SPE) methods (C18, HLB, MAX)) from recovery, easy operation, time cost, and organic solvent usage aspects. Finally, Poly-Sery HLB cartridge was confirmed as the most appropriate material for its high recovery and time cost merits. We are also introducing two-dimensional liquid chromatography (2DLC) method for the simultaneous determination of five major proteins and seven food additives in milk powders. Optimization of switching mode, choice of columns, mobile phase, and flow speed was discussed. We also compared limit of detection (LOD), recovery, and sample treatment with the results of high-performance liquid chromatography (HPLC). Results show that 2DLC is simpler, faster, and more accurate than the HPLC method.

Keywords: additives, milk powders, sample treatment, 2DLC, proteins

1. Introduction

Dairy products are beneficial to human health, especially for formula-fed newborns. India and the European Union both produced around 160 Mt milk in 2016 [1]. People are very concerned about the nutritional ingredients and illegal additives of the milk products. As we all know, bovine milk includes 80% caseins (CN) and 20% whey proteins. Caseins consist of α_{s1}-CN, β-CN, α_{s2}-CN, and κ-CN in an approximate 4:4:1:1 weight ratio. Whey proteins mainly consist of β-lactoglobulin (β-LgA, β-LgB) and α-lactalbumin (α-Lac) in a 3:1 weight ratio [2]. The quantity of milk fraction proteins is related to the development of the baby. As many literatures reported, preservatives and artificial sweeteners may be harmful to people's health [3–5].

According to the regulation of FDA and China national food safety standard, food additives such as benzoic acid, sorbic acid, natamycin, lysozyme, saccharin sodium, and aspartame are not permitted to be added to milk powder. So, establishment of accurate and convenient methods for the analysis of these food additives in milk powder is critical to people's health.

Here, I'll introduce two works of our groups to readers: the comparison of six sample preparation methods for the analysis of four preservatives and two artificial sweeteners in milk powders [6] and two-dimensional liquid chromatography (2DLC) for determination of five major proteins and seven additives in milk powders.

2. Comparison of six sample preparation methods for analysis of four preservatives and two artificial sweeteners in milk powders

It is not very easy to determine the trace residues or contaminants in infant milk powder for its complex matrix [7]. So, the sample pretreatment is the key step in the whole analytical procedures. According to the literatures, there are two kinds of sample preparation methods for analysis of contaminants in dairy products. One is to extract the targeted analytes, and the other is to remove the interferents. Usually, solid-phase extraction had been widely used as a milk sample preparation method. Sometimes, liquid-liquid extraction followed by a SPE cleanup step was served to remove the macromolecular protein prior to determination of the target analytes [8]. Removal of the protein in milk could be done by precipitating them with heavy metallic salt [9] or sodium tungstate [10].

Our group developed six sample preparation methods based on the literatures, that is, liquid-liquid extraction, organic precipitation, heavy precipitation, and three different solid-phase extraction methods (C18, HLB, MAX). In order to obtain the higher recovery and reduce the time cost and organic solvent dosage, the six different sample preparation methods were compared.

2.1 Sample preparation and extraction

2.1.1 Method A (liquid-liquid extraction)

This method is based on the study of preservatives in cheeses [11]. Around 2.0 g milk powder was mixed with 4.0 mL of deionized water (60°C). After 10 min of ultrasonication, 5.0 mL of ethyl acetate and 1.0 mL 10 mmol L^{-1} of formic acid were added. The samples were extracted for 40 min on a rotary mixer at 400 rpm. After that, they were centrifuged for 5 min at 3200 rpm. The supernatant was transferred to another tube, and the sediment was extracted with 5.0 mL of ethyl acetate once again. The second supernatant obtained was combined with the one from the first extraction. Then they were filtered and evaporated to dryness at ambient temperature. The residues were dissolved in 500 μL mixture solution (0.1 M acetate buffer: methanol = 2:1, v/v) and vortexed for 20 s.

2.1.2 Method B (precipitation based on sodium tungstate)

This method is based on the study of five macrolide antibiotics in milk [10]. The sample dissolving steps were the same as method A (liquid-liquid extraction). After 10 min of ultrasonication, this solution was centrifuged at 6000 rpm for 15 min. The defatted milk was transferred to a new centrifuge tube, and then 1.0 mL 10% sulfuric acid and 5.0 mL 10% sodium tungstate solutions were added. The resulting

solution was vigorously shaken for 2 min and diluted to 10 mL with water and centrifuged at 4000 rpm for 10 min. The supernatant was filtered and evaporated to dryness at ambient temperature. The residues were dissolved in 500 μL mixture solution (0.1 M acetate buffer:methanol = 2:1, v/v) and vortexed for 20 s.

2.1.3 Method C (precipitation based on potassium ferrocyanide)

This method is based on the detection of adulteration of milk with soy milk [9]. The protocols of method C were the same as method B, only except the precipitants of 3.0 mL 0.085 mol L^{-1} $K_4[Fe(CN)_6]$ and 3.0 mL 0.25 mol L^{-1} $ZnSO_4$ solutions employed in this method.

2.1.4 Method D: F (solid-phase extraction)

This method is based on the study of fluoroquinolones in milk [12] and determination of 20 pharmacologically active substances in various milk samples [13]. After 10 min of ultrasonication, 7.0 mL 1% trichloroacetic acid (TCA) and 3.0 mL acetonitrile were added. The resulting solution was vigorously shaken for 2 min and centrifuged at 4000 rpm for 10 min. The supernatant obtained extracted by SPE. Three kinds of cartridges (CNWBOND LC-C18 SPE, Poly-Sery HLB SPE, and Poly-Sery MAX SPE) were examined. The SPE protocol on three types of cartridges was consisted of the following steps: (1) activation of the cartridges with 3 mL methanol first and then conditioned with 3 mL deionized water, (2) sample loading, and (3) sample elution with 3 mL 20 mmol L^{-1} ammonium sulfate and 3 mL 80% acetonitrile. After the eluates were filtered, the steps of evaporation and residues dissolving were the same as method A (liquid-liquid extraction).

2.2 Comparison of sample preparation methods

For method A (liquid-liquid extraction), the extraction time of 10, 15, 20, 25, 30, 35, 40, 45, and 50 min was investigated. The results are illustrated in **Figure 1**, indicating 40 min was the best choice. The different extraction temperatures from 25 to 40°C were tested, and the results showed that there were no obvious differences in recoveries of each preservative. For method B (precipitation based on sodium tungstate) and method C (precipitation based on potassium ferrocyanide), the results showed that there were no significant differences between these two methods. The recoveries of benzoic acid, sorbic acid, and saccharin sodium were more than 80%. However, the recovery of lysozyme (<30%) indicated that both of the precipitate-based methods were not suitable for enzyme [14] and it might be coprecipitated with the proteins in milk powder. For method D-F (SPE), three kinds of cartridges (CNWBOND LC-C18 SPE, Poly-Sery HLB SPE, and Poly-Sery MAX SPE) and their elution solvents applied were investigated. Porous silica particles surface bonded with C18 was the most commonly used sorbents. Poly-Sery HLB is the kind of polymeric sorbents that was reported as being superior to the silica-based C18. The major difference between the HLB and MAX sorbents is the presence of the anion-exchange groups that provide high selectivity for acidic compounds. So, the three kinds of cartridges were tested to evaluate their applicability. Four elution solvents with different polarity were tested: 70% acetonitrile, 80% acetonitrile, 90% acetonitrile, and 100% acetonitrile. The recoveries of each food additive showed that Poly-Sery HLB with 80% acetonitrile provided the best results.

Figure 2 shows the recovery results of each food additive obtained by the six different abovementioned methods, respectively. Each sample was analyzed in triplicate. Both method A (liquid-liquid extraction) and method D (Poly-Sery HLB SPE)

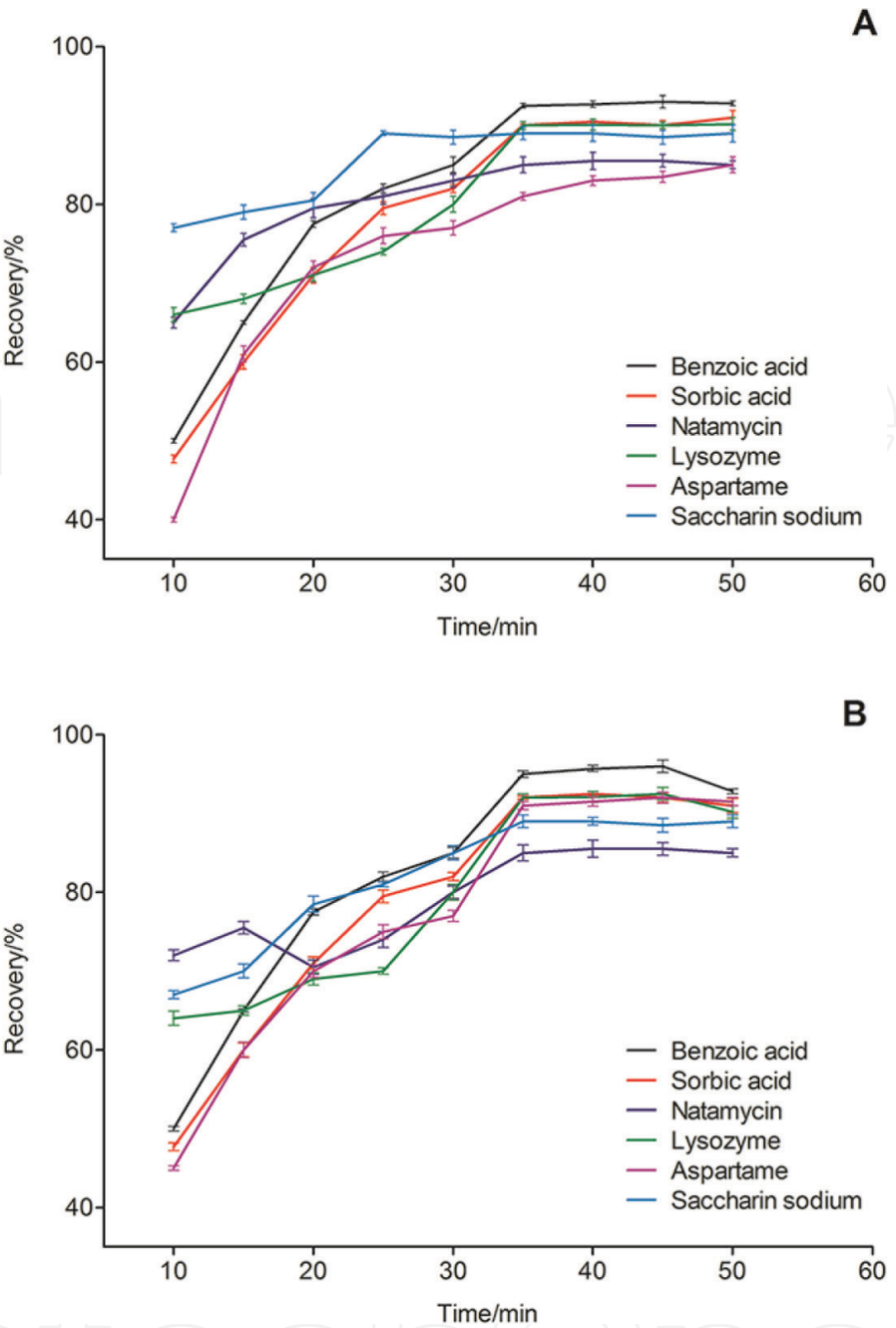


Figure 1.
Effects of extraction time on the six food additives recovery obtained with method A in the two milk samples (n = 3). (A) Whole milk powder and (B) skimmed milk powder (SMP). The six food additives: benzoic acid, sorbic acid, natamycin, lysozyme, aspartame, and saccharin sodium.

have good recoveries (>80%) for all six analytes, but results of our study showed that the average RSD% for method A of the six analytes were all between 3.7 and 5.4%, which is more than that of HLB SPE method (2.3–3.8%). Considering the environmental and economic costs, method D (Poly-Sery HLB SPE) was employed in our further study.

2.3 SPE (Poly-Sery HLB)-HPLC-DAD method validation

The proposed SPE (Poly-Sery HLB)-HPLC-DAD method was validated in terms of linearity, limit of detection, limits of quantity (LOQ), within- and between-day precision, and accuracy. **Figure 3** shows the chromatograms of the mixed standard solutions. We can see that the six preservatives and sweeteners were

baseline separated and had a good resolution ($R \geq 2.6$) under the chromatographic condition.

The linearity of the method was evaluated under 210 nm with the mixed standard solutions, pooled whole milk powder matrices,and pooled skimmed milk powder matrices. The LODs and LOQs in each case were estimated based on S/N = 3 and 10, respectively. The results including the regression equations, the linear ranges, and regression coefficients are summarized in **Table 1**.

The precision and accuracy were tested in two milk powder matrices, that is, the whole milk powder and the skimmed milk powder. **Figures 4** and **5** showed the

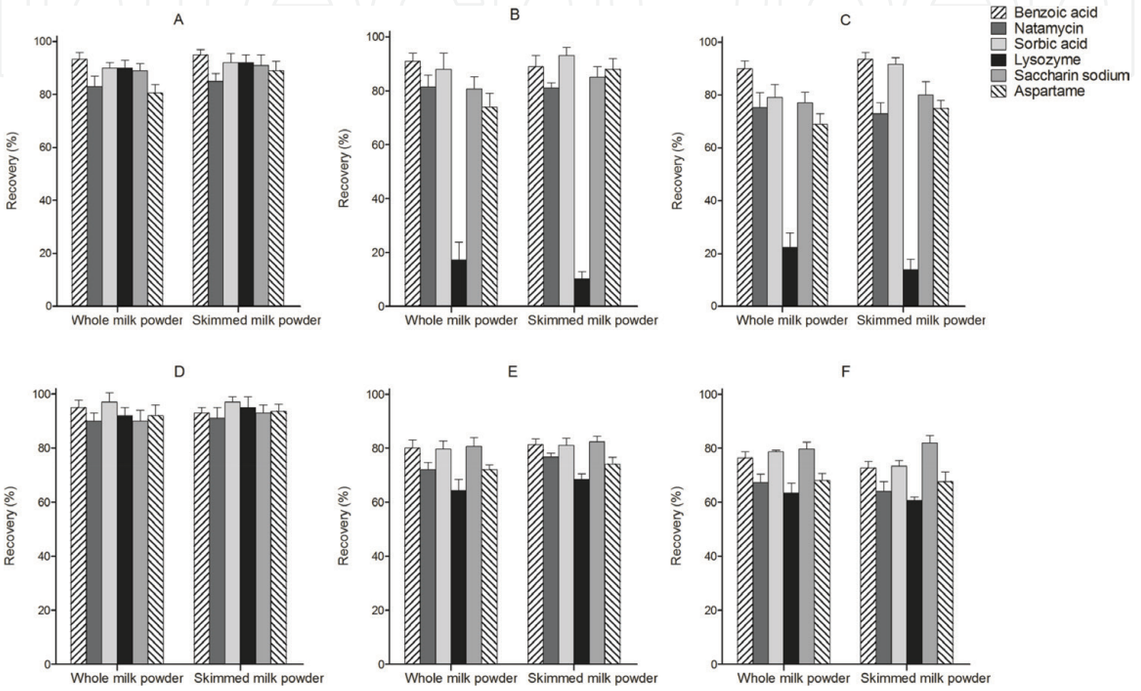


Figure 2.
Recovery results of the six different sample preparation methods. The left group in A–F represents the whole milk powder matrix, and the right group in A–F represents the skimmed milk powder matrix. The six food additives: benzoic acid, sorbic acid, natamycin, lysozyme, aspartame, and saccharin sodium. (A) Method A (liquid-liquid extraction). (B) Method B (precipitation based on sodium tungstate). (C) Method C (precipitation based on potassium ferrocyanide). (D–F) Method D-F (solid phase extraction): (D) Poly-Sery HLB SPE, (E) CNWBOND LC-C18 SPE, and (F) Poly-Sery MAX SPE.

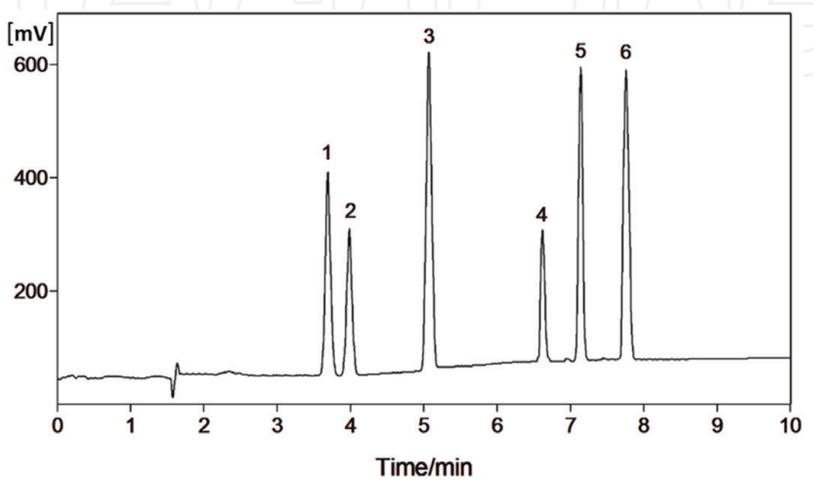


Figure 3.
Chromatogram of mixed standard solutions. (1) Benzoic acid, (2) sorbic acid, (3) saccharin sodium, (4) natamycin, (5) aspartame, and (6) lysozyme.

representative chromatograms. All chromatographic peaks were separated completely and had a good resolution ($R \geq 1.5$). Recovery studies of benzoic acid, sorbic acid, natamycin, lysozyme, saccharin sodium, and aspartame were evaluated by analysis of blank pooled samples spiked with 10 μg g^{-1} (lower level), 50 μg g^{-1} (middle level), and 100 μg g^{-1} (upper level) of each analyte. And the data were calculated based on the matrix-matched regression curves and summarized in **Table 2**. The intraday precision was studied at lower, middle, and upper concentration levels (n = 5). The interday precision was analyzed with spiked samples at 50 μg g^{-1} for six-day determinations (**Table 3**).

	Amounts of chemical reagents used	Time cost[e]
Method A[a]	Ethyl acetate: 5.0 + 5.0 mL Formic acid solution (10 mmol L^{-1}): 1.0 mL	>60 min
Method B[b]	Sulfuric acid solution (10%): 1.0 mL Sodium tungstate solution (10%): 5.0 mL	≈50 min
Method C[c]	$K_4[Fe(CN)_6]$ solution (0.085 mol L^{-1}): 3.0 mL $ZnSO_4$ solution (0.25 mol L^{-1}): 3.0 mL	≈50 min
Method D–F[d]	TCA solution: 7.0 mL Acetonitrile: 3.0 mL Methanol: 3.0 mL Ammonium sulfate solution: 3.0 mL Acetonitrile: 3.0 mL	≈25 min

[a]*Method A (LLE).*
[b]*Method B (precipitation based on sodium tungstate).*
[c]*Method C (precipitation based on potassium ferrocyanide).*
[d]*Method D–F (SPE).*
[e]*Time cost is the average value of the three experiments.*

Table 1.
Comparison of the cost-effectiveness of six methods.

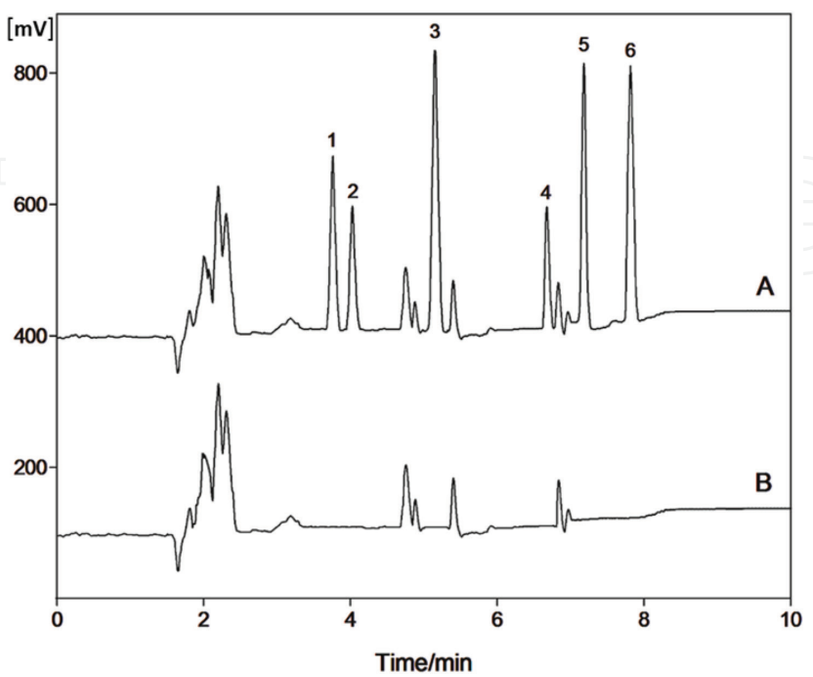


Figure 4.
Chromatograms of the blank whole milk powder sample and spiked whole milk powder sample. (A) The spiked whole milk powder sample: (1) benzoic acid, (2) sorbic acid, (3) saccharin sodium, (4) natamycin, (5) aspartame, and (6) lysozyme (50 μg g^{-1} of each food additives). (B) The blank whole milk powder sample.

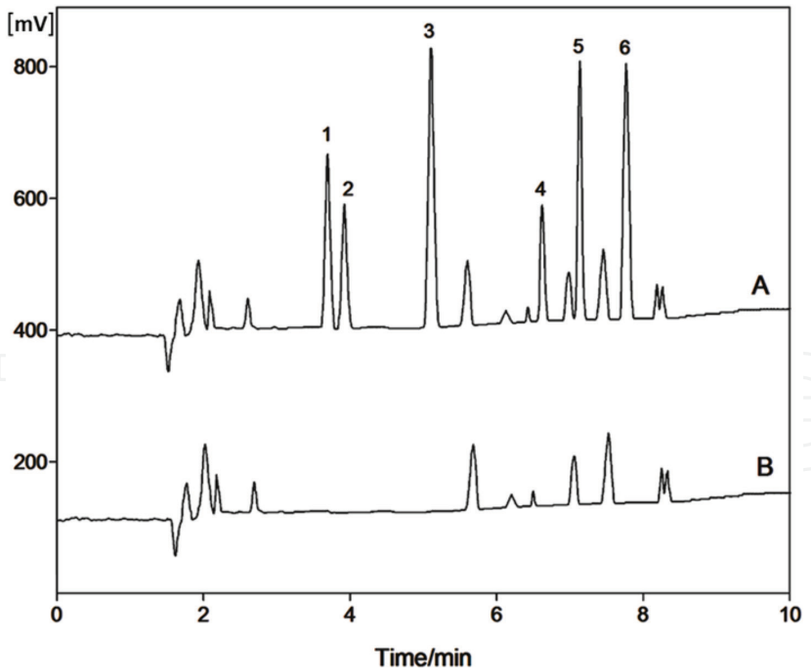


Figure 5.
Chromatograms of the blank skimmed milk powder sample and spiked skimmed milk powder sample. (A) The spiked skimmed milk powder sample: (1) benzoic acid, (2) sorbic acid, (3) saccharin sodium, (4) natamycin, (5) aspartame, and (6) lysozyme (50 μg g^{-1} of each food additives). (B) The blank skimmed milk powder sample.

Analytes	Sample matrix	Regression equation[b] y = ax ± b	Regression coefficient	Linear range (μg L^{-1})	LOD (μg L^{-1})	LOQ (μg L^{-1})
Benzoic acid	A[a]	y = 0.582x − 0.035	0.9999	100–20,000	45	95
	B[a]	y = 0.497x − 0.028	0.9998	250–20,000	70	200
	C[a]	y = 0.501x + 0.037	0.9998	250–20,000	60	200
Sorbic acid	A[a]	y = 0.362x + 0.017	0.9998	150–25,000	60	130
	B[a]	y = 0.297x + 0.028	0.9994	300–25,000	90	255
	C[a]	y = 0.500x − 0.054	0.9995	300–25,000	90	240
Natamycin	A[a]	y = 0.313x + 0.039	0.9999	200–30,000	60	135
	B[a]	y = 0.364x + 0.023	0.9997	500–30,000	80	215
	C[a]	y = 0.288x + 0.060	0.9997	500–30,000	90	230
Saccharin sodium	A[a]	y = 0.405x + 0.097	0.9999	100–25,000	30	75
	B[a]	y = 0.329x − 0.010	0.9992	200–25,000	50	120
	C[a]	y = 0.525x − 0.091	0.9995	200–25,000	70	150
Aspartame	A[a]	y = 0.617x − 0.043	0.9999	100–30,000	50	95
	B[a]	y = 0.577x + 0.033	0.9996	200–30,000	60	165
	C[a]	y = 0.505x − 0.023	0.9997	200–30,000	70	180
Lysozyme	A[a]	y = 0.405x + 0.097	0.9997	100–30,000	45	95
	B[a]	y = 0.381x + 0.059	0.9995	250–30,000	60	170
	C[a]	y = 0.370x + 0.068	0.9996	250–30,000	60	170

[a] *(A) Aqueous, (B) pooled whole milk powder, and (C) pooled skimmed milk powder.*
[b] *y is the average peak area of each analyte (n = 3), and x is the mass concentration of the analyte in μg L^{-1}.*

Table 2.
Linearity and LOD of the developed method (n = 3).

Analytes	Added ($\mu g\ g^{-1}$)	Whole milk powder		Skimmed milk powder	
		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Benzoic acid	10	93	4.8	95	3.3
	50	95	3.5	93	2.9
	100	93	1.4	95	2.5
Sorbic acid	10	94	3.7	94	4.1
	50	97	1.5	97	3.0
	100	92	1.2	99	2.2
Natamycin	10	89	4.9	90	3.9
	50	90	2.0	91	3.5
	100	96	1.0	99	2.7
Lysozyme	10	91	4.5	92	4.4
	50	92	5.0	95	1.9
	100	101	2.8	103	2.3
Saccharin sodium	10	95	3.3	89	4.6
	50	90	4.5	93	4.1
	100	97	2.0	98	2.4
Aspartame	10	90	2.9	93	3.6
	50	92	4.5	94	1.1
	100	94	2.4	94	1.2

Table 3.
Precision and accuracy of the assay for whole milk powder and skimmed milk powder analysis (n = 3).

2.4 Conclusion

Among the six different sample extraction methods, two precipitate-based methods (method B and method C) were not suitable for the low recovery of lysozyme. Both method A and method D obtained good recoveries of six food additives simultaneously, but the major problem of method A is the lower reproducibility and much more time cost than method D. SPE was a simple and rapid method for the extraction of six food additives. From the results of method D–F, Poly-Sery HLB cartridge was confirmed as the most appropriate material for its high recovery.

3. 2DLC for determination of five major proteins and seven additives in milk powders

Two-dimensional liquid chromatography has been used in many aspects. Herein, a 2DLC method was introduced for the simultaneous determination of five major proteins and seven additives in milk powders. Considering the macromolecular proteins, C4 column was placed in the first dimension (1D), and C18 column was placed in the second dimension (1D) for analysis of the seven additives. Finally, the five proteins in milk powders were separated completely on the 1D column, and the seven additives can be simultaneously analyzed on the 2D column. In the middle of 1D and 2D, a trapping column and a ten-port switching valve was served. This method was compared with the conventional one-dimensional liquid

chromatography (1DLC) in terms of sample preparation, limit of detection (LOD), and recovery.

3.1 1DLC separation of additives

3.1.1 Sample preparation for 1DLC

0.5 g milk powder samples were diluted in 2.0 mL ultrapure water. After 10 min of ultrasonication, 0.5 mL of Carrez I solution (500 mM aqueous potassium ferrocyanide), 0.5 mL of Carrez II solution (500 mM aqueous zinc acetate), and 1 mL of ACN were added in order to precipitate the proteins.

3.1.2 1DLC analysis

Maltol, ethyl maltol, vanillin, ethyl vanillin, benzoic acid, sorbic acid, and saccharin sodium were separated on a 2010 AT chromatographic instrument from Shimadzu Corporation (Kyoto, Japan). A C18 analytical column (4.6 × 150 mm, 5 μm) was used. The mobile phases were ammonium acetate buffer (25 mM, pH 6.6) (solvent A) and ACN/water = 90/10 (v/v) (solvent B); the following gradient program was used: 0–2.8 min, 0% ACN; 2.8–5 min, progressing linearly to 45% ACN; and 5–15 min, maintaining at 45% ACN. The flow rate was 1.0 mL/min, and the column temperature was maintained at 40°C. The injection volume was 5 μL, and the detection wavelength was 214 nm. The peak area was calculated for quantification, and each sample or standard was injected in triplicate.

3.2 2DLC separation of proteins and additives

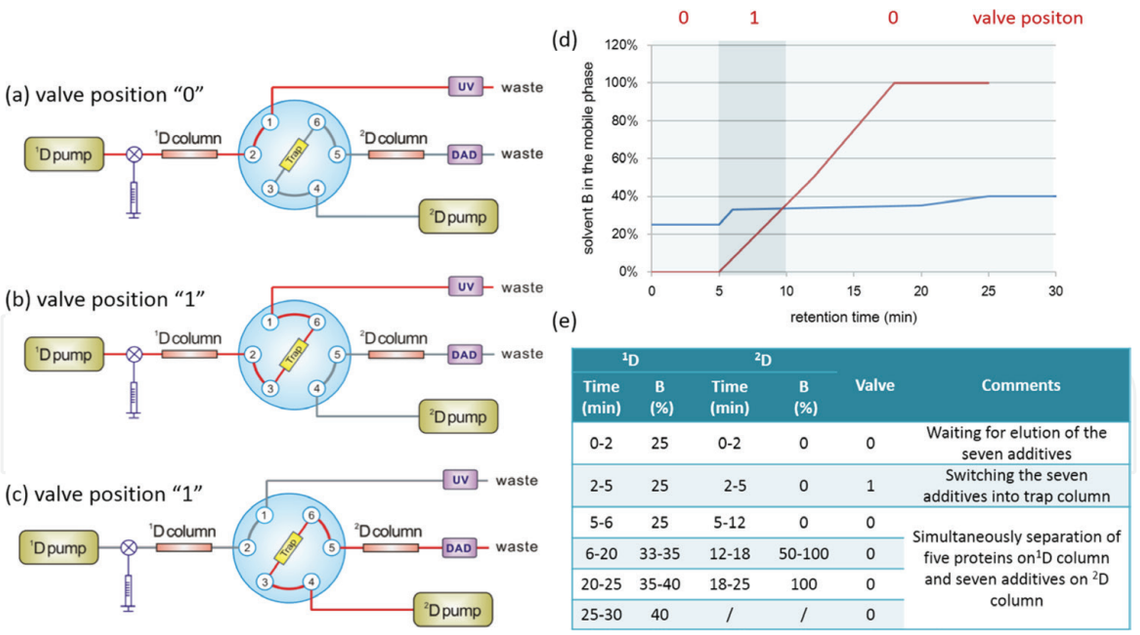
3.2.1 Sample preparation for 2DLC

Around 0.2 g of the milk powder samples were dissolved for 10 min in 5 mL of buffer (6 M urea, 0.5% OG). The samples were then filtered through a 0.45 μm nylon membrane before injected into the 2DLC system for analysis.

3.2.2 2DLC analysis

The 2DLC system consisted of two LC-20AB binary gradient pumps (Shimadzu Technologies), one six-port two-position switching valve (VICI Valco Instruments, Houston, TX, USA), a SIL-20A autosampler, two DGU-20A3 degassers, a CTO-20A column oven, and two SPD-M20A diode array detectors.

A scheme of the 2DLC system is shown in **Figure 6**. For the first dimension (1D), a Venusil XBP-C4 analytical column (4.6 mm × 100 mm, 5 μm) coupled with a C4 guard column was used. One aspect is for separation of proteins and additives, and the other is for five proteins analysis. The target fractions (polar substances) from 1D were enriched by a trapping column (ODS C18, 4.6 mm × 50 mm, 5 μm) and switched into the 2D through a six-port valve. A Hypersil ODS-2 C18 column (4.6 × 150 mm, 5 μm) was used to completely separate the seven food additives in 2D. The mobile phase consisted of ACN/water/TFA (10/90/0.1, v/v/v) (solvent A) and ACN/water/TFA (90/10/0.1, v/v/v) (solvent B) for 1D and ammonium acetate (25 mM, pH 6.6) (solvent A) and ACN/water (50/50, v/v, pH 7.2) with 25 mM ammonium acetate (solvent B) for 2D. The column temperature was 40°C. The detection wavelengths for 1D was 214 nm, for 2D were 254 nm and 278 nm. The eluted program is shown in **Figure 6**. The injection volume was 5 μL, and each sample or standard was injected in triplicate.



^{1}D		^{2}D		Valve	Comments
Time (min)	B (%)	Time (min)	B (%)		
0-2	25	0-2	0	0	Waiting for elution of the seven additives
2-5	25	2-5	0	1	Switching the seven additives into trap column
5-6	25	5-12	0	0	Simultaneously separation of five proteins on ^{1}D column and seven additives on ^{2}D column
6-20	33-35	12-18	50-100	0	
20-25	35-40	18-25	100	0	
25-30	40	/	/	0	

Figure 6.
Schematic representation (a–c) and gradient, flow rates, and switching times (d and e) of the stop-flow heart-cutting 2DLC system.

3.3 Optimization of chromatographic conditions

In order to accurately quantify the five proteins and seven additives in milk powder samples using the 2DLC method, some important parameters were optimized, including the stationary phase, mobile phase, and switching time.

According to the literature, a shorter switching time in the 2DLC system means better shape of the peaks in the 2D chromatogram [15]. Because the milk powder matrix is so complex, the ideal 1D column would be able to separate the proteins and additives into two groups. The additives with higher polarity were concentrated within a short period of time and eluted rapidly, while the proteins were separated completely after the elution of additives by adjusting the mobile phase. In order to achieve this goal, two columns were tested: a Venusil XBP-C4 column (4.6 mm × 100 mm, 5 μm) and a Venusil XBP-C8 column (4.6 mm × 100 mm, 5 μm). These two types of columns could separate the seven additives and five proteins as two groups. The seven additives were concentrated at 2.0–5.0 min on the C4 column and 2.0–6.0 min on the C8 column. Therefore, the Venusil XBP-C4 column was chosen as the 1D column because of the shorter switching time. **Figure 7A** shows the chromatogram of the seven additives and five major proteins. The seven additives were focused at the first minutes, and the five major proteins could be separated later. For 2D separation, Hypersil ODS-2 C18 column showed better separation performance for the seven additives. A trapping column was used as the interface between 1D and 2D, which should result in better enrichment of the targets [16]. For online 2DLC, the choice of the mobile phase is very important. Because of protein separation, ACN was chosen as the organic mobile phase. 0.1% v/v TFA was added to all mobile phases to improve the protein separation effect.

The mobile phases for 1D were A1, ACN/water (10/90 v/v, 0.1% TFA), and B1, ACN/water (90/10 v/v, 0.1% TFA). Solvent B1 was set at 25% from 0 to 5 min in order to elute the additives quickly. Due to the little polarity difference of proteins, a gentle gradient of 0.14% B min^{-1} was used to achieve good separation of the five proteins, which was consistent with the literature [17–19]. As shown in **Figure 7**, the proteins were eluted in the following order: α_{s2}-CN, α_{s1}-CN, α-Lac, β-CN, β-LgB, and β-LgA. It should be noted that there were no standards for α_{s1}-CN and α_{s2}-CN

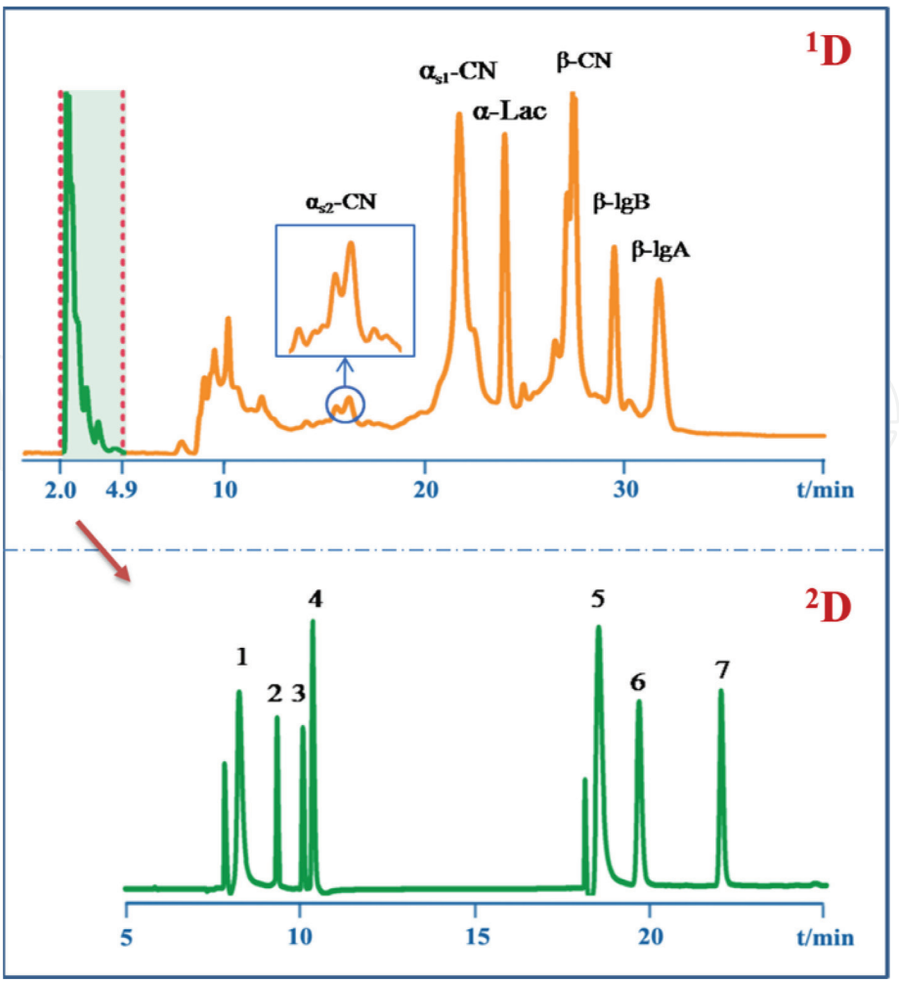


Figure 7.
The 2DLC chromatogram of the 12 mixed standards substances. The 1D chromatogram of seven additives (2.0–5.0 min) and five proteins on the 1D C4 column (4.6 mm × 100 mm, 5 μm) (up) and the 2D chromatogram of the seven additives on the C18 analytical column (down). (1) Maltol, (2) saccharin sodium, (3) benzoic acid, (4) sorbic acid, (5) ethyl maltol, (6) ethyl vanillin, and (7) vanillin.

proteins, only for their mixture [10]. The chromatographic profiles showed no carryover effects of these proteins. A shoulder for the α_{s1}-CN standard can be seen due to the presence of its two variants (α_{s1}-CN and α_{s2}-CN), which are very difficult to separate completely. From the different findings from previous reports, the monomorphic α-Lac was eluted firstly than β-CN [17–19]. The three shoulders of β-CN corresponded to its variants. As previously reported, γ-CN is the proteolytic product of β-CN, so they could be eluted together [18]. For β-Lg, variant B eluted before variant A, which is consistent with the literature [18]. During the process of quantitative analysis, α_{s1}-CN and α_{s2}-CN were quantified together, as for the three variants of β-CN.

Acetic ammonia is often used as the modifier in liquid chromatography separation. To obtain better separation of the seven additives in 2D, a series of acetic ammonia concentrations (15, 20, 25, 30 mM) were tested. When 25 mM acetic ammonia was added, the baseline was much more stable, and the peak shape was greatly improved. Therefore, the 2D mobile phase were as follows: A2, 25 mM acetic ammonia, and B2 ACN/water (50/50 v/v) with 25 mM acetic ammonia. The gradient program is shown in **Figure 6**. The initial mobile phase of 1D was optimized and set at 25% B1. If lower than 25% B1, elution of the seven additives would be taken too long in the 1D column, which could lead to sample loss in the trapping column before switching; if higher than 25% B1, maltol and saccharin sodium could be separated incompletely in 2D because of ACN in the trapping column.

The switching time is a key parameter in this method. Three switching time (2.0–4.5, 2.0–5.0, and 2.0–5.5 min) were tested. When the switching time was between 2.0 and 4.5 min, maltol and saccharin sodium were separated incompletely, and the sorbic acid peak was less sharp than that for 2.0–5.0 min; when between 2.0 and 5.5 min, some analytes were lost in the trapping column. Therefore, 2.0–5.0 min was chosen as the final switching time for the experiment. **Figure 7** shows the chromatogram of the 12 mixed standard substances using the optimized 2DLC method. In **Figure 7A**, the seven additives were eluted between 2.0 and 5.0 min due to their higher polarity, and the proteins were separated on the 1D column (8.0–30.0 min); **Figure 7B** shows the 2D chromatogram of the seven additives that were switched from the 1D column at 2.0–5.0 min. The whole analysis process was less than 30 min, which provide a highly efficient analysis method.

3.4 Comparison of analysis parameters for 1DLC and 2DLC

The matrix effect, linearity, LOD, intra- and interday precision, and accuracy were validated under the optimized conditions for 1DLC and 2DLC.

The method validation parameters of 1DLC and 2DLC were shown in **Table 4**. The correlation coefficient values (R^2) for both methods are higher than 0.9988 for all the additives. And R^2 of ethyl maltol in 1DLC is lower than that in 2DLC.

Considering the complexity of milk powder, the possibility of a matrix effect was investigated by comparing the slope ratio of the calibration curves for the seven additives obtained in the presence and absence of blank milk powder [20]. For example, the slope ratio is closer to 1.0, which means a lower matrix effect in the method. The results in **Table 4** show that 2DLC (slope ratio: 0.94–1.09) had a lower matrix effect than 1DLC (slope ratio: 0.84–1.21). The sample matrix effect for the determination of the seven additives for both 1DLC and 2DLC can be seen in **Figure 8**. The milk powder sample matrix chromatogram of 2DLC (b′) is much clean and flat than that in 1DLC (a′), and there has no interference peak for the analytes. Although the matrix effect of 2DLC is low, we still chose the matrix-matched standard curve for the sample analysis [19]. The LOD values of the 2DLC method were higher than that of the 1DLC method, as the peak width obtained with the new method is broader than that with the conventional method. Those are the advantages and disadvantages of these two methods.

Table 5 shows the precision and recovery results of 1DLC and 2DLC. The intra-day and interday data showed that the precision of the two methods is satisfactory. However, the recovery of the 2DLC method (89.6–103.5%) was much better than that for the 1DLC method (65.5–99.2%), which is mainly benefit from the "one-step" sample preparation method. Analytes may be lost during the processes of traditional sample pretreatment (such as solid-phase extraction, liquid–liquid extraction, and precipitation). In this method, the whole analysis time was less than 1 h. So, 2DLC is much more efficient than 1DLC. Overall considering the environmental protection and time saving, the automation offered by 2DLC possesses more advantages.

3.5 Commercial sample analysis

Four different commercial milk and milk powder samples purchased from local supermarkets were analyzed using the developed 2DLC method. The chromatograms are shown in **Figure 9**. **Figure 9A** and **B** were infant formula milk powder (IFMP), **Figure 9C** was skimmed milk powder, and **Figure 9D** was fresh bovine milk. Benzoic acid and ethyl vanillin were detected only in the IFMP 1 sample. α-CN, β-CN, and α-Lac were detected in the four milk products. β-LgB and β-LgA were detected in the IFMP 2 and SMP samples.

Analytes	Methods	Sample matrix	Regression equation[a] y = ax ± b	R^2	Slope ratio (matrix/blank)	Linear range (μg mL^{-1})	LOD (μg mL^{-1})	LOQ (μg mL^{-1})
MAL	1DLC	Blank	y = 34636x − 34306	0.9996	1.21	0.28–28	0.051	0.21
		Matrix	y = 41933x − 77788	0.9995	/	1.0–100	0.41	1.02
	2DLC	Blank	y = 33548x + 367540	0.9991	1.09	0.28–28	0.065	0.187
		Matrix	y = 36410x + 354210	0.9990	/	0.28–28	0.105	0.25
Saccharin sodium	1DLC	Blank	y = 24706x + 43056	0.9998	0.97	0.22–22	0.026	0.10
		Matrix	y = 23945x + 96114	0.9998	/	0.5–50	0.067	0.21
	2DLC	Blank	y = 4328x + 7786	0.9991	0.94	0.22–22	0.044	0.11
		Matrix	y = 4086x + 10206	0.9991	/	0.22–22	0.074	0.19
Benzoic acid	1DLC	Blank	y = 31513x − 244	1.0000	1.08	0.25–25	0.018	0.051
		Matrix	y = 34007x − 3087	1.0000	/	0.5–50	0.18	0.42
	2DLC	Blank	y = 4497x + 318	0.9999	1.06	0.25–25	0.09	0.25
		Matrix	y = 4757x − 485	0.9999	/	0.3–30	0.10	0.28
Sorbic acid	1DLC	Blank	y = 147925x − 1384	1.0000	0.89	0.2–20	0.01	0.025
		Matrix	y = 131050x − 3021	1.0000	/	0.5–50	0.056	0.136
	2DLC	Blank	y = 57114x + 186559	0.9992	0.96	0.2–15	0.054	0.133
		Matrix	y = 54623x + 228303	0.9990	/	0.25–18	0.075	0.20
EMA	1DLC	Blank	y = 34036x − 197820	0.9977	1.04	2.5–50	0.14	0.42
		Matrix	y = 35360x − 87395	0.9985	/	5–100	1.14	3.33
	2DLC	Blank	y = 82117x + 167643	0.9996	1.07	0.5–50	0.165	0.50
		Matrix	y = 87714x + 47480	0.9995	/	1–100	0.18	0.56

Analytes	Methods	Sample matrix	Regression equation[a] y = ax ± b	R^2	Slope ratio (matrix/blank)	Linear range (μg mL^{-1})	LOD (μg mL^{-1})	LOQ (μg mL^{-1})
EVA	1DLC	Blank	y = 45172x + 459	1.0000	0.90	0.2–20	0.017	0.055
		Matrix	y = 40683x + 521	1.0000	/	0.5–50	0.043	0.15
	2DLC	Blank	y = 42319x + 4981	0.9996	1.01	0.2–20	0.016	0.051
		Matrix	y = 42898x − 1620	0.9995	/	0.1–10	0.019	0.056
VAN	1DLC	Blank	y = 65925x + 54232	0.9999	0.84	0.2–20	0.015	0.048
		Matrix	y = 55386x + 47261	0.9998	/	0.5–50	0.039	0.12
	2DLC	Blank	y = 45492x + 4220	0.9994	1.06	0.2–20	0.018	0.049
		Matrix	y = 48056x – 1552	0.9997	/	0.1–10	0.018	0.054
α-CN	1DLC	Blank	y = 1596833x + 58163	0.9984	/	100–5000	50	92.7
α-Lac	1DLC	Blank	y = 4334341x – 13906	0.9997	/	10–500	3.0	9.9
β-CN	1DLC	Blank	y = 2982340x + 12008	1.0000	/	16–780	4.0	10.2
β-LgB	1DLC	Blank	y = 707669x + 1006	0.9997	/	15–750	5.1	18.4
β-LgA	1DLC	Blank	y = 2393184x + 4246	1.0000	/	15–750	3.8	12.4

[a]y is the average peak area of each additive (n = 3), and x is the mass concentration of the additive in mg mL^{-1}.

Table 4.
Method validation parameters of 1DLC and 2DLC (n = 3).

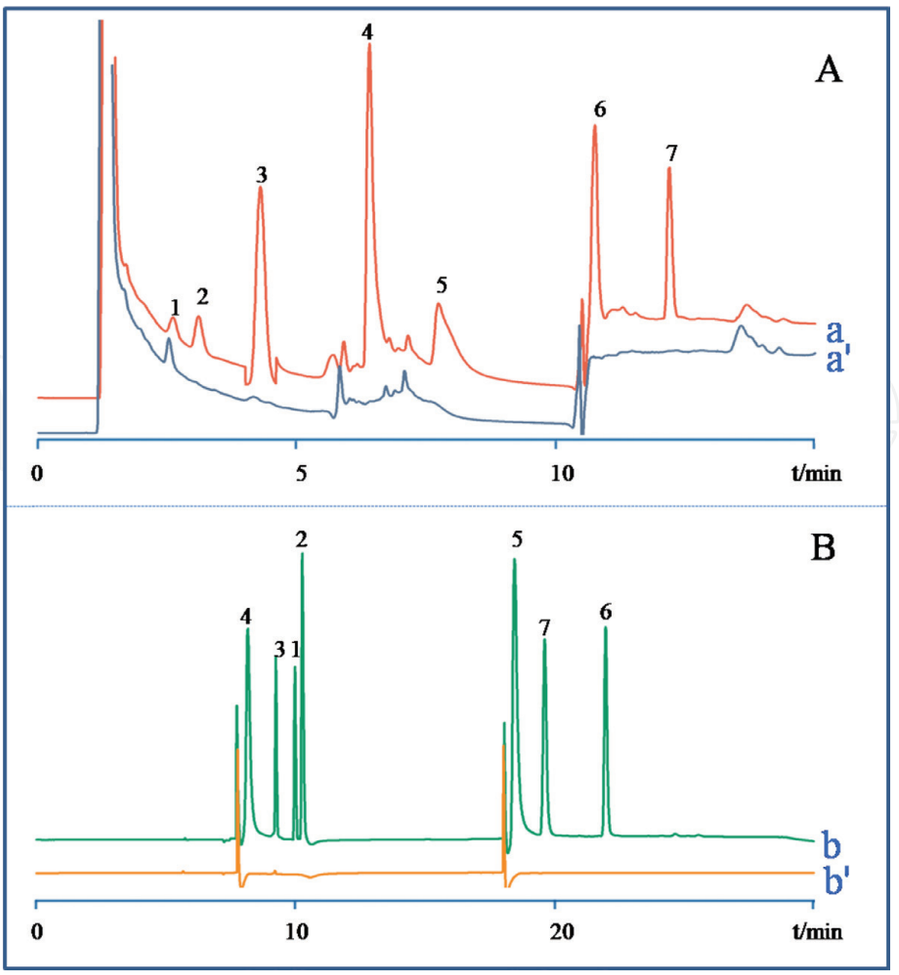


Figure 8.
1DLC (A) and 2DLC (B) chromatograms for testing sample matrix effect. (a' and b') Sample matrix without standard substances. (a and b) Sample matrix with standard substances. Chromatographic peaks: (1) benzoic acid, (2) sorbic acid, (3) saccharin sodium, (4) maltol, (5) ethyl maltol, (6) vanillin, and (7) ethyl vanillin.

	Concentration ($\mu g\ mL^{-1}$)		Precision				Spiked (ng)		Recovery			
			Intraday RSD		Interday RSD				Recovery (%)		RSD	
	1D	2D	1D	2D	1D	2D	1D	2D	1D	2D	1D	2D
Maltol	1.25	0.5	2.93	2.86	3.85	2.93	3	1.4	73.6	92.1	0.55	3.66
	3	6.25	0.85	0.19	0.43	2.17	9	7	72.6	97.5	2.07	1.68
	18.75	18.75	0.72	2.70	1.19	2.73	45	21	72.1	103.5	1.14	3.92
Saccharin sodium	1	0.4	0.12	1.44	1.13	1.43	0.7	1.1	67.5	93.4	2.75	2.36
	2.4	5	0.77	0.58	2.04	3.28	5	5.5	70.1	93.9	0.33	2.84
	15	15	0.42	1.93	0.77	2.11	22.5	16.5	78.4	91.2	0.80	1.78
Benzoic acid	1	0.5	1.03	1.99	1.18	3.29	0.7	1.5	65.5	90.6	3.42	3.09
	2.4	6.25	0.11	1.88	0.46	4.96	4	7.5	71.0	95.2	0.42	0.34
	15	18.75	0.13	2.53	0.40	1.87	20	22.5	79.1	91.1	0.36	0.34
Sorbic acid	1	0.4	0.06	2.25	1.54	5.44	1.25	1.25	71.2	100.6	0.08	1.09
	2.4	5	0.19	0.90	0.25	3.74	5	6.25	72.2	102.8	0.16	0.79
	15	15	0.21	2.22	0.20	4.82	22.5	18.75	75.7	94.5	0.18	2.65

	Concentration ($\mu g\ mL^{-1}$)		Precision				Spiked (ng)		Recovery			
			Intraday RSD		Interday RSD				Recovery (%)		RSD	
	1D	2D	1D	2D	1D	2D	1D	2D	1D	2D	1D	2D
Ethyl maltol	2.5	2	3.72	0.90	3.48	0.73	9	5	71.6	105.4	4.00	0.58
	6	25	0.76	0.98	4.80	2.90	22.5	25	70.1	98.2	0.85	2.26
	37.5	75	0.16	1.28	1.89	2.98	45	75	80.2	96.5	0.87	1.25
Ethyl vanillin	1	0.4	0.41	0.38	2.65	1.52	2	0.51	82.8	92.1	0.05	3.36
	2.4	5	0.12	0.83	1.05	4.31	4	2.55	84.5	92.6	0.17	3.92
	15	15	0.04	1.00	0.29	1.84	20	7.65	79.3	98.0	0.09	0.55
Vanillin	1	0.4	1.81	2.24	1.82	1.68	2.5	0.49	81.7	92.6	1.36	1.14
	2.4	5	0.70	0.33	1.21	0.84	7	2.45	86.5	93.7	0.69	2.66
	15	15	0.69	0.11	1.29	0.47	22.5	7.35	85.5	98.4	1.46	3.00
α-CN	200	/	4.55	/	4.28	/	506	/	86.6	/	3.07	/
	1200	/	0.46	/	4.14	/	1210	/	99.2	/	1.12	/
	5000	/	0.15	/	6.45	/	4050	/	94.7	/	3.73	/
α-Lac	20	/	1.99	/	2.48	/	49	/	87.7	/	3.07	/
	120	/	0.51	/	3.39	/	120	/	86.4	/	1.49	/
	500	/	0.33	/	4.11	/	395	/	88.3	/	2.75	/
β-CN	30	/	3.70	/	3.71	/	78	/	105.2	/	1.66	/
	180	/	0.42	/	2.68	/	188	/	94.0	/	2.28	/
	780	/	0.41	/	1.12	/	392	/	101.7	/	1.93	/
β-LgB	30	/	2.57	/	4.60	/	73.5	/	101.1	/	2.22	/
	180	/	1.55	/	3.24	/	176.4	/	99.2	/	3.24	/
	750	/	0.67	/	3.08	/	588	/	83.5	/	2.78	/
β-LgA	30	/	2.93	/	3.10	/	52	/	99.4	/	3.39	/
	180	/	1.06	/	1.14	/	125	/	98.2	/	1.99	/
	750	/	0.03	/	0.86	/	600		91.7	/	4.84	/

Table 5.
Accuracy of the two methods (n = 6).

Table 6 showed the contents of the five major proteins and the seven additives. The contents of α-CN and β-CN were much higher than that of α-Lac, β-LgB, and β-LgA in all the milk products. The contents of α-Lac, β-LgB, and β-LgA were lower in the infant formula milk powder than that in the skimmed milk powder. The results are consistent with those from the literature [17], which is probably due to the denaturation of the thermosensitive whey proteins [21] or intentional removal of β-LgB to prevent allergic reactions [22].

In order to evaluate the accuracy of protein determination using the proposed method in this work, four brands of commercial milk products were analyzed using both the 2DLC and Kjeldahl methods. **Table 7** shows the total major protein contents in the various milk matrices determined by these methods, 2DLC, the Kjeldahl method, and TPC, as given by the manufacturers. The RSD of the three groups were less than 3%, which means that the milk protein contents were similar for our method and the Kjeldahl method as well as that given by the manufacturers.

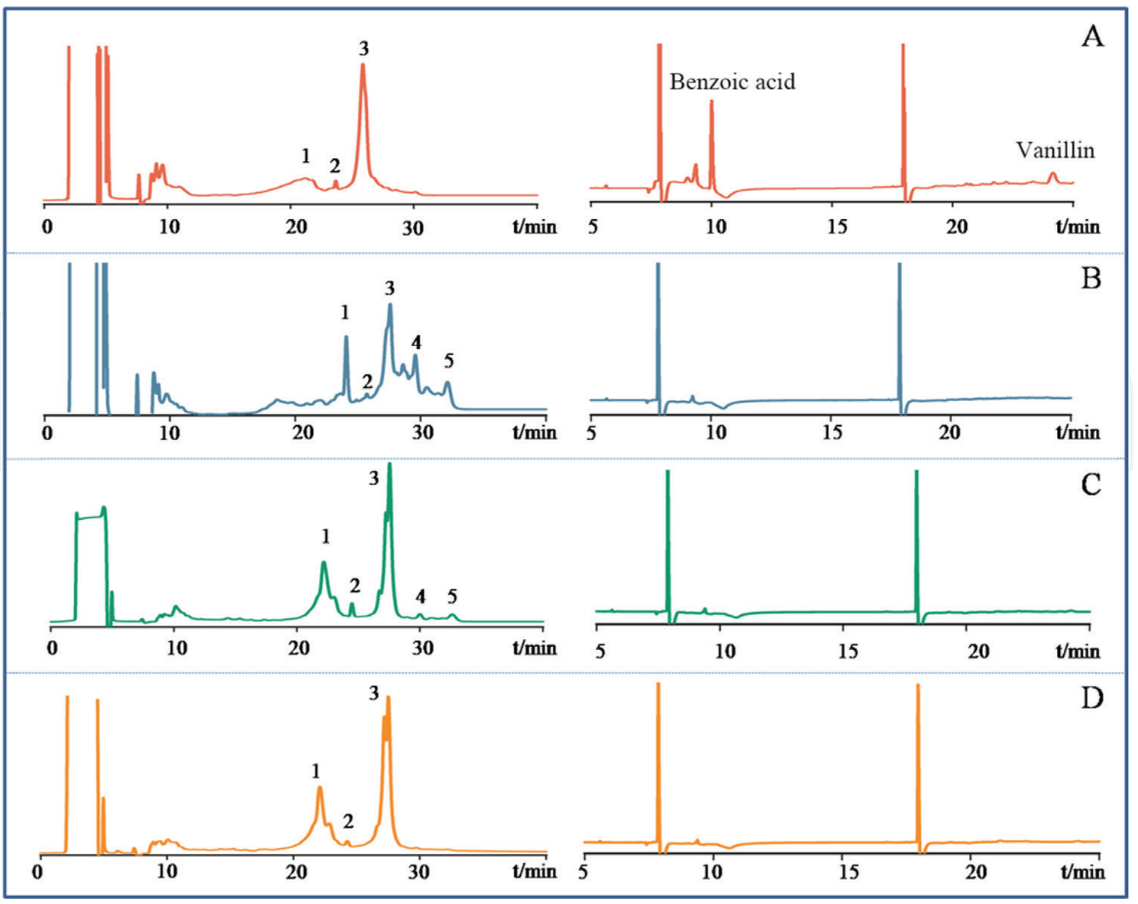


Figure 9.
Chromatograms of four brands of commercial milk and milk powders. (A) Infant formula milk powder 1, (B) infant formula milk powder 2, (C) skimmed milk powder, and (D) bovine milk. (1) α-Casein (α-CN), (2) α-lactalbumin (α-Lac), (3) β-casein (β-CN), (4) β-lactoglobulin B (β-LgB), and (5) β-lactoglobulin A (β-LgA).

Sample (μg g^{-1})	IFPM[a]1	IFPM2	SMP[a]	BM[a]
α-CN	43.11 ± 0.45[c]	67.59 ± 0.60	225.41 ± 3.00	22.65 ± 0.30
α-Lac	0.87 ± 0.02	2.48 ± 0.05	4.47 ± 0.14	0.28 ± 0.01
β-CN	56.38 ± 0.52	27.91 ± 0.32	98.59 ± 1.89	9.00 ± 0.11
β-LgB	ND[b]	4.97 ± 0.28	5.52 ± 0.19	ND
β-LgA	ND	3.75 ± 0.12	6.19 ± 0.16	ND
MAL	ND	ND	ND	ND
Saccharin sodium	ND	ND	ND	ND
Benzoic acid	1553.00 ± 0.04	ND	ND	ND
Sorbic acid	ND	ND	ND	ND
EMA	ND	ND	ND	ND
EVA	ND	ND	ND	ND
VAN	20.51 ± 0.24	ND	ND	ND

[a]*IFPM, infant formula powder milk; SMP, skimmed milk powder; BM, bovine milk.*
[b]*ND, not detected.*
[c]*The values of the concentration are means ± SD (n = 3).*

Table 6.
Contents of food additives determined in milk powder samples by 2DLC (n = 3).

	TMPC[a] with 2DLC	Kjeldahl method	TPC[b] indicated by manufacturers	RSD[d]
IFMP 1	10.04 ± 0.10[c e]	9.77 ± 0.09	10.4	0.03
IFMP 2	10.67 ± 0.14	10.83 ± 0.07	11.4	0.03
SMP	32.85 ± 0.54	34.19 ± 0.35	33.0	0.02
Milk	3.19 ± 0.04	2.96 ± 0.08	3.1	0.03

[a]*TMPC, the total major protein concentrations.*
[b]*TPC, the total protein concentration.*
[c]*Powder milks in g/100 g and liquid milks in g/100 ml.*
[d]*RSD among the data determined by the two methods and indicated by manufacturers.*
[e]*The values of the concentration are means ± SD (n = 3).*

Table 7.
Comparison between the total major protein concentrations (TMPC) in the various milks determined with 2DLC method and the total protein concentration (TPC) determined with Kjeldahl method and TPC given by the manufacturers.

4. Conclusions

In this chapter, two kinds of analysis methods for common additives are introduced. One is HPLC, and the other is 2DLC. Poly-Sery HLB cartridge was confirmed as the most appropriate material for HPLC because of the higher recovery. As to 2DLC, the sample preparation method is much easier, time-saving, and efficient, and this method possesses much higher recovery of food additives by avoiding the sample loss; and the analysis process was performed on an automated instrument within 30 mins. Therefore, it is simpler, faster, and more accurate than current standard methods.

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Conflict of interest

Sicen Wang on behalf of other authors declares that all authors of this article have no conflict of interest. This article does not contain any studies with human or animal subjects.

Abbreviations

HPLC	high-performance liquid chromatography
RPLC	reversed-performance liquid chromatography
2DLC	two-dimensional liquid chromatography

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