FOOD COMPOSITION AND ADDITIVES

Determination of Bovine Lactoferrin in Food by HPLC with a Heparin Affinity Column for Sample Preparation

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An HPLC method was developed for the quantitative determination of bovine lactoferrin (bLF) in sterilized milk, modified milk, fermented milk, infant formula, adult formula, rice cereal, vitamin function drink, and protein powder products. bLF was first extracted with a phosphate buffer (pH 8), underwent cleanup in a heparin affinity column, and was detected by HPLC with a C_4 column and diode-array detector at a wavelength of 280 nm. The proposed method provided a linear detection range of 10.0–1000 µg/mL with an LOD of 0.6 mg/100 g in liquid samples and 3 mg/100 g in solid samples and an LOQ of 2 mg/100 g in liquid samples and 10 mg/100 g in solid samples. In addition, the method showed good recovery for various samples, ranging from 76 to 96%. The method had several remarkable advantages, including ease of handling, high sensitivity and accuracy, good reproducibility, and low-cost detection. Based on the distinctive properties presented here, we believe the proposed HPLC assay holds great promise for the oversight and detection of bLF in testing organizations, dairy enterprises, and regulatory authorities.

Bovine lactoferrin (bLF) is a natural protein that is widely found in cow's milk. It is an iron-binding glycoprotein with a single polypeptide chain of 689 amino acids and molecular weight of 77 kDa. Recent studies have provided increasing evidence that bLF not only has physiological functions as an antimicrobial and/or antiviral, immunomodulatory agent, and antioxidant, but can also pharmacologically suppress tumor metastasis (1–7). The European Union has approved bLF as a new type of food ingredient (8), whereas Chinese governmental authorities have specified that the bLF concentration limit in dairy products should be 1.0 g/kg and that bLF dosage in infant formula is 1.0 g/L (9, 10). However, at present, there is no standard method for the determination of bLF in dairy or other food products. Various methods have been developed for bLF determination, including ELISA (11), sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE), capillary electrophoresis (CE; 12, 13), HPLC (14–16), and LC-MS (17). ELISA is widely used because of its good sensitivity and universality, however, it has poor reproducibility and detection is expensive. SDS-PAGE requires laborious sample pretreatment, sophisticated and time-consuming procedures, and large amounts of sample, and is not very stabile or sensitive. CE is widely used in protein analysis, however, it is limited by capillary, voltage, and other conditions, which may not be sensitive enough for the separation of proteins with small differences in molecular weight. In addition, CE has poor reproducibility for the high-throughput analysis of bLF. LC-MS is highly sensitive and selective, but cannot be widely applied for the routine detection of bLF in product quality control (QC) because the costs of detection are prohibitive. In our previous patented method for bLF detection in dairy products based on an HPLC method (18), our experimental results demonstrated that the proposed HPLC method was not robust as a standard method for the daily QC testing of bLF. With authorization from the Ministry of Health and Family Planning Commission of China, we further improved the HPLC method to develop a simple, universal, and robust method for the accurate determination of bLF in various products during QC, governmental quality supervision, import–export product testing, and risk assessment. The major challenge in developing this method was the separation, and accurate testing, of a low-concentration target protein from other dominant proteins in dairy or other food products. In this study, we used a heparin affinity column to efficiently enrich bLF from a complex sample matrix, used a C_4 column for the analytical column, and optimized detection conditions for the HPLC method. The proposed method was successfully applied for the quantitative analysis of bLF in eight products: sterilized milk, modified milk, fermented milk, infant formula, infant rice cereal, adult formula, protein powder, and vitamin function drink samples.

Experimental

Reagents and Materials

bLF was donated by Tatua Co-operative Dairy Co. (Batch No. SP113101). Cellulose membrane (0.45 µm) was

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purchased from ANPEL (ANPEL Scientific Instrument, Shanghai, China). All chemical reagents were AR grade and purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). HPLC grade acetonitrile was purchased from Merck (Darmstadt, Germany). All mobile phase and standard solutions were prepared in water treated with a Nanopure water system (Thermo Fisher Scientific Co., Ltd, Dubuque, IA). A HiTrap Heparin HP column (1 mL) was purchased from GE Healthcare Life Sciences (Uppsala, Sweden). Solutions were as follows: buffer A was composed of 0.2 mol/L Na_2HPO_4 (pH 8) and buffer B of 0.05 mol/L Na_2HPO_4 and 1.0 mol/L NaCl (pH 8).

Instrumentation

All experiments were carried out on an Agilent HPLC 1200 series instrument (Agilent Technologies, Waldbronn, Germany) equipped with a diode-array detector (DAD) and ChemStation Software. A C_4 analytical column (250 × 4.6 mm, 5 µm) was purchased from ANPEL and a C_{18} guard column (4.6 × 50 mm, 5 µm) was purchased from Waters (Waters Technologies, Ireland). The experimental centrifuge used was an MR23i purchased from Thermo Fisher (Thermo Fisher Scientific, Nantes, France).

Methods

(a) *Sample extraction.*—(*1*) *Liquid sample.*—Liquid sample (5 g) was transferred into a 50 mL centrifuge tube. Buffer A solution (5 mL) was added and the contents mixed on a vortex mixer for 0.5 min and centrifuged for 10 min at 10 000 rpm at 4°C. The supernatant was transferred to a new 50 mL centrifuge tube. Buffer A solution (5 mL) was added to the first 50 mL centrifuge tube and the above process repeated to collect supernatant. The two supernatants were then merged to become the sample extraction solution.

(*2*) *Solid sample.*—Accurately weighed solid sample (1 g) was transferred into a 50 mL centrifuge tube. Buffer A solution (10 mL) was added and the content mixed on a vortex mixer for 0.5 min, followed by the same procedures described above for the liquid sample.

(b) *Sample cleanup.*—The heparin affinity column was rinsed with 5 mL buffer A solution at a rate of 1.0 mL/min. The column was flushed out with sample extraction solution, washed with 10 mL buffer A solution, and then eluted with 3.0 mL buffer B solution. The effluent was passed through a 0.45 µm cellulose membrane prior to injection into the HPLC system. Preparation of the bLF standard solution for quantitative analysis should follow the same procedures noted above for the heparin affinity column.

(c) *LC conditions.*—A C_4 column was used as the analytical column, coupled with a C_{18} guard column. The column temperature was set to 30°C, the DAD was set at 280 nm, and the injection volume was set at 50 µL, with a flow rate of 1.0 mL/min. Line A in the mobile phase was pure CH_3CN, whereas line B was 0.1% (v/v) trifluoride acetic acid in water. Gradients were set as shown in Table 1. The LOD was calculated as 3× the SD of the noise level. All raw data were processed with ChemStation Software.

Table 1. Optimization of gradient elution

Time, min	Line A, %	Line B, %	Flow rate, mL/min
0	30	70	1.0
5	55	45	1.0
10	60	40	1.0
12	30	70	1.0
16	30	70	1.0

Results and Discussion

Optimization of Chromatographic Conditions

A detection wavelength of 280 nm was selected based on the maximum absorption of bLF. In our patented HPLC method (18), C_{18} was used as the analytical column. Our experimental results showed that the baseline was unstable and retention time (RT) of bLf changed after 20 injections. Because these problems may have resulted from a high salt concentration in the sample solution, we tried using the C_4 column as the analytical column instead of the C_{18} column to potentially provide more stability and higher separation efficiency. As shown in Figure 1, the gradient elution was optimized to achieve the best peak shape and minimum amount of interference from the sample matrix.

Optimization of Sample Pretreatment

The food sample matrix was very complex, containing various chemicals and biomaterials, such as protein, carbohydrates, vitamins, minerals, and additional supplementary materials. To accurately determine the amount of bLF in the food samples, an appropriate sample pretreatment method had to be established to eliminate any interference from the sample matrix and to preconcentrate the bLF analyte.

Affinity chromatography, based on the reaction between the target protein molecule and the ligand on the column, is an effective protein purification method. The target protein is retained on the column as other proteins are eluted. Heparin is an acidic, negative-charged polysaccharide with a functional group of sulfate, which has been used to purify bLF by affinity chromatography (19–22). Thus, we used a HiTrap Heparin column to clean up the sample matrix as shown in the Figure 2.

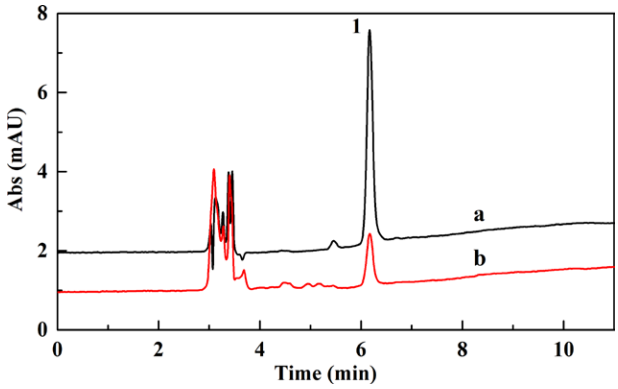

Figure 1. HPLC chromatograms of bLF. (a) Standard chromatography and (b) infant formula sample with cleanup. Peaks 1 is bLF. For conditions, *see Experimental* section.

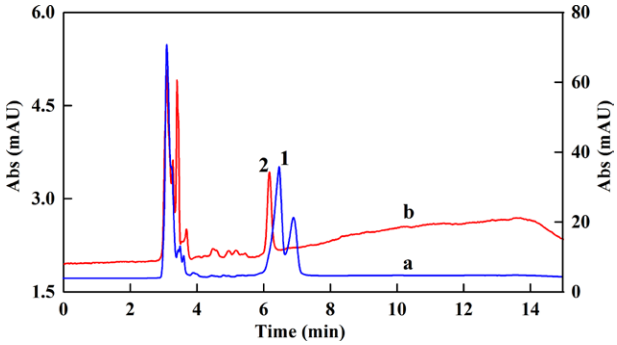

Figure 2. HPLC Chromatogram of bLF with and without affinity column cleanup. (a) Infant formula sample without cleanup and (b) infant formula sample with cleanup. (Peak 1) bLF with interference and (peak 2) bLF. The RT for peaks 1 and 2 was 6.1 min. Chromatogram a (without cleanup) corresponds to the *y*-axis on the right and chromatogram b (with cleanup) to the *y*-axis on the left. For conditions, *see Experimental* section.

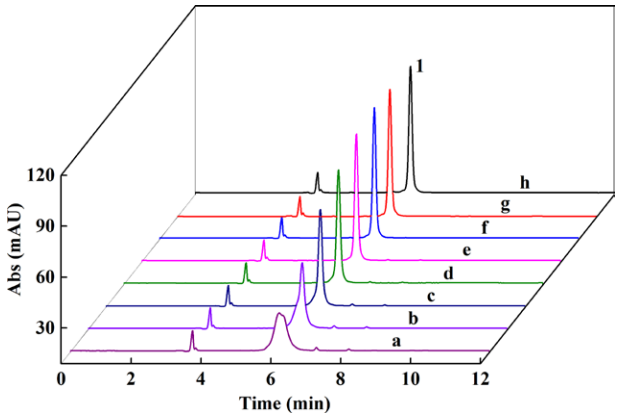

Figure 4. Chromatogram of bLF in water baths at different temperatures for 1 h: (a) 90°C, (b) 80°C, (c) 70°C, (d) 60°C, (e) 50°C, (f) 40°C, (g) 30°C, and (h) 20°C. Peak 1 is bLF at an RT of 6.1 min. For conditions, *see Experimental* section.

The infant formula sample matrix could be effectively removed after treatment of the HiTrap Heparin affinity column.

Solvable proteins can usually be extracted with water or ingredient-specific buffer. Buffer concentration, pH, and environmental temperature are important factors to consider in the extraction efficiency of a solvable protein. It should be noted that the biological activity of bLF should be maintained during sample pretreatment. In this study, spiked bLF in powdered milk was extracted with water and different concentrations of phosphate buffer. As shown in Figure 3, 200 mM phosphate buffer almost fully extracted bLF from milk powder, with a recovery of 96.0%. The extraction efficiencies of the phosphate buffer at different pH (5–9) were almost the same. In this study, pH 8 was selected for subsequent experiments.

As a protein, bLF is denatured at high temperature. It has been reported that denaturation of bLF begins at 40°C, becomes rapidly denatured at 70°C, and then 95% bLF is denatured at 85°C (23). Although higher temperatures would improve extraction efficiency, at high temperatures, protein denaturation significantly changes bLF chromatography and results in deviations in the test results. In this study, 1 mL of 200 µg/mL standard solution in Eppendorf PCR tubes was extracted at different temperatures—20, 30, 40, 50, 60, 70, 80, and 90°C—and then injected directly into the HPLC system. As shown in the Figure 4, higher temperatures resulted in the denaturation of bLF, and the chromatographic characteristics of bLF were significantly changed. In addition, the denatured bLF could not be retained on the HiTrap Heparin column and, thus, could not be detected during HPLC. Therefore, for convenience, room temperature was selected as the optimum temperature.

Finally, ion strength and elution volume were optimized to elute the target analyte from the affinity column. As shown in Figure 5, a higher concentration of NaCl in the buffer would improve elution efficiency, therefore, 1.0 M NaCl would almost fully elute bLF in milk powder. The elution curve in Figure 6 shows that 3.0 mL effluent was enough to elute bLF from a 1 mL affinity column.

Analytical Parameters and Validation

Specificity.—Specificity was investigated by comparing the chromatograms of eight different kinds of samples spiked with bLF or bLF standard. As shown in Figure 7, the bLF standard and spiked samples exhibited sharp and symmetric peaks at the

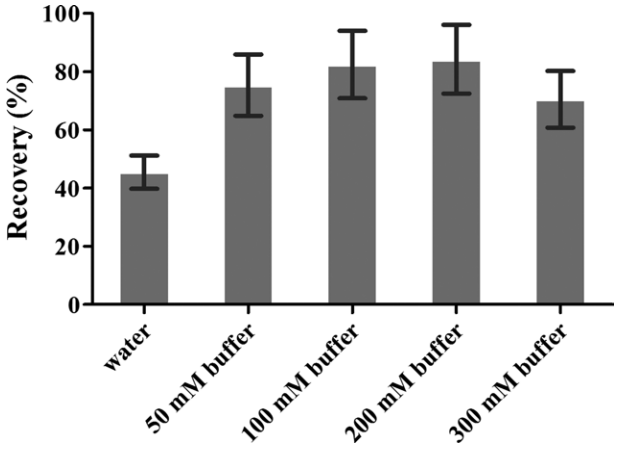

Figure 3. Recovery of bLF by applying different sample extraction solutions. Error bars were calculated from six independent experiments.

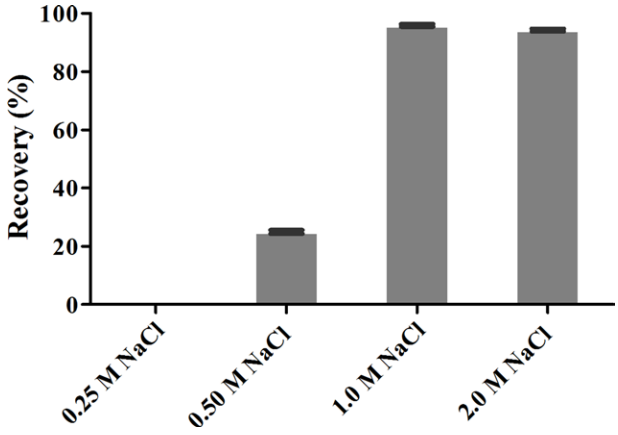

Figure 5. Recovery of bLF by applying different concentrations of NaCl. Error bars were calculated from six independent experiments.

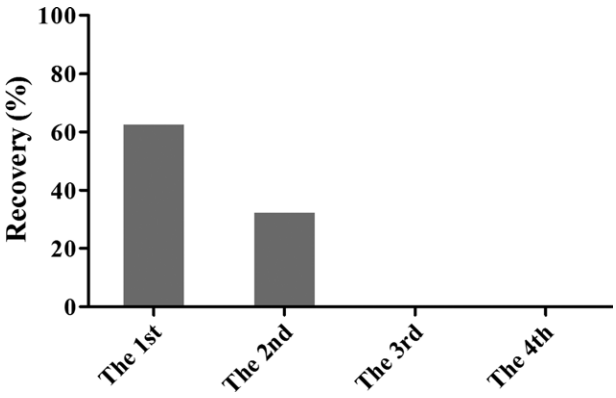

Figure 6. Elution volume optimization The total volume is 4 mL. The 1st is the first 1mL, the 2nd is the second 1 mL, the 3rd is the third 1 mL, the 4th is the forth 1mL.

same RT. In a sample without bLF, no peak was observed at the specific RT. These results indicated that the bLF analyte was obtained specifically from the pretreated sample.

Linearity.—Linearity was demonstrated by running standards over a range of 10.0–1000 µg/mL. Linearity was checked at regular intervals. The linear correlation coefficient (r) for all standard curves had to be ≥0.999.

Accuracy.—Accuracy of the method was determined by overspike recoveries at approximately 100% of the fortified levels or at levels within the analytical range of the method if the product did not contain inherent levels of or had not been fortified with analytes. The products were spiked with bLF for 3 days (Table 2). On each day, spiked solutions containing bLF were added to ready-to-feed or reconstituted powders and mixed well. Each spiked product was prepared and analyzed in duplicate.

Precision.—Precision was determined as intermediate precision, which is defined as the agreement between the test results obtained using the same method on identical test material in the same laboratory by the same operators using the same equipment over a period of several days. It was expressed in percentage RSD (Table 3).

LOD and LOQ.—The LOD and LOQ were determined by calculating the concentrations corresponding to 3 and 10 times the S/N, respectively. The LOD was 0.6 mg/100 g in liquid samples and 3 mg/100 g in solid samples, whereas the LOQ was 2 mg/100 g in liquid samples and 10 mg/100 g in solid samples.

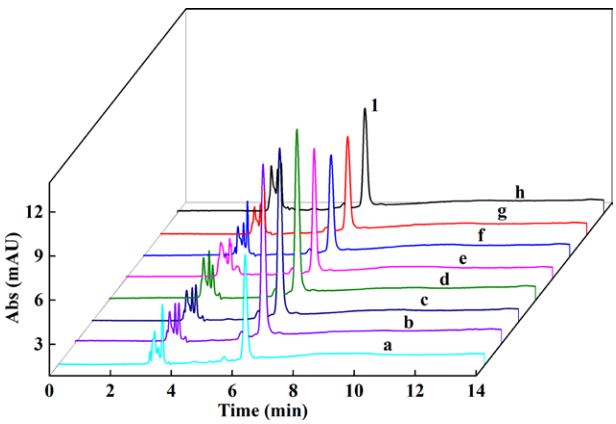

Figure 7. HPLC chromatogram of bLF in the eight different matrixes: (a) sterilized milk, (b) modified milk, (c) fermented milk, (d) vitamin function drink, (e) infant formula, (f) infant rice cereal, (g) adult formula, and (h) protein powder. Peak 1 is bLF at an RT of 6.2 min. For conditions, *see Experimental* section.

Interlaboratory Validation Test

Five laboratories in China joined in the interlaboratory validation of this study, including Shanghai Municipal Supervisory Institute of Veterinary Drugs and Feedstuff (Shanghai, China); Shanghai Municipal Center for Disease Control and Prevention (Shanghai, China); Shanghai Entry-Exit Inspection and Quarantine Bureau (Shanghai, China); Technology Center Bright Dairy and Food Co., Ltd (Shanghai, China); and Institute for Agri-Food Standards and Testing Technology (Shanghai, China). Three samples were delivered to each laboratory, and the same experimental procedure was followed. The results are listed in Table 4.

Table 2. Recovery test results in the eight different kinds of samples (*n* = 5)

Matrix	Spiking levels, mg/100 g	Mean recovery, %	RSD, %
Sterilized milk	2	82	9.1
	5	87	4.1
	10	88	2.4
	20	91	2.9
Modified milk	2	85	7.7
	5	86	3.0
	10	90	4.3
	20	90	4.5
Fermented milk	2	83	9.7
	5	86	5.6
	10	85	6.6
	20	89	4.3
Vitamin function drink	2	90	2.0
	5	96	1.2
	10	96	2.8
	20	95	1.7
Infant formula	10	88	6.2
	30	92	1.7
	50	90	3.5
	100	94	4.1
Infant rice cereal	10	79	8.9
	30	81	6.0
	50	86	4.5
	100	90	3.1
Adult formula	10	81	9.6
	30	84	8.7
	50	86	4.4
	100	87	3.9
Protein powder	10	94	1.6
	30	91	2.5
	50	94	1.8
	100	95	2.6

Table 3. Results of the spike test in the eight different kinds of samples (*n* = 12)

Matrix	Spiking levels, mg/100g	Mean recovery, %	RSD, %
Sterilized milk	10	89	3.6
Modified milk	10	92	5.4
Infant formula	10	88	4.8
Vitamin function drink	10	96	2.1
Protein powder	10	93	3.3
Adult formula	10	82	7.7
Infant rice cereal	10	80	6.7
Fermented milk	10	84	6.1

Table 5. Comparison of the proposed HPLC method and the LC-MS/MS method

Matrix No.	Mix process technology	HPLC method mg/100 g	LC-MS/MS method mg/100 g	Amount added mg/100 g
1	Dry-mix	37.3	61.7	30–50
2	Dry-mix	36.8	56.1	30–50
3	Dry-mix	420	507	400–500
4	Dry-mix	490	491	400–500
5	Wet-mix	6.82	184	200
6	Wet-mix	7.57	200	200
7	Wet-mix	10.0	230	200
8	Wet-mix	7.23	246	200

Comparison of LC and LC-Tandem MS (MS/MS) Methods

As shown in Table 5, there was a substantial deviation between the proposed HPLC and LC-MS/MS methods (17). To test for bLF in infant formula products that were produced in a dry-mix process (24), the testing results of the proposed HPLC method fell into the added-bLF range, whereas higher results were achieved with the LC-MS/MS method. However, the proposed method had very low results when infant formula products were produced with a wet-mix process. During a wet-mix process, bLF is exposed to high temperatures and, thus, the majority of bLF would be denatured in the final product, subsequently failing to be retained on the affinity column and thereby not detectable. Because the LC-MS/MS method was based on a signature peptide and multiple reaction monitoring mode, the method could not distinguish natural bLF from denatured bLF, therefore, denatured bLF was included in the final results. The LC method provided accurate natural bLF results, whereas the LC-MS/MS results included both natural and denatured bLF.

Conclusions

In this study, an accurate and robust HPLC method was developed for the determination of bLF in eight different kinds of products. The key points in this method were sample cleanup with a heparin affinity column and the optimization of extraction and eluent conditions. bLF was effectively extracted from samples with 200 mM phosphate buffer at pH 8.0, retained by the heparin affinity column, and eluted with a phosphate buffer and 1.0 M NaCl. All analytical parameters of this method complied with regulatory requirements (25). It should be pointed out that once bLF was thermally denatured, its chromatographic characteristics were significantly changed and, thus, bLF could not be retained by the heparin affinity column. This method can accurately detect natural bLF because thermally denatured bLF is washed out as sample matrix.

Table 4. Comparison of real samples in the five laboratories (*n* = 3)

	Matrix					
	Infant formula 1		Infant formula 2		Modified milk	
Lab No.	Detected content, mg/100 g	RSD, %	Detected content, mg/100 g	RSD, %	Detected content, mg/100 g	RSD, %
1	30.9	2.5	31.8	1.7	5.3	2.9
2	30.9	2.6	32.1	2.1	5.4	3.7
3	30.6	3.7	32.5	2.2	5.2	3.8
4	31.7	2.7	32.5	2.2	5.2	3.8
5	31.5	1.8	32.3	2.1	5.4	3.2

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