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Quantification of lactoferrin in dietary supplements using a Heparin affinity clean-up column by HPLC PDA detector

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Abstract

The use of dietary supplements containing lactoferrin is a growing trend. As a result, there is a need for a simple and reliable analytical method to determine the content of lactoferrin in these supplements to ensure food quality. In this study, an HPLC method was developed and validated for determining lactoferrin in dietary supplements using a HiTrap Heparin clean-up column in combination with HPLC. The study involved selecting optimal conditions for extracting lactoferrin from the sample matrix, followed by chromatography conditions using a C4 column (150 mm × 4.6 mm, 3.5 µm). The limit of detection (LOD) and limit of quantification (LOQ) were determined, with the achieved values being 1.2 mg/100g and 4.0 mg/100g, respectively. The method has shown good recovery (99.5–103.4%) and precision (RSD 1.76–3.44%), meeting the method performance requirements outlined in AOAC SMPR 2020.005. The validated method was applied to determine the contents of lactoferrin in dietary supplement samples.

Keywords: *Lactoferrin, dietary supplement, HPLC, affinity column, clean-up, Heparin.*

1. INTRODUCTION

Lactoferrin is a glycoprotein with iron-binding properties belonging to the transferrin family. Its structure comprises a single polypeptide chain consisting of approximately 600 - 700 amino acids, with a molecular weight of approximately 80 kDa [1]. Owing to its ability to sequester iron, lactoferrin plays crucial roles in the immune system, including anti-inflammatory and antibacterial functions, inhibition of tumor proliferation and metastasis, intestinal iron absorption, and anti-inflammatory, antiviral, and antibacterial activities [2, 3].

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The concentration of lactoferrin in milk undergoes significant variations during lactation and across species. Among these, breast milk and bovine milk are particularly rich sources of lactoferrin. Despite sharing structural similarities of up to 70%, the lactoferrin content in breast milk surpasses that found in bovine milk [4, 5]. Consequently, there is a growing interest in supplementing lactoferrin in nutritional products, especially in the form of milk-based nutritional supplements designed for infants and young children.

For the purpose of product evaluation and quality control, the development of a lactoferrin analysis method is important and necessary. Currently, several methods have been established using various techniques, such as enzyme-linked immunosorbent assay (ELISA) [6], surface plasmon resonance (SPR) based immune-sensors [7], capillary electrophoresis (CE) [8], liquid chromatography-tandem mass spectrometry (LC-MS/MS) [9], and reversed-phase high-performance liquid chromatography (RP-HPLC) [10, 11]. Although ELISA exhibits high selectivity and sensitivity, it suffers from low reproducibility. Moreover, result accuracy depends on the quality of antibodies and antigens, leading to potential false-negative or lower-than-expected results. CE is widely used for protein analysis but is influenced by conditions such as conductivity and voltage. LC-MS/MS, with high sensitivity, relies on identifying characteristic peptides originating from the tryptic hydrolysis process. However, this method demands advanced technical expertise and incurs significant costs associated with equipment, making it challenging for widespread application in laboratories in Vietnam. Meanwhile, RP-HPLC can be employed for the determination of natural lactoferrin. However, the complex composition of supplementary food matrices, including other whey proteins like β-lactoglobulin, α-lactalbumin, and serum albumin, may impact the method's sensitivity if the lactoferrin content in the sample is low. Due to the high affinity between lactoferrin and heparin [12, 13], the HiTrap Heparin column was selected for the purification and enrichment of lactoferrin in this study.

Heparin, a sulfated glycosaminoglycan extracted from natural proteoglycans, comprises units of uronic acid and D-glucosamine. Heparin possesses a high negative charge density and exhibits a strong affinity for lactoferrin. Conversely, modified whey proteins can be precipitated by adjusting the sample pH to 4.6 and subsequently separated from the sample matrix through centrifugation [12]. Therefore, this study investigates sample processing conditions and the purification of lactoferrin using a Heparin column, followed by high-performance liquid chromatography (HPLC) analysis.

2. MATERIALS AND METHOD

2.1. Chemicals and reagents

2.1.1. Chemicals

Lactoferrin standard (97 %) with lot number: SLCH5940 was provided by Sigma-Aldrich, USA. Stock standard solution (1000 µg/mL) was used to prepare working standard solutions. The concentration of the working standard solution ranges from 4–400 µg/mL.

The chemicals used in the study include sodium chloride (NaCl), disodium hydrophosphate (Na_2HPO_4), trifluoroacetic acid (TFA), acetonitrile (ACN) from Merck, with high-performance liquid chromatography (HPLC) purity, and purified water for chromatography.

Blank samples, selected for research and to confirm the applicability of the method, consists of powdered and liquid milk supplements intended for infants aged 0 - 36 months, without lactoferrin and colostrum.

Spike samples involve adding 1.0 mL of a standard solution with a concentration of 400 µg/mL to 5.0 g of the test sample (equivalent to 16.0 mg/100g) for examination.

Real samples include 10 powdered and 10 liquid milk-based nutritional supplement (TPBS) samples purchased in Hanoi.

2.1.2. Instrumental analysis

The high-performance liquid chromatography (HPLC) system Alliance - Waters e2695 using a 2998 PDA detector (Waters, USA). Some other equipment includes HiTrap Heparin 1 mL affinity column (Cytiva); analytical balance (with accuracy to 0.0001 g) XS105 (Metler Toledo); pH meter (Metler Toledo); reciprocating shaker (IKA); ultrasonic bath (Elma); centrifuge (Hermle); XBridge Protein BEH C4 column (150 mm × 4.6 mm, 3.5 µm) from Waters.

2.2. Method

2.2.1. Sample processing method

The subject of the study is nutritional supplements derived from milk, which typically have complex matrices containing significant amounts of casein, proteins, sugars, as well as various other nutrients, while lactoferrin is present in low concentrations. Therefore, the sample processing procedure needs to purify impurities and eliminate some co-eluting proteins during chromatography [15].

Several investigations have been conducted to optimize the analysis process and enhance the efficiency of lactoferrin extraction from the nutritional supplement matrix, including (1) investigation of sample extraction conditions, such as solvent selection, lipid removal conditions, extraction frequency, and extraction technique; (2) examination of sample treatment through the Heparin column, involving solvent selection and sample loading, as well as solvent and elution volume.

The anticipated sample processing procedure and the Heparin column cleaning process are illustrated in Figure 1. The method was evaluated on blank powdered samples and validated for both powdered and liquid sample matrices.

2.2.2. Analysis method

The research employed an HPLC method with a PDA detector, and optimal conditions were investigated, including the solvent program. Based on references [13, 14] and the available laboratory conditions, the Waters XBridge Protein BEH C4 chromatographic column (150 mm × 4.6 mm, 3.5 µm) with a mobile phase consisting of two channels A: 0.1% TFA in water, B: 0.1% TFA in ACN was utilized for lactoferrin determination. Detection and quantification were performed at a wavelength of 280 nm.

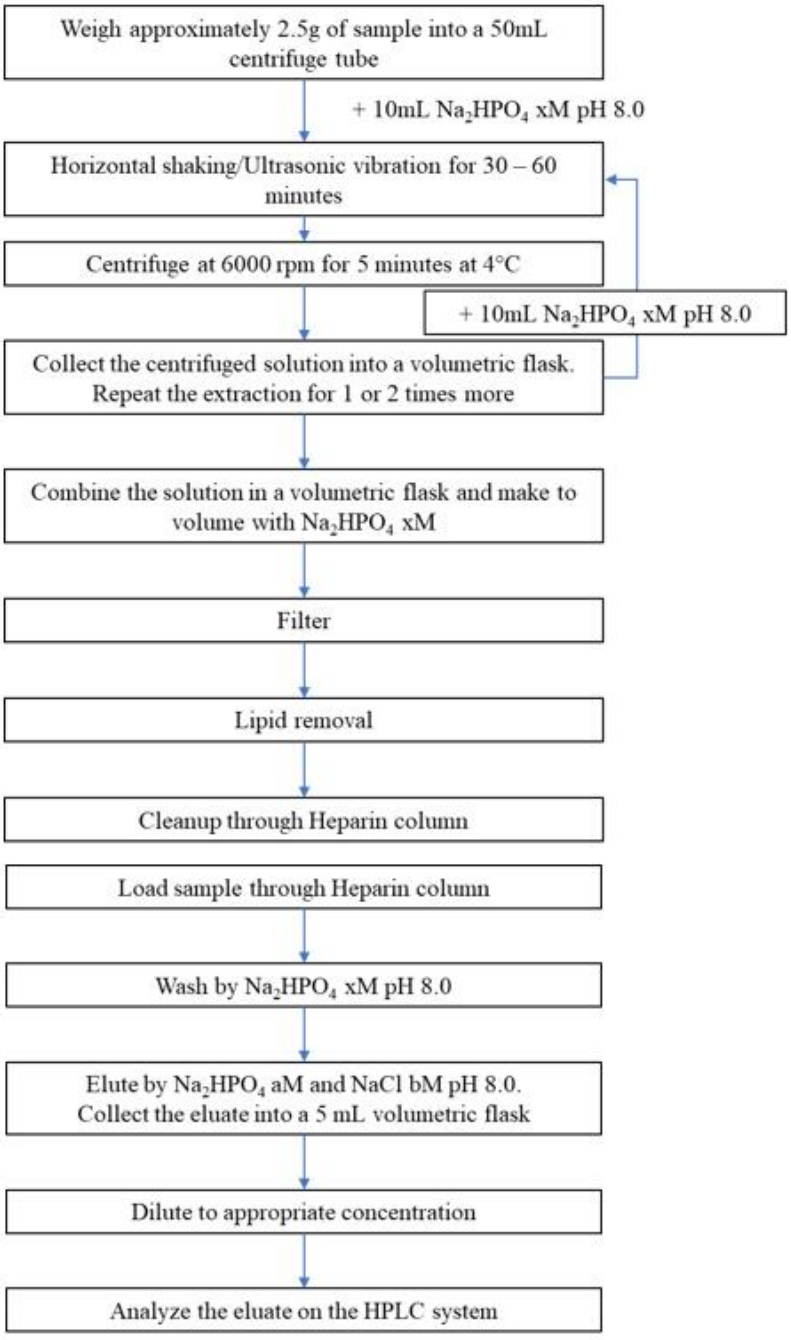

Figure 1. *Proposed sample preparation*

3. RESULTS AND DISCUSSION

3.1. Optimizing of chromatography conditions

Based on references [13, 14] and a preliminary examination in the laboratory, it was observed that the C4 chromatographic column demonstrated better separation efficiency and stability compared to the C18 column. This could be attributed to the influence of high salt concentrations in the sample solution. A solvent program investigation was conducted by analyzing a lactoferrin standard solution with a concentration of 40 µg/mL. The elution

regime was studied with a mobile phase consisting of channel A: 0.1% TFA in water and channel B: 0.1% TFA in ACN, maintaining constant flow rates at the following ratios:

Program 1: 60% A – 40% B

Program 2: 62% A – 38% B

Program 3: 65% A – 35% B

The results of the analysis are presented in Figure 2.

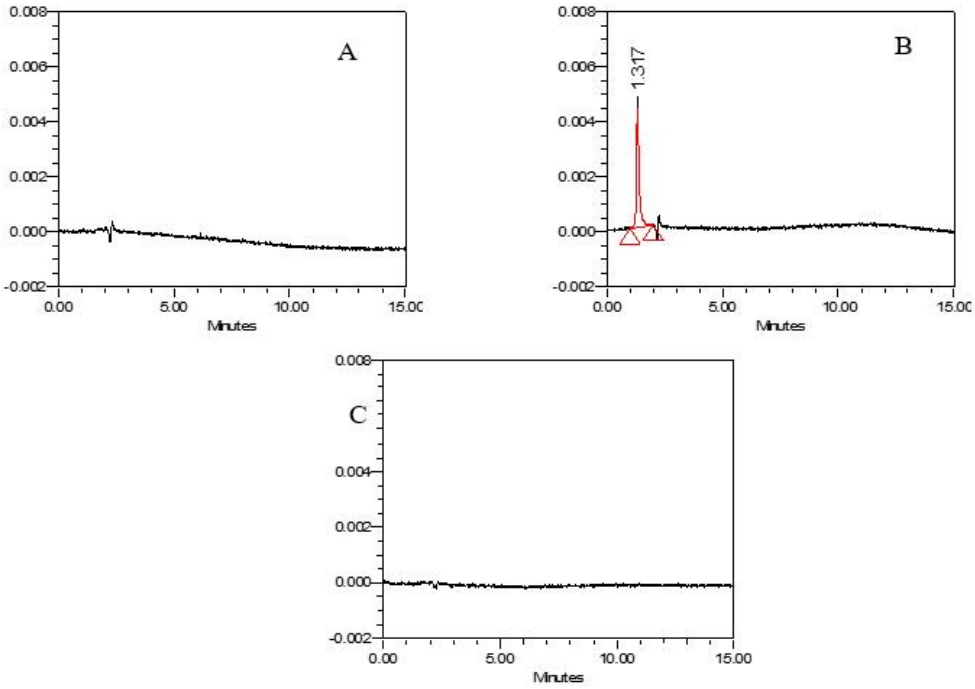

Figure 2. *Chromatogram of solvent program investigation: A – Program 1, B - Program 2, C - Program 3*

From the chromatographic analysis, it is evident that lactoferrin is eluted from the separation column only when the solvent channels achieve a ratio of 62:38. However, the short retention time (1.317 minutes) may be influenced by solvent peaks or other impurities. The chromatogram is presented in Figure 3.

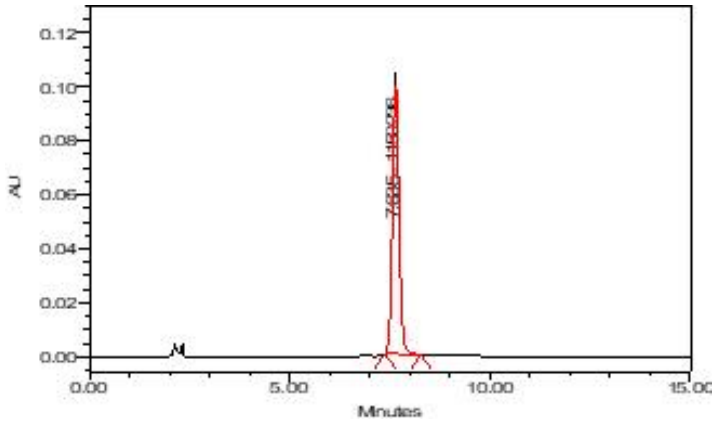

Figure 3. *Chromatogram of lactoferrin analysis according to selected solvent program*

The chromatogram results in Figure 3 indicate that the selected gradient program provides an appropriate retention time and balanced peak shape. This is suitable for application in sample analysis. The gradient elution program consists of 0.1% TFA in water (A) and 0.1% TFA/ACN (B): 0 → 2.0 minutes: 30% B; 2.0 → 5.0 minutes: 30 → 40% B; 5.0 → 7.0 minutes: 40% B; 7.0 → 8.0 minutes: 40 → 30% B; 8.0 → 15.0 minutes: 30% B. The flow rate is set at 1.0 mL/minute. The injection volume is 50 µL.

3.2. Sample Processing Conditions Investigation

3.2.1. Sample processing using Heparin Column

In the study, the use of a Heparin column facilitates sample purification and enrichment, saving sample processing time and simplifying the sample processing. The results in Figure 4 show the chromatogram of lactoferrin in milk without passing through the Heparin column, indicating a sample with numerous impurities, an unstable baseline, and a risk of column fouling. Lactoferrin in milk after passing through the Heparin column no longer contains impurities, and the baseline is stable. Therefore, the research opted for sample processing through the Heparin column.

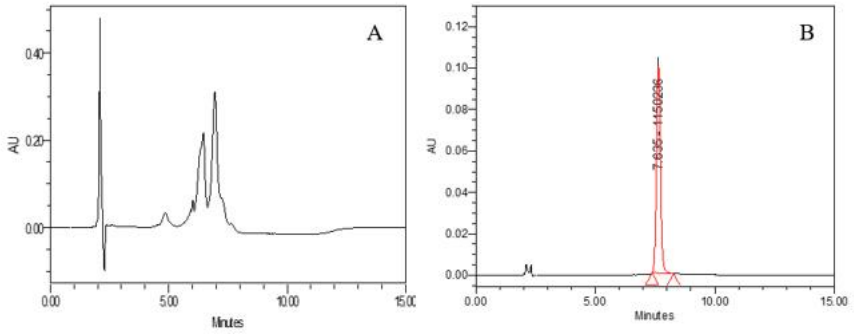

Figure 4. *Results of sample processing conditions investigation: A-not using Heparin column; B-using Heparin column*

3.2.2. Investigation of extraction solvents

Following the anticipated sample processing procedure (Figure 1), the sample is extracted using an appropriate solvent and loaded through the Heparin column. The solvents investigated include: (1) H_2O; and phosphate buffer solutions with pH 8.0 and concentrations of Na_2HPO_4 (2) 0.05 M; (3) 0.1 M; (4) 0.2 M; (5) 0.3 M. The results are depicted in Figure 5.

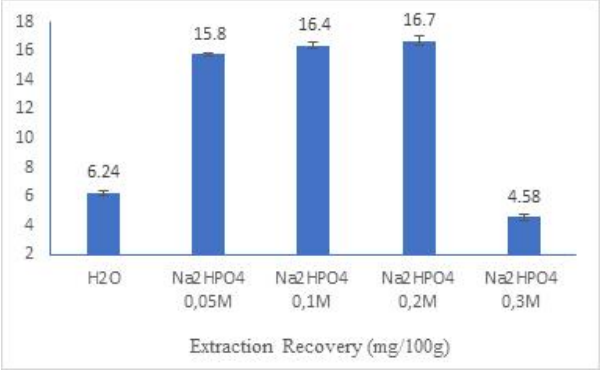

Figure 5. *Results of the Investigation and Selection of Extraction Solvents*

From the results in Figure 5, it is observed that the extraction efficiency is low when water is used as the sample extraction solvent. The investigation results for different phosphate concentrations show that a concentration of 0.2 M achieves the best extraction efficiency. As the phosphate buffer concentration increases, the lactoferrin content decreases significantly. This aligns with previous research findings, suggesting that the electrostatic interaction between lactoferrin and heparin may be influenced by ion concentration [14].

3.2.3. Investigation of Lipid Removal Conditions

To perform lipid removal, after being extracted three times and quantified using the chosen extraction solvent, the sample is divided into three portions and investigated under the following conditions:

(a): Centrifugation at 6000 rpm for 5 minutes;

(b): Storage at 2 - 4°C overnight, followed by centrifugation at 6000 rpm for 5 minutes;

(c): Storage at -2 - 4°C overnight, followed by centrifugation at 12000 rpm for 10 minutes.

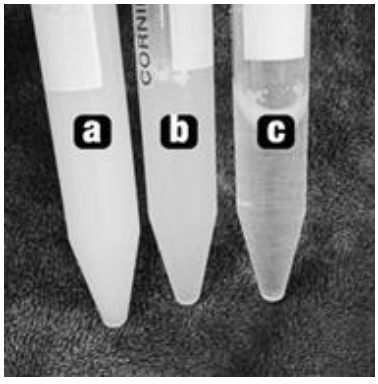

Figure 6. *Results of the Investigation on the Lipid Removal Process*

As the results from Figure 6, the obtained solution is clear, with no visible lipid portion after refrigeration and centrifugation at 12000 rpm for 10 minutes. Therefore, the lipid component is effectively removed by refrigeration and centrifugation at 12000 rpm for 10 minutes. Procedure (c) for the lipid removal is selected, and further studies are conducted accordingly.

3.2.4. Investigation of Elution Solvents

The concentration of NaCl in the elution solvent is a crucial parameter that can influence the elution process of lactoferrin from heparin. Research by Ounis et al. (2008) has demonstrated that different NaCl concentrations in the elution solvent yield different efficiencies. An investigation into the NaCl concentration in the elution solvent is conducted. The sample, after lipid removal, is eluted using 5 mL of buffer solution at pH 8.0 containing 0.05 M Na_2HPO_4 and NaCl with concentrations of (1) 0.25 M; (2) 0.5 M; (3) 1.0 M; (4) 2.0 M. The analysis results are presented in the chart in Figure 7.

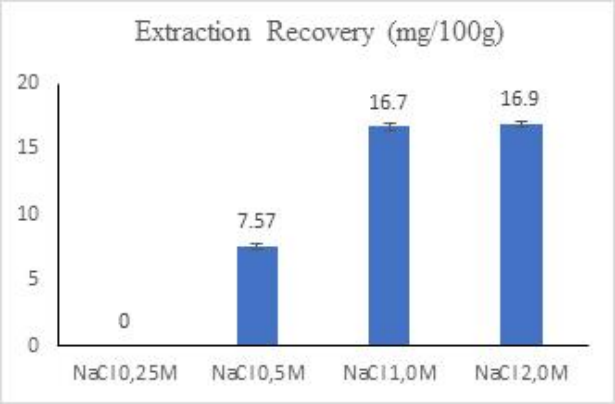

Figure 7. *Results of eluent solvent investigation*

The results from Figure 7 indicate that the phosphate buffer solution containing 1.0 M NaCl provides the best elution efficiency, and this efficiency decreases as the NaCl concentration increases. This phenomenon may be attributed to the reduced interaction between lactoferrin and heparin at higher salt concentrations [13], leading to decreased extraction efficiency. Therefore, the phosphate buffer solution with 0.05 M Na_2HPO_4 and 1.0 M NaCl at pH 8.0 is chosen for subsequent experiments.

3.2.5. Investigation of Other Sample Processing Conditions

The extraction method, number of extractions, and elution volume also impact the sample extraction efficiency (results not presented in the paper). An investigation is conducted on sample extraction using ultrasound and shaking techniques, with 1-2 extractions, each with 10 mL of solvent, and elution volumes ranging from 1 to 5 mL. The results indicate that the shaking technique with two extractions and a 5 mL elution volume demonstrates good recovery of lactoferrin. This aligns with some previous studies [12-14] as the ultrasonic technique may cause heating, leading to lactoferrin denaturation, affecting its interaction with Heparin and reducing extraction efficiency. Chromatographic analysis results show that the third extraction solution no longer exhibits lactoferrin signals.

After optimizing the conditions, the sample processing procedure is summarized as follows:

A. Sample Extraction: Weigh an accurate amount of approximately 5.0 g of the sample into a 50 mL centrifuge tube. Add 10 mL of 0.2 M Na_2HPO_4 pH 8.0 solution, and shake horizontally at 210 strokes/minute for 60 minutes. Centrifuge at 6000 rpm for 5 minutes at 4°C. Transfer the supernatant to a 25 mL volumetric flask. Repeat the extraction for the second aliquot similarly. Combine the extracts in the volumetric flask and bring them to volume with the extraction buffer. Filter the solution and clean through the Heparin column.

B. Sample Cleaning through Heparin Column:

- Load the sample through the Heparin column.
- Wash impurities with at least 10 mL of 0.2 M Na_2HPO_4 pH 8.0.

- Elute lactoferrin with a solution containing 0.05 M Na_2HPO_4 and 1 M NaCl pH 8.0 into a 5 mL volumetric flask.

- Rinse the column with at least 5 mL of 0.05 M Na_2HPO_4 and 2 M NaCl pH 8.0.

- Wash the column with at least 5 mL of purified water twice.

- Rinse and store the Heparin column with at least 5 mL of 20% ethanol following the manufacturer's instructions [16].

C. HPLC chromatographic Conditions:

- Analytical column: XBridge Protein BEH C4 (150 mm × 4.6 mm, 3.5 μm)
- Column temperature: 35°C
- Injection volume: 50 μL
- Flow rate: 1.0 mL/min
- Analysis time: 15 minutes
- Mobile phase program: Table 1

Table 1. *Lactoferrin analytical mobile phase program*

Time (minute)	***TFA 0.1%/H_2O (%)***	***TFA 0.1%/ ACN (%)***
0.0	70	30
2.0	70	30
5.0	60	40
7.0	60	40
8.0	70	30
15.0	70	30

3.3. Method Validation

3.3.1. Selectivity of the Method

Determine the selectivity of the method as follows:

- Standard sample: Use a lactoferrin standard solution with a concentration of 40 μg/mL.

- Blank solvent sample: Use a solution of 0.05 M Na_2HPO_4 and 1 M NaCl pH 8.0.

- Blank sample: Weigh 5.0 g of the powdered dietary supplement sample without lactoferrin. Perform the analysis according to the previously studied procedure.

- Spiked sample: Weigh 5.0 g of the blank sample. Add a lactoferrin standard solution with a concentration of 400 μg/mL. Perform the analysis according to the previously studied procedure.

The results are presented in Figure 8.

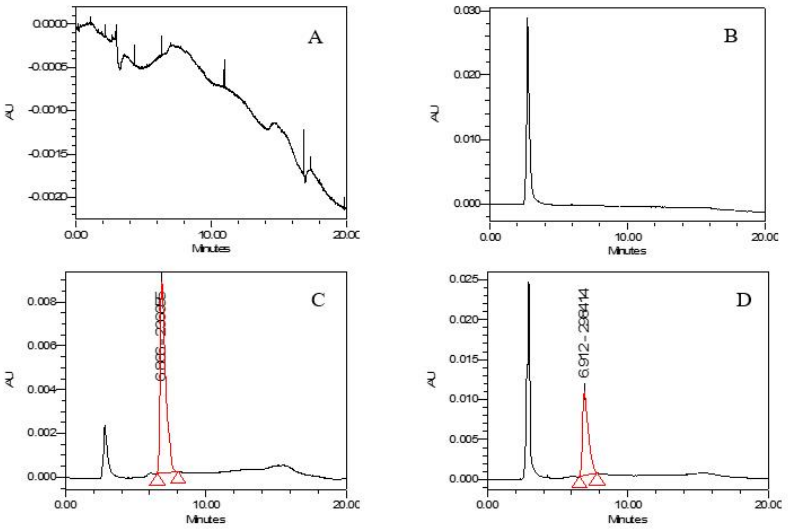

Figure 8. *Results of the method selectivity validation: A: blank solvent sample; B: blank powdered sample; C: standard solution; D: Spiked sample.*

The results from Figure 8 indicate that the chromatograms of the blank solvent sample and the blank sample do not show peaks at the retention time of lactoferrin. The chromatogram of the spiked sample exhibits a peak that matches the peak in the chromatogram of the standard sample. Therefore, the method investigated meets the requirements for selectivity.

3.3.2. Standard curve of lactoferrin

The standard solutions of lactoferrin were prepared with concentrations ranging from 4 to 400 µg/mL, comprising 7 standard points with concentrations of 4 µg/mL, 8 µg/mL, 16 µg/mL, 40 µg/mL, 80 µg/mL, 160 µg/mL, and 400 µg/mL. These solutions were analyzed using HPLC, and a calibration curve was constructed based on the peak area and the corresponding concentration of the analyte. Table 2 presents the results of the lactoferrin standard curve analysis.

Table 2. *Dependence of peak area on lactoferrin concentration*

Concentration (µg/mL)	***Area***	***$C_{calculated}$ (µg/mL)***	***Bias (%)***
400	1150236	400	-0.04
160	461014	160	0.14
80.0	231365	80.4	0.48
40.0	115689	40.2	0.41
16.0	44252	15.3	-4.20
8.00	23529	8.12	1.54
4.00	11485	3.94	-1.59

The equation of the standard curve is y = 2876,31*x + 163,23 with the linear correlation coefficient $R^2 = 1$. In the investigated concentration range, lactoferrin is linear in the range of 4 - 400 µg/mL, bias per standard point <15%. Therefore, the investigated method has a satisfactory linear range from 4 - 400 µg/mL.

3.3.3. Limit of detection, limit of quantification (LOD, LOQ)

According to SMPR 2020.005 [18], the quantification limit for the method should be 4 mg/100g. To achieve this, 100 µL of a lactoferrin standard solution with a concentration of 1000 µg/mL was added, and the analysis was conducted following the established procedure. The signal-to-noise ratio (S/N), calculated using the Empower software of the instrument, indicated that S/N was ≥10 for both sample backgrounds (37.6 for the powdered sample background and 35.9 for the liquid sample background) (Figure 9). Thus, the method exhibits sufficient sensitivity to determine the limit of quantification (LOQ) at 4.0 mg/100g, with an LOQ of 1.2 mg/100g for both sample backgrounds.

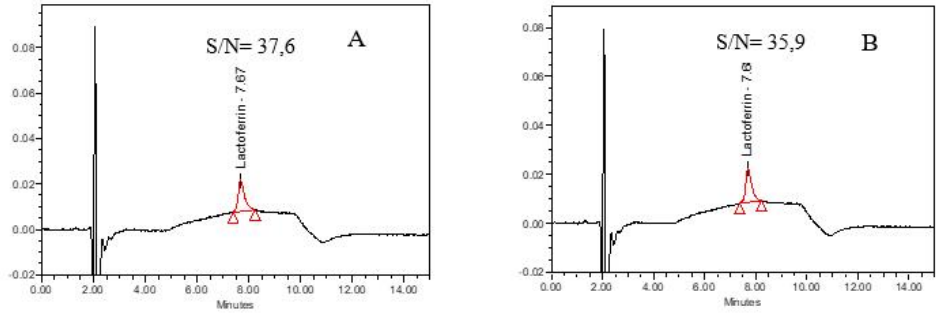

Figure 9. *Lactoferrin analytical chromatogram at LOQ level: A, powder sample matrix; B: liquid sample matrix*

3.3.3. Accuracy and precision

The accuracy and precision of the method were determined through recovery, repeatability, and internal reproducibility. The blank sample was spiked with lactoferrin standard at three concentration levels: 4, 20, and 100 mg/100g (equivalent to LOQ, 5*LOQ, 25*LOQ), and the analysis was carried out according to the established procedure. Each concentration level was repeated six times. Internal reproducibility was conducted similarly on different days. The validation results indicate that the method meets the requirements for accuracy and precision for both sample matrices (Table 3).

Table 3. *Repeatability and reproducibility of lactoferrin in powdered and liquid dietary supplement matrices*

Matrix	Content (mg/100 g)	Accuracy (% mean recovery)	Precision	
			Repeatability (RSD_r %)	Reproducibility (RSD_R %)
Powdered samples	4.0	99.5 – 103.4	3.44	2.96
	20	97.8 – 100.7	2.30	2.56
	100	97.2 – 102.7	1.98	2.39
Liquid samples	4.0	97.0 – 104.0	2.87	2.68
	20	97.4 – 102.7	2.29	2.62
	100	97.1 – 102.0	1.76	2.02

The results obtained from Table 3 demonstrate that the method complies with SMPR 2020.005 [18] criteria, with a recovery of 90–110%, repeatability below 6%, and internal reproducibility below 9%. The validation results indicate that the method meets the performance requirements for deployment and application in the analysis of real samples.

3.3. Application of real sample analysis

The results of the analysis of lactoferrin content in 20 samples of powdered and liquid dietary supplements purchased on the market are shown in Table 4.

Table 4. *Results of analysis of food supplement samples*

Matrix	*Code*	*Content (mg/100g)*	*Label product (mg/100g)*	*Matrix*	*Code*	*Content (mg/100mL)*	*Label product (mg/100mL)*
Powdered product	R01	8.33	10.0	**Liquid product**	L01	4.97	5.00
	R02	8.53	10.0		L02	4.04	5.00
	R03	10.1	10.0		L03	6.58	8.00
	R04	8.96	10.0		L04	5.94	5.00
	R05	15.9	15.0		L05	5.67	5.00
	R06	4.81	5.00		L06	6.72	8.00
	R07	45.2	50.0		L07	5.02	5.00
	R08	52.0	50.0		L08	5.90	5.00
	R09	9.61	10.0		L09	8.84	8.00
	R10	8.67	10.0		L10	5.94	5.00

From the results obtained in Table 4, among the 20 analyzed milk samples, the lowest lactoferrin content is 4.81 mg/100g for powdered product and 4.04 mg/100mL for liquid milk, while the highest content is 52.0 mg/100g for powdered product and 8.84 mg/100mL for liquid milk. All analysis results show differences from the labeled content of less than 20%. The method exhibits appropriate quantification limits and linear ranges for determining lactoferrin content in various dietary supplement samples currently available in the market.

The approach using the Heparin column for lactoferrin quantification shows potential in complex milk samples cleanup. However, the current research only extends to the analysis of dietary supplement samples. Additionally, there are various milk products such as yogurt, other lactoferrin-enriched supplements, etc., that need further exploration and refinement. In the future, the research team plans to continue developing this method for application to a broader range of sample matrices.

4. CONCLUSIONS

The study has developed and validated a method for quantifying lactoferrin in dietary supplements using a HiTrap Heparin cleanup column and HPLC analysis. The limit of detection, limit of quantification are determined to be 1.2 mg/100g and 4.0 mg/100g, respectively, for both powdered and liquid sample matrices. The method demonstrates good recovery (99.5 - 103.4%) and precision (RSD is 1.76-3.44%), meeting the performance requirements outlined in AOAC SMPR 2020.005. The method has been successfully applied for lactoferrin analysis at the National Institute for Food Control.

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Định lượng lactoferrin trong thực phẩm bổ sung bằng HPLC và làm sạch qua cột ái lực Heparin

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Tóm tắt

Thực phẩm bổ sung chứa lactoferrin đang có xu hướng ngày càng tăng. Do đó, một phương pháp phân tích đơn giản, đáng tin cậy để xác định lactoferrin đã được xây dựng, giúp kiểm soát chất lượng thực phẩm. Trong nghiên cứu này, phương pháp xác định lactoferrin trong thực phẩm bổ sung sử dụng cột làm sạch HiTrap Heparin kết hợp với HPLC đã được phát triển và thẩm định. Nghiên cứu đã lựa chọn các điều kiện tối ưu để chiết lactoferrin ra khỏi nền mẫu, sau đó tách sắc ký trên cột C4 (150 mm × 4,6 mm, 3,5 µm). Giới hạn phát hiện và giới hạn định lượng được tiến hành trên mẫu thực có hàm lượng thấp, giá trị LOD và LOQ đạt được lần lượt là 1,2 mg/100g và 4,0 mg/100g. Phương pháp có độ thu hồi (99,5– 03,4%) và độ chụm tốt (RSD 1,76–3,44 %) đáp ứng yêu cầu về hiệu năng phương pháp theo AOAC SMPR 2020.005. Phương pháp sau khi thẩm định đã được áp dụng để xác định lactoferrin trong mẫu thực phẩm bổ sung.

Từ khóa: *Lactoferrin, thực phẩm bổ sung, HPLC, cột ái lực, làm sạch, Heparin.*