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Evaluation of Different RP-HPLC Methods for Lactoferrin Quantification in Goat Milk

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ABSTRACT

Objective: This study aimed to develop and validate a reliable method for the quantification of lactoferrin in goat milk. It also addressed the challenges associated with applying standard heparin-based methods, originally developed for cow's milk, to goat milk due to differences in fat and casein composition.

Materials and Methods: Different Reverse-phase High-performance Liquid Chromatography (RP-HPLC) methods for lactoferrin quantification were evaluated and validated. Goat milk samples were prepared using an optimized protocol that included two rounds of centrifugation at 4000 rpm, followed by filtration and high-speed centrifugation at 10,000 rpm. Defatting was identified as a critical step in sample preparation. In addition, the study investigated the use of cation-exchange resin with larger bead size as an alternative to conventional heparin-based beads for lactoferrin isolation.

Results: The optimized sample preparation method improved the clarity and suitability of goat milk samples for analysis. The use of larger cation-exchange resin beads facilitated better sample flow through the column and prevented clogging, which is a common issue with heparin-based columns. The validated RP-HPLC method showed good accuracy and robustness for lactoferrin quantification in goat milk.

Conclusion: A robust and accurate method for lactoferrin determination in goat milk was successfully developed. The combination of optimized sample preparation and the use of larger cation-exchange resin beads improve analytical performance and overcomes limitations of conventional heparin-based methods. This approach provides a reliable alternative for industrial and research applications involving goat milk.

INTRODUCTION

Lactoferrin is an iron-binding glycoprotein that belongs to the transferrin family[1]. It exhibits several important biological activities, including antibacterial, anti-inflammatory and antitumor effects[2,3]. Due to these multifunctional health benefits, lactoferrin is widely used in the nutraceutical industry. It is naturally present in various biological secretions such as tears, mucus, saliva and milk[4]. The concentration of lactoferrin in human milk varies during lactation, with levels exceeding 5 g/L in colostrum and decreasing to 2 to 4 g/L in mature milk[5-7].

Structurally, lactoferrin is a single polypeptide chain with a molecular weight of approximately 80 kDa[8,9]. It consists of two globular lobes, the carboxyl (C) lobe and the amino (N) lobe, which are connected by an alpha helix. Each lobe is further divided into two domains, C1 and C2 and N1 and N2, respectively and these domains are mainly composed of beta-sheet structures[8,11]. Lactoferrin has a strong ability to bind ferric iron. Based on its iron-binding state, it exists in three forms: apo-lactoferrin, mono-lactoferrin and holo-lactoferrin. Apo-lactoferrin contains no ferric ions, mono-lactoferrin contains one ferric ion and holo-lactoferrin contains two ferric ions, representing the fully saturated form. Native lactoferrin is typically a mixture of these three forms[9,11].

Due to the increasing demand for lactoferrin, non-human sources are being explored for large-scale production. Cow's milk contains approximately 0.1-0.2 g/L of lactoferrin[12]. In addition, goat milk has attracted attention as an alternative source for lactoferrin extraction. Mature goat milk contains lactoferrin in the range of approximately 17.5-120 mg/mL or higher[13]. Studies have shown that lactoferrin from goat milk has functional properties similar to those of human milk lactoferrin. Goat milk is also known for its higher digestibility and beneficial immunological properties, making it a suitable source for infant formula production. Furthermore, the oligosaccharide composition of goat milk is similar to that of human milk and the glycosylation pattern of goat milk lactoferrin closely resembles that of human lactoferrin[14].

Given the growing commercial interest in goat milk lactoferrin, there is a need for accurate and reliable analytical methods for its quantification. Common methods used for lactoferrin analysis in cow's milk include electrophoresis, Enzyme-linked Immunosorbent Assay (ELISA) and high-performance liquid chromatography (HPLC)[15]. However, limited studies have focused on the analysis of lactoferrin in goat milk and there is a lack of comprehensive validation of analytical methods and sample preparation procedures[16,17]. The relatively higher fat content of goat milk compared to human and cow's milk makes its analysis more challenging when using standard methods[18]. Therefore, this study aims to develop and validate an optimized analytical method, including appropriate sample preparation, for the determination of lactoferrin in goat milk using reverse-phase HPLC techniques.

MATERIALS AND METHODS

Goat milk samples were obtained from a goat farm in the Meredith region, Australia. All chemicals used in this study were purchased from Sigma-Aldrich Australia and the ion exchange resins were obtained from Cytiva Australia.

Lactoferrin in goat milk was quantified using four different chromatographic methods. The first method (Method 1, Drackova method) involved reverse-phase chromatography of goat whey samples[16]. Goat milk samples were centrifuged at 4000×g for 20 min to remove fat. After fat removal, casein was precipitated by adjusting the milk sample to pH 4.5 with 1 N HCl. The precipitated casein was separated by centrifugation at 4000× g for 15 min. The resulting whey supernatant was used for HPLC analysis. Chromatographic analysis was performed using an Agilent 1260 Infinity II HPLC system with Agilent ChemStation software. An Agilent Poroshell 300 SB-C8 column (5 µm, 2.1×75 mm) was used. A flow rate of 1 mL/min was maintained with a linear gradient system. Mobile phase A consisted of water, acetonitrile and trifluoroacetic acid in a ratio of 95:5:0.1 (v/v/v), while mobile phase B consisted of water, acetonitrile and trifluoroacetic acid in a ratio of 5:95:0.1 (v/v/v). The column temperature was maintained at 45 °C and the injection volume was 10 µL.

The second method used for lactoferrin quantification involved SP-functionalized cation exchange resin chromatography. In this method, a Tricorn column packed with Sepharose Big Beads was used to isolate lactoferrin from goat milk. Before loading onto the SP Big Beads column, milk samples were centrifuged at 4000 rpm followed by 10000 rpm to remove fat and other solid particles. After isolation of lactoferrin from goat milk, quantification was performed using the GB method.

The third method used for lactoferrin quantification was based on a heparin affinity column (HiTrap Heparin HP column, Cytiva, 1 mL)[19]. Goat milk samples were centrifuged at 4000 rpm for 20 min at 4 °C. Approximately 5 g of the centrifuged sample was collected and mixed with buffer A (28.4 g dissolved in 1000 mL water, pH 7.5). The mixture was centrifuged again at 10000 rpm for 8 minutes at 4°C. Lactoferrin isolation was carried out using a HiTrap Heparin HP affinity column (1 mL). Before sample loading, the column was equilibrated with 5 mL of buffer A. Subsequently, 10 mL of sample was loaded onto the column at a flow rate of 1 mL/min. After sample loading, the column was washed with 10 mL of buffer A. Bound proteins were then eluted with 5 mL of buffer B containing 7.1 g disodium hydrogen phosphate and 58.4 g sodium chloride dissolved in 900 mL water (pH 8.0). The eluted fraction containing lactoferrin was quantified using the modified GB RP-HPLC method. Chromatographic analysis was performed using an Agilent 1260 Infinity II HPLC system with Agilent ChemStation software. A ProSphere C4 column (300 Å, 150 mm×4.6 mm) was used for the analysis.

The fourth method used for lactoferrin quantification was similar to Method 2 but the goat milk sample preparation prior to heparin affinity separation was modified. Whole goat milk was first centrifuged at 4000 rpm for approximately 8 min and the fat layer was removed from the surface. The sample was then centrifuged again at the same speed. After the second centrifugation, the sample was filtered through a 1 µm filter. The double-centrifuged and filtered sample was mixed with buffer solution and the subsequent procedure was carried out as described in Method 3, including RP-HPLC analysis.

The fifth method used to validate the chromatographic methods described above was SDS-PAGE analysis. Two different protein sources were analyzed by SDS-PAGE: (1) Endogenous lactoferrin present in goat whey samples isolated by acid precipitation at pH 4.6 and (2) Commercial bovine lactoferrin (bLF) obtained from Merck Life Science, which was used as a quantitative control. The bLF standards were prepared in deionized water at six concentrations: 0.0625, 0.125, 0.25, 0.5, 0.75 and 1.0 mg/mL.

Prior to electrophoresis, the goat whey samples were filtered through a 0.45 µm filter. Protein separation was performed under both reducing and non-reducing conditions according to the standard protocol provided by Thermo Fisher Scientific. Following electrophoresis, proteins in the gel were stained with 0.1% Coomassie Brilliant Blue. The stained gels were subsequently analyzed using ImageJ software.

RESULTS AND DISCUSSION

Quantification of lactoferrin in goat milk: The RP-HPLC chromatogram of goat milk whey obtained using Method 1 is shown in Fig. 1. Method 1 was used to characterize lactoferrin in goat milk whey samples. The concentration of lactoferrin was determined from the standard calibration curve generated using purified lactoferrin analyzed under the same RP-HPLC conditions. Using this method, the lactoferrin concentration in goat milk was determined to be 132 mg/L.

The RP-HPLC chromatogram obtained using Method 2, based on SP Big Bead separation, is shown in Fig. 2. Lactoferrin concentration was calculated using a standard sample analyzed by the GB method described in the Materials and Methods section. The concentration of lactoferrin determined by this method was 130 mg/L.

Goat milk samples analyzed using Method 3 produced the RP-HPLC chromatogram shown in Fig. 3. The lactoferrin concentration determined using this method was 76 mg/L.

The modified chromatographic procedure used in Method 4 also successfully quantified goat milk lactoferrin and the corresponding chromatogram is presented in Fig. 4. The concentration of lactoferrin obtained using Method 4 was 126 mg/L.

To compare the chromatographic methods, a non-chromatographic approach was also employed. In this study, SDS-PAGE analysis was used to quantify lactoferrin in goat milk samples. The purity and migration pattern of bovine lactoferrin standards were first evaluated to establish a quantitative reference for comparative analysis. The bovine lactoferrin standards produced a single distinct band with a molecular mass of approximately 80-85 kDa[9] (Fig. 5), indicating structural integrity of the protein and the absence of fragmentation or degradation. Details of the SDS-PAGE bands and representative samples are presented in Table 1.

Densitometric analysis performed using ImageJ demonstrated a linear relationship between integrated band intensity and protein concentration over the tested range of 0.0625-1.0 mg/mL (Table 2). The resulting standard curve showed a coefficient of determination (R^2) of 0.9869.

Analysis of isolated goat whey samples identified endogenous goat lactoferrin through co-migration with the bovine lactoferrin standard (Fig. 5), which is consistent with

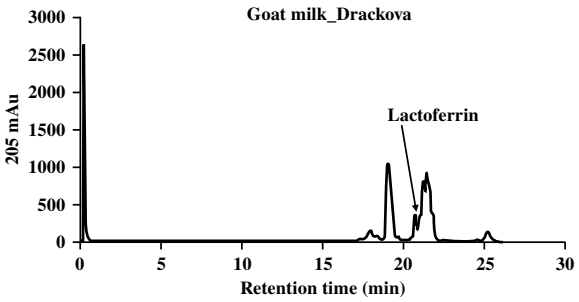

Fig. 1: RP HPLC chromatogram of goat milk whey

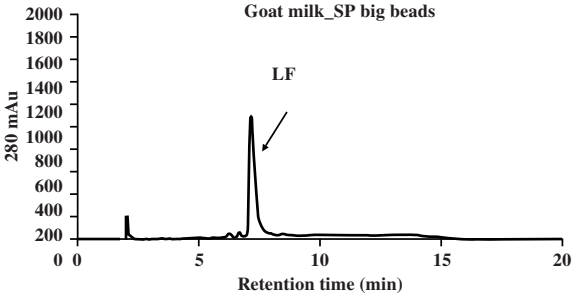

Fig. 2: RP-HPLC of SP big beads isolated lactoferrin

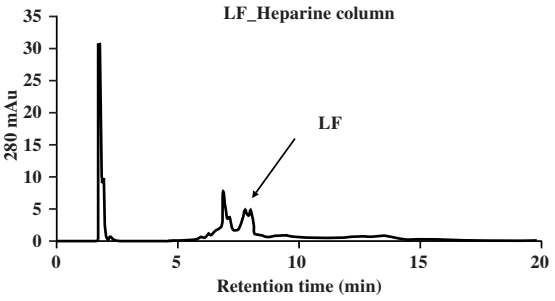

Fig. 3: RP HPLC of lactoferrin elution sample

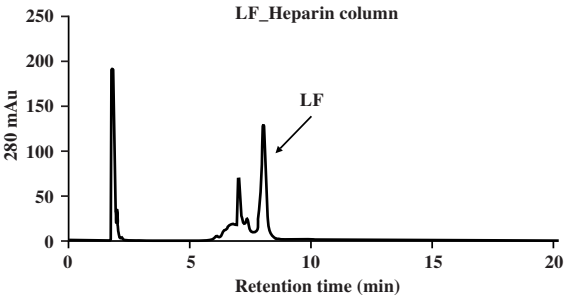

Fig. 4: RPHPLC of the lactoferrin elution sample from Method 4

previously reported migration patterns for lactoferrin from these species[20]. The electrophoretic separation provided sufficient resolution to isolate the goat lactoferrin fraction for further analysis. Densitometric quantification of the non-reduced goat milk sample (Lane 4) showed a lactoferrin concentration of 134 mg/mL.

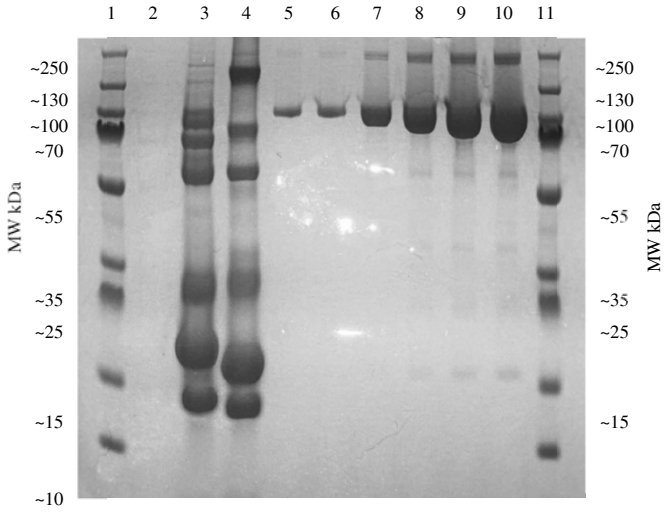

Fig. 5 : SDS PAGE analysis on the goat milk and lactoferrin standards

Table 1: Details of each SDS-PAGE band and the corresponding representative samples

Well No.	Sample name	Volume (µL)
1	Ladder	5
2	Blank	-
3	Goat milk (reduced) (10× dilution)	10
4	Goat milk (non-reduced) (10× dilution)	10
5	0.00625% LF standard (reduced)	10
6	0.0125% LF standard (reduced)	10
7	0.025% LF Standard (reduced)	10
8	0.05% LF standard (reduced)	10
9	0.075% LF Standard (Reduced)	10
10	0.1% LF Standard (Reduced)	10
11	Ladder	5

A summary of lactoferrin concentrations obtained from the same batch of goat milk samples using the different analytical methods is presented in Table 3. Comparison of the results showed that the lactoferrin concentration in goat milk was approximately 130±4 mg/L. Methods 1, 2 and 4, as well as the SDS-PAGE analysis from Method 5, produced comparable results. In contrast, Method 3 yielded a substantially lower concentration.

Method 3 was based on heparin affinity chromatography, which is commonly used for bovine lactoferrin purification. In Method 4, the sample preparation procedure was modified by including additional centrifugation and filtration steps before chromatography. This modified preparation resulted in lactoferrin concentrations comparable to those obtained using the other methods. The lower concentration observed in Method 3 may therefore be associated with insufficient removal of fat from goat milk samples prior to loading onto the heparin column. The smaller bead size of the heparin resin, compared with the SP Big Beads, may make the column more susceptible to blockage or masking of binding sites by residual fat, thereby reducing lactoferrin binding efficiency.

Table 2: Integrated peak areas from ImageJ

Well No.	Sample name	Area
1	Ladder	-
2	Blank	-
3	Milk reduced	11855.212
4	Milk non-reduced	6577.740
5	LF -0.00625%	4963.882
6	LF -0.0125%	8776.823
7	LF -0.025%	20434.735
8	LF -0.05%	29091.848
9	LF -0.075%	36196.266
10	LF -0.1%	42349.236
11	Ladder	-

Table 3: Lactoferrin concentration in goat milk samples measured using different methods

HPLC Method used	LF (mg/L)
Drackova method (Method 1)	132
SP big beads/GB (Method 2)	130
Heparin/GB (Method 3)	76
Heparin /GB modified (Method 4)	126
SDS PAGE analysis	134

When the same goat milk sample was re-analyzed using the heparin column after extended centrifugation, a higher lactoferrin concentration was obtained. These findings suggest that proper sample preparation is essential before chromatographic analysis of goat milk. In particular, goat milk samples should be thoroughly centrifuged and filtered to ensure effective fat removal. Furthermore, SP Big Beads or other larger ion-exchange chromatographic resins may be more suitable for goat milk analysis because of their larger bead size relative to heparin affinity resins.

CONCLUSION

This study developed a reliable and accurate method for the quantification of lactoferrin in goat milk. Among the methods evaluated, the heparin affinity chromatography

method was successfully applied for lactoferrin quantification when appropriate sample preparation was performed. Effective sample preparation was found to be critical for accurate analysis. Goat milk samples required two rounds of centrifugation at 4000 rpm, followed by filtration and high-speed centrifugation at 10000 rpm to achieve sufficient fat removal before chromatographic separation. The results demonstrated that proper defatting of goat milk is essential for efficient lactoferrin isolation, particularly when using heparin affinity columns. In comparison with heparin resin, cation exchange resins with larger bead sizes provided advantages for lactoferrin isolation from goat milk. The larger bead size allowed the milk samples to pass through the resin bed more effectively without clogging, even when standard defatted sample preparation was used. These findings indicate that larger bead ion-exchange resins may be more suitable for routine lactoferrin analysis in goat milk samples.

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