Determination of bovine lactoferrin in dairy products by ultra-high performance liquid chromatography–tandem mass spectrometry based on tryptic signature peptides employing an isotope-labeled winged peptide as internal standard

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HIGHLIGHTS

- A UHPLC–MS/MS method for quantification of bovine lactoferrin was developed.
- Tryptic fragment LRPVAAEIYGTK was chosen as signature peptide of bovine lactoferrin.
- A winged peptide containing isotopically-labeled signature peptide was designed as internal standard.
- The method for determining lactoferrin does not discriminate between the different forms of lactoferrin.
- Meet the growing demand to quantify bovine lactoferrin in different dairy products.

GRAPHICAL ABSTRACT
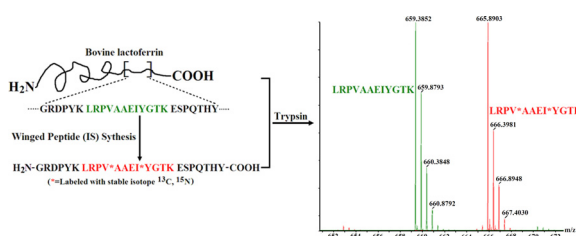
ABSTRACT

A new and sensitive determination method was developed for bovine lactoferrin in dairy products including infant formulas based on the signature peptide by ultra high-performance liquid chromatography and triple-quadruple tandem mass spectrometry under the multiple reaction monitoring mode. The simple pretreatment procedures included the addition of a winged peptide containing the isotope-labeled signature peptide as internal standard, followed by an enzymatic digestion with trypsin. The signature peptide was chosen and identified from the tryptic hydrolyzates of bovine lactoferrin by ultra high-performance liquid chromatography and quadrupole-time-of-flight tandem mass spectrometry based on sequence database search. Analytes were separated on an ACQUITY UPLC BEH 300 C18 column and monitored by MS/MS in seven minutes. Quantitative result bias due to matrix effect and tryptic efficiency was corrected through the use of synthetic isotope-labeled standards. The limit of detection and limit of quantification were 0.3 mg/100 g and 1.0 mg/100 g, respectively. Bovine lactoferrin within the concentration range of 10–1000 nmol L^{-1} showed a strong linear relationship with a linear correlation coefficient (r) of >0.998. The intra- and inter-day precision of the method were RSD < 6.5% and RSD < 7.1%, respectively. Excellent repeatability (RSD < 6.4%) substantially supported the application of this method for the determination of bovine lactoferrin in dairy samples. The present

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method was successfully validated and applied to determination of bovine lactoferrin in dairy products including infant formulas.

1. Introduction

Lactoferrin is an iron-binding glycoprotein and classified as a member of transferrin family. It predominantly exists in mammalian milk including human and bovine milk [1]. The published literature indicates that lactoferrin plays multiple biological and pharmacological roles such as intestinal iron uptake and regulation, antibacterial, antiviral, antioxidant and anti-inflammatory activity, immune response, suppression of tumors growth and metastasis [2–7]. Lactoferrin content is considered as species and lactation period-dependent. Lactoferrin is present in human milk and colostrums with significantly higher levels compared to the bovine equivalent [1,8]. These factors stimulated an increasing trend of lactoferrin supplementation in foods for infants and an increasing commercial interest in exploiting the therapeutic value of lactoferrin throughout the world [9]. However, the efficacy of lactoferrin supplementation depends on the manufacturing process because thermal exposure may compromise protein structure and function [10]. These changes of protein structure may affect the applicability and accuracy of the traditional analytical methods. For nutritional assessment and quality control, it is necessary to establish reliable analytical methods for determination of lactoferrin at endogenous level in raw milk, at fortified level in lactoferrin-fortified products and at pharmaceutical level in milk protein isolates.

Currently, analytical techniques for the determination of lactoferrin are reported using the methods of immunochemical techniques [11,12], enzyme linked immunosorbent assay (ELISA) [13,14], reversed phase high-performance liquid chromatography (RP-HPLC) [15–17], surface plasmon resonance (SPR)-based immunosensors and capillary electrophoresis (CE). The immunodiffusion techniques have inherently poor precision and low sensitivity inspite of its easy operation and simple instrumentation. ELISA methods are more selective and sensitive, but their effectiveness depends on the quality of antigen and the antibodies. Furthermore, the potential modification of the target proteins as antigen during the manufacturing process of food and dairy may affect the binding affinity of the antigen and antibodies, which may further lead to false-negative or underestimated results in ELISA analysis. Recent developments in label-free, real-time optical biosensor techniques based on SPR-immunoassay have been used for the analysis of lactoferrin in milk and infant formulas [10,18–21]. However, SPR-based biosensor immunoassays are critically influenced by temperature, sample components and specificity of reversible interaction between antibody and antigen. RP-HPLC methods using a gradient elution has been reported for the determination of lactoferrin in goat milk, bovine whey samples and simulated gastrointestinal fluids, but they suffered from insufficient resolution and sensitivity for analyzing lactoferrin in low concentration. Furthermore, lactoferrin of three different forms (apo-, native- and holo-lactoferrin) because of the presence or absence of iron in different environmental conditions might affect their physicochemical properties [22], which in turn might affect their separation performance. Compared to other analytical techniques, CE is widely accepted to have advantages in protein analysis. It was employed to determining bovine lactoferrin in cheese whey concentrates and infant formulas [23,24]. Nevertheless, analysis of bovine lactoferrin using CE methods is very difficult to achieve because of poor reproducibility, low sample recovery and poor separation of lactoferrin from other whey proteins. To the best of our knowledge, thus far, neither official and confirmatory methods nor certified reference materials could be used to support a harmonized approach to the quantitative analysis of bovine lactoferrin in dairy products, especially in infant formulas.

In recent years, liquid chromatography–mass spectrometry (LC–MS) techniques, which combine high selectivity and sensitivity with accurate quantification, have been employed to characterize, identify, or quantify proteins on the basis of entire proteins or tryptic peptides. The analysis of major milk proteins including casein, β-lactoglobulin and α-lactalbumin has been investigated with LC–MS [25–27], but the quantitative determination of bovine lactoferrin was not reported.

In the present work, the aim was to develop and validate a simple, robust, sensitive and nonimmunological method for the rapid quantification of bovine lactoferrin based on specific peptide. The analytical procedure encompasses a simple enzymatic digestion of samples spiked with a winged peptide as internal standard to evaluate the digestion efficiency. Centrifugation and filtration are used to remove the insoluble residues after tryptic digestion. A signature peptide is selected from the tryptic lactoferrin solution as the representative of bovine lactoferrin protein. The isotopically-labeled signature peptide from the tryptic winged peptide is employed as the actual isotopically-labeled internal standard of the lactoferrin signature peptide during the quantitative analysis. Subsequent analysis is performed by liquid chromatography–electrospray ionization–tandem mass spectrometry (LC–ESI–MS/MS) in the multiple reaction monitoring (MRM) mode under the positive ionization mode. The contents of bovine lactoferrin in dairy products are calculated based on the equimolar relationship between lactoferrin protein and lactoferrin signature peptide. Recoveries, precision and measurement uncertainty were evaluated by replicate analysis and the satisfactory results were achieved. Finally, the validated LC–MS/MS method was applied to the determination of bovine lactoferrin contents in various dairy products including infant formulas and whey protein concentrates.

2. Materials and methods

2.1. Chemicals

Ammonium bicarbonate (NH_4HCO_3), dithiotheritol (DTT), iodoacetamide (IAA) and calcium chloride ($CaCl_2$) were analytical grade and purchased from Sigma–Aldrich (St. Louis, MO, USA). Formic acid (FA) and acetonitrile (ACN) of HPLC grade were obtained from Merck (Darmstadt, Germany). Lactoferrin from bovine milk (≥85%), Fmoc-Val-OH-$^{13}C_5,^{15}N$, Fmoc-Ile-OH-$^{13}C_6,^{15}N$ and Fmoc-Leu-OH-$^{13}C_6,^{15}N$ (98% isotopic enrichment) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Sequencing grade modified trypsin was from Worthington Biochemical Corporation (Freehold, NJ, USA). All chemical agents were prepared using 50 mM NH_4HCO_3 and without further purification. Ultrapure water was obtained by a Milli-Q Gradient A 10 water purification system (Millipore, Bedford, MA, USA) during all the experiments.

2.2. Synthetic peptide standards

The signature peptide LRPVAAEIYGTK, VDSALYLGSR (corresponding to amino acid residues 93–104 and 333–342 of bovine lactoferrin, respectively), stable isotope-labeled signature peptide LRPV*AAEI*YGTK (V*, Val-OH-$^{13}C_5,^{15}N$; I*, Ile-OH-$^{13}C_6,^{15}N$),

VDSAL*YL*GSR (L*, Leu-OH-$^{13}C_6$,^{15}N) and internal standard GRDPYKLRPV*AAEI*YGTKESPQTHY, ALGFLRIPSKVDSAL*YL*GS-RYLTTLKNLRE were synthesized by ChinaPeptides Co., Ltd. (Shanghai, China). All the peptide standards were synthesized with purity of more than 95%.

2.3. Preparation of tryptic hydrolysates

Prior to tryptic hydrolysis, 1.0 g of samples were dissolved with 50 mM NH_4HCO_3 and diluted to the final concentration of total protein at approximately 1 mg mL^{-1} determined. Aliquots of 500 μL sample solution were spiked with 100 μL of 2 μM stable isotope-labeled internal standard GRDPYKLRPV*AAEI*YGTKESPQ-THY and then mixed with 400 μL of 50 mM NH_4CO_3. The mixtures were reduced by adding 10 μL of 500 mM DTT solution in 50 °C water bath for 30 min. An alkylation was performed with 30 μL of 500 mM IAA solution for 30 min at room temperature in the dark. Subsequently, add 10 μL of 100 mM $CaCl_2$ solution and enough trypsin solution (trypsin to protein ratio of 1:50–1:100, w/w). Mix gently and incubate overnight in 37 °C water bath. After terminating the digestion reaction by adding 10 μL FA, the digested mixture was diluted to 2 mL using ACN-ultrapure water (10:90, v/v) with 0.1% FA and centrifuged at 15,000 g for 10 min at room temperature. The supernatant was analyzed by LC–MS/MS after passing through a 0.22 μm nylon filter.

2.4. Liquid chromatography

Tryptic hydrolysates were separated using an ACQUITY UPLC System equipped with ACQUITY UPLC binary solvent manager, sample manager, and column manager (Waters, Milford, MA, USA). The analytical column was an ACQUITY UPLC BEH 300 C18 column (1.7 μm particle size, 2.1 × 100 mm, 300 Å) equipped with a guard column of the same material. The autosampler temperature was kept at 10 °C, and column temperature was kept at 40 °C. The mobile phase consisted of 0.1% FA aqueous solution (Solvent A) and ACN with 0.1% FA (Solvent B). A binary solvent gradient was run at 0.3 mL min^{-1} to elute the tryptic hydrolysates. The LC elution gradient started with 10% B for 1 min, followed by a gradient to 32% B in 2.2 min and another gradient to 100% in 1 min. The column was equilibrated at 100% B for 0.6 min and then back to 10% B for 2 min before the next injection. The injection volume was 10 μL and the total run time for each injection was 7 min. The effluent from the UHPLC system was directed into the electrospray ion (ESI) source of mass spectrometer (MS).

2.5. Mass spectrometry

Search and identification of signature peptide for bovine lactoferrin was performed on a Synapt G2 High Definition Mass Spectrometer equipped with ESI source (Waters, Milford, MA, USA). The conditions were as follows: capillary voltage, 3.0 kV; sampling cone voltage, 30 V; extraction cone voltage, 4.0 V; source temperature, 100 °C; desolvation temperature, 400 °C; cone gas, 50 L h^{-1} undefine nitrogen; desolvation gas, 600 L h^{-1} nitrogen. The instrument was operated in the electrospray positive ion (ESI$^+$) mode. The quadrupole time-of-flight (Q-TOF) mass analyzer was calibrated in MS/MS mode on a daily basis using Leu-Enkephalin in the range m/z 50–1500. The data acquired with MS^E continuum mode were processed using MassLynx 4.1 (Waters) and analyzed by searching sequence databases using ProteinLynx Global Server 2.5 software (PLGS 2.5, Waters). The search parameters were trypsin enzyme, fixed modification site of carboxymethyl and a maximum of one missed cleavage. The specificity of the signature peptide selected for bovine lactoferrin was confirmed by online BLAST search in UniProt (www.uniprot.org) and NCBI (www.ncbi.nlm.nih.gov).

All quantitative data were obtained using a Xevo TQ MS with ESI source (Waters, Milford, MA, USA) by multiple reaction monitoring (MRM) method. The mass spectrometer was operated in the electrospray positive ion (ESI$^+$) mode between 200 and 1800 m/z. The instrument conditions were adjusted to maximize analytical specificity and sensitivity. Relevant instrument parameters were set as follows: capillary voltage, 3.50 kV; cone voltage, 30 V; source temperature, 150 °C; desolvation gas temperature, 350 °C; cone gas flow, 50 L h^{-1} nitrogen; desolvation gas flow, 900 L h^{-1} nitrogen; and argon collision gas pressure to 3×10^{-3} mba for MS/MS analysis. Mass transitions monitored in the method were m/z 659.4 > 737.4, 659.4 > 850.5 for the peptide LRPVAAEIYGTK, m/z 540.8 > 595.3, 540.8 > 866.5 for VDSALYLGSR, m/z 665.8 > 742.9, 665.8 > 863.2 for LRPV*AAEI*YGTK and m/z 547.8 > 602.1, 547.8 > 880.3 for VDSAL*YL*GSR, respectively. The acquired data were processed with MassLynx 4.1 software.

2.6. Method validation

The established method was validated by evaluation of specificity, linearity, sensitivity, repeatability, recovery and precision (intra- and inter-day). The specificity was demonstrated by comparing the retention time of the synthetic signature peptide standard, natural peptide from tryptic samples and samples without enzymatic digestion. A standard curve with seven different concentrations in the range of 10–1000 nM (each containing a fixed concentration of stable isotope-labeled signature peptide) was obtained by the internal standard method. The slope, intercept and linearity of the standard curve were evaluated by linear regression analysis. Sensitivity was determined by evaluation of limit of detection (LOD) and limit of quantification (LOQ). The LOD and LOQ were determined as the concentrations of the target analytes after all steps of sample preparation at which their signal-to-noise (S/N) ratio of 3:1 and 10:1, respectively. Repeatability was evaluated by calculating the percentage of relative standard deviation (RSD) for detected results of samples with bovine lactoferrin at low, medium and high concentration levels on the same day. Recovery of the present method was evaluated by employing the standard addition method. The spiking levels of bovine lactoferrin equaled to the original levels in fresh milk, infant formula and whey protein concentrate, respectively. The recovery rates are calculated as the result of the measured value minus the original level divided by the spiked value. Intraday precision was calculated by eleven replicate analyses of each sample with three different concentration levels on the same day. For inter-day precision, the same samples were prepared and determined on seven consecutive days. Precision was calculated in terms of RSD of the measured results.

3. Results and discussion

3.1. Selection and synthesis of signature peptide standard for bovine lactoferrin

The method of tryptic peptides-based protein identification and quantitation using MS detector is gaining wider acceptance [26–29]. The development of proteomics and bioinformatics tools is emerging to assist in the computational prediction of tryptic products. For the future development of a MRM-based quantitative method for determination of bovine lactoferrin in dairy, the choice of good signature peptides to represent bovine lactoferrin is essential and crucial. The candidate peptides were selected based on several critical factors such as their specificity of amino acid sequences to bovine lactoferrin, their reproducibility between sample preparation, the abundance of their ions in proteolytic raw milk and dairy, the relative intensity of their MS signal, and
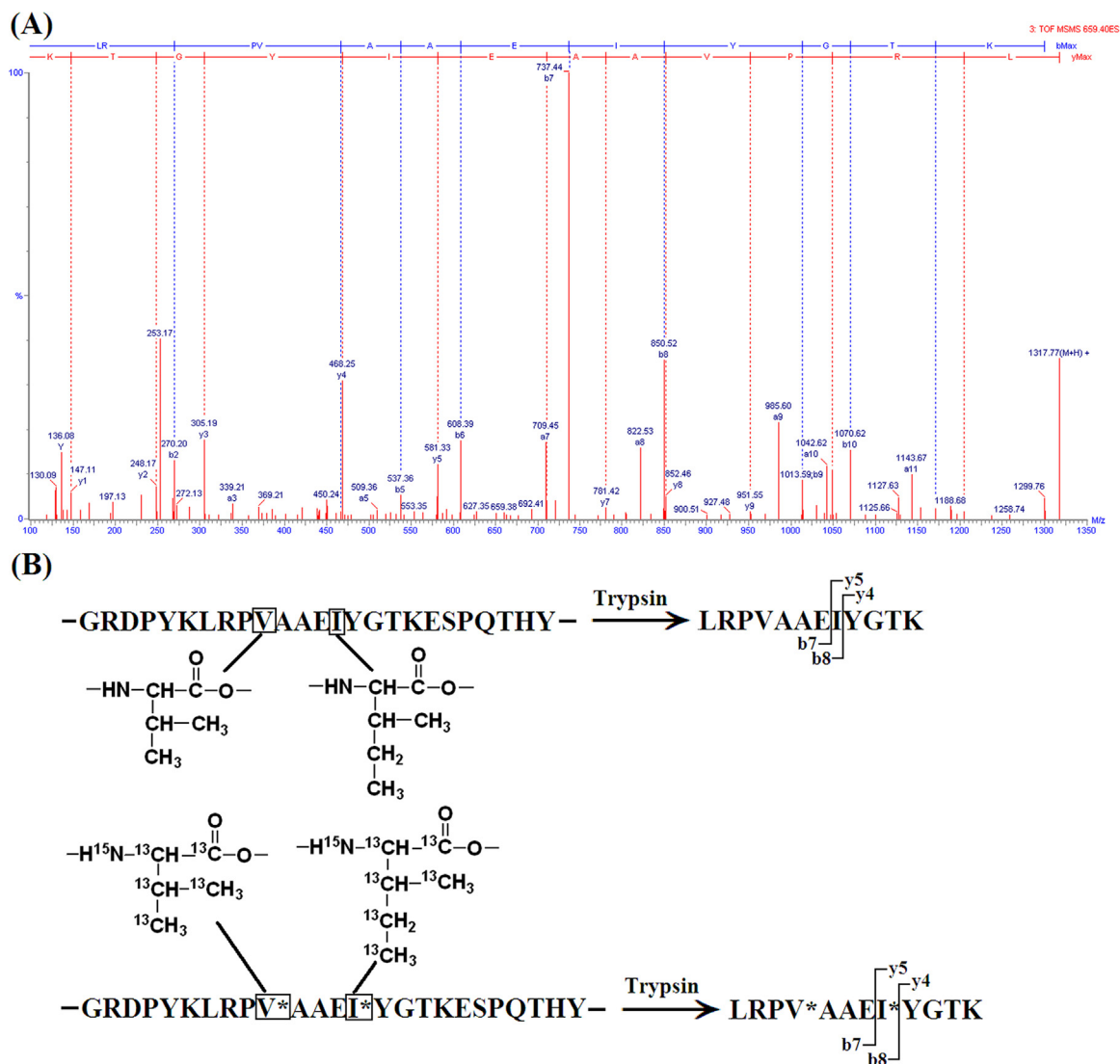
Fig. 1. Fragment ions and amino acid sequences of bovine lactoferrin signature peptide LRPVAAEIYGTK and its corresponding isotope-labeled analog LRPV*AAEI*YGTK: (A) fragment ions of bovine lactoferrin signature peptide LRPVAAEIYGTK; (B) amino acid sequences of bovine lactoferrin signature peptide LRPVAAEIYGTK and its corresponding isotope-labeled analog LRPV*AAEI*YGTK.

preferably they should not contain amino acids susceptible to chemical modification.

The specific peptides for bovine lactoferrin were chosen and identified by comparing the endogenous and theoretical peptides from tryptic bovine lactoferrin. The endogenous tryptic peptides were acquired by analysis of the tryptic digests of bovine lactoferrin standard, raw milk, whey protein concentrates and infant formulas. The theoretical tryptic cleavage products of bovine lactoferrin were obtained by computational prediction by Waters Biolynx softwares and online PeptideMass tools (http://web. expasy.org/peptide_mass). By UHPLC-Q-TOF analysis and sequence database search, eleven peptides were identified from tryptic bovine lactoferrin and dairy. They showed the same charged state distribution and corresponding molecular weight with the theoretical tryptic cleavage products. Three peptides of them were selected as specific biomarkers for bovine lactoferrin on the basis of their specificity and relative intensity. The results of online BLAST search in UniProt (www.uniprot.org) and NCBI (www.ncbi. nlm.nih.gov) showed the three peptides only exist in lactoferrin. Their amino acid sequences corresponding to isoform P24627 were LRPVAAEIYGTK (residues 93–104), FFSASCVPCIDR (residues 171–182) and VDSALYLGSR (residues 333–342), respectively. The acidic

solution during MS analysis tends to protonate all the available basic residues including amino-terminal amine and the basic side groups in a peptide. Tryptic peptides tend to be doubly charged because trypsin cleaves peptides at the carboxy-terminal side of lysine (K) and arginine (R) [30,31]. The doubly charged ions of the three tryptic peptides were preferred based on their high signal intensity in the tryptic dairy samples during MS analysis. They were confirmed to be absent in the undigested dairy samples during MRM analysis. However, they were present in the tryptic dairy products. In our further study, the second proposed peptide marker FFSASCVPCIDR (residues 171–182) was not considered because it had two cysteine (C) susceptible to carboxymethyl modification. Finally, the peptide LRPVAAEIYGTK (residues 93–104 of isoform P24627) was selected and synthesized as the signature peptide of bovine lactoferrin due to its highest intensity, abundance and sensitivity. Moreover, advantages of the use of peptide LRPVAAEIYGTK for quantitative measurement of bovine lactoferrin are the quantitative yield of the peptide from tryptic digestion and the absence in its sequence of amino acids prone to post-translation modification. Mass transitions were selected as m/z 659.4 > 737.4 and m/z 659.4 > 850.5 from product ion mass spectra of the synthetic peptide LRPVAAEIYGTK, corresponding to
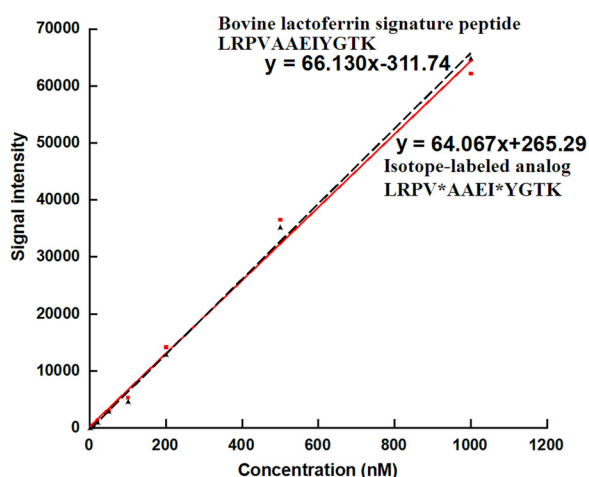
Fig. 2. Linear response of bovine lactoferrin signature peptide LRPVAAEIYGTK and its corresponding isotope-labeled analog LRPV*AAEI*YGTK during the UHPLC–MS/MS analysis.

b7 and b8 fragment ion, respectively (Fig. 1). The specificity and selectivity of the synthesized peptide LRPVAAEIYGTK were also confirmed by analyzing the dairy samples before and after trypsin cleavage.

3.2. Optimization and synthesis of isotopically-labeled signature peptide and internal standard

Liquid chromatography–tandem mass spectrometer is widely accepted as a highly sensitive and selective tool for protein quantification. However, its accuracy is prone to be affected by ionization efficiency and susceptibility to collision induced dissociation of different peptides. Furthermore, tryptic peptides-based methods for endogenous protein quantification rely on the extreme variability from target protein to proteolytic cleavage peptides between experiments. In an attempt to minimize the ionization efficiency and digestion variability, we employed a novel approach with a winged peptide as an internal standard. The sequence of the winged peptide is GRDPYKLRPV*AAEI*YGTKESPQ-THY. It is composed of a stable isotope-labeled analog of the signature peptide and six or seven amino acid residues of the sequence of bovine lactoferrin concatenated at each end. The sequence of the stable isotope-labeled analog of the selected signature peptide is LRPV*AAEI*YGTK. Its mass transitions were optimized as m/z 665.8 > 742.9 and m/z 665.8 > 863.2 from product ion mass spectra, corresponding to b7 and b8 fragment ion, respectively (Fig. 1). The two fragment ions showed 6 and 13 Da-shifted ions compared with b7 and b8 fragment ions of the signature peptide LRPVAAEIYGTK, respectively. The differences were because of the isotope-labeled valine and isoleucine residues.

When the winged peptide as internal standard was digested using the same enzymatic cleavage protocol for bovine lactoferrin during the sample preparation, the stable isotope-labeled analog of the signature peptide would be released. The analysis results indicated that the winged peptide as internal standard was absent in dairy samples before and after tryptic digestion and the stable isotope-labeled analog of the signature peptide was absent before tryptic digestion of spiked samples. However, it was present in the tryptic cleavage products of spiked samples with the winged peptide as internal standard. The peptide LRPV*AAEI*YGTK, a stable isotope-labeled analog of the signature peptide showed the similar chromatographic performance and linear response to the signature peptide (LRPVAAEIYGTK) of bovine lactoferrin during the UHPLC–MS/MS analysis (Figs. 2 and 3). The tryptic digestion

efficiency of bovine lactoferrin or the internal standard was evaluated using the corresponding tryptic amount compared to the known amount of bovine lactoferrin or the winged peptide as internal standard, respectively (Fig. 4). The digestion efficiency was 91.1–99.2% and 90.4–98.8% for bovine lactoferrin and its synthetic internal standard, respectively, when they were spiked into mobile phase and different dairy products. In addition, the tryptic digestion efficiencies in different dairy samples spiked with bovine lactoferrin standard or the synthetic internal standard were evaluated by a t-test assay. No significant difference was found within the 95% confidence intervals ($p > 0.05$). The use of a structurally matched internal standard containing amino acids beyond the tryptic cleavage sites led to improved assay precision, likely because the internal standard underwent tryptic cleavage in a fashion similar to that of the intact bovine lactoferrin in dairy samples. A similar approach with a winged peptide as internal standard was recently applied to measure thyroglobulin in serum and plasma by Kushnir et al. [29]. The good consistency indicated that the synthetic internal standard could best mimic the analytical behavior of intact bovine lactoferrin.

In order to further check if this chosen signature peptide (LRPVAAEIYGTK) and its corresponding internal standard would suffice to provide accurate and reproducible results, another signature peptide VDSALYLGSR (residues 333–342), the corresponding isotope-labeled signature peptide VDSAL*YL*GSR and its winged peptide internal standard (IS) ALGFLRIPSKVDSAL*YL*GS-RYLTTLKNLRE were synthesized and employed in the supplementary experiments. The peptide VDSALYLGSR showed the similar linear response to the bovine lactoferrin standard over the concentration range of 10–1000 nM. No significant difference was found within the 95% confidence intervals ($p > 0.05$) between the tryptic digestion efficiencies of the winged peptide internal standard (ALGFLRIPSKVDSAL*YL*GSRYLTTLKNLRE) and bovine lactoferrin standard (data not shown). Based on the similar linear response and tryptic digestion efficiency, the two winged internal standards (GRDPYKLRPV*AAEI*YGTKESPQTHY and ALGFL-RIPSKVDSAL*YL*GSRYLTTLKNLRE) were then spiked into six samples for determination of bovine lactoferrin. After tryptic digestion pretreatment and analysis by UHPLC–MS/MS, the contents of lactoferrin in these samples were calculated according to the equimolar relationship of the signature peptides and lactoferrin protein (Table 1). The results determined by the two different winged internal standards showed consistency and no
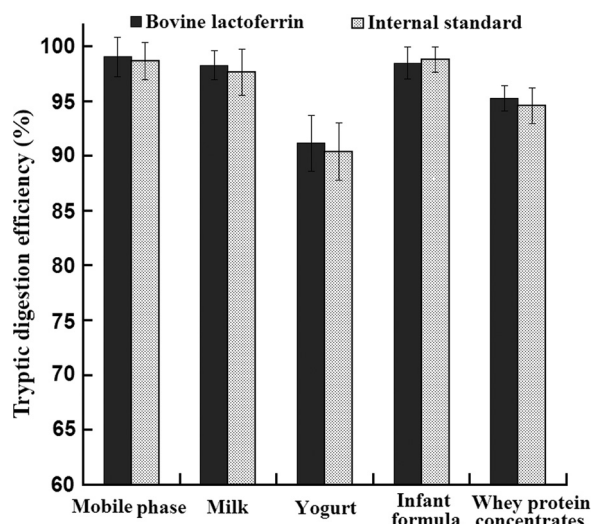

Fig. 3. Tryptic digestion efficiency of bovine lactoferrin and its isotope-labeled internal standard in different matrices.
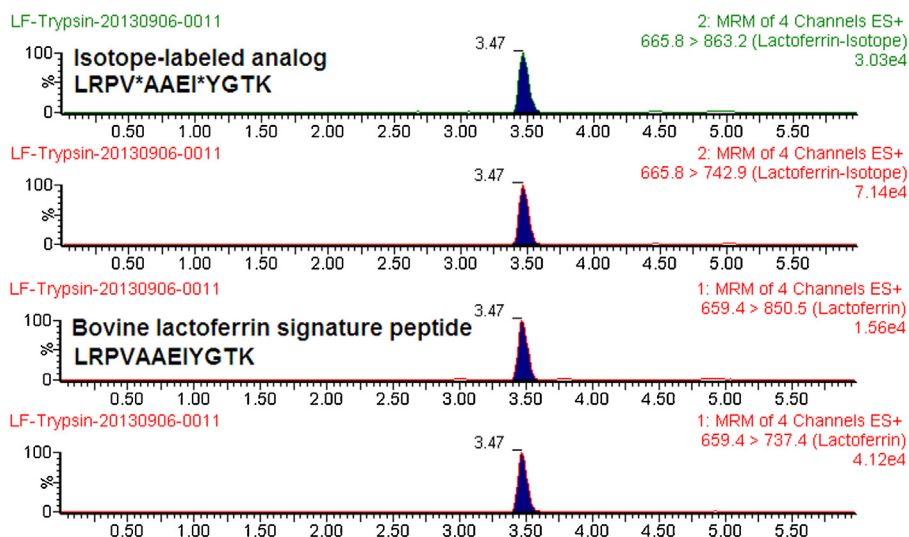
Fig. 4. UHPLC–MS/MS chromatograms of bovine lactoferrin signature peptide LRPVAAEIYGTK (B) and its corresponding isotope-labeled analog LRPV*AAEI*YGTK (A) in a tryptic dairy sample.

significant difference by a t-test ($p = 0.41 > 0.05$). All the experiment results illustrated that the chosen signature peptide (LRPVAAEIYGTK) and its corresponding internal standard (GRDPYKLRPV*AAEI*YGTKESPQTHY) could suffice to provide accurate and reproducible quantitative results of lactoferrin.

3.3. Method validation

3.3.1. Specificity

The specificity was assessed by comparing the chromatograms of the peptide standards with the corresponding tryptic samples spiked with internal standard. Both the synthetic peptide standards and the selected signature peptide from tryptic samples showed a sharp and symmetric peak at 3.47 ± 0.02 min. In samples without tryptic digestion, no peak was observed at the retention time. All these results indicated that there were no interferences from the matrix components on the retention time of the peptide standards.

3.3.2. Linearity, sensitivity and repeatability

The assay exhibited linearity between the analyte/IS peptide area ratio (y) versus analyte/IS concentration ratio (x) in the range of 10–1000 nM. The typtical linear regression equation obtained was $y = 66.13x - 311.74$ ($n = 3$). The correlation coefficient (r) of the standard curve was greater than 0.998. The LOD (found at S/N = 3) and LOQ (found at S/N = 10) were 0.3 mg/100 g and 1 mg/100 g, respectively. The sensitivity could fully meet the quantification requirements of bovine lactoferrin in various dairy products. The repeatability expressed as the RSD was obtained from the results

from multiple measurements ($n = 6$) of each sample. The RSD was 2.1–6.4%, which demonstrated that the developed UHPLC–MS/MS method was reproducible.

3.3.3. Recovery, intra- and inter-day precision

The spiking recovery rates were 87.8–104.7% with the RSD of 3.4–4.7% (Table 2). The RSDs of intra- and inter-day precision were determined as 2.7–6.5% and 1.9–7.1%, respectively. All the results demonstrated that the current method had a good recovery and precision. The established method fully satisfied the requirements for the quantification of bovine lactoferrin in dairy products.

3.4. Method application

To verify the applicability of the established method, the popular dairy products were randomly sampled from the local retailers (Hangzhou, China) for analysis in this work, which mainly included liquid milk, yogurt, whole milk powder, skimmed milk powder, infant formula and whey protein concentrates. Also, six lactoferrin powder with different purity degrees as ingredients in infant formulas were obtained from different manufacturers. All samples were pretreated and subjected to UHPLC–MS/MS analysis according to the aforementioned method and procedures. The selected signature peptide from bovine lactoferrin and its corresponding isotope-labeled signature peptide from spiked internal standard were successfully detected in the tryptic cleavage products of all samples (A typical chromatogram is shown in Fig. 4). The results indicated that the contents of bovine lactoferrin in different samples were robustly measured (Table 3). In this work, the measured contents of bovine lactoferrin were 1.9–6.2 mg/100 g in liquid milk. The lactoferrin contents were 62.2–112.5 mg/100 g in whole milk powder and skimmed milk

Table 1
Contents of bovine lactoferrin in samples measured by two different winged internal standard ($n = 3$).

No.	Detected content by two different winged internal standard (mg/100 g)	
	Internal standard 1[a]	Internal standard 2[b]
1	4.1 ± 0.3	4.3 ± 0.2
2	3.7 ± 0.4	3.9 ± 0.3
3	51.5 ± 3.7	49.2 ± 2.2
4	52.9 ± 1.4	56.4 ± 2.4
5	115.9 ± 7.8	116.7 ± 8.9
6	106.6 ± 8.5	103.8 ± 5.6
t-test	$p = 0.41 > 0.05$	

[a] Internal standard 1 = GRDPYKLRPV*AAEI*YGTKESPQTHY.
[b] Internal standard 2 = ALGFLRIPSKVDSAL*YL*GSRYLTTLKNLRE.

Table 2
Spiked recovery test of the present UHPLC–MS/MS method for determination of bovine lactoferrin ($n = 6$).

Original level (mg/100 g)	Spiked level (mg/100 g)	Determined level (mg/100 g)	Recovery rate[a] (%)	RSD (%)
3.1 ± 0.2	3.0	5.7 ± 0.3	87.8 ± 4.1	4.7
48.8 ± 3.6	50.0	97.1 ± 5.7	96.5 ± 3.8	3.9
106.8 ± 5.6	100.0	211.5 ± 9.2	104.7 ± 3.6	3.4

[a] Recovery rates = (Determined level − Original level) × 100%/Spiked level.

Table 3
Detected contents of bovine lactoferrin in different dairy products by the developed UHPLC–MS/MS method (n = 3).

Sample type	No.	Detected content (mg/100 g)	Sample type	No.	Detected content (mg/100 g)
Liquid milk (n = 6)	1	6.1 ± 0.4	Whey protein concentrates (n = 6)	19	73.1 ± 4.61
	2	3.6 ± 0.2		20	71.6 ± 1.4
	3	3.2 ± 0.2		21	74.9 ± 2.7
	4	1.9 ± 0.1		22	56.4 ± 1.9
	5	4.1 ± 0.3		23	65.2 ± 4.7
	6	6.2 ± 0.5		24	58.1 ± 2.1
Yogurt (n = 4)	7	4.3 ± 0.3	Infant formula (n = 12)	25	59.7 ± 3.4
	8	3.9 ± 0.2		26	72.5 ± 2.8
	9	4.9 ± 0.3		27	48.3 ± 1.8
	10	3.6 ± 0.1		28	61.2 ± 2.5
Whole milk powder (n = 4)	11	82.2 ± 3.7		29	51.5 ± 2.2
	12	79.8 ± 6.58		30	67.1 ± 2.6
	13	87.5 ± 3.1		31	63.2 ± 3.5
	14	62.2 ± 4.3		32	61.9 ± 4.8
Skimmed milk powder (n = 4)	15	93.2 ± 2.6		33	68.2 ± 2.3
	16	99.3 ± 0.5		34	50.4 ± 2.8
	17	98.4 ± 2.6		35	64.2 ± 1.2
	18	112.5 ± 8.7		36	49.6 ± 2.0

powder, respectively. Samples of infant formulas and whey protein concentrates contained bovine lactoferrin in the range of 49.6–74.9 mg/100 g. The contents of bovine lactoferrin were obviously different in different kinds of dairy products. For the same kind of dairy product, the lactoferrin contents in samples from different manufacturers were also very different. One of the potential impact factors of the difference in contents may be various industrial processing techniques of different manufacturers. Moreover, different raw milk as ingredients may also influence the final lactoferrin contents in dairy products because the content of lactoferrin in milk is determined by the different genetic factors, feeding system, lactation stage, age of cows and even environmental conditions.

4. Conclusions

In the present study, a UHPLC–MS/MS method in MRM mode for the quantitative determination of bovine lactoferrin is developed based on the signature peptide derived from the tryptic hydrolyzates of bovine lactoferrin. The sample preparation procedures include the addition of a winged peptide containing the isotopically-labeled signature peptide as internal standard, followed by an enzymatic digestion with trypsin. The signature peptide was derived and selected from the tryptic digestion products. Its specificity was identified based on comparing the endogenous and theoretical peptides from tryptic bovine lactoferrin. Furthermore, it was also proved by sequence database search on the basis of UHPLC-Q-TOF analysis. The present method was successfully applied to determination of bovine lactoferrin in dairy products including infant formulas and milk protein isolates.

To the best of our knowledge, this is the first time that a mass spectrometric method at the peptide level has been employed to quantitatively analyze bovine lactoferrin in dairy products.

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