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Multiple reaction monitoring-based determination of bovine α-lactalbumin in infant formulas and whey protein concentrates by ultra-high performance liquid chromatography–tandem mass spectrometry using tryptic signature peptides and synthetic peptide standards

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ABSTRACT

The determination of α-lactalbumin in various dairy products attracts wide attention in multidiscipline fields because of its nutritional and biological functions. In the present study, we quantified the bovine α-lactalbumin in various infant formulas and whey protein concentrates using ultra-high performance liquid chromatography coupled to tandem mass spectrometer in multiple reaction monitoring mode. Bovine α-lactalbumin was quantified by employing the synthetic internal standard based on the molar equivalent relationship among the internal standard, bovine α-lactalbumin and their signature peptides. This study especially focused on the recovery rates of the sample preparation procedure and robust quantification of total bovine α-lactalbumin in its native and thermally denatured form with a synthetic internal standard KILDKVGINNYWLAHKALCSE. The observed recovery rates of bovine α-lactalbumin ranged from 95.8 to 100.6% and the reproducibility was excellent (RSD < 6%) at different spiking levels. The limit of quantitation is 10 mg/100 g for infant formulas and whey protein concentrates. In order to validate the applicability of the method, 21 brands of infant formulas were analyzed. The acquired contents of bovine α-lactalbumin were 0.67–1.84 g/100 g in these infant formulas in agreement with their label claimed values. The experiment of heat treatment time showed that the loss of native α-lactalbumin enhanced with an increasing intensity of heat treatment. Comparing with Ren's previous method by analysis of only native bovine α-lactalbumin, the present method at the peptide level proved to be highly suitable for measuring bovine α-lactalbumin in infant formulas and whey protein concentrates, avoiding forgoing the thermally induced denatured α-lactalbumin caused by the technological processing.

1. Introduction

α-Lactalbumin isolated from sweet whey is an acidic (pI 4–5), strong Ca^{2+}-binding globular protein which is present in all mammals' milk [1,2]. It is a single polypeptide chain with four disulphide bonds consisting of 123 amino acids in the milk of human and cow. The high degree of sequence homology (more than 72%) exists between human and bovine α-lactalbumin, corresponding to a molecular mass of 14,070 Da and 14,178 Da, respectively [3–5]. α-Lactalbumin has been reported to have various physiologic and nutritional functionalities. Besides its well-acknowledged roles of participating in lactose biosynthesis in the lactating mammary gland as a component of lactose synthase, recent researches demonstrate that α-lactalbumin can stimulate the absorption of essential minerals and regulate gut microflora and immune function [1,2,4–6]. α-Lactalbumin, a predominant whey protein in human milk (25–35% of the total protein), is a potential well-balanced supply source of essential amino acids (particularly tryptophan and cysteine) and benefits the growth of infants. Unfortunately, it only comprises 2–5% of the total protein (20% of the whey protein) in mature bovine milk [4,6]. In contrast, β-lactoglobulin is a predominant whey protein in mature bovine milk, but is absent from human milk. Infant formulas with supplementation of α-lactalbumin-rich whey protein concentrates have therefore been prepared to make the protein composition close to that in human milk [4]. Considering the different industrial processing techniques and corresponding preparative α-lactalbumin products with different purity degree may influence the protein compositions in infant formulas, it is important and essential to

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develop the reliable quality control methods for the quantitative measurement of α-lactalbumin.

The analytical methods available for the determination of α-lactalbumin (either α-lactalbumin alone or concomitant with other whey proteins) include gel electrophoresis (GE) [7], capillary electrophoresis (CE) [8] and liquid chromatographic techniques [9–12]. Immunological detection methods are commonly described because of their inherent specificity and sensitivity for native protein conformation [13–15]. However, the potential problems of low accuracy, poor reproducibility, cross-reactivity and the tedious process of antibodies' development limit their applications in actual dairy production. By far, high performance liquid chromatography (HPLC) is still the well-documented method for determination of whey protein in the dairy industry though it spends a long time in the separation of the target proteins.

In recent years, the quantification of proteins in complex biological matrices employs liquid chromatography–mass spectrometer (LC–MS) technique because of the versatility, high resolution and short analysis time of the method [16–21]. The protein quantitation by LC–MS can be achieved by detecting the intact protein (at the protein level) or signature peptides after protein digestion (at the peptide level) [22–25]. The quantitation method at the protein level directly analyzes the intact protein after isolation and extraction procedures. The major problem of this strategy is the availability of a suitable internal standard (IS) though it avoids the potential digestion efficiency problem. Theoretically, the most optimal IS is an isotope-labeled analogue of the target protein. However, it requires a complex and laborious procedure of protein expression using the isotopically enriched media to obtain such an optimal IS [16,23,26]. An alternative option is employing a protein variant of the high sequence homology with the analyzed protein or a modified form of the target protein as the IS. Based on this approach, the bovine β-lactoglobulin content in different dairy products was determined by LC–MS using β-lactoglobulins from caprine and bubaline as the IS at the protein level [23]. Ren et al. [26] developed a robust and accurate LC–MS method for simultaneous determination of native α-lactalbumin and two variants of β-lactoglobulin in infant formulas employing human α-lactalbumin as the IS. Unfortunately, these methods are only feasible to measure the native form of the target protein rather than the total α-lactalbumin including the denatured form, particularly the thermally induced denatured form. In view of the influence of various technological processing steps such as fermentation or heat treatment on the amount of native α-lactalbumin [27–29], it is urgent and necessary to develop a quantitation method to determine total α-lactalbumin (both native and thermally denatured). The strategy of peptide level can meet the quantitation requirement by analyzing a tryptic signature peptide whose sequence is specific for the analyzed protein. The major problems of the methodology are to find a specific signature peptide for the target protein and a suitable IS peptide.

In the present study, a reliable LC–MS/MS method based on tryptic product peptides and synthetic peptide standards is established for determination of total α-lactalbumin in infant formulas and whey protein concentrates. The quantitation of the α-lactalbumin content was carried out by digesting the infant formula or whey protein concentrates followed by MRM-based LC–MS/MS analysis at the peptide level. A specific signature peptide of bovine α-lactalbumin was found and an extended peptide precursor (assigned as EPP) was optimized and synthesized as the IS from which the IS peptide was released during the tryptic digestion to compensate for losses of α-lactalbumin during the sample preparation procedure including the digestion step. The developed methodology should provide detailed preference information for the analysis of other proteins in complex matrices and was applied to the determination of bovine total α-lactalbumin contents in various infant formulas and whey protein concentrates.

2. Materials and methods

2.1. Chemicals

Ammonium bicarbonate (NH_4HCO_3), dithiotheritol (DTT), iodoacetamide (IAA), calcium chloride ($CaCl_2$) and acetic acid (AA) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile (ACN) and formic acid (FA) were purchased from Merck (Darmstadt, Germany). All the reagents used were analytical or HPLC grade. Proteomics grade trypsin was from Agilent Technologies, Inc. (Santa Clara, CA, USA). All chemicals were prepared using 50 mM NH_4HCO_3 and without further purification. Ultrapure water was passed through a Milli-Q Gradient A 10 system (Millipore, Bedford, MA, USA) during all the experiments.

2.2. Synthetic peptide standards

The signature peptide standard VGINYWLAHK (corresponding to amino acid residues 99–108 of bovine α-lactalbumin) and internal standard peptides VGINYFLAHK, VGINYPLAHK, VGINYHLAHK, VGINYWGAHK, VGINNY WLAHK were synthesized by ChinaPeptides Co., Ltd. (Shanghai, China). The internal standards KILDKVGINYFLAHKALCSE, KILDKVGINYPLAHKALCSE, KILDKVGINYHLAHKALCSE, KILDKVGINYWGAHKALCSE, and KILDKVGINNYWLAHKALCSE were synthesized by the same company. The purity of all the synthetic peptides was >95%.

2.3. Preparation of tryptic hydrolysates

Prior to trypsin hydrolysis, 1.0 g of commercial infant formulas were dissolved and diluted to 100 mL with deionized water, with the final concentration of total protein at approximately 1 mg mL^{-1} determined by Bradford assay (Sigma–Aldrich, USA). Aliquots of 0.5 mL solution were spiked with 20 μL 12.5 μM IS KILDKVGINNYWLAHKALCSE and then mixed with 0.48 mL 50 mM NH_4HCO_3. The mixtures were reduced with 10 μL of 500 mM DTT solution in 50 °C water bath for 30 min. An alkylation was performed by adding 30 μL of 500 mM IAA in the dark for 30 min at room temperature. Subsequently, 10 μL of 100 mM $CaCl_2$ and 30 μL of 200 μg mL^{-1} trypsin were added and incubated overnight at 37 °C. The digestion reaction was terminated by adding 20 μL FA. After incubation for 1 h at room temperature, the digested mixture was diluted to 10 mL using ACN-ultrapure water (10:90, v/v) with 0.1% FA and centrifuged at 2500 g for 10 min. The supernatant was passed through a 0.22 μm nylon filter before analysis by LC–MS/MS.

2.4. UHPLC–MS/MS conditions

The liquid chromatography (LC) was carried out using the ACQUITY UPLC System equipped with ACQUITY UPLC binary solvent manager (BSM), ACQUITY UPLC sample manager, and ACQUITY UPLC column manager (Waters, Milford, MA, USA). Separation of peptides was performed on a narrow-bore Acquity UPLC BEH 300 C18 column (1.7 μm particle size, 2.1 × 100 mm) from Waters (Milford, MA, USA), equipped with a guard column of the same material. A binary solvent gradient was run at 0.3 mL min^{-1} to analyze the tryptic hydrolysates. The 0.1% FA aqueous solution (Solvent A) and ACN in 0.1% FA aqueous solution (Solvent B) were used for the mobile phases. The injection volume was 5 μL while column temperature was 40 °C. The LC elution gradient started with 5% B for 1.2 min, followed by a gradient to 60% B in 1.8 min, another gradient to 80% in 1 min and a third gradient to 100% B in 0.2 min. The column was equilibrated at 100% B for 1.4 min and then back to 5% B for 2.2 min before the next injection (total run time 8 min for each injection).

Table 1
MS/MS parameters on the precursor and product ions and collision energy of signature peptide and internal standard peptide.

Name	Sequence	Molecular weight (Da)	Precursor ion (*m/z*)		Product ion (*m/z*)	Collision energy (eV)
			$[M+H]^+$	$[M+2H]^{2+}$		
Signature peptide	VGINYWLAHK	1199.7	1200.2	600.5	283.9	27
					354.7*	22
Internal standard peptide	VGINNYWLAHK	1314.5	1315.5	657.4	283.9	27
					354.7*	22

* Quantitative ion.

All data were acquired using Xevo TQ triple quadrupole mass spectrometer (MS) equipped with an electrospray ion (ESI) source (Waters, Milford, MA, USA). The mass spectrometer was operated under the electrospray positive ion (ESI^+) mode between 200 and 1800 *m/z*. The conditions used for the electrospray source were 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} mbar for MS/MS analysis. The precursor ions *m/z* with high abundance of the corresponding peptides were confirmed by direct injection. Based on the confirmation of precursor ions, more than two product ions were selected under the daughter scan mode as the analysis was performed by a low resolution LC–MS. Based on comparison of the sensitivity and specificity, the final selection of precursor ions and product ions and their optimal collision energy during the subsequent multiple reaction monitoring (MRM) analysis are shown in Table 1.

2.5. Linearity and calibration

The stock standard solutions of the synthetic peptides (200 μM) were prepared as in ultrapure water. All stock solutions were kept in the dark at −20 °C for approximately one month. The calibration curve ranged from 10 to 1000 nM was constructed to span the range of the amount of α-lactalbumin digested in the sample. Seven standard concentrations were employed containing 10, 25, 50, 100, 250, 500 and 1000 nM synthetic signature peptide VGINYWLAHK of bovine α-lactalbumin, as well as 250 nM IS peptide VGINNY WLAHK in each concentration by diluting the stock solutions step by step with 0.1% FA. All LC–MS/MS measurements were performed in triplicate.

The amounts of the tryptic signature peptide from the digested samples can be obtained from the standard curve made of the synthetic signature peptide. Finally, the contents of bovine α-lactalbumin in samples were calculated based on the molar equivalent relationship between the signature peptide VGINYWLAHK and bovine α-lactalbumin.

3. Results and discussion

3.1. Selection and synthesis of tryptic signature peptide of α-lactalbumin

Selection of suitable signature peptides is a crucial challenge for developing LC–MS/MS approach for proteins quantitation based on tryptic peptides. The sequence of a suitable signature peptide should be specific to the analyzed protein (not too short) and avoid containing susceptible amino acid such as cysteine and methionine to chemical modifications [22,23]. The selected peptides can be reproducibly observed between sample preparations and detectable in every digested sample [30]. In addition, the very long peptides should be avoided because they are difficult and expensive to synthesize and may possess unfavorable properties for LC–MS/MS analysis. The selected signature peptides significantly affect the specificity and sensitivity of LC–MS/MS analysis since various peptides have different ionization efficiencies and susceptibilities in MS scans [22]. The MS-based approach for protein identification and quantitation by analysis of proteotypic peptides is widely acknowledged and various bioinformatics softwares and tools are emerging to assist in the computational prediction of the frequently observed proteotypic peptides of proteins in MS/MS analysis [16,17,22,30]. However, additional experiments are still required to optimize and verify the suitable signature peptides, the experimental methods and MS instrumentation for the development of LC–MS/MS assays.

With the development of genomics and proteomics, bovine α-lactalbumin has been sequenced and widely studied. Its complete amino acid sequence is available in the publicly accessible protein databases [30]. The theoretical tryptic peptides of bovine α-lactalbumin were obtained by computational prediction using Waters Biolynx softwares. The endogenous peptides from the tryptic hydrolysates of bovine α-lactalbumin standard, infant formulas and whey protein concentrates were separated and identified by LC coupled to MS. The signature peptides of bovine α-lactalbumin were obtained by comparing the theoretical and endogenous peptides. For the initial experiments, three tryptic peptides were selected from a list of moderate to high abundance peptides reproducibly detectable by UHPLC–MS/MS analysis of the trypsin digests. The three peptide candidates during UHPLC–MS/MS analysis showed charged state distribution and corresponding molecular weight in good agreement with the theoretical values. The singly charged ions of the three peptide fragments were *m/z* 488, 618 and 1200. The corresponding sequences were ILDK, EQLTK and VGINYWLAHK (Fig. 1).

Since β-lactoglobulin and casein proteins are the major interferences for quantitation of α-lactalbumin in infant formulas, the selected peptide fragments were compared with the theoretical and endogenous tryptic peptides of bovine β-lactoglobulin, α_{S1}-, α_{S2}-, β- and κ-casein. Furthermore, various rice-based and soy-based infant formulas without supplement of whey protein were digested and analyzed to verify their specificity and selectivity. All the results illustrated that the three selected peptides were specific to bovine α-lactalbumin. Finally, the peptide VGINYWLAHK corresponding to residues 99–108 of bovine α-lactalbumin was selected and synthesized as the signature peptide of bovine α-lactalbumin for MRM assays because of its highest abundance, intensity and sensitivity in all the LC–MS/MS analyses. Moreover, the signature peptide VGINYWLAHK was also confirmed to be absent in the undigested sample matrices by mass spectrometer analysis. Thus, there is no interference occurred during the determination of bovine α-lactalbumin when the peptide VGINYWLAHK was chosen as the specific peptide of bovine α-lactalbumin. Trypsin is the commonly used protease for protein digestion and the effects of different digestion conditions on peptide recovery has been well studied. Recoveries were calculated using the known amount of bovine α-lactalbumin standard in the digestion, compared to the amount of the tryptic signature peptide found from the standard curve made of the synthetic signature peptide. The results (Fig. 2) showed that the tryptic digestion was more than 96% and one molar of bovine α-lactalbumin released one molar of the tryptic signature peptide VGINYWLAHK.

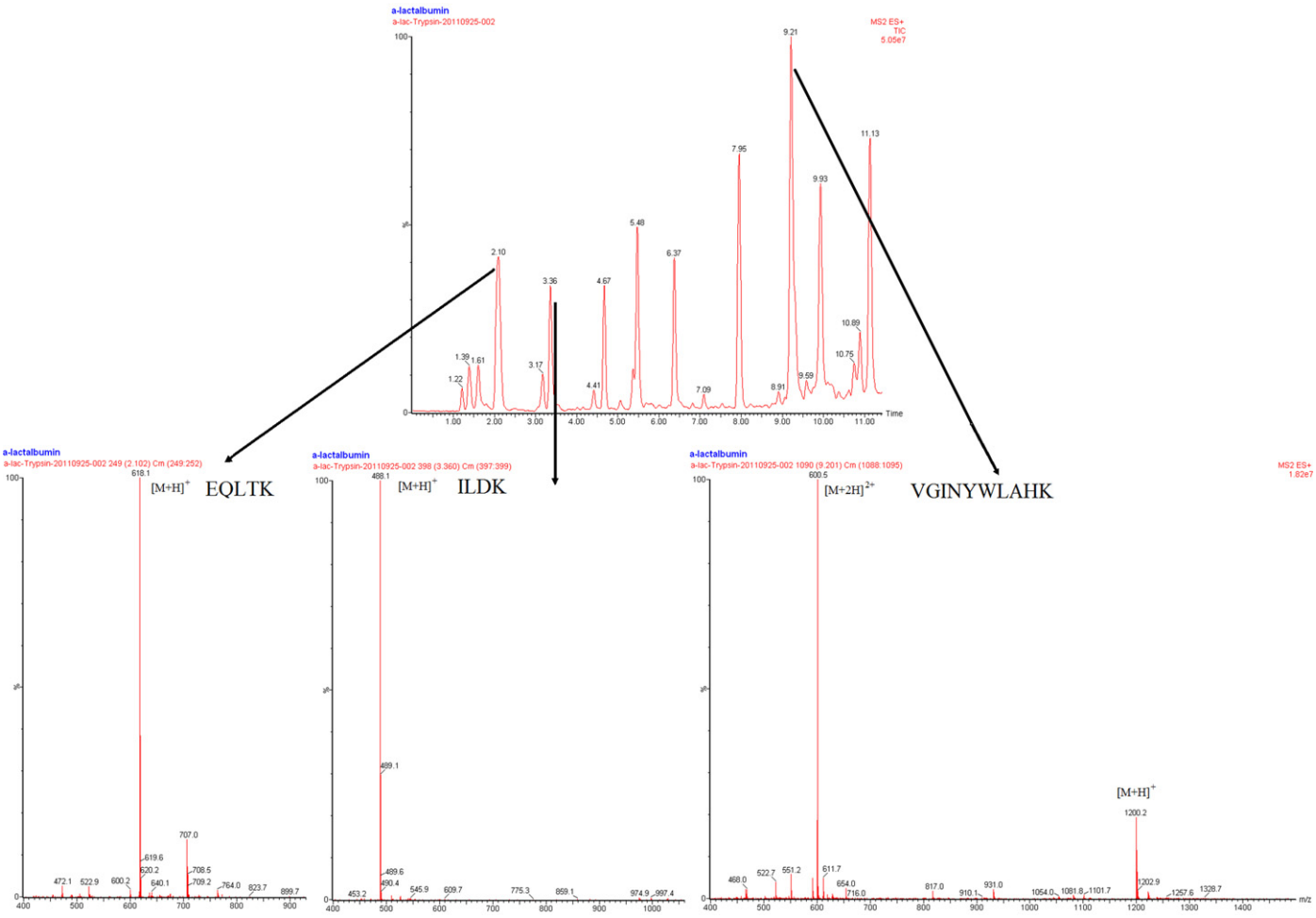

Fig. 1. Chromatogram and extracted ions of tryptic product peptides of bovine α-lactalbumin.

3.2. Optimization and synthesis of internal standard

Though LC–MS is a highly sensitive and selective tool for protein quantification, the accuracy of the technology may be influenced by the different ionization efficiency of the analytes in various matrices [16,26]. Moreover, the LC–MS/MS methodology for protein quantification by analysis of peptides relies on the complete digestion of the analyzed protein into cleavage peptides so that the peptide selected as the signature peptide is accurately quantified. The problem can be circumvented by adding a proper internal standard to the samples to correct the recovery losses caused by ionization efficiency and sample preparation [23]. The synthetic internal standard peptides work best with an incorporated stable isotope labeling so that the reference peptide and corresponding endogenous peptide share the same physicochemical properties. Such isotope-labeled synthetic analogues of the tryptic signature peptides have been employed as internal standards to quantify plasma proteins and membrane proteins [18,31,32]. However, in some cases, the isotopically labeled internal standard is not stable. In addition, the different behavior between the isotope-labeled synthetic internal standard peptide and the intact protein during the sample digestion could result in different losses of analyte during sample preparation [23,26]. The behavior difference was the result of the different tryptic digestion efficiency. Theoretically, the optimal internal standard is the isotopically labeled full-length protein analogue of the analyzed protein, which has identical physicochemical properties and digestion behavior with the target protein. It is not feasible in routine detection and quality control in dairy factories because it is a very difficult and expensive procedure to obtain such internal standards. One potential strategy to circumvent the problem involves the use of an extended peptide precursor as the internal standard. The extended peptide precursor should contain the internal standard peptide and the same tryptic cleavage site with the analyzed protein. On the

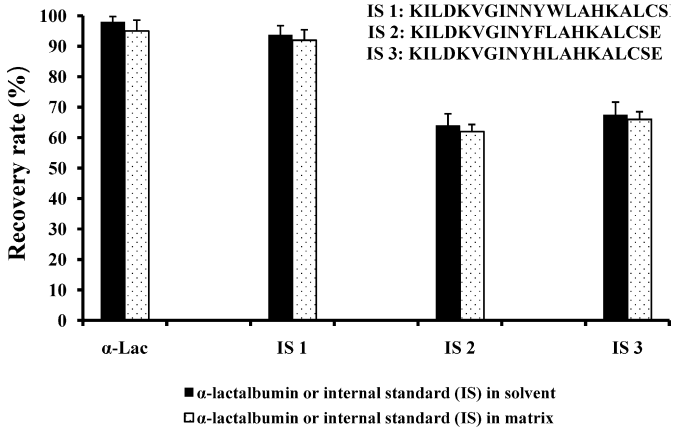

Fig. 2. Tryptic digestion efficiency of bovine α-lactalbumin (α-Lac) and three internal standards (IS) in solvent or in infant formula matrix.

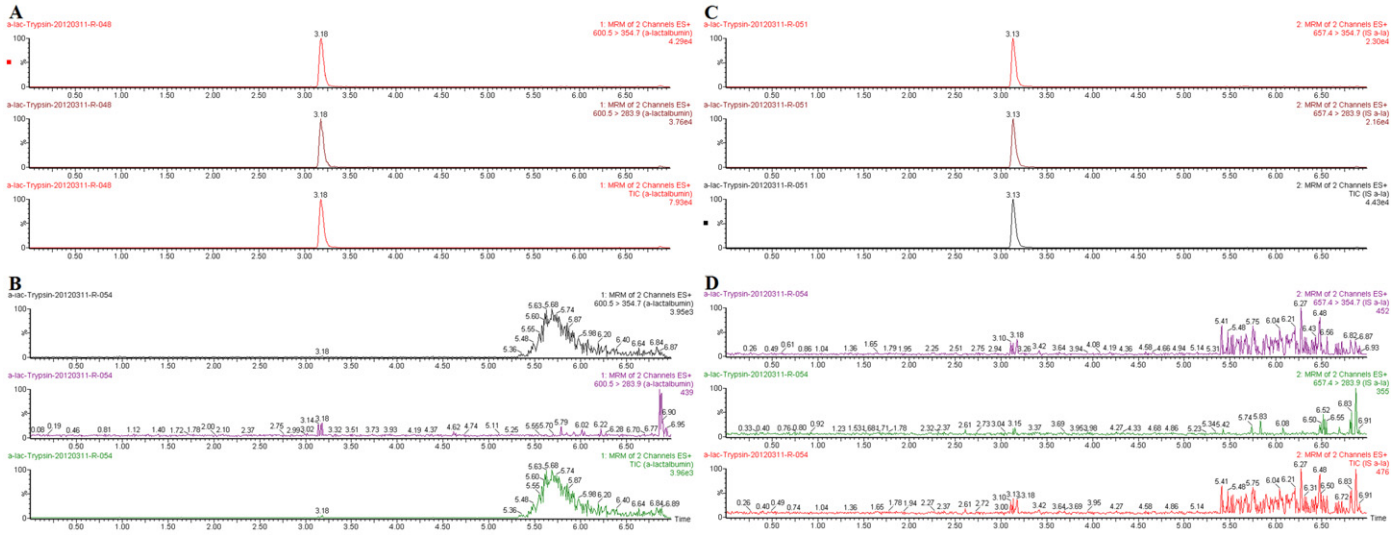

Fig. 3. LC–MS/MS chromatograms in MRM mode of the tryptic signature peptide VGINYWLAHK and internal standard (IS) peptide VGINNYWLAHK from tryptic products of the unspiked and spiked sample with IS. (A) Presence of the tryptic signature peptide VGINYWLAHK in unspiked sample after digestion; (B) absence of the IS peptide VGINNYWLAHK in unspiked sample after digestion; (C) presence of the IS peptide VGINNYWLAHK in spiked sample after digestion; (D) absence of the IS peptide VGINNYWLAHK in spiked sample before digestion.

basis, five extended peptide precursors as the internal standards were synthesized and optimized to assess the tryptic digestion efficiency. These synthetic peptides (KILDKVGINYFLAHKALCSE, KILDKVGINYPLAHKALCSE, KILDKVGINYHLAHKALCSE, KILDKVGINYWGAHKALCSE, and KILDKVGINNYWLAHKALCSE) mimicking the corresponding 20 or 21 residues of bovine α-lactalbumin contain the synthetic analogue of the selected signature peptide and the same tryptic cleavage sites as that in the native α-lactalbumin. The surrogates as internal standards were digested using the same digestion protocol with trypsin for bovine α-lactalbumin and analyzed using UHPLC–MS/MS. The results showed the complete absence of the internal standard peptides before tryptic digestion and the presence of the corresponding tryptic internal standard peptides after digestion (Fig. 3). Furthermore, the three IS peptides VGINNYWLAHK, VGINYFLAHK and VGINYHLAHK which were released from KILDKVGINNYWLAHKALCSE, KILDKVGINYFLAHKALCSE and KILDKVGINYHLAHKALCSE, respectively, showed the similar linear response and chromatographic behavior to the selected signature peptide (VGINYWLAHK) of α-lactalbumin during the UHPLC–MS/MS analysis (Figs. 3 and 4). The tryptic internal standard peptides were quantified using the corresponding standard curve made from dilutions of their corresponding synthetic internal standard peptides, respectively. Recoveries were calculated using the known amount of each extended peptide precursors as the internal standard in the digestion, compared to the amount of the corresponding tryptic internal standard peptide found from the standard curve. The data in Fig. 2 illustrated that the digestion efficiency was quite similar to that of bovine α-lactalbumin with recoveries of 93.6 ± 2.3% for KILDKVGINNYWLAHKALCSE, compared to the recoveries of 64.0 ± 3.8% to 67.5 ± 4.2% for the other two precursors. In addition, a *t*-test was performed on the different data of recoveries in different infant formulas spiked with bovine α-lactalbumin standard or the three internal standards. The results demonstrated that the bias was not significantly different from zero at 95% confidence ($p > 0.05$) when KILDKVGINNYWLAHKALCSE was employed. These results indicate that the analytical behavior of internal standard KILDKVGINNYWLAHKALCSE can best mimic that of bovine α-lactalbumin. Thus, it was chosen as the internal standard for quantification of bovine α-lactalbumin in this method system.

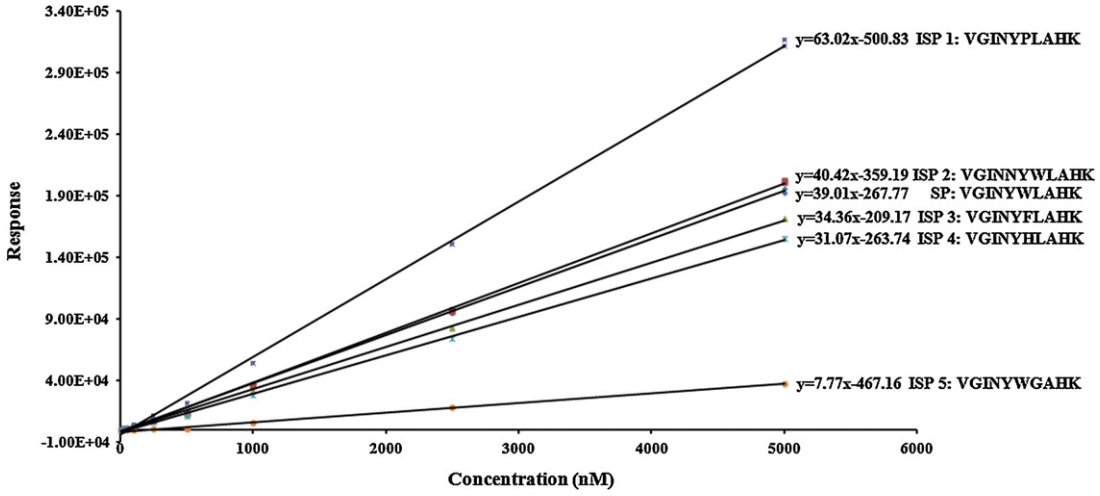

Fig. 4. Linear response of synthesized signature peptide (SP) of bovine α-lactalbumin and internal standard peptides (ISPs).

Table 2
Recovery test of the UHPLC–MS/MS method ($n = 6$).

	Spiked standard content (g/100 g)	Detected content (g/100 g)	Recovery rate (%)	RSD (%)
1	1.0	0.96	95.8	5.1
2	3.0	2.98	99.3	3.5
3	5.0	5.03	100.6	4.3

3.3. Method validation of the present UHPLC–MS/MS method

3.3.1. Linearity and sensitivity

The internal standard method was used to calibrate the system for bovine α-lactalbumin quantitation. The calibration curve was established after injection of the standard solutions at seven concentrations. The calibration curve showed good linearity with the correlation coefficient R^2 of 0.999 over the range of 10–1000 nM. Because there was no negative sample of bovine α-lactalbumin, the linear regression based on blank matrix was not validated.

The limit of quantification estimated as the concentration whose signal intensity is ten times higher than the standard deviation of the background noise, was 10 mg/100 g.

3.3.2. Recovery test

Recovery studies were performed by employing the standard addition method. Twenty four portions of premix base powder (the background amount of approximately 1 g/100 g bovine α-lactalbumin) with internal standard were spiked with bovine α-lactalbumin standard at four concentration levels (six portions per concentration level) of 0.0 (control), 1.0 (low spiking level), 3.0 (intermediate spiking level) and 5.0 g/100 g (high spiking level), respectively. The spiked samples were prepared and subjected to LC–MS/MS analysis. The results showed that the current method had good accuracy with 95.8%, 99.3% and 100.6% of spiked recoveries at low, intermediate and high spiking levels, respectively (Table 2). The RSD values for bovine α-lactalbumin at three concentration levels were 5.1%, 3.5% and 4.3%, respectively. Therefore, this method is able to quantify bovine α-lactalbumin in infant formulas and whey protein concentrates with good recovery and precision.

3.4. Method comparison between the current method and Ren's previous method

In previous studies, LC-ESI–MS methods have been reported for the determination of native α-lactalbumin or β-lactoglobulin at the intact protein level, either in full scan mode or in selected area monitoring mode [23,26]. Different sensitivity, specificity and reproducibility were observed from different methods when they were applied to protein quantification in complex matrices. Besides, only the quantification of native forms of proteins by these methods was reported. The limitation of such quantification methods indicate the native protein do not completely reflect the quality and nutrition value of dairy products. To circumvent the problematic issues, another strategy based on MRM was established to measure tryptic product peptides. It is well known that the MRM scan mode of tandem MS (MS/MS) is capable of rapid, specific and sensitive determination of analytes in complex matrices. The quantification of MRM is highly reproducible when an isotope-labeled internal standard is employed in the analysis workflows [30]. Sequence and mass spectrometer parameters of signature peptide and IS peptide used in the present UHPLC–MS/MS method were shown in Table 1. In order to test the advantages of the developed method for determination of bovine α-lactalbumin (both native and thermally denatured form), fresh milk of heat treatment time was prepared and measured by using the present method and previous validated method [26], respectively. Fresh milk in 12 test tubes (approximately 5 mL per test tube) was heated at 80 °C in water bath for 0, 15, 30, 40, 50, 60, 70, 80, 90, 100, 110 and 120 min, respectively. In the unheated fresh milk, the contents of bovine α-lactalbumin determined by this method and previous established method were 1.57 and 1.53 mg kg^{-1}, respectively (Fig. 5). After the fresh milk was heated 120 min at 80 °C, the contents obtained by the two methods were 1.56 and 0.53 mg kg^{-1}, respectively. The analysis results illustrated that the two methods are capable of determining the native α-lactalbumin and they obtain the similar amount in the unheated fresh milk. The amounts of α-lactalbumin acquired by the present method had no significant difference, though the loss of native α-lactalbumin increased when the fresh milk was continuously heated. However, previous validated method [26] was not capable of measuring the thermally induced denatured α-lactalbumin and the amounts determined by their method decreased with the increasing heat treatment intensity.

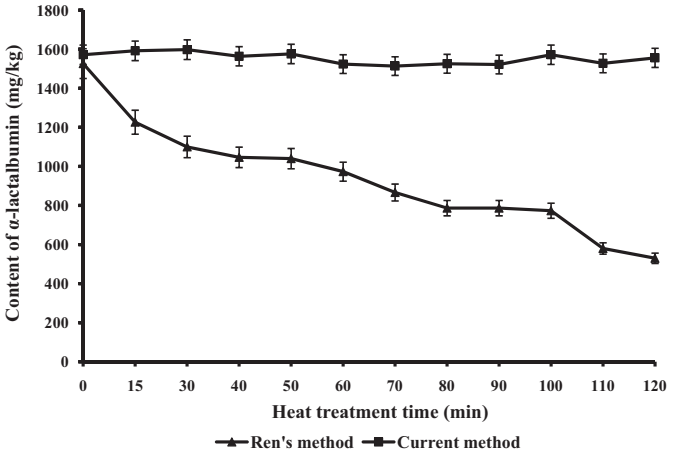

Fig. 5. Dynamics of bovine α-lactalbumin in heated fresh milk by the present method and Ren's previous method. Each data point used in the dynamics was generated from a mean of the contents of four repeat measurements. The error bar of each of those four measurements is plotted as plus and minus one standard deviation.

3.5. Analysis of α-lactalbumin in infant formulas and whey protein concentrates

In order to further verify the applicability of the established analytical method, 21 different brands of infant formulas were randomly sampled from different supermarkets in Hangzhou while eight whey protein concentrates were obtained from different manufacturers with different purity degrees. All the samples were pretreated and subjected to UHPLC–MS/MS analysis using current optimized method. The results showed that the selected signature peptide for bovine α-lactalbumin was detected in the tryptic digests of all samples and the contents of bovine α-lactalbumin in different samples were reliably determined (Table 3). Results indicated that bovine α-lactalbumin was fortified by artificially added whey protein concentrates because of the bioactive and nutritional properties of α-lactalbumin. The label claimed values of infant formulas were ranged from 0.6 to 1.9 g/100 g and stated on side of their packets or cans. The contents of bovine α-lactalbumin measured in this study were 0.67–1.84 g/100 g for infant formulas and 1.47–12.19 g/100 g for whey protein concentrates. Various industrial processing techniques may be the potential impact factors of the differences of bovine α-lactalbumin contents in the whey protein concentrates from different manufacturers. Generally speaking, the measured values of bovine α-lactalbumin in infant formulas are in agreement with their label claimed values. Thus, this UHPLC–MS/MS method based on analysis of tryptic peptides

Table 3
Label claim levels and detected contents of bovine α-lactalbumin in infant formulas and whey protein concentrates by UHPLC–MS/MS analysis.

Sample	Sample No.	Label claim (g/100 g)	Detected content (g/100 g)
Infant formula (IF)	IF-1	1.7	1.68
	IF-2	1.8	1.76
	IF-3	1.2	1.15
	IF-4	1.2	1.23
	IF-5	1.2	1.18
	IF-6	1.7	1.65
	IF-7	1.9	1.84
	IF-8	1.8	1.82
	IF-9	1.26	1.30
	IF-10	1	1.04
	IF-11	1.8	1.84
	IF-12	0.64	0.67
	IF-13	0.7	0.72
	IF-14	1	1.05
	IF-15	1.25	1.29
	IF-16	1.2	1.18
	IF-17	1.5	1.53
	IF-18	1.2	1.17
	IF-19	1.2	1.22
	IF-20	–	1.34
	IF-21	–	1.07
Whey protein concentrate (WPC)	WPC-1	–	2.26
	WPC-2	–	2.72
	WPC-3	–	2.25
	WPC-4	–	2.08
	WPC-5	–	12.19
	WPC-6	–	11.83
	WPC-7	–	1.47
	WPC-8	–	2.11

can be successfully used to quantify bovine α-lactalbumin in infant formulas and whey protein concentrates.

4. Conclusions

A robust UHPLC–MS/MS method at peptide level was developed for the determination of bovine α-lactalbumin in infant formulas and whey protein concentrates. The tryptic fragment VGINYWLAHK was selected and validated as the signature peptide of bovine α-lactalbumin. A synthetic internal standard KILDKVGINNYWLAHKALCSE has been proved to have similar analytical behavior and tryptic digestion efficiency to bovine α-lactalbumin, and it was spiked with infant formula or whey protein concentrate prior to the sample preparation procedure to compensate for the analyte losses. The contents of bovine α-lactalbumin in samples were measured by employing the synthetic internal standard based on the molar equivalent relationship between internal standard peptide (VGINNYWLAHK) and the signature peptide of bovine α-lactalbumin. Comparing with the previous methods by analysis of only native bovine α-lactalbumin, the present method with mass spectrometer with MRM mode offered lower detection limit and satisfactory accuracy and reproducibility. An additional advantage of the current method was a measure of the whole bovine α-lactalbumin including the native form and thermally induced denatured form. Finally, it was successfully applied to determine bovine α-lactalbumin in 21 different infant formulas and eight whey protein concentrates. Since the established method could determine native and thermal denatured bovine α-lactalbumin, as well as soluble and therefore functional bovine α-lactalbumin, its application might promote the development of nutrient investigation and quality control of infant formulas and other dairy products containing α-lactalbumin.

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