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Quantification of lactoferrin in breast milk by ultra-high performance liquid chromatography–tandem mass spectrometry with isotopic dilution

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Abstract

We developed a LC–MS/MS method for quantification of human lactoferrin in breast milk based on tryptic peptides and synthetic isotopic peptide standard. The signature peptides were obtained from tryptic breast milk. They were screened by the computational prediction by Biolynx software, and confirmed by database searching after analysis of liquid chromatography-quadrupole-time-of-flight tandem mass spectrometry (LC-Q-TOF-MS). The winged isotopic-labeled signature peptide was used as internal standard to compensate the matrix effect. The spiking recovery of human lactoferrin ranged from 92.1% to 97.5%, and the relative standard deviation (RSD) was 3.4-4.7%. The limit of detection (LOD) and limit of quantitation (LOQ) were 1 mg/100g and 3 mg/100g in tryptic breast milk, respectively. The present testing method was sensitive and selective, which was successfully applied to human breast milk at different lactation stages in Beijing, China. The data revealed that levels of secreted lactoferrin decreased with the extension of lactation.

Key words: Breast milk; Lactoferrin; Absolute quantification; LC-MS/MS

1. Introduction

Lactoferrin, a member of the transferrin family, has diverse physiological functions, such as antimicrobial/antiviral activities, immunomodulatory activity and antioxidant activity [1-3]. It can be found in milk of most mammalian species [4]. As functional protein in breast milk, lactoferrin accounts for about 25% of the whey [5, 6]. Noticeably, the level of lactoferrin is affected by many factors, for example lactation stage and individual characteristic [7-9]. So, accurate and fast quantification method in breast milk is of great interest for nutritional research and commercial design of infant formula.

Common quantification methods can be classified into immunological and non-immunological methods. Reported immunological methods were nephelometric immunoassays [10], enzyme-linked immunosorbent assay (ELISA) [11] and surface plasmon resonance (SPR)-based immunosensors [12]. Reverse phase high performance liquid chromatography (RP-HPLC) [13, 14] and capillary electrophoresis (CE) [15] were not based on the immunological technique. Immunological methods are high selective and sensitive, but their quantitative reproducibility may be not very well. Furthermore, the degeneration of protein in immunological analysis may cause false negative. RP-HPLC is usually used by a gradient elution on silica-based column with the UV detector. CE has a good resolution to tested proteins based on ratios of their charge to mass. However, the separation of proteins by CE may result in poor reproducibility and low recovery due to lack of electroosmotic flow control and protein adsorption onto the capillary walls

[16].

In recent years, methods for protein quantification by LC–MS/MS with multiple reaction monitoring (MRM) and tryptic product peptides have been reported [17-21]. MRM has emerged as a promising technique for precise protein quantification based on specific peptides [22, 23]. The synthetic peptide segment as a biological marker can be used as the quantification standards. However, there is obvious signal interference in mass spectrometry due to complicated biological matrix. The usage of isotopic internal standard may compensate for the matrix effect and analyte loss in sample preparation.

In this study, we aim to develop a LC–MS/MS method for determination of human lactoferrin in breast milk based on tryptic peptides. Specific or signature peptides were predicted by Biolynx software (Waters, Milford, MA, USA), and confirmed by database searching after liquid chromatography-quadrupole-time-of-flight tandem mass spectrometry (LC-Q-TOF-MS) analysis. The isotopic-labeled signature peptide as internal standard was employed for avoiding matrix interference in mass spectrometry. The sample preparation of tryptic digestion was also optimized for the accurate quantification. The developed method was accurate, sensitive and efficient in the application of human lactoferrin quantification.

2. Materials and methods

2.1. Chemicals

Ammonium bicarbonate (NH_4HCO_3), dithiotheritol (DTT), iodoacetamide (IAA)

and hydrochloric acid (HCl, 37%) 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. Sequencing grade modified trypsin was from Shanghai Yaxin Biotechnology Co., Ltd (Shanghai, China). All chemical agents were prepared using ultrapure water 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. Sample collection

Human breast milk samples were from mothers of full-term infants (> 36th gestational week) in the city of Beijing, China. Mothers who have suspicion of infection or history of smoking were excluded. Sample collection was performed with a manual suction pump in an aseptic fashion.

Twenty six colostrum samples were collected from the 1st to the 7th day post-partum. Forty one transitional milk samples were collected from the 8th to the 16th day. Forty three mature milk samples were collected from 17th to 330th days of lactation. All samples were kept frozen (−20 °C) until analysis. Each participant presented written informed consent for participating in the study. The study was approved by the institutional review board.

2.3. Synthetic peptide standards

The signature peptide VPSHAVVAR (corresponding to amino acid residues

269–277 of human lactoferrin), stable isotope-labeled signature peptide VPSHAV*V*AR, (V*, Val-OH-^{13}C5, ^{15}N), and internal standard FKDCHLARVPSHAV*V*ARSVNGKE were synthesized by ChinaPeptides Co., Ltd. (Shanghai, China). All the peptide standards were synthesized with purity of more than 95%.

2.4. Preparation of tryptic hydrolysates

Prior to tryptic hydrolysis, sample portions of 0.2 g were weighed into 10-mL volumetric flask and diluted to constant volume with deionized water. A 100 μL aliquot of diluent were spiked with 100 μL of 200 nmol/L stable isotope-labeled internal standard and then mixed with 665 μL deionized water. A 10 μL aliquot of 50 mmol/L DTT solution were added to the mixtures and reduced in 80 °C water bath for 30 min at this stage. In the next step, an alkylation was performed by adding 10 μL of 150 mmol/L IAA in the dark for 30 min at room temperature. Subsequently, 100 μL of 500 mmol/L NH_4HCO_3 and 10 μL of 400 μg/mL trypsin (freshly prepared) were added and incubated 30 min at 37 °C. The reaction was terminated by addition of 5 μL formic acid. The insoluble substances in tryptic hydrolysates were removed by centrifuging at 13000 g for 10 min. The supernatant was analyzed by LC–MS/MS after passing through a 0.22 μm nylon filter.

2.5. 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). Chromatographic separation was carried out on a narrow-bore Acquity UPLC BEH 300 C_{18} column (1.7 μm, 2.1 mm × 100 mm) maintained at 40 °C, equipped with a guard column of the same material (Waters, Milford, MA, USA). The aqueous solution (0.1% FA) (Solvent A) and ACN solution (0.1% FA) (Solvent B) were used for the mobile phases. Gradient elution was: 3% B to 32% B for 5min; 100% B for 1 min; re-equilibration at the initial conditions for 2 min. The flow rate for separations was maintained at 0.3 mL/min and a 10.0 μL injection volume was used for all standards and samples.

2.6. Mass spectrometry

Time of flight mass spectrometry (TOF-MS) detection was performed on a Synapt G2 HDMS equipped with an electrospray ion (ESI) source (Waters, Milford, MA, USA). All data were acquired in the electrospray positive ion (ESI^+) mode with MS^E mode. Details of TOF conditions were as follows: capillary voltage, 3 kV; sampling cone voltage, 25 V; extraction cone voltage, 4 V; source temperature, 100°C; desolvation temperature, 400°C; cone gas flow, 30 l h^{-1}; desolvation gas flow, 800 l h^{-1}; ramp trap collision energy, 15–35 V; and lockspray reference compound, leucine-enkephalin. The acquired data were analysed using ProteinLynx Global Server version 2.5 software with the followed settings: mode, electrospray-MS^E; lockmass for charge 1, 556.2771 Da; minimal fragment ion matches per peptide, 2; minimal fragment ion matches per peptide, 5; allowed missed cleavage, 1; fixed

modifications, carbamdomethyl C; and variable modifications, oxidation M. The databank was imported from UniProt Knowledgebase (http://www.uniprot.org).

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 positive electrospray (ESI^+) mode with both quadrupoles tuned for unit resolution. Selected operating parameters were capillary voltage (3.5 kV), desolvation temperature (500 °C), desolvation gas (800 L/h), cone gas (150 L/h), argon collision gas pressure (3×10^{-3} mbar). Two multiple reaction monitoring (MRM) transitions were monitored for each compound, with cone voltages and collision energies were optimized for each transition. The precursor ion, product ion and their optimal MRM parameters are shown in Table 1.

3. Results and discussion

3.1. Signature peptide and internal standard of human lactoferrin

Selection of suitable signature peptides for accurate quantitation of targeted protein is a crucial challenge for developing LC–MS/MS approach. Bioinformatics tool is usually adopted to assist the computational prediction of tryptic products [21]. The 52 theoretical tryptic cleavage peptides of human lactoferrin were obtained by computational prediction by Biolynx softwares and online PeptideMass tools provided by UniProt (http://web.expasy.org/peptide_mass).

For further confirmation of signature peptides, UPLC-Q-TOF-MS was applied for comparing the endogenous and theoretical peptides from tryptic lactoferrin. The

26 peptides were identified detected in tryptic breast milk after comparing between acquired data and results of sequence database search. The coverage of the searched peptides from the total human lactoferrin sequence is 53.55%. The candidate peptides were selected based on several critical factors such as specificity of amino acid sequences, reproducibility in sample preparation, intensity of their MS signal [24]. Furthermore, the peptides with more amino acids (>14) may be not used due to their expensive synthesize and unfavorable LC properties [24]. Therefore, 5 peptides (VPSHAVVAR, THYYAVAVVK, EDAIWNLLR, DGAGDVAFIR and YLGPQYVAGITNL) were selected as possible signature peptides (Table 1).

3.2. Selection of targeted signature peptide

In order to find targeted signature peptide with the property of easy tryptic digestion and high MS intensity, modified experiments of the variable of digestive time were designed. The digestive time were ranged from 0.25 to 8 h at 37 °C. The acquired data of the 8 h pretreatment were considered as the 100% efficiency. The data showed that five candidate peptides had the different cleavage efficiency (Fig. 1). The peptide of VPSHAVVAR and THYYAVAVVK can be completely digested within 1 hour. Other three peptides, DGAGDVAFIR, EDAIWNLLR and YLGPQYVAGITNLK, had more enzymolysis time. Meanwhile, different MS intensity of five peptides was observed (Fig. 2). Peptide VPSHAVVAR had the largest MS intensity. Accordingly, it was selected and synthesized as the targeted signature peptide of human lactoferrin. Our selected signature peptide with high cleavage

efficiency can be obtained by less enzymolysis time (<1h) than reported by previous literatures (>4h) [17, 18].

Efficiencies of tryptic hydrolysis within five peptides were different. Human lactoferrin is composed of 703 amino acid residues, and it is considered as a large molecular protein [25]. Although disulfide bonds of lactoferrin were sheared by DTT and IAA in the pretreatment process, the secondary structure main composed of hydrogen bond and van der Waals forces still existed. So, the long digestive time of DGAGDVAFIR, EDAIWNLLR and YLGPQYVAGITNLK may be caused by the wrapped restriction sites.

Besides the selection of signature peptide, proper internal standard is necessary for the method accuracy. Some researchers have reported a winged internal standard which is composed of a stable isotope-labeled signature peptide and other non-targeted amino acid residues on both ends of peptide [18-20]. The use of winged internal standard may improve method precision due to its similar tryptic digestion comparing with ananlyzed protein in samples [19]. Accordingly, in this study, isotope-labeled signature peptide VPSHAV*V*AR and its winged peptide internal standard FKDCHLARVPSHAV*V*ARSVNGKE were employed. The results showed that this method was accurate and reproducible for lactoferrin quantification.

3.3. Optimization of preparation procedure for targeted signature peptide

The reduction reaction is the first step of sample preparation, which can damage the tertiary structure of analyted protein. Generally, the concentration of DTT in the

reaction solution is 0.5-5.0 mmol/L, and the reaction temperature below 60 ℃ in this process [18-21]. However, Lactoferrin is difficult to be reduced and digested due to the larger molecular weight and structure [26, 27]. Therefore, stronger reducing conditions should be needed to destroy the structure of the protein.

The conditions of reduction reaction on concentration of DTT and reaction temperature were optimized by the orthogonal experiments. The concentrations of DTT in the reaction solution and the reduction reaction temperatures were designed as 0.5, 1.0, 2.5, 5.0 mmol/L and 50, 60, 70, 80, 90 ℃, respectively. Levels of IAA solution in the reaction solution were three times of concentrations of DDT. Our results showed that lactoferrin detection values were decreased with the increasing concentrations of DTT, and improved with the increasing of reduction reaction temperature. Reduction reaction temperature was main factor that influencing the reduction reaction. The detected values reached the peak when the temperature was at 80 ℃ (Fig. 3). So, the optimized conditions for reduction reaction with orthogonal experiments are 0.5 mmol/L DTT in the reaction solution at 80 ℃.

3.3. Method validation

3.3.1. Specificity

The selected signature peptide VPSHAVVAR only in lactoferrin was showed by the data of online BLAST search in UniProt (www.uniprot.org). The chromatograms of the selected signature peptide from tryptic breast milk spiked with internal standard showed the sharp and symmetric peaks without interferences at 2.27±0.05 min (Fig.

239 4). Without tryptic digestion, there was no targeted peak appeared in the breast milk.
240 All these results indicated that the selected signature peptide VPSHAVVAR had a
241 high specificity for quantitation of human lactoferrin.
242
243 *3.3.2. Linearity and sensitivity*
244 The linear regression equation for levels of targeted signature peptide ranged
245 from 16 to 160 nmol/L was y=1.06023x-0.129415. It had good linearity and
246 coefficient of determination ($r^2 > 0.997$). The amounts of the tryptic signature peptide
247 from the digested samples can be obtained from the standard curve made of the
248 synthetic signature peptide. The contents of human lactoferrin in samples were
249 calculated based on the molar equivalent relationship between the signature peptide
250 VPSHAVVAR and human lactoferrin.
251 The LOD and LOQ calculated as the lowest concentration that provided a
252 signal-to-noise ratio of 3 and 10 were 1 mg/100g and 3 mg/100g, respectively. The
253 linear range and sensitivity could satisfy the quantification requirements for the
254 different lactoferrin concentrations in various breast milk samples.
255
256 *3.3.3. Recovery, intra- and inter-day precision*
257 The synthesized signature peptide standards were spiking to the control diluent
258 of breast milk at 120, 240 and 360 mg/100g, respectively. After pre-treatment
259 analogously with the above optimal preparation, the concentrations of control and
260 spiked samples were quantified and calculated using the theoretical concentrations for

the recovery test. The results showed that the current method had good accuracy with 92.1-97.5% of spiked recoveries and 3.4–4.7% of RSD (Table 2). To evaluate the intra-day and inter-day precision of the method, a diluent of breast milk sample was pre-treated with the above optimal preparation each day on four consecutive days. The RSDs of intra- and inter-day precision were 2.59-4.82% and 2.32-3.89%, respectively.

3.4. Method application

All the samples of collection were pretreated and subjected to UHPLC–MS/MS analysis using current optimized method. The results revealed that there were significant differences ($P<0.05$) between colostrum (282.6 mg/100g), transition milk (174.8 mg/100g) and mature milk ferritin milk (94.2 mg/100g) (Fig. 5). Levels of secreted lactoferrin in breast milk decreased with the extension of lactation. The change of lactoferrin levels in breast milk from Beijing city, China was consistent to previous report [28]. The immune factor, lactoferrin, in human milk undergoes remarkable changes, which may represent adaptations for the recipient infant [29].

4. Conclusions

In this study, an UPLC-TQ-MS/MS method for the absolute quantification of human lactoferrin was developed based on the signature peptides derived from the tryptic hydrolysis. The tryptic fragment peptide VPSHAVVAR was selected and validated as the signature peptide of human lactoferrin due to its high cleavage efficiency and intensity. A winged peptide containing the isotopically-labeled

283 signature peptide was finally chosen as internal standard to compensate ionization
284 suppression and analyte losses in sample preparation. Furthermore, a fast preparation
285 method was established by optimizing the reduction reaction. The results showed that
286 the method was accurate, sensitive and selective. It was successfully applied to
287 routine determination of human lactoferrin in a group of different stages of breast
288 milk in Beijing, China. The data revealed that secretion levels of lactoferrin have a
289 significant decrease with the extension of lactation.
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343

344 **Table 1** MRM parameters of the precursor ion, product ion, cone voltage, collision
345 energy and type of fragment for each candidate signature peptide and IS.

Peptide	Precursor ion *(m/z)*	Cone voltage *(V)*	Product ion *(m/z)*	Collision energy *(V)*	Fragmentation pattern
VPSHAVVAR	312.30	15	326.60	13	y6
			418.70[a]	10	y8
VPSHAV*V*AR	316.30	15	333.10	13	y6
			424.60[a]	10	y8
DGAGDVAFIR	510.89	25	172.90[a]	25	b1
			506.10	20	y4
EDAIWNLLR	565.42	25	515.20	20	y4
			701.30[a]	18	y5
THYYAVAVVK	575.85	25	239.05[a]	20	b2
			912.20	20	y8
YLGPQYVAGITNLK	769.08	30	645.40	30	y6
			716.10[a]	25	y7

346 [a], Quantitative ions

347

348 **Table 2** Recovery test of the UHPLC–MS/MS method (n = 20).

Original level (mg/100 g)	Spiked level (mg/100 g)	Determined level (mg/100 g)	Recovery rate* (%)	RSD (%)
168.7±5.5	120.0	285.7±7.5	97.5	2.6
	240.0	400.2±9.4	96.5	2.4
	360.0	500.1±12.7	92.1	2.5

349 *Recovery rates = (Determined level - Original level) ×100%/Spiked level

350

Figure captions

Fig. 1. Hydrolysis degree curve of different candidate peptides

Fig. 2. Quantitative channels of candidate peptides in breast milk after preparation

Fig. 3. Optimization of DTT concentration and temperature in reduction reaction

Fig. 4. MRM chromatograms of human lactoferrin signature peptide VPSHAVVAR and its corresponding isotope-labeled analog VPSHAV*V*AR from a tryptic human milk

Fig. 5. The content changes of lactoferrin in different duration of lactation

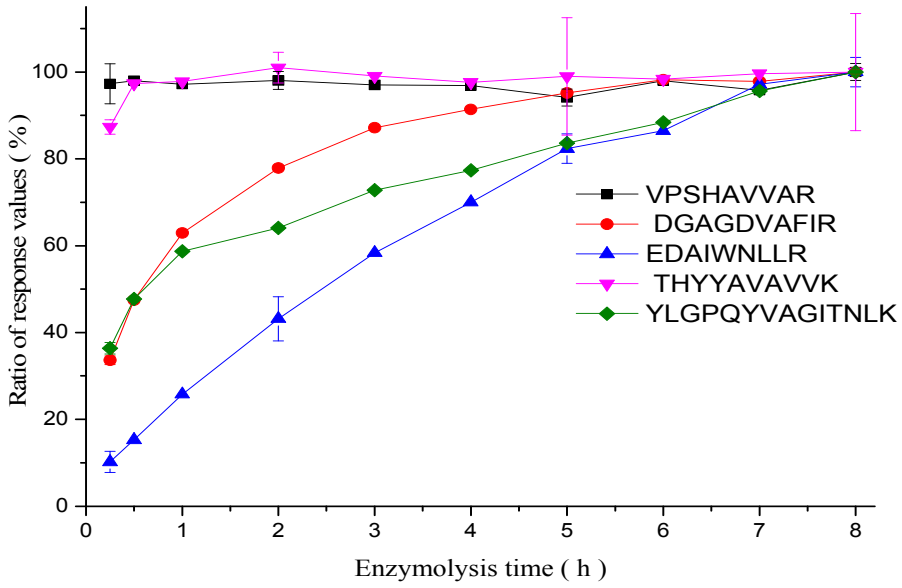

Fig. 1. Hydrolysis degree curve of different candidate peptides

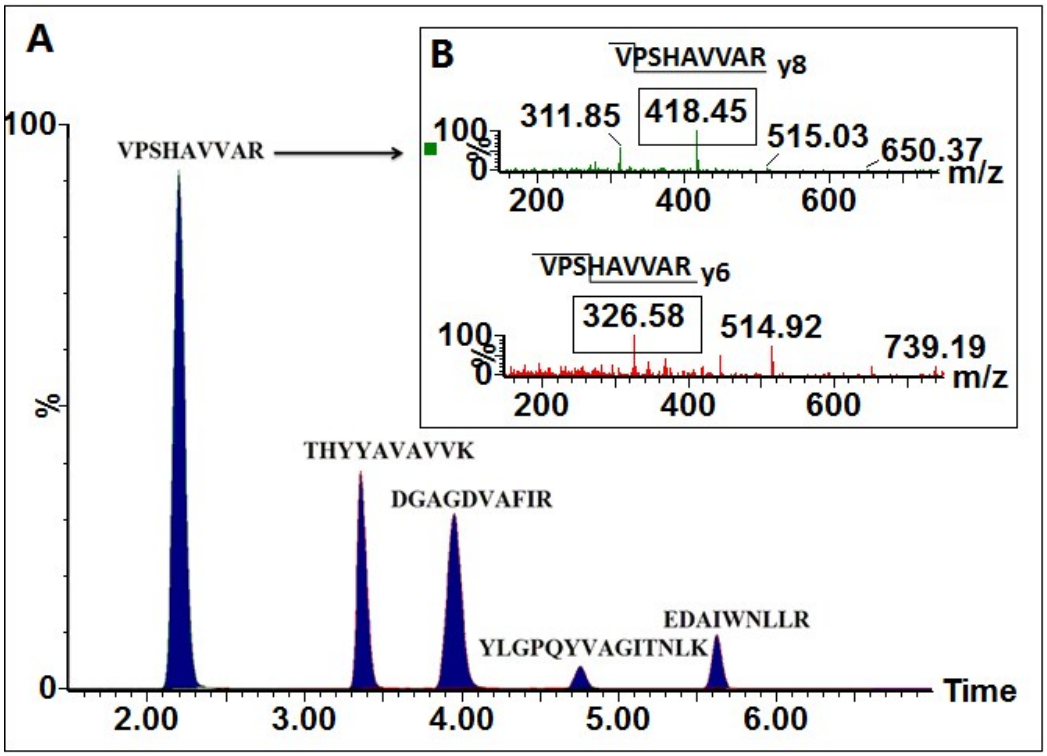

Fig. 2. Quantitative channels of candidate peptides in breast milk after preparation (A) and the fragmentation pattern of targeted signature peptide VPSHAVVAR (B)

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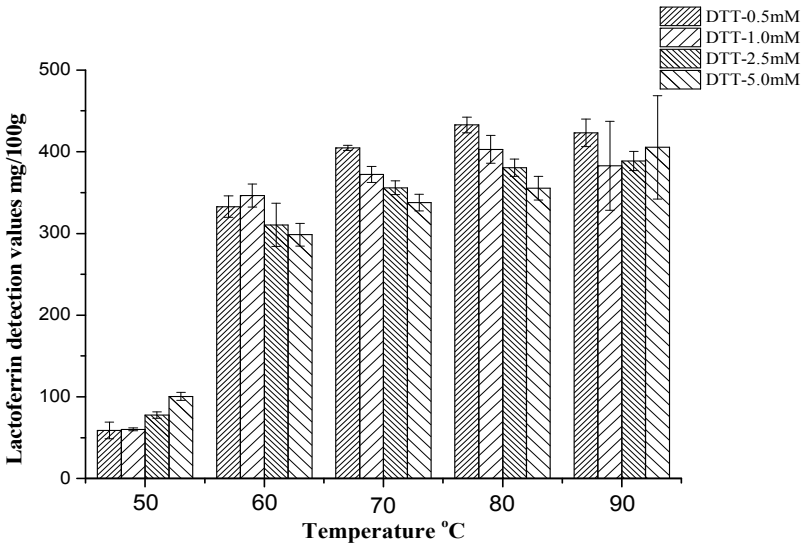

370

371 **Fig. 3.** Optimization of DTT concentration and temperature in reduction reaction

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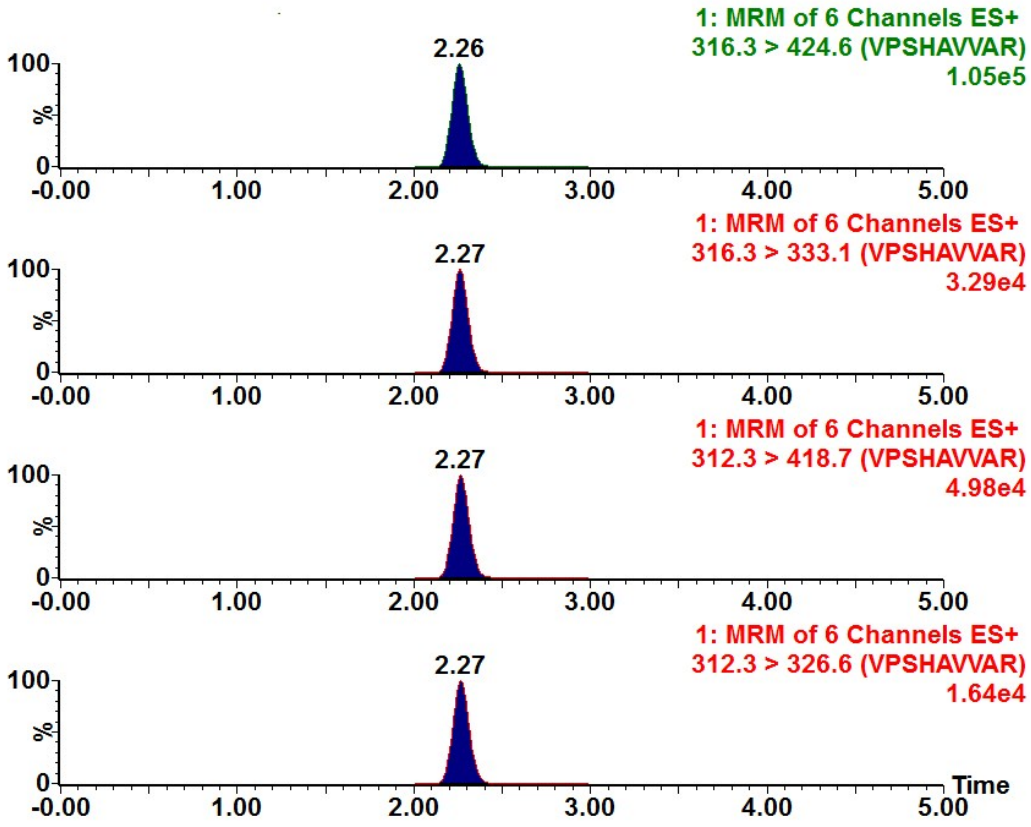

374

375 **Fig. 4.** MRM chromatograms of human lactoferrin signature peptide VPSHAVVAR and its
376 corresponding isotope-labeled analog VPSHAV*V*AR from a tryptic human milk

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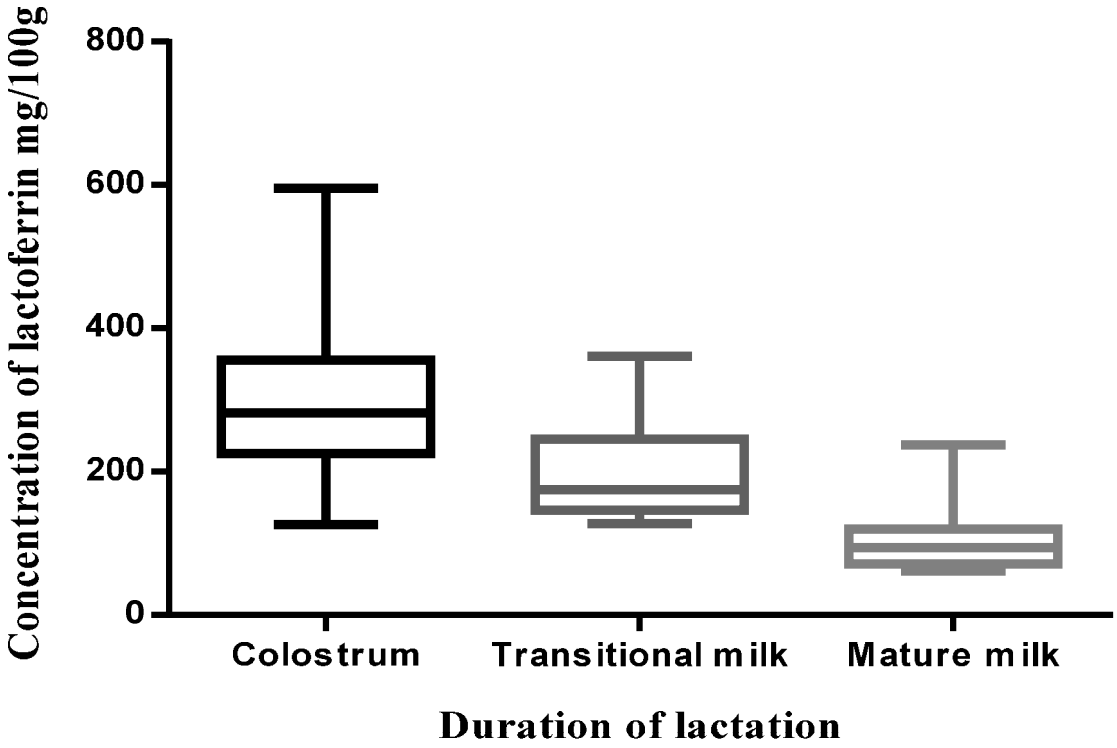

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382 **Fig. 5.** The content changes of lactoferrin in different duration of lactation

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