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Development of a liquid chromatography-tandem mass spectrometry method for simultaneous detection of the main milk allergens

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ABSTRACT

The goal of this study is development and validation of a method to confirm and quantify milk allergens in food products based on liquid chromatography-tandem multiple reactions-monitoring, mass spectrometry (LC-MRM/MS). Here, emphasis was placed on two whey proteins, α -lactalbumin (α -La) and β -lactoglobulin (β -Lg), plus a third, α _{s1}-casein (α _{s1}-CN), known to be the main allergenic components of milk. Five marker peptides (one for α -La, two for β -Lg and two for α _{s1}-CN) and three quantitative marker peptides from the digestion of standard milk proteins were identified using matrix-assisted laser desorption/ionization with tandem time-of-flight mass spectrometry (MALDI-TOF/TOF MS). Optimization of enzymatic hydrolysis conditions were defined as 37 °C for 16 h. The linearity ranges for the three allergenic proteins (α -La, β -Lg and α _{s1}-CN) were 0.97–31.25 μ g/mL, 0.48–31.25 μ g/mL and 0.48–31.25 μ g/mL, respectively. The assays were validated for absolute quantification of three milk proteins with satisfactory results, which indicates that the established mass method is suitable for the expression of levels in daily food.

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1. Introduction

Food allergies have become a serious public health concern, especially within the past few years. According to a recent survey, up to 4%–5% of the total human population in industrialized countries suffers from a clinically-proven food allergy (Mehta, Ramesh, Feuille, Groetch, & Wang, 2014). Cow's milk allergy (CMA) has become a common disease in early childhood, its prevalence ranging from 1.6% to 2.8% among children younger than 2 years of age (Monaci, Tregoat, van Hengel, & Anklam, 2006). The most abundant milk proteins belong to the main classes of caseins and whey accounting for 80% and 20% of the total milk proteins, respectively (J. M. Wal, 1998). Casein (CN) represents the main fraction of milk proteins and is subdivided into a number of families (α , β , κ). They are characterized by a central hydrophobic part and a hydrophilic layer with phosphorylated sites and a hardly clear tridimensional structure suggesting the presence of preferentially

linear epitopes (J. Wal, 2001). α _{s1}-Casein (α _{s1}-CN), a major cow's milk allergen, has nearly 70% unordered structure, with only a small amount of secondary structure, such as α -helix or β -sheets (Chatchatee, Järvinen, Bardina, Beyer, & Sampson, 2001; Cocco, Järvinen, Sampson, & Beyer, 2003). The whey protein α -lactalbumin (α -La) is an important cow's milk allergen (J. M. Wal, 1998), comprising a water-soluble monomeric globular domain primarily α -helical in nature with some α -helix and β -sheet and a hydrophobic core well protected from the solvent in the interior by the rigid packing of side chains (Acharya, Stuart, Phillips, & Scheraga, 1990). β -Lactoglobulin (β -Lg) is the major whey protein of ruminant species and is also present in the milks (Kontopidis, Holt, & Sawyer, 2004), which consists of six different short fragments of the polypeptide chain, which are located especially in the β strands, covering a flat area on the allergen surface (Niemi et al., 2007).

Several analytical approaches for the detection of food allergens are available to-date. Mostly they target the allergenic proteins that indicate the presence of the allergenic food ingredient (Diaz-Amigo, 2010; Monaci, Brohée, Tregoat, & van Hengel, 2011). So immunoassays are currently commonly used for screening

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purposes because antibodies can recognize specific proteins in a complex mixture of compounds of different nature. Some target the DNA fragments of allergenic proteins to indirectly evaluate the allergenic food ingredient (Sun et al., 2015). The development of instruments and methods for accurate sequence-specific DNA analysis is essential for the efficient use of genomic information. Biosensors are applied in evaluation of allergenicity of food ingredient, through establishing the cell-based electrochemical sensor (Jiang et al., 2013) or cell-based fluorescence sensor (Jiang et al., 2015) et al. The enzymatic hydrolysis is incompetent in elimination or reduction of the allergenicity of food products (Panda, Tetteh, Pramod, & Goodman, 2015). In practice enzyme hydrolysis has often not reduced the allergenicity of complex foods and can result in an increase in allergenicity because of exposure of new antigenic epitopes due to protein breakdown (Lee et al., 2007). So the allergenic protein-based detection methods would not be suitable for evaluation of food allergens.

Methods based on mass spectrometry (MS) have advanced significantly in terms of sensitivity and specificity, having improved the identification, characterization, and determination of food allergens (Monaci, Pilolli, De Angelis, Godula, & Visconti, 2014; Shefcheck & Musser, 2004). LC–MS/MS has superior characteristics, improved reproducibility, recovery, sensitivity, dynamic range, and quantification (Koeberl, Clarke, & Lopata, 2014; Weber, Raymond, Ben-Rejeb, & Lau, 2006). In this sense, selected reaction monitoring (SRM) is a highly specific quantitative methodology based on the measurement of specific proteotypic peptide masses (peptide precursor and precursor fragments masses) corresponding to the protein of interest in a triple quadrupole (QQQ) mass spectrometer (Manes, Mann, & Nita-Lazar, 2015; Parker et al., 2015). A proteotypic peptide is defined as a peptide that identifies a protein uniquely; thus, this protein can be specifically quantified by peptide measurement.

This study explored a peptide-based LC–MRM/MS method for simultaneously sensitive determination of multi-allergens in milk samples and milk related food samples. The mainly allergenic protein, α -La, β -Lg and α_{s1} -CN, were treated with enzymolysis of trypsin, before conducted with MS analysis. With optimization of the enzymatic hydrolysis conditions and the design of peptides design, the mass detection method was of implementation with stability, precision and accuracy analysis. After the method verification, the real sample test was conducted with modified mass method.

2. Material and methods

2.1. Chemicals

Milk, cookie, cake and other samples were purchased from the Auchan supermarket (Wuxi, Jiangsu province, China). From Sigma-Aldrich Co. (Shanghai, China), α -lactalbumin (α -La, $\geq 90\%$), β -lactoglobulin (β -Lg, $\geq 85\%$) and α_{s1} -casein (α_{s1} -CN, $\geq 70\%$) were purchased. The peptide fragments used (purity $\geq 85\%$) were obtained from Sangon Biological Technology (Shanghai, China). Trypsase (for HPLC purification) was purchased from Promega (USA). Dithiothreitol (DTT, for HPLC purification) and iodoacetamide (IAA, also for HPLC purification) were purchased from Sigma-Aldrich Co. (Shanghai, China).

1.5 mg/mL standard protein stock solution, composed of 9 mg each α -La, β -Lg and α_{s1} -CN, were added to 6 mL of 25 mmol/L NH_4HCO_3 solution, then stored at -20°C . Next, 0.5 mg/mL standard protein in-process solution was prepared, which was composed of 1 mL standard protein stock solution of α -La, β -Lg and α_{s1} -CN 1 mg/mL synthetic peptides stock solution, containing 1 mg peptides powder, was dissolved in water/acetonitrile (95/5, v/v) solution,

sub-packaged and stored at -20°C . Stock standard solutions of synthetic peptides (1 mg/mL) were prepared in water/acetonitrile (95/5, v/v) and stored at -20°C . Working solutions were freshly prepared by appropriate dilution of the stock solutions. 20 mM Tris-HCl (pH = 8.2) and 25 mM ammonium bicarbonate (pH = 8.0) were prepared with ultrapure water. Trypsin solution of 0.01 $\mu\text{g}/\mu\text{L}$ was prepared by 30 mM acetic acid and stored for approximately 1 month at 20°C . Ultrafiltration was performed with Amicon ultra-15 Centrifugal Filter Devices with a volume of 15 mL and a 3 kDa cutoff (Merck Millipore Ltd. Ireland).

2.2. Sample preliminary treatment

For whole protein extraction from daily food samples (cookie, cake, etc.), 2 g of each sample was diluted with 30 mL of extraction buffer (20 mM Tris-HCl, pH = 8.2). Then the samples were vortexed and incubated at 60°C in a water bath for 2 h with vigorous shaking. The mixture was centrifuged at 14,000 rcf for 25 min at 4°C . The bottom layer was removed, and the supernatant was centrifuged at 14,000 rcf for another 25 min. The supernatant was ultra-filtered through a 3 kDa molecular weight cutoff filter (MWCO) centrifugal filter and washed 3 times with 8 mL of 25 mM Tris-HCl (pH = 8.2). The retained concentrate was collected and the filter washed with 20 mM Tris-HCl (pH = 8.2). The total protein concentrations of extracted fractions were determined by Enhanced BCA Protein Assay Kit. Proteins from milk were separated by gel electrophoresis. Polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 12% separation gel, SDS running buffer, following the manufacturer's instructions (Wilson, Martinez-Villaluenga, & De Mejia, 2008).

2.3. Trypsin digestion

In gel digestion protocol: The gel bands (milk sample) were cut into spot, washed with water for twice. The gel spot were destained two times with 25 mM ammonium bicarbonate in 50% acetonitrile (ACN) followed by reduction with 1 mM Dithiothreitol (DTT) in 25 mM ammonium bicarbonate at 57°C for 1 h. The sample was then allowed to room temperature, iodoacetamide (IAA) solution (25 mM NH_4HCO_3 , 50 mM IAA) were added for alkylation at room temperature in the dark for 30 min. The gel spot then dehydrated with CAN, 20 μL digest solution (0.01 $\mu\text{g}/\mu\text{L}$, trypsin) were added at 37°C for 1 h, the gels were extracted once with 50 μL extraction buffer (5% TFA, 67% ACN) at 37°C for 30 min. The peptides mixture was stored at -20°C for MALDI-TOF-TOF MS identification (Cutillas, Timms, Cutillas, & Timms, 2010).

In solution digestion was performed as follows: 50 μL of the final extract solution was transferred into a 2 mL tube and DTT (1 M in 25 mM ammonium bicarbonate) was added for protein reduction (final concentration 10 mM). The sample was incubated at 60°C for 30 min. The sample was then cooled to room temperature, and IAA (1 M in 25 mM ammonium bicarbonate) were added into the mixture for protein alkylation, and the sample was placed at room temperature in the dark for 30 min. Trypsin digestion was done by adding prepared trypsin (0.01 $\mu\text{g}/\mu\text{L}$) to each sample (1:30, trypsin/protein ratio) and incubated for 16 h at 37°C while mixing. Then 1% FA was added to the mixture to stop digestion. Finally the samples were centrifuged at 12,000 rcf for 20 min at 4°C ; the supernatant was transferred to another micro-centrifuge tube and stored at -20°C before LC–MRM/MS analysis.

2.4. Identification of maker peptides by MALDI-TOF-TOF MS

Characterization of the peptides mixture was achieved using a matrix-assisted laser desorption ionization (MALDI) mass

spectrometer equipped with an electrospray Z spray source and a 355 nm pulsed nitrogen laser. MALDI-TOF-TOF MS (AB SCIEX, USA) analysis was performed in the positive reflector mode with an acceleration voltage of 20 kV. The spectra were obtained in the mass ranges from 500 to 4000 m/z. Based on combined MS and MS/MS spectra, proteins were successfully identified based on 95% or higher confidence interval of their scores in the MASCOT V2.1 search engine (Matrix Science Ltd., London, U.K.), using the following search parameters: Swissprot or NCBI database; taxonomy: Mammalia; trypsin as the digestion enzyme; two missed cleavage site; fixed modifications of Carbamidomethyl; 50 Da for precursor ion tolerance and 0.2 Da for fragment ion tolerance (Sasanya, Abd-Alla, Parker, & Cannavan, 2010).

In the likely event that effect of processing such as high pressure and temperature is found to affect allergen detection by means of MS analysis, it is of importance to establish a link between the presence of specific peptides and the processing history of milk. Commercial food sample purchased from market were chosen for analysis as they undergo treatment of high pressure and temperature. After extraction and SDS-PAGE separation of proteins from the milk samples, the gel digest were performed for the above MALDI-TOE-TOF MS (Lendoiro et al., 2012).

2.5. Liquid chromatography-triple quadrupole mass

Quantification of the peptides were carried out by an ultra-performance liquid chromatography system coupled with a triple quadrupole time of flight mass spectrometer (ACQUITY UPLC/TQD, Waters, USA). The chromatography was performed using a gradient elution with a reversed phase column to separate the complex biological matrix (digested proteins of food samples) from the peptides of interest (Li, Palandra, Nemirovskiy, & Lai, 2009). The chromatographic system consist of a BEH300 C₁₈ analytical column, 2.1 × 100 mm, 1.7 μm particle size (waters, Corp., Milford, USA) for peptides separation and equipped with an auto sampler. The

injection volume was 1 μL. Elution was performed at a flow rate of 0.3 mL/min with: acetonitrile as eluent A and water containing 0.1% FA as eluent B. The solvent gradient used for the analysis of the tryptic digests was as follows: initial: 10% A, 90% B; 0.1 min: 10% A, 90.06% B; 4 min: 100% A, 0.06% B (hold for 2 min); 6.5 min: 10% A, 90.06% B (Miliotis et al., 2011).

MS/MS experiments were carried out on a quadrupole-time-of-flight mass spectrometer. The mass spectrometer was operated in positive ion electrospray ionization with a full scanning range of 20–2000 m/z. Instrumental parameters for mass spectral acquisition were as follows: capillary voltage: 3.5 kV, cone voltage: 35 V, RF: 0.1 V, source temperature: 140 °C, desolvation temperature: 380 °C, desolvation gas flow: 800 L/h, Cone gas flow: 50 L/h, Collision gas flow: 0.10 mL/min (Monaci, Losito, Palmisano, & Visconti, 2011).

Marker peptides, derived from α_{s1}-CN, β-Lg and α-La and their internal peptides (IPs) were detected specifically by monitoring UPLC elution times and ion pairs corresponding to the precursor and specific product ion mass/charge ratios (m/z) on a triple quadrupole mass spectrometer operated in the positive ion MRM mode (Ansari, Stoppacher, Rudolf, Schuhmacher, & Baumgartner, 2011; Kamiie et al., 2008). Transitions, cone voltage and collision energy were manually optimized by direct infusion of each marker peptide and their IPs (2 μg/mL in 30% acetonitrile in water). Two transitions per peptide were monitored simultaneously for quantitative and qualitative analysis of α_{s1}-CN, β-Lg and α-La. Data acquisition and integration of the peak areas of the MRM signal for marker peptides derived from α_{s1}-CN, β-Lg and α-La their IPs were processed with Masslynx software version 4.1 (waters, Corp., Milford, USA).

2.6. Preparation of calibration curves and QCs

To develop a quantitative method using non-isotopic IS, it is important to prepare a blank control containing no targeted

β-lactoglobulin					
LIVTQTMKGL	DIQKVAGTWY	SLAMAASDIS	LLDAQSAPLR	VYVEELKPTP	50
LIVTQTMKGL	DIQKVAGTWY	SLAMAASDIS	LLDAQSAPLR	VYVEELKPTP	50
EGDLEILLQK	WENDECAQKK	IILAETKIPA	VFKIDALNEN	KVLVLDTDYK	100
EGDLEILLQK	WENDECAQKK	IILAETKIPA	VFKIDALNEN	KVLVLDTDYK	100
EYLLFCMENS	AEPEQSLVCQ	CLVRTPEVDD	EALEKFDKAL	KALPMHIRLS	150
EYLLFCMENS	AEPEQSLVCQ	CLVRTPEVDD	EALEKFDKAL	KALPMHIRLS	150
FNPTQLEEQC	HI Bovine β-Lg A				162
FNPTQLEEQC	HI Bovine β-Lg B				162
α-lactalbumin					
EQLTKCEVFR	ELKDLKGYGG	VSLPEWVCTT	FHTSGYDTQA	IVQNNDSTEY	50
GLFQINNKEW	CKDDQNPSS	NICNISCDF	LDDDLTDDIM	CVKKILDKYG	100
INYLWLAHKAL	CSEKLDQWLC	KEL			123
α-s1casein					
RPKHPKHQGG	LPQEVINENL	LRFFVAPFPE	VFGKEKVNEL	SKDIGSESTE	50
DQAMEDIKQM	EAESISSEE	IVPNSVEQKH	IQKEDVPSE	YLGYLEQLLR	100
LKKYKVPQLE	IVPNSAEERL	HSMKEGIHAQ	QKEPMGVNQ	ELAYFYPELF	150
RQFYQIDAYP	SGAWYYVPLG	TAYTDAPSFS	DIPNPIGSEN	SEKTTMPLW	199

Fig. 1. Amino acid sequence of α-La, β-Lg and α_{s1}-CN, and peptides identified by MALDI-TOF are highlighted in bold, underlined residues overlap with known IgE epitopes.

Table 1
The selected peptide sequence of the three protein.

Protein	Calc. mass	Obsrv. mass	±ppm	Peptide sequence	Start-end
α_{s1} -CN	1267.7045	1267.7233	15	<u>YLGYLEQLLR</u>	106–115
	1384.73	1384.748	13	FFVAPFPPEVFGK	38–49
	1759.9449	1759.9652	12	<u>HQGLPQEVLENLLR</u>	23–37
	1871.9861	1872.0027	9	YKVPQLEIVPNSAEER	119–134
	1336.6270	1336.6735	–4	HIQKEDVPSEER	95–105
	2235.2356	2235.2500	6	HPIKHQGLPQEVLENLLR	19–37
	2332.1318	2332.1384	2	EPMIGVNQELAYFYPELFR	148–166
	3207.593	3207.585	–2	EGIHAAQQKEPMIGVNQELAYFYPELFR	140–166
	932.5365	932.5216	–7	<u>LIVTQTMK</u>	1–8
	1586.9066	1586.9325	3	LIVTQTMKGLDIQK	1–14
β -Lg	2707.376	2707.2468	10	VAGTWYSLAMAASDISLLDAQSAPLR	15–40
	1634.7721	1634.7675	4	<u>TPEVDDEALEKFDK</u>	125–138
	2818.2666	2818.123	–51	YLLFCMENSAAPEQSLACQCLVR	152–174
	1200.6525	1200.6412	–9	<u>VGINNYWLAHK</u>	99–108
α -La	1204.6031	1204.6238	17	LDQWLCEKL	115–123
	1715.7504	1715.7336	–10	FLDDDLTDDIMCVK	80–93
	2003.8182	2003.8193	1	DDQNPHSSNICNSCDK	63–79

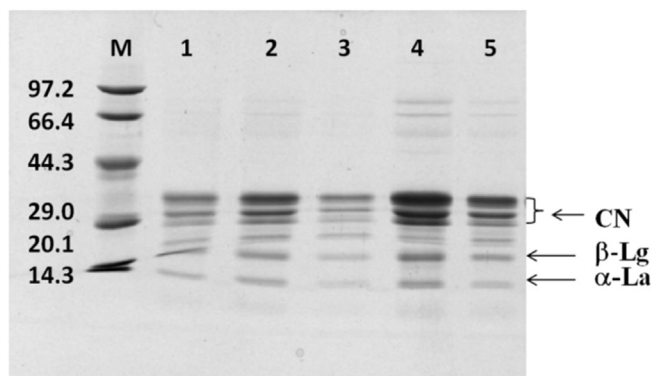


Fig. 2. SDS-PAGE profiles of milk allergens in milk and milk products, 1: modulation whole milk; 2: UHT milk; 3: milk cookies; 4: pasteurized milk; 5: milk powder; M: protein marker (14.3–97.2 kDa).

proteins. For this purpose, the extracted wheat protein fraction was used as the blank matrix to prepare standard solutions (α_{s1} -CN, β -Lg and α -La) for calibration curve and quality control samples (QCs). 500 μ L of three standard solutions (1.5 mg/mL for α_{s1} -CN, β -Lg, and α -La, respectively) was prepared by mixing wheat protein fraction

with the required amount of standard α_{s1} -CN, β -Lg and α -La from bovine milk. Then a mixed stock solution (500 μ g/mL each protein) for calibration curve was prepared by mixing 200 μ L standard solutions (1.5 mg/mL) together. For the determination of the concentration of α_{s1} -CN, β -Lg, α -La in the food samples, the LC-MS/MS method in MRM mode was calibrated by analyzing 12 levels of mixture: 0.1, 0.5, 1, 5, 15, 20, 25, 30, 35, 40, 50 and 100 μ g/mL. The quality control samples (QCs) were prepared by mixing the blank matrix with standard proteins at 3 concentration levels: 1, 25 and 50 μ g/mL. The concentration of IPs: YLGYLEQLLR, TPEVDDEALEK, VGINNYWLAHK were optimized to ensure appropriate mass signal response to the target peptide of α_{s1} -CN, β -Lg, α -La. On each day of analysis, calibration curves and QC samples mixed with the stable internal peptides (final concentration: 1.5 μ g/mL) were freshly prepared as mentioned above.

3. Results and discussion

3.1. Selection of specific peptide for allergen protein

After enzymolysis of α -La, β -Lg and α_{s1} -CN with standard protease, characterization of the peptide fragments was conducted using MALDI-TOF/TOF MS. Amino acid sequences from three kinds of milk allergen proteins appear in Fig. 1, which illustrates that the

Table 2
Q1/Q3 transitions of 8 target peptides and Inter standard selected for the MRM experiments.

Protein name	Retention time (min)	Peptides sequence	Precursor ions (Q1)	Product ions (Q3)	Cone voltage (V)	Collision energy (eV)
β -Lg	1.98	<u>LIVTQTMK</u>	467.5	199.20	25.0	19.0
				227.20 ^a	25.0	15.0
	1.84	TPEVDDEALEK	623.8	199.20 ^{a/q}	35.0	35.0
				1048.20	35.0	30.0
	1.84	TPEVDDEALEK	659.3	199.20	40.0	30.0
α -La				608.40 ^{a/q}	40.0	25.0
	2.23	VGINNYWLAHK	601.1	931.5	37.0	25.0
				654.4 ^{a/q}	37.0	25.0
	2.21	<u>VGINNYWLAHK</u>	658.2	931.5	37.0	28.0
				284.3 ^{a/q}	37.0	27.0
α_{s1} -CN	2.23	HQGLPQEVINENLLR	587.6	436.30 ^a	25.0	20.0
				758.20	25.0	20.0
	2.67	<u>YLGYLEQLLR</u>	634.6	249.20 ^{a/q}	30.0	25.0
				991.30	30.0	25.0
	2.60	YLGYLEQLLR	627.8	758.00 ^{a/q}	35.0	26.0
				978.00	35.0	26.0

a, selected "label candidate peptides" for further MS detection.

q, selected "label candidate peptides" for quantification of allergen protein.

Sequence with underline for known allergen protein epitopes.

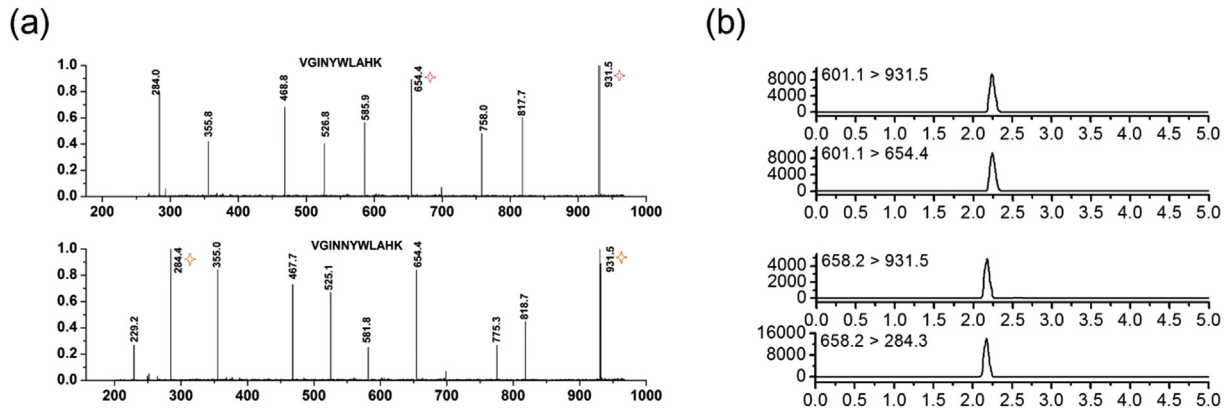


Fig. 3. (a) MS/MS spectra of QDPs and IPs, highlights the product ions selected for MRM and (b) extracted MRM ion chromatogram of the marker peptides and internal peptides of allergenic protein α -La.

coverage rates of characterized peptide sequences were approximately 37% for α -La, 45% for β -Lg and 46% for α _{s1}-CN. In total, 17 peptide sequences were identified, including four peptide sequences from α -La, five peptide sequences from β -Lg and eight peptide sequences from α _{s1}-CN. The identified peptide sequences partly included published allergen epitopes, which represent significant allergen protein detection (Ansari et al., 2011). If the specific peptide sequences detected by LC-MRM/MR are allergen epitopes, the analysis results might be used evaluate food allergenicity. To establish a stable MRM quantitative detection method, the specific peptides selected from the identified enzymatic hydrolysis peptides must have excellent stability and sensitivity as characteristic peptides. Those peptides of greater abundance were selected as “label candidate peptides” for further specificity analysis. For specificity analysis of “label candidate peptides,” the protein blast tool from the NCBI database (NCBI, 2014) (<http://blast.ncbi.nlm.nih.gov>) was applied, based on the principle that the selected “label candidate peptides” are unique to the corresponding protein, and do not exist in other proteins. The selected “label candidate peptides” are VGINYWLAHK (α -La), LIVTQTMK (β -Lg), TPEVDDEALEK (β -Lg) and HQGLPQEVINENLLR (α _{s1}-CN),

YLGYLEQLLR (α _{s1}-CN), in Table 1. As inter-species homologues, the selected label candidate peptides are also present in corresponding proteins of non-human species, mainly ruminants. However, we found no effect from species non-specificity on the detection results. Considering that protein structure can be affected by food processing, allergenic milk proteins extracted from milk and biscuit sample were separated by SDS-PAGE, Fig. 2. Throughout collection, enzymatic hydrolysis, and peptide sequence analyses, the selected label candidate peptides illustrated excellent stability.

3.2. Optimization of MRM conditions

To define optimal MRM conditions, synthetic peptide standard solutions were directly injected into the mass spectrometer. First, the precursor ions of peptide fragments were confirmed under optimized cone voltage, then collision energy parameters were optimized for further MS/MS analysis of peptides (Hammad, Saleh, Novotny, & Mechref, 2009). Two stable and sensitive characteristic ions were selected from the product ions fragmentation to establish MRM-detecting ions. Under the optimized MRM conditions, these peptides were detected for qualification and quantification of

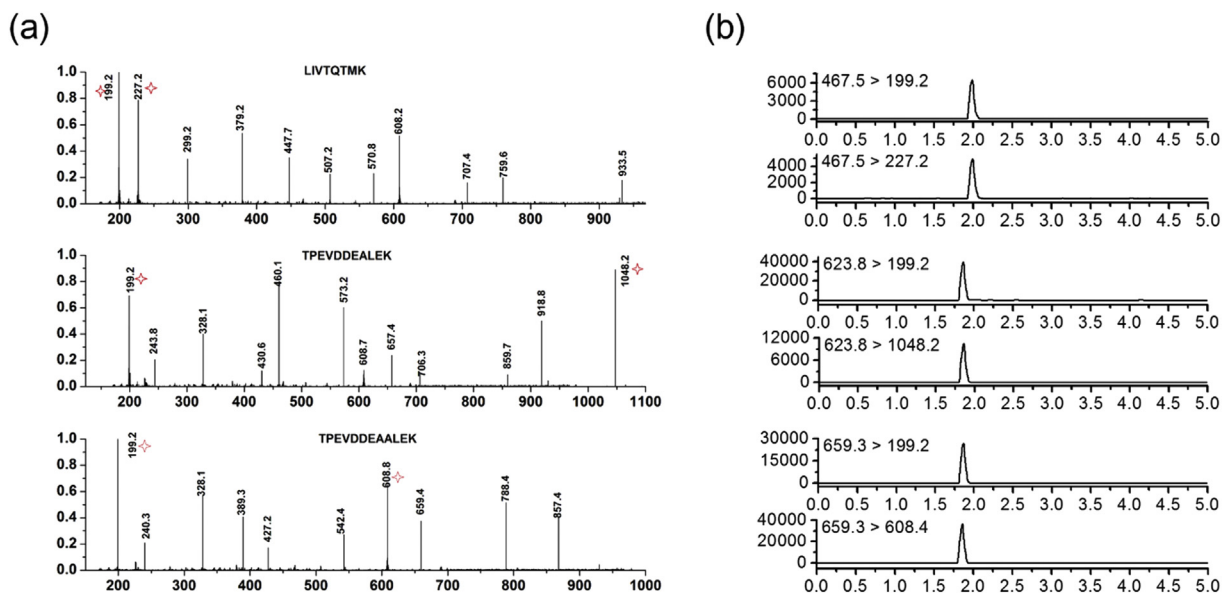


Fig. 4. (a) MS/MS spectra of QDPs and IPs, highlights the product ions selected for MRM and (b) extracted MRM ion chromatogram of the marker peptides and internal peptides of allergenic protein β -Lg.

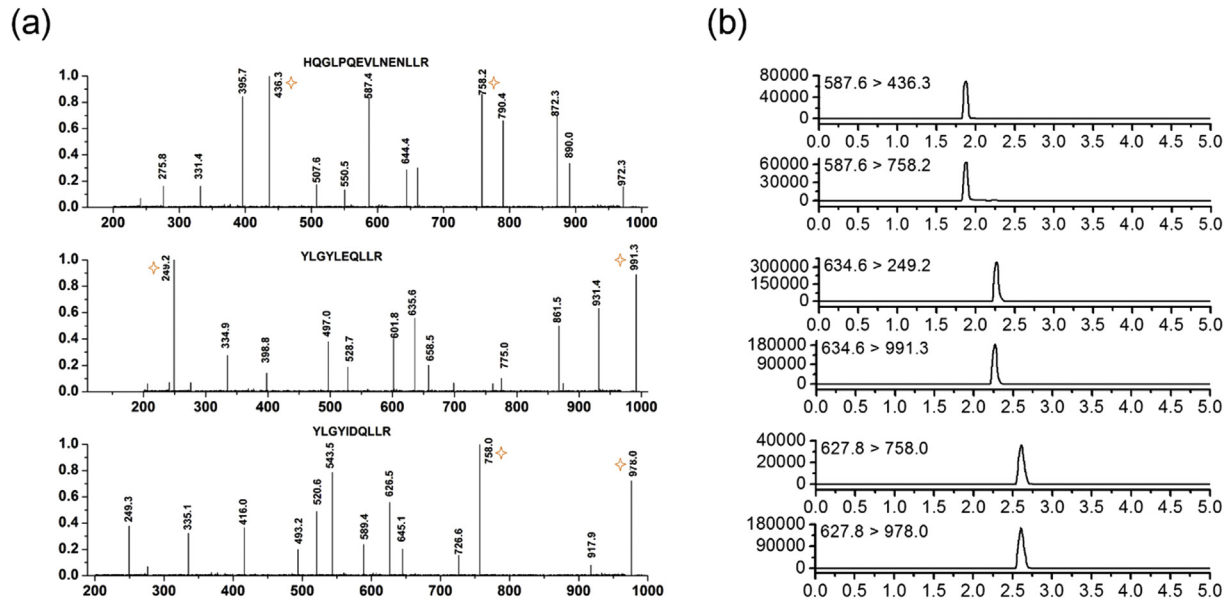


Fig. 5. (a) MS/MS spectra of QDPs and IPs, highlights the product ions selected for MRM and (b) extracted MRM ion chromatogram of the marker peptides and internal peptides of allergenic protein α_{s1} -CN.

allergen proteins. Table 2 describes the optimized experimental parameters. Although retention times of some peptide sequences were arrived at by superposition, their molecular weights were diverse, above 20 Da, and do not generate interference under MS detection.

3.3. Internal peptides design

In order to minimize the matrix effect on the LC-MRM detection results, internal peptides (IPs) were designed. Isotope-labeled and label-free quantitative protein detection

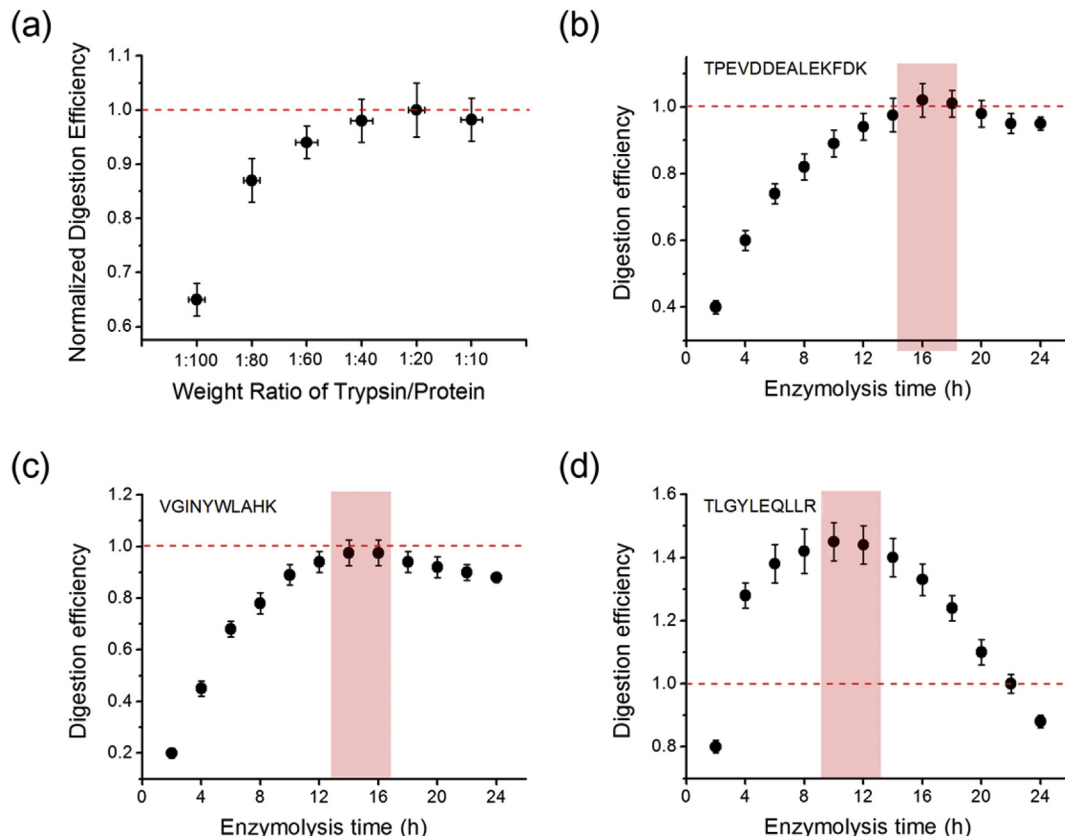


Fig. 6. (a) The optimization of weight ratio of trypsin/protein; The optimization of enzymolysis time of β -Lg (b), α -La (c), and α_{s1} -CN (d).

Table 3

The quantification equation, LOD and LOQ of the three proteins.

Protein name	QDPs and IPs	Linear range	Quantification equation	LOD (S/N > 10) µg/mL	LOQ (S/N > 10) µg/mL	R ² value
β-Lg	TPEVDDEALEK	0.48–31.25	y = 0.213x – 0.0345	0.20	0.48	0.9983
α-La	TPEVDDEALEK VGINYWLAHK VGINNYWLAHK	0.97–31.25	y = 0.142x – 0.266	0.39	0.97	0.9921
α _{s1} -CN	YLGYLEQLLR	0.48–31.25	y = 0.1965x + 0.1378	0.20	0.48	0.9934

Table 4

Comparison with other published allergen detection methods.

Methods	Targets	Linear range µg/mL	LOD (S/N > 10) µg/mL	LOQ (S/N > 10) µg/mL	Ref.
Peptide-based LC-MRM/MS	β-Lg, α _{s1} -CN peptides	0.97–31.25	0.20	0.48	This work
Electrochemical immunosensor	Casein protein	0.1–10	0.05	–	(Cao et al., 2011)
ELISA	Caseins protein	1.6–25.6	1	–	(Monaci, Brohé, et al., 2011)
LC-MS/MS	Lactalbumin protein	5–40	1	4	(Monaci & van Hengel, 2008)
Peptide-based LC-MRM/MS	α _{s1} -CN peptides	–	10	–	(Heick, Fischer, & Pöpping, 2011)
DNA sensor	Ara h1 gene	3.91 × 10 ^{−17} –1.25 × 10 ^{−15} mol/L	1.3 × 10 ^{−17} mol/L	–	(Sun et al., 2015)

were the two main strategies applied here. Label-free quantitative detection was conducted by replacing amino acids to design internal peptides, whose detection results possessed precision similar to isotope-labeled quantitative detection (Tang et al., 2013). The respective quantitative marker peptides (QMPs) for β-Lg (Fig. 3), α-La (Fig. 4) and α_{s1}-CN (Fig. 5) proteins are TPEVDDEALEK (623.8/199.2, 623.8/1048.2), VGINYWLAHK (601.1/931.5, 601.1/654.4) and YLGYLEQLLR (634.6/249.2, 634.6/991.3). Referring to the published literature (Chen, Chen, & Hsieh, 2015; Nongonierma & Fitzgerald, 2015), we obtained respective IPs for the three mentioned proteins thus: TPEVDDEALEK (659.3/199.2, 659.3/608.4), VGINNYWLAHK (658.2/931.5, 658.2/284.3) and YLGIDQLLR (627.8/758.0, 627.8/978.0). To evaluate the specificity of the designed IPs, BLAST was exploited. IPs used to deduce the matrix effect on experimental error need to be equipped with properties similar to the intended analyte. The behaviors of QMPs and IPs under chromatography and mass spectrometry were studied, which illustrate that the QMPs and IPs behave similarly under chromatography and mass spectrometry, including ion fracture mode and retention time. As well, the retention times of the three IPs did not create interference, which indicates that the three IPs are suitable for further quantification detection (Ansari, Stoppacher, & Baumgartner, 2012).

3.4. Optimization of enzymatic hydrolysis conditions

Digestion efficient using trypsin is crucial in LC-MRM/MS method for absolute quantification of protein in biological matrices. Generally, the inefficient tryptic digestion could result in inaccurate quantification, when LC-MRM/MS is used to detect the peptides as the alternative of target protein. In our work purified α-casein were used as calibration standards to confirm the complete digestion. To achieve the optimum digestion of α-casein of interest, digestion of the purified α-casein were investigated at the presence of blank matrix at a series weight ratio of trypsin to protein (1:10, 1:20, 1:40, 1:60 1:80 and 1:100) for 15 h at 37 °C, followed by LC-MRM/MS analyses. Fig. 6(a) demonstrated that the optimal weight ratio of trypsin to protein for efficient digestion of α-casein (an example) in blank matrix is ~1:20. Sample α-La, β-Lg and α_{s1}-CN mixtures of approximately 30 µg/mL each were subjected to trypsin

enzymatic hydrolysis in series for 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 h. By analyzing the abundance of the three peptides, VGINYWLAHK, TPEVDDEALEK and YLGYLEQLLR, the degrees of enzymatic hydrolysis of α-La, β-Lg and α_{s1}-CN proteins were characterized. Fig. 6(b–d) illustrates that the optimal enzymolysis time for VGINYWLAHK, TPEVDDEALEK is 16 h, at which point α-La, β-Lg completely enzymolyzed. Due to differences in protein structure, it takes approximately 12 h to complete enzymolysis of α_{s1}-CN protein. With comprehensive consideration, the optimal enzymatic hydrolysis conditions are 37 °C, for 16 h.

3.5. Accuracy analysis

The specific MRM transitions based on the chromatographic retention time and MS spectrum were used to detect and quantify target protein by LC-MRM/MS. Using the optimized MRM acquisition parameters and the previously optimized digest condition, calibration curve for quantitation was carried out by standard protein in blank matrix. For each peptide analyzed by LC-MRM/MS, the peak area was normalized to that of the internal standard peptide. Eight-point calibration curve (analyte-to-IPs peak area ratio to concentration) was conducted by linear regression analysis with 1/x² (x = analyte concentration)

Table 5

Intra-day and Inter-day precision and accuracy validation.

Days	Protein name	Concentration of quality samples (µg/mL)					
		1 ± 0.05		20 ± 0.1		40 ± 0.1	
		RE%	RSD%	RE%	RSD%	RE%	RSD%
1 (n = 3)	β-Lg	−0.89	4.48	7.33	1.86	7.67	10.22
	α-La	−2.11	2.89	−2.64	3.63	1.4	5.36
	α _{s1} -CN	−1.01	3.56	3.71	3.38	3.27	1.84
2 (n = 3)	β-Lg	3.11	7.28	−0.73	6.74	−5.12	4.43
	α-La	4.78	3.98	5.6	2.91	−0.4	3.7
	α _{s1} -CN	2.78	5.24	−2.04	4.17	3.95	3.21
3 (n = 3)	β-Lg	1.15	7.42	6.51	6.69	−1.64	7.27
	α-La	6.22	9.50	3.73	3.98	4.74	4.99
	α _{s1} -CN	−0.33	4.42	0.82	0.53	4.98	11.11
Totally (n = 9)	β-Lg	0.7	2.06	4.7	4.53	0.43	7.78
	α-La	2.96	4.32	2.22	4.22	1.91	2.56
	α _{s1} -CN	4.81	2.01	8.29	2.85	4.73	1.86

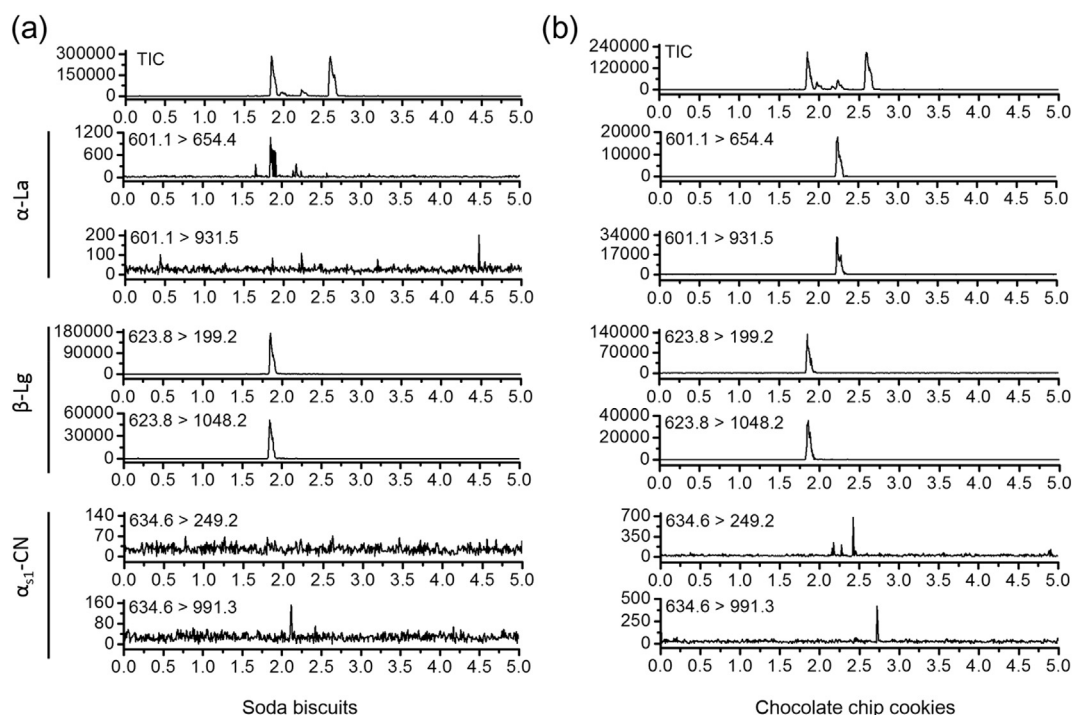


Fig. 7. The real sample test. The LC-MRM/MS of (a) soda biscuits and (b) chocolate chip cookies.

weighting over a calibration range of 0.49–50 $\mu\text{g/mL}$, Fig. S1. The calibration curve calculation was displayed in Table 3, and the comparison between the published allergen detection methods and the established LC-MRM/MS method is shown in Table 4. Three samples from each group, at high, medium and low concentration, were enzymolyzed and subjected to MRM detection to evaluate the intra-day and inter-day accuracy over three days. Protein concentration was calculated with a freshly-prepared standard curve, as shown in Table 5. Results indicate that the intra-day and inter-day precision values for quality control samples at three different concentrations were below 20%.

3.6. The real sample test

To detect allergenic proteins from milk, biscuit and other food,

samples were purchased from a local supermarket named Auchan, Wuxi, Jiangsu province, China. After the pretreatment process mentioned above, the sample solution was diluted for chromatography-mass spectrometry analysis. Fig. 7 depicts the extracted total ion chromatograms and QMPs and IPs chromatograms for soda biscuits and chocolate chip cookies. As observed from said figure, despite abundant impurities in the real samples, the MRM detection mode was able to remove interference by impurity ions through the ion selection process. Table 6 shows the allergenic protein detection results of food samples through QMP concentration detection. No target peptides were generated in the ion channel of wheat flour extract, which was selected as a blank matrix. The value of allergenic protein/extracted total protein was calculated to describe the allergenic protein content. The detection results for the three target allergenic proteins of food samples

Table 6
Concentrations of α -La, β -Lg and α s1-CN ($\mu\text{g/g}$ total protein) detected in real food samples.

Food names	Detected α -La expression levels (Mean $\pm$ SD $\mu\text{g/mL}$)			Detected β -Lg expression levels (Mean $\pm$ SD $\mu\text{g/mL}$)			Detected α s1-CN expression levels (Mean $\pm$ SD $\mu\text{g/mL}$)		
	601.1 > 931.5	601.1 > 654.4	Content of α -La ($\mu\text{g/g}$)	623.8 > 119.2	623.8 > 1048.2	Content of β -Lg ($\mu\text{g/g}$)	634.60 > 249.2	634.60 > 249.2	Content of α s1-CN ($\mu\text{g/g}$)
Milk power	3.59 $\pm$ 0.21	5.12 $\pm$ 0.37	3.46 $\pm$ 0.31	28.66 $\pm$ 0.34	10.07 $\pm$ 0.91	75.32 $\pm$ 0.48	24.77 $\pm$ 0.17	11.09 $\pm$ 1.07	247.92 $\pm$ 30.65
Chocolate chip cookies	2.44 $\pm$ 0.16	3.76 $\pm$ 0.54	2.54 $\pm$ 0.29	18.44 $\pm$ 0.11	6.72 $\pm$ 0.44	48.46 $\pm$ 0.27	NP	NP	ND
Soda biscuits	NP	NP	ND	22.37 $\pm$ 0.19	7.14 $\pm$ 0.64	58.79 $\pm$ 0.51	NP	NP	ND
Stuffing of yolk pie	<0.97	<0.97	ND	<0.48	<0.48	ND	<0.48	<0.48	ND
Surface of yolk pie	NP	NP	ND	NP	NP	ND	NP	NP	ND
Patisseries	<0.97	<0.97	ND	2.56–14.78	<0.48	ND	<0.48	<0.48	ND
Waffles	<0.97	<0.97	ND	<0.48	<0.48	ND	<0.48	<0.48	ND
Peanut jam	NP	NP	ND	NP	NP	ND	NP	NP	ND
Wheat meal	NP	NP	ND	NP	NP	ND	NP	NP	ND

NP: No Peaks, under the LOD.

ND: Not detective, under the LOQ.

coincide with the label components, which confirms that the developed LC-MRM/MS-based method can be applied to confirm and quantify milk allergens in food products.

4. Conclusion

An LC-MRM/MS-based method was developed and validated to confirm and quantify three main milk allergens in food products: α -lactalbumin (α -La), β -lactoglobulin (β -Lg) and α _{s1}-casein (α _{s1}-CN). After optimization of enzymatic hydrolysis conditions, the three milk allergen proteins were analyzed using MALDI-TOF/TOF MS. After confirmation of precursor ions and product ions, cone voltage and collision energy, the optimized MRM parameters for the selected peptides to detect α -La and β -Lg and α _{s1}-CN were determined. The assays were validated for absolute quantification of three milk proteins with satisfactory results, which indicated that the established mass method could be applied to the expression of levels in daily food.

Notes

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.foodcont.2016.11.030>.

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Supplementary Data

Development of a Liquid Chromatography-Tandem Mass Spectrometry Method for **Simultaneous** Detection of the Main Milk Allergens

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24 **Figures and Tables:**

25 Figure S1. The quantification equation of the three protein

26 Table S1 Results of Stability Tests

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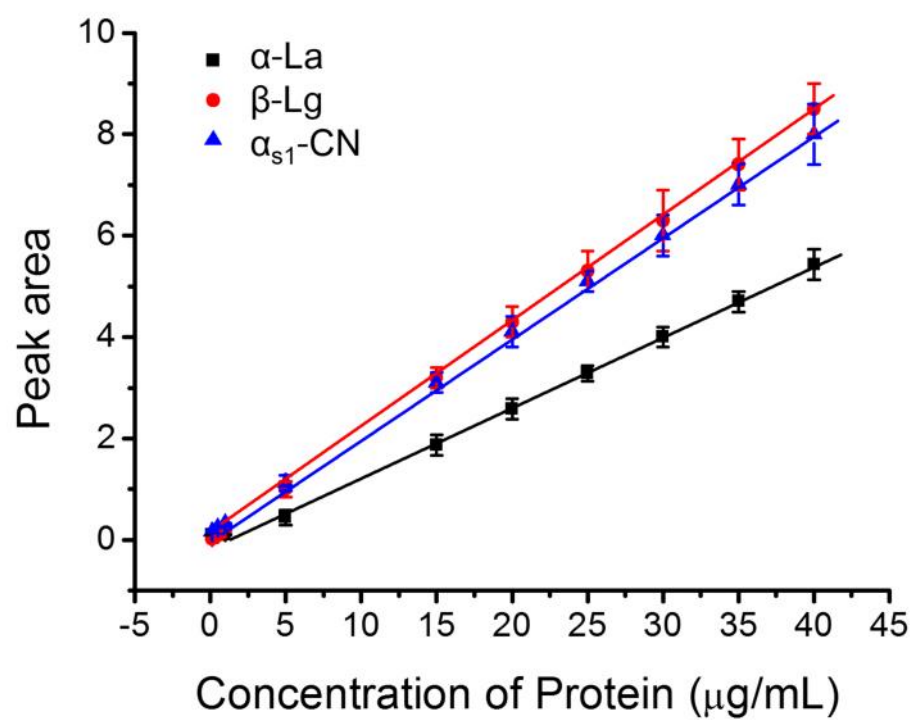


Figure S1. The quantification equation of the three protein

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Table S1 Results of Stability Tests

Protein Name	Short-term Stability 25 °C for 2 h	Automatic sampler Stability 4 °C for 24 h	Enzymolysis Stability 37 °C for 16 h
β -Lg	91.13-96.08	97.4-102.5	87.0-105.9
α -La	86.9-100.5	101.2-112.3	91.0-98.3
α_{s1} -CN	92.4-109.7	103.4-118.9	94.7-110.4

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