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RESEARCH PAPER

Quantitative analysis of cow whole milk and whey powder adulteration percentage in goat and sheep milk products by isotopic dilution-ultra-high performance liquid chromatography-tandem mass spectrometry

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Abstract The aim of the study was to develop a method for quantification of cow's whey and whole milk powder in goat or sheep milk products including infant formula. A ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) method was established for simultaneous quantification of four caseins and two major whey proteins by detecting their signature peptides, which were able to act as markers for differentiating goat or sheep from cow whey and whole milk powder in infant formulas. The signature peptides were screened based on 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 isotopic-labeled signature peptide was used as internal standard to compensate the matrix effect. The limits of quantification were 0.01–0.05 g/100 g for target proteins. The observed recovery rates ranged from 82.3 to 116.6 % and the reproducibility was excellent (RSD <12 %) at different spiking levels. The RSDs of intra- and inter-day precision were 2.8–6.2 and 3.3–9.8 %, respectively. The multiple reaction monitoring method was successfully applied to milk powder with different composition, showing high specificity and accuracy in detection of species involved. The calculating formula was designed to assess the composition of adulteration in the actual detection of infant formulas. These results highlight applicability of this method for the detection of infant formulas with complicated matrix.

Keywords Adulteration · Quantification · Goat and sheep · Infant formula

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Introduction

Raw milk is used to prepare a multitude of dairy products that are characterized by different nutritional values and specific features, depending on the originating dairy animal species and technological processes. Cow milk contains more than 20 allergenic proteins, in which casein and β-lactoglobulin are reported to cause most allergenic effects [1, 2]. Goat and sheep milk products appear to be potentially less allergenic and have consequently become important as a substitute for people (including children) suffering from cow milk intolerance [3]. Furthermore, whey proteins in goat and sheep milk themselves are increasingly being recognized for their bioactivity or health-promoting benefits, such as immunomodulatory, anti-microbial, and transfer of passive immunity activities [4]. Infant formula made by goat milk is considered as an appropriate alternative for infants who cannot be served with breast milk [5].

However, the seasonal availability and the low yield of goat and sheep milk, together with the low price of bovine milk make goat and sheep milk products attractive targets for deliberate adulteration [6]. Apart from economic loss, the adulterations may have serious consequences because consumers

may be exposed to invisible allergens such as cow milk proteins containing β-lactoglobulin [7]. Such a circumstance has prompted research in new advanced methods for detecting species origin of dairy products.

For milk and dairy products, the common identifiable techniques can be classified into two kinds of methods based on DNA analysis and milk proteins, respectively. Polymerase chain reaction (PCR) was a useful qualitative tool for the quality control in food industry. PCR-based methods were designed and applied to detect cow milk in goat and sheep cheeses [8, 9]. Recently, different protein-based methods for species identification in milk and dairy products have been developed using electrophoresis, immunological strategies, chromatography, or mass spectrometry. The most commonly employed electrophoretic techniques are polyacrylamide gel electrophoresis [10–13], capillary isoelectric focusing [11], two-dimensional electrophoresis [14], and capillary electrophoresis (CE) [15]. Enzyme-linked immunosorbent assay (ELISA) is a rapid, sensitive, and specific method for the detection of cow milk in adulterated samples. The native cow milk proteins in adulterated goat and sheep milk products have been successfully detected by ELISA [16, 17]. Liquid

Table 1 Precursor ion, product ion, cone voltage, collision energy, and fragment type for each candidate signature peptide and its corresponding internal standard

Peptide	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (*V*)	Fragmentation pattern	Retention time (min)
CEVFR	355.8	175.4[a]/322.4[b]	18/15	y1/y2	4.62
CEV*F*R	363.9	332.4[a]/437.5[b]	18/15	y2/y3	4.62
NICNISCDK	562.2	228.1[a]/896.6[b]	18/15	b2/y8	4.65
NI*CNI*SCDK	569.3	235.4[a]/903.4[b]	18/15	b2/y8	4.65
LSFNPTQLEEQCHI	858.6	462.2[a]/685.1[b]	26/27	b4/y5	7.40
LSFNPTQL*EEQCHI*	865.7	462.2[a]/693.5[b]	26/27	b4/y5	7.40
LAFNPTQLEGQCHV	807.5	600.2[a]/1168.4[b]	26/27	y5/y10	6.95
LAFNPTQL*EGQCHV*	814.0	606.2[a]/1181.4[b]	26/27	y5/y10	6.95
GPFPIIV	742.4	441.3[a]/625.4[b]	27/27	y4/b6	9.50
GPFPI*I*V	756.5	455.5[a]/639.2[b]	25/30	y4/b6	9.50
GPFPILV	742.4	441.3[a]/625.4[b]	27 / 27	y4/b6	9.75
GPFPI*L*V	756.5	455.5[a]/639.2[b]	25/30	y4/b6	9.75
FFSDK	322.3	349.2[a]/496.1[b]	15/10	y3/b4	4.69
F*F*SDK	332.3	349.2[a]/262.1[b]	15/15	y3/y2	4.69
FFDDK	336.3	262.3[a]/524.5[b]	15/10	y2/y4	4.86
F*F*DDK	346.1	262.3[a]/534.3[b]	15/15	y2/y4	4.86
FFVAPFPEVFGK	692.9	295.1[a]/394.2[b]	27/22	b2/b3	10.24
FFV*APFPEV*FGK	699.2	295.1[a]/400.0[b]	25/20	b2/b3	10.24
FVVAPFPEVFR	654.4	891.5[a]/962.9[b]	27/22	y7/y8	9.53
FVV*APFPEV*FR	660.6	897.5[a]/968.4[b]	25/20	y7/y8	9.53
VNELSK	345.5	234.0[a]/590.4[b]	15/15	y2/y5	3.60
ENINELSK	473.7	147.2[a]/234.3[b]	15/15	y1/y2	9.65
ENLCSTFCK	579.5	244.2[a]/642.8[b]	20/17	b2/y5	5.44
LCTTSCEEVVR	677.3	274.1[a]/878.4[b]	27/22	y2/y7	5.01
NAVPITPTLNR	598.3	285.5[a]/912.0[b]	25/20	b3/y8	5.95
NAGPFTPTVNR	587.3	494.8[a]/687.4[b]	15/15	y9/y6	5.56
FALPQYLK	490.2	219.2[a]/761.6[b]	20/24	b2/y6	7.29
F*ALPQYL*K	499.1	229.2[a]/655.8[b]	15/15	b2/y5	7.29
FAWPQYLK	526.8	648.4[a]/834.4[b]	20/24	y5/y6	7.75
F*AWPQYL*K	535.6	655.4[a]/229.4[b]	15/15	y5/b2	7.75
TVYQHQK	452.5	201.1[a]/364.2[b]	18/15	b2/b3	5.62
TVDQHQK	428.2	201.0[a]/316.2[b]	18/24	b2/b3	4.98

*V** Val-OH-$^{13}C_5$,^{15}N, *F** Phe-OH-$^{13}C_9$,^{15}N, *I** Ile-OH-$^{13}C_6$,^{15}N, *L** Leu-OH-$^{13}C_6$,^{15}N

[a] Quantitative ions

[b] Qualitative ions

chromatography methods have also been reported for separating major proteins from bovine, goat, and sheep milk [11]. Furthermore, liquid chromatography-tandem mass spectrometry (LC-MS) [18, 19] and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) [20, 21] have been applied for the qualitative and quantitative analysis of signature proteins and peptides.

Although the above-mentioned approaches are effective in certain instances, their applications were mainly focused on the identification of simple adulteration, for example, the adulteration of goat and sheep cheeses with common cow milk. However, few studies have been focused on the method development and validation for analyzing the adulteration of infant formula, which possesses more complicated matrix and consists of whey and milk powder with certain proportion. In fact, the addition of cow's whey powder in goat infant formula powder is not illegal on the premise of clear species label in China. Current available methods have difficulty to identify the species of whey powder and whole milk powder, and to quantify their percentage in further. Consequently, the goat milk infant formula becomes a vulnerable product, and it is easily adulterated by cow's whey or whole milk powder with the false or misleading labeling.

In this work, we aim to develop a UHPLC-MS/MS method for quantification of cow's whey and whole milk powder percentage in goat or sheep milk products including infant formula by measuring four caseins and two major whey proteins based on their signature peptides as markers. The specific peptides markers of the target proteins 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 isotope-labeled signature peptides as internal standard were employed for avoiding matrix interference during mass spectrometry analysis. The goat milk infant formula samples were pretreated and subjected to UHPLC-MS/MS analysis using the established method. The acquired data were finally put into the calculation formula for assessing different composition of adulteration in the samples.

Materials and methods

Chemicals and samples

Goat, sheep, and cow milk whey and whole milk powder were obtained from domestic manufacturers. Eleven of goat milk infant formula from different commercial brands were purchased from local supermarkets. Dithiothreitol (DTT), iodoacetamide (IAA), ammonium bicarbonate (NH_4HCO_3), and hydrochloric acid (HCl, 37 %) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Table 2 Specific peptide candidates of the target proteins

Protein	Peptide	Cow marked	Goat marked	Sheep marked	Position
α-lactalbumin	CEVFR	√	×	×	25–29
	NICNISCDK	×	√	√	90–98
β-lactoglobulin	LSFNPTQLEEQCHI	√	×	×	167–180
	LAFNPTQLEGQCHV	×	√	√	167–180
β-casein	GPFPIIV	√	×	×	217–224
	GPFPILV	×	√	√	217–224
κ-casein	FFSDK	√	×	×	38–42
	FFDDK	×	√	√	38–42
α_{s1}-casein	FFVAPFPEVFGK	√	×	×	38–49
	FVVAPFPEVFR	×	√	√	38–48
	VNELSK	√	×	×	52–57
	ENINELSK	×	√	√	50–57
α_{s2}-casein	ENLCSTFCK	√	×	×	49–57
	LCTTSCEEVVR	×	√	√	51–61
	NAVPITPTLNR	√	×	×	131–141
	NAGPFTPTVNR	×	√	√	131–141
	FALPQYLK	√	×	×	190–197
	FAWPQYLK	×	√	√	190–197
	TVYQHQK	√	×	×	198–204
	TVDQHQK	×	√	√	198–204

HPLC-grade acetonitrile (ACN) and formic acid (FA) were supplied by Merck (Darmstadt, Germany). Sequencing grade recombinant trypsin was supplied by Shanghai Yaxin Biotechnology Co., Ltd. (Shanghai, China). For all experiments, ultrapure water generated by a Milli-Q Gradient A 10 water purification system (Millipore, Bedford, MA, USA) was used.

Synthetic peptide standards

The selective signature peptides and their stable isotope-labeled signature peptides of α-lactalbumin, β-lactoglobulin, β-casein, α_{s1}-casein, α_{s2}-casein, and κ-casein (Table 1) were synthesized by ChinaPeptides Co., Ltd. (Shanghai, China). All the peptide standards were synthesized with purity of more than 95 %.

Preparation of tryptic hydrolysates

The presented pretreatment procedure was based on our previous study [22]. Before tryptic digestion, 0.1 g of samples were dissolved and diluted to 10 mL with ultrapure water in volumetric flask. Aliquots of 50 μL diluted milk were mixed with 100 μL stable isotope-labeled internal standard and 590 μL ultrapure water. The mixtures were reduced with 10 μL aliquot of 100 mmol/L DTT in 70 °C water bath for 30 min and then alkylated with 10 μL of 300 mmol/L IAA in the dark for 30 min at room temperature. After mixing with

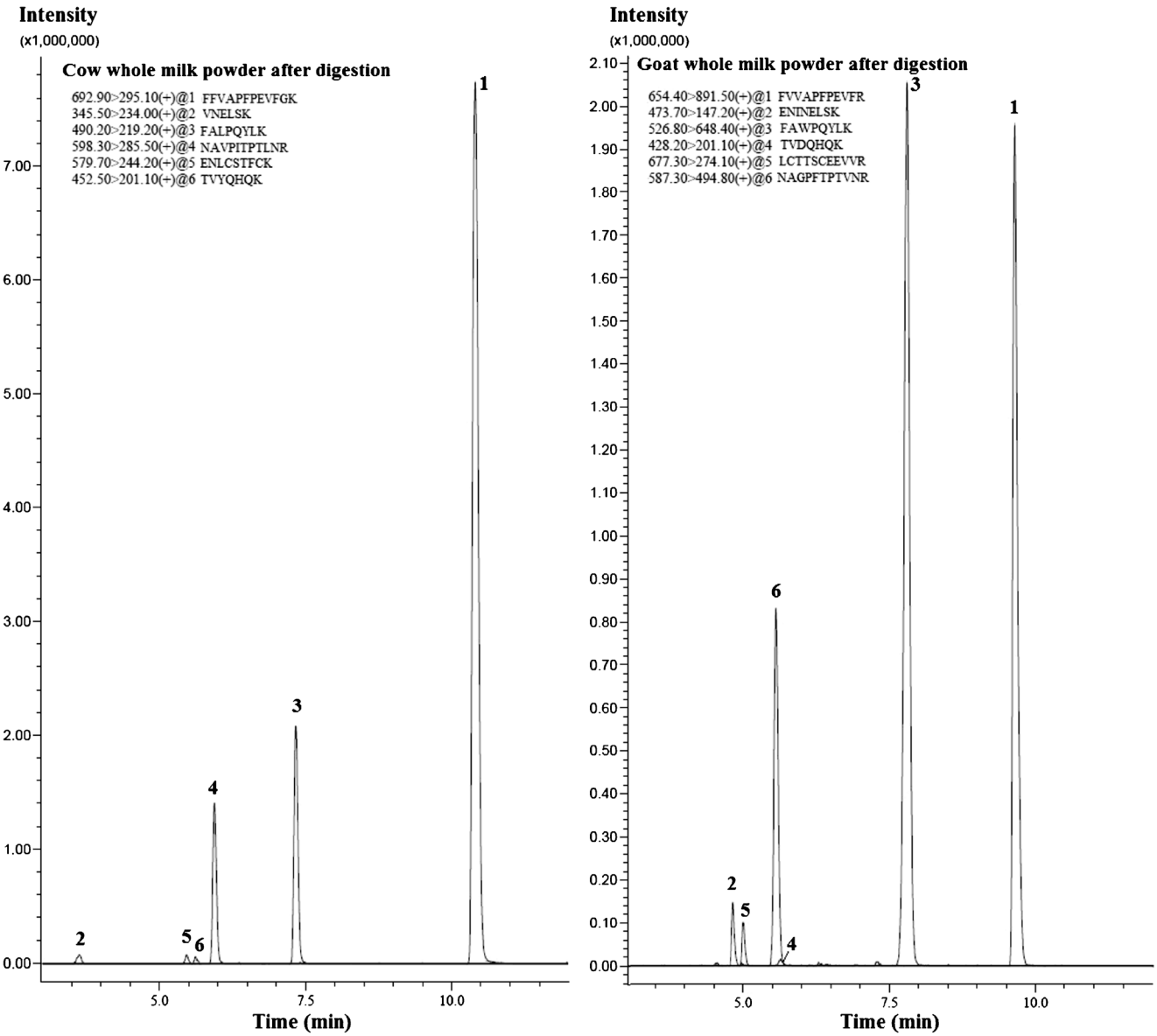

Fig. 1 UHPLC-MS/MS chromatograms of candidate peptides in pretreated cow and goat whole milk powder by tryptic digestion

200 μL of 500 mmol/L NH_4HCO_3 and 30 μL of 1 mg/mL trypsin (freshly prepared), the mixtures were incubated at 37 °C for 3 h. The enzymatic digestion reaction was stopped by adding 10 μL formic acid. After centrifuging the digested mixtures at 13,000 g for 10 min, the supernatants were obtained for analysis by UHPLC-MS/MS.

Liquid chromatography

Separation of tryptic hydrolysates was performed on an UHPLC System equipped with LC-30AD binary solvent manager, SIL-30AC sample manager, and CTO-30A column manager (Shimadzu, Kyoto, Japan). The analytical column was a narrow-bore Acquity UPLC BEH 300 C_{18} column (1.7 μm, 2.1 mm × 100 mm) equipped with a guard column of the same material (Waters, Milford, MA, USA). The 0.1 % formic acid in water and ACN were used for the mobile phases A and B, respectively. The column temperature was 40 °C and LC flow rate was at 0.3 mL/min. The elution program was shown as follows: linear step to 25 % B from initial 3 % B in 5 min; linear step to 30 % B in 2 min; linear step to 40 % B in 2 min; increasing to 100 % B in 1 min and holding for 2 min before re-equilibration at the initial conditions. The injection volume was 10 μL.

Mass spectrometry

The presented parameters of mass spectrometry were based on our previous study [22]. Search of signature peptide by TOF-MS was performed on a Synapt G2 High Definition Mass Spectrometer equipped with ESI source (Waters, Ltd.). The data were acquired in the electrospray positive ion (ESI^+) mode with MS^E mode and analyzed by searching sequence databases using ProteinLynx Global Server version 2.5

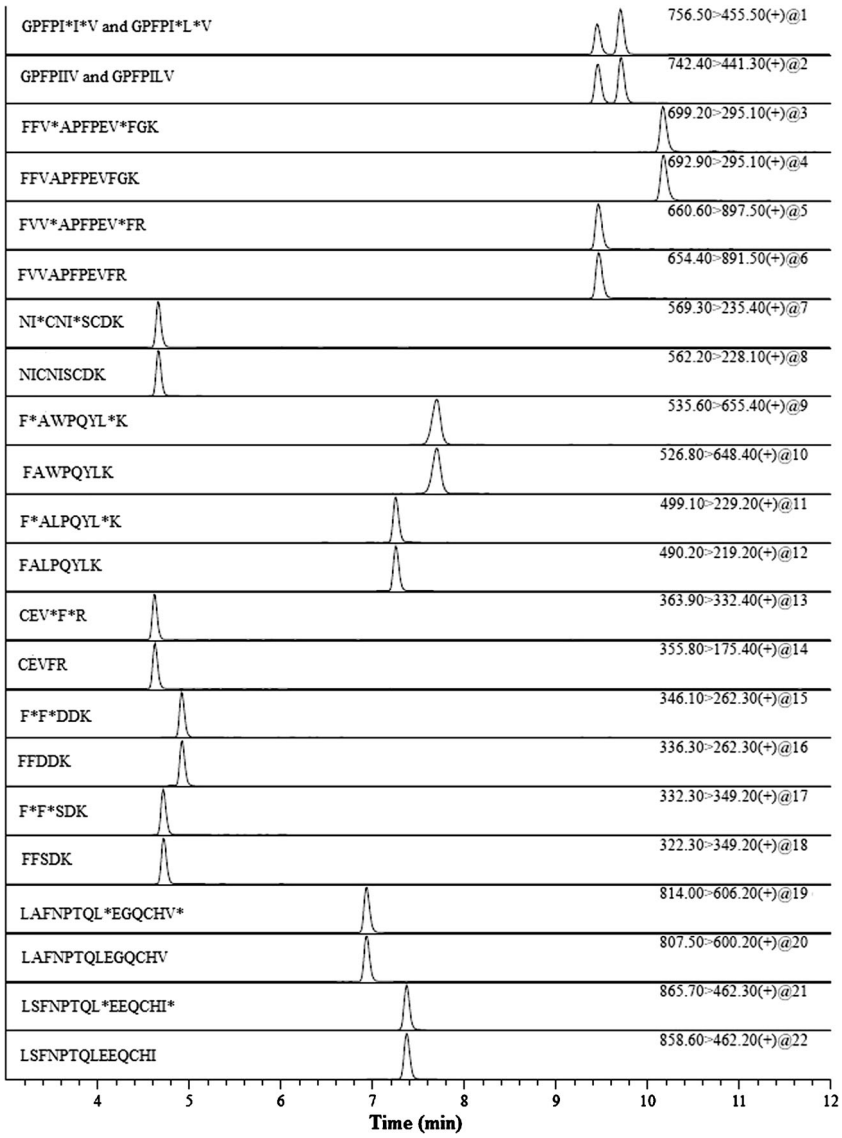

Fig. 2 UHPLC-MS/MS chromatograms of selective signature peptides and their corresponding isotope-labeled signature peptides in the standard mixture

Table 3 Composition of milk powder made with goat, sheep, and cow milk powder

Item	Composition, %										
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
Goat milk powder	100	0	0	80	60	40	20	0	0	0	0
Sheep milk powder	0	100	0	0	0	0	0	80	60	40	20
Cow milk powder	0	0	100	20	40	60	80	20	40	60	80

software. Relevant instrument and software parameters were set 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; desolvation gas flow, 800 L/h; ramp trap collision energy, 15–35 V; lockspray reference compound, leucine-enkephalin; 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).

Data acquisition were performed on a Shimadzu LCMS-8050 plus equipped with an electrospray ionization (ESI) source by multiple reaction monitoring (MRM) method. The positive electrospray (ESI^+) mode with both quadrupoles tuned for unit resolution was operated with the followed settings: capillary voltage, 3.5 kV; desolvation line (DL) temperature, 250 °C; heat block temperature, 400 °C; nebulizing gas flow, 3.0 L/min; and drying gas flow, 10.0 L/min. Two MRM transitions for each compound were monitored after their parameters were optimized (Table 1).

Results and discussion

Selection of proteins and signature peptide

The principal proteins in sheep and goat milk are about the same as in cow milk [23]. The casein fraction, including β-casein, α_{s1}-casein, α_{s2}-casein, and κ-casein, constitutes about 70–80 % of the total protein in milk. The major whey proteins are β-lactoglobulin and α-lactalbumin that approximately account for 70–80 % of total whey protein [23, 24]. Consequently, the four caseins and two major whey protein were selected for assessing the contents of milk powder and whey powder from different milk species in infant formula.

In order to differentiate the proteins of goat or sheep with cow in infant formula, specific peptides of target proteins were screened by the computational prediction using Biolynx software and online PeptideMass tools provided by UniProt (http://web.expasy.org/peptide_mass), and further confirmed by database searching after liquid chromatography-quadrupole-time-of-flight tandem mass spectrometry (LC-Q-TOF-MS) analysis. The specific peptides were selected usually based on several critical criteria such as specificity of amino acid sequences, intensity of their MS signal, and reproducibility in sample preparation [25]. Methionine is one of the most readily oxidized amino acid in proteins [26], therefore the peptides comprising methionine should be excluded. Furthermore, the peptides with more than 14 amino acids may generally be not used due to their considerable cost for the synthesis and unfavorable LC properties [25]. In the present work, we focused on the assessment of cow milk adulterate because of economic motivation, and the differentiating goat milk with sheep milk was not considered. That is to say, the tryptic-specific peptides shared by goat and sheep milk proteins were selected for their quantification.

Consequently, the candidate peptides were initially screened according to the above criteria (Table 2), and their MRM conditions were optimized, respectively (Table 1). The couple of tryptic-specific peptides of the same protein for differentiating goat/sheep milk with cow milk were selected as the near position of target protein's sequence as possible. It might largely ensure that the tryptic-specific peptides for differentiating target proteins possess similar physicochemical properties in the pretreatment. The unique suitable peptides were found and synthesized for respective quantification of β-lactoglobulin, α-lactalbumin, β-casein, and κ-casein. Several candidate peptides of α_{s1}-casein and α_{s2}-casein were compared by intensity of their MS signal, and the peptides (FFVAPFPEVFGK, FVVAPFPEVFR, FALPQYLK, and FAWPQYLK) having the largest MS intensity were selected

Table 4 Composition of formula milk powder made with goat, cow milk powder, and whey powder

Item	Composition, %					
	S12	S13	S14	S15	S16	S17
Goat milk powder	0	0	10	10	10	10
Cow milk powder	0	0	10	10	10	10
Goat milk whey powder	100	0	70	50	30	10
Cow milk whey powder	0	100	10	30	50	70

Table 5 Quantitative results of the targeted proteins in different samples

Species	g/100 g	Sample																
		S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17
Cow	α-lactalbumin	–	–	0.63	0.12	0.12	0.25	0.24	0.38	0.38	0.49	0.49	–	1.26	0.19	0.43	0.68	0.90
	β-lactoglobulin	–	–	2.38	0.45	0.48	0.93	0.94	1.46	1.53	2.01	1.99	–	6.15	0.90	2.05	3.35	4.39
	β-casein	–	–	6.38	1.20	1.18	2.42	2.40	3.70	3.65	4.75	4.76	–	–	0.64	0.68	0.72	0.74
	α_{s1}-casein	–	–	6.20	1.27	1.31	2.60	2.60	3.85	3.84	5.06	5.08	–	–	0.72	0.74	0.73	0.75
	α_{s2}-casein	–	–	2.19	0.45	0.43	0.86	0.87	1.34	1.31	1.78	1.74	–	–	0.25	0.25	0.25	0.26
	κ-casein	–	–	1.97	0.40	0.36	0.78	0.75	1.21	1.19	1.60	1.52	–	–	0.21	0.21	0.21	0.22
Goat/Sheep	α-lactalbumin	0.65	0.15	–	0.47	0.12	0.38	0.09	0.25	0.06	0.13	0.03	0.60	–	0.48	0.37	0.26	0.12
	β-lactoglobulin	2.06	2.96	–	1.46	2.44	1.22	1.83	0.75	1.23	0.38	0.61	4.75	–	3.68	2.71	1.68	0.73
	β-casein	9.22	8.60	–	7.07	6.85	5.34	5.13	3.49	3.44	1.77	1.68	–	–	0.95	0.95	0.91	0.90
	α_{s1}-casein	2.82	6.30	–	2.11	4.86	1.59	3.75	1.04	2.52	0.54	1.21	–	–	0.29	0.29	0.29	0.28
	α_{s2}-casein	2.55	1.78	–	1.90	1.48	1.48	1.13	0.99	0.78	0.53	0.40	–	–	0.28	0.29	0.29	0.28
	κ-casein	2.11	1.49	–	1.68	1.31	1.28	1.00	0.87	0.65	0.42	0.33	–	–	0.22	0.22	0.21	0.22

and synthesized as the signature peptides (Fig. 1). It is noteworthy that the peptides GPFPIIV and GPFPILV of β-casein could be completely separated by UHPLC (Fig. 2) though they had the similar parameters of MS.

Synthesis of isotopically labeled signature peptides

Though UHPLC-MS/MS is a highly sensitive and selective tool for peptides quantification, the accuracy of the technology may be influenced by the different ionization efficiency of the analytes in various matrices and susceptibility to collision-induced dissociation of different peptides [27, 28]. In the study of authentic assessment of dairy products, the accurate quantification of various proteins from ambiguous samples is a crucial challenge. The use of isotopically labeled internal standard may minimize the ionization efficiency. Accordingly, the isotope-labeled signature peptides were employed as internal standard in this study (Table 1).

In order to assess the accuracy of established method, two different experiments were designed to respectively simulate the adulteration of goat or sheep whole milk powder and goat milk infant formula: the first mixing sheep or goat whole milk powder with cow whole milk powder (Table 3), and the second mixing goat and cow whole milk powder with goat and cow milk whey powder (Table 4). A quantitative test was run with promising results, as the quantitative values of targeted proteins (Table 5) showed good linear correlation ($r > 0.99$) between additional percentages of species milk powder. The linear correlation (Table 6) of whole milk powder for different species was showed by average correlation of six signature proteins in the first experiment. And the linear correlation (Table 6) of whey powder were calculated by average correlation of β-lactoglobulin and α-lactalbumin after deducting the blank of whole milk powder in the second experiment.

Method validation

Specificity

The chromatograms of the selected signature peptide from corresponding species' tryptic milk showed the steep and symmetric peaks without interferences. In fact, when this multianalyte MRM method was applied to tryptic milk samples made with single-specie milk (milk powder for cow, sheep, or goat), the spectra

Table 6 Linear correlation for different percentages of milk powder from different species

Item	Composition percentage, %						Linear correlation ($n = 6$)
Goat milk powder	100	80	60	40	20	0	0.9962 ± 0.0030
Sheep milk powder	100	80	60	40	20	0	0.9982 ± 0.0026
Cow milk powder	100	80	60	40	20	0	0.9985 ± 0.0009
Goat milk whey powder	100	70	50	30	10	0	0.9991 ± 0.0003
Cow milk whey powder	100	70	50	30	10	0	0.9991 ± 0.0001

showed unambiguously only specie-specific markers for corresponding species' protein (Fig. 3). Without tryptic digestion, there was no targeted peak appearing in the chromatogram for milk sample (Fig. 3).

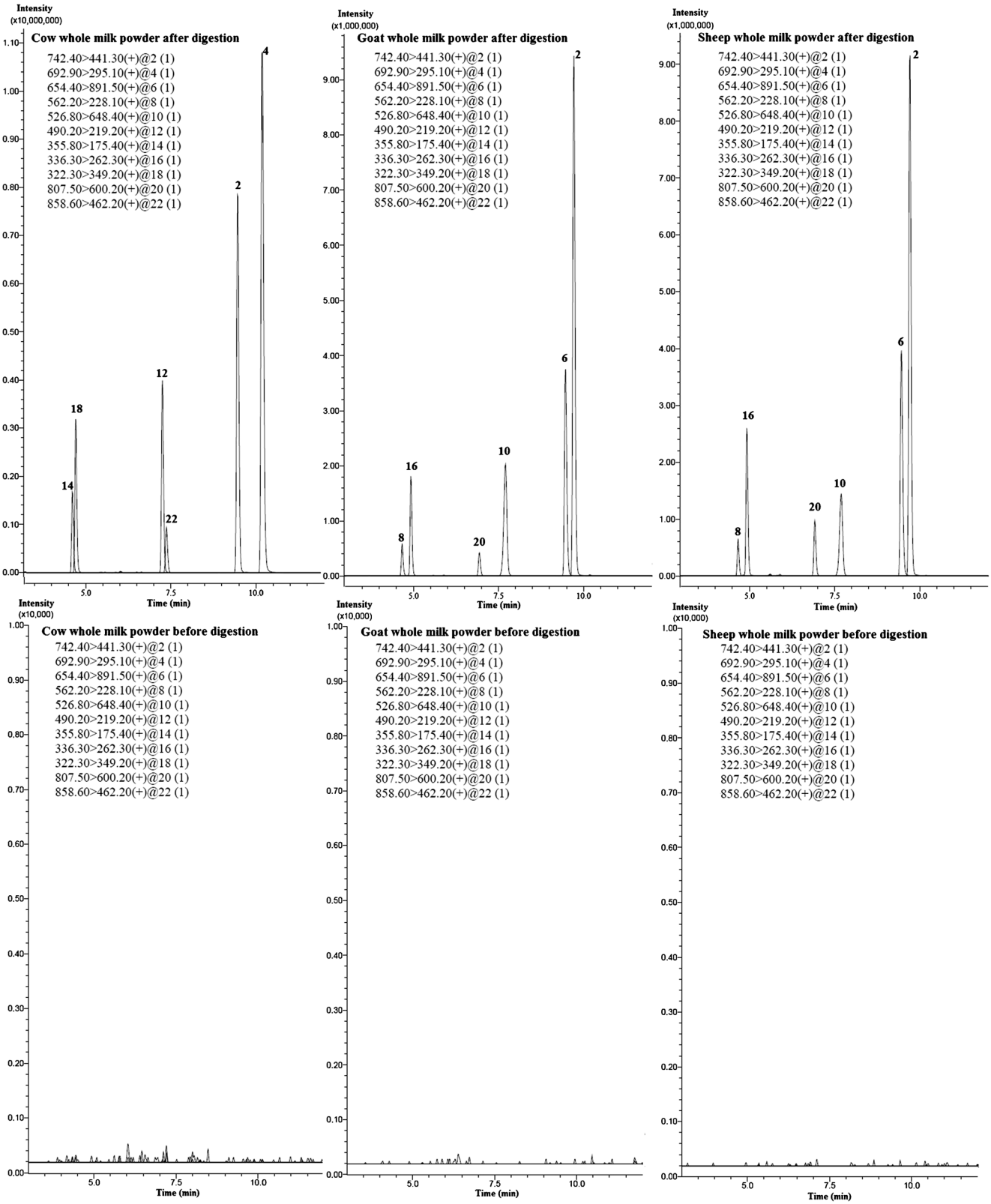

Fig. 3 UHPLC-MS/MS chromatograms of selective signature peptides in pretreated/unpretreated cow, goat, and sheep whole milk powder by tryptic digestion

Linearity and sensitivity

The linear regression equation for eight levels of targeted signature peptides had good linearity and coefficient of correlation ($r^2 > 0.99$) (Table 7). The amounts of the tryptic signature peptides from the digested samples can be obtained from the standard curve by using the synthetic signature peptides as calibration standards. The contents of targeted proteins in samples were calculated based on the molar equivalent relationship between the signature peptides and original proteins.

The LOQ for each analyte was calculated as the lowest concentration providing a signal-to-noise ratio of 10 (Table 7), respectively. The linear range ($r > 0.99$) and sensitivity (LOQs of 0.01–0.05 g/100 g) could satisfy the quantification requirements for different targeted protein concentrations in various ambiguous adulterate milk samples. In fact, the chromatograms of tryptic sample showed the steep and

Table 7 Linearity, LOQ, and spiked recovery of the present UHPLC-MS/MS method ($n = 20$)

Species	Protein	Linear range (nmol/L)	Linear regression equation	Linear correlation	LOQ (g/100 g)	Spiked level (nmol/L)	Recovery[a] (%)	RSD (%)
Cow	α-lactalbumin	15–1200	$y = 2.80608*x + 2.66928$	0.995	0.01	30	101.4	6.9
						60	100.8	4.6
						120	100.1	5.8
	β-lactoglobulin	45–3600	$y = 0.77274*x - 0.368623$	0.996	0.05	90	82.3	6.8
						180	87.4	7.3
						360	83.2	5.8
	β-casein	65–5200	$y = 2.00076*x - 1.39976$	0.999	0.05	130	116.6	11.1
						260	107.9	8.9
						520	105.7	5.4
	α_{s1}-casein	70–5600	$y = 0.778426*x - 1.53835$	0.995	0.05	140	107.9	4.0
						280	105.5	3.9
						560	105.2	3.9
	α_{s2}-casein	35–2800	$y = 0.896697*x - 1.06593$	0.997	0.05	70	115.4	9.5
						140	111.3	5.6
						280	108.2	6.5
	κ-casein	35–2800	$y = 0.517689*x - 0.756008$	0.995	0.05	70	95.0	3.2
						140	94.5	3.1
						280	94.9	3.0
Goat/Sheep	α-lactalbumin	15–1200	$y = 1.19739*x - 1.25912$	0.996	0.01	30	100.1	4.3
						60	97.2	7.0
						120	99.0	4.9
	β-lactoglobulin	45–3600	$y = 2.79003*x - 0.568108$	0.993	0.05	90	106.2	9.2
						180	104.2	6.9
						360	107.4	5.2
	β-casein	35–2800	$y = 0.755795*x + 0.0196128$	0.997	0.05	70	100.3	4.0
						140	97.0	5.2
						280	95.6	3.6
	α_{s1}-casein	75–6000	$y = 1.46689*x - 1.53099$	0.996	0.05	150	115.6	3.3
						300	113.9	4.1
						600	110.4	5.7
	α_{s2}-casein	50–4000	$y = 0.655691*x - 0.665907$	0.998	0.05	100	110.3	2.9
						200	105.2	3.4
						400	104.5	5.0
	κ-casein	50-4000	$y = 0.79938*x - 0.476862$	0.998	0.05	100	96.2	4.4
						200	95.6	3.3
						400	97.4	3.1

[a] Recovery = determined level / spiked level × 100 %

symmetric peaks without any interference even if the sample consisted of 0.1 % cow whole milk powder and 99.9 % goat whole milk powder (Fig. 4).

Recovery, intra- and inter-day precision

The synthesized signature peptides standards for goat or sheep milk proteins were spiked into the control diluent of cow milk, while the synthesized signature peptides standards for cow milk proteins were spiked into the control diluent of goat milk (Table 7). The spiked samples were pretreated and analyzed with the above-established method. The recovery test was carried out by comparing the measured concentrations of control and spiked samples with the theoretical concentrations. The spiking recoveries were 82.3–116.6 % with 2.9–11.1 % of RSD (Table 7). Aliquots of cow, goat, and sheep milk mixture was pretreated with the above optimal preparation each day for four consecutive days to evaluate the intra-day and inter-day precision of the method. The RSDs of intra- and inter-day precision were observed as 2.8–6.2 and 3.3–9.8 %, respectively. All the results showed that the current method had good accuracy and precision.

Method application

As caseins exists only in whole milk powder other than whey powder, they can serve as the markers for whole milk powder. In this paper, the pure cow's (goat's) raw whole milk powder and whey powder were chosen as the standard substance. The addition amount of whole

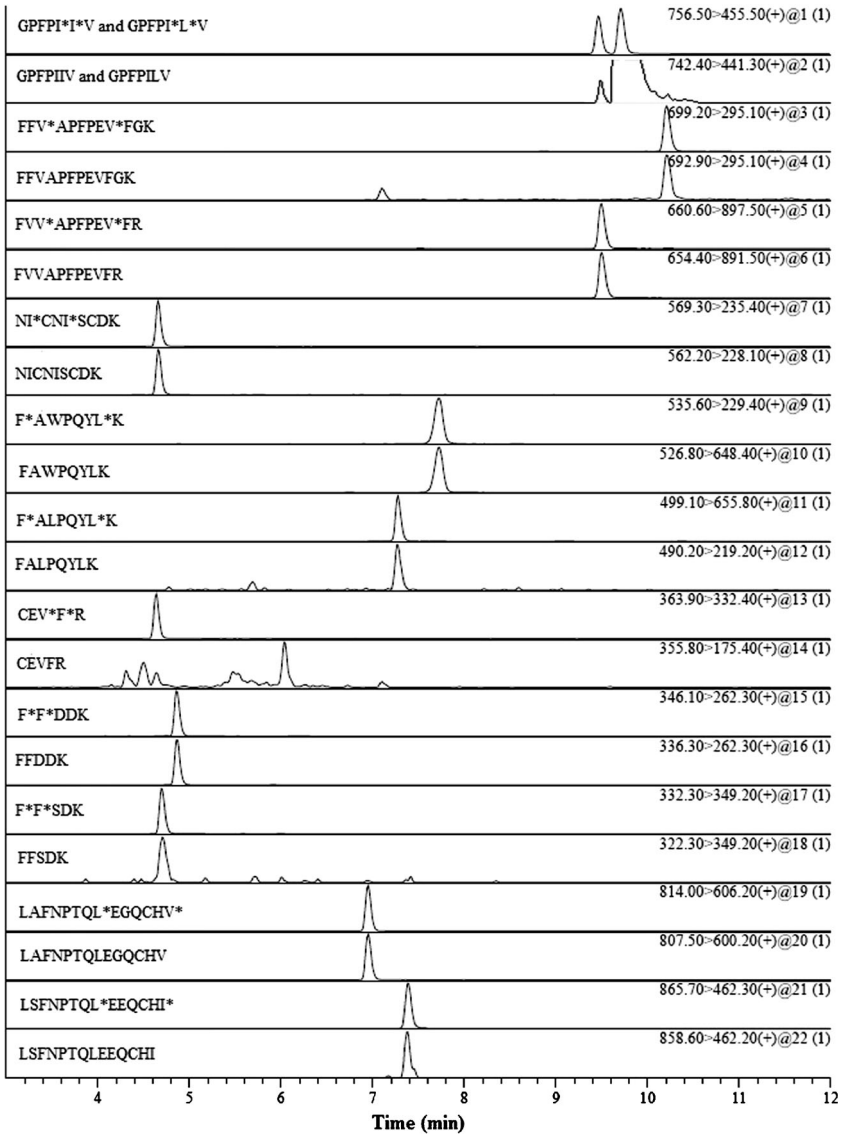

Fig. 4 UHPLC-MS/MS chromatograms of selective signature peptides and their corresponding isotope-labeled signature peptides in the tryptic sample which consisted of 0.1 % cow whole milk powder and 99.9 % goat whole milk powder

Table 8 Quantitative values and data analysis of the targeted proteins in samples of different commercial brands

Species	g/100 g	Sample														
		S1	S12	S3	S13	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11
Cow	α-lactalbumin	–	–	0.63	1.26	–	–	–	1.04	1.34	1.05	1.08	1.05	0.64	0.72	1.38
	β-lactoglobulin	–	–	2.38	6.15	–	–	–	3.11	3.83	3.54	2.73	2.57	3.08	2.42	2.59
	β-casein	–	–	6.38	–	–	–	–	–	–	–	–	–	–	1.68	0.50
	α_{s1}-casein	–	–	6.20	–	–	–	–	–	–	–	–	–	–	0.45	0.47
	α_{s2}-casein	–	–	2.19	–	–	–	–	–	–	–	–	–	–	0.37	0.18
	κ-casein	–	–	1.97	–	–	–	–	–	–	–	–	–	–	1.74	0.18
	Whole milk powder	–	–	100.00	–	–	–	–	–	–	–	–	–	–	25.33	7.95
	Whey powder	–	–	–	100.00	–	–	–	56.01	69.77	61.94	51.42	48.85	50.20	31.64	49.32
Goat	α-lactalbumin	0.65	0.60	–	–	0.42	0.94	0.38	0.15	0.13	0.04	0.22	0.16	0.14	0.14	0.25
	β-lactoglobulin	2.06	4.75	–	–	2.17	2.34	1.13	0.39	0.42	0.13	0.59	0.51	0.35	0.39	0.49
	β-casein	9.22	–	–	–	1.49	0.77	1.97	0.65	0.57	0.25	1.06	0.86	0.43	0.65	4.66
	α_{s1}-casein	2.82	–	–	–	1.14	0.81	1.62	0.69	0.53	0.18	1.09	0.71	0.52	0.64	0.73
	α_{s2}-casein	2.55	–	–	–	1.08	0.68	1.37	0.48	0.43	0.16	0.85	0.61	0.39	0.47	0.96
	κ-casein	2.11	–	–	–	4.72	2.89	5.46	3.27	2.80	1.24	4.91	4.05	1.89	2.33	0.78
	Whole milk powder	100.00	–	–	–	50.48	30.84	62.40	30.48	25.93	10.96	47.37	37.31	19.34	24.49	42.69
	Whey powder	–	100.00	–	–	22.84	45.69	–	–	–	–	–	–	–	–	–

milk powder in samples were assessed by the ratio of sum caseins in samples to that in standard substance of whole milk powder. The whey proteins are simultaneously present in whole milk powder and whey powder, thus the sum of β-lactoglobulin and α-lactalbumin after deducting the part from whole milk powder were divided by the value in standard substance of whey powder to estimate the additive amount of whey powder. The details of calculating formula is given below:

$$M_a = \frac{C_1}{C_2} \times 100$$

$$M_b = \left(\frac{W_1}{W_3} - \frac{C_1}{C_2} \times \frac{W_2}{W_3}\right) \times 100$$

Where M_a: addition ratio of whole milk powder; M_b: addition ratio of whey powder; C_1: sum of caseins in sample; C_2: sum of caseins in standard substance of whole milk powder; W_1: sum of β-lactoglobulin and α-lactalbumin in sample; W_2: sum of β-lactoglobulin and α-lactalbumin in standard substance of whole milk powder; W_3: sum of β-lactoglobulin and α-lactalbumin in standard substance of whey powder; units of all values for caseins, β-lactoglobulin, and α-lactalbumin were g/100 g.

Eleven of goat infant formula milk powder with different commercial brands were pretreated and subjected to UHPLC-MS/MS analysis using current optimized method. The results revealed that there were different percentage of adulteration in the samples (Table 8). The samples with brand 1–3 were consisted of pure goat whey and whole milk powder with certain proportion which conformed to their labels. Although the labels of the samples with brand 3–9 did not give clear indication of species of whey powder, the results of data analysis indicated that their principal components were cow whey powder and goat whole milk powder. Surprisingly, we confirmed that the samples with brand 10 and 11 were adulterated by cow's whey and whole milk powder with false labeling.

Conclusions

In this study, an analytical method was developed for quantifying the percentage of cow's whey and whole milk powder in goat or sheep milk products including infant formula. In detail, an UHPLC-MS/MS method for simultaneous quantification of four caseins and two whey proteins was established by detecting their signature peptides, which were able to act as markers for differentiating cow from goat or sheep whey and whole milk powder in infant formula. The relevant tryptic fragment peptides were selected and validated as the specific markers. The isotopic-labeled signature peptide as internal standard was employed for avoiding matrix interference in mass spectrometry. The accuracy, sensitivity, and selectivity of current method were validated via the calibration curves, LOD and LOQ, intra- and inter-day precision, and recovery. The specific calculating formula was established to estimate the addition of whey and whole milk powder from cow and goat. It was successfully applied to routine determination of goat infant formula milk powder with different commercial brands ($n = 11$), and the results revealed that there were

different composition and percentage of adulteration in the samples we analyzed.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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