

Selection of possible marker peptides for the detection of major ruminant milk proteins in food by liquid chromatography-tandem mass spectrometry

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Abstract The aim of this work was the determination of peptides, which can function as markers for identification of milk allergens in food samples. Emphasis was placed on two casein proteins (α - and β -casein) and two whey proteins (α -lactalbumin and β -lactoglobulin). In silico tryptic digestion provided preliminary information about the expected peptides. After tryptic digestion of four milk allergens, the analytical data obtained by combination of reversed-phase high performance liquid chromatography and quadrupole tandem mass spectrometry (LC-MS/MS) led to the identification of 26 peptides. Seven of these peptides were synthesized and used for calibration of the LC-MS/MS system. Species specificity of the selected peptides was sought by BLAST search. Among the selected peptides, only LIVTQTMK from β -lactoglobulin (m/z 467.6, charge 2+) was found to be cow milk specific and could function as a marker. Two other peptides, FFVAPF-PEVFGK from α -casein (m/z 693.3, charge 2+) and GPFPIIV from β -casein (m/z 742.5, charge 1+), occur in water buffalo milk too. The other four peptides appear in the milk of other species also and can be used as markers for ruminant species milk. Using these seven peptides, a multianalyte MS-based method was developed. For the establishment of the method, it was applied at first to different dairy samples, and then to chocolate and blank samples, and the peptides could be determined down to

1 ng/mL in food samples. At the end, spiked samples were measured, where the target peptides could be detected with a high recovery (over 50%).

Keywords Milk allergens · α -Casein · β -Casein · α -Lactalbumin · β -Lactoglobulin · Liquid chromatography-tandem mass spectrometry

Introduction

Milk is one of the most common and widespread allergenic foods. Milk allergy is an adverse immunological reaction to milk proteins of different mammalian species. The milk of different ruminant species (e.g. cow, buffalo, sheep and goat) as well as that of humans, contains the same or closely homologous proteins which share the same structural, functional and biological properties [1].

About 3.2% of milk is protein, which is subdivided into two fractions: casein accounts for 80% of the milk protein and whey for the other 20% [2]. The caseins (α -casein 42% and β -casein 28%) are found in the micelles, giving milk its cloudy appearance [3]. The whey fraction contains two major components: β -lactoglobulin (BLG; 10%) and α -lactalbumin (ALA; 5%). Studies have shown that due to the great variability of human IgE response, no single allergen or particular structure can account for a major part of milk allergenicity, and polysensitization to several proteins is also common. All milk proteins, even proteins present at low concentrations, appear to be potential allergens; however, the caseins, BLG and ALA are major allergens (50–65% of patients), and sensitization has been demonstrated to be closely linked [1, 4–6].

It seems that even trace amounts of milk allergens can induce allergic reactions in patients and result in mild to

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fatal health problems. However, available data and reports from controlled food challenges do not permit the establishment of validated threshold doses for specific allergic reactions or the determination of a level of exposure that could protect allergic consumers against a reaction to allergens present in their food [1, 7, 8]. The threshold levels vary from patient to patient, depending on the food concerned to the sensitivity of selected allergic individuals. Due to the fact that currently the only effective treatment is total avoidance of the offending food, EU regulation enforces the declaration (Directive 2007/68/EC amending Annex IIIa to Directive 2000/13/EC) of 14 food allergens, including milk and products thereof. However, the detection of allergens in food products can be very difficult because often only trace amounts are present or they are masked by the food matrix. Thus, reliable, sensitive and specific analytical methods allowing accurate identification of milk or milk components in food samples are sought. These approaches could target either the allergen itself or a marker that indicates the presence of the offending food and food component [9]. The methods for the detection of proteins in general include almost exclusively immunoanalytical methods, which use specific antibodies for the detection of target proteins, such as immunoblotting or enzyme-linked immunosorbent assay [10]. However, heating and technological food processing might lead to changes in target-protein structure, affecting final antibody-based detection. In order to overcome these drawbacks, mass-spectrometry (MS)-based methods have been developed which are not dependent on antibodies and could therefore offer a complementary tool, useful for final confirmation of the presence of an allergen in different commodities [11].

MS analysis has been applied in the last several decades in broad diverse objectives, including the determination of molecular weight for complete protein and sequencing of peptides from enzymatic mapping for structural characterization [12]. More specific analysis comprises the digestion of the sample with an enzyme such as trypsin, followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis to derive the amino acid sequences of individual peptides of the proteolytic digest (bottom-up approach) [2, 13]. The analytical throughput and efficiency of top-down proteomics are still limited for large-scale proteome analysis. Peptide-level measurements are generally more effective for protein identification than mass spectrometric analysis of intact proteins, and the accuracy of mass detection is more precise for small molecules. Also, specificity and selectivity is a major requirement, which can be increased for the whole analysis method with the choice of many target peptides (multianalyte method) [10]. Most of the publications, which deal with the detection of milk allergens, analyzed intact milk proteins [e.g. 8]. Monaci and van Hengel [7] developed a method based on LC

coupled with electrospray ionization (ESI)-MS detection to identify and quantify, again, intact allergenic whey proteins in mixed fruit juices with a limit of detection down to 1 $\mu\text{g/mL}$.

On the one hand, different MS techniques were used for the analysis of milk proteins of different ruminant species [e.g. 14, 15] or for the determination of the sequences of the tryptic peptides derived thereof [e.g. 16, 17]. On the other hand, numerous allergenic IgE and IgG binding epitopes of each of the four milk allergens were investigated to determine the allergenicity of the milk allergens [1, 3–6, 16]. Despite these two facts, literature is scarce in the field of mass spectrometric detection of allergenic milk proteins at low concentrations in food products and specially the combining of two above-mentioned techniques (using peptides as marker for the identification of whole milk allergens by MS). Very recently, Monaci et al. [18, 19] used ESI-Q-TOF MS for the identification of peptides derived from casein. In the first publication [18], they detected some peptides only from caseins (α and β) at 100 and 1,000 $\mu\text{g/mL}$ in fined white wine. In a technical note [19], they detected two peptides derived from α -casein as markers. As they rely on a comparison of retention times in LC-MS full scan data with compound confirmation by data-dependent MS/MS product ion scan data of standard and matrix samples, the risk of negative influence of unknown co-eluting matrix components on the signals from chosen diagnostic tryptic peptides cannot be excluded.

In this work, mass spectrometric techniques have been used for the detection of seven peptide markers arising from both casein (α and β) and whey milk proteins (α -lactalbumin and β -lactoglobulin). A multianalyte method was developed, which is capable to detect these diagnostic tryptic peptides from different milk proteins in a single analysis run. For the establishment of the method, it was applied at first to different dairy, blank and chocolate samples and then to spiked samples, wherein the target peptides could be detected with a high recovery. This study is the first step to establish a possible alternative and comparative method to immunoanalytical methods, which could be used as a reference method for the determination of milk allergens in different food samples.

Material and methods

Materials, chemicals and reagents

Milk and dairy products (parmesan, mozzarella, yogurt, sheep yogurt and whey drink) and also chocolate products were purchased from local markets. The proteins (all from bovine milk) α -casein (minimum 70% α_s -casein), β -casein (minimum 90%), α -lactalbumin ($\geq 85\%$) and β -lactoglobulin (approximately 90%) and also the chemicals ammonium

acetate (MS grade) and iodoacetamide were obtained from Sigma-Aldrich (Steinheim, Germany). Ammonium hydrogen carbonate ($\geq 99\%$), sodium hydrogen carbonate ($\geq 99.5\%$), 1,4-dithiothreitol ($\geq 99\%$), milk powder, acetonitrile (HPLC gradient grade) and formic acid ($\geq 98\%$) were purchased from Carl Roth GmbH (Karlsruhe, Germany). Trypsin (sequencing grade, modified) was from Roche Applied Science (Mannheim, Germany). Urea and di-sodium hydrogen phosphate dihydrate were from Merck (Germany). Sodium hydroxide was from Fluka (Buchs, Switzerland). Water was drawn from a Milli-Q plus System from Millipore (Molsheim, France).

Target peptides Seven peptides have been chosen (one for α -lactalbumin and two for each other selected protein, which showed the highest MS signal) and ordered in lyophilized form from 'Peptide 2.0' (Chantilly, VA, USA).

If not stated otherwise, procedures were performed at room temperature.

Sample preparation (extraction, digestion and spiking)

For extraction, 0.4 g of sample was mixed with 4 mL of extraction buffer (50 mM NaHCO_3 , pH 9.6). The mixture was heated at 60 °C for 15 min in a water bath (GFL, Burgwedel, Germany) and centrifuged for 20 min at 6,500 $\times$ g and 4 °C using a Conical Tube Fixed-angle Rotor (C1015) and an ALLEGRA® X-22 centrifuge (Beckman Coulter, Inc., Fullerton, CA, USA). The supernatant was filtered through both prefilter RW06, 47MM and membrane filter 0.45 μm HA from Millipore (Millipore Corporation, Billerica, MA, USA) and glass microfiber MN85/70 BF from Macherey-Nagel (Macherey-Nagel GmbH & Co., Düren, Germany). The filtrate was dried completely overnight using a vacuum centrifuge, Model VR-1/120/240 (Heraeus Instruments, Denmark).

Proteolytic digestions with trypsin were carried out using a protocol for in-solution digestion [20]: In short, the extracted proteins were resuspended in a 100 mM NH_4HCO_3 buffer pH 8 containing 6 M urea. One hundred-microlitre aliquots were reduced and alkylated using 200 mM DTT and 200 mM iodoacetamide in 100 mM NH_4HCO_3 . One hundred-microlitre of a trypsin solution (200 ng/ μL trypsin in 100 mM NH_4HCO_3) was added to the samples and incubated at 37 °C overnight. The reaction was stopped by addition of formic acid.

For spiking, both milk and mixtures of synthesized peptides were used. For determining the effect of procedures, the samples were spiked either before or after extraction, prior to digestion. For milk spiking before extraction, different volumes of milk was added to 1 g ground peanut puffs. For milk spiking after extraction, 100 μL milk were added to 900 μL peanut puffs extracted with 50 mM NaHCO_3 , pH 9.6. Also, for testing the matrix effect, 24 μL of standard peptide mixture with different

concentrations (37, 111, 370 and 1,111 ng/mL) were added to 80 μL of a 1:100 diluted digested food sample. The volume was adjusted with 50% aqueous acetonitrile (v/v) containing 5 mM ammonium acetate to 240 μL .

High performance liquid chromatography (HPLC)

Chromatographic separation of tryptic digested samples was carried out at 25 °C on a Zorbax SB-C₁₈ reversed phase column, 150 $\times$ 2.1 mm, 3.5 μm particle size (Agilent, Waldbronn, Germany), equipped with a C₁₈ 4 $\times$ 3 mm i.d. security guard cartridge (Phenomenex, Torrance, CA, USA) and using an HPLC series 1100 (Agilent, Waldbronn, Germany). Injection volume was 5 μL , and the flow rate was 0.35 mL/min. Eluent A consisted of 10% aqueous acetonitrile (v/v), and eluent B was 95% aqueous acetonitrile (v/v). Both HPLC eluents contained 5 mM ammonium acetate. The following HPLC program was used: initial hold of 0.5 min, gradient from 0% to 100% eluent B within 10.5 min, 3.5 min hold, switchback of the mobile phase to eluent A within 2 min and equilibration of the column for 12.5 min.

Electrospray tandem mass spectrometry

A QTrap 4000 LC-MS/MS system (Applied Biosystems, Foster City, CA, USA) equipped with a 'Turbo V Ion Spray' ESI source was used. Screening and identification of tryptic peptides, as well as optimization of SRM parameters for profiling of selected tryptic peptides, were carried out by directly infusing of tryptic digested milk proteins (concentration, 25–50 mg/L) or of peptide standard solutions (with a concentration of 140–330 $\mu\text{g/L}$), respectively, into the mass spectrometer at a flow rate of 10 $\mu\text{L/min}$; all were dissolved in 50% aqueous acetonitrile (v/v) and 0.1% formic acid (v/v).

The ESI source was used in positive ionization mode with the following parameters: curtain gas, 69 kPa (10 psi); ion source gas 1 (sheath gas), 207 kPa (30 psi); ion source gas 2 (drying gas), 345 kPa (50 psi); source temperature, 150 °C; and ion spray voltage, +4,000 V. Collision gas was set to high. Nitrogen was used for all gases. For each SRM transition, a dwell time of 50 ms was chosen, and the pause between mass ranges was 5 ms. The optimized SRM parameters (Table 1) were used for profiling of tryptic peptides of milk allergens by LC-MS/MS.

For the use of the QTrap4000 in LC-MS/MS experiments, the ESI source was used with the following settings: gas 1, 345 kPa (50 psi); gas 2, 345 kPa (50 psi); and source temperature, 535 °C. For the other parameters, the same values were chosen as for the SRM optimizations. Chromatographic and (tandem) mass spectrometric data were evaluated using the Analyst™ software version 1.5.

MS full scans were performed in the Q1-MS mode (the first quadrupole scans, the other quadrupoles transfer the

Table 1 Parameters of the LC-MS/MS method in SRM mode for profiling of tryptic peptides of milk allergen proteins

Protein		MW (Da)	Q1 (<i>m/z</i>)	Q1 ion	Q3 (<i>m/z</i>)	Q3 ion	DP (V)	CE (V)	CXP (V)	
α -Lactalbumin	VGINYWLAHK ^a	1,199.6	601.2	[M+2 H] ²⁺	284.4	y ₂	81	41	14	Q
	11/131 ^b		601.2		355.4	y ₃	81	41	30	
	3,704/43,764 ^c		601.2		654.4	y ₅	81	33	8	
β -Lactoglobulin	IPAVFK ^a	673.5	338.0	[M+2 H] ²⁺	282.0	b ₃	41	21	14	Q
	1/30 ^b		338.0		294.4	y ₂	41	27	18	
	1,111/30,115 ^c		338.0		561.6	y ₅	41	19	8	
α -Casein	LIVTQTMK ^a	932.5	467.6	[M+2 H] ²⁺	227.4	b ₂	46	21	14	Q
	4/73 ^b		467.6		608.6	y ₅	46	23	8	
	1,111/21,756 ^c		467.6		707.6	y ₆	46	21	10	
α -Casein	YLGYLEQLLR ^a	1,266.7	634.8	[M+2 H] ²⁺	249.4	a ₂	56	35	12	Q
	1/20 ^b		634.8		771.8	y ₆	56	33	12	
	3,704/67,128 ^c		634.8		991.7	y ₈	56	31	16	
β -Casein	FFVAPFPEVFGK ^a	1,383.7	693.3	[M+2 H] ²⁺	267.3	a ₂	56	47	18	Q
	1/18 ^b		693.3		676.6	y ₆	56	43	10	
	3,704/61,455 ^c		693.3		920.8	y ₈	56	29	16	
β -Casein	GPFPPIV ^a	741.4	742.5	[M+H] ⁺	441.5	y ₄	101	41	12	Q
	1/35 ^b		742.5		512.4	b ₅	101	41	16	
	3,704/117,654 ^c		742.5		625.6	b ₆	101	35	10	
β -Casein	VLPVPQK ^a	779.5	780.6	[M+H] ⁺	213.2	b ₂	101	61	12	Q
	1/34 ^b		780.6		372.4	y ₃	101	51	10	
	3,704/111,919 ^c		780.6		568.6	y ₅	101	45	16	

Working ranges were obtained in a calibration analysis: seven peptides dissolved in organic solvent (standard mixture, injected at eight concentration levels). The protein concentrations were calculated from the concentration of according peptide masses

LOD limit of detection, LR linear range, Q1 precursor ion, Q3 product ion, DP declustering potential, CE collision energy, CXP cell exit potential, V volts, Q quantifier

^aTryptic peptide

^bLOD: peptide/protein (ng/ml)

^cLR: peptide/protein (ng/ml)

ions to the detector) in the mass range 350–1,500 and 1,500–2,800 Da for a duration of 1.5 min each (corresponding to 45 accumulated scans each). Charge states of the precursor ions of intact tryptic peptides were confirmed by measurements in the enhanced resolution (ER) scan mode, hereby scanning a range of 15 Da around the precursors (*m/z* of precursor ± 15 Da). In the EPI mode, the precursor ions were fragmented with a collision energy (CE) of 50 ± 20 V collision energy spread, and the resulting product ions were scanned at a speed of 4,000 Da/s in the mass range 50 Da to *m/z* of precursor ion +20 Da, corresponding to 30 accumulated scans. Declustering potential for MS full scans and EPI was 70 V. Dwell time for each SRM transition was chosen 50 ms.

Calibration of the LC-MS/MS method in SRM mode

For the determination of the concentration of the peptides in the tryptic digested food samples, the LC-MS/MS method in SRM mode was calibrated by analyzing eight dilution

levels of a mixture containing seven synthetic standard peptides (concentration of each peptide, 111.1 $\mu\text{g/mL}$). This mixture was diluted with 50% acetonitrile (*v/v*) and 5 mM ammonium acetate in nine steps (concentration range from 1.1 to 3,704 ng/mL per peptide). The calculated concentrations agreed well with expected peptide concentrations (the arithmetic mean of accuracy was $102 \pm 26\%$). These results were obtained from seven replications. Coefficients of determination of calibration curves were between 0.9962 and 1.

Results and discussion

Identification of marker for milk allergens

Searching for possible markers by *in silico* digestion

As the sequences of the four milk proteins are known, *in silico* tryptic digestions of them were performed. This

facilitates the searching for peptides, which could subsequently be used as markers in MS measurements. For this purpose, 'PeptideMass' [21] was used. The sequences of milk proteins available in the database (with accession numbers P02662, α -S1-casein precursor; P02666, β -casein precursor; P00711, α -lactalbumin precursor; and P02754, β -lactoglobulin precursor) were theoretically digested with trypsin, and the masses of peptides were calculated. The obtained peptides served as candidate list for further MS experiments.

Peptide identification and characterization by MS and MS/MS

After digestion of four selected milk proteins with trypsin, the tryptic peptides in the samples were detected selectively by MS/MS. In short, the peptides, which were found by *in silico* digestions, were searched in MS full scan spectra of respective digested milk protein. The accurate masses of those, which were found in full scan, were determined with scan mode enhanced resolution (ER). Then, these compounds were fragmented in enhanced product ion mode (EPI) scan mode and their structures confirmed.

The analytical strategy for the peptide LIVTQTMK from BLG is described hereafter and was applied in the same way to the other tryptic peptides (data not shown). Screening and structural characterization was carried out by directly infusing diluted tryptic digests of analytical standards of milk allergen proteins into the mass spectrometer. First, MS full scan experiment of BLG was performed in the mass range of m/z 350–1,500 and 1,500–2,800 Da. MS signals of putative singly, doubly and triply charged ions of tryptic peptides, calculated from the data of the *in silico* digestion of the corresponding allergen proteins (in this case BLG), were searched in the MS spectra. For BLG, ten peptides were found, some of them appearing in two different charge states (Fig. 1). Also, the MS analysis of the digests of the other milk proteins yielded several ions of peptides. Subsequently, the charge states of the peptides were confirmed by individual measurements of the respective ions in the enhanced resolution (ER) scan mode (data not shown). The instrument accumulates the intact ions in the LIT and scans them to the detector at a low speed, thus providing more accurate mass values and better mass resolution than the Q1-MS full scan mode (data not shown). Then, the candidate ions were analyzed by MS/MS in enhanced product ion (EPI) scan mode, to determine their amino acid sequences. In this measurement mode of the QTrap 4000 instrument, precursor ions at a defined m/z ratio are selected in the quadrupole Q1, fragmented by collision-induced dissociation (CID) in the collision cell, the generated product ions trapped in the quadrupole Q3 operated as LIT and scanned at high sensitivity to the detector. Figure 2 shows the MS/MS product ion spectrum of the $[M+H]^+$ precursor ion

at m/z 933.6, of which the entire amino acid sequence of the tryptic peptide LIVTQTMK can be deduced. As in this case, the fragmentation predominantly yielded product ions of the y -ion series; the amino acid sequence of the peptide has to be read from right to left. The tandem MS/MS data were confirmed by analysis of a peptide standard and by a theoretical MS/MS fragmentation of the putative peptide sequence carried out with the software 'Peptide Fragment Ion Analyser' (PFIA-II) [23].

In total, the structures of ten tryptic peptides of BLG were determined by MS/MS, as well as five peptides of ALA, six peptides of α -casein and five peptides of β -casein. From these peptides, a total of seven peptides (one from ALA and two for each other chosen milk protein), which exhibited the highest signal intensities in the above-mentioned LC-MS/MS experiments (in EPI mode), were selected as standard peptides for the development of an LC-MS/MS profiling method and synthesized. The synthetic peptides were purchased as lyophilized material with high purity (from 97% for LIVTQTMK to 100% for IPAVFK). They were dissolved in methanol with the concentration of 1 mg/mL as stock solutions.

Development of an LC-MS/MS profiling method

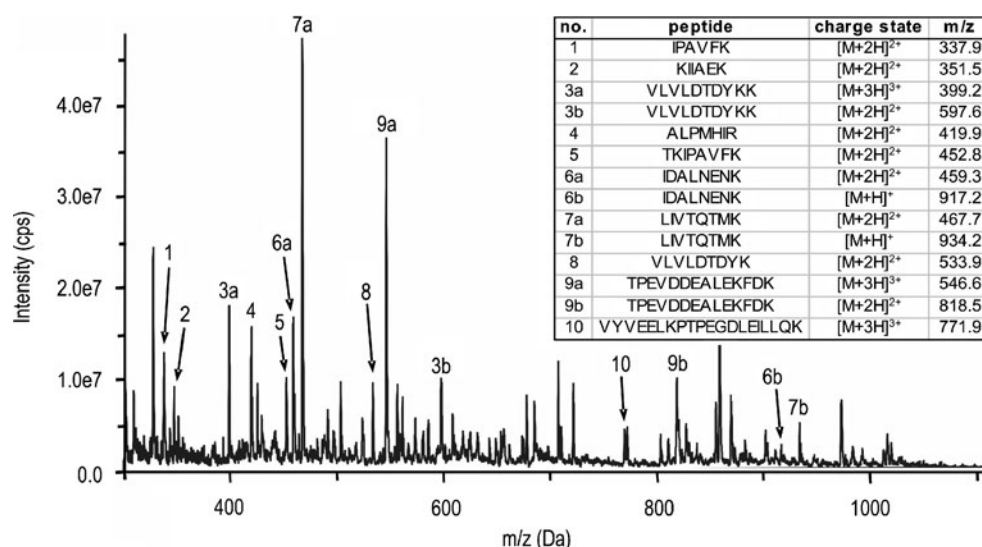
The establishment of a profiling method is described here only for the tryptic peptide LIVTQTMK of BLG. It was carried out in the same way for the other six peptides. For this purpose, the MS/MS analysis mode 'Selected Reaction Monitoring' (SRM) of the QTrap 4000 was employed. The SRM chromatogram represents only product ions of a particular m/z ratio, which have been generated by fragmentation of a selected precursor ion. Therefore, this method has a very high specificity and sensitivity for the respective compound.

Analyte-dependent SRM parameters were optimized by direct infusion of synthetic peptides into the mass spectrometer. After combining SRM parameters with an LC gradient program, a selective LC-MS/MS method was obtained that allowed the specific determination of one or more analytes. This method was extended to a multianalyte method by including the SRM parameters for the individual tryptic peptides of the milk allergens listed in Table 1. Each analyte can be identified by using three specific SRM transitions, attaining their signal maximum at the same retention time. For each of the peptides, the most intense SRM transition served as quantifier (Q), whereas the second and third SRM transitions were chosen as qualifiers for the identification of the target compound.

Determination of species specificity by BLAST search

Since the same or closely homologous proteins and their variants are present in milk of different ruminant species—

Fig. 1 MS full scan spectrum of a tryptic digest of BLG, directly infused into the mass spectrometer, containing diagnostic peptide ions, which were listed in the table (insert). The charge states of the peptides have been confirmed by MS measurements in the enhanced resolution (ER) scan mode



for example, cow milk caseins occur with 80% to 90% sequence homologies in the other species as well [1]—a BLAST search was applied (BLAST 2.2.18, first access 28.10.2008) [22] in order to determine the species specificity of these selected tryptic peptides.

The results of BLAST search for all of the seven peptides are summarized in Table 2. The BLAST search showed that all the peptide sequences were found in *Bos taurus* (cattle). The peptide sequence FFVAPFPEVFGK of α -casein appeared additionally in α -casein of *Bubalus bubalis* (water buffalo). YLGYLEQLLR, also from α -casein, was found in α -casein of cattle, water buffalo, *Capra hircus* (goat) and *Ovis aries* (sheep). Similarly, GPFPIIV from β -casein was found in β -casein of water buffalo as well as in cattle. The second selected peptide of β -casein, VLPVPQK, seems to be

a highly conserved region in mammals protein, as it was found in β -casein of cattle, water buffalo, goat, sheep, *Bos indicus* (zebu), *Giraffa camelopardalis* (giraffe) and even in *Tragulus napu* (greater mouse-deer), a species of even-toed ungulate which is found in Southeast Asia.

Like VLPVPQK, the peptide VGINYWLAHK from ALA could be found in ALA of various species, e.g. cattle, water buffalo, goat, sheep, zebu, *Bos grunniens* (yak) and also in *Cervus canadensis xanthopygus* (Manchurian wapiti), a subspecies of elk, native to eastern Asia. The peptide IPAVFK from BLG was found in BLG of cattle, water buffalo, goat, sheep, and *O. aries musimon* (European mouflon). However, LIVTQTMK, also from BLG, seems to be cattle specific and could not be found in the other species. These results are data bank-dependent; currently, no

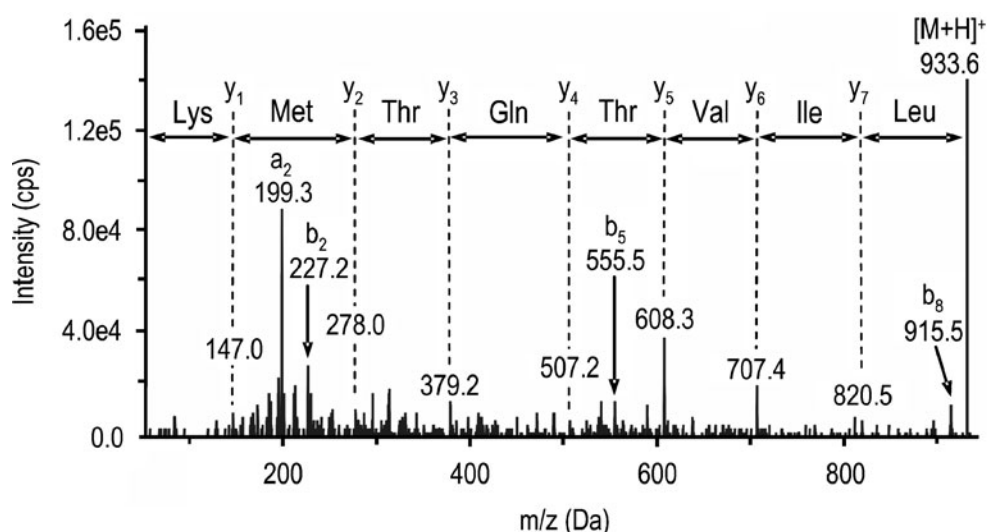


Fig. 2 MS/MS spectrum of the tryptic peptide LIVTQTMK from BLG. As fragment ions of the y-ion series occur in the spectrum, the amino acid sequence must be read from right to left

Table 2 The results of BLAST search for selected standard peptides of four milk allergens

Species	α -Lactalbumin	β -Lactoglobulin		α -Casein		β -Casein	
	VGINYWLAHK	LIVTQTMK	IPAVFK	FFVAPFPEVFGK	YLGYLEQLLR	VLPVPQK	GPFPPIV
Cattle	✓	✓	✓	✓	✓	✓	✓
Water buffalo	✓		✓	✓	✓	✓	✓
Goat	✓		✓		✓	✓	
Sheep	✓		✓		✓	✓	
Zebu	✓					✓	
Giraffe						✓	
Greater mouse-deer						✓	
Yak	✓						
Manchurian wapiti	✓						
Mouflon			✓				

Only accessions with 100% query coverage were considered

more proteins could be found, but the data bank should be searched routinely to find out if there are any new entries.

Consequently, LIVTQTMK was the only peptide which could be used as a specific marker for detection of cow milk in the samples. The two other peptides, namely, GPFPPIV from β - and FFVAPFPEVFGK from α -casein, could be used as marker for detection of milk from cow and water buffalo; however, the probability of contamination of cow milk with water buffalo milk is low. The other peptides could be used as markers for identification of ruminant milk in general, where cow milk is one of them.

Allergenicity studies have shown the existence of numerous allergenic IgE and IgG binding epitopes, which are widely spread along each of the four milk allergens [1, 3–6, 16]. All of our seven selected peptides are within these epitopes, for example, a study carried out on sera of 46 patients allergic to milk showed that LIVTQTMK and IPAVFK, both from BLG, could be recognized by 65.4% and 28.3% of the sera, respectively [5].

Additionally, it has to be taken into consideration that the LC-MS/MS method is not able to distinguish between isobaric peptides such as LIVTQTMK from cattle and IIVTQTMK from European mouflon. Both of them have a molecular weight of 933.54 Da, but differ in their sequences due to the isobaric amino acids Leu and Ile in the first position.

Identification of milk allergens in food samples by the LC-MS/MS profiling method

In calibration analysis with synthetic peptide standards, the lowest determined concentrations were between 1 and 11 ng/mL (signal-to-noise ratio=3; Table 1), dependent on the peptide. For most of the peptides, e.g. for the BLG-derived peptide LIVTQTMK, the lowest concentration was

found to be 1.11 ng/mL; the linear range extends up to 1,111 or 3,704 ng/mL, respectively. Only for VGINYWLAHK (from ALA), the concentration was as high as 11.1 ng/mL; it exhibited poor SRM signal response, although it showed through five candidate peptides from α -lactalbumin the highest signal intensities in the MS full scan to MS/MS product ion scans prior to purchase of standards.

Using these synthetic peptides and their calibration curves, the presence and also the concentration of natural milk proteins in different food samples could be determined (Table 3). First, samples like milk and dairy products (yoghurt, various different cheese sorts, e.g. parmesan and mozzarella and whey drink) and also milk powder were used to develop and establish the method. At the next step, the method was applied to different samples as yoghurt from sheep's milk and chocolate samples, where milk belonged to ingredients such as country crisp, milky bites, nutella and Neapolitan wafer, and also blank samples, for example, sponge fingers, peanut puffs and ice cream cones. The samples were diluted to different concentrations before being analyzed with the LC-MS/MS method in SRM mode, which consisted of parameters for the seven tryptic peptides specifying four milk proteins. Characteristic peak patterns (profiles) were obtained, indicating significant differences between the concentrations of the individual peptides in the samples (Fig. 3).

The method showed good sensitivity, and the results could be repeated. All of the selected peptides could be detected and measured with a concentration down to 1 ng/mL in milk powder, milk and dairy products, as expected; in cheese varieties and also in yoghurt, the lowest detectable concentration of whey proteins is lower than casein proteins as estimated. It seems that fermentation did not disturb the detection. In whey drink, all tryptic peptides

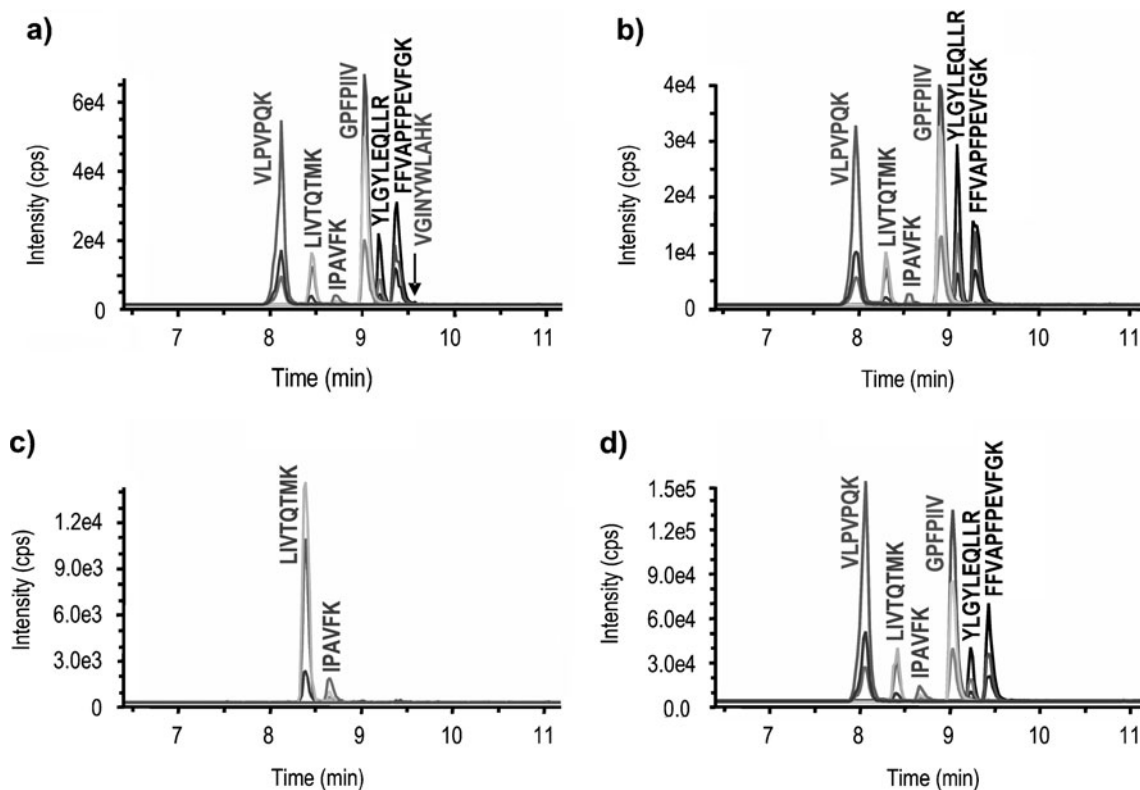
Table 3 Lowest detectable concentration for different food samples and blanks

Food samples (replications)	Lowest detectable concentration (ng/mL)						
	ALA	BLG		α -Casein		β -Casein	
	VGINYWLAHK	IPAVFK	LIVTQTMK	YLGYLEQLLR	FFVAPFPEVFGK	GPFPPIV	VLPVPQK
Milk (6)	3.8	3.4	1.0	3.0	1.1	2.1	1.2
Yoghurt (5)	9.6	5.3	7.6	23.9	21.2	16.3	10.1
Mozzarella (4)	1.3	3.2	4.4	198.0	182.0	114.0	68.8
Parmesan (4)	15.5	5.0	1.1	12.4	7.6	5.3	3.3
Whey drink (4)	17.3	2.4	2.8	1.1	1.8	1.8	1.5
Milk powder (4)	21.5	11.2	10.2	41.8	82.4	57.3	31.6
Sheep's yoghurt (2)	14.0	4.4	15.7	25.2	2.8	25.0	26.5
Country crisp (4)	17.7	9.4	10.4	42.8	47.6	40.0	22.8
Milky bites (3)	5.8	3.3	2.5	14.7	16.2	12.1	6.0
Hazelnut spread (2)	No peak	3.9	1.1	4.4	7.5	4.3	2.2
Neapolitan wafer (2)	11.1	7.7	4.9	20.8	22.3	16.2	5.5
Sponge fingers (2)	No peak	No peak	No peak	No peak	1.5	2.0	1.2
Peanut puffs (2)	No peak	No peak	No peak	No peak	No peak	No peak	No peak
Ice cream cones (2)	No peak	No peak	No peak	1.6	1.4	No peak	1.2

The number of replications was given in parentheses. The measurements were performed in different dilution

of whey and casein proteins could be detected with the concentration down to 1.1 ng/mL. The lowest detectable concentration for milk powder is much higher than for milk. It could be based on the fact that milk powder could

not be resuspended well in aqueous buffer. All of the peptides could be detected in sheep's yoghurt, too. However, regarding BLAST search (Table 2), three of seven selected milk peptides should not appear in sheep

**Fig. 3** Profiling of milk (a) and three milk-containing food samples: (b) parmesan, (c) whey drink and (d) yogurt by LC-MS/MS in SRM mode

milk. It could be evidence either for food adulteration (mixing cow milk with sheep) or cross-contamination during production.

In milk chocolate samples, again all peptides could be determined with the lowest detectable concentration down to 1.1 ng/mL. Only VGINYWLAHK from ALA could not be detected in hazelnut spread. It seems that with this method also, the detection of probably masked proteins by polyphenols is possible compared to immuno-based methods, where special extraction efforts have to be taken into consideration for extraction of proteins from samples including chocolate to avoid protein loss by polyphenols appearing in chocolate.

Although in the ingredients of sponge fingers and ice cream cones no milk was listed, in both of them, three peptides could be detected, however, in low concentrations; it could be an evidence of cross-contamination

during processing. Only peanut puffs proved to be a real blank sample, which was further used for spiking experiments.

Recovery calculation for spiked samples

The spiking experiments could be divided into two parts: standard addition and recovery calculations. The standard addition of peptides mixtures to food samples was used to test the matrix effect. For this purpose, at first, all 14 digested food samples as well as four used concentrations of peptide mixtures were separately measured by MS, and then the spiked samples after standard addition. The standard additions were calculated by dividing the calculated concentrations of spiked samples (from peak area) by the sum of the concentrations of peptide mixture and food samples (Table 4a). For calculating the results in Table 4,

Table 4 Spiked samples

Food and blank samples	ALA	BLG		α -Casein		β -Casein	
	VGINYWLAHK	IPAVFK	LIVTQTMK	YLGYLEQLLR	FFVAPFPEVFGK	GPFPIIV	VLPVPQK
Standard addition (%)							
Milk ^a	77±2	85±5	97±0	106±4	92±7	95±0	98±0
Yoghurt ^a	82±2	87±0	85±20	71±1	99±1	88±4	95±4
Mozzarella ^a	80±20	82±12	85±23	97±5	98±2	97±1	92±9
Parmesan ^a	87±6	79±10	84±15	91±13	92±5	93±4	86±4
Whey drink ^a	68±7	69±13	81±9	96±10	96±4	93±8	84±10
Milk powder ^a	78±5	94±0	103±4	112±13	96±3	97±3	92±7
Sheep's yoghurt ^a	83±11	77±13	105±8	91±9	92±6	97±1	92±3
Country crisp ^a	70±3	92±4	85±7	101±5	92±3	87±1	84±3
Milky bites ^a	82±5	79±21	69±7	92±7	95±4	96±1	83±4
Hazelnut spread ^a	86±7	80±23	70±16	85±11	88±9	87±6	87±7
Neapolitan wafer ^a	89±13	74±10	78±10	80±9	96±3	96±5	85±9
Sponge fingers ^a	95±6	76±4	72±21	85±2	94±4	84±3	89±6
Peanut puffs ^a	91±9	50±1	54±3	84±12	93±8	95±2	95±1
Ice cream cones ^a	89±5	80±7	57±25	70±14	89±0	90±5	84±4
Recovery (%)							
Blank extract spiked with milk ^b	Not possible	98±10	52±1	102±1	97±6	89±4	72±5
Blank extract spiked with peptides mixture ^b	59±7	93±6	80±3	102±7	112±5	128±12	79±5
Blank spiked with 1 ml of milk ^c	89±4	80±22	72±11	103±15	109±4	94±4	98±5
Blank spiked with 500 µl of milk ^c	92±11	129±4	102±1	110±7	128±10	98±8	113±1
Blank spiked with peptides mixture ^c	89±7	100±11	90±12	99±15	98±9	129±14	101±2

^aStandard addition; four concentration of peptide mixtures were added to the food samples after digestion (the mean values of four concentrations are given in the table)

^bBlank sample (peanut puffs) spiked with milk or peptides mixture after extraction, before digestion

^cBlank sample (peanut puffs) spiked with milk or peptides mixture before extraction

The recoveries were calculated from two replications (for b and c)

all spike levels were considered, and arithmetic means were calculated. The results were satisfying because all the peptides were found again with high percentage (over 50%).

Alternatively, to determine the influence of extraction and digestion procedure, the selected blank sample (peanut puffs) was spiked with either milk or peptide mixture, directly before and after extraction, then digested with trypsin, measured with MS and again recoveries were calculated (Table 4b). The calculations were performed by dividing the concentrations of spiked samples (measured by peak area) by the sum of the concentrations of blank (which should be zero) and milk, or peptide mixture, respectively.

The calculated recoveries for spiked blank samples showed that the digestion procedure only has an influence on the already identified critical peptide VGINYWLAHK from ALA, as this was the only peptide which was not detectable. The recoveries for the other peptides with over 52% were satisfying. The measurements including the extraction procedure also showed very good reproducibility from 72 to 129% for all peptides which indicates that in this case, the extraction procedure and the tryptic digest do not have a negative influence on the following MS measurements, and there are no losses of peptides detectable; there is also no significant difference between spiking with milk or peptides mixture here.

Conclusion

Seven peptides (chain length, 5–12 amino acids) derived from tryptic digestion of four allergenic milk proteins were selected, according to data obtained from evaluation of MS/MS spectra, as potential markers for the detection of these proteins (α -casein, β -casein, α -lactalbumin and β -lactoglobulin) in food samples. The BLAST search has shown that only one peptide (LIVTQTMK from BLG) could be used as a specific marker for detection of cow milk in the samples; two other peptides, FFVAPFPEVFGK (α -casein) and GPFPIIV (β -casein), could be used as marker for detection of milk from cow and water buffalo; and four other peptides could be used as markers for identification of ruminant milk in general. Using these peptides as the standards, an LC-MS/MS multianalyte method based on SRM for the simultaneous determination of the peptides was developed, optimized and applied to tryptic digestion of different dairy and processed food samples, and also to blanks and spiked samples. The results showed that the method was able to detect the selected peptides in samples with concentrations down to 1 ng/mL and yielded peak patterns characteristic for the individual samples; however, for further trace amount determination, the method has to undergo a complex validation procedure.

The HPLC method itself is very rapid as the run lasts only 20 min. The LC-MS/MS in combination with the selected reaction monitoring (SRM) is a very selective method with good quantification properties. Although sample preparation is more complex compared to immuno-based assays, it could be shown by spiking experiments that the extraction and digestion procedures do not have a negative influence on the results. Therefore, the used LC-MS/MS method could be a possible candidate in the process of reference method search for food allergen detection.

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