SPECIAL GUEST EDITOR SECTION

Development and Validation of a Method for the Quantification of Milk Proteins in Food Products Based on Liquid Chromatography with Mass Spectrometric Detection

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The protection of allergic consumers is crucial to the food industry. Therefore, accurate methods for the detection of food allergens are required. Targeted detection of selected molecules by MS combines high selectivity with accurate quantification. A confirmatory method based on LC/selected reaction monitoring (SRM)-MS/MS was established and validated for the quantification of milk traces in food. Tryptic peptides of the major milk proteins β-lactoglobulin, β-casein, αS2-casein, and κ-casein were selected as quantitative markers. Precise quantification was achieved using internal standard peptides containing isotopically labeled amino acids. For each peptide, qualifier and quantifier fragments were selected according to Commission Decision 2002/657/EC. A simple sample preparation method was established without immunoaffinity or SPE enrichment steps for food matrixes containing different amounts of protein, such as baby food, breakfast cereals, infant formula, and cereals. Intermediate reproducibility, repeatability, accuracy, and measurement uncertainty were determined for each matrix. LOD values of 0.2–0.5 mg/kg, e.g., for β-lactoglobulin, were comparable to those obtained with ELISA kits. An LOQ of approximately 5 mg/kg, expressed as mass fraction skim milk powder, was validated in protein-rich infant cereals. The obtained validation data show that the described LC/SRM-MS/MS approach can serve as a confirmatory method for the determination of milk traces in selected food matrixes.

Cow's milk is one of the most common causes of allergic reactions in the early years of life. Approximately 2.5% of infants show adverse reactions, ranging from mild to severe, to cow's milk (1–3). Cow's milk contains approximately 3.5% protein, which is usually divided into the casein fraction that represents approximately 80% of the total protein and the whey fraction that represents the remaining 20%. Among these proteins, eight have been characterized as being allergenic, including αS1-casein (*Bos* d 8 α-S1), αS2-casein (*Bos* d 8 α-S2), β-casein (*Bos* d 8 b), and κ-casein (*Bos* d 8 k) from the casein fraction, and α-lactalbumin (*Bos* d 4), β-lactoglobulin (*Bos* d 5), bovine serum albumin (*Bos* d 6), and lactoferrin from the whey fraction (4).

Undeclared allergens can be inadvertently introduced into a food during manufacturing due to the use of common production lines and equipment. Analysis of food samples for the presence of undeclared allergens is an integral part of food allergen management, particularly for validating equipment-cleaning procedures as well as monitoring finished foods for traces of allergenic ingredients.

Currently, ELISA, PCR, and real-time PCR are the methods of choice for detection of many food allergens. These techniques are relatively fast and easy to use in routine analysis. ELISA can detect the allergenic protein, while PCR allows only an indirect determination of the presence of an allergenic food commodity via DNA markers (5, 6). For ELISA, the effectiveness of the analysis depends on the quality of the antigen used as the target molecule and the quality of the antibodies directed against the antigen. Furthermore, food processing can modify these target proteins (7, 8) and may affect their allergenic potential and binding affinity to antibodies (9), and may lead to false-negative or underestimated results in ELISA testing. Currently, there are no official and confirmatory methods and no certified reference materials that could be used to support a harmonized approach to food allergen detection.

In recent years, MS-based techniques have been used to characterize, identify, or quantify food allergen proteins (10–13). However, the analytical strategies vary in terms of sample preparation or the choice of the target molecules, which were entire proteins or peptides derived by enzymatic digestion. For milk allergens, the first MS-based quantification of whey proteins was described by Huber and Premstaller (14). They used selected ion monitoring in combination with external calibration for

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Table 1. LC gradient used for the separation of milk peptides with an Agilent 1200 HPLC instrument; solvent A: 0.1% formic acid in LiChrosolv purified water and solvent B: 0.1% formic acid in LiChrosolv acetonitrile

Time, min	Solvent A, %	Solvent B, %
0	98	2
5	98	2
45	50	50
48	35	65
50	0	100
53	0	100
55	98	2
65	98	2

α-lactalbumin, β-lactoglobulin B, and β-lactoglobulin A. Internal standards (ISs) were used by Czerwenka et al. (15) for the quantification of β-lactoglobulin in different cow's milk products using RP-HPLC protein separation and MS detection of the entire proteins in the full-scan mode. Species variants of bovine β-lactoglobulin were used as ISs. The authors showed an increasing loss of β-lactoglobulin with increasing heat treatment due to milk processing. Nano-LC/MS/MS analysis of tryptic digested matrixes subjected to further purification has been developed for the detection of milk allergens in cookies (16). MS, especially the targeted detection of selected molecules known as selected reaction monitoring (SRM) or multireaction monitoring, combines high selectivity with accurate quantification. This approach is frequently used for quantification of small molecules and is now more and more adapted to protein and peptide quantification. Direct MS determination of cow's milk proteins without prior enzymatic digestion was established in mixed-fruit juices using SPE and LC coupled to quadrupole MS with full-scan and multiple-ion monitoring acquisition modes (17). The multiple-ion monitoring acquisition mode allowed detection of milk protein traces down to 1 μg/mL. This approach was improved in order to detect peptides arising from α and β casein in fined white wine down to 100 and 1000 μg/mL, respectively (18, 19).

This paper describes details of the development and validation of a sensitive and nonimmunological method for the quantification of milk traces based on selective determination of peptides specific for β-lactoglobulin A and B, αS2-casein, β-casein, and κ-casein. It is a confirmatory method and complementary to ELISA. The analytical procedure described encompasses a simple extraction of the food sample using a buffered solution containing ammonium bicarbonate and urea, without any additional enrichment or solid-phase purification step. Centrifugation is used to remove insoluble residues before enzymatic digestion of the supernatant using porcine trypsin. Subsequent analysis by LC-electrospray ionization (ESI)-MS/MS in SRM using the positive ionization mode is done after addition of the IS solution containing the respective [$^{13}C_6$, $^{15}N_2$]-lysine or [$^{13}C_6$, $^{15}N_4$]-arginine stable isotope-labeled synthetic peptide homologs of β-lactoglobulin, αS2-casein, β-casein, and κ-casein derived peptides. Positive identification of selected peptides in the sample and validation of the method were conducted according to the criteria defined in European Union (EU) Commission Decision 2002/657/EC (20). Recoveries, precision, and measurement uncertainty were calculated from the analysis of replicate extractions from infant formula, infant cereals, breakfast cereals, and baby food matrixes, as well as rinse water spiked with skim milk powder (SMP) at different fortification levels covering the range from 1 to 150 mg/kg. Highest sensitivity was achieved in baby food, with an LOD for SMP of <1 mg/kg and an LOQ of 2 mg/kg. Food matrixes with higher protein concentrations, like breakfast cereals and infant cereals, as well as rinse water resulted in an LOQ of 5 mg/kg, while soy-based infant formula had an LOQ of 20 mg/kg, probably due to strong matrix suppression effects.

Experimental

Reagents

Ammonium bicarbonate was purchased from Fluka (Buchs, Switzerland). Urea, formic acid 98–100%, acetonitrile (LiChrosolv® hypergrade for LC/MS), and LiChrosolv water hypergrade for LC/MS were from Merck (Darmstadt, Germany). Trypsin (sequencing grade modified, V511 20 μg lyophilized enzyme) was purchased from Promega (Madison, WI). Veratox ELISA for determination of milk was from Neogen (Lansing, MI) and Ridascreen β-lactoglobulin ELISA kit from R-Biopharm (Darmstadt, Germany).

Purified proteins β-casein, κ-casein, β-lactoglobulin (BLG), and β-lactoglobulin A and B (all bovine) were obtained from Sigma (Steinheim, Germany); casein was obtained from Fluka.

SMP samples used were nonfat milk powder, National Institute of Standards and Technology, Gaithersburg, MD, standard reference material (No. 1549, protein amount 34.7%), Fluka SMP (protein amount 33.8%), and SMP from Hochdorf Holding AG (Hochdorf, Switzerland, protein amount 22.9%).

Materials and Apparatus

All analyses were performed using an Agilent 1200 HPLC instrument coupled to a 6460 triple quadrupole mass spectrometer equipped with an Electrospray Jet Stream ionization source (all from Agilent Technologies, Geneva, Switzerland). Peptides were separated on a Waters Corp. (Milford, MA) Symmetry 300™

Table 2. Transition reactions with collision energies in eV (given within parentheses) of target and IS peptides[a]

Peptide	Fragmentor energy, V	Transition reactions, *m/z*, used for: Quantification (quantifier ion)	Analyte confirmation (qualifier ion)	Relative intensity (Ratio of qualifier:quantifier ion), % Calibration solution	Infant formula	Breakfast cereals	Infant cereals	Baby food	Rinse water	Peak area ratio limit, %
β-casein AVPYPQR	110	415.5 → 400.2 (15)	415.5 → 660.3 (11)	86 ± 2	77 ± 4	83 ± 2	82 ± 1	84 ± 1	85 ± 1	±20
β-casein IS AVPYPQR [$^{13}C_6$, $^{15}N_4$]		420.5 → 410.2 (15)	420.5 → 670.3 (11)	56 ± 5	74 ± 1	62 ± 1	66 ± 1	59 ± 0.4	58 ± 2	±20
β-lactoglobulin TPEVDDEALEK	165	623.5 → 819.4 (22)	623.5 → 918.4 (22)	76 ± 6	88 ± 12	80 ± 3	78 ± 2	80 ± 2	82 ± 2	±20
β-lactoglobulin IS TPEVDDEALEK [$^{13}C_6$, $^{15}N_2$]		627.5 → 827.4 (22)	627.5 → 926.4 (22)	62 ± 1	81 ± 5	74 ± 2	77 ± 2	72 ± 1	65 ± 3	±20
β-lactoglobulin VLVLDTDYK	145	533.3 → 853.4 (9)	533.3 → 754.4 (9)	44 ± 1	45 ± 2	43 ± 1	42 ± 1	43 ± 2	43 ± 1	±25
β-lactoglobulin IS VLVLDTDYK [$^{13}C_6$, $^{15}N_2$]		537.3 → 861.4 (9)	537.3 → 762.4 (9)	43 ± 1	43 ± 1	45 ± 0.5	44 ± 0.5	45 ± 2	43 ± 1	±25
αS2 casein ALNEINQFYQK	155	684.3 → 827.4 (16)	684.3 → 940.5 (15)	47 ± 2	48 ± 7	45 ± 2	43 ± 3	45 ± 0.4	46 ± 1	±25
αS2 casein IS ALNEINQYQK[$^{13}C_6$, $^{15}N_2$]		688.3 → 835.4 (16)	688.3 → 948.5 (15)	41 ± 1	45 ± 5	48 ± 4	46 ± 1	45 ± 0.3	42 ± 4	±25
αS2 casein FALPQYLK	140	490.1 → 648.4 (7)	490.1 → 761.5 (7)	55 ± 1	55 ± 5	55 ± 1	57 ± 2	55 ± 1	56 ± 0.4	±20
αS2 casein IS FALPQYLK [$^{13}C_6$, $^{15}N_2$]		494.1 → 656.4 (7)	494.1 → 769.5 (7)	54 ± 1	59 ± 3	52 ± 2	54 ± 1	52 ± 2	53 ± 3	±20
κ-casein YIPIQYVLSR	155	626.3 → 975.6 (10)	626.3 → 765.4 (20)	51 ± 1	65 ± 5	62 ± 1	64 ± 1	64 ± 0.4	61 ± 1	±20
κ-casein IS YIPIQYVLSR [$^{13}C_6$, $^{15}N_4$]		631.3 → 985.6 (10)	631.3 → 775.4 (20)	57 ± 1	64 ± 2	65 ± 3	62 ± 2	61 ± 2	62 ± 3	±20

[a] Parent ions are $[M+2H]^{2+}$ ions, and fragment ions $[M+H]^{1+}$ ions. Ratios of qualifier:quantifier ions were determined for calibration solutions on 7 different days, and SMP-fortified food matrixes at 50, 100, and 150 mg/kg levels in replicate analyses. Peak area ratio limits are fixed according to EU criteria (ref. 20).

Table 3. Approximate retention times and mobile phase B (0.1% formic acid in LiChrosolv acetonitrile) elution concentrations

Protein	Peptide	Retention time, min	Mobile phase B, %
β-Casein	AVPYPQR	19	19
β-Lactoglobulin	TPEVD-DEALEK	22	22
β-Lactoglobulin	VLVLDTDYK	26	27
αS2-Casein	ALNEINQ-FYQK	27	29
αS2-Casein	FALPQYLK	30	32
κ-Casein	YIPIQYVLSR	32.5	35

C_{18} column (3.5 µm particle size, 2.1 × 150 mm, 300 Å) equipped with a Symmetry 300™ C_{18} 3.5 µm, 2.1 × 10 mm guard column. For sample preparation and extraction, the following materials were used: a rotating shaker (Janke & Kunkel IKA-Labortechnik, Staufen, Germany); a refrigerated centrifuge with rotor adapted for 50 mL tubes (Eppendorf AG, Hamburg, Germany); a laboratory blender (Waring, Bender and Hobein, Zurich, Switzerland); a ball mill with steel milling jars and balls (Retsch, Haan, Germany); and 50 mL conical centrifugation tubes (polypropylene; Falcon, Le Pont de Claix, France). All peptide solutions and sample extracts were prepared in LoBind Safe Lock reaction tubes (Eppendorf) using Milliex® low protein-binding, syringe-driven filter units with a Durapore® PVDF membrane, 0.45 µm (Millipore, Billerica, MA) for robustness testing. For protein quantification, a SPRINT™ Rapid Protein Analyzer (CEM, Matthews, NC) was used according to the manufacturer's instructions.

IS and Calibration Standard Solutions

Synthetic peptides and stable isotope-labeled homologs for β-casein (AVPYPQR and AVPYPQR[$^{13}C_6$,$^{15}N_2$]); β-lactoglobulin A and B (TPEVDDEALEK, TPEVDDEALEK[$^{13}C_6$,$^{15}N_2$]; VLVLDTDYK, VLVLDTDYK[$^{13}C_6$,$^{15}N_2$]); αS2-casein (ALNEINQFYQK, ALNEINQFYQK[$^{13}C_6$,$^{15}N_2$], FALPQYLK, FALPQYLK[$^{13}C_6$,$^{15}N_2$]); and κ-casein (YIPIQYVLSR, YIPIQYVLSR[$^{13}C_6$,$^{15}N_2$]) were purchased individually prepared as solutions in 5% (v/v) acetonitrile from ThermoFisher Scientific (Biopolymers, Ulm, Germany). Purity of these IS peptides was approximately 97% according to the supplier. The concentration of the individual solutions was determined by amino acid analysis as described in Pharm. Eu. 2.2.56 (6.00; 21). Peptides were hydrolyzed for 24 h to their individual amino acid constituents in the presence of 6 M HCl at 110°C. Following hydrolysis, amino acids were covalently labeled with the 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AccQ-Fluor reagent, Waters Corp.) using a precolumn derivatization process according to the supplier's instructions and detected by fluorescence at 395 nm after separation on a C_{18} RP-HPLC column at Protagen AG (Dortmund, Germany).

Afterwards, stable isotope-labeled peptides were mixed and diluted with 0.1% (v/v) formic acid to give a working solution of 1.88 pmol/µL for the peptide β-casein (AVPYPQR and AVPYPQR[$^{13}C_6$, $^{15}N_4$]) and 0.63 pmol/µL for the other peptides. The same procedure was done for nonlabeled synthetic peptides. For IS spiking purposes, the resulting working solutions of the stable isotope labeled peptides were further diluted 125-fold with either 0.1 or 0.2% (v/v) formic acid, resulting in a final concentration of 15 fmol/µL for β-casein AVPYPQR[$^{13}C_6$, $^{15}N_4$] and 5 fmol/µL for the other peptides.

For preparation of the calibration solutions, working solutions of both the stable isotope-labeled peptide and the nonlabeled peptide working solutions were further diluted 31.25-fold with 0.1% (v/v) formic acid, resulting in final concentrations of 60 fmol/µL for β-casein peptides and 20 fmol/µL for the other peptides. The nonlabeled peptides were made up with an increasing concentration, and the stable isotope labeled-peptides were added at a constant concentration. The concentrations of the peptides in 0.1% (v/v) formic acid were 0, 0.75, 1.5, 3.0, 4.5, 7.5, and 15.0 fmol/µL for β-casein AVPYPQR; 0, 0.25, 0.5, 1, 1.5, 2.5, and 5 fmol/µL for the other nonlabeled peptides; 7.5 fmol/µL for β-casein AVPYPQR[$^{13}C_6$, $^{15}N_4$]; and 2.5 fmol/µL for the other stable isotope-labeled peptides.

Table 4. MW values and mass fractions of proteins in milk powders used for the expression of results as mass fraction skim milk powder/kg sample[a]

	αS2-Casein	β-Casein	κ-Casein	β-Lactoglobulin
Molecular weight, kDa	25.3	24	19	18
Mass fraction in dried liquid milk, %	3.5	10	3.5	3
Mass fraction in skim milk powder, %	2.7	8.5	3.1	3.1

[a] *See* refs 24 and 25.

Food Samples

Laboratory-finished product samples used for fortification with SMP were either baby food puree based on carrot and potato (0.7% protein); hypoallergenic soy-based infant formula (14% protein); milk- and lactose-free infant cereals (15.5% protein); and extruded, sweetened breakfast cereal flakes (8.4% protein). The baby food puree laboratory sample was

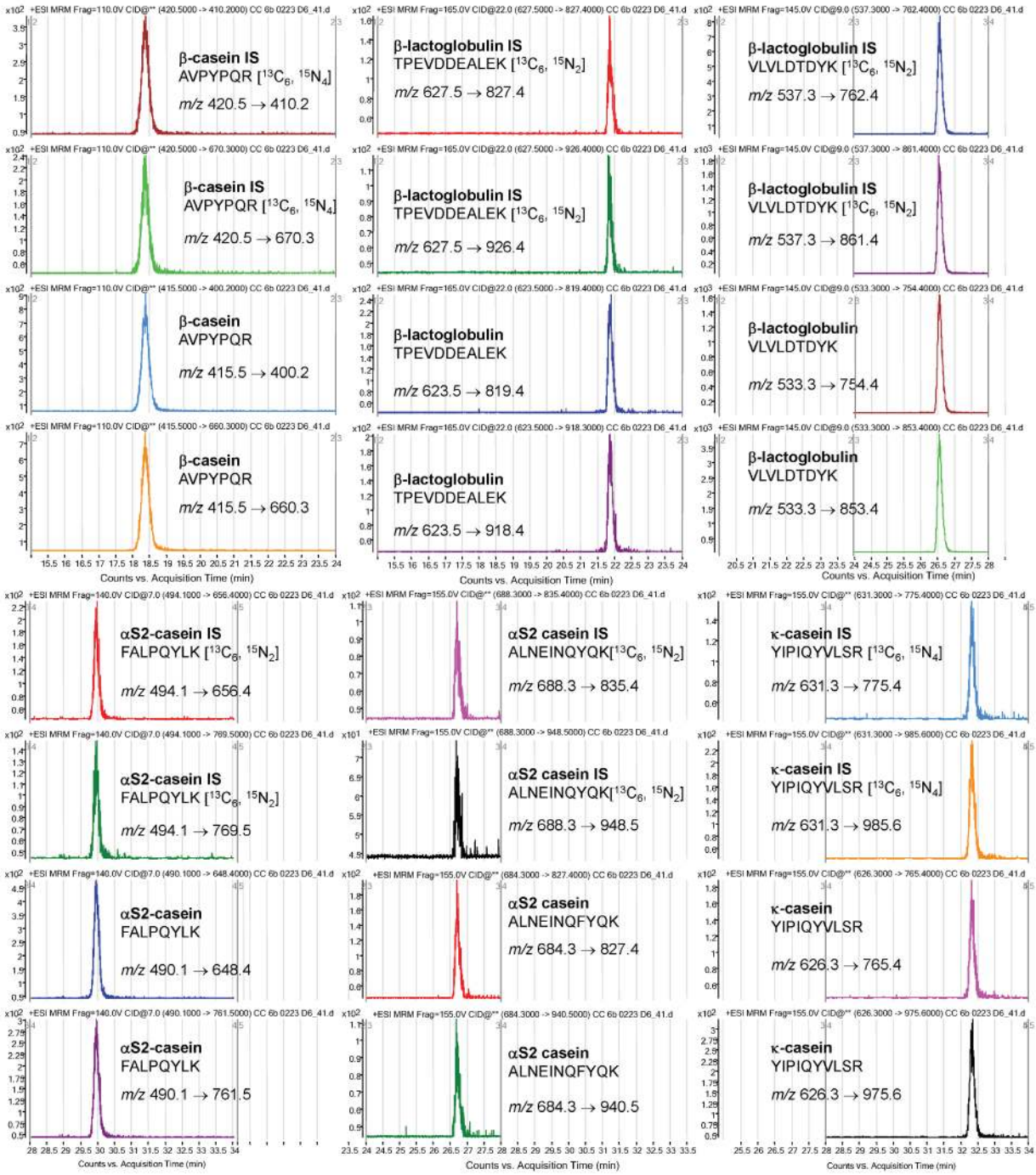

Figure 1. LC/ESI-MS/MS chromatograms of the marker peptides and their related isotopically labeled homologs from calibration solutions (5 fmol/μL). Monitored quantifier and qualifier ions are shown.

rehomogenized, and breakfast cereal flakes were milled using a laboratory blender before weighing the test portions. All samples were tested for the absence of milk traces using commercially available ELISA kits (β-lactoglobulin, Ridascreen, r-Biopharm; Veratox Total Milk, Neogen) according to the manufacturers' instructions provided with the kits. No milk traces were found in these samples with respect to the given detection limits of the ELISA kits (LOD β-lactoglobulin and r-Biopharm = 0.63 mg/kg, and LOD Total Milk, Neogen = 1 mg/kg SMP; data not shown). Food commodities used for selectivity testing were purchased from local supermarkets or provided by Nestlé factories: almond, cashew, hazelnut, peanut, Brazil nut, walnut,

Table 5. Results of BLAST homology searches for the selected marker peptides with 100% query coverage (✓) and nonhomology (×)

Peptide sequence	Protein	Cow	Water buffalo	Goat	Sheep	Reindeer	Human
AVPYPQR	β-Casein	✓	✓	×	×	×	×
TPEVDDEALEK	β-Lactoglobulin	✓	✓	×	×	×	×
VLVLDTDYK	β-Lactoglobulin	✓	✓	✓	✓	✓	×
ALNEINQFYQK	αS2-Casein	✓	✓	✓	✓	✓	×
FALPQYLK	αS2 Casein	✓	×	×	×	×	✓
YIPIQYVLSR	κ-Casein	✓	✓	✓	✓	✓	×

pine nut, pecan nut, pistachio, sesame seeds (black and white), poppy seeds, pumpkin seeds, sunflower seeds, cocoa, wheat flour, barley flour, rye flour, oat flakes, buckwheat flour, corn flour, rice (white), chick peas, peas, soy beans, split peas, lima beans, beef meat, frozen cooked shrimp, dried salmon granules, whole egg powder, lecithin, milk whole powder, demineralized whey powder, sweet whey powder, potassium caseinate, sodium caseinate, milk fat globule membrane enriched protein whey concentrate, acid whey, yogurt powder, fresh yogurt, sweet concentrated milk, ultra-heat-treated (UHT) milk (all bovine), fresh goat's milk, fresh sheep's milk, and buffalo milk powder.

SMP used for sample fortification was reconstituted to give protein concentrations of 10, 1, and 0.1 mg/mL (depending on the spiking level) in purified water, and solutions were gently shaken for 1 h in order to completely dissolve the proteins. Other dairy ingredients were either reconstituted or diluted in extraction buffer (50 mM NH_4HCO_3, 1 M urea, pH 8.0) to give a final protein concentration of approximately 0.3% (w/v) and allowed to resolubilize for 15 min on a rotary shaker, and liquid samples were diluted. If required, total protein quantification was performed using SPRINT™ Rapid Protein Analyzer (CEM) compliant with AOAC **967.12** (22).

Sample Extraction

Sample extraction was performed with respect to the protein amount of the analyzed sample. Samples were categorized into groups with a protein mass fraction of (A) 0–2%, (B) 2.1–4%, (C) 4.1–8.5%, and (D) 8.6–16%.

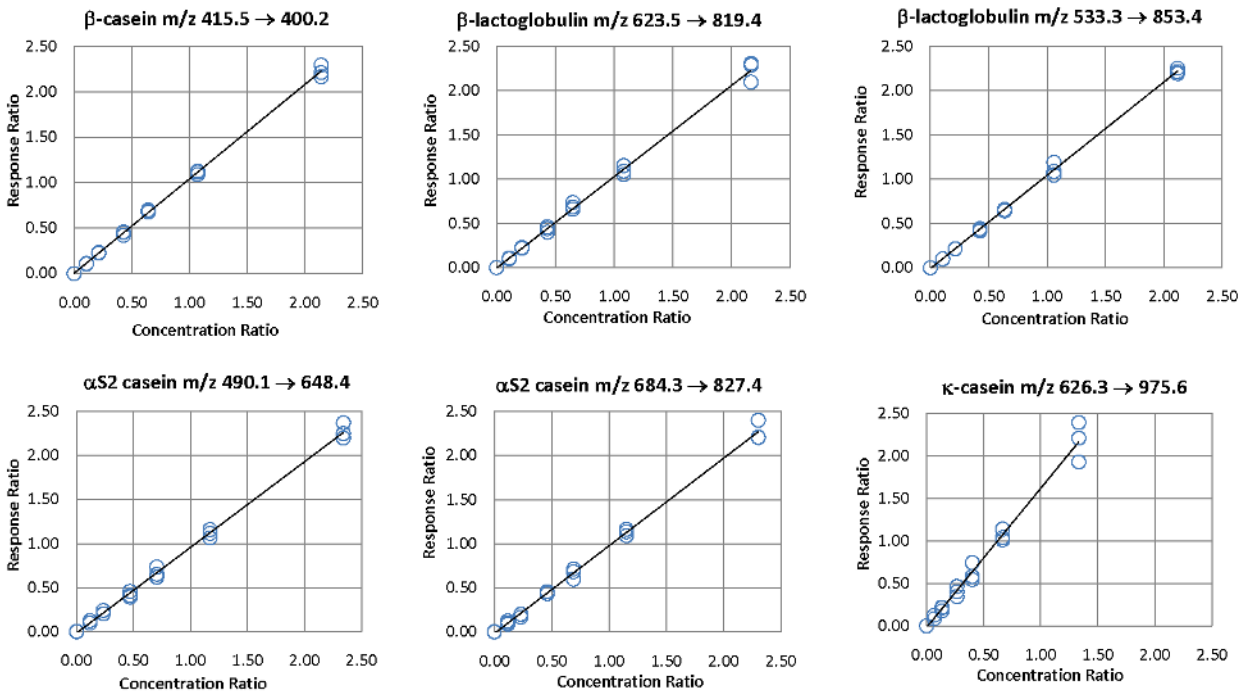

Figure 2. Calibration curves of individual synthetic peptides. Slopes, intercepts, and R^2 values for calibration curves formed from representative data in triplicate determination (different days) are given in Table 6.

Table 6. Slopes, intercepts, and R^2 values of the calibration curves[a]

Analyte	Range of ratio area ST/area IS		Slope	Intercept	Coefficient of determination (R^2)	SD of residuals
	Minimum	Maximum	Central value	Central value		
β-Casein *m/z* 415.5 → 400.2	0	2.14	1.04	0.0027	0.999	0.026
β-Lactoglobulin *m/z* 623.5 → 819.4	0	2.16	1.03	–0.0033	0.996	0.046
β-Lactoglobulin *m/z* 533.3 → 853.4	0	2.12	1.05	–0.0078	0.999	0.026
αS2-Casein *m/z* 684.3 → 827.4	0	2.30	1.00	–0.0162	0.997	0.045
αS2-Casein *m/z* 490.1 → 648.4	0	2.34	0.97	–0.0143	0.997	0.042
κ-Casein *m/z* 626.3 → 975.6	0	1.33	1.64	–0.0224	0.986	0.090

[a] Peptide concentrations in the calibration solutions range from 0 to 15.0 fmol/μL for β-casein, and 0 to 5 fmol/μL of the other nonlabeled peptides. These concentrations correspond to 18–360 ng/mL β-casein, 6.3–127 ng/mL αS2-casein, 4.5–90 ng/mL β-lactoglobulin, and 4.8–95 ng/mL κ-casein.

Samples of 8 g from category A and 4 g samples from categories B, C, and D were extracted in 40 mL freshly prepared and preheated (60 ± 3°C) extraction buffer (50 mM NH_4HCO_3, 1 M urea, pH 8.0), and mixed with a vortex mixer. After shaking samples for 15 min on a rotator shaker, the resulting extracts were centrifuged for 10 min (5000 × *g*; 4–8°C). Aliquots of supernatants were transferred into low-protein binding reaction tubes.

Trypsin Digestion

A 20 mg amount of lyophilized trypsin was reconstituted in 100 μL extraction buffer (50 mM NH_4HCO_3, 1 M urea, pH 8.0). Trypsin addition and sample dilution were carried out with respect to the total protein concentration in the sample extract in order to obtain an approximate protein:trypsin ratio of 50:1. To an aliquot of 200 μL sample extract (A and B), 80 μL reconstituted trypsin solution (16 μg) was added. For samples with an initial protein amount of 4.1–8.5% (C), 100 μL sample extract was supplemented with 85 μL (17 μg) trypsin solution. For samples with an initial protein amount of 8.6–16% (D), 70 μL sample extract was supplemented with 98 μL (19.6 μg) trypsin solution. Proteins were digested overnight at 37 ± 1°C in a water bath. The resulting solution was centrifuged for 10 min at approximately 16 000 × *g* at room temperature.

Protein digests were transferred into HPLC vials. Samples initially containing 4.1–8.5% protein (C) were diluted 1.5-fold, and samples with 8.6 –16% protein (D) were diluted two-fold with 0.1% formic acid prior to the IS addition. Samples with initial protein amount below 4.1% (A and B) were supplemented directly with the IS solution. To this end, the respective peptide solutions (A and B) were diluted 1:1 with the IS solution in 0.1 or 0.2% formic acid. Samples were stored for maximum of 1 day at 2–8°C.

Operating Procedure and Determination

(**a**) *HPLC and mass spectrometer conditions*.—HPLC analysis was performed by means of an RP Symmetry300™ C_{18} HPLC column with a Symmetry300 C_{18} guard column using an Agilent 1200 binary pump system. The mobile phase consisted of solvent A, 0.1% formic acid in LiChrosolv purified water; and solvent B, 0.1% formic acid in LiChrosolv acetonitrile. Injected peptide solutions were separated at 23 ± 3°C with a flow rate of 0.2 mL/min using the gradient described in Table 1. Peptides were transferred into the mass spectrometer in the interval from 15 to 34 min using a diverter valve.

MS detection was done on an Agilent 6460 LC/MS triple quadrupole mass spectrometer with Jet Stream technology in the positive ESI mode under the following conditions: gas temperature 350°C; gas flow, 10 L/min; nebulizer gas pressure, 30 psi; sheath gas temperature, 350°C; sheath gas flow, 10 L/min; capillary voltage, 3 kV; and nozzle voltage, 300 V. The quantitative analysis was performed using tandem MS in the SRM mode alternating two transition reactions for each peptide, two transition reactions for the related IS peptides and a dwell time of 20 ms (Table 2). Resolution of 0.7 amu for quadrupole 1 (Q1) and 0.7 or 1.2 amu for quadrupole 3 (Q3) were chosen. UV spectra were recorded at 214 nm for each sample. Data were processed using Masshunter Quant software B.04.00. MS tuning was performed in the positive ESI mode by infusing a 3.5 pmol/μL solution of each individual peptide with an HPLC flow made

Table 7. Slopes, R^2 values, LOD, and LOQ for the individual peptides in food matrixes[a]

		β-Casein *m/z* 415.5 → 400.2	β-Lactoglobulin *m/z* 623.5 → 819.4	β-Lactoglobulin *m/z* 533.3 → 853.4	αS2-Casein *m/z* 684.3 → 827.4	αS2-Casein *m/z* 490.1 → 648.4	κ-Casein *m/z* 626.3 → 975.6
Soy-based infant formula	Range, mg/kg SMP	50–150	50–150	10–150	n.q.[b]	20–150	50–150
	Slope	0.87	0.86	0.76	—	1.68	0.60
	R^2	0.996	0.979	0.996	—	0.995	0.989
	LOD, mg/kg SMP	20	20	5	50	5	20
	LOQ, mg/kg SMP	50	50	10	150	20	50
Breakfast cereals	Range, mg/kg SMP	5–150	5–150	5–150	5–150	5–150	10–150
	Slope	1.37	1.11	1.26	1.85	4.78[c]	1.34
	R^2	0.993	0.992	0.992	0.985	0.973	0.976
	LOD, mg/kg SMP	1	2	1	2	1	2
	LOQ, mg/kg SMP	5	5	5	5	5	10
Infant cereals	Range, mg/kg SMP	5–150	5–150	5–150	5–150	5–150	10–150
	Slope	1.06	1.13	0.92	1.03	1.26	0.45
	R^2	0.998	0.998	0.999	0.983	0.995	0.995
	LOD, mg/kg SMP	1	2	1	2	1	2
	LOQ, mg/kg SMP	5	5	5	5	5	10
Baby food	Range, mg/kg SMP	1–50	2–50	2–100	2–50	2–50	2–50
	Slope	1.14	0.95	0.76	0.92	1.31	0.67
	R^2	0.998	0.995	0.999	0.986	0.998	0.995
	LOD, mg/kg SMP	<1	1	1	1	1	1
	LOQ, mg/kg SMP	1	2	2	2	2	2
Rinse water	Range, mg/kg SMP	5–75	5–100	2–100	5–50	2–150	5–75
	Slope	0.99	0.86	0.86	0.86	1.86	0.54
	R^2	0.99	0.993	0.993	0.984	0.995	0.975
	LOD, mg/kg SMP	2	1	1	1	1	2
	LOQ, mg/kg SMP	5	5	2	5	2	5

[a] Samples were fortified with SMP from 1 to 150 mg/kg including a blank matrix. Depending on matrix suppression effects, the working range of the analyzed samples was limited. LOD and LOQ are expressed as mass fraction of SMP.

[b] n.q. = Below LOQ.

[c] Low extraction efficiency of αS2-casein peptide (*m/z* 490.1 → 648.4) in breakfast cereals matrix.

of solvents A and B corresponding to its approximate concentration for elution (Table 3) using a T-connector. The flow rate was set to 200 µL/min with other settings as given above. Optimum collision energies for each peptide transition were obtained using the voltage ramping function.

(**b**) *Sample analysis*.—Amounts of 20 µL each of sample solution, reagent blanks, and control samples were injected into the column. The maximum protein amount on the column was chosen to be not higher than 40 µg in order to avoid column saturation. Blank runs [0.1% (v/v) formic acid] were performed after each sample with high protein amounts, like infant formula or infant cereals, or when a baseline shift was observed in the UV spectrum recorded for each sample at 214 nm in order to remove potentially remaining peptides from the column.

(**c**) *Identification and confirmation*.—β-lactoglobulin and the respective casein peptides are considered positively identified in the sample when confirmation criteria, as defined in EU Commission Decision 2002/657/EC (20), are fulfilled. The individual peptides were considered positively identified in the matrixes when (*1*) the ratio of the chromatographic retention time of the analyte to that of the corresponding IS corresponded to that of the averaged retention time of the calibration solutions within a ± 2.5% tolerance; (*2*) a signal was present at each of the two diagnostic transition reactions for the respective peptide and at each of the two transition reactions for its corresponding IS peptides; and (*3*) the peak area ratio

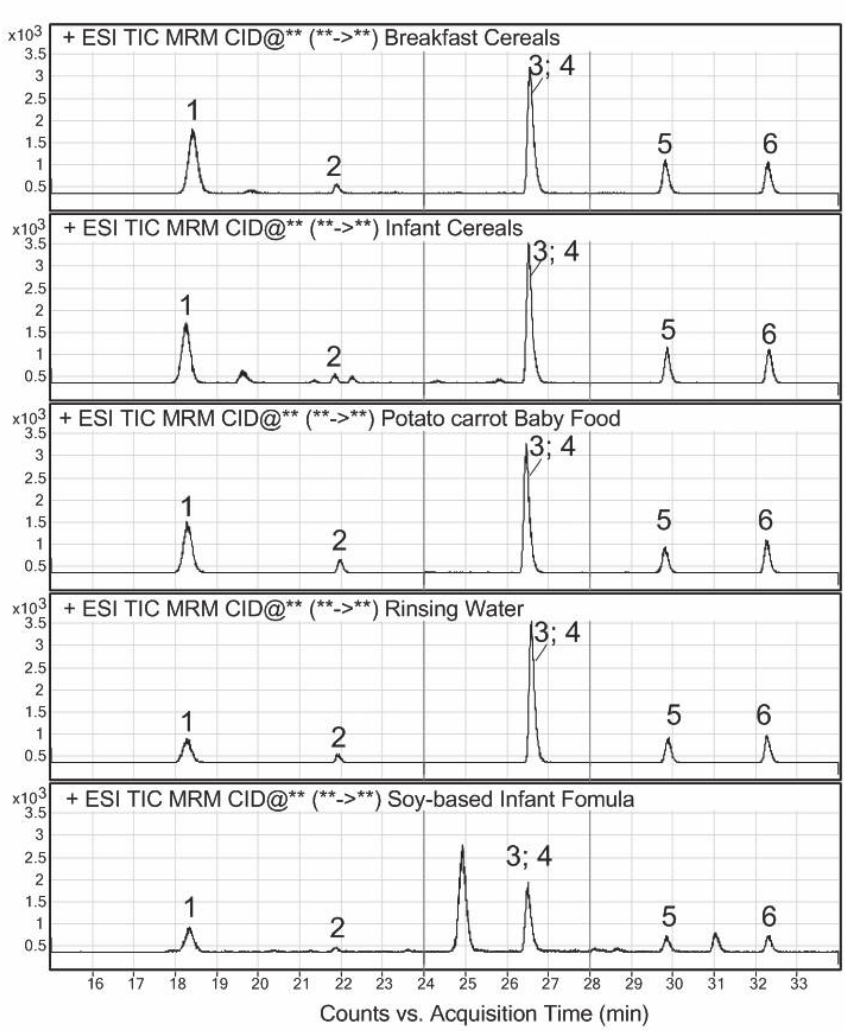

Figure 3. Example of LC/ESI-MS/MS TIC chromatograms of milk peptide extract from different matrixes: breakfast cereals, infant cereals, carrot-based baby food, rinse water, and soy-based infant formula. Spiking level: SMP, 15 mg/kg (1 = β-casein AVPYPQR, 2 = β-lactoglobulin TPEVDDEALEK, 3 = β-lactoglobulin VLVLDTDYK, 4 = αS2-casein ALNEINQFYQK, 5 = αS2-casein FALPQYLK, and 6 = κ-casein YIPIQYVLSR).

from the different transition reactions recorded for both the unlabeled and labeled species was within the tolerances fixed by the EU criteria.

(**d**) *Quantification*.—Each tryptic peptide was quantified by means of IS addition and calibration curves [analyte/IS peptide area ratio (= y) versus analyte/IS concentration ratio (= x)]. Amounts of 20 µL calibration standard solutions (0–15.0 fmol/µL β-casein peptide AVPYPQR and 0–5 fmol/µL of the other nonlabeled peptides, each containing a fixed concentration of 7.5 fmol/µL β-casein AVPYPQR[$^{13}C_6$, $^{15}N_4$] and 2.5 fmol/µL of the other stable isotope-labeled peptides) were injected into the column in replicates. One series of calibration standards was injected prior to and one after the samples to be analyzed. Calibration curves were calculated from the average values of the two series. The calibration standards covered 18–360 ng/mL for β-casein, 6.3–127 ng/mL for αS2-casein, 4.5–90 ng/mL for β-lactoglobulin, and 4.8–95 ng/mL for κ-casein.

The linearity of MS responses was checked by calculating the RSD(r) of the average of response factors (RF = y/x), which should be below 15% (23). Calibration curves were not constructed in the different food matrixes analyzed. S/N values were determined using the algorithms implemented in the Masshunter software using the following parameters: peak-to-peak without enhancement (factor = 1). Noise regions were adjusted close to the expected retention time of the target molecules for each food matrix individually. The obtained analysis results were expressed as mass fraction of the individual milk proteins, and as mass fraction of SMP equivalents in the different food matrixes. Values were calculated using molecular masses of the milk proteins as well as

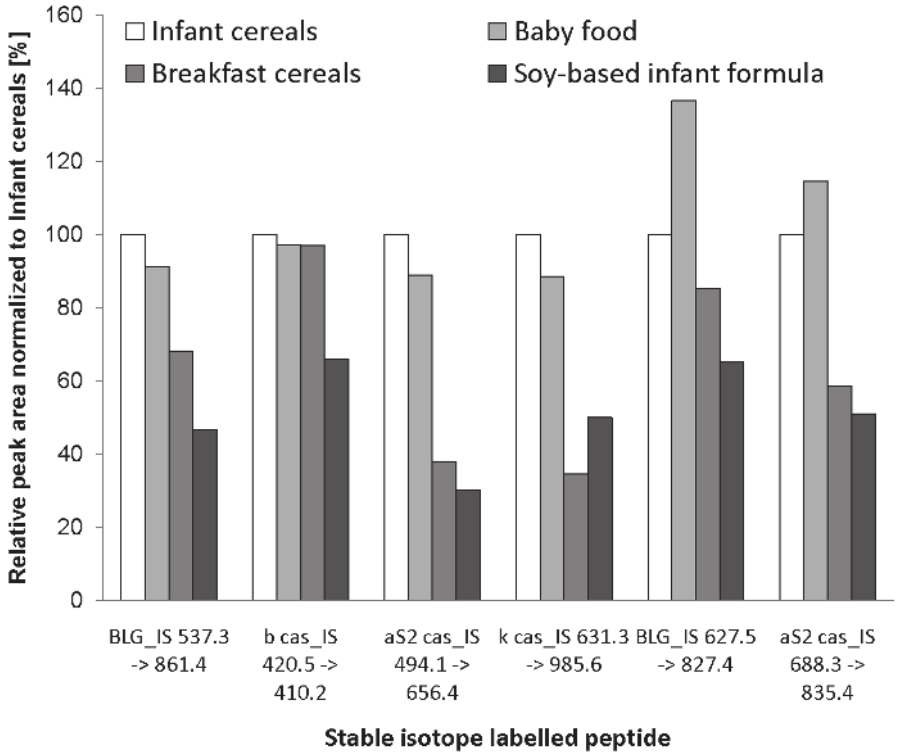

Figure 4. Impact of matrix suppression on signal intensities of isotopically labeled IS peptides in different food matrixes. Signal intensities have been normalized to those obtained in infant cereals.

their mean mass fractions in SMP based on literature data presented in Table 4 (24, 25).

(**e**) *Method validation and confirmation criteria.*—Method validation was conducted according to the Commission Decision 2002/657/EC (20) and Abbott et al. (26). Recovery rates and within-day [SD(r)] and within-laboratory [SD(iR)] precisions were calculated according to the International Organization for Standardization (27) from the analysis of aliquots from five blank matrixes (soy-based infant formula, lactose-free infant cereals, extruded and sweetened breakfast cereals, carrot/potato puree baby food, and water) fortified with SMP at three fortification levels (5.0, 10.0, and 15.0 mg/kg). Analyses were performed by three operators on 6 to 7 different days, each analyzing two replicate samples of each fortification level at least twice. Values were calculated for each milk protein-derived tryptic peptide and were also expressed as mass fraction skimmed milk powder (24, 25). Measurement uncertainties were estimated based on existing validation data as proposed by Barwick and Ellison (28). Confirmation criteria for MS techniques were applied according to Commission Decision 2002/657/EC (20).

Linearity was determined for the calibration standard solutions as well as for the responses obtained for food matrixes fortified with SMP at 1–150 mg/kg levels. Selectivity for selected typical dairy ingredients was determined, as well as potential cross-reactivity towards selected food commodities, as suggested for validation of ELISA methods for determination of milk traces (29).

Results and Discussion

Selection of Peptides and Mass Spectrometer Parameter Optimization

Suitable peptides for quantification of milk traces were selected by a combination of different approaches. First, theoretical preselection of proteins and peptides was performed based on their high abundance in milk in order to ensure sensitivity of the method. Furthermore, these peptides were compared to proteins from other food commodities and with milk proteins from other ruminant species like goat, sheep, and buffalo using the Basic Local Alignment Search Tool (BLAST; 30) against entries in the NCBInr database (Version of 05/2009). The selected proteins, β-lactoglobulin, β-casein, αS1-casein, αS2-casein, and κ-casein, were theoretically digested with trypsin in order to select peptides as potential candidates for quantification using the following criteria: suitable hydrophilicity under the above-mentioned HPLC conditions [estimated using GPMAW (General Protein Mass Analysis for Windows, version 8.21, Lighthouse data, Odense, Denmark)], peptide size between 7 and 15

Table 8. Repeatability, intermediate reproducibility limits, and standard and expanded uncertainty at a 95% confidence interval level

Analyte	SMP fortification level, mg/kg	Days × replicates	Median	RSD(r), %	r, %	RSD(iR), %	iR, %	Relative standard uncertainty, %	Expanded standard uncertainty, %
				Baby food					
β-Casein AVPYPQR 415.5 → 400.2	5	6 × 2	5.14	1.6	4.5	9.1	25.3	10	20
	10	7 × 2	10.38	3.4	9.5	9.5	26.3	10	20
	15	7 × 2	15.77	3.5	9.6	5.5	15.2	6	13
β-Lactoglobulin VLVLDTDYK 533.3 → 853.4	5	6 × 2	3.32	5.5	13.5	45.7	127	67	134
	10	7 × 2	7.6	5.0	13.8	16.7	46.3	24	48
	15	6 × 2	11.08	2.9	8	19.4	53.7	31	62
β-Lactoglobulin TPEVDDEALEK 623.5 → 819.4	5	6 × 2	4.39	8.2	22.9	11.2	30.9	15	31
	10	7 × 2	9.24	2.5	6.9	15.5	42.9	17	33
	15	7 × 2	13.58	6.4	17.8	17	47.2	18	36
αS2-Casein ALNEINQFYQK 684.3 → 827.4	5	7 × 2	3.91	13.2	36.5	38.6	107	41	82
	10	6 × 2	7.68	3.8	10.6	8.3	23	20	39
	15	7 × 2	11.12	16.7	46.3	28.0	78	34	69
αS2-Casein FALPQYLK 490.1 → 648.4	5	7 × 2	6.13	8.2	22.8	55.3	153	44	88
	10	7 × 2	11.27	9.8	27.1	30.7	85	33	65
	15	7 × 2	16.19	9.5	26.4	42.1	117	45	90
κ-Casein YIPIQYVLSR 626.3 → 975.6	5	4 × 2	2.36	7.8	21.6	51.7	143	—	—
	10	7 × 2	3.79	9.1	25.3	41.9	116	93	187
	15	6 × 2	6.75	5.2	14.4	23.1	64	66	132
				Breakfast cereals					
β-Casein AVPYPQR 415.5 → 400.2	5	7 × 2	4.71	26.5	73.4	69.1	192	74	147
	10	6 × 2	9.56	9.5	26.3	11.9	33	13	25
	15	5 × 2	18.44	4.4	12.1	40.7	113	19	38
β-Lactoglobulin VLVLDTDYK 533.3 → 853.4	5	6 × 2	4.97	5.3	14.6	30	83	32	65
	10	6 × 2	8.42	17.3	48	56.1	155	67	134
	15	6 × 2	14.38	10.6	29.5	26.1	72	28	56
β-Lactoglobulin TPEVDDEALEK 623.5 → 819.4	5	6 × 2	5.55	16.2	44.8	24.8	69	26	53
	10	6 × 2	8.66	10.8	29.9	38.7	107	42	83
	15	6 × 2	15.24	13.1	36.3	24.8	69	27	53
αS2-Casein ALNEINQFYQK 684.3 → 827.4	5	6 × 2	6.85	4.4	12.1	31.1	86	34	67
	10	6 × 2	10.54	10.7	29.8	20.2	56	22	43
	15	6 × 2	14.02	20.8	57.5	57.4	159	62	123
αS2-Casein FALPQYLK 490.1 → 648.4	5	6 × 2	8.26	10.5	29	23.6	66	32	64
	10	4 × 2	14.20	2.92	8.1	10.5	29	45	90
	15	4 × 2	19.04	5.3	14.7	8.9	25	67	134
κ-Casein YIPIQYVLSR 626.3 → 975.6	5	4 × 2	2.51	15.4	41.7	22.0	61	—	—
	10	4 × 2	4.91	13.3	38.7	29.1	81	95	191
	15	6 × 2	6.33	20.6	57.2	66.1	183	99	198
				Infant cereals					
β-Casein AVPYPQR 415.5 → 400.2	5	7 × 2	5.23	5.0	13.9	75.2	209	80	161
	10	6 × 2	9.35	5.6	15.5	70.6	196	76	152
	15	7 × 2	14.55	6.3	17.6	29.2	81	31	62

Table 8. (*continued*)

Analyte	SMP fortification level, mg/kg	Days × replicates	Median	RSD(r), %	r, %	RSD(iR), %	iR, %	Relative standard uncertainty, %	Expanded standard uncertainty, %
β-Lactoglobulin VLVLDTDYK 533.3 → 853.4	5	6 × 2	4.59	11.8	32.6	96.9	269	105	209
	10	6 × 2	8.29	8.4	23.3	42.3	117	46	91
	15	7 × 2	13.00	6.0	16.8	18.5	51	20	39
β-Lactoglobulin TPEVDDEALEK 623.5 → 819.4	5	7 × 2	5.19	29.5	81.8	128.5	356	137	274
	10	6 × 2	8.52	23.4	64.8	32.5	90	34	69
	15	7 × 2	15.5	11.1	30.7	43.7	121	47	93
αS2-Casein ALNEINQFYQK 684.3 → 827.4	5	4 × 2	8.99	31.4	87	62.0	172	—	—
	10	4 × 2	12.58	10.3	28.5	39.4	109	—	—
	15	7 × 2	14.27	33.2	92.1	44.9	125	47	94
αS2-Casein FALPQYLK 490.1 → 648.4	5	6 × 2	10.74	13.7	38	37.6	104	46	92
	10	5 × 2	15.60	8.9	24.6	48.4	134	—	—
	15	7 × 2	20.26	12.3	34.1	40.7	113	43	87
κ-Casein YIPIQYVLSR 626.3 → 975.6	5	6 × 2	3.55	14.5	40.1	49.2	136	53	106
	10	4 × 2	7.43	15.9	44	23.5	65	—	—
	15	6 × 2	7.92	4.1	11.4	79.5	220	97	194
Infant formula									
β-Lactoglobulin VLVLDTDYK 533.3 → 853.4	10	6 × 2	6.87	10.8	30	79.1	219	85	171
	15	7 × 2	11.03	4.3	11.9	53.1	147	57	113
Rinse water									
β-Casein AVPYPQR 415.5 → 400.2	5	7 × 2	1.04	19.2	53.1	71.8	199	205	410
	10	6 × 2	1.99	12.9	35.7	18.7	52	197	395
	15	6 × 2	4.75	7.8	21.7	18.6	52	110	219
β-Lactoglobulin VLVLDTDYK 533.3 → 853.4	5	6 × 2	1.75	6.3	17.5	53.5	148	110	219
	10	7 × 2	3.36	2.5	6.9	24.3	67	102	204
	15	6 × 2	5.36	8.0	22.2	25.4	70	94	188
β-Lactoglobulin TPEVDDEALEK 623.5 → 819.4	5	6 × 2	2.05	13.3	36.9	29.4	81	79	157
	10	6 × 2	4.53	5.6	15.4	14.1	39	62	123
	15	6 × 2	6.73	14.7	40.8	12.9	36	63	126
αS2-Casein ALNEINQFYQK 684.3 → 827.4	5	6 × 2	3.57	13.9	38.6	34.6	96	42	84
	10	7 × 2	7.56	5.3	14.6	13.3	37	22	43
	15	6 × 2	9.54	16.8	46.6	18.6	52	35	69
αS2-Casein FALPQYLK 90.1 → 648.4	5	6 × 2	4.50	12.6	34.9	24.2	67	26	52
	10	7 × 2	8.07	4.7	13	37.3	103	40	80
	15	6 × 2	12.65	8.2	22.9	21	58	23	45
κ-Casein YIPIQYVLSR 626.3 → 975.6	5	5 × 2	1.19	12.4	34.3	16.6	46	—	—
	10	6 × 2	2.16	17.0	47.1	19.1	53	183	365
	15	6 × 2	2.44	12.5	34.6	44	122	262	525

amino acids; no presence of Met, Cys, Trp amino acid residues within the sequence of the peptide; and none to few known post-translational modifications or known modifications of amino acids during food processing (e.g., 31, 32).

After preselection of the potential marker peptides, purified β-lactoglobulin A and B, β-casein, casein-mix, and κ-casein were digested with trypsin and analyzed by HPLC with both UV and MS/MS detection in order to identify and select peptides that showed good ionization

Table 9. Quantification of individual proteins in three different skim milk powder (SMP) samples expressed as protein mass fraction/100 g sample and protein mass fraction/100 g total protein (single-day determination)[a]

SMP sample and true protein (TP) amount	β-Casein	β-Lactoglobulin		αS2-Casein		κ-Casein
	m/z 415.5 → 400.2	*m/z* 623.5 → 819.4	*m/z* 533.3 → 853.4	*m/z* 684.3 → 827.4	*m/z* 490.1 → 648.4	*m/z* 626.3 → 975.6
Protein amount, g/100 g sample						
SMP Fluka (34.3% TP)	11.8	2.7	2.7	3.8	5.5	1.8
SMP NIST (34.7% TP)	10.7	2.5	2.3	5.0	4.9	1.4
SMP Hochdorf (22.9% TP)	8.0	1.8	1.9	3.5	3.9	1.1
Theoretical value (refs. 24, 25)	9–11	2–4		2–4		3–4
Protein amount, g/100 g total protein						
SMP Fluka (34.3% TP)	34.5	7.9	7.8	11.0	16.0	5.3
SMP NIST (34.7% TP)	30.7	7.3	6.7	14.6	14.3	4.2
SMP Hochdorf (22.9% TP)	34.8	7.9	8.1	15.3	17.2	4.9
Theoretical value (refs. 24, 25)	25–35	7–12		8–11		8–15

[a] TP amounts (total protein) were determined according to AOAC Method **967.12** (ref. 21) using a SPRINT protein analyzer.

characteristics [high signal intensity (total ion current; TIC) for the doubly charged parent ions and singly charged fragment ions]. The selected peptides were purchased as synthetic peptides of both nonlabeled and stable isotope-labeled species: β-casein AVPYPQR and AVPYPQR[$^{13}C_6$, $^{15}N_4$]; β-lactoglobulin TPEVDDEALEK and TPEVDDEALEK [$^{13}C_6$, $^{15}N_2$]; β-lactoglobulin VLVLDTDYK and VLVLDTDYK [$^{13}C_6$, $^{15}N_2$]; αS2-casein ALNEINQFYQK and ALNEINQFYQK [$^{13}C_6$, $^{15}N_2$]; αS2-casein FALPQYLK and FALPQYLK [$^{13}C_6$, $^{15}N_2$]; and κ-casein YIPIQYVLSR and YIPIQYVLSR [$^{13}C_6$, $^{15}N_4$].

Optimum parameters for the Agilent 6460 mass spectrometer were obtained by syringe-infusion of each peptide standard solution at a flow rate of 0.2 mL/min. The MS conditions were described previously. Optimum collision energies for each peptide fragment were obtained by ramping the energy level, and are given together with the respective fragment masses for quantification and analyte confirmation in Table 2. Parent ions were all doubly charged $[M+2H]^{2+}$ ions, and fragment ions were all singly charged $[M+H]^{1+}$ ions (Figure 1). Relative intensities (ratio of qualifier:quantifier ion) were determined for the calibration solutions from analyses performed on 7 different days, covering concentrations of 0.25–5 fmol/μL (0.75–15 fmol/μL for β-casein) for the nonlabeled peptides and at a fixed concentration of 2.5 (7.5) fmol/μL for the respective isotopically labeled peptides. Relative intensities were also determined in SMP-fortified food matrixes at 50, 100, and 150 mg/kg in replicates. Peak area ratios calculated for all fortified food matrixes and the calibration standards met the limits fixed according to EU criteria Commission Decision 2002/657/EC (20; Table 2).

Selectivity and Cross-Reactivity

Selectivity of the selected peptides was assessed in silico using BLAST (30) searches and by analyzing typical dairy ingredients and milk sample from species other than bovine as well as typical food commodities (raw materials) as proposed in Abbott et al. (26) for potential cross-reactivity. The detection capabilities of the method were elucidated for typical dairy ingredients like milk whole powder, demineralized whey powder, sweet whey powder, potassium and sodium caseinate, MFGM-enriched protein whey concentrate, acid whey, yogurt powder, fresh yogurt, sweet concentrated milk, UHT milk (all bovine), fresh goat's milk, fresh sheep's milk, and buffalo milk powder. In all bovine-derived dairy samples, the presence of all marker peptides was confirmed. Casein samples showed minor amounts of remaining whey protein β-lactoglobulin, while the casein peptides were detected in trace amounts in the whey powder samples. The confirmed presence of all maker peptides in fresh yogurt and yogurt powder, as well as in sweet concentrated and UHT milk, demonstrated that the method is compatible with fermented and intensively heat-treated dairy samples as well. This is an important advantage compared to most ELISA-based assays that are often specific either for the native or denatured protein species but rarely for both. Furthermore, the method can also quantify milk protein originating from sheep, goat, and buffalo based on the species homology of some selected peptides (Table 5). All chosen peptides are specific for *Bos taurus*; all peptides except αS2-casein FALPQYLK allow determination of *Bubalus bubalis* (water buffalo) protein; κ-casein YIPIQYVLSR, αS2-casein ALNEINQFYQK, and β-lactoglobulin VLVLDTDYK can also be used to detect and quantify traces of milk from sheep, goat, and

Table 10. Trueness of the method determined by the calculation of the mean recovery rates obtained for the individual food matrixes spiked with SMP at 5, 10, and 15 mg/kg levels, analyzed on 6 to 7 different days in two replicates each[a]

		αS2-Casein *m/z* 490.1 → 648.4	αS2-Casein *m/z* 684.3 → 827.4	β-Casein *m/z* 415.5 → 400.2	β-Lactoglobulin *m/z* 533.3 → 853.4	β-Lactoglobulin *m/z* 623.5 → 819.4	κ-Casein *m/z* 626.3 → 975.6
Baby food	Rec_a [%]	151 ± 15	99 ± 2.6	122 ± 2.1	66 ± 10	87 ± 2.1	35 ± 3.9
	Rec_b [%]	117 ± 12	76 ± 2.0	103 ± 1.8	69 ± 11	90 ± 2.2	43 ± 4.9
Breakfast cereals	Rec_a [%]	188 ± 25	145 ± 29	121 ± 17	91 ± 7.2	97 ± 12	36 ± 3.7
	Rec_b [%]	145 ± 19	112 ± 22	103 ±14	94 ± 7.4	100 ± 12	45 ± 4.6
Soy-based infant formula	Rec_a [%]	n.q.[b]	n.q.	n.q.	69 ± 3.3	n.q.	n.q.
	Rec_b [%]	n.q.	n.q.	n.q.	71 ± 3.4	n.q.	n.q.
Infant cereals	Rec_a [%]	203 ± 28	157 ± 51	102 ± 18	84 ± 4.3	94 ± 10	53 ± 9
	Rec_b [%]	157± 22	133 ± 43	98 ± 5.6	87 ± 4.4	97 ± 11	66 ± 12
Rinse water	Rec_a [%]	110 ± 6.0	91 ± 7.9	29 ± 7.5	34 ± 1.0	42 ± 2.3	16 ± 2.9
	Rec_b [%]	85 ± 4.6	70 ± 6.1	24 ± 6.4	35 ± 1.1	44 ± 2.4	21 ± 3.9

[a] Recovery rates were calculated based on the following reported mass fractions of the individual milk proteins (a) for skim milk powder and (b) for dried liquid milk: $w_{\alpha S2\text{-}casein}$: (a) 2.7 and (b) 3.5%; $w_{\beta\text{-}casein}$: (a) 8.5 and (b) 10%; $w_{\kappa\text{-}casein}$: (a) 3.1 and (b) 3.5%; $w_{\beta\text{-}lactoglobulin}$: (a) 3.1 and (b) 3.0%; (refs. 24, 25).

[b] n.q. = < LOQ.

reindeer origin. LC/MS/MS analysis of buffalo, goat, and sheep milk confirmed this theoretical assignment. Reindeer milk was not analyzed.

Cross-reactivity was estimated based on the presence and absence of signal in the respective SRM chromatograms of the individual peptides. Signals with S/N <3 were considered as "not present/not cross-reacting (n.d. = not detectable)." The following food ingredients were analyzed: almond, cashew, hazelnut, peanut, Brazil nut, walnut, pine nut, pecan nut, pistachio, sesame seeds (black and white mixed 1:1), poppy seeds, pumpkin seeds, sunflower seeds, cocoa, wheat, barley, rye, oat, buckwheat, corn, rice (white), chick peas, peas, soy beans, split peas, lima beans, beef meat, frozen fresh shrimps, salmon granules, egg, and lecithin. None of the samples showed any cross-reactivity towards the selected marker peptides except rye meal and corn meal. Rye meal samples showed significant amounts of all milk peptides, and corn meal samples showed significant amounts of all milk peptides except for β-casein. However, BLAST searches did not indicate any homology of the milk peptides to these food commodities. Therefore, a real cross-contamination of these samples was concluded and confirmed by ELISA analysis (Veratox Total Milk Assay, data not shown). Individual peptides were detected with minor signal intensities with S/N <3 in peanut and white rice (β-lactoglobulin *m/z* 533.3 → 819.4), lima bean (β-casein *m/z* 415.5 → 400.2), and split peas (κ-casein *m/z* 626.3 → 975.6 and *m/z* 626.3 → 765.4) showing minor signals at retention times of the milk peptides. However, none of these matrixes showed signals for all milk peptides. Furthermore, these signals were considered as interferences due to missing confirmation by the qualifier ion or other milk proteins.

Linearity, LOD, and LOQ

For the standard curve, linear relationships were analyzed for each quantifier transition. The concentrations of the seven level calibration standards (including the blank) range from 0.75 to 15 pmol/μL (corresponding to 18–360 ng/mL) for β-casein AVPYPQR, and from 0.25 to 5 fmol/μL of the other nonlabeled peptides (corresponding to 6.3–127 ng/mL αS2-casein, 4.5–90 ng/mL β-lactoglobulin A and B, and 4.8–95 ng/mL κ-casein; Figure 2). The corresponding ratios for IS and standard concentration are calculated based on peak areas. All peptides showed linear relationship between theoretical and observed concentration ratios. The slopes were determined for representative curves obtained on 3 different days in triplicate. Slopes had average values close to 1.0 with coefficients of determination (R^2) > 0.995, except for κ-casein with a slope of 1.64 and R^2 of 0.990 (Table 6). The deviation of κ-casein may be a result of an unidentified impurity in one of the synthesized peptide solutions or an error in the determined concentration thereof.

In addition to the evaluation of the calibration curves, linear correlations of signal intensities to SMP fortification level in the food matrixes were determined and summarized in Table 7. For example, LC/ESI-MS/MS TIC chromatograms of milk peptides extracted from breakfast cereals, infant cereals, carrot-based baby food, rinse water, and soy-based infant formula fortified with

Table 11. Robustness analysis using six-level Plackett-Burman statistical design[a]

	β-Casein *m/z* 415.5 → 400.2			β-Lactoglobulin *m/z* 533.3 → 853.4			β-Lactoglobulin *m/z* 623.5 → 819.4		
	Critical difference: ± 9.50			Critical difference: ± 5.38			Critical difference: ± 7.37		
Parameter	Mean Level 1, mg/kg	Mean Level 2, mg/kg	Δ	Mean Level 1, mg/kg	Mean Level 2, mg/kg	Δ	Mean Level 1, mg/kg	Mean Level 2, mg/kg	Δ
Extraction method	55.27	54.51	+0.76	30.53	31.73	–1.2	42.54	42.59	–0.05
Extraction temperature	54.79	54.98	–0.19	30.00	32.26	–2.3	41.23	43.90	–2.67
Sample filtration	54.66	55.11	–0.45	31.40	30.86	+0.54	42.65	42.49	+0.16
Trypsin addition	54.63	55.14	–0.51	27.62	34.65	–7.03	40.37	44.76	–4.39
Digestion time	54.05	55.72	–1.68	38.75	23.51	+15.3	45.01	40.12	+4.89
Column temperature	53.80	55.97	–2.17	30.66	31.60	–0.95	42.53	42.61	–0.08
	αS2-Casein *m/z* 684.3 → 827.4			αS2-Casein *m/z* 490.1 → 648.4			κ-Casein *m/z* 626.3 → 975.6		
	Critical difference: ± 8.51			Critical difference: ±11.26			Critical difference: ± 5.02		
Parameter	Mean Level 1, mg/kg	Mean Level 2, mg/kg	Δ	Mean Level 1, mg/kg	Mean Level 2, mg/kg	Δ	Mean Level 1, mg/kg	Mean Level 2, mg/kg	Δ
Extraction method	63.50	66.63	–3.13	63.50	66.63	–3.13	28.84	29.11	–0.27
Extraction temperature	62.63	67.49	–4.86	62.63	67.49	–4.86	29.28	28.68	+0.6
Sample filtration	62.22	67.91	–5.69	62.22	67.91	–5.69	28.46	29.49	–1.03
Trypsin addition	64.27	65.86	–1.59	64.27	65.86	–1.59	33.07	24.88	+8.19
Digestion time	61.39	68.73	–7.34	61.39	68.73	–7.34	18.00	39.96	–21.96
Column temperature	66.71	63.41	+3.30	66.71	63.41	+3.30	30.98	26.97	+4.01

[a] Level 1 conditions were used throughout method validation; Level 2 conditions represent slight variations, as described in the text.

SMP at 15 mg/kg level are shown in Figure 3. Suppression of peptide signals was found to be different in the food matrixes and to have a significant impact on the LOD and LOQ. Highest sensitivity was achieved in low protein-containing carrot- and potato-based baby food. An LOD for SMP could be achieved at 1 mg/kg for all diagnostic peptides. The corresponding LOQ was 2 mg/kg for all peptides. For breakfast cereals, infant cereals, and rinse water, the LOQ was 1 mg/kg SMP for a minimum of two out of six diagnostic peptides, or 2 mg/kg if all peptides were considered. The LOQ was 5 mg/kg for five out of six peptides in these matrixes. Higher LOD (5 mg/kg) and LOQ (20 mg/kg) were obtained for SMP-fortified soy-based infant formula, probably because of strong matrix suppression effects. In order to illustrate matrix effects, mean absolute areas of stable isotope-labeled IS peptides, spiked at fixed concentrations into the matrixes, were compared after normalization on signal intensities obtained for infant cereal matrix and shown in Figure 4. Signal suppression occurred neither in a similar strength for all milk derived peptides in the same matrix nor in a similar strength for the same peptide in the different analyzed matrixes. This figure clearly demonstrates the importance of IS addition for quantification purposes in order to compensate for such suppression effects and to ensure reliable results.

Repeatability, Intermediate Reproducibility, and Measurement Uncertainty

The absolute difference between two independent single test results obtained on identical test material by the same operator (corresponding to the repeatability limit, r, at the 95% confidence level) and the absolute difference between two independent single test results obtained using the same method on identical test material by different operators using different equipment on different days (corresponding to the reproducibility limit, R, at the 95% confidence level) are summarized in Table 8.

Recovery

Calculation of recovery rates turned out to be difficult due to unavailable certified reference material, e.g., in the form of purified individual proteins, milk samples with known protein composition, or naturally incurred food samples. Therefore, recovery rates were estimated based on self-spiked food samples and theoretical values for the protein composition of skimmed milk powder and dried liquid milk (24, 25). Furthermore, three SMP samples were analyzed as such, and the concentrations for milk proteins obtained compared with theoretical values. All analyzed proteins met the expected values except κ-casein, which represents only approximately 50% (Table 9). Similar low recovery rates (16–66%) were found in the SMP-fortified food matrixes. This deviation from the theoretical values can be a result of an incomplete digestion; deviation of the given concentration of the synthetic labeled and nonlabeled peptides, used for calibration; or instability of these peptides. The obtained mean slope (1.64) of the calibration curve for κ-casein supports the assumption of an incorrect concentration or impurity of the labeled and/or the nonlabeled synthesized standard peptide that leads to an underestimation of the κ-casein concentration in the samples (Table 10). Recovery rates were also low in SMP-fortified rinse water (16–42%). This can be explained by the phenomenon that proteins diluted to low concentrations in purified water tend to adsorb to surfaces in a complex process, which is driven by different protein-surface interaction forces (33). Protein adsorption is usually only partially reversible because proteins may undergo structural changes due to adsorption on the surface and, therefore are not available for the applied detection method (34).

Robustness

The robustness of the method was examined by applying a two-level, six factor Plackett-Burman statistical experimental design (35). Six method variables that are sensitive to variation were evaluated for their influence on the system suitability criteria set in the method procedure. The method variables were investigated by using different equipment or in a specified range above or below (= Level 2) the nominal method conditions (= Level 1). These included the extraction method [centrifugation tubes (Level 1) versus volumetric flask (Level 2)], the extraction temperature [60°C (Level 1) versus room temperature (Level 2)], application of sample filtration prior to HPLC injection [without (Level 1) versus with protein low-binding PVDF 0.45 μm filter units (Level 2)], trypsin addition [one step (Level 1) versus two step interval (Level 2)], digestion time [overnight (Level 1) versus 6 h (Level 2)], and HPLC column temperature [25°C (Level 1) versus 30°C (Level 2)]. All experiments were carried out using baby food fortified with SMP at 50 mg/kg as a representative sample with an assigned overall method precision RSD(r) of 10%. Level 1 are the conditions used throughout validation; Level 2 are the tested variables (Table 11).

It was found that only the peptide VLVLDTDYK from β-lactoglobulin and YIPIQYVLSR from κ-casein are sensitive to the trypsin addition procedure and digestion time. None of the other studied variables significantly (*t*-test, $\alpha = 0.05$) affected the final results.

Conclusions

An LC/SRM-MS/MS method for quantification of trace amounts of milk proteins was established for food matrixes containing low, medium, and high amounts of protein. The analytical strategy was based on direct quantification

using selected tryptic peptides from β-lactoglobulin, α-, β-, and κ-casein, and synthesized tryptic peptide homologs containing the N-terminal isotopically labeled amino acids [$^{13}C_6$, $^{15}N_2$]-lysine or [$^{13}C_6$, $^{15}N_4$]-arginine as ISs. This method showed less interferences caused by food processing than ELISA because target proteins are denatured and then enzymatically digested before quantification, and IS addition compensates for matrix effects. The highest sensitivity was achieved in baby food with an LOD for SMP of <1 mg/kg and LOQ of 2 mg/kg. Food matrixes with high protein concentrations, such as breakfast cereals and infant cereals, as well as rinse water resulted in an LOQ of 5 mg/kg. In contrast, a high protein food, soy-based infant formula, had an LOQ of 20 mg/kg, which was affected by strong matrix suppression.

Performance characteristics obtained during Commission Decision 2002/657/EC compliant validation qualifies this LC/MS/MS approach as a potential confirmatory method for milk allergen detection. Furthermore, the simultaneous quantification of four different proteins in one run allows multiallergen determination with the possibility to extend to more food allergens.

References

(1) Host, A., & Halken, S. (1990) *Allergy* **45**, 587–596
(2) Bischoff, S.C. (2007) *Curr. Treat. Opt. Gastroenterol.* **10**, 34–43
(3) Skripak, J.M., Matsui, E.C., Mudd, K., & Wood, R.A. (2007) *J. Allergy Clin. Immunol.* **120**, 1172–1177
(4) Wal, J.M. (1998) *Allergy* **53**, 1013–1022
(5) Van Hengel, A.J. (2007) *Anal. Bioanal. Chem.* **389**, 111–118
(6) Poms, R.E., Klein, C.L., & Anklam, E. (2004) *Food Addit. Contam.* **21**, 1–31
(7) Hau, J., & Bovetto, L. (2001) *J. Chromatogr. A* **926**, 105–112
(8) Fenaille, F., Parisod, V., Tabet, J.-C., & Guy, P.A. (2005) *Proteomics* **5**, 3097–3104
(9) Prescott, V.E., Campbell, P.M., Moore, A., Mattes, J., Rothenberg, M.E., Foster, P.S., Higgins, T.J.V., & Hogan, S.P. (2005) *J. Agric. Food Chem.* **53**, 9023–9030
(10) Sancho, A.L., & Mills, E.N.C. (2010) *Regul. Toxicol. Pharm*. **58**, S42–S46
(11) Rigby, N.M., Marsh, J., Sancho, A.I., Wellner, K., Akkerdaas, J., van Ree, R., Knulst, A., Fernandez-Rivas, M., Brettlova, V., Schilte, P.P., Summer, C., Pumphrey, R., Shewry, P.R., & Mills, E.N.C. (2008) *Mol. Nutr. Food Res.* **52**, S251–S261
(12) Kirsch, S., Fourdrilis, S., Dobson, R., Scippo, M.L., Maghuin-Rogister, G., & De Pauw, E. (2009) *Anal. Bioanal. Chem.* **395**, 57–67
(13) Trujillo, A.J., Casals, I., & Guamis, B. (2000) *J. Chromatogr. A* **870**, 371–380
(14) Huber, C.G., & Premstaller, A. (1999) *J. Chromatogr. A* **849**, 161–173
(15) Czerwenka, C., Maier, I., Potocnik, N., Pittner, F., & Lindner, W. (2007) *Anal. Chem.* **79**, 5165–5172
(16) Weber, D., Raymond, P., Ben-Rejeb, S., & Lau, B. (2006). *J. Agric. Food Chem.* **54**, 1604–1610
(17) Monaci, L., & van Hengel, A.J. (2008) *J. Chromatogr. A* **1192**, 113–120
(18) Monaci, L., Losito, I., Palmisano, F., & Visconti, A. (2010) *J. Chromatogr. A* **1217**, 4300–4305
(19) Monaci, L., Norgaard, J.V., & van Hengel, A.J. (2010) *Anal. Methods* **2**, 967–972
(20) EC Decision 2002/657 (Aug. 12, 2002) *Off. J. Eur. Commun.* **L221**, 8–36
(21) *European Pharmacopoeia* (2007) 6th Ed., Method 01/2008:20256 (2.2.56) Council of Europe, European Directorate for the Quality of Medicines & HealthCare, Strasbourg, France
(22) *Official Methods of Analysis* (2000) 17th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **967.12**
(23) Rodriguez, M., & Orescan, D.B. (1998) *Anal. Chem*. **70**, 2710–2717
(24) *Food Chemistry* (1996) 3rd Ed., O.R. Fennema (Ed.), Marcel Dekker Inc., New York, NY
(25) Farrell, H.M. Jr, Jimenez-Flores, R., Bleck, G.T., Brown, E.M., Butler, J.E., Creamer, L.K., Hicks, C.L., Hollar, C.M., Ng-Kwai-Hang, K.F., & Swaisgood, H.E. (2004*) J. Dairy Sci*. **87**, 1641–1674
(26) Abbott, M., Godefroy, S.B., Yeung, J.M., Popping, B., Ulberth, F., Roberts, J., Musser, S., Wehling, P., Taylor, S., Hayward, S., & Poms, R. (2010) *J. AOAC Int.* **93**, 442–450
(27) ISO 5725-2 (1994) *Accuracy (Trueness and Precision) of Measurement Methods and Results—Part 2: Basic Method for the Determination of Repeatability and Reproducibility of a Standard Measurement Method*, International Standards Organization, Geneva, Switzerland
(28) Barwick, V.J., & Ellison, S.L.R. (1998) *Valid Analytical Measurement (VAM) Project: Development and Harmonization of Measurement Uncertainty Principles, Part D: Protocol for Uncertainty Evaluation from Validation Data*, Teddington, UK
(29) Mauron, J. (1990) *J. Nutr. Sci. Vitaminol. (Tokyo)* **36** Suppl. 1, S57–69
(30) BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi, National Center for Biotechnology Information, National Library of Medicine, Bethesda, MD
(31) Augustin, M.A., & Udabage, P. (2007) *Adv. Food Nutr. Res*. **53**, 1–38
(32) Fenaille, F., Morgan, F., Parisod, V., Tabet, J.C., & Guy, P.A. (2004) *J. Mass Spectrom.* **39**, 16–28
(33) Nakanishi, K., Sakiyama, T., & Imamura, K. (2001) *J. Biosci. Bioeng.* **91**, 233–244
(34) Kleijn, M., & Norde, W. (1995) *Heterogen. Chem. Rev.* **2**, 157–172
(35) Plackett, R.L., & Burman, J.P. (1946) *Biometrika* **33**, 305–325