

Liquid Chromatographic Techniques in Food Sciences

Regular Article

Quantification of Casein in Baked Food Products by Selective Analysis of Phosphorylated Peptides Using Fluorous Derivatization with Liquid Chromatography-Tandem Mass Spectrometry Method

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Casein is one of the allergen proteins present in milk. Therefore, a quantification method for the selective analysis of casein using fluorous derivatization with LC-tandem mass spectrometry (LC-MS/MS) was developed. After two allergen proteins (α_{S1} -casein and β -casein) extracted from baked sugar cookies were tryptic digested, the obtained phosphorylated peptides were selectively derivatized by β -elimination with $\text{Ba}(\text{NO}_3)_2$ under basic condition and Michael addition with perfluoroalkylthiol (1*H*,1*H*,2*H*,2*H*-perfluorooctanethiol, PFOT). In this study, YKVPQLEIVPN(pSer)AQQR (104–119 fragment from α_{S1} -casein) and FQ(pSer)EEQQTEDELQDK (33–48 fragment from β -casein) obtained by tryptic digestion were selected as target peptides. The phosphorylated serine residue in each peptide was converted to a perfluoroalkyl group by derivatization. The obtained fluorous-derivatized peptides were analyzed by LC-MS/MS, to which a fluorous LC column was connected. Therefore, it was possible to analyze casein without being affected by the matrix components in the baked food sample. When the present method was applied to cookies with arbitrary amounts of α_{S1} -casein and β -casein, the obtained quantification values were in good agreement with the arbitrary amounts spiked. The quantification limits of α_{S1} - and β -casein in cookie analysis were 246 and 152 ng/g, respectively. Hence, this method can be used to analyze trace amounts of allergen proteins present in the baked food.

Key words fluorous derivatization; milk allergy; α_{S1} -casein; β -casein; LC-tandem mass spectrometry (LC-MS/MS); cookie sample

Introduction

Milk allergy is one of the most common food allergies in childhood and can progress into adulthood, often requiring a diet free of milk allergens.¹⁾ Raw milk is processed into array of products, including cheese, yogurt, and whey powder. These products are frequently added to various food products, raising a concern that unexpected milk allergens may be mixed or retained in these food products.^{2,3)} Casein is one of the milk proteins that cause milk allergy. Casein is heat resistant, when heated, the structure of the protein hardly changes, and therefore heating casein does not influence occurrence of allergy. Moreover, casein does not decompose easily during fermentation. Hence, processed foods, such as yogurt and cheese require the same attention.^{4–6)} It is difficult to remove casein from food during post-processing, and thus it is important to test for it before shipment.

The European Union (EU), U.S.A., Japan, and other developed countries have adopted mandatory labeling for food allergens.⁷⁾ The two main methods to detect allergens in food are the antibody-based enzyme-linked immunosorbent assay (ELISA)^{8,9)} and DNA-based PCR.^{10,11)} However, the ELISA technique can be affected by cross-reactivity, leading to false-positive results. Furthermore, unexpected effects

owing to food processing may lead to epitope masking.¹²⁾ The DNA-based methods, such as PCR, are specific and sensitive; however, they cannot directly detect proteins, leading to false-negative results.¹³⁾

Thus, MS-based analysis overcomes the problems encountered in ELISA and PCR because it can selectively detect proteins or peptides digested with enzymes.¹⁴⁾ Due to the ability of MS to identify and detect mass, multiple allergens can be detected in a single analysis.^{15–17)} In addition, protein allergen analysis using trypsin can analyze hidden allergens in foods with good reproducibility.¹⁸⁾ However, in general, food-based analysis is affected by the complex matrix components of the sample, reducing the efficiency of tryptic digestion and interfering with MS detection.¹⁹⁾ Moreover, the background complexity remains unresolved although these dispersions can be corrected by adding stable isotopic proteins or peptides.²⁰⁾

In recent years, fluorous derivatization LC-MS methods almost unaffected by matrix components, such as biological samples and food samples, have been reported.^{21–26)} The derivatization method utilizes the specific affinity of perfluoroalkyl groups. Therefore, selective analysis with a reduced background is possible by derivatizing the analytes with fluorous reagents and separating them on a fluorous LC column. In

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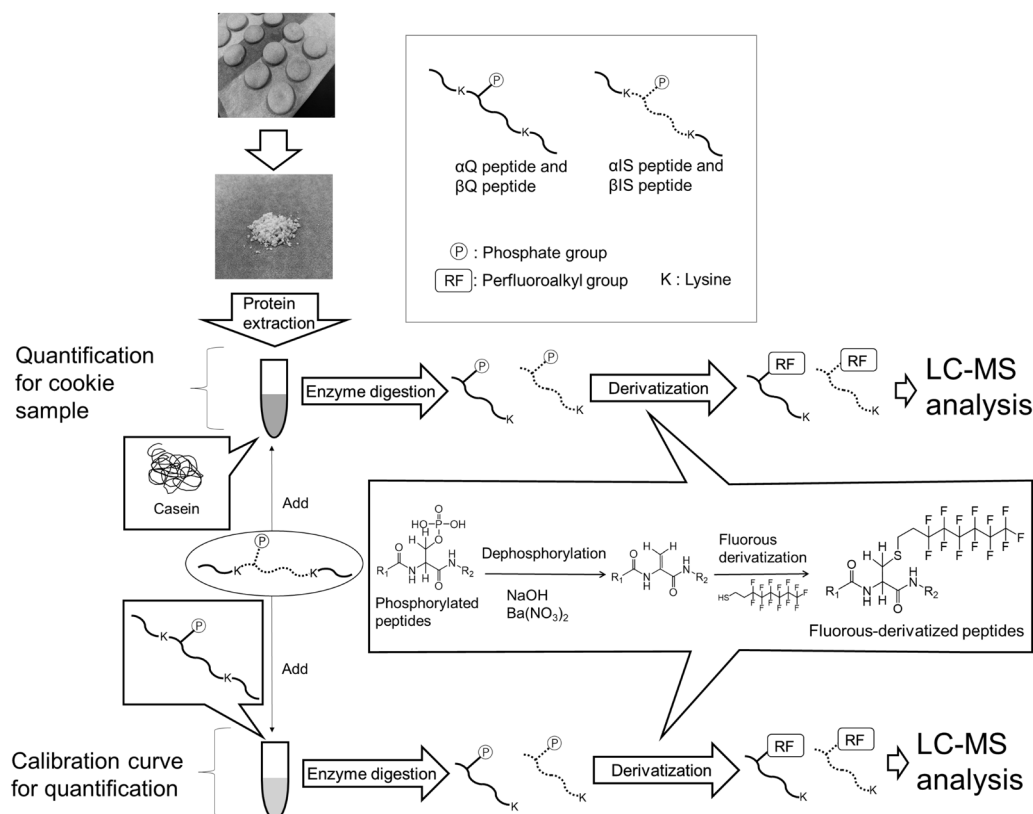


Fig. 1. Outline of Casein Analysis in Baked Sugar Cookies Used in the Present Study

addition, selective fluororous derivatization of phosphate groups of phosphoproteins using β -elimination and Michael addition reactions has been developed.²⁷⁾ In this study, by focusing on the phosphate groups in caseins, a fluororous derivatization LC-tandem mass spectrometry (LC-MS/MS) method selective for the phosphate group was applied for the analysis of casein in baked sugar cookies. For the quantification of casein, a QconCAT method^{28,29)} using synthetic peptides (wherein a peptide containing a stable isotope amino acid was linked) were used as internal standards (Fig. 1). Therefore, the background complexity owing to matrix components in the sample can be reduced by applying the present method to allergen analysis. Moreover, samples containing minute amounts of allergens can be accurately analyzed with high reliability. Thus, we confirmed the advantages of the fluororous derivatization LC-MS/MS method by preparing milk-free and quality-controlled baked sugar cookies and acquiring validation data. This method was also applied to commercially available cookie analysis.

Results and Discussion

Optimization of Fluororous Derivatization Reaction We reoptimized the fluororous derivatization reaction based on a previous study²⁷⁾ as all target phosphorylated peptides in this study have only one phosphate group in the molecule. 1*H*,1*H*,2*H*,2*H*-Perfluorooctanethiol (PFOT), optimal for one phosphate-based peptide in the molecule,²⁷⁾ was used as the derivatization reagent. The overall reaction conditions were optimized after selecting the fluororous reagent. A tryptic digested peptide mixture was used to consider the effect of the solution in the derivatization reaction. Optimization studies included varying reagent concentrations of PFOT (0.1, 0.2, 0.4,

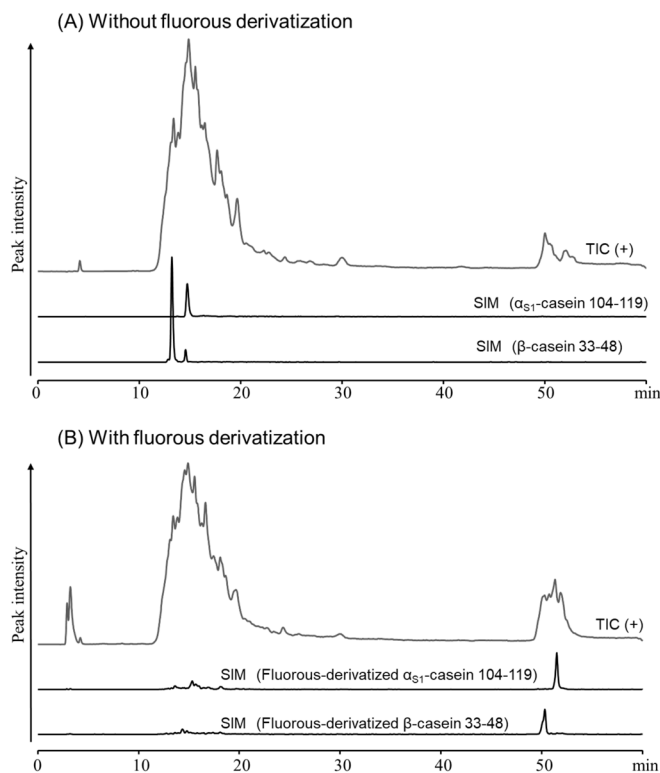


Fig. 2. Chromatograms of Caseins Spiked Blank Cookie Sample

The chromatograms were obtained by analysis without fluororous derivatization (A) and with fluororous derivatization (B). The total ion chromatograms (TICs) were between m/z 500 and 1500. Selected ion monitoring (SIM) chromatograms were set to m/z 977.05 (underivatized α_{S1} -casein 104–119), m/z 688.30 (underivatized β -casein 33–48), m/z 745.70 (fluororous-derivatized α_{S1} -casein 104–119), and m/z 782.35 (fluororous-derivatized β -casein 33–48).

0.8, and 1 M), barium nitrate ($\text{Ba}(\text{NO}_3)_2$) (0.01, 0.05, 0.1, 0.2 M, and ≥ 0.3 M (saturated)), and sodium hydroxide (NaOH) (0.05, 0.1, 0.2, 0.5, 0.7, and 1 M), reaction temperature (25, 40, and 60°C), and time (3, 5, 10, 30, and 60 min). The optimized reagent concentrations were 1 M PFOT, saturated $\text{Ba}(\text{NO}_3)_2$, and 0.7 M NaOH . The optimum reaction temperature and time were 60°C and 10 min, respectively.

Optimization of Protein Extraction The protein extractions were repeated one to three times owing to the poor recovery of caseins. As a result, the amounts of caseins obtained did not change with the number of extractions. Therefore, the number of protein extractions was set to one.

Separation of Phosphorylated Peptides from Matrix Components of Baked Sugar Cookies In the present method, phosphorylated peptides obtained from caseins by tryptic digestion were selectively fluorously-derivatized and analyzed on a fluorous LC column to achieve separation from the matrix components of the sample, allowing accurate analysis. However, it is necessary to confirm whether the target peptides are selectively retained on the fluorous LC column and separated from the matrix component of the baked sugar cookies. Therefore, caseins were added to each blank cookie sample after protein extraction and then tryptic digestion was carried out. Subsequently, the samples were analyzed without and with derivatization (Figs. 2A and 2B, respectively). The matrix components of the samples were eluted between 10–20 min in both chromatograms. The underivatized phosphorylated peptides were eluted around the retention time of the matrix component (Fig. 2A). On the other hand, it was

confirmed that the derivatized phosphorylated peptides were eluted around 50 min and could be analyzed without interference from the matrix components (Fig. 2B). Other peaks that appeared at approximately 50 min in Fig. 2B were considered as other phosphorylated peptides following fluorous derivatization. In the present study, these peaks did not affect the detection of casein fragments, however, even if they gave strong peak intensities, it is possible to separate them by modifying the mobile phase conditions. Therefore, the chromatograms obtained confirmed that the phosphorylated peptides were separated and could be analyzed without any interference from the matrix components of the baked sugar cookies.

Quantitativity The α_{S1} -casein peptide (αQ peptide) and β -casein peptide (βQ peptide) were used for quantification. These peptides in which 3 or 4 residue amino acids are extended the *N*- and *C*-terminals of the fragment peptides (αF and βF peptides, respectively) to be detected digested by trypsin as well as casein proteins. Using these peptides as standards and peptides of the same sequence containing the isotopic amino acid as internal standards (ISs), it is possible to correct for differences in trypsin digestion efficiency between samples. Moreover, the linearity of the calibration curves and the precision at three points were confirmed when the QconCAT method^{28,29)} was combined with the present method. The results are shown in Table 1. The linearity (r^2) of the obtained calibration curves was greater than 0.999, indicating that the quantitativity was sufficient. In addition, the relative standard deviation (RSD) of the intra-day repeated analysis for the three concentrations of casein was less than

Table 1. Linearity of Calibration Curve, Precision, LOD, and LOQ

	Linearity ^{a)} (r^2)	Precision ^{b)} (% , $n = 6$)			LOD ^{c)} (fmol)	LOQ ^{d)} (ng/g)
		10 $\mu\text{mol/kg}$	20 $\mu\text{mol/kg}$	100 $\mu\text{mol/kg}$		
α_{S1} -Casein	0.9992	5.0	2.3	1.1	30.7	246
β -Casein	0.9999	1.9	2.0	1.4	18.9	152

a) Calibration curve in the range of 2–1000 $\mu\text{mol/kg}$. *b)* Relative standard deviation of the peak area for 10, 20, and 100 $\mu\text{mol/kg}$ were treated as the standard procedure. *c)* Defined as the amount on the column, yielding a signal-to-noise ratio of 3. *d)* Defined as the sample concentration, yielding a signal-to-noise ratio of 10.

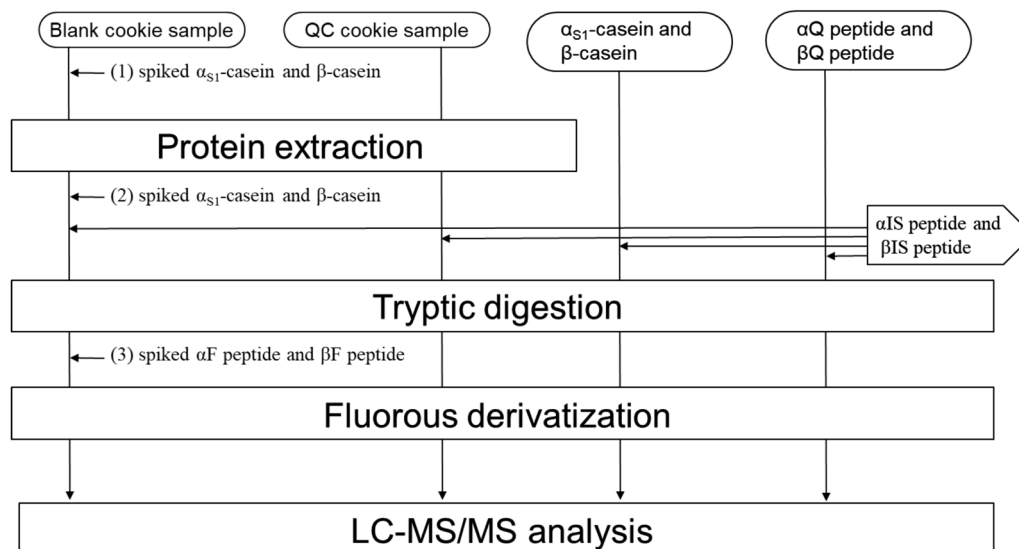


Fig. 3. The Experimental Procedure for Validation Study and Quantification for QC Samples

The quantification values obtained by spiking (1) and (2) are defined as BC_{sp1} and BC_{sp2} , respectively. The quantification values obtained by analyzing the casein standards are defined as STs. BC_{sp3} -samples without spiking and the BC_{sp3} -sample spiked with the fragment peptides (3) were prepared for calculation of matrix effects.

5.0%, and the precision was sufficiently good even when the tryptic digestion procedure was included. The detection and quantification limits (LODs and LOQs) are shown in Table 1. LOQ of commercially available ELISA kits for α_{S1} -casein is 300–5000 ng/g. In comparison, LOQs of the present method for α_{S1} -casein and β -casein were 152–246 ng/g, which were sensitive enough to analyze allergens.

Validation of Casein Quantification in Baked Sugar Cookies The amounts of caseins were quantified using the QconCAT method after the spiked samples were prepared, and each parameter was calculated, as shown in Fig. 3. The results are shown in Table 2. In the present study, the protein extraction (R) was 64.9 to 72.9%, and the recoveries were almost the same as those in a previous study.¹⁶⁾ The accuracy (A) and matrix effect when corrected by IS (M_{IS}) were 97.4 to 103% and 99.1 to 102%, respectively. Additionally, it was confirmed that the QconCAT method satisfactorily corrected the variation between each sample and quantified it accurately. The matrix effect when uncorrected (M_{AQ}) was 94.9 to 101%, which is almost similar to that of M_{IS} . These results indicate that the background did not interfere with detection. Moreover, even if caseins are present only at the limit of quantification, it can be accurately quantified without false-negative results.

Quantification of Casein in Quality Control Samples The present method was used to quantify the three different concentrations of casein in quality control (QC) samples to which α_{S1} -casein and β -casein were added. The QconCAT method was used for the quantification. Protein extraction was corrected using the calculated R . Furthermore, each quantification was repeated six times, and the mean and standard deviation were used as the quantification values. The results are presented in Table 3, together with the preparation values. In all the cases, the preparation and quantification values were in agreement, confirming that the present method could be used for accurate quantification.

Quantification of Casein in Commercially Available Baked Sugar Cookies Caseins in baked sugar cookies com-

mercially available in Japan were quantified using the present method. The results are shown in Table 4. α_{S1} -Casein and β -casein were detected in all cookies, and each of them could be quantified.

Conclusion

In this study, a fluoruous derivatization LC-MS/MS method for phosphorylated peptides was applied for the selective analysis of α_{S1} -casein and β -casein milk allergens in cookies. As the fluoruous-derivatized phosphopeptides were selectively retained on a fluoruous LC column, they could be detected without any interference from matrix components in the cookies. The QconCAT method was applied to quantify casein in cookies. Therefore, the present method could correct the dispersion during the pretreatment procedure among samples. Furthermore, by analyzing the QC samples, the present method confirmed that the quantification was accurate. This indicates that casein can be accurately quantified even at limit of quantification without any false-negative results. False-negatives in food allergen analysis can be fatal for people with food allergies. Thus, the present method is useful in allergen analysis because false-negatives can be prevented.

Experimental

Reagents and Materials PFOT, α_{S1} -casein, and β -casein were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). NaOH was obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Acetonitrile and formic acid were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Boric acid, sodium tetraborate decahydrate, $\text{Ba}(\text{NO}_3)_2$, and trypsin were obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). All aqueous solutions were prepared using ultrapure water purified using a Merck KGaA Milli-Q® Gradient-A10 system (Darmstadt, Germany). All other organic solvents and reagents were of LC-MS grade. The synthesized peptides, α_{S1} -casein peptides [α_{S1} -casein: 101-123, LKKYKVPQLEIVPN(pSer)AEERLHSM (α Q peptide) and LKKYKVPQ{Leu(¹³C₆, ¹⁵N)}EIVPN(pSer)AEERLHSM (α IS peptide) for QconCAT; 104-119, YKVPQLEIVPN(pSer)AEER (α F peptide) which is a fragment peptide obtained by tryptic digestion] and β -casein peptides [β -casein: 30-52, IEKFQ(pSer)EEQQQTEDELQDKIHPF (β Q peptide) and IEKFQ(pSer)EEQQQTEDE{Leu(¹³C₆, ¹⁵N)}QDKIHPF (β IS peptide) for QconCAT; 33-48, FQ(pSer)EEQQQTEDELQDK (β F peptide) which is a fragment peptide obtained by tryptic digestion], were used as standard and internal standard (IS) peptides and

Table 2. Validation Study for Spiked Cookie Sample

	R^a (%)	A^b (%)	M_{IS}^c (%)	M_{AQ}^d (%)
α_{S1} -Casein	72.9	103	99.1	94.9
β -Casein	64.9	97.4	102	101

a) The recovery of protein extraction. b) The accuracy based on trypsin digestion, fluoruous derivatization, and LC-MS/MS analysis. c) Matrix effect when corrected by IS d) Uncorrected matrix effect.

Table 3. Comparison of the Quantification Value ($n = 6$) and the Preparation Value for QC Sample

		QC _A ($\mu\text{g/g}$)	QC _B ($\mu\text{g/g}$)	QC _C ($\mu\text{g/g}$)
α_{S1} -Casein	Preparation value	57.0	28.5	5.76
	Quantification value	57.9 $\pm$ 0.9	28.2 $\pm$ 0.5	5.93 $\pm$ 0.2
β -Casein	Preparation value	109	54.2	11.0
	Quantification value	109 $\pm$ 0.8	54.3 $\pm$ 0.7	10.7 $\pm$ 0.3

Table 4. Quantification Values of Caseins in Commercially Available Cookies Analyzed by the Present Method ($n = 3$)

	Cookie A ($\mu\text{g/g}$)	Cookie B ($\mu\text{g/g}$)	Cookie C ($\mu\text{g/g}$)	Cookie D ($\mu\text{g/g}$)
α_{S1} -Casein	13.5 $\pm$ 0.2	1.98 $\pm$ 0.02	72.9 $\pm$ 0.4	82.9 $\pm$ 0.3
β -Casein	4.23 $\pm$ 0.1	2.89 $\pm$ 0.04	43.0 $\pm$ 0.5	10.7 $\pm$ 0.4

obtained from GenScript Japan Inc. (Tokyo, Japan).

Casein solution and stock solutions of peptides were prepared based on their respective purities to obtain 250 $\mu\text{mol/kg}$ and 1 mmol/kg, respectively, with 100 mM borate buffer (pH 8.7) and stored at -20°C .

Quality Control Sample Preparation The preparation of QC samples was based on Lamberti's method.¹³⁾ The blank dough of baked sugar cookie was prepared by mixing 70 g canola oil, 90 g sucrose, and 200 g soft wheat flour. The spiked dough of baked sugar cookies was prepared by mixing the blank dough of baked sugar cookies and casein solution to detect levels of casein-contaminated cookies (QC_A: α_{S1} -casein 57.0 $\mu\text{g/g}$, β -casein 109 $\mu\text{g/g}$; QC_B: α_{S1} -casein 28.5 $\mu\text{g/g}$, β -casein 54.2 $\mu\text{g/g}$; QC_C: α_{S1} -casein 5.76 $\mu\text{g/g}$, β -casein 11.0 $\mu\text{g/g}$). Finally, all the cookies were baked for 12 min at 180°C in a conventional ventilated oven.

Protein Extraction The protein extraction procedure was based on Taylor's method.³⁰⁾ Baked sugar cookies were crushed into a fine powder using a mortar and pestle. Accurately weighed approximately 250 mg of the obtained powder and 1.5 mL of 1% sodium dodecyl sulfate (SDS) (100 mM borate buffer, pH 8.5) were added to a 2-mL polypropylene tube and vortexed at 60°C for 20 min. Subsequently, the mixture was centrifuged at $18000 \times g$ for 20 min at 4°C and, the supernatant was transferred to a 15-mL polypropylene tube. Further, 5 mL of methanol and chloroform mixture (2:1, v/v) was added to the supernatant, and the mixture was vortexed for 1 min. Subsequently, 3.5 mL of chloroform was added to the mixture and vortexed for 1 min. The tube was centrifuged at $3500 \times g$ for 30 min at room temperature. This procedure generated a triphasic solution containing a protein interphase. The upper and lower phases were carefully discarded, and the remaining wet interphase was transferred to a new 2-mL polypropylene tube. The resulting intermediate phase was dried under reduced pressure for 60 min.

Tryptic Digestion For tryptic digestion, 890 μL of borate buffer (100 mM, pH 8.7), accurately weighed approximately 10 mg of IS, and 100 μL of 0.01% trypsin solution (100 mM borate buffer, pH 8.7) were added to the dried sample. Subsequently, mixture was incubated at 37°C for 24 h with vortexing. The tryptic digests were desalted using a solid-phase extraction (SPE) cartridge (StrataTM-X 33 μm polymeric reversed-phase, 60 mg/3 mL; Phenomenex, Torrance, CA, U.S.A.) by gravity as follows:

The SPE cartridges were conditioned with 3 mL of methanol with 3 mL of ammonium formate solution (5 mM) in 1% (v/v) formic acid (H_2O). The tryptic digests were loaded onto the cartridge and further washed with 1 mL of ammonium formate solution (5 mM) in 1% (v/v) formic acid (H_2O). Peptides were eluted using 1 mL of ammonium formate solution (5 mM) in 1% (v/v) formic acid [70% (v/v) methanol] into a fresh 1.5-mL polypropylene tube. The eluent was dried under

reduced pressure without heat, and the residue was dissolved in 10 μL of H_2O .

Derivatization of Phosphorylated Peptides For derivatization, 40 μL of 1 M PFOT in acetonitrile and 50 μL of 0.7 M NaOH in saturated $\text{Ba}(\text{NO}_3)_2$ were added to 10 μL of the standard peptide solution or the solution after digestion by trypsin. The mixture was heated at 60°C for 10 min. Subsequently, 20 μL of 20% formic acid in H_2O (v/v) was added, and 5 μL of the mixture was injected into the LC-MS.

Instrumentation and LC-MS/MS Conditions LC was performed using a Nexera HPLC system (Shimadzu Scientific Instruments, Inc., Kyoto, Japan) equipped with a system controller (CBM-20A), a binary solvent delivery system (LC-30AD), a degasser (DGU-20A), an autosampler (SIL-30AC), and a column heater oven (CTO-20A). An LCMS-8050 triple quadrupole mass spectrometer (Shimadzu Scientific Instruments, Inc.) equipped with an electrospray ionization (ESI) interface was used in this study. LabSolutions LCMS software (Shimadzu Scientific Instruments, Inc.) was used to control the instruments and process the data. The mass spectrometer was operated in positive ESI mode in either the precursor ion scan or selected reaction monitoring (SRM) mode.

A Fluofix-II 120E (250×2.1 mm i.d., 5 μm ; flow rate: 0.2 mL/min; FUJIFILM Wako Pure Chemical Corporation) was used as the LC column. Solvent A [5 mM ammonium formate and 1% (v/v) formic acid in water] and solvent B [5 mM ammonium formate and 1% (v/v) formic acid in methanol] were used as mobile phases for gradient elution. The gradient program was as follows:

The concentration of mobile phase B was maintained at 1% (v/v) for 0–5 min, linearly changed to 57% (v/v) for 5–15 min, and then maintained at 57% (v/v) for 15–45 min, at 100% (v/v) for 45–55 min, and at 1% (v/v) for 55–60 min. The column oven temperature was set to 40°C , and the injection volume was 5 μL . The MS/MS conditions were set as follows: ion spray voltage, 4.0 kV; desolvation line temperature, 250°C ; heat block temperature, 400°C ; drying gas flow rate, 10.0 L/min; nebulizer gas flow rate, 3.0 L/min; and collision-induced dissociation gas pressure, 450 kPa. The precursor ion (Q1), product ion (Q3), and collision energy (CE) of the target peptides with derivatization in the SRM transition are listed in Table 5.

Linearity of Calibration Curve, Precision, and Detection Limit The samples for the calibration curve were prepared at six concentrations of 2, 10, 20, 100, 200, and 1000 $\mu\text{mol/kg}$. Stock peptide solutions of the αQ and βQ peptides and IS solutions of the αIS and βIS peptides were each diluted with 100 mM borate buffer (pH 8.7). Approximately 40 mg of calibration sample solution and 10 mg of IS solutions of αIS peptide and βIS peptide were accurately mixed by weight with a semi-microbalance. Subsequently, the tryptic digestion and the derivatization were performed according to standard procedures. Calibration curves were created using the peak

Table 5. Precursor and Product Ions (Q1 and Q3) and CE for the SRM Method in This Study

Target peptide		Q1 (<i>m/z</i>)	Q3 (<i>m/z</i>)	CE (eV)
α_{S1} -Casein (αF peptide)	Natural peptide	745.70	582.90	–22.0
	Isotope-labeled peptide	748.05	582.90	
β -Casein (βF peptide)	Natural peptide	782.35	684.45	–20.0
	Isotope-labeled peptide	784.65	684.45	

area ratio of the sample peptide for the calibration curve and isotope-labeled peptide. The intra-day precision values of peak area were assessed by performing each analysis of 10, 20, and 100 $\mu\text{mol/kg}$ six times on the same day. LOD and LOQ were defined as the amount on the column and the concentration of cookie sample those provided signal-to-noise (S/N) ratios of 3 and 10, respectively.

Validation Study Using Spiked Samples We used milk-free baked sugar cookies (BC samples) to calculate the recovery of protein extraction (R), accuracy based on tryptic digestion, fluoruous derivatization, and LC-MS/MS analysis (A), matrix effect when corrected by IS (M_{IS}) and uncorrected matrix effect (M_{AQ}). The amount of spiked casein and fragment peptide were 2 nmol in each case. The experimental procedure for the validation study is shown in Fig. 3. The quantification values obtained were defined as BC_{sp1} and BC_{sp2} , where caseins were spiked before protein extraction (1) and before tryptic digestion (2), respectively. The quantification value obtained by analyzing the standard caseins was defined as STs. R and A were calculated using the following equations:

$$R(\%) = \frac{BC_{sp1}}{BC_{sp2}} \times 100$$

$$A(\%) = \frac{BC_{sp2}}{ST} \times 100$$

In addition, a BC-sample without spiking and a BC_{sp3} -sample spiked with fragment peptides after tryptic digestion (3) were prepared for calculation of matrix effects. M_{IS} and M_{AQ} were calculated by the following formulas using the quantification values (BC_{sp3BC} , BC_{sp3H_2O}) and peak area values (PA_{sp3BC} , PA_{sp3H_2O}) when the BC_{sp3} -sample was diluted 10-fold with the BC-sample (BC_{sp3BC} and PA_{sp3BC} were obtained) and diluted with 50% acetonitrile (BC_{sp3H_2O} and PA_{sp3H_2O} were obtained).

$$M_{IS}(\%) = \frac{BC_{sp3BC}}{BC_{sp3H_2O}} \times 100$$

$$M_{AQ}(\%) = \frac{PA_{sp3BC}}{PA_{sp3H_2O}} \times 100$$

Quantification for Quality Control and Real Samples

Three QC samples (QC_A , QC_B , and QC_C) were prepared and quantified using the present method. Each quantification was repeated six times, and the obtained quantification values were compared to the preparation values. Casein lost during protein extraction was corrected using R value obtained from the validation study.

Caseins present in four baked sugar cookies that are commercially available in Japan (Cookies A–D) were quantified using this method. In this quantification, casein lost during protein extraction was corrected using the R value obtained in the validation study.

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Conflict of Interest The authors declare no conflict of interest.

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