

Development of an SI-Traceable HPLC–Isotope Dilution Mass Spectrometry Method To Quantify β -Lactoglobulin in Milk Powders

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Supporting Information

ABSTRACT: β -Lactoglobulin (β -LG) is one of the major allergenic proteins in milk. There is an urgent demand for an accurate and traceable method to develop β -LG certified reference material (CRM). In this work, β -LG was enzymatically digested and a specific peptide was chosen for quantitation by isotope-dilution mass spectrometry (IDMS). With amino acid CRMs as standards, the results could be traced to SI unit. By the proposed method, the recovery ranged from 86.0% to 118.3% with CVs <9.0%. The LOD and LOQ were 4.8×10^{-5} g/g and 1.6×10^{-4} g/g of β -LG in milk powder, respectively. Ten samples from domestic market were analyzed with CVs <5.6%, and the relative expanded uncertainties ranged from 4.2% to 5.9% ($k = 2$). With the CRMs, it is expected that the comparability of β -LG quantitation results will be improved among different laboratories.

KEYWORDS: β -lactoglobulin, liquid chromatography isotope dilution mass spectrometry, HPLC–IDMS, milk powder, allergen

■ INTRODUCTION

In recent years, the issue of food safety is attracting more attention. Milk is one of the most commonly consumed beverages for protein intake, and is one of the main allergenic foods. Milk contains more than 20 proteins/allergens that can cause allergic reactions.¹ The main whey protein in milk is β -LG, and it is very stable while the N-end is attached.² Because of its stability, β -LG cannot be digested by pepsin, and it readily passes from the gastrointestinal tract into the blood circulation system. In sensitive individuals, this causes a hypersensitive reaction that stimulates the immune system.³ Therefore, there is an urgent demand for an accurate and traceable method to develop β -LG CRM to harmonize and improve the quantification comparability among different laboratories.

There are many technologies for β -LG detection, including enzyme linked immunosorbent assays (ELISA),^{4–7} high performance liquid chromatography (HPLC),^{8,9} and capillary electrophoresis (CE).^{9–12} By capillary zone electrophoresis, the main proteins in milk, such as β -LG, α -lactalbumin, and caseins, were successfully separated within 60 min, and satisfactory linearity, reproducibility, and precision have been achieved.¹⁰ Mass spectrometry (MS) has played a pivotal role in proteomics research and has proved to be a powerful analytical technique for both protein and peptide analyses.^{13,14} Special

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attention has been paid to new methods such as those based on multidimensional systems comprising liquid chromatography (LC) to separate peptides or proteins and MS to analyze them.¹⁵ Chromatography and MS-based techniques have become much more widely used for analyses of proteins and peptides.^{16–35}

IDMS is a primary method in the metrological field. It allows highly accurate and precise measurements of various substances and has been used widely since its development in the 1980s.^{17–19} It has been used to detect and quantify drugs, hormones, pesticides and veterinary drug residues in serum, and other organic small molecules with high accuracy. With the development of the life science and biotechnology industries, IDMS methods have been further developed to quantify proteins,^{21–35} nucleic acids,³⁶ and other biological macromolecules.^{37–40} Especially, IDMS has been successfully applied into analyses with milk powders and shows several advantages. Zhang et al. developed an IDMS method to quantify 12 mycotoxins in milk-based infant formula and foods. The average recoveries in fortified milk, milk-based infant formula, milk powder, and baby yogurt ranged from 89% to 126% with CVs of <20%. The validated method provides sufficient selectivity, sensitivity, accuracy, and reproducibility to screen for aflatoxin M1 at nanogram per kilogram concentrations and other mycotoxins, without using standard addition or matrix-matched calibration to compensate for matrix effects.²⁰ In 2008, Burkitt et al. first reported the accurate quantification of a protein in solution by an IDMS method.²¹ In that study, the absolute concentration of a protein was determined using an SI-traceable peptide quantification method in which a standard peptide was quantified by amino acid analysis with IDMS, and amino acid standards of known purity were used. This allowed fully SI-traceable measurements of protein concentration. However, this method was not used to determine the protein content in mixtures or complex matrices. In another study, researchers enzymatically digested a protein into polypeptides; then, using an isotope-labeled peptide as an internal standard, they measured the specific peptide content by an IDMS method and further calculated the total protein content. This allowed them to accurately determine the protein content in mixtures and matrices.²²

In recent years, LC–MS/MS and IDMS have been used to detect protein allergens in food. Weber et al. developed a LC–MS/MS method of the tryptic digest of a cleaned-up food matrix extract for the detection of milk allergens.²³ Careri et al. developed MS-based technology for the detection and quantification of contaminant or allergen traces.²⁴ Cryar developed IDMS method for the analysis of lysozyme in wine and the production of CRMs.²⁵ To date, there have been no reports on quantitation of β -LG in milk powders by HPLC–IDMS. Development of an SI-traceable quantification method for β -LG in milk powders can improve the accuracy and reliability of β -LG detection. Such a method would benefit the development of a β -LG CRM and improve the comparability of β -LG quantification results among different laboratories.

MATERIALS AND METHODS

Chemicals and Reagents. DL-Dithiothreitol (DTT), iodoacetamide (IAM), ammonium bicarbonate, D₃-leucine, perfluoroheptanoic acid, β -LG, and trypsin were purchased from Promega Co.. The specific activity of the trypsin is 18704 U/mg (BAEE as substrate). The trypsin is diluted in 50 mM acetic acid with a protein concentration of 0.5 mg/mL. RapiGest SF was obtained from Waters

Co. Ltd. (Milford, MA, USA). The peptides of TPEVDDEALEK (TK-11) and isotope-labeled TPEVDDEAL*EK (L-TK-11, where L* is D₃-Leu) were synthesized by GL Biochem. Co. Ltd. (Shanghai, China). The CRMs of proline, valine, leucine, and phenylalanine were obtained from the National Institute of Metrology (NIM, Beijing, China). Milk powder samples were obtained from domestic manufacturers.

Reagent Preparation. To prepare 50 mmol/L ammonium bicarbonate (pH = 8.0), 0.179 g of ammonium bicarbonate was dissolved in 45 mL of water, and then the pH was adjusted to 8.0 with aqueous ammonia. To prepare 1 mg/mL RapiGest SF, approximately 1 mg of solid RapiGest SF was dissolved in 1 mL of 50 mmol/L ammonium bicarbonate buffer. To prepare 0.1 mol/L DTT: 0.154 g of DTT was dissolved in 0.01 mol/L NaOAc (pH = 5.2) and then filtered through a 0.22 μ m filter. To prepare 0.1 mol/L IAM: 0.185 g of IAM was dissolved in 10 mL of pure water. The peptides and β -LG solutions were prepared as follows: each peptide/protein (TK-11, L-TK-11, and β -LG) was dissolved in pure water containing 0.1% formic acid to a final concentration of 1 mg/mL. The peptide solutions were distributed into 1.5 mL centrifuge tubes and stored at –80 °C until analysis.

Peptide or Protein Hydrolysis. 1 mg of TK-11 or β -LG was weighed with a balance capable of 1 μ g imprecision, and it was dissolved in about 1 g of 0.1 mol/L HCl. The mass of the HCl was weighed with a balance capable of 0.01 mg imprecision. 100 μ L of TK-11 (1 mg/mL) or β -LG (1 mg/mL) was added to a 2 mL ampule and lyophilized. Then 500 μ L of 6 mol/L HCl was added. The solution was flushed with nitrogen for 2 min before sealing. Then the ampules were put into an oven and hydrolysis was carried out at 110.0 $\pm$ 0.5 °C for 48 h for TK-11 and for 24 h for β -LG. Isotope-labeled amino acids were added to the mixture after hydrolysis, and then the mixture was flushed with nitrogen and dried. Finally, it was reconstituted with 0.1% formic acid and filtered for instrumental analysis.

Enzymatic Digestion. Approximately 2.7 mg of β -LG was dissolved in 600 μ L of RapiGest SF buffer. Then, 30 μ L of 0.1 mmol/L DTT was added and the mixture was incubated in a water bath at 60 °C for 30 min before addition of 60 μ L of 0.1 mol/L IAM. The mixture was cooled to room temperature and then kept in the dark for 40 min. Then, 30 μ L of trypsin was added and the mixture was kept at 37 °C for 48 h for digestion. An additional 5 μ L of trypsin was added to the digestion mixture every 24 h. The proteolysis reaction was terminated by adding 200 μ L of 0.1 mmol/L HCl. The mixture was kept at –80 °C until instrumental analysis.

Protein Identification. The digested samples were analyzed by a Synapt G1 mass spectrometer (UBA171, Waters) with a capture column (5 μ m Symmetry C₁₈, 180 μ m $\times$ 20 mm) and an analytical column (1.7 μ m BEH130 C₁₈, 100 μ m $\times$ 100 mm). The column temperature was 35 °C and the sample temperature was 8 °C during analysis. The injection volume was 2 μ L, and the flow rate was 0.5 μ L/min. The mobile phase A was pure water from a Milli Q system with 0.1% formic acid, and the mobile phase B was MeCN. The sample loading time was 3 min, and the trapping flow rate was 5 μ L/min. The separation was performed as per the gradient shown in Table S1 in the Supporting Information. The total runtime was 60 min.

The operating parameters of the nano-ESI source were set as follows: positive ionization mode; capillary voltage, 3.0 kV; source temperature, 100 °C; desolvation nitrogen gas flow rate, 500 L/h; nano gas pressure, 2.0 bar; sampling cone voltage, 30 V. The mass to charge ratios of the target fragment ions were captured from m/z 100 to m/z 2000. The data were processed with MassLynx 4.1, and the data acquisition time was from 8 to 37 min.

Protein identification was carried out with Proteinlynx, with the following parameters: databank was Swiss-Prot; fixed modification was carbamidomethyl C; variable modifications were deamidation N, deamidation Q, and oxidation M; minimum fragment ion matches per peptide was 3; minimum fragment ion matches per protein was 7; minimum peptide matches per protein was 1.

Determination of the Mass Fraction of Pure TK-11 or β -LG in Solid Powder by Acid Hydrolysis. The mass fraction of pure peptide or pure protein in the solid powder was determined by HPLC–IDMS after acid hydrolysis. The exact mass fraction of proline,

valine, leucine, or phenylalanine in the hydrolyzed mixture was quantitatively determined by HPLC–IDMS with amino acid CRMs as standards and relative isotope-labeled amino acids as internal standards. An Agilent ZORBAX SB-Aq column (3.5 μ m, 2.1 $\times$ 150 mm) was used for separation. The column temperature was 40 $^{\circ}$ C during analysis. The injection volume was 20 μ L, and the flow rate was 0.2 mL/min. The mobile phase A was 0.8 mmol/L perfluoroheptanoic acid with 0.05% TFA, and the mobile phase B was MeCN. The gradient is shown in Table S2 in the Supporting Information.

A Thermo-Finnigan TSQ quantum triple quadrupole mass spectrometer (San Jose, CA, USA) equipped with an ESI ion source was used for detection. The ESI ionization was operated in positive mode, and the multiple reaction monitor (MRM) mode was used for detection of Pro (116 $\rightarrow$ 70), L-Pro (121 $\rightarrow$ 74), Val (118 $\rightarrow$ 72), L-Val (123 $\rightarrow$ 76), Leu (132 $\rightarrow$ 86), L-Leu (142 $\rightarrow$ 96), Phe (166 $\rightarrow$ 120), and L-Phe (175 $\rightarrow$ 128). The flow rate of sheath gas and auxiliary gas was 30 and 10 arbitrary units (au), respectively. The capillary temperature was set at 270 $^{\circ}$ C, and the spray voltage was set at 3000 V.

The mass fraction of each amino acid was calculated according to formula 1:^{22,27,41}

$$c = \frac{Pm_s[R_s(I_1 - I_2) - (I_1R_2 - I_2R_1)]}{M(R_1 - R_2)} \quad (1)$$

where c is the mass fraction of each amino acid (g/g); m_s is the absolute mass of isotope-labeled amino acid in the sample solution (g); R_s is the ratio of the peak area of an amino acid to its isotope-labeled one of the sample; I_1 is the mass ratio of an amino acid to its isotope-labeled one of the high-level standard; I_2 is the mass ratio of an amino acid to its isotope-labeled one of the low-level standard; R_1 is the peak area ratio of an amino acid to its isotope-labeled one of the high-level standard; R_2 is the peak area ratio of an amino acid and its isotope-labeled one of the low-level standard; M is the absolute mass of the sample (g); and P is the purity of the amino acid CRM.

The mass fraction of pure TK-11 or pure β -LG in the solid was calculated according to formula 2, as follows:

$$c = \frac{c_1 \cdot MW_p}{c_0 \cdot MW_{AA} \cdot N} \quad (2)$$

where c is the mass fraction of the pure peptide or protein in the solid matrix; c_1 is the mass fraction of each amino acid (g/g); MW_p is the molecular mass of the peptide or protein; MW_{AA} is the molecular mass of the amino acid; N is the number of specific amino acids in one peptide or protein molecule; and c_0 is the gravimetric prepared solid concentration (g/g).

Determination of the Mass Fraction of β -LG in Milk Powders by Proteolysis. 0.1 g of milk powder was dissolved in 0.8 mL of commercial extraction buffer (R-Biopharm, R4902). The solution was incubated in a water bath at 60 $^{\circ}$ C for 15 min and was shaken every 2 min. Then 15.2 mL of 50 mmol/L ammonium bicarbonate was added into the sample and was centrifuged at 8437g for 20 min. 3 mL of the supernatant was taken out and diluted three times with 50 mmol/L of ammonium bicarbonate. 2.7 g of the diluted sample solution was added into the ultrafiltration tube with the cutoff molecular mass of 3 kDa, and was centrifuged at 6500g for 15 min to remove the extraction buffer. Then the sample was rinsed 5 times by adding 1 mL of 50 mmol/L ammonium bicarbonate and centrifuging. Finally, 100 μ L of L-TK-11 solution was added into the ultrafiltration tube and mixed well with the sample. 100 μ L of the solution was taken out for proteolysis according to the protocol described in Enzymatic Digestion except the proteolysis time was 16 h.

A ZORBAX SB-Aq column (3.5 μ m particle size, 2.1 $\times$ 150 mm; Agilent, Palo Alto, CA, USA) was used to separate the digested mixture. The column temperature was 40 $^{\circ}$ C during the analysis. The injection volume was 10 μ L, and the flow rate was 0.2 mL/min. The mobile phase A was water containing 0.1% formic acid, and the mobile phase B was acetonitrile containing 0.1% formic acid. The gradient is shown in Table S3 in the Supporting Information.

The Thermo-Finnigan TSQ equipped with an ESI ion source was used for IDMS analysis. The ESI ionization was operated in positive mode, and selected ion mode (SIM) was used to monitor TK-11 (m/z = 623) and L-TK-11 (m/z = 625). The flow rate of sheath gas and auxiliary gas was 30 and 10 arbitrary units (au), respectively. The capillary temperature was set at 270 $^{\circ}$ C, and the spray voltage was set at 3000 V.

The mass fraction of TK-11 in the proteolysis mixture was calculated according to formula 1, where c is the mass fraction of TK-11 in the proteolysis mixture (g/g); m_s is the mass of L-TK-11 added to the sample (g); R_s is the peak area ratio of TK-11 to L-TK-11 of the sample; I_1 is the mass ratio of TK-11 to L-TK-11 of the high-level standard; I_2 is the mass ratio of TK-11 to L-TK-11 of the low-level standard; R_1 is the peak area ratio of TK-11 to L-TK-11 of the high-level standard; R_2 is the peak area ratio of TK-11 to L-TK-11 of the low-level standard; M is the mass of the proteolysis sample (g); and P is the mass fraction of pure TK-11 determined by the HPLC–IDMS method.

The mass fraction of β -LG in the solid matrix was calculated according to formula 3:

$$c = \frac{c_1 \cdot MW_p}{c_0 \cdot MW_{pep}} \quad (3)$$

where c is the mass fraction of β -LG in the solid matrix (g/g); c_1 is the mass fraction of TK-11 (g/g); MW_{pep} is the molecular mass of TK-11; MW_p is the molecular mass of β -LG; and c_0 is the gravimetric prepared milk concentration (g/g).

RESULTS AND DISCUSSION

Selection of a Specific Peptide for β -LG Quantification. As different protein molecules may produce the same peptides after proteolysis, a specific peptide is generally filtered and selected for quantification to avoid interference from other peptides. To select a peptide specific to β -LG, pure β -LG was digested with trypsin and the peptides were analyzed. The total ion chromatogram and identified TK-11 are shown in Figure S1 in the Supporting Information. The identified peptides covered 59% for the amino acid sequence of the target protein. Among all the identified peptides, TK-11 (TPEVDDEALEK) had the strongest signal intensity and the peptide sequence was only found in the Swiss-Prot entries of LACB_BOVIN (P02754) and LACB_BUBBU (P02755), which show a difference of two amino acid residues. Therefore, TK-11 was chosen for peptide-based quantification of β -LG.

Optimization of Hydrolysis Times for TK-11 and β -LG. The mass fraction of TK-11 or β -LG was determined by amino acid analysis coupled with the IDMS method. However, the hydrolysis time should be optimized to ensure the complete hydrolysis of the target peptide or protein. TK-11 and β -LG were hydrolyzed according to the above protocol. The ratio of each amino acid to its isotope-labeled one vs hydrolysis time was plotted to determine the optimum hydrolysis time (Figure S2a,b in the Supporting Information).

The ratio rapidly increased at the early stage of the hydrolysis reaction (Figure S2 in the Supporting Information). For TK-11, the ratio reached a plateau after 48 (Figure S2 in the Supporting Information), and 48 h was therefore selected as the optimum time for TK-11 hydrolysis. Similarly, 24 h was selected as the best time for β -LG hydrolysis.

Determination of the Mass Fraction of Pure TK-11 or β -LG in a Solid Matrix. We used nine vials of TK-11 or β -LG for mass fraction determination by the IDMS procedure. The average mass fraction of TK-11 was 79.1% with a relative standard deviation of 5.6%, and the average mass fraction of β -LG was 82.9% with a relative standard deviation of 3.7%.

Optimization of Proteolysis Conditions for β -LG. The proteolysis reaction reaches a chemical equilibrium. To ensure that the reaction proceeded efficiently, we optimized the following conditions: digestion time, amount of denaturing agent and reductive alkylation reagent, and time and temperature of reductive alkylation.

To optimize the digestion time, β -LG was digested according to the above protocol for various times. The ratio of TK-11 to L-TK-11 vs proteolysis time was plotted to determine the optimum time for proteolysis (Figure 1). As shown in Figure 1,

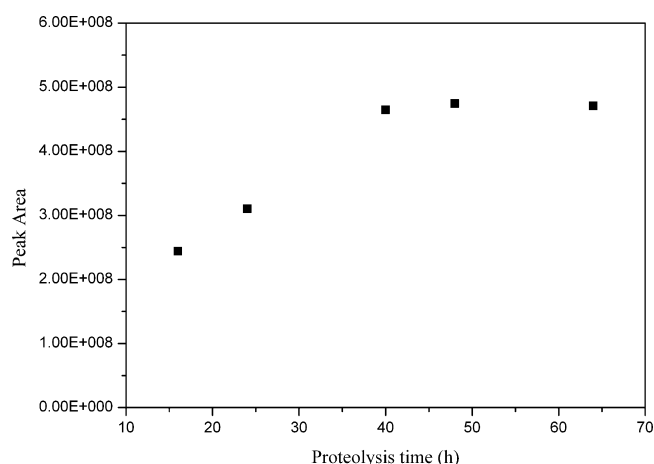


Figure 1. Optimization of proteolysis time for β -LG.

the proteolysis reaction occurred rapidly in the early stages, but reached a plateau after 48 h. Therefore, the proteolysis time of 48 h was used for proteolysis of β -LG.

Next, we optimized the amounts of denaturing agent and reductive alkylation reagent. We conducted four parallel experiments with different amounts of denaturing agent and reductive alkylation reagent (Table 1). As shown in Figure S3

Table 1. Optimization of Amounts of Denaturing Agent and Reductive Alkylation Reagent for Hydrolysis Reaction

reagent	amount of reagent (μ L)			
	expt 1	expt 2	expt 3	expt 4
0.1% RapiGest SF	100	100	$\times^a$	$\times$
DTT	5	$\times$	5	$\times$
IAM	10	$\times$	10	$\times$
trypsin	5	5	5	5

^a $\times$: Reagent omitted.

in the Supporting Information, the maximum peak area was observed in Experiment No. 3, indicating that those experimental conditions gave the best proteolysis efficiency for β -LG. Experiment No. 3 contained reducing reagents but lacked denaturants.

Then, we optimized the amount of reductive reagent by digesting β -LG according to the above protocol with various amounts of DTT. The peak area of TK-11 vs quantity of the reducing reagent was plotted to determine the optimum amount of DTT (Figure S4 in the Supporting Information).

As shown in Figure S4 in the Supporting Information, the peak area of TK-11 increased with increasing amounts of DTT. The maximum peak area was observed in the reaction

containing 35 μ L of DTT solutions; therefore, this amount of DTT was used for the proteolysis reactions.

Finally, we optimized the temperature by conducting the reductive alkylation reaction at different temperatures for various times (Figure S5 in the Supporting Information). As shown in Figure S5 in the Supporting Information, the optimum temperature and duration of the reductive alkylation reaction were 60 $^{\circ}$ C and 15 min, respectively.

Determination of Proteolytic Efficiency. As the proteolysis reaction reaches a chemical equilibrium, or the inhibitors may exist in the extraction buffer, the proteolysis efficiency of β -LG cannot reach 100% even in the optimized digestion conditions.

To determine the proteolytic efficiency of pure β -LG, the mass fraction of β -LG in solid was simultaneously determined by amino acid based IDMS and peptide-based IDMS. As amino acid analysis is a traditional method for protein quantitation, the proteolytic efficiency can be calculated by comparing the ratio of the mass fraction obtained by peptide-based IDMS with that obtained by amino acid based IDMS, assuming complete hydrolysis. The proteolytic efficiency was determined to be 75.7% with a relative standard deviation of 3.0% for seven repeats.

However, the inhibitors in the extraction buffer will depress the proteolytic efficiency greatly. Therefore, ultrafiltration was performed to remove the extraction buffer with 50 mmol/L ammonium bicarbonate to avoid the inhibitory effect of the extraction buffer, which was described in Determination of the Mass Fraction of β -LG in Milk Powders by Proteolysis. In order to determine the proteolytic efficiency of β -LG in milk powders when extraction buffer was applied, some amount of pure β -LG was taken out and treated as the sample according to the protocol described in the section Determination of the Mass Fraction of β -LG in Milk Powders by Proteolysis. Then the proteolytic efficiency can be calculated by comparing the ratio of the mass fraction obtained by peptide-based IDMS to the gravimetric prepared value. However, due to the different β -LG concentrations and different operations, the proteolytic efficiency varied for each run. Nine runs for 3 β -LG levels (0.01 g/g, 0.05 g/g, 0.10 g/g) were carried out for proteolytic efficiency investigation. The proteolytic efficiency was from 6.1% to 15.1% with an average of 11.0% (Table 2). Compared with the proteolytic efficiency of 38% of pure β -LG with digestion time of 16 h, the depression of the inhibitors was also significant even after buffer exchange.

Method Performance of the Peptide-Based β -LG Quantitation by HPLC-IDMS. The performance of the

Table 2. The Spiked Recoveries of Three Milk Powders with Different β -LG Levels

sample no.	level (g/g)	recovery (%) (n = 7)	CV (%) (n = 7)	proteolytic effc (%)
1	0.01	90.0–108.5	8.4	10.7
	0.05	86.2–98.3	4.1	15.1
	0.1	108.1–117.7	2.8	11.1
2	0.01	92.7–111.6	7.1	10.2
	0.05	86.0–98.6	5.1	12.8
	0.1	89.4–108.0	6.5	6.1
3	0.01	96.9–118.3	9.0	14.6
	0.05	96.2–108.7	5.2	10.0
	0.1	96.2–110.1	5.5	8.8

peptide-based HPLC–IDMS was evaluated for quantitation of both pure β -LG and β -LG in milk powder.

For the quantitation of pure β -LG in solution, a pure β -LG CRM developed by NIM was used to evaluate the accuracy and repeatability of the method. The bias, which can reflect the accuracy of the method, was calculated according to the formula 4

$$A = \frac{|c - c_0|}{c_0} \times 100\% \quad (4)$$

where c is the measured mass fraction of β -LG by TK-11 coupled with the IDMS method (g/g); c_0 is the certified value of β -LG (g/g); and A is the relative bias. The relative bias was determined to be 1.9%, and the relative standard deviation was 3.0% for seven repeats. The limits of detection (LOD) and quantification (LOQ) were calculated based on the S/N ratio of 3 and 10 by single point calibration shown in formula 5, which were 4.8×10^{-8} g/g and 1.6×10^{-7} g/g pure β -LG in solution, respectively. The relative expanded uncertainty was evaluated⁴² and it was 4.2% with a coverage factor of 2, which corresponded to a 95% confidence level.

$$m_{\text{sample}} = \frac{R_{\text{h-sample}}}{R_{\text{h-STD}}} \cdot R_{\text{m-STD}} \cdot M_{\text{sample}} \quad (5)$$

where $R_{\text{m-STD}}$ is the mass ratio of TK-11 to L-TK-11 in the standard solution; $R_{\text{h-STD}}$ is the peak height ratio of TK-11 to L-TK-11 of the standard solution; M_{sample} is the mass of L-TK-11 in the sample solution (g); m_{sample} is the mass of TK-11 in the sample solution (g); and $R_{\text{h-sample}}$ is the ratio of signal-to-noise.

For quantitation of β -LG in milk powder, the pure β -LG was added into the native milk powders. And it was treated following the protocol of Determination of the Mass Fraction of β -LG in Milk Powders by Proteolysis. The recovery was calculated as the ratio of the measured concentration difference of the spiked and native milk samples to the concentration of the addition. Therefore, the recovery can be obtained although there is β -LG in native milk powder. As the proteolytic efficiency varied from sample to sample, the proteolytic efficiency for each sample was measured for correction with pure β -LG in extraction buffer as described in Determination of Proteolytic Efficiency. Then the recovery can be obtained by formulas 6 and 7.

$$c_0 = \frac{m_0}{m_s} \cdot P \quad (6)$$

$$R = \frac{c_1 - c_2}{E \cdot c_0} \times 100\% \quad (7)$$

where m_0 is the mass of the pure β -LG added into the spiked sample (g); P is the mass fraction of pure β -LG; m_s is the mass of the sample (g); c_0 is the β -LG concentration added into the sample (g/g); c_1 and c_2 are the measured concentrations of the β -LG in spiked and native samples, respectively (g/g); E is the proteolytic efficiency; and R is the recovery.

Three different brands of milk powders with 3 different spiked levels of each brand were analyzed in the recovery study. The recoveries ranged from 86.0% to 118.3% with maximum CV of 9% (Table 2). The LOD and LOQ were calculated based on the S/N ratio of 3 and 10 by single point calibration, which were 4.8×10^{-5} g/g and 1.6×10^{-4} g/g β -LG in milk powder, respectively. The relative expanded uncertainty was evaluated,⁴²

and it was 5.9% with a coverage factor of 2, which corresponded to 95% confidence level.

Compared with previously reported methods, the LOD of the proposed method is comparable to that by instrumental methods, such as CE-MS (10^{-6} g/g), LC-MS (10^{-6} g/g), CE (10^{-5} g/g), and HPLC (10^{-6} g/g).^{9,11,16} However, the LOD of the proposed method is considerably higher than LOD values reported for ELISA methods (10^{-8} to 10^{-12} g/g).^{4,7} However, the proposed HPLC–IDMS method is a potential primary method, which has advantages for a better recovery of 86.0–118.3% and a lower average standard deviation compared with other methods reported.^{4,16}

Determination of the Mass Fraction of β -LG in Milk Powders by HPLC–IDMS. Ten samples from the domestic market were analyzed, covering defatted, full cream, infant formula, and partially hydrolyzed formula milk powders. All samples were analyzed according to the protocol described in Determination of the Mass Fraction of β -LG in Milk Powders by Proteolysis. Two subsamples were taken out for each sample, and 3 repeat analyses were performed for each subsample. The results are shown in Table 3, and the typical

Table 3. The Mass Fractions of β -LG in 9 Milk Powder Matrices by HPLC–IDMS

sample no.	β -LG content (g/g)	CV (%)
1	0.03	1.9
2	0.02	1.4
3	0.04	1.4
4	0.02	1.4
5	0.03	1.6
6	0.03	5.6
7	0.04	2.8
8	0.13	3.7
9	0.04	5.1

total ion and extracted ion chromatograms are shown in Figure 2. All of the ten samples were successfully analyzed by the proposed IDMS method with CVs of <5.6%. The contents of β -LG ranged from 0.02 g/g to 0.04 g/g for most samples except one brand had a higher β -LG content of 0.13 g/g.

As no β -LG free milk was available, the extraction buffer was used as the blank and negative control. No TK-11 peak was detected of the negative control. To reduce the chance of the false positive, all the samples were treated identically without digestion and analyzed by HPLC–IDMS. No TK-11 peak was detected for all the samples except partially hydrolyzed formula milk powder, demonstrating that no free TK-11 or similar m/z interferences with the same retention time of TK-11 appeared in the samples except partially hydrolyzed formula milk powder. As the proposed method cannot differentiate the intact β -LG from β -LG segments, the proposed method is not suitable for partially hydrolyzed formula milk samples. Though the β -LG content can be calculated for the partially hydrolyzed formula milk sample, it did not reflect the real content; therefore, the result of the partially hydrolyzed formula milk sample was not included in Table 3. In addition, the single charged TK-11 ($m/z = 1245$) and L-TK-11 ($m/z = 1248$), instead of double charged TK-11 ($m/z = 623$) and L-TK-11 ($m/z = 625$), were also captured by HPLC–IDMS to confirm the results of the ten samples, and no clear difference was observed, which demonstrates the reliability of the proposed method.

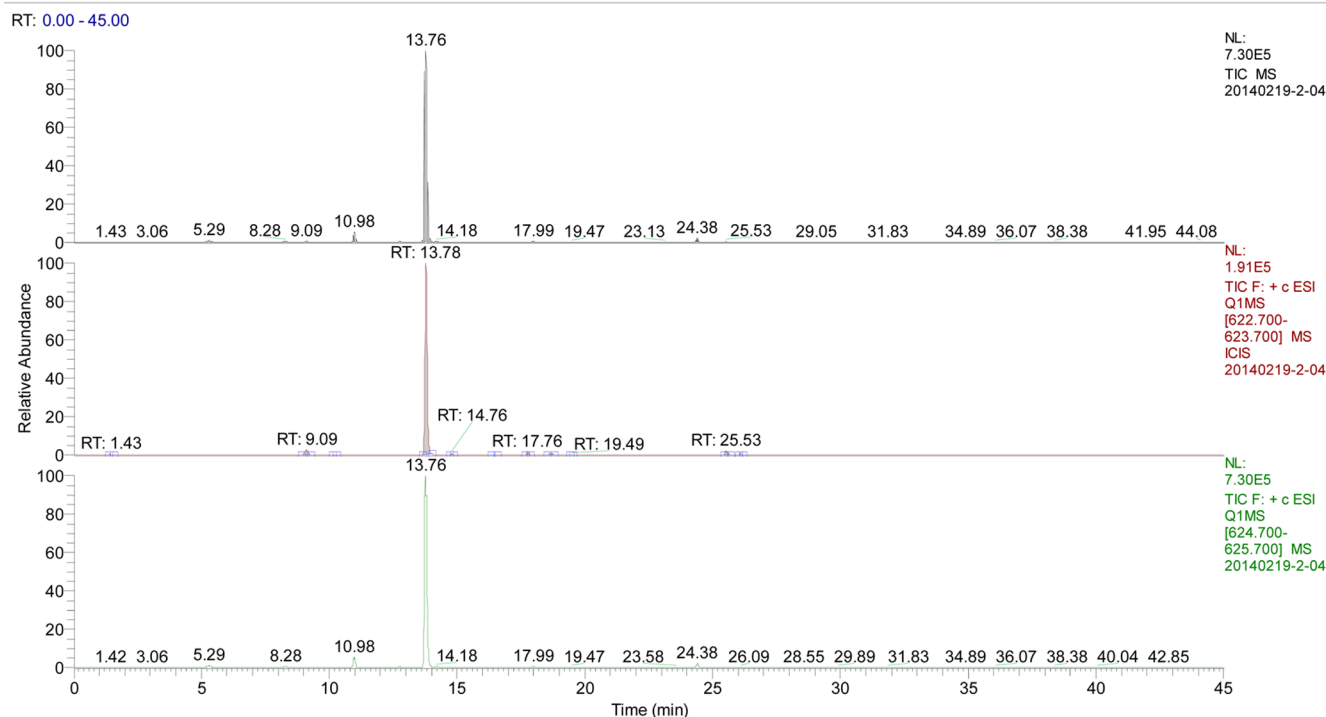


Figure 2. The typical total ion and extracted ion chromatograms of a real sample by HPLC–IDMS.

The quantification results are SI-traceable via the amino acid CRM and IDMS methods (Figure 3). This method is accurate

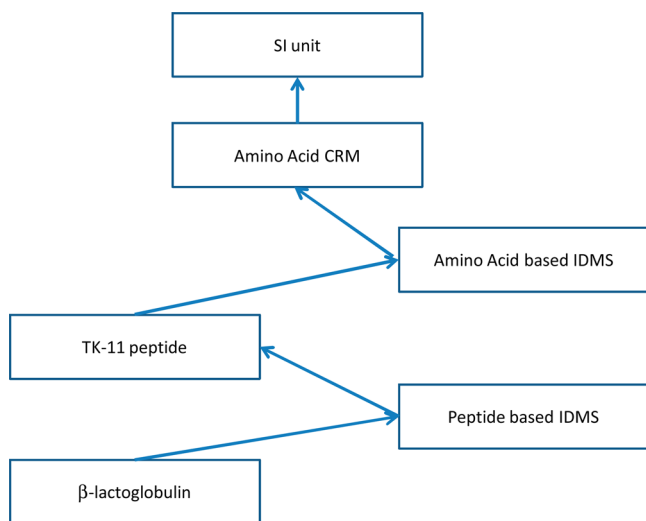


Figure 3. Traceability chain for β -LG quantification.

and sufficiently sensitive to detect β -LG in milk powders. It will be useful to develop both pure β -LG CRM and CRM of β -LG in milk powders. The method is also expected to improve the comparability of β -LG quantification results among different laboratories.

■ ASSOCIATED CONTENT

Supporting Information

Table S1, gradient of mobile phases for separation of the digested peptides from pure β -LG by UPLC-Q-TOF; Table S2, gradient of mobile phases for separation of Pro, Val, Leu, and

Phe in the amino acid mixture by HPLC–IDMS; Table S3, gradient of mobile phases for separation of TK-11 from other digested peptides within 45 min by HPLC–IDMS; Figure S1a, the total ion chromatogram of protein identification by UPLC-Q-TOF; Figure S1b, the fragments of TK-11 corresponded to its sequence; Figure S2, optimization of hydrolysis time for TK-11 (a) and β -LG (b) according to the peak area ratio of each amino acid to its isotope-labeled one; Figure S3, comparison of the different amount of denaturing agent and reductive alkylation reagent addition: effect on the proteolysis efficiency; Figure S4, comparison of the different amount of DTT: effect on the peak area of TK-11; Figure S5, comparison of the different temperature and duration of reductive alkylation reaction: effect on peak area of TK-11. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

β -lactoglobulin, β -LG; CRM, certified reference material; IDMS, isotope dilution mass spectrometry; TK-11, peptide with the sequence TPEVDDEALEK; NIM, National Institute

of Metrology; CV, coefficient of variation; LOQ, limit of quantitation; LOD, limit of detection; DTT, dithiothreitol; IAM, iodoacetamide

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Supplementary material

Tables

Table S1. Gradient of mobile phases for protein identification by UPLC-Q-TOF

Table S2 Gradient of mobile phases for analysis of amino acid mixture by

HPLC-IDMS

Table S3 Gradient of mobile phases for analysis of digested peptides by HPLC-IDMS

Figures

Figure S1 The total ion chromatogram and identified TK-11.

Figure S2 Optimization of hydrolysis times for TK-11 (a) and β -lactoglobulin (b)

Figure S3 Effect of amount of denaturing agent and reductive alkylation reagent on hydrolysis reaction (experiment no. corresponds to that shown in Table 1)

Figure S4 Effect of amount of DTT on peak area of TK-11 in reaction product.

Figure S5 Effects of temperature/duration of reductive alkylation reaction on product formation.

Table S1. Gradient of mobile phases for protein identification by UPLC-Q-TOF

Time (min)	Flow (μL/min)	A (%)	B (%)
0	0.5	97.0	3.0
30	0.5	60.0	40.0
35	0.5	15.0	85.0
37	0.5	15.0	85.0
38	0.5	97.0	3.0

Table S2 Gradient of mobile phases for analysis of amino acid mixture by

HPLC-IDMS

Time (min)	Flow rate (mL/min)	A (%)	B (%)
0	0.2	100	0
4	0.2	100	0
9	0.2	86	14
14	0.2	84	16
24	0.2	84	16
32	0.2	70	30
34	0.2	40	60
37	0.2	40	60
39	0.2	0	100
65	0.2	0	100

Table S3 Gradient of mobile phases for analysis of digested peptides by HPLC-IDMS

Time (min)	Flow rate (mL/min)	Mobile phase A (%)	Mobile phase B(%)
0	0.2	97	3
2	0.2	97	3
20	0.2	70	30
25	0.2	3	97
30	0.2	3	97
35	0.2	97	3
45	0.2	97	3

Figure S1a

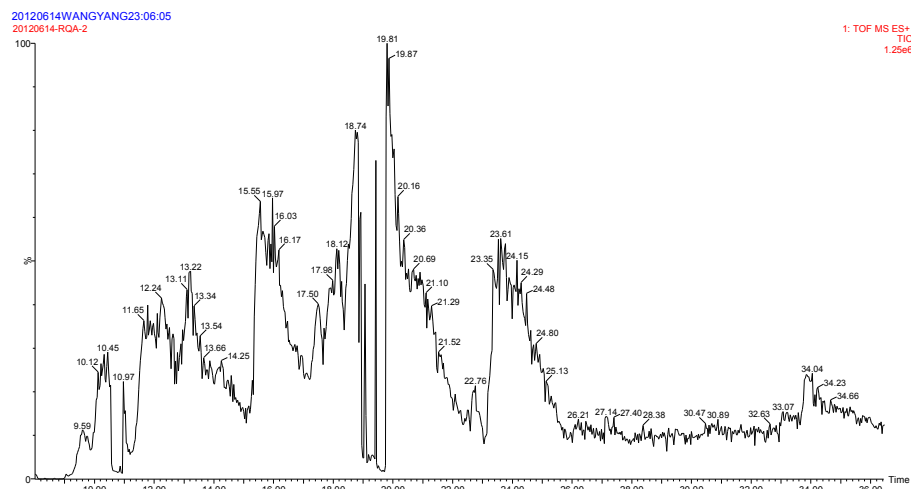


Figure S1b

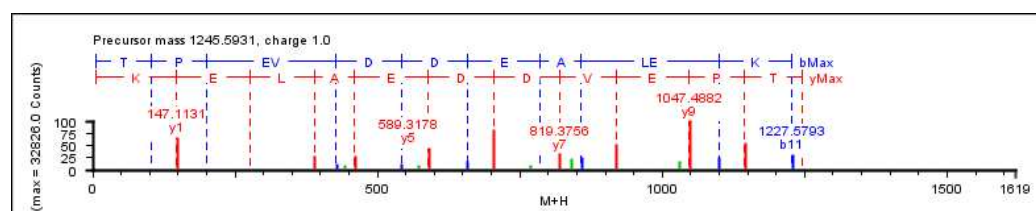


Figure S1 The total ion chromatogram and identified TK-11.

Figure S2a

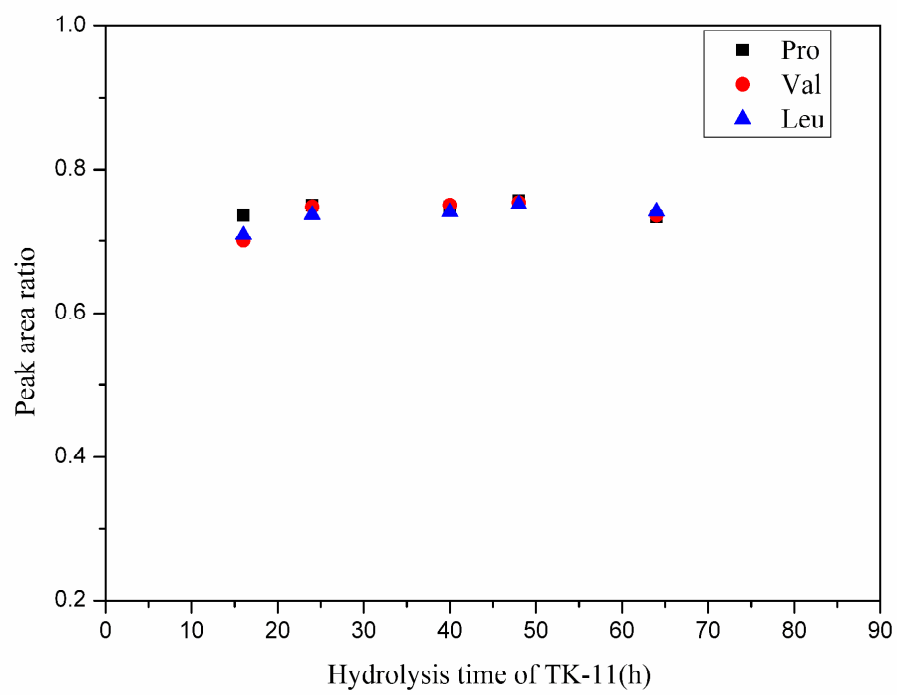


Figure S2b

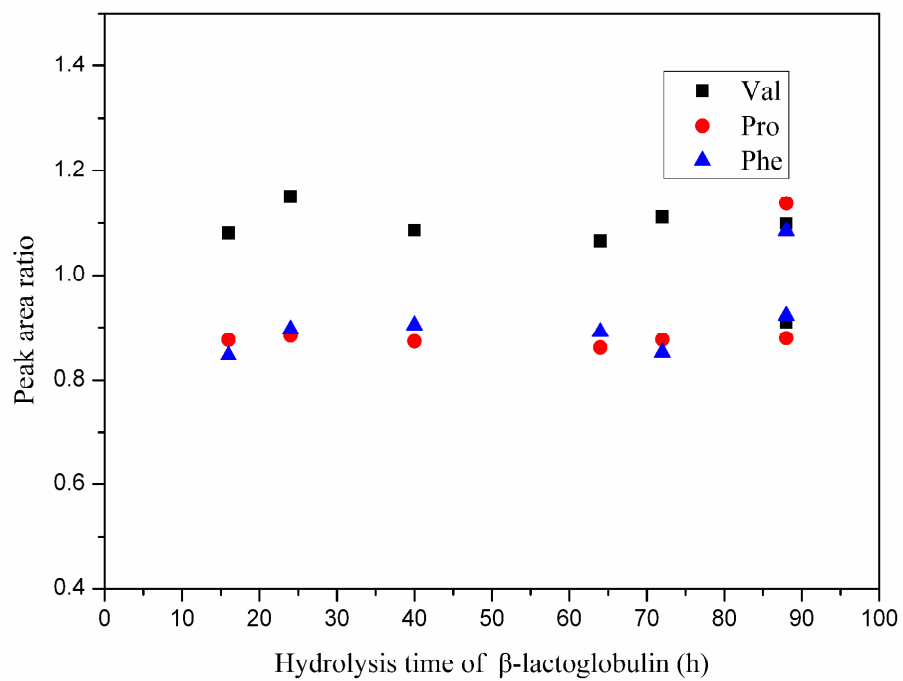


Figure S2 Optimization of hydrolysis time for TK-11 (a) and β-lactoglobulin (b)

Figure S3

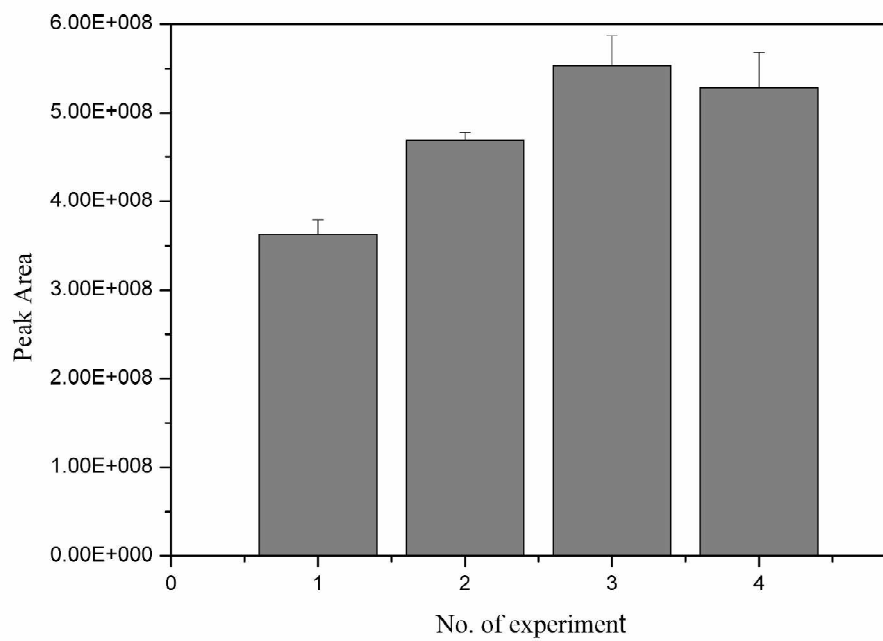


Figure S3 Effect of amount of denaturing agent and reductive alkylation reagent on hydrolysis reaction (experiment no. corresponds to that shown in Table 1)

Figure S4

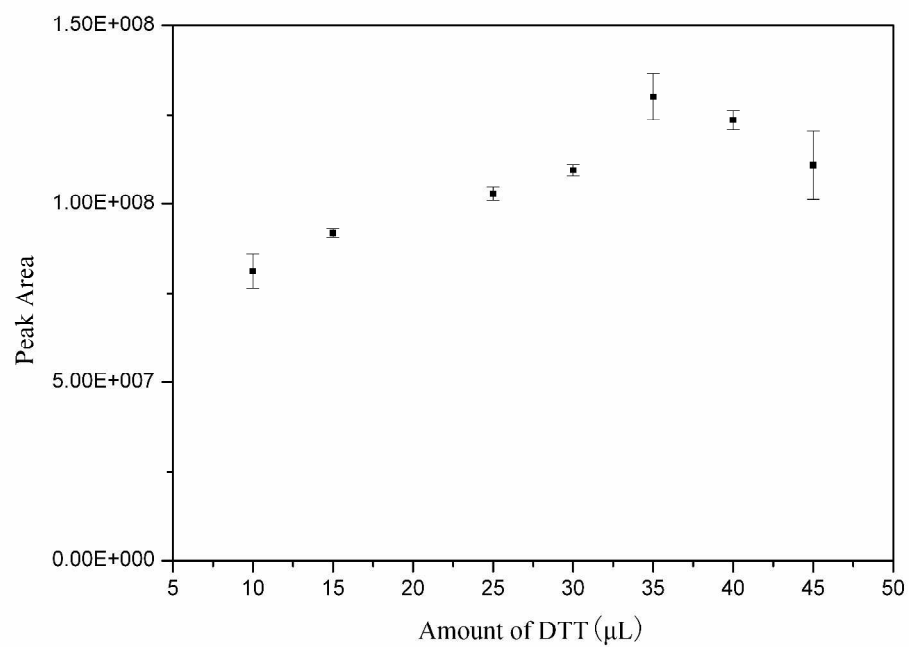


Figure S4 Effect of amount of DTT on peak area of TK-11 in reaction product.

Figure S5

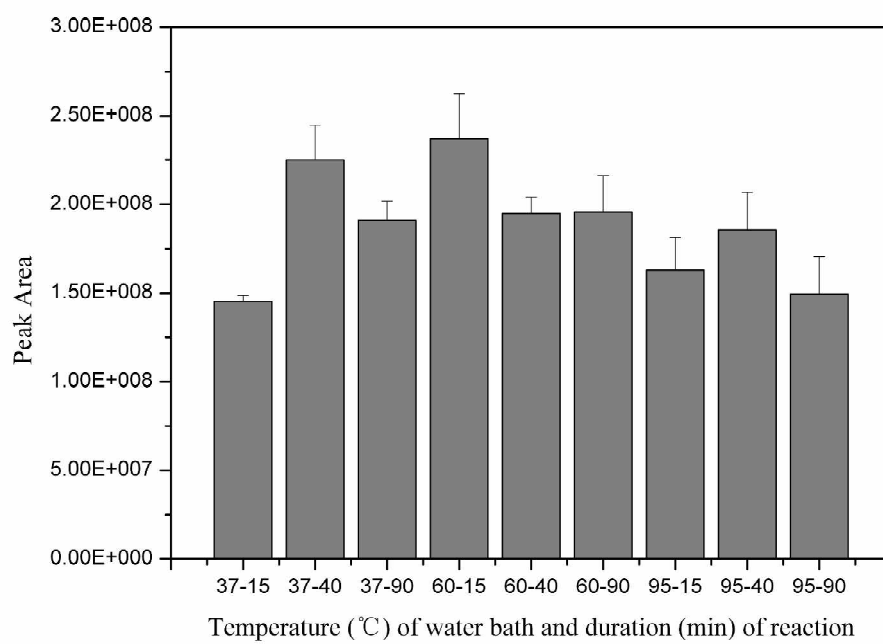


Figure S5 Effects of temperature/duration of reductive alkylation reaction on product formation.